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A Mouse Model for the Transition of *Streptococcus pneumoniae* from Colonizer to Pathogen upon Viral Co-Infection Recapitulates Age-Exacerbated Illness

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Abstract

Streptococcus pneumoniae (pneumococcus) is an asymptomatic colonizer of the nasopharynx in most individuals but can progress to a pulmonary and systemic pathogen upon influenza A virus (IAV) infection. Advanced age enhances host susceptibility to secondary pneumococcal pneumonia and is associated with worsened disease outcomes. The host factors driving those processes are not well defined, in part due to a lack of animal models that reproduce the transition from asymptomatic colonization to severe clinical disease.

This paper describes a novel mouse model that recreates the transition of pneumococci from asymptomatic carriage to disease upon viral infection. In this model, mice are first intranasally inoculated with biofilm-grown pneumococci to establish asymptomatic carriage, followed by IAV infection of both the nasopharynx and lungs. This results in bacterial dissemination to the lungs, pulmonary inflammation, and obvious signs of illness that can progress to lethality. The degree of disease is dependent on the bacterial strain and host factors.

Importantly, this model reproduces the susceptibility of aging, because compared to young mice, old mice display more severe clinical illness and succumb to disease more frequently. By separating carriage and disease into distinct steps and providing the opportunity to analyze the genetic variants of both the pathogen and the host, this *S. pneumoniae*/IAV co-infection model permits the detailed examination of the interactions of an important pathobiont with the host at different phases of disease

progression. This model can also serve as an important tool for identifying potential therapeutic targets against secondary pneumococcal pneumonia in susceptible hosts.

Introduction

Streptococcus pneumoniae (pneumococcus) are Gram-positive bacteria that asymptomatically reside in the nasopharynx of most healthy individuals[1,2]. Promoted by factors that are not completely defined, pneumococci can transition from benign colonizers of the nasopharynx to pathogens that spread to other organs resulting in serious infections, including otitis media, pneumonia, and bacteremia[3]. Pneumococcal disease presentation is, in part, dependent on strain-specific differences, including the serotype, which is based on the composition of capsular polysaccharides. There have been over 100 serotypes characterized so far, and some are associated with more invasive infections[4,5]. Several other factors increase the risk of pneumococcal disease. One such factor is viral infection, where the risk of pneumococcal pneumonia is increased 100-fold by IAV[6,7]. Historically, *S. pneumoniae* is one of the most common causes of secondary bacterial pneumonia following influenza and is associated with worse outcomes[8]. Another major risk factor is advanced age. In fact, *S. pneumoniae* is the leading cause of community-acquired bacterial pneumonia in elderly individuals above 65 years old[9,10]. Elderly individuals account for the majority (>75%) of deaths due to pneumonia and influenza, indicating that the two risk factors-aging and IAV infection-synergistically worsen disease susceptibility[11,12,13,14]. However, the mechanisms by which viral infection prompts the transition of pneumococci from asymptomatic colonizer to invasive pathogen and how this is shaped by host factors remain poorly defined. This is largely due to the absence of a small animal model that recapitulates the transition from asymptomatic pneumococcal colonization to critical clinical disease.

Co-infection studies have classically been modeled in mice inoculated with pneumococci directly into the lungs 7 days following influenza infection[15,16]. This reproduces the susceptibility to secondary bacterial pneumonia and is ideal for studying how antiviral immune responses impair antibacterial defenses[17]. However, longitudinal studies in humans have demonstrated that pneumococcal carriage in the nasopharynx, where the bacteria can form asymptomatic biofilms[18], is uniformly associated with invasive diseases[19,20]. Bacterial isolates from infections of the middle ear, lung, and blood are genetically identical to those found in the nasopharynx[20]. Thus, to study the transition from asymptomatic carriage to invasive disease following IAV infection, a model was established in which mice were intranasally administered biofilm-grown pneumococci followed by IAV infection of the nasopharynx[21,22]. Viral infection of the upper airway led to changes in the host environment that led to the dispersal of pneumococci from biofilms and their spread to the lower airways[21]. These dispersed bacteria had upregulated expression of virulence factors important for infection, converting them from colonizers to pathogens[21]. These observations highlight the complex interaction between the virus, host, and bacteria and demonstrate that the changes to the host triggered by viral infection have a direct impact on the pneumococcal behavior, which, in turn, alters the course of bacterial infection. However, this model fails to recapitulate the severe

signs of illness observed in humans, likely because the virus is limited to the nasal cavity, and the systemic effects of viral infection on host immunity and lung damage are not recapitulated.

We recently established a model that incorporates the complex interaction between the host and pathogens but also more closely mimics the disease severity observed in humans[23]. In this model, mice are first infected intranasally with biofilm-grown pneumococci to establish asymptomatic carriage, followed by IAV infection of both the nasopharynx and lungs. This resulted in bacterial dissemination to the lungs, pulmonary inflammation, and illness that progressed to lethality in a fraction of young mice[23]. This previous study demonstrated that both viral and bacterial infection altered host defense: viral infection promoted bacterial dissemination, and prior bacterial colonization impaired the ability of the host to control pulmonary IAV levels[23]. Examining the immune response revealed that IAV infection diminished the antibacterial activity of neutrophils, while bacterial colonization blunted the type I interferon response critical to antiviral defense[23]. Importantly, this model reproduced the susceptibility of aging. Compared to young mice, old mice displayed signs of disease earlier, showed more severe clinical illness, and succumbed to infection more frequently[23]. The work presented in this manuscript shows that the degree of disease is also dependent on the bacterial strain, because invasive pneumococcal strains display more efficient dissemination upon IAV infection, show more overt signs of pulmonary inflammation, and result in accelerated rates of disease compared to non-invasive strains. Thus, this *S. pneumoniae*/IAV co-infection model permits the detailed examination of both pathogen and host factors and is well-suited for studying immune responses to polymicrobial infections at the different phases of disease progression.

Protocol

All animal studies were performed in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee.

1. Preparing chemically defined media (CDM)

1. Prepare the stocks as follows:
    1. Dissolve the mix I compounds listed in **Table 1** in 100 mL of ultrapure water while stirring. Store in 200 µL aliquots at −20 °C.
    2. Dissolve the mix II compounds listed in **Table 1** in 20 mL of 0.1 M NaOH while stirring. Store in 100 µL aliquots at −20 °C.
    3. Dissolve the mix III compounds listed in **Table 1** in 1 mL of ultrapure water while stirring. Store in 10 µL aliquots at 4 °C.
    4. Dissolve the mix IV compound listed in **Table 1** in 1 mL of ultrapure water while stirring. Store in 10 µL aliquots at −20 °C.
    5. Dissolve the compounds listed in **Table 2** initially in 15 mL of ultrapure water while stirring. Adjust the pH to 7.0 with a few drops of 0.1 M NaOH and adjust the final volume to 20 mL using ultrapure water. Store in 1 mL aliquots at −20 °C.
    6. Dissolve the compounds listed in **Table 3** in 90 mL of ultrapure water on a hot plate at 50 °C while stirring. Adjust the pH to 7.0 with 0.1 M NaOH, and then

adjust the final volume to 100 mL using ultrapure water. Store in 5 mL aliquots at −20 °C.

2. Make the starter stock freshly every time by dissolving the compounds in **Table 4** in 70 mL of ultrapure water while stirring.
3. To the fresh starter stock, add the following mix stocks in order: 200 µL of Mix I stock (**Table 1**), 80 µL of Mix II stock (**Table 1**), 10 µL of Mix III stock (**Table 1**), 10 µL of Mix IV stock (**Table 2**), 1 mL of Vitamin stock (**Table 3**), and 5 mL of Amino Acid stock (**Table 4**).
4. Once the stocks have been added, adjust the final volume to 100 mL by adding 30 mL of ultrapure water to the beaker.
5. Supplement the CDM with compounds from **Table 4**. Once mixed thoroughly, filter-sterilize and store at 4 °C for a maximum of 2 weeks.

2. Growing the *S. pneumoniae* biofilm

1. Prepare RP-10 medium by mixing 445 mL of RPMI 1640 with 50 mL of heat-inactivated fetal bovine serum (FBS) and 5 mL of penicillin/streptomycin at 10,000 U/mL and 10,000 µg/mL, respectively.
2. Grow the NCI-H292 (H292) mucoepidermoid carcinoma cell line. Add the cells from one purchased vial to 5 mL of RP-10 medium in a T-25 tissue culture-treated flask. Incubate at 37 °C/5% CO_2 for 3-5 days to reach 100% confluence.
3. Check the cells under a light microscope using 10x magnification to assess confluency.
   NOTE: When all the cells are in contact with other cells and there are no gaps in between, then the desired 100% confluency is reached.
4. Wash the cells 2x in 5 mL of room-temperature PBS. Ensure the buffer is calcium free to avoid chelating the EDTA in the following step.
5. Add 1 mL of trypsin-EDTA to the flask and incubate at 37 °C/5% CO_2 for 5-10 min until the cells detach. Neutralize with 4 mL of RP-10 medium. Gently mix by pipetting up and down and transfer to a 50 mL conical tube.
6. Add 500 µL of the cell suspension per well to a tissue culture-treated 24-well plate. From a confluent T-25 flask, expect 2×10^6-4×10^6 cells/mL.
7. On the following day, check the cells under a light microscope to make sure that they are confluent, as in step 2.3. If they are not, then incubate for longer.
8. Once the H292 cells are 100% confluent in the 24-well plate, gently wash the cells 3x with 1 mL of room-temperature PBS to ensure that no medium containing antibiotics or debris remains.
9. After washing the cells, add 250 µL/well of 4% paraformaldehyde to fix the cells. Incubate for either 1 h on ice or overnight at 4 °C.
10. The night prior to cell fixation, streak the *S. pneumoniae* strain of interest on blood agar plates and incubate overnight at 37 °C/5% CO_2.
    NOTE: The data presented here are with the following *S. pneumoniae* strains obtained *via* collaborative exchange: serotype 19F otitis media isolate EF3030[24], classical serotype 2 Avery strain D39[25], and serotype 4 bacteremia isolate TIGR4[26]. The strains are also available from public collections referenced in the **Table of Materials**.
11. Prepare CDM plus oxyrase (0.15 U/mL) by adding 100 µL of oxyrase (30 U/mL) to 20 mL of CDM.

NOTE: Oxyrