Sharpening nature's tools for efficient tuberculosis control: A review of the potential role and development of host-directed therapies and strategies for targeted respiratory delivery.

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Sharpening nature’s tools for efficient tuberculosis control – a review of the potential role and development of host-directed therapies and strategies for targeted respiratory delivery.

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Abstract

Centuries since it was first described tuberculosis (TB) remains a significant global public health issue. Despite ongoing, holistic measures implemented by health authorities and a number of new oral treatments reaching the market there is still a need for an advanced, efficient TB treatment. An adjunctive, host directed therapy designed to enhance endogenous pathways and hence compliment current regimens could be the answer. Integration of drug repurposing, including synthetic and naturally occurring compounds, with a targeted drug delivery platform is an attractive development option. In order for a new anti-tubercular treatment to be produced in a timely manner, a multidisciplinary approach should be taken from the outset including stakeholders from academia, the pharmaceutical industry and regulatory bodies keeping the patient as the key focus. Pre-clinical considerations for the development of a targeted host directed therapy are discussed here.

Key words

Anti-tubercular, immunomodulation, vitamin, targeted drug delivery, microparticle, particle engineering, inhalation, adjunctive therapy, in vitro, in vivo, pre-clinical testing, multidisciplinary.
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1. Introduction

Tuberculosis (TB) infection, caused by the pathogen *Mycobacterium tuberculosis* (*Mtb*), represents a global public health crisis traversing centuries. Recent figures published by the World Health Organisation (WHO) cite TB as the leading cause of death by infection alongside Human Immunodeficiency Virus (HIV), responsible for 1.5 million deaths annually [1, 2]. Compounding this crisis is the rising number of drug resistant cases of Mtb infection. Multi-drug resistant TB (MDR-TB), defined as resistance to at least the two first-line anti-bacterials (isoniazid and rifampicin) and extensively drug resistant Mtb (XDR-TB), characterised by additional resistance to second-line therapeutics, have complicated the already arduous treatment regimens [3]. Despite reductions in the global burden of TB following the WHO’s introduction of directly observed treatment, short-course (DOTS) in the 1990s, and recently the adoption of a more holistic approach by incorporating patient care, policy and research, the stated aim of reducing TB deaths and incidence by 90% and 80%, respectively, between 2015 and 2030 remains a substantial challenge [1, 4]. The TB Alliance, established in 2000, has placed significant emphasis on the development of new combinatory regimens, and crucially, new drug candidates, by bringing organisations together using its product development partnership approach (PDP) [5]. Two new drugs, delamanid and bedaquiline, have recently been licensed for the treatment of MDR-TB. However, as with conventional anti-mycobacterials, acquired resistance to these novel agents has already been reported, emphasising the limitation of pathogen-directed therapies in treating this heterogeneous and dynamic disease [6].

Our natural defences, however, should not be underestimated. Despite one third of the world’s population living with latent tuberculosis infection (LTBI), just 9.6 million people developed active disease in 2014 [1]. The host immune response to TB infection, therefore, is capable of successfully limiting infection in the majority of individuals. Thus, a logical question is how can the immune response be enhanced in patients where active disease takes hold? The concept of ‘host directed therapies’ (HDT), whereby therapeutics targeting the human host's immune response to infection for the purpose of augmenting beneficial and reducing harmful features, have garnered significant international interest, evidenced by the recent publication of several high-quality reviews on the topic [7-9]. The most obvious attraction of this approach is the lower likelihood of development of treatment-resistant strains of Mtb in comparison to conventional pathogen-directed therapies. Additionally, HDTs hold potential in a myriad of settings – as vaccine adjuncts, as prophylactic therapies for close
contacts of cases, as strategies to limit infectivity and shorten treatment duration, as well as improving overall survival outcome and reducing the lung tissue damage resulting from excessive inflammation. Furthermore, “drug repurposing” of currently licensed medications has already been highlighted as an approach to tackling the TB pandemic, given the benefits of faster market access and reduced development costs [10, 11]. Several drugs currently licensed for other indications (some of which are also off-patent) are showing promise as potential HDTs, offering affordable provision to the underdeveloped economies that most require novel TB therapies.

The host immune response to Mtb infection, however, is layered with complexity. Many immune functions that are vital early in host defence prove detrimental in advanced infection [12]. Consequently, in addition to agents that enhance the natural host immune response to TB, a large number of anti-inflammatory agents that work through attenuation of the destructive effects of excessive immune responses are also under investigation as potential HDTs. The temporal events of infection and disease progression must be understood and considered in order to maximise efficacy of new therapies and, crucially, to prevent undesired harmful outcomes. Additionally, several promising HDTs – including vitamins which are discussed in detail in this review – have been demonstrated to influence host metabolism, therefore variable host nutritional status may be important in determining the efficacy of various approaches to manipulation of immune response. Genetic variations in host immunity are also likely to impact individual response to HDT to a far greater extent than conventional pathogen-targeted antimicrobials [13], potentially suggesting a role for integration of pharmacogenomics and a “personalised medicine” approach into the development of HDTs, tailoring therapies to an individual’s “immune signature” [14, 15]. Although such measures at present are still considered novel and consequently costly for the treatment, accompanying diagnostics and multidisciplinary care, if governments and policy makers truly wish to overcome this pandemic then the potential of this approach must be evaluated [15]. These adjunctive therapies would need rationalisation in order to be efficaciously integrated into standard regimens and with that, provisions for accessibility, bearing in mind that if successful this approach could save money in the long term freeing up resources for alternative needs.

In parallel with the rise in interest in HDT and drug repurposing has been the ongoing interest in alternative methods of drug delivery in tuberculosis treatment. The use of advanced engineering and formulation tools to allow targeted delivery of anti-tubercular drugs via the pulmonary route has been
the focus of many studies worldwide using carriers designed to target the alveolar macrophage [16-24]. Our group has showed that Poly(Lactide -co-Glycolide) (PLGA) microparticles encapsulating standard anti-tubercular drugs can be efficiently taken up by THP-1 derived macrophages and cause bacillary killing in an in vitro infection model of tuberculosis [25]. Numerous crucial factors must be taken into consideration when developing an inhaled treatment ranging from the physico-chemical properties of formulation to device compatibility in order to ensure the formulation efficiently reaches the site of action. Various methods of formulation manufacture will be discussed later in this review, meanwhile device design technology for pulmonary delivery of treatments in chronic conditions such as cystic fibrosis continues to advance, particularly in the administration of labile proteins, an aspect which may prove crucial in the development of HDT for TB [26].

It is clear, therefore, that a true multidisciplinary approach must be taken to enable the development of new anti-tubercular treatments. If a novel cargo can be combined with more efficient, targeted drug delivery a number of attractive opportunities could develop [8]. Figure 1 shows a number of the key stakeholders required in the process of HDT drug development: clinicians for first-hand experience and patient interaction, research scientists including immunologists, microbiologists, geneticists, pharmaceutical scientists, chemists, and bioengineers for idea generation and preclinical testing. Once collected, the data can be disseminated to industry for manufacture scale-up, device design and clinical trials with the aim of securing a marketing authorisation). A substantial amount of time will pass before any new treatment can be brought before policy makers, integrated into protocols and eventually reaching the patient. A new chemical entity (NCE) can take 10 – 20 years to reach the market under the U.S Food and Drug Administration (FDA) regulations, a timeline that the TB community cannot afford therefore with the use of repurposed drugs, including HDT’s, could be the answer.
Fig.1. Multidisciplinary commitment is required for host-directed drug development. The patient must be the focus of all development strategies.

In this review, we discuss a number of the multidisciplinary aspects important in the pre-clinical development of HDT, including drug selection and delivery as well as design of both in vitro and in vivo models for evaluation of efficacy. With a primary focus on vitamins as HDTs, we discuss the co-development of potential pulmonary delivery systems for an efficient new therapy for Mtb infection.

### 2.0 Host-directed therapies (HDT) for Tuberculosis

*Mycobacterium tuberculosis* (Mt) has a tendency to reside in oxygen rich tissues of the lungs where it is phagocytosed by alveolar macrophages (AM) and readily manipulates the host environment for its own gain. Inflammation and granuloma formation follows infection allowing containment of the bacteria, however, a fine balance of this immune response is required to limit dissemination and development of active disease whilst simultaneously maintaining lung structure and function [9, 27].

HDT for Mtb infection include a wide range of compounds targeting a plethora of different (sometimes opposing) pathways, reflecting the intricacy of the human immune response to the bacillus. When
discussing possible compounds, whether for targeted, pulmonary delivery or otherwise, it is important to consider the following points:

1. TB necessitates the administration of multiple anti-bacterials simultaneously in order to rapidly reduce bacterial burden and, in some cases, treat extra-pulmonary sites of infection [28-30]. Therefore any new formulation developed is likely to be an adjunctive therapy to current regimens.

2. With the aid of appropriate diagnosis, current regimens for TB are generally effective with a treatment success rate of 86% for all newly diagnosed cases in 2013 [1]. The greatest threat to this statistic is MDR-TB and so a therapy aimed at treating or preventing the escalation of MDR-TB by, for example shortening current regimens, would be most beneficial [30].

3. The active ingredient(s) should not cause serious interactions with current effective treatments or produce undesirable effects that would lead to further non-compliance.

4. The chosen HDT should be cost effective and readily available - 95% of TB-related deaths happen in low to middle income countries therefore the cost of the treatment and formulation must not be prohibitive[1]. A fact that might encourage investment from multinational pharmaceutical companies.

5. Due to the complex nature of the immune response to infection, it is important that we have a comprehensive understanding of the mechanism of action of any HDT in order to ensure that we tailor clinical trial design accordingly to avoid underestimating efficacy.

With this in mind, we discuss some promising approaches to HDT for TB infection.

2.1 Vitamins as host-directed therapies

Epidemiological data has shown that body mass index (BMI), and hence nutritional status, effects the host response to TB [31]. With 95% of TB cases worldwide occurring in low to middle-income countries, the WHO have guidelines in place to support undernourished patients of all ages throughout treatment with standard regimens including the prescribing of nutrient-rich foods where required [32]. In addition to the observed link between micro- and macro-nutritional status and TB is the relationship between worsening lung function and vitamin C and D deficiency in smokers [33, 34]. This may provide partial explanation for the epidemiological finding that smoking is a major risk factor
for TB infection, associated with an inappropriate immune response [35]. Prior to the development of antibacterial agents, micro-nutrients such as vitamin A and vitamin D were administered to patients in the form of cod liver oil as ‘anti-infective’ therapies for numerous conditions, including TB. Although little was known of the mode of action, these micro-nutrients were thought to enhance the immune system and, thereby helping to fight infection [36, 37]. Interest in nutrients subsequently waned with the introduction of specific anti-tubercular drugs. However, the rise in resistance patterns seen in recent decades has resulted in renewed attention being directed towards these alternative chemotherapies. Here, we review the evidence regarding the use of several key vitamins as HDT for TB.

