TOWARDS HOST-DIRECTED DRUG THERAPIES FOR INFECTIOUS AND NON-COMMUNICABLE DISEASES

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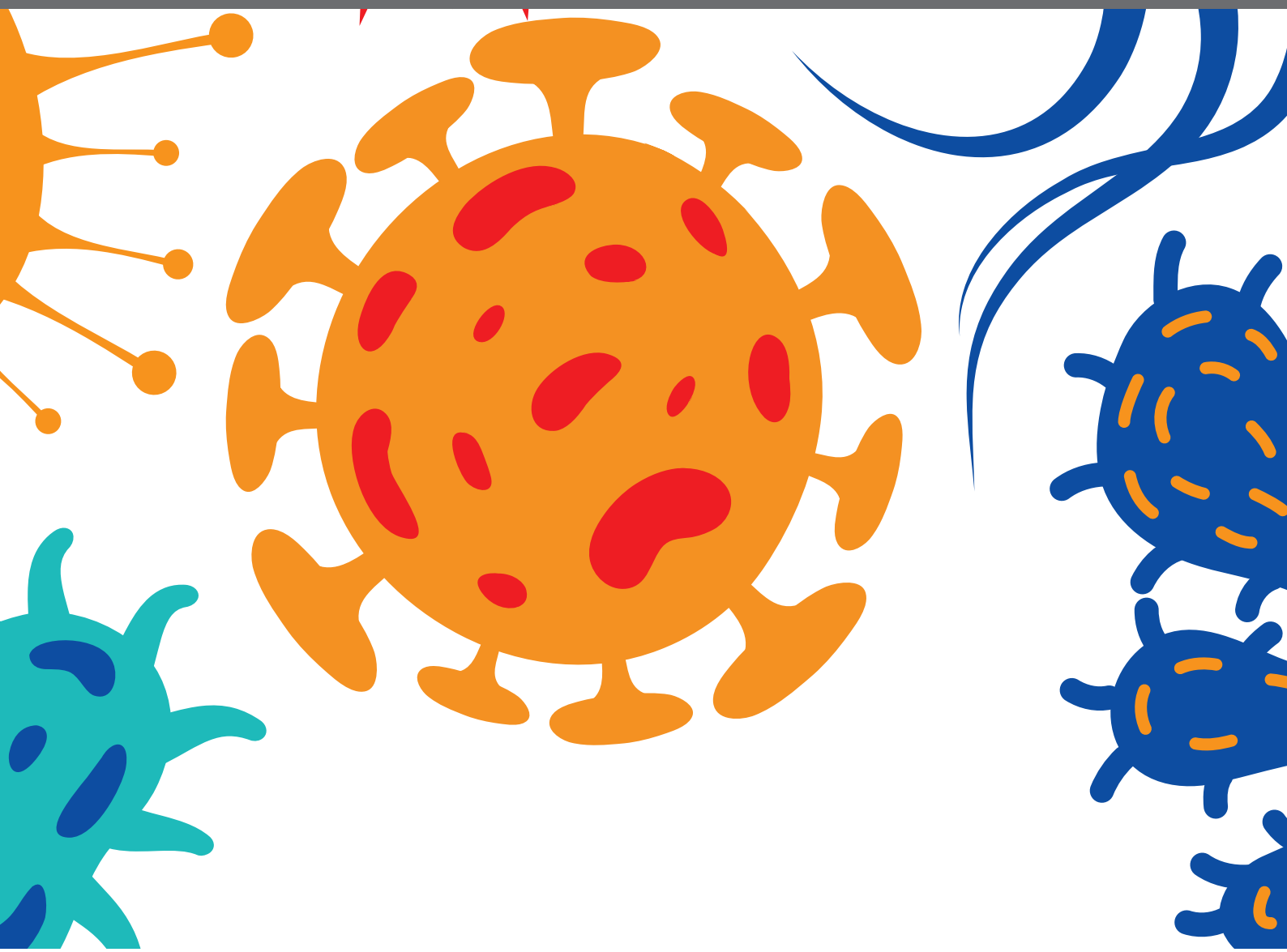

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TOWARDS HOST-DIRECTED DRUG THERAPIES FOR INFECTIOUS AND NON-COMMUNICABLE DISEASES

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Table of Contents

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REVIEW
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Principles of Immunotherapy: Implications for Treatment Strategies in Cancer and Infectious Diseases

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The advances in cancer biology and pathogenesis during the past two decades, have resulted in immunotherapeutic strategies that have revolutionized the treatment of malignancies, from relatively non-selective toxic agents to specific, mechanism-based therapies. Despite extensive global efforts, infectious diseases remain a leading cause of morbidity and mortality worldwide, necessitating novel, innovative therapeutics that address the current challenges of increasing antimicrobial resistance. Similar to cancer pathogenesis, infectious pathogens successfully fashion a hospitable environment within the host and modulate host metabolic functions to support their nutritional requirements, while suppressing host defenses by altering regulatory mechanisms. These parallels, and the advances made in targeted therapy in cancer, may inform the rational development of therapeutic interventions for infectious diseases. Although "immunotherapy" is habitually associated with the treatment of cancer, this review accentuates the evolving role of key targeted immune interventions that are approved, as well as those in development, for various cancers and infectious diseases. The general features of adoptive therapies, those that enhance T cell effector function, and ligand-based therapies, that neutralize or eliminate diseased cells, are discussed in the context of specific diseases that, to date, lack appropriate remedial treatment; cancer, HIV, TB, and drug-resistant bacterial and fungal infections. The remarkable diversity and versatility that distinguishes immunotherapy is emphasized, consequently establishing this approach within the armory of curative therapeutics, applicable across the disease spectrum.

Keywords: immunotherapy, T cell therapy, antibody therapy, cancer, infectious diseases

INTRODUCTION

Numerous host factors which constitute the immune system influence treatment outcomes and are accountable for disease progression or regression. Immunotherapy is collectively defined as a therapeutic approach that targets or manipulates the immune system (Papaioannou et al., 2016). Ultimately, immunotherapy aims to harness the host's adaptive and innate immune response to effectuate long-lived elimination of diseased cells and can be categorized broadly into passive

(including adoptive and antibody-based) and active (including vaccine therapy and allergen-specific) approaches. Passive-mediated immunotherapy involves the administration of *ex vivo*-generated immune elements (antibodies, immune cells) to patients and does not stimulate the host immune response, while active immunotherapy induces the patient's immune response and results in the development of specific immune effectors (antibodies and T cells) (Tur and Barth, 2014).

Consequently, these targeted therapies advance host cellular responses to disease by stimulating immune responses, targeting disease-causing virulence factors and improving immunological memory (Zumla et al., 2016b). As such, several types of targeted therapies have been approved for cancer treatment and act by blocking regulatory biochemical pathways or mutant proteins, essential to tumor growth and maintenance and have reduced toxic side effects as they act with precision on diseased cells with little collateral tissue damage (Wykes and Lewin, 2018). As such, "immunotherapy" is almost exclusively, and somewhat biasedly, associated with the treatment of cancer. In this review, clinically relevant examples of adoptive therapies are provided to emphasize the versatility of their corresponding general principles of action for both non-communicable and infectious diseases.

T CELL THERAPIES

T cells initiate potent and specific immune responses against foreign antigens. Strategies that activate these immune responses, broadly define T cell therapies and are often developed in combination with monoclonal antibodies (mAbs) (**Figure 1**).

Vaccines

Vaccination represents the first form of host-directed immunotherapy to be introduced and includes various categories (**Table 1**). Most vaccines work by introducing a non-infectious version of a disease-causing microbe into an individual, thereby providing a better stimulus for the activation of disease-specific T cells and the development of immunological memory. Memory immune cells are able to rapidly kill microbes and prevent infection. While this type targeted therapy has been able to eradicate smallpox and drastically reduce the disease burden of multiple infectious agents such as rabies, typhoid, cholera, hepatitis and more, it has been far less effective against cancer and chronic infectious diseases such as human immunodeficiency virus (HIV). The combination of therapeutic vaccines and other immune-based therapies may provide improved efficacy (Waldmann, 2003).

Following the discovery of Edward Jenner's smallpox vaccine in 1796 (Nabel, 2013) vaccination, especially against viral infections, has been the leading practice of infection prevention. Developed in 1971, an example of a modern viral vaccine success is the MMR (mumps, measles and rubella) vaccine (Larson et al., 2018). This live-attenuated vaccine contains variants of all three pathogens. MMR can be transported in lyophilized form and is usually administered subcutaneously. Developing vaccines for other viral pathogens remains challenging despite enormous technological advancements. This pertains particularly to HIV, where in spite of more than 30 years of research, no reports of a completely effective prophylactic or therapeutic vaccine exist (Dangeti, 2014). The lack of efficacy of candidate HIV vaccines has been attributed to HIV escape from vaccine specific CD8+ T cells, lack of CD8+ T cells replenishment and inability to target latently infected cells (reviewed by Perreau et al., 2017). Presently, it is accepted that a successful HIV vaccine would comprise of an optimized vector and/or immunogen proficient in stimulating broad, long-lived and safe immune responses (Esparza and Van Regenmortel, 2014). Up until 2016 only 6 HIV-vaccine candidates reached clinical trials and most of these failed to produce protective broadly neutralizing antibodies thus changing the focus of vaccine strategies to elicit CTL responses (Stephenson et al., 2016). Effective T cell-based vaccine can limit the initial viral replication and lower viral load set point, thereby preventing the initial infection or/and slow disease progression (Haynes, 2015). The first CTL based vaccine trial was the step study (HVTN502) which also did not show any protection and actually induced higher infection rates in a subset of participants (Cao et al., 1995; Duerr et al., 2012). Eventually, the RV144 trial, which used a prime-boost strategy with a canarypox viral vector and envelope protein gp120 demonstrated a 31% efficacy rate (Rerks-Ngarm et al., 2009). Results from the RV144 trail demonstrated that even non-neutralizing antibodies which match the infecting strain also have the ability to prevent infection (NCT01435135) primarily via ADCC and Fc mediated responses. The major concern with such a vaccine strategy is the enormous diversity of HIV-1 strains (Stephenson et al., 2016). To improve on the RV144 results, current strategies are focused on understanding immune correlates of protection that would increase the breadth of ADCC and FcR activities (Haynes, 2015). Although these trials have been mostly disappointing, they are informative and highlight the remarkable struggle associated in developing effective HIV vaccines and provide essential information to support pre-clinical development of other novel T cell vaccine strategies.

Unlike viral-targeted vaccines, cancer vaccines do not prevent disease but rather stimulate the immune system to attack an already existing disease. Many cancer vaccines consist of cancer cells, parts of cells, or pure antigens. Often, a patient's immune cells are isolated and exposed to cancer antigens, and once activated, these immune cells are re-introduced into the patient's body and are better able to suppress cancer cells. Sipuleucel-T (Provenge®) (Sip-T) is the first FDA (United States Food and Drug Administration)-approved therapeutic cancer vaccine. It is an autologous vaccine used for the treatment of patients with asymptomatic or minimally symptomatic castration-resistant metastatic prostate cancer (mCRPC) and who are not responsive to hormone therapy (Cheever and Higano, 2011). Sip-T elicit anti-tumor activity via activation of T cells that are specific for prostatic acid phosphatase (PAP), an enzyme found on the surface of 95% of prostate cancer cells (Alteri et al., 2018). The vaccine is formulated to comprise of a patient's mature antigen-presenting cells (APCs) expressing PAP after *ex vivo* exposure to a granulocyte macrophage colony stimulating factor (GM-CSF)-and PAP fusion protein (Gardner et al., 2012).

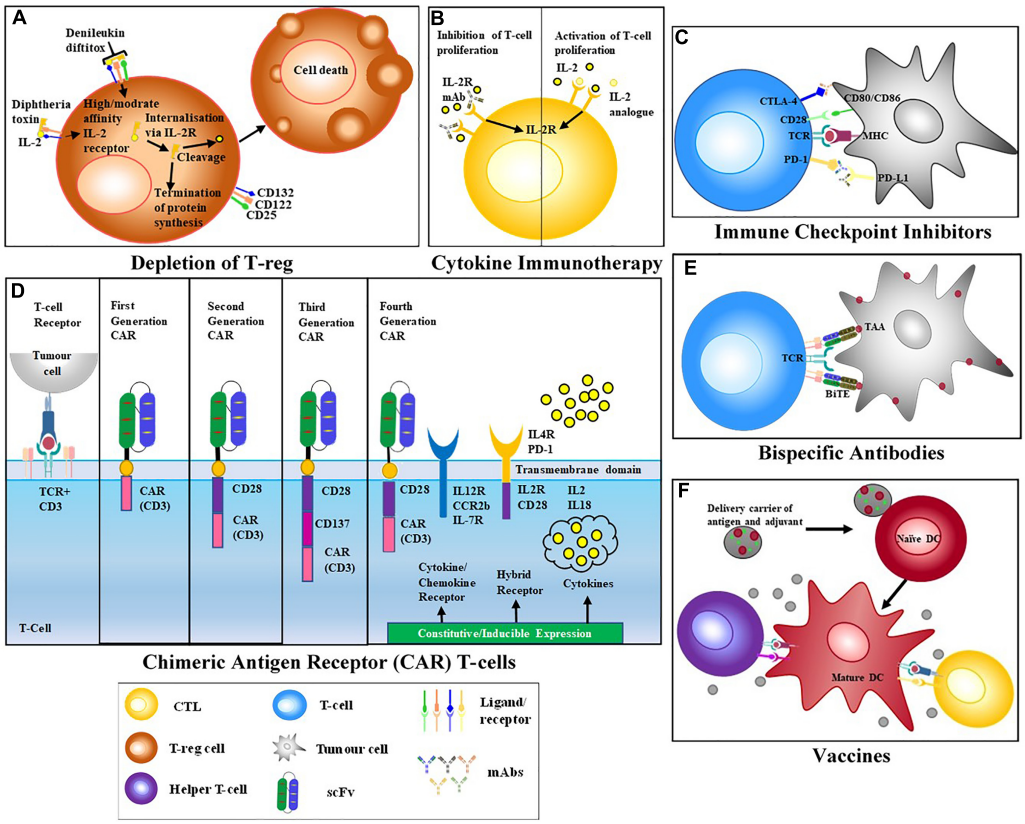

FIGURE 1 | T cell-activating Therapeutic Strategies. **(A)** Treg depletion – biologics such Denileukin diftitox (DD) bind to target receptors on suppressor cells and initiate apoptosis via down-stream signaling. **(B)** Cytokine therapy – addition of pro-inflammatory cytokines increases immune activation while the addition of anti-inflammatory cytokines reduces immune activation. MAbs specific for cytokine receptors may also be used to block cytokine stimulation of the immune system. **(C)** Immune checkpoint blockade – mAbs block the interaction of inhibitory receptors CTLA-4 and PD-1, resulting in the activation of effector T cells (QYResearch) **(D)** Chimeric antigen receptors (CARs) T cells are modified T cells with a recombinant receptor; usually a scFv that redirects the specificity of effector T cells. First generation CARs that only comprised an activation domain were prone to anergy. Due to this signaling failure, second and third generation CARs, incorporating a CD3 chain and cytoplasmic domain of a co-stimulatory receptor, like CD28 were generated. Fourth generation CARs also included constitutive or inducible expression of co-receptors or soluble cytokines together with T cell activating CAR (Golubovskaya and Wu, 2016). **(E)** Bispecific antibodies containing two binding arms one specific for a target antigen and a second arm specific for CD3, thereby bringing T cells into close proximity to target cells and activating T cells while bypassing the need for MHC restricted engagement. **(F)** Vaccines – Introduction of non-infectious component to stimulate activation of T cells and development of memory immune cells.

There is still no clinically approved vaccine for fungal infections; however, there are a growing number of candidates in pre-clinical development and at various phases of clinical trials (Health, 2012). Fungal vaccine strategies have mainly prioritized CD4+ T cell and B cell stimulation, thereby enhancing protection mediated by these defense mechanisms (Nanjappa and Klein, 2014). This involves targeting common antigens that are shared among a variety of medically relevant fungi. One example is the β-1,3-D-glucan, a key component of the fungal cell wall (Armstrong-James et al., 2017). Mice immunized with this glucan, conjugated to diphtheria toxin, elicit strong antibody responses that are protective against models of aspergillosis, candidiasis and cryptococcosis. Moreover, immunizing mice with antigen encapsulated in glucan, also stimulate antigen-specific antibody and T cell responses. Preclinical studies involving the vaccination of mice with an attenuated strain of *Blastomyces dermatitidis* showed protection against subsequent challenge from virulent strains (Wüthrich et al., 2003). Even upon CD4+ T cell depletion, protection was seen due to the emergence of protective CD8+ T cells. More recently, the focus of fungal vaccines has been on subunit vaccines and the two containing *Candida albicans*-derived proteins, currently in phase I/II clinical trials, were found to confer immunogenicity (De Bernardis et al., 2012; Schmidt et al., 2012).

Majority of FDA approved vaccines work by stimulating a CD4+ T cell dependent antibody response. However, for cancer and intracellular infections a CD8+ cytotoxic T cell (CTL) response is required for protection. Thus far, the primary limitation of such vaccines has been a lack of ability to prime and activate disease specific-CD8+ T cells necessary for protection. This is attributed to the inherent mechanism by which external foreign antigens administered in a vaccine are presented to the immune system via MHCII, thus stimulating

TABLE 1 | Vaccine types and examples.

Type	Description	Advantages	Disadvantages	Disease	Reference
Live attenuated	Less pathogenic strain of microbe	Induction of long lived responses	Adverse effects in immune-compromised	MMR, Smallpox	World Health Organization, 2017
Inactivated	Pathogens killed through chemical treatment or heat	Cannot replicate	Often induces weaker immune responses than other methods	Cholera	Bi et al., 2017
Subunit	A vaccine designed to induce immune responses to the most dominant epitopes of a pathogen	High level of safety	Multiple doses are usually required	Hepatitis B	Van Den Ende et al., 2017
Toxoid	Induces an immune response to the pathogens toxin	Strong antibody response and long-lasting antigen specific memory	Booster doses are often required	Diphtheria	World Health Organization, 2017
Conjugate	A strong antigen (often a protein) covalently attached to a weak antigen (often a bacterial polysaccharide)	Safe for use in infants. Long lasting immune responses	Expensive to produce	Bacterial Meningitis	Wasserman et al., 2018
DNA	Fragments of DNA encoding antigens for specific pathogens are injected for endogenous production	Non-infectious, no cold chain required	Limited to protein antigen production	Experimental	Vetter et al., 2018
Recombinant	Recombinant DNA delivered through bacterial or viral vaccine vectors	Strong immune responses	Anti-vector immunity can lead to adverse effects	HPV	Sipp et al., 2018

CD4+ based responses. Recently, these challenges have been addressed by photochemical internalization (PCI) which has been shown to deliver antigenic peptides to the cytosol of antigen presenting cells by disruption of the endosomal membranes using a co-endocytosed photosensitizer (Haug et al., 2018). The 30-fold increase in MHCI presentation and resulting 30–100-fold increase in disease specific CD8+ activation in comparison to antigen stimulation alone, is promising. Furthermore, preclinical *in vivo* experiments involving the induction of antigen-specific CTL responses against cancer antigens in mice confirmed the efficacy of PCI as a peptide-based vaccine. Strategies such as these are not only applicable to cancer by have great potential to improve various peptide vaccines especially for diseases like HIV where an appropriate CTL response is required for protection.

Enhancing T Cell Activation

Successful T cell activation requires two signals: T cell receptor (TCR) binding to peptide-MHC complex and binding of T cell co-receptors with counter-receptors on APCs. T cell exhaustion is a state of T cell dysfunction that arises during persistent antigen exposure and/or inflammation and is associated with many chronic infections and cancer. It is characterized by persistent expression and diversity of inhibitory receptors, progressive and hierarchical loss of effector cytokines, metabolic imbalances, altered expression and function of transcription factors, failure to convert to quiescence and inability to acquire antigen-independent memory T cell homeostasis (Wherry, 2011; Schietinger and Greenberg, 2014). Thus, T cell exhaustion is a mechanism of immune evasion essentially leading to the inefficient control of infection and tumors. Importantly, exhausted T cells are not inert but sustain suboptimal, essential functions that encumber ongoing pathogen infection or tumor progression (Wherry and Kurachi, 2015).

This state of T cell dysfunction was initially described in the murine lymphocytic choriomeningitis virus (LCMV) model (Zajac et al., 1998), and has since been observed in animal and human models during chronic viral infections such as HIV (Kaufmann et al., 2007), Hepatitis C virus (HCV), Hepatitis B virus (HBV) (Guidotti and Chisari, 2006), simian immunodeficiency virus SIV (Zeng et al., 2011), along with various cancers (Lee et al., 1999), malaria infections (Illingworth et al., 2013) and *Mycobacterium tuberculosis* infection (Khan et al., 2017). Major advances have been made in three significant areas including inhibitory receptors and negative regulatory pathways, the absence of canonical memory T cell properties and maintenance, and the origin and homeostasis of exhausted T cells (Kim and Ahmed, 2010; Paley et al., 2012; Crawford et al., 2014). As such, there has been considerable interest in avoiding or reversing this dysfunctional state of exhaustion, and consequently, restoring or augmenting immune responses to effectively control infection or malignancies (Pauken and Wherry, 2015).

Modulating Intrinsic Inhibitory Receptors

To minimize tissue damage, negative pathways of immunoregulation such as those used in immune checkpoint inhibition offer a plethora of inhibitory pathways that are essential for preserving self-tolerance and moderating the duration and magnitude of immune responses in peripheral tissues (Pardoll, 2012). These pathways are inherently involved in T cell exhaustion and involve cell surface inhibitory receptors that modulate autoreactivity and immunopathology (Sharpe et al., 2007). Inhibitory receptors are transiently expressed in functional effector T cells, however, increased and prolonged expression can be considered a hallmark of exhausted T cells (Wherry, 2011). Pathogens and tumors promote immune checkpoint-mediated inhibitory interactions to escape immune

control. Many of these checkpoint proteins have already been studied extensively and will be addressed in relation to cancer, HIV, TB, and malaria infections.

Within cancer immunotherapy, cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein 1 (PD-1) are the canonical immune-checkpoint receptors that illustrate ligand–receptor interactions between T cells and APCs that modulate the T cell response to antigen (MHC- mediated molecule complexes that are recognized by the TCR). CTLA-4 is a co-inhibitory receptor expressed on T cells that competes with CD28, a T cell co-stimulatory receptor, for binding on shared identical ligands CD80 (B7.1) and CD86 (B7.2) (Pardoll, 2012). Binding of CTLA-4 inhibits the activation of T cells, whereas binding of CD28 to CD80/CD86 activates the T cell responses (Dyck and Mills, 2017). CTLA-4 expression on activated T cells reduces CD28 co-stimulation by; outcompeting CD28 for binding to B.7 ligands as it has a greater affinity for the B7 ligands and, possibly also by depletion of CD80/CD86 via *trans*-endocytosis (Qureshi et al., 2011). Subsequently, CTLA-4 controls the activation state of effector T cells and initiates tolerance by inhibiting cytokine production and activating transcription factor STAT5 (Srahna et al., 2006). Furthermore, CTLA-4 plays a physiological role in the negative modulation of helper T cell activity, stimulating the suppressive function of T regulatory (Treg) cells (Topalian et al., 2015).

PD-1 has also been extensively studied and predominantly modulates effector T cell activity within tissue and tumors and is therefore more widely expressed than CTLA-4; it is induced on activated T cells, B cells, macrophages, dendritic cells and Tregs. The potent inhibitory signal is provided through its interaction with programmed death-ligand 1 (PD-L1) and/or PD-L2, whereby PD-1 inhibits kinases involved in T cell activation (Latchman et al., 2001; Francisco et al., 2009; Araki et al., 2013).

CTLA-4 is constitutively expressed on all cancer cells but found to be highly expressed on breast tumor cells and is implicated in immune dysregulation and associated with poor prognosis (Mao et al., 2010; Yu et al., 2015; Yuan et al., 2015). PD-L1 is expressed on a majority of tumor cells, and tumor-derived APCs, signifying the principal role of the PD-1 pathway in tumor immune evasion associated with poor prognosis (Hirano et al., 2005; Currie et al., 2009). There is gathering evidence that inhibitory receptors have a significant role in modulating T cell responses during chronic viral infections, including HIV, as well as during acute phase malarial infections (Trautmann et al., 2006; Kaufmann et al., 2007; Crawford and Wherry, 2009; Gonçalves-Lopes et al., 2016). Furthermore, CTLA-4 is expressed on Tregs and CD8+ T cells, while PD-1 is expressed on neutrophils, Tregs and natural killer (NK) cells of patients infected with *M. tuberculosis*, however, over a course of standard anti-TB therapy, this expression declines as responsiveness of effector T cells to antigenic stimulation improves (Merlo et al., 2001; Li et al., 2015; Zumla et al., 2016a).

Lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin-3 (TIM-3), band T lymphocyte attenuator (BTLA) and T-cell immunoglobulin and ITIM domain (TIGIT) are highly expressed on exhausted T-cells and represent the next generation of immune checkpoints that are under evaluation for the treatment of chronic diseases including cancer and chronic viral infections such as HIV (Topalian et al., 2015; Chew et al., 2016; Dyck and Mills, 2017; Grabmeier-Pfistershammer et al., 2017; Manieri et al., 2017). Interestingly, Anderson et al. (2016) recently proposed a model describing LAG-3, TIM-3, and TGIT as second tier co-inhibitory molecules that play a specific role in regulating immune responses at sites of tissue inflammation, distinct to first tier co-inhibitory receptors CTLA-4 ad PD-1 which are primarily involved in maintaining self-tolerance. This hierarchal ranking of co-inhibitory receptors provides a basis for selecting synergistic receptor blockade combinations that will result in improved T cell and NK cell effector function (Anderson et al., 2016).

Immune checkpoint inhibitors

As immune checkpoints are mediated by ligand–receptor connections, these pathways can be easily blocked by monoclonal antibodies (mAbs) or regulated by recombinant forms of ligands or receptors (Pardoll, 2012). As such, PD-1 and CTLA-4 present ideal targets for immune checkpoint inhibition therapies, one of the most noteworthy immunomodulatory therapies of current times. In contrast to vaccines which activate immune cells to attack specific targets, this type of therapy removes the natural disruptions of the immune system (Postow et al., 2015).

Subsequent to the clinical success of Ipilimumab, an anti-CTLA4 mAb for the treatment of advanced melanoma which acts by blocking the binding of CTLA-4 expressed on T cells to CD80/86 ligands expressed on tumor cells (Kyi and Postow, 2014), an onslaught of immune checkpoint inhibitors have received accelerated FDA-approval. This includes Pembrolizumab and Nivolumab, both recently approved mAbs which bind the inhibitory receptor PD-1, blocking its interaction with ligands PDL-1/PDL-2, thus releasing T cells for cytotoxic action (Nguyen and Ohashi, 2015). These immune-checkpoint inhibitors are effective for the treatment of melanoma, renal cell carcinoma, and non-small lung cancer (Robert et al., 2015a,b). In addition to targeting inhibitory receptors, blockade of the ligand PD-L1, by Atezolizumab, Durvalumab, and Avelumab, has demonstrated remarkable efficacy in lung, bladder, urothelial carcinoma and other cancers (Massard et al., 2016; Balar et al., 2017; Hsu et al., 2017; Rittmeyer et al., 2017).

However, most patients do not respond to immune checkpoint monotherapy; attributed to the absence of tumor-infiltrating effector T cells and tumor microenvironment properties. As checkpoint inhibitors target ligands/receptors of immune regulation, expression of these targets on target cell membranes is an essential prerequisite for the treatment to be effective. More than 50% of cancers induce a quiescent immune tumor microenvironment which is naturally depleted of immune effector cells and thus, lacks sufficient targets for checkpoint immunotherapy to be effective. Recently, the high levels of circulating exosomal PD-L1 was shown to correlate with immune dysfunction, suggests a role as a predictive biomarker for patient responses to anti-PD-1 therapy (Chen et al., 2018). Furthermore, combining checkpoint therapies with cancer vaccine strategies like PCI has the potential to significantly enhance the efficacy of

both therapies. Cancer vaccines stimulate CD8+ T cells which express checkpoint targets; consequently the normal inhibitory effects of checkpoints would be reduced by checkpoint therapy, thereby further enhancing CD8+ activation. The synergistic effects of cancer vaccines and checkpoint combination therapy has been demonstrated (Duraiswamy et al., 2013; Karyampudi et al., 2014; Fu et al., 2015; Soares et al., 2015). The positive pre-clinical results of some of these studies has initiated 2 clinical trials assessing various vaccines in combination with nivolumab for the treatment of pancreatic cancer (Kleponis et al., 2015).

In addition to cancer vaccines, combinations of other immunotherapies with checkpoint inhibitors, holds promise for the rational development of curative therapies. The simultaneous blocking of the CTLA-4 and PD-1 pathways, given their distinct and non-overlapping mechanisms of action, has been the most successful combination tested thus far, resulting in tumor regression in patients with advanced melanoma, demonstrating the synergistic effects of combining checkpoint inhibitors (Wolchok et al., 2013; Hodi et al., 2016; Weber et al., 2016). The safety and efficacy of various combinations of immune checkpoint inhibitors with cytotoxic chemotherapy (referred to as immunochemotherapy), with small-molecule inhibitors or with radiation therapy are also under intense clinical investigation[1] (Mahoney et al., 2015; Li et al., 2017). Recently, prolonged progression-free survival of patients with metastatic triple-negative breast cancer treated with a combination of the chemotherapy drug nab-paclitaxel and the PDL-1 inhibitor Atezolizumab, was demonstrated in a Phase 3 trial (Schmid et al., 2018).

The development of primary or acquired resistance remains clinically pertinent. Recent studies suggest that inactivation of JAK1 and JAK2 by cancer cells results in resistance to interferon gamma which contributes to checkpoint resistance and immune escape. In addition, resistance is likely driven by multiple other mechanisms including loss of function of CD8+ T cells due to a lack of mutational or shared antigens required for T cell recognition, reduced antigen presenting components, tumor cell induced T-cell signaling inhibition and depressed sensitivity to apoptotic inducting molecules (Shin et al., 2016; Zaretsky et al., 2016). Methods of reducing these types of resistances would need to be considered for the development of new checkpoint therapies.

In addition, as with other therapies, immune checkpoint therapies are associated with a high rate of toxicities, characterized as immune-related adverse events (IRAEs) and include diarrhea/colitis and hepatic/dermatologic/endocrine toxicities. These adverse effects, although severe, are manageable with immunosuppressive agents such as corticosteroids, which in turn, may also be associated with other short-term side effects (Michot et al., 2016; Spain et al., 2016).In chronic infection, the preclinical development of checkpoint inhibitors has had mixed results, suggesting intrinsic differences in the mechanisms of T cell dysfunction between chronic infection and cancer. In *in vitro* settings, CTLA-4 blockade enhanced HIV-specific CD4+ T cell function, PD-1/PD-L1 blockade improved HIV-, HCV-, and HBV-specific CD8+ T cell function, while CTLA-4/PD-1 combination blockade reversed HCV-specific CD8+ T cell exhaustion (Kim and Ahmed, 2010). However, in chronic LCMV-infected mice, neither CTLA-4 nor LAG-3 blockade improved T cell function or viral load, whereas PD-1 blockade alone and in combination with LAG-3 blockade effectively salvaged T cells from exhaustion and decreased the virus load (Barber et al., 2006). In the SIV macaque model, CTLA-4 blockade did not enhance viral-specific T cell responses, but rather amplified viral replication at mucosal sites, whereas a recent study demonstrated anti-PD-1 treatment delayed viral rebound after combined antiretroviral therapy (cART) was withdrawn (Cecchinato et al., 2008; Gill et al., 2016). The *ex vivo* dual blockade of co-inhibitory receptor TIGIT and ligand PD-L1 restored HIV-specific CD8+ T cell effector responses (Chew et al., 2016). In HIV-infected patients, treatment with Ipilimumab resulted in reactivation of latently virus-infected CD4+ T-cells, rendering these cells discernable to the immune system (Wightman et al., 2015) (**Figure 2**). Furthermore, a recent Phase I trial demonstrated enhanced HIV-specific CD8+ T cell responses following treatment with an anti-PD-L1 antibody (Gay et al., 2017). Overall, these studies suggest a role for immune checkpoints in limiting T cell responses during HIV infection and the subsequent potential of immune checkpoint inhibitors. Although the anti-PD-1 therapy Nivolumab effectively reduced viral load in HCV-infected individuals, it has been proposed that established virus-specific T cells in the liver is crucial for enhanced T cell responses by PD-1 blockade (Fuller et al., 2013; Gardiner et al., 2013). In patients with pulmonary TB, PD-L1 is highly expressed on *M. tuberculosis*-infected macrophages, and *in vitro* blockade with mAbs induces IFN-γ-initiated killing of infected cells by autologous peripheral blood T cells (Zumla et al., 2016a).

While checkpoint inhibitors have revolutionized cancer immunotherapy there are clearly also many drawbacks associated with this therapy at this stage. The many combination immunotherapy studies involving checkpoint inhibitors will provide much needed information on the molecular mechanisms of resistances and toxicity associated with checkpoint therapy and subsequently assist in improving efficacy. Despite the many issues that still require addressing there have been overwhelming positive results associated with PD1/PDL-1 and CTLA-4 checkpoint inhibition studies demonstrating the potential of checkpoint inhibitors to greatly improve treatment outcomes not only in cancer patients but with other diseases as well, especially in combination with other immunotherapies.

Cytokine Therapy

Cytokines are protein mediators involved in essentially all important biological processes including immunity, cell proliferation and inflammation, wound healing and repair, cell migration, fibrosis and angiogenesis. Cytokines provide crucial signals for fundamental processes involved in a plethora of diseases thus it is intuitive that manipulation of cytokines may alter diseased states both positively and negatively. With the field of cancer, multiple mice studies have demonstrated the anti-tumor effects of cytokines leading to cytokine-based therapies

[1] www.clinicaltrials.gov

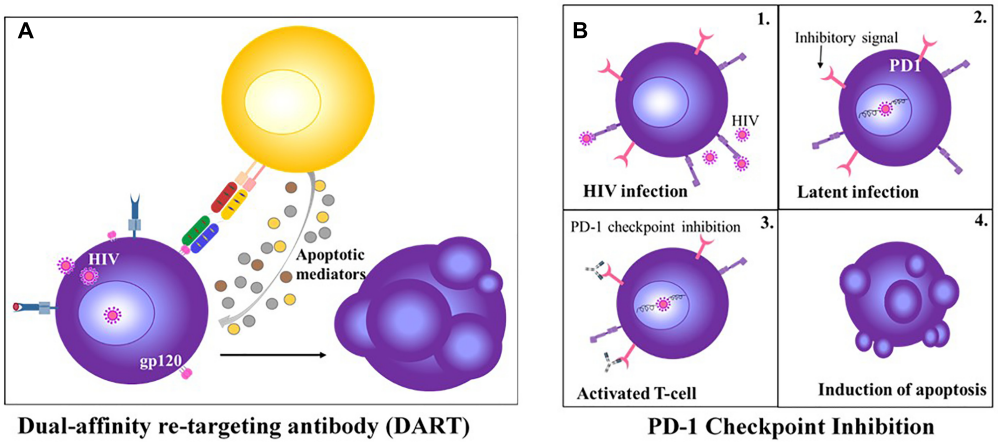

FIGURE 2 | HIV-targeting T cell Therapies. **(A)** Anti-CD3 and anti-gp120 DART treatment redirects CD8+ T cell to kill HIV infected CD4+ T cells (Perreau et al., 2017). **(B)** PD-1 check point inhibition of latently infected CD4+ T cell results in re-activation of the T cell and induction of apoptosis (Wykes and Lewin, 2018).

for cancer treatment. Cytokines such as IL-1, IL-12, IL-15, IL-18, IL-21, and GM-CSF are just some examples of cytokines that have entered clinical trials for treatment of patients with advanced cancer (Feldmann, 2008). Recently, administration of pro-inflammatory cytokines IL-12 and IL-18 demonstrated a reversal in MHC-1 deficient tumor induced NK cell anergy. Treatment increase NK cell activity which mediated antitumor responses resulting in improved survival of mice challenged with MHC-1 deficient tumors (Ardolino and Raulet, 2016).

Neutralization of suppressive cytokines like TGF-β and IL-10 have shown promising results and will the remain the focus of this section. IL-10 is expressed by diverse cell types such as dendritic cells (DCs), monocytes and/or CD4+ T cells, and its upregulation is associated with disease progression during chronic infections as well as poor prognosis in cancer patients (Said et al., 2010; Richter K. et al., 2013). Furthermore, IL-10 has been implicated in promoting human visceral leishmaniasis, by restricting Th1 cell-type responses and/or neutralizing parasitized tissue macrophages and, in parallel, compromising responsiveness to chemotherapy (Murray et al., 2002). During *in vivo M. tuberculosis* infection, IL-10 has been shown to be a negative regulator of the immune response, contributing to chronic infection (Pitt et al., 2012). In addition, fungal clearance is impaired by IL-10, as demonstrated by *Cryptococcus neoformans* infections, the leading cause of fatal mycosis in HIV+ individuals (Murdock et al., 2014).

Blockade of the IL-10 receptor (IL-10R) with anti-IL-10 mAbs enhances control of LCMV infection and restores T cell responses (Brooks et al., 2006; Ejrnaes et al., 2006), while simultaneous blockade of IL-10 and PD-1 reverses CD8+ T cell exhaustion, improving HIV control (Wherry, 2011). Furthermore, simultaneous IL-10 blockade during therapeutic DNA vaccination enhanced clearance of chronic LCMV infection (Ni et al., 2015). In the mouse model, the use of antimony, the conventional chemotherapy for visceral leishmaniasis, in conjunction with anti-IL-10R treatment permitted a considerable reduction in duration and dosage of treatment (Weiner et al., 2012). During *M. tuberculosis* infection, antibody blockade of IL-10R specifically during BCG vaccination improves Th1 and Th17 responses, and IFN-γ and IL-17A expression *in vivo*, resulting in significantly enhanced protection against aerogenic challenge with *M. tuberculosis*, compared to BCG vaccination alone (Pitt et al., 2012). In the murine model for *C. neoformans* infection, IL-10 blockade amplified various effector mechanisms, including improved accumulation of CD4+ T cells, B cells, Th1 and Th17 cells, but not CD8+ T cells, and, furthermore, effectively abrogated dissemination of infection to the brain (Murdock et al., 2014).

TGFβ is often over-expressed in several tumors and has been implicated in promoting growth, progression, and metastatic potential of advanced cancers (Jakowlew, 2006; Tian et al., 2011; Drabsch and Ten Dijke, 2012). Various TGFβ pathway inhibitors including small molecules, anti-TGFβ antibodies and synthetic small peptides have been reported to restore immune response and increase the efficacy of combination immunotherapy (Neuzillet et al., 2015). During chronic viral infection, both murine and human, TGFβ signaling has been implicated in T cell exhaustion (Wherry and Kurachi, 2015). However, in contrast to cancer therapy, the systemic administration of antibody- and inhibitor-based strategies to prevent TGFβ signaling during chronic viral infection have proven futile, at least in the murine model, the reasons for which remain unclear (Boettler et al., 2012; Garidou et al., 2012).

Given that cytokines play such a multifunctional role in different biological systems, systemic inhibition of any given cytokine will have wanted and unwanted effects, necessitating the need to make this therapy more targeted. Cytokines have differing signaling cascades that may be exploited for selecting a target to inhibit their functions. TNF is an ideal example; its two forms, membrane-bound or secreted, each interact with distinct receptors, TNFR1 or TNFR2, differentially expressed on multiple cell types (Drutskaya et al., 2018). Depending on the source of cells secreting TNF, and the neighboring cytokines, it may act as pro-inflammatory or anti-inflammatory. Thus, targeting

selected downstream pathways may provide a more controlled treatment option than systemic TFN inhibition. TNFR1 is responsible for majority of TNF pro-inflammatory functions which signals via NF-κB and AP-1 resulting in expression of other pro-inflammatory cytokines like IL-1 and IL-6 (Simmonds and Foxwell, 2008). A TNFR1-specific antibody, ASTROSAB has demonstrated effective inhibition of TNF induced IL-6 and IL-8 secretion in cell culture (Richter F. et al., 2013). A second study in chimeric humanized mice demonstrated that the inhibition if TNFR1 by a human TFNR1 silencer (TROS) lowered TNFR1-induced proinflammation reactions but did not alter TNFR2 signaling (Steeland et al., 2015). Studies looking at the source of TNF found that TNF produced by myeloid cells play a role in pathogenesis of experimental arthritis and autoimmune encephalomyelitis (Grivennikov et al., 2005; Kruglov et al., 2011). Contrastingly TNF secreted by T cells demonstrated protection against *M. tuberculosis* infections (Allie et al., 2013). TNF perfectly demonstrates the pleiotropic functionality typical of pro-inflammatory cytokines, balancing regulatory functions and pro-inflammatory signaling to maintain homeostasis of the immune system. Thus, systemic knock-out of TNF while beneficial in controlling certain inflammatory and autoimmune diseases, has major drawbacks like increased risk of severe infections, cardiovascular, neurological and immunological complications. Currently antibody-based immunotherapies with dual affinities for TNF and specific cell types are in development; one example is a myeloid-specific TFN inhibitor (MYSTI) that binds to both myeloid cells and TNF thus only restricting myeloid-secreted TNF activity. MYSTI demonstrated better protection against LPS/D-gal hepatotoxicity in humanized mice compared to anti-TNF treatment (Efimov et al., 2016).

Multiple other recombinant cytokines have already been approved for treatment of patients; IL-2 for cancer (Baron and Narula, 1990; Dutcher, 2002), several IFNα derivatives for cancer and viral infections (Syed and Ahmadpour, 1998; Melian and Plosker, 2001; Marcellin et al., 2004), IL-11 thrombocytopenia induced by chemotherapy (Isaacs et al., 1997), IFNβ for multiple sclerosis and IFNγ for osteoporosis and cancer (Cutler and Brombacher, 2005). Understanding the molecular mechanisms of action of different cytokines in the context of a specific disease will be an essential prerequisite for developing more targeted approaches to anti-cytokine/cytokine therapy. Targeting specialized processes rather than systemic cytokines has the added benefit of restricting toxicity and reducing interference with non-related biological processes. With the exponential development in antibody technologies it is likely that cytokine therapies in the near future might all be targeted to specific functions rather than cytokines overall.

T Cell Engineering

Recruitment and Activation of Cytotoxic T Cells

Bispecific antibodies (BsAbs) have two binding sites, each with a unique antigen specificity. Multiple formats of bsAbs exist including bi-specific T cell engagers (BiTEs), dual-affinity re-targeting antibodies (DARTs) and Tandem Diabodies. The most commonly used format is a tandem scFv, of which BiTEs are the most clinically advanced in oncology and are distinguished for their binding specificity to the TCR complex. These antibodies comprise both an antigen-specific arm and a T cell activating arm via generic CD3-interaction, which bypasses the requirement for MHC-I-restricted engagement. The activated T cells are redirected to target cells through the recognition of a pathogen/tumor-specific antigen by BiTE antibodies (Wolf et al., 2005; Bargou et al., 2008). BsAb mechanism of action is via creation of a temporary cytolytic synapse between the T cell and the target cell and the ensuing activation and proliferation of T cells elicits target cell lysis (Offner et al., 2006). The immunological synapses created by BiTEs are indistinguishable from those induced during natural CTL responses. The size of the synapse is determined by the distance between anti-CD3 and anti-antigen moieties and is a vital measure of cytotoxic potency; the smaller the synapse the closer the T cell membrane is to the target cell and since delivery of apoptotic mediators, such as granzyme proteases and perforin, is conducted by passive diffusion, this results in more efficient target-cell lysis (Moore et al., 2011).

BsAbs have been posited for the treatment of malignancies and inflammatory conditions for decades but have only recently begun to yield results (Fan et al., 2015). As such, catumaxomab (Removab) was the first bispecific trifunctional drug approved for the treatment of malignant ascites (Linke et al., 2010; Sedykh et al., 2018). Thereafter, blinatumomab (Blincyto®), a CD19 × CD3 BiTE, gained accelerated FDA-approval for the treatment of relapsed and/or refractory (R/R) acute lymphoblastic leukemia (ALL), and numerous other BiTE candidates are currently in phase I clinical trials for the treatment of various cancers (Przepiorka et al., 2015). Furthermore, as T cell exhaustion due to high antigen load has been suggested, concurrent PD-1 or PD-L1/2 blockade is under investigation (Köhnke et al., 2015). Studies are now focused on the efficacy of BiTEs for solid tumor malignancies, in which penetration of the tumor by BiTEs and T cells are at the forefront (Yuraszeck et al., 2017).

Given the success of bsAbs in cancer immunotherapy, similar strategies to eliminate pathogen-infected cells have been adopted. Following early studies on natural HIV receptor CD4 and an anti-CD3 binding moiety, no further development of bsAbs ensued for more than 20 years (Berg et al., 1991). Recently, DARTs, an alternative tandem ligand format, have been exploited for T cell–mediated cytolysis of latently HIV-infected cells (**Figure 2**). These bsAbs comprise anti-Env and anti-CD3 arms which simultaneously bind to HIV-infected cells and CD3-expressing polyclonal T cells, subsequently inducing CD8+ T cell elimination of HIV-infected CD4+ T cells *in vitro*, reducing virion levels *ex vivo* and mediating clearance of latent HIV reservoirs from resting CD4+ T cells (Sloan et al., 2015; Sung et al., 2015). The efficacy of DARTs on the HIV-1 reservoir *in vivo* remains undetermined. Apart from DARTs, BiTEs that target the HIV-1 envelope protein gp120 and CD4 have been recently described with potent antiviral activity *in vitro* and *ex vivo* (Brozy et al., 2018). In addition to HIV, bsABs have been developed to redirect effector T cells to HBV- and

human cytomegalovirus (HCMV)-infected cells, *in vitro* (Meng et al., 2018).

The appeal of BsAbs lies in their synergistic effects attributed to their ability to simultaneously target several antigens (Sedykh et al., 2018). Although a promising therapeutic strategy, BsAbs are not without their challenges. In clinical settings, continuous delivery is necessary due to the rapid clearance of these agents, related to their small size and consequently, short half-life. Furthermore, blinatumomab is associated with severe adverse events including neurotoxicity and cytokine release syndrome, which although reversible and manageable by treatment with corticosteroids and/or Tocilizumab, an interleukin-6 receptor antagonist, discontinuation of use by patients is still common (Thakur et al., 2018). In addition, clinical success in solid tumors remains to be demonstrated, which may provide insights into the applicability of these agents against intracellular pathogens.

Reprogramming T Cells

Chimeric antigen receptor (CAR) T cells are modified T cells with a recombinant receptor, comprising a scFv that redirects the specificity of effector T-lymphocytes, fused to a transmembrane and signaling domain that mediates T cell activation without MHC engagement. By bypassing the standard kinetics of active immunization, CARs rapidly generate tumor-targeting T cells and modulate T cell proliferation and persistence in the tumor microenvironment (Ho et al., 2003; Sadelain et al., 2003). The therapy involves the *ex vivo* expansion of the patient's peripheral blood T cells, genetically modifying the cells to express the CAR and re-infusing the engineered T cells back into the patient (Gattinoni et al., 2006).

First generation CAR T cells comprising a targeting scFv and chimeric CD3/FcεRIγ have undergone phase I clinical trials for the treatments of ovarian cancer (scFv-FcεRIγ) (Sadelain et al., 2003), refractory follicular lymphoma (scFv-CD3ζ) (Ho et al., 2003), neuroblastoma and melanoma (scFv-CD3ζ) (NCT00085930) (Zhao et al., 2006; Birkholz et al., 2009). In theory, any cell surface molecule, even those that elicit tolerance due to self-reactivity, may be targeted by CAR modified T cells, thereby increasing the natural T cell repertoire. Transgenic mice studies with first generation CARs recognized that these CAR T cells were prone to anergy (Brocker and Karjalainen, 1995). Thus, second and third generation CARs comprising an activating CD3 domain and cytoplasmic domain co-stimulatory receptors like CD28, 4-1BB, DAP10, ICOS or OX40 were generated. These CARs demonstrated superior activity as confirmed by a comparative study in patients treated with CD28/CD3ζ or CD3ζ-only CAR (Savoldo et al., 2011).

Chimeric antigen receptor T cell immunotherapy has achieved impressive clinical outcomes for the treatment of hematological malignancies, specifically B cell acute lymphoblastic leukemia (ALL) and lymphoma (Kochenderfer and Rosenberg, 2013; Maude et al., 2014). As such, two CD19-targeted CAR T cell therapies, Tisagenlecleucel (2nd generation) and Axicabtagene Ciloleucel (3rd generation) were recently FDA-approved (Neelapu et al., 2017; Schuster et al., 2017). Majority of cancer-related morbidity and mortality is associated to solid malignancies, however, attempts to apply CAR T cell therapy to solid tumors have been less effective compared to hematologic cancers. This has been attributed to a number of obstacles; the lack of suitable tumor-specific antigens and the subsequent toxicity to normal tissue, inefficient CAR T cell infiltration of tumor barriers, such as the extracellular matrix (ECM), as well as the immunosuppressive tumor microenvironment (Zhukovsky et al., 2016; Srivastava and Riddell, 2018).

As such, genetically modified fourth generation CARs also known as TRUCKs (T cell redirected for universal cytokine-mediated killing) with the ability to secret pro-inflammatory and pro-proliferative cytokines have been developed (Golubovskaya and Wu, 2016). This ability to induce cytokine expression is highly advantageous in the setting of solid tumors where T-cell penetration is poor and due to high phenotypic diversity, many cancer cells remain invisible to penetrating T-cells. The expression of cytokines such as IL-12 induced by TRUCKs provides a cytokine gradient and initiates universal cytokine mediated killing of cancer cells which eradicates cancer cells lacking antigen at the tumor site (Li et al., 2018). Recently, a TRUCK system utilizing the synNotch receptor was developed to express a variety of payloads in response to target antigens (Roybal et al., 2016; Roybal and Lim, 2017). In addition to inducible cytokines this synNotch system can induce adjuvants, checkpoint, bispecific and pro-tumor cytokine antibodies. With this system more anti-tumor cytokines and factors with the potential to remodel the suppressive tumor environment may be co-administered.

The unique obstacles imposed by solid tumors are also associated with brain tumors, although further exacerbated by additional biological complexities. With regards to glioblastoma, the most common malignant brain tumor in adults, recent developments in CAR T cells have addressed these challenges which include penetration through the blood–brain barrier (BBB), inadequate neoantigen presentation and the presence of glioma stem cells (GSCs) and their intrinsic role in drug and radiation resistance [reviewed by (Bagley et al., 2018)]. The poor homing of CAR T cells to target sites (Ager et al., 2016) has recently been addressed by the rational reengineering of CD6 to generate cytotoxic homing system (HS) T cells that readily infiltrate the otherwise restrictive BBB (Samaha et al., 2018). Studies such as these may provide significant insight into engineering T cells to target elusive infectious agents.

While most CAR T cell studies conducted to date are in the context of cancer immunotherapy, Kumaresan and colleagues developed a fungal-specific Dectin-1 C-type lectin receptor. The resulting D-CAR T cell specific for β-1.3-D-glucan showed effective killing of *Aspergillus fumigatus in vitro* and *in vivo* (Kumaresan et al., 2014). This proof-of-principle study shows that CAR technology can encompass C-type lectins, a key innate pattern recognition receptor with broad repertoires against fungal pathogens and thus, may be applied to other infectious diseases (Armstrong-James et al., 2017).

As with other immunotherapies, adverse effects are associated with CAR T cells. These include the on-target toxic effects of cytokine release syndrome (CRS) and neurotoxicity which can be managed by treatment with corticosteroids and/or Tocilizumab (Gauthier and Turtle, 2018). In a recent phase I/II clinical

trial, CRS severity was found to be associated with infectious complications (Hill et al., 2018). Reducing the adverse effects of treatment, improved models to evaluate pre-clinical efficacy and importantly, the ultimate cost implications to patients, will determine the extent to which CAR T cell therapy can be applied (Srivastava and Riddell, 2018). The advances in CAR T cell therapy for solid tumors offers promise for intracellular infectious agents, such as *M. tuberculosis*, for which the inherent heterogeneity of granulomas accounts for poor antimicrobial drug penetration and accumulation (Kiran et al., 2016).

Depletion of Suppressor Cell Populations

Tregs are a subpopulation of T cells that modulate the immune system by reducing activation of effector T cell populations during prolonged inflammatory responses and inducing tolerance to benign molecules and self-antigens. Modern cancer immunotherapy approaches include biologics that selectively deplete suppressor cell populations, like Tregs. One such agent, a diphtheria toxin (DT) (Liedtke et al., 2008)-related interleukin 2 (IL-2) fusion protein, known as DD depletes cells with high expression levels of CD25 (Kiyokawa et al., 1991). DD is comprised of 3 domains; the DT catalytic domain, the DT membrane translocation domain and a substituted human IL-2 instead of the native DT-receptor. The IL-2 moiety allows for selective targeting of IL-2R bearing cells. Upon IL-2 and IL-2R binding, the fusion protein is internalized and transported to the cell cytosol where the catalytic domain inhibits protein synthesis resulting in downstream alterations that ultimately lead to apoptosis (Zahaf and Schmidt, 2017). DD has been approved for treatment of cutaneous T cell lymphoma and subsequent human studies. Results from these studies indicate that in addition to killing CD25+ lymphoma T cells, DD also transiently depleted Tregs (Litzinger et al., 2007). More recent studies assessing DD treatment in Leishmanian-infected mice verified a reduction of lesional Tregs, better lesion resolution and reduced parasite burden (Divanovic et al., 2011).

During *M. tuberculosis* infection, bacilli are engulfed by dendritic cells in the lung and transported to lymph nodes where CD4+ and CD8+ T cells are activated. Tregs have an opposing effect that limit potential protective responses and facilitate bacterial replication (Kursar et al., 2007). Additional studies using specific antibodies to deplete Tregs in *M. tuberculosis*-infected mice showed decreased bacterial burden (Gupta et al., 2017). Similarly to Tregs, another suppressor cell type, myeloid-derived suppressor cell (MDSC) populations also increase during *M. tuberculosis* infection contributing to T cell dysfunction and exuberant inflammation (Knaul et al., 2014). As with Tregs, specific antibody depletion of MDSCs ameliorated pathology and reduced bacterial replication *in vivo*. In addition to the suppressive effect of these cell populations, both Tregs and MDSCs express IL-2R, rendering these cell populations susceptible to DD treatment. *M. tuberculosis*-infected mice treated with DD monotherapy, or in conjunction with standard anti-TB treatment of indicated the effective reduction of Treg and MDSC populations resulting in reduced bacterial burdens (Gupta et al., 2017). While no studies have yet been conducted in the patients with TB, these studies suggest that IL-2 receptor targeting might be a viable treatment option in the future and should be validated in clinical trials.

Although the role of Tregs in the pathogenesis of HIV and HCV infections remains controversial, several studies have indicated Treg-mediated suppression of effector T cell responses in these diseases (Li et al., 2008). As such, the Treg depletion mouse model (DEREG), which expresses the diphtheria toxin (DT) receptor under the control of the *Foxp3* promoter, has shown the reactivation of virus-specific CD8+ T cells following the transient depletion of Tregs during chronic viral infection (Kim et al., 2007; Lahl et al., 2007; Dietze et al., 2011; Berod et al., 2012). The limitations to DEREG, including DT-mediated toxicity, warrants further studies to investigate the role of Tregs during chronic viral infection (Christiaansen et al., 2014).

ANTIBODY/LIGAND-BASED THERAPIES

Selecting the Ideal Ligand

The variety of mechanisms of action that exist to accomplish ligand-mediated control of disease suppression designates this approach an attractive one. The distinct structure of mAbs/natural ligands define their highly efficient function in immunity and underpins antibody/ligand engineering and design. The aim of ligand-targeted therapeutics (LTT) is to neutralize or eliminate pathogenic infections and/or malignant cells (**Figure 3**). This is done by three major mechanisms of action: (a) inhibiting the function of certain molecules, (b) targeting particular cells or (c) acting as signaling molecules (Brekke and Sandlie, 2003).

The complex set of interaction that occur between antibodies/ligands and their cognate receptors, marks choosing an appropriate targeting ligand a crucial step in developing successful targeting applications. Multiple variables should be accounted; the level of receptor expression, the binding affinity of the targeting molecule (Damelin et al., 2015), a role for receptor-mediated internalization, the use of non-antibody based ligands and full antibody or antibody-fragment (Kovtun et al., 2006). For most therapies, the target antigen/receptor should ideally be expressed in high density on the target cell surface to permit accurate selectivity (Beck et al., 2017). In addition, avoidance of a high degree of target heterogenicity may limit off-target effects. Importantly, targets which are shed are not favored as free targets may compete with cell-bound targets thus reducing the therapeutic effects of a treatment (Allen, 2002).

Non-antibody ligands such as Fas, tumor necrosis factor (TNF) and TFN-related apoptosis-inducing ligand (TRIAL) are often easily available, relatively cheap to manufacture and easy to handle (Mathew and Verma, 2009). The main disadvantage is their relatively non-selective expression on diseased cells, making antibodies the preferred targeted delivery agent (Allen, 2002). Furthermore, recent advances in antibody engineering and phage display have improved the identification and selection of antibodies/antibody derivatives with a high degree of specificity and wide range of binding affinity for target tissue/cells (Zhao et al., 2016).

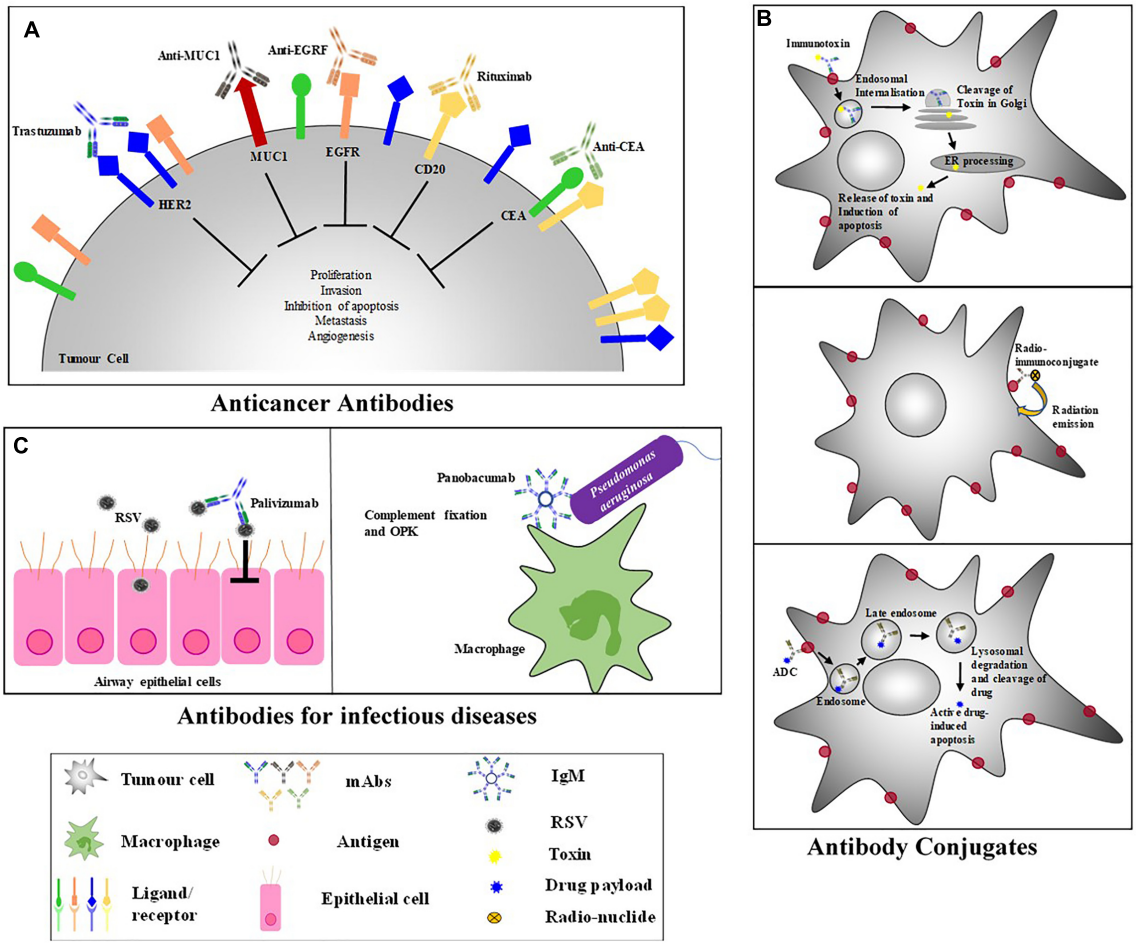

FIGURE 3 | Antibody-Based Therapeutic Strategies. **(A)** Anticancer antibodies eliminate cancer cells and cause tumor destruction by targeting cancer antigens. **(B)** Antibody-conjugates – (i) Immunotoxins: bind to a surface receptor of an infected cell, undergo endocytosis and intracellular trafficking to the cytosol where most toxins induce cell death; (Becker and Benhar, 2012) (ii) ADCs: combine the specificity of mAbs with the cytotoxic potential of drugs and binds to internalizing receptors on target cell and are taken up by endocytosis; Once in the cell, ADCs undergo cellular trafficking to a lysosome where lysosomal degradation results in the cleavage and release of the active drug into the cellular cytoplasm where the drug induces apoptosis; (Scotti et al., 2015) (iii) Radioimmunoconjugates: antibodies attached to a radioactive molecule, once the antibody binds the target cell, the radio-particle's radiation interacts with target cells, resulting in cell death. **(C)** Anti-viral antibodies – to eliminate a viral inhibition of cell infection, viral replication, cell-cell transmission, viral release as well as mediated killing of infected cells needs to occur; Palivizumab is a neutralizing antibody that binds to RSV preventing virus-host cell interactions (Groothuis and Nishida, 2002). Most antibacterial therapeutic mAbs function by inducing complement fixation and opsonophagocytic killing (OPK) of target bacteria; Panobacumab induces macrophage OPK of *Pseudomonas aeruginosa* (Que et al., 2014).

The use of whole antibodies is advantageous, allowing increased binding avidity that occurs through multiple binding sites on one molecule. Additionally, antibodies possess an Fc domain which engages with other immune cells that potentiate the natural immune response by antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Whole antibodies may also be more stable that antibody fragments over prolonged storage periods (Allen, 2002). Despite the advantages, whole antibodies also have their limitations; they commonly bind to normal tissues via Fc receptors, specifically on macrophages resulting in high liver and spleen uptake that can cause increased immunogenicity. Fab and scFv (single-chain variable fragments) lack both an Fc domain and complement-activating region thus reducing the potential for immunogenicity. Their smaller size also permits better cellular penetration which is a desirable effect of certain treatments. Furthermore, scFvs are attractive choices due to ease of identification and production, for example using phage display or *Escherichia coli* fermentation, as well as their reduced immunogenicity (Ahmad et al., 2012).

Therapeutic Monoclonal Antibodies

Monoclonal Antibody (mAb) therapy has been studied and developed for the treatment of cancer, more so than for any other disease area. Subsequently, the trials and errors and ensuing solutions encountered in the cancer field, have paved the way and advanced antibody-based therapies. These include the initial complications associated with administering mouse

hybridoma-derived antibodies to humans and the human anti-mouse antibody (HAMA) response which was subsequently resolved by chimerization and humanization, using recombinant DNA technology (Morrison et al., 1984; Jones et al., 1986). Thus, after years of research, a variety of mAb-based approaches that mediate their antitumor effects through diverse mechanisms have been developed (Weiner, 2015). Attesting to the success of these strategies, numerous mAbs have received clinical approval for the treatment of various cancers, as reviewed by (Kim and Kim, 2015).

The inhibitory activity of mAbs is conducted by preventing soluble mediators like growth factors and cytokines from reaching their target receptors. This is achieved by binding to the mediator or its cognate receptor. Targeting involves directing antibodies to a particular cell population where Fc receptor functions are elicited or to which effector moieties are transported. Subsequently, antibody signaling is conducted by cross linking of receptors related to programmed cell death or by specific receptors that function as agonists for activation of specific cell types (Brekke and Sandlie, 2003).

Antibody therapy for infectious diseases can be traced back more than 100 years ago to serum therapy for the neutralization of bacterial toxins (Graham and Ambrosino, 2015). In comparison to neutralization of toxins, prevention of viral diseases with the use of mAbs is more complex. To eliminate a viral infection a number of events need to occur, these include inhibition of: cell infection, viral replication, cell–cell transmission, viral release as well as mediated killing of infected cells (Parren and Burton, 2001; Burton, 2002). A typical immune response to such an infection results in the generation of specific polyclonal antibodies. Some of these antibodies are neutralizing others are not. The overall outcome, with a combination of blocking, neutralizing and eliminating antibodies might indeed be effective protection. The anti-RSV mAb Palivizumab is approved for high risk infants, and is clinically effective prophylactically, but not therapeutically (Hu and Robinson, 2010). As such, super-antibodies are a new generation of highly potent and/or broadly cross-reactive mAbs that offer prophylactic and therapeutic possibilities for viral infections. This includes highly antigenically variable viruses such as HIV, influenza virus, and Ebola virus, the treatments of which by super-antibodies, are under clinical evaluation (Walker and Burton, 2018).

As with most antibodies, surface antigens are attractive targets for antibacterial mAbs. The key features of surface-specific antibacterial mAbs are complement fixation for engagement of the host immune system and opsonophagocytic killing (OPK). Panobacumab, one of the most clinically advanced antibacterial mAbs, is a human anti-*Pseudomonas aeruginosa* LPS serotype O11 (Horn et al., 2010; Que et al., 2014). This mAb showed OPK activity *in vitro* and exhibited decreased bacterial burden and improved survival in infection models (Horn et al., 2010). In a phase II clinical trial, Panobacumab was used adjunctively with standard of care antibiotics resulting in shortened duration to disease resolution in *P. aeruginosa* 011 pneumonia patients (Que et al., 2014). While strain-specific mAbs have shown promising results, its activity is limited to strains that express its target receptors. Contrastingly, a protective mAb targeting *P. aeruginosa* type III secretion protein, PcrV plays an important role in transporting virulence factors into host cells (Frank et al., 2002). This led to the development of KB001, a phase II mAb for the prevention of *P. aeruginosa* ventilator-associated pneumonia (Baer et al., 2009).

In addition, the secreted virulence factor, alpha toxin (AT) which causes cellular death by the formation permeable pores in the plasma membrane, is targeted by two anti-staphylococcal mAbs currently undergoing efficacy trials (Berube and Wardenburg, 2013; Oganesyan et al., 2014). This prospective therapy has demonstrated prophylactic protection in *Staphylococcus aureus* pneumonia mouse models (Hua et al., 2014; Sause et al., 2016).

Furthermore, scFvs to the spirochete *Borrelia* have been found to have direct bactericidal effects (Larocca et al., 2008). Similarly, an anti-idiotype antibody that mimics yeast killer toxin has also been described to have direct cell-killing effects against an array of broad spectrum bacteria along with yeast, fungi and mycobacteria (Conti et al., 2000). Such antibodies may overcome the necessity for a competent host immune response for efficacy. More often, however, antibacterial antibodies rely on recruitment of varying antibody-directed immune effector functions such as; initiating antibody-directed cellular cytotoxicity (ADCC), activating the complement cascade, and inducing oxidative bursts. Moreover, all antibody variable regions are able to catalyze redox reactions resulting in the generation of potent oxidizing agents that directly harm bacteria (Wentworth et al., 2003). This process causes inflammation which further enhances the immune response by recruitment of immune system elements (Babior et al., 2003).

Antibody-Conjugates

Different antibody formats consisting of an antibody binding fragment conjugated to varying effector molecules like radionuclides (radioimmunotherapy/RITs), drugs (antibody-drug conjugates/ADCs), toxins (immunotoxins) and enzymes (antibody-directed enzyme prodrug therapy/ADEPT) are referred to as antibody conjugates. Conjugated mAbs are used to deliver one of these effector molecules specifically to infected cells while limiting the side effects of off-target drug delivery (Weiner et al., 2012).

Radioimmunotherapy/radioimmunoconjugates

The first effective form of targeted cancer therapy was ^{131}I, a β-particle emitter that also emits small amounts of γ-radiation. It was used for treatment of thyroid cancer because of its specificity for elemental iodine in the thyroid. The success of this treatment led researchers to explore various carrier molecules including mAbs which could be utilized for targeted delivery of radioisotopes to infected cells (Weiner, 2015). The first 2 FDA approved conjugated antibody therapies, were radioimmunotherapies, both for the treatment of hematological malignancies. These RITs were ^{90}Y-ibritumomab tiuxetan and ^{131}I-tositumomab, both targeting CD20 for treatment of relapsed and/or rituximab-refractory follicular or low-grade lymphomas. There are a number of other RITs in development, several of which target solid tumors (Weiner, 2015). Surface receptors

(non-internalizing) are sufficient for treatment with RITs as internalization is not required by all RITs to induce killing of target cells (Illidge and Johnson, 2000). In addition, while most antibody-based therapies rely on targets with high binding affinity, RITs may be more effective against diseased cells with lower binding affinity targets; therapeutic proteins with high binding affinity will likely bind the first target receptor it contacts, predicted to be on the periphery of the tumor, and given its high affinity, will not penetrate deeper into the tumor thus resulting in radiation exposure to a higher number of healthy cells circulating around the tumor. However, lower binding affinity targets may permit better tumor penetration by allowing the therapeutic protein to move from one target expressing cell to the next, within the tumor microenvironment (Allen, 2011).

Whilst the use of mAbs has significantly improved specificity of radiotherapies many challenges persist limiting its widespread clinical application. Radioisotopes are in a continual state of decay harming healthy tissue during circulation or taken up non-specifically (Pouget et al., 2011). Bone marrow in particular is highly sensitive to radiation and is therefore affected by circulating RITs. In addition, the kidney and liver are exposed to high doses of radiation due to their roles in clearing RITs and free radioisotopes, resulting in a limited amount of radiation being delivered to target sites, even with the use of very specific targeting agents (Pouget et al., 2011). Further, the actual production of RITs is highly complex and requires experienced nuclear-medicine physicians due to the dangers of radiation poisoning. To overcome these challenges, many ongoing studies are assessing novel radioisotopes like α-emitters which deliver ionizing radiation adjacent, within up to a few cell diameters, to diseased cells consequently restricting radiation exposure to target cells and reducing exposure to neighboring benign cells (Allen, 2011). Current clinical and preclinical studies on such α-particles are ongoing and have the potential to significantly enhance RITs (Weiner, 2015).

There are presently no clinically approved RITs for treatment of infectious diseases, but studies have demonstrated promising progress in this field. The potential efficacy of RIT against *C. neoformans* (CN) has been of particular focus (Dadachova et al., 2003). Due to the current HIV pandemic, CN has emerged as a major fungal disease-causing life-threatening meningoencephalitis in 6–8% of AIDS patients. Further, CN infections are usually incurable in immunocompromised patients as current drug treatments are unable to eradicate the infection in this population (Casadevall et al., 1993; Currie and Casadevall, 1994). The α-particle emitter Bismuth-213 (^{213}Bi) has been proposed for use in certain solid cancers and single-cell disorders and is currently in phase I/II clinical trials for treatment of leukemia patients (Boll et al., 1997; Sgouros et al., 1999; Adams et al., 2000; Mcdevitt et al., 2000). Consequently, a RIT comprising either of two radioisotopes, a high-energy β-emitter Rhenium-188 (^{188}Re) or ^{213}Bi and the capsule-specific mAb 18B7, were assessed with the CN mouse models. 18B7 has no inherent fungicidal activity by itself, but when conjugated to either ^{188}Re or ^{213}Bi, resulted in decreased fungal burden and prolonged survival in RIT treated mice (Dadachova et al., 2003).

The positive results of the CN study has prompted researchers to conducted a second investigation assessing the RIT approach in animal models of *Streptococcus pneumonia* (Dadachova et al., 2004). In this study the ^{213}Bi radioisotope was conjugated to mAb D11, which has specific binding of pneumococcal capsular polysaccharide 8 (PPS8) and is protective against this serotype in multiple strains of mice and infection models (Zhong et al., 1999; Burns et al., 2003). ^{213}Bi was specifically chosen over β-emitters for its short half-life that allows delivery of substantial amounts of radiation in a shorter time which is imperative when targeting rapidly dividing cells like pneumococci. Treatment of *S. pneumonia* with ^{213}Bi-D11 RIT demonstrated bacterial killing in a dose-dependent manner and increased survival in *S. pneumonia*-infected C57BL/6 mice (Dadachova et al., 2004).

Furthermore, RIT has been reported to significantly decrease the number of HIV-infected cells *in vivo*, via conjugation of ^{213}Bi and ^{188}Re radioisotopes to gp41 (HIV-envelope protein)-specific mAb 246-D (Dadachova et al., 2006). A major advantage of using RIT as a treatment for viruses is that the RIT itself does not neutralize the virus, consequently reducing the probability of applying selection pressure that may result resistant strains forming (Dadachova et al., 2006). Recently, the potential therapeutic utility of RIT in combination with antiretroviral drugs (ARVs) has been demonstrated as a novel treatment for the eradication of HIV (Dadachova and Casadevall, 2014).

The morphological similarities observed between granuloma formation and that of a solid tumor, a highly fibrotic and hypoxic microenvironment, suggests this type of treatment may prove useful in TB patients (Datta et al., 2015). Furthermore, granulomas and solid tumors have functionally abnormal vasculature resulting in impaired small molecule distribution, exhibiting a wide range of variation in spatial drug distribution with majority of drugs accumulating in the periphery of the tumor/granuloma and not penetrating into the central diseased tissue. There are currently no RITs approved for the treatment of TB but given that granuloma penetration is a major challenge, RITs with a wider radiation field may be able to bind to the cells on the outside of the granuloma and still elicit killing of bacilli within the granuloma. Additionally, other forms of immunotherapy used for treatment of solid tumors may be applicable to TB granulomas; one important example is bevacizumab, an anti-VEGF antibody used to normalize tumor vasculature in solid tumors. A study conducted in rabbit TB models demonstrated the ability of bevacizumab to normalize the abnormal vasculature of granulomas, resulting in reduced hypoxia and increased small molecule drug penetration into the central granuloma (Datta et al., 2015).

Antibody-drug conjugates (ADCs)

Antibody-drug conjugates combine the specificity of mAbs with the cytotoxic potential of drugs (Weiner, 2015). Currently there are two FDA- approved chemotherapeutic ADCs, Ado-rastuzumab, a HER2^{+}-targeting conjugate that exerts cytotoxicity via inhibition of microtubule assembly, and Brentuximab Vedotin, a CD30-targeting mAb conjugated to an antimitotic drug for the treatment of Hodgkin (NCT00848926)

and anaplastic large cell lymphoma (NCT00866047) (Amiri-Kordestani et al., 2014; Herrera et al., 2018). This CD30-specific ADC, although approved for cancer treatment, has also been tested as an anti-viral ADC for the treatment of HIV (Hogan et al., 2018). CD30 is part of the TFN receptor superfamily, expressed on tumor cells and on a small population of lymphocytes in healthy individuals (Stein et al., 1985; Falini et al., 1995). Additionally, CD30 has been associated with HIV disease progression, with cell-associated HIV-1 RNA being considerably enriched in CD30 expressing CD4+ T cells. The significant reduction in HIV-1 DNA in PBMCs from HIV-infected donors by the ADC, suggests CD30 maybe a potential therapeutic target for HIV (Hogan et al., 2018).

Unlike RITs all ADC's require internalizing targets to elicit their effector functions thus only targets which undergo receptor-mediated endocytosis may be used for ADC treatment (Trail et al., 2017). The distribution of receptors on diseased cells and tissue is also an important consideration when choosing a target for ADC's. For diseases where penetration is not an issue, such as hematological cancers and other blood borne diseases, targeted therapeutics may easily bind to their target cells thus even a moderately expressed target may be sufficient for effective killing. However, for solid tumors, TB granulomas and other difficult to penetrate diseases it is important that the therapeutic protein have an increased serum half-life to allow sufficient time for uptake into diseased tissue (Allen, 2002). Furthermore, the frequency of target receptor internalization/recycling may also affect the binding of the therapeutic protein. For targets with fast turnover rates, changes in surface receptor expression have little impact on the accumulation of the ADC in target cells whereas targets with slow turnover rates show reduced efficacy with decrease surface receptor expression (Sadekar et al., 2015).

Recently, an antibody-antibiotic conjugate (AAC), Thiomab combining essential characteristics of a mAB and an antibiotic has been shown to be an effective therapy against *S. aureus* (Lehar et al., 2015). This ACC works similarly to ADC's in that it uses and antibody to deliver an antibiotic payload to bacteria (Mariathasan and Tan, 2017). As with ADC's the antibody, linker and payload used in the design of the ACC play critical roles in the binding, stability, and effector functions, respectively. A mAb, directed against β-*N*-acetylglucosamine cell-wall teichoic acid (β-GlcNAc) residues on the cell wall of *S. aureus* was chosen as this target is highly expressed on *S. aureus* but not on mammalian cells. For this ACC, a dimethyl DNA31, referred to as dmDNA31, was the chosen antibiotic as it possesses a functional group amenable to conjugation, it is active against *S. aureus* in both its dormant and active state and has potent bactericidal activity. Finally, the linker selected for the ACC was a MC-ValCit-PABQ linker consisting of caproic acid and maleimide for attachment to the antibody, a protease-cleavable dipeptide, valine citrulline and a novel salt, PABQ for attachment of the antibiotic. Thiomab effector function comprises killing of intracellular bacteria via intracellular release of dmDNA31 and internalization of extracellular bacteria through rapid opsonization. Given the rapid emergence of antibiotic resistance and lack of discovery of new antibiotics the ACC avenue provides opportunity for developing novel therapeutics to treat infectious diseases (Mariathasan and Tan, 2017).

Immunotoxins

A classical IT is comprised of a binding domain and a toxic protein domain originally attached by chemical conjugation and later recombinantly fused. The binding domain of an IT may be natural ligands (growth factors, surface receptors), an antibody, or a recombinant derivative (Barth, 2009). To be effective, an IT needs to bind to a surface receptor of an infected cell, undergo endocytosis and intracellular trafficking of its catalytically active subunit to the cytosol where it induces cell death. Additionally, the natural binding domains of the toxins need to be removed so that cellular uptake may be controlled by the specificity of the mAb alone. Historically, bacterial- or plant-derived toxins have first been used to generate recombinant ITs (rITs). Their recognition as foreign antigens by the human immune system, has resulted in induction of neutralizing antibody responses in patients. To reduce immunogenicity, (a) B and T cell epitopes have been removed from the protein toxins, and (b) the bacterial/plant toxins have been replaced by human apoptosis-inducing enzymes which are non-toxic until delivered intracellularly to the target cell (Jordaan et al., 2018). As such, targeted human cytolytic fusion proteins represent the 4th and most recent generation of anticancer immunotherapeutics, with a plethora of candidates such as granzyme B, angiogenin, DAP-K and Map-tau currently under preclinical investigation (Hristodorov et al., 2013; Amoury et al., 2016; Lilienthal et al., 2016; Akinrinmade et al., 2017).

Majority of the focus on developing ITs has been for treatment of cancer, however, there are also those that target viruses (Spiess et al., 2016). Anti-viral ITs interfere with the infection cycle of viruses by targeting virus entry, intracellular replication, viral particle formation and cellular extrusion or by modulating the anti-viral cellular immune responses (Zhu et al., 2015). In addition, anti-viral ITs have a capacity to kill viral infected cells (Spiess et al., 2015; Zhu et al., 2015) and because target molecules are often encoded by the virus itself, off-target killing of cells expressing low levels of the target receptor is greatly reduced compared to anti-cancer immunotoxins. Furthermore, interference of protein synthesis in infected cells prevents viral dissemination and destroys reservoirs of latently infected cells (Geoghegan et al., 2015) which pertains to diseases such as HIV, where current therapies only target active viral infections and the lack of eradication of latently infected cells results in patients needing lifelong therapy (Kennedy et al., 2006). ITs comprising anti-HIV capsid protein gp120 mAb linked to a truncated form of *Pseudomonas exotoxin A* (PE) display specific and potent cytotoxicity against HIV replication in monocyte-derived macrophages and PBMC's (Ashorn et al., 1991; Berger and Pastan, 2010). Mice studies utilizing gp120-specific ITs in combination with standard therapy showed significant improvement over standard therapy alone but was unable to eradicate the disease (Goldstein et al., 2000), probably as a result of immunogenicity-related neutralization. A major complication of immunotoxin-based treatment of HIV is the

limited expression of HIV-proteins in latently infected cells, engendering this cell population invisible to the immunotoxin. Combination therapy of ADC's with drugs such as latency-revering agents, may increase the target receptor expression on latently infected cells enhancing susceptibility to ADC-induced toxicity (Perreau et al., 2017). Furthermore, two PE-based ITs YC15-PE38 and 2014-PE38, have been developed for the specific killing of Kaposi's sarcoma-associated herpesvirus (KSHV)-infected cells and target KSHV lytic glycoproteins. Both ITs impede the construction of infectious KSHV particles and specifically kill KSHV-infected cells (Cai and Berger, 2011; Chatterjee et al., 2012).

In recent times, antibodies isolated from members of the camelid family have been used to develop ITs directed against herpes-simplex virus 2 (HSV-2) (Geoghegan et al., 2015). These antibodies are termed VHH as they consist of the variable domains of antibody heavy-chains and lack light chain and CH1 domains (Hamers-Casterman et al., 1993). VHH share a large degree of homology with mammalian variable domains and have enhanced tissue penetration due their smaller size (Cortez-Retamozo et al., 2002). A VHH, R33 antibody with specific binding to viral cell surface glycoprotein D on HSV-2 was identified using phage display. R33 alone did not have any neutralizing effect on HSV-2, however, in combination with the cytotoxic domain of PE, this IT demonstrated extremely efficient killing of HSV-2-infected cells *in vitro*.

In addition to anti-viral ITs, the targeted delivery of granzyme B (Araki et al., 2013) to *Plasmodium falciparum* provided the first evidence of granzyme B's anti-parasitic effects on malaria (Kapelski et al., 2015). The merozoite surface protein 4 (MSP4)-targeting IT induced enhanced inhibition of parasite growth in infected erythrocytes and although the exact mechanism of action is unknown the evidence strongly suggests that malaria-specific ITs are valuable drug candidates for treatment of multidrug resistant *P. falciparum* strains.

Antibody drug conjugates has significantly improved in efficacy over the last few years, however, its therapeutic application is still limited and require further improvements. All ADC's currently approved for therapy target tumor associated antigens not tumor specific antigens thus resulting in undesired toxicity that limits its therapeutic application. New generation ADC's should focus on disease-specific targets and will most likely require payload that do not interact with cells unless conjugated to an antibody. The major challengers in developing ADC's are identifying appropriate targets, designing chemically stable linkers and choosing an appropriately cytotoxic drug (Lin et al., 2018). All of these factors require extensive optimization to fully improve the efficacy and tolerability of ADC's thus understanding each of these components is key to developing new generation ADCs.

CONCLUSION

In spite of the conventional association of the term "immunotherapy" with the treatment of cancer, indeed publication records are indicative of this bias, this approach is fast-establishing itself within the armory of therapeutics applicable across the disease spectrum. Herewith, the versatility of the strategies that distinguish the immunotherapeutic approach have been described, with an emphasis on therapies that enhance T cell effector function as well as ligand-based therapies that neutralize or eliminate diseased cells. Although not within the scope of this review, natural killer (NK) cell immunotherapies, particularly CAR-modified NK cells and bispecific killer cell engagers (BiKEs), are rapidly gaining attention for application in cancer and HIV (Gleason et al., 2014; Rezvani and Rouce, 2015; Schmohl et al., 2016; Liu et al., 2017). Although the development of vaccines, whether therapeutic or prophylactic, still remains a major focus for infectious diseases such as HIV, based on the number and progress of preclinical studies, the development of immune checkpoint inhibitors and antibody-based therapies are emerging as promising approaches. T cell engineering has transformed adoptive therapies; CAR T cells in particular have gained massive recognition in the cancer field and although not initially successful in clinical trials, second-generation anti-HIV CAR T cells are being explored for their potential to provide long-term protection (Zhen et al., 2017; Wagner, 2018). In addition, it is evident that advances in antibody-conjugates against cancer have paved the way for similar strategies in infectious diseases, especially HIV and various bacterial and fungal infections. In this regard, an onslaught of novel agents and a significant increase in pre-clinical studies in various disease models can be expected.

Despite these encouraging results, ultimately, the high cost of these agents will necessitate comprehensive evaluations of the economic sustainability on healthcare systems, particularly in low- and middle-income countries where the burden of diseases such as HIV and TB remain significant. In addition, the common adverse effects amongst T cell-based therapies, specifically neurotoxicity and CRS, although manageable, remains a major challenge during patient care. Importantly, not all patients respond to immunotherapy, and in cancer, this is attributed to various resistance mechanisms. Combination immunotherapy may provide a solution; a multipronged approach to a curative treatment that has been heralded as the next wave of therapeutic strategies for cancer, proving successful with the recent case of complete durable regression of metastatic breast cancer (Morrissey et al., 2016; Zacharakis et al., 2018). Combinatorial strategies that promote an immunogenic tumor microenvironment by introducing existing chemotherapeutic agents with immune checkpoint inhibitors are fast gaining clinical recognition (Sharma and Allison, 2015). Recently, a mathematical model was developed to predict the efficacy of the combination of a cancer vaccine with an immune checkpoint inhibitor (Lai and Friedman, 2017). The establishment of such tools will be essential to provide rational insight to the advancement of combination immunotherapy. Clinical studies evaluating the configuration of these combinatorial regimens will be equally important (Schmid et al., 2018). Certainly, a combinatorial approach is

central to achieving an HIV cure (Perreau et al., 2017). Of note, despite the incredibly diverse application of mAbs for infectious diseases, its relative underutilization in comparison to cancer may be attributable to the high cost compared with small molecule drugs or vaccines. Consequently, it is possible that immunotherapies may be relegated to adjunct or interim intervention strategies.

The age of immunotherapies has revolutionized cancer treatment. However, there is still much room left for improvement and efforts are ongoing to address better patient outcomes (Mohindra, 2018). With the growing understanding of the biological etiology of diseases, the current portfolio of immunotherapies is expected to expand significantly and may be, just as groundbreaking in combating infectious diseases.

AUTHOR CONTRIBUTIONS

KN and TN contributed equally to the development and writing of the paper, reviewing relevant literature, and preparation of figures in the paper. SC contributed to the writing of the paper and provided the table on vaccines. SB provided substantial, direct, and intellectual contribution to the work. All authors consented for publication.

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REVIEW
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Host-Directed Drug Therapies for Neglected Tropical Diseases Caused by Protozoan Parasites

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The neglected tropical diseases (NTDs) caused by protozoan parasites are responsible for significant morbidity and mortality worldwide. Current treatments using anti-parasitic drugs are toxic and prolonged with poor patient compliance. In addition, emergence of drug-resistant parasites is increasing worldwide. Hence, there is a need for safer and better therapeutics for these infections. Host-directed therapy using drugs that target host pathways required for pathogen survival or its clearance is a promising approach for treating infections. This review will give a summary of the current status and advances of host-targeted therapies for treating NTDs caused by protozoa.

Keywords: leishmaniasis, Chagas disease, Human African Trypanosomiasis, host-directed therapy, treatment

INTRODUCTION TO NEGLECTED TROPICAL DISEASES

The neglected tropical diseases (NTDs) comprise a group including 20 different illnesses which currently affect over a billion individuals and amount to approximately 12% of the total global health burden across 149 tropical and subtropical countries (Chappuis et al., 2007; Ready, 2014; Mello et al., 2017). In humans, NTDs impair cognitive and physical development, cause development of chronic physical or emotional conditions, and could result in an increased mortality and morbidity having a significant economic impact on the economy in developing countries (Centers for Disease Control and Prevention, 2017; World Health Organization, 2018).

In this review we focus on three NTDs caused by different but related to protozoa which account for the highest death toll amongst all NTDs (Hotez et al., 2007): Leishmaniasis, caused by multiple species of the *Leishmania*; Chagas disease, caused by *Trypanosoma cruzi*; and Human African trypanosomiasis (HAT), caused by either *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense*. According to the World Health Organization (WHO), these three NTDs require "Innovative and Intensified Disease Management (IDM)" approaches due to lacking research and development investments as well as a deficiency of effective control tools in endemic areas (Hotez et al., 2007).

Many NTDs are not a major concern in the most developed countries, but they continue to persist in areas where people live with poor sanitation and hygiene and remain in close contact with insect vectors and infected reservoir hosts. Tourists and military personnel traveling to endemic areas are also exposed to these infections and pose a risk of contracting them. Reports show that hundreds of US troops deployed in endemic areas such as Iraq have contracted cutaneous or visceral leishmaniasis (Weina et al., 2004). Additionally, immigration and increased exchanges of economic

and social nature between different countries have contributed to the globalization of some NTDs such as Chagas disease (Kalil-Filho, 2015).

Leishmania and *Trypanosoma* not only infect humans but they also infect wild and domesticated animals, which serve as reservoirs for these diseases. Carnivores, rodents and lagomorphs have been identified as reservoirs for leishmaniasis in Europe, but the dog remains the main domestic reservoir, especially for *L. infantum* (Millan et al., 2014). Relocated or traveling dogs have been shown to bring *L. infantum* to non-endemic areas, spreading the disease throughout Europe (Maia and Cardoso, 2015). Along with the Mediterranean area, zoonotic leishmaniasis can be found in the Middle East, West Africa, Central Asia and the Americas. Here again wild animals and dogs are mainly responsible for *L. infantum* transmission (Harhay et al., 2011). Similarly, *Trypanosoma* can also infect a wide variety of domesticated and wild animals. For example, the presence of a widespread *T. cruzi* infection has been reported along the Texas-Mexico border in government working dogs (Meyers et al., 2017). In Uganda, the spread of sleeping sickness caused by *T. brucei rhodesiense* has been traced to infected cattle movement (Selby et al., 2013). Additionally, cases of horses infected with *T. evansi*, causative agent of the chronic wasting disease surra, have been reported in Brazil and other areas (Elshafie et al., 2013; Parreira et al., 2016). The widespread infection of livestock and other animals increases the risk of transmission to humans, severely impacting whole regions with the potential for global effect. Furthermore, animal infection can lead to infertility and loss of livestock resulting significant economic losses in many African, Asian and American countries (Giordani et al., 2016).

Despite the high prevalence of these three diseases, currently only a handful of treatments available against these parasites and many of those exhibit high toxicity due to the biomolecular similarities between eukaryotic parasites and mammalian cells, as well as to the accumulation of toxic derivative products of the therapeutic compounds. For instance, it is known that the toxicity of benznidazole and nifurtimox, established drugs for Chagas disease, is due to the metabolic conversion occurring after enzymatic reduction of nitro-groups (Bermudez et al., 2016). Another problem associated with some of these drugs is the increasing parasitic resistance as a result of adaptation. Resistance can arise due to different mechanisms such as target modifications, decreased drug uptake or increased efflux and augmented drug metabolism (Yasinzai et al., 2013). Several of these mechanisms, and a combination of them, have been documented in *Leishmania* parasites resistant to antimonials (Sb^{III}, trivalent form reduced from the pentavalent form), miltefosine and amphotericin B (Ponte-Sucre et al., 2017). For example, reduced expression of the Sb^{III} transporter AQP1 leads to increased resistance to antimonials. Additionally, the uptaken Sb^{III} can be bound by the thiol compound trypanothione, present in *Leishmania,* and either sequestered or expelled from the cell via specific efflux pumps (Ponte-Sucre et al., 2017). Furthermore, increased expression of ABCG-like transporter TcABCG1 in *T. cruzi* confers resistance to benznidazole (Zingales et al., 2015).

Because of these concerns, it is imperative to find new therapeutics with low toxicity for the human host while maintaining high anti-parasitic efficacy. This review focuses on host-targeted approaches to treat NTDs caused by these three protozoan parasites.

HOST-TARGETED THERAPEUTICS

Host-targeted drugs bypass many of the problems encountered by treatments that specifically target parasites, by acting directly on host molecules or pathways that are redundant for the host but critical for pathogen invasion, survival and replication. Such approaches are likely to have a less chance of developing resistance as the host molecules and processes mutate at lower rates than most pathogens. Additionally, because these drugs act on the host, and not on specific pathogens, these treatments may be broad-spectrum and effective against several pathogens.

Different strategies have been employed to identify new host targets. The broader and more general approaches are transcriptomic and proteomic analysis as well as the assessment of microRNA, small interfering RNA (siRNA) and short hairpin RNA expression profiles (Prudencio and Mota, 2013; Krishnan and Garcia-Blanco, 2014). RNA interference in *Drosophila* has been previously used to identify several host factors manipulated by pathogens to their own advantage. This method was adopted to identify the host factors important for resistance to *Listeria monocytogenes* and *Chlamydia caviae* (Prudencio and Mota, 2013). Functional genomics have also been used to study gain or loss of function by over-expressing cDNA or iRNA respectively in mammalian cells to investigate the effects of different phenotypes on pathogenesis of intracellular pathogens. Additionally, hybrid interaction screens can be used to study protein-protein interaction between the host and the pathogen and can help identify potential host targets for drug therapy. Another method used to identify protein-protein as well as protein-RNA interactions is affinity chromatography (Krishnan and Garcia-Blanco, 2014).

Recent studies have identified several host-targeted therapeutics which show promise as novel drugs for treating neglected tropical parasitic infections. These approaches include the use of immuno-modulators, kinase inhibitors, and also natural compounds, which activate pro-inflammatory transcription factors like NF-Kb. Of these treatments, immuno-modulators are promising therapeutics not only used by themselves but also in combination with other drugs (Tiuman et al., 2011). In this review we focus on the host-targeted therapy and possible approaches to treat *Leishmania, T. brucei*, and *T. cruzi* infections.

LEISHMANIASIS

Leishmaniasis is a group of tropical diseases caused by protozoan parasites of the genus *Leishmania* and transmitted via the bite of female Phlebotomine sandflies. This disease affects approximately 12 million people in more than 80 tropical and

subtropical countries with incidence of reported cases rising rapidly at 2 million cases annually (McGwire and Satoskar, 2014). There are more than 20 different species of *Leishmania* distributed in both the Old and New World (Centers for Disease Control and Prevention, 2013; World Health Organization, 2018). The clinical manifestations of leishmaniasis depend on many factors including, interactions between the infecting *Leishmania* species and the immune response of the host, localized or disseminated parasite infection, genetic profile of the parasite and mammalian host, stress and also nutritional status of the host (Locksley et al., 1999; McCall et al., 2013). Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis, which manifests as localized skin lesions that can become chronic, leading to significant tissue destruction and disfigurement. Other forms of infections are mucosal leishmaniasis (ML), or life-threatening visceral leishmaniasis (VL), which is the second most fatal parasitic infection after malaria and is characterized by dissemination of the parasites to liver, spleen and bone marrow (The Center for Food Security and Public Health, 2017).

Although macrophages serve as the preferred host cell for *Leishmania*, this parasite can also infect other cells like dendritic cells (Woelbing et al., 2006; Contreras et al., 2014), mast cells (Bidri et al., 1997; Lu and Huang, 2017), fibroblasts (Hespanhol et al., 2005) and neutrophils (Mougneau et al., 2011). In particular, neutrophils are recruited within hours of the infection and delay the recruitment of dendritic cells, important for antigen presentation (Hurrell et al., 2015). Neutrophils seem to play an ambivalent role in leishmaniasis; on one hand they serve as host cells, propagating the infection and indirectly hindering antigen presentation (Peters et al., 2008; Hurrell et al., 2015), on the other hand they have been shown to contribute to parasite killing by releasing neutrophil extracellular traps (Mougneau et al., 2011).

HOST IMMUNOLOGY

A protective immune response against leishmaniasis is characterized by a CD4+ Th1-polarized immune response (Centers for Disease Control and Prevention, 2013). Upon interaction with parasites, antigen presenting cells (APCs) activate T-cells by direct contact as well as by the release of disease-protective cytokines such as interleukin-12 (IL-12). These cytokines prime naïve T-helper cells to differentiate into Th1 cells, the main producers of interferon-γ (IFN-γ) (Naman et al., 2018). Natural killer (NK) cells is an another important source of IFN-y that contributes to Th1 cell differentiation and ultimately to disease resolution (Mougneau et al., 2011). IFN- γ stimulates phagocytes to produce reactive oxygen- and nitrogen-species resulting in parasite killing (Pace, 2014). IFN-γ, IL-12 and TNF-α are crucial mediators of protection against various forms of *Leishmania* infection (Naman et al., 2018; World Health Organization, 2018). While a polarized Th1 response is associated with resolution of CL, susceptibility to CL is associated with an induction of a Th2 immune response. A Th2-polarized immune response is characterized by the production of interleukin-4 (IL-4), IL-13 and interleukin-10 (IL-10). These cytokines, along with TGF-β suppress protective immune response and promote parasite and exacerbation of disease (Odiit et al., 1997). In contrast to CL, the resolution of VL requires the production of interleukin-4 (IL-4) and interleukin-13 (IL-13) as well as IL-4 α signaling pathway, which induces mature granuloma formation and promotes parasite clearance (Satoskar et al., 1995; Alexander et al., 2000; Stager et al., 2003; McFarlane et al., 2011).

Several drugs are currently used to treat leishmaniasis; but there is no vaccine available for disease prevention. The standard in treatment of leishmaniasis in most endemic countries involves use of pentavalent antimonials such as sodium stibogluconate (SSG). Other drugs, including liposomal amphotericin B, azoles, imiquimod, miltefosine, paromomycin and pentamidine, have also been used with variable success. Unfortunately, the current treatments have several drawbacks, including poor patient compliance due to prolonged treatment duration, high toxicity and emergence of drug resistant parasites (Eugene, 1987; Bray et al., 2003; Torrado et al., 2008; Jhingran et al., 2009; Olliaro and Sundar, 2009; Tiuman et al., 2011; McGwire and Satoskar, 2014; Lamotte et al., 2017; Ponte-Sucre et al., 2017). In this section, we will discuss novel host-targeted drugs to treat leishmaniasis.

HOST-TARGETED THERAPEUTICS AND APPROACHES FOR TREATMENT OF LEISHMANIASIS

Imatinib is an inhibitor of Abl/Arg kinase family of tyrosine kinases, which can directly remodel the actin-based cytoskeleton to mediate phagocytosis (Greuber and Pendergast, 2012; Zhang and Kima, 2016). The Abl/Arg kinases have been previously shown to play a role in phagocytosis of *Leishmania amazonensis* promastigotes by macrophages (Wetzel et al., 2012). Although treatment with imatinib did not significantly alter cytokine production, reduced the uptake of both opsonized and non-opsonized parasites and led to reduced lesion severity in mice (Wetzel et al., 2012).

Phosphoinositide 3-kinase γ (PI3Kγ) is part of a family of enzymes with the function of phosphorylating lipids containing phosphotidylinositol. PI3Kγ is expressed in leukocytes and mediates cell migration by initiating actin cytoskeletal reorganization. Because cytoskeletal rearrangement is also critical to phagocytosis, blocking or deleting PI3Kγ results in a significant impairment of parasite entry into phagocytic host cells *in vitro* and *vivo*. This decreased phagocytosis, along with an impaired recruitment of cells at the infection site, conferred increased resistance against *L. mexicana* in C57BL/6 mice. Furthermore, AS-605240, an isoform-selective PI3kγ inhibitor, was therapeutically as effective as aforementioned standard anti-leishmanial drug SSG in treating *L. mexicana* infection (Cummings et al., 2012). A recent study used AS101 (ammonium trichloro [1,2-ethanediolato-O,O']-tellurate), a tellurium-based immunomodulator for the treatment of *L. donovani* infection. Along with a direct effect on promastigotes, AS101 was also shown to reverse T-cell anergy, promote NO and antibody production, and more importantly inhibit the

STAT3/IL-10 pathway by blocking the PI3k/Atk signaling in infected macrophages. This could further promote MAPK and NF-κB activity (Vishwakarma et al., 2018). A recent study by Khadem et al. (2017), showed that administration of PI3K p110δ inhibitors CAL-101 and IC87114 resulted in a decrease in parasitic burden in both a CL and VL murine model. This result was accompanied by increased cytokine production in the spleen, livers and footpads of infected mice. The authors suggest the use of these inhibitors along with amphotericin B for even better outcomes (Khadem et al., 2017).

Ibrutinib is a small inhibitor currently used for the treatment of chronic lymphocytic leukemia and other B-cell malignancies due to its action as an irreversible inhibitor of Bruton's tyrosine kinase (BTK) found on B-cells (Pan et al., 2007; Harrison, 2012). Because of its homology with BTK, IL-2 inducible kinase (ITK) found on both Th1 and Th2 cells is also inhibited by ibrutinib (Dubovsky et al., 2013). Previous studies have identified ibrutinib as a clinically relevant drug not only against cancer, but also for the treatment of infectious disease using the cutaneous model of leishmaniasis caused by *L. major*. This beneficial effect was due to a Th1 polarized response characterized by the production of disease protective cytokines such as IFN-γ (Dubovsky et al., 2013).

Berberine chloride is a quaternary isoquinoline that acts via phosphorylation of protein p38 in the MAP kinase pathway. This compound upregulates NO production and IL-12 expression, both disease protective, while downregulating expression of disease exacerbating IL-10 in macrophages infected with *L. donovani* (Saha et al., 2011). Berberine also alters AMP-activated protein kinase (AMPK) signaling, leading to increased activation of macrophage inflammasomes (Casey et al., 2015; Li et al., 2017).

Statins are HMG-CoA reductase inhibitors commonly orally administered to decrease low density lipoprotein (LDL) levels in hyperlipidemic individuals by preventing the synthesis of cholesterol in the liver (Sirtori, 2014). During *L. donovani* infection, this statin-dependent reduction in cholesterol levels resulted in decreased attachment of infectious promastigotes to macrophages, which is critical for parasite invasion. Consequently, macrophages treated with lovastatin had fewer intracellular amastigotes (Kumar et al., 2016). Furthermore, topical application of simvastatin to ear and footpad lesions of *L. major*- infected BALB/c and C57BL/6 mice reduced lesion size as well as parasitic burdens in draining lymph nodes (Parihar et al., 2016).

Naloxonazine is an opioid-receptor antagonist that up-regulates expression of the vacuolar ATPase (vATPase) proton pump and actin related genes, mediating the formation and maturation of phagolysosomes. The vATPase proton pump has been shown to be critical in acidification of parasitophorous vacuoles within phagocytes. The general understanding is that promastigotes are more sensitive to acidic pH than amastigotes. This observation suggests that acidification of vacuole before the transformation of promastigotes into amastigotes can potentially lead to increased parasite killing (De Muylder et al., 2016).

Pentalinonsterol (cholest-4,20,24-trien-3-one) is a natural product isolated from the roots of *Pentalinon andrieuxii*, a native plant of the Yucatan peninsula. Recent studies have shown that Pentalinonsterol can stimulate macrophages by activating the NF-kB pathway. This activation resulted in an upregulation of NO, critical for parasitic killing, as well as pro-inflammatory cytokines TNF-α and IL-12 in macrophages and bone marrow derived macrophages (BMDMs) *in vitro*. Pentalinonsterol treatment also increased antigen presentation and expression of costimulatory molecules, which ultimately resulted in augmentation of both the responses *in vivo*. Because of its immunomodulatory properties, pentalinonsterol has been proposed as a potential adjuvant in vaccination against infectious diseases (Oghumu et al., 2017).

Oleuropein is a glycosylated seco-iridoid that can be cheaply derived from numerous plants, in particular the olive tree, *Olea europaea L.* (Oleaceae). This natural bioactive compound was shown to promote Th1 type immune responses and increase the oxidative stress within the host, both important for protection against *Leishmania* infection. Balb/c mice infected with *L. donovani* and subsequently treated with oleuropein showed a Th1 polarization characterized by expression of genes like TGF-β1 and IFN-γ as well as transcription factors like GATA3 (Kyriazis et al., 2016). This immunomodulatory effect was believed to be due to the inhibition of IL-1β which promotes disease progression and non-healing phenotypes in *Leishmania major* infections (Voronov et al., 2010; Charmoy et al., 2016). In addition to these properties, oleuropein treatment increased the production of ROS in both *in vitro* and *in vivo* models of *L. donovani* infection (Kyriazis et al., 2016). Oleuropein has also been shown to inhibit extracellular signal related kinase (ERK1/2) (Abe et al., 2011). This is relevant because activation of ERK1/2 enhances expression of IL-10 and reduction of IL-12 which resulted in decreased p38 MAPK activation and increased parasite survival (Feng et al., 1999; Mathur et al., 2004). Interestingly, oleuropein has stimulatory effects on the AMPK pathway similar to berberine chloride (Andreadou et al., 2014). Because of its immunomodulatory actions and low toxicity, oleuropein could be employed to complement other treatments.

Mahanine is a carbazole alkaloid isolated from a medicinal plant native to the Indian subcontinent. *In vivo* studies have shown that mahanine induces apoptosis of both antimony sensitive and resistant *L. donovani* (Roy et al., 2017). Mahanine augments NO and ROS generation, thereby causing parasitic apoptosis due to oxidative stress. Along with its effect on NO and ROS, mahanine affects Th1 cytokines by acting on the STAT pathway. First employed in the treatment of various types of cancer, mahanine has been effectively repurposed against VL (Roy et al., 2017). Mahanine inhibits JAK1 and Src which subsequently promotes the degradation of STAT3, an important transcription factor in macrophages that causes upregulation of IL-10 expression and suppression of Th1 responses (Biswas et al., 2011; Lee et al., 2011; Das et al., 2014). While mahanine possesses its own unique immunomodulatory effects, several other natural compounds stimulate ROS and NO production *in vitro* including but not limited to, lupeol from *Sterculia villosa*, dehydroabietic acid from *Pinus elliottii*, and oil extracts from

Nectandra species (da Costa-Silva et al., 2015; Bosquiroli et al., 2017; Das et al., 2017; Goncalves et al., 2018). For example, *Punica granatum*, commonly known as pomegranate, has been shown to have antiparasitic and antioxidant properties (Kaur et al., 2006). A recent study has demonstrated that oral treatment with *P. granatum* juice significantly reduced the lesion sizes of mice infected with *L. major,* compared to untreated mice. The anti-leishmanial activity is attributed to the presence of flavonoid and phenolic compounds including ellagitannins and luteolin. Luteolin in particular inhibits the extracellular promastigote growth (Mittra et al., 2000). In contrast, ellagitannins enhance non-specific immunity via macrophage activation and by inducing the production of NO, IFN-γ and TNF-α, which increase the oxidative stress on the parasites. The study showed that *P. granatum* juice has the potential to be an effective, safe and easily administrable treatment against CL (Alkathiri et al., 2017).

Fucoidan is a multi-sulfated polysaccharide isolated from the sporophylls of the brown algae *Undaria pinnatifida*. Fucoidan enhances dendritic cell (DC) maturation through increased expression of MHC-II and co-stimulatory molecules such as CD80/86 and CD40 (Jin et al., 2014). Fucoidan treatment of DCs induced secretion of IL-6, IL-12, TNF-α and IFN-γ with notable increases in NO production and decreases in production of the anti-inflammatory cytokines IL-10 and TGF-β (Yang et al., 2008; Kar et al., 2011; Jin et al., 2014). Over 93% inhibition of *L. donovani* amastigote replication was achieved *in vitro* and parasites were cleared from both the liver and spleen in 6 weeks *in vivo*; the reduction of parasite burden was observed in both antimony susceptible and resistant *L. donovani* strains (Kar et al., 2011). Fucoidan activates p38 and ERK1/2 associated NF-κB signaling in *L. donovani*-infected macrophages (Sharma et al., 2014).

Artemisinin is a natural compound found in *Artemisia annua*, a traditional Chinese medicinal herb that is the mainstay for treatment of malaria. Treatment with Artemisinin-loaded poly lactic co-glycolic acid (ALPLGA) nanoparticles was shown to increase levels of IL-2 and IFN-γ and decrease levels of IL-4 and IL-10 in BALB/c mice infected with *L. donovani* (Want et al., 2015). Artemisinin treated macrophages secreted increased levels of IL-12 *in vitro* through inhibition of JNK signaling (Cho et al., 2012). Treatment of *L. donovani*-infected BALB/c mice with artemisinin-conjugated nanoparticles and liposomal preparations resulted in approximately 80% reduction of spleen and liver parasite burdens after one administration (Want et al., 2015; Want et al., 2017). Furthermore, treatment of mice with the semi-synthetic derivative, dihydroartemisinin, has been shown to decrease the number of T-regulatory cells, which are important mediators of *Leishmania* pathogenesis present in the spleen, in addition to having Th1 polarizing effects (Belkaid, 2003; Noori and Hassan, 2011). Oral administration of *Artemisia annua* powder in gelatin capsules drastically reduced lesion size and improved appearance compared to no treatment in hamsters infected with *L. panamensis*; 5 out 6 hamsters treated with *A. annua* capsules daily for 30 days were completely cured and 2 clinical patients taking *A. annua* capsules were cleared of infection within 45 days without adverse reactions or reoccurrence 24 months after completion of therapy (Mesa et al., 2017).

Eugenol is a component of *Syzygium aromaticum,* a species of clove native of Australia and tropical regions of Central and South America. *S. aromaticum* has anti-bacterial, anti-trypanosomal and anti-malarial activity. These properties are due to the immunomodulatory effects of this compound on both humoral and cell-mediated immune responses. Treatment of *L. donovani*-infected BALB/c mice with eugenol increased Th1 polarization, characterized by high levels of IFN-γ and IL-2, with a concomitant decrease in the Th2 immune response (Charan Raja et al., 2017). This treatment also induced nitric oxide production in infected macrophages and proliferation of both CD4+ and CD8+ T-cells. Overall, eugenol treatment resulted in decreased parasitic burdens in the spleen and livers of *L. donovani*-infected mice most likely due to both leishmanicidal and immunomodulatory properties (Islamuddin et al., 2016; Charan Raja et al., 2017).

Brazilian propolis has recently been investigated for the treatment of American tegumentary leishmaniasis. Propolis is a bee product composed mainly of di-triterpens, phenolic compounds and essential oils. This natural product has previously been shown to have anti-inflammatory, anti-oxidant and immunomodulatory properties (Miranda et al., 2015). In a study from 2015, it was shown that treatment with NO in combination with Brazilian propolis mediated a decrease in number of parasitized cells, leading to reduced inflammation and tissue damage in a *L. amazonensis* murine model (Miranda et al., 2015). More recently, the immunomodulatory properties of Brazilian propolis was investigated in human-derived peripheral blood mononuclear cells (PBMC) isolated from *L. braziliensis* infected patients. This study found that in both healthy and infected donors, propolis was able to increase the levels of IL-4 and IL-17 while decreasing IL-10, showing an overall decrease in inflammation which could promote the control of the parasites (Dos Santos Thomazelli et al., 2017).

Phospholipase A_2 (PLA_2) are a family of enzymes with the function of reacting with various phospholipids to produce lysophospholipids, a class of lipid mediators, as well as arachidonic acid, a precursor of eicosanoids (Murakami and Kudo, 2002; Moreira et al., 2014). It has been previously shown that PLA_2 activates NF-kB in isolated peritoneal macrophages (Moreira et al., 2014). Additionally, treatment with liposome encapsulated PLA_2 isolated from the venom of *Bothrops jararacussu,* resulted in a significant rise in TNF-α and NO production in the cutaneous lesions as well as lymph nodes of *L. amazonensis*-infected BALB/c mice (de Barros et al., 2018).

Leptin is an adipocyte hormone that plays a role in thymic homeostasis and has been shown to mediate a pro-inflammatory response in animal models. In particular, leptin induces Th1 immune responses while suppressing Th2 responses (Maurya et al., 2016). Leptin induced Th1 polarization, characterized by IFN-γ, IL-12 and IL-1β, resulted in decreased parasitic burdens of C57BL/6 mice infected with *L. donovani* (Dayakar et al., 2016; Maurya et al., 2016). Leptin treatment also mediated NO production in antigen-presenting cells (Maurya et al., 2016). In particular, leptin activates macrophages by promoting the

phosphorylation of ERK1/2 and Akt, which is usually inhibited during VL infection (Dayakar et al., 2016).

MicroRNAs (miRNAs) are short, single-stranded non-coding RNAs that bind to gene transcripts to regulate protein translation (Bartel, 2009; Geraci et al., 2015). Because of their role in mediating post-transcriptional repression, miRNAs are linked to the regulation of host processes involved in the development and activity of innate and adaptive immune responses (Geraci et al., 2015; Drury et al., 2017). The immunomodulatory functions of miRNAs make them promising host targets for developing new therapeutics for infectious disease (Drury et al., 2017). miRNAs have been implicated in visceral leishmaniasis infection in macrophages and dendritic cells *in vitro* (Geraci et al., 2015). Additionally, certain miRNAs have been involved in T-reg specialization and stability in an *L. major* model, and in autophagy in both *L. major* and *L. donovani* models (Kelada et al., 2013; Frank et al., 2015; Singh et al., 2016). *L. donovani* infection was found to increase the stability of microRNA ribonucleoprotein (miRNP) in infected macrophages. miRNP works to restrict the production of pro-inflammatory cytokines, detrimental for parasite survival within the host cell (Chakrabarty and Bhattacharyya, 2017). For these reasons, miRNAs can serve as drug targets to manipulate the host immune response to pathogens. We summarized the compounds in this section and their targets in **Table 1** and their detailed mechanism of action in **Figure 1**.

CHAGAS DISEASE (AMERICAN TRYPANOSOMIASIS)

Trypanosoma cruzi is the causative agent of American trypanosomiasis (Chagas disease). This infection has a prevalence of nearly 6-8 million people worldwide (Aiga et al., 2009) and causes about 12,000 deaths annually. Although Chagas disease is endemic in certain areas in Central and South America, it is found worldwide through the migration of chronically infected individuals. Additionally, there is a low incidence of parasite-infected triatomine bugs in the lower 20 states in the United States which occasionally infect humans (Bern et al., 2011; Klotz et al., 2014).

Trypanosoma cruzi infection is most often initiated during blood feeding of parasite-infected triatomine bugs which defecate near the feeding site. Metacyclic trypomastigotes present in the insect feces enter the wound and establish infection. Metacyclic trypomastigotes are able to invade any nucleated host cells before transforming into intracellular amastigotes and replicating (Rassi et al., 2010). Amastigotes eventually differentiate into blood-stage trypomastigotes and exit the host cell. Trypomastigotes and intracellular amastigote-laden host cells disseminate within the host and can infect multiple organ systems. Key end organ tropisms for parasites are cardiac and gastrointestinal smooth-muscle. The life cycle is complete when uninfected triatomine bugs feed on infected mammalian hosts and ingest parasites, which grow and differentiate in the insect gastrointestinal tract and eventually migrate to the hindgut. The majority of Chagas infections occur through insect vector-borne transmission; however, the disease can also be transmitted transplacentally, through blood and tissue transplantation, through the consumption of parasite-laden meat or contaminated freshly squeezed fruit juice and through accidental laboratory exposure (Tyler and Engman, 2001; de Souza et al., 2010; Bern et al., 2011).

The disease has two distinct stages, acute and chronic. The acute stage lasts for 4–8 weeks and generally goes unnoticed or it presents with mild symptoms such as fever, headache, fatigue, and/or rash. Once the infection is established, most patients undergo chronic infection. Among chronically infected patients, 60–80% of individuals will develop an indeterminate chronic stage without showing any symptoms. The remaining 20–40% will eventually develop significant cardiac or gastrointestinal symptoms including arrhythmias and cardiomyopathy resulting in congestive heart failure, and gastrointestinal tract dysmotility syndromes leading to symptoms associated with achalasia, megacolon and mega-esophagus (Sanchez-Guillen Mdel et al., 2006; Bern et al., 2011).

Only two drugs available for treatment of Chagas disease are nifurtimox and benznidazole (Bern et al., 2007). These drugs are not completely effective and their use is difficult due to toxic side effects (Castro et al., 2006). Thus, there is an urgent need to develop new drugs and vaccines for the treatment and control of Chagas disease. Host-targeted therapy could provide an alternative approach to treat Chagas disease in the future. Here, we focus on some of the possible host molecular targets that can be exploited to treat Chagas disease.

HOST IMMUNOLOGY

Trypanosoma cruzi invasion of host cells, intracellular growth and parasite release eventually elicits a rise in parasitemia, which in turn induces pro-inflammatory responses by macrophages and natural killer cells and results in strong CD8+ T cell immune responses (Tarleton, 2015). However, these immune responses can only control the infection partially, as a low level of infection persists for the entire life of the host. During the acute phase of infection, the Th1 response is involved in protection (Tarleton et al., 2000). Driven by interleukin 2 (IL-2) and interferon gamma (IFN-γ) produced by Th1 cells, this type of immune response is important in resistance against *T. cruzi* infection, whereas a Th2 polarized response mediates parasite persistence. IFN-γ-mediated protection is regulated by the transcription factor STAT-1 (signal transducer and activator of transcription 1), and the lack of STAT-1 has been shown to increase susceptibility to *T. cruzi* infection in mice (Stahl et al., 2014; Kulkarni et al., 2015).

Glycosylphosphatidylinositol (GPI)-anchored mucin-like glycoprotein from *T. cruzi* plays a crucial role in macrophage activation, mediating stimulation of pro-inflammatory cytokines such as TNF-α, IL-12, and also inducing NO synthesis in innate immune cells (Camargo et al., 1997). A recent study shows that Th17, a subset of CD4+ T cells, provides a stronger protective response than Th1 cells against *T. cruzi* infection. Th17-dependent protection is due to the phagocytic respiratory burst as well as the activation of CD8+ T cells (Cai et al., 2016).

TABLE 1 | Host-targeted therapeutics for leishmaniasis.

Host-targeted Drug	Classification	Mode of action	Reference
Imatinib	Abl/Arg kinase inhibitor	Plays a role in phagocytosis	Wetzel et al., 2012
Phosphoinositide 3-kinase γ (PI3Kγ) inhibitor	PI3Kγ inhibitor	Inhibits actin cytoskeletal reorganization	Cummings et al., 2012
Ibrutinib	Bruton tyrosine kinase (BTK), and IL-2–inducible kinase (ITK) inhibitor	Results in a Th1 polarized immune response	Dubovsky et al., 2013
Berberine chloride	Hydrochloride salt	Up-regulates nitric oxide and IL-12 production while downregulating IL-10 production	Casey et al., 2015; Li et al., 2017
Statins	HMG CoA reductase inhibitors	Leads to reduced numbers of promastigotes attached to host cells	Kumar et al., 2016; Parihar et al., 2016
Naloxonazine	Opioid receptor antagonist	Up-regulates the expression of the vacuolar ATPase (vATPase) proton pump to acidify the vacuole	De Muylder et al., 2016
Pentalinosterol	Natural compound – cholest-4,20,24-trien-3-one	Activates macrophages to up-regulate pro-inflammatory cytokine and nitric oxide production	Oghumu et al., 2017
Oleuropein	Natural compound – glycosylated seco-iridoid	Promotes a Th1 type immune response and increases oxidative stress	Voronov et al., 2010; Abe et al., 2011; Charmoy et al., 2016; Kyriazis et al., 2016
Mahanine	Natural compound – carbazole alkaloid	Modulates Th1 cytokines and promotes oxidative stress	Biswas et al., 2011; Lee et al., 2011; Das et al., 2014, 2017; da Costa-Silva et al., 2015; Bosquiroli et al., 2017; Roy et al., 2017; Goncalves et al., 2018
Punica granatum (pomegranate)	Natural compound – flavonoids and phenolic compounds	Activates macrophages to increase oxidative stress by inducing Th1 cytokines	Kaur et al., 2006; Mittra et al., 2000; Alkathiri et al., 2017
Fucoidan	Natural compound – multi-sulfated polysaccharide	Enhances DCs maturation and stimulates production of pro-inflammatory cytokines while down-regulating anti-inflammatory cytokines	Yang et al., 2008; Kar et al., 2011; Jin et al., 2014; Sharma et al., 2014
Artemisinin	Natural compound – sesquiterpene lactone containing a peroxide bridge	Increases levels of IL-2 and IFN-γ and decreases levels of IL-4 and IL-10	Belkaid, 2003; Noori and Hassan, 2011; Cho et al., 2012; Want et al., 2015, 2017
Eugenol	Natural compound – phenylpropene	Mediates Th1 polarized response	Islamuddin et al., 2016; Charan Raja et al., 2017
Propolis	Natural compound – mainly flavonoids, aromatic acids and benzopyranes	Decreases inflammation	Miranda et al., 2015; Dos Santos Thomazelli et al., 2017
Phospholipase A_2	Enzyme that produces lipid mediators	Promotes activation of NF-kB in macrophages and results in increased nitric oxide and TNF-α production	Murakami and Kudo, 2002; Moreira et al., 2014; de Barros et al., 2018
Leptin	Adipocyte hormone	Induces a Th1 polarized response and augments nitric oxide production	Dayakar et al., 2016; Maurya et al., 2016
Micro RNA targets (miRNA)	mRNA	Act as immunomodulators	Bartel, 2009; Kelada et al., 2013; Frank et al., 2015; Geraci et al., 2015

Thus, the combined effect of cell-mediated immune unbalanced response associated continuous subpatent parasite antigens may play a significant role in the development of the pathogenesis of Chagas disease. Therefore, host-directed drugs modulating host immune response could be a viable therapeutics for managing Chagas disease.

HOST-TARGETED THERAPEUTICS AND APPROACHES FOR TREATMENT OF CHAGAS DISEASE

While host-targeted therapy in *T. cruzi* infection has not been well studied, there are several studies that show different host

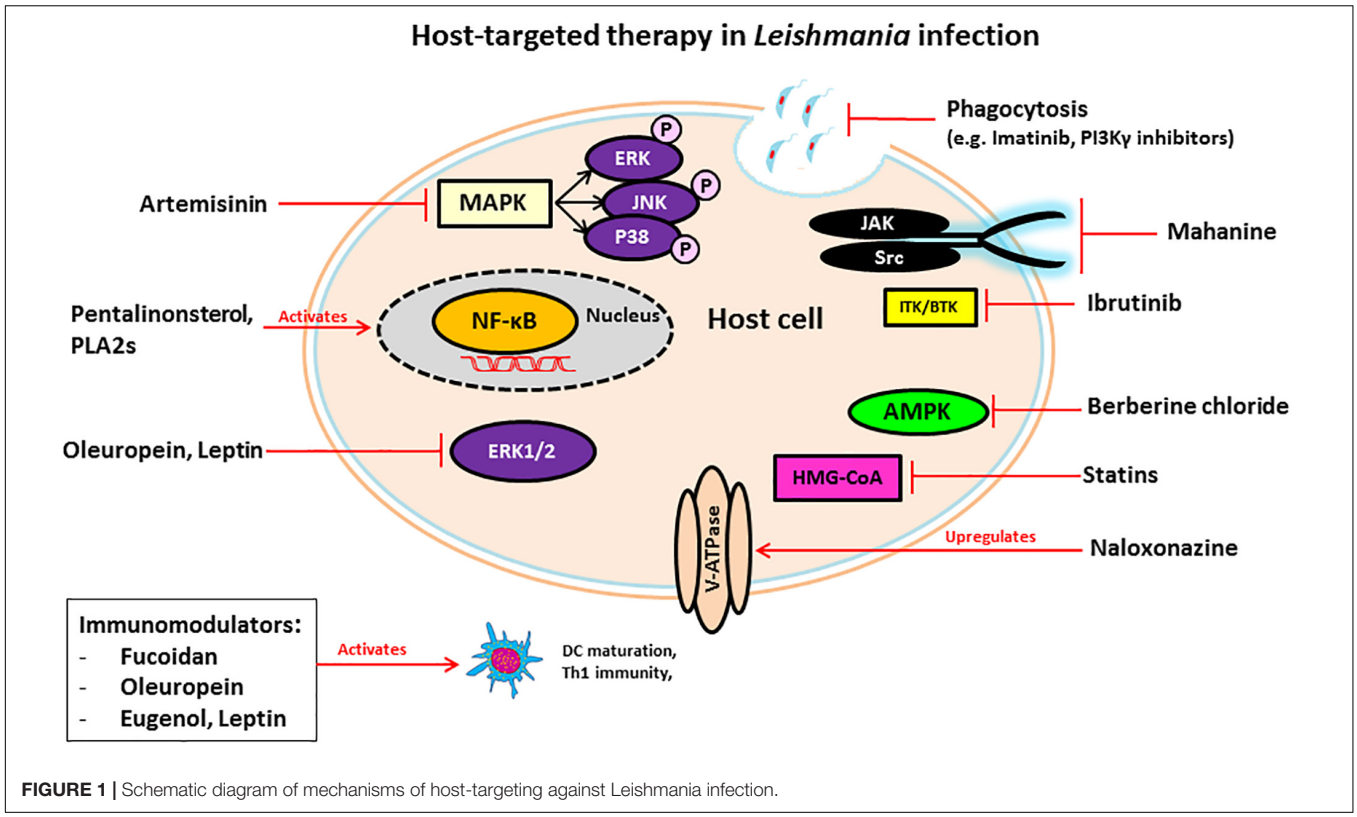

FIGURE 1 | Schematic diagram of mechanisms of host-targeting against Leishmania infection.

molecules are critical for the establishment of *T. cruzi* infection, and the inhibition of these molecules may reduce or ablate infection.

G-protein coupled receptors are a family of receptors that utilize G-proteins to transduce signals into the cell and control diverse functions, including regulation of gene transcription, cellular motility, and metabolic enzymes. *T. cruzi* trypomastigotes invade host cells through association with various GPCRs including platelet-activating factor receptor (Kawano et al., 2011), bradykinin receptor B1 and B2 (Scharfstein et al., 2000; Todorov et al., 2003) and muscarinergic 2 receptor (Wallukat et al., 2010). Several inhibitors of GPCRs have been shown to prevent *T. cruzi* entry and infection and mediate protection against Chagas disease. Cannabinoids, a family of potent immunosuppressive agents, inhibit G-protein signaling and invasion of cardiac myoblasts by *T. cruzi* in mice (Croxford et al., 2005). It is known that parasite-derived thromboxane A2 (TXA2) is important for disease progression in Chagas disease (Villalta et al., 2009). Intracellular amastigotes release TXA2 and initiate signaling after binding with TXA2 receptor (TP). Binding of TXA2 and TP activates a variety of cell types including dendritic cells, monocytes, platelets, cardiac myocytes and endothelial cells, resulting in apoptosis of cells, vasoconstriction, dilated cardiomyopathy, enhanced platelet adherence and aggregation (Ashton et al., 2007). The TXA2 receptor antagonist SQ29548 has been shown to inhibit *T. cruzi* infection mediated through TP (Ashton et al., 2007; Silva et al., 2016).

Carvedilol is a non-selective β-adrenergic receptor blocker used to manage congestive heart failure. It has been shown to improve Chagas cardiomyopathy in combination with renin-angiotensin inhibitors (Botoni et al., 2007).

SB-431542 compound is an inhibitor of the TGF-β type I receptor kinase. It has been shown that host TGF-β is increased during *T. cruzi* infection (Silva et al., 1991) and is taken up by amastigotes to modulate the life cycle of *T. cruzi* (Araujo-Jorge et al., 2002; Waghabi et al., 2005). Recently, it has been reported that elevated TGF-β causes the heart fibrosis and severe cardiomyopathy in Chagas disease. These findings suggest that the treatment of cardiomyocytes with SB-431542, can inhibit the effect of TGF-β-mediated amastigote proliferation and cardiac myopathy in Chagas disease. Experimental evidence suggests that treatment with this drug lowers the penetration of trypomastigotes into cardiomyocytes, decreases intracellular amastigote multiplication and trypomastigote release from the cells, reducing the severity of infection and mortality of mice (Waghabi et al., 2007, 2009).

Terpenoides possess anti-trypanosomal activity. Treatment with terpenoid compounds, such as cumanin and psilostachyin, reduces parasitemia and mortality of parasite-infected mice and intracellular amastigote replication in Vero cells (Sulsen et al., 2013). Terpenoides are a widespread group of natural products and potent inhibitors of NF-κB signaling which mediates TNF-α-induced cell death. During early stages of infection, *T. cruzi* invade hepatocytes, macrophages, and Kupffer cells and increase TNF-α production that causes apoptotic cell death of infected

hepatocytes (Ronco et al., 2010). Furthermore, infection of *T. cruzi* leads to activation of host cell NF-κB signaling that protects infected cells from undergoing apoptosis (Petersen et al., 2006). As terpenoides are such potent inhibitors of NF-κB, treatment might result in robust apoptotic cell death of infected cells that release intracellular parasites outside of the cells. Although the exact mechanism of the trypanocidal effect of terpenoides is still unknown, they may target released parasites from apoptotic host cells directly and engage in anti-trypanosomal activity. In contrast, da Silva et al. (2013) showed that the sesquiterpene lactones-psilostachyin and cynaropicrin did not have efficacy in the mouse model of acute *T. cruzi* infection when comparing benznidazole (da Silva et al., 2013).

Immuno-modulators are compounds that modify immune responses. Extracts from *Lycopodium clavatum*, a spore bearing vascular plant, act as an immuno-modulator to induce Th1 immune responses. *T. cruzi* infected rats treated with *Lycopodium clavatum* extract have reduced progression of GI tract Chagas disease (Brustolin Aleixo et al., 2017). A recent study by Otta et al. (2018) showed that K777 the extract lead compound induces prominent proinflammatory responses modulation by interleukin -10-positive $CD4^+/CD8^+$ T cells and this contributed to the protection against the Chagas disease. In addition, diet supplementation with fish oil led to increase the resistance to *T. cruzi* infection through modulating various immunological factors (Lovo-Martins et al., 2017).

Inhibition of β-oxidation: The replication of intracellular amastigotes is largely supported by parasite scavenging of host metabolic network, including host cell fatty acid metabolism in cardiac and smooth muscle (Combs et al., 2005). It has been shown that long chain fatty acid oxidation is the key source of nutrients for intracellular amastigotes. Long chain fatty acids are oxidized in the peroxisome to produce short chain fatty acids that are transported to the mitochondria by acyl-CoA dehydrogenase for β-oxidation. Recent study of siRNA screen shows that the enzymes of β-oxidation are essential for growth of amastigotes inside the host cells and alteration of fatty acid metabolism and β-oxidation inhibits the intracellular growth of amastigotes (Caradonna et al., 2013). Although several β-oxidation inhibitors have been identified as, for example etomoxir (Paumen et al., 1997), mildronate (Liepinsh et al., 2006), trimetazidine, and ranolazine (Sabbah and Stanley, 2002), more studies are needed to find which can treat Chagas disease. We summarized the compounds in this section and their targets in **Table 2** and their detailed mechanism of action in **Figure 2**.

HUMAN AFRICAN TRYPANOSOMIASIS (SLEEPING SICKNESS)

Human African trypanosomiasis (HAT) is caused by two subspecies of *Trypanosoma brucei*, *T. b. gambiense,* and *T. b. rhodesiense*. *T. b. gambiense* is the causative agent of a chronic form of the disease often referred to as West African trypanosomiasis, which is prevalent in Western and Central Africa and affects 24 countries. *T. b. rhodesiense* causes a more acute form of the disease found in Eastern and Southern Africa affecting 13 countries, and is often referred to as East African trypanosomiasis (Franco et al., 2014).

Of the two subspecies, *T. b. gambiense* is the more prevalent threat, as humans are considered the primary reservoir for the parasite. *T. b. rhodesiense* primarily infects animal reservoirs, and humans are incidentally infected (Franco et al., 2014). According to the World Health Organization (WHO), the majority of approximately 2,000 new cases of Western African trypanosomiasis reported in 2016 were in the Congo, while only about 50 new cases were found in Eastern Africa (World Health Organization, 2017). Currently, there are estimated to be fewer than 20,000 active cases of sleeping sickness, with 65 million at risk. The majority of new cases occur in the Democratic Republic of Congo, contributing 84% of new cases in 2015 (Franco et al., 2014). HAT is slated for elimination as a public health threat in 2020, with a downward trend in new cases and a drop of yearly DALY from 2,734 DALY in the year 2000 to a DALY of 372 in year 2015 (World Health Organization, 2013, 2016, 2017).

Both subspecies of *T. brucei* are transmitted by Tsetse flies harboring metacyclic trypomastigotes, while feeding on mammalian hosts. Once inside the host, trypomastigotes migrate to the blood stream and lymphatics disseminate throughout the host, at which point they begin to multiply through binary fission. (Centers for Disease Control and Prevention, 2016). In acute human trypanosomiasis, parasites disseminate throughout the lymphatics and blood stream of the host. Parasites eventually breach the blood-brain barrier, leading to infection of the central nervous system, and eventual death in essentially 100% of untreated patients (Brun et al., 2010).

HOST IMMUNOLOGY

Upon entering the human host, metacyclic *T. brucei* immediately encounter the host's innate immune defense mechanisms. These parasites, however, are covered in a thick, highly dense coat of variable surface glycoproteins (VSGs) which protect parasites from the mounting host humoral response, as well as from host complement-mediated lysis (Horn, 2014). Parasites undergo switching of antigenically different VSGs in order to circumvent antibody-mediated killing. This switching of VSGs results in undulating waves of parasitemia in which parasites possessing older VSG coats are subject to immune clearance and clones expressing neo-VSG escape immune surveillance and replicate. This mechanism allows the trypanosomes to replicate and survive at sub-lethal levels of infection.

Human susceptibility, disease progression and outcome of HAT are linked to differences in individual genetic predisposition for varying cytokine levels and T cell differentiation. Once infected, certain individuals can control infection and become asymptomatic carriers without any specific intervention. These asymptomatic people have increased quantities of IL-6, IL-8 and TNFα, as well as decreased levels of IL-12, indicating that the ability to control infection relies upon a controlled Th1 response. However, those individuals showing high levels of

TABLE 2 | Host-targeted therapeutics for Chagas disease.

Host-targeted Drug	Classification	Mode of action	Reference
Cannabinoids, SQ29548	inhibitors	G protein couple receptor	Scharfstein et al., 2000; Todorov et al., 2003; Croxford et al., 2005;Ashton et al., 2007; Wallukat et al., 2010; Kawano et al., 2011
Carvedilol	β-adrenergic receptor blocker	β-adrenergic receptor	Botoni et al., 2007
SB-431542 compound	inhibitor	TGF-β type I receptor kinase	Silva et al., 1991; Araujo-Jorge et al., 2002; Waghabi et al., 2005; Waghabi et al., 2007
Terpenoides	Inhibitors	NF-κB signaling	Petersen et al., 2006; Ronco et al., 2010; da Silva et al., 2013; Sulsen et al., 2013
Lycopodium clavatum	immunomodulator	Induces Th1 immune responses	Brustolin Aleixo et al., 2017; Lovo-Martins et al., 2017; Otta et al., 2018

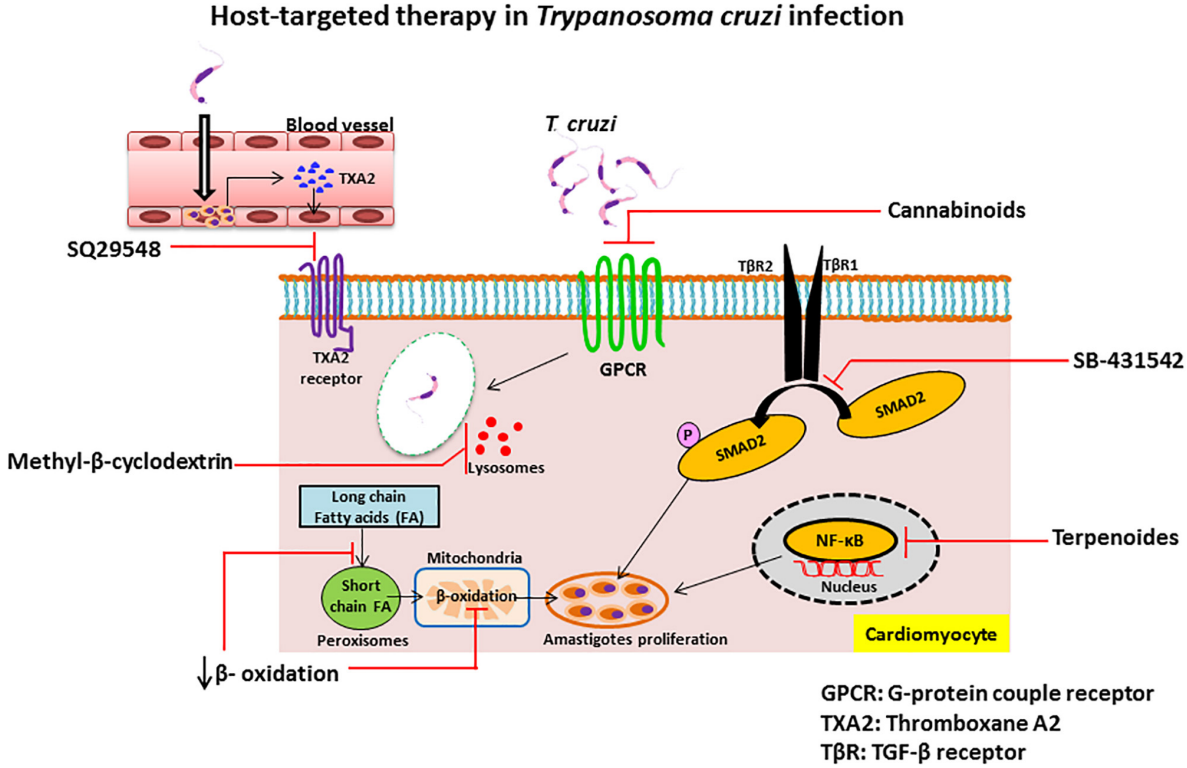

FIGURE 2 | Schematic diagram of mechanisms of host-targeting against *Trypanosoma cruzi* infection.

IL-6, IL-8, TNFα, and IL-10 are at risk of developing disease once infected (Courtin et al., 2006; Kato et al., 2015, 2016). In contrast, the individuals who show more susceptibility to uncontrolled infection produce higher amounts of IL-2 and IL-4. Control of late infection once it has been established, relies upon the immune system being able to effectively switch from a Th1 to a Th2 immune response (Ilboudo et al., 2014). While the Th1 response is more beneficial during the initial stages of infection, more anti-inflammatory Th2 responses are implicated in trypano-tolerance once an infection has been established. IFN-γ expression is responsible for early resistance and control against initial infection (Hertz et al., 1998). While IFN-γ is beneficial to the host during initial stages of infection, the lack of effective switching to a more polarized Th2 response during the late stages of infection may lead to hyper-inflammation in the CNS, ultimately overwhelming the host (Shi et al., 2003).

In addition to the Th1 and Th2 response, high concentrations of VSG released by the parasites in the blood stream play an important role in inducing host immune response. Glycosylphosphatidyl-inositol (GPI)-phospholipase C induced release of GPI-linked VSGs exposes macrophages to previously masked regions of the GPI-tails of VSG. GPI-recognition by macrophages induces MyD88 dependent activation of the NF-κB cascade resulting in a massive release of TNF-α, IL-1, and IL-12 (Leppert et al., 2007; Cheung et al., 2016; Stijlemans et al., 2016). This release of pro-inflammatory cytokines alongside parasite components results in the induction of classically activated macrophages. These macrophages release ROS and

NO which can be detrimental to parasites, however, they are also damaging to the host, causing physiological and cellular destruction (Stijlemans et al., 2016).

HOST-TARGETED THERAPEUTICS AND APPROACHES FOR TREATMENT OF HAT

Current treatment approaches of HAT consist of 5 drugs, each one specific for different stages of the infection. Pentamidine and suramin are used to treat the first stage of the disease, whereas melarsoprol and a combination of nifurtimox-eflornithine are employed to treat the second stage of the disease. All these drugs have significant potential side effects. Pentamidine treatment is fairly ineffective in combating the second stage of infection by *T. b. gambiense* and both stages of *T. b. rhodesiense,* suramin treatment is only effective against the first stage of *T. b. rhodesiense* infection (Buscher et al., 2017).

Melarsoprol is employed to treat the late-stage of infections caused by both *T. b. gambiense* and *T. b. rhodesiense*, and is the only drug used for late-stage infection caused by *T. b. rhodesiense*. However, significant toxicity, the mode of administration and the lack of its availability in endemic areas, hinder widespread usage of this drug. Although nifurtimox and eflornithine are individually effective against *T. b. gambiense* infection, combination therapy with these drugs has better efficacy and diminished side effects (Barrett et al., 2007; Priotto et al., 2009; Babokhov et al., 2013).

There are currently three drugs under development for HAT, as well as a few host target molecules which have shown promising results in the reduction of parasitemia.

Diamidine derivatives, including pafuramidine (DB289), which is administered orally, is well tolerated and mediates parasite clearance in late-stage infections of both human *T. brucei* subspecies. However, after stage III clinical trials, DB289 was abandoned due to high nephrotoxicity. Despite this, DB289 has spurred the development of other diamidine derivative drugs (Kennedy, 2013).

Benzoxaborole drugs, such as SCYX-7158, are capable of crossing the blood-brain barrier *in vivo* and can clear infection of both *T. brucei* subspecies. This drug can be administered orally and also has a long half-life, allowing it to be used as a single dose. SCYX-7158 successfully completed stage I clinical trials and has been approved to move forward with stage II/III clinical trials (Jacobs et al., 2011). While no specific proteins or enzyme have yet been shown to play a role in the method of action of benzoxaboroles, evidence strongly suggests that drugs impede upon the ability of *T. brucei* to properly metabolize methionine (Steketee et al., 2018).

Fexinidazole, a nitroimidazole compound which is effective against both parasites and CNS disease, is safe and well tolerated in early studies. A recent study by Mesu et al. (2018), has reported that stage II/III clinical trials of fexinidazole against HAT have been completed and showed that oral fexinidazole is effective and safe for the treatment of *T. b*. gambiense infection compared with nifurtomox-eflornithine combination therapy in late-stage HAT patients. No method of action for nitroimidazoles has yet been elucidated, though it is known to be a substrate for a type I nitro-reductase and is theorized to function *in vivo* as a pro-drug (Wyllie et al., 2016; Papadopoulou et al., 2017).

Tyrosine kinase inhibitors, such as lapatinib and a few of its derivatives have shown some activity in controlling *T. brucei*

TABLE 3 | Host-targeted and anti-parasitic therapeutics for HAT.

Host-targeted drug	Classification	Mode of action	Reference
Pafuramidine (DB289)	Diamidine derivative	Interferes with the nuclear mechanisms, inhibiting synthesis of DNA, RNA	Kennedy, 2013
Acoziborole (SCYX-7158)	Benzoxaborole drug	Negatively impacts methionine metabolism	Jacobs et al., 2011; Steketee et al., 2018
Fexinidazole	Nitroimidazole compound	Nitroreductase substrate pro-drug	Wyllie et al., 2016; Papadopoulou et al., 2017; Mesu et al., 2018
Dactolisib (NVP-BEZ235)	Kinase inhibitor	PI3K/mTOR inhibitors	Diaz-Gonzalez et al., 2011; Seixas et al., 2014; Fernandez-Cortes et al., 2017
Lapatinib	Kinase Inhibitor	TbLBPK 1-4 inhibition	Guyett et al., 2017
S-(2-boronoethyl)-L-cysteine (BEC)	Arginase inhibitor	Reduction in quantity of growth factors available for trypanosomes	Nzoumbou-Boko et al., 2017; Onyilagha et al., 2018
Curcumin, gallic acid, quercetin, resveratrol	Phenolic or flavonoid compounds	Increases oxidative stress against the parasites, while offering oxidative protection against the host	Wolkmer et al., 2013; Smith et al., 2016; Baldim et al., 2017
Vitamins C, A, E, and D3	Antioxidant vitamins	Enhances host immune function, protects host from oxidative damage	Chekwube et al., 2014; Ibrahim et al., 2016; Jamal et al., 2016
Naphthyridine derivatives, thiosemicarbazone	Chelating compounds	Antioxidant and anti-inflammatory properties, transition metal chelation	Ellis et al., 2015; Wall et al., 2018

infection (Behera et al., 2014; Woodring et al., 2015). Lapatanib mediates antiparasitic activity against trypanosomes by inhibiting four separate protein kinases leading to changes in flagellar topology and an inhibition in the parasite endocytosis. These four kinases have been termed TbLBPK 1-4 and their interruption results in the dephosphorylation of BILBO-1, kinesins, and Rab in *T. brucei* (Guyett et al., 2017).

PI3K/mTOR inhibitors, such as NVP-BEZ235 have efficacy in combating *T. brucei* infection using a mouse model (Diaz-Gonzalez et al., 2011). Through inhibition of several kinase cascades necessary for bloodstream trypanosomes to thrive, effects are seen in the stability of parasite flagellum, in the ability of trypanosomes to mount a proper stress response to complement-mediated and osmotic-lysis, and in endocytosis (Seixas et al., 2014; Fernandez-Cortes et al., 2017).

Arginase Inhibitors, namely *S*-(2-boronoethyl)-L-cysteine (BEC), have been shown to reduce the parasitic burden both *in vivo* and *in vitro*. By blocking Arginase-1 and reducing available growth factors released by macrophages, proliferation of parasites is reduced. *Tb*KHC1 has also been identified as a candidate protein involved with this immunomodulation (Nzoumbou-Boko et al., 2017). Furthermore, it has been shown that preventing the function of Arginase-1 results in the prevention of myeloid-derived suppressor cells inhibiting $CD4^+$ T-cell proliferation, as well as the production of IFN-γ, both of which aid in suppression of trypanosomes (Onyilagha et al., 2018).

Phenolic or flavonoid compounds are a group of plant derived antioxidants which have shown some trypanocidal effects *in vitro* and include the compounds curcumin, gallic acid, quercetin, and resveratrol. Curcumin in particular has been shown to have immunomodulatory effects on the host, preventing damage caused by the generation of ROS (Wolkmer et al., 2013). Gallic acid and quercetin both have also been shown to have prooxidant effects, leading to a direct trypanocidal effects by generation of excess ROS while also maintaining the host protective antioxidant effects (Baldim et al., 2017). Several of these compounds have also been identified as having inhibitory effects against *T. brucei* RNA triphosphatase (Smith et al., 2016). Few *in vivo* studies have been carried out in relation to these plant derived phenolic or flavonoid antioxidant compounds, making this a target area of interest for future research.

Antioxidant vitamins, in particular vitamin C, but also vitamins E and A, have protective effects for the host during *T. brucei* infection through *in vivo* studies. These vitamins are presumed to function as antioxidant to reduce parasitemia and largely reduce organ damage associated with *T. brucei* infections (Ibrahim et al., 2016). Furthermore, vitamin C has been shown to potentiate the trypanocidal effects of diminazene aceturate in a co-administration trial (Chekwube et al., 2014). More recently, vitamin D3 has shown some efficacy in protecting the host during *T. brucei* infection, however the mechanism of action is not yet known (Jamal et al., 2016).

Chelating compounds have myriad positive effects for the host including anti-inflammatory and antioxidant properties. 1,8-naphthyridine derivative compounds are particularly of interest, as they have been shown to possess anti-trypanosomal activity via their ability to chelate Zn^{2+}, Cu^{2+} and Fe^{2+} which are necessary for trypanosomes to thrive in the host (Wall et al., 2018). Thiosemicarbazone has also shown to have some efficacy in ridding a host of trypanosomes through chelation of iron (Ellis et al., 2015).

Presently, no host-targeted drugs or vaccines are described for the treatment or prevention of HAT. The presence of VSGs and the parasites' ability to undergo antigenic variation represent a major challenge for vaccine discovery and unfortunately there has been no development of drugs to target the VSG gene switching mechanism. Potential host-targeting drugs include those affecting host immunomodulation and those which can affect a polarized shift toward a Th1 response, while increasing the production of IFN-γ to drive the clearance of the parasites before they are able to invade the CNS. We summarized the compounds in this section and their targets in **Table 3**.

CONCLUSION

Leishmaniasis, Chagas disease and HAT cause the highest number of deaths amongst all NTDs (Hotez et al., 2007). One of the major confounding issues to eliminating these NTDs is that they are often present in small endemic areas; they have relatively limited global disease burdens and are effectively ignored by the community at large. This is further compounded in that these endemic areas are often subject to political and military turmoil, in areas which lack infrastructural support or effective health care systems. Finally, climate change has also had an impact on the spread of NTDs, as the warmer weather increases the development of insect vectors, which escalates the transmission of these diseases (Hotez, 2017).

Because these infections are difficult to eradicate, it is imperative that more attention is directed toward finding preventive and curative therapeutics to control their spread. There are currently no prophylactic vaccines for the above-mentioned protozoan diseases and the available treatments are antiquated and have significant toxicities. Development of novel agents or utilization of existing host-targeted therapeutics is a promising avenue for the treatment of protozoan NTDs. Despite the numerous advances in the immunology and cell biology fields, we are still far from eradicating these diseases. We believe that moving forward it will be crucial to allocate more funds toward pre-clinical and especially clinical research focused on developing and testing new host-targeted therapeutics for NTDs. Unfortunately, many NTDs affect remote and rural areas of low-and middle income countries (LMICs) with limited resources, infrastructures, and medical personnel. In these areas it is challenging to follow the Good Clinical Practices (GCPs) outlined by WHO and International Conference of Harmonization (ICH) to conduct meaningful clinical trials (Boelaert and Consortium, 2016; Ravinetto et al., 2016). We hope that raising awareness about NTDs and their burden will fuel the already ongoing mobilization of resources on a global scale to aid the development of more infrastructures to conduct

clinical trials as well as screening and treatment in endemic areas.

AUTHOR CONTRIBUTIONS

SV, BJ, GV, NR, GH, and OH reviewed the literature and wrote sections and first draft of the manuscript. AS and BM contributed to conception of the manuscript, wrote sections of the manuscript and revised the manuscript. All authors read and approved the submitted version.

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MINI REVIEW
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Cellular Targets for the Treatment of Flavivirus Infections

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Classical antiviral therapy targets viral functions, mostly viral enzymes or receptors. Successful examples include precursor herpesvirus drugs, antiretroviral drugs that target reverse transcriptase and protease, influenza virus directed compounds as well as more recent direct antiviral agents (DAA) applied in the treatment of hepatitis C virus (HCV). However, from early times, the possibility of targeting the host cell to contain the infection has frequently re-emerged as an alternative and complementary antiviral strategy. Advantages of this approach include an increased threshold to the emergence of resistance and the possibility to target multiple viruses. Major pitfalls are related to important cellular side effects and cytotoxicity. In this mini-review, the concept of host directed antiviral therapy will be discussed with a focus on the most recent advances in the field of Flaviviruses, a family of important human pathogens for which we do not have antivirals available in the clinics.

Keywords: flavivirus, antiviral, host-directed therapy, screening tools, mechanism of action

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INTRODUCTION

The discovery of acyclovir about 40 years ago marked a new era in the field of antivirals (Elion, 1993). Acyclovir acts as a prodrug being activated by the viral thymidine kinase, but targeting the viral DNA polymerase. Therefore, the drug is highly specific with a narrow range of action that includes herpes simplex, varicella zoster, and cytomegalovirus. High specificity leads to the emergence of viruses encoding a thymidine kinase resistant to acyclovir. Acute episodes of herpes infection are manageable with acyclovir, unless prolonged treatment, as in immune compromised patients where resistance usually emerges, is required (Palù et al., 1992). In such cases, drugs with alternative targets are needed. This scheme of high specificity, low toxicity and emergence of resistant viruses is a concern associated with every antiviral treatment of the last few decades. Therefore, there is a constant pressure to develop combined therapies against different viral targets to decrease viral fitness. This concept is particularly relevant for life-long therapies for chronic infections, such as the human immune deficiency virus (HIV) (Van Lint et al., 2013). Recently, a combination of novel inhibitors of viral functions achieved the remarkable objective of eradication of chronic HCV infection (Pawlotsky et al., 2015). Success stories like these certainly reinforce the validity of the approach and the importance of targeting viral functions for the development of successful antivirals. Nevertheless, other approaches targeting cellular functions, collectively called host directed (antiviral) therapies (HDT) are being considered either to enhance the activity of direct drugs, or to cover more infectious agents at the same time.

Before the advent of direct antiviral agents (DAA), the therapy for hepatitis C was based on a combination of recombinant interferon-α (IFNα) and ribavirin, a nucleoside analog with broad antiviral activity and poor specificity. Interferons are natural cellular proteins that trigger an innate antiviral response of the cell, as well as, stimulate the adaptive immune response.

The combination of IFNα and Ribavirin results in higher sustained virological response rates (McHutchison et al., 1998). Hence, this is a perfect example of combination of a HDT, IFNα, with an antiviral agent, Ribavirin, although the latter may be targeting host factors as well, thus explaining its broad-spectrum antiviral activity. The treatment is not as effective as using DAAs and has some important side effects, but has been the only option for HCV treatment since decades. Paradoxically, the molecular mechanisms of antiviral activity of IFNα emerged only lately, after being used extensively in the clinics, not only as an antiviral, but also in the treatment of other diseases. IFNα is a signaling molecule that binds to its receptor and triggers a kinase cascade and transcription factor activation, leading to the induction of several interferon-stimulated genes (ISGs) with antiviral activity. ISG or a combination of ISGs, directly responsible for particular virus inhibition, is currently an area of intense investigation (Schoggins and Rice, 2011).

HCV belongs to the family Flaviviridae, genus Hepacivirus, while a large number of viruses of human interest belong to the genus Flavivirus (Lindenbach et al., 2007; Baud et al., 2017; Carletti et al., 2017). These include arthropod-borne viruses (arboviruses) delivered by mosquitoes and ticks such as Dengue virus (DENV), Yellow fever virus (YFV), West-Nile virus (WNV), Japanese encephalitis virus (JEV), Zika virus (ZIKV), and tick-borne encephalitis virus (TBEV). These viruses represent a threat to the population particularly in areas naïve to the infection and lately, due to climate change and increased movement of people, became responsible of sudden outbreaks outside endemic areas. Given their increasing importance for human health and the lack of treatment options, these viruses represent a challenge for the development of novel direct antivirals. Current approaches at the pre-clinical stage include targeting the viral enzymes such as: RNA-dependent RNA polymerase, methyltransferase, protease and helicase. However, Flavivirus is a large family and it is difficult to predict which of the virus would be responsible for the next epidemic. Therefore, HDT could represent a strategy for pan-Flavivirus agents, either blocking essential host cell pathways, required by the virus for replication, or activating cellular intrinsic antiviral programs that are common among family members. Only a handful of HDT antiviral drugs have been evaluated up to clinical trials so far and all of them are being used in the treatment of DENV as a result of repurposing. In this review we will focus exclusively on antiviral HDT as an alternative option in treating Flavivirus infection, leaving drugs aimed at viral targets to other readings.

FLAVIVIRUS STRUCTURE AND LIFE CYCLE

Flavivirus virions are composed of a nucleocapsid (C protein), protecting a single-stranded RNA genome (vRNA) of approximately 11 kb with positive polarity, surrounded by a lipid bilayer containing two envelope glycoproteins: E (envelope) and M (membrane) (Lindenbach et al., 2007). Flavivirus genomes are modified with a 5′ m^7G cap structure but lack the 3′ polyadenylated tail that is characteristic of most cellular mRNAs. A single long open reading frame (ORF), flanked by 5'and 3' non-coding regions (NCRs), encodes a polyprotein, which is cleaved into different viral proteins by host and viral proteases. The structural proteins capsid (C), pre-membrane (prM), and envelope (E) precede the non-structural proteins NS1, NS2A, NS2B, NS3 (helicase and protease), NS4A, NS4B, and NS5 (RNA-dependent RNA polymerase and methyltransferase). A complete understanding of the process whereby Flaviviruses attach and enter into mammalian and mosquito cells has not been reached, although for certain members (i.e., DENV) more detailed information is available (Muñoz et al., 1998; Acosta et al., 2009; Piccini et al., 2015). It has been proposed that attachment factors on the cell surface are responsible for the first low affinity contact of the virus. Concentration of the virus on the cell surface serves to facilitate binding to specific receptors that eventually promote effective entry in the target cells (Navarro-Sanchez et al., 2003; Miller et al., 2008; Meertens et al., 2012). The uncoating of the nucleocapsid in the cytosol is followed by a Cap-dependent first-round of translation of viral RNA. The multi-transmembrane domain polyprotein precursor localizes on the endoplasmic reticulum (ER), where it is cleaved by cellular and viral enzymes. The NS5 polymerase synthesizes a complementary RNA strand, which then serves as a template for the asymmetric synthesis of additional vRNA. Flavivirus replication occurs in virus-induced vesicles that appear as spherical invagination of the endoplasmic reticulum (ER) and may serve to limit diffusion of viral/host proteins and to protect replication intermediates from the surveillance of cellular cytoplasmic receptors (Miorin et al., 2013; Romero-Brey and Bartenschlager, 2014). The genomic vRNA is believed to be extruded from these replication compartments and assembled with the C protein on the ER membrane, which then buds into the ER lumen. Newly synthesized virions are transported via the secretory pathway, where glycan modification of E and prM as well as cleavage of prME occurs, followed by release at the cell surface (Lindenbach et al., 2007).

OMICS BASED APPROACH FOR HDT TARGET SELECTION

Viruses encode only few essential genes and heavily rely on the host cells to complete their replication cycle. The cell responds to infection by activating antiviral pathways. Therefore, hundreds of host factors are required for either supporting or limiting viral infection, and hence, their modulation could represent optimal targets for HDT. A number of loss-of-function genetic screens based on RNA interference (RNAi), on insertional mutagenesis in human haploid cells (HAP1) or on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology have been conducted over the years to provide an unbiased and comprehensive strategy to uncover host factors that promote or restrict virus replication (Krishnan et al., 2008; Sessions et al., 2009; Marceau et al., 2016; Zhang et al., 2016). A limited number of gain-of-function screens, based on overexpression of a set of putative antiviral effectors or of cellular microRNAs (miRNA), have also been performed (Schoggins et al., 2011; Smith et al.,

2017). Other screens based on viral protein-protein or viral RNA-protein interaction provided information on the cellular pathways engaged by the infection, albeit only after proper functional validation (Ward et al., 2011; Heaton et al., 2016; Phillips et al., 2016). Genetic screens and interactome analysis are valuable instruments to understand the viral replication cycle, although often similar screens poorly overlap in the output, indicating high variability in their execution. Bottom-up approaches, such as those outlined above, are complemented by top-down strategies that take advantage of libraries of compounds of various sources, such as herbal drugs from traditional medicine or drugs approved for the treatment of other pathologies (Sood et al., 2015; Barrows et al., 2016; Wang et al., 2017). Repurposing strategies are particularly attractive because, if successful, may provide ready-to-use drugs that have already passed several steps in the approval process for human treatment. Drug repurposing, also known as drug re-profiling or drug repositioning, is the identification of new therapeutic indications for drugs that are in the clinics or compounds that failed at some stage of the clinical trials. Drug repurposing can be considered a first line approach for neglected infectious diseases primarily occurring in developing countries, where an effective treatment is urgently needed. This strategy reduce time and risks intrinsic to the drug discovery process to quickly advance a drug-candidate to late-stage development, therefore it is very attractive for pharmaceutical companies as it may open new markets for proprietary compounds.

Top-down approaches suffer from difficulties in the definition of the active principle and from the lack of knowledge of the target, except in the case of repurposing where the target has already been identified in most cases. To note, however, that the inhibitory concentration of a drug, repurposed for Flaviviruses infection, could be very different to that used therapeutically for the original disease. This has two important implications, first that the active target might be different, second that the clinical trials should be repeated at the new active concentrations.

The results from such efforts and from "educated guess" approaches resulted in a number of compounds at various levels of testing. To note, however, that Flaviviruses have dual hosts: mosquitoes/ticks or vertebrates (birds, mammals). Therefore, the analysis that follows will be limited to mammalian cells in view of human treatment. To this end, the lifecycle of Flaviviruses has been divided in four steps: (i) attachment and entry, (ii) translation and polyprotein processing, (iii) replication, and (iv) egress. Attention has been put on druggable host factors at the various steps of the viral life cycle and on promising HTDs that have been discovered as a result of repurposing or screenings. A scheme illustrating the approaches to identify HTD for Flavivirus infection is shown in **Figure 1**, while **Table 1** summarizes the antiviral HTD drugs for Flavivirus treatment proposed to date.

ATTACHMENT AND ENTRY

The E protein coating the virion is responsible for attachment to the cell surface through low-affinity receptors and co-receptors. It is likely that Flaviviruses require multiple co-receptors that define tissue tropism and pathogenesis (Perera et al., 2008; Perera-Lecoin et al., 2013). A number of receptors, for several members of the family, have been proposed *in vitro*. For example, antagonists of the chemokine co-receptor CCR5, developed for HIV-1 therapy, were effective in DENV treatment (Marques et al., 2015). However, a complete picture of (co)-receptors for Flavivirus entry is yet not available and identification of HDT targets of attachment awaits further studies. After attachment, the virus enters the cell by endocytosis, which could be either clathrin-dependent or independent based on the virus and cell type analyzed (Kalia et al., 2013). Nevertheless, acidification of the late endosome, mediated by the vacuolar-type H + ATPase (vATPase) complex, is a common essential step for all Flaviviruses that is enhanced by the transmembrane protein ribonuclease kappa (RNASEK) (Perreira et al., 2015). Acidification induces conformational changes in the E protein that expose hydrophobic peptides. This permits the fusion of the viral and vesicle membranes to allow nucleocapsid access to the cytoplasm (Kaufmann and Rossmann, 2011). As an example of repurposing, the anti-malaria drug Chloroquine is capable of alkalinizing intracellular organelles, such as endosomes and lysosomes. With such mechanisms, chloroquine is suitable to block entry of flaviviruses that require a pH-mediated fusion step in endosomes. *In vitro,* this drug inhibited DENV replication in a dose-dependent manner at non-toxic concentrations, with the additional benefit of lowering the activation of pro-inflammatory cytokines (Tricou et al., 2010). However, treatment of patients had no favorable effect on viremia or fever. The final step of the entry, that is uncoating, is least understood. However, several reports have demonstrated that it requires ubiquitin-mediated destabilization of the capsid, which allows ribosomes to access viral RNA (Krishnan et al., 2008; Byk et al., 2016).

TRANSLATION AND POLYPROTEIN PROCESSING

The viral RNA is capped to allow initiation of translation by host factors. However, DENV has been shown to be able to switch from cap-dependent to cap-independent RNA translation when host cell translation is inhibited experimentally (Edgil et al., 2006). Lack of a poly(A) tail appears to be compensated by direct binding of the poly(A) binding protein (PABP) to the 5′-end of the viral genome (Polacek et al., 2009). In addition, recent findings showed that ZIKV and DENV suppress host cell translation, while translation of their RNA is maintained (Roth et al., 2017; Reid et al., 2018). Ribosomal proteins are required for core ribosome activities and for translation of specific subsets of mRNA and possibly vRNA. RPS25, RPL18, RPLP1/2 have been shown to be required for Flavivirus infectivity and likely required for translation of vRNA (Le Sommer et al., 2012; Cervantes-Salazar et al., 2015; Marceau et al., 2016; Campos et al., 2017). Flavivirus translation occurs at the ER membrane, most probably through the signal recognition particle (SRP) pathway. Indeed, several screens identified proviral components of the SRP pathway and translocon complex (Sessions et al.,

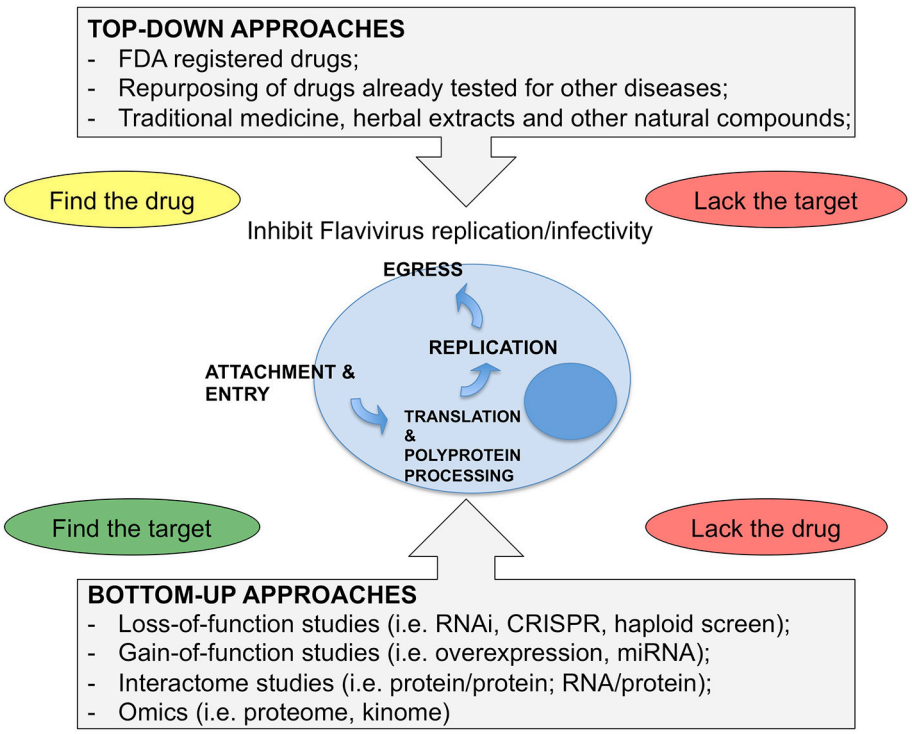

FIGURE 1 | Strategies for antiviral HDT. **(Top-Down)** approaches consider drugs from various sources as starting material for a screen of antiviral activity. These approaches can identify drugs with both viral and cellular targets. However, when starting from complex mixtures, it is not easy to define the active compound, while screenings of Food and Drug Administration (FDA) registered drugs have the advantage of knowing the drug in advance and, in most cases, the target. Bottom-up approaches are based on unbiased screens by depleting or overexpressing host factors or miRNAs. Here the critical issue is to find the drug for the target that is identified by the screen. **(Bottom-Up)** approaches also include various approaches where the network of virus-host interactions is studied or where the response of the cell to the infection is analyzed as a whole. These methods require functional validation and only after that it is possible to proceed further and select the drugs.

2009; Le Sommer et al., 2012; Marceau et al., 2016; Zhang et al., 2016). Antiviral host factors include the TIA/R proteins that bind the TBEV genomes to inhibit translation (Albornoz et al., 2014). The viral polyprotein is cleaved by NS3 and its cofactor NS2B (NS2B/3 complex) and cellular proteases. Three subunits (SPCS1/2/3) of the signal peptidase complex (SPC) have been shown to be required for DENV and WNV infectivity (Zhang et al., 2016). Intriguingly, dependence on SPC appears to be a peculiarity of Flaviviruses not shared by any other RNA virus making it a promising target for therapy. Protein chaperons have been shown to be important for Flavivirus infection at multiple steps of the viral life cycle. HSP70 appears to be required for NS5 folding, but also associates with the capsid and is involved in assembly and possibly in entry (Ye et al., 2013; Taguwa et al., 2015). Protein chaperones, such as HSP70 or HSP90, are considered generally pro-viral as they promote protein folding. These could represent important targets for antiviral therapy, particularly in association with direct antiviral agents to decrease the threshold for mutagenesis leading to resistance (Geller et al., 2007; Anderson et al., 2014). The small molecule HS-72 has been shown to inhibit the stress-inducible form of HSP70 leading to a defect in DENV entry (Howe et al., 2016).

REPLICATION

Replication of Flavivirus genomes occurs in replication vesicles spatially separated from sites of translation (Welsch et al., 2009; Gillespie et al., 2010; Miorin et al., 2013). This probably allows the switch from two incompatible processes targeting the same vRNA: translation and RNA synthesis as well as protecting viral dsRNA from innate sensing (Overby et al., 2010; Miorin et al., 2012). Several host RNA-binding proteins have been involved in vRNA synthesis: exoribonuclease family member 3 (ERI3) localizes to sites of viral replication and enhances RNA synthesis of DENV and YFV (Ward et al., 2016). The AU-rich binding factor 1, p45 isoform (AUF1 p45) and its cofactor arginine methyltransferase (PRMT1) have been shown to promote WNV vRNA cyclization, which is required for vRNA synthesis (Friedrich et al., 2014, 2016). Several other host factors have been shown to bind the viral RNA, but their mechanism of action still remains elusive. Replication vesicles contain both non-structural viral proteins and vRNA and are connected to the cytosol by a pore. The RNA-dependent RNA polymerase NS5 catalyzes the synthesis of the minus-strand vRNA that becomes the template for multiple rounds of positive-stranded vRNA, which then could either be translated,

TABLE 1 | Antiviral HDT for Flaviviruses.

Viral step	Target	Proposed drug and mechanism	Comments	References
Attachment	CCR5 co-receptor	Met-RANTES (CCR5/CCR1 receptor antagonist). Competes for CCR5 binding	Requires treatment before infection to be effective. Limited to *in vitro* studies	Marques et al., 2015
Entry	Lowers the pH of endosomes and lysosomes	Chloroquine and Mefloquine (repurposing of antimalarial drugs) Alkalinization of intracellular organelles impairs virus entry	Effective *in vitro* and in a primate model of infection but not effective in DENV patients	Tricou et al., 2010; Barrows et al., 2016
	Inducible heat-shock protein 70 (Hsp70i)	HS-72 (N-(1-Propyl-1H-benzimidazol-2-yl)-1-(2-pyrazinyl)-3(S)-piperidinecarboxamide). Disrupts the association of Hsp70i with the DENV receptor complex	Limited to *in vitro* studies	Howe et al., 2016
	host cell kinases AP2-associated protein kinase 1 (AAK1) and cyclin G–associated kinase (GAK)	Sunitinib and Erlotinib (repurposing of anticancer drugs) potent tyrosine kinase inhibitors that act as broad-spectrum antivirals. Host kinases AAK1 and GAK regulate entry, but also assembly and/or release of multiple RNA viruses through phosphorylation of membrane trafficking adaptors	Synergistic effect of both drugs on DENV infection. Active *in vitro* and in mice	Bekerman et al., 2017
	dopamine D2 receptor (D2R)	Prochlorperazine (repurposing of an antiemetic drug) D2R antagonist also targeting DENV entry	Active *in vitro* and in animal models	Simanjuntak et al., 2015
	N-methyl-d-aspartate (NMDA) receptors	Memantine (blockade NMDA receptors) is an amantadine derivative used for the treatment of neurological disorders and Alzheimer. Prevents neuronal death caused by ZIKV	Active *in vitro* and in animal models	Costa et al., 2017
	Unknown	Nanchangmycin (polyether produced by *Streptomyces nanchangensis*) blocks an early step in the entry process of ZIKV, discovered in a screening of FDA-approved drugs	Active *in vitro* on several cell types and against several viruses including DENV, WNV, CHIKV, SINV	Rausch et al., 2017
	Membrane function	Daptomycin (antibiotic) lipopeptide that disrupts cell membranes rich in phosphatidylglycerol (PG) suggesting an effect on late endosomal membranes, which are critical for ZIKV entry	Mechanism still unclear. Limited to *in vitro* studies	Barrows et al., 2016
Replication	Oligosaccharyltransferase (OST) complex	NGI-1 (N-linked Glycosylation Inhibitor-1) Blocks DENV and ZIKV RNA synthesis independently of its activity on glycosylation	Limited to *in vitro* studies	Puschnik et al., 2017
	Inosine monophosphate (IMP) dehydrogenase	MPA (mycophenolic acid) used as immunosuppressant but with broad antimicrobial activities. Blocks replication of DENV RNA by depleting intracellular guanosine levels	Limited to *in vitro* studies	Diamond et al., 2002
	Importin α/β	Ivermectin (broad-spectrum anti-parasite drug). Proposed to block nuclear import of DENV NS5 replicase	Shown also to target YFV NS3 helicase Limited to *in vitro* studies	Mastrangelo et al., 2012; Wagstaff et al., 2012; Barrows et al., 2016
	Cyclophilin A (CyPA) peptidyl-prolyl isomerases	Cyclosporin (immunosuppressant). Affects RNA synthesis by targeting the interaction of CyPA with WNV NS5	Limited to *in vitro* studies	Qing et al., 2009
	Bcr-Abl kinase	Imatinib and Nilotinib (repurposing of anticancer drugs) inhibit the tyrosine kinase Bcr-Abl and have shown activity for DENV replication. Derivative GNF-2 also targeting DENV E protein	Derivative GNF-2 also targeting DENV E protein. Limited to *in vitro* studies	Clark et al., 2016

(Continued)

TABLE 1 | Continued

Viral step	Target	Proposed drug and mechanism	Comments	References
	Bcr-Abl kinase	AZD0530 and Dasatinib (repurposing of anticancer drugs) inhibit DENV RNA replication	Inhibition mechanism toward DENV *via* the Fyn kinase. Limited to *in vitro* studies	de Wispelaere et al., 2013
	Eukaryotic translation	Nitazoxanide (thiazolid, anti-protozoan drug) Licensed drug effective in the treatment of gastrointestinal infections and proposed as a broad-spectrum antiviral agent. Inhibits translation by activation eIF2α	Activity *in vitro* against DENV, JEV, YFV	Rossignol, 2014
	Unknown	Hippeastrine hydrobromide (*Amaryllidaceae* plant extract) reported to anti- HCV and anti-avian influenza H5N1 activity. Active against ZIKV, discovered in a screening of FDA-approved drugs	Active *in vitro* in neuronal progenitors, organotypic infection models and in animal models	Zhou et al., 2017
	Unknown	Azithromycin (macrolide antibiotic) reduced viral proliferation and virus induced cytopathic effects in glial cell lines and human astrocytes	Stage of infection inhibition not known. Limited to *in vitro* studies	Retallack et al., 2016
Assembly and Egress	Acetyl-CoA Carboxylases	TOFA (5-(tetradecyloxy)-2-furoic acid) and MEDICA 16 (3,3,14,14-tetramethylhexadecanedioic acid). Reduces the synthesis of lipids affecting membrane rearrangements during WNV infection	May also affect virus replication in addition to assembly. Limited to *in vitro* studies	Merino-Ramos et al., 2016
	chymotrypsin-like activity of the proteasome	Bortezomib (proteasome inhibitor). The mechanism is not clear but inhibits virus egress *in vivo*	Limited to *in vitro* studies	Choy et al., 2015; Barrows et al., 2016
	Serine-Arginine-rich protein kinase	SFV785 (1-[2-(1-azacyclooctanyl)-5-(trifluoromethyl)] phenyl-3-nicotinoylthiourea). Affect assembly-associated ER compartments	Limited to *in vitro* studies	Anwar et al., 2011
	ER α-glucosidases	CM-9-78 and CM-10-18 (Oxygenated alkyl imino sugar derivatives). Inhibitors of a-glucosidases I and II	Increased efficiency in combination with Ribavirin. Limited to *in vitro* studies	Chang et al., 2011a,b
	ER α-glucosidases	Celgosivir (6-O-butanoyl castanospermine) inhibits glycosylation of viral protein E and NS1	Active *in vitro* and *in vivo* but failed in in a phase 1b clinical trial (CLADEN)	Rathore et al., 2011; Low et al., 2014; Watanabe et al., 2016
	ER α-glucosidases	Deoxynojirimycin (DNJ) is a natural iminosugar extracted from Mulberry leaves. DNJ and derivatives inhibit glycosylation of viral protein E and NS1	Active for DENV *in vitro* and *in vivo* (AG129 mice)	Wu et al., 2002
	glucocorticoid receptor agonist	Prednisolone (Corticosteroid) Corticosteroids are highly effective anti-inflammatory agents that have been proposed for DENV HSS	Does not affect DENV infection *in vitro* and is not active in patients	Tam et al., 2012
	3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase	Lovastatin (repurposing of a statin drug). Decreases cholesterol/isoprenoid synthesis and glycosylation affecting entry and the release of infectious particles from the infected cell	Lovastatin di not affect viremia nor virus clearance *in vivo* and in clinical trials	Rothwell et al., 2009; Martinez-Gutierrez et al., 2014; Whitehorn et al., 2016

HTD drugs obtained by a variety of top-down and bottom-up strategies that showed inhibitory potential toward Flaviviruses are listed together to their target(s) and proposed mechanism of action.

further replicated or assembled in new virions. ER membrane rearrangement is a characteristic hallmark of all Flaviviruses, but the molecular details of the process, which likely requires several host factors, are still not understood. The ER associated oligosaccharyltransferase (OST) complex was recently found to be associated with viral non-structural proteins and required for DENV RNA synthesis. The catalytic function of the OST complex is not required for DENV replication, suggesting that

the complex may have a structural role in the formation of replication complex (Marceau et al., 2016). Both STT3A and STT3B subunits of the OST complex are required for DENV replication, but only STT3A is required for YFV, ZIKV, and WNV infectivity, highlighting differences in the requirement for OST complex variants among Flaviviruses. Interestingly, the OST inhibitor NGI-1 has pan-Flavivirus antiviral activity highlighting the critical importance of this cellular pathway, which could be exploited for antiviral HDT (Puschnik et al., 2017). Other processes that induce ER membranes rearrangements have been implicated in Flavivirus replication. DENV, ZIKV, and YFV strongly depend on the ER membrane complex (EMC) for their replication (Savidis et al., 2016). The EMC is involved in transmembrane proteins processing, maturation and is also associated to the OST complex. During Flavivirus replication, an abundant non-coding RNA fragment (termed sfRNA for subgenomic flaviviral RNA) has been detected in infected cells (Pijlman et al., 2008). The sfRNA is derived from incomplete degradation of the viral 3′ NCR by the cellular 5′-3′ exonuclease Xrn1 and has been proposed to regulate various cellular pathways to facilitate flaviviral pathogenicity and to inhibit the interferon/stress response (Bidet et al., 2014; Manokaran et al., 2015). Flaviviral RNA is also edited by host methyltransferases for N6-adenosine methylation (m6A), which modulates viral replication (Gokhale et al., 2016; Lichinchi et al., 2016). Generation of sfRNA and m6A-vRNA are peculiar aspects of the Flavivirus life cycle dependent on host factors that could be targeted for HDT.

ASSEMBLY AND EGRESS

Assembly of virions initiate at the ER membrane by association between the capsid and vRNA, which results in the formation of nucleocapsid. Electron microscopy images show assembly occurring juxtaposed to the replication vesicles, possibly to facilitate the process of extruding the vRNA from the vesicles pore and binding to the capsid (Romero-Brey and Bartenschlager, 2014). Association of the nucleocapsid with E and prM heterodimers, inserted into the ER membrane, precedes budding of immature viral particles into the ER lumen. Viral particles are then transported along the secretory pathway to the Golgi where maturation and N-linked glycosylation of prM and E proteins takes place. A reduction in pH triggers a conformational change in prME spikes, which involves the same vATPase required for entry (Duan et al., 2008). The cellular protease Furin cleaves prM in this acidified compartment, converting the immature viral particle into a fully infectious virus that is subsequently released from the cell by vesicular fusion with the plasma membrane. Lipid droplets were shown to play a role in DENV assembly, either as storage for capsid protein or for nucleocapsid formation (Samsa et al., 2009). Several host factors have been involved in capsid localization to lipid droplets indicating the importance of this compartment (Iglesias et al., 2015). Cholesterol is also involved in the formation of lipid droplets required for DENV replication. Lovastatin inhibits DENV by reducing the release of infectious particles from the infected cell, but also by inhibiting virus entry (Rothwell et al., 2009; Martinez-Gutierrez et al., 2014). *In vivo* and in clinical trials, Lovastatin did not affect viremia nor virus clearance (Whitehorn et al., 2016). Corticosteroids may alleviate symptoms such as, capillary permeability and hemorrhage that are exacerbated in severe Dengue infection. Corticosteroids do not inhibit, neither enhance, Dengue infection *in vitro* and in patients these treatments were not efficacious (Tam et al., 2012). A key druggable enzyme for lipid synthesis is the acetyl-CoA carboxylase and its inhibitors have been shown to reduce WNV replication significantly (Merino-Ramos et al., 2016). However, targeting lipid metabolism could affect several steps of the virus life cycle. The host helicase DDX56 binds capsid and may facilitate transfer of viral RNA from the replication vesicles to the ER membrane, which is the site of WNV assembly (Xu and Hobman, 2012). Moreover, druggable targets for antiviral therapy could be the Src kinases that have been shown to be involved in the late stages of DENV life cycle and in promoting WNV trafficking through the secretory pathway (Hirsch et al., 2005). Proteins of the endosomal sorting complex required for transport (ESCRT) localize to the sites of DENV and JEV assembly, and their depletion inhibited the production of infectious virus (Tabata et al., 2016). Furthermore, the proteasome inhibitor Bortenzomib was also shown to inhibit DENV production *in vitro* and *in vivo* (Choy et al., 2015) as well as ZIKV. However, the mechanism for the latter was not described (Barrows et al., 2016). Other factors such as the Ras-related in brain protein Rab8b and the ADP-ribosylation proteins Arf4/5 were shown to promote Flavivirus egress (Kudelko et al., 2012; Kobayashi et al., 2016). Disruption of the KDEL-based recycling process was shown to be required for DENV egress by associating with prM (Li et al., 2015). The kinase inhibitor SFV785 targets the recruitment and assembly of the nucleocapsid during DENV assembly and reduces the production of infectious DENV (Anwar et al., 2011). An important process that occurs in the secretory pathway is the glycosylation of viral proteins. Within Flavivruses, the targets of glycosylation are the prM, E and NS1 proteins. Flaviviruses strongly depend on glycosylation for infectivity (Sessions et al., 2009). Several iminosugar derivatives, that inhibit glycosylases, demonstrated potent antiviral activity against Flaviviruses, either alone or in combination with Ribavirin (Chang et al., 2011a). Celgosivir (6-O-butanoyl castanospermine) is an inhibitor of ER α-glycosidases, active against DENV with *in vitro* EC_{50} in the sub-micromolar range and good selective index. Oral Celgosivir is active in a model of lethal Dengue in mice with reduction of viremia and protection against virus-induced mortality (Rathore et al., 2011; Watanabe et al., 2016). However, in a phase 1b clinical trial (CELADEN), celgosivir failed to show a significant antiviral effect, although modest antiviral trends were observed in patients with secondary infection (Low et al., 2014). Celgosivir antiviral activity against Dengue is both cell and virus-strain dependent and timing of treatment appears to be critical. Although Celgosivir failed in clinical trials, approaches targeting viral protein glycosylation appear very promising because, in addition to affecting directly virus assembly and egress, it also induce ER stress and the unfolded protein response, which

could potentiate antiviral activity through apoptosis or the innate signaling pathway (Smith, 2014).

CONCLUDING REMARKS

The road toward HDT for treatment of Flavivirus infections is still long, but investing in a detailed understanding of the virus lifecycle at the molecular levels could eventually lead to novel and more potent strategies for treatment.

Current progress outlined in this review identified a panel of compounds with different cellular targets that have a potential inhibitory activity against Flaviviruses. In particular, two pathways are showing great promise: the ER associated OST complex required for RNA synthesis and the induction of ER stress that may induce innate signaling pathways. Studies need to move quickly to *in vivo* and to clinical trials when appropriate, but even if compounds result not as effective as expected, they remain very informative on the cellular pathways most relevant to Flavivirus infection. Concerning repurposing approaches, the most promising drugs are probably the kinase inhibitors that are already being used for the treatment of inflammation and cancer. These drugs could quickly move to the clinics for antiviral HDT.

The near future would hopefully witness broad-range treatments that will provide a first line pharmaceutical coverage in the case of new epidemics of members of this family of viruses.

AUTHOR CONTRIBUTIONS

AM drafted the manuscript. TC and MKZ reviewed the manuscript, added references, and prepared the figure.

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MINI REVIEW
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Targeting IL-13 as a Host-Directed Therapy Against Ulcerative Colitis

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The role of interleukin-13 in mediating ulcerative colitis remains under scrutiny. Compelling evidence from both man and mouse suggests that IL-13 not only contributes to the pathology associated with disease but is also involved in mediating the inflammatory response. These studies have led to the approach of targeting IL-13 as a promising treatment strategy in alleviating ulcerative colitis disease. Despite this evidence, recent clinical trial data suggests that specifically blocking the receptor through which IL-13 signals, IL-4 receptor-alpha (IL-4Rα) in ulcerative colitis patients, is insufficient in protecting them from disease outcome. This challenges the importance of IL-13 as a therapeutic target. This review describes the role of IL-13 in ulcerative colitis and current treatment strategies that target IL-13. The potential role of IL-13 signaling independently of IL-4Rα in mediating ulcerative colitis is highlighted as an important consideration when targeting the signaling mechanisms of IL-13 for therapeutic approaches.

Keywords: ulcerative colitis, interleukin-13, IL-4 receptor-alpha, drug targets, Inflammatory Bowel Disease, T-helper type 2 immune response

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INTRODUCTION

Since the discovery of IL-13 it has been shown to be a key cytokine in controlling pathogens such as helminthic parasites, but also as a prominent feature in allergic and inflammatory diseases. IL-13 is a 10–14 kDa immune-regulatory cytokine first described as a protein preferentially produced by activated T helper-Type (Th) 2 cells (Brown et al., 1989; McKenzie et al., 1993; Minty et al., 1993; Hershey, 2003). However, it has since been established that IL-13 is in-fact produced by a wide variety of cell types, including innate immune cells, with diverse biological activities (Wynn, 2003; Mannon and Reinisch, 2012). These include basophils, eosinophils, mast cells, natural killer cells, epithelial cells, smooth muscle cells, fibroblasts, and NK T cells. This highlights the role of cells other than T and B cells that produce IL-13; however the role of these cells specifically in the gut is yet to be established. Once produced IL-13 initiates a cascade of immunological process which aid in parasite clearance. Here IL-13 can act directly on macrophages, driving the differentiation toward the M2 phenotype resulting in a Th2 response (Gordon, 2003). Furthermore, IL-13 drives beneficial responses such as, the IgE isotype switch, eosinophil recruitment, mucus production and muscle contraction. However, these are the very responses that contribute to pathology in an inflammatory response. Our own studies have shown that blocking IgE in an animal model of ulcerative colitis reduces the severity of disease (Hoving et al., 2012). This was associated with reduced mast cell activation as described below. The pleiotropic nature of IL-13 quickly became evident by the myriad of diseases in which it plays either a beneficial or detrimental role. IL-13 is a prominent mediator of allergic lung disease, including pulmonary inflammation, asthma and anaphylaxis (Gour and Wills-Karp, 2015). More recently, IL-13 has been linked to enhancing brain function in mice by increasing cognitive memory (Brombacher et al., 2017). Here, IL-13 was able to stimulate primary astrocytes

to produce brain-derived neurotrophic factor, known to enhance cognitive function. Furthermore, a new SNP in the regulatory region of *il13* (rs1881457C) has been associated with an increased risk of Coronary Artery Disease in a Chinese Han co-hort (Zha et al., 2018). Here the functional mechanisms remain unknown.

IL-13 AND ITS ROLE IN COLITIS

IL-13 is an interesting cytokine in the role it plays in mediating Th2 inflammatory diseases. Initially IL-13 was a target for host directed therapy for asthma, dermatitis and other allergic diseases. However, IL-13 has also been linked to mediating the host inflammatory cascade responsible for the pathogenesis of ulcerative colitis. Combining evidence from mice and man, the mechanism of IL-13 mediated colitis is illustrated in **Figure 1**. Essentially, a defect in defect in antigen recognition triggers an inappropriate and exaggerated immune response. This is further aggravated by the disruption of epithelial tight junctions, increasing permeability of the intestinal epithelium and resulting in an increase in the uptake of luminal antigens. In a mouse model of ulcerative colitis using the hapten, oxazolone to induce a transient disease phenotype, blocking IL-13 (Heller et al., 2002) or using IL-13 gene-deficient mice (Weigmann et al., 2008) has been shown to ameliorate or prevent disease induction. While IL-13 production by NK T cells has been shown to play a major role in mediating disease, our own studies have implicated additional components of the immune response that contribute to the onset and maintenance of disease. These include IL-4Rα-responsive $CD4^+$ T cells and IgE production by B cells which contribute to oxazolone-induced pathology in mice. Depleting IgE was linked to a reduction in the number of activated mast cells and reduced pathology (Hoving et al., 2012).

Increasing evidence demonstrates that IL-13 is responsible for initiating the detrimental inflammatory cascade in colitis. While orchestrating an inflammatory response by immune cells IL-13 can also act directly on epithelial cells. In ulcerative colitis, IL-13 has been described as a key effector cytokine acting on epithelial cell function and initiating apoptosis (Heller et al., 2005, 2008). The addition of IL-13 *in vitro* to HT-29 epithelial cell monolayers causes an increased expression of the pore-forming tight junction protein claudin-2 (Rosen et al., 2011). The increased expression of this protein was associated with increased epithelial barrier permeability. As a consequence, small antigens enter the gut and the loss of ions and water into the intestinal lumen leads to diarrhea. Independent to the role on claudin-2, IL-13 was recently shown to downregulate tricellulin expression. Tricellulin is a protein essential for the barrier against macromolecules

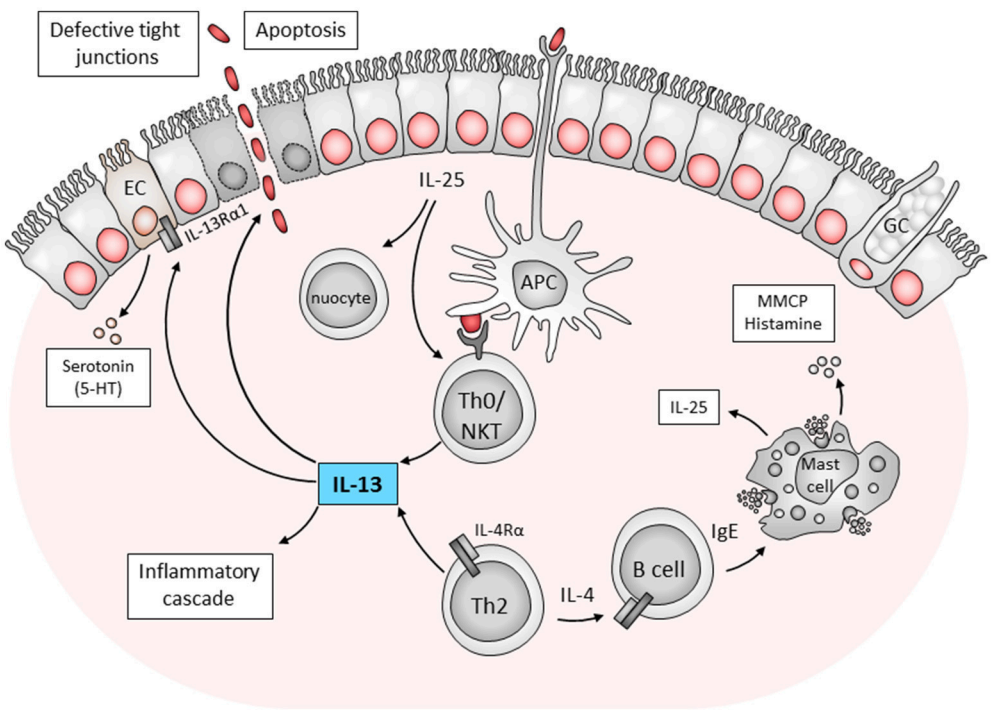

FIGURE 1 | Immune components contributing to ulcerative colitis. The mechanisms of pathogenesis is postulated as follows; a defect in antigen sampling by antigen presenting cells (APCs) or direct stimulation from epithelial cells activates Th0 cells or NK T cells to drive a Th2/type 2 response. Here IL-25 production by epithelial cells was also linked to disease pathology through nuocytes and NK T cells. NK T cells produce IL-13 which is toxic to epithelial cells. Furthermore, conventional CD4+ Th2 cells which produce IL-4 can also stimulate B cells to drive inflammation in an IgE-dependent manner. These findings indicate the complex interaction of host cells in the development of ulcerative colitis. APC, antigen presenting cell; EC, enterochromaffin cell; GC, goblet cell; IL, interleukin; MMCP, murine mast cell protease; NKT, natural killer T cell; Th0, T helper type; 5-HT- serotonin 5-hydroxytryptamine (Heller et al., 2002; Ikeda et al., 2003; Ghia et al., 2009; Camelo et al., 2012; Hoving et al., 2012).

and is reduced in ulcerative colitis but not Crohn's disease (Krug et al., 2017). While IL-13Rα1 upregulates claudin-2 in ulcerative colitis, IL-13Rα2 downregulates tricellulin, allowing macromolecule uptake.

Additional studies have expanded on the current understanding of the role IL-13 plays in colitis and describe additional mechanisms associated with IL-13 during colitis. For example, in the oxazolone colitis mouse model, blocking IL-25 derived from intestinal epithelial cells improved the clinical aspects of disease (Camelo et al., 2012). This was associated with reduced IL-13 production by lamina propria cells, fewer NKT cells, and nuocytes infiltrating the mucosa and a decrease in serum IgE levels. Interestingly, mast cells have previously been shown to be potent producers of IL-25 (Ikeda et al., 2003), which could in turn also contribute to the downstream immunological cascade seen in ulcerative colitis. Therefore, not only could IL-25 be involved in initiating disease, but also maintaining the detrimental Th2 response in established disease. Interestingly, in the Dextran sulfate sodium (DSS) hapten-induced mouse model of ulcerative colitis, serotonin production by enterochromaffin cells of the mucosa was implicated in disease (Ghia et al., 2009). More recently, this serotonin production was linked to IL-13, and highlights the interaction between the immune-endocrine axis in IL-13-mediated gut inflammation (Shajib et al., 2013). These mechanistic insights into disease pathogenesis could provide additional host directed IL-13 drug targets to alleviate the symptoms of ulcerative colitis.

IL-13 SIGNALING MECHANISMS

Both IL-4 and IL-13 cytokines use the IL-4Rα chain as a component of their receptors (**Figure 2**). This was shown in mice treated with anti-IL-4Rα antibodies or IL-4 antagonists (Aversa et al., 1993), which specifically blocked responses of both IL-4 and IL-13 (Zurawski et al., 1993, 1995; Hilton et al., 1996). The IL-4Rα consists of a 140-kDa IL-4Rα chain which is a component of both the type I and type II IL-4 receptors. The IL-4Rα is expressed in relatively low numbers on numerous cell types. The type I IL-4 receptor results from association of IL-4Rα with the gamma common (γc) chain, which is also a component of the receptors for IL-2, IL-7, IL-9, and IL-15 (McKenzie et al., 1993). The type II IL-4/IL13 receptor results from association of IL-4Rα with IL-13Rα1. The type II receptor is composed of the IL-4Rα chain and the 65-70 kDa IL-13Rα1 chain and serves as an alternative receptor for IL-4 (**Figure 2**). By itself, IL-13Rα1 binds IL-13 with low affinity but when paired with IL-4Rα, it binds IL-13 with high affinity and forms a functional unit that signals (McKenzie et al., 1993; Miloux et al., 1997). IL-13Rα1 is expressed on the majority of cell types tested with the exception of human or mouse T cells (Hershey, 2003). Although IL-13 signals via the IL-13Rα1, it has a higher binding affinity to the α2 chain of the IL-13 receptor (IL-13Rα2), which has previously been considered as a decoy receptor for IL-13 with no signal transduction. IL-13Rα2 is a 55–60 kDa protein closely related to IL-13Rα1 except that the cytoplasmic domain has no signaling motifs or binding sequences for signaling molecules (Donaldson et al., 1998). However, more

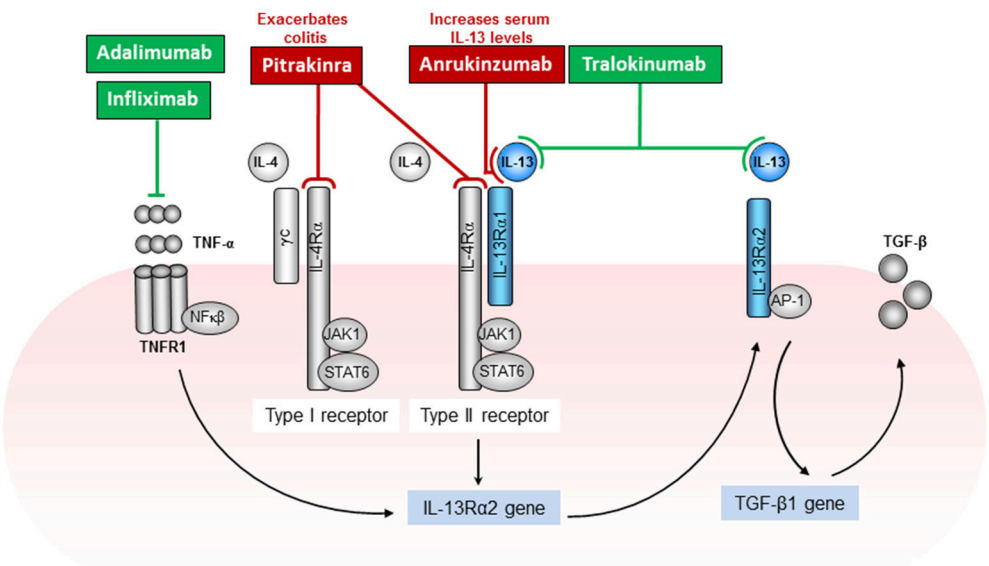

FIGURE 2 | IL-13 signaling mechanisms and associated host directed targets in the treatment of ulcerative colitis. IL-13 signals through the type II (IL-4Rα and IL-13Rα1) receptor complex and activates the JAK1/STAT6 pathway. In addition, IL-13 has also been shown to signal through IL-13Rα2, activating AP-1 to induce the secretion of TGF-β. This pathway is, in part dependent on the production of TNFα. Various drug targets have been directed at IL-13 including the IL-4Rα signaling pathway to block the immune response that mediate Th2-driven inflammatory diseases such as allergy and colitis. Drug treatments that were beneficial or improved disease outcome are depicted in green and treatments that were unsuccessful or exacerbated disease outcome are depicted in red (Rutgeerts et al., 2005; Wenzel et al., 2007; Levin and Shibolet, 2008; Reinisch et al., 2011, 2015; Mannon and Reinisch, 2012; Sandborn et al., 2012; Verma et al., 2013; Colombel et al., 2014; Feagan et al., 2014; Danese et al., 2015; Palamides et al., 2016; Hoving et al., 2017; Popovic et al., 2017).

recent publications have highlighted a possible signaling pathway for IL-13 through the IL-13Rα2. IL-13 signaling through the IL-13Rα2 was shown to be involved in the induction of TGF-β1 production or mediating fibrosis in a chronic mouse model of Crohn's disease (Fichtner-Feigl et al., 2006, 2008).

IL-4 and IL-13 not only share common subunits, they also share common signaling pathways (**Figure 2**). The components of both receptor complexes are associated with Janus kinases (JAK). JAK1 has been proposed to associate with the IL-4Rα chain, γc with JAK3 and IL-13Rα1 with JAK2 (Nelms et al., 1999). The signal transducer and activator of transcription 6 (STAT6) is recruited to the phosphorylated IL-4Rα where it also becomes phosphorylated by JAKs (Nelms et al., 1999). Studies using STAT6-deficient mice have determined that IL-13 signaling uses the JAK/STAT6 pathway (Takeda et al., 1996). In fact, in the oxazolone colitis model in which an increased epithelial cell, T cell, macrophage, and NKT cell STAT6 phosphorylation was observed, STAT6-deficient mice demonstrated a reduced disease phenotype (Rosen et al., 2013). Until recently the IL-13Rα2, which binds IL-13 with high affinity, was thought to relay no signal. Fichtner-Feigl and colleagues have shown that IL-13 signaling through IL-13RαI/IL-4Rα together with TNF-α signaling through TNFR1, up regulates IL-13Rα2 surface expression on macrophages. IL-13 binding this receptor activates AP-1 to induce the secretion of TGF-β (Fichtner-Feigl et al., 2006).

HOST DIRECTED TREATMENT STRATEGIES AGAINST IL-13

Considering the compelling link between IL-13 and ulcerative colitis, various clinical trials have been implemented to target IL-13 as a treatment strategy. A previous review by Mannon and Reinisch elegantly summarized drug targets for IL-13 in the treatment of colitis, however at the time most of these trials were ongoing and the outcomes unknown (Mannon and Reinisch, 2012). Conflicting results from recent clinical trials taint the optimism for using anti-IL-13 treatments for ulcerative colitis. However, a better understanding of the signaling mechanism of IL-13 and associated drug target sites could provide a useful approach for treatment strategies. Anrukinzumab (IMA-638) is a humanized monoclonal antibody which binds IL-13 and prevents the cytokine from binding IL-4Rα but maintains the ability to bind both IL-13Rα1 and IL-13Rα2 (**Figure 2**). In a multicenter, randomized, double-blind, placebo-controlled study, patients with active UC received anrukinzumab or placebo treatment (Reinisch et al., 2015). The primary endpoint was fold change from baseline in fecal calprotectin, a protein released into the intestine and recognized as a marker for active inflammatory bowel disease. IL-13 levels increased in treated patients, and no improvement was reported. Considering that IL-13 would still be able to bind other IL-13 receptors, this trial indicates the potential of IL-13 to mediate colitis independently of the IL-4Rα. In fact the mean fecal calprotectin levels in the patients receiving the highest dose of 600 mg anrukinzumab was actually increased at week 14. The authors conclude that there was no significant therapeutic effect of anrukinzumab on patients with active UC and that the study had a high drop-out rate due to the lack of efficacy.

Another recent candidate in ulcerative colitis treatment is tralokinumab, a human IL-13-neutralizing IgG4 monoclonal antibody. This monoclonal antibody has a very high affinity for IL-13 and is at stage III clinical trials for both allergic asthma and atopic dermatitis. A recent study characterizing the structure of tralokinumab Fab in complex with IL-13 demonstrates the inhibition of binding to both IL-13Rα1 and IL-13Rα2 (Popovic et al., 2017). Analyzing the structure in detail defined the mechanism of interactions and demonstrated that tralokinumab inhibits the formation of the tertiary complex among IL-13, IL-13Rα1, and IL-4Rα and inhibits the complex formation between IL-13 and IL-13Rα2. In a randomized, double-blind, placebo-controlled, phase IIa study for the treatment of ulcerative colitis, tralokinumab did not significantly improve the clinical response (Danese et al., 2015). These results were discouraging; however patients did have a higher clinical remission rate and improved mean partial Mayo score. Furthermore, patients in the study were not classified according to their base-line mucosal IL-13 levels. Considering that UC patients have been shown to have differential expression profiling of downstream inflammatory cytokines depending on the severity of disease (Verma et al., 2013), there may be the potential to identify specific patient groups with increased IL-13 as a biomarker for which tralokinumab treatment would be most beneficial.

Many treatment approaches against ulcerative colitis target the IL-4Rα signaling pathway. This is based on the fact that both IL-4 and IL-13 signal through the IL-4Rα. However, in light of recent studies describing the potential role of IL-13Rα2 in IL-13 signaling, this treatment approach may not be appropriate in ulcerative colitis (Fichtner-Feigl et al., 2006, 2008). Pitrakinra is a recombinant human IL-4 protein, (rather than a monoclonal antibody such as anrukinzumab and tralokinumab) which is mutated and therefore prohibits complex formation between IL-4Rα and IL-2Rγ or IL-13Rα1, but has no known effect on IL-13Rα2 (Wenzel et al., 2007). In a promising study of asthma, two phase IIa clinical trials demonstrated improved control over asthma symptoms after treatment with Pitrakinra (Wenzel et al., 2007). Based on this success, Pitrakinra treatment was used in a mouse model of ulcerative colitis (Palamides et al., 2016). To overcome some of the caveats of using chemically-induced animal models of colitis, the authors described a new model of ulcerative colitis. Essentially, NOD-scid IL2Rγnull mice reconstituted with peripheral blood mononuclear cells derived from UC-affected individuals develop colitis-like symptoms when challenged with ethanol. In this model pitrakinra showed no therapeutic benefit. In fact, treatment was associated with exacerbated symptoms and pathological manifestations. This outcome supports our own data from the oxazolone mouse model in which mice deficient of IL-4Rα presented with significantly exacerbated disease phenotype (Hoving et al., 2017). Here, mice that do not produce IL-13 (IL-4Ra/IL-13-deficient mice) are protected from colitis. However, the disease phenotype in the adoptive transfer model mentioned above was not directly associated with IL-13 and it would be interesting to know the outcome of pitrakinra treatment using the oxazolone colitis

model. Thus, attempts to block the IL-4Rα signaling pathway may actually exacerbate disease outcome.

Lastly, anti-TNFα has proven to be effective in patients that do not respond to convention treatment strategies. In fact this therapeutic approach was the first approved for inflammatory bowel disease treatment more than 50 years ago. As TNFα production is traditionally associated with a Th1 response, it is therefore not a likely candidate for ulcerative colitis treatment. None-the-less, monoclonal antibodies against TNFα such as infliximab and adalimumab have shown promising outcomes (Rutgeerts et al., 2005; Levin and Shibolet, 2008; Reinisch et al., 2011; Sandborn et al., 2012; Colombel et al., 2014; Feagan et al., 2014). Studies describing the effect of anti-TNFα treatment on patient IL-13 production are very limited. It can be postulated that the success of anti-TNFα therapy in ulcerative colitis could be linked to the mechanism behind IL-13Rα2 signaling (**Figure 2**). Fichtner-Feigl and colleagues, elegantly describe the signaling pathway during the activation of the IL-13Rα2 (**Figure 2**). Here IL-13Rα2 gene expression was shown to be dependent on TNFα and could provide, at least in part, a link between the IL-4Rα-independent signaling of IL-13 and successful anti-TNFα treatment in ulcerative colitis patients (Fichtner-Feigl et al., 2006).

CONCLUSION

The potential benefits of ameliorating IL-13 production in ulcerative colitis remains an interesting approach in treating disease. Furthermore, these treatment strategies could potentially be extended beyond the pathology of colitis. Targeting IL-13 in allergic diseases and dermatitis has already proven to be promising. If the optimal treatment strategy and correct targets can be identified for ulcerative colitis, this could even translate to preventative treatment of colitis-associated colorectal cancer. The link between ulcerative colitis and colorectal cancer is widely accepted, however the extent of the risk is difficult to determine as reports describe widely varying rates (Eaden et al., 2001; Lakatos and Lakatos, 2008). However, the severity of colitis and duration of inflammation are contributing risk factors. Therefore, reducing either of these by targeting IL-13 could consequently reduce the risk of cancer. Indeed, Schiechl and colleagues provide strong evidence to validate this preventative approach (Schiechl et al., 2011) as blocking IL-13 or depleting NKT cells reduced inflammation, tumor size and tumor number. In summary, new treatment approaches that specifically target IL-13 and differentiate IL-4 from IL-13 signaling mechanisms could be key in developing a successful treatment strategy in a subset of patients identified with a specific biomarker, for example increased mucosal IL-13.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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The Immune Mechanisms of Lung Parenchymal Damage in Tuberculosis and the Role of Host-Directed Therapy

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Impaired lung function is common in people with a history of tuberculosis. Host-directed therapy added to tuberculosis treatment may reduce lung damage and result in improved lung function. An understanding of the pathogenesis of pulmonary damage in TB is fundamental to successfully predicting which interventions could be beneficial. In this review, we describe the different features of TB immunopathology that lead to impaired lung function, namely cavities, bronchiectasis, and fibrosis. We discuss the immunological processes that cause lung damage, focusing on studies performed in humans, and using chest radiograph abnormalities as a marker for pulmonary damage. We highlight the roles of matrix metalloproteinases, neutrophils, eicosanoids and cytokines, like tumor necrosis factor-α and interleukin 1β, as well as the role of HIV co-infection. Finally, we focus on various existing drugs that affect one or more of the immunological mediators of lung damage and could therefore play a role as host-directed therapy.

Keywords: tuberculosis, lung damage, host-directed therapy, cavity, pulmonary function, matrix metalloproteinase, neutrophils, immune mechanisms

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INTRODUCTION

In 2016, an estimated 10.4 million people developed tuberculosis (TB) worldwide. Although effective diagnosis and treatment saved about 53 million lives between 2000 and 2016, TB remains a major threat worldwide: 16% of TB cases die from the disease, corresponding to 1.7 million deaths in 2016 (World Health Organization, 2017). Among those who are cured successfully, residual pulmonary impairment is common. Various studies have looked at lung function in patients with a known history of TB; they found abnormal lung function in 34 – 94% of patients, varying in severity from mild to severe (Willcox and Ferguson, 1989; Plit et al., 1998; de Valliere and Barker, 2004; Chung et al., 2011; Vecino et al., 2011; Akkara et al., 2013; Baez-Saldana et al., 2013; Ralph et al., 2013a; de la Mora et al., 2015; Nihues Sde et al., 2015; Manji et al., 2016). It results in considerable medical costs (Jordan et al., 2010) and decreased quality of life (Ralph et al., 2013a; de la Mora et al., 2015).

Impaired lung function is associated with chest radiograph (CXR) abnormalities in most of the studies. It can easily be measured using spirometry, which measures air volumes and airflow rates of the lung. Forced vital capacity (FVC) is the maximal volume of air exhaled by a patient from the position of maximal inspiration, by means of a rapid, maximally forced expiration; forced expiratory volume in 1 s (FEV1) is the amount of air exhaled during the first second of the FVC maneuver. The nature and severity of pulmonary impairment can be categorized by combining these two measurements: obstruction is defined as a FEV1/FVC ratio < 70%, restriction is suggested by a low FVC (<80% of the predicted value). Obstruction, low FVC, and mixed defects have all been reported in patients with previous TB.

PURPOSE OF REVIEW

The aim of TB treatment is to kill the causative mycobacteria with anti-mycobacterial agents. Because of the lengthy duration of the treatment, the possibilities of drug toxicity, and increasing drug resistance, host-directed therapies (HDT), have gained attention (Hawn et al., 2013; Wallis and Hafner, 2015; Zumla et al., 2015). HDTs are agents that can augment host defense mechanisms, modulate excessive inflammation or both, by manipulating the hosts response to a pathogen rather than targeting the pathogen itself. This may lead to improved clinical treatment outcomes such as reduced morbidity, mortality, and end-organ damage, and long-term functional recovery. Supplementing anti-TB treatment with drugs that reduce pulmonary damage could result in improved pulmonary function. To predict which interventions could be beneficial, an understanding of the pathogenesis of pulmonary damage in TB is important. What are the immunological processes leading to lung damage in humans? Where and how in the process could we intervene to prevent or reduce lung damage? How much damage is already done at diagnosis and how much still occurs during treatment?

WHAT DOES PULMONARY DAMAGE IN HUMAN TB LOOK LIKE?

The established paradigm positions the caseating granuloma as the characteristic lesion of TB. However, this paradigm originates from animal studies in the late 20th century, when data on histology of human TB had become rare. Studies done before the 1950s describe two characteristic presentations in human pulmonary TB: the caseous granuloma and the tuberculous pneumonia. They divide lung pathology into primary and post-primary TB. Primary TB is the infection that occurs when people first encounter *Mycobacterium tuberculosis (Mtb)*. Post-primary TB occurs later, as a result of reactivation of latent TB or reinfection, and causes the majority of clinical TB (Hunter, 2011). The two differ with regard to their location in the lung, the host immune response and their histopathology. Primary TB typically occurs mainly in the lower zones of the lung. It is usually self-limiting but leads to consolidative pneumonia or lymphadenitis in a small proportion of individuals. It is characterized by a greater bacillary load and reduced lipid accumulation in the alveoli and the interstitium compared to post-primary TB, as well as an acute inflammatory response; cavitation however, is rare. Post-primary TB is said to develop mainly in the apices of the lung. It is characterized by obstructive pneumonia, which is frequently asymptomatic in its early stages. Endobronchial spread from the small peripheral airways can lead to necrotic caseous pneumonia, associated with progressive tissue necrosis and cavity formation or fibrocaseous disease (Long et al., 1998; Hunter, 2016). TB typically heals with persisting cavities, scarring, and pleural adhesions, as observed in autopsies of persons with previous TB who died of other causes (Theegarten et al., 2006). However, abnormal findings need not be present and viable TB can be found in both macroscopically normal and abnormal appearing lung tissue (Kuhne and Willgeroth, 1988).

Chest radiographs are commonly used to visualize pulmonary damage. Radiologists distinguish primary and post-primary TB as the two typical patterns in active TB. Primary TB is characterized by lymphadenopathy and air space consolidation often in the middle or lower lobes, with or without an accompanying pleural effusion. Post-primary TB consists of consolidation and/or nodules, frequently in the upper lobes or apices of the lower lobes, with or without cavitation (Nachiappan et al., 2017). CXRs of people with previous TB show abnormalities in 14–100%, including fibrosis, bronchiectasis, and persisting cavities, the latter occurring more often in re-treatment patients or those with multi-drug resistant TB (Meghji et al., 2016). All these abnormalities are associated with impaired lung function.

Computed tomography (CT) scans are more sensitive than CXRs, especially for imaging of centrilobular small nodules or the so-called tree-in-bud sign; these classical features of early endobronchial spread of TB are often underestimated on a CXR (Skoura et al., 2015); [^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) with CT combines anatomic imaging with imaging of metabolic activity of lesions. It has been used in TB to follow the evolution of lung lesions during treatment (Martinez et al., 2012; Malherbe et al., 2016) and, importantly, has shown that metabolically active lung lesions may be present before the onset of clinical disease (Esmail et al., 2016), and persist after treatment completion (Malherbe et al., 2016).

WHAT HAPPENS AFTER MTB ENTERS THE LUNG?

After *Mtb* enters the lung, the bacilli are taken up by alveolar macrophages, dendritic cells, and neutrophils, or occasionally epithelial cells; the latter possibly resulting in limited early bacterial growth. Infected cells start producing and secreting antimicrobial peptides, cytokines (like interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-12, and IL-6) and chemokines. Other immune cells and permissive macrophages are attracted to the site of infection (O'Garra et al., 2013). *Mtb* itself, using multiple strategies, directs the recruitment of macrophages and triggers granuloma formation (Ndlovu and Marakalala, 2016).

Secondary granulomas are formed by infected macrophages departing the primary granuloma or when a granuloma ruptures. While *Mtb* replicates freely in the macrophages, dendritic cells migrate to the local lymph nodes, to activate T cells. The arrival of *Mtb* specific T-cells in the lung usually does not happen until 14–21 days after initiation of the infection (Gallegos et al., 2008). Their production of TNF-α and interferon-γ (IFN-γ) stimulates killing activities by macrophages. Moreover, T-cells complete granuloma formation by forming the lymphocytic cuff surrounding it (O'Garra et al., 2013).

The balance between the eicosanoids prostaglandin E2 (PGE2) and lipoxin A4 (LXA4) affects the mode of death of infected macrophages. LXA4 promotes macrophage necrosis, resulting in cell lysis of the macrophage, thereby allowing *Mtb* to escape and spread to neighboring cells. PGE2 stimulates apoptosis, leaving the macrophage plasma membrane intact, containing the bacilli, and enhancing immunity (Chen et al., 2008). Leukotriene (LT) B4, through regulation of TNF-α production (Tobin et al., 2012) and possibly attraction of neutrophils (Lammermann et al., 2013), is also involved, with both high and low levels of LTB4 inducing macrophage necrosis (Tobin et al., 2012).

In only 10% of individuals, progressive primary disease occurs; in the remaining 90% the initial infection is contained and latent infection is established (O'Garra et al., 2013). Current thinking views active and latent TB on a spectrum of tuberculosis disease, rather than as two distinct disease states as historically classified. (Barry et al., 2009).

GRANULOMAS

Most human granulomas are composed of a center of infected macrophages, with the ability to differentiate, for example into epithelioid cells, multi-nucleated giant cells, and foamy macrophages. An outer layer of lymphocytes surrounds these cells, and many other cells, including neutrophils, dendritic cells, natural killer (NK) cells and fibroblasts may form part of the granuloma. The granuloma contains the mycobacteria, preventing their spread, but at the same time serves as a site of replication and persistence for *Mtb* (Ndlovu and Marakalala, 2016). Different types of granuloma exist: cellular, suppurative, fibrotic, or caseous (Canetti, 1955). Caseous necrosis occurs when cells within the granuloma undergo necrosis (O'Garra et al., 2013); alternatively, it has been suggested that – in post-primary TB - granulomas form in response to existing areas of necrotic caseous pneumonia (Hunter, 2016). Caseous necrosis happens in conjunction with extracellular matrix (ECM) destruction. In the classical paradigm, tissue destruction occurs as a result of caseous necrosis (O'Garra et al., 2013). However, an alternative theory proposes that collagen destruction precedes caseation and, therefore, ECM destruction is the initial pathological event (Al Shammari et al., 2015).

Diverse types of granulomas can be present in one lung at the same time, ranging from small cellular granulomas to multiple caseous granulomas that coalesce and expel their contents to form large cavities; they behave independently of each other, and different immunologic profiles exist between (Ulrichs et al., 2005; Subbian et al., 2015) and within (Marakalala et al., 2016) granulomas. Granulomas can be stable, or either resolve or progress. Clinically, the behavior of a few or even a single poorly controlled granuloma can determine the outcome of the disease on a host level (Flynn, 2018).

CAVITIES, BRONCHIECTASIS AND FIBROSIS

The lung consists of both cellular and extracellular components. The ECM is comprised of the interstitial connective tissue matrix, which forms the parenchyma of the lung, surrounding cells and providing structural scaffolding, and the basement membrane, which separates the alveolar epithelium or endothelium from the surrounding stroma. Support of the alveoli by the ECM is needed for normal lung function; destruction or abnormal remodeling of the ECM occurs in many pulmonary diseases and leads to pulmonary impairment (Elkington and Friedland, 2006). The ECM of the lung is mainly made up of type I collagen and elastin. Type III and IV collagen are important components of the alveolar wall and basement membrane. Large fibers are connected by smaller fibrils. Dissemination of mycobacteria from the lung parenchyma into the airways as well as formation of cavities requires destruction of the ECM through cleavage of both small fibrils and large fibers. Collagens, however, are highly resistant to cleavage by proteolytic enzymes; only matrix metalloproteinases (MMPs) are capable of completely degrading the ECM (Elkington and Friedland, 2006). Consequently, MMPs play an important role in the development of cavities, bronchiectasis as well as fibrosis.

The development of cavities in TB has been studied extensively in rabbits, using *Mycobacterium bovis*. In these studies, cavities developed from liquefied caseating granulomas, that contained large numbers of actively growing bacteria. Bacteria release high amounts of tuberculin-like products causing a tissue-damaging delayed-type hypersensitivity reaction (Dannenberg, 2006). This T-cell mediated immune reaction is important; cavities developed mainly in pre-sensitized rabbits and desensitization or immune suppression could prevent cavity formation (Yamamura et al., 1968; Yamamura et al., 1974). Cavities are formed when expanding granulomas ruptures their caseous contents into a bronchus (Dannenberg, 2006).

Histologic studies in humans show a different picture of cavity formation that challenges the paradigm described in rabbits (Hunter, 2016): cavities do not develop from liquefied caseating granulomas, but from a caseous pneumonia. Host lipids and mycobacterial antigens accumulate in the alveoli, but only small numbers of bacteria are present. Similar to the rabbit model, sudden necrosis related to a delayed-type hypersensitivity reaction against mycobacterial antigens occurs (Hunter, 2016). However, an alternative yet controversial theory, based on the small numbers of bacteria observed and several observations related to autoimmunity

seen in patients with TB, proposes a role for autoimmunity: mycobacteria induce inappropriate host responses to self-antigens, causing autoimmune inflammation (Elkington et al., 2016). A considerable overlap in gene expression signatures between TB and autoimmune diseases, greater than seen with other infectious diseases, supports this theory (Clayton et al., 2017).

The lipid-rich necrotic material in granulomas does not have the enzymatic activity to degrade collagen and consequently, its build-up is only one component of cavity formation. Extracellular matrix breakdown takes place and involves MMPs. Indeed, increased concentrations of MMPs have been found in TB cavities in rabbits (Kubler et al., 2015) and in humans (Sakamoto et al., 2013; Ong et al., 2015). Neutrophils have also been found in cavities (Ong et al., 2015).

Bronchiectasis, an irreversible dilatation of the bronchi, is caused by an ongoing inflammatory process (like TB), which results in damage to the airway epithelium, leading to an inability to clear secretions, as well as destruction of the elastin in the airway walls (Milliron et al., 2015). Similar to cavity formation, MMPs have been implicated in the development of bronchiectasis, with increased levels being found in sputum, bronchoalveolar lavage fluid (BALF), and the lamina propria of patients with bronchiectasis (Sepper et al., 1995; Zheng et al., 2002; Guan et al., 2015). Neutrophils, together with macrophages and T-cells, are the dominant cell type in bronchiectatic inflammation (King, 2009). Alternatively, traction bronchiectasis can occur, secondary to scarring of the adjacent parenchyma or narrowing of more proximal bronchi (Milliron et al., 2015).

Fibrosis results from the excessive deposition of components of the ECM such as collagen and fibronectin in and around inflamed or damaged tissue by myofibroblasts. Its pathogenesis is complicated (Wynn and Ramalingam, 2012), with many innate and adaptive immune cells and cytokines playing a role. Transforming growth factor (TGF-β), produced by macrophages, lung epithelial cells, and fibroblasts, is one of the key players (Wynn and Ramalingam, 2012) and indeed, higher levels of TGF-β in serum and BALF correlate with an increase in fibrosis seen on high-resolution CT scan in patients with TB 6 months after the start of treatment (Ameglio et al., 2005). TNF-α, IL-β, and IL-17-induced neutrophil recruitment also seems to play a crucial role in the development of fibrosis (Wynn and Ramalingam, 2012). MMPs appear to be involved: some MMPs reduce fibrosis, but others – perhaps counterintuitively – promote it (Giannandrea and Parks, 2014). In a Taiwanese study, patients with an *MMP-1 (-1607G)* gene polymorphism, leading to excessive MMP-1 production, were more likely to have moderate to advanced fibrosis on CXR 1 year after completion of TB treatment (Wang et al., 2010).

WHAT ARE THE IMMUNOLOGICAL MEDIATORS AND PROCESSES LEADING TO LUNG DAMAGE?

Much of our recent knowledge of immunological processes in TB comes from animal models. Mice, rabbits, guinea pigs, and zebra-fish have all been used to study TB. However, none of these models completely replicate the immunopathology seen in human TB. More recently, non-human primates have also been used, exhibiting a spectrum of pathology closely resembling TB in humans (Flynn et al., 2015).

For this review, we included studies done in humans, where serum and BALF markers are commonly used to assess the immunological processes in the lung. Serum measurements reflect systemic responses and do not represent what happens in individual granulomas, as was shown by a difference in gene expression patterns between granuloma and blood (Subbian et al., 2015). BALF more closely reflects responses taking place in the lung, however, even BALF only reflects processes taking place in the airways and not necessarily those in the lung parenchyma. Histology is the only way to assess the immunological processes occurring within a granuloma; however, histological samples are more difficult to obtain and, therefore, most study findings in humans are built on assumptions using available body fluid. Studies that do include histological samples cannot present longitudinal data.

When conducting our review, we searched for studies that assessed inflammatory mediators, and associated them with radiological abnormalities as a marker for pulmonary damage (**Figure 1**).

MATRIX METALLOPROTEINASES

There are 23 MMPs in humans. They can be secreted by a variety of cells, including macrophages/monocytes, neutrophils, and lung epithelial cells. Their generation is tightly regulated. They are not stored requiring gene transcription immediately before secretion; exceptions being MMP-8 and -9 stored in neutrophils. Once activated, they are regulated by endogenous inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). Expression of MMPs is increased by prostaglandin and several cytokines (including IL-1β, IL-17 (Singh et al., 2018), TNF-α, and IFN-γ) (Elkington P.T. et al., 2011); hypoxic conditions, present in TB lesions, also increase expression and secretion of MMP-1 through the induction of hypoxia-inducible factor 1α (Belton et al., 2016). A recent study has demonstrated a role for platelets in MMP-1 upregulation in *Mtb*-infected monocytes, in addition to upregulation of IL-1β and IL-10 (Fox et al., 2018).

As described above, degradation of collagens and elastins by MMPs during active TB leads to the formation of cavities. Strong evidence of the role of MMPs in lung damage comes from studies in transgenic mice expressing human MMP-1. Wildtype mice do not express the ortholog of MMP-1 in lung and do not develop caseous necrosis or cavities in response to *Mtb*; in human MMP-1 transgenic mice, however, infection with TB leads to collagen destruction and caseous necrosis (Elkington P. et al., 2011; Al Shammari et al., 2015). MMPs also play a role in granuloma formation (Parasa et al., 2017).

Several MMPs are upregulated in blood, sputum, and BALF of patients with active TB, primarily MMP-1, -3, -7, -8, and -9 (Elkington P.T. et al., 2011). MMP-1 is the dominant

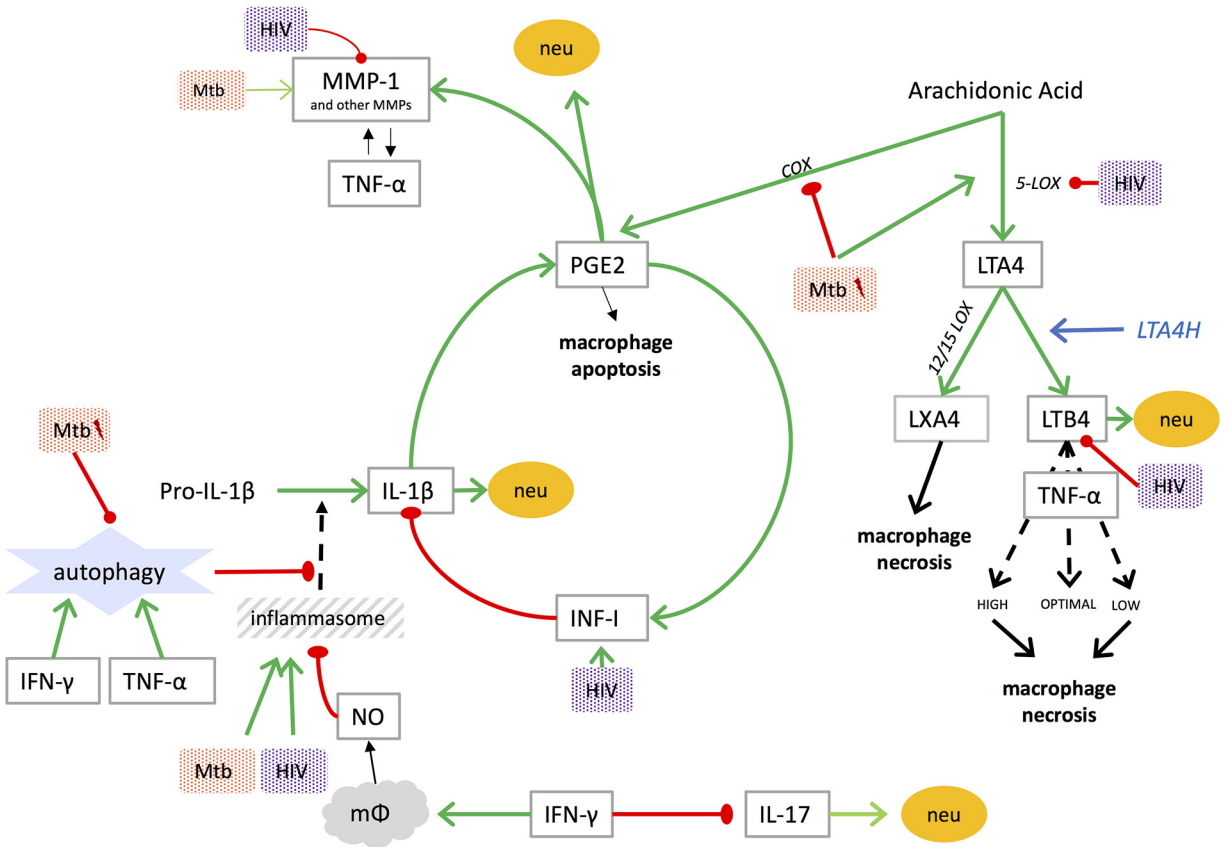

FIGURE 1 | Mediators of lung damage in TB and interplay with [lightning flash cartoon], virulent *Mtb;* COX, cyclooxygenase; IFN-I, type I interferon; IFN-γ, interferon gamma; IL, interleukin; LOX, lipoxygenase; LT, leukotriene; LTA4H, leukotriene A4 hydrolase; LX, lipoxin; mφ, macrophage; MMP, matrix metalloproteinase; Mtb, mycobacterium tuberculosis; neu, neutrophil recruitment; NO, nitric oxide; PGE2, prostaglandin E2; TNF, tumor necrosis factor. NO inhibits assembly of the NLRP3 inflammasome (Mishra et al., 2013).

collagenase in TB (Elkington P. et al., 2011); its secretion is driven by *Mtb* directly by activation of multiple intracellular signaling pathways and by intercellular networks (Ong et al., 2014). Corresponding TIMPs are not similarly upregulated by *Mtb*, leading to a matrix-degrading phenotype in TB (Price et al., 2001). In a zebrafish model, using *M. marinum* to study granuloma formation, mycobacterial-derived ESAT-6 induced MMP-9 secretion, enhancing monocyte recruitment to granulomas (Taylor et al., 2006; Volkman et al., 2010).

Increased levels of MMPs correlate with pulmonary damage: sputum levels of MMP-1, -2, and -8 were elevated in patients with cavities and correlated positively with the extent of infiltrates on CXR (Walker et al., 2012; Ong et al., 2015). Similarly, sputum levels of membrane type-1 MMP (a membrane-bound collagenase expressed on monocytes), plasma concentrations of procollagen III N-terminal propeptide (PIIINP, a degradation product of collagen type III), BALF levels of MMP-3, -7, and -8, and serum concentrations of MMP- 1, -8, and -9, correlated with more extensive CXR abnormalities in patients with TB from several different countries (Hrabec et al., 2002; Seddon et al., 2013; Singh et al., 2014a; Sathyamoorthy et al., 2015; Sigal et al., 2017). These findings suggest a central role for MMPs and extracellular matrix degradation in the development of lung damage in TB.

NEUTROPHILS

Neutrophils are abundant in the airways of humans with active TB (Eum et al., 2010). Their role in TB appears dichotomous: high numbers of neutrophils in the blood at the time of exposure are associated with lower likelihood of infection (Martineau et al., 2007). Conversely later in TB their numbers in blood were associated with worse patient outcomes (Barnes et al., 1988; Lowe et al., 2013). Various soluble mediators (amongst others Il-1β, IL-8, IL-17, PGE2, LTB4, and granulocyte colony-stimulating factor) promote neutrophil recruitment (Lowe et al., 2012); others, like IFN-γ and nitric oxide (NO), reduce neutrophil recruitment and survival, partly via inhibition of IL-17 (Nandi and Behar, 2011), IL-1β and 12-lipooxygenase (12-LOX) (Mishra et al., 2017).

At the time of presentation with active TB, neutrophils are associated with lung damage: a neutrophil-driven, IFN-inducible whole-blood transcript signature (Berry et al., 2010), higher blood (Abakay et al., 2015; Panteleev et al., 2017) and BALF

(Nolan et al., 2013) neutrophil counts, and higher serum levels of S100 proteins (a protein produced by neutrophils, promoting their own recruitment) (Gopal et al., 2013; Berrocal-Almanza et al., 2016) in patients with active TB all relate with the extent of lung radiographic disease. Lung damage is thought to be contributed to by their indiscriminate killing mechanisms, which can result in significant bystander damage to surrounding host tissue. Moreover, neutrophils are the only cells that store MMPs (Ong et al., 2015), while they do not synthesize TIMPs, thus allowing for unrestrained effects of MMPs (Masure et al., 1991). Removing infected or dying neutrophils is necessary to protect the surrounding tissue. Removal of apoptotic neutrophils by macrophages promotes subsequent killing of *Mtb*, whereas removal of necrotic neutrophils allows for mycobacterial survival and proliferation inside the macrophages. *Mtb* drives neutrophil necrosis, a process that requires neutrophil-derived reactive oxygen species (ROS) (Dallenga et al., 2017b). Inhibition of ROS-production could restore growth control of *Mtb* by macrophages (Dallenga et al., 2017a).

EICOSANOIDS

The eicosanoids PGE2, LXA4, and LTB4 are all metabolites of arachidonic acid (AA). Cyclooxygenase (COX) converts AA into PGE2, while 5-lipooxygenase (5-LOX) generates LTA4, which is again converted into either LXA4 by 12-LOX, or LTB4 by leukotriene A4 hydrolase (LTA4H) (Dietzold et al., 2015). As mentioned previously, the balance between these eicosanoids influences the mechanism of macrophage death (Chen et al., 2008). Macrophage apoptosis leads to an early immune response with better control of the infection and minimal immunopathology, while macrophage necrosis leads to a delayed immune response, inadequate control of infection and greater immunopathology (Divangahi et al., 2013). Virulent strains of *Mtb* promote LXA4 production, thereby stimulating necrosis and mycobacterial spread (Chen et al., 2008). To our knowledge, no studies have correlated PGE2 or LXA4 with pulmonary function in human TB; one can speculate that tipping the eicosanoid-balance toward PGE2 may result in less lung damage. Findings in mice and latent TB in humans, however, show that levels of PGE2 were low early in the infection and increased later in and during active TB (Rangel Moreno et al., 2002; Shu et al., 2013; Mayer-Barber et al., 2014; Lee et al., 2015). This underlines the complex and poorly elucidated role of PGE2 in TB infection and may even suggest a changing role for PGE2 during the course of the disease. LTB4, which is generated by LTA4H, has been correlated with severity of TB on CXRs in one study (el-Ahmady et al., 1997).

CYTOKINES

Various studies have assessed the association between cytokines (including IFN–γ and TNF–α, and several pro- and anti-inflammatory interleukins) and CXR abnormalities in TB (Dlugovitzky et al., 1997; Sodhi et al., 1997; Casarini et al., 1999; Tsao et al., 1999, 2000, 2002; van Crevel et al., 2000; Mazzarella et al., 2003; Ameglio et al., 2005; Wu et al., 2007; Berry et al., 2010; Su et al., 2010; Walker et al., 2012; Nolan et al., 2013; Chowdhury et al., 2014; Fan et al., 2015; Sigal et al., 2017). The different measuring methods used and the fact that several cytokines are not limited to a single effector function make comparison and interpretation challenging.

Only TNF-α and IL-1β in both blood and BALF seem to unambiguously correlate with CXR abnormalities. Higher levels of TNF-α and IL-1β correlate with the presence or size of cavities (Tsao et al., 2000; Ameglio et al., 2005; Chowdhury et al., 2014; Sigal et al., 2017) and with the extent of pulmonary involvement (Casarini et al., 1999; Walker et al., 2012). Moreover, lower levels of these cytokines were found in patients with an early radiological response to TB treatment (improved CXR after 2 months of treatment) compared to those with a later (at 6 months) response (Su et al., 2010). In animal models, the effect of TNF-α seems to be dose dependent, where both high and low doses lead to tissue destruction (Bekker et al., 2000; Tobin et al., 2012). LTA4H polymorphism, and subsequently eicosanoid patterns, play a role in its regulation (Tobin et al., 2012). Both TNF-α and IL-1β affect secretion of MMPs and MMPs in their turn can play a role in the release, activation or inactivation of TNF-α and IL-1β (Elkington and Friedland, 2006). IL-1β also associates with activation of fibroblasts (Borthwick, 2016) and the recruitment of neutrophils (Lowe et al., 2012; Mishra et al., 2017), which all associate with lung damage.

AUTOPHAGY

Autophagy is an intracellular self-digestion process: cytosolic material is engulfed by a double-membrane vesicle called the autophagosome, that delivers it to lysosomes for degradation and subsequently releases the degraded products back to the cytosol. Autophagy can be used by the host to eliminate intracellular pathogens and plays an important role in defense against *Mtb* (Gutierrez et al., 2004); both IFN-γ and TNF-α can induce autophagy (Songane et al., 2012). It can also downregulate IL-1β production mediated through the inflammasome (an intracellular multiprotein complex that triggers formation of proinflammatory cytokines), by removing large inflammasome complexes or damaged mitochondria - which, through production of ROS, trigger the inflammasome (Rathinam et al., 2012). Virulent *Mtb* can inhibit autophagy (Gupta et al., 2016), subsequently leading to increased IL-1β production (Songane et al., 2012). It was found that patients infected by *Mtb* strains with poor *in vitro* autophagy-inducing ability displayed more severe radiographic extent of disease (Li et al., 2016). Consequently, inducing autophagy could limit lung damage.

THE MODULATING ROLE OF HIV

Globally, 13 percent of people with active TB who know their HIV status are co-infected with HIV-1 (World Health Organization,

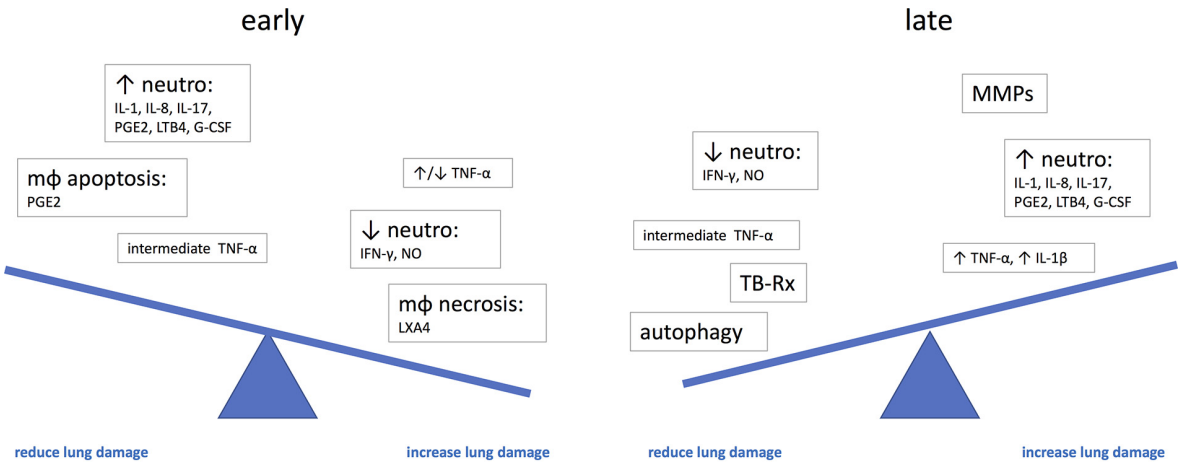

FIGURE 2 | The potential effect of immune mediators on the development of lung damage at different stages of disease. G-CSF, granulocyte-colony stimulating factor; IL, interleukin; LTB4, leukotriene B4; LXA4, lipoxin A4; mφ, macrophage; neutro, neutrophils; NO, nitric oxide; PGE2, prostaglandin E2; TNF, tumor necrosis factor.

2017). Although TB is also a risk factor for airflow obstruction in patients with HIV (Samperiz et al., 2014; Pefura-Yone et al., 2015; Gupte et al., 2017), in HIV positive patients with a low CD4 count (CD4 < 200/mm^3) TB often presents with atypical CXR findings or even normal CXRs, while cavitation is 4-fold less common (Kwan and Ernst, 2011). These findings suggest that TB-related pulmonary damage might be reduced in HIV co-infected patients and the host immune response, necessary for protection against TB, is required for the development of cavities. Indeed, several of the factors previously discussed and implicated in pulmonary damage, are affected by HIV co-infection. For example, sputum levels of MMP-1, -2, -8, and -9 are reduced in HIV-TB co-infected patients, compared to patients without HIV (Walker et al., 2012, 2017) as is the activity and life span of neutrophils (Lowe et al., 2015). The effect of HIV co-infection on the levels of several of the other cytokines is variable across studies and thus it is difficult to interpret a clear trend (Zhang et al., 1994; Elliott et al., 1999; de Castro Cunha et al., 2005; Riou et al., 2012; Walker et al., 2012; Mihret et al., 2014; Kassa et al., 2016).

Paradoxical TB-associated immune reconstitution inflammatory syndrome (TB-IRIS) develops in approximately 18% (95% CI 16–21%) of patients on treatment for HIV-associated TB, usually within the first few weeks after starting ART (Namale et al., 2015). It results in new or recurrent TB signs and symptoms, commonly involving the lungs, such as cough, chest pain, and worsening radiographic pulmonary infiltrates. TB-IRIS is associated with increased levels of several cytokines, particularly IL-6, TNF-α and IFN-γ (Tadokera et al., 2011; Conesa-Botella et al., 2012; Lai et al., 2015; Ravimohan et al., 2015) and inflammasome activation (Lai et al., 2015). It results in increased neutrophil recruitment (Nakiwala et al., 2018), and up-regulation of MMP-1, -3, -7, -8, and -10 (Tadokera et al., 2014; Ravimohan et al., 2016; Walker et al., 2017). LT4AH also appears to play a role, as more severe TB-IRIS has been reported in patients with mutant (TT and CT) LTA4H genotypes (Narendran et al., 2016).

These findings suggest that TB-IRIS could result in pulmonary damage and impaired lung function. To date, only one study has explored the relationship between TB-IRIS and lung function in 14 patients with HIV-associated TB, 3 of whom developed TB-IRIS (Ravimohan et al., 2016). The study found that an increase in MMP-8 between baseline pre-ART and 4 weeks post-ART initiation strongly associated with impairment in lung function, but the small sample size limits definitive conclusions.

WHERE CAN WE INTERVENE TO PREVENT OR REDUCE LUNG DAMAGE?

There are several uncertain areas around therapies to prevent or limit lung damage in TB. Changes in the lungs start to develop before clinical symptoms appear (Esmail et al., 2016; Zak et al., 2016; Scriba et al., 2017), and therefore, a large proportion of lung damage may already have occurred by the time the patient presents; several mediators of lung damage may have different roles at different stages of the disease; granulomas in various stages can be present at the same time in a single individual, and only a single or a few progressive granulomas can determine the outcome of the disease. Therefore, it remains uncertain what happens for example to the contained granulomas if we systemically treat the patient with potentially immunosuppressive therapy or what the right time is to intervene (**Figure 2**).

ANTITUBERCULOUS THERAPY AS HOST-DIRECTED THERAPY

Sputum *Mtb* load is associated with systemic inflammation and, combined with pre-treatment C-reactive protein levels,

inversely correlates with CXR improvement 60 days after start of treatment (Mesquita et al., 2016). Time between first TB symptoms and start of treatment (de Valliere and Barker, 2004; Baez-Saldana et al., 2013), duration of treatment (Chung et al., 2011), and smear positivity (Chung et al., 2011) are associated with impaired pulmonary function, suggesting that prompt diagnosis and treatment will limit lung damage. In addition to a direct anti-mycobacterial effect, *in vitro* studies suggest that some antimycobacterial agents may have immunomodulatory action. Pyrazinamide directly reduces levels of TNF-α, IL-6 and IL-1β (Manca et al., 2013), quinolones downregulate MMP-1, -3, and -9 (Singh et al., 2014a), and rifampicin downregulates MMP-3 production by bronchial epithelial cells (Singh et al., 2014a) and inhibits PGE2 production (Yuhas et al., 2007). P-aminosalicylic acid (PAS), which is an aspirin derivate, suppresses PGE2-dependent MMP-1 production (Rand et al., 2009). Both isoniazid (INH) and pyrazinamide (PZA) enhance autophagy (Kim et al., 2012).

MEDICINES USED IN OTHER HUMAN DISEASES AS HOST-DIRECTED THERAPY FOR TB

In an adjunctive approach to TB therapy, treatment could be supplemented with host-directed therapies. Several readily available drugs affect cytokines, MMPs or eicosanoids and therefore potentially reduce pulmonary damage (**Table 1**).

TABLE 1 | Host-directed therapies potentially inhibiting lung damage and/or promoting lung repair.

Host-directed inhibiting lung damage	Potential mechanism
Steroids	↓ INF-γ, TNF-α, IL-1β (and IL-6, IL-10, IL-12p40, and IP-10 in TB-IRIS)
	↓ MMP-7 (in TB-IRIS)
Doxycycline	↓ MMP-1, -3, and -9
Vitamin D	↓ MMP-7 and -9
	↓ IFN-γ, IL-6, IL-10, TNF-α
	↑ autophagy
Rapamycin, everolimus	↓ MMP-1 and -3
	↑ autophagy
NSAIDs	↓ PGE2[1] and ↑ LXA4
Zileuton	↓ 5-LOX
Phosphodiesterase-4 inhibitors	↓ TNF-α
	↓ neutrophil recruitment
Metformin	↓ TNF-α
	↑ autophagy
Statins	↑ autophagy
TNF-α blockers	↓ TNF-α
PGE2	↑ PGE2[1]
IFN-γ	↑ IFN-γ
Mesenchymal stromal cells	Control inflammation and mediate tissue repair

[1] *The effect of inhibiting or increasing PGE2 on lung damage could vary depending on the stage of the disease.*

Steroids have been used as adjunctive treatment in TB for several decades (Dooley et al., 1997; Critchley et al., 2013), mainly in TB meningitis, pericarditis, and TB-IRIS, even though corticosteroid use without concomitant TB treatment increases the risk of developing TB (Jick et al., 2006). Two recent reviews concluded that there is no high quality evidence that steroid treatment significantly affects mortality or sputum conversion rate in pulmonary TB (Critchley et al., 2014; Schutz et al., 2018). An earlier review – including mostly studies done in the 1960s and patients not on rifampicin-based TB treatment – did find a beneficial effect of steroids on radiographic resolution and regression of cavities (Smego and Ahmed, 2003). A meta-regression analysis of 12 studies found steroids do accelerate sputum TB culture conversion (Wallis, 2014) – which is inversely associated with development of airflow obstruction (Radovic et al., 2016); however, high doses (134 mg prednisone daily) for an extended period (2 months) are required to reach clinically relevant outcomes (Wallis, 2014). Moreover, the only two studies in this analysis in which patients were on rifampicin-based treatment show contradicting results.

Corticosteroids inhibit various cytokines in TB (IFN-γ, TNF-α, IL-1β) and TB-IRIS (IL-6, IL-10, IL-12p40, TNF-α, IFN-γ, and IP-10) (Mahuad et al., 2004; Mayanja-Kizza et al., 2005; Meintjes et al., 2012; Bongiovanni et al., 2015). In patients with tuberculous meningitis, the effect of corticosteroids was found to be LTA4H genotype modulated, with only patients with the mutant TT genotype, leading to a higher inflammatory response, benefitting from steroid treatment (Tobin et al., 2012). In patients with TB-IRIS, however, this difference in genotype on the effect of steroid treatment was not confirmed (Narendran et al., 2016). The effect of corticosteroid treatment and TB-IRIS on pulmonary function is being assessed in a substudy of the PredART trial (Meintjes et al., 2017).

Little evidence is available for other **TNF-α blocking therapies**. A trial of 16 patients with HIV-associated TB treated with etanercept (but no ART) showed a tendency to greater CXR improvement from baseline to 6 months compared to a placebo group, although this was not statistically significant ($p = 0.2$) (Wallis et al., 2004). Case reports describe successful treatment of paradoxical TB reactions or TB-IRIS – involving the pleura, lymph nodes or brain – with infliximab (Blackmore et al., 2008; Jorge et al., 2012; Hsu et al., 2016), or adalimumab (Wallis et al., 2009; Lee et al., 2012). Although only one case refers to pulmonary TB-IRIS [occurring after interruption of prior anti-TNF-α treatment (Wallis et al., 2009)], these case reports support the possible benefits of TNF-α blockers in the treatment of (complicated) TB. Restarting TNF-α blockers during or after TB treatment was safe and only led to one recurrence of TB in a cohort of 22 patients in Turkey followed for a median of 53 months (Ozguler et al., 2016).

Doxycycline is the only licensed **MMP-inhibitor** for use in humans. It suppresses MMP-1, -3, and -9 secretion by *Mtb* infected human macrophages and bronchial epithelial

cells (Walker et al., 2012). Other agents also inhibit MMPs *in vitro*: prednisone – in patients with TB-IRIS – suppresses MMP-7 gene expression (Tadokera et al., 2014), vitamin D inhibits secretion of MMP-7 and -9 (Anand and Selvaraj, 2009; Coussens et al., 2009), and **rapamycin** (an mTOR-inhibitor and a known autophagy inducer that can also affect macrophage polarization (Mercalli et al., 2013)) inhibits MMP-1 and MMP-3 (Singh et al., 2014b). Use of the latter in TB is limited by the interaction with rifampicin. In mice, broad spectrum inhibition of MMPs enhances the efficacy of INH and RIF treatment (Xu et al., 2018). Conceptually, inhibition of MMPs may lead to less pulmonary damage, but so far, no clinical trials have directly assessed this. Currently, everolimus, a rapamycin derivate, is being tested as HDT in patients with moderate to far advanced pulmonary tuberculosis (together with vitamin D, auranofin [a gold complex with antimicrobial activity used in rheumatoid arthiritis], and CC-11050 [a phosphodiesterase 4 (PDE4) inhibitor]), using rifabutin-based anti-TB treatment (ClinicalTrials.gov NCT02968927); with change in FEV1 being one of the secondary outcomes. Both rapamycin, its derivates, and vitamin D could theoretically reduce lung damage through inhibition of MMPs, although the effect of vitamin D treatment on CXR abnormalities is variable (see below). PDE4 inhibitors, in combination with INH treatment, have been shown to reduce TB-associated lung damage in rabbits (Subbian et al., 2011) and pulmonary bacillary load in mice (Maiga et al., 2015). Doxycycline is being investigated for its potentially modulating effect on tissue destruction in pulmonary TB (ClinicalTrials.gov NCT02774993).

NSAIDs inhibit the enzyme cyclooxygenase (COX), thereby inhibiting PGE2 production and enhancing LXA4 production. An adjunctive role for NSAIDs in treatment of human TB has only been shown for acetylsalicylic acid in reducing PZA-induced arthralgia (Petty and Dalrymple, 1964; Horsfall et al., 1979) and possibly in TB meningitis (Misra et al., 2010; Schoeman et al., 2011; Mai et al., 2018). Negative effects have been described: a Taiwanese study found an association between NSAID use (both traditional NSAIDs and selective COX-2 inhibitors) and an increased risk of active TB (Wu et al., 2017). However, it is not clear whether this association is causative (i.e., decreased apoptosis at the very early stages of TB) or merely reflects an increased use of NSAIDs early during TB. In mice, inhibition of PGE2 by the NSAID ibuprofen was shown to affect lung pathology: inhibition early in the disease process leads to an increase in pulmonary inflammation and pathology (Rangel Moreno et al., 2002), whereas inhibition later during disease decreased lung pathology and neutrophil influx (Rangel Moreno et al., 2002; Vilaplana et al., 2013). Increasing **PGE2** by early (day one post infection) administration of exogenous PGE2 (dinoproston – normally used for induction of labor) and/or the 5-lipo-oxygenase inhibitor zileuton (used in the treatment of asthma) to IL-1 deficient mice resulted in less necrotic lung pathology by TB (Mayer-Barber et al., 2014). No studies with dinoproston or zileuton have been performed in human TB to date. A pilot study is currently investigating the effect of ibuprofen added to multi-drug resistant TB treatment on radiological improvement of TB, amongst other endpoints (ClinicalTrials.gov NCT02781909).

In *in vitro* models, **metformin,** a widely used antidiabetic agent, has been shown to inhibit TNF production by monocytes (Arai et al., 2010), affect macrophage polarization (Nadella et al., 2017), and promote autophagy (Singhal et al., 2014). It affects Th1 responses, but data are conflicting: in mice infected with TB, metformin treatment promotes the expansion of *Mtb*-specific IFN-γ secreting T cells in the lungs (Singhal et al., 2014), whereas in human THP-1 cells (not infected with *Mtb*) metformin suppressed the production of Th1-related cytokines (Chen et al., 2018). Metformin use in patients with diabetes mellitus on treatment for TB was associated with decreased mortality compared to patients using other anti-diabetic drugs in two retrospective observational cohorts (Singhal et al., 2014; Degner et al., 2018). A retrospective cohort study of TB patients with diabetes mellitus showed that those using metformin at diagnosis and during TB treatment had fewer cavities and fewer CXR abnormalities compared to those using other anti-diabetic drugs (Singhal et al., 2014). Another retrospective study, however, showed increased cavitatory disease in patients using metformin (Degner et al., 2018).

Vitamin D3 induces autophagy (Campbell and Spector, 2012) and inhibits the secretion of MMP-7, -9 (Anand and Selvaraj, 2009; Coussens et al., 2009), and several cytokines, for example IFN-γ, TNF-α, IL-6, and IL-10 (Vidyarani et al., 2007; Harishankar et al., 2014) *in vitro*. However, its effect on radiological outcomes are ambiguous: three trials comparing vitamin D3 as adjunctive therapy demonstrated no effect on CXR score (Martineau et al., 2011; Ralph et al., 2013b; Mily et al., 2015) or pulmonary function (Ralph et al., 2013b), while one study found more CXR improvement in the vitamin-D3 treated group (Salahuddin et al., 2013).

Statins are widely used inhibitors of cholesterol biosynthesis. They induce autophagy *in vitro* (Parihar et al., 2014) with broad anti-inflammatory effects, although not directly demonstrated in TB (Hennessy et al., 2016). Their use has been associated with a reduced risk of developing active TB in some studies (Lai et al., 2016; Liao et al., 2017; Su et al., 2017), but not in all (Kang et al., 2014). No studies have been performed in humans assessing statins in relation to pulmonary damage in TB; in mice, statins have been found to reduce lung pathology (Parihar et al., 2014). A future study will look at the effect of pravastatin added to standard TB treatment on pulmonary function (NCT03456102).

Several studies looked at the effect of **IFN-γ** as adjunctive therapy for TB (Gao et al., 2011). The studies were small, and most were performed in patients with multi-drug resistant TB. Aerosolized IFN-γ in combination with TB treatment resulted in better CXR outcomes compared to TB treatment alone. This contradicts the finding in mice, where adding IFN-γ resulted in worse pulmonary outcomes (Sakai et al., 2016). The authors conclude that IFN-γ might be beneficial as adjunctive therapy in TB, but larger trials are needed to confirm this.

Mesenchymal stromal cells are tissue-resident non-hematopoietic adult progenitor cells. They are believed to facilitate organ homeostasis and tissue repair and can modulate immune responses; they have been used in treatment of graft-versus-host-disease and autoimmune diseases (Parida et al., 2015). In a phase 1 trial in patients with drug resistant TB, infusions of autologous mesenchymal stromal cells, 4 weeks after starting TB treatment, was safe and resulted in CXR improvement in 25/36 patients compared to 15/36 controls (Skrahin et al., 2016).

CONCLUSION

The immune mechanisms of parenchymal lung damage in human TB are complex and incompletely understood. The difference between pulmonary damage in animal models (mostly occurring as a result of primary TB) and humans (mostly occurring as a result of post-primary TB) further complicates study of this phenomenon. Processes taking place in the lung are heterogeneous, with granulomas with varying degrees of mycobacterial control existing next to each other and inflammatory cells and cytokines appearing to have different effects at different time points. MMPs seem to play an important role and consequently, inhibition of MMPs may lead to reduction in pulmonary damage, however, this remains to be proven in clinical trials. Neutrophils are another key mediator of pulmonary damage, whose recruitment could potentially be inhibited by NSAIDs. The role of other effectors is less clear and better insight into their effects over the course of TB infection and disease is needed to be able to guide potential intervention. Future studies of human TB and (host-directed) therapy should include radiographically assessed lung damage and pulmonary function as an outcome.

AUTHOR CONTRIBUTIONS

CS wrote the first draft of the manuscript. GM, BA, NW, RW, and LL critically revised the manuscript. All authors read and approved the submitted version.

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frontiers in Microbiology

REVIEW
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The Prospects of an Active Vaccine Against Asthma Targeting IL-5

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Allergen-specific T helper type 2 (Th2) responses followed by eosinophilic inflammation of the lung are important causes of allergic asthma. Interleukin-5 (IL-5) is a master regulator of eosinophil differentiation as well as activation. Blocking IL-5 using monoclonal antibodies (mAbs) against IL-5 is a powerful way to improve asthmatic symptoms in patients with an eosinophilic component of the disease. We have previously shown that vaccination against IL-5 can abrogate eosinophilic inflammation of the lung in allergic mice. More recently, we have demonstrated that eosinophil-mediated skin disease in horses with insect bite hypersensitivity can be strongly reduced by vaccination against IL-5. Here we would like to propose the development of a similar vaccine for the treatment of asthma in humans.

Keywords: virus-like particle, IL-5, vaccine, asthma, cytokine, vaccination

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ASTHMA – A SIGNIFICANT CHRONIC RESPIRATORY DISEASE THAT REPRESENTS A GROWING CHALLENGE

Asthma is a globally significant chronic respiratory disease with upward of 300 million patients experiencing considerable morbidity ranging from occasional breathing difficulties (shortness of breath, coughing, wheezing, and chest tightening) to more severe exacerbations (asthma attack) requiring clinical interventions (Network, 2018). Inflammation in the lungs can be triggered by a variety of non-specific stimuli such as cold air and exercise, as well as by allergens or infectious microorganisms such as viruses. In turn, these events can activate a combination of innate and adaptive immune responses and cellular inflammation, resulting in symptoms like bronchial smooth muscle hyper-reactivity, excess mucus production from goblet cells within epithelia, and potential for bronchial remodeling and airway narrowing. Given the remarkable heterogeneity in both stimuli and responses, it is not surprising to see the involvement and overlap of several distinct soluble mediators and cell types. Key amongst these are cytokines involved in immunoglobulin isotype switching (IgE synthesis by IL-4), mast cell proliferation (IL-9), airway hyper-responsiveness and mucus production (IL-13), and eosinophil inflammation (IL-5). In addition to type 2 T helper cell (Th2) allergic responses, also Th1, Th17, and Th9 cellular subsets have been implicated (Holgate and Polosa, 2008; Lambrecht and Hammad, 2015).

In many patients, the disease can be controlled by a combination of anti-inflammatory immunosuppressants (such as inhaled corticosteroids) and by bronchodilators that relax constricted airway smooth muscle (e.g., β_2-adrenergic agonists). However, between 5–10% of patients are refractory to corticosteroid treatments thus complicating management of their asthma and often leading to serious exacerbations requiring hospital intervention. This has encouraged the

development of several biological drug alternatives based on monoclonal antibodies (mAbs) for those individuals where conventional small molecule drugs have failed.

mAbs HAVE CHANGED THE WAY WE TREAT CHRONIC DISEASES BY ALLOWING THE TARGETING OF PROTEIN-PROTEIN INTERACTIONS OUTSIDE THE CELL

Generation of mAbs was described more than 40 years ago in mice (Kohler and Milstein, 1975), and over the past 20 years, the technology has started to change clinical practice (Cui et al., 2017). In contrast to small molecular drugs, which classically target intracellular proteins, enzymes, ion channels, and G-coupled proteins, mAbs have allowed the blocking of extracellular protein-protein interactions. They may inhibit receptor–ligand interactions or specifically target cell surface receptors. Blocking protein–protein interactions with high specificity has made it possible to inhibit cytokines, chemokines and growth factors implicated in otherwise intractable disease conditions while targeting cell surface receptors is often followed by antibody-dependent cellular cytotoxicity (ADCC) and is generally used for the elimination of cancer cells. Due to these unique properties, mAbs have become the most rapidly growing revenue source for the pharmaceutical industry. The top-in-class antibody Humira® was the world's best-selling drug 2015 with annual global expenditure reaching US$14 billion. Moreover, 6 of the 10 biggest blockbuster drugs are mAbs[1].

The flip-side of the argument is the high costs that are associated with the generation of these biomolecules, cutting a large fraction of the world's population off from the benefits brought by this new treatment modality. In addition, despite mAbs high efficiency, treatment involves injections of very substantial amounts of protein that may cause local side-effects such as pain and irritation or even severe anaphylactic reactions. An additional potential issue associated with the use of mAbs is the induction of anti-drug antibodies that may neutralize or eliminate the injected mAbs and may accelerate local or systemic adverse events during injection (Steenholdt et al., 2011, 2012).

Many of these problems could be tackled by moving on from the current passive vaccination strategies with mAbs to active vaccination approaches that instruct the body to generate its own antibody responses. Potential issues with this new approach are that it may be difficult to (i) reach sufficient levels of antibodies and (ii) the response may be less controllable; in particular, the potential irreversibility of the induced antibody responses is an obvious issue. However, recent developments in vaccine design, genetics, and clinical research are converging to pave the way for the clinical development of such auto-vaccines. It was recently shown that anti-cytokine vaccination could reach critical levels in humans (Lauwerys et al., 2014; Cavelti-Weder et al., 2016; Ducreux et al., 2016) and other target species (Bachmann et al., 2018; Fettelschoss-Gabriel et al., 2018b), and that the induced antibody responses are indeed reversible. The present review will examine recent findings and outline how these findings may be applied to the development of a vaccine against asthma targeting IL-5.

[1]https://labiotech.eu/top-10-best-selling-biologicals-blockbusters-2015/

HARNESSING THE IMMUNE SYSTEM: HOW TO INDUCE SELF-SPECIFIC ANTIBODY RESPONSES DIRECTED AGAINST CYTOKINES

Long-lived IgG responses require activation of specific B as well as (follicular) Th cells. However, the immune system is usually tolerant to self-proteins, and induction of cytokine-specific antibody responses, therefore, requires overcoming or bypassing B cell as well as Th cell tolerance. In general, soluble proteins do not cause B cell tolerance and antibody responses against soluble self-proteins are usually controlled by Th cell tolerance only (Adelstein et al., 1991). As a case in point, we have recently shown that introduction of a single virus-derived Th cell epitope into IL-1β was able to overcome B cell unresponsiveness and immunization with this modified cytokine formulated in adjuvants readily resulted in strong and specific IgG responses (Spohn et al., 2014). Hence, linking cytokines to a source of Th cell epitopes is able to drive specific B cell responses. This is similar to classical carbohydrate conjugate vaccines where B cells recognize the carbohydrate and Th cells recognize the carrier protein to which the carbohydrates are linked (Mond et al., 1995) (**Figure 1A**).

Conventional protein-cytokine conjugate vaccines have struggled to induce sufficient levels of antibodies to reach clinical efficacy (Ratsimandresey et al., 2009). Indeed, while the use of strong adjuvants in preclinical experiments may help to increase the efficiency in murine model diseases, translation to the clinic is difficult, as such adjuvants cannot be used in humans. A case in point is a vaccine against TNF (Dalum et al., 1999), which successfully treated rheumatoid arthritis in mice but failed to reach efficacy in humans. One reason for this failed translation was the use of complete Freund's adjuvants (CFA) in the murine experiments, an adjuvant that under no circumstances could be used in humans, due to the induction of local granuloma or systemic reactogenicity, hypersensitization to mycobacteria (tuberculin) and heightened risk of autoimmune disease.

Virus-like particles (VLPs) may offer an elegant solution to this problem. By displaying cytokines on the surface of VLPs, it is possible to induce high levels of neutralizing antibodies without the use of adjuvants. Several factors account for the high immunogenicity of antigens displayed on VLPs. One important parameter is the repetitive display of the antigen, causing efficient cross-linking of B cell receptors (BCRs) on B cells, constituting a strong activation signal (**Figure 1B**). Also, repetitive epitopes efficiently recruit components of the innate humoral immune system in particular complement and natural IgM (Bachmann and Jennings, 2010). This enhances B cell activation via CD21 and facilitates B cell-mediated antigen deposition on follicular

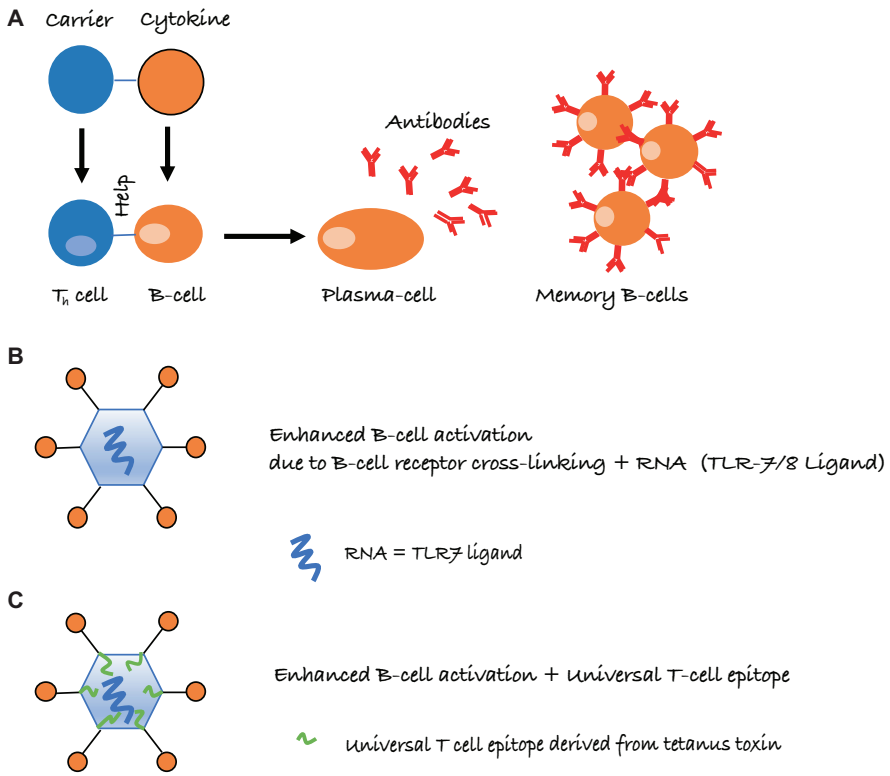

FIGURE 1 | Schematic representation of intermolecular help for the induction of cytokine-specific antibody responses. **(A)** Cytokines are conjugated to a carrier protein, resulting in cytokine-specific B cells receiving, however, carrier specific T-cell help. **(B)** Conjugation to virus-like particles (VLPs) further increases antibody responses, due to repetitive display of the antigen and packaged non-coding RNA, which is a ligand for toll-like receptor 7/8 (TLR7/8) and present in many RNA virus-derived VLPs. **(C)** Presence of a universal T cell epitope may further improve Th cell dependent IgG responses, in particular if the epitope is derived from tetanus toxin (TT), as essentially everybody harbors memory Th cells against TT.

dendritic cells (FDCs), which present native antigen to B cells (Link et al., 2012). Antigen on FDCs is essential for the formation of germinal centers, which in turn are essential for the generation of memory B and long-lived plasma cells (Liu et al., 1992). Hence, the ability to recruit natural IgM and complement greatly increases immunogenicity. An additional reason for the strong immunogenicity of antigens displayed on VLPs is the bacterial RNA packaged by many VLPs produced in bacteria. RNA is a ligand for TLR7/8 and serves as "packaged adjuvant" enhancing IgG as well as IgA responses (Jegerlehner et al., 2007; Bessa et al., 2009; Hou et al., 2011) (**Figure 1B**). For this reason, cytokines on the surface of VLPs can induce strong and neutralizing antibody responses that show preclinical and clinical efficacy. As an example, formulation of an IL-1β-VLP conjugate vaccine in Alum was sufficient to induce neutralizing anti-IL-1 antibodies in at least a fraction of human type II diabetes patients (Cavelti-Weder et al., 2016). We have recently optimized the immunogenicity of a VLP derived from the plant-specific cucumber mosaic virus. By introducing a universal T cell epitope derived from tetanus toxin, we generated a VLP optimized for the elderly ($CuMV_{TT}$) (Zeltins et al., 2017) since essentially all individuals have been immunized multiple times against tetanus and harbor specific memory Th cells (**Figure 1C**). Using this $CuMV_{TT}$, we have been able to treat atopic dermatitis in dogs and horses by immunizing against IL-31 and IL-5, respectively (Bachmann et al., 2018; Fettelschoss-Gabriel et al., 2018b). Hence, cytokines displayed on VLPs hold promise to induce clinically relevant antibody levels for the treatment of chronic diseases.

It is important to note that maintenance of T cell tolerance for self-antigens is key, so vaccines targeting self-molecules should avoid breaking tolerance completely. Otherwise, there may be a risk of undesired auto-immune complications. An important safety aspect of VLP-based vaccines is that they confer self-antigens with repetitive presentation on their surface [an immunogenic feature of many pathogens, also called pathogen-associated structural pattern (PASP) (Bachmann and Jennings, 2010)]. Hence, VLPs place the self-antigen in an immunologically active context and link it to T-cell help directed toward the VLP carrier, features that are absent from endogenous self-antigens (see **Figure 1**). Therefore, the B cells receive bystander T cell help for the self-antigen only when coupled to the VLP, whereas uncoupled self-antigens will not receive bystander T cell help. Reassuringly in preclinical studies for psoriasis (a chronic inflammatory skin disorder), a VLP-vaccine targeting the pro-inflammatory cytokine IL-17, we demonstrated that induced anti-IL-17 antibody levels reversed after cessation of

immunization and stimulating an endogenous flare of the cytokine using imiquimod (a TLR7/8 ligand and potent IL-17 activator) was unable to influence antibody responses, as T cell help from the VLP was missing (Rohn et al., 2006; Zeltins et al., 2017). This is fundamentally different from classical prophylactic vaccines that are designed to trigger the immune system to respond upon re-encountering the target pathogen.

ASTHMA AND THE ROLE OF IL-5

Allergic asthma is characterized by chronic inflammation of the airways and manifests as episodes of airway obstruction and wheezing. Roughly half of severe asthma clinical cases appear to present with a non-allergic etiology. However, a key feature for a subset of asthma patients is increased levels of granulocytes called eosinophils, which are very toxic cells in the body (Yancey et al., 2017). Chronic eosinophilic airway inflammation is a particularly severe form of asthma, which eventually causes structural changes of the airways. This disease progression is summarized as airway remodeling that may result in bronchial hyperresponsiveness to non-specific stimuli, a characteristic of intrinsic (non-allergic) asthma (Cohn et al., 2004).

Standard therapies for asthma are non-specific and involve corticosteroids and long- or short-acting β-adrenoceptor agonists. These medications, however, only suppress symptoms but fail to treat the underlying cause of the disease. Furthermore, compliance is generally low, in particular for twice-daily inhalation regimens (Bender et al., 1997; Onyirimba et al., 2003). Non-specific immunotherapy using VLPs loaded with a ligand for TLR9 is a promising option for the treatment of asthma but requires further investigation (Beeh et al., 2013; Rank et al., 2013). In contrast, specific immunotherapy (SIT) represents a potential disease-modifying therapy that is, as the name implies, more specific than the classical drugs (Nagata and Nakagome, 2010). SIT, however, is only possible for a limited number of allergens, time-consuming and cannot be performed with patients suffering from severe asthma (Creticos, 1992; Walker et al., 1995). Drugs specifically interfering with the allergic immune response are promising new alternatives. A key cell type in asthma is the allergen-specific Th2 cells, which produce IL-4, causing generation of IgE followed by activation of mast cells. In addition, Th2 cells release IL-13, increasing obstructing mucus production and IL-5, as well as eotaxin, recruiting eosinophils. An alternative to the non-specific standard therapy with corticosteroids or SIT, is therefore to specifically target the cells and factors involved in asthma. Four such FDA approved drugs are mAbs against IgE (Omalizumab), IL-5 (Mepolizumab, Reslizumab) (Quirce et al., 2017), and recently IL-5 receptor (Benralizumab) (Kupczyk and Kuna, 2018). Omalizumab neutralizes IgE and may even remove it from mast cells. Hence, blocking IgE mostly results in reduced mast cell activity, most important during the early asthma response. In contrast, therapies blocking IL-5 aim at reducing blood and tissue eosinophils thereby being more potent at halting later phases of the response, including airway remodeling. Mepolizumab, Reslizumab, and Benralizumab are therefore mostly used for the treatment of severe eosinophilic asthma, a chronic condition requiring life-long treatment. GlaxoSmithKline (GSK) is currently negotiating with the authorities for approval of Mepolizumab in chronic obstructive pulmonary disease (COPD) subsequent to a successful phase III study in the indication (Pavord et al., 2017). Nonetheless, adverse reactions such as urticaria, anaphylaxis or serum sickness have been reported with passive administration of antibodies and repeated injections are required to maintain effectiveness. With an excellent safety profile (Leung et al., 2017) and the requirement for long-term if not life-long treatment, IL-5 might be an ideal target for the development of an active vaccine against asthma and potentially COPD.

PRECLINICAL AND CLINICAL DATA SUPPORTING THE DEVELOPMENT OF A VACCINE AGAINST IL-5

IL-5 is a soluble low abundant protein and tolerance at the B cell level may therefore not be expected (Bachmann and Dyer, 2004). Indeed, the coupling of IL-5 to VLPs derived from the bacteriophage Qβ readily induced strong and neutralizing antibody responses in mice in the absence of an adjuvant (Zou et al., 2010). Neutralizing antibodies were able to reduce numbers of eosinophils in peripheral blood and reduced eosinophil infiltration in the lung of asthmatic mice by more than 95%. Hence, vaccination against IL-5 resulted in protection against eosinophilic infiltration, the same principal biological effect observed for commercial anti IL-5 antibody therapies. Similar observations have been made with a DNA based vaccine (Hertz et al., 2001). DNA vaccines, however, are usually not immunogenic in humans and therefore difficult to translate into clinical application. More recently, we have demonstrated that VLP-based vaccines are efficacious in a more real-life setting, namely insect bite hypersensitivity (IBH) in horses. IBH is an allergic response to bites of midges (*Culicoides*) and results in strong inflammation of the skin by eosinophils. Similarly as seen in allergic asthma in humans (Price et al., 2016), blood eosinophil counts correlated with disease severity (Fettelschoss-Gabriel et al., 2018b). In a double-blind, placebo-controlled study, IBH horses were immunized against IL-5 using a conjugate vaccine consisting of IL-5 conjugated to the immune optimized $CuMV_{TT}$-VLP (Zeltins et al., 2017). IBH is caused by insect-bites and therefore seasonal with no symptoms during the winter. Horses were vaccinated with IL-5-$CuMV_{TT}$ in the absence of adjuvants before and during the season. The vaccine was well tolerated with no signs of adverse events after repeated doses, and no difference in helminth burden could be detected. Strikingly, symptoms of IBH in immunized horses were strongly reduced compared to the previous untreated season of the same horses as well as to the placebo horses (Fettelschoss-Gabriel et al., 2018b). Anti-IL-5 responses declined in all horses with defined half-lives of a few months and thus were reversible. More recently, we have shown that horses can be immunized over multiple seasons and that protection against IBH remains stable (Fettelschoss-Gabriel et al., 2018a).

Hence, vaccination of horses against IL-5 results in strong protection against IBH, an eosinophil-mediated inflammatory disease. By analogy, we are confident that a human version of the vaccine will protect against severe asthma, with an eosinophil-mediated pathology for the disease of the lung.

CONCLUSION

IL-5 is an excellent target for the treatment of allergic and non-allergic asthma with an eosinophilic component. We propose to develop a vaccine against IL-5 for the treatment of asthma in humans to broaden patient access to the highly effective therapy and to facilitate long-term treatment.

AUTHOR CONTRIBUTIONS

Each author contributed to the writing of the manuscript.

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frontiers in Cellular and Infection Microbiology

MINI REVIEW
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Translational Potential of Therapeutics Targeting Regulatory Myeloid Cells in Tuberculosis

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Despite recent advances in tuberculosis (TB) drug development and availability, successful antibiotic treatment is challenged by the parallel development of antimicrobial resistance. As a result, new approaches toward improving TB treatment have been proposed in an attempt to reduce the high TB morbidity and mortality rates. Host-directed therapies (HDTs), designed to modulate host immune components, provide an alternative approach for improving treatment outcome in both non-communicable and infectious diseases. Many candidate immunotherapeutics, designed to target regulatory myeloid immune components in cancer, have so far proven to be of value as repurposed HDT in TB. Several of these studies do however lack detailed description of the mechanism or host pathway affected by TB HDT treatment. In this review, we present an argument for greater appreciation of the role of regulatory myeloid cells, such as myeloid-derived suppressor cells (MDSC), as potential targets for the development of candidate TB HDT compounds. We discuss the role of MDSC in the context of Mycobacterium tuberculosis infection and disease, focussing primarily on their specific cellular functions and highlight the impact of HDTs on MDSC frequency and function.

Keywords: regulatory myeloid cells, myeloid-derived suppressor cells, Mycobacterium tuberculosis, host-directed therapy, immunotherapy

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INTRODUCTION

The global TB health concern is exacerbated by the emergence of drug-resistant *Mycobacterium tuberculosis (Mtb)* strains. Other considerations, such as the substantial economic burden imposed by the length of TB treatment and the associated drug toxicity, favor the development of novel TB drugs (Islam et al., 2017). Surprisingly, the current pipeline for the development of new antibiotic compounds against *Mtb* remains slim. TB therapeutic research is now focused on the establishment of novel treatment strategies, such as host-directed therapies (HDTs), as an adjunctive approach to the current treatment regimen. HDTs aimed at modulating host immune homeostasis to ensure eradication of the invading pathogen, whilst simultaneously limiting tissue pathology, appears most promising. Similar HDT approaches correcting aberrant host pathways by way of targeting immune checkpoints, have shown huge success in cancer treatment plans. While immunotherapeutics has placed much emphasis on active enhancement of adaptive immune cell function through direct targeting of T-cell checkpoints, myeloid cells have recently emerged as equally attractive immune targets (Burga et al., 2013). Regulatory myeloid cells, such

as myeloid-derived suppressor cells (MDSC), constitute a key innate immune checkpoint that impedes protective immunity in cancer (Young et al., 1987; Gabrilovich and Nagaraj, 2009). Common signaling pathways and similarities in immune regulation in malignancy and infectious disease, support the idea that cancer immunotherapeutic discoveries, can guide TB HDT strategies focused on pharmacological modulation of regulatory myeloid cells. We discuss the unfavorable role of regulatory myeloid cells in oncology, efforts to target MDSC in cancer clinical trials, knowledge on their negative contribution to *Mtb* control and highlight TB HDT compounds with potential to manipulate MDSC.

REGULATORY MYELOID CELLS IN TUBERCULOSIS: MYELOID-DERIVED SUPPRESSOR CELLS

While the role of immunosuppressive regulatory T-cells have been demonstrated (Singh et al., 2012; Larson et al., 2013), the involvement of regulatory myeloid cells in TB, is not yet fully appreciated. In this regard, one of the mechanisms accounting for inadequate T-cell responses, is through defective engagement of innate immunity (Daker et al., 2015). Therefore, identification of new targets that regulate innate immune cell function and promote optimal activity of protective anti-TB immune responses, are likely to contribute to development of effective HDT targets.

Myeloid cells are the first responders to *Mtb* challenge during pulmonary infection and are critically involved in the induction of adaptive immunity, containment of bacilli and orchestration of inflammation. The key contribution of innate immunity in the initiation and regulation of adaptive immunity has led to the design of immunotherapies modulating innate cells, aimed at controlling diseases such as cancer (Qin et al., 2015). While MDSC are considered crucial in curbing inflammation-induced pathology, chronic or excess inflammation results in accumulation of MDSC (Ostrand-Rosenberg and Sinha, 2009). Overabundant MDSC, in turn, produce inflammatory mediators which recruit additional MDSC, thereby exacerbating inflammation (Cheng et al., 2008; Sinha et al., 2008). MDSC have also gained attention in the TB field due to their host immunosuppressive potential and ability to harbor Mtb bacilli (Knaul et al., 2014). MDSC frequencies are significantly expanded in the blood of TB patients, but decrease in number following successful TB chemotherapy (du Plessis et al., 2013). Several lines of evidence demonstrate the detrimental effect of MDSC on anti-TB immunity, including T-cell activation, proliferation, trafficking, regulatory T-cell induction and T-cell cytokine responses (du Plessis et al., 2013; Obregón-Henao et al., 2013; Knaul et al., 2014; Daker et al., 2015). MDSC may also impair phagocyte responses through production of IL-10 and TGF-β, inhibiting DC and macrophage function, and polarizing these cells toward a Th2 phenotypic response, as shown in tumor biology (Knaul et al., 2014). Such impairments are likely to affect Mtb control mechanisms, as well as the initiation and maintenance of effective adaptive immunity. MDSC are not only capable of regulating the intensity of T-cell responses to particular antigens, but also determine the numbers and activity of other immuno-regulatory cells. Given this immuno-modulatory capacity, MDSC should be considered as potential targets for fine-tuning the host response to *Mtb*. The major value of MDSC-immunotherapeutic strategies, is that these agents may be combined with traditional TB treatment and other HDT options to improve and optimize pathogen clearance.

PHARMACOLOGICAL TARGETING OF REGULATORY MYELOID CELLS

The phenotypic and functional diversity of cellular subsets present within the myeloid compartment remains underappreciated and poorly investigated in the context of TB. The complexity of innate phagocytes in the lungs of TB patients is particularly striking, suggesting that detailed characterization is imperative to understanding the mechanisms of TB susceptibility or protection (Silver et al., 2016). MDSC, purposed to regulate inflammation, have gained attention due to their central role in prevention of host anti-tumor immunity and subsequent immune escape (Lesokhin et al., 2012). MDSC not only support tumor cell metastasis, proliferation and angiogenesis, but also create an immunosuppressive environment for cancer cell evasion of host immunity and chemotherapy-induced senescence, thereby promoting disease and treatment resistance (Gabrilovich et al., 2012; Bronte et al., 2016; Kumar et al., 2016). An area of intense research in the oncology field, is the identification and targeting of MDSC mechanisms and molecules supporting tumor escape. Reversal of MDSC function, inhibition of MDSC recruitment or depletion of MDSC numbers, have all shown promise in enhancing the activity of cancer vaccines and therapies in preclinical models, with growing evidence from clinical trials (Di Mitri et al., 2015; Draghiciu et al., 2015a).

STRATEGIES FOR REVERSING MDSC IMPACT ON ANTI-TB IMMUNITY

Regulatory myeloid cells such as MDSC have been successfully depleted with anti-Gr1+ antibodies in murine cancer models, with an associated reduction in tumor burden (Condamine et al., 2014). The Gr1+ antigen is, however, not present in humans, and is also a non-specific granulocyte marker, making this MDSC depletion strategy highly contentious (Xing et al., 2016). These findings do, however, suggest that depletion of MDSC is an effective immunotherapeutic approach. Other MDSC depletion strategies have shown greater success as cancer immunotherapy at pre-clinical and clinical trial level, and should be considered as repurposed treatment options for TB (Draghiciu et al., 2015a). MDSC targeting strategies can be categorized as approaches to (1) block MDSC inducing factors, (2) reverse MDSC functionality, and (3) differentiate MDSC into non-suppressive cells (**Figure 1** and **Table 1**). There is, however, considerable cross-talk and overlap between these pathways, with numerous feedback mechanisms necessitating investigations in each stage of the TB disease spectrum.

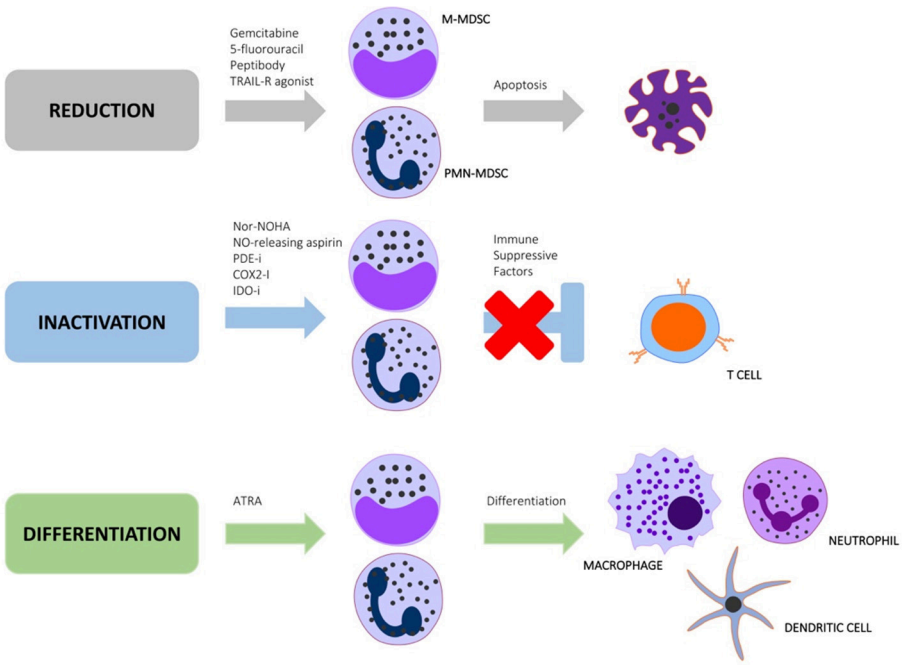

FIGURE 1 | Immunotherapeutic strategies aimed at targeting regulatory myeloid cell pathways to reduce, inactivate or differentiate MDSC.

INHIBITION OF MDSC EXPANSION AND RECRUITMENT

Cytokines

In cancer, mediators known to enhance the expansion of MDSC include interleukin-6 (IL-6), tumor necrosis alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), cyclooxygenase-2 (COX2), prostaglandins, stem-cell factor (SCF), macrophage colony-stimulating factor (M-CSF), vascular-endothelial growth factor (VEGF), IL-1β and IL-17 (Shipp et al., 2016). A recent study has shown that blocking of the IL-6 receptor (IL-6R) or TGF-β in tumor-bearing mice, decreases both monocytic and granulocytic MDSC subsets, alongside a reduction in tumor growth and improvement in T-cell function (Sumida et al., 2012). In contrast, results with GM-CSF treatment have been variable (Ma et al., 2017). Mice deficient in IL-17R and IFN-γR, also demonstrated lower MDSC levels and increased T-cells that were associated with a reduction in tumor development (He et al., 2010).

In TB, some of these cytokines are also being investigated as potential targets in HDT approaches, but none have investigated the effect on MDSC specifically. Instead, adjunctive cytokine treatment as intervention in TB has, however, largely focused on supplementation of mediators which activate macrophages to promote mycobacterial killing or blockade of pro-inflammatory cytokine signaling to limit lung damage (Condos et al., 1997, 2003; Vogt and Nathan, 2011). The majority of studies report on IFN-γ treatment of TB and demonstrate a reduction in pro-inflammatory cytokine production, enhancement of TB-specific CD4 T-cell responses, enhanced sputum conversion and reduced radiological involvement, although some inconsistent outcomes have been reported (Park et al., 2007; Dawson et al., 2009). IFN-γ treatment has also shown promise as HDT in a small study on Cuban patients infected with non-tuberculous mycobacterial lung disease, such as *M. avium* (Suárez-Méndez et al., 2004). This suggests that some HDTs may also be beneficial in the treatment of NTM. It was shown that IFN- γ improved the extent and clearance rate of pulmonary and radiological symptoms of these patients by the month 18 time point (Suárez-Méndez et al., 2004). Pre-clinical data on anti-TNF-α, anti-VEGF and IL-6R blockers as TB HDT in TB animal models and case studies of severe pulmonary TB patients have been encouraging (Okada et al., 2011; Datta et al., 2015). It remains crucial to appreciate the complex role of cytokines in TB immune regulation, necessitating in depth characterization of the optimal cytokine dose and timing to determine the cytokine's effect on MDSC induction, and resultantly on disease modulation.

Enzymes and Transcription Factors

In cancer, several of the cytokine mediators targeted by TB HDTs, trigger activation of the transcription factor, signal transducer and activator of transcription 3 (STAT3), which activates the signaling pathway mediating tumor-MDSC induction (Gao et al., 2007). In cancer, STAT3 is critically involved in MDSC expansion by stimulating expression of immature myeloid cell (IMC) genes involved in MDSC development (Tartour et al., 2011; Sansone and Bromberg, 2012; **Table 1**, **Figure 1**). STAT3 is phosphorylated by the tyrosine kinase Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway (Nicolas et al., 2012). Agents targeting these kinases in cancer

TABLE 1 | Agents affecting regulatory myeloid cell pathways have been tested as immunotherapeutics in cancer, some of which have also shown promise when evaluated in TB.

Host-directed therapy	Regulatory myeloid response to host-directed therapy in cancer	References	Host-directed therapy in TB?	References
(1) INHIBITION OF MYELOID-DERIVED SUPPRESSOR CELL EXPANSION AND RECRUITMENT				
Anti-IL-6R	Reduction of both granulocytic and monocytic MDSC subsets, reduction in tumor growth and improved T cell functions. **Murine model.**	Sumida et al., 2012	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Troublesome results being found in *M.tb* infection model where blockade of IL-6R results in an increase in susceptibility to infection in mice. **Murine infection model.**	Okada et al., 2011
Etanercept Anti-TNF-α	Reduced MDSC frequencies in the blood with simultaneous delayed tumor growth and volume. Potentially a CD8 T cell-dependent mechanism. **Murine and human model.**	Bayne et al., 2012; Atretkhany et al., 2016	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Troublesome risk of anti-TNF-α treatment resulting in reactivation of active disease, especially in latent infection cases. **Human infection model.**	Wallis, 2009
Anti-GM-CSF	Variable results. These included the impairment of GM-CSF-mediated MDSC differentiation in the supernatant of cancerous lesions following treatment with neutralizing anti-GM-CSF antibodies, as well as reduced MDSC accumulation in the spleen following GM-CSF knockout. **Murine model.**	Dolcetti et al., 2010; Ma et al., 2017	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. GM-CSF appears to confer a protective role in TB owing to its activation of macrophages to inhibit intracellular *M.tb* growth, therefore, a GM-CSF targeted therapy may prove detrimental to the host. **Murine and human infection model.**	Robinson, 2017; Rothchild et al., 2017
Anti-VEGF	Reduced numbers of circulating VEGFR1-expressing MDSC which may restore immunocompetency. **Murine and human model.**	Kusmartsev et al., 2008	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. In a TB model, anti-VEGF treatment promotes vascular normalization, reduces hypoxic areas within the TB granuloma and thereby provide improved delivery mechanisms for current anti-Tuberculosis therapies. **Murine and human infection model.**	Datta et al., 2015
Anti-IL-17R + Anti-IFN-γR	Reduced MDSC numbers, increased number of T cells, and reduced tumor development. **Murine model.**	He et al., 2010	Experimental stages. Anti-IL-17R has been shown to reduce the granulocytic subset of MDSC in a murine TB model. **Murine infection model.**	Freches et al., 2013; Lombard et al., 2016; Segueni et al., 2016
Sunitinib Tyrosine kinase inhibitor (multitargeted)	Inhibition of STAT3 through the inhibition of the Jak/Stat pathway reverses MDSC expansion. **Murine and human model.**	Chen et al., 2015a; Draghiciu et al., 2015b,c; Ko et al., 2009; Xin et al., 2009	Effect on MDSC, in the context of TB, yet to be evaluated.	N/A.
Gefitinib Tyrosine kinase inhibitor (targets EGFR mutations)	S100A9^{+} MDSC-derived macrophages in the tumor microenvironment mediate resistance to tyrosine kinase inhibitors targeting EGFR mutations. **Human model.**	Maemondo et al., 2010; Feng et al., 2018	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Gefitinib has been shown to inhibit STAT3 which is crucial for the expansion of MDSC, making it a promising target for directed therapies. **Murine and human infection model.**	Stanley et al., 2014; Sogi et al., 2017
Imatinib Tyrosine kinase inhibitor (targets ABL family)	Reduces the number of MDSC, as well as the levels of arginase 1, to those of healthy control patients. **Human model.**	Giallongo et al., 2014	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Imatinib reduced the number of granulomatous lesions and bacterial load in a murine TB model. **Murine and human model.**	Napier et al., 2011; Kalman et al., 2017
5-Fluorouracil (5-FU) Antimetabolite – Thymidylate synthase inhibitor	Reduces the number of MDSC without affecting other lymphocyte and myeloid population frequencies, except those of B cell population frequencies which are increased. **Murine model.**	Vincent et al., 2010	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Potential mycobacterial resistance threatens further investigation of this therapy in the context of TB. ***in vitro* infection model.**	Singh et al., 2015
Gemcitabine Antimetabolite – DNA synthesis inhibitor		Suzuki et al., 2005; Le et al., 2009	Effect on MDSC, in the context of TB, yet to be evaluated. Potentially increases the risk of reactivation of *M.tb* infection or increases susceptibility to *M.tb* infection in cancer patients.	Not Applicable.

(Continued)

TABLE 1 | Continued

Host-directed therapy	Regulatory myeloid response to host-directed therapy in cancer	References	Host-directed therapy in TB?	References
MiR-155 Inhibitor	Reduces the expansion of the MDSC population within tumor-bearing mice and tumor growth. **Murine and human mode.**	Fabani et al., 2010; Li et al., 2014	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Inconclusive evidence exists that MiR-155 is indeed disease-promoting and needs to be investigated further. **Murine and human infection model.**	Huang et al., 2015; Iwai et al., 2015; Wagh et al., 2017; Etna et al., 2018
MiR-21 Inhibitor		Li et al., 2014; Chen et al., 2015b; Drakaki et al., 2017	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. **Murine infection model.**	Wu et al., 2012
(2) INHIBITION OF MYELOID-DERIVED SUPPRESSOR CELL FUNCTION				
Nor-NOHA Arginase-1 Inhibitor	Delays tumor growth by reversing MDSC function. **Murine model.**	Rodriguez et al., 2003, 2004	Experimental stages. Nor-NOHA inhibits ARG1 in phagocytes, resulting in the reduction of mycobacterial growth and lowering of IL-10 production. **Murine and human infection model.**	Talaue et al., 2006; de Oliveira Fulco et al., 2014; Mason et al., 2015
NO-aspirin NOS and COX-2 Inhibitors	Reverse MDSC-mediated immunosuppression in both *in vitro* and *in vivo* cancer models, with marked reductions in MDSC frequencies and delayed tumor growth. **Murine and human model.**	Wu and Morris, 1998; Fiorucci et al., 2000; Bronte and Zanovello, 2005; Corazzi et al., 2005; Molon et al., 2011	NO-aspirin yet to be tested. Conflicting aspirin data. NO inhibitor findings contradict NO-aspirin data as NO inhibitors resulted in heightened bacterial burdens, increased lung pathology and reactivation of latent infection. **Murine infection model.**	Chan et al., 1995; Botha and Ryffel, 2002; Byrne et al., 2007
IDO Inhibitors	Successful inhibition of MDSC expansion in tumors and reduced immunosuppressive effects. **Human model.**	Wang et al., 2014; Holmgaard et al., 2015	Experimental stages. Effect of IDO-i have yet to be evaluated. Blocking of IDO does, however, reduce clinical manifestations of TB and alter granuloma organization. **Murine, macaque and human infection model.**	Hirsch et al., 2016; Gautam et al., 2018
PDE-5 Inhibitors Sildenafil and Tadalafil	Reversal of MDSC functions and augmentation of anti-tumor immunity via the inhibition of the degradation of cyclic guanosine monophosphate (cGMP). This results in a reduction of ARG1 and iNOS expression. **Murine and human model**.	Serafini et al., 2006; Noonan et al., 2014	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. **Murine and Rabbit infection model**.	Subbian et al., 2011, 2016; Maiga et al., 2013, 2015
COX2 Inhibitors Indomethacin and Etoricoxib	Downregulation of the production of ARG1 and iNOS by MDSC, resulting in the reduction of suppressive MDSC functions. May also reduce MDSC numbers or block MDSC activation. **Murine model.**	Rodriguez et al., 2005; Veltman et al., 2010; Fujita et al., 2011	Experimental stages. COX2-i enhance Th1 immunity and downregulate the frequency of *M.tb*-induced Tregs, but their MDSC-specific effects have not yet been evaluated. **Murine and human infection model**.	Hernández-Pando et al., 2006; Tonby et al., 2016
PD-L1 Inhibitors	MDSC-mediated immunosuppression is abrogated. **Murine and human model**.	Pilon-Thomas et al., 2010; Kleffel et al., 2015; Sharma and Allison, 2015b; Kleinovink et al., 2017	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Promising results have been shown in a TB model with the restoration of T cell responsiveness, cytokine secretion and proliferation, however immune reactivation responses are troublesome. **Human infection model.**	Hassan et al., 2015; Reungwetwattana and Adjei, 2016
Calprotectin (S100A8/9) Inhibitor	Inhibition of MDSC function resulting in reduced tumor growth. **Murine model.**	Sinha et al., 2008	Experimental stages—effect on MDSC, in the context of TB, yet to be evaluated. Upregulation of calprotectin in serum of TB patients is known to correlate with disease severity and pathology. Calprotectin is, therefore, a promising target for directed therapeutics. **Murine infection models.**	Kang et al., 2011; Gopal et al., 2013
(3) MATURATION/DIFFERENTIATION OF MYELOID-DERIVED SUPPRESSOR CELLS INTO NON-SUPPRESSIVE CELLS				
ATRA Retinoid-activated transcriptional regulator activator	Maturation of early myeloid cells into fully differentiated, non-immunosuppressive cells via the upregulation of GSH which reverses MDSC suppressive functions. **Murine and human models.**	Kuwata et al., 2000; Gabrilovich et al., 2001; Luo and Ross, 2006; Nefedova et al., 2007; Nakanishi et al., 2008; Gabrilovich and Nagaraj, 2009	Experimental stages. Effect of ATRA on MDSC in a TB model has been somewhat successful with an observed restoration of T cell numbers, reduced bacterial burden and lung pathology. **Murine infection models.**	Knaul et al., 2014

patients are being investigated in an attempt to reverse MDSC expansion. For example, the tyrosine kinase inhibitor (TKI) sunitinib, blocks MDSC expansion in cancer patients and tumor bearing mice (Chen et al., 2015a; Draghiciu et al., 2015b,c). Furthermore, STAT3 overexpression in myeloid cells trigger expansion of MDSC and upregulate S100A9, which directs MDSC accumulation (Cheng et al., 2008; Wu et al., 2011). In TB, protein kinase inhibitors have also emerged as attractive candidates in the development of antimicrobial drugs. The TKI, gefitinib, a FDA-approved compound for the treatment of non-small-cell lung carcinoma, has recently been tested in human TB due to its ability to inhibit the epidermal growth factor receptor (EGFR) and activate autophagy to restrict bacterial growth. Gefitinib demonstrated *in vitro* and *in vivo* efficacy against Mtb infection, although the involvement of MDSC has not been considered (Stanley et al., 2014; Sogi et al., 2017). The TKI, imatinib, has also shown therapeutic efficacy in TB mouse models, leading to the initiation of a pre-clinical study on Mtb infection in a non-human primate model, but again, the role of MDSC was not investigated (Napier et al., 2011; Kalman et al., 2017). Inhibitors of protein kinase R are also being screened in TB mouse models, whereas other anti-cancer kinase inhibitors such as sunitinib, malate and curcumin analogs with known effects on tumor-derived MDSC induction, remains to be tested in TB.

Cytotoxic Agents

In cancer, selected cytotoxic cancer agents have also been used to deplete MDSC through a yet undefined mechanism(s). For example, 5-fluorouracil (5-FU) and gemcitabine treatment reduces the number of MDSC without affecting the frequency of T-cells, dendritic cells, NKT- or NK-cells, yet increases the B-cell population (Le et al., 2009; Vincent et al., 2010). In TB, the effect of 5-FU on host immunity, more specifically, MDSC, has not been tested, and although its direct bactericidal action against Mtb has been reported, mycobacterial resistance to 5-FU continues to be a concern (Singh et al., 2015). Efforts evaluating 5-FU as TB HDT has been directed to investigation on inhibitors of phosphodiesterase-5 (PDE-5), which has a known effect on MDSC function (see section Phosphodiesterase Inhibitors).

Micro-RNA

With antisense technology improving, targeting of microRNAs (MiR) by immunotherapeutics is increasingly considered (Li et al., 2014). In cancer, MiR-155 and MiR-21 are critically required for the expansion of MDSC in tumor-bearing mice and to facilitate tumor growth; miR 93-106b cluster regulate expression of PD-L1 on MDSC; while a specific role for miR-142-3p has recently been suggested in cancer through preventing differentiation of myeloid cells (Li et al., 2014; Chen et al., 2015b). The role of MiR in TB continues to be unraveled, as reports emerge of Mtb facilitating expression of MiR-155 to disrupt the process of autophagy (Wagh et al., 2017; Etna et al., 2018). Although MiR-155 is responsive to TB therapy, it remains inconclusive if its expression is disease promoting, as MiR-155 knockout mice are susceptible to Mtb infection (Iwai et al., 2015; Wagh et al., 2017). MiR-21 is also upregulated following Mtb infection, reportedly to escape the host immune response by downregulating the genes for TNF-α and IL-6 (Wu et al., 2012). Also in TB, HDT manipulation of other miR, controlling key myeloid functions, has also been proposed. Although not yet tested in clinical trials, Mtb induced expression of miR-142-3p targets an actin-binding protein leading to reduced phagocytosis in primary human macrophages (Bettencourt et al., 2013). MiR-106b-5p was also specifically upregulated in human macrophages following Mtb infection to reduce function by lowering cathepsinS expression and favoring Mtb survival (Pires et al., 2017). Greater understanding of MiR function and regulation, also in the context of regulatory myeloid cells is, however, required before therapeutic targeting of host MiR becomes a reality. This is likely to be accompanied by the requirement of cell-specific delivery techniques, to avoid potential off-target immunological effects (Iannaccone et al., 2014).

INHIBITION OF MDSC FUNCTION

Enzyme Inhibitors

Arginase-1

In cancer, a critical mechanism whereby MDSC induce lymphocyte suppression is by local depletion of essential amino acids required for T-cell proliferation. Tumor-MDSC highly express the enzyme arginase-1 (ARG1) which catabolizes l-arginine to urea and ornithine (Wu and Morris, 1998; Bogdan, 2001), thereby inhibiting T-cell proliferation through decreased CD3-theta chain expression (Rodriguez et al., 2003, 2004). In lung cancer, treatment with the arginase inhibitor $N^{(\omega)}$-hydroxy-L-arginine (nor-NOHA) delays tumor growth by reversing MDSC function (Rodriguez et al., 2004). **In TB,** Nor-NOHA inhibition of ARG1 in phagocytes has also shown promise, through its reduction of mycobacterial growth and lowering of IL-10 production *in vitro* (Talaue et al., 2006; de Oliveira Fulco et al., 2014).

iNOS

In cancer, MDSC also generate oxidative stress by increasing levels of reactive oxygen species (ROS) and inducible nitric oxide (iNOS) with resultant immunosuppressive effects (Bronte and Zanovello, 2005; Youn et al., 2008). ROS and iNOS activity also steers the production of harmful peroxynitrites, H202 and NO (Schmielau and Finn, 2001; Youn et al., 2008; **Table 1**, **Figure 1**). Although NO is crucial, in TB, to mycobacterial control, nitrogen and oxygen intermediates suppress T-cell function by nitration of the T-cell receptor (Nagaraj et al., 2007), induction of T-cell apoptosis (Mannick et al., 1999), reduction of MHC expression(Harari and Liao, 2004) and reduction of CD3-theta chain expression (Schmielau and Finn, 2001). NOS inhibitors have been shown to reverse MDSC-mediated immunosuppression in both *in vitro* and *in vivo* cancer models (Wu and Morris, 1998; Bronte and Zanovello, 2005). For example, nitro-aspirin (NO-aspirin), a new molecule in which aspirin is covalently linked to a NO-group, suppresses the production of ROS and provides feedback inhibition to iNOS (Fiorucci et al., 2000). *In vitro* and *in vivo* models of NO-aspirin treatment have also been shown to reduce MDSC

numbers, reverse MDSC induced inhibition of T-cell responses by reducing CCL2 chemokine nitration and delay tumor growth (Molon et al., 2011). NO-aspirin also inhibits the COX-2 enzyme, another MDSC inducer (Corazzi et al., 2005). In TB, information on aspirin as HDT has been conflicting, but the effect of NO-aspirin remains to be investigated (Byrne et al., 2007). In spite of this, findings from murine TB emphasize NO as vital component in innate immune control of Mtb, and argues against the use of NO inhibitors as these result in heightened bacterial burden, increased lung pathology and mortality (Chan et al., 1995) and reactivation of latent Mtb infection (Botha and Ryffel, 2002).

Phosphodiesterase Inhibitors

Phosphodiesterase-5 (PDE-5) inhibitors (PDE-5-i), such as sildenafil and tadalafil, have good safety profiles as these have been used for decades to treat pulmonary hypertension, cardiac hypertrophy and erectile dysfunction. PDE-5-i were shown to reverse MDSC function in cancer patients and augment anti-tumor immunity by inhibiting the degradation of cyclic guanosine monophosphate (cGMP), leading to reduction in ARG1 and iNOS expression (Serafini et al., 2006; Noonan et al., 2014). Promising pre-clinical findings demonstrate that treatment with PDE-5-i improve T-cell responses, delay tumor growth and abrogate Treg proliferation in several cancer types (Serafini et al., 2006). This has resulted in a number of clinical trials on PDE-5-i in cancer, including a study evaluating whether treatment of oropharyngeal carcinoma patients with tadalafil could enhance T-cell tumor infiltration (NCT00843635); whether tadalafil can improve responses to dexamethasone chemotherapy (NCT01374217), if sildenafil treatment improves the outcome of non-small cell lung carcinoma (NCT00752115), or if tadalafil in combination with a novel vaccine and gemcitabine chemotherapy or radiation therapy, improves cancer outcome (NCT01342224).

In TB, the severe side effects associated with thalidomide, has led to the consideration of analogs, such as PDE-i, with similar potential to inhibit TNF-α (Aragon-Ching et al., 2007). PDE-i have been shown to decrease TB disease severity, reduce lung pathology and bacillary load in mouse models (Subbian et al., 2011, 2016; Maiga et al., 2013, 2015). The effect of PDE-i on immune cell phenotypes and function during human Mtb infection and TB disease remains poorly defined, but future and ongoing trials could cast more light on the impact of PDE-i on TB host immune responses, and necessitate evaluation of the effect on MDSC (NCT02968927).

Cyclooxygenase Inhibitors

Prostaglandin-E2 (PGE-2) has both pro-inflammatory and immune-suppressive properties and is synthesized by cyclo-oxygenase-2 (COX2). MDSC highly express the PGE-2 receptor, E-prostanoid 4, which, upon binding, induces ARG1 (Rodriguez et al., 2005). In cancer, Treatment of MDSC with COX inhibitors (COX2-i) have shown to down-regulate their production of ARG1 and iNOS, thereby reducing MDSC suppressive function (Veltman et al., 2010). COX2-i may thus target MDSC on multiple levels, either by reducing their numbers or by blocking their activation (Rodriguez et al., 2005; Fujita et al., 2011). In TB, COX2-i also significantly improved host immunity to Mtb in animal models by enhancing Th1 immunity (Hernández-Pando et al., 2006). *In vitro* treatment of TB patients' blood samples with the COX2-i, indomethacin, a nonsteroidal anti-inflammatory drug, downregulated the frequency of Mtb-induced Tregs and impaired T-cell proliferation and antigen-specific cytokine responses (Tonby et al., 2016). Although this study did not consider the effect on regulatory myeloid cells, an ongoing trial evaluating the impact of the COX2-i, etoricoxib, on immune-mediated host clearance of Mtb, will allow for assessment of MDSC functionality (NCT02503839).

