REPURPOSED DRUGS AS IMMUNE-MODULATORS TO COMBAT INFECTIOUS DISEASES

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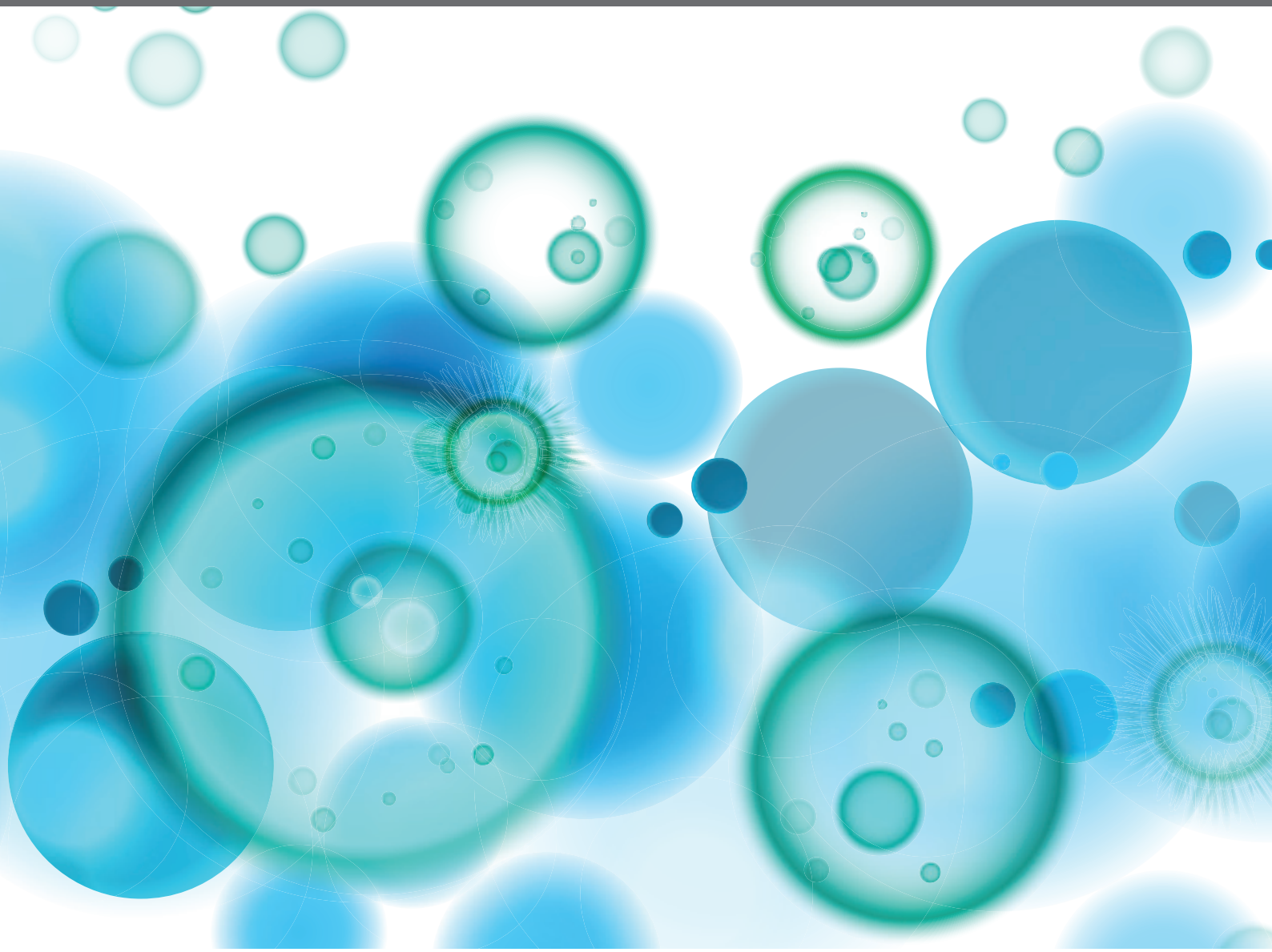

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REPURPOSED DRUGS AS IMMUNE-MODULATORS TO COMBAT INFECTIOUS DISEASES

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EDITORIAL
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Editorial: Repurposed Drugs as Immune-Modulators to Combat Infectious Diseases

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Editorial on the Research Topic

Repurposed Drugs as Immune- Modulators to Combat Infectious Diseases

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Repurposed drugs offer efficient treatment options as monotherapy or adjunctive for diseases that have fewer or no therapeutic interventions. In this Research Topic, we collected new data on the potential of repurposed drugs against infectious diseases, including tuberculosis and COVID-19.

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (Mtb)*, a bacterium responsible for the maximum number of deaths before SARS-CoV-2. Given severe toxicity, low efficacy, and the duration (minimum six months) often affect patients' adherence leading to the emergence of drug resistance. Generating new antibiotics to overcome resistance is a too costly and time-consuming process as evident with the development of new antibiotics Bedaquiline and Delamide, to treat drug-resistant TB, required approximately 20-30 years. In this Research Topic, Fatima et al. discussed the beneficial use of drugs such as sulfonamides, sulfanilamide, sulfadiazine, clofazimine, linezolid, amoxicillin, carbapenems, metformin, verapamil, fluoroquinolones, statins and NSAIDs repurposed to treat TB. This also included mechanisms of action with emphasis on their immunomodulatory effects on the host to attain both host- and pathogen-directed therapies for the potential synergistic effect as adjunctive TB therapies.

The success of *Mtb* has been related to its ability to manipulate the host effector mechanisms, including phagosome escape and/or maturation, autophagy, antigen presentation, and metabolic pathways. In this Research Topic, Nienaber et al. highlighted another aspect of HDT, which is based on the modulation of the TB-associated inflammatory response through the manipulation of lipid mediators metabolism. The authors summarized mainly preclinical studies, about the beneficial effects of Non-steroidal anti-inflammatory drugs (NSAIDs) and omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) on the outcome of TB treatment. By reducing the host exacerbated inflammation, the lipid manipulators contributed to the decrease of bacterial burden and ameliorated the treatment of the infection. In agreement, Hayford et al. showed that indeed co-administration of ibuprofen (short-term) and n-3 LCPUFA as an adjunct decreased mycobacterial loads and improved lung tissue pathology by decreasing pro-inflammatory cytokines

in C3HeB/FeJ mice. These studies suggested that LCPUFA is a suitable candidate as an adjunct to frontline TB drugs. However, clinical trials are needed to confirm the benefit/safety of patients.

The balance between the timing and levels of pro- and anti-inflammatory responses plays a key role in the fate of *Mtb* infection. An excessive pro-inflammatory response may cause an enlargement of granuloma and tissue damage, which may prolong the length of TB treatment and permanently diminish the lung function of TB survivors. The review by Krug et al. provided comprehensive information on drugs that target inflammatory (corticosteroids, MMPs, PARP1, TNF antagonists) or anti-inflammatory (Tregs and MDSCs) pathways to control lung damage and bacterial growth in preclinical models. However, the beneficial/detrimental role of these drugs is dependent on the time of usage during the disease. Consequently, therapies that modulate this spectrum of immune responses at the appropriate time may have the potential to improve the treatment of TB or reduce the permanent lung damage after a microbiological cure. The repurposed drugs known to modulate such responses may improve the future of TB therapy, however, the adverse effect of drug-drug interaction and their bioavailability may be a limiting factor for such host-directed therapies.

The ideal host-directed therapeutics for TB should potentiate the antimycobacterial defenses while preventing excessive inflammation and tissue injury. While the conventional anti-TB therapy uses a combination of antibiotics to maximize clearance of *Mtb* by targeting the pathogen metabolism, ion channel blockers could enhance bacillary clearance by targeting both the pathogen and host immune responses. Ion channel blockers alter cell physiology by attenuating ion exchange across the cellular and subcellular membrane commonly used to treat diseases such as hypertension. It is notable that several FDA-approved ion channel blockers have shown promise at both restricting *Mtb in vitro* and attenuating inflammation *in vivo*. Additionally, some ion channel blockers have direct antimycobacterial activity. In this Research Topic, Mitini-Nkhoma et al. provided a review of the literature on the clinically approved ion channel blockers that demonstrated anti-tuberculosis activity in *Mtb*-infected macrophages and/or in the animal model proposed as potential HDTs against drug-sensitive and -resistant TB.

The lung macrophages are one of the major myeloid cells that *Mtb* colonizes to establish infection. *Mtb* infected macrophages are more permissive to generate HIV viral particles rendering HIV-TB coinfection very challenging to treat. Protease inhibitors (PIs), which targets the viral replication cycle, are in clinical use to treat HIV. However, their effectiveness to treat HIV-TB coinfection is of great research interest. *Mtb* inhibits macrophage phagosome maturation and downregulates the lysosomal hydrolases such as Cathepsin S and H. Chemical scaffolds that relieve this blockade have the potential to boost *Mtb* killing and limit HIV replication during coinfections. In this Research Topic, Pires et al. showed that Saquinavir (SQV), a PI sold as Invirase/Fortovase to treat patients with HIV, increases lysosomal Cathepsin S protease activity, which increases the killing of *Mtb* in HIV coinfected human macrophages. Interestingly, by yet unidentified mechanisms, SQV enhances the expression of the MHC II at the cell surface; increases T cell priming, proliferation, and IFN-γ production. This study establishes the potential of SQV as a potential candidate for HDT against TB. Although there are no experimental models to test the effectiveness of SQV in HIV-TB coinfections, further studies could offer more insights into this clinically approved drug as an HDT for TB.

HDT also has the potential to modulate host-immune responses which can improve the efficacy of BCG vaccine or frontline TB drugs. Bouzeyen et al. showed that co-administration of MK-2206, an inducer of apoptosis, enhanced the ability of BCG to impart protection against *Mtb* in mice and guinea pigs through multiple mechanisms; FOXO3 activation, enhanced BCG induced apoptosis, inhibition of macrophage IL-10 secretion, and increased BCG induced effector/memory T cells. Furthermore, the lung tissue damage in animals immunized with BCG/MK-2206 was reduced in comparison to BCG alone immunization. In addition to MK-2206, Berberine, a plant-derived natural compound, is historically used to treat diabetes and hypertension. Ozturk et al. showed that as an adjunct, it improved the efficacy of frontline TB drugs. The administration of berberine with isoniazid (INH) and rifampicin (RIF) increased *Mtb* killing in murine and human macrophages. This approach also decreased lymphoid, myeloid cells recruitment and production of inflammatory cytokine/chemokine such as CXCL-10, IL-1β, and CCL3 in the lungs of mice. The availability of toxicology data for these studies would reduce the development timeline to be evaluated in Phase II/III clinical trials.

Besides TB, Nontuberculous mycobacterial (NTM) infections present a serious challenge to clinical management due to several innate resistance mechanisms of mycobacteria. Additionally, the current NTM treatment regimen presents significant clinical side effects in patients. In this Research Topic, Crilly et al. proposed host-directed immune-modulatory therapies for treating *Mycobacterium avium* complex (MAC). The authors included and discussed pathways; autophagy and PD-1/PD-L1 as viable HDT targets. In addition to targeting excessive pathological inflammation using anti-TNF antibodies, the authors described compounds with broad activity like statins and metformin. Future research to develop MAC HDT presents major opportunities with significant challenges and better treatment options for NTM patients.

Sepsis represents a major clinical problem and cause of death for patients in intensive care units worldwide. Among the pro-inflammatory mediators known to drive the pathogenesis of sepsis, plasma IL-1β level is associated with poor prognosis. Experimental models of LPS induced septic shock have shown the role of NLRP3 inflammasome activation in regulating IL-1β production and driving acute inflammatory pathologies. Both genetic and pharmacological inhibition of NLRP3 inflammasome ameliorates the acute inflammation and tissue damage caused during sepsis. In this Research Topic, Luo et al. investigated the role of the FDA approved Fat mass and

obesity-related protein (FTO) inhibitor "Entacapone" as a potential therapeutic for sepsis. FTO is the primary N^6-methyladenosine demethylase, well studied in obesity, however, the role in inflammatory diseases remains unclear. The knockdown (siRNA) and inhibition (entacapone) of FTO, hindered macrophage activation, tissue damage and improved survival in LPS-induced endotoxic shock in mice. Mechanistically, the ablation of FTO inhibited the NLRP3 inflammasome regulated IL-1β production through FOXO1/NF-kB signaling in macrophages. Therefore, targeting FTO offers potential treatment options for life-threatening sepsis and similar pathologies.

In 2020, the COVID-19 pandemic has ravaged healthcare systems around the world. Patients infected with SARS-CoV-2 present diverse inflammatory sequelae with the progression of the disease. The terminally ill patients present severe lung inflammation, cytokine storm, and extensive lung damage that ultimately affects the air exchange and leads to mortality. While antivirals may be beneficial at the early stages of COVID-19, anti-inflammatory drugs such as dexamethasone have been effective in limiting the severity of disease by targeting the overwhelming inflammatory response and cytokine storm. Burrage et al. discussed the potential and clinical data from the use of various immunomodulatory agents targeting cytokines (IL-1, IL-6, TNF), inflammasome (NLRP3 inhibitor, colchicine), systemic inflammation (corticosteroids). While there are multiple clinical trials are underway, this review will help design randomized double-blinded clinical trials to find evidence-based therapies in the context of this global pandemic. For instance, Caracciolo et al. showed the use of Canakinumab (anti-IL-1β monoclonal antibody) in the case of an 85-year-old male, treated for the severe form of SARS-CoV-2 infection for compassionate use. The condition of the patient deteriorated with acute respiratory distress syndrome, cardiac and renal failure after 25 days of hospitalization, was intubated. Subsequently, diuresis recovered, and condition improved: high IL-6 levels and NK cells expressing $CD56^{bright}$ (associated with cytokine release) were significantly reduced giving rise to NK $CD56^{dim}$. Unfortunately, the patient did not survive after day 58 owing to persistent SARS-CoV-2 infection and pulmonary bacterial superinfection. Therefore, canakinumab rescued a high-risk, very elderly patient, from multiorgan damage complicating COVID-19. It may represent a useful treatment in severe cases; however, further studies are warranted.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Immunomodulatory Drugs in the Management of SARS-CoV-2

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With the onset of the global pandemic in 2020 of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), there has been increasing research activity around certain disease-modifying drugs that are used for the management of inflammatory disorders such as rheumatoid arthritis, spondyloarthrosis, psoriatic arthritis, systemic lupus erythematosus, and inflammatory bowel disease for managing coronavirus symptoms. In the conditions mentioned, many people are on long-term treatment with agents including hydroxychloroquine, tumor necrosis factor alpha (TNFα) inhibitor drugs, other biologic agents such as monoclonal antibodies to IL-6 and Janus kinase inhibitors including baricitinib and tofacitinib, which are used to control inflammatory responses in their respective auto-immune condition. There is emerging data that immunomodulatory drugs could be protective at reducing certain features of SARS-CoV-2 and improving recovery. In addition, it is important to understand if subjects being treated with the immunomodulatory agents described have a less severe SARS-CoV-2 infection, as they are deemed some protection from their immunomodulatory treatment, or if they develop infections similar to non-immunocompromised patients. There is a huge unmet clinical need to advise patients responsibly about whether they should remain on their immunomodulatory treatment or not in light of Covid-19 infection. In this article we will discuss potential treatment options for SARS-CoV-2 using immunomodulatory drugs and at what stage of the condition they may be beneficial. Viable treatment options during the global coronavirus pandemic are a much-needed and an intensely active area of research.

Keywords: SARS-CoV-2, hyperinflammation, biologics, cytokines, immunomodulators

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INTRODUCTION

The global pandemic of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), which originated in China in late 2019, has spread rapidly throughout the world to become a global pandemic. The emergence of this very infectious virus has placed huge burdens on populations worldwide, infecting millions and causing deaths in thousands of people across the globe. There is currently no cure for coronavirus. Although a diagnostic test is available for PCR testing of the virus by nasopharyngeal swab, there are cases in which clinical features are apparent, but a swab test may be negative, including cough, shortness of breath, temperature, often accompanied by laboratory changes such as lymphopenia, raised serum C-Reactive Protein (CRP), ferritin levels and pulmonary infiltrates on chest radiographs.

Coronavirus is primarily a respiratory illness affecting the lungs, which can lead to high temperatures, cough, headache, sore throat, shortness of breath, arthralgia, myalgia, chest pain, altered taste, and confusion. The condition can cause a rapid inflammatory response in the body, with the release of cytokines and acute deterioration. The coronavirus outbreak has led to new opportunities to study the immune response to coronavirus and to consider novel therapeutics for this condition.

Due to the lack of availability of a cure, there is a huge international effort to develop potential vaccines and pharmacotherapies to treat SARS-CoV-2. Among the candidate treatments, immunomodulatory agents have been proposed to target the inflammatory reaction that is induced in the lungs of affected patients and also the cytokine storm which affects people in severe cases. A number of agents more commonly used in inflammatory conditions, including corticosteroids, hydroxychloroquine, biologic inhibitors of IL-6 and IL-1, such as tocilizumab and anakinra respectively, TNFα inhibitors and janus kinase inhibitors have all been proposed as potential therapies for SARS-CoV-2 (1, 2), some of which are already in clinical trials, such as the RECOVERY trial (3). The RECOVERY trial, which is being co-ordinated in Oxford, has already recruited more than 11,500 participants from over 175 NHS hospitals in the UK and includes low dose corticosteroids, hydroxychloroquine, and tocilizumab, which are treatments commonly used to treat inflammatory arthritis (3). Other treatment arms in the trial include lopinavir-ritonavir, azithromycin, and convalescent plasma.

It is now apparent that SARS-CoV-2 infection has two clear clinical phases of infection: the former, which involves the viral infection and replication (4) and the inflammatory phase which often leads to rapid deterioration and worsening respiratory symptoms, requiring hospital admission in many cases to avoid deterioration (4, 5). Although corticosteroids are not routinely recommended and may exacerbate COVID-19-associated lung injury (4), in hyperinflammation, immunosuppression is likely to be beneficial. Mehta et al. reported features of a cytokine storm syndrome in a subgroup of COVID-19 patients (5). Further data indicate that an elevated ferritin (a hallmark feature of secondary hemophagocytic lymphohistiocytosis) and elevated IL-6 are predictors of fatality. It has been proposed that by screening for hyperinflammation to identify at risk groups, targeted immunomodulation could improve mortality (5). The current state of play of potential therapeutics that could be used to directly target the virus, or reduce its effects on the host response, are summarized in **Figure 1**.

In this review, we discuss the rationale for the potential use of immunomodulator therapies in the management of SARS-CoV-2. In particular, we will explore which patient subgroups with respect to infection severity and systemic response, the immunomodulators may be beneficial.

CYTOKINE-BASED THERAPIES

Targeted biologic therapies against specific cytokines have become the treatment of choice in active rheumatic inflammatory conditions. Over the last few decades, improved understanding of the immunology of inflammatory diseases, coupled with the advancement of technologies allowing mass production of biologic therapies, has transformed the management of conditions including rheumatoid arthritis, ankylosing spondylitis, and inflammatory bowel disease with cytokine-targeted biologic therapies.

Data from several groups has shown that cytokine levels are elevated in people hospitalized with SARS-CoV-2 infection, with a rapid release of cytokines such as IL-1, IL-6, and TNF alpha (3, 6–8). In the context of other concomitant risk factors such as male gender, increased age, immunocompromise, and obesity (9–13), rapid onset of the cytokine storm requires urgent treatment to prevent multi-organ failure and death.

It has been noted that severity of SARS-CoV-2 and increased deaths have been associated with several risk factors, including older age (9), male gender (10), black or minority ethnic origin (11), obesity (12), diabetes mellitus (13), and cardiovascular disease (9). Such observations have led to hypotheses that genetic risk factors for cytokine release syndrome (CRS) or cytokine storm (CS) may be at play. For example, conditions including Familial Mediterranean Fever (FMF) or TRAPS (Tumor Necrosis Factor Associated Periodic Fever Syndromes) are known to be more prevalent in specific ethnic groups, including Mediterranean, Arab, Jewish, Turkish, Armenian, North African descent with some mutations found in Asian populations.

Such observations, as highlighted above, have led to the concept that therapies targeted to IL-6, IL-1, and TNF alpha may have a role to play in the post-infection stage of SARS-CoV-2. In the post-infective stage, an accelerated inflammatory response sets in, which has important implications for the management of SARS-CoV-2 infection.

IL-6 Cytokine Inhibitors

Biologics targeted to IL-6, such as tocilizumab, a humanized monoclonal antibody generated to the IL-6 receptor, are licensed for the management of active rheumatoid arthritis, juvenile idiopathic arthritis, and replasing or refractory giant cell arteritis (GCA) (6). They are also licensed for the treatment of cytokine release syndrome. IL-6 is a key cytokine in the mediation of fever and the acute phase reponse, including C-reactive protein and ferritin.

Tocilizumab has already been used in the context of severe Covid-19 infection. A recent retrospective study reported outcomes for 21 patients in China (7). Tocilizumab has been used in people with severe features of Covid-19, including in subjects with severe infection, having a respiratory rate $\geq$ 30 breaths/min, $SpO_2 \leq 93\%$ while breathing room air and a $PaO_2/FiO_2 \leq 300$ mmHg. In this uncontrolled study, 21 patients with severe or critical Covid-19 pneumonia were treated with tocilizumab 400 mg intravenously (7). In many of the subjects treated, the fever returned to normal within a few days, 15 out of 20 lowered their oxygen requirement and one patient needed no further oxygen therapy. In 19 out of 20 subjects, there was an improvement in Computerized Tomography (CT) scans of the chest.

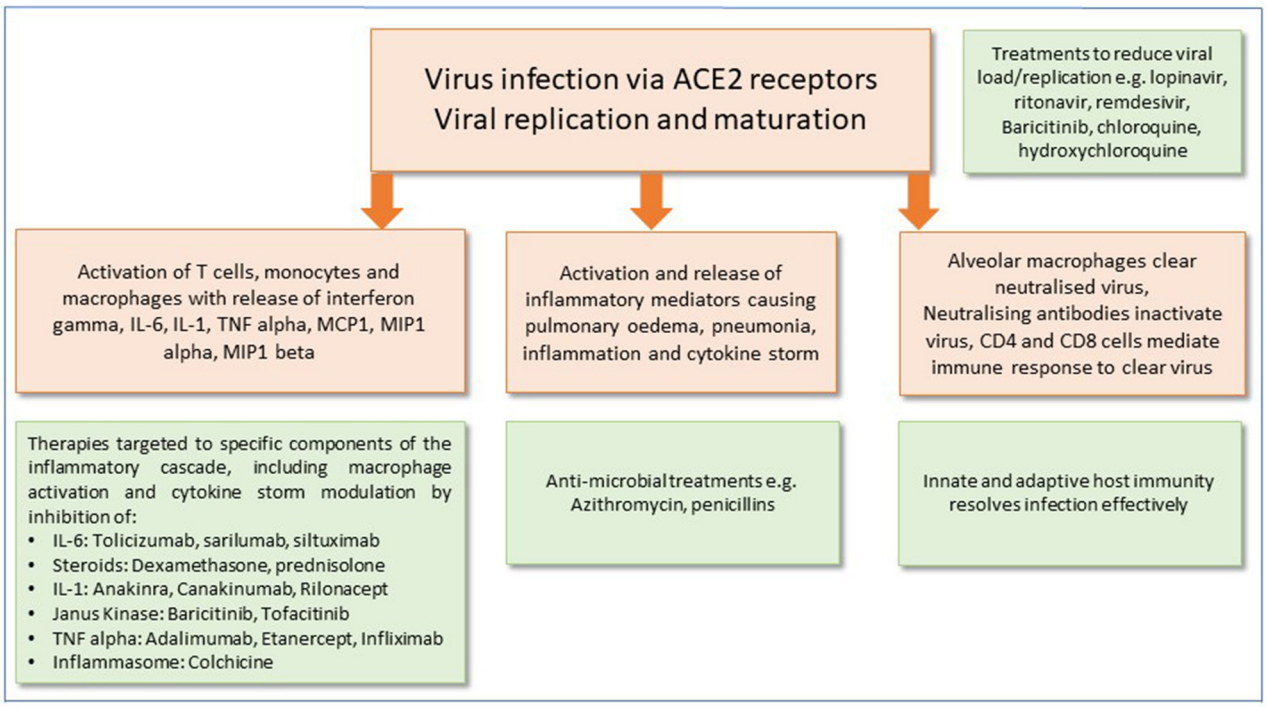

FIGURE 1 | Potential therapeutic approaches for the management of SARS-CoV-2 infection. Mechanisms of cell injury and damage are shown in peach boxes. Green boxes show potential therapeutic targets and immune responses to modify and alleviate infection.

Case History

We treated a case of severe Covid-19 in London in April 2020 with tocilizumab which was provided on a compassionate use basis. Compassionate drug use refers to use of this drug for an unapproved indication to treat seriously ill patients when no other treatments are available. A 54-year-old Kurdish woman attended the emergency department at St George's Hospital, London, with 7 days of gradually worsening headache, fever, a new productive cough and loss of taste.

The patient had a history of asthma (well-controlled with regular budesonide 200 micrograms and formoterol 6 mg combination inhaled twice a day and salbutamol 100 micrograms inhaled up to four times daily as required), subarachnoid hemorrhage (due to an anterior communicating artery aneurysm, managed with coiling), chronic headaches (managed with co-codamol 30/500 1–2 tablets up to four times daily as required, tramadol 50 mg orally twice daily and pregabalin 25 mg orally daily) and gastro-esophageal reflux disease (managed with lansoprazole 15 mg orally daily and sodium alginate with potassium bicarbonate 10 mL up to four times daily as required). She was a lifelong non-smoker, with no alcohol intake.

Her temperature was 37.8°C, heart rate 86 beats per minute, respiratory rate 18 breaths per minute, blood pressure 146/94 mmHg and peripheral oxygen saturation 99% breathing room air. On examination she had crepitations at the right lung base.

Her blood neutrophil count was 7.8 $\times$ 10^9/L, lymphocyte count of 0.9 $\times$ 10^9/L and C-reactive protein 82 mg/L. Her chest radiograph demonstrated bi-basal opacities, more pronounced on the right-hand side (**Figure 2**). A respiratory swab was sent for SARS-CoV-2 RNA.

She was diagnosed with probable COVID-19 and initiated on doxycycline 200 mg once only, then to continue 100 mg daily for possible community acquired pneumonia. She was discharged with advice to self-isolate and a plan for review in the ambulatory medical care unit the next day.

On review the following day her peripheral oxygen saturations were 90% breathing room air with respiratory rate 17–21 breaths/min. Her nasopharyngeal swab detected SARS-CoV-2 RNA (Abbot Realtime SARS-CoV-2 assay). The Abbott RealTime SARS-CoV-2 assay is a real-time (rt) reverse transcriptase (RT) polymerase chain reaction (PCR) test used for the qualitative detection of nucleic acids from the SARS-CoV-2 in nasopharyngeal (NP) and oropharyngeal (OP) swabs from patients (14).

A decision was made to admit her for oxygen therapy, initially requiring 2 L/min oxygen to achieve oxygen saturations greater than 94%, and to continue doxycycline for possible supra-added bacterial pneumonia.

On day 3 of admission she continued to have fevers and felt increasingly breathless, with peak temperature 39.5°C, respiratory rate of 20–30 breaths per minute and oxygen requirement of 4 L/min. Her blood neutrophil count was 9.2 $\times$ 10^9/L, blood lymphocyte counts 0.6 $\times$ 10^9/L, and C-reactive protein 302 mg/L. A repeat chest radiograph showed patchy areas of consolidation within the lung peripheries bilaterally and retrocardiac left lower zone opacification with air bronchograms

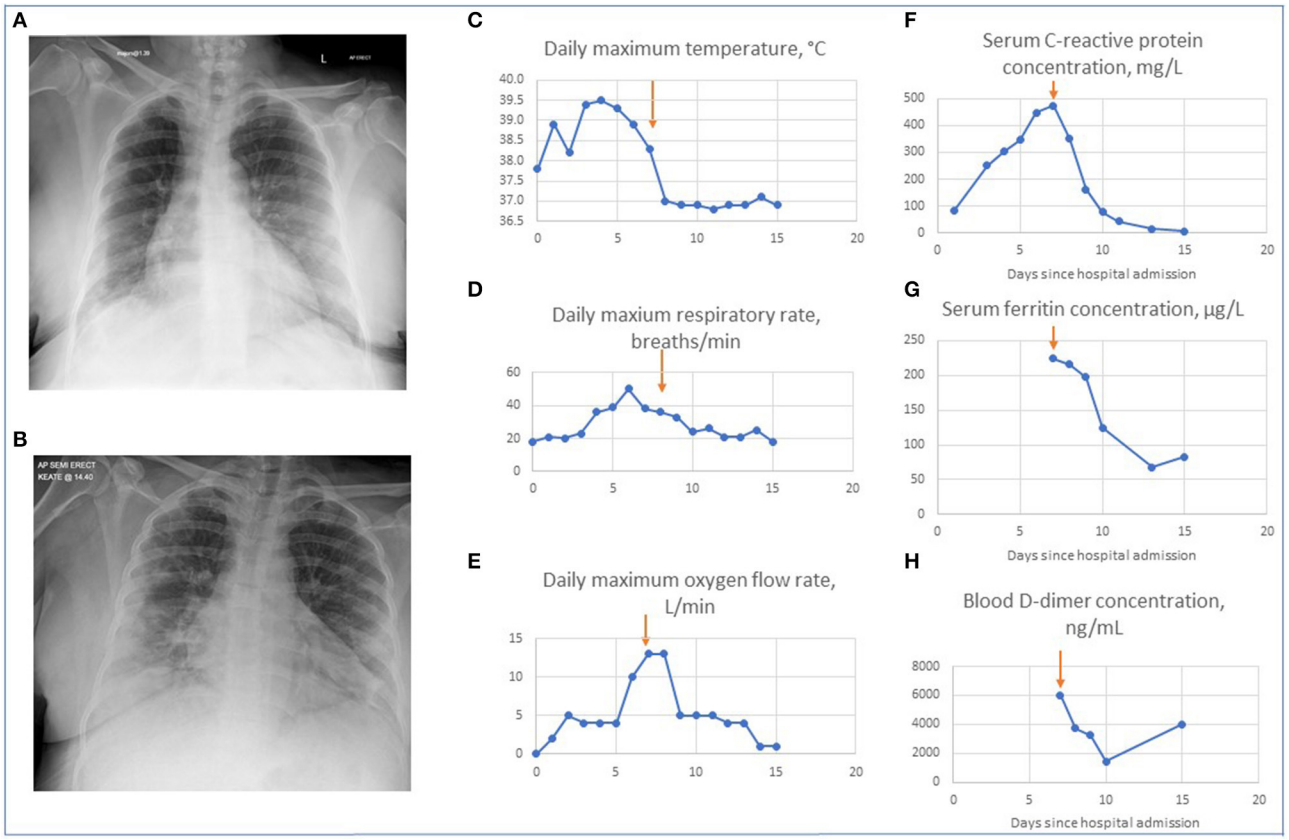

FIGURE 2 | Changes in patient's clinical, radiographic and biochemical parameters on treatment with tocilizumab. **(A)** Chest radiograph on admission. This demonstrates minor bi-basal opacity more pronounced on the right. **(B)** Chest radiograph on day 3. This demonstrates patchy areas of consolidation within the lung peripheries bilaterally with retrocardiac left lower zone opacification with air bronchograms. Graphs showing changes in the maximal daily values of parameters for patient's hospital admission for temperature **(C)**, respiratory rate **(D)** oxygen requirement **(E)**, serum C reactive protein levels **(F)**, ferritin levels **(G)**, and D-dimers **(H)**. Arrows indicate Day 7 when tocilizumab was given.

(**Figure 2**). Benzylpenicillin 1.2 g 4-hourly intravenously was commenced in addition to doxycycline to treat for possible supra-added severe pneumonia.

By day 7 of admission her work of breathing and oxygen requirements had continued to increase, with a respiratory rate of 32–38 breaths per minute and oxygen requirement of 13 L/min, with peak temperature 38.3°C. Her blood neutrophil count was 9.5×10^9/L, blood lymphocyte counts 0.6×10^9/L, C-reactive protein 474 mg/L, D-dimer >6,000 ng/mL, and ferritin 224 µg/L. A full and comprehensive infection screen was completed with no focus of bacterial infection: mycoplasma serology and urinary legionella and pneumococcal antigens were not detected and there was no bacterial growth on sputum, urine, or blood cultures. A decision was made to administer a single dose of intravenous tocilizumab, dosed at 8 mg/kg, accessed through a compassionate use off-license scheme, to treat for cytokine storm secondary to coronavirus infection, and she was reviewed for consideration of admission to critical care.

On day 8 of admission, within 12–24 h of tocilizumab administration, her oxygen requirements had reduced to 5 L/min and her respiratory rate had improved to 22–33 breaths per minute. Over the course of the following week she showed continuous improvement. By day 15 of admission she was discharged with respiratory rate of 18 breaths per minute and oxygen saturations 92% breathing room air, with no recorded fevers for 7 days. Two weeks after discharge her symptoms had continued to improve although she noted persistent loss of taste and occasional cough. Her imaging and improvement in biochemical parameters during her admission are shown in **Figure 2.** The clinical course of the disease and rapid response to treatment with tocilizumab suggested that our patient had cytokine storm secondary to SARS-CoV-2 infection, which was responsive to IL-6 inhibition.

Our case highlights that in severe Covid-19 infection, where subjects may be exhibiting features of cytokine storm with little reponse to full supportive care, there may be a case for treatment with a single dose of intravenous tocilizumab to reverse the effects of the cytokine storm and to prevent the positive feedback loop of release of pro-inflammatory cytokines which leads to rapid clinical deterioration and death in many cases.

There are several points to be noted from our case and in general from the pharmacology of tocilizumab treatment. Anti-IL-6 treatment often leads to a very rapid reduction in CRP levels as it is a strong suppressor of acute phase reactants produced by the liver. It is therefore important to monitor patients closely for other intercurrent infections e.g., bacterial, since they may fail to mount a full response as a result of inhibition of inflammatory pathways. Larger trials of IL-6 inhibitors for Covid-19 are now underway and will be important to establish the clinical scenarios in which it will be of optimal use (3, 8). Current data suggests that it may best be used in severely ill Covid-19 patients, to reduce the likelihood of subjects requring critical care or to prevent catastrophic cytokine storm features. In rheumatoid arthritis, IL-6 inhibitors are usually used as weekly or monthly injections. However, in the setting of acute Covid-19 related inflammation, a single dose may be adequate. Current trials are also testing repeated use in severe cases. In people with rheumatoid arthritis treated with IL-6 inhibitors, long-term monitoring for raised lipid levels and development of lower intestinal perforation are closely monitored, with patients who have underlying diverticular disease considered a relative contraindication for treatment in the rheumatoid arthritis setting. It remains to be seen whether in the acute infection setting of Covid-19 subjects are prone to the development of the side-effects previously observed for IL-6 inhibitors in other disease indications.

IL-1 Cytokine Inhibitors

Interleukin-1 is a very active pro-inflammatory cytokine which is released during inflammatory processes including sepsis and chronic inflammation. It can lower pain thresholds but also cause sustained tissue damage (15). Monotherapy using the IL-1 receptor antagonist, anakinra, is already proven in several autoinflammatory syndromes including rheumatoid arthritis, hereditary systemic autoinflammatory diseases such as Familial Mediterranean Fever (FMF), Cryopyrin-associated periodic syndrome (CAPS), and TNF receptor-associated periodic syndrome (TRAPS). There are several commercially available inhibitors of IL-1 which are licensed, including the IL-1 receptor anatonist anakinra, the soluble decoy receptor rilonacept and the neutralizing monoclonal antibody to IL-1 beta, canakinumab.

IL-1 modulators are often extremely effective in conditions where there are sustained fevers and a marked systemic inflammatory response. For example, we recently treated a case of unexplained fevers, weight loss and night sweats, in a patient with no known infection, who had genetic sequencing that showed a mutation in intron 4 of the gene for TNF receptor superfamily 1A (TNFRSF1A), c.473-72 G > A, which demonstrated the diagnosis of tumor necrosis factor-associated periodic fever syndrome (TRAPS) (16). Our patient underwent treatment with the IL-1 receptor antagonist anakinra at 100 mg daily subcutaneously and within 2 days he had symptomatic improvement, suppression of CRP and serum amyloid A levels began to normalize (16).

There are centers across the world that are currently using anakinra for CRS and CS-related features of Covid-19. The importance of release of IL-1 and IL-6 pro-inflammatory cytokine released by lung tissue in response to toll-like receptor activation during SARS-CoV-2 infection is recognized and are valid treatment targets (17). It remains to be seen if there are specific clinical differences in outcome between IL-1 or IL-6 inhibition in the setting of severe SARS-CoV-2 infection. It may be that IL-6 inihibitors may be preferred as a single injection that has sustained effect over a longer period of time, in comparison to IL-1 inhibitors, which since they are usually given as a daily injection and therefore may require repeated dosing.

TNF Alpha Cytokine Inhibitors

The advent of biologic therapies targeted at the inhibition of TNFα in the 1990s led to a step change in the management of many inflammatory conditions for which the drugs are licensed, including rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, juvenile arthritis, and inflammatory bowel disease. Currently a wide variety of formulations of TNF inhibitors are used, including fully humanized biologics targeted to TNFα that include adalimumab, etanercept, and infliximab. The demonstration that TNFα is a key cytokine that is produced in a wide range of conditions causing inflammation, both in the acute and chronic phase, has been borne out by its success as a treatment in a broad range of conditions. In conditions such as rheumatoid arthritis, blockade of TNFα leads to a subsequent decrease in IL-1 and IL-6, adhesion molecules and angiogenic factors such as vascular endothelial growth factor (VEGF). The rationale for the use of TNF inhibitors in hospitalized patients with SARS-CoV-2 has been proposed (18). In people with inflammatory arthritis and inflammatory bowel disease, screening for tuberculosis (TB) and malignancy are performed and subjects with a history of latent or active TB are commenced on TB eradication treatment before starting TNF inhibitors. In addition, people with a cancer history within the previous 5 years are not usually given TNF inhibitors. Such considerations may be overriden in the acute setting of infection with Covid-19, but may have long-term consequences and should be considered in study designs.

CORTICOSTEROIDS

Corticosteroids have the ability to suppress inflammation by acting on reducing the activation of several inflammatory mediators produced by the body during infection and inflammation (19). Corticosteroids bind to a corticosteroid receptor (CR) and the complex translocates to the nucleus where it binds to the glucocorticoid response element (GRE). This complex increases the transcription of a number of anti-inflammatory genes, including those encoding inhibitory (I)-κB, which inhibits the activation of nuclear factor (NF)-κB, genes encoding cytokines IL-4, IL-10, IL-13, and TGFβ (19, 20). The corticosteroid-CR complex inhibits binding of transcription factors (AP)-1 and (NF)-κB to their response elements, thereby reducing the production of pro-inflammatory cytokines IL-1β and TNFα in activated macrophages. Corticosteroids also increase the synthesis of lipocortin-1, which inhibits the precursor of eicosanoids, platelet activating factor and phospholipase A_2. The multiple mechanisms of action of glucocorticoids make them effective at suppressing inflammatory

responses at several sites, including the lung tissue, joint, and systemic inflammation.

Infection with SARS-CoV-2 infection induces destruction within lung cells, which triggers a local immune response by activation of macrophages and monocytes, cytokine release and induce T and B cell responses. The innate and adapative immune response is usually sufficient to clear the virus-induced damage in most cases. However, in some people an altered immune reponse occurs, with development of severe lung and systemic pathology. Due to their effects on multiple aspects of inflammation, corticosteroids can be used in the early stages of cytokine storm and macrophage activation syndrome (MAS), when there is an overwhelming inflammatory response in the body, often in response to an infectious trigger. Several studies have shown there is a positive effect by corticosteroids in reducing immunopathological damage (21). However, other studies have shown that viral RNA concentrations of SARS-CoV-2 can increase with corticosteroid treatment compared with placebo (22). It may be more prudent to use corticosteroids in a peri-intensive care setting, when subjects may be entering a cytokine storm, rather than in treating ambulatory patients or those only requiring routine care for their infection. Indeed initial analysis from the RECOVERY trial of 2,104 patients randomized to receive dexamethasone 6 mg once per day fro 10 days (orally or intravenously) has demonstrated a reduction in 28-day mortality in ventilated patients and patients requiring oxygen compared to those receiving usual care (23) There was no benefit for patients who did not require respiratory support. Peer review publication of this data is awaited.

CHLOROQUINE AND HYDROXYCHLOROQUINE

Chloroquine and hydroxychloroquine are used widely across the world as antimalarials. They also have a role in the treatment of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and other inflammatory rheumatic diseases. Chloroquine and hydroxychloroquine are weak bases (24). They have a broad volume of distribution and a half-life of approximately 50 days (25). They have multiple mechanisms of action, including altering cell pH, affecting lysosomal activity, autophagy, signaling pathways, and inhibition of cytokine production and co-stimulatory molecules (26). Recently, chloroquine was identified as having potent activity against SARS-CoV-2 (27). Although clinical trials of chloroquine and hydroxychloroquine are currently underway in the treatment of SARS-CoV-2, there are questions that currently remain unanswered. These include the optimal timing of using the drug; some reports suggest early use to inhibit viral replication may be optimal, whereas several clinical trials are using chloroquines at high dose for patients with symptoms severe enough to require hospital admission (3), ranging from 500 to 1,000 mg per day. The potential longer-term toxicity effects of the chloroquines in the context of SARS-CoV-2, e.g., myocarditis, arrythmias, retinal toxicity, are not known in the context of randomized controlled trials (28). However, emerging data from the RECOVERY trial from a total of 1,542 patients randomized to hydroxychloroquine compared to 3,132 patients randomized to usual care has not shown hydroxychloroquine to be effective in reducing mortality or hospital stay duration (29).

JANUS KINASE INHIBITORS

The Janus kinase inhibitors (JAKis) are also known as targeted synthetic disease-modifying anti-rheumatic drugs (tsDMARDs) (30). JAKis block cytokine signaling by inhibiting the phosphorylation of activated cytokine receptors. When activated, the phosphorylated cytokine receptors recruit STAT transcription factors which modulate gene transcription.

They are currently the only licensed tsDMARDs for the management of active rheumatoid arthritis. Drugs included in the JAKi group include baricitinib, tofacitinib, perficitinib, filgotinib, upadacatinib, and fostaminib. In the management of RA, a safety signal reported has been the increased risk of herpes zoster infection, especially in Japanese and Korean patients with RA (31). It has been argued that JAKis may not be useful in the early stages of infection with SARS-CoV-2, since the activity of interferons, which are often the major mediators of viral clearance in the body, are mediated via the JAK-STAT signaling pathway. JAKis have been proposed as a treatment in severe coronavirus infection with features akin to cytokine storm (32). Recently, an open-label study testing the effect of baricitinib was published of 12 patients in Italy treated for SARS-CoV-2 infection (33). There were 10 males and 2 females in the study group, with a mean age of 63.5. Fever, oxygen saturations, oxygen requirements and C-reactive protein significantly improved in the baricitinib group compared with controls. A transfer to the Intensive Care Unit was requested in 33% (4/12) of controls and in none of the baricitinib-treated patients ($p = 0.093$). Discharge at week 2 occurred in 58% (7/12) of the baricitinib-treated patients vs. 8% (1/12) of controls ($p = 0.027$). However, this small trial of 12 subjects was open-label and not randomized. Larger randomized controlled trials are now underway to assess the value of baricitinib in the management of SARS-Cov-2 infection. Several clinical trials are underway of baricitinib therapy in comparison to anti-viral therapies (NCT04320277 and NCT04321993), but have not reported so far. In a recent study reported from the USA in 86 subjects who developed SARS-CoV-2 and also had an immune-mediated inflammatory condition, 62% of subjects were on a biologic drug or JAKi, but of those only 7% of those were hospitalized (34). The US case series data in people who developed SARS-CoV-2 suggests that being on an immunomodulator did not appear to increase the risk of developing SARS-CoV-2 features that led to serious infection or death in this case series.

INFLAMMASOME

Colchicine is a microtubule inhibitor drug widely used in the management of gout and conditions that involve localized inflammation including serositis e.g., Behcet's disease, Systemic Lupus Erythematosus (SLE), and pericarditis (35, 36).

Myocardial injury is recognized in SARS-CoV-2 infection, with an imbalance of oxygen supply and demand due to Adult Respiratory Distress Syndrome (ARDS) and acute lung injury. Histologically proven myocarditis has been found in SARS-CoV-2 infection, and the additional injury caused to cadiac tissue by activation of a cytokine storm, with vascular inflammation, endothelial dysfunction, and arrhythmias have been observed (37). It has been suggested that the NLRP3 inflammasome activation, which is initiated by viroporin E, is a component of SARS-CoV-2 (38), thereby inducing an inflammatory response. Since colchicine has been shown to inhibit the NLRP3 inflammasome (39), it is a potential valid target for the use of colchicine in Covid-19 infection. There are already 4 clinical trials announced that will be investigating the use of colchicine in SARS-CoV-2 with endpoints including need for hospitalization or death. Some trials are designed as colchicine monotherapy in addition to standard clinical care (ClinicalTrials.gov Identifier: NCT04322682, ClinicalTrials.gov Identifier: NCT04326790, ClinicalTrials.gov Identifier: NCT04322565), whereas other trials are designed with concomitant administration of anti-viral therapy including lopinavir/rotinavir (ClinicalTrials.gov Identifier: NCT04328480).

CONCLUSIONS

Our review has discussed the wide range of clinical features with which SARS-CoV-2 infection can present. Recognizing which clinical features are most likely to be targeted by specific therapies will be crucial to establish optimal therapeutics for treating infection. For example, anti-viral agents may be needed to target prevention of viral entry and replication, whereas immunomodulatory drugs are most likely to play a role in cytokine storm and macrophage activation in patients who are at high risk of requiring intensive care in order to prevent uncontrolled inflammation and death. There is a huge need to conduct well-designed, randomized controlled trials in the context of SARS-CoV-2 infection, so that true signal outcomes for efficacy are determined that lead to evidence-based therapies for the global pandemic.

AUTHOR CONTRIBUTIONS

NS conceived and wrote the manuscript. SK collated references and assisted in writing the manuscript. DB wrote the case history in the manuscript and managed the patient with NS. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Misra DP, Agarwal V, Gasparyan AY, Zimba O. Rheumatologists' perspectives on coronavirus (COVID-19) and potential therapeutic targets. *Clin Rheumatol*. (2020) 39:2055–62. doi: 10.1007/s10067-020-05073-9
2. Ferner RE, Aronson JK. Chloroquine and hydroxychloroquine in covid-19. *BMJ*. (2020) 369:m1432. doi: 10.1136/bmj.m1432
3. https://www.recoverytrial.net/files/recovery-protocol-v6-0-2020-05-14.pdf
4. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med*. (2020) 46:846–8. doi: 10.1007/s00134-020-05991-x
5. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson J, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet*. (2020) 395:1033–4. doi: 10.1016/S0140-67362030628-0
6. European Medicines Agency. *EPAR RoActemra*. EMEA/H/C/000955 - II/0086/G (2019).
7. Xu X, Han M, Li T, Sun W, Wang D, Fu B. Effective treatment of severe COVID-19 patients with tocilizumab. *Proc Natl Acad Sci USA*. (2020) 117:10970–5. doi: 10.1073/pnas.2005615117
8. CORIMUNO-19 study. Available online at: https://clinicaltrials.gov/ct2/show/NCT04331808
9. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell LF, Chernyak Y, et al. Factors associated with hospitalization and critical illness among 4,103 patients with COVID-19 disease in New York City. *MedRxiv [Preprint]*. (2020). doi: 10.1101/2020.04.08.20057794
10. Jin JM, Bai P, He W, Wu F, Liu XF, Han DM, et al. Gender differences in patients with COVID-19: focus on severity and mortality. *Front Public Health*. (2020) 8:152. doi: 10.3389/fpubh.2020.00152
11. Pareek M, Bangash MN, Pareek N, Pan D, Sze S, Minhas JS, et al. Ethnicity and COVID-19: an urgent public health research priority. *Lancet*. (2020) 395:1421–2. doi: 10.1016/S0140-6736(20)30922-3
12. Lighter J, Phillips M, Hochman S, Sterling S, Johnson D, Francois F, et al. Obesity in patients younger than 60 years is a risk factor for Covid-19 hospital admission. *Clin Infect Dis*. (2020) 71:896–7. doi: 10.1093/cid/ciaa415
13. Huang I, Lim MA, Pranata R. Diabetes mellitus is associated with increased mortality and severity of disease in COVID-19 pneumonia–a systematic review, meta-analysis, and meta-regression. *Diabetes Metab Syndr*. (2020) 14:395–403. doi: 10.1016/j.dsx.2020.04.018
14. https://www.molecular.abbott/int/en/products/infectious-disease/RealTime-SARS-CoV-2-Assay
15. Dinarello CA, Simon A, van der Meer JWM. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov*. (2012) 11:633–52. doi: 10.1038/nrd3800
16. Youngstein T, Koh MBC, Ros Soto J, Rowczenio D, Trojer H, Lachmann H, et al. Adult-onset tumour necrosis factor-receptor associated periodic syndrome presenting as transfusion-dependent refractory haemophagocytosis. *Rheumatology*. (2018) 57:582–3. doi: 10.1093/rheumatology/kex427
17. Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, et al. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by coronavirus-19 (COVID-19 or SARS-CoV-2): anti-inflammatory strategies. *J Biol Regul Homeost Agents*. (2020) 34:1 doi: 10.23812/CONTI-E

18. Hussell T, Pennycook A, Openshaw PJ. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. *Eur J Immunol*. (2001) 31:2566–73. doi: 10.1002/1521-4141200010931:92566::AID-IMMU25663.0.CO;2-L
19. Ramamoorthy S, Cidlowski JA. Corticosteroids – Mechanisms of action in health and disease. *Rheum Dis Clin North Am*. (2016) 42:15–31. doi: 10.1016/j.rdc.2015.08.002
20. Barnes PJ. Mechanisms and resistance in glucocorticoid control of inflammation. *J Steroid Biochem Mol Biol*. (2010) 120:76–85. doi: 10.1016/j.jsbmb.2010.02.018
21. Russell B, Moss C, Rigg A, Van Hemelriick M, COVID-19 and treatment with NSAIDs and corticosteroids: should we be limiting their use in the clinical setting. *Ecancermedicalscience*. (2020) 14:1023. doi: 10.3332/ecancer.2020.1023
22. Wong SSY, Yuen K-Y. The management of coronavirus infections with particular reference to SARS. *J Antimicrob Chemother*. (2008) 62:437–41. doi: 10.1093/jac/dkn243
23. https://www.recoverytrial.net/news/low-cost-dexamethasone-reduces-death-by-up-to-one-third-in-hospitalised-patients-with-severe-respiratory-complications-of-covid-19
24. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/009768s037s045s047lbl.pdf
25. Tett SE, Cutler DJ, Day RO, Brown KF. Bioavailability of hydroxychloroquine tablets in healthy volunteers. *Br J Clin Pharmacol*. (1989) 27:771–9.
26. Meyerowitz EA, Vannier AGL, Friesen MGN, Schoenfeld S, Gelfand JA, Callahan MV, et al. Rethinking the role of hydroxychloroquine in the treatment of COVID-19. *FASEB J*. (2020) 34:6027–37. doi: 10.1096/fj.202000919
27. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell Res*. (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
28. Lane JCE, Weaver J, Kostka K, Duarte-Salles T, Abrahao MTF, Alghoul H, et al. Safety of hydroxychloroquine, alone and in combination with azithromycin, in light of rapid widespread use for Covid-19: a multinational, network cohort and self-controlled case series study. *medRxiv*. (2020) 2020.04.08.20054551. doi: 10.1101/2020.04.08.20054551
29. https://www.recoverytrial.net/news/statement-from-the-chief-investigators-of-the-randomised-evaluation-of-covid-19-therapy-recovery-trial-on-hydroxychloroquine-5-june-2020-no-clinical-benefit-from-use-of-hydroxychloroquine-in-hospitalised-patients-with-covid-19
30. Taylor PC. Clinical efficacy of launched JAK inhibitors in rheumatoid arthritis. *Rheumatology*. (2019) 58(Suppl. 1):i17–26. doi: 10.1093/rheumatology/key225
31. Sepriano A, Kerschbaumer A, Smolen JS, van der Heijde D, Dougados M, van Vollenhoven R, et al. Safety of synthetic and biological DMARDs: a systematic literature review informing the 2019 update of the EULAR recommendations for the management of rheumatoid arthritis. *Ann Rheum Dis*. (2020) 79:760–70. doi: 10.1136/annrheumdis-2019-216653
32. Richardson PJ, Corbellino M, Stebbing J. Baricitinib for COVID-19: a suitable treatment. *Lancet Infect Dis*. (2020). doi: 10.1016/S1473-3099(20)30270-X. [Epub ahead of print].
33. Cantini F, Niccoli L, Matarrese D, Nicastri E, Stobbione P, Goletti D. Bariticinib therpay in COVID-19: a pilot study on safety and clinical impact. *J Infect*. (2020). doi: 10.1016/j.jinf.2020.04.017. [Epub ahead of print].
34. Haberman R, Axelrad J, Chen A, Castillo R, Yan D, Izmirly P, et al. Covid-19 in immune-mediated inflammatory diseases – Case series from New York. *N Engl J Med*. (2020) 383:85–8. doi: 10.1056/NEJMc2009567
35. Morel N, Bonjour M, Le Guern V, Le Jeunne C, Mouthon L, Piette JC, et al. Colchicine: a simple and effective treatment for pericarditis in systemic lupus erythematosus? A report of 10 cases. *Lupus*. (2015) 24:1479–85. doi: 10.1177/0961203315593169
36. Imazio M, Trinchero R, Adler Y. Colchicine for the treatment of pericarditis. *Future Cardiol*. (2008) 4:566–607. doi: 10.2217/14796678.4.6.599
37. Guzik TJ, Mohiddin SA, Dimarco A, Patel V, Savvatis K, Marelli-Berg FM, et al. COVID-19 and the cardiovascular system: implications for risk assessment, diagnosis and treatment options. *Cardiovas. Res*. (2020) 116:1666–87. doi: 10.1093/cvr/cvaa106
38. Castaño-Rodriguez C, Honrubia JM, Gutiérrez-Álvarez J, Diego ML, Nieto-Torres JL, jimenez-Guardeño JM, et al. Role of severe acute respiratory syndrome coronavirus viroporins E, 3a, and 8a in replication and pathogenesis. *MBio*. (2018) 9:e02325. doi: 10.1128/mBio.02325-17
39. Misawa T, Takahama M, Kozaki T, Lee H, Saitoh T, Akira S. Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. *Nat Immunol*. (2013) 14:454–60. doi: 10.1038/ni.2550

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CASE REPORT
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Case Report: Canakinumab for the Treatment of a Patient With COVID-19 Acute Respiratory Distress Syndrome

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Severe cases of COVID-19 present with serious lung inflammation, acute respiratory distress syndrome and multiorgan damage. SARS-CoV-2 infection is associated with high cytokine levels, including interleukin-6 and certain subsets of immune cells, in particular, NK, distinguished according to the cell surface density of CD56. Cytokine levels are inversely correlated with lymphocyte count, therefore cytokine release syndrome may be an impediment to the adaptive immune response against SARS-CoV-2 infection. Canakinumab, a monoclonal antibody targeting IL-1β is under investigation for the treatment of severe SAR-CoV-2 infection. An 85 year old male presenting in our hospital with COVID-19, whose condition was complicated by acute respiratory distress syndrome and cardiac and renal failure (with oliguria) after 25 days of hospitalization, was intubated and received canakinumab for compassionate use. On the next day, diuresis recovered and conditions improved: high IL-6 levels and NK cells expressing $CD56^{bright}$ (associated with cytokine relase) were significantly reduced giving rise to NK $CD56^{dim}$. Patient died on day 58 with pulmonary bacterial superinfection and persistent SARS-CoV-2 positivity. In conclusion, canakinumab rescued a high risk, very elderly patient, from multiorgan damage complicating COVID-19. It may represent an useful treatment in severe cases.

Keywords: COVID-19, SARS-CoV-2, canakinumab, IL-1, IL-6, cytokine storm, natural killer, acute respiratory distress syndrome

INTRODUCTION

SARS-CoV-2 is responsible for the current pandemic of coronavirus disease 2019 (COVID-19). Patients present with fever, dry cough, dyspnea, and pneumonia (1). Some patients (approximately 15%), prevalently elderly and with comorbidities, develop serious multiple organ inflammation and acute respiratory distress syndrome (ARDS) (2–4) and require intensive care unit (ICU) admission (3, 4).

The immune response, including the release of pro-inflammatory cytokines and activation of T cells, are essential for controlling the viral spread, inflammation, and tissue renewal

(5, 6). The damged host cell releases proteins induce the production of pro-inflammatory cytokines by nearby cells. Monocytes, macrophages and T cells are attracted to the site of infection, establishing a pro-inflammatory feedback circuit. When the immune response is hampered, the excessive pro-inflammatory cytokines in the lungs are responsible for lung tissue damage and the cytokine storm (CS), or macrophage activation syndrome (MAS), leading to multi-organ damage.

Severe cases with CS progress to ARDS (7, 8). The mechanisms leading to such complications are complex and still under investigation. Many features of COVID-19 resemble MAS triggered by viral infection (8, 9). In fact, SARS-CoV-2 infection is associated with high levels of cytokines and, interestingly, levels of IL-6 have been reported to be correlated with mortality (10). Furthermore, in severe cases, a reduction of natural killer cells and other T lymphocytes, has been observed. Cytokine levels are inversely correlated with lymphocyte count, therefore CS may be an impediment to the adaptive immune response against SARS-CoV-2 infection (6, 11). Moreover, the severity of COVID-19 is correlated with NK subsets distinguished according to the cell surface density of CD56. In fact, $CD56^{bright}$ subset increases with severity. $CD56^{dim}$ NK cells physiologically comprise around 90% of NK cells in peripheral blood and are frequently described as the most cytotoxic, whereas $CD56^{bright}$ NK cells are abundant cytokine producers (12, 13).

Interleukin-1 (IL-1) activates the expression of several pro-inflammatory genes. IL-1β induces inflammation during infection and autoimmunity (14). IL-1β is released by various cell types, including macrophages (15, 16). Canakinumab, a monoclonal antibody targeting IL-1β, is approved for use in rheumatologic disorders[1]. Based on the mechanism of action of canakinumab, the drug is under investigation for the treatment of severe SAR-CoV-2 infection.

We present a case of an 85 year old male presenting with COVID-19, complicated by ARDS and cardiac and renal failure, rescued by canakinumab. An indication for compassionate use for COVID-19 during the current pandemic and approval from the local ethics committee was obtained in our center.

CASE DESCRIPTION

Patient was admitted to hospital on March 23, 2020, presenting with fever (38.5°C), hypoxemia (p02 = 61 mmHg), cough, and dyspnea. Medical history revealed only mild arterial hypertension treated with amlodipine and prostatic hypertrophy not requiring treatment. SARS-CoV-2 swab was positive. Chest X-ray showed an interstitial lung pattern and small left pleural effusion. Renal and liver biochemistry were normal. Noteworthy, the patient presented lymphopenia. Reactive C Protein (RCP) was 139 mg/L. Coagulation tests were normal except for Fibrinogen 619 mg/dL and D-dimers 409 ng/mL. He was at first treated in the COVID ward and received broad spectrum antibiotics, hydroxychloroquine, and oxygen therapy with Venturi mask with 30% FiO2 setting. On day 3, a chest computerized tomography (CT) without contrast showed severe lung injury (**Figure 1**).

On day 4, though the fever had subsided, his respiratory condition deteriorated and continuous positive airway pressure (CPAP) non-invasive ventilation with 40% FiO2 setting and positive end-expiratory pressure (PEEP) 10 cmH_20 was initiated, together with azitromicin, enoxaparin sodium and lopinavir/ritonavir. On day 5, tocilizumab 8 mg/kg was administered intravenously (within a clinical trial) repeated after 12 h, while continuing hydroxychloroquine, azitromicin and enoxaparin. On day 23, his conditions precipitated with presentation of ARDS, a PaO_2/FiO_2 ratio (PF) of 103 ($Fi0_2$ setting 60%, $p0_2$ 62 mmHg) and severe arterial hypertension. He was transferred to the Intensive Care Unit (ICU) in an obnubilated and non-collaborative condition, so that he was sedated with dexmedetomidine while continuing CPAP ventilation. On day 24, patient presented oliguria with acute renal and cardiac failure and progressive respiratory failure. He was intubated and received assisted mechanical ventilation together with furosemide continuous intravenous infusion and vasopressor amines.

On day 25, the patient's son was informed of the severity of the patient's clinical conditions and of the risks and benefits of canakinumab treatment. He signed informed consent to administer treatment, to process and publish all relevant clinical research data and potentially identifying information. Canakinumab was administered at a single 300 mg s.c. dose on days 25 and 31.

DIAGNOSTIC ASSESSMENT

To evaluate the biochemical effects of canakinumab, general laboratory chemistry, IL-6, and immunophenotype were collected before and after first and second administration.

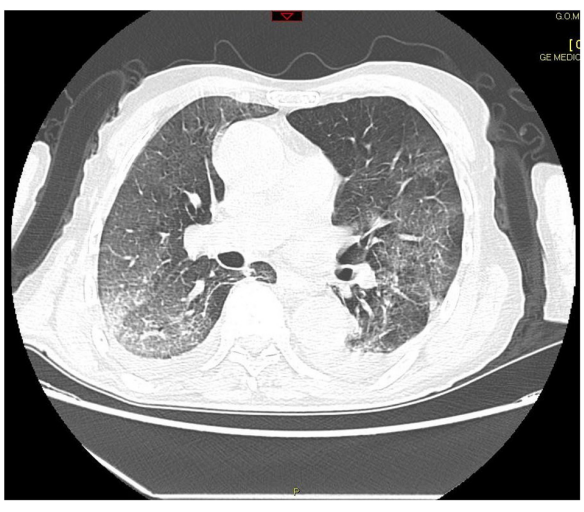

FIGURE 1 | CT scan on day 3 showed bilateral, patchy alveolar opacities progressing to diffuse consolidations, with a "white lung" appearance and widespread ground-glass opacities and moderate bilateral pleural effusions.

[1]https://www.drugs.com/pro/ilaris.html#s-34067-9

The drug was well tolerated in the short term, and on the day following the first administration, the patient's diuresis normalized and renal function improved gradually without full recovery (on day 53, creatinine level reached 88 μmol/L). The findings are summarized in **Table 1**.

During hospitalization, the patient underwent periodical microbiological surveillance tests. SARS-Cov-2 genome was evaluated by the Microbiology and Virology laboratory of our hospital. Samples from upper (nasopharyngeal) and lower (bronchoalveolar, bronchoaspirate, and tracheal aspirate) airways were collected and processed within 24 h. RNA-COVID 19 was evaluated using an Allplex 2019-nCoV assay that identifies three different target genes: E (envelope), RdRp (RNA-dependent RNA polymerase), and N (nucleoprotein gene). Based on the interpretation criteria, detection of one or more genes was interpreted as positive COVID-19. There was a high viral replication persisting on Day 43.

TABLE 1 | The laboratory findings before (day 23) and after the First (day 28) and Second (day 42) administrations of canakinumab.

Variable	Before	After First	After Second
Hemoglobin (Hb) g/dL	12.0	11.3	8.7
White Blood Cell count (WBC) × 10^9/L	4.4	6.5	12.4
Neutrophils-bands (Neutroph) × 10^9/L	3.4	5.8	10.3
Lymphocytes (Lymph) × 10^9/L	0.5	0.2	0.5
Platelet count (PLT) × 10^9/L	135	107	291
D-dimer (D-d) nmol/L	2.1	1.9	3.2
Creatinine (Cr) μmol/L	44	124	97
CRP mg/L	3.1	10.2	156.0
Lactate dehydrogenase (LDH) μkat/L	5.0	3.8	3.8
Alkaline phosphatase μkat/L	1.6	1.9	1.9
Alanine aminotransferase (ALT) μkat/L	1.0	0.5	0.2
Aspartate aminotransferase (AST) μkat/L	0.5	0.4	0.4
γ-Glutamyltransferase (GGT) μkat/L	0.4	0.4	0.5
Serum IL-6, IU/ml	424.6	46.2	75.2
Immunophenotype, cells/μL			
Lymphocyte T			
CD3+	402	172	114
CD3+CD4+	309	117	74
CD3+CD8+	95	56	41
Lymphocyte B CD19+	31	32	34
Lymphocyte NK			
CD16+CD56+	111	77	18
NK $CD56^{DIM}$	57	66	109
NK $CD56^{BRIGHT}$	42	1	2
CD4/CD8 Ratio	3	2	2

On day 31, as the respiratory conditions did not improve significantly, the Film Array Pneumonia detected the presence of bacterial infection caused by Acinetobacter C.B. Complex – 107 copies/mL – and Pseudomonas Aeruginosa – 106 copies/mL, treated initially with the association of Piperacillin + Tazobactam together with Cotrimoxazole provided intravenously QID, followed by Colistin 3000000 aerosol BID, then with Ceftazidime/Avibactam intravenous TID and finally with Doxycycline 100 mg intravenous BID (the latter ongoing).

The initial chest CT scan on Day 13 and X-rays performed before First administration (Day 23) and after Second administration (Day 38) are shown in **Figure 2**.

DISCUSSION

Canakinumab is an IL-1 antagonist indicated to treat autoinflammatory disorders. Severe COVID-19 cases show symptoms associated with an excessive release of cytokines (17, 18).

The IL-1 cytokine family plays an important role in regulating inflammation and is produced in response to inflammatory stimuli and infections. IL-1 production requires inflammasome/Caspase-1-dependent processing. It mediates its effects by binding to its receptor to activate downstream signaling which activates MAPKs and NF-kappa B, leading to the expression of pro-inflammatory mediators that drives the IL-6 signaling pathway.

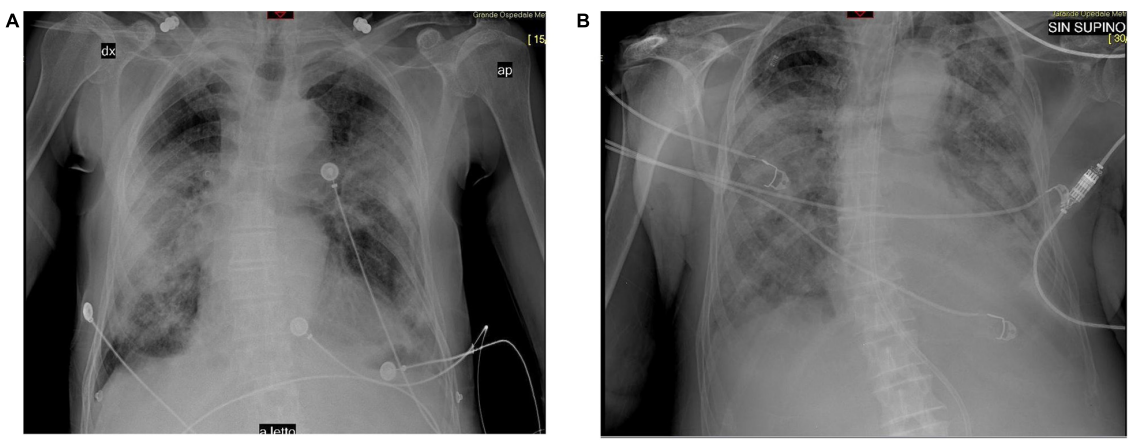

FIGURE 2 | (A) Before the first administration of canakinumab, the chest X-ray shows interstitial changes, ground glass opacities, and multifocal and bilateral effusions. **(B)** After second administration, the chest X-ray shows the extensive bilateral opacities and bilateral effusions, concomitant to bacterial superinfection.

IL-6 significantly contributes to MAS. Its levels increase with the severity of COVID-19 (9, 19, 20) and the area of pulmonary infiltration (≥50%) in patients with ARDS, together with specific lymphocyte subsets (21). Though the present case had received tocilizumab prior to canakinumab, IL-6 level remained high, postulating that tocilizumab be insufficient to rescue the patient from the subsequent cytokine storm. In our patient, IL-6 and NK CD56bright both decreased after treatment with canakinumab, suggesting that canakinumab, by interfering with IL-1, reduces IL-6. Following the Second administration, IL-6 and CRP levels increased which can be explained with the development of superimposed pulmonary bacterial infection.

Several immunotherapeutic drugs are promising for the treatment of the cytokine storm associated with COVID-19 (22). Amongst these, anakinra, an IL-1 receptor antagonist, saltuximab, an IL-6 antagonist, and sarilumab, an IL-6 receptor antagonist, are undergoing Phase 3 stage development (23).

However, canakinumab treatment is associated with adverse events, mainly an increased incidence of serious infections. In fact, IL-1β physiologically contributes to host defense against infection by enhancing the antimicrobial action of phagocytes and inducing Th1 and Th17 adaptive immune responses (24). In our case, the patient survived the MAS, but developed bacterial pulmonary superinfection, which was the final cause of death on day 58. This case represents the first published report of canakinumab for the treatment of multiorgan damage associated with COVID-19.

PATIENT PERSPECTIVE

Excessive cytokine release induces severe complications and worsens the prognosis in COVID-19. There are numerous ongoing trials to find treatments that target virus and/or inflammation. Until an effective treatment is found, in this scenario, Canakinumab due to its blocking action of proinflammatory activity by IL-1β can constitute a potential useful treatment in the modulation of hyperinflammatory symptoms, for a subgroup of patients with COVID-19, that resemble the cytokine storm in patients with MAS. The active involvement of immunologists in the clinic and in clinical trials may improve patient outcomes.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Sezione Sud – Regione Calabria. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MC, EO, and DL drafted the initial manuscript. CG performed flow cytometry. FD'A performed microbiological testing. MM performed cytokine tests. All authors reviewed and revised the manuscript and have read and agreed to the published version of the manuscript to the work reported.

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REFERENCES

1. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3.
2. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2.
3. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061–9.
4. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20.
5. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat Rev Immunol.* (2014) 14:36–49. doi: 10.1038/nri3581
6. Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, et al. Coronavirus infections and immune responses. *J Med Virol.* (2020) 92:424–32.
7. George MR. Hemophagocytic lymphohistiocytosis: review of etiologies and management. *J Blood Med.* (2014) 5:69–86.
8. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis.* (2020) 12:ciaa248.
9. Wan S, Yi Q, Fan S, Lv J, Zhang X, Guo L, et al. Characteristics of lymphocyte subsets and cytokines in peripheral blood of 123 hospitalized patients with 2019 novel coronavirus pneumonia (NCP). *MedRxiv.* (2020) [Preprint]. doi: 10.1101/2020.02.10.20021832
10. Perricone C, Triggianese P, Bartoloni E, Cafaro G, Bonifacio AF, Bursi R, et al. The anti-viral facet of anti-rheumatic drugs: lessons from COVID-19. *J Autoimmun.* (2020) 17:102468. doi: 10.1016/j.jaut.2020.102468
11. Pedersen F, Ho YC. SARS-CoV-2: a storm is raging. *J Clin Invest.* (2020) 130:2202–5.
12. Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, et al. Coronavirus infections and immune responses. *J Med Virol.* (2020) 92:424–32.
13. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol.* (2001) 22:633–40.
14. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol.* (2008) 9:503–10.
15. Dinarello CA. Therapeutic strategies to reduce IL-1 activity in treating local and systemic inflammation. *Curr Opin Pharmacol.* (2004) 4:378–85.
16. Watkins LR, Milligan ED, Maier SF. Glial proinflammatory cytokines mediate exaggerated pain states: implications for clinical pain. *Adv Exp Med Biol.* (2003) 521:1–21.
17. Ren K, Torres R. Role of interleukin-1beta during pain and inflammation. *Brain Res Rev.* (2009) 60:57–64. doi: 10.1016/j.brainresrev.2008.12.020
18. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult in patients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62.
19. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. HLH across speciality collaboration, UK. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 395:1033–4.
20. Lee CR, Kang JA, Kim HE, Choi Y, Yang T, Park SG. Secretion of IL-1β From imatinib-resistant chronic myeloid leukemia cells contributes to BCR-ABL mutation-independent imatinib resistance. *FEBS Lett.* (2016) 590:358–68.

21. Crayne CB, Albeituni S, Nichols KE, Cron RQ. The immunology of macrophage activation syndrome. *Front Immunol.* (2019) 10:119.
22. Wang W, He J, Lie P, Huang L, Wu S, Lin Y, et al. The definition and risks of cytokine release syndrome-like in 11 COVID- 19-infected pneumonia critically ill patients: disease characteristics and retrospective analysis. *MedRxiv*. (2020) [Preprint]. doi: 10.1101/2020.02.26.20026989
23. Bonam SR, Kaveri SV, Sakuntabhai A, Gilardin L, Bayry J. Adjunct immunotherapies for the management of severely Ill COVID-19 Patients. *Cell Rep Med.* (2020) 1:100016. doi: 10.1016/j.xcrm.2020.100016
24. Van de Veerdonk FL, Netea MG, Dinarello CA, Joosten LAB. Inflammasome activation and IL-1β and IL-18 processing during infection. *Trends Immunol.* (2011) 32:110–6.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
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The New Frontier of Host-Directed Therapies for *Mycobacterium avium* Complex

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Mycobacterium avium complex (MAC) is an increasingly important cause of morbidity and mortality, and is responsible for pulmonary infection in patients with underlying lung disease and disseminated disease in patients with AIDS. MAC has evolved various virulence strategies to subvert immune responses and persist in the infected host. Current treatment for MAC is challenging, requiring a combination of multiple antibiotics given over a long time period (for at least 12 months after negative sputum culture conversion). Moreover, even after eradication of infection, many patients are left with residual lung dysfunction. In order to address similar challenges facing the management of patients with tuberculosis, recent attention has focused on the development of novel adjunctive, host-directed therapies (HDTs), with the goal of accelerating the clearance of mycobacteria by immune defenses and reducing or reversing mycobacterial-induced lung damage. In this review, we will summarize the evidence supporting specific adjunctive, HDTs for MAC, with a focus on the repurposing of existing immune-modulatory agents targeting a variety of different cellular pathways. We also highlight areas meriting further investigation.

Keywords: nontuberculous mycobacteria (NTM), *Mycobacterium avium* complex, host-directed therapy, *Mycobacterium tuberculosis*, drug repurposing

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INTRODUCTION

Nontuberculous mycobacteria (NTM), including organisms of the *Mycobacterium avium* complex (MAC), represent a significant and growing threat to human health worldwide. Since the beginning of the AIDS epidemic in the 1980s, the prevalence of MAC infection has increased substantially worldwide (1). MAC is widely distributed in the environment, including in water and soil, and is transmitted via inhalation into the respiratory tract and via ingestion into the GI tract (2). The most common clinical syndromes caused by MAC are pulmonary infection in patients with underlying lung disease, as well as disseminated disease in the severely immunocompromised (3, 4). A recent review of MAC pulmonary disease worldwide reported a five-year all-cause mortality rate of 27% (5).

In addition to the virulence factors common to all mycobacteria, MAC possesses several unique features which may contribute to pathogenesis. For example, MAC demonstrates increased resistance

to phagosome-lysosome fusion and oxidative damage in murine macrophages, suggesting a unique ability to survive within activated macrophages (6). MAC can escape from macrophages undergoing apoptosis and survive extracellularly, evading the cytotoxic response necessary to eliminate intracellular bacteria (7). MAC also expresses several unique glycopeptolipids, which may modulate macrophage signaling cascades, thereby preventing an effective inflammatory response (8).

Treatment of MAC is challenging. Current treatment recommendations vary depending on the underlying conditions, severity of disease, and *in vitro* susceptibility profile. Macrolide-susceptible pulmonary disease is generally treated with a three-drug regimen, which includes a macrolide, ethambutol and a rifamycin, for at least 12 months after negative sputum-culture conversion (9). MAC often exhibits resistance to first-line antibiotics, and *in vitro* susceptibility testing for non-macrolide drugs has poor correlation with clinical efficacy. MAC pulmonary infection can present as cavitary disease with long-term respiratory sequelae. A milder form of the disease, which manifests as fibronodular bronchiectasis has a slower progression, but has been linked to increased mortality (10).

In the face of the increasing prevalence, high mortality, and treatment challenges associated with MAC infections, new therapeutic options are urgently needed. A promising avenue of research is that of host-directed therapies (HDTs). HDTs are adjuncts to antimicrobial therapy, differing from the latter in that they target host processes rather than the pathogen itself. The goal of HDTs is to boost protective immune responses, especially those inhibited or otherwise modified by the pathogen, and prevent excessive pathological inflammation (11, 12). Unlike novel antibacterial agents, they also confer the advantage of not contributing to drug resistance or cross-resistance to conventional antibiotics (12). Although HDTs are an active area of investigation in the therapy of tuberculosis (TB), as well as many non-mycobacterial infectious diseases (11–15), there has been a relative dearth of research into the potential of HDTs as adjunctive therapies for disease caused by MAC (16).

In the current review, we summarize HDT agents which are currently under investigation for MAC disease, as well as other HDTs and potentially targetable host pathways, which have not been investigated directly for MAC, but which show promise for future research.

IMPROVEMENT OF ANTIMYCOBACTERIAL IMMUNITY

Enhancing Autophagy: mTOR Inhibitors

Autophagy is a key self-degradative process in which the cytoplasmic contents of a cell are taken up by autophagosomes, trafficked to the lysosome, and digested (17). Although basal levels of this process occur in every cell, stress conditions, such as nutrient deficiency or pathogen infection, induce autophagy as a way of establishing homeostasis (18, 19). Autophagy plays a role in multiple physiological and pathological pathways, including the clearance of mycobacteria and other intracellular pathogens (17).

Initiation of autophagy is dependent on the Unc-51-like kinase-1 (ULK1) complex. This initiator complex is, in turn, regulated by the master regulator of autophagy, mammalian target of rapamycin (mTOR). mTOR plays a critical role in cellular metabolism, promoting anabolism and suppressing catabolic processes, such as autophagy (20). mTOR signaling is complex and can be activated or inhibited by a wide variety of molecules and signaling pathways. Nutrient states, particularly amino acid levels at the cellular level, serve as the main signal for mTOR activation. In nutrient-rich states, mTOR exerts an inhibitory effect on the ULK1 complex, leading to suppression of autophagy (21). Because of its important role in metabolism and cell growth, mTOR inhibition is a therapeutic target for a number of diseases, including autoimmune disorders and cancer (22). Rapamycin and other analogs directly inhibit mTOR activity, and vitamin D blocks upstream signaling to activate mTOR (22, 23). During *Mycobacterium tuberculosis* (Mtb) infection, the activation of both intracellular or extracellular surface pattern recognition receptors (PRRs) by certain unique Mtb-associated molecules, such as lipomannan, lipoarbinomannan, phthiocerol dimycocerosate (PDIM), lipoproteins, mycolic acid and *Mtb* DNA/RNA, induces autophagy (24, 25). Given that autophagy plays an important role in mycobacterial clearance, and MAC can survive intracellularly by blocking phagosome-lysosome fusion, enhancing autophagy through inhibition of the mTOR pathway appears to be an attractive HDT strategy (26, 27).

To date, there has been little research on targeting autophagy to improve host control of MAC infection. Early *et al.* reported that induction of autophagy by lactoferrin increases MAC killing by macrophages and renders the bacteria more susceptible to ethambutol, suggesting that autophagy is worthy of further investigation as an HDT target (28). Although they have not been studied in the context of MAC infection, mTOR inhibitors have been explored as HDTs for Mtb, with mixed results (29). Most data from *in vitro* studies have suggested that mTOR inhibition may result in enhanced intracellular killing of Mtb, however there is also some contrasting evidence to suggest that induction of autophagy results in increased Mtb growth, especially in the context of Mtb/HIV co-infection (30, 31). Vitamin D, an upstream inhibitor of mTOR signaling, also has shown some promise as an HDT for TB, although clinical trials do not show a consistent benefit, and it has not been investigated specifically against MAC (32).

Aside from autophagy, mTOR is involved in multiple metabolic and immunological pathways, which could affect mycobacterial pathogenesis and immunity. As a whole, the role of mTOR and autophagy in MAC infection remains largely unexplored, and further research is required to evaluate its suitability as an HDT target.

Blocking the PD-1/PD-L1 Pathway: Anti-PD-1/PD-L1 Therapy

The Programmed Cell Death Protein-1 (PD-1) and its ligand, PD-L1, are the major components of the PD-1/PD-L1 pathway, an immune checkpoint, which regulates peripheral immune tolerance and suppresses inflammation (33). PD-1 is expressed

on multiple cell types, including activated T cells, B cells, natural killer cells, and macrophages. PD-L1 is expressed on nonlymphoid cells. Binding of PD-1 to PD-L1 inhibits proliferation and effector functions of T and B cells, preventing self-reactivity (34). PD-L1 is highly expressed on tumor cells and virus-infected cells, conferring resistance to cell-mediated immunity. PD-L1 is also expressed on macrophages and plays a role in regulating immunosuppressive and pro-inflammatory activity. PD-L1 signaling in tumor-associated macrophages induces an immunosuppressive phenotype (35). Recently, the PD-1/PD-L1 pathway has become the subject of extensive research in cancer immunotherapy, as PD-1/PD-L1 antibody blockade has demonstrated efficacy in inducing cell-mediated immunity against multiple cancer types. Treatment of tumor-associated macrophages with anti-PD-L1 antibodies confers a pro-inflammatory phenotype, with increased expression of inducible nitric oxide synthase (iNOS), MHC II, TNF-α, and CD40 (36, 37). This is particularly important, since TNF-α and iNOS are critical effector mechanisms in the killing of intracellular mycobacteria, including MAC by macrophages (38). In patients with MAC pulmonary disease, expression of PD-1 by CD4 T cells is directly correlated with disease severity (39). An analysis of peripheral blood mononuclear cell (PBMC) function in such patients found that expression of PD-1 and PD-L1 were increased in lymphocytes of infected patients, which correlated with increased lymphocyte apoptosis compared to lymphocytes from healthy controls (40). Treatment of PBMCs obtained from MAC patients with anti-PD-1 and PD-L1 antibodies resulted in increased IFN-γ production and reduced T-cell apoptosis compared to PBMCs from healthy controls (40). These data suggest that PD-1/PDL-1 therapy could rescue immune cells from an immunosuppressive phenotype, allowing an improved immune response against MAC.

Although anti-PD-1 therapy may hold promise for treatment of MAC, there is some evidence that PD-1 is necessary for mycobacterial immunity, particularly against Mtb. Thus, mice deficient in PD-1 are more susceptible to Mtb infection (41). In Mtb granulomas, PD-1 is expressed in stable, cellular granulomas, but not in caseating ones, suggesting that it plays a role in granuloma maintenance. In a three-dimensional cell culture model, PD-1 inhibition led to increased Mtb growth, possibly due to excessive TNF-α expression (42).

The potential of anti-PD-1/PDL-1 therapy to improve the immune response to MAC remains to be investigated, both *in vitro* and *in vivo*. As anti-PD-1/PD-L1 therapy becomes more common in cancer therapy, retrospective analyses of its effect on patient susceptibility to MAC disease and clinical outcomes following MAC therapy may be useful.

Heme Oxygenase Inhibition

Heme oxygenase (HO-1) is an antioxidant enzyme that catalyzes the conversion of heme into carbon monoxide, biliverdin and iron (43, 44). Apart from its role in cytoprotection, HO-1 has been shown to regulate cell proliferation, differentiation, and apoptosis (44). The induction of pulmonary HO-1 is associated with TB disease (45), suggesting its potential utility as a diagnostic biomarker. Although its role in TB pathogenesis is not fully understood, experimental data in Mtb-infected mice have shown that lung bacterial loads decrease following HO-1 inhibition by the metalloporphyrin, SnPPIX (45). The same study found that a combination of an HO-1 inhibitor, SnPPIX and antimycobacterial therapy enhanced T-cell-dependent pathogen clearance. Clinical data have shown that plasma HO-1 levels decline following successful TB treatment (46).

As in the case of Mtb infection, HO-1 has been found to be elevated during MAC infection in BALB/c mice (47). Consistent with a host protective role in resisting MAC infection, mycobacterial burden in the liver, lungs and spleen was significantly higher and the disease was more likely to be disseminated in mice with HO-1 deficiency compared to HO-1 homozygous or heterozygous mice (47, 48). Further investigation is required to determine how HO-1 activity is regulated during MAC infection, and whether HO-1 inhibition is a promising HDT in the context of MAC.

IFN-γ Therapy

IFN-γ plays a significant role in immunity against Mycobacterium infections. In contrast to type I IFNs (α and β), which are made by virus-infected cells, IFN-γ is produced by activated T cells, NK cells, and macrophages, leading to the activation of phagocytes, stimulation of antigen presentation to T cells, and regulation of several other cellular functions, including proliferation, apoptosis, and cell adhesion (49). In particular, IFN-γ induces the expression of iNOS (50) and the respiratory burst enzyme NADPH-dependent phagocyte oxidase (51), thereby enhancing the mycobactericidal activity of macrophages. Mice with mutations in the IFN-γ receptor have been shown to have increased susceptibility to intracellular pathogens (52). Pre-treatment of intestinal and peritoneal-derived macrophages with IFN-γ produced both bactericidal and bacteriostatic activity against MAC following infection of these cells (53, 54). Although *in vivo* treatment of beige and Swiss-Webster mice with recombinant murine IFN-γ did not alter the course of visceral MAC infection (55), the bactericidal activity of clofazimine against MAC was enhanced in beige mice pre-treated with IFN-γ (54).

Mutations in the IFN-γ receptor gene or anti-IFN-γ autoantibodies confer increased susceptibility to disseminated NTM infections in humans (56–58). IFN-α, which, like IFN-γ, signals through STAT1, activating many common downstream effector genes, has shown some promise in treating patients with IFN-γ signaling defects and disseminated mycobacterial disease (59). In a study of 7 patients with disseminated MAC infection, subcutaneous administration of IFN-γ, in combination with conventional medical treatment, resulted in improvement in symptoms, and pathological and radiological findings, and also reduced the need for medical procedures, such as paracentesis following 8 weeks of treatment (60). Aerosolized IFN-γ has shown some promise in treating patients with TB and idiopathic pulmonary fibrosis, and is worthy of study in patients with pulmonary MAC (61).

PREVENTION OF EXCESSIVE AND PATHOLOGICAL INFLAMMATION

Suppressing Excessive TNF-α Activation: Anti-TNF Antibodies

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine which is upregulated during MAC and Mtb infection and plays an essential role in antimycobacterial immunity (62). During mycobacterial infection, T cells, macrophages, and dendritic cells produce TNF-α in response to multiple signaling pathways (63). TNF-α signaling is complex, and the cytokine serves multiple functions, including in the formation and maintenance of granulomas, as evidenced by the observation that mice deficient in TNF-α or receiving anti-TNF-α therapy produce defective granulomas following mycobacterial infection (64, 65). TNF-α also promotes killing of intracellular mycobacteria by macrophages, as the TNF blockers adalimumab and infliximab suppressed phagosome maturation in primary human PBMCs in the presence or absence of IFN-γ (66) Moreover, TNF-α serves macrophage antimicrobial functions by activating reactive oxygen and nitrogen species (67). Treatment with anti-TNF-α antibody has been associated with decreased resistance to MAC infection in mice (68).

Although TNF-α is required for an effective immune response, excessive TNF-α production has deleterious pathological effects. Thus, when its production is properly regulated, TNF-α induces apoptosis of Mtb-infected infected cells by recruiting Fas-associated protein with death domain (FADD) and subsequent activation of effector caspases and signal-regulating kinase 1 (ASK1), thus favoring mycobacterial clearance (63, 69, 70). However, when produced in excessive amounts, TNF-α results in necrosis of Mtb-infected macrophages and hyperinflammation through activation of serine/threonine-protein (RIP)1/3 kinases and mitochondrial reactive oxygen species (ROS) production (70–72). TNF-α also induces necroptosis, a highly inflammatory form of cell death, which could contribute to pathological inflammation (73).

Because of its roles in mycobacterial immunity and pathology, TNF-α has been a focus of HDT investigation. Multiple anti-TNF antibodies and TNF soluble receptors have been approved for use in humans to block TNF-α activity, and are primarily used to treat autoinflammatory conditions, such as rheumatoid arthritis. TNF blockers have shown some promise as HDTs for mycobacterial infections. Combined use of the TNF-α receptor inhibitor etanercept with antibiotics decreased the lung burden of Mtb and reduced TB-associated lung pathology in infected mice compared to antibiotics alone (74). However, the role of anti-TNF therapy in clinical cases of mycobacterial infection is controversial. Patients receiving anti-TNF therapy are at increased risk for developing disease due to Mtb and MAC (75–77). After a diagnosis of TB or MAC disease is made, anti-TNF therapy is usually halted at least until anti-mycobacterial therapy has been initiated and the infection is under control. On the other hand, there are several reports of TB patients experiencing clinical exacerbation upon discontinuation of anti-TNF treatment, and improvement of disease following its reinstitution (78–80). In addition, a subset of MAC-infected patients show favorable outcomes if anti-TNF therapy is maintained throughout treatment (76). However, it is uncertain in these cases whether anti-TNF therapy contributed as an adjunctive HDT or by ameliorating the underlying autoimmune disease.

The roles of TNF-α in mycobacterial immunity and disease are complex, and the therapeutic potential and risk of inhibiting TNF-α function during MAC infection require further investigation. Given the relatively long half-lives of most TNF blockers relative to antibiotics, there is concern over sudden stoppage of all treatment by patients, resulting in the unopposed anti-TNF activity and possible worsening of infection (81). Since TNF-α interacts with multiple other signaling pathways, further research is also needed to identify other cytokines which, if targeted in tandem with TNF-α, could hold promise as HDTs.

Broad Suppression of Inflammation: Nonsteroidal Anti-Inflammatory Drugs and Corticosteroids

Excessive and chronic inflammation is an important factor in the progression of mycobacterial disease (82). Thus, the broad inhibition of the inflammatory response by non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids is an attractive HDT strategy. NSAIDs have been well-studied as adjunctive therapies for TB, with a protective effect, both in animal models and in human disease, when used in conjunction with antibiotics (83). There are multiple proposed mechanisms for these effects. NSAIDs suppress the excessive recruitment of neutrophils to granulomas, which can be responsible for destructive inflammation (84, 85). By reducing prostaglandin E2 (PGE2) expression, NSAIDs also inhibit phagocytosis and killing of mycobacteria during late TB (86). NSAIDs have anti-thrombotic effects, which may prevent the hypercoagulable state occasionally observed with severe TB (87, 88). Despite their relatively well-characterized role as an adjunctive therapy for TB, there has been little research into NSAIDs as HDTs for MAC. The NSAID diclofenac sodium modulates multiple cytokines in MAC-stimulated macrophages but does not improve bacterial clearance by macrophages or infected mice (89). Although NSAIDs can prevent destructive inflammation, they might also inhibit an effective immune response. This is especially concerning for MAC, since an immunocompromised state is a major risk factor for disseminated MAC disease (10). NSAIDs have not been causally linked to MAC disease, but long-term NSAID use has been identified as a possible predisposing factor in at least one case (90).

Corticosteroids are some of the earliest HDTs used for mycobacterial disease and may be useful in treating patients with late-stage and extrapulmonary TB (91, 92). In particular, short-term steroid use, by reducing inflammation caused by antibiotic-mediated killing of mycobacteria and accompanying increased intracranial pressure, has been shown to improve mortality by as much as 25% in patients with tuberculous meningitis (93). Similar to NSAIDs, the beneficial effect of corticosteroids is primarily attributed to the suppression of pathological inflammation. Corticosteroids exert their anti-inflammatory effects through a variety of mechanisms, including

by inducing transcription of anti-inflammatory genes, such as annexin-1, IL-10 and IκB-α (inhibitor of NF-κB), by direct interacting with NF-κB, AP-1 and other immunomodulatory transcription factors, inhibiting maturation and differentiation of antigen presentation cells with reduced sensitivity to T cell regulation, and promoting the formation of macrophages with anti-inflammatory properties (94).

The use of corticosteroids as an HDT for MAC disease is somewhat controversial, due to their immunosuppressive effects and the lack of controlled studies (95–97). Although there is a significant body of research on the use of corticosteroids in reducing inflammation due to a variety of infectious diseases, their specific role as an adjunctive HDT for MAC disease has not been studied. Further research is required to understand the effects of corticosteroids on MAC infection on the molecular, cellular, and organismal level, to determine whether their use is justified or contraindicated in specific stages of MAC disease.

MULTIPLE MECHANISMS OF ACTION

Targeting Lipid Metabolism and Inducing Autophagy: Statins

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are a class of lipid-lowering medications, which have shown promise as HDTs for TB (98). PBMCs from patients with familial hypercholesterolemia receiving statin therapy demonstrate resistance to *ex vivo* Mtb infection compared to those from untreated donors (99). Adjunctive therapy with simvastatin enhanced the bactericidal activity of the first-line anti-mycobacterial regimen in a mouse model of chronic TB and shortened the duration of curative treatment in a murine model of TB relapse (100, 101). Consistent with a class effect of statins, pravastatin adjunctive therapy showed a dose-dependent reduction in bacillary lung burden and decreased lung inflammation in conjunction with front-line chemotherapy in a mouse model of chronic TB (102). Mechanistically, statins reduce the formation of lipid droplets in foamy macrophages, which may serve as a nutrient source for intracellular Mtb and contribute to antibiotic tolerance (99, 103). However, the primary HDT mechanism of action of statins likely involves the promotion of phagosome maturation and autophagy, thereby improving clearance of Mtb by infected macrophages (99). Statins enhance autophagy of Mtb-infected macrophages by blocking mTORC1, activating AMP-activated protein kinase (AMPK) and favoring nuclear translocation of transcription factor EB (TFEB) (104). Although the role of lipid-laden, foamy macrophages in MAC pathogenesis is less well understood than in TB, morphologically similar phenotypes have also been described in MAC-infected macrophages, and it is possible that statins could have similar HDT effects (105).

Activation of AMPK and Potentiation of Macrophage Effector Function: Metformin

Multiple studies have found that use of the anti-hyperglycemic drug metformin reduces the risk of TB and improves clinical outcomes in patients with diabetes mellitus (106, 107). Experimental evidence indicates that metformin has multiple host-directed effects, which may promote clearance of MAC. The drug enhances mycobacterial killing in human PBMCs by promoting autophagy and phagosome-lysosome fusion, as well as by selectively increasing mitochondrial ROS production (108). Metformin has a dose-dependent inhibitory effect on intracellular replication of mycobacteria through activation of the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway (109). Metformin also suppresses TNF-α expression in human monocytes (110). In Mtb-infected mice, metformin adjunctive therapy is associated with reduced chronic lung inflammation, enhanced immune responses, and improved efficacy of antibiotics (111, 112). In contrast, Dutta *et al.* showed that adjunctive therapy with human-equivalent doses of metformin did not enhance the bactericidal or sterilizing activities of the first line antitubercular regimen in Mtb-infected BALB/c mice (111). Given the widespread use of metformin and the high prevalence of MAC disease, retrospective analyses of the effect of metformin on MAC microbiological and clinical outcomes would be useful to gauge its promise as an adjunctive HDT for MAC.

Immunomodulation and Antimicrobial Properties: Clavanin-MO

Clavanin-MO is a naturally occurring antimicrobial peptide which possesses immunomodulatory properties (113). Both *in vitro* and *in vivo*, clavanin-MO stimulates production of inflammatory mediators, including IFN-γ, granulocyte-macrophage-stimulating factor, and monocyte chemoattractant protein-1, while suppressing the pro-inflammatory cytokines IL-12 and TNF-α (113). Clavanin-MO protects animal models from infection by both gram-positive and gram-negative bacteria (113). Although clavanin-MO has not been tested against mycobacteria, its immunomodulatory effects could potentially improve the immune response against MAC while blocking pathological inflammation, especially since it affects both IFN-γ and TNF-α, which are targets of other promising HDTs.

Potentiation of Macrophage Effector Function and Antimicrobial Activity: Thioridazine

Thioridazine is a neuroleptic drug, which has both direct antimycobacterial and host-directed effects (114, 115). The drug acts directly against Mtb by inhibiting antibiotic efflux pumps, thereby enhancing antibiotic susceptibility *in vitro* (116). Thioridazine also affects the host by inhibiting mammalian efflux pumps in the macrophage, leading to acidification of the phagosome and improving mycobacterial clearance (114, 117). Although its efficacy as an adjunctive therapy in murine models of chronic TB is controversial (118, 119), thioridazine was found to reduce the emergence of isoniazid-resistant mutants in Mtb-infected mouse lungs following co-administration with the standard anti-TB regimen (120). Thioridazine has been suggested as an adjunctive therapy for MAC, but research in this area has been limited (121–123). A short course of thioridazine and moxifloxacin was sufficient to clear MAC from infected monocytes (122). However, the pharmacokinetics of

thioridazine may prevent it from reaching effective concentrations in the lung, thus limiting its clinical utility in MAC pulmonary disease (121, 123).

HDTs WITH UNKNOWN OR POORLY UNDERSTOOD MECHANISMS OF ACTION

Poloxamer CRL-1072

Poloxamer CRL-1072 is a surfactant which makes mycobacteria more susceptible to some antibiotics, possibly through disruption of mycobacterial surface lipids (124). Its effects are especially pronounced in macrophages and mice compared to broth culture, suggesting that it has an effect on the host response to mycobacterial infection (124). The mechanisms of action of CRL-1072 are poorly understood. The surfactant induces production of nitric oxide in cultured human macrophages, leading to improved clearance of MAC (125). In addition, CRL-1072 induces production of IL-8 in human macrophages, a chemotactic factor which attracts neutrophils and T cells to the site of infection (126). To date, there has been little research on CRL-1072, and much remains unknown about its potential as an HDT. An important consideration is that, as a surfactant, CRL-1072 would likely have to be delivered topically to the lungs via inhalation. There is precedent for inhaled therapies for MAC with the recently FDA-approved Amikacin Liposome Inhalation Suspension (ALIS) (127).

Picolinic Acid

Picolinic acid is a degradation product of L-tryptophan with metal-chelating properties (128). An oral formulation, chromium(III) picolinate is safe and available as a nutritional supplement (129–131). Experimentally, it has both antimicrobial and host-directed effects against MAC. Specifically, picolinic acid potentiates the antimicrobial effects of clarithromycin, rifampicin, and some fluoroquinolones against both extracellular and intracellular MAC, suggesting that it has direct antimicrobial activity, which may be due to its iron-chelating properties (132). When used together with IFN-γ, picolinic acid also triggers apoptosis of MAC-infected mouse macrophages, thereby inhibiting intracellular mycobacterial growth (133, 134). Picolinic acid may also increase expression of TNF-α and interleukin-1, improving macrophage effector function (135). On the other hand, picolinic acid does not upregulate production of β-defensin-1, free fatty acids, or reactive oxygen and nitrogen intermediates (136). Therefore, its potentiation of macrophage effector functions remains poorly understood.

HDT TARGET PATHWAYS FOR FUTURE INVESTIGATION

HIF-1α

Hypoxia-inducible factor-1 alpha (HIF-1α) is a key regulator of cellular metabolism in hypoxic environments and is involved in the immune response, even under normoxic conditions (137). HIF-1α is thought to play an important role in immunity to mycobacterial infection. In zebrafish, stabilization of HIF-1α protects against *M. marinum* infection (138). The protective effect is related to upregulation of IL-1β in macrophages, which results in increased nitric oxide production by neutrophils (139). There is also evidence that HIF-1α plays multiple roles in the macrophage response to Mtb infection by mediating IFN-γ-dependent genes, regulating immune effectors, shifting metabolism to aerobic glycolysis, and blocking excessive inflammation (140–142). In general, HIF-1α promotes a pro-inflammatory state, which may improve mycobacterial clearance early in infection, but also induces pathological inflammation and immune exhaustion during chronic infection.

HIF-1α has not been well-studied in the context of MAC infection. However, research on other mycobacteria suggests that HIF-1α is a double-edged sword. Whereas induction of HIF-1α promotes a pro-inflammatory state, which may improve mycobacterial clearance early during the course of infection, it can also lead to pathological inflammation and immune exhaustion during chronic infection (140, 143). Targeting the HIF-1α pathway (and its timing) as an HDT strategy for MAC remains to be investigated.

