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A Modified BPaL Regimen for Tuberculosis Treatment replaces Linezolid with Inhaled Spectinamides

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Abstract

The Nix-TB clinical trial evaluated a new 6-month regimen containing three-oral- drugs; bedaquiline (B), pretomanid (Pa) and linezolid (L) (BPaL regimen) for treatment of tuberculosis (TB). This regimen achieved remarkable results as almost 90% of the multidrug resistant (MDR) or extensively drug resistant (XDR) TB participants were cured but many patients also developed severe adverse events (AEs). The AEs were associated with the long-term administration of the protein synthesis inhibitor linezolid. Spectinamide 1599 is also a protein synthesis inhibitor of *Mycobacterium tuberculosis* with an excellent safety profile but which lacks oral bioavailability. Here, we propose to replace L in the BPaL regimen with spectinamide (S) administered via inhalation and we demonstrate that inhaled spectinamide 1599, combined with BPa —BPaS regimen—has similar efficacy to that of BPaL regimen while simultaneously avoiding the L-associated AEs. The BPaL and BPaS regimens were compared in the BALB/c and C3HeB/FeJ murine chronic TB efficacy models. After 4-weeks of treatment, both regimens promoted equivalent bactericidal effect in both TB murine models. However, treatment with BPaL resulted in significant weight loss and the complete blood count suggested development of anemia. These effects were not similarly observed in mice treated with BPaS. BPaL and BPa, but no the BPaS treatment, also decreased myeloid to erythroid ratio suggesting the S in the BPaS regimen was able to recover this effect. Moreover, the BPaL also increased concentration of proinflammatory cytokines in bone marrow compared to mice receiving BPaS regimen. During therapy both regimens improved the lung lesion burden, reduced neutrophil and cytotoxic T cells counts while increased the number of B and helper and regulatory T cells. These combined data suggest that inhaled spectinamide 1599 combined with BPa is an effective TB regimen that avoids L-associated AEs.

Imporatance

Tuberculosis (TB) is an airborne infectious disease that spreads via aerosols containing *Mycobacterium tuberculosis* (Mtb), the causative agent of TB. TB can be cured by administration of 3-4 drugs for 6-9 months but there are limited treatment options for

patients infected with multidrug (MDR) and extensively resistant (XDR) strains of Mtb. BPaL is a new all-oral combination of drugs consisting of Bedaquiline (B), Pretomanid (Pa) and Linezolid (L). This regimen was able to cure ~90% of MDR and XDR TB patients in clinical trials but many patients developed severe adverse events (AEs) associated to the long-term administration of linezolid. We evaluated a new regimen in which Linezolid in the BPaL regimen was replaced with inhaled spectinamide 1599. In the current study, we demonstrate that 4-weeks of treatment with inhaled spectinamide 1599 in combination with Bedaquiline and Pretomanid has equivalent efficacy to the BPaL drug combination and avoids the L-associated-AEs.

eLife assessment

In this **useful** study, the authors report the efficacy, hematological effects, and inflammatory response of the BPaL regimen (containing bedaquiline, pretomanid, and linezolid) compared to a variation in which Linezolid is replaced with the preclinical development candidate spectinamide 1599, administered by inhalation in tuberculosis-infected mice. The authors provide **convincing** evidence that supports the replacement of Linezolid in the current standard of care for drug-resistant tuberculosis. The work will be of interest to those studying tuberculosis treatment regimens.

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Introduction

Tuberculosis (TB) remains one of the leading causes of death initiated by an infectious agent. In 2021, the World Health Organization reported 10.6 million new TB cases worldwide and among those, 450,000 cases were also diagnosed as multidrug resistant (MDR) or extensively drug resistant (XDR) TB (1). Treatment of MDR- and XDR- TB patients is lengthy and is often poorly tolerated due to significant associated side effects (2).

Recently, a 6-month novel treatment regimen of three-oral-drugs: bedaquiline (B), pretomanid (Pa) and linezolid (L) referred to as the BPaL regimen was approved. Preclinical studies demonstrated the better efficacy of BPaL regimen for drug sensitive TB compared to the standard TB chemotherapy (1,3,4) and thereafter, the BPaL regimen was tested in the Nix-TB clinical trial conducted in South Africa (5). This trial enrolled patients with XDR-TB and treatment-intolerant or non-responsive MDR-TB, including HIV positive patients with a CD4 count of 50 or higher. The results were remarkable as 95 out of 107 patients were cured though many patients had a high rate of treatment-associated adverse events (AEs).

The long-term administration of linezolid (an oxazolidinone antibiotic) was likely the causative agent resulting in bone marrow myelosuppression (48%), peripheral neuropathy, optic neuritis (81%) and anemia (37%) in patients treated with the BPaL regimen (5). Subsequently, the ZeNix trial adjusted the BPaL regimen to a linezolid dose of 600mg. This trial also had remarkable results; it cured 84-91% of patients (9 to 26 weeks of therapy, respectively) and resulted in fewer AEs than those observed in the Nix- TB trial (6). Apart from AEs, several studies have also raised awareness of high doses of linezolid leading to development of linezolid-resistant *Mycobacterium tuberculosis* (Mtb) (7–9). At present, the TB-drug development field is working to modify the BPaL regimen to maintain or improve its efficacy while diminishing treatment-associated AEs (10).

Spectinomycin, an aminocyclitol antibiotic, is a broad spectrum antibiotic used mainly for the treatment of *Neisseria gonorrhoeae* (11). It inhibits bacterial protein synthesis (12) and has an acceptable safety profile with no known ototoxicity and nephrotoxicity (13,14). The activity of spectinomycin against Mtb is very poor but its structural modification led to the development of a new series of semisynthetic analogs called spectinamides (12,15,16). Spectinamides bind selectively with the bacterial 30S ribosomes and importantly, unlike linezolid, they do not bind to the mitochondrial 30S in mammalian cells. The latter represents a great advantage for reduced potential for side effects, such as ototoxicity and myeloid suppression that are commonly associated with other protein synthesis inhibitors such as amikacin and linezolid, respectively (17,18). Spectinamides' potent anti-tubercular activity is attributed to its ability to evade drug efflux by Rv1258c major facilitator superfamily transporter present on the surface of Mtb (19,20). Spectinamides have shown excellent activity against MDR- and XDR-Mtb strains (16,21) h owever, their poor oral availability has limited their usage to injectable forms.

One of the lead spectinamides, 1599, has demonstrated promising results *in vitro* and *in vivo* and was shown to lack cross-resistance with existing anti-TB drugs (16,19–23). 1599, delivered subcutaneously, proved an effective partner agent when combined with rifampin and pyrazinamide and also with bedaquiline, pretomanid or moxifloxacin in TB mouse efficacy models of increasing complexity (21).

One of the limitations of using 1599 as injectable is a potential risk for poor patient compliance (24) and direct administration of aerosolized antibiotics to the lungs has been studied for decades as an alternative to systemic drug administration via injection. Aerosolized administration of 1599 has been tested in preclinical *in vivo* studies using liquid formulation of the drug. These studies have shown that inhaled 1599, used in monotherapy or in combination with pyrazinamide, is efficacious and well tolerated in murine TB efficacy models (25,26). A comparative study assessing biodistribution of the drug in relation to the administration route demonstrated that 1599 showed 48 times higher exposure in mouse lungs via inhalation compared to equivalent dosages administered by subcutaneous injection; the latter may explain the increased efficacy of this drug following intrapulmonary aerosol (22,26). Moreover, 1599 was shown to be amenable to dry powder formulation and delivery, suggesting a pathway to a more patient-friendly delivery system (27). Therefore, in this study, we hypothesized that combining BPa with inhaled S will maintain equivalent efficacy to the BPaL regimen while avoiding the accompanying toxicities that occurred with long-term BPaL administration to human MDR/XDR-TB patients. Based on the diversity of outcomes observed during human TB disease (22) and as no single animal model recapitulates the wide spectrum of human TB pathology (28), we chose the BALB/c and C3HeB/FeJ murine TB models. The BALB/c chronic TB model is representative of a long-term Mtb chronic infection that develops homogenous lung granulomatous lesions restraining the bacilli within intracellular compartments (29) of macrophages and foamy macrophages. In contrast, low-dose aerosol Mtb infection of C3HeB/FeJ mice also results in a chronic infection, but their lungs exhibit a heterogenous spectrum of lesions including granulomas similar to those seen in BALB/c chronic TB model in addition to caseous necrotic lesions surrounded by a fibrotic rim (30–32). The caseum of these necrotic granulomas creates a hypoxic environment and contains abundant extracellular bacilli (31–33) in a similar fashion to necrotic granulomas found in some human TB patients. It is believed that the fibrotic, necrotic and hypoxic environment of these granulomas creates barriers to drug penetration, alters bacterial phenotype, and all together challenge therapeutic outcomes (34–37). Therefore, to understand the implications of drug efficacy and drug associated AEs in scenario without (BALB/c) and with (C3HeB/FeJ) necrotic granulomas, both murine TB efficacy models were employed.

Results

Linezolid and spectinamide 1599 show similar efficacy in monotherapy

To compare the efficacy of L or S in monotherapy, Mtb-infected C3HeB/FeJ (n=7) and BALB/c (n=4-6) mice received L (administered 5/7 days per week orally at 100 mg/Kg) or S (administered 3/7 days per week on alternate days via intrapulmonary aerosol delivery at 100 mg/Kg and 50 mg/Kg, respectively) for 4 weeks. At the end of treatment, the animals were sacrificed, and the CFU in lungs and spleen was enumerated (**Figure 1A-D**). Treatment of C3HeB/FeJ mice with L (7.30 ± 0.45 $\log_{10}$) or S (6.90 ± 0.48 $\log_{10}$) for 4 weeks resulted in an average of 0.51 and 0.91 $\log_{10}$ CFU reduction in the lungs respectively, compared to untreated (UnRx) control (7.81 ± 0.36 $\log_{10}$) (**Figure 1A**). This difference failed to achieve statistical significance owing to the larger standard deviation associated with heterogenous advanced lung pathology observed in this model (26,31,32). In contrast, L (4.49 ± 0.10 $\log_{10}$) treated C3HeB/FeJ mice had significantly lower spleen bacterial burden compared to UnRx (5.43 ± 0.27 $\log_{10}$), with no significant difference between the L and S (4.94 ± 0.11 $\log_{10}$) treatment arm (**Figure 1C**). In BALB/c mice, L or S treatment was found to promote significant reduction in lung bacterial burden (0.83 and 0.77 $\log_{10}$ respectively) compared to UnRx or the vehicle only controls (**Figure 1B**). There was no significant difference in lung bacterial burden after L or S treatment in BALB/c mice and there was no change in spleen bacterial burden compared to UnRx control (**Figure 1D**).