2.1.1 Vitamin A

Vitamin A is a fat soluble vitamin currently licenced in the treatment of acute promyelocytic leukaemia (APL) and acne vulgaris [38]. With food as the source, vitamin A can enter the body as all-trans-retinol, β-carotene or retinyl esters and undergoes a series of reactions before being converted to the active metabolite all-trans-Retinoic Acid (atRA) [39]. AtRA then exerts its effect through binding to the nuclear receptor family retinoic acid receptor (RAR) which in turn heterodimerises with retinoid X receptor (RXR) [39]. In 1989, Crowle et al examined the effects of atRA on virulent Mtb survival in vitro with cultured human macrophages treated both before and after Mtb infection. Successful reduction in bacterial viability up to 7 days was evident in both instances, with treatment post-infection exhibiting the greatest effect [36]. Other studies have shown anti-bacterial effects on mycobacteria in culture, indicating both pathogen-targeted and host-targeted effects [40, 41]. Anti-bacterial properties of atRA in vivo have been demonstrated in Mtb infected Wister-Lewis rats alongside promising histopathology results [42].

More recently, detailed studies on the immunomodulatory and host-directed actions of atRA have been conducted, including investigation of its influence on the induction of phagocytosis of Mtb and autophagy [43]. This is likely due to effect on the down-regulation of tryptophan-aspartate containing coat (TACO) protein gene transcription, allowing intracellular motility and thus phagosome-lysosome fusion to proceed, resulting in the eventual degradation of Mtb [44-46]. Cholesterol has been highlighted as an essential amenity for Mtb within activated alveolar macrophages, providing energy to enable the persistence of the pathogen [47]. A recent study has revealed that atRA can reduce cellular cholesterol levels, thus increasing lysosomal acidification via the Niemann-Pick Disease Type
2C (NPC2) gene and leading, at least in part, to enhanced degradation of the bacillus [48]. Furthermore, atRA is thought to play a part in controlling iron metabolism, another essential nutrient for Mtb survival [49, 50]. Our group has demonstrated the effect of alveolar macrophage derived atRA on the stimulation of regulatory T Cells (Tregs) in the lungs through the transcription factor FoxP3 in conjunction with TGF-β1, leading to downregulation of the pro-inflammatory effects of the innate immune system and upregulation of anti-inflammatory proteins – namely interleukin-10 (IL-10) [51]. Although Toll-Like Receptor 2 (TLR2) is required for the balance between apoptosis and necrosis during Mtb infection [52], atRA has been shown to down regulate TLR2 signalling and dampening of TNF-α, IL-1RA, IL-6, IL-8, IL-10 leading to a reduction of inflammation in other disease models (Propionibacterium acnes) [53, 54]. This immune regulation by atRA could make it an ideal adjunct in the treatment of Mtb to reduce unwanted tissue damage if administered at the correct stage of disease. AtRA is relatively cheap and readily available, allowing accessibility for formulation and manufacture scale-up.

Recognised for its effects on growth and differentiation as well as apoptosis, atRA treatment has proven efficacy in a variety of clinical settings, currently licensed for use for both dermatologic and oncologic indications as previously mentioned [55, 56]. Massaro and Massaro further demonstrated the diversity of atRA treatment by demonstrating its ability to induce alveolar regeneration in a model of experimentally-induced emphysema [57]. Estrella et al have also shown atRA, in combination with vitamin D, to stimulate the formation of multinucleated giant cells (MNGC) [43]. The vitamin pre-treatment resulted in significantly decreased bacterial viability compared to phorbol-12-myristate-13-acetate (PMA) differentiated THP-1 cells and increased survival of infected MNGC lasting for 60 days post-infection [43]. MNGC’s are a component of TB granuloma formation and although debate surrounds the specific role of MNGC’s in the granuloma process [58, 59] the structure supports the containment of mycobacteria after infection [60, 61]. As well as anti-bacterial effects associated with atRA (data not shown), we have demonstrated the formation of MNGCs when THP-1 derived macrophages are treated with atRA post-infection (see Fig. 2). Thus, it would be reasonable to postulate that the use of atRA as a HDT for TB would possibly provide a host-protective effect by aiding granuloma formation through promotion of MNGCs and reduction in cholesterol-laden (foamy) macrophage formation as well as modulation of the inflammatory response.
Figure 2. All-trans-Retinoic-acid treated THP-1 derived macrophages leads to the development of multinucleated giant cells (MNGC). THP-1 cells differentiated into macrophages (using PMA) were treated with atRA in the presence or absence of Mtb infection (H37Ra) and viewed 72 hours later using an Olympus IX51 microscope. Phalloidin-FITC (green) and Hoechst (blue) were added to allow visualisation of the cell actin and nucleus respectively.

Despite this evidence suggesting atRA as a promising adjunctive therapy for Mtb infection, results of pre-clinical trials have not been reflected in the clinic. Studies have found that Vitamin A supplementation had no effect on tuberculosis outcomes, with and without co-morbidities such as HIV and diabetes [62, 63]. These outcomes could be due in part to the complex metabolism of vitamin A, depending on what form it enters the body or the stage of infection. AtRA-associated toxicities are well documented from teratogenic effects to liver and dermatologic events [55] therefore targeted delivery of the active metabolite could potentially overcome both issues leading to a more effective treatment.
2.1.2 Vitamin D

Vitamin D is an endogenous vitamin requiring sunlight for activation currently licenced for treatment of osteoporosis and, in topical form, treatment of psoriasis [64]. Once metabolised from 7-dehydrocholesterol to 25-dihydroxyvitamin D3 and subsequently to 1,25-dihydroxyvitamin D3 (1,25D), the fat soluble vitamin acts on the vitamin D receptor, heterodimerizing with the same nuclear retinoid X receptor family as atRA, which is often studied in parallel [39]. Anti-mycobacterial action has been documented since the 1980s, with slowed intracellular Mtb growth demonstrated in human macrophages treated with 1,25D [65, 66] and in mycobacterial culture using Mycobacteria Growth Indicator Tube (MGIT) and BACTEC™ culture systems [40, 41].

As described above, 1,25D in combination with atRA has effects on differentiation of monocytes, induction of phagocytosis and autophagy [43], as well as TACO gene transcription and consequent phagolysosomal maturation [44]. Distinct from atRA, 1,25D exerts its antimicrobial activity via cathelicidin antimicrobial peptide (CAMP), a 37-amino acid protein generated by monocytes, as well as other immune cells [67]. CAMP creates pores in cell membranes rendering the bacteria susceptible to degradation [68]. Liu et al demonstrate that this process relies upon TLR1 and TLR2 stimulation to aid in the conversion of vitamin D to the active form, followed by vitamin D receptor (VDR) expression for the release of cathelicidin [69]. A transcriptomics-led study indicates a link between pro-adipogenic peroxisome proliferator-activated receptor gamma (PPARγ), VDR and lipid metabolism, echoing the effect of atRA on nutrient availability for Mtb residing within granuloma [70]. Finally, 1, 25D has host-protective properties, blocking Th1 pro-inflammatory responses and stimulating Th2 responses thus balancing cytokine release post-infection [39].

With lower levels of vitamin D associated with higher mortality [71] and an increase in human cathelicidin following treatment with vitamin D3 [72] there is convincing evidence of the role of vitamin D in tuberculosis control. Nonetheless, clinical studies involving the supplementation of vitamin D have failed to meet expectations [73, 74] with few showing any benefit [75]. A major determinant in vitamin D effectiveness is exposure to sunlight which can leading to variability across populations. Phototherapy has been used in the treatment for TB for some time. In 1903, Niels Finsen won a Nobel prize by exploiting the use of high intensity light to treat patients with various forms of TB before a reason of its efficacy was known [76]. As overexposure to phototherapy can be dangerous and impractical therefore a targeted form of the active vitamin D metabolite could avoid such
administration difficulties. To summarise, vitamin D boasts host protective abilities, however, its potential in the clinic requires thorough pre-clinical and clinical investigations to determine optimal dose, treatment schedule and whether co-administration with another micronutrient such as atRA would be more beneficial.

2.1.3 Antioxidant vitamins

Vitamins B, C and E as well as vitamin A are classified as antioxidants – that is, agents that prevent oxidation and thus reduce cell damage caused by free radicals [39]. Obtained from various foods sources or supplementation, antioxidants have garnered interest for the treatment of TB for protective properties. Although not traditionally considered as anti-bacterial or immunomodulatory agents, antioxidants have protective properties that may render them useful as HDTs.

Pyridoxine (vitamin B6) has been routinely prescribed alongside isoniazid (INH) with the intention of reducing the risk of peripheral neuropathy. This undesirable effect of INH treatment is caused by its ability to inactivate or inhibit the production of pyridoxal 5’ phosphate (active), an essential co-enzyme in many metabolic reactions [77]. Other antioxidants have potential in TB therapy such as vitamin C (ascorbic acid), an essential reducing agent for maintaining a healthy body, negating lipid peroxidation & decreasing genetic mutations [34]. In Mtb infection specifically, vitamin A, C and E levels are decreased, concurrent with increased oxidative stress. Administration of these antioxidants could, therefore, reduce oxidative stress in infected patients and balance the immune response [78]. Two recent studies depict the anti-mycobacterial role of vitamin C (i) as an anti-oxidant, through gene transcription modulation which slows growth, promoting mycobacterial dormancy [79] and (ii) as a pro-oxidant via the Fenton reaction, causing an increase in ferrous ions and (as well as altered phospholipid metabolism) damaging the bacterial DNA, an effect which is specific to Mtb and prevents development of resistance [80].

Vitamin E protects against atherosclerosis but may have a role in TB treatment due to its anti-inflammatory properties as it is known to reduce pro-inflammatory cytokine production IL-1, IL-6, IL-8, IFN-γ and TNF-α as well as inhibit the reactive oxygen species (ROS)-driven induction of the NFκB inflammatory response [39]. The mechanism of action of vitamin E could possibly reduce tissue damage in tuberculosis pathogenesis.
2.1.4 Practicalities of vitamins as HDT

One significant and long-recognised challenge associated with the therapeutic use of vitamins is their inherent instability and the consequent difficulties faced in maintaining efficacy through the processes of manufacture, storage and administration. Vitamins A, C and D are susceptible to air, heat, light, moisture and pH [81], thus careful manufacturing procedures will be required for any vitamin-based HDT. One strategy for maintenance of stability and prevention of degradation following administration is targeted therapy through micro- (spray-drying) or Nano encapsulation, as discussed later in this review.

Further studies are required on anti-inflammatory properties of vitamins from cytokine analysis through to histology post-treatment in pre-clinical models as well as lung function in clinical trials. This would hopefully provide an indication of suitable dose and formulation for pulmonary delivery.

None of the vitamins discussed here interact with first-line TB treatment [82]. Apart from vitamin A, which is teratogenic in pregnancy and therefore is contra-indicated, other vitamins might prove important in the treatment of special populations such as paediatric tuberculosis or when the patient is pregnant. Finally, vitamins are reasonably priced (depending on dose) and widely available, altogether making nature’s own tonic an attractive adjunctive therapy to Mtb infection.

2.2 Other host-directed therapies

There are a large number of other agents under investigation as potential HDTs for TB infection. Here, we discuss some of the important pathways of the host immune response that may serve as potential targets and review those agents that are generating current interest.