Broadly Protective HDT Targets Against Intracellular Pathogens

A recent study screened FDA-approved drugs to identify HDT targets with broad protection against multiple intracellular pathogens (14). Three targets were identified which broadly protect THP-1 cells from intracellular bacteria: antagonizing G protein receptor (GPCR) signaling, interfering with intracellular calcium signaling, and disrupting membrane cholesterol distribution (14). Although mycobacteria have been shown to manipulate G-protein-coupled receptors to suppress epithelial signaling pathways (144) and to inhibit intracellular calcium signaling, leading to reduced phagosome-lysosome fusion and increased mycobaceterial survival within human macrophages (145), these cellular pathways have not been directly targeted by therapies, and represent an area of potential future investigation.

CONCLUSIONS

Although HDTs represent a promising tool to improve MAC clinical outcomes, they have been the subject of little research to date. Looking to the future, there are several major challenges and opportunities in MAC HDT research which remain to be met. Two specific research needs are a better understanding of MAC pathophysiology to identify HDT targets, and improved model systems to allow investigation of potential HDTs.

An improved understanding of the host-pathogen interactions during MAC disease could reveal additional HDT targets. To date, the majority of HDTs against MAC fall into two general categories: improving immune effector function or modulating pathologic inflammation. The mechanism of several HDTs are not completely understood. A better mechanistic understanding of their function could improve our knowledge of MAC pathophysiology and identify new pathways to be targeted by

HDTs. For example, the efficacy of statins in improving TB clinical outcomes suggests that the metabolism of mycobacterial-infected cells may be a promising area of investigation (102).

A lack of *in vitro* and *in vivo* experimental models of MAC infection has been a major barrier to research. Current model systems are not standardized, and do not always yield replicable or clinically useful results (146). Cell cultures cannot entirely recapitulate a disease which involves long-term, complex interactions between multiple cell types, tissues and organs, while murine models of NTM differ from human disease in their immune responses and granuloma structure, and generally do not sustain chronic infection unless immune suppression is induced (147). These deficiencies are especially important for investigating HDTs, which may target complex or human-specific pathways. Recent advances in model systems will inform future HDT research. *In silico* models could identify promising HDTs prior to the expense and difficulty of *in vitro* and *in vivo* experimentation. Recent developments in organoid models promise to allow better *in vitro* investigation of complex pathways involving interactions between multiple cell types and the extracellular matrix. For example, a three-dimensional granuloma model has recently been developed for Mtb and could be a valuable tool for investigating HDTs if adapted for MAC (148).

Finally, there is an unexplored need to investigate the use of HDTs in combination. To date, most studies have examined a particular HDT in isolation or in combination with antibiotics. Investigation of HDTs with potentially complementary mechanisms could identify therapeutic combinations that have a greater effect than the sum of their parts.

MAC is an emerging infectious disease of particular concern due to its rising prevalence, resistance to frontline antibiotics, and associated chronic morbidity and mortality (1, 5, 10). HDTs against MAC represent a promising but underexplored avenue of research, which could hold great potential in improving microbiological and clinical outcomes.

AUTHOR CONTRIBUTIONS

NC and PK conceived the work. NC, SA, and PK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Brode SK, Daley CL, Marras TK. The epidemiologic relationship between tuberculosis and non-tuberculous mycobacterial disease: a systematic review. *Int J Tuberc Lung Dis* (2014) 18:1370–7. doi: 10.5588/ijtld.14.0120
2. Lande L, George J, Plush T. Mycobacterium avium complex pulmonary disease: new epidemiology and management concepts. *Curr Opin Infect Dis* (2018) 31:199–207. doi: 10.1097/QCO.0000000000000437
3. Karakousis PC, Moore RD, Chaisson RE. Mycobacterium avium complex in patients with HIV infection in the era of highly active antiretroviral therapy. *Lancet Infect Dis* (2004) 4:557–65. doi: 10.1016/S1473-3099(04)01130-2
4. Chen C, Chen H, Chou C, Huang C, Lai C, Hsueh P. Pulmonary infection caused by nontuberculous mycobacteria in a medical center in Taiwan, 2005–2008. *Diagn Microbiol Infect Dis* (2012) 72:47–51. doi: 10.1016/j.diagmicrobio.2011.09.009
5. Diel R, Lipman M, Hoefsloot W. High mortality in patients with Mycobacterium avium complex lung disease: a systematic review. *BMC Infect Dis* (2018) 18:206–x. doi: 10.1186/s12879-018-3113-x
6. Gomes MS, Paul S, Moreira AL, Appelberg R, Rabinovitch M, Kaplan G. Survival of Mycobacterium avium and Mycobacterium tuberculosis in acidified vacuoles of murine macrophages. *Infect Immun* (1999) 67:3199–206. doi: 10.1128/IAI.67.7.3199-3206.1999
7. Early J, Fischer K, Bermudez LE. Mycobacterium avium uses apoptotic macrophages as tools for spreading. *Microb Pathog* (2011) 50:132–9. doi: 10.1016/j.micpath.2010.12.004
8. Rocco JM, Irani VR. Mycobacterium avium and modulation of the host macrophage immune mechanisms. *Int J Tuberc Lung Dis* (2011) 15:447–52. doi: 10.5588/ijtld.09.0695
9. Daley CL, Iaccarino JM, Lange C, Cambau E, Wallace RJ, Andrejak C, et al. Treatment of Nontuberculous Mycobacterial Pulmonary Disease: An Official ATS/ERS/ESCMID/IDSA Clinical Practice Guideline: Executive Summary. *Clin Infect Dis* (2020) 71:e1–e36. doi: 10.1093/cid/ciaa241
10. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. *Am J Respir Crit Care Med* (2007) 175:367–416. doi: 10.1164/rccm.200604-571ST
11. Palucci I, Delogu G. Host Directed Therapies for Tuberculosis: Futures Strategies for an Ancient Disease. *Chemotherapy* (2018) 63:172–80. doi: 10.1159/000490478
12. Zumla A, Rao M, Wallis RS, Kaufmann SH, Rustomjee R, Mwaba P, et al. Host-directed therapies for infectious diseases: current status, recent progress, and future prospects. *Lancet Infect Dis* (2016) 16:47. doi: 10.1016/S1473-3099(16)00078-5
13. Kolloli A, Subbian S. Host-Directed Therapeutic Strategies for Tuberculosis. *Front Med* (2017) 4:171. doi: 10.3389/fmed.2017.00171
14. Czyż DM, Potluri L, Jain-Gupta N, Riley SP, Martinez JJ, Steck TL, et al. Host-Directed Antimicrobial Drugs with Broad-Spectrum Efficacy against Intracellular Bacterial Pathogens. *mBio* (2014) 5:e01534–14. doi: 10.1128/mBio.01534-14
15. Frank DJ, Horne DJ, Dutta NK, Shaku MT, Madensein R, Hawn TR, et al. Remembering the Host in Tuberculosis Drug Development. *J Infect Dis* (2019) 219:1518–24. doi: 10.1093/infdis/jiy712
16. Bento CM, Gomes MS, Silva T. Looking beyond Typical Treatments for Atypical Mycobacteria. *Antibiot (Basel)* (2020) 9:18. doi: 10.3390/antibiotics9010018
17. Mizushima N. Autophagy: process and function. *Genes Dev* (2007) 21:2861–73. doi: 10.1101/gad.1599207
18. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol* (2013) 15:713–20. doi: 10.1038/ncb2788
19. Ohsumi Y. Historical landmarks of autophagy research. *Cell Res* (2014) 24:9–23. doi: 10.1038/cr.2013.169
20. Kim J, Guan K. mTOR as a central hub of nutrient signalling and cell growth. *Nat Cell Biol* (2019) 21:63–71. doi: 10.1038/s41556-018-0205-1
21. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* (2017) 168:960–76. doi: 10.1016/j.cell.2017.02.004
22. Zheng Y, Jiang Y. mTOR Inhibitors at a Glance. *Mol Cell Pharmacol* (2015) 7:15–20.
23. Lisse TS, Hewison M. Vitamin D. *Cell Cycle (Georgetown Tex)* (2011) 10:1888–9. doi: 10.4161/cc.10.12.15620

24. Delgado M, Singh S, De Haro S, Master S, Ponpuak M, Dinkins C, et al. Autophagy and Pattern Recognition Receptors in Innate Immunity. *Immunol Rev* (2009) 227:189–202. doi: 10.1111/j.1600-065X.2008.00725.x
25. Oh JE, Lee HK. Pattern Recognition Receptors and Autophagy. *Front Immunol* (2014) 5:300. doi: 10.3389/fimmu.2014.00300
26. Frehel C, de Chastellier C, Lang T, Rastogi N. Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic Mycobacterium avium. *Infect Immun* (1986) 52:252–62. doi: 10.1128/IAI.52.1.252-262.1986
27. Crowle AJ, Dahl R, Ross E, May MH. Evidence that vesicles containing living, virulent Mycobacterium tuberculosis or Mycobacterium avium in cultured human macrophages are not acidic. *Infect Immun* (1991) 59:1823–31. doi: 10.1128/IAI.59.5.1823-1831.1991
28. Silva T, Moreira AC, Nazmi K, Moniz T, Vale N, Rangel M, et al. Lactoferricin Peptides Increase Macrophages' Capacity To Kill Mycobacterium avium. *mSphere* (2017) 2:301. doi: 10.1128/msphere.00301-17
29. Singh P, Subbian S. Harnessing the mTOR Pathway for Tuberculosis Treatment. *Front Microbiol* (2018) 9:70. doi: 10.3389/fmicb.2018.00070
30. Andersson A, Andersson B, Lorell C, Raffetseder J, Larsson M, Blomgran R. Autophagy induction targeting mTORC1 enhances Mycobacterium tuberculosis replication in HIV co-infected human macrophages. *Sci Rep* (2016) 6:28171. doi: 10.1038/srep28171
31. Floto RA, Sarkar S, Perlstein EO, Kampmann B, Schreiber SL, Rubinsztein DC. Small Molecule Enhancers of Rapamycin-Induced TOR Inhibition Promote Autophagy, Reduce Toxicity in Huntington's Disease Models and Enhance Killing of Mycobacteria by Macrophages. *Autophagy* (2007) 3:620–2. doi: 10.4161/auto.4898
32. Hawn TR, Matheson AI, Maley SN, Vandal O. Host-Directed Therapeutics for Tuberculosis: Can We Harness the Host? *Microbiol Mol Biol Rev* (2013) 77:608–27. doi: 10.1128/mmbr.00032-13
33. Hu J, Zhang W, Zuo W, Tan H, Bai W. Inhibition of the PD-1/PD-L1 signaling pathway enhances innate immune response of alveolar macrophages to mycobacterium tuberculosis in mice. *Pulm Pharmacol Ther* (2020) 60:101842. doi: 10.1016/j.pupt.2019.101842
34. Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat Immunol* (2013) 14:1212–8. doi: 10.1038/ni.2762
35. Hartley GP, Chow L, Ammons DT, Wheat WH, Dow SW. Programmed Cell Death Ligand 1 (PD-L1) Signaling Regulates Macrophage Proliferation and Activation. *Cancer Immunol Res* (2018) 6:1260–73. doi: 10.1158/2326-6066.CIR-17-0537
36. Xiong H, Mittman S, Rodriguez R, Moskalenko M, Pacheco-Sanchez P, Yang Y, et al. Anti–PD-L1 Treatment Results in Functional Remodeling of the Macrophage Compartment. *Cancer Res* (2019) 79:1493–506. doi: 10.1158/0008-5472.CAN-18-3208
37. Zhang Y, Du W, Chen Z, Xiang C. Upregulation of PD-L1 by SPP1 mediates macrophage polarization and facilitates immune escape in lung adenocarcinoma. *Exp Cell Res* (2017) 359:449–57. doi: 10.1016/j.yexcr.2017.08.028
38. Bekker LG, Freeman S, Murray PJ, Ryffel B, Kaplan G. TNF-alpha controls intracellular mycobacterial growth by both inducible nitric oxide synthase-dependent and inducible nitric oxide synthase-independent pathways. *J Immunol* (2001) 166:6728–34. doi: 10.4049/jimmunol.166.11.6728
39. Shu C, Pan S, Feng J, Wang J, Chan Y, Yu C, et al. The Clinical Significance of Programmed Death-1, Regulatory T Cells and Myeloid Derived Suppressor Cells in Patients with Nontuberculous Mycobacteria-Lung Disease. *J Clin Med* (2019) 8:736. doi: 10.3390/jcm8050736
40. Shu C, Wang J, Wu M, Wu C, Lai H, Lee L, et al. Attenuation of lymphocyte immune responses during Mycobacterium avium complex-induced lung disease due to increasing expression of programmed death-1 on lymphocytes. *Sci Rep* (2017) 7:42004. doi: 10.1038/srep42004
41. Tousif S, Singh Y, Prasad DVR, Sharma P, Kaer LV, Das G. T Cells from Programmed Death-1 Deficient Mice Respond Poorly to Mycobacterium tuberculosis Infection. *PLoS One* (2011) 6:19864. doi: 10.1371/journal.pone.0019864
42. Tezera LB, Bielecka MK, Ogongo P, Walker NF, Ellis M, Garay-Baquero DJ, et al. Anti-PD-1 immunotherapy leads to tuberculosis reactivation via dysregulation of TNF-α. *Elife* (2020) 9:52668. doi: 10.7554/eLife.52668
43. Scharn CR, Collins AC, Nair VR, Stamm CE, Marciano DK, Graviss EA, et al. Heme Oxygenase-1 Regulates Inflammation and Mycobacterial Survival in Human Macrophages during Mycobacterium tuberculosis Infection. *J Immunol* (2016) 196:4641–9. doi: 10.4049/jimmunol.1500434
44. Grochot-Przeczek A, Dulak J, Jozkowicz A. Haem oxygenase-1: non-canonical roles in physiology and pathology. *Clin Sci (Lond)* (2012) 122:93–103. doi: 10.1042/CS20110147
45. Costa DL, Namasivayam S, Amaral EP, Arora K, Chao A, Mittereder LR, et al. Pharmacological Inhibition of Host Heme Oxygenase-1 Suppresses Mycobacterium tuberculosis Infection In Vivo by a Mechanism Dependent on T Lymphocytes. *mBio* (2016) 7:1675. doi: 10.1128/mbio.01675-16
46. Rockwood N, Costa DL, Amaral EP, Du Bruyn E, Kubler A, Gil-Santana L, et al. Mycobacterium tuberculosis Induction of Heme Oxygenase-1 Expression Is Dependent on Oxidative Stress and Reflects Treatment Outcomes. *Front Immunol* (2017) 8:542. doi: 10.3389/fimmu.2017.00542
47. Silva-Gomes S, Appelberg R, Larsen R, Soares MP, Gomes MS. Heme Catabolism by Heme Oxygenase-1 Confers Host Resistance to Mycobacterium Infection. *Infection Immun* (2013) 81:2536–45. doi: 10.1128/iai.00251-13
48. Saunders BM, Cooper AM. Restraining mycobacteria: Role of granulomas in mycobacterial infections. *Immunol Cell Biol* (2000) 78:334–41. doi: 10.1046/j.1440-1711.2000.00933.x
49. Reljic R. IFN-gamma therapy of tuberculosis and related infections. *J Interferon Cytokine Res* (2007) 27:353–64. doi: 10.1089/jir.2006.0103
50. Kamijo R, Harada H, Matsuyama T, Bosland M, Gerecitano J, Shapiro D, et al. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* (1994) 263:1612–5. doi: 10.1126/science.7510419
51. Cassatella MA, Bazzoni F, Flynn RM, Dusi S, Trinchieri G, Rossi F. Molecular basis of interferon-gamma and lipopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components. *J Biol Chem* (1990) 265:20241–6.
52. Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, et al. Immune response in mice that lack the interferon-gamma receptor. *Science* (1993) 259:1742–5. doi: 10.1126/science.8456301
53. Hsu N, Young LS, Bermudez LE. Response to stimulation with recombinant cytokines and synthesis of cytokines by murine intestinal macrophages infected with the Mycobacterium avium complex. *Infect Immun* (1995) 63:528–33. doi: 10.1128/IAI.63.2.528-533.1995
54. Gomez-Flores R, Tucker SD, Kansal R, Tamez-Guerra R, Mehta RT. Enhancement of antibacterial activity of clofazimine against Mycobacterium avium-Mycobacterium intracellulare complex infection induced by IFN-gamma is mediated by TNF-alpha. *J Antimicrob Chemother* (1997) 39:189–97. doi: 10.1093/jac/39.2.189
55. Squires KE, Murphy WF, Madoff LC, Murray HW. Interferon-gamma and Mycobacterium avium-intracellulare infection. *J Infect Dis* (1989) 159:599–600. doi: 10.1093/infdis/159.3.599
56. Wu U, Holland SM. Host susceptibility to non-tuberculous mycobacterial infections. *Lancet Infect Dis* (2015) 15:968–80. doi: 10.1016/S1473-3099(15)00089-4
57. Glosli H, Stray-Pedersen A, Brun AC, Holtmon LW, Tønjum T, Chapgier A, et al. Infections due to various atypical mycobacteria in a Norwegian multiplex family with dominant interferon-gamma receptor deficiency. *Clin Infect Dis* (2008) 46:23. doi: 10.1086/525855
58. Jouanguy E, Altare F, Lamhamedi S, Revy P, Emile JF, Newport M, et al. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guérin infection. *N Engl J Med* (1996) 335:1956–61. doi: 10.1056/NEJM199612263352604
59. Bax HI, Freeman AF, Ding L, Hsu AP, Marciano B, Kristosturyan E, et al. Interferon alpha treatment of patients with impaired interferon gamma signaling. *J Clin Immunol* (2013) 33:991–1001. doi: 10.1007/s10875-013-9882-5
60. Holland SM, Eisenstein EM, Kuhns DB, Turner ML, Fleisher TA, Strober W, et al. Treatment of refractory disseminated nontuberculous mycobacterial infection with interferon gamma. A preliminary report. *N Engl J Med* (1994) 330:1348–55. doi: 10.1056/NEJM199405123301904
61. Smaldone GC. Repurposing of gamma interferon via inhalation delivery. *Adv Drug Deliv Rev* (2018) 133:87–92. doi: 10.1016/j.addr.2018.06.004
62. Appelberg R. Protective Role of Interferon Gamma, Tumor Necrosis Factor Alpha and Interleukin-6 in Mycobacterium tuberculosis and M. avium

Infections. *Immunobiology* (1994) 191:520–5. doi: 10.1016/S0171-2985(11)80458-4
63. Dorhoi A, Kaufmann SHE. Tumor necrosis factor alpha in mycobacterial infection. *Semin Immunol* (2014) 26:203–9. doi: 10.1016/j.smim.2014.04.003
64. Kindler V, Sappino A, Grau GE, Piguet P, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell* (1989) 56:731–40. doi: 10.1016/0092-8674(89)90676-4
65. Kaneko H, Yamada H, Mizuno S, Udagawa T, Kazumi Y, Sekikawa K, et al. Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. *Lab Invest* (1999) 79:379–86.
66. Harris J, Hope JC, Keane J. Tumor Necrosis Factor Blockers Influence Macrophage Responses to Mycobacterium tuberculosis. *J Infect Dis* (2008) 198:1842–50. doi: 10.1086/593174
67. Blaser H, Dostert C, Mak TW, Brenner D. TNF and ROS Crosstalk in Inflammation. *Trends Cell Biol* (2016) 26:249–61. doi: 10.1016/j.tcb.2015.12.002
68. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity* (1995) 2:561–72. doi: 10.1016/1074-7613(95)90001-2
69. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, et al. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. *Infect Immun* (1997) 65:298–304. doi: 10.1128/IAI.65.1.298-304.1997
70. Vassalli P. The Pathophysiology of Tumor Necrosis Factors. *Annu Rev Immunol* (1992) 10:411–52. doi: 10.1146/annurev.iy.10.040192.002211
71. Kundu M, Pathak SK, Kumawat K, Basu S, Chatterjee G, Pathak S, et al. A TNF- and c-Cbl-dependent FLIP(S)-degradation pathway and its function in Mycobacterium tuberculosis-induced macrophage apoptosis. *Nat Immunol* (2009) 10:918–26. doi: 10.1038/ni.1754
72. Keane J, Remold HG, Kornfeld H. Virulent Mycobacterium tuberculosis strains evade apoptosis of infected alveolar macrophages. *J Immunol* (2000) 164:2016–20. doi: 10.4049/jimmunol.164.4.2016
73. Declercq W, Vanden Berghe T, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. *Cell* (2009) 138:229–32. doi: 10.1016/j.cell.2009.07.006
74. Skerry C, Harper J, Klunk M, Bishai WR, Jain SK. Adjunctive TNF inhibition with standard treatment enhances bacterial clearance in a murine model of necrotic TB granulomas. *PLoS One* (2012) 7:e39680. doi: 10.1371/journal.pone.0039680
75. Winthrop KL, Chang E, Yamashita S, Iademarco MF, LoBue PA. Nontuberculous Mycobacteria Infections and Anti–Tumor Necrosis Factor-α Therapy. *Emerging Infect Dis* (2009) 15:1556–61. doi: 10.3201/eid1510.090310
76. Yoo J, Jo K, Kang B, Kim MY, Yoo B, Lee C, et al. Mycobacterial diseases developed during anti-tumour necrosis factor-α therapy. *Eur Respir J* (2014) 44:1289–95. doi: 10.1183/09031936.00063514
77. Wallis RS. Tumour necrosis factor antagonists: structure, function, and tuberculosis risks. *Lancet Infect Dis* (2008) 8:601–11. doi: 10.1016/S1473-3099(08)70227-5
78. Wallis RS, van Vuuren C, Potgieter S. Adalimumab treatment of life-threatening tuberculosis. *Clin Infect Dis* (2009) 48:1429–32. doi: 10.1086/598504
79. Arend SM, Leyten EMS, Franken WPJ, Huisman EM, van Dissel JT. A patient with de novo tuberculosis during anti-tumor necrosis factor-alpha therapy illustrating diagnostic pitfalls and paradoxical response to treatment. *Clin Infect Dis* (2007) 45:1470–5. doi: 10.1086/522993
80. Garcia Vidal C, Rodríguez Fernández S, Martínez Lacasa J, Salavert M, Vidal R, Rodríguez Carballeira M, et al. Paradoxical response to antituberculous therapy in infliximab-treated patients with disseminated tuberculosis. *Clin Infect Dis* (2005) 40:756–9. doi: 10.1086/427941
81. Yew WW. Clinically significant interactions with drugs used in the treatment of tuberculosis. *Drug Saf* (2002) 25:111–33. doi: 10.2165/00002018-200225020-00005
82. Ehlers S. Immunity to tuberculosis: a delicate balance between protection and pathology. *FEMS Immunol Med Microbiol* (1999) 23:149–58. doi: 10.1111/j.1574-695X.1999.tb01234.x
83. Kroesen VM, Gröschel MI, Martinson N, Zumla A, Maeurer M, der Werf v, et al. Non-Steroidal Anti-inflammatory Drugs As Host-Directed Therapy for Tuberculosis: A Systematic Review. *Front Immunol* (2017) 8:772. doi: 10.3389/fimmu.2017.00772
84. Vilaplana C, Marzo E, Tapia G, Diaz J, Garcia V, Cardona P. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J Infect Dis* (2013) 208:199–202. doi: 10.1093/infdis/jit152
85. Marzo E, Vilaplana C, Tapia G, Diaz J, Garcia V, Cardona P. Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tubercul (Edinb)* (2014) 94:55–64. doi: 10.1016/j.tube.2013.09.004
86. Ivanyi J, Zumla A. Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *J Infect Dis* (2013) 208:185–8. doi: 10.1093/infdis/jit153
87. Schafer AI. Effects of nonsteroidal antiinflammatory drugs on platelet function and systemic hemostasis. *J Clin Pharmacol* (1995) 35:209–19. doi: 10.1002/j.1552-4604.1995.tb04050.x
88. Schoeman J, Mansvelt E, Springer P, van Rensburg AJ, Carlini S, Fourie E. Coagulant and fibrinolytic status in tuberculous meningitis. *Pediatr Infect Dis J* (2007) 26:428–31. doi: 10.1097/01.inf.0000261126.60283.cf
89. Sano C, Shimizu T, Sato K, Kawauchi H, Kawahara S, Tomioka H. Therapeutic Effects of Benzoxazinorifamycin KRM-1648 Administered Alone or in Combination with a Half-Sized Secretory Leukocyte Protease Inhibitor or the Nonsteroidal Anti-Inflammatory Drug Diclofenac Sodium against Mycobacterium avium Complex Infection in Mice. *Antimicrob Agents Chemother* (1999) 43:360–4. doi: 10.1128/AAC.43.2.360
90. Yang D, Chang W, Cheng M, Lai J, Chang D, Chen C. Peripheral arthritis caused by Mycobacterium avium-intracellulare in a patient with ankylosing spondylitis. *J Clin Rheumatol* (2009) 15:323–4. doi: 10.1097/RHU.0b013e3181bbbcfb
91. Dolecek R. [Certain steroids in the treatment of pulmonary tuberculosis]. *Cas Lek Cesk* (1951) 90:1160–3.
92. Critchley JA, Young F, Orton L, Garner P. Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *Lancet Infect Dis* (2013) 13:223–37. doi: 10.1016/S1473-3099(12)70321-3
93. Prasad K, Singh MB, Ryan H. Corticosteroids for managing tuberculous meningitis. *Cochrane Database Syst Rev* (2016) 2016:2244. doi: 10.1002/14651858.CD002244.pub4
94. Schutz C, Davis AG, Sossen B, Lai RP, Ntsekhe M, Harley YX, et al. Corticosteroids as an adjunct to tuberculosis therapy. *Expert Rev Respir Med* (2018) 12:881–91. doi: 10.1080/17476348.2018.1515628
95. Goetz MB. Are corticosteroids useful adjunctive agents in the treatment of disseminated Mycobacterium avium complex infection associated with human immunodeficiency virus infection? *Clin Infect Dis* (1998) 26:687–8. doi: 10.1086/514598
96. Dorman SE, Heller HM, Basgoz NO, Sax PE. Adjunctive corticosteroid therapy for patients whose treatment for disseminated Mycobacterium avium complex infection has failed. *Clin Infect Dis* (1998) 26:682–6. doi: 10.1086/514597
97. Kobashi Y, Matsushima T. Clinical analysis of pulmonary Mycobacterium avium complex disease in association with corticosteroid treatment. *J Infect Chemother* (2003) 9:68–74. doi: 10.1007/s10156-002-0216-4
98. Yang C. Advancing host-directed therapy for tuberculosis. *Microb Cell* (2017) 4:105–7. doi: 10.15698/mic2017.03.565
99. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdayal R, Mhlanga MM, et al. Statin therapy reduces the mycobacterium tuberculosis burden in human macrophages and in mice by enhancing autophagy and phagosome maturation. *J Infect Dis* (2014) 209:754–63. doi: 10.1093/infdis/jit550
100. Skerry C, Pinn ML, Bruiners N, Pine R, Gennaro ML, Karakousis PC. Simvastatin increases the in vivo activity of the first-line tuberculosis regimen. *J Antimicrob Chemother* (2014) 69:2453–7. doi: 10.1093/jac/dku166
101. Dutta NK, Bruiners N, Pinn ML, Zimmerman MD, Prideaux B, Dartois V, et al. Statin adjunctive therapy shortens the duration of TB treatment in mice. *J Antimicrob Chemother* (2016) 71:1570–7. doi: 10.1093/jac/dkw014
102. Dutta NK, Bruiners N, Zimmerman MD, Tan S, Dartois V, Gennaro ML, et al. Adjunctive Host-Directed Therapy With Statins Improves Tuberculosis-Related Outcomes in Mice. *J Infect Dis* (2020) 221:1079–87. doi: 10.1093/infdis/jiz517

103. Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, et al. Foamy Macrophages from Tuberculous Patients' Granulomas Constitute a Nutrient-Rich Reservoir for M. tuberculosis Persistence. *PLoS Pathog* (2008) 4:204. doi: 10.1371/journal.ppat.1000204
104. Bruiners N, Dutta N, Guerrini V, Salamon H, Yamaguchi KD, Karakousis PC, et al. The anti-tubercular activity of simvastatin is mediated by cholesterol-driven autophagy via the AMPK-mTORC1-TFEB axis. *J Lipid Res* (2020) 61:1617–28. doi: 10.1194/jlr.RA120000895
105. Caire-Brändli I, Papadopoulos A, Malaga W, Marais D, Canaan S, Thilo L, et al. Reversible Lipid Accumulation and Associated Division Arrest of Mycobacterium avium in Lipoprotein-Induced Foamy Macrophages May Resemble Key Events during Latency and Reactivation of Tuberculosis. *Infection Immun* (2014) 82:476–90. doi: 10.1128/iai.01196-13
106. Zhang M, He J. Impacts of metformin on tuberculosis incidence and clinical outcomes in patients with diabetes: a systematic review and meta-analysis. *Eur J Clin Pharmacol* (2020) 76:149–59. doi: 10.1007/s00228-019-02786-y
107. Degner NR, Wang J, Golub JE, Karakousis PC. Metformin Use Reverses the Increased Mortality Associated With Diabetes Mellitus During Tuberculosis Treatment. *Clin Infect Dis* (2018) 66:198–205. doi: 10.1093/cid/cix819
108. Lachmandas E, Eckold C, Böhme J, Koeken, Valerie ACM, Marzuki MB, et al. Metformin Alters Human Host Responses to Mycobacterium tuberculosis in Healthy Subjects. *J Infect Dis* (2019) 220:139–50. doi: 10.1093/infdis/jiz064
109. Yew WW, Chang KC, Chan DP, Zhang Y. Metformin as a host-directed therapeutic in tuberculosis: Is there a promise? *Tubercul (Edinb)* (2019) 115:76–80. doi: 10.1016/j.tube.2019.02.004
110. Arai M, Uchiba M, Komura H, Mizuochi Y, Harada N, Okajima K. Metformin, an antidiabetic agent, suppresses the production of tumor necrosis factor and tissue factor by inhibiting early growth response factor-1 expression in human monocytes in vitro. *J Pharmacol Exp Ther* (2010) 334:206–13. doi: 10.1124/jpet.109.164970
111. Dutta NK, Pinn ML, Karakousis PC. Metformin Adjunctive Therapy Does Not Improve the Sterilizing Activity of the First-Line Antitubercular Regimen in Mice. *Antimicrob Agents Chemother* (2017) 61:652. doi: 10.1128/aac.00652-17
112. Singhal A, Jie L, Kumar P, Hong GS, Leow MK, Paleja B, et al. Metformin as adjunct antituberculosis therapy. *Sci Transl Med* (2014) 6:263ra159. doi: 10.1126/scitranslmed.3009885
113. Silva ON, de la Fuente-Núñez C, Haney EF, Fensterseifer ICM, Ribeiro SM, Porto WF, et al. An anti-infective synthetic peptide with dual antimicrobial and immunomodulatory activities. *Sci Rep* (2016) 6:35465. doi: 10.1038/srep35465
114. Amaral L, Viveiros M. Thioridazine: A Non-Antibiotic Drug Highly Effective, in Combination with First Line Anti-Tuberculosis Drugs, against Any Form of Antibiotic Resistance of Mycobacterium tuberculosis Due to Its Multi-Mechanisms of Action. *Antibiot (Basel)* (2017) 6:3. doi: 10.3390/antibiotics6010003
115. Dutta NK, Karakousis PC. Thioridazine for treatment of tuberculosis: promises and pitfalls. *Tubercul (Edinb)* (2014) 94:708–11. doi: 10.1016/j.tube.2014.09.001
116. Machado D, Couto I, Perdigão J, Rodrigues L, Portugal I, Baptista P, et al. Contribution of Efflux to the Emergence of Isoniazid and Multidrug Resistance in Mycobacterium tuberculosis. *PLoS One* (2012) 7:e34538. doi: 10.1371/journal.pone.0034538
117. Machado D, Pires D, Perdigão J, Couto I, Portugal I, Martins M, et al. Ion Channel Blockers as Antimicrobial Agents, Efflux Inhibitors, and Enhancers of Macrophage Killing Activity against Drug Resistant Mycobacterium tuberculosis. *PLoS One* (2016) 11:e0149326. doi: 10.1371/journal.pone.0149326
118. Dutta NK, Pinn ML, Karakousis PC. Sterilizing activity of thioridazine in combination with the first-line regimen against acute murine tuberculosis. *Antimicrob Agents Chemother* (2014) 58:5567–9. doi: 10.1128/AAC.03408-14
119. Dutta NK, Pinn ML, Zhao M, Rudek MA, Karakousis PC. Thioridazine lacks bactericidal activity in an animal model of extracellular tuberculosis. *J Antimicrob Chemother* (2013) 68:1327–30. doi: 10.1093/jac/dkt037
120. Dutta NK, Pinn ML, Karakousis PC. Reduced emergence of isoniazid resistance with concurrent use of thioridazine against acute murine tuberculosis. *Antimicrob Agents Chemother* (2014) 58:4048–53. doi: 10.1128/AAC.02981-14
121. Ruth MM, Pennings LJ, Koeken, Valerie ACM, Schildkraut JA, Hashemi A, et al. Thioridazine Is an Efflux Pump Inhibitor in Mycobacterium avium Complex but of Limited Clinical Relevance. *Antimicrob Agents Chemother* (2020) 64:181. doi: 10.1128/AAC.00181-20
122. Srivastava S, Deshpande D, Sherman CM, Gumbo T. A 'shock and awe' thioridazine and moxifloxacin combination-based regimen for pulmonary Mycobacterium avium-intracellulare complex disease. *J Antimicrob Chemother* (2017) 72:i43–7. doi: 10.1093/jac/dkx308
123. Deshpande D, Srivastava S, Musuka S, Gumbo T. Thioridazine as Chemotherapy for Mycobacterium avium Complex Diseases. *Antimicrob Agents Chemother* (2016) 60:4652–8. doi: 10.1128/AAC.02985-15
124. Jagannath C, Emanuele MR, Hunter RL. Activities of Poloxamer CRL-1072 against Mycobacterium avium in Macrophage Culture and in Mice. *Antimicrob Agents Chemother* (1999) 43:2898–903. doi: 10.1128/AAC.43.12.2898
125. Jagannath C, Sepulveda E, Actor JK, Luxem F, Emanuele MR, Hunter RL. Effect of poloxamer CRL-1072 on drug uptake and nitric-oxide-mediated killing of Mycobacterium avium by macrophages. *Immunopharmacology* (2000) 48:185–97. doi: 10.1016/s0162-3109(00)00203-4
126. Jagannath C, Pai S, Actor JK, Hunter RL. CRL-1072 enhances antimycobacterial activity of human macrophages through interleukin-8. *J Interferon Cytokine Res* (1999) 19:67–76. doi: 10.1089/107999099314432
127. Daley CL, Olivier KN. ALIS (Amikacin Liposome Inhalation Suspension): The Beginning of a Wonderland? *Am J Respir Crit Care Med* (2018) 198:1473–5. doi: 10.1164/rccm.201810-1901ED
128. Evans GW, Johnson PE. Characterization and quantitation of a zinc-binding ligand in human milk. *Pediatr Res* (1980) 14:876–80. doi: 10.1203/00006450-198007000-00007
129. Ganguly R, Sahu S, Ohanyan V, Haney R, Chavez RJ, Shah S, et al. Oral chromium picolinate impedes hyperglycemia-induced atherosclerosis and inhibits proatherogenic protein TSP-1 expression in STZ-induced type 1 diabetic ApoE-/- mice. *Sci Rep* (2017) 7:45279. doi: 10.1038/srep45279
130. Vincent JB. Chromium: celebrating 50 years as an essential element? *Dalton Trans* (2010) 39:3787–94. doi: 10.1039/b920480f
131. Stout MD, Nyska A, Collins BJ, Witt KL, Kissling GE, Malarkey DE, et al. Chronic Toxicity and Carcinogenicity Studies of Chromium Picolinate Monohydrate Administered in Feed to F344/N Rats and B6C3F1 Mice for 2 Years. *Food Chem Toxicol* (2009) 47:729–33. doi: 10.1016/j.fct.2009.01.006
132. Cai S, Sato K, Shimizu T, Yamabe S, Hiraki M, Sano C, et al. Antimicrobial activity of picolinic acid against extracellular and intracellular Mycobacterium avium complex and its combined activity with clarithromycin, rifampicin and fluoroquinolones. *J Antimicrob Chemother* (2006) 57:85–93. doi: 10.1093/jac/dki418
133. Pais TF, Appelberg R. Induction of Mycobacterium avium growth restriction and inhibition of phagosome-endosome interactions during macrophage activation and apoptosis induction by picolinic acid plus IFNgamma. *Microbiol (Reading)* (2004) 150:1507–18. doi: 10.1099/mic.0.26815-0
134. Pais TF, Appelberg R. Macrophage control of mycobacterial growth induced by picolinic acid is dependent on host cell apoptosis. *J Immunol* (2000) 164:389–97. doi: 10.4049/jimmunol.164.1.389
135. Blasi E, Mazzolla R, Pitzurra L, Barluzzi R, Bistoni F. Protective effect of picolinic acid on mice intracerebrally infected with lethal doses of Candida albicans. *Antimicrob Agents Chemother* (1993) 37:2422–6. doi: 10.1128/aac.37.11.2422
136. Tomioka H, Shimizu T, Tatano Y. Effects of picolinic acid on the antimicrobial functions of host macrophages against Mycobacterium avium complex. *Int J Antimicrob Agents* (2007) 29:460–4. doi: 10.1016/j.ijantimicag.2006.12.010
137. Hellwig-Bürgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann W. Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions. *J Interferon Cytokine Res* (2005) 25:297–310. doi: 10.1089/jir.2005.25.297
138. Elks PM, Brizee S, van der Vaart M, Walmsley SR, van Eeden FJ, Renshaw SA, et al. Hypoxia inducible factor signaling modulates susceptibility to mycobacterial infection via a nitric oxide dependent mechanism. *PLoS Pathog* (2013) 9:e1003789. doi: 10.1371/journal.ppat.1003789
139. Ogryzko NV, Lewis A, Wilson HL, Meijer AH, Renshaw SA, Elks PM. Hif-1α-Induced Expression of Il-1β Protects against Mycobacterial Infection in Zebrafish. *J Immunol* (2019) 202:494–502. doi: 10.4049/jimmunol.1801139

140. Braverman J, Sogi KM, Benjamin D, Nomura DK, Stanley SA. HIF-1α Is an Essential Mediator of IFN-γ-Dependent Immunity to Mycobacterium tuberculosis. *J Immunol* (2016) 197:1287–97. doi: 10.4049/jimmunol.1600266
141. Braverman J, Stanley SA. Nitric Oxide Modulates Macrophage Responses to Mycobacterium tuberculosis Infection through Activation of HIF-1α and Repression of NF-κB. *J Immunol* (2017) 199:1805–16. doi: 10.4049/jimmunol.1700515
142. Resende M, Ferreira CM, Barbosa AM, Cardoso MS, Sousa J, Saraiva M, et al. Myeloid HIF-1α regulates pulmonary inflammation during experimental Mycobacterium tuberculosis infection. *Immunology* (2020) 159:121–9. doi: 10.1111/imm.13131
143. Guan R, Wang J, Li Z, Ding M, Li D, Xu G, et al. Sodium Tanshinone IIA Sulfonate Decreases Cigarette Smoke-Induced Inflammation and Oxidative Stress via Blocking the Activation of MAPK/HIF-1α Signaling Pathway. *Front Pharmacol* (2018) 9:263. doi: 10.3389/fphar.2018.00263
144. Alaridah N, Lutay N, Tenland E, Rönnholm A, Hallgren O, Puthia M, et al. Mycobacteria Manipulate G-Protein-Coupled Receptors to Increase Mucosal Rac1 Expression in the Lungs. *J Innate Immun* (2017) 9:318–29. doi: 10.1159/000453454
145. Malik ZA, Denning GM, Kusner DJ. Inhibition of Ca(2+) signaling by Mycobacterium tuberculosis is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. *J Exp Med* (2000) 191:287–302. doi: 10.1084/jem.191.2.287
146. Andréjak C, Almeida DV, Tyagi S, Converse PJ, Ammerman NC, Grosset JH. Characterization of mouse models of Mycobacterium avium complex infection and evaluation of drug combinations. *Antimicrob Agents Chemother* (2015) 59:2129–35. doi: 10.1128/AAC.04841-14
147. Bernut A, Herrmann J, Ordway D, Kremer L. The Diverse Cellular and Animal Models to Decipher the Physiopathological Traits of Mycobacterium abscessus Infection. *Front Cell Infect Microbiol* (2017) 7:100. doi: 10.3389/fcimb.2017.00100
148. Tezera LB, Bielecka MK, Chancellor A, Reichmann MT, Shammari BA, Brace P, et al. Dissection of the host-pathogen interaction in human tuberculosis using a bioengineered 3-dimensional model. *Elife* (2017) 6:21283. doi: 10.7554/eLife.21283

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MINI REVIEW
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The Manipulation of the Lipid Mediator Metabolism as Adjunct Host-Directed Therapy in Tuberculosis

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Host-directed therapies (HDTs) enhance the host response to tuberculosis (TB) infection to reduce disease severity. For instance, the manipulation of lipid mediator production diminishes the hyperactive immune response which is a known pathological feature of TB that generates lung tissue damage. Non-steroidal anti-inflammatory drugs (NSAIDs) and omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) are examples of such HDTs. In this mini-review, we recapitulate the literature available on the effects of NSAIDs and n-3 LCPUFA in TB as well as the immunological pathways underpinning these effects. Many NSAIDs have a great deal of data describing their effects and safety and in many jurisdictions are inexpensive, and sold over the counter in neighborhood convenience stores and supermarkets. The potential benefits of NSAIDs in TB are well-documented in pre-clinical studies. The reduction of pro-inflammatory lipid mediator production by inhibiting cyclooxygenase (COX) pathways with NSAIDs has been found to improve lung histopathology, bacterial control, and survival. Additionally, n-3 LCPUFA and its novel bioactive metabolites produced by COX and lipoxygenase (LOX) have been identified as safe and effective pro-resolving and antibacterial pharmaconutrients. Nevertheless, heterogeneous results have been reported in pre-clinical TB studies. Recently, the importance of the correct timing of NSAIDs and n-3 LCPUFA administration in TB has also been highlighted. This mini-review will provide a better understanding of the potential contribution of these therapies toward reducing inflammatory lung damage and improving bactericidal activity, especially during later stages of TB infection. It further highlights that clinical trials are required to confirm benefit and safety in TB patients.

Keywords: cyclooxygenase, lipoxygenase, non-steroidal anti-inflammatory drugs, omega-3 polyunsaturated fatty acids, tuberculosis, lipid mediators, pharmaconutrition

INTRODUCTION

Tuberculosis (TB) remains one of the leading causes of death globally (1). Additionally, multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) patients are burdened by long, costly treatments, with substantial adverse and drug interaction effects and poor cure rates (2, 3). To facilitate treatment, host-directed therapies (HDTs) have been under investigation to augment traditional anti-tubercle treatment regimes. HDTs attempt to modify the host's immune response to reduce tissue damage and indirectly aid bacterial killing, therefore, it should not select drug resistance (4–7). The main objectives of such treatments are to reduce treatment times, post-treatment lung pathology and TB relapse rates (8).

Inflammation is important in host defense, but TB elicits a hyperactive inflammatory response and is characterized by chronic non-resolving inflammation. This exacerbated inflammation results in lung tissue necrosis and cavitation, also facilitating TB transmission (9, 10). Lipid mediators (LMs) are hormone-like substances enzymatically produced from polyunsaturated fatty acids (PUFA) via cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) pathways. A balance between pro-inflammatory and inflammation resolving LM production is of utmost importance from the initiation of the immune response to the resolution of TB infection (11). The manipulation of LMs can be useful as part of immunomodulatory therapy in TB and work synergistically or additively with other standard treatments (12, 13). The use of non-steroidal anti-inflammatory drugs (NSAIDs) has been investigated in this regard (14, 15).

A recent meta-analysis of clinical trials showed that anti-inflammatory medication and pharmaconutrition therapy (vitamin D) may aid in inflammation resolution and improved disease progression outcomes (16). Additionally, the pharmaconutrient omega-3 long-chain PUFA (n-3 LCPUFA) also modify LM production and may be an emerging therapy to consider (17). In this mini-review, we aim to summarize the literature available on the effects of NSAIDs and n-3 LCPUFA in TB as well as the immunological pathways supporting these effects.

CHRONIC NON-RESOLVING INFLAMMATION IN TUBERCULOSIS

One of the key pathological features of TB is that immune cells are recruited to pulmonary spaces, leading to the development of lung granuloma and alterations in lung tissue (lesion formation) (18–20). Granuloma formation is not only intended to separate the TB-infected macrophages from surrounding healthy tissues, but also to keep them in close contact with T cells (21, 22). However, under the direction of the TB pathogen, a hyperactive and non-resolving host immune and inflammatory response are elicited which eventually facilitate lung tissue damage (9, 21). Cavity formation from liquefied granuloma is the most destructive form of TB (21). This results partly from the host's exacerbated inflammatory response, where higher concentrations of plasma IFN-γ, TNF-α, IL-17, and IL-1β have been associated with cavitary TB (9). Unfortunately, in 14–100% of patients, cavities, scarring (fibrosis), and pleural adhesions persist, contributing to persistent abnormal lung function even after TB cure and the resultant lower quality of life (23–27). Therefore, controlling the prolonged exacerbated inflammatory response may benefit clinical outcomes. There is also a close connection between cytokine and LM networks in TB which will be discussed in more detail in the following section.

LIPID MEDIATORS IN TUBERCULOSIS

PUFA are hydrolyzed from membrane phospholipids by phospholipase A_2, to release free fatty acids locally at the site of infection or to be transported to the inflammatory site extracellularly (28–31). Released fatty acids give rise to LMs, by enzymatic pathways, to facilitate pro-inflammatory or inflammation-resolving responses (28, 31). In **Figure 1**, the LMs and their biosynthesis pathways are illustrated. Arachidonic acid (AA) is the main substrate for LM synthesis owing to its high concentrations in cell membranes (11). The LMs produced from AA include the lipoxins (LX), 4-series leukotrienes (LT), 2-series prostaglandins (PG), hydroxyeicosatetraenoic acids, and thromboxanes (TX) by CYP450, COX and LOX enzymes (**Figure 1**) (32, 33). The LMs derived from AA mostly signal pro-inflammatory responses, except for LX, which also display anti-inflammatory and pro-resolving effects (20, 34, 35). The n-3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) also serve as precursors for LMs by COX and LOX activity. These LMs are referred to as specialized pro-resolving mediators (SPMs), including resolvins, protectins and maresins that promote anti-inflammatory pathways and actively contribute to inflammation resolution and tissue functioning restoration (36–40).

Recent studies have highlighted the impact of TB infection on systemic concentrations of different LMs, which persists even after anti-TB treatment (41–43). Apart from the important functions of LMs in the inflammatory response in TB, they also influence TB pathogenesis (41, 42). As such, LMs play a fundamental role in determining the fate of macrophages and their phagocytic ability, as well as immune cell recruitment (44, 45).

The functions of individual LMs in TB remain controversial but more recent research suggests that the balance and timing of the production of specific LMs during the TB disease course are essential for good treatment outcomes (12, 41, 44, 46). For example, the essential action of PGE_2 in the innate immune response of human TB and how a balance in PGE_2/LTB_4 prevents severe inflammation and immunopathology (44, 47). Additionally, the AA-derived LXA_4 has been positively correlated with inflammation and bacterial burden in TB patients (41). Furthermore, how PGE_2, as well as other LM concentrations, affect outcomes may vary during the different stages of TB infection and, therefore, the specific roles of LMs may change during the disease progression (47–49).

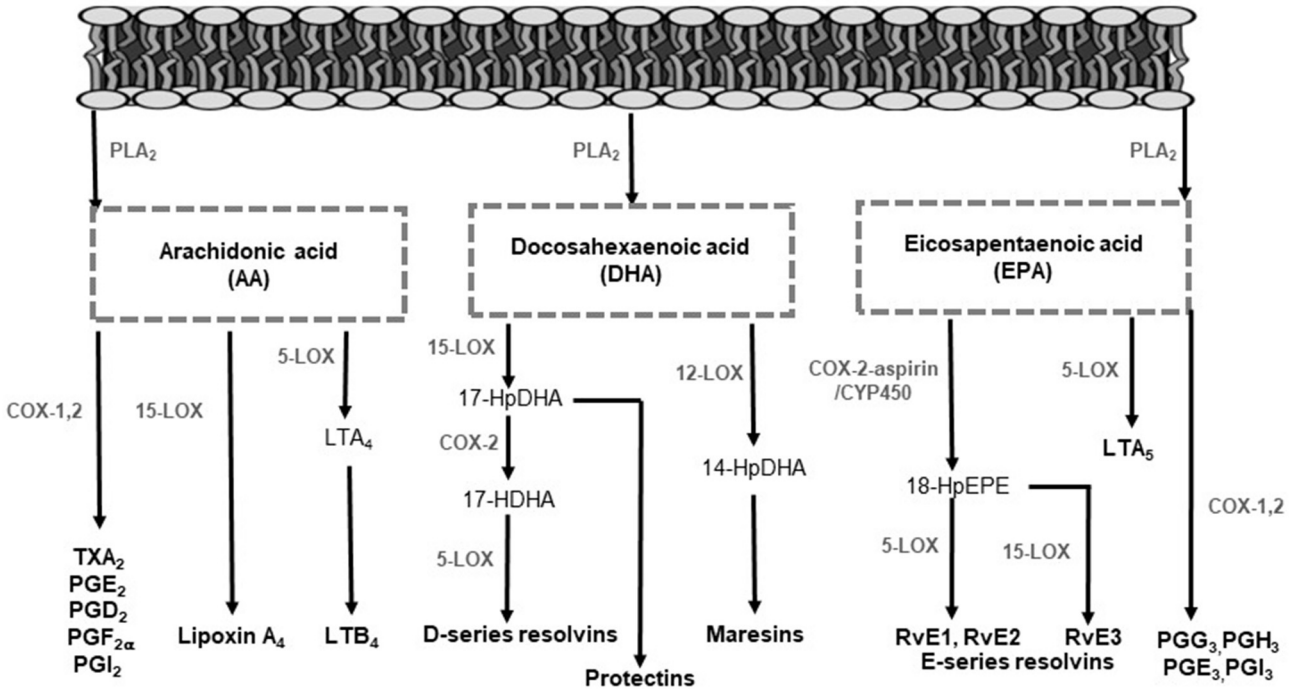

FIGURE 1 | Lipid mediator biosynthesis pathways. In response to infection, polyunsaturated fatty acids are hydrolyzed from membrane phospholipids by phospholipase enzymes to release free fatty acids for lipid mediator production. Arachidonic acid serves as a substrate to form lipoxins, 4-series LTs, 2-series PGs, and TXs. The enzymes 5-LOX, 12-LOX, and 15-LOX produce LTs and lipoxins. Additionally, COX enzymes mediate the production of PGs and TXs. Eicosapentaenoic acid serves as a substrate for the intermediate 18-HPEPE by either COX-2 or CYP450 enzyme activity. From 18-HPEPE the E-series resolvins (RvE1, RvE2, and RvE3) are produced by 5- and 15-LOX. Eicosapentaenoic acid is also converted by 5-LOX to form the less inflammatory LTs. Docosahexaenoic acid is metabolized to form the D-series resolvins and protectins by 5- and 15-LOX and the maresins by 12-LOX. COX, cyclooxygenase; CYP450, cytochrome P450; HDHA, hydroxydocosahexaenoic acid; HpDHA, hydroxyperoxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HpEPE, hydroxyperoxy-eicosapentaenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; PLA_2, phospholipase A_2; Rv, resolvins; TX, thromboxane.

Although research on the role of LM production and its manipulation as HDT in TB has focused mainly on AA-derived LMs, there is a growing interest in SPMs in TB. The plasma metabolomics of newly diagnosed human TB patients has revealed a pro-resolving plasma LM profile, including higher concentrations of the D-series resolvins (50). Furthermore, Colas et al. (12) reported that a pro-resolving LM profile (specifically resolvins) was correlated with 80-day survival, whilst lower levels of SPMs were linked to more severe disease in adults with TB meningitis (12). The reasons for this is that, apart from their inflammation resolving properties, maresins, resolvins and protectins have been implicated to enhance phagocytosis and anti-bacterial activity in TB (51). More studies are needed to describe the role of SPMs in TB and their immunotherapeutic properties. Nevertheless, the importance of LMs in TB regulation, together with the connection between cytokine and LM networks, accentuates the possibilities of LMs as immunotherapy targets in TB (52, 53). However, a time-dependant approach should be considered as the timing of the manipulation of these pathways may influence outcomes in TB disease (47, 48).

PRECLINICAL TRIALS ON CYCLOOXYGENASE- AND LIPOXYGENASE-MODULATING DRUGS IN TUBERCULOSIS

The therapeutic effects of NSAIDs are mainly ascribed to their ability to reduce the production of pro-inflammatory LMs by inhibiting COX-1 and COX-2 activity (48, 54–56), but inadvertently the metabolism of pro-resolving LMs are also inhibited. In essence, they mitigate the conversion of AA to PGE_2 and TXA_2, thereby reducing pain, inflammation, fever, platelet aggregation and vasoconstriction (14, 48, 57). However, the major effects of NSAIDs in TB are ascribed to reduced PGE_2 production, as PGE_2 may inhibit phagocytosis while promoting bacterial growth and tissue damage in the late stages of TB-infection (47, 48). Aspirin (acetylsalicylic acid) and ibuprofen are frequently used NSAIDs (14). In murine models, low-dose aspirin (3 mg/kg/day) lower lung pathology and improve bacillary control thereby increasing survival (15, 58). This is ascribed to its anti-inflammatory effects at both systemic and local lung tissue level, together with lower neutrophil recruitment (by increased LXA_4 and reduced LTB_4 production) and enhanced

T-helper1-(Th1) cell responses (15, 39, 58, 59). Although aspirin has been implicated in enhancing the antibacterial activity of pyrazinamide, it may display an antagonistic effect on isoniazid (55, 60). On the other hand, it seems that ibuprofen may be a better anti-inflammatory agent option, displaying no interference with anti-tuberculosis therapy in rodent models (60). Furthermore, when provided in the absence of conventional TB treatment, improvements in lung histopathology, survival and bacillary load have been reported when administering ibuprofen (80 mg/kg/day) in TB-infected mice (57).

Other NSAIDs displaying COX-inhibiting characteristics include indomethacin and diclofenac. In an earlier study, Hernandez-Pando et al. (61) found that when administering 5 mg/kg/day indomethacin to BALB/C mice with TB-induced lung granulomas, the T cell imbalances, that are characteristic of TB infection, were reversed and the harmful cell-mediated and humoral immunity lessened (61). In an *in vitro* study in blood samples of TB patients, COX-2 was found to be upregulated. However, the COX-1/2 inhibitor indomethacin reduced cytokine responses and T cell proliferation by modulating Th1 effector and T regulatory cells (62). Additionally, indomethacin enhanced the response to immunization with *M. vaccae* (63). Similarly, diclofenac treatment has been shown to reduce lung lesions and bacillary load and increase survival in murine models (64, 65). The new generation NSAID celecoxib also selectively inhibits COX-2 but has fewer side effects (44). It can increase the sensitivity of bacteria to antibacterial treatment and reverse MDR-TB (66, 67). This is ascribed to COX-2 regulating the MDR protein 1 (MDR-1) gene expression. Therefore, the administration of celecoxib blocks the MDR efflux pump and increases drug accumulation (66).

In preclinical TB studies, COX inhibition by NSAID therapy has also had some unfavorable effects. Both ibuprofen and celecoxib treatment increased bacterial burden and ibuprofen decreased survival in *Mtb*-infected mouse models (68). The detrimental properties of NSAID therapy could be attributed to its effects on the adaptive immunity impairing Th1 cell responses and mitigating IFN-γ expression (68). However, it seems that the infection route may influence outcomes as earlier preclinical studies showing promising results infected mice intravenously causing acutely high systemic bacterial loads and inflammation (68). Furthermore, the timing of NSAID administration is important. When administering ibuprofen to *Mtb*-infected mice on day one following infection, lung pathology and inflammation were increased which was linked to PGE_2 inhibition early in the onset of the disease. Conversely, inhibition later in the disease (60 days after infection) reduced neutrophil inflow and, thereby, lessened lung pathology (69). Therefore, COX inhibition may be detrimental to host resistance early in TB infection (48, 70).

With regards to modulating LOX pathways, inhibiting 5-LOX reduces lung pathology, whilst improving bactericidal activity and survival rates (44, 49, 71). Furthermore, 5-LOX deficient mice also show increased IFN-γ, IL-12 and nitric oxide synthase mRNA levels since LX negatively regulates Th1 cell responses (71). When 5-LOX deficient mice were treated with LTB_4 susceptibility toward TB, lung inflammation and tissue damage were worsened, demonstrating the key role of LTB_4 on TB progression and disease outcomes (44). Various LOX-inhibiting therapies exist, such as selective redox-based inhibitors, iron ligand inhibitors e.g., zileuton, and thiazoles e.g., Zeneca ZD2138, but whether they can be successfully repurposed as HDT in TB is to be determined.

CLINICAL TRIALS INVESTIGATING THE USE OF CYCLOOXYGENASE-INHIBITING DRUGS IN TUBERCULOSIS

There are several limitations when translating animal research findings to humans, therefore, the success of the use of COX-inhibiting therapy in preclinical trials prompted the initiation of clinical trials. Observational research has caused concern that NSAID use may increase the risk of the development of active TB. In case reports and an unadjusted analysis of a case-control study, NSAID treatment positively associated with an increased risk of active TB (72–75). However, it is unclear whether this association was causal or rather related to the fact that individuals with subclinical, diagnosed, or undiagnosed active TB are known to have increased NSAID use (75). Furthermore, in the case-control study, COX-inhibition was not associated with active TB in an adjusted analysis. The results were also not replicated in rheumatoid arthritis patients where NSAID therapy was not associated with the risk of active TB (75, 76). Supporting this, in a phase 1 *ex vivo* study in healthy human whole blood inoculated with *Mtb*, celecoxib did not affect whole-blood bactericidal activity (77). Therefore, these findings should be interpreted carefully and more controlled trials are required.

There is a paucity in randomized clinical trials exploring the use of NSAIDs as an adjunct treatment during active TB. In older studies, low-dose aspirin reduced some of the side effects of pyrazinamide treatment in TB patients (78, 79). Aspirin has also been investigated as adjunctive treatment in TB meningitis patients, where different dosages of aspirin daily (81, 150, or 1,000 mg) ensued fewer strokes and lower 3-month mortality rates (80, 81). The beneficial effect was ascribed to aspirin inhibiting TXA_2 and increasing protectin concentrations in cerebral spinal fluid (81). In 2019 a randomized controlled phase 2 trial of the efficacy and safety of using adjunctive ibuprofen in XDR-TB (NCT02781909) was completed, however, the results of this trial remain to be published. Two other trials are registered in this regard including a phase 1 trial administering etoricoxib to MDR-TB patients (NCT02503939) and a randomized controlled clinical trial administering meloxicam to TB patients to determine its ability to modulate or prevent TB-immune reconstitution inflammatory syndrome (IRIS) (NCT02060006). A third three site EDCTP-funded trial is about to start recruiting randomizing drug sensitive and drug resistant TB patients to ibuprofen, aspirin or placebo. NSAIDs have well-known side effects (48). However, as is the case for most other anti-inflammatory drugs, no serious adverse effects related to NSAIDs have been reported in clinical trials in TB patients (14, 16). Compared to traditional antibiotic treatment, NSAIDs are not subject to bacterial resistance and some may even aid in improving bacterial sensitivity to antibiotics (44). Nevertheless,

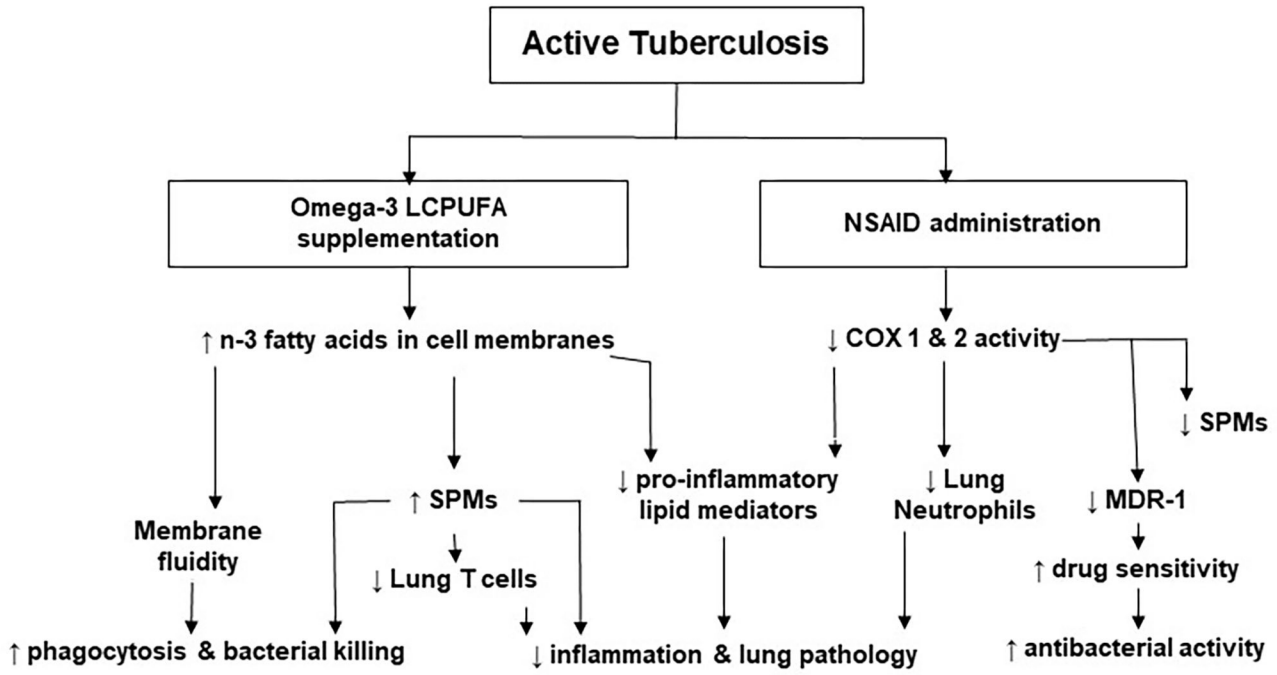

FIGURE 2 | The effects of non-steroidal anti-inflammatory drugs and omega-3 long-chain polyunsaturated fatty acids in tuberculosis. COX, cyclooxygenase; LCPUFA, long-chain polyunsaturated fatty acid; MDR, multi-drug resistant; NSAID, non-steroidal anti-inflammatory drug; n-3, omega-3; SPMs, specialized pro-resolving lipid mediators.

the newer generation NSAIDs may be a safer option to consider. Furthermore, the results of the clinical trials that are pending, will provide greater clarity on the safety and efficacy of NSAID therapy in TB (44). Prospective randomized clinical trials should focus on the dosage, timing and duration that provide the best results when administering NSAIDs adjunct to TB treatment.

FATTY ACID MANIPULATION AS PHARMACONUTRITION THERAPY IN TUBERCULOSIS

Apart from the possibility of using drug therapy to modulate COX and LOX activity, a therapeutic nutritional approach to alter the substrate for COX and LOX pathways may be a promising way to get the same results with fewer side effects. This could be possible through the use of n-3 LCPUFA as pharmaconutrition therapy. Previous studies on the role of n-3 LCPUFA in TB are limited. Some of these studies have raised awareness that supplementation may cause an increased active TB susceptibility and reduced ability of the host to control the infection (82–85). Bonilla et al. (83) found that *fat-1* mice with a genetically higher n-3 PUFA status were more susceptible to active TB and that bacterial loads positively associated with n-3 PUFA levels. The authors ascribed this to the macrophages of these mice which were deficient in various important functions (83). Supporting this, n-3 LCPUFA-fed *Mtb*-infected guinea pigs had a higher bacterial burden when compared with their n-6 PUFA-fed counterparts (84, 85). In addition to these studies, Bazinet et al. (86) found that n-3 PUFA supplementation in piglets, increased the levels of antibodies in response to TB immunization (86).

Contrasting with these results, n-3 LCPUFA supplementation has been shown to lower bacterial load, compared with n-6 PUFA-supplemented or control groups in *Mtb*-infected mice (87). Recently, it was also found that EPA and DHA supplementation initiated 1 week after *Mtb* infection induced a more pro-resolving lung LM profile, and exerted both local lung and systemic anti-inflammatory effects, whilst enhancing bactericidal activity and improving anemia of infection in C3HeB/FeJ mice (17). The reason for inconsistent findings may be related to the timing of the administration of n-3 LCPUFA. When administered after the initial inflammatory response to the infection, beneficial effects were found, whilst providing it before or early in *Mtb* infection worsened the outcomes. Differences in EPA and DHA dosages may also have contributed, where a higher EPA content seems beneficial (17, 87). Lastly, due to the preclinical nature of the studies, the type and species of animals used may have influenced results (83–85). The safety and efficacy of n-3 LCPUFA as therapy adjunct to standard TB treatment and how this compares to other anti-inflammatory treatments are still to be determined in preclinical trials. However, preliminary results from a TB mouse model study conducted by our group show that n-3 LCPUFA does not interfere with the efficacy of standard TB medication (unpublished data).

Only two clinical trials have been conducted to ascertain the effect of n-3 LCPUFA in TB. The first supplemented a combination of fish oil (350 mg n-3 PUFA), vitamin A (1,500 UI) and Zinc (10 mg) with standard TB drug treatment, in pediatric TB patients. The group receiving supplementation for 1 month had lower TNF-α concentrations and an improved body mass index, compared with a group that received standard drug treatment only (88). In the second trial, n-3 LCPUFA (300 mg) was supplemented in combination with Zinc sulfate (15 mg) once per day for 1 month in a small number ($n = 20$) of adult Indonesian TB patients receiving standard TB treatment (89). Supplementation caused non-significant, reduced sputum smear conversion rates and mediated significant improvements in body weight and $CD4^+$ counts compared with the control group (89). However, in both studies, the timing of the initiation of supplementation was not mentioned and n-3 LCPUFA was supplemented in combination with other nutrients. Although there is a paucity in clinical trials on n-3 LCPUFA supplementation in TB, it has been found safe in animal TB models and clinical trials in other inflammatory diseases (17, 32). As pharmaconutrition therapy, n-3 LCPUFA supplementation is also safe for long term use and not subject to bacterial resistance like antibiotics. Bearing in mind the side effects of other anti-inflammatory drugs the nutritional modulation of inflammatory pathways may be a safer approach. However, as clinical evidence is lacking, future randomized clinical trials should provide n-3 LCPUFA as single pharmaconutrient adjunct to standard TB treatment. Furthermore, the appropriate timing, duration and dosage of such supplementation need to be investigated as the manipulation of LM concentrations may produce different outcomes depending on the stage of TB disease.

DISCUSSION

Published data suggest that LMs regulate inflammatory and immune responses and that their roles vary at different stages of the disease. For example, high concentrations of PGE_2 may worsen disease progression and down-regulate cell-mediated immunity in later stages of TB infection (69). Altering LM concentrations by modulating COX and LOX activity is a novel HDT approach in TB. **Figure 2** represents the effects of NSAIDs and n-3 LCPUFA in TB as well as the underlying mechanisms supporting them. Prescribing NSAIDs as analgesic and anti-inflammatory medication is common worldwide. These medications have shown promising results in pre-clinical TB studies by inhibiting COX activity to reduce the production of pro-inflammatory and sometimes immunosuppressive LMs (14, 15, 60, 65, 90). This aids in attenuating inflammation-induced tissue damage and improves the antibacterial actions of the host with active TB. Additionally, COX-inhibitors can aid in improving the concentrations of certain drugs and drug sensitivity, by the manipulation of MDR-1 (66, 77). Therefore, the synergistic effects of TB treatment and NSAIDs may benefit TB outcomes (14, 55, 62, 91). Preclinical trials on NSAID therapy in TB have also highlighted that the timing of administration is important, where NSAIDs at later stages of the disease may be more beneficial (48, 68, 69). Although favorable results regarding the anti-inflammatory and antibacterial activity of COX-inhibition therapy in TB have been found in preclinical trials, more randomized controlled clinical trials are needed to determine the efficacy and safety in patients with active TB (14, 15, 55, 57, 58, 60, 92). More definite recommendations are anticipated upon completion and publication of clinical trials that are currently ongoing.

Apart from beneficial effects, NSAIDs also carry well-known side effects, such as the risk of gastrointestinal ulcers, bleeding and renal injury (48). Another HDT option is n-3 LCPUFA which facilitate pro-resolving and anti-inflammatory pathways by altering the membrane phospholipid fatty acid composition of blood and tissue cells that are important in immune responses (93–95). These fatty acids partially replace AA in membranes as the substrate for pro-inflammatory LMs (94, 96, 97). Furthermore, they also serve as precursors for SPMs, which have inflammation resolving properties (36–40). They alter immune cell recruitment by halting neutrophil infiltration and lowering T cell proliferation (31, 32, 98). Also, SPMs have direct effects to stimulate monocytes to migrate and differentiate into macrophages for phagocytic activity, and to enhance bacterial phagocytosis and killing (99–102). The few available studies on n-3 LCPUFA in TB have portrayed mixed results with some showing benefit concerning bacterial killing and pulmonary inflammation (17, 83, 87), whilst others reported harm (82–85, 103). A recent study in *Mtb*-infected mice highlighted the importance of the timing of n-3 LCPUFA supplementation, where supplementation after the initial inflammatory response seems to be beneficial (17). Preclinical studies combining n-3 LCPUFA with standard TB drug treatment are still required. In the only two clinical trials that have been conducted on n-3 LCPUFA therapy in TB patients, a positive effect was found on sputum smear conversion, body weight gain, inflammation resolution, and $CD4^+$ T cell count (88, 89). As n-3 LCPUFA were combined with other nutrients in these clinical trials, more randomized controlled trials are required to determine the correct dosage and timing of supplementation in patients with active TB. Another possible HDT in TB is LOX-manipulating therapy, however, clinical trials on repurposing drugs such as zileuton are still lacking.

CONCLUSION

Both NSAIDs and n-3 LCPUFA may help to reduce excessive inflammatory lung damage and improve bactericidal activity, especially during later stages of TB disease. However, more human data, particularly randomized controlled clinical trials are required to confirm the clinical benefit and safety of these HDT approaches in patients with active TB.

AUTHOR CONTRIBUTIONS

LM and AN contributed to the conception of the mini-review. AN was responsible for text writing and figure assembly of the first draft. AN, LM, FH, EV, and NM revised and edited the text and figures. All authors contributed to the article and approved the submitted version.

REFERENCES

1. WHO. *Global Tuberculosis Report. Geneva: World Heatlh Organization* (2019).
2. Dheda K, Gumbo T, Maartens G, Dooley KE, McNerney R, Murray M, et al. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. *Lancet Respir Med.* (2017) 5:291–360. doi: 10.1016/S2213-2600(17)30079-6
3. Lan Z, Ahmad N, Baghaei P, Barkane L, Benedetti A, Brode SK, et al. Drug-associated adverse events in the treatment of multidrug-resistant tuberculosis: an individual patient data meta-analysis. *Lancet Respir Med.* (2020) 8:383–94. doi: 10.1016/S2213-2600(20)30047-3
4. Baindara P. Host-directed therapies to combat tuberculosis and associated non-communicable diseases. *Microb Pathogen.* (2019) 130:156–68. doi: 10.1016/j.micpath.2019.03.003
5. Kim Y-R, Yang C-S. Host-directed therapeutics as a novel approach for tuberculosis treatment. *J Microbiol Biotechnol.* (2017) 27:1549–58. doi: 10.4014/jmb.1705.05032
6. Palucci I, Delogu G. Host directed therapies for tuberculosis: futures strategies for an ancient disease. *Chemotherapy.* (2018) 63:172–80. doi: 10.1159/000490478
7. Stek C, Allwood B, Walker NF, Wilkinson RJ, Lynen L, Meintjes G. The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy. *Front Microbiol.* (2018) 9:2603. doi: 10.3389/fmicb.2018.02603
8. Tsenova L, Singhal A. Effects of host-directed therapies on the pathology of tuberculosis. *J Pathol.* (2020) 250:636–46. doi: 10.1002/path.5407
9. Kumar NP, Moideen K, Banurekha VV, Nair D, Babu S, editors. Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. *Open Forum Infect Dis.* (2019) 6:ofz257. doi: 10.1093/ofid/ofz257
10. Kumar P. Adult pulmonary tuberculosis as a pathological manifestation of hyperactive antimycobacterial immune response. *Clin Transl Med.* (2016) 5:38. doi: 10.1186/s40169-016-0119-0
11. Calder PC. Metabolism of polyunsaturated fatty acids by cells of the immune system. In: Burdge GC, editor. *Fatty Acid Metabolism*. London: Elsevier Inc. (2018). p. 136–51.
12. Colas RA, Nhat LTH, Thuong NTT, Gómez EA, Ly L, Thanh HH, et al. Proresolving mediator profiles in cerebrospinal fluid are linked with disease severity and outcome in adults with tuberculous meningitis. *FASEB J.* (2019) 33:13028–39. doi: 10.1096/fj.201901590R
13. Fullerton JN, O'Brien AJ, Gilroy DW. Lipid mediators in immune dysfunction after severe inflammation. *Trends Immunol.* (2014) 35:12–21. doi: 10.1016/j.it.2013.10.008
14. Kroesen VM, Gröschel MI, Martinson N, Zumla A, Maeurer M, van der Werf TS, et al. Non-steroidal anti-inflammatory drugs as host-directed therapy for tuberculosis: a systematic review. *Front Immunol.* (2017) 8:772. doi: 10.3389/fimmu.2017.00772
15. Kroesen VM, Rodríguez-Martínez P, García E, Rosales Y, Díaz J, Martín-Céspedes M, et al. A beneficial effect of low-dose aspirin in a murine model of active tuberculosis. *Front Immunol.* (2018) 9:798. doi: 10.3389/fimmu.2018.00798
16. Hayford FEA, Dolman RC, Blaauw R, Nienaber A, Smuts CM, Malan L, et al. The effects of anti-inflammatory agents as host-directed adjunct treatment of tuberculosis in humans: a systematic review and meta-analysis. *Resp Res.* (2020) 21:223. doi: 10.1186/s12931-020-01488-9
17. Nienaber A, Baumgartner J, Dolman RC, Ozturk M, Zandberg L, Hayford FE, et al. Omega-3 fatty acid and iron supplementation alone, but not in combination, lower inflammation and anemia of infection in mycobacterium tuberculosis-infected mice. *Nutrients.* (2020) 12:2897. doi: 10.3390/nu12092897
18. Ernst JD. The immunological life cycle of tuberculosis. *Nature Rev Immunol.* (2012) 12:581. doi: 10.1038/nri3259
19. Kaplan G, Post FA, Moreira AL, Wainwright H, Kreiswirth BN, Tanverdi M, et al. Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity. *Infect Immun.* (2003) 71:7099–108. doi: 10.1128/IAI.71.12.7099-7108.2003
20. Robinson RT, Orme IM, Cooper AM. The onset of adaptive immunity in the mouse model of tuberculosis and the factors that compromise its expression. *Immunol rev.* (2015) 264:46–59. doi: 10.1111/imr.12259
21. Dorhoi A, Kaufmann S. Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. *Semin Immunopathol.* (2016) 38:153–66. doi: 10.1007/s00281-015-0531-3
22. Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity.* (2008) 28:10. doi: 10.1016/j.immuni.2007.12.010
23. De La Mora IL, Martinez-Oceguera D, Laniado-Laborin R. Chronic airway obstruction after successful treatment of tuberculosis and its impact on quality of life. *Int J Tuberc Lung Dis.* (2015) 19:808–10. doi: 10.5588/ijtld.14.0983
24. Manji M, Shayo G, Mamuya S, Mpembeni R, Jusabani A, Mugusi F. Lung functions among patients with pulmonary tuberculosis in Dar es Salaam–a cross-sectional study. *BMC Pulm Med.* (2016) 16:58. doi: 10.1186/s12890-016-0213-5
25. Meghji J, Simpson H, Squire SB, Mortimer K. A systematic review of the prevalence and pattern of imaging defined post-TB lung disease. *PLoS ONE.* (2016) 11:e0161176. doi: 10.1371/journal.pone.0161176
26. Nihues SdSE, Mancuzo EV, Sulmonetti N, Sacchi FPC, Viana VdS, Martins Netto E, et al. Chronic symptoms and pulmonary dysfunction in post-tuberculosis Brazilian patients. *Braz J Infect Dis.* (2015) 19:492–7. doi: 10.1016/j.bjid.2015.06.005
27. Theegarten D, Kahl B, Ebsen M. Frequency and morphology of tuberculosis in autopsies: increase of active forms. *Dtsch Med Wochenschr.* (2006) 131:1371–6. doi: 10.1055/s-2006-946581
28. Basil MC, Levy BD. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nature Rev Immunol.* (2016) 16:51–67, doi: 10.1038/nri.2015.4
29. Dennis EA, Cao J, Hsu Y-H, Magrioti V, Kokotos G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem Rev.* (2011) 111:6130–85. doi: 10.1021/cr200085w
30. Kasuga K, Yang R, Porter TF, Agrawal N, Petasis NA, Irimia D, et al. Rapid appearance of resolvin precursors in inflammatory exudates: novel mechanisms in resolution. *J Immunol.* (2008) 181:8677–87. doi: 10.4049/jimmunol.181.12.8677
31. Serhan CN, Chiang N, Dalli J. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Mol Aspects Med.* (2017) 64:1–17. doi: 10.1016/j.mam.2017.08.002
32. Calder PC. Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance. *Biochim Biophys Acta Mol Cell Biol Lipids.* (2015) 1851:469–84. doi: 10.1016/j.bbalip.2014.08.010
33. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest.* (2001) 108:15–23. doi: 10.1172/JCI200113416
34. Kaufmann SHE, Dorhoi A. Inflammation in tuberculosis: interactions, imbalances and interventions. *Curr Opin Immunol.* (2013) 25:441–9, doi: 10.1016/j.coi.2013.05.005
35. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. *Chem Rev.* (2011) 111:5922–43. doi: 10.1021/cr100396c
36. Barden AE, Mas E, Mori TA. n-3 Fatty acid supplementation and proresolving mediators of inflammation. *Curr Opin Lipidol.* (2016) 27:26–32. doi: 10.1097/MOL.0000000000000262
37. Jaudszus A, Gruen M, Watzl B, Ness C, Roth A, Lochner A, et al. Evaluation of suppressive and pro-resolving effects of EPA and DHA in human primary monocytes and T-helper cells. *J Lipid Res.* (2013) 54:923–35. doi: 10.1194/jlr.P031260
38. Mas E, Barden A, Burke V, Beilin LJ, Watts GF, Huang R-C, et al. A randomized controlled trial of the effects of n-3 fatty acids on resolvins in chronic kidney disease. *Clin Nutr.* (2016) 35:331–6. doi: 10.1016/j.clnu.2015.04.004
39. Morris T, Stables M, Hobbs A, de Souza P, Colville-Nash P, Warner T, et al. Effects of low-dose aspirin on acute inflammatory responses in humans. *J Immunol.* (2009) 183:2089–96. doi: 10.4049/jimmunol.0900477

40. Serhan CN. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *FASEB J.* (2017) 31:1273–88. doi: 10.1096/fj.201601222R
41. Kumar NP, Kadar Moideen AN, Viswanathan V, Shruthi BS, Shanmugam S, Hissar S, et al. Plasma eicosanoid levels in tuberculosis and tuberculosis-diabetes co-morbidity are associated with lung pathology and bacterial burden. *Front Cell Infect Microbiol.* (2019) 9:335. doi: 10.3389/fcimb.2019.00335
42. Vinhaes CL, Oliveira-de-Souza D, Silveira-Mattos PS, Nogueira B, Shi R, Wei W, et al. Changes in inflammatory protein and lipid mediator profiles persist after antitubercular treatment of pulmonary and extrapulmonary tuberculosis: a prospective cohort study. *Cytokine.* (2019) 123:154759. doi: 10.1016/j.cyto.2019.154759
43. Shivakoti R, Dalli J, Kadam D, Gaikwad S, Barthwal M, Colas RA, et al. Lipid mediators of inflammation and resolution in individuals with tuberculosis and tuberculosis-Diabetes. *Prostagl Other Lipid Mediat.* (2020) 147:106398. doi: 10.1016/j.prostaglandins.2019.106398
44. Sorgi CA, Soares EM, Rosada RS, Bitencourt CS, Zoccal KF, Pereira PAT, et al. Eicosanoid pathway on host resistance and inflammation during *Mycobacterium tuberculosis* infection is comprised by LTB4 reduction but not PGE2 increment. *BBA-Mol Basis Dis.* (2020) 1866:165574. doi: 10.1016/j.bbadis.2019.165574
45. Behar SM, Divangahi M, Remold HG. Evasion of innate immunity by *Mycobacterium tuberculosis*: is death an exit strategy? *Nat Rev Microbiol.* (2010) 8:668. doi: 10.1038/nrmicro2387
46. Colas RA, Shinohara M, Dalli J, Chiang N, Serhan CN. Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. *Amer J Physiol-Cell Physiol.* (2014) 307:C39–54. doi: 10.1152/ajpcell.00024.2014
47. Pellegrini JM, Tateosian NL, Morelli MP, Rolandelli A, Amiano NO, Palmero DJ, et al. Immunosuppressive role of PGE2 during human tuberculosis. *bioRxiv.* (2020). doi: 10.1101/2020.08.04.236257
48. Ivanyi J, Zumla A. Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *J Infect Dis.* (2013) 208:185–8. doi: 10.1093/infdis/jit153
49. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature.* (2014) 511:99. doi: 10.1038/nature13489
50. Frediani JK, Jones DP, Tukvadze N, Uppal K, Sanikidze E, Kipiani M, et al. Plasma metabolomics in human pulmonary tuberculosis disease: a pilot study. *PLoS ONE.* (2014) 9:e108854. doi: 10.1371/journal.pone.0108854
51. Ruiz A, Sarabia C, Torres M, Juárez E. Resolvin D1 (RvD1) and maresin 1 (Mar1) contribute to human macrophage control of *M. tuberculosis* infection while resolving inflammation. *Int Immunopharmacol.* (2019) 74:105694. doi: 10.1016/j.intimp.2019.105694
52. Dietzold J, Gopalakrishnan A, Salgame P. Duality of lipid mediators in host response against *Mycobacterium tuberculosis*: good cop, bad cop. *F1000Prime Rep.* (2015) 7:29. doi: 10.12703/P7-29
53. Mayer-Barber KD, Sher A. Cytokine and lipid mediator networks in tuberculosis. *Immunol Rev.* (2015) 264:264–75. doi: 10.1111/imr.12249
54. Amann R, Peskar BA. Anti-inflammatory effects of aspirin and sodium salicylate. *Euro J Pharmacol.* (2002) 447:1–9. doi: 10.1016/S0014-2999(02)01828-9
55. Byrne ST, Denkin SM, Zhang Y. Aspirin and ibuprofen enhance pyrazinamide treatment of murine tuberculosis. *J Antimicrob Chemother.* (2006) 59:313–6. doi: 10.1093/jac/dkl486
56. Ohdo S, Ogawa N, Song JG. Chronopharmacological study of acetylsalicylic acid in mice. *Euro J Pharmacol Environ Toxicol Pharmacol.* (1995) 293:151–7. doi: 10.1016/0926-6917(95)00011-9
57. Vilaplana C, Marzo E, Tapia G, Diaz J, Garcia V, Cardona P-J. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J Infect Dis.* (2013) 208:199–202. doi: 10.1093/infdis/jit152
58. Marzo E, Vilaplana C, Tapia G, Diaz J, Garcia V, Cardona P-J. Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tuberculosis.* (2014) 94:55–64. doi: 10.1016/j.tube.2013.09.004
59. Tobin DM, Roca FJ, Oh SF, McFarland R, Vickery TW, Ray JP, et al. Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell.* (2012) 148:434–46. doi: 10.1016/j.cell.2011.12.023
60. Byrne ST, Denkin SM, Zhang Y. Aspirin antagonism in isoniazid treatment. *Antimicrob Agents Chemother.* (2007) 51:794. doi: 10.1128/AAC.01145-06
61. Hernandez-Pando R, Orozco H, Mancilla R. T-cell lung granulomas induced by sepharose-coupled *Mycobacterium tuberculosis* protein antigens: immunosuppressive phenomena reversed with cyclophosphamide and indomethacin. *Immunology.* (1995) 86:506.
62. Tonby K, Wergeland I, Lieske NV, Kvale D, Tasken K, Dyrhol-Riise AM. The COX-inhibitor indomethacin reduces Th1 effector and T regulatory cells in vitro in *Mycobacterium tuberculosis* infection. *BMC Infect Dis.* (2016) 16:1–12. doi: 10.1186/s12879-016-1938-8
63. Shroff K, Sainis K, Sengupta S, Kamat R. Role of antigen-presenting cells in variation in immunogenicity of mycobacteria. *Clin Exp Immunol.* (1990) 79:285–90. doi: 10.1111/j.1365-2249.1990.tb05192.x
64. Dutta N, Kumar KA, Mazumdar K, Dastidar SG, Ray R, Chakrabarty A. *In vitro* and *in vivo* antimycobacterial activity of antiinflammatory drug, diclofenac sodium. *Indian J Exp Biol.* (2004) 42:922–7.
65. Dutta NK, Mazumdar K, Dastidar SG, Park J-H. Activity of diclofenac used alone and in combination with streptomycin against *Mycobacterium tuberculosis* in mice. *Int J Antimicrob Agents.* (2007) 30:336–40. doi: 10.1016/j.ijantimicag.2007.04.016
66. Kalle AM, Rizvi A. Inhibition of bacterial multidrug resistance by celecoxib, a cyclooxygenase-2 inhibitor. *Antimicrob Agents Chemother.* (2011) 55:439–42. doi: 10.1128/AAC.00735-10
67. Salunke SB, Azad AK, Kapuriya NP, Balada-Llasat J-M, Pancholi P, Schlesinger LS, et al. Design and synthesis of novel anti-tuberculosis agents from the celecoxib pharmacophore. *Bioorg Med Chem.* (2015) 23:1935–43. doi: 10.1016/j.bmc.2015.03.041
68. Mortensen R, Clemmensen HS, Woodworth JS, Therkelsen ML, Mustafa T, Tonby K, et al. Cyclooxygenase inhibitors impair CD4 T cell immunity and exacerbate *Mycobacterium tuberculosis* infection in aerosol-challenged mice. *Commun Biol.* (2019) 2:1–10. doi: 10.1038/s42003-019-0530-3
69. Rangel Moreno J, Estrada Garcia I, De La Luz García Hernández M, Aguilar Leon D, Marquez R, Hernández Pando R. The role of prostaglandin E2 in the immunopathogenesis of experimental pulmonary tuberculosis. *Immunology.* (2002) 106:257–66. doi: 10.1046/j.1365-2567.2002.01403.x
70. Divangahi M, Chen M, Gan H, Desjardins D, Hickman TT, Lee DM, et al. Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. *Nat Immunol.* (2009) 10:899. doi: 10.1038/ni.1758
71. Bafica A, Scanga CA, Serhan C, Machado F, White S, Sher A, et al. Host control of *Mycobacterium tuberculosis* is regulated by 5-lipoxygenase–dependent lipoxin production. *J Clin Invest.* (2005) 115:1601–6. doi: 10.1172/JCI23949
72. Brennan M. Activation of latent tuberculosis by nonsteroidal anti-inflammatory agents. *Can Med Assoc J.* (1980) 122:400.
73. Brennan M. Latent tuberculosis and anti-inflammatory agents. *Can Med Assoc J.* (1982) 126:21.
74. Tomasson HO, Brennan M, Bass M. Tuberculosis and nonsteroidal anti-inflammatory drugs. *Can Med Assoc J.* (1984) 130:275.
75. Wu C-W, Wu J-Y, Lee M-TG, Lai C-C, Wu I-L, Tsai Y-W, et al. Risk of incident active tuberculosis disease in patients treated with non-steroidal anti-inflammatory drugs: a population-based study. *BMC Pulm Med.* (2017) 17:82. doi: 10.1186/s12890-017-0425-3
76. Brassard P, Lowe AM, Bernatsky S, Kezouh A, Suissa S. Rheumatoid arthritis, its treatments, and the risk of tuberculosis in Quebec, Canada. *Arthritis Care Res.* (2009) 61:300–4. doi: 10.1002/art.24476
77. Naftalin CM, Verma R, Gurumurthy M, Hee KH, Lu Q, Yeo BCM, et al. Adjunctive use of celecoxib with anti-tuberculosis drugs: evaluation in a whole-blood bactericidal activity model. *Sci Rep.* (2018) 8:1–8. doi: 10.1038/s41598-018-31590-4
78. Petty TL, Dalrymple GV. Inhibition of pyrazinamide hyperuricemia by small doses of acetylsalicylic acid. *Ann Intern Med.* (1964) 60:898–900. doi: 10.7326/0003-4819-60-5-898
79. Horsfall P, Plummer J, Allan W, Girling D, Nunn A, Fox W. Double blind controlled comparison of aspirin, allopurinol and placebo in the management of arthralgia during pyrazinamide administration. *Tubercle.* (1979) 60:13–24. doi: 10.1016/0041-3879(79)90051-5

80. Misra U, Kalita J, Nair P. Role of aspirin in tuberculous meningitis: a randomized open label placebo controlled trial. *J Neurol Sci.* (2010) 293:12–7. doi: 10.1016/j.jns.2010.03.025
81. Mai NT, Dobbs N, Phu NH, Colas RA, Thao LT, Thuong NT, et al. A randomised double blind placebo controlled phase 2 trial of adjunctive aspirin for tuberculous meningitis in HIV-uninfected adults. *Elife.* (2018) 7:e33478. doi: 10.7554/eLife.33478.026
82. Bonilla DL, Ly LH, Fan Y-Y, Chapkin RS, McMurray DN. Incorporation of a dietary omega 3 fatty acid impairs murine macrophage responses to mycobacterium. *PLoS ONE.* (2010) 5:e10878. doi: 10.1371/journal.pone.0010878
83. Bonilla DL, Fan Y-Y, Chapkin RS, McMurray DN. Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *J Infect Dis.* (2010) 201:399–408. doi: 10.1086/650344
84. McFarland CT, Fan Y-Y, Chapkin RS, Weeks BR, McMurray DN. Dietary polyunsaturated fatty acids modulate resistance to *Mycobacterium tuberculosis* in guinea pigs. *J Nutr.* (2008) 138:2123–8. doi: 10.3945/jn.108.093740
85. Paul KP, Leichsenring M, Pfisterer M, Mayatepek E, Wagner D, Domann M, et al. Influence of n-6 and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. *Metabolism.* (1997) 46:619–24. doi: 10.1016/S0026-0495(97)90003-2
86. Bazinet RP, Douglas H, McMillan EG, Wilkie BN, Cunnane SC. Dietary 18: 3ω3 influences immune function and the tissue fatty acid response to antigens and adjuvant. *Immunol Lett.* (2004) 95:85–90. doi: 10.1016/j.imlet.2004.06.007
87. Jordao L, Lengeling A, Bordat Y, Boudou F, Gicquel B, Neyrolles O, et al. Effects of omega-3 and-6 fatty acids on *Mycobacterium tuberculosis* in macrophages and in mice. *Microbes Infect.* (2008) 10:1379–86. doi: 10.1016/j.micinf.2008.08.004
88. Nenni V, Nataprawira HM, Yuniati T. Role of combined zinc, vitamin A, and fish oil supplementation in childhood tuberculosis. *Southeast Asian J Trop Med Public Health.* (2013) 44:854–61.
89. Durry FD, Wirjatmadi B, Adriani M. The role of zinc sulphate and omega 3 on the improvement of weight and speed conversion conversion on tuberculosis parent patients at Surabaya Park Hospital, 2015. *J Ilm Kedokt Wijaya Kusuma.* (2018) 7:62–74. doi: 10.30742/jikw.v7i1.313
90. Maitra A, Evangelopoulos D, Chrzastek A, Martin LT, Hanrath A, Chapman E, et al. Carprofen elicits pleiotropic mechanisms of bactericidal action with the potential to reverse antimicrobial drug resistance in tuberculosis. *J Antimicrob Chemother.* (2020) 75: 3194–201. doi: 10.1093/jac/dkaa307
91. Dutta NK, Annadurai S, Mazumdar K, Dastidar SG, Kristiansen JE, Molnar J, et al. Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. *Int J Antimicrob Agents.* (2007) 30:242–9. doi: 10.1016/j.ijantimicag.2007.04.018
92. Critchley JA, Young F, Orton L, Garner P. Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *Lancet Infect Dis.* (2013) 13:223–37. doi: 10.1016/S1473-3099(12)70321-3
93. Calder PC. Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem Soc Trans.* (2017) 45:1105–15. doi: 10.1042/BST20160474
94. Browning LM, Walker CG, Mander AP, West AL, Madden J, Gambell JM, et al. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish–. *Am J Clin Nutr.* (2012) 96:748–58. doi: 10.3945/ajcn.112.041343
95. Jakiela B, Gielicz A, Plutecka H, Hubalewska M, Mastalerz L, Bochenek G, et al. Eicosanoid biosynthesis during mucociliary and mucous metaplastic differentiation of bronchial epithelial cells. *Prostaglandins Other Lipid Mediat.* (2013) 106:116–23. doi: 10.1016/j.prostaglandins.2013.05.001
96. Calder PC. Functional roles of fatty acids and their effects on human health. *J Parenter Enter Nutr.* (2015) 39(1_suppl.):18S−32. doi: 10.1177/0148607115595980
97. Healy D, Wallace F, Miles E, Calder P, Newsholme P. Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids.* (2000) 35:763–8. doi: 10.1007/s11745-000-0583-1
98. Fielding BA, Calder PC, Irvine NA, Miles EA, Lillycrop KA, von Gerichten J, et al. How does polyunsaturated fatty acid biosynthesis regulate T-lymphocyte function? *Nutr Bull.* (2019) 44:350–55. doi: 10.1111/nbu.12404
99. Chiang N, Fredman G, Bäckhed F, Oh SF, Vickery T, Schmidt BA, et al. Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature.* (2012) 484:524. doi: 10.1038/nature11042
100. Codagnone M, Cianci E, Lamolinara A, Mari V, Nespoli A, Isopi E, et al. Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. *Muc Immunol.* (2018) 11:35. doi: 10.1038/mi.2017.36
101. Lee CR, Zeldin DC. Resolvin infectious inflammation by targeting the host response. *N Engl J Med.* (2015) 373:2183–5. doi: 10.1056/NEJMcibr1511280
102. Spite M, Norling LV, Summers L, Yang R, Cooper D, Petasis NA, et al. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature.* (2009) 461:1287. doi: 10.1038/nature08541
103. Mayatepek E, Paul K, Leichsenring M, Pfisterer M, Wagner D, Domann M, et al. Influence of dietary (n-3)-polyunsaturated fatty acids on leukotriene B 4 and prostaglandin E 2 synthesis and course of experimental tuberculosis in guinea pigs. *Infection.* (1994) 22:106–12. doi: 10.1007/BF01739016

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Repurposing Saquinavir for Host-Directed Therapy to Control Mycobacterium Tuberculosis Infection

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Despite the available antibiotics, tuberculosis (TB) has made its return since the 90's of the last century as a global threat mostly due to co-infection with HIV, to the emergence of drug resistant strains and the lack of an effective vaccine. Host-directed strategies could be exploited to improve treatment efficacy, contain drug-resistant strains, improve immune responses and reduce disease severity. Macrophages in the lungs are often found infected with *Mycobacterium tuberculosis* (Mtb) and/or with HIV. The long-term survival of lung macrophages infected with Mtb or with HIV, together with their ability to produce viral particles, especially during TB, makes these niches major contributors to the pathogenicity of the infection. Among the available drugs to control HIV infection, protease inhibitors (PIs), acting at post-integrational stages of virus replication cycle, are the only drugs able to interfere with virus production and release from macrophages during chronic infection. For Mtb we recently found that the pathogen induces a general down-regulation of lysosomal proteases, helping bacteria to establish an intracellular niche in macrophages. Here we found that the PI saquinavir, contrary to ritonavir, is able to induce an increase of endolysosomal proteases activity especially of cathepsin S in Mtb infected macrophages and during co-infection with HIV. Our results indicate that saquinavir treatment of infected macrophages led not only to a significant intracellular killing of Mtb but also: (i) to an improved expression of the HLA class II antigen presentation machinery at the cell surface; (ii) to increased T-lymphocyte priming and proliferation; and (iii) to increased secretion of IFN-γ. All together the results indicate saquinavir as a potential host directed therapy for tuberculosis.

Keywords: saquinavir, protease inhibitors, tuberculosis, HIV-co-infection, host directed therapies

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INTRODUCTION

Mycobacterium tuberculosis (Mtb) the causative agent of tuberculosis (TB) and the AIDS-associated human immunodeficiency virus (HIV), have in common macrophages (Mø) as immune cell reservoir. Both pathogens alter the Mø microbicidal and immune-activating functions and convert these cells into intracellular survival niches (1). In the case of Mtb, the WHO estimates that one quarter of the human population is latently infected and among these, 10% will develop the active disease. From the latently infected group about 600,000 people are estimated to be carriers of

multidrug-resistant (MDR) and extensively drug-resistant Mtb strains (XDR) (2). Main contributors to TB activation from latency are immunosuppressive conditions, especially HIV co-infection, malnutrition and aging.

While for HIV the infection became a chronic controlled situation with the available medicines, for TB and particularly during co-infection with HIV the scenario is a global threat for humankind. This includes, as aforementioned, (A) the increased MDR and XDR strains to current available antibiotics; (B) the condition that Mtb exacerbates HIV infection and vice versa leading to TB activation from latency; and (C) the fact that co-infected individuals contribute to viral spread and to MDR and XDR strains transmission (2–4). All together this led us to search for host targets that may be manipulated during infection to boost the immune responses blocked by the pathogens as an alternative therapeutic strategy to current antimicrobials. In this context, the repositioning of drugs represents a useful strategy in the search for new fast therapeutic approaches for TB control. Furthermore host-directed strategies could be exploited to improve treatment efficacy and outcome and reduce disease severity and mortality.

Combined antiretroviral therapies have been applied in HIV-infected patients for more than two decades and include a cocktail of nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTIs (nNRT1s), protease inhibitors (PIs), and integrase inhibitors (5). These were shown to efficiently suppress HIV replication, leading to partial immune restoration and turning AIDS into a chronic infection. A threat to this controlled situation arises from the fact that HIV in addition to infect $CD4^+$ T-lymphocytes also infects Mø. While the absolute number of infected Mø in the body is relatively low compared to $CD4^+$ T-cells this is not the case for HIV infected Mø in the lungs (6) particularly during co-infection with Mtb (7). Furthermore alveolar Mø simultaneously infected with HIV and Mtb, were isolated from a patient co-infected with both pathogens (8). The long-term survival of lung Mø infected with these pathogens, together with their ability to exacerbate the infection by each other, turns these viral reservoirs into a challenge to HIV eradication since they continue producing virus in this tissue despite antiretroviral therapy (3, 4). Among the available drugs to control HIV infection, protease inhibitors (PIs), acting at post-integrational stages of virus replication cycle (9), are the only drugs able to interfere with virus production and release from Mø during chronic infection (10). The anti-viral activity of PIs is based on inhibition of the HIV aspartic protease, responsible for the cleavage of the Gag/Pol polypeptide and the structural viral core proteins leading to the production of immature viral particles, the inhibition of viral replication and cell-to-cell spreading (10–12).

PI were recently shown to directly act as modulators of endolysosomal proteases activity, namely of cysteine cathepsins in human $CD4^+$ T-cells and in antigen presenting cells (APCs) as dendritic cells and Mø (13). Curiously, while saquinavir (SQV) activates omni-cathepsins enzymatic activity (omnicathepsins includes cathepsins B, L and S), ritonavir (RTV) displayed the opposite effect on cells obtained from non-infected individuals.

