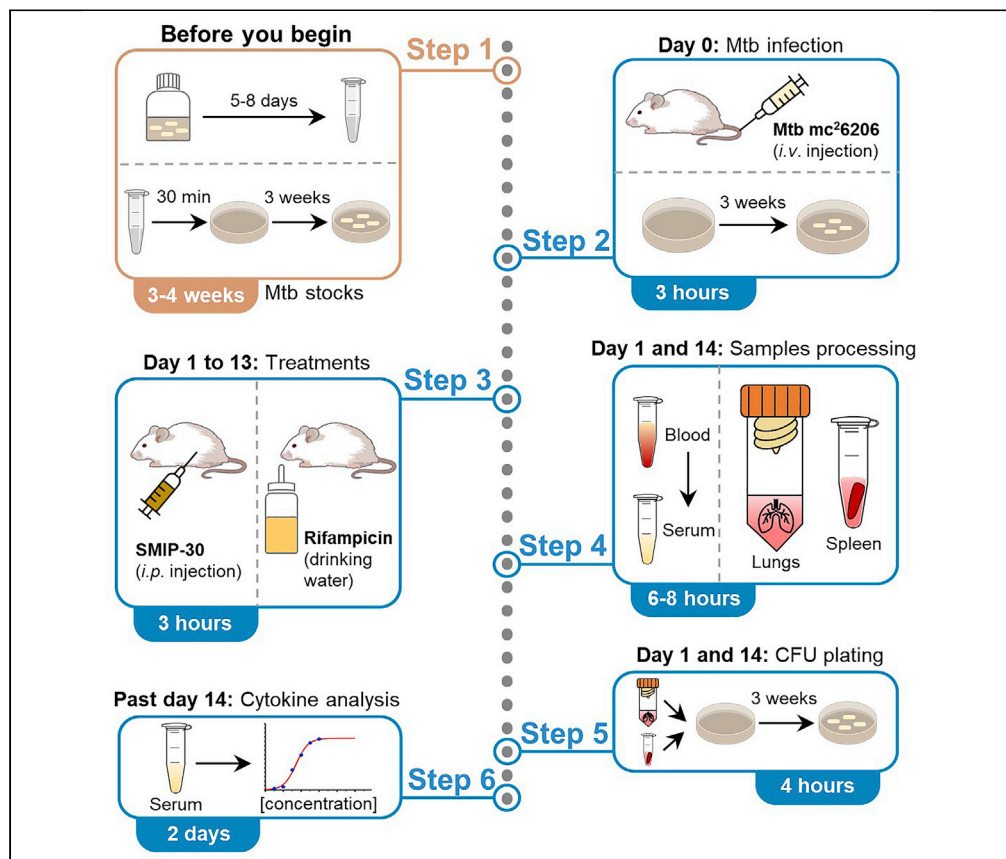


Protocol

An *in vivo* biosafety-level-2-compatible model of *Mycobacterium tuberculosis* infection for drug susceptibility testing



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Highlights

BSL-2 murine model of drug susceptibility testing against *Mycobacterium tuberculosis*

Auxotrophic strain of *Mycobacterium tuberculosis* retaining all virulence genes

Parallel measurement of bacterial burden and cytokine profiling in infected mice

Easily adaptable to test combinations of antibiotics and host-directed compounds

Available mouse models for tuberculosis drug susceptibility testing requires using virulent biosafety-level-3 (BSL-3) *Mycobacterium tuberculosis* (Mtb) strains, or attenuated BSL-2 strains that lack virulence genes. Here, we present a BSL-2-compatible mouse model for tuberculosis drug susceptibility testing using the auxotrophic Mtb mc²6206 strain, which retains all virulence genes. Using rifampicin and a new autophagy-boosting compound, SMIP-30, we provide a step-by-step guide for the infection, drug administration, and evaluation of Mtb burden and cytokine profiles. This protocol is easily adaptable for testing of other antibiotics and host-directed compounds.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

An *in vivo* biosafety-level-2-compatible model of *Mycobacterium tuberculosis* infection for drug susceptibility testingYi Chu Liang,^{1,4} Stefania Berton,^{1,4,5} Courtney Reeks,³ and Jim Sun^{1,2,6,*}¹Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H 8M5, Canada²Centre for Infection, Immunity and Inflammation, University of Ottawa, Ottawa, ON K1H 8M5, Canada³Animal Care and Veterinary Service, University of Ottawa, Ottawa, ON K1H 8M5, Canada⁴These authors contributed equally⁵Technical contact: sberton@uottawa.ca⁶Lead contact*Correspondence: jim.sun@uottawa.ca<https://doi.org/10.1016/j.xpro.2022.101575>

SUMMARY

Available mouse models for tuberculosis drug susceptibility testing requires using virulent biosafety-level-3 (BSL-3) *Mycobacterium tuberculosis* (Mtb) strains, or attenuated BSL-2 strains that lack virulence genes. Here, we present a BSL-2-compatible mouse model for tuberculosis drug susceptibility testing using the auxotrophic Mtb mc²6206 strain, which retains all virulence genes. Using rifampicin and a new autophagy-boosting compound, SMIP-30, we provide a step-by-step guide for the infection, drug administration, and evaluation of Mtb burden and cytokine profiles. This protocol is easily adaptable for testing of other antibiotics and host-directed compounds.

For complete details on the use and execution of this protocol, please refer to Berton et al. (2022).

BEFORE YOU BEGIN

The immune response to tuberculosis (TB) is critical in controlling infection and preventing or resolving disease. TB therapy strategies should ideally contain both an antibiotic component as well as a host-directed component to both directly kill the pathogen and stimulate immune defenses together in order to achieve maximal clearance. *In vitro* drug testing models only partially capture the complexity of Mtb infection, which raises the need for *in vivo* models that can more accurately represent a real infection experienced by the host.

The protocol below describes the use of a murine Mtb infection model for drug susceptibility testing of the TB antibiotic rifampicin and the host-directed compound SMIP-30 to examine their effects on Mtb survival within the mouse and their effects on the cytokine response of the immune system. This protocol provides an *in vivo* infection model using a BSL-2-compatible *M. tuberculosis* H37Rv derivative, originally described and characterized in (Jain et al., 2014). This strain is auxotrophic for both leucine and pantothenate and is fully antibiotic-sensitive (Jain et al., 2014). This strain also behaves similarly to H37Rv in macrophages (Mouton et al., 2019), further supporting its use as a safer alternative model to virulent strains. We recently provided proof of concept that auxotroph Mtb strains can be effectively used for *in vivo* drug susceptibility testing (Berton et al., 2022). Our protocol is intended to provide an example of drugs that could be tested using this model and an overview of the steps and timeline needed.



All media and solutions must be prepared before starting the experiments, according to recipes in the materials and equipment section. It is also possible to store solutions as indicated in the footnotes of the recipes.

△ **CRITICAL:** It is important to consider the age, sex, and background of the mice used in the experiments. Our experiments are performed using 6- to 8-week-old female BALB/c mice. While cohorts of 4–6 mice were necessary and sufficient to evaluate significant differences for most of our studies, we recommend a preliminary sample size calculation before starting (Charan and Kantharia, 2013). All mice used in this protocol are housed in a pathogen-free biosafety level 2 animal facility and allowed to acclimate for 2 weeks before starting the experiments.

Institutional permissions

All experiments and procedures in this protocol were approved by the Animal Care Committee of the University of Ottawa (institutional animal protocol #3234) and supported by the Animal Care and Veterinary Service. Housing and experimental procedures were in accordance with the institutional guidelines, the Animals for Research Act, and the Canadian Council on Animal Care. Researchers will need to obtain approval for animal research and ethics from their relevant institutions.

Preparation of Middlebrook 7H10 agar plates

⌚ Timing: 3 h

△ **CRITICAL:** Plates are prepared in a biological safety cabinet to ensure sterility.