2.2.1 Autophagy Inducers

Autophagy, the intracellular pathway of lysosomal degradation, is important for the degradation of Mtb within the macrophage [83-86], however virulent Mtb strains successfully arrest phagosome-lysosomal fusion in order to evade eradication [87-90]. Agents that induce autophagy, therefore, have garnered interest as potential HDTs.

Rapamycin, a potent inducer of autophagy through inhibition of the mammalian target of rapamycin (mTOR), reduces intracellular replication of Mtb within RAW 264.7 murine macrophages [84]. This
immunosuppressant is currently used in clinical practice to prevent transplant rejection, however concerns regarding adverse reactions [8], as well as negative effects on cell-mediated immunity at high doses [91], warrant a cautious approach to its potential use as HDT. Stage of disease may be of particular importance when considering this pathway as a prospective HDT target, as several case reports implicate rapamycin in reactivation of LTBI [92-94]. However, observations of enhanced pro-inflammatory cytokine response in dendritic cells [84], coupled with work by Jagannath et al. demonstrating enhanced antigen presentation by C57BL/6 murine dendritic cells in the presence of rapamycin, may support a role for this agent as a vaccine adjunct [95]. Furthermore, recent reports of superior efficacy of rapamycin delivered in inhalable particles compared to rapamycin in solution in terms of enhancing mycobacterial killing suggests an alternative route to oral administration may be advantageous[96], which should limit unwanted systemic effects.

A range of other inducers of autophagy have been identified [97], and a number of clinically-licensed agents observed to restrict Mtb intracellular growth impact autophagy [98, 99]. Several of these agents, including licensed anticonvulsants carbamazepine and valproate, have yielded promising preclinical results in a zebrafish model of infection [99]. Other autophagy-inducing compounds, such as fluoxetine (a selective serotonin reuptake inhibitor clinically licensed for the treatment of depression) and gefitinib (an epidermal growth factor receptor inhibitor used in the treatment of breast and lung cancers), have seen success in a J774 murine macrophage model, while the autophagy-inducing peptide Tat-beclin 1 restricted viral replication in vivo in C57BL/6J mice [98, 100]. Interestingly, there may exist a degree of strain-specificity in terms of efficacy of some of these agents, notably carbamazepine [99], which again underscores the need for careful planning with regard to trial design and future clinical introduction of this agent as a HDT.

The oral biguanide metformin commonly prescribed for the treatment of Type II diabetes mellitus, has received attention following reports by Singhal et al. [101] of AMPK-mediated induction of enhanced phagosome-lysosomal fusion in Mtb infection of primary human monocyte-derived macrophages (MDM) and the human monocytic cell line THP1. Widespread use of this medication worldwide, low cost and acceptable safety profile render it an attractive option as a HDT. Singhal et al. also report metformin to increase generation of reactive oxygen species (ROS) in infected primary human MDM, though this is not supported by experiments using lipopolysaccharide (LPS)-stimulated macrophages [102], and potential direct anti-mycobacterial effects have also been suggested [103]. Given the
metabolism-sensitive nature of AMPK, and of autophagy itself [104], possible impact of host nutritional status on efficacy of metformin as a HDT should be considered in future research.

### 2.2.2 Metabolic Regulators

A further reminder of the strong link between autophagy and host metabolism is the observation that statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors in clinical use as lipid-lowering agents, reduced Mtb intracellular survival, both in primary human PBMCs and MDM taken from hypercholesterolemic patients, as well as in vivo infection of C57BL/6 mice, through inducing progression of phagosome-lysosomal fusion [105]. Statins also decrease serum hypercholesterolemia, which has been linked to Mtb susceptibility [47], and a study involving 388 bacteraemic patients showed a significant decrease in mortality with statin therapy (6% v 28%, p=0.002) [106]. However, a retrospective analysis of diabetic patients found no benefit of statin therapy [107]. The possibility of host metabolic status impacting upon efficacy of this approach to HDT should again be considered in this context.

The complexity of metabolically-targeted HDTs is further emphasised by work involving peroxisome proliferator activated receptor gamma (PPAR-γ). PPAR-γ antagonist GW9662 enhanced BCG clearance by peritoneal macrophages from C56BL/6 mice [108], as did PPAR-γ knock-down in human MDM infected with M. tuberculosis [109], and recently antimicrobial effects of Vitamin D have been linked to PPAR-γ modulation, as described above [70]. Conversely, clinically licensed PPAR-γ agonists rosiglitazone (an anti-diabetic drug) and telmisartan (an anti-hypertensive agent) have been postulated as potential HDTs also, believed to reduce airway inflammation thus reducing infection-induced lung tissue damage [8, 110]. Again, this emphasises the complex interplay of both pro- and anti-inflammatory pathways that must be elucidated in the search for new HDTs for Mtb.

Despite the significant intricacy of metabolically-targeted HDTs, the recent upsurge in immunometabolism research is likely to generate a host of novel targets for manipulation of the host immune response in the near future [111, 112]. β-glucan, for example, a fungal cell wall constituent that confers non-specific protection against infection (termed “trained immunity”) through dectin-1-mediated epigenetic reprogramming of myeloid cells [113], thus holding potential as a HDT adjunct to vaccination, has been shown to mediate its effects through manipulation of intracellular glucose metabolism in macrophages [114]. However, as with many potential HDT targets, possible impact of
host immunogenetics on treatment response must be anticipated, with a loss-of-function polymorphism in the Dectin-1 gene occurring at a general allele frequency of 6 – 8 % in Caucasian populations and 4% in African populations, and several alternative splice isoforms of Dectin-1 have also having been described [115, 116]. Consequently, further elucidation of these pathways in the setting of Mtb infection is vital in order to harness this growing field in developing new HDTs, with consideration of patient subgroups likely and unlikely to benefit from metabolic manipulation is equally important.

2.2.3 Eicosanoid Manipulation

The eicosanoid signalling pathway, made up of prostaglandins and leukotrienes derived from the lipid arachidonic acid, exerts effects on cell death, adaptive immunity, mycobacterial clearance and cytokine production [117-120]. Briefly, lipoxigenase-generated lipoxin A4 (LXA4) and leukotriene B4 (LTB4) are thought to favour mycobacterial survival while cyclooxygenase-generated prostaglandin E2 (PGE2) favours elimination of the pathogen [117, 119-121]. However, as with other aspects of the immune response to Mtb infection, the role of these lipid mediators varies with stage of disease, with early PGE2 playing an essential role in intracellular bacillary killing but elevated PGE2 later in infection associated with decreased cell-mediated immunity and disease progression [122]. Furthermore, evidence suggests that skewing eicosanoid pathways too far in either direction may be detrimental in the setting of Mtb infection, with both under- and over-production of LTB4 associated with poorer outcome in patients with tuberculous meningitis [123]. Notwithstanding these challenges, manipulation of eicosanoid metabolism offers several promising HDTs that deserve consideration, and importantly which are already licensed for clinical use for other purposes, as outlined below.

Zileuton, a 5-lipoxygenase inhibitor that is used clinically for the treatment of asthma, has been suggested as a novel HDT following findings by Mayer-Barber et al. [124] that administration following Mtb infection improved survival and reduced lung colony-forming units (c.f.u.) in C57BL/6 mice, as did twice-weekly administration of intranasal PGE2. Conversely, cyclooxygenase inhibitor ibuprofen, a licensed anti-inflammatory analgesic that works to decrease PGE2 production, also improved survival in a C3HeB/FeJ mouse model of infection through reductions in lesion size and bacillary load [125], and diclofenac sodium has been shown to reduce c.f.u. in spleen and liver of infected Swiss albino mice both alone and in combination with streptomycin [126, 127]. Thus, manipulation of eicosanoid balance presents a complex area to navigate in development of HDTs. It has also been suggested
that different mycobacterial strains may influence eicosanoid balance in different ways to facilitate survival [7], and host genetic variation is likely to play a significant role in determining the benefit of eicosanoid-targeted HDTs [13].

2.2.4 Corticosteroids

Corticosteroids have been used as a HDT in the treatment of Mtb since the 1950s [128], and a recent meta-analysis of 41 trials found a 17% reduction in mortality (RR 0.83, 95% CI 0.74–0.92) associated with steroid administration [129], likely due to reductions in tissue damage caused by excessive inflammation. Furthermore, a phase 2 clinical trial involving 187 eligible patients demonstrated that prednisolone co-administered with standard anti-tuberculous therapy significantly increased sputum conversion at four weeks in HIV-associated TB patients, though associated with substantial adverse events likely to preclude clinical use at this dose [130]. However, host immune genetics again must be considered in relation to the role of corticosteroids in the treatment of TB. Tobin et al. observed that a single nucleotide polymorphism in the leukotriene A4 hydrolase (LTA4H) promoter underlay two divergent causes for poor outcome in tuberculous meningitis – namely, insufficient inflammation and excessive inflammation – and thus impacted response to adjunctive corticosteroid therapy [13, 123]. This emphasises that empirical initiation of HDT without consideration of immunogenetics of individual patients could result in amplification of the detrimental outcome – possibly presenting an argument for a “personalised medicine” approach.

2.2.5 Cytokine Modulation

Cytokines play critical roles in both in innate and adaptive immune response to Mtb infection in humans. As with other components of the immune response to infection, the roles of various cytokines may fluctuate depending on the stage of disease, host genetic and environmental factors, and mycobacterial strain, emphasising the importance of mechanistic understanding of each HDT. Despite this complexity, a number of recombinant cytokines and agents that modulate cytokine production show promise as HDT’s.

The pro-inflammatory cytokine interferon-γ (IFN-γ) plays an essential role in the intracellular killing of Mtb, by overcoming the autophagic block, as well as activation of the Th1 response [84, 131-133]. A clinical trial of 77 included patients with cavitary pulmonary TB randomised to DOTS alone, DOTS with nebulised recombinant IFN-γ1β (Actimmune® – a recombinant form of IFN-γ that is currently
FDA-approved for the treatment of chronic granulomatous disease, osteopetrosis and Friedrich's ataxia[134-136]) or DOTS with subcutaneous recombinant IFN-γ1β over 4 months found significant increase in sputum smear conversion at 4 weeks with nebulised IFN-γ1β with DOTS compared to the other two groups (60% vs 36%, p=0.03). There was also non-significant trend towards increased sputum culture conversion at 4 weeks (32% vs 18%, p=0.15), despite no difference in radiological outcome, suggesting potential utility of this HDT in decreasing duration of infectivity [137]. This observation has since been supported by meta-analysis of five trials, of which only one was randomised, using aerosolised recombinant IFN-γ [138]. A number of small, uncontrolled, off-label trials using recombinant IFN-γ in the treatment of drug resistant tuberculosis have consistently reported radiological improvement following completion of IFN-γ therapy, though results in terms of sputum conversion differ between studies [139-141]. Adjuvant administration of interleukin-12 (IL-12), a potent inducer of IFN-γ release from T cells [142] was also reported to improve outcome in a patient with progressive pansensitive Mtb infection [143]. This is in line with observations of this cytokine's important role in the immune response to Mtb, with IL-12 receptor β1 deficiency recognised as the most common form of Mendelian susceptibility to mycobacterial infection [144]. Interestingly, we have recently described a role for the MyD88-adaptor-like (Mal) signalling protein in INF-γ signalling within the macrophage [145]. Mal activity is abrogated by a single nucleotide polymorphism at position S180 in its coding gene, and homozygosity for this SNP confers increased susceptibility to Mtb infection [146]. It is therefore conceivable that genetic variation in Mal genotype may impact upon efficacy of IFN-γ as a HDT for Mtb infection, and failure to consider this moving forward could cause underestimation of efficacy.