Combination therapy with BPaL or BPaS have similar efficacy

We further tested and compared the efficacy of L or S when used in combination therapy with BPa. Mtb-infected C3HeB/FeJ mice were treated with either BPaL (B= 25mpk; Pa= 100 mg/Kg and L= 100 mg/Kg all administered 5/7 a week via oral gavage) or BPaS (BPa as in BPaL and S= 100 mg/Kg administered 3/7 a week on alternate days via intrapulmonary aerosol delivery) for 4 weeks. The comparative analysis from combined data of three independent studies is shown in **Figure 1E**&G (data from individual studies are shown in Figures S1-3). Two of three studies contained an extra group of BPa treated mice as a reference control. Compared to UnRx (7.48 ± 0.12 $\log_{10}$) control, mice in the BPa (5.49 ± 0.42 $\log_{10}$), BPaL (4.76 ± 0.38 $\log_{10}$) and BPaS (4.98 ± 0.35 $\log_{10}$) treatment groups had significantly reduced the lung bacterial burden by 1.99, 2.72, and 2.50 $\log_{10}$ respectively (**Figure 1E**). Although a higher CFU reduction was observed in the lungs of C3HeB/FeJ mice treated with BPaL or BPaS, these differences failed to achieve statistical significance compared to BPa backbone regimen. All three regimens proved highly effective at reducing spleen bacterial burden in C3HeB/FeJ mice, with most mice returning no CFU within the limit of detection (LOD) employed herein (**Figure 1G**).

The effect of BPaL and BPaS (S= 50 mg/Kg) combination therapy on the bacterial burden in lungs and spleen of Mtb-infected BALB/c mice was determined at the end of 4- weeks of treatment. **Figure 1F**&H show the combined lung and spleen CFU data from two independent studies (data for each study is shown in Figures S8-9). The combined result demonstrated that compared to UnRx (5.24 ± 0.17 $\log_{10}$) control, mice in the BPaL (1.29 ± 0.13 $\log_{10}$) and BPaS (1.44 ± 0.17 $\log_{10}$) treatment groups returned significantly fewer CFU in the lungs, with most mice returning no CFU within the LOD employed herein (**Figure 1F**). As in C3HeB/FeJ TB model, no significant difference was observed in the lung CFU of BALB/c mice treated with either the BPaL or BPaS regimen. BPaL and BPaS therapy reduced BALB/c spleen bacterial burden to below the LOD of the assay with no CFU recovered for any treated mice (**Figure 1H**). In summary, these results

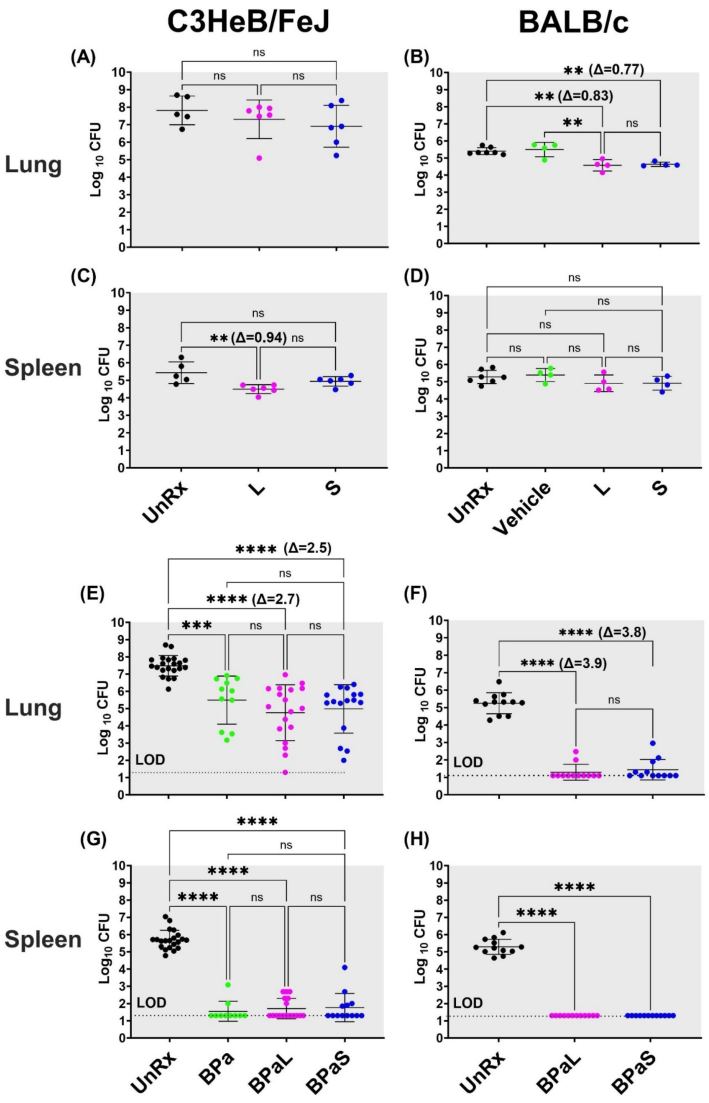

Figure 1.

Bactericidal effect of BPaL and BPaS in TB mouse models after 4-weeks of treatment BALB/c and C3HeB/FeJ female mice were chronically infected with a low dose aerosol infection of Mtb Erdman strain to deliver ~75 and ~100 bacilli respectively. Post-infection, BALB/c and C3HeB/FeJ mice were rested for 4 and 8-9 weeks respectively until they were randomly assigned to the study groups. The mice were treated with monotherapy of linezolid or spectinamide 1599 or combination therapy of BPaL, BPa or BPaS for 4 weeks. Bedaquiline (B), pretomanid (Pa) and linezolid (L) were administered at 25, 100 and 100 mg/kg respectively by oral gavage for 5/7 a week while spectinamide 1599 at 50 and 100 mg/kg in BALB/c and C3HeB/FeJ TB models respectively for 3/7 a week on the alternate days via intrapulmonary aerosol delivery. On the third day of the last treatment, the mice were euthanized, and their lungs and spleen were collected. The organs were homogenized, serially diluted and plated on 7H11 agar with 4% charcoal (to avoid drug carry-over effect) to determine bacterial burden in the form of colony forming units (CFU) in each sample. CFU were enumerated after 4-6 weeks of incubation at 37 °C and expressed as log_{10}.C3HeB/FeJ and BALB/c TB models showing efficacy of monotherapy (A-D) and combination therapy (E-H). The C3HeB/FeJ graphs (E&G) represent the pooled data from three independent studies (n=3-8 each, Figures S1-3) and two of the three studies contained BPa as a reference control. The BALB/c graphs (F&H) represent the pooled data from two independent studies (n=5 each, Figures S8-9). Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$ was considered significant and ** = p<0.001, *** = p<0.0001, **** = P<0.0001. UnRx = untreated, L = linezolid, S = spectinamide 1599, LOD: limit of detection.

support our hypothesis and demonstrate that both BPaL, BPaS (and BPa in the C3HeB/FeJ TB model) multidrug regimens show equivalent bactericidal effects in C3HeB/FeJ and BALB/c chronic TB efficacy models.

Monitoring of adverse events

Five approaches were employed to monitor treatment-associated AEs in mice in this study including 1) changes in the body weight of mice; 2) lung histopathology and lesion scoring; 3) evaluation of complete blood count (CBC); 4) clinical pathology to study myelosuppression in bone marrow and 5) changes in content of immune cells in the lungs, spleen, bone marrow and blood.

BPaL therapy decreases the body weight of mice

No significant difference in the body weight among the treatment groups in either C3HeB/FeJ or BALB/c mice was observed following 4-weeks of treatment with S or L alone (Figure S4A and S10A respectively). On the other hand, C3HeB/FeJ or BALB/c mice treated with BPa, BPaL or BPaS showed marginal loses of body weight, ranging from 2.37-5.13% and only mice receiving the BPaL regimen, when compared to the UnRx control, reached statistically significant loses in body weight by the end of treatment (Figure S4B and S10B respectively).

BPaL and BPaS therapy result in significant lower lung lesion burden

The lesions in Mtb-infected UnRx C3HeB/FeJ mice (**Figure 2A** &B) showed a spectrum of diverse granuloma types ranging from aggregations of macrophages and lymphocytes to highly organized granulomas with collagen encapsulation and a region of central caseous necrosis that resembles to those found in some human patients (31,32). By comparison, lung lesions of Mtb-infected UnRx BALB/c mice (**Figure 2C** &D) consisted of granulomas with a very homogeneous structure (31) formed also by aggregations of macrophages and lymphocytes without a necrotic core. Mice treated for 4 weeks with the BPaL or BPaS regimen presented with significant reduction in the number and size of granulomas in both C3HeB/FeJ and BALB/c TB models compared to their respective UnRx control, with no significant difference in lesion burden score between the combination drug treatment groups (**Figure 2E** &F).

Association of L with altered blood profile and mild anemia in mice

The effect of L in the blood profile of humans and mouse has been reported (38–42) but the same has not been reported for S. Therefore, a complete blood count (CBC) profile was performed on Mtb-infected C3HeB/FeJ mice at the end of 4-weeks of treatment to quantify treatment-associated hematological changes. The results obtained from mice treated with L or S alone are summarized in **Figure 3A**. Of 20-blood parameters evaluated, two blood parameters were affected during treatment. When compared to UnRx control and S treated mice, L treatment significantly increased the red blood cell distribution width standard deviation (RDWs), while both L and S treatment were associated with a significant decrease in the mean corpuscular hemoglobin concentration (MCHC) compared to UnRx control (**Figure 3A**).

