MACROPHAGE KILLING ASSAY

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BACKGROUND

This protocol is used to determine a macrophage population's ability to kill bacteria. It works by incubating macrophages and bacteria together so the macrophages can phagocytose the bacteria. The macrophages are then placed in sterile water in order to lyse them. Bacteria inside the macrophages will then be in solution, which can be plated and counted.

EQUIPMENT

- Accutase (Sigma-Aldrich #A6964)
- Tryptic Soy Broth (TSB)
- Spectrophotometer
- Vortex
- Cell lifter
- Trypan Blue
- Hemocytometer
- Double-distilled water (ddH_20)
- PBS
- Incubator
- Blood plates (5% defibrillated Sheep's blood, 10 ug/mL Neomycin)

PROTOCOL:

Day 0: Preparing for the killing assay:

Making blood plates (5% defibrinated Sheep's blood, 10 ug/mL Neomycin)

S. pneumoniae naturally produces a high concentration of H_2O_2 during growth, thus culturing the bacteria on a plate requires a source of catalase to neutralize this produced compound. Red blood cells can be utilized as a source of catalase because *S. pneumoniae* are alpha-hemolytic and the enzyme is released into the agar plate as they burst the red blood cells.

1. Add 15 g of tryptic soy broth powder and 7.5 g of agar to 500 mL of ddH_2O. Autoclave the mixture.

2. Cool the tryptic soy agar mixture at room temperature for 45 minutes – 1 hour. The mixture should reach a temperature where it is comfortable to touch the bottle for 10 seconds with your hands in order to prevent the blood from burning.
3. Add 25 mL of defibrillated sheep's blood (Cedarlane Labs) and 500 uL of neomycin [10 mg/mL] (Sigma-Aldrich) to the tryptic soy agar mixture. Mix well.
4. Quickly pour the blood plate mixture into 100mm x 15 mm petri dishes to prevent the blood from burning.
5. Cool the blood plates under a BSL 2 cabinet for 1 hour to allow for solidification and to prevent condensation on the plates.
6. Store at 4 °C until further use.

Preparation of tubes:

Time interval of 15 minutes was determined to be optimal in capturing a high resolution of the bacterial killing kinetics over the course of the assay. 6 time points (0, 15, 30, 45, 60, 75 minutes) is recommended.

1. Prepare three 1.5 mL Eppendorf tubes containing 900 uL of autoclaved ddH_2O for each time point per sample. **(Note: We recommend preparing these aliquots the day before your experiment)**
    a. Number of tubes required = number of samples x number of time points x number of dilutions
        i. Example: Experiment with treated cells (n = 3) vs positive control (n = 3) would require

        6 x 6 x 3 = 108 tubes.
2. Label the tubes with the dilution factor, timepoint, and sample identification.

Labeling the blood plates:

In order to account for any plate to plate variability (E.x. thickness of blood plates, degree of blood burned while making the blood plates) we recommend dividing each blood plate into 6

equal sections, with half the blood plate containing the positive control dilution and the other half containing your test group. **(See illustration 1)** This can be done by drawing lines with a Sharpe on the back of the blood plates.

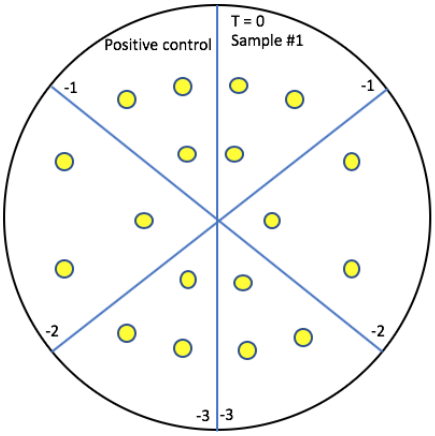


Illustration 1. Plating map of *S. pneumoniae* on blood agar plates

Alternatively, the variability within a sample group can be minimized by dividing the plate into 9 equal sections, with each 1/3 of the plate containing a replicate. **(See illustration 2)**

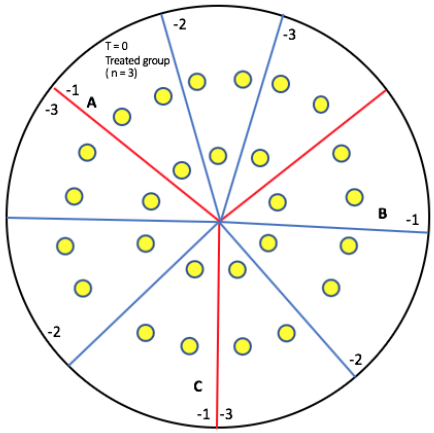


Illustration 2. Alternative plating map of *S. pneumoniae* on blood agar plates

Day 1: Conducting the killing assay

Step 1: Preparation of *S. pneumoniae*

1. Thaw 1 mL frozen aliquot of *S. pneumoniae* (~$8x10^7$ Colony Forming Unit [CFU])

2. Gently add *S. pneumoniae,* in a drop-wise manner, to a falcon polystyrene tube containing 4 mL TSB. Prepare a separate falcon polystyrene tube containing 5 mL TSB as a blank for the spectrophotometer.
3. Place both polystyrene tubes in the incubator at 37°C and 5% CO_2 until *S. pneumoniae* reaches an OD_{600} of 0.5. (Estimate time of growth: 2 ½ - 3 hours) Use the tryptic soy broth as a blank when reading the OD_{600}.
4. Take 1 mL of the bacterial culture and centrifuged at 15,000 rpm for 1 minute.
    a. 1 mL of this mixture is approximately 1 $x10^8$ CFU
5. Remove the supernatant and resuspend the pellet in 1mL cold PBS. Keep the bacteria on ice.
6. Titre the *S. pneumoniae* by performing 1:10 serial dilutions (8x). Plate 10 uL (3x) on a blood agar plate for every dilution.

Step 2: Harvesting MØ from plates/Treating the cells with a compound

In order to minimize the duration of the assay, it is recommended that the cells be lifted while the *S. pneumoniae* is growing. While trypsin is frequently utilized to dissociate adhesive cells, it has been known to cleave cell surface proteins and lead to proteome alterations within mammalian cells. This may affect the receptors on the membrane surface required to internalize the pathogens for killing. Therefore, we recommend lifting MØs using accutase (Sigma-Aldrich), a gentler, less toxic protease cocktail, leading to less alterations to the outer membrane proteome of MØs.

For samples requiring pretreatment with a compound for 30 minutes

1. When the *S. pneumoniae* culture reached an OD_{600} of 0.25 (approximately an hour into bacterial culture), remove the media from the plate containing MØ and wash with PBS to remove any dead cells.

For samples requiring pretreatment with a compound for 1 hour

1. When the *S. pneumoniae* culture reached an OD_{600} of 0.20 (approximately 45 minutes into bacterial culture), remove the media from the plate containing MØ and wash with PBS to remove any dead cells.

a. Start lifting protocol at OD_{600} of ~0.3 if you are not treating the cells with a compound

2. For a 150 mm plate containing MØ, add 10 mL of accutase solution for 10 minutes at 37˚C and 5% CO_2.
3. Using a cell lifter, gently lift residual cells adhered to the plate.
4. Place the cell suspension into a 50-mL falcon tube containing 30 mL of warm DMEM – 10 media.
5. Centrifuge at 500g for 5 minutes at 4˚C.
6. Resuspend the pellet in PBS and determine the concentration of cells using trypan blue and a hemocytometer.
7. Aliquot $5x10^5$ cells into a 1.5 mL Eppendorf tube for every sample.
8. Centrifuge at 500g for 5 minutes at 4˚C
9. Wash with PBS and spin down again
10. **Experiments requiring pretreatment of cells with a compound:** Remove the supernatant and re-suspend the cells in media containing the compound of interest and place on nutator at 37˚C for duration of treatment.
    a. Skip to step 13 if no pretreatment is required
11. Centrifuge the cells at 500g for 5 minutes at 4˚C
12. Remove the supernatant and wash the cells with PBS. Spin down the cells again.
13. Resuspend the cells in 500 uL of PBS.