For Mtb we previously demonstrated that during infection of human Mø, a general down-regulation of cathepsins gene expression, concomitant with a decreased protease activity, occurs either in resting M0 or in IFN-γ M1 activated cells (14, 15). This may be a strategy used by the pathogen to manipulate the host microbicidal responses in order to survive intracellularly in these immune cells and to prevent antigen presentation. Here we found that SQV, contrary to RTV, is able to enhance the omnicathepsins protease activity including a very significant increase in cathepsin S activity in Mtb infected Mø. The enhancement of the catalytic activity was able to overcome the enzymatic inhibition induced by the pathogen in a three-fold magnitude. The same was observed during HIV co-infection. Our results indicate that SQV treatment during Mtb infection led not only to an exacerbated intracellular killing of the bacteria but also to an improved expression of the HLA class II antigen presentation machinery at the cell surface, to $CD4^+$ T-lymphocyte priming and proliferation as well as to increased secretion of IFN-γ. All together the results indicate SQV as a potential host directed therapy for tuberculosis.

MATERIALS AND METHODS

Cells and Culture Conditions

Human monocyte-derived Mø were obtained by isolating $CD14^+$ monocytes from buffy coats of healthy blood donors provided by the national blood institute (Instituto Português do Sangue e da Transplantação, Lisbon, Portugal) following a protocol established between Dr. Anes (Faculty of Pharmacy, University of Lisbon) and the Portuguese Institute for Blood, allowing access to buffy coats from healthy blood donors, for scientific research and academic purposes. The supplier provided no personal details from the donors. The cells were isolated using the MACS cell separation system (Miltenyi Biotec). Briefly, the mononuclear cell fraction was isolated using Ficoll-Paque PLUS (GE Healthcare) density gradient medium. This fraction was incubated with anti-CD14 magnetic beads and then passed across the MACS magnetic columns for positive monocyte selection. To induce differentiation to Mø, the isolated monocytes were allowed to adhere to 48- or 96-well plates at 1.5×10^5 or 5×10^4 cells per well, respectively for 2 h at 37°C, 5% CO_2, in RPMI-1640 medium (HyClone, GE Healthcare). Following adherence, the medium was supplemented to achieve a final concentration of 10% (v/v) FBS (HyClone, GE Healthcare), 1 mM sodium pyruvate (HyClone, GE Healthcare), 10 mM HEPES (HyClone, GE Healthcare), 0.1% β-mercaptoethanol (Gibco), and 20 ng/mL recombinant human M-CSF (Biolegend). Differentiation lasted for 7 days and medium was renewed every three to four days until day 7. Purity of the isolated culture was verified by flow cytometry.

Bacterial Cultures and HIV Isolates

Mycobacterium tuberculosis H37Rv (ATCC 27294), H37Rv GFP-expressing strain and *Mycobacterium bovis* BCG Pasteur (ATCC 35734) were grown in Middlebrook's 7H9 medium

supplemented with 10% OADC enrichment (Difco), 0.02% glycerol and 0.05% tyloxapol at 37°C (15). Preceding the infections, bacterial cultures on exponential grown phase were centrifuged and washed in phosphate-buffered saline (PBS). Bacteria were resuspended in RPMI-1640 medium (Mø culture medium) without antibiotics. In order to dismantle bacterial clumps, the bacterial suspension was treated by ultrasonic bath for 5 min. Residual clumps were removed by 1-minute centrifugation at 500 × *g*. Single-cell suspension was verified by fluorescence microscopy and quantified by optical density at 600 nm.

Primary HIV-1 isolate UCFL1032 was obtained after cocultivation of infected patient's peripheral blood mononuclear cells (PBMCs) with PHA-stimulated PBMCs from uninfected individuals. Viral stocks were established in PBMCs from low-passaged supernatants of original cultures, aliquoted and maintained at -80° C until used. Viral concentration was measured by reverse transcriptase (RT) activity using an enzyme linked immunosorbent assay (Lenti-RT kit, Caviditech, Uppsala, Sweden). HIV-$1_{UCFL1032}$ was characterized both genetically and phenotypically: it belongs to subtype B and uses CXCR4 coreceptor to enter host cells. It has the ability to enter Mø that produce low amounts of viral progeny upon infection, a phenotype similar to what is described during the course of patients Mø infection (16). This isolate is part of viral library created and maintained in our laboratory since the late Eighties, where a significant amount of HIV-1 and HIV-2 were characterized (17).

All experimental procedures using live Mtb and HIV were performed in the Biosafety Level 3 laboratory at the Faculty of Pharmacy of the University of Lisbon, respecting the national and European academic containment level 3, laboratory management and biosecurity standards, based on applicable EU Directives. All procedures have been approved by the faculty's biological safety committee.

Treatment and Infection of Mø

Prior to infection, Mø were treated for 1 h with selected concentrations of saquinavir (SQV) (Merck Life Science) or ritonavir (RTV) (Merck Life Science) previously reconstituted in DMSO. Following pretreatment, the bacterial/viral suspension was added without removing the inhibitors. Mø were infected with a MOI of 1 of bacteria and inoculated with the equivalent of 1 ng of RT of HIV-$1_{UCFL1063}$. After 3 h of infection at 37°C, 5% CO_2, the cells were washed with PBS to remove free bacteria/virus and cultivated in fresh complete medium supplemented with SQV or RTV. The controls were treated with the same concentration of DMSO as carried during treatments.

Phagocytosis of the bacteria was evaluated by flow cytometry using *M. tuberculosis* H37Rv GFP-expressing strain and following the procedures below. Monitorization of HIV infection was performed by fluorescence microscopy. Macrophages were fixed with 4% paraformaldehyde 4% sucrose solution in PBS for 1 h and quenched by incubating with 50 mM NH_4Cl in PBS. Cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked with 1% BSA in PBS for 30 min. Cells were stained with anti-Gag antibody 1:100 (KC57, Beckman Coulter) in PBS BSA 1% for 1 hour, washed and then incubated with Alexa Fluor 555 Goat anti-Mouse IgG secondary antibody 1:1000 (Cell Signaling Technology) for 30 minutes. Coverslips were mounted using ProLong Gold Antifade Mountant (ThermoFisher Scientific) and visualized on a Zeiss Axioskop 40 fluorescence microscope. Analysis was performed on ImageJ software **(Supplemental Figure 1)**. To further confirm that the cell culture was infected with HIV, integration of the viral DNA into host genome was confirmed using nested polymerase chain reaction (PCR) as described (18). Briefly, a first round of PCR amplification was done using an *Alu*-specific sense primer in combination with a *gag* antisense HIV-1 specific primer; the PCR products were then subjected to a second amplification reaction targeting the HIV-1 R/U5 region of LTR, leading to an amplicon with 391 bp **(Supplemental Figure 1)**.

Macrophage Viability

Macrophages seeded in 96-well plates were treated with SQV, RTV or DMSO for 3 days. Next, samples were washed and incubated with PrestoBlue (Invitrogen) resazurin-based solution at 37°C, 5% CO_2, according to the manufacturer's instructions. After 3 h of incubation, fluorescence emission was analyzed in a Tecan M200 spectrofluorometer. Non-treated cells were used as reference and cells treated with RTV 100 µg/mL were used as control for cell death.

Enzymatic Activity of Cathepsins

Following 24 h of treatment and infection with *M. tuberculosis* H37Rv, or co-infection with HIV, Mø in a 96-well plate were washed with PBS and incubated in PBS with omnicathepsin (Z-FR-AMC, Z-Phe-Arg-AMC) (Enzo Life Sciences) or cathepsin S (Z-VVR-AFC) (BioVision) fluorogenic substrate for 1,5 h at 37°C in a Tecan M200 spectrofluorometer. Fluorescence readings were performed every 5 min. Essay specificity was verified by treating the cell lysates with general protease inhibitor E-64d or with specific cathepsin S inhibitor, provided in the kit.

Bacteria Intracellular Survival

When required, infected cells in 96-well plates were lysed in 0.05% Igepal solution for 15 min. Serial dilutions of the resulting bacterial suspension were plated in Middlebrook 7H10 with 10% OADC (Difco) and incubated for 2-3 weeks at 37 °C before colonies were observable and counted under the microscope.

Bacteria Growth Curves in Broth Medium

M. tuberculosis H37Rv in single-cell suspension were incubated in bacteria culture medium with selected concentrations of SQV, RTV and DMSO at 37°C, 5% CO_2 for 15 days. The optical density at 600 nm was measured at discrete time points. Isoniazid (INH) was used as control for inhibition of growth.

Flow Cytometry

Following 24 h of treatment and infection with *M. tuberculosis* H37Rv, Mø in 48-well plates were recovered with HyQTase cell detachment solution (HyClone, GE Healthcare). For the

identification of apoptotic and necrotic cells Annexin V Apoptosis Detection Kit with PI (Cat # 640914, Biolegend) was used following the manufacturer's instructions. Cells were incubated with annexin V and propidium iodide for 20 minutes, washed with the appropriate kit buffer and fixed in 4% paraformaldehyde for 1 h. Following fixation, cells were washed again in buffer and analyzed. For surface staining of HLA molecules, detached cells were promptly fixated for 1 h. Following fixation, cells were washed and incubated with Human TruStain FcX Fc receptor blocking solution (Biolegend) for 10 minutes and then stained for 20 min with antibodies specific for human HLA class I (Cat # 311422, Biolegend) and HLA class II (Cat # 361716, Biolegend) molecules. Samples were analyzed in Guava easyCyte™ 5HT flow cytometer.

$CD4^+$ T-Lymphocytes Proliferation

Autologous $CD4^+$ T-lymphocytes were obtained from the same healthy PPD^+ donors according to the isolation protocol described above. Positive selection of the $CD4^+$ lymphocytes was performed using anti-CD4 magnetic beads (Miltenyi Biotec). Isolated lymphocytes were cultivated in 75 cm^2 flask at 2×10^6 cells per mL in RPMI-1640 medium (HyClone, GE Healthcare) supplemented with 15% (v/v) FBS (HyClone, GE Healcthcare), 1 mM sodium pyruvate (HyClone, GE Healcthcare), 10 mM HEPES (HyClone, GE Healcthcare) and 20 UI/ml of human recombinant Interleukin 2 (Biolegend) for 3 days prior to the experiment. Immediately before the experiment the lymphocytes were stained with Carboxyfluorescein diacetate succinimidyl ester (Cat # 423801, Biolegend) following the manufacturer's instructions. Macrophages infected with *M. tuberculosis* H37Rv or *M. bovis* BCG and treated for 24 h were washed and cocultivated with the lymphocytes at a ratio of 5 lymphocytes per macrophage for 5 days. $CD4^+$ lymphocytes were recovered after 5 days of coculture and analyzed using Guava easyCyte™ 5HT flow cytometer.

IFN-γ Quantification

Supernatants from the previous assays were recovered following 24 h of infection and treatment and following an additional 5 days of coculture with $CD4^+$ lymphocytes and stored at -80 °C for posterior analysis of interferon-γ (IFN-γ) secretion. The quantification was performed by Sandwich Enzyme-Linked Immunosorbent Assay using ELISA Max Deluxe Set Human for IFN-γ (Cat # 430104, Biolegend) kits and following the manufacturer's instructions. Absorbance was measured by Tecan M200 spectrofluorometer at 570 nm.

Statistical Analysis

Data are presented as mean ± standard error except if stated otherwise. Statistical analysis was performed using SigmaPlot 12. Multiple group comparisons were made using ANOVA one parameter tests followed by pairwise comparisons of the groups using Holm-Sidak test. Two group comparisons were made using Student's t-test. All the prerequisites of the tests were verified. The considered nominal alpha criterion level was 0.05 below which differences between samples were deemed significant.

RESULTS

Treatment With Saquinavir Impacts Cysteine Cathepsins Enzymatic Activity in Mø Infected With Mtb and HIV

Protease Inhibitors (PI) prescribed to HIV-infected patients were previously found to directly manipulate the proteolytic activity of endolysosomal cysteine cathepsins in APCs isolated from healthy non-infected donors (13). Here in the context of infected Mø with Mtb or during co-infection with HIV, we first aimed to assess the effect of HIV PIs saquinavir (SQV) and ritonavir (RTV) on omnicathepsin proteolytic activities (which measure the combined activities of cathepsin B, L, and S). Cathepsins B, L and S are all involved in intracellular killing of pathogens internalized by Mø through phagocytosis/endocytosis (14). Cathepsin S, in addition, is strongly expressed in APCs and also operates in the endocytic pathway with proteolytic activities required for antigen and MHC class II processing (15, 19, 20).

The selected concentrations of SQV and RTV ranging from 5 to 20 μg/mL were based in previous studies (5, 13) concerning the average levels found in the plasma of people treated with a single daily dose of 5 to 10 μg/mL (13, 21–23). Here, Mø were treated with SQV or RTV one hour before infection (as detailed in methods) and the drugs were left in contact with cells during the whole assay. The cleavage of a peptidase-specific fluorogenic peptide substrate was measured over 1.5 hours, 24 h post-infection. Cells treated with omnicathepsin inhibitor E-64d were used as control.

Figure 1A (upper panels) shows the effects of PIs on omnicathepsin proteolytic activities in Mø infected with Mtb or co-infected with HIV relatively to non-treated infected cells. Treatment with SQV led to a very significant increase of the proteolytic activity in a dose-dependent manner, while no effects were observed for RTV in all conditions tested. In non-infected cells (**Figure 1A** upper panels left and right) the effects of SQV on cathepsin kinetics was more exacerbated than in infected ones reinforcing our previous results that Mtb infection results in an overall decrease of cathepsins activity (14, 15). The kinetics when using the concentration of 10 μg/mL was near the saturation level by the end of 60 min treatment (**Figure 1A** upper right panel).

In parallel, we assessed the PIs effect on kinetics of cathepsin S activity alone using a cathepsin S cleavage-specific fluorogenic peptide substrate. As depicted in **Figure 1A** (lower panel) SQV strongly enhanced the proteolytic activity of cathepsin S in a dose dependent manner during Mtb infection and during co-infection with HIV contrary to RTV that presented kinetics similar to the control.

To confirm the effect of SQV and RTV in cell viability/cytotoxicity we performed the resazurin assay (**Figure 1B** upper panel) with results indicating cytotoxic effects on Mø viability at a concentration of 40 and 20 μg/mL for SQV and RTV, respectively,

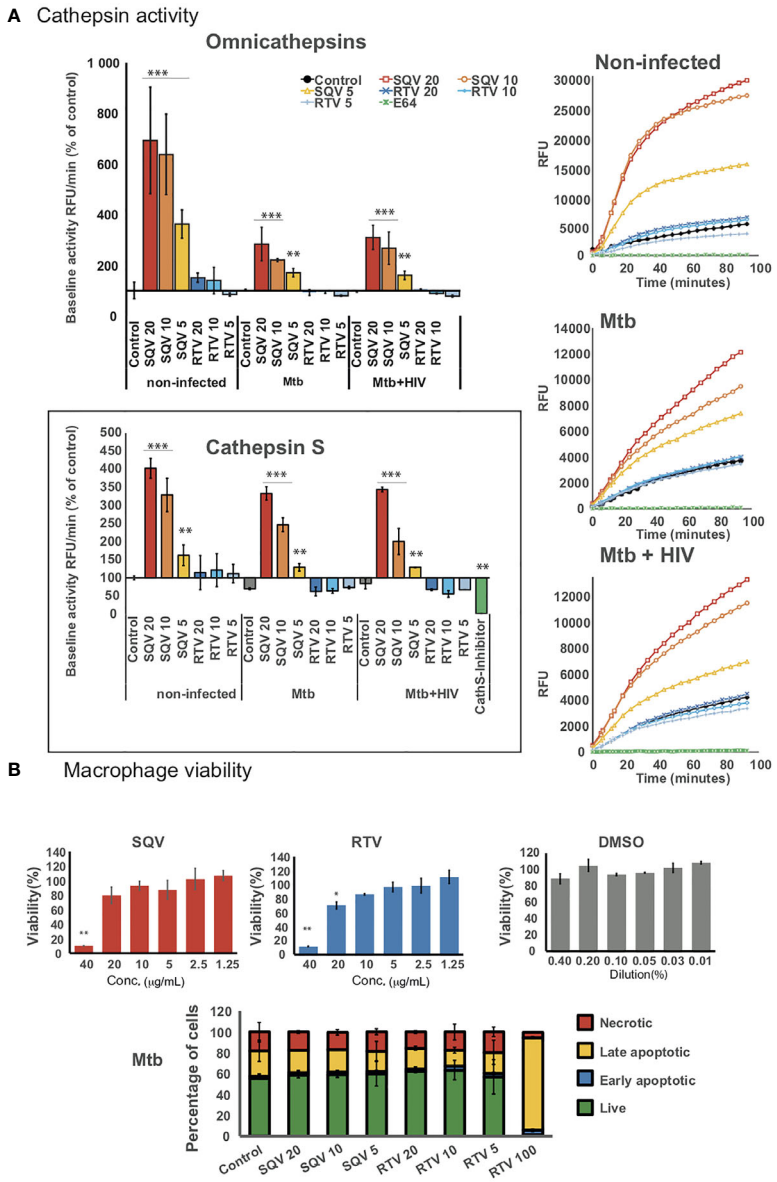

FIGURE 1 | HIV PIs alter cathepsins' activity in human macrophages infected with Mtb. **(A)** Omnicathepsin activity or cathepsin S activity alone were monitored with a specific fluorogenic substrate every 5 min in live cells pretreated with DMSO, RTV, SQV, or with specific inhibitors (E-64d or ZFL-COCHOO for cathepsin S). The slope of fluorescence emission in the presence of DMSO was represented as 100%, and the effect of each PI was calculated as a percentage of the DMSO control. Data are represented as average from three independent experiments and donors and data dispersion represented by the error bars as standard error (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relatively to control). **(B)** Cell viability (upper bar-plots) was measured in non-infected cells treated for 3 days with the PIs and using PrestoBlue resazurin-based solution by quantifying the emission of fluorescence in a plate reader. Cell death (lower bar-plot) was measured by flow cytometry after 24 h of infection using FITC-Annexin V and propidium iodide. Values show the average of three biological replicates from one representative experiment performed in triplicate while error bars depict standard deviation (*$P < 0.05$, **$P < 0.001$ relatively to control).

but without effects when using therapeutic concentrations of 5- 10 μg/mL. To further evaluate the impact on programmed cell death in infected Mø we used annexin-V and propidium iodide staining as markers for apoptosis and necrosis. We detected no increased toxicity on infected cells treated with concentrations of PIs ranging from 5 to 20 μg/mL, relatively to the control (**Figure 1B** lower panel; **Supplemental Figure 2**).

Treatment With HIV PI Saquinavir Results in Increased Mtb Killing in Primary Human Mø During Mono-Infection and During HIV Co-Infection.

Once established that PI treatment of infected cells did not impact apoptosis neither necrosis in the experimental conditions used and, therefore, would not interfere with the

amount of live bacteria recovered from infected Mø, we next tested the effects of SQV and RTV on Mtb intracellular killing. Our hypothesis is that SQV strongly increasing the proteolytic activity of omnicathepsins may reverse and largely compensate the induced inhibitory effect observed during Mtb infection (14).

As shown in **Figure 2A** (upper and lower panel) pre-treatment with SQV in therapeutic concentrations significantly enhances the intracellular killing of Mtb during mono-infection or during HIV co-infection, in a dose-dependent manner ($P < 0.001$). No effects were observed using therapeutic concentrations of RTV. Since cells were pre-treated before infection and the PIs were added again just once after bacteria internalization into Mø, the impact on bacteria killing was mainly observed during the first 3 days of infection with a concomitant recover of the intracellular replication afterwards. The effects on intracellular killing of Mtb were similar to those achieved using pyrazinamide (PZA) at a minimal inhibitory concentration estimated *in vitro* of 100 μg/mL (**Figure 2A**, lower panel).

To confirm that PI treatment did not impact on the ability of Mø to internalize bacteria, we assessed Mø containing GFP expressing bacteria by flow cytometry (**Figure 2B**, **Supplemental Figure 2**). The results show that approximately 50% of Mø were infected (left panel) and the infected population was loaded with similar amounts of bacilli (right panel), independent of the concentration of PIs used.

Finally in order to disregard a microbicidal effect of the PIs directly in Mtb we evaluated the effect of higher concentrations of SQV and RTV than those used in *ex vivo* assays directly on bacteria replication in liquid media. Using turbidimetry assays at an OD of 600 nm, the *in vitro* growth of Mtb was similar to samples treated with 20 μg/mL either with SQV or RTV (**Figure 2C**). Isoniazid at a concentration of 0.1 μg/mL was used as control.

Altogether our results suggest that the Mtb intracellular killing effects of SQV are not attributed to a direct bactericidal effect of the drug but rather to an improved activity of omnicathepsins in the endocytic pathway. This is in accordance with our previous published results indicating that the limited non-toxic treatment with the omnicathepsin inhibitor E-64d helped Mtb survival in a 3 fold magnitude (14, 15).

Treatment With HIV PI Saquinavir Results in Increased Surface Expression of HLA Class II Antigen Presentation Machinery and CD4$^+$ T-Lymphocyte Proliferation

Appropriate innate immune responses lead to destruction of pathogens during phagocytosis but also to adaptive immune responses that are crucial to control infections. SQV was demonstrated to enhance cathepsin S activity in non-infected cells (13) and here we show that SQV significantly enhances cathepsin S activity in infected cells. Since SQV regulates the activity of cathepsin S it may also be implicated in endosomal antigen and HLA class II processing required for appropriate antigen presentation (19, 20). Previously we hypothesized that the noticeable Mtb-induced decrease in cathepsin S expression during infection might be linked to poor antigen processing and presentation, compromising the adaptive immunity response to infection (15). Here we further analyzed if SQV or RTV interfere with HLA class II antigen presentation machinery during infection, thus helping to improve the adaptive immune responses.

For this, we first evaluated the effects of PIs during Mtb infection or during HIV co-infection and analyzed changes in the surface expression of HLA class II molecules compared to non PI-treated infected cells. For all concentrations tested, SQV treatment led to a significant increase of HLA class II expression at the cell surface as measured by flow cytometry (**Figure 3A** upper panel, **Supplemental Figure 3).** RTV used at the maximum therapeutic concentration found in plasma of treated patients achieved after a single dose administration (5 μg/mL), did not show any changes relatively to the control. In contrast, the lowest concentration of SQV (5 μg/mL) induced a significant increase of HLA class II presentation of endogenous antigens in non-infected cells, as observed in **Figure 3B**.

Cathepsin S, was demonstrated to be implicated in partial antigen processing for cross-presentation to CD8$^+$ T-lymphocytes (24) but without affecting the levels of HLA class I expression at the cell membrane (5, 25). Therefore as control we tested the expression of HLA class I at the cell surface by flow cytometry. The results indicate no changes in HLA class I expression in any treated samples when compared to control, during Mtb mono-infection. However, during co-infection with HIV, we observed a significant increase in the expression of HLA class I in control cells, relatively to control cells mono-infected with Mtb. This result confirms the overall induced effect of cytosolic viral peptides in increasing the expression level of HLA class I molecules (**Figure 3A** lower panel). The results also indicate that the treatment with SQV and RTV induced no differences on the expression of the antigen presentation machinery.

Since BCG has been used as TB vaccine for more than one century, and since it has been losing the efficacy to protect from infection, we next tested the effect of PIs in improving the HLA class II expression at the cell surface, required to improve antigen presentation. As observed in **Figure 3C** for BCG infected cells we noticed a highly significant ($P < 0.01$) increase in antigen presentation levels.

To further evaluate the consequences of PIs treatment on antigen presentation we performed cocultures of treated and infected Mø with autologous CD4$^+$ T-lymphocytes obtained from the same healthy PPD$^+$ donors and evaluated their ability to induce T-cell proliferation (**Figure 3D**). Following the same pattern of HLA surface expression, treatment with SQV in Mtb or BCG-infected cells induced a significant T-cell proliferation relatively to the control, after 5 days post-cocultures as evaluated by flow cytometry (**Figure 3D**). No changes were observed in cells treated with RTV.

We inferred that the induced T-cell proliferation would be concomitant with enhanced IFN-γ secretion and lately to indirect Mø activation and again the potentiation of the bactericidal effect. Therefore we performed quantification of

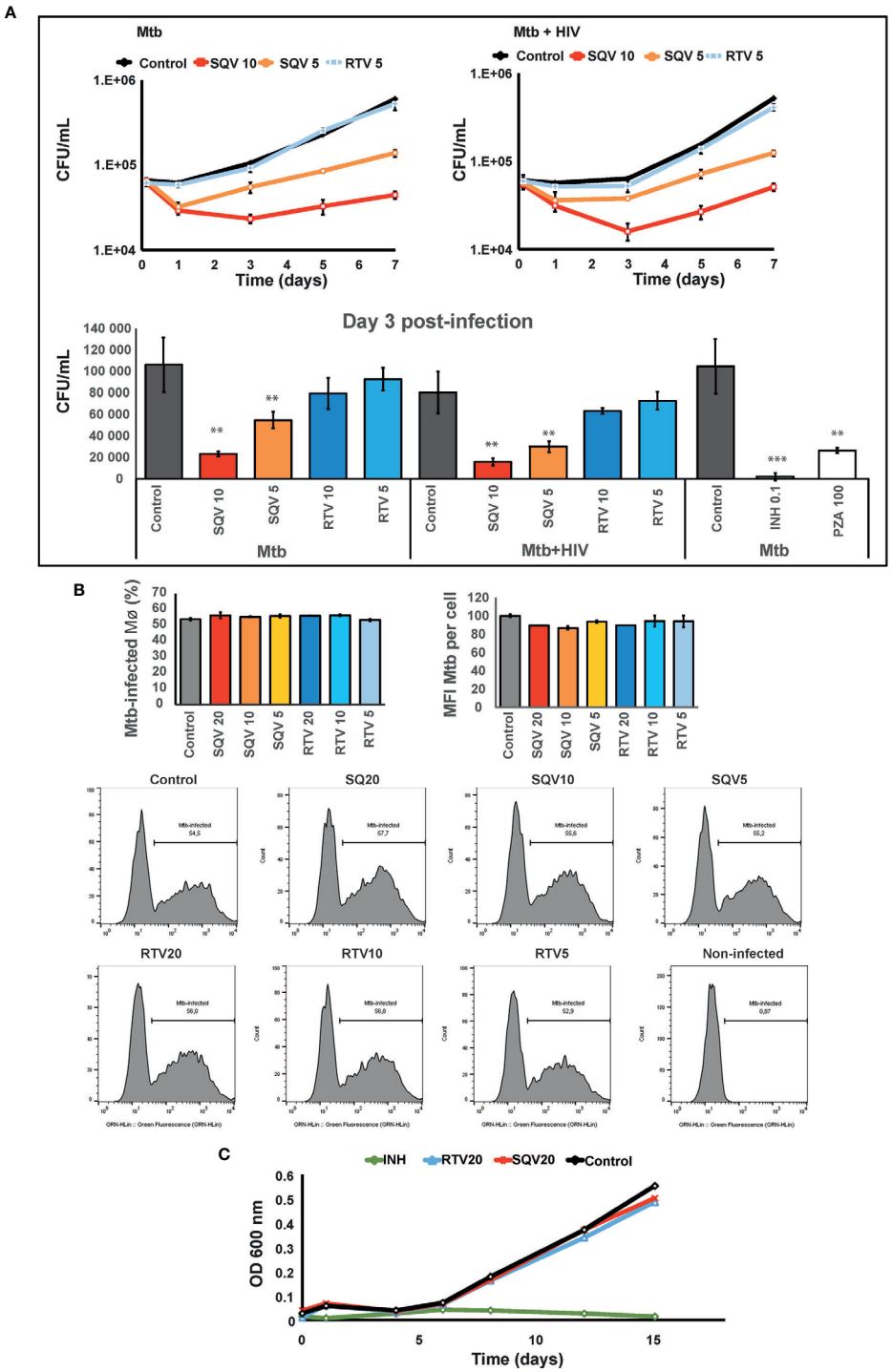

FIGURE 2 | SQV decreases the intracellular survival of *Mycobacterium tuberculosis* (Mtb). **(A)** Intracellular survival of Mtb during mono- or co-infection with HIV along 7 days of infection. Data represents colony forming units (CFU) of intracellular bacteria recovered from Mø treated with the PI or DMSO control. Culture medium was changed on day 3 p.i. without addition of fresh PIs. Values depict mean CFU representative of three biological replicates from one representative experiment performed in triplicate. Error bars depict the standard deviation (*P*** < 0.01; ****P* < 0.001 relatively to control). **(B)** Percentage of Mø infected with Mtb and median fluorescence intensity of Mtb per Mø were measured by flow cytometry in Mø pre-treated with the PIs and after 3 h of infection with GFP-expressing Mtb. Bar-plots depict the average of three biological replicates and the error bars depict the standard error. Raw values from one representative replicate are presented in the fluorescence intensity histograms. **(C)** Mtb growth curves in broth medium treated with PIs and incubated for 15 days. Values represent the optical density measured at discrete time points from one representative experiment performed twice. Isoniazid (INH) was used as control for inhibition of growth.

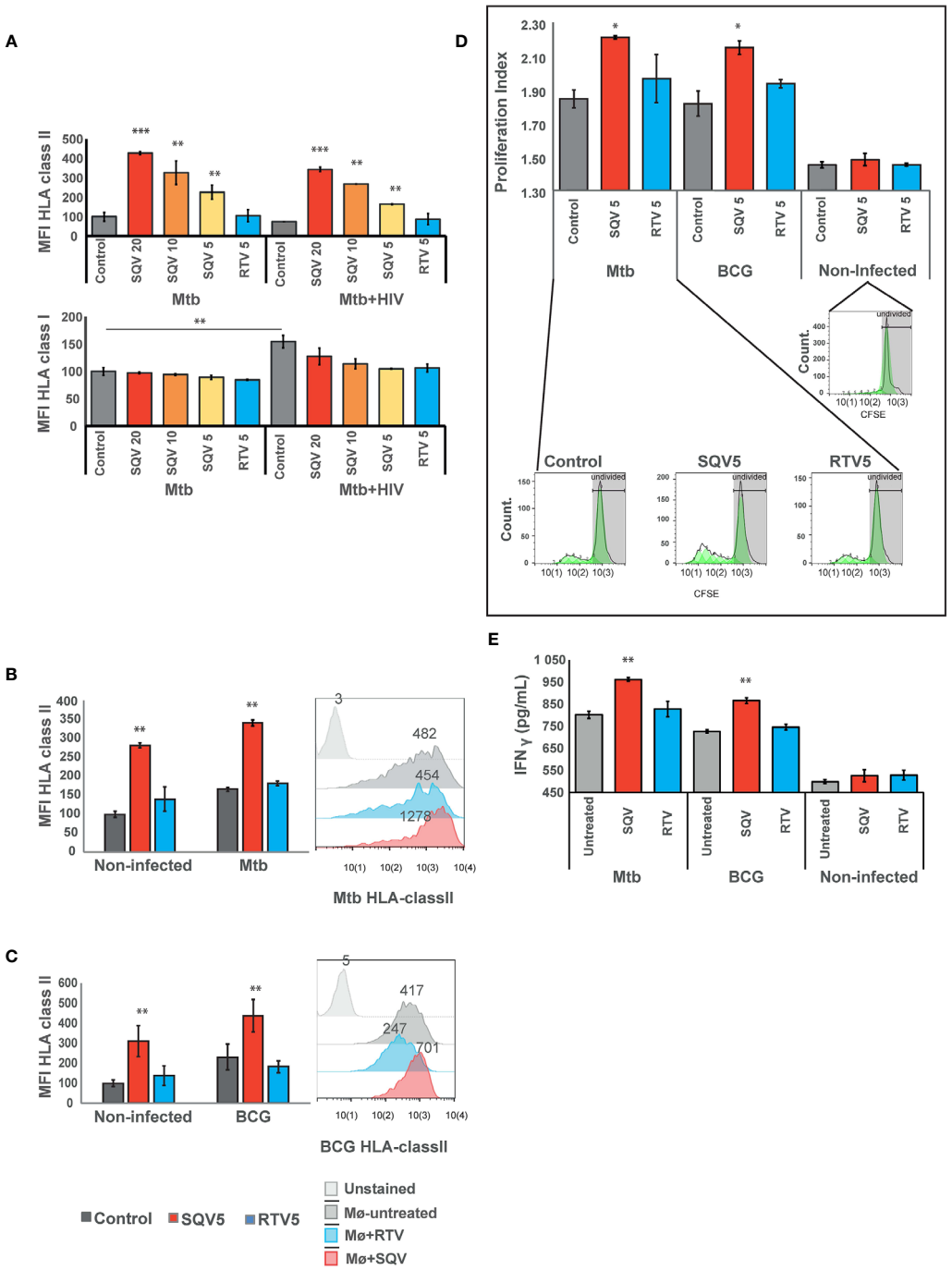

FIGURE 3 | SQV results in increased expression of HLA class II antigen presentation machinery **(A)** Surface expression of human leukocyte antigen (HLA)-class II or class I on Mø infected with Mtb or co-infected with HIV. **(B)** Surface expression of human leukocyte antigen (HLA)-class II on Mø infected with Mtb or **(C)** BCG compared to non-infected Mø. HLAs were measured by flow cytometry after 24 h of infection. Values in bar plots represent the average of median fluorescence intensity measured on three biological replicates from one representative experiment performed in triplicate relative to control. Error bars depict standard deviation (**$P < 0.001$, relatively to control). Raw values from one representative replicate are presented in the fluorescence intensity histograms. **(D)** CD4$^+$ T-cell proliferation after 5 days of coculture with Mtb or BCG-infected Mø. Following 24 h of the infection, CFSE stained CD4$^+$ T-cells were added to the infected Mø culture. After 5 days of coculture, CD4$^+$ T-cells CFSE fluorescence was measured by flow cytometry. Values in bar plots represent the proliferation index (average number of divisions per cell) of CD4$^+$ T-cell (*$P < 0.01$, relatively to control). Histograms from one representative replicate of the different treatments infected with Mtb are presented in the bottom. The green areas represent the CD4$^+$ T-cell populations after each division as modeled by the software. **(E)** IFN-γ was quantified in the supernatant after 5 days of cocultures of Mø with CD4$^+$T-lymphocytes by ELISA. Values depict mean concentration of three biological replicates from one representative experiment performed in duplicate. Error bars depict the standard deviation (**$P < 0.01$; ***$P < 0.001$, relatively to control).

IFN-γ secretion from non-infected cells and compared to infected ones treated or not with SQV. We observed in agreement with T-cell proliferation increased secretion of IFN-γ in cocultures supernatants of Mtb or BCG infection exacerbated in conditions treated with SQV **(Figure 3E)**; no significant alterations in IFN-γ secretion were detected in non-infected cocultures.

DISCUSSION

The purpose of our study was to decipher whether the first-generation HIV protease inhibitor, SQV, could be repurposed as a host-directed therapy for tuberculosis especially during co-infection with HIV. Tuberculosis, the so call white plague disease until the beginning of the twentieth-century, remains a leading cause of mortality worldwide due to an infectious agent. While in the last hundred years the vaccine BCG and the introduction of antibiotics helped to control the disease, since 1980 with the emergence of HIV this scenario has completely changed. HIV co-infection exacerbates Mtb infection helping reactivation from latency (2–4). Moreover due to increased drug resistance strains, co-infection impacts the transmission of MDR.

Accordingly, there is an urgent need to develop new medicines to control resistant bacteria and to redirect the immune responses of the host to effectively control the infection and the inflammatory responses. Within this context repurposing approved drugs will speed the process of improve therapy for the outcome of TB.

SQV was one of the first drugs developed to control HIV infection (26). It acts as an aspartic protease inhibitor interfering with HIV protease activity and therefore prevents the cleavage of Gag-Pol protein precursors. This inhibition ultimately blocks the infectivity of nascent virions and cell to cell spreading (10–12). It is likely that for other pathogens dependent on proteases for their life cycle, SQV and other PIs could be repurposed to control the respective infections. In fact they showed inhibitory effects against a wide spectrum of pathogens such as *Plasmodium falciparum* (27), *Trypanosoma cruzei* (28), the fungi *Fonsecaea pedrosoi* (29) and SARS-CoV and avian influenza viruses (30).

It is expectable that HIV PI may also interfere with the host proteases. Among host proteases with relevance during immune responses to infections are cathepsins in the endocytic pathway and threonine proteases of the proteosome. Consistently, HIV PIs were shown to alter cathepsin activity of antigen presenting cells (13) and to interfere with proteosome peptide processing leading to accumulation of polyubiquitinated products (13, 21). Accordingly, HIV PIs designed to inhibit the HIV aspartyl protease were described to alter the activity of aspartyl proteases like cathepsin D and E, as well as cysteine proteases, such as cathepsin S (13).

Our previous results indicated the ability of Mtb to down-regulate the activity of cathepsins in order to successful survive in human Mø (14). Mtb infection leads to a strong inhibition of cathepsins B, S and L (14, 15) all of them involved in crucial activities during innate and adaptive immune responses. These results lead us to hypothesized that SQV, by inducing an increased activity of these cathepsins in non-infected immune cells, could be repurposed in the TB context to revert the blockade induced by the pathogen.

Here we demonstrated that SQV is able to increase omnicathepsins proteolytic activity during Mtb infection and during co-infection with HIV **(Figure 1A)**. In Mø, these endolysosomal enzymes are enrolled in pathogen killing as one of the first innate immune responses to infections. Likewise we observed a significantly intracellular killing of Mtb in human Mø treated with SQV **(Figure 2A)**. Since either the infection with these pathogens and the treatment of host cells with SQV are inducers of apoptosis (13, 21, 31, 32), we disregard this programmed cell death as inducer of pathogen killing by adjusting the experimental conditions **(Figure 1B)**. We may conclude that SQV induced pathogen killing was due to an increased activity of cathepsins along the endocytic pathway coincident with the same compartment of Mtb.

Cathepsin S contributes to the antigen presentation machinery by processing pathogen peptides and by generating of HLA-classII epitopes. Likewise, in infected cells treated with SQV a significant increase in HLA class II molecules were detected at the plasma membrane of infected cells leading to increased T-lymphocyte priming and proliferation **(Figures 3A, D)**. This was particularly relevant during BCG infection indicating that SQV improves the capabilities of presenting vaccine antigens **(Figures 3C, D)**. Since the population in Portugal has been vaccinated for decades with BCG until 2017, most of the population are PPD+. Thus we expect the blood from healthy donors to carry a significant population of memory/effector T cells that responded to the challenge of Mtb-infected macrophages (33, 34).

Here we found that SQV treatment induces higher levels of T-cell-secreted IFN-γ in a context that mimics bacteria replication during active TB **(Figure 3E).** This increased secretion of IFN-γ may have a dual effect: (1) activation of Mø to a more bactericidal state and, (2) indirectly contributing to a decreased IL-1β secretion. In fact it was previous demonstrated that pretreatment of *M. tuberculosis*–infected Mø with IFN-γ specifically inhibited the release of IL-1β suggesting that during TB IFN-γ may suppress lung immunopathology induced by dysregulation of IL-1β (35). These results suggest the cytokine environment might help achieve a better control of the immunopathology in the lungs, in accordance to published studies performed in murine models of Mtb mono-infection (35).

Moreover SQV has been referred to possess anti-inflammatory effects especially in the lungs (36). This was attributed to the suppression of TLR4 signaling pathways of high-mobility group box 1 (HMGB1). The beneficial effects were linked to decreased levels of circulating and lung tissue inflammatory cytokines, such as IL-6, IL-1β, TNF-α, and iNOS.

Cathepsins S and L have been demonstrated to regulate autophagy (37). Mtb and HIV are known to inhibit autophagy: upon infection of Mø in the lungs, inhibition of the autophagic pathway by the first invader will likely benefit the second or induce a similar behavior in neighboring cells (38). It could be that an SQV-induced increase of the proteolytic activities of

cathepsins S and L would improve autophagy. This would in turn help infected cells to eliminate not only of the pathogens but also of cytosolic aggregates and inflammatory signaling molecules, contributing to decreased tissue inflammation (39).

Altogether our data and relevant literature indicates SQV as a potential host directed therapy for Tuberculosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: EA, DP, JA-P. Methodology, acquisition and analysis: DP, EA. Investigation: DP, SV, MC, MM. Writing: EA. Supervision: EA. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.647728/full#supplementary-material

REFERENCES

1. Bell LCK, Noursadeghi M. Pathogenesis of HIV-1 and Mycobacterium tuberculosis co-infection. *Nat Rev Microbiol* (2018) 16:80–90. doi: 10.1038/nrmicro.2017.128
2. World Health Organization. *Global tuberculosis report 2020*. Geneva: World Health Organization (2020). Available at: https://apps.who.int/iris/bitstream/handle/10665/329368/9789241565714-eng.pdf?ua=1.
3. Souriant S, Balboa L, Dupont M, Pingris K, Kviatcovsky D, Cougoule C, et al. Tuberculosis Exacerbates HIV-1 Infection through IL-10/STAT3-Dependent Tunneling Nanotube Formation in Macrophages. *Cell Rep* (2019) 26:3586–3599.e7. doi: 10.1016/j.celrep.2019.02.091
4. Mancino G, Placido R, Bach S, Mariani F, Montesano C, Ercoli L, et al. Infection of human monocytes with Mycobacterium tuberculosis enhances human immunodeficiency virus type 1 replication and transmission to T cells. *J Infect Dis* (1997) 175:1531–5. doi: 10.1086/516494
5. Kourjian G, Xu Y, Mondesire-Crump I, Shimada M, Gourdain P, Le Gall S. Sequence-specific alterations of epitope production by HIV protease inhibitors. *J Immunol* (2014) 192:3496–506. doi: 10.4049/jimmunol.1302805
6. Aquaro S, Caliò R, Balzarini J, Bellocchi MC, Garaci E, Perno CF. Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir. *Antiviral Res* (2002) 55:209–25. doi: 10.1016/s0166-3542(02)00052-9
7. Toossi Z, Johnson JL, Kanost RA, Wu M, Luzze H, Peters P, et al. Increased replication of HIV-1 at sites of Mycobacterium tuberculosis infection: potential mechanisms of viral activation. *J Acquir Immune Defic Syndr* (2001) 28:1–8. doi: 10.1097/00042560-200109010-00001
8. Mwandumba HC, Russell DG, Nyirenda MH, Anderson J, White SA, Molyneux ME, et al. Mycobacterium tuberculosis resides in nonacidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. *J Immunol* (2004) 172:4592–8. doi: 10.4049/jimmunol.172.7.4592
9. Arts EJ, Hazuda DJ. HIV-1 antiretroviral drug therapy. *Cold Spring Harb Perspect Med* (2012) 2:a007161. doi: 10.1101/cshperspect.a007161
10. Titanji BK, Aasa-Chapman M, Pillay D, Jolly C. Protease inhibitors effectively block cell-to-cell spread of HIV-1 between T cells. *Retrovirology* (2013) 10:161–11. doi: 10.1186/1742-4690-10-161
11. Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, et al. Rational design of peptide-based HIV proteinase inhibitors. *Science* (1990) 248:358–61. doi: 10.1126/science.2183354
12. Craig JC, Duncan IB, Hockley D, Grief C, Roberts NA, Mills JS. Antiviral properties of Ro 31-8959, an inhibitor of human immunodeficiency virus (HIV) proteinase. *Antiviral Res* (1991) 16:295–305. doi: 10.1016/0166-3542(91)90045-s
13. Kourjian G, Rucevic M, Berberich MJ, Dinter J, Wambua D, Boucau J, et al. and Cross-Presentation. *J Immunol* (2016) 196:3595–607. doi: 10.4049/jimmunol.1600055
14. Pires D, Marques J, Pombo JP, Carmo N, Bettencourt P, Neyrolles O, et al. Role of Cathepsins in Mycobacterium tuberculosis Survival in Human Macrophages. *Sci Rep* (2016) 6:32247. doi: 10.1038/srep32247
15. Pires D, Bernard EM, Pombo JP, Carmo N, Fialho C, Gutierrez MG, et al. Mycobacterium tuberculosis Modulates miR-106b-5p to Control Cathepsin S Expression Resulting in Higher Pathogen Survival and Poor T-Cell Activation. *Front Immunol* (2017) 8:1819. doi: 10.3389/fimmu.2017.01819
16. Honeycutt JB, Thayer WO, Baker CE, Ribeiro RM, Lada SM, Cao Y, et al. HIV persistence in tissue macrophages of humanized myeloid-only mice during antiretroviral therapy. *Nat Med* (2017) 23:638–43. doi: 10.1038/nm.4319
17. Calado M, Matoso P, Santos-Costa Q, Espirito-Santo M, Machado J, Rosado L, et al. Coreceptor usage by HIV-1 and HIV-2 primary isolates: the relevance of CCR8 chemokine receptor as an alternative coreceptor. *Virology* (2010) 408:174–82. doi: 10.1016/j.virol.2010.09.020
18. Kumar R, Vandegraaff N, Mundy L, Burrell CJ, Li P. Evaluation of PCR-based methods for the quantitation of integrated HIV-1 DNA. *J Virol Methods* (2002) 105:233–46. doi: 10.1016/s0166-0934(02)00105-2
19. Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* (1998) 93:1135–45. doi: 10.1016/s0092-8674(00)81458-0
20. Lautwein A, Burster T, Lennon-Duménil A-M, Overkleeft HS, Weber E, Kalbacher H, et al. Inflammatory stimuli recruit cathepsin activity to late endosomal compartments in human dendritic cells. *Eur J Immunol* (2002) 32:3348–57. doi: 10.1002/1521-4141(200212)32:12<3348::AID-IMMU3348>3.0.CO;2-S
21. Pajonk F, Himmelsbach J, Riess K, Sommer A, McBride WH. The human immunodeficiency virus (HIV)-1 protease inhibitor saquinavir inhibits proteasome function and causes apoptosis and radiosensitization in non-HIV-associated human cancer cells. *Cancer Res* (2002) 62:5230–5.
22. Acosta EP, Kakuda TN, Brundage RC, Anderson PL, Fletcher CV. Pharmacodynamics of human immunodeficiency virus type 1 protease inhibitors. *Clin Infect Dis* (2000) 30 Suppl 2:S151–9. doi: 10.1086/313852
23. van Heeswijk RP, Veldkamp AI, Mulder JW, Meenhorst PL, Lange JM, Beijnen JH, et al. Once-daily dosing of saquinavir and low-dose ritonavir in HIV-1-infected individuals: a pharmacokinetic pilot study. *AIDS* (2000) 14:F103–10. doi: 10.1097/00002030-200006160-00003

24. Shen L, Sigal LJ, Boes M, Rock KL. Important Role of Cathepsin S in Generating Peptides for TAP-Independent MHC Class I Crosspresentation In Vivo. *Immunity* (2004) 21:155–65. doi: 10.1016/j.immuni.2004.07.004
25. Wang B, Niu D, Lai L, Ren EC. p53 increases MHC class I expression by upregulating the endoplasmic reticulum aminopeptidase ERAP1. *Nat Commun* (2013) 4:2359–11. doi: 10.1038/ncomms3359
26. Baker R. FDA approves 3TC and saquinavir. Food and Drug Administration. *BETA* (1995) 5:9.
27. Nsanzabana C, Rosenthal PJ. In vitro activity of antiretroviral drugs against Plasmodium falciparum. *Antimicrob Agents Chemother* (2011) 55:5073–7. doi: 10.1128/AAC.05130-11
28. Castilho VVS, Gonçalves KCS, Rebello KM, Baptista LPR, Sangenito LS, Santos HLC, et al. Docking simulation between HIV peptidase inhibitors and Trypanosoma cruzi aspartyl peptidase. *BMC Res Notes* (2018) 11:825–7. doi: 10.1186/s13104-018-3927-z
29. Palmeira VF, Goulart FRV, Granato MQ, Alviano DS, Alviano CS, Kneipp LF, et al. Fonsecaea pedrosoi Sclerotic Cells: Secretion of Aspartic-Type Peptidase and Susceptibility to Peptidase Inhibitors. *Front Microbiol* (2018) 9:1383. doi: 10.3389/fmicb.2018.01383
30. Savarino A. Expanding the frontiers of existing antiviral drugs: possible effects of HIV-1 protease inhibitors against SARS and avian influenza. *J Clin Virol* (2005) 34:170–8. doi: 10.1016/j.jcv.2005.03.005
31. Behar SM, Divangahi M, Remold HG. Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? *Nat Rev Microbiol* (2010) 8:668–74. doi: 10.1038/nrmicro2387
32. Welin A, Eklund D, Stendahl O, Lerm M. Human macrophages infected with a high burden of ESAT-6-expressing M. tuberculosis undergo caspase-1- and cathepsin B-independent necrosis. *PloS One* (2011) 6:e20302. doi: 10.1371/journal.pone.0020302
33. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* (2014) 14:24–35. doi: 10.1038/nri3567
34. Nguipdop-Djomo P, Heldal E, Rodrigues LC, Abubakar I, Mangtani P. Duration of BCG protection against tuberculosis and change in effectiveness with time since vaccination in Norway: a retrospective population-based cohort study. *Lancet Infect Dis* (2016) 16:219–26. doi: 10.1016/S1473-3099(15)00400-4
35. Mishra BB, Rathinam VAK, Martens GW, Martinot AJ, Kornfeld H, Fitzgerald KA, et al. Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1β. *Nat Immunol* (2013) 14:52–60. doi: 10.1038/ni.2474
36. Yu Z, Tong Y, Zhang R, Ding X, Li Q. Saquinavir Ameliorates Liver Warm Ischemia-Reperfusion-Induced Lung Injury via HMGB-1- and P38/JNK-Mediated TLR-4-Dependent Signaling Pathways. *Mediators Inflammation* (2017) 2017:7083528. doi: 10.1155/2017/7083528
37. Chen K-L, Chang W-SW, Cheung CHA, Lin C-C, Huang C-C, Yang Y-N, et al. Targeting cathepsin S induces tumor cell autophagy via the EGFR-ERK signaling pathway. *Cancer Lett* (2012) 317:89–98. doi: 10.1016/j.canlet.2011.11.015
38. Espert L, Beaumelle B, Vergne I. Autophagy in Mycobacterium tuberculosis and HIV infections. *Front Cell Infect Microbiol* (2015) 5:49. doi: 10.3389/fcimb.2015.00049
39. Lim J, Park H, Heisler J, Maculins T, Elife MR-G. Autophagy regulates inflammatory programmed cell death via turnover of RHIM-domain proteins. *elifesciencesorg* (2019) 8:e44452. doi: 10.7554/eLife.44452.001

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REVIEW
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Repurposing Immunomodulatory Drugs to Combat Tuberculosis

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Tuberculosis (TB) is an infectious disease caused by an obligate intracellular pathogen, *Mycobacterium tuberculosis (M.tb)* and is responsible for the maximum number of deaths due to a single infectious agent. Current therapy for TB, Directly Observed Treatment Short-course (DOTS) comprises multiple antibiotics administered in combination for 6 months, which eliminates the bacteria and prevents the emergence of drug-resistance in patients if followed as prescribed. However, due to various limitations viz., severe toxicity, low efficacy and long duration; patients struggle to comply with the prescribed therapy, which leads to the development of drug resistance (DR). The emergence of resistance to various front-line anti-TB drugs urgently require the introduction of new TB drugs, to cure DR patients and to shorten the treatment course for both drug-susceptible and resistant populations of bacteria. However, the development of a novel drug regimen involving 2-3 new and effective drugs will require approximately 20-30 years and huge expenditure, as seen during the discovery of bedaquiline and delamanid. These limitations make the field of drug-repurposing indispensable and repurposing of pre-existing drugs licensed for other diseases has tremendous scope in anti-DR-TB therapy. These repurposed drugs target multiple pathways, thus reducing the risk of development of drug resistance. In this review, we have discussed some of the repurposed drugs that have shown very promising results against TB. The list includes sulfonamides, sulfanilamide, sulfadiazine, clofazimine, linezolid, amoxicillin/clavulanic acid, carbapenems, metformin, verapamil, fluoroquinolones, statins and NSAIDs and their mechanism of action with special emphasis on their immunomodulatory effects on the host to attain both host-directed and pathogen-targeted therapy. We have also focused on the studies involving the synergistic effect of these drugs with existing TB drugs in order to translate their potential as adjunct therapies against TB.

Keywords: *Mycobacterium tuberculosis*, directly observed therapy short course, repurposed approved drugs, immunomodulators, T cells (Th1/Th2), vaccine, immunotherapy

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INTRODUCTION

Mycobacterium tuberculosis (*M.tb*) is a deadly pathogen, which infects a large cluster of the population globally and is the cause of the maximum number of deaths due to a single infectious agent (1). The World Health Organization (WHO) has reported that around 10 million people across the globe suffer from active TB infection; with the mortality rate of around 1.3 million (2). Nearly one-fifth of the mortality due to TB is because of the emergence of drug-resistant strains, which do not respond to the frontline anti-TB drugs. The rise in drug-resistance is an alarming situation and has made the control of TB even more challenging. Current treatment of TB involves administration of multiple antibiotics for a minimum period of 6 months for drug-susceptible TB and requires more than two years of treatment in case of drug-resistant TB. This treatment regime is lengthy and is associated with severe side effects such as dampening of the immune system, organ toxicity and emergence of drug-resistance (3).

To mitigate the challenges of dealing with TB, we are in urgent need of novel drugs with an action mechanism that can treat as well as shorten the treatment regime for both drug-susceptible and drug-resistant strains, which may be better tolerated and may increase adherence to the therapy. Recently, three new drugs, bedaquiline, delamanid and pretomanid have been approved by the Food and Drug Administration (FDA) against TB (4, 5). However, it is very difficult and tedious to develop new efficacious TB treatment regime owing to the time taken and the cost incurred in various processes involved. Therefore, the pharmaceutical industries as well as the researchers are focusing on identifying novel drug and target interactions using pre-existing drugs which have been used in the treatment of different diseases, a practice called drug repurposing.

Drug repurposing, also known as drug repositioning involves identifying new therapeutic usages of already established and approved drugs. This strategy encompasses a lower risk of failure, reduces the time required for new drug development, involves comparatively less investment and may lead to the discovery of novel targets which may be used for further research in the pharmaceutical field (6). The most successful example of drug repurposing is Sildenafil (7). It was initially developed for use as an anti-hypertensive drug but has gained huge popularity as a medication against erectile dysfunction. Its repurposed use as an inhibitor of phosphodiesterase 5 is a consequence of a serendipity (7). Later, drugs such as thalidomide and its derivatives like lenalidomide were successfully used as repurposed drugs for the treatment of diseases such as Erythema Nodosum Leprosum (ENL) and multiple myeloma (8). The success of these drugs by serendipity has led to the ongoing serious efforts by researchers in discovering new roles of pre-existing drugs in different disease contexts. In TB, drug repurposing offers a very attractive strategy to deal with the emerging challenge of drug resistance and to discover drug combinations that may shorten the duration of treatment ultimately preventing the development of resistance and promoting adherence to the treatment. Drug repurposing is by far transforming translational research by assuring total safety and efficacy while cutting short the time invested in passing the regulatory hurdles and thus ensuring that the drug reaches the clinic within 3-4 years. Moreover, there has been ongoing research on the use of immuno-modulators as an adjunct therapy with the conventional DOTs (Directly Observed Treatment, Short-course) treatment with the sole purpose of reducing the duration of treatment as well as pulmonary toxicity (9). This adjunct therapeutics has been designed so as to prevent reinfection and also reactivation of the TB disease. Many of the repurposed drugs are an attractive target to be used as immuno-modulators as there is no safety concern involved with them. Their use as immune modulators along with the standard anti-TB regimen may achieve total elimination of the pathogen in a short time. The chemical structure of the various drugs repurposed for TB has been shown in **Figure 1**. Immunomodulators are natural or synthesized compounds that activate or suppress the immune system by the release of either pro-inflammatory or anti-inflammatory cytokines in order to help the immune system deal with a pathogen more effectively. Pro-inflammatory responses by cytokines released by T cells such as IFN-γ, TNF-α in association with IL-6, IL- 1 and chemokines such as CCL5, CCL9, CXCL10, and CCL2 attracts immune cells at the site of infection and lead to the effective elimination of the pathogen (10). The pro-inflammatory cytokine response is mainly responsible for initiating a cascade of events that ultimately leads to the killing of *M.tb*. The immunomodulators act on different immune cells such as neutrophils, macrophages, lymphocytes, natural killer (NK) cells to exert their effector responses aimed at clearing the bacteria from the host. The mechanism of action of immuno-modulators has been shown in **Figure 2**.

These immunomodulators have gained tremendous attention in anti-TB therapy as these compounds when administered together with the DOTS regime helps in the early clearance of the infection as well as aids in the prevention of drug-resistance development. Many of the immunomodulators help mask the side effects of the harsh anti-TB antibiotic therapy.

Here, in this review, we discuss drugs, which display promising effects against TB and hence have been repurposed for use against TB. The drugs that we have discussed appear to be the most significantly studied in case of TB. We also highlight their mechanism of action along with any study if present for their use as immuno-modulators as an adjunct therapy against TB (**Table 1**).

REPURPOSED DRUGS FOR ANTI-TB TREATMENT AND THEIR IMMUNOMODULATORY PROPERTIES

With very slow development in the addition of novel drugs against TB and the fast emergence of drug resistance among TB patients, there is a need to focus on repurposing drugs for better treatment outcome against TB. WHO has recommended the inclusion of repurposed drugs such as fluoroquinolones, linezolid, clofazimine, and carbapenems, among many others, for the treatment of drug-resistant TB. Here, we discuss each of these repurposed drugs along with their mechanism of action

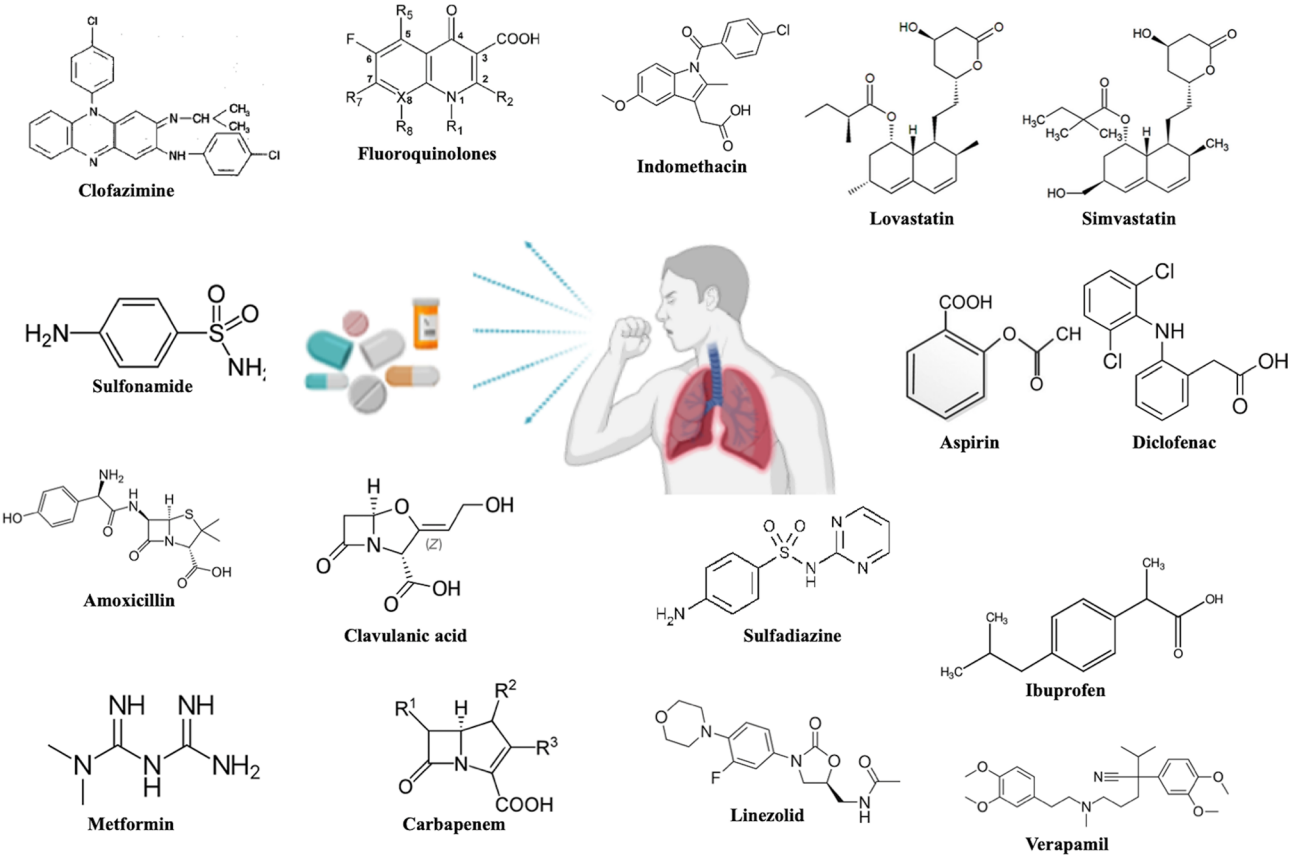

FIGURE 1 | Chemical structures of the proposed repurposed drugs under investigation against TB disease.

and any immunomodulatory role if known to date. Different approaches used to repurpose the drugs against different diseases have been summarized in **Figure 3**.

Clofazimine

Clofazimine, a riminophenazine antibiotic was discovered in Dublin in the 1950s and was originally introduced in 1969 for the treatment of leprosy (11). Recently, it has been repurposed for the treatment of drug-resistant TB after a study which stated that including clofazimine in anti-TB regime could treat MDR-TB in 9-12 months and has been recommended by WHO as a second-line drug along with other first line treatments (11). Clofazimine works like a pro-drug, which releases reactive oxygen species (ROS) upon re-oxidation by oxygen after initially being reduced by NADH dehydrogenase (NDH-2) (12). Clofazimine apparently competes with menaquinone (MK-4) for its reduction by NDH-2 (13). Clofazimine also exerts its anti-mycobacterial as well as anti-inflammatory properties by a Ca^{2+}-independent increase in mycobacterial PLA2 and by its effects on potassium channels (14, 15). However, it is independent of the C-type phospholipases of *M.tb* (16). Owing to its efficacy and negligible toxicity in the treatment of drug-resistant strains in mice model studies and in clinical trials, it comes out as a promising drug candidate for TB management (17, 18). In different clinical trials in Bangladesh and China, clofazimine has shown to reduce the treatment duration of MDR-TB (17) and is recommended by WHO for treatment of drug-resistant TB along with other drugs. Clofazimine is reported to display immuno-modulatory properties by enhancing T_{CM} (Central Memory T cells) responses while reducing T_{EM} (Effector Memory T cells) population by blocking $KV1.3^{+}$ potassium ion channel on the surface of T_{EM} (18, 19). Depending upon their differentiation state, homing potential, duration of survival and production of three major cytokines-IFN-γ, TNF-α and IL-2 (20). Memory T cells help in providing protective immunity against TB. Clofazimine is inexpensive, compared with other drugs in an MDR-TB drug-regimen and seems to be very promising as drug of future, for TB.

Statins

Statins are HMG-CoA reductase inhibitors that have recently been explored for their anti-tubercular effects (21). Statins have been prescribed to hyperlipidemic patients in order to reduce the risk of stroke and other cardiovascular diseases. Recent studies have demonstrated that statins have anti-inflammatory and

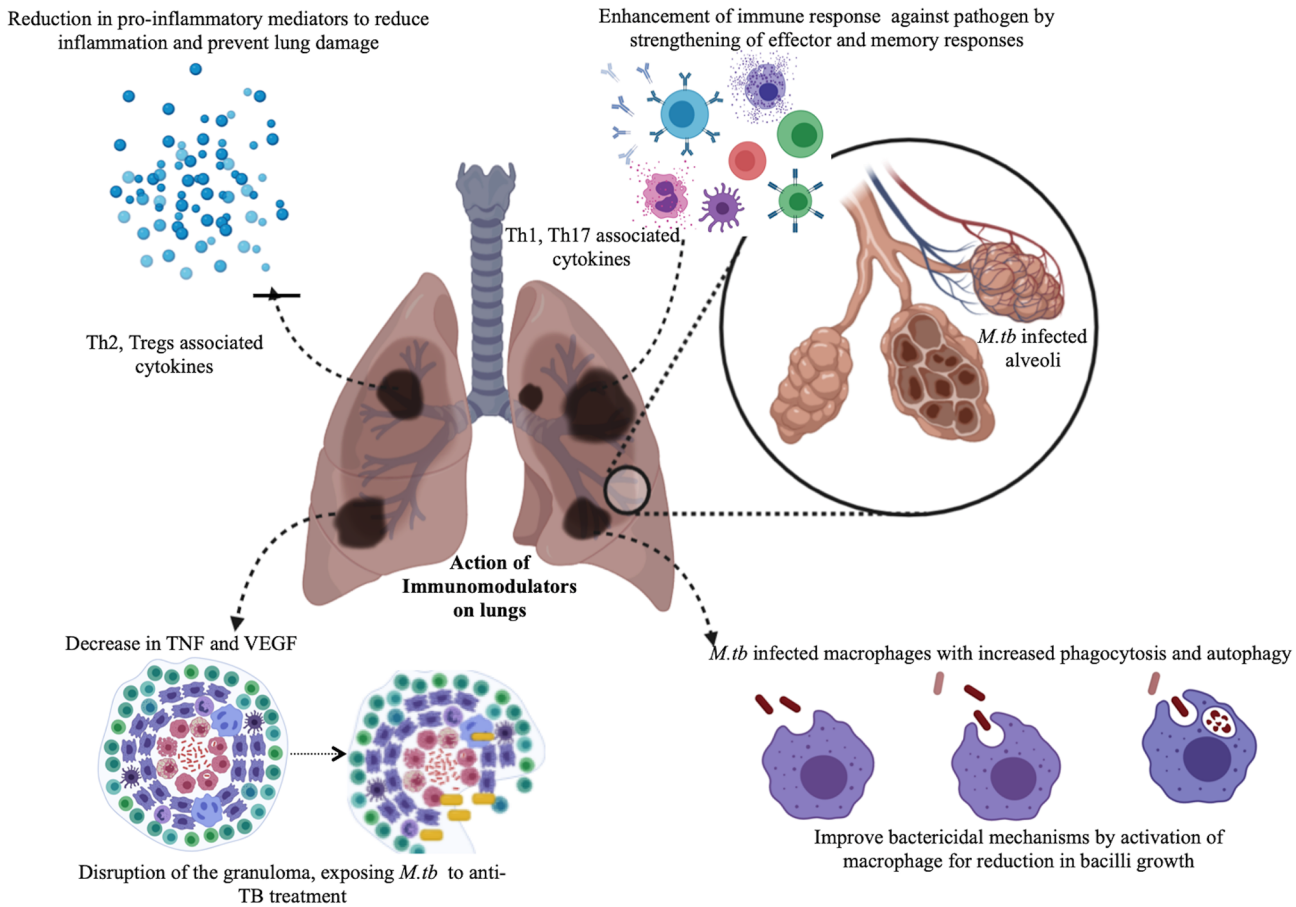

FIGURE 2 | Mechanism of action of immuno-modulatory drugs.

TABLE 1 | Repurposed drugs with their year of introduction and status in TB treatment.

Name of drug	Year of introduction	Status of the drug	Properties and efficacy against TB
Clofazimine	In 1969 for the treatment of leprosy	Approved	Reduces the treatment length for drug-resistant TB and displays immuno-modulatory properties
Statins	In 1959 for cardiovascular diseases	Phase 2 clinical trials	Anti-inflammatory and immuno-modulatory
NSAIDs	In 1969 for the treatment of rheumatoid arthritis	Phase 3 clinical trials	Anti-inflammatory and immuno-modulatory
Fluoroquinolones	In 1962 for the treatment of bacterial infection	Approved f	By inhibiting the replication and transcription of bacterial DNA
Linezolid	In 1990s for vancomycin- resistant *Enterococcus faecium* infections	Approved	Acts as a protein synthesis inhibitor
Verapamil	In 1968 for treating blood pressure	Phase 2 clinical trial	Calcium efflux blocker, which reduces the duration of TB therapy.
Metformin	In 1922 to treat diabetes	Phase 2b clinical trial	Immunomodulatory
Amoxicillin/clavulanic acid	In 1974 for treatment of bacterial infections	Phase 2 clinical trial	Prevents bacterial cell wall synthesis
Carbapenems	In 1976 to inhibit beta lactamase enzyme	Phase 2 clinical trials	Target the cell wall of *M.tb* bacteria
Sulphonamides and their derivatives	In 1956 against gram positive and gram negative bacteria	Approved	Used as combination therapy against drug resistant TB

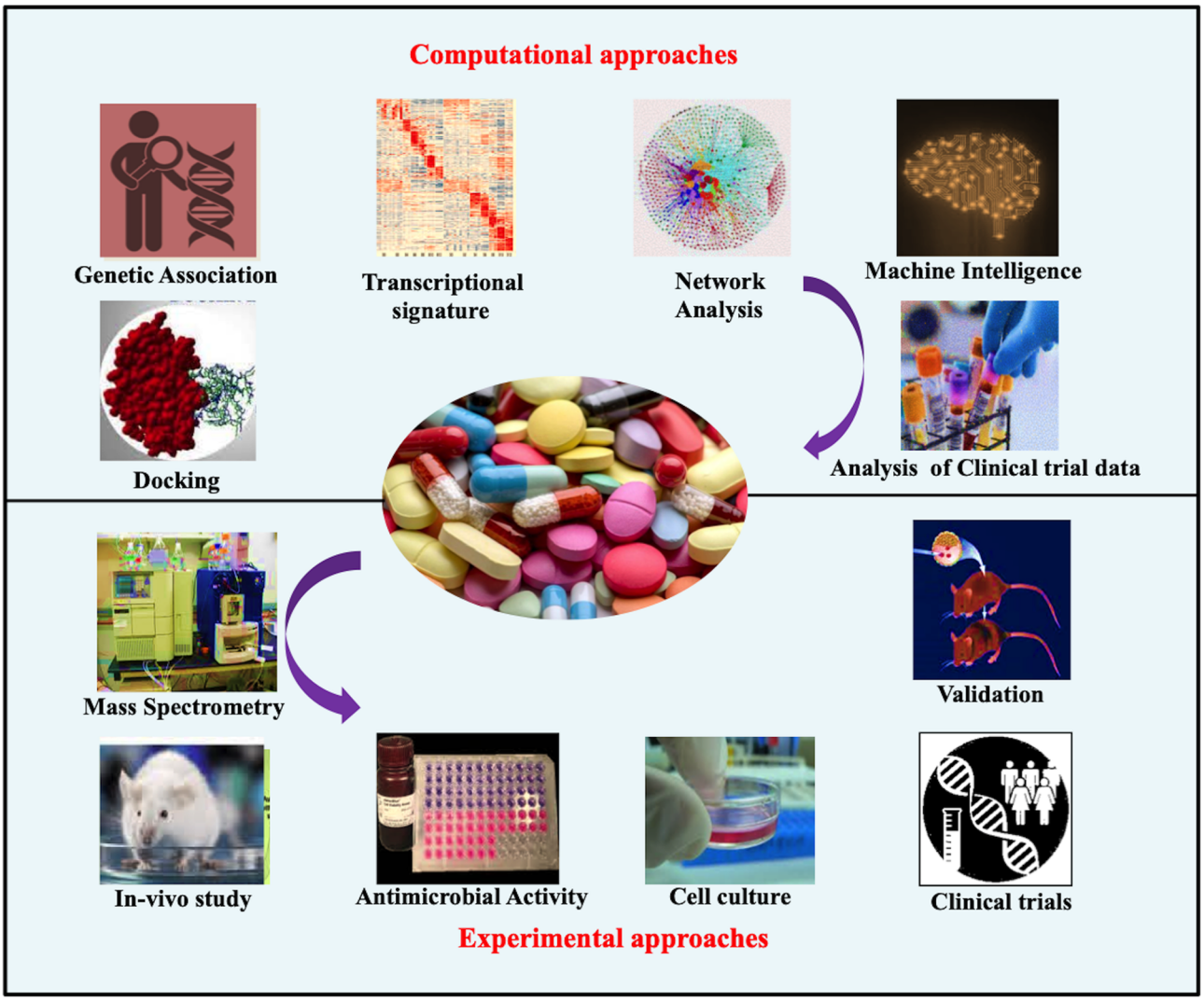

FIGURE 3 | Different computational and experimental approaches used in drug repurposing.

immunomodulatory properties as well (22). Statins prevent TB by blocking the HMG-CoA reductase thereby reducing cholesterol synthesis and accumulation. Also, statin inhibits the process of phagocytosis, which is essential for the uptake of *M.tb* inside macrophages (23). The pioneering study to establish the effect of statin on TB infection was conducted 20 years ago where it was observed that statins, in particular, fluvastatin plays an immunomodulatory role by modulating the Th1 and Th2 cytokine responses, inducing the release of pro-inflammatory cytokines, IL-1β, IL-18 and IFN-γ, and also leads to as the activation of autophagy and apoptosis (24).

Recently, Parihar et al. reported that PBMCs and monocyte-derived macrophages (MDMs) isolated from familial hypercholesterolemia patients undergoing statin therapy (for a minimum period of six months) were more resistant to *M.tb* infections then those from patients not on statin treatment (25). It was also reported that statins treatment reduced TB pathogenesis and disease severity in mice. Simvastatin treated bone marrow-derived macrophages (BMDMs) displayed significantly reduced bacterial burden compared to the untreated cells owing to increased phagocytosis and autophagy. In the mice model, treatment with statins showed a 10-fold reduction in the bacterial load in the major organs infected (spleen, liver, and lungs) as compared to the untreated mice (25). Later in the same year, it was reported that atorvastatin and simvastatin reduced mycobacterial burden up to 75 percent and showed a synergistic effect with front-line anti-TB drug rifampicin (RIF) in the murine model of TB (26, 27).

Dutta et al., in 2016 studied the effect of simvastatin with front-line anti-TB drugs as an adjuvant for its role in reducing the duration of treatment in mice model of TB (28). Simvastatin was observed to significantly increase the anti-mycobacterial

activity of first-line antibiotics while significantly reducing the time required to achieve sterile clearance in the lungs hinting at its use as an adjuvant with anti-TB treatment.

Apart from the anti-mycobacterial activity, studies have also shown the immunomodulatory activity of this drug against TB (29). Simvastatin has been shown to increase the number of natural killer (NK) T cells, induce the secretion of pro-inflammatory cytokines and enhance the expression of co-stimulatory molecules on monocytes together with an increase in autophagy and apoptosis which ultimately leads to a steady decrease in bacterial load (29). In 2019, Dutta et al. performed another study with HMG-CoA inhibitors such as pravastatin and fluvastatin along with simvastatin with first-line anti-TB drugs to evaluate their potential as adjunct agents. They concluded that of all the statins tested; pravastatin was the most potential to be used as an adjunct with the least toxicity (30). They also concluded that the addition of statins to first-line drugs reduce the duration of the therapy thereby proposing this therapy for the treatment of TB in human patients.

Recently it has been reported that during TB treatment, greater than 99% of the bacteria clear up within 3 weeks of treatment. However, less than 1% of bacteria become non-responsive to conventional antibiotics and remains in a metabolically inactive state. These non-responsive bacteria hibernate in Mesenchymal Stem Cells (MSCs), and are refractive to conventional antibiotics. Upon infection in MSCs, the *M.tb* population migrates to the cytosol where they induce lipid synthesis. Finally, *M.tb* slides into lipid droplets where they hibernate and use the host's lipids as a carbon source. A macrophage is a natural host for active TB, whereas MSCs are the host for dormant TB. *M.tb* in MSCs can be killed by inducing autophagy or by inhibition of lipid synthesis (31). Therefore, it has been shown that the addition of rapamycin or statin along with conventional antibiotics dramatically reduce the length of TB treatment eliminating both replicating and hibernating dormant bacteria, in turn reducing the possibility of generating drug resistance. The statin, pravastatin, is in Phase 2b clinical trials. Despite, more than two decades of research on the use of statins as an anti-TB agent, the initial results of clinical studies are very uncertain. However, considering the promising results in mice models, further clinical trials to investigate the effect of Statins in the treatment of TB are recommended.

NSAIDs

Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are a class of drugs that are used to treat inflammation, pain and fever (32). They reduce inflammation by inhibiting the synthesis of prostaglandins, which mediate the inflammatory process. NSAIDs target the Cyclooxygenase enzymes, COX1 and COX2, which synthesize the prostaglandins from arachidonic acid. NSAIDs were initially used as analgesic, antipyretic and anti-inflammatory drugs. However, their effect has recently been explored in cancer and neurodegenerative diseases (33). The main NSAIDs used in TB treatment in mice model are diclofenac, Ibuprofen and Aspirin, and Indomethacin. The main mechanism, through which NSAIDs work during TB treatment, is by reducing the inflammation caused by the influx of monocytes, lymphocytes and neutrophils (34). As these cells produce a high amount of prostaglandins (PGE2), which causes inflammatory effects, NSAIDs attenuate the disproportionate inflammatory response caused by migration of these cells during active TB and thus may help in the improvement in the disease outcome (35).

Diclofenac, mostly used to treat arthritis and gout, has recently been used as an antimicrobial drug. A study by Dutta et al. showed that Diclofenac treated mice displayed reduced bacterial burden and disease pathogenesis as compared to the control group (36). Diclofenac also shows a synergistic effect with Streptomycin in mice model of TB (37). Diclofenac has been known to dampen the host immune system by inhibition of Kv1.3 expression in activated macrophages and T lymphocytes. Diclofenac treatment in macrophages leads to decreased iNOS levels thereby hindering their activation (38). However, no immune study involving diclofenac has been conducted in respect to TB.

Indomethacin is COX-inhibitor, which does not differentiate between COX-1 and COX-2. Recently its use as an immunomodulator to balance the T cell phenotype during TB has come to the fore-front because of its immunosuppressive nature. Since, TB is a disease characterized by both infection and inflammation; anti-inflammatory drugs such as NSAIDs improve the disease outcome in severely ill patients. Hernandez-Pando et al. reported the use of indomethacin in regulating T cell imbalance in the granuloma during the course of the disease (39). In another study, in mice immunized with *M. vaccae*, pre-treatment with indomethacin induced better response than in non-treated animals (40).

Ibuprofen, like indomethacin, is an indiscriminating COX-inhibitor. Ibuprofen has been reported to promote survival of *M.tb* infected mice while decreasing the number and size of lung lesions because of the low bacterial burden (41). Moreover, there was reduced infiltration of neutrophils in ibuprofen treated mice as compared to the control group. As reported by Vilaplana et al., combine therapy with Ibuprofen and isoniazid reduced the neutrophilic invasion but aspirin showed the opposite effect. Therefore, they suggested the use of ibuprofen and recommended not using aspirin during TB infection (41). Another group (Byrne et al.) also confirmed the same results (42). However, Byrne et al. in yet another follow-up study confirmed that both ibuprofen and aspirin can be used along with the first-line anti-TB drugs to shorten the treatment course (43). In a study in TB patients who had already been treated by first-line anti-TB drugs, aspirin significantly lowered the serum uric acid concentrations to almost normal levels during the treatment of arthralgia (44). A few years later, Horsfall et al. showed that while treating the arthralgia patients with pyrazinamide, along with anti-arthralgia drugs (aspirin or allopurinol), the aspirin-treated group showed better disease outcome (45). The potential role of aspirin has been investigated in another randomized human trial, in the early treatment of TB meningitis along with the immune-suppressant, dexamethasone. This study established the adjunct potential of

aspirin as host-directed therapy that inhibits thromboxane-A2 to reduce new brain infarcts (46). To conclude, all studies on NSAIDs establish their potential as immuno-modulators that can be favorable if given as co-therapy during TB treatment. Their protective potential is facilitated by their anti-inflammatory properties. They work by improving the effectiveness of antibiotics and have some bactericidal potential as well.

Fluoroquinolones

Fluoroquinolones (FQs) are antibiotics, which kill the bacteria by binding to and inhibiting the function of topoisomerase II and IV enzymes. FQs penetrate into the lipid bilayer of the bacteria to exert their functions (47). WHO has recommended the use of FQs (moxifloxacin, gatifloxacin, levofloxacin), for treating MDR-TB, as second-line anti-TB drugs (48). Moreover, FQs also mediate change in the host immune responses (49). Riesbeck et al. discovered that FQs induce the secretion of IL-2 (50) in mice through the activation of transcription factor NFAT-1 (51). Some FQs, (ciprofloxacin, moxifloxacin, levofloxacin, trovafloxacin, and grepafloxacin) induce the expression of IL-2 in monocytes stimulated by LPS while preventing the expression of TNF-α (52). FQs also suppress the production of pro-inflammatory cytokines. Katsuno et al. reported that in the presence of IL-18, FQs reduce the secretion of IFN-γ (53). Recently, it has been reported that by reducing the expression of CD40, norfloxacin lowers the production of IFN-γ in Langerhans cells (54).

Similar findings have been reported in different diseases such as cancer and viral infections in the mice model (55, 56). Healthy individuals consuming ciprofloxacin and moxifloxacin display decreased IFN expression in the lymphocytes (57, 58). IL-12 is an essential cytokine for Th1 cell responses (59). It has been documented that in patients with TB, levofloxacin and inhibits IL-12 production (58).

Matsui et al. have reported that treatment with norfloxacin leads to the reduction in Th2 responses by limiting IL-4 production (54). FQs such as moxifloxacin and ciprofloxacin reduce IL-4 expression in PBMCs from healthy individuals (57). On the contrary, in diseases, FQ treatment leads to the increased production of IL-4 and IL-10 (60–65). Although FQs are known to exert anti-inflammatory functions, limited work has been done to explore their immunomodulatory properties in TB.

Linezolid

Linezolid is a synthetic antibiotic, which are used to treat several gram-positive bacterial infections. They inhibit bacterial protein synthesis by preventing the formation of the translation initiation complex (66). Linezolid is the first member of the oxazolidinone class of antibiotics, which were initially used against plant infections. A few years later, their antibacterial properties were documented (67). Due to the ineffectiveness of DOTs therapy in treating drug-resistant bacteria, WHO has recommended the use of linezolid as a potential repurposed drug to treat patients infected with MDR-TB or XDR-TB (68) after it being accepted by the US Food and Drug Administration (FDA or USFDA) for antibacterial use in 2000. Several studies demonstrate the *in-vitro* and *in-vivo* effect of linezolid in the treatment of MDR-TB both in humans and mice which proved its effectiveness in treating DR-TB (69–77); though some studies also report that it exhibits various side effects such as neurotoxicity and blood toxicity (78). Nevertheless, linezolid administration has better adherence, better efficacy and is well tolerated by the DR patients (79). A recent report states that combination therapy of bedaquiline with linezolid is safe for treatment of pregnant DR patients with no reported toxicity in the fetus (80). Therefore, in spite of the drawbacks such as neurotoxicity and blood toxicity, linezolid could be used to treat drug resistance in patients where survival is a priority. However, the dose and duration of treatment need more optimization.

Several *in-vitro* and *in-vivo* studies have established the immunomodulatory nature of linezolid. It has anti-inflammatory effects as it suppress the phagocytic ability of macrophages (THP-1) after infection with heat-killed *E. coli* (81). In the mice model of diseases, such as pneumonia and sepsis, the immunomodulatory effects of linezolid have been extensively studied and almost all studies report that linezolid reduces the damage caused due to excessive inflammation by long term production of pro-inflammatory cytokines. Moreover, it reduces the production of cytokines such as interleukin-1β (IL-1β), IL-6, IL-8, IFN-γ, and TNF-α and reduces the infiltration of neutrophils and monocytes at the infection site as demonstrated by various mice and human studies (82–87).

In a study conducted on 52 patients infected with Methicillin-resistant *Staphylococcus aureus* (MRSA), it was reported that the majority of the patients showed a significant decrease in fever in 3 days, despite being culture positive when treated with linezolid, as compared to the untreated cases, which take a week for the reduction in fever (88). This may be due to the anti-inflammatory properties of linezolid. Another report by Danin et al. studied the effect of linezolid on cytokines production in periapical tissues of teeth (89). They reported that linezolid had a different effect on proinflammatory cytokines. While IL-1ra level was decreased, IL-6, and TGF-β level remained the same. These studies establish that linezolid has significant immunomodulatory properties. However, the effect of linezolid treatment on the host immune system during TB is highly understudied.

Verapamil

Verapamil is an efflux pump inhibitor (calcium ion channel inhibitor), which is used to treat patients with high blood pressure and cardiac disorders. It inhibits the entry of calcium into the calcium channels present in the heart muscle cells and those in arteries (90). This causes relaxation of heart muscles and vasodilation. It also improves the delivery of oxygen to the heart and thus helps in treating angina patients (91). An initial study by Gupta et al. suggested that verapamil together with standard anti-TB therapy improves the bacterial clearance in *M.tb* infected mice, reduces the time of treatment and decreases the disease relapse rates to a much greater extent than in mice undergoing standard treatment, suggesting an adjunct role for verapamil in anti-TB therapy (92). Gupta et al. also suggested that administration of verapamil together with bedaquiline reduces

the bacterial load in *M.tb* infected mice and therefore calcium efflux blockers can be explored as adjuncts in TB therapy (93). After this pioneering work, many follow up studies also confirmed the protective role of verapamil in TB therapy (94–96). These studies demonstrated other mechanisms of protection conferred by verapamil such as by increasing the bioavailability of bedaquiline (94) and by disturbing the mycobacterial membrane energetics (95). It also displayed a protective adjunct effect in combination with front-line anti-TB drugs in rifampicin-resistant strains of *M.tb* (96). However, there is not much literature on the effect of verapamil on the immune system and therefore studies are needed to establish the role of verapamil as an immunomodulator in TB despite it showing promising results in TB treatment.

Metformin

Metformin is a very old drug, which is used to treat type 2 diabetes. Metformin acts by decreasing the production of glucose in the liver, minimizing the absorption of glucose and increasing its peripheral utilization. Metformin functions by AMP-activated protein kinase (AMPK) dependent and independent mechanisms. The other proposed mechanisms are by inhibiting mitochondrial respiration by blocking NADH: ubiquinone oxidoreductase (Complex I) of the mitochondrial electron transport chain or by targeting the mitochondrial glycerophosphate dehydrogenase (97). In the mitochondria, upon inhibition of Complex I, activation of 5′- adenosine monophosphate-activated protein kinase (AMPK) takes place. AMPK upon activation tries to restore the energy balance of the cell by activating catabolic pathways (energy-generating) for ATP-generation and, stopping the functioning of the anabolic mechanisms (energy-consuming). Metformin increases AMPK activation, which in turn inhibits the mammalian target of rapamycin (mTORC1), which eventually shifts the cellular state to catalytic form and leads to fast utilization of glucose to maintain the energy homeostasis in the cell (97).

Singhal et al. (98) reported the use of metformin as an adjunct therapy against TB. In THP-1 cells and human monocyte-derived macrophages (hMDMs), treatment with metformin reduced mycobacterial growth, which was AMPK dependent as cells deficient of AMPK did not show this effect (98). In the *in-vivo* mice model, metformin treatment increased the efficacy of standard anti-TB drugs and showed reduced disease pathology compared to those treated with isoniazid (INH) and ethionamide alone. Treatment with metformin enhanced the protective immune response and increased ROS production. The drug proved effective in eliminating drug-resistant bacterial strains as well by promoting efficient phagosome-lysosome fusion. In human studies, treatment with metformin improved the disease severity in both the two cohorts tested and provided better elimination of the bacteria. This study indicated that metformin could be used as adjunctive therapy for improving the effectiveness of the standard treatment course of TB.

Another recent study by the same group shed some light on the mechanism of protection conferred by metformin (99). Their work demonstrates that metformin educates $CD8^+$ T cells and enhances their anti-mycobacterial capacity as the mice infected after being adoptively transferred with metformin-treated $CD8^+$ T cells showed a significant reduction in *M.tb* load in the lungs as compared to the control mice. Also, there was a significant difference in the size of the lung between the two groups. They reported that metformin treatment has a major effect on $CD8^+$ T cells, which expand to form memory like $CD8^+CXCR3^+$ T cells in mice, which confer long-term by enhancing BCG elicited $CD8^+$ T-cell responses. These host protective $CD8^+CXCR3^+$ T cells helped in achieving better clearance of the bacteria and prevented disease reactivation in a major percentage of the population. These results were also confirmed in the human PBMCs. Therefore this study establishes the role of metformin as an agent which educates the $CD8^+$ T cell compartment to undergo metabolic programming to form memory like $CXCR3^+$ T cells which have better homing capacity and protective potential.

Other groups have also proposed the use of metformin as an adjunct therapy since, in human cohorts studies, there was a significant reduction in the mortality rate in patients receiving both metformin and DOTs treatment (100–103). Other studies also establish the role of metformin in manipulating the host immune response against TB. For instance, metformin affects the number of total neutrophils and white blood cells with an increase in the ratio of monocytes to lymphocytes in the circulation (104). Treatment with metformin leads to up-regulation of genes for ROS and causes culture conversion through the process of autophagy (98,103,https://www.frontiersin.org/articles/10.3389/fmicb.2020.00435/full#B19).

Amoxicillin/Clavulanic Acid

Back in the 1940s, it was discovered that penicillin, a β-lactam antibiotic, was non-inhibitory to *M.tb in vitro* (104). Further research revealed that *M.tb* is impervious to β-lactams *in-vitro* due to the presence of *M.tb* penicillinase, which is encoded by BlaC gene (105). Over the years, research has revealed that a β-lactam antibiotic when combined with a β-lactamase inhibitor maintains its potency (106). So, amoxicillin, an antibiotic of the beta-lactam family of antibiotics, in combination with clavulanate, a beta-lactamase inhibitor has been widely prescribed for oral administration as a broad-spectrum antibiotic for treating a variety of bacterial infections (105). Combining amoxicillin with clavulanate has widened the spectrum of usage of amoxicillin against β-lactamase-mediated resistant bacterial strains such as *M.tb* (106). The peptidoglycan cell wall synthesis requires the action of DD-transpeptidases enzymes that are basically penicillin-binding proteins (PBP). Amoxicillin works by binding to these enzymes thereby blocking the peptidoglycan cell wall synthesis, which eventually leads to bacterial death (107). Clavulanic acid has no antimicrobial activity of its own and works by stopping the bacteria from destroying amoxicillin (108).

Subsequently, a number of studies have been carried out all around the globe to understand and examine the *in vitro* efficacy of amoxicillin-clavulanate against clinical *M.tb* isolates. Owing to the absence of established baseline sensitivity breakpoints of

amoxicillin-clavulanate against *M.tb*, the ratio of MICs in these studies have been quite varied, varying from 2:1 amoxicillin to clavulanate concentration (109) in one study to almost 13:1 amoxicillin to clavulanate concentration (110) in another. However, it was established that even at the lowest oral dosage of 375mg, amoxicillin-clavulanate were concentrated in bronchial mucosa, most likely producing lung tissue levels enough to inhibit common respiratory pathogens (111). Researchers have also studied correlations between amoxicillin-clavulanate efficacies on various resistance strains of *M.tb*. Amoxicillin/clavulanic acid have been proposed in combination therapy with second-line anti-TB drugs for the treatment of DR-TB owing to its low cost and fewer side effects by WHO and is chosen to be included in group 5 antibiotics (112). Hugonnet et al. in 2009, have reported the efficient role of clavulanate against XDR-TB (113). A recent study by Diacon et al. reported the combination use of amoxicillin/clavulanic acid with carbapenems, which led to the reduction in *M.tb* burden (114). Despite the successful use of this beta-lactam antibiotic in TB, there is not much work on its effect on the immune system or on its immunomodulatory properties. Therefore, this area needs further research to successfully exploit this antibiotic as a repurposed drug against TB.

Carbapenems

Carbapenems are beta-lactam antibiotics, which are unique in being impervious to being hydrolyzed by most beta-lactamases and being able to inhibit the PBP enzymes. The first beta-lactam antibiotic was isolated from *Streptomyces clavuligerus* followed by the development of clavulanic acid and thienamycin (115, 116). Thienamycin is considered the parent carbapenem, which has been modified to form all the subsequently discovered carbapenems. Of all beta-lactams synthesized, carbapenems have the broadest range of activity against both gram-positive and gram-negative bacteria making them "the drugs of last resort" (117, 118). To improve stability, thienamycin was chemically modified over time into other more stable derivatives such as imipenem (119). Later, more stable derivatives with a broader spectrum such as biapenem, meropenem, doripenem, and ertapenem were synthesized (120–125). Carbapenems target the PBP enzymes inhibiting peptidoglycan synthesis *via* crosslinking. Eventually, the bacterial wall weakens, leading to the death of the bacteria due to high osmotic pressure. Imipenem and panipenem act better against gram-positive bacteria whereas biapenem, meropenem and doripenem kill gram-negative bacteria efficiently (126–129). A combination study by Hugonnet et al., 2009 states that meropenem together with clavulanic acid kills MDR *M.tb* efficiently (113). Another study by Tiberi et al. reported that carbapenems when given intravenously are extremely helpful in treating XDR-TB strains (130). Veziris et al., in 2011 had previously shown that even though less efficient compared to INH, treatment with a combination of carbapenem together with clavulanate in *M.tb* infected mice increased the survival of the mice while reducing the bacterial load (131). As discussed earlier, a combination of amoxicillin/clavulanic acid and carbapenem has been studied in clinical trials and seem very promising for the treatment of DR-TB (114). In spite of being proposed as an adjunct therapy for DR-TB, there is almost no information on the effect of these drugs on the immune system and needs serious research.

Sulfonamides and their Derivatives

The sulfonamides or sulfa drugs are wide spectrum bacteriostatic antibiotics, which work against most gram-positive and gram-negative bacteria. Sulfonamides and their derivatives were used from the 1930s up to the 1950s as a monotherapy (132) but were later discontinued due to their low efficacy compared to INH and streptomycin and high toxicity (133). It was believed that *M.tb* is resistant to trimethoprim-sulfamethoxazole (TMP-SMX). However, in 2009 a study in humans reported that the use of TMP-SMX on immuno-compromised patients provided better outcome and the drug worked on the *M.tb* strains isolated from the same infected patients (134). In another study conducted in 2014 in HIV-TB co-infected patients, who were being treated with TMP-SMX in order to protect them from *Pneumocystis jirovecii* infection, TMP-SMX proved to be quite effective in preventing TB (135). In another study conducted in Nigeria, in patients co-infected with HIV and MDR-TB, the time required for sputum conversion reduced significantly upon administration of TMP-SMX (136). Sulfadiazine, a sulfa drug used for the treatment of leprosy has been repurposed to treat DR-TB and proved to be more effective and safe than other sulfa drugs for the treatment of TB (137, 138). These drugs can be tested further to be included in TB treatment through more research using random human cohorts as subjects. Regarding the way they affect the immune system, this area needs more extensive study, as there are very limited information available which may establish their role as immunomodulators.

CONCLUSIONS AND FUTURE PERSPECTIVE

Drug repurposing is indisputably a smart strategy to develop a new treatment regime for TB within a short period of time and also to treat drug-resistant pathogens. Some of the repurposed drugs have shown great promise for future treatment of TB and have been extensively studied. However, we still need to repurpose as many drugs as we can through various approaches such as computational and experimental biology to explore the potential of already existing thousands of drugs in order to minimize the time for novel drug discovery as the incidence of resistance in the *M.tb* population is occurring at a very fast pace and we urgently need a new improved treatment regime. Such studies should be organized in the human cohorts. As the influence of the host-protective immune system continues to gain attention in the advancement of host-directed therapies so we should also aim to study how each of the repurposed drugs affects the balance of the host immune system and deals with infection and inflammation. This would enable better designing of combination therapies that would help achieve the goal of TB eradication program by shortening of the treatment regime and preventing drug resistance while being cost-effective for the populations.