The Nix-TB trial associated the long-term administration of L within the BPaL regimen as the causative agent resulting in anemia in patients treated with the BPaL regimen (5). Thus, the effect of combination therapy with the BPaL or BPaS regimen on CBC profile was analyzed at 1-, 2- and 4-weeks of treatment (**Figure 3B**). None of the 20 parameters of CBC changed during the first 2 weeks of treatment. However, out of the 20-blood parameters evaluated, a total of four blood parameters were affected at 4- weeks-of treatment. L-containing BPaL regimen was again associated with a significant increase in the RDWs, and lower hemoglobin (HGB) compared to UnRx control after 4 weeks. This effect was not observed in mice treated with BPa or BPaS (**Figure 3B**). However, as in monotherapy, there was a trend towards lower overall MCHC in mice

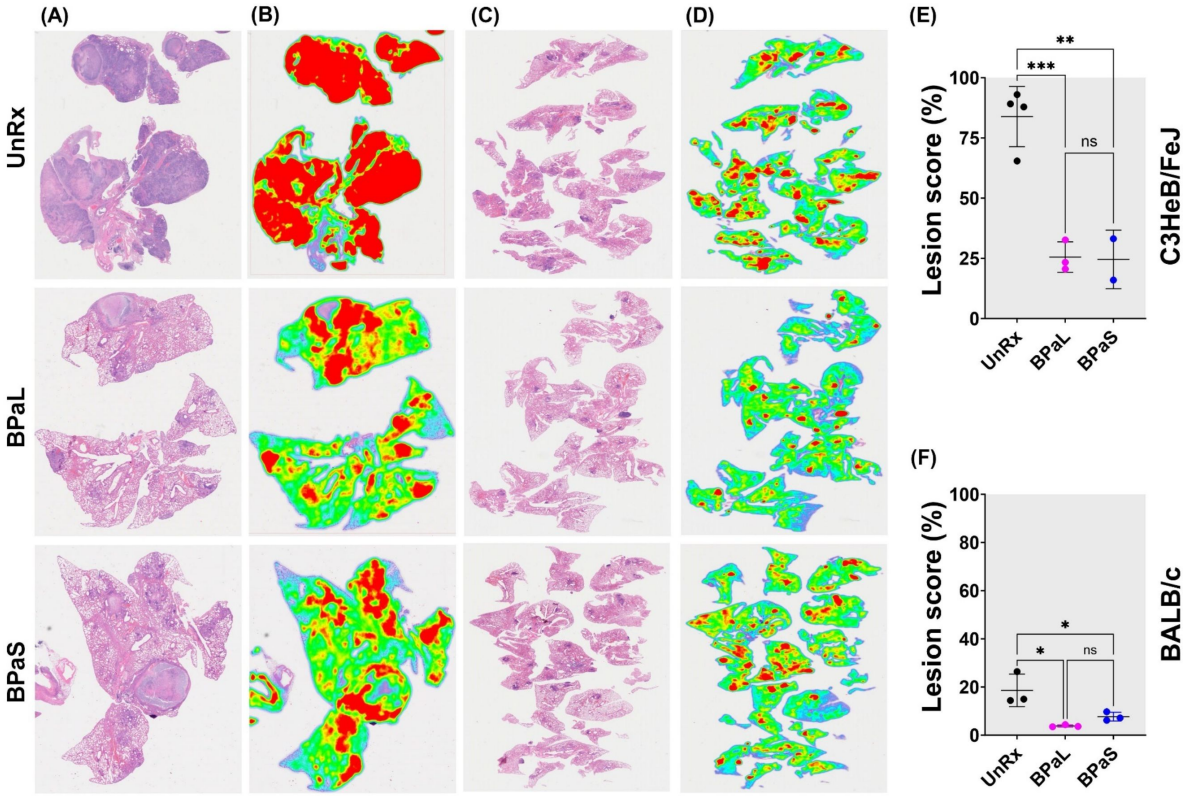

Figure 2.

Effect of therapy on lung histopathology of TB mouse models after 4- weeks of treatment.

At the end of therapy, the mice were euthanized, and their lungs were collected and processed for histopathology and lesion scoring. FFPE sections were cut at 5 µm, stained with hematoxylin and eosin (H&E) and imaged at 40x (A: C3HeB/FeJ, C: BALB/c)). Lesion maps (B: C3HeB/FeJ, D: BALB/c) show the infected areas in red color while green color represents the uninvolved parenchymal tissue. Lesion scores (E: C3HeB/FeJ, F: BALB/c) were calculated as the proportion of infected area over the total lung area per animal. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison test, and $p < 0.05$ was considered significant and ** = $p<0.001$, *** $P<0.0001$. UnRx = untreated

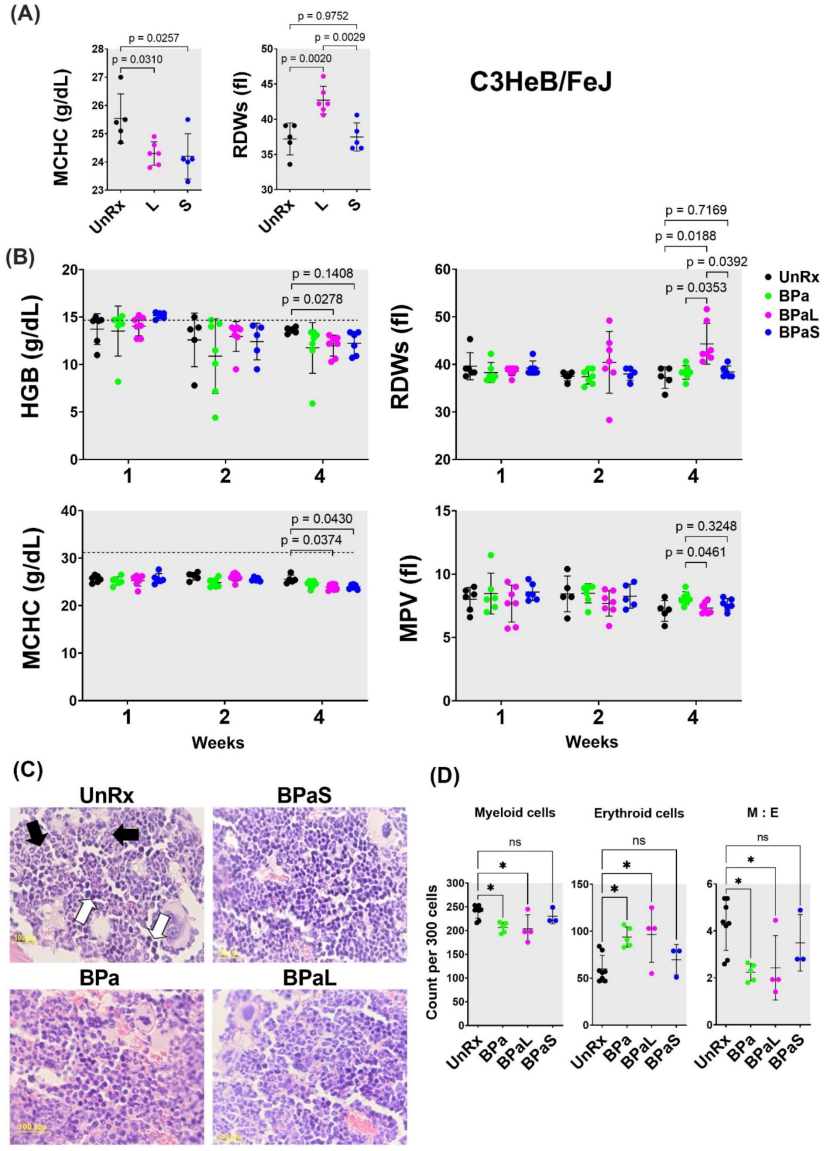

Figure 3.

Complete blood count profiling and bone marrow histopathology of Mtb infected C3HeB/FeJ TB model during 4-weeks of therapy "During therapy of mice in **Figure 1**, the blood was collected at 1-, 2- and 4-weeks of treatment. The complete blood count was collected in VETSCAN® HM5 hematology analyzer (Zoetis)". (A) monotherapy, (B) multidrug therapy. The MCHC (mean corpuscular hemoglobin concentration) and RDWs (red blood cell distribution width- standard deviation) along with the HGB (hemoglobin concentration) and MPV (mean platelet volume) are shown. A horizontal dotted line indicates the lower end of the reference interval for C3HeB/FeJ mice. The sternum, femur and tibias bones from each mouse were collected, fixed in 4% PFA, and processed for histology (C&D). Sections were cut at 5 µm, stained with hematoxylin and eosin (H&E) and imaged at 40x. (C) Representative photos of bone marrow sections showing myeloid (black arrows) and erythroid (white arrows) cells in bone marrow of untreated (UnRx) and treatment (BPa, BPaS and BPaS) groups. (D) The number of myeloid (M) and erythroid (E) among a total of 300 cells in 5 different regions were counted for each group and M:E was calculated. Statistical significance was calculated using one-way ANOVA with Tukey's test for multiple comparisons. $p < 0.05$ was considered significant and ** = p<0.001, *** = P<0.0001.

treated with either BPaL or BPaS. The mean platelet volume (MPV) was marginally higher at 4 weeks in mice treated with BPa compared to BPaL or BPaS, albeit not significantly different from UnRx control (**Figure 3B**). Given that no difference in HGB or RDWs was observed between UnRx control and their comparator BPa and BPaS regimens, we concluded that the significant HGB decrease and RDWs increase (often observed during development of anemia) were associated with inclusion of L in BPaL regimen.

Spectinamide 1599 recovers the altered ratio of myeloid to erythroid cells in bone marrow

To further evaluate if L was associated with myelosuppressive effect, we performed hematopathology analysis on bone marrow from Mtb-infected C3HeB/FeJ and BALB/c mice at the end of treatment. For C3HeB/FeJ mice, the number of myeloid (M) and erythroid (E) precursor cells were calculated from H&E stained sections and their myeloid to erythroid ratio (M:E) was determined by counting 300 bone marrow cells in 5 different regions (**Figure 3C**). The BPa and BPaL treatment significantly decreased myeloid cells while increasing the proportion of erythroid cells (**Figure 3D**). Hence, the corresponding ratio in bone marrow of animals treated with BPaL or BPa was lower compared to UnRx control. Importantly, the BPaS treatment did not show any difference in the content of myeloid or erythroid cells when compared to UnRx control suggesting that S in the BPaS was able to recover this effect. In Mtb-infected BALB/c mice, the number of myeloid and other cell types were counted, however, no significant difference was found among the control and treatment groups (data not shown).

BPaL therapy increases proinflammatory cytokine response in bone marrow

A comparative analysis for concentration of cytokines and chemokines in the bone marrow, plasma, and lung samples from Mtb-infected C3HeB/FeJ mice treated with BPaL or BPaS regimen was also conducted. The bone marrow samples demonstrated a significant difference between the BPaL and BPaS groups, with appreciably higher level of pro-inflammatory cytokines and chemokines (IL-1β, IL-12p70, IL-23, TNFα, GROα (CXCL1), MP-2α (CXCL2), IP-10 (CXCL10), MP-1α (CCL3), RANTES (CCL5), MCP-3 (CCL7) and Eotaxin (CCL11)) in the BPaL- compared to BPaS-treated mice (**Figure 4B**). The plasma and lung samples, however, had similar cytokine and chemokine contents between the treatment groups except for MCP-3 (CCL7) in plasma which was significantly higher in BPaS compared to BPaL group (Figure S13). We also performed a correlation analysis of bone marrow cytokine and chemokine content with lung CFU obtained from treatment (BPaL or BPaS) and UnRx groups (**Figure 4C**). The analysis suggested that compared to UnRx control, there was a strong correlation between the profound reduction of lung bacterial burden and profound reduction in bone marrow cytokine and chemokine contents observed in mice from BPaL or BPaS groups. Similar correlations were found between lung CFU and content of cytokines and chemokines in lung (Figure S14) and plasma (Figure S15).