1. For a final volume of 1 liter (including supplements), suspend 19 g of Middlebrook 7H10 agar base in 900 mL demineralized water and add glycerol to 0.5% final concentration.
2. Sterilize by autoclaving at 121°C for 15 min or following established laboratory procedures.
3. Cool the Middlebrook 7H10 agar to 45°C and aseptically add OADC, leucine and pantothenic acid to a final concentration of 10%, 50 µg/mL and 24 µg/mL, respectively.

Note: To avoid premature solidification of the 7H10 agar medium, pre-warm the OADC, leucine, and pantothenic acid briefly at 37°C before adding them to the medium.

Note: Antibiotic resistance can be added to the Mtb mc²6206 strain by transformation with an appropriate plasmid. If you are using a modified Mtb strain with antibiotic resistance, add the specific antibiotic to the agar.

4. Mix by swirling the contents and dispense 25 mL per plate.

Note: Swirl the bottle regularly throughout the preparation process to prevent solidification.

5. Allow plates to solidify. Store them upside down in the dark at 4°C.

△ **CRITICAL:** Avoid preparing large batches of plates. Store the plates for no more than 2 weeks.

Alternatives: To optimize resources and storage space, petri dishes divided in quadrants can be used. These quadrant plates would allow up to four different dilutions or samples to be plated together, reducing the number of plates needed. To prepare quadrant plates, dispense 6 mL of agar into each quadrant. When inoculating the samples for colony-forming units (CFU), plate 25 µL per quadrant.

Preparation of Mtb stocks for *in vivo* infection

⌚ Timing: 30 min to thaw stock and prepare culture, 5–8 days for culture to grow, 1 h to prepare stocks and plate CFU, 3 weeks for colonies to grow

⚠ **CRITICAL:** Mtb work is performed in a biological safety cabinet.

6. Start a new culture of Mtb mc²6206 strain:
 - a. Pre-warm the 7H9 complete medium to 20°C–25°C.
 - b. Thaw one vial of Mtb and transfer the volume in a 30 mL square PETG media bottles with closure (leak-proof screw-cap culture bottle) containing 5 mL of 7H9 complete media. If using a modified Mtb strain with antibiotic resistance, add the appropriate amount of antibiotic to the growth medium.

Note: We usually prepare 1 mL glycerol cryo-stocks of Mtb when the culture OD₆₀₀ is around 0.7. Therefore, when stocks are diluted in 5 mL of 7H9 complete media, the initial OD is around 0.1.

- c. Incubate the culture at 37°C with slow shaking (50 rpm) using a secondary container.
 - d. Monitor the culture growth by checking the OD₆₀₀ daily with a spectrophotometer.

⚠ **CRITICAL:** Use a second autoclavable plastic container to grow and transport the Mtb culture. This will ensure the appropriate containment in case of biohazardous spills. Moreover, before opening the Mtb culture bottle, wait for 5 min to allow any aerosol to settle.

7. When OD₆₀₀ reaches 0.7–0.8 (3–4 days), add 25 mL of 7H9 complete medium to expand the culture.
8. When OD₆₀₀ reaches 0.5–0.8 (2–4 days), prepare the glycerol stocks of the Mtb culture.
 - a. Add sterile glycerol to 20% final concentration into the culture bottle and mix well. We recommend using a sterile autoclaved 60% glycerol solution and add it to the Mtb culture in proportion.
 - b. Aliquot the glycerol-bacteria culture into screw-cap cryo-tubes (1 mL/tube) and freeze all stocks at –80°C.

⚠ **CRITICAL:** When pipetting large volumes of Mtb culture, make sure to avoid the generation of aerosols. Pipette the culture gently and allow aerosols to settle and deposit before re-opening the culture bottle.

9. Establish the CFU of the cryo-stocks:
 - a. Thaw out one cryo-stock of Mtb.
 - b. Calculate the expected number of bacteria (CFU) in the stock based on the original OD₆₀₀ of the culture at the time of freezing using a conversion of OD₆₀₀ 1.0 = 3 × 10⁸ CFU/mL.

Note: This calculation will provide a rough approximation of the real CFU, which will guide the researcher in narrowing down the number of serial dilutions to prepare to assess the real CFU number (step 9c). If the glycerol stocks were prepared as described in step 8a (i.e., adding 1/3 volume of a 60% glycerol solution to a culture to achieve a 20% final glycerol concentration), be sure to account for the diluted OD when making the calculation.

- c. Using 7H9 complete media, prepare 10-fold serial dilutions until you obtain a dilution with an expected CFU count of 50–200 per 100 μL (example shown in [Figure 1A](#)). Plate (in triplicates) 100 μL of this dilution on 7H10 agar plates.

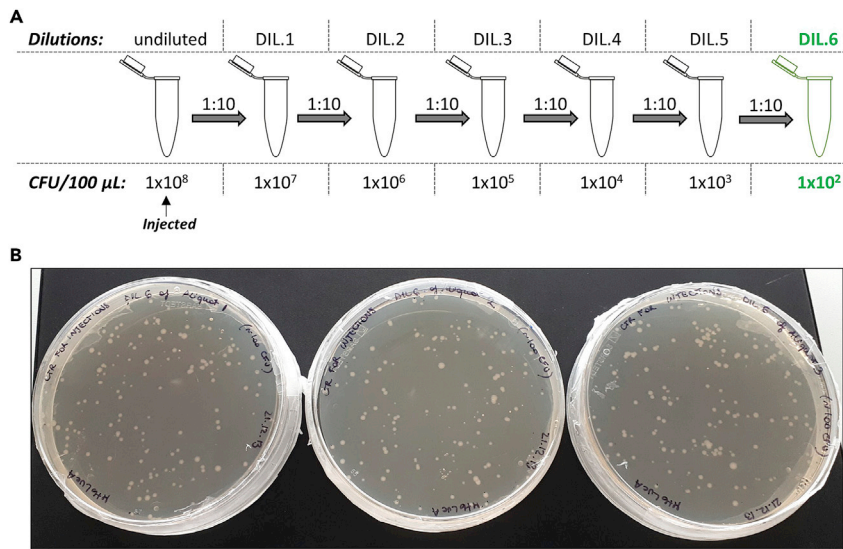


Figure 1. Evaluation of the *i.v.* injected CFU

(A) The scheme shows the ideal serial dilutions to prepare when $\sim 10^8$ CFU are injected *in vivo*. In green, the serial dilution that should be plated to enable easy counting of the colonies.

(B) Examples of plates with a good number of colonies are shown.

10. Incubate the plates at 37°C for 2–3 weeks until colonies are visible (Figure 1B). Count the colonies and convert the number to CFU/mL according to the inoculated dilution.