AUTHOR CONTRIBUTIONS

SF wrote the manuscript. SF, AB, and VD edited the manuscript. AB and VD conceived of the hypothesis. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Cohen A, Mathiasen VD, Schon T, Wejse C. The global prevalence of latent tuberculosis: a systematic review and meta-analysis. *Eur Respir J* (2019) 54 (3):1900655. doi: 10.2139/ssrn.3289796
2. World Health Organization. *Global Tuberculosis Report 2019*. (2019). Available at: www.who.int/TB/Data.
3. Tousif S, Singh DK, Ahmad S, Moodley P, Bhattacharyya M, Van Kaer L, et al. Isoniazid induces apoptosis of activated CD4+ T cells: implications for post-therapy tuberculosis reactivation and reinfection. *J Biol Chem* (2014) 289(44):30190–5. doi: 10.1074/jbc.C114.598946
4. Field SK. Bedaquiline for the treatment of multidrug-resistant tuberculosis: great promise or disappointment? *Ther Adv Chronic Dis* (2015) 6(4):170–84. doi: 10.1177/2040622315582325
5. Conradie F, Diacon AH, Ngubane N, Howell P, Everitt D, Crook AM, et al. Treatment of Highly Drug-Resistant Pulmonary Tuberculosis. *N Engl J Med* (2020) 382(10):893–902. doi: 10.1056/NEJMoa1901814
6. Pushpakom S, Iorio F, Eyers PA, Escott KJ, Hopper S, Wells A, et al. Drug repurposing: progress, challenges and recommendations. *Nat Rev Drug Discovery* (2019) 18(1):41–58. doi: 10.1038/nrd.2018.168
7. Moreland RB, Goldstein II, Kim NN, Traish A. Sildenafil Citrate, a Selective Phosphodiesterase Type 5 Inhibitor: Trends Endocrinol Metab. *Trends Endocrinol Metabol* (1999) 10(3):97–104. doi: 10.1016/s1043-2760(98) 00127-1
8. Singhal S, Mehta J, Desikan R, Ayers D, Roberson P, Eddlemon P, et al. Antitumor activity of thalidomide in refractory multiple myeloma. *N Engl J Med* (1999) 341(21):1565–71. doi: 10.1056/NEJM199911183412102. Erratum in: N Engl J Med 2000 Feb 3;342(5):364. PMID: 10564685.
9. Fatima S, Dwivedi VP. Revisiting Host-Directed Adjunct Therapies in Tuberculosis. *J Bacteriol Mycol* (2020) 7(4):1139. doi: 10.1093/cid/civ027
10. Domingo-Gonzalez R, Prince O, Cooper A, Khader SA. Cytokines and chemokines in *Mycobacterium tuberculosis* infection. *Microbiol Spectr* (2016) 4:TBTB2–0018-2016. doi: 10.1128/microbiolspec.TBTB2-0018-2016
11. Van Deun A, Maug AK, Salim MA, Das PK, Sarker MR, Daru P, et al. Short, highly effective, and inexpensive standardized treatment of multidrug-resistant tuberculosis. *Am J Respir Crit Care Med* (2010) 182(5):684–92. doi: 10.1164/rccm.201001-0077OC
12. Yano T, Kassovska-Bratinova S, Teh JS, Winkler J, Sullivan K, Isaacs A, et al. Reduction of clofazimine by mycobacterial type 2 NADH:quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species. *J Biol Chem* (2011) 286(12):10276–87. doi: 10.1074/jbc.M110.200501
13. Lechartier B, Cole ST. Mode of Action of Clofazimine and Combination Therapy with Benzothiazinones against Mycobacterium tuberculosis. *Antimicrob Agents Chemother* (2015) 59(8):4457–63. doi: 10.1128/ AAC.00395-15
14. Steel HC, Matlola NM, Anderson R. Inhibition of potassium transport and growth of mycobacteria exposed to clofazimine and B669 is associated with a calcium-independent increase in microbial phospholipase A2 activity. *J Antimicrob Chemother* (1999) 44(2):209–16. doi: 10.1093/jac/44.2.209
15. Cholo MC, Boshoff HI, Steel HC, Cockeran R, Matlola NM, Downing KJ, et al. Effects of clofazimine on potassium uptake by a Trk-deletion mutant of Mycobacterium tuberculosis. *J Antimicrob Chemother* (2006) 57(1):79–84. doi: 10.1093/jac/dki409
16. Bopape MC, Steel HC, Cockeran R, Matlola NM, Fourie PB, Anderson R. Antimicrobial activity of clofazimine is not dependent on mycobacterial C-type phospholipases. *J Antimicrob Chemother* (2004) 53(6):971–4. doi: 10.1093/jac/dkh215
17. Tang S, Yao L, Hao X, Liu Y, Zeng L, Liu G, et alClofazimine for the treatment of multidrug-resistant tuberculosis: prospective, multicenter, randomized controlled study in China. *Clin Infect Dis* (2015) 60:1361–7. doi: 10.1093/cid/civ027
18. Singh DK, Dwivedi VP, Ranganathan A, Bishai WR, Van Kaer L, Das G. Blockade of the Kv1.3 K+ Channel Enhances BCG Vaccine Efficacy by Expanding Central Memory T Lymphocytes. *J Infect Dis* (2016) 214:1456–64. doi: 10.1093/infdis/jiw395
19. Ahmad S, Bhattacharya D, Gupta N, Rawat V, Tousif S, Van Kaer L, et al. Clofazimine enhances the efficacy of BCG revaccination via stem cell-like memory T cells. *PloS Pathog* (2020) 16(5):e1008356. doi: 10.1371/ journal.ppat.1008356
20. Tonaco MM, Moreira JD, Nunes FFC, Loures CMG, Souza LR, Martins JM, et al. Evaluation of profile and functionality of memory T cells in pulmonary tuberculosis. *Immunol Lett* (2017) 192:52–60. doi: 10.1016/j.imlet. 2017.10.014
21. Banerjee D, Bhattacharyya R. Statin therapy may prevent development of tuberculosis in diabetic state. *Med Hypotheses* (2014) 83:88–91. doi: 10.1016/ j.mehy.2014.04.002
22. Alffenaar JC, Akkerman OW, van Hest R. Statin Adjunctive Therapy for Tuberculosis Treatment. *Antimicrob Agents Chemother* (2016) 60:7004. doi: 10.1128/AAC.01836-16
23. Loike JD, Shabtai DY, Neuhut R, Malitzky S, Lu E, Husemann J, et al. Statin inhibition of Fc receptor-mediated phagocytosis by macrophages is modulated by cell activation and cholesterol. *Arterioscler Thromb Vasc Biol* (2004) 24:2051–6. doi: 10.1161/01.ATV.0000143858.15909.29
24. Montero MT, Hernández O, Suárez Y, Matilla J, Ferruelo AJ, Martínez-Botas J, et al. Hydroxymethylglutaryl-coenzyme A reductase inhibition stimulates caspase-1 activity and Th1-cytokine release in peripheral blood mononuclear cells. *Atherosclerosis* (2000) 153:303–13. doi: 10.1016/s0021-9150(00)00417-2
25. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdayal R, Mhlanga MM, et al. Statin therapy reduces the mycobacterium tuberculosis burden in human macrophages and in mice by enhancing autophagy and phagosome maturation. *J Infect Dis* (2014) 209:743–63. doi: 10.1093/infdis/jit550
26. Lobato LS, Rosa PS, Ferreira Jda S, Neumann Ada S, da Silva MG, do Nascimento DC, et al. Statins increase rifampin mycobactericidal effect. *Antimicrob Agents Chemother* (2014) 58:5766–74. doi: 10.1128/AAC.01826-13
27. Skerry C, Pinn ML, Bruiners N, Pine R, Gennaro ML, Karakousis PC. Simvastatin increases the in vivo activity of the first-line tuberculosis regimen. *J Antimicrob Chemother* (2014) 69:2453–7. doi: 10.1093/jac/ dku166

28. Dutta NK, Bruiners N, Pinn ML, Zimmerman MD, Prideaux B, Dartois V, et al. Statin adjunctive therapy shortens the duration of TB treatment in mice. *J Antimicrob Chemother* (2016) 71(6):1570–7. doi: 10.1093/jac/dkw014
29. Guerra-De-Blas PDC, Bobadilla-Del-Valle M, Sada-Ovalle I, et al. Simvastatin enhances the immune response against Mycobacterium tuberculosis(2019). (Accessed February 27, 2020).
30. Dutta NK, Bruiners N, Zimmerman MD, Tan S, Dartois V, Gennaro ML, et al. Adjunctive host-directed therapy with statins improves tuberculosis-related outcomes in mice. *J Infect Dis* (2019) pii:jiz517. doi: 10.1093/infdis/jiz517
31. Fatima S, Kamble SS, Dwivedi VP, Bhattacharya D, Kumar S, Ranganathan A, et al. Mycobacterium tuberculosis programs mesenchymal stem cells to establish dormancy and persistence. *J Clin Invest* (2020) 130(2):655–61. doi: 10.1172/JCI128043
32. Ivanyi J, Zumla A. Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *J Infect Dis* (2013) 208(2):185–8. doi: 10.1093/infdis/jit153
33. Klegeris A, McGeer PL. Non-steroidal anti-inflammatory drugs (NSAIDs) and other anti-inflammatory agents in the treatment of neurodegenerative disease. *Curr Alzheimer Res* (2005) 2(3):355–65. doi: 10.2174/1567205054367883
34. Dheda K, Booth H, Huggett JF, Johnson MA, Zumla A, Rook GA. Lung remodeling in pulmonary tuberculosis. *J Infect Dis* (2005) 192:1201–9. doi: 10.1086/444545
35. Marzo E, Vilaplana C, Tapia G, Diaz J, Garcia V, Cardona PJ. Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tuberc (Edinb)* (2014) 94(1):55–64. doi: 10.1016/j.tube.2013.09.004
36. Dutta NK, Kumar KA, Mazumdar K, Dastidar SG. In vitro and in vivo anti-mycobacterial activity of antiinflammatory drug, diclofenac sodium. *Indian J Exp Biol* (2004) 42(9):922–7.
37. Dutta NK, Mazumdar K, Dastidar SG, Park JH. Activity of diclofenac used alone and in combination with streptomycin against Mycobacterium tuberculosis in mice. *Int J Antimicrob Agents* (2007) 30(4):336–40. doi: 10.1016/j.ijantimicag.2007.04.016
38. Villalonga N, David M, Bielańska J, González T, Parra D, Soler C, et al. Immunomodulatory effects of diclofenac in leukocytes through the targeting of Kv1.3 voltage-dependent potassium channels. *Biochem Pharmacol* (2010) 80(6):858–66. doi: 10.1016/j.bcp.2010.05.012
39. Hernandez-Pando R, Orozco H, Mancilla R. T-cell lung granulomas induced by sepharose-coupled Mycobacterium tuberculosis protein antigens: immunosuppressive phenomena reversed with cyclophosphamide and indomethacin. *Immunology* (1995) 86(4):506–11.
40. Shroff KE, Sainis KB, Sengupta SR, Kamat RS. Role of antigen-presenting cells in variation in immunogenicity of mycobacteria. *Clin Exp Immunol* (1990) 79(2):285–90. doi: 10.1111/j.1365-2249.1990.tb05192.x
41. Vilaplana C, Marzo E, Tapia G, Diaz J, Garcia V, Cardona PJ. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J Infect Dis* (2013) 208(2):199–202. doi: 10.1093/infdis/jit152
42. Byrne ST, Denkin SM, Zhang Y. Aspirin antagonism in isoniazid treatment of tuberculosis in mice. *Antimicrob Agents Chemother* (2007) 51(2):794–5. doi: 10.1128/AAC.01145-06
43. Byrne ST, Denkin SM, Zhang Y. Aspirin and ibuprofen enhance pyrazinamide treatment of murine tuberculosis. *J Antimicrob Chemother* (2007) 59(2):313–6. doi: 10.1093/jac/dkl486
44. Petty TL, Dalrymple GV. Inhibition of pyrazinamide hyperuricemia by small doses of acetylsalicylic acid. *Ann Intern Med* (1964) 60:898–900. doi: 10.7326/0003-4819-60-5-898
45. Horsfall PA, Plummer J, Allan WG, Girling DJ, Nunn AJ, Fox W. Double blind controlled comparison of aspirin, allopurinol and placebo in the management of arthralgia during pyrazinamide administration. *Tubercle* (1979) 60(1):13–24. doi: 10.1016/0041-3879(79)90051-5
46. Mai NT, Dobbs N, Phu NH, Colas RA, Thao LT, Thuong NT, et al. A randomised double blind placebo controlled phase 2 trial of adjunctive aspirin for tuberculous meningitis in HIV-uninfected adults. *eLife* (2018) 7:e33478. doi: 10.7554/eLife.33478
47. Pranger AD, van der Werf TS, Kosterink JGW, Alffenaar JWC. The Role of Fluoroquinolones in the Treatment of Tuberculosis in 2019. *Drugs* (2019) 79(2):161–71. doi: 10.1007/s40265-018-1043-y
48. Falzon D, Schűnemann HJ, Harausz E, González-Angulo L, Lienhardt C, Jaramillo E, et al. World Health Organization treatment guidelines for drug-resistant tuberculosis, 2016 update. *Eur Respir J* (2017) 49(3):1602308. doi: 10.1183/13993003.02308-2016
49. Dalhoff A. Immunomodulatory activities of fluoroquinolones. *Infection* (2005) 2:55–70. doi: 10.1007/s15010-005-8209-8
50. Riesbeck K, Bredberg A, Forsgren A. Ciprofloxacin enhances the synthesis of interleukin-2 in human lymphocytes, while other cytokines are inhibited. *Eur J Clin Microbiol Infect Dis* (1991) 1991:387–88. doi: 10.1007/BF01979471
51. Riesbeck K, Forsgren A. Increased interleukin 2 transcription in murine lymphocytes by ciprofloxacin. *Immunopharmacology* (1994) 2:155–64. doi: 10.1016/0162-3109(94)90050-7
52. Dalhoff A, Shalit I. Immunomodulatory effects of quinolones. *Lancet Infect Dis* (2003) 6:359–71. doi: 10.1016/S1473-3099(03)00658-3
53. Katsuno G, Takahashi HK, Iwagaki H, Mizuno K, Yagi T, Mori S, et al. The immunosuppressive effects of ciprofloxacin during human mixed lymphocyte reaction. *Clin Immunol* (2006) 1:110–19. doi: 10.1016/j.clim.2005.12.004
54. Matsui K, Kashima A, Motegi A. Norfloxacin, a fluoroquinolone antibiotic, inhibits Langerhans cell-mediated Th1 and Th2 cell development. *J Pharm Pharm Sci* (2019) 1:122–30. doi: 10.18433/jpps30335
55. Xue H, Field C, Sawyer M, Dieleman L, Baracos V. Prophylactic ciprofloxacin treatment prevented high mortality, and modified systemic and intestinal immune function in tumour-bearing rats receiving dose-intensive CPT-11 chemotherapy. *Br J Cancer* (2009) 10:1581. doi: 10.1038/sj.bjc.6605051
56. Enoki Y, Ishima Y, Tanaka R, Sato K, Kimachi K, Shirai T, et al. Pleiotropic effects of levofloxacin, fluoroquinolone antibiotics, against influenza virus-induced lung injury. *PloS One* (2015) 6:e0130248. doi: 10.1371/journal.pone.0130248
57. Williams AC, Galley HF, Watt AM, Webster NR. Differential effects of three antibiotics on T helper cell cytokine expression. *J Antimicrob Chemother* (2005) 3:502–06. doi: 10.1093/jac/dki251
58. Serebryakova V, Urazova O, Novitsky V, Vengerovskii A, Kononova T. In vitro study of the modulatory effects of levofloxacin and BCG on secretion of proinflammatory cytokines in infiltrative pulmonary tuberculosis. *Bull Exp Biol Med* (2018) 2:225–28. doi: 10.1007/s10517-018-4319-z
59. Wojno ED, Hunter CA, Stumhofer JS. The immunobiology of the interleukin-12 family: room for discovery. *Immunity* (2019) 50(4):851–70. doi: 10.1016/j.immuni.2019.03.011
60. Loron G, Olivier P, See H, Le Sache N, Angulo L, Biran V, et al. Ciprofloxacin prevents myelination delay in neonatal rats subjected to E. coli sepsis. *Ann Neurol* (2011) 2:341–51. doi: 10.1002/ana.22190
61. Blasi F, Tarsia P, Mantero M, Morlacchi LC, Piffer F. Cefditoren versus levofloxacin in patients with exacerbations of chronic bronchitis: serum inflammatory biomarkers, clinical efficacy, and microbiological eradication. *Ther Clin Risk Manage* (2013) 9:55. doi: 10.2147/TCRM.S41131
62. Saini H, Chhibber S, Harjai K. Azithromycin and ciprofloxacin: a possible synergistic combination against Pseudomonas aeruginosa biofilm-associated urinary tract infections. *Int J Antimicrob Agents* (2015) 4:359–67. doi: 10.1016/j.ijantimicag.2014.11.008
63. Gupta PV, Nirwane AM, Belubbi T, Nagarsenker MS. Pulmonary delivery of synergistic combination of fluoroquinolone antibiotic complemented with proteolytic enzyme: A novel antimicrobial and antibiofilm strategy. *Nanomed Nanotechnol Biol Med* (2017) 7:2371–84. doi: 10.1016/j.nano.2017.06.011
64. Jantova S, Paulovičova E, Paulovičova L, Janoškova M, Panik M, Milata V. Immunobiologicalefficacy and immunotoxicity of novel synthetically prepared fluoroquinolone ethyl 6-fluoro-8-nitro-4-oxo-1, 4-dihydroquinoline-3-carboxylate. *Immunobiology* (2018) 1:81–93. doi: 10.1016/j.imbio.2017.10.008
65. Badari MS, El-Fatah SGA, Kamel SI, Mohamed A. Immunomodulatory action of levofloxacin on cytokine production in adults with community-acquired pneumonia. *Med J Cairo Univ* (2014) 82(2):127–32.
66. Batts DH. Linezolid-a new option for treating Gram-positive infections. *Oncology* (2000) 14(8 Suppl 6):23–9.

67. Ford CW, Zurenko GE, Barbachyn MR. The discovery of linezolid, the first oxazolidinone antibacterial agent. *Curr Drug Targets Infect Disord* (2001) 1 (2):181–99. doi: 10.2174/1568005014606099
68. Zhang X, Falagas ME, Vardakas KZ, Wang R, Qin R, Wang J, et al. Systematic review and meta-analysis of the efficacy and safety of therapy with linezolid containing regimens in the treatment of multidrug-resistant and extensively drug-resistant tuberculosis. *J Thorac Dis* (2015) 7(4):603–15. doi: 10.3978/j.issn.2072-1439.2015.03.10
69. Agyeman AA, Ofori-Asenso R. Efficacy and safety profile of linezolid in the treatment of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis: a systematic review and meta-analysis. *Ann Clin Microbiol Antimicrob* (2016) 15(1):41. doi: 10.1186/s12941-016-0156-y
70. Maartens G, Benson CA. Linezolid for treating tuberculosis: a delicate balancing act. *EBioMedicine* (2015) 2(11):1568–9. doi: 10.1016/j.ebiom.2015.10.014
71. Ramírez-Lapausa M, Pascual Pareja JF, Carrillo Gómez R, Martínez-Prieto M, González-Ruano Pérez P, Noguerado Asensio A, et al. Retrospective study of tolerability and efficacy of linezolid in patients with multidrug-resistant tuberculosis (1998–2014). *Enferm Infecc Microbiol Clin* (2016) 34 (2):85–90. doi: 10.1016/j.eimc.2015.04.003
72. Bhuniya S, Mohapatra PR, Panigrahi MK, Behera P, Pradhan G, et al. Linezolid in drug-resistant tuberculosis: haste makes waste. *Eur Respir J* (2015) 46(6):1843–4. doi: 10.1183/13993003.01162-2015
73. Wasserman S, Meintjes G, Maartens G. Linezolid in the treatment of drug-resistant tuberculosis: the challenge of its narrow therapeutic index. *Expert Rev Anti Infect Ther* (2016) 14(10):901–15. doi: 10.1080/14787210.2016.1225498
74. Lee M, Cho SN, Barry CE 3rd, Song T, Kim Y, Jeong I, et al. Linezolid for XDR-TB – final study outcomes. *N Engl J Med* (2015) 373(3):290–1. doi: 10.1056/NEJMc1500286
75. Sotgiu G, Pontali E, Migliori GB. Linezolid to treat MDR-/XDR-tuberculosis: available evidence and future scenarios. *Eur Respir J* (2015) 45(1):25–9. doi: 10.1183/09031936.00145014
76. Bolhuis MS, Tiberi S, Sotgiu G, De Lorenzo S, Kosterink JG, van der Werf TS, et al. Linezolid tolerability in multidrug-resistant tuberculosis: a retrospective study. *Eur Respir J* (2015) 46(4):1205–7. doi: 10.1183/13993003.00606-2015
77. Liu Y, Bao P, Wang D, Li Y, Tang L, Zhou Y, et al. Clinical outcomes of linezolid treatment for extensively drug-resistant tuberculosis in Beijing, China: a hospital-based retrospective study. *Jpn J Infect Dis* (2015) 68 (3):244–7. doi: 10.7883/yoken.JJID.2014.222
78. Tang S, Yao L, Hao X, Zhang X, Liu G, Liu X, et al. Efficacy, safety and tolerability of linezolid for the treatment of XDR-TB: a study in China. *Eur Respir J* (2015) 45:161–70. doi: 10.1183/09031936.00035114
79. Schecter GF, Scott C, True L, Raftery A, Flood J, Mase S. Linezolid in the treatment of multidrug-resistant tuberculosis. *Clin Infect Dis* (2010) 50:49–55. doi: 10.1086/648675
80. Jaspard M, Elefant-Amoura E, Melonio I, De Montgolfier I, Veziris N, Caumes E. Bedaquiline and linezolid for extensively drug-resistant tuberculosis in pregnant woman. *Emerg Infect Dis* (2017) 23:1731–2. doi: 10.3201/eid2310.161398
81. Ballesta S, Pascual A, Garcia I, Perea EJ. Effect of linezolid on the phagocytic functions of human polymorphonuclear leukocytes. *Chemotherapy* (2003) 49:163–6. doi: 10.1159/000071139
82. Franks Z, Campbell RA, Vieira de Abreu A, Holloway JT, Marvin JE, Kraemer BF, et al. Methicillin-resistant Staphylococcus aureusinduced thrombo-inflammatory response is reduced with timely antibiotic administration. *Thromb Haemost* (2013) 109:684–95. doi: 10.1160/TH12-08-0543
83. Pichereau S, Moran JJ, Hayney MS, Shukla SK, Sakoulas G, Rose WE. Concentration-dependent effects of antimicrobials on Staphylococcus aureus toxin-mediated cytokine production from peripheral blood mononuclear cells. *J Antimicrob Chemother* (2012) 67:123–9. doi: 10.1093/jac/dkr417
84. Lambers C, Burian B, Binder P, Ankersmit HJ, Wagner C, Muller M, et al. Early immunomodulatory effects of linezolid in a human whole blood endotoxin model. *Int J Clin Pharmacol Ther* (2010) 48:419–24. doi: 10.5414/CPP48419
85. Takahashi G, Sato N, Yaegashi Y, Kojika M, Matsumoto N, Kikkawa T, et al. Effect of linezolid on cytokine production capacity and plasma endotoxin levels in response to lipopolysaccharide stimulation of whole blood. *J Infect Chemother* (2010) 16:94–9. doi: 10.1007/s10156-009-0012-5
86. Kaku N, Morinaga Y, Takeda K, Kosai K, Uno N, Hasegawa H, et al. Antimicrobial and immunomodulatory effects of tedizolid against methicillin-resistant Staphylococcus aureus in a murine model of hematogenous pulmonary infection. *Int J Med Microbiol* (2016) 306:421–8. doi: 10.1016/j.ijmm.2016.05.010
87. Jacqueline C, Broquet A, Roquilly A, Davieau M, Caillon J, Altare F, et al. Linezolid dampens neutrophil-mediated inflammation in methicillin-resistant Staphylococcus aureus-induced pneumonia and protects the lung of associated damages. *J Infect Dis* (2014) 210:814–23. doi: 10.1093/infdis/jiu145
88. Yoshizawa S, Tateda K, Saga T, Ishii Y, Yamaguchi K. Virulence-suppressing effects of linezolid on methicillin-resistant Staphylococcus aureus: possible contribution to early defervescence. *Antimicrob Agents Chemother* (2012) 56:1744–8. doi: 10.1128/AAC.05430-11
89. Danin J, Linder L, Lundqvist G, Wretlind B. Cytokines in periradicular lesions: the effect of linezolid treatment. *Surg Med Pathol Radiol Endod* (2003) 96:492–8. doi: 10.1016/S1079-2104(03)00059-3
90. Angus JA, Brazenor RM, Le Duc MA. Verapamil: a selective antagonist of constrictor substances in dog coronary artery: implications for variant angina. *Clin Exp Pharmacol Physiol Suppl* (1982) 6:15–28.
91. Hangai-Hoger N, Tsai AG, Friesenecker B, Cabrales P, Intaglietta M. Microvascular oxygen delivery and consumption following treatment with verapamil. *Am J Physiol Heart Circ Physiol* (2005) 288(4):H1515–20. doi: 10.1152/ajpheart.00955.2004
92. Gupta S, Tyagi S, Almeida DV, Maiga MC, Ammerman NC, Bishai WR. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. *Am J Respir Crit Care Med* (2013) 188(5):600–7. doi: 10.1164/rccm.201304-0650OC
93. Gupta S, Cohen KA, Winglee K, Maiga M, Diarra B, Bishai WR, et al. Efflux inhibition with verapamil potentiates bedaquiline in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* (2014) 58(1):574–6. doi: 10.1128/aac.01462-13
94. Xu J, Tasneen R, Peloquin CA, Almeida DV, Li SY, Barnes-Boyle K, et al. Verapamil Increases the Bioavailability and Efficacy of Bedaquiline but Not Clofazimine in a Murine Model of Tuberculosis. *Antimicrob Agents Chemother* (2017) 62(1):e01692–17. doi: 10.1128/AAC.01692-17. Published 2017 Dec 21.
95. Chen C, Gardete S, Jansen RS, Shetty A, Dick T, Rhee KY, et al. Verapamil Targets Membrane Energetics in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* (2018) 62(5):e02107–17. doi: 10.1128/AAC.02107-17
96. Caleffi-Ferracioli KR, Cardoso RF, de Souza JV, Murase LS, Canezin PH, Scodro RB, et al. Modulatory effects of verapamil in rifampicin activity against Mycobacterium tuberculosis. *Future Microbiol* (2019) 14:185–94. doi: 10.2217/fmb-2018-0277
97. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia* (2017) 60(9):1577–85. doi: 10.1007/s00125-017-4342-z
98. Singhal A, Jie L, Kumar P, Hong GS, Leow MK, Paleja B, et al. Metformin as adjunct antituberculosis therapy. *Sci Transl Med* (2014) 6(263):263ra159. doi: 10.1126/scitranslmed.3009885
99. Böhme J, Martinez N, Li S, Lee A, Marzuki M, Tizazu AM, et al. Metformin enhances anti-mycobacterial responses by educating CD8+ T-cell immunometabolic circuits. *Nat Commun* (2020) 11(1):5225. doi: 10.1038/s41467-020-19095-z
100. Lee YJ, Han SK, Park JH, Lee JK, Kim DK, Chung HS, et al. The effect of metformin on culture conversion in tuberculosis patients with diabetes mellitus. *Kor J Intern Med* (2018) 33:933–40. doi: 10.3904/kjim.2017.249
101. Degner NR, Wang J-Y, Golub JE, Karakousis PC. The effect of diabetes and comorbidities on tuberculosis treatment outcomes. A62. Treatment of tuberculosis. *Am Thorac Soc* (2017) 195:A7587. doi: abs/10.1164
102. Degner NR, Wang JY, Golub JE, Karakousis PC. Metformin use reverses the increased mortality associated with diabetes mellitus during tuberculosis treatment. *Clin Infect Dis* (2018) 66:198–205. doi: 10.1093/cid/cix819

103. Novita BD, Ali M, Pranoto A, Soediono EI, Mertaniasih NM. Metformin induced autophagy in diabetes mellitus – tuberculosis co-infection patients: a case study. *Indian J Tuberc* (2019) 66:64–9. doi: 10.1016/j.ijtb.2018.04.003
104. Abraham EP, Chain E, Fletcher CM, Florey HW, Gardner AD, Heatley NG, et al. Further observations on penicillin. *Lancet* (1941) 238(6155):177–89. doi: 10.1016/S0140-6736(00)72122-2
105. Kurz SG, Wolff KA, Hazra S, Bethel CR, Hujer AM, Smith KM, et al. Can inhibitor resistant substitutions in the Mycobacterium tuberculosis β-lactamase BlaC lead to clavulanate resistance? A biochemical rationale for the use of β-lactam β-lactamase inhibitor combinations. *Antimicrob Agents Chemother* (2013) 57(12):6085–96. doi: 10.1128/AAC.01253-13
106. Janez K, Jerneja O. A new amoxicillin/clavulanate therapeutic system: Preparation in vitro and pharmacokinetic evaluation. *Int J Pharm* (2007) 335:106–13. doi: 10.1016/j.ijpharm.2006.11.007
107. Brogden RN, Carmine A, Heel RC, Morley PA, Speight TM, Avery GS. Amoxycillin/clavulanic acid: a review of its antibacterial activity, pharmacokinetics and therapeutic use. *Drugs* (1981) 22(5):337–62. doi: 10.2165/00003495-198122050-00001
108. Wise R, Andrews JM, Bedford KA. In vitro study of clavulanic acid in combination with penicillin, amoxycillin, and carbenicillin. *Antimicrob Agents Chemother* (1978) 13(3):389–93. doi: 10.1128/AAC.13.3.389
109. Nadler JP, Berger J, Nord JA, Cofsky R, Saxena M. Amoxicillin-clavulanic acid for treating drug-resistant Mycobacterium tuberculosis. *Chest* (1991) 99 (4):1025–6. doi: 10.1378/chest.99.4.1025
110. Zhang D, Wang Y, Lu J, Pang Y. In vitro activity of beta-lactams in combination with beta-lactamase inhibitors against multidrug-resistant Mycobacterium tuberculosis isolates. *Antimicrob Agents Chemother* (2016) 60(1):393–9. doi: 10.1128/AAC.01035-15
111. Gould IM, Harvey G, Golder D, Reid TM, Watt SJ, Friend JA, et al. Penetration of amoxycillin/clavulanic acid into bronchial mucosa with different dosing regimens. *Thorax* (1994) 49:999–1001. doi: 10.1136/thx.49.10.999
112. Cassir N, Rolain JM, Brouqui P. A new strategy to fight antimicrobial resistance: the revival of old antibiotics. *Front Microbiol* (2014) 5:551. doi: 10.3389/fmicb.2014.00551
113. Hugonnet JE, Tremblay LW, Boshoff HI, Barry CE, Blanchard JS. Meropenem-clavulanate is effective against extensively drug-resistant Mycobacterium tuberculosis. *Science* (2009) 323:1215–8. doi: 10.1126/science.1167498
114. Diacon AH, van der Merwe L, Barnard M, von Groote-Bidlingmaier F, Lange C, García-Basteiro AL, et al. beta-Lactams against Tuberculosis–new trick for an old dog? *N Engl J Med* (2016) 375:393–4. doi: 10.1056/NEJMc1513236
115. Papp-Wallace KM, Endimiani A, Taracila AM, Bonomo RA. Carbapenems: Past, Present, and Future. *Antimicrob Agents Chemother* (1976) 4943–60.
116. Kohler J, Dorso KL, Young K, Hammond GG, Rosen H, Kropp H, et al. Vitro Activities of the Potent, Broad-Spectrum Carbapenem MK-0826 (L-749,345) against Broad-Spectrum β-Lactamase-and Extended-Spectrum β-Lactamase-Producing Klebsiella pneumoniae and Escherichia coli Clinical Isolates. *Antimicrob Agents Chemother* (1999) 43: (5):1170–6.
117. Paterson DL. Recommendation for treatment of severe infections caused by Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs). *Clin Microbiol Infect* (2000) 6:460–3. doi: 10.1046/j.1469-0691.2000.00107.x
118. Torres JA, Villegas MV, Quinn JP. Current concepts in antibiotic-resistant gram-negative bacteria. *Expert Rev Anti Infect Ther* (2007) 5:833–43. doi: 10.1586/14787210.5.5.833
119. Miyadera T, Sugimura Y, Hashimoto T, Tanaka T, Iino K, Shibata T, et al. Synthesis and in vitro activity of a new carbapenem, RS-533. *J Antibiot (Tokyo)* (1983) 36:1034–9. doi: 10.7164/antibiotics.36.1034
120. Hashihayata T, Sakoh H, Goto Y, Hirose M, Sakuraba S, Imamura H, et al. Diastereoselective synthesis of (2R,4R)-2-aryl- 4-hydroxypyrrolidine: preparation of the side chain of novel carbapenem. *Chem Pharm Bull (Tokyo)* (2001) 49:1500–2. doi: 10.1248/cpb.49.1500
121. Hashihayata T, Sakoh H, Goto Y, Yamada K, Morishima H. Synthesis of the side chain of a novel carbapenem via iodine-mediated oxidative cyclization of (1R)-N-(1-aryl-3-butenyl)acetamide. *Chem Pharm Bull (Tokyo)* (2002) 50:423–5. doi: 10.1248/cpb.50.423
122. Lee JH, Lee KS, Kang YK, Yoo KH, Shin KJ, Kim DC, et al. Synthesis and biological activity of novel 1-methylcarbapenems with oxyiminopyrrolidinylamide moiety. *Bioorg Med Chem Lett* (2003) 13:4399–403. doi: 10.1016/j.bmcl.2003.09.039
123. Lee KS, Kang YK, Yoo KH, Kim DC, Shin KJ, Paik YS, et al. Novel 1-methylcarbapenems with isoxazoloethenyl moieties containing carboxylic acid sodium salt. *Bioorg Med Chem Lett* (2005) 15:231–4. doi: 10.1016/j.bmcl.2004.09.092
124. Sunagawa M, Sasaki A, Yamaga H, Shinagawa H, Sumita Y, Nouda H, et al. Novel quaternary ammonium carbapenems: 1-methyl-2-(5-substituted pyrrolidinylthio) carbapenems. *J Antibiot (Tokyo)* (1994) 47:1337–40. doi: 10.7164/antibiotics.47.1337
125. Ueda Y, Vinet V. Synthesis and in vitro activity of novel quaternary ammonium carbapenems: 2-pyridiniopropyl and 1-pyridinioethyl carbapenems. *J Antibiot (Tokyo)* (1992) 45:940–53. doi: 10.7164/antibiotics.45.940
126. Bassetti M, Nicolini L, Esposito S, Righi E, Viscoli C. Current status of newer carbapenems. *Curr Med Chem* (2009) 16:564–75. doi: 10.2174/092986709787458498
127. Goa KL, Noble S. Panipenem/betamipron. *Drugs* (2003) 63:913–26. doi: 10.2165/00003495-200363090-00005
128. Nix DE, Majumdar AK, DiNubile MJ. Pharmacokinetics and pharmacodynamics of ertapenem: an overview for clinicians. *J Antimicrob Chemother* (2004) 53(Suppl. 2):ii23–8. doi: 10.1093/jac/dkh205
129. Perry CM, Ibbotson T. Biapenem. *Drugs* (2002) 62:2221–35. doi: 10.2165/00003495-200262150-00005
130. Tiberi S, D'Ambrosio L, De Lorenzo S, Viggiani P, Centis R, Sotgiu G, et al. Ertapenem in the treatment of multidrug-resistant tuberculosis: first clinical experience. *Eur Respir J* (2016) 47:333–6. doi: 10.1183/13993003.01278-2015
131. Veziris N, Truffot C, Mainardi JL, Jarlier V. Activity of carbapenems combined with clavulanate against murine tuberculosis. *Antimicrob Agents Chemother* (2011) 55(6):2597–600. doi: 10.1128/AAC.01824-10
132. Nayer H, Steinbach M. Sulfanilamide in clinical tuberculosis. *Am Rev Tuberc* (1939) 40:470–2.
133. Spies HW, Lepper MH, Blatt NH, Dowling HF. Tuberculous meningitis treatment with streptomycin, para-aminosalicylic acid and promizole, isoniazid and streptomycin, and isoniazid. *Am Rev Tuberc* (1954) 69:192–204. doi: 10.1164/art.1954.69.2.192
134. Forgacs P, Wengenack NL, Hall L, Zimmerman SK, Silverman ML, Roberts GD. Tuberculosis and trimethoprim-sulfamethoxazole. *Antimicrob Agents Chemother* (2009) 53(11):4789–93. doi: 10.1128/AAC.01658-08
135. Hasse B, Walker AS, Fehr J, Furrer H, Hoffmann M, Battegay M, et al. Cotrimoxazole prophylaxis is associated with reduced risk of incident tuberculosis in participants in the Swiss HIV Cohort Study. *Antimicrob Agents Chemother* (2014) 58:2363–8. doi: 10.1128/AAC
136. Oladimeji O, Isaakidis P, Obasanya OJ, Eltayeb O, Khogali M, Van den Bergh R, et al. Intensive-phase treatment outcomes among hospitalized multidrug-resistant tuberculosis patients: results from a nationwide cohort in Nigeria. *PloS One* (2014) 9:e94393. doi: 10.1371/journal.pone.0094393
137. Brouqui P, Aubry C, Million M, Drancourt M, Raoult D. Totally resistant tuberculosis: Will antileprosy drugs be helpful? *Int J Antimicrob Agents* (2013) 42:584–5. doi: 10.1016/j.ijantimicag.2013
138. Ameen SM, Drancourt M. In vitro susceptibility of Mycobacterium tuberculosis to trimethoprim and sulfonamides in France. *Antimicrob Agents Chemother* (2013) 57:6370–1. doi: 10.1128/AAC.01683-13

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MINI REVIEW
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Host-Directed Therapies: Modulating Inflammation to Treat Tuberculosis

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Following infection with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), most human hosts are able to contain the infection and avoid progression to active TB disease through expression of a balanced, homeostatic immune response. Proinflammatory mechanisms aiming to kill, slow and sequester the pathogen are key to a successful host response. However, an excessive or inappropriate pro-inflammatory response may lead to granuloma enlargement and tissue damage, which may prolong the TB treatment duration and permanently diminish the lung function of TB survivors. The host also expresses certain anti-inflammatory mediators which may play either beneficial or detrimental roles depending on the timing of their deployment. The balance between the timing and expression levels of pro- and anti-inflammatory responses plays an important role in the fate of infection. Interestingly, *M. tuberculosis* appears to manipulate both sides of the human immune response to remodel the host environment for its own benefit. Consequently, therapies which modulate either end of this spectrum of immune responses at the appropriate time may have the potential to improve the treatment of TB or to reduce the formation of permanent lung damage after microbiological cure. Here, we highlight host-directed TB therapies targeting pro- or anti-inflammatory processes that have been evaluated in pre-clinical models. The repurposing of already available drugs known to modulate these responses may improve the future of TB therapy.

Keywords: tuberculosis, PARP inhibition (PARPi), MMPs (metalloproteinases), immunotherapy, diphtheria fusion protein toxin, MDSCs, host-directed therapies

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INTRODUCTION

Tuberculosis (TB) is a devastating communicable disease caused by *Mycobacterium tuberculosis* (*M.tb*) that is responsible for approximately 10 million infections and 1.4 million human deaths every year (1). Global TB control is complicated by long treatment durations and emerging drug resistance (1). Interestingly, most people infected with *M.tb* develop lifelong latent TB without ever experiencing signs and symptoms of disease. Successful containment is the result of a multifaceted immune response that restricts bacterial expansion but may fail to completely eliminate the pathogen (2). When sterilization is not achieved, the host may nevertheless successfully contain

the infection by forming granulomas. However, in individuals who progress to active TB, granulomatous containment breaks down, resulting in lesion expansion, necrosis and liquefaction accompanied by bacterial proliferation and lung damage (2). This granulomatous inflammation during active TB may permanently diminish lung function even after completion of TB therapy (3).

The host utilizes both anti- and pro-inflammatory mechanisms in an effort to contain the infection: during latent *M.tb* infection, the immune response is successfully balanced but during active disease, this homeostatic balance is lost and disease progression occurs. Anti-inflammatory responses, mediated by regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), M2-polarized macrophages and cytokines such as interleukin (IL)-10, are observed during active TB and may antagonize the bactericidal effects of the immune system (4). Despite the presence of these immuno-tolerizing cells, host pro-inflammatory responses during active TB are often inappropriately expressed at high levels, either spatially or temporally, resulting in lung damage. Consequently, host-directed therapies (HDTs) that modify these non-productive immunologic responses may offer potential benefit as adjunctive agents alongside antimicrobial TB therapy (5). In this mini-review, we highlight FDA-approved drugs as well as select agents in development that have immunomodulatory activity and are under study as HDTs for TB in pre-clinical models and/or human clinical trials.

IMPROVING TB THERAPY BY MODULATING PRO-INFLAMMATORY RESPONSES

In immunocompetent patients with active TB, pro-inflammatory immune responses are often robust but fail to contain bacterial proliferation, leading to tissue damage and nonproductive inflammation. Nearly half of all active TB patients suffer from persistent or even progressive pulmonary dysfunction and face an increased risk of chronic lung disease even after microbiologically successful cure (3, 6–9). Post-TB lung defects (PTLD) include obstructive or restrictive lung disease, both of which may lead to chronic dyspnea, cough, reduced exercise tolerance, and a heightened risk for infections (3). In addition to shortening the duration of therapy, a parallel goal for TB HDTs is to avoid the development of irreversible lung damage from nonproductive inflammatory responses and to concomitantly improve the quality of life of TB survivors (3, 10). In this section, we discuss several classes of HDTs that may reduce nonproductive inflammation and PTLD (**Figure 1**, left; **Table 1**, top).

MMP Inhibitors

Tissue-degrading matrix metalloproteinases (MMPs), in particular MMP-1, -3 and -9, are major drivers of TB-associated lung damage (51–55). While extracellular matrix remodeling is important for immune cell migration and granuloma formation,

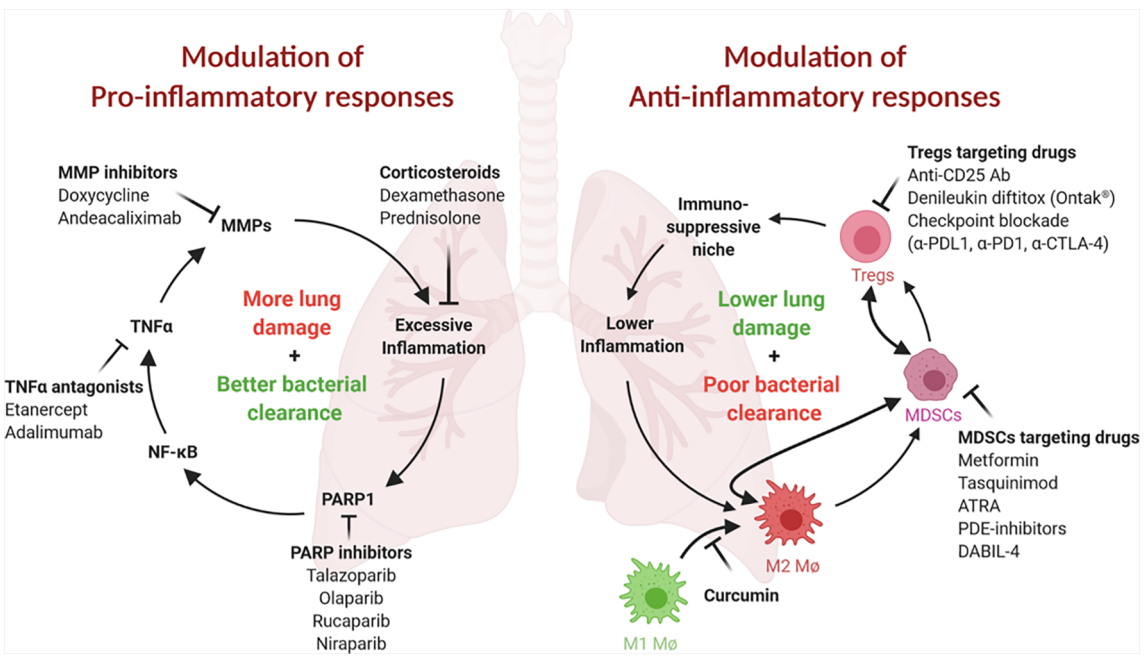

FIGURE 1 | Both pro- and ani-inflammatory responses play critical roles in TB pathogenesis. (Left) Proinflammatory responses and tissue remodeling in TB are important for bacterial clearance but may lead to excessive inflammation and persisting lung damage. Adjunct modulation of lung remodeling (for example, *via* TNFα or MMP inhibition) or inflammation (for example, by corticosteroids) may improve the outcome of TB therapy. Inhibition of PARP1, an essential NF-κB, TNFα and MMP cofactor and driver of lung inflammation, may be similarly beneficial. (Right) Anti-inflammatory responses safeguard against tissue damage but may result in less than desirable bacterial clearance. These responses are often mediated by immunosuppressive cell populations, such as MDSCs, Tregs and M2 macrophages. Inhibition or elimination of these cell types may be achieved using the inhibitors shown. This figure was created using BioRender.

TABLE 1 | Immune-modulatory drugs that may improve TB therapy.

Drug	HDT Class	Host Target	Applications	Preclinical data in TB	Ref.
Doxycycline	MMP Inhibitors	Multiple MMPs	Bacterial infections	Improved TB containment in cells, guinea pigs; Phase II trial ongoing (NCT02774993)	(11)
Marimastat	MMP Inhibitors	Multiple (MMP-1, -2, -7, -9, -14)	Cancer (discontinued)	Improved TB containment in mice	(12, 13)
Andecaliximab	MMP Inhibitors	MMP-9	Cancer, auto-inflammatory disorders (in development)	Reduced relapse rates in mice	(14, 15)
Cipemastat	MMP Inhibitors	MMP-1, -8, -13	Rheumatoid arthritis (discontinued)	Increased lung damage and death in mice; no effect in rabbits	(16, 17)
Etanercept	TNF antagonists	TNFα	Arthritis (various forms), ankylosing spondylitis	Accelerated bacterial clearance, reduced relapse rates in mice; may improve outcome in TB-HIV patients (Phase I) or severely ill TB patients; risk of impaired bacterial containment without adequate anti-TB therapy	(15, 18–22)
Dexamethasone/ Prednisolone	Corticosteroids	Broad-spectrum anti-inflammatory effects *via* modulation of glucocorticoid/mineralocorticoid receptor signaling	Inflammatory and immune-mediated disorders (numerous)	Modest improvements in lung function; recommended for TB meningitis (survival benefit) but not for pulmonary TB	(23–31)
Talazoparib	PARP inhibitors	PARP1/2; PARP3, PARP4, TNKS1, TNKS2	Cancer	May reduce inflammation and TB lung damage in mice	(32–36)
Olaparib	PARP inhibitors	PARP1/2; PARP3, PARP4, PARP16, TNKS1, TNKS2	Cancer	N/A	(33, 34, 36)
Rucaparib	PARP inhibitors	PARP1/2, PARP3, PARP10, TNKS1, TNKS2	Cancer	N/A	(33, 34, 36)
Niraparib	PARP inhibitors	PARP1/2, PARP3, PARP4, PARP12	Cancer	N/A	(33, 34, 36)
Metformin	MDSCs	HIF1α, CD39, CD73, AMPK-DACHi-CXCL1	Diabetes	Reduced severity and mortality in diabetic patients	(37, 38)
Tasquinamod	MDSCs	S100A9	Cancer	Decreased lung and spleen bacillary burden in mice	(39)
ATRA	MDSCs	Upregulates glutathione synthase	Cancer	Decreased lung bacillary burden and pathology in mice and rats	(40–42)
DABIL-4	MDSCs	IL-4R	Preclinical model of breast cancer	Decreased lung bacillary burden in mice	(43)
Sildenafil	MDSCs	PDE-5i	Erectile dysfunction and pulmonary hypertension	Reduced lung bacillary burden, pathology and severity in mice	(44)
Roflumilast and CC-11052	MDSCs	PDE-4i	COPD	Improved lung function in mice	(45, 46)
Denileukin Diftitox (Ontak®)	Tregs	IL-2R	Refractory cutaneous T-cell lymphoma	Reduced lung bacillary burden in mice	(47)
Checkpoint blockade therapy	Tregs	CTLA4, PD1	Cancer	*Mtb*-infected macaques overexpress CTLA-4	(48)
Curcumin	M2 macrophages	IL-10	Preclinical models of cancer	Modest efficacy in mice	(49)
Anti-IL-10 antibody	Tregs	IL-10	Preclinical model of cancer	Reduced lung bacillary burden in mice	(50)

MMP, matrix metalloproteinases; TNKS, tankyrase; PDE, phosphodiesterase.

MMP levels in TB patients remain elevated even after treatment completion and thus may drive progressive lung dysfunction (55, 56). Consequently, adjunctive MMP inhibition has been studied as an HDT to improve TB outcome. **Doxycycline**, a well-known antibacterial agent, also is known to have MMP inhibitory properties, making it the only currently FDA-approved MMP inhibitor. Doxycycline has been shown to not only inhibit TB-induced MMP activation but also to contain mycobacterial growth in cells and guinea pigs (11). Results from a phase II pilot study (NCT02774993) that evaluated the efficacy of adjuvant doxycycline as a novel HDT for pulmonary TB are pending and may offer insights into the safety and efficacy of this approach.

Excess MMP activity is observed in a number of human degenerative diseases and hence several targeted MMP inhibitors have been developed and evaluated in human studies. While adverse effects hindered early MMP inhibitors, there is newfound optimism that this may be overcome with a newer generation of inhibitors (57). For example, the broad-spectrum MMP inhibitor **marimastat** (BB-2516) reduced granuloma formation and bacterial growth *in vitro* and increased the efficacy of TB antibiotics in mice but its clinical development was discontinued due to its side effects (12, 13). However, the humanized monoclonal MMP-9 antibody **andecaliximab** is in late-stage development for cancer and auto-inflammatory disorders (14) and might improve TB outcome since the addition of an anti-MMP-9 antibody has been shown to reduce TB relapse rates in mice (15). In contrast, the MMP-1 inhibitor **cipemastat** increased immunopathology and death in *M.tb*-infected C3HeB/FeJ mice and failed to prevent *M.tb*-mediated cavity-generation in a rabbit model (16, 17). Nonetheless, the next generation of MMP inhibitors with improved selectivity, specificity and safety is a promising class of drugs that warrants consideration for HDT activity in TB.

TNF Antagonists

An alternative to direct MMP inhibition is to modulate the factors that promote MMP expression and TB inflammation, such as tumor necrosis factor α (TNFα) and the transcription factor NF-κB (55). TNFα is an important driver of TB lung damage by enhancing granuloma progression, cavitation, and MMP expression, and its expression levels are inversely correlated with the resolution of lung lesions during TB therapy (58–61). Correspondingly, HIV-positive TB patients generally have less lung damage than HIV-negative TB patients, and TB-immune reconstitution inflammatory syndrome (TB-IRIS) following antiretroviral therapy is associated with increased lung damage and reduced lung function (8, 62–64). TNFα also contributes to restrictive and obstructive airflow deficits by promoting fibrogenesis (18, 65, 66). Adjuvant administration of the TNFα antagonist **etanercept** accelerated bacterial clearance and reduced relapse rates in mice, and a promising phase I trial showed that etanercept may improve lung involvement and treatment responses in TB-HIV patients (15, 19, 20). There have also been case reports of TNFα inhibitors being used successfully to improve the clinical course of patients with advanced drug-susceptible TB who were doing poorly (21). In contrast, however, TNFα inhibitors are well-known to impair bacterial containment when used without accompanying multidrug anti-TB therapy (18, 22). While TNFα antagonists have the potential to improve TB therapy when used as adjunctive agents, there have been concerns about their expense, their need to be given parenterally, and the potential for disease worsening if administered without adequate anti-TB chemotherapy, and due to these concerns advanced clinical trials to test them as adjunctive HDTs for TB have not been performed (18, 60, 67).

Corticosteroids

Corticosteroids are another class of anti-inflammatory drugs that have garnered attention as potential TB-HDTs (68). In pulmonary TB, adjunctive corticosteroids, including the broadly immunosuppressive agents **dexamethasone** and **prednisolone**, have been studied for their ability to reduce post-treatment morbidity. Indeed, while some studies have demonstrated modest improvements in clinical outcomes, such as preservation of lung vital capacity, major improvements in the prevention of lung disability have not been shown (23–28). Thus, corticosteroids are not recommended in current TB treatment guidelines for the management of pulmonary TB (29, 30). Corticosteroids have also been evaluated in the management of tuberculous pericarditis, but they do not appear to change outcomes and are currently not recommended in that setting (69). In contrast, well-controlled studies have demonstrated a clear-cut survival benefit for use of corticosteroids in TB meningitis, and hence corticosteroids are considered mandatory in the treatment of that form of TB (31).

PARP Inhibitors

Poly(ADP-ribose) Polymerase (PARP) inhibitors (PARP-*I*s) are a new class of anticancer drugs introduced in the last decade, and four such agents are already FDA-approved. The PARP family of enzymes, comprised of at least 17 members, regulates wide-ranging cellular functions *via* the post-translational modification of mono- or poly(ADP-ribosyl)ation (70–74). PARP1, the founding member of the PARP family, is a eukaryotic master regulator particularly important for inflammatory processes and stress responses and accounts for at least 85% of cellular poly-ADP-ribose (PAR) formation (75). Importantly, PARP1 amplifies and sustains chronic inflammation by inducing inflammatory mediators that further stimulate its own activation (75, 76). Consequently, PARP1 contributes to disorders such as endotoxic shock, sepsis, asthma, COPD and ARDS, and PARP-*I*s have been shown to reduce inflammation and disease severity in numerous inflammatory conditions (75–78). PARP1 is an essential NF-κB, TNFα and MMP cofactor, and PARP-*I*s protect against tissue degradation by inhibiting multiple MMPs (71, 79–86). Therefore, PARP-*I*s have been proposed as HDTs for reducing TB-induced inflammation and lung disease (32). There are currently four FDA-approved PARP-*I*s for cancer therapies, **talazoparib** (Talzenna, Pfizer), **olaparib** (Lynparza, AstraZeneca), **rucaparib** (Rubraca, Clovis Oncology) and **niraparib** (Zejula, GlaxoSmithKline), with many more in various phases of development looking to expand their application in cancer therapy and beyond (33–35). Since it has been shown that PARP1 inhibition can ameliorate numerous inflammatory conditions, including rheumatoid arthritis, asthma, atherosclerosis and allergy-, toxicity- and injury-induced inflammation, the addition of a PARP inhibitor might similarly improve TB therapy by reducing inflammation and lung damage (75, 79, 87).

IMPROVING TB THERAPY BY MODULATING ANTI-INFLAMMATORY RESPONSES

An important theme in TB pathogenesis research in recent decades has been the observation that *M.tb* carries virulence

traits that subvert normal host immune responses and lead to pathogen survival and/or proliferation. One such mechanism is the recruitment of immunosuppressive or tolerizing cells to the site of infection, resulting in blunted bactericidal responses and the expression of elevated levels of IL-10 which further promotes anti-inflammatory responses (88). Indeed, it has recently been shown that the microbial polypeptide ESAT6 is one mediator that promotes the differentiation of M1 macrophages into anti-inflammatory M2 macrophages (89). Other tolerizing, immunosuppressive cells that are recruited to the site of infection include MDSCs, Tregs and M2-polarized macrophages. In this section, we highlight the major cell types involved in these anti-inflammatory responses and discuss drugs that target them and may be candidate TB HDTs (**Figure 1**, right; **Table 1**, bottom).

Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs represent an immunosuppressive cell population increasingly recognized as an important driver of TB pathogenesis. MDSCs are comprised of two distinct subsets: polymorphonuclear MDSCs (PMN-MDSCs) and mononuclear MDSCs (M-MDSCs). In mice, PMN-MDSCs are defined as $CD11b^+$ $Ly6G^+$ $Ly6C^{low}$ and M-MDSCs as $CD11b^+$ $Ly6G^-$ $Ly6C^{High}$. In humans, MDSCs are identified as $CD11b^+$ $CD33^+$ $HLA\text{-}DR^{low/neg}$ cells (90, 91), and these are further subdivided into PMN-MDSCs by the markers $CD14^-$ $CD66b^+$ $CD15^+$, and M-MDSCs as $CD14^+$ (92–95). While the role of MDSCs in suppressing inflammation has been extensively studied in cancer, it is becoming increasingly evident that MDSCs play an important role in the establishment of chronic infections including TB. Clinical studies have revealed that levels of MDSCs are high in the blood and sputum of active TB patients at the time of diagnosis and that they decline in response to successful chemotherapy (96–98). This association suggests that MDSCs may play an important role in the pathogenesis of active TB pathology and its dysfunctional inflammatory processes. Further evidence comes from murine studies where the relative abundance of MDSCs has also been found to correlate with the TB susceptibility of a given mouse strain. Relatively high levels of MDSCs are observed in susceptible mouse strains, such as 129S2 and C3HeB/FeJ, while lower MDSC levels are found in relatively resistant strains, such as BALB/c and C57BL/6 (99). Multiple HDTs have been tested in both pre-clinical and clinical settings that (1) inhibit the recruitment, expansion or function of MDSCs; or (2) specifically or non-specifically deplete their population.

Metformin. The widely used diabetes drug **metformin** inhibits the frequency and recruitment of MDSCs in cancer by modulating the expression and activity of HIF-1α, CD39, and CD73 and the AMPK-DACH1-CXCL1 axis (100, 101). A widely cited study in 2014 revealed that metformin reduced disease severity and inflammation in mice and was retrospectively associated with a lower degree of disease severity in diabetic patients with active TB who happened to be taking metformin during TB treatment (37). Another retrospective study showed that metformin therapy reduces the elevated TB mortality observed in diabetics (38). In spite of these observations, long-term chemotherapy studies in mice have failed to demonstrate a significant beneficial effect of adjunctive metformin together with standard TB chemotherapy (102). Clearly, prospective human studies are needed, and the NIH has recently funded a prospective Phase 2A study of metformin in patients with TB (103).

Tasquinimod is an experimental quinoline-3-carboxamide drug that has been studied in human prostate cancer (104). It has been shown to slow tumor growth in murine cancer models and to reduce MDSC tumor infiltration (105). It is believed to act by binding to and inhibiting the activity of the S100A9 protein; S100A9 together with S100A8 are known to modulate myeloid cell activity though TLR4 binding (104, 106). Because of its anti-MDSC properties, tasquinimod has been tested in murine TB models, and it has been shown not only to deplete MDSCs but also to decrease the relative bacterial burden in both lungs and spleens of infected animals (39).

All-trans Retinoic Acid (ATRA, tretinoin, a vitamin A derivative) is an FDA-approved drug which has been tested extensively in cancer models and has been shown to deplete MDSCs and slow tumor growth. While its precise mechanism of action is unknown, ATRA upregulates glutathione synthase (GSS), neutralizes high levels of reactive oxygen species (ROS) and induces differentiation of myeloid cells away from the MDSC phenotype (107). Importantly, however, ATRA has pleotropic effects on numerous cell types so in instances where it was found to be effective, one cannot be certain that its efficacy was through MDSC inhibition. Multiple groups have tested the effects of ATRA in murine TB models both as a monotherapy and in combination with standard TB therapy. In *M. tb.*-infected mice and rats, ATRA has been shown to reduce relative bacterial burden and lung pathology in a manner that correlates with MDSC depletion. The drug also exhibits anti-mycobacterial activity *in vitro* (96, 108).

In addition to the non-specific depletion of MDSCs, our group has recently tested the diphtheria toxin-related IL-4 fusion protein, **DABIL-4**, as a targeting agent against MDSCs which are known to express the IL-4 receptor, CD124. In an acute murine model of TB, DABIL-4 administration depleted $IL\text{-}4R^+$ MDSCs, $IL\text{-}4R^+$ M2 macrophages and $IL\text{-}4R^+$ lymphocytes. Depletion of these cell populations coincided with a significant reduction in the lung bacillary burden at day 21 post infection (43). We have also tested DABIL-4 in a murine breast cancer model and demonstrated that targeted depletion of MDSCs results in slower tumor growth and reduced splenomegaly and metastasis (109).

Phosphodiesterase inhibitors. Sildenafil, an FDA-approved type 5 phosphodiesterase-selective inhibitor (PDE-5i), is used in human patients for the treatment of erectile dysfunction and pulmonary hypertension. The drug downregulates arginase-1 and nitric oxide synthase-2 (NOS2) in a cGMP-dependent fashion, thereby hampering the immunosuppressive potential of MDSCs (110). Maiga et al. showed that the combination of sildenafil and **cilostazol** (an FDA-approved PDE-3 inhibitor) reduced pathology, disease severity and bacterial burden in murine TB; however, monotherapy with sildenafil alone showed no statistically significant benefit in the same mouse

model (44, 111). PDE-4 inhibitors, such as **roflumilast** and **CC-11052**, a Celgene PDE4 inhibitor in development, have also shown promising activity against TB in animal models (45, 46). A clinical trial evaluating CC-11052 as an adjunctive HDT alongside standard therapy has been conducted (NCT02968927), and preliminary results suggest that use of CC-11052 was associated with improvements in lung function (112).

Regulatory T-Cells (Tregs)

Tregs comprise an immunosuppressive $CD4^+$ T-cell population which express CD25 and FoxP3. $CD8^+$ Tregs also exist but their role in TB has not been extensively studied. Classic $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs are anti-inflammatory cells which keep effector T-cell function in check while promoting MDSC recruitment and maturation to further facilitate immunosuppression. Their presence in active TB is believed to inhibit anti-bacterial immune responses and to contribute to disease progression (113). Consistent with this, elevated Treg levels have been described in the blood and pleural fluid in pulmonary TB patients compared with healthy controls, and Treg levels were observed to decline to healthy control levels after successful TB chemotherapy (114).

Treg-depleting immunotherapies. The administration of **anti-CD25 monoclonal antibodies** in various cancer models has not only depleted Tregs but also slowed tumor progression (115). Anti-CD25 antibodies have been tested in the mouse TB model and were found to reduce relative bacillary loads in the lung and spleen and to improve lung pathology (116). **Denileukin diftitox (Ontak®),** a diphtheria toxin-related IL-2 fusion protein that was previously approved by the FDA for the treatment of refractory cutaneous T-cell lymphoma, is known to have potent Treg-depleting activity and has also been tested in murine TB models (47, 117). Ontak® monotherapy not only decreased Treg and MDSC frequencies in lungs and spleens but also significantly reduced relative bacterial CFU counts in a short-term TB mouse model. Additionally, the fusion protein toxin when combined with standard TB therapy significantly accelerated bacterial clearance in mice (47, 117).

Checkpoint Blockade Immunotherapy

Checkpoint blockade therapies, such as anti-PD-1 and anti-CTLA4 antibodies, have revolutionized the field of immunotherapy and have become an essential part of standard care for various human malignancies (118). In *M.tb*-infected macaques, Tregs have been shown to express CTLA-4, suggesting that anti-CTLA-4-directed checkpoint inhibitors may offer a potential HDT TB treatment (48). However, several groups have reported TB reactivation in cancer patients treated with checkpoint blockade therapy (119–121). While this does not necessarily indicate that checkpoint inhibitors given as adjuvants alongside appropriate anti-TB chemotherapy will fail to accelerate TB cure, more studies will be needed reach a conclusion regarding the efficacy of checkpoint blockade therapy as HDT for TB.

Anti-IL-10 Therapies

IL-10 is a key anti-inflammatory cytokine secreted by $CD4^+$ T cells, macrophages and MDSCs that suppresses T-cell function, blunts inflammatory responses, and promotes TB disease progression (50). IL-10 has been implicated in the M2-polarization of macrophages and this may further contribute to anti-inflammatory responses. An abundance of M2-polarized macrophages has been described in human lung granulomas (122), although it remains unclear if these M2 macrophages are causal in granuloma formation or rather a secondary consequence. IL-10 inhibitors would be expected to inhibit the direct anti-inflammatory effects of IL-10 and also prevent conversion of M1 macrophages into M2 macrophages. Indeed, IL-10 inhibitors have been tested both in cancer models and also in models of TB. **Curcumin** (diferuloylmethane), one of the active compounds found in turmeric, has been shown to modulate IL-10 levels and the frequency of M2 macrophages (123). Preparations of curcumin have been shown to drive a therapeutic benefit in a murine metastatic breast cancer model (124). In the context of TB, curcumin has also been shown to control the growth of *M.tb* in THP-1 macrophages and in primary alveolar macrophages derived from healthy human controls (125). More recently, a nanoparticle preparation of curcumin was tested in a murine TB model where it showed modest activity as monotherapy and more potent activity in combination with isoniazid (49). Direct inhibition of IL-10 with an **anti-IL-10-receptor antibody** in a murine TB model was shown to reduce bacterial CFU counts although it had little impact on the lung pathology (50).

DISCUSSION

Host-directed therapies have the potential to improve the treatment of TB by modulating either pro- or anti-inflammatory immune mechanisms. Interference with certain pro-inflammatory mechanisms offers the potential to reduce lung damage, increase antibiotic efficacy and shorten treatment duration. On the other hand, modulation of certain immunosuppressive immune responses may enhance the innate bactericidal activity of the immune system and thus accelerate bacterial clearance. Repurposing drugs that are safe and approved for human use is an approach that may fast-track the clinical development of new host-directed TB treatment regimens. Here, we reviewed HDTs of interest for TB that target pro- or anti-inflammatory immune mechanisms (**Figure 1**; **Table 1**). On the proinflammatory side, we highlighted MMP inhibition, TNFα antagonists, corticosteroids and PARP inhibition to reduce TB-associated lung damage and inflammation. However, immune modulation in TB should be approached with caution as disrupting the intricate host-pathogen relationship can also increase the risk for disease progression or exacerbate inflammation. It is important that the dosing, frequency and timing of TB-HDTs are carefully optimized to minimize potentially harmful effects. Moreover, HDTs should be primarily evaluated as treatment adjuvants to be utilized alongside fully active traditional anti-TB chemotherapy. A related concern is that of drug-drug interactions and the potential for one agent to reduce the circulating concentration of another.

Even though TB-associated persistent lung dysfunction is a common disability in TB survivors, there are currently no guidelines for the diagnosis or management of PTLDs, and it is unclear to what extent they contribute to the economic burden of TB (8, 126). Reducing TB-associated lung dysfunction has the potential to greatly improve the quality of life after TB by reducing morbidity and loss of income. While pulmonary function testing in early TB carries some risk of TB transmission, it has been safely implemented in numerous clinical trials. We therefore recommend that more consideration should be given to the routine assessment of lung function in TB clinical trials. In addition to HDTs, non-pharmacological interventions, such as pulmonary rehabilitation, may improve lung function after completion of TB therapy and should be considered in the management of TB patients on a case-by-case basis (127). Importantly, we hope to increase awareness that the fight against TB does not end with microbiological cure.

AUTHOR CONTRIBUTIONS

SK and SP contributed equally to this mini-review. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. World Health Organization. *Global tuberculosis report 2019*. Geneva: World Health Organization (2019). Licence: CC BY-NC-SA 3.0 IGO.
2. Huynh KK, Joshi SA, Brown EJ. A delicate dance: host response to mycobacteria. *Curr Opin Immunol* (2011) 23(4):464–72. doi: 10.1016/j.coi.2011.06.002
3. Ravimohan S, Kornfeld H, Weissman D, Bisson GP. Tuberculosis and lung damage: from epidemiology to pathophysiology. *Eur Respir Rev* (2018) 27 (147):170077. doi: 10.1183/16000617.0077-2017
4. Cicchese JM, Evans S, Hult C, Joslyn LR, Wessler T, Millar JA, et al. Dynamic balance of pro- and anti-inflammatory signals controls disease and limits pathology. *Immunol Rev* (2018) 285(1):147–67. doi: 10.1111/imr.12671
5. Zitko J. Doležal M: Old Drugs and New Targets as an Outlook for the Treatment of Tuberculosis. *Curr Medicinal Chem* (2018) 25(38):5142–67. doi: 10.2174/0929867324666170920154325
6. Chushkin MI, Ots ON. Impaired pulmonary function after treatment for tuberculosis: the end of the disease? *Jornal brasileiro pneumologia publicacao oficial da Sociedade Bras Pneumologia e Tisilogia* (2017) 43(1):38–43. doi: 10.1590/s1806-37562016000000053
7. Manji M, Shayo G, Mamuya S, Mpembeni R, Jusabani A, Mugusi F. Lung functions among patients with pulmonary tuberculosis in Dar es Salaam - a cross-sectional study. *BMC Pulm Med* (2016) 16(1):58–8. doi: 10.1186/s12890-016-0213-5
8. Meghji J, Lesosky M, Joekes E, Banda P, Rylance J, Gordon S, et al. Patient outcomes associated with post-tuberculosis lung damage in Malawi: a prospective cohort study. *Thorax* (2020) 75(3):269–78. doi: 10.1136/thoraxjnl-2019-213808
9. Malherbe ST, Shenai S, Ronacher K, Loxton AG, Dolganov G, Kriel M, et al. Persisting positron emission tomography lesion activity and Mycobacterium tuberculosis mRNA after tuberculosis cure. *Nat Med* (2016) 22(10):1094–100. doi: 10.1038/nm.4177
10. Plit ML, Anderson R, Van Rensburg CE, Page-Shipp L, Blott JA, Fresen JL, et al. Influence of antimicrobial chemotherapy on spirometric parameters and pro-inflammatory indices in severe pulmonary tuberculosis. *Eur Respir J* (1998) 12(2):351–6. doi: 10.1183/09031936.98.12020351
11. Walker NF, Clark SO, Oni T, Andreu N, Tezera L, Singh S, et al. Doxycycline and HIV infection suppress tuberculosis-induced matrix metalloproteinases. *Am J Respir Crit Care Med* (2012) 185(9):989–97. doi: 10.1164/rccm.201110-1769OC
12. Parasa VR, Muvva JR, Rose JF, Braian C, Brighenti S, Lerm M. Inhibition of Tissue Matrix Metalloproteinases Interferes with Mycobacterium tuberculosis-Induced Granuloma Formation and Reduces Bacterial Load in a Human Lung Tissue Model. *Front Microbiol* (2017) 8:2370. doi: 10.3389/fmicb.2017.02370
13. Xu Y, Wang L, Zimmerman MD, Chen KY, Huang L, Fu DJ, et al. Matrix metalloproteinase inhibitors enhance the efficacy of frontline drugs against Mycobacterium tuberculosis. *PloS Pathog* (2018) 14(4):e1006974. doi: 10.1371/journal.ppat.1006974
14. Shah MA, Yanez Ruiz EP, Bodoky G, Starodub A, Cunningham D, Yip D, et al. A phase III, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of andecaliximab combined with mFOLFOX6 as first-line treatment in patients with advanced gastric or gastroesophageal junction adenocarcinoma (GAMMA-1). *J Clin Oncol* (2019) 37(4_suppl):4–4. doi: 10.1200/JCO.2019.37.4_suppl.4
15. Ordonez AA, Pokkali S, Kim S, Carr B, Klunk MH, Tong L, et al. Adjunct antibody administration with standard treatment reduces relapse rates in a murine tuberculosis model of necrotic granulomas. *PloS One* (2018) 13(5): e0197474. doi: 10.1371/journal.pone.0197474
16. Ordonez AA, Pokkali S, Sanchez-Bautista J, Klunk MH, Urbanowski ME, Kubler A, et al. Matrix Metalloproteinase Inhibition in a Murine Model of Cavitary Tuberculosis Paradoxically Worsens Pathology. *J Infect Dis* (2019) 219(4):633–6. doi: 10.1093/infdis/jiy373
17. Urbanowski ME, Ihms EA, Bigelow K, Kubler A, Elkington PT, Bishai WR. Repetitive Aerosol Exposure Promotes Cavitary Tuberculosis and Enables Screening for Targeted Inhibitors of Extensive Lung Destruction. *J Infect Dis* (2018) 218(1):53–63. doi: 10.1093/infdis/jiy127
18. Tsenova L, O'Brien P, Holloway J, Peixoto B, Soteropoulos P, Fallows D, et al. Etanercept Exacerbates Inflammation and Pathology in a Rabbit Model of Active Pulmonary Tuberculosis. *J Interferon Cytokine Res* (2014) 34 (9):716–26. doi: 10.1089/jir.2013.0123
19. Skerry C, Harper J, Klunk M, Bishai WR, Jain SK. Adjunctive TNF inhibition with standard treatment enhances bacterial clearance in a murine model of necrotic TB granulomas. *PloS One* (2012) 7(6):e39680. doi: 10.1371/journal.pone.0039680
20. Wallis RS, Kyambadde P, Johnson JL, Horter L, Kittle R, Pohle M, et al. A study of the safety, immunology, virology, and microbiology of adjunctive etanercept in HIV-1-associated tuberculosis. *AIDS (London England)* (2004) 18(2):257–64. doi: 10.1097/00002030-200401230-00015
21. Wallis RS, van Vuuren C, Potgieter S. Adalimumab treatment of life-threatening tuberculosis. *Clin Infect Dis an Off Publ Infect Dis Soc America* (2009) 48(10):1429–32. doi: 10.1086/598504
22. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *New Engl J Med* (2001) 345(15):1098–104. doi: 10.1056/NEJMoa011110
23. Malik SK, Martin CJ. Tuberculosis, corticosteroid therapy, and pulmonary function. *Am Rev Respir Dis* (1969) 100(1):13–8. doi: 10.1164/arrd.1969.100.1.13
24. Angel JH, Chu LS, Lyons HA. Corticotropin in the treatment of tuberculosis. A controlled study. *Arch Internal Med* (1961) 108:353–69. doi: 10.1001/archinte.1961.03620090025004
25. Marcus H, Yoo OH, Akyol T, Williams MH Jr. A randomized study of the effects of corticosteroid therapy on healing of pulmonary tuberculosis as judged by clinical, roentgenographic, and physiologic measurements. *Am Rev Respir Dis* (1963) 88:55–64. doi: 10.1164/arrd.1963.88.1.55

26. Dooley DP, Carpenter JL, Rademacher S. Adjunctive corticosteroid therapy for tuberculosis: a critical reappraisal of the literature. *Clin Infect Dis an Off Publ Infect Dis Soc America* (1997) 25(4):872–87. doi: 10.1086/515543
27. Critchley JA, Young F, Orton L, Garner P. Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *Lancet Infect Dis* (2013) 13(3):223–37. doi: 10.1016/S1473-3099(12)70321-3
28. Wallis RS. Corticosteroid Effects on Sputum Culture in Pulmonary Tuberculosis: A Meta-Regression Analysis. *Open Forum Infect Dis* (2014) 1(1):ofu020. doi: 10.1093/ofid/ofu020
29. Nahid P, Dorman SE, Alipanah N, Barry PM, Brozek JL, Cattamanchi A, et al. Official American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America Clinical Practice Guidelines: Treatment of Drug-Susceptible Tuberculosis. *Clin Infect Dis an Off Publ Infect Dis Soc America* (2016) 63(7):e147–95. doi: 10.1093/cid/ciw376
30. Nahid P, Mase SR, Migliori GB, Sotgiu G, Bothamley GH, Brozek JL, et al. Treatment of Drug-Resistant Tuberculosis. An Official ATS/CDC/ERS/IDSA Clinical Practice Guideline. *Am J Respir Crit Care Med* (2019) 200 (10):e93–e142. doi: 10.1164/rccm.201909-1874ST
31. Thwaites GE, Nguyen DB, Nguyen HD, Hoang TQ, Do TT, Nguyen TC, et al. Dexamethasone for the treatment of tuberculous meningitis in adolescents and adults. *New Engl J Med* (2004) 351(17):1741–51. doi: 10.1056/NEJMp048227
32. Krug S, Kumar P, Kang BG, Dawson TM, Dawson VL, Bishai WR. Pyrazinamide inhibits the host enzyme PARP1 to reduce inflammation and accelerate bacterial clearance. In: *Tuberculosis: Science Aimed at Ending the Epidemic (eSymposia)*. Keystone, CO, USA: Virtual Keystone Symposia (2020).
33. Berger NA, Besson VC, Boulares AH, Burkle A, Chiarugi A, Clark RS, et al. Opportunities for the repurposing of PARP inhibitors for the therapy of non-oncological diseases. *Br J Pharmacol* (2018) 175(2):192–222. doi: 10.1111/bph.13748
34. Curtin NJ, Szabo C. Therapeutic applications of PARP inhibitors: anticancer therapy and beyond. *Mol aspects Med* (2013) 34(6):1217–56. doi: 10.1016/j.mam.2013.01.006
35. Pfizer. *TALZENNA™ (talazoparib) capsules, for oral use [prescribing information]*. FDA (2018).
36. Thorsell A-G, Ekblad T, Karlberg T, Löw M, Pinto AF, Trésaugues L, et al. Structural Basis for Potency and Promiscuity in Poly(ADP-ribose) Polymerase (PARP) and Tankyrase Inhibitors. *J Medicinal Chem* (2017) 60(4):1262–71. doi: 10.1021/acs.jmedchem.6b00990
37. Singhal A, Jie L, Kumar P, Hong GS, Leow MK-S, Paleja B, et al. Metformin as adjunct antituberculosis therapy. *Sci Trans Med* (2014) 6(263):263ra159–263ra159. doi: 10.1126/scitranslmed.3009885
38. Degner NR, Wang JY, Golub JE, Karakousis PC. Metformin Use Reverses the Increased Mortality Associated With Diabetes Mellitus During Tuberculosis Treatment. *Clin Infect Dis* (2018) 66(2):198–205. doi: 10.1093/cid/cix819
39. Gupta S, Krug S, Pokkali S, Leanderson T, Isaacs JT, Srikrishna G, et al. Pharmacologic Exhaustion of Suppressor Cells with Tasquinimod Enhances Bacterial Clearance during Tuberculosis. *Am J Respir Crit Care Med* (2019) 199(3):386–9. doi: 10.1164/rccm.201805-0820LE
40. Coleman MM, Basdeo SA, Coleman AM, Cheallaigh CN, Peral de Castro C, McLaughlin AM, et al. All-trans Retinoic Acid Augments Autophagy during Intracellular Bacterial Infection. *Am J Respir Cell Mol Biol* (2018) 59(5):548–56. doi: 10.1165/rcmb.2017-0382OC
41. Wheelwright M, Kim EW, Inkeles MS, De Leon A, Pellegrini M, Krutzik SR, et al. All-trans retinoic acid-triggered antimicrobial activity against Mycobacterium tuberculosis is dependent on NPC2. *J Immunol (Baltimore Md 1950)* (2014) 192(5):2280–90. doi: 10.4049/jimmunol.1301686
42. Kusmartsev S, Cheng F, Yu B, Nefedova Y, Sotomayor E, Lush R, et al. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res* (2003) 63 (15):4441–9.
43. Parveen S, Lun S, Urbanowski ME, Cardin M, Murphy JR, Bishai WR. Effective host-directed therapy for tuberculosis by targeted depletion of myeloid-derived suppressor cells using a diphtheria toxin-based fusion protein. *bioRxiv* (2020) 2020.2012.2010.420224. doi: 10.1101/2020.12.10.420224
44. Maiga M, Ammerman NC, Maiga MC, Tounkara A, Siddiqui S, Polis M, et al. Adjuvant host-directed therapy with types 3 and 5 but not type 4 phosphodiesterase inhibitors shortens the duration of tuberculosis treatment. *J Infect Dis* (2013) 208(3):512–9. doi: 10.1093/infdis/jit187
45. Maiga MC, Ahidjo BA, Maiga M, Bishai WR. Roflumilast, a Type 4 Phosphodiesterase Inhibitor, Shows Promising Adjunctive, Host-Directed Therapeutic Activity in a Mouse Model of Tuberculosis. *Antimicrob Agents Chemother* (2015) 59(12):7888–90. doi: 10.1128/AAC.02145-15
46. Subbian S, Tsenova L, Holloway J, Peixoto B, O'Brien P, Dartois V, et al. Adjunctive Phosphodiesterase-4 Inhibitor Therapy Improves Antibiotic Response to Pulmonary Tuberculosis in a Rabbit Model. *EBioMedicine* (2016) 4:104–14. doi: 10.1016/j.ebiom.2016.01.015
47. Gupta S, Cheung L, Pokkali S, Winglee K, Guo H, Murphy JR, et al. Suppressor Cell-Depleting Immunotherapy With Denileukin Diftitox is an Effective Host-Directed Therapy for Tuberculosis. *J Infect Dis* (2017) 215 (12):1883–7. doi: 10.1093/infdis/jix208
48. Green AM, Mattila JT, Bigbee CL, Bongers KS, Lin PL, Flynn JL. CD4(+) regulatory T cells in a cynomolgus macaque model of Mycobacterium tuberculosis infection. *J Infect Dis* (2010) 202(4):533–41. doi: 10.1086/654896
49. Tousif S, Singh DK, Mukherjee S, Ahmad S, Arya R, Nanda R, et al. Nanoparticle-Formulated Curcumin Prevents Posttherapeutic Disease Reactivation and Reinfection with Mycobacterium tuberculosis following Isoniazid Therapy. *Front Immunol* (2017) 8:739. doi: 10.3389/fimmu.2017.00739
50. Beamer GL, Flaherty DK, Assogba BD, Stromberg P, Gonzalez-Juarrero M, de Waal Malefyt R, et al. Interleukin-10 promotes Mycobacterium tuberculosis disease progression in CBA/J mice. *J Immunol (Baltimore Md 1950)* (2008) 181(8):5545–50. doi: 10.4049/jimmunol.181.8.5545
51. Elkington P, Shiomi T, Breen R, Nuttall RK, Ugarte-Gil CA, Walker NF, et al. MMP-1 drives immunopathology in human tuberculosis and transgenic mice. *J Clin Invest* (2011) 121(5):1827–33. doi: 10.1172/JCI45666
52. Ong CW, Elkington PT, Brilha S, Ugarte-Gil C, Tome-Esteban MT, Tezera LB, et al. Neutrophil-Derived MMP-8 Drives AMPK-Dependent Matrix Destruction in Human Pulmonary Tuberculosis. *PloS Pathog* (2015) 11(5): e1004917. doi: 10.1371/journal.ppat.1004917
53. Arpino V, Brock M, Gill SE. The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biol J Int Soc Matrix Biol* (2015) 44-46:247–54. doi: 10.1016/j.matbio.2015.03.005
54. Kübler A, Luna B, Larsson C, Ammerman NC, Andrade BB, Orandle M, et al. Mycobacterium tuberculosis dysregulates MMP/TIMP balance to drive rapid cavitation and unrestrained bacterial proliferation. *J Pathol* (2015) 235 (3):431–44. doi: 10.1002/path.4432
55. Sabir N, Hussain T, Mangi MH, Zhao D, Zhou X. Matrix metalloproteinases: Expression, regulation and role in the immunopathology of tuberculosis. *Cell Proliferation* (2019) 52(4):e12649. doi: 10.1111/cpr.12649
56. Ugarte-Gil CA, Elkington P, Gilman RH, Coronel J, Tezera LB, Bernabe-Ortiz A, et al. Induced sputum MMP-1, -3 & -8 concentrations during treatment of tuberculosis. *PloS One* (2013) 8(4):e61333. doi: 10.1371/journal.pone.0061333
57. Fields GB. The Rebirth of Matrix Metalloproteinase Inhibitors: Moving Beyond the Dogma. *Cells* (2019) 8(9):984. doi: 10.3390/cells8090984
58. Elkington PT, Green JA, Emerson JE, Lopez-Pascua LD, Boyle JJ, O'Kane CM, et al. Synergistic up-regulation of epithelial cell matrix metalloproteinase-9 secretion in tuberculosis. *Am J Respir Cell Mol Biol* (2007) 37(4):431–7. doi: 10.1165/rcmb.2007-0011OC
59. Elkington PTG, Emerson JE, Lopez-Pascua LDC, O'Kane CM, Horncastle DE, Boyle JJ, et al. Mycobacterium tuberculosis Up-Regulates Matrix Metalloproteinase-1 Secretion from Human Airway Epithelial Cells via a p38 MAPK Switch. *J Immunol* (2005) 175(8):5333–40. doi: 10.4049/jimmunol.175.8.5333
60. Mootoo A, Stylianou E, Arias MA, Reljic R. TNF-alpha in tuberculosis: a cytokine with a split personality. *Inflammation Allergy Drug Targets* (2009) 8 (1):53–62. doi: 10.2174/187152809787582543
61. Su W-L, Perng W-C, Huang C-H, Yang C-Y, Wu C-P, Chen J-H. Association of Reduced Tumor Necrosis Factor Alpha, Gamma Interferon, and Interleukin-1β (IL-1β) but Increased IL-10 Expression

with Improved Chest Radiography in Patients with Pulmonary Tuberculosis. *Clin Vaccine Immunol* (2010) 17(2):223–31. doi: 10.1128/CVI.00381-09
62. Ravimohan S, Tamuhla N, Kung S-J, Nfanyana K, Steenhoff AP, Gross R, et al. Matrix Metalloproteinases in Tuberculosis-Immune Reconstitution Inflammatory Syndrome and Impaired Lung Function Among Advanced HIV/TB Co-infected Patients Initiating Antiretroviral Therapy. *EBioMedicine* (2016) 3:100–7. doi: 10.1016/j.ebiom.2015.11.040
63. Chamie G, Luetkemeyer A, Walusimbi-Nanteza M, Okwera A, Whalen CC, Mugerwa RD, et al. Significant variation in presentation of pulmonary tuberculosis across a high resolution of CD4 strata. *Int J Tuberc Lung Dis* (2010) 14(10):1295–302.
64. Meintjes G, Lawn SD, Scano F, Maartens G, French MA, Worodria W, et al. Tuberculosis-associated immune reconstitution inflammatory syndrome: case definitions for use in resource-limited settings. *Lancet Infect Dis* (2008) 8(8):516–23. doi: 10.1016/S1473-3099(08)70184-1
65. Wynn TA. Integrating mechanisms of pulmonary fibrosis. *J Exp Med* (2011) 208(7):1339–50. doi: 10.1084/jem.20110551
66. Barlo NP, van Moorsel CH, Korthagen NM, Heron M, Rijkers GT, Ruven HJ, et al. Genetic variability in the IL1RN gene and the balance between interleukin (IL)-1 receptor agonist and IL-1β in idiopathic pulmonary fibrosis. *Clin Exp Immunol* (2011) 166(3):346–51. doi: 10.1111/j.1365-2249.2011.04468.x
67. Arbués A, Brees D, Chibout SD, Fox T, Kammüller M, Portevin D. TNF-α antagonists differentially induce TGF-β1-dependent resuscitation of dormant-like Mycobacterium tuberculosis. *PloS Pathog* (2020) 16(2): e1008312. doi: 10.1371/journal.ppat.1008312
68. Young C, Walzl G, Du Plessis N. Therapeutic host-directed strategies to improve outcome in tuberculosis. *Mucosal Immunol* (2020) 13(2):190–204. doi: 10.1038/s41385-019-0226-5
69. Mayosi BM, Ntsekhe M, Bosch J, Pandie S, Jung H, Gumedze F, et al. Prednisolone and Mycobacterium indicus pranii in tuberculous pericarditis. *New Engl J Med* (2014) 371(12):1121–30.
70. David KK, Andrabi SA, Dawson TM, Dawson VL. Parthanatos, a messenger of death. *Front Biosci (Landmark edition)* (2009) 14:1116–28. doi: 10.2741/3297
71. Gibson BA, Kraus WL. New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. *Nat Rev Mol Cell Biol* (2012) 13(7):411–24. doi: 10.1038/nrm3376
72. Langelier MF, Zandarashvili L, Aguiar PM, Black BE, Pascal JM. NAD(+) analog reveals PARP-1 substrate-blocking mechanism and allosteric communication from catalytic center to DNA-binding domains. *Nat Commun* (2018) 9(1):844. doi: 10.1038/s41467-018-03234-8
73. Swindall AF, Stanley JA, Yang ES. PARP-1: Friend or Foe of DNA Damage and Repair in Tumorigenesis? *Cancers (Basel)* (2013) 5(3):943–58. doi: 10.3390/cancers5030943
74. Amé J-C, Spenlehauer C, de Murcia G. The PARP superfamily. *BioEssays* (2004) 26(8):882–93. doi: 10.1002/bies.20085
75. Ba X, Garg NJ. Signaling mechanism of poly(ADP-ribose) polymerase-1 (PARP-1) in inflammatory diseases. *Am J Pathol* (2011) 178(3):946–55. doi: 10.1016/j.ajpath.2010.12.004
76. Bai P, Virag L. Role of poly(ADP-ribose) polymerases in the regulation of inflammatory processes. *FEBS Lett* (2012) 586(21):3771–7. doi: 10.1016/j.febslet.2012.09.026
77. Ke Y, Wang C, Zhang J, Zhong X, Wang R, Zeng X, et al. The Role of PARPs in Inflammation-and Metabolic-Related Diseases: Molecular Mechanisms and Beyond. *Cells* (2019) 8(9):1047. doi: 10.3390/cells8091047
78. Giansanti V, Dona F, Tillhon M, Scovassi AI. PARP inhibitors: new tools to protect from inflammation. *Biochem Pharmacol* (2010) 80(12):1869–77. doi: 10.1016/j.bcp.2010.04.022
79. Rosado MM, Bennici E, Novelli F, Pioli C. Beyond DNA repair, the immunological role of PARP-1 and its siblings. *Immunology* (2013) 139 (4):428–37. doi: 10.1111/imm.12099
80. Liu L, Ke Y, Jiang X, He F, Pan L, Xu L, et al. Lipopolysaccharide activates ERK-PARP-1-RelA pathway and promotes nuclear factor-kappaB transcription in murine macrophages. *Hum Immunol* (2012) 73(5):439–47. doi: 10.1016/j.humimm.2012.02.002
81. Liaudet L, Pacher P, Mabley JG, Virag L, Soriano FG, Hasko G, et al. Activation of poly(ADP-Ribose) polymerase-1 is a central mechanism of lipopolysaccharide-induced acute lung inflammation. *Am J Respir Crit Care Med* (2002) 165(3):372–7. doi: 10.1164/ajrccm.165.3.2106050
82. Vuong B, Hogan-Cann AD, Alano CC, Stevenson M, Chan WY, Anderson CM, et al. NF-kappaB transcriptional activation by TNFalpha requires phospholipase C, extracellular signal-regulated kinase 2 and poly(ADP-ribose) polymerase-1. *J Neuroinflamm* (2015) 12:229. doi: 10.1186/s12974-015-0448-8
83. Mishra M, Kowluru RA. Role of PARP-1 as a novel transcriptional regulator of MMP-9 in diabetic retinopathy. *Biochim Biophys Acta Mol basis Dis* (2017) 1863(7):1761–9. doi: 10.1016/j.bbadis.2017.04.024
84. Nicolescu AC, Holt A, Kandasamy AD, Pacher P, Schulz R. Inhibition of matrix metalloproteinase-2 by PARP inhibitors. *Biochem Biophys Res Commun* (2009) 387(4):646–50. doi: 10.1016/j.bbrc.2009.07.080
85. Chen T, Wang W, Li J-R, Xu H-Z, Peng Y-C, Fan L-F, et al. PARP inhibition attenuates early brain injury through NF-κB/MMP-9 pathway in a rat model of subarachnoid hemorrhage. *Brain Res* (2016) 1644:32–8. doi: 10.1016/j.brainres.2016.05.005
86. Ghorai A, Sarma A, Chowdhury P, Ghosh U. PARP-1 depletion in combination with carbon ion exposure significantly reduces MMPs activity and overall increases TIMPs expression in cultured HeLa cells. *Radiat Oncol* (2016) 11(1):126. doi: 10.1186/s13014-016-0703-x
87. Gupte AN, Paradkar M, Selvaraju S, Thiruvengadam K, Shivakumar SVBY, Sekar K, et al. Assessment of lung function in successfully treated tuberculosis reveals high burden of ventilatory defects and COPD. *PloS One* (2019) 14(5):e0217289. doi: 10.1371/journal.pone.0217289
88. de Martino M, Lodi L, Galli L, Chiappini E. Immune Response to Mycobacterium tuberculosis: A Narrative Review. *Front Pediatr* (2019) 7:350. doi: 10.3389/fped.2019.00350
89. Refai A, Gritli S, Barbouche MR, Essafi M. Mycobacterium tuberculosis Virulent Factor ESAT-6 Drives Macrophage Differentiation Toward the Pro-inflammatory M1 Phenotype and Subsequently Switches It to the Anti-inflammatory M2 Phenotype. *Front Cell Infect Microbiol* (2018) 8:327. doi: 10.3389/fcimb.2018.00327
90. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol (Baltimore Md 1950)* (2001) 166(1):678–89. doi: 10.4049/jimmunol.166.1.678
91. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. *Clin Cancer Res* (2007) 13(2 Pt 2):721s–6s. doi: 10.1158/1078-0432.CCR-06-2197
92. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res* (2005) 65(8):3044–8. doi: 10.1158/0008-5472.CAN-04-4505
93. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, et al. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol Off J Am Soc Clin Oncol* (2007) 25(18):2546–53. doi: 10.1200/JCO.2006.08.5829
94. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* (2009) 9(3):162–74. doi: 10.1038/nri2506
95. Condamine T, Ramachandran I, Youn JI, Gabrilovich DI. Regulation of tumor metastasis by myeloid-derived suppressor cells. *Annu Rev Med* (2015) 66:97–110. doi: 10.1146/annurev-med-051013-052304
96. Knaul JK, Jörg S, Oberbeck-Mueller D, Heinemann E, Scheuermann L, Brinkmann V, et al. Lung-residing myeloid-derived suppressors display dual functionality in murine pulmonary tuberculosis. *Am J Respir Crit Care Med* (2014) 190(9):1053–66. doi: 10.1164/rccm.201405-0828OC
97. du Plessis N, Loebenberg L, Kriel M, von Groote-Bidlingmaier F, Ribechini E, Loxton AG, et al. Increased frequency of myeloid-derived suppressor cells during active tuberculosis and after recent mycobacterium tuberculosis infection suppresses T-cell function. *Am J Respir Crit Care Med* (2013) 188(6):724–32. doi: 10.1164/rccm.201302-0249OC
98. Tsiganov EN, Verbina EM, Radaeva TV, Sosunov VV, Kosmiadi GA, Nikitina IY, et al. Gr-1dimCD11b+ immature myeloid-derived suppressor cells but not neutrophils are markers of lethal tuberculosis infection in mice. *J Immunol (Baltimore Md 1950)* (2014) 192(10):4718–27. doi: 10.4049/jimmunol.1301365

99. du Plessis N, Kotze LA, Leukes V, Walzl G. Translational Potential of Therapeutics Targeting Regulatory Myeloid Cells in Tuberculosis. *Front Cell Infect Microbiol* (2018) 8:332. doi: 10.3389/fcimb.2018.00332
100. Li L, Wang L, Li J, Fan Z, Yang L, Zhang Z, et al. Metformin-Induced Reduction of CD39 and CD73 Blocks Myeloid-Derived Suppressor Cell Activity in Patients with Ovarian Cancer. *Cancer Res* (2018) 78(7):1779–91. doi: 10.1158/0008-5472.CAN-17-2460
101. Qin G, Lian J, Huang L, Zhao Q, Liu S, Zhang Z, et al. Metformin blocks myeloid-derived suppressor cell accumulation through AMPK-DACH1-CXCL1 axis. *Oncoimmunology* (2018) 7(7):e1442167. doi: 10.1080/2162402X.2018.1442167
102. Dutta NK, Pinn ML, Karakousis PC. Metformin Adjunctive Therapy Does Not Improve the Sterilizing Activity of the First-Line Antitubercular Regimen in Mice. *Antimicrob Agents Chemother* (2017) 61(8):e00652-17. doi: 10.1128/AAC.00652-17
103. Kornfeld H, Singhal A, Wallis R. *Trial of Metformin for TB/HIV Host-directed Therapy*. National Institute of Health (NIH) (2019).
104. Williamson SC, Hartley AE, Heer R. A review of tasquinimod in the treatment of advanced prostate cancer. *Drug Des Devel Ther* (2013) 7:167–74. doi: 10.2147/DDDT.S31500
105. Shen L, Pili R. Tasquinimod targets suppressive myeloid cells in the tumor microenvironment. *Oncoimmunology* (2019) 8(10):e1072672. doi: 10.1080/2162402X.2015.1072672
106. Yoshioka Y, Mizutani T, Mizuta S, Miyamoto A, Murata S, Ano T, et al. Neutrophils and the S100A9 protein critically regulate granuloma formation. *Blood Adv* (2016) 1(3):184–92. doi: 10.1182/bloodadvances.2016000497
107. Nefedova Y, Fishman M, Sherman S, Wang X, Beg AA, Gabrilovich DI. Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res* (2007) 67(22):11021–8. doi: 10.1158/0008-5472.CAN-07-2593
108. Mourik BC, Leenen PJ, de Knegt GJ, Huizinga R, van der Eerden BC, Wang J, et al. Immunotherapy Added to Antibiotic Treatment Reduces Relapse of Disease in a Mouse Model of Tuberculosis. *Am J Respir Cell Mol Biol* (2017) 56(2):233–41. doi: 10.1165/rcmb.2016-0185OC
109. Parveen S, Siddharth S, Cheung LS, Kumar A, Murphy JR, Sharma D, et al. IL-4 receptor targeting as an effective immunotherapy against triple-negative breast cancer. *bioRxiv* (2020), 2020.2008.2005.238824. doi: 10.1101/2020.08.05.238824
110. Leukes V, Walzl G, du Plessis N. Myeloid-Derived Suppressor Cells as Target of Phosphodiesterase-5 Inhibitors in Host-Directed Therapeutics for Tuberculosis. *Front Immunol* (2020) 11:451. doi: 10.3389/fimmu.2020.00451
111. Maiga M, Agarwal N, Ammerman NC, Gupta R, Guo H, Maiga MC, et al. Successful shortening of tuberculosis treatment using adjuvant host-directed therapy with FDA-approved phosphodiesterase inhibitors in the mouse model. *PloS One* (2012) 7(2):e30749. doi: 10.1371/journal.pone.0030749
112. Wallis RS, Ginindza S, Beattie T, Arjun N, Likoti M, Edward V, et al. Preliminary Results of an Experimental Medicine Trial of Adjunctive Host-Directed Therapy in Adults with Moderately or Far-Advanced Rifampin-Susceptible Pulmonary Tuberculosis. *Am J Respir Crit Care Med* (2019) 199:A7388. doi: 10.1164/ajrccm-conference.2019.199.1_MeetingAbstracts.A7388
113. Cardona P, Cardona P-J. Regulatory T Cells in Mycobacterium tuberculosis Infection. *Front Immunol* (2019) 10:2139. doi: 10.3389/fimmu.2019.02139
114. Chen X, Zhou B, Li M, Deng Q, Wu X, Le X, et al. Zhang H *et al*: CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease. *Clin Immunol* (2007) 123(1):50–9. doi: 10.1016/j.clim.2006.11.009
115. Arce Vargas F, Furness AJS, Solomon I, Joshi K, Mekkaoui L, Lesko MH, et al. Fc-Optimized Anti-CD25 Depletes Tumor-Infiltrating Regulatory T Cells and Synergizes with PD-1 Blockade to Eradicate Established Tumors. *Immunity* (2017) 46(4):577–86. doi: 10.1016/j.immuni.2017.03.013
116. Ozeki Y, Sugawara I, Udagawa T, Aoki T, Osada-Oka M, Tateishi Y, et al. Transient role of CD4+CD25+ regulatory T cells in mycobacterial infection in mice. *Int Immunol* (2010) 22(3):179–89. doi: 10.1093/intimm/dxp126
117. Kumar P, Kumar A, Parveen S, Murphy JR, Bishai W. Recent advances with Treg depleting fusion protein toxins for cancer immunotherapy. *Immunotherapy* (2019) 11(13):1117–28. doi: 10.2217/imt-2019-0060
118. Sanseviero E, O'Brien EM, Karras JR, Shabaneh TB, Aksoy BA, Xu W, et al. Anti-CTLA-4 Activates Intratumoral NK Cells and Combined with IL15/IL15Rα Complexes Enhances Tumor Control. *Cancer Immunol Res* (2019) 7(8):1371–80. doi: 10.1158/2326-6066.CIR-18-0386
119. Barber DL, Sakai S, Kudchadkar RR, Fling SP, Day TA, Vergara JA, et al. Tuberculosis following PD-1 blockade for cancer immunotherapy. *Sci Transl Med* (2019) 11(475):eaat2702. doi: 10.1126/scitranslmed.aat2702
120. Fujita K, Terashima T, Mio T. Anti-PD1 Antibody Treatment and the Development of Acute Pulmonary Tuberculosis. *J Thorac Oncol* (2016) 11(12):2238–40. doi: 10.1016/j.jtho.2016.07.006
121. Reungwetwattana T, Adjei AA. Anti-PD-1 Antibody Treatment and the Development of Acute Pulmonary Tuberculosis. *J Thorac Oncol* (2016) 11(12):2048–50. doi: 10.1016/j.jtho.2016.10.008
122. Huang Z, Luo Q, Guo Y, Chen J, Xiong G, Peng Y, et al. Mycobacterium tuberculosis-Induced Polarization of Human Macrophage Orchestrates the Formation and Development of Tuberculous Granulomas In Vitro. *PloS One* (2015) 10(6):e0129744. doi: 10.1371/journal.pone.0129744
123. Abdollahi E, Momtazi AA, Johnston TP, Sahebkar A. Therapeutic effects of curcumin in inflammatory and immune-mediated diseases: A nature-made jack-of-all-trades? *J Cell Physiol* (2018) 233(2):830–48. doi: 10.1002/jcp.25778
124. Shiri S, Alizadeh AM, Baradaran B, Farhanghi B, Shanehbandi D, Khodayari S, et al. Dendrosomal curcumin suppresses metastatic breast cancer in mice by changing m1/m2 macrophage balance in the tumor microenvironment. *Asian Pac J Cancer Prev* (2015) 16(9):3917–22. doi: 10.7314/APJCP.2015.16.9.3917
125. Bai X, Oberley-Deegan RE, Bai A, Ovrutsky AR, Kinney WH, Weaver M, et al. Curcumin enhances human macrophage control of Mycobacterium tuberculosis infection. *Respirology* (2016) 21(5):951–7. doi: 10.1111/resp.12762
126. van Kampen SC, Wanner A, Edwards M, Harries AD, Kirenga BJ, Chakaya J, et al. International research and guidelines on post-tuberculosis chronic lung disorders: a systematic scoping review. *BMJ Global Health* (2018) 3(4):e000745. doi: 10.1136/bmjgh-2018-000745
127. Singh SK, Naaraayan A, Acharya P, Menon B, Bansal V, Jesmajian S. Pulmonary Rehabilitation in Patients with Chronic Lung Impairment from Pulmonary Tuberculosis. *Cureus* (2018) 10(11):e3664–4. doi: 10.7759/cureus.3664

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Longer-Term Omega-3 LCPUFA More Effective Adjunct Therapy for Tuberculosis Than Ibuprofen in a C3HeB/FeJ Tuberculosis Mouse Model

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Advancement in the understanding of inflammation regulation during tuberculosis (TB) treatment has led to novel therapeutic approaches being proposed. The use of immune mediators like anti-inflammatory and pro-resolving molecules for such, merits attention. Drug repurposing is a widely used strategy that seeks to identify new targets to treat or manage diseases. The widely explored nonsteroidal anti-inflammatory drug (NSAID) ibuprofen and a more recently explored pharmaconutrition therapy using omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs), have the potential to modulate the immune system and are thus considered potential repurposed drugs in this context. These approaches may be beneficial as supportive therapy to the already existing treatment regimen to improve clinical outcomes. Here, we applied adjunct ibuprofen and n-3 LCPUFA therapy, respectively, with standard anti-TB treatment, in a C3HeB/FeJ murine model of TB. Bacterial loads, lung pathology, lung cytokines/chemokines and lung lipid mediators were measured as outcomes. Lung bacterial load on day 14 post-treatment (PT) was lower in the n-3 LCPUFA, compared to the ibuprofen group (p = 0.039), but was higher in the ibuprofen group than the treated control group (p = 0.0315). Treated control and ibuprofen groups had more free alveolar space initially as compared to the n-3 LCPUFA group (4 days PT, p= 0.0114 and p= 0.002, respectively); however, significantly more alveolar space was present in the n-3 LCPUFA group as compared to the ibuprofen group by end of treatment (14 days PT, p = 0.035). Interleukin 6 (IL-6) was lower in the ibuprofen group as compared to the treated control, EPA/DHA and untreated

control groups at 4 days PT (p = 0.019, p = 0.019 and p = 0.002, respectively). Importantly, pro-resolving EPA derived 9-HEPE, 11-HEPE, 12-HEPE and 18-HEPE lipid mediators (LMs) were significantly higher in the EPA/DHA group as compared to the ibuprofen and treated control groups. This suggests that n-3 LCPUFAs do improve pro-resolving and anti-inflammatory properties in TB, and it may be safe and effective to co-administer as adjunct therapy with standard TB treatment, particularly longer-term. Also, our results show host benefits upon short-term co-administration of ibuprofen, but not throughout the entire TB treatment course.

Keywords: ibuprofen, C3HeB/FeJ mice, tuberculosis adjunctive treatment, repurposed drugs, pharmaconutrition, host-directed therapies, n-3 LCPUFAs

INTRODUCTION

Tuberculosis (TB) was the leading cause of death from a single infectious agent worldwide until the 2019 outbreak of the Covid-19 disease, surpassing human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) in 2018 (1). Tuberculosis causes an inflammatory reaction that damages the surrounding tissue, causing significant morbidity (2, 3). Host cellular immune responses are exploited (4) to promote the accumulation of permissive phagocytic cells while delaying activation of the acquired responses in TB (5, 6). Thus, therapy targeted towards modulating host inflammatory pathways to reduce abnormal or unwarranted inflammation and lung tissue destruction is a plausible host-directed intervention in TB treatment (7–9). Host-directed intervention administered in conjunction with conventional TB treatment can aid in tissue damage repair, preserve lung function, enhance the effectiveness of TB drug therapy and shorten treatment duration (9).

Recent animal model work suggests that the manipulation of host modulators with repurposed drugs, including pharmaconutrients, may lead to the improvement of TB outcomes associated with common TB morbidities (7, 10–12). Due to the limited advancement in novel TB drug development for more than 40 years now, except for the recent approval of bedaquiline, delamanid and pretomanid (13–15), the use of repurposed drugs to augment current TB therapy has gained much interest. This has led to an interest in exploring the use of ibuprofen as a repurposed drug, and more recently also long-chain omega-3 polyunsaturated fatty acids (n-3 LCPUFAs) as possible adjunct therapies in TB treatment (16–18). Ancillary treatments for diseases like TB associated with inflammation, using repurposed drugs, may ameliorate morbidity and possibly reduce mortality, and are likely to have additionally significant economic, social and survival benefits. Therefore, immune modulating drugs and pharmaconutrients are potentially important agents worth investigating to offer novel host adjunct therapies in TB management (19, 20).

Ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID), inhibits both COX 1 and 2, which metabolise both pro- and anti-inflammatory mediators. This drug, in the absence of TB drug treatment, reduced the percentage of affected lung area, bacillary load and granuloma formation, and increased survival in a C3HeB/FeJ mouse TB model (10, 21). Ibuprofen has also been shown to have no detrimental interaction with either rifampicin (R) or isoniazid (H), neither when used concomitantly with rifampicin, isoniazid, pyrazinamide and ethambutol (RHZE) during anti-tuberculosis therapy in mice (22, 23). However, long-term use of ibuprofen is associated with certain gastrointestinal, low risk cardiovascular, renal and hepatic adverse effects, although these side effects are associated with dose, associated medications, and the patient population (24, 25). Nevertheless, ibuprofen is still considered to have a relatively favourable safety profile among NSAIDs (24).

A possible safer anti-inflammatory or pro-resolving alternative to ibuprofen may be n-3 LCPUFAs. Long-chain n-3 PUFAs are present in oily fish and supplements in the form of EPA and DHA. Their ability to downregulate several aspects of inflammation suggests that these fatty acids might be important in controlling the development and severity of inflammatory diseases, and subsequently, have possible use as a component of a novel therapy approach to various diseases (26). We previously demonstrated that EPA/DHA supplementation lowered systemic and lung inflammation, and decreased lung bacterial burden in C3HeB/FeJ mice infected with *Mycobacterium tuberculosis (Mtb)* in the absence of standard TB treatment (17). However, there is currently no evidence on the interaction of a therapeutic dose of n-3 LCPUFAs with the first-line of anti-TB drugs.

In the present study, we hypothesised that co-administration of ibuprofen or n-3 LCPUFAs, together with standard TB antibiotics, are safe and would potentially improve the clinical outcome and pathology associated with TB. To this end, we investigated the effects of EPA/DHA and ibuprofen, administered as an adjunct to the standard antibiotic treatment-regimen in *Mtb*-infected C3HeB/FeJ mice. Our study is the first to have administered ibuprofen and n-3 LCPUFAs together with standard TB antibiotics, namely rifampicin, isoniazid, pyrazinamide and ethambutol, in the intensive phase, and also in conjunction with rifampicin and isoniazid in the continuation phase in a murine model, in line with TB treatment in humans. We found that EPA/DHA and ibuprofen as adjunct therapy reduce *Mtb* burden and suppress pulmonary immunopathology associated with TB, as compared to untreated controls, and may have the potential to limit TB associated inflammation and improve other clinical outcomes.

MATERIALS AND METHODS

Experimental Design and Dietary Conditioning of Animals

Forty-eight mice, aged 10-12 weeks, were conditioned on a standardised AIN-93G purified rodent diet for six weeks before infection. The mice were then infected with *Mtb* (50-70 CFU) by means of aerosol inhalation. Two weeks post-infection (PI), the mice were randomly allocated to the four treatment groups: 1) the treated control group received Rifafour® for 3 days (n = 12), followed by rifampicin and isoniazid for 11 days (n = 6); 2); the EPA/DHA group received an EPA/DHA-enriched diet plus Rifafour® for 3 days (n = 12), followed by the EPA/DHA-enriched diet plus rifampicin and isoniazid for 11 days (n = 6); 3); the ibuprofen group received ibuprofen plus Rifafour® for 3 days (n = 12), followed by ibuprofen plus rifampicin and isoniazid for 11 days (n = 6); and 4) the untreated control group received no treatment throughout the entire duration of the experiment (n = 12). Treatment was administered in two phases. In the first phase, ibuprofen and Rifafour® (150 mg rifampicin + 75 mg isoniazid + 400 mg pyrazinamide + 275 mg ethambutol) were administered by oral gavage, and in the second phase, ibuprofen as well as rifampicin and isoniazid (RH) were administered in drinking water (Cornell model). Treatment duration of phase one lasted for three days, representing the initial 2 months intensive phase TB treatment equivalent in humans with the same four medications, Rifafour®. Whilst phase two continued for another 11 days, representing the four months less intensive TB treatment phase (continuous phase) humans equivalence with two medications RH (27, 28). Each group consisted of 6 mice, in two independent experiments. All treatment groups received a standard AIN-93 diet; with the exception of the EPA/DHA-supplemented group that received the AIN-93 diet enriched with Incromega oil *ad libitum* as shown in **Figure 1** *(See* **Supplementary File 1** *for the detailed description of how the experiment was conducted).*

Experimental Mice Model and Ethics Statement

Male and female C3HeB/FeJ mice (obtained from Jackson Laboratory, Bar Harbor, ME), between 10 and 12 weeks of age, were randomly placed in groups of six in a standard type 2 long individually ventilated cage with filter tops, transparent red plastic mouse houses, dried wood shavings and shredded filter paper as floor coverings after infection. Mice were housed in a biosafety level III facility and were exposed to a temperature range set at 22 to 24°C and 12-to-12 hour light cycles. The study was approved by the AnimCare Animal Research Ethics Committee of North-West University, South Africa (ethics number: NWU-00055-19-S5), and the Animal Research Ethics Committee of the University of Cape Town, South Africa (ethics number: FHS AEC 019-023).

Aerosol Infection

The virulent *Mtb* H37Rv strain was cultured and stocks were prepared and stored at -80°C, as previously described (29). Mice were infected by nebulising with 6 ml of a suspension that contained 2.4 x 10^7 live bacteria in an inhalation exposure system (model A4224, Glas-Col) for 40 minutes. Four mice were euthanized to confirm the infection dose a day after infection, with each mouse being infected with around 50-70 *Mtb* colony-forming units (CFU).

Treatment Administration

All drugs used for treatment were either dissolved or suspended in distilled water and administered either by oesophageal gavage or in the drinking water. The following doses were used: in phase one (intensive phase), each mouse received 0.2 mL of antibiotic consisting of Rifafour® (150 mg rifampicin + 75 mg isoniazid + 400 mg pyrazinamide + 275 mg ethambutol) dissolved in 30 ml distilled water through oral gavage administration. In phase two (continuation phase), isoniazid (0.1g/L) and rifampicin (0.1g/L) were delivered to mice in drinking water (30). For the adjunct ibuprofen group, 0.05 g/L of ibuprofen (Nurofen® cherry flavour, purchased from local pharmacy) was administered *ad libitum* in phases one and two. Because of the bitter taste of rifampicin-isoniazid together with ibuprofen, 1% sucrose was added to drinking water for three groups in phase 2. The water consumption was measured in phase two to confirm equal drug intake in all three groups.

Blood and Tissue Collection

At the end of day 4 and 14 post-treatment, mice were euthanized by exposure to halothane, after which blood was collected *via* cardiac puncture. The blood was collected into EDTA-coated Microtainer® tubes (K2EDTA, 1000 µl, Becton Dickinson), then centrifuged at 8000 rpm. The peripheral blood mononuclear cells (PBMC) were collected from buffy coats and used for fatty acid (FA) analysis. The liver and lung lobes were removed aseptically and weighed before preparation. The left lung lobe was homogenized in saline and 0.04% Tween-80 for the analysis of the bacillary load and lung cytokines. The right superior and post-caval lung lobes were snap-frozen in liquid nitrogen and stored at -80°C for lung LM analyses. The right middle lobe was fixed in 10% neutral buffered formalin for histological analysis.

Bacteria Loads and Lung Histopathology

Lung lobes and spleen were aseptically removed, homogenized and serial dilutions prepared. Dilutions were plated onto Difco™ Middlebrook 7H10 Agar medium (BD Biosciences, Johannesburg, South Africa), with oleic acid-albumin-dextrose-catalase (OADC) supplementation and 0.5% glycerol. The CFU were counted 21 days after incubation at 37°C. The results were expressed as $\log_{10}$CFU. For the assessment of lung pathology, the right middle lobes of the lungs were fixed in 10% buffered formalin and embedded in paraffin wax after processing in Leica TP 1020 Processor for 24 hours. 3-µm-thick sections (three sections with 30 µm-apart) of the embedded tissues were cut and stained with hematoxylin-eosin (H&E) stain. The images were acquired in Nikon Eclipse 90i microscope and analysed with NIS-Elements AR software (Nikon Corporation, Tokyo, Japan) to determine the granulomatous area and free alveolar space as a percentage of the total lung tissue (31).

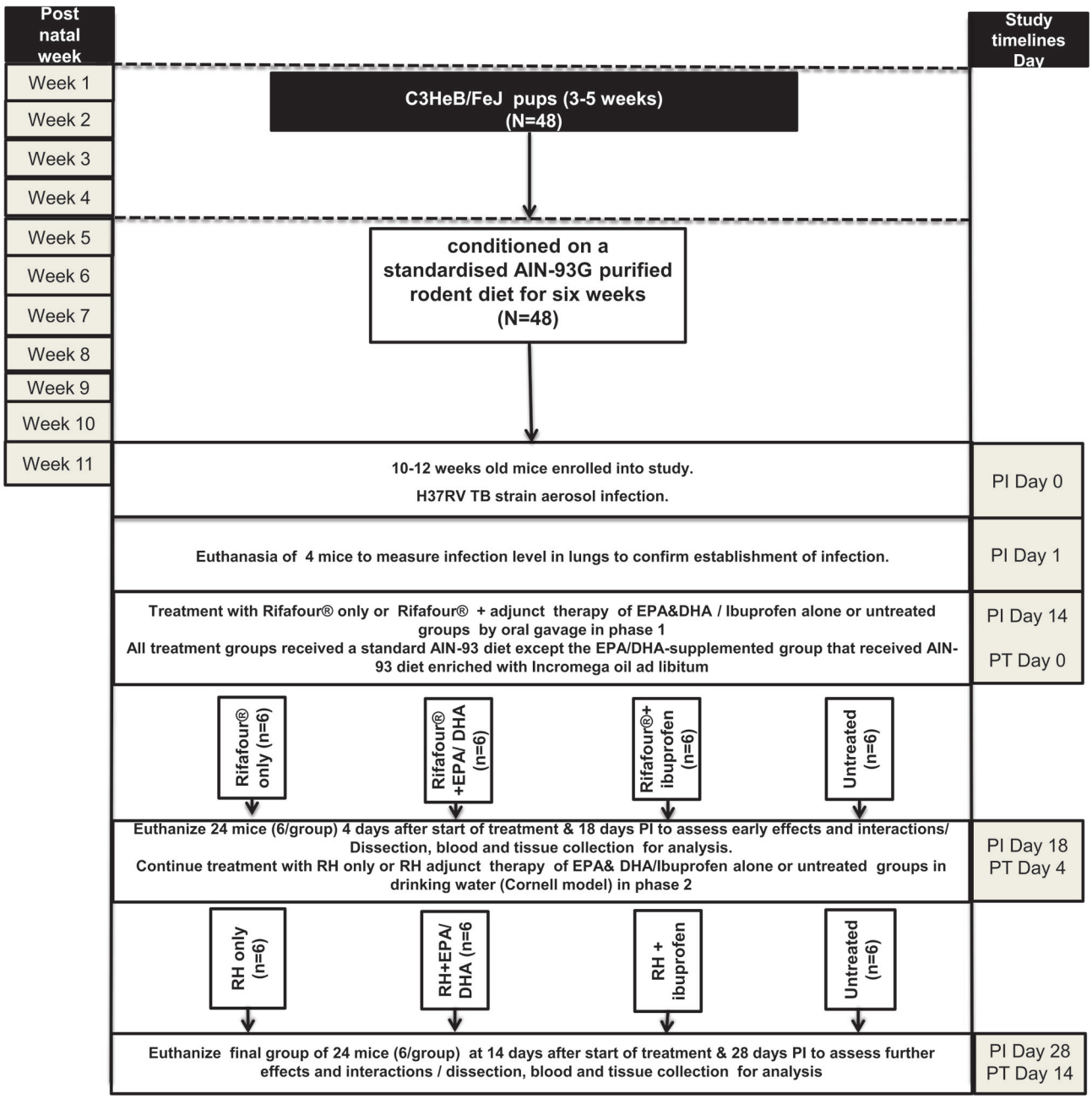

FIGURE 1 | Experimental design of the study. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Rifafour®, 150 mg rifampicin + 75 mg isoniazid + 400 mg pyrazinamide + 275 mg ethambutol; RH, rifampicin and isoniazid; N, number of mice; PT: post treatment; PI: post infection; AIN-93G formulation containing soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg diet and 8 g Fe/kg (40 ppm Fe); EPA/DHA supplemented diet contains soybean oil at 70 g/kg diet, coconut oil at 27 g/kg diet, Incromega TG4030 oil DHA 500 TG SR (minimum 44% of FA as EPA; minimum 28% of FA as DHA); untreated group received no treatment throughout the entire duration of the experiment.

Determination of Total Phospholipid Fatty Acid Composition

Total phospholipid FA composition was analysed by gas chromatography-tandem mass spectrometry as previously described (17). Fatty acids were extracted from ~200 µL PBMC with chloroform:methanol (2:1, v:v; containing 0.01% BHT) by a modification of the method of Folch et al. (32). The composition of EPA (20:5n-3), DHA (22:6n-3), arachidonic acid (AA, 20:4n-6), osbond acid (22:5n-6), total n-3 LCPUFA, total omega-6 long-chain polyunsaturated fatty acid (n-6 LCPUFA) and total

n-6/n-3 LCPUFA ratio as a percentage of total phospholipid fatty acids were determined.

Extraction and Quantification of Lipid Mediators

Lipid mediators in crude lung homogenates were extracted and analysed by liquid chromatography-tandem mass spectrometry. Lipid mediators were extracted from lung tissue, in 10 µl/mg homogenization buffer (phosphate-buffered saline), with solid-phase extraction using Strata-X (Phenomenex, Torrance, CA). The method was modified for Strata-XSPE columns from a previously described method (33). Data were quantified with Masshunter B0502, using external calibration for each compound and internal standards [PGD2-d4, PGE2-d4, PGF2-d4 and 5- and 12-HETE-d8; 1000 pg of each (Cayman Chemicals, Ann Arbor, MI)] to correct for losses and matrix effects. Extracted and quantified LMs included: DHA-derived pro-resolving 17-hydroxydocosahexaenoic acid (17-HDHA) and protectin D1 (PD1); EPA-derived pro-resolving LM intermediates 2-, 5-, 9-, 11-, 15- and 18-hydroxyeicosapentaenoic acid (HEPE); AA-derived pro-inflammatory intermediates 5-, 8-, 9-, 11-, 12- and 15-hydroxyeicosatetraenoic acid (HETE); AA-derived prostaglandin D1 (PGD1), prostaglandin E2 (PGE2), prostaglandin F2α (PGFα2); and thromboxane B2 (TBXB2).

Measurement of Cytokine/Chemokine in Lung Homogenates

Lung homogenates were centrifuged at 3000g and supernatants were stored at -80°C until further analysis. Interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, monocyte chemoattractant protein 1 (MCP-1), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1-alpha (MIP-1 α) and regulated on activation normal T-cell expressed and secreted (RANTES) levels were measured using the Quansys Biosciences Q-Plex™ Mouse Cytokine Screen (West Logan, UT, USA) 16-plex array for mouse cytokines according to manufacturer's instructions. Arrays were analyse using the Q-View Imager Pro and Q-View Software.

Statistical Analyses and Data Representation

Statistical analyses were computed using IBM SPSS Statistics software version 23 and GraphPad Prism Software version 8.2 (GraphPad Software Inc., La Jolla, CA, USA). A minimum sample size of 6 per treatment group was calculated for a two-sided alpha of 0.05 and a power of 80% as previously described (34). The normality of the data was evaluated by histogram visual inspection and Kolmogorov-Smirnov test. All data are presented as mean ± standard error of the mean (SEM). Treatment effects were examined using one-way analysis of variance (ANOVA) and the Tukey post-hoc test. Statistically significant differences are designated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

RESULTS

Ibuprofen Adversely Affected Bactericidal Efficacy of TB Drugs, However, EPA/DHA Did Not

We investigated whether EPA/DHA increased the efficacy of TB drugs. Mice were infected with *Mtb* for 2 weeks and then administered with TB drugs to euthanize at 4 and 14 days post-treatment (**Figure 2**). Bacillary load in lung and spleen was determined to ascertain whether EPA/DHA or ibuprofen co-administration interfered with the standard TB treatment, Rifafour and RH. Lung and spleen bacterial burdens were similar between standard TB treatment and adjunct EPA/DHA treatment groups (**Figures 2A, B**). Interestingly, lung bacterial loads were lower on day 14 PT in the EPA/DHA group, compared to the ibuprofen group ($p = 0.039$). This suggests that it may be safe to co-administer EPA/DHA as adjunct therapy with standard TB treatment, particularly in the longer term. However, lung burdens were higher in the adjunct ibuprofen group, as compared to the standard treatment control group on day 14 PT ($p = 0.032$), with a similar trend on 4 PT ($p = 0.051$), suggesting it may not be suitable to co-administer ibuprofen during standard TB treatment with regards to lung bacterial burdens. However, spleen burden was lower in the ibuprofen group, compared to the other treatment groups, although not significantly. Both lung and spleen burdens in the non-treated controls remained higher during the infection, as expected (**Figures 2A, B**).

Ibuprofen and EPA/DHA as an Adjunct Therapy Improved Lung Pathology

Lung histology sections were stained with H&E to determine tissue pathology and quantitate the percentage of free alveolar space. We found significantly more free alveolar space in the treated control and ibuprofen groups than in the EPA/DHA group at the initial stage (day 4 PT; $p = 0.011$ and $p = 0.002$, respectively), but significantly more alveolar space in the EPA/DHA and the treated control groups than in the ibuprofen group after 14 days of treatment ($p = 0.035$ and $p < 0.001$, respectively). This indicates that adjunctive ibuprofen may be beneficial in the early phase, whereas EPA/DHA may be more beneficial in the longer term, considering the improvement to tissue pathology. As expected, the untreated control group had significantly less free alveolar space than the treated groups throughout entire course of the study (**Figures 2C, D**).

Dietary EPA/DHA Supplementation and Ibuprofen Treatment Altered Lung Inflammatory Cytokines During Co-Administration With Standard TB Therapy

The concentrations of cytokines and chemokines in the lung homogenates were measured to assess local inflammatory effects. Cytokines/chemokines are responsible for recruitment of polymorphonuclear cells (PMN), monocytes and lymphocytes, which are involved in inflammatory reactions, mediate adaptive immune cell responses, act as anti-inflammatory agents to

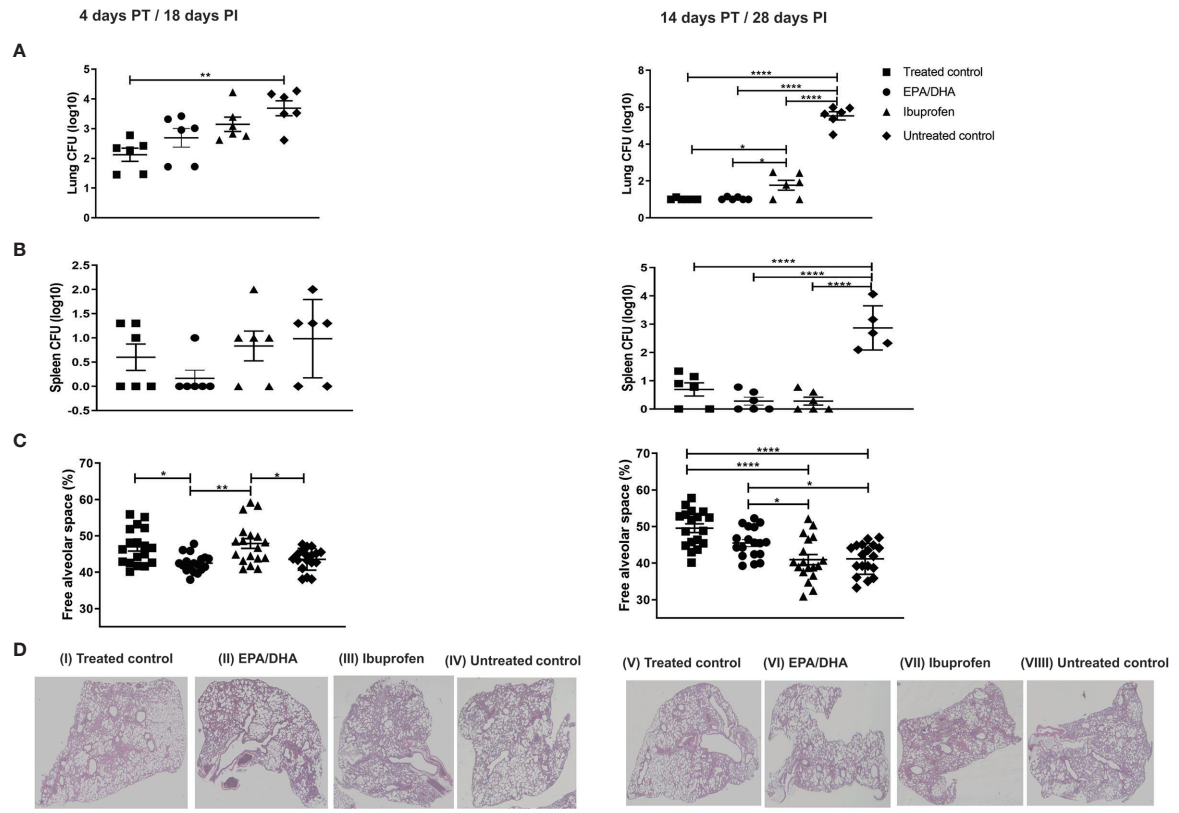

FIGURE 2 | Effect of adjunctive treatment on lung, spleen bacillary load and lung histopathology. **(A)** Lung bacillary load, **(B)** spleen bacillary load, **(C)** percentage of free alveolar space, and **(D)** haematoxylin-eosin stained sections of the lungs of selected representative group, after 18 [D(I-IV)] and 28[D(V-VIII)] days PI treatment period. All mice except untreated controls were on standard TB antibiotics Rifafour® for 4 days of treatment, then rifampicin and isoniazid (RH) for 10 days. The data represented as mean ± SEM of n=6 mice/group and representative of two independent experiments. One-way ANOVA followed by the Tukey post-hoc test was used to compare means, significance at $^{*}P < 0.05$, $^{**}P < 0.01$, ****P < 0.0001. CFU, colony-forming units; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid PT, post-treatment; PI, post-infection.

resolve inflammation and limit tissue damage during *Mtb* infection (35). Interferon γ (IFN-γ) was significantly lower in all the treated groups at 4 days (treated control, $p < 0.001$; EPA/DHA, $p = 0.003$ and ibuprofen, $p < 0.001$) and 14 days (treated control, $p = 0.005$; EPA/DHA, $p = 0.003$ and ibuprofen, $p = 0.017$) PT, compared to the untreated control mice (**Figure 3A**). Interleukin 1 alpha (IL-1α) was significantly higher in the EPA/DHA group at 4 days PT ($p = 0.001$) and lower in the EPA/DHA and ibuprofen groups at 14 days PT ($p = 0.012$ and $p = 0.028$, respectively), compared to the untreated control mice (**Figure 3B**). Interleukin 1 beta (IL-1β) was significantly lower in the EPA/DHA group at 14 days PT ($p = 0.033$), compared to the untreated control mice, while no difference was observed at the earlier time point (**Figure 3C**). Interleukin 6 (IL-6) was lower in the ibuprofen group at 4 days PT, compared to the treated control, EPA/DHA and untreated control groups ($p = 0.019$, $p = 0.019$ and $p = 0.002$ respectively). Subsequently, IL6 was lower in both the EPA/DHA and ibuprofen groups by day 14 PT than in the treated control group ($p = 0.012$ and $p = 0.012$, respectively, **Figure 3D**). Concentrations of IL2 were lower in the EPA/DHA and ibuprofen groups than in the treated control group by day 14 PT ($p = 0.002$ and $p = 0.039$, respectively, **Figure 3E**). This suggests that the effect of adjunct EPA/DHA administration on the lung inflammatory cytokine profile was more profound at the later phase of treatment consistent with lower lung burdens, whereas, ibuprofen adjunct treatment decreased inflammatory cytokines in the early phase of treatment.

Granulocyte-macrophage colony stimulating factor (GM-CSF) was lower in the EPA/DHA group, compared to the ibuprofen group by day 14 PT ($p = 0.003$) and higher in the ibuprofen group than in the treated controls ($p = 0.025$, **Figure 3F**), which suggests an interesting regulation between GM-CSF secretion and COX1/COX2 inhibition. Macrophage inflammatory protein 1-alpha (MIP1-α) was lower in the EPA/DHA group by day 14 PT ($p = 0.048$, **Figure 3G**). Monocyte chemoattractant protein-1(MCP1) was higher in the EPA/DHA and ibuprofen groups, compared to the treated controls at day 4 PI ($p = 0.002$ and 0.012, respectively), and higher in the treated controls and EPA/DHA group than in the untreated controls by day 14 PT ($p = 0.009$ and 0.008 respectively, **Figure 3H**). These results support varied effects of ibuprofen and EPA/DHA adjunct treatment on inflammatory chemokines.

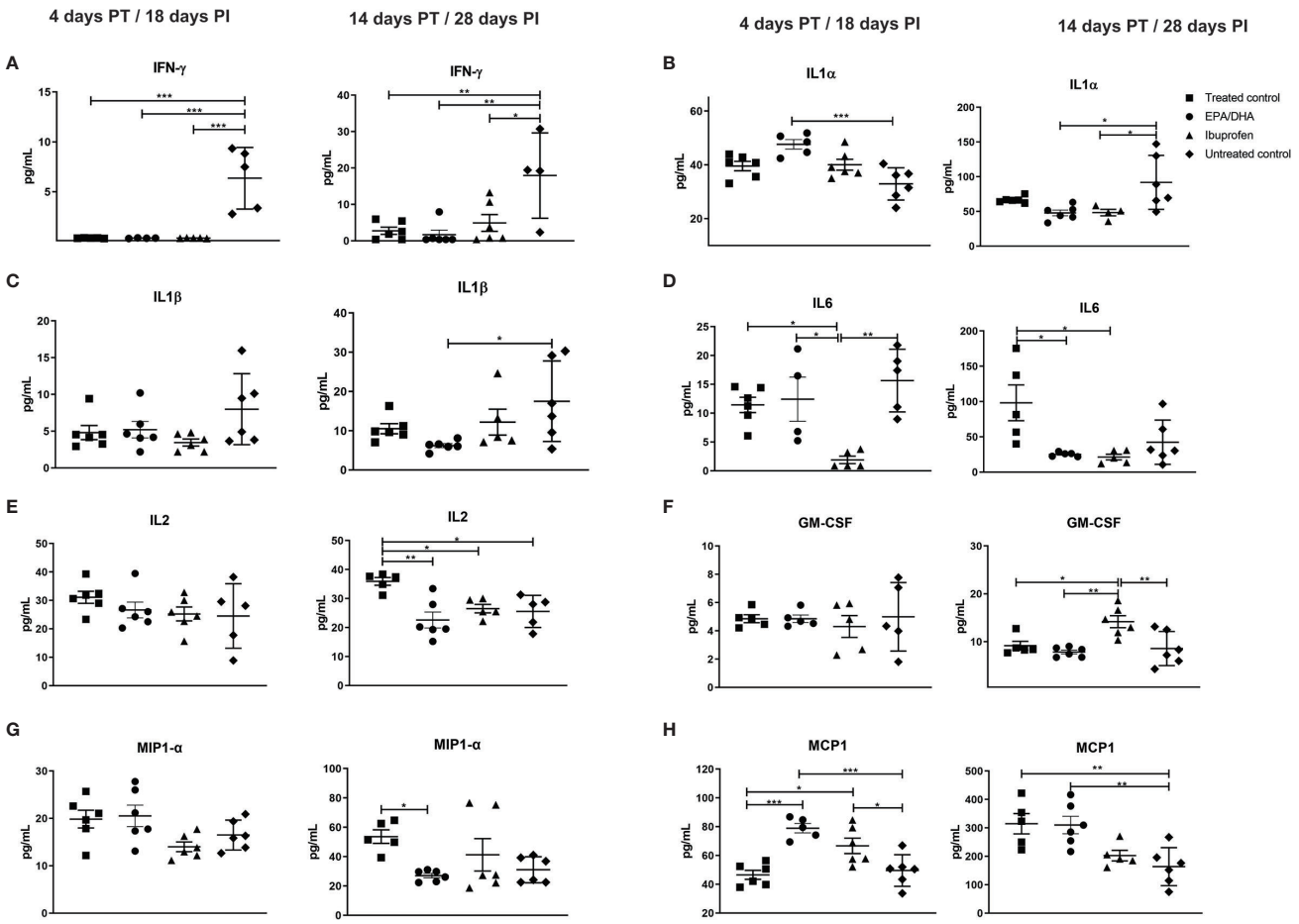

FIGURE 3 | Treatment effects on lung cytokine/chemokines levels. **(A)** IFN-γ, **(B)** IL-1α, **(C)** IL-1β, **(D)** IL6, **(E)** IL2, **(F)** GM-CSF, **(G)** MIP-1 α and **(H)** MCP-1. All data are presented in pg/mL. All mice except untreated controls were on standard TB antibiotics Rifafour® for 4 days of treatment, then rifampicin and isoniazid (RH) for 10 days. All values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test was used to compare means, *P < 0.05, **P < 0.01, ***P < 0.001. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; IL, interleukin; IFN- γ, interferon γ; IL-1α, interleukin 1 alpha; IL-1β, Interleukin 1 beta, GM-CSF, granulocyte-macrophage colony-stimulating factor; MIP-1 α, macrophage inflammatory protein 1-alpha and MCP-1, Monocyte chemoattractant protein-1; PT, post-treatment; PI, post-infection.

Interleukin-5 (IL5) was higher in the treated controls and ibuprofen group than untreated controls on days 4 PT ($p = 0.034$ and $p = 0.016$, respectively, **Supplementary Figure 1A**). Interleukin-10 (IL-10) was lower in the ibuprofen than in the treated control ($p = 0.044$, **Supplementary Figure 1B**), while IL-12 was lower in the EPA/DHA group than in the untreated controls ($p = 0.023$, **Supplementary Figure 1C**) both at day 14 PT. Interleukin-4 (IL-4), IL-17, TNF-α, IL-3 and RANTES all showed lower trends in the EPA/DHA treatment groups than in the other treatment groups by day 14 PT, although not significantly (**Supplementary Figures 1D–H**).

Effects of Treatments on PBMC Fatty Acid Composition

Table 1 shows the phospholipid FA composition of PBMC of four groups of mice measured at 4 and 14 days PT. The change in membrane phospholipid FA composition of immune cells plays a vital role in immune and inflammatory responses (16). The EPA/DHA group had a significantly higher EPA composition than all other groups (day 4 PT, all $p < 0.001$ and day 14 PT, all $p < 0.001$), while DHA was higher in the ibuprofen group than in the untreated controls at day 18 PI ($p = 0.007$). The composition of the total n-3 LCPUFA in the EPA/DHA treatment group was lower than that of the untreated controls (day 4 PT, $p = 0.003$), but was higher in the EPA/DHA treatment group after 14 days of treatment ($p = 0.039$). Regarding n-6 PUFAs, the ibuprofen treatment group had higher total n-6 LCPUFAs (day 4 PT, $p = 0.052$ and day 14 PT, $p < 0.001$) and total n-6/n-3 LCPUFA ratio (day 4 PT, $p = 0.009$ and day 14 PT, $p = 0.001$) compositions, compared to the EPA/DHA treatment groups over the course of infection at different time points. These trends were similar for the AA (day 4 PT, p=0.006 and day 14 PT, $p < 0.001$) and osbond acid (day 4 PT, $p = 0.19$ and day 14 PT, $p = 0.003$) compositions, respectively. Consistent with n-3 levels, the

TABLE 1 | Phospholipid fatty acid composition of PBMC in *Mtb*-infected C3HeB/FeJ mice receiving EPA/DHA or ibuprofen adjunct treatment at different time points[#].

	4 days post-treatment				
Fatty acids	**Rifafour**	**Rifafour + EPA/DHA**	**Rifafour + ibuprofen**	**untreated control**	**P- value**
20:5n-3 (EPA)	0.40 ± 0.02[b]	0.60 ± 0.02[a]	0.35 ± 0.01[b]	0.41 ± 0.02[b]	<0.001
22:6n-3 (DHA)	11.38 ± 0.32[b]	11.54 ± 0.19[b]	12.00 ± 0.20[a]	12.67 ± 0.21[a,b]	0.007
Total n-3 LCPUFA	12.56 ± 0.32[b]	12.99 ± 0.17[b]	13.12 ± 0.19[a,b]	14.05 ± 0.22[a]	0.003
20:4n-6 (AA)	17.72 ± 0.37[b]	18.38 ± 0.38[a,b]	19.31 ± 0.26[a]	17.83 ± 0.06[b]	0.006
22:5n-6 (osbond)	0.93 ± 0.60	0.89 ± 0.04	0.92 ± 0.03	1.03 ± 0.02	0.19
Total n-6 LCPUFA	22.22 ± 0.50[b]	23.22 ± 0.46[a,b]	23.82 ± 0.27[a]	22.83 ± 0.16[a,b]	0.052
Total n-6/n-3 LCPUFA ratio	1.77 ± 0.05[a]	1.79 ± 0.03[a]	1.82 ± 0.03[a]	1.63 ± 0.02[b]	0.009
	14 days post-treatment				
	RH	**RH + EPA/DHA**	**RH + ibuprofen**	**untreated control**	**P- value**
20:5n-3 (EPA)	0.32 ± 0.0[b]	0.74 ± 0.08[a]	0.33 ± 0.00[b]	0.35 ± 0.00[b]	<0.001
22:6n-3 (DHA)	11.87 ± 0.12	12.19 ± 0.37	12.50 ± 0.13	12.26 ± 0.21	0.23
Total n-3 LCPUFA	13.12 ± 0.11[b]	14.12 ± 0.38[a]	13.90 ± 0.19[a,b]	13.76 ± 0.24[a,b]	0.039
20:4n-6 (AA)	18.26 ± 0.13[a]	17.24 ± 0.24[b]	18.83 ± 0.13[a]	18.40 ± 0.08[a]	<0.001
22:5n-6 (Osbond)	1.18 ± 0.03[a,b]	1.07 ± 0.03[b]	1.24 ± 0.03[a]	1.27 ± 0.04[a]	0.003
Total n-6 LCPUFA	23.69 ± 0.15[b]	22.51 ± 0.22[c]	24.56 ± 0.17[a]	23.99 ± 0.16[a,b]	<0.001
Total n-6/n-3LCPUFA ratio	1.81 ± 0.01[a]	1.60 ± 0.05[b]	1.77 ± 0.02[a]	1.75 ± 0.02[a]	0.001

[#]*Values are reported as mean ± SEM percentage of total fatty acids from one experiment shown as representative of two independent experiments (n = 6 per group). Means in a row without common superscript letters differ significantly, P < 0.05. One-way ANOVA, with Tukey post hoc test was used to test effects between groups. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; N, total number of mice used in experiment; PBMC, peripheral blood mononuclear cell; Rifafour, rifampicin 150 mg + isoniazid 75 mg + pyrazinamide 400 mg + ethambutol 275 mg; RH, rifampicin and isoniazid.*

effects of EPA/DHA treatment on n-6 levels were more profound on day 28 PI. The higher levels of pro-resolving and anti-inflammatory FAs, such as the n-3 LCPUFAs, present in the membrane phospholipid of the EPA/DHA treatment group, together with the reduced amounts of pro-inflammatory FAs, such as AA, noted in later time points, suggests the EPA/DHA adjunct treatment in TB may be more effective than ibuprofen co-treatment in terms of decreasing exaggerated inflammation.

Dietary EPA/DHA Treatment Elevated Pro-Resolving and Reduced Pro-Inflammatory Lung Lipid Mediators, as Compared to Ibuprofen

Crude lung homogenate LMs were measured in order to determine the various treatment effects at the site of disease (the lung). The concentration of EPA-derived PGE3 in the EPA/DHA treated group was comparatively higher than the ibuprofen and the treated control groups on both day 4 PT ($p = 0.002$ and $p = 0.014$, respectively) and day 14 PT ($p = 0.046$ and $p = 0.008$, respectively; **Figure 4A**). Similarly, higher concentrations were observed in the EPA/DHA group for EPA-derived pro-resolving LM intermediates 9-HEPE (day 4 PT, EPA/DHA vs ibuprofen, $p = 0.017$), 11-HEPE (day 4 PT, EPA/DHA vs ibuprofen, $p = 0.005$; EPA/DHA vs treated control, $p = 0.011$ and EPA/DHA vs untreated, $p = 0.022$), 12-HEPE (day 4 PT, EPA/DHA vs ibuprofen, $p = 0.035$), and 18-HEPE (day 4 PT, EPA/DHA vs ibuprofen, $p = 0.005$ and EPA/DHA vs treated control, $p = 0.006$) (**Figures 4B–E**).Trends of higher concentrations of DHA-derived pro-resolving PD1 (**Figure 4F**) and 17HDHA (**Supplementary Figure 2A**) LMs were seen more in the EPA/DHA group than in the other treatment groups, although not significantly.

Pro-inflammatory AA-derived PGD2 was significantly lower in the ibuprofen group than the untreated control group after 14 days of treatment (**Figure 4G**), while PGE2 was significantly higher in all the treatment groups than the untreated control group after 4 days of treatment (**Figure 4H**). No significant differences were seen in the treatment effects of AA-derived pro-inflammatory intermediates 5-, 8-, 9-, 11-, 12- and 15-HEPE among the treatment groups (**Supplementary Figures 2D–H**).

DISCUSSION

Improved host-directed therapies (HDTs) to augment standard TB treatment has gained attention of late, due to its potential to overcome the obstacles faced by current antibiotic therapies (9). This approach, amongst other mechanisms, aims to improve the host defence mechanisms, and/or modulate excessive inflammation, by altering the host's response, rather than targeting the *Mtb* itself (36, 37). Recently, much research was done determining the effects of various repurposed drugs as adjunctive agents (4, 38, 39), including n-3 LCPUFAs (17, 40, 41) and ibuprofen (10, 42), with varying results in the field of TB.

In the current investigation we determined that the co-administration of EPA/DHA, together with standard TB antibiotic treatment, did not interfere with the bactericidal effects of antibiotics. Moreover, this adjunct treatment approach reduced the percentage of affected lung area compared to standard therapy after two weeks of treatment. EPA/DHA lowered lung inflammation while increasing the production of pro-resolving LMs. This suggests that it may be safe and effective against tuberculosis to co-administer EPA/DHA as adjunct therapy with standard TB treatment. However, ibuprofen adjunct therapy appears to have attenuated the effect

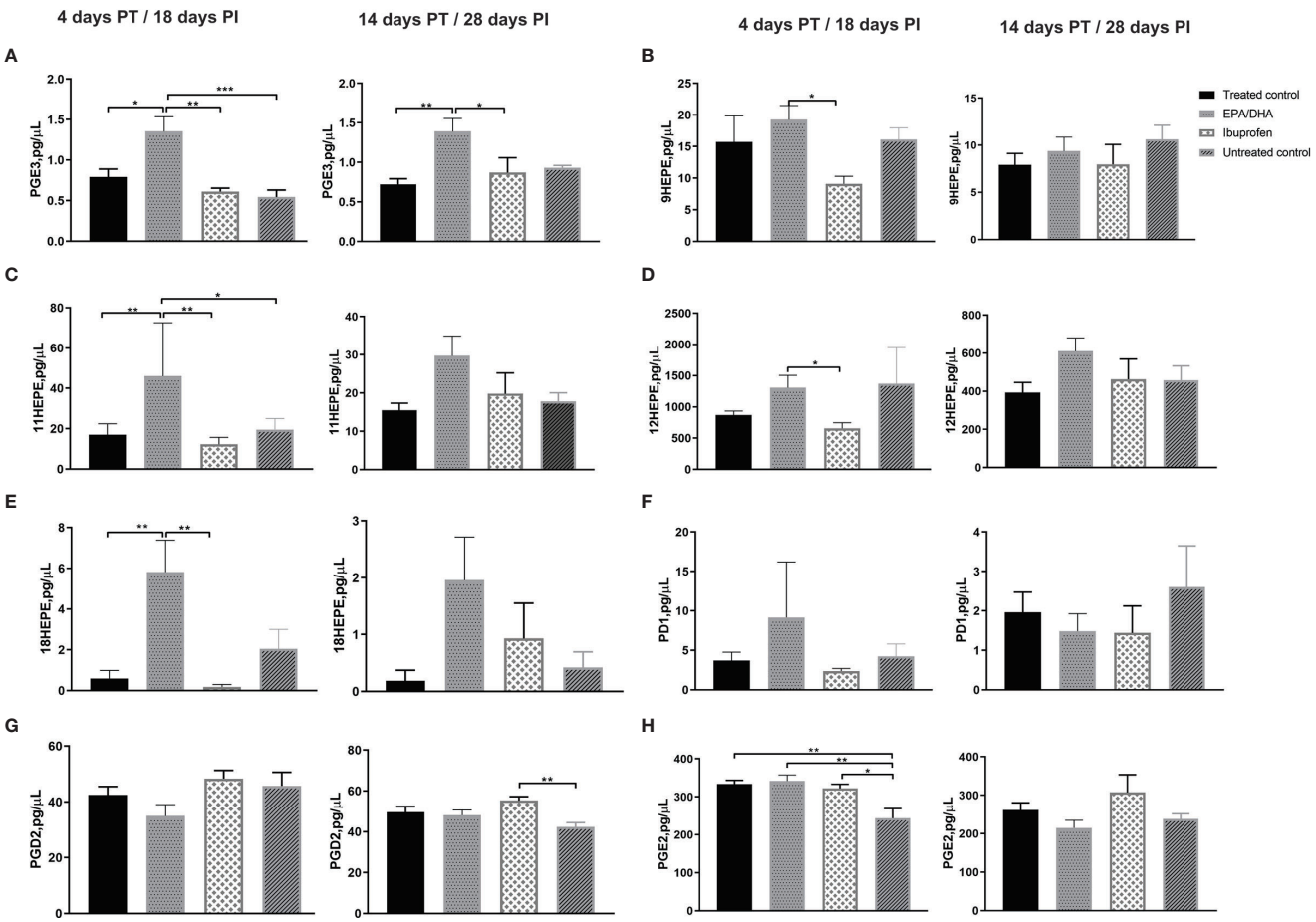

FIGURE 4 | Treatment effects of lipid mediators in crude lung homogenate at the local site of intervention. **(A)** PGE3, **(B)** 9-HEPE, **(C)** 11-HEPE, **(D)** 12-HEPE, **(E)** 18-HEPE, **(F)** PD1, **(G)** PGD2 and **(H)** PGE2. All data are presented in pg/µL. All mice, except untreated controls, were on standard TB antibiotics Rifafour® for 4 days of treatment, then rifampicin and isoniazid (RH) for 10 days. All values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=6 per group). One-way ANOVA followed by Tukey's post-hoc test was used to compare means, *P < 0.05, **P < 0.01 and ***P < 0.001. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; PGD2, prostaglandin D2; PGE3, Prostaglandin E3; PD1, Protectin D1 and PGE_2, prostaglandin E_2; PT, post treatment; PI, post infection.

of the TB antibiotics on lung bacterial burden and resulted in a reduced free alveolar space after the initial fourteen days of co-administration, already showing a trend towards the former effect at four days. Nonetheless, ibuprofen did suppress TB lung pathology by decreasing inflammatory cytokines, markedly IL6, in the early phase of treatment which also likely caused alveolar space to be higher in the ibuprofen group compared to the EPA/DHA group at day 4. This suggests that ibuprofen may be beneficial in reducing inflammation, thus preventing TB associated inflammatory pathology in the early phase of treatment. However, considering ibuprofen's possible interference with TB drug efficacy, its suitability as adjunct long-term treatment seems limited.

Morbidity and mortality in TB are associated with a failure to resolve lung immunopathology, due to the local inflammatory response of the host to ongoing *Mtb* infection (43, 44). This could be resolved with adjunctive therapy by improving the antibiotic efficacy (45). Concerning lung pathology, EPA/DHA together with TB antibiotics in the current study significantly reduced bacteria burden and mitigated excess pulmonary inflammatory damage in C3HeB/FeJ mice when compared to the ibuprofen treatment group, as indicated by the significantly less free alveolar space observed in the lungs of the ibuprofen group, compared to the EPA/DHA and treated control groups after 14 days of treatment. A similar observation was made in our previous work, where EPA/DHA supplementation alone, in the absence of TB antibiotics, resulted in enhanced bactericidal effects and inflammation resolution (17). This could be related to the enhanced phagocytic ability of immune cells *via* special pro-resolving mediators (SPMs): resolvins and protectins (46). These SPMs have been found to stimulate phagocytosis of bacteria, both killing and clearing (47, 48). The inflammation lowering effects observed in our study further demonstrates the beneficial effect of n-3 LCPUFAs as immuno-resolvents (49) with anti-inflammatory properties (50). Earlier murine macrophage-like cell lines and animal models, however, have

reported mixed results. Similarly to our findings, 8-week old female BALB/c mice on n-3 LCPUFA-supplemented diet (EPA content 1.5% and DHA 1.1% of total energy), in the absence of TB antibiotics, showed reduced bacterial loads (CFU) in the lung and spleen at 21 post-infection (40). However, on the contrary murine macrophage-like cell lines infected with virulent H37Rv *Mtb* and treated with DHA only, had higher bacterial loads compared with the control group at 3 days post-infection (51). A possible reason for these differences may be due to the one experiment using cell lines; the other was done using an animal model. However, fat-1 transgenic mice, which endogenously produce n-3 PUFAs, infected with virulent H37Rv Mtb *via* the aerosol route, also had increased bacterial loads in the spleen at 4-, 8- and 12-weeks post-infection (41). This observation could partly be explained by diminished activation, recruitment and anti-mycobacterial immune response in the fat-1 mice, resulting in reduced resistance to tuberculosis (52, 53). In our model, dietary supplementation of EPA/DHA slightly shifted the n-6/n-3 ratio towards n-3 LCPUFAs but not as exaggerated as seen in fat-1 transgenic mice (41). Since excess amounts of n-3 LCPUFAs can be unfavourable when treating bacterial infection, more subtle changes by dietary supplementation of EPA/DHA, may serve better for reducing the inflammation and bacterial load.

The results also show that ibuprofen treatment appears to have attenuated the effect of the TB antibiotics in the lungs for the first 14 days of treatment. These findings were in congruence with the findings of Mortensen et al., who observed that treatment with the cyclooxygenase inhibitor (COXi); ibuprofen did not reduce the bacterial burden in the lungs and spleen after aerosol infection in a CB6F1 mouse model. They also observed little or no impact on inflammation (54). The researchers argued that the route of infection, rather than the dose of ibuprofen was a cause for the observed outcome, as a similar dose of *Mtb* Erdman intravenously infected CB6F1 mice, showed a reduced pulmonary bacterial burden, as well as decreased lung infiltration of neutrophils, using the same intervention. Although the administration of ibuprofen alone was shown to reduce the percentage of affected lung area by alleviating excessive inflammation, and also reduce the bacillary load in mice infected with the intravenous route (10), it has also been demonstrated to have no direct bactericidal activity against *Mtb* (22, 42). Notwithstanding, our findings do suggest that ibuprofen does confer some advantage to the host by limiting TB-associated inflammation in the short-term.

The supplementation of n-3 LCPUFAs in this study resulted in a comparatively elevated cell membrane composition of pro-resolving lung LM, most profoundly noticeable at 4 days post treatment (as indicated by elevated concentrations of the less inflammatory EPA-derived PGE3 and EPA-derived pro-resolving LM intermediates: 9-HEPE, 11-HEPE, 12-HEPE and 18-HEPE, compared to the ibuprofen and the treated control groups). Additionally, a decreased trend of the lung pro-inflammatory AA-derived lipid mediators PGE2 and PGF2α in the EPA/DHA treatment, comparative to the treated control and the ibuprofen groups after 14 days (although not statistically significant) was observed. A study by Mancuso et al. reported a similar finding, where fish oil altered pro-resolving lipid mediators in the bronchoalveolar lavage fluids (55). There was also a corresponding significant reduction in the pro-inflammatory cytokine concentrations of IL-2 and IL-6 with EPA/DHA supplementation, as compared to the treated controls. In turn, reduced IL-2 levels can result in lower T cell proliferation and decreased activation of $CD8^+$ effector T cells (56). Reduced IL-2 levels can also favour memory T cell formation over effective T cells, which in turn can lead to a dampening of excessive inflammatory responses (57, 58). Similarly, increased levels of IL-6 have been shown to correlate to human disease progression due to its role in inflammation and tissue damage (59, 60). There was a similar observation with the ibuprofen treatment group, where IL-2 and IL-6 were reduced, compared to the treated controls. Worth noting was the significantly reduced concentrations of IL-6 observed in the ibuprofen treatment group at four days post-treatment, compared with the EPA/DHA supplemented group, supporting the previously observed association of ibuprofen treatment on IL-6 levels, as was seen in cystic fibrosis patients (61). Likewise, the pro-inflammatory lipid mediators 5-, 8-, 9-, 12- and 15- HETE were also reduced in ibuprofen treatment after four days, although not significantly (data not shown).

Initially, there was a higher production of DHA-derived pro-resolving 17HDHA and PD1 in the EPA/DHA group, compared to the other treatment groups. This could possibly explain the lowering of the pro-inflammatory mediator PGE_2 (62) and inhibition of Th1 type cytokine IFN-γ (63). PD1 has demonstrated to be a potent agonist of resolution of inflamed tissues and might have therapeutic potential when sustained inflammation and/or impaired resolution are constituents of pathologic pathways (64). PD1 also blocks airway hyper-responsiveness, counter-regulating signalling in an allergic airway, leading to a possible new therapeutic strategy for modulating inflammation in the asthmatic lung (62).

Monocyte chemotactic protein-1 (MCP-1) was significantly higher with EPA/DHA and ibuprofen treatments, in the initial stages of treatment when compared to the treated controls, and continued to increase over the treatment period. MCP-1 has, however, been shown to recruit and direct leukocyte movement during inflammation, and may have a negative influence on T-cell immunity (65). Furthermore, tuberculosis severity is associated with higher levels of MCP-1, since MCP-1 can induce recruitment of permissive monocytes and *Mtb* localisation to the lung parenchyma (66, 67). However, in the presence of antibiotic pressure, the lung bacterial burden is reduced, which may override any negative impact of elevated MCP-1 levels in the lungs. In a recent study, it has been shown that MCP-1 can also polarise alveolar macrophages to egress from the airway and interact with other immune cells around TB granulomas to gain killing effector functions (68).

Even though most of the inflammatory cytokine profiles were similar in the EPA/DHA group when compared to the other treatment groups, a large change was seen in the pro-resolving LM levels. Hence, our findings support the assertion that n-3

LCPUFAs have both inflammation and pro-resolving properties, and supplementation with this will not inhibit the host's natural immune and inflammatory responses necessary for protection against *Mtb*. This is in agreement with a study by Serhan et al., which showed that SPMs, which are derivatives of EPA/DHA, are not immunosuppressive and do not block inflammation, but instead produce pro-resolving effects (46). Likewise, the effect of ibuprofen on the inflammatory cytokines IL-6 and IL-2, thus supports its ability to reduce inflammation and resolve host-mediated immune pathology (54, 69) *via* actions of cyclooxygenase (COX) 1 and/or 2 inhibition or modulation.

Our results are further strengthened by the fact that we used a murine model that is already well established and was successfully used before to investigate the interaction between TB lesion pathology and treatment (70). It also reflects human pulmonary TB well, as it forms human-like lesions during *Mtb* infection (71). This model has been previously reported to be a relevant disease model that can be used to explore new TB therapies (70, 71). Furthermore, this study used an experimental design that mimicked the different phases of human TB treatment, i.e. the initial intensive treatment phase, followed by the continuous treatment phase, and was also designed to simulate the acute immune response induced by *Mtb* infection. We additionally analysed local markers of inflammation, and administered the treatment (EPA/DHA and ibuprofen) together with standard TB antibiotics, in order to better understand the possible outcome in a clinical situation.

This study was aimed to primarily determine whether n-3 LCPUFA and ibuprofen were suitable candidates for use as an adjunct therapy in TB treatment, as well as a possible target for host HDTs. Even though the potential benefits of ibuprofen and n-3 LCPUFAs have been demonstrated in TB treatment, our study is the first to our knowledge to have administered the ibuprofen or n-3 LCPUFAs together with standard TB antibiotics, and these effects being assessed at different time points. This study was also aimed at contributing to preclinical data needed to precede human studies, providing evidence of whether there are any interactions between the standard TB antibiotics and a therapeutic dose of n-3 LCPUFAs or ibuprofen.

CONCLUSION

Collectively, our results support the use of EPA/DHA supplementation during TB treatment, as it does not interfere with standard TB regimen, it functions to reduces *Mtb*-elicited immunopathology and also promote pro-resolving LM production, especially considering the long treatment duration of TB treatment. Though ibuprofen did elicit anti-inflammatory effects and protected the host by limiting TB-associated immunopathology, it may be more appropriately used transiently as an adjunct therapy, since it appeared to attenuate the effect of the TB antibiotics over the long term, and should, therefore, be used acutely and with care. Our study demonstrates that EPA/DHA as adjunct therapy reduces *Mtb* burden and suppresses TB pulmonary immunopathology in the long term. Thus, EPA/DHA and ibuprofen show promise to limit TB associated inflammation and improve other clinical outcomes. If successful in human application, these treatment options may assist to improve TB clinical outcomes in the near future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The AnimCare Animal Research Ethics Committee of the North-West University, Potchefstroom, South Africa (ethics number: NWU-00055-19-S5), and the Animal Research Ethics Committee of the University of Cape Town, Cape Town, South Africa (FHS AEC 019_023).

AUTHOR CONTRIBUTIONS

LM headed the project. FH, LM, RD, RB and AN conceptualised and planned the experiments. FH, LM, MO and SP investigated and performed the experiments. FH, LM and MO analysed the data. FH, LM, MO and SP contributed to the interpretation of the results. FH took the lead in writing the manuscript. RD, FB and SP were involved in acquiring resources and funding for the experiment. All authors (LM, RD, RB, AN, DL, CS, FB, MO and SP) provided critical feedback and helped to shape the research, analysis and manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.659943/full#supplementary-material

REFERENCES

1. World Health Organization. *Global Tuberculosis Report 2019. WHO Tb Report* (2019). Available at: https://www.who.int/tb/publications/global_report/en/ (Accessed Assessed May 28, 2020).
2. Hawn TR, Matheson AI, Maley SN, Vandal O. Host-Directed Therapeutics for Tuberculosis: Can We Harness the Host? *Microbiol Mol Biol Rev* (2013) 77:608–27. doi: 10.1128/MMBR.00032-13
3. Zumla A, Rao M, Parida SK, Keshavjee S, Cassell G, Wallis R, et al. Inflammation and Tuberculosis: Host-Directed Therapies. *J Internal Med* (2015) 277:373–87. doi: 10.1111/joim.12256
4. Abreu R, Giri P, Quinn F. Host-Pathogen Interaction as a Novel Target for Host-Directed Therapies in Tuberculosis. *Front Immunol* (2020) 11:1553. doi: 10.3389/fimmu.2020.01553
5. Cooper AM, Torrado E. Protection Versus Pathology in Tuberculosis: Recent Insights. *Curr Opin Immunol* (2012) 24:431–7. doi: 10.1016/j.coi.2012.04.008
6. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The Immune Response in Tuberculosis. *Annu Rev Immunol* (2013) 31:475–527. doi: 10.1146/annurev-immunol-032712-095939
7. Ivanyi J, Zumla A. Nonsteroidal Antiinflammatory Drugs for Adjunctive Tuberculosis Treatment. *J Infect Dis* (2013) 208:185–8. doi: 10.1093/infdis/jit153
8. Dheda K, Booth H, Huggett JF, Johnson MA, Zumla A, Rook GA. Lung Remodeling in Pulmonary Tuberculosis. *J Infect Dis* (2005) 192:1201–10. doi: 10.1086/444545
9. Zumla A, Chakaya J, Hoelscher M, Ntoumi F, Rustomjee R, Vilaplana C, et al. Towards Host-Directed Therapies for Tuberculosis. *Nat Rev Drug Discov* (2015) 14:511–2. doi: 10.1038/nrd4696
10. Vilaplana C, Marzo E, Tapia G, Diaz J, Garcia V, Cardona PJ. Ibuprofen Therapy Resulted in Significantly Decreased Tissue Bacillary Loads and Increased Survival in a New Murine Experimental Model of Active Tuberculosis. *J Infect Dis* (2013) 208:199–202. doi: 10.1093/infdis/jit152
11. Kroesen VM, Rodríguez-Martínez P, García E, Rosales Y, Díaz J, Martín-Céspedes M, et al. A Beneficial Effect of Low-Dose Aspirin in a Murine Model of Active Tuberculosis. *Front Immunol* (2018) 9:798. doi: 10.3389/fimmu.2018.00798
12. Chandrasekaran P, Saravanan N, Bethunaickan R, Tripathy S. Malnutrition: Modulator of Immune Responses in Tuberculosis. *Front Immunol* (2017) 8:1316. doi: 10.3389/fimmu.2017.01316
13. Osborne R. First Novel Anti-Tuberculosis Drug in 40 Years. *Nat Biotechnol* (2013) 31:89–91. doi: 10.1038/nbt0213-89
14. Sotgiu G, Pontali E, Centis R, D'Ambrosio L, Migliori GB. Delamanid (OPC-67683) for Treatment of Multi-Drug-Resistant Tuberculosis. *Expert Rev Anti Infect Ther* (2015) 13:305–15. doi: 10.1586/14787210.2015.1011127
15. Keam SJ. Pretomanid: First Approval. *Drugs* (2019) 79:1797–803. doi: 10.1007/s40265-019-01207-9
16. Calder PC. Omega-3 Fatty Acids and Inflammatory Processes: From Molecules to Man. *Biochem Soc Trans* (2017) 45:1105–15. doi: 10.1042/BST20160474
17. Nienaber A, Baumgartner J, Dolman RC, Ozturk M, Zandberg L, Hayford FEA, et al. Omega-3 Fatty Acid and Iron Supplementation Alone, But Not in Combination, Lower Inflammation and Anemia of Infection in Mycobacterium Tuberculosis-Infected Mice. *Nutrients* (2020) 12:2897. doi: 10.3390/nu12092897
18. Kroesen VM, Gröschel MI, Martinson N, Zumla A, Maeurer M, van der Werf TS, et al. Non-Steroidal Anti-inflammatory Drugs As Host-Directed Therapy for Tuberculosis: A Systematic Review. *Front Immunol* (2017) 8:772. doi: 10.3389/fimmu.2017.00772
19. Paton NI, Chua YK, Earnest A, Chee CB. Randomized Controlled Trial of Nutritional Supplementation in Patients With Newly Diagnosed Tuberculosis and Wasting. *Am J Clin Nutr* (2004) 80:460–5. doi: 10.1093/ajcn/80.2.460
20. Young C, Walzl G, Du Plessis N. Therapeutic Host-Directed Strategies to Improve Outcome in Tuberculosis. *Mucosal Immunol* (2020) 13:190–204. doi: 10.1038/s41385-019-0226-5
21. Marzo E, Vilaplana C, Tapia G, Diaz J, Garcia V, Cardona PJ. Damaging Role of Neutrophilic Infiltration in a Mouse Model of Progressive Tuberculosis. *Tuberculosis* (2014) 94:55–64. doi: 10.1016/j.tube.2013.09.004
22. Byrne ST, Denkin SM, Zhang Y. Aspirin Antagonism in Isoniazid Treatment of Tuberculosis in Mice. *Antimicrob Agents Chemother* (2007) 51:794–5. doi: 10.1128/AAC.01145-06
23. K Baxter and CL Preston eds. *Stockley's Drug Interactions*. London: Pharmaceutical Press (2010). p. 495.
24. Varrassi G, Pergolizzi JV, Dowling P, Paladini A. Ibuprofen Safety At the Golden Anniversary: Are All NSAIDs the Same? A Narrative Review. *Adv Ther* (2020) 37:61–82. doi: 10.1007/s12325-019-01144-9
25. Ahmadi A, Khalili M, Olama Z, Karami S, Nahri-Niknafs B. Synthesis and Study of Analgesic and Anti-Inflammatory Activities of Amide Derivatives of Ibuprofen. *Mini Rev Med Chem* (2017) 17:799–804. doi: 10.2174/1389557516666161226155951
26. Calder PC. Omega-3 Polyunsaturated Fatty Acids and Inflammatory Processes: Nutrition or Pharmacology? *Br J Clin Pharmacol* (2013) 75:645–62. doi: 10.1111/j.1365-2125.2012.04374.x
27. Dutta S, Sengupta P. Men and Mice: Relating Their Ages. *Life Sci* (2016) 152:244–8. doi: 10.1016/j.lfs.2015.10.025
28. World Health Organization & Stop TB Initiative. *Treatment of Tuberculosis: Guidelines WHO/HTM/TB/2009.420 Ed*, *4th Edition*. World Health Organization (2010).
29. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdayal R, Mhlanga MM, et al. Statin Therapy Reduces the Mycobacterium Tuberculosis Burden in Human Macrophages and in Mice by Enhancing Autophagy and Phagosome Maturation. *J Infect Dis* (2014) 209:754–63. doi: 10.1093/infdis/jit550
30. Guler R, Parihar SP, Spohn G, Johansen P, Brombacher F, Bachmann MF. Blocking IL-1α But Not IL-1β Increases Susceptibility to Chronic Mycobacterium Tuberculosis Infection in Mice. *Vaccine* (2011) 29:1339–46. doi: 10.1016/j.vaccine.2010.10.045
31. Parihar SP, Ozturk M, Marakalala MJ, Loots DT, Hurdayal R, Maasdorp DB, et al. Protein Kinase C-delta (Pkcδ), a Marker of Inflammation and Tuberculosis Disease Progression in Humans, is Important for Optimal Macrophage Killing Effector Functions and Survival in Mice. *Mucosal Immunol* (2018) 11:496–511. doi: 10.1038/mi.2017.68
32. Folch J, Lees M, Stanley GS. A Simple Method for the Isolation and Purification of Total Lipides From Animal Tissues. *J Biol Chem* (1957) 226:497–509. doi: 10.1016/S0021-9258(18)64849-5
33. Malan L, Baumgartner J, Zandberg L, Calder PC, Smuts CM. Iron and a Mixture of DHA and EPA Supplementation, Alone and in Combination, Affect Bioactive Lipid Signalling and Morbidity of Iron Deficient South African School Children in a Two-by-Two Randomised Controlled Trial. *Prostaglandins Leukot Essent Fatty Acids* (2016) 105:15–25. doi: 10.1016/j.plefa.2015.12.005
34. Ricci C, Baumgartner J, Malan L, Smuts CM. Determining Sample Size Adequacy for Animal Model Studies in Nutrition Research: Limits and Ethical Challenges of Ordinary Power Calculation Procedures. *Int J Food Sci Nutr* (2020) 71:256–64. doi: 10.1080/09637486.2019.1646714
35. Domingo-Gonzalez R, Prince O, Cooper A, Khader SA. Cytokines and Chemokines in Mycobacterium Tuberculosis Infection. Tuberculosis and the Tubercle Bacillus (2017) 1:33–72. doi: 10.1128/9781555819569.ch2
36. Tobin DM, Roca FJ, Oh SF, McFarland R, Vickery TW, Ray JP, et al. Host Genotype-Specific Therapies can Optimize the Inflammatory Response to Mycobacterial Infections. *Cell* (2012) 148:434–46. doi: 10.1016/j.cell.2011.12.023
37. Guler R, Brombacher F. Host-Directed Drug Therapy for Tuberculosis. *Nat Chem Biol* (2015) 11:748–51. doi: 10.1038/nchembio.1917
38. Kolloli A, Subbian S. Host-Directed Therapeutic Strategies for Tuberculosis. *Front Med* (2017) 4:171. doi: 10.3389/fmed.2017.00171
39. Ndlovu H, Marakalala MJ. Granulomas and Inflammation: Host-Directed Therapies for Tuberculosis. *Front Immunol* (2016) 7:434. doi: 10.3389/fimmu.2016.00434
40. Jordao L, Lengeling A, Bordat Y, Boudou F, Gicquel B, Neyrolles O, et al. Effects of Omega-3 and-6 Fatty Acids on Mycobacterium Tuberculosis in Macrophages and in Mice. *Microbes Infect* (2008) 10:1379–86. doi: 10.1016/j.micinf.2008.08.004
41. Bonilla DL, Fan YY, Chapkin RS, McMurray DN. Transgenic Mice Enriched in Omega-3 Fatty Acids are More Susceptible to Pulmonary Tuberculosis: Impaired Resistance to Tuberculosis in Fat-1 Mice. *J Infect Dis* (2010) 201:399–408. doi: 10.1086/650344

42. Byrne ST, Denkin SM, Zhang Y. Aspirin and Ibuprofen Enhance Pyrazinamide Treatment of Murine Tuberculosis. *J Antimicrob Chemother* (2007) 59:313–6. doi: 10.1093/jac/dkl486
43. Kiran D, Podell BK, Chambers M, Basaraba RJ. Host-Directed Therapy Targeting the Mycobacterium Tuberculosis Granuloma: A Review. *Semin Immunopathol* (2016) 38:167–83. doi: 10.1007/s00281-015-0537-x
44. Reeme AE, Robinson RT. Dietary Vitamin D3 Suppresses Pulmonary Immunopathology Associated With Late-Stage Tuberculosis in C3HeB/FeJ Mice. *J Immunol* (2016) 196:1293–304. doi: 10.4049/jimmunol.1500931
45. Skerry C, Harper J, Klunk M, Bishai WR, Jain SK. Adjunctive TNF Inhibition With Standard Treatment Enhances Bacterial Clearance in a Murine Model of Necrotic TB Granulomas. *PloS One* (2012) 7:e39680. doi: 10.1371/journal.pone.0039680
46. Serhan CN, Chiang N, Dalli J. New Pro-Resolving N-3 Mediators Bridge Resolution of Infectious Inflammation to Tissue Regeneration. *Mol Aspects Med* (2017) 64:1–7. doi: 10.1016/j.mam.2017.08.002
47. Chiang N, Fredman G, Bäckhed F, Oh SF, Vickery T, Schmidt BA, et al. Infection Regulates Pro-Resolving Mediators That Lower Antibiotic Requirements. *Nature* (2012) 484:524–8. doi: 10.1038/nature11042
48. Spite M, Norling LV, Summers L, Yang R, Cooper D, Petasis NA, et al. Resolvin D2 is a Potent Regulator of Leukocytes and Controls Microbial Sepsis. *Nature* (2009) 461:1287–91. doi: 10.1038/nature08541
49. Serhan CN. Treating Inflammation and Infection in the 21st Century: New Hints From Decoding Resolution Mediators and Mechanisms. *FASEB J* (2017) 31:1273–88. doi: 10.1096/fj.201601222R
50. Yaqoob P. Fatty Acids and the Immune System: From Basic Science to Clinical Applications. *Proc Nutr Soc* (2004) 63:89–105. doi: 10.1079/PNS2003328
51. Bonilla DL, Ly LH, Fan YY, Chapkin RS, McMurray DN. Incorporation of a Dietary Omega 3 Fatty Acid Impairs Murine Macrophage Responses to Mycobacterium Tuberculosis. *PloS One* (2010) 5:e10878. doi: 10.1371/journal.pone.0010878
52. Nowak J, Weylandt KH, Habbel P, Wang J, Dignass A, Glickman JN, et al. Colitis-Associated Colon Tumorigenesis is Suppressed in Transgenic Mice Rich in Endogenous N-3 Fatty Acids. *Carcinogenesis* (2007) 28:1991–5. doi: 10.1093/carcin/bgm166
53. Eicher SD, McVey DS. Dietary Modulation of Kupffer Cell and Splenocyte Function During a Salmonella Typhimurium Challenge in Mice. *J Leukoc Biol* (1995) 58:32–9. doi: 10.1002/jlb.58.1.32
54. Mortensen R, Clemmensen HS, Woodworth JS, Therkelsen ML, Mustafa T, Tonby K, et al. Cyclooxygenase Inhibitors Impair CD4 T Cell Immunity and Exacerbate Mycobacterium Tuberculosis Infection in Aerosol-Challenged Mice. *Commun Biol* (2019) 2:1–10. doi: 10.1038/s42003-019-0530-3
55. Mancuso P, Whelan J, DeMichele SJ, Snider CC, Guszcza JA, Karlstad MD. Dietary Fish Oil and Fish and Borage Oil Suppress Intrapulmonary Proinflammatory Eicosanoid Biosynthesis and Attenuate Pulmonary Neutrophil Accumulation in Endotoxic Rats. *Crit Care Med* (1997) 25:1198–206. doi: 10.1097/00003246-199707000-00023
56. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs That Promote the Differentiation of Effector Cytolytic T Cells. *Immunity* (2010) 32:79–90. doi: 10.1016/j.immuni.2009.11.012
57. Kaartinen T, Luostarinen A, Maliniemi P, Keto J, Arvas M, Belt H, et al. Low Interleukin-2 Concentration Favors Generation of Early Memory T Cells Over Effector Phenotypes During Chimeric Antigen Receptor T-Cell Expansion. *Cytotherapy* (2017) 19:689–702. doi: 10.1016/j.jcyt.2017.03.067
58. Barber DL, Mayer-Barber KD, Feng CG, Sharpe AH, Sher A. CD4 T Cells Promote Rather Than Control Tuberculosis in the Absence of PD-1–Mediated Inhibition. *J Immunol* (2011) 186:1598–607. doi: 10.4049/jimmunol.1003304
59. Ilonidis G, Parapanisiou E, Anogeianaki A, Giavazis I, Theofilogiannakos EK, Tsekoura P, et al. Interleukin-1beta (IL-1 Beta), Interleukin 6 (IL-6) and Tumor Necrosis Factor (TNF) in Plasma and Pleural Fluid of Pneumonia, Lung Cancer and Tuberculous Pleuritis. *J Biol Regul Homeost Agents* (2006) 20:41–6.
60. Sasindran SJ, Torrelles JB. Mycobacterium Tuberculosis Infection and Inflammation: What is Beneficial for the Host and for the Bacterium? *Front Microbiol* (2011) 2:2. doi: 10.3389/fmicb.2011.00002
61. Chmiel JF, Konstan MW, Accurso FJ, Lymp J, Mayer-Hamblett N, VanDevanter DR, et al. Use of Ibuprofen to Assess Inflammatory Biomarkers in Induced Sputum: Implications for Clinical Trials in Cystic Fibrosis. *J Cyst Fibros* (2015) 14:720–6. doi: 10.1016/j.jcf.2015.03.007
62. Levy BD, Kohli P, Gotlinger K, Haworth O, Hong S, Kazani S, et al. Protectin D1 is Generated in Asthma and Dampens Airway Inflammation and Hyperresponsiveness. *J Immunol* (2007) 178:496–502. doi: 10.4049/jimmunol.178.1.496
63. Wallace FA, Miles EA, Evans C, Stock TE, Yaqoob P, Calder PC. Dietary Fatty Acids Influence the Production of Th1-but Not Th2-type Cytokines. *J Leukoc Biol* (2001) 69:449–57. doi: 10.1189/jlb.69.3.449
64. Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and Protectin D1 Activate Inflammation-Resolution Programmes. *Nature* (2007) 447:869–74. doi: 10.1038/nature05877
65. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *J Interferon Cytokine Res* (2009) 29:313–26. doi: 10.1089/jir.2008.0027
66. Hasan Z, Cliff JM, Dockrell HM, Jamil B, Irfan M, Ashraf M, et al. CCL2 Responses to Mycobacterium Tuberculosis Are Associated With Disease Severity in Tuberculosis. *PloS One* (2010) 4:e8459. doi: 10.1371/journal.pone.0008459
67. Cambier CJ, O'Leary SM, O'Sullivan MP, Keane J, Ramakrishnan L. Phenolic Glycolipid Facilitates Mycobacterial Escape From Microbicidal Tissue-Resident Macrophages. *Immunity* (2017) 47:552–65. doi: 10.1016/j.immuni.2017.08.003
68. Dunlap MD, Howard N, Das S, Scott N, Ahmed M, Prince O, et al. A Novel Role for C–C Motif Chemokine Receptor 2 During Infection With Hypervirulent Mycobacterium Tuberculosis. *Mucosal Immunol* (2018) 11:1727–42. doi: 10.1038/s41385-018-0071-y
69. Kaufmann SH, Dorhoi A, Hotchkiss RS, Bartenschlager R. Host-Directed Therapies for Bacterial and Viral Infections. *Nat Rev Drug Discov* (2018) 17:35. doi: 10.1038/nrd.2017.162
70. Lanoix JP, Lenaerts AJ, Nuermberger EL. Heterogeneous Disease Progression and Treatment Response in a C3HeB/FeJ Mouse Model of Tuberculosis. *Dis Models Mech* (2015) 8:603–10. doi: 10.1242/dmm.019513
71. Lenaerts A, Barry CEII, Dartois V. Heterogeneity in Tuberculosis Pathology, Microenvironments and Therapeutic Responses. *Immunol Rev* (2015) 264:288–307. doi: 10.1111/imr.12252

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Targeted Inhibition of FTO Demethylase Protects Mice Against LPS-Induced Septic Shock by Suppressing NLRP3 Inflammasome

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Sepsis refers to the systemic inflammatory response syndrome caused by infection. It is a major clinical problem and cause of death for patients in intensive care units worldwide. The Fat mass and obesity-related protein (FTO) is the primary N^6-methyladenosine demethylase. However, the role of FTO in the pathogenesis of inflammatory diseases remains unclear. We herein show that nanoparticle-mediated *Fto*-siRNA delivery or FTO inhibitor entacapone administration dramatically inhibited macrophage activation, reduced the tissue damage and improved survival in a mouse model of LPS-induced endotoxic shock. Importantly, ablation of FTO could inhibit NLRP3 inflammasome through FoxO1/NF-κB signaling in macrophages. In conclusion, FTO is involved in inflammatory response of LPS-induced septic shock and inhibition of FTO is promising for the treatment of septic shock.

Keywords: FTO, N^6-methyladenosine, entacapone, inflammasome, sepsis

INTRODUCTION

Recently, there are more than 18 million cases of severe sepsis worldwide each year. This disease refers to systemic inflammatory response syndrome caused by infections (1, 2). Infectious factors in sepsis activate the mononuclear macrophage system and other inflammatory response cells, resulting in the production and release of multiple inflammatory mediators. Sepsis is a major clinical problem and the leading cause of death in patients in intensive care units worldwide (3, 4). Thus, the development of novel effective treatments for sepsis is urgently needed.

NLRP3 inflammasome, causing the maturation and secretion of interleukin-1β (IL-1β), plays a critical role in the inflammatory response (5, 6). Upon normal circumstances, the active NLRP3 inflammasome helps to recruit immune cells to the site of infection and trigger the adaptive immune response (7). However, under pathological conditions, the aberrant activation of NLRP3 inflammasome can lead to the occurrence of inflammatory diseases, including septic shock. Numerous studies have shown that NLRP3 inflammasomes are associated with lipopolysaccharide (LPS)-induced septic shock (8–10). The level of IL-1β in plasma is associated

with the severity of shock (11). In addition, targeting NLRP3 inflammasomes through gene editing can alleviate the acute inflammation of LPS-induced endotoxin shock (12). Considering the important role of inflammasome in the pathogenesis of sepsis, strategies aimed at regulating its activation may be beneficial for sepsis treatment.

N^6-methyladenosine (m^6A) is the most abundant internal modification of messenger RNA (mRNA) and non-coding RNA in eukaryotic cells (13–15). The Fat mass and obesity-related protein (FTO) belongs to the family of Fe^{2+} and α-ketoglutarate-dependent oxygenase, which mainly catalyze the m^6A demethylation (16). It is involved in multiple mRNA-related processes, including transcriptional stability, alternative splicing, mRNA translocation and protein translation (17–21). In the recent years, FTO has been widely regarded as an attractive biological target owing to its function on the mRNA modification. Targeted inhibition of FTO has been found to reduce body weight and regulate liver gluconeogenesis in diet-induced obese mice (22). However, it is unclear whether targeting FTO can be used to treat inflammatory diseases, especially for septic shock. Entacapone was previously considered to be a catechol-O-methyltransferase (COMT) inhibitor for the treatment of Parkinson's disease (23). It has recently been identified as an effective chemical inhibitor of FTO. Structural and biochemical studies demonstrated that entacapone can directly bind to FTO and inhibit the demethylation activity (22). Therefore, entacapone was used as FTO inhibitor for treatment in this study.

In the recent years, FoxO1 has been confirmed to participate in regulating the production of IL-1β by macrophages, suggesting that FoxO1 signaling through NF-κB participates in pro-inflammatory cytokine production (24). Moreover, it has been found that FoxO1 can mediate the activation of NLRP3 inflammasome. Inhibition of FoxO1 by using of the molecule inhibitors could block NLRP3 inflammasome assembly and activation (25). Thus, we hypothesize that FTO is involved in LPS-induced septic shock and targeted inhibition of FTO demethylase might protect mice against LPS-induced septic shock by suppressing NLRP3 inflammasome *via* FoxO1/NF-κB signaling.

MATERIALS AND METHODS

Human Samples

Twenty-four healthy volunteers and 15 septic patients were enrolled and classified according to the criteria of the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) (1). Peripheral blood samples were collected after receiving a written informed consent from septic patients. The experiment was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology.

Reagents

LPS and nigericin were purchased from Sigma-Aldrich (St. Louis, MO). NF-kB inhibitor, QNZ (EVP4593), was supplied by MedChemExpress (New Jersey, USA). Antibodies against FTO, NLRP3, FoxO1, P65, p-P65, IL-1β and Cleaved-IL-1β (Asp117) were obtained from Cell Signaling Technologies (Beverly, MA). ELISA kits of IL-1β, interleukin-6 (IL-6), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10) and interleukin-12(p70) (IL-12(p70)) were purchased from eBioscience (San Diego, CA). Brilliant Violet 421™ anti-mouse F4/80 antibody, PE anti-mouse/human CD11b antibody, FITC anti-mouse I-A/I-E antibody, APC anti-mouse CD80 antibody, PE/Cy7 anti-mouse CD86 antibody, FITC anti-mouse Ly-6G antibody and APC anti-mouse CD40 antibody were obtained from BioLegend (San Diego, CA, USA). Lipidoid (C12-200) was supplied by Xinjiahecheng Medical Chemistry Corporation (Wuhan, Hubei, China). mPEG2000-DEG was purchased from NOF Corporation (Tokyo, Japan).

Mice

C57BL/6 mice (6–8 weeks old) were purchased from the Jackson's Laboratory (Bar Harbor, ME, USA) and maintained in a specific pathogen free facility. Male mice were used in all animal studies. All experimental mice were housed individually in ventilated cages in a pathogen-free facility with a 12 h light/dark cycle and were fed with a standard mouse chow diet. siRNA-loaded liposomes were injected into mice 2 days before LPS challenge (15 mg/kg). Survival rate of mice were monitored. Other groups of mice were injected with 10 mg/kg entacapone before the systemic injection of LPS. All procedures involving animals were approved by the Tongji Hospital Animal Care and Use Committee in accordance with the National Institutes of Health guidelines.

Cell Culture

Murine bone marrow derived macrophages (BMDMs) were differentiated with macrophage colony stimulating factor as previously reported (26). Briefly, the mouse bone marrow cells were flushed out from the femur and tibia with a syringe. Then the cells were filtered through a 70 μm filter. The red blood cells were lysed. Then, the cells were cultured with a medium containing macrophage colony stimulating factors. Finally, the differentiated BMDMs were treated with the indicated stimulation, and collected for quantitative RT-PCR and Western blot analyses.

Activation of NLRP3 Inflammasome

First, the indicated concentration of entacapone and the same amount of DMSO were added to the cell culture medium. Then stimulate the BMDMs with 1 μg/mL LPS for 5 h. Nigericin (20 μM) was added to the cell culture medium for 30 min to induce inflammasome activation.

siRNA Transfection

The siRNA against *Fto* was purchased from RiboBio Co., Ltd (Guangzhou, P. R. China). The sequences for the *Fto* siRNA are as follows: sense strand 5′-GGCAGAGATCCTGATACTT-3′. Then, Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was used to perform siRNA transfection as previously

described (27). A *scramble* siRNA duplex served as the negative control.

In Vivo Biodistribution of the Liposomes

DiI-loaded liposomes were prepared as previously described (28). The mice were intraperitoneally injected with liposomes and anesthetized at different time points. The peritoneal fluid was collected for fluorescence analysis.

Preparation and Characterization of siRNA-Loaded Liposomes

siRNA-loaded liposomes were prepared as described previously (29, 30). Briefly, lipoid, cholesterol, DSPC, and mPEG-DMG were dissolved in ethanol at a specific molar ratio. At the same time, siRNA was dissolved in citrated buffer (10 mM, pH 3). Then, the liposomes and siRNA were mixed rapidly by vortex.

Western Blot Analysis

Western blot analysis was conducted by using established techniques (27, 31). Briefly, the cells were lysed on ice with RIPA lysis buffer (Biyuntian, Shanghai, China). Then, the Western blot analysis was performed using indicated primary antibodies. β-actin served as a loading control.

Quantitative RT-PCR Analysis

Total RNA was isolated from human monocytes or murine BMDMs using the Trizol™ reagent (Takara, Japan). Real-time PCR was performed using the SYBR Green PCR master mix (Applied Biosystems, South San Francisco, CA, USA) in the ABI Prism 7500 Sequence Detection System (Applied Biosystems, South San Francisco, USA). The following primers were used: human *IL-1β* forward, 5′-CCACAGACCTTCCAGGAGAATG-3′ and reverse, 5′- GTGCAGTTCAGTGATCGTACAGG-3′; human *FTO* forward, 5′-ACTTGGCTCCCTTATCTGACC-3′ and reverse, 5′-TGTGCAGTGTGAGAAAGG CTT-3′; human *18S* forward, 5′-GTAACCCGTTGAACCCCATT-3′ and reverse, 5′-CCATCCAATCGGTAGTAGCG-3′; mouse *IL-1β* forward, 5′-GGATGAGGACAT GAGCACCT-3′ and reverse, 5′-GGAGCCTGTAGTGCAGTTGT-3′; mouse *Fto* forward, 5′-TCACAGCCTCGGTTTAGTTC-3′ and reverse, 5′-GCAGGATCAAAGGATTTCAACG-3′; and mouse *β-actin* forward, 5′-AGCCATGTA CGTAGCCATCC-3′ and reverse, 5′- CTCCAGCTGTGGTGGTGAA-3′. The relative RNA amount was normalized with *18S* or *β-actin* RNA.

Quantification of Total m^{6}A Level

Total mRNA m^{6}A levels were detected by EpiQuik™ m^{6}A RNA Methylation Quantification Kit (Epigentek). Total RNA was isolated from human monocytes or murine BMDMs using the Trizol™ reagent (Takara, Japan) and the concentration was detected using a NanoDrop spectrophotometer 2000 (Thermo Fisher Scientific). Measurements were performed to the manufacturer's instructions by using colorimetric ELISA assays. The input RNA amount was 200 ng per reaction. m^{6}A % was calculated to quantify the relative m^{6}A RNA methylation levels of different RNA samples.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) was carried out using a One-Step TUNEL Apoptosis Assay Kit (Biyuntian, Shanghai, China). The slides were treated with 20 μg/mL DNase-free protease K for 20 min at room temperature. Then, the slides were washed with PBS. The TUNEL reaction mixture was added to the sample and incubated for 60 min at 37 °C. DAPI was used to stain nuclei simultaneously. The TUNEL-positive cells were detected under a fluorescence microscope.

Flow Cytometry Analysis

The mice were euthanized and the cells were obtained from the peritoneum and spleens. The flow cytometric analysis was performed using fluorophore-conjugated antibodies as previously described (32). Data were analyzed using FlowJo V10 software.

Statistical Analysis

The Kaplan-Meier method was used for survival analysis. Other results were expressed as the mean ± SEM, and comparisons were accomplished by the Student's *t* test or ANOVA as appropriate. In all cases, $P < 0.05$ was considered statistically significant. All *in vitro* studies were conducted with at least three replications. Statistical analyses of the data were conducted using the GraphPad Prism 7.00 software (GraphPad Software Inc., San Diego, CA).

RESULTS

FTO Expression Is Correlated With IL-1β Expression in Peripheral Blood Monocytes of Septic Shock Patients

First, we want to investigate whether FTO expression and m^{6}A level are related to the pathogenesis of sepsis in humans. Compared with healthy volunteers, FTO expression in monocytes of patients with sepsis was significantly reduced (**Figure 1A**). The m^{6}A level in septic patients was elevated (**Figure 1B**). Interestingly, FTO expression was correlated with higher IL-1β expression in monocytes of septic patients (**Figure 1C**). The m^{6}A level was significantly lower correlated with higher IL-1β expression (**Figure 1D**). In addition, LPS stimulation can decrease the expression of FTO in murine primary macrophages with significantly higher m^{6}A level (**Figures 1E–G**). However, the FTO expression was increased after QNZ, the NF-kB inhibitor, treatment (**Figure 1H**).

We hypothesized that the lower FTO expression level would attenuate the disease progression but FTO expression might be inhibited during the phase of sepsis as a feedback effect. Thus, silencing of *Fto* may herald a better treatment outcome.

Preparation of *Fto* siRNA-Loaded Liposomes

For *in vivo* experiments, we designed *Fto* siRNA sequence to inhibit FTO expression in macrophages to verify the hypothesis. Firstly, we examined the biodistribution of *Fto* siRNA-loaded

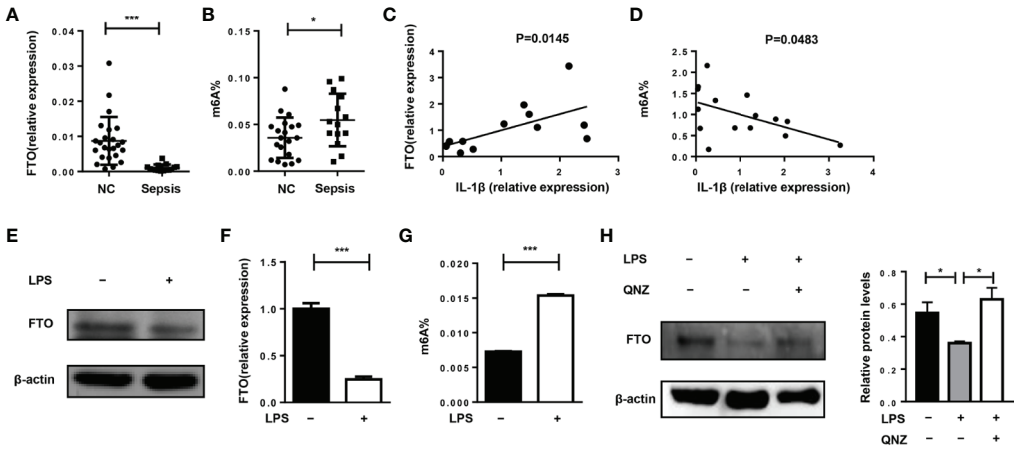

FIGURE 1 | FTO expression is correlated with IL-1β expression in monocytes of septic shock patients. **(A, B)** qPCR analysis of expression of FTO and the m^6A level in peripheral monocytes of septic patients (n = 15) and healthy volunteers (n = 24). **(C)** Correlation between FTO gene expression (n = 12) and m^6A level (n = 15) **(D)** in peripheral monocytes from human patients. Murine BMDMs were treated or untreated with 500 ng/ml LPS. FTO expression was analyzed by western blot **(E)** and qPCR **(F)**. **(G)**The m^6A level was measured by colorimetric ELISA assay. **(H)** LPS-stimulated BMDMs were pretreated with or without 5 μM QNZ(EVP4593) prior to the LPS treatment. FTO expression was measured by western blot. β-actin was used as the loading control. Data are shown as mean ± SEM; *P < 0.05; ***P < 0.001 (two-tailed unpaired t-test). Pearson's correlation analysis was performed in **(C, D)**.

liposomes in C57BL/6 mice (**Figure 2A**). DiI is a lipophilic membrane dye used to label the lipid mixture. The DiI-loaded liposomes were intraperitoneally injected into mice and the peritoneal fluid was collected for fluorescence analysis. Then we measured the fluorescence intensity of DiI in $F4/80^+CD11b^+$ macrophages. The result suggested siRNA-loaded liposomes efficiently targeted macrophages (**Figure 2B**). Next, we detected the DiI^+ cells by flow cytometry to figure out the cellular localization of liposomes. Surprisingly, the majority of DiI^+ cells were $F4/80^+CD11b^+$macrophages but not other monocytes, such

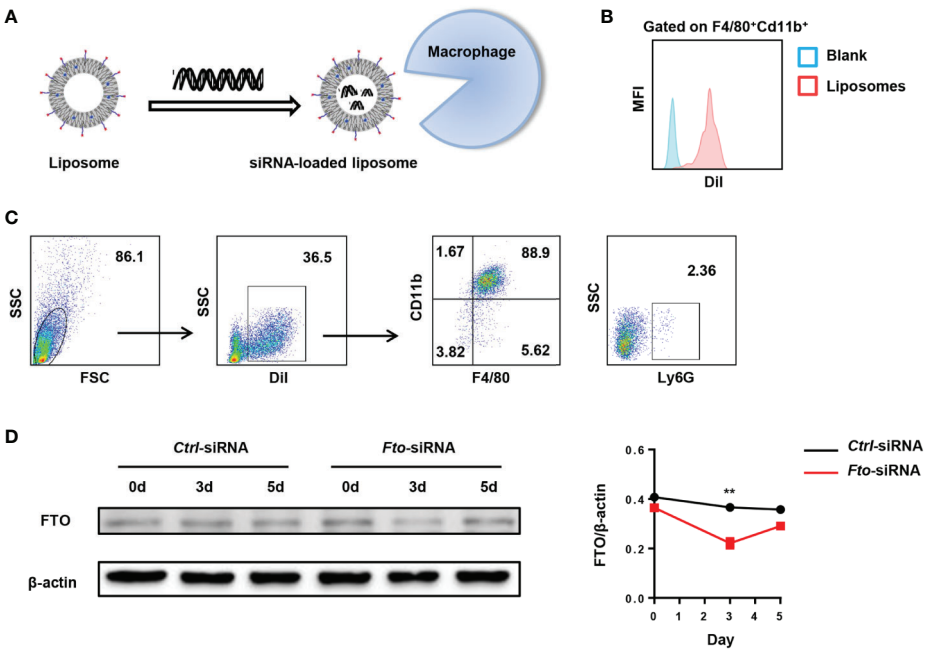

FIGURE 2 | Preparation of *Fto* siRNA-loaded liposomes. DiI-loaded liposomes were intraperitoneally injected in mice and the peritoneal fluid was collected for fluorescence analysis. **(A)** Schematic diagram of the preparation process of *Fto* siRNA-loaded liposomes. **(B)** The mean fluorescence intensity of DiI in $F4/80^+CD11b^+$ macrophages. **(C)** Flow cytometry analysis of the liposomes distribution in the mouse peritoneal fluid. **(D)** Temporal changes in FTO expression in the peritoneal macrophages after *Fto* siRNA-loaded liposomes injection. Data are shown as mean ± SEM; **P < 0.01 (two-tailed unpaired t-test).

as dendritic cells and neutrophils (**Figure 2C**). To further clarify the appropriate time interval for the treatment of siRNA-loaded liposomes, we evaluated the FTO expression in the peritoneal macrophages after injection of *Fto* siRNA-loaded liposomes. We found that the expression of FTO decreased significantly on the 3rd day after liposomes administration, while the expression gradually increased on the 5th day (**Figure 2D**). Western blot results showed that the best time interval between administrations was less than 3 days, which suggested us to give injection of liposomes 2 days before LPS treatment. Therefore, the *Fto* siRNA had the highest interference efficiency during the onset of the disease.

In summary, the above results confirmed that liposomes could selectively target peritoneal macrophages, which made them suitable for the treatment of LPS-induced septic shock.

Intraperitoneal Administration of *Fto* siRNA-Loaded Liposomes Protects Mice Against LPS-Induced Septic Shock

To evaluate whether FTO could directly participate in the inflammatory response, we tested whether *in vivo* silencing of *Fto* by means of siRNA reduced systemic inflammation and lethal shock in mouse models of sepsis. Nanoparticle-mediated delivery of *Fto* siRNA can silence FTO expression *in vivo*. Then, we injected a lethal dose of LPS intraperitoneally into the mice to induce shock and monitored the lethality rate between the mice pretreated with *scrambled* siRNA (*ctrl*-siRNA) or *Fto*-siRNA before LPS administration (**Figure 3A**).

The mice pretreated with *Fto* siRNA-loaded liposomes showed significantly higher resistance to the lethal effects of LPS in contrast to the ctrl mice that showed 100% lethality within 60 h after 15 mg/kg LPS injection. Although surviving mice pretreated with *Fto* siRNA-loaded liposomes showed shock symptoms at the beginning, they gradually recovered afterwards, indicating a potent protective effect on LPS-induced septic shock (**Figure 3B**). The concentrations of IL-1β, IL-6, IFN-γ and TNF-α in the serum were significantly reduced in mice pretreated with the *Fto* siRNA-loaded liposomes (**Figure 3C**), whereas the amounts of IL-12(p70) and IL-10 remained unchanged (**Figure 3D**). High-mobility group box 1 (HMGB1) was recognized as a late-stage mediator of endotoxin lethality and aggravated the septic shock induced by LPS in mouse models (33, 34). Remarkably, mice treated with *Fto* siRNA-loaded liposomes displayed significantly lower levels of serum HMGB1 (**Figure 3E**). We checked the cytokine intracellular levels in peritoneal macrophages. The results showed *Fto* silencing could reduce the cytokine intracellular levels (**Figure S3**). Consistently, we observed a decrease in the level of immune cell infiltration in the liver, kidney, lung and heart of mice administrated with *Fto* siRNA-loaded liposomes (**Figure 3F**). To examine the protective effect of liposomes on liver damage, tissue sections were treated with TdT and labeled nucleotides and then provided immunofluorescence assay. The TUNEL assay results demonstrated that silencing of *Fto* attenuated LPS induced liver injury and cell apoptosis (**Figure 3G**). Taken together, we could conclude that siRNA-silencing of *Fto* protected mice from LPS-induced endotoxic shock and decreased the inflammatory response *in vivo*.

Intraperitoneal Administration of Fto-siRNA Liposomes Attenuates Macrophage Activation *in Vivo*

The inflammatory cytokine storm is mainly triggered by macrophages and neutrophils. Therefore, we tested the activation of macrophages and neutrophils in the peritoneum and spleen after LPS stimulation. Administration of *Fto*-siRNA liposomes inhibited MHCII, CD80 and CD86 expression in macrophages (F4/80$^+$CD11b$^+$) compared to control group (**Figures 4A–D**). However, the activation marker CD40 in Ly6G$^+$ neutrophils did not show a significant difference (**Figures 4E, F**). These results suggested that silencing of *Fto* by siRNA attenuates macrophage activation in the peritoneum and spleen, although the neutrophil profiles were not affected.

The Knocking Down of the *Fto* Gene Expression Inhibits NLRP3 Inflammasome-Mediated IL-1β Secretion Through FoxO1/NF-κB Signaling in Macrophages

To further investigate the role of FTO in IL-1β expression, we used siRNA to treat the primary macrophages for the *in vitro* study. Transfection of primary macrophages with *Fto*-siRNA led to a pronounced reduction in the *Fto* mRNA level (**Figure 5A**). Next, we stimulated siRNA-transfected macrophages with LPS and the NLRP3 inflammasome activator, nigericin, to measure IL-1β secretion (**Figure 5B**). Whereas *scrambled* siRNA-transfected macrophages secreted IL-1β, the production of IL-1β was impaired in *Fto* siRNA-transfected cells (**Figure 5B**).

Since NF-κB signaling plays an important role in the inflammatory response induced by LPS, the effect of NF-κB on primary macrophages was determined by Western blot analysis. We found that LPS stimulation markedly induced the phosphorylation of p65 and pretreatment with *Fto*-siRNA significantly suppressed this process (**Figure 5C**). This indicated that silencing *Fto* could inhibit the activation of NF-κB, thereby regulating the expression of pro-inflammatory genes in macrophages. It was noted that *Foxo1* mRNA is a direct substrate of FTO. FoxO1 signaling through NF-κB was involved in coupling pro-inflammatory cytokine production (24). Thus, we want to assess the inhibitory effect of FTO on FoxO1 expression. As expected, treatment with *Fto* siRNA markedly inhibited FoxO1 expression in primary macrophages (**Figure 5C**). Collectively, our data supported that silencing *Fto* suppressed NLRP3 inflammasome-mediated IL-1β production through FoxO1/NF-κB signaling in macrophages.