BPaL and BPaS therapies reduce inflammation-associated cells

We further assessed the environment of immune cells using flow cytometry in bone marrow, lungs, and blood from each group of Mtb-infected C3HeB/FeJ mice (**Figure 5**). The bone marrow (**Figure 5A**) results revealed that as compared to UnRx control, there was a significant decrease in the percentage of inflammatory myeloid phenotypes (CD45+CD3-CD11b+CD11c-Ly6C+CCR2+, CD45+CD3-CD11b+CD11c-Ly6C+CCR2+MHC-II+ and CD45+CD3-D14+CCR2+) in response to therapy with either BPa, BPaL, or BPaS. In contrast, neutrophils (CD45+CD3-CD11b+CD11c- Ly6C+Ly6G^{high}), precursor T cells (CD45+CD3+) and B cells (CD45+CD3-CD19+B220-) were significantly increased in either BPa, BPaL, or BPaS treatment groups compared to UnRx control in bone marrow. This reduced inflammatory response in treatment groups is also consistent in blood shown by significantly reduced inflammatory myeloid cells (CD45+CD3-CD11b+CD11c-Ly6C+CCR2+) (**Figure**

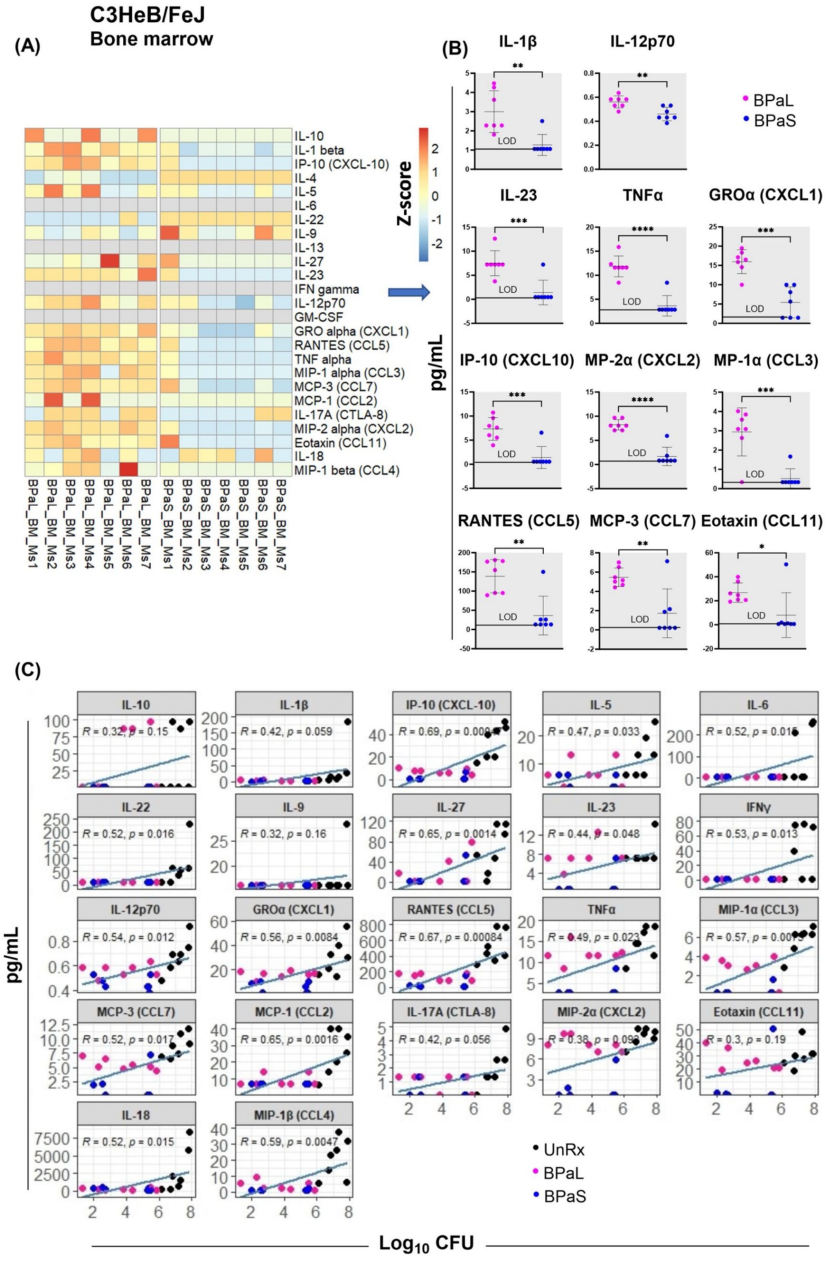

Figure 4.

Bone marrow cytokine and chemokine profiling in Mtb infected C3HeB/FeJ TB model after 4-weeks of treatment Femur bones from selected studies in mice in **Figure 1** were collected to harvest the bone marrow. The bone marrow was resuspended in PBS, centrifuged and the supernatant was collected for the evaluation of cytokine's content. BPaL and BPaS therapy showed profile of 26 cytokines and chemokines in bone marrow and the data were converted to Z score and represented as a heatmap (A) and graphically (B). (C) Spearman's correlation analysis of bone marrow cytokines and chemokines (Y axis: pg/ml) with the lung bacterial burden (X axis; log_{10}CFU). Statistical significance was calculated using the t test. $p < 0.05$ was considered significant and ** = p<0.001*** = p<0.0001, **** = P<0.00001.

5B). Interestingly, the response to therapy in the lungs (**Figure 5C**) was manifested by a significant increase of CD3+CD4+ T helper cells and B-1 cells (CD3-CD19+) and a reverse trend for CD3+CD8+ and γδ-T cells (CD3+CD8+γδTCR+).

Furthermore, we also assessed changes in the distribution of immune cells in the lungs from Mtb-infected C3HeB/FeJ mice in response to therapy using multiplex fluorescent immunohistochemistry (mfIHC). A 7-color composite image for cell markers (B220, CD4, CD8, Foxp3, F4/80 and Ly6G) along with DAPI is shown in **Figure 6A**, while their single-color staining is shown in **Figure 6B**. **Figure 6A** shows a typical necrotic TB granuloma comprised of central necrosis, peripheral rim and lung parenchyma. The analysis of mfIHC images revealed that the BPaL and BPaS treatments significantly and dramatically lowered the number of neutrophils (count based on Ly6G+) compared to UnRx control, however, F4/80+ cells were observed significantly higher in BPaS compared to UnRx control (**Figure 6C**, S16). Interestingly, the spearman's correlation plot (**Figure 6D**) shows that a significant decrease in neutrophils was positively correlated with the corresponding increase in most of the other immune cells.

Discussion

We used two preclinical chronic TB murine efficacy models (34–36) to investigate BPa, BPaL and BPaS, for efficacy and any associated AEs during the course of 4- weeks of treatment. Overall, our antimicrobial data are in accordance with the granuloma spectrum of both mouse models used where a more robust reduction in lung bacterial burden was observed in the absence (BALB/c) versus the presence (C3HeB/FeJ) of necrotic lesions. Both multidrug regimens as BPaL or BPaS significantly reduced lung bacterial burden by 3.8-4.0 $\log_{10}$ and 2.5-2.7 $\log_{10}$ in BALB/c and C3HeB/FeJ TB models, respectively (**Figures 1E-H**). We therefore conclude that both regimens promote similar bactericidal effects in murine models lacking, or featuring, advanced pulmonary pathology. Furthermore, the potent antimicrobial effect of the BPaL and BPaS regimens also resulted in improvement of the pathological outcome during chronic infection with Mtb but only the L-containing BPaL regimen, not BPaS, was associated with a significant decrease in the body weight at week 4 in Mtb- infected BALB/c and C3HeB/FeJ mice (Figure S4B and S10B). Future studies will determine if prolonged treatment with BPaL will continue affecting the body weight of the animals. The extent of weight loss is an important preclinical and clinical parameter in TB patients because it determines the severity of disease progression (43), and it is also an indicator of *in vivo* drug efficacy (44). The bactericidal effects of S (as monotherapy) (45) and BPaL (46) in mice observed in these studies is in line with previous reports and similar inverse relationship between body weight and L exposure was recently reported in human patients (47).

Among L-associated hematological side effects, the incidence of anemia is reported up to 62.5% in MDR and XDR-TB patients (48–50) and the onset of this effect can occur 2 weeks to 2 months post L administration (38). In TB patients treated with anti- TB drugs (51–53), increased RDWs and lower HGB are associated with anemia and these parameters serve as markers of disease prognosis. Using a CBC profile of 20 peripheral blood parameters, our preclinical study failed to detect any difference between L or S 4-weeks monotherapy in terms of total red blood cells, white blood cells, platelets, or hemoglobin (HGB) concentration compared to UnRx control (data not shown). However, L treatment alone increased the RDWs and both L and S decreased the MCHC (**Figure 3A**). Likewise, none of the 20-blood parameters evaluated showed any change after the first 2 weeks of therapy with BPa, BPaL, or BPaS (**Figure 3B**). However, by 4 weeks, a significant drop in HGB and an increase in the RDWs was apparent for the BPaL group (**Figure 3B**). This is interpreted to mean that mild hematological effects observed in mice treated for 4 weeks with L or BPaL are dependent on the number of L-doses administered and are thus, time-dependent. Overall, the onset of these hematological effects when testing an L-containing regimen (BPaL) is in agreement with previous studies (38,39) in human patients.

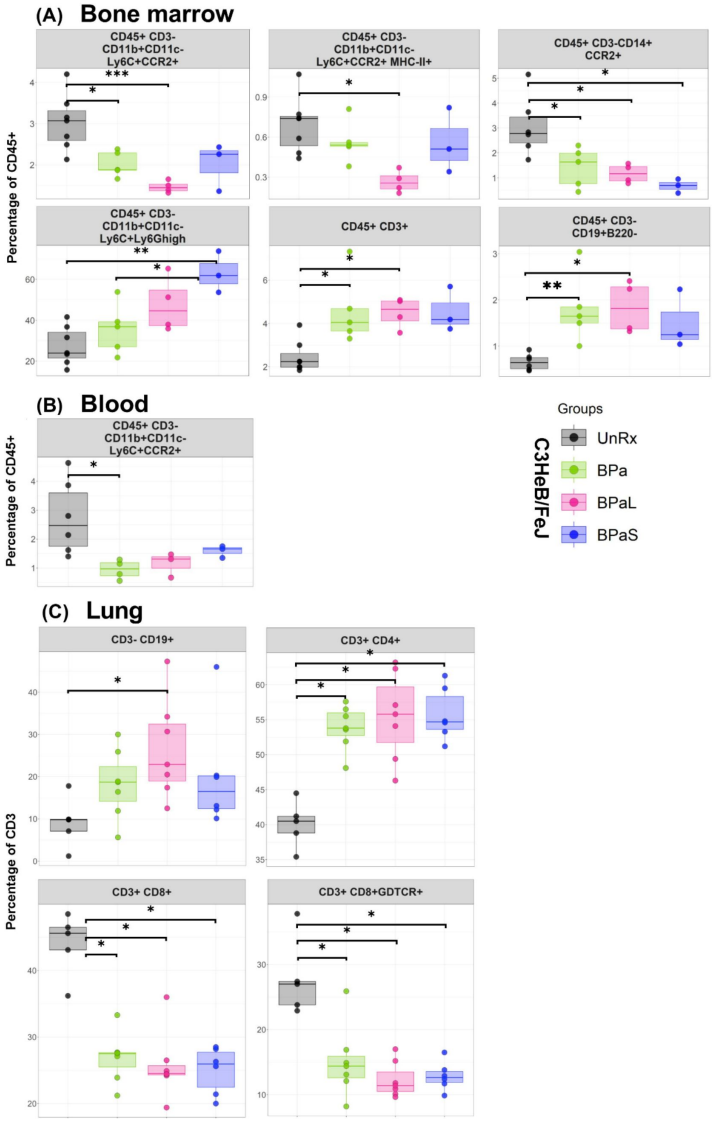

Figure 5.

