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***In vitro* model of mycobacteria and HIV-1 co-infection for drug discovery**

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Abstract

Tuberculosis (TB) has become a global health threat in the wake of the Human Immunodeficiency Virus (HIV) pandemic and is the leading cause of death in people with HIV/AIDS. Treatment of patients with *Mycobacterium tuberculosis* (*Mtb*)/HIV co-infection is complicated by drug interactions and toxicity that present huge challenges for clinical intervention. Discovery efforts to identify novel compounds with increased effectiveness and decreased drug-drug interactions against *Mtb*, HIV-1, or both, would be greatly aided by the use of a co-infection model for screening drug libraries. Currently, inhibitors of *Mtb* are screened independently in mycobacterial cell cultures or target based biochemical screens and less often in macrophages or peripheral blood leukocytes. Similarly, HIV-1 drugs are screened *in vitro* independently from anti-mycobacterial compounds. Here, we describe an *in vitro* model where primary human peripheral blood mononuclear cells or monocyte-derived macrophages are infected with *Mycobacterium bovis* BCG and HIV-1, and used to evaluate drug toxicity and activity in a co-infection setting. Our results with standard compounds (e.g. Azidothymidine, Rifampicin) demonstrate the utility of this *in vitro* model to evaluate drug effectiveness relevant to cellular toxicity, HIV-1 replication, and intracellular mycobacterial growth, through the use of ELISA, bacterial enumeration, and multi-

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Competing interests

The authors have no financial conflict of interest.

variate flow cytometry. This model and associated assays have great value in accelerating the discovery of compounds for use in *Mtb*/HIV-1 co-infected patients.

Keywords

HIV; TB; *In vitro* model; Drug screening; Drug interactions

1. Introduction

The prevalence of tuberculosis (TB) has caused a global health crisis due to the re-emergence and the rapid development of drug resistant *Mtb* isolates. The rising incidence of drug resistant isolates has made development of more effective clinical treatments a top priority. HIV-1 infection is a significant contributing factor in both the re-emergence of TB and the alarming rate of *Mtb* drug resistance development [refer to reviews²⁻⁴]. Infection with HIV-1 increases the susceptibility to new TB infection, increases the risk of reactivation of latent TB infection, and can promote an aggressive course of TB in those with active TB. An estimated 14 million people worldwide have *Mtb*/HIV-1 co-infection and TB is the leading cause of death in people with HIV/AIDS.^{1,5} Treatment of patients with *Mtb*/HIV-1 co-infection is further complicated by host, pathogen, and drug interactions that are very poorly understood and thus present huge challenges for clinical intervention.⁶

In most clinical settings, TB is treated prior to HIV-1 in co-infected patients for several reasons. Firstly, anti-retrovirals (ARV) work poorly in people with active TB infection because the immune activation by *Mtb* actually aids viral replication.^{7,8} The control of *Mtb* replication relies heavily on cell-mediated immunity and the effectiveness of TB drugs depends on complementary action among the patient immune system and drug action. Therefore, TB drugs are much less effective in people with compromised immune systems; TB treatment in someone with HIV/AIDS or other immune compromising ailments is extended and often less effective.^{9,10} Secondly, simultaneous treatment with ARV and TB chemotherapy is often challenging due to drug-drug interactions that lead to a reduction in efficacy of ARV as well as toxicity. This is primarily due to effects of the Rifamycin (e.g. rifampicin, RIF) class of compounds, which are to date the most effective TB drugs. RIF induces CYP3A4 enzymes that accelerate metabolism of HIV-1 protease inhibitors and non-nucleoside reverse transcriptase inhibitors, consequently reducing the effective dose of these two anti-viral drugs.^{11,12} Lastly, since ARV therapy is needed for a lifetime and TB treatment is required for several months to years, long term simultaneous use of these drugs may result in serious adverse effects.^{2,6,9} Therefore, there is a significant and urgent need to identify novel compounds that are more effective, have fewer interactions, and potentially have dual activity (anti-retroviral and anti-mycobacterial) with a shorter duration of chemotherapy.

Anti-TB compounds are currently screened independently from HIV-1 compounds in mycobacterial cultures, and less often, in macrophages or peripheral blood leukocytes (PBL). Compounds with anti-retroviral activity are screened with HIV-1-infected cell lines or human PBL and drug effectiveness determined by changes in production of viral capsid

p24 or reductions in viral RNA using real-time PCR. More definitive characterization requires costly studies in non-human primates or clinical trials in patients. Importantly, these stand-alone experiments do not account for polymicrobial interactions including cell death and accelerated microbial growth as well as biological effects of compounds that may be additive or synergistic when combined.

Here, we describe the development of a novel *in vitro* co-infection model for simple, inexpensive evaluation of drug activity with human peripheral blood mononuclear cells (PBMC) or monocyte-derived macrophages (MDM). Using this model and standard drugs used for the treatment of HIV-1 and TB [e.g. antiretroviral Azidothymidine (AZT), anti-mycobacterial Isoniazid (INH)], we demonstrate how biological variables of co-infection and compound exposure can be rapidly evaluated *in vitro* using traditional techniques such as ELISA and CFU enumeration as well as the potential for multi-variate flow cytometric analysis of bacteria, virus, and cells in a single tube. Importantly, we can measure biological effects of mycobacteria and HIV-1 co-infection, including greater cell death and increased mycobacterial replication. This model and the associated assays will facilitate screening of novel or existing drug libraries to identify compounds to treat *Mtb*, HIV, or both pathogens in a co-infection setting.

2. Materials and Methods

2.1 HIV and mycobacterial isolates

Dual-tropic (R5/X4) HIV-1_{89.6} is an HIV-1 laboratory strain originally isolated from infected individuals. The original preparation was prepared from a molecularly cloned virus, and grown in CEMx174 cells¹³ and donated to the NIH AIDS Research and Reference Reagent Program. We purchased HIV-1_{89.6} from the Virology Core Facility, Center for AIDS Research at Baylor College of Medicine, Houston, TX, which was prepared and propagated in human PBMCs. A *Mycobacterium bovis* (*M. bovis*) BCG strain expressing a red fluorescent protein (tdtomato, with a spectrum similar to Texas Red) and previously described^{14,15} was used. The *M. bovis* BCG working stock used for the studies were cultured in our lab using standard BCG growth media which was made from DIFCO 7H9 Broth (Becton Dickinson, San Diego, CA) as previously described.¹⁶ Briefly, cultures were grown in a shaker at 100 rpm, 37°C for 10–14 days and kanamycin (50 ng/ml) was added to the stock once per 5 days to maintain the plasmid expressing td tomato. When the OD value of the growing stock reached 0.8–1.0, the stocks were frozen in the storage medium (long term storage) or PBS (for infections) at –80°C.

2.2 Preparation and activation of peripheral blood mononuclear cells

Peripheral blood was obtained from healthy donors as approved by the UTMB Institutional Review Board. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood using Accuprep (Accurate chemicals, New York, NY) and density centrifugation as previously described.¹⁷ An RBC Lysis buffer from Sigma (St. Louis, MO) was used to remove any remaining red blood cells according to the manufacturer recommendation. Cell viability of the isolated PBMC population was determined with flow cytometry using the live/dead fixable aqua cell viability assay (Invitrogen, New York, NY).

Cells were cultured in cRPMI at 10^6 cells/ml of media in 5% CO₂ at 37°C as previously described.¹⁷ In some experiments, peripheral blood monocytes were isolated by magnetic bead-conjugated antibody separation using AutoMacs as previously described¹⁸ and used to generate monocyte-derived macrophages (MDM) following 5–6 days of culture with 50 ng/ml of recombinant human M-CSF (R&D systems).

2.3 Flow cytometry to detect intracellular HIV-1 and *M. bovis* BCG infection

The mAbs against FITC-conjugated HIV p24 was purchased from Beckman Coulter (Indianapolis, IN). Following infection and treatment, PBMC or MDM were harvested at appropriate time points for flow cytometric analysis. After harvest, cells were incubated with CD16/CD32 Fc Block (BD Biosciences, San Jose, CA,) to reduce non-specific binding of antibodies. Cells were permeabilized using the BD Cytotfix/Cytoperm kit (BD Pharmingen, CA), then labeled with a FITC-conjugated antibody specific to HIV-1 (KC-67, Beckman-Coulter) as we have previously described.¹⁷ Samples were then incubated for 48 hours in 4% formaldehyde (Polysciences Inc, Warrington, PA) diluted in PBS prior to acquisition. A total of 50,000 gated events (based on expected leukocyte side scatter/forward scatter characteristics) were collected using a BD LSR II (Fortessa) flow cytometer (BD Biosciences). Analysis of data was performed by FCS Express 4 (De Novo, Los Angeles, CA) software. To control for background and to establish thresholds for gating positive cells, an isotype-matched FITC-labeled antibody was used.

2.4 ELISA to detect secreted HIV-1 p24 protein

The levels of secreted HIV-1 p24 in culture supernatants were measured at day 7 post infection using an ELISA kit purchased from Zeptometrix (Buffalo, NY). Secreted protein was converted to pg/ml based on the standard curve generated from known HIV-1 p24 standards included in the kit as recommended by the manufacturer.

2.5 CFU enumeration of mycobacteria

PBMC were disrupted with 0.067% SDS and lysates were used to determine bacterial growth following culture with control or standard compounds. The bacterial load was measured by colony forming unit (CFU) enumeration by limiting dilution and determining growth on selective agar (7H11) as we have previously described.¹⁶

3. Results and Discussion

New co-infection models and assays are needed in the ongoing effort to develop and optimize clinical interventions for people infected with both *Mtb* and HIV-1. Here, we describe the development of an *in vitro* mycobacteria/HIV-1 co-infection model using human peripheral blood PBMCs or MDM. As outlined in Fig. 1, PBMC were infected with HIV-1_{89.6} (dual tropic isolate of HIV-1) for 5 hr and washed to remove residual virus and viral proteins in the PBMC cultures as previously reported.¹⁷ Productive infection with HIV-1 was confirmed by detection of HIV-1 p24 protein secretion into the culture supernatant as measured by ELISA (Fig. 2A), while p24 was not detected in mock-infected culture supernatant (not shown). The level of HIV-1 p24 produced by infected cultures was within the standard range recommended by the manufacturer for detection of HIV infection

using human biological samples. Viral replication and drug activity was detectable though the numbers of virus-infected cells were low (Fig. 4A).

Significantly, this assay more closely mimics the physiologically relevant infection level in comparison to traditional drug screening performed in cell lines manipulated to maximize HIV-1 infection (e.g. receptor transgenic). AZT treatment reduced p24 production in PBMC infected with HIV-1 alone compared to control cultures treated with the vehicle (Fig. 2A). Co-infection of PBMC with BCG did not impact HIV-1 replication as indicated by p24 production in both vehicle- and AZT-treated cultures. Similarly, anti-mycobacterial compounds INH and RIF did not markedly alter AZT anti-retroviral activity *in vitro* at these concentrations. This is expected with regard to RIF as Rifamycin family member interactions with ARV occur due to alterations in liver enzyme levels;¹² measurable effects would presumably require an *in vivo* model.

As expected, *M. bovis* BCG replicated in infected PBMC cultures treated with gentamicin (50 Pg/ml) to eliminate extracellular bacteria (Fig. 2B). Treatment with standard anti-mycobacterial drugs INH and RIF dramatically reduced intracellular CFU at 7 day post-infection (Fig. 2B). Though the effects of anti-mycobacterial drugs were consistently observed, there was marked variability in the baseline bacterial CFU measured in PBMC from different donors (Fig. 2B). Donor variability in the level of intracellular bacterial infection is a recurring issue in performance of *in vitro* assays which requires optimization.^{16,18} To normalize the data across multiple donors, the CFU numbers of non-treated, non-HIV-infected cultures were set to 100% and the data re-analyzed accordingly (Fig. 2C). Following normalization, increased intracellular *M. bovis* BCG replication due to co-infection of cultures with HIV-1 was observed (Fig. 2B, C) as previously reported with *Mtb*-infected cultures.¹⁹ Co-infection with HIV-1 increased mycobacterial growth to 150% of the control growth (Fig. 2C) while treatment with AZT to reduce the viral load brought mycobacterial growth back to levels observed in the absence of HIV-1 infection. These data demonstrate that the effect of HIV-1 to alter mycobacterial growth can be reproduced in this *in vitro* assay while also assessing inhibition of these effects with antimicrobial compounds. Importantly, the demonstration that these effects can be observed with BCG means that preliminary compound screening can be performed within a Biosafety Level 2 (BSL2) laboratory instead of BSL3. This will facilitate adaptation of assays to evaluate drug effectiveness that are higher throughput and can incorporate more sophisticated technologies.

Drug toxicity and interactions issues are high priority considerations in *Mtb*/HIV-1 endemic areas and contribute to poor clinical outcomes.^{6,9,11} An optimum assay for screening compounds for use in *Mtb*/HIV-1 co-infected patients should account for both pathogen and drug effects on cell viability. Here, we used a cell viability reagent from Invitrogen (fixable aqua live/dead viability marker) illustrated in Fig. 3 because this approach is not compromised by the formaldehyde fixation procedures required for pathogen inactivation. The differences in cell viability due to mono- or dual-infection and drug exposure were easily visualized (Fig. 3A). *M. bovis* BCG infection promoted greater cell death in cultures, and co-infection with HIV-1 exacerbated this effect in some donors (Fig. 3B). These data

demonstrate that the additive effects of mycobacteria, HIV-1, and standard compounds, to reduce PBMC viability, can be assessed in this co-infection assay.

A novel feature of our assay is that two fluorescent probes were used to measure virus and bacterial growth, respectively. This facilitates rapid readouts adaptable to high throughput assays in order to screen compound libraries and approximate effective dose for *in vitro* anti-viral and anti-mycobacterial activity. A simplified example is shown in Fig. 4 using fluorescence-based detection of HIV-1 and *M. bovis* BCG in co-infected cultures by flow cytometry. The data presented in Fig. 4A (bottom panel) were obtained using a red (tdtomato) fluorescent *M. bovis* BCG construct and a FITC-labeled antibody to HIV-1 p24. Intracellular infection with HIV-1 (7.5%), BCG (34%) and both (2.7 %) was detected following 7 days of culture (Fig. 4A). Interestingly, these data demonstrate that most cells are mono-infected, though there are likely biological effects mediated indirectly that can impact mycobacterial growth as demonstrated in Fig. 2. This is not surprising as several HIV-1 proteins have been shown to mediate immunomodulatory and immunosuppressive effects in the absence of direct viral infection.^{20–22} The application of this flow cytometric assay for measuring cellular infection following drug exposure or other interventions is illustrated in Fig. 4B. A reduction of intracellular HIV-1 and BCG was clearly observed following treatment with anti-retroviral AZT and anti-mycobacterial RIF, respectively. By using a multivariate flow cytometry assay, an additional fluorescent channel could be assigned for measurement of cell viability (e.g. Fig. 3) or several other parameters of interest in a single tube or 96 well plate. Further optimization of this flow cytometric approach would facilitate high throughput screening of large compound libraries, as validated by measurement of viral titers and mycobacterial numbers using ELISA and CFU enumeration.