△ CRITICAL: We usually need to incubate plates for 3 weeks until colonies are ready to count. However, the time needed for growth may vary depending on the CFU that was plated. We recommend to begin checking plates at 2 weeks of incubation. Do not incubate plates for too long as colonies may overgrow and merge.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>Mycobacterium tuberculosis</i> mc ² 6206 (Δ panCD Δ leuCD)	Gift from W. Jacobs, Albert Einstein College of Medicine	N/A
Biological samples		
Mouse blood and serum	(Berton et al., 2022)	N/A
Mouse lungs	(Berton et al., 2022)	N/A
Mouse spleens	(Berton et al., 2022)	N/A
Chemicals, peptides, and recombinant proteins		
Middlebrook 7H9 broth	VWR	Cat#CA90003-876
Middlebrook 7H10 agar	VWR	Cat#CA90003-728
OADC	VWR	Cat#CA90000-418
L-leucine	Fisher Scientific	Cat#AAJ6282422
D-pantothenic acid	VWR	Cat#A16609-22
Tween 80	Fisher Scientific	Cat#AC278632500
PBS	MilliporeSigma	Cat#D8537-500ml
Rifampicin	Fisher Scientific	Cat#AAJ6083603
SMIP-30	(Berton et al., 2022)	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
LEGENDplex™ Mouse Inflammation Panel (13-plex). BioLegend	CEDARLANE	Cat#740446
Experimental models: Organisms/strains		
Mouse: BALB/c (female, 6–8 weeks old, strain code: 028)	Charles River Laboratories	N/A
Software and algorithms		
LEGENDplex™ Data Analysis Software Suite	BioLegend	https://www.biolegend.com/en-us/legendplex/software
GraphPad Prism	GraphPad	RRID: SCR_002798; https://www.graphpad.com/scientific-software/prism/
Other		
Square PETG Media Bottles with Closure	Fisher Scientific	Cat#03-311-1V
Sterile Microcentrifuge Tubes with Screw Caps	Fisher Scientific	Cat#02-681-373
Miltenyi GentleMACS M tubes	Miltenyi	RRID: SCR_020269
Miltenyi GentleMACS GentleMACS	Miltenyi	RRID: SCR_020280
CytoFLEX Flow Cytometer	Beckman Coulter	RRID: SCR_019627
BD Micro-Fine™ IV Insulin Syringes	Fisher Scientific	Cat#14-829-1A
BD 1 mL TB Syringe Slip Tip	Fisher Scientific	Cat#B309659
BD PrecisionGlide™ 25G × 5/8 (0.5 mm × 16 mm) needles	Fisher Scientific	Cat#14-826AA
Fisherbrand™ RNase-Free Disposable Pellet Pestles	Fisher Scientific	Cat#12-141-364

MATERIALS AND EQUIPMENT

Autoclaved 7H9 broth

Reagent	Final concentration	Amount
7H9 broth powder	n/a	4.7 g
Glycerol (60%)	0.2%	3.33 mL
Water	n/a	up to 900 mL
Total	n/a	900 mL

Autoclave 7H9 broth to sterilize. Once autoclaved, broth can be stored at 20°C–25°C for up to 3 months.

7H9 complete media

Reagent	Final concentration	Amount
Autoclaved 7H9 broth	n/a	268.65 mL
OADC	10%	30 mL
Tween 80 (20%)	0.05%	750 µL
L-leucine (50 mg/mL)	50 µg/mL	300 µL
D-pantothenic acid (24 mg/mL)	24 µg/mL	300 µL
Total	n/a	300 mL

We use sterile-filtered Tween 80, leucine, and pantothenic acid. We also sterile filter the prepared media and store it at 4°C for a maximum of 2 months.

Autoclaved 7H10 agar

Reagent	Final concentration	Amount
7H10 agar powder	n/a	19 g
Glycerol (60%)	0.5%	8.3 mL
Water	n/a	up to 900 mL
Total	n/a	900 mL

Autoclave 7H10 agar medium to sterilize. Use it for the preparation of agar plates right after autoclaving.

7H10 agar plates

Reagent	Final concentration	Amount
Autoclaved 7H10 agar	n/a	898 mL
OADC	10%	100 mL
L-leucine (50 mg/mL)	50 µg/mL	1 mL
D-pantothenic acid (24 mg/mL)	24 µg/mL	1 mL
Total	n/a	1000 mL

Before adding all the components to the freshly autoclaved 7H10 agar medium, allow it to cool down to 45°C. We use sterile-filtered leucine and pantothenic acid. Store plates at 4°C in the dark for no more than 2 weeks.

PBS with 0.05% Tween (PBS-Tween)

Reagent	Final concentration	Amount
PBS (1 ×, sterile)	n/a	99.75 mL
Tween 80 (20%)	0.05%	250 µL
Total	n/a	100 mL

We use sterile-filtered Tween 80. Prepare PBS-Tween fresh on the day of use.

Reaction buffer

Reagent	Final concentration	Amount
HEPES	25 mM	0.297 g
Sodium chloride	100 mM	0.292 g
Autoclaved water	n/a	Up to 50 mL
Total	n/a	50 mL

We sterile-filter the reaction buffer and store it at 4°C for no more than 2 weeks.

STEP-BY-STEP METHOD DETAILS

Infection of mice with Mtb – Day 0

⌚ Timing: ~3 h

In this step, we infect mice with 10^7 – 10^8 CFU of Mtb using lateral tail vein injection.

1. Based on the previously evaluated CFU number (see “before you begin” section), thaw the appropriate number of bacterial stocks. Pool the stocks together and centrifuge them at $10,000 \times g$ for 2 min at 20°C–25°C.
2. Remove supernatant and resuspend the bacteria pellet using PBS-Tween to obtain a suspension of the desired CFU in 100 µL. We recommend infecting each mouse with 10^7 – 10^8 CFU.

Note: A high CFU *i.v.* infection is required to deposit sufficient Mtb in lungs and spleen to assess the effects of a treatment. The auxotroph strain Mtb mc²6206 used in this protocol does not proliferate *in vivo*. Therefore, the CFU deposited post-infection will only decrease over the course of the experiment.

3. Prepare at least $n + 3$ aliquots of 110 µL (100 µL of bacterial suspension effectively injected + 10 µL of extra volume to account for the syringe dead space), where n is the number of mice to be injected.

Note: Each aliquot will be used for a single mouse/injection. The extra aliquots are used as (1) backup and (2) for agar plates as control to verify the injected CFU.

4. To calculate the exact injected CFU, make serial dilutions of one or more of the aliquots prepared in step 3 and inoculate the appropriate dilution(s) in 7H10 agar plates. The CFU are usually very consistent with what was determined in steps 9 and 10 of the “before you begin” section.

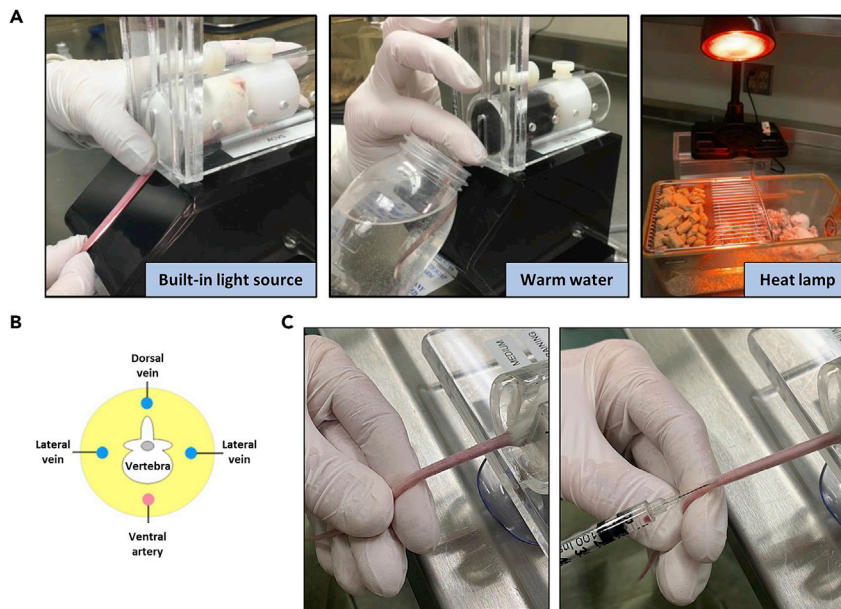


Figure 2. Lateral tail vein injection of mice for Mtb infection

(A) The tail of the mouse can be warmed using methods such as a light source, warm water, or a red heat lamp for vasodilation and ease of injection.

(B) The scheme indicates the position of the veins in the mouse tail.

(C) Hold the tail taut between the thumb and index finger (left), then inject. If the needle is in the vein, a flash of blood may be seen (right) then proceed with the injection.