Immune cell populations in the bone marrow, lung and blood of C3HeB/FeJ TB model after 4-weeks of treatment The bone marrow, lung and blood from selected studies from **Figure 1** were evaluated by flow cytometry. The samples were processed for a panel of 17-color antibodies and the data were analyzed by FlowJo software using manual gating strategy. The myeloid and lymphoid phenotypes present in the untreated (UnRx) and treatment (BPa, BPaL or BPaS) groups are shown. Statistical significance was calculated using one-way ANOVA with Tukey's test for multiple comparisons. $p < 0.05$ was considered significant and ** = p<0.001*** = p<0.0001, **** = P<0.00001.

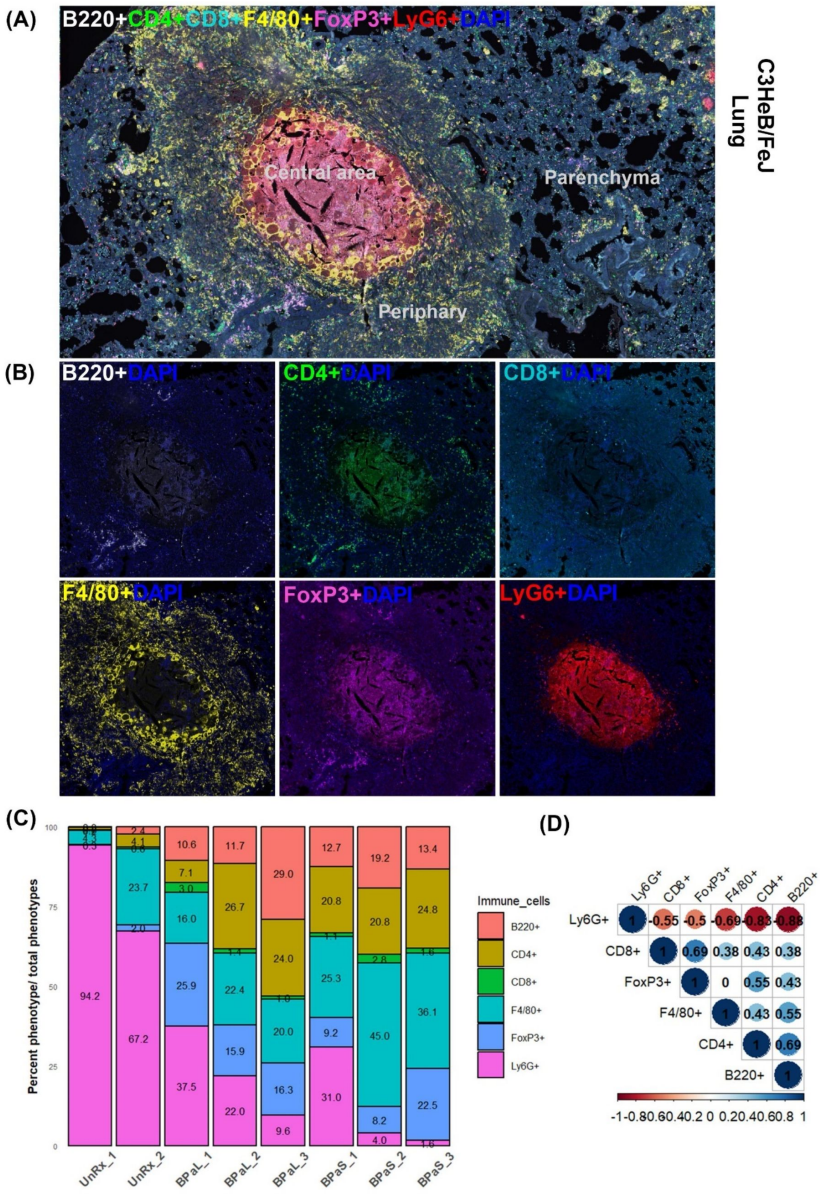

Figure 6.

Immune cell populations in the lungs of Mtb infected C3HeB/FeJ TB model after 4-weeks of treatment Selected mice from those shown in **Figure 1** were processed for multiplex fluorescence immunohistochemistry (mfIHC). The mfIHC was performed for a panel of 6-color antibodies + DAPI using Opal-plex Tyramide Signal Amplification (TSA). Slides were scanned using multispectral automated PhenoImager™ (Akoya Biosciences) and analyzed for different immune cell populations using the inForm® tissue Finder and Phenochart software (Akoya Biosciences). (A) The lung mfIHC full composite image displays B220, CD4, CD8, F4/80, FoxP3 and Ly6G markers along with DAPI staining for nuclei in the TB granuloma. The central and peripheral regions of a TB granuloma and the parenchyma of lung are also shown. (B) Single color composite image of individual markers with DAPI showing distribution of each immune cell population in the TB granuloma. (C) Cell populations (%) of several immune cells per total number of phenotypes calculated in untreated (UnRx: n = 2 mice) and treatment (BPaL and BPaS: n = 3 mice each) groups based on a panel of 6-color antibodies + DAPI. (D) Spearman's correlation matrix for several immune cell populations (B220, CD4, CD8, F4/80, FoxP3, Ly6G) showing all relationships. A coefficient with a value of either +1 (blue), 0 (white), or -1 (red) indicates a perfect association, no association, and a perfect negative association of ranks, respectively. Numbers indicate the correlation coefficient.

The mechanism of L-induced toxicity is attributed to its binding with the host mitochondrial ribosomes leading to mitochondrial toxicities (17). The latter results in activation of Nlrp3 inflammasome (54) and subsequently results in L-mediated bone marrow myelosuppression (55); a phenomenon consistent with the hematologic anomalies seen in patients treated with L for extended time periods. Because spectinamides do not bind to mitochondrial ribosomes there is reduced potential for similar side effects (16,18). To conclusively test this hypothesis, we performed bone marrow histopathology to quantify the myeloid to erythroid ratio (M:E), a parameter that provides information about the relative proportions of myeloid lineage (granulocytes, monocytes, and their precursors) to erythroid lineage (56). The BPa and BPaL regimens altered M:E in the C3HeB/FeJ TB model by suppressing myeloid and inducing erythroid lineages (**Figure 3C**&D) whereas no such difference was observed in mice treated with BPaS compared to untreated control. L was previously shown to impact M:E ratio, although an opposite trend was observed in those studies, which employed 12-days of L administration and a different strain of otherwise healthy mice (54). Time course studies using a single consistent assay method are needed to resolve this discrepancy.

Elevation of interleukin 1β (IL-1β) levels and activation of Nlrp3 inflammasome have been previously linked to myelosuppression (55). A 26-plexed immunoassay on bone marrow samples from Mtb-infected C3HeB/FeJ mice revealed that most of the proinflammatory cytokines and chemokines including IL-1β, IL-12p70 and TNF-α were present at significantly higher concentrations in animals treated with BPaL compared to BPaS (**Figure 4A**&B). The presence of elevated IL-1β was previously reported during monotherapy with L (54,57). Studies from UnRx Mtb-infected C3HeB/FeJ mice also revealed highly elevated levels of cytokines and chemokines (**Figure 4C**). As expected, the positive therapeutic effect of BPaL and BPaS (as seen by reduction in bacterial and lesion burden of lungs) also correlated with decreased level of proinflammatory cytokines in bone marrow, lung, and plasma (**Figures 4C**, S14 and S15 respectively).

Similar to changes observed for cytokine profile, the flow cytometry data of bone marrow, blood, and lungs along with quantification of mfIHC lung image analysis revealed a significant change in the percentage of myeloid and lymphoid phenotypes during treatment compared to UnRx control (**Figures 5** and **6**). Most notably, the therapeutic effect of the BPa, BPaL and BPaS treatments reduced inflammatory myeloid cells expressing CCR2 in blood and bone marrow and reduction in cytotoxic T cells (CD3+CD8+) and γδT cells (CD3+CD8+γδTCR+) in lungs. In addition, BPa, BPaL and BPaS therapy significantly increased the influx of helper T cells (CD3+CD4+), regulatory T cells (Foxp3+) and B cells (CD3-CD19+) in lungs. These results suggest that the combination therapy promotes the immune system's equilibrium by reducing inflammation and enhancing adaptive immune responses.

An additional striking finding from this study was a strong concordance between the decrease in lung and spleen bacterial burden and a corresponding decrease in the number of cells expressing the neutrophil associated marker (Ly6G) (**Figure 6C**&D). An implication of this finding is that the favorable treatment outcomes may promote a corresponding decline in Ly6G neutrophil population as suggested before (58). Among all immune cell phenotypes studied, no differences were found between BPaL and BPaS treated animals and only the F4/80+ cells in the BPaS (but not in the BPaL) treated animals showed significant increase when compared to the UnRx control.

To conclude, the TB drug development field is working towards developing shorter and safer therapies with a common goal of developing new multidrug regimens of low pill burden that are accessible to patients, of short duration (ideally 2-3 months) and consist of 3-4 drugs of novel mode-of-action with proven efficacy, safety, and limited toxicity. Here we present initial results for new multidrug regimens containing inhaled spectinamide 1599 that are in line with these goals. It is proposed that human use of spectinamides 1599 will be administered using a dry powder formulation delivered by the RS01 Plastiape dry powder inhaler. We already reported on the

aerodynamic properties of dry powder spectinamide 1599 within #3 HPMC capsules and delivered from a RS01 Plastiape inhaler device (59). Future studies are already under consideration for funding by NIH-NIAID to understand the pharmacokinetics of mono, binary and ternary combinations of BPaS. These studies also aim to identify the optimal dose level and dosing frequency of each regimen along with their efficacy and relapse free-sterilization potential. Studies are also planned using a model-based pharmacokinetic-pharmacodynamic (PKPD) framework, guided by an existing human BPa PKPD model (61) to find allometric human dose levels, dosing frequencies and treatment durations that will inform the experimental design of future clinical studies.

