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Advances in Host-Directed Therapies Against Tuberculosis

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Preface

Tuberculosis (TB) has caused at least 1 billion deaths over the past two centuries, more than the combined number of deaths from malaria, smallpox, HIV/AIDS, cholera, plague, and influenza. The WHO estimates that there are more than 2 billion people latently infected with *Mycobacterium tuberculosis* (Mtb). TB is the leading cause of death among infectious diseases and one of the top 10 causes of death worldwide. In 2016, TB caused an estimated 1.7 million deaths including over 400,000 in persons living with HIV/AIDS. An estimated 10.4 million new TB cases occurred in 2016, 10% of which were among individuals living with HIV infection. About 4.1% of the new cases were multidrug-resistant (MDR) TB, with 6.2% of those cases identified as extensively drug-resistant (XDR) TB. Current treatment has major limitations, including long duration with poor adherence, high rates of intolerability and toxicity, and frequent drug interactions. One result of these drawbacks is the steady emergence of MDR/XDR TB, which is notoriously difficult and expensive to treat. Because of limited profitability, development of new agents by pharmaceutical companies has been slow and sporadic. With increasing resistance to current antibiotics and reduced investment in the development of new antimicrobial drug classes, the World Health Organization has warned that we are at risk of entering a "post-antibiotic era."

To meet the challenges of increasing antimicrobial resistance, the infectious disease community needs innovative therapeutics. The field of oncology has addressed complex treatment challenges by developing approaches to reverse disruptions in cell regulatory mechanism that cause malignancies and often lead to suppression of immune processes. Effective and far less toxic new therapeutic approaches are based on discoveries of basic pathogenic mechanisms of malignancies. These strategies include "precision medicine" (targeted reversal of cell pathway disruptions caused by mutations as pathogenic drivers) and novel "immuno-oncology" interventions, for example, immune checkpoint reversal, now transforming cancer therapeutics. Pathogens target many of the same regulatory pathways as they modulate host cell regulatory and metabolic functions to promote their own survival by impeding host immunity. The same knowledge, tools, and interventions now revolutionizing oncology can be adapted for improved therapy of many infections.

Host-directed therapy (HDT) is intended to enhance microbial killing and lessen detrimental inflammation/tissue damage by targeting host regulatory molecules and pathways modulated by pathogenesis. Preclinical studies have identified drugs with promising HDT benefits for adjunctive TB treatment. Choosing candidate agents for evaluation needs to be based on knowledge of the host regulatory signaling pathways disrupted by pathogens and identifying specific pathway molecules that can be therapeutically targeted. Promising host-based interventions already in clinical use and in evaluation for TB therapy include tyrosine kinase and phosphodiesterase inhibitors; agents to restore disruptions in immunometabolism, regulatory pathways, and effector mechanisms; modulation of immune suppressive cells (especially MDSCs); immune co-receptor-based checkpoint interventions; epigenetic agents; and combinations of these agents. New HDT strategies are urgently needed for TB meningitis to reduce the associated high mortality and morbidity.

HDT goals include reversing TB-induced immune defects to achieve increased bacillary killing to shorten treatment duration and improve MDR treatment outcomes, both by directly improving immune function and also by decreasing excessive tissue inflammation and death (and reversing inhibition of autophagy and apoptotic pathways) to improve drug and immune cell penetration and function. Another important goal is prevention of inflammatory lung tissue damage with loss of pulmonary function. Impaired pulmonary function (both obstructive and restrictive) often persists after TB treatment. Even mild decreases in FEV1 have been associated with significantly increased mortality in very large cohort studies of broadly inclusive populations. One key aspect of HDT target discovery research is distinguishing between detrimental immune cell regulation changes and tissue damage caused by pathogen molecular drivers and adaptive changes that are necessary for enhancing antimicrobial control and killing. Thus, some HDTs could lead to an over-reactive immune response, causing excessive tissue damage. Both preclinical and clinical HDT studies must carefully address and monitor for this risk. Successful adoption of HDT for infectious diseases also requires careful research into how to identify which patient subpopulations, types of agents, timing of initiation, and dosing regimens/duration will result in the most benefit and least harm.

Re-purposing of HDT drugs for many non-infectious diseases provides a practical approach to address the lack of incentive in developing novel antimicrobial agents due to the high cost and lengthy development time needed for approval of new drug classes relative to the limited financial return on investment. In order to develop innovative new HDT strategies for infectious diseases based on precision medicine/immunotherapy principles, multi-disciplinary teams of researchers will be needed with expertise in microbiology/clinical infectious diseases, classical immunology, and the still-emerging field of molecular biology of cell regulation. One initial step in this process is to facilitate collaboration among researchers studying key regulatory signaling pathways and targeted interventions for non-communicable diseases and those in the infectious diseases community. These

approaches will create a transformative new paradigm for the treatment and prevention of TB and a wide variety of other infectious diseases, and will have a high global health impact, particularly in the face of progressive emergence of antimicrobial resistance.

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About the Editors

Petros C. Karakousis, M.D., is an infectious diseases-trained Physician Scientist and Professor of Medicine at the Johns Hopkins University School of Medicine. His research focus is on host–pathogen interactions contributing to *Mycobacterium tuberculosis* persistence and antibiotic tolerance. His laboratory is actively investigating the repurposing of clinically available agents with immune-modulatory properties as adjunctive host-directed therapy, in order to shorten the duration of TB treatment and improve lung pathology.

Maria Laura Gennaro, M.D., is Professor of Medicine at Rutgers New Jersey School of Medicine. Her laboratory studies mechanisms of adaptation expressed by *Mycobacterium tuberculosis* and by the host macrophage during infection, with the goal of finding targets for therapeutic intervention. She has a specific interest in macrophage lipid metabolism, which is altered following *M. tuberculosis* infection, thereby promoting bacterial survival.

Richard Hafner, M.D., is an infectious diseases-trained Physician and Chief of the TB Clinical Research Branch in the Division of AIDS at NIAID/NIH. Throughout his career, he has had a long-standing interest in advancing innovative host-directed therapies for infections. He has been involved in several clinical trials, authored various articles, and hosted multiple scientific meetings related to research to develop targeted HDTs for TB.

Part I
Introduction

Chapter 1
Introduction: An Overview of Host-Directed Therapies for Tuberculosis

Daniel J. Frank and Robert N. Mahon

Host-Directed Therapy: Purpose and History

Despite the widescale success of the antibiotic era in mitigating a plethora of bacterial infectious diseases, tuberculosis (TB) "the white death" remains a public health scourge claiming approximately 1.3 million lives annually, with estimates of nearly a third of the world's population infected with its causative agent *Mycobacterium tuberculosis* (Mtb) [1]. While the first antibiotics, sulfonamides and penicillin, proved to be ineffective at controlling Mtb infection, the advent of streptomycin in 1943 created chemotherapeutic treatment options for this disease. Streptomycin and para-aminosalicyclic acid, the two effective anti-TB chemotherapeutic drugs, rapidly induced resistance by Mtb when either agent was given alone [2], a harbinger of the multidrug-resistant (MDR) TB strains that would eventually develop. Isoniazid (INH), developed a few years later, was a much more potent and caused fewer toxic side effects. The development of drug resistance is a recurring problem in TB treatment, as Mtb has developed ways to circumvent nearly every antibiotic.

Host-directed therapy (HDT) offers the potential to combat these drug resistance issues. First, by focusing on host, rather than bacterial targets, to empower the immune system to clear the mycobacterial infection, the agents do not directly apply selective pressure on the bacteria. Second, HDT agents may be employed in combination with standard anti-mycobacterial therapy potentially shortening treatment, and thereby improving adherence and limiting the emergence of resistance arising due to incomplete treatment. An added benefit of many of these HDT agents is they also have anti-inflammatory effects that ameliorate the lifelong inflammatory

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pulmonary tissue damage caused by active TB infection, improving the quality of life and possibly long-term survival, for cured patients [3–6].

HDT has its roots as some of the oldest TB therapy. Prior to the chemotherapeutic era, all TB treatments by necessity were "host directed." Ascertaining the impact on patients is difficult because no adequate comparison has ever been performed [7]. A systematic review of 564 patients admitted to New York State sanatoria found [8] a mortality rate of 37%, an improvement over models indicating a mortality rate between 53% and 86% [9] for TB cases not in sanatoria. The effect may be due to the host-directed benefits of a healthy diet, proper rest, mild exercise regimen, and sunlight often included in the sanatoria setting [10]. Indeed, evidence suggests that reclining in a supine position reduced Mtb bacilli growth [11].

The Antibiotic Era

By the mid-1950s, the development of effective TB drugs, like INH and PAS, the focus on the host diminished, and the sanatoria quickly closed. New classes of anti-TB drugs were discovered, and combination treatment regimens employed to impede the emergence of drug-resistant TB. However, increasingly drug-resistant TB remained a problem. By 2006, the first reports of extensively drug-resistant (XDR) TB appeared, revealing strains resistant to the two major first line TB drugs, INH and rifampicin, as well as aminoglycosides and quinolones [12, 13], highlighting the need for alternative treatment strategies for TB.

Complicating progress is a lack of funding for TB drug development, with the World Health Organization estimating $3.5 billion shortfall for TB implementation in 2018, and as much as a $2 billion per year research shortfall as well as limited profit motivation for pharmaceutical companies [14]. HDT strategies can take advantage of investments in other fields, such as in oncology and autoimmune diseases, to re-purpose drugs already in use or in development that may modulate the immune system to improve TB outcomes [15]. Therapies already proven safe and effective for other disorders have a streamlined and more cost-effective pathway to approval for TB clinical use. Understanding how Mtb dysregulates the host immune system to create a hospitable environment, and how HDT agents may improve immune functions to more rapidly cure TB and decrease excess tissue damage is the key to developing clinically impactful HDT.

Host Response to Mtb

The growth of Mtb in the lung has long been tied into the state of the immune response [16]. After the first few weeks of infection, as Mtb rapidly replicates within macrophages, its growth substantially decreases upon the arrival of T cells. A functional immune response controls, but does not eradicate Mtb, leading to a latent

infection classically defined by the formation of a granuloma and tuberculin reactivity. Active disease occurs when immunity is unable to control Mtb growth, either soon after infection, or after immunity is compromised during latency, leading to granuloma breakdown and bacterial proliferation in tissues [16]. While no proven HDT targets have been identified, many potential targets within several subsets of immune cells have either been proposed or are being tested. From these results, we can begin to home in on specific cellular pathway targets for optimal therapeutic benefit from HDTs.

T cells are vitally important to the control of Mtb pathogenesis, although the exact mechanisms remain unclear. While IFN-γ production was thought to be of primary importance in T cell functionality [17], recent studies have suggested that it is not required and likely detrimental to control Mtb growth within the lung [18]. Without a deeper understanding of how T cells control TB, knowing how to target them with HDTs is difficult. Immunomodulatory agents used to treat autoimmune disorders are well known to increase the risk of reactivation in latently infected TB patients. Work with the immune checkpoint inhibitor PD-1 has also shown that immune activating agents can lead to detrimental results during TB disease [19, 20]. Initial murine studies utilizing PD-1 knockout mice showed significantly increased lethality during Mtb infection. Knockout mice had increased cytokine levels and inflammatory cells present in the lung, indicating that maintaining balanced negative regulation of T cell immunity is essential to control TB. TB reactivation has been subsequently reported in several cancer patients being treated with a PD-1 checkpoint inhibitor [21]. Further supporting the role of the T cell response during TB disease is a recent study that reported harmful effects when T cell metabolism was modulated [22]. Initially thought to be an ameliorative HDT target due to its role in Mtb-induced necrosis in macrophages [23], knockout studies of the mitochondrial matrix protein cyclophilin D, had heightened T cell responses that increased cytokine levels without a change in Mtb burden, and led to the death of most of the mice within 3 months of Mtb challenge.

The Inflammatory Response

One of the drivers of utilizing HDTs is a desire to lessen the inflammatory and tissue damaging effects caused by active TB on the host. Even after successful TB treatment, Mtb infected patients are at an increased risk to develop chronic pulmonary dysfunctions (COPD) [24] making immuno-modulatory agents candidates for HDT development. Corticosteroids were one of the first agents evaluated as an HDT for TB. While benefits have been observed as an adjunctive therapy for tuberculous meningitis, non-physiological concentrations were required for an effect on pulmonary TB with serious adverse events reported at lower concentrations [25]. Several non-steroidal anti-inflammatory drugs have been, or are currently, being tested as potential HDTs ranging from over the counter drugs (e.g. aspirin or ibuprofen) to prescription arthritis medications [5]. While targeting acute inflammation mediators

has been therapeutically beneficial for some autoimmune disorders, there is an open question on whether stopping inflammation is the best course of action in infectious disease induced inflammatory situations as these interventions may not have favorable effects in treatment of infections. The inflammatory process has three stages: onset, resolution, and post-resolution [26]. The resolution phase occurs after the onset of acute inflammation when apoptosis of inflammatory cells occurs, cytokines and other mediators are removed from the extracellular environment through decoy receptors, pro-inflammatory signaling pathways are turned off, and macrophages are reprogrammed to produce anti-inflammatory cytokines and pro-resolution mediators. Instead of only inhibiting inflammation, an alternative course of action could be enhancing resolution. For example, eicosanoids, including prostaglandins and resolvins, promote resolution by suppressing TLR and NF-κB signaling [27]. Prostaglandins are specifically involved in the cross-regulation of IL-1 and Type I interferon during TB disease. Prostaglandins are synthesized from arachidonic acid via cyclo-oxygenase (COX) that competes with 5-lipoxygenase (5-LO) for available arachoidonic acid. Zileuton, an inhibitor of 5-LO, increases prostaglandins synthesis and when administered 1 month after Mtb infection, when the onset of inflammation has likely dissipated, can significantly increase survival in mice [28]. A key factor for the development of HDTs is timing: a therapeutic agent that has beneficial affects during the early stages of Mtb infection may have no benefit, or even be harmful, during latency or late stages of infection.

A key component of resolution is the induction of apoptosis and the clearance of dead cells. Neutrophils, primary drivers during the onset of inflammation, are induced to go through apoptosis by a series of pro-apoptotic factors and then phagocytosed by macrophages by efferocytosis [29]. When this process is perturbed during uncontrolled inflammation, neutrophils instead go through necrosis, a poorly regulated form of cell death. Necrotic cells release damage-associated molecular patterns (DAMPs) and other pro-inflammatory molecules that further exacerbate pathogenesis. Mtb actively induces necrosis of infected cells and blocks apoptosis. Several studies have shown that infected apoptotic neutrophils activate macrophages leading to phagosomal maturation and significantly decreased Mtb burden [30, 31]. Mtb, by way of ESAT-6 and its secretion system ESX-1, instead induces necrosis of neutrophils, releasing viable Mtb into the extracellular environment where it can be phagocytosed by neighboring macrophages. An attenuated strain of Mtb lacking ESX-1 secretion system stays within the apoptotic neutrophil as it phagocytosed by the macrophage unable to block phagosome maturation [31]. Better understanding the mechanisms of Mtb-induced necrosis in order to identify potential HDT targets is a priority. Two that have been identified are reactive oxygen species (ROS) that are required for Mtb induced necrosis in neutrophils, and peroxisome proliferator-activated receptor (PPAR)γ a nuclear receptor known to be necessary for Mtb pathogenesis by limiting apoptosis. Early *in vitro* studies of inhibitors of ROS and Mcl-1, a downstream effector of PPARγ, in macrophages have decreased Mtb levels compared to untreated controls [31, 32].

Immunosupression

An important question needing to be addressed by HDT is whether negative regulatory immune cells and pathways utilized by Mtb to subvert host immunity and by the host to protect against deleterious inflammatory responses can be targeted therapeutically. As highlighted above, blocking or removing brakes, "checkpoint inhibition"- on T cell responses has had no beneficial effect on Mtb burden, but increases inflammation and tissue damage in the lung [19, 20, 22]. The concept of "disease tolerance" whereby the host dampens the inflammatory and adaptive immune response to the presence of a persistent infectious pathogen so as to protect against tissue damage has started to be explored in the context of TB [33]. Utilizing HDTs that are meant to induce host immunity in this context may have deleterious effects, particularly in the absence of an effective antimicrobial agent. An example of this is a recent study testing a matrix metalloproteinase (MMP) inhibitor as a single therapy HDT for TB [34]. Expecting to observe decreased pulmonary cavitation, the authors instead reported an increased cavitation, heightened immunopathology, and decreased survival. A second study that used other MMP inhibitors and included antibiotics was able to show a significant decrease in bacterial burden in mice given antibiotics with MMP inhibitors compared to antibiotics alone [35]. Thus, the context of when and how an HDT is used is an essential component of their development.

Myeloid-derived suppressor cells (MDSCs) are a regulatory cell population that acts to resolve inflammation and return to homeostasis [36]. They produce anti-inflammatory cytokines (e.g. IL-10), generate ROS and nitric oxide, suppress T cell proliferation by removing arginine from the extracellular environment, and recruit Tregs. The cancer field has been at the forefront of MDSC research, identifying new phenotypic markers, describing cellular functions, and identifying ways that they are used by tumors to grow and metastasize [37]. Initial observations during Mtb infection have found that MDSC levels rise in the blood during active disease and decrease after successful therapy [38]. Intriguingly, Mtb may be phagocytosed by MDSCs and can evade host immunity within these cells [39]. As a potential HDT target, all-trans retinoic acid (ATRA) differentiates MDSCs into mature macrophages, DCs, and neutrophils, decreased Mtb burden, and improved lung function in mice. While extensive research is needed to characterize the role of MDSCs at different stages of infection, exploring them as a potential HDT target has a strong rationale.

Immunometabolism

All cells require energy to function and replicate and immune cells are no exception. Over the past few years, there has been a renewed interest and appreciation in the metabolic activity of immune cells and how its directly intertwined with their functionality [40, 41]. A naïve T cell upon activation requires the energy and biosynthetic

molecules needed for proliferation, while a long-term resident memory T cell lives in a more quiescent state with energy requirements focused on long-term metabolic stability. Proliferating T cells utilize aerobic glycolysis for their energy needs that is an inefficient source of ATP but allows for the synthesis of needed biomolecules (e.g. amino acids, fatty acids). Memory cells use the more efficient oxidative phosphorylation as their energy source. Other immune cells, including macrophages and dendritic cells, go through similar metabolic reprogramming in response to immune function changes. Primary drivers of this metabolic programming are the signaling molecules mTOR and AMPK. Signaling through mTOR places the cell in an anabolic state, while AMPK alerts the cell to low ATP levels and reprograms the cell into a catabolic state [42]. The role of these signaling molecules, and their potential as HDT targets, is currently being studied with both an mTOR inhibitor targeting drug (everolimus) and metformin a drug with several reported mechanisms including AMPK activation.

Additional aspects of immunometabolism are also being tested as HDT targets. Tryptophan is an essential amino acid that humans obtain through diet and is needed by proliferating T cells [43]. To suppress T cell activity tryptophan can be removed and metabolized by neighboring cells. Tryptophan deprivation has been proposed as a driver of immune suppression in the tumor microenvironment. Indoleamine-2,3-dioxygenase (IDO) produces kyneurenine among other metabolites from tryptophan and has become the focus of therapeutically targeting tryptophan metabolism [43]. In Mtb granulomas, IDO levels are elevated, and studies have indicated a link between bacterial burden and IDO levels [44]. In macaques, the use of the IDO inhibitor 1-methyl-tryptophan resulted in decreased Mtb growth, improved pulmonary pathology, and increased T cell numbers [45]. Also, granulomas were reorganized, allowing for T cells to migrate into the granuloma. The results suggest that HDTs that allow for improved penetration into granulomas may be promising agents.

How the metabolic activity of an immune cell correlates with its functionality is an open and important question for immunometabolism, although the mitochondria and the generation of ROS are known to be involved [46]. Some metabolites may also directly signal within the cell. For example, the TCA metabolite itaconate inhibits the release of both IL-1β and Type I Interferons linking itaconate to the known role of the two cytokines in the regulation of inflammation during TB [47]. Studies with immune-responsive gene 1(Irg1), a mitochondrial enzyme that produces itaconate, indicate that in the absence of itaconate mice quickly succumb to Mtb infection with increased levels of inflammation and pathology [48].

The Modern HDT Clinical Pipeline

For an HDT agent to go into clinical development, animal data showing an improvement in bacterial load, immunopathology, and overall survival should be necessary. The question of exactly which animal model(s) are most appropriate is an open debate. The vast majority of *in vivo* HDT research has been done in small animal models, particularly murine. Imatinib, statins, metformin, MMP inhibitors, --the

phosphodiesterase-4 (PDE-4) inhibitor CC-3052, and zileuton have all been studied in mice with some also tested in guinea pigs. Only a few potential HDTs have been studied in non-human primates (NHPs) while several have gone on to clinical development without NHP data. Furthermore, the zebrafish model has also identified a few potential HDTs [49, 50]. None of the models produce an infection identical to what is observed in human tuberculosis, thus determining what type of animal studies should be necessary for further development as an HDT is difficult. Many questions remain unanswered. If a potential HDT does not show a benefit in small animal models, should the agent not be studied in the costlier NHP model? What criteria should be used to advance agents to clinical studies? As research into HDTs develops and allows correlation of findings from clinical trials with animal models, we will be better able to answer these questions.

The current pipeline of HDT development has three segments. Agents (a) in clinical development (b) being tested in small vertebrate animals and monkeys or (c) being tested *in vitro*. The HDTs in some form of clinical development include statins, imatinib, metformin, everolimus, and CC-3052. How this group was first identified and tested is enlightening for how future HDTs may be developed. Metformin was originally identified based off an *in vitro* screen of 13 autophagy and AMPK-activating drugs in BCG challenged THP-1s, a human macrophage cell line. While metformin was not the only drug screened found to have an ameliorative effect on bacterial burden, it has been in wide clinical use for decades, so it was selected for further development [51]. Statins were initially tested in human PBMCs and macrophages and then in mice because of the known role of host cholesterol in Mtb pathogenesis, and statins immunomodulatory capabilities [52]. Imatinib was identified through a focused analysis of the role of receptor tyrosine kinases in TB [53]. As a well described targeted anti-inflammatory, CC-3502 was tested *in vivo* in the presence of INH [54]. Everolimus was also initially tested as a well-established anti-inflammatory and inducer of autophagy [55]. Clinical evaluation of these drugs for TB treatment is ongoing, thus extrapolating on their ultimate effectiveness as HDTs is not possible. However, these studies have laid a foundation for how potential HDTs can be identified and developed for clinical testing.

Most of the current HDTs in clinical development were chosen from an *in vitro* testing, either as a targeted study of a specific drug or class, or from a screening of several drug classes. These studies usually utilize either monocyte-derived macrophages from humans or mice, or a macrophage cell line, primarily human (e.g. THP-1s). While these assays have resulted in identification of promising candidates, several have produced false positive results (23). Reliance on monocellular *in vitro* assays to establish initial evidence on the potential of HDT is problematic and should be replaced with multicellular assays. Several *in vitro* human granuloma models have been developed and are starting to be used to test antibiotics and HDTs [56]. While these assays do not completely recapitulate the *in vivo* human granuloma environment, they do provide additional complexity over standard *in vitro* models through the addition of multiple types of human immune cells, and fibroblasts. Thus, making them an extremely useful model for HDT development. They may aid in improving the translational quality of *in vitro* discoveries.

When to stop developing a potential HDT is a pertinent question for determining the progression of drug candidates into clinical development. Selecting agents approved as safe for use for other diseases will help mitigate risk. However, understanding the impact of drug-drug interactions between the HDT agent and TB and HIV treatment drugs is also critical. Positive or negative results for an HDT agent given without concomitant TB treatment should not be used to make critical decisions concerning further evaluation.

Conclusion

Mtb actively disrupts host immune cellular pathways to create a favorable environmental niche as it establishes infection. The overarching goal of HDT is to reverse or compensate for this immune dysregulation to allow the host immune system to improve TB treatment outcomes. As you will read throughout this book, HDT candidates represent a broad spectrum of agents targeting a variety of cells and pathways, complicating their clinical development and direct comparison. They all are intended to restore balanced regulation among immune cell metabolic pathways, between pro- and anti-inflammatory pathways, necrosis and apoptosis, and activation and inhibition of specific immune cell populations. Achieving such balance is the key to harnessing the potential of HDT for infectious diseases.

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Part II
Targeting Immunometabolism

Chapter 2
Sirtuin Deacetylases: Linking Mycobacterial Infection and Host Metabolism

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Introduction

Cellular metabolism involves a balance between energy intake, utilization, and storage. This balance is finely controlled by an evolutionarily conserved program regulated by three energy sensors: Mechanistic (formerly mammalian) Target of Rapamycin (mTOR, a serine/threonine kinase); AMP-activated protein kinase (AMPK, a serine/threonine kinase); and sirtuins (nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases) [1–4]. These energy sensors are known to either cooperate or oppose each other's actions [5, 6] to regulate energy homeostasis, cellular function, and immuno-metabolic health during basal and physiologically stressed conditions, including infections [7–9]. The crosstalk between mTOR and AMPK signaling with cellular immunity during infections has been intensively studied [7, 10, 11]. Recent evidence supports the role of sirtuins in linking metabolic, mitochondrial bioenergetic pathways, and immune responses [7, 12]. Here we provide a brief overview of sirtuin signaling with a focus on inflammation and fibrosis that are the main drivers of morbidity and mortality in tuberculosis (TB). We further discuss how sirtuin circuits can be harnessed by host-directed therapies (HDTs) to accelerate sterilization of TB and promote the non-fibrotic resolution of pulmonary TB disease.

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The Sirtuin Family

The sirtuin family of proteins were originally characterized in *Saccharomyces cerevisiae* as the yeast silent information regulator 2 (Sir2) [4, 13]. The *SIR2* gene was shown to be a transcriptional silencer of mating-type loci in budding yeast, yeast telomers, and ribosomal RNA [13–15], where it could extend lifespan by repressing genome instability [13, 16]. Later, Sir2 was shown to be a type III NAD^+-dependent histone deacetylase and to have ADP-ribosyltransferase activity [17, 18]. Since the discovery of *SIR2* in yeast, the gene was found to be a member of a large family of conserved genes present in multiple organisms including *Caenorhabditis elegans*, *Drosophila,* and mammals [13]. Importantly, the *Drosophila* and *C. elegans* Sir2 orthologs induced by calorie restriction also regulate health and longevity [19–22].

In mammals, the sirtuin family has seven members (Sirt1-7) subdivided into four classes (Class I-IV), which are categorized by their highly conserved NAD^+-binding and catalytic domain (the sirtuin core) [23]. Despite their conserved NAD^+ binding domain, sirtuins differ by their N and C termini, subcellular localization, enzymatic activity, and deacetylase targets (Table 2.1). Although the yeast Sir2 protein was originally described as a type III NAD^+-dependent histone deacetylase [17], mammalian sirtuins deacetylate a range of histones and non-histone proteins, thereby regulating the activity of multiple cellular pathways including central metabolic pathways and inflammation [4, 13]. Since sirtuins deacetylate histone proteins, they belong to class III histone deacetylases (HDACs) [24]. The main enzymatic reaction of sirtuins involves deacetylation at lysine residues and hydrolysis of NAD^+, producing nicotinamide adenine mononucleotide (NAM) [13, 17]. NAD^+, a regulator of sirtuin activity, is a key metabolite in multiple cellular pathways, and cellular pools of NAD^+ are tightly regulated in response to stresses such as caloric restriction [1, 25–28]. In addition to the levels of cellular NAD^+, sirtuins are also regulated by NAM through non-competitive inhibition. NAM is recycled back to NAD^+ by the action of the rate-limiting enzyme nicotinamide adenine mononucleotide phosphoribosyltransferase (Nampt), which is responsive to cellular stress and nutrient availability, and is also a regulator of sirtuins, particularly Sirt1 [26, 29, 30]. Although mammalian sirtuins are protein deacetylases, some members have the capacity to remove other post-translation protein modifications, including malonyl and succinyl moieties [31, 32]. The enzymatic reaction, protein targets, and physiological function of each mammalian sirtuin are highlighted in Table 2.1.

Sirtuins, Mitochondria, and Inflammation

Inflammation is a biological response important for the maintenance of cellular homeostasis and pathogen eradication, and plays an important role in diseases. An excessive inflammatory response, however, have been shown to be detrimental to the host and contributes to tissue injury [33]. The inflammatory response links

Table 2.1 Physiological function and subcellular localization of mammalian sirtuins

Sirtuin	Activity	Localization	Targets	Physiological function	References
Sirt1	Deacetylation	Nucleus, Cytosol	FOXO1, FOXO3, PGC1α, NF-κB, HIF-1α, LXR, SREBP1c, FXR	Metabolism, cell stress, inflammation,	[4, 13, 25, 40, 92]
Sirt2	Deacetylation	Cytosol, Nucleus	FOXO1, p65, HIF-1α,Tubulin, PEPCK	Genome integrity, cell cycle progression inflammation, neurological disorders	[4, 13, 93, 94]
Sirt3	Deacetylation	Mitochondria	LCAD, IDH2, HMGCS2, GDH, SOD2, AceCS2, OXPHOS subunits, others	Metabolism, mitochondrial function, oxidative stress	[4, 13, 95–98]
Sirt4	ADP-ribosylation, Deacetylase, Lipoamidase	Mitochondria	GDH, MCD	Fatty acid oxidation, insulin secretion	[99–102]
Sirt5	Deacetylation, Demalonylation, Desuccinylation	Mitochondria	CPS1, HMGCS2	Urea cycle, Fatty acid metabolism	[103, 104]
Sirt6	Deacetylation, ADP-ribosylation	Nucleus	H3K9, H3K56	Glucose metabolism, Inflammation	[4, 49, 105]
Sirt7	Deacetylase Desuccinylation	Nucleolus	RNA pol I	Genome integrity	[4, 13, 106]

immune pathways to metabolic and mitochondrial bioenergetic pathways, all of which are regulated, at least in part, by sirtuins [34]. Sirtuins, particularly Sirt1, Sirt3, Sirt4, and Sirt6, sense and respond to cellular nutrient changes and NAD^+ availability, resulting in cellular metabolic reprogramming and mitochondrial adaptations that are coupled to cellular responses under stress [35]. In monocytes, Sirt1 and Sirt6 play a critical role in switching the inflammatory response from the acute to adaptive phase, a process involving inactivation of Nuclear Factor-κB (NF-κB) RelA/p65 and activation of NF-κB RelB, transcription factors that function as master regulators of immune responses in macrophages (Mϕ) [36–38]. Sirt1 deacetylates lysine 310 of RelA/p65 and deacetylates histone 1 lysine 27 (H1K27), allowing for recruitment of RelB to the promoters of inflammatory genes [36, 39, 40]. During chronic inflammation, when Sirt1 levels are decreased, NF-κB signaling (most likely due to RelA/p65 hyperacetylation) is increased, resulting in a hyperinflammatory state [41–43]. Mice with myeloid-specific *SIRT1* deletion showed dysregulated NF-κB signaling and uncontrolled cytokine production when stimulated with various stimuli including bacterial endotoxin and dietary lipids [41]. In line with

these observations, myeloid-specific *SIRT1*$^{-/-}$ mice were found to be susceptible to *Listeria monocytogenes* [44] and *Mycobacterium tuberculosis* (*Mtb*) [42]. Conversely, *SIRT1* transgenic mice are protected against inflammatory response induced by diabetes or high-fat feeding [45–47], indicating that Sirt1 is an important bridge linking cell metabolism, immune response, and inflammation.

Sirt6 works in coordination with Sirt1 to modulate NF-κB activity and regulate immune responses by interacting with RelA/p65 and deacetylating histone 3 lysine 9 (H3K9) at NF-κB target gene promoters, which decreases RelA/p65 promoter occupancy at target genes [35, 41, 48]. Sirt1 and Sirt6 also integrate glycolysis in monocytes with acute inflammatory responses by altering hypoxia-inducible factor (HIF)-1α levels/stability, which stimulates genes involved in glycolysis [12, 35, 49, 50] (Fig. 2.1). Moreover, HIF-1α is destabilized and inactivated through direct binding and deacetylation by Sirt1 [50] and Sirt2 [51]. In addition, Sirt1 epigenetically regulates HIF-1α by deacetylating histone 3 lysine 14 (H3K14) at the promoter of

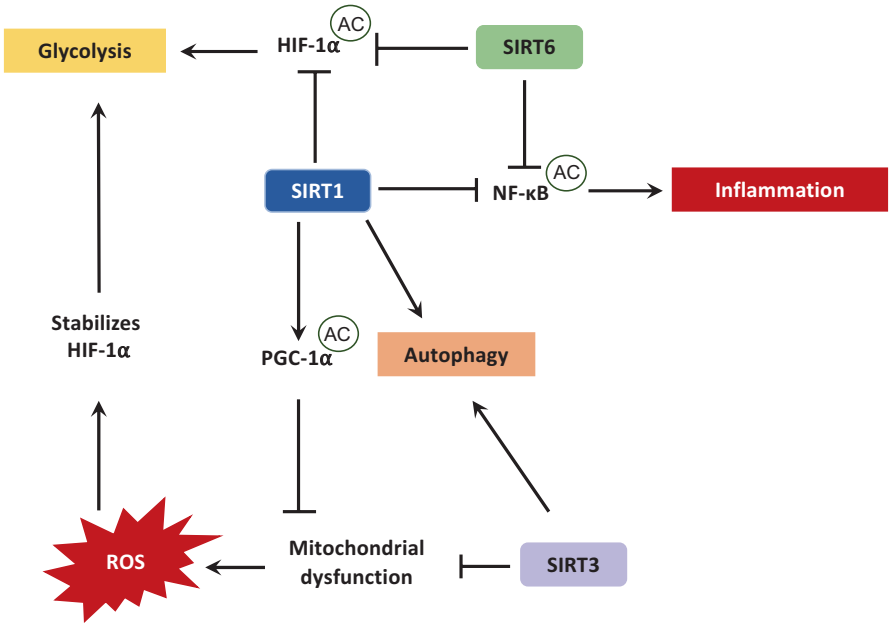

Fig. 2.1 Role of sirtuins in regulating inflammation, autophagy and glycolysis. Sirt1, Sirt3 and Sirt6 regulate cellular pathways involved in the immune response, metabolism, and mitochondrial function, all of which are intrinsically linked. The inflammatory response and glycolysis are negatively regulated by Sirt1 and Sirt6 through their deacetylation activities on Nuclear Factor (NF)-κB and hypoxia-inducible factor-1 alpha (HIF-1α). Sirt1, by regulating the activity of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and Sirt3, together maintains mitochondrial function and autophagy. Mitochondrial perturbation in turn leads to the production of reactive oxygen species (ROS), which stabilizes HIF-1α and further activates the glycolytic fate of the cell. Under stress, such as from *Mtb* infection, sirtuin activity is compromised resulting in mitochondrial dysfunction and enhanced inflammation and glycolytic activity. By targeting sirtuins, host cell metabolic activity could be restored

the *HIF1A* gene, which results in reduced transcription of HIF-1α [52]. Also, the mitochondrial sirtuin Sirt3 has been shown to metabolically reprogram tumor cells by destabilizing and inactivating HIF-1α [53]. However, unlike Sirt1, which directly binds and deacetylates HIF-1α [50], Sirt3 indirectly destabilizes HIF-1α through Sirt3-mediated suppression of reactive oxygen species (ROS) [53, 54] (Fig. 2.1).

Linked to their role in cellular metabolism, mitochondria are important in proinflammatory signaling [55]. Mitochondria are the site of cellular ATP production and for signaling pathways such as production of ROS, mitophagy and mitochondrial DNA repair, which not only regulate mitochondrial homeostasis but modulate various cellular processes [56]. Mitochondrial ROS (mtROS) can also provoke mitochondrial dysfunction leading to cellular stress, inflammation, and injury [55–57]. Sirt3 mediates mitochondrial protective effects, which are altered in experimental models of inflammation [58–60]. *SIRT3*$^{-/-}$ mice exhibit enhanced inflammatory response to endotoxin challenge, which is associated with increased production of proinflammatory cytokines and mtROS; these responses can be ameliorated with pharmacological activation of Sirt3 [58]. In addition, Sirt3 promotes defense against *Mtb* infection, in part due to its role in maintaining mitochondrial function [60]. Sirt4, another mitochondrial sirtuin, is also important in the host metabolic response to inflammation as it promotes the resolution of acute inflammation in endotoxin-treated human monocytes by coordinately reprogramming cellular metabolism and bioenergetics [61]. Sirt4 also suppresses the inflammatory response in human endothelial cells by preventing the nuclear translocation of NF-κB, thereby interfering with NF-κB signaling [62, 63]. Altogether, Sirt1, Sirt3, Sirt4, and Sirt6 work in conjunction to regulate the inflammatory response by integrating mitochondrial bioenergetics with Mϕ metabolic reprogramming (Fig. 2.1). Thus, sirtuin signaling is an attractive target for the next generation of TB HDTs.

Sirtuins and Fibrosis

Fibrosis (scar formation due to excess collagen deposition) is a possible outcome of resolving inflammation. It becomes detrimental to the host when it impairs vital organs, as is the case for pulmonary or pericardial fibrosis after TB. Pulmonary fibrosis (PF) results from the accumulation of fibroblasts, leading to enhanced extracellular matrix deposition. Furthermore, damage of alveolar epithelial cells may result in decreased epithelial function and epithelial to mesenchymal transition, a biological process in which the epithelial nature of alveolar epithelial cells is lost [64, 65]. Sirtuins have been shown to play a complex role in the progression of PF [64, 66–68]. Sirt1, Sirt3, and Sirt7 are attenuated in murine models of PF as well as in lung tissue from patients with idiopathic and systemic sclerosis-associated PF, particularly within the fibrotic regions of the lungs [66, 67]. Activation of Sirt1 by the natural activator resveratrol attenuates lung fibroblast differentiation and diminishes the severity of experimental lung fibrosis by modulating the TGF-β/p300

fibrotic signaling cascade [56, 64, 69, 70]. Like Sirt1, Sirt7 exerts its antifibrotic effects by regulating TGF-β fibrotic signaling, particularly by regulating levels of Smad3, the downstream mediator of TGF-β receptor signaling. Sirt7 overexpression reduces Smad3, while attenuating Sirt7 expression increases Smad3 and enhances fibrogenesis [64, 66]. Sirt3 exerts its antifibrotic effects by maintaining mitochondrial ROS and cellular redox homeostasis, which is important for fibroblast homeostasis [58, 67]. In addition to Sirt1, Sirt3, and Sirt7, Sirt6 can also modulate cardiac [71] and liver fibrosis [72]. Additionally Sirt6 counteracts the development of PF by altering the epithelial to mesenchymal transition by targeting fibrotic signaling pathways involving Smad3 [64, 73]. Overall, sirtuins play an important role in diverse fibrotic responses, suggesting their potential value as targets for anti-fibrotic therapies.

Crosstalk of Sirtuins and Mycobacterial Infection

Mycobacterial infection triggeres a complex interplay between the host immune response and *Mtb* pathogenic mechanisms. Since immune response and metabolic programs are intrinsically linked [74], targeting metabolism in chronic conditions such as cancer, uncontrolled inflammation, and chronic infections such as TB have been proposed [1, 75]. A comparative transcriptome analysis of Mϕ infected with various bacterial species, including *Mtb*, showed an upregulation of a core genes associated with a classically activated Mϕ signature, correlating with a general metabolic reprograming such as increased glycolytic flux and decreased oxidative phosphorylation, which illustrates the intimate link between metabolic fate and function of immune cells during infection [76–78]. It has been shown that *Mtb* infection perturbs sirtuin activity in Mϕ [42, 60]. This could be due to the depletion of cellular NAD^+ pools, possibly by a secreted *Mtb* toxin that promotes cell death [79]. In addition to reducing NAD^+ levels, *Mtb* infection reduces expression of *SIRT1* mRNA and protein, an effect that is associated with exacerbated immunopathology [42]. Pharmacological activation of Sirt1 inhibits intracellular growth of *Mtb* by stimulating autophagy and reduces chronic inflammation by deacetylating RelA/p65 [42]. Evidence that *SIRT1* mRNA expression is reduced in blood of TB patients and increases following anti-TB therapy [42] supports a role for Sirt1 in the host response to TB.

Sirt3 is also important for the host defense against *Mtb*, as it coordinates mitochondrial function and autophagy that are required for an appropriate antimycobacterial response [60]. Infection of Sirt3 null mice with *Mtb* results in greater inflammation, mitochondrial damage, and oxidative stress compared to wildtype [60]. Pharmacological activation of Sirt3 in *Mtb*-infected wildtype macrophages restored host mitochondrial function, reduced cellular oxidative stress and promoted autophagy [60]. Both Sirt1 and Sirt3 are important regulators of autophagy, a key cellular defense against intracellular pathogens [42, 80, 81]. Sirt1 deacetylates LC3 (central protein in autophagy pathway and autophagosome biogenesis) at K49

and K51 [82], while Sirt3 promotes autophagy through mechanisms including regulation of Peroxisome Proliferator-Activated Receptor alpha (PPARα), a transcriptional regulator of autophagy and inflammation [83, 84]. Furthermore, similar to *SIRT1*, *SIRT3* mRNA expression is decreased in peripheral blood mononuclear cells of TB patients [60]. The host-protective role of Sirt3 against infection appears to be restricted to TB since *SIRT3*$^{-/-}$ mice do not differ from wild type mice in terms of cytokine production, bacterial burden, and survival, when subjected to endotoxemia, *Escherichia coli* peritonitis, *Klebsiella pneumoniae* pneumonia, listeriosis, or candidiasis [85].

Although Sirt1 and Sirt3 mediate protective effects against *Mtb* infection, no such phenotype was identified in *Mtb*-infected mice with myeloid-specific *SIRT2* deletion [86]. However, Sirt2 seems to play a detrimental role against other bacterial infections such as those caused by *Listeria monocytogenes*, *Helicobacter pylori* and *Staphylococcus spp.* [87–89]. During *L. monocytogenes* infection, Sirt2 deacetylates histone 3 lysine 18 (H3K18) thereby up-regulating genes required for infection; moreover, *SIRT2*$^{-/-}$ mice showed impaired *L. monocytogenes* growth, relative to wild type mice [87, 90]. Although the potential role of Sirt5 in TB progression has yet to be analyzed, Sirt5 does not impact the innate immune defense against endotoxemia, *Escherichia coli* peritonitis, *Streptococcus pneumoniae* pneumonia, *Klebsiella pneumoniae*, or listeriosis [91].

Conclusions and Perspectives

Host metabolic programs and immune response are inextricably linked, and it is becoming clear that immuno-metabolic circuits are particularly important in chronic infectious and non-infectious inflammatory diseases. Sirtuins, which are regulated in a tissue, cell and organelle-specific manner, are important arbiters of these circuits. Their expression and localization are regulated by proteins that themselves are sirtuin substrates, suggesting complex feedback network(s) in the crosstalk between inflammatory response, cellular metabolism, and mitochondrial bioenergetics. Targeting sirtuins may have the capability to restore the balance towards protective immunity by reducing pathological inflammation and enhancing host response. Available evidence suggest the potential for Sirt1 and Sirt3 agonists for HDT against TB. In contrast to some immunosuppressive HDT candidates, sirtuin agonists have the potentially unique capacity to directly modulate tissue-damaging inflammation and suppress fibrosis during resolution, while also promoting antibacterial effector function. Given the multiplicity of targets and pathways regulated by sirtuins, additional preclinical studies are needed to clarify their mechanism(s) of protective efficacy and to identify compounds with high activity and bioavailability before making the next step to clinical trials in TB.

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Chapter 3
The Mammalian Target of Rapamycin Complex 1 (mTORC1): An Ally of *M. tuberculosis* in Host Cells

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The mTOR-Containing Complexes

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) is a conserved member of the phosphoinositide 3-kinase (PI3K)-related kinase family. It is the key component of two distinct multi-subunit complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Besides mTOR, the catalytic subunit, the two complexes share two additional subunits: the mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL), which stabilizes mTOR, and the DEP-domain-containing mTOR-interacting protein (Deptor), which inhibits mTOR. Moreover, mTORC1 is characterized by the presence of the regulatory-associated protein of mTOR (Raptor, also known as RPTOR), which is involved in mTORC1 localization and substrate recruitment, and the proline-rich AKT substrate of 40 kDa (PRAS40, also known as AKT1S1), an insulin-responsive mTORC1 inhibitor. mTORC2 contains mSIN1, Protor, and the rapamycin-insensitive companion of mTOR (Rictor), a protein that may have analogous function to Raptor. Among the most recent reviews on the structure and function of the two complexes, the reader is referred to [1]. The primary functions of mTORC1 and mTORC2 are distinct: mTORC1 controls cell growth, while mTORC2 controls cell survival and proliferation [2]. Here, we focus on mTORC1, which has been studied in the context of tuberculosis pathogenesis.

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mTORC1 Regulation

mTORC1 senses growth factors, amino acids, energy status, stress, and oxygen levels to regulate many biological processes required for cell growth and proliferation. Activation of mTORC1 requires two distinct signaling pathways. The first, which is initiated by growth factors and cellular stress signals, ultimately activates the small GTPase Rheb by regulating its nucleotide state. When Rheb is bound to GTP (as opposed to GDP), it resides on the lysosomal membrane and directly stimulates mTOR kinase activity by inducing conformational changes [3] and by favoring mTOR phosphorylation by upstream kinases [4]. Phosphorylated mTOR catalyzes subsequent phosphorylation of itself and other components of the mTORC1 complex [4]. Moreover, in order to interact with Rheb, mTORC1 must be recruited to the lysosomal membrane by the Rag GTPases. These heterodimeric enzymes, which are composed of RagA or RagB and RagC or RagD, are competent to recruit mTORC1 only when RagA or RagB is bound to GTP and RagC or RagD is bound to GDP. The nucleotide state of the Rags is regulated by a second signaling pathway that is initiated by nutrients, such as amino acids and glucose. Thus, mTORC1 is activated only when both growth factors and nutrients are present, leading to the colocalization of the mTORC1 recruitment complex and an mTORC1 activator at the lysosomal surface. The requirement for coordinated intracellular and extracellular cues ensures that mTORC1 is activated only when resources are plentiful and cellular growth can be sustained. A detailed description of nutrient and growth factor signaling to mTORC1 can be found in recent reviews [5, 6].

The Functions of mTORC1

The activation of mTOR is key to achieve cell growth and proliferation. To do so, mTORC1 initiates a cascade of events supporting anabolism and blocking catabolic processes (reviewed in [7–9]). The anabolic program includes enhancing the synthesis of proteins, lipids, nucleotides, and other macromolecules. The catabolic processes blocked by mTORC1 are (i) autophagy, a mechanism of protein degradation aimed at restoring the lysosomal pool of amino acids, and (ii) lysosome biogenesis. Thus, mTORC1 controls autophagy flux and the ability of the lysosome to degrade cellular constituents.

Protein synthesis is promoted by mTORC1 through the direct phosphorylation of two of its major targets, the translational regulators, eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1). Phosphorylated 4E-BP1 cannot bind eIF4E, which enables it to form the complex required for the initiation of cap-dependent translation. Moreover, phosphorylated (active) S6K1 controls various downstream effectors, leading to increased ribosome and mRNA biogenesis, and translational initiation and elongation. In addition of transcription by RNA polymerase (Pol) II, mTORC1 also

induces Pol I activation and ribosomal RNA (rRNA) production through S6K1, and releases Pol III from inhibition, thereby promoting transcription of 5S rRNA and transfer RNA (tRNA).

Lipid biosynthesis is regulated by mTORC1 through several mechanisms (reviewed in [10]). One is the induction of the sterol regulatory element-binding protein 1/2 (SREBP1/2) transcription factors that control the expression of genes involved in fatty acid and cholesterol synthesis. SREBP1/2 induction by mTORC1 occurs at multiple levels (expression, processing, and translocation to the nucleus), some of which are controlled through S6K1 (see also [11]). In addition, mTORC1 phosphorylates Lipin-1, a phosphatidic acid phosphatase, and prevents its nuclear entry and negative regulation of SREBP1/2 abundance [12]. Moreover, mTORC1 utilizes multiple mechanisms, some of which remain to be elucidated, to increase expression and activity of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor that controls the expression of genes required for fatty acid synthesis, uptake, and esterification (reviewed in [13]). Furthermore, S6K2, an effector of mTORC1 that is highly homologous to S6K1, suppresses PPARα, a nuclear receptor that induces fatty acid oxidation and ketogenesis [14], by inducing the nuclear localization of nuclear corepressor 1 (NCoR1).

mTORC1 also regulates the nucleotide pool, which is critical for DNA replication and rRNA biosynthesis in proliferating cells. *De novo* purine biosynthesis is driven by mTORC1 through the direct activation of activating transcription factor 4 (ATF4), which induces expression of key genes in purine biosynthesis and supporting pathways such as the pentose phosphate pathway [15], while pyrimidine biosynthesis is promoted through S6K1-mediated phosphorylation of key enzymes in the pathway [16, 17].

Since the mechanisms it induces consume energy, mTORC1 positively regulates energy metabolism in multiple ways (reviewed in [8]). It increases transcription and translation of the transcription factor hypoxia inducible factor 1α (HIF1α) (reviewed in [7]), which reprograms cellular energy metabolism from oxidative phosphorylation toward glycolysis [18]. Moreover, mTORC1 increases mitochondrial oxidative function to maintain energy homeostasis in response to nutrient and hormonal signals [8], in part by driving the formation of a complex between PPARγ-coactivator 1a (PGC1a) and the transcription factor Ying-Yang 1 (YY1), which positively regulates mitochondrial biogenesis and oxidative functions [19]. In addition, mTORC1 enhances translation of nuclear-encoded mitochondrial genes through 4E-BP to expand the ATP production capacity of the cell [20].

A critical component of mTORC1's role in promoting cellular growth and proliferation lies in its ability to shut down catabolic processes. Two intertwined catabolic processes, lysosomal biogenesis and autophagy, are regulated by mTORC1. Autophagy is the major intracellular degradation system by which cytoplasmic materials are delivered to and degraded in the lysosome [21]. The lysosome is the primary site of the breakdown of proteins, polysaccharides, and complex lipids into building blocks that can be utilized for cellular growth [22]. These functions, together, serve as a dynamic recycling system to produce new building blocks and energy for cellular renovation and homeostasis. When mTORC1 is inactive,

non-phosphorylated microphthalmia-associated transcription factor (MiTF), transcription factor EB (TFEB), and the related transcription factor E3 (TFE3) can translocate to the nucleus and activate genes related to lysosomal biogenesis and autophagy (reviewed in [5, 9]). Newly formed lysosomes then break down proteins and release constituent monomers back to the cytoplasm to regenerate the pool of cellular amino acids, leading to reactivation of the mTORC1 pathway and its growth-promoting functions. This feedback loop between mTORC1 and the lysosome is crucial for metabolic homeostasis and cell survival [5]. Indeed, the very localization of mTORC1 activation at the surface of the lysosome is a testament to the central importance of the mTORC1-lysosome link [5, 6]. In addition, mTORC1 phosphorylates and inhibits two key autophagy initiators, unc-51-like autophagy-activating kinase 1 (ULK1) and autophagy-related protein 13 (ATG13) [5, 6, 9], which are required for autophagosome formation [21]. mTORC1 also blocks autophagosome and endosome maturation by inhibiting the interaction of its target UV radiation resistance-associated gene (UVRAG) protein with the homotypic fusion and protein sorting (HOPS) complex (reviewed in [9]), which assist in trafficking, autophagosome-lysosome fusion, and Rab7 activation [21, 23]. Thus, mTORC1 negatively regulates both early and late stages of autophagy.

mTORC1 and Tuberculosis

mTORC1 Regulation of Immune Cells

M. tuberculosis positively regulates the mTORC1 pathway in immune cells. For example, activation of mTORC1 in alveolar macrophages [24], and mTORC1 pathway upregulation have been observed in human tuberculous lungs [25]. Furthermore, human primary macrophages infected in vitro with *M. tuberculosis* show increased phosphorylation of mTORC1 and its targets S6K and 4EBP-1 [25, 26]. The signals activating mTORC1 during *M. tuberculosis* infection are understudied. Various extracellular signals might contribute to mTORC1 activation in immune cells during *M. tuberculosis* infection, including growth factors, Toll-like receptor (TLR) ligands, cytokines, and lipid mediators of inflammation [27]. For example, blocking the biological action of TNFα by using anti-TNF receptor-blocking antibodies decreases mTORC1 activation status in macrophages infected in vitro [25]. Except for TNFα, the role of signaling molecules in mTORC1 activation in the context of tuberculosis is poorly understood.

Once activated, mTORC1 participates in the regulation of metabolic and immune functions of immune cells during *M. tuberculosis* infection.

Autophagy Autophagy has an important role during *M. tuberculosis* infection, since its induction by physiological or pharmacological stimuli [28, 29] positively regulates several protective immune functions. These include: (i) maturation and

acidification of *M. tuberculosis*-containing phagosomes, which counter the *M. tuberculosis*-mediated block of phagosome-lysosome fusion [30–33]; (ii) release of antigenic fragments from lysosome-degraded bacteria and antigen presentation [34]; and (iii) control of inflammatory response to avoid excess inflammation [35, 36] and promote bacterial clearance [37]. The protective effects of autophagy in tuberculosis are further supported by the observation that transgenic autophagy-defective mice infected with *M. tuberculosis* exhibit higher bacillary burden and more severe lung pathology than wild-type animals [30].

M. tuberculosis infection results in inhibition of the autophagic flux [38–40], and some mycobacterial factors express anti-autophagic activity [41–43]. Inhibition of autophagy by *M. tuberculosis* infection occurs concurrently with mTORC1 activation, suggesting the possibility that mycobacteria activate mTORC1 to block or slow down autophagy [44]. Indeed, nutrient starvation (which leads to mTORC1 inactivation) and treatment with rapamycin (a chemical inhibitor of mTORC1) reduces *M. tuberculosis* growth in macrophages [30].

Foam cell formation Foam cells form through dysregulated lipid metabolism in mammalian macrophages. When lipid content exceeds the homeostatic capacity of macrophages, the excess lipids are stored in quasi-organelles called lipid droplets. Lipid droplet accumulation results in the foamy appearance of the macrophage [45]. Foam cells are associated with chronic inflammation in metabolic, infectious, and autoimmune diseases, and in certain cancers [46]. Formation of foam cells typically impairs macrophage immune function and contributes to pathogenesis [47]. In tuberculosis, caseous lung granulomas are characterized by aggregates of various immune cells distributed around a lipid-rich necrotic center. Foam cells are present in the areas immediately adjacent to the necrotic core, and the proportion of foam cells correlates with the extent of lesion necrosis and tissue damage. Moreover, the chronic and nonresolving inflammatory state associated with foam cell accumulation and death generates further tissue damage. Damage of the lung parenchyma results in loss of pulmonary function, while granuloma liquefaction and cavitation into the airway ultimately leads to the release of extracellular bacilli into the external environment and transmission of infection [46]. Thus, foam cell formation contributes to worsening of the histopathology and disease outcome.

Foam cells in tuberculous lung lesions and primary macrophages infected *ex vivo* with *M. tuberculosis* accumulate triglycerides. In vitro mechanistic studies by Guerrini et al. have shown that triglyceride accumulation in human macrophages infected with *M. tuberculosis* is mediated by TNF receptor signaling through downstream activation of the caspase cascade and mTORC1 [25]. While the mTORC1 targets responsible for tuberculous foam cell formation are presently unknown, several cellular functions regulated by mTORC1 might contribute to triglyceride accumulation. These include PPARɣ and SREBP-1c [75, 76], which induce lipogenesis and triglyceride biosynthesis, as described above. Additionally, mTORC1 may increase cellular lipid content by inhibiting autophagy [78, 79], which promotes lipid catabolism by delivering triglycerides stored in lipid droplets to lysosomes

(a mechanism called lipophagy) [80]. mTORC1-dependent block of autophagy can also activate the caspase cascade through accumulation of p62, which binds and activates caspase 8. Caspase activation induces mitochondrial dysfunction [76], which is associated with reduced fatty acid utilization and consequent lipid accumulation [77].

Central carbon metabolism A metabolic shift to aerobic glycolysis (the Warburg effect) has been observed during *M. tuberculosis* infection [48]. In a murine model of tuberculosis, transcriptomic analyses of infected lungs revealed gene expression changes that were consistent with the Warburg effect [49]. These included increased expression of genes encoding glucose transporters and glycolytic enzymes, and downregulation of genes involved in the TCA cycle and oxidative phosphorylation (OXPHOS). In addition, immunohistochemistry showed increased abundance of glycolytic enzymes in macrophages and T cells in lung granulomas from the same animals [49]. Consistent with these observations, accumulation of lactate, the product of glycolysis, was observed in *M. tuberculosis*-infected mouse lungs by metabolomics [50]. Gene expression changes consistent with the Warburg effect were also reported in tuberculous rabbits [51] and in lung granulomas of individuals with active tuberculosis [52].

A few mechanistic and functional studies have been performed on aerobic glycolysis in immune cells during *M. tuberculosis* infection. Induction of aerobic glycolysis has been reported with several types of macrophages infected with *M. tuberculosis* in vitro [26, 53]. Using human peripheral blood mononuclear cells (PBMC), it has been shown that the *M. tuberculosis*-induced shift toward aerobic glycolysis occurs in a TLR2-dependent and NOD2-independent manner [26]. The same study also showed that this metabolic reprogramming is mediated in part through activation of Protein kinase B (AKT)-mTORC1 pathway in *M. tuberculosis*-infected human and mouse macrophages [26]. Moreover, since HIF-1α expression is increased in tuberculous lesions in mouse lungs [49], it has been proposed that HIF-1α is a key regulator of the metabolic reprogramming occurring in immune cells during *M. tuberculosis* infection [26, 49]. Thus, mTORC1 is implicated in the metabolic remodeling of immune cells during tuberculosis.

The metabolic rewiring of immune cells induced by *M. tuberculosis* infection has functional implications, as it has been associated with a proinflammatory macrophage phenotype and infection outcome [26, 48, 53]. Inhibition of the metabolic shift from OXPHOS to aerobic glycolysis by 2-deoxyglucose resulted in decreased levels of the pro-inflammatory cytokine IL-1β, which is required for host resistance to *M. tuberculosis* [54], and increased levels of the anti-inflammatory cytokine IL-10 in *M. tuberculosis*-infected macrophages [53]. Consistent with these data, the expression of genes encoding glycolysis or TCA cycle enzymes in PBMC differed among healthy donors, latently infected individuals, and active tuberculosis patients [26]. Murine studies and in vitro mechanistic experiments with murine macrophages showed that the HIF-1α-dependent metabolic shift toward glycolysis constitutes a defense mechanism of macrophages during *M. tuberculosis* infection [55]. Furthermore, studies in zebrafish infected with *Mycobacterium marinum*

(a fish model of tuberculosis) also demonstrated a role for HIF-1α in antimycobacterial responses, leading to the proposal of stabilization of HIF-1α as a potential target for therapeutic intervention against tuberculosis [56].

Further insight into the relationship between the metabolic and functional state of macrophages and infection outcome derives from studies utilizing various *M. tuberculosis* strains. Transcriptomic analysis of murine bone marrow-derived macrophages (BMDM) infected with the CDC1551 and HN878 strains of *M. tuberculosis* revealed that, while a similar set of Warburg effect-associated genes was induced by both strains, macrophages infected with the CDC1551 strain were characterized by high glycolytic flux and high expression of inflammatory and antimicrobial effector molecules, while macrophages infected with the HN878 strain had elevated glucose uptake and lower glycolytic flux [57]. Since these strains induce differential immune responses in BMDM [57], a link may exist between host metabolic state and immune response.

mTORC1 and BCG

In addition to its regulatory function of immune cells during *M. tuberculosis* infection, mTORC1 has been implicated in "trained immunity", i.e., the long-term functional reprogramming of innate immune cells (myeloid cells and natural killer cells) that is evoked by a certain stimulus (e.g., exposure to β-glucan from fungi) and increases responsiveness to subsequent, unrelated stimuli [58, 59]. The protective and long-lasting effects of the vaccine strain *Mycobacterium bovis* Calmette Guerin (BCG) against tuberculosis in murine models of infection [60] and the non-specific beneficial effects of BCG administration against certain forms of malignancy and infections with unrelated pathogens [61–68] have been attributed to trained immunity.

At least two tightly intertwined mechanisms have been associated with trained immunity [59, 69–71]; one is epigenetic reprogramming [70], and the second is aerobic glycolysis [59]. mTORC1 has been involved in both. For example, the AKT-mTORC1-HIF-1α signaling pathway is required for increased aerobic glycolysis in β-glucan–trained monocytes [59]. Furthermore, mice lacking myeloid-specific HIF-1α, a major target of mTORC1, were unable to express trained immunity in response to sepsis [59]. With respect to BCG-induced trained immunity, BCG induction of trained immunity in human and murine monocytes is accompanied by increased glycolysis and glutamine metabolism, and required activation of the AKT-mTORC1 pathway [72]. Furthermore, histone marks of gene activation were found at the promoters of mTOR and key glycolytic genes [72], linking metabolic and epigenetic changes.

The mTORC1 pathway has also been associated with the efficacy of BCG vaccination via mechanisms other than trained immunity. For example, autophagy induced by rapamycin (an mTORC1 inhibitor) enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells [73].

mTORC1 and HDT Against Tuberculosis

Overall, in tuberculosis, activation of mTORC1 is beneficial for the pathogen and detrimental to the host, since both mTORC1-mediated inhibition of autophagy and lipid accumulation promote intracellular bacterial survival. Consequently, one might propose inhibition of mTORC1 as a host-directed therapeutic approach. This may be achieved in multiple ways. One is the direct inhibition of mTORC1 with FDA-approved mTOR inhibitors such as rapamycin (sirolimus) and rapamycin derivatives such as everolimus, which decrease *M. tuberculosis* load of macrophages [30, 73]. Another approach is the use of activators of AMP-activated protein kinase (AMPK), which inhibits mTORC1 [74–76]. These drugs include, for example, metformin, which is also FDA-approved (see below). A third approach is based on the recent work of Bruiners et al. [77] showing that mTORC1 activation and mTORC1-mediated inhibition of autophagy can be dampened by drugs, such as statins, that reduce the intracellular levels of cholesterol. This effect is explained by mechanistic links between cholesterol trafficking and mTORC1 activation, which both occur at the lysosome [78, 79] and by AMPK activation by statins. As a result, statins are anti-mycobacterial [80, 81]. Thus, mTORC1 can be inhibited in multiple ways to obtain anti-mycobacterial effects.

The choice of mTORC1 as an HDT target for tuberculosis treatment, however, is not straightforward. In addition to its "pro-bacterial" effects through autophagy inhibition and induction of triglyceride accumulation in macrophages, mTORC1 also favors the host since mTORC1-induced metabolic remodeling is necessary to control *M. tuberculosis* infection. The contrasting effects of mTORC1 on the macrophage antimicrobial capability, which can be explained by the effects of the activation of various mTORC1 targets on macrophage phenotype, might underlie, at least in part, the macrophage heterogeneity observed in tuberculous granulomas. A summary of the effects of mTORC1 signaling during *M. tuberculosis* infection and the main HDT approaches targeting mTORC1 is shown in Fig. 3.1.