△ CRITICAL: It is very important to add a cohort to evaluate the deposited CFU at 24 h post infection. This will allow for assessing how variable the infection procedure is and to determine the actual number of CFU deposited before any treatments begin.

5. Prepare a separate syringe with needle for each animal, drawing all 100 μ L of Mtb suspension into the needle and syringe. Ensure that the bacteria are not deposited at the bottom of the tube by vortexing or pipetting the Mtb suspension before drawing it into the syringe.
6. Warm the tail to cause vasodilation using a heat source such as the restrainer's built-in light source, by soaking the tail in warm water (max temperature of 40°C) or using a heat lamp (Figure 2A).

Note: Heat lamps with red bulbs are more likely to cause hyperthermia and animals must be carefully monitored. The time under the lamp should be limited to 1–2 min and the lamp should be placed no closer than 1–2 feet from the cage. Signs of hyperthermia include red extremities, rapid respiration or panting, and decreased activity.

7. With the mouse placed in the restraint device, locate one of the two lateral tail veins in the middle third of the tail by rotating the tail slightly to either side (Figure 2B). The veins appear as blue lines down either side of the tail.
8. Restrain the tail:
 - a. Gently pull the tail straight toward you, holding it between your thumb and first finger approximately 2/3 of the way down the length of the tail. The tail should have no slack.
 - b. Position your thumb lower than your first finger with the tail slightly bent (Figure 2C, left).

△ CRITICAL: Do not squeeze or bend the tail too much or the vein will blanch and potentially cause injury.

9. With the bevel of the needle facing up and the needle parallel to the vein, steady the hub of the needle on top of your thumb and slide the needle forward into the tail vein until the bevel is fully inserted.
10. Confirm the location by gently applying negative pressure to the plunger; if the needle is in the vein, you may see a flash of blood (Figure 2C, right). If you do not see this, check the angle of the needle while maintaining negative pressure and redirect the needle until you see a flash of blood.
11. Once proper needle placement is confirmed, proceed with the injection by smoothly depressing the plunger until the solution has been fully administered.

Note: If the needle is in the vein, there will be no resistance while injecting and the vein itself will blanch. If the needle is not in the vein, resistance will be felt, and the fluid will cause discoloration around the vein or a subcutaneous bleb.

12. Pull the needle straight out and discard the syringe/needle directly into a biohazardous sharps container without recapping. Use a new needle and syringe for each animal.
13. Loosen the restraint device and apply light pressure to the puncture site with a dry piece of gauze until the bleeding has stopped. Remove the mouse from the restraint device and place it back into its cage.
14. Begin the treatments 24 h after the mice are infected.
 - a. For the 24 h cohort, do not administer any treatments. Instead, proceed to cardiac puncture and serum collection, as well as lung and spleen collection and CFU plating.

Treatment of mice with rifampicin and SMIP-30 – Day 1–13

⌚ Timing: ~3 h every 2 days until the end of the experiment

In this step, we prepare solutions of rifampicin and SMIP-30 and administer them through drinking water and intraperitoneal injection, respectively.

Note: We usually conduct experiments within a 14-day period. Due to its auxotrophic nature, this strain does not replicate in mice, and it is cleared by the immune system over time. A 14-day period allows enough remaining bacteria to perform CFU analyses and to evaluate possible differences among the tested treatments. As such, the steps below are optimized for a regimen of treatments being administered in a 14-day period, starting on day 1 post-infection. We stop treatments 24 h before the endpoint on day 14, to reduce the risk of drug carry-over during the CFU analysis. Depending on the regimen and compounds to be tested, you may choose your own treatment frequencies.

Note: In this experiment, we administer (i) rifampicin through drinking water *ad libitum*, changing the water bottle to refresh the antibiotic every 2 days, and (ii) SMIP-30 through intraperitoneal injection every second day. Other methods of drug delivery such as oral gavage can also be used, though we have not tested other methods. Adapt the steps according to your specific experiment.

15. Prepare a solution of SMIP-30 in order to inject a dose of 25 mg/kg in a volume of 200 μ L. Prepare at least $n + 3$ aliquots of SMIP-30 and vehicle, where n is the number of mice to be injected. Each aliquot will be used for a single mouse/injection. The extra aliquots are used as backup.

Note: To be precise in the drug dosage, weigh the animals at day 0 (before the Mtb infection) to estimate the average weight. For female BALB/c of 8–10 weeks of age, we considered an average weight of 20 g.

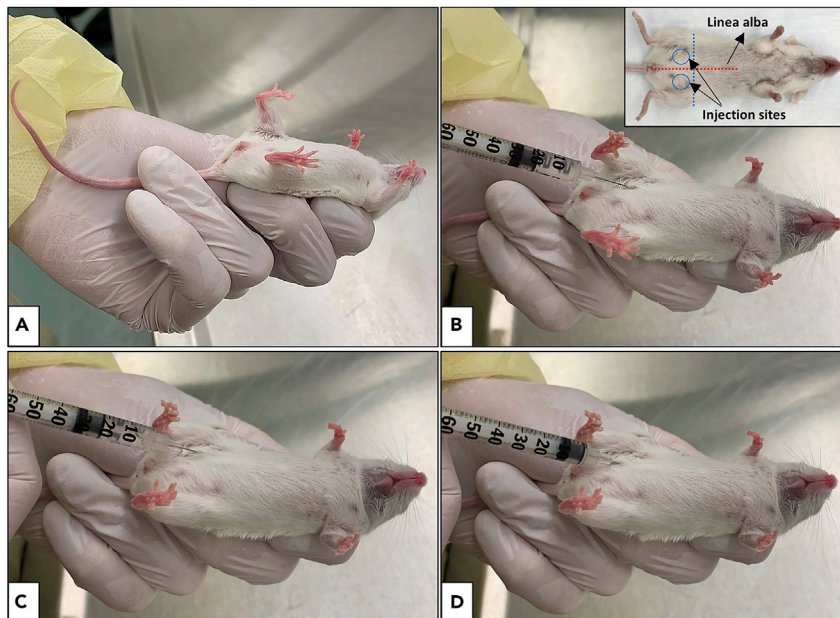


Figure 3. Intra-peritoneal administration of compound treatment

(A) Restrain the mouse by its scruff tilting its head down and hold it stretched with the tail pinned by a finger.

(B) Inject the mouse in the lower abdominal quadrant. The injection sites are indicated in the inside panel.

(C and D) When negative pressure is observed (C), proceed with the injection (D).

16. Prepare 200 mL of rifampicin at 20 mg/L directly in the drinking bottle of each cage of mice to be treated.

Note: We observed that each mouse drinks ~2–3 mL of water every 2 days. This water consumption is observed for BALB/c, 8–10-week-old females, 20 g body weight. We prepared 200 mL of rifampicin water to ensure that more than enough is available. If you are using compounds that are expensive and cannot be wasted, adjust the volume of water accordingly.

Note: A rifampicin dose of 20 mg/L administered *ad libitum* in drinking water is a subtherapeutic dose for Mtb mc²6206, since it does not significantly affect the *in vivo* CFU at 2 weeks of treatment when used as monotherapy. We selected this dose in order to appreciate any potential effects with combination treatment. The mc²6206 strain does show sensitivity to rifampicin as we have tested doses of 80 and 160 mg/L, which does result in dose-dependent reduction in CFU (see [Expected Outcomes](#)).

Note: If higher doses of rifampicin or other antibiotics are administered via drinking water, it might be helpful to add a sweetener like sucrose (3%), in order to make the water more palatable. Indeed, many antibiotics display degrees of bitterness, which prevent the mice from drinking properly. If any sweetener is added to the antibiotic solution, remember to add the same amount of sweetener in the vehicle bottles.

17. Administer 200 μ L of SMIP-30 or vehicle to each mouse using intra-peritoneal injections.
 - a. Prepare a separate syringe with needle for each animal. A syringe with 27-gauge needle was used for our experimental compound.
 - b. Draw the required volume of test compound or vehicle into the needle and syringe. Rotate the needle so that the bevel points “up”.

- c. Restrain the mouse with its head tilted towards the ground so that its head is lower than its hind end (Figure 3A). This allows the abdominal viscera to shift cranially and minimize accidental puncture of abdominal organs at the injection site.
- d. Identify anatomical landmarks. The preferred injection site is the lower left or right abdominal quadrant to avoid damage to the urinary bladder, cecum and other abdominal organs (Figure 3B, insight).
- e. Insert needle parallel to the linea alba at a 30°–45° angle horizontal with the bevel up until the entire bevel is within the abdominal cavity (Figure 3B).
- f. Pull back on the plunger to ensure there is negative pressure prior to injecting. If negative pressure is observed (Figure 3C), proceed with the injection (Figure 3D).

Note: Aspiration of green material, yellow liquid or blood may indicate that the intestinal tract, the bladder, or an abdominal vessel has been punctured. If so, remove the needle from the animal, discard the syringe and repeat the injection.

18. Administer rifampicin by placing the drinking bottle with rifampicin solution into the cage. Due to its light sensitivity, use foil to wrap the water bottles containing rifampicin.
19. Repeat the drug administrations every 2 days, with the final administration at day 13 post-infection.
20. At day 14 post-infection, proceed to cardiac puncture, lung and organ harvesting, and CFU plating for all remaining mice.

△ CRITICAL: Body weight measurements and visual examinations must be conducted during the course of the experiment to assess the health and wellbeing of the mice and highlight potential adverse effects caused by the drugs or by the procedures. We consider any loss of body weight greater than 15% to be an endpoint. Make sure that your protocol considers and specifies potential endpoints due to toxic effects or adverse reactions.

Blood collection by intra-cardiac puncture – Day 1 and 14

⌚ Timing: ~3 h

In this step, we perform cardiac puncture to collect blood from mice.

Note: We perform cardiac puncture and collect the blood for isolation of serum from the 24 h cohort of mice on day 1 and all remaining mice on day 14. The steps are the same.

Note: This procedure is designed to obtain a single and large blood sample of good quality. It is a non-survival method (end-point procedure) and requires general anesthesia.

21. Anesthetize the mouse with isoflurane (or any alternative deep anesthesia procedure permitted in your animal usage protocol) and place it in the dorsal recumbency position, while inhaling the anesthetic through the isoflurane nozzle (Figure 4A).
22. Continue with the procedure only when the mouse is deeply anesthetized. The anesthetic depth must be confirmed with one of the accepted methods, such as the lack of pedal reflex, Figure 4B.
23. Spray the bodies with 70% ethanol.
24. Locate the xiphoid process of the sternum and insert a 25G needle with 1 cc syringe bevel up into the left ventricle of the heart (Figure 4C).
25. Insert the syringe with the plunger slightly pulled, in order to have negative pressure inside the syringe. If the puncture hits the heart properly, the blood will automatically start filling the syringe (Figure 4D).
26. Once steady blood flow into the needle has been confirmed, stabilize the needle hub and withdraw as much blood as necessary (Figure 4E). Usually, it is possible to collect up to 0.8–1 mL of blood.

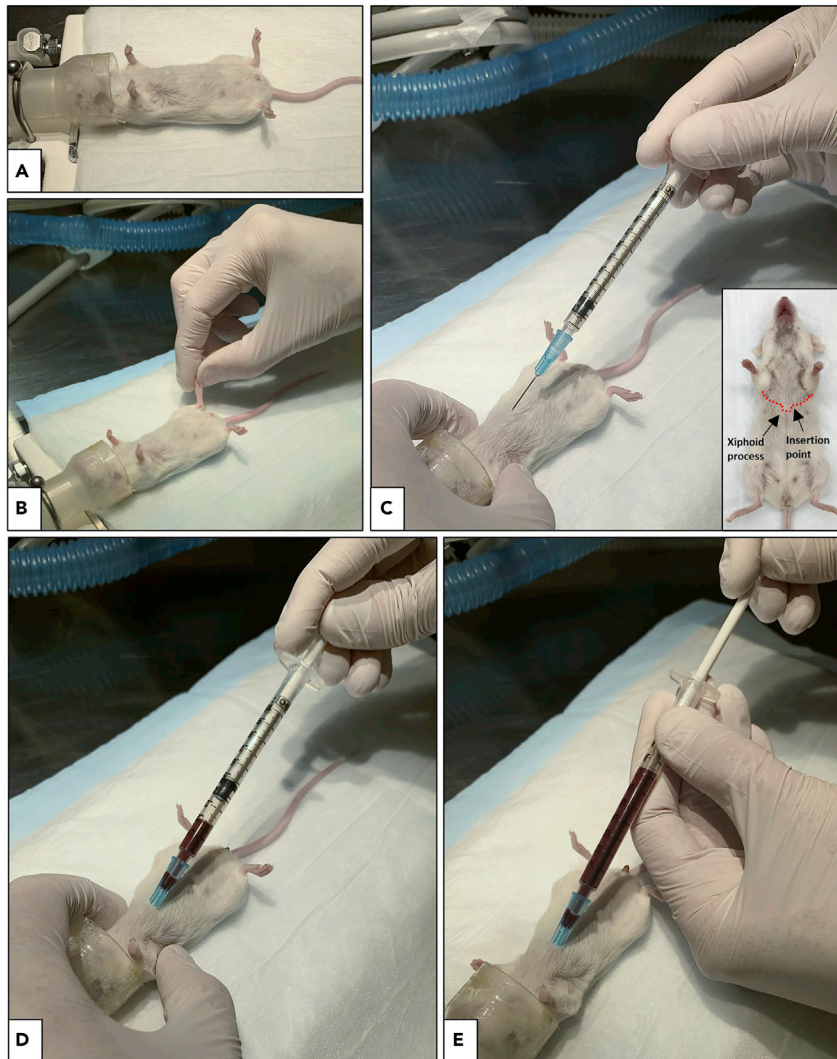


Figure 4. Blood withdrawal by cardiac puncture (terminal procedure)

- (A) Anesthetize the mouse with isoflurane.
 (B) Confirm the mouse is deeply anesthetized by checking for the lack of the pedal reflex by pinching its foot. No reactions should be noticed.
 (C) Locate the xiphoid process (indicated in the inside panel) and insert the needle (with plunger slightly pulled to create negative pressure) into the left ventricle of the heart.
 (D) If the heart is successfully pierced, blood will automatically begin to fill the syringe as a result of the negative pressure.
 (E) Pull on the plunger to collect the blood.

Note: It can be difficult to reach the heart the first time you insert the needle. You may need to move the needle at different angles. If no blood is drawn, remove your needle and insert again at a different angle. If the needle gets clogged, change the syringe.

27. Release the collected blood into a 1.5 mL Eppendorf tube (Figure 5A). To reduce hemolysis, it is recommended to carefully remove the needle from the syringe before releasing the blood into the tube. Allow the blood to coagulate at 20°C–25°C for 30 min or at 4°C for 1–2 h.
28. Observe for the absence of cardiac or respiratory cycles and perform a second physical method of euthanasia (e.g., cervical dislocation).

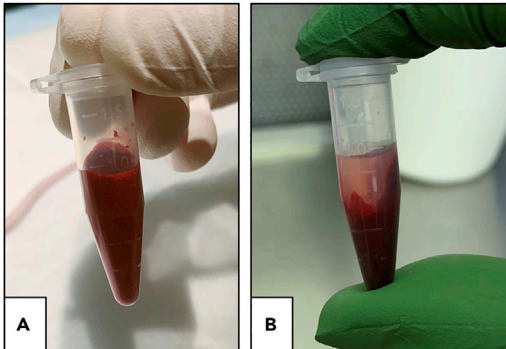


Figure 5. Processing of blood samples to collect serum

(A) The withdrawn blood is collected in 1.5 mL tubes and incubated at room temperature for 30 min to allow coagulation.