Materials and Methods

Female C3HeB/FeJ and BALB/c mice at 6-8 weeks of age were purchased from the Jackson Laboratories. All protocols and use of these animals were approved by the Institutional Animal Care and Use Committee (IACUC) at CSU. Animals were infected with a low dose aerosol infection of Mtb (Erdman strain; ATCC 35801) using an inhalation exposure system (Glas-Col, Terre Haute, IN) calibrated to deliver ~50-100 colony forming units (CFU) to the lungs (22). Clinical observations (e.g., inactivity, rough fur, hunched posture, increased respiratory rate or effort) were monitored daily and their body weights were taken weekly.

Drug preparation and treatment

Bedaquiline fumarate (B) and linezolid (L) were obtained from LKT laboratories, pretomanid (Pa) from ChemShuttle and 1599 (S: dihydrochloride) was provided by Dr. Lee at St. Jude Children's Research Hospital. B was administered at 25 mg/kg. Pa and L were administered at 100 mg/kg each and S was delivered by inhalation in liquid form at 50 and 100 mg/kg to BALB/c and C3HeB/FeJ mice, respectively. The drugs were formulated in weekly batches according to the body weight of the animals, aliquoted for single daily dosing, and stored at 4 °C in the dark.

The drugs were prepared and administered as reported previously (21,22). Drug treatment was started 4 weeks post-aerosol infection for BALB/c and at 8-9 weeks post- aerosol infection for C3HeB/FeJ mice to allow time for lung pathology to fully develop.

All drugs were administered once daily for 5 days/week for 4 weeks by oral (gavage) administration except S which was administered 3 days/week by intrapulmonary aerosol delivery using the Penn Century microsprayer as reported previously (22). B was administered in the morning, Pa one hour after B and, L and S at least 4 hours after Pa.

Necropsy

After 4-weeks of treatment, C3HeB/FeJ and BALB/c mice were humanely euthanized by CO_2 narcosis. Blood, lungs, spleen, femur and tibia bones were collected from each mouse for further processing and analysis.

Assessment of Efficacy

The efficacy of the treatment was assessed by determining changes in bacterial burden [measured as colony forming units (CFU)] in the lungs and spleen of animals at necropsy. The lungs and spleen were homogenized and prepared as reported previously (22). The lung homogenates were plated onto 7H11 agar plates supplemented with 0.4% activated charcoal to reduce the carryover effect of drugs and incubated at 37 °C for 6-8 weeks before the final CFU count. The remaining lung homogenate was centrifuged, and the supernatant was collected and stored at -80 °C for evaluation of cytokines and chemokines.

Histopathology and lesion scoring

The lungs were fixed in 4% paraformaldehyde (PFA) for 48 hours then embedded in paraffin for histopathology. Sections from formalin fixed and paraffin embedded (FFPE) tissues were cut at 5 µm, stained with hematoxylin and eosin (H&E) and scanned at 40X magnification using multispectral automated PhenoImager (Akoya Biosciences) for histopathological evaluation. The extent of lung lesion burden was quantified in blinded digital images using an open-source QuPath software for image analysis as described previously (62). For each tissue section, a region of interest (ROI) was generated at low magnification with a custom tissue detecting algorithm using decision forest training and classification to differentiate tissue versus background based on color and area. Lesions were identified within tissue ROIs at high magnification with an additional custom-made algorithm using decision forest training and classification based on staining intensity, color normalization and deconvolution, area, and morphological features. Percent lesion calculations were integrated into the same algorithm and calculated from tissue area and lesion area as designated by the ROI and lesions detected.

The sternum and one femur were fixed in 4% PFA and processed for histology. To evaluate the myelosuppressive effect of the drugs, bone sections were cut at 5 µm and stained with H&E. The number of myeloid and erythroid cells from 5 different regions of the bone were blinded and then counted by a veterinary clinical pathologist.

Bone marrow collection

Briefly, a 0.6 mL sterile Eppendorf tube punctured at the bottom with the help of a 26-gauge needle was inserted into a 1.5 mL sterile Eppendorf tube. One end of the epiphysis of the long bones was cut open to expose the bone marrow and placed down into the small Eppendorf tube-system. The tubes were centrifuged at 10,000 x g for 15 seconds and the marrow was collected from the base of the large Eppendorf tube. The bone marrow was resuspended in PBS and centrifuged again. Thereafter, the supernatant was collected and stored at -80 °C for evaluation of cytokines and chemokines while the bone marrow cells were saved in 4% PFA and freezing media for further use in clinical pathology analysis and flow cytometry, respectively.

Processing of blood

For complete blood count (CBC) analysis of C3HeB/FeJ animals during the treatment, blood was collected in K2-EDTA tubes via submandibular vein puncture as described previously (63). The blood was immediately analyzed in VETSCAN® HM5 hematology analyzer (Zoetis).

At the time of necropsy, whole blood was collected via cardiac puncture in K2- EDTA containing tubes. After adding an equal volume of PBS, the samples were centrifuged at 800 × g for 10 min at 25 °C with the brake off (deceleration = 0). The top plasma layer was collected and stored at -80 °C for evaluation of their cytokine's content. The buffy coat was collected, washed and the erythrocytes were lysed using Miltenyi RBC lysis buffer (Miltenyi, CA). The cells were washed and resuspended in 500 µL of complete DMEM media and prepared for flow cytometry analysis.

Cytokine quantification

Multiplex immunoassay was performed using a Luminex bead-based multiplex ELISA kit (ProcartaPlex Mouse Cytokine & Chemokine Panel 1 26plex, reference # EPXR260-26088-901, Invitrogen). Each sample was normalized to the total protein concentration determined by Bicinchoninic acid (BCA) assay (Thermo Fisher). The BCA and Luminex assay were performed according to the manufacturer's instructions and the final stained samples were fixed with 4% PFA prior to acquisition. Sample data were acquired on a MAGPIX instrument running xPONENT 4.3 software (Luminex Corp.).

Heatmaps were generated using R pheatmap package. Correlation analysis of cytokine contents in bone marrow, plasma and lungs with the lung bacterial burden was performed using the corrplot package in R.

Immune cell populations analysis

Single cell suspension of bone marrow, blood, and lungs from C3HeB/FeJ mice was prepared as described previously (62). Cells counting, viability staining and cell staining (Table S1) was performed accordingly (62). Samples were acquired using Cytek Aurora^{TM} 4-Laser spectral flow cytometer where 100,000 events were recorded. Data were analyzed in FlowJo software (BD Biosciences) using manual gating (64).

Multiplex fluorescent immunohistochemistry

Five µm sections of FFPE lung tissues were stained for multiplex fluorescent immunohistochemistry (mfIHC) by the Imaging Core at the University of Colorado, Anschutz Medical Campus, Denver. The mfIHC was performed for a panel of 6-color antibodies + DAPI using Opal-plex Tyramide Signal Amplification (TSA) technique using a Leica Bond III autostainer. The details of antibodies and Opal fluorophores used are given in Table S2. Each antibody was optimized using Opal 3-Plex Anti-Rb Detection Kit (Akoya Biosciences Inc. cat# NEL830001KT) and stained with automated LabSat^{TM} Research (Lunaphore Technologies SA, Epredia). Slides were scanned using multispectral automated PhenoImager^{TM} (Akoya Biosciences) and analyzed for several immune cell populations using the inForm$^{®}$ tissue Finder (Version 2.4.8) and Phenochart^{TM} (Version 1.0.12) software (Akoya Biosciences).

Statistical analysis

Bacterial burden data were expressed as CFU which were Log_{10}- transformed and analyzed using GraphPad Prism version 9.5.1 (GraphPad software, La Jolla, CA). The statistical analysis was performed using a Tukey's multiple comparison test as part of either one-way or two-way ANOVA and mixed-model effect where necessary. The correlation analysis was performed using the spearman's correlation test. Flow cytometry and mfIHC data were graphed in R studio and statistical evaluation was performed using stats package in R.

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Editors

Reviewing Editor
Bavesh Kana
University of the Witwatersrand, Johannesburg, South Africa

Senior Editor
Bavesh Kana
University of the Witwatersrand, Johannesburg, South Africa

Reviewer #1 (Public Review):

Summary:
The manuscript entitled A Modified BPaL Regimen for Tuberculosis Treatment replaces Linezolid with Inhaled Spectinamides by Malik Zohaib Ali et al. is an extension of previous studies by this group looking at the new drug spectinamide 1599. The authors directly compare therapy with BPaL (bedaquiline, pretomanid, linezolid) to a therapy that substitutes spectinamide for linezolid (BPaS). The Spectinamide is given by aerosol exposure and the BPaS therapy is shown to be as effective as BPaL without adverse effects. The work is rigorously performed and analyses of the immune responses are consistent with curative therapy.

Strengths:

1. This group uses 2 different mouse models to show the effectiveness of the BPaS treatment.
   2)Impressively the group demonstrates immunological correlates associated with Mtb cure with the BPaS therapy.
   3)Linezolid is known to inhibit ribsomes and mitochondria whereas spectinaminde does not. The authors clearly demonstrate the lack of adverse effects of BPaS compared to BPaL.

Weaknesses:

1. Although this is not a weakness of this paper, a sentence describing how the spectinamide would be administered by aerosolization in humans would be welcomed.

https://doi.org/10.7554/eLife.96190.2.sa3

Reviewer #2 (Public Review):

Summary:
Replacing linezolid (L) with the preclinical development candidate spectinamide 1599, administered by inhalation, in the BPaL standard of care regimen achieves similar efficacy, reduces hematological changes and por-inflammatory responses.

Strengths:
The authors not only measure efficacy but also quantify histological changes, hematological responses and immune responses, to provide a comprehensive picture of treatment response and the benefits of the L to S substitution.

The authors generate all data in two mouse models of TB infection, each reproducing different aspects of human histopathology.

Extensive supplementary figures ensure transparency.

Weaknesses:
Articulation of objectives and hypotheses can be improved, as suggested below.

https://doi.org/10.7554/eLife.96190.2.sa2

Reviewer #3 (Public Review):

Summary:
In this paper, the authors sought to evaluate whether the novel TB drug candidate, spectinamide 1599 (S), given via inhalation to mouse TB models, and combined with the drugs B (bedaquiline) and Pa (pretomanid), would demonstrate similar efficacy to that of BPaL regimen (where L is linezolid). Because L is associated with adverse events when given to patients longterm, and one of those is associated with myelosuppression (bone marrow toxicity) the authors also sought to assess blood parameters, effects on bone marrow, immune parameters/cell effects following treatment of mice with BPaS and BPaL. They conclude that BPaL and BPaS have equivalent efficacy in both TB models used and that BPaL resulted in weight loss and anemia (whereas BPaS did not) under the conditions tested, as well as effects on bone marrow.