Step 3: Starting the killing assay

14. Add 500 uL of *S. pneumoniae* [OD_{600} of 0.5] suspended in PBS to the $5x10^5$ aliquot of MØ in 500 uL for a total volume of 1 mL *S. pneumoniae*/macrophage mixture [multiplicity of infection (MOI) of 100]
    a. **Note:** MOI is defined as the ratio of infectious agents to the number of the target cells. In order to capture the subtle changes in the kinetics of macrophages-mediated killing of *S. pneumoniae*, MOI was optimized to increase the overall bacterial load over the course of the assay. **(See supplementary figure 1)**
15. Co-incubate *S. pneumoniae* and MØ for 30 minutes at 37°C with gentle nutation of the mixture to allow for internalization of the bacteria by MØ. **(Important:** For the negative control, co-incubate *S. pneumoniae* and MØ for 30 minutes at 4°C to inhibit internalization of the pathogen by preventing actin polymerization. The negative control is kept at 4°C for the whole duration of the assay)
16. Centrifuge the sample at 500g for 5 minutes and remove the supernatant containing unbound bacteria.
17. Resuspend the pellet in 1 mL PBS and vortex well.
18. Add 100 uL to a tube containing 900 uL ddH_2O. (10^{-1} dilution)
19. Place the remaining 900 uL containing MØ with internalized *S. pneumoniae* onto a nutator within a 37°C fridge and set a timer for 15 minutes
    a. To generate a high-resolution bacterial kill curve, it is recommended that a sample is taken every 15 minutes to capture any subtle changes in the rate of bacterial killing.

20. Perform two additional serial dilutions by removing 100 uL from the 10^{-1} sample and adding it to a tube containing 900 uL of ddH_2O (10^{-2}). Repeat for the 10^{-3} dilution.
    a. **Important:** Vortex the sample well prior and following each serial dilution step to ensure all MØ are lysed by the osmotic pressure. Change pipette tips for each step.
21. Plate 10 uL (x3) of each dilution into the appropriate section of the blood plate.
    a. Working backwards from 10^{-3} to 10^{-1} dilution is recommended in order to save time not changing tips
22. Let the plate dry under a BSL level 2 hood for 15 minutes uncovered.
23. Place the plate upside down in the incubator at 37°C and 5% CO_2 for 24 hours.
24. Repeat steps 18 - 23 for each time point. (15, 30, 45, 60 , 75 minutes)

Day 2: Counting the colonies

1. After 24 hours, count the number of colonies in each plated droplet for a single dilution and calculate the average of the 3 drops per dilution. Multiply the average by the dilution factor and 10^2 in order to obtain the CFU/mL.
    a. To reduce variability between samples, count the colonies on the same dilution factor for every time point if possible (E.x. 10^2 dilution was utilized to count the colonies at time point 0. Therefore 10^2 dilution was utilized for every subsequent time point) If in the latter time points there is a low/no colonies in the designated dilution factor, move to the next subsequent dilution. (E.x. No growth on 10^2 dilution at T = 60 , count colonies in the 10^1 dilution)

Sample calculation:

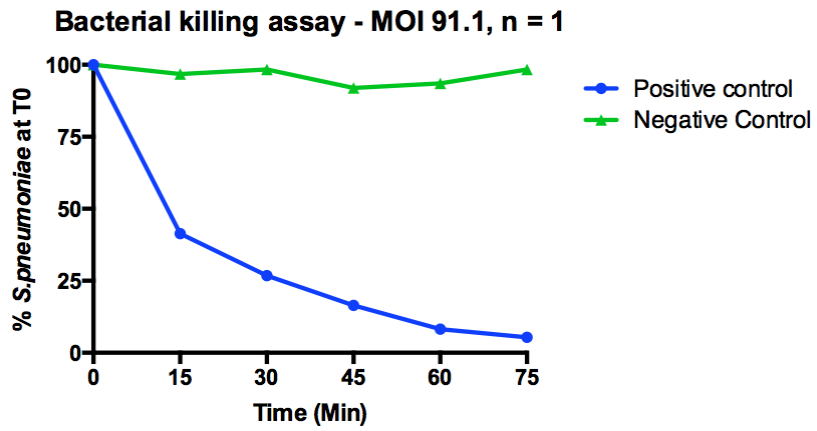
At the 15-minute time point, the 10^3 dilution had 10, 15, 12 colonies.

Average of the colonies = (10 + 15 + 12)/3 = 12.33 colonies

CFU calculation: 12.33 x 10^2 x 10^3 = 1.23 x 10^6 CFU/mL

b. The rate of killing is normalized to the CFU at T0 to account for differences in internalized *S. pneumonia* between the samples. Normalization generates a bacterial kill curve showing the percentage of viable *S. pneumoniae* remaining in the phagosomes of MØ relative to T0.

2. Graph the Data.



Example graph of killing assay

ANALYSIS

Slope calculation:

The slope [%Killed/min] at each time interval was calculated using the following formula:

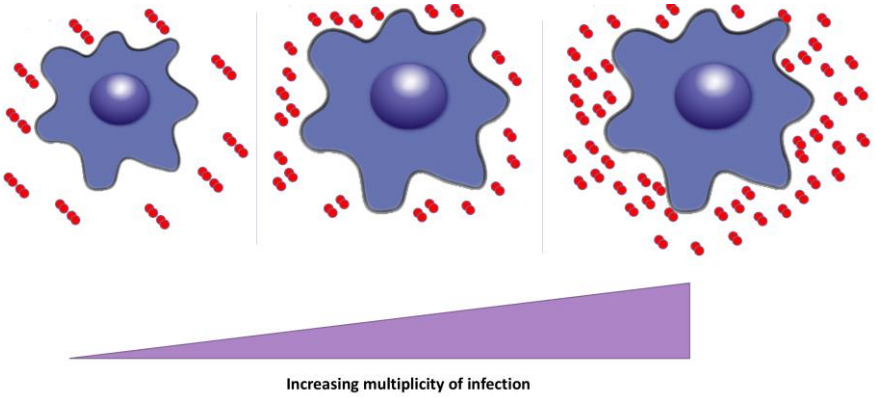
[%bacteria remaining (Time 2) - %bacteria remaining (Time 1)]/ [Time 2- Time 1]

Area under the curve calculation:

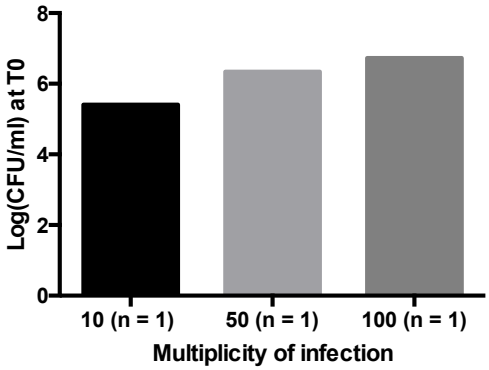
The bacterial killing efficacy is inversely related to the area under curve of kill curves that were normalized to the CFU at T = 0.

The trapezoid method was utilized to calculate the area underneath the curve of the kill curve.

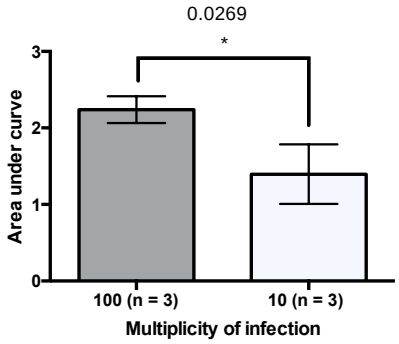
SUPPLEMENTARY FIGURES



A)

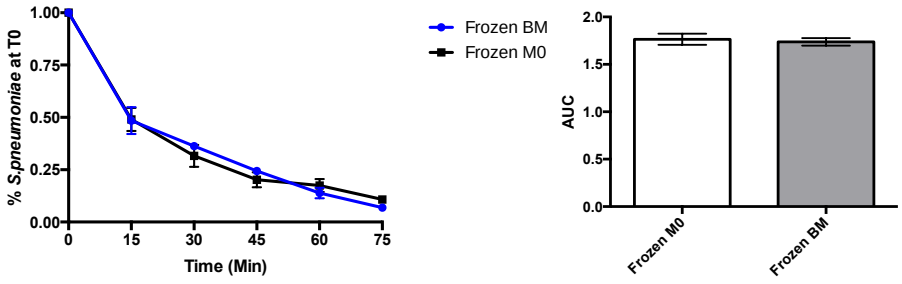


B)

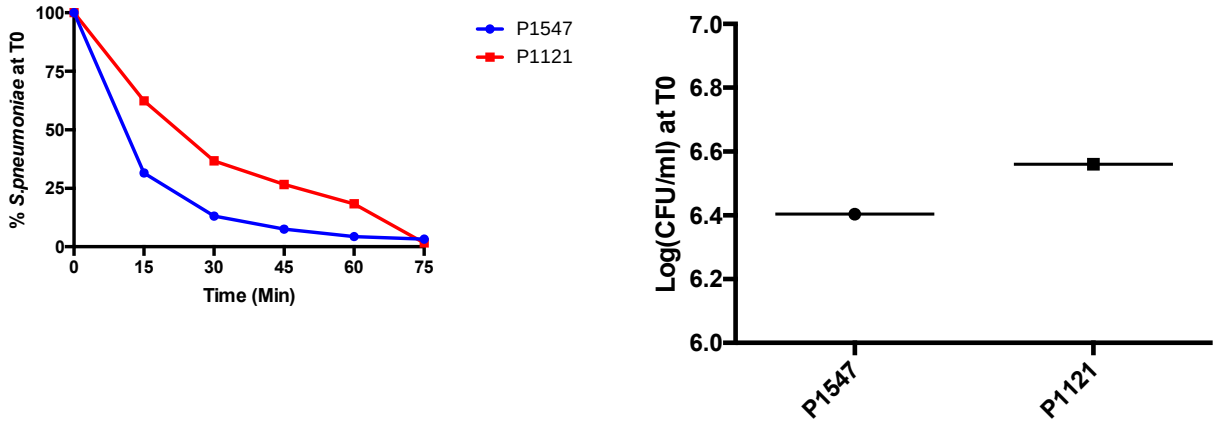


C)

Supplementary Figure 1. *Optimization of MOI for the bacterial killing assay.* Bone marrow progenitors from female C57BL/6 mice were cultured in RPMI media + LCM for 8 days. A) Illustration depicting increasing multiplicity of infection. B) There was a greater quantity of bound/internalized *S. pneumoniae* at T0 with increasing MOI. There was a 1.17x increase in T0 bacterial load when the MOI was increased from 10 to 50 and a 1.06x increase in T0 bacterial load when the MOI was increased from 50 to 100. C) A bacterial killing assay with an MOI of 100 had a higher AUC compared to one conducted with an MOI of 10. Error bars represent SD of the mean. An unpaired parametric t test was performed to determine statistical significance. p = 0.0269



Supplementary Figure 2 *S. pneumoniae (P1547) killing does not differ between freshly differentiated mouse MØ and thawed mouse MØ (Previously stored in liquid nitrogen with 10% DMSO FBS)*



Supplementary Figure 3 *There are differences in killing and T0 internalization/binding between P1547 and P1121*