Entacapone Targeting of FTO Demethylase Protects Against LPS-Induced Septic Shock

To confirm the protection provided by the blockade of FTO, we tested the therapeutic effect of specific FTO activity inhibitor, entacapone, on the LPS-induced septic shock model. Mice were injected with 10 mg/kg entacapone or the same amount of DMSO before the systemic injection of LPS (**Figure 6A**). Consistent with

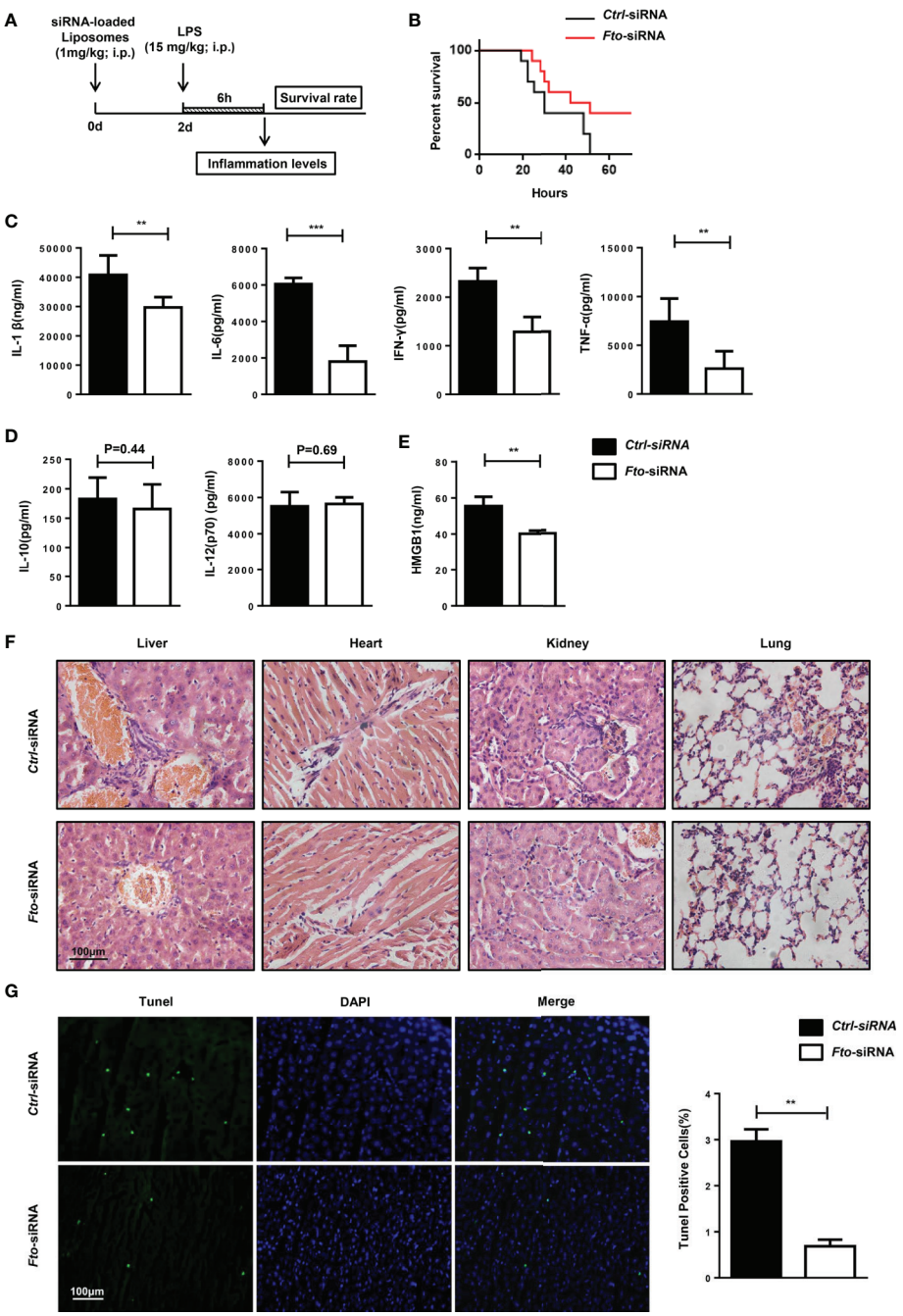

FIGURE 3 | Intraperitoneal administration of *Fto* siRNA liposomes protects mice against LPS-induced septic shock. **(A)** Nanoparticle-mediated *Fto*-siRNA or *ctrl*-siRNA were injected into C57BL/6 mice 2 days before LPS challenge. **(B)** Survival rate of mice injected with siRNA loaded-liposomes (n = 16). **(C, D)** Serum levels of IL-1β, IL-6, TNF-α, IFN-γ, IL-10 and IL-12(p10) were measured 6 h after LPS injection with pretreatment of *Fto*-siRNA or *ctrl*-siRNA liposomes. Plasma cytokine concentrations were measured by ELISA(n = 4 in each group). **(E)** Analysis of serum HMGB1 levels by ELISA. **(F)** Histology of representative tissues stained with hematoxylin and eosin (size bar, 50 μm). **(G)** The TUNEL assay of liver tissue sections. Data are shown as mean ± SEM; **P < 0.01; ***P < 0.001 (two-tailed unpaired t-test).

the results in the *Fto*-siRNA pretreated mice, the mice treated with entacapone before LPS injection showed significantly higher survival rate than the control group (**Figure 6B**). Besides, 20% of entacapone treated mice survived at 36 h after LPS challenge (**Figure 6B**). This result was in sharp contrast with the control group mice that died within 36 hours after LPS administration, and clearly showed that inhibition of FTO had a positive effect on the survival of endotoxin shock. Based on the results, HE staining showed that the immune cell infiltration in the colon, liver, kidney, and lung of the control group mice was more severe than that of

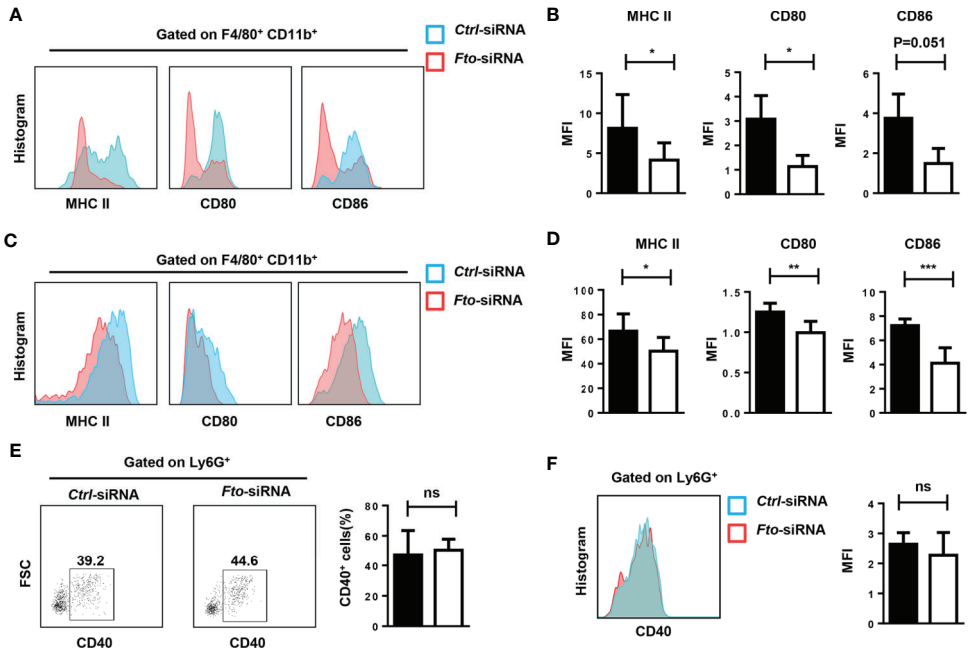

FIGURE 4 | *Fto* knock down attenuates macrophage activation in LPS induced-septic shock. The peritoneal cells **(A, B, E)** and splenocytes **(C, D, F)** were harvested 6 h after intraperitoneal injection of LPS. **(A–D)** Representative flow cytometry data for analysis of CD80, CD86 and MHC II expression in macrophages, and the mean fluorescence intensity (MFI) values of MHC II,CD80 and CD86 and the expression in F4/80^{+}CD11b^{+} macrophages are shown as bar graphs. **(E, F)** Representative flow cytometry data for analysis of CD40 expression in neutrophils, and the mean fluorescence intensity values of CD40 expression in Ly6G^{+} neutrophils are shown in bar graphic figures. Data are shown as mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed unpaired t-test). ns, not significant.

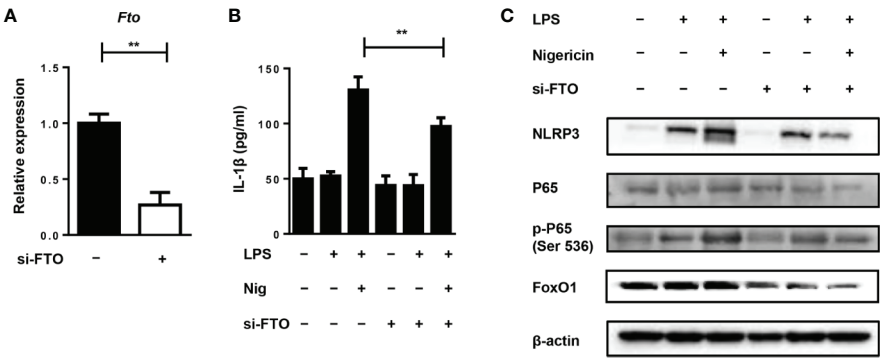

FIGURE 5 | Silencing of Fto inhibits NLRP3 inflammasome-mediated IL-1β secretion through FoxO1/NF-κB signaling pathway in macrophages. **(A)** qPCR results showed treatment of *Fto*-siRNA down-regulated target genes at the mRNA level. **(B)** BMDMs were pre-incubated with *Fto*-siRNA or *ctrl*-siRNA and the NLRP3 inflammasome activation was induced by the treatment of LPS and nigericin. The concentration of IL-1β in culture medium was measured by ELISA. **(C)** Immunoblot analysis of NLRP3, p65, phospho-p65 (Ser 536) and FoxO1 expression in BMDMs stimulated with LPS and nigericin. β-actin was used as a loading control. Data are shown as mean ± SEM; **P < 0.01 (two-tailed unpaired t-test).

the entacapone-pretreated mice (**Figure 6C**). Consistent with this observation, the serum concentrations of IL-1β, IL-6, IFN-γ and TNF-α in mice were significantly reduced by pretreatment with entacapone (**Figure 6D**). The serum HMGB1 level substantially decreased in entacapone treated mice (**Figure 6E**). Thus, these results suggested that FTO inhibition by using the specific inhibitor might be beneficial for septic shock treatment.

Entacapone Treatment Attenuates Macrophage Activation in LPS-Induced Septic Shock

Next, we tested whether entacapone had an effect on the activation of macrophages and neutrophils in mice treated with LPS. During the inflammatory processes, LPS stimulation significantly increased the expression of CD86 and MHCII in

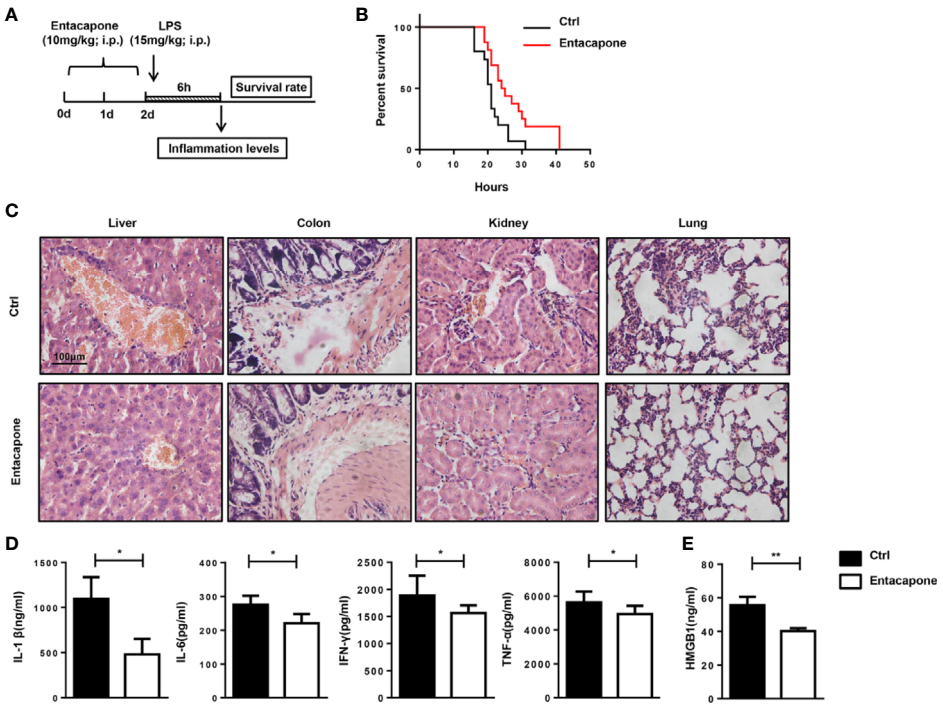

FIGURE 6 | Entacapone targeting of FTO demethylase protects against LPS-induced septic shock *in vivo*. C57BL/6 mice were pre-treated with or without entacapone (10 mg/kg) 2 days prior to LPS injection and 1 h before LPS treatment (15 mg/kg). **(A, B)** Survival rate was monitored continuously (n = 15 in ctrl group; n = 16 in entacapone group). **(C)** Histology of representative tissues stained with hematoxylin and eosin for each experimental group (size bar, 50 μm; n = 4 in each group). **(D)** Serum levels of IL-1β, IL-6, TNF-α, and IFN-γ were measured 6 h after LPS injection with or without entacapone. Plasma cytokine concentrations were measured by ELISA (n = 4 in each group). **(E)** Analysis of serum HMGB1 levels by ELISA. Data are shown as mean ± SEM; *P < 0.05, **P < 0.01. (two-tailed unpaired t-test).

macrophages, and pretreatment with entacapone significantly attenuated LPS-induced macrophage activation in the peritoneum (**Figures 7A, B**) and spleen (**Figures 7C, D**). However, entacapone administration did not affect the neutrophil activation in both ctrl and entacapone treated mice (**Figures 7E, F**).

Entacapone Inhibits NLRP3 Inflammasome-Mediated IL-1β Secretion

It has been found that entacapone could inhibit FTO activity by directly binding to FTO (22). For *in vitro* study, we treated macrophages with entacapone before LPS administration, which significantly increased the m^6A methylation level compared to the non-treated group (**Figure 8A**). To verify the effect of entacapone on the activation of NLRP3 inflammasomes, we stimulated murine primary macrophages with LPS and inflammasome activator, nigericin, with or without entacapone. The results showed that entacapone significantly reduced secretion of IL-1β induced by nigericin (**Figure 8B**). Consistently, the reduction of NLRP3 and cleaved-IL-1β levels by entacapone treatment was confirmed by immunobloting (**Figure 8C**). Moreover, NF-κB signaling pathway is involved in the process of inflammasome activation. We found that entacapone had a significant inhibitory effect on the phosphorylation of NF-κB enhanced by nigericin treatment. Considering that entacapone elicited its effects on FTO-FoxO1 regulatory axis, we further investigated whether entacapone suppressed the activation of NF-κB signaling *via* the inhibition of FoxO1. Indeed, entacapone dramatically down-regulated the expression of FoxO1 (**Figure 8C**), which was similar to the effect of *Fto*-siRNA treatment. In summary, the results suggested that entacapone inhibited NLRP3 inflammasome-mediated IL-1β secretion through downregulation of FoxO1/NF-κB signaling pathway.

DISCUSSION

It is worth noting that sepsis is a major clinical problem and the development of novel effective treatments for sepsis is urgently needed. In previous studies, FTO is considered to be associated with the risk of obesity. Recently, FTO has been identified as a m^6A eraser and plays a critical role in multiple inflammatory disorders. The *Fto* gene has been reported to respond to LPS and to serve as a link between inflammation and metabolic responses. The dominant point mutation of *Fto* gene can reduce fat mass, increase energy expenditure and improve white adipose tissue inflammation (35). Importantly, FTO has been found to be related with the levels of C-reactive protein (36). Greater adiposity conferred by FTO SNPs leads to higher C-reactive

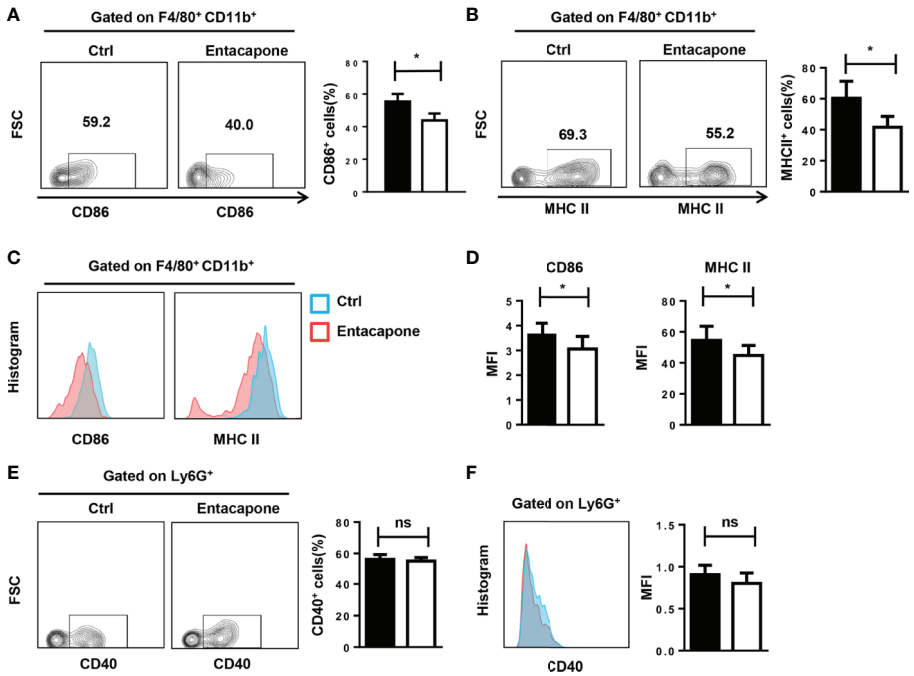

FIGURE 7 | Entacapone treatment attenuates macrophage activation in LPS-induced septic shock. C57BL/6 mice were pre-treated with or without entacapone (10 mg/kg) 2 days prior to LPS injection and 1 h before LPS treatment (15 mg/kg). The peritoneal cells **(A, B, E)** and splenocytes **(C, D, F)** were harvested 6 h after intraperitoneal injection of LPS. **(A–D)** Representative flow cytometry data for analysis of CD86 and MHC II expression in macrophages, and the mean fluorescence intensity values of CD86 and MHC II expression in F4/80$^+$CD11b$^+$ macrophages are shown as bar graphs (n=8 for each group). **(E, F)** Representative flow cytometry data for analysis of CD40 expression in neutrophils, and the mean fluorescence intensity values of CD40 expression in Ly6G$^+$ neutrophils are shown as a bar graph. Data are shown as mean ± SEM; *P < 0.05 (two-tailed unpaired t-test). ns, not significant.

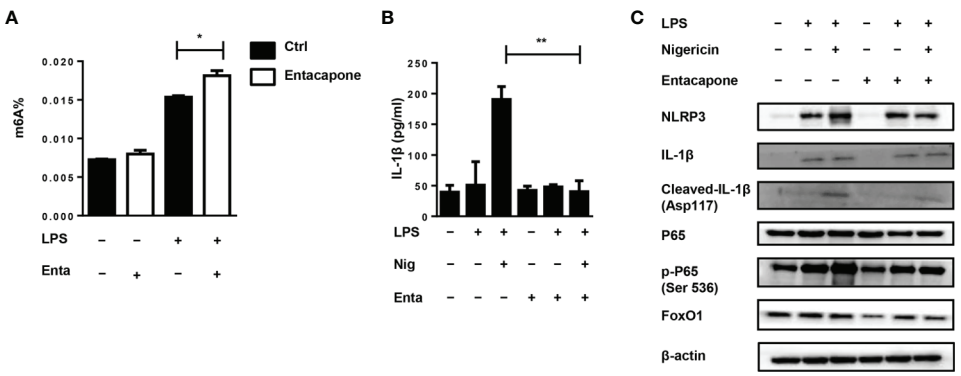

FIGURE 8 | Blockade of FTO inhibits NLRP3 inflammasome-mediated IL-1β secretion. **(A)** BMDMs were pre-incubated with or without entacapone 1 h before LPS stimulation. Then the total RNA was collected after 12 h and the mRNA m^{6}A levels were detected by colorimetric ELISA assay. **(B)** BMDMs were pre-incubated with or without entacapone. Then, LPS and nigericin were added to activate the NLRP3 inflammasome. The concentration of IL-1β in culture medium was measured by ELISA. **(C)** Immunoblot analysis of NLRP3, IL-1β, Cleaved-IL-1β (Asp117), FoxO1, P65 and phospho-P65 (Ser 536) expression. β-actin was used as a loading control. Data are shown as mean ± SEM; *P < 0.05; **P < 0.01 (two-tailed unpaired t-test).

protein levels (37). In addition, the present study demonstrated that FTO expression level is higher in the liver of patients with non-alcoholic steatohepatitis (38). Although these reports have shown that FTO is associated with inflammatory disorders, the role of FTO in LPS-induced endotoxin shock remains unknown. In the study, we evaluated the effect of *Fto*-siRNA on the host inflammatory response to LPS-induced endotoxin shock and found that *Fto*-siRNA treated mice showed a higher survival rate compared with control mice. In addition, serum pro-inflammatory cytokines were also significantly reduced in mice

pretreated with *Fto*-siRNA, indicating that nanomedicine-based gene therapy can be used as a potential treatment strategy for endotoxin shock.

The production of IL-1β mediated by NLRP3 inflammasomes undergoes a two-step signaling process. First, in the priming phase, the synthesis of pro-IL-1β and NLRP3 depends on the activation of NF-κB. Pathogen-related molecular patterns, such as LPS, are recognized by Toll-like receptors and induce inflammatory response (39). In the secondary signals, NLRP3 inflammasome activation can be triggered by various inducers to promote the mature IL-1β production. Therefore, NLRP3 inflammasome has become an attractive target to reduce inflammation. It has been demonstrated that targeting NLRP3 inflammasomes can alleviate the acute inflammation of LPS-induced endotoxin shock (12). In this study, we evaluated the effect of FTO on macrophages after LPS stimulation and revealed the anti-inflammatory function of *Fto*-siRNA at the LPS priming stage. *Fto*-siRNA reduces IL-1β secretion by inhibiting NF-κB activation and suppressing NLRP3 inflammasome activation. Therefore, the protective effect of targeted FTO inhibition is the combined inhibitory effect of the NF-κB pathway and the activation of NLRP3 inflammasome, and ultimately reduces the production of mature IL-1β.

FoxO1 is a forkhead transcription factor involved in mediating the insulin signaling pathway. The m^6A sites on *Foxo1* mRNA can be demethylated by FTO to up-regulate FoxO1 expression (22). The FoxO1 pathway regulates multiple cellular processes, such as inflammatory responses, gluconeogenesis, and apoptosis (40–42). It is well established that FoxO1 promotes the production of pro-inflammatory cytokines in insulin resistant hepatocytes (43). FoxO1 has also been confirmed to participate in the regulation of IL-1β production in macrophages (24), suggesting a critical role of FoxO1 signaling in inflammatory process. Moreover, it has been found that FoxO1 can mediate the activation of NLRP3 inflammasome. Inhibition of FoxO1 by using of the molecule inhibitors could block NLRP3 inflammasome assembly and activation (25). Notably, our data demonstrated that silencing *Fto* could inhibit NLRP3 inflammasome activation by down-regulating the expression of FoxO1. However, whether the protective effect of FTO inhibition involves other signaling pathways is currently under investigation.

Entacapone was initially approved by the FDA as a COMT inhibitor for the treatment of Parkinson's disease (44). Interestingly, it has found that entacapone exhibits its anti-inflammatory properties through anti-oxidation and anti-inflammatory mechanisms in Ang II-induced kidney damage, rather than changes in renal dopaminergic tension induced by COMT inhibition (45). Therefore, elucidating its in-depth anti-inflammatory mechanism is important to explore the role of entacapone in some inflammatory diseases. Recently, entacapone has been identified as a selective inhibitor of FTO activity and can be used as a "tool compound" to study the function of FTO *in vivo* (22). In our study, we demonstrated convincing evidence at entacapone, targeting FTO, could suppress the activation of NLRP3 inflammasome and reduce the release of mature IL-1β in murine primary macrophages, which induced by LPS and the inflammasome activator. Entacapone administration

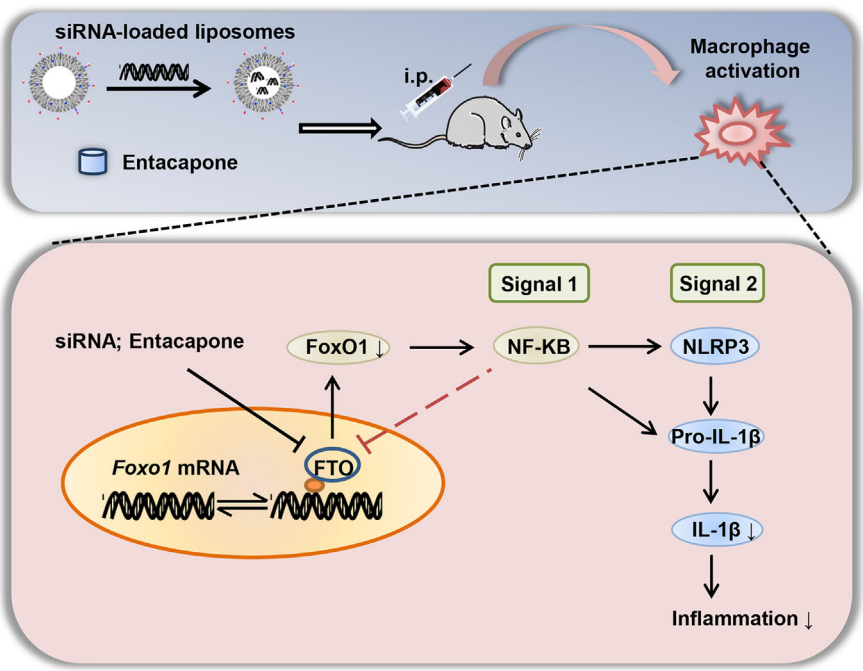

FIGURE 9 | A diagram shows that targeted inhibition of FTO demethylase protects against LPS-induced septic shock by suppressing NLRP3 inflammasome.

dramatically inhibited macrophage activation, reduced the tissue damage, and delayed the death in a mouse model of LPS-induced septic shock. Therefore, our study provides a new mechanism for the protective effect of entacapone on LPS-induced endotoxic shock and suggests that entacapone may be a promising therapeutic strategy for sepsis in clinical settings.

Our research still has limitations. Since we have confirmed the role of targeted inhibition of FTO in LPS-induce septic shock, we should use conditional knockout mice for further verification. Besides, we have clarified FTO inhibition could suppress NLRP3 inflammasome activation through down-regulating the expression of FoxO1. Therefore, additional experiments should be conducted in animals with FoxO1 overexpression to reversely verify this result.

In summary, we elucidated that FTO is involved in inflammatory response of LPS-induced septic shock. The nanoparticle-mediated *Fto*-siRNA delivery or entacapone administration dramatically inhibited macrophage activation, reduced the tissue damage, and improved survival in a mouse model of LPS-induced endotoxic shock (**Figure 9**). Mechanistically, inhibition of FTO could inhibit NLRP3 inflammasome through FoxO1/NF-κB signaling in macrophages. Therefore, targeting FTO is promising for the treatment of sepsis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Tongji Hospital Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JLu conducted most of the studies and drafted the manuscript. FW and FS contributed to the study design. FW provided help with the flow cytometry analyses. TY, QZ, and CY jointly performed some of the experiments. SR was involved in animal breeding. PY, FX, QY, and SZ contributed to the study design and review of the manuscript. C-YW and JLi designed the research, interpreted the data, and revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.663295/full#supplementary-material

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* (2016) 315(8):801–10. doi: 10.1001/jama.2016.0287
2. Seymour CW, Liu VX, Iwashyna TJ, Brunkhorst FM, Rea TD, Scherag A, et al. Assessment of Clinical Criteria for Sepsis: for the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* (2016) 315(8):762–74. doi: 10.1001/jama.2016.0288
3. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of Severe Sepsis in the United States: Analysis of Incidence, Outcome, and Associated Costs of Care. *Crit Care Med* (2001) 29(7):1303–10. doi: 10.1097/00003246-200107000-00002
4. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock: 2012. *Crit Care Med* (2013) 41(2):580–637. doi: 10.1097/CCM.0b013e31827e83af
5. Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G. The Inflammasome: A caspase-1-activation Platform That Regulates Immune Responses and Disease Pathogenesis. *Nat Immunol* (2009) 10(3):241–7. doi: 10.1038/ni.1703
6. Keller M, Rüegg A, Werner S, Beer H-D. Active Caspase-1 is a Regulator of Unconventional Protein Secretion. *Cell* (2008) 132(5):818–31. doi: 10.1016/j.cell.2007.12.040
7. Martinon F, Mayor A, Tschopp J. The Inflammasomes: Guardians of the Body. *Annu Rev Immunol* (2009) 27:229–65. doi: 10.1146/annurev.immunol.021908.132715
8. Mao K, Chen S, Chen M, Ma Y, Wang Y, Huang B, et al. Nitric Oxide Suppresses NLRP3 Inflammasome Activation and Protects Against LPS-induced Septic Shock. *Cell Res* (2013) 23(2):201–12. doi: 10.1038/cr.2013.6
9. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential Activation of the Inflammasome by Caspase-1 Adaptors ASC and Ipaf. *Nature* (2004) 430(6996):213–8. doi: 10.1038/nature02664
10. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, et al. Mice Deficient in IL-1β-Converting Enzyme are Defective in Production of Mature IL-1β and Resistant to Endotoxic Shock. *Cell* (1995) 80(3):401–11. doi: 10.1016/0092-8674(95)90490-5
11. Saperstein S, Chen L, Oakes D, Pryhuber G, Finkelstein J. Il-1beta Augments TNF-alpha-mediated Inflammatory Responses From Lung Epithelial Cells. *J Interferon Cytokine Res* (2009) 29(5):273–84. doi: 10.1089/jir.2008.0076

12. Xu C, Lu Z, Luo Y, Liu Y, Cao Z, Shen S, et al. Targeting of NLRP3 Inflammasome With Gene Editing for the Amelioration of Inflammatory Diseases. *Nat Commun* (2018) 9(1):4092. doi: 10.1038/s41467-018-06522-5
13. Wei C-M, Gershowitz A, Moss B. Methylated Nucleotides Block 5′ Terminus of HeLa Cell Messenger RNA. *Cell* (1975) 4(4):379–86. doi: 10.1016/0092-8674(75)90158-0
14. Wei CM, Gershowitz A, Moss B. 5'-Terminal and Internal Methylated Nucleotide Sequences in HeLa Cell Mrna. *Biochemistry* (1976) 15(2):397–401. doi: 10.1021/bi00647a024
15. Esteller M, Pandolfi PP. The Epitranscriptome of Noncoding Rnas in Cancer. *Cancer Discov* (2017) 7(4):359. doi: 10.1158/2159-8290.CD-16-1292
16. Shi H, Wei J, He C. Where, When, and How: Context-Dependent Functions of RNA Methylation Writers, Readers, and Erasers. *Mol Cell* (2019) 74(4):640–50. doi: 10.1016/j.molcel.2019.04.025
17. Dominissini D, Moshitch-Moshkovitz S, Schwartz S, Salmon-Divon M, Ungar L, Osenberg S, et al. Topology of the Human and Mouse M6a RNA Methylomes Revealed by M6a-Seq. *Nature* (2012) 485(7397):201–6. doi: 10.1038/nature11112
18. Slobodin B, Han R, Calderone V, Vrielink JAFO, Loayza-Puch F, Elkon R, et al. Transcription Impacts the Efficiency of Mrna Translation Via Co-transcriptional N6-Adenosine Methylation. *Cell* (2017) 169(2):326–37.e12. doi: 10.1016/j.cell.2017.03.031
19. Meyer KD, Saletore Y, Zumbo P, Elemento O, Mason CE, Jaffrey SR. Comprehensive Analysis of mRNA Methylation Reveals Enrichment in 3' UTRs and Near Stop Codons. *Cell* (2012) 149(7):1635–46. doi: 10.1016/j.cell.2012.05.003
20. Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, et al. N6-Methyladenosine-Dependent Regulation of Messenger RNA Stability. *Nature* (2014) 505(7481):117–20. doi: 10.1038/nature12730
21. Wang X, Zhao BS, Roundtree IA, Lu Z, Han D, Ma H, et al. N(6)-Methyladenosine Modulates Messenger RNA Translation Efficiency. *Cell* (2015) 161(6):1388–99. doi: 10.1016/j.cell.2015.05.014
22. Peng S, Xiao W, Ju D, Sun B, Hou N, Liu Q, et al. Identification of Entacapone as a Chemical Inhibitor of FTO Mediating Metabolic Regulation Through FOXO1. *Sci Trans Med* (2019) 11(488):eaau7116. doi: 10.1126/scitranslmed.aau7116
23. Nissinen E, Lindén IB, Schultz E, Pohto P. Biochemical and Pharmacological Properties of a Peripherally Acting catechol-O-methyltransferase Inhibitor Entacapone. *Naunyn-Schmiedeberg's Arch Pharmacol* (1992) 346(3):262–6. doi: 10.1007/bf00173538
24. Su D, Coudriet GM, Hyun Kim D, Lu Y, Perdomo G, Qu S, et al. FoxO1 Links Insulin Resistance to Proinflammatory Cytokine IL-1beta Production in Macrophages. *Diabetes* (2009) 58(11):2624–33. doi: 10.2337/db09-0232
25. Kim DH, Kim SM, Lee B, Lee EK, Chung KW, Moon KM, et al. Effect of Betaine on Hepatic Insulin Resistance Through FOXO1-induced NLRP3 Inflammasome. *J Nutr Biochem* (2017) 45:104–14. doi: 10.1016/j.jnutbio.2017.04.014
26. Guo Y-C, Zhang M, Wang F-X, Pei G-C, Sun F, Zhang Y, et al. Macrophages Regulate Unilateral Ureteral Obstruction-Induced Renal Lymphangiogenesis Through C-C Motif Chemokine Receptor 2–Dependent Phosphatidylinositol 3-Kinase-Akt–Mechanistic Target Of Rapamycin Signaling and Hypoxia-Inducible Factor-1α/Vascular Endothelial Growth Factor-C Expression. *Am J Pathol* (2017) 187(8):1736–49. doi: 10.1016/j.ajpath.2017.04.007
27. Yang P, Zhang Y, Pang J, Zhang S, Yu Q, He L, et al. Loss of Jak2 Impairs Endothelial Function by Attenuating Raf-1/Mek1/Sp-1 Signaling Along With Altered eNOS Activities. *Am J Pathol* (2013) 183(2):617–25. doi: 10.1016/j.ajpath.2013.04.007
28. Pan T, Zhou Q, Miao K, Zhang L, Wu G, Yu J, et al. Suppressing Sart1 to Modulate Macrophage Polarization by siRNA-loaded Liposomes: A Promising Therapeutic Strategy for Pulmonary Fibrosis. *Theranostics* (2021) 11(3):1192–206. doi: 10.7150/thno.48152
29. Whitehead KA, Dorkin JR, Vegas AJ, Chang PH, Veiseh O, Matthews J, et al. Degradable Lipid Nanoparticles With Predictable In Vivo siRNA Delivery Activity. *Nat Commun* (2014) 5:4277. doi: 10.1038/ncomms5277
30. Wang Y, Zhang L, Wu G-R, Zhou Q, Yue H, Rao L-Z, et al. MBD2 Serves as a Viable Target Against Pulmonary Fibrosis by Inhibiting Macrophage M2 Program. *Sci Adv* (2020) 7(1):eabb6075. doi: 10.1126/sciadv.abb6075
31. Ran L, Yu Q, Zhang S, Xiong F, Cheng J, Yang P, et al. Cx3cr1 Deficiency in Mice Attenuates Hepatic Granuloma Formation During Acute Schistosomiasis by Enhancing the M2-type Polarization of Macrophages. *Dis Models Mech* (2015) 8(7):691–700. doi: 10.1242/dmm.018242
32. Wang F, Sun F, Luo J, Yue T, Chen L, Zhou H, et al. Loss of Ubiquitin-Conjugating Enzyme E2 (Ubc9) in Macrophages Exacerbates Multiple Low-Dose Streptozotocin-Induced Diabetes by Attenuating M2 Macrophage Polarization. *Cell Death Dis* (2019) 10(12):892. doi: 10.1038/s41419-019-2130-z
33. Scaffidi P, Misteli T, Bianchi ME. Release of Chromatin Protein HMGB1 by Necrotic Cells Triggers Inflammation. *Nature* (2002) 418(6894):191–5. doi: 10.1038/nature00858
34. Li L, Lu YQ. The Regulatory Role of High-Mobility Group Protein 1 in Sepsis-Related Immunity. *Front Immunol* (2020) 11:601815. doi: 10.3389/fimmu.2020.601815
35. Church C, Lee S, Bagg EAL, McTaggart JS, Deacon R, Gerken T, et al. A Mouse Model for the Metabolic Effects of the Human Fat Mass and Obesity Associated FTO Gene. *PLoS Genet* (2009) 5(8):e1000599–e. doi: 10.1371/journal.pgen.1000599
36. Ligthart S, Vaez A, Hsu Y-HInflammation Working Group of the CC, Pmi Wg XCP and LifeLines Cohort S, et al. Bivariate Genome-Wide Association Study Identifies Novel Pleiotropic Loci for Lipids and Inflammation. *BMC Genomics* (2016) 17:443. doi: 10.1186/s12864-016-2712-4
37. Welsh P, Polisecki E, Robertson M, Jahn S, Buckley BM, de Craen AJM, et al. Unraveling the Directional Link Between Adiposity and Inflammation: A Bidirectional Mendelian Randomization Approach. *J Clin Endocrinol Metab* (2010) 95(1):93–9. doi: 10.1210/jc.2009-1064
38. Lim A, Zhou J, Sinha RA, Singh BK, Ghosh S, Lim K-H, et al. Hepatic FTO Expression is Increased in NASH and its Silencing Attenuates Palmitic Acid-Induced Lipotoxicity. *Biochem Biophys Res Commun* (2016) 479(3):476–81. doi: 10.1016/j.bbrc.2016.09.086
39. Takeuchi O, Akira S. Pattern Recognition Receptors and Inflammation. *Cell* (2010) 140(6):805–20. doi: 10.1016/j.cell.2010.01.022
40. Nakae J, Kitamura T, Silver DL, Accili D. The Forkhead Transcription Factor Foxo1 (Fkhr) Confers Insulin Sensitivity Onto glucose-6-phosphatase Expression. *J Clin Invest* (2001) 108(9):1359–67. doi: 10.1172/JCI12876
41. de Candia P, Blekhman R, Chabot AE, Oshlack A, Gilad Y. A Combination of Genomic Approaches Reveals the Role of FOXO1a in Regulating an Oxidative Stress Response Pathway. *PLoS One* (2008) 3(2):e1670–e. doi: 10.1371/journal.pone.0001670
42. Park S-J, Sohn H-Y, Yoon J, Park SI. Down-Regulation of FoxO-dependent c-FLIP Expression Mediates TRAIL-induced Apoptosis in Activated Hepatic Stellate Cells. *Cell Signall* (2009) 21(10):1495–503. doi: 10.1016/j.cellsig.2009.05.008
43. Miao H, Zhang Y, Lu Z, Liu Q, Gan L. FOXO1 Involvement in Insulin Resistance-Related Pro-Inflammatory Cytokine Production in Hepatocytes. *Inflamm Res* (2012) 61(4):349–58. doi: 10.1007/s00011-011-0417-3
44. Schrag A. Entacapone in the Treatment of Parkinson's Disease. *Lancet Neurol* (2005) 4(6):366–70. doi: 10.1016/S1474-4422(05)70098-3
45. Helkamaa T, Finckenberg P, Louhelainen M, Merasto S, Rauhala P, Lapatto R, et al. Entacapone Protects From Angiotensin II-induced Inflammation and Renal Injury. *J Hypertens* (2003) 21(12):2353–63. doi: 10.1097/00004872-200312000-00025

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Co-Administration of Anticancer Candidate MK-2206 Enhances the Efficacy of BCG Vaccine Against *Mycobacterium tuberculosis* in Mice and Guinea Pigs

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The failure of *M. bovis* BCG to induce long-term protection has been endowed to its inability to escape the phagolysosome, leading to mild activation of $CD8^+$ mediated T cell response. Induction of apoptosis in host cells plays an important role in potentiating dendritic cells-mediated priming of $CD8^+$ T cells, a process defined as "cross-priming." Moreover, IL-10 secretion by infected cells has been reported to hamper BCG-induced immunity against Tuberculosis (TB). Previously, we have reported that apoptosis of BCG-infected macrophages and inhibition of IL-10 secretion is FOXO3 dependent, a transcription factor negatively regulated by the pro-survival activated threonine kinase, Akt. We speculate that FOXO3-mediated induction of apoptosis and abrogation of IL-10 secretion along with *M. bovis* BCG immunization might enhance the protection imparted by BCG. Here, we have assessed whether co-administration of a known anti-cancer Akt inhibitor, MK-2206, enhances the protective efficacy of *M. bovis* BCG in mice model of infection. We observed that *in vitro* MK-2206 treatment resulted in FOXO3 activation, enhanced BCG-induced apoptosis of macrophages and inhibition of IL-10 secretion. Co-administration of *M. bovis* BCG along with MK-2206 also increased apoptosis of antigen-presenting cells in draining lymph nodes of immunized mice. Further, MK-2206 administration improved BCG-induced $CD4^+$ and $CD8^+$ effector T cells responses and its ability to induce both effector and central memory T cells. Finally, we show that co-administration of MK-2206 enhanced the protection imparted by *M. bovis* BCG against *Mtb* in aerosol infected mice and guinea pigs. Taken together, we provide evidence that MK-2206-mediated activation of FOXO3 potentiates BCG-induced immunity and imparts protection against *Mtb* through enhanced innate immune response.

Keywords: BCG vaccine, Akt inhibitor, innate immunity, apoptosis, tuberculosis

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INTRODUCTION

Despite global efforts and advances in healthcare, Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is still the leading cause of mortality from a single infectious agent. Globally, 10 million new cases and 1.4 million deaths due to TB were reported in 2019 (1). Bacilli Calmette-Guérin (BCG) is the most widely used and only available prophylactic TB vaccine (2). Although it is effective against the severe forms of disseminated and pulmonary forms of TB in children, BCG confers only limited and variable protection against the disease in adolescents and adults who account for the majority of TB transmission (3). The protection imparted by BCG is in the range of 0% to 80% and is dependent on the individual's age and incident rates of TB. Mangtani et al., have reported that in a population at high risk for TB, immunization with BCG was able to show protection for at least 10 years. In comparison, in population at low-risk for TB, BCG immunization was able to impart protection for at least 20 years (4). Hence, there is an urgent need for better control and prevention strategies to stop disease transmission and sustain a long-term TB control (5). Several other strategies such as live attenuated, heat killed, viral vector based, protein subunits and DNA vaccines have been employed to develop alternate TB vaccines (6). However, it still remains elusive to replace BCG because of its safety and only few candidate vaccines have significantly showed better protection than BCG in either animal studies or clinical trials (7). Host-directed procedures that amplify the BCG-elicited protective response might prove useful to control disease transmission and enhance the protection imparted by BCG vaccine.

The available data from experimental and clinical studies suggest that an effective immune response against TB relies mainly on a robust effector and memory T cells responses (8). Consequently, an efficient TB vaccine should be able to induce the formation of antigen-specific polyfunctional type-1 $CD4^+$ and $CD8^+$ T cells through peptides presented by MHC-II and MHC-I, respectively, on the surface of infected macrophages (9, 10). The decrease of BCG effectiveness has been endowed to the defect in antigen processing which subsequently results in diminished Th1 responses. It has been demonstrated that immunization with BCG is unable to induce effective $CD8^+$ and $CD4^+$ T cells responses and this deficiency might be one of the factors accounting for its poor efficacy (11, 12). In fact, BCG bacilli sequester within phagosomes of macrophages and phagocytes subsequently fuse with lysosomes for degradation and generation of peptides (11). Thereafter, peptides bound to MHC-II complex are exported to the plasma membrane for presentation to $CD4^+$ T cells. Further, BCG also secretes proteins that are either cleaved within the phagosomes or processed for presentation along with MHC-I to $CD8^+$ T cells (11). Previous studies have reported two defects in antigen processing which affects the ability of BCG-infected phagocytes to prime T cells. It has been shown that BCG residing within immature phagosomes might not expose its repertoire of antigens for lysosomes mediated degradation. Moreover, it was reported that limited cleavage of Ag85B and its assembly into MHC-II complex occurs within antigen presenting cells phagosomes (13). This eventually results in inaccessibility of BCG antigens to both the endocytic MHC-II and cytosolic MHC-I antigen presentation pathways and suboptimal activation of T cells and Th1 immunity (14).

It has been reported that apoptosis of infected host cells facilitates mycobacterial antigen presentation to T lymphocytes through MHC-I and CD1 (15). In accordance, vaccination with apoptotic vesicles, purified from BCG-infected macrophages, was able to confer better protection, than BCG, against challenge with *Mtb* in mice. Such higher protection was associated with the enhancement of dendritic cell-mediated cross-priming of $CD8^+$ T cells, termed the "Detour pathway" (16). In agreement, it has been demonstrated that the improved vaccine efficacy of the recombinant BCG *ΔureC hly*$^+$ vaccine in comparison to the parental strain was associated with higher cross-priming as a consequence of enhanced apoptosis (17). Furthermore, vaccination with the *ΔsecA2ΔlysA* and *ΔnuoG* mutant strains of *Mtb* also triggered higher levels of apoptosis in comparison to BCG immunized animals and this resulted in better protection against *Mtb* infection (18, 19). Also, immunization with DNA vaccine co-expressing the Ag85A and the human pro-apoptotic caspase 3 increased protection against *Mtb* infection in comparison to immunization with DNA vaccine encoding Ag85A only (20). In comparison to immunization with BCG, rBCG strain expressing the human pro-apoptotic protein BAX also triggered macrophages apoptosis and elicited predominantly a Th1 protective response (21). Taken together, these studies suggest that enhancing apoptosis in host cells, during BCG vaccination, might be useful to improve its efficacy.

Previous studies have shown that IL-10 negatively regulates the immune response to mycobacterial infection as it affects the adaptive immune response by impeding the functions of macrophages and DC (22, 23). In agreement, it has been reported that BCG inhibits the expression of MHC-II through IL-10 induction, therefore affecting the optimal activation of $CD4^+$ T cells (22). This impaired activation of $CD4^+$ T cells subsequently reduces long term persistence and proliferative activity of $CD8^+$ T cells, their distribution from lymph nodes to distant organs and consequently delayed acquisition of immune protection (24). It has been shown that immunization with BCG in IL-10–deficient mice results in stronger DC activation through increased expression of the surface presenting molecules (25). IL-10 also suppresses the innate immune responses of BCG infected macrophages by inhibiting toll-like receptor-mediated signaling and activating the pro-survival pathway PI3K/Akt (26, 27). In concordance, inhibition of macrophages apoptosis by *M. bovis* also correlates with increased IL-10, Bcl2 and decreased TNF-α production (28). Moreover, induction of IL-10 production in both human and mice upon BCG vaccination limits its protective efficacy (29). Interestingly, it was proposed that BCG-induced Th1 responses progressively switches to Th2, however, in the absence of IL-10, the host immunity leans towards Th1 response post BCG vaccination. In agreement with these findings, IL-10 KO mice

vaccinated with BCG presented higher numbers of T cells secreting IFN-γ, IL-17, and TNF-α in comparison to BCG vaccinated wild type mice (30). As expected, the blockade of IL-10 signaling during BCG vaccination resulted in enhanced, sustainable Th1 and Th17 responses and better protection in mice infected with *Mtb* (31). Taken together, these observations suggest that inhibition of IL-10 signaling along with induction of apoptosis during BCG immunization might be a promising strategy to drive stronger Th1/Th17 mediated response and promote long-term immunity against TB.

Previously, we reported that both, apoptosis of BCG-infected macrophages and inhibition of the associated secretion of IL-10, relies on activation of FOXO3, a transcription factor negatively regulated by the pro-survival activated threonine kinase Akt (32, 33). Based on these observations, we hypothesize that activation of FOXO3, using an Akt inhibitor, would enhance BCG-induced host cells apoptosis and inhibit IL-10 production, leading to better protection against TB. To test this hypothesis, we assessed the combination of an anti-cancer candidate Akt inhibitor, MK-2206, and *M. bovis* BCG to impart protection against *Mtb* in aerosol infected mice and guinea pigs. We observed that treatment of macrophages with MK-2206 resulted in enhanced BCG-induced apoptosis and inhibition of IL-10 secretion. The administration of *M. bovis* BCG along with MK-2206 inhibitor increased apoptosis of antigen presenting cells (APCs) in lymph nodes. Further, MK-2206 co-administration improved BCG-induced $CD4^+$ and $CD8^+$ effector T cells responses and enhanced the ability of BCG to induce both effector and central memory T cells. Interestingly, we also found that co-administration of MK-2206 strengthened the protection conferred by *M. bovis* BCG against *Mtb* in aerosol infected mice and guinea pigs. Taken together, we provide evidence that FOXO3 activation by MK-2206 potentiates BCG-induced immunity and protection against *Mtb* through enhanced apoptosis of host cells and abrogation of IL-10 secretion.

MATERIALS AND METHODS

Cell Culture and Infection

The mouse macrophage cell lines J774A.1 (ATCC TIB-67) and RAW264.7 (ATCC TIB-71) were maintained and cultured in DMEM (Hyclone, USA) supplemented with 5% fetal bovine serum (FBS) (Thermo Fisher, USA) (34). For infection experiments, macrophages were infected with a single cell suspension of *M. bovis* BCG Pasteur strain 1173P2, at a multiplicity of infection (MOI) of 1:10 for 3 h. Subsequently, the extracellular bacteria were removed by washing twice with 1× PBS and cells were overlaid with DMEM medium containing MK-2206 (Selleckchem, USA).

Apoptosis Assay and Measurement of Caspase Activity

Apoptosis assay was performed according to the manufacturer`s recommendation. Briefly, cells were infected with BCG and subsequently treated with different concentrations of MK-2206. At designated time points, cells were harvested, washed with 1× PBS, stained with APC-Annexin V- and 7AAD (BD Pharmingen, US) and apoptosis was analyzed using FACS Canto flow cytometer (BD Biosciences, US). The cytometric detection of activated caspase-3 and caspase-7 in apoptotic cells was assayed using CellEvent™ Caspase-3/7 Flow Cytometry assay kit using 503/530 nm filter (FITC channel) (Thermo Fischer, USA).

Western Blotting

For immunoblot analysis, macrophages were lysed in 1× Laemmli buffer and the protein concentration was estimated using Bicinchoninic acid protein assay kit (BCA, Sigma Aldrich, USA). For immunoblot analysis, equal amounts of whole-cell lysate were resolved by electrophoresis on 10% SDS-PAGE gel, transferred to PVDF membrane and subsequently probed with the respective antibodies as per manufacturer recommendations. The primary antibodies used in the study were purchased from either Sigma Aldrich, Merck [FKHRL1-D12 (p-FOXO3Thr32), FKHRL- 1 (FOXO3), β-actin or Cell Signaling Technology, USA (Akt and p-AktSer473).

Ethics Statement

The animal experiments involving mice and guinea pigs were approved from animal ethics committee from Translational Health Science and Technology Institute (THSTI) and International Centre for Genetic Engineering and Biotechnology (ICGEB). The animal experiments were performed as per the guidelines mentioned by the committee for the purpose of control and supervision of experiments on animals.

Immunization of Mice and Guinea Pigs

For preparation of vaccine stocks, *M. bovis* BCG (Pasteur stain 1173P2) was cultured in Middlebrook 7H9 medium supplemented with 10% oleic acid albumin dextrose, 0.05% Tween 80 and 0.5% glycerol. The cultures were grown till mid-log phase, harvested by centrifugation, washed, resuspended in 1× PBS and stored as 1,000 µl aliquots at −80°C till further use. The bacterial viability was determined by plating 10.0-fold serial dilutions on Middlebrook 7H11 plates and plates were incubated at 37°C for 3 to 4 weeks. For animal studies, MK-2206 was purchased from Merck and formulated in 30% w/v captisol solution (Cydex pharmaceuticals) as per manufacturer recommendations and previous reports (35, 36). Captisol is a chemically modified β-cyclodextrin that enhances safety, stability, solubility and dermal absorption of the drug (37, 38). For mice experiments, BALB/c mice (female, 6–8 weeks) were subcutaneously immunized and divided into the following 4 groups: (i) sham immunized, (ii) vaccinated with 10^6 CFU of *M. bovis* BCG, (iii) vaccinated with 10^6 CFU of *M. bovis* BCG followed by administration of MK-2206 (25 mg/kg) at day 2 and day 3 post-immunization at the same site of vaccination and (iv) administration of MK-2206 at day 2 and day 3 along with the adjuvant. Guinea pigs were immunized *via* intradermal route

and divided into 3 groups; (i) sham immunized group, (ii) vaccinated with 10^5 CFU of *M. bovis* BCG and (iii) BCG immunized and boosted with administration of MK-2206, at day 2 and day 3 post-immunization at the same site of vaccination.

Preparation of Splenocytes and Lymph Nodes Single Cells

For single cell preparation, spleens and inguinal lymph nodes were treated with complete RPMI medium containing 1 mg/mL of collagenase D (Roche) and 10 U of DNase I (Sigma) at 37°C for 30 min. Subsequently, single cell preparation of collagenase treated organs were prepared and filtered through a 70-µm cell strainer. The single cell suspension was centrifuged, washed with 1× PBS (without Ca-Mg), resuspended in RPMI and enumerated using trypan blue exclusion method.

Flow Cytometry Analysis

For *ex-vivo* apoptosis assay, live cells from lymph nodes (1×10^6) were incubated for 10 min with anti-CD16/CD32 to block nonspecific antibody binding. Subsequently, cells were stained with Annexin V-PE, anti-CD11b-BV510, anti-CD11c-APC for 30 min at 4°C as per manufacturer's recommendations (BD Biosciences). The antibody-stained cells were washed twice, suspended in FACS staining buffer and subsequently analyzed using flow cytometry (FACS Canto II, BD Biosciences). For T cell responses, single-cell suspension of spleens was seeded at a density of 1×10^6 cells per well in a 48-well plate and stimulated for 48 h with 10 µg/ml Purified Protein Derivative (PPD; Statens Serum Institut). Subsequently, cells were harvested, washed, incubated for 15 min with anti-CD16/CD32 and subsequently stained for extracellular markers with the following anti-mouse antibodies: anti-CD3-FITC, anti-CD4-Percp, anti-CD8-APCH7, anti-CD44-PE and anti-CD62L-BV421 for 30 min at 4°C as per manufacturer's recommendations (**Supplementary Table 1**).

For intracellular cytokine staining, spleen cells were *ex vivo* stimulated for 48 h with PPD in the presence of Golgi plug containing Brefeldin A (BD Biosciences) for the last 6 h. Following this, cells were harvested, washed, incubated for 10 min with anti-CD16/CD32 and stained for extracellular markers with anti-CD3-FITC, anti-CD4 -Percp, anti-CD8-APCH7 for 30 min at 4°C (BD Biosciences). The intracellular staining of cells was performed using a cytofix/cytoperm kit with anti-TNF-α-BV421, anti-IFN-γ-Pecy7 and anti–IL-17-BV510 (**Supplementary Table 1**). The stained samples were washed twice, resuspended in FACS staining buffer and data was collected by flow cytometry (FACS Canto II, BD Biosciences). The acquired data was analyzed using Flow Jo software.

Cytokine Assays

Cytokines levels in the supernatants of PPD stimulated splenocytes were quantified using specific ELISA kits (BD PharMingen, USA). The levels of TNF-α, IFN-γ, IL-2, IL-4, IL-10, IL-12, GM-CSF, and IL-5 in filtered supernatants of lung homogenates were measured using Bio-plex pro mouse cytokine Th_1/Th_2 assay kit (Bio-Rad laboratories).

Protection Studies Against Infection With *Mtb*

Prior to aerosol challenge few animals were euthanized at both 3 weeks and 8 weeks post-immunization to check for residual viable BCG bacilli. As expected, we did not observe any bacterial counts in lungs of immunized animals at both time points. For aerosol challenge, *Mtb*, H37Rv was grown till $OD_{600\ nm} \sim 1.0$ and washed twice with 1× PBS. Subsequently, for mice experiment, single cell suspension containing 5 $x10^8$ bacilli was prepared in nebulizer. The infection of immunized mice *via* aerosol route using Inhalation Exposure System (Glas-Col, LLC) resulted in implantation of 300 CFU in lungs. In the case of guinea pigs, single cell suspension containing 1 $x10^8$ bacilli was prepared in nebulizer. The infection of immunized guinea pigs *via* aerosol route using Inhalation Exposure System (Glas-Col, LLC) resulted in implantation of 100 CFU in lungs. The disease progression was determined at 30- and 75- days post-infection. For CFU determination, lungs and spleens were homogenized in 2 ml of normal saline and 100 µl of 10-fold serial dilutions were plated on MB7H11 medium at 37°C for 3 to 4 weeks. For histopathology analysis of lungs, 10% formalin fixed lung tissues were stained with hematoxylin, eosin and extent of tissue damage was determined by a pathologist as previously described (39).

Statistical Analysis

Statistical analyses and generation of graphs were performed with GraphPad Prism version 7 (GraphPad Software Inc., USA). Data are expressed as the mean ± standard error of the mean (SEM). Comparison between groups was performed by paired t-test and differences with a p value less than 0.05 were considered as statistically significant.

RESULTS

MK-2206 Enhances Apoptosis of BCG-Infected Macrophages *In Vitro*

We have previously reported that FOXO3 activation mediates apoptosis of BCG infected macrophages, an important strategy for host to eliminate intracellular bacteria (33). We have also recently reported that pharmacological inhibition of Akt with MK-2206 induces the activation of FOXO3 transcription factor in BCG-infected macrophages. This results in strong inhibition of IL-10 expression through FOXO3 binding on IL-10 promoter (32). Moreover, such negative regulation of IL-10 secretion favored the establishment of strong M1/Th1 immune responses and inhibited the intracellular replication of mycobacteria. In the present study, we first verified whether the MK-2206–mediated activation of FOXO3 could induce macrophage apoptosis. Murine J774A.1 macrophage cell line was treated with different concentrations of MK-2206 and the percentage of apoptotic cells was evaluated at 24 and 48 h post treatment as described in *Materials and Methods*. As shown in **Figure S1A**, treatment of

macrophages with MK-2206 resulted in a dose-and time dependent increase in the percentage of apoptosis compared to vehicle-treated controls. Moreover, we observed that MK-2206–induced apoptosis was caspase-dependent, as shown by the increase in expression levels of effectors, caspase 3/7 activity in drug-treated samples (**Figure S1B**). In comparison to DMSO-treated cells, a 4.0-fold increase in caspase3/7 activity was observed in macrophages upon exposure to 10 μM of MK-2206 for 48 h. We also observed induction of apoptosis by MK-2206 was accompanied with decreased expression levels of both p-Akt and p-FOXO3 in a time dependent manner (**Figure S1C**). Taken together, these observations suggest that decreased phosphorylation of Akt in the presence of MK-2206 promotes FOXO3 dependent intrinsic apoptosis in macrophages. Noteworthy, we have also confirmed our previous data regarding the inhibition, by MK-2206, of the BCG-induced secretion of IL-10 in murine macrophages (**Figure S1D**) (32).

To further verify if MK-2206 treatment enhances apoptosis of mycobacteria-infected macrophages, cells were infected with BCG at MOI of 1:10, treated with MK-2206 and subsequently apoptosis was evaluated. As shown in **Figure 1A**, we noticed that infection did not induce a significant apoptosis compared to uninfected cells at 24 h post-infection. However, the percentage of Annexin V+ increased by approximately 2-fold at 48 h post-infection in comparison to uninfected cells (**Figure 1A**). Furthermore, treatment with the MK-2206 enhanced BCG-induced apoptosis in a dose and time dependent manner upon 24 h (30% and 42% increase of Annexin V+ cells for 5 and 10 μM of MK-2206, respectively) and 48 h (20% and 32% increase of Annexin V+ cells for 5 and 10 μM of MK-2206, respectively) of treatment, in comparison to BCG-infected cells (**Figure 1A**). We also investigated the relative activation of caspases 3/7 in BCG-infected cells post 24 and 48 h of MK-2006 treatment (**Figure 1B**). We found that treatment with MK-2206 increased the activity of caspases 3/7 in a dose and time dependent manner as compared to BCG infection alone. As shown in **Figure 1B**, we observed 15% and 30% increase of caspases 3/7 activity at 24 h for 5 and 10 μM MK-2206 treatment, respectively. Further, 30% and 41% increase of caspases 3/7 activity was observed for 5 μM and 10 μM of MK-2206 treatment, respectively, at 48 h (**Figure 1B**). Taken together, these results implicate that activation of FOXO3 through Akt inhibition post BCG infection results in enhancement of apoptotic cell death.

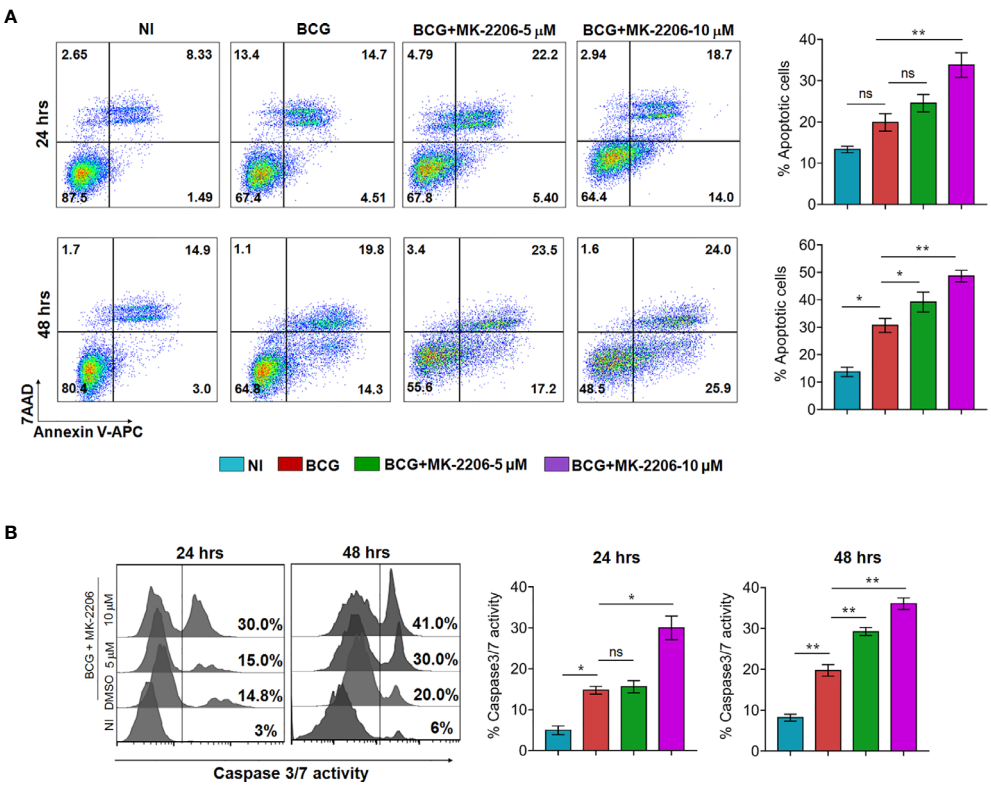

FIGURE 1 | MK-2206 enhances apoptosis in BCG infected macrophages. J774A.1 macrophages were infected with BCG (MOI ~ 10) and subsequently treated with different concentrations of MK-2206 (5 or 10 μM) for 24 and 48 h. Subsequently, macrophages were harvested at the indicated time points and assessed for apoptosis induction and caspase 3/7 activation. **(A)** Representative dot plots show the frequency of apoptotic cells (revealed by the sum of Annexin V^+/$7AAD^+$ cells) at 24 and 48 h post MK-2206 treatment. Histograms depict the percentage of apoptotic cells at 24 and 48 h upon the indicated treatments. The values are presented as mean ± SEM of two experiments performed in triplicates. **(B)** To evaluate caspases activation, cells were stained with 2 μM of cell event caspase-3/7 green detection reagent and the samples were analyzed by flow cytometry. Histograms show representative data from two experiments performed in triplicates. The data shown is mean ± SEM obtained from two experiments performed in triplicates. Statistical differences were obtained between the indicated groups *$p < 0.05$; **$p < 0.01$; ns, non-significant; NI, non-infected.

MK-2206 Administration Enhances Apoptosis of BCG-Infected Cells *In Vivo* and Triggers APCs Maturation and Their Trafficking to DLNs

In an attempt to verify the ability of Akt inhibitor, MK-2206, to induce apoptosis of phagocytes *in situ* post BCG immunization, mice were immunized with BCG followed by administration of MK-2206 as described in *Materials and Methods* (**Figure 2A**). It is worth to note that injection of the Akt inhibitor was performed at days 2 and 3 post BCG vaccination to avoid the blockade of mycobacteria phagocytosis, which relies on PI3K/Akt pathway (40). Administration of MK-2206 (25 mg/kg) followed the homologous route of BCG priming in order to target the same DLNs, which has been shown to provide a more effective boost (**Figure 2A**) (41). Following vaccination, BCG resides within mononuclear phagocytes such as dermal macrophages and resident DCs (42, 43). We speculated that the administration of MK-2206 would result in the induction of apoptosis of the infected cells at the local site. Surface exposure of phosphatidylserine molecules in apoptotic cells helps in the efferocytosis of infected apoptotic cells by DCs and macrophages which triggers APCs maturation and their trafficking to DLNs (43). As the first step to elucidate the effect of MK-2206 on apoptosis post BCG vaccination, we performed flow cytometry analysis on lymph nodes cells from the different groups of animals. The subsets of APCs were identified based on the surface expression of CD11c and CD11b using a gating strategy as shown in **Figure S2A**. We found a significant increase in apoptosis at early time points in DLNs of mice vaccinated with BCG/MK-2206 in comparison to sham-immunized and BCG-immunized mice (**Figure 2B**). The induced apoptosis in immunized mice was further characterized in lymph nodes phagocytes using macrophage and dendritic cell-associated markers. As shown in **Figure 2C**, the percentage of apoptotic cells among the $CD11b^+$ subsets increased from 18% (BCG group) to 25% (BCG/MK-2206 group), while the frequency of Annexin V^+ cells among the $CD11c^+$ populations increased from 17% (BCG group) to 22% (BCG/MK-2206).

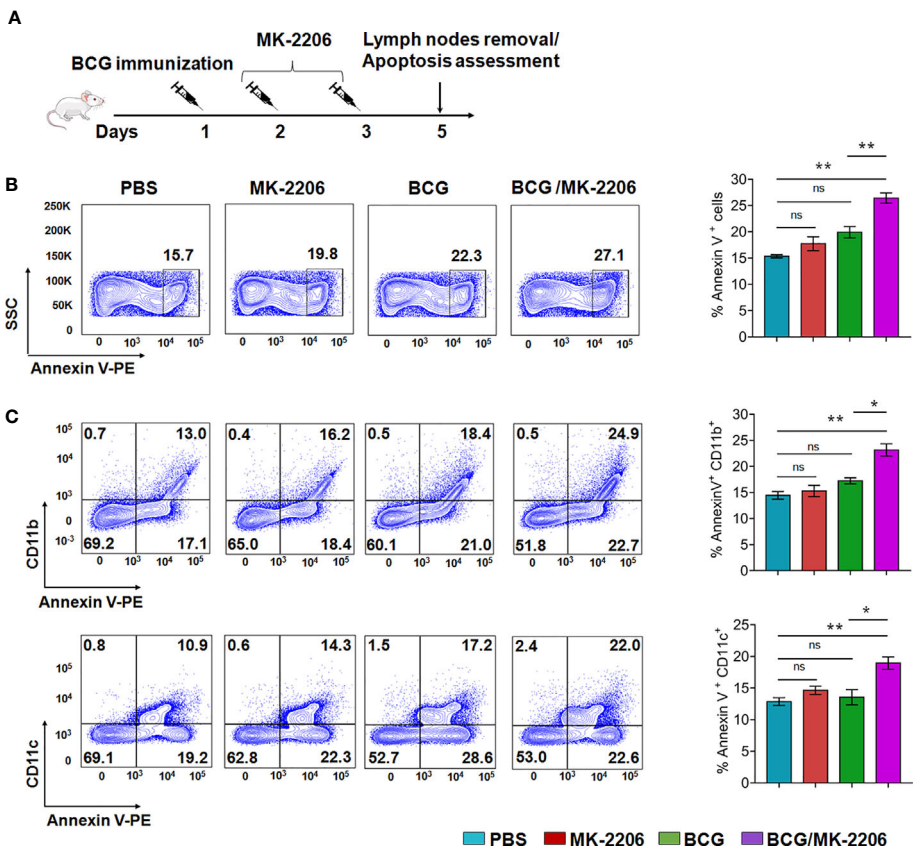

FIGURE 2 | MK-2206 enhances apoptosis in lymph nodes of BCG-vaccinated mice. **(A)** Schematic design of homologous route prime-boost vaccination strategy in mice. Animals were mock treated or primed subcutaneously with 1×10^6 BCG at day 1. Two and three days later, two groups of mock and BCG-vaccinated mice were administered MK-2206 (25 mg/kg) *via* subcutaneous route. Five days after administration, mice (n = 4–5/group) from a single experiment were sacrificed and apoptosis was measured by flow cytometry in draining lymph nodes using Annexin V staining. **(B)** Representative dot plots of apoptotic cells (Annexin V+) in lymph nodes from different groups are shown. **(C)** Frequencies of Annexin V positive cells among $CD11b^+$ and $CD11c^+$ subsets were measured in DLNs. The data shown in these panels is mean ± SEM of the results obtained from a single experiment (n = 4–5 mice/group). Statistical differences were obtained between the indicated groups *$p < 0.05$; **$p < 0.01$; ns, non-significant.

We next checked the infiltration of leukocytes within lymph nodes tissue which were phenotyped and quantified by flow cytometry (**Figure S2A**). $CD11c^+CD11b^+$ were designated as myeloid DCs, $CD11c^-CD11b^+$ were designated as monocytes/macrophages and $CD11c^+CD11b^-$ as recruited macrophages based on previously described functional and morphologic characteristics (44, 45). We observed an enhanced infiltration of $CD11c^+/CD11b^-$, $CD11c^-/CD11b^+$ and $CD11c^+/CD11b^+$ subsets in DLNs of BCG/MK-2206 immunized mice in comparison to BCG-immunized mice (**Figures S2B, C**). These data suggest that administration of MK-2206 in BCG immunized mice triggered the expansion and accumulation of activated and matured APCs in DLNs.

MK-2206 Augments the Proportion of Multifunctional T Cells in BCG-Vaccinated Mice

It is well established that innate responses associated with macrophages apoptosis is associated with an enhanced DC-dependent cross-priming and better antigen-specific $CD4^+$ and $CD8^+$ T cells in lymphatic tissues (46). Moreover, multifunctional Th1 cells (producing IFN-γ and TNF-α) along with Th17 are known to define a correlate of vaccine-mediated protection (47). Therefore, we next compared the T cell response between BCG/MK-2206 prime boost immunized and BCG immunized mice. As described in *Materials and Methods*, spleens were harvested at 21 days post-immunization and single cell suspension was prepared (**Figure S3A**). Splenocytes were stimulated with PPD and the frequency of $CD4^+$ and $CD8^+$ T cells producing the signature of cytokines associated with Th1 (IFN-γ, TNF-α) and Th17 (IL-17) was determined by flow cytometry. As shown in **Figure 3A**, we observed that immunization with BCG followed by administration of MK-2206 resulted in significant higher proportion of $CD4^+$ T cells producing IFN-γ (100% increase) or TNF-α (65% increase) or IL-17 (127% increase), in comparison to BCG immunized mice. Likewise, we observed that the frequency of $CD8^+$ T cells, expressing either IFN-γ or TNF-α or IL-17, was higher in mice primed with BCG and boosted with MK-2206 as compared to mice vaccinated with BCG alone (**Figure 4A**). Further, we characterized the Ag-specific multifunctional T cells based on their ability to produce two or three cytokines. We found that mice vaccinated with BCG followed by MK-2206 administration induced significantly higher multifunctional

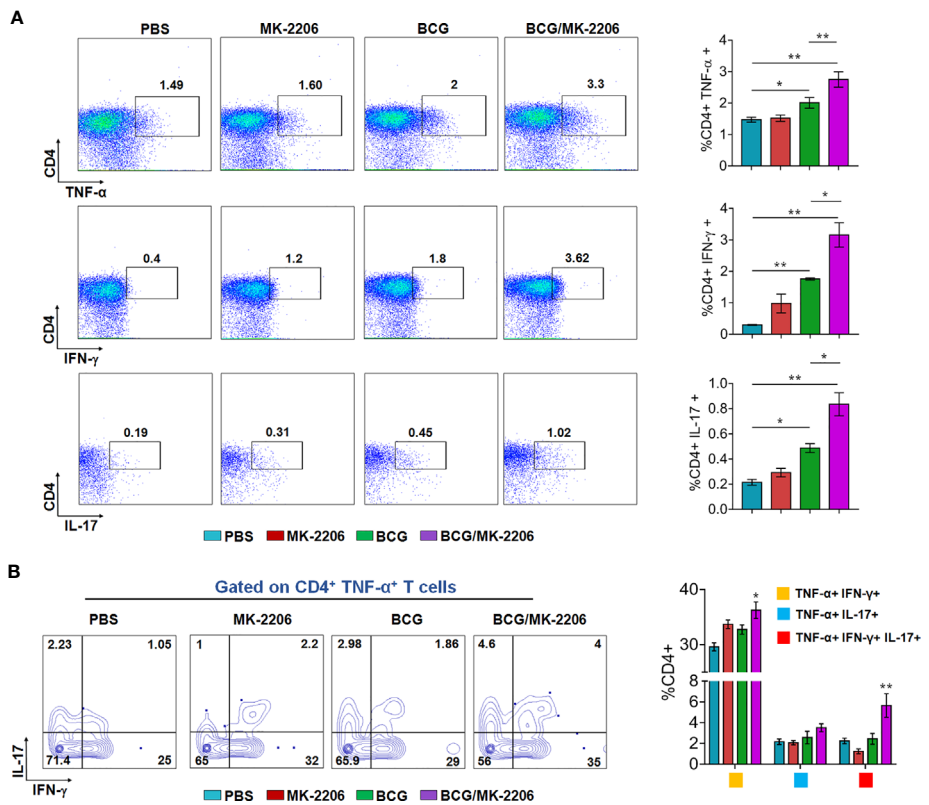

FIGURE 3 | MK-2206 increases Ag-specific multifunctional $CD4^+$ T cell responses in vaccinated mice. Each group of mice was vaccinated as described in *Materials and Methods* section. Twenty-one days after immunization, mice from each group (n=5) were euthanized and their splenocytes were incubated *ex-vivo* with PPD for 48 h in the presence of Golgi stop. **(A)** Representative dot plots showing the frequency of PPD-specific $CD4^+$ cells producing TNF-α or IFN-γ or IL-17. Data was analyzed by multicolor flow cytometry by gating on $CD3^+CD4^+$ lymphocytes. **(B)** Multifunctional cells were identified by gating on $CD4^+$ TNF-α^+, T cells. Representative FACS plots show the frequencies of $CD4^+$ T cells co-expressing TNF-α and/or IFN- γ and/or IL-17. The data shown in this panel is mean ± SEM obtained from a single experiment (n=5 mice/group). Statistical differences were obtained for the indicated groups. *p < 0.05; **p < 0.01.

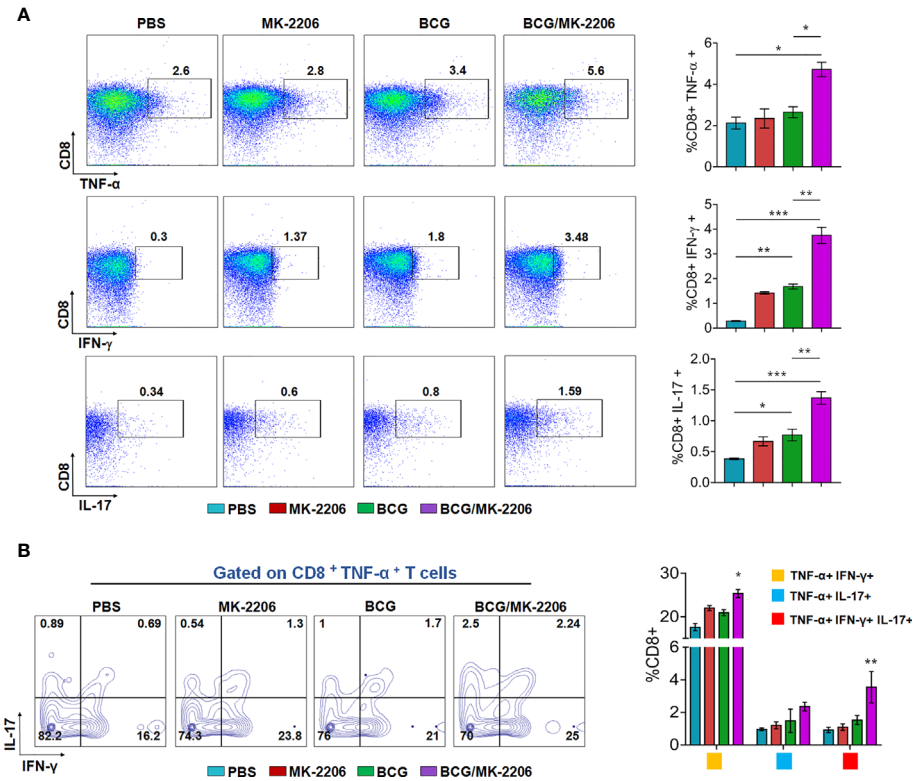

FIGURE 4 | MK-2206 increases antigen-specific multifunctional CD8$^+$ T cell responses in vaccinated mice. Each group of mice was vaccinated as described in *Materials and Methods* section. Twenty-one days post -immunization, mice from each group (n=5) were euthanized and their splenocytes were incubated *ex-vivo* with PPD for 48 h in the presence of Golgi stop. **(A)** Representative dot plots showing the frequency of PPD-specific CD8$^+$ cells producing TNF-α or IFN-γ or IL-17 were analyzed by multicolor flow cytometry by gating on CD3$^+$CD8$^+$ lymphocytes. **(B)** Multifunctional cells were identified by gating on CD8$^+$TNF-α$^+$ T cells: Representative FACS plots show the frequencies of CD8$^+$ T cells co-expressing TNF-α and/or IFN- γ and/or IL-17. Results are displayed as mean ± SEM obtained from a single experiment (n = 5 mice/group). Statistical differences were obtained for the indicated groups. *p < 0.05; **p < 0.01; ***p < 0.001.

CD4$^+$ T and CD8$^+$ T cells in comparison to BCG immunized mice (**Figures 3B**, **4B**). Interestingly, we noticed that multifunctional triple positive T cells (i.e. expressing IFN-γ and TNF-α and IL-17) were prominent in the responding CD4$^+$ and CD8$^+$ populations in spleens from mice with BCG/MK-2206 prime boost regimen in comparison to BCG immunized mice (**Figures 3B**, **4B**). These results indicate that MK-2206 co-administration during BCG immunization significantly promoted priming of multifunctional T cells and increased Th1 and Th17 protective responses.

BCG/MK-2206 Prime Boost Regimen Enhances Th1 Responses in Mice

In order to better characterize BCG/MK-2206–mediated increase of specific type 1 responses in vaccinated mice, we performed *ex-vivo* PPD re-stimulations of spleen cells and quantified the levels of the secreted cytokines by ELISA. We observed that cells from mice immunized with the combination of BCG and MK-2206 secreted higher concentrations of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-12, and IL-2 at day 21 post-immunization in comparison to animals immunized with BCG alone (**Figure 5A**). Concomitantly, by day 60 following immunization, the concentrations of these cytokines were sustained and slightly increased in BCG/MK-2206 immunized mice in comparison to BCG immunized group (**Figure 5B**). The production of Th2 related cytokine such as IL-10 and IL-4 were higher in the PPD stimulated spleen cells from BCG immunized group in comparison to mice immunized with BCG/MK-2206 at 21 days post-immunization (**Figure 5A**). As expected, in comparison to BCG immunized group, a significant decrease in IL-10 production was intensified over 60 days post vaccination in cells from BCG/MK-2206 vaccinated group (**Figure 5B**). However, we observed that IL-4 levels were increased in BCG/MK-2206 immunized group in comparison to the BCG immunized one (**Figure 5B**). Our data suggest that administration of MK-2206 to BCG immunized mice enhanced Th1 response.

MK-2206 Improves the Memory Responses in BCG-Vaccinated Mice

Long-term vaccine efficacy primarily depends upon the memory response generated during infection (8). Until recently, memory T cells are subdivided into two main subsets, T cells expressing high level of CD62L, termed T central memory (TCM), which

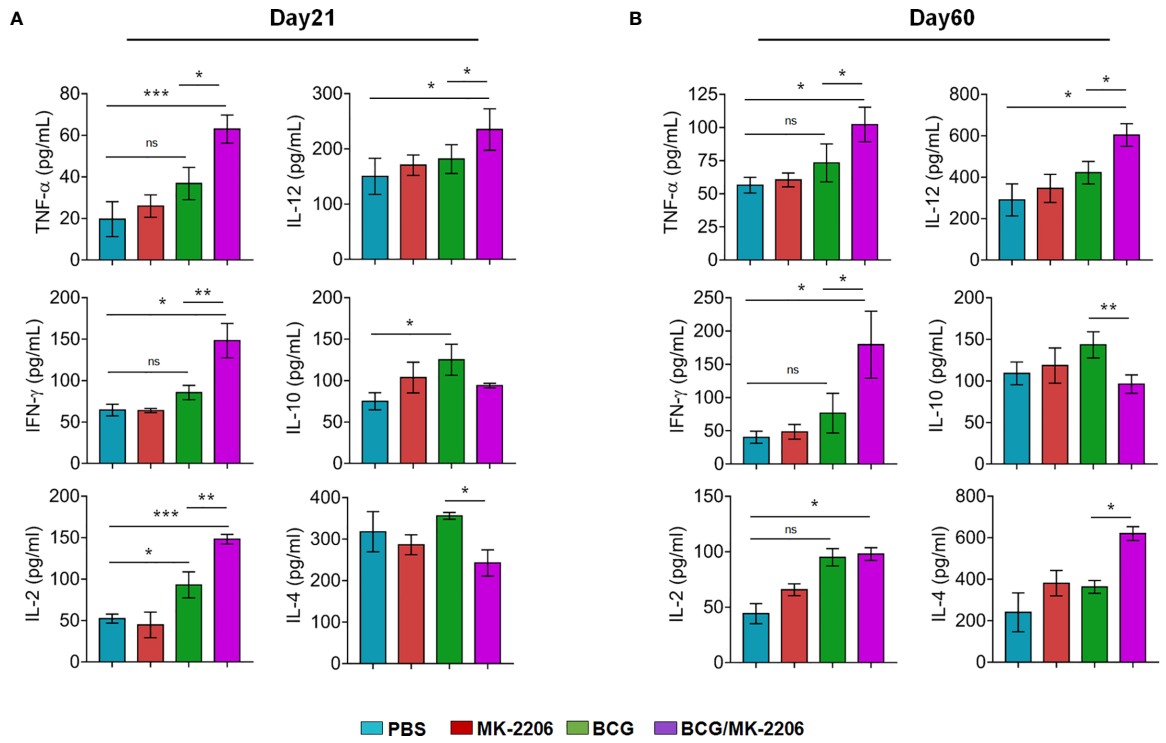

FIGURE 5 | BCG/MK-2206 prime boost regimen enhances Th1 responses in mice. Splenocytes were isolated at 21 days **(A)** and 60 days **(B)** post-immunization and were stimulated *ex-vivo* with PPD for 48 h as described in *Materials and Methods*. The concentrations of representative Th1 cytokines (TNF-α, IFN-γ, IL-2, IL-12) and Th2 cytokines (IL-10, IL-4) were measured in the culture supernatants by ELISA. The data shown is mean ± SEM obtained from a single experiment (n = 5 animals/group). ns, non-significant; Statistical differences were obtained for the indicated groups. *p < 0.05; **p < 0.01; ***p < 0.001. ns, non-significant.

reside in lymphoid organs and T cells expressing low level of CD62L named T effector memory (TEM), which survey the site of infection (48, 49). Previous studies have shown that immunization with BCG does not induce sufficient levels of TCM cells in lungs and spleens (50). Moreover, there is a growing evidence regarding the lack of the ability of BCG to protect adults because of its failure to generate long lasting central memory T cells, including $CD8^+$ memory T cells (51). Therefore, a strategy that enhances TCM responses would provide improved vaccine efficacy. To elucidate the effect of BCG and MK-2206 combination on memory response, we quantified the proportions of TCM and TEM cells by flow cytometry at 60 days post immunization as per the gating strategy (**Figure 6A**). As shown in **Figure 6B**, we observed that the proportion of $CD4^+$ TEM ($CD44^{high}$ $CD62L^{low}$) doubled in mice treated with BCG (from 12% to 26%), compared to sham immunized mice. Interestingly, similar increase was also seen in MK-2206–treated mice in comparison to sham-immunized mice (from 12% to 25.4%). Further, administration of MK-2206 in BCG immunized mice resulted in slight but significant 20% increase in the proportion of $CD4^+$ TEM, in comparison to BCG immunized mice. We observed similar findings when we studied the effect of the indicated treatments on the proportion of $CD8^+$ TEM ($CD44^{high}$ $CD62L^{low}$). As shown in **Figure 6C**, administration of MK-2206 alone augmented the proportion of $CD8^+$ TEM by 35% in comparison to sham-immunized mice. Further, administration of MK-2206 in BCG immunized mice resulted in 27% increase in $CD8^+$ TEM levels in comparison to BCG immunized mice (**Figure 6C**). The detailed analysis of TCM cells ($CD44^{high}CD62L^{high}$) showed ~42% increase in the proportion of both $CD4^+$ and $CD8^+$ TCM populations in BCG immunized, in comparison to sham-immunized mice (**Figures 6B, C**). Further, in comparison to BCG-immunized mice, administration of MK-2206 resulted in 36% increase in the number of $CD4^+$ TCM (**Figure 6B**). The effect of MK-2206 on BCG-immunized mice was even higher for the proportion of $CD8^+$ TCM population resulting in 40% increase (**Figure 6C**). These results indicate that MK-2206 enhances BCG-induced memory responses, especially the pool of TCMs within $CD4^+$ and $CD8^+$ cells. Based on these observations, we hypothesized that augmentation of TCM and TEM populations, induced by administration of Akt inhibitor in BCG immunized mice might result in better protection against TB.

Combining MK-2206 to BCG Improves the Vaccine-Induced Protection Against *Mtb* Infection in Mice

In order to assess the impact of MK-2206–mediated enhancement of effective and memory T cells responses on the protective efficacy of BCG, in terms of reduction in bacterial load

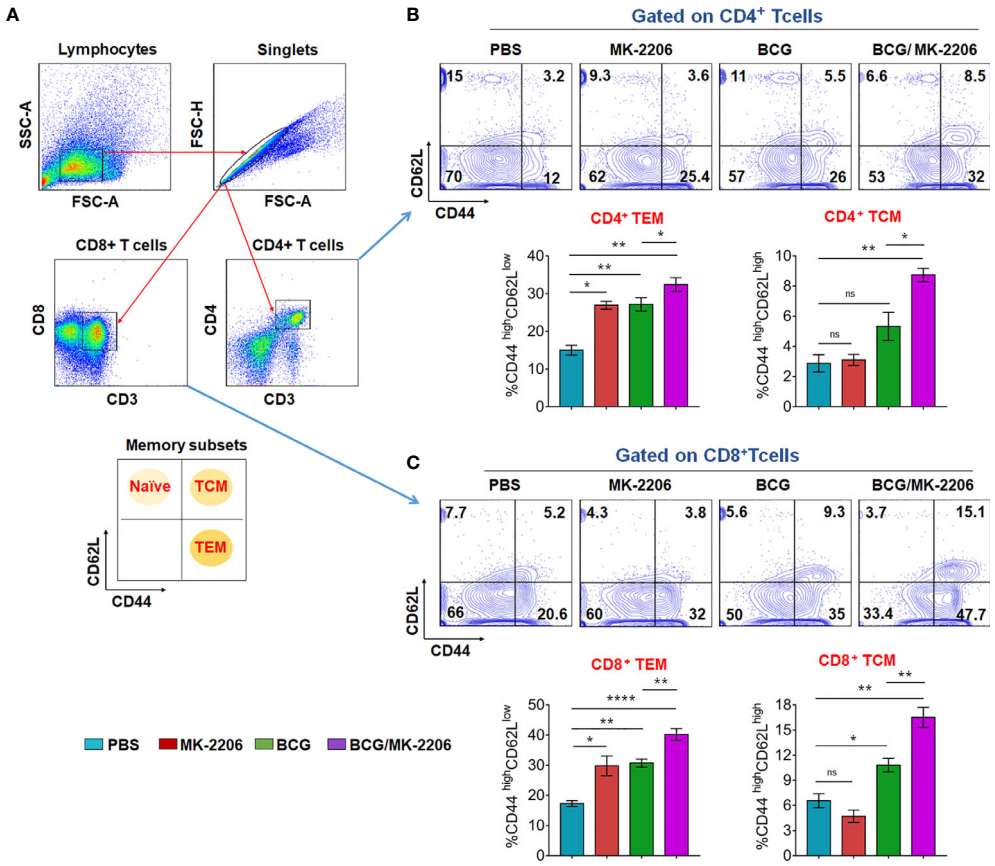

FIGURE 6 | BCG/MK-2206 prime boost regimen induces expansion of effector and central memory T cells. Sixty days post immunization, mice from each group were sacrificed and single cells suspension from spleens were prepared as described in *Materials and Methods*. Splenocytes were stimulated *in vitro* with PPD for analysis of antigen specific memory T cells responses. Live cells were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD44 and anti-CD62L, data was acquired by flow cytometry and subsequently analyzed by FlowJo software. **(A)** Gating strategy used to define $CD4^+$ and $CD8^+$ memory T cells. Debris were excluded based on FSC and SSC parameters and doublets were excluded in FSC-H versus FSC-A plots. After $CD3^+$ gating, the gated populations of $CD4^+$ and $CD8^+$ T cells were further checked for the expression of memory markers of naïve ($CD62L^{high}$ $CD44^{low}$), central memory ($CD62L^{high}$ $CD44^{high}$) and effector memory ($CD62L^{low}CD44^{high}$) T cells. **(B, C)** Representative dot plots showing the percentage of TEM and TCM within $CD4^+$ **(B)** and $CD8^+$ **(C)** cells. The histograms represent total number of TEM and TCM within $CD4^+$ **(B)** and $CD8^+$ **(C)** T cells. Data shown in these panels is mean ± SEM obtained from a single experiment (n=5 mice/group). Statistical differences were obtained for the indicated groups. ns, non-significant; *p < 0.05; **p < 0.01; ****p < 0.0001; ns, non-significant.

in mice, we immunized mice as indicated above and determined the bacterial loads over 30 and 75 days post *Mtb* challenge (**Figure 7A**). As expected, at 30 days post infection, the lungs and splenic bacillary loads in BCG-vaccinated mice were significantly reduced by 2.34- and 7.5-fold, respectively in comparison to the sham-immunized mice (**Figures 7B, C**). Similar reduction in bacterial counts was observed at 75 days post *Mtb* challenge. The reduction of bacterial loads was even much higher in lungs (4.8-fold reduction) and spleen (22.5-fold reduction) of BCG/MK-2206 immunized animals, compared to sham-immunized mice (**Figures 7B, C**) at 30 days post challenge. In agreement, the lung and splenic bacillary load was reduced by 9-fold and 6.3-fold, respectively, in BCG/MK-2206 immunized mice compared to sham vaccinated animals at 75 days post challenge. Interestingly, administration of MK-2206 in BCG immunized mice has further augmented the BCG-induced protection by reducing the lung bacillary loads by 2- and 3.5-fold at 30 days and 75 days, respectively, post *Mtb* challenge (**Figures 7B, C**). Moreover, the splenic bacillary load was reduced by 3.0-fold at both time points in BCG/MK-2206 immunized animals as compared to BCG vaccinated mice. It is worth to note, that the sole administration of MK-2206 also reduced *Mtb* burden in the lungs of mice, but with a less pronounced trend in spleens (**Figures 7B, C**).

In order to further characterize the immune responses underlying the better protection observed in BCG/MK-2206 vaccinated mice, we quantified the levels of *in vivo* cytokines in lung homogenates at 75 days post-challenge. We found that vaccination with BCG and co-administration of MK-2206 induced a significant higher magnitude of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-2, and GM-CSF in comparison to BCG immunized mice (**Figure 7D**). The pro-

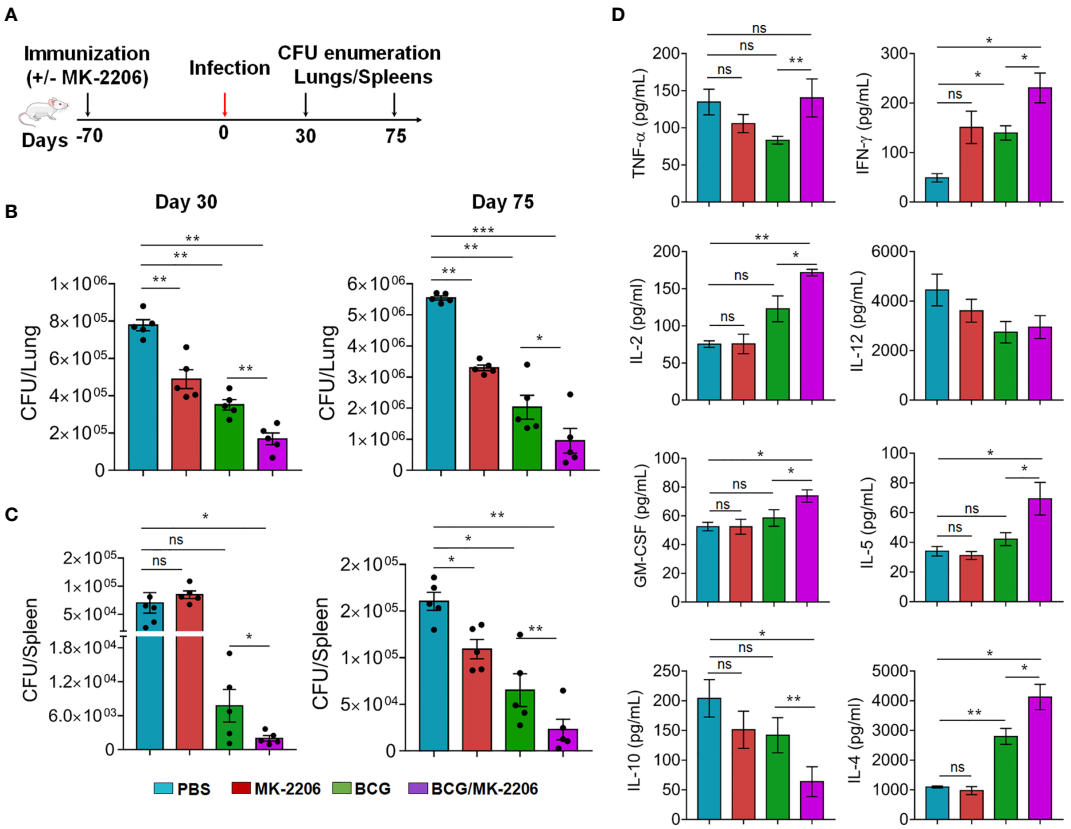

FIGURE 7 | MK-2206 improves BCG elicited protection against TB in mice. **(A)** Schematic representation of protection study in mice. Animals were primed with BCG vaccine and given two boosts with MK-2206 (25 mg/kg) as described in *Materials and Methods*. **(B, C)**. At 70 days post-immunization, animals were infected *via* aerosol route with a high dose of 300 CFU of *Mtb* H37Rv. The lungs **(B)** and splenic **(C)** bacillary loads were determined at day 30 and day 75 post-challenge. For bacterial enumeration, homogenates were diluted 10.0-fold and 100 µl was plated on 7H11 agar at 37°C for 3 to 4 weeks. The data shown in these panels is representative of two experiments and is mean ± SEM CFU obtained from five to six animals per group per time point. **(D)** The levels of cytokines were measured in filtered lung homogenates by Luminex assay. The data shown in this panel is means ± SEM obtained from n = 5–6 mice per group. Statistical differences were obtained for the indicated groups. *p < 0.05; **p < 0.01; ***p < 0.001; ns, non-significant.

inflammatory dominant response observed in sham-immunized mice is required for the activation of innate immune cells and increases the recruitment of additional leukocytes into the site of infection for the formation of granuloma and *Mtb* clearance. These observations are consistent with previous reports that showed the importance of Th1 immune response in controlling bacterial infections (47). Concomitantly, IL-10 expression was significantly diminished in mice vaccinated with BCG/MK-2206 in comparison to BCG immunized animals. The observed low expression levels of IL-10 in BCG/MK-2206 vaccinated mice in comparison to BCG immunized mice might explain the improved protection seen in these animals. Paradoxically, we also observed increased expression of IL-4 and IL-5 in the lung homogenates of mice that received BCG/MK-2206 prime boost regimen relative to BCG primed animals (**Figure 7D**). These observations suggest that there is a need to keep a balance between pro- and anti-inflammatory immune responses in order to avoid tissue damage. Since, IL-5 and IL-4 are important for B-cells growth and activation, the observed significant increase in expression of these cytokines in lung homogenates of mice vaccinated with BCG/MK-2206 may reflect a controlled B-cell activation, which might help in controlling *Mtb* infection (52). These observations are in concordance with previous studies suggesting that both cell-mediated and humoral immunity have a role in preventing TB disease (53).