Strengths:
The authors used two mouse models of TB that are representative of different aspects of TB in patients (which they describe well), intending to present a fuller picture of the activity of the tested drug combinations. They conducted a large body of work in these infected mice to evaluate efficacy and also to survey a wide range of parameters that could inform the effect of the treatments on bone marrow and on the immune system. The inclusion of BPa controls (in most studies) and also untreated groups led to a large amount of useful data that has been collected for the mouse models per se (untreated) as well as for BPa - in addition to the BPaS and BPaL combinations which are of particular interest to the authors. Many of these findings related to BPa, BPaL, untreated groups etc corroborate earlier findings and the

authors point this out effectively and clearly in their manuscript. To go further, in general, it is a well written and cited article with an informative introduction.

Weaknesses:
The authors performed a large amount of work with the drugs given at the doses and dosing intervals stated, but there is no exposure data available at this time. The authors intend to evaluate exposure-effect relationships in future work. An understanding of the exposures at which the efficacy and adverse effects are seen will assist in the translation of these findings to the clinic.
In addition, it is always challenging to interpret findings for combinations of drugs and for now, the data available cannot attribute confidence to the weight loss seen for only the BPaL group to L specifically, as opposed to a PK interaction leading to an elevated exposure and weight loss due to B or Pa. It is not yet possible, then to state that what is seen are "L-associated AEs" - this is assumed only.
The evaluations of activity in the BALB/c mouse model as well as the spleens of the Kramnik model resulted in CFU below/at the limit of detection so comparisons between BPaL and BPaS cannot be made and so the conclusion of equivalent efficacy in BALB/c is not supported with the data shown. There is no BPa control in the BALB/c study, therefore it is not possible to discern whether L or S contributed to the activity of BPaL or BPaS. The same is true for the assessment of lesions - unfortunately, there was no BPa control meaning that even where equivalency is seen for BPaL and BPaS, the reader is unable to deduce whether L or S made a contribution to this activity.
Although these weaknesses limit what we can learn from the current body of data, the authors note that further studies will be done to increase understanding of the points above.

https://doi.org/10.7554/eLife.96190.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *In this useful study, the authors report the efficacy, hematological effects, and inflammatory response of the BPaL regimen (containing bedaquiline, pretomanid, and linezolid) compared to a variation in which Linezolid is replaced with the preclinical development candidate spectinamide 1599, administered by inhalation in tuberculosis-infected mice. The authors provide convincing evidence that supports the replacement of Linezolid in the current standard of care for drug-resistant tuberculosis. However, a limitation of the work is the lack of control experiments with bedaquiline and pretomanid only, to further dissect the relevant contributions of linezolid and spectinamide in efficacy and adverse effects.*

We acknowledge a limitation in our study due to lack of groups with monotherapy of bedaquiline and pretomanid however, similar studies to understand contribution of bedaquiline and pretomanid to the BPaL have been published already (references #4 and #60 in revised manuscript). Our goal was to compare the BPaS versus the BPaL with the understanding that TB treatment requires multidrug therapy. We omitted monotherapy groups to reduce complexity of the studies because the multidrug groups require very large number of animals with very intensive and complex dosing schedules. Even if B or Pa by themselves have better efficacy than the BPa or BPaL combination, patients will not be treated with only B or Pa because of very high risk of developing drug resistance to B or/and PA. If drug resistance is developed for B or Pa, the field will lose very effective drugs against TB.

> *Although the manuscript is well written overall, a re-formulation of some of the stated hypotheses and conclusions, as well as the addition of text to contextualize translatability, would improve value.*

Manuscript has been edited to address these critiques. Answers to individual critiques are below.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *This manuscript is an extension of previous studies by this group looking at the new drug spectinamide 1599. The authors directly compare therapy with BPaL (bedaquiline, pretomanid, linezolid) to a therapy that substitutes spectinamide for linezolid (BPaS). The Spectinamide is given by aerosol exposure and the BPaS therapy is shown to be as effective as BPaL without adverse effects. The work is rigorously performed and analyses of the immune responses are consistent with curative therapy.*
>
> *Strengths:*
>
> *(1) This group uses 2 different mouse models to show the effectiveness of the BPaS treatment.*
>
> *(2) Impressively the group demonstrates immunological correlates associated with Mtb cure with the BPaS therapy.*
>
> *(3) Linezolid is known to inhibit ribosomes and mitochondria whereas spectinaminde does not. The authors clearly demonstrate the lack of adverse effects of BPaS compared to BPaL.*
>
> *Weaknesses:*
>
> *(1) Although this is not a weakness of this paper, a sentence describing how the spectinamide would be administered by aerosolization in humans would be welcomed.*

We already reported on the aerodynamic properties of dry powder spectinamide 1599 within #3 HPMC capsules and its delivery from a RS01 Plastiape inhaler device (reference #59 in revised manuscript). To address this critique, we added a last paragraph in discussion “It is proposed that human use of spectinamides 1599 will be administered using a dry powder formulation delivered by the RS01 Plastiape dry powder inhaler" (reference #59 in revised manuscript).

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Replacing linezolid (L) with the preclinical development candidate spectinamide 1599, administered by inhalation, in the BPaL standard of care regimen achieves similar efficacy, and reduces hematological changes and proinflammatory responses.*
>
> *Strengths:*
>
> *The authors not only measure efficacy but also quantify histological changes, hematological responses, and immune responses, to provide a comprehensive picture of treatment response and the benefits of the L to S substitution.*

> *The authors generate all data in two mouse models of TB infection, each reproducing different aspects of human histopathology.*
>
> *Extensive supplementary figures ensure transparency.*
>
> *Weaknesses:*
>
> *The articulation of objectives and hypotheses could be improved.*

We edited to "The AEs were associated with the long-term administration of the protein synthesis inhibitor linezolid. Spectinamide 1599 (S) is also a protein synthesis inhibitor of *Mycobacterium tuberculosis* with an excellent safety profile, but which lacks oral bioavailability. Here, we propose to replace L in the BPaL regimen with spectinamide administered via inhalation and we demonstrate that inhaled spectinamide 1599, combined with BPa —BPaS regimen—has similar efficacy to that of BPaL regimen while simultaneously avoiding the L-associated AEs.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In this paper, the authors sought to evaluate whether the novel TB drug candidate, spectinamide 1599 (S), given via inhalation to mouse TB models, and combined with the drugs B (bedaquiline) and Pa (pretomanid), would demonstrate similar efficacy to that of BPaL regimen (where L is linezolid). Because L is associated with adverse events when given to patients long-term, and one of those is associated with myelosuppression (bone marrow toxicity) the authors also sought to assess blood parameters, effects on bone marrow, immune parameters/cell effects following treatment of mice with BPaS and BPaL. They conclude that BPaL and BPaS have equivalent efficacy in both TB models used and that BPaL resulted in weight loss and anemia (whereas BPaL did not) under the conditions tested, as well as effects on bone marrow.*
>
> *Strengths:*
>
> *The authors used two mouse models of TB that are representative of different aspects of TB in patients (which they describe well), intending to present a fuller picture of the activity of the tested drug combinations. They conducted a large body of work in these infected mice to evaluate efficacy and also to survey a wide range of parameters that could inform the effect of the treatments on bone marrow and on the immune system. The inclusion of BPa controls (in most studies) and also untreated groups led to a large amount of useful data that has been collected for the mouse models per se (untreated) as well as for BPa - in addition to the BPaS and BPaL combinations which are of particular interest to the authors. Many of these findings related to BPa, BPaL, untreated groups, etc corroborate earlier findings and the authors point this out effectively and clearly in their manuscript. To go further, in general, it is a well-written and cited article with an informative introduction.*
>
> *Weaknesses:*
>
> *The authors performed a large amount of work with the drugs given at the doses and dosing intervals started, but at present, there is no exposure data available in the paper. It would be of great value to understand the exposures achieved in plasma at least (and in the lung if more relevant for S) in order to better understand how these relate to clinical exposures that are observed at marketed doses for B, Pa, and L as well as to understand the exposure achieved at the doses being evaluated for S. If available as historical data this could be included/cited. Considering the great attempts made to*

> *evaluate parameters that are relevant to clinical adverse events, it would add value to understand what exposures of drug effects such as anemia, weight loss, and bone marrow effects, are being observed. It would also be of value to add an assessment of whether the weight loss, anemia, or bone marrow effects observed for BPaL are considered adverse, and the extent to which we can translate these effects from mouse to patient (i.e. what are the limitations of these assessments made in a mouse study?). For example, is the small weight loss seen as significant, or is it reversible? Is the magnitude of the changes in blood parameters similar to the parameters seen in patients given L? In addition, it is always challenging to interpret findings for combinations of drugs, so the addition of language to explain this would add value: for example, how confident can we be that the weight loss seen for only the BPaL group is due to L as opposed to a PK interaction leading to an elevated exposure and weight loss due to B or Pa?*

We totally agree with this critique but the studies suggested by the reviewer are very expensive and

logistically/resource intensive. Data reported in this manuscript was used as preliminary data in a RO1 application to NIH-NIAID that included studies proposed above by this reviewer. The authors are glad to report that the application got a fundable score and is currently under consideration for funding by NIH-NIAID. The summary of proposed future studies is included in the last paragraph of the discussion in this revised manuscript.

> *Turning to the evaluations of activity in mouse TB models, unfortunately, the evaluations of activity in the BALB/c mouse model as well as the spleens of the Kramnik model resulted in CFU below/at the limit of detection and so, to this reviewer's understanding of the data, comparisons between BPaL and BPaS cannot be made and so the conclusion of equivalent efficacy in BALB/c is not supported with the data shown. There is no BPa control in the BALB/c study, therefore it is not possible to discern whether L or S contributed to the activity of BPaL or BPaS; it is possible that BPa would have shown the same efficacy as the 3 drug combinations. It would be valuable to conduct a study including a BPa control and with a shorter treatment time to allow comparison of BPa, BPaS, and BPaL.*

We agree with the reviewer these studies need to be done. Some of them were recently published by our colleague Dr. Lyons (reference #60 in revised manuscript). The studies proposed by the reviewer will be performed under a new award under consideration for funding by the NIH-NIAID, the summary of future studies is included in the last paragraph of the discussion in this revised manuscript.