(B) After centrifugation at $1,000 \times g$ for 20 min, the serum (top layer) is transferred to a new tube and immediately frozen at -80°C .

⚠ **CRITICAL:** Depending on the number of mice and staff support, it may take a long time to perform the cardiac puncture on all the animals. Ensure as best as possible that all blood samples are incubated for the same amount of time. If needed, you can centrifuge and collect the serum in batches.

29. Centrifuge the tubes at $1,000 \times g$ for 20 min.
30. Transfer supernatant (Figure 5B) to clean tubes and immediately freeze at -80°C until ready to perform the cytokine analysis. Ideally, each sample should be aliquoted in smaller volumes to avoid multiple freeze/thaw cycles.

Note: If the blood is maintained and processed at 4°C or the withdrawal was performed too fast, it is possible to observe hemolysis (Figure 5B). To avoid this, perform all the steps at 20°C – 25°C , but freeze the serum immediately after centrifugation.

31. Proceed to lung and spleen harvesting and CFU plating immediately.

Harvest of lungs and spleens and CFU assay – Day 1 and 14

⌚ **Timing:** ~8 h

In this step, we collect the lungs and spleens from the Mtb-infected mice and homogenize them for CFU plating.

Note: We perform organ harvesting and CFU plating from the 24 h cohort of mice on day 1 post infection and all remaining mice on day 14. The steps are the same.

Note: Prepare the GentleMacs and 1.5 mL Eppendorf tubes at the same time you prepare the tubes for cardiac puncture. Organs will need to be collected immediately following the cardiac puncture.

⚠ **CRITICAL:** Mouse work, organ homogenization, and CFU plating are done in a biological safety cabinet to maintain sterility and prevent contamination. All the tubes and reagents must be sterile and handled aseptically.

32. Prepare (n = number of mice) GentleMacs M tubes with 2 mL of PBS-Tween to collect the lungs.
33. Prepare (n = number of mice) empty 1.5 mL tubes to collect the spleens.
34. Spray your scissors and forceps with 70% ethanol before use and between each mouse.
35. Using a sharp pair of scissors, remove the skin from the abdomen to chest.
36. Carefully cut open the peritoneum and collect the spleen.

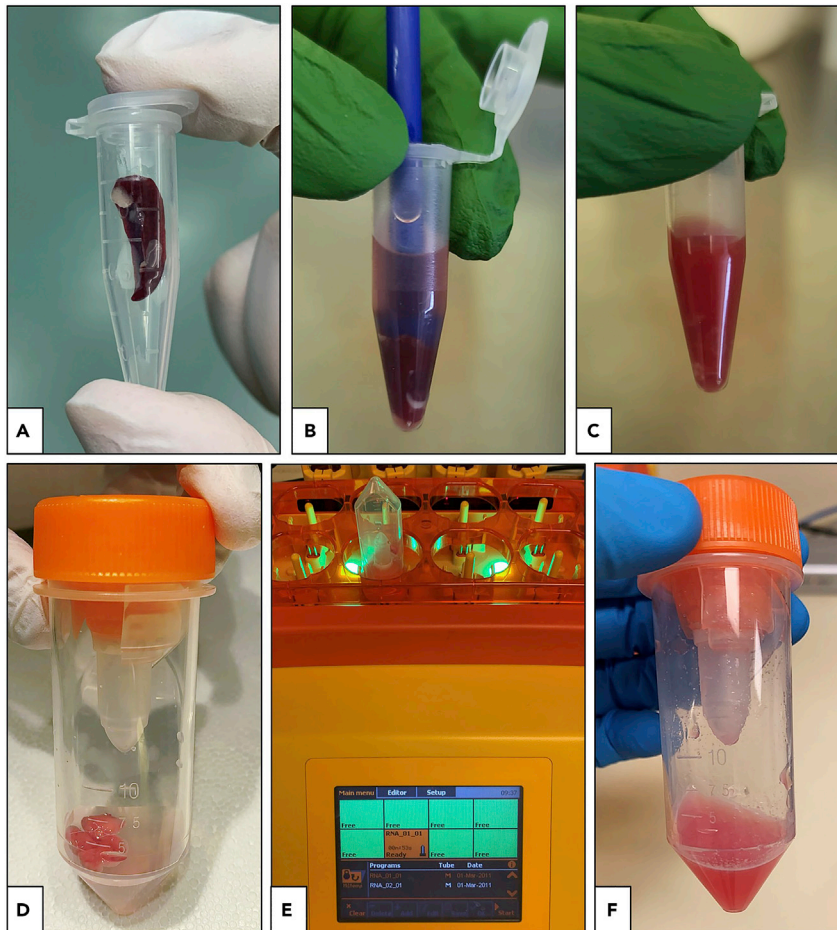


Figure 6. Homogenization of lungs and spleens for CFU assay

(A–C) Collect the spleen into a tube (A), weigh it, add 500 μ L PBS-Tween and begin homogenizing with a sterile pestle (B) until you obtain a uniform homogenate (C).

(D–F) Collect the lungs into a GentleMacs M tube with 2 mL PBS-Tween (D), homogenize with the GentleMacs dissociator (E) to obtain a uniform homogenate (F).

△ CRITICAL: Be careful not to damage the intestines to reduce the risk of microbial contamination.

37. Using forceps and scissors, open the diaphragm, carefully cut the trachea and remove the lungs.
38. Once the organs are collected, proceed with their processing and homogenization.

39. Spleens:

- a. Collect the spleen inside a 1.5 mL Eppendorf tube (Figure 6A).
- b. Measure the weight of each spleen using an identical empty Eppendorf tube to tare the scale.

Note: Considering the small size of mouse spleens, a precision scale is recommended in order to be more accurate in the evaluation of differences amongst the samples.

- c. Add 500 μ L of PBS-Tween to each of the tubes containing the spleens.
- d. Homogenize spleens using sterile pestles (Figure 6B) until you obtain a uniform homogenate (Figure 6C).

Table 1. Serial dilutions of each organ from each endpoint to be plated for CFU assay

Endpoint (d.p.i.)	Organ	Serial dilutions				
		Undiluted	DIL 1 (1:10)	DIL 2 (1:10)	DIL 3 (1:10)	DIL 4 (1:10)
1	Lungs	✗	✓	✓		
1	Spleens	✗	✗	✗	✓	✓
14	Lungs	✓	✓			
14	Spleens	✗	✗	✓	✓	

The green checkmarks identify the serial dilutions that we usually seed in 7H10 plates when 10^7 – 10^8 CFU are *i.v.* injected in each mouse. d.p.i.: days post infection.

Note: If the spleen is very large, more PBS-Tween (i.e., 1 mL) and a larger tube might be required.

40. Lungs:
 - a. Place each lung sample (lobes not separated) in a GentleMacs M tube containing 2 mL PBS-Tween (Figure 6D).
 - b. Run the appropriate GentleMacs protocol to homogenize the lungs (Figure 6E). In our experiments, we used the “RNA_01” protocol and ran the protocol twice, to obtain a uniform homogenate (Figure 6F).
41. Once the organs are homogenized, prepare 1.5 mL tubes with 900 μ L PBS-Tween for the serial dilutions to inoculate for CFU assay (Table 1).
42. Prepare serial dilutions by transferring 100 μ L of homogenate into the tubes containing 900 μ L PBS-Tween.
43. Plate 100 μ L of the appropriate dilution on to 7H10 agar plates (Table 1, green checkmarks).