MK-2206 Improves BCG Elicited Protection Against TB in Guinea Pigs

We further performed guinea pig experiments to determine whether BCG/MK-2206 can confer a similar level of better protection in comparison to BCG in a more susceptible animal model (54, 55). The guinea pigs were immunized as previously described and animals were challenged *via* aerosol infection that resulted in implantation of 100 bacilli in lungs. Bacterial burdens of each group of animals were assessed over 75 days post-challenge (**Figure 8A**). As shown in **Figure 8B**, vaccination with BCG resulted in 4.5-fold and 2.0-fold reduction of lung bacillary loads at 30 and 75 days post challenge, respectively, in comparison to sham-immunized animals. We observed that co-

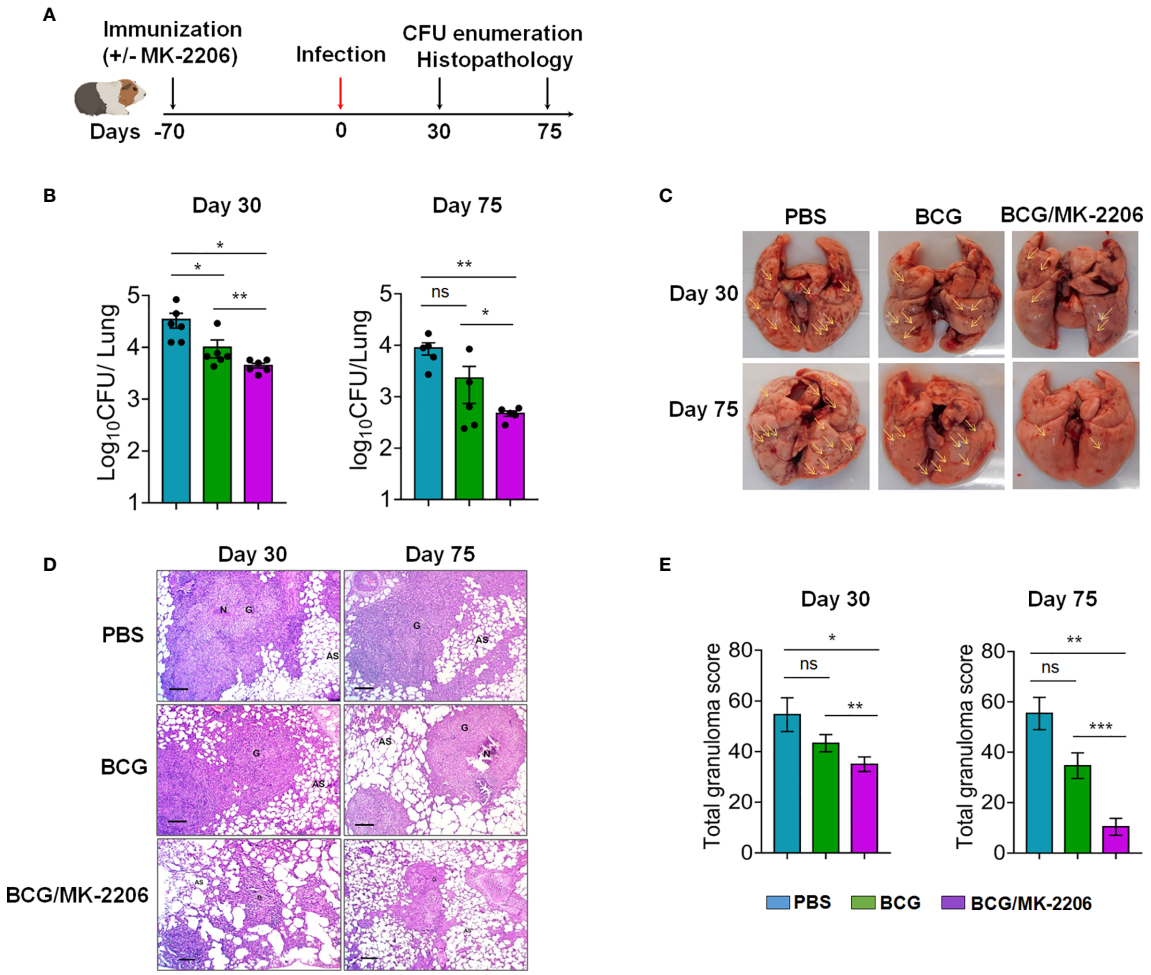

FIGURE 8 | MK-2206 ameliorates BCG elicited protection against TB in guinea pigs. **(A)** Schematic design of protection studies in guinea pigs. The guinea pigs were immunized as described in *Materials and Methods*. After 70 days post-immunization, the animals were infected *via* aerosol route with H37Rv strain **(B)** The lung bacillary load was determined in animals at day 30 and day 75 post *Mtb* challenge. For CFU enumeration, 10.0-fold serial dilution of homogenates are plated on 7H11 agar at 37°C for 3 to 4 weeks. The data shown is mean ± SEM of $\log_{10}$ CFU obtained from a single experiment (n = 5–6 animals per group per time point). **(C)** Representative photographs of lung lobes from a representative animal for each group are shown. Yellow arrows highlight the lesions seen in these images as characterized by hemorrhages, swelling or exudative inflammation. **(D)** The section of lungs was stained with Hematoxylin-eosin (HE) and viewed at 40× magnification. A representative section from each group is shown. Scale bar 200 μm. **(E)** The total granuloma score obtained from n = 5–6 animals per group was determined. The data shown in these panels is mean ± SEM of total granuloma score obtained from a single experiment (n = 5–6 animals per group) at day 30 and day 75 post-infection. Statistical differences were obtained for the indicated groups. *p < 0.05; **p < 0.01; ***p < 0.001; ns, non-significant.

administration of MK-2206 in BCG immunized guinea pigs resulted in ~ 15.0- fold and 13.0- fold CFU reduction of the viable bacteria in the lungs of guinea pigs at 30 and 75 days, respectively, in comparison to sham-immunized group (**Figure 8B**). We also observed that immunization with BCG followed by administration of MK-2206 significantly reduced lung bacillary load by 5.0-fold at 75 days post-challenge in comparison to BCG immunized group (**Figure 8B**). As shown in **Figure S4A**, we noticed that in comparison to BCG immunized group, animals belonging to BCG-primed MK-2206 group showed reduction in spleen bacillary load by ~ 6.5-fold at 30 days post-challenge. However, no difference has been observed between the splenic bacillary loads in BCG immunized and BCG/MK-2206 immunized groups at 75 days post-challenge. We observed that in both groups, the bacterial loads in spleens of immunized animals were below the limit of detection (**Figure S4A**).

We further substantiated CFU data by evaluating lung pathology and inflammation on histological preparations from different groups. As described in *Materials and Methods*, lung sections were fixed and stained with H&E and the affected area of inflammation relative to the total lung area were analyzed as described previously (56). In sham-immunized and BCG-immunized group, we noticed that *Mtb* infection induced severe pathology that was characterized by the presence of larger size and number of granuloma (**Figures 8C–E**). Importantly, compared to sham- and BCG vaccinated groups, guinea pigs primed with BCG and boosted with MK-2206 had remarkably decreased granuloma like lesions in both lungs and

spleens (**Figures 8C** and **S4B**). Interestingly the BCG primed, MK-2206 boosted group exhibited markedly ameliorated histopathology at both 30 days and 75 days post-challenge as manifested by smaller size of granuloma and larger alveolar spaces in comparison to sham- and BCG-immunized animals (**Figure 8D**). In agreement with CFU data, total granuloma score was also reduced by 3.0-fold in BCG-primed MK-2206 group in comparison to BCG only immunized group (**Figure 8E**). These data imply that administration of MK-2206 in BCG immunized animals conferred prevention of severe and extensive lung inflammation compared to that observed in BCG immunized mice after aerosol infection with *Mtb*.

DISCUSSION

The only available TB vaccine, BCG, provides variable efficacy to pulmonary TB. Therefore, new strategies for the development of a safe and an effective TB vaccine are urgently needed (3). Recent clinical studies on new vaccine candidates for *Mtb* failed to show better protection than the parental vaccine, and a number of these vaccine candidates still use BCG in some form (7). Therefore, development of strategies that enhance the magnitude of existing BCG-mediated protection may overcome the disadvantage of its variable and limited protection (7). One of the key features of an efficient TB vaccine is the priming of antigen-specific T cells to generate memory recall responses, upon encounter with *Mtb* that result in long lasting protection from the infection (9). However, the nature and magnitude of antigen-specific adaptive immune response depends mainly on the baseline immune signature post-vaccination (51). Innate immunity plays a key role in TB vaccine responses and the modulation of innate immune responses upon vaccination results in better protection in animal models (57). Studies have shown that early activation of macrophages upon vaccination, as part of the innate immune mechanisms, contributes to the control of mycobacterial infection. The activation of innate immune response upon BCG vaccination is also essential to shape the subsequent adaptive immune response (29). Moreover, it has been shown that the BCG vaccination site is often prone to an IL-10 driven-Th2 response that inhibits protective Th1, which might be responsible for the failure of BCG vaccine in adults (29).

In the contrary, the blockade of IL-10 following BCG vaccination resulted in an increased Th1, Th17 responses and vaccine mediated-protection against *Mtb* in both susceptible and resistant strains of mice (31). Moreover, apoptosis of mycobacteria-infected phagocytes and efferocytosis promote the recruitment and activation of macrophages and DC which subsequently cross-prime antigen-specific T cells thereby establish protective immune response against aerogenic *Mtb* infection (29, 43). Hence, increasing apoptosis along with the inhibition of IL-10 secretion during vaccination may constitute a promising approach for better priming of the BCG-induced anti-TB immunity. Previously, we have shown that activation of the host FOXO3 transcription factor in mycobacteria-infected macrophages, through the inhibition of Akt by MK-2206, dampened the levels of IL-10 secretion and increased the expression of co-stimulatory molecules in BCG-infected macrophages, leading to a potent M1/Th1 typical immune response (32). Moreover, we have also shown that BCG-induced apoptosis of infected macrophages also relies on FOXO3 activation (33). In the present study, we investigated the impact of FOXO3 activation, using the anticancer candidate Akt inhibitor MK-2206, on BCG-induced immune response and its ability to impart protection against *Mtb* challenge in mice and guinea pigs.

For analysis of Akt inhibition-mediated effects *in vitro*, we investigated the apoptotic functions of MK-2206 using J774A.1 murine macrophage cell line. We observed that inhibition of Akt resulted in activation of endogenous FOXO3 levels and promoted caspase dependent-apoptosis of BCG-infected macrophages. These results are in accordance to previous observations that Akt inhibition promotes the activation of FOXO3 pro-apoptotic functions and increase caspases activity (58). Further studies showed that BCG activates the PI3K/Akt, to gain survival advantages which in turn results in the release of IL-10, inhibition of phagosome maturation and down regulation of antimicrobial responses (59). IL-10 negatively regulates apoptosis *via* STAT3 activation to promote the transcription of anti-apoptotic genes (60). In addition, it has also been demonstrated that transgenic mice expressing human IL-10 under the histocompatibility complex class II promoter, strongly inhibited anti-mycobacterial response of macrophages such as apoptosis and the production of pro-inflammatory cytokines and nitric oxide (61). It has also been reported that induction of cell apoptosis post-BCG immunization resulted in clearance of intracellular bacteria and increased antigen processing leading to improved Th1 responses and better protection (29).

We have previously shown that FOXO3 activation, through inhibition of Akt signaling, at the primary site of infection dampens IL-10 release, increases antigenic presentation of APCs and results in intracellular clearance of BCG (32). Thus, we hypothesized that local administration of apoptosis inducer (FOXO3 activator, MK-2206) at the site of BCG vaccination might result in apoptosis of infiltrating phagocytes and increased protection. A key question in evaluating efficacy of BCG-booster vaccine is when to administer a booster to avoid immunological interference. Here, intradermal vaccination with BCG allowed the phagocytosis of live bacilli before administration of Akt inhibitor at the site of BCG inoculation, as Akt signaling is also involved in the engulfment of bacteria by murine and human phagocytes (62). Moreover, it was previously reported that the homologous route of prime-boost vaccinations targeting DLNs at the local site would provide a more effective boost than the heterologous routes of vaccination targeting distant lymph nodes (41).

We observed co-administration of MK-2206 in BCG immunized mice resulted in a strong influx of DC subsets at early time point in DLNs. Moreover, we noticed that at five days post BCG immunization, numbers of apoptotic cells remained unchanged in DLNs compared to sham-immunized mice. However, the administration of BCG/MK-2206 significantly increased the number of apoptotic cells in DLNs compared to

BCG-vaccinated and unvaccinated animals. It is important to note that the administration of MK-2206 alone did not induce cell death and none of the mice died during the experiment or showed any clinical symptoms suggesting the non-toxic effect of the local injection of MK-2206. In addition, we avoided the continuous administration of MK-2206 because Akt kinase has been shown to play an essential role in promoting maturation, migration and survival of APCs (63). Interestingly, Abadie et al., reported that during the first three days post BCG inoculation, phagocytes remained concentrated at the injection site and subsequently these are not observed at early time points in DLNs (42). Our data, suggest that inhibition of Akt promotes DCs activation and their migration to DLNs. This observation is in accordance with previous report showing that the activation of PI3K/Akt pathway upon Leishmania infection subverts DCs immuno-stimulatory abilities and impair the transcription of pro-inflammatory cytokines (64). In addition, it was shown that the inhibition of Akt signaling activates FOXO1, which stimulates the expression of adhesion molecules, ICAM1 and CCR7, required for DCs activation, migration to DLNs and their re-circulation to infected tissues (65). Studies have also shown that IL-10 affects DC trafficking from the site of infection to local DLNs in order to drive polarization of T cells toward Th1 (66). Apoptosis has been described as a defense mechanism against TB infection and has been shown to promote anti-TB protective immunity (67). Indeed, cell death of mycobacteria-infected macrophages deliver antigens to DCs that subsequently cross-prime antigen-specific T cells which subsequently expand and migrate to the lungs to impart protection against *Mtb* infection in mice (68). In the current study, the BCG/MK-2206 boost strategy induced the generation of a high frequency of antigen-specific multifunctional T-cells including IFN-γ, TNF-α and IL-17 triple positive $CD4^+$ and $CD8^+$ T cells, compared with BCG immunized mice. Previously it has been shown that the magnitude of vaccine-induced polyfunctional T cells have been shown to correlate with vaccine-induced protection against TB (69). Recombinant TB vaccines and subunit vaccines inducing a higher frequency of multifunctional $CD4^+$ T cells are shown to confer both short and long term protection against *Mtb* post-challenge (70). However, most protective vaccines elicit a mixture of antigen-specific $CD4^+$ and $CD8^+$ T cell responses.

The magnitude of $CD8^+$ T cell responses is a key immune parameter for optimum control of mycobacterial infection (71). Importantly, the BCG prime/MK-2206 boost approach elicited polyfunctional $CD8^+$ T cells producing cytokines, which were amplified in comparison to BCG-immunized group. Presumably, the induction of apoptosis by BCG/MK-2206 vaccine resulted in higher $CD8^+$ T cell responses, since enhanced apoptosis is associated with increased cross-presentation of antigens to $CD8^+$ T cells and improved immunity against *Mtb* infection *in vivo* (72). Several studies have implicated the involvement of $CD8^+$ T cells in protective immunity against TB (24, 73). Indeed, the depletion of $CD8^+$ T cells in mice and macaques resulted in failure of BCG to impart protection and increased bacterial burden suggesting that these cells are crucial for control of disease progression (74, 75). In another study, it has been shown that the pro-apoptotic *ΔnuoG Mtb* strain was more effective in priming $CD8^+$ T cells in animals and therefore might more effective in clearing intracellular *Mtb* in comparison to the parental BCG vaccine (19).

Our study also revealed that MK-2206 co-administration significantly increased the BCG-induced TCM and TEM pool within $CD4^+$ and $CD8^+$ T cells. The efficacy of a vaccine relies on the induction of memory responses, which result from the expansion of antigen-specific lymphocytes. Post-infection, TEM appear early at the affected site and have effector functions such as cytokines secretion and provide immediate protection, while TCM home to the DLNs where they proliferate and generate new waves of effector cells upon antigen re-exposure (73). Our results clearly demonstrate that vaccination with BCG/MK-2206 further increased the BCG-elicited protection in lungs of mice and guinea pigs challenged with *Mtb* at 30- and 75 days post-infection. The enhanced efficacy of BCG/MK-2206 in comparison to BCG in mice was associated with an increased expression levels of proinflammatory cytokines such as TNF-α, IFN-γ and IL-2 and a significant decrease in IL-10 production. Among Th1 cytokines, IFN-γ, TNF-α, and IL-2 are classically considered as important components of the antimycobacterial cytokine cascade and are associated with protection against TB (76). These cytokines work synergistically to activate macrophages and maintain the integrity of granuloma (77). The increase in IL-10 levels supports the mycobacterial survival in the host and reduces the protective response to *Mtb* in mice (31). However, the level of IL-4 was increased in BCG/MK-2206 vaccinated mice. These observations are in accordance with previous reports suggesting that IL-4 plays a key role in inducing Th1 cell differentiation by promoting IL-12 and inhibiting IL-10 production by APCs (78). In contrast to the concept that IL-4 acts primarily as an anti-inflammatory molecule, it has been reported that IL-4 has an important role in B cell proliferation, isotype secretion and induction of MHC-II surface expression on these cells, which is important to enhance the activation and recruitment of T cells and eosinophils (79). Moreover, mature Th17 cells are resistant to the effect of IL-4 and are able to secrete IL-17 in Th2 environment (80). The enhanced IL-4 levels in mice immunized with BCG/MK-2206 provides an explanation for the lack of exacerbated inflammation as evident with the reduced immunopathology in the lungs of guinea pigs primed with BCG and followed by MK-2206 administration. These observations suggest that the balance of pro- and anti-inflammatory cytokines plays a critical role in determining the outcome of lung host defenses against *Mtb*. In addition, Th2 cytokines, IL-4 and IL-5 promote B cell class switching to neutralizing antibodies such as IgG1 and they further regulate the magnitude of Th1 cytokines (81). We also found that vaccination with BCG/MK-2206 further increased production of GM-CSF in lung homogenates. GM-CSF overexpression during acute *Mtb* infection contributes to an efficient M1 response, while interfering with GM-CSF pathway might impair the host inflammatory response against *Mtb* (82). This is also in accordance with previous reports showing that GM-CSF based adjuvant formulation is effective to improve BCG immunogenicity (83).

In summary, our study reveals that FOXO3 activation, during BCG vaccination, results in induction of apoptosis and a decrease in

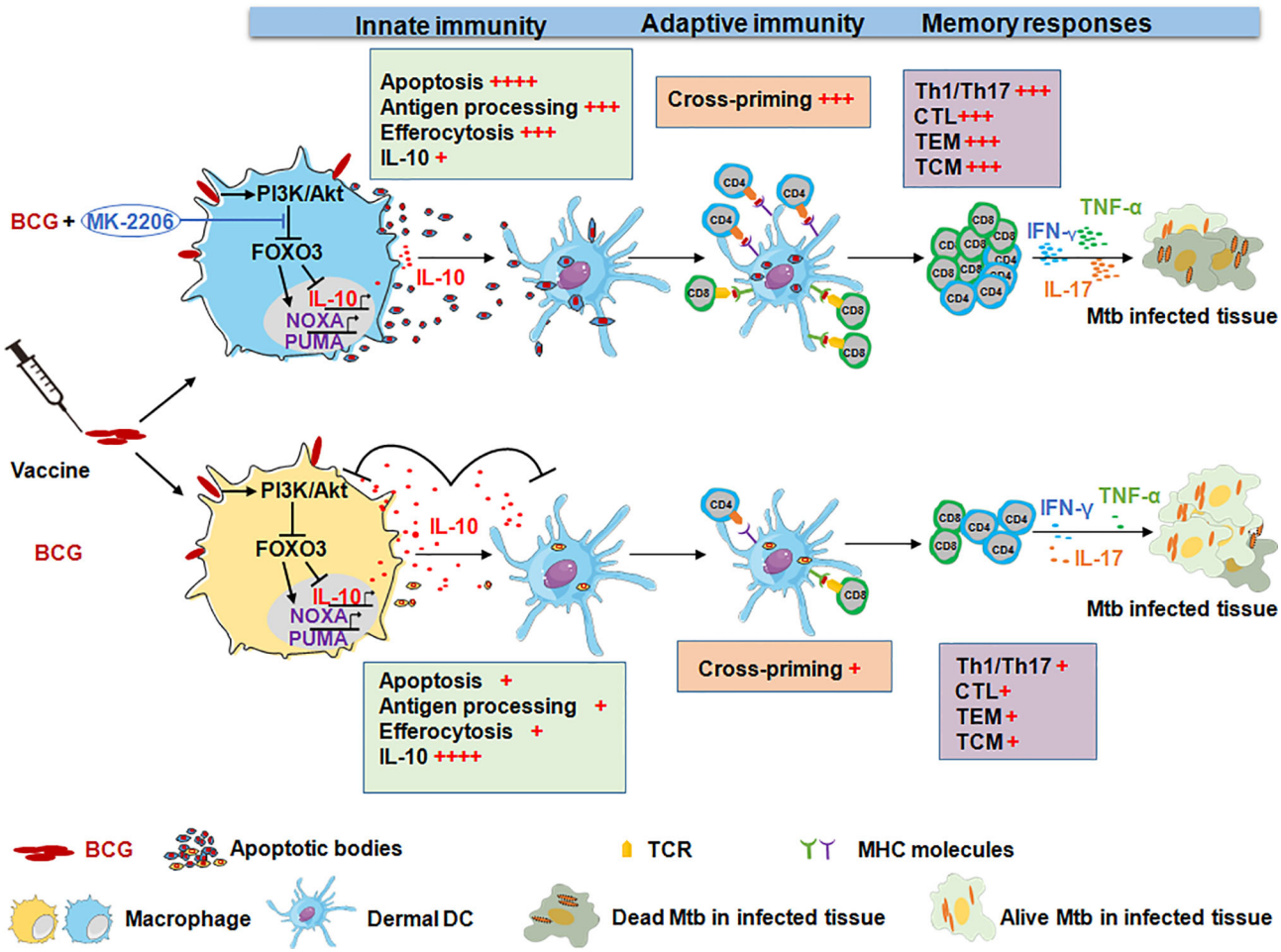

FIGURE 9 | Administration of the Akt kinase inhibitor MK-2206 post BCG vaccination confers higher protection against aerogenic infection. Immunization with BCG induces IL-10 production and also promotes macrophage survival by activating the PI3K/Akt signaling pathway. The activated Akt translocate into the nucleus and phosphorylates FOXO3. The phosphorylation of FOXO3 results in cytoplasmic accumulation and its subsequent degradation. This subsequently results in suppression of Th1 immune response and host antimicrobial pathways, which favors bacterial intracellular replication. In contrast, inhibition of BCG-mediated Akt activation by MK-2206 subsequently results in FOXO3 activation which triggers apoptosis and reduces IL-10 secretion. This eventually results in disruption of growth niche for intracellular replication of BCG, promotes recruitment of APCs and efferocytosis, an important source of antigen presentation. Finally, this leads to optimal induction of antigen specific T cells and the generation of higher memory T cell response that translates into better protection against challenge with *Mtb* in mice and guinea pigs.

IL-10 secretion, leading to stronger efferocytosis and higher recruitment of APCs, important sources of Ag for cross-presentation of T cells by DCs. Accordingly, this biological activity induces Ag specific T cells and enhances Th1/Th17 cells, which promotes a robust effective and memory immune response that confer higher protection against *Mtb* infection (**Figure 9**). Our data also support the notion that boosting of the innate immune response further strengthens the BCG-induced protection against *Mtb*. Future experiments would evaluate whether BCG/MK-2206 combination can impart better protection then BCG in Nonhuman primate model. Taken together, enhancing the formulation of BCG vaccine to harness innate immunity offers novel insights into approaches to design vaccines for future TB and non-tuberculous diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Translational Health Science and Technology Institute and International Centre for Genetic Engineering and Biotechnology.

AUTHOR CONTRIBUTIONS

ME, RB, and RS conceived and designed the work plan. RB, SC, and TG performed experiments. RB, ME, and RS analyzed the data, interpreted them, and wrote the paper as well. M-RB, KR, and MH contributed with providing reagents and discussing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.645962/full#supplementary-material

REFERENCES

1. World Health Organization. *Global Tuberculosis Report 2020* (2020). Available at: https://www.who.int/publications/i/item/9789240013131 (Accessed November 18, 2020).
2. Dockrell HM, Smith SG. What Have We Learnt About BCG Vaccination in the Last 20 Years? *Front Immunol* (2017) 8:1134. doi: 10.3389/fimmu.2017.01134
3. Scriba TJ, Mizrahi V. Previews Renewing the Fight Against TB With an Old Vaccine. *Cell* (2020) 180:829–31. doi: 10.1016/j.cell.2020.02.024
4. Mangtani P, Nguipdop-Djomo P, Keogh RH, Trinder L, Smith PG, Fine PEM, et al. Observational Study to Estimate the Changes in the Effectiveness of Bacillus Calmette-Guérin (BCG) Vaccination With Time Since Vaccination for Preventing Tuberculosis in the UK. *Health Technol Assess* (2017) 21:5–53. doi: 10.3310/hta21390
5. Hatherill M, White RG, Hawn TR. Clinical Development of New Tb Vaccines: Recent Advances and Next Steps. *Front Microbiol* (2020) 10:3154. doi: 10.3389/fmicb.2019.03154
6. Méndez-Samperio P. Current Challenges and Opportunities for Bacillus Calmette-Guérin Replacement Vaccine Candidates. *Scand J Immunol* (2019) 90:1–6. doi: 10.1111/sji.12772
7. Kaufmann SHE. Vaccination Against Tuberculosis: Revamping BCG by Molecular Genetics Guided by Immunology. *Front Immunol* (2020) 11:316. doi: 10.3389/fimmu.2020.00316
8. Andersen P, Scriba TJ. Moving Tuberculosis Vaccines From Theory to Practice. *Nat Rev Immunol* (2019) 19:550–62. doi: 10.1038/s41577-019-0174-z
9. Behar SM, Woodworth JSM, Wu Y. Next Generation: Tuberculosis Vaccines That Elicit Protective CD8 + T Cells. *Expert Rev Vaccines* (2007) 6:441–56. doi: 10.1586/14760584.6.3.441
10. Lindestam Arlehamn CS, Lewinsohn D, Sette A, Lewinsohn D. Antigens for CD4 and CD8 T Cells in Tuberculosis. *Cold Spring Harbor Perspect Med* (2014) 4:a018465. doi: 10.1101/cshperspect.a018465
11. Bakhru P, Sirisaengtaksin N, Soudani E, Mukherjee S, Khan A, Jagannath C. BCG Vaccine Mediated Reduction in the MHC-II Expression of Macrophages and Dendritic Cells is Reversed by Activation of Toll-like Receptors 7 and 9. *Cell Immunol* (2014) 287:53–61. doi: 10.1016/j.cellimm.2013.11.007
12. Ryan AA, Nambiar JK, Wozniak TM, Roediger B, Shklovskaya E, Britton WJ, et al. Antigen Load Governs the Differential Priming of CD8 T Cells in Response to the Bacille Calmette Guérin Vaccine or Mycobacterium Tuberculosis Infection. *J Immunol* (2009) 182:7172–7. doi: 10.4049/jimmunol.0801694
13. Khan A, Bakhru P, Saikolappan S, Das K, Soudani E, Singh CR, et al. An Autophagy-Inducing and TLR-2 Activating BCG Vaccine Induces a Robust Protection Against Tuberculosis in Mice. *NPJ Vaccines* (2019) 4:1–19. doi: 10.1038/s41541-019-0122-8
14. Moliva JI, Turner J, Torrelles JB. Immune Responses to Bacillus Calmette-Guérin Vaccination: Why do They Fail to Protect Against Mycobacterium Tuberculosis? *Front Immunol* (2017) 8:407. doi: 10.3389/fimmu.2017.00407
15. Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K, et al. Apoptosis Facilitates Antigen Presentation to T Lymphocytes Through MHC-I and CD1 in Tuberculosis. *Nat Med* (2003) 9:1039–46. doi: 10.1038/nm906
16. Winau F, Weber S, Sad S, De Diego J, Hoops SL, Breiden B, et al. Apoptotic Vesicles Crossprime CD8 T Cells and Protect Against Tuberculosis. *Immunity* (2006) 24:105–17. doi: 10.1016/j.immuni.2005.12.001
17. Farinacci M, Weber S, Kaufmann SHE. The Recombinant Tuberculosis Vaccine rBCG Δurec:: Hly+ Induces Apoptotic Vesicles for Improved Priming of CD4+ and CD8+ T Cells. *Vaccine* (2012) 30:7608–14. doi: 10.1016/j.vaccine.2012.10.031
18. Hinchey J, Jeon BY, Alley H, Chen B, Goldberg M, Derrick S, et al. Lysine Auxotrophy Combined With Deletion of the secA2 Gene Results in a Safe and Highly Immunogenic Candidate Live Attenuated Vaccine for Tuberculosis. *PloS One* (2011) 6:e15857. doi: 10.1371/journal.pone.0015857
19. Gengenbacher M, Nieuwenhuizen N, Vogelzang A, Liu H, Kaiser P, Schuerer S, et al. Deletion of Nuog From the Vaccine Candidate Mycobacterium Bovis BCG Δurec:: Hly Improves Protection Against Tuberculosis. *mBio* (2016) 7:1–10. doi: 10.1128/mBio.00679-16
20. Gartner T, Romano M, Suin V, Kalai M, Korf H, De Baetselier P, et al. Immunogenicity and Protective Efficacy of a Tuberculosis DNA Vaccine Co-Expressing Pro-Apoptotic Caspase-3. *Vaccine* (2008) 26:1458–70. doi: 10.1016/j.vaccine.2007.12.056
21. Li G, Liu G, Song N, Kong C, Huang Q, Su H, et al. A Novel Recombinant BCG-expressing Pro-Apoptotic Protein BAX Enhances Th1 Protective Immune Responses in Mice. *Mol Immunol* (2015) 66:346–56. doi: 10.1016/j.molimm.2015.04.003
22. Sendide K, Deghmane A-E, Pechkovsky D, Av-Gay Y, Talal A, Hmama Z. Mycobacterium Bovis BCG Attenuates Surface Expression of Mature Class II Molecules Through IL-10-Dependent Inhibition of Cathepsin S. *J Immunol* (2005) 175:5324–32. doi: 10.4049/jimmunol.175.8.5324
23. Couper KN, Blount DG, Riley EM. Il-10: The Master Regulator of Immunity to Infection. *J Immunol* (2008) 180:5771–7. doi: 10.4049/jimmunol.180.9.5771
24. Wang J, Santosuosso M, Ngai P, Zganiacz A, Xing Z. Activation of CD8 T Cells by Mycobacterial Vaccination Protects Against Pulmonary Tuberculosis in the Absence of CD4 T Cells. *J Immunol* (2004) 173:4590–7. doi: 10.4049/jimmunol.173.7.4590

25. Xu H, Jia Y, Li Y, Wei C, Wang W, Guo R, et al. IL-10 Dampens the Th1 and Tc Activation Through Modulating DC Functions in BCG Vaccination. *Mediators Inflammation* (2019) 2019:8616154. doi: 10.1155/2019/8616154
26. Méndez-Samperio P, Trejo A, Pérez A. Mycobacterium Bovis Bacillus Calmette-Guérin (BCG) Stimulates IL-10 Production *Via* the PI3K/Akt and P38 MAPK Pathways in Human Lung Epithelial Cells. *Cell Immunol* (2008) 251:37–42. doi: 10.1016/j.cellimm.2008.03.002
27. Bai W, Liu H, Ji Q, Zhou Y, Liang L, Zheng R, et al. TLR3 Regulates Mycobacterial RNA-Induced Il-10 Production Through the PI3K/AKT Signaling Pathway. *Cell Signall* (2014) 26:942–50. doi: 10.1016/j.cellsig.2014.01.015
28. Rodrigues MF, Barsante MM, Alves CCS, Souza MA, Ferreira AP, Amarante-Mendes GP, et al. Apoptosis of Macrophages During Pulmonary Mycobacterium Bovis Infection: Correlation With Intracellular Bacillary Load and Cytokine Levels. *Immunology* (2009) 128:e691. doi: 10.1111/j.1365-2567.2009.03062.x
29. Schaible UE, Linnemann L, Redinger N, Patin EC, Dallenga T. Strategies to Improve Vaccine Efficacy Against Tuberculosis by Targeting Innate Immunity. *Front Immunol* (2017) 8:1755. doi: 10.3389/fimmu.2017.01755
30. Redford PS, Murray PJ, O'Garra A. The Role of IL-10 in Immune Regulation During M. Tuberculosis Infection. *Mucosal Immunol* (2011) 4:261–70. doi: 10.1038/mi.2011.7
31. Pitt JM, Stavropoulos E, Redford PS, Beebe AM, Bancroft GJ, Young DB, et al. Blockade of IL-10 Signaling During Bacillus Calmette-Guerin Vaccination Enhances and Sustains Th1, Th17, and Innate Lymphoid IFN- and IL-17 Responses and Increases Protection to Mycobacterium Tuberculosis Infection. *J Immunol* (2012) 189:4079–87. doi: 10.4049/jimmunol.1201061
32. Bouzeyen R, Haoues M, Barbouche MR, Singh R, Essafi M. Foxo3 Transcription Factor Regulates Il-10 Expression in Mycobacteria-Infected Macrophages, Tuning Their Polarization and the Subsequent Adaptive Immune Response. *Front Immunol* (2019) 10:2922. doi: 10.3389/fimmu.2019.02922
33. Haoues M, Refai A, Mallavialle A, Barbouche MR, Laabidi N, Deckert M, et al. Forkhead Box O3 (FOXO3) Transcription Factor Mediates Apoptosis in BCG-infected Macrophages. *Cell Microbiol* (2014) 16:1378–90. doi: 10.1111/cmi.12298
34. Mehta PK, King CH, White EH, Murtagh JJ, Quinn FD. Comparison of *In Vitro* Models for the Study of Mycobacterium Tuberculosis Invasion and Intracellular Replication. *Infect Immun* (1996) 64:2673–9. doi: 10.1128/iai.64.7.2673-2679.1996
35. Agarwal E, Chaudhuri A, Leiphrakpam PD, Haferbier KL, Brattain MG. Akt Inhibitor MK-2206 Promotes Anti-Tumor Activity and Cell Death by Modulation of AIF and Ezrin in Colorectal Cancer. *BMC Cancer* (2014) 14:1–12. doi: 10.1186/1471-2407-14-145
36. Zhao YY, Tian Y, Zhang J, Xu F, Yang YP, Huang Y, et al. Effects of an Oral Allosteric AKT Inhibitor (MK-2206) on Human Nasopharyngeal Cancer *In Vitro* and *In Vivo*. *Drug Des Dev Ther* (2014) 8:1827–37. doi: 10.2147/DDDT.S67961
37. Martins PS, Ochoa R, Pimenta AMC, Ferreira LAM, Melo AL, da Silva JBB, et al. Mode of Action of β-Cyclodextrin as an Absorption Enhancer of the Water-Soluble Drug Meglumine Antimoniate. *Int J Pharm* (2006) 325:39–47. doi: 10.1016/j.ijpharm.2006.06.014
38. Pal A, Roy S, Kumar A, Mahmood S, Khodapanah N, Thomas S, et al. Physicochemical Characterization, Molecular Docking, and *In Vitro* Dissolution of Glimepiride-Captisol Inclusion Complexes. *ACS Omega* (2020) 5:19968–77. doi: 10.1021/acsomega.0c01228
39. Singh R, Singh M, Arora G, Kumar S, Tiwari P, Kidwai S. Polyphosphate Deficiency in Mycobacterium Tuberculosis Is Associated With Enhanced Drug Susceptibility and Impaired Growth in Guinea Pigs. *J Bacteriol* (2013) 195:2839–51. doi: 10.1128/JB.00038-13
40. Lv Y, Fang L, Ding P, Liu R. Pi3k/Akt-Beclin1 Signaling Pathway Positively Regulates Phagocytosis and Negatively Mediates NF-κb-Dependent Inflammation in Staphylococcus Aureus-Infected Macrophages. *Biochem Biophys Res Commun* (2019) 510:284–9. doi: 10.1016/j.bbrc.2019.01.091
41. Nandakumar S, Kannanganat S, Dobos KM, Lucas M, Spencer JS, Amara RR, et al. Boosting BCG-primed Responses With a Subunit Apa Vaccine During the Waning Phase Improves Immunity and Imparts Protection Against Mycobacterium Tuberculosis. *Nat Publ Group* (2016) 6:25837. doi: 10.1038/srep25837
42. Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJM, Tanguy M, et al. Neutrophils Rapidly Migrate *Via* Lymphatics After Mycobacterium Bovis BCG Intradermal Vaccination and Shuttle Live Bacilli to the Draining Lymph Nodes. *Blood* (2005) 106:1843–50. doi: 10.1182/blood-2005-03-1281
43. Penteado L de A, Dejani NN, Verdan FF, Orlando AB, Niño VE, Dias FDN, et al. Distinctive Role of Efferocytosis in Dendritic Cell Maturation and Migration in Sterile or Infectious Conditions. *Immunology* (2017) 151:304–13. doi: 10.1111/imm.12731
44. Wolf AJ, Linas B, Trevejo-Nuñez GJ, Kincaid E, Tamura T, Takatsu K, et al. Mycobacterium Tuberculosis Infects Dendritic Cells With High Frequency and Impairs Their Function In Vivo. *J Immunol* (2007) 179:2509–19. doi: 10.4049/jimmunol.179.4.2509
45. Lai R, Jeyanathan M, Afkhami S, Zganiacz A, Hammill JA, Yao Y, et al. Cd11b + Dendritic Cell–Mediated Anti– Mycobacterium Tuberculosis Th1 Activation Is Counterregulated by CD103 + Dendritic Cells *Via* IL-10. *J Immunol* (2018) 200:ji1701109. doi: 10.4049/jimmunol.1701109
46. Behar SM. Antigen-Specific CD8+ T Cells and Protective Immunity to Tuberculosis. *Adv Exp Med Biol* (2013) 783:141–63. doi: 10.1007/978-1-4614-6111-1_8
47. Zeng G, Zhang G, Chen X. Th1 Cytokines, True Functional Signatures for Protective Immunity Against TB ? *Nat Publ Group* (2017) 15:206–15. doi: 10.1038/cmi.2017.113
48. Henao-Tamayo MI, Ordway DJ, Irwin SM, Shang S, Shanley C, Orme IM. Phenotypic Definition of Effector and Memory T-lymphocyte Subsets in Mice Chronically Infected With Mycobacterium Tuberculosis. *Clin Vaccine Immunol* (2010) 17:618–25. doi: 10.1128/CVI.00368-09
49. Ahlers JD, Belyakov IM. Memories That Last Forever: Strategies for Optimizing Vaccine T-Cell Memory. *Blood* (2010) 115:1678–89. doi: 10.1182/blood-2009-06-227546
50. Rai PK, Chodisetti SB, Zeng W, Nadeem S, Maurya SK, Pahari S, et al. A Lipidated Peptide of Mycobacterium Tuberculosis Resuscitates the Protective Efficacy of BCG Vaccine by Evoking Memory T Cell Immunity. *J Trans Med* (2017) 15:201. doi: 10.1186/s12967-017-1301-x
51. Brazier B, McShane H. Towards New TB Vaccines. *Semin Immunopathol* (2020) 42:315–31. doi: 10.1007/s00281-020-00794-0
52. Essone PN, Lebouenyi M, Maloupazoa Siawaya AC, Alame-Emane AK, Aboumegone Biyogo OC, Dapnet Tadatsin PH, et al. M. Tuberculosis Infection and Antigen Specific Cytokine Response in Healthcare Workers Frequently Exposed to Tuberculosis. *Sci Rep* (2019) 9:8201. doi: 10.1038/s41598-019-44294-0
53. Tanner R, Villarreal-Ramos B, Vordermeier HM, McShane H. The Humoral Immune Response to BCG Vaccination. *Front Immunol* (2019) 10:1317. doi: 10.3389/fimmu.2019.01317
54. Kashino SS, Napolitano DR, Skobe Z, Campos-Neto A. Guinea Pig Model of Mycobacterium Tuberculosis Latent/Dormant Infection. *Microbes Infect* (2008) 10:1469–76. doi: 10.1016/j.micinf.2008.08.010
55. Clark S, Hall Y, Williams A. Animal Models of Tuberculosis: Guinea Pigs. *Cold Spring Harbor Perspect Med* (2015) 5. doi: 10.1101/cshperspect.a018572
56. Singh R, Singh M, Arora G, Kumar S, Tiwari P, Kidwai S. Polyphosphate Deficiency in Mycobacterium Tuberculosis Is Associated With Enhanced Drug Susceptibility and Impaired Growth in Guinea. *J Bacteriol* (2013) 195:2839–51. doi: 10.1128/JB.00038-13
57. Khader SA, Divangahi M, Hanekom W, Hill PC, Maeurer M, Makar KW, et al. Targeting Innate Immunity for Tuberculosis Vaccination. *J Clin Invest* (2019) 129:3482–91. doi: 10.1172/JCI128877
58. Das TP, Suman S, Alatassi H, Ankem MK, Damodaran C. Inhibition of AKT Promotes FOXO3a-dependent Apoptosis in Prostate Cancer. *Cell Death Dis* (2016) 7:e2111. doi: 10.1038/cddis.2015.403
59. Hussain T, Zahid S, Shah A, Zhao D, Sreevatsan S, Zhou X. The Role of IL-10 in Mycobacterium Avium Subsp. Paratuberculosis Infection. *Cell Commun Signaling* (2016) 14:29. doi: 10.1186/s12964-016-0152-z
60. Donnelly RP, Dickensheets H, Finbloom DS. The Interleukin-10 Signal Transduction Pathway and Regulation of Gene Expression in Mononuclear Phagocytes. *J Interferon Cytokine Res* (1999) 573:563–73. doi: 10.1089/107999099313695

61. Feng CG, Kullberg MC, Jankovic D, Cheever AW, Caspar P, Coffman RL, et al. Transgenic Mice Expressing Human Interleukin-10 in the Antigen-Presenting Cell Compartment Show Increased Susceptibility to Infection With Mycobacterium Avium Associated With Decreased Macrophage Effector Function and Apoptosis. *Infect Immun* (2002) 70:6672–9. doi: 10.1128/IAI.70.12.6672-6679.2002
62. Lovewell RR, Hayes SM, O'Toole GA, Berwin B. Pseudomonas Aeruginosa Flagellar Motility Activates the Phagocyte PI3K/Akt Pathway to Induce Phagocytic Engulfment. *Am J Physiol - Lung Cell Mol Physiol* (2014) 306: L698–L707. doi: 10.1152/ajplung.00319.2013
63. Hee J, Heung T, Hee K, Kim S, Lee Y, Woo K, et al. Enhancement of DC Vaccine Potency by Activating the PI3K/AKT Pathway With a Small Interfering RNA Targeting PTEN. *Immunol Lett* (2010) 134:47–54. doi: 10.1016/j.imlet.2010.08.008
64. Neves BM, Silvestre R, Resende M, Ouaissi A, Cunha J, Tavares J, et al. Activation of Phosphatidylinositol 3-Kinase/Akt and Impairment of Nuclear Factor-κb: Molecular Mechanisms Behind the Arrested Maturation/ Activation State of Leishmania Infantum-Infected Dendritic Cells. *Am J Pathol* (2010) 177:2898–911. doi: 10.2353/ajpath.2010.100367
65. Graves DT, Milovanova TN. Mucosal Immunity and the FOXO1 Transcription Factors. *Front Immunol* (2019) 10:2530. doi: 10.3389/ fimmu.2019.02530
66. Mihret A. The Role of Dendritic Cells in Mycobacterium Tuberculosis Infection. *Virulence* (2012) 3:654. doi: 10.4161/viru.22586
67. Lam A, Prabhu R, Gross CM, Riesenberg LA, Singh V, Aggarwal S. Role of Apoptosis and Autophagy in Tuberculosis. *Am J Physiol - Lung Cell Mol Physiol* (2017) 313:L218–29. doi: 10.1152/ajplung.00162.2017
68. Winau F, Kaufmann SHE, Schaible UE. Apoptosis Paves the Detour Path for CD8T Cell Activation Against Intracellular Bacteria. *Cell Microbiol* (2004) 6:599–607. doi: 10.1111/j.1462-5822.2004.00408.x
69. Lewinsohn DA, Lewinsohn DM, Scriba TJ. Polyfunctional CD4+ T Cells as Targets for Tuberculosis Vaccination. *Front Immunol* (2017) 8:1262. doi: 10.3389/fimmu.2017.01262
70. Lindenstrøm T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, et al. Tuberculosis Subunit Vaccination Provides Long-Term Protective Immunity Characterized by Multifunctional Cd4 Memory T Cells. *J Immunol* (2009) 182:8047–55. doi: 10.4049/jimmunol.0801592
71. Wu Y, Woodworth JS, Shin DS, Morris S, Behar SM. Vaccine-Elicited 10-Kilodalton Culture Filtrate Protein-Specific CD8 + T Cells are Sufficient to Mediate Protection Against Mycobacterium Tuberculosis Infection. *Infect Immun* (2008) 76:2249–55. doi: 10.1128/IAI.00024-08
72. Boom WH. New TB Vaccines: Is There a Requirement for CD8+ T Cells? *J Clin Invest* (2007) 117:2092–4. doi: 10.1172/JCI32933
73. Nieuwenhuizen NE, Kaufmann SHE. Next-Generation Vaccines Based on Bacille Calmette-Guérin. *Front Immunol* (2018) 9:121. doi: 10.3389/ fimmu.2018.00121
74. Hu Z, Wong K, Zhao H, Wen H, Ji P, Ma H, et al. Sendai Virus Mucosal Vaccination Establishes Lung-Resident Memory Cd8 T Cell Immunity and Boosts BCG-Primed Protection Against TB in Mice. *Mol Ther* (2017) 25:1222–33. doi: 10.1016/j.ymthe.2017.02.018
75. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, et al. A Critical Role for CD8 T Cells in a Nonhuman Primate Model of Tuberculosis. *PloS Pathog* (2009) 5:e1000392. doi: 10.1371/journal.ppat.1000392
76. Choi HG, Kwon KW, Choi S, Back YW, Park HS, Kang SM, et al. Antigen-Specific IFN-γ/IL-17-Co-Producing CD4+ T-Cells Are the Determinants for Protective Efficacy of Tuberculosis Subunit Vaccine. *Vaccines* (2020) 8:1–22. doi: 10.3390/vaccines8020300
77. Cavalcanti YVN, Brelaz MCA, Neves JKDAL, Ferraz JC, Pereira VRA. Role of TNF-alpha, IFN-Gamma, and IL-10 in the Development of Pulmonary Tuberculosis. *Pulm Med* (2012) 2012:745483. doi: 10.1155/2012/745483
78. Yao Y, Li W, Kaplan MH, Chang CH. Interleukin (IL)-4 Inhibits IL-10 to Promote IL-12 Production by Dendritic Cells. *J Exp Med* (2005) 201:1899–903. doi: 10.1084/jem.20050324
79. Silva-Filho JL, Caruso-Neves C, Pinheiro AAS. IL-4: An Important Cytokine in Determining the Fate of T Cells. *Biophys Rev* (2014) 6:111–8. doi: 10.1007/ s12551-013-0133-z
80. Cooney LA, Towery K, Endres J, Fox DA. Sensitivity and Resistance to Regulation by IL-4 During Th17 Maturation. *J Immunol* (2011) 187:4440–50. doi: 10.4049/jimmunol.1002860
81. Chin'Ombe N, Bourn WR, Williamson AL, Shephard EG. Oral Vaccination With a Recombinant Salmonella Vaccine Vector Provokes Systemic HIV-1 Subtype C Gag-Specific CD4+ Th1 and Th2 Cell Immune Responses in Mice. *Virol J* (2009) 6:87. doi: 10.1186/1743-422X-6-87
82. Benmerzoug S, Marinho FV, Rose S, Mackowiak C, Gosset D, Sedda D, et al. Gm-CSF Targeted Immunomodulation Affects Host Response to M. Tuberculosis Infection. *Sci Rep* (2018) 8(1):8652. doi: 10.1038/s41598-018-26984-3
83. Wang J, Zganiacz A, Xing Z. Enhanced Immunogenicity of BCG Vaccine by Using a Viral-Based GM-CSF Transgene Adjuvant Formulation. *Vaccine* (2002) 20:2887–98. doi: 10.1016/S0264-410X(02)00241-4

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REVIEW
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Something Old, Something New: Ion Channel Blockers as Potential Anti-Tuberculosis Agents

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Tuberculosis (TB) remains a challenging global health concern and claims more than a million lives every year. We lack an effective vaccine and understanding of what constitutes protective immunity against TB to inform rational vaccine design. Moreover, treatment of TB requires prolonged use of multi-drug regimens and is complicated by problems of compliance and drug resistance. While most *Mycobacterium tuberculosis* (Mtb) bacilli are quickly killed by the drugs, the prolonged course of treatment is required to clear persistent drug-tolerant subpopulations. Mtb's differential sensitivity to drugs is, at least in part, determined by the interaction between the bacilli and different host macrophage populations. Therefore, to design better treatment regimens for TB, we need to understand and modulate the heterogeneity and divergent responses that Mtb bacilli exhibit within macrophages. However, developing drugs *de-novo* is a long and expensive process. An alternative approach to expedite the development of new TB treatments is to repurpose existing drugs that were developed for other therapeutic purposes if they also possess anti-tuberculosis activity. There is growing interest in the use of immune modulators to supplement current anti-TB drugs by enhancing the host's antimycobacterial responses. Ion channel blocking agents are among the most promising of the host-directed therapeutics. Some ion channel blockers also interfere with the activity of mycobacterial efflux pumps. In this review, we discuss some of the ion channel blockers that have shown promise as potential anti-TB agents.

Keywords: mycobacterium, tuberculosis, host-directed therapies, ion channel blocker, efflux pump, drug-repurposing

INTRODUCTION

Tuberculosis (TB) is an airborne infection contracted by inhalation of droplet nuclei containing viable *Mycobacterium tuberculosis* (Mtb) that are released into the air by a person with active pulmonary TB. The disease has been a major cause of morbidity and mortality for several millennia (1). In 2019 alone, 10 million people developed active TB and 1.4 million of them died of the disease (2).

Most of the TB cases in 2019 were in South-East Asia (44%), Africa (25%) and western Pacific (18%) (2).

TB is challenging to treat even though there are now more than 20 first- and second-line anti-TB drugs in clinical use (3). Current anti-TB treatment regimens utilize combinations of no less than 3 drugs that must be taken for at least 6 months (3). The lengthy treatment duration and side effects of the drugs often lead to poor compliance with treatment, unfavorable outcomes and development of drug-resistant Mtb strains (4). In 2019, more than 0.5 million people developed multidrug-resistant (MDR) or rifampicin (RIF)-resistant (RR) TB worldwide (2). Treatment of drug-resistant TB requires longer and more complex drug regimens, and often causes more serious adverse effects than treatment of drug-susceptible TB (5). Current TB drugs target the pathogen and function by compromising the structural integrity or metabolic machinery of Mtb. In the last few years, host-directed therapy (HDT) targeting macrophages has emerged as a promising therapeutic strategy for both drug-susceptible TB and MDR-TB.

In the lung, alveolar macrophages (AMs) are among the most important innate defenses against Mtb. They phagocytose and eliminate bacteria through various pathways including phagosome maturation, autophagy and apoptosis. However, Mtb has evolved to survive inside macrophages by corrupting macrophage antimicrobial responses. HDTs for TB aim to rectify or circumvent the corrupted antimycobacterial responses.

Ion channel blockers are among the most promising potential HDTs for TB (**Table 1**). They are a diverse group of compounds that alter cell physiology by attenuating ion currents across cellular and subcellular membranes, and are most commonly used to treat noncommunicable diseases such as hypertension. Several Food and Drug Administration (FDA)-approved ion channel blocking agents have shown promise at both enhancing Mtb clearance by the immune system and attenuating inflammation *in vitro* and in animal models of TB (**Figure 1**). Additionally, some ion channel blocking agents have direct antimycobacterial activity. Here we review ion channel blocking agents that have demonstrated anti-tuberculosis activity in Mtb-infected macrophages and/or in animal models of TB.

ION CHANNEL BLOCKERS WITH POTENTIAL AS ANTI-TUBERCULOSIS AGENTS

Calcium Channel Blockers

Calcium ions (Ca^{2+}) act as second messengers in several signal transduction pathways (24). Calcium is more abundant in the extra cellular fluid (ECF) than in the cytosol (25). In the cell, most of the $Ca^{2+}$are sequestered in endoplasmic reticuli (ER) (25). Cell activation signals induce the flow of Ca^{2+} from the ER and ECF into the cytosol through channels such as inositol-1,4,5-trisphosphate receptors (IP_3Rs), ryanodine receptors (RyRs), voltage-gated calcium channels (VGCC) and calcium release-activated calcium (CRAC) channels.

Ca^{2+} signaling is important in antimycobacterial responses, including autophagy, phagosome maturation and apoptosis. In general, high cytosolic concentrations of Ca^{2+} promote phagosome maturation and acidification of mycobacteria-containing phagosomes, necrosis and apoptosis; while cell autophagy can be both upregulated or downregulated by Ca^{2+} (26–28). Whether Ca^{2+} influx upregulates or downregulates autophagy depends on factors such as the biological context and the ion channel conducting the Ca^{2+} current. For example, Ca^{2+} currents through the ATP-gated cation channel $P2X_7$ receptor enhanced autophagy and intracellular killing of *M. bovis*-BCG in human macrophages, while currents through VGCCs inhibit autophagy (26, 28).

TABLE 1 | Progress towards clinical use of ion channel blockers as anti-tuberculosis agents.

Year	Milestone	Reference
1990	Crowle and May demonstrated that chloroquine inhibits Mtb growth in macrophage cultures and potentiates streptomycin, pyrazinamide and isoniazid	(6)
1992	Crowle and colleagues observed that chlorpromazine was more active against Mtb in macrophage cultures than in broth	(7)
1993	Klemens and colleagues reported that clofazimine was effective against an MDR-TB strain in mice	(8)
1994	Gollapudi and colleagues demonstrated that verapamil improves accumulation of INH in Mtb-infected macrophages and promotes sensitivity of Mtb to INH	(9)
1996	Grange and Snell demonstrated that ambroxol has antimycobacterial activity in macrophages	(10)
2003	Esiobu and Hoosein observed that sodium valproate inhibits growth of *Mycobacterium smegmatis* in broth	(11)
2007	Byrne and colleagues observed that ketoconazole was synergistic with rifampicin-isoniazid-pyrazinamide	(12)
2010	van Deun and colleagues successfully used clofazimine as part of a 9-month MDR-TB treatment regimen in a clinical trial	(13)
2013	Smolarz and colleagues demonstrated that resveratrol has antitubercular activity in broth	(14)
2014	Stanley and colleagues demonstrated that fluoxetine promotes autophagic control of Mtb in macrophages	(15)
2015	Schiebler and colleagues successfully reduced the bacteria burden in mice infected with MDR-TB using carbamazepine and valproic acid	(16)
2016	Machado and colleagues successfully used verapamil, thioridazine and chlorpromazine to decrease bacteria burden in Mtb-infected macrophages	(17)
2016	WHO conditionally recommended a short course MDR-TB treatment regimen containing clofazimine	(18)
2018	Choi and colleagues demonstrated that ambroxol promotes autophagy and potentiates rifampicin in murine models of TB	(19)
2018	Rao and colleagues demonstrated that sodium valproate has antimycobacterial activity in broth and in macrophages in culture, and enhances activity of rifampicin and isoniazid	(20)
2019	Roca and colleagues demonstrated that dantrolene inhibits necrotic death and promotes Mtb control in Mtb-infected macrophages	(21)
2019	Yang and colleagues demonstrated that resveratrol has antitubercular activity in mice	(22)
2021	Lee and colleagues observed that the use of calcium channel blockers was associated with a 32% decrease in the risk of active tuberculosis	(23)

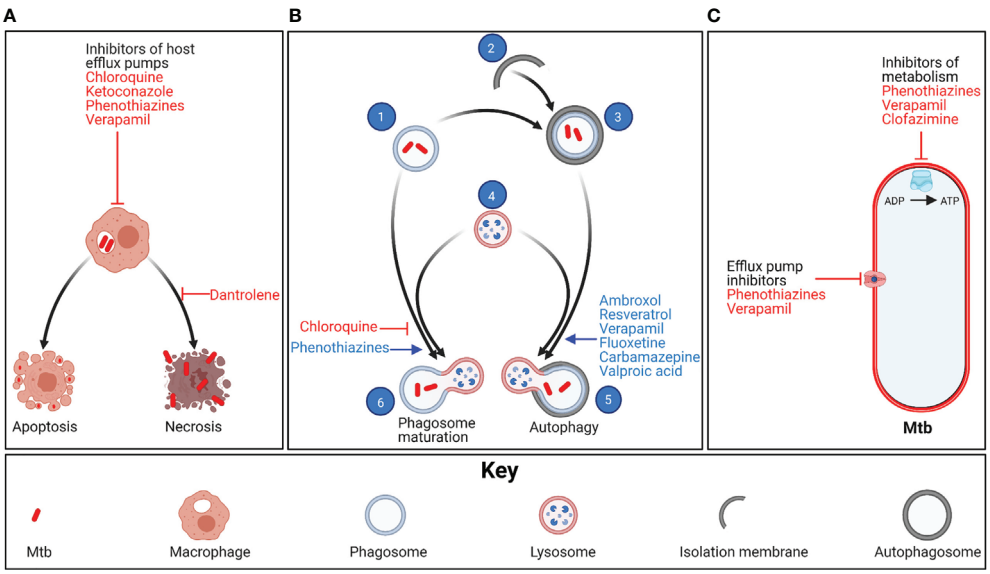

FIGURE 1 | Mechanism of action of ion channel blockers. **(A)** Chloroquine, ketoconazole, phenothiazines and verapamil inhibit eukaryotic efflux systems, allowing anti-TB drugs to achieve higher concentrations inside Mtb-infected host cells. Mtb promotes necrotic death of infected macrophages, leading to release of the bacteria into the extracellular space, where the bacteria continue to proliferate in the necrotic cells or infect new cells. In contrast, apoptotic cell death leads to enzymatic degradation of most of the bacteria. Dantrolene prevents necrotic death of Mtb-infected macrophages. **(B)** Phagocytosed Mtb (1) can also be eliminated through phagosome maturation and autophagy. In autophagy, isolation membranes (2) elongate and engulf phagosomes. The autophagosome-sequestered phagosomes (3) then fuse with lysosomes (4) to form autophagolysosomes (5), following which the lysosomal enzymes degrade the phagocytosed bacteria. Ambroxol, resveratrol, verapamil, fluoxetine, carbamazepine and valproic acid promote autophagy. In phagosome maturation, Mtb phagosomes fuse with lysosomes to form phagolysosomes (6). Phenothiazines promote acidification of phagolysosomes, thus enhancing activity of the lysosomal enzymes. In contrast, chloroquine inhibits phagosome maturation, thus preventing redox-induced Mtb drug tolerance, making the bacteria more susceptible to anti-TB drugs. **(C)** Phenothiazines and verapamil can also inhibit Mtb metabolism and efflux pump activity. Clofazimine, a second-line anti-TB agent, also inhibits Mtb metabolism. Created with BioRender.com.

A recent population-based analysis investigated whether the use of calcium channel blockers modifies the risk of active TB among patients with heart failure or cerebrovascular diseases in the clinical setting (23). The analysis included 8164 new active TB patients and 816,400 controls treated with or without calcium channel blockers compared with β-blockers or loop diuretics. Overall, the use of calcium channel blockers was associated with a 32% decrease in the risk of active TB [relative risk (RR), O.68 (95% CI, 0.58-0.78)] after adjustment with disease risk score. Analysis of the effect of different types of calcium channel blockers revealed that use of dihydropyridine calcium channel blockers was associated with a lower risk of TB [RR, 0.63 (95% CI, 0.53-0.79)] than non-dihydropyridine calcium channel blockers [RR, 0.73 (95% CI, 0.54-0.94)]. β-blockers or loop diuretics were not associated with lower risk of TB [RR, 0.99 (95% CI, 0.83-1.12)] and [RR, 0.88, (95% CI. 0.62-1.26)], respectively (23). This is the first large population-based study to confirm that calcium channel blockers modify and reduce the risk of active TB in humans. Therefore, modulating Ca^{2+} signaling using calcium channel blockers is an attractive host-directed therapeutic strategy for TB. Dantrolene, resveratrol and verapamil are the calcium channel blockers that have shown the most promise as potential anti-TB agents.

Dantrolene

Dantrolene is a RyR antagonist clinically approved for treatment of malignant hyperthermia. RyRs are intracellular Ca^{2+} channels that mediate the release of Ca^{2+} from the ER in response to elevated cytosolic Ca^{2+} levels (29). They are important in physiological and pathological processes, including necrosis and apoptosis. Apoptosis involves enzymatic degradation of intracellular contents including most of the phagocytosed bacteria, and their packaging into fragments called apoptotic bodies (30, 31). In contrast, necrosis involves swelling of organelles, loss of plasma membrane integrity and release of intracellular contents into the extracellular space (30). Mtb is able to continue growing inside necrotic macrophages, promoting lung inflammation and parenchymal injury (32). Mtb inhibits apoptotic cell death and promotes death of infected macrophages by necrosis (32). Roca et al. demonstrated the importance of ER-mitochondria signaling relay involving RyR and plasma membrane L-type Ca^{2+} channels for TNF-mediated necrosis of Mtb-infected macrophages in a zebrafish model of TB. Dantrolene reduced TNF-induced necrotic death of Mtb-infected macrophages by more than 50% by attenuating RyR activity and the surge in cytosolic Ca^{2+} that normally precedes necrosis, attesting to its potential as a HDT for TB (21). Inhibition of RyR activity

with dantrolene has also been shown to promote autophagy (33), although this has not been demonstrated in Mtb-infected macrophages.

Resveratrol

Resveratrol (3, 5, 4′-trihydroxystilbene) is a natural polyphenol produced by plants including grapes and berries and widely used as a food supplement (34, 35). It interacts with and modulates the activity of at least 20 mammalian proteins including CRAC channels and VGCCs (36). Resveratrol attenuates Mtb-induced inflammatory responses and enhances elimination of Mtb in macrophages, at least in part by upregulating the expression of host sirtuin 1 (37–39). Sirtuin 1 is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase that deactivates ReLA, the p65 subunit of nuclear factor kappa B (NF-κB) (40). NF-κB is a transcription factor important in the maturation of dendritic cells, M1 polarization of macrophages, differentiation of Th0 cells to the Th1 phenotype and expression of pro-inflammatory cytokines including IL-1, IL-8 and TNF-α (41, 42). Mtb down-regulates expression of host sirtuin 1 in monocytes/macrophages, in mouse models of TB and in TB patients with active disease, leading to overexpression of NF-κB (40). While NF-κB expression is generally associated with anti-TB responses, NF-κB also inhibits apoptosis and autophagy, two of the pathways most effective at eliminating intracellular Mtb (43). Recently, Cheng et al. reported a log fold decrease in bacteria load after treating THP-1 cells infected with W148, an MDR-TB strain, with resveratrol for 3 days (40).

Verapamil

Verapamil is an L-type calcium channel (LTCC) blocker widely used for the treatment of hypertension, angina and abnormal heart rhythms. LTCCs are a subfamily of VGCCs, and are expressed on the plasma membrane of most cell types. Ca^{2+} currents through LTCCs inhibit release of ER calcium stores in macrophages, thus attenuating Ca^{2+}-dependent signaling processes including macrophage activation (44). A previous study demonstrated that Mtb induces up-regulation of VGCCs in macrophages and dendritic cells to circumvent immune responses (44). Inhibiting LTCC currents in Mtb-infected macrophages with verapamil increases the concentration of Ca^{2+} in the cytosol, leading to upregulation of autophagy and Mtb killing (45–47).

The LTCC blockers verapamil and nifedipine also modulate iron metabolism by mobilizing iron from tissues, reducing intra-macrophage iron concentration and enhancing urinary iron excretion (48, 49). Iron is a cofactor in numerous biochemical reactions and is an essential nutrient for growth, replication and pathogenicity of many intracellular pathogens including Mtb (50). Further, prolonged iron overload promoted insulin resistance in skeletal muscle cells *in vitro* and *in vivo* in a mouse model of iron overload by inhibiting mTORC1 activation on autolysosomes and interfering with autophagic lysosomal regeneration (51). Therefore, by limiting iron availability, LTCC blockers promote a key pathway to enhance host resistance and clearance of intracellular pathogens such as Mtb.

In addition to enhancing host mycobacterial responses, Verapamil is bactericidal to both replicating and non-replicating Mtb in broth (52). It also inhibits both host and bacterial efflux pumps and is synergistic with RIF and isoniazid (INH) in broth, in macrophage cultures and in mouse models of TB (17, 52, 53). Gupta and colleagues reported that supplementing standard TB therapy with verapamil yielded an extra 1.15 log CFU reduction in pulmonary bacterial load in a murine model of TB (54).

Progress Towards Clinical Use of Calcium Channel Blockers as Anti-Tuberculosis Agents

While evidence from *in vitro* and animal models of TB indicates that calcium channel blockers have anti-TB activity, there has been no progress in transitioning from pre-clinical findings to clinical practice. The use of dantrolene as an anti-TB agent could prove challenging due to its numerous adverse effects which include muscle weakness, sedation, visual disturbances and hallucinations (55). Compared to dantrolene, verapamil and resveratrol are generally well tolerated and would be the preferred compounds for repurposing as anti-TB agents. Therefore, there is need for human clinical trials to assess the efficacy of verapamil and resveratrol as clinically relevant adjunct HDTs for TB.

Potassium Channel Blockers

While Ca^{2+} directly modulate antimycobacterial responses, potassium (K^+), sodium (Na^+) and chloride (Cl^-) ions primarily modulate macrophage responses by modulating Ca^{2+} currents.

K^+ currents promote autophagy and other anti-TB responses in Mtb-infected macrophages (28). K^+ is more abundant in the cytosol than the ECF while Ca^{2+}, Na^+ and Cl^- are more abundant in the ECF than the cytosol. This creates an overall negative charge inside the cell relative to the ECF. The electrochemical gradient between the cytosol and ECF facilitates movement of calcium into the cytosol during macrophage activation. Outward K^+ currents help sustain Ca^{2+} entry and macrophage activation by preventing plasma membrane (PM) depolarization and maintaining an electrical gradient between the ECF and cytosol (56, 57). Several K^+ channel antagonists have shown promise as potential anti-TB drugs, but most appear to promote macrophage anti-TB responses by mechanisms remote from ion channel blockade. Such compounds include chloroquine, ketoconazole and clofazimine.

Chloroquine

Chloroquine has been in use as an antimalarial agent for over 5 decades. It also suppresses the activity of mammalian delayed rectifier K^+ channels (Kv1.3) in leukocytes and lymphocyte production of pro-inflammatory cytokines (58). Chloroquine has anti-inflammatory and immunomodulatory properties and is used to treat autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus. In addition, chloroquine has engendered interest as a potential HDT against several viral diseases.

Mishra and colleagues observed a modest reduction in growth of intracellular Mtb following exposure to chloroquine alone, but a five-fold increase in the activity of INH when the two compounds were used together (59). The combination of chloroquine with INH eliminated Mtb within 8 weeks in a murine model of TB, while INH alone only reduced bacterial load by 2 logs during the same timeframe. Additionally, chloroquine eradicated drug-tolerant Mtb, ameliorated lung pathology and reduced post-treatment TB relapse in *in vivo* mouse models of TB (59).

Chloroquine enhances the activity of INH through at least two divergent pathways. First, it inhibits phagosome acidification, thus reducing redox-induced Mtb drug tolerance (59). Second, chloroquine increases the intramacrophage concentration of INH by inhibiting the activity of p-glycoprotein and breast cancer resistance protein-1 (BCRP-1) (60, 61). P-glycoprotein and BCRP-1 are mammalian efflux pumps that are overexpressed in Mtb infected macrophages, where they extrude anti-TB drugs into the ECF, thus protecting the intracellular bacteria from the antibiotics (62). Together, these studies suggest potential for repurposing chloroquine to shorten the duration of current TB treatment and to achieve relapse-free cure.

Ketoconazole

Ketoconazole is an azole antifungal used to treat cutaneous and systemic fungal infections. It kills fungi by inhibiting synthesis of ergosterol, an essential component of the fungal PM (63). Ketoconazole also inhibits the activity of voltage-gated potassium channels ($K_v1.5$, $K_v11.1$) and other mammalian proteins (63, 64).

The azole class of antifungals has been reported to possess anti-TB activity. Byrne and colleagues reported that ketoconazole inhibited growth of Mtb in broth (12). Furthermore, they observed a 3.42 log CFU reduction in bacterial load in lungs of Mtb-infected mice that were treated with ketoconazole-RIF-INH-pyrazinamide (PZA), and a 3.08 log CFU reduction in mice that were treated with RIF-INH-PZA, indicating that ketoconazole is synergistic with current first-line anti-TB drugs (12). Ketoconazole enhances the activity of anti-TB drugs, at least in part, by inhibiting pregnane X receptor (PXR), a promiscuous ligand-dependent transcriptional factor that is activated by steroid and xenobiotic agents (65, 66). PXR modulates expression of mammalian drug efflux and metabolism genes and reduces the efficacy of rifamycins against intracellular Mtb (67). The role of ketoconazole in the treatment of TB should be explored further.

Clofazimine

Clofazimine is a riminophenazine dye that is used as a first line agent in the treatment of leprosy, in combination with RIF and dapsone (68). It kills mycobacteria by disrupting multiple physiological processes, including respiration and K^+ transport across the PM (68). It was originally developed as an anti-TB drug more than five decades ago, but proved to be inferior to RIF and INH (68). The use of clofazimine to treat TB was revisited recently, and clofazimine has been listed as a second line anti-TB agent (2). The efficacy of various clofazimine-containing regiments against MDR-TB is currently being assessed in the BEAT TB, endTB-Q and TB-PRACTECAL clinical trials (2). Furthermore, pre-clinical studies show that clofazimine could reduce the duration of treatment of drug-susceptible TB. Tyagi and colleagues successfully sterilized Mtb-infected mice with a 3-month course of clofazimine-RIF-INH-PZA-ethambutol (EMB), but achieved a similar outcome with RIF-INH-PZA-EMB after treatment for 5 months (69). CLO-FAST, a phase 2 clinical trial is currently assessing the efficacy of a 3-month anti-TB regimen containing clofazimine and rifapentine against drug-susceptible TB (2).

In addition to its direct antimycobacterial activity, clofazimine has recently been shown to enhance host antimycobacterial responses by inhibiting mammalian Kv1.3 K^+ channels, which are highly expressed on effector memory T (Tem) lymphocytes (70, 71). Singh and colleagues demonstrated that inhibition of Kv1.3 channels on Tem cells by clofazimine during BCG vaccination in mice enhanced vaccine efficacy by promoting selective expansion of central memory T (Tcm) cells, a T-cell subset that is associated with protective anti-TB responses. Mice that received clofazimine also exhibited significantly enhanced resistance against TB (71). These reports suggest Kv1.3 K^+ channel blockade as a promising approach to enhance BCG vaccine efficacy in humans.

Progress Towards Clinical Use of Potassium Channel Blockers as Anti-Tuberculosis Agents

While clofazimine has now been adopted as an anti-TB agent, there has been no progress towards bringing ketoconazole and chloroquine into clinical use against TB. Azole antifungals such as ketoconazole and fluconazole are commonly used to treat concomitant candida or cryptococcal infections in HIV-infected TB patients but the impact of azole treatment on TB outcomes has not been assessed. Ketoconazole is generally well tolerated, but can cause serious adverse effects such as hepatotoxicity (72). Rifampicin, isoniazid and pyrazinamide can also cause drug-induced hepatitis (73), therefore, concomitant use of these anti-TB drugs with azole antifungals may increase the risk of hepatotoxicity. The safety of ketoconazole when used as adjunct treatment for TB requires further investigation.

Sodium Channel Blockers

There is paucity of data on the role of Na^+ channels in anti-TB responses. However, opening of PM Na^+ channels leads to influx of Na^+ into the cytosol down its chemical gradient, thus reducing the electrical gradient between the ECF and cytosol. This reduces the driving force for Ca^{2+} entry. Most PM Na^+ channels may therefore inhibit host antimycobacterial responses, and several Na^+ channel antagonists including ambroxol, carbamazepine and valproic acid promote host anti-TB responses (16, 19).

Ambroxol

Ambroxol is an inhibitor of voltage-gated sodium channel (Na_v) 1.8 and is primarily used as a mucolytic agent (74). It is a potent inducer of autophagy and has garnered interest as a potential therapeutic agent to hasten degradation of misfolded proteins in

proteinopathies including Parkinson's disease and primary alveolar proteinosis (75, 76). It has no direct antimycobacterial activity (19, 53), but it induces dose-dependent autophagic control of Mtb *in vitro* and *in vivo* and promotes mycobacterial killing in Mtb-infected primary mouse macrophages (19). Additionally, Choi and colleagues observed that ambroxol potentiated the antimycobacterial activity of rifampicin in a murine TB model, resulting in a three-fold decrease in bacterial load in mice treated with ambroxol and RIF relative to mice treated with RIF alone (19). Ambroxol warrants further evaluation as a HDT to augment and enhance the efficacy of current chemotherapy for TB in humans.

Carbamazepine

Carbamazepine is used to treat epilepsy, schizophrenia and bipolar disorder. It inhibits the activity of Na_v 1.5, thus indirectly inhibiting the uptake of inositol through Na^+-dependent inositol transporters on the PM (16). Inositol is a carbocyclic sugar upstream to biosynthesis of inositol-1,4,5-trisphosphate (IP_3), a lipid second messenger that activates IP_3Rs on ER. Blockade of inositol uptake by carbamazepine therefore reduces cytosolic levels of IP_3, leading to decreased Ca^{2+} release from the ER and upregulation of autophagy (16). Treatment of MDR-TB-infected mice with carbamazepine for 30 days resulted in a ten-fold decrease in pulmonary bacterial load, improved lung pathology and stimulated adaptive immunity. This was achieved through induction of autophagic killing of intracellular Mtb, mediated by cellular depletion of inositol and independent of mTOR (16).

Valproic acid

Valproic acid is an inhibitor of Na_vs, and is used to treat epilepsy, migraine and bipolar disorder (77, 78). It is active against Mtb in broth through mechanisms that have not been fully elucidated (20). Rao and colleagues observed a 1.5 log CFU reduction in bacterial load following treatment of intracellular Mtb with valproic acid or INH, and a 2 log CFU reduction when the two drugs were used together (20). Like carbamazepine, valproic acid promotes autophagy by interfering with biosynthesis of IP_3 (79). In addition, it inhibits host histone deacetylase 1 (HDAC1), a protein that is usually upregulated in Mtb-infected macrophages (80, 81). HDACs suppress gene expression by promoting chromatin packaging, thus making a segment of DNA inaccessible to the cellular transcription machinery (82). Upregulating HDAC1 in Mtb-infected macrophages reduces expression of IL-12β, a cytokine important in the initiation of Th1 responses (80). Therefore, repurposing of carbamazepine and valproic acid as adjunct HDTs to enhance intracellular killing of Mtb by current anti-TB drugs should be explored as a treatment option for human TB.

Progress Towards Clinical Use of Sodium Channel Blockers as Anti-Tuberculosis Agents

The use of ambroxol, carbamazepine and valproic acid against TB has not been tested in a clinical trial setting. Carbamazepine and valproic acid are used widely as treatment for epilepsy in low- and middle-income countries where the burden of TB is high. However, the impact of concomitant use of sodium channel blockers and anti-TB treatment on TB outcomes has not been evaluated. Furthermore, both carbamazepine and valproic acid have been shown to cause hepatotoxicity in some individuals (83, 84), but there are no clinical trial data on the safety of carbamazepine and valproic when used in combination with existing anti-TB drugs.

Other Ion Channel Blockers

Fluoxetine

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) and is primarily used as an anti-depressant. However, fluoxetine also has antiviral, antibacterial and immunomodulatory properties (85–87). In addition to inhibiting the uptake of serotonin into pre-synaptic neurons, fluoxetine modulates the activity of VGCCs, K2P, Na_vs and 5-hydrotryptamine 3 (5-HT3) (88–90).

Schump and colleagues observed a 50% reduction in growth of intracellular Mtb following treatment with fluoxetine, even though it had limited activity against Mtb in broth (91). Several mechanisms for this have been described, including accumulation inside macrophages and induction of autophagy (15, 91). In another study, Stanley and colleagues demonstrated that fluoxetine promoted secretion of TNF-α, induced autophagy and inhibited growth of intracellular Mtb by 75% in J774 cells (15). These observations merit evaluation of the anti-TB activity of fluoxetine in clinical studies of human TB.

Phenothiazines

Phenothiazines are a large group of heterocyclic molecules most widely used as antipsychotics and antihistamines due to their ability to modulate dopamine signaling (92). Most phenothiazines bind to and modulate the activity of multiple mammalian proteins, including ligand-gated ion channels, ion pumps, G protein-coupled receptors and efflux pumps (92, 93).

The antimycobacterial properties of phenothiazines have been known for decades, but they were overshadowed by the current first line anti-TB compounds, to which they are inferior (7). However, the rise of MDR-TB has rekindled interest in the use of phenothiazines against Mtb. Most phenothiazines are active against extracellular Mtb at concentrations that cannot be achieved safely *in vivo*. However, they are generally active against intracellular Mtb at much lower concentrations (94). Some phenothiazines are concentrated by macrophages to at least 10 times their plasma concentrations, which may partly explain their potency against intracellular Mtb (94–96). Thioridazine, chlorpromazine, promethazine, methyldiazine and trifluoperazine are among the phenothiazines that have shown the most promise as potential anti-TB agents.

Thioridazine was once a popular drug for schizophrenia and psychosis but has largely been replaced by the newer generation of neuroleptics. It kills extracellular Mtb by disrupting ATP synthesis (17, 97). Machado and colleagues demonstrated that thioridazine promotes acidification of Mtb phagosomes, and reported an 88% increase in Mtb killing by thioridazine-treated macrophages (17). As thioridazine has multiple eukaryotic protein targets, the mechanism through which it promotes phagosome acidification has not been elucidated. However, one

possible explanation is that it inhibits the efflux of ions from the phagolysosome, leading to indirect acidification (98).

In addition, thioridazine inhibits mycobacterial drug efflux systems, reduces resistance levels of different strains of MDR-TB to first and second-line anti-TB agents, and hastens clearance of drug-susceptible Mtb by first-line anti-TB agents (17, 95, 99). Dutta and colleagues were able to sterilize lungs of mice infected with drug-susceptible Mtb with a 4 month course of thioridazine-RIF-INH-PZA, but achieved the same with RIF-INH-PZA in 5 months (95).

Progress Towards Clinical Use of Other Ion Channel Blockers as Anti-Tuberculosis Agents

Fluoxetine and phenothiazines are currently not used as anti-TB drugs in clinical practice. However, thioridazine is relatively well tolerated, and has received more attention as a potential anti-TB agent than any other phenothiazine. There is need for clinical trials to determine the efficacy of thioridazine as part of anti-TB treatment regimens in humans.

SUMMARY AND CONCLUDING REMARKS

Ion channel blockers have the potential to contribute to the treatment of TB to reduce morbidity and mortality from the disease. Their ability to enhance the activity of first-line anti-TB drugs could help hasten clearance of Mtb from lungs of individuals with pulmonary TB disease, reduce transmission of infection, emergency of drug-resistant mutants and relapse rates. The ideal host-directed therapeutics for TB should potentiate the immune system's antimycobacterial defenses while preventing excessive inflammation and tissue injury. In addition to enhancing clearance of Mtb, ion channel blockers generally attenuate host inflammatory responses and may reduce tissue injury in TB patients. In the absence of a single agent that can eliminate Mtb, combination therapy will remain the mainstay of TB treatment. While current drug combinations are designed to maximize clearance of Mtb by targeting the pathogen, ion channel blockers could enhance bacillary clearance by targeting both the pathogen and the host immune response. The reduction in the risk of active TB associated with the use of dihydropyridine calcium channel blockers is a cause for optimism and may pave the way for clinical trials of ion channel blockers as adjunct treatment for human TB.

AUTHOR CONTRIBUTIONS

SM-N wrote the manuscript with input from HM and DT. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OYC, Gernaey AM, et al. Detection and Molecular Characterization of 9000-Year-Old *Mycobacterium tuberculosis* From a Neolithic Settlement in the Eastern Mediterranean. *PLoS One* (2008) 3(10):e3426. doi: 10.1371/journal.pone.0003426
2. World Health Organization. *Global Tuberculosis Report 2020*. Geneva: World Health Organisation (2020). Licence: CC BY-NC-SA 3.0 IGO.
3. Sotgiu G, Centis R, D'Ambrosio L, Migliori GB. Tuberculosis Treatment and Drug Regimens. *Cold Spring Harb Perspect Med* (2015) 5(5):a017822. doi: 10.1101/cshperspect.a017822
4. Mekonnen HS, Azagew AW. Non-Adherence to Anti-Tuberculosis Treatment, Reasons and Associated Factors Among TB Patients Attending at Gondar Town Health Centers, Northwest Ethiopia. *BMC Res Notes* (2018) 11(1):691. doi: 10.1186/s13104-018-3789-4
5. Yang TW, Park HO, Jang HN, Yang JH, Kim SH, Moon SH, et al. Side Effects Associated With the Treatment of Multidrug-Resistant Tuberculosis at a Tuberculosis Referral Hospital in South Korea: A Retrospective Study. *Medicine (Baltimore)* (2017) 96(28):e7482. doi: 10.1097/MD.0000000000007482
6. Crowle AJ, May MH. Inhibition of Tubercle Bacilli in Cultured Human Macrophages by Chloroquine Used Alone and in Combination With Streptomycin, Isoniazid, Pyrazinamide, and Two Metabolites of Vitamin D3. *Antimicrob Agents Chemother* (1990) 34(11):2217–22. doi: 10.1128/AAC.34.11.2217
7. Crowle AJ, Douvas GS, May MH. Chlorpromazine: A Drug Potentially Useful for Treating Mycobacterial Infections. *Chemotherapy* (1992) 38(6):410–9. doi: 10.1159/000239036
8. Klemens SP, DeStefano MS, Cynamon MH. Therapy of Multidrug-Resistant Tuberculosis: Lessons From Studies With Mice. *Antimicrob Agents Chemother* (1993) 37(11):2344–7. doi: 10.1128/AAC.37.11.2344
9. Gollapudi S, Reddy M, Gangadharam P, Tsuruo T, Gupta S. *Mycobacterium Tuberculosis* Induces Expression of P-glycoprotein in Promonocytic U1 Cells Chronically Infected With HIV Type 1. *Biochem Biophys Res Commun* (1994) 199(3):1181–7. doi: 10.1006/bbrc.1994.1355
10. Grange JM, Snell NJC. Activity of Bromhexine and Ambroxol, Semi-Synthetic Derivatives of Vasicine From the Indian Shrub *Adhatoda Vasica*, Against *Mycobacterium tuberculosis In Vitro*. *J Ethnopharmacol* (1996) 50(1):49–53. doi: 10.1016/0378-8741(95)01331-8
11. Esiobu N, Hoosein N. An Assessment of the In Vitro Antimicrobial Effects of Two Antiepileptic Drugs - Sodium Valproate and Phenytoin. *Antonie van Leeuwenhoek* (2003) 83(1):63–8. doi: 10.1023/A:1022992224594
12. Byrne ST, Denkin SM, Gu P, Nuermberger E, Zhang Y. Activity of Ketoconazole Against *Mycobacterium tuberculosis* In Vitro and in the Mouse Model. *J Med Microbiol* (2007) 56(Pt 8):1047–51. doi: 10.1099/jmm.0.47058-0
13. Van Deun A, Maug AKJ, Salim MAH, Das PK, Sarker MR, Daru P, et al. Short, Highly Effective, and Inexpensive Standardized Treatment of Multidrug-Resistant Tuberculosis. *Am J Respir Crit Care Med* (2010) 182 (5):684–92. doi: 10.1164/rccm.201001-0077OC
14. Smolarz HD, Swatko-Ossor M, Ginalska G, Medynska E. Antimycobacterial Effect of Extract and its Components From *Rheum Rhaponticum*. *J AOAC Int* (2013) 96(1):155–60. doi: 10.5740/jaoacint.12-010
15. Stanley SA, Barczak AK, Silvis MR, Luo SS, Sogi K, Vokes M, et al. Identification of Host-Targeted Small Molecules That Restrict Intracellular

Mycobacterium Tuberculosis Growth. *PLoS Pathog* (2014) 10(2):e1003946. doi: 10.1371/journal.ppat.1003946
16. Schiebler M, Brown K, Hegyi K, Newton SM, Renna M, Hepburn L, et al. Functional Drug Screening Reveals Anticonvulsants as Enhancers of mTOR-Independent Autophagic Killing of *Mycobacterium Tuberculosis* Through Inositol Depletion. *EMBO Mol Med* (2015) 7(2):127–39. doi: 10.15252/emmm.201404137
17. Machado D, Pires D, Perdigão J, Couto I, Portugal I, Martins M, et al. Ion Channel Blockers as Antimicrobial Agents, Efflux Inhibitors, and Enhancers of Macrophage Killing Activity Against Drug Resistant *Mycobacterium tuberculosis*. *PLoS One* (2016) 11(2):e0149326. doi: 10.1371/journal.pone.0149326
18. World Health Organisation. *WHO Treatment Guidelines for Drug-Resistant Tuberculosis : 2016 Update*. Geneva: World Health Organisation (2016).
19. Choi SW, Gu Y, Peters RS, Salgame P, Ellner JJ, Timmins GS, et al. Ambroxol Induces Autophagy and Potentiates Rifampin Antimycobacterial Activity. *Antimicrob Agents Chemother* (2018) 62(9):e01019–18. doi: 10.1128/aac.01019-18
20. Rao M, Valentini D, Zumla A, Maeurer M. Evaluation of the Efficacy of Valproic Acid and Suberoylanilide Hydroxamic Acid (Vorinostat) in Enhancing the Effects of First-Line Tuberculosis Drugs Against Intracellular *Mycobacterium tuberculosis*. *Int J Infect Dis* (2018) 69:78–84. doi: 10.1016/j.ijid.2018.02.021
21. Roca FJ, Whitworth LJ, Redmond S, Jones AA, Ramakrishnan L. TNF Induces Pathogenic Programmed Macrophage Necrosis in Tuberculosis Through a Mitochondrial-Lysosomal-Endoplasmic Reticulum Circuit. *Cell* (2019) 178(6):1344–1361.e11. doi: 10.1016/j.cell.2019.08.004
22. Yang H, Hu J, Chen YJ, Ge B. Role of Sirt1 in Innate Immune Mechanisms Against *Mycobacterium Tuberculosis* Via the Inhibition of TAK1 Activation. *Arch Biochem Biophys* (2019) 667:49–58. doi: 10.1016/j.abb.2019.04.006
23. Lee CC, Lee MTG, Hsu WT, Park JY, Porta L, Liu MA, et al. Use of Calcium Channel Blockers and Risk of Active Tuberculosis Disease: A Population-Based Analysis. *Hypertension* (2021) 77(2):328–37. doi: 10.1161/HYPERTENSIONAHA.120.15534
24. Newton AC, Bootman MD, Scott JD. Second Messengers. *Cold Spring Harb Perspect Biol* (2016) 8(8):a005926. doi: 10.1101/cshperspect.a005926
25. Clapham DE. Calcium Signaling. *Cell* (2007) 131(6):1047–58. doi: 10.1016/j.cell.2007.11.028
26. Biswas D, Qureshi OS, Lee WY, Croudace JE, Mura M, Lammas DA. ATP-Induced Autophagy Is Associated With Rapid Killing of Intracellular Mycobacteria Within Human Monocytes/Macrophages. *BMC Immunol* (2008) 9:35. doi: 10.1186/1471-2172-9-35
27. Kondratskyi A, Kondratska K, Skryma R, Prevarskaya N. Ion Channels in the Regulation of Apoptosis. *Biochim Biophys Acta* (2015) 1848(10 Pt B):2532–46. doi: 10.1016/j.bbamem.2014.10.030
28. Kondratskyi A, Kondratska K, Skryma R, Klionsky DJ, Prevarskaya N. Ion Channels in the Regulation of Autophagy. *Autophagy* (2018) 14(1):3–21. doi: 10.1080/15548627.2017.1384887
29. Hamilton SL. Ryanodine Receptors. *Cell Calcium* (2005) 38(3-4):253–60. doi: 10.1016/j.ceca.2005.06.037
30. Molloy A, Laochumroonvorapong P, Kaplan G. Apoptosis, But Not Necrosis, of Infected Monocytes Is Coupled With Killing of Intracellular Bacillus Calmette-Guérin. *J Exp Med* (1994) 180(4):1499–509. doi: 10.1084/jem.180.4.1499
31. Kumar S, Birge RB. Efferocytosis. *Curr Biol* (2016) 26(13):R558–9. doi: 10.1016/j.cub.2016.01.059
32. Divangahi M, Behar SM, Remold H. Dying to Live: How the Death Modality of the Infected Macrophage Modulates Immunity to Tuberculosis. *Adv Exp Med Biol* (2013) 783:103–20. doi: 10.1007/978-1-4614-6111-1_6
33. Vervliet T. Ryanodine Receptors in Autophagy: Implications for Neurodegenerative Diseases? *Front Cell Neurosci* (2018) 12:89. doi: 10.3389/fncel.2018.00089
34. Weiskirchen S, Weiskirchen R. Resveratrol: How Much Wine do You Have to Drink to Stay Healthy? *Adv Nutr* (2016) 7(4):706–18. doi: 10.3945/an.115.011627
35. Koushki M, Amiri-Dashatan N, Ahmadi N, Abbaszadeh HA, Rezaei-Tavirani M. Resveratrol: A Miraculous Natural Compound for Diseases Treatment. *Food Sci Nutr* (2018) 6(8):2473–90. doi: 10.1002/fsn3.855
36. McCalley AE, Kaja S, Payne AJ, Koulen P. Resveratrol and Calcium Signaling: Molecular Mechanisms and Clinical Relevance. *Molecules* (2014) 19(6):7327–40. doi: 10.3390/molecules19067327
37. Coimbra ES, Santos JA, Lima LL, Machado PA, Campos DL, Pavan FR, et al. Synthesis, Antitubercular and Leishmanicidal Evaluation of Resveratrol Analogues. *J Braz Chem Soc* (2016) 27(12):2161–9. doi: 10.5935/0103-5053.20160107
38. Vestergaard M, Ingmer H. Antibacterial and Antifungal Properties of Resveratrol. *Int J Antimicrob Agents* (2019) 53(6):716–23. doi: 10.1016/j.ijantimicag.2019.02.015
39. Zumla A, Rao M, Dodoo E, Maeurer M. Potential of Immunomodulatory Agents as Adjunct Host-Directed Therapies for Multidrug-Resistant Tuberculosis. *BMC Med* (2016) 14:89. doi: 10.1186/s12916-016-0635-1
40. Cheng CY, Gutierrez NM, Marzuki MB, Lu X, Foreman TW, Paleja B, et al. Host Sirtuin 1 Regulates Mycobacterial Immunopathogenesis and Represents a Therapeutic Target Against Tuberculosis. *Sci Immunol* (2017) 2(9):eaaj1789. doi: 10.1126/sciimmunol.aaj1789
41. Liu T, Zhang L, Joo D, Sun SC. NF-κB Signaling in Inflammation. *Signal Transduct Target Ther* (2017) 2:17023. doi: 10.1038/sigtrans.2017.23
42. Aronica MA, Mora AL, Mitchell DB, Finn PW, Johnson JE, Sheller JR, et al. Preferential Role for NF-κB/Rel Signaling in the Type 1 But Not Type2 T Cell-Dependent Immune Response In Vivo. *J Immunol* (1999) 163(9):5116–24.
43. Bai X, Feldman NE, Chmura K, Ovrutsky AR, Su WL, Griffin L, et al. Inhibition of Nuclear Factor-Kappa B Activation Decreases Survival of *Mycobacterium Tuberculosis* in Human Macrophages. *PLoS One* (2013) 8(4):e61925. doi: 10.1371/journal.pone.0061925
44. Gupta S, Salam N, Srivastava V, Singla R, Behera D, Khayyam KU, et al. Voltage Gated Calcium Channels Negatively Regulate Protective Immunity to *Mycobacterium tuberculosis*. *PLoS One* (2009) 4(4):e5305. doi: 10.1371/journal.pone.0005305
45. Paik S, Kim JK, Chung C, Jo EK. Autophagy: A New Strategy for Host-Directed Therapy of Tuberculosis. *Virulence* (2019) 10(1):448–59. doi: 10.1080/21505594.2018.1536598
46. Juárez E, Carranza C, Sánchez G, González M, Chávez J, Torres M, et al. Autophagy Inducing Drugs Contribute to Control Mycobacterium Tuberculosis Infection. *Front Immunol*. doi: 10.3389/conf.fimmu.2015.05.00160. Conference Abstract: IMMUNOCOLOMBIA2015 - 11th Congress of the Latin American Association of Immunology - 10o. Congreso de la Asociación Colombiana de Alergia, Asma e Inmunología.
47. Juárez E, Carranza C, Sánchez G, González M, Chávez J, Sarabia C, et al. Loperamide Restricts Intracellular Growth of *Mycobacterium Tuberculosis* in Lung Macrophages. *Am J Respir Cell Mol Biol* (2016) 55(6):837–47. doi: 10.1165/rcmb.2015-0383OC
48. Shan HL, Wang Y, Wu JW, Hang PZ, Li X, Sun LH, et al. Verapamil Reverses Cardiac Iron Overload in Streptozocin-Induced Diabetic Rats. *Naunyn Schmiedebergs Arch Pharmacol* (2013) 386(7):645–50. doi: 10.1007/s00210-013-0863-2
49. Ludwiczek S, Theurl I, Muckenthaler MU, Jakab M, Mair SM, Theurl M, et al. Ca2+ Channel Blockers Reverse Iron Overload by a New Mechanism Via Divalent Metal Transporter-1. *Nat Med* (2007) 13(4):448–54. doi: 10.1038/nm1542
50. Zhang L, Hendrickson RC, Meikle V, Lefkowitz EJ, Ioerger TR, Nierderweis M. Comprehensive Analysis of Iron Utilization by *Mycobacterium Tuberculosis*. *PLoS Pathog* (2020) 16(2):e1008337. doi: 10.1371/journal.ppat.1008337
51. Jahng JWS, Alsaadi RM, Palanivel R, Song E, Hipolito VEB, Sung HK, et al. Iron Overload Inhibits Late Stage Autophagic Flux Leading to Insulin Resistance. *EMBO Rep* (2019) 20(10):e47911. doi: 10.15252/embr.201947911
52. Chen C, Gardete S, Jansen RS, Shetty A, Dick T, Rhee KY, et al. Verapamil Targets Membrane Energetics in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* (2018) 62(5):e02107. doi: 10.1128/AAC.02107-17
53. Mitini-Nkhoma SC, Fernando N, Ishaka GKD, Handunnetti SM, Pathirana SL. Ion Transport Modulators as Antimycobacterial Agents. *Tuberc Res Treat* (2020) 2020:1–7. doi: 10.1155/2020/3767915
54. Gupta S, Tyagi S, Almeida DV, Maiga MC, Ammerman NC, Bishai WR. Acceleration of Tuberculosis Treatment by Adjunctive Therapy With Verapamil as an Efflux Inhibitor. *Am J Respir Crit Care Med* (2013) 188(5):600–7. doi: 10.1164/rccm.201304-0650OC

55. Wedel DJ, Quinlan JG, Iaizzo PA. Clinical Effects of Intravenously Administered Dantrolene. *Mayo Clin Proc* (1995) 70(3):241–6. doi: 10.4065/70.3.241
56. Lewis RS, Cahalan MD. Potassium and Calcium Channels in Lymphocytes. *Annu Rev Immunol* (1995) 13:623–53. doi: 10.1146/annurev.iy.13.040195.003203
57. Hou P, Zhang R, Liu Y, Feng J, Wang W, Wu Y, et al. Physiological Role of Kv1.3 Channel in T Lymphocyte Cell Investigated Quantitatively by Kinetic Modeling. *PLoS One* (2014) 9(3):e89975. doi: 10.1371/journal.pone.0089975
58. Kazama I. Targeting Lymphocyte Kv1.3-Channels to Suppress Cytokine Storm in Severe COVID-19: Can it be a Novel Therapeutic Strategy? *Drug Discov Ther* (2020) 14(3):143–4. doi: 10.5582/ddt.2020.03046
59. Mishra R, Kohli S, Malhotra N, Bandyopadhyay P, Mehta M, Munshi M, et al. Targeting Redox Heterogeneity to Counteract Drug Tolerance in Replicating *Mycobacterium Tuberculosis*. *Sci Transl Med* (2019) 11(518):eaaw6635. doi: 10.1126/scitranslmed.aaw6635
60. Matt U, Müller R, Müller D, Stenziger A, Zinkernagel A, Seelig A, et al. Chloroquine as a Potential Adjunctive Therapy in Tuberculosis. *Eur Respir J* (2014) 44(Suppl 58):1896.
61. Matt U, Selchow P, Dal Molin M, Strommer S, Sharif O, Schilcher K, et al. Chloroquine Enhances the Antimycobacterial Activity of Isoniazid and Pyrazinamide by Reversing Inflammation-Induced Macrophage Efflux. *Int J Antimicrob Agents* (2017) 50(1):55–62. doi: 10.1016/j.ijantimicag.2017.02.022
62. te Brake LHM, de Knegt GJ, de Steenwinkel JE, van Dam TJP, Burger DM, Russel FGM, et al. The Role of Efflux Pumps in Tuberculosis Treatment and Their Promise as a Target in Drug Development: Unraveling the Black Box. *Annu Rev Pharmacol Toxicol* (2018) 58:271–91. doi: 10.1146/annurev-pharmtox-010617-052438
63. Zonios DI, Bennett JE. Update on Azole Antifungals. *Semin Respir Crit Care Med* (2008) 29(2):198–210. doi: 10.1055/s-2008-1063858
64. Dumaine R, Roy ML, Brown AM. Blockade of HERG and Kv1.5 by Ketoconazole. *J Pharmacol Exp Ther* (1998) 286(2):727–35.
65. Pavek P. Pregnane X Receptor (PXR)-Mediated Gene Repression and Cross-Talk of PXR With Other Nuclear Receptors Via Coactivator Interactions. *Front Pharmacol* (2016) 7:456. doi: 10.3389/fphar.2016.00456
66. Bhagyaraj E, Tiwari D, Ahuja N, Nanduri R, Saini A, Kalra R, et al. A Human Xenobiotic Nuclear Receptor Contributes to Nonresponsiveness of *Mycobacterium tuberculosis* to the Antituberculosis Drug Rifampicin. *J Biol Chem* (2018) 293(10):3747–57. doi: 10.1074/jbc.M117.818377
67. Shehu AI, Li G, Xie W, Ma X. The Pregnane X Receptor in Tuberculosis Therapeutics. *Expert Opin Drug Metab Toxicol* (2016) 12(1):21–30. doi: 10.1517/17425255.2016.1121381
68. Cholo MC, Steel HC, Fourie PB, Germishuizen WA, Anderson R. Clofazimine: Current Status and Future Prospects. *J Antimicrob Chemother* (2012) 67(2):290–8. doi: 10.1093/jac/dkr444
69. Tyagi S, Ammerman NC, Li SY, Adamson J, Converse PJ, Swanson RV, et al. Clofazimine Shortens the Duration of the First-Line Treatment Regimen for Experimental Chemotherapy of Tuberculosis. *Proc Natl Acad Sci U S A* (2015) 112(3):869–74. doi: 10.1073/pnas.1416951112
70. Ren YR, Pan F, Parvez S, Fleig A, Chong CR, Xu J, et al. Clofazimine Inhibits Human Kv1.3 Potassium Channel by Perturbing Calcium Oscillation in T Lymphocytes. *PLoS One* (2008) 3(12):e4009. doi: 10.1371/journal.pone.0004009
71. Singh DK, Dwivedi VP, Ranganathan A, Bishai WR, Van Kaer L, Das G. Blockade of the Kv1.3 K+ Channel Enhances BCG Vaccine Efficacy by Expanding Central Memory T Lymphocytes. *J Infect Dis* (2016) 214(9):1456–64. doi: 10.1093/infdis/jiw395
72. Heiberg JK, Svejgaard E. Toxic Hepatitis During Ketoconazole Treatment. *Br Med J (Clin Res Ed)* (1981) 283(6295):825–6. doi: 10.1136/bmj.283.6295.825
73. Askgaard DS, Wilcke T, Dossing M. Hepatotoxicity Caused by the Combined Action of Isoniazid and Rifampicin. *Thorax* (1995) 50(2):213–4. doi: 10.1136/thx.50.2.213
74. Leffler A, Reckzeh J, Nau C. Block of Sensory Neuronal Na+ Channels by the Secreolytic Ambroxol Is Associated With an Interaction With Local Anesthetic Binding Sites. *Eur J Pharmacol* (2010) 630(1-3):19–28. doi: 10.1016/j.ejphar.2009.12.027
75. Oda N, Tamai K, Suzuki Y, Yoshimatsu H, Matsuoka H, Matsumoto Y, et al. Marked Improvement in Autoimmune Pulmonary Alveolar Proteinosis With Severe Hypoxemia in a Patient Treated With Ambroxol: A Case Report. *J Med Case Rep* (2015) 9:100. doi: 10.1186/s13256-015-0588-6
76. Mullin S, Smith L, Lee K, D'Souza G, Woodgate P, Elflein J, et al. Ambroxol for the Treatment of Patients With Parkinson Disease With and Without Glucocerebrosidase Gene Mutations: A Nonrandomized, Noncontrolled Trial. *JAMA Neurol* (2020) 77(4):427–34. doi: 10.1001/jamaneurol.2019.4611
77. Ghodke-Puranik Y, Thorn CF, Lamba JK, Leeder JS, Song W, Birnbaum AK, et al. Valproic Acid Pathway: Pharmacokinetics and Pharmacodynamics. *Pharmacogenet Genomics* (2013) 23(4):236–41. doi: 10.1097/FPC.0b013e32835ea0b2
78. Zanatta G, Sula A, Miles AJ, Ng LCT, Torella R, Pryde DC, et al. Valproic Acid Interactions With the NavMs Voltage-Gated Sodium Channel. *Proc Natl Acad Sci U S A* (2019) 116(52):26549–54. doi: 10.1073/pnas.1909696116
79. Ji MM, Wang L, Zhan Q, Xue W, Zhao Y, Zhao X, et al. Induction of Autophagy by Valproic Acid Enhanced Lymphoma Cell Chemosensitivity Through HDAC-Independent and IP3-Mediated PRKAA Activation. *Autophagy* (2015) 11(12):2160–71. doi: 10.1080/15548627.2015.1082024
80. Chandran A, Antony C, Jose L, Mundayoor S, Natarajan K, Kumar RA. *Mycobacterium tuberculosis* Infection Induces HDAC1-Mediated Suppression of IL-12B Gene Expression in Macrophages. *Front Cell Infect Microbiol* (2015) 5:90. doi: 10.3389/fcimb.2015.00090
81. Soria-Castro R, Schcolnik-Cabrera A, Rodríguez-López G, Campillo-Navarro M, Puebla-Osorio N, Estrada-Parra S, et al. Exploring the Drug Repurposing Versatility of Valproic Acid as a Multifunctional Regulator of Innate and Adaptive Immune Cells. *J Immunol Res* (2019) 2019:9678098. doi: 10.1155/2019/9678098
82. Gatla HR, Muniraj N, Thevkar P, Yavvari S, Sukhavasi S, Makena MR. Regulation of Chemokines and Cytokines by Histone Deacetylases and an Update on Histone Decetylase Inhibitors in Human Diseases. *Int J Mol Sci* (2019) 20(5):1110. doi: 10.3390/ijms20051110
83. Kalapos MP. Carbamazepine-Provoked Hepatotoxicity and Possible Aetiopathological Role of Glutathione in the Events: Retrospective Review of Old Data and Call for New Investigation. *Adverse Drug React Toxicol Rev* (2002) 21(3):123–41. doi: 10.1007/BF03256188
84. Gayam V, Mandal AK, Khalid M, Shrestha B, Garlapati P, Khalid M. Valproic Acid Induced Acute Liver Injury Resulting in Hepatic Encephalopathy- A Case Report and Literature Review. *J Community Hosp Intern Med Perspect* (2018) 8(5):311–4. doi: 10.1080/20009666.2018.1514933
85. Di Rosso ME, Palumbo ML, Genaro AM. Immunomodulatory Effects of Fluoxetine: A New Potential Pharmacological Action for a Classic Antidepressant Drug? *Pharmacol Res* (2016) 109:101–7. doi: 10.1016/j.phrs.2015.11.021
86. Karine de Sousa A, Rocha JE, Gonçalves de Souza T, Sampaio de Freitas T, Ribeiro-Filho J, Melo Coutinho HD. New Roles of Fluoxetine in Pharmacology: Antibacterial Effect and Modulation of Antibiotic Activity. *Microb Pathog* (2018) 123:368–71. doi: 10.1016/j.micpath.2018.07.040
87. Bauer L, Manganaro R, Zonsics B, Strating JRPM, El Kazzi P, Lorenzo Lopez M, et al. Fluoxetine Inhibits Enterovirus Replication by Targeting the Viral 2C Protein in a Stereospecific Manner. *ACS Infect Dis* (2019) 5(9):1609–23. doi: 10.1021/acsinfecdis.9b00179
88. Poulin H, Bruhova I, Timour Q, Theriault O, Beaulieu JM, Frassati D, et al. Fluoxetine Blocks Nav1.5 Channels Via a Mechanism Similar to That of Class 1 Antiarrhythmics. *Mol Pharmacol* (2014) 86(4):378–89. doi: 10.1124/mol.114.093104
89. Bétry C, Etiévant A, Oosterhof C, Ebert B, Sanchez C, Haddjeri N. Role of 5-HT3 Receptors in the Antidepressant Response. *Pharmaceuticals* (2011) 4(4):603–29. doi: 10.3390/ph4040603
90. Dong YY, Pike ACW, Mackenzie A, McClenaghan C, Aryal P, Dong L, et al. K2P Channel Gating Mechanisms Revealed by Structures of TREK-2 and a Complex With Prozac. *Science* (2015) 347(6227):1256–9. doi: 10.1126/science.1261512
91. Schump MD, Fox DM, Bertozzi CR, Riley LW. Subcellular Partitioning and Intramacrophage Selectivity of Antimicrobial Compounds Against *Mycobacterium Tuberculosis*. *Antimicrob Agents Chemother* (2017) 61(3):e01639–16. doi: 10.1128/AAC.01639-16
92. Jaszczyszyn A, Gąsiorowski K, Świątek P, Malinka W, Cieślik-Boczula K, Petrus J, et al. Chemical Structure of Phenothiazines and Their Biological

Activity. *Pharmacol Rep* (2012) 64(1):16–23. doi: 10.1016/S1734-1140(12)70726-0

93. Wu CH, Bai LY, Tsai MH, Chu PC, Chiu CF, Chen MY, et al. Pharmacological Exploitation of the Phenothiazine Antipsychotics to Develop Novel Antitumor Agents-A Drug Repurposing Strategy. *Sci Rep* (2016) 6:27540. doi: 10.1038/srep27540
94. Viveiros M, Amaral L. Enhancement of Antibiotic Activity Against Poly-Drug Resistant *Mycobacterium tuberculosis* by Phenothiazines. *Int J Antimicrob Agents* (2001) 17(3):225–8. doi: 10.1016/S0924-8579(00)00343-5
95. Dutta NK, Pinn ML, Karakousis PC. Sterilizing Activity of Thioridazine in Combination With the First-Line Regimen Against Acute Murine Tuberculosis. *Antimicrob Agents Chemother* (2014) 58(9):5567–9. doi: 10.1128/AAC.03408-14
96. Jiang C, Yang W, Wang C, Qin W, Ming J, Zhang M, et al. Methylene Blue-Mediated Photodynamic Therapy Induces Macrophage Apoptosis Via ROS and Reduces Bone Resorption in Periodontitis. *Oxid Med Cell Longev* (2019) 2019:1529520. doi: 10.1155/2019/1529520
97. Amaral L, Kristiansen JE, Abebe LS, Millett W. Inhibition of the Respiration of Multi-Drug Resistant Clinical Isolates of *Mycobacterium Tuberculosis* by Thioridazine: Potential Use for Initial Therapy of Freshly Diagnosed Tuberculosis. *J Antimicrob Chemother* (1996) 38(6):1049–53. doi: 10.1093/jac/38.6.1049
98. Amaral L, Viveiros M. Thioridazine: A Non-Antibiotic Drug Highly Effective, in Combination With First Line Anti-Tuberculosis Drugs, Against Any Form of Antibiotic Resistance of *Mycobacterium tuberculosis* Due to its Multi-Mechanisms of Action. *Antibiotics* (2017) 6(1):3. doi: 10.3390/antibiotics6010003
99. van Soolingen D, Hernandez-Pando R, Orozco H, Aguilar D, Magis-Escurra C, Amaral L, et al. The Antipsychotic Thioridazine Shows Promising Therapeutic Activity in a Mouse Model of Multidrug-Resistant Tuberculosis. *PLoS One* (2010) 5(9):e12640. doi: 10.1371/journal.pone.0012640

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Evaluation of Berberine as an Adjunct to TB Treatment

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Tuberculosis (TB) is the global health problem with the second highest number of deaths from a communicable disease after COVID-19. Although TB is curable, poor health infrastructure, long and grueling TB treatments have led to the spread of TB pandemic with alarmingly increasing multidrug-resistant (MDR)-TB prevalence. Alternative host modulating therapies can be employed to improve TB drug efficacies or dampen the exaggerated inflammatory responses to improve lung function. Here, we investigated the adjunct therapy of natural immune-modulatory compound berberine in C57BL/6 mouse model of pulmonary TB. Berberine treatment did not affect *Mtb* growth in axenic cultures; however, it showed increased bacterial killing in primary murine bone marrow-derived macrophages and human monocyte-derived macrophages. *Ad libitum* berberine administration was beneficial to the host in combination with rifampicin and isoniazid. Berberine adjunctive treatment resulted in decreased lung pathology with no additive or synergistic effects on bacterial burdens in mice. Lung immune cell flow cytometry analysis showed that adjunctive berberine treatment decreased neutrophil, $CD11b^{+}$ dendritic cell and recruited interstitial macrophage numbers. Late onset of adjunctive berberine treatment resulted in a similar phenotype with consistently reduced numbers of neutrophils both in lungs and the spleen. Together, our results suggest that berberine can be supplemented as an immunomodulatory agent depending on the disease stage and inflammatory status of the host.