> *In the Kramnik lungs, as the authors rightly note, the studies do not support any contribution of S or L to BPa - i.e. the activity observed for BPa, BPaL, and BPaS did not significantly differ. Although the conclusions note equivalency of BPaL and BPaS, which is correct, it would be helpful to also include BPa in this statement;*

We edited and now included in lines #191 as requested

> *It would be useful to conduct a study dosing for a longer period of time or assessing a relapse endpoint, where it is possible that a contribution of L and/or S may be seen - thus making a stronger argument for S contributing an equivalent efficacy to L. The same is true for the assessment of lesions - unfortunately, there was no BPa control meaning that even where equivalency is seen for BPaL and BPaS, the reader is unable to deduce whether L or S made a contribution to this activity.*

Added in the future plans in the last paragraph of discussion

"Future studies are already under consideration for funding by NIH-NIAID to understand the pharmacokinetics of mono, binary and ternary combinations of BPaS. These studies also aim to identify the optimal dose level and dosing frequency of each regimen along with their efficacy and relapse free-sterilization potential. Studies are also planned using a model-based pharmacokinetic-pharmacodynamic (PKPD) framework, guided by an existing human BPa PKPD model (reference #61 in revised manuscript), to find allometric human dose levels, dosing frequencies and treatment durations that will inform the experimental design of future clinical studies.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Although this is not a weakness of this paper, a sentence describing how the spectinamide would be administered by aerosolization in humans would be welcomed.*

Last paragraph of discussion was added "It is proposed that human use of spectinamides 1599 will be administered using a dry powder formulation delivered by the RS01 Plastiape dry powder inhaler". We already reported on the aerodynamic properties of dry powder spectinamide 1599 within #3 HPMC capsules and delivered from a RS01 Plastiape inhaler device (reference #59 in revised manuscript)

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Major comments*
>
> *The Abstract lacks focus and could more clearly convey the key messages.*

Edited as requested

> *The two mouse models and why they were chosen need to be described earlier. Currently, it's covered in the first section of the Discussion, but the reader needs to understand the utility of each model in answering the questions at hand before the first results are described, either in the introduction or in the opening section of the results.*

Thank you for suggestion, we agree. We moved the first paragraph in discussion to last paragraph in Introduction.

> *Line 130: Please justify the doses and dosing frequency for S. A reference to a published manuscript could suffice if compelling.*

The dosing and regimens were previously reported by our groups in ref 21 and 22 in revised manuscript.-

(21) Robertson GT, Scherman MS, Bruhn DF, Liu J, Hastings C, McNeil MR, et al. Spectinamides are effective partner agents for the treatment of tuberculosis in multiple mouse infection models. J Antimicrob Chemother.

(22) Gonzalez-Juarrero M, Lukka PB, Wagh S, Walz A, Arab J, Pearce C, et al. Preclinical Evaluation of Inhalational Spectinamide-1599 Therapy against Tuberculosis. ACS Infect Dis. 2021;7(10):2850–63.

> *Figures 1 E to H: several "ns" are missing, please add them.*

Edited as requested

> *Line 184 to 190: suggest moving the body weight plots to a Supplemental Figure, and at least double the size of the histology images to convey the message of lines 192-203.*
>
> *Please include higher magnification insets to illustrate the histopathological findings. In that same section, please add a sentence or two describing the lesion scoring concept/method. It is a nice added feature, not widespread in the field, and deserves a brief description.*

Edited as requested. We added detailed description for scoring method in M&M under histopathology and lesion scoring

> *Line 206: please add an introductory sentence explaining why one would expect S to cause (or not) hematological disruption, and why MCHC and RDW were chosen initially (they are markers of xyz). The first part of Figure 3 legend belongs to the Methods.*

To address this critique we added in #225-226 “The effect of L in the blood profile of humans and mouse has been reported (references #38-42 in revised manuscript) but the same has not been reported for S” . In line #229-230 we added “Of 20-blood parameters evaluated, two blood parameters were affected during treatment”.

> *The first part of Figure 3 legend belongs to the Methods.*

We edited Figure 3 to “During therapy of mice in Figure 1, the blood was collected at 1, 2- and 4-weeks posttreatment. The complete blood count was collected in VETSCAN® HM5 hematology analyzer (Zoetis)”.

> *Line 218: please explain why the 4 blood parameters that are shown were selected, out of the 20 parameters surveyed.*

We added an explanation in line 239-240 “out 20-blood parameters evaluated, a total of four blood parameters were affected at 2 and 4-weeks-of treatment”.

> *Line 243 and again Line 262 (similar to comment Line 206): please add an introductory paragraph explaining the motivation to conduct this analysis and the objective. Can the authors put the experiment in the context of their hypothesis?*

To address this critique, we added in line #235-237 “The Nix-TB trial associated the long-term administration of L within the BPaL regimen as the causative agent resulting in anemia in patients treated with the BPaL regimen (5).”

> *Figure 4C (and the plasma and lung equivalent in the SI). This figure needs adequate labeling of axes: X axis = LOG CFU? Please add tick marks for all plots since log CFU is only shown for the bottom line. Y axes have no units: pg/mL as in B?*

Figure legend were edited to add (Y axis:pg/ml) and (X axis; log10CFU).

> *Line 255-256: please remove "pronounced" and "profound". There is a range of CFU reduction and cytokine reduction, from minor to major. The correlation trend is clear and those words are not needed.*

Edited as requested

> *Line 277-289, Figure 6: given the heterogeneity of a C3HeB/FeJ mouse lung (TB infected), and the very heterogeneous cell population distribution in these lungs (Fig. 6A), the validity of whole lung analysis on 2 or 3 mice (the legend should state what 1, 2 and 3 means, individual mice?) is put into question. "F4/80+ cells were observed significantly higher in BPaS compared to UnRx control": Figure S14 suggests a statistically significant difference, but nothing is said about the other cell type, which appears just as much reduced in BPaS compared to UnRx as F4/80+. Overall, sampling the whole lung for these analyses should be mentioned as a limitation in the Discussion.*

We agree with the reviewer that "visually" it appears as other populations in addition to F4/80 have statistical significance. We run again the two way Anova with Tukey test and only the BPaS and UnRx for F4/80 is significant.

We edited figure S16 (previously S14) to add ns for every comparation.

In Figure 6A was edited ; N=2 are 2 mice for Unrx and n=3 mice for BPaL/BPaS each.

> *Line 355-360: "The BPa and BPaL regimens altered M:E in the C3HeB/FeJ TB model by suppressing myeloid and inducing erythroid lineages" This suggests that altered M:E is not associated with L, putting into question the comparison between BPaS, BPaL, and UnRx. Can the authors comment on how M:E is altered in BPa and not in BPaS?*

Our interpretation to this result was that addition of S in our regimen BPsS was capable of restoring the M:E ratio altered by the BPa and BPaL. This interpretation was included in main text in line #263-264 and is also now added to abstract

> *Line 379: discuss the limitations of working with whole lungs.*

Sorry we cannot understand this request. In our studies we always work with whole lungs if the expected course of histopathology/infection among lung lobes is very variable (as is the case of C3HeB/Fej TB model)

> *Concluding paragraph: "Here we present initial results that are in line with these goals." If such a bold claim is made, there needs to be a discussion on the translatability of the route of administration and the dose of S. Otherwise, please rephrase.*

We added the following last paragraph to discussion:

To conclude, the TB drug development field is working towards developing shorter and safer therapies with a common goal of developing new multidrug regimens of low pill burden that are accessible to patients, of short duration (ideally 2-3 months) and consist of 3-4 drugs of novel mode-of-action with proven efficacy, safety, and limited toxicity. Here we present initial results for new multidrug regimens containing inhaled spectinamide 1599 that are in line with these goals. It is proposed that human use of spectinamides 1599 will be administered using a dry powder formulation delivered by the RS01 Plastiape dry powder inhaler. We already reported on the aerodynamic properties of dry powder spectinamide 1599 within #3 HPMC capsules and delivered from a RS01 Plastiape inhaler device (reference #59 in revised manuscript). Future studies are already under consideration for funding by NIHNIAID to understand the pharmacokinetics of mono, binary and ternary combinations of BPaS. These studies also aim to identify the optimal dose level and dosing frequency of each regimen along with their efficacy and relapse free-sterilization potential. Studies are also planned using a model-based pharmacokinetic-pharmacodynamic (PKPD) framework, guided by an existing human BPa PKPD model (references #60 and 61 in revised manuscript) , to find

allometric human dose levels, dosing frequencies and treatment durations that will inform the experimental design of future clinical studies.

> *Minor edits*
>
> *Adverse events, not adverse effects (side effects)*

Edited as requested

> *BALB/c (not Balb/c, please change throughout).*

Edited as requested

> *Line 92: replace 'efficacy' with potency or activity.*

Edited as requested

> *"Live" body weight: how is that different from "body weight"? Suggest deleting "live" throughout, or replace with "longitudinally recorded" if that's what is meant, although this is generally implied.*

Edited as requested

> *The last line of Figure 2 legend is disconnected.*
>
> *Line 331: delete "human".*

Edited as requested

> ***Reviewer #3 (Recommendations For The Authors):***

We thank the reviewer for these suggestions. The data presented in this manuscript with 4 weeks of treatment along with monitoring of effects of therapy in blood, bone marrow and immunity have been submitted for a RO1 application to NIH-NIAID, which have received a fundable score and is under funding consideration. All the points suggested by the reviewer(s) here are included in the research proposed in the RO1 application including manufacturing and physico-chemically characterize larger scale of dry powders of spectinmides and evaluation of their aerodynamic performance for human or animal use; Pharmacokinetics and efficacy studies to determine the optimal dose level and dosing frequency for new multidrug regimens containing spectinamides. These studies include mono, binary and ternary combinations of each multidrug regimen along with their efficacy and relapse free- sterilization potential. These studies will also develop PK/PD simulation-based allometric scaling to aid in human dose projections inhalation. We hope the reviewer will understand all together these studies will last 4-5 years.

> *Although I truly appreciate the great efforts of the authors, I suggest that in order to better evaluate the contribution of S versus L to BPa in these models, repeat studies be run that:*
>
> *(a) include BPa groups to allow the contribution of S and L to be assessed. Included in research proposed RO1 application mentioned above*
>
> *(b) use shorter treatment times in BALB/c to allow comparisons at end of Tx CFU above the LOD. We have added new data for 2 weeks treatment with BPaL and BPaS in Balb/c mice infected with MTb that was removed from previous submission of this manuscript*

> *(c) use longer treatment times and ideally a relapse endpoint in Kramnik to allow*
>
> *assessment of L and S as contributors to BPa (i.e. give a chance to see better efficacy of BPaL or BPaS versus BPa) and also measure plasma exposures of all drugs (or lung levels if this is the translatable parameter for S) to allow detection of any large DDI and also understand the translation to the clinic. Related to the safety parameters, it would be really great to understand whether or not the observations for BPaL would be labeled adverse in a toxicology study/in a clinical study, and it would be useful to include information on the magnitude of observations seen here versus in the clinic (eg for the hematological parameters).*

The research proposed in the RO1 application mentioned above included extensive PK, extended periods of treatment beyond 1 month of treatment (2-5 months as needed to reach negative culturable bacterial from organs) and of course relapse studies.

> *Minor point: I suggest rewording "high safety profile" when describing spectinomides in the intro - or perhaps qualify the length of dosing where the drug is well tolerated*

"high safety profile" was replaced by “an acceptable safety profile”

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