Note: The number of serial dilutions to prepare depends mainly on the injected CFU, the time post-infection of organ harvesting and the expected therapeutic effect of your tested drugs. See Table 1 for more details about the serial dilutions that we usually prepare for our experimental conditions. See also expected outcomes for more details about the *in vivo* clearance of Mtb mc²6206.

44. Incubate plate at 37°C for 2–3 weeks until colonies are visible.
45. Count the colonies and calculate the CFU (per organ) by multiplying the colony number by the plated serial dilution and the total volume of the organ homogenate.

Note: In our experience, the volume of the lungs does not significantly vary among the cohorts of Mtb mc²6206 infected mice. In contrast, the size of spleens can significantly change between the cohorts, affecting the final volume of the spleen homogenate. Therefore, for a more accurate calculation of the CFU/spleen, precisely measure the final volume of the homogenate or use the spleen weight to approximate the final volume (i.e., 500 μ L of PBS-Tween + spleen weight). Whatever methods you use, be consistent for all the samples.

Cytokine and chemokine profiling assay

⌚ Timing: 6 h (assay preparation and protocol), 2 h (data acquisition), 2–3 h (data analysis)

Note: The preparation steps, the assay protocol, and the data acquisition by flow cytometry should be performed in the same day. The data analysis can be performed any time after the data acquisition.

In this step, we analyze the cytokine and chemokine profiles of the mice using the collected serum. We used the bead-based multiplex immunoassay LEGENDplex, from BioLegend. More specifically, we perform the mouse serum analysis using the predefined Mouse Inflammation Panel (13-plex) with V-bottom plates. Here below we briefly describe the protocol as we applied it. For more details, please see [manufacturer's manual](#).

Note: Many different multiplex or ELISA kits can be used to evaluate the serum concentration of cytokines and chemokines of interest. The choice for the appropriate analysis should be based on the expected response of the *in vivo* experiments. In our case, we opted for a panel of inflammatory cytokines and chemokines to evaluate the inflammatory response induced by (1) Mtb infection, (2) drug treatments and (3) the combination of the two stimuli.

46. Read the manual carefully.
47. We suggest setting up the flow cytometer accordingly before beginning the experiment. This will allow for proper reading of the samples after completing the protocol without any delay. To access the setup instructions, visit: www.biolegend.com/legendplex and click on the Instrument Setup tab to select the instructions for the specific flow cytometer that will be used.
48. Prepare the reagents as described in the manual. The main components that must be prepared or processed before starting the experiment are the following: the Mouse Inflammation Panel Premixed Beads, the wash buffer and the Matrix C.
49. Prepare the standards by reconstituting the lyophilized Mouse Inflammation Panel Standard Cocktail as described in the manual.

Note: The top standard concentrations of analytes in this panel are set at various concentrations but may be subject to change from lot to lot. Refer to the lot-specific Certificate of Analysis for this information (provided along with the kit or downloadable from the BioLegend website).

50. Thaw the serum vials on ice and centrifuge at $8,000 \times g$ for 2 min at 4°C to remove any particulates.

Note: According to the manufacturer, the serum samples should be diluted 1:2 to ensure accuracy and avoid clogging of the filter paper. Since we do not use the filter paper option (V-bottom plates instead) and the analytes in our samples are mainly in lower concentration ranges, we do not dilute our samples.

51. Allow all reagents to warm to 20°C – 25°C before use.
52. Keep the plate upright during the entire assay procedure to avoid losing beads and keep the plate wrapped with aluminum foil for all the incubation steps.
53. Standards and samples should be run and loaded in the plate as recommended to facilitate the following data analysis.
54. In the standard wells, combine 25 μL of Matrix C and 25 μL of each standard. In the sample wells, combine 25 μL of Assay Buffer and 25 μL of mouse serum.
55. Vortex the mixed beads for 30 s and add 25 μL of mixed beads to each well (final volume 75 μL per each well). Be sure to mix the beads regularly during this step to avoid bead settling.
56. Seal the plate, cover it with aluminum foil and shake it at 800 rpm on a plate shaker for 2 h at 20°C – 25°C .
57. Centrifuge the plate at 1050 rpm ($\sim 250 \times g$) for 5 min.
58. Immediately after centrifugation, remove the supernatant either by pipetting with a multi-channel pipette or by dumping it in the sink by plate inversion.

Note: Since we experienced some bead loss with the inversion method, we opted for pipetting the supernatant instead.

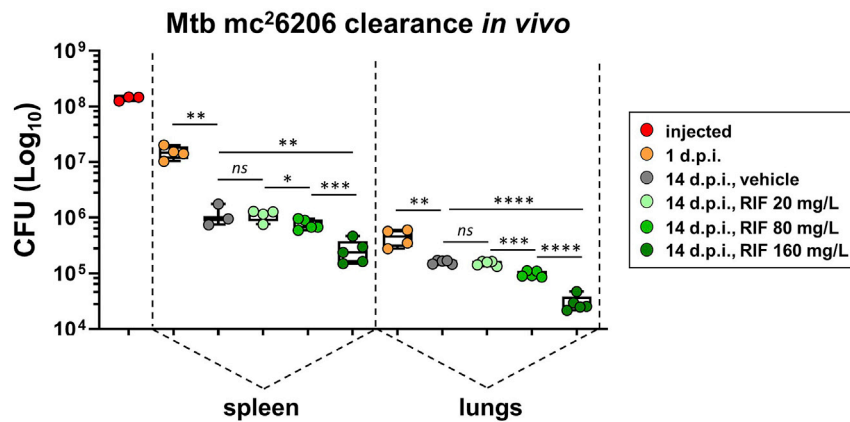


Figure 7. Clearance and effect of rifampicin on *Mtb mc²6206* in vivo

The graph shows the CFU obtained *in vivo* in mice infected with $\sim 10^8$ CFU of *Mtb mc²6206* via *i.v.* injection. The CFU analysis is performed in spleen and lungs, as indicated. The 1 day post infection (d.p.i.) data represent the initially deposited CFU, whereas the 14 d.p.i. data points show the natural *Mtb* clearance by the immune system (vehicle) or the reduction of *Mtb* burden due to treatment with rifampicin. While a 20 mg/L dose of rifampicin (via drinking water) is subtherapeutic, higher doses of 80 and 160 mg/L of rifampicin induce a significant dose-dependent reduction of *Mtb* burden. Data are shown as the mean \pm SD, where each point represents one mouse. Statistical significance is calculated using the unpaired Student's *t*-test. *ns*: not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001

59. Wash the plate once with 200 μ L of wash buffer into each well and shake the plate at 800 rpm for 1 min. Repeat steps 57 and 58.
60. Add 25 μ L Detection Antibodies to each well.
61. Seal the plate, cover it with aluminum foil and shake it at 800 rpm on a plate shaker for 1 h at 20°C–25°C.
62. At the end of the incubation, add 25 μ L of SA-PE to each well directly, without washing the plate.
63. Seal the plate, cover it with aluminum foil and shake it at 800 rpm on a plate shaker for 30 min at 20°C–25°C.
64. Repeat steps 57 and 58.
65. Add 150 μ L of 1 \times Wash Buffer to each well. Resuspend the beads by pipetting and read the samples on a flow cytometer. We used a Beckman Coulter CytoFLEX, transferring the samples in a flat-bottom 96 well plates and reading directly using the autosampler.

Note: Despite the instrument being set up to shake each well before analyzing the sample, we experienced instrument clogging. To minimize the problem, we analyze 2 columns at a time and resuspended the beads by pipetting often during the reading (pausing the cytometer). We also added a wash step for the flow cytometer with water every 16 samples.