Additional considerations must be taken into account when tuberculosis is associated with a comorbidity, as the effects of mTORC1 inhibition may vary with the accompanying disease. One example is HIV, a major tuberculosis comorbidity (https://www.who.int/tb/areas-of-work/treatment/risk-factors/en/), since treatment with mTORC1 inhibitors of macrophages co-infected in vitro with *M. tuberculosis* and HIV results in increased *M. tuberculosis* growth [82]. Since it has been reported that rapamycin facilitates viral infections [83], it can be hypothesized that, mTORC1 inhibitors may favor HIV replication and, hence, the (direct or indirect) growth-promoting effects that HIV is known to have on *M. tuberculosis* [83]. In the case of diabetes mellitus, which is another major tuberculosis comorbidity and a risk factor for the establishment and outcome of tuberculosis [84, 85], treatment with metformin, an anti-diabetes drug that functions as an AMPK activator, is associated with reduced risk of developing active tuberculosis and with improved clinical outcomes [86–92]. Indeed, the concurrent effects of metformin on mTOR signaling and anti–*M. tuberculosis* responses [93] suggest that the antimicrobial effect of metformin may be due to mTORC1 inhibition. Another example is cancer, as treatment of various types of cancer with mTORC1 inhibitors have been

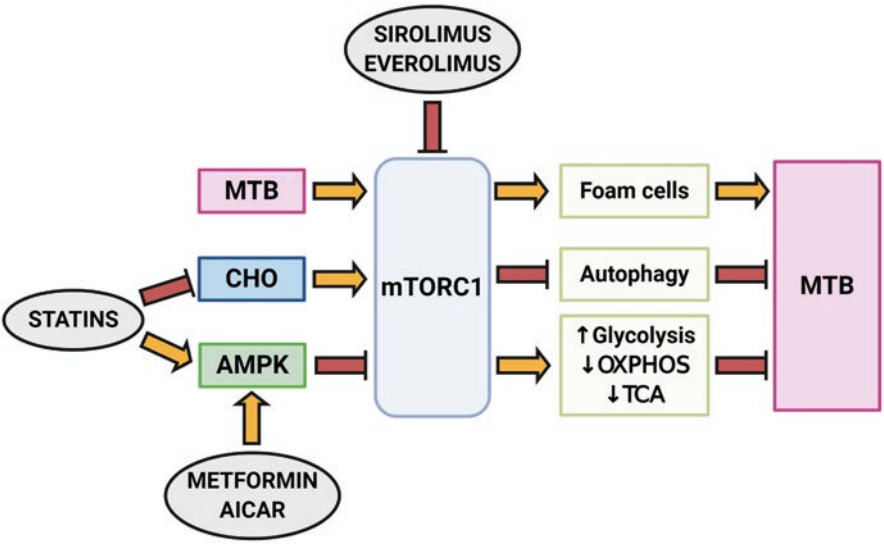

Fig. 3.1 Targeting mTORC1 for host-directed therapies against tuberculosis. *M. tuberculosis* infection induces activation of the mTORC1 signaling pathway. This results in: (1) formation of foam cells; (2) inhibition of autophagy; and (3) a shift in glucose metabolism favoring aerobic glycolysis over the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS). While most of the mTORC1-induced effects facilitate *M. tuberculosis* survival in the host cell, metabolic reprogramming of immune cells is implicated in the antimycobacterial response. AMPK and mTORC1 pathways inhibit each other to modulate autophagy, cellular metabolism, and immune responses during infection. Cellular cholesterol promotes mTORC1 recruitment from the cytosol to the lysosomal membrane, where mTORC1 triggers downstream effects favoring *M. tuberculosis* survival. Host-directed drugs (grey circles) inhibit mTORC1 activation during *M. tuberculosis* infection

reported to induce tuberculosis reactivation [94, 95]. Thus, in conclusion, the use of drugs targeting the mTORC1 pathway as new adjunctive host-directed therapies against tuberculosis must be carefully evaluated, particularly in the presence of comorbidities.

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Chapter 4
HIF-1α as a Potential Therapeutic Target for Tuberculosis Treatment

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Hypoxia-inducible factors (HIFs) represent a family of master regulators that respond to hypoxia in the cellular microenvironment. At the molecular level, HIFs are heterodimers comprising oxygen-sensitive α and -stable β subunits. In mammals, each subunit has three isoforms (HIF-1α, -2α, and -3α or -1β, -2β, and -3β). Among them, HIF-1α has been intensively investigated due to its diverse functions in cellular and tissue stress responses [8]. However, the mechanisms by which HIF-1α regulates cell immune responses and metabolism, and their implications for therapeutic intervention in various disease settings remain to be fully understood.

Structure of HIF-1α - the Basis of Function

HIF-1 was discovered as a hypoxia-inducible master transcription factor that operates ubiquitously in mammalian cells [117]. It is a heterodimeric complex with hypoxia-inducible DNA-binding activity consisting of a 120 kDa α subunit and a 91–94 kDa β subunit [118, 147, 148]. The latter, named HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed and non-inducible [143]. The HIF-1α subunit has two transactivation domains [101], an N-terminal transactivation domain (NTAD), and a C-terminal transactivation domain (CTAD). It also contains an intervening inhibitory oxygen-dependent degradation domain (ODDD) that localizes between the NTAD and CTAD. This ODDD can repress the transcriptional activities of the other domains [11, 51, 105].

The regulatory mechanism of HIF-1α is highly conserved. Under normal conditions, at least one of the two proline residues near the NTAD remains hydroxylated

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by members of the prolyl hydroxylase domain-containing enzyme (PHDs) family. Hydroxylation of HIF-1α exposes a binding site for the ubiquitin ligase complex pVHL, the product of the von Hippel Lindau (VHL) tumor suppressor gene [82]. Subsequently, HIF-1α is poly-ubiquitinated, followed by proteasomal degradation. Other than O_2, these hydroxylation reactions require cofactors such as α-ketoglutarate, ferrous iron, and ascorbate [116]. In addition, factor inhibiting HIF 1 (FIH1) hydroxylates the asparagine residue within the HIF-1α CTAD using coactivators such as p300 and CREB-binding protein (CBP), resulting in blockade of transcription complex formation [113]. In contrast, stress conditions, including hypoxia and inflammation, promote HIF-1α transcription and translation, protein stability, and/or transactivation [96]. Once the HIF-1α protein is stabilized, it heterodimerizes with HIF-1β via the basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) domains, thereby enabling DNA binding to targeted genes containing hypoxia-response-element (HRE) sequences in their promotors [152]. Through multiple pathways, HIF-1α activation contributes to pathogenesis of diseases such as cancer, diabetes, and tuberculosis [9, 80, 112, 139].

The Immunoregulatory Role of HIF-1α in Cancer and Other Diseases

The immunoregulatory role of HIF-1α is evidenced by its intensive crosstalk with the cellular inflammatory response and metabolism via multiple pathways, including: (1) activation of NF-κB signaling directly upregulates HIF-1α mRNA expression [32]; (2) NF-κB impairs the activity of PHD by reducing cytosolic free iron availability, leading to the inhibition of HIF-1α hydroxylation [127]; (3) other pro-inflammatory molecules, including NO, IFN-γ, IL-1β, IL-6, and TNF-α, can induce the transcriptional expression of HIF-1α mRNA, by either increasing reactive oxygen species or other HIF-1α-dependent pathways, such as ERK/MAPK, JAK/STAT, and PI3K/Akt/mTOR [41, 134, 136]; (4) accumulated tricarboxylic acid (TCA) cycle intermediates, such as succinate and fumarate, serve as important regulators of HIF-1α activation [98, 132, 137]; and (5) hormones (angiotensin II, insulin, thrombin) and growth factors, including platelet-derived growth factor, insulin-like growth factor 1 (IGF-1), IGF-2, TGF-β1, and IL-3, induce HIF-1α mRNA expression [30, 69, 154].

One of the characteristic features of all solid tumors is the expression of HIF-1α. Observations that VHL is involved in HIF-1α activation originally highlighted the importance of HIF-1α in cancer progression [47, 48, 82]. Since then, numerous studies have explored the significance of HIF-1α in cancer pathogenesis and have led to the development of potential anti-cancer therapies [17, 102, 110, 114]. By initiating angiogenesis and altering cellular metabolism, HIF-1α promotes tumor growth and metastasis [70]. In addition, HIF-1α is frequently associated with activation of NF-κB, leading to the production of pro-inflammatory mediators in tumor

cells and tumor-associated immune cells [20]. Inflammation can further activate HIF-1α in a hypoxia-independent manner, worsening the tumor microenvironment [21]. HIF-1α also enhances tumor glycolytic flux by triggering the expression of several genes encoding glycolytic proteins/enzymes, such as glucose transporter 1 (GLUT1), hexokinases, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), and lactate dehydrogenase A (LDHA) [77]. Together, by modulating pathways in inflammation and glucose metabolism, HIF-1α exerts its effects on gene transcription, DNA repair, cell migration and apoptosis inhibition, thus favoring tumor development [77, 86, 141].

HIF-1α activation also occurs during many bacterial infections. Infection of mice and/or human cell lines by *Yersinia enterocolitica, Salmonella enterica* subsp. enterica, and *Enterobacter aerogenes* leads to a robust HIF-1α response, presumably by reducing the iron content of host cells via bacterial siderophores, which inhibits prolyl hydroxylase activity [40]. Moreover, murine macrophages infected by group B *Streptococcus, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Salmonella typhimurium*, also up-regulate production of antimicrobial peptides, nitric oxide (NO) and TNF-α, concurrent with the increase of HIF-1α and glycolysis [18, 101]. These observations suggest that in the context of bacterial infection, HIF-1α is of pivotal importance in the intracellular killing of bacterial pathogens, probably through the upregulation of glycolytic metabolism that leads to antimicrobial and pro-inflammatory responses in immune cells.

While the involvement of HIF-1α in immune response activation has been well characterized, a role for HIF-1α in immune suppression is also emerging in multiple diseases. For example, HIF-1α protects the host from excessive inflammation by decreasing NF-κB and pro-inflammatory cytokines, and by suppressing the inflammatory response of macrophages in murine apical periodontitis [42]. In addition, HIF-1α diminishes the proliferation and effector function of $CD4^+$ T cells, inhibits the differentiation of naïve $CD4^+$ T cells toward the Th1 phenotype, and promotes the immunosuppressive function of regulatory T cells in a mouse model of colitis-associated colon cancer [153]. The type 1 regulatory T cells (Tr1), which are $Foxp3^-$ regulatory $CD4^+$ cells, are metabolically reprogrammed to aerobic glycolysis through HIF-1α-dependent mechanisms. Proliferation of T cells towards Tr1 cells could also exert immune protection by IL-10 production [79]. T-lymphocyte infiltration is reduced in mice with diabetic cardiomyopathy related to HIF-1α upregulation [68]. B cell-specific deletion of HIF-1α compromises IL-10 expression, whereas in wild-type B cells, HIF-1α upregulates downstream glycolytic genes, such as *Glut1*, *Pkm2* (pyruvate kinase M2), *Hk2* (hexokinase 2), and *Ldha*, to boost cellular glycolytic activity. Additionally, by binding to the HRE regions (HRE 1 and HRE 2) of the *Il10* promoter, HIF-1α activates *Il10* transcription to exert protective effects in a murine model of autoimmune disease [83]. Furthermore, despite the fact that elevated HIF-1α induces inflammation in macrophages, a myeloid HIF-1α knockout in mice results in exacerbated gastritis in response to *Helicobacter pylori* infection via increased inflammation [81]. These observations imply that HIF-1α might play a pivotal role in controlling redundant immunological responses via its anti-inflammatory effector functions.

The protective role of HIF-1α is not limited to immune cells. LPS-induced IL-6 synthesis is suppressed by HIF-1α activation via upregulation of suppressors of cytokine signaling in human dental pulp cells [33]. In human bronchial epithelial cells, HIF-1α suppresses the expression of inflammatory mediators via TLR stimulation by microbial factors [104]. Furthermore, stabilization of HIF-1α by pharmacological inhibition of PHD enhances HIF-1α-dependent glycolysis, thereby promoting arterial oxygenation and host protection from neutrophil-LPS-induced ATP decline and death of mouse alveolar epithelial cells [140]. These results are consistent with HIF-1α dampening acute lung inflammation by increasing the glycolytic capacity of mouse alveolar epithelial cells [26]. The above observations are somewhat unexpected, since HIF-1α is known for its pro-inflammatory role, especially during bacterial infections [2, 121]. Thus, it is very likely that HIF-1α plays diverse and complex roles in inflammation and cellular metabolism, presumably depending on cell type, specific disease, and stages of disease progression.

Role of HIF-1α in *Mycobacterium tuberculosis* Infection

In *Mycobacterium tuberculosis (Mtb)* infection, at least three signaling pathways contribute to HIF-1α activation. First, production of ROS and TCA cycle metabolites during immune cell activation leads to upregulation of HIF-1α and metabolic remodeling to glycolysis, at least in macrophages and T cells [19, 61, 119, 124]. Second, hypoxia in the cellular microenvironment, caused by increased immune and metabolic activities and/or aggregation of immune cells in granulomas/lesions, stimulates increased expression of HIF-1α [5, 144]. Third, the downregulation of PHDs and FIH during *Mtb* infection also contributes to increased HIF-1α [121, 124]. Recently, HIF-1α-mediated glycolysis, which is similar to the metabolic state of cancer cells, has been associated with *Mtb* infection [2, 9, 36, 121, 124]. HIF-1α appears to play a critical role during *Mtb* infection, as mice with *Hif1a* deficiency in myeloid cells lost control of *Mtb* growth in mouse lungs. Bone marrow-derived macrophages (BMDMs) from mice with *Hif1a* deficiency in the myeloid lineage also have significant defects in IL-1β mRNA production during *Mtb* infection [9, 92]. The IFN-γ/HIF-1α axis has been proposed as a crucial component of the macrophage defense mechanism against *Mtb* infection [9, 10]. The IFN-γ/HIF-1α pathway is also essential for lipid droplet formation and the consequent production of prostaglandin E2 in murine BMDMs [56]. In addition, a metabolic shift to aerobic glycolysis is essential for optimal control of infection in INF-γ-activated BMDMs, suggesting a role for HIF-1α in the metabolic remodeling of activated macrophages [92]. However, emerging evidence indicates that increased HIF-1α expression and glycolysis during *Mtb* infection are independent of INF-γ [92, 122]; rather, INF-γ further stimulates HIF-1α-mediated metabolic remodeling program, leading to an enhanced anti-microbial response against the pathogen [92, 108]. Elevated levels of HIF-1α expression and glycolysis are also observed in infected rabbit lungs at late chronic stages of disease, and in lung granulomas of humans with active tuberculosis

[5, 44, 131]. These observations suggest that the role of HIF-1α in tuberculosis is dependent on the stage of infection, which is discussed in the following sections.

In animal models of tuberculosis, the early phase of *Mtb* infection is normally recognized as about 3–4 weeks of logarithmic growth of the pathogen after infection, which is followed by the control of bacterial growth due to the onset of host adaptive immunity [123, 135]. It is characterized by an intense inflammatory response that might influence the final outcome of infection. Early events in response to *Mtb* infection include activation of immune cells (DCs, macrophages and natural killer cells), production of cytokines, and expression of pattern recognition receptors. Induction of HIF-1α in this stage is normally caused by non-hypoxic factors, and its induction is associated with the ability of host immune cells to control the infection. For example, HIF-1α absence in myeloid cells drastically downregulates cellular glycolytic capacity, leading to a reduced ATP pool and impairment of myeloid cell aggregation, viability, invasiveness, and anti-bacterial activity [18]. HIF-1α also maintains effector functions of human and murine neutrophils by protecting them from apoptosis [146]. Moreover, specific deletion of HIF-1α in mouse T cells suppresses the effector functions of $CD8^+$ T cells [97]. Similarly, HIF-1α promotes maturation of murine DCs and reduces allogeneic T-cell proliferation and differentiation into regulatory T cells in inflammatory tissues [31, 49]. At early stages of *Mycobacterium marinum* infection, stabilization of host HIF-1α reduces the bacterial burden by induction of leukocyte-inducible nitric oxide synthase (NOS2) [28, 29]. This protective role of NOS2 is related to HIF-1α-mediated IL-1β signaling, as zebrafish with IL-1β blockade fail to control bacterial growth, despite activation of HIF-1α [90]. This observation further suggests that HIF-1α primes host immunity to facilitate bacterial clearance at early stages of *Mtb* infection. Due to the physiological demand for an active immune response and the pro-inflammatory role of HIF-1α, it is plausible to assume that optimal control of *Mtb* can be achieved by promoting HIF-1α stabilization and activation during the early phases of infection.

When the host fails to clear *Mtb* during early infection, the infection progresses to the late chronic phase. This stage of the infection is characterized by elevated level of inflammation with increased size of granulomas/lesions, development of hypoxia and/or necrosis in the microenvironment, and elevated expression of HIF-1α and glycolysis in animal models of tuberculosis and in humans with tuberculosis disease [25, 121]. In patients with pulmonary tuberculous granulomas, HIF-1α and NF-κB are up-regulated, which might contribute to lung destruction and cavitation by increasing the secretion of matrix metalloproteinases from macrophages and neutrophils [5, 91]. In addition, exaggerated expression of pro-inflammatory markers, including TNF-α, IL-6, and IFN-γ, have been identified in tuberculous granulomas in humans and animal models [89, 106]. Moreover, inflammatory DCs can be responsible for granulomatous dissemination by migration and spreading granulomatous inflammation at late chronic stages of the infection in mice [39]. Proteomic analysis also reveals that pro-inflammatory pathways are activated in the caseous and cavitary centers of human tuberculous granulomas [76]. Taken together, these observations imply that the elevated expression of HIF-1α in

the late chronic phase of tuberculosis elicits a detrimental outcome in tissues by promoting deleterious inflammation and modulating cellular metabolism. Therefore, suppressing the HIF-1α expression might attenuate excessive inflammation and limit tissue damage in the host.

Targeting HIF-1α as a Therapeutic Approach in Tuberculosis Treatment

Considering the metabolic and immunological functions of HIF-1α and its up-regulation during *Mtb* infection, we postulate that HIF-1α plays a dual, temporally dynamic role in the host antibacterial immune response. During the early stages of *Mtb* infection, HIF-1α-mediated metabolic reprogramming and the induction of innate immunity in myeloid cells are required for the host to prime adaptive immunity and control infection. As infection progresses to the late chronic stage, with the exacerbation of lung pathology, prolonged and elevated expression of pro-inflammatory cytokines and HIF-1α in the hypoxic environment in granulomas/lesions strongly suggest that HIF-1α continues to act as a pro-inflammatory mediator. Thus, inhibition of HIF-1α may attenuate overt inflammatory responses and avoid tissue damage and disease exacerbation. Indeed, efficacy of temporal tuberculosis treatment is shown in a rodent model in which HIF-1α blockade during early infection exacerbates disease progression, whereas it reduces pulmonary bacillary load and tissue damage during the late chronic stages of infection [2]. Based on its dual role in tuberculosis, different approaches targeting HIF-1α and/or related factors at different phases of the infection can be adopted as adjunct host-directed therapies (HDTs) for tuberculosis control.

Early Stage: Boosting of HIF-1α to Enhance the Antimicrobial Response

HIF-1α overexpression is associated with stimulation of macrophage glycolysis metabolism and its polarization toward the inflammatory M1 phenotype [149]. Similar observations have been obtained in cancer research where enhancement of HIF-1α activity leads to a metabolic shift toward glycolysis in tumor-associated immune cells and enhancement of their anti-cancer pro-inflammatory response in the microenvironment [16, 84, 86]. Thus, factors that induce HIF-1α expression and promote glycolysis and inflammation can be potentially adopted to enhance bacterial clearance at early stages of *Mtb* infection.

A multitude of studies have been conducted on various factors to promote HIF-1α expression. Hypoxia-mimetic $CoCl_2$ and androgens help maintain HIF-1α mRNA

levels at post-transcriptional and translational levels in the presence of HuR, an RNA-binding protein that has been implicated in the stability, subcellular shuttling and translation of HIF-1α mRNA in human T cells [120] and HeLa cells [35]. On the protein level, vasoactive hormones (angiotensin II and thrombin) can stabilize HIF-1α protein through induction of H_2O_2 production, which reduces the availability of a PHD cofactor, ascorbate, and downregulates hydroxylation, thus leading to decreased pVHL binding in vascular smooth muscle cells [94, 95]. To stabilize HIF-1α by inhibiting hydroxylation, salts of Co^{2+}, Cu^{2+}, and Ni^{2+} can also be used to antagonize Fe^{2+}, a cofactor of PHDs [109]. Iron chelators, including deferoxamine [87], N-propyl gallate [142], 1,10-phenanthrolines [126], and quercetin [50], have been shown to induce HIF-1α by repressing PHD activity. Inhibitors that act directly on PHDs are currently in development [52]. Several recent clinical trials have the tested the disease-modulating activity of various PHD inhibitors, including roxadustat [4], daprodustat [1], vadadustat [100], molidustat [7], and desidustat [54]. These treatments/compounds could potentially be repurposed and employed as adjunct HDTs to improve clinical outcomes in the treatment of pulmonary tuberculosis (Table 4.1).

Late Chronic Stage: Suppression of HIF-1α to Improve Treatment Outcome

As infection progresses to late chronic stages, HIF-1α continues to play a regulatory role in mediating inflammation and metabolism, likely compounded by the hypoxic environment in the tuberculosis granulomas/lesions [5]. In the meantime, concurrent prolonged inflammation can lead to immune exhaustion and deleterious tissue damage [2, 129, 167]. In granulomas of human and animal models, pro-inflammatory compartments are located in the center, surrounded by a cellular periphery possessing anti-inflammatory signatures. This phenomenon suggests that the host is controlling the progression of excessive inflammation in granulomas with a ring of anti-inflammatory activity at the rim of the lesion [23, 76]. Thus, attenuating excessive inflammation by targeting HIF-1α offers perspectives for tuberculosis treatment during the late chronic stage of the infection.

In immune cells, macrophage- and neutrophil-specific HIF-1α ablation is associated with reduced glycolysis and downregulation of the inflammatory response [18]. HIF-1α also drives $CD8^+$ T-cell effector function by promoting glycolytic metabolism [97]. Similarly, knockdown of HIF-1α leads to a significant glycolytic inhibition in DCs [49]. Lower expression of HIF-1α in dental mesenchymal stem cells can also enhance the production of monocyte chemoattractant protein-1 under inflammatory conditions, which promotes recruitment of macrophages. Intriguingly, the recruited macrophages function like M2 macrophages, with high IL-10 and low TNF-α production after LPS/TRL4 activation [78]. Therefore, from a therapeutic

Table 4.1 Manipulation of HIF-1α and implications for tuberculosis therapy

Agents	mechanism	Implications for TB therapy	References
Activation			
Angiotensin II	HIF-1α mRNA	Sp1 gene stimulation	[95]
CoCl2	HIF-1α mRNA stabilization	Promote mRNA binding to RNA-binding proteins	[35]
Androgens	HIF-1α mRNA stabilization	Promote mRNA binding to RNA-binding proteins	[120]
Thrombin	HIF-1α protein stabilization	Reduce hydroxylation	[94]
Salts of Co^{2+}, Cu^{2+}, and Ni^{2+}	HIF-1α protein stabilization	Inhibit hydroxylation	[109]
Deferoxamine	HIF-1α protein stabilization	Inhibit hydroxylation	[87]
N-propyl gallate	HIF-1α protein stabilization	Inhibit hydroxylation	[142]
1,10-phenanthrolines	HIF-1α protein stabilization	Inhibit hydroxylation	[126]
Quercetin	HIF-1α protein stabilization	Inhibit hydroxylation	[50]
Roxadustat	HIF-1α protein stabilization	Inhibit hydroxylation	[4]
Daprodustat	HIF-1α protein stabilization	Inhibit hydroxylation	[1]
Vadadustat	HIF-1α protein stabilization	Inhibit hydroxylation	[100]
Molidustat	HIF-1α protein stabilization	Inhibit hydroxylation	[7]
Desidustat	HIF-1α protein stabilization	Inhibit hydroxylation	[54]
Inhibition			
2-methoxyestradiol	HIF-1α protein translation	Anti-inflammation	[71, 161]
Wortmannin	HIF-1α mRNA	Anti-inflammation, blocking NF-κB	[74, 111]
Rapamycin	HIF-1α mRNA	Anti-inflammation, mTOR inhibition	[60, 165]
Aminoflavone	HIF-1α mRNA	Anti-inflammation, M2 polarization	[138]
Glyceollins	HIF-1α protein translation, stability	Anti-inflammation by blocking NF-κB	[55, 65, 103]
Topotecan	HIF-1α protein translation	Inhibiting ERK, increase glucose metabolism	[6, 22, 93, 107]
Geldanamycin	HIF-1α protein degradation	Anti-inflammation, HSP-90 inhibition	[72, 150]
Vorinostat	HIF-1α protein translation	HSP-90 inhibition, anti-inflammation, increase Treg	[15, 158]

(continued)

Table 4.1 (continued)

Agents	mechanism	Implications for TB therapy	References
Cardiac glycosides	HIF-1α protein translation	Anti-inflammation, reduce glycolysis	[34, 159]
GDC-0941	HIF-1α protein translation	PI3K inhibition, reduce glycolysis	[12, 160]
PP242	HIF-1α protein translation	mTOR inhibition, anti-inflammation	[59, 155]
YC-1	HIF-1α protein translation	Inhibition of NF-κB, anti-inflammation	[66, 67]
PX-478	HIF-1α mRNA	Anti-inflammation, reduce glycolysis	[57, 133, 151]
Acriflavine	HIF-1α dimerization	Inhibition of NF-κB, anti-inflammation,	[14, 64]
Echinomycin	HIF-1α DNA binding	Reduce glycolysis	[58, 156]
Chetomin	HIF-1α transcriptional activity	Anti-inflammation, reduce glycolysis	[128, 130, 145]
Bortezomib	HIF-1α transcriptional activity	Anti-inflammation	[38, 53, 85]
Triptolide	HIF-1α transcriptional activity	Anti-inflammation	[43, 163, 164]
LW6	HIF-1α protein stability	Reduce glycolysis, prevent energy collapse	[27, 63]
Cryptotanshinone	HIF-1α protein stability	Anti-inflammation	[62, 73, 162]
17-DMAG	HIF-1α protein stability	Inhibition of NF-κB, anti-inflammation	[45, 125]
17-AAG	HIF-1α protein stability	Anti-inflammation	[24, 46, 168]
CAY10585	HIF-1α protein stability transcriptional activity	Anti-inflammation	[37]

point of view, these investigations shed light on the possibility of inhibiting HIF-1α to manipulate the metabolism of immune cells for better treatment outcomes. Indeed, HIF-1α has been proposed as a critical target for pharmaceutical therapy in cancer, and many of its inhibitors have previously been tested in tumor models and in clinical trials [115, 157]. For example, rapamycin, an mTOR inhibitor, has anti-mycobacterial and anti-inflammatory activities by suppressing the pro-inflammatory cytokines IL-12 and TNF-α in *Mtb*-infected human and murine cells [60, 165]. These effects are likely due to the inhibition of mTOR activity, which, together with AKT, is required for the metabolic switch toward aerobic glycolysis and the antimicrobial response of activated macrophages [60, 75]. Aminoflavone, a well-known biflavonoid present in various plants, has been long suggested as a potential antitubercular compound [3]. Prolonged treatment of aminoflavone (>41 days) is related to M2 macrophage polarization in a murine model of mammary cancer [13],

potentially by inhibiting HIF-1α mRNA expression [138]. Wortmannin, a fungal metabolite that acts as an inhibitor of PI3K, blocks HIF-1α transcription in both inflammatory and hypoxic conditions [111], presumably by inhibiting the activation of NF-κB [74]. In addition, 2-methoxyestradiol can downregulate HIF-1α at the post-transcriptional level [71], attenuate inflammatory responses [161], and reduce bacillary loads at late chronic stages of tuberculosis [2]. Detailed information of HIF-1α inhibitors with potential application for tuberculosis therapy is summarized in Table 4.1.

Concluding Remarks

Emerging evidence indicates that HIF-1α-mediated metabolic remodeling and its association with the effector functions of host immune cells serve as one of the hallmarks of host-pathogen interactions in tuberculosis. A better understanding of the immunometabolic features of host immune cells, especially in the context of different pathologies at different stages of the infection, will not only reveal novel insights into the mechanisms of tuberculosis pathogenesis, but will also provide strategies for the development of potential adjunct HDTs to improve treatment efficacy and clinical outcomes [88, 166]. One of the important considerations is to target HIF-1α by repurposing existing compounds and/or drugs that are confirmed to have the expected effects on host immune cells. Since the majority of HDTs in tuberculosis research are focused only on limiting or dampening the prolonged hyper-inflammation of active TB [99, 166], repurposing compounds and/or drugs by targeting HIF-1α signaling to boost the anti-microbial response of host immune cells provides an attractive and important strategy to prevent worsening disease during early stages of the infection. This approach would be especially important for preventing the reactivation of previously acquired latent infection to clinical disease, the most common scenario in areas of the world with low tuberculosis incidence, such as the US.

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Chapter 5
Nuclear Receptors in Host-Directed Therapies against Tuberculosis

Eun-Kyeong Jo

Introduction

Tuberculosis (TB) is a serious infectious disease with high morbidity and mortality worldwide, and mainly caused by *Mycobacterium tuberculosis* (Mtb) [1]. Due to increasing threats with drug-resistant cases [2], there is a pressing need for development of host-directed therapies against TB. Understanding the complicated host-pathogen interaction in TB research is essential for approaches to develop potential therapeutics and preventives against TB including drug-resistant infections.

Nuclear receptors (NRs) are ligand-sensing transcriptional factors that function in the regulation of target gene expression [3, 4]. So far, 48 NRs have been reported in humans, although half of all NRs lacks traditional ligands, and are classified as 'orphan family members' [4]. Among NRs, vitamin D receptor (VDR) is the best studied NR in host-directed therapies against TB [5–7]. Vitamin D deficiency and VDR polymorphism are closely linked to host susceptibility to Mtb infections, either TB or extrapulmonary TB [8–11], although the underlying mechanisms are not fully understood. In addition, peroxisome proliferator-activated receptors [PPARα, β, γ; NR1C1, 2, 3] are well-known lipid-sensing NR that recognize endogenous fatty acids, thereby primarily regulating lipid metabolism [4]. Furthermore, liver X receptors (LXRs) and farnesoid X receptor (FXR) can recognize cholesterol metabolites and bile acids to function in regulation of cholesterol metabolism and bile acid homeostasis, respectively. In this chapter, we will discuss the function of VDR, PPARs, and LXR in the advances of antimicrobial immunity during mycobacterial infection.

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VDR Signaling and Antimicrobial Immune Defense in TB

VDR is expressed in a variety of immune cell subsets, including monocytes/macrophages, dendritic cells, and naïve CD4+ T cells [12]. VDR activation through interaction with the bioactive ligand, 1,25-dihydroxyvitamin D_3 (1,25-D3), triggers the induction of chromatin remodeling to regulate the expression of distinct genes [12]. VDR hetero-dimerization with the retinoic acid receptor (RXR) results in the regulation of target gene expression through binding to the vitamin D response elements (VDREs) of the target genes [12, 13]. Immune-relevant target genes containing VDREs include the genes of antimicrobial proteins such as cathelicidin [14–16] and β-defensin (DEFB4) [17]. In addition, VDR signaling is critically involved in pleiotrophic immune functions, including myeloid differentiation [18], macrophage chemotactic ability [19], and expression of proinflammatory cytokines [20]. However, high-dose supplementation of vitamin D accelerates the resolution of proinflammatory responses during chemotherapy of pulmonary infections [21]. In the context of Mtb infection, an interaction between VDR and TLR2 signaling is essentially required for the induction of human cathelicidin expression in monocytes/macrophages to further enhance antimicrobial responses against Mtb [22–24]. Furthermore, VDR-mediated antimicrobial activity is mediated through reactive oxygen intermediates [25], nitric oxides [26], antimicrobial peptides [22, 23, 27], and autophagy [28].

Autophagy, a catabolic process through lysosomal degradation, is well-known for its host defensive ability against Mtb infection [29]. Importantly, 1,25-D3 treatment of human monocytes/macrophages activates VDR-mediated antimicrobial responses through the autophagy pathway [27, 30]. In addition, TLR2/1 activation by mycobacterial lipoprotein LpqH can induce antibacterial autophagy and antimycobacterial responses through AMPK-p38 MAPK pathways via C/EBP-β-dependent Cyp27b1 expression and cathelicidin induction [31]. Moreover, vitamin D-mediated signaling cooperates with Th1 responses to enhance autophagy and promote antimicrobial responses against TB [32, 33]. Importantly, vitamin D treatment induces autophagic flux and the production of human cathelicidin mirobial peptide to inhibit HIV replication and intracellular mycobacterial growth [34], suggesting that VDR signaling-based therapeutics are promising approaches against dual infection with both HIV and Mtb, not only to single infection.

PPARs and Antimicrobial Host Defense During Mtb Infection

PPAR-α is a member of lipid-sensing PPAR family that primarily regulates the expression of genes involved in fatty acid β-oxidation, ketogenesis, and inflammatory responses [35–37]. Previous studies showed that PPAR-α plays an essential role in host protection against Mtb infection through transcriptional activation of

autophagy and lysosomal biogenesis, and promotion of lipid catabolism [38]. In addition, PPAR-α-activating agents upregulate transcription factor (TF)-EB, which is crucial for lysosomal biogenesis and lipid metabolism, in brain cells and macrophages [38, 39]. Combined with a key role for TFEB in antimicrobial host defense against Mtb infection [40], these studies strongly suggest that PPAR-α-TFEB activation promotes anti-mycobacterial host defense through enhancement of phagosomal maturation and lysosomal activity.

PPAR-γ is another member of PPARs, and has a similar function as PPAR-α in lipid metabolism and regulation of inflammation in innate immune cells [41, 42]. During Mtb infection, PPAR-γ functions as a key regulator in immune responses and nutrient metabolism [43, 44]. Mtb infection, particularly the 19-kDa lipoprotein [45], can trigger the induction of PPAR-γ gene expression in macrophages [43, 46]. Mechanistically, PPAR-γ is required for Mcl-1 induction during Mtb infection, thereby promoting intracellular Mtb growth [46]. It was noted that PPAR-γ-dependent inhibition of host defense was detected only in lung macrophages but not in bone marrow-derived macrophages or peritoneal macrophages, suggesting that Mtb-induced PPARγ plays an important role primarily in the macrophages located in the lung compartment [47]. It was also shown that BCG infection induced PPAR-γ expression through TLR2 and that PPAR-γ inhibition enhanced intracellular killing activity against BCG [48]. In addition, the pathogenic effects of PPARγ in host protection are, at least, partly mediated through defective Th1 cell responses, since earlier studies showed that PPARγ deletion in alveolar macrophages resulted in the activation of Th1 responses [49]. Moreover, the interactions between lipids derived from Mtb-host cells and the lipid-sensing NR PPARγ may contribute to the intracellular survival of Mtb through modulation of macrophage function [50]. Thus, PPARγ may play pathogenic functions and favor the establishment of chronic Mtb infection [51].

Importantly, vitamin D treatment of macrophages downregulates PPAR-γ expression and lipid droplet accumulation during Mtb infection [52]. PPAR-γ agonists counteracted the antimicrobial effects through VDR signaling, suggesting a cross-talk between PPAR-γ and VDR signaling pathways [52]. Another recent study showed that vitamin B1 had a beneficial effect upon Mtb infection through regulation of PPAR-γ-dependent signaling [44]. Vitamin B1 effects of limitation of Mtb were mediated through down-regulation of PPAR-γ activity. The number of intracellular Mtb in macrophages were suppressed by vitamin B1, and these effects were abrogated by PPAR-γ agonists, suggesting a negative role for PPAR-γ in innate host defense against Mtb infection [44]. In addition, an aptamer (ZXL1) that binds to mannose-capped lipoarabinomannan of Mtb showed anti-Mtb effects through inhibition of PPARγ expression [53]. However, in an opposite direction, GM-CSF-mediated antimicrobial responses against Mtb was dependent on PPARγ expression in peritoneal macrophages [54]. Together, PPARα and PPARγ may have opposing effects in innate host defenses against Mtb infection, although their functions may vary with cell type and immune status.

LXR and Host Defense During Mtb Infection

LXRs, LXRα and LXRβ, play an important role in transcriptional regulation of lipid homeostasis and inflammatory responses in macrophages [55]. Earlier studies showed that Mtb infection induced the expression of LXRs and LXR target genes in lung tissues and cells [56]. LXR-deficient mice had defective protective responses in early infection, and had dysregulated inflammatory factors in the lung cells. In addition, LXR agonists TO901317 and GW3965 were beneficial in antimicrobial responses, because they decreased lung bacterial burden and increased Th1/Th17 responses in vivo [56]. These data suggest that LXR signaling is crucial for host resistance to Mtb infection and could be a novel target for anti-TB agents. Future studies are warranted to clarify the role of LXR in clinical samples from human TB.

Conclusions

So far, with the exception of VDR, the functions of most NRs in TB are still largely uncharacterized. Understanding the functions of NRs may contribute to the development of host-directed therapeutics against TB. Given numerous reports on VDR-induced host protection against Mtb infection, VDR is the best characterized and promising target for host-directed, adjunctive therapy against TB. Additionally, PPARs and LXRs are actively induced by Mtb infection, and importantly involved in host pathogenesis/protection during Mtb infection. LXR exerts pivotal roles in innate host defense with antimicrobial effects against Mtb infection. However, the exact roles of these NRs are largely unknown in human samples and clinical studies. Future studies are warranted to examine the possibility to include NR agonists in adjunctive chemotherapy against human TB.

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Part III
Enhancing Anti-mycobacterial Mechanisms

Chapter 6
Autophagy as a Target for Host-Directed Therapy Against Tuberculosis

Surbhi Verma, Raman Deep Sharma, and Dhiraj Kumar

Introduction

Tuberculosis (TB) is the most deadly infectious disease, killing about 1.5 million people annually. According to the WHO, about 1.7 billion people are estimated to have latent TB and each year ten million people develop active TB worldwide. Among several factors, the emergence of drug-resistant strains of the causative pathogen *Mycobacterium tuberculosis* (*Mtb*) has significantly contributed to the high mortality and morbidity due to TB. The evolution of drug resistance despite having an effective therapeutic regimen underscores the limitations of conventional antibiotic-based therapy against TB. It also highlights the importance of searching for alternative therapies that may shorten treatment and reduce antimicrobial resistance.

Host-directed therapy (HDT) is an attractive approach to limit pathogenic infections by modulating host factors/pathways [1–4]. The underlying principle is that most pathogens are known to rely upon a set of host factors in order to establish the infection and cause disease. Identifying and targeting such factors not only benefit against a particular pathogen, but also against other pathogens, which may share the same host pathways for pathogenicity. Infection of host cells by *Mtb* is known to hijack/utilize several host physiological processes including signaling, trafficking, metabolism, gene regulation, apoptosis, phago-lysosomal pathways, and autophagy [5, 6]. While host factors involved in each of the above-mentioned physiological processes have been explored, autophagy has emerged as a promising pathway of choice when targeting intracellular bacteria, particularly *Mtb,* by HDT approaches. Autophagy is a conserved intracellular catabolic process, which delivers undesirable cytoplasmic content, like misfolded proteins, damaged organelles such

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as mitochondria (mitophagy), peroxisomes (pexophagy), ER (ER-phagy) or intracellular pathogens (xenophagy) to lysosomes for their degradation. Intracellular bacteria like *Mtb* can be controlled by autophagy (or xenophagy) by targeting the bacteria to the lysosomal compartment for degradation via autophagy, highlighting the role of this process in defense against *Mtb*.

The housekeeping functions of autophagy, also referred as the homeostatic arm of autophagy, regulates quality control of damaged organelles and other cytosolic cargos to help regulate cellular inflammation, and, therefore, are essential for cellular survival [7]. Interestingly, the core autophagy machinery involved in basal autophagy and xenophagy is mostly shared. Thus, identifying regulatory factors, which selectively target anti-bacterial autophagy, i.e., xenophagy, without compromising the homeostatic functions of autophagy will be more useful to develop any therapeutic strategy.

The primary focus of this chapter is on autophagy, which is a key innate defense pathway that cooperates with other antimicrobial defense mechanisms to combat the infection. We will discuss diverse mechanisms, which have been shown to regulate autophagy during mycobacterial infections. Finally, we also discuss a variety of small molecule modulators, which have been tested against *Mtb* infection in diverse infection models, and the possibility of their development as adjunct therapeutic candidates against tuberculosis.

Antibiotic Resistance in TB and the Need for Host-Directed Therapy

The current standard treatment for drug-sensitive TB involves a cocktail of several first-line antibiotics, including isoniazid (INH), rifampicin (RIF), pyrazinamide and ethambutol, which are administered for a minimum of 2 months followed by additional 4 months of INH and RIF. In the case of patients developing drug resistance, second- and third-line drugs are used instead. Together, a total of ~14 antimicrobial drugs are currently being prescribed for tuberculosis based on the drug resistance profile of the infecting strain, the latest additions being bedaquiline and delamanid [8, 9].

Each of these drugs is an antibiotic, meaning it targets distinct vital functions in the bacteria, like mycobacterial cell-wall biosynthesis, DNA or RNA metabolism, protein synthesis and ATP synthase [10]. The antibiotics exert huge selection pressure on the bacteria, which often results in the emergence of drug resistance. In tuberculosis, drug-resistant infections can be resistant to a single drug resistant, multi-drug resistant (MDR, resistant to INH, RIF and any other first line drug) or extensively drug resistant (XDR, resistant to INH or RIF and 2 second line drugs – a quinoline and an injectable agent). While identification and development of new antibiotics can address the problem of drug-resistance, all new and future antibiotics also suffer from the risk of consequent development of resistance. There is therefore

a consensus now that unconventional approaches must be adopted to target existing and future drug resistance.

One key factor contributing to emergence of drug-resistance in TB is the long duration of treatment. In the case of MDR-TB, the treatment regimen could take more than 20 months, which also results in severe off-target effects like, liver toxicity, development of diabetes, pulmonary and chronic renal diseases etc. [11]. The prolong treatment results in non-compliance which further aid emergence of novel drug-resistance. Alternate approaches are therefore also sought which if combined with the existing treatment regimens, should help shorten the duration of treatment, thereby dramatically reducing the chances of emergence of drug resistance.

An exciting alternative approach, which has emerged in the past couple of decades, aims to target function of the host cell factors rather than the bacterial components. The strategy behind HDT is to target and reverse the host mechanisms exploited by the pathogen for their replication or persistence, and/or boost protective host immune responses against the pathogen in animal models [3]. They can be used as adjuvants to increase the efficiency of current anti-TB regimen [12]. In the case of *Mtb* infection, several host factors have been implicated whose modulation increases the clearance of *Mtb* from the cells [2, 13]. These include manipulating membrane trafficking processes like autophagy [14], targeting eicosanoids [15]), lipoxygenase [16], vitamin D signaling [17–19], anti-inflammatory pathways [20] and use of agents including calcium channel blockers [21], tyrosine kinases inhibitors [20, 22], phosphodiesterase inhibitors [23, 24], histone modification [25] and cytokine modulation [25, 26]. In addition, anti-VEGF inhibitors like bevacizumab [27], antihyperglycemic drugs like metformin [28], N-acetyl cysteine [29] and beta defensins [30] also tend to control the infection. Though there are numerous targets for HDT, the subsequent sections highlight the recent advances on the regulation of autophagy, especially during *Mtb* infection and the emerging autophagy based anti-tuberculosis therapies.

Autophagy: The Conserved Cellular Process of Degradation

Autophagy was discovered as a cellular response pathway to stresses like nutrient starvation [31]. After the seminal discovery of the genes regulating this pathway during the 1990s [32], related research escalated and revealed that cells also exhibit basal levels of autophagy independent of nutrient deprivation or stress, which is required for the maintenance of cellular homeostasis [33]. In addition, defects in the components of the autophagy machinery have been implicated in a myriad of diseases like neurodegenerative disorders [34], cancer [35], lysosomal storage disorders [36], Crohn's disease [37], liver diseases [38], diabetes [39], muscular dystrophy [40] and aging [41], underscoring its relevance in maintaining the cellular equilibrium.

The pathway of autophagy initiates with the activation of Unc-51 like kinase-1 (ULK1) complex [42], comprising the serine/threonine protein kinase ULK1, focal

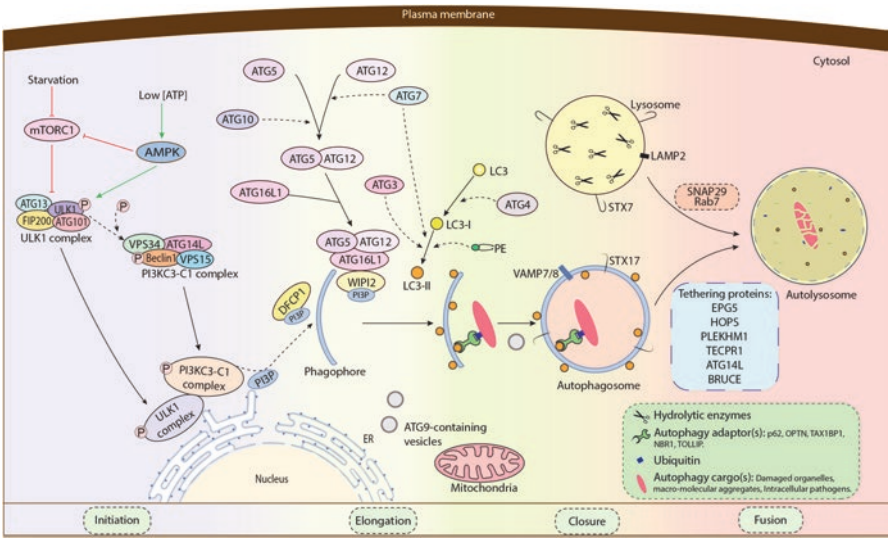

Fig. 6.1 Basic autophagy pathway and its core machinery. Various stresses like nutrient starvation and low intracellular ATP level serves as inducer for autophagy to happen. In nutrient-rich environment mTORC1 interacts and inhibits ULK1 complex, but upon nutrient starvation this interaction is altered due to inhibition of mTORC1, allowing ULK1 complex to initiate autophagy. ULK1 is phosphorylated by AMPK (AMP-activated protein kinase), which is positively regulated by low ATP level. Active ULK1 complex phosphorylates Beclin 1 of PI3KC3-C1 complex, complex responsible for the production of PI3P. PI3P leads to nucleation of an isolated membrane, known as phagophore, originated from various intracellular compartments like ER, Golgi, others. After initiation step, the elongation step requires additional autophagy-related proteins like ATG3, ATG 4, ATG 5, ATG 7, ATG 12 with some other factors, and translocation of LC3 for its lipidation. Presence of LC3 on phagophore allows cargo recruitment by interacting with various adaptor proteins. The closure of phagophore assembly requires to fuse ATG9-containing vesicles to form a double membrane vesicle, autophagosome. The final step in autophagy process is the fusion of cargo-encapsulated autophagosome with the hydrolase rich-lysosome. Various tethering and SNARE proteins are involved in the fusion to form a single membrane vesicle, called autolysosome, where encapsulated cargo is degraded by highly efficient hydrolytic enzymes. Abbreviations: mTOR1 mammalian Target Of Rapamycin Complex 1, ATG Autophagy-related protein, ULK1 Unc-51 Like autophagy activating Kinase, P Phosphate, ATP Adenosine Triphosphate, AMPK AMP-activated Protein Kinase, VPS Vacuolar Protein Sorting, FIP200 FAK family kinase-Interacting Protein Of 200 KDa, LC-3 microtubule-associated proteins 1A/1B Light Chain 3, PI3KC3-C1 Class III Phosphatidylinositol 3-Kinase Complex I, PI3P Phosphatidylinositol 3-Phosphate, ER Endoplasmic Reticulum, DFCP1 Double FYVE domain-Containing Protein, WIPI2 WD repeat domain, Phosphoinositide Interacting 2, PE Phosphatidylethanolamine, LAMP2 Lysosome-Associated Membrane Protein 2, STX Syntaxin, VAMP Vesicle-Associated Membrane Protein, SNAP Synaptosomal Nerve-Associated Protein, EPG5 Ectopic P-Granules autophagy protein 5 Homolog, HOPS Homotypic fusion and Protein Sorting protein complex, PLEKHM1 Pleckstrin Homology domain-containing family M member 1, TECPR1 Tectonin Beta-Propeller Repeat containing 1, BRUCE BIR Repeat-containing Ubiquitin-Conjugating Enzyme, OPTN Optineurin, TAX1BP1 Tax1 Binding Protein 1, NBR1 Neighbor of BRCA1, TOLLIP Toll Interacting Protein, FUNDC1 FUN14 Domain-Containing protein 1, SMURF1 Smad Ubiquitination Regulatory Factor 1, TRIM Tripartite Motif, STBD1 Starch-Binding Domain-containing protein 1

adhesion kinase family-interacting protein of 200 kDa (FIP200), ATG13 and ATG101 [43] (Fig. 6.1). Its downstream effector, class III phosphatidylinositol 3-kinase complex (PI3KC3) [44], consisting of vacuolar protein sorting 34 (VPS34), Beclin 1, ATG14L and VPS15 [45], triggers the biogenesis of phagophore assembly site (PAS) at omegasomes, by the production of phosphatidylinositol 3-phosphate (PI3P) [46]. The further recruitment of PI3P-binding proteins, such as double FYVE domain-containing protein 1 (DFCP1) and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) at the PAS promotes the expansion of the phagophore [47, 48]. This leads to the formation of most apical feature of the autophagy process, the autophagosomes, which surrounds the cargo to be degraded via the process. The growth of the autophagosomes also relies on two conserved ubiquitin-like conjugation systems, ATG3-ATG7-ATG10 and ATG5-ATG12-ATG16L1. These systems aid in enriching these double-membrane structures with lipidated ATG8 proteins [49, 50]. There are two subfamilies of ATG8, microtubule-associated protein 1 light chain 3 (MAP1LC3) and γ-aminobutyric acid receptor-associated proteins (GABARAP), which are further classified as MAP1LC3A, MAP1LC3B, MAP1LC3B2, MAP1LC3C, GABARAP, GABARAPL1 and GABARAPL2 [51]. The incorporation of these proteins seals the autophagosomes and drives their maturation (Fig. 6.1).

The autophagosomes fuses with either endosomes or directly with the lysosomes for their maturation. The concerted action of three sets of proteins brings in this process. These are Rab GTPases, tethering factors and soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) [52, 53]. Among Rab GTPases, Rab7 has been studied to have a potential role in the fusion [54, 55]. Several of its effectors, like Rab-interacting lysosomal protein (RILP) [56], FYVE and coiled-coil domain containing protein 1 (FYCO-1), derive the mobility of the autophagosomes, whereas other effector proteins, like Pleckstrin homology domain-containing family member 1 (PLEKHM1) [57] and ectopic P-granules autophagy protein 5 homolog (EPG5) [58], enhance vesicle fusion. Tethering factors involved in bridging the two vesicles include homotypic fusion and protein sorting (HOPS) [59], Tectonin beta-propeller repeat-containing protein 1 (TECPR1) [60] and ATG14 [61]. Finally, the Q-SNAREs syntaxin 17 and SNAP29 on autophagosomes and R-SNARE Vamp7/8 on lysosomes mediates the fusion [62]. The degradation in the autolysosome follows the activation of lysosomal hydrolases through vacuolar acidifcation, which require action of lysosomal ATPase channels to pump in the H^+ ions [63, 64]. The lysosomal enzymes then act on the autophagy cargo for its degradation, which could be recycled or serve as substrates for anabolic pathways. The dependence of autophagic degradation of cargos on vacuolar acidification is elegantly used to assess the rate at which autophagy-mediated degradation is taking place in a cell at any given time, also known as autophagy flux. Autophagy flux is assessed by comparing ATG8 levels in the presence and absence of vacuolar ATPase inhibitors (bafilomycin A1 or chloroquine) or lysosomal hydrolase inhibitors like E64D and pepstatin. Since ATG8, which is part of all autophagosomes, also get degraded in the lysosomes, inhibition of lysosomal degradation results in the accumulation of ATG8 proteins, suggesting an active degradation process in the autolysosomes. This assay has also been modified and adapted to measure cargo

specific autophagy flux, i.e., rate of degradation of specific cargo via autophagy (for example, mitophagy flux, xenophagy flux, etc.) [65].

The process of autophagy can be selective or non-selective. Selective autophagy differs from bulk (non-selective) degradation in the way that the specific cargos are identified by a dedicated cohort of autophagy adaptors (selective autophagy receptors). The recognition by the adaptors in most instances is through the ubiquitin chains, which are tagged on the cargos by various E3 ubiquitin ligases. Once bound to the cargo, the adaptors load them onto the autophagosomes via binding to the LC3 through their LC3-interacting domain (LIR). The major autophagy adaptors that are known are sequestosome-1 (SQSTM1/p62), optineurin (OPTN), nuclear dot protein of 52 kDa (NDP52), tax1 binding protein 1 (TAX1BP1), neighbor of BRCA1 gene 1 (NBR1) and Toll-interacting protein (TOLLIP). A detailed description of cargo tagging and recognition by the autophagy adaptors is provided elsewhere [66].

Autophagy as a Defense Mechanism Against *Mycobacterium tuberculosis*

Besides homeostatic functions of autophagy, like stress response/starvation/protein-organelle control, this process is also implicated in controlling the intracellular survival of diverse bacterial pathogens. The first report to show that autophagy is induced upon pathogenic infection came in 1984, from the work of Rikihisa, in which guinea pig polymorphonuclear leukocytes (PMNs) were observed to phagocytose *Rickettsiae* in autophagosomes [67]. Currently, most of the intracellular bacterial pathogens are known to be targeted by autophagy, including *Salmonella typhimurium* [68], *Shigella flexneri* [69], Group A *streptococcus* [70], *Legionella pneumophila* [71], *Rickettsia conorii*, *Listeria monocytogenes* [72] and *Mtb* [73], highlighting its importance in the innate immune response.

The role of autophagy during mycobacterial infections first gained attention when it was reported that induction of autophagy by rapamycin led to the suppression of *M. bovis* BCG and *Mtb* growth in macrophages [73]. This was followed by a series of studies showing how autophagy could serve as a host defense mechanism by overcoming the phagosome maturation block caused by *Mtb* in the macrophages [74–76]). Then, through a genome-wide siRNA screening study, ~74 host-dependent factors for intra-macrophage survival of *Mtb* were identified, many of which seemed to act via regulation of autophagy [2]. Subsequently, polymorphisms in autophagy-related genes, such as immunity-related guanosine triphosphatase family M protein (IRGM), vitamin D receptor (VDR), IFNγR, toll like receptor 8 (TLR8) and ATP receptor P2X7R, showed a high association with susceptibility to mycobacterial infections [77, 78]. Together, the studies mentioned above resulted in establishing the fact that autophagy was a major defense mechanism and helped in controlling mycobacterial infections. It is also evident now that understanding mechanistic

underpinnings of autophagy during mycobacterial infection could potentially allow the development of novel host-directed therapeutic strategies.

Regulation of Autophagy During *Mycobacterium tuberculosis* Infection

Diverse mammalian cell types can be infected by *Mtb*, including phagocytic cells like macrophages [79], dendritic cells [80] and neutrophils [81], and some non-phagocytic cells, like fibroblasts [82], stem cells [83] and airway epithelial cells like type II alveolar cells [84]. Upon infection, signaling from various surface or intracellular pattern recognition receptors (PRRs) helps in the initiation of innate responses. Pathogen-associated molecular patterns (PAMPs) of *Mtb* like lipomannan (LM), lipoarbinomannan (LAM), its mannosylated form (ManLAM), phthiocerol dimycocerosate (PDIM), lipoproteins, mycolic acid and *Mtb* DNA/RNA are the primary ligands for such receptors. Activation of the PRRs, like toll-like receptors (TLRs) [85] and nod-like receptors (NLRs) then drives the induction of autophagy [86, 87]. Shin et al. showed that autophagy is activated by TLR1/2/CD14 signaling stimulated by mycobacterial lipoprotein LpqH [88]. These signaling events involve the downstream activation of VDR, which increases the expression of ATG5 and BECN1, as well as cytosolic production of the antibacterial peptide, cathelicidin, which are critical for the defense [89]. Additionally, binding of NOD2 to MDP increases the recruitment of IRGM, ATG16L1 and LC3 in *Mtb*-infected alveolar macrophages [90].

Mtb-derived DNA/RNA or c-di AMP, when secreted into the host cytosol via the ESX-1 secretion system, triggers recognition by cGAS to induce the stimulator of IFN genes (STING) pathway for initiating ubiquitin-mediated selective autophagy [91]. The E3 ubiquitin ligase, Parkin can mark *Mtb* with ubiquitin, which further recruits NDP52 and p62 autophagy adaptors for the targeting of *Mtb* to the autophagy machinery (Fig. 6.2) [92]. Another E3 ubiquitin ligase, SMURF1 also functions in a similar manner for mycobacterial targeting [93]. In addition, there is an emerging role of TRIM proteins (class of E3 ligases) in controlling mycobacterial infections. TRIM16 cooperates with galectin-3 for the recruitment of the core autophagy proteins ULK1, Beclin 1, and ATG16L1 for combating *Mtb* invasion [94]. Moreover, it also promotes the nuclear translocation of transcription factor EB (TFEB), a key modulator of lysosome biogenesis. Another protein of the same class, TRIM22, enhances clearance of *Mtb* by targeting the NF-κB/beclin 1 pathway [95]. However, other reports shows autophagy targeting of *Mtb* independent of E3 ligases. In a very recent finding, direct recruitment of the ubiquitin to the *Mtb* surface protein Rv1468c was observed to trigger xenophagy in a p62-dependent manner [96]. Besides NDP52 and p62, other autophagy adaptors are also observed to target *Mtb.* In a study from zebrafish embryos, p62 or OPTN deficiency increased their susceptibility to *M. marinum* infection, whereas the overexpression of these proteins increased the autophagic targeting of *M. marinum* [97]. In another report, TAX1BP1 and NBR1

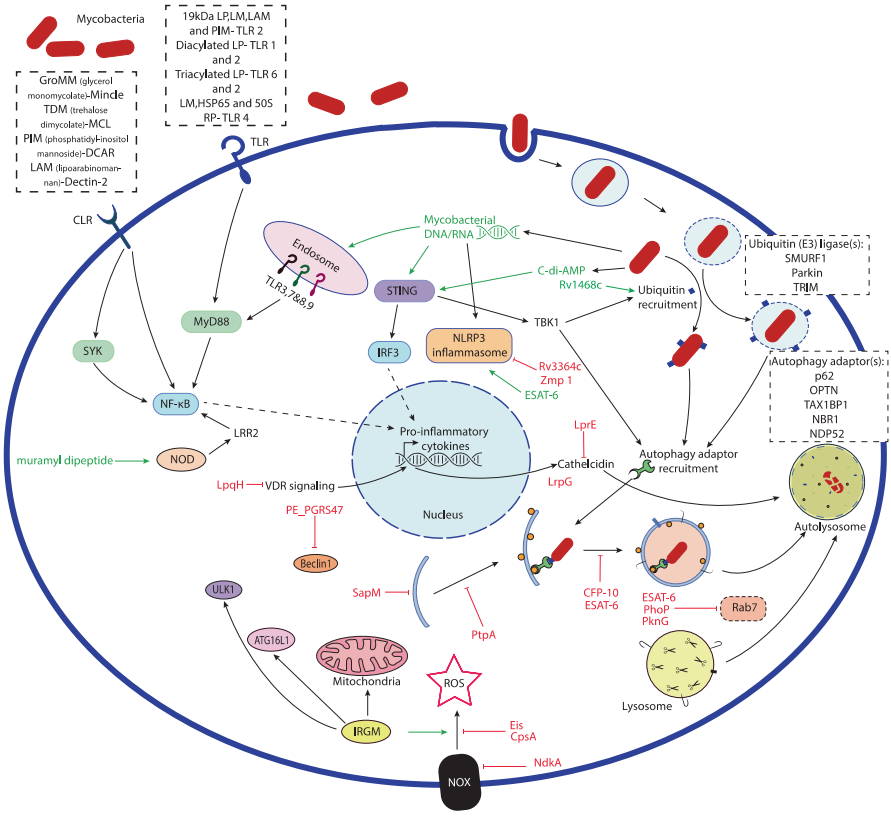

Fig. 6.2 Schematic representation of recognition, engulfment, tagging, targeting and host intracellular signaling events during *Mtb* pathogenesis. Abbreviations: Host: STING Stimulator of Interferon Genes, Syk Spleen tyrosine kinase, MyD88 Myeloid Differentiation primary response 88, NOD Nucleotide-binding Oligomerization Domain, LRR Leucine-Rich Repeat, NOX NADPH Oxidase, ROS Reactive Oxygen Species, IRGM Immunity-related GTPase family M protein, ULK1 Unc-51 Like autophagy activating Kinase, ATG16L1 Autophagy related 16 Like 1, VDR Vitamin D Receptor, NLRP NOD, Leucine rich Repeat and Pyrin domain, TBK1 TANK-Binding Kinase 1 SMURF1 Smad Ubiquitination Regulatory Factor 1, TRIM Tripartite Motif, OPTN Optineurin, TAX1BP1 Tax1 Binding Protein 1, NBR1 Neighbor of BRCA1, NDP52 Nuclear Dot Protein 52, IRF3 IFN Regulatory Factor 3, NF-κB Nuclear Factor kappa-light-chain-enhancer of activated B cells. Bacterial: Zmp1 Zinc metalloprotease 1, ESAT-6 6kD-Early Secretory Antigenic Target, SapM Secreted acid phosphatase M, PtpA protein tyrosine phosphatase, CFP-10 10-kDa Culture Filtrate Protein, PknG Protein kinase G, Eis Enhanced intracellular survival, NdkA Nucleoside diphosphate kinase A

were also observed to colocalize with *Mtb* upon infection in macrophages [98]. Apart from direct recruitment to *Mtb*, p62 was also observed to deliver ribosomal and bulk ubiquitinated cytosolic proteins to autolysosomes, where they are proteolytically converted into products capable of killing *Mtb* [99]. These findings reflect the unique bactericidal properties of the autophagy adaptors.

Besides driving the recruitment of autophagy components towards *Mtb,* signaling cascades from PRRs increase the production of inflammatory cytokines, which in turn crosstalk with autophagy for shaping the outcome of *Mtb* infection. Numerous studies highlight the role of IFN-γ, TNFα, and IL-1β in autophagy regulation [100]. In fact, a synergistic role of IFN-γ and TNFα has been observed for initiating the autophagic anti-mycobacterial response. Furthermore, IRGM, the downstream effector of IFN-γ, is a strong mediator of inflammation and helps in the secretion of various cytokines like IL-4, IL-6 and IL-1β [101]. In addition, it plays an essential role in induction of mycobacterial xenophagy, serving as scaffold for major components of the machinery like ULK1, Beclin 1 and ATG16L1 [102, 103]. Interestingly, autophagy is now understood to be an anti-inflammatory process and its inhibition results in increased inflammation [104]. The classical activation of macrophages allows them to acquire inflammatory potential via inhibiting autophagy. At the same time, the overproduction of mitochondrial ROS due to inhibition of mitophagy helps in bacterial killing [105, 106].

Modulation of Autophagy as Preferred Mechanisms for Survival by Mycobacteria

As discussed in the above sections, autophagy acts as a defense mechanism against *Mtb* infection. *Mtb* has also evolved strategies to evade or circumvent the autophagy pathway. Multiple host factors get modulated by the arsenal of molecular effectors of *Mtb* to directly or indirectly impede autophagy. Among the various mycobacterial virulence factors, the role of the ESX1 machinery in regulating autophagy has been studied. Regulation of autophagy by *Mtb* may occur at multiple levels, like expression of autophagy-related genes, regulation of inflammation and inflammatory cytokines, and recruitment of specific factors to the autophagosomes needed for maturation and degradation of cargos. The virulence factors ESAT-6 and CFP10 impact host cell expression of ATG8, a critical autophagy protein, thereby helping to evade autophagic targeting of *Mtb* [107]. Another ESX1-secreted protein, EspB, suppresses IFN-γ-induced autophagy in murine macrophages [108]. The effect of ESAT-6 is diverse, as it modulates multiple host factors, including activating the NLRP3 inflammasome in human macrophages to suppress autophagy [109]. However, *Mtb* Zinc metalloprotease 1 (Zmp1) can prevent inflammasome activation and IL-Iβ processing, thereby lowering their targeting to autophagy [110]. While a large number of studies focus on autophagic targeting of *Mtb* and the bacterial evasion of such targeting as the tug-of-war between the host and the pathogen, there are also parallel observations, which highlight that targeting *Mtb* to autophagosomes alone is not sufficient for bacterial killing. Since autophagic degradation of cargos is achieved upon maturation of autophagosomes into autolysosomes, bacteria present in autophagosomes that are able to block the maturation process will evade killing. The virulence factor ESAT-6, along with the response regulator PhoP, inhibit the recruitment of RAB7, a small GTPase important for lysosomal fusion, thereby

blocking the targeting of *Mtb* to the lysosomes [111]. More importantly, the exclusion of RAB7 from the autophagosomes is highly selective and is restricted to only *Mtb*-positive autophagosomes or xenophagosomes. Thus, *Mtb* can selectively modulate xenophagy flux without disturbing the basal autophagy flux in the cells [7]. The significance of selective xenophagy is apparent, considering the role of autophagy in cellular homeostasis. The bacteria benefits from selectively blocking xenophagy flux, as it also ensures prolonged survival of the host cells, which may otherwise get compromised in case the autophagy block is global/non-selective. Exclusion of RAB7 from *Mtb* autophagosomes is also facilitated by the secreted acid phosphatase (SapM), which binds to RAB7 and inhibit autophagosome-lysosome fusion [112]. Expression of antimicrobial peptides, like cathelicidin, is perturbed by *Mtb* factors, like LprE, which also helps suppress autophagy and promote bacterial survival [113]. The maturation of autophagosomes is also dependent on host phasphatidylinositols (PI3Ps), a common requirement even during phagosome maturation. The mycobacterial cell wall glycolipids ManLAM and LM mimic mammalian phosphatidylinositols, thereby competitively targeting PI3KC3 and hampering the autophagy pathway [114]. Yet another mode of regulating autophagy by *Mtb* involves reworking cellular redox homeostasis and NADPH metabolism. The enhanced intracellular survival (Eis) protein of *Mtb* upregulates the cellular ROS (NADPH-derived) and proinflammatory cytokine levels, suppressesing autophagy [115, 116]. *Mtb* is also capable of sensing and inhibiting non-canonical autophagy pathways, like LC3-associated phagocytosis (LAP). The *Mtb* protein CpsA is involved in its escape from LAP by inhibiting the recruitment of NADPH oxidase 2 to the mycobacterial phagosome [117]. In fact, several newer loci of *Mtb*-mediated intervention in host autophagy are also being discovered. For example, in a genome wide gain of function screen, *PE_PGRS47* (Rv2741) loci of *Mtb* was implicated in suppressing autophagy, as well as in antigen presentation [118]. Altogether, we now have staggering evidence of direct involvement of *Mtb* factors in regulating autophagy in host cells upon infection (Fig. 6.2).

Subversion of Autophagy by *Mtb* Via Targeting RNA Metabolism

Transcriptional and post-transcriptional regulation of autophagy and associated genes upon *Mtb* infection can eventually shape the autophagy response to the bacteria. Autophagy genes can be regulated at the gene level expression or isoform level expression, leading to a novel means of regulation [119]. However, one aspect of RNA-mediated regulation of autophagy during *Mtb* infection that has strongly evolved in the recent past, is the role of non-coding RNAs [120]. The non-coding RNA contingent of the host includes micro-RNAs (miRNA), long noncoding RNA (lncRNA) and circular RNA.

miRNAs: The miRNAs work at several targets in the core and extended autophagy machinery to control cellular autophagy pathways. In most cases, mycobacterial infection is believed to induce the expression of certain specific miRNAs, which then target the corresponding autophagy genes for post-transcriptional gene regulation. In the core autophagy pathway, miR-155 has opposing activities, since it targets RHEB in macrophages, a negative regulator of autophagy to help bacterial clearance [121], but, at the same time, also targets ATG3 expression in dendritic cells and helps bacterial survival [122]. The expression of miR-155 increases upon *Mtb* infection, but its precise role during in vivo infection remains unclear. Other core autophagy proteins targeted via mi-RNAs during *Mtb* infection include regulation of UVRAG by miR-125a [123], ATG4b by miR-129-3p [124], BECN1 by miR-30a [125], and ATG5 by miR1958 [126]. In addition, factors closely associated with the regulation of autophagy but not directly part of the core autophagy machinery, like DRAM2, CACNA2D3, and ATM are regulated by miR-144, miR-27a and miR-18a, respectively [127–129].

Besides miRNA, LncRNA are also reported to impact host responses during *Mtb* infection. While some evidence indicates lncRNA-mediated regulation of autophagy during *Mtb* infection, they require more characterization to establish direct involvement *in vivo* (Table 6.1) [136, 137].

Autophagy Modulators as Therapeutic Candidates Against Tuberculosis

Through the preceding sections, it is apparent that numerous host and pathogen factors regulate the process of autophagy during *Mtb* infection. Not surprisingly, therefore, serious efforts have been made to target the autophagy pathway in order to cripple the bacterial manipulation of host defense and help killing of *Mtb*. It is important to highlight here that the involvement of autophagy in other chronic diseases, such as neurodegeneration and cancer, has already resulted in a massive interest in identifying regulators of this process with potential therapeutic benefits [171, 172]. Large chemical screenings involving FDA-approved drugs, small molecule libraries, and natural product-based libraries have resulted in identification of several molecules of interest [173, 174]. Targeted studies subsequent to identification of any new autophagy modulator have revealed the mechanism of action of most such compounds. Broadly, autophagy regulators could act either directly on core autophagy pathways, like ULK1, BECN1, ATG5, or feed upstream to regulate the core autophagy molecules directly or indirectly, such as AMP-activated protein kinase (AMPK), *mammalian target of rapamycin (*mTOR), SRC, AKT, and PI3K, among others (Table 6.2).

The mTOR-dependent and independent regulators: The mTOR complex is the primordial regulator of autophagy [175]. Hence, its inhibition by rapamycin is

Table 6.1 miRNAs regulating *Mtb* intracellular survival through the autophagy pathway

miRNA	Specific host target	Mechanism	Reference
miR-30a	BECN1	Downregulates BECN1 and ATG5 expression	[125]
miR-144	DRAM2	Reduces the DRAM2 levels (DRAM2 interacts with BECN1 and UVRAG)	[127]
miR-33	Multiple autophagy genes	Lowers ATG5, ATG12, LC3B, LAMP1 and PRKAA1 levels	[130]
miR-27a	Calcium transporter CACNA2D3	Inhibits autophagosome formation via downregulating calcium signaling	[128]
miR-125a	UVRAG	Hinders autophagy by targeting UVRAG	[123]
miR-18a	ATM	Suppresses autophagy via downregulating ATM protein kinase	[129]
miR-20a	ATG16L1 and ATG7	Inhibits autophagic response by silencing the ATG16L1 and ATG7 expression	[131]
miR-26a	KLF4	Upregulates KLF4 which induces Mcl-1 expression thereby suppressing autophagy	[132]
miR-155	ATG3	Subverts autophagy by targeting ATG3	[122]
miR-155	Rheb	Promotes autophagy by binding to Rheb (negative regulator of autophagy)	[121]
miR-1958	Atg5	Regulates autophagy by reducing Atg5 expression and LC3 puncta	[126]
miR-889	TNF-like weak inducer of apoptosis (TWEAK)	Inhibits autophagy in latent tuberculosis via suppression of TWEAK (TWEAK induces autophagy through AMPK activation)	[133]
miR-1303	Atg2B	Represses Atg2B translation to regulate autophagy	[134]
miR-17-5p	Mcl-1and STAT3	Reduces expression of Mcl-1 and STAT3. Also reduces the interaction of Mcl-1 and BECN1, therby lowering autophagy flux. (During mycobacterial infection downregulation of miR-17-5p has been observed)	[135]

among the earliest autophagy inducers used to show a direct effect on *Mtb* survival via autophagy [73]. One of the analogs of rapamycin, everolimus, has also been investigated to control infection in a similar fashion [148]. Other compounds that have been tested to induce autophagy to clear mycobacteria via mTOR targeting includes small molecule enhancers of rapamycin (SMERs) [165]; nitazoxanide, the anti-protozoal drug [157] and baicalin, a herbal medicine [141]. Most of these compounds work through targeting the PI3K/Akt/mTOR axis. However, mTOR is at the center of diverse cellular processes, including cellular bioenergetics, protein synthesis, and nutrient stress, among others [176], rendering it a less attractive target for development of drugs against TB. This limitation can be overcome, though, by mTOR-independent regulators of autophagy. In a FDA-approved drug screen by

Table 6.2 Modulators of autophagy against *Mycobacterium tuberculosis*

Compounds	Description	Role in autophagy signaling pathways	Reference
AICAR	Analog of AMP	Increases the phosphorylation of AMPK. AMPK-PPARGC1A pathway induces the expression of multiple autophagy genes.	[138]
Ambroxol	Active mucolytic agent	Increases LC3B punctae and TFEB nuclear translocation. Enhances the antimycobacterial action of rifampin.	[139]
AZD0530 (Saracatinib)	Src TK inhibitor	Increases autophagy flux	[140]
Baicalin	Natural occurring flavanoid	Induces autophagy via the PI3K/Akt/mTOR pathway.	[141]
Bazedoxifene	Selective estrogen receptor modulator	Increased ROS production and AKT/mToR signaling	[142]
Carbamazepine	FDA approved anticonvulsant	Induces Inositol triphosphate depletion and activates AMPK. Increases the expression of IRGM and ATG16L1	[143, 144]
Curcumin	Diarylheptanoid (natural occurring phenol)	Increases LC3B punctae. Inhibits PI3K/Akt/mTOR signaling. Activates AMPK.	[145, 146]
DHEA	Steroid hormone	Induces autophagosome formation.	[147]
Everolimus	Analog of rapamycin	Inhibits mTORC1	[148]
Fluoxetine	Selective serotonin reuptake inhibitor	Augments TNFα production	[149]
GABA	Inhibitory neuro-transmitter	Increases expression of autophagy-related genes and genes for TLR. Increases autophagy flux	[150]
Gefitinib	EGFR inhibitor	Increases autophagy by inhibiting p38.	[149]
GSK4112	NR1D1 agonist	Increases autophagy flux and TFEB levels.	[151]
GW7647	PPARα agonist	Induces the expression of multiple autophagy related genes like TFEB, LAMP2 and RAB7	[152]
Honokiol	SIRT3 activator	Activates SIRT3-PPARA-TFEB signaling.	[153]
Imatinib	Abl TK inhibitor	Increases autophagy through BECN1 and ATG5	[154, 155]
Loperamide	Opioid-receptor agonist	Induces autophagy flux. Increases the expression of ATG16L1 and LC3	[144]
Metformin (phenformin)	Antidiabetic drug	Activates AMPK.	[28]

(continued)

Table 6.2 (continued)

Compounds	Description	Role in autophagy signaling pathways	Reference
Nilotinib	TK inhibitor	Induces autophagy by attenuating c-ABL dependent PI3k/Akt/mTOR signaling pathway. Activates E3 ubiquitin ligase, parkin	[156]
Nitazoxanide	Antiprotozoal drug	Inhibits mTOR signaling	[157]
Nortriptyline	Tricyclic antidepressant	Induces autophagosomes formation and enhance lysosomal acidity.	[158]
NSC 18725	Nitroso containing pyrazole derivative	Increases BECN1 and ATG3 expression.	[159]
Ohmyungsamycins A and B	Cyclic peptides	Triggers AMPK activation.	[160]
Pasakbumin A	Herbal medicine	Activates the NF-kB and ERK1/2-mediated signaling pathways.	[161]
4-Phenylbutyrate	Histone deacetylase inhibitor	Enhances the expression of BECN1 and ATG5	[162]
Resveratrol	Phenolic compound	Upregulates SIRT1 and SIRT3 and activates AMPK phosphorylation.	[163, 164]
SMERs (SMERs 10, 18 and 28)	Small molecule enhancers of rapamycin	Induces autophagy independent of mTOR signaling	[165]
SRT1720	Sirtuin activator	Upregulates lipidated levels of LC3 via activating SIRT1	[163]
Statins	Cholesterol-lowering drug	Reduces mTOR activity.	[166, 167]
Tat-Beclin	Peptide derived from Beclin 1 linked to HIV Tat protein	Increases autophagy via ATG5	[168]
Thiostrepton	Thiopeptide antibiotic	Induces ER stress-mediated autophagy.	[169]
Trehalose	Naturally occurring disaccharide	Increases xenophagy flux. Induces lysosomal calcium ion release for the translocation of TFEB to the nucleus.	[168, 170]
Valproic acid	FDA approved anticonvulsant	Increases autophagosome formation through ATG12. Increases the expression of IRGM and ATG16L1	[143, 144]
Verapamil	Calcium channel blocker	Increases Mtb colocalization with LC3	[144]
Vitamin D3	Cholecalciferol	Induces cathelicidin production. Increases the expression of BECN1 and ATG5	[89]
Wy14643	PPARα agonist	Induces TFEB signaling	[152]

Schiebler and colleagues, two anticonvulsant drugs, carbamazepine (CBZ) and valproic acid showed the ability to restrict the growth of both sensitive and drug-resistant *Mtb* in macrophages [143]. The effect was primarily driven by the induction of autophagy through inositol 1,4,5-trisphosphate (IP3) depletion and AMPK activation [143]. Two additional drugs, loperamide and verapamil also work independently of mTOR to induce autophagy and restrict *Mtb* growth in monocyte-derived macrophages as well as murine alveolar cells [144]. The search for mTOR-independent regulators of autophagy continues since such compounds can have fewer undesired effects. In this context, another mTOR-independent autophagy inducer, trehalose, assumes significance, considering its less complex nature, availability, and recent demonstration of efficacy in controlling infection in animal models of TB [168]. In *ex vivo* conditions, trehalose can also kill *Mtb* and non-tuberculous mycobacterial strains (NTMs) through autophagy induction, making it an attractive candidate for further exploration as an HDT [168]. It is important to highlight that trehalose is currently under clinical trial for neurodegenerative disorders, mostly due to its ability to induce autophagy [177–179].

AMPK and SIRT regulators: AMPK is an upstream kinase, which activates autophagy by sensing the cellular energetics via phosphorylating the target protein ULK1 [180]. AMPK activity can be modulated by trans-resveratrol, metformin and the AMP-mimetic 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), each of them are shown to restrict the *Mtb* growth via inducing autophagy, [28, 138, 164]. However, resveratrol can induce autophagy through multiple possible mechanisms, including up-regulation of SIRT3 and SIRT1 activity [163, 164]. In fact, multiple additional regulators of SIRT3 and SIRT1 are known and can help control *Mtb* infection. For example, the biphenolic compound, honokiol, recently identified as SIRT3 activator, enhances increased LC3B punctae formation during *Mtb* infection in BMDMs [153]. Similarly, another activator of SIRT1, SRT1720, helps in *Mtb* clearance via autophagy [163]. Recently identified cyclic peptides, ohmyungsamycins A and B, have been shown to trigger AMPK activation for activating autophagy in *Mtb*-infected BMDMs. They also tend to control bacteria load in *M. marinum*-infected Drosophila melanogaster [160].

Nuclear receptors agonists as regulators of autophagy during *Mtb* infection: Nuclear receptors are transcription factors, which are activated upon binding specific ligand like steroid hormones and lipid mediators etc. and regulate variety of cellular functions [181]. Signaling through VDR activates autophagy [182] and its role in controlling *Mtb* infection is experimentally validated [183]. Treatment with 1,25-dihydroxyvitamin D3, the active form of vitamin D3, induces autophagy in *Mtb*-infected macrophages via induction of the cathelicidin-derived antimicrobial peptide, LL-37 [89]. In a similar fashion, 4-phenylbutyrate increases anti-microbial activity against *Mtb* in macrophages [162]. Apart from VDR, several other nuclear receptors tend to play effective roles during mycobacterial infection.

The metabolically active form of vitamin A, all-trans retinoic acid, also promotes autophagy via STING/TBK1/IRF3 axis and helps in the clearance of *Mtb* [184]. The adopted orphan nuclear receptor peroxisome proliferator-activated receptor α (PPARα) agonists promote autophagy to suppress *Mtb* and *M. bovis* in BMDMs by regulating several genes like Rab7, Lamp2 and Tfeb [152]. Estrogen-related receptor α (ERRα), another orphan nuclear receptor, which is a key regulator of energy homeostasis and mitochondrial function, helps coordinate innate immune function against *Mtb* involving autophagy. Mechanistically, ERRα cooperates with SIRT1 and post translationally regulates several proteins, like ATG5, BECN1, and ATG7 for promoting autophagy [185]. Moreover, selective estrogen receptor modulators (SERMs), commonly used for the treatment of breast cancers, like tamoxifen and, more recently, bazedoxifene can also control *Mtb* survival within macrophages. At least for bazedoxifene, the mechanism involves induction of autophagy [142]. While some of the agonists and modulators for nuclear receptors mentioned above are being explored for potential anti-TB therapeutic strategy, it is also intriguing that such diverse nuclear receptors, known for distinct cellular functions, could regulate autophagy especially during bacterial infection to play a role in host defense.

Protein kinases and other signaling mediators: In a chemical screen performed by Stanley and group in 2014, fluoxetine, a serotonin reuptake inhibitor, and gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR), were noted to stimulate autophagy for improved *Mtb* clearance during *in vivo* infection [149]. Signaling through cell-surface receptors can elicit a variety of host responses and usually such targets may seem less suitable for developing anti-TB therapeutics. In this context, several host protein kinases, not directly known to regulate autophagy, have also emerged to influence autophagy, at least during mycobacterial infection. Among them, the protein tyrosine kinase (PTK) SRC has emerged as an attractive target to achieve bacterial killing. A possible role of SRC kinase was uncovered using a comparative transcriptomic approach and has consistently shown protective effects in *ex vivo* and *in vivo* models. Mechanistically, inhibition of SRC family kinase by the specific inhibitor AZD0530 increases xenophagy flux to help kill *Mtb* in THP-1 macrophages and also reduces inflammation and pathology in the animal models of tuberculosis [140]. Another PTK inhibitor, nilotinib, that specifically attenuates Abelson (c-ABL) TK, has been shown to promote autophagic degradation of *M. bovis* and *M. avium subspecies paratuberculosis* [156]. Other compounds tested in the context of autophagy includes statins, commonly used cholesterol lowering drugs [166], gamma-aminobutyric acid (GABA) [150], haloperidol [158], pasakbumin A [161], ambroxol [139], thiostrepton [169] and NSC 18725 [159] amongst others. Furthermore, in a very recent approach, depletion of BTF3a (Basic transcription factor 3a) [186] and downregulation of receptor expressed in lymphoid tissues-like protein 1 (RELL1) [187] notably reduces mycobacterial survival, thereby providing new targets for *Mtb* treatment.

Future Perspective

Anti-bacterial autophagy during *Mtb* infection can be regulated by a plethora of mechanisms, as described above. Inducers of autophagy, which could activate the anti-bacterial arm of autophagy, i.e., xenophagy, have serious potential for development as host-directed anti-TB therapeutics. Since there are so many ways autophagy could be regulated during *Mtb* infections, determining which ones are better candidates for developing therapeutic strategies is a critical priority. Whether it could be via targeting the core autophagy machinery or upstream regulators like nuclear receptors, signaling kinases, or small non-coding RNAs remains to be established. So far, the majority of autophagy inducers identified, either through high-content screening or through targeted approaches, seem to activate both the defense arm and the homeostatic arm of autophagy. Since the homeostatic arm of autophagy intrinsically promotes survival and has anti-inflammatory implications, it may not be assumed to be a very serious roadblock in developing autophagy-focused HDT against TB. However, it is also important to realize that inflammation is a part of natural host defense, and therefore activating a patently anti-inflammatory process could complicate the host response. An interesting silver lining has emerged recently through studies which report that autophagy inhibitors like chloroquine, which typically blocks lysosomal acidification may actually help the host by making phagosome-resident *Mtb* more sensitive to existing anti-TB drugs [188]. By simple extrapolation, this could also mean that autophagy inducers may help enhance the activity of drugs to which the bacteria are tolerant.

While no direct evidence of this kind exists, it certainly raises the question that instead of looking at any means of inducing global autophagy, we should rather focus on only the defense arm, i.e., xenophagy. However, there is little information on any such pathway or modulator, which targets xenophagy without affecting the basal autophagy rates. Another level of complexity is that several core autophagy proteins also have functions independent of regulating just autophagy. For example, ATG5 has an autophagy-independent role in neutrophil recruitment and tissue pathology [189]. These diverse facets of the core homeostatic machinery of autophagy must be further explored. Currently, at least three potential candidates that act via regulating autophagy are at different phases of clinical trials for adjunct therapy against tuberculosis- Vitamin D3, Metformin and a statin (pravastatin). With better clarity on mechanisms for selective xenophagy regulation, more nuanced host-directed therapeutic candidates are likely to be developed in the future.

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Chapter 7
Metformin: A Leading HDT Candidate for TB

Amit Singhal and Hardy Kornfeld

Introduction

Inadequacies of current antibiotic therapies to clear persistent *Mycobacterium tuberculosis* (*Mtb*) infection and emergence of drug-resistance are major challenges for eliminating TB [1, 2]. Disappointing results from the REMOxTB [3] and RIFAQUIN [4] treatment shortening trials highlight the need for novel interventions to cure TB [1, 5, 6]. This led to the emergence of a new paradigm in TB treatment *vis-à-vis* host-directed therapy (HDT), which focuses on the activation of host factors subverted by the pathogens in order to mitigate damaging immune pathology and to hasten lesion sterilization in combination with antimicrobials [7–11].

An effective HDT agent for tuberculosis (TB) could potentially (i) augment antibiotic efficacy treatment, (ii) shorten treatment regimens for drug-susceptible and/or drug-resistant *Mtb* infections, (iii) reduce immune-mediated lung injury and fibrotic resolution associated with TB, and (iv) promote development of immunological memory that could protect against relapse and reinfection. We were the first to propose metformin for TB-HDT, reporting that it reduces lung bacterial load, immune pathology, and gene expression pathways associated with fibrosis in mice [12]. Metformin, a biguanide and an indirect activator of 5′ adenosine monophos-

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phate-activated protein kinase (AMPK), is on the World Health Organization's list of essential medicines. It is considered to be one of the most effective therapeutics for treating type 2 diabetes (T2D) since it reduces hepatic gluconeogenesis with no effect on insulin secretion or weight gain and without causing hypoglycaemia when used as monotherapy for T2D [13]. Our original report was followed by a series of retrospective studies showing that T2D patients taking metformin have lower risk for *Mtb* infection, progression from infection to TB disease, TB mortality, and TB recurrence [14–24]. This suggests the promise of metformin as an adjunct therapy for TB. However, the molecular and cellular mechanism of metformin's HDT efficacy remains unclear. It is possible that as with T2D, metformin efficacy in TB is associated with its well-known pleiotropicity, as also shown in cancer [25] and atherosclerosis [26]. Here we review the potential host targets responsible for metformin's HDT effect and provide the current state of the retrospective and prospective human data demonstrating metformin's efficacy in TB patients.

Mechanistic Targets of Metformin

Since the introduction of metformin in the clinic for T2D (Europe – 1950s; USA – 1995), considerable effort has been made to better understand the mechanism behind its anti-hyperglycemic effect. In addition to the direct action of metformin on liver, a myriad of indirect effects play an important role of the drug on lipid metabolism, meta-inflammation, and immune cells, leading to immuno-glucoregulatory network regulation. It has been shown that a handful of host intracellular proteins may be responsible for the pleiotropic effects of metformin. However, the exact nature of molecular interaction between the drug and its targets for T2D remain unknown.

1. **AMPK** - AMPK is a top candidate target for metformin. This mammalian serine/threonine protein kinase is activated during metabolic stress and controls energy homeostasis [27]. Metformin activates hepatic AMPK, leading to the reduction of gluconeogenic gene transcription, induction of fatty acid oxidation (FAO), and suppression of sterol regulatory element-binding protein (SREBP)-1, a key lipogenic transcription factor [28]. Activation of AMPK, which results from the phosphorylation of Thr172, is controlled by liver kinase B1 (LKB1) and is required for metformin-mediated (i) inhibition of glucose production by hepatocytes [28] and (ii) regulation of glucose uptake in skeletal muscle cells [29]. In a cell-free assay, metformin did not influence LKB1-mediated Thr172 phosphorylation, indicating that AMPK may not be a direct target of metformin [30]. The anti-inflammatory and anti-bacterial effects of metformin are also thought to depend on AMPK activation [31, 32]. Metformin reduces intracellular growth of *L. pneumophila* [32] and *Mtb* [33] through activation of mitochondrial ROS (mtROS) in an AMPK-dependent manner. PBMCs from healthy individuals who took metformin for 6 days showed increased Thr172 phosphorylation of

AMPK, and these PBMCs demonstrated increased phagocytic capacity and decreased TNFα, IL1β, IL6 and IL17 release when stimulated with *Mtb* lysate [34], suggesting relevance of AMPK in metformin's anti-*Mtb* properties. Moreover since AMPK activates autophagy via inhibiting Mammalian Target of Rapamycin (mTOR), AMPK activators have been considered as a promising TB-HDT [35].

2. **Mitochondrial electron transport chain (ETC) complex I** - Evidence that metformin-mediated activation of AMPK is secondary to the direct effect of metformin on mitochondria suggested that a mitochondrial protein could be metformin's primary target [30]. In 2000, two independent groups demonstrated that mitochondrial ETC complex I (a redox enzyme complex consisting of 44 subunits which is also known as NADH:ubiquinone oxidoreductase) is the primary target of metformin [36, 37] and that the metabolic effects of the drug was preserved in liver-specific AMPK-deficient mice [37]. Complex I inhibition by metformin results in the suppression of ATP production and decrease in [ATP]:[ADP] and [ATP]:[AMP] ratios [36, 37], leading to an increase in Thr172 phosphorylation of AMPK and a switch to AMPK-dependent catabolic pathways that generate ATP. This also results in downregulation of mRNA expression of gluconeogenic enzymes [38] and inhibition of acetyl-CoA carboxylase 1 (ACC1) and ACC2 activities [39]. Effects on mitochondria have also been suggested by the notion that metformin is a positively charged molecule that accumulates within mitochondria to concentrations up to 1000-fold higher than in the cytoplasm [40]. Of note, anti-tumor effects of metformin depend on inhibition of mitochondrial complex I [41]. Metformin is a non-competitive complex I inhibitor and interacts with subunit ND3, but does not alter the structural integrity of complex I [42]. Interestingly, a recent study in normal and diabetic rats showed that metformin at clinically-relevant plasma concentrations inhibits hepatic gluconeogenesis by a redox-dependent manner, which was independent of the inhibition of complex I and ACC activity [43]. This revealed a complex I-independent mechanism of metformin, which is most likely AMPK-independent as well.
3. **Mitochondrial glycerophosphate dehydrogenase -** Controversies in (i) AMPK-dependent or AMPK-independent effects of metformin, (ii) drug doses that have been used in *in vitro* and *in vivo* experiments, and (iii) acute effects of metformin administration that differ from the AMPK activator A-769662 [40, 44, 45], resulted in the identification of the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase (mGPDH/GPD2) as a non-competitive inhibitor of metformin [44]. Rats and mice that do not express GPD2 exhibited abrogated metformin-mediated decrease in plasma glucose and inhibition of endogenous glucose production [44]. GPD2, via the glycerol-3-phosphate shuttle, connects oxidative phosphorylation (OXPHOS) and glycolysis, and regulates macrophage inflammatory responses to bacterial lipopolysaccharide (LPS) [46]. In fact, LPS-stimulated macrophages demonstrate an induction of aerobic glycolysis (Warburg effect), which has also been observed in *Mtb*-infected macrophages and mice [47, 48], and in cancer [49]. Targeting the Warburg effect and restoring energy yield is currently being pursued as a cancer treatment [50].

Since different metabolic states of macrophages are implicated in the control or progression of intracellular bacterial infections [51], and metformin has been demonstrated to inhibit the Warburg effect in order to exert an anti-tumor effect [52], it can be reasoned that metformin inhibits aerobic glycolysis in TB. Notably, it was recently shown that metformin targets GPD2 to control growth of thyroid cancer *in vitro* and *in vivo* [53]. In addition to GPD2, metformin also inhibits liver glucose production by inhibiting fructose-1-6-bisphosphate [54], a glycolytic/gluconeogenic enzyme. Taken together, these observations support the concept that manipulating metabolic circuits may be exploited as an adjunct for future TB HDT strategies.

4. **Growth differentiation factor 15 (GDF15)** - GDF15 (also known as macrophage inhibitory cytokine -1 [MIC-1]) is a biomarker of cellular stress that belongs to the TGFβ superfamily. It is expressed in various tissues in response to inflammation and binds to its receptor GDNF family receptor alpha like (GFRAL), the expression of which is thought to be restricted to selected regions of the brainstem. GDF15 promotes anti-inflammatory responses of adipose tissue macrophages, and macrophages with defects in OXPHOS lose their ability to secrete GDF15 [55]. Bacterial and viral infections induce GDF15, which promotes metabolic adaptation to systematic inflammation, indicating GDF15 as an inflammation-induced central mediator of tissue tolerance [56]. Furthermore, GDF15 confers a survival benefit in sepsis [56]. In an elegant study, Poll *et al* recently showed that metformin elevates circulating levels of GDF15, which is necessary to obtain its beneficial effects on energy balance and body weight [57]. This also suggests a role for metformin in endocrine-immune crosstalk. Overall, these studies indicate that the anti-inflammatory and anti-TB activity of metformin may involve GDF15.

Studies Demonstrating Metformin's Anti-TB Properties

1. **Animal data** - Interest in metformin's anti-*Mtb* properties was initiated by our discovery in 2014 that metformin enhances control of *Mtb* replication in macrophages by inducing phagosome-lysosome fusion and mtROS production, and in the tissues of aerosol infected C57BL/6J (B6) mice [33]. Metformin enhanced the efficacy of anti-TB drugs, ameliorated lung pathology, and reduced inflammation [33]. A subsequent study by Dutta et al. [58] using Balb/c mice concluded that metformin does not improve the sterilizing activity of first-line antimicrobial TB treatment, despite trends for lower CFU at 2 months ($P = 0.54$) and 3.5 months ($P = 0.039$) of adjunctive treatment. The differences in the two studies could reflect differences in mouse strain and methodologies. The study by Dutta et al. [58] did not include assessment of immune pathology, whereas the immunomodulatory effects of metformin might provide a more clinically significant benefit than accelerated sputum conversion. Indeed, metformin has been shown to reinvigorate immunity in *Mtb*-infected B6 mice by reprogramming

$CD8^+$ T cell metabolism [59]. In addition to programming of $CD8^+$ T cells in *Mtb*-infected mice, the Singhal lab recently found that metformin-educated $CD8^+$ T cells from uninfected mice have enhanced FAO, OXPHOS, survival capacity, and anti-*Mtb* properties (Bohme et al., Nat Comm, 2020, In press). These studies together indicate the important role of $CD8^+$ T cells in metformin-derived host immunity against *Mtb*. Moreover, an unpublished study in *Mtb*-infected guinea pigs supports the hypothesis that metformin promotes host resistance to infection during chronic TB (~90 days post-infection) by maintaining metabolic homeostasis of immune cells (Haugen Frankel et al., R. Basaraba, Colorado State University, personal communication). Of note, metformin treatment was started 4 weeks before *Mtb* challenge of guinea pigs and continued throughout the study. Taken together, these animal studies indicate the important role of metformin-programmed host immunity towards *Mtb* infection and pathology.

2. **Retrospective data from TB-T2D cohorts -** The large population of individuals with T2D living in high TB burden regions and treated with metformin or other anti-diabetic therapies has provided an opportunity for data mining to identify potential HDT efficacy. The initial report of metformin treatment in *Mtb*-infected mice included human data from the Singapore TB control program indicating that metformin reduces the risk for cavitary TB and mortality in adults with T2D [12]. Subsequent retrospective studies supported that observation, providing evidence that adults treated with metformin for T2D had lower prevalence of *Mtb* infection [14, 23], reduced progression from latent infection to active TB disease [15, 16, 18, 20, 24, 60], accelerated sputum conversion [19, 22], lower all-cause mortality in TB [17], and lower risk of recurrent TB after cure [21] as compared to those treatment with non-metformin antidiabetic regimens. Altogether, these studies reported protective effects in 293,081 individuals treated with metformin. A major caveat of these results is that they reflect metformin efficacy in people living with T2D and may not be generalized to the normoglycemic population. Some of these studies addressed that question, finding that protective effects of metformin for TB were not associated with better glycemic control. Degner et al. [17] reported that in a cohort of 2416 T2D patients treated for TB, those taking metformin had significantly lower odds of death (hazard ratio 0.56, 95% confidence interval 0.39–0.82) despite significantly higher mean glycohemoglobin (HbA1c) level. While HbA1c data were not available to Pan et al. [16], they reported significantly lower TB progression with metformin use. This effect was dose-dependent, while clinical factors reflecting diabetic severity did not differ between those taking metformin and those in the group taking sulfonylureas. Lee et al. [19] reported that use of metformin was associated with faster sputum conversion in diabetic patients with cavitary TB, but HbA1c (and other potential confounders) were no different between the metformin and non-metformin groups in that cohort. Together with evidence that metformin benefits metabolically normal mice infected with *Mtb* and that it promotes nominally TB-protective shifts of immunological parameters in healthy adults, these find-

ings strongly suggest that the TB-HDT efficacy of metformin might extend to the general population.

3. **Prospective data from TB-T2D cohorts -** The lack of prospective studies evaluating the effects of metformin in people with TB-DM comorbidity reflects a major knowledge gap. In the prospective Effects of Diabetes on Tuberculosis Severity (EDOTS) study, participants with pulmonary TB and T2D treated with metformin had significantly lower plasma levels of several matrix metalloproteinases (MMP-1, 2, 3, 7, 9 and 12) at the time of TB diagnosis as compared to those treated with non-metformin antidiabetic regimens [61]. Overall, MMP levels positively correlated with HbA1c, but no significant difference in HbA1c levels was detected between participants in the metformin and non-metformin groups. It could be hypothesized that reduced MMP levels result in reduced lung damage with TB [62]; however, future prospective studies including assessment of lung function are required to support this hypothesis. One clinical trial of metformin (METRIF) is reportedly underway [63] while two others have been funded, with accrual pending (Table 7.1). The latter two include the NIH-funded METHOD trial (#5U01AI134585; PIs - Kornfeld, Singhal and Wallis) and the DRTB-HDT trial (#847465; PI - Wallis) funded by the European Union Horizon 2020 programme. Each of these trials involve different patient populations. The METRIF study will compare metformin added to standard treatment for drug-sensitive pulmonary TB in non-diabetic adults without HIV in India. The METHOD trial will compare metformin added to standard treatment for drug-sensitive pulmonary TB in non-diabetic, HIV-positive adults in South Africa. Finally, the DRTB-HDT study will compare antimicrobial regimens for rifampin-resistant pulmonary TB with or without the addition of metformin or CC11050 in non-diabetic adults with or without HIV in Germany, Romania, Georgia, and

Table 7.1 Clinical trials testing the efficacy of metformin in TB patients

Trial	Approach & population	Clinical site(s)	Endpoints	Drug & dose
METRIF	Randomized open-label; non-diabetic HIV- with newly diagnosed drug-susceptible PTB	Five sites in India	Time to sputum culture conversion	Metformin 1000 mg daily for first 2 months
METHOD	Randomized open-label; non-diabetic HIV+ with newly diagnosed drug-susceptible PTB	Single site in South Africa	Safety & tolerability; time to culture conversion; lung function; occurrence of IRIS	Metformin; 1000 mg daily for first 3 months
DRTB-HDT	Randomized open-label; non-diabetic ±HIV with RIF-resistant PTB	Germany, Moldova, Romania, Georgia, Mozambique, South Africa	Time to culture conversion; lung function	Metformin 2000 mg daily or CC11050 200 mg twice daily; for 6 months

Moldova. Outcomes for all three trials include time to sputum conversion, while the METHOD and DRTB-HDT trials will also assess lung function. None of these studies are powered to provide a strong indication of the potential for treatment shortening. However, evidence of safety and tolerability, along with trends for outcomes indicative of HDT efficacy, should provide a foundation for future large-scale efficacy trials.

Can Metformin Rewire Fibrotic Resolution in TB?

Lung matrix destruction and fibrotic remodeling are clinically significant features of TB pathology, reflecting damage from host immunity. Respiratory failure and hemoptysis are the leading proximal causes of death in TB [64], while pulmonary impairment after TB afflicts roughly half of all TB survivors (reviewed in [62]). In the pre-antibiotic era, fibrosis of diseased lung benefitted the host by reducing dead space ventilation and creating an environment unfavourable for relapse. However, this response becomes detrimental when infection is sterilized by antibiotics. Fibrosis also hampers antibiotic penetration during TB treatment, reducing efficacy and promoting resistance. Since goals for HDT are accelerated lesion sterilization and mitigation of lung damage, this raises the question of metformin mediated reprograming of fibrosis in TB. Metformin decreases lung inflammation in *Mtb*-infected mice [33] and guinea pigs (R. Basaraba, personal communication), including reduced expression of matrix metalloproteinases (MMPs) that are implicated in TB-related lung injury [65]. Importantly, elevated MMP levels are associated with impaired lung function in TB [66], and metformin use is associated with reduced plasma MMP levels in diabetic TB patients [61]. A recent study demonstrated antifibrotic effects of metformin in the mouse bleomycin model [67, 68], where it deactivated myofibroblasts in an AMPK-dependent manner and reversed established fibrosis. Metformin was also shown to mitigate radiation-induced pulmonary fibrosis in rats [69]. Recent clinical studies found that a restrictive ventilatory defect indicative of fibrosis was the most common lung function abnormality in TB survivors [70, 71], supporting the idea that antifibrotic effects of metformin could be clinically relevant.

Safety and Drug-Drug Interactions During Metformin Usage in TB Patients

Metformin is the most widely prescribed treatment for T2D worldwide and has an excellent record of safety. Nonetheless, metformin carries a black box warning about lactic acidosis (LA), with a mortality rate approaching 50%. The black box warning in the FDA-approved metformin package insert states in part: “The

reported incidence of LA in patients receiving metformin hydrochloride is very low (approximately 0.03 cases/1000 patient-years, with approximately 0.015 fatal cases/1000 patient-years). There were no reports of LA in more than 20,000 patient-years exposure to metformin in clinical trials. Reported cases have occurred primarily in diabetic patients with significant renal insufficiency, including both intrinsic renal disease and renal hypoperfusion, often in the setting of multiple concomitant medical/surgical problems and concomitant medications". Despite the black box warning, the strength and even the existence of this association has been questioned. Some authorities hold that LA occurring in patients taking metformin is attributable to other factors commonly associated with diabetes (sepsis, dehydration, renal failure). A Cochrane review of 347 comparative trials and cohort studies with a combined 70,490 patient-years of metformin exposure identified no cases of fatal or non-fatal LA [51].

Conclusions

A substantial body of retrospective studies have described protective effects of metformin against several infectious diseases (both viral and bacterial) in animal models and in humans, leading to the consideration of metformin as an adjunctive therapy for use in combination with antimicrobials [72]. Based on the preclinical mouse and guinea pig data and retrospective human data, metformin is considered a top candidate in the TB-HDT pipeline. While there is a sufficient basis of preclinical and retrospective clinical evidence to justify the prospective clinical trials listed in Table 7.1, a detailed mechanistic understanding of its protective effects in TB is lacking. Filling this knowledge gap will strengthen the interpretation of trial data and likely identify pathways that may be more specifically targeted with agents having better efficacy and tolerability than metformin.

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Chapter 8
Statins as Host-Directed Therapy for Tuberculosis

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Introduction

Statins are a class of drugs that inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway. Since cholesterol biosynthesis is one of the branches of this pathway, statins lower cholesterol levels. In particular, these drugs are effective in lowering low-density lipoprotein (LDL) cholesterol and are commonly used for secondary prevention in patients with a history of myocardial infarction, as well as for primary prevention in people at high risk for cardiovascular disease. Statins also possess broad immunomodulatory and anti-inflammatory properties, and their use has been associated with reduced dengue virus replication and reduced risk of lung cancer [1–4]. Retrospective cohort studies also have shown that statin users experienced reduced sepsis-related morbidity and mortality [5, 6], reduced incidence of community-acquired pneumonia (CAP) [7, 8], and increased survival following CAP [9]. Currently, a number of statins are on the market, including atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. Several of these statins have been shown to enhance host cell clearance of intracellular bacteria. For example, atorvastatin and lovastatin reduce the growth of *Chlamydia pneumonia* and *Salmonella enterica* in macrophages and in mouse models of infection [10, 11]. Simvastatin increases host defenses against listeriosis in mice by targeting LLO-dependent escape of *Listeria monocytogenes* [12]. Lovastatin and simvastatin contribute to reducing *Borrelia burgdorferi* burden and altering the murine immune response to favor clearance of spirochetes in a mouse model of Lyme disease [13].

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Host-Dependent, Anti-TB Activity of Statins: Preclinical Studies

In early studies, fluvastatin was found to induce the release of Th1 cytokines in *Mycobacterium tuberculosis* (Mtb)-infected and uninfected macrophages, and to promote the activation of caspase 1 [14]. In 2009, Lü et al. investigated the effect of endogenous cholesterol on lipid raft formation and activation of gamma-delta (γδ) T cells in human peripheral blood mononuclear cells (PBMCs) with and without Mtb antigen stimulation [15]. They observed that lovastatin and fluvastatin inhibited tyrosine phosphorylation and expression of monosialotetrahexosylganglioside (GM1) and CD69 in γδ T cells stimulated *in vitro* with Mtb antigens. This stimulation yielded higher release of Th1 cytokines and promoted the activation of caspase 1, suggesting that inhibition of HMG-CoA reductase may be immune-protective with therapeutic potential against TB.

However, none of these studies evaluated the effect of statins on mycobacterial growth *in vitro* and *in vivo* models. In 2014, Parihar et al. reported that mononuclear cells and macrophages isolated from patients receiving statin therapy for familial hypercholesterolemia showed increased resistance to Mtb infection [16]. Further, simvastatin therapy in Mtb-infected mice reduced dissemination from the lungs and bacterial burdens in the spleen and liver were significantly (up to ten-fold) lower relative to those in untreated control animals. The lungs of infected animals showed a 1.5-fold reduction in bacterial burden following simvastatin therapy for 6 weeks, which could be further improved to a three-fold reduction when rosuvastatin, which has a longer half-life, was used. In the same study, statins were found to enhance macrophage-based killing of Mtb by promoting phagosomal maturation and autophagy [16]. Corroborating the findings of Parihar et al., Skerry et al. found that simvastatin lacks direct tuberculocidal activity, but promotes killing of intracellular Mtb by macrophages and enhances the bactericidal activity of INH in macrophages [17]. Moreover, simvastatin and atorvastatin were shown to increase rifampin-mediated killing of Mtb in macrophages [18]. Another study found that very high concentrations of simvastatin showed anti-Mtb activity (MIC = 100 μg/ml), which was enhanced with vancomycin 10 μg/ml (MIC = 50 μg/ml) [19].

Recently, Dutta et al. conducted a preclinical study aimed at comparing the bactericidal activities of the standard TB regimen (rifampin, isoniazid, pyrazinamide and ethambutol given at human-equivalent doses; RHZE) with or without escalating doses of pravastatin against chronic TB infection in BALB/c mice. Antibiotics were given five times weekly by esophageal cannulation for 8 weeks (corresponding to the intensive phase of TB treatment in humans) beginning 6 weeks after infection. Treatment with RHZE plus pravastatin at doses ranging from 30 to 180 mg/kg demonstrated a dose-dependent increase in bactericidal activity, reducing lung bacillary counts by 0.2–0.6 $\log_{10}$, 0.3–0.6 $\log_{10}$ and 0.3–0.8 $\log_{10}$ compared to RHZE alone after treatment for 2 weeks, 4 weeks and 8 weeks, respectively. The degree of lung inflammation correlated with the bactericidal activity of each drug regimen after 8 weeks of treatment.

In the same study, the adjunctive antitubercular activities of simvastatin and pravastatin were tested in C3HeB/FeJ mice, which, unlike BALB/c and C57BL/6 mice, develop human-like necrotic granulomas in the lungs following aerosol infection with Mtb [20–24]. The mice were infected with ~50 bacilli of Mtb H37Rv and 6 weeks later were treated with one of the following regimens: (1) No treatment (negative control); (2) Human-equivalent doses of the first-line regimen (RHZE) (positive control); (3) RHZE + simvastatin 90 mg/kg; (4) RHZE + pravastatin 50 mg/kg; (5) RHZE + pravastatin 90 mg/kg. After 8 weeks of treatment, mice receiving human-like exposures of statin adjunctive therapy had significantly reduced lung bacillary burdens relative to control mice receiving RHZE alone. Thus, relative to the control regimen, adjunctive therapy with simvastatin 90 mg/kg, pravastatin 90 mg/kg, and pravastatin 50 mg/kg further reduced lung bacterial counts by 1.28 $\log_{10}$ ($p < 0.0001$), 1.16 $\log_{10}$ ($p < 0.01$), and 0.78 $\log_{10}$ ($p < 0.05$), respectively [24–26].

The treatment-shortening potential of statin adjunctive therapy has been tested in the standard mouse model of TB chemotherapy [27]. After 6 weeks of treatment, simvastatin adjunctive therapy led to a 1.4 $\log_{10}$ greater reduction in lung bacillary counts relative to the standard regimen alone (rifampin/isoniazid/pyrazinamide, RHZ) given at human-equivalent doses. The addition of simvastatin shortened the time required to achieve lung-culture negativity from 4.5 months to 3.5 months. After 3.5 months of treatment relapse rates were 50% (5/10 mice) and 20% (2/10 mice) in the RHZ and RHZ + simvastatin groups, respectively. No relapses were observed in either group after 4.5 months of treatment.

Retrospective Clinical Cohort Studies

A recent review summarizes the evidence from retrospective clinical studies investigating the effects of statin use on TB incidence [28]. In 2014, Kang et al. evaluated the effect of statins on the risk of developing TB among patients with newly diagnosed diabetes mellitus type 2 (DM2) in South Korea [29]. Relative to non-TB patients, statin use was less frequent among TB patients (19.2% vs. 33.6%). However, after adjustment for potential baseline confounders (age, sex, history of silicosis, malignancy, HIV/AIDS, chronic kidney disease, use of systemic corticosteroids, comorbidities [e.g., dyslipidemia, hypertension, angina, myocardial infarction, cerebrovascular disease, peripheral artery disease, and retinopathy], and history of hospitalization), statin use did not alter the risk of developing TB in patients with DM2 (hazard ratio [HR], 0.98; 95%CI 0.89–1.07).

In 2015, Lee et al. studied TB incidence in Taiwanese patients with DM2, hypertension, and dyslipidemia, as well as any potential effects of therapies for these conditions [30]. Cox proportional hazard regression models determined that statin users had a lower risk of developing active TB, with a risk ratio of 0.76 (95% CI, 0.60–0.97). The level of adherence to statin therapy and the statin dose received by patients were not reported in this study.

In 2016, Lai et al. performed a nested case-control study to examine whether statin therapy decreases the risk of incident TB using the Taiwan national health insurance program database [31]. In this study, the duration of statin use was inversely associated with the risk of active TB. Certain potentially confounding variables, such as body mass index and smoking and their effects, could not be evaluated in this database.

In 2017, Liao et al. conducted an age- and sex- matched case-control study of patients aged ≥20 years with recently diagnosed pulmonary TB in Taiwan [32]. Multivariable logistic regression analysis showed that subjects receiving atorvastatin had a lower probability of developing active TB (0.56, 95% CI 0.46, 0.68), although the data could not fully account for the effects of residual confounding variables (e.g., socioeconomic status).

Another retrospective cohort study conducted in Taiwan in 2017 revealed a reduced risk of TB disease among statin users by multivariate analysis (HR: 0.53; 95% CI, 0.47–0.61; $P < .001$) [33]. Compared with the matched control group, statin use showed a dose-response relationship with risk of incident TB (<180 cumulative defined daily doses [cDDDs]: HR, 1.06; 95% CI, 0.91–1.24; $P = .477$; 180–365 cDDDs: HR, 0.57; 95% CI, 0.45–0.72; $P < .001$; >365 cDDDs: HR, 0.27; 95% CI, 0.22–0.33; $P < .001$).

A retrospective study by Yeh et al. in 2018 investigated the effects of statin use on risk of TB and CAP among patients with reversible and nonreversible obstructive airways disease in Taiwan [34]. Cox proportional regression analysis with time-dependent variables showed that statin users had a lower risk of TB (adjusted hazard ratio (aHR) 0.49, 95% confidence interval (CI) 0.34–0.70) and pneumonia (HR 0.52, 95% CI 0.41–0.65) than nonusers, regardless of age, sex, comorbidities, and inhaled corticosteroid or oral steroid use.

Finally, Pan et al. investigated the use of statin vs. non-statin lipid-lowering agents on the risk of various infectious diseases in patients with diabetes [35]. They found that compared with non-statin drugs, statin use was specifically associated with a decreased risk of TB (low-potency statin users, aHR: 0.692; 95% CI: 0.455–1.053; high-potency users, aHR: 0.491; 95% CI: 0.241–0.999).

A recent meta-analysis of data from nine cohort studies [29–34, 36–38] reported that statin use was associated with reduced active TB disease (risk ratio [RR]: 0.60, 95% confidence interval [CI]: 0.45 to 0.75, $p < 0.001$) [39]. However, the observational studies described above have several limitations. First, inherent to all retrospective data analyses, it is possible that the authors did not adequately control for all confounding factors, potentially leading to erroneous rejection of the null hypothesis regarding statin use vs. nonuse. Second, all studies were based in Taiwan or South Korea, and the findings may not be readily applicable to other populations. Finally, despite the widespread use of statins, there are currently no retrospective data available on the relationship between statin use and microbiological or clinical outcomes in patients receiving treatment for active TB.

The Mode of Action of Statins as Adjunctive Therapy for TB

Although the molecular mechanisms responsible for the anti-TB, host-directed activities of statins require further investigation, this class of drugs appears to have pleiotropic effects on the vascular and immune systems [40].

Simvastatin was shown to inhibit receptor for advanced glycation end-products (RAGE) expression in atherosclerotic plaques by decreasing MPO-dependent AGE generation [41]. Statins also increase soluble RAGE level by inducing RAGE shedding, which might help to prevent the development of RAGE-mediated pathogenesis. Statins improve cholesterol efflux from foam cells of the arterial wall and block the harmful effects of AGE on macrophages by suppressing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity through inhibition of geranylgeranylation of Rac-1 [42]. Statins also influence heme oxygenase 1 (HO-1) activation in cardiovascular diseases by regulating multiple signaling pathways, such as activator protein (AP)-1, protein kinase G (PKG), extracellular matrix-regulated kinase (ERK), p38 MAPK or NFκB in vascular wall cells [43]. Finally, statins were shown to modulate oxidized LDL-mediated histone modifications and gene expression in cultured human endothelial cells [42].

Recent reports suggest that statin-mediated effects are not limited to cardiovascular diseases, and that these drugs might be used to treat osteoporosis, Alzheimer disease, rheumatoid arthritis, acute lung injury, and chronic obstructive pulmonary disease (COPD) [44]. The pleiotropic effects exerted by statins occur in various cell types of the immune system, and some effects have been described that could paradoxically attenuate or inhibit the immune response [45]. Statins alter the function of innate and adaptive immune cells, including macrophages, dendritic cells, T cells, and endothelial cells [46], and also prevent induction of trained immunity [47]. In addition, they affect endothelial cell cytoskeletal rearrangement, NADPH oxidase activity, and nitric oxide generation, as well as endothelial cell gene expression, which are relevant to the pathobiology of acute lung injury [42]. By inhibiting mevalonate synthesis, statins also inhibit the production of other isoprenoid molecules, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These molecules serve as lipid labels for the post-translational modification of several proteins, including G protein gamma subunits and small GTP-binding proteins, such as Ras, Rho, Rab, Rac, Ral or Rap [48–50]. Statins also inhibit IκB degradation, MMP-9 activity, TNF-α production, and cell spreading by the upregulation of tetraspanins, especially CD9 [51].

The precise mechanism(s) by which statins exert their host cell-mediated, antitubercular activity is a topic of intense investigation. Cholesterol biosynthesis and transport may play a crucial role in the adaptation of Mtb within host tissues [52, 53], and its inhibition by statins could potentially alter protective immunity, thus altering disease outcome in the infected host [16]. In *ex vivo* studies, statins reduced intracellular Mtb burden in human macrophages [16]. The process of autophagy is important for control of Mtb growth *in vivo*, as well as for preventing excessive inflammation in the host [54]. Previous reports have highlighted the importance of

autophagy for the full activity of isoniazid and pyrazinamide against intracellular Mtb through the release of reactive oxygen species (ROS) by infected host cells [55, 56]. Exposure of Mtb-infected macrophages to simvastatin promotes phagosome-lysosome fusion and autophagy [16]. Recent data by Dutta et al. showing reduced phagosome acidification and proteolysis of macrophages following pravastatin exposure are consistent with an activated phenotype of pravastatin-treated macrophages. Decreased proteolysis can promote antigen presentation [57, 58], which is beneficial for bacterial clearance, while the observed delay in acidification reflects a balance in the production of ROS (which consume protons) and phagosomal acidification upon immune cell activation [57, 59]. Guerra-de-Blas et al. recently observed that simvastatin promotes apoptosis, autophagy, and the expression of co-stimulatory molecules in monocytes and increases the proportion of natural killer T cells [60]. Statins have also been shown to inhibit transforming growth factor beta (TGF-β) [61, 62]. Both production and bioactivation of TGF-β take place at sites of Mtb infection, suggesting that inhibitors of TGF-β signaling might have a role as adjunctive anti-TB therapies [63]. Bruiners et al. recently demonstrated that simvastatin inhibits mechanistic target of rapamycin complex 1 (mTORC1) activity and regulates transcription factor EB (TFEB) nuclear translocation to induce autophagy and lysosomal biogenesis [64].

Conclusion

Retrospective clinical data suggest that statin use is associated with a reduced risk of incident TB, and preclinical data suggest that adjunctive statin therapy may shorten the duration of curative treatment for drug-susceptible pulmonary TB, perhaps by promoting clearance of intracellular bacilli by macrophages. However, a number of important questions remain to be addressed before introducing statins as adjunctive host-directed therapy for TB in the clinical setting, including: (1) whether the anti-TB activity is a general class effect or statin-specific; (2) the precise dosing of statin, particularly given the known drug interactions with the key sterilizing drug rifampin; and (3) their potential utility in patients with HIV co-infection and other forms of immune deficiency. A two-stage randomized clinical trial, Statin Adjunctive Therapy for TB. (StAT-TB; National Clinical Trial (NCT) Identifier # NCT03456102), is currently underway to determine: (1) the safety/tolerability and pharmacokinetics of pravastatin co-administered with the first-line regimen to patients with drug-susceptible, pulmonary TB; and (2) the potential adjunctive activity of pravastatin in these patients using microbiological and clinical endpoints, including median time to sputum-culture negativity and lung function during TB treatment.

Potential Conflicts of Interest
All authors: No reported conflicts of interest.
All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Chapter 9
Antimycobacterial Attributes of Mitochondria: An Insight into Host Defense Mechanisms

Rikesh K. Dubey and Apoorva Narain

Introduction

Tuberculosis (TB) is a major global health problem caused by *Mycobacterium tuberculosis* (MTB). According to the World Health Organization (WHO), nearly one third of the global population is infected with TB [1]. MTB is inhaled in the form of aerosols and primarily infect the alveolar macrophage inside the lungs. As the bacteria have co-evolved with humans over the course of thousands of years, MTB has developed several mechanisms to escape from the host defense machinery and replicate and survive intracellularly. MTB secrete various virulence factors, which modulate macrophage functions by preventing phagosome-lysosome fusion, blocking phagosomal acidification, manipulating host cell proteins, and, perhaps most importantly, by regulating cell death pathways through targeting of cellular organelles, such as mitochondria [2, 3].

In recent years, mitochondria have emerged as one of the important target organelles for pathogenic bacteria. The mitochondrion is a semi-autonomous organelle, which is present in almost all eukaryotic cells and is responsible for numerous cellular processes, such as ATP production via oxidative phosphorylation, ion homeostasis, calcium storage, biosynthesis of fatty acids and regulation of cell death pathways. Although little is known about the role of mitochondria in MTB pathogenesis, their involvement has been reported in many chronic diseases, such as diabetes mellitus, Alzheimer's disease and cancer. Modulation of host cellular activity by pathogens represents a common virulence strategy. In the context of TB, alveolar macrophages use various anti-mycobacterial defenses, including phagosomal maturation, phagolysosomal fusion, oxidative stress and induction of apoptotic cell death pathways to limit the growth of MTB, but the mycobacteria have evolved various

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strategies to overcome most host defenses in order to survive chronically inside the lungs. The fate of the infected cell depends on the fine balance between anti-mycobacterial responses of the host macrophage and the various evasion mechanisms of MTB, which is highly adapted to its human host.

Apoptosis

After MTB infection, two forms of cell death are commonly observed, necrosis and apoptosis. Necrosis is defined by cell lysis and generally used by the MTB to evade host defenses and spread the infection. In contrast, apoptosis is a programmed cell death pathway and an important innate immune mechanism to limit the growth and dissemination of MTB. Apoptosis is characterized by condensation of the nucleus and cytoplasm, membrane blebbing and endonucleosomal DNA damage. Apoptosis plays a pivotal role in the progression of diseases like cancer, inflammation, neurodegenerative disorders and viral diseases. A study by Molloy et al. has very methodically demarcated the differences between necrosis and apoptosis and how these two cell death pathways are induced by the mycobacteria. Peripheral blood monocytes were exposed separately to hydrogen peroxide (necrosis inducer) and ATP (to induce apoptosis) and infected with viable *Mycobacterium bovis* BCG. Infected macrophages were killed in both conditions, but the viability of the mycobacteria was only reduced in apoptotic cells. This study made it evident that the two forms of cell death have completely different outcomes during mycobacterial infection [4].

Mitochondria play a pivotal role in the regulation of apoptotic cell death [5]. Apoptosis is initiated either through extrinsic (death receptor-mediated) or intrinsic (mitochondria-mediated) pathway [6]. The extrinsic pathway is activated by death signaling ligands, such as TRAIL, through death receptors DR4 and DR5, which ultimately activate the initiator caspase-8, thereby propagating the apoptotic signal by cleaving downstream effector caspases, like caspase-3 [7, 8]. In contrast, the intrinsic pathway involves the release of apoptogenic factors, i.e., cytochrome C (CytC), Smac/DIABLO, Omi/HtrA2 or endonuclease G from the intermembrane space of the mitochondria to the cytosol, which promotes apoptosis [9, 10]. The released CytC forms a complex or apoptosome with APAF1 and caspase-9. This complex cleaves and activates caspase-9 by hydrolyzing ATP, which in turn activates caspase-3, ultimately resulting in apoptotic cell death [11]. Both of these pathways converge upon the same effector pathway, i.e., caspase-3 activation. A large number of cellular stimuli, such as DNA damage, ischemia, oxidative stress and microbial infection trigger apoptosis by the mitochondria-mediated intrinsic pathway. Cleavage and activation of BID (BCL-2 protein) is a characteristic of the intrinsic apoptotic pathway. However, it is also known that BID is cleaved and activated by caspase-8, thus allowing cross-talk between the intrinsic and extrinsic pathways. The BCL-2 group of proteins play a significant role in mitochondrial biology by regulating the permeability of mitochondria through their proapoptotic and antiapoptotic actions [12].

MTB Infection and Modulation of Mitochondrial Functions

The mitochondria is a multifaceted organelle with control over many vital cellular pathways. Mitochondria play significant roles in cell cycle control, metabolic regulation, aging, development and in the immune response to various diseases. Several viral and bacterial proteins are known to modulate mitochondrial functions to promote microbial survival and dissemination of infection. The effects of mycobacterial proteins and their interactions with the mitochondrial network are largely unknown. A study conducted to investigate the effect of MTB infection on mitochondria in A549 alveolar epithelial cells has shown that MTB infection alters mitochondrial morphology, mass, fragmentation and distribution. In contrast, the avirulent strain *M. bovis BCG* was unable to induce the same effects [13]. Using an ESAT-6 deletion mutant of MTB, this study showed that these mitochondrial effects were mediated by ESAT-6, an important superantigen of MTB. A similar pattern of mitochondrial disruption and network fragmentation was also observed in epithelial cells infected with *Listeria monocytogenes* [14]. Recently, a host immune-responsive gene, *irg*1 (also called *acod*1), which encodes a mitochondrial enzyme induced under inflammatory conditions to produce a TCA cycle intermediate, itaconate, has been found to be regulated by MTB [15]. Deletion of the *irg*1 gene enhanced the pathological inflammatory response in mice infected with recombinant MTB strain, causing severe lung injury and acclerated death [15]. The MTB protein Cpn60.2 has been shown to interact with the mitochondrial protein mortalin to inhibit apoptosis [16]. Although apoptosis adversely impacts the long-term survival of MTB inside alveolar macrophages, a few studies have reported that, under certain circumstances, MTB also promotes apoptosis through host-pathogen interactions involving 19 kDa, lipoarabinomanan (LAM), TLR2, PE_PGRS33 and HBHA [3, 17, 18]. MAV2054, a *M. avium* protein, on being overexpressed in *M.smegmatis*, has been also shown to promote apoptosis in bone marrow-derived macrophages by dissipating the mitochondrial membrane potential and increasing ROS production [19].

Mitochondrial proteome analysis of MTB H37Rv-infected cells has revealed inhibition of mitochondrial-mediated apoptosis through manipulation of the host's cellular machinery. Validating the same, attenuation of mitochondrial function was coupled with vigorous activation of bactericidal mechanisms in MTB H37Ra-infected cells [20]. Loss of mitochondrial membrane potential (MMP) by release of proapoptotic factors Cyt C and Smac/DIABLO (which translocate from the intermembrane space to the cytosol), apoptosis inducing factor (AIF) and endonuclease G (EndoG) (which play an important role in apoptotic cell death), leads to the arrest of normal cellular biosynthetic and bioenergetic functions [21]. Any mechanism which is responsible for MMP loss leads to structural and functional collapse of the mitochondria and cell death. Various studies have shown that MTB infection affects the MMP, thereby deciding the fate of the cell. MTB H37Rv infection has been shown to cause an increased MMP, in comparison to infection with its avirulent counterpart H37Ra.

After MTB infection of cells, mycobacterial proteins PE_PGRS33, HBHA and MAV2054 localize to the mitochondria and affect mitochondrial functions, such as altering MMP, energy production and promotion or inhibition of apoptosis [17–19]. Studies have shown that virulent MTB causes disruption of the inner membrane of mitochondria, leading to necrosis. The fate of the MTB-infected macrophage concerning apoptosis and necrosis is dependent on the intramitochondrial concentration of ions, especially Ca^{2+}. When exposed to a calcium ionophore (which enhances intramitochondrial calcium concentration), MTB-infected cells did not proceed towards necrosis; instead the mitochondrial permeability transition (MPT) was stabilized and activation of cytochrome C and caspases was reduced, thus favoring antimycobacterial activity of the cells. Upon treatment with a calcium uniporter inhibitor, the MTB-infected cells infected showed increased mitochondrial swelling, release of cytochrome C, decreased MPT, and compromised antimycobacterial activity [22]. Therefore, mitochondrial membrane integrity is a critical factor in determining the host's defense against MTB.

Other Mitochondrial Factors Involved in Antimycobacterial Mechanisms

Cyclophilin D

Cyclophilin D (CypD) belongs to the cyclophilin class of proteins, which have prolyl isomerase activity and catalyze isomerization at proline residues (trans to cis), thereby assisting in protein folding. PPIase is a cis-trans isomerase present in the mitochondrial membrane matrix, which has been implicated in the production of ROS by modulating the mitochondrial permeability transition pore (MPTP) size [23].

Upon mycobacterial infection of the host, T lymphocytes, which are primarily involved in preventing the dissemination of MTB, switch from an OXPHOS state to aerobic glycolysis. This change of state from resting to active, even in the presence of oxygen, is known as "Warburg effect". This increased glycolysis also leads to increased ATP production by T lymphocytes [24, 25]. CypD lowers ATP levels by increasing MPTP, which depolarizes the mitochondrial inner membrane and enhances ROS production. This process promotes necrosis, which is beneficial to MTB and its survival inside the host. CypD regulates mitochondrial OXPHOS by making use of the proton motive force generated by complexes I-IV. Many mitochondrial proteins, such as p53, PPARα, GSK-3β, BCL2 bind directly to Cyp D. Studies have shown that inhibition of CypD activity renders mice more susceptible to MTB infection [25]. AMP-activated protein kinase (AMPK) controls activated T lymphocytes by monitoring levels of glucose. T cells deficient in AMPKα1 show reduced response to pathogenic challenge and glucose limitation [24].

mROS

Mitochondrial ROS (mROS) are generated on the inner membrane of the mitochondria during oxidative phosphorylation. Although excessive amounts of ROS are damaging to mitochondrial components and reduced amounts are associated with the metabolic response towards hypoxia, mROS are involved in many different cellular signaling pathways, most importantly in autophagy and inflammation [26]. mROS facilitate the cellular response towards any anomaly encountered, such as sterile damage, metabolic imbalance and infection. Following the engulfment of bacteria, the oxidative burst inside antigen-presenting cells (APCs) leads to the fusion of phagosome and lysosome and bacterial killing due to the highly acidic pH inside the phagolysosome [26]. MTB specifically targets the process of phagosome lysosome fusion inside the macrophage, and adapts its metabolism in order to survive in the infected host for prolonged periods of time.

In order to eradicate TB infection, consideration must be given to supplement standard antimycobacterial therapy with host-directed immunotherapy. Metformin (MET), the subject of another chapter of this book, could be one such potential candidate. Two parallel studies conducted in 2015 predicted two different targets for MET. One reported its role in targeting MTB by enhancing host immune mechanisms, and the other a potential role in directly targeting the bacteria [26, 27]. MET may be accumulated within mitochondria 1000-fold more than in the extracellular medium. The drug suppresses the function of mitochondrial complex I (NADH dehydrogenase) of the respiratory chain, leading to changes in AMP: ATP, ADP: ATP and NAD^+: NADH ratio [28]. This increases the generation of mROS in the host. Such high concentrations of redox ions have bactericidal activity against mycobacteria. In addition, autophagy, which is essential in containing intracellular bacteria like MTB, is regulated by AMPK signaling, which is induced my MET. AMPK signaling restricts the immunopathology of TB by enhancing the bactericidal activity of other antimycobacterial drugs like isoniazid and ethionamide [29]. Two of the first line TB drugs, namely, pyrazinamide and isoniazid, stimulate the generation of mROS, thereby activating autophagy in the infected host. The ROS-scavenging agents N-acetyl cysteine and glutathione have been shown to suppress the activity of MET and increase the intracellular growth of MTB [26].

Mitochondrial Unfolded Protein Response

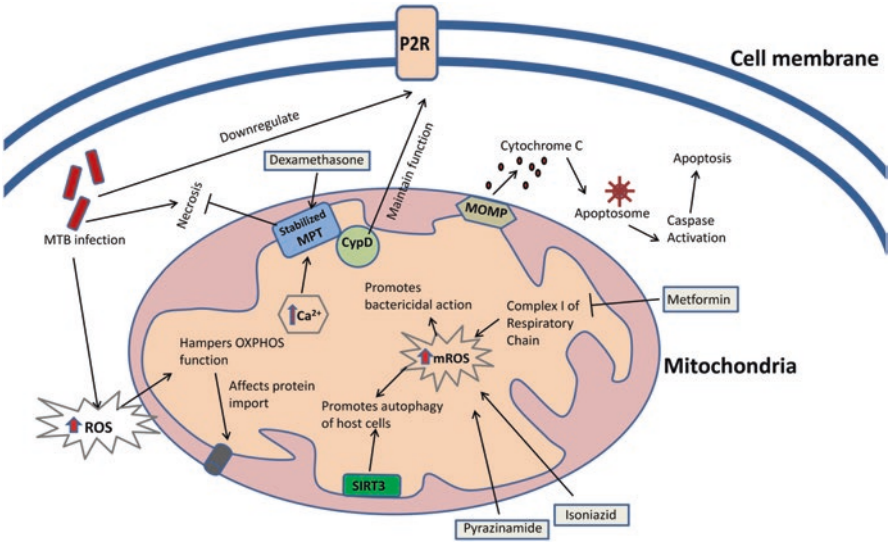
Mitochondrial unfolded protein response (upr^{MT}) is a transcriptional factor that repairs defective mitochondria and also promotes cell survival. It relieves mitochondria of its metabolic stress by functioning in two different ways. First, it increases the breakdown of amino acids and glycolysis, and second, it dampens expression of genes encoding TCA cycle and OXPHOS factors [30]. Mitochondria in a healthy state are the powerhouse of the cell, regulating a cascade of cellular signaling,

including apoptosis. However, in some scenarios, mitochondrial integrity may be compromised. For example, MTB infection leads to excessive ROS generation, which hampers the functioning of OXPHOS genes required for the four respiratory complexes and ATP. Defective OXPHOS negatively affects the import rate of mitochondrial proteins by either interfering with mitochondrial chaperones or by disturbing the proton gradient [30, 31]. In *Caenorhabditis elegans,* a model organism frequently used to study the function of upr^{MT}, this transcriptional factor was shown to be regulated by another transcription factor, ATFS-1 [30, 31].

Mitochondria against Mycobacteria

The role of mitochondria as an anti-mycobacterial organelle is poorly characterized. However, some recent findings have provided preliminary insights. Kim et al. found that the mitochondrial protein SIRT3 (sirtuin3) coordinates mitochondrial function and autophagy activation to promote defense against mycobacteria through peroxisome proliferator activated receptor alpha (PARA). *sirt3*$^{-/-}$ mice infected with MTB or *M. bovis* BCG had increased bacillary load and granulomatous lesions in the lungs, as well as increased mortality [32]. Structural and functional restoration of mitochondria limits intracellular MTB survival and increases host clearance of bacilli [33]. Similarly, stabilization of MPT keeps mitochondria from releasing cytochrome C into the cytosol, enhancing the antimycobacterial activity of MTB H37Ra-infected macrophages [34]. MPT protects mitochondria from mycobacterial damage by favoring apoptosis [34]. The precise role of intact mitochondrial membrane integrity during MTB-induced cell death as a defense against mycobacteria is not well understood.

Purinergic receptors (P2Rs) are plasma membrane molecules that are involved in the wide array of cellular functions, like apoptosis, vascular reactivity, cytokine secretion. Studies performed with MTB so far have elaborated the role of P2X as a host defense against mycobacteria. Two other P2Rs, P1 and P2Y, are G-protein-coupled receptors (GPCRs), whereas P2X are ligand-gated ion channels, which are activated by ATP. P2X receptors play an important role in macrophage activation and apoptosis. MTB downregulates the functioning of P2Rs [34]. As discussed above, increased intramitochondrial calcium ion concentration $[Ca^{2+}]_m$ stabilizes MPT, lowers caspase activation, and decreases mitochondrial cytochrome C to prevent necrosis, which is a mechanism used by MTB to evade host immune defenses [22]. CypD, a mitochondrial matrix protein, also plays a pivotal role in preventing necrosis by regulating MPTP. Genetic blockade of CypD in the zebrafish model of TB prevented macrophage necrosis and enhanced antimycobacterial immunity [35]. A study conducted by Grab et al. has shown that dexamethasone (a corticosteroid), the only adjunctive TB chemotherapy in common clinical use, inhibits necrotic cell death in MTB-infected cells [36]. The abrogation of necrotic cell death in MTB-infected cells is mediated by MPT through mitogen-activated protein kinase phosphatase 1 (MKP-1)-dependent dephosphorylation of p38 MAPK [36].



Schematic representation of antimycobacterial aspects of mitochondria: ROS Reactive oxygen species, CypD Cyclophilin D, MOMP Mitochondrial outer membrane permeabilization, MPT Mitochondrial membrane permeability transition, mROS Mitochondrial reactive oxygen species, P2R Purinergic receptors, Ca^{2+} Mitochondrial calcium concentration, SIRT3 Sirtuin 3, mitochondrial protein, OXPHOS Oxidative phosphorylation system, Metformin Proposed as a candidate adjunctive host-directed therapy for tuberculosis, Dexamethasone Only adjunct anti-tuberculosis drug, Isoniazid and Pyrazinamide First line anti-tuberculosis drugs

Future Perspectives

The role of mitochondria in viral and neurodegenerative diseases is well established, although their role in host defense against tuberculosis remains relatively unexplored. Emerging evidence supports the fact that mitochondria regulate innate immune responses against intracellular pathogens; however, the mechanisms underlying this function are largely unknown. Mitochondrial morphology and function appear to play a crucial role in antimycobacterial defense. The available literature shows that MTB infection targets mitochondria in a number of ways, like altering mitochondrial size and shape, release of apoptotic factors, mitochondrial membrane potential and ATP levels. Mitochondrial membrane integrity appears to be one of the main targets of pathogenic bacteria. Loss of membrane integrity is thought to be involved in cell death. Identification of molecules or pathways able to restore mitochondrial structural and functional integrity could yield novel host directed-therapies for the management of TB. However, further

studies are required to discover the mechanisms used by mitochondria to control MTB infection.

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Part IV
Targeting Immune Cells

Chapter 10
Conventional and Unconventional Lymphocytes in Immunity Against *Mycobacterium tuberculosis*

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Introduction

Invasion of the human body by microorganisms leads to a myriad of immune cells being activated through diverse pathogen-derived stimuli. Understanding the cellular responses that are triggered and the involved interactive networks is essential for the design of new vaccines to enhance host protection and decrease immunopathology, as well as to the design of new treatment strategies such as host-directed therapies (HDT). Tuberculosis (TB) is the leading infectious disease in terms of overall global mortality [295]. Although it is well known that T cells play a crucial role in protection from TB disease and its pathogenicity, the exact T cell subpopulations involved remain incompletely defined. In addition, B cells, antibodies, and non-T cell lymphocyte populations such as NK and ILC cells play an additional role in the immune response to *Mycobacterium tuberculosis* (Mtb). In this chapter, we will discuss how these lymphocyte populations participate in the immune response to Mtb and how they may be involved in protective immunity. First, we briefly review recent studies focusing on conventional lymphocytes, including classically MHC-restricted $CD4^+$ T cells and $CD8^+$ T cells, as well as B cells. Thereafter, we will briefly discuss the recently discovered roles of NK and ILC cells and finally focus on the role of so-called unconventional or donor-unrestricted T lymphocytes [92]. These cells include: HLA-E-restricted T cells; MR1-restricted T cells (also known as mucosal associated invariant T (MAIT) cells, discussed in more detail in Chap. 15); CD-1-restricted T cells; and TCRγδ T cells.

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Unconventional T cells, as opposed to classical CD4+ and CD8+ T cells, recognize peptide-, lipid-, phospho- or metabolite-derived antigens presented via non-polymorphic molecules, and exist as relatively large populations capable of eliciting relatively rapid responses, positioning these cells at the interface of innate and adaptive immunity [54, 92, 135]. The activation of these unconventional lymphocytes has already been shown to play a significant role in protective immunity against TB. Unconventional immunity has drawn significant interest from vaccine and immunotherapy development not only in the field of infectious diseases but also in cancer immunology, allowing for an interdisciplinary approach to promote discovery and evaluation of novel vaccine and treatment strategies including HDT, *e.g.* by immune checkpoint inhibition [93, 135, 209, 255].

Repertoire and Functions of Conventional Lymphocytes in TB

MHC Class II-Restricted CD4+ T Cells in TB

The role of T cells as indispensable players in the control of Mtb infection in humans became apparent when the depletion of CD4+ T cells as a consequence of human immunodeficiency virus (HIV) infection appeared to correlate with reduced immunity against Mtb and high TB reactivation rates [154]. Even though not sufficient, activation of CD4+ T helper 1 (Th1) cells and the production of IFN-γ and TNFα is necessary for protective immunity against Mtb in mice and in humans, as evidenced by IL-12- or IFN-γ receptor signaling-deficient patients with increased susceptibility to non-tuberculous mycobacterial infections [12, 131, 172, 207, 293]. Largely based on these insights, IFN-γ and TNFα production by CD4+ T cells have been used widely as readout of successful immunity, e.g. following infection (INF-γ release assays (IGRAs)) or vaccination [76, 98, 129, 144, 202]. However, other studies have shown that IFN-γ production by Th1 cells is not sufficient for complete protection against Mtb-induced disease; indeed, numbers of circulating IFN-γ producing T cells are not an appropriate predictor of effective immunity after vaccination and could even be a correlate of bacterial load [18, 52, 77, 83, 136]. Supporting an important role for non-IFN-γ dependent mechanisms of protection, "long-term TB resister" individuals were found to recognize Mtb antigens by non IFN-γ-producing T cells, showing they were infected while remaining IGRA negative [168]. This data supports earlier work by Coppola *et al* in which *in vivo*-expressed (IVE)-TB antigens elicited significant T cell responses in latently infected (LTBI) individuals in the absence of IFN-γ production [51]. Furthermore, in the MVA85A vaccine (modified Vaccinia Ankara virus expressing antigen 85A (Ag85A)) phase 2B clinical trial in South Africa, BCG vaccinated infants received a booster vaccination with MVA85A. No additional protection against TB disease was observed in infants, even though they showed enhanced frequencies of IFN-γ-producing CD4+ T cells against Ag85A [273, 274]. Intriguingly, both placebo and MVA85A vaccinated infants who developed TB disease showed higher HLA-DR expression on CD4+ T cells before vaccination, likely as a result of inflammation-driven

activation, and this correlate of risk was validated in a cohort of Mtb-infected adolescents [75]. Similar results were obtained in rhesus macaques (RM) vaccinated with an Ag85A-expressing adenovirus which elicited robust and sustained antigen-specific IFN-γ-producing T cell responses in the lung without conferring protection to Mtb infection [55]. From recent mouse models, it has become clear that IFN-γ production by $CD4^+$ T cells represents only 30% of the total $CD4^+$ T cell-mediated control of bacterial loads in the lungs of mice, and that excess IFN-γ can even be detrimental to the control of TB disease [243, 246]. Increased numbers of circulating IFN-γ-producing Mtb-specific $CD4^+$ T cells were also observed in humans who developed TB reactivation after cancer immunotherapy with anti-immune checkpoint (PD-1) specific monoclonal antibodies, further associating a rise in IFN-γ production with increased severity of TB disease [9], and suggesting the importance of balanced inflammation and IFN-γ production in optimal control of Mtb infection and TB disease.

Recent studies in animal models have indeed identified non-IFNγ correlates and mechanisms of protection in Mtb infection. In Dijkman *et al*, IL-17 production by $CD4^+$ bronchoalveolar (BAL) cells and IL-10 production by Mtb-specific BAL cells were identified as the strongest correlates of protective immunity induced in the lungs of pulmonary-BCG vaccinated rhesus macaques that were challenged with Mtb under repeated limiting-dose conditions [67]. In mice, IL-17 and IL-22 production increased Mtb control by recruiting T cells within lymphoid follicles in the lung, leading to optimal macrophage activation [80, 83, 95, 146]. In humans, consistent with findings in animal models, active TB was associated with a weaker production of IL-17 by circulating $CD4^+$ T cells as compared with LTBI [126, 215, 251]. Whether this was due to cellular sequestration at the site of infection or reduced systemic activity could not be discerned. Recently, CD153 was identified as a TNF superfamily molecule expressed on the surface of Mtb-specific $CD4^+$ but not $CD8^+$ T cells [247]. Expression of CD153 was associated with protection against Mtb infection in mice and correlated with diminished bacterial loads in granulomas in non-human primates (NHP). Also in humans, active TB disease was associated with a reduced frequency of antigen-specific $CD4^+$ T cells expressing CD153 compared to individuals with controlled latent Mtb infection. This identifies CD153-expressing $CD4^+$ T cells as an important correlate and potential mechanistic mediator of protective immunity during Mtb infection, and suggests that cytolytic activity of $CD4^+$ T cells might contribute to their role in orchestrating protective immunity against Mtb [247].

MHC Class Ia Restricted $CD8^+$ T Cells in TB

The contribution of classical MHC class-Ia-restricted $CD8^+$ T cells to immunity against Mtb remains somewhat uncertain and subject of debate. Induction of $CD8^+$ T cell responses was associated with an elevated bacterial burden in mice and with an elevated risk of TB disease in infants [20, 198]. On the other hand, $CD8^+$ T cell depletion in a NHP model resulted in a weakened BCG vaccine-induced immune

control of Mtb, indicating a critical role for these cells in protection against TB [42]. In NHP co-infected with Mtb and SIV, protective immunity mediated by $CD8^+$ T cells correlated with control of TB disease [78]. Similarly, depletion of $CD8^+$ T cells in latently infected mice resulted in an increase in bacterial burden, suggesting a more important role for $CD8^+$ T cells during late stages of infection [219].

The use of peptide libraries and selected peptide-loaded HLA-A*0201 tetramers allowed the detection of polyfunctional $CD8^+$ T cells recognizing newly defined Mtb-derived epitopes during LTBI [276]. However, vaccine-induced high frequency of highly activated and cytotoxic Mtb-specific $CD8^+$ T cells provided no protection against Mtb challenge in mice [166]. Interestingly, immunodominant $CD8^+$ T cell responses to Mtb antigens can fail to recognize Mtb-infected target cells in both mice and in humans, suggesting a bacterial immune evasion mechanism [204, 300]. Indeed, a recent study proposed that the frequency of T cells with capacity to recognize infected macrophages is a more appropriate alternative correlate of protective immunity [213]. Increased frequencies of $CD8^+$ T cells expressing the regulatory markers CD25, CD39 and Foxp3 were observed in BCG vaccinees with low skin inflammation response upon vaccination, indicating a possible role for $CD8^+$ T cells in regulating BCG-induced immune responses [21]. In addition, expression of exhaustion marker KLRG1 in $CD8^+$ T cells was increased following BCG vaccination, and this correlated with poor $CD8^+$ T cell proliferation [22]. RNA sequencing of Mtb-stimulated conventional $CD8^+$ T cells from peripheral blood showed downregulation of IL-2R (CD25), a suggestive sign of senescence [120]. Thus, the specific contribution of conventional $CD8^+$ T cells in the host defense against Mtb infection needs more definitive investigation.

Mobilizing Conventional T Cells by TB Vaccines: Results from TB Vaccine Clinical Trials

Efforts in the field of TB vaccine discovery have actively focused on the development of subunit vaccines based on specific immunogenic components of the bacillus being administered as a purified antigen or expressed as a genetic insert in a viral vector, such as the candidate vaccine MVA85A. Whole cell-attenuated Mtb vaccines, such as MTBVAC, are intended as safe and immunogenic live vaccines following deletion of essential virulence genes from the Mtb genome. Parallel strategies aim to improve the current BCG vaccine by inserting additional antigens or by genetic manipulation to improve its immunogenicity. Alternative delivery routes using existing vaccines, such as mucosal or intravenous administration of BCG, might prove advantageous by inducing local mucosal immunity at the site of infection, while immunity is also induced systemically [56, 140].

Correlates of protective immunity induced by TB vaccination would be of value in promoting and accelerating TB vaccine evaluation [208]. However, biomarker discovery is hampered by the high complexity of immune responses in the different stages of TB disease, as well as the significant inter- and intra-individual varia-

tions in immune responses to Mtb infection [252]. Better biomarkers are necessary to improve the prediction of vaccine efficacy in preclinical and clinical trials [163, 309]. MVA85A, which was designed to boost BCG protective efficacy and was immunogenic and protective in animal studies, did not confer any additional protection against TB in infants in the above discussed phase 2b clinical trial despite its significant immunogenicity driving $CD4^+$ polyfunctional T cells [273, 274], again highlighting the need for alternative correlates to polyfunctional $CD4^+$ T cells.

Recently, a novel promising vaccine candidate containing a fusion protein of two immunogenic Mtb antigens in combination with the AS01 adjuvant system (M72/$AS01_E$) was tested in Mtb-infected adults in a phase 2b trial, and showed 49,7% vaccine efficacy against TB disease during 3 years of follow-up in an LTBI population [180, 272]. Also recently, BCG revaccination in adolescents was evaluated in a phase 2 trial showing a 45% efficacy in protecting against sustained IGRA conversion [201]. Both studies show there is significant potential for TB vaccine boosting of protective immune responses. In a recent immune correlate-based comparison of six novel TB vaccine candidates M72/$AS01_E$ was identified as the highest inducer of antigen-specific $CD4^+$ T cells [236].

In contrast to subunit vaccines, whole cell vaccines, such as the live-attenuated MTBVAC and the recombinant BCG VPM1002, are designed to induce broad immune responses to many diverse antigens. This might present an advantage to TB protection as a result of stimulating a wider and more diverse repertoire of immune cell populations, including unconventional and innate lymphocytes. The development of an *in vitro* mycobacterial growth inhibition assay allowing definition of the ability of immune cells, including T cells, to control outgrowth of Mtb *in vitro*, could be useful in facilitating the identification of correlates of protection upon vaccination or infection [134, 277]. The availability of biosamples from the two mentioned successful phase 2B trials should, for the first time, allow identification of immune correlates of vaccine-induced protection, which in turn are necessary to better evaluate TB vaccine candidates.

Role of B Cells and Antibody Responses in TB

Several studies have provided evidence for the presence of B cell dysfunction during Mtb infection in humans and NHP, which might jeopardize host immunity. Active TB in humans was correlated with impaired proliferation of B cells and reduced B cell frequencies, antibody and cytokine production, and antigen presentation capacity compared to healthy and treated individuals [133]. In a second study, individuals with LTBI exhibited a reduction in the frequency of circulating B cells, and this was linked to inflammation-enhanced myelopoiesis rather than migration of B cells from the circulation to the site of infection [49]. In NHP, protection from reactivation was associated with expanded B-cell follicles, and the depletion of B cells resulted in increased bacterial burden and altered granulomatous responses,

implying a role for B cells in Mtb control [78, 216]. Indeed, protection against TB disease in NHP was associated with inducible bronchus-associated lymphoid tissue (iBALT) which is dependent on CXCL13 and B-cell function [72, 141, 147, 171]. In humans, B cells as well as antibody-producing plasma cells were shown to be present in tuberculous granulomas suggesting a role for B cells in containment of Mtb infection in the lung [285, 286].

Latent Mtb infection and active tuberculosis disease are associated with distinct humoral responses which may modulate pathogenesis [133]. Antibodies from latently infected individuals showed superior avidity and enhanced opsonization capacity compared to antibodies from patients with active disease, and –importantly, promoted elimination of intracellular bacteria by macrophages [167]. Individuals highly exposed to Mtb while remaining persistently tuberculin skin test (TST) and IGRA negative, presented with potentially "protective" antibodies with enhanced avidity and distinctive IgG Fc-glycosylation profiles when compared to exposed individuals who established a latent infection (TST or IGRA conversion) [4, 164, 168]. Furthermore, in the above mentioned NHP TB-model, elevated levels of IgA in the lung were associated with protection against Mtb infection and disease [67]. Altogether, B cells and antibody responses may play an important role in protection against infection with Mtb and may be involved in sustaining latency of Mtb infection and in preventing TB reactivation.

Innate Lymphocytes Involved in Immunity Against Mtb

Innate lymphoid cells (ILCs) share characteristics with both innate and adaptive immune cells and play an important role in lung tissue repair upon infection [150, 186]. Lacking rearranging antigen-specific receptors, three major subsets of ILCs have been defined by their response to cytokine stimulation. ILC1s, similar to NK cells, secrete IFN-γ in response to IL-12, IL-15 and IL-18 while ILC2s secrete type-2 cytokines in response to IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). ILC3s on the other hand are considered the innate counterpart of Th17 cells as they produce IL-22 and IL-17 in response to IL-23 and IL-1β [211]. ILCs were shown to be depleted from the blood of TB patients compared to healthy controls and analysis in mice and humans infected with Mtb showed rapid accumulation of ILC3s in the lungs preceding infection control [6]. Using mouse models, this study demonstrated the importance of ILC3s in the early immune control of Mtb through IL-17 and IL-22 signaling, allowing the recruitment of alveolar macrophages and iBALT organization.

NK cells, an innate cytotoxic population, were shown to be one of the main producers of IFN-γ in infants and children after BCG vaccination, supporting the importance of innate immunity in the early immune response to, and possibly control of Mtb infection [307]. A recent comprehensive analysis of immune cell subsets across multiple cohorts showed that NK cell frequencies were increased in the blood of individuals with LTBI, decreased in patients with active TB, and normal-

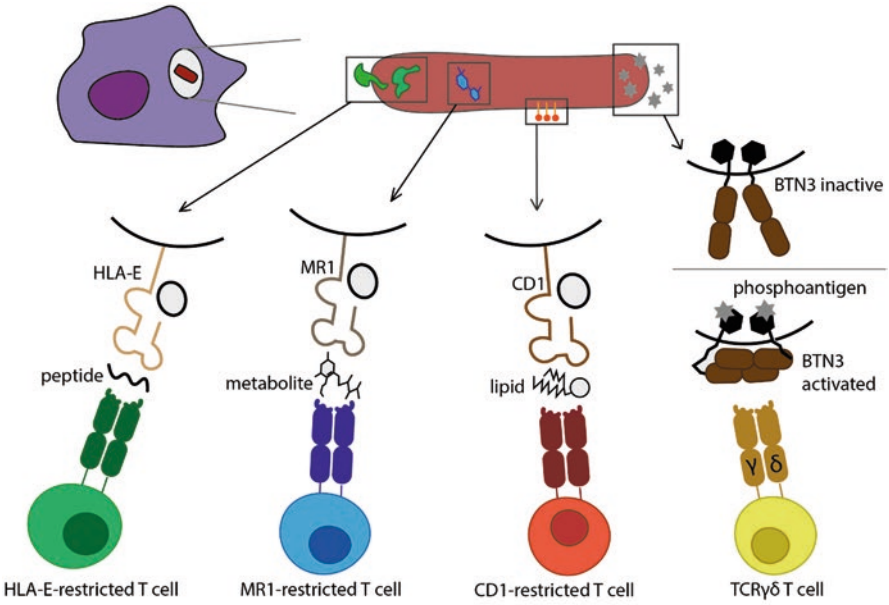
ized after treatment and clinical cure to baseline levels observed in healthy uninfected controls. NK cells from LTBI showed enhanced targeted cytotoxicity compared to NK cells from uninfected controls, suggesting an important role for NK cells in the successful immune control of Mtb infection. Longitudinal analysis showed that NK cell abundance in peripheral blood could serve as a correlate of reduced risk of disease progression, or correlate of treatment-induced recovery [49].

Repertoire and Functions of Unconventional Lymphocytes in TB

The earlier disappointing results obtained in clinical studies of TB vaccine candidates such as MVA85A, designed to target conventional $CD4^+$ and $CD8^+$ T cells, motivated the search for new vaccine approaches and strategies in TB, including the targeting of complementary immune cells and mechanisms such as unconventional T cells. Unconventional T cells can be classified according to the nature of the antigen presenting molecule that triggers them, or by the expression of αβ T-cell receptor (TCR) or γδ TCR. Among the TCRαβ-expressing unconventional T cells, HLA-E-, MR1- and CD1-restricted T cell populations can be discriminated. TCRγδ-expressing T cells are typically reactive to phosphoantigens presented via butyrophilin molecules but can also be CD1-restricted (see Fig. 10.1). Transcriptional profiling of non-MHC-restricted unconventional T cells indicated that they cluster together as innate immune cells which commonly share preferential transcription of genes encoding for effector function while decreasing their proliferative capacity [105]. We will discuss the principal characteristics of each unconventional T cell population and their potential to improve current vaccine strategies against Mtb.

(a) **HLA-E restricted T cells.**

There is increasing evidence that HLA-E-restricted T cells play an important role in protective immunity to Mtb [19, 109]. HLA-E is an HLA class Ib molecule characterized by limited polymorphism and reduced cell surface expression compared to HLA class Ia molecules. In contrast to the large genetic polymorphism and resulting molecular diversity of HLA class Ia (A, B, C) molecules, HLA-E has only two coding allelic variants differentiated by a single amino acid difference at position 107, where HLA-E*01:01 contains an arginine and HLA-E*01:03 a glycine [90]. These two variants do not present structural differences and are equally present in the human population [175, 266]. Characterization of TCR sequences recognizing cytomegalovirus (CMV)-derived peptides presented via HLA-E identified a preferential usage of Vβ16 gene segment [118], but this preferential TCR expression was not observed in the context of HLA-E-restricted T cells specific for Mtb-derived peptides [222]. Preliminary data from our group indicate that the TCR repertoire of Mtb-specific HLA-E-restricted T cells is more diverse than would be expected from



	HLA-E-restricted T cells	MR1-restricted T cells			CD1-restricted T cells			TCRγδ T cells
		Classical MAIT cells	Non-classical MAIT cells	Atypical MR1-restricted T cells	Group 1 (CD1a, CD1b, CD1c)	iNKT (invariant or type I NKT) (CD1d)	Diverse (type II NKT) (CD1d)	
Recognized antigen	Peptide: - HLA class I signal - Pathogen or tumor derived peptides	Metabolic derivatives: - 6-FP (folate metabolite, antagonist) - 5-OE-RU, 5-OP-RU and FO (riboflavin metabolites, agonists) - PLI, PLIII (photolumazines)			Foreign lipids: - CD1a: lipopeptides - CD1b: MA, glycolipids, Mtb SGLs (lysosome loading) - CD1c: Isoprenoid glycolipids and mycoketides	- Ceramide lipids: α-GalCer, PIM	- Sulfatides and glycosylceramides (no endosomal loading) - Phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol	Presentation via BTN3 - Phosphoantigen: IPP, TUBag1-4, HMBPP, BrHPP, mGLP - CD1d-presented lipids, MICA/MICB, ULBP, EPCR, CD1c
					Self lipids: sphingomyelins, phospholipids (ER loading)			
Additional markers	$CD8^+$	CD161, CD26, IL-18R, $PLZF^+$		$CD161^{+/-}$, $CD26^{+/-}$, $PLZF^-$	$CD4^+$, $CD8^+$, $CD4^-CD8^-$			$CD4^-CD8^-$
TCR characteristics	Diverse TCRαβ	Semi-invariant TCRαβ		Diverse TCRαβ	Polyclonal TCRαβ, among others:	Conserved TCRαβ or TCRγδ	Diverse TCRαβ	Diverse TCRγδ
		TRAV1-2 TRAJ33/12/20 TRBV6/20	$TRAV1-2^{neg}$ TRAJ34/37 TRBV28 TRBJ2-5	$TRAV1-2^{neg}$ Unknown	TRAV1-2, TRAJ9 TRAV17 TRBV4-1 TRBV6-2 (LDN5-like) (GEM T cells)	TRAV10 TRAJ18 TRBV25		Vγ9Vδ2 TCR in phosphoantigen-specific T cells
Functional properties in the context of BCG/Mtb infection and contribution to protection	Th1 cytokine production (IFN-γ and TNF-α) - Mtb growth inhibition Th2 cytokine production (IL-4, IL-5, IL-13) - Cytotoxic function - Regulatory function Multi functional - Mtb growth inhibition	- Stimulation via TCR signaling (early time points) and TCR-independent via IL-12 + IL-18 signaling (later time points) - Cytotoxic function: granzyme B - IL-2, IL-12, IL-18 production			Th1-like cytokine profile and granulysin-mediated cytotoxicity - IFN-γ, TNF-α, CD40L and IL-2 production - Mtb growth inhibition	- TCR-independent activation via IL-12 and IL-18 signaling - Rapid innate-like activation - Strong IFN-γ and IL-4 production - GM-CSF production - Mtb growth inhibition	Unknown	- IFN-γ, TNF-α and GM-CSF production - Production of Th17 cytokines - Cytotoxic function - Mtb growth inhibition - Helper function
Frequencies in peripheral blood	Increased in active TB and TB/HIV compared to LTBI and healthy controls	Reduced in active TB, HIV and TB/HIV compared with LTBI and healthy controls			Associated with Mtb infection control	Increased in LTBI compared with active TB and healthy controls	Unknown	Increased in children with bacterial meningitis

Fig. 10.1 Schematic representation of unconventional lymphocytes being activated after recognition of different pathogen-derived stimuli presented via their corresponding non-polymorphic molecules. The table below lists the main characteristics of each unconventional lymphocyte. HLA-E-restricted T cells, triggered by peptide antigen, can exhibit cytotoxic or regulatory functions leading to Mtb growth inhibition. MR-1-restricted MAIT cells recognize metabolite-derived antigens and can be further classified according to their TCR gene usage. Although their precise role in the immune response against Mtb infection is not yet established, they might be important for mucosal immunity during early timepoints of infection. On the other hand, the multiple subsets of CD-1-restricted T cells, activated upon presentation of lipid antigens, have shown the capacity to control Mtb growth and are thought to be associated with the control of Mtb infection. TCRγδ T cells are mostly restricted to phosphoantigen presentation via BTN3 and, upon activation, are able to inhibit Mtb growth by executing cytotoxic and helper functions

T cells reactive to a monomorphic antigen-presenting molecule such as HLA-E (manuscript in preparation).

HLA-E has a high degree of functional and structural conservation among species, with the most frequently studied HLA-E homologues being Qa-1^b in mice and Mamu-E in rhesus macaques (RM) [107, 235, 296]. Despite a higher allelic variation in Mamu-E compared to humans, there is high genetic conservation between Mamu-E and HLA-E molecules [24, 296]. Both have been reported to bind the same peptide sequences, and cross-species recognition by Mamu-E-restricted $CD8^+$ T cells of HLA-E-presented peptides has been demonstrated, making the RM a suitable model for studying HLA-E-restricted T cell function *in vivo* [296].

Exploiting the unique capacity of CMV-vectors to induce long-lasting and prevalent effector $CD4^+$ and $CD8^+$ T cells, the Picker group vaccinated RM with simian immunodeficiency virus (SIV) antigens genetically inserted in a modified rhesus CMV (RhCMV) vector. The vaccine induced complete protection in half of all SIV challenged animals, which was dependent on unconventional $CD8^+$ T cell responses genetically restricted by either MHC-E or MHC-II molecules [108]. A preclinical trial in which RM were vaccinated subcutaneously with RhCMV vectors encoding 6 to 9 Mtb antigens followed by Mtb challenge, also led to a reduction of Mtb infection and disease by 68%, although in this model, MHC-E restricted $CD8^+$ T cells seemed dispensable for this protection and a more prominent role for $CD4^+$ T cells was reported [109]. A more recent study with a RhCMV vector expressing *Plasmodium knowlesi* antigens delayed parasitemia upon sporozoite challenge. In this study, MHC-restriction of $CD8^+$ T cell responses was exclusively by MHC-E and MHC-II, further supporting the potential of unconventionally-restricted $CD8^+$ T cells as vaccine target cellular populations in infectious diseases [110].

HLA-E and Qa-1^b display only a 65% overall amino acid sequence identity although the peptide binding specificity and specialized function are maintained between the two molecules as a result of the relatively more conserved peptide binding region [128, 182]. Studies with Qa-1^b-deficient mice have shown that the genetic lack of this antigen presenting molecule leads to a reduced protection against severe Mtb infection as a result of, amongst others, altered immune regulation of $CD8^+$ T cell responses [19]. Collectively these reports provide evidence that HLA-E/MHC-E/Qa-1^b-restricted $CD8^+$ T cells could potentially contribute to immunity and protection against Mtb infection.

HLA-E-Dependent Antigen Presentation

For successful homeostatic expression on the cell surface, HLA-E requires binding to nonameric peptides, typically derived from HLA-class Ia signal sequences [25, 162]. The HLA-E/signal peptide complex was initially described to interact with C-type lectin CD94/NKG2A (inhibitory) and CD94/NKG2C (activating) receptor complexes which are expressed on the natural killer (NK) cell surface. NKG2A binds HLA-E with higher affinity compared to NKG2C and when engaged by HLA-E inhibits NK cell responses and cytolytic activity. This mechanism enables NK cells to monitor the integrity of HLA-class Ia surface expression [5, 26, 31, 137,

161]. Many tumors have been reported to downregulate HLA-class Ia expression, and as a result become susceptible to NK cell-mediated killing because the supply of HLA-E binding peptides and consequently HLA-E cell surface expression is inhibited [88]. Certain pathogens, such as CMV, escape from cellular immunity by manipulating HLA-class Ia expression and function: CMV impairs the presentation of peptides via HLA-class Ia molecules by downregulating cell surface expression; by inducing degradation of HLA-class Ia molecules; by blocking peptide loading onto HLA molecules; by inducing conformational changes on transporter associated with antigen processing (TAP); and by preventing peptide/HLA complex stabilization through interaction with tapasin [220, 255]. However, in order to counteract the resulting risk of NK cell-mediated cytotoxicity triggered by the absence of HLA class Ia peptides, CMV has evolved to encode a peptide within the leader sequence of glycoprotein UL40 which is identical to the HLA-E binding canonical HLA-class Ia signal peptide. When this UL40 peptide binds HLA-E, the latter is presented on the surface of infected cells through a TAP-independent pathway, which leads to inhibition of NK cell function, allowing CMV to escape from NK immunity [280, 282]. CD94/NKG2A is also expressed on a small proportion of activated $CD8^+$ T cells and might play a role in regulating cytolytic T cell responses to avoid tissue damage [27, 178, 185].

However, HLA-E can also present peptides to TCR molecules on $CD8^+$ T cells. The TCR-mediated $CD8^+$ T cell recognition of self-peptides presented by Qa-1^b was demonstrated in the context of tumor cells with antigen processing deficiencies. When the classical MHC-class Ia antigen-processing pathway was impaired, Qa-1^b bound MHC-class Ia signal peptides could be replaced by alternative, tumor-derived antigenic peptides which could be sensed by the TCR, leading to activation of cytotoxic T cells with anti-tumor activity [59, 206]. Similar studies in the context of Mtb, CMV and *Salmonella enterica* serotype Typhi (S.Typhi) infections later supported HLA-E as a presentation molecule for TCR-mediated recognition of pathogen-derived peptides, extending the function of HLA-E antigen presentation to adaptive immune responses [115, 118, 132, 181, 217, 218, 244]. These observations underscore the potential importance of HLA-E for the design of vaccines, including against TB, with the capacity to stimulate unconventional protective $CD8^+$ T cell responses. The enriched expression of HLA-E in Mtb phagosomes further supports the hypothesis that HLA-E could have a unique capacity to capture and present Mtb-derived peptides to unconventional $CD8^+$ T cells [100, 112]. Within the current TB epidemics, HIV co-infection considerably increases the probability of developing active TB disease [295]. The fact that HLA-E is not downregulated by HIV infection further supports the targeting of this molecule for the design of novel improved vaccine approaches [200]. Only recently, downregulation of HLA-E surface expression was observed on $CD4^+$ T cells infected with primary HIV-1, while infection with cell-line adapted strains maintained HLA-E expression [265]. Further investigation is required to establish this effect on macrophages, the primary target cell of Mtb.

Initial work identified only relatively few nonameric peptides as HLA-E binders using a somewhat strict predictive peptide/HLA-E binding motif that was largely

developed based on the sequences of HLA-E-binding HLA-class Ia leader sequences [153, 182, 205]. However, especially following attempts to identify Mtb-derived peptide sequences capable of eliciting HLA-E restricted $CD8^+$ T cell responses, increasing evidence accumulated that the repertoire of HLA-E-binding peptides is more extensive than initially thought. Multiple nonameric peptide sequences across the Mtb genome that were predicted *in silico* to bind HLA-E could induce $CD8^+$ T cell proliferation upon presentation via HLA-E *ex vivo* [132]. In the context of Qa-1^b, the repertoire of tumor-derived peptides that was presented was also found to be broader than initially thought [107, 206]. Additionally, peptide elution studies identified HLA-E binding peptides of varying lengths, ranging between 8 and (non-canonical) 20 amino acids, as well as peptides with modifications such as glycosylation, acetylation, and oxidation, further increasing the diversity of HLA-E binding peptides [113, 152, 156, 179]. Surprisingly, differences were also observed in the peptide repertoires presented by HLA-E*01:01 and HLA-E*01:03 which might reflect unanticipated differential functionality [36]. A recent structural analysis demonstrated the conformational plasticity of peptides bound to HLA-E, which seems to tolerate a broader array of hydrophobic and polar amino acid in the primary pockets than classical HLA-class Ia molecules, despite the presence of preferred anchor residues [290]. A diverse repertoire of SIV-derived epitopes presented by MHC-E was also observed and MHC-E-restricted "supertopes", which were recognized by all animals tested, were identified [108]. Additional efforts are underway towards the identification of novel HLA-E-binding peptides with improved immunogenicity which could be deployed in improved vaccine strategies.

Multifunctional Properties of HLA-E Restricted T Cells

Although the search for pathogen-derived peptides with an optimal HLA-E-restricted $CD8^+$ T cell immunogenic capacity is still ongoing, several studies have taken advantage of already identified HLA-E-binding peptides to shed light on the functional properties of this unconventional T cell population. HLA-E-restricted $CD8^+$ T cells produce Th1 cytokines such as IFN-γ and TNF-α, normally associated with inhibition of Mtb growth, but surprisingly can also express IL-4, IL-5 and IL-13 cytokines typical of a Th2-like phenotype, both at the monoclonal and at the polyclonal level [179, 181, 222]. HLA-E-restricted $CD8^+$ T cell clones with a Th2 cytokine profile have cytotoxic as well as regulatory functions and were capable of inhibiting intracellular mycobacterial growth [132, 181, 222]. Multifunctional HLA-E-restricted $CD8^+$ T cells co-expressing CD107, IFN-γ and TNF-α, as well as IL-17A-producing HLA-E-restricted $CD8^+$ T cells, were also observed long-term after immunization with S.Typhi [245], and were more frequent in adults and older pediatric participants than in younger children, suggesting an age-associated maturation of this unconventional T cell population [308]. In TB, the expression of the transcription factor GATA3 and the cytokine IL-13 among all investigated cytolytic Mtb-specific HLA-E-restricted $CD8^+$ T cells clones suggested that the polyfunc-

tional Th2/cytolytic/regulatory phenotype might play an important role in anti-mycobacterial immunity, and could mediate effector as well as anti-inflammatory functions. In addition, B-cell function was also activated by Mtb-specific HLA-E-restricted $CD8^+$ T cell clones via IL-4. The broad functional capacity of HLA-E-restricted $CD8^+$ T cells could be of interest to the improvement and enrichment of current vaccine strategies to enhance Mtb-inhibitory capacity while also regulating Th1 immunity and thus preventing excessive inflammation.

The use of tetramers to detect Mtb-specific HLA-E-restricted $CD8^+$ T cells allowed the demonstration of this population in the peripheral blood of TB patients and LTBI, and revealed increased frequencies in patients with active TB and TB/HIV co-infection compared with LTBI and healthy controls. This work further supported the importance of the poly-functionality of these cells in the circulation of Mtb infected individuals [181, 222]. The regulatory function of Mtb-specific Qa-1^b-restricted $CD8^+$ T cells was also observed in mice, and mechanistically was found to be important in preventing exacerbated T cell activation and inflammation, likely underlying their more effective protection against severe TB in which inflammation is an important component of its pathogenesis [19].

Altogether, these studies support the notion that HLA-E-restricted T cells are important contributors in immunity and probably also protective immunity against Mtb. Definition of improved peptide triggers and a deeper understanding of the mechanisms leading to protective immunity are areas in need of further investigation.

(b) **CD1-restricted T cells.**

Given the high abundance and molecular diversity of lipids in the cell wall of Mtb, CD1-restricted lipid-specific T cells might be important in sensing infection and in immune protection against Mtb. Bacterial lipids are distinct from lipids present in mammalian cells and are crucial for bacterial survival and membrane integrity, making them an interesting target for vaccine development.

The human CD1 system is composed of non-polymorphic molecules structurally similar to MHC class Ia, albeit with a larger antigen-binding groove which is adapted to position lipid antigens deep within the groove, partially closing on the lipid which is exposed through small openings named portals [122, 194, 305]. Five different isoforms are expressed in humans, classified into group 1 (CD1a, CD1b and CD1c), group 2 (CD1d), and group 3 (CD1e) CD1 family members, each with distinct functions and patterns of tissue expression. Group 1 and 2 CD1 molecules are specialized in presenting self or foreign lipid antigens on the surface of antigen-presenting cells for specific recognition by TCRαβ and TCRγδ T cells [13, 193]. Although structurally highly similar, the presentation of lipid antigens via group 1 and 2 CD1 molecules leads to the specific activation of different T-cell populations in terms of TCR diversity and functional capacity. Group 3 CD1 expression is strictly intracellular where CD1e acts as a lipid transfer protein mediating CD1-dependent lipid antigen presentation [85].

CD1 molecules bind self-lipids, such as sphingomyelins and phospholipids, in the endoplasmic reticulum, which confers stability to CD1 molecules by facilitating

correct refolding, before they can be transported to and expressed at the cell surface [53, 79, 106, 121]. Once cell-surface expressed, group 1 and 2 CD1 molecules recycle and accumulate in lysosomes where they encounter and can bind exogenous lipids from phagocytosed bacteria for presentation, followed by recycling to the cellular surface [127]. Tyrosine-containing motifs in the cytoplasmic tail of CD1b regulate this recycling mechanism and localize CD1b to late endosomes and lysosomes where acidic conditions are optimal for lipid loading [124, 225, 268]. CD1c and CD1d instead survey different intracellular compartments by binding adaptor protein 2 (AP-2) and localizing to early and late endosomes [30, 269], whereas CD1a recycles via early endosomes [38].

Group 1 CD1-Restricted T Cells

Group 1 CD1-restricted T-cells have been described mainly in the context of mycobacterial infections. Presentation of the structurally diverse immunogenic lipids is enabled via the CD1b superchannel of interconnected pocket tunnels that forms the binding groove and which accommodates the various lipids' long acyl chains [11]. Mycolic acid (MA) and glycolipids, major pathogenic components of the mycobacterial cell wall, were among the first lipid antigens described to activate T cells in a CD1b-restricted manner [13, 96, 259]. The additional identification of glucose monomycolate (GMM) as a CD1b-presented lipid antigen led to the proposal of a CD1b antigen motif in which a polar cap determines highly specific T cell recognition [188, 189]. This hypothesis was later confirmed through crystal structures which showed how the antigen anchors its fatty acyl groups within the hydrophobic pockets of CD1, while exposing its hydrophilic part upward for TCR recognition [11, 82]. The synthesis of GMM involves the interaction of mycobacterial mycolate with glucose from the infected host, leading to specific T-cell activation in the precise context of active mycobacterial infection [190]. Experiments with modified TCRs helped define specific residues within the TCR β CDR3 that are required for MA recognition [96]. Sulfoglycolipids (SGLs), which are expressed only by Mtb, were shown to activate $CD8^+$ T cells from Mtb infected patients but not PPD negative donors, in a CD1b-dependent fashion, triggering IFN-γ production and efficient recognition of infected cells to allow killing of intracellular mycobacteria [91]. Glycerol monomycolate (GroMM), on the other hand, was found to stimulate CD1b-restricted $CD4^+$ T cells in vaccinated and latently infected donors but not active TB patients [159].

Identified lipid ligands for CD1a and CD1c are less numerous compared to CD1b. CD1a, the isoform with the smallest groove, can present lipopeptides for TCRαβ-mediated T cell activation [192, 304]. Isoprenoid glycolipids and mycoketide antigens instead are known to specifically bind the CD1c isoform and to induce TCR-mediated T-cell responses [174, 191, 249].

The knowledge on lipid antigens led to the development of CD1a, CD1b and CD1c tetramers for the detection of CD1-restricted polyclonal T-cells in blood from

Mtb-infected patients [138, 139, 170]. The use of CD1b tetramers loaded with GMM allowed the discovery of germline-encoded mycolyl reactive (GEM) T cells. These cells presented increased intra- and inter-donor conservation of TCR sequences encoded by *TRAV1-2* joined to *TRAJ9*, and a preferential usage of *TRBV6-2*, illustrating their donor-unrestricted characteristics [227, 230]. Structural analysis showed that the GEM TCR recognition mechanism involves simultaneous specific interactions with both CD1b and GMM [97]. LDN5-like T cells are another example of polyclonal T-cells with a semi-invariant TCR combination, which were seen in multiple donors by using CD1b-GMM tetramers. These cells express a TCRα chain encoded by *TRAV17*, paired with a *TRBV4-1* encoded β chain, similar to a previously described T cell clone isolated from a *Mycobacterium leprae*-infected skin lesion, known as LDN5 [188, 228]. More recently, however, TCR sequence analysis of CD1b-GMM-specific T cells revealed a more complex and diverse clonally-expanded repertoire despite the conservation of the antigen-presenting molecule [63]. Further insights into the different CD1-restricted TCR repertoires are expected from newly developed CD1 tetramers, *e.g*. CD1b tetramers loaded with MA or SGLs [125, 229].

Specificity of CD1-restricted T cell recognition is not only determined by the antigenic region in direct contact with the TCR but also by the distal hydrophobic regions of the lipid antigens buried deep within the CD1 cleft [104, 130, 177]. CD1b tetramers loaded with different forms of MA showed diverse TCR cell binding, highlighting the influence of such distal groups on T cell recognition [229]. The immunogenicity of diverse naturally occurring and synthetic MA forms was found to be dependent on the length or presence of functional groups on the lipid chain, which is also of importance in the context of infections with other bacterial families closely related to Mtb, and also differed between varying MA constructs. In the context of SGLs, longer acyl chains led to increased stimulatory capacity [89]. Also important for immunogenicity are specific residues on the CD1b molecule surface, probably by ensuring proper SGL binding and positioning [86, 125].

Functional analyses of CD1-restricted T cell clones *in vitro* initially showed granulysin-mediated Mtb-specific cytotoxicity and a Th1-like cytokine profile [237, 264]. Mycobacterial lipids are able to stimulate CD1b-restricted T cells leading to IFN-γ and TNF-α production, a response which was demonstrated to be important for early as well as late, memory human immune responses to Mtb infection [184, 227]. Expansion of these cells correlated with pathogen burden as they were detected in patients with active TB but not in BCG-vaccinated healthy individuals [184]. A more recent *ex vivo* profiling of CD1-restricted T cells in a TB endemic population showed polyfunctional GMM-specific $CD4^+$ T cell responses with simultaneous expression of CD40L, IL-2, TNFα and IFN-γ which were unrelated to protein-specific T cell responses [254]. Importantly, the frequency of circulating CD1-restricted T cells specific for mycobacterial lipid antigens was associated with Mtb infection control [33, 284]. Bronchoalveolar lavage cells obtained from patients with latent TB contained lipid-responsive polycytotoxic T cells which co-expressed perforin, granzyme B and granulysin [33]. Additionally, an intronic polymorphism

identified in *CD1A* was associated with increased susceptibility to tuberculosis, emphasizing the potential importance of CD1a lipid antigen presentation for TB immunity [253].

The development of a human CD1b transgenic mouse model prompted adoptive T cell transfer studies which showed that CD1b-restricted MA-specific T cells with polyfunctional capacities, participated in control of mycobacterial proliferation and conferred protection to animals in a Mtb challenge model *in vivo* [73, 306]. In the guinea pig model, which naturally expresses group 1 CD1 molecules, aerosol Mtb challenge of animals vaccinated with mycobacterial lipids showed reduced bacterial burden and lung pathology [57, 58, 117]. Altogether, these studies underscore the potential protective effect of CD1-restricted lipid-reactive T cells and prompt the inclusion of mycobacterial lipids to enhance vaccine strategies. Significant hurdles accompany the development of lipid-based vaccines such as the technological limitations to synthetically produce lipids on a large scale, and the labor-intensive purification procedures. Novel live mycobacterial vaccine candidates (discussed in Section "Repertoire and Functions of Conventional Lymphocytes in TB") are a possible way to induce lipid-reactive T cells while overcoming these difficulties.

iNKT: Invariant or Type I Natural Killer T Cells

Invariant or type I natural killer T (iNKT) cells typically recognize ceramide lipids such as α-galactosylceramide (α-GalCer) presented by group 2 CD1 (CD1d) [14, 15, 28, 143]. TCR-mediated stimulation of these cells leads to rapid innate-like activation, strong IFN-γ and IL-4 cytokine production and cytotoxic activity. In addition, TCR independent IL-12- and IL-18-driven activation mechanisms have been described that can activate iNKT cells [29, 173]. iNKT cells express conserved TCR sequences with the α chain predominantly encoded by *TRAV10* joined to *TRAJ18*, and the β chain preferentially using *TRBV25* [61, 221]. The role of iNKT cells was widely studied in mice in the context of bacterial infections such as *Streptococcus pneumoniae* [142], *Pseudomonas aeruginosa* [149, 203], and *Mycobacterium spp* [40, 66, 267], amongst others, which collectively suggested a protective role for iNKT cells during early infection. α-GalCer as a highly potent agonist of iNKT cells has potential in vaccine optimization and immunotherapy [39, 81, 148, 288]. Mycobacteria-cell wall-derived phosphatidylinositol mannoside (PIM) was defined as a CD1d antigen recognized by a subpopulation of iNKT cells. PIM presented by CD1d triggered T cell cytotoxic activity and the production of IFN-γ but not IL-4, although the latter is typically associated with strong iNKT cell activation [74]. Guinea pigs immunized with PIM and SGL, the latter lipid antigen mediating CD1b-restricted T cell responses, showed reduction in bacterial load and lung pathology upon Mtb challenge to a degree comparable to that obtained when using protein antigens in the same model [101, 158]. These promising results are

encouraging for the development of vaccine combinations which include lipid antigens for targeting unconventional CD1d-restricted iNKT cells to enhance protective immunity to Mtb.

Even though the antigenic stimulus was not explored, iNKT cell-dependent CD1d-restricted protection against Mtb was confirmed *in vivo* in CD1d knockout mice and was shown to require CD1d expression for recognition of infected cells, as well as IL-12 and IL-18 signaling [242]. Human iNKT cells expressing IFN-γ and granulysin exhibited antimycobacterial activity *in vitro* [84]. Another study in mice showed that iNKT cell-mediated control of Mtb replication was dependent on granulocyte-macrophage colony-stimulating factor (GM-CSF) in the absence of IL-12, IL-18 or IFN-γ driven responses [238]. This alternative antimicrobial effector function driven by GM-CSF can be mediated not only by iNKT cells, but also by TCRγδ T cells and conventional $CD4^+$ and $CD8^+$ T cells, and showed protective efficacy in mouse studies *in vitro* [94, 233, 239, 271]. GM-CSF was also prominently found to be induced by Mtb antigens in human studies [51].

Corroborating findings in mice, studies in NHP have also suggested a protective role of $CD8^+$ iNKT cells in Mtb infection [41]. Their frequency was increased in the circulation of infected animals which showed improved control of the infection and better disease outcome. Conversely, several studies in humans provided evidence for a reduction of iNKT cells in the blood during active Mtb infection [123, 145, 187, 260, 270]. More recently, latently Mtb infected individuals showed increased frequencies of iNKT cells compared with uninfected individuals or patients with active TB, together suggesting a possible role for iNKT cells in preventing disease progression. Active TB was associated with increased expression of exhaustion marker PD-1 on iNKT cells, further supporting their importance for immune control of Mtb [212].

Diverse or Type II Natural Killer T Cells

Diverse, or type II NKT cells are also CD1d restricted but express a diverse TCRαβ repertoire and lack reactivity to α-GalCer [48, 279]. These cells recognize CD1d molecules presenting sulfatide and glycosylceramide species without requiring endosomal loading, and phenotypically are similar to type I NKT cells, [7, 48, 199]. Mtb-derived phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylinositol were able to stimulate type II NKT hybridomas upon presentation via CD1d [279]. Highly abundant in the human bone marrow during steady state [70], type II NKT cells were enriched in the liver and showed a Th1-like cytokine profile during hepatitis C virus infection [71]. Type II NKT cells have been shown to inhibit antitumor immune responses in a murine B-cell lymphoma model, and they seem to have also a suppressive role in autoimmune diseases while being proinflammatory in parasitic infections [17, 226]. Surprisingly, very little is known about the recognition of (mycobacterial) antigens by type II NKT cells, and their relevance in TB.

(c) **TCR gamma-delta (γδ) T cells**

TCR gamma-delta (γδ) T cells are a distinct subset of $CD3^+$ T cells featuring Vγ and Vδ encoded TCR segments which are enriched in epithelial tissues. TCRγδ T cells are involved in immune surveillance of cellular stress responses and display characteristics of both innate and adaptive immunity [23, 46, 114]. Contrary to αβ T cells, which are activated upon antigen presentation by MHC molecules, TCRγδ T cells are not restricted by MHC molecules for presentation of antigens but recognize antigens presented via butyrophilin-3 (BTN3), also known as CD277 [275], although a minor population also recognizes lipid antigens in the context of CD1d as discussed above. Comparison of CDR3 lengths indicated that γδ TCRs are more similar to immunoglobulins (Igs) than to αβ TCRs, revealing a structural explanation for the quite different mechanisms of antigen recognition by the two types of TCRs [234]. Indeed, TCRγδ T cells are triggered by various cellular stress-induced stimuli including certain CD1d-presented lipids [8, 169, 283] and MHC class I-like proteins such as MHC class I polypeptide-related sequence A (MICA) and B (MICB) [99, 297], UL-16 binding protein (ULBP) [151], endothelial protein C receptor (EPCR) [294], and CD1c [240, 261], among others. TCRγδ T cells have been shown to also recognize ATP synthase and apolipoprotein A-1 complex on the surface of tumor cells leading to cytolytic activity [250].

Relevant in the context of Mtb infection, TCRγδ T cells can also be reactive against phosphoantigens, which are non-peptidic phosphorylated small intermediates of metabolic pathways in mammalian cells and pathogens such as Mtb [10, 50, 103]. Phosphoantigen-specific T cells, characterized by the expression of Vγ9Vδ2 TCR, are the most predominant subpopulation of TCRγδ T cells and represent up to 5% of peripheral T cells in healthy individuals. They can expand to as much as 20–50% in patients with diverse bacterial and parasitic infections [16, 197]. Recognition of phosphoantigens by TCRγδ T cells is independent of presentation via MHC but requires TCR recognition, cell-to-cell contact, and BTN3 [32, 111, 157, 195, 210, 287, 291]. The BTN3 subfamily consists of BTN3A1, BTN3A2 and BTN3A3, transmembrane proteins composed of extracellular Ig domains with the capacity to stimulate Vγ9Vδ2 T cells [231]. BTN3A1 is able to mediate phosphoantigen-dependent Vγ9Vδ2 T cell activation through the binding of its B30.2 intracellular domain to phosphoantigen [60, 111, 287, 291, 292]. By contrast, BTN3A3 does not bind phosphoantigen as a result of a single amino acid difference within the B30.2 binding pocket which disrupts complementarity with the phosphoantigen [248]. These findings led to the hypothesis of an inside-out signaling model in which intracellular binding of phosphoantigen to B30.2 leads to conformational changes on the extracellular domain of BTN3A1, allowing Vγ9Vδ2 T cell recognition [102, 103]. However, there is still controversy on the precise molecular mechanism driving Vγ9Vδ2 T cell activation by BTN3 and some recent studies have suggested that all three isoforms have the capacity to induce a Vγ9Vδ2 T cell response [2, 232]. A recent study of the BTN3 structures identified the loss of multiple H-bonds interactions between phosphoantigen and BTN3A3 compared to BTN3A1, providing a structural explanation for the differential phosphoantigen

recognition by BTN3 isotypes leading to inside-out signaling for Vγ9Vδ2 T cells activation [302].

The discovery of accumulating TCRγδ T cells in human granulomatous leprosy skin lesions led to the identification of isopentenyl pyrophosphate (IPP) as a natural mycobacterial phosphoantigen recognized by TCRγδ T cells, which was mediated via the core carbon chain and pyrophosphate moiety [183, 196, 275]. Subsequently, TUBag1-4 were the first Mtb-derived phosphoantigens identified with the capacity to stimulate Vγ9Vδ2 T cells with increased potency compared to IPP [50]. Other identified potent phosphoantigens found to drive TCRγδ T cells were (E)-4-hydroxy-3-methyl-but-e-enylpyrophosphate (HMBPP) and bromohydrin pyrophosphate (BrHPP) [3, 69, 116]. Recently, 6-O-methylglucose lipopolysaccharides (mGLP) were identified as specific Mtb-derived antigens capable of expanding Vγ9Vδ2 T cells which control intracellular mycobacterial replication [299].

TCRγδ T cells are associated with a protective immune response to Mtb infection and great efforts have been made in dissecting the precise mechanisms underlying this protection [44]. TCRγδ T cells stimulated by Mtb phosphoantigen responded by producing IFN-γ and TNF-α to enhance DC maturation [35, 62]. Indeed, when cocultured *in vitro* with Mtb-infected APCs, TCRγδ T cells showed higher production of IFN-γ compared to TCRαβ T cells [160, 281]. Several studies have shown that IL-2 and IL-15 induce Th1 cytokine responses, including expression of IL-5 and IL-13, in human TCRγδ T cells upon phosphoantigen stimulation [87, 289]. Production of IL-17 by TCRγδ T cells has been shown to be driven by phosphoantigen stimulation in the presence of IL-6, IL-23, IL-1β, and TNFα and plays a role in expansion of Vγ9Vδ2 T cells in rhesus macaques [256]. Circulating TCRγδ T cells from TB patients also showed a strong and rapid production of proinflammatory cytokine IL-17 [47, 214]. Notably, the frequency of IL-17$^+$ Vγ9Vδ2 T cells was shown to be increased in peripheral blood and the cerebrospinal fluid of children with bacterial meningitis [34, 256]. Interestingly, *in vitro* stimulation with Mtb or BCG, as opposed to purified phosphoantigens, activated more oligoclonal protective subsets of Vγ9Vδ2 T cells which not only produced IFN-γ, but also showed cytolytic activity and inhibited extracellular and intracellular mycobacterial growth through the production of granulysin and perforin [64, 65, 262]. Granzyme A was also shown to be involved in the suppression of intracellular mycobacterial growth through the induction of TNF-α production in infected monocytes [263]. In a recent study, IL-12 was shown to influence the early expansion of Vγ9Vδ2 T cells upon phosphoantigen stimulation in the presence of TNFα, leading to differentiation of polyfunctional effector Vγ9Vδ2 T cells expressing antimicrobial cytokines IFN-γ, TNF-α, GM-CSF, and cytotoxic molecules granzyme B, granulysin, and perforin. Notably, these cells were shown to inhibit intracellular mycobacterial growth *in vitro* [301]. In addition to their cytolytic properties, Vγ9Vδ2 T cells perform also helper functions by enhancing the Mtb-control capacity of Mtb-specific TCRαβ T cells through CD40-CD40L interactions [1].

The important role of TCRγδ T cells in protection against Mtb infection and TB disease is further supported by animal studies. Administration of phosphoantigen in

combination with IL-2 generated early *in vivo* expansion of Vγ9Vδ2 T cells with multifunctional effector phenotypes and enhanced protection by reducing Mtb burden and attenuating TB lesions in the lung of NHP [43]. More recently, a study involving adoptive transfer of autologous Vγ9Vδ2 T cells further demonstrated their protective role against TB disease in NHP, in which Vγ9Vδ2-expressing T cells are prevalent [223]. Consistent with these results, expansion of Vγ9Vδ2 T cells was observed in blood and lungs of BCG-vaccinated NHP and was associated with clearance of bacteremia upon BCG revaccination and Mtb challenge, suggesting that Vγ9Vδ2 T cells contribute to control of mycobacterial infection [155, 258]. The inclusion of phosphoantigens in a prime-boost strategy to immunize NHP against Mtb resulted in the immediate expansion of TCRγδ T cells with a strong production of Th1 cytokines upon the initial immunization [37]. IL-22-producing T cells, involved in granuloma formation during Mtb infection in NHP, have been shown to be regulated by Vγ9Vδ2 T cells, and IL-22 was shown to be induced during severe TB in NHP [224, 303]. In mice, TCRγδ T cells were shown to be the main producers of GM-CSF during early Mtb infection contributing to resistance against Mtb, and GM-CSF-producing Mtb-specific TCRγδ T cells were detected in peripheral blood of infected patients [239]. Further research on the function of TCRγδ T cells should assess mechanisms by which these cells are able to reduce mycobacterial burden, either by direct effector activity and/or by indirectly contributing to the activation and regulation of alternative key immune players. BCG vaccination in infants resulted in an expansion in the number of circulating TCRγδ T cells, a population which was shown to be one of the main early producers of IFN-γ [176, 278, 307]. In adults, BCG vaccination promotes the *in vitro* expansion of TCRγδ T cells upon stimulation with Mtb, hinting to a memory-like phenotype [119]. Recruitment of Vγ9Vδ2 T cells with an HMBPP-producing *Listeria monocytogenes* (Lm) vaccine vector alone was shown to induce a protective effector memory response against Mtb in NHP, suggesting pathogen cross-reactivity [241, 257].

High-throughput sequencing of the TCRγδ CDR3 repertoire in the context of Mtb infection showed reduced TCR diversity in TB patients compared to healthy controls, suggesting clonal expansion [165]. Screening of epitopes for these clonally expanded CDR3 sequences allowed the identification of Mtb protein Rv0002 as a novel ligand for TCRγδ. A previous study showed that TCRγδ T cells are able to recognize protein ligands through a dual recognition mechanism involving both the TCRγδ and NKG2D receptors [151]. The Rv0002 Mtb protein was able to stimulate circulating TCRγδ T cells from TB patients, but the precise mechanism leading to the recognition of this protein still needs to be elucidated [165]. Several other TCRγδ T cell-specific Mtb-derived protein antigens have been identified which might induce protective responses [45, 68, 298]. Therefore, TCRγδ T cells reactive to Mtb phosphoantigens or Mtb protein antigens may be promising immune cells to mobilize and engage in novel immunization strategies against Mtb.

Concluding Remarks

The current challenge in the fight against tuberculosis is characterized by a lack of understanding of the precise immune responses essential to protection against infection or disease progression. Complex infectious pathogens generally require involvement and integration of both innate and adaptive immune cells and molecules. The studies compiled in this chapter evidence influential roles for unconventional lymphocytes in host defence to TB, in integration with conventional T cells and B cells, as well as key cells and molecules of the innate immune system. HLA-E-restricted T cells, MR-1 restricted MAIT cells, CD1-restricted T cells and TCRγδ T cells are all activated upon presentation of different mycobacterial pathogen specific stimuli via non-polymorphic molecules, allowing for an integrated response. Whole cell vaccines such as MTBVAC and VPM1002, currently under evaluation in clinical trials, are promising candidates carrying antigens to potentially elicit conventional as well as unconventional T cells. Specific strategies to boost whole cell vaccine-induced activation of unconventional T-cells may further increase protective immunity. Understanding how these responses are triggered and regulated is essential to be able to optimally harness them to the advantage of TB control.

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Chapter 11
Targeting Inhibitory Cells Such as Tregs and MDSCs in the Tuberculous Granuloma

Sadiya Parveen, John R. Murphy, and William R. Bishai

Need for Host-Directed Therapies for Tuberculosis Treatment

Mycobacterium tuberculosis (Mtb) is one of the most successful obligate human pathogens in human history, infecting more than one-fourth of the world's population. The World Health Organization reported that ten million humans contracted active TB disease, and 1.3 million died in 2017 alone worldwide, making tuberculosis one of the most significant global health challenges. New approaches to therapy are needed for many reasons. The long duration of existing treatment regimens contributes to poor adherence and drug-related toxicities, especially in developing countries. And the emergence of multidrug and extensively drug-resistant Mtb strains limits the utility of current drugs. These issues raise the need for the development of novel TB drugs that are more effective, less toxic, and act over shorter treatment periods. Efforts to identify novel antibacterial compounds against Mtb have progressed slowly with only two new drug classes specifically for TB introduced since the 1960s. Hence, novel treatment strategies such as host-directed therapies (HDTs), aimed a modulating/boosting host immunity, have attracted attention as a means to bolster the TB treatment armamentarium. HDTs also offer the advantage of potentially reducing TB associated tissue damage. Moreover, HDTs are unlikely to be compromised by the emergence of drug resistance in the same manner as antimicrobials are. With several novel immunomodulatory agents becoming available for neoplastic and autoimmune diseases, it may be possible to repurpose such agents as HDTs for tuberculosis. HDT approaches that modulate the host immune pathways by targeting T-cell checkpoints have resulted in a paradigmatic shift in the treatment of many malignant diseases.

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In this chapter, we will review the roles of immunosuppressive cells in tuberculosis. As is the case with cancer, cells of both lymphoid and myeloid origin, T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs) respectively, have been implicated in generating a permissive environment for Mtb survival and proliferation [16, 57]. We will discuss both the biology of these immunosuppressive cells as well as the strategies to target them with inhibitors.

Tuberculous Granulomas: Morphology and Heterogeneity

The hallmark lesion of TB pathology is the tuberculous granuloma. Granulomas are a compact mass of immune cells comprised of lymphocytes, macrophages (both infected and uninfected), foamy macrophages, epithelioid cells, and Langerhans cells. Historically, granuloma formation has been considered a primary host defense mechanism to contain Mtb infection in a localized region. Histological analyses of autopsy samples from lungs of TB patients demonstrated higher bacterial burden in areas with caseation necrosis as opposed to the areas of cellular infiltrates lacking necrosis [151, 181]. Similarly, studies performed in zebrafish revealed that granulomas with necrosis were associated with uncontrolled bacterial proliferation [151, 202]. However, in recent decades, it has become apparent that the granulomatous lesion represents an incomplete immune response, since eradication of Mtb is rarely achieved. From this point of view, rather than a hostile microenvironment for the microbe, the granuloma may be seen as a bacterial subversion mechanism whereby the bacteria prevent immune killing and generate a permissive niche for their survival, proliferation, and spread. It is also clear that Mtb can modulate its growth dynamics within the granuloma. These changes include entry into a persistent non-replicating state defined by slow growth and concomitant metabolic changes rendering the bacilli resistant to various environmental stresses [151, 222]. Structurally and immunologically, there are key similarities between the tuberculous granuloma and the tumor microenvironment present during cancer (Fig. 11.1)**.**

Interestingly, different stages of tuberculosis are also characterized by differences in the cellular composition and architecture of the granuloma: (1) during active and latent TB, classic granulomas with a central necrotic region comprised of dead/apoptotic macrophages and other immune cells are present; (2) during active disease, necrotic neutrophilic granulomas, fibrotic granulomas, and non-necrotic granulomas composed almost entirely of macrophages with a few lymphocytes are found, while (3) during latent TB (and occasionally in active disease), granulomas are largely fibrotic. During active TB, Mtb replicates in the peripheral, oxygenated regions of hypoxic granulomas. In contrast, during latent TB infection, the central region of the granuloma is believed to harbor small numbers of Mtb bacilli present in a persistent state.

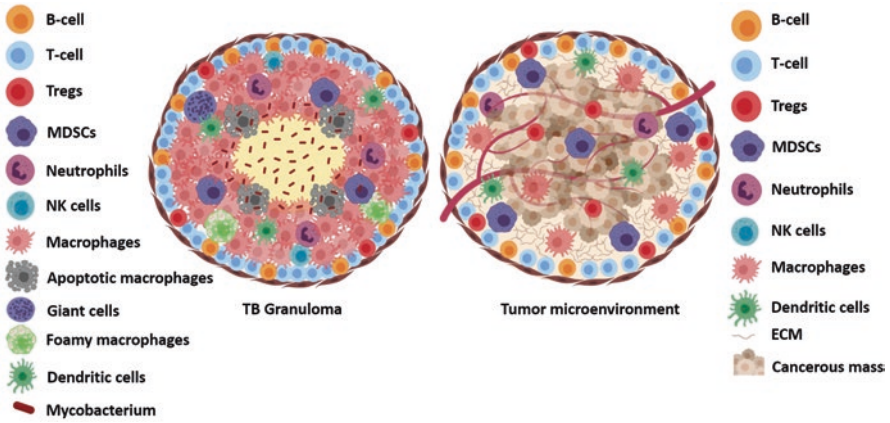

Fig. 11.1 Structure and composition of the tuberculous granuloma and a solid tumor microenvironment. Both pathological sites share similar features including the abundance of immunosuppressive cell populations of MDSCs, Tregs and M2-polarized macrophages which prevent mounting of an effective Th2 response, despite the presence of the necessary immune components. The innermost region also experiences severe hypoxia and, at times, undergo necrosis. Intriguingly, these seemingly hostile environments remain permissive and often serve as a favorable niche for either Mtb replication or tumor growth. In several cases, granuloma and solid tumors are also encircled by a lymphatic layer serving as an outermost protective layer

Role of Immune Cells in the Granuloma Formation and Dissemination

Both host and bacterial factors drive formation of granulomas in the lungs of TB patients [205, 240]. In the early stages of infection immediately following inhalation, Mtb infects alveolar macrophages and replicate inside them. Uninfected macrophages from the surrounding area are recruited by both host and bacterial factors. For example, the secreted Mtb protein ESAT-6 induces epithelial cells to secrete matrix metalloproteinase-9 (MMP-9), which then recruits additional macrophages into the granuloma [46, 212, 216]. At the site of infection, local macrophages undergo metabolic reprogramming and differentiate into specialized myeloid cell types including giant cells, foamy macrophages, and epithelioid macrophages [168, 170]. Some infected macrophages migrate away from the initial site of infection and enter lymphatics and the vasculature, resulting in a re-seeding of infection to remote organs and other sites in the lung, and secondary granulomas [46].

Beside macrophages, dendritic cells (DCs) are one of the first immune responders to arrive at the site of the infection. DCs gain access to bacterial antigens and serve to trigger acquired cell-mediated immune responses [227]. DCs may also become infected, and infected DCs have been demonstrated to contribute to granuloma reformation by interacting with Mtb-specific T-cells at developing foci [87]. Upon infection, mycobacteria modulate DC behavior by impairing their antigen presentation capability and their ability to migrate; this serves to delay the onset of

acquired immunity, which is required to prevent bacterial proliferation [226, 227]. Additionally, polymorphonuclear neutrophils (PMNs) also play important roles in the formation of early granulomas in a chemokine-mediated process [189]. Through studies in experimental mouse models, PMNs have been shown to play a role in T-cell priming [14, 62, 103, 106, 189]. Another myeloid cell population, myeloid-derived suppressor cells (MDSCs), have been shown to accumulate at the lesion site and cooperate with the cells residing in granuloma, mediating inflammation and suppressing various T-cell responses [54].

T- and B-cells arrive at the infection site in the final stages of granuloma formation and constitute the outermost lymphocytic layer of the granuloma [60, 82, 144, 174, 210]. The arrival of these cells coincides with the cessation of bacterial proliferation and entry of the bacilli into a state of persistence [34, 39, 67, 68, 169, 182, 183]. Numerous T-cell subsets including $CD4^+$, $CD8^{+,}$ and CD4/CD8 double-negative cells are known to assemble during this phase of granuloma formation [43, 148, 184]. In subjects who develop progressive, active TB, a dominant feature within the core of the lesion is macrophage necrosis, with bacilli being released extracellularly for subsequent rounds of infection and necrosis. This cycle leads to the formation of the hypoxic granuloma core abundant in lipids known as the caseum [109].

Pre-clinical Animal Models for Immunotherapy

For drug discovery, murine models of disease have remained the most convenient and widely used system. However, it should be noted that most mice strains such as Balb/c, C57BL/6, and 129S2 develop granulomas that are cellular, devoid of necrosis, fibrosis, and hypoxia, and do not recapitulate the human pathology. There are a few mouse strains that develop necrotic TB lung lesions, particularly the C3HeB/FeJ strain. C3HeB/FeJ mice, as well as C57BL/6-sst1S mice, exhibit greater susceptibility to tuberculosis than their wild type counterparts and develop necrotic, human-like granulomas [132, 166]. Both C3HeB/FeJ and C57BL/6-sst1S strains have been used to study granuloma biology and to perform preclinical drug testing. A transgenic mouse strain overexpressing IL-13 has also been shown to form necrotic granulomas. The strain was developed by Heitmann et al., and the necrotic granuloma pathology emphasizes the role of IL-13/IL-4 axis in tuberculosis progression [91, 112].

Myeloid-Derived Suppressor Cells (MDSCs): The Double-Edged Swords?

MDSCs represent a subset of immature heterogenous progenitor innate immune cells which are known to suppress both T-cell and NK-cell functions. MDSCs are comprised of two major subpopulations; monocytic MDSCs (M-MDSCs) and

polymorphonuclear MDSCs (PMN-MDSCs). In mice, M-MDSCs are defined as $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{high}$ cells while PMN-MDSCs are $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$. In humans, MDSCs have been classically described as $CD11b^{+}$ $CD33^{+}$ $HLA\text{-}DR^{low/neg}$ cells [5, 157]. Further, PMN-MDSCs are classified as $CD14^{-}$ $CD66b^{+}$ $CD15^{+}$, while M-MDSCs as $CD14^{+}$ [38, 66, 71, 236]. A third subset of early murine MDSCs (e-MDSCs) has been described but they have not been detected in human samples to date [20].

Despite the above definitions, a comprehensive marker set to discriminate MDSCs from other granulocytic and monocytic cell populations is yet to be defined [38, 72]. As a result, the above-listed cell surface markers should be used in conjunction with functional assays such as T-cell activity/proliferation inhibition assays to accurately identify MDSCs [20]. In cancer, PMN-MDSCs constitute up to 80% of the MDSC population in the tumor microenvironment, and both M-MDSCs and PMN-MDSCs have been shown to induce host-driven T-cell tolerance and to promote immunosuppression [66, 139]. Similar observations for M-MDSCs and PMN-MDSCs have also been made in chronic infections [22, 149]. Moreover, M-MDSCs are also known to differentiate into PMN-MDSCs [143]. However, the two subsets possess differential immunosuppressive activity, and M-MDSCs have consistently shown higher immunosuppressive potential than PMN-MDSCs in tumor models [143].

The Emerging Role of MDSCs in Tuberculosis and Tuberculous Granuloma

The earliest evidence for the involvement of regulatory myeloid cell populations resulted from studies of immune responses following *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) infection [136]. These studies showed that BCG infection induced expansion of myeloid progenitor cells in the bone marrow and that these cells subsequently migrate to the peritoneal cavity and spleen [9, 10]. Later studies demonstrated that BCG administration expands both bone marrow-derived and splenic suppressor cells [105, 110]. Accumulation of such suppressive cell populations was also observed in mice exposed to Complete Freund's adjuvant (CFA), which contains inactivated mycobacteria [221]. These studies also established that these suppressive cells blocked T-cell proliferation, dampened T-cell activation, and showed no cytotoxic activity against mycobacteria [105, 110, 221].

Later investigations demonstrated the presence of MDSCs in the blood of both BCG vaccinated mice [142] and in humans with active TB [56]. Interestingly, the level of MDSCs has also been demonstrated to increase in recently exposed household contacts of TB patients [107]. Also, the levels of MDSCs seem to be higher in necrotic granuloma-prone murine strains such as $NOS2^{-/-}$, C3HeB/FeJ, 129S2 compared to strains which are resistant to necrotic granuloma formation [111, 209]. However, the frequency of MDSCs and MDSC subset populations have still not been correlated with various disease factors such as bacterial burden, lung radiological involvement, and degree of AFB smear positivity. M-MDSCs have been

mainly identified in pleural effusions, while PMN-MDSCs have been shown to be abundant in bronchoalveolar lavage (BAL) of pulmonary TB patients [61]. While it is not directly known, the relative abundance of MDSC subsets in such biological compartments may be related to disease stage in humans. Indeed, in murine models of acute TB infection, MDSCs initially appear in the lung and subsequently spread to other compartments such as the pleural, bronchi (BAL fluid), and peripheral blood as the disease progresses [209].

In addition to suppressing T-cell responses, lung residing M-MDSCs serve as a favorable niche for Mtb replication through IL-4/IL4R dependent mechanisms [111]. One contributing factor could be insufficient antibacterial activity of M-MDSCs against Mtb. Secondly, in tumor models, MDSCs present in the tumor microenvironment have been shown to consume oxygen at a higher rate and utilize fatty acid oxidation as the primary source of energy [94]. This altered metabolic state of M-MDSCs may also be driving Mtb growth inside M-MDSCs, as in the case of alveolar macrophages, since bacilli have been shown to use host cholesterol and fatty acids [96]. However, no studies have yet established a link between fatty acid oxidation and Mtb proliferation. Additionally, there is also no clarity on the metabolic state or the subcellular localization of Mtb inside M-MDSCs. Interestingly, ex vivo derived human M-MDSCs do not support robust Mtb replication as well as macrophages [2]. Thus, much remains to be learned.

Immunosuppressive Mechanisms Mediated by MDSCs

As shown in Fig. 11.2, in many cancers MDSCs have been shown to exhibit potent immunosuppressive functions in the tumor microenvironment via multiple mechanisms: (1) blocking of lymphocyte homing; (2) depletion of metabolites important for T cell function; (3) production of reactive oxygen and nitrogen species; (4) expression of exoenzymes that regulate adenosine metabolism; and (5) expression of inhibitory immune checkpoint molecules.

Inhibition of T-Cell Lymphocyte Homing and Tissue Infiltration

Perhaps the most important immunosuppressive characteristic of MDSCs is to block both antigen-dependent and -independent activation of $CD4^+$ and $CD8^+$ T-cells. This blockade is caused either by impairing lymphocyte homing or depleting the metabolites essential for T-cell function. MDSCs have been shown to induce down-regulation of several cell adhesion molecules in T-cells that are critical for cell migration and infiltration of infected tissue sites. For example, splenic MDSCs downregulate the expression of the cell adhesion molecule L-selectin (CD62L) on T- and B-cells, reducing the homing and antigen-dependent activation of $CD8^+$ cells in lymph nodes [113, 162]. This downregulation was found to be

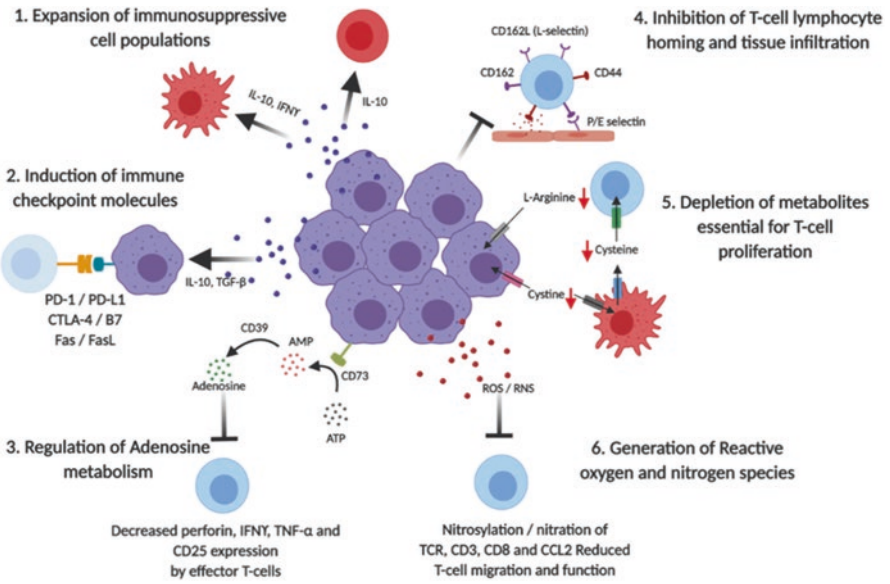

Fig. 11.2 Schematic representation of prominent MDSC-mediated immunosuppressive mechanisms. These include (1) expansion of immunosuppressive cell populations of Tregs and M2-polarized macrophages; (2) induction of immune checkpoint molecules such as PD-L1, CTLA-4 and FasL etc.; (3) Regulation of adenosine metabolism by overexpressing ectoenzymes (CD39 and CD73) and expanding the exhausted effector T cell population; (4) inhibition of T-cell lymphocyte homing and infiltration into the affected tissues by downregulating the expression of one or more adhesion molecules; (5) depletion of the metabolites such as arginine and cysteine crucial for the proliferation of effector T-cells and; (6) generation of reactive oxygen and nitrogen species killing the effector T-cells. Both M-MDSCs and PMN-MDSCs utilize slightly different immunosuppressive mechanisms in various cancer models [143]. Several of these mechanisms also need to be verified in the context of TB infection

inversely correlated with MDSC levels in mice and is likely driven by metalloprotease ADAM 17 (TACE), which is expressed on the surface of MDSCs [86]. In vitro, the M-MDSC subset, which is NO-dependent, has been shown to offset the activation-induced changes in CD44, CD62L, and CD162 expression by T-cells, leading to impaired T-cell infiltration of tissues [187].

Inhibition of T-Cell Proliferation by Depleting Essential Metabolites

MDSCs can also modulate the immune system by creating a depletion of essential metabolites in the tumor microenvironment by secreting a battery of four different enzyme categories: (1) nitric oxide synthases (NOS1, NOS2, and NOS3), (2) arginases (ARG-1 and ARG–2), (3) arginine-glycine amidino transferase, and (4)

L-arginine decarboxylase. In addition, MDSCs have been shown to deplete L-arginine by increasing CAT-2B transporter mediated uptake, which is accompanied with increased ARG-1 expression in these cells [180]. The scarcity of extracellular L-arginine may inhibit the proliferation of activated T cells and reduce the expression of the TCR-ζ chain critical for the process of antigen recognition and signal transduction [19]. It should be noted, however, that it was recently reported that ARG-1 expression is not crucial for MDSC-mediated immunosuppression, although ARG-1 expression could be induced by activated T cells [13]. In this setting, direct cell-cell contact was necessary for MDSCs to inhibit T cell proliferation [13]. Since multiple studies verified the contribution of ARG-1 to MDSC function, further investigations will be critical to understand its precise role in MDSC function.

T cells are also known to lack cystathionine γ-lyase and certain cystine transporters, making cysteine an essential amino acid for their homeostasis [77]. Accordingly, T-cells depend upon macrophages and dendritic cells, which take up cystine, convert it into cysteine, and secrete it into the extracellular space through alanine–serine–cysteine (ASC) transporters. Since MDSCs express the SLC7A11 transporter, they are able to deplete cystine from the tumor microenvironment and thereby impair T cell functions [198]. Depletion of cysteine also compromises the resistance of immune cells to reactive oxygen species by limiting the generation of glutathione, an important antioxidant molecule [161].

Generation of Reactive Oxygen and Nitrogen Species

MDSCs also secrete toxic reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, hydrogen peroxide, and singlet oxygen, which serve to (1) inhibit or kill local susceptible immune cells including lymphocytes, (2) promote MDSC expansion and, (3) recruit additional MDSCs probably via VEGF receptors present on MDSCs [117, 130]. It has been shown that MDSCs isolated from NOX2 knockout mice generate lower amounts of ROS and, as a result, were unable to inhibit IFN-γ secretion or proliferation of antigen-specific $CD8^+$ T cells [40]. Also, as ROS production decreases, MDSCs tend to differentiate into $F4/80^+$ $Gr1^-$ macrophages or $CD11c^+$ $CD11b^+$ dendritic cells [40]. It has also been shown that MDSCs can partially protect themselves from the adverse effects of ROS by adopting metabolic changes such as by expressing Nrf2, a transcription factor mediating the cellular antioxidant responses [11], or by increasing glycolysis, which leads to the cytosolic accumulation of the anti-oxidative intermediate phosphoenolpyruvate (PEP) [101]. PEP has been shown to not only prevent ROS-induced apoptosis but also to promote MDSCs accumulation [101]. In a mouse ovarian cancer model, increased expression of VEGFR-1 and -2 on intra-tumoral MDSCs was linked to their recruitment to the tumor site in a STAT3 dependent fashion [93, 153, 219, 238].

In addition to ROS, MDSCs have also been shown to generate high levels of reactive nitrogen species (RNS), primarily nitric oxide (NO), by activating iNOS

[172]. Elevated NO levels then lead to the upregulation of multiple immunosuppressive markers including IDO, IL-10, ARG-1 in ex-vivo-derived MDSCs [156]. In combination with low L-arginine levels, iNOS was shown to induce the production of peroxynitrites (ONOO-), a highly reactive ion that causes T cell apoptosis and nitration of T cell receptors (TCRs), thereby inhibiting T cell activation. Earlier studies have also suggested that iNOS-dependent NO production by MDSCs adversely affects different FcR-mediated functions of NK cells [200]. In both PMN-MDSCs and neutrophils, high levels of myeloperoxidase (MPO) have been shown to induce production of free radicals [233]. The level of MPO also increases in the plasma of renal cell carcinoma patients, and is perhaps produced by PMN-MDSC [179].

Modulation of Other Immune Cell Populations

MDSCs have been shown to induce the *de novo* generation of Tregs in vivo via IFN-γ, TGF-β, and IL-10-dependent mechanisms [95, 121]. M-MDSCs derived from hepatocellular carcinoma patients induced formation of Tregs when co-cultured with matched T cells [239]. In turn, Tregs potentiate MDSC activity by inducing the expression of ligands related to programmed cell death ligand 1 (PD-L1, B7-H1), and production of IL-10 [69]. M-MDSCs have also been shown to facilitate the recruitment of Tregs to the tumor microenvironment by a CCR5 dependent process [185]. MDSCs were also found to facilitate the reprograming of macrophages towards an M2-like phenotype, thereby promoting tumor growth [12].

Several studies have shown that MDSCs can impair NK cell function by down-regulating the expression of NK cell-activating receptors (NKp46, NKp44 and NKG2D), by down-regulating the production of perforin which mediates target cell apoptosis, or by interfering with their ability to sense and respond to IL-2.

Expression of Ectoenzymes Regulating Adenosine Metabolism

Extracellular ATP functions as a Danger-Associated Molecular Pattern (DAMP) and promotes both innate and adaptive immune responses. MDSCs are known to dampen this response by high expression of ectonucleotidases (most prominently CD39 and CD73) on their cell surface. These ectonucleotidases progressively dephosphorylate ATP into adenosine and thereby inhibit T cell function [127]. In ovarian carcinoma patients, the administration of metformin, which downregulates the expression of CD39 and CD73 in MDSCs, was found to enhance overall survival rates accompanied by a decrease in total count of MDSCs and improvement in effector T-cell function [128].

Expression of Immune Checkpoint Molecules

PD-L1 is a well-known negative regulator of T effector cell function that mediates immunosuppression [102]. PD-L1 induces anergy and apoptosis by binding to its cognate receptor PD-1 on the T cell surface. Interestingly, various reports have demonstrated that MDSCs upregulate PD-L1 expression in cancer patients and murine tumor models [230]. The induction of PD-L1 on MDSCs is likely to be mediated by soluble factors M-CSF, VEGF, and IFN-γ [98].

MDSC Immunosuppressive Mechanisms During TB Infection

As described above, the immunosuppressive mechanisms mediated by MDSCs have been well studied in cancer biology. However, their roles in TB infection remain less defined. Myeloid cells induce adaptive immunity, are the first responders to Mtb aerosol challenge, and play critical roles in the containment of the bacilli. These first-responder myeloid cells also serve to prevent excess inflammation [57]. In active TB lesions, however, there is a pathologic progression of local inflammation, which is associated with MDSC accumulation [162]. To date, studies on the roles of MDSCs in TB pathogenesis have mostly focused on MDSCs involvement in T-cell suppression [57].

As described above, it is known that MDSCs interact extensively, both through direct cell/cell interactions, and indirectly through other immune cell populations such as macrophages, dendritic cells, and regulatory B- and T-cells [124, 167, 220]. This network of interactions remains poorly characterized in TB. Despite the recent observation that MDSCs are correlated with reduced T-cell function in TB patients, there is still no clarity on the mechanism by which MDSCs impair lymphocyte function during TB. The effects perhaps could be mediated either by inhibition of cytokine production, impaired T-cell activation, reduced T-cell mobility and trafficking, or dampened T-cell priming leading to suppressed polyclonal T-cell proliferation [58, 142]. In the case of BCG vaccinated mice, iNOS is known to mediate suppression of lymphocytes, with the concomitant *in situ* co-expression of ARG1 and iNOS in lung lesions [111]. Furthermore, in experimental TB studies, MDSC function has been shown to be regulated by several cell surface molecules. For instance, direct cell-to-cell contact between tmTNF-α expressing MDSCs and TNFR2-expressing CD4 T-cells has been shown to regulate MDSC activity in mice with mycobacterial pleurisy [27]. Also, TB infection has been shown to up-regulate PD-L1 on human MDSCs *in vitro* [2] and restrict T-cell proliferation [37, 237]. This expression may contribute to the profound immunosuppression observed in end-stage TB patients.

Additionally, the role of various enzymes/factors crucial to MDSC functions has not been established in the context of TB. Prominent examples are NADPH-oxidase-mediated ROS production [20], Cox-2-mediated production of prostaglandins [140], and the upregulation of autophagy molecules in MDSCs [3]. The impact of

MDSCs on other immunosuppressive cell populations such as Tregs, Bregs, and NK cells also remains unknown. It will be important to understand the interaction between MDSCs and macrophages, which are preferred intracellular niche of Mtb bacilli in the host.

MDSCs have been shown to be critical for granuloma stability by either modulating or producing cytokines such as IFN-γ, TNF-α, IL-10, and IL-6. In murine models of TB infection, MDSCs have also been shown to harbor the bacilli (preferentially M-MDSCs) and promote tissue damage. Targeting of MDSCs, either alone or in conjunction with standard TB chemotherapy, reduces bacillary load and improves prognosis [83, 111, 142, 209]. Moreover, in a non-human macaque primate model, a population of macrophages co-expressing nitric oxide synthase and arginase-1 were detected specifically in necrotic granulomas [144]. However, in TB patients, the interaction(s) of human MDSCs with Mtb and their impact upon granuloma formation and pathology remains in need of further study.

It is known that T-cells secrete the protective cytokines, IFN-γ and TNF-α, which are critical to the maintenance of granuloma structure, which may serve to contain bacterial replication and prevent reactivation of latent TB [2]. Along these lines, MDSCs have been proposed to play a role in TB reactivation by blocking the production of protective cytokines by T cells and exacerbating immunosuppression in the tuberculous granuloma.

In cancer biology, the role of PDL-1/PD-1 axis and its blockade has been shown to restore T-cell activation and the down-regulate IL-6 and IL-10 [154]. However, to date PD-L1-mediated suppression of effector T-cell function by MDSCs has not been demonstrated [120, 234]. In TB, Agarwal et al. have shown upregulation of PD-L1 on Mtb-infected MDSCs as well as an increased frequency of PD-L1 positive cells in "in vitro granuloma like structures (IVGLSs)" [2]. However, PD-L1 neutralization using anti-PD-L1 antibody did not restrict mycobacterial growth although it promoted infiltration of $CD3^+$ cells. Studies in humans receiving antimicrobial therapy for TB have revealed that, while PD-L1 levels are elevated at the time of initial diagnosis, there is down-regulation of PD-1, PD-L1, and PD-L2 expression during treatment [89]. Studies in murine models and active TB patients indicate that PD-1 inhibitors inhibit the immunosuppressive activity of MDSCs primarily by interfering with PD-1/PD-L2 cross-talk [2, 208]. A few studies, however, have demonstrated granuloma formation and development of tuberculosis in cancer patients that were treated with checkpoint blockade therapy [7, 70, 175]. The latter observation, if confirmed, would question the efficacy of checkpoint blockade as host-directed immunotherapy for tuberculosis.

Therapeutic Targeting of MDSCs in TB

Over the last decade, MDSCs have emerged as a novel and promising therapeutic target for adjunctive host-directed therapies (HDT) for TB. The major focus of this approach has been to alleviate the immunosuppression conferred by MDSCs on

T-cells by employing strategies that either inhibit MDSC induction/activation or block MDSC functions. MDSC-targeting strategies have shown promise in cancer both in preclinical and clinical studies and may be valuable HDT treatment options for TB [55]. These strategies may be broadly classified as approaches to (1) inhibit MDSC expansion and recruitment, (2) block MDSC function, and (3) promote differentiation of MDSCs into other non-immunosuppressive cells.

Inhibition of MDSC Expansion and Recruitment

Several cytokines, such as vascular endothelial growth factor (VEGF), TNF-α, and IL-6, have been shown to be critical for the expansion of MDSCs. Approaches targeting these cytokines have shown encouraging results in animal models of TB and in patients with severe cases of pulmonary TB [44, 159]. In Mtb-infected mice, administration of anti-TNF-α antibodies led to higher bacterial burden in liver, spleen, and lungs, and decreased survival. Interestingly, anti-TNF-α treatment also results in the occurrence of immature granulomas in the same organs. Since TNF-α is required for granuloma formation, anti-TNF-α antibody treatment most likely destabilized tuberculous granulomas and their ability to contain bacterial proliferation, thereby leading to bacterial expansion. Treatment with the anti-VEGF monoclonal antibody bevacizumab has been shown to induce vascularization of granulomas and to alleviate hypoxia in a rabbit model of TB [44]. Also, granuloma re-vascularization may help improve the delivery of the chemotherapeutic drug to the lesion and thereby improve the efficacy of the available treatment regimen. Both TNF-α and anti-VEGF therapies has been shown to reduce MDSC frequencies in cancer models [178, 217].

Transcription factors such as STAT3 and STAT3-induced S100A9 proteins have been also found to be critical for MDSC expansion and development [30, 196, 203]. Drugs targeting the kinases responsible for STAT3 phosphorylation have shown promise in murine cancer models and patients [28, 55]. Studies from our laboratory showed that the FDA-approved JAK-STAT inhibitor tofacitinib reduced the time to organ sterilization when added to standard anti-TB drug therapy in a mouse model of TB [137]. Along similar lines, the FDA-approved drug gefitinib, which inhibits the tyrosine kinase activity of the EGFR, exhibited both in vitro and in vivo efficacy against Mtb infection [197, 199]. Another drug, imatinib, which inhibits the tyrosine kinase activity of the Abelson proto-oncogene protein ABL1 as well as that of the platelet-derived growth factor receptor, has also shown efficacy against TB in murine models [57, 150]. However, the role of MDSCs was not specifically investigated. Cytotoxic agents, such as 5-fluorouracil and gemcitabine, which are known to deplete MDSCs in cancer models and also may have direct antibacterial activity against Mtb, may offer a potential dual role as mixed antibacterial and host-directed therapies [119, 195, 215]. However, their significant bone marrow suppressive activity may limit their utility.

The serum DAMP molecules of S100A8 and S100A9, which are produced by myeloid cells, are heavily expressed in tuberculous granulomas and are thought to

play a role in MDSC trafficking to the granuloma. Their expression has also been associated with disease severity, neutrophilic inflammation, and lung pathology in animal TB models [80, 103]. Tasquinimod, a drug that binds S100A9 and appears to block it from engaging cell surface receptors, has been used to block MDSC trafficking [193]. In a murine model, administration of tasquinimod resulted in significant decline in MDSCs population in lung and spleen after 21 days of the treatment, along with significant reduction in the bacillary burden [84]. This suggests that agents that reduce MDSC trafficking may have beneficial HDT activity in TB.

Blockade of MDSC Function

Treatment of phagocytes with the Arginase-1 inhibitor N^{ω}-hydroxy-L-arginine (nor-NOHA) resulted in decreased bacterial burden and lowered IL-10 production by mycobacteria-challenged macrophages in vitro [48]. Similarly, phosphodiesterase-5 inhibitors (PDE-5-i), such as sildenafil and tadalafil, were shown to decrease MDSCs functionality by reducing iNOS and Arg1 expression, thereby compromising tumor growth [155, 190]. In TB, treatment with phosphodiesterase inhibitors (PDE-i), including FDA-approved agents, resulted in reduced bacterial load, improved lung pathology, and decreased disease severity in mouse models [138, 201]. Cyclooxygenase-2 (COX2)-inhibitors also have shown promising results in improving Th1 immune responses to Mtb infection [92]. Treatment of blood samples from TB patients with indomethacin, a COX2 inhibitor, reduced Mtb-specific Treg frequencies, T-cell proliferation and cytokine production; however, this study did not investigate the effect on MDSCs [207]. An ongoing trial with another COX2 inhibitor, etoricoxib, is currently being evaluated for its impact upon the myeloid cell populations during human TB [59]. In macaques, administration of the small molecule inhibitor 1-methyltryptophan, which blocks indoleamine 2,3-dioxygenase (IDO), a host enzyme known to activate MDSC and Treg function, decreased TB disease severity, reduced bacterial burden, and improved pathological symptoms, presumably by perturbing granuloma organization [74]. Related experiments in IDO deficient mice, however, did not reveal an effect during Mtb infection [15]. Neither IDO study reported specific analyses of the effect on MDSC function, therefore further studies are needed.

Promoting Differentiation of MDSCs into Other Non-immunosuppressive Cells

In cancer models, all trans-retinoic acid (ATRA), a naturally occurring isomer of retinoic acid (a vitamin A metabolite), works as a differentiation factor, since it promotes conversion of MDSCs into mature dendritic cells, macrophages, and granulocytes both in vitro and in vivo [116]. It also downregulates the expression of immunosuppressive genes including PD-L1, IL-10, and IDO in MDSCs [206]. In

TB, ATRA and other retinoic acids exhibited antibacterial activity in Mtb infected phagocytes in vitro [41, 224]. In a murine TB model, ATRA treatment led to reduced MDSC frequencies, improved T-cell population, reduced bacillary burden, and improved pathology [111]. ATRA has also been shown to augment autophagy of Mtb bacilli in alveolar macrophages of both murine and human origin [35].

Regulatory T Cells (Tregs): Friend or Foe?

Regulatory T-cells (Tregs) are an immunosuppressive subset of T cells that are often classified as $CD4^+$ $CD25^+$ $FoxP3^+$ cells. These cells can be divided into two subsets: (1) natural Tregs (nTregs), which develop in the thymus as a result of interactions with self-antigens, and (2) induced or adaptive Tregs (iTregs/aTregs), which accumulate in the peripheral blood and other organs upon chronic exposure to antigens [147]. Abbas et al. has recently recommended to rename nTregs as 'Thymus-derived Tregs (tTregs)' and iTregs/aTregs as 'Peripherally-derived Treg (pTreg)' based on the anatomical location of their development [1]. tTreg generation strongly depends on TCR and CD28 signals and cytokines such as IL-2, IL-15, and TGFβ, which increase FoxP3 expression [186]. In contrast, pTreg generation is stimulated in an anti-inflammatory milieu and is a dendritic cell-dependent process [51, 214]. Both Treg subsets share many suppressive mechanisms and cell surface molecules, such as CTLA-4, LAG, and NRP1, which mediate both contact-dependent inhibition and secretion of soluble cytokines such as IL-10 and TGF-β [32]. Tregs have also been classified based on the cytokine production. For example, the T3 subset produces TGFβ, the Tr1 subset produces IL-10, and the Tr35 subset produces IL-35. These subsets elicit their suppressive functions in a contact-independent fashion [6]. Tregs were initially identified as cell populations that prevented autoimmune diseases [223, 232]. Further, the significance of Tregs to human diseases was clearly established by the discovery that individuals who carry mutations in their FoxP3 locus and have non-functional Tregs develop a fatal systemic autoimmunity, also known as immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX), which can only be managed by transplantation of bone marrow [218].

The Emerging Role of Tregs in Tuberculosis and Tuberculosis Granuloma

The role of Tregs in TB and their effect on disease outcome is still incompletely understood. Several reports describe the expansion of Tregs in the blood, lungs, and other tissues of patients with active TB, and have associated their accumulation with a role in T cell immunosuppression and disease progression [85, 90]. Several other studies, however, found no increase of Tregs in TB patients [31, 152]. It has been

proposed that increased Tregs may provide an anti-inflammatory counterbalance response during TB to limit pro-inflammatory tissue destruction [52]. This down modulation may be beneficial to the host during acute infections, but in chronic infections, particularly when the pathogen is not rapidly destroyed, may adversely affect the host [115]. An additional consideration is that mounting an effective Th1 cellular response during TB is critical to the eradication of the bacilli. However, certain regulatory mechanisms, including Treg functions, may down modulate Th1 response to prevent exacerbation of disease pathology at the expense of compromised pathogen clearance [52, 118].

Moreover, it remains unclear whether Treg expansion in TB causes or is caused by disease progression. During Mtb infection, the expansion of Tregs is believed to be induced by IL-10 and TGF-β, which are known to be abundantly secreted in active TB patients [52]. Ribeiro-Rodrigues et al. reported that Treg frequencies increased in PBMCs of active TB patients and did not decrease even after 6 months of standard antibiotic therapy. Thus, it has been proposed that Treg persistence following treatment may contribute to sustained suppression of IFN-γ production [177]. Another study in pulmonary TB patients by Chen et al. found a positive correlation between the decreased levels of Treg and improved prognosis in patients after 2 years of chemotherapy [29].

Major Immunosuppressive Mechanisms Mediated by Tregs

An understanding of the mechanism of Treg function is critical not only to understanding peripheral tolerance but also to identify putative therapeutic targets. In cancer, immunosuppressive mechanisms of Tregs can be classified into four broad categories: (1) secretion of immunomodulatory cytokines such as TGFβ, IL-10, and IL-35; (2) granzyme- and perforin-mediated cytolysis of effector T cells; (3) metabolic disruption by inhibiting either IL-2R-dependent proliferative responses or cAMP-mediated and A2 adenosine receptor-mediated metabolic responses; and (4) affecting the function and maturation of dendritic cells (Fig. 11.3).

Secretion of Immunomodulatory Cytokines

Tregs secrete multiple immunomodulatory cytokines, such as IL-10, TGFβ, and IL-35, which affect various immune cells expressing the receptors for these cytokines and polarize immune responses [6].

IL-10 is a central immunomodulatory cytokine that can act by both paracrine and autocrine signaling [125, 146]. IL-10 is known to down-regulate Th1 responses and IFN-γ production by inhibiting the production of inflammatory cytokines such as IL-12 [131]. IL-10 also prevents tyrosine phosphorylation of the co-stimulatory molecule CD28 and adversely impacts the interaction of effector T-cells with

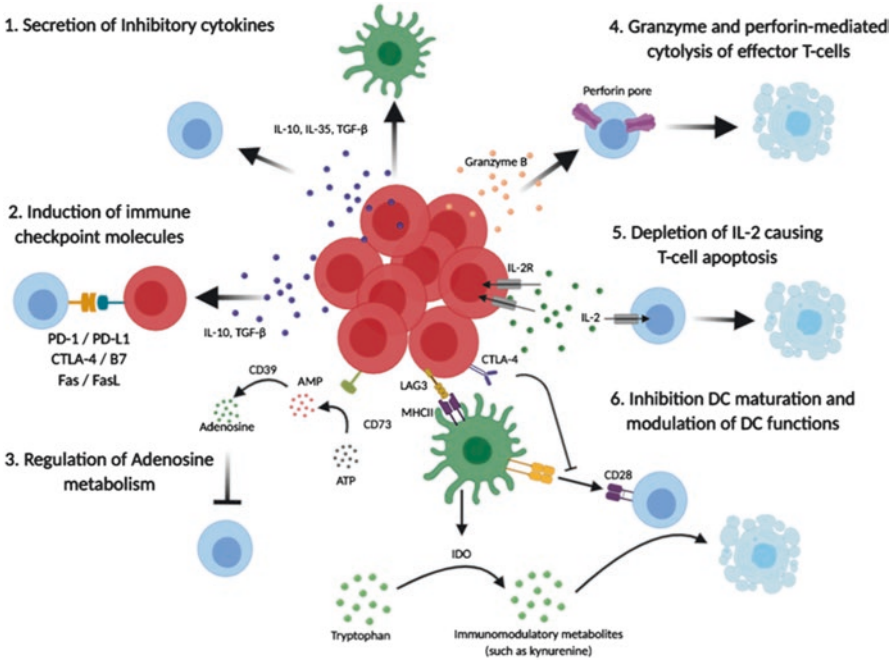

Fig. 11.3 Schematic representation of major Treg-mediated immunosuppressive mechanisms. These include (1) secretion of inhibitory cytokines such as IL-10, IL-35 and TGF-β blunting anti-TB responses mediated by effector T-cells and dendritic cells; (2) induction of immune checkpoint molecules such as PD-L1, CTLA-4 and FasL etc.; (3) Regulation of adenosine metabolism expanding the exhausted effector T cell population; (4) direct cytotoxic killing of effector T-cells via granzyme and perforin mediated pathways; (5) depletion of IL-2 by upregulating IL-2R. Deprived of IL-2, T-cells cannot proliferate and eventually undergo apoptosis; (6) inhibition of both dendritic cell maturation and its function via both contact-dependent and -independent mechanisms. Immunosuppressive mechanisms in cancer models needs to be verified in the context of TB infection

antigen-presenting cells [165, 204]. IL-10 also induces SOCS3 expression in monocytes thereby inhibiting the NF-κB-induced factor MyD88 and the production of IL-1β, IL-6, and TNF-α [26, 204].

TGF-β has been shown to act by several mechanisms including (i) suppression of effector T cell differentiation; (ii) promotion of the differentiation of naıve T cells into Tregs or Th17 cells; (iii) inhibition of T and B cell proliferation; (iv) inhibition of macrophage, dendritic cell, and NK cell activities; (v) regulation of dendritic cell maturation and differentiation; (vi) inhibition of IL-2 production; and (vii) modulation of cell proliferation by regulating the expression of cell cycle-regulating factors such as cyclin-dependent kinases (CDKs such as p15, p21, and p27) and cell cycle promoters (such as cMYC, cyclin D2, CDK2, and cyclin E) [134].

IL-35, a heterodimeric cytokine, acts via several mechanisms including (i) suppressing T helper cell proliferation; (ii) promoting the differentiation of naive T cells into Tregs subset secreting IL-35 (iTr35), and (iii) inducing the conversion of B lymphocytes into B regulatory cells [36, 218].

Granzyme- and Perforin-Mediated Cytolysis of Effector T Cells

Tregs produce granzyme B, a serine protease that induces apoptosis in effector T cells [23, 32, 78, 79]. Tregs secrete granules containing granzymes and perforins via directed exocytosis to the extracellular milieu. Once released, perforin molecules insert into the target cell membrane and create a pore through which granzymes enter the cell. Granzyme can also be endocytosed in a mannose-6-phosphate receptor-mediated process and released from the endosome into the cytosol in a perforin-dependent manner [63, 129]. Additionally, granzyme recruitment is also induced by the membrane damage caused by perforins. Within the affected cell, granzyme B induces apoptosis by caspase-dependent and independent processes [100, 126, 135]. Granzyme B has also been shown to suppress $CD4^+$ $CD25^+$ effector cells in a perforin-independent manner [23, 79]. By cytolysis, Tregs effectively decrease the population of pro-inflammatory immune cells thus skewing immunity in an anti-inflammatory direction [6].

Metabolic Disruption

Tregs control the activity and proliferation of T effector cells and other immune cells both non-specifically by consuming IL-2 and specifically, by cAMP release and/or 2A adenosine receptor-mediated signaling pathways. Each of these three pathways serves to modulate effector T cell responses.

IL-2 is the main cytokine secreted by T-cells upon antigenic stimulation and is essential for T-cell proliferation [8]. By constitutively over-expressing high-affinity IL-2 receptors, Tregs deplete IL-2 from the intracellular milieu. This competition for IL-2 deprives T effector cells and interrupts their ability to proliferate [21].

cAMP is a second messenger that also regulates the functions of T effector and antigen presenting cells. Tregs accumulate high cAMP levels in the cytoplasm by activating several adenylyl cyclases such as AC7 and AC9 [4, 225]. Tregs are then able to transfer cAMP to the target cells via gap junctions. This results in the activation of protein kinase A (PKA), which functions in multiple signaling pathways to: (1) prevent proximal activation of T-cell receptor by inactivating lymphocyte specific protein tyrosine kinase (Lck); (2) inactivate NF-κB, which induces the transcription of proinflammatory cytokines (TNF-α, IL-6, IL-8, VEGF, IL-1b) and metalloproteases (for example, MMP-1, −2, −3 and −13) and; (3) regulate the activation of Rap-1 GTPase to inhibit T cell proliferation and differentiation [33, 50, 213].

Tregs also overexpress the surface-bound ectoenzymes CD39 and CD73, which promote the conversion of ATP to adenosine. Adenosine then interacts with adenosine receptors on the membranes of T-cells, B-cells, NK-cells, macrophages, dendritic cells, and granulocytes [158]. This interaction activates a signaling cascade regulating the transcription of several inflammatory genes resulting in suppression of pro-inflammatory cytokines (such as TNF-α) and promotion of anti-inflammatory

cytokines (such as IL-10) [49, 64, 88, 145]. Adenosine can also act as autocrine factor and modulate the anti-inflammatory response of Tregs. Adenosine can also compromise the ability of dendritic cells to express co-stimulatory molecules and inhibit the activation of effector cells [88, 158].

Adverse Effect upon the Function and Maturation of Dendritic Cells

Dendritic cells (DCs) govern many aspects of the immune response through antigen presentation and activation of resting helper T cells. Upon interaction with Tregs, DCs adopt a tolerogenic behavior inducing Treg cell expansion thereby favoring an immunosuppressive microenvironment. Furthermore, Tregs compete with T effector cells for ligands, such as CTLA-4 and LAG3, on the surface of DCs [6, 122]. The Treg-DC interaction via CTLA-4 results in IFN-γ production, which in turn induces indoleamine 2,3-dioxygenase (IDO). IDO, which metabolizes tryptophan, creates a local deficit of this amino acid thereby arresting growth of effector cells. IDO-mediated tryptophan degradation also produces multiple immunomodulatory metabolites, such a 3-hydroxyanthranillic acid, quinolinic acid, kynurenine and 3-hydroxykynurenine. These metabolites induce apoptosis in T effector cells either by direct activation of caspases or by inducing oxidative stress and depleting glutathione, the main antioxidant in animal cells [18, 81, 97, 229].

Treg-Mediated Immunosuppressive Mechanisms During TB Infection

In murine TB models, Mtb infection induces pathogen-specific Tregs that delay $CD4^+$ and $CD8^+$ lymphocytes priming in pulmonary lymph nodes, and adversely affect their recruitment into the lung [191]. Tregs also prevent pathogen clearance in lung tissue and granulomas [115, 188]. Mtb-induced Tregs also delay the onset of adaptive immunity, facilitating Mtb proliferation as the infection establishes in the lung [39, 163, 191]. The depletion of Tregs and other $CD25^+$ cells, soon after infection, reduces the bacterial burden and granuloma formation [164]. TB progression also positively correlates with the accumulation of $CD8^+$ Tregs producing IL-10 in granulomas [42]. $CD4^+$ $Foxp3^+$ and $CD4^+$ $CTLA\text{-}4^+$ Tregs also increase in patients with consistently positive IGRA tests compared to the consistently negative TB case contacts, suggesting Tregs expansion during latent TB infection [73]. Accordingly, circulating Treg levels may serve as an indicator of therapeutic response in different states of TB disease. For example, Treg frequencies decline in pulmonary TB patients treated with chemotherapy [90, 99, 194] while their levels tend to increase during extra-pulmonary TB treatment [65, 90]. Interestingly,

Treg levels did not change in TB patients who developed MDR-TB during the course of the therapy [194].

The involvement of Tregs in extrapulmonary TB and extrapulmonary dissemination has also been reported [47]. For example, pleural TB patients showed enrichment of Tregs in pleural fluid relative to peripheral blood [75, 228]. Pleural Tregs suppressed production of IFN-γ by lymphocytes [75]. Pleural $CD39^+$ Treg (i.e., activated Tregs) blocked the generation of Th17 cells in a TGF-β-dependent fashion [231]. Additionally, Yuan et al. demonstrated that adhesion molecule present on the surface of pleural mesothelial cells, promoted non-antigen-specific expansion of Tregs, inhibited leukocytes homing, and dampened anti-Mtb T-cell responses [235]. These adhesion molecules include intercellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1(VCAM-1).

In juvenile patients presenting with TB lymphadenitis, Treg accumulation, as well as high levels of TGF-β and IL-13, but not IFN-γ, TNF-α, or IL-17, has been reported [173]. Similarly, military TB patients exhibit enrichment of both Treg and IL-10 in peripheral blood, pleural fluid, and bronchoalveolar lavage [192]. Increased levels of IL-10 and TGF-β mediate Treg expansion during Mtb infection in active TB patients [17, 29, 53, 176]. Some studies have reported a preferential suppressive capacity of Tregs against $IFN\text{-}\gamma^+$ cells compared to $IL\text{-}17^+$ cells, which may cause accumulation of $IL\text{-}17^+$ cells at the site of infection and promote Mtb dissemination [108, 141, 211]. Other studies showed an abundance of Tregs after 2 months of TB treatment, contradicting a role for Tregs in down-regulating inflammation. However, the abundance of Tregs declined as the treatment progressed [52]. Moreover, MDR- and XDR-TB patients exhibit an altered immunophenotype with abundant Tregs both in the circulation and in pulmonary lesions, compared to patients with drug-susceptible TB or latent TB infection [45, 76]. Tregs isolated from these patients could suppress T-lymphocyte proliferation, impair their function, and reduce bacillary containment in macrophages through a cell-to-cell contact independent mechanism [45, 76]. Geffner et al. also showed that Tregs isolated from the blood of MDR-TB patients suppress anti-Mtb-immune responses by inducing IL-10 production, inhibiting IFN-γ secretion and CTL degranulation [76].

Therapeutic Targeting of Tregs in Tuberculosis

Historically, Tregs have been an attractive therapeutic target due to their crucial role in TB pathogenesis and progression [24]. Multiple research groups observed therapeutic benefits upon Tregs depletion in murine TB models. Ozeki et al. showed that depletion of $CD25^+$ cells with an anti-CD25 monoclonal antibody before Mtb aerosol infection in a DBA/2 mouse model reduced bacillary burden in lungs and spleen as well as improved histopathology in the lungs [164]. However, the effect was only sustained for 2 weeks post-infection. Depletion of $CD25^+$ cells during the chronic phase of infection showed no significant improvement in outcomes. In

another study using a bone marrow chimera system in which all FoxP3$^+$ cells expressed Thy1.1, FoxP3$^+$ cells could be depleted using an anti-Thy1.1 antibody. Despite an approximately 1 log reduction in bacillary load of lungs, there was no significant change in the numbers of pathogen-specific effector T cells in this study. The result could potentially be caused by the reduced abundance of Mtb antigens [188]. Another study by Quinn et al. used an anti-CD25 antibody to deplete the Tregs in vivo 3 days before aerosol infection with BCG or Mtb. This study also observed significantly decreased CD25 expression on CD4$^+$ T-cells in the peripheral blood, spleen, and lungs, with a concomitant increase in IFN-γ$^+$ and IL-2$^+$ cells. However, the authors did not detect significant differences in the lung bacterial burden or histopathology. Together, the data implied that, depletion of natural Tregs causes only small changes in cytokine production and does not adversely affect the course of Mtb or BCG infection [171].

In a more recent study by Gupta et al., a diphtheria toxin-related IL-2 fusion protein known as denileukin diftitox (Ontak®) was used to deplete Tregs in mice challenged with Mtb [83, 114]. Monotherapy with denileukin diftitox reduced the frequency of both Tregs and MDSCs in lungs and spleen and caused a substantial reduction of Mtb CFU counts at 3 weeks post-therapy, compared with untreated controls. In the same study, the addition of denileukin diftotox to standard anti-TB chemotherapy resulted in the reduction of bacterial CFU counts compared with standard anti-TB therapy alone [83, 114].

In contrast to these studies, several Treg depletion studies in vivo have also shown contradictory results and emphasized host-protective functions of Tregs. For example, a study with two closely related mouse strains, C3HeB/FeJ and C3H/HeN, which share similar haplotypes but display different susceptibility to Mtb infection, showed that the more resistant strain, CeH/HeN, displayed higher numbers of Tregs upon mycobacterial challenge [25]. Moreover, depletion of CD25$^+$ cells with an anti-CD25 antibody in the more resistant C3H/HeN strain resulted in more severe lung pathology [123]. Another study compared a hyper-susceptible mouse strain, I/StSnEgYCit, with a relatively resistant strain, C57BL/6Jcit, and found that the susceptible strain had lower Treg levels in mediastinal lymph nodes. In contrast, Tregs levels increased in the resistant C57BL/6Jcit strain, implying that Tregs may have host protective roles [104]. Yet another study by Leepiyasakulchai et al. showed higher numbers of Tregs in the lungs of resistant mouse strains such as C57BL/6 and BALB/c relative to the susceptible strain DBA/2 following Mtb infection [123]. Interestingly, the hypervirulent Mtb strain HN878 induces elevated CD4$^+$ CD25$^+$ FoxP3$^+$ CD223$^+$ IL-10$^+$ Tregs populations, which appear to suppress Th1 responses [160].

The therapeutic approach of Treg depletion has also been tested in combination with BCG vaccination. When C57BL/6 mice were treated with a murine IL-28B expressing adenoviral vector with BCG and a subunit vaccine booster, no additive protective effects were seen, although Tregs were downregulated [133]. Besides, anti-CD25 monoclonal antibody administration did not improve BCG effectiveness [171]. Thus, Treg depletion does not appear to augment BCG vaccine effectiveness.

The discrepancy among the studies showing contradictory results may potentially be explained by: (1) inadequate Treg depletion methods; (2) plasticity of Tregs depending upon the disease site and stage and; (3) differences in the markers used to identify the population.

Conclusions and Future Perspectives

During the course of Mtb infection, the complex interplay of Mtb bacilli and host immune system aids in suppressing anti-TB immune responses and facilitates Mtb proliferation and dissemination in the host. Targeting major immunosuppressive cell populations such as MDSCs and Tregs may boost the host immune system to mount an effective anti-TB response that counteracts the Mtb persistence mechanisms. This approach might lead to the clearance of bacilli from the affected tissues without causing excessive tissue damage, as seen in the case of chemotherapeutic approaches. However, our current knowledge of Mtb mediated immunomodulatory mechanisms and the roles played by MDSCs/Tregs is limited. Future studies are needed to understand these mechanisms at the cellular and molecular levels, and may lead to the development of effective host-directed therapies for the treatment of TB either as stand-alone agents or in combination with current antimicrobial regimens.

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Chapter 12
Targeting Suppressor T Cells

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Suppressor T Cells

Several types of T cells have suppressive activity, like the interleukin (IL)-10 induced and producing Tr1 cells, transforming growth factor (TGF)-β producing Th3 cells, natural killer T (NKT) cells, the CD8+FoxP3- T cell population, γ/δ TCR+ cells and natural FoxP3+ regulatory T cells (Tregs) [1–3]. CD4+CD25+ Tregs expressing the transcription factor forkhead box P3 (FoxP3) are considered 'professional' Tregs as they already have suppressive functions prior to encountering antigens and retain their suppressive function after clonal expansion while continuing to express FoxP3 [4]. Suppressor T cells constitute approximately 10% of CD4+ T cells, and co-express CD25.

Suppressor T cells play a pivotal role in the regulation of adaptive immune responses and in mediating immunological tolerance through the down-regulation of the effector functions of CD4+ or CD8+ T cells. Tregs further suppress the activation, proliferation, and effector functions of antigen-presenting cells (APCs), NK cells, and B cells [5]. These functions are important for preventing autoimmune disease and maintaining immune tolerance. Most importantly, these cells maintain immunological homeostasis, but also limit immune responses required to achieve sterilizing cure during parasitic, viral, and bacterial infections by preventing, down-regulating, or delaying the initiation of adaptive immune responses [6, 7].

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Circulating frequencies of Tregs are higher in patients with drug-sensitive tuberculosis (TB) with a higher bacterial burden [8, 9]. They accumulate at even higher frequencies in bronchoalveolar fluids in patients with pulmonary TB [9, 10], suggesting compartmentalization to the site of disease. Subsequently, after successful TB treatment, the frequency of these cells decreases. *Ex vivo*, Tregs suppress T cell proliferation and *M. tuberculosis*-specific interferon gamma (IFNγ) production [9, 11]. Knockdown of FoxP3 in mice infected with *M. tuberculosis* resulted in reduced bacterial replication [12, 13]. Patients with extensively drug-resistant (XDR)-TB, who failed treatment, have higher frequencies of circulating Tregs compared to patients with drug-sensitive TB and those who are latently infected with *M. tuberculosis*, and even more so in the lung compared to peripheral blood [14]. Tregs from XDR-TB patients also suppress T cell proliferation and impair the ability of macrophages to contain *M. tuberculosis* growth *in vitro* in coculture experiments with autologous effector T cells and/or Tregs.

XDR-TB is a major threat to the global TB control efforts and contributes 6.2% of all global multidrug-resistant (MDR) TB cases [15]. Given that treatment options for XDR-TB is still limited, host-directed therapies targeting Treg functions may advance treatment strategies for this devastating form of TB, or might become an adjunctive form of therapy, even in drug sensitive infection, in attempts to shorten treatment regimens. The therapeutic benefit of this approach will have to be evaluated, since the depletion of FoxP3+ Tregs will most likely be met with increased inflammation and tissue pathology, requiring that effective immunological responses against XDR-TB and destructive immunopathology be carefully balanced.

Regulatory Mechanisms of Adaptive Immune Responses

The exact mechanisms of Treg-mediated suppression remain controversial. Conventionally, it was thought that Tregs mediate their inhibitory activity through cell-to-cell contact as *in vitro* culture experiments indicated that Tregs could not suppress the proliferation of T effector cells when the two cell populations were separated using semi-permeable Transwell™ membranes [16]. In contrast, the suppression of T effector cell proliferation by Tregs was independent of cell-to-cell contact in XDR-TB patients; moreover, the containment of *M. tuberculosis* was not affected when IL-10, TGF-β and CLTA-4 proteins were neutralized [14]. This highlights the different mechanisms by which Tregs exert their suppressive functions.

Tregs can also suppress T helper cell proliferation by increasing the expression of the IL-2 co-receptor CD25. This allows Tregs to utilize IL-2 and deprive T helper cells of IL-2, which is required for proliferation [17]. By doing so, CD4+CD25+ Tregs limit strong Th1 responses induced by microbial antigens during infections and prevent excessive inflammation and tissue damage in the host [2]. During TB, the suppressive effect mediated by Tregs on Th1 responses has been shown to be restricted to CD4+ T cells, which are the most important IFNγ-producing cells involved in controlling *M. tuberculosis* replication [18].

Tregs also produce inhibitory cytokines and growth factors, including but not limited to IL-10, IL-35, and TGF-β, which limits IFNγ production and effector T cell proliferation and function [19, 20]. Elevated IL-10 concentrations in active TB is associated with the suppression of Th1 and Th17 immune responses [21], decreased antigen processing and presentation functions [22], and altered granuloma formation [23]. Patients with active TB who have increased frequencies of Tregs in their circulation also have decreased IFNγ responses [11]. Delayed activation of CD4+ and CD8+ cells in draining lymph nodes caused by Tregs also delays the migration of the cells to the lung, which is the site of infection [6].

Tregs further suppress immune responses by killing effector T cells through expressing the serine protease granzymes A and B, which induce apoptosis in autologous target cells such as activated CD4+ and CD8+ T cells, and APCs (monocytes and dendritic cells) [24].

Potential Host-Directed Therapeutic Targets

Strategies for modulating Treg cell responses in combination with different TB vaccines have so far yielded contrasting results. A prime-boost vaccine strategy including BCG and the 65,000 molecular weight heat-shock protein (hsp65) DNA vaccine resulted in decreased CD4+FoxP3+ cell numbers in the lungs of mice infected with *M. tuberculosis* and conferred protection and reduced lung pathology [25]. The protective efficacy of the BCG/DNA-hsp 65 is associated with an increased ratio of CD4+/CD4+FoxP3+ cells in both spleen (post vaccination) and lungs (post challenge) of *M. tuberculosis* infected mice. It is therefore hypothesized that the vaccine induces strong CD4+ T cell responses and weak Treg responses. Inhibiting both Th2 and Treg cells during BCG vaccination with Suplatast tosylate and D4476 (small molecule inhibitor), respectively, improved *M. tuberculosis* clearance in mice and therefore vaccine efficacy by inducing a superior Th1 responses and long-lived protective central memory T cell responses [26]. Protective responses in mice were not enhanced by using anti-CD25 and reducing the number of Treg cells prior to BCG vaccination and prior to administering the ESAT-6 subunit TB vaccine concurrently with IL-28B [27, 28]. These results highlight the important, yet incompletely understood, role of suppressor T cells in regulating effective immune responses against *M. tuberculosis.*

Increased Indoleamine 2,3-dioxygenase (IDO) activity following MVA85A vaccination was associated with reduced immunogenicity in South African versus UK subjects [29]. Immune responses to *M. tuberculosis* are modulated by IDO, which also blocks T cell proliferation. Drugs inhibiting IDO, like epacadostat, significantly decrease Treg cell proliferation and increase the activity of cytotoxic T lymphocytes *in vitro*, suggesting that vaccine responses could be improved by combining the vaccine with IDO inhibitors [30]. Strategies employed in cancer that target the IDO enzyme, such as vaccine components

directed at IDO epitopes or small hairpin RNA plasmids that silence the IDO gene, could be potentially exploited for TB treatment and vaccination [29, 31].

Tyrosine kinase inhibitors also reduce Treg cells. Imatinib, a treatment used for chronic myelogenous leukemia, reduces the bacterial load and disease pathology in *M. tuberculosis* infected mice, and works synergistically with anti-TB drugs [32].

CD4+CD25+ T cells furthermore express the glucocorticoid-induced TNF receptor (GITR). The GITR ligand (GITR-L) can be induced in a wide range of cells as a result of strong inflammatory signals [33]. Signalling through this receptor temporarily turns off the suppressive function of these cell, at least *in vitro* [34, 35]. How this interaction down-regulates Treg function *in vivo* remains to be determined, but it could be exploited to temporarily suppress Treg function and achieve sterilization during treatment.

Dupilumab, a monoclonal antibody that targets the IL-4-receptor-α chain, has already been approved for the use of atopic dermatitis [36]. It is also currently being tested as treatment for persistent asthma [37]. Agents such as dupilumab might also represent potential immunotherapeutics in TB.

Since Tregs utilize the IL-10 and TGF-β pathway to facilitate their immunosuppressive function [19], these pathways could also be targeted to assist the clearance of the *M. tuberculosis* bacilli. Large clinical trials are, however, needed to investigate the effect of inhibiting these pathways.

Lastly, the adjuvant immunomodulatory drug, levamisole, has been shown to decrease sputum conversion time and improve radiological findings during TB treatment [38]. The immunomodulatory effect induced by levamisole treatment is a shift from a Th2 or mixed Th1/Th2 response to a predominant Th1 type response through the induction of IL-18 [38, 39]. Adjuvant immunomodulation with levamisole, therefore, has the potential for shortening the total duration of TB treatment.

Conclusion

Complete blocking of FoxP3+ regulatory T cells might carry the risk of unacceptable levels of immunopathology. Nonetheless, in cancer biology, success was achieved with approaches that interfere with immune checkpoints (such as programmed cell death protein-1) to promote aggressive anti-cancer host responses. The question therefore is whether a similar approach lends itself to the treatment of TB, and in particular XDR-TB or totally drug resistant TB. Could specific suppressor T cells be targeted by new immunotherapeutics or vaccines to improve the course and outcome of drug resistant TB? Given the devastation caused by such forms of TB, efforts need to be made to explore not only new antibiotics for TB, but also host-directed therapies. It is clear that such interventions in TB would have to be made cautiously, as the exact role of suppressor T cells, the contributions of different subtypes, and the best timing of such interventions during the treatment phase are not well understood. In the context of cancer therapy outcomes, various Treg

populations are associated with improved or worsened therapy outcomes [40]. CD4+CD25+FoxP3+ regulatory T cells, although a major player in suppression, are not the only suppressive cells that may play a role during *M. tuberculosis* infection; therefore, further research is required to determine which regulatory pathways and which Treg populations should be targeted. Nevertheless, targeting immune suppressive T cell populations offers potentially exciting new strategies for adjunctive treatment or even prevention of TB, either to shorten treatment regimens or in infections with drug resistant strains.

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Chapter 13
Neutrophil-Mediated Mechanisms as Targets for Host-Directed Therapies Against Tuberculosis

Tobias K. Dallenga and Ulrich E. Schaible

Introduction

Neutrophils play a critical role in quickly recognizing and eliminating invading pathogens. During their short life span, after leaving the bone marrow, neutrophils represent the predominant leucocyte population in circulation. Upon recognition of inflammation, neutrophils attach to the vessel wall and extravasate into tissues to enter the site of infection; they then eliminate the pathogen by phagocytosis and by releasing microbicidal effectors into the phagosome during its maturation (from the formation of the phagosomal cup through the maturation and subsequent acidification of the enclosed phagosome). Neutrophil effectors, which are delivered to the phagosomal cup by secretory granules, comprise antimicrobial peptides, such as cathelicidin and beta defensins, NADPH oxidase and myeloperoxidase, which generate reactive oxygen species, and hydrolytic enzymes degrading proteins, lipids and sugars [22]. Another antimicrobial mechanism is the release of neutrophil extracellular traps (NETs) in the context of a cell death process called NETosis, in which pathogens are entangled in nets of decondensed chromatin decorated with antimicrobial effectors [23]. Dying neutrophils are then removed by other phagocytes. When neutrophils undergo apoptosis, they are taken up by macrophages in a highly regulated process called efferocytosis [17]. When neutrophils die by necrosis, the recognition and uptake of necrotic material are distinct from the removal of apoptotic material. This process has been called necrophorocytosis [5]. Since neutrophils carry highly cytotoxic and tissue-damaging effectors, their accumulation, activation, and clearance need to be tightly controlled and efficient. However, proliferation and persistance of bacterial pathogens may lead to uncontrolled influx of neutrophils attracted to the site of infection by chemokines such

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as IL-8 (CXCL8), CXCL1 and CXCL2 [1, 25]. Neutrophil over-activation, which is typically accompanied by massive cell death and insufficient clearance of dead neutrophils, can lead to the release of large amounts of alarmins, such as S100A8/9, and toxic molecules causing inflammation and tissue damage [29].

Neutrophils in Tuberculosis

Mycobacterium tuberculosis has developed multiple strategies to evade killing by phagocytes. Upon phagocytosis by macrophages, *M. tuberculosis* blocks phagosomal maturation, acidification and fusion with degradative lysosomes, a mycobacterial escape mechanism not yet studied in neutrophils [2]. *M. tuberculosis* secretes catalase, an enzyme that quickly breaks down H_2O_2 into water and oxygen and, thus, can protect from the reactive oxygen species generated by neutrophils [27]. Moreover, secreted mycobacterial peptidases as well as the thick, waxy and hydrophobic mycobacterial cell wall that is coated by a phosphatidylinositol mannoside capsule further protects *M. tuberculosis* from neutrophil-mediated damage due to antimicrobial peptides and hydrolases [9, 11]. Furthermore, after surviving the initial attack of the neutrophil's bactericidal armamentarium, *M. tuberculosis* induces necrotic cell death in human neutrophils as early as 6 h post infection in vitro [4, 5]. Induction of necrotic cell death of neutrophils protects *M. tuberculosis* from prolonged exposure to neutrophilic antibacterial effector molecules and reduces its killing [4]. Induction of neutrophil necrosis requires the small mycobacterial virulence factor called early secretory antigenic target-6 (ESAT-6) and a functional type VII ESAT-6 secretion system (ESX-1) [5]. The 6 kDa ESAT-6 is thought to be constitutively secreted as a heterodimer together with the 10 kDa culture filtrate protein-10 (CFP-10). Some evidence exists that ESAT-6 can interact with host cell membranes, compromise the integrity of the phagosomal membrane [3], and potentially open gateways for additional virulence factors or for leaking of neutrophil toxic effector molecules into the cytosol. Accordingly, *M. tuberculosis* mutants that lack the ESAT-6-coding gene are unable to induce necrosis in neutrophils [5]. However, necrotic cell death of *M. tuberculosis*-infected neutrophils also depends on the generation of reactive oxygen species by the neutrophils [5]. Indeed, neutrophils from patients suffering from chronic granulomatous disease that do not have a functional NADPH oxidase do not die by necrosis during *M. tuberculosis* infection [4].

Neutrophil necrosis results in the leakage of highly toxic molecules and enzymes into the extracellular space as well as of alarmins and other pro-inflammatory stimuli, causing tissue destruction and recruitment of additional neutrophils as well as monocytes to the site of infection [29]. Even these uninfected infiltrating neutrophils most likely succumb to secondary necrosis, as efficient efferocytosis after apoptosis and tissue regeneration are not achieved effectively [6]. Thus, a vicious circle of necrosis ensues, in which *M. tuberculosis* infection leads to necrotic neutrophil cell death, tissue damage, further influx of neutrophils, and more necrosis,

which exacerbate pathology. Consequently, a necrotic center develops within the granuloma, which can subsequently break open into alveoli and bronchioli, resulting in the typical symptoms of tuberculosis, including cough that facilitates transmission of aerosolized mycobacteria [24]. A prominent role of neutrophils in tuberculosis pathogenesis is confirmed by the finding that neutrophils represent the predominant cell population infected with *M. tuberculosis* in sputum and bronchoalveolar lavages obtained from active tuberculosis patients [8]. Moreover, massive accumulation of neutrophils has been observed in resected sections of tuberculous lungs [10]. The association of neutrophils with active tuberculosis and exacerbating pathology correlates with observations in mouse models resembling the human lung pathology. When infected with *M. tuberculosis* by aerosol, susceptible mouse strains show significantly more neutrophils in lung infiltrates than resistant ones [15]. Moreover, comparative blood transciptome analyses of susceptible mice and tuberculosis patients revealed a shared type I interferon signature and recruitment and activation of neutrophils in addition to a lack of efficient B cell, T cell, and natural killer cell responses [21].

Targeting Neutrophil-Mediated Mechanisms as a Host-Directed Therapy in Tuberculosis

In the face of the current rise of *M. tuberculosis* isolates multidrug resistant to Isoniazide and Rifampicin (MDR) or additional antibiotics (XDR), new approaches need to be developed to support faster and more effective treatments. Disease exacerbation in active tuberculosis is partially driven by pathological host responses causing tissue damage; moreover, long-term sequelae such as cavernous lesions and impaired lung function persist even after successful therapy [13]. Several mechanisms in the interaction between neutrophils and *M. tuberculosis* may represent toeholds for a host-directed therapy adjunct to antibiotic treatment. For instance, when the necrotic cell death of *M. tuberculosis*-infected human neutrophils is prevented by pharmacological inhibition of the neutrophilic myeloperoxidase, the fate of infected cells shifts from necrosis towards apoptosis [5] (Fig. 13.1). Consequently, *M. tuberculosis* becomes tightly enwrapped in apoptotic neutrophils, which avoids spillage of toxic effector molecules into the extracellular space, and reduces the risk of tissue damage. Enwrapping also limits the interactions between mycobacterial products and host cell factors. For example, when macrophages ingested necrotic infected neutrophils in which *M. tuberculosis* were only loosely associated with the necrotic material, the mycobacteria could directly interact with the macrophages to promote proliferation within the macrophages [5]. Subsequent mycobacterial growth resulted in necrosis of these macrophages and release of the initial mycobacterial inoculum. Such events likely represent the initiation and maintenance of active tuberculosis in patients. In contrast, when uninfected human macrophages ingest *M. tuberculosis*-infected apoptotic neutrophils, the macrophages can control replication of *M. tuberculosis*, curbing further spread.

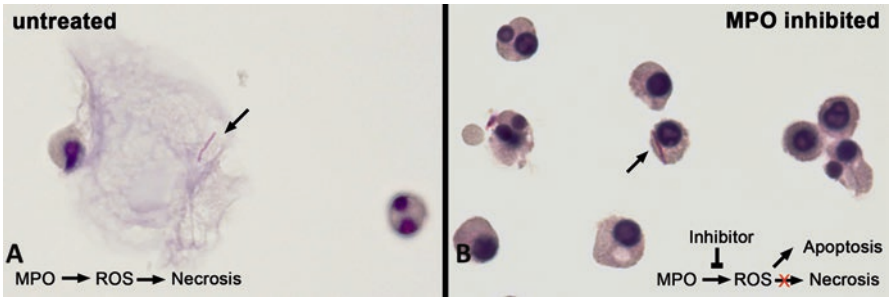

Fig. 13.1 *M. tuberculosis* (pink, arrow) infected neutrophils succumbed to a necrotic cell death (membrane rupture, lysis, debris) mediated by the reactive oxygen species hypochlorous acid generated by myeloperoxidase (**a**). When myeloperoxidase was specifically inhibited, *M. tuberculosis*-infected neutrophils did not succumb to a necrotic cell death but underwent default apoptosis (chromatin condensation and cleavage, nucleus rounding) (**b**). Downstream uptake of *M. tuberculosis*-infected neutrophils by macrophages either promoted or controlled mycobacterial growth depending on whether neutrophils were necrotic or necrosis was inhibited

Another host-oriented treatment strategy involves controlling the influx of naïve neutrophils into infectious lesions. Studies in murine tuberculosis models showed that reduced numbers of neutrophils infiltrating the sites of infection were associated with limited lung pathology, lower mycobacterial counts, and increased survival rates. For example, the tuberculosis-resistant mouse strain BL/6 becomes more susceptible when the autophagy-associated protein ATG5 is knocked out in myeloid cells. The phenotype was not associated with impaired autophagy, rather with a yet uncharacterized ATG5-mediated mechanism that reduces neutrophil infiltration, mycobacterial numbers, tissue pathology, and death rates in wild type mice [14]. Moreover, it was long thought that the toxic properties of nitric oxide reduced *M. tuberculosis* loads during infection by directly acting on the mycobacteria or inducing apoptosis of infected macrophages [12]. However, Mishra and colleagues found that nitric oxide repressed NLRP3 inflammasome–dependent IL-1β production and 12-hydroxyeicosatetraenoic acid (12-HETE)-mediated neutrophil recruitment, which promote mycobacterial replication [20]. In humans, 12-HETE is synthesized by *Alox12,* which is expressed in cavitary lesions of active TB patients; moreover, a single nucleotide polymorphism enhancing *alox12* expression was associated with active tuberculosis [16]. Infiltrating and necrotic neutrophils may also create a micronutrient-rich environment for *M. tuberculosis* growth [20]. Furthermore, neutrophil infiltration is not only induced by chemokines, but also by small lipid mediators, including 12-Hete, prostaglandin E_2 (PGE_2), and leukotriene B_4 (LTB_4), which are synthesized by 12-lipoxygenase (alox12), cyclooxygenases 1 and 2 and 5-lipoxygenase, respectively. All three pathways can be targeted by clinically approved drugs. Indeed, interference with the synthesis of small lipid mediators reduced neutrophilic cell counts at sites of mycobacterial lung infection. Nonsteroidal anti-inflammatory drugs Ibuprofen and Aspirin block cyclooxygenases 1 and 2, leading to reduced levels of prostaglandin H2 and other small lipid

mediators, such as PGE_2, PGD_2, and thromboxane A_2 [16, 26]. Ibuprofen treatment of *M. tuberculosis*-infected mice of the susceptible C3HeB/FeJ strain down modulated neutrophil influx and reduced mycobacterial counts, granuloma sizes, and histopathology, and increased survival rates [26]. Low-dose Aspirin led to a less pronounced amelioration of disease [16]. Zileuton, an inhibitor of alox5-mediated synthesis of lipoxin A_4 (LXA_4) and LTB_4, which enhances infected macrophage necrotic cell death and neutrophil recruitment, was successfully used to reduce bacterial loads and immunopathology by promoting protective IL-1β and limiting disease-associated type 1 IFN production in mice [18]. It should however be noted that LXA_4 and LTB_4 exhibit opposing effects on the production of TNF-α, a central cytokine in tuberculosis that is both beneficial (activation of anti-*M. tuberculosis* effector functions of macrophages and maintenance of granuloma formation in latent tuberculosis infection) and detrimental (exacerbated clinical TB, cell necrosis and cachexia) [7, 19]. Thus, the effects of inhibitors of small lipid mediators need to be carefully evaluated. In addition to drug-mediated modulation of neutrophil influx into tuberculosis granulomas, direct antibody-mediated targeting of neutrophils or neutrophil attracting chemokines such as IL-8 might be considered as potential therapeutic approaches.

Conclusion

Neutrophils represent a promising target for host-directed therapies that target immune cell populations, since they represent one of the main contributors to pathology in many different infectious and non-infectious diseases. Although neutrophils also play an important role in tissue repair after injury, mostly by clearing tissues from microbes, it is thought that as long as neutrophils infiltrate in large numbers, tissue damage and extensive scarring occurs [28]. Resolution of inflammation and wound healing does not commence as long as neutrophils infiltrate the tissue, degrade extracellular matrix proteins, release toxic molecules, and accumulate as (secondary) necrotic cellular graveyard. Careful pharmacological interference with neutrophil-recruiting pathways and effectors may be beneficial in chronic disease, when they fail to remove pathogens from tissues but rather promote pathology.

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Chapter 14
Type I Interferon and Interleukin-1 Driven Inflammatory Pathways as Targets for HDT in Tuberculosis

Katrin D. Mayer-Barber and Christopher M. Sassetti

Over the last decade, innate cytokines, in particular interleukin-1 (IL-1) and type I Interferons (IFNs), have received considerable attention for their role in host resistance to *Mtb* infection and may represent druggable targets for dampening detrimental inflammation that could impair control of infection [1–3]. Here, we will review briefly our current understanding of IL-1 and type I IFNs in TB pathogenesis and expand on more recent findings that point to the IL-1 and type I IFN pathways as promising targets for HDT.

Type I IFN Pathway

Interferons are a group of cytokines best known for their potent anti- viral properties. IFNs can be separated into three sub-families, designated as type I-III IFNs, with type I IFNs being composed of 13 IFNα subtypes, IFNβ, IFNε, IFNκ and IFNω; type II IFN represented by one cytokine, IFNγ; and type III IFNs comprising members of the IFNλ family, which includes IFNλ1 (also known as IL-29), IFNλ2 (also known as IL-28A) and IFNλ3 (also known as IL-28B) [2, 4]. Type 1 IFNs bind the ubiquitously expressed IFNAR receptor complex, consisting of an IFNAR1 and an IFNAR2 chain, which, when activated together, engage Tyk2 and Jak1. This classic activation leads to signal transducer and activator of

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transcription (STAT) 1-STAT2 hetero-dimer formation and subsequent translocation to the nucleus, where dimeric STATs recruit additional transcriptional factors that ultimately lead to the induction of hundreds of IFN-stimulated genes (ISGs) [5]. In addition to STAT activation, IFNAR1 signaling can activate the mammalian target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K) and multiple mitogen-activated protein kinase (MAPK) pathways [6]. The ability to activate multiple signaling cascades contributes to the diversity of type I IFN-driven responses, which allows for transcription of genes beyond those intended for viral control, for example pro-apoptotic and anti-apoptotic molecules, cytokines, chemokines, and enzymes involved in lipid metabolism [5, 7].

Interleukin-1 Pathway

IL-1, in contrast, is most widely known in host resistance to infection for protection against acute bacterial infections, in which rapid inflammatory responses and IL-1-induced chemokines are required for optimal neutrophil-dependent control [8, 9]. There are two IL-1 species, IL-1α and IL-1β, respectively, which both bind and activate the IL-1R complex, comprising the IL-1R1 and IL-1RAP chains expressed by all cells to varying degrees [10]. A third ligand for the IL-1R1 complex is the endogenous IL-1R antagonist (IL-1RA), which competes with IL-1α and IL-1β for binding, thereby blocking productive IL-1R signaling. Once the IL-1R complex is activated, myeloid differentiation primary response gene 88 (Myd88) is recruited, allowing for oligomerization with interleukin-1 receptor-associated kinase (IRAK) to form the Myddosome complex, which serves as a platform to phosphorylate IRAKs [11, 12]. IRAK phosphorylation leads to the recruitment and oligomerization with tumor-necrosis factor-associated factor 6 (TRAF6), resulting in NF-κB activation. Importantly, IL-1R engagement leads to highly potent pro-inflammatory signals, and, thus, requires complex negative regulation at multiple intersection points to prevent inflammation-induced tissue pathology and auto-inflammatory diseases. In addition to the endogenous IL-1RA, soluble IL-1R1 and membrane bound IL-1R2 can act as decoy receptors. IL-1β expression is also tightly regulated at both the transcriptional and post-translational level via the inflammasome (extensively reviewed in [13]). Briefly, IL-1β is generated in an inactive pro-form that requires proteolytic cleavage by caspase-1 to convert the immature 31kD pro-IL-1β polypeptide to the 17kD mature IL-1β, which exhibits optimal biological activity [9, 13, 14]. Inflammasome complexes typically consist of a NOD-like receptor (NLR), such as NLRP3 or AIM2, and adaptor molecules, such as Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which then recruit and activate caspase-1.

Rationale for Targeting Type I IFNs and IL-1 Based on Clinical TB Studies

Interest in type I IFN as a target for host-directed therapy (HDT) of tuberculosis was catalyzed through a seminal transcriptomic study by Anne O'Garra and colleagues revealing that patients with active tuberculosis exhibit a prominent type I IFN-inducible gene signature in whole blood [15]. This study provided the first evidence for a role of type I IFNs in the pathogenesis of human tuberculosis. Importantly, blood transcriptional profiles of patients with active tuberculosis were enriched in a type I IFN-inducible gene signature that correlated with the extent of radiographic lung disease and was reduced following successful antitubercular treatment [15]. Since then, several studies verified and extended these findings in additional patient cohorts from different geographic regions with diverse host genetic and epidemiological TB backgrounds [16–24]. Furthermore, a subset (10–25%) of latently infected patients who were asymptomatic also presented with an elevated type I IFN-inducible peripheral blood signature, suggesting that these patients might be more likely to progress to active disease [15, 25]. This notion was confirmed and extended in an elegant adolescent cohort study, where patients were followed longitudinally for 2 years prior to diagnosis with active TB disease [24, 26]. Overexpression of specific sets of IFN response genes were detected early in tuberculosis contacts who went on to progress to active disease, demonstrating that peripheral activation of the type I IFN response actually precedes the onset of active disease and clinical TB symptoms [24–27]. In addition, TB reactivation has been described in several case reports, in which type I IFN (IFN-alpha) was administered to patients undergoing chronic viral hepatitis treatment [28–34]. Finally, a recent study described a genetic variant in the *IFNAR1* gene, which impaired type I IFN signaling and was associated with increased TB resistance [35]. Taken together, these clinical studies provide strong evidence that type I IFN signaling correlates with impaired control of *M. tuberculosis* and increased risk of tuberculosis disease in humans.

A similar, though less robust, set of clinical observations associate the IL-1 pathway with TB disease. IL-1β production is increased in the lungs of patients with pulmonary TB [36], and plasma markers of IL-1 and inflammasome signaling are enhanced during TB meningitis [37]. These markers are also increased during particularly inflammatory manifestations of TB, such as HIV-associated immune reconstitution syndrome (IRIS) [38–40]. A number of genetic association studies further implicate IL-1β production in TB disease progression [41–43]. In particular, Zhang et al. described a common genetic variant in the human IL1B promoter region that alters the association of the C/EBPb and PU.1 transcription factors and controls *Mtb*-induced IL-1β production. The high IL-1β expressing genotype was associated with the development of active tuberculosis, severe pulmonary disease and poor treatment outcome [43]. These observations suggest that enhanced IL-1β expression can promote disease. Currently, there are

no genetic studies that specifically address the effect of decreased IL-1 signaling in TB, and limited conclusions can be drawn from clinical experience with inhibitors of this pathway. Despite individual case reports of TB reactivation during treatment with recombinant human IL-1Ra (Anakinra) [44, 45], this treatment is associated with a remarkably low risk of TB reactivation, especially compared to TNFα blockade [46–48]. It is possible that these observations are related to the short half-life of Anakinra, which may limit its potency. Evaluating the effects of a newer neutralizing monoclonal antibody targeting IL-1β (Canakinumab) is not straightforward, as trials of this agent excluded individuals with latent TB infection or TB risk factors [49, 50]. While the consequent low number of TB cases limits the conclusions that can be drawn, Canakinumab treatment was not found to increase TB incidence compared a control cohort [49, 50]. In sum, the existing clinical data indicate that both IFN and IL-1 pathways are associated with TB disease, and the enhanced or inappropriate production of either cytokine could promote disease.

Molecular Mechanisms of IL-1 and Type I IFN Induction by *Mtb*

IL-1 and type I IFN pathways have been studied extensively *in vitro* during *Mtb* infection of macrophages to gain insight into the molecular mechanisms of IL-1 and type I IFN induction. Well-designed *in vitro* studies revealed that the bacterial ESX-1 type VII secretion system was required for both type I IFN expression as well as inflammasome activation [51–53]. The nucleotidyltransferase cyclic GMP-AMP synthase (cGAS), a central component in the cytosolic surveillance pathway, recognizes bacterial and mitochondrial DNA in the cytosol leading to STING activation, type 1 interferon induction and autophagy in a *Mtb* lineage-dependent manner [53–57]. Of note, the hyper-virulence of clinical *Mtb* isolates was linked to their ability to potently induce type I IFN *in vitro* [58, 59]. The acquisition of drug resistance-conferring single nucleotide polymorphisms in *Mtb* has been shown to modulate host macrophage metabolic reprogramming and IL-1 and type IFN induction [60]. *In vitro*, cytosolic *Mtb* DNA was shown to activate AIM2, a NLR, that triggers the inflammasome and IL-1βprocessing [56, 61–63]. Similar experiments have demonstrated that ESX1 is required for NLRP3-inflammasome activation and IL-1β processing in response to *Mtb* infection [62, 64–66]. In contrast, *Mtb*-infected mice deficient in caspase-1/11, ASC or NLRP3, showed unimpaired IL-1β production and importantly, were considerably less susceptible to infection than IL-1β deficient mice [64, 65, 67] suggesting that IL-1 β can be induced and processed *in vivo* by an inflammasome-independent mechanism. Taken together, various *Mtb* strains activate distinct cytosolic PRR systems, namely the cGAS-IFN-axis vs. the AIM2/NLRP3-IL-1β-pathway, and the extent of activation is likely related to ESX-1 activity [53, 54, 56, 57, 68].

Role of IL-1 and Type I IFNs During *Mtb* Infection in Experimental Animal Models

Based on *Mtb* infection of mouse strains engineered to lack IL-1 or type I IFN pathway activation, IL-1 and type I IFNs seem to play largely opposing roles in host resistance against *Mtb in vivo* [12, 14]. Type I IFN responses have been associated with the virulence of *M. tuberculosis* strains and increased host susceptibility in mice. Several studies have reported reduced bacterial loads and/or improved host survival in IFNAR1-deficient mice after *Mtb* infection, demonstrating a functional and host-detrimental role of type I IFNs in tuberculosis [2, 3]. Excessive and/or sustained induction of type I IFNs by either direct cytokine instillation, administration of the double-stranded RNA homologue poly-ICLC, or co-infection with influenza virus have all been reported to dramatically reduce host resistance to *M. tuberculosis* infection in a type I IFN-dependent manner [58, 69–71]. Mouse models of increased susceptibility to *Mtb*, such as the 129S2 mouse or the C3HeB/FeJ mouse, the latter of which carries the "supersusceptibility to tuberculosis 1" (Sst1) allele, are often used in pre-clinical pharmacokinetic or drug efficacy studies because they more closely recapitulate the range of pulmonary pathology observed in humans, including granulomas with necrotic cores [72, 73]. Importantly, a very recent study convincingly demonstrated that type I IFNs are the cause of exacerbated disease in the susceptible B6 mouse strain carrying the *Sst1* allele ($B6^{Sst1}$) [74]. Ji et al. showed that Sst1-mediated early susceptibility was abrogated in the absence of *Ifnar1* signaling, while only partially reduced when STING signaling was inhibited. Likewise, a prior study found that relative susceptibility of 129S2 mice, compared to the more resistant C57BL/6 strain, was largely attributable to type I IFN signaling [75].

Proposed mechanisms to explain the pathogenic effects of type I IFNs include direct suppression of host-protective cytokines and mediators, such as IL-12, IL-1 and PGE2, and indirect suppression of these factors via type I IFN-dependent induction of IL-10, which has been observed in murine and human cells *in vitro* [60, 76–80], as well as in mouse models [75, 77, 81–83]. In addition, Type I IFNs have been shown to promote expansion of bacterial growth-permissive myeloid cells, which are thought to contribute to the spread of infection and pulmonary inflammation [69, 75, 84, 85]. However, in the absence of IFNγ signaling, type I IFNs have been suggested to play a protective role early after *M. tuberculosis* infection in mice, indicating that the dominant antimicrobial activity of type II IFNs may mask potential protective functions of type I IFNs [86, 87].

Both IL-1α and IL-1β are critically and non-redundantly required for host resistance to *Mtb* infection in the murine model of *Mtb* infection [1, 67, 77, 88–91]. Mice deficient in IL-1α, IL-1β or both, and IL-1R1-deficient mice are highly susceptible to pulmonary tuberculosis, as reflected by an increased mortality and an enhanced mycobacterial growth in lungs and spleens [77, 92]. While the exact mechanisms by which IL-1 mediates protection against *Mtb* infection are still incompletely understood, protection requires cooperative IL-1R responsiveness in cells of both

hematopoietic and non-hematopoietic origin [92–94]. Interestingly, these observations also argue that infected cell-intrinsic and cell-autonomous roles for IL-1R1 expression are dispensable for anti-mycobacterial effector functions and that IL-1-dependent signals protect infected cells in trans [93]. One major protective role of IL-1 during *Mtb* infection was shown to be linked to its ability to trigger arachidonic acid-derived lipid mediator prostaglandin E2 (PGE2) synthesis and COX-2 activation [78]. Mice deficient in IL-1 or IL-1 signaling displayed major defects in PGE2 production in the lungs and PGE2 supplementation reduced pulmonary *Mtb* loads and extended survival [78]. In addition to PGE2, IL-1 is also required for induction of IL-17, a cytokine shown to be important during *Mtb* infection and during vaccination [77, 95].

While IL-1 signaling is critical for protective immunity to a number of pathogens, including *Mtb*, the persistent or inappropriate activation of this pathway causes inflammatory pathology [9, 96]. Consistent with the potentially detrimental effects of IL-1 signaling, the production of mature IL-1β is regulated at multiple levels as *Mtb* infection progresses. The processing of IL-1β by the inflammasome is inhibited by reactive oxygen species produced by the NADPH-dependent phagocyte oxidase, and loss of this regulation exacerbates inflammatory disease in *Mtb*-infected mice [97]. As the infection progresses and the adaptive response emerges, lymphocyte-derived IFNγ plays a particularly important role in restricting pathological IL-1-dependent inflammation via the production of nitric oxide, which inhibits inflammasome-dependent production of IL-1β [98, 99]. In the absence of this regulatory mechanism, uncontrolled IL-1 signaling promotes both pathology and bacterial replication via the recruitment of disease-promoting granulocytes [99, 100]. Thus, while it is clear that IL-1 signaling is critical for protective immunity to *Mtb*, it appears to be equally important that the activity of this pathway is controlled throughout persistent infection.

IL-1 and Type I IFN Cross-Talk in Tuberculosis

When dysregulated, both the IL-1 and type I IFNs pathways have extreme inflammatory capacity and inborn errors of innate immunity revealed key roles for both IL-1 and type I IFNs in auto-inflammatory syndromes and interferonopathies in infants and adolescents [101, 102]. Mutual antagonism between IL-1 and type I IFN has been reported in the settings of viral hepatitis and in autoimmune inflammatory disorders, such as multiple sclerosis, systemic lupus erythematosus and systemic-onset juvenile idiopathic arthritis, and is extensively reviewed elsewhere [12, 14]. Initial studies carried out in the context of viral infections established that type I IFNs potently up-regulate IL-1RA, now considered an IFN-inducible gene, thereby limiting IL-1 signaling [12, 14]. The strong counter-regulation of the IL-1 and type I IFN pathways observed in these diverse disease settings suggests that the two cytokine networks each represent a major, functionally distinct class of innate inflammation. How cross-regulation of IL-1 and type I IFNs affects pathogen

clearance and pathology through both the innate and adaptive immunes systems will likely be dependent on the local environment and stage of infection.

Since IL-1R1- and IFNAR1-deficient mice display opposite outcomes in host resistance and survival after *Mtb* infection, multiple studies have explored whether and how these two major innate cytokines axes may be cross-regulating each other in tuberculosis [12, 14, 74, 77–79]. Indeed, during *Mtb* infection, type I IFNs inhibit IL-1α and IL-1β production, both in infected human and mouse myeloid cells and in mouse models [75–77, 79, 82]. This inhibition was partially due to type I IFN-dependent induction of IL-10, an important anti-inflammatory cytokine [77, 82]. Type I IFNs also potently upregulated expression of the endogenous IL-1Ra during *Mtb* infection *in vitro* and *in vivo*, further decreasing IL-1 activity [74, 77, 78]. Of note, a very recent study by Vance and colleagues elegantly showed that IL-1RA upregulation alone could account for the majority of the pro-bacterial, host-detrimental effects of type IFN in the susceptible $B6^{Sst1}$ mouse model, even in the presence of high levels of IL-1β cytokine itself [74]. When crossed to IL-1Ra-deficient mice, $B6^{Sst1}$ animals no longer displayed type I IFN-driven increased bacterial loads and survival was significantly extended [74]. In addition to modulating IL-1RA and IL-1α and IL-1β expression, type I IFNs also induce expression of the soluble decoy receptor IL-1R2 [78]. Finally, type I IFN-mediated inhibition of IL-1 cytokine production was confirmed *in vivo* in *Mtb* infected lungs where type I IFN acted directly on IL-1-producing myeloid subsets to limit their IL-1α and IL-1β cytokine production [77].

Conversely, both IL-1α and IL-1β directly suppressed IFN β mRNA and protein induction during *Mtb* infection [78]. In addition, IL-1 -dependent PGE2 inhibited type I IFN protein expression by human and murine cells *in vitro* [78]. IL-1 mediated type I IFN inhibition was evident *in vivo*, because IL-1R1-deficient animals exhibited increased levels of type I IFN in the lungs after *Mtb* infection. The significance of IL-1 type I IFN cross-regulation for biological outcomes was revealed through the generation of mice doubly deficient in IL-1R1 and type IFN signaling, which displayed significantly prolonged survival compared to mice deficient in IL-1R1 alone [78]. Thus, IL-1-mediated host resistance is at least in part due to its potent ability to limit detrimental type IFN expression, directly through IL-1R1 mediated signals and indirectly through PGE2.

HDT Approaches Based on IL-1 and Type I IFN in Tuberculosis

Evidence from experimental animal models, clinical whole blood transcriptomic signatures, and genetic association studies suggests that limiting type I IFN-driven inflammation represents a promising approach for TB HDT. However, conventional C57BL6 mice infected with *Mtb* are fairly resistant to *Mtb* infection and induce little type I IFN when compared to the more susceptible 129S2 or $B6^{Sst1}$ mouse strains, making experimental preclincial studies targeting type IFN-driven disease

challenging. In contrast, when administered the double-stranded RNA homologue pICLC, C57BL6 mice (pICLC-B6) succumb rapidly to *Mtb* infection in a type I IFN- and IL-10 dependent manner [69, 78]. Employing the pICLC-B6 model of type I IFN-driven TB disease exacerbation, Sher and colleagues provided an *in vivo* proof-of-concept study for type I IFN targeting HDTs in a mammalian experimental animal model [78]. The treatment approach was based on findings from IL-1 and type I IFN crosstalk, where IL-1-induced PGE2 was found to potently limit type I IFN. Type I IFNs were indirectly targeted by intranasal administration of PGE2 itself and by increasing PGE2 levels through 5-lipoxygenase (5-LO) blockade with zileuton. Zileuton is an FDA approved, clinically licensed 5-LO inhibitor for the treatment of asthma. The combined therapy of both zileuton and PGE2 boosted cytoprotective IL-1β production and reduced detrimental type I IFN in the pICLC-B6 model [78]. Importantly, manipulation of the eicosanoid balance in favor of PGE2 rendered pICLC-B6 mice completely resistant to type I IFN-induced weight loss and 100% of the mice survived type I IFN-driven disease exacerbation with lower bacterial loads and reduced pulmonary necrosis. Administration of multiple agents was not required because either PGE2 alone or zileuton treatment alone protected against both weight loss and mortality and correlated with increased PGE2 in the lungs of mice [78]. In addition, protection correlated with decreased pulmonary bacterial loads, tissue necrosis and reduced type I IFN and IL1Ra production following treatment. The effect of PGE2 on bacterial burden was likely due to its anti-inflammatory effects, as neither zileuton nor PGE2 directly inhibited growth of *Mtb in vitro*. Co-administration of the drugs neither interfered nor synergized with standard antibiotic therapy to reduce bacterial loads in the lungs, arguing that potential therapeutic effects could be observed even in settings of drug treatment failure [78]. Finally, the therapeutic (as opposed to protective) efficacy of 5-LO blockade was demonstrated when zileuton treatment was delayed in pICICL-B6 mice until 30 days post-infection, a time point when pICICL-B6 animals already display a 10–20% weight loss. This delayed treatment with zileuton resulted in significantly enhanced survival, with 40% of the animals remaining alive at the end of the study. Thus, manipulation of the eicosanoid balance towards protective PGE2 can both prevent and therapeutically ameliorate disease exacerbation associated with high type I IFN expression during *Mtb* infection in mice and may represent a feasible HDT to pursue in patients.

Recently, a second HDT targeting detrimental type I IFN-driven disease was described in the $B6^{Sst1}$ model [74]. This HDT strategy also builds on mechanistic insights gained from IL-1 and type I IFN crosstalk during *Mtb* infection. Vance and colleagues revealed that susceptibility in the $B6^{Sst1}$ model was due to increased type I IFN-mediated induction of IL-1Ra, resulting in decreased functional IL-1 signaling. Consistent with this model, monoclonal antibody therapy to neutralize IL-1Ra reduced bacterial loads, decreased weight loss and reduced tissue pathology in $B6^{Sst1}$ mice. Thus, antibody-mediated reduction of IL-1Ra was able to rescue type I IFN-driven susceptibility to *Mtb* in $B6^{Sst1}$ mice without producing overt detrimental immunopathology [74]. Indeed, induction of high levels of IL-1Ra has been associated with active disease in TB patients [78, 103] when activation of type I and type

II IFN pathways are prominent [3]. The potent induction of the endogenous IL-1Ra by type I IFNs and potentially simultaneous inhibition of IL-1β expression and processing by type II IFN, could suggest a reduced dependency on IL-1 signaling in established infection, which could explain the low reactivation rate and overall good safety profile of IL-1-blocking agents during *Mtb* infection in humans. Taken together, pre-clinical data from two mouse models of type I IFN driven disease, the pICICL-B6 and $B6^{Sst1}$ models, respectively, provide compelling small animal data that modulating the type I IFN response can ameliorate TB-driven lung disease and limit tissue pathology. In addition to indirectly modulating type I IFNs, via PGE or IL-1Ra, one could also envision directly targeting IFNAR1 with monoclonal antibodies as adjunctive HDT in *Mtb*-infected individuals, and a fully human IgG1 monoclonal antibody against IFNAR1 (anifrolumab) is currently in development for treatment of systemic lupus erythematosus [104].

HDT based on IL-1 blockade could also be envisioned as an adjunctive therapy that reduces pathological inflammation. In the context of pulmonary TB, it is clear that excessive IL-1 signaling is associated with the extent of radiological disease, both before and after chemotherapy [43]. Pulmonary impairment after tuberculosis (PAIT) has a significant impact on long-term outcomes [105], and preventing this condition could have a profound effect on TB disease burden [106]. The rationale for IL1 blockade may be even stronger for particularly inflammatory manifestations of TB. For example, nonspecific anti-inflammatories are often used as adjunctive therapies for TB meningitis TBM and TB/HIV IRIS, but these conditions remain difficult to treat. IL1 and inflammasome signaling are associated with both diseases [37–40], suggesting that a more specific IL-1 therapy could be useful. Based on this anti-inflammatory rationale, Flynn and colleagues investigated IL-1 inhibition in conjunction with antibiotic therapy in models of pulmonary TB in mice and nonhuman primates [107]. In these studies, IL-1 receptor blockade by means of Anakinra administration in nonhuman primates was found to decrease the fraction of lesions that became necrotic. Notably, IL-1 blockade did not impair the host response to *Mtb* or the efficacy of antibiotic therapy in these settings. Further studies are necessary to determine if the observed anti-inflammatory effects are sufficient to preserve lung function, and whether this strategy is efficacious in other, more IL-1-associated clinical settings such as TB meningitis or TB-IRIS.

In summary, the intimate and prolonged interaction between *Mtb* and host immunity makes TB a particularly attractive disease for host-directed therapies. The simple fact that most humans are able to control this infection suggests that HDT alone may be capable of promoting a protective immune response. Even if this lofty goal remains out of reach, the use of HDT as an adjunct to antimicrobial therapy could improve outcomes either by accelerating bacterial clearance or by ameliorating the lung pathology that contributes to transmission and can lead to long-term disability. The IFN/IL-1 axis remains central to these efforts, as these mediators are integral to both bacterial control and the development of pathology. Despite the promise of these strategies, we are only beginning to understand the complexity of these pathways in the context of natural infection. A major complication with any HDT is the immunological heterogeneity observed between individuals, sites of infection, and

manifestations of disease. While identifying the settings where an individual HDT has the greatest therapeutic effect remains a challenge, this review highlights a promising path forward by incorporating both clinical data and experimentally tractable animal models that encompass this heterogeneity.

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Chapter 15
H. Mucosal-Associated Invariant and Vγ9Vδ2 T Cells

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Conventional Versus Innate-Like T Cell Immunity in Tuberculosis Vaccinology

Targeting conventional T cell memory with Bacille Calmette-Guérin (BCG) vaccination has demonstrated variable efficacy and non-durable protection against active Tuberculosis (TB) disease [1–4]. Despite mimicking natural peptide-specific $CD4^+$ and $CD8^+$ T cell immunity acquired during chronic *Mycobacterium tuberculosis* infection, BCG as well as several investigational candidate vaccines have largely failed to confer reproducible, life-long protection in adults [5]. Importantly, individuals cured of active TB can still develop recurrent active infection, demonstrating that natural immunity may not be consistently protective [6–8].

Developing an effective TB vaccine has proven challenging as the immune correlates of protection are not well understood [9]. For example, induction of polyfunctional *Mtb*-specific $CD4^+$ T cells is common among immunogenic vaccine candidates but may not be associated with efficacy [10]. Recent successes in preventing culture-confirmed active infection with M72 fusion protein/AS01e adjuvant vaccine candidate [11] or sustained interferon γ release assay (IGRA) conversion

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with BCG revaccination [12] have revitalized interest in protective peptide-specific T cell immunity in certain populations, though more comprehensive analysis of alternative immune correlates in these studies is warranted [10, 13].

While a new generation of vaccine approaches seek to optimize the systemic induction of conventional T cell immunity [14–18], one relatively unexplored strategy in humans harnesses innate-like T cells within the lung [18–23]. Mucosal-associated invariant (MAIT) and Vγ9Vδ2 (also known as Vγ2Vδ2) T cells are the two most abundant innate-like lymphocytes in the αβ and γδ T cell subsets, respectively. They are considered "innate-like" due to both conserved T cell receptors (TCRs) and *non-peptide* ligands derived from microbes, including *Mtb*. In contrast to the delayed responses of conventional, peptide-specific T cells, MAIT and Vγ9Vδ2 T cell are activated within hours after antigen exposure in vitro and may contribute to mycobacterial clearance [24, 25]. Recent studies of their early immune responses to *Mtb* exposure and infection identify them as potentially important targets for TB host-directed therapies [25–28].

MAIT Cells

Activation and Function

MAIT cells are abundant in human peripheral blood, ranging from <1–18% of T cells, and are also detectable in lung, liver, gut, and other mucosal tissues [24, 26, 29]. They recognize small molecule intermediates of Vitamin B metabolism presented by the oligomorphic MHC I-related molecule, MR1, using a conserved TCR α chain (TRAV1–2/Vα7.2, humans; TRAV1/Vα19, mice) with oligoclonal Vβ chain usage of TRBV6 or TRBV20 [26, 30, 31]. Importantly, MR1 and the MAIT TCR were highly selected for during mammalian evolution to detect products of microbial metabolism [29, 32].

MAIT cells can respond to bacteria [33–35] and fungi [36, 37] in an MR1-dependent manner, but also to bacterial superantigens [38] and viruses in a TCR-independent manner through IL12 and IL18 [39]. They can undergo polyclonal expansion during bacterial infection [40] and may be polyfunctional, producing IFNγ, TNFα, IL17, GMCSF, IL2, and various granzymes [24, 41]. In a transcriptional analysis of a gradient of "innateness" which is defined by rapid effector function at the expense of clonal proliferation, MAIT cells were most similar to $CD8^+$ conventional T cells and invariant Natural Killer T cells [42].

Emerging data demonstrate that MR1 can bind diverse metabolites that may distinguish between microbes [37, 43, 44] or that are unrelated to Vitamin B biosynthesis, such as non-steroidal anti-inflammatory drugs [45]. MAIT cell abundance and function in autoimmune lesions, tumors, and graft versus host disease raise the possibilities that MR1 ligands may be trafficked to sites distant from microbial niches or may include unidentified self-antigens [26, 46–56].

MAIT Cell Subset Heterogeneity

While the majority of MAIT cells express CD8, there are also $CD4^+$, $CD4^-CD8^-$, and $CD4^+CD8^+$ subsets, which may represent different lineages and functional archetypes [24, 41, 57–59]. Several studies support that $CD8^+$ and $CD4^-CD8^-$ MAIT cells are primarily cytotoxic through synthesis of granzymes, IFNγ and TNFα [24, 41, 58], whereas the roles of $CD4^+$ and $CD4^+CD8^+$ MAIT cells are less understood [26]. One study reports increased IL4 and IL13 production in $CD4^+$ MAIT cells (identified by TRAV1–2/Vα7.2 antibodies rather than tetramers) relative to $CD8^+$ MAIT cells [26, 41].

Responses to Mtb *in Animal Models*

MAIT cells compose a significant portion of the $CD8^+$ *Mtb*-reactive T cell pool and can be activated by and kill *Mtb*-infected cells in vitro [33–35]. Recent attempts to understand MAIT cell responses to *Mtb* in animal models have been limited by low baseline abundance in laboratory mice and non-human primates [60–62]. In vivo evidence for MAIT cell control of *Mtb* is limited to MR1 deficient and transgenic mouse models, where MR1 deficient mice were shown to have a mild increase in pulmonary *Mtb* colony-forming units (cfu) compared to TRAV1/Vα19 transgenic mice that overexpress the murine MAIT TCR α chain [35, 62]. However, *Mtb* aerosol infection in both wild type laboratory mice and rhesus macaques demonstrate limited MAIT cell pulmonary enrichment that occurs during the first 2 weeks of infection [61–63].

Several publications demonstrate that MAIT cell abundance and function may be enhanced through priming. One study observed enhanced intradermal MAIT cell responses at BCG vaccination sites in macaques that were subsequently challenged with systemic BCG infection [64]. Further investigation in mice using antigen-specific priming of MAIT cells through intranasal inoculation of the potent MR1 ligand 5–2(2-oxopropylideneamino-6-D-ribitylaminouracil (5-OP-RU) in conjunction with toll-like receptor (TLR) ligands, resulted in robust enrichment of MAIT cells in lung that was sustained weeks after the initial priming event [65, 66]. Importantly, intranasal priming with TLR 2/6 ligand, Pam2Cys, with 5OPRU prior to respiratory inoculation of *Legionella longbeacheae* decreased colony-forming units compared to wild type mice who received TLR ligand alone or MR1 deficient mice receiving intranasal priming with Pam2Cys + 5OPRU [67]. In the same study, MAIT cell adoptive transfer in RAG-deficient mice lacking lymphocytes rescued them from lethal infection. Additionally, MAIT cell adoptive transfer prior to murine influenza infection conferred protection against lethal disease [68]. Protection against *Legionella* was dependent upon MR1, IFNγ and GM-CSF, but not IL17A, TNFα or perforin, whereas protection against influenza infection was MR1-independent. Taken together, these studies support the idea that MAIT cell priming could be effective in ameliorating pulmonary infection, a strategy that has not yet been tested in models of *Mtb* infection.

MAIT Cell Responses to Mtb *Infection in Humans*

Several studies demonstrate human MAIT cell responses to *Mtb*. MAIT cells are depleted in the peripheral blood of both adults [35, 69] and children [70] with active TB as well as recently exposed household contacts [24]. HIV/TB co-infection exacerbates MAIT cell depletion and is associated with elevated levels of the programmed death receptor, PD-1 [71, 72]. MAIT cells can also undergo oligoclonal expansion in the airways of persons with active TB [31]. One study identified a polymorphism in the human MR1 locus that was associated with susceptibility to tuberculous meningitis [73].

Heterogeneous responses have been observed among human MAIT cell subsets during *Mtb* exposure and infection. Peripheral blood $CD4^+$ MAIT cells were relatively enriched in recently infected household contacts whereas $CD8^+$ MAIT cells were depleted [24]. Further, MAIT cells of exposed but uninfected household contacts demonstrated preserved granzyme B and depressed IFNγ responses. Increased peripheral blood $CD25^+CD4^+$ MAIT cells were also detected in recently exposed healthy household contacts relative to community controls. In a separate study, peripheral blood $IL17^+CD8^+$ MAIT cell abundance was associated with healthy household TB contacts who did not develop latent infection [25]. Taken together, these studies implicate MAIT cell immunity early after *Mtb* exposure.

Several gaps remain in our understanding of how MAIT cells can be targeted as immunotherapy against *Mtb* [28]. Three recent studies performed MAIT cell priming in murine lung with TLR agonists +5OPRU prior to aerosol *Mtb* infection [74–76] and together convincingly demonstrate that MAIT cell enrichment in lung is not sufficient to attenuate Mtb infection, as measured by bacterial load. Emerging data would suggest that adjunctive cytokine stimulation with IL12/IL18/IL23 may enhance MAIT cell activity against mycobacterial infection, but have yet to be tested in vivo against *Mtb* [77, 78]. While MAIT cell priming was not effective as a preventive strategy, one of these studies demonstrate that 5OPRU treatment during the chronic phase of infection significantly reduced lung *Mtb* bacterial load in an IL17A-dependent manner [75]. Together, these data suggest that MAIT cells may be targeted as an adjuvant immunotherapy in *Mtb*-infected patients.

Vγ9Vδ2 T Cells

Activation, Function, and Subset Heterogeneity

Vγ9Vδ2 T cells compose >50% of γδ T lymphocytes and are highly abundant in the peripheral blood and mucosal tissues of primates, but not other mammals [79, 80]. These cells respond to small molecule pyrophosphate antigens through a non-MHC mechanism that senses isoprenoid intermediates such as (e)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) in a non-MHC, butyrophilin-dependent manner [81]. The majority of Vγ9Vδ2 T cells are $CD4^-CD8^-$, however subsets of $CD4^+$,

$CD8^+$ and $CD4^+CD8^+$ are also present [24]. Little is known about their functional heterogeneity [24]. Like MAIT cells, Vγ9Vδ2 T cells can execute pleiotropic effector functions, secreting IFNγ, TNFα, perforin, and granzymes [82–85].

Responses to Mtb *in Animal Models*

Vγ9Vδ2 T cells are activated by and can kill *Mtb*-infected cells in a granulysin-dependent manner [86–88]. Extensive in vivo investigation of Vγ9Vδ2 T cell responses during *Mtb* infection has been conducted using the rhesus macaque model. It was first shown that Vγ9Vδ2 T cells expand in BCG-vaccinated macaques, in which *M. tuberculosis* infection was attenuated [89]. In contrast, SIV/*Mtb* co-infection inhibited *Mtb*-specific Vγ9Vδ2 T cell responses [90]. Selective targeting of Vγ9Vδ2 T cells in macaques demonstrate that both in vivo expansion with HMBPP/IL2 and adoptive transfer of in vitro expanded Vγ9Vδ2 T cells result in robust pulmonary Vγ9Vδ2 T cell enrichment that protects against high dose *Mtb* infection (500 cfu) in a perforin and IFNγ-dependent manner [83, 84].

More recently, respiratory immunization of macaques was performed with HMBPP-producing attenuated *Listeria monocytogenes* (*Lm*) vector *Lm* Δ*actA* ΔprfA*, a strain under development as a cancer vaccine vector [91]. *Lm* Δ*actA* delta Δ*prfA** immunization induced sustained expansion of Vγ9Vδ2 in bronchoalveolar lavage fluid and to a lesser degree, blood, for up to 3 months post-immunization compared to delta *Lm* Δ*gcpE* Δ*actA* Δ*prfA* which does not express HMBPP-synthase [92]. These cells produced more IFNγ in lung than blood that was detectable ex vivo without re-stimulation. After moderate dose *Mtb* infection (80 cfu instilled by bronchoscope), selective Vγ9Vδ2 priming with *Lm* Δ*actA* ΔprfA* controlled *Mtb* growth, attenuated lung pathology, and inhibited dissemination to spleen, liver, and kidney.

Importantly, selective Vγ9Vδ2 T cell immunization recruited conventional $CD4^+$ and $CD8^+$ αβ T cells to the lung earlier than controls. In a separate study, Vγ9Vδ2 cells were shown to antagonize T_{reg} populations in an IFNγ-specific manner [93]. Taken together, these data suggest that selective Vγ9Vδ2 T cell immunization may orchestrate a more expansive immune response to control *Mtb* growth. Despite this robust anti-mycobacterial activity, *Mtb* inhibits Vγ9Vδ2 T cell differentiation into effector cells [94], which may explain why Vγ9Vδ2 cells only begin to proliferate during the adaptive phase of the immune response in *Mtb*-infected macaques [89].

Responses to Mtb *in Human Studies*

Humans with active TB infection demonstrate reduced lung and peripheral blood Vγ9Vδ2 T cell with depressed functional responses [94–97]. Vγ9Vδ2 T cells of active TB patients undergo TCR selection evidenced by sequencing of complementarity determining region 3 (CDR3) [79, 98]. In healthy children, Vγ9Vδ2 responses

to phosphoantigens were robust in purified protein derivative (PPD) positive individuals but not in PPD negative donors [99]. There is also evidence of $CD4^+$ γδ T cell expansion observed in healthy, recently exposed contacts with latent infection [24, 100]. Taken together, these findings suggest that Vγ9Vδ2 activation and proliferation after *Mtb* exposure may be a biomarker of early infection.

Future Directions: Harnessing Conserved Antigen-Recognition for Immunotherapy

Dissecting the Functional Heterogeneity of Immune Responses

Despite their evolutionarily conserved antigen-recognition systems, MAIT and Vγ9Vδ2 T cells demonstrate marked heterogeneity in surface receptors, transcription factors, and effector responses [24–26, 30, 41, 59, 97]. Optimal targeting of MAIT and Vγ9Vδ2 T cell immunity against *Mtb* will require a more sophisticated understanding of antigen/cytokine-specific oligoclonal responses within these populations [37, 44, 101, 102].

This will entail reconciling differences in lineage markers in humans and animal models. While most murine MAIT cells are $CD4^-CD8^-$, primate MAIT cells are predominantly $CD8^+$ [24, 64]. Minor populations of $CD4^+$ and $CD4^+$ $CD8^+$ MAIT cells have also been described and their function is not well understood [24, 30, 41]. Similarly, the majority of primate Vγ9Vδ2 T cell are $CD4^-CD8^-$ or $CD8^+$ [103], however rare $CD4^+$ and $CD4^+$ $CD8^+$ populations have also been observed in the human repertoire [24, 100]. Many studies of innate-like T cell responses to *Mtb* do not distinguish between these subsets [61, 64, 97, 104]. Some studies suggest that lineage markers CD4 and CD8 may identify MAIT or Vγ9Vδ2 T cells with distinct functions during *Mtb* infection [24, 26, 41]. Future investigations can help to elucidate differences in abundance and function of subpopulations of MAIT and Vγ9Vδ2 T cells that may guide targeting specific effectors.

Characterizing Innate-Like T Cell Memory

Due to their recognition of evolutionarily conserved antigens, priming of MAIT or Vγ9Vδ2 T cells against *Mtb* may confer the additional benefit of heterologous protection against other pathogens. This may parallel observations of trained immunity in classical innate lineages such as monocytes and natural killer cells after BCG vaccination [40, 105, 106]. Animal models have demonstrated sustained MAIT and Vγ9Vδ2 enrichment in lungs of mice and rhesus macaques months after initial antigen-specific immunization [65, 67, 92], which may reflect epigenetic changes

similar to those observed in trained innate cells that can modulate subsequent homologous and heterologous immune responses [21, 105, 106].

There are also potential limitations to such a vaccination approach. Though conserved, the degree of antigen experience will vary per donor and may predict the amplitude of response to subsequent vaccination. This may explain the marked heterogeneity in baseline peripheral MAIT and Vγ9Vδ2 T cell abundance observed in healthy donors [24, 25]. Further, the durability of innate T cell responses after antigen recognition has not been well-defined. Due to their dependence on endogenous microbial metabolites during ontogeny [55, 56, 98, 107], it will be important to consider the influence of gut microbial composition on innate T cell immunity [24]. Ligands derived from different microbes may differentially bind MR1 [37, 43, 45] or butyrophilin [88] and modulate subsequent activation or inhibition of MAIT and Vγ9Vδ2 T cells. As MAIT and Vγ9Vδ2 T cell responses may be non-specific, future studies can help to measure effects on non-targeted bacteria as well as healthy cells presenting MR1 ligand during priming that may lead to idiosyncratic adverse events such as microbial dysbiosis or autoimmunity.

Targeting Innate-Like T Cell Immunity Throughout the *Mtb* Lifecycle

In summary, MAIT and Vγ9Vδ2 T cells are promising targets to be considered in the development of the first pulmonary mucosal TB vaccine. In addition to harnessing MAIT and Vγ9Vδ2 T cells as a preventive strategy against infection, it will also be instructive to test selective priming during latency and active infection, either alone, or as adjuvant immunotherapy with conventional medical regimens. Ongoing pre-clinical studies and future early phase clinical trials in humans will determine the safety, feasibility, and efficacy of harnessing innate-like T cell immunity against *Mtb* infection.

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Chapter 16
Alveolar Epithelial Cells

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Alveolar Epithelial Cells (ATs) as One of the First Lines of Defense Against *Mycobacterium tuberculosis* Infection

Tuberculosis (TB) is still one of the most important infectious diseases worldwide, caused by *Mycobacterium tuberculosis* (*M.tb*), a single pathogen that is responsible for over one billion deaths in the last 200 years [1, 2]. Despite a broad spectrum of potential outcomes after *M.tb* infection leading to pulmonary, extrapulmonary, or disseminated active TB, latent *M.tb* infection, or even clearance by the host, it is critical to consider the primary site of infection after *M.tb* is deposited in the alveolar space [3, 4]. *M.tb* possess multiples strategies for establishing infection in the lung after reaching the alveolar space [5]. Alveolar macrophages (AMs), neutrophils, and dendritic cells can phagocytize *M.tb* [4, 6, 7]; however, *M.tb* also may be recognized and taken up by nonprofessional phagocytic cells that line the alveolar epithelium [8, 9]. There are two types of alveolar epithelial cells (ATs) [10]. Type I ATs are the most dominant cell type, providing the structured shape of the alveolus and allowing gas exchange [9, 10]. However, type II ATs (ATIIs) play an essential role in host defense by maintaining alveolar integrity, preventing microbial dissemination, and secreting lung alveolar mucosa [11]. The lung alveolar mucosa is composed of a lipid phase called surfactant and an aqueous hypophase called alveolar lining fluid (ALF). Alveolar surfactant is mainly composed of phospholipids, and

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dipalmitoyl-phosphatidylcholine (DPPC) is the most abundant one (~70% of the total surfactant lipids) [12]. ALF is composed of host innate soluble components including hydrolytic enzymes (hydrolases), antimicrobial peptides (AMPs), surfactant proteins (SPs), mucosal antibodies (Abs), and cytokines, among others. These components induce agglutination or modifications to the cell envelope of microbes, enhancing their recognition and phagocytosis by alveolar compartment cells [11–16]. ATs are an alternative mechanism to control the infection without the activation of an adaptive immune response [9, 17, 18]. AT infection could be beneficial to *M.tb* bypassing the professional phagocyte response, providing the bacterium with a protective intracellular niche that favors its replication, cell envelope remodeling, and metabolic adaptation to the host. Nonetheless, *M.tb*-infected ATs trigger a local inflammatory response by releasing pro-inflammatory cytokines, recruiting immune cells into the infection site, and eventually contributing to granuloma formation and control of the spread of the infection [3, 13, 19].

AT Mechanisms in Response to *M.tb* Infection

ATIIs surface integrins [e.g., CD51 (vitronectin) and CD29 (B1 integrin)], carbohydrate receptors [e.g., heparin, hyaluronic acid, laminin, collagen], and extracellular matrix (ECM) components, as well as pattern recognition receptors (e.g., C-type lectins and TLRs), are involved in receptor-mediated endocytosis of *M.tb* and in modulating the AT immune response during infection [4, 20]. The expression of these AT receptors is polarized, coordinated, and highly specific, sometimes working synergistically together (e.g., hyaluronic acid binding to ECM driving *M.tb* invasion of ATIIs) to facilitate recognition, binding, and uptake of *M.tb* by the alveolar epithelium and enhancing ATs susceptibility to invasion. In this context, *M.tb* antigens (Ags) released to the alveolar environment from the *M.tb* cell envelope surface by the action of host ALF hydrolases, by the bacterium itself, and/or after infected host cells death, can interact with the alveolar epithelium, changing the dynamics of the infection [21, 22]. Indeed, *M.tb* Ag ESAT-6, phosphatidyl-*myo*-inositol mannosides (PIMs), and mannose-capped lipoarabinomannan (ManLAM) among other *M.tb* Ags induce ATIIs cytotoxicity [23–27]. Moreover, *M.tb* heparin-binding hemagglutinin (HBHA), malate synthase, Ag 85 complex, and fibronectin-attachment proteins (FAPs) bind to sulphated glycoconjugates and ECM promoting *M.tb* adherence and crossing of the alveolar epithelium barrier and thus, *M.tb* dissemination [23, 28].

Of the 11 TLRs that ATs express on their surface, TLR-2, −4, −6, and −9 participate in *M.tb*-AT interactions [4, 18, 20]. TLR-2, the most studied TLR in ATIIs, interacts with *M.tb* PIMs, ManLAM, lipomannan (LM,) and the 19-KDa lipoprotein among others, leading to an increased ATIIs secretion of AMPs, HβD-2, cathelicidin, and the neutrophil chemoattractant CXCL-5, all involved in modulating *M.tb* infection outcome in ATIIs [18, 29]. The most studied C-type lectin in ATs is the C-type lectin domain family 7 member A (CLEC7A or Dectin-1). CLEC7A binds

to *M.tb* through an undefined mechanism; however, this binding results in ATs reactive oxygen species (ROS) production, as well as of TNF, IL-6, IL-8, IL-12 and AMPs, causing better *M.tb* growth control in ATs [4, 18, 30]. Nevertheless, *M.tb*-induced inhibition of CLEC7A in ATIIs downregulates the immune response, limiting recruitment of cells to the infection site, delaying granuloma formation, and control of the infection [4]. Within ATIIs, *M.tb* bacilli traffic to non-acidified late endosomes, allowing *M.tb* to evade killing and replicate. Alternatively, *M.tb* uses the autophagy pathway to its advantage to grow in infected ATIIs and avoid elimination [26, 31].

Some innate soluble components secreted by ATIIs are involved in controlling *M.tb* infection. Studied in detail are SP-A, SP-D, complement component 3 (C3), AMPs, Abs, and ALF hydrolases, among others. Upon deposition in the alveolar space, ALF hydrolases alter the *M.tb* cell envelope, and to subsequently release *M.tb* cell envelope fragments into the alveolar milieu [13, 32, 33]. Some of these fragments potentially can bind and block the function of ALF innate soluble components such as SP-A/-D, C3, and AMPs, but the effects on *M.tb* uptake by ATIIs are still uncertain. Importantly, the oxidative status of ALF links to the functionality of these ALF innate components [14, 34]. Thus, successful *M.tb* infection may depend on the inflammatory and oxidative status of the host ALF. High ALF oxidation and dysfunctionality of its soluble components could facilitate *M.tb* infection in the lung.

AT Targeted Host-Directed Therapeutic Strategies for TB

Although many host response mechanisms involved in *M.tb* infection are characterized, treatment for TB remains challenging. The field is rapidly delineating host-pathogen interactions in order to develop TB host-directed therapeutics (HDT) agents [35, 36]. The main HDT strategies, rather than acting directly on *M.tb*, manipulate or modulate critical host pathways involved in controlling *M.tb* invasion and growth, including, but not limited to enhancing host cell responses, making the local environment less favorable for *M.tb*, optimizing inflammatory responses (immunomodulatory) for cell recruitment or even modifying lung inflammation and pathology [35, 37]. Studies have mainly focused on the induction of $CD4^+$ T cell responses and release of TNF, IL-12, and IFN cytokines to restrict *M.tb* infection [38], decreasing cholesterol levels in the membrane to promote phagosomal maturation and autophagy, inhibiting PD-1 expression to promote $CD8^+$ T cell-mediated immune responses. Inhibiting 5-lipoxygenase to suppresses leukotriene production and reduce lung pathology. Neutralizing vascular endothelial growth factor (VGEF) to facilitate drug entry into granulomas, and neutralizing TNF to disrupt granulomas and reduce lung pathology, among others [39].

However, further studies are needed to identify promising HDT strategies targeting effector cells, for instance ATs. In order to enhance intracellular *M.tb* killing, decrease inflammation and lung tissue damage, enhance T cell responses, and to

ultimately suppress *M.tb* invasion and growth (Fig. 16.1a–e). Indeed, during infection, *M.tb* induces the expression of matrix metalloproteinases (MMPs) by macrophages and ATs, causing inflammatory tissue damage and progression of the disease [40–43]. Thus, inhibition of MMP activity in infected mice by SB-3CT in combination with conventional anti-TB drugs suppresses MMP-9 activity levels showing better control of the infection and reduced disease progression [39, 44] (Fig. 16.1c). Another example is the use of CpG oligodeoxynucleotides to induce host phospholipase D (PLD) activation, and subsequently, PLD-dependent phagolysosome maturation and ROS production, leading to intracellular *M.tb* killing in the ATs and macrophages [35, 45, 46] (Fig. 16.1c).

During *M.tb* infection, CXCL5 produced by ATs [29] drives neutrophil recruitment to the infection site. However, excessive neutrophil accumulation is detrimental, causing a destructive cellular inflammation driving pulmonary TB [29]. Thus, a promising HDT targeting ATs is the host modulation of microRNAs (e.g., miR-223) targeting CXCL chemoattractants (including CXCL2, CCL3, and IL-6), which regulate leukocyte chemotaxis, leading to more controlled destructive neutrophilic inflammation [47, 48]. In addition, miR-223 suppresses activation of the master regulator NF-kB in ATs, dampening neutrophil activation, restricting cell inflammation, and tissue destruction [49] (Fig. 16.1d).

The use of 1,25-dihydroxy-vitamin D3 (vitamin D) as an HDT agent also has several applications in TB treatment, including enhancing and accelerating inflammatory responses, inducing autophagy of *M.tb*-infected cells, and reducing tissue injury [39]. Vitamin D induces *M.tb*-infected macrophages to secrete IL-1β, which activates ATs to produce AMPs such as *DEFB4*/hBD2 (β-Defensin 2), allowing better control of *M.tb* infection [48, 50](Fig. 16.1d). Vitamin D also upregulates the expression of other cathelicidin-related AMPs, such as LL37, in ATs, preventing *M.tb* colonization [51, 52](Fig. 16.1d). The production of hBD-2 by ATs and AMs is also considered an important mechanism to inhibit *M.tb* proliferation [25, 53], and should be considered as a potential target of HDT.

Syndecans (Sdcs) are transmembrane cell surface proteoglycans, which mediate host cell adhesion matrix ligand-receptor interactions and contribute to the regulation of cell morphology, migration, and signaling [54]. Scd1 and Scd4 host receptors are detected by mycobacterial HBHA allowing *M.tb* attachment and internalization into the non-professional phagocytes [17, 55] and consequent extrapulmonary dissemination [56]. Further studies are needed to identify promising HDT strategies to block the interaction between the host Sdcs and mycobacterial HBHA. A study reported that the blockage of Sdcs by anti-Sdc-1 or anti-Sdc-4 antibodies could reduce mycobacterial internalization by ATs [55] (Fig. 16.1e).

M.tb has been reported to induce cholesterol-dense regions of the plasma membrane in ATs, known as lipid rafts (LR) [57]. Various pathogens, including *M.tb,* induce the formation of LR aggregates to facilitate bacteria internalization, hijacking of the host cell, and worsen survival [17, 57]. Treatment of ATs with a sterol-binding agent, Filipin III, disrupts mycobacterial-induced LR aggregation and significantly reduces *M.tb* infection in ATs [57](Fig. 16.1e).

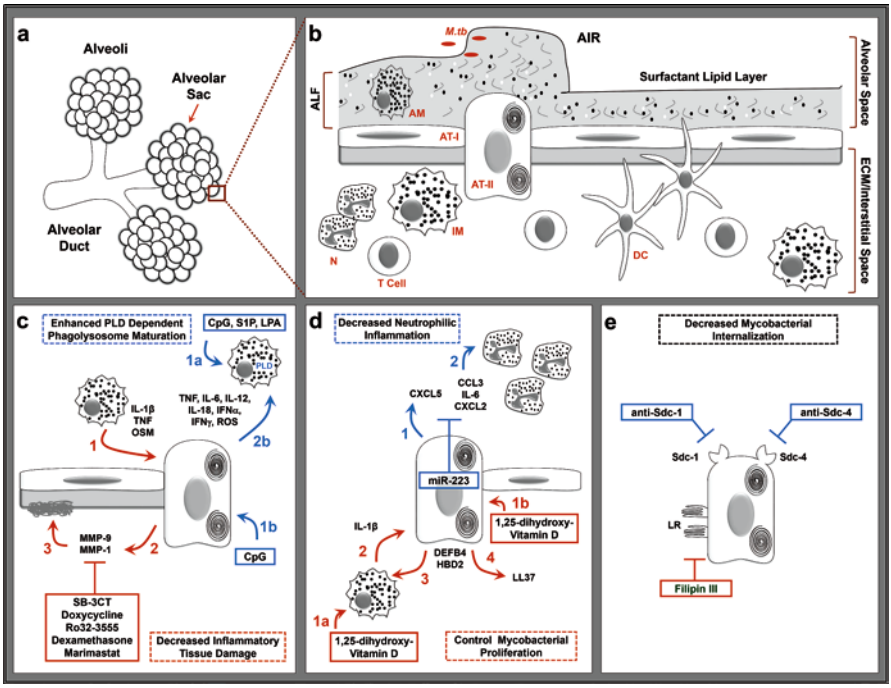

Fig. 16.1 Potential AT-targeted host-directed therapeutic strategies against *Mycobacterium tuberculosis* (*M.tb*). (**a**) *M.tb* is deposited into the distal portion of the lung, *i.e.*, the alveolus. (**b**) In this environment, *M.tb* encounters alveolar lining fluid (ALF) that allows recognition by phagocytes, including alveolar macrophages (AMs), neutrophils (Ns), and/or dendritic cells (DCs). However, *M.tb* recognition and uptake can also take place by nonprofessional phagocytic cells that line the alveolar surface, including alveolar epithelial (ATs). (**c**–**e**) AT-targeted host-directed therapeutic strategies include enhancing intracellular *M.tb* killing, suppressing inflammation and lung tissue damage, and enhancing T cell responses. (**c**) AMs secrete pro-inflammatory mediators (red arrow 1) driving ATs to secrete metalloproteinases (MMPs) (red arrow 2), which in turn degrade the extracellular matrix, causing tissue destruction (red arrow 3). SB-3CT and doxycycline among other drugs, inhibit MMP activity in infected ATs, thus suppressing inflammation and lung tissue damage. Conversely, CpG oligodeoxynucleotides induce directly (blue arrow 1a) or indirectly (blue arrows 1b and 2b) AMs phospholipase D (PLD) activation and subsequently, PLD-dependent phagolysosome maturation, leading to intracellular *M.tb* killing. (**d**) 1,25-dihydroxy-vitamin D3 induces paracrine macrophage epithelial signaling (red arrows 1a and 2) or directly induces ATs (red arrow 1b) to better control infection, upregulating the expression of antimicrobial peptides such as DEFB4/HBD2 (red arrow 3) and LL37 in ATs (red arrow 4). Targeting CXCL chemoattractants via miR-223 also decreases destructive cellular inflammation caused by neutrophils (blue arrows 1 and 2). (**e**) Blocking interactions between host cell receptors (Sdc-1/Sdc-4) and/or lipid rafts (LR) with mycobacterial adhesins (*i.e.* HBHA) reduces mycobacterial binding and internalization by ATs. Abbreviations: ALF alveolar lining fluid, AM alveolar macrophage, ATs alveolar epithelial cells, N neutrophil, IM interstitial macrophage, DC dendritic cell, ECM extracellular matrix, CpG CpG oligodeoxynucleotides, S1P sphingosine 1-phosphate, LPA lysophosphatidic acid, PLD phospholipase D, MMP matrix metalloproteinases, ROS reactive oxygen species, Sdc syndecan, LR lipid raft. Cytokines/Chemokines: IL-1β, TNF, OSM, IL-6, IL-12, IL-18, IFNα, IFNϒ, CCL3, CXCL2, CXCL5. Antimicrobial peptides: DEFB4, HBD2, LL37

Considering that ATs constitute one of the main structural defensive barriers in the lung alveolar space and play essential roles in cell communication and recruitment, controlling cellular inflammation, and the release of innate antimicrobials against *M.tb*, it is imperative to identify protective HDT agents to target these host cells. HDT targeting of ATs could address the improvement of drug efficacy, decreasing both treatment duration and potential drug toxicity, and, accordingly, achieve faster control of active TB disease.

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Part V
Preclinical Models for Assessing HDTs

Chapter 17
In Vitro Models of Human Granuloma Formation to Analyze Host-Directed Therapies

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The Human Tuberculosis (TB) Granuloma

Mycobacterium tuberculosis (Mtb) is an obligate pathogen of man and the formation of granulomas has long been recognised as the key interface between the infectious agent and the host immune response. Granulomas were identified even before Koch's discovery of tuberculous bacilli [1]. They are complex multicellular structures, often characterized by central caseating necrosis and surrounded by macrophages and multinucleated giant cells, with an outer layer of T-cell infiltrates during active infection, which is pathognomonic of human tuberculosis. Once healed, granulomas tend to calcify, a typical feature seen on chest radiography. Although the granuloma is thought traditionally to contain infection, recent seminal studies in the zebrafish model of TB have identified the granuloma as a place where mycobacteria can proliferate [2].

Therefore, the granuloma provides the key host-bacterial interface where host directed therapy may exert beneficial effects. However, granulomas are also the site of TB pathology, whereby breakdown of collagen and other extracellular matrix components leads to lung tissue destruction that precipitates cavitation, resulting in transmission of infection [3]. The potential deleterious effects of any intervention

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therefore cannot be ignored, hence both protective and possible pathological processes need to be considered when assessing host-directed therapies.

Challenges in Modeling Human TB Granulomas *In Vitro*

As multicellular organized structures, TB granulomas form and evolve over time. Therefore, ideal *in vitro* modeling should reflect this scenario spatially and temporally. Due to this major challenge, *in vivo* systems such as the mouse, guinea pig, rabbit, and, more recently, the zebrafish model have been the mainstay of studying granuloma function in human TB [4]. Whilst these models have generated a huge body of data and valuable insights into host-pathogen interactions, they are limited by lack of human cells. Mtb has undergone prolonged co-evolution with humans [5], and therefore any other mycobacterial/host pairing makes the assumption that the fundamental biology is the same. *Mycobacterium bovis* and Mtb share a genetic identity of 99.7% [6], and yet *M. bovis* does not cause the same disease in humans, but instead is a pathogen of cows and deer. This illustrates how even a small change in mycobacterial DNA may affect pathogenicity and the importance of the appropriate host-pathogen pairing in experimental studies.

Considering the features of human disease, certain criteria can be defined that are necessary for an ideal model of human TB [7]. Essentially, the *in vitro* model should be based on primary human cells, as TB is a disease of humans. Furthermore, pathogenic Mtb is required, as attenuated strains may not replicate disease biology. The model should also reflect the host-pathogen interaction in three-dimensional space [8], including how cell-cell and cell-matrix interactions modulate the biological outcome [9]. The presence of extracellular matrix acts as a cell survival factor, and the degradation of matrix may further modify cell biology. The granuloma composition is multicellular and dynamic over time, and so ideally cellular migration within and out of granulomas would also be modeled in the *in vitro* system.

Finally, genetic variation in the host-pathogen interaction in TB has been observed, with different Mtb strains optimally evolved for different human genetic populations [10, 11]. Therefore, the modeling of Mtb and the genetic makeup of host cells needs consideration. In addition, all these studies need to be performed in containment level III facilities, creating a logistical challenge when generating additional readouts, such as live cell imaging, flow cytometry and high throughput testing more difficult.

In Vitro Models of Human TB Granulomas Developed to Date

Given the characteristics of the host-pathogen interactions outlined above, we focus here on human granuloma models widely used to date. The first *in vitro* model developed was from Altare's group, and so can be regarded as a pioneer system

developed several years ahead of the others [12]. Their group demonstrated cellular aggregation over time and the potential to investigate specific features of the host-pathogen relationship in an *in vitro* system. The initial model used microspheres coated with partially purified protein derivative of Mtb (PPD), and progressed to Mtb within 2-dimensional culture, showing progressive cellular aggregation [13]. In-depth study of the Mtb-macrophage relationship later highlighted the significant role of Mtb in macrophage differentiation within the granuloma [14]. The group has also used this model in high-throughput screening technology to evaluate novel anti-tuberculosis therapies [15].

This approach to modeling human TB granulomas has been further developed and adapted by several groups. Seitzer et al. demonstrated formation of larger peripheral blood mononuclear cell (PBMC) spheroids infected with pathogenic Mtb more than uninfected PBMCs, suggesting cell aggregation is a dynamic process derived by the presence of mycobacteria [16]. This was further confirmed by Birkness et al., who demonstrated the chemotactic effect of Mtb infection on PBMCs involved in granuloma formation [17]. Most recently, this model has been further refined by the Schlesinger group [18], who have compared cells from patients not exposed to Mtb, patients exposed to Mtb, and patients with other granulomatous conditions, such as sarcoidosis, to probe human and Mtb determinants of the host immune response. Therefore, this model and its further development has given widespread insights into pathogenesis.

The next major development in granuloma modeling was to incorporate extracellular matrix. An alternative model system using this approach was investigated by Lerm's group, who refined a cellular layering system, which allows fibroblasts, epithelial cells and macrophages to be studied on a backbone of collagen [19]. This has been used to study the airway and can therefore allow modelling of early events in the host-pathogen interaction during initial mycobacterial infection [20]. This model is limited to studying initial innate events, due to MHC incompatibility, and consequently does not include T cells. Kapoor and colleagues have also developed a 3-dimensional cell culture model incorporating collagen and fibronectin gels, allowing cell aggregation and granuloma formation [21]. Using this model, the group investigated factors resulting in Mtb resuscitation out of dormancy. This system has also been used by Arbués and colleagues to investigate the effect of different monoclonal antibodies targeting cytokines, including TNF-α, IL-17A and IL-12-p40 in tuberculosis pathology [22]. These human TB granuloma models have made clear advances, but a central challenge of the models is the downstream analysis of cells once embedded in collagen and the addition of new cells to replenish the granuloma.

Our group has used a bioengineering approach to generate microspheres within which primary human PBMCs, collagen and Mtb can be incorporated. Like the models above, it has both benefits and limitations. The main component of the microsphere matrix is alginate, which permits cell migration but is not natural to the human body, being derived from seaweed [23, 24]. We optimised the parameters for stable microsphere generation [25], and produced multiparameter readouts to probe the host-pathogen interaction. Firstly, we demonstrated that the incorporation of

collagen into the matrix improves cell survival in microspheres, supporting findings from transgenic mouse studies [9], and permitting longer term experiments than standard 2-D cell culture. Next, we demonstrated the potential to investigate host-directed therapies, such as modulation of the cytokine pathway and prostaglandin pathway [26], where the addition of PGE_2 reduced Mtb growth but increased secretion of chemokines that may cause greater pathology. We have also shown that doxycycline inhibits expression of matrix metalloproteinases and prevents extracellular matrix breakdown [27], demonstrating the potential to investigate host-directed therapy. Another key finding is that Mtb is sensitive to pyrazinamide in our 3-dimensional microsphere system, mimicking that seen in patients, whereas Mtb in 2-dimensional cell culture is not pyrazinamide-sensitive [28], confirming that the system recapitulates mycobacterial antibiotic sensitivity in patients. Most recently, we have used this system to model the increase in Mtb growth observed in patients with cancer treated with anti-PD-1 therapy, as well as to dissect the excessive cytokine secretion that leads to progressive Mtb growth [29]. Therefore, the system can identify both protective and harmful outcomes of each intervention.

Limitations of Current Models and Development Needs

All the models described above have advantages over standard 2-dimensional monolayer cell culture, but at the same time fall short of the complexity of events *in vivo*. However, each *in vitro* system only needs to replicate the pathophysiological events that one is attempting to model to achieve enough complexity. There are several features of human TB disease, which current models do not reflect but have the potential to be developed. Each *in vitro* system could be modulated to truly imitate a feature of TB, and identification of that specific TB pathology during the temporal progression of the granuloma is critical. There is always an inherent tension between ease of use and throughput of each model versus further layers of complexity, and there is no additional benefit in simply developing more complex model systems, unless they yield a greater understanding of the underlying biology.

Firstly, although the models permit longer infection than current standard two-dimensional systems, the longest experimental duration is approximately 4 weeks, which is still relatively short compared to the host-pathogen interaction *in vivo*. Therefore, further optimization to permit genuinely long-term experiments, such as the incorporation of cells derived from stem cells, could potentially generate a system to assess the long-term effects of host-directed therapies. Secondly, cell recruitment into and cell egress out of the granuloma occurs over time, requiring easy movement of cells within the model. This is not possible in most current systems, which incorporate extracellular matrix. Likewise, pharmacokinetic modeling to investigate the mode of action of synthetic or natural potential therapeutic chemicals is complex. Here, integration of microfluidic systems permits continuous exchange of fluid around granulomas, as shown for rifampin pharmacokinetics during *in vitro* modeling [28]. The advantage of incorporating extracellular matrix in

these systems is that the cells are not eluted under flow conditions. The cellular ingress in any system will also require diverse matrices that allow the adhesion and migration of cells to reflect matrices *in vivo*. Finally, the modelling of diverse matrix regions with different mechanotransduction signals, for instance fibrous and non-fibrous areas, represent a new and unchartered bioengineering challenge.

Within human TB lesions there are a variety of microenvironments, such as hypoxia [30]. This can be modeled by increasing granuloma size, thus creating an oxygen gradient and producing a microsphere where the centre is hypoxic. Alternatively, multiple granulomas could be simultaneously cultured in incubators set at normoxic or hypoxic conditions, then mixed to reproduce a range of granulomas subjected to different oxygen concentrations. TB is exacerbated with specific comorbidities, including diabetes and smoking [31]. These comorbidities could be modeled within granulomas, but it does present challenges around sample size and power calculations. Finally, within the same individual, some TB lesions will progress while others regress, demonstrating that the outcome of the host-pathogen interaction is determined at a local, rather than a systemic level. Mimicking these essential local processes within the granuloma is a major hurdle yet to be overcome.

Use of *In Vitro* Models to Assess Host-Directed Therapies

The currently available models have the benefit of permitting analysis of the host-pathogen interaction in a system containing primary human cells and virulent Mtb. Even without further modification, a range of different host-directed therapies can be assessed in these models, such as showing the benefits and limitations of modulating the prostaglandin pathway within an advanced cell culture system or the effect of doxycycline in reducing extracellular matrix turnover. If these models represent the correct host-pathogen pairing, there is significant potential for the findings to be translated to the clinic. However, it is important that multiparameter readouts of various events are considered. If a host-directed therapy results in a reduction in Mtb growth, this must be balanced against the effect of any single intervention on other parameters, such as host cell survival, pro-inflammatory and anti-inflammatory cytokine secretion, tissue destructive matrix metalloproteinase activity and collagen destruction. Therefore, no single outcome should be taken as a definitive sign of therapeutic benefit because it may come at a cost of harm in a second equally important outcome. Human granuloma models should enable highly detailed mechanistic analysis, which may provide novel insights that are not feasible in clinical studies or animal experiments.

In the future, integration with single cell sequencing and proteomic readouts may provide powerful “multi-omic” analyses of the effects of each intervention and mechanistic insights into how subtle changes in gene expression at a single cell level affect the host-pathogen relationship at the granuloma level. Ultimately, the critical determinant of whether human granuloma modeling is worthwhile will be how many new therapies investigated at an experimental level translate to clinical

use. This is unknown and unpredictable, but the authors propose that the use of *in vitro* models to refine and define the readouts of clinical trials will help maximize the chance of success of emerging host-directed therapies, and ensure prioritization of agents with the optimal effect profile.

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Chapter 18
C3HeB/FeJ as a Key Mouse Strain for Testing Host-Directed Therapies Against Tuberculosis

Pere-Joan Cardona and Cristina Vilaplana

Introduction

The use of the C3HeB/FeJ strain as a laboratory animal for tuberculosis (TB) modeling was described for the first time by Yamamoto and Kakinuma. In their work on the induction of a pulmonary granulomatous response after intravenous inoculation of oil-associated BCG cell wall vaccine, this strain was defined as a "low responder" as opposed to C57BL/6 mice [1]. However, it was Igor Kramnik's team that studied it more in depth in their work on mapping a new locus that had a major effect on *M. tuberculosis* (Mtb) infection, the *sst1* (susceptibility to tuberculosis 1) locus. In particular, they used the C3HeB/FeJ mouse because, as an Nramp1-resistant, Mtb-susceptible inbred strain, it showed a "dramatic pathology, including pneumonitis and necrosis" in comparison with the C57BL/6J strain, which showed interstitial inflammation with very few living bacilli after 6 months of infection [2]. The hypoxic nature of the lesions with cavities and liquefaction induced in this strain attracted the attention of drug developers at Colorado State and Johns Hopkins Universities, where teams started working with it in order to improve TB chemotherapy [3]. Nevertheless, there was some initial hesitation, as all pre-clinical TB chemotherapy had been conducted in BALB/c, C57BL/6 or Swiss mouse strains [4].

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Excessive Inflammatory Response is a Hallmark of TB Progression

Influenced by the "damage framework" theory of Casadevall and Pirofsky [5, 6], our group started to work with the concept that in the majority of cases, progression towards TB was caused by an exaggerated immune response against Mtb [7]. Our team was deeply influenced by the data obtained in the minipig model, and, in particular, the rapid encapsulation process taking place around minimal granulomas (around 1 mm of diameter), due to the presence of interlobular septa [8]. This supported the idea of a rapid and potent inflammatory response to avoid the encapsulation process. For this reason, we started to investigate which mice strains developed liquefaction. Although we had already observed some sporadic cavity formation in CD1 outbred mice, and were able to induce lung cavities in the model of reactivation generated in SCID mice [9], we continued to search for a robust and reproducible model, i.e., one using a non-immunocompromised, inbred strain, to ascertain the mechanism involved. Due to the histological parallels we observed in the lesions of SCID mice and those described by Kramnik [2], we started to work with the C3HeB/FeJ strain.

Fibrosis Versus Neutrophils

The initial work done was based on trying to set a balance in which the fibrotic reaction was a key defense mechanism to stop the liquefaction process, leading to a type of "holistic" theory [10], which in turn led to a series of failed experiments based on the use of profibrotic agents, such as bleomycin or doxycycline. Soon after publication, we realized how erroneous this hypothesis was. There was an "elephant in the room" which we had not wanted to see, which were the neutrophils. Even though we observed them and even included them in our "*in silico*" models [11], they were included only to fill up space! At that time, neutrophils were mostly dismissed in the pathogenesis of TB, and we received the criticism that the C3HeB/FeJ mouse was not a real TB model, but a type of "TB abscess model". This led us to look at a genuine abscess model induced by *Staphylococcus aureus* infection, and to test heparin as an inhibitor of this process [12], which we showed to have a protective effect against TB progression [13].

Neutrophils as Hallmark of TB Progression. The Origin of Host-Directed-Therapies

Soon we realized that heparin had an inhibitory effect against neutrophils [14], and accordingly we decided to test the effect of anti-inflammatory agents, such as ibuprofen or aspirin. We realized that by stopping the infiltration of granulomas

by neutrophils we could halt their progression towards liquefaction and cavitation [13, 15]. The accumulation of neutrophils promoted extracellular overgrowth of Mtb, which led to a rapid increase in size of the granulomas, the induction of new peripheral lesions, and their eventual coalescence, leading to an extensive intragranulomatous response that liquefies the necrotic tissue, a process which fits the "bubble-model" [16]. At that time, the dogma was that liquefaction was a later phase in TB progression, which led to extracellular bacilli growth, fueled by cavity formation [17]. This reaction was compatible with the need for a rapid growth of the lesions surpassing the strong encapsulation mechanism noted in big mammals like humans, with the pulmonary parenchyma fragmented by a structure of interlobular septa. This was confirmed by evaluation of TB patients prior to receiving chemotherapy and analysis of their lung lesions. In particular, Medlar clearly differentiated between exudative lesions, which tend to enlarge and have a high infiltration of neutrophils, and proliferative lesions, which are characterized by cellular infiltration with macrophages and lymphocytes [18, 19].

The C3HeB/FeJ model has been of great utility in evaluating mechanisms to counterbalance the damage induced by an excessive Th17 response linked to the stimulation of neutrophil infiltration into Mtb granulomas [20, 21], as well as the induction of a Treg response, by oral administration of heat-killed environmental mycobacteria [22]. It has also been useful to characterize the protective mechanisms triggered by the administration of aspirin [23] or to support the choice of pravastatin from amongst other statins as adjunctive therapy for active TB [24]. Additionally, a model of latent tuberculosis reactivation has been developed by neutralizing TNF, which has more similarities to the human condition, compared with the traditional Cornell model using C57Bl/6 mice [25].

The Importance of the Challenge Route in C3HeB/FeJ

The route of infection is very important. In the original studies by Kramik and our group, the mice were infected via the intravenous route. Using this approach, we were able to induce liquefaction in lesions in a very reproducible and predictable manner. By contrast, aerosol challenge generates a more complex scenario [4, 26], leading to the induction of both human-like lesions with liquefaction (i.e., exudative), with a homogeneous (Type I) and irregular development, and a fibrotic "honey comb" pattern (Type II), but characterized by a strong infiltration with neutrophils and a large amount of intragranulomatous necrosis. Type III lesions are the ones usually formed in C57BL/6 or BALB/c mice, and are characterized by a scarcity of neutrophils, a greater abundance of macrophages, with an evolution towards lymphocytic cuffing infiltration and a robust presence of foamy macrophages in the periphery. The problem is that the induction of "human-like" lesions (type I and II) is not predictable. In our experience, even when challenging with multiple consecutive aerosol challenges (up to 8 in a week) Type I or II lung lesions were mainly absent, even 16 weeks post-aerosol infection [27].

The intravenous route of infection is controversial, as several groups have reported that they are unable to reproduce the robustness and predictability shown in our team's experience (personal communication, various groups). Recently, we have been able to provide a possible explanation for this phenomenon. We believe it is related to the quality of the inoculum, as it is very important to prepare inoculum that includes some corded bacilli in order to achieve reproducibility of the lung lesions [28].

Conclusion: The Importance of Mouse Strain in Testing Host-Directed Therapies

As described above, we believe that the use of the C3HeB/FeJ strain following intravenous challenge with corded Mtb is the most reliable and clinically relevant mouse model for testing HDT, as it provides a robust and reproducible system in which we can ascertain the presence of rapidly progressing human-like lesions with liquefaction. It can provide reliable information for the use of HDT as prophylaxis or as an adjuvant therapeutic tool, by halting the progression of existing lesions. In contrast, the use of C57BL/6 or BALB/c strains does not give this information, as these models provide a sort of tolerant response [7] characterized by the presence of very low levels of inflammation and progressive infiltration of the lung parenchyma primarily with foamy macrophages, with the ability to stabilize the bacillary load in the lung but keeping it very high in the spleen [29, 30]. These findings do not mirror the evolution of TB-induced pathology in humans and may yield results which are not clinically applicable. For example, Byrne et al. showed with the BALB/c model that aspirin and ibuprofen enhanced treatment with pyrazinamide, while aspirin antagonized isoniazid treatment [31, 32]. Another example is the study by Mortensen et al. [33] investigating the use of a Cox-2 inhibitor (celecoxib) in a CB6F1 mice strain, which showed an increase or decrease of the lung bacillary load depending on the challenge route. These results are difficult to interpret in the context of "tolerant" C57BL/6-BALB/c lesions.

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Chapter 19
The Rabbit Model for Assessing Host-Directed Therapies for Tuberculosis

Selvakumar Subbian and Gilla Kaplan

Background

Since the early 1900s, the rabbit (*Oryctolagus cuniculus*) has been used as a model to study mycobacterial diseases, including tuberculosis (TB). Rabbits recapitulate the pathologic features, ranging from active progressive disease to latent TB infection (LTBI), as seen in human patients [1, 2]. The model was established in the 1920s primarily by Lurie; Lurie used *Mycobacterium bovis* (Mbo) and *Mycobacterium tuberculosis* (Mtb) to infect rabbits with known genealogies, of which one was "resistant," and another "susceptible" or "intermediary-resistant" [3]. The inbred "susceptible" rabbits infected with Mbo Ravenel developed progressive lung disease characterized by high numbers of large, cavitary lesions with hematogenous spread of bacilli to other organs. In contrast, the "intermediary-resistant" rabbits showed a spectrum of outcomes, ranging from active, progressive disease to non-progressive infection. Moreover, pulmonary infection of "susceptible" rabbits with the virulent laboratory strain Mtb H_{37}Rv, did not result in the extensive progressive disease pathology seen in the Mbo-infected animals. These observations demonstrated the range of rabbit lung responses to infection with different mycobacterial strains, and also showed that rabbits, similar to humans, are relatively resistant to progressive Mtb infection; nearly 90% of Mtb-infected immune-competent humans do not develop active TB [4]. These studies also set the stage for future work on the genetic predisposition of the host to Mtb infection. Since the extinction of Lurie's original "resis-

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tant" and "susceptible" lineages of rabbits, the out = bred New Zealand White rabbits became the most commonly used in TB research.

Pulmonary *Mycobacterium tuberculosis* Infection in Rabbits

Disease pathology, cellular architecture, and differential evolution of individual granulomas are noted in rabbits with active pulmonary TB, similar to what is seen in human TB [5]. Within the same lung lobe small, non-progressive, "healing-type" granulomas can appear with central mineralization surrounded by a thick fibrotic capsule, while others can be of variable size and at different stages of maturation, including central caseation and cavitation [6]. Moreover, rabbit lung granulomas are characterized by central hypoxia, which is believed to be a crucial micro environment encountered by Mtb in human TB lesions [7].

Exposing rabbits to an Mtb-containing aerosol or inoculating Mtb directly into the airway has been the standard technique used to establish pulmonary infection. We and others have shown that the outcome following infection of New Zealand White rabbits with clinical Mtb isolates depends on the specific nature of the infecting strain and the inoculum dose used for infection [8]. Using a "snout-only" aerosol exposure system, we have reported the kinetics of progressive and non-progressive Mtb infection in the rabbits [6]. Rabbit lungs implanted with about 3.5 $\log_{10}$ colony-forming units (CFU) of the hyper-virulent Mtb strain HN878 had 7 $\log_{10}$ CFU by 4 weeks post-infection. At similar numbers of inoculated bacteria (about 3.5 $\log_{10}$), the initial growth of the hyper-immunogenic Mtb CDC1551 strain was significantly lower (5.5 $\log_{10}$) in the lungs of infected rabbits. Furthermore, the HN878-infected animals sustained a high bacillary load until 12 weeks, before increasing slightly at 16 weeks, when lung cavitation occurs. HN878 infection was associated with bacillary dissemination to the liver and spleen [9]. In contrast, the reduced growth of CDC1551 was maintained until 8 weeks, followed by a further reduction in CFU, until no cultivable bacilli could be isolated from the lungs of infected animals at 16 weeks post-infection, consistent with latent Mtb infection (LTBI) [10]. However, since reactivation of LTBI could be achieved when CDC1551-infected animals were subjected to 4 weeks of treatment with immunosuppressive agents starting at 16 or 20 weeks post-infection, the animals were not sterilizing the infection [10].

The differential kinetics and nature of the rabbit immune response to infection with the different Mtb strains were confirmed by measuring the disease pathology and immune cell activation in the lungs, lymph node, and spleen [9, 10]. These studies established that progressive Mtb infection is associated with exacerbated early inflammation consisting of neutrophils, which interfere with the macrophage-mediated innate immune response, and contribute to a delayed onset of a sub-optimal T-cell response. In this failed host protective response, the replicating bacteria cause destructive disease pathology, including cavitation of the lungs [9, 10]. In contrast, dampened neutrophil recruitment to the site of infection, associated with an early and optimal macrophage response to Mtb infection, facilitated a robust

and efficient T-cell response. In these animals, we observed limited bacterial growth and controlled disease pathology, resulting a in non-progressive disease. Thus, early differential inflammation plays a crucial role in determining the subsequent course of Mtb infection in the rabbit model [11]. This observation also suggests that modulating the host inflammatory response can have an impact on treatment outcomes for TB.

Rabbit Model of Tuberculous Meningitis

The resemblance of clinical signs, disease progression, and the immunopathology between human tuberculous meningitis (TBM) and the rabbit model of central nervous system (CNS) infection was established in the early 1900s [12]. Another classical study compared the pathology of TBM between children and the rabbit model and demonstrated that dissemination/inoculation of Mtb into the meningeal space caused progressive TBM in both the rabbit model and humans [13]. The clinical findings in the cerebrospinal fluid (CSF) in the rabbit TBM model are very similar to human disease, with a predominance of lymphocytes, high protein levels, and viscous CSF. Moreover, enhancement of the basal meninges and hydrocephalus are common anomalies seen both in the rabbit model of TBM and in human TBM cases [14]. The cellular response underlying brain infection by Mtb is also similar between humans and the rabbit model of TBM. The microglial cells play an essential role in eliciting an immune response against Mtb in TBM [15, 16]. These cells produce elevated levels of pro-inflammatory molecules, including TNF-α, IFN-γ, IL-6, IL-1β, CCL2, CCL5, and CXCL10, that can also be measured in the whole blood and CSF of TBM patients [16, 17]. Similar to these observations in humans, infection of microglial cells/macrophages by pathogenic mycobacteria leads to increased production of pro-inflammatory cytokines in the rabbit model of TBM [18].

We have demonstrated that New Zealand White rabbits infected either intrathecally by Mbo Ravenel strain or by the Mtb Beijing strains HN878 and W4 developed progressive disease pathology in the brain, associated with encephalopathy and loss of coordination by 3 weeks post-infection [19]. The infected rabbits had severe meningitis with prolonged inflammation, necrotizing vasculitis, and leptomeningeal thickening. In these animals, a persistently large number of bacilli were observed in the CSF and brain that also disseminated to the lungs and liver with time. In contrast, intrathecal infection with the laboratory strain Mtb $H_{37}Rv$ or the clinical isolate Mtb CDC1551 yielded an attenuated disease pathology in the brain with fewer clinical manifestations and protracted lower levels of bacillary growth [19, 20]. Thus, rabbit TBM displays Mtb strain-specific immunopathology, which can be utilized to expand our understanding of protective versus permissive host responses to this infection.

The current TBM treatment strategy for humans includes the administration of standard anti-TB drugs in-combination with corticosteroids [21]. Even though standard first-line antibiotic therapy is effective in reducing mortality among TBM

cases, it is less effective in curing the disease. This is primarily due to the poor penetration of antibiotics across the blood-brain-barrier (BBB) and exacerbation of inflammation associated with the antibiotic-mediated killing of Mtb. Although anti-TB treatment of rabbits with progressive TBM significantly reduced the bacillary load in CSF and brain, the chemotherapy failed to alleviate the clinical signs and pathology in these animals. Moreover, rabbits with active TBM had severe inflammation in the brain due to excessive TNF-α production that damaged brain function [20].

Host-Directed Therapy Studies in Rabbits

The current treatment regimen for drug-sensitive pulmonary TB includes isoniazid (INH), rifampicin (RIF), prescribed for a minimum of 6 months, and pyrazinamide (PZA), and ethambutol (ETH) used for the first 2 months of therapy. The prolonged duration of multi-drug treatment, combined with drug-induced toxicities, contribute to medical nonadherence and result in the development of multi- and extreme- drug-resistant Mtb strains (MDR and XDR). Adjunctive host-directed therapy (HDT) is an emerging modality in TB treatment, in which small molecule inhibitors fine-tune the host response to Mtb infection. This host immune modulation, in combination with standard antibiotics, can improve the clinical outcome of treatment [22]. This approach has the potential to either shorten the treatment duration and/or improve the lung function of cured TB patients. Since HDT drugs, unlike antibiotics, function by directly perturbing host cell functions, the development of Mtb resistance to these drugs does not occur. Promising data from pre-clinical animal models, including rabbits, have shown that adjunct HDT can improve the clinical outcomes of conventional, antibiotics-based TB treatment.

During the last decade, several structural analogs of thalidomide have been synthesized and evaluated for their potency as selective TNF-α inhibitors [23, 24]. Ultimately, two classes of novel compounds with TNF-α inhibitory activity were identified: Selective Cytokine Inhibitory Drugs (SelCIDs) and Immunomodulatory Drugs (IMiDs) [25]. Both SelCIDs and IMiDs are more potent inhibitors of TNF-α than the parent drug thalidomide. Recently, the rabbit model of pulmonary TB was used to evaluate the inhibitors of host phosphodiesterase-4 (PDE4), an enzyme that converts cyclic AMP into AMP, as an adjunct to antibiotics in improving TB treatment outcome [26]. These molecules have been shown to facilitate the improved killing of Mtb by antibiotics while dampening the inflammation caused by Mtb infection. We have demonstrated that CC-3052 and CC-11050, two of the PDE4 inhibitors (PDE4i) tested, significantly reduced the bacillary load when administered to rabbits in combination with INH, compared to treatment with INH alone [27, 28]. The PDE4i-treated animals also showed reduced TNF-α driven inflammation and dampened macrophage activation in the lungs. Similar observations were reported by others and our group in murine models of TB, which showed the potential of PDE4i in improving anti-TB therapy [29, 30]. Mtb-infected rabbit lungs also

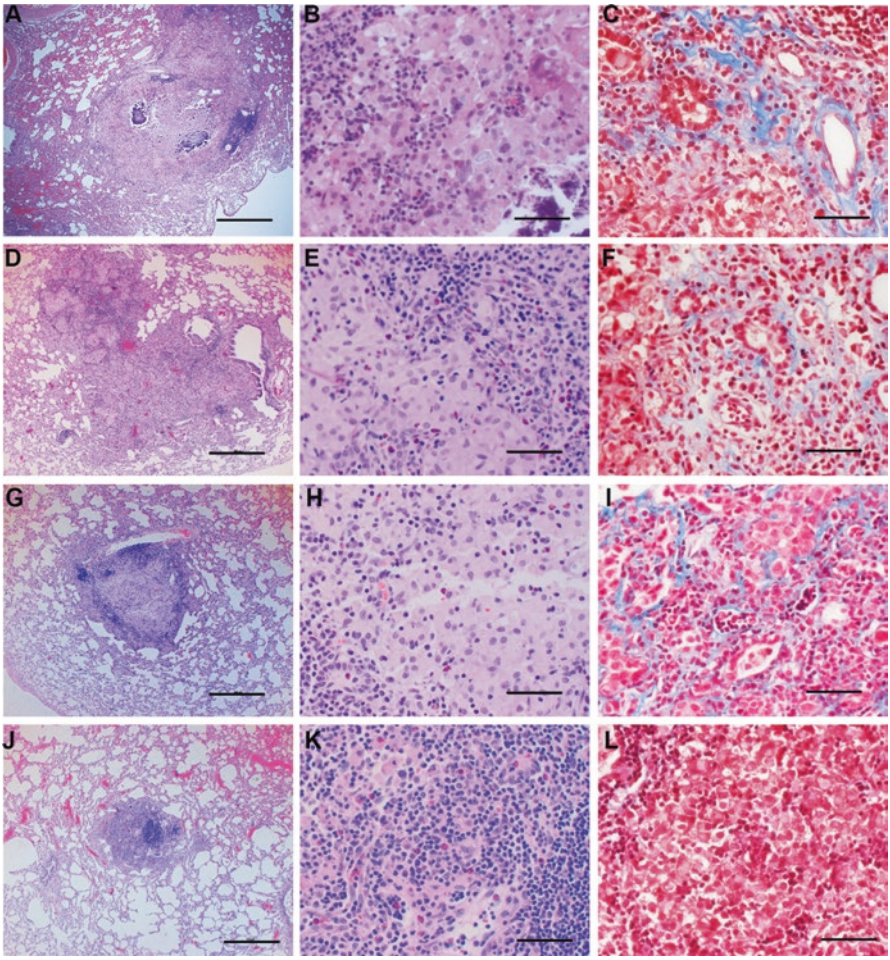

Fig. 19.1 Adjunctive treatment with CC-11050 in combination with antibiotics reduces pulmonary pathology and fibrosis in a rabbit model of tuberculosis. Image shows hematoxylin and eosin- (**a**, **b**, **d**, **e**, **g**, **h**, **j**, **k**) and trichrome- (**c**, **f**, **i**, **l**) stained lung sections of rabbits with pulmonary tuberculosis without any treatment (**a**–**c**) or treated with CC-11050 alone (**d**–**f**) or isoniazid alone (**g**–**i**) or CC-11050 in combination with isoniazid (**j**–**l**). The blue color in **c**, **f**, **i**, **l** shows collagen deposition, a marker of fibrosis. Magnification: 40× (**a**, **d**, **g**, **j**) and 400× (**b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**). Scale bar: 500 μm (**a**, **d**, **g**, **j**) and 50 μm (**b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**)

demonstrated a significant reduction in the fibrotic response during adjunct PDE4i plus INH treatment, compared to infected rabbits treated with INH alone (Fig. 19.1) [27, 28]. These results paved the way for a Phase-II clinical trial in patients with pulmonary TB to evaluate the potential of CC-11050 along with anti-TB treatment in improving clinical outcomes [31].

TBM continues to have unacceptably high morbidity and mortality rates, particularly in young children. The efficacy of the standard use of corticosteroids as

adjunctive therapy for TBM patients is limited. Therefore, there is an urgent need to develop alternative, efficient adjunctive HDT treatment modalities for this devastating manifestation of TB. Our anti-TB drug studies in a rabbit model of sub-acute CNS infection showed poor improvement in the clinical deterioration; about 50% of animals had a progressive neurologic disease and died of disease pathology despite a reduction in bacillary load in the CSF and brain [18–20, 32]. This detrimental disease pathology was associated with elevated TNF-α levels in the CSF. Treatment of these rabbits with an adjunctive IMiD3 improved the clinical outcome and reduced mortality from TBM. The beneficial effect of IMiD3 has been attributed to its ability to dampen TNF-α, IL-1β, IL-6, and other inflammatory cytokines. Additionally, the IMiD3 has been shown to cross the BBB effectively and accumulates in CSF at sufficient concentrations without interfering with the penetration of anti-TB drugs into the CSF [18–20, 32]. Importantly, adjunctive immune-modulating therapy with analogs of thalidomide (IMiD3) in combination with anti-TB drugs not only improved bacillary clearance but also reversed the disease pathology and restored brain function [6, 19]. However, the IMiDs, similar to thalidomide, are teratogenic and therefore are not the treatment of choice for an infectious disease such as TBM. In contrast to the IMiDs, SelCIDs are non-teratogenic, selectively inhibit TNF-α expression and suppress lymphocyte proliferation; they have minimal effect on the production of other monocyte pro-inflammatory cytokines [23]. The efficacy of PDE4i, while evaluated extensively in preclinical models as well as in humans with newly diagnosed pulmonary TB, has not been tested as adjunctive therapy for TBM.

Summary and Conclusion

The rabbit model of pulmonary TB has contributed extensively to our understanding of the pathogenesis of human TB and facilitated the exploration of better therapeutic strategies, including HDT. Molecular genetic tools have been developed to explore the immunological changes in the host immune response during Mtb infection in this model [33–35]. The rabbit model of TBM has also provided a useful tool for expanding our understanding of the pathogenesis of CNS infection and disease. This preclinical animal model can facilitate the selection of optimal candidate adjunctive therapies to control disease pathology and improve the outcome of Mtb infection in the lungs and CNS of humans.

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Part VI
Clinical Trials of HDTs and Special Considerations for Study Endpoints

Chapter 20
Clinical Trials of TB-HDT Candidates

Robert S. Wallis

Introduction

Tuberculosis (TB) drug development proceeds from pre-clinical studies through phase 1, 2, and 3 clinical trials that progressively increase in size and duration. This progression minimizes medical risk to participants and financial risk to sponsors. Trial endpoints typically differ according to study phase (Table 20.1). There is a similar progression of endpoints in HDT trials, but in many trials these have been supplemented by measures of lung function and inflammation, exercise capacity, and symptom scores. In some cases, these novel endpoints have replaced microbiological endpoints as primary study outcomes. The use of these alternative outcome measures is discussed in the sections that follow for each trial.

Trials of Corticosteroids and Anti-TNF Agents

The first wave of TB-HDT studies dates to the early 1960s, in trials of corticosteroids conducted shortly after these drugs were introduced into routine clinical use. Although a review by Dooley in 1997 accurately described this literature as "extensive although largely forgotten" [1], their impact has since undergone a reappraisal. Several of these early studies included spirometry as a trial endpoint [2–4]. Many included assessments of fever, serum albumin, sedimentation rate, cavity closure, and other clinical parameters. One study, which included 5 years of post-treatment follow-up, found fewer relapses and improved long-term survival among steroid recipients [5]. A meta-analysis by Critchley in 2013 of 41 trials reported that corticosteroids reduced on-treatment mortality by 17%, but the finding was heavily

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Table 20.1 TB trial endpoints

	Phase				
	1	2a	2b	2c	3
Typical TB drug trial endpoints					
Safety					
PK					
WBA					
CFU/TTP					
Culture conversion					
Failure/relapse					
Additional HDT trial endpoints					
Immune phenotype and function					
Imaging (PET/CT)		?			
Symptom score					
Spirometry			?		
Exercise capacity					

PK **pharmacokinetics,** *WBA* **mycobactericidal activity in ex vivo whole blood culture,** *CFU* **colony forming unit analysis,** *TTP* **time to positivity in automated liquid culture systems,** *PET* **positron emission tomography,** *CT* **computed tomography**

influenced by effects in patients with central nervous system disease [6]. A meta-analysis by Wallis in 2014 of 12 trials found a dose-dependent effect of corticosteroids on sputum culture conversion, but noted that in only one trial did the effect size approach that required for treatment shortening [7, 8]. In that trial, conducted from 1998 to 2000 in Uganda, 187 HIV-1-infected sputum smear positive TB patients with CD4+ T-cell counts gt;200/μl were randomized to receive prednisolone 2.75 mg/kg/d or placebo for the first 4 weeks of standard TB treatment. The dose was selected in a pilot study as that required to reduce TNF production by half; it is the largest corticosteroid dose studied in patients with TB. At week 4, sputum cultures of 62% of prednisolone recipients had converted to negative, *vs* 37% of those receiving placebo ($P = 0.001$). The effect corresponds to a hazard ratio of approximately 1.83, similar to that seen in patients receiving high-dose rifampin (35 mg/kg/d) in the PanACEA MAMS-TB trial [9]. However, prednisolone at this dose was not well tolerated, with frequent serious adverse events including hyperglycemia, fluid retention, and hypertension.

Only one controlled trial has been conducted to date of a specific anti-TNF agent in patients with TB. In that study, 16 HIV-1/TB co-infected patients received eight doses of etanercept (sTNFR:Fc) 25 mg given twice weekly beginning on day 4 of tuberculosis therapy. The study shared entry criteria and CD4-matched control subjects with the high-dose prednisolone trial described above. Trends towards superior responses to TB treatment were evident in etanercept-treated subjects in body mass, performance score, number of involved lung zones, cavitary closure, and time to

sputum culture conversion. Etanercept treatment resulted in a 25% increase in CD4 cells by week 4 ($P = 0.1$ compared with controls). The change in CD4 cell count was inversely related to the change in serum neopterin, a marker of macrophage activation. There was no effect on plasma HIV RNA.

It is now recognized that etanercept has little effect on granulomatous inflammation in patients with Crohn's disease or sarcoidosis [10, 11], and, compared to anti-TNF monoclonal antibodies, is relatively inefficient in reactivating latent TB infection [12–15]. Although numerous case reports describe therapeutic responses to anti-TNF mAbs in patients with severe or life-threatening TB and TB-IRIS [16–19], no randomized clinical trials of anti-TNF mAbs have yet been conducted.

Aurum TB-HDT Study

In 2014, a workshop jointly sponsored by US NIH and the Bill and Melinda Gates foundation helped transform the field of TB-HDT research [20]. Drawing inspiration from advances of the past decade in cancer immunotherapy, it identified cellular targets and molecular HDT candidates, and charted clinical development pathways. Two main HDT objectives were identified: enhancing the antimicrobial activity of phagocytic cells, thereby shortening treatment; and reducing lung inflammation, thereby preventing permanent lung injury and limiting post-TB morbidity and mortality.

Several funding opportunities emerged from the workshop. The first was an award from the Gates foundation to the Aurum Institute. This phase 2 trial was of an experimental medicine design, with 4 HDT arms: CC-11050 (a type 4 phosphodiesterase inhibitor); everolimus (an mTOR inhibitor); auranofin (an orally bioavailable gold salt with anti-TB and anti-inflammatory properties); and ergocalciferol (vitamin D). It was the first trial specifically recruiting 'hard-to-treat' patients with TB, those at increased risk of poor outcomes based on initial chest radiography or sputum microbiology. In the pre-chemotherapy era, these factors predicted mortality [21]. In the modern era, they predict relapse and permanent FEV1 loss [22, 23]. The experimental treatments were given for the first 4 months of rifabutin-substituted standard therapy (2HRbZE/4HRb), as everolimus and CC-11050 are metabolized by CYP3A4 and are incompatible with rifampin. Adherence was assessed using rifabutin pill counts; patients whose counts indicated <83% of rifabutin tablets taken were excluded from the per protocol population. The study recently completed 12 months of post-treatment follow-up on all participants.

Spirometry Patients in the Aurum study underwent spirometric testing at multiple time points, including on the first day of study participation. Performance was graded according to ATS/ERS criteria [24], with grades A-E considered acceptable. On day 1, all but 1 of 200 patients met this criterion, although more than 30% were grade E, failing to meet a criterion that the two highest volumes differ by no more than 200 ml (required for grade D). This proportion declined progressively during

treatment, dropping below 10% by month 4. A key preliminary finding of the trial, presented as a late-breaking abstract at the 2019 meeting of the American Thoracic Society, was that two treatments, CC-11050 and everolimus, resulted in superior recovery of FEV1 at month 6 compared to control, whereas auranofin and ergocalciferol were ineffective [25]. The difference from control for both CC-11050 and everolimus at month 6 was approximately 200 ml, twice that considered the minimum clinically meaningful treatment effect in COPD [26]. A secondary spirometry analysis restricted to grades A-D reached similar conclusions. The finding that this difference emerged at the end of treatment, 2 months after HDTs had been discontinued, appeared to indicate effects on post-inflammatory airway remodeling. Patients are presently undergoing 1 year of post-treatment follow-up to determine the durability of this effect.

FEV1 is an independent predictor of all-cause mortality in the general population, even in persons with only mild to moderate impairment [27]. In low-income countries where TB is prevalent, mortality risk doubles as FEV1 declines to 70% of predicted, and doubles again as it further declines to 50% [27]. Some of the patients enrolled in the Aurum trial may therefore expect a fourfold increase in long-term all-cause mortality due to permanent loss of FEV1. This prediction is consistent with that observed in a recent meta-analysis of post-TB mortality in which the excess deaths appeared to be due to unexpected cardiovascular and respiratory illness [28, 29]. Interventions to protect the lung in TB may therefore confer significant long-term survival benefits and address important unmet medical needs.

PET/CT ^{18}F-FDG PET/CT scans were performed in the Aurum study on days 1 and 56. Patients underwent whole-body imaging after a single dose of propranolol to inhibit glucose uptake by brown fat [30]. Regions of interest were outlined manually using MIM image analysis software (mimsoftware.com) to include both lungs but exclude mediastinal and other thoracic structures. PET signals are generally quantified as standardized uptake value adjusted to body weight (SUVbw). Three PET parameters were analyzed: total glycolytic activity (SUVbw*ml, where ml refers to the volume of the region of interest), maximum SUVbw (the activity of the single most active spot), and peak SUVbw (the activity of the most active 1 cm sphere). All were log-transformed to improve normality. Radiodensity (CT) was assessed as modified total Hounsfield Units (mHU). The HU scale assigns air a value of −1000 and water a value of zero. This scale was modified by adding 1000 and dividing by 1000, yielding a numerical result similar to SUV. The total mHU of a region of interest was calculated as mHU*ml; it also was log-transformed. Everolimus alone reduced glycolytic activity compared to control; the other treatments, including CC-11050, had no effect. Peak and maximum SUV measures appeared more sensitive than total SUV in detecting the everolimus effect. None of the drugs affected radiodensity, although a trend toward reduced total mHU was apparent for everolimus. This appears to indicate highly treatment-specific effects on PET, possibly related to mechanism of action or types of cells that are targeted.

NAC-TB (N-Acetylcysteine)

Reactive oxygen species (ROS) are generated in excess in TB by *M. tuberculosis*-infected cytokine-activated macrophages and neutrophils. Glutathione (GSH) ordinarily protects tissues against damage by ROS, through the reaction $2GSH + O^{-} \rightarrow GSSG + H_2O$. However, GSH is consumed in this reaction; levels become markedly depleted in blood in TB, both in animal models [31] and in TB patients [32]. The lowest GSH levels have been reported in TB patients who go on to develop TB drug-induced liver injury (TB-DILI) [33]. Cysteine availability is the rate-limiting step in cellular GSH synthesis. Cysteine can be replenished by orally administered N-acetylcysteine (NAC), a WHO-designated essential medicine for the prevention of fatal liver injury after paracetamol (acetaminophen) poisoning. One small study in elderly TB patients found NAC 600 mg BID fully protected against TB-DILI [34]. NAC protects against necrosis in *M. tuberculosis*-infected macrophages *in vitro* and in the lungs of TB-infected guinea pigs [31, 35]. NAC also has been described as having anti-inflammatory and antimicrobial properties [35, 36], although its MIC against *M. tuberculosis* is not achieved in plasma after oral dosing.

The NAC-TB trial is the first to examine the effects of NAC on the lungs in TB. The study is being conducted in 110 Tanzanian patients as an interventional sub-study of TB-SEQUEL, a TB cohort study examining the long-term consequences of pulmonary TB supported by the German Ministry of Education and Research (BMBF). Like the Aurum TB-HDT study, the trial recruits patients with moderately advanced or far-advanced disease by chest X-ray and a heavy burden of infection based on sputum Xpert cycle threshold (Ct). The NAC dose is 1200 mg BID. The study is currently actively recruiting subjects. Unique study endpoints include measurements of GSH and WBA.

GSH Levels of total and oxidized glutathione are measured in whole blood samples using a colorimetric kit (Arbor Assays). Measured values mainly reflect the high concentrations in blood cells. Reduced glutathione (GSH) is calculated as the difference between the two measured values. Baseline measurements show GSH levels approximately one-quarter of those of healthy Europeans.

WBA The bactericidal activity against intracellular *M. tuberculosis* H37Rv is measured in *ex vivo* whole blood cultures [37]. Blood samples are obtained prior to and at intervals after dosing on specified study days. On day 1, blood is obtained before and after the first NAC dose, to assess its direct anti-TB activity. At later time points, NAC and chemotherapy are administered together, to assess the contribution of GSH replenishment and the combined effects of the full regimen.

NAC will also be studied in the panTB-HM trial to be supported by EDCTP. The trial will test three experimental 4-month regimens with a background of bedaquiline and delamanid, plus sutezolid at two dose levels, and sutezolid plus NAC. The experimental regimens will be independently compared to standard 6-month therapy in a phase 2c design. The hypothesis is that by restoring GSH, NAC can prevent

hepatotoxicity and promote recovery of lung function. The proposal is presently in the grant-agreement stage. If funded, recruitment is anticipated to start in 2021.

IMPACT-TB (Imatinib)

Optimal doses of repurposed TB-HDTs may differ from those of their original indications. This is most evident for imatinib. In preclinical studies, maximal antimycobacterial activity of imatinib occurred at exposures approximately ¼ of those used to treat CML, and corresponded to that producing maximum myelopoiesis [38]. Imatinib is metabolized by CYP3A4; although the magnitude of its PK interactions with rifabutin are presumably reduced compared to rifampin, they have not yet been studied.

This NIH-supported project therefore includes two studies. The first, in healthy volunteers, will evaluate the safety, pharmacokinetics, and effects of imatinib on myelopoiesis in adults when given with and without isoniazid and rifabutin. Participants will be enrolled into one of two cohorts, which differ in the order in which drugs are introduced. WBA will serve as an exploratory endpoint. The objective of the study is to select two imatinib doses to advance to a phase 2 study in RIF-S-TB patients, to be given with rifabutin-substituted standard therapy. The dose selection/PK DDI study is presently recruiting subjects at Emory University. The phase 2 trial is anticipated in 2021, to be conducted by the Aurum Institute.

METHOD (Metformin)

This NIH-supported study will examine the safety and preliminary efficacy of metformin in 112 HIV+ RIF-S-TB patients. Efficacy endpoints will include sputum culture conversion, spirometry, chest X-ray, and symptoms, and will include exploratory ‘omics and PK/PD analyses. The project will include 12 weeks of metformin treatment, reaching a dose of 1000 mg daily, using extended release tablets. Enrollment is anticipated to start late in 2020.

DRTB-HDT (AMG-634 and Metformin)

This Horizon 2020 project will examine the effects of CC-11050 (now AMG-634) and metformin in a 3-arm trial in 330 patients with RIF-R-TB. The study will be conducted at clinical sites in Germany, Romania, Moldova, Georgia, Mozambique, and South Africa. TB HDTs will be given for 6 months, and patients followed for 12 additional months. All patients will also receive treatment for RIF-R-TB and HIV (if so infected) as per country and WHO guidelines. The study has two co-primary

endpoints: FEV1 at 6 months (a superiority comparison) and the hazard ratio for stable sputum culture conversion (initially a non-inferiority comparison). Experimental treatments will be considered successful only if both co-primary endpoints are met. All isolates will have phenotypic and genetic drug susceptibility testing, performed centrally. The study is expected to begin enrollment in 2020. The project is led by the Aurum Institute.

SMA-TB (Ibuprofen)

This project, also funded by Horizon 2020, will focus on development of biomarkers to predict response to adjunctive ibuprofen given at two dose levels for the first 8 weeks of TB treatment. Recruitment of 300 subjects is planned at sites in South Africa and Georgia. The primary endpoints will be median time to a reduction in symptom score (TBScore), and the hazard ration for stable culture conversion. The project is anticipated to start in 2020.

Statins as Adjunctive Therapy for TB (StAT-TB) Trial

Preclinical studies have shown that simvastatin adjunctive therapy statin enhances the first-line anti-TB regimen's antimicrobial activity and shortens the time required to achieve cure in a standard mouse model of chronic TB infection (PMID: 24855121; 26903278). Subsequent preclinical studies reported that, relative to seven other statins tested, pravastatin showed the most favorable therapeutic window and exhibited dose-dependent adjunctive activity in the standard mouse model of TB chemotherapy and in a mouse model of human-like necrotic TB lung granulomas (PMID: 31605489). Although exposures of simvastatin and simvastatin acid in humans are greatly reduced (by 90%) by rifampin (PMID: 11180018), pravastatin exposures are reduced by only 30–50% during co-administration with rifampin (PMID: 14748817), and pravastatin can be co-administered with antiretrovirals (with drug and dose selection).

Statins as Adjunctive Therapy for TB (StAT-TB) is a 2-stage, Phase 2b clinical trial. Stage 1 is a 14-day safety/PK study to determine pravastatin exposures over 24 hours when given together with first-line treatment (HRZE) in adults with drug-susceptible, pulmonary TB and to ensure the combination is safe and well-tolerated. Stage 2 is 6-month efficacy study to determine the potential role of pravastatin adjunctive therapy on time to sputum culture conversion and various objective and subjective measures of lung function. StAT-TB began enrolling in March, 2020.

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Chapter 21
Outcomes for Clinical Trials of Host-Directed Therapies for Tuberculosis

Akshay N. Gupte, Sara C. Auld, William N. Checkley, and Gregory P. Bisson

Introduction

Clinical trials of chemotherapeutic drugs for tuberculosis (TB) have primarily studied microbiological outcomes such as culture conversion and declines in bacterial burden in clinical specimens. However, a focus on microbiological cure does not account for pulmonary morbidity associated with TB [1]. Despite cure, up to half of successfully treated TB patients have evidence of impaired pulmonary function [2]. The large global burden of the TB epidemic combined with relatively high rates of treatment completion in drug-sensitive cases mean that millions of adults each year will develop defects in lung health. In addition to functional disability, low lung function has been associated with a higher risk of mortality in large population-based studies. Treated TB patients have higher mortality rate than the general population and post-TB lung disease may be an important contributor [3].

While a major goal of host-directed therapies (HDTs) for TB is to improve the microbiologic efficacy of treatment, an additional goal is to decrease the morbidity and mortality associated with the disease. HDTs already have an established role in decreasing the risk of certain adverse events associated with TB, exemplified by the use of corticosteroids as adjuvant therapy to improve outcomes in TB meningitis [4]. Yet, in pulmonary TB, the most common form of the disease, identifying effective HDTs capable of definitively reducing respiratory morbidity has remained

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elusive. The challenges inherent in identifying such HDTs are substantial. First, the pathogenesis of TB-associated lung injury is unclear, preventing the identification of potentially modifiable immune pathways for intervention. Second, the host immune response to TB is dynamic over the course of the disease and prior pharmacokinetic/pharmacodynamic analysis of TB drugs have suggested quadratic relationships with a time-dependent "U-shaped" curve for the drug-outcome association [5]. Therefore, the optimal timing and dosing strategies of a HDT intervention will depend on the candidate drug and its intended use; e.g., addressing the excess inflammatory response in acute lung injury or addressing chronic lung impairment by targeting pro-fibrotic pathways.

In addition to the complexities of selecting agents capable of safely addressing the inflammation and fibrosis associated with human TB, clinically meaningful measurements of long-term lung health can be challenging to incorporate into the relatively short follow-up period of most randomized trials. However, a variety of assessments of respiratory health could be incorporated into future HDT trials. These metrics are only weakly correlated with each other, indicating that different indices are measuring distinct pulmonary health constructs and that multiple measures should be used for a multi-dimensional assessment of lung health. Even within each category of assessment (e.g., symptom screens), multiple options exist. While this armamentarium of tools provides options, the United States Food and Drug Administration (FDA) guidance for chronic obstructive pulmonary disease (COPD) trials recommends measurement of specific respiratory health constructs likely to be affected by the candidate drug. Yet, the relationships between specific respiratory health measures and the highly heterogeneous lung damage mechanisms of TB remain unclear, making those choice of assessments difficult in practice. Unlike COPD where several lung health measures have been associated with clinical outcomes, including survival, the association of most candidate lung health endpoints for TB HDT trials with long-term clinical outcomes have not been well studied.

In this section, outcomes of respiratory health for pulmonary TB will be discussed to facilitate the development of HDT trials. While a wide variety of conventional and novel lung health metrics are available, the most established objective and subjective measures will be discussed. References to websites with listings of other options will be included to facilitate selection of measures.

Respiratory Health Outcomes

Objective Measures of Respiratory Health

An objective measure is one that is not based on patient perception. This includes, but is not limited to, pulmonary function testing, exercise testing, and lung imaging.

Pulmonary Function Testing

Pulmonary function tests (PFTs) include a combination of evaluations such as spirometry, measurement of lung volumes by plethysmography or multi-breath dilution techniques, and diffusion capacity of the lungs for carbon monoxide (DLCO). Together, these tests can quantify airflow dynamics, lung volumes, and alveolar physiology, and are commonly used objective measures of respiratory function in clinical trials of new treatments for most chronic lung diseases, including asthma, COPD and idiopathic pulmonary fibrosis (IPF). The American Thoracic Society (ATS) and the European Respiratory Society (ERS) have published detailed guidelines on the conduct and interpretation of PFTs. These are critically important because the measurements are both effort- and operator-dependent [6–10]. PFTs can provide both absolute values for airflow and lung volume, e.g., liters/second and liters, as well as percent of predicted values, (absolute values as a percent of expected values from apparently healthy individuals of the same age, sex and height). In addition, distribution-based methods such as z-score standardized lung function parameters and the lower limit of normal (LLN), defined as the fifth percentile of a lung function parameter using reference equations from apparently healthy populations, are widely used [11]. The main outcomes of spirometry testing, are the forced expiratory volume in the first second (FEV_1) and the vital capacity (VC), typically measured as a slow vital capacity (SVC) or by forced respiratory maneuvers (forced vital capacity [FVC]). Lower FEV_1 relative to VC (i.e. the FEV_1 to VC ratio) is suggestive of airflow obstruction while a lower VC is suggestive of restriction [7]. In diseases with airflow obstruction as the primary pathology, such as in COPD and asthma, the change in FEV_1 in response to therapy and the FEV_1 at a certain time point after randomization are considered potentially important and appropriate outcomes by the FDA. In restrictive lung diseases characterized by difficulty filling the lungs with air, such as in interstitial lung diseases or systemic sclerosis, change in VC in response to therapy or the VC at a certain time point of follow-up are established trial endpoints.

Numerous additional measures can be obtained from PFTs, such as the peak expiratory flow rate (PEFR), total lung capacity (TLC), and DLCO. Additionally, the magnitude of change in FEV_1 after administering a short-acting bronchodilator and the post-bronchodilator FEV_1/VC ratio can help in identifying "fixed" airflow obstruction and inform clinical decisions for long-term bronchodilator therapy among treated TB cases [7]. In addition to being validated with long-term clinical outcomes such as disease exacerbations, hospitalizations, and survival in multiple chronic lung diseases, certain PFT metrics, such as FEV_1, have minimum clinically important differences (MCIDs) established for respiratory conditions, most notably COPD [12]. MCID data are required for appropriate sample size calculations. Specifically, trials should hypothesize that a candidate HDT improves a parameter by the MCID or more, in order to increase chances that use of the HDT will be utilized by clinicians.

Several challenges remain to using PFTs in HDT trials in pulmonary TB. Perhaps most notable, is the marked heterogeneity of TB disease manifestations in the lung. This heterogeneity can result in reduced FEV_1, FVC, both, or neither at initial presentation and/or treatment completion, complicating reliance on any single measure for HDT trials [13]. For example, if a study uses FEV_1 as the primary outcome due to a hypothesized effect on airflow, and at randomization half of enrolled patients have defects in FEV_1 and another half have defects in FVC, then the study may be underpowered to detect the proposed effect size in difference in FEV_1. While an obvious way to address this would be to only enroll patients with specific patterns of lung function impairment, this complicates trial enrollment and compromises generalizability of study results. Furthermore, conducting high-quality PFTs in patients with newly diagnosed TB is challenging due to coughing and other aspects of chronic illness, such as cachexia and generalized weakness. Strict infection control practices should be observed while performing PFTs in TB cases to ensure patient safety and prevent nosocomial TB transmission. PFTs can also change rapidly during TB treatment, and defects observed at the time of diagnosis may be markedly different a few weeks after treatment initiation. In addition, lung function likely continues to evolve after TB treatment completion. While some studies suggest stabilization during the 6 months after cure, others have suggested progressive and accelerated lung function declines beyond 6 months after cure in patients with a history of TB [14]. However, these data come from a few selected studies, and the generalizability of these findings are uncertain. Thus, the optimal timing of endpoint assessment using PFTs is unknown. Also, in pulmonary TB, specific PFT metrics have not been validated with long-term clinical outcomes, such as progressive lung disease and survival. Finally, PFT variability can relate to various issues, including the degree of encouragement given to patients during the procedure and the number of spirometry trials performed at a site, indicating that multi-site studies must develop strict procedural standardization to minimize variability in PFT performance and quality across institutions. Of note, these strengths and weaknesses are also broadly relevant to physiologic assessments in non-pulmonary disease, such as in echocardiographic assessments of heart function and flow in pericardial TB.

In summary, PFTs, as objective physiologic assessments of organ function, exemplify an important outcome in HDT trials. However, the long-term clinical relevance and the complexity of lung function assessment over time in TB complicates their use and interpretation. Limitations of current data in TB result in a need to extrapolate the rationale for PFT inclusion as trial endpoints from other chronic lung diseases, which remains to be validated. To address this gap, including PFT assessments in clinical trials, in coordination with other measures of respiratory health, is needed to build the evidence-base relating lung function measurements to overall health and pulmonary disease outcomes. Pairing lung functional assessments with symptoms, biomarkers of inflammation and fibrosis can help inform mechanisms of lung damage in TB, facilitating future investigations.

Exercise Testing

PFTs aim to assess lung function directly, but lung function does not necessarily predict physiologic limitations that relate to respiratory health. In TB studies, correlations between lung function assessed by PFTs and exercise testing are often modest at best. In addition, TB, even when confined to a single organ system, is a disease characterized by systemic inflammation, endocrine disorders, catabolic metabolism, fatigue and musculoskeletal wasting. Measurement of lung function in the resting state may fail to capture adverse effects of TB on organ systems other than the lung, as well as potential extrapulmonary benefits of HDTs. Clinical exercise testing aims to provide a more holistic and dynamic evaluation of patients during submaximal and peak exercise efforts. While exercise testing in isolation (i.e., without corresponding questionnaires) cannot produce information specifically about pulmonary function, it does provide a global assessment of the patient during the specified effort. As a result, tissue involvement at any anatomic site, including in the pulmonary, cardiovascular and musculoskeletal systems, can affect test results. These tests range from simple assessments meant to reflect activities of daily living (e.g., stair steps) to strenuous and sophisticated protocols involving treadmills and measurements of blood gas oxygenation, metabolism and cardiac function. One practical, simple and common exercise testing procedure used in chronic lung disease is the 6-minute walk test (6MWT), which evaluates the distance (6MWD) patients can walk during that time interval while exerting a self-paced, submaximal effort on a flat, hard pre-specified track [15]. The 6MWT can be further enhanced to measure point-of-care oxygen saturation (spO_2) before and after the test, providing a simple assessment of lung physiology.

Similar to PFTs, exercise capacity testing is attractive as another type of objective outcome in TB HDT trials, in that lower capacity is associated with clinical outcomes in various chronic lung diseases and congestive heart failure, and MCIDs for several pulmonary conditions have been studied [16]. In addition, the ATS/ERS and other medical societies have published detailed guidelines for exercise testing, which facilitates standardization of procedures [17]. Furthermore, simple tests such as the 6MWT can be done in the clinic, are acceptable to patients, and are suitable for resource-limited settings. Exercise capacity is also relevant to TB, as many patients with pulmonary TB have decreased 6MWD values at diagnosis and lower exercise capacity tends to correlate with the extent of radiographic involvement of the lung. Furthermore, TB treatment is associated with improvements in the 6MWD, and some adults have abnormally low 6MWD values after TB treatment completion [18]. Moreover, exercise capacity testing is conceptually important as, unlike PFTs, it measures capacity for activity that has inherent clinical relevance to patients.

However, many of the same limitations and complexities related to use of PFTs extend to exercise capacity testing. First, 6MWD measurement has not been associated with long-term clinical outcomes such as survival in TB. Second, while 30 m has been proposed as the MCID for the change in 6MWTD in COPD (improvement or decline), these data were derived generally from older male cigarette smokers,

who likely do not reflect the exercise capacity or symptomatic experience of younger patientstypically affected by TB globally [16]. Studies of sub-maximal exercise capacity in patients with pulmonary TB, who are often in their 20s and 30s, may find "ceiling" effects [19]. Third, as for PFTs, exercise testing is both operator/supervisor and patient–dependent. Even minor changes to the protocol or encouragement are associated with measurable differences in distance walked. Thus, careful standardization across sites and over time within sites is a critical issue. Finally, the 6MWT may not be appropriate for some forms of extrapulmonary TB, such as musculoskeletal or central nervous system disease that may directly impair ambulation. Conversely, as a global assessment affected by numerous physiologic factors, 6MWT may be insensitive to the effects of HDTs that target highly organ-specific mechanisms. For example, an HDT that only affects ciliary function in the lung may have a disproportionately low impact on 6MWT distance.

Despite these limitations, carefully conducted exercise testing appropriate to the population under study should be employed as complimentary measures to other assessments of health in TB HDT trials. As with PFTs, these measures ideally will be paired with symptom and biomarker assessments to inform mechanisms of exercise capacity limitations, which in turn should inform both biological plausibility of any observed effects as well as the design of future studies.

Lung Imaging

Chest radiography (CXR) is widely used in TB clinical care and research, and can be assessed qualitatively and quantitatively. Qualitative assessments include the presence or absence of lung cavities, mediastinal lymphadenopathy, miliary involvement, type and number of lung zones affected, and other measures. Several studies have developed quantitative CXR scoring systems comprised of the percent of lung involvement and the number and size of cavities. For instance, the Timika CXR score divides the lungs into several zones and the percent of lung involvement is expressed from 0% to 100% with an additional 40 points for cavitary disease. The total Timika CXR score therefore ranges from 0 to 140 points [20]. However, there are important aspects of the CXR to consider prior to its use as a clinical trial endpoint. First, CXR assessments have substantial between- and within-reader variability, and require standardized protocols with at least two independent readers and further consensus adjudication to ensure high quality data. Second, CXRs lack sufficient resolution to identify structural changes in the lungs. For instance, CXRs may be unable to differentiate between airway or parenchymal pathology, and can be relatively insensitive to changes over the course of treatment. Finally, quantitative CXR scores correlate weakly with PFTs or other functional outcomes and their role in measuring long-term pulmonary morbidity remains unclear [18, 21]. Nevertheless, CXRs are easy to perform, inexpensive, and widely available, making them an appealing outcome measure for HDT trials in resource limited settings.

Multi-detector computed tomography (CT) has provided novel insights into the pathophysiology of chronic lung diseases and, has been widely used in patient

management and research. However, the use of CT imaging in TB research, especially in clinical trials, has historically been low. Analysis of CT imaging can be performed qualitatively, semi-quantitatively, or quantitatively using novel metrics. Qualitative assessment is done by trained radiologists using the Fleischer Society Guidelines to minimize variability and standardize outcome reporting [22]. Semi-quantitative assessment of CT images can be done by measuring the radio-density of lung tissue expressed in Hounsfield-units (HU), where water is arbitrarily assigned 0 HU and air is −1000 HU. Using these benchmarks, diseased lung parenchyma can be assigned a HU score, which can be aggregated over lung zones to provide a global scoring system [23, 24]. CT images can then be semi-quantitatively compared across intervention arms or within the same individual over time. Quantitative CT assessments include measuring large and small airway wall thickness, emphysema scores, and parametric response mapping (PMR) i.e. 3-dimentional comparison of the lung architecture between end-inspiratory and end-expiratory scans [25, 26]. Quantitative CT analysis, while providing an unprecedented level of detail, is relatively new and is yet to be implemented in clinical practice. However, several large COPD studies have demonstrated associations between quantitative CT measures and clinically relevant health outcomes such as lung function decline, exacerbations, hospitalization, and mortality [27–29]. Overall, CT imaging can identify changes to the lung architecture and can serially quantify the evolution or resolution of lung pathology over time. Within the context of TB HDT trials, CT imaging can be used to describe the structural phenotype of TB-related lung injury and quantify its resolution in response to an intervention. Importantly, CT findings often precede overt symptomatic disease, enabling the study of sub-clinical or incipient phenotypes of lung disease [30, 31].

However, CT imaging is not without its challenges. CT facilities are expensive and may not be widely available at the point of care, especially in areas where TB burden is high. Analysis of CT images requires computationally sophisticated image analysis software. Further, appropriate safety and radiation dosing protocols should be observed while performing serial evaluations with high-resolution CT imaging during TB treatment to minimize radiation risk. Finally, MCIDs of semi-quantitative and quantitative CT metrics have not been established. Therefore, the most relevant key CT metrics and their quantitative differences to TB-associated lung injury are unclear. Nevertheless, CT imaging remains a promising avenue of investigation and a potentially important pulmonary outcome for TB HDT trials.

An added advantage of CT imaging is with the use of PET tracers to measure pulmonary inflammation. The most widely used tracer is fluorodeoxyglucose (FDG-18), a marker of cellular activity extrapolated to function as a non-specific marker of inflammation. Glycolytic activity on FDG PET-CT has recently been shown to be associated with pulmonary function and symptoms in adults with HIV/TB and provides a quantitative readout of local lung inflammation that may be useful in HDT studies [32]. Newer PET tracers that specifically bind to TB bacilli, drug molecules, and key inflammatory cells are currently under investigation and may prove to be promising and highly specific outcome measures in future HDT trials [33].

Subjective Measures of Respiratory Health

Subjective measures rely on patient's ability to report how they feel or perceive their symptoms and health status. These include symptom questionnaires and quality of life assessments.

Quality of Life Questionnaires

The most holistic of these subjective measures relate to quality of life, defined as "an individual's satisfaction or happiness with life in domains he or she considers important." Within quality of life, there are numerous domains, including health. Health-related quality of life (HRQoL) is itself comprised of several distinct domains as they relate to illness (or its absence). Low HRQoL has been related to TB disease symptoms and severity, and therefore HRQoL plausibly could be improved by HDTs for TB. Moreover, although TB symptoms often improve rapidly on treatment, a history of TB and attendant pulmonary morbidity are associated with reductions in quality adjusted life-years. Thus, measurement of HRQoL is a potentially important outcome of TB HDT studies as it can provide insights into the possibility that these novel therapies reduce the impact of a disease on an individual's life.

A variety of tools are available to assess HRQoL, with a comprehensive list available on the ATS website [34]. Existing measures include instruments evaluating overall health (e.g., the SF-36 and the shorter SF-12) and instruments assessing specific organ systems or diseases, such as the Modified Medical Research Council dyspnea scale (mMRC), Chronic Respiratory Disease Questionnaire (CRQ), the COPD Assessment Test (CAT), and the St. George's Respiratory Questionnaire (SGRQ) [35, 36]. Symptom scores, such as the Borg dyspnea scale, are more focused and generally do not attempt to assess overall impact of disease on patient health. Respiratory disease-specific instruments such as the CAT and the SGRQ capture the patient-graded severity of symptoms such as cough, sputum production, and wheezing, in addition to relating disease status to the patient's general perception of well-being. Additionally, CAT and SGRQ are widely used in clinical practice to determine therapeutic strategies and prognosis [37]. These instruments are therefore plausibly important in pulmonary TB, are generally easy to administer to patients, have MCIDs determined in other pulmonary diseases (e.g., 4-points or more for the total SGRQ score), are responsive to interventions, and have been associated with long-term outcomes such as survival in COPD [38]. Importantly, in contrast to both PFTs and exercise testing, HRQoL can be obtained even from patients with very severe disease as long as they can communicate with study staff. Furthermore, HRQoL scores often correlate only weakly or moderately with PFT and exercise testing results, suggesting that they are measuring distinct characteristics of pulmonary disease not captured by physiologic measures. Finally, the more established instruments have been translated into numerous languages.

The major limitations of PFTs and exercise testing – a lack of data validating scores with survival and uncertain MCIDs in TB – also apply to these tools. In addition, CAT and SGRQ scores, being subjective assessments, can vary within patients during TB treatment and make the ideal timing of data collection unclear. More fundamentally, HRQoL instruments have not been developed in TB and their interpretation must be extrapolated from other diseases. Despite these limitations, the FDA considers various HRQoL measures as valid and important outcomes for trials of new drugs for COPD and IPF, and numerous registration trials have incorporated these measures as primary or secondary endpoints. Moreover, the systemic nature of TB-associated inflammation supports the approach of measuring global effect of disease. Furthermore, respiratory questionnaires are sensitive to change in disease status over a relatively shorter duration of follow-up, such as the course of standard TB treatment, and are an important step toward patient-centered outcomes in clinical care [39, 40]. Thus, trials of TB HDTs should also include one or more of these measures.

The ATS website has extensive lists of available instruments [34]. Limitations discussed above for both objective and subjective measures apply to these measures as well.

Microbiological Outcomes

The importance of lung injury in TB is not limited to post-TB lung disease. Lung cavitation has been associated with poor drug penetration and higher risk of poor microbiological outcomes, including recurrent disease [41, 42]. Conversely, rapid reductions in bacterial load may reduce the host inflammatory response and limit tissue damage in the lungs secondary to lower antigenic stimulus. Therefore, TB clinical trials should comprehensively evaluate pulmonary outcomes in concert with conventional microbiological endpoints. Microbiological outcomes for TB HDT trials have been discussed in previous chapters of this book. Here, we will briefly summarize key microbiological outcomes that should be considered along with respiratory outcomes for TB HDTs.

Early Bactericidal Activity

Early bactericidal activity (EBA) is a commonly used outcome for early phase (Phase-IIA) trials of new TB drugs. EBA of experimental drugs is assessed by comparing the rate of decline in bacillary load during the first 14 days of intervention. Bacillary load is measured by the colony count on Lowenstein-Jensen (LJ) solid culture or time to culture positivity on Mycobacterial Growth Indicator Tube (MGIT). More recently, *Mycobacterium tuberculosis* DNA amplification cycle thresholds (Ct) from the Xpert MTB/Rif assay have been used as a surrogate

marker for bacterial load. However, EBA poorly correlates with long-term microbiological outcomes and recent studies have found minimal overlap between Phase-IIA and Phase-IIB/III regimens. Furthermore, 14 days may be a relatively short duration of exposure to a HDT intervention to detect any meaningful antibacterial effect. Nevertheless, EBA outcomes continue to be used as endpoints in early phase trials [43].

Time to Culture Conversion

Comparing the proportion of participants with negative cultures at 8 weeks of treatment has widely been used as an endpoint for Phase-IIB trials. Culture conversion at 8 weeks is simple to use and, historically, has been shown to correlate with long-term outcomes such as relapse-free cure [44, 45]. However, recent Phase-III trials of fluoroquinolone-based regimens for TB treatment shortening found higher relapse rates in the fluoroquinolone arm compared to standard-of-care despite promising Phase-IIB data on 2-month culture conversion [46]. Further, the ability of 2-month culture conversion to predict long-term outcomes may be different for regimens without rifampicin and is likely influenced by the drug-class under investigation. Finally, the binary endpoint of proportion with 2-month culture conversion is distinct from the quantitative endpoint of EBA common in Phase-IIA trials. This has prevented the linkage of drug efficacy across the early phase trials. To address these limitations, newer approaches have employed intensive sputum sampling during early treatment to assess the time to culture positivity and time to stable culture conversion as alternative endpoints for Phase-IIB trials [47]. Such quantitative approaches, while lacking long-term validation with relapse-free cure similar to 2-month culture conversion, have facilitated the design of more efficient trials requiring smaller sample sizes.

Treatment Failure and Recurrence

The primary microbiological outcomes for Phase-III trials of TB drugs are treatment failure and recurrence, measured independently or as a composite endpoint along with mortality. Treatment failure is typically defined as microbiological evidence of TB disease during the final 1 or 2 months of treatment. In the absence of microbiological confirmation, clinical failure judged by the presence of symptoms and signs suggestive of TB disease is considered an unfavorable outcome. Recurrent TB disease as a primary endpoint is typically measured during the 12 months after treatment completion and is defined as microbiological evidence of TB disease among individuals who did not fail treatment. TB recurrence can be further divided into relapse (i.e. resurgence of the same *M. tuberculosis* strain as the initial TB episode) or re-infection (i.e. infection with a new *M. tuberculosis*

strain) using strain genotyping. At least 18 months of follow-up after randomization is recommended to ascertain these microbiological outcomes in Phase-III trials. However, relatively low rates of relapse and long durations of follow-up, both requiring large sample sizes to offset, are practical challenges to conducting robust Phase-III trials.

Mortality

Death is the ultimate outcome in clinical research. All-cause and TB-specific mortality should be assessed in TB HDT trials. Since poor lung function, especially FVC, has been associated with a higher risk of mortality, careful mortality ascertainment will be particularly important for HDTs that aim to prevent or limit TB-associated lung injury [48, 49].

Putting It All Together: A Conceptual Approach

While there are limitations to the use of objective and subjective measures in characterizing respiratory outcomes in TB, a thoughtful incorporation of selected tools into TB HDT trials will considerably enhance the value of these studies beyond microbiologic assessments alone. For example, if a trial fails to demonstrate a shorter time to culture conversion, but meaningfully improves PFT parameters and HRQoL, the HDT would still present a compelling case for consideration in clinical care. Pasipanodya et al. documented that most of the reduction in HRQoL associated with TB is not due to TB-associated mortality but rather to pulmonary impairment after TB treatment completion, underscoring the importance of these measures. But how do researchers choose which measures to include, and when and how often are assessments done? The following approach is meant as a guide to aid in future study design.

1. *Consider mechanism-outcome associations:* As mentioned above, and similar to what has been emphasized in FDA guidance on evaluation of new COPD drugs, HDT trials in TB should link outcomes with proposed biologic mechanisms wherever possible. For example, candidate HDTs that appear to affect autophagy theoretically should decrease inflammation by rapid clearance of the pathogen, but could also increase paradoxical reactions if enhanced pathogen killing results in immune activation. Thus, a trial could enroll patients with a certain degree of pulmonary damage radiographically at baseline, and follow-up could conduct serial PFT assessments, CT imaging, FDG PET-CT, and mechanistic biomarkers or *in vitro* assays as endpoints. While some patients would not produce usable PFT data at baseline due to cough or severe illness, the majority would likely be able to complete the assessment at later time

points, allowing for an evaluation of imbalances in lung function across randomized arms. While precise mechanisms for many agents may not be known, this effort should facilitate development of rich datasets to enable meaningful exploratory analyses.

2. *Understand sample size calculations and MCIDs:* As mentioned above, MCIDs in pulmonary diseases other than TB have been proposed for many assessments discussed above. Sample sizes for HDT trials should be based either on published data from TB trials that become available in the future, on pilot data specifically collected for the proposed HDT study or, least desirable, on analogies drawn from pulmonary diseases other than TB. Numerous reviews of the process of derivation of MCIDs for various relevant measures have been published. Briefly, the MCID is the smallest difference in the proposed primary endpoint associated with an effect that is either perceived by the patient as important or with a future clinically relevant outcome, such as hospital admission or death. One example of this approach, in absence of published or pilot data in TB, would be to use the MCID of the SGRQ of 4 as established in COPD, in a trial hypothesizing that a candidate HDT improves HRQoL. The ATS website has an useful list of MCIDs for various tools, with references [50].
3. *Consider timing of endpoint assessment:* Existing data suggest that tissue damage from TB may accelerate the trajectory of lung function decline over time, with greater declines observed over 1 year after treatment completion [14, 51, 52]. Therefore, collection of endpoint data could focus simply on evaluating outcomes at, for example, 12 months after candidate therapy completion. However, HDTs under study may initially be given for much shorter periods, such as for 8 or 12 weeks, when microbiologic and safety endpoints are typically evaluated in phase-II trials. Assessing changes in pulmonary endpoints in response to HDT at earlier timepoints and subsequently at the completion of HDT therapy could provide valuable data supporting an HDT's beneficial (or harmful) effects. Future studies could evaluate such drugs when given over longer intervals, if benefits are observed during drug administration.
4. *Consider multiple pulmonary outcomes:* As discussed above, pulmonary impairment in TB is highly heterogenous and may continue to evolve during and after treatment. While choosing one primary outcome measure can simplify study design and analysis, a multi-dimensional assessment of respiratory health should be considered as secondary outcomes. Such an approach has the potential to capture the full spectrum of TB-associated lung disease, which otherwise may be missed with isolated tests. We propose a conceptual framework for multi-dimensional respiratory health assessments in Fig. 21.1.

Summary

Potential endpoints for HDT studies of TB include clinical outcomes and surrogate markers using both objective and subjective assessments. Despite gaps in our understanding of how these endpoints perform in the context of TB, measurement of

Fig. 21.1 Conceptual framework for a multi-dimensional assessment of respiratory health in TB

these physiologic and patient-oriented outcomes can enhance the scientific output of HDT trials, increasing the knowledge base for future HDT development.

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Chapter 22
Pharmacological Considerations for Clinical Trials of Host-Directed Therapies for Tuberculosis

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Pharmacologic Considerations for HDT

Pharmacokinetic-Pharmacodynamic and Pharmacokinetic-Toxicodynamics in Clinical Trials: General Considerations

The pharmacokinetics (PK) of a drug is commonly explained as 'what the body does to the drug' and is typically described in terms of drug disposition—that is: absorption, distribution, metabolism, and excretion (ADME) of the drug. Pharmacologists are keenly interested in characterizing sources of variability in the parameters used to describe PK, either the primary parameters (rate of absorption (k_a), volume of distribution (V_d) and clearance (CL)) or the more intuitively understood secondary PK parameters (maximum concentration (Cmax), area under the concentration-time curve (AUC), or time above a parameter of interest for that drug (e.g., time above minimal inhibitory concentration (MIC), T > MIC). Linking PK to pharmacodynamics (PD), loosely 'what the drug does to the body,' or drug effect, is critically important for understanding exposure-response (or PK-PD) relationships. As all drugs are potential poisons, rational drug use and dose optimization must also take into account relationships between PK and toxic effects, or toxicodynamics (TD). A full characterization of PK-PD and PK-TD relationships helps define the therapeutic index, defined by Encyclopaedia Britannica as the 'margin of safety that exists between the dose of a drug that produces the desired effect and the dose that produces unwanted and possibly dangerous side effects.'

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Challenges in Assessing PK-PD and PK-TD Relationships in HDT

In tuberculosis (TB) disease, assessing PK-PD relationships and employing them in decision-making along the drug development pathway can be challenging for several reasons. First, there is significant heterogeneity in patient characteristics and, therefore, responses to treatment, owing to differences in disease presentation (e.g. large lung cavities, with reduced blood supply and/or large numbers of *Mycobacterium tuberculosis (M.tb.)* bacilli) and in immune contribution to cure (e.g. HIV or diabetes mellitus related effects). Second, surrogate microbiologic markers of treatment response such as time to sputum culture negativity correlate imperfectly with relapse-free cure [1]. At some point during treatment, the patient stops producing culture-positive sputum, but live 'persister' bacilli are still present. This sub-population may not grow using standard culture methods and are the hardest to kill. Lastly, drugs are given in combination, and one must disentangle the contributions of individual drugs and quantify synergies or antagonisms between drugs. Despite these issues, assessing *antimicrobial* agents for TB is relatively straightforward—the approach is to link plasma PK (or site-of-disease PK, see below) with anti-mycobacterial activity (change in sputum bacterial load and MIC) at similar points in time over the course of treatment. Additionally, experts agree that carefully collecting drug concentrations (PK) and conducting PK-PD and PK-TD analyses are critical to effective drug development [2, 3].

For HDT, these relationships are more complicated [1]. There may be a delay between drug administration and its effect, depending on the mechanism of action of the drug [2]. The effect of the drug may be indirect, affecting inflammatory processes which, in turn, impact drug distribution to the site of disease [3]. HDT agents may have unpredictably complex, non-linear dose/effect relationships [4]. MIC is of limited relevance for HDT agents, and so PK-PD assessments will not include specific information about the susceptibility profile of the infecting strain (e.g. AUC/MIC or Time > MIC) [5]. The drug may affect different clinical outcomes, such as lung function, reinfection, and death, compared to microbiologic measures important for antimicrobial agents [6]. The timing of administration of HDT agents is critical during the course of treatment, and identification of the optimal window is not always straightforward [7]. Adverse effects will not typically result from direct effects of the drug on target organs but rather from effects of intermediary effector cells on that organ. PK-PD and PK-TD approaches must be adjusted from the outset to account for these differences, lest important drug toxicities be missed (See below).

Translational Modeling: Can this Help Us?

In Chap. 5, we learned about preclinical approaches to understanding HDT, from *in vitro* granuloma formation to *in vivo* small and large animal models. While one would expect immune responses and, therefore, responses to immunomodulators to differ among species, when the influence of cytokines and other inflammatory

markers on TB outcomes is known and is present in both animal models and humans, harvesting data from preclinical models to inform PK-PD models in humans can be useful [4–6]. One must recognize though, that metabolism differs between mice and humans, so PK-PD translation must take into account differences in parent and metabolite ratios. The main metabolite of simvastatin, for example, is active against intracellular *M.tb* and circulates at high concentrations in mice, but not humans [7]. Preclinical assessments may give a general sense of the PK parameters that correlate best with treatment response (e.g. Cmax or time above a relevant concentration), and this information can be used to guide design of PK components of clinical studies. Recent murine models have demonstrated the potentiating effect that matrix metalloproteinase (MMP) inhibitors have on first-line TB drugs [8]. Inflammatory markers, changes in cell populations, and surface markers that correlate both with drug PK and with treatment effect in preclinical models can be used to guide choice of surrogate outcomes to measure in clinical studies. The potency of various promising PDE4 inhibitors to lessen inflammation caused by tuberculosis and potentiate the effect of isoniazid was first demonstrated in a series of murine and rabbit models, which later informed the dosing selection for human trials [9, 10]. In some cases, PK-toxicity correlates are similar in animal models and humans and can be used to guide PK-TD components of human trials [11]. Some toxicities, in theory, can be predicted with organ-on-a-chip technologies [12]. For drugs early in development for which clinical safety has not been established or for repurposed drugs with known and narrow therapeutic margins, exposure-response in animals may help eliminate drugs for clinical development wherein exceptionally high drug exposures are needed to see modest effect.

Multiscale models that include interlinking complex information about organism, host characteristics, drug mechanisms and effect, and time are needed, with translational components that allow us to bridge preclinical and clinical data. Some progress with this is being made with TB therapeutics generally, employing data science to develop models that include mycobacterial growth function, adaptive immune response effect on bacterial growth, specific patient pathology, and drug effect [13]. Adding in HDT effects, both on host immunology and pathology and bacterial growth or death, can be helpful insomuch as the immune mediators are well-characterized and included in the system. Organoids (eventual organ-on-a-chip technology with three-dimensional complex human cell granuloma that includes all relevant immune effector cells) coupled with mathematical modeling may not just be theoretical [14]. However, effects of HDT on immune effector cells and immune function in model systems will not exactly parallel what is seen in humans. As similar PD markers are measured in clinical studies as were studied in preclinical systems, these translational models can then be iteratively adjusted to incorporate new information and updated to improve preclinical-clinical links.

Strategies to Consider (Learning from HDT in Other Therapeutic Areas)

Immunotherapy has transformed cancer therapeutics, and this success provides the best example to date of the use of integrative pharmacology to guide preclinical work, clinical translation, patient selection, dosing, and even combination regimens for host-directed therapeutics [15, 16]. Modulation of immune pathways, whether inhibitory or stimulatory, is the intended outcome of immunotherapy, and explanatory PK-PD evaluations in immuno-oncology take into account target expression profiles, checks and balances in immune regulation, complexities of the tumor microenvironment, and acquired resistance [15, 16]. This type of modeling is sophisticated and requires full mechanistic understanding of drug effect via its effects on the immune system. However, less complex models may still yield important information provided the desired clinical or biomarker effect is defined and measurable. Examples include immunotherapy for treatment of allergen-associated asthma (endpoint as frequency of rescue inhaler use) [17] and monoclonal antibodies for rheumatoid arthritis (indirect PK-PD modeling using the endpoint of c-reactive protein (CRP), a correlate of disease activity, and direct PK-PD modeling using the endpoint of disease activity score in 28 joints (DAS28), a patient-relevant clinical outcome) [18]. Cases in the field of infectious diseases are also illustrative. For hepatitis C virus (HCV) infection, for example, plasma HCV levels drop below the limits of detection long before the virus is eradicated from the liver. Development of a comprehensive viral kinetic model that explained cure was a prerequisite for successful drug development [19]. Based on this, a model was built that incorporated early viral kinetics and PEG-IFN concentrations to estimate the concentration that decreases HCV production by 50% (the EC50) for each individual patient, and the EC50 was predictive of the desired outcome, sustained virologic response [20]. In hepatitis B virus (HBV) infection, measuring HBV in the blood fails to account for viral antigens that lead to negative effects on immune responses that, in turn, impact treatment response, and so evaluation of pharmacodynamics of candidate treatments is necessarily multidimensional [21].

Examples in HDT PK-PD or PK-TD in TB

To recap, HDT for TB is directed at several different elements in the pathogen-host-disease interface (see Fig. 22.1). Examples include phosphodiesterase inhibitors (e.g. PDE4i) that decrease lung pathology via inhibition of degradation of cyclic adenosine monophosphate (cAMP) and lowering of inflammatory cytokines like IL-8 [22]; 3-hydroxy-e-methyl-glutaryl-CoA reductase (HMG-CoA reductase) inhibitors (aka statins) that reduce accumulation of lipids in macrophages and enhance intracellular killing of *M.tb* [7]; metformin, an anti-diabetes medication that enhances macrophage activity, autophagy, cell-mediated immune responses,

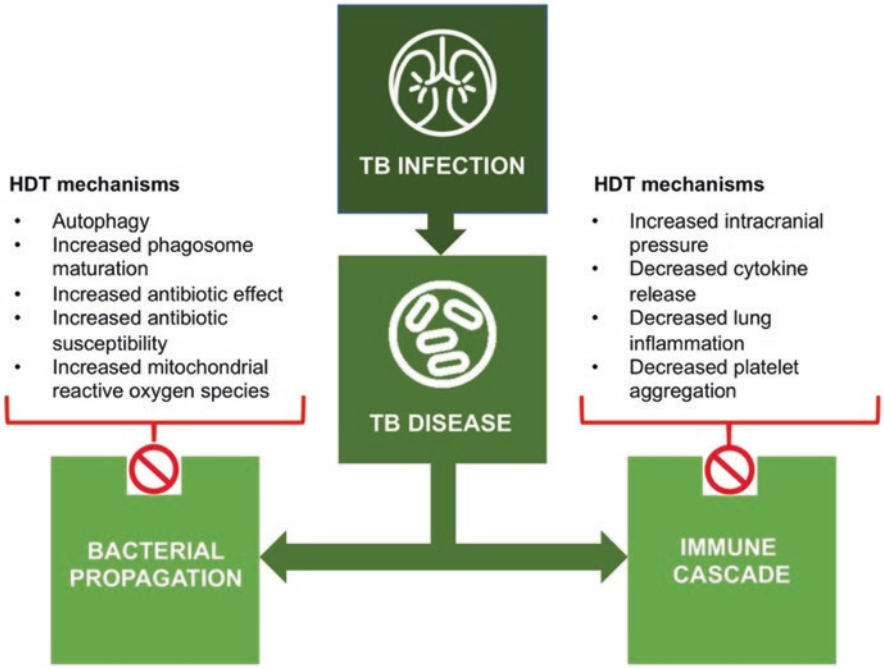

Fig. 22.1 Mechanisms of host-directed therapy (HDT) in tuberculosis disease

and oxidative stress and decreases lung pathology [23, 24]; tyrosine kinase inhibitors (TKI, such as imatinib) that promote phagosome maturation and acidification and autophagy and/or augment TH1 immunity [25, 26]; and agents that are potent immunomodulators and/or target cytokines (IL-4, IL-2, TNF-α, IFN-γ) such as thalidomide, pascolizumab (NCT01638520), CC-11050 [9], ibuprofen, and others [27] (Table 22.1). In some cases, there is a putative link between drug therapy, an observable and measurable intermediate biomarker, and clinical effect that would be amenable to exposure-response assessments (Chap. 6).

There have been few attempts at PK-PD or PK-TD assessments of HDT in TB. In some studies, though, PK were collected, alongside immunologic and microbiologic data. In one mouse study, doses of PDE4i were given at doses known to have anti-inflammatory properties in mice, and for those that proved to be effective (cilostazol and sildenafil, but not rolipram and cilomilast), PK were assessed and reported, though not linked to microbiologic or immunologic effects [22]. In patients with HIV (but not TB), use of CC-11050, an experimental PDE4i, was associated with lower levels of IL-8, lower percentage of NK cells, and higher IL-6, though exposure-response relationships were not explored, and there were no clear clinical implications of these changes in cytokines (no change in CD4 counts or plasma viremia) [28]. In mice infected with *M.tb*, there was no link between simvastatin use and cholesterol abundance in plasma or lung lesions, the latter assessed quantitatively by MALDI-MSI [7], suggesting that cholesterol will

not be a useful surrogate marker of anti-inflammatory treatment effect among TB patients; simvastatin and its metabolite were measured but PK-cholesterol or PK-outcomes relationships were not assessed. Fortunately, there are multiple current randomized controlled clinical trials of HDT for TB that offer the opportunity to assess PK-biomarker and PK-outcomes associations prospectively (Table 22.1). For repurposed drugs or drugs being developed for other indications, PK-TD relationships may be well-characterized from studies outside the TB field [29], with limited need for additional work in patients with TB (other than assessing overlapping toxicities of particular clinical concern). The novel agent CC-11050 has recently been suggested (along with everolimus) to shorten time to culture conversion and improve lung function when combined with standard treatment in a HDT trial [30]. Given that CC-11050 is primarily metabolized by CYP3A4, careful inclusion of PK measures into future studies is critical to determine composition of safe combination therapy.

Special Considerations—Site-of-Disease PK and Severity and Location of Disease

In specific cases, namely TB sepsis and central nervous system (CNS) TB, the inflammation associated with TB disease causes significant morbidity and high risk of mortality [31, 32]. In these clinical scenarios, HDT has the potential to contribute even more meaningfully to TB therapeutics. In one study, hospitalized patients who died from TB had immune signatures dominated by mediators of chemotactic signaling and the innate immune system, different from patients who survived, a discovery made possible by bioinformatics (hierarchical cluster and principal components analysis) [31]. The soluble inflammatory mediators in this 'immune signature' could be relevant intermediate markers in PK-PD assessments of HDT targeting this critically ill population. With CNS TB, inflammation in the brain and meninges leads to vasculitis and strokes, with sequelae including cranial nerve deficits, paresis, and death [33].

Though infections of the CNS are among the most devastating manifestations of TB, there are inherent challenges to the study of optimal drug penetrance, including candidate agents for HDT. In general, *antimicrobial* agents must cross the blood-brain barrier (BBB) to access the brain and meninges, where *M.tb.* resides; to some extent, penetration across the blood:cerebrospinal fluid barrier (BCSFB) may also be necessary to eradicate organisms in TB meningitis (TBM) [34, 35]. It is possible to estimate the penetration coefficient of a given drug into CSF by measuring drug in plasma and CSF matrices directly and to compare CSF concentrations to minimal inhibitory concentrations (MIC) of the organism. Microdialysis is a tool that can be used to measure drug in the brain [36]; it is an invasive procedure, though one that is increasingly part of standard of care in neuro-critical care settings and may be more reflective of drug concentrations at the site of disease than CSF [35, 37–39]. Given that the concentrations of inflam-

matory molecules differ considerably between ventricular and lumbar CSF among patients with TBM, there remain outstanding questions about optimal site of measurement to inform PK-PD assessments [40].

Ultimately, understanding PK-PD relationships can be critical for drug and dose optimization [41, 42], particularly for a relatively rare disease such as TBM, for which enrollment into large trials is challenging [43]. With HDT for CNS-TB, PK at the site of the disease becomes even more complicated in that drug effect may be direct or indirect, via its effect on immune modulators. As previously mentioned, we can learn much from the cancer literature about immunotherapeutic drugs that have activity in brain metastases. The brain, once thought to be immunologically 'privileged', is now known to undergo immunosurveillance with the help of its resident myeloid cells (microglia). BBB damage, caused by tumor or infection, may increase CNS drug exposures [44]. Therefore immunotherapy may have both indirect effects via alterations in systemically circulating immune cells which then cross into sequestered sites, as well as direct local effects that enhance *M.tb* killing or dampen inflammation. All of these potential effects will warrant close ongoing study as therapeutic options are explored [45–47]. The class with the strongest support for adjunctive HDT for TBM is corticosteroids, though there is some evidence that this benefit is restricted to certain host genotypes [48]. Thalidomide had shown promise as adjunctive therapy for TB meningitis in children. A higher dose (24 mg/kg/day) was selected for a randomized trial and resulted in high rates of excess adverse events compared to standard treatment, therefore the trial was terminated early [49]. Lower dosing strategies, however, have shown promise in several case series including children and adults [50, 51]. Given that strokes are a potentially devastating complication of TBM, standard and high-dose aspirin has been studied in three trials to date with mixed results [52–54], though additional studies are upcoming (NCT03927313, NCT04145258).

Though CNS TB may be the disease entity for which HDT has the most profound effect, it remains a rare manifestation by comparison to pulmonary TB. This more common presentation faces other challenges. Among patients with cavitary pulmonary TB, drug effect must extend into the necrotic, avascular lesions where large numbers of bacilli reside [55, 56]. Our understanding of immune characteristics of cavitary lung lesions is green, yet recent studies have provided a putative pathophysiological map of these complex lesions [57]. Spatial intralesional mapping that co-locates and quantifies drug and the immune modulators they aim to influence may be the way of the future.

Advice and Future Directions

Outlining the pharmacology of HDT for TB poses many challenges, as described, yet remains vital to this field. As interest in this field grows and repurposed or novel drugs are explored as HDT agents in TB, comprehensive understanding of

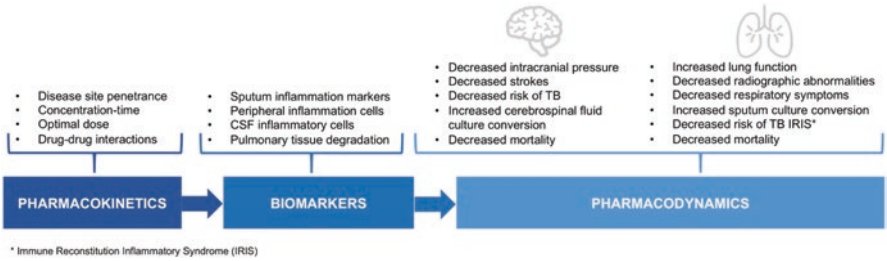

Fig. 22.2 Potential measures of pharmacokinetics, biomarkers, and pharmacodynamics relevant to the pharmacology of host-directed therapy for tuberculosis

the PK-PD and PK-TD relationships will provide those early signals on which agents to advance efficiently and correctly, at the right dose, for the right patients (see Fig. 22.2, Table 22.1).

There are many current or recent clinical trials investigating promising agents for HDT, such as azithromycin, everolimus, auranofin, vitamin D3, CC-11050, ibuprofen, n-acetyl cystine, and pravastatin. While this is encouraging for those who are optimistic about these adjunctive therapies, the heterogeneity in study designs reflects the lack of consensus about how to best measure the PK, PD, and therapeutic windows for these drugs in the context of TB treatment. Ideally, all of these trials would include robust PK coupled with established and novel markers of drug activity at the site of disease, but many have only one dose level [58, 59] (NCT02968927). This is particularly problematic given that some of these novel agents have the potential for significant drug-drug interactions with standard TB treatment (e.g. CC-11050) [10].

An ideal HDT trial from a pharmacology perspective would include certain features, such as different dosing levels, which would permit assessment of dose-response and selection of the optimal dose. Hysteresis is a clinically important feature of certain drug-disease pairs and is best described by collecting longitudinal PK and PD data. Mathematical models at any stage should be built with this phenomenon in mind [60]. The numerous ongoing preclinical studies and clinical trials of HDT for TB offer the opportunity to imbed PK collections longitudinally and may shed light on PK-biomarker and PK-PD relationships. As with all biomarker research, we must think critically about the cascade of events any HDT should affect, and whether these truly correlate with clinically important outcomes. Animal studies are particularly valuable in identifying these relationships to inform later human study design, but have limited utility in identifying drug-drug interactions (DDI). Human trials of HDT must include assessment of possible DDI, especially given that some of our best drugs include rifamycins (potent and broad inducers) and bedaquiline (CYP3A substrate). The HDT field should also consider other more patient-centered clinical outcomes in our PK-PD assessments. These might include correlating HDT PK with various lung function parameters such as forced expiratory volume over 1 second (FEV1), which is important to long term patient health status as well being as a correlate of disease activity [61]. Certain intermediate

Table 22.1 Host directed therapies, preclinical and clinical assessments, with focus on biomarkers and pharmacology

Agent	Ongoing trial: design	HDT effect	Animal data	PK measures	PD measures	Biomarker	Knowledge gaps
Metformin [1–3]	METRIF (upcoming): 2 months RHZE plus metformin 1000 mg versus SOC; difference in time to culture conversion, PK, PGx, DDI, safety, tolerability, autophagy, immune response	Induction of mitochondrial reactive oxygen species, facilitation of phagosome-lysosome fusion, promotion of autophagy, reduced inflammatory cytokine/chemokine production (TNFa, IL-1b, IL-6, MCP-1, IAM-1)	Improved pulmonary lesions, reduced bacillary burden in mouse model	Well established in diabetes, no PK studies for TB but no apparent dose-dependent effect from observational studies; METRIF will collect intensive-sparse PK at week 4 (sampling strategy unknown) for AUC and Cmax, as well as DDI rifampicin	Systematic review showing decreased risk of TB among diabetic patients taking metformin (one study with dose-proportional risk reduction), reduced mortality among TB patients, and increase in 2 month sputum culture conversion	Reduced neutrophil to lymphocyte ratio, reduced cytokine CCL11 among non-TB patients; METRIF collecting biomarkers (not otherwise specified)	Biomarkers in TB; best PD measures, dose-response relationship
Pravastatin [4–8]	NCT03882177 (StAT-TB dose-finding study); NCT03456102 (StAT-TB efficacy trial) – will include PK, PD (serial sputa), safety/tolerability, DDI with RHZE regimen	Promote intracellular Mtb killing, promote phagosome maturation and autophagy, potentiate rifampin in macrophages	Shortens time to culture conversion and improves relapse rate when added to first-line regimen in mouse model	Human PK well-described from prior studies; robust PK including dose-escalation with DDI planned for StAT-TB	Dose-dependent increase in bactericidal activity for pravastatin (in mice); observational studies show decreased risk of active TB (duration-dependent); Robust PK-PD planned for StAT-TB	Cholesterol does not appear to be reliable biomarker	Optimal dosing, rifamycin interactions

(continued)

Table 22.1 (continued)

Agent	Ongoing trial: design	HDT effect	Animal data	PK measures	PD measures	Biomarker	Knowledge gaps
Corti-costeroids [8–11]	NCT03100786 – testing dexamethasone; NCT03092817 – dex for TBM	Multimodal effect, suppresses humoral immune response, dampening cytokine production	None	Generally described, but not able to find studies correlating exposure for TB disease	Improved radiographic lesions, decreased symptoms, faster time to culture conversion, reduced TB-IRIS among patients with HIV, reduced mortality for TBM	Unknown, possibly reduced TNF-alpha, reduced IFN-gamma	Optimal biomarker, role in pulmonary TB?
Aspirin [12–15]	NCT03927313 (LASER TBM) – study of 100 HIV+ persons, testing safety and PK of SOC plus dexamethasone versus SOC plus high-dose rifampicin + linezolid, with/without high-dose aspirin 1000 mg daily	Antiplatelet, anti-inflammatory, anti-oxidant properties thought to reduce rates of thrombotic events with TBM; mixed results from prior treatment trials but some evidence of dose-dependent reduction in new stroke and death; anti-inflammatory properties with higher (>600 mg) doses by inhibiting prostaglandins and thromboxane A2	Enhanced sterilizing effect of PZA in mouse model; low-dose improved survival, reduced lung pathology, decreased bacillary load, reduced systemic inflammation (IL-6, IL-1B, TNF-a), reduced disease site (lung) inflammation (fewer neutrophils, reduced tissue factor)	Upcoming LASER TBM trial plans to collect CSF and serum PK but only for LZD/RIF (not ASA)	LASER TBM will estimate change in radiographic appearance, CSF culture conversion, occurrence of TBM IRIS, bleeding events, toxicity	NCT02237365 demonstrated dose-dependent reduction in thromboxane A2	Optimal dose, risk/benefit calculus, PK-PD for HDT

	NCT04145258 (INTENSE-TBM) – study of 768 persons with TBM, comparing outcomes of standard WHO regimen, intensified regimen (addition of linezolid, increased rifampicin dose), both with/without aspirin 200 mg daily			Upcoming INTENSE-TBM will have subset with CSF and serum PK (for LZD/RIF and DTG when relevant, not ASA)	INTENSE-TBM will collect data on death, new neurologic events, AEs, disability, culture conversion (rate and time to), in vitro bactericidal activity		
Imatinib [16–19]	Animal: rhesus macaques (added to background of moxi-pza-emb)	Enhanced myelopoeisis, phagosome maturation and acidification, autophagy	Reduced granulomas and bacterial burden in mice with drug-sensitive and rifampicin-resistant TB lesions; synergistic with rifampicin and rifabutin	CYP3A4 substrate (385% increased clearance with rifampicin co-administration), wide PK variability (Cmin)	In CML, inverse dose-response relationship (enhanced effect and reduced AE with lower doses <400 mg)	In vitro: active cathepsin D (detected by fluorescent substrate pepstatin-bodipy, BPF) correlates well as marker for acidic lysosomal environment among imatinib-treated cells	Role of neutrophil-induced lung damage, biomarkers for TB; optimal dose selection

(continued)

Table 22.1 (continued)

Agent	Ongoing trial: design	HDT effect	Animal data	PK measures	PD measures	Biomarker	Knowledge gaps
Thalidomide [20–24]	N/A (now focused on analogues without teratogenicity)	TNF-a inhibition, activates NK cells, inhibits lymphocyte apoptosis, induces Th2 activity	TNFa in CSF correlates with disease activity in rabbit TBM model, survival with thalidomide treatment	Well defined in healthy volunteers, limited PK from patients with TB; minimal CYP metabolism including no altered clearance with rifampicin	Clinical improvement (cessation of seizures, resolution of ataxia, resumption of walking), radiographic improvement	CSF TNFa concentration (reduced in CSF of TBM patients treated with thalidomide, serum TNFa correlated less well)	Dose selection
Azithromycin [25]	NCT03160638: daily azithromycin 250 mg in addition to RHZE, compared to RHZE alone	Enhances bactericidal activity of epithelial fluid lining, reduced pulmonary inflammation for various lung diseases possibly by reducing MMP gene expression and secretion in the lungs	No animal models of efficacy in TB	NCT03160638 will collect PK (? details)	NCT03160638 will collect sputum inflammatory cell counts, serum WBC counts, sputum cytokines, serum cytokines, pulmonary tissue degradation (sputum and serum markers), pulmonary tissue remodeling (sputum and serum markers), time to sputum conversion	Unknown, possibly reduced MMP-1 and MMP-3 in lung fluid?	Dose selection, DDI, PK-PD for TB HDT

Everolimus [26–28]	NCT02968927: Comparison of 4 HDT agents when combined with experimental regimen RbHZE	Rapamycin analog that inhibits mTOR, promoting autophagy	Promotes survival, restores T cell function, enhances CD8 response to vaccines in mice	Well established from transplant and oncology, substrate of CYP3A4/PgP	Sputum culture conversion; change in FEV1; PET CT uptake; serum neopterin; QuantiFERON gold; gene expression profile; PD-1 expression on CD4 and CD8 cells	Influenza vaccine titers used in elderly patients to demonstrate immune enhancement	Dose selection; DDI with first-line regimen; eventual nanoparticle formulation?
Auranofin [29–31]		Inhibition of thioredoxin reductase (TrxR), disrupting cell protection against reactive oxygen species, active against replicating and dormant bacilli	Effective in murine model of staph aureus infection, no available data on Mtb animal model	Well tolerated clinically from rheumatoid arthritis literature, PK established in recent phase 1 trial as anti-parasitic		Unknown	Biomarker
Vitamin D3 [32–35]		Required for cathelidicin (antimicrobial peptide) formation, later involved in autophagy; treatment trials mixed and mostly show benefit in sputum culture rate for *tt* genotype of Vitamin D receptor	Calcitriol enhances activity of PZA in mice	Prior trials obtained serum 25(OH)D concentration		Unknown	Role in TB therapy remains unclear; combination with phenylbutyrate promising but dose selection unclear (see below)

(continued)

Table 22.1 (continued)

Agent	Ongoing trial: design	HDT effect	Animal data	PK measures	PD measures	Biomarker	Knowledge gaps
CC-11050 [36–38]		Inhibition of phosphodiesterase 4 (PDE4i) (involved in cytokine/ chemokine release)	Improved lung pathology/ fibrosis, decreased bacillary burden in rabbit model (plus additive effect with INH), marked difference in gene expression pathways of interest (inflammatory cytokines, lung inflammation, macrophage activation, lung fibrosis)	Phase 1 study in 19 HIV+ persons complete, reported no interaction with efavirenz but based on single measure at baseline and week 4; M15 is major metabolite and inhibits CYP2C9, 2C19, 3A4		Phase 1 study of HIV+ persons showed reduced IL-8 but no altered trajectory and no change in TNF-a, IL-6, IL-10, IFN-g, sCD14, CRP, d-dimer	DDI with rifamycins (given it is CYP3A4 substrate); biomarkers; PK-PD for TB

Phenyl-butyrate plus Vitamin D3 [39–42]	None ongoing	Phenylbutyrate is histone deacetylase inhibitor, increases cathelidicin LL-37 expression (antimicrobial peptide that requires Vitamin D3)	None specific to this combination	20 g daily approved for urea cycle disorders; dose-finding studies in healthy volunteers used antimycobacterial activity in monocyte-derived macrophages (MDM) to demonstrate greatest effect with 500 mg twice daily phenylbutyrate and 5000 IU once daily Vitamin D3 though this group had highest baseline Vitamin D3 concentrations and no PK on phenylbutyrate collected; later trial in TB patients collected vitamin D concentration (not overall time-concentration exposure)	NCT01580007 (RCT of PB plus D3 versus placebo) showed regimen was well-tolerated with some improvement in composite TB score and enhanced culture conversion rates at week 4 but no statistical significance at week 8 compared to placebo, with levels of LL-37 and clinical benefit correlated by some measures; NCT01698476 showed some benefit in TB score but no effect on culture conversion	Possibly cathelidicin LL-37	Optimal dosing, PK-PD for HDT in TB patients with PK of phenylbutyrate included

(continued)

Table 22.1 (continued)

Agent	Ongoing trial: design	HDT effect	Animal data	PK measures	PD measures	Biomarker	Knowledge gaps
Pasco-lizumab [43, 44]	NCT01638520 – safety/efficacy of blocking IL-4 as adjunctive therapy for patients with pulmonary TB on SOC	IL-4 impairs clearance of Mtb, blockade might hasten cure	Murine mAb was synthesized then humanized with specificity to human and monkey T cell response to IL-4 (no TB animal studies)	Phase 1 trial showed single IV dose well tolerated with long half life (2 weeks), later multidose phase II trial (for asthma) terminated early for lack of benefit	None for TB, no data since 2017 on clinicaltrials.gov	IL-4?	Dose-finding in humans with TB
NSAID (e.g. ibuprofen) [13, 45–47]	NCT02781909 – completed trial of ibuprofen as HDT for XDR TB (results pending); NCT02060006 – no update since 2014; NCT02503839 – COX2 inhib for MDR, +/− with TB vaccine	COX inhibition, preventing prostaglandin and leukotriene formation thereby curbing inflammatory response	Increased survival, reduced bacillary loads, and enhancement of PZA activity in murine model of TB given ibuprofen	None for TB treatment, well-defined generally	NCT02781909: microbiologic and radiographic improvement (month 2 and 6) including time to culture conversion, also collecting 'immune responses' but details not available	Unknown	Optimal dosing, biomarker selection

N-acetyl-cysteine [48–52]	NCT03702738 – recruiting, randomizing moderate-severe pulmonary TB patients to NAC 1200 mg twice daily for 4 months or placebo plus RHZE; NCT03281226 recruiting patients with HIV and pulmonary TB to receive NAC 600 mg twice daily or placebo plus RHZE	TB (and HIV and ?DM2) deplete glutathione in lymphocytes, leading to increased TNF and free radical formation; NAC (glutathione precursor) improved MTB control by reducing IL-1, TNF-a, IL-6 and increasing IFN-gamma in vitro	NAC decreases bacillary load of spleen and degree of lung necrosis in Mtb infected guinea pigs	None for TB treatment, well-defined generally	Invitro study using human monocyte-derived macrophages infected with TB showed dose-dependent decrease in Mtb growth and activity; NCT03702738 will monitor time to stable sputum conversion and whole blood bactericidal activity (WBA); NCT03281226 will describe inflammatory cytokine profiles; prior small RCTs showed increased glutathione levels, greater weight gain, and faster culture conversion	NCT03702738 will collect change in reduced glutathione (GSH) in blood cells, ratio of GSH to oxidized glutathione (GSSG)	Biomarker, optimal dosing, PK-PD

(continued)

Table 22.1 (continued)

Agent	Ongoing trial: design	HDT effect	Animal data	PK measures	PD measures	Biomarker	Knowledge gaps
Doxy-cycline [53–55]	NCT02774993 – completed trial of healthy volunteers and TB patients randomized to placebo or doxycycline (plus RHZE for TB patients), results not available	Inhibits MMP; evidence of in vitro synergy with amikacin against drug-resistant TB strains	Guinea pig pulmonary TB model showed reduced bacillary load and improved imaging, but not correlated with MMP levels	None for TB treatment, well-defined generally	Dose-dependent suppression of MMP-1 and MMP-3 in Mtb infected human macrophages	MMP-1, 2, 3 concentration in induced sputum? NCT02774993 collected serum procollagen III N-terminal peptide (PIIINP)	Duration, PK-PD, biomarker
Infliximab [56–60]	N/A	TNF-a antagonist	None specific to infliximab for HDT in TB, but general animal models support TNF-a targeted therapy	Unknown	Supportive evidence for use in TBM in 1 pediatric case, 6 adult cases	Unknown	Optimal dose selection, timing, biomarkers

outcomes such as lung function may in fact be more affected by HDT than antimicrobial agents and trial outcomes for HDT agents should be designed accordingly [62]. Preliminary results from NCT02968927 show improved lung function among participants randomized to receive CC-11050 or everolimus in addition to rifabutin-based TB therapy [30]. When, where, and how to measure PK of HDT agents are important considerations with profound implications for the success of these adjunct therapies. The site of disease PK and outcome measurements vary by drug and by disease state (pulmonary versus CNS TB, for example). HDT for TB meningitis, in which inflammation is particularly harmful, represents a critical clinical concern, and urgent study of PK-PD relationships is needed in order to promote integration of promising agents into clinical care. For all TB therapeutics research, including HDT, expert pharmacometrics is critically important, as is careful study of immunologically-based therapies for other diseases. The relationships among drug, immunologic cascade, lesions, and outcomes are complex and bioinformatics will help us better understand these phenomena.

As is true across the TB drug development pipeline, the success of HDT will require inclusion of translational and clinical pharmacology at every step, with constant feedback to iteratively improve these integrative models. Clinical pharmacology of HDT for TB is completely novel, so current investigators will define the standards and tone of this discipline. There is unprecedented enthusiasm for this field, coupled with availability of emerging and repurposed agents. Careful study of HDT, supported by well-defined pharmacology, offers the potential to shorten therapy, enhance treatment effectiveness, improve patient function, and reduce morbidity and mortality.

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