In summary, we have developed and characterized an *in vitro* model to facilitate screening of novel compounds for potential use in treatment of TB and HIV-1 in the setting of co-infection. This model could be applied to prioritization of drug candidates selected from large compound libraries, followed by evaluation of safety and efficacy in animal models. In the future, we anticipate the potential to additionally incorporate the use of humanized mice in translational studies to characterize novel drugs identified through high throughput *in vitro* systems. Humanized mice are increasingly being applied to evaluate anti-retroviral therapies^{23,24} and were recently used to establish an animal model of TB.²⁵ Ideally, compounds selected based on performance in an *in vitro* co-infection model described here could eventually progress to *in vivo* characterization in *Mtb*/HIV-1 co-infected humanized mice.

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Experimental Design

Figure 1.

Experimental design for mycobacteria/HIV-1 co-infection of PBMC. Healthy human donor peripheral blood was used to isolate PBMC, and in some experiments to derive MDM, for use in *M. bovis* BCG and HIV-1_{89.6} co-infection assays. The flow chart describes the experimental approach for isolation, infection, and assessment of co-infected PBMC and MDM following treatment with anti-retroviral and anti-mycobacterial compounds.

Figure 2.

Effect of drugs on mycobacteria and HIV-1 replication in co-infected cultures. PBMCs were isolated from peripheral blood of healthy human donors, infected with mock (media), HIV-1_{89.6} for 5 h and/ or *M. bovis* BCG (tdtomato) for 1 h. Samples were treated with gentamicin (50 ng/ml) to eliminate extracellular bacteria, washed, and cultured for 7 days in media or with various drug treatments: AZT (5.0 µg/mL); RIF (5.0 µg/mL); INH (0.1 µg/mL); or DMSO (1%). A, ELISA analysis of HIV-1 p24 secretion into the media from infected PBMC, (n=6 for DMSO, BCG, AZT groups and n=4 for groups with RIF). B, CFU enumeration of BCG-infected PBMCs as affected by single and dual drug treatments (n=5 except RIF and INH groups where n=2–3 were used). C, intracellular growth of *M. bovis* BCG normalized to growth in the absence of HIV-1 infection or drug treatment per

individual donor. The dotted grey line indicates baseline growth of BCG in the absence of HIV-1 or drug treatment. Values shown are the means $\pm$ SEM. Statistically significant decreases in p24 secretion (A) or CFU (B, C) due to drug treatment compared to treatment with vehicle (DMSO) are designated as follows: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. A statistically significant increase in CFU due to HIV-1 co-infection is designated by ††, $p < 0.01$.

Figure 3.

Viability of PBMC following mycobacteria/HIV-1 infection and drug exposure. PBMC were isolated from peripheral blood of healthy human donors. A, flow cytometric analysis of side scatter and forward scatter characteristics of isolated cells and representative dot plots of the PBMC viability as determined by detection of the live/dead fixable aqua marker of cell death. B, summarized data of cell viability as affected by vehicle (DMSO), infection with HIV-1, BCG, or both, in compound vehicle (DMSO), and following exposure to standard drug compounds diluted in DMSO: AZT (5.0 $\mu\text{g/mL}$); RIF (5.0 $\mu\text{g/mL}$); INH (0.1 $\mu\text{g/mL}$); or DMSO (1%). Values shown are the means $\pm$ SEM of results from 3 individual donors, performed in duplicate. Statistically significant increases in cell death due to drug treatment compared to treatment with vehicle (DMSO) are designated as follows: *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$.

Figure 4.

Flow cytometric analysis of drug effects in MDM cultures infected with mycobacteria and HIV-1. PBMC were isolated from peripheral blood of healthy human donors and used to derive MDM (Mφ). A, flow cytometric analysis of side scatter and forward scatter characteristics of enriched macrophages (Gate 1) and a representative plot showing the % of BCG (PE-Texas Red) and HIV-1 (FITC) co-infected macrophages in the selected (Gate 1) population. B, representative plots demonstrating the % of macrophages positive for the intracellular BCG (tdtomato fluorescence) or intracellular HIV-1 (FITC fluorescence) infection as affected by drug treatment [AZT (5.0 μg/mL), RIF (5.0 μg/mL) or DMSO (1%)]. Results shown are representative of data from 3 individual donors.