Similarly, the pro-inflammatory cytokine interleukin-2 (IL-2) is fundamental in the host response to Mtb infection[147] through activation and expansion of T cells, facilitating adaptive immune responses [148, 149]. Though early, non-randomised trials indicated potential efficacy as a HDT in multidrug-resistant (MDR)-TB [150, 151], a subsequent randomised trial involving 110 patients with pansensitive pulmonary TB found no effect [148]. However, as Geffner et al.[152] demonstrate in a study including 25 MDR and 20 pansensitive TB patients, MDR strains induce greater Th2 and decreased Th1 responses when compared to pansensitive strains. Thus the mechanism of action of recombinant IL-2 may render it a uniquely efficacious HDT for the treatment of MDR-TB rather than pansensitive disease. A recent multi-centre randomised trial conducted in China involving 50 patients with MDR-TB
reported both microbiological and radiological improvement with recombinant IL-2 therapy [149]. These data may warrant further trials of recombinant IL-2 for use as HDT in MDR-TB.

In contrast to IFN-γ and IL-2, pro-inflammatory cytokine tumour necrosis factor-α (TNF-α), despite its essential role in maintenance of granuloma integrity in latent TB infection (LTBI) [153, 154], has been suggested to exacerbate the effects of inflammation in severe disease. Accordingly, monoclonal antibodies directed against TNF-α have demonstrated efficacy as HDTs in several case reports [155-157]. Similarly, a number of case reports suggested a role for thalidomide, an agent which downregulates TNF-α while simultaneously inducing IFN-γ and IL-2 [158, 159], as an adjunctive HDT in TB treatment [160, 161]. Thalidomide is currently used to treat erythema nodosum leprosum, the type-2 immunologic reaction associated with Mycobacterium leprae infection, again believed to mediate its effects through downregulation of TNFα, as well as upregulation of IL-2 [162-164]. However, a randomised controlled trial involving 47 children with tuberculous meningitis found no benefit of adjunctive thalidomide and was terminated early due to increased adverse events and deaths in the thalidomide arm [165]. Phosphodiesterase (PDE) inhibitors, including agents currently licensed for other clinical uses such as cilostazol and sildenafil, also regulate TNF-α production through decreasing cAMP activation and have shown promising results in preclinical murine and rabbit studies [166-170].

Thus, cytokine modulation remains a viable option in terms of novel HDT development. Kaufmann et al. provide a comprehensive review of recombinant cytokines and cytokine inhibitors currently under pre-clinical and clinical investigation as TB HDTs [9], which we are likely to hear more of in the future.

2.2.6 Protein Kinase Inhibitors

Mtb utilises a range of cell surface receptors to gain entry to the macrophage [171], including tyrosine kinases [172], for which a number of inhibitors are used clinically for treatment of chronic myeloid leukaemia (CML) [173]. Imatinib, one such tyrosine kinase inhibitor, has been shown to reduce bacterial uptake as well as increase intracellular killing of Mtb by enhancing acidification of the phagosome in murine macrophages [174, 175]. Furthermore, at low doses imatinib augments myelopoiesis, which may further reduce mycobacterial load [176]. A number of other tyrosine kinase inhibitors currently licensed for treatment in CML may also have anti-mycobacterial effects, warranting investigation.
2.2.7 Matrix Metalloproteinase Inhibitors

Likewise, the matrix metalloproteinase (MMP) inhibitor doxycycline is also licensed for clinical use, both as an antimicrobial agent and (at lower doses) to reduce tissue damage in periodontal disease [177]. MMPs have been implicated in the lung tissue destruction evident in pulmonary TB [178], and doxycycline suppresses MMP-1 and -9 secretion in Mtb infection, both in vitro and in vivo, resulting in reduced lung c.f.u. 8 weeks post-infection in a guinea pig model [179]. As with many of the other host immune pathways that may be amenable to manipulation by HDTs, MMP signalling is also influenced by gene polymorphisms [180], thus strategic patient selection may be required to maximise efficacy of doxycycline or other MMP inhibitors as HDTs.

2.2.8 MicroRNAs (miRs)

MicroRNAs – short, non-coding RNAs that are involved in the post-transcriptional regulation of mRNAs – are recognised to play an important role in controlling protein expression in order to regulate diverse intracellular pathways, including several of the pathways under investigation as potential HDT targets [181, 182]. Made up of short nucleotide sequences, miRNAs can be readily targeted using engineered oligonucleotides, as evidenced by the effect of miR-122 inhibitor miravirsen which has shown efficacy in chronic Hepatitis C infection and is currently being evaluated in a phase 2 clinical trial[14, 183]. Several miRNAs are upregulated in Mtb infection [184-186], including those involved in regulation of important anti-mycobacterial processes such as autophagy [187, 188], apoptosis [189, 190] and cytokine production[191]. Given the relative ease of designing inhibitors to miRNAs, it is likely that they will provide an attractive target for manipulating the host response to Mtb infection in future HDT development [182].

3. Potential advanced formulations for targeted, respiratory delivery of host-directed therapies

Treating by the same route as infection may further enhance the potential of HDTs as a viable therapy for Mtb infection. Drug delivery by inhalation is a popular method of drug administration used since ancient Egyptian times with the first inhaler described in the 1600's [192]. Aerosolised treatments require a droplet size of approximately 1 - 5µm for deposition in the lower airways but ongoing advances in formulation and device design it seems reasonable that treatment regimens of pulmonary diseases would include at least the option of local delivery of medications. A study examining the
pharmacokinetics of the first-line anti-tubercular drug rifampicin in guinea-pigs when administered by various routes by Garcia-Contreras et al delivered fundamental evidence as to why an inhaled treatment option for TB should be seriously considered [193]. The plasma versus time curves in figure 3 (A) and (B) show faster initial absorption of rifampicin when administered via the pulmonary route (IRPP) followed by similar plasma concentration levels, over a timeframe of 12 hours, in comparison to oral administration (ORPP) despite only half of the oral dose administered via insufflation. Analysis of bronchoalveolar lavage (BAL) samples taken from the same animals revealed lung concentrations of rifampicin 7 -9 fold higher in the pulmonary treated group, maintaining levels above the minimum inhibitory concentrations for rifampicin for the duration of the study [193]. Results such as these exhibited by Garcia-Contreras et al make inhaled therapies a promising alternative for both pulmonary and extra pulmonary TB. By increasing the level of active pharmaceutical ingredient (API) at the site of action and achieving comparable plasma levels to oral treatment with lower doses an inhaled treatment could facilitate reduced dosage regimens and decreased systemic side effects, thus encouraging compliance. To date, a great deal of research into targeted formulations for the treatment of Mtb infection has been carried out using standard anti-tubercular drugs [194], therefore a novel HDT cargo may provide an ideal synergy of drug and design to get a new treatment to market.
Figure 3. The absorption of rifampicin following administration by intravenous, oral and pulmonary routes. Plasma concentrations in male Dunkin-Hartley guinea pigs were monitored over a 12 hour period post-treatment with (Figure 3(A)): an intravenous solution (IV, 10mg/kg), oral suspension (ORS, 40mg/kg), oral suspension of porous particles (ORPP, 40mg/kg) and a dry powder of rifampicin porous particles by inhalation (IRPP, 20mg/kg). Figure 3(B) displays the data presented in Figure 3(A) corrected by dose to allow a comparison of treatment groups [193]. Data kindly reproduced by permission of the publisher American Chemical Society.

3.1 Formulation experience with HDT in clinical and non-clinical settings

Reformulation of the first-line anti-tubercular agents began some time ago [194, 195]. Interest has also arisen in the formulation of second-line TB treatments such as the injectables (Aminoglycosides) for inhalation with the aim of reducing undesirable effects[194]. Little has been published on HDT inhalation therapy thus far apart from recombinant Interferon-γ (IFN-γ) administered off label as an adjunctive treatment for TB, as previously mentioned. IFN-γ has been used in the clinic for both drug
sensitive and drug resistant TB for a number of years - administered as a nebulised solution in normal saline with improved patient outcomes [137, 138, 196, 197]. One study delivered a nebulised solution with a mass median aerodynamic diameter (MMAD) of 3.2µm, however, > 50% of the dose was lost in the upper airways, a result that could be attributed to heterogenous particle sizes and/or the pathophysiology associated with TB infected lungs [196]. Nonetheless, with just 40% reaching the lungs levels of IFN-γ were significantly increased in the bronchoalveolar (BAL) fluid (1hr post treatment) of patients with pulmonary mycobacterial disease. In addition, aerosolised IFN-γ was well tolerated, reducing clinical and bacteriologic signs of mycobacterial disease after incorporation into standard regimens which is promising for administration of similar protein based HDT’s [196]. Small interfering RNA (siRNA) was successfully administered to Mtb infected mice as a solution via the intrapulmonary route [198] and an inhaled formulation of simvastatin for use in a pressurised Metered Dose Inhaler (pMDI) was developed with the aim of treating inflammatory airways conditions. A stable formulation of simvastatin and ethanol as a co-solvent was produced with a fine particle fraction of 30.77 ± 2.44% [199].

Vitamins have been formulated for a range of indications despite several difficulties due to their physiochemical instabilities [81]. AtRA has so far been incorporated into micro- and nanoparticles, liposomes and niosomes for oncology indications with sizes suitable for lower lung deposition, an encapsulation efficiency generally >50% and controlled release of drug for over 30 days in physiological medium [200-203]. Successful in vivo administration of atRA by intratracheal instillation resulted in a longer half-life of drug compared to intravenous administration (IV) [204]. Inhaled atRA-liposomes were safely administered to a patient with extensive emphysema [205]. Vitamins B and C were successfully spray dried to maintain stability and controlled release in food processing yielding microparticles that would potentially be suitable for inhalation [81], while vitamin E metabolites have also been formulated into many preparations, including liposomes, decreasing mortality in an in vivo lung hypoxia model by half [206]. Nanoparticles made of PLGA and chitosan-coated PLGA for enhanced bioavailability incorporating vitamin E [207]and flaxseed oil were the basis of a nebulised solution examined for the potential treatment of lung injury post fire smoke inhalation [208].

Finally, another option for development could be a multiple payload formulation including more than one drug in a delivery system. One review articles lists numerous combinations that have been tested using standard anti-tubercular drugs in liposomes, microparticles and nanoparticles with the aim to
potentially reducing the burden of treatment to the patient [194]. Depending on the physico-chemical characteristics of the HDT to be formulated, there are many choices available some of which will be discussed later.

3.2 Inhalation devices for HDT

Medical devices, for any indication, must be designed to the highest quality in order to maintain physico-chemical properties of the formulation, generate reproducible doses, allow ease of administration for the patient and be cost effective. The choice of inhalation device should be considered as early in the product development process as possible to ensure formulation and manufacture of the API are tailored effectively for the device.

There are three types of devices available for administration by inhalation, first, the pressurised metered dose inhaler (pMDI) which comprises of the drug formulation suspended in a propellant that is expelled as a measured dose from device once activated by patient. While commonly used in conditions such as asthma, pMDI’s require effective patient counselling to ensure precise coordination of the actuation and hence inhalation of the dose [209]. With their portability and quick use, avoiding the need to load doses as required with other devices, pMDI’s are ideal for the treatment of exacerbations [210]. Although API’s, not usually administered by inhalation, have been successfully formulated for administration via a pMDI, for the treatment of inflammatory lung diseases including clarithromycin [211], theophylline [212] and the potential HDT simvastatin [199], the pMDI can be limited with regard to volume making them unlikely to be used for tuberculosis treatment.

Alternatively, dry powder inhalers (DPI) generally rely on the patients breath to activate and draw out each dose out of system making them easier to use than pMDI’s if the patients inspiratory flow is sufficient [209, 210]. An example of a dry powder inhaler can be seen in figure 4 (A), the T-326 which is used in conjunction with PulmoSphere™ technology – porous microparticles carrying tobramycin described later (section 3.4.1). The device, and complementary formulation (Tobi™ Podhaler™), is indicated for cystic fibrosis (CF) patients where lengthy courses of high dose antibiotics as prescribed recurrently. In the past, CF patients were restricted to the use of nebulizers but the T-326 allows high doses of antibiotics to be administered efficiently through the use of re-fillable capsules containing the dry powder formulation, a key property of any potential inhaled TB treatment where large doses are likely to be required [213, 214]. DPI’s have great potential for inhaled TB treatment due to improved
stability of the actives in the dry powder form and cost as the product including formulation can be sold as one.

Nebulisers emit a gentle mist under compressed air that can be taken in by mouth and or nose via a mask suitable for all ages and patient conditions. Large doses of one or more drugs can be administered via a nebuliser leading to their essential role in chronic conditions such as CF [210]. Nebulisers can be divided into three categories depending on how the aerosol is generated: jet nebuliser (Venturi effect), ultrasonic nebuliser (piezo-electric transducer) and vibrating mesh (piezo-electric crystal and plate) [194]. The modern vibrating mesh nebulisers are lightweight, portable, silent and offer improved stability of solution and, hence, active ingredient making them attractive for long term treatments in conditions such as TB [26, 209]. A review of inhaled TB treatments shows that the majority of human trials involving off label inhaled medications for TB were carried out by aerosol using a nebuliser [194]. The Aeroneb Go nebuliser system (Aerogen, Galway, Ireland) shown in Figure 4 (B) is an example of a homecare device based on the vibrating mesh technology. The disadvantages of nebuliser devices, which may affect TB treatment in certain regions, include the need for power, water for reconstitution of product and cleaning of the device and the formulations often require cold-chain transport and storage.
3.3 Particle properties and their influence on the effectiveness of inhaled therapies

Whilst API's can be formulated and administered as a solution or suspension via a nebuliser as discussed, an alternative method is to develop a delivery platform encapsulating the drug within inhalable particles for administration using a dry powder inhaler. Particles are fabricated using suitable excipients that can protect and maintain the stability of the cargo (API) and increase the targeting capacity to specific cells or organs. Release of the cargo can also be manipulated using these drug delivery systems (DDS) with the aim of improving administration schedules. This is a particularly attractive option as it could enable spatial and temporal control of inhaled delivery for emerging HDT's including increased targeting and corresponding immunomodulation required for defence against mycobacterial infection [21, 215-217]. Studies assessing inhaled formulations for TB are continually being reviewed [194, 195, 209]. Here, along with highlighting the essential properties of inhaled formulations, we discuss alternative as well as advanced particle engineering methods available for the potential development of targeted HDT.

The basic requirement for any form of inhaled therapeutic is that the formulation can be dispersed and deposited effectively at the site of action within the pulmonary system. This requires fine control over the aerodynamic properties of the drug-loaded particles. For solid spherical particles there is a restricted particle diameter window in which the particles neither impact on the throat to be swallowed nor are they expelled in the patients' breath. The optimum diameter size for solid spherical particles to deposit in the lungs is considered to be approximately 1 - 5 µm [218]. However nano-sized particles have also been studied, either as standalone particles or in composite systems, due to the high-surface area, encapsulation efficiency and their ability to escape mucociliary clearance [219, 220]. Particle size also impacts the degree of phagocytosis of inhaled particles by alveolar macrophages. Nanoparticles are typically engulfed by macrophages through two main endocytosis pathways - phagocytosis or pinocytosis - whereas microparticles are thought to be primarily phagocytosed [221].
This is a particularly important facet of formulation design for inhaled TB therapies aimed at targeting these cells via inhalation. The structural features of particles including their density can impact their Mass Median Aerodynamic Diameter (MMAD) with large porous structures displaying aerodynamic behaviour that is similar to smaller particles [222]. The size and shape of the final particle in addition to the material it is formed from (e.g. composition, molecular weight) and pH of the surrounding environment all have implications for the drug release profile and particle degradation in the lungs [223, 224]. High surface area structures such as nanoparticles typically display more rapid drug release and particle degradation rates. Additionally the surface topography and particle morphology will influence phagocytosis by macrophages. Modifications such as surface coatings or fibrous strands on the particle surface, for example through PEGylation [225] will affect MMAD and/or macrophage uptake.

The choice of material used to manufacture the particle can have a significant effect on the overall efficacy of the inhaled therapy. The high humidity within the lungs favours the use of hydrophobic materials to prevent aggregation of the particles due to wetting; this would cause them to lose their aerodynamic properties [226]. The particle degradation in the lungs and drug release profile are also heavily influenced by the material forming the particle matrix. A key feature of particulate delivery systems is that they allow more sensitive API’s to be utilised as they can protect the API from enzymatic degradation and oxidation [209], of particular importance with many labile HDT’s.

The technique employed to produce particles suitable for inhaled therapies also depends heavily on the materials and API involved. Production methods which require harsh conditions e.g. extreme temperatures to produce the final particle may result in adverse effects to sensitive molecules during fabrication. In addition each method may present different degrees of control over the final particle size, morphology and other properties.

3.4 Particle engineering approaches

3.4.1 Microparticle based Drug Delivery Systems

Microparticle-based therapies are one of the most studied technologies for the delivery of anti-tuberculcular treatments by inhalation due to their size and ability to deliver treatments directly to the site of infection within the alveolar macrophage [17, 18, 25]. Evidence of immune simulation may provide additional benefits of this treatment option over standard oral administration [216]. Previous work in
this area has indicated that when inhaled microparticles are phagocytised they may induce classical bactericidal activation [227-229]. In this manner, inhaled microparticles can offer multiple modes of action towards the treatment of TB from the delivery of therapeutics and the activation of the infected macrophages.

For example, microparticles containing large anti-tubercular drug payloads (isoniazid and rifabutin) have been examined for their effect on cytokine responses in the bronchoalveolar lavage (BAL) fluid of mice compared to orally administered treatments. An upregulation of the pro-inflammatory cytokine TNF-α, an essential component of the innate immune response to Mtb, was found in the groups treated with inhaled microparticles only (~0.5mg/Kg of each drug) whereas TNF-α levels were similar to uninfected and untreated controls when isoniazid and rifabutin were administered orally (4.5mg/Kg of each drug), with and without the addition of inhaled microparticles. These results support the use of a vehicle as a key element for resultant immune modulation and potentially reduced dosage requirements in early disease management in particular where TNF-α is crucial in host defence [217].

Our group has also shown the significant effect of unloaded microparticles on reducing bacterial viability through the induction of the transcription factor NFκB and autophagy [216], proving the value of microparticles as a new treatment platform for TB.

There are numerous techniques employed to produce drug-loaded microparticles for potential treatments. Each technique offers advantages and drawbacks resulting in a variety of particle properties. We have selected some of the commonly used techniques with interesting variations that might be suitable for a novel inhalable HDT.

Spray drying relies on controlled evaporation of aerosolised droplets, containing the dissolved API and excipients, dispersed by an inert gas flow into an enclosed drying chamber before being deposited in a collection chamber [230]. Whist a large quantity of starting material is required making it costly for some APIs, there are numerous advantages of spray drying including: medium scale production in a quick one-step process, high encapsulation of cargo and good reproducibility [230].

Spray drying is a common method used in studies involving standard anti-tubercular drugs and the FDA G.R.A.S listed polymer Poly (Lactide -co-glycolide) (PLGA) [231] and also been used to encapsulate the potential HDT, nitric oxide. Nitric oxide production is an essential component of the innate immune response which can be halted by Mtb, by delivering nitric oxide donors to the site of infection Verna et al were able to decrease bacterial viability in THP-1 derived macrophages and
control release of NO for up to 72 hours in comparison to nitric oxide donors in solution [21]. There are many other options available to adapt the spray-drying method and DDS for specific cargos depending on the desired outcome. Durham et al prepared spray dried pyrazine acid leucine/ammonium salt microparticles. These particles displayed spherical morphology, with the different counter-ions affecting the final morphology and parameters required for inhalation therapy [232]. Kwok et al have produced mannitol-based particles with a MMAD of 2.2-3.1 µm suitable for inhalation therapies. These particles were loaded with antimicrobial peptides, D-LAK120-HP13 and D-LAK120-A synthetic peptides, intended for treatments of drug resistant TB [233]. Similarly, Contreras et al examined the behaviour of rifampicin/ l-leucine thin-walled porous particles in the lungs of guinea pigs which displayed in increased bioavailability compared to oral dosing [193]. Spray drying and spray freeze drying have both been employed for the production of peptide-based pH responsive powders for the delivery of therapeutic nucleic acids to the lungs. Due to their pH responsive nature after phagocytosis they can escape the phagolysosomal maturation before degradation takes place and they release their payload [234]. Collapsed sphere microparticles with a diameter of 2.88 ± 0.8 µm and a MMAD of 1 µm containing the HDT rapamycin were examined by Gupta et al. These particles were taken up by macrophages within 3 hours and were more effective in clearing intracellular mycobacteria compared to rapamycin in solution [96]. Rifampicin loaded PLGA based nanoparticles within mannitol microspheres were prepared by Ohashi et al through a single step four nozzle spray drying technique for inhaled tuberculosis treatment targeting alveolar macrophages [235]. Treatment of the chronic pulmonary disease cystic fibrosis (CF), caused by infection with Pseudomonas aeruginosa, has been greatly advanced by the introduction of PulmoSphere™ technology for use in a dry powder inhaler. PulmoSphere™ particles are porous and in the context of CF contain the antibiotic tobramycin. The sponge-like, drug loaded particles are prepared by an emulsion based spray-drying method resulting in physico-chemical characteristics required for efficient pulmonary targeting such as spherical shape, low micron size and reduced agglomeration [213, 214].

Solvent evaporation, using single or double emulsions, is another commonly used method in the production of particles for anti-tubercular treatment [16, 25] whereby an emulsion (o/w or w/o/w) containing the active ingredients, polymer and surfactants such as poly vinyl alcohol is homogenised before allowing any organic solvents to evaporate. Micro- or nanosized particles are subsequently
collected by lyophilisation. More complex structures are also possible using these methods to modulate size, encapsulation efficiency and release rate and lung deposition patterns as seen in the study by Ungaro et al through a combination of solvent evaporation and spray drying methods [236]. To overcome issues with NP deposition in the lungs due to their submicron size Ungaro et al modified the PLGA NPs to achieve optimal size, zeta potential, drug loading of the antibiotic used (tobramycin) and subsequently spray-dried the NPs with lactose to form nano-embedded microparticles (NEM). By varying the composition of NPs, and hence NEMs, differences in aerosolisation properties and lung deposition were achieved suitable for a potential inhaled treatment for respiratory infections [236]. Rifampicin loaded PLGA micropsheres have been frequently examined in the literature for their treatment of TB. Mycobacterial mycolic acids have been coated onto the surface of isoniazid-loaded PLGA-based nanoparticles via a double emulsion solvent evaporation technique where they provide an additional targeting function to the inhaled therapeutic. The addition of a mycolic acid (MA) ligand resulted in increased phagocytic uptake of the NPs, in comparison to NPs without MA, allowing phagolysosomal maturation and delivery of the cargo directly to residing mycobacteria within the macrophage [237].

3.4.2 Lipid based Drug Delivery Systems

Vitamins have been incorporated into lipid based carriers as referenced earlier, this is likely due to the hydrophobic nature of Vitamins A and E [203, 206] but there are many options available for similar hydrophobic HDT compounds. Liposomes are an alternative carrier system to polymeric particles available for controlled pulmonary delivery with desirable biocompatibility owing to their construction from phospholipids which are a normal constituent of lung surfactant [209]. These submicron sized liposomal carriers are formed when various phospholipid combinations are dissolved in an organic solvent producing a film which is hydrated in an aqueous solution resulting in an aqueous core surrounded by one or more bilayers forming small or large unilamellar vesicles (SUV and LUV) or multilamellar vesicles (MLV) [238, 239]. Drugs can be entrapped in either the aqueous phase or a lipid bilayer depending on the hydrophilicity of the API. Conventional anti-tubercular drugs have been encapsulated within liposomes [239] leading to reduced bacterial load when delivered in vivo [240, 241]. Liposomes are, however, relatively unstable systems and undergo fusion and aggregation over time. As pulmonary delivery of a therapeutic is inherently dependent on the physical features of the dispersed particles prolipsomes, dry free-flowing powder particles that form a liposomal dispersion on
contact with water made from freeze dried or spray dried liposomes, are preparation possibilities [242]. Pyrazinamid-based proliposomes were prepared by spray-drying for delivery of therapeutics to the alveolar region and resulted in corrugate spherical particles, depending on the ratios of and crystalline nature of the involved components, with a MMAD of 4.26-4.39 µm, a fine particle fraction of 20-30% and 26-45% encapsulation efficiency. These loaded proliposomes were shown to be less toxic to pulmonary cells (Calu-3, A549 and NR8383) than pyrazinamide alone in vitro as well as showing no liver or renal toxicity in vivo following intratracheal administration to male Wistar rats [242]. In a comparable study, levofloxacin-based proliposomes have shown similar physical features and toxicity with anti-mycobacterial activity against Mycobacterium bovis and Mycobacterium tuberculosis, although the latter was comparable to levofloxacin alone [243].

Recently, interest in solid lipid nanoparticles (SLN’s) has developed where the core is a solid lipid in contrast to the aqueous core of the liposomes. SLNs are composed of lipids such as diglycerides, fatty acids and waxes that are melted allowing the addition of an API and then homogenised in the presence of an aqueous phase including a surfactant for stabilisation of the core. The SLN’s can be collected following cooling of the nano-emulsion [244, 245]. A comparison study of SLNs composed of two different lipids (glyceryl dibehenate and glyceryl tristearate) show good drug loading with encapsulation efficiencies of 81.0 ± 9.6% to 89.9 ± 5.1%, stability at high temperatures, compatibility with A549 and Calu-3 cell lines as well as efficient uptake by THP-1 derived macrophages with internalisation of SLNs evident just one hour after treatment [245]. Pandey et al also presented promising in vivo efficacy data when rifampicin, isoniazid and pyrazinamide loaded SLNs provided sufficient controlled release of the cargo and significantly decreased bacterial viability in Laca mice infected with H37Rv following oral administration in comparison to free drugs [244].

Nanostructured lipid carriers (NLCs) employ a blend of both solid and liquid lipids and Song et al utilised this technique to produce cationic mannosylated NLCs for the targeted delivery of rifampicin to alveolar macrophages. With an encapsulation efficiency of >90% and a diameter of approximately 160 nm, mannosylated rifampicin NLCs displayed greater macrophage targeting and uptake than unmodified rifampicin NLCs in both a NR8383 cell line and in vivo after intravenous administration without causing undue inflammation or toxicity [246]. Equally, solid lipid particles can be formed as solid lipid microparticles (SLMs) for suitability in a range of applications. These SLMs can provide acceptable aerodynamic for pulmonary delivery and a negative surface charge to promote
phagocytosis as shown in the murine macrophages cell line J774 [19]. Research has been carried out to combine a solid polymer-based core with an external lipid coating thereby combining the benefits of each system such as the biological compatibility of the external lipid coating with the increased stability of the polymer core to protect any delivered therapeutics. Rose et al examined the fabrication of lipid modified PLGA-based nanoparticles which displayed immune responses similar those induced by liposomes of the base lipids and highlights the additional potential of lipids for modifying their biological properties of inhaled particles [247]. Bhardwaj et al also examined lipid-polymer hybrid nanoparticles encapsulating isoniazid and ciprofloxacin which displayed increased uptake compared to the free drugs and remained stable over a 6 month period which was attributed to the polymer core [248].

3.4.3 Alternative Particle Engineering Strategies

Despite the commonly used particle manufacturing methods described above providing good drug loading, controlled release and many options for targeting of AMs to date there are more diverse techniques for particle preparation currently being developed by researchers. The possibility of controlling particle production resulting in monodisperse batches of pre-determined measurements, using methods suitable for labile APIs, allowing straightforward scale-up whilst simultaneously reducing wastage would provide an array of benefits for HDT product development. The combination of a host-directed cargo with precision particle production could potentially allow sufficient fine-tuning of treatments to tackle the issue MDR-TB. Examples of these sophisticated methods are as follows:

Electrohydrodynamic methods have recently become more prevalent in the production of particles and other micro-structured scaffolds with biological applications. Biological polymers, such as chitosan and alginate, and biologically compatible polymers, such as PCL, PLA, PLGA, coupled with variations in the setup, operation parameters, concentration and polymer chain entanglements can produce particles with a range of different morphologies. These include various shapes of single particles, coated particles, multiple particle layers, nanoparticle encapsulated inside micro particles and multi-compartment with a focus towards the development of multifunctional particles capable of targeted drug delivery [249]. Electrohydrodynamic methods provide a gentler approach than some previously described processes that require high temperatures, such as spray drying, therefore maintaining the integrity and bioactivity of sensitive cargos such as proteins and vitamins[250]. Methods such as electro spraying could also reduce product wastage by optimising the method of
particle collection. This modification along with the production of monodisperse batches leading to less product yield being lost to filters, again like spray drying, is especially important when expensive cargos are used [251]. Lastly, electro spraying can be readily scaled-up by increasing the number of nozzles whereas scale-up is a key restraint of a number of the conventional methods mentioned above, particularly solvent evaporation [249].

Preparation of microstructures from sacrificial template methods provide an avenue for more variations in and control of morphology. Tscheka et al employed such a method to produce cylindrical polysaccharide agarose rods 10.24 ± 1.47 µm in length with a width of 1.99 ±0.08 µm. These were formed through the cross-linking of 500 nm diameter silica beads within the 10 µm thickness and pore size 2 µm holes etched into polycarbonate membrane. These rods offer advantages due to their uniform shape in that they are easily dispersed, similar in structure to Mycobacterium tuberculosis and displayed a direction dependent rate of rod uptake with the phagocyte moving around the rod in order to reach an adequate orientation to achieve invagination increasing the residence time of the inhaled treatment [252].

PRINT (Particle Replication In Non-wetting Template) is another particle fabrication technique that is beginning to see more frequent use for the production of inhaled nano- and micro-sized therapeutics. This method is comprised of soft-lithographic techniques adapted from the semi-conductor industry and has shown more precise control over particle size, shape, composition and surface chemistry [253]. Roberts et al utilised this technique to fabricate PLGA, PEG and hydrogel (polyethylene glycol derivatives) particles sized similar to common bacteria, virus, platelets and red blood cells (80x320 nm, 1 µm, 1.5 µm and 6 µm) and in a variety of shapes such as rods, cylinders and donuts. The aim of the study was to allow the prediction of baseline inflammatory outcomes from controller-defined batches therefore provide a well-defined platform for which suitable cargoes can be loaded. Along with uptake by murine macrophages in vitro, these precision-made particles did not stimulate any detectable levels of the inflammatory cytokines TNF-α, IL-6 or IL-1β and did not show signs of toxicity when assessed in bone marrow derived macrophages, results which were echoed in vivo by a lack of immune cell recruitment. The level of endotoxin contamination in the particles was also tested using a Limulus amebocyte lysate (LAL) assay. The results were negative indicating that the use of PRINT technology is potentially a ‘cleaner’ process to solvent evaporation, for example, which involves numerous steps in an open system leaving the formulation susceptible to contamination [253].
Although the results of this study conflict studies discussed earlier where PLGA particles stimulate immune response [217] PRINT technology may provide an alternative avenue for the treatment of late stage disease where excess inflammation severely impacts the lung. It is imperative that if a carrier is used to deliver a potential HDT by inhalation that the immunogenic effect of that system is fully understood to allow synergy with a suitable cargo.

4.0 Preclinical efficacy models specific to HDT

Debate arises regarding the benefit of pre-clinical testing for established, repurposed drugs apart from dose optimisation, nevertheless it important to understand cellular mechanisms of treatments, particularly in HDT, and so work continues in research community. Detailed descriptions of the pathways manipulated by HDTs were discussed earlier and in the literature in [7-9]. Rather than an exhaustive review of TB models here we discuss some important points regarding model choice for both *in vitro* and *in vivo* screening of potential HDT for Mtb infection bearing in mind that it is not possible to fully recapitulate the complex and variable host-pathogen interactions which occur in human TB disease in any one model.

4.1 *Mycobacterium tuberculosis* (Mtb) strain selection

For both *in vitro* and *in vivo* testing the bacterial strain must be chosen carefully depending on the experimental end point sought. Mtb is characterised by phenotypic heterogeneity, giving rise to the variable traits and metabolic states – active and latent. These ‘states’ of activity can make it difficult to screen drugs depending on the mode of action. Furthermore, the number of resistant strains of Mtb are growing and so should be considered when testing efficacy of treatments. Bacille Calmette-Guerin (BCG ) has been used for research purposes for many years as it is the attenuated strain used in the current vaccine, however, BCG lacks ESAT-6, a protein central to replication in macrophages and DNA sensing in the cytosol which is required for induction of autophagy [254]. The virulent and attenuated stains, H37Rv and H37Ra respectively, are now commonly used for *in vitro* and *in vivo* assays. Although arising from same parent stain H37, quite a significant genetic variation exists owing mainly to additional genes in H37Ra as opposed to the large number of gene deletions seen in BCG
In addition, secretion of ESAT6 is greatly reduced in H37Ra due to a point mutation in the transcriptional regulator PhoP [256]. It should also be noted that H37Rv carries a mutation in whiB6, which regulates ESX-1 expression resulting in reduced secretion of ESAT6 when compared to the laboratory strains GC1237 and Erdman and many clinical isolates when cultured in vitro [257]. The mutation does not influence the virulence of the laboratory strains in the mouse model of infection although H37Rv has been shown to be less virulent than GC123, HN878 and Erdman in the guinea pig infection model [258]. Ideally, most treatment models would utilise virulent strains, however, it is often more practical to commence research with a non-virulent strain before progressing to virulent or even resistant strains which require biosafety level three containment measures.

4.2 In vitro models of mycobacterial infection

4.2.1 Macrophages

Cells of the monocyte/macrophage lineage phagocytose Mtb and play a pivotal role in Mtb infection and host defence. As a result single cell macrophage cultures are the most commonly studied in vitro models of infection and many studies have utilized these cells to answer questions about the basic molecular and biochemical pathways involved in innate immune responses to mycobacterial infection and, by extension, have resulted in the identification of a large number of potential targets for HDTs. Macrophages are a heterogeneous group of cells which can adopt a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype in response to cues from their immediate environment, with IFN-γ being an important inducer of activated (M1) macrophages. Activated macrophages possess a sophisticated armoury of anti-bacterial weapons directed against intracellular pathogens – including phagocytosis, production of reactive oxygen and nitrogen species, trafficking of ingested bacteria to degradative lysosomes, autophagy, secretion of chemokines and cytokines. Mycobacteria have evolved ways to counteract some of these protective responses including phagolysosomal maturation and autophagy, to survive for long periods of time within macrophages, possibly in a latent or dormant state.

Alveolar macrophages are likely to be the first phagocytes to be infected with Mtb [259] and constitute a distinct self-renewing population of macrophages that is maintained in the lung independent of circulating monocytes [260, 261]. Alveolar macrophages obtained by bronchoalveolar lavage can be purified by plastic adherence within a few hours [262]. Inflammatory monocytes, recruited to the site
of Mtb infection from the circulation, also differentiate into macrophages (and dendritic cells) and are crucial for the production of protective cytokines through induction of eicosanoids [124] and for eliciting an adaptive immune response [263].

They are considered to be alternatively activated or M2-like in vivo in the tolerant environment in the lung but there are several reports of alveolar macrophages displaying a pro-inflammatory gene expression signature immediately after harvest [264, 265]. Therefore it may be advisable to culture the cells for a day or two before use. Cigarette smoking can alter the response of alveolar macrophages to Mtb infection [35] and should be controlled for if possible. As discussed earlier, we have shown that human alveolar macrophages can be specifically targeted by PLGA microparticles [266] which could be engineered to deliver encapsulated HDTs directly to the lung.

Monocyte-derived macrophages are often used as surrogates for alveolar macrophages due to ease of access. Peripheral blood monocytes can be differentiated to form macrophages by culturing them in medium containing either human serum or FBS supplemented with the survival factors M-CSF or GM-CSF for 5 to 7 days. However, alveolar macrophages have been reported to inhibit intracellular growth of BCG and H37Ra to a greater extent than MDMs in vitro [267] therefore caution should be used when interpreting results obtained with these cells. Nonetheless, inflammatory monocytes are recruited to the lungs in the mouse infection model, where they differentiate into macrophages and dendritic cells (DCs) [263], which are crucial for the production of protective cytokines through induction of eicosanoids [124] and for eliciting an adaptive immune response [263], thus providing a rationale for using MDMs as an in vitro model to assess HDTs. A recent study, using MDMs infected with GFP-expressing BCG as a model, combined the results of a high content screening assay evaluating a library of HDTs (most of which are FDA approved) with a genome-wide siRNA screen to identify nortriptyline, haloperidol and prochlorperazine edisylate as inhibitors of intracellular growth of mycobacteria via stimulation of autophagy and endosomal trafficking [268].

The use of cell lines is more practical for studies requiring large numbers of macrophages, for example high throughput screening of HDTs [98] and avoids the heterogeneity in macrophage responses between donors. When treated with PMA the THP-1 and U937 monocytic cell lines behave similarly to MDMs with increased adherence and increased surface expression of macrophage specific markers [269]. Unlike U937 cells, the response of THP-1 cells to TLR stimulation is similar to that of MDMs [270] and in many respects PMA-treated THP1 cells respond to Mtb infection similarly
to alveolar macrophages [271]. The THP-1 model was used by Maiga et al [169] to determine whether phosphodiesterase inhibitors could modulate Mtb-induced cAMP levels in macrophages before commencing a mouse study which showed that cilostazol combined with conventional TB drugs shortens the time required to eradicate infection compared to the standard short-course chemotherapy regimen alone. Similarly Dutta et al [272] used THP-1 cells infected with Mtb H37Rv to show that simvastatin enhances the activity of first-line anti-TB drugs before demonstrating that adjunctive treatment with the statin in a mouse model of infection reduced the time required to achieve sterility in the lung compared to conventional drugs alone. It should be noted that THP-1 cells behave more like monocytes than primary macrophages in response to inflammasome activation [273] thus it is important that results obtained with these cell lines are confirmed in primary human macrophages.

The murine cell lines J774A.1 and RAW 264.7 as well as bone marrow derived macrophages (BMDMs) are often used to predict the effects of treatments on mice in vivo [274]. Stanley et al [98] performed high throughput imaging-based screening of a library of small molecules from the Broad Institute bioactives collection and 159 kinase inhibitors using GFP-tagged H37Rv and J774A.1 cells and the hits, several of which overlapped with those identified in the screen carried out in MDMs by Sundaramurthy and colleagues [268] were verified in BMDMs. Cultured primary murine bone marrow-derived macrophages (BMDM) from wild type and knock out mice are frequently used to evaluate the contribution of host genes to the immune response to mycobacteria yet there are significant differences between the response of primary murine and human macrophages to Mtb which influence their response to HDTs, particularly with regard to the role of reactive nitrogen species. For example, when activated by IFN-γ murine peritoneal and bone marrow-derived macrophages restrict the intracellular growth of Mtb via iNOS and nitric oxide production. In contrast, IFN-γ pre-treatment does not influence intracellular growth of mycobacteria in human alveolar macrophages or MDMs under standard cell culture conditions [267, 275]. In another example, treatment with TLR2 agonists improves the ability of both murine and human macrophages to control Mtb proliferation in vitro yet the biochemical pathways responsible for this microbicidal effect differ between the two species; in mouse macrophages it is mediated by nitric oxide [276]. In contrast, in cultured human macrophages, monocytes and DCs infected with H37Rv, a study which rekindled
interest in the use of Vitamin D supplementation as an adjunctive therapy showed that TLR2 signalling, in combination with Vitamin D3, upregulates expression of cathelecidin which co-localises with the phagosome to kill the bacilli in an iNOS-independent manner [69]. Recently, iNOS has been shown to be regulated by the NOD2 PRR in human MDM infected with BCG and H37Rv [265, 277] identifying NOD2 as a potential target of host directed therapies.

4.2.2 Dendritic cells

Upon phagocytosis of bacteria immature dendritic cells (DCs) undergo a maturation process which includes upregulation of co-receptors, migration to lymph nodes, release of cytokines such as IL-12 and presentation of antigen to CD4+ and CD8+ T cells. Purification of primary human DC subsets from human peripheral blood requires laborious cell sorting which yields low numbers of cells. Immature DCs can be more easily generated from peripheral blood monocytes by culturing them in medium containing the cytokines GM-CSF and IL-4 for 5-6 days. These monocyte-derived DCs (mo-DC) mature after phagocytosis of Mtb and are capable of eliciting specific T helper and cytotoxic T cell responses. Several reports suggest that cultured mo-DCs can also control the growth of intracellular Mtb although others have not observed this microbicidal effect [278-281]. Their main function in TB is probably trafficking of live mycobacteria to the lymph nodes to present antigen to T cells. Notwithstanding this, the study by Liu et al mentioned above showed that vitamin D treatment in combination with TLR2 agonists can stimulate killing of Mtb by cultured mo-DCs [69] suggesting that the microbicidal activity of DCs could be boosted in vivo by HDTs. However, it is unclear whether mo-DCs generated in vitro accurately represent inflammatory monocyte-derived DCs and it is unlikely that they are representative of primary DC subsets. The most obvious translational application of research into DC-Mtb interactions is directed towards designing a better vaccine to prevent TB; in fact rapamycin treatment improves the protection afforded by BCG vaccination in the mouse TB model via induction of autophagy [95]. Given their important role in T cell polarization, targeting DCs using HDTs such as the autophagy inducers discussed in section 2.2.1 may also assist in the production of a more appropriate T cell response in patients with in active disease. Further investigation of the effects of autophagy inducing drugs on the function of primary human DC subsets will be necessary to establish the feasibility of such an approach in humans.

4.2.3 Airway epithelial cells
Alveolar epithelial cells are the first cells to encounter inhaled Mtb bacilli. Examination of autopsy samples from individuals with TB has revealed that these cells can be infected with Mtb in vivo [282]. Infection of alveolar epithelium triggers host protective responses such as secretion of chemokines and cytokines, nitric oxide production and antigen presentation to Mtb-specific non-classical CD8+ T cells [282]. With regard to HDTs, research has focused on modulating MMP activity of alveolar epithelial cells. In the zebrafish model of TB epithelial cells have been shown to secrete MMP 9 which degrades collagen to cause tissue damage, attracts infected macrophages to the site of infection and promotes dissemination from the early granuloma [283]. It has been proposed that tissue damage due to MMP activity promotes cavitation into the airways and dissemination to other human hosts [284].

Primary cultures of human polarised type I and type II alveolar pneumocytes can be grown on transwell inserts but this is a time consuming and expensive process and the cells have a finite lifespan. Although for practical reasons the A549 adenocarcinoma cell lines, which displays some of the characteristics of type II pneumocytes, is often used to study Mtb interactions with the airway epithelium it may not be an appropriate model for MMPs. A549 cells do not upregulate MMP9 when exposed to supernatant from infected human macrophages, unlike primary normal human bronchial epithelial cells (NHBE) [285]. Treatment with MMP inhibitors reduces MMP activity in of Mtb-infected NHBE cells and has been proposed as a HDT to reduce immunopathology due to destruction of collagen [284, 285].

4.2.4 Neutrophils

Neutrophils can phagocytose Mtb and are the most commonly infected phagocyte found in sputum and BAL from patients with active TB [284]. The role of neutrophils in TB pathogenesis is not well understood; they may be protective in the early stages of infection but in chronic TB infected neutrophils appear to promote a type I interferon response that correlates with disease severity [286, 287]. Peripheral blood neutrophils can be isolated from buffy coats and infected in vitro with Mtb. These are short-lived cells as they undergo apoptosis within 12-24 hours in culture and are therefore difficult to work with in vitro. Nevertheless, a recent in vitro study using cultured human neutrophils revealed that they secrete MMP8, which correlates with results found in vivo. MMP8 probably contributes to tissue damage resulting in cavitation and dissemination and is therefore a target for MMP inhibitors such as doxycycline [288]. Given their association with immunopathology in the lung
and the fact that they are the predominant infected cell in the airways of patients with active TB [289]. Further investigation of these cells as potential targets of HDTs is warranted.

4.2.5 3-D models of Mtb infection

Although the single cell in vitro models of Mtb infection outlined above have revealed a wealth of information about the basic interactions of Mtb with innate immune cells allowing investigators to identify potential HDTs, they are not representative of the complex environment of the lung. The majority of infected individuals develop latent TB with the bacilli contained in granuloma which constrain their growth pushing them into a dormant state. Upon reactivation, the structure of the granuloma can break down releasing bacteria, stimulating the formation of new lesions and possibly spreading Mtb to other individuals. The granuloma is an organised aggregate of cells consisting of infected macrophages in the centre surrounded by uninfected macrophages, epithelioid macrophages, multinucleated giant cells and T and B lymphocytes. The granuloma may become calcified or surrounded by a fibrotic capsule to add another level of complexity. In addition, caesous granulomas, which contain a core of necrotic cells and extracellular bacilli, can be formed. As well as presenting a physical barrier which may impede the diffusion of drugs into areas where infected cells or extracellular mycobacteria reside, adapting to the microenvironment of the granuloma can force the bacilli to undergo genetic or phenotypic changes which alter their susceptibility to anti-microbial agents [27, 290]. Furthermore several types of granulomas can be found within the same lung, possibly exerting divergent pressures on the bacilli within them, which may result in differing susceptibility to drugs. In short, the complexity and diversity of granulomas presents a challenge for investigators attempting to model the effects of host-directed therapies in vitro.

A model of the early granuloma can be generated in vitro by infecting PBMCs with mycobacteria. After a couple of days aggregates of infected monocytes/macrophages surrounded by B and T lymphocytes are formed [291, 292]. This model has been utilised to study host-pathogen interactions [293] and to demonstrate differing susceptibilities of mycobacteria contained within the granuloma to conventional chemotherapeutic drugs compared to single cell macrophage cultures [294]. Parasa et al [295] recently developed a co-culture model consisting of polarised 16HBE14o- human bronchial epithelial cells grown on transwell filters at an air-liquid interface, with MRG5 human lung fibroblast embedded in a layer of collagen on the basolateral surface, to mimic the architecture of the alveolus and into which they introduced macrophages which had been infected with GFP-expressing H37Rv.
This model recapitulates some of the characteristics of granulomas observed in animal models of TB including dependence on ESAT6 for granuloma formation and the induction of necrosis. Multicellular 3-D in vitro models of Mtb infection are likely to be more widely used in the future and may prove to be more predictive of the clinical efficacy of HDTs than traditional 2-D monocultures.

4.2.6 Testing HDTs in combination with conventional anti-tubercular drugs

Regardless of the in vitro models used to test the effects of adjunctive therapies it will be important to assess their effect in combination with conventional chemotherapeutic drugs such as rifampicin to check for detrimental drug-drug interactions. In addition, as highlighted in a recent review [290], care will also have to be taken to ensure that HDTs under investigation do not merely drive Mtb bacilli into a non-replicative state leading to increased resistance to the effects of those anti-tubercular drugs, which preferentially act on replicating bacilli. Developing appropriate in vitro pre-clinical models to rigorously test the efficacy and safety of host directed therapies will be a challenge for immunologists in the future.

4.3 In vivo models of mycobacterial infection

Similar to in vitro modelling of Mtb infection, in vivo assessment of potential treatments requires careful consideration regarding host animal and Mycobacterium tuberculosis strain in addition to appropriate route of administration of the experimental treatment. The aim of in vivo testing is generally to provide basic safety and efficacy data regarding a formulation thus preparing for subsequent clinical studies. In vivo studies, therefore, should mimic the human environment where feasible. An extensive review of model systems of various Mtb infections was published by Franzblau et al, here we will focus mainly focus on the possibilities for HDT efficacy testing [296].

4.3.1 In vivo model selection

Animal studies generally begin with small rodents such as mice, rats and guinea pigs for a number of reasons: small quantities of formulation can be administered therefore scale-up of manufacturing may be kept to a minimum, lower cost than larger animals and a considerable amount of data is required from in vivo studies which must be statistically significant and so smaller animals generally allow greater power for studies [297]. The disadvantages of using rodents relate mainly to differences in physical, physiological, genetics, and hence immune response, as eluded to in discussion regarding
the suitability of murine versus human macrophages for \textit{in vitro} efficacy testing (4.2.1) making extrapolation of results difficult at times [298]. Subsequent to model selection, the method of administration for the formulation being examined must be decided upon. Direct and passive methods of administration can be employed depending on the nature and quantity of formulation to be administered [298]. Formulations designed for inhalation can be administered via a number specifically designed devices, for dry powders for example, a Dry Powder Insufflator™ can be used [298] – similar to patients, device is crucial to ensure targeted delivery of the active ingredients, inefficient delivery can lead to misinterpretation of results. Lastly, stage of disease progression and treatment timelines must also be considered accordingly to mimic clinical events as close as possible.

4.3.2 \textit{In vivo} models suitable for HDT screening in mycobacterial infection

As the granuloma is the key component of the innate immune response to TB an \textit{in vivo} model in which granuloma development follows infection is central to successful HDT screening. Guinea pigs and rabbits and develop granulomas similar to humans but can be expensive and suffer mixed drug tolerability hence a lot of early studies start incorporate mouse models. Common mice strains such as BALB/C, C3H/HeJ and C57/BL6 do not develop necrotic lesions compared to C3HeB/FeJ mice that do in fact develop necrotic lesions following Mtb infection [299]. The C3HeB/FeJ (Kramnik) strain has increased susceptibility to Mtb infection but otherwise a standard immune response including granuloma development. Kramnik mice are less responsive to therapy than BALB/c due to development of necrotic lung lesions, ideal for modelling difficult to treat cases [300]. Gupta et al used the Kramnik model (H37Rv) to examine efficacy of HDT verapamil in combination with rifampicin. Despite requiring a dose increase due to interaction rifampicin, one study demonstrated that verapamil could hasten bacterial clearance and reduce relapse rates compared to standard treatment alone [301]. A simvastatin study showed antibacterial effects \textit{in vitro} but did not translate \textit{in vivo} when used alone which may be due to using BALB/c (CDC1551) mouse model, it did however show an additive effect when administered alongside standard anti-tubercular treatments [302]. A more recent study, also using a BALB/c (H37Rv) mouse model, showed significant synergistic effect when simvastatin was administered alongside rifampicin, isoniazid and pyrazinamide with a coinciding reduction in relapse rates, albeit using an increased dose of simvastatin and lengthy study period. Interestingly, no significant difference in cholesterol levels were found in the plasma or lung lesions
post-treatment in this case [272]. Singhal et al found a marginal reduction in bacterial load when metformin was used in combination with first and second line ATDs but the non-necrotising C57/BL6 (H37Rv) was used - a greater effect may have ascertained a in an alternative host model? [101]. A diet rich in vitamin D₃ proved of little benefit in controlling infection when fed to C3HeB/FeJ for one month prior to H37Rv infection in a notable study by Reeme and Robinson. Nonetheless, modulation of the inflammatory immune response including limiting of immunopathological events were evident highlighting importance of carefully selecting experimental end-points and study design when evaluating HDTs [303]. Zebrafish is another useful model of granuloma development used for efficacy testing. Susceptible to Mycobacterium marinum, zebrafish show similar immune responses to humans infected with Mycobacterium tuberculosis and, when the zebrafish larvae are employed, allows visualisation of disease progression through optical transparency [123, 304]. A non-human primate (NHP) model of tuberculosis infection has also been successfully developed. The model, utilising macaques, can exhibit complex pathology of latent and active disease including both caseous and non-necrotising granulomas providing translational results and therefore lots of opportunity for HDT testing. Drawbacks to the NHP model are the cost and facilities required [305].

One in vivo model suitable for assessing all HDT’s may never be attainable but it is important to use genetic variations where possible and be aware of the limitations when analysing results from efficacy studies.

4.3.3 Obtaining and Interpretation of results following HDT treatment

As the effect of HDT often occurs through an indirect action on the bacteria studies should not rely on typical read outs such as measuring the bacterial viability (by colony forming units) post treatment. Additional assessments such as chemokine and cytokines levels, histopathology, cell recruitment, antigen presentation and cytotoxicity might be more beneficial where feasible. Experimental time points should also be carefully chosen as the impact of a HDT will not necessarily be evident as soon as treatment as with conventional, antibacterial therapies. Nonetheless, pharmacokinetic and pharmacodynamic studies to understand how HDT’s and repurposed API’s act when administered by various routes are essential. Similarly, the design of clinical trials involving HDT’s will in will require careful selection of comparators and end-points as not all patients will respond to HDTs in the same way and may be genotype specific [13] therefore need robust biomarkers and methods of assessment. Dose optimisation studies are important for repurposed treatments as it will not
necessarily correlate with other indications. Finally, stage of disease progression and dosing schedule will dictate efficacy of any treatment but is of particular importance in relation to HDT’s. Many immune functions that are vital early in host defence prove detrimental in advanced infection, such as TNFα production which has been associated with both resistance and susceptibility to infection [12].

5. Concluding remarks

Development of host-directed therapies presents an intricate challenge, where temporal events of infection and host immunogenetics play a far greater role in determining efficacy compared to conventional antimicrobials. Nonetheless, this approach to treating disease holds the potential to generate novel treatments targeting the ever-growing problem of drug resistant strains, shortening burdensome treatment duration [7], and possibly enhancing vaccine efficacy [9], as well as reducing immune-mediated damage which contributes largely to the pathology of infection [8]. With nutritional sufficiency central to combating tuberculosis then perhaps vitamin therapy may prove to be a promising avenue of exploration in this regard. Technologies exist to deliver new HDT to the site of action, maximising their efficacy and minimising toxicities. As we proceed, we will require a detailed understanding of the mechanism of action of the proposed agents using appropriate in vitro and in vivo models, strategic development of suitable delivery systems, and careful clinical trial design to fully evaluate these therapies. A multi-disciplinary approach is vital, therefore, in order to maximally exploit this innovative field.
6. References


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