Keywords: tuberculosis, host-directed therapy, berberine, anti-inflammatory, C57BL/6 and C3Heb/FeJ Kramnik mice

INTRODUCTION

Tuberculosis (TB) is currently the second deadliest infectious disease worldwide caused by a single pathogen with an estimated 10 million cases and 1.4 million deaths reported in 2019 (1). Although it has been previously stated that between one-third to one-fourth of the world population has latent tuberculosis infection (LTBI) according to the epidemiological modeling studies, this notion has been challenged recently (2, 3). There is still an alarmingly large reservoir of active TB cases due to a 5-10% risk of LTBI progression (4). In addition to ongoing transmission in communities, the treatment for TB is long and complex with considerable side effects. Poor compliance, inadequate health infrastructure for drug monitoring has resulted in the increasing prevalence of multidrug (MDR) and extensively drug-resistant (XDR) TB. A recent study forecasted that by 2040, 32.5% of incident TB cases in Russia and 12.4% in India will be MDR-TB even though acquired drug resistance rates have been dropping (5). TB also impinges on adults during their economically productive life period furthermore, it customarily affects impoverished and socially disadvantaged communities to a greater extent. It is estimated that TB will cost the global economy $983 billion between 2015-2030 (6). Therefore, the development of novel treatment options with a lower toxicity profile that can synergize with the existing first-line and second-line antibiotics to decrease treatment duration is the focus of attention. There has been substantial progress in the anti-mycobacterial field with the recent approvals of bedaquiline, delamanid and pretomanid. Besides antimycobacterials; adjunct host-directed therapies (HDT) are also in the spotlight as alternative approaches to exploit host-pathogen interplay. Improving bacterial killing mechanisms, reinforcing immune and memory responses, disrupting TB granuloma structure and balancing inflammatory responses can be targeted for HDT (7, 8). One of the hallmarks of pulmonary TB is inadequately regulated inflammatory responses which exacerbate tissue damage, necrosis and eventual lung cavitation (9). An immunomodulatory HDT that fine-balances the host inflammatory pathway can dampen excess host inflammatory response and long term lung damage.

Berberine is the bioactive ingredient extracted from roots, barks, rhizomes of medicinal plant families Berberidaceae (barberry), Ranunculaceae (goldenseal), Rutaceae (cork tree) and Annonaceae (African whitewood). Berberine is an isoquinoline alkaloid with antimicrobial, antidiabetic, anti-tumor and anti-inflammatory properties (10–14). As a Chinese and Native American traditional medicine, it has been used for the treatment of gastroenteritis and dysentery (15). The diverse pleiotropic actions of berberine are mainly ascribed to its immunomodulatory properties through inhibition of nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK) signaling pathways and inhibition of pro-inflammatory cytokine production (16). Berberine can also affect cell proliferation, cell death and inhibit prolonged activation of immune cells. In the experimental autoimmune neuritis model, berberine treatment ameliorated the development of the autoimmune disease by inhibiting $CD4^+$ T cell proliferation (17). In the collagen-induced arthritis model, berberine treatment induced apoptosis of IL-12 producing mature dendritic cells in spleen and lymph nodes of mice that can result in subsequent restriction of chronic activation of T cells (18). In dextran sulfate sodium (DSS)-induced colitis model, berberine induced apoptosis of colonic macrophages and decreased pro-inflammatory cytokine production from colonic macrophages and colon epithelial cells (19). In trinitrobenzene sulfonic acid (TNBS)-induced colitis model, berberine treatment shifted macrophages into tissue repair and remodelling associated M2 phenotype rather than tissue destructive, pro-inflammatory M1 phenotype (20).

The immunomodulatory mechanisms of berberine in diverse inflammatory disease models prompted us to investigate the potential of berberine as an adjunct therapy in C57BL/6 murine model of tuberculosis. In the present study, we sought out synergistic effects of berberine with first-line antimycobacterials rifampicin and isoniazid in well-established murine models. Our results suggest that berberine treatment decreases tissue pathology without any additive or synergistic effects on the bacterial burden. The decrease in tissue pathology correlates with a decreasing number of inflammatory neutrophils, recruited macrophages and $CD11b^+$ dendritic cells in the C57BL/6 model when the treatment started at earlier stages of infection. However; the effect on decreased inflammatory cells was not observed once berberine treatment started at later stages of C57BL/6 *Mtb* infection. Our results suggest that berberine adjunctive treatment can exert its beneficial effects depending on the inflammatory stage of the host during tuberculosis.

MATERIALS AND METHODS

Mice

8-10 weeks old C57BL/6 mice were kept under specific-pathogen-free conditions in a biosafety level 3 containment facility individually ventilated cages (5 mice per cage) with filter tops (type 2 long), as well as dried wood shavings and shredded filter paper as floor coverings. The temperature range was set at 22–24°C and 12h dark–12h light cycles. All experiments were performed in accordance with the South African National Guidelines and the University of Cape Town of practice for laboratory animal procedures. The protocol was approved by the Animal Ethics Committee (AEC Permit Number: 015/040), Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa. Similarly, C3HeB/FeJ (Kramnik) mice were kept under specific-pathogen-free conditions in individually ventilated cages at the animal resource facility at Albany Medical College, Albany, New York. All experiments were performed in accordance with the IACUC guidelines at the Albany Medical College, New York.

Mtb Growth Assay

Two-fold diluted concentrations of berberine (Sigma Aldrich, B3251) were screened for their anti-mycobacterial activity in 96-well,

black, clear-bottom microplates (Greiner Bio-One, Germany), as previously reported (21). Single-cell suspension of constitutively GFP expressing H37Rv Mtb strain from frozen glycerol (15%) stock with a working concentration of $1x10^6$ colony-forming unit (CFU)/mL was prepared in Middlebrook 7H9 (Difco™, BD Biosciences) supplemented with 25 μg/mL kanamycin (Sigma Aldrich), 10% Middlebrook Oleic Acid-Albumin-Dextrose-Catalase (OADC) (v/v), 0.05% Tween 80 (w/v) and 0.2% glycerol (v/v). 100 μl of H37Rv-GFP was added to each experimental well followed by 100 μl 2X concentrated of berberine prepared in 7H9 broth as described above to test the 3.9 μM to 250 μM concentration range. Fluorescence (485ex/520em nm) was measured at days 0, 4, 6, 8, 10 and 12, with a BMG Labtech Omega Plate Reader (Germany).

Bone Marrow-Derived Macrophage (BMDM) and Monocyte-Derived Macrophage (MDM) Generation and Intracellular CFU Assay

Bone marrow-derived macrophages were generated from 8-12 weeks old C57BL/6 mice. Generation of BMDM was performed as described previously (22). BMDM were seeded in tissue culture-treated 96-well flat-bottom plates (Costar®, Corning) at a concentration of $1x10^6$ cells/ml. The cells were treated with 30μM berberine overnight, followed by H37Rv infection. In other sets of experiments, BMDM were rested overnight and infected with H37Rv. After 4 hours of infection, media was supplemented with berberine or vehicle (DMSO final concentration 0.1%).

Monocyte-derived macrophages were generated from Leukopak obtained from Western Province Blood Service. Briefly, Leukopak was diluted 1:1 with phosphate buffered saline (PBS) containing 2% fetal bovine serum (Gibco, ThermoFisher) and centrifuged at 500g for 25 minutes with brakes off in Leucosep (Greiner Bio-one) tubes with Histopaque 1077 (Sigma Aldrich). The buffy coat is removed by Pasteur pipette and washed twice with PBS+2%FBS at 120g to remove platelets. Peripheral blood mononuclear cells were counted and subjected to $CD14^+$ positive selection (Miltenyi) according to the manufacturer's instructions. Isolated monocytes were seeded in 60 mm Nunc cell culture dishes (ThermoFisher) at a concentration of $1x10^6$ cells/ml in RPMI 1640 media (Gibco, ThermoFisher) supplemented with 10% human AB serum (Sigma-Aldrich), 50 U/ml penicillin G (ThermoFisher), 50 μg/ml streptomycin (ThermoFisher) and 50 ng/ml recombinant human M-CSF (Peprotech) for 7 days. MDM were harvested by 20 minutes of incubation in Accutase® (Sigma-Aldrich) solution. MDM were seeded in tissue culture-treated 96-well flat-bottom plates (Costar®, Corning) at a concentration of $1x10^6$ cells/ml without the antibiotics. The cells were then treated with 30μM berberine overnight, followed by H37Rv infection.

BMDM and MDM were infected with a multiplicity of infection (MOI) 1. At 4 hours, 2 days and 5 days post-infection, the cells were washed once with sterile PBS and lysed in 0.1% Triton X-100. The cell lysates were diluted 10- and 100-fold and plated in Middlebrook 7H10 (Difco™, BD Biosciences). 7H10 plates incubated at 37°C for 14 days and colonies are counted under Nikon SMZ800N stereomicroscope.

BMDM Activation and Reactive Oxygen Species (ROS) Assay

$1x10^6$ BMDM were infected with H37Rv for 2 days in 12 well plates. The media was removed, and cells were washed once with PBS and incubated for 10 min in 0.5 mg/ml lidocaine and 10 mM EDTA in PBS at 37°C. Cells were lifted by pipetting and washed with PBS. The flow cytometry staining protocol mentioned below was followed. 575V Viability Dye, CD11b-PerCPCy5.5 (Clone M1/70), F4/80-PeCy7 (Clone BM8), MHCII-AlexaFluor700 (Clone M5/114.15.2), CD80-BV421 (Clone 16-10A1) were used for staining. BMDM were seeded on 96 well black/clear bottom plates (ThermoFisher) and infected with H37Rv for two days. Infected BMDM were incubated with 5 μM CellROX Green Reagent (ThermoFisher) according to manufacturer's instruction. Uninfected BMDM were used as blank wells. The fluorescence was measured (485 nm excitation/525 nm emission) on Spectramax iD3 multi-mode reader (Molecular Devices).

Western Blot Analysis

$3x10^6$ BMDM were seeded in 6-well plates and pretreated with berberine (30 μM) or vehicle (DMSO 0.1%) overnight. BMDM were infected H37Rv for 30, 60 and 120 minutes and washed with cold PBS before lysing with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) including cOmplete Protease inhibitor and PhosSTOP phosphatase inhibitor cocktail (Roche). Cell lysate protein content was determined using the BCA Protein Assay Kit (ThermoFisher). 30 μg of protein was loaded to 10% resolving acrylamide gel and wet WB was performed onto a nitrocellulose membrane. The membrane was probed with either SAPK/JNK Antibody, Phospho-SAPK/JNK (Thr183/Tyr185) (G9), NFκB p65 (D14E12), Phospho-NFκB p65 (Ser536) (93H1) (Cell Signaling Technology) or GAPDH (Santa Cruz Biotechnology) primary antibodies and either with goat anti-rabbit IgG H&L (HRP) pre-absorbed or goat anti-mouse IgG H&L (HRP) pre-absorbed (Abcam) secondary antibodies. Immunoblots were developed using the KPL LumiGLO ® Reserve Chemiluminescent Substrate Kit (SeraCare) on the iBright FL1000 Imaging System (Thermo Fisher).

Aerosol Infection and Treatment of Mice

Mycobacterium tuberculosis H37Rv was grown in Middlebrook 7H9 broth as described previously (23). For infection of the C3HeB/FeJ mice, *Mtb* Erdman strain was used. Prior to infection, stock solutions of *Mtb* were thawed, washed once with phosphate-buffered saline and inoculum was prepared in sterile saline containing 0.05% Tween 80. Aerosol infection was performed using an inhalation exposure system (model A4224, Glas-Col). To infect mice with a low dose of 100 CFU/lung, animals were exposed for 40 min to an aerosol generated by

nebulizing approximately 6 ml of a suspension containing $2.4x10^7$ live bacteria. The infection dose was checked at one day post-infection by determining the bacterial load in the lungs of four infected mice. One-week post-infection, four groups of mice were left untreated, treated with berberine (1 mg/ml), isoniazid/rifampicin (both 0.1 mg/ml), isoniazid/rifampicin/berberine (both 0.1mg/ml and 1 mg/ml, respectively) in drinking water. Berberine concentration was determined according to previously published reports (24). Drinking water was supplemented with 1% glucose in all four groups due to decreased water intake in the isoniazid/rifampicin/berberine triple-drug group. Glucose supplemented drug treatment groups showed similar water intake. Drinking water was changed twice a week and volume was measured to estimate the average drug intake. On average, berberine-treated groups received 5.5 mg berberine per day and isoniazid/rifampicin groups received 0.6 mg rifampicin/isoniazid (RIF/INH). At the experimental endpoint, mice were euthanized with halothane and cardiac puncture was performed for confirmation of death by exsanguination. The blood was left at room temperature for 30 min to clot and centrifuged at 1500g for 10 min at 4°C. The serum was collected to measure liver ALT and AST enzyme levels at National Health Laboratory Services (NHLS) diagnostic laboratory.

Determination of Mycobacterial Loads and Lung Histopathology

Mycobacterial loads in the lungs and spleen of *Mtb*-infected mice were determined as previously described (25). The right superior lobes were fixed with 10% neutral-buffered formalin, and tissue was processed with the Leica TP 1020 benchtop processor for 24 h and embedded in paraffin wax. Four 3 μm thick sections with 30 μm distance apart were cut in Leica Sliding Microtome 2000R, deparaffinized and subsequently stained with the hematoxylin & eosin (H&E) stain. The lung images were acquired in Nikon 90i Eclipse widefield microscope and free alveolar space was quantified using NIS elements (Nikon Corporation, Japan). Briefly, the images were converted to binary, and H&E positive area was measured. Fill holes function of NIS elements was employed to measure the complete lung area including the alveolar spaces. Free alveolar spaces were calculated by subtracting H&E positive area from the complete lung area and presented as a percentage to the complete lung area.

Lung and Spleen Immune Cell Populations

Single-cell suspensions of the left lobes of the lung were prepared as previously described (26). For late-onset berberine treatment experiments, half of the spleen was mechanically digested sequentially through 100 μm and 70 μm cell strainers (SPL Life Sciences). The cells were washed once with media (DMEM+10% FCS) and red blood cells were lysed by ACK lysis buffer (150 mM NaCl, 10 mM $KHCO_3$ and 0.1mM Na_2EDTA) for 5 min incubation at room temperature. The cells were washed once with media again and counted with CytoSMART (Corning) automated cell counter. Briefly, $1x10^6$ cells were washed once with PBS and stained with dead cell marker (575V Viability Dye, BD Biosciences) for 15 min at room temperature. The staining was later quenched and washed with 0.5%BSA in PBS and cells were then subjected to staining for B cells ($CD3^-CD19^+$), CD4 T cells ($CD19^-CD3^+CD4^+$), CD8 T cells ($CD19^-CD3^+CD8^+$), alveolar macrophages ($SiglecF^+CD11c^+CD64^+$), neutrophils ($CD11b^+Ly6G^+$), $CD11b^+$ dendritic cells ($CD64^-CD11b^+CD11c^+MHCII^+$) and inflammatory macrophages ($CD64^+CD11c^-CD11b^+SiglecF^-$) and monocyte-derived DCs ($CD64^+CD11c^+CD11b^+$) in the presence of 10% heat-inactivated rat serum and 10% FcγR blocker for 30min on ice. Similarly, neutrophils ($CD11b^+Ly6C^+Ly6G^+$), inflammatory monocytes ($CD11b^+Ly6C^+Ly6G^-$) and T cells ($CD11b^-CD3e^+Ly6C^-Ly6G^-$) were identified in the lungs of C3HeB/FeJ mice. Spleen myeloid populations were identified as neutrophils ($CD11b^+Ly6G^+$), monocytes ($CD11b^+Gr\text{-}1^+CD11c^-Ly6G^-$), $CD169^+$ macrophages ($CD11b^+CD169^+CD11c^-$), red pulp macrophages ($F4/80^+CD11b^{low}CD169^-CD11c^-$), CD11b DC ($CD11c^+MHCII^+CD11b^+CD8^-$) and CD8 DC($CD11c^+MHCII^+CD8^+CD11b^-$). Antibodies used for flow cytometry analysis were as follows: CD64-PeCy7 (Clone X54-5/7.1), Ly6C-PerCPCy5.5 (Clone AL-21), CD11b-V450 (Clone M1/70), MHCII-APC (Clone M5/114.15.2), CD11c-A700 (Clone HL3), SiglecF-APCCy7 (Clone E5-2440), Ly6G-FITC (Clone 1A8), CD4-BV510 (Clone RM4-5), CD3-A700 (Clone 500A2), CD19-PerCPCy5.5 (Clone 1D3) and CD8-APC (Clone 53-6.7), Gr-1 Biotin (Clone RB6-8C5), CD169 APC-eFluor780 (Clone Ser-4) and Streptavidin-PECF594 purchased from BD Biosciences and eBioscience. Cells were washed then fixed in 2% paraformaldehyde overnight and acquired by BD LSRII (BD Pharmingen) and analysed by FlowJo V9 (TreeStar, US). Marker positive stained cells are calculated as a percentage of live cells and later these percentages are back-calculated to cell counts obtained from Trypan Blue exclusion method and finally back-calculated by multiplying the ratio of total lung weight to the left lobe weight.

Analysis of Cytokines in Tissue Homogenates and Culture Supernatants

The cell-free lung and spleen homogenate was spun at 3000g for 5 min and stored at -80°C until ELISA analysis, samples were thawed and double filtration (0.2 μM) was performed before transporting the supernatant from biosafety level 3 facility. Lung homogenates were analysed for the IFNγ, IL-12p40, GM-CSF (BD Biosciences), IFN-β, IL-10 (BioLegend), IL-1β, CXCL10, and CCL3 (R&D Systems) by ELISA according to manufacturers' instructions. Similarly, supernatants from *Mtb*-infected macrophages were collected and stored at -80°C until further analysis. After thawing, the supernatants were filtered through Corning FiltrEX 96-well low protein binding filter plates and the supernatant was transported out of BSL3. Nitrite concentrations were measured by Griess assay. IL-1α (R&D Systems), IL-6 (BD Biosciences) and TNF (Biolegend) ELISA were performed according to the manufacturers' instructions.

Gene Expression in Total Lung Tissue

Lung middle lobes were washed with cold PBS and immersed in RNAprotect Tissue reagent (Qiagen) overnight at 4°C and the

next day transferred to -80oC for long-term storage. On the day of RNA purification, the sample was thawed, transferred in RLT buffer and homogenized by sonification. RNA extraction was performed by RNeasy Mini Kit (Qiagen), total RNA was transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit (ThermoFisher) according to the manufacturer's instructions. Real-time qPCR was performed with SYBR Green PCR Master Mix (ThermoFisher) in QuantStudio 7 Pro Real-Time PCR System (ThermoFisher). Quantitative expression analyses of Ifngr1and Csf2ra were normalized against the housekeeping gene Hprt. qPCR primers as follows; Hprt forward 5'- GTTGGATATGCCCTTGAC-3', reverse 5'- AGGACTAGAACACCTGCT-3'; Ifngr1 forward 5'- TACAGGTAAAGGTGTATTCGGGT-3', reverse 5'- ACCGT GCATAGTCAGA TTCTTTT-3'; Csf2ra forward 5'- CCTGCT CTTCTCCACGCTAC-3', reverse 5'- CAACCGAAGGG CGAGACT-3'.

Statistics

Data are represented as mean values ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey *post-hoc* test if the data points are normally distributed. The normality is checked through the Shapiro-Wilk normality test and the Anderson Darling test. If the data did not fit into Gaussian distribution Kruskal-Wallis test was performed. Statistical differences in all groups are shown as significant *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. All the data were plotted using the GraphPad Prism 8 software.

RESULTS

Berberine Decreased Growth of *Mycobacterium tuberculosis* in Primary Macrophages and Modulated Responses to the Infection

We investigated whether berberine has an impact on the survival of *Mycobacterium tuberculosis* (*Mtb*) in liquid broth and macrophages. First, we assessed whether berberine has a direct anti-mycobacterial effect in a liquid culture medium supplemented with a range of concentrations for 12 days. We found that berberine had no mycobactericidal effect on the axenic growth of *Mtb* in a concentration range from 3.9-250 μM (**Figure 1A**). We then determine the cytotoxic effect of berberine on primary murine macrophages by Cell Titer Blue assay. BMDM were pre-treated with 2-fold concentrations of berberine from 0 to 2000 μM for 6 days. Macrophages treated with berberine at 62.5 μM and above displayed a significant cytotoxic effect. The highest concentration which had no

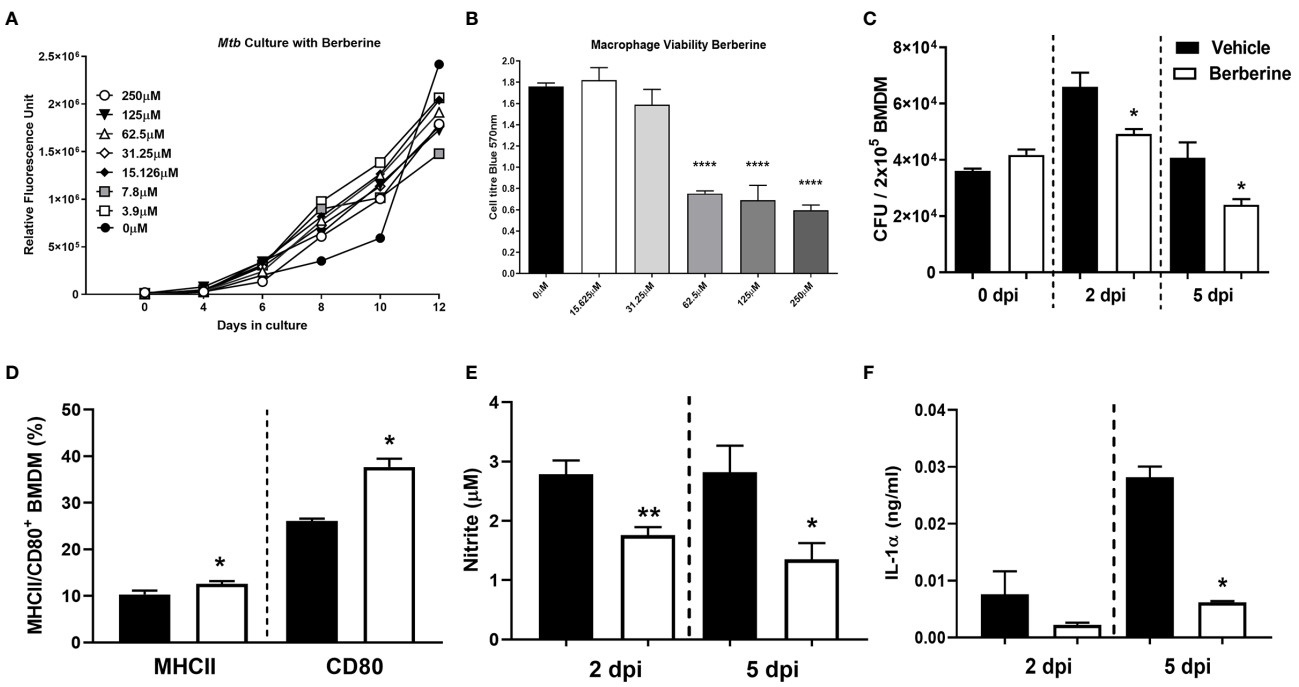

FIGURE 1 | Cytotoxic effect of berberine on *Mycobacterium tuberculosis* (*Mtb*) and macrophages. **(A)** *Mtb* was cultured in 7H9 culture broth at the indicated concentrations of berberine to determine the mycobactericidal activity. **(B)** C57BL/6 murine BMDMs were treated with indicated concentrations of berberine for 3 days to measure cellular viability using Cell Titer Blue assay. **(C)** Murine macrophages were pre-treated with berberine (30 μM) overnight. Cells were then infected with *Mtb* (MOI=1) to determine the growth in a time-dependent manner. **(D)** BMDM were infected with *Mtb* for two days and stained for MHCII and CD80 antibodies to measure macrophage activation in the presence of berberine. **(E, F)** Cell culture supernatants were analysed for nitrite by Griess Reagent Assay and IL-1α by ELISA. Data represented as mean ± SEM of triplicates and representative of two experiments, analysed by one-way ANOVA with Tukey *post-hoc* test **(A, B)** or two-tailed unpaired Student's t-test **(C, D)** or Mann-Whitney test **(E, F)** defining differences in all groups as significant *$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq 0.0001$.

cytotoxic effect was 31.25 μM and we used 30 μM used for subsequent intracellular assays (**Figure 1B**). Mouse BMDM were pre-treated with 30 μM overnight to determine the intracellular growth of *Mtb* in a time-kinetic manner. We found that berberine treatment had no effect on the uptake of Mtb (day 0) and showed a significant decrease in *Mtb* growth at 2 and 5 days post-infection (**Figure 1C**). The increased mycobacterial killing ability of macrophages was similar in macrophages treated with berberine after phagocytosis of *Mtb* (**Supplementary Figure 1A**). To further probe *Mtb* infected macrophage modulation upon berberine treatment, interestingly resulted in a higher percentage of MHCII and CD80 (**Figure 1D**) and higher expression of CD80 (**Supplementary Figure 1B**). Furthermore, berberine treatment resulted in decreased levels of nitric oxide (NO) (**Figure 1E**), pro-inflammatory cytokines IL-1α (**Figure 1F**), IL-6 (**Figure 2A**); while it did not effect TNFα (**Figure 2B**) levels *in vitro*. Berberine can exert its antibacterial effects through the increase of reactive oxygen species (ROS), although there are studies also reporting antioxidant activities of berberine (27–29). We have measured intracellular ROS levels of berberine-treated murine macrophages which showed no change in ROS accumulation (**Figure 2C**). In line with the previous reports, we observed decreased phosphorylation of p65 subunit of NF-κB and c-Jun N terminal kinase (JNK) (**Figure 2D**). Furthermore, we assessed the influence of berberine on the intracellular growth of *Mtb* in human monocyte-derived macrophages (MDM). Considering the sex differences in monocyte or MDM response (30), we measured the bacterial killing effect of berberine supplementation in female and male donor-derived MDM. Similar to mouse macrophages, both female and male MDM significantly decreased intracellular *Mtb* growth upon berberine treatment (**Figure 2E**). These results show that berberine treatment decreased *Mtb* growth in mouse and human macrophages possibly by modulating the antimicrobial capacity of the host; while paradoxically downregulating pro-inflammatory responses.

Berberine as an Adjunctive Therapy Decreased the Lung Pathology During *Mtb* Infection in Mice

To investigate the biological relevance of our findings in macrophages, we determined whether berberine as an adjunctive therapy will have a host-protective effect *in vivo*. Oral administration of berberine is deemed safe since no mortality was observed in mice administered with as high as 20.8 g/kg of body weight due to extremely poor absorption in the gut, rapid and extensive metabolism (31, 32). Owing to low oral

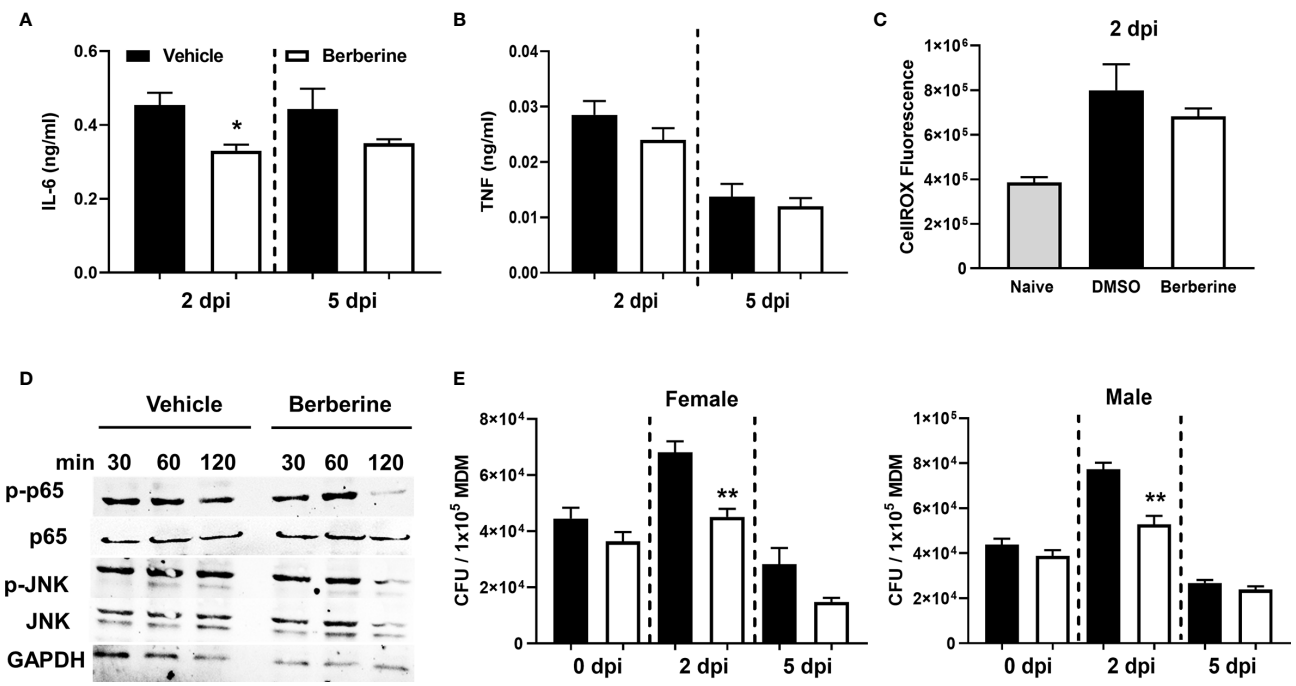

FIGURE 2 | Anti-inflammatory effects of berberine in *Mtb* infected BMDM. Berberine or vehicle-treated BMDM isolated from C57BL/6 mice were infected with *Mtb*. **(A)** IL-6, **(B)** TNF measured in the supernatants 2 days and 5 days post-infection and **(C)** accumulation of intracellular ROS was measured by CellROX Green reagent at 2 days post-infection. **(D)** Ser536 phosphorylated p65, total p65, Thr183/Tyr185 phosphorylated JNK, total JNK, and housekeeping control GAPDH levels were assayed by Western Blot in berberine or vehicle pre-treated BMDM at 30, 60 and 120 minutes post *Mtb* infection. **(E)** Human monocyte-derived macrophages from male and female donors (n = 3 each) were infected to determine mycobacterial growth at the indicated time points. Data represented as mean ± SEM of four replicates and representative of two experiments. Statistical significance was analysed by a two-tailed unpaired Student's t-test defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01.

bioavailability, short-term and chronic berberine administration have not resulted in concerning adverse effects in the clinic (33). To evaluate the potential of berberine in TB therapy, mice were infected with a low dose (100CFU, *Mtb* H37Rv) by aerosol inhalation. One week after infection, mice were treated with berberine and rifampicin/isoniazid alone or in combination for 4 weeks *ad libitum* (**Figure 3A**). Berberine treatment groups consumed slightly less water; however, had no differences in body weight change (**Supplementary Figure 2A**). Moreover, serum alanine transaminase (ALT) and aspartate transaminase (AST) were similar between the groups after 4 weeks of treatment, indicating that berberine had no liver toxicity though there was a reduced trend of AST and ALT levels in the Rif/Inh/Ber group (**Supplementary Figure 2B**). After 4 weeks of treatment, mice were euthanized to determine mycobacterial burdens and lung pathology. We found that berberine alone or as an adjunctive to rifampicin/isoniazid had no effect on the lung and spleen mycobacterial burdens (**Figure 3B**). However, lung sections revealed that berberine used as an adjunct to first-line anti-TB drugs, INH and RIF, significantly decreased the lung tissue pathology (**Figure 3C**). Furthermore, we quantified the free alveolar spaces in these sections indeed revealed that berberine as an adjunct significantly increased the non-inflamed alveolar spaces in the lungs when compared to mice treated with INH/RIF alone or untreated mice (**Figure 3D**). These results demonstrate that berberine as an adjunct to first-line drugs decreased lung pathology during tuberculosis.

Berberine as an Adjunctive Therapy Modulates Immune Cell Recruitment in the Lungs During *Mtb* Infection in Mice

We then assessed whether berberine as an adjunct treatment influences lung immune cell recruitment during *Mtb* infection. A single-cell suspension of lungs was prepared to analyse immune cell recruitment by flow cytometry. We found that mice treated with Rif/Inh or Rif/Inh/Ber showed decreased lung cell numbers (**Figure 4A**). The cell suspension was subjected to flow antibody staining using markers for phenotyping of lymphocytes and myeloid cell compartments. Berberine as an adjunct therapy decreased $CD4^+$ and $CD8^+$ T cells (**Figures 4B, C**) to the same level as Rif/Inh group. Similar to T cells, B cells (**Figure 4D**) were also decreased in mice treated with Rif/Inh/Ber to a similar extent of Rif/Inh group. Amongst myeloid cells, berberine as an adjunct decreased alveolar macrophages when compared to untreated, similar to rifampicin/isoniazid treated mice (**Figure 4E**). Neutrophils are a major contributor to lung

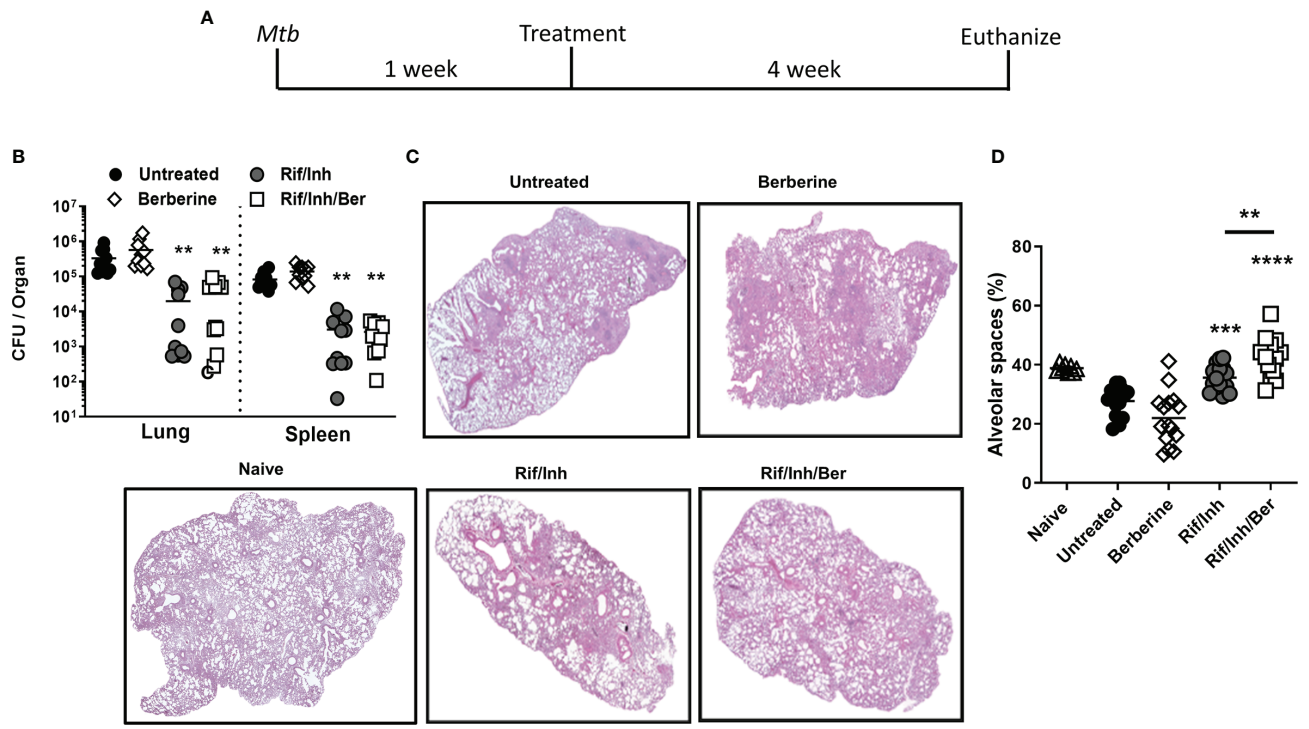

FIGURE 3 | Berberine as an adjunctive therapy against *Mtb* infection in C57BL/6 mice. **(A)** Animals were infected with *Mtb* (100CFU) *via* aerosol inhalation. After 1 week of infection, mice were either left untreated or treated with berberine (1 mg/ml) or rifampin/isoniazid (0.1 mg/ml) and rifampin/isoniazid in combination with berberine in drinking water for 4 weeks as shown in the layout. **(B)** Mycobacterial burdens in the lung and spleen. **(C)** Representative lung section for pathology. **(D)** Quantification of free alveolar air spaces in the lungs at 5 weeks post-infection. Data is shown from pooled two experiments (n=10 per group) and the line denotes mean value, analysed by one-way ANOVA with Tukey *post-hoc* test defining differences in all groups as significant $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$. Asterisks without the line show the significance compared to the untreated group.

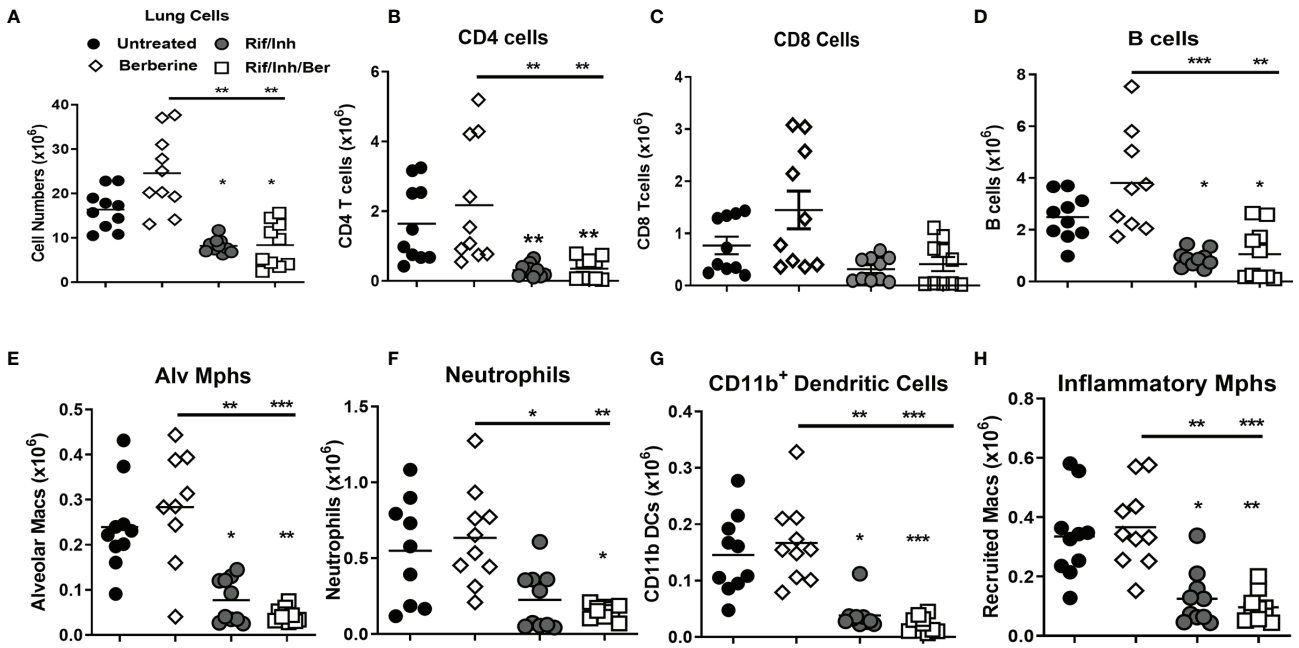

FIGURE 4 | Effect of berberine on lung immune cells populations as an adjunctive therapy during *Mtb* infection in C57BL/6 mice. Single-cell suspension of lungs was analyzed for immune cell populations by flow cytometry at 5 weeks post-infection; **(A)** Total lung cells, **(B)** CD4 T cells ($CD19^-CD3^+CD4^+$), **(C)** CD8 T cells ($CD19^-CD3^+CD8^+$), **(D)** B cells ($CD19^+CD3^-$), **(E)** Alveolar macrophages ($Siglec\text{-}F^+CD11c^+$), **(F)** Neutrophils ($Gr1^+Siglec\text{-}F^-Cd11c^-$), **(G)** Dendritic cells ($CD11c^+CD11b^+MHCII^+$) and **(H)** inflammatory macrophages ($CD11b^+CD11c^-MHCII^+$). Data is shown from pooled two experiments (n = 10 per group) and the line denotes mean value analysed by Kruskal-Wallis test with Dunn's multiple comparisons correction defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Asterisks without the line below show the significance compared to the untreated group.

pathology in tuberculosis (34, 35). Mice treated with rifampicin/isoniazid decreased neutrophils in the lungs (**Figure 4F**), most probably due to reduced lung burdens. Berberine combined with rifampicin/isoniazid further significantly decreased the neutrophils when compared to mice treated with berberine alone (**Figure 4F**). This may explain the observed reduced lung pathology in this group of mice. Furthermore, mice treated with rifampicin/isoniazid alone and antibiotics with berberine displayed reduced conventional $CD11b^+$ dendritic cells (**Figure 4G**) and inflammatory macrophages (**Figure 4H**) when compared to untreated or berberine alone animals. These results suggested that berberine as an adjunct to antibiotic therapy decreased lymphoid and myeloid cell recruitment in the lungs during tuberculosis.

Berberine as an Adjunctive Therapy Decreases Certain Inflammatory Cytokines in the Lungs During *Mtb* Infection in Mice

We next assessed the cytokine levels in lung homogenates by ELISA at 4 weeks post-treatment. The analysis revealed that mice treated with berberine as an adjunct significantly reduced CXCL-10 (**Figure 5A**), while there was a decreasing trend towards IL-1β (**Figure 5B**) and CCL3 levels (**Figure 5C**) when compared to antibiotics alone. CXCL10 is an inflammatory chemokine that activates cells to increase inflammatory lung damage and is highly expressed in patients with active TB (36). Rif/Inh treatment decreased IL-12p40 levels drastically; however adjunct berberine treatment did not promote the reduction of IL-12p40 levels (**Figure 5D**). Moreover, adjunct berberine treatment did not affect IFNγ, GM-CSF, IFN-β and IL-10 levels in the lung (**Figures 5E–H**). Interestingly, CCL3 and IFNγ levels showed a bimodal distribution similar to lung CFU (**Figure 3B**), indicating that lung bacterial burdens are the major driver in the production of these cytokines/chemokines. Together, these results suggest that berberine as an adjunct decreases certain chemokine/cytokine production in the lungs during *Mtb* infection.

Onset of Late Berberine Treatment Does Not Effect Its Adjuvant Potential While Exerting Stronger Responses in the Spleen

H37Rv *Mtb* strain grows exponentially during the first three-four weeks of infection in the lungs of C57BL/6 mice and bacterial burden stabilizes after this stage which coincides with the arrival of antigen-specific T cells in the lungs (37, 38). We, therefore, were prompted to adapt adjunct berberine treatment at peak bacterial burdens levels in the lung i.e. 3 weeks after infection (**Figure 6A**). Berberine alone treatment or adjunct berberine treatment did not affect lung and spleen bacterial burdens (**Figure 6B**); however, the adjunctive treatment resulted in decreased tissue involvement (**Figures 6C, D**) similar to berberine treatment started at 1 week after infection. However, adjunctive treatment resulted in increased lung and spleen total

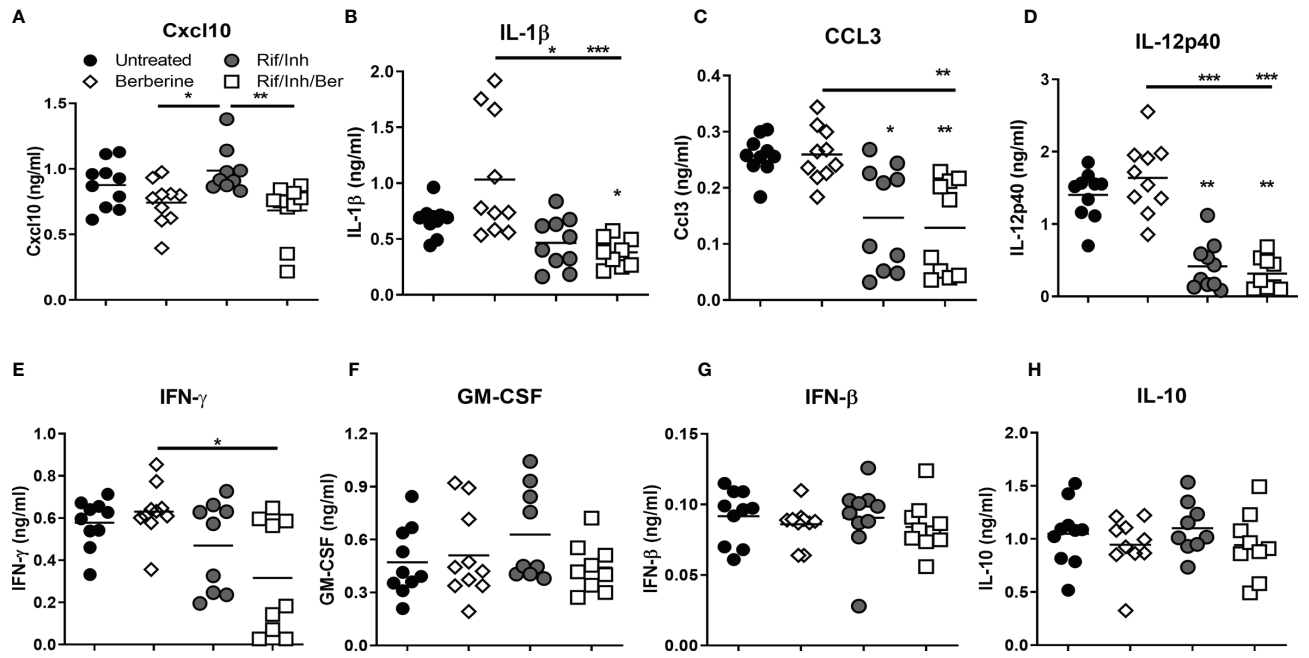

FIGURE 5 | Effect of berberine on lung cytokines as an adjunctive therapy during *Mtb* infection in C57BL/6 mice. Lung homogenates were analyzed for the cytokines and chemokine by ELISA at 5 weeks post-infection; **(A)** CXCL10, **(B)** IL-1β, **(C)** CCL3, **(D)** IL-12p40, **(E)** IFNγ, **(F)** GM-CSF, **(G)** IFN-β and **(H)** IL-10. Data is shown from pooled two experiments (n = 10 per group) and the line denotes mean value, analysed by Kruskal-Wallis test with Dunn's multiple comparisons correction defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Asterisks without the line below show the significance compared to the untreated group.

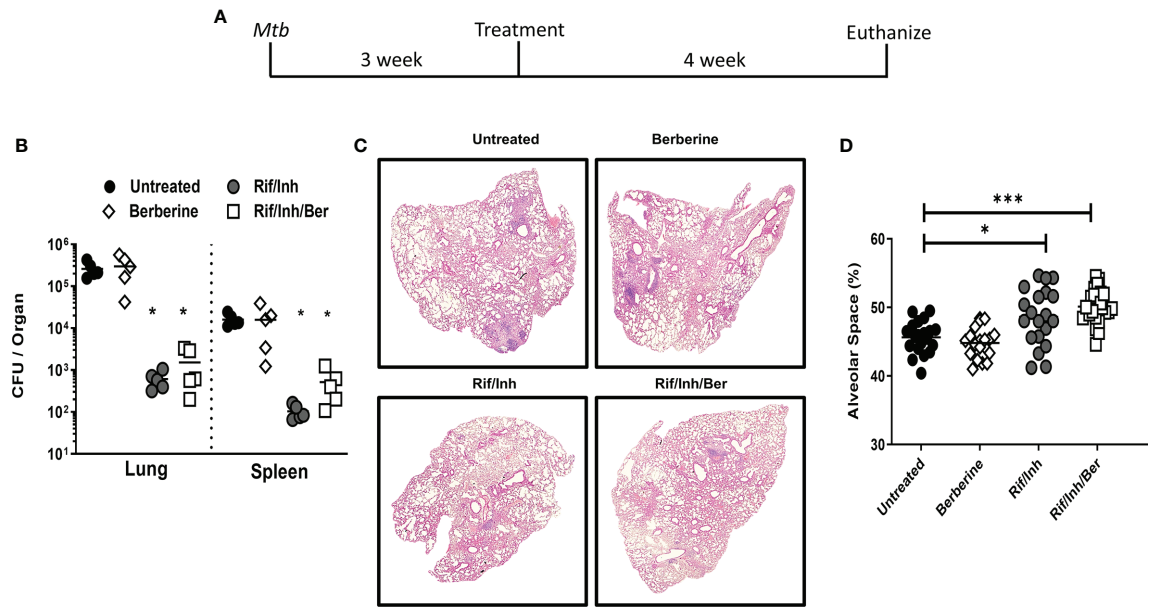

FIGURE 6 | Late-onset of berberine adjunctive therapy against *Mtb* infection in C57BL/6 mice. **(A)** Animals were infected with *Mtb* (100CFU) *via* aerosol inhalation. After 3 weeks of infection, mice were either left untreated or treated with berberine (1 mg/ml) or rifampin/isoniazid (0.1 mg/ml) and rifampin/isoniazid in combination with berberine in drinking water for 4 weeks as shown in the layout. **(B)** Mycobacterial burdens in the lung and spleen. **(C)** Representative lung section for pathology. **(D)** Quantification of free alveolar air spaces in the lungs at 4 weeks post-treatment. Data is shown representative of two experiments (n = 5 per group) and the line denotes mean value, analysed by Brown-Forsythe and Welch ANOVA test with Tamhane T2 *post-hoc* test defining differences in all groups as significant *P ≤ 0.05; ***P ≤ 0.001. Asterisks without the line below show the significance compared to the untreated group.

cell numbers (**Figure 7A**) which are driven mainly by increased CD4 T cells (**Figure 7B**) and B cell numbers (**Figure 7C**). There were also expanded CD4 T cell and CD8 T cell and B cell numbers in the spleens of the adjunctive treatment group and a similar trend was observed in the berberine alone treatment group (**Figures 7B, D**). Similar to earlier adjunct treatment, we observed decreased neutrophil numbers in the spleens and lungs of the adjunctive group (**Figure 7E**); however, conventional $CD11b^+$ dendritic cells (**Figure 7F**) remain unaffected. To recapitulate *in vitro* macrophage activation results, we investigated MHCII expression levels in lung and spleen myeloid subsets. In the lungs, lower percentages of recruited interstitial macrophages in the adjunct group were MHCII positive, but $CD169^+$ macrophages, red pulp macrophages and monocytes of berberine alone and adjunctive treatment groups consistently showed increased MHCII expression (**Supplementary Figure 2C**). It is shown that berberine influences caspase 3 activation and downstream apoptosis events in a cell-specific manner (39); we sought out *in vivo* activated caspase-3 levels in lung and spleen cells. The adjunctive treatment group showed decreased caspase-3 positive cells in the spleen, but this effect was not observed in the lungs (**Supplementary Figure 2D**). CXCR3 is the receptor for CXCL-10 and regulates the migration of antigen-specific Th1 cells into the lung (40). $CXCR3^+$ CD4 T cells are efficient in localizing lung parenchyma around the lymphocytic cuff of TB granulomas and rarely in the myeloid core (41). We found that lung CD4 T cells in the antibiotic-treated groups have increased frequencies of $CXCR3^+$ subsets and the adjunctive berberine treatment decreased the frequency of $CXCR3^+$ CD4 T cells in the spleen compared to Rif/Inh group (**Supplementary Figures 3A, E**). Late onset of berberine adjunctive treatment did not change lung alveolar macrophage, recruited interstitial macrophage, lung monocyte-derived DC, spleen $CD169^+$ macrophage, spleen red pulp macrophage or spleen $CD8\alpha^+$ DC cell numbers (**Supplementary Figures 3B–D, F–H**). In terms of lung and spleen cytokine/chemokine levels; lung IFNγ, lung and spleen CXCL10, lung GM-CSF, lung and spleen IFNβ levels remain unchanged (**Supplementary Figures 4A, D–F**). Antibiotic treatment groups showed reduced spleen IFNγ, spleen IL-1β and lung IL-12p40 levels (**Supplementary Figures 4A–C**). Interestingly, the berberine alone group increased lung and spleen IL-1β levels (**Supplementary Figure 4B**); a similar trend was also observed in the early treatment onset of berberine (**Figure 5B**). Aside from CXCL10 signaling, we also investigated whether IFNγ and GM-CSF signaling was affected by gene expression levels of IFNγ (Ifngr1) and GM-CSF receptors (Csf2ra) in total lung tissue by qPCR. Berberine alone suppressed Ifngr1 expression levels while Csf2ra expression did not change among different groups (**Supplementary Figure 4G**). Our data suggest that late-onset of berberine treatment still reduced neutrophil recruitment and beneficial against lung pathological responses, while spleen tissue was more affected by adjunctive berberine treatment.

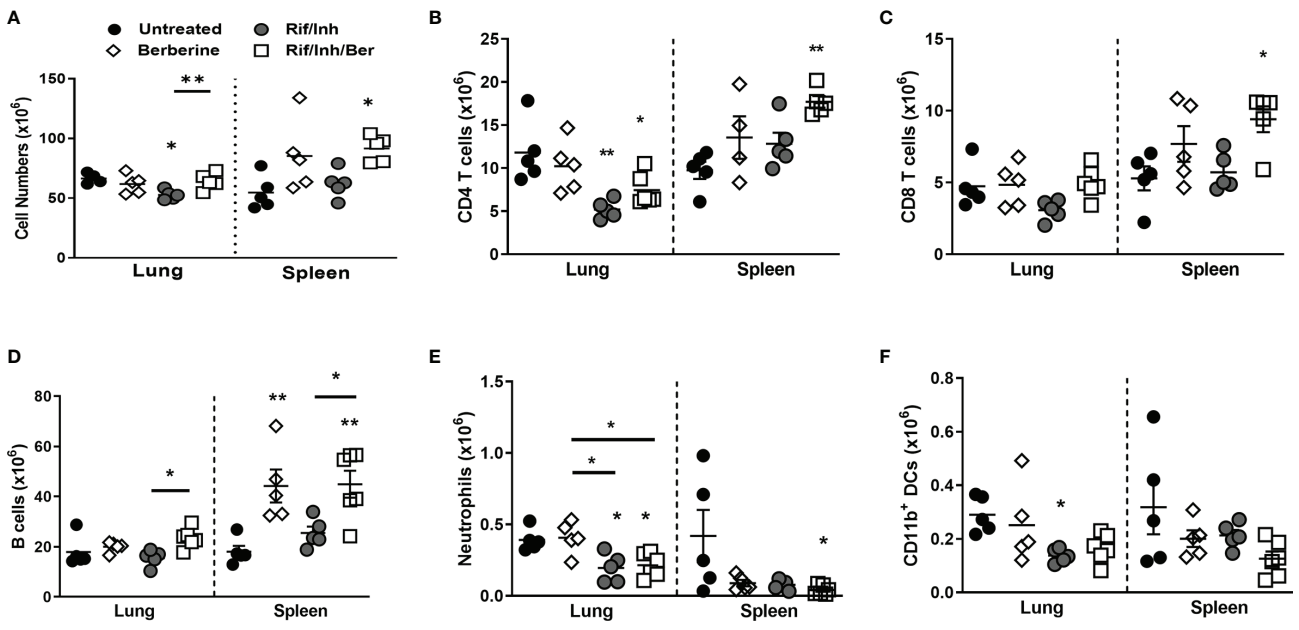

FIGURE 7 | Effect of late-onset berberine adjunctive therapy on lung and spleen immune cell populations during *Mtb* infection in C57BL/6 mice. Single-cell suspension of lungs and spleens were analyzed for immune cell populations by flow cytometry at 4 weeks post-treatment; **(A)** Total lung and spleen cells, **(B)** CD4 T cells ($CD19^-CD3^+CD4^+CD8^-$), **(C)** CD8 T cells ($CD19^-CD3^+CD8^+CD4^-$), **(D)** B cells ($CD19^+CD3^-$), **(E)** Neutrophils ($CD11b^+Ly6G^+$), **(F)** CD11b Dendritic cells counts ($CD11c^+CD11b^+MHCII^+$). Data is shown representative of two experiments (n = 5 per group) and the line denotes mean value, analysed by one-way ANOVA with Tukey *post-hoc* test **(A–D)** and Kruskal-Wallis test with Dunn's multiple comparisons correction **(E, F)** defining differences in all groups as significant *$P \leq 0.05$; **$P \leq 0.01$. Asterisks without the line below show the significance compared to the untreated group.

Berberine Decreases Bacterial Load and Pathology During *Mtb* Infection in C3HeB/FeJ Mice

We further investigated the host protective role of berberine in a more relevant model of tuberculosis. We used C3HeB/FeJ (Kramnik) mice, which recapitulates the progressive lung pathology of patients with TB. Mice were infected with *Mtb* Erdman *via* aerosol inhalation to deposit 100CFU in the lungs. After 3 weeks of infection, cohorts of mice were treated with either berberine or antibiotics or berberine combined with antibiotics (**Supplementary Figure 5A**). Four weeks following treatment, mice were euthanized to analyze the lung mycobacterial burden as CFU, the extent of inflammation as measured by immune cell recruitment and histopathological lung damage. The bacterial burden was similar between antibiotics and berberine as an adjunctive (**Supplementary Figure 5B**). Interestingly, berberine alone also significantly decreased lung mycobacterial burdens in Kramnik mice as opposed to C57BL/6 mice (**Supplementary Figure 5B**). Furthermore, H&E staining revealed that lung pathology is decreased in cohorts treated with berberine alone or in combination with antibiotics (**Supplementary Figure 5C**). Lung alveolar space quantification revealed that berberine alone did not effect lung pathology to a significant extent, however, berberine as adjunctive therapy increased the lung alveolar free space when compared to berberine alone or untreated group (**Supplementary Figure 5D**). We then assess the recruitment of immune cells in the lungs of these mice. In line with late onset in C57BL/6 mice, berberine as an adjunct significantly increased total cells harvest when compared to mice treated with antibiotics alone (**Supplementary Figure 5E**). Similarly, T cells (**Supplementary Figure 5F**), polymorphonuclear cells (**Supplementary Figure 5G**) and recruited monocytes (**Supplementary Figure 5H**) were significantly increased in mice treated with berberine as an adjunct when compared to animals treated with antibiotics alone. This may explain increased total lung cells harvested. Berberine, alone had no effect on neutrophil influx to the lung suggesting that this compound modulates inflammation without perturbing these innate cell recruitment. In contrast to C57BL/6 model, berberine treatment combined with antibiotics significantly increased neutrophil recruitment to the lung when compared to animals treated with antibiotics alone (**Supplementary Figure 5G**). Collectively, these results indicate that berberine as adjunctive therapy may offer enhanced protection against tuberculosis infection in an experimental model of progressive pulmonary TB.

DISCUSSION

Our findings demonstrate that berberine as an adjunct to rifampicin/isoniazid against tuberculosis decreased lung pathology in an experimental murine model of TB. The pleiotropic actions of berberine are mainly associated with its immunomodulatory properties through inhibition of NF-κB, MAPK and inhibition of pro-inflammatory cytokine production (16). We have also observed similar effects of berberine in *Mtb*-infected mouse macrophages *in vitro*. Intriguingly, berberine treatment pre- or post-*Mtb* infection increased the bactericidal activity of macrophages despite they released decreased levels of nitric oxide, IL-6, and IL-1α. Similar to murine macrophages, human macrophages also reduced mycobacterial growth following berberine treatment. The limitation is macrophages were not generated from CD16-negative selected monocytes which may result in mixed M1 and M2 macrophages, however, markers of classical and alternative activation are not very clear in human macrophages. Increased ROS levels contribute to the bacterial killing, we confirmed that decreased bacterial growth in macrophages was independent of ROS. In contrast, berberine had no direct inhibitory effect on *Mtb* but modulates host responses to mediate antimycobacterial effects. Our findings are in line with the report showed enhanced macrophage antibacterial activity even though berberine inhibits mRNA expression of iNOS, COX-2, IL-1β, TNF and IL-6 in LPS-stimulated inner medullary collecting duct-3 cells by reducing NF-kB activity (42). In atherosclerosis, berberine treatment inhibits inflammation in mouse macrophages (J774A.1) by inducing autophagy through AMPK/mTOR pathway (43) and uncoupling protein 2 in mice (44). Moreover, berberine inhibits the formation of foamy macrophages by enhancing LXRalpha-ABCA1-dependent cholesterol efflux in macrophages (45). Additionally, a berberine derivative is shown to induce lysosomal acidification through activation of transcription factor EB in methicillin-resistant *Staphylococcus aureus* and enteroinvasive *Escherichia coli* infected BMDM (46). Therefore, it is feasible that berberine treatment decreases the intracellular survival of *Mtb* in macrophages. Activation of macrophages by increased CD80 expression and increased frequencies of $MHCII^+$ BMDM or splenic macrophages *in vivo* can be context-specific to *Mtb* infection. It has been previously reported that berberine pre-treatment did not change cell surface expression of CD80 and MHCII in naïve macrophages (47) or decreased CD80 expression in lipopolysaccharide (LPS) stimulated bone marrow-derived dendritic cells (18). Increased macrophage activation and macrophage *Mtb* killing mechanisms, while decreased proinflammatory gene expression and NF-κB and JNK kinase activation shows that berberine has pleiotropic and context-specific functions in macrophages.

Augmenting macrophage activation and bacterial killing mechanisms while reducing inflammation could increase the immunomodulatory potential of berberine during TB disease. Moreover, berberine immunotherapy as an adjunct had a beneficial effect for the host by decreasing lung pathology in the treated C57BL/6 after 4 weeks of therapy. However, berberine alone or in combination did not affect lung mycobacterial burdens. Within 2 weeks *Mtb* disseminates from the lungs to other organs for example spleen and liver (48). Considering this berberine may target dissemination to other organs during treatment. Similar to lungs, berberine had no effect on mycobacterial burdens in the spleen. Interestingly, we found that berberine showed stronger effects on macrophage activation and B lymphocyte numbers in the spleen. Increased B cell

numbers in the spleen were also reported in the mouse leukemia model before (49). It will be interesting to study whether berberine affects B-cell development in the bone marrow or reduces B-cell turnover. Overall, this study revealed that berberine treatment reduced deleterious lung pathological consequences during TB.

Berberine also has anti-parasitic (50), -viral (51), -fungal (52) and -helminth activity (53). Previously, administration of berberine with isoniazid protects against liver injury caused by oxidative stress and inflammation in rats by suppressing NF-kB, iNOS, the pro-inflammatory cytokines and upregulating PPAR-γ (54). We found that berberine has an adjunctive effect on rifampicin/isoniazid-induced control of lung inflammation in *Mtb*-infected mice. The synergistic effects of berberine have also been reported with commonly used antibiotics against Methicillin-Resistant *Streptococcus aureus* (MRSA) (55) and fluconazole-resistant *Candida albicans* (56). Berberine has been proven for its antibacterial activity against a broad spectrum of microbial pathogens (57) and exhibited sub-MICs on conventional antimicrobial agents such as ampicillin, azithromycin, cefazolin, and levofloxacin (58). The primary antibacterial mechanism of berberine is due to inhibition of the cell division protein FtsZ (59). Berberine has a synergistic effect with some common antibiotics especially with linezolid, cefoxitin, and erythromycin (60). The direct microbicidal effect of berberine appears to inhibit biofilm formation (61) and synthesis of DNA (57). In our study, we could not detect ROS levels *in vivo* due to the short half-life of ROS; however, *in vitro* BMDM showed that berberine does not show antioxidant properties during *Mtb* infection. Berberine increases the antibacterial properties of macrophages and we observed a similar trend in the susceptible Kramnik mouse model when the bacterial burdens were high in the lung. We ruled out the possibility of hepatotoxic effects of berberine by probing serum ALT and AST levels. The stronger effects of berberine were seen in the lung pathology in C57BL/6 mice. The decreased lung pathology was associated with fewer neutrophil recruitment both in the lungs and spleen. Additionally, berberine showed immunomodulatory properties *in vivo* by increased macrophage activation and B cell numbers. Overall, the data suggest that the effects of adjunctive berberine treatment are pleiotropic during *Mtb* infection.

We then further validated our findings in a susceptible and highly inflammatory Kramnik (C3HeB/FeJ) mice, a highly relevant model, which recapitulates human lung lesions and a comprehensive model to study tuberculosis immunopathology (62). Remarkably in these animals, berberine alone was able to significantly reduce lung mycobacterial burden, which might be attributed to higher susceptibility of this animal model in comparison to C57BL/6 mice. However, no adjunct effect on bacterial load was observed in mice treated with a combination of berberine and antibiotics; the latter was consistent with the C57BL/6 model. Moreover, rifampicin/isoniazid alone significantly increased lung alveolar air spaces compared to mice treated with berberine alone. However, mice treated with rifampicin/isoniazid in combination with berberine did not further increase lung alveolar spaces when compared to antibiotic only group. Furthermore, mice treated with rifampicin/isoniazid in combination with berberine showed increased lung cell numbers, T cells, polymorphonuclear cells and recruited monocytes in the lungs. These findings mirrored our observations with C57BL/6 mice when treated 3 weeks post *Mtb* infection except for the effect on neutrophils. This indicated that berberine had an adjunct but not additive or synergistic effect on lung inflammation in C57BL/6 mice but not susceptible Kramnik mice.

The absence of the adjunctive effect of berberine in Kramnik mice points out that preventing neutrophilic inflammation is the main driver of anti-inflammatory effects of berberine in C57BL/6 mice. Previously, forward genetics analyses revealed that Kramnik mice have a susceptible allele of super susceptibility to tuberculosis (sst1) locus on mouse chromosome 1, unlike C57BL/6 counterparts which harbor the resistant allele (63) Sp140 gene was recently identified to confer resistance to C57BL/6 mouse and it is found to negatively regulate Type I IFN responses (64). Type I IFNs are shown to drive pathological inflammation in tuberculosis especially in Kramnik mice through neutrophilia (35, 65). In our experiments, we have seen that the early or late onset of adjunctive berberine treatment did not effect IFNβ levels in C57BL/6 mice, hence Type-1 IFN dominated neutrophilia was probably not inhibited in Kramnik mice. Targeting Type I IFN network by neutralizing antibodies with berberine adjunctive therapy would be a future study to understand the extent of berberine immunomodulation and anti-inflammatory effects in adjuvant therapy in Kramnik mice.

In summary, we report here that berberine as an adjunctive therapy decreased lung inflammation by targeting immune cell recruitment and reducing inflammatory cytokines. The lack of the development of granulomatous lung pathology with caseous necrosis resembling the human TB disease in the C57BL/6 model and Kramnik experiment further warrants studies starting treatment in much later time points in Kramnik mice or nonhuman primates (NHP) before testing in patients. Another important drawback of berberine adjunctive therapy can be attributed to its low absorption rates in the gut (66). Therefore, future studies with hyperinflammatory clinical Mtb strains in Kramnik mouse and NHP models are warranted to consider berberine or its derivatives with increased oral bioavailability as a potential adjunct therapy to current first-line anti-TB drugs in the clinic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee (Permit Number: 015/040), Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa.

AUTHOR CONTRIBUTIONS

MO, JC, RH, MS, RM, RG, BM, and SP: designing research studies, conducting experiments, acquiring data, analyzing data. BM, HS, and FB: resources and funding for the research. SP and MO writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.656419/full#supplementary-material

Supplementary Figure 1 | Bactericidal and immunomodulatory effects of berberine on *Mtb* infected macrophages. **(A)** BMDM from C57BL/6 mice were either pre-treated with berberine (30 μM) overnight or treatment started 4 hours post-infection. Cells were infected with *Mtb* (MOI = 1) to determine the growth in 2 days and 5 days post-infection (dpi); 4 hours post-infection CFU was assayed to measure the differences in bacterial uptake. **(B)** MHCII and CD80 mean fluorescence intensity (MFI) was measured in vehicle or berberine treated *Mtb* infected BMDM to check protein abundance on the surface. Data represented as mean ± SEM of four replicates and representative of two experiments, analysed by a two-tailed unpaired Student's t-test defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01, ***P ≤ 0.001.

Supplementary Figure 2 | Immunomodulatory effects of berberine as an adjunctive therapy during *Mtb* infection in C57BL/6 mice **(A)** Water consumption was measured after every water change to estimate drug uptake per mouse per day. **(B)** Serum ALT and AST levels were measured 4 weeks post TB treatment to infer liver cytotoxicity and hepatoprotective effects of berberine. **(C)** MHCII expressing lung alveolar macrophages, interstitial macrophages and monocyte-derived DCs; spleen $CD169^+$ macrophages, red pulp macrophages and monocytes after 4 weeks treatment in late-onset of berberine adjunctive therapy. The data is presented as the percentage of the parent population. **(D)** Activated caspase 3 flow cytometry as a marker of apoptosis. Data is shown as Caspase-3^+ total live cells in the lungs and spleen of mice 4 weeks post TB treatment in the late-onset adjunctive berberine treatment. Data is shown representative of two experiments (n = 5 per group) and the line denotes mean value, analysed by Kruskal-Wallis test with Dunn's multiple comparisons correction **(A, B)** and one-way ANOVA with Tukey *post-hoc* test **(C, D)** defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01. Asterisks without the line below show the significance compared to the untreated group.

Supplementary Figure 3 | Effect of late-onset berberine adjunctive therapy on lung and spleen immune cells populations during *Mtb* infection in C57BL/6 mice. **(A)** Lung $CXCR3^+$ CD4 and CD8 T cell frequencies, **(B)** lung alveolar macrophage ($SiglecF^+CD11c^+CD64^+$), **(C)** lung interstitial macrophages ($CD64^+CD11b^+CD11c^-SiglecF^-$), **(D)** lung monocyte-derived DC counts ($CD64^+CD11b^+CD11c^+$), **(E)** spleen $CXCR3^+$ CD4 and CD8 T cell frequencies, **(F)** spleen $CD169^+$ macrophage ($CD11b^+CD169^+CD11c^-$) **(G)** spleen red pulp macrophage ($F4/80^+CD11b^{low}CD169^-CD11c^-$), **(H)** spleen CD8 DC counts ($CD11c^+MHCII^+CD8^+CD11b^-$). Data is shown representative of two experiments (n = 5 per group) and the line denotes mean value, analysed by one-way ANOVA with Tukey *post-hoc* test **(A–C, E–F)** and Kruskal-Wallis test with Dunn's multiple comparisons correction (D, G) defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Asterisks without the line below show the significance compared to the untreated group.

Supplementary Figure 4 | Effect of late-onset of berberine adjunctive therapy on lung and spleen cytokines/chemokines and receptor gene expression during *Mtb* infection in C57BL/6 mice. Lung and spleen homogenates were analyzed for the cytokines and chemokines by ELISA at 4 weeks post-treatment; **(A)** IFNγ, **(B)** IL-1β, **(C)** IL-12p40, **(D)** CXCL10, **(E)** GM-CSF, and **(F)** IFN-β. **(G)** Total RNA was extracted from lung tissue and qPCR was performed to measure Ifngr1 and Csf2ra levels. Hprt housekeeping gene was used for normalization and fold change differences were calculated by using untreated as the reference sample. Data is shown representative of two experiments (n = 5 per group) and the line denotes mean value, analysed by one-way ANOVA with Tukey *post-hoc* test **(A–F)** and Kruskal-Wallis test with Dunn's multiple comparisons correction **(G)** defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Asterisks without the line below show the significance compared to the untreated group.

Supplementary Figure 5 | Berberine as an adjunctive therapy against *Mtb* infection in C3HeB/FeJ (Kramnik) mice. **(A)** Mice were infected with *Mtb* Erdman (100 CFU) *via* aerosol inhalation. After 3 weeks of infection, mice were either left untreated or treated with berberine (1 mg/ml) or rifampin/isoniazid (0.1 mg/ml) and rifampin/isoniazid with berberine in drinking water for 4 weeks as shown in the layout. **(B)** Mycobacterial burdens in the lungs. **(C)** Representative lung section for pathology evaluated by H&E staining. **(D)** Quantification of free alveolar air spaces. Single-cell suspension of the lung was analyzed for immune cell populations by flow cytometry. **(E)** Total lung cells, **(F)** T cells ($CD3^+CD11b^-Ly6G^-Ly6C^-$), **(G)** polymorphonuclear cells ($CD11b^+Ly6G^+Ly6C^+$) and **(H)** inflammatory monocytes ($CD11b^+Ly6G^-Ly6C^+$) in the lungs at 4 weeks post-treatment. Data represented as mean ± SEM of n = 3-4 mice per group from one experiment, analysed by one-way ANOVA with Tukey *post-hoc* test defining differences in all groups as significant *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

REFERENCES

1. Organization, W.H. Global Tuberculosis Report 2020. In: *Global Tuberculosis Programme*. Geneva: World Health Organization (WHO) (2020).
2. Houben RMGJ, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-Estimation Using Mathematical Modelling. *PloS Med* (2016) 13(10):e1002152. doi: 10.1371/journal.pmed.1002152
3. Behr MA, Edelstein PH, Ramakrishnan L. Revisiting the Timetable of Tuberculosis. *BMJ* (2018) 362:k2738. doi: 10.1136/bmj.k2738
4. Salgame P, Geadas C, Collins L, Jones-López E, Ellner JJ. Latent Tuberculosis Infection – Revisiting and Revising Concepts. *Tuberculosis* (2015) 95(4):373–84. doi: 10.1016/j.tube.2015.04.003
5. Sharma A, Hill A, Kurbatova E, van der Walt M, Kvasnovsky C, Tupasi A, et al. Estimating the Future Burden of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis in India, the Philippines, Russia, and South Africa: A Mathematical Modelling Study. *Lancet Infect Dis* (2017) 17(7):707–15. doi: 10.1038/nchembio.1917
6. *Price of a Pandemic*. Available at: https://docs.wixstatic.com/ugd/309c93_2709b6ff47c946fe97b94a72fdfd94e3.pdf?index=true.

7. Guler R, Brombacher F. Host-Directed Drug Therapy for Tuberculosis. *Nat Chem Biol* (2015) 11(10):748–51. doi: 10.1038/nchembio.1917
8. Young C, Walzl G, Du Plessis N. Therapeutic Host-Directed Strategies to Improve Outcome in Tuberculosis. *Mucosal Immunol* (2020) 13(2):190–204. doi: 10.1038/s41385-019-0226-5
9. Dorhoi A, Kaufmann SHE. Pathology and Immune Reactivity: Understanding Multidimensionality in Pulmonary Tuberculosis. *Semin Immunopathol* (2016) 38(2):153–66. doi: 10.1007/s00281-015-0531-3
10. Kuo CL, Chi CW, Liu TY. The Anti-Inflammatory Potential of Berberine *In Vitro* and *In Vivo*. *Cancer Lett* (2004) 203(2):127–37. doi: 10.1016/j.canlet.2003.09.002
11. Musumeci R, Speciale A, Costanzo R, Annino A, Ragusa S, Rapisarda A, et al. Berberis Aetnensis C. Presl. Extracts: Antimicrobial Properties and Interaction With Ciprofloxacin. *Int J Antimicrob Agents* (2003) 22(1):48–53. doi: 10.1016/S0924-8579(03)00085-2
12. Cui G, Qin X, Zhang Y, Gong Z, Ge B, Zang YQ. Berberine Differentially Modulates the Activities of ERK, P38 MAPK, and JNK to Suppress Th17 and Th1 T Cell Differentiation in Type 1 Diabetic Mice. *J Biol Chem* (2009) 284 (41):28420–9. doi: 10.1074/jbc.M109.012674
13. Ayati SH, Fazeli B, Momtazi-Borojeni AA, Cicero AFG, Pirro M, Sahebkar A. Regulatory Effects of Berberine on Micrornome in Cancer and Other Conditions. *Crit Rev Oncol Hematol* (2017) 116:147–58. doi: 10.1016/j.critrevonc.2017.05.008
14. Kumar A, Ekavali , Chopra K, Mukherjee M, Pottabathini R, Dhull DK. Current Knowledge and Pharmacological Profile of Berberine: An Update. *Eur J Pharmacol* (2015) 761:288–97. doi: 10.1016/j.ejphar.2015.05.068
15. Vuddanda PR, Chakraborty S, Singh S. Berberine: A Potential Phytochemical With Multispectrum Therapeutic Activities. *Expert Opin Investigational Drugs* (2010) 19(10):1297–307. doi: 10.1517/13543784.2010.517745
16. Ehteshamfar S-M, Akhbari M, Afshari JT, Seyedi M, Nikfar B, Shapouri-Moghaddam A, et al. Anti-Inflammatory and Immune-Modulatory Impacts of Berberine on Activation of Autoreactive T Cells in Autoimmune Inflammation. *J Cell Mol Med* (2020) 24(23):13573–88. doi: 10.1111/jcmm.16049
17. Li H, Li XL, Zhang M, Xu H, Wang CC, Wang S, et al. Berberine Ameliorates Experimental Autoimmune Neuritis by Suppressing Both Cellular and Humoral Immunity. *Scand J Immunol* (2014) 79(1):12–9. doi: 10.1111/sji.12123
18. Hu Z, Jiao Q, Ding J, Liu F, Liu R, Shan L, et al. Berberine Induces Dendritic Cell Apoptosis and has Therapeutic Potential for Rheumatoid Arthritis. *Arthritis Rheum* (2011) 63(4):949–59. doi: 10.1002/art.30202
19. Yan F, Wang L, Shi Y, Cao H, Liu L, Washington MK, et al. Berberine Promotes Recovery of Colitis and Inhibits Inflammatory Responses in Colonic Macrophages and Epithelial Cells in DSS-Treated Mice. *Am J Physiol Gastrointest Liver Physiol* (2012) 302(5):G504–14. doi: 10.1152/ajpgi.00312.2011
20. Li C, Xi Y, Li S, Zhao Q, Cheng W, Wang Z, et al. Berberine Ameliorates TNBS Induced Colitis by Inhibiting Inflammatory Responses and Th1/Th17 Differentiation. *Mol Immunol* (2015) 67(2, Part B):444–54. doi: 10.1016/j.molimm.2015.07.013
21. Hlaka L, Rosslee M-J, Ozturk M, Kumar S, Parihar SP, Brombacher F, et al. Evaluation of Minor Groove Binders (MGBs) as Novel Anti-Mycobacterial Agents and the Effect of Using non-Ionic Surfactant Vesicles as a Delivery System to Improve Their Efficacy. *J Antimicrobial Chemother* (2017) 72 (12):3334–41. doi: 10.1093/jac/dkx326
22. Tamgue O, Gcanga L, Ozturk M, Whitehead L, Pillay S, Jacobs R, et al. Differential Targeting of C-Maf, Bach-1, and Elmo-1 by microRNA-143 and microRNA-365 Promotes the Intracellular Growth of Mycobacterium Tuberculosis in Alternatively IL-4/IL-13 Activated Macrophages. *Front Immunol* (2019) 10(421). doi: 10.3389/fimmu.2019.00421
23. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdayal R, Mhlanga MM, et al. Statin Therapy Reduces the Mycobacterium Tuberculosis Burden in Human Macrophages and in Mice by Enhancing Autophagy and Phagosome Maturation. *J Infect Dis* (2013) 209(5):754–63. doi: 10.1093/infdis/jit550
24. Cao H, Song S, Zhang H, Zhang Y, Qu R, Yang B, et al. Chemopreventive Effects of Berberine on Intestinal Tumor Development in Apcmin/+ Mice. *BMC Gastroenterol* (2013) 13:163–3. doi: 10.1186/1471-230X-13-163
25. Parihar SP, Ozturk M, Marakalala MJ, Loots DT, Hurdayal R, Maasdorp DB, et al. Protein Kinase C-Delta (Pkcδ), a Marker of Inflammation and Tuberculosis Disease Progression in Humans, is Important for Optimal Macrophage Killing Effector Functions and Survival in Mice. *Mucosal Immunol* (2018) 11(2):496–511. doi: 10.1038/mi.2017.68
26. Nienaber A, Baumgartner J, Dolman RC, Ozturk M, Zandberg L, Hayford FEA, et al. Omega-3 Fatty Acid and Iron Supplementation Alone, But Not in Combination, Lower Inflammation and Anemia of Infection in Mycobacterium Tuberculosis-Infected Mice. *Nutrients* (2020) 12(9). doi: 10.3390/nu12092897
27. Du G-F, Le Y-J, Sun X, Yang X-Y, He Q-Y. Proteomic Investigation Into the Action Mechanism of Berberine Against Streptococcus Pyogenes. *J Proteomics* (2020) 215:103666. doi: 10.1016/j.jprot.2020.103666
28. Kassab RB, Vasicek O, Ciz M, Lojek A, Pereck T. The Effects of Berberine on Reactive Oxygen Species Production in Human Neutrophils and in Cell-Free Assays. *Interdiscip Toxicol* (2017) 10(2):61–5. doi: 10.1515/intox-2017-0010
29. Ma X, Deng J, Cui X, Chen Q, Wang W. Berberine Exhibits Antioxidative Effects and Reduces Apoptosis of the Vaginal Epithelium in Bacterial Vaginosis. *Exp Ther Med* (2019) 18(3):2122–30. doi: 10.3892/etm.2019.7772
30. Beenakker KGM, Westendorp RGJ, de Craen AJM, Chen S, Raz Y, Ballieux BEPB, et al. Men Have a Stronger Monocyte-Derived Cytokine Production Response Upon Stimulation With the Gram-Negative Stimulus Lipopolysaccharide Than Women: A Pooled Analysis Including 15 Study Populations. *J Innate Immun* (2020) 12(2):142–53. doi: 10.1159/000499840
31. Kheir MM, Wang Y, Hua L, Hu J, Li L, Lei F, et al. Acute Toxicity of Berberine and its Correlation With the Blood Concentration in Mice. *Food Chem Toxicol* (2010) 48(4):1105–10. doi: 10.1016/j.fct.2010.01.033
32. Wang K, Feng X, Chai L, Cao S, Qiu F, et al. The Metabolism of Berberine and Its Contribution to the Pharmacological Effects. *Drug Metab Rev* (2017) 49 (2):139–57. doi: 10.1080/03602532.2017.1306544
33. Liu C-S, Zheng Y-R, Zhang Y-F, Long X-Y. Research Progress on Berberine With a Special Focus on its Oral Bioavailability. *Fitoterapia* (2016) 109:274–82. doi: 10.1016/j.fitote.2016.02.001
34. Mishra BB, Lovewell RR, Olive AJ, Zhang G, Wang W, Eugenin E, et al. Nitric Oxide Prevents a Pathogen-Permissive Granulocytic Inflammation During Tuberculosis. *Nat Microbiol* (2017) 2:17072–2. doi: 10.1038/nmicrobiol.2017.72
35. Moreira-Teixeira L, Stimpson PJ, Stavropoulos E, Hadebe S, Chakravarty P, Ioannou M, et al. Type I IFN Exacerbates Disease in Tuberculosis-Susceptible Mice by Inducing Neutrophil-Mediated Lung Inflammation and NETosis. *Nat Commun* (2020) 11(1):5566. doi: 10.1038/s41467-020-19412-6
36. Bhattacharyya C, Majumder PP, Pandit B. CXCL10 is Overexpressed in Active Tuberculosis Patients Compared to M. Tuberculosis-Exposed Household Contacts. *Tuberculosis (Edinb)* (2018) 109:8–16. doi: 10.1016/j.tube.2018.01.005
37. Kim WS, Kim J-S, Cha SB, Han SJ, Kim H, et al. Virulence-Dependent Alterations in the Kinetics of Immune Cells During Pulmonary Infection by Mycobacterium Tuberculosis. *PloS One* (2015) 10(12):e0145234–e0145234. doi: 10.1371/journal.pone.0145234
38. Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura TJ, et al. Initiation of the Adaptive Immune Response to Mycobacterium Tuberculosis Depends on Antigen Production in the Local Lymph Node, Not the Lungs. *J Exp Med* (2007) 205(1):105–15. doi: 10.1084/jem.20071367
39. Agnarelli A, Natali M, Garcia-Gil M, Pesi R, Tozzi MG, Ippolito C, et al. Cell-Specific Pattern of Berberine Pleiotropic Effects on Different Human Cell Lines. *Sci Rep* (2018) 8(1):10599. doi: 10.1038/s41598-018-28952-3
40. Groom JR, Luster AD. CXCR3 in T Cell Function. *Exp Cell Res* (2011) 317 (5):620–31. doi: 10.1016/j.yexcr.2010.12.017
41. Kauffman KD, Sallin MA, Sakai S, Kamenyeva O, Kabat J, Weiner D, et al. Defective Positioning in Granulomas But Not Lung-Homing Limits CD4 T-Cell Interactions With Mycobacterium Tuberculosis-Infected Macrophages in Rhesus Macaques. *Mucosal Immunol* (2018) 11(2):462–73. doi: 10.1038/mi.2017.60
42. Kim DG, Choi JW, Jo IJ, Kim MJ, Lee HS, Hong SH, et al. Berberine Ameliorates Lipopolysaccharide–Induced Inflammatory Responses in Mouse Inner Medullary Collecting Duct–3 Cells by Downregulation of NF–κb Pathway. *Mol Med Rep* (2020) 21(1):258–66. doi: 10.3892/mmr.2019.10823
43. Fan X, Wang J, Hou J, Lin C, Bensoussan A, Chang D, et al. Berberine Alleviates Ox-LDL Induced Inflammatory Factors by Up-Regulation of

Autophagy *via* AMPK/mTOR Signaling Pathway. *J Trans Med* (2015) 13 (1):92. doi: 10.1186/s12967-015-0450-z
44. Wang Q, Zhang M, Liang B, Shirwany N, Zhu Y, Zou M-H. Activation of AMP-Activated Protein Kinase Is Required for Berberine-Induced Reduction of Atherosclerosis in Mice: The Role of Uncoupling Protein 2. *PloS One* (2011) 6(9):e25436. doi: 10.1371/journal.pone.0025436
45. Lee T-S, Pan C-C, Peng C-C, Kou Y-R, Chen C-Y, Ching L-C, et al. Anti-Atherogenic Effect of Berberine on Lxrα-ABCA1-Dependent Cholesterol Efflux in Macrophages. *J Cell Biochem* (2010) 111(1):104–10. doi: 10.1002/jcb.22667
46. Liu X, Zhang N, Liu Y, Liu L, Zeng Q, Yin M, et al. MPB, a Novel Berberine Derivative, Enhances Lysosomal and Bactericidal Properties *via* TGF-β–Activated Kinase 1-Dependent Activation of the Transcription Factor EB. *FASEB J* (2019) 33(1):1468–81. doi: 10.1096/fj.201801198R
47. Kim TS, Kang BY, Cho D, Kim S-H. Induction of Interleukin-12 Production in Mouse Macrophages by Berberine, a Benzodioxoloquinolizine Alkaloid, Deviates CD4+ T Cells From a Th2 to a Th1 Response. *Immunology* (2003) 109(3):407–14. doi: 10.1046/j.1365-2567.2003.01673.x
48. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium Tuberculosis is Influenced by Host Factors and Precedes the Initiation of T-Cell Immunity. *Infect Immun* (2002) 70(8):4501–9. doi: 10.1128/IAI.70.8.4501-4509.2002
49. Yu F-S, Yang J-S, Lin H-J, Yu C-S, Tan T-W, et al. Berberine Inhibits WEHI-3 Leukemia Cells *In Vivo*. *In Vivo* (2007) 21(2):407–12. doi: 10.1096/fasebj.21.6.A1189-a
50. Saha P, Bhattacharjee S, Sarkar A, Manna A, Majumder S, Chatterjee M. Berberine Chloride Mediates Its Anti-Leishmanial Activity *via* Differential Regulation of the Mitogen Activated Protein Kinase Pathway in Macrophages. *PloS One* (2011) 6(4):e18467. doi: 10.1371/journal.pone.0018467
51. Warowicka A, Nawrot R, Goździcka-Józefiak A. Antiviral Activity of Berberine. *Arch Virol* (2020) 165(9):1935–45. doi: 10.1007/s00705-020-04706-3
52. Mahmoudvand H, Ayatollahi Mousavi SA, Sepahvand A, Sharififar F, Ezatpour B, Gorohi F, et al. Antifungal, Antileishmanial, and Cytotoxicity Activities of Various Extracts of Berberis Vulgaris (Berberidaceae) and Its Active Principle Berberine. *ISRN Pharmacol* (2014) 2014:602436. doi: 10.1155/2014/602436
53. Dkhil MA. Role of Berberine in Ameliorating Schistosoma Mansoni-Induced Hepatic Injury in Mice. *Biol Res* (2014) 47(1):8–8. doi: 10.1186/0717–6287–47–8
54. Mahmoud A, Germoush M, Soliman A. Berberine Attenuates Isoniazid-Induced Hepatotoxicity by Modulating Peroxisome Proliferator-Activated Receptor ?, Oxidative Stress and Inflammation. *Int J Pharmacol* (2014) 10:451–60. doi: 10.3923/ijp.2014.451.460
55. Liang RM, Yong XL, Duan YQ, Tan YH, Zeng P, Zhou ZY, et al. Potent *In Vitro* Synergism of Fusidic Acid (FA) and Berberine Chloride (BBR) Against Clinical Isolates of Methicillin-Resistant Staphylococcus Aureus (MRSA). *World J Microbiol Biotechnol* (2014) 30(11):2861–9. doi: 10.1007/s11274-014-1712-2
56. Li D-D, Xu Y, Zhang D-Z, Quan H, Mylonakis E, Hu D-D, et al. Fluconazole Assists Berberine To Kill Fluconazole-Resistant Candida Albicans. *Antimicrobial Agents Chemother* (2013) 57(12):6016–27. doi: 10.1128/AAC.00499-13
57. Peng L, Kang S, Yin Z, Jia R, Song X, Li L, et al. Antibacterial Activity and Mechanism of Berberine Against Streptococcus Agalactiae. *Int J Clin Exp Pathol* (2015) 8(5):5217–23.
58. Zuo GY, Li Y, Han J, Wang GC, Zhang YL, Bian ZQ. Antibacterial and Synergy of Berberines With Antibacterial Agents Against Clinical Multi-Drug Resistant Isolates of Methicillin-Resistant Staphylococcus Aureus (MRSA). *Molecules* (2012) 17(9):10322–30. doi: 10.3390/molecules170910322
59. Boberek JM, Stach J, Good L. Genetic Evidence for Inhibition of Bacterial Division Protein FtsZ by Berberine. *PloS One* (2010) 5(10):e13745. doi: 10.1371/journal.pone.0013745
60. Wojtyczka RD, Dziedzic A, Kępa M, Kubina R, Kabała-Dzik A, Mularz T, et al. Berberine Enhances the Antibacterial Activity of Selected Antibiotics Against Coagulase-Negative Staphylococcus Strains *In Vitro*. *Molecules* (2014) 19(5):6583–96. doi: 10.3390/molecules19056583
61. Chu M, Zhang M-B, Liu Y-C, Kang J-R, Chu Z-Y, Yin K-I, et al. Role of Berberine in the Treatment of Methicillin-Resistant Staphylococcus Aureus Infections. *Sci Rep* (2016) 6(1):24748. doi: 10.1038/srep24748
62. Kramnik I, Beamer G. Mouse Models of Human TB Pathology: Roles in the Analysis of Necrosis and the Development of Host-Directed Therapies. *Semin Immunopathol* (2016) 38(2):221–37. doi: 10.1007/s00281-015-0538-9
63. Kramnik I, Dietrich WF, Demant P, Bloom BR. Genetic Control of Resistance to Experimental Infection With Virulent Mycobacterium Tuberculosis. *Proc Natl Acad Sci USA* (2000) 97(15):8560–5. doi: 10.1073/pnas.150227197
64. Ji DX, Witt KC, Kotov DI, Margolis SR, Louie A, Chevee V, et al. Role of the Transcriptional Regulator SP140 in Resistance to Bacterial Infections *via* Repression of Type I Interferons. *Elife* (2021) 10. doi: 10.7554/eLife.67290
65. Ji DX, Yamashiro LH, Chen KJ, Mukaida N, Kramnik I, Darwin KH, et al. Type I Interferon-Driven Susceptibility to Mycobacterium Tuberculosis is Mediated by IL-1ra. *Nat Microbiol* (2019) 4(12):2128–35. doi: 10.1038/s41564-019-0578-3
66. Godugu C, Patel AR, Doddapaneni R, Somagoni J, Singh M. Approaches to Improve the Oral Bioavailability and Effects of Novel Anticancer Drugs Berberine and Betulinic Acid. *PloS One* (2014) 9(3):e89919. doi: 10.1371/journal.pone.0089919

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