66. Using the template previously setup following the Flow Cytometer Setup Guide, set the flow rate to low and set the number of beads to be acquired to about 300 per analyte (4000 beads for a 13-plex assay), creating a large gate to include both Beads A and Beads B (gate A+B).
67. Read the samples following the recommendations in the manual.
68. When all the samples are acquired, proceed with the data analysis using the BioLegend's LEGENDplex™ data analysis software, provided with the kit.

EXPECTED OUTCOMES

Based on our previous work (Berton et al., 2022), we have observed that while there is variability in the absolute CFU between experiments, the deposited CFU at 24 h post-infection is consistently proportional to the injected inoculum. Based on CFU analysis of the day 1 post-infection cohort and of the stock that was injected, lungs show a deposited to injected CFU ratio of 0.25%–0.3%, and spleens show a ratio of 10%–11%. There is also a consistent decrease of approximately 1-log

in CFU in the organs between day 1 and day 14 post-infection (due to immune clearance), which leaves sufficient bacteria to evaluate the efficacy of antibiotics and/or host-directed compound treatments (Figure 7). Given that we developed this protocol to test a combination of antibiotic and host-directed compound, the doses we used should be subtherapeutic when administered individually. Indeed, we observed that 25 mg/kg SMIP-30 and 20 mg/L of rifampicin did not affect bacterial burden by themselves (Berton et al., 2022). To validate the use of this auxotroph strain as a model for drug susceptibility testing, we also confirmed (Berton et al., 2022) that higher doses of rifampicin (80 and 160 mg/L) did dose-dependently reduce Mtb burden as expected. The relevant data are summarized and shown in Figure 7. Depending on the treatment(s) you want to test and if you are interested in combination therapies, you should plan the doses accordingly.

We also observed that the inflammatory cytokines MCP-1 and IFN- γ , high in mouse serum 24 h post-infection, decrease in the mice over the course of the two-week infection period, which was consistent across all mice independent of the SMIP-30 or rifampicin treatments. Furthermore, levels of other cytokines including IL-6, IL-10, TNF- α and IL-1 β were also not affected by any treatments (Berton et al., 2022). However, depending on the drug you test, different outcomes may be expected.

QUANTIFICATION AND STATISTICAL ANALYSIS

The analysis of the cytokine data is performed using the LEGENDplex™ Data Analysis Software Suite. All the statistical analyses are performed with GraphPad Prism 9 and the statistical significance is calculated using the unpaired Student's t-test (CFU counts) or one-way ANOVA (cytokine analysis). The graphs show individual data points for better visualization of data distribution. Values of $p < 0.05$ are considered to be significant. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

LIMITATIONS

In contrast to *M. bovis* Bacillus Calmette-Guérin (BCG), which contains a natural RD1 deletion (Behr et al., 1999) that removes virulence factors such as early secreted antigenic target-6 kDa (ESAT-6) and culture filtrate protein-10 kDa (CFP-10), the attenuated double auxotrophic strain mc²6206 (Δ leuCD/ Δ panCD) of *M. tuberculosis* offers significant advantages. A comprehensive comparison of *M. tuberculosis* H37Rv and the double auxotroph strain demonstrated similar *in vitro* and intramacrophage replication rates, similar responses to anti-TB agents and whole genome sequence conservation (Mouton et al., 2019). In order to proliferate, the growth medium must be supplemented with both leucine and pantothenate (Sampson et al., 2004). The main advantage of Mtb mc²6202 is its excellent biosafety profile *in vivo*, as it is fully attenuated even in immunocompromised hosts that succumb to BCG challenge (Jain et al., 2014; Sampson et al., 2004, 2011). A similar auxotroph strain has been found safe in simian immunodeficiency virus (SIV) infected infant macaques, supporting its biosafety and potential for vaccination in HIV-infected infants (Jensen et al., 2012).

Despite the advantages offered by a BSL-2 compatible Mtb strain, a few limitations should be considered for *in vivo* experiments. Due to the inability of Mtb mc²6202 to grow and replicate in mice and the natural clearance by the host immunity, this strain is not suitable for prolonged *in vivo* infection studies. In immunocompetent mice, the natural clearance accounts for ~1–1.5 log reduction in the CFU at 2 weeks post-infection, which complicates the interpretation of Mtb burden in treatment regimens longer than 3–4 weeks. The lack of *in vivo* replication also limits the possibility to discriminate between bactericidal and bacteriostatic drug effects *in vivo*, unless supplementation of leucine and pantothenate can be successfully administered to the animal to support *in vivo* Mtb proliferation. As such, pure bacteriostatic drug effects will be challenging to model with this BSL-2 compatible Mtb infection mouse model. To our knowledge, *in vivo* supplementation to support the growth of auxotrophic Mtb strains has not been investigated.

TROUBLESHOOTING

Problem 1

Contaminated plates (steps 41–45).

Potential solution

To minimize the risk of contamination from other bacteria that may have entered the mice, antibiotics such as the PANTA antibiotic mixture (BD BBL MGIT PANTA Antibiotic Mixture) at 0.2% can be added to your agar plates or to the PBS-Tween that you use to homogenize and prepare serial dilutions. Furthermore, try to clean the nozzle and breathing apparatus that is used to deliver the isoflurane to the mice as much as possible, since they will breathe deeply and any microbes that may be present on the apparatus would be delivered to the lungs. Minimize the exposure time of the mice to outside air to reduce the risk of the animal breathing in any airborne bacteria or fungi that may establish itself in the lungs, and make sure to thoroughly decontaminate your pipettes, tips, and tools for the anesthesia, necropsy and organ collection.

Problem 2

Slow growth of the Mtb agar plates (step 10 in “before you begin”; steps 4 and 45).

Potential solution

The growth of the bacteria on the agar plates will depend on the seeding density. If too many bacteria are plated, the individual colonies will grow much more slowly as the bacteria compete for nutrients. It is helpful to plate multiple dilutions of your organ homogenates to ensure that you can obtain at least 1 dilution that contains a sufficient amount of bacteria while not being too dense. With the 24 h samples, we typically plate the third and fourth 10-fold dilution of the spleens and the first and second 10-fold dilution of the lungs. With the day 14 samples, we typically plate the second and third 10-fold dilution of the spleens and the undiluted and first 10-fold dilution of the lungs (Table 1). With these dilutions, we have sometimes observed that they contain too many bacteria to grow properly on the plates. To be safe, the next two 10-fold dilutions can also be prepared and plated. When calculating the CFU per organ, be consistent with which dilution you use to perform the calculation across the samples.

Problem 3

Variability in CFU counts between mice and between experiments (steps 41–45).

Potential solution

The amount of CFU that will be deposited in the mice is dependent on how much was injected, and whether the injection was performed properly. To make sure that numbers are consistent, the injection should be performed by someone well-trained and experienced in intravenous mouse injections. If you notice that a mouse was not properly injected, be sure to record which one so you are aware that the potential outlier in CFU is due to human error and not the mouse/treatment itself. It is therefore essential to check the CFU of the stocks used for injection as well as the 24 h post-infection controls to know what CFU was actually injected into the mice.

Problem 4

Non-successful cardiac puncture (steps 24–26).

Potential solution

If the cardiac puncture is not successful, blood can be collected from the thoracic cavity right after euthanasia. In general, this method allows to collect less blood volume than the cardiac puncture, but usually it is enough to perform the cytokine and chemokine profiling assay.

Problem 5

The cytokine concentrations in the serum are too high in the multiplex assay (steps 46–68).

Potential solution

If the concentration of the tested cytokines and chemokines result in out of range signals in the multiplex assay, dilute the serum accordingly and repeat the assay.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jim Sun (jim.sun@uottawa.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, Y.C.L., S.B., C.R., and J.S.; visualization, Y.C.L., S.B., and J.S.; writing – original draft, Y.C.L. and S.B.; writing – review & editing, Y.C.L., S.B., C.R., and J.S.; supervision, funding acquisition, and resources, J.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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