Indoleamine 2,3-Dioxygenase Inhibitors

Monocytic MDSC also express high levels of the enzyme indoleamine 2,3-dioxygenase (IDO) that mediates immunosuppression through a mechanism involving regulatory T-cells (Tregs) (Holmgaard et al., 2015). In cancer, inhibition of IDO successfully blocks expansion of MDSC in tumors, as well as reducing the immunosuppressive effects of MDSC in IDO deficient mice (Yang, 2009; Wang et al., 2014; Holmgaard et al., 2015). Mtb is also a potent inducer of IDO (Hirsch et al., 2016). In TB, even though the mycobacterial burden in IDO-deficient mice is comparable to those of wild type mice (Blumenthal et al., 2012), a recent report demonstrated that blocking of IDO decreases both the clinical manifestations of TB as well as microbial and pathological correlates in macaques by altering granuloma organization (Gautam et al., 2018). Additionally, IDO inhibitors may reduce the number of MDSC in the lungs of TB patients, however this remains to be tested.

Checkpoint Inhibitors

In cancer, tumor-derived MDSC highly express programmed death ligand-1 (PD-L1), which engages the PD-1 receptor on T-cells, resulting in an exhausted phenotype (Jiang et al., 2015; O'Donnell et al., 2017). Blocking of PD-L1 abrogated MDSC-induced immune suppression in a murine melanoma model (Kleffel et al., 2015; Kleinovink et al., 2017).

In TB, the use of a PD-L1 checkpoint inhibitor has shown some promise in an *in vitro* model through its restoration of T-cell responsiveness to TB antigens, cytokine secretion and proliferation (Sharma and Allison, 2015a). Similarly, TB treatment has been shown to reduce expression of the genes responsible for PD-L1 expression on T-cells and natural killer cells (Hassan et al., 2015). However, by reactivating the immune system through treatment with this checkpoint inhibitor, two cancer patients have developed active TB disease (Reungwetwattana and Adjei, 2016). This highlights the complexities associated with modulation of immune regulatory molecules, such as PD-L1, which are upregulated on multiple immune cell subsets in a range of disease conditions (Shen et al., 2016). Even so, inhibition of PD-L1 on immune regulatory cells such as MDSC in TB, merits further investigations.

In cancer, the calcium-binding pro-inflammatory alarmin, calprotectin (S100A8/9), is highly expressed on murine tumor-derived MDSC and contributes to the induction of MDSC (Sinha et al., 2008). Interference with S100A8/A9 signaling inhibits MDSC function, leading to decreased tumor growth

(Sinha et al., 2008). In TB patients, serum abundance of S100A8/9 correlates with disease severity while also mediating neutrophilic inflammation and lung pathology in Mtb-infected experimental animals (Kang et al., 2011; Gopal et al., 2013). Given the association of S100A8/9 with MDSC, current TB treatment strategies and lung inflammation may thus benefit from targeting this molecule, with the expectation that it could limit immunosuppression and neutrophilic influx.

MATURATION/DIFFERENTIATION OF MDSC IN NON-SUPPRESSIVE CELLS

Differentiation of MDSC into mature myeloid cells without immunosuppressive functionalities is another promising immunotherapeutic strategy. Various studies have shown enhanced MDSC levels in the bone marrow and spleen of vitamin-A deficient mice (Kuwata et al., 2000; Walkley et al., 2002).

All-Trans Retinoic Acid

All-trans retinoic acid (ATRA) is a vitamin-A metabolite that activates retinoid-activated transcriptional regulators (Nakanishi et al., 2008). These factors activate target genes resulting in the maturation of early tumor-associated myeloid cells into fully differentiated, non-immunosuppressive cells (Nefedova et al., 2007). In cancer, ATRA treatment also up-regulates glutathione (GSH), a ROS scavenger, thereby reversing MDSC immunosuppressive function (Nefedova et al., 2007). Furthermore, ATRA treatment of MDSC led to their differentiation into mature DC, granulocytes and monocytes, through their upregulation of differentiation markers such as HLA-DR (Lathers et al., 2004; Gabrilovich and Nagaraj, 2009). On the other hand, ATRA also increases expression of the transcription factor FoxP3, thereby inducing the development of Tregs which are considered to be detrimental to anti-tumor and anti-TB immunity alike (Ma et al., 2014). Despite this potential deleterious side effect, several clinical trials are evaluating ATRA as treatment option to modulate MDSC in cancer patients (NCT00601796; NCT00618891).

In TB, ATRA and other retinoic acids have shown to enhance anti-mycobacterial immune functions in phagocytes upon *in vitro* Mtb infection, which was dependent on its ability to reduce total cellular cholesterol and increase lysosomal acidification (Crowle and Ross, 1989; Wheelwright et al., 2014). Although vitamin-A deficiency strongly predicts risk of incident TB among HHC of TB patients, pre-clinical trials of vitamin-A supplementation has not been promising, likely due to the complex metabolic route required for conversion of vitamin-A to the active ingredient, ATRA (Lawson et al., 2010; Visser et al., 2011). The therapeutic impact of ATRA on MDSC has been evaluated in a TB mouse model, demonstrating that MDSC ablation restores T-cell numbers, reduces Mtb burden and decrease lung pathology (Knaul et al., 2014). More recently it was also shown that ATRA augments autophagy of Mtb in human and murine alveolar macrophages(Coleman et al., 2018) Further experiments are however required to fully establish the impact of ATRA on MDSC function in TB patients when employed as adjunctive therapy.

TARGETING MDSC IN NON-TUBURCULOUS INTRACELLULAR INFECTIONS

In addition to their involvement in Mtb infections, MDSC have versatile roles in other intracellular pathogenic infections (Dorhoi and Du Plessis, 2018). Their effect during these infections are governed by the pathogen species and the course of infection. Targeting of MDSC in other intracellular infections could thus involve either the reduction or expansion of their suppressive capacity and/or frequency, depending on their beneficial or detrimental impact on disease outcome. For example, MDSC are increased in both clinical and experimental viral infections such as HIV, SIV, and LP-BM5, contributing to pathogen survival through TH1 immunosuppression as observed by the correlation with viral load and CD4 T-cell count (Gama et al., 2012; Vollbrecht et al., 2012; Garg and Spector, 2014; O'Connor et al., 2016). Similar detrimental effects have been reported for cytomegalovirus (CMV) infections (Garg et al., 2017), with MDSC impairing viral clearance (Daley-Bauer et al., 2012). MDSC accumulation has been reported also for various non-tuberculous intracellular bacterial infections. *Staphylococcus aureus* infections are sustained by MDSC promoting an immunosuppressive environment and impairing macrophage responsiveness (Heim et al., 2015; Tebartz et al., 2015), whereas MDSC frequencies in *Francisella tularensis* infection correlate with the extent of tissue pathology, loss of pulmonary function and host mortality (Periasamy et al., 2016). Above mentioned depletion strategies might thus be applicable to a broad range of intracellular bacterial infections, although the timing of administration might require careful consideration.

CONCLUSION

Tumors escape immune attack by a variety of mechanisms, often complementary in their ability to induce immunosuppression. Molecular interventions targeting innate immune cell regulatory pathways are making great advances in the immune-oncology field. Immune based cancer therapies are now also being recognized for their ability to potentiate anti-pathogen immunity when used in combination with classical treatment approaches. As for cancer, TB is described as a chronic inflammatory disease, characterized by a dysregulated immune profile. Data from cancer research suggest that MDSC can also be disarmed at pre-determined time points to redefine the outcome of disease. Therapeutic targeting of regulatory myeloid cells, such as MDSC and their molecular drivers, are, therefore, considered to be an exciting new strategy to help ameliorate TB via more effective and less-toxic strategies. Nevertheless, amongst the several approaches of TB HDT being sought, targeting of MDSC have not been explored, or their mechanistic involvement in the success of selected HDT, not appreciated. There is,

however, a pressing need to study key signaling pathways and intermediates involved in the induction and function of regulatory myeloid cells, to allow pre-clinical screening of re-purposed drugs showing promise in oncology trials. For instance, the impact of metabolic pathways on MDSC function has only recently been recognized and requires investigation in the TB context. It will also be important to identify markers specific to MDSC, in particular, the predominant monocytic subset, to advance identification and development of suitable MDSC-targeting TB immunotherapies. We propose that MDSC remain an under investigated regulatory myeloid cell population that holds great promise in the TB HDT field.

AUTHOR CONTRIBUTIONS

GW and NDP conceptualized the manuscript. NDP, LAK, and VL designed and drafted the manuscript with input from GW.

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Vitamin B1 Helps to Limit *Mycobacterium tuberculosis* Growth *via* Regulating Innate Immunity in a Peroxisome Proliferator-Activated Receptor-γ-Dependent Manner

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It is known that vitamin B1 (VB1) has a protective effect against oxidative retinal damage induced by anti-tuberculosis drugs. However, it remains unclear whether VB1 regulates immune responses during *Mycobacterium tuberculosis* (MTB) infection. We report here that VB1 promotes the protective immune response to limit the survival of MTB within macrophages and *in vivo* through regulation of peroxisome proliferator-activated receptor γ (PPAR-γ). VB1 promotes macrophage polarization into classically activated phenotypes with strong microbicidal activity and enhanced tumor necrosis factor-α and interleukin-6 expression at least in part by promoting nuclear factor-κB signaling. In addition, VB1 increases mitochondrial respiration and lipid metabolism and PPAR-γ integrates the metabolic and inflammatory signals regulated by VB1. Using both PPAR-γ agonists and deficient mice, we demonstrate that VB1 enhances anti-MTB activities in macrophages and *in vivo* by down-regulating PPAR-γ activity. Our data demonstrate important functions of VB1 in regulating innate immune responses against MTB and reveal novel mechanisms by which VB1 exerts its function in macrophages.

Keywords: *Mycobacterium tuberculosis*, vitamin B1, macrophages, peroxisome proliferator-activated receptor-γ, adjuvant

INTRODUCTION

Tuberculosis, caused by the bacterium *Mycobacterium tuberculosis* (MTB), remains a major global health challenge and is the leading cause of mortality among infectious diseases worldwide. MTB is estimated to infect one-third of the world's population, but only 10% of infected individuals show symptoms and develop clinical disease (1). Upon infection with MTB, several factors contribute to the disease outcome, with cell-mediated immunity representing one of the most critical determinants (2). As the first line of immune defense against MTB, macrophages provide a major habitat for MTB to remain dormant in the host for several years. Triggered upon MTB infection, multiple inflammatory signaling pathways in macrophages are activated to initiate a tailored

immune response toward the invading pathogen (3). Depending on phenotype and function, macrophages can polarize to several macrophage subsets, such as classically activated macrophages (M1 macrophages), alternatively activated macrophages (M2 macrophages), regulatory macrophage, tumor-associated macrophages, and myeloid-derived suppressor cells (4). The M1 phenotype displays an inflammatory profile such as expressing CD86 and MHC-II, secreting pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), and producing iNOS-dependent reactive nitrogen intermediates (4). M2 macrophages can be further subdivided into three subgroups: M2a, M2b, and M2c (5) and are marked by expressing CD206 and arginase 1 (6). The productions of pro-inflammatory cytokines TNF-α and IL-6 play a crucial role in pathogen clearance (7).

However, active tuberculosis occurs when the infection is no longer contained by the immune system. Tuberculosis can lead to weight loss and micronutrient deficiencies by increasing nutritional requirements, changing metabolic processes, decreasing appetite, and reducing food intake (8). Poor nutritional status is more common in people with active tuberculosis than in people without tuberculosis. Likewise, tuberculosis is more common in individuals with poor nutritional status (9). Nutrient deficiencies can result in immunosuppression and dysregulation of immune responses. Particularly, deficiencies in certain nutrients can impair phagocytic function in innate immunity including cytokine production, as well as cell-mediated immunities (10, 11), which can increases the susceptibility to active tuberculosis and delays recovery (12).

Vitamins are organic compounds and essential nutrients required by an organism in limited amounts. An increasing number of studies have begun to explore the mechanisms by which vitamins regulate immunity and their effects as adjuvant to treat tuberculosis (13, 14). Vitamin (V) A, D, and E are the most widely studied, and the mechanisms by which they regulate immunity have been partly elucidated (15, 16). Vitamin B1 (VB1) (also known as thiamin or thiamine) is needed for the metabolism of carbohydrates, but cannot be produced in humans, and thus it is an essential nutrient. It is known that vitamin B1 had a protective effect against oxidative retinal damage induced by antituberculotics (17). However, it is unclear whether VB1 participates in the immune regulation process during MTB infection.

Peroxisome proliferator-activated receptor γ (PPAR-γ), a member of the lipid-activated nuclear receptor family, has been implicated in the differentiation, and lipid metabolism of innate immune cells including macrophages and involved in inflammatory responses (18). In macrophages, PPARs integrate metabolic and inflammatory signaling to PPAR-γ function as an important "molecular switch" in regulating immune responses and nutrient metabolism during MTB infection (18–20).

In this study, we demonstrated that VB1 promoted the protective immune response in mice to enhance their resistance to MTB infection *via* regulating macrophage function. VB1 promoted the polarization of macrophages into strongly microbicidal, classically activated phenotype, and increased their expression of TNF-α and IL-6 *via* regulating NF-κB signaling in a PPAR-γ-dependent manner during MTB infection.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6J mice, 6 weeks old, were purchased from the Experimental Animal Center of Southern Medical University. PPAR-$\gamma^{floxp/floxp}$ (PPAR-$\gamma^{fl/fl}$) and Lyz2-cre mice were purchased by Shanghai Research Center for Model Organisms (Shanghai, China). All animal experiments in this study were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental protocols were reviewed and approved by the Medical Ethics Board and the Biosafety Management Committee of Southern Medical University (approval number L2015123).

Infection of Mice and Colony-Forming Units (CFUs)

6-week-old female and male mice were exposed to 1×10^7 CFUs of MTB H37Rv (ATCC 27294, the same below) in an Inhalation Exposure System (Glas-Col, USA), which delivers ~200 bacteria to the lung per animal. At 24 h after infection, bacterial titers in the lungs of at least two mice were determined to confirm the dose of MTB H37Rv inoculation. After infection, VB1 (Sigma-Aldrich, USA), isoniazid (INH), or water were orally administered daily until the indicated times. The VB1 solution (7.3 g/L) was prepared by dissolving 220 mg VB1 in 30 mL water. The INH solution (2.0 g/L) and water serve as positive and negative controls, respectively. Oral administration was started from 1 day after infection and performed every day until the specified time. For VB1-treated mice, the VB1 dose (200 μL/mouse) was equivalent to 20 μg VB1/100 g body weight. INH or water was given in the same manner as that for VB1-treated mice. Rosiglitazone (Sigma-Aldrich, USA) was administered intraperitoneally in 125 μL of corn oil at 20 mg/kg. Sham mice received corn oil only. Bacterial burden was determined by plating serial dilutions of spleen and lung homogenates onto 7H10 agar plates (BD Biosciences, USA) with 10% OADC. Plates were incubated in 5% CO_2 at 37°C for 3~4 weeks before counting colonies.

Lung Cells Isolation

Lung cell suspensions were prepared by perfusing cold saline containing heparin through the heart, removed, and sectioned in ice-cold medium. Dissected lung tissue was incubated in 0.7 mg/mL collagenase IV and 30 μg/mL DNase [Sangon Biotech (Shanghai), China] at 37°C for 30 min. Digested lungs were disrupted by passage through a 70-μm nylon cell strainer, treated with red blood cell lysis buffer, and processed over a 40:80% Percoll (GE Healthcare) gradient. The resulting cell suspension was washed and counted.

Culture of Bone Marrow-Derived Macrophages (BMDMs), Mycobacterial Infection, and Stimulation of VB1

Bone marrow cells were taken from C57BL/6J mice and placed on cell culture dishes (96 mm × 22 mm; CELLTER, China) at 37°C/5% CO_2 in DMEM (Corning, USA) containing 10% fetal

bovine serum (FBS; Corning, USA). The cells differentiated into macrophages induced by granulocyte macrophage colony-stimulating factor (100 ng/mL; PeproTech, USA) until the seventh day. BMDMs were placed on a 12-well cell culture plates (CELLTER) for 48 h at 37°C/5% CO_2 in DMEM containing 10% FBS. Then cells were persistently infected with MTB H37Rv until the indicated time. VB1 (20 μM) was added every 24 h.

Fluorescence-Activated Cell Sorting (FACS) Analysis

For surface staining, BMDMs or lungs cells were harvested, washed, and stained for 30 min on ice with mixtures of fluorescently conjugated mAbs or isotype-matched controls. mAbs of mice were as follows: FITC-anti-F4/80, APC-anti-CD80, PE-Cy7-anti-CD86, PE-anti-MHC-II, Percp-Cy5.5-anti-CD11b, Pacific Blue-anti-Gr-1 (eBioscience, USA). Cell phenotype was analyzed by flow cytometry on a flow cytometer (BD LSR II) (BD Biosciences, USA). Data were acquired as the fraction of labeled cells within a live-cell gate and analyzed using FlowJo software (Tree Star). All gates were set on the basis of isotype-matched control antibodies.

Enzyme-Linked Immunosorbent Assay (ELISA)

Lungs were homogenized in 2 mL PBS + 0.05% Tween 80. Homogenized tissue supernatants were filtered (0.22 μm). Cell culture supernatants were collected and assayed for cytokines. Cytokine production was measured by ELISA of mouse TNF-α and IL-6 (ExCell Bio, China) according to the manufacturer's protocol.

Real-Time PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's recommendations. For mRNA, first-strand cDNA synthesis was performed using RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific). An Eppendorf Master Cycle Realplex2 and SYBR Green PCR Master Mix (Applied Biosystems, USA) were used for real-time PCR (40 cycles). PCR products were then separated by electrophoresis through a 1% agarose gel and were visualized by being stained with ethidium bromide. The forward primer and reverse primer for mTNF-α were 5′-CACAGAAAGCATGATCCGCGAC-3′ and 5′-TGCCACAAGCAGGAATGAGAAGAG-3′. The forward primer and reverse primer for mIL-6 were 5′-GTCCGGAGAGGAGACTTCAC-3′ and 5′-CTGCAAGTGCATCATCGTTGT-3′. The forward primer and reverse primer for mβ-Actin were 5′-GATTACTGCTCTGGCTCCTAGC-3′ and 5′-GACTCATCGTACTCCTGCTTGC-3′.

Western Blotting

Cells were washed three times with ice-cold PBS and then lysed in lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich, USA), and 1 mM DTT. Equal amounts (20 mg) of cell lysates were resolved using 8–15% polyacrylamide gels transferred to PVDF membrane. Membranes were blocked in 5% non-fat dry milk in PBST and incubated overnight with the respective primary antibodies at 4°C. These respective primary antibodies list are as follows: Phospho-NF-κB p65 (Ser536) (Clone: 93H1; CST, USA), NF-κB p65 (Clone: D14E12; CST), Phospho-Akt (Ser473) (Clone: D9E; CST), Akt (Clone: C67E7; CST), Phospho-p38 MAPK (Thr180/Tyr182) (Clone: D3F9; CST), p38 MAPK (Clone: D13E1; CST), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Clone: D13.14.4E; CST), p44/42 MAPK (Erk1/2) (Clone: 137F5; CST), Phospho-JNK (Thr183/Tyr185) (Clone: G9; CST), SAPK/JNK (CST), GAPDH (Clone: D16H11; CST), PPARγ (Clone:81B8; CST), and SUMO-1 (Clone: C9H1, CST). The membranes were incubated at room temperature for 1 h with appropriate HRP-conjugated secondary antibodies and visualized with Plus-ECL (PerkinElmer, CA, USA) according to the manufacturer's protocol.

Oxygen Consumption Rate (OCR) Analysis

Mitochondrial OCR in intact cells was measured using the XF-24 analyzer (Seahorse Bioscience, USA) as described in the manufacturer's instructions. Briefly, BMDMs were seeded into XF-24 microplates and then maintained at 37°C in a non-CO_2 incubator for at least 1 h before assay. ATP turnover and maximal uncoupled OCRs were determined by treating the cells with oligomycin (1 mmol/L) (Sigma-Aldrich) or carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 1 mmol/L) (Sigma-Aldrich), respectively. Rotenone and antimycin A (1 mmol/L each) (Sigma-Aldrich) were used to inhibit complex 1- and complex 3-dependent respiration. OCR was normalized to protein content. Each experimental condition was analyzed using four to six biological replicates.

Lipid Body Staining and Enumeration

Cells and infection were performed as above. The cells were fixed with 4% paraformaldehyde for 5 min, stained with 0.5% Oil red O for 30 min at room temperature, and counterstained with hematoxylin to stain nuclei. Cells were rinsed with PBS, mounted on glass slides, and imaged. Lipid bodies ere enumerated by light microscopy with a 3,100 objective lens for 50 consecutive macrophages on each slide.

Co-Immunoprecipitations

Bone marrow-derived macrophages were seeded on 100-mm dishes at 1×10^6 cells per dish. Cells were treated with VB1 for 24 h. Cells were lysed in 1% digitonin (Calbiochem) buffer (20 mM Tris–HCl, 150 mM NaCl, 1% digitonin) containing protease inhibitors (Roche). Cleared supernatants were incubated with 10 mg of anti-PPAR-γ antibody, followed by incubation with immobilized protein G (Pierce). The beads were washed four times by 1% digitonin lysis buffer and immunoprecipitates were eluted with SDS sample buffer by boiling for 5 min.

In Vitro MTB Killing Assay

Bone marrow-derived macrophages were allowed to adhere to 12-well flat bottom plates at 5×10^5 cells per well and infected with MTB H37Rv at an MOI of 5 for 1 h at 37°C with 5% CO_2, then wells were extensively washed with pre-warmed PBS to remove non-adherent bacteria. The cells were incubated at 37°C

with 5% CO_2 for indicated time, and then were lysed in 1 mL of distilled water. Bacterial burden was determined by plating serial dilutions onto 7H10 agar plates supplemented with 10% OADC. Plates were incubated at 37°C in 5% CO_2 for 3 weeks before counting colonies. All infections were performed in triplicate.

Statistics

All experiments were performed at least twice. When shown, multiple samples represent biological (not technical) replicates of mice randomly sorted into each experimental group. No blinding was performed during animal experiments. Determination of statistical differences was performed with Prism 5 (Graphpad Software, Inc.) using unpaired two-tailed *t*-tests (to compare two groups with similar variances), or one-way ANOVA with Bonferonni's multiple comparison test (to compare more than two groups).

RESULTS

VB1 Led to Decreased Mycobacterial Growth in Mice *via* Regulating Function of Macrophages

Because the role of VB1 in antibacterial growth was unclear, we first investigated whether VB1 might affect MTB infection *in vivo*. We infected wild-type mice with MTB H37Rv and orally administrated either VB1, isoniazid (INH), or water (control group, Ctrl) into the infected mice for 1, 2, and 4 weeks, followed by measurement of MTB burden in the lungs and spleens of infected mice. As expected, INH treatment greatly reduced MTB CFU in mice at all the times examined. Importantly, MTB CFUs were constantly lower in VB1-treated mice than control mice at all indicated time points (**Figure 1**), suggesting that VB1 treatment enhanced containment of MTB growth was significantly suppressed by VB1 treatment.

We next wanted to explore how VB1 promoted an effective immune response against MTB infection. Because VB1 treatment reduced MTB burden at 1 week after infection when adaptive immunity has not been activated during MTB infection (21), we hypothesized that VB1 might affect innate immune immunity against MTB. Since macrophages and neutrophils are the major types of immune cells that kill and eliminate MTB at an early stage of infection, we determined the percentage of immune cells in the lungs of MTB-infected mice treated with VB1 or water 7 days after infection by FACS analysis. We found that VB1 had no effect on the percentages and numbers of $CD11b^+Gr1^-$ monocyte–macrophages and $CD11b^+Gr1^+$ polymorphonuclear cells in lungs from MTB-infected mice (**Figures 2A,B**; Figure S1A in Supplementary Material). However, we found that macrophages showed increased expression levels of CD86 and MHC-II, which are characteristic features of classically activated macrophages, but decreased expression levels of CD206, which is the characteristic feature of alternatively activated macrophages, from VB1-treated mice infected with MTB H37Rv compared

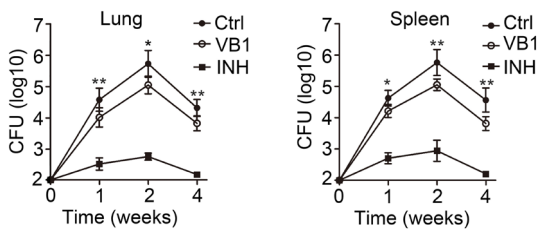

FIGURE 1 | The anti-bacillus effect of vitamin B1 (VB1) in mice with *Mycobacterium tuberculosis* infection. C57BL/6J mice were infected with H37Rv (~200 bacteria/mouse). Oral administration with water (Ctrl), VB1, and INH (n = 15 mice/group) was started from the day after infection (day 1) and continued for 1, 2, and 4 weeks alternatively. The lungs and spleens were analyzed at indicated time. Colony-forming units (CFUs) were obtained from the lung and spleen cell lysates by serial dilution and plating on 7H10 agars in triplicate. The colonies were counted after 4 weeks. Data shown are the mean ± SD. *P < 0.05 and **P < 0.01. Data are representative of three independent experiments with similar results.

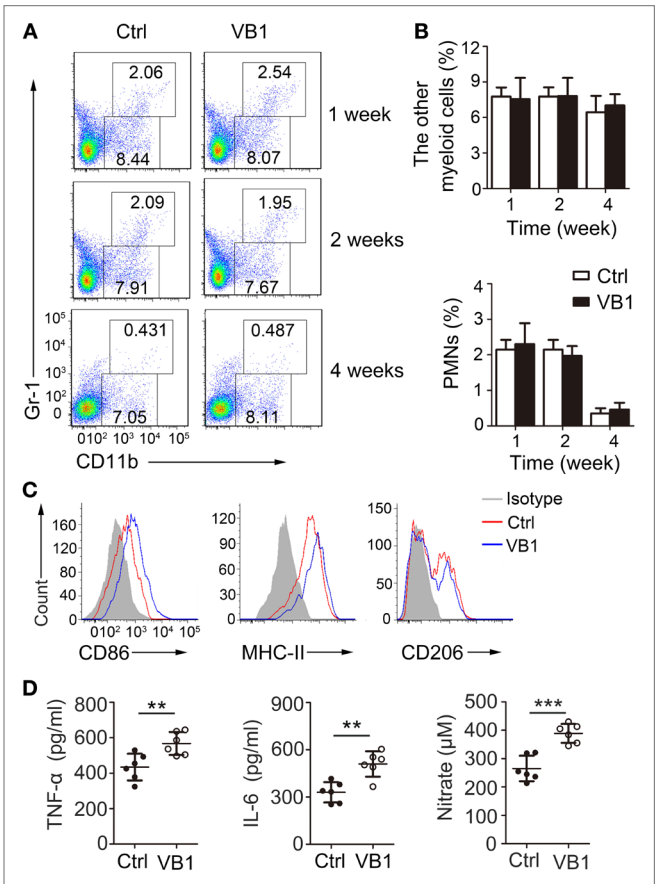

FIGURE 2 | The pro-inflammatory effect of vitamin B1 (VB1) in lungs of *Mycobacterium tuberculosis* (MTB)-infected mice. Lung cells from H37Rv-infected mice treated with VB1 or untreated were harvested at 1 week, 2 weeks, and 4 weeks after infection. **(A)** The percentage of myeloid cells is displayed as dot plots. **(B)** The percentages of other myeloid cells and polymorphonuclear cells in lungs were shown. **(C)** The expressions of CD86, MHC-II, and CD206 were detected *via* flow cytometry at 4 weeks. **(D)** Concentration of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) in lungs (homogenized in 2 mL PBS and 0.05% Tween 80) from mice with MTB infection at 1 weeks were detected by enzyme-linked immunosorbent assay. Data shown are the mean ± SD. **P < 0.01 and ***P < 0.001. Data are representative of three independent experiments with similar results.

to those obtained from control mice (**Figure 2C**; Figure S1B in Supplementary Material). The increased levels of TNF-α, IL-6, and nitrate were detected in lung homogenates of VB1-treated mice (**Figure 2D**). Together these observations indicate that VB1 could regulate the functions of macrophages to promote the protective immune response of the mice during MTB infection.

VB1 Promoted the Innate Immune Response of Macrophages After Mycobacterial Infection *In Vitro*

To determine if VB1 affected the function of macrophages directly during MTB infection, we pretreated BMDMs with VB1 *in vitro* for 24 h, followed by MTB H37Rv infection. VB1-treated BMDMs displayed enhanced upregulation of CD86 and MHC-II expression (**Figure 3A**; Figure S2A in Supplementary Material), increased TNF-α and IL-6 expression at both mRNA (**Figure 3B**) and protein (**Figure 3C**) levels, and increased nitrate production (Figure S2B in Supplementary Material) compared with PBS-treated group, indicating enhanced M1 polarization. By contrast, CD206 expression and the activity of arginase-I were decreased in VB1-treated BMDMs at 24 h after MTB infection (**Figure 3A**; Figure S2B in Supplementary Material), suggesting reduced M2 polarization. Overall, these results demonstrate that VB1 promotes classically activated polarization and pro-inflammatory cytokine production of macrophages after mycobacterial infection *in vitro*.

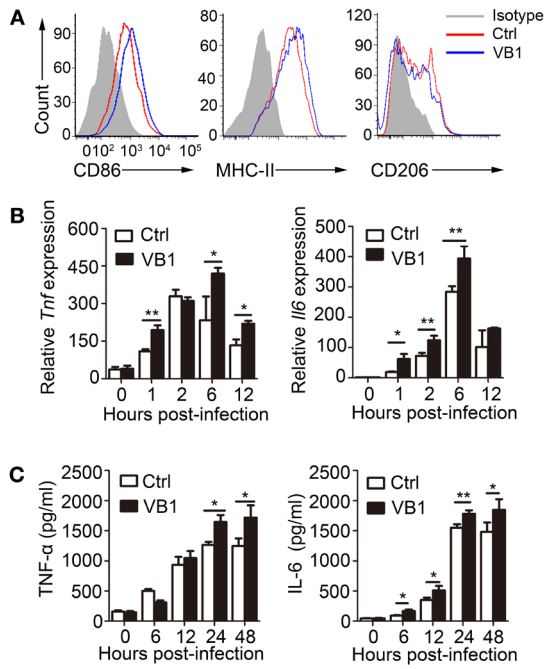

FIGURE 3 | The pro-inflammatory effect of vitamin B1 (VB1) on bone marrow-derived macrophages (BMDMs) after *Mycobacterium tuberculosis* (MTB) infection. BMDMs were pretreated with VB1 or phosphate buffer saline (Ctrl) for 24 h followed by MTB H37Rv infection (MOI 5). **(A)** Expressions of CD86, MHC-II, and CD206 were detected *via* flow cytometry after infection at 24 h. **(B)** Tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) mRNA expression determined by real-time PCR. **(C)** TNF-α and IL-6 secretion for indicated time points was measured by enzyme-linked immunosorbent assay. Data shown are the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are representative of three independent experiments with similar results.

VB1 Regulates the NF-κB Signal After Mycobacterial Infection

Polarization and cytokine expression of macrophages are known to be regulated by NF-κB-, PI3K-AKT-, and MAPK- (ERK1/2, p38, and JNK) dependent signaling pathways. We evaluated the role of VB1 in regulating these signaling pathways during mycobacterial infection. We found that phosphorylation of NF-κB p65 was enhanced but phosphorylation of AKT appeared reduced in VB1-treated macrophages (**Figure 4A**; Figure S3 in Supplementary Material). However, VB1 treatment did not obviously affected ERK1/2, c-Jun N-terminal kinase (JNK), or p38 phosphorylation (**Figure 4A**; Figure S3 in Supplementary Material). These results demonstrate that VB1 mainly regulates the NF-κB signal in macrophages after mycobacterial infection.

VB1 Regulated Mitochondrial Respiration and Lipid Metabolism in Macrophages

Vitamin B1 is reported to be indispensable for metabolism in its active form thiamine pyrophosphate. Furthermore, a previous report suggested that metabolic signaling regulates inflammatory signaling. Therefore, we examined whether VB1 regulated NF-κB signal by mediating the metabolism in macrophages. First, we investigated whether VB1 affected the metabolism of macrophages. Indeed, basal and ATP-dependent OCR readings indicated that BMDMs treated with VB1 had significantly higher OCR than untreated group (**Figure 4B**). In addition, VB1-treated BMDMs contained less lipid bodies than control BMDMs (**Figure 4C**). Thus, VB1 increases mitochondrial respiration and lipid metabolism in macrophages. Because fatty acids have been shown to modulate the regulation of innate immune response, our data suggest that VB1 may impact macrophage function after MTB infection by regulating lipid metabolism.

VB1-Mediated Innate Immune Responses Are Mediated Through Modulation of PPAR-γ Signaling

PPARs integrate metabolic and inflammatory signaling in macrophages (19). PPAR-γ, in particular, is known to function as an important "molecular switch" in regulating macrophage immune responses to MTB (20). PPAR-γ, activated by it endogenous ligands, such as polyunsaturated fatty acids or fatty acid derivatives, sequesters the p65 subunit of NF-κB complex and prevents NF-κB-dependent regulation of genes (19). To determine whether VB1 regulation of the NF-κB signal depends on PPAR-γ, we used rosiglitazone, an agonist of PPAR-γ, to activate PPAR-γ after VB1 treatment in macrophages with H37Rv infection. We found that rosiglitazone neutralized the activation of NF-κB p65 induced by VB1 (**Figure 5A**; Figure S4 in Supplementary Material). The PCR products of IL-6 and TNF-α were no longer increased in VB1-treated BMDMs after rosiglitazone addition (**Figure 5B**). Furthermore, increased CD86 and MHC-II expression induced by VB1 were abrogated after rosiglitazone treatment, while CD206

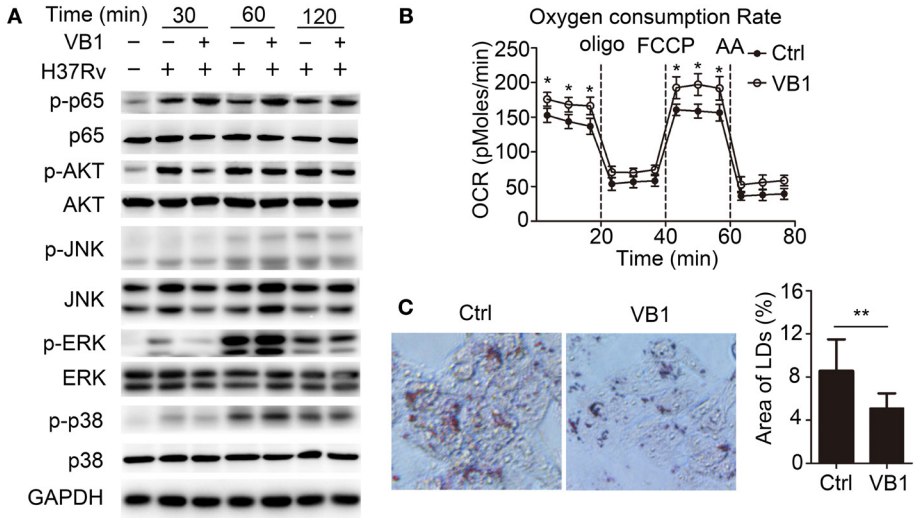

FIGURE 4 | The role of vitamin B1 (VB1) in regulating signaling pathways and mitochondrial respiration. Bone marrow-derived macrophages (BMDMs) were pretreated with VB1 or phosphate buffer saline (Ctrl) for 24 h followed by *Mycobacterium tuberculosis* H37Rv infection (MOI 5). **(A)** Western blot analysis of the phosphorylation status of p65, AKT, JNK, ERK, and p38. GAPDH is as an internal control. These results are from a representative experiment ($n = 3$). **(B)** Oxygen consumption rate (OCR) of BMDMs. O_2 consumption was normalized to protein content. oligo, oligomycin; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; AA, antimycin A. **(C)** BMDMs were stained with Oil Red O to stain the lipid droplets. Staining was assessed by bright field microscopy and Image J software was used to calculate the total area of Oil Red O-stained droplets per cell. Original magnification ×600. Values are mean ± SD of all cells in 20 randomly selected micrographs from each group. $^*P < 0.05$ and $^{**}P < 0.01$. Data are representative of three independent experiments with similar results.

expression was recovered after rosiglitazone treatment (**Figure 5C**; Figure S5A in Supplementary Material). Likewise, the amount of nitrate and activity of arginase-I would be not different between VB1-treated and mix of VB1 and rosiglitazone-treated groups (Figure S5B in Supplementary Material). A previous study showed that ligand-dependent SUMOylation mediates the initial step of transrepression of inflammatory response genes by PPAR-γ (22). Our results showed that the expression of PPAR-γ was not changed after VB1 treatment in BMDMs (**Figure 5D**—input), but SUMOylation of PPAR-γ was suppressed (**Figure 5D**—IP; Figure S6A in Supplementary Material).

To assess whether the regulatory function of VB1 in macrophages is dependent on PPAR-γ, we generated and analyzed BMDMS from PPAR-$\gamma^{fl/fl}$-Lyz2-Cre mice. We found that phosphorylation level of NF-κB p65 was enhanced in BMDMs from PPAR-γ-Lys2-cre mice after MTB H37Rv infection, but it was not different between VB1-treated and control groups (**Figure 5E**; Figure S6B in Supplementary Material). Similarly, TNF-α and IL-6 mRNA levels were increased in PPAR-γ-deficient BMDMs, but they were not further increased with VB1 treatment (**Figure 5F**). These results suggest that VB1-mediated innate immune responses are dependent on PPAR-γ.

VB1 Limits Mycobacterial Growth in Macrophages

The data we have shown thus far indicated that VB1 regulated functions of macrophages in a PPAR-γ-dependent manner. We further examined the role of VB1 in inhibiting MTB H37Rv growth in macrophages. Treatment of BMDMs with VB1 during MTB H37Rv infection reduced mycobacterial CFU inside the cells 1, 2, and 3 days post-infection (**Figure 6A**). The number of intracellular viable bacilli in mock-treated BMDMs was approximately 1.5-fold greater than in VB1-treated cells on 3 days post-infection (**Figure 6A**). However, BMDM survival was not obviously affected by VB1 treatment (Figure S7A in Supplementary Material). Such negative effects of VB1 on MTB growth/survival was diminished when BMDMs were simultaneously treated with rosiglitazone (**Figure 6B**). Moreover, BMDMs pretreated with VB1 showed similar abilities of phagocytosis of fluorescently labeled MTB H37Rv, suggesting that VB1 had no major effects on cell association of MTB H37Rv with macrophages (Figure S7B in Supplementary Material).

To investigate if VB1 exerted its protective role in a PPAR-γ-dependent manner, we examined MTB H37Rv burden in the lung after oral administration of VB1 with or without rosiglitazone for 2 weeks. We found that rosiglitazone treatment increased MTB burden in the lung and diminished the protective effect of VB1 on MTB *in vivo* (**Figure 6C**). Furthermore, mice with PPAR-γ-deficiency in myeloid cells showed stronger antibacterial ability than WT control mice, but VB1 did not additively enhance protective immune responses in PPAR-γ-deficiency mice against MTB (**Figure 6D**). Collectively, these results indicate that VB1 is instrumental in limiting growth of intracellular mycobacteria in macrophages and *in vivo* that is dependent on proper PPAR-γ activity.

DISCUSSION

Since the development of anti-tuberculosis drugs, adjunct tuberculosis therapies, including therapeutic vaccines, vitamin

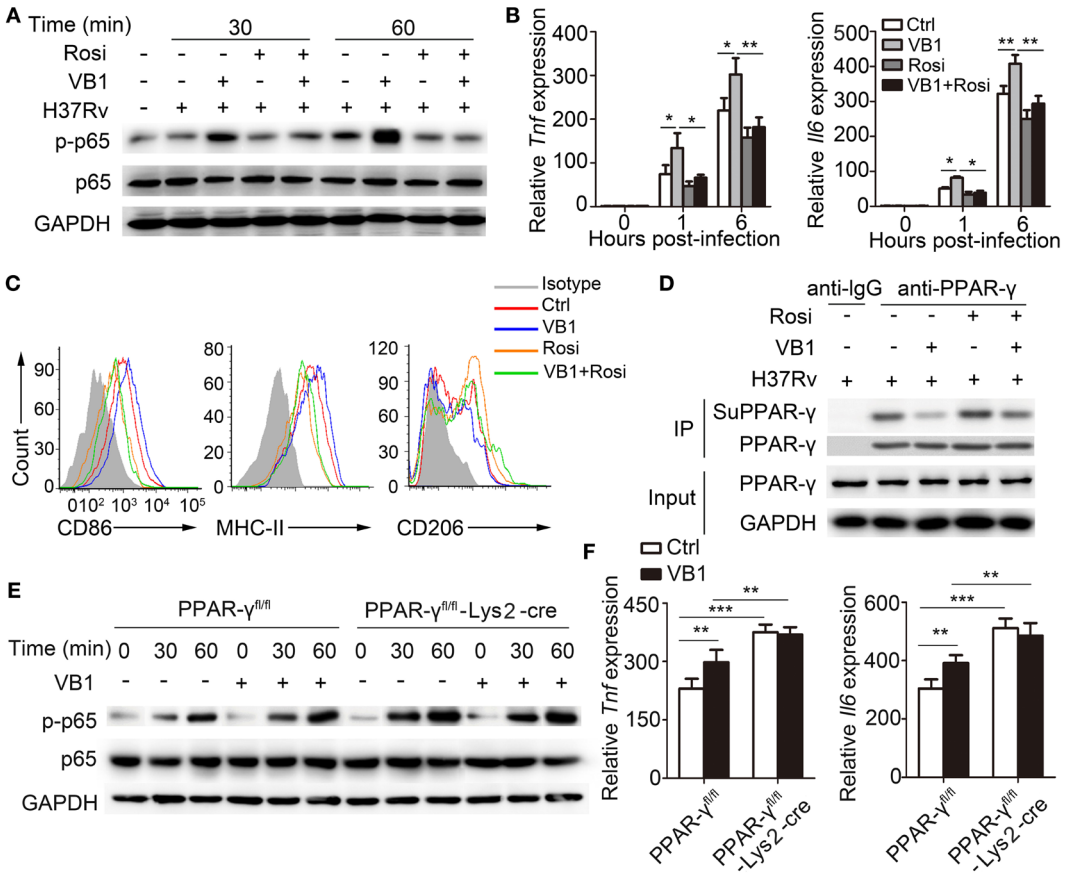

FIGURE 5 | Vitamin B1 (VB1) promoted the innate immune response *via* suppressing SUMOylation of peroxisome proliferator-activated receptor (PPAR-γ). Bone marrow-derived macrophages (BMDMs) were pretreated with phosphate buffer saline, VB1, Rosi, or mixture of VB1 and Rosi for 24 h and then challenged with *Mycobacterium tuberculosis* H37Rv (MOI 5) for indicated time. **(A)** Western blot analysis of the phosphorylation status of p65. GAPDH is as an internal control. **(B)** Tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) mRNA expression determined by real-time PCR. **(C)** The expressions of CD86, MHC-II, and CD206 were detected *via* flow cytometry after infection at 24 h. **(D)** Immunoprecipitation and immunoblots were performed with indicated antibodies. **(E)** Western blot analysis of the phosphorylation status of p65 in BMDMs from PPAR-$\gamma^{fl/fl}$ and PPAR-$\gamma^{fl/fl}$-Lys2-cre mice. GAPDH is as an internal control. **(F)** TNF-α and IL-6 mRNA expression determined by real-time PCR in BMDMs from PPAR-$\gamma^{fl/fl}$ and PPAR-$\gamma^{fl/fl}$-Lys2-cre mice. Data shown are the mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Data are representative of three independent experiments with similar results.

supplementation, and/or repurposing of drugs targeting biologically and clinically relevant molecular pathways, have received considerable attention (13, 15). Previous studies showed that thiamine pyrophosphate, a VB1 derivative, protects retinal tissues from ethambutol-induced oxidative damage (17), that VB1 inhibits the production of cytokines and increases the anti-inflammatory activity of a corticosteroid in a chronic model of inflammation induced by complete Freund's adjuvant (23), and that VB1 reduced serum pro-inflammatory cytokines in adjuvant-induced arthritis and DEN-induced hepatic cancer (24, 25). However, no study examining the immune regulatory mechanism of VB1 in MTB infection has been reported. We observed increased TNF-α in lung homogenate of VB1-treated mice with MTB infection, which did not agree with the results of previous studies. We also found that VB1 supplementation promoted NF-κB signaling and IL-6 and TNF-α production in macrophages. Thus, the function of VB1 in different cell types may differ. Further studies are required to clarify this possibility.

A hallmark of MTB infection is the differentiation of infected macrophages into lipid-rich foam cells (26). These cells accumulate lipid droplets, which are lipid storage organelles required for intracellular bacillary growth (27). A previous study showed that VD treatment abrogates infection-induced accumulation of lipid droplets of infected macrophages and is beneficial for suppressing the growth of MTB in macrophages (28). Thiamine pyrophosphate, the active form of VB1, is a cofactor present in all living systems and is indispensable for metabolism (29). Our results showed that VB1 supplementation in macrophages increased mitochondrial respiration and lipid metabolism. Thus, it is possible that VB1 exerts its protective roles during MTB infection by affecting macrophage lipid metabolism.

Peroxisome proliferator-activated receptor-γ integrates metabolic and inflammatory pathways (19) and functions as an important "molecular switch" in regulating macrophage immune responses to MTB (20, 30). In this study, we found that VB1

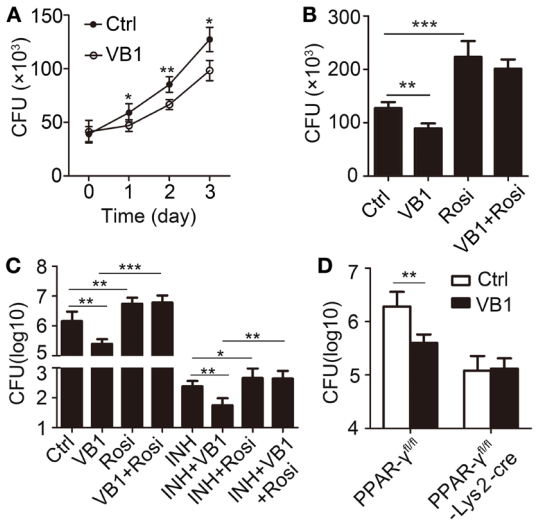

FIGURE 6 | The anti-bacillus effect of vitamin B1 (VB1) in macrophages. **(A,B)** Bone marrow-derived macrophages were pretreated with phosphate buffer saline, VB1, Rosi, or mixture of VB1 and Rosi for 24 h and then challenged with *Mycobacterium tuberculosis* H37Rv (MOI 5) for 1 h. **(A)** Intracellular viable bacteria were detected with colony-forming unit (CFU) assays at 0, 1, 2, 3 day post-infection. **(B)** Intracellular viable bacteria were detected with CFU assays at 72 h post-infection. **(C,D)** PPAR-$\gamma^{fl/fl}$ or PPAR-$\gamma^{fl/fl}$-Lys2-cre mice were infected with H37Rv (~200 bacteria/mouse). Oral administration with water (Ctrl), VB1, INH, or Rosi (n = 5 mice/group) was started from the day after infection (day 1) and continued for 2 weeks. CFUs were obtained from the lung cell lysates by serial dilution and plating on 7H10 agars in triplicate. The colonies were counted after 4 weeks. **(C)** C57BL/6J mice. **(D)** PPAR-$\gamma^{fl/fl}$ or PPAR-$\gamma^{fl/fl}$-Lys2-cre mice. Data shown are the mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Data are representative of three independent experiments with similar results.

regulated the NF-κB signal in a PPAR-γ-dependent manner. VB1 supplementation suppressed the activation of PPAR-γ, whereas an agonist of PPAR-γ neutralized the antimycobacterial effect of VB1. This is consistent with the results of a previous study showing that activation of PPAR-γ decreased TNF production and promoted the intracellular growth of MTB (31, 32). In addition, we found that VB1 prompted the polarization of macrophages into M1 macrophages (classically activated macrophages) *in vitro* and *in vivo*, which is beneficial for suppressing mycobacteria growth (33). A previous study showed that PPAR-γ has a vital role in the polarization of macrophages with MTB infection (31, 34). In agreement with their study, we found that the agonist of PPAR-γ reduced the increased CD86 and MHC-II expression but promoted CD206 expression.

Taken together, these findings indicate that VB1 can significantly inhibit MTB growth *in vitro* and *in vivo* by regulating innate immunity. VB1 may exert its immune regulation function during MTB infection *via* multiple mechanisms that include modulating NFκB signaling and lipid metabolism. We suggest that, in clinical trials involved with VB1 supplement, the immune regulation effects of VB1 should be monitored.

ETHICS STATEMENT

All animal experiments in this study were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental protocols were reviewed and approved by the Medical Ethics Board and the Biosafety Management Committee of Southern Medical University (approval number L2015123).

AUTHOR CONTRIBUTIONS

SH and LM designed research; SH, WH, XD, YF, YY, CH, SL, QSW, and YH conducted research; SH, WH, XZ, CZ, QW, and LM analyzed data; SH, X-PZ, and LM wrote the paper. LM had primary responsibility for final content. All authors read and approved the final manuscript.

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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.2018.01778/full#supplementary-material.

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METHODS
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The Deconstructed Granuloma: A Complex High-Throughput Drug Screening Platform for the Discovery of Host-Directed Therapeutics Against Tuberculosis

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Mycobacterium tuberculosis (Mtb) continues to be a threat to Global Public Health, and its control will require an array of therapeutic strategies. It has been appreciated that high-throughput screens using cell-based assays to identify compounds targeting Mtb within macrophages represent a valuable tool for drug discovery. However, the host immune environment, in the form of lymphocytes and cytokines, is completely absent in a chemical screening platform based on infected macrophages alone. The absence of these players unnecessarily limits the breadth of novel host target pathways to be interrogated. In this study, we detail a new drug screening platform based on dissociated murine TB granulomas, named the Deconstructed Granuloma (DGr), that utilizes fluorescent Mtb reporter strains screened in the host immune environment of the infection site. The platform has been used to screen a collection of known drug candidates. Data from a representative 384-well plate containing known anti-bacterial compounds are shown, illustrating the robustness of the screening platform. The novel deconstructed granuloma platform represents an accessible, sensitive and robust high-throughput screen suitable for the inclusive interrogation of immune targets for Host-Directed Therapeutics.

Keywords: tuberculosis, pulmonary, *Mycobacterium tuberculosis*, high-throughput screening assays, host-directed therapeutics, macrophages

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INTRODUCTION

Mycobacterium tuberculosis, the causative agent of human tuberculosis (TB), poses an ongoing threat to human health. The escalating incidence of drug resistant strains provides a new impetus to understand this complex pathogen and to identify novel therapeutics. Ginsberg and Spigelman (Ginsberg and Spigelman, 2007) list the goals and challenges for improved TB therapy, one of which is to "discover and develop new drugs that have novel mechanisms of action and are effective against persistent bacilli," as well as to "develop new preclinical approaches to identify optimized drug combinations…." As a first step toward these aims, we have directed our efforts to developing improved drug screening tools. Traditional drug discovery programs for the identification of

new anti-microbial compounds have focused on either target-based screening against enzymatic activities that have been shown genetically to be essential for bacterial survival (Mdluli and Spigelman, 2006; Lamichhane, 2011), or phenotypic screens exploring bacterial survival in rich broth (Collins and Franzblau, 1997; Pethe et al., 2010). Both approaches have had limited success in recent times, calling into question whether we should continue to devote our major efforts down these paths. Whilst complicated, the most frequently cited reasons for the failure of these approaches are limited drug permeability in the former approach, and the inappropriate nature of rich bacterial broth to mirror the physiology within the host in the latter approach (Koul et al., 2011; VanderVen et al., 2015; Zuniga et al., 2015).

There is also the sense that the "low-hanging fruit" have already been picked, and compounds with known modes of action continue to re-emerge from ongoing screens. For these reasons we feel that it is critical to develop new, innovative approaches to drug discovery that explore and exploit the host-derived pressures on *Mycobacterium tuberculosis* (Mtb) to identify compounds with enhanced efficacy within the host.

Host-direct therapy (HDT) is an emerging theme in the treatment of infectious diseases; small molecules are utilized to modulate or perturb host responses, to reduce replication or persistence of pathogens, to limit tissue damage, and to enhance the efficacy of current treatments (Kim and Yang, 2017; Kolloli and Subbian, 2017). Current HDT approaches for Mtb target an array of pathways, including: disintegration of granuloma structure, autophagy, anti-inflammatory response, and, more recently, checkpoint blockage to unleash the immune suppression induced by Mtb infection in the lung (Kolloli and Subbian, 2017; Kaufmann et al., 2018). However, our knowledge of immune protection against TB is heavily reliant on studies based on failed immunity in genetically-modified animals and catastrophic human genetic lesions, and this has likely limited the development of both vaccines and new host-dependent therapies (Huang and Russell, 2017). Recent work has indicated that, in addition to immune-mediated control, host macrophage ontogeny also plays a major role in expansion of bacterial numbers and disease progression (Huang et al., 2018). Furthermore, the efficacy of existing anti-tuberculosis drugs *in vivo* is markedly different from their *in vitro* activity, and is impacted by the immune status of the host tissue and cellular environment (Liu et al., 2016; Russell, 2017). These layers of host-dependent complexity represent an increased challenge to development of effective new drugs, but they also represent an un-mined opportunity if we can develop appropriate *in vitro* screens that incorporate this biology. Therefore, to accelerate anti-TB drugs and HDT discovery, we need a discovery platform with minimal assumptions that provides a more holistic representation of the complex biology of the infected host.

With this aim in mind we have developed a high throughput screening (HTS) assay utilizing cells recovered from Mtb-infected mouse lungs (*D*econstructed *G*ranuloma, DGr), which we are using to screen compounds with efficacy against Mtb within the context of host-derived immune cells. These cells, which comprise mainly of resident alveolar macrophages, monocyte-derived macrophages, neutrophils and T- and B-lymphocytes, have been recruited and educated by the infection environment and therefore reflect the host immune pressures active within the TB granuloma. The DGr platform has been adapted to an HTS-compatible, 384-well format and generates a robust reproducible readout with an extensive dynamic range. We believe that this novel HTS platform will reveal new avenues toward the manipulation of the host immune environment to restrict bacterial growth and survival, and to act in concert with existing drug treatments and increase their *in vivo* potency.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. Mice were used at 6–8 week old. All mice were maintained in a specific pathogen-free biosafety level-3 facility at Cornell University. Animal care was in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Mtb Cultivation

Mtb strains were cultured in Middlebrook 7H9 liquid medium supplemented with 10% v/v OADC (oleic acid, bovine serum albumin, D-glucose, catalase, sodium chloride; Becton Dickinson) and 0.05% w/v Tyloxapol, or on solid Middlebrook 7H10 agar supplemented with 10% v/v OADC at 37°C, with 25 μg/ml kanamycin or 30 μg/ml hygromycin B where appropriate. Cultures for HTS were grown for 5 days in 100 ml roller cultures (7H9/OADC/Tyloxapol).

Mtb Reporter Strains

The Erdman background of Mtb was used to generate all reporter strains, this is a reference strain commonly used for studies of pathogenesis. We chose to use two different fluorescent reporter strains for the initial infection (mCherry) and the *ex-vivo* challenge (mKO) to allow us to identify cells infected from each of the separate infections. We found that after incubation, following the *ex-vivo* challenge, the intensity of mKO signal increased, however the mCherry readings remained constant. We favor mKO as a reporter for the final plate assay over mCherry because it has a greater range/sensitivity and a lower background. (1) mCherry strain: For mouse infection we used a *smyc*'::mCherry strain, which has been previously described (Tan et al., 2013); mice were inoculated intranasally with ∼1,000 CFU of Erdman(*smyc*'::mCherry) in 30 μl PBS containing 0.05% Tween 80. The inoculum dosage was confirmed by plating different dilutions on 7H10 plates. Plates were incubated at 37°C and colonies enumerated after 3 weeks. (2) monomeric Kusabira Orange (mKO) strain: For the pilot HTS screen we created a tetracycline (tet) inducible mKO Mtb reporter strain, Erdman(P_{606}'::mKO-tetON), by introduction of a replicating plasmid containing the tet-ON regulator upstream of P_{606} driven mKO, in the destination Gateway vector pDE43-MEK. The fluorescent protein Kusabira Orange was originally isolated from

the stony coral *Fungia concinna* (Karasawa et al., 2004), and the mKO derivative used here was codon-optimized for Mtb by GenScript. (3) Dual mCherry-mKO strain: For imaging purposes we generated a dual reporter strain, Erdman(P_{606}'::mKO-tetON, *smyc*'::mCherry), which constitutively expressed the mCherry fluorescent protein under the *smyc* promoter (Tan et al., 2013), and also inducibly expressed mKO upon the addition of exogenous tetracycline, on the replicating Gateway plasmid pDE43-MEK backbone.

BMDM and J774 Preparation

BMDM were isolated from C57BL/6J mice and cultured in DMEM (Corning Cellgro) supplemented with 10% FBS (Thermo Scientific), 20% L929-conditioned media, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (Corning Cellgro) at 37°C, 5% CO_2, for 9 days prior to seeding for experiments. J774 cells were obtained from ATCC. Cells were maintained in DMEM +10% FBS + 2 mM L-glutamine + 1 mM sodium pyruvate (complete DMEM) + 1% penicillin/streptomycin at 37°C, 5% CO_2, until 90% confluence, in T300 flasks. Cells were scraped from the flasks into cold, sterile PBS, centrifuged at 700 rpm for 10 mins, and re-suspended at 1 x 10^6/ml in complete DMEM. 30 μl of the cell suspension was then seeded into each well of 384-well optically clear, black plates (~3×10^4 cells/well) and incubated overnight at 37°C, 5% CO_2.

Generation of Deconstructed Granuloma (DGr) Cell Suspensions

Mice were infected with Mtb, and sacrificed after 3 weeks. In the actual drug screen we process and pool cells from 5 infected mice to generate 6 × 384 well plates. The pooling of the granuloma cells minimizes variation and renders the screen much more reproducible from run to run by limiting mouse-to-mouse variation. Mouse lungs lungs were removed and placed into Miltenyi GentleMACS C-tubes, containing 5 ml lung dissociation buffer (5% FBS/PBS solution containing 250 U/ml collagenase IV and 20 U/ml DNase). Lungs were processed using the GentleMACS Lung 37C_m_LDK_1 program, with 1 set of lungs per tube. Cells were then pooled and re-suspended in 10 ml 5% FBS/PBS per set of lungs, passed through a 70 μm cell strainer, and then pelleted at 1,200 rpm for 10 min. 3 ml of ACK buffer (Lonza) was used to re-suspend the cell pellet and incubated at room temperature for 5 min to lyse red blood cells, following which 10 ml 5% FBS/PBS was added and the cell suspension passed through a 40 μm cell strainer. After a final spin at 1,200 rpm for 10 min the resulting pellet was re-suspended in 20 ml complete DMEM and cell counts performed. The cells were diluted to 3.33×10^6/ml in complete DMEM with Amphotericin B (final concentration 20 μg/ml). 30 μl of cells were seeded into each well of a 384 well optically clear, black plates (~10^5 cells/well). Lung cells were then followed by a secondary *ex vivo* infection with a desired fluorescent Mtb reporter strain at MOI 0.1 as detailed below.

Test Compounds

Test drugs were arrayed from a 2 mM stock solution in DMSO via acoustic dispense (ECHO 550 liquid handler; Labcyte Inc.) into 384-well Axygen P-384-120SQ-C-S plates, sealed and stored frozen prior to use. Each well contained a final volume of 500 nl 1.5 mM compound. Pre-spotted assay plates were thawed and 24.5 μl of incomplete DMEM was added to each well to give a final drug concentration of 30 μM/well. These compound plates were centrifuged at 1,000 rpm and stored at 4°C prior to addition to assay plates.

High-Throughput Screen Protocol

Erdman(P_{606}'::mKO-tetON) was grown for a week in 100 ml supplemented Middlebrook 7H9 broth with kanamycin. On the day of infection, 50 ml of culture was harvested by centrifugation at 3,000 rpm for 10 mins, re-suspended in 1 ml sterile cold basal uptake buffer (25 mM dextrose, 0.5% bovine serum albumin, 0.1% gelatin (Sigma), 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ in PBS), passed through a tuberculin needle 6 times, and re-suspended in complete DMEM media at a final OD_{600} of 0.2. DGr cells were then infected with 10 μl/well of this bacterial suspension using a Perkin Elmer Janus liquid handling robot. Plates were returned to incubator (37°C, 5% CO_2) for 1 hr to allow bacteria uptake by host cells. Following this incubation, the DGr cells received 10 μl /well complete DMEM with 60 μg/ml Amphotericin B (final concentration in assay 20 μg/ml) and 0.075 μg/ml isoniazid (INH), where appropriate (final concentration in assay 0.0125 μg/ml). Plates were returned to the incubator following this addition. Finally, test inhibitor compounds were transferred to the assay plates, with each well receiving 10 μl of 30 μM test compound (final compound concentration 5 μM). Plates were incubated at 37°C, 5% CO_2. On day 4, 10 μl of 1.4 μg/ml anhydrotetracycline (ATc) was added per well (final concentration 200 ng/ml). Assay plates were returned to the incubator at 37°C, 5% CO_2 for a further 3 days. On day 7, the fluorescence was read from the bottom on a Perkin Elmer Envision plate reader—excitation 530/8 nm, dichroic 555 nm, emission 579/25 nm.

The inclusion of Amphotericin B is a potential confounder because of its known immune-modulatory capacity (Hedges et al., 2015) but, because the lung is not a sterile environment, we needed to add the Amphotericin B as an antifungal. We have a series of counter screen assays to verify and validate hits from the primary screen therefore spurious positives will be removed from those compounds advanced in further analysis.

Flow Cytometry

Lung cells were incubated with Fc block (eBioscience) for 15 min. Cells were then counted and incubated for 30 min in the dark with fluorophore-conjugated antibodies. Fluorophore-conjugated mAb specific to mouse CD45 (30-F11; BD Biosciences), CD11b (M1/70; BD Biosciences), CD64 (X54-5/7.1; Biolegend), CX_3CR1 (SA011F11; BioLegend), Ly6G (1A8; BioLegend), MerTK (DS5MMER), SiglecF (E50-2440; BD Biosciences), CD4 (GK1.5), EpCAM (G8.8) were purchased from eBioscience unless otherwise indicated. Fixable viability dye was purchased from eBioscience. Cells were analyzed with a Becton Dickenson LSRII flow cytometer and data analyzed with FlowJo software (Tree Star).

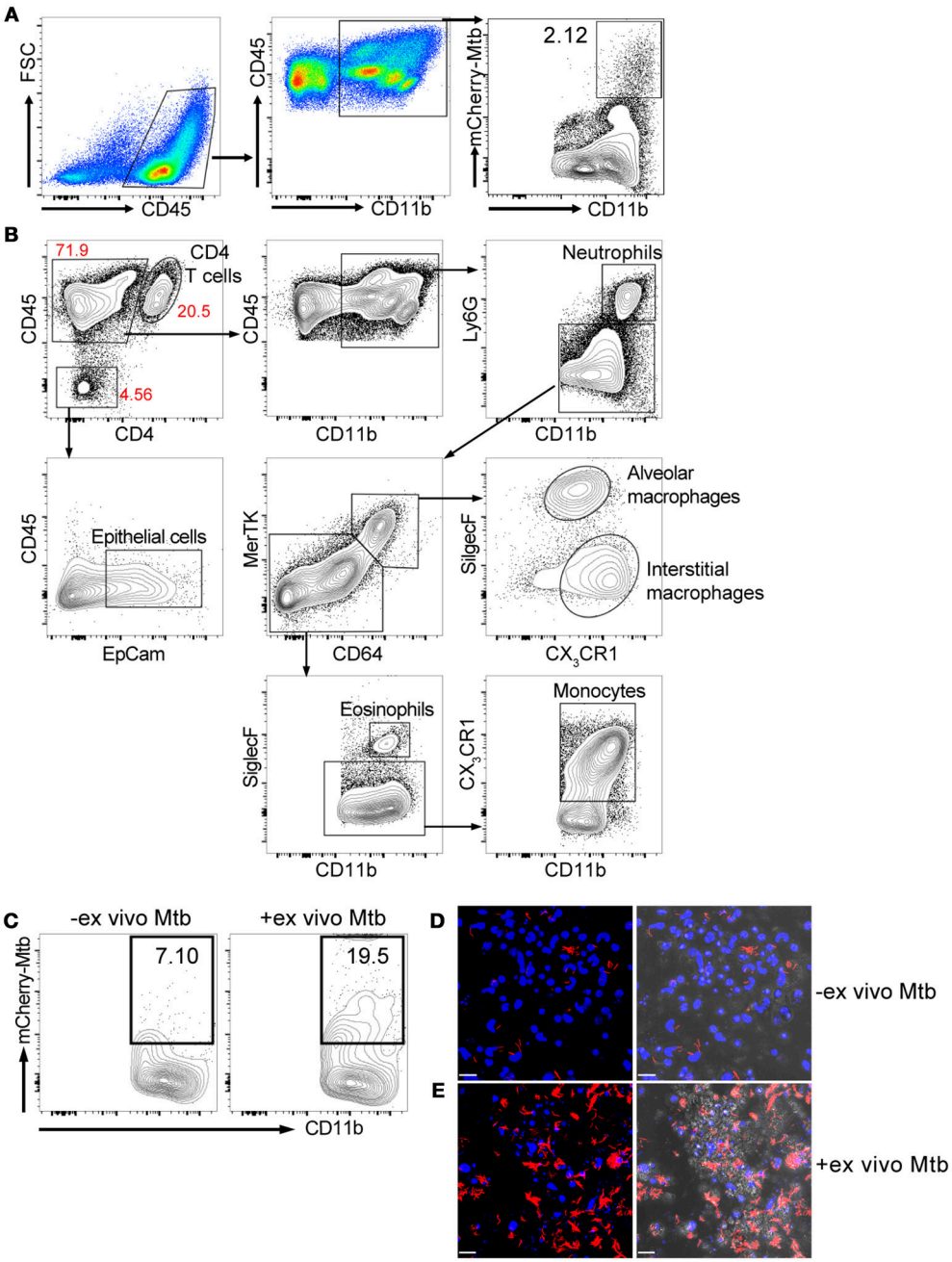

FIGURE 1 | *Ex vivo* challenge with the fluorescent Mtb strain increases the numbers of infected myeloid cells. Mice were infected with ~1,000 CFU Erdman(*smyc'*::mCherry) for 3 weeks. Lung single cell suspensions were isolated for the analysis. **(A)** Gating strategy and flow cytometric analysis of percentage of Mtb-infected myeloid cells ($CD45^+CD11b^+$) in the mouse lung after 3 weeks infection. **(B)** Multi-color flow cytometry analysis of myeloid populations in Mtb infected mouse lung at 3 weeks post infection. **(C)** Flow cytometry analysis of percentage of Mtb-infected lung myeloid cells before and after *ex vivo* challenge with mCherry Mtb at MOI 0.1 for 24 h. **(D,E)** Confocal images of cultured lung cells isolated from mCherry Mtb-infected mice without **(D)** or with **(E)** *ex vivo* challenge. The left panels of **(D,E)** show the fluorescent images and the right panels show the same fluorescent images overlaid with the brightfield images. Scale bar = 25 μm in **(D,E)**. The experiments were repeated at least two times.

Confocal Microscopy

Cells were imaged using a Leica SP5 confocal microscope and images were exported and analyzed using Volocity software (PerkinElmer).

Statistical Analysis

Results were statistically analyzed using Student's *t*-test, or one-way ANOVA test with multiple comparisons where appropriate, with Prism 6.0 (GraphPad Software).

RESULTS AND DISCUSSION

Increased Number of Infected Myeloid Cells After Challenge With Fluorescent Mtb

We isolated lung cells from C57BL/6J mice challenged with 1000 CFU of Erdman(*smyc'*::mCherry) for 3 weeks, to examine the distribution of mCherry Mtb in the infected mouse lung, and investigate whether the fluorescent bacterial signal in the isolated lung cells could be detected by a plate reader. Three weeks post infection was chosen because this is the transition period from innate immunity to the initiation of adaptive immunity in the lung during Mtb infection (Urdahl, 2014). We found that although Mtb predominately infects $CD45^+CD11b^+$ myeloid cells, only 2.12% of the $CD45^+CD11b^+$ cells carried Mtb (**Figure 1A**). We also developed a comprehensive multi-color flow cytometry analysis and further revealed different myeloid populations in the Mtb infected murine lung, including neutrophils, monocytes, alveolar and interstitial macrophages (**Figure 1B**). The tissue homogenization process releases these immune cells preferentially, and we observe only a relatively modest contribution from epithelial lung cells, fewer than 5% CD45- cells, in the resulting suspension (**Figure 1B**). Unfortunately, mCherry fluorescence was below the level of detection by the plate reader due to the low penetrance of bacteria in the lung myeloid cell population. To overcome this issue, we subjected the cells to an *ex vivo* challenge with reporter Mtb to further enhance the signal. Indeed, the percentage of mCherry Mtb infected $CD45^+CD11b^+$ cells was significantly increased 3 days post *ex vivo* challenge (**Figure 1C**), and this was further confirmed by confocal microscopy (**Figures 1D,E**). We conclude that the lung myeloid cells isolated from Mtb-infected mice are highly phagocytic, enabling an *ex vivo* challenge to markedly increase the number of infected cells and enhance fluorescent signal.

Validation of Tet-ON Inducible Mtb Reporter Strain

Fluorescent reporter-based assays have been used extensively to test and screen antimicrobial compounds against mycobacteria (Stanley et al., 2012; VanderVen et al., 2015; Gupta et al., 2017). To achieve a higher sensitivity and an extended dynamic range, we constructed a dual inducible/constitutive fluorescent Mtb strain that constitutively expresses mCherry, and expresses mKO upon induction by tetracycline (or its derivative ATc), to enable the specific detection of viable bacteria. The rationale behind the construction of this strain is consistent with previous reports on the use of inducible fitness reporters in mycobacterium (Martin et al., 2012; Mouton et al., 2016). The performance of the new Mtb reporter strain was tested in murine bone marrow-derived macrophages (BMDM) and in J774 cells, a murine macrophage-like cell line. Both mCherry and mKO signal were readily detected by confocal microscopy upon ATc induction of infected cells. In contrast, mKO signal was completely absent in the presence of the anti-tuberculosis drug rifampicin (Rif) in both BMDM and J774 cells (**Figure 2A**). Analysis of the mKO fluorescence signal intensity with a plate reader demonstrates the robust dynamic range of the reporter strain in J774 cells, in the presence or absence of Rif (**Figure 2B**). This new inducible Mtb reporter strain therefore represents a highly sensitive tool suitable for HTS.

Deconstructed Granuloma Model in 96- and 384-Well Platforms

We next combined the *ex vivo* challenge with a inducible tet-ON mKO reporter to validate the assay in 96- and 384-well plates and test its performance in a HTS format. We created single cell suspensions of the lungs removed from mice infected for 3 weeks with mCherry Mtb. These cells include the large numbers of innate immune cells that infiltrate into the lung, along with the developing lymphocyte response. The mKO Mtb strain was added *ex vivo* to lung cell cultures established in 96- or 384-well plates. Both Rif and INH were included in the test plates in control wells. Upon ATc induction, mKO signal was detected by the plate reader in the control wells but not in the control wells containing anti-tuberculosis drugs (**Figure 3**). The magnitude of the response likely reflects both bacteria numbers and bacterial fitness (ability to detect and respond to ATc).

Analysis and Presentation of Data From the Primary Drug Screen

The DGr has been utilized in a single dose drug screen of 10,000 bioactive compounds, where each compound was tested at 5 μM against the tet-ON mkO strain. The raw fluorescent data from a representative 384-well plate is shown in **Figure 4A**. These data were transformed into z-scores, calculated as $z = x - m/s$ where $m =$ median of sample plate, and $s =$ sample standard deviation; a z-score of -2 or lower was considered to indicate a "hit" (**Figure 4B**). Known anti-bacterial compounds present in this particular plate e.g., solithromycin and sparfloxacin, (**Figure 4C**) were included to validate the platform. Both of these compound classes have been reported to have activity, albeit less than frontline drugs, against Mtb (Rastogi et al., 2000; Pranger et al., 2011). These data demonstrated that the DGr platform represents a viable, sensitive, and robust method for screening compounds for anti-tuberculosis activities. The inclusion of all the immune cell subsets from the infection site in the assay suggests that this platform has the capacity to identify anti-tuberculosis activities that are dependent on a diverse array of host pathways.

Hits from the screen that we have completed have to be validated, and modes of action have to be determined, nonetheless we see considerable value in documenting the DGr platform in depth because this approach has broad application for the interrogation of chemical, genetic and immunological perturbation of the host components from the Mtb site of infection.

CONCLUDING REMARKS

We have established an *in vitro* drug screening platform that incorporates the diverse cell populations that are recruited and educated within the murine TB granuloma. In contrast to existing screens on Mtb-infected macrophage mono-cultures, which have already proven useful (Christophe et al., 2010; Stanley et al.,

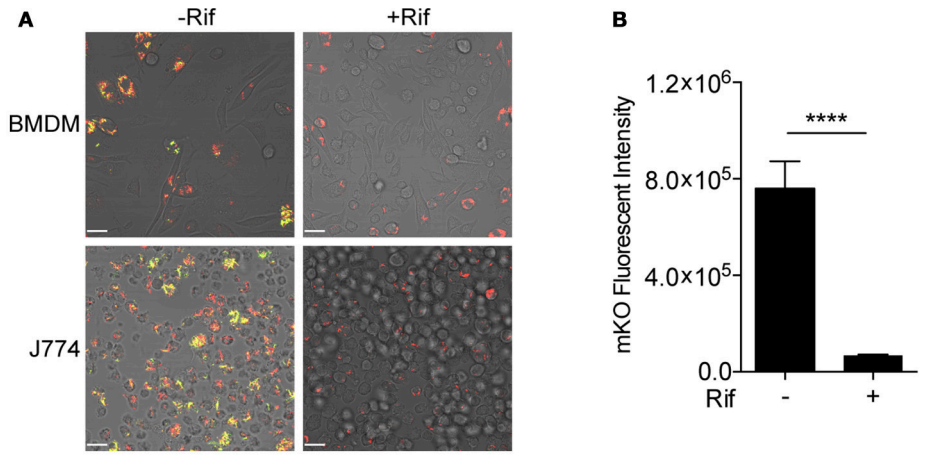

FIGURE 2 | Validation of tet-ON inducible Mtb strain. **(A)** Confocal images of tet-ON inducible mKO/mCherry infected BMDM (MOI 3) and J774 (MOI 4) cells with or without Rif treatment. Rif (5 μM) was added to Mtb infected cells 3 h post infection. ATc (200 ng/ml) was added to cultures on day 4, and confocal images were taken on day 7. **(B)** The fluorescence signal of mKO was obtained with a plate reader. Data are shown as mean ± standard deviation. The experiments were repeated at least two times. *P*-value was calculated using Student's *t*-test. ****$P < 0.0001$.

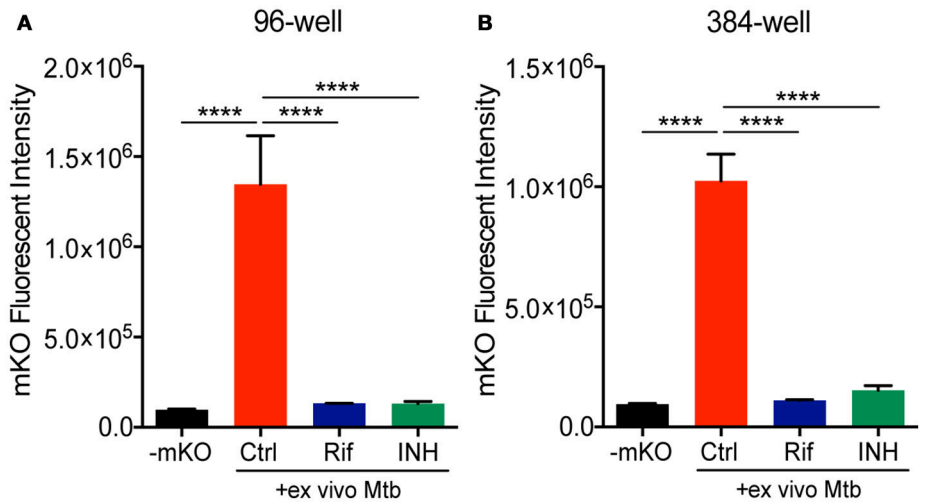

FIGURE 3 | Deconstructed granuloma assay in 96- and 384-well platforms. Lung cells isolated from infected mice were cultured in **(A)** 96-well or **(B)** 384-well plates. Tet-ON inducible mKO/mCherry Mtb were added to the cultures at MOI 0.1 with or without addition of Rif and INH. The fluorescence signal of mKO was acquired with a plate reader. Data are shown as mean ± standard deviation. The experiments were repeated at least two times. *P*-value was calculated using one-way ANOVA test with Tukey's multiple comparisons. ****$P < 0.0001$.

2012; VanderVen et al., 2015), this new platform encompasses the broad range of immune pressures active in *in vivo* infection and therefore provides an increased number of potential targets for host-directed therapeutics. Although previous assays on heterologous cell types challenged *in vitro* have been detailed (Silva-Miranda et al., 2015), this current platform utilizes cells selected and programed by *in vivo* infection, thus making fewer assumptions regarding cell type and immune status. We have demonstrated that the platform is amenable to a HTS in a 384 well plate format and have called it the DGr (*D*econstructed *Gr*anuloma) platform. Through the development process we found that an *ex vivo* challenge was required to amplify the bacterial signal for detection by plate reader. During optimization of the platform we used two different fluorescent reporter Mtb strains: a mCherry strain was used for the initial mouse infection, to generate the immune response in the animal, followed by *ex vivo* infection with a tet-ON mKO strain, employed for detection purposes in the HTS assay. We have performed a HTS screen with this DGr platform and provide data from a representative plate to demonstrate the robustness of the assay. Final assessment of the platform and its value as an HTS to discover novel HDTs is dependent on the analysis of the "hits" from the screening of this compound collection, which is ongoing.

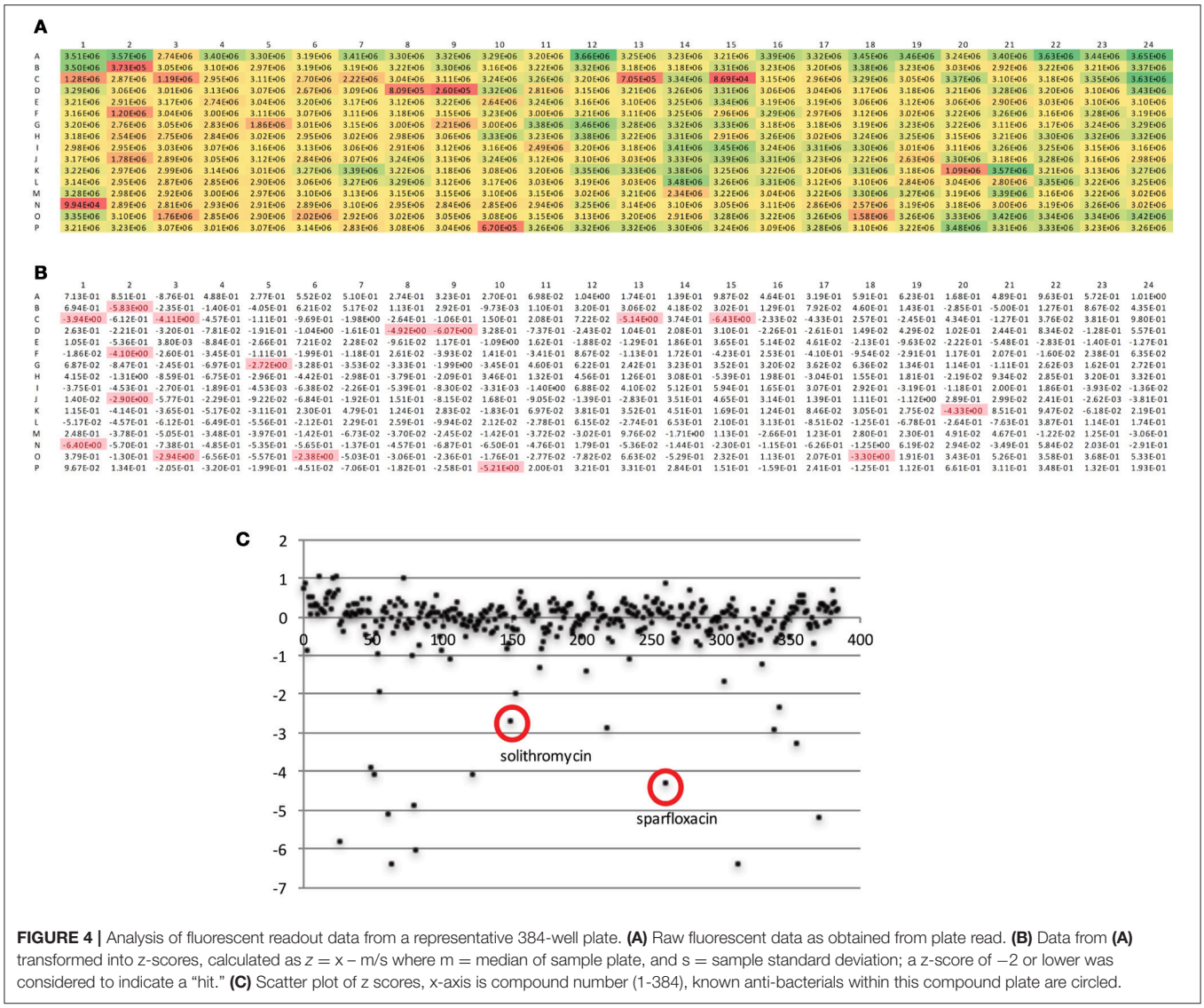

FIGURE 4 | Analysis of fluorescent readout data from a representative 384-well plate. **(A)** Raw fluorescent data as obtained from plate read. **(B)** Data from **(A)** transformed into z-scores, calculated as $z = x - m/s$ where m = median of sample plate, and s = sample standard deviation; a z-score of −2 or lower was considered to indicate a "hit." **(C)** Scatter plot of z scores, x-axis is compound number (1-384), known anti-bacterials within this compound plate are circled.

In summary, we have developed a robust new, biologically-rich platform for screening for compounds with anti-tuberculosis activities. We believe that this complex platform encompasses much of the immune environment of the infection site, and is of particular value in the pursuit of Host-Directed Therapeutics. We also suggest that similar approaches using cells dissociated from the infected tissue harvested from experimental *in vivo* infections with other microbial pathogens might have comparable value to other drug discovery efforts.

AUTHOR CONTRIBUTIONS

LH and DR conceived the study, conducted the initial experiments and interpreted data; LH, NK, MT, and AB were responsible for conducting the experiments, data acquisition and interpretation. ST conceived of the use of mKO in Mtb, and generated and carried out initial tests on the inducible mKO strain. DP provided critical reagents. CM and HP provided the compound collection and assisted in the design and interpretation of the screen data. LH, AB and DR wrote the manuscript. All authors critically read and edited the manuscript.

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Evaluating New Compounds to Treat *Burkholderia pseudomallei* Infections

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Burkholderia pseudomallei is the causative agent of melioidosis, a disease that requires long-term treatment regimens with no assurance of bacterial clearance. Clinical isolates are intrinsically resistant to most antibiotics and in recent years, isolates have been collected that display resistance to frontline drugs. With the expanding global burden of *B. pseudomallei*, there is a need to identify new compounds or improve current treatments to reduce risk of relapse. Using the Pathogen Box generated by Medicines for Malaria Venture, we screened a library of 400 compounds for bacteriostatic or bactericidal activity against *B. pseudomallei* K96243 and identified seven compounds that exhibited inhibitory effects. New compounds found to have function against *B. pseudomallei* were auranofin, rifampicin, miltefosine, MMV688179, and MMV688271. An additional two compounds currently used to treat melioidosis, doxycycline and levofloxacin, were also identified in the screen. We determined that the minimal inhibitory concentrations (MIC) for levofloxacin, doxycycline, and MMV688271 were below 12 μg/ml for 5 strains of *B. pseudomallei*. To assess persister frequency, bacteria were exposed to 100x MIC of each compound. Auranofin, MMV688179, and MMV688271 reduced the bacterial population to an average of 4.53×10^{-6}% compared to ceftazidime, which corresponds to 25.1% survival. Overall, our data demonstrates that auranofin, MMV688197, and MMV688271 have the potential to become repurposed drugs for treating melioidosis infections and the first evidence that alternative therapeutics can reduce *B. pseudomallei* persistence.

Keywords: *Burkholderia*, antibiotic resistance, persistence, pathogen box, treatment

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INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, a disease originally of importance in Southeast Asia and Northern Australia (Currie et al., 2000; Sarovich et al., 2012a,b; Hatcher et al., 2015). However, a recent report found that global distribution of the pathogen is severely underreported and estimated that annually, *B. pseudomallei* causes 165,000 human infections and 89,000 deaths worldwide (Limmathurotsakul et al., 2016). Aside from being classified as a Tier 1 Select Agent due to its bioavailability and high potential of aerosolization, *B. pseudomallei* is a multidrug-resistant pathogen that is susceptible to very few antibiotics (Sarovich et al., 2012a,b; Ahmad et al., 2013). Depending on the clinical manifestations of the disease, treatment for *B. pseudomallei* is usually biphasic, starting with 10–14 days of intravenous therapy, followed by

weeks to months of oral eradication therapy. The most commonly administered intravenous drugs consist of ceftazidime with or without trimethoprim-sulfamethoxazole (TMP-SMX), amoxicillin–clavulanic acid, imipenem, and cefoperazone/sulbactam (Estes et al., 2010). The second phase of treatment consists of a minimum of 3 months of oral chloramphenicol, TMP-SMX, and doxycycline, or amoxicillin-clavulanic acid (Estes et al., 2010).

Treatment failure has been reported in some cases due to antibiotic resistance. Clinical reports show that all *B. pseudomallei* species possess resistance against many classes of antibiotics and some isolates are also resistant to front-line antibiotics, such as ceftazidime and TMP-SMX, making treatment options limited (Sadiq et al., 2016; Cummings and Slayden, 2017). In addition to antibiotic resistance and the requirement for an extensive treatment regimen, an additional hurdle is that infection relapse occurs in 13–23% of patients (Chaowagul et al., 1993; Currie et al., 2000; Maharjan et al., 2005; Suntornsut et al., 2016). Though speculation has been made that these repeat cases could be due to re-infection, a recent study found that 75% of these recurrent cases are due to relapse, while only 25% are due to re-infection (Maharjan et al., 2005).

The ability of *B. pseudomallei* to generate persistent populations is thought to be a major contributor to latent infections which can recrudesce when the immune system is compromised (Chaowagul et al., 1993). Bacterial persistence is well documented to be associated with chronic infections and infection relapse (Zhang, 2014; Byndloss and Tsolis, 2016). Persistence is a mechanism by which a portion of an antibiotic susceptible population enters a dormant-like state, rendering antibiotics ineffective. Many bacterial genes have been identified to play a role in persistence, however, very no compounds have been developed to target persister populations. Additionally, new drug discovery cost an average of 802 million U.S. dollars and requires approximately 10 years from start of development to use in the clinic, and implementing new compounds into current treatment regimens is complicated (Adams and Brantner, 2006). To reduce economic burden and advance the speed at which novel drugs can be tested, many groups are investigating the potential for repurposing Food and Drug Administration (FDA)-approved drugs. This method involves screening large panels of FDA-approved compounds for efficacy against off-label conditions, such as those associated with infectious diseases. Importantly, the diversity of compounds included in these panels allows for testing non-traditional treatments against a wide variety of organisms. Here, we took the drug repurposing approach to explore new treatment options for melioidosis and demonstrate efficacy with anti-rheumatic and anti-kinetoplastid compounds against *B. pseudomallei*.

MATERIALS AND METHODS

Ethics Statement

All manipulations of *B. pseudomallei* were conducted in CDC/USDA-approved and registered biosafety level 3 (BSL3) facilities at the University of Texas Medical Branch (UTMB), and experiments with select agents were performed in accordance with BSL3 standard operating practices. The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (IACUC #0503014D) was approved by the Animal Care and Use Committee of the UTMB.

Bacterial Strains and Culture Conditions

B. pseudomallei strain K96243 was obtained from BEI Resources (Manassas, VA, USA). *B. pseudomallei* 576 was obtained from the Defense Science and Technology Laboratory (DSTL, UK); *B. pseudomallei* NCTC13178 and NCTC13179 were obtained from the Health Protection Agency Centre for Infections (HPA), UK; and *B. pseudomallei* MX2013 was obtained from CDC, USA. For all experiments, bacterial strains were stored at −80°C. Prior to use, strains were streaked onto Luria-Bertani agar with 4% glycerol (LBG) and grown for 36–48 h prior to use. For liquid cultures, 3–5 colonies were inoculated into LBG broth and grown for 16 h at 37°C.

Pathogen Box

The Pathogen Box was obtained from Medicines for Malaria Venture (MMV, Geneva, Switzerland). The bacterial culture of *B. pseudomallei* K96243 was prepared as described above. Compounds were solubilized as directed by MMV. Briefly, compound plates were allowed to thaw at room temperature and 90 μl of DMSO (Sigma) was added to reach a concentration of 1 mM and further diluted with PBS to reach 100 μM. DMSO was also diluted with PBS and served as a negative control. Bacteria were adjusted to 5×10^5 CFU per ml and treated with a final concentration of 2, 5, 10, or 50 μM of each compound or DMSO control (0.2–1%). Plates were incubated at 37°C. At 16 and 24 h post incubation, turbidity was checked visually and wells with little or no growth were recorded. The 2 μM concentration for each compound correlated to the following: doxycycline 888.88 μg/ml, MMV688179 806.74 μg/ml, MMV688271 806.74 μg/ml, levofloxacin 722.5 μg/ml, rifampicin 1,645.9 μg/ml, auranofin 1,357 μg/ml, and miltefosine 815.14 μg/ml. For the remainder of the experiments the concentration was expressed as μg/ml. The list of all compounds tested can be found at https://www.pathogenbox.org/about-pathogen-box/composition.

Minimum Inhibitory Concentrations (MICS)

Compounds were serially diluted two-fold in PBS to generate a suitable range of doses for testing. Overnight cultures were grown and adjusted to 1×10^6 CFU/ml. One-hundred μl of bacterial suspension was mixed with 100 μl of compound suspended in culture media to yield a final bacterial concentration of 5×10^5 CFU/ml. *B. pseudomallei* can effectively grow in 5% DMSO, so 2% was the highest final concentration of DMSO utilized for the MIC assays. Plates were incubated at 37°C and examined for growth inhibition at 24 and 48 h. An initial study was conducted examining a range from 1 to 400 μg/ml, and then further adjusted and repeated for MIC determination. The MIC was calculated as the lowest concentration that visually inhibited bacterial growth.

The experiments were performed in duplicate with 2 biological replicates.

Kill Curve

Overnight cultures were adjusted to 5×10^5 CFU/ml and 1x MIC (**Figure 1A**) or 1–5x MICs (**Figure 1B**) of compound was added with or without 5% DMSO (**Figure 2**). Plates were incubated at 37°C and samples collected at 2, 4, 6, 8, 10, 12, and 24 h for CFU enumeration.

Persister Assay

To determining the persister frequency, bacteria were grown for 16 h and adjusted to 1×10^8 CFU/ml in media containing 1, 5, 10, or 100x MIC in triplicate. After 24 h of incubation at 37°C, the surviving bacteria were assayed by serial diluting and plating on LB agar. Persistence was quantified by normalizing the surviving bacteria to the input concentrations and was expressed as percent survival. For each assay, a DMSO vehicle control only group was included to examine the potential effect of DMSO

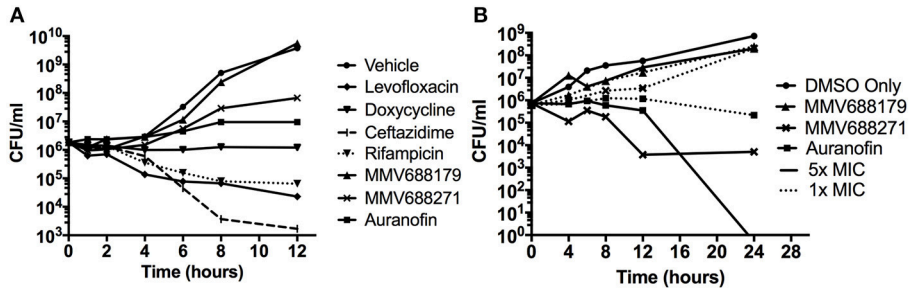


FIGURE 1 | Characterization of Novel Melioidosis Treatment Compounds. **(A)** *B. pseudomallei* was cultured with 1x MIC at 37°C for 12 h. Over the course of time, CFUs were enumerated to determine if the compounds have a bacteriostatic or bactericidal activity. **(B)** *B. pseudomallei* was cultured with 1-5x MICs in the presence of 5% DMSO at 37°C for 24 h. A vehicle control was included to show the effect of 5% DMSO on growth.

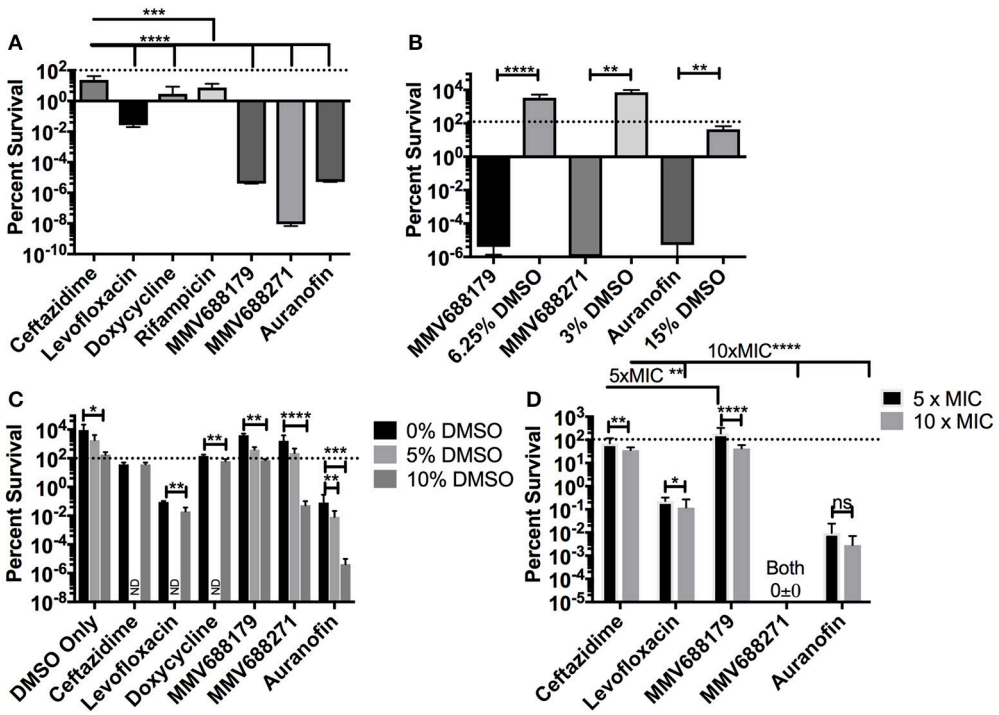


FIGURE 2 | Determination of Persistence Frequency. **(A)** Persister frequency represented as percent survival of 1×10^8 CFU/ml of *B. pseudomallei* exposed to 100x MIC of each compound. DMSO vehicle controls (15, 3, or 6.25%) and associated experimental conditions were tested in **(B)**. **(C)** To determine the effect of DMSO, persister assays were performed with 5x MIC of the compound and 0, 5, or 10% DMSO. Groups not investigated are labeled ND. All assays were grown at 37°C for 24 h and data presented as percent survival. **(D)** Persister assays were repeated at 5x MIC and 10x MIC to assess dose dependency on percent survival. Error bars indicate standard deviation and asterisks indicate a statistically significant difference between treatments ($P < 0.05$, $^*P < 0.001$, $^{**}P < 0.001$, $^{***}P < 0.0001$,****; ns, no statistically difference).

at the same concentrations used in the experimental groups. One-Way ANOVA with Kruskal-Wallis correction was used to determine if each compound had significantly different results compare to ceftazidime exposure (**Figures 2A,C,D**). *T*-test with a Mann-Whitney correction was used to compare each compound against the DMSO control (**Figure 2B**), and also to identify the significance of DMSO or dose effect for one compound (**Figures 2C,D**).

RESULTS

MMV Pathogen Box

In this study, we used the MMV Pathogen Box to screen for compounds that inhibit *B. pseudomallei* K96243. Compounds were identified and further tested if they inhibited growth of the bacteria *in vitro*, as determined by reduced or lack of turbidity in comparison to vehicle-control-treated bacteria. To fully determine compound efficacy, we tested each compound at 2, 5, 10, and 50 μM/ml for growth inhibition. Of the 400 compounds tested, levofloxacin, doxycycline, auranofin, rifampicin, MMV688271, and MMV68817 were able to inhibit growth at all concentrations (**Table 1**). Miltefosine exposure resulted in consistent growth reduction and was further investigated to determine its MIC (**Table 2**). For all further studies, ceftazidime was used as a positive control since it is the most commonly recommended treatment for melioidosis (Estes et al., 2010).

Minimal Inhibitory Concentration (MIC)

As the first step in examining the efficacy of these compounds, we determined their minimum inhibitory concentrations (MIC) against 5 clinical isolate strains of *B. pseudomallei* from Australia, Thailand, or Mexico (**Table 2**). MMV688271, rifampicin, levofloxacin, and doxycycline were effective at concentrations below 50 μg/ml for all strains. The anti-kinetoplastid compound MMV688271 had an MIC ranging from 6 to 12 μg/ml. Rifampicin exhibited MICs below 50 μg/ml with a wide range (18–45 μg/ml) for the 5 strains, and was generally higher as compared to other compounds (e.g., ceftazidime). As expected, doxycycline, levofloxacin, and ceftazidime exhibited consistently low MICs and all have been previously used to treat patients with melioidosis (**Table 2**).

On the other hand, MMV688179, auranofin, and miltefosin had MIC $\geq$ 50 μg/ml. The MMV688179 compound exhibited an MIC of 12.5 μg/ml for *B. pseudomallei* K96243, although it was unable to inhibit growth up to 100 μg/ml for the remaining 4 stains. Due to limited availability of MMV688179, we did not evaluate higher concentrations. Auranofin exhibited a consistent MIC of 150 μg/ml for all strains. In our initial screen, miltefosine reduced the turbidity of the bacterial culture; however, when the concentration was increased to 1,600 μg/ml, miltefosine still could not fully inhibit bacterial growth and, therefore, was not further investigated in these studies.

Kill Curves

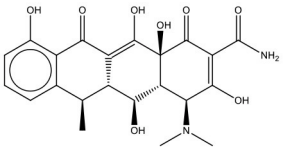
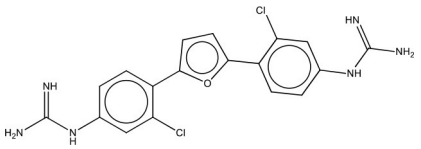
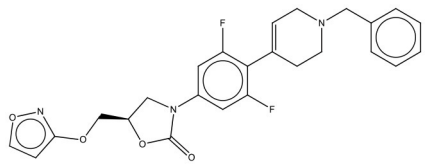
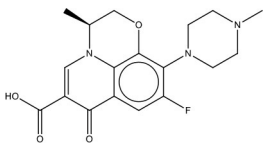
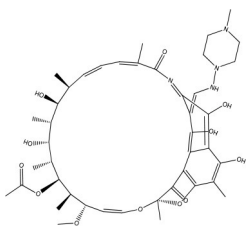
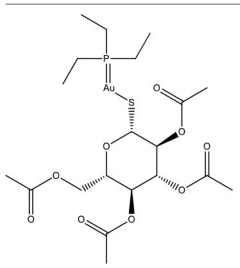
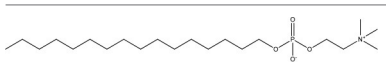
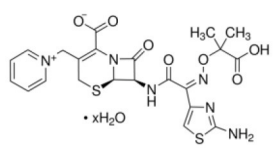
To determine whether the identified compounds had bacteriostatic or bactericidal activity, we conducted kill curves by treating bacterial cultures with low doses of the compounds normalized by their MIC. When treated with 1x MIC for 12 h (**Figure 1A**), we found that levofloxacin, ceftazidime, and rifampicin had bactericidal properties that began as early as 2 h and continued steadily until approximately 8 h post-treatment. At 8 h, levofloxacin, ceftazidime, and rifampicin treatment reduced the number of bacteria to 4.58 ± 6.48%, 0.26 ± 0.11%, and 5.58 ± 5.19%, respectively. Consistent with the literature, doxycycline exhibited bacteriostatic properties.

Auranofin treatment at 1x MIC inhibited growth in a bacteriostatic manner, while both anti-kinetoplastids were unable to inhibit growth. Because the anti-kinetoplastids and auranofin are DMSO-soluble, we also assayed for bacteriostatic or bactericidal properties in the presence of 5% DMSO (**Figure 1B**). As a control, *B. pseudomallei* was treated with 5% DMSO alone and we observed that the bacteria grew at three orders of magnitude above the input. With 5% DMSO and 1x MIC of auranofin combined, a bactericidal effect was observed and increased at 5x MICs, confirming its inhibitory properties. Both anti-kinetoplastids compounds did not show any effect with 5% DMSO and 1x MIC. However, when increased to 5x MICs, MMV688271 treatment had a bactericidal effect, reducing the mean bacterial concentration to 0.489 ± 0.68%, whereas MMV688179 did not show any effect. As a side note, we observed that MMV688179 requires more DMSO for full solubilization, which was corroborated by the visual presence of precipitated compound at 5% DMSO, and might be the reason of the lack of antibacterial activity.

Persister Assays

For many diseases, the generation of latent infection has been attributed to establishment of persister cell populations (Wood et al., 2013). It has been reported that *B. pseudomallei* K96243 has a persister frequency of 10^{-1} (percent survival of 10%) in the presence of 100x MIC of ceftazidime (400 μg/ml) (Butt et al., 2014). To test if our compounds of interest had an improved ability to inhibit the formation of persister cells compared to ceftazidime, we treated bacteria with 100x MIC for 24 h and assessed the persistence frequency by CFU enumeration normalized by input (**Figure 2A**). The results with levofloxacin and ceftazidime (both 400 μg/ml) are consistent with the literature (Butt et al., 2014), resulting in frequencies of 2.67 × 10^{-2} ± 6.99 × 10^{-3}% and 23.87 ± 18.65%, respectively. Doxycycline (100 μg/ml) has a frequency of 2.911 ± 5.67%, while rifampicin, a drug not commonly used clinically due to high rates of resistance, had a persister frequency of 7.56 ± 7.563% (**Figure 2A**). Meanwhile, treatment with auranofin (1.5 mg/ml), MMV688179 (1,250 μg/ml), and MMV688271 (600 μg/ml) resulted in nearly complete eradication of the bacteria. In the few instances when bacteria was observed growing, only 1–4 CFUs were recovered on the 10^{-1} plate. The overall persister frequency and standard deviation for MMV688179 was 3.98 × 10^{-6} ± 7.89 × 10^{-6}%, and for auranofin was 5.24 × 10^{-6} ± 1.48 × 10^{-5}%. The MMV688271 compound inhibited bacterial survival to the greatest extent, with a persister frequency of 9.109 × 10^{-9} ± 2.25 × 10^{-9}%. As indicated above, auranofin, MMV688179, and MMV688271

TABLE 1 | Identification of novel Melioidosis treatment compounds.

Compound	Compound ID	Position	Trivial name	Total molecular weight	Molecular weight parent molecule	Molecular formula
	MMV000011	Plate B E04	Doxycycline	480.9	444.44	$C_{22}H_{24}N_2O_8$
	MMV688179	Plate C F03	NA	476.19	403.27	$C_{18}H_{16}N_6OCl_2$
	MMV688271	Plate D E10	NA	476.19	403.27	$C_{18}H_{16}N_6OCl_2$
	MMV687798	Plate E A05	Levofloxacin (-)-ofloxacin	361.37	361.37	$C_{18}H_{20}N_3O_4F$
	MMV688775	Plate E A06	Rifampicin	822.94	822.94	$C_{43}H_{58}N_4O_{12}$
	MMV688978	Plate E H05	Auranofin	678.48	678.48	$C_{20}H_{34}AuO_9PS$
	MMV688990	Plate E H06	Miltefosine	407.57	407.57	$C_{21}H_{46}NO_4P$
	Added as control	NA	Ceftazidime	546.58	546.58	$C_{22}H_{22}N_6O_7S_2 \cdot xH_2O$

were solubilized with DMSO, which was carried over into the persister assays. To separately examine the effect of DMSO, vehicle controls with the equivalent concentration of the solvent alone were included (**Figure 2B**). The concentrations of DMSO at 3 and 6% contained bacteria counts that were higher than the input, showing that bacterial growth still occurred. At 15% DMSO, 54% bacteria remained, suggesting bacterial death. As expected, each control group had significantly higher bacterial

TABLE 2 | Minimal Inhibitory Concentrations.

Minimal inhibitory concentrations	Levofloxacin (μg/ml)	Ceftazidime (μg/ml)	Doxycycline (μg/ml)	Auranofin (μg/ml)	Rifampicin (μg/ml)	Miltefosine (μg/ml)	MMV688271 (μg/ml)	MMV688179 (μg/ml)
B. pseudomallei K96243	4	4	1	150	45	>1600	6	12.5
B. pseudomallei 576	10	4	2.5	150	18	>1600	12	>100
B. pseudomallei NCTC13178	6	4	3	150	18	>1600	10	>100
B. pseudomallei NCTC13179	6	6	2.5	150	25	>1600	8	>100
B. pseudomallei MX2013	6	3	2.5	150	18	>1600	12	>100

counts compared to its associated treatment group, suggesting an effect of the compound on bacterial persistence and not just the DMSO (**Figure 2B**).

To further determine whether DMSO had an effect on compound efficacy, we exposed *B. pseudomallei* to 5x MIC of compound in the presence of 0, 5, or 10% DMSO (**Figure 2C**). For the vehicle control, only 10% DMSO significantly impacted growth of the bacteria but levels were not reduced below the bacterial input dose. At 0% DMSO, all compounds, except MMV688179, were significantly different ($P < 0.03$) from the DMSO only control. At 5% DMSO, only auranofin significantly reduced the bacteria count. Lastly, all compounds significantly ($P \leq 0.0001$) reduced bacterial recovery compared to the 10% DMSO controls.

Lastly, to assess dose dependence on bacterial clearance, we adjusted each compound concentration to 5 and 10x MIC and enumerated the bacteria after 24 h (**Figure 2D**). At these concentrations, all drugs except MMV688271 showed a dose dependent decrease in efficacy between 5 and 10x MIC. When comparing all 5x MIC conditions, MMV688271 and auranofin showed improved killing compared to ceftazidime, while MMV688179 was less effective. The same was true when comparing all 10x MIC treatments vs. ceftazidime treatment.

DISCUSSION

B. pseudomallei is an important environmental bacterium that is able to cause severe infection and death if left untreated, and still causes a mortality rate of 40% when treatment is provided (Limmathurotsakul et al., 2016). With increasing global travel, *B. pseudomallei* can be introduced into previously non-endemic areas and persist in the environment (Limmathurotsakul et al., 2016). Today, in addition to Asia and Australia, the endemic presence of the pathogen has been confirmed in South America and the Caribbean. Of particular interest to the USA, reports indicate that Florida and certain regions of Texas are environmentally suitable for *B. pseudomallei*, further, increasing the threat of successful introduction into this country. Aside from the threat to North America, the increasing burden of this disease and isolation of antibiotic resistant strains could result in increased disease relapse, which is estimated to occur in 13–23% of cases (Limmathurotsakul et al., 2016).

This study aimed to identify and assess new treatment options or compounds that can complement existing melioidosis therapeutics. By screening the MMV Pathogen Box, we identified several compounds with different levels of efficacy against *B. pseudomallei*. Of the compounds identified, doxycycline is currently widely-used. Doxycycline is most efficacious when used for localized infections and for multi-drug treatment during systemic disease (Perumal Samy et al., 2017). Additionally, *B. pseudomallei* studies showed low rates of resistance to doxycycline when testing against 50 strains (2%) (Thibault et al., 2004). Levofloxacin is a promising compound that has been tested clinically due to its low MIC and high rate of bacterial killing. Here, we showed that of the available drugs, levofloxacin generated a smaller persister population than those reported for doxycycline and ceftazidime (Thibault et al., 2004; Estes et al., 2010; Butt et al., 2014). However, levofloxacin and other fluoroquinolones are generally not recommended for melioidosis due to high rates of relapse (Perumal Samy et al., 2017). Studies have also shown that among 50 *B. pseudomallei* isolates, 52% were resistant to levofloxacin, making treatment difficult unless new analogs with broader efficacy *in vivo* are generated (Thibault et al., 2004). Rifampicin also exhibits a moderate MIC and strong bactericidal activity against *B. pseudomallei*; however, like levofloxacin, resistance has been identified in 88% of the clinical isolates (Thibault et al., 2004).

The remaining three candidates (auranofin, MMV688179, and MMV688271) are novel agents with activity against *B. pseudomallei*. Although auranofin has a high MIC, all three were extremely effective at reducing persistent populations *in vitro*. This is particularly relevant, as persistent populations are important therapeutic targets due to their association with latent infection and relapse (Fauvart et al., 2011). Most importantly, these three drugs resulted in a significantly reduced frequency of persistent bacteria compared to all other compounds tested, including the antibiotics used in the clinic. This marks a milestone in therapeutic approaches in which novel compounds have been identified that can reduce *Burkholderia* persistent populations to 10^{-8}–10^{-10} CFU/ml. Although the mechanisms

of action against *B. pseudomallei* remain unclear, we have shown that auranofin and MMV688271 display bactericidal activity and are the best candidates for further detailed study.

Auranofin is a FDA approved compound that is used to treat rheumatoid arthritis and has been repurposed for other diseases such as HIV, cancer, parasitic, and bacterial infections (Harbut et al., 2015; Roder and Thomson, 2015; Thangamani et al., 2016; Wang et al., 2017). The known mechanism of action for auranofin is enzyme inhibition by irreversibly binding to thiol or selenol groups, resulting in the disruption of selenium metabolism and selenocysteine synthesis. Selenocysteine is required for the synthesis of glycine, proline, and thioredoxin reductases which are important for energy production (Asghari et al., 2017). In both *Clostridium difficile* and *Enterococcus faecalis* infection, auranofin has been shown to reduce selenium concentration and prevent production of selenoproteins, like in the case of glycine reductases (Jackson et al., 2006; Srivastava et al., 2011; Roder and Thomson, 2015). The major challenge moving forward with auranofin is its low bioavailability and high MIC. While generally administered orally, only 25% of the compound is absorbed and the peak concentration of 6–9 μg/ml is achieved after 20 min, which is far below the required MIC observed in this work (Roder and Thomson, 2015). In an attempt to increase availability, in a separate study we administered the compound to mice intraperitoneally at 10 mg/kg/day, which is just below the levels previously used in other treatments (Mirabelli et al., 1985; Ashino et al., 2011). We found that auranofin treatment alone did not provide significant protection against infection and further studies are needed to determine whether synergistic effects could be achieved when administered in combination with other compounds (data not shown). Alternatively, auranofin analogs are available and are being tested for other infectious diseases (Aguinagalde et al., 2015; Roder and Thomson, 2015). Such auranofin analogs offer the possibility of a lower MIC while maintaining inhibition of persister cell formation *in vitro*, which makes these compounds attractive candidates.

Both MMV688179 and MMV688271 are analogs to furamidine, which is an amphipathic diamine antiprotozoal drug. Pentamidine is as an aromatic diamidine used against human African *trypanosomiasis*. When Pentamidine's phenyl ring has been replaced with a furan ring, furamidine is generated and this drug is effective against human African trypanosomiasis. Furamidine in the form of a pro-drug is Pafuramidine, which is currently undergoing phase III clinical trials as a treatment for African trypanosomiasis (Ming et al., 2009; Pohlig et al., 2016). Furamidine has also shown efficacy against some Gram-positive cocci in wounds and burns, confirming their antibacterial potential and warranting the generation of analogs to test against a wide range of pathogens (Bichowsky-Slomnitzki, 1948). Although the mechanism of action of MMV688271 and MMV688179 remains unclear, it is known that these compounds interact with DNA at AT-rich sites and are believed to inhibit replication (Wang et al., 2000). Similar to Furamidine, analogs of these compounds have been shown to be effective against some Gram-positive cocci in wounds and burns, confirming their antibacterial potential (Bichowsky-Slomnitzki, 1948). We found that MMV688271 and MMV688179 can nearly eradicate *B. pseudomallei* at 100x MIC (600 and 1250 μg/ml, respectively). MMV688179 visibly precipitated at concentrations lower than 10% DMSO, making it a more unstable compound and potentially the reason for the lower efficacy. In contrast, MMV688271 proved to be more stable in solution and more effective at lower concentrations of DMSO.

Together, our data provides a strong rationale for further studies with the anti-kinetoplastid compounds, auranofin, or analogs that display improved solubility and lower MIC. Studies examining combination treatments with current antibiotics could be useful to generate novel persister eradication therapies. Future examination of such combinations and identifying synergistic or additive effects may be leveraged to improve current treatment plans. Overall, a drug repurposing approach for the testing of compounds against melioidosis showed that *B. pseudomallei* is resistant to many drugs compared to other bacteria tested against the same pathogen box; however, we successfully identified new compounds to be considered as anti-persister drugs. Importantly, this approach can be expanded to include additional platforms for compound discovery, in order to evaluate non-conventional therapies against *B. pseudomallei*.

AUTHOR CONTRIBUTIONS

BR and AT designed research. BR performed the research with assistance by JM, DT, and LM, analyzed data, wrote the manuscript and was edited by JM, LM, and AT.

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High Throughput Screening for Natural Host Defense Peptide-Inducing Compounds as Novel Alternatives to Antibiotics

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A rise in antimicrobial resistance demands novel alternatives to antimicrobials for disease control and prevention. As an important component of innate immunity, host defense peptides (HDPs) are capable of killing a broad spectrum of pathogens and modulating a range of host immune responses. Enhancing the synthesis of endogenous HDPs has emerged as a novel host-directed antimicrobial therapeutic strategy. To facilitate the identification of natural products with a strong capacity to induce HDP synthesis, a stable macrophage cell line expressing a luciferase reporter gene driven by a 2-Kb avian β-defensin 9 (*AvBD9*) gene promoter was constructed through lentiviral transduction and puromycin selection. A high throughput screening assay was subsequently developed using the stable reporter cell line to screen a library of 584 natural products. A total of 21 compounds with a minimum Z-score of 2.0 were identified. Secondary screening in chicken HTC macrophages and jejunal explants further validated most compounds with a potent HDP-inducing activity in a dose-dependent manner. A follow-up oral administration of a lead natural compound, wortmannin, confirmed its capacity to enhance the *AvBD9* gene expression in the duodenum of chickens. Besides *AvBD9*, most other chicken HDP genes were also induced by wortmannin. Additionally, butyrate was also found to synergize with wortmannin and several other newly-identified compounds in *AvBD9* induction in HTC cells. Furthermore, wortmannin acted synergistically with butyrate in augmenting the antibacterial activity of chicken monocytes. Therefore, these natural HDP-inducing products may have the potential to be developed individually or in combinations as novel antibiotic alternatives for disease control and prevention in poultry and possibly other animal species including humans.

Keywords: host defense peptides, antimicrobial peptides, defensins, high throughput screening, HDP inducers, wortmannin, host-directed antimicrobial therapy, antimicrobial resistance

INTRODUCTION

Antimicrobial resistance is posing a major threat to public health. While it is necessary to continue the development of antibiotics with direct antimicrobial activities, host-directed therapies have emerged as attractive alternative strategies to combating infectious and non-communicable diseases (Zumla et al., 2016). Host defense peptides (HDPs), also known as antimicrobial peptides, are represented by a large diverse group of small peptides that are synthesized primarily by phagocytic cells and epithelial cells lining the gastrointestinal, respiratory, and urogenital tracts (Zasloff, 2002). With antimicrobial, immunomodulatory and barrier protective activities, HDPs constitute an important phylogenetically conserved first line of defense in virtually all species of life (Hilchie et al., 2013; Mansour et al., 2014; Robinson et al., 2015). Two main HDP families, namely defensins and cathelicidins, exist in vertebrate animals (Zanetti, 2004; Selsted and Ouellette, 2005). One cathelicidin known as LL-37, six α-defensins, and a minimum of 39 β-defensins exist in humans (Wang, 2014), whereas four cathelicidins (CATH1-3 and CATH-B1) and 14 avian β-defensins (AvBD1-14) are present in chickens (Cuperus et al., 2013; Zhang and Sunkara, 2014).

While HDPs are being directly explored as novel antimicrobials or vaccine adjuvants against drug-resistant infections (Choi et al., 2012; Hilchie et al., 2013; Mansour et al., 2014), modulating the synthesis of endogenous HDPs has shown promise in the treatment of shigellosis, pulmonary tuberculosis, cholera, and enteropathogenic *E. coli*-induced diarrhea (Raqib et al., 2012; Al-Mamun et al., 2013; Mily et al., 2015; Sarker et al., 2017). In fact, a number of small-molecule compounds such as butyrate, vitamin D_3, bile acids, and histone deacetylase inhibitors have been shown to induce HDP synthesis in humans without provoking inflammation (Campbell et al., 2012; Van Der Does et al., 2012; Lyu et al., 2015; Yedery and Jerse, 2015). A high throughput screening (HTS) luciferase reporter assay was recently developed to identify multiple compounds with the ability to induce human *LL-37* gene expression (Nylen et al., 2014).

To facilitate the identification of HDP-inducing compounds for use in other animal species, particularly in poultry, here we report the establishment of a cell-based HTS assay. Our earlier studies revealed that, among multiple chicken HDPs, *AvBD9* is the most readily inducible gene in response to butyrate and several other compounds in chickens (Sunkara et al., 2011, 2014). Here we constructed a stable chicken macrophage cell line integrated permanently with a lentiviral luciferase reporter vector under control of chicken *AvBD9* gene promoter. Such a stable cell line was further employed to screen a library of 584 natural products. Multiple *AvBD9*-inducing compounds were identified and further validated for their HDP-inducing activities *in vitro*, *ex vivo*, and *in vivo*. We confirmed several natural compounds such as wortmannin to have a strong ability to enhance HDP gene expression with good potential for further development as novel antibiotic alternatives for application in poultry and possibly other animal species.

MATERIALS AND METHODS

Chemicals

Cell culture media and supplements such as RPMI 1640, DMEM, PBS, and antibiotics (penicillin, streptomycin, and puromycin) were purchased from Lonza (Allendale, NJ), Fisher Scientific (Pittsburgh, PA) or Santa Cruz Biotechnology (Dallas, TX). Heat-inactivated fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Flowery Branch, GA). Sodium butyrate and sanguinarine were procured from Sigma-Aldrich (St. Louis, MO). Trichostatin A (TSA), apicidin, HC toxin, LY294002, PX866, CAL-101, MK2206, Triciribine, GDC0068, Rapamycin, AZD8055, and BEZ235 were obtained from Cayman Chemical (Ann Arbor, MI). Tetrandrine was acquired from Santa Cruz Biotechnology, and (–)-depudecin was purchased from BioVision (Milpitas, CA) and MyBioSource (San Diego, CA). Datiscetin was ordered from BOC Sciences (Shirley, NY), while wortmannin and CUDC-907 were procured from Selleck Chemicals (Houston, TX).

Cell Culture

Chicken HTC macrophage cells (Rath et al., 2003), kindly provided by Dr. Narayan C. Rath of USDA-ARS, were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stable HTC cell lines (HTC/*AvBD9-luc*) transduced with the *AvBD9*-driven luciferase gene were maintained in the same complete medium supplemented additionally with 0.5 μg/ml puromycin. Human 293T embryonic kidney epithelial cells (HEK 293T) were obtained from ATCC (Manassas, VA) and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C and 5% CO_2 and subcultured every 3–4 days.

Construction of the *AvBD9* Luciferase Reporter Plasmids

Chicken genomic DNA was extracted from the liver of a Cobb broiler chicken using Quick-gDNA Microprep Kit (Zymo Research, Irvine, CA) according to the manufacturer's recommendations. A series of *AvBD9* gene promoter constructs were cloned from chicken genomic DNA using CloneAmp HiFi PCR Premix (Takara Bio USA, Mountain View, CA) with different forward primers paired with a common reverse primer (**Table 1**). It is noted that the 5′-end of gene-specific reverse primer begins at the third nucleotide upstream of the start codon of the *AvBD9* mRNA (GenBank accession number NM_001001611). PCR products were then cloned into a *Kpn*I-linearized luciferase reporter vector, pGL4.21[*luc2P*/Puro] (Promega, Madison, WI), using a ligation-independent In-Fusion HD PCR Cloning Kit (Takara Bio USA). The presence of the insert in each recombinant plasmid was confirmed with direct Sanger sequencing. Recombinant plasmids were propagated in Stellar *E. coli* HST08 competent cells (Takara Bio USA) and purified with QIAprep Spin Plasmid Miniprep Kit (Qiagen, Germantown, MD) for transient transfections as described below.

TABLE 1 | Primers used in this study[a,b].

Name	Sequence	Size (bp)
AvBD9 FORWARD PRIMERS		
AvBD9-120-F	TGGCCTAACTGGCCGGTACCGTCCAGACCCACAGCCTTTA	118
AvBD9-300-F	TGGCCTAACTGGCCGGTACCTCTCTGGGTGCAGCCCA	298
AvBD9-399-F	TGGCCTAACTGGCCGGTACCCAACACCATGTCCAAGAGCCAC	397
AvBD9-611-F	TGGCCTAACTGGCCGGTACCAGATATCAAGGACAGGGATGGG	609
AvBD9-950-F	TGGCCTAACTGGCCGGTACCCCTCAAGAGTGGCATTTCTCAG	948
AvBD9-1999-F	TGGCCTAACTGGCCGGTACCGTGGATGCTGTTATTGCCTGGA	1,997
AvBD9-2998-F	TGGCCTAACTGGCCGGTACCGAGATCTGCAGGAAAGCAGCT	2,996
AvBD9-3948-F	TGGCCTAACTGGCCGGTACCAAACAGGAATTTCCACATGGCAG	3,946
AvBD9-1999-Lenti-F	TTTTATCGATGAATTCGTGGATGCTGTTATTGCCTGGA	1,997
AvBD9 REVERSE PRIMERS		
AvBD9-3-R	CCGGATTGCCAAGCTTTTGTCCTCTGCTGTGGAATAG	
AvBD9-3-Lenti-R	TACACGCCTAACTAGTTTGTCCTCTGCTGTGGAATAG	

[a] *Each forward primer consists of a common linker sequence at the 5′-end and a KpnI site in the middle (as underlined) and a gene-specific sequence at the 3′-end, whereas the reverse primer included a different linker sequence at the 5′-end and a HindIII site in the middle (as underlined) and a gene-specific sequence at the 3′-end. The exceptions are AvBD9-1999-Lenti-F and AvBD9-3-Lenti-R that are composed of different linker sequences and restriction sites.*

[b] *The number associated with each primer indicates the upstream position relative to the start codon of the AvBD9 mRNA reference sequence (GenBank accession no. NM_001001611).*

Transient Transfection and Luciferase Assay

HTC cells were seeded overnight in 24-well tissue culture plates before being transfected with 50 ng/well of different *AvBD9* promoter-driven luciferase reporter plasmids using FuGENE HD Transfection Reagent (Promega). After 24 h, cells were stimulated in duplicate with or without 8 mM sodium butyrate for another 24 h. Luciferase activity was measured by adding an equal volume of Steady-Glo Substrate to each well for 10 min using Steady-Glo Luciferase Assay System (Promega) according to the manufacturers' instructions. The luminescence was detected using Modulus Single-Tube Luminometer (Turner Biosystems, Sunnuvale, CA).

Development of a Stable HTC/*AvBD9-luc* Luciferase Reporter Cell Line

A 2.0-Kb *AvBD9* gene promoter fragment was cloned into a lentiviral luciferase reporter vector, pGreenFire1-mCMV-Puro (System Biosciences, Palo Alto, CA) using In-Fusion HD PCR Cloning Kit (Takara Bio USA) and gene-specific primers (**Table 1**). The PCR product in the recombinant plasmid was confirmed by Sanger sequencing. Pseudolentiviral particles were packaged by transfecting HEK 293T cells in a 10-cm tissue culture dish with 1 μg of recombinant *AvBD9* reporter lentivector and 5 μg of the pPACKH1 plasmid mix (System Biosciences) using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The cell culture medium containing pseudolentiviral particles was collected 48 h after transfection and stored at −80°C. For viral transduction, HTC cells were seeded at 1×10^5 cells/well in a 6-well plate overnight and then incubated with 2 ml HEK 293T cell culture medium containing the pseudolentiviruses for 4 h before being replenished with 4 ml fresh cell culture medium. After 3 days of incubation, transduced HTC cells were expanded to 10-cm dishes in complete RPMI 1640 medium containing 0.5 μg/ml puromycin for a week of selection, with medium change every 2–3 days. Single cell clones were obtained by limiting dilution of stable cells in 96-well plates in complete RPMI 1640 medium in the presence of 0.5 μg/ml puromycin. After 10–14 days, individual cell clones were gradually expanded and assessed for their responsiveness to sodium butyrate. The most responsive cell clones, named HTC/*AvBD9-luc*, were chosen for the development of a high-throughput screening (HTS) assay.

Optimization of a Cell-based HTS Assay For *AvBD9*-Lnducing Compounds

Stable HTC/*AvBD9-luc* cells were grown overnight at different densities in the presence or absence of FBS in a 96-well white tissue culture plate with clear bottom (Santa Cruz Biotechnology). Cells were stimulated with 8 mM butyrate for 24 h, followed by luminescence detection with Steady-Glo Luciferase Assay System (Promega) on L-Max II Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA). To assess the robustness of the HTS assay, Z'-factor (Zhang et al., 1999) was used, which is expressed as $Z' = 1 - \frac{(3\sigma_p + 3\sigma_n)}{|\mu_p - \mu_n|}$, where σ_p and σ_n are standard deviations of positive and negative controls, while μ_p and μ_n are the mean luciferase activity of positive and negative controls, respectively. To assess the Z'-factor, HTC/*AvBD9-luc* cells were grown at 4×10^4 cells/well overnight in 96-well white plates in 50 μl complete RPMI 1640 medium, followed by stimulated with or without 8 mM butyrate in 48 technical replicates for another 24 h. Luciferase activity was measured with Steady-Glo Luciferase Assay System on L-Max II Luminescence Microplate Reader.

Screening of Natural Product Libraries

HTC/*AvBD9-luc* cells were seeded at 4×10^4 cells/well overnight in 96-well white tissue culture plates in complete

RPMI 1640 medium containing 0.5 μg/ml puromycin. The natural products and rare natural products libraries consisting of 584 compounds were previously purchased from BIOMOL International (Plymouth Meeting, PA) (Davenport et al., 2014), dissolved in DMSO at 10 mg/ml, and further diluted in RPMI 1640–0.2 mg/ml. Compounds were then added to individual wells to a final concentration of 20 μg/ml for 24 h, followed by luciferase assay with Steady-Glo Luciferase Assay System (Promega) on L-Max II Luminescence Microplate Reader (Molecular Devices). Cell viabilities were also assessed by adding alamarBlue Reagent (Thermo Fisher Scientific, Waltham, MA) to cell culture to a final concentration of 0.2% 4 h before luciferase assay. Fluorescence was detected on FLx800 Multi-Detection Microplate Reader (BioTek, Winooski, VT) at the excitation/emission wavelengths of 570 nm and 590 nm, respectively. The relative luciferase activity was determined for each compound after normalization to the cell viability. For selection of positive compounds, Z-score (Curtis et al., 2016) was calculated, which is defined as $Z = \frac{x-\mu}{\sigma}$, where x is relative luciferase activity of an individual compound, μ is the mean luciferase activity of all test compounds, and σ is the standard deviation of all test compounds in a 96-well plate. A compound with a minimum Z-score of 2.0, meaning that its luciferase activity is two standard deviations above that of the mean, was considered a hit (Curtis et al., 2016).

Secondary Screening of the Hit Compounds

Dose-response experiments were conducted in 96-well plates seeded with HTC/*AvBD9-luc* cells and treated with three different concentrations (5, 20, and 80 μg/ml) of all hits in duplicate for 24 h. Cell viability and luciferase assays were conducted as described above. For those compounds showing a robust dose-dependent response, their HDP-inducing activities were further validated in parental HTC cells (6×10^5/well) at different concentrations in 12-well plates. After 24 h stimulation, cells were subjected to RNA isolation and real-time RT-qPCR as described below.

RNA Extraction and RT-qPCR

After stimulation, cells were directly lysed in RNAzol RT (Molecular Research Center, Cincinnati, OH), followed by total RNA extraction. Maxima First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) or iSCRIPT RT Supermix (Bio-Rad) was used for cDNA synthesis and qPCR was performed using QuantiTect SYBR Green qPCR Master Mix (Qiagen, Valencia, CA) or iTaq Universal SYBR Green Supermix (Bio-Rad) as described (Sunkara et al., 2011, 2012, 2014). The expression levels of various chicken HDP genes as well as a house-keeping gene, glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*), were evaluated using gene-specific primers, and relative fold changes in gene expression were calculated using the ΔΔCt method as described (Sunkara et al., 2011, 2012, 2014).

Intestinal Explant Culture

Chicken jejunal explants were prepared as described (Sunkara et al., 2014). Briefly, an approximately 10-cm jejunal segment was collected from 1- to 2-week-old broiler chickens, washed thoroughly in cold PBS containing 100 μg/ml of gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin, and dissected into a series of small segments (approximately 5 × 5 mm). Jejunal segments were then placed individually in 6-well plates containing 4 ml RPMI 1640 medium supplemented with 10% FBS, 20 mM HEPES, 100 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin. The segments were treated in triplicate with different concentrations of a compound and then incubated in a Hypoxia Chamber (StemCell Technologies, Vancouver, BC, Canada) flushed with 95% O_2 and 5% CO_2 at 37°C for 24 h. Total RNA isolation and RT-qPCR analysis of chicken HDP gene

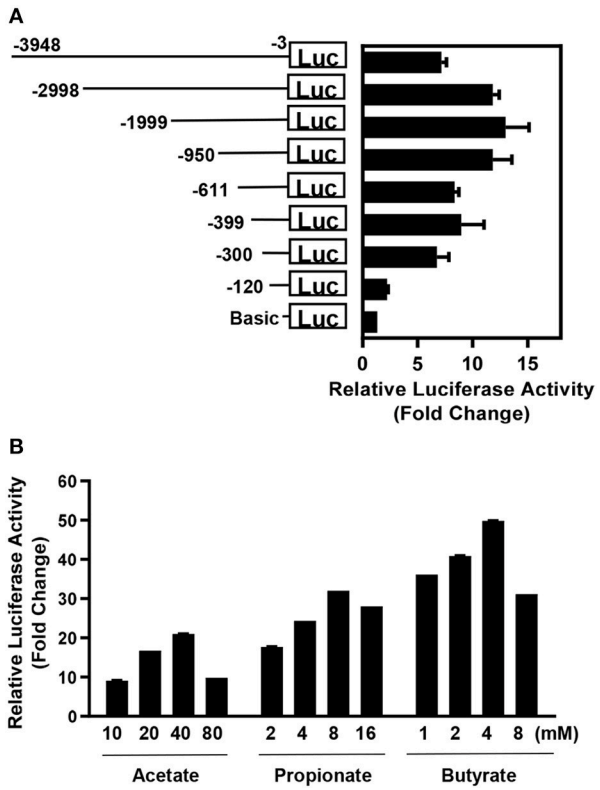

FIGURE 1 | Promoter analysis of the *AvBD9* gene. **(A)** *AvBD9* gene promoter constructs of different lengths were cloned into pGL4.21[*luc2P*/Puro] luciferase reporter vector and transfected into chicken HTC macrophage cells, followed by stimulation with 8 mM sodium butyrate for 24 h. The fold change in luciferase activity of the cells transfected with each *AvBD9* promoter construct in response to butyrate relative to that of the cells transfected with the promoterless basic vector is shown. **(B)** Chicken HTC cells were transfected with the pGL4.21[*luc2P*/Puro] luciferase reporter vector driven by a 2-Kb *AvBD9* gene promoter construct, followed by stimulation with or without different concentrations of short-chain fatty acids for 24 h. For each short-chain fatty acid, fold change in luciferase activity of stimulated cells was calculated relative to that of the non-stimulation control. The results are means ± SEM of 2–3 independent experiments.

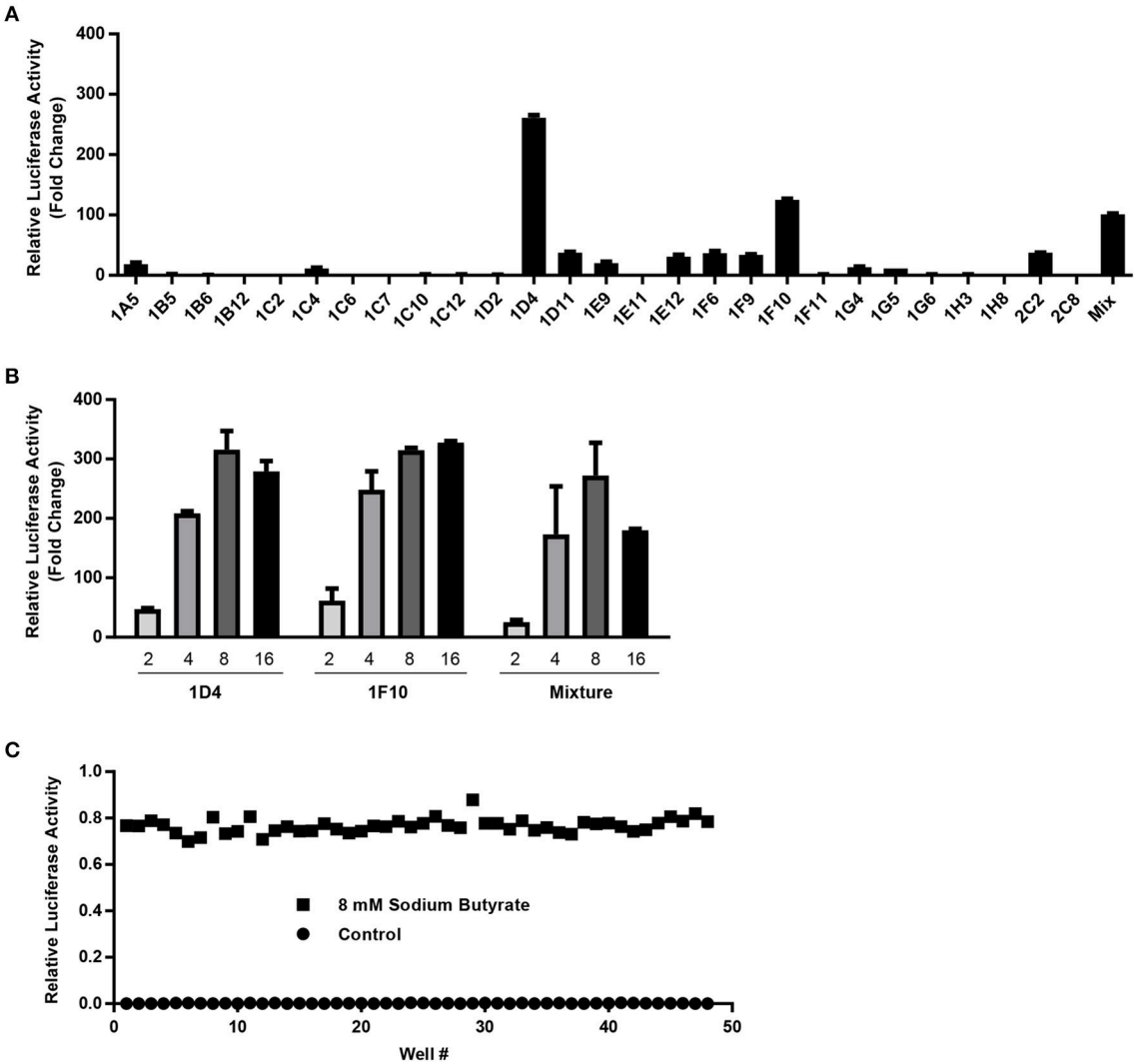

FIGURE 2 | Characterization and optimization of stable HTC/*AvBD9-luc* luciferase reporter cells for high-throughput screening. **(A)** The fold change in luciferase activity of each cell clone in response to 8 mM butyrate relative to that of non-stimulation control. **(B)** Dose-dependent response of two selected stable cell clones and stable cell mixture to sodium butyrate (mM). The results in **(A,B)** are means ± SEM of two independent experiments. **(C)** Relative luciferase activities of stable reporter cells in the presence or absence of 8 mM sodium butyrate for calculation of the Z'-factor.

expression were performed with jejunal explants after stimulation.

Oral Gavage of HDP-Inducing Compounds to Chickens

A total of 72 newly hatched male broiler chickens were obtained from Cobb-Vantress Hatchery (Siloam Springs, AR), housed on floor cages, divided randomly into groups of 6, and provided *ad libitum* access to a commercial antibiotic-free diet (DuMOR Chick Starter/Grower 20%) and tap water. After 3 days of acclimation, for each treatment group, 12 chickens in two cages were orally gavaged every 12 h for three times with 0.5 ml of PBS alone or PBS containing 5, 10, 20, or 40 μM wortmannin or 40 mM sodium butyrate. After 36 h of initial gavage, all birds were euthanized with carbon dioxide and cervically dislocated. A segment of the mid-duodenum was collected, snap frozen in liquid nitrogen, and stored at −80°C for future homogenization in RNAzol RT and RNA extraction. This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition (2010), by Federation of Animal Science Societies. The protocol was approved by the Institutional Animal Care and Use Committee of Oklahoma State University under protocol number AG1610.

Minimum Inhibitory Concentration (MIC) Assay

The MICs of wortmannin and butyrate were determined using a standard broth microdilution assay as recommended by the Clinical and Laboratory Standards Institute

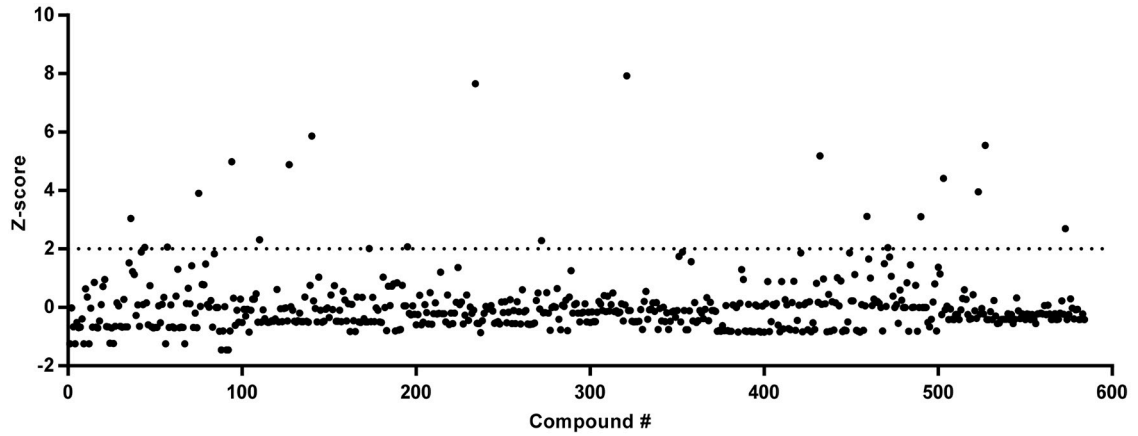

FIGURE 3 | Z-scores of 584 natural products following a primary screening. Stable HTC/*AvBD9-luc* luciferase reporter cells were stimulated with 20 μg/ml of natural products for 24 h in 96-well plates, followed by the luciferase assay. The alamarBlue Dye was added 4 h before the luciferase assay to measure cell viability. The luciferase activity of each compound was normalized to cell viability before the Z-score was calculated.

TABLE 2 | The Z-scores and major functions of 21 hits at 20 μg/ml from primary screening of the Natural Products Library.

Compound name	CAS number	Mass (g/mol)	Z score	Structural family	Major function[a,b]
Sanguinarine	5578-73-4	332.1	7.92	Benzophenanthridine alkaloid	Inducer of DNA damage
Datiscetin	480-15-9	286.2	7.66	Hydroxylated flavonoid	?
Wortmannin	19545-26-7	428.4	5.86	Steroid	Inhibitor of PI3K/DNA-PK and DNA repair
HC toxin	83209-65-8	436.5	5.55	Cyclic tetrapeptide	HDAC inhibitor
Hypocrellin B	123940-54-5	528.5	5.18	Perylenequinone	Inducer of DNA strand breakage
Parthenolide	20554-84-1	248.3	4.99	Sesquiterpene lactone	HDAC inhibitor
Tetrandrine	518-34-3	622.8	4.88	Bisbenzylisoquinoline alkaloid	Calcium channel blocker
Apicidin	183506-66-3	623.8	4.42	Cyclic tetrapeptide	HDAC inhibitor
(–)-Depudecin	139508-73-9	212.2	3.95	Polyketide	HDAC inhibitor
Isotetrandrine	477-57-6	622.8	3.91	Bisbenzylisoquinoline alkaloid	Calcium channel blocker
Silibinin	22888-70-6	482.4	3.11	Flavonolignan	STAT3, cyclo- and lipoxygenase inhibitor
Sclerotiorin	549-23-5	390.9	3.1	Azaphilone	HSP90 and lipoxygenase inhibitor
Cytochalasin D	22144-77-0	507.6	3.04	Alkaloid	Actin polymerization inhibitor
Trichostatin A	58880-19-6	302.4	2.7	Hydroxamic acid	HDAC inhibitor
Radicicol	12772-57-5	364.8	2.31	Polyketide	HSP90 and DNA topoisomerase II inhibitor
Tamarixetin	603-61-2	316.3	2.29	O-methylated flavonoid	?
Carminic acid	1260-17-9	492.4	2.07	Glucosidal hydroxyanthrapurin	?
Forskolin	66575-29-9	410.5	2.06	Labdane diterpene	Adenylyl cyclase agonist
Dihydroergocristine mesylate	24730-10-7	707.8	2.06	Ergot alkaloid	Serotonin receptor antagonist
Brassinin	105748-59-2	236.4	2.04	Indole phytoalexin	STAT3 and PI3K/Akt/mTOR inhibitor
Robinetin	490-31-3	302.2	2.01	Hydroxylated flavonoid	?

[a] *Although there is little information on their biological activities, datiscetin, tamarixetin, and robinetin are expected to have similar epigenetic functions in histone acetylation to structurally related quercetin.*

[b] *PI3K, phosphatidylinositide 3-kinases; DNA-PK, DNA-dependent protein kinase; HDAC, histone deacetylase; STAT3, signal transducer and activator of transcription 3; HSP90, heat shock protein 90; AKT, protein kinase B; mTOR, mechanistic target of rapamycin.*

(National Committee for Clinical Laboratory Standards, 2003) as we previously described (Xiao et al., 2006, 2009; Bommineni et al., 2007). Briefly, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076) and *Escherichia coli* (ATCC 25922) were streaked onto trypticase soy agar (Fisher Scientific) plates, followed by subculture of 2–3 individual colonies in trypticase soy broth (Fisher Scientific) with shaking at 37°C for 3 h to reach the mid-log phase. Bacteria were then diluted to 5×10^5 CFU/ml in Mueller Hinton Broth (Fisher Scientific). After dispensing 90 μl/well in a 96-well tissue culture plate, 10 μl of wortmannin were added in duplicate to final concentrations of 5, 10, 20, 40, 80, 160, and 320 μM with or without 4 mM sodium

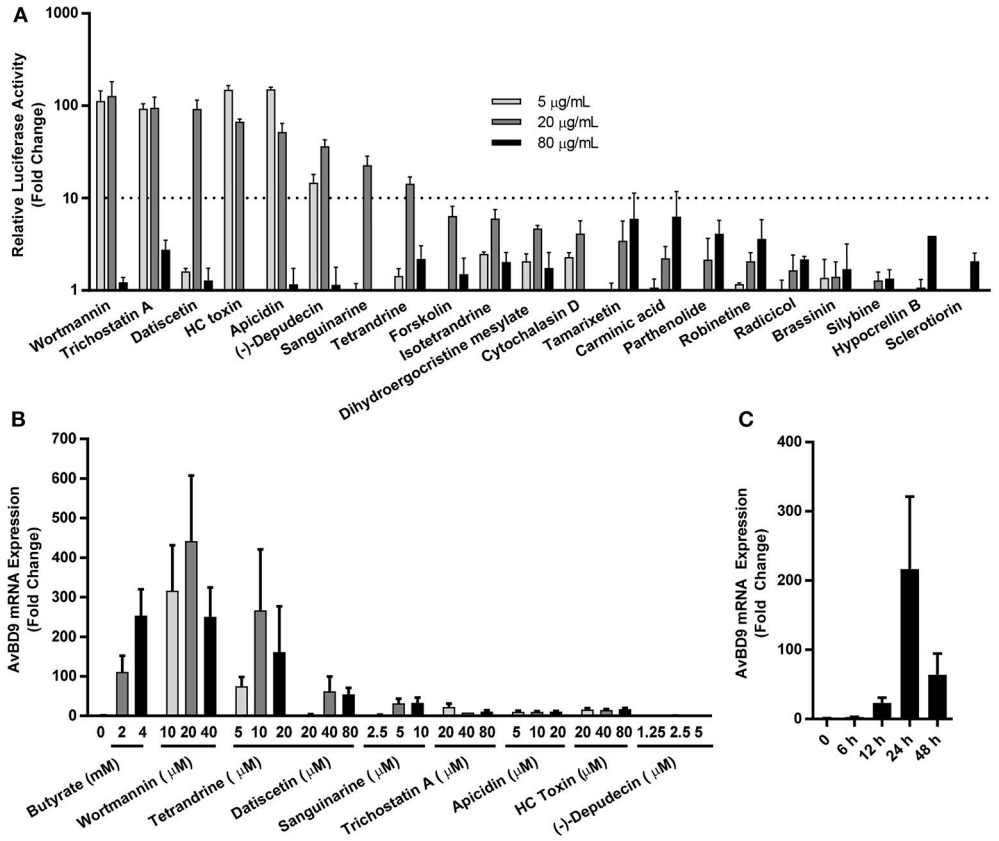

FIGURE 4 | Secondary screening of newly identified natural HDP-inducing compounds. **(A)** Dose-dependent changes in luciferase activity in stable HTC/*AvBD9-luc* luciferase reporter cells in response to 21 hits identified in the primary screening. **(B)** Dose-dependent induction of *AvBD9* mRNA expression in parental HTC cells stimulated with selected compounds for 24 h by RT-qPCR. **(C)** Time-dependent changes in *AvBD9* mRNA expression levels in HTC cells treated with 10 μM wortmannin by RT-qPCR. The results are means ± SEM of three independent experiments.

butyrate. MIC was determined as the lowest concentration of the compound or compound combination that gave no visible bacterial growth after overnight incubation at 37°C.

Antimicrobial Activity of Chicken Monocytes

The antibacterial activities of chicken monocytes treated with wortmannin, butyrate or their combination was assessed as described previously (Schauber et al., 2006; Sunkara et al., 2011) with slight modifications. In brief, peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated venous blood of 1- to 4-week-old male Cobb broilers through gradient centrifugation using Histopaque 1077 (Sigma-Aldrich). Monocytes were obtained by seeding PBMCs at 3×10^7 cells/well in complete RPMI 1640 medium containing 10% FBS, 20 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin in 6-well plates overnight and washing off non-adherent cells twice with calcium- and magnesium-free Hank's balanced salt solution (HyClone, Pittsburgh, PA). Monocytes were replenished with fresh complete RPMI 1640 medium and stimulated in duplicate with 40 μM wortmannin in the presence or absence of 4 mM butyrate. After 24 h, cells were scraped, washed twice with calcium- and magnesium-free Hank's balanced salt solution, and resuspended in 100 μl water. Cells were then frozen at −80°C for 20 min, thawed, and sonicated for 30 s, followed by centrifugation at 12,000 × *g* for 10 min at 4°C. Cell supernatants were collected and 20 μl of the supernatants were incubated with 80 μl of *S. enteritidis* (ATCC 13076) at 2.5×10^5 CFU/ml in 20% trypticase soy broth containing 1 mM NaH_2PO_4 and 25 mM $NaHCO_3$ in a 96-well plate at 37°C. Bacterial turbidity was measured at OD_{600} using SpectraMax M3 (Molecular Devices, Sunnyvale, CA) at 3, 6, 9, and 24 h.

Statistical Analysis

Data are expressed as means ± SEM. Statistics was performed with GraphPad Prism (San Diego, CA) using unpaired Student's two-tailed *t*-test. $P < 0.05$ was considered significant.

RESULTS

Selection of Appropriate *AvBD9* Gene Promoter Constructs to Establish a Stable Cell Line

AvBD9 has been shown to be the most inducible HDP gene in response to butyrate and several other small-molecule compounds in chickens (Sunkara et al., 2011,

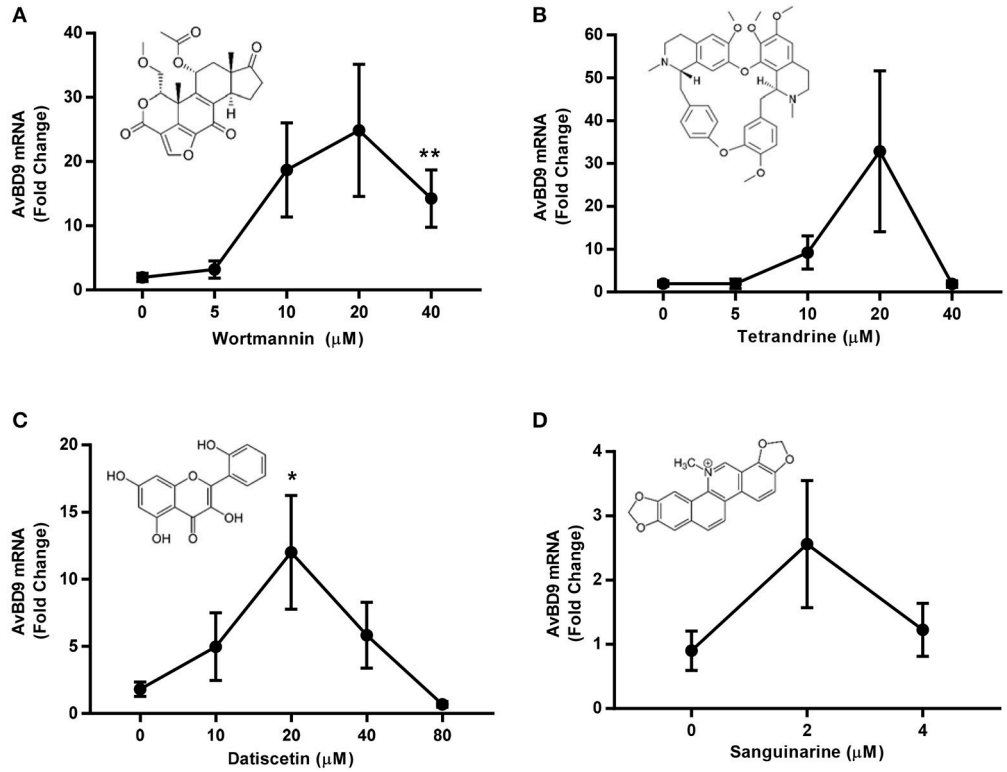

FIGURE 5 | Dose-dependent induction of *AvBD9* mRNA expression in response to wortmannin, tetrandrine, datiscetin, and sanguinarine in chicken jejunal explants. Jejunal explants were prepared and treated with different concentrations of each compound for 24 h, followed by RT-qPCR analysis of *AvBD9* mRNA expression. The results are means ± SEM of 2–3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ (by unpaired Student's *t*-test).

2014). Therefore, the *AvBD9* gene promoter was chosen to drive luciferase reporter gene expression. In order to select an appropriate promoter segment to provide maximum luciferase activation, eight *AvBD9* promoter constructs of varying lengths were cloned into a luciferase reporter vector, pGL4.21[*luc2P*/Puro] (Promega). The recombinant vectors were separately transfected into chicken HTC macrophage cells and stimulated with 8 mM sodium butyrate for 24 h. Luciferase assay revealed that *AvBD9* promoter activity was clearly length-dependent, with the 2-Kb promotor construct giving a maximum 13-fold increase in luciferase activity relative to a basic promoterless construct (**Figure 1A**). Consequently, the 2-Kb *AvBD9* construct was used for subsequent stable cell line development. Besides butyrate, the 2-Kb *AvBD9* construct was also confirmed to respond to two other short-chain fatty acids, namely acetate and propionate, in a dose-dependent manner after transfection into HTC cells (**Figure 1B**).

It is noted that omission of either of two promoter regions (120–300 or 611–950 bp) upstream of the *AvBD9* start codon resulted in greatly diminished luciferase activity in response to butyrate (**Figure 1A**), implying the presence of consensus binding sites for critical transcription factors in these two regions. Conversely, inclusion of a 950-bp segment upstream of the 2,998-bp region obviously suppressed the luciferase activity (**Figure 1A**), suggesting the existence of the binding site for a negative regulator. A preliminary scanning for putative transcription factor binding sites in those regions revealed several candidate transcription factors (data not shown), which are currently being experimentally verified.

Establishment of a Cell-Based HTS Assay to Identify *AvBD9*-Inducing Compounds

To establish a stable luciferase reporter cell line driven by the *AvBD9* gene promoter, the 2-Kb *AvBD9* promoter construct that gave the highest fold increase in response to butyrate (**Figure 1A**) was cloned into a lentiviral luciferase reporter vector, pGreenFire1-mCMV-Puro (System Biosciences). Pseudoviruses were generated in 293T cells and subsequently used to infect chicken HTC macrophages. After 1 week of selection in puromycin, a portion of surviving cells were subjected to limiting dilution in 96-well plates. Individual cell clones were gradually expanded, followed by evaluation of their responsiveness to butyrate. Among 27 cell clones analyzed, 1D4 and 1F10 showed the highest fold increase, and both were superior to the mixture of cells prior to limiting dilution (**Figure 2A**). These two cell clones were further confirmed to contain the 2-Kb transgene by PCR (data not shown) and gave a similar 300-fold increase in luciferase activity following 24-h stimulation with 8 mM sodium butyrate (**Figure 2B**). Therefore, these two stable reporter cell

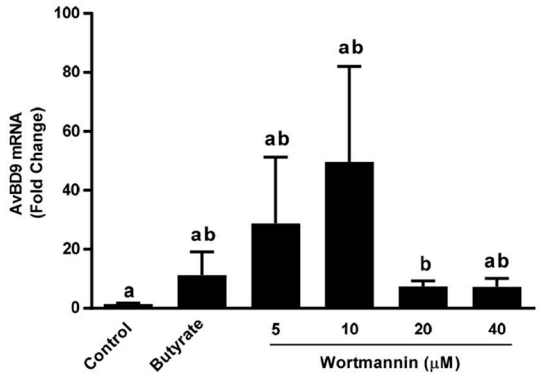

FIGURE 6 | *In vivo* induction of *AvBD9* mRNA expression in the duodenum of chickens by wortmannin. Different concentrations of wortmannin or 40 mM sodium butyrate were administered to 3-day-old chickens ($n = 12$) by oral gavage every 12 h for 36 h. A segment of the mid-duodenum was collected and subjected to total RNA isolation and RT-qPCR analysis of *AvBD9* mRNA expression. Fold changes were calculated relative to the control chickens receiving an equal volume of saline three times. The bars without common superscript letters denote statistical significance (by unpaired Student's *t*-test).

clones, named HTC/*AvBD9-luc,* were used interchangeably and 8 mM butyrate was used as positive control in subsequent HTS assays. To further evaluate the robustness of the HTS assay, we assessed the Z'-factor (Zhang et al., 1999) by measuring luciferase activity of stable cells stimulated with or without 8 mM butyrate in a 96-well plate. Positive controls (8 mM butyrate) were clearly separated from negative controls (no stimulation) (**Figure 2C**), and the Z'-factor was calculated to be 0.80, indicating that the HTS assay is excellent (Zhang et al., 1999).

Identification and Validation of Natural HDP-Inducing Compounds

To identify natural small-molecule compounds with the ability to induce *AvBD9*, natural product and rare natural product libraries of 584 compounds were screened at a final concentration of 20 μg/ml in 96-well plates. The Z-scores of all compounds tested were shown in **Figure 3**. Using a Z-score of 2.0 as the threshold (Curtis et al., 2016), 21 hits were identified and they represent a structurally diverse group of natural products, with a majority being flavonoids and alkaloids (**Table 2**). To our surprise, many compounds are involved in epigenetics by regulating histone modification and DNA repair (**Table 2**). It is noted that none of the compounds had a Z-score of less than −2.0, suggesting that none had a strong activity to suppress *AvBD9* gene expression.

To further validate the *AvBD9*-inducing activity of the 21 hits identified in the primary screening, dose-response experiments were conducted in stable HTC/*AvBD9-luc* cells. When applied at 5, 20, and 80 μg/ml for 24 h, all compounds showed an obvious dose-dependent change in luciferase activity, and at least one concentration of each compound resulted in an increased luciferase activity (**Figure 4A**), indicative of the validity of our primary screening. Out of 21 compounds, eight including datiscetin, wortmannin, tetrandrine, trichostatin A, HC toxin, (−)-depudecin, apicidin, and sanguinarine had a higher than 10-fold increase in luciferase activity, and were consequently chosen for further confirmation of mRNA expression in parental HTC cells by RT-qPCR. As expected, all compounds, except for (−)-depudecin, induced *AvBD9* mRNA expression (**Figure 4B**), signifying the reliability of the HTS assay in identifying *AvBD9*-inducing compounds. It is currently unknown why (−)-depudecin, a known HDP inducer in human cells (Kallsen et al., 2012), purchased from two different vendors including BioVision (Milpitas, CA) and MyBioSource (San Diego, CA) failed to work. It is likely because of a variation in structural integrity among difference sources as (−)-depudecin is chemically instable due to the presence of two oxirane rings separated by a *trans* double bond (Kwon et al., 1998).

Among the compounds that induced *AvBD9* mRNA expression, wortmannin, tetrandrine, datiscetin, and sanguinarine were the most potent (**Figure 4B**). Wortmannin and tetrandrine, when used in the μM range, showed a comparable, if not superior, fold increase to 2 or 4 mM butyrate that gave a maximum 100- to 250-fold *AvBD9* mRNA induction. We further confirmed in a time-course experiment that wortmannin gave a peak induction of *AvBD9* mRNA expression at 24 h (**Figure 4C**). It is worth mentioning that higher doses of most compounds showed diminished *AvBD9* induction, suggesting the existence of a negative feedback mechanism.

Ex Vivo and *in Vivo* Confirmation of *AvBD9* Induction

To verify the ability of individual compounds to induce *AvBD9* expression in the intestinal tract, chicken jejunal explants were prepared and stimulated with different concentrations of wortmannin, tetrandrine, datiscetin, and sanguinarine for 24 h. All four compounds showed an obvious dose-dependent induction of *AvBD9* in jejunal explants. The optimal dose for wortmannin, tetrandrine, and datiscetin was 20 μM with a 15- to 40-fold induction of *AvBD9* mRNA, while 2 μM sanguinarine gave a peak induction of approximately 2.5-fold (**Figure 5**). To further confirm whether wortmannin is capable of inducing *AvBD9 in vivo*, 3-day-old broiler chickens were given 5, 10, 20, or 40 μM wortmannin or 40 mM butyrate by oral gavage every 12 h for 36 h. RT-qPCR analysis of *AvBD9* gene expression in the duodenum revealed that 5 and 10 μM wortmannin increased *AvBD9* mRNA expression by approximately 30- and 50-fold, respectively, which was superior to 40 mM butyrate showing a 10-fold increase (**Figure 6**).

Induction of Multiple Chicken HDP Genes by Natural Compounds and Their Synergy With Butyrate

Besides *AvBD9*, 13 other β-defensins and 4 cathelicidins exist in chickens (Cuperus et al., 2013; Zhang and Sunkara, 2014) and butyrate can induce more than a half number of them (Sunkara et al., 2011). To examine how other chicken HDP

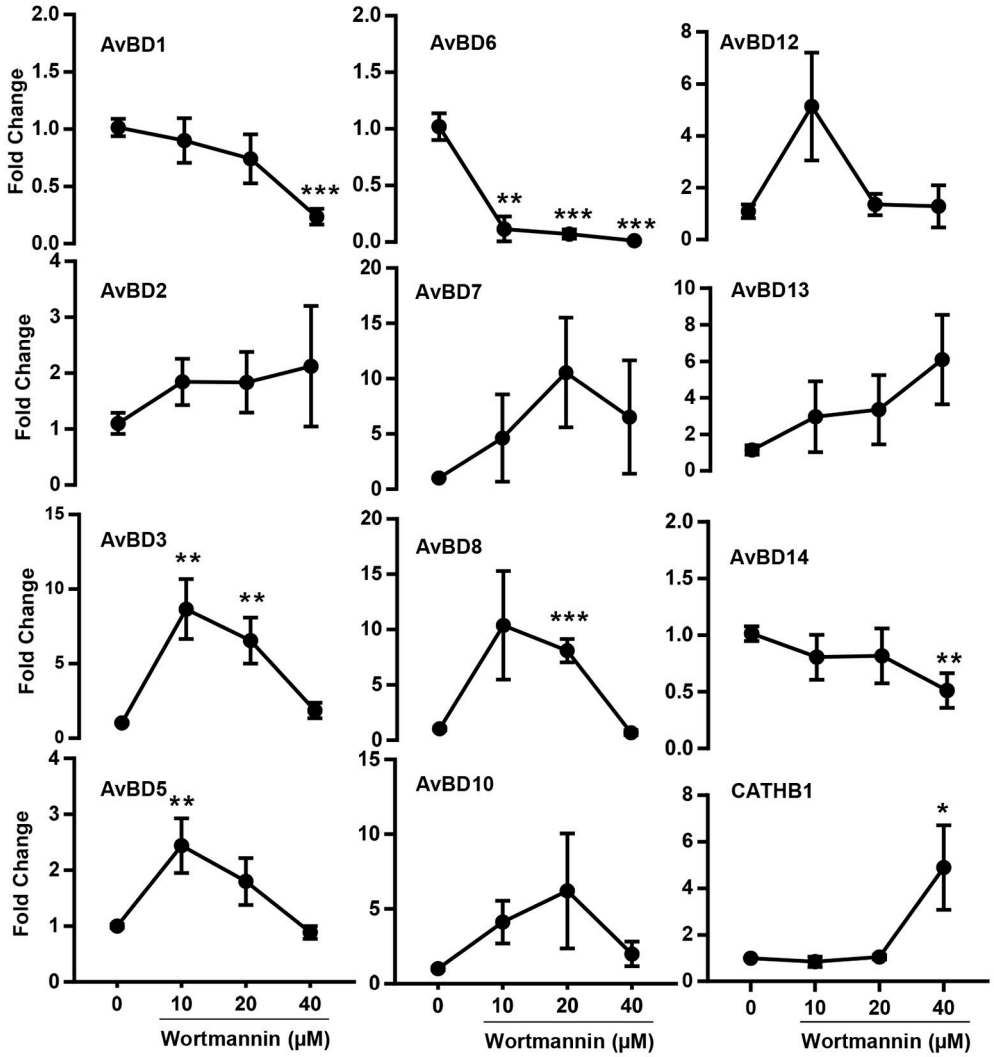

FIGURE 7 | Dose-dependent changes in multiple chicken HDP mRNA expression levels in HTC cells by wortmannin. Chicken HTC cells were treated with or without three different concentrations of wortmannin for 24 h, followed by RT-qPCR analysis of mRNA expression of all chicken HDP genes that can be detected in HTC cells. The results are means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (by unpaired Student's *t*-test).

genes are regulated by wortmannin, HTC cells were stimulated with or without three different doses (10, 20, and 40 μM) of wortmannin for 24 h, followed by RT-qPCR of individual β-defensins and cathelicidins. Among those HDP genes that can be detected in HTC cells, all but three were obviously induced by wortmannin, albeit with a reduced magnitude of induction relative to *AvBD9* (**Figure 7**). Clearly, different HDP gene showed different patterns of induction. Wortmannin dose-dependently increased *AvBD2, AvBD13,* and *CATHB1* gene expression, while *AvBD3, 5, 7, 8, 9, 10,* and *12* had a peak induction at 10 or 20 μM (**Figure 7**). On the other hand, *AvBD1, 6*, and *14* was dose-dependently suppressed by wortmannin.

Intrigued by the synergy between butyrate with other small-molecule compounds such as vitamin D_3 (Schauber et al., 2008), lactose (Cederlund et al., 2013), and forskolin (Sunkara et al., 2014), we sought to evaluate a possible synergistic action between butyrate and several newly-identified HDP-inducing compounds. A dramatic synergy was observed between butyrate and any of wortmannin, tetrandrine and datiscetin in HTC cells, but not between butyrate and sanguinarine (**Figures 8A–D**). For example, 4 mM butyrate and 40 μM wortmannin individually enhanced *AvBD9* expression by approximately 200- and 250-fold, respectively, while a combination of 4 mM butyrate and 40 μM wortmannin induced *AvBD9* expression by approximately 15,500-fold, which reflected an additional 60-fold increase over either compound alone (**Figure 8A**). Similarly, the butyrate/tetrandrine (**Figure 8B**) and butyrate/datiscetin combinations (**Figure 8C**) also displayed a strong synergy separately. However, no synergy was observed

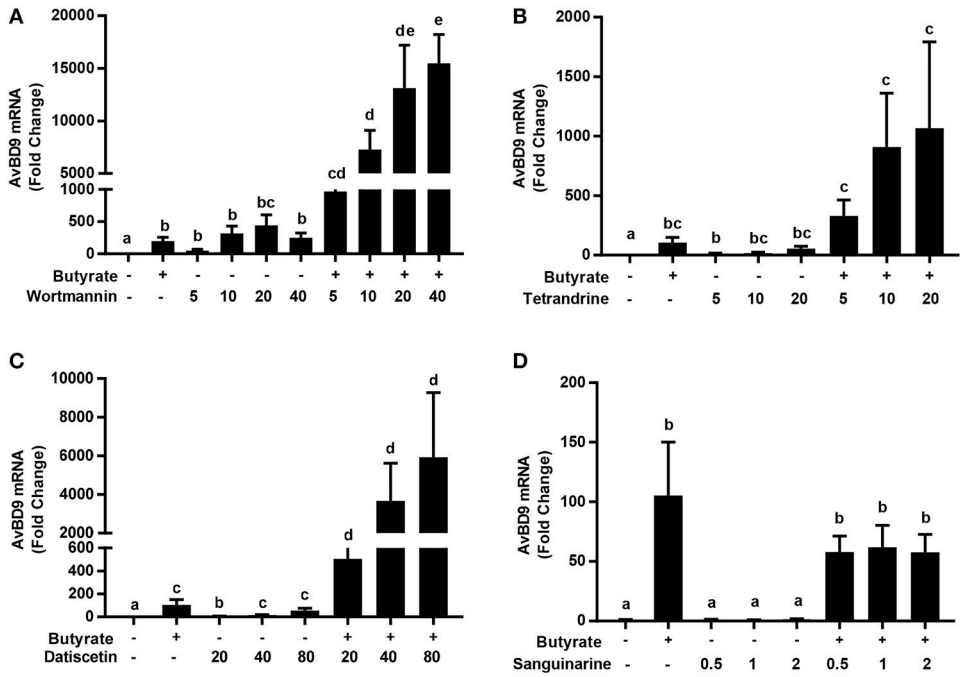

FIGURE 8 | Synergistic induction of *AvBD9* mRNA expression in HTC cells between butyrate and three newly identified natural products. Chicken HTC cells were treated with 4 mM butyrate in the presence or absence of different concentrations of wortmannin **(A)**, tetrandrine **(B)**, datiscetin **(C)**, or sanguinarine **(D)** for 24 h, followed by RT-qPCR analysis of *AvBD9* mRNA expression. The results are means ± SEM of 2–3 independent experiments. The bars without common superscript letters denote statistical significance (by unpaired Student's *t*-test). It is noted that an obvious synergy in AvBD9 gene expression was observed between butyrate and any of wortmannin, tetrandrine, datiscetin, but not between butyrate and sanguinarine.

between butyrate and sanguinarine (**Figure 8C**), suggesting the mechanisms of action among different compounds are likely to be different.

Augmentation of the Antibacterial Activity of Chicken Monocytes by Wortmannin

HDP inducers such as butyrate and vitamin D_3 are capable of enhancing the antibacterial activity of host cells (Schauber et al., 2006, 2008; Liu et al., 2007; Sunkara et al., 2011; Rekha et al., 2015) and alleviate disease symptoms (Sarker et al., 2011; Zhao et al., 2014; Mily et al., 2015).To confirm whether wortmannin or the combination of wortmannin and butyrate can augment the antibacterial activity of host cells, chicken monocytes were isolated and stimulated with 40 μM wortmannin, 4 mM sodium butyrate or their combination for 24 h, followed by incubation of the cell lysate with *S. enteritidis* (ATCC 13076) and measurement of the bacterial turbidity (Schauber et al., 2006; Sunkara et al., 2011). Consistent with our earlier observation (Sunkara et al., 2011), butyrate-treated monocytes exhibited an obviously enhanced ability to suppress bacterial growth (**Figure 9**). Wortmannin also improved the ability of monocytes to kill bacteria. Importantly, a combination of wortmannin and butyrate resulted in nearly complete suppression of bacterial growth up to 24 h, suggestive of their synergistic activity.

To rule out the possibility that augmented bacterial killing of chicken monocytes is due to direct antibacterial activity of wortmannin or butyrate, a standard broth microdilution assay (National Committee for Clinical Laboratory Standards, 2003) was performed using two reference bacterial strains, *S. enteritidis* (ATCC 13076) and *E. coli* (ATCC 25922) exposed to wortmannin in serial 2-fold dilutions in the presence or absence of 4 mM butyrate. Wortmannin alone or in combination with 4 mM butyrate showed no obvious antibacterial activity, with the MIC beyond 320 μM, the highest concentration that we tested (data not shown), implying that wortmannin, particularly the wortmannin/butyrate combination, could enhance HDP synthesis and bacterial clearance without exerting selective pressure on bacteria, thus reducing the likelihood of triggering bacterial resistance against HDP inducers.

Involvement of the PI3K/AKT/mTOR Pathway in *AvBD9* Gene Induction

Phosphoinositide 3-kinases (PI3K) are a family of structurally related enzymes that are involved in a variety of cellular functions that often signal through protein kinase B (also known as AKT) and mechanistic target of rapamycin (mTOR) (Polivka and Janku, 2014; Fruman et al., 2017). Wortmannin is a well-known inhibitor of PI3K (Ui et al., 1995) and brassinin is a newly identified AvBD9-inducing compound (**Table 2**) also with a reported PI3K inhibitory activity (Izutani et al., 2012). To examine whether the PI3K/AKT/mTOR pathway is involved in *AvBD9* gene induction, specific inhibitors to PI3K (PX-866, LY294002, and CAL-101), AKT (MK2206, GDC0068, triciribine) or mTOR (Rapamycin, AZD8055) or dual inhibitors

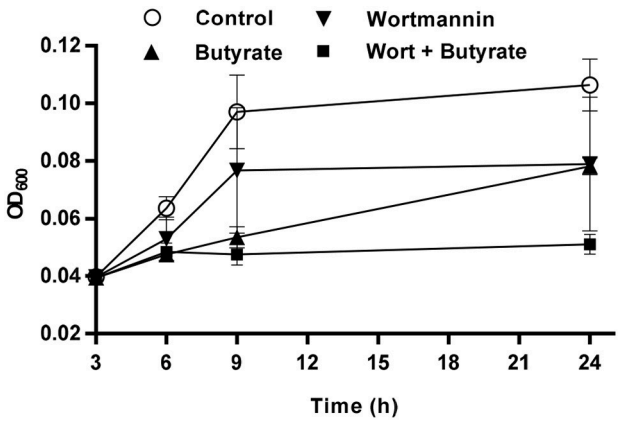

FIGURE 9 | Augmentation of the antibacterial activity of chicken monocytes by wortmannin and butyrate. Chicken monocytes were stimulated with 40 μM wortmannin, 4 mM butyrate or their combination for 24 h. Cell lysates were then incubated with *Salmonella enteritidis* (ATCC 13076) at 37°C. The bacterial turbidity was measured at OD_{600} at 3, 6, 9, and 24 h. The results are means ± SEM of two independent experiments.

to PI3K/mTOR (BEZ235) or PI3K/HDAC (CUDC-907) were applied to HTC cells separately for 24 h, followed by RT-qPCR analysis of *AvBD9* gene expression. Among all four PI3K inhibitors, only pan-inhibitors, wortmannin and its structural analog PX-866, gave a robust *AvBD9* induction, while another pan-inhibitor (LY294002) and an isoform-specific inhibitor (CAL-101) showed a minimum or no activity (**Figure 10**), suggesting that specific inhibition of PI3K may have a limited effect on *AvBD9* induction. The reason that wortmannin and PX-866 work well is likely due to their non-specific activities. Wortmannin is highly efficient in suppressing PI3K in the low nanomolar range, but can non-specifically inhibit several other PI3K-related kinases such as DNA-dependent protein kinase (DNA-PK) at higher concentrations (Wymann et al., 1996). In this study, the micromolar concentrations of wortmannin are needed to induce chicken HDP genes, and no appreciable HDP gene induction was observed when wortmannin was used below 1 μM (data not shown). Therefore, it is likely that PI3K inhibition alone is insufficient for robust HDP gene induction. In agreement, none of the three AKT inhibitors or mTOR inhibitors had an obvious ability to induce *AvBD9* expression (**Figure 10**), suggesting a minimum involvement of the PKA/AKT/mTOR signaling pathway in chicken *AvBD9* induction. Interestingly, dual inhibition of PI3K/mTOR or PI3K/HDACs gave obvious *AvBD9* expression in chicken HTC cells, albeit with a much reduced fold increase relative to wortmannin or PX-866 (**Figure 10**).

DISCUSSION

Increased resistance to conventional antibiotics necessitates the development of novel antimicrobial strategies. With no or reduced likelihood of triggering resistance, host-directed antimicrobial therapies are gaining increased attention, with a number of products being approved for human use or evaluated at different stages of clinical trials (Zumla et al., 2016). Moderating the synthesis of endogenous HDPs has shown promise in reducing infections and alleviating clinical infections and is being actively explored as an alternative approach to antimicrobial therapy (Campbell et al., 2012; Van Der Does et al., 2012; Lyu et al., 2015). A number of small-molecule compounds such as butyrate and vitamin D_3 have been identified to induce HDP synthesis in humans and other animal species (Campbell et al., 2012; Van Der Does et al., 2012; Lyu et al., 2015). A cell-based HTS assay was recently developed by employing human HT-29 intestinal epithelial cells transfected with a fusion of a 4-Kb human cathelicidin *LL-37* gene promoter and its entire open reading frame with a luciferase reporter gene (Nylen et al., 2014). Such an approach has led to discovery of a group of compounds with the ability to induce *LL-37* gene expression; however, a majority of the compounds are weak relative to many known LL-37 inducers such as butyrate (Nylen et al., 2014). To facilitate the identification of additional, perhaps more potent HDP inducers particularly for poultry applications, we developed a HTS assay by fusing a luciferase gene with a 2-Kb *AvBD9* gene promoter, followed by lentiviral transduction into a chicken macrophage cell line. After optimization, we obtained a Z'-factor of 0.80 for our HTS assay, which suggested that it is a robust system (Zhang et al., 1999) and equivalent to the previously reported human HTS assay that had a Z'-factor of approximately 0.70 (Nylen et al., 2014). By employing such a HTS assay, we identified 21 natural compounds with a strong ability to boost *AvBD9* gene expression after a screening of 584 natural products.

Out of the 21 HDP-inducing compounds identified in this study, only forskolin, trichostatin A (TSA), apicidin, and (–)-depudecin have been reported earlier with the ability to induce HDP gene expression in humans and chickens (Yin and Chung, 2011; Kallsen et al., 2012; Sunkara et al., 2014). The 17 remaining compounds are linked with a role in HDP induction for the first time. To our surprise, 14 out of 21 HDP-inducing compounds are involved in epigenetic modifications of DNA, histones or non-histone proteins (**Table 2**). TSA, HC toxin, parthenolide, apicidin, and (–)-depudecin are known histone deacetylase (HDAC) inhibitors (Bassett and Barnett, 2014), which contribute to histone hyper-acetylation, chromosomal relaxation, and often enhanced gene expression (Chen et al., 2015). Consistently, human HDPs such as LL-37, β-defensin-1, and β-defensin-2 have been found to be upregulated by HDAC inhibitors such as butyrate, TSA, apicidin, sulforaphane, curcumin, MS-275, and resveratrol and its analogs (Yedery and Jerse, 2015). Although it has not been definitively confirmed, datiscetin, tamarixetin, and robinetin are all structurally similar to quercetin, a natural flavonol with the ability to regulate the activities of histone acetyltransferases, sirtuins, and classical HDACs (Lee et al., 2011; Xiao et al., 2011; Trevino-Saldana and Garcia-Rivas, 2017). Therefore, it is very likely these quercetin-like flavonoids have histone modifying functions as well. Besides histone acetylation, four compounds including sanguinarine, wortmannin, hypocrellin B, and radicicol are known to induce DNA damage, inhibit DNA repair or DNA topoisomerase II activity (Xu et al., 1998; Hashimoto et al., 2003; Barker et al., 2006; Matkar et al., 2008), which has been recently revealed to exert a

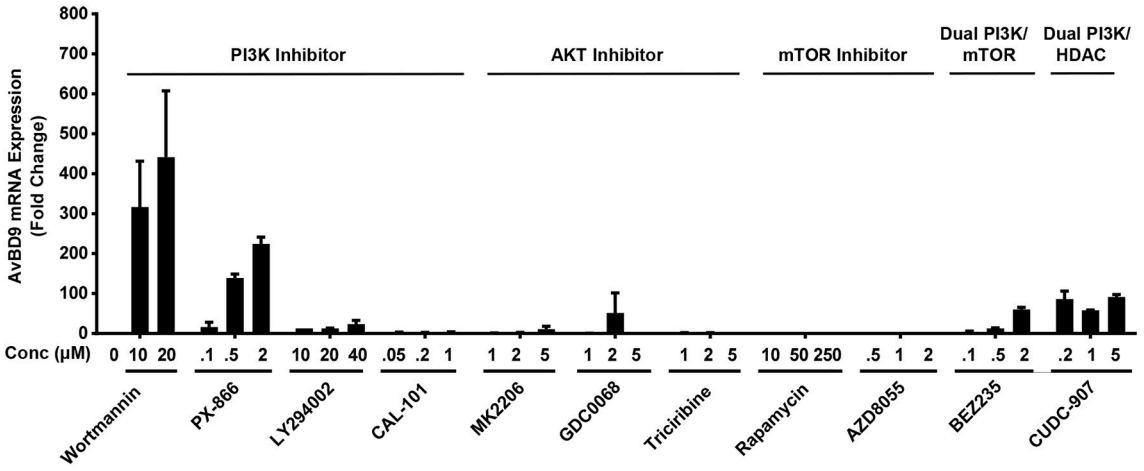

FIGURE 10 | Involvement of the PI3K/AKT/mTOR signaling pathway in AvBD9 mRNA induction. Chicken HTC cells were treated with or without different concentrations of specific inhibitors to PI3K (Wortmannin, PX-866, LY294002, and CAL-101), AKT (MK2206, GDC0068, triciribine) or mTOR (Rapamycin, AZD8055), dual PI3K/mTOR inhibitor (BEZ235) or dual PI3K/HDAC inhibitor (CUDC-907) for 24 h, followed by RT-qPCR analysis of *AvBD9* gene expression. The results are means ± SEM of 2–3 independent experiments.

positive role in initiation of gene transcription (Pommier et al., 2016; Vitelli et al., 2017). Additionally, radicicol, sclerotiorin, and sanguinarine are capable of inhibiting heat shock protein 90 (HSP90) (Davenport et al., 2014; Kabbaj et al., 2015; Khandelwal et al., 2016), which is known to interact with DNA topoisomerase II (Barker et al., 2006) and whose activity is regulated by reversible acetylation (Prodromou, 2016). Thus, inhibition of DNA repair or HSP90 could positively impact the transcription of a subset of genes perhaps including many HDPs.

Wortmannin, tetrandrine, datiscetin, and sanguinarine are among the four most potent *AvBD9*-inducing compounds identified in this study. Wortmannin is a fungal metabolite and a well-known inhibitor of phosphoinositide 3-kinases (PI3K) (Ui et al., 1995), which are critically involved in a variety of cellular metabolism and immune functions (Fruman et al., 2017). However, the PI3K/AKT/mTOR pathway alone appears to play a minimum role in *AvBD9* gene induction because most specific inhibitors to PI3K, AKT and mTOR are largely ineffective in inducing *AvBD9*. The reason that wortmannin is highly efficient is likely due to its dual inhibitory role to both PI3K and DNA-PK (Hashimoto et al., 2003), with the latter being required to repair double-strand DNA breaks via the non-homologous end joining pathway (Davis et al., 2014; Blackford and Jackson, 2017). Inhibition of both PI3K and DNA repair perhaps creates a synergistic effect on HDP gene induction.

Among the other three potent *AvBD9* inducers, tetrandrine is a *bis*-benzylisoquinoline alkaloid extracted from the root of a Chinese herb, *Stephania tetrandra* S. Moore (Bhagya and Chandrashekar, 2016; Liu et al., 2016). Datiscetin is a plant-derived flavonoid and structurally related to quercetin that is known to have epigenetic functions (Lau and Chang, 2015) and a strong ability to induce chicken HDP genes (data not shown). Therefore, it is of little surprise that datiscetin is capable of inducing *AvBD9*. Sanguinarine is a benzophenanthridine alkaloid extracted from the bloodroot plant *Sanguinaria canadensis* (Selvi et al., 2009) that can cause DNA damage (Matkar et al., 2008), which could subsequently lead to an increase in *AvBD9* gene transcription. However, the mechanism by which tetrandrine induces AvBD9 expression remains unknown. Tetrandrine is a well-known calcium channel blocker and has been used as a Chinese traditional medicine for decades to treat hypertensive and arrhythmic conditions, inflammation, fibrosis, and silicosis (Bhagya and Chandrashekar, 2016; Liu et al., 2016). Whether tetrandrine augments *AvBD9* expression by acting as a calcium channel blocker warrants further investigations. We observed a strong synergy in *AvBD9* gene induction between butyrate, a well-studied pan-HDAC inhibitor, and any of wortmannin, datiscetin and tetrandrine, but not between butyrate and sanguinarine. The mechanism behind their synergy needs to be further investigated.

Although structurally divergent from each other, a common feature among wortmannin, tetrandrine, and sanguinarine is that they are all anti-inflammatory, antioxidative, anti-proliferative, and pro-apoptotic (Ui et al., 1995; Selvi et al., 2009; Bhagya and Chandrashekar, 2016; Liu et al., 2016). So is likely the case with datiscetin because of the anti-inflammatory and antioxidative properties associated with structurally-related quercetin (Chirumbolo, 2010). The ability to enhance HDP gene expression and antioxidative response without triggering inflammation makes these compounds desirable for further development as host-directed therapeutics for disease control and prevention. The fact that some of these compounds have demonstrated synergistic actions among each other suggests the potential of employing these compounds or their combinations as alternatives to antibiotics for poultry applications. Additionally, due to a lack of direct antimicrobial activities, these HDP inducers augment host immunity and disease resistance with a minimum risk of triggering resistance.

Furthermore, because many HDP inducers have been found to promote HDP synthesis across animal species (Yedery and Jerse, 2015), it is likely that a few, if not all, of these newly-identified compounds are capable of enhancing HDP synthesis and disease control and prevention beyond chickens.

AUTHOR CONTRIBUTIONS

GZ and WL: conceived and designed the experiments; WL, ZD, LS, SB, and KR: performed the experiments; WL and GZ: analyzed and interpreted the data; RM: provided the reagents; WL and GZ: drafted and revised the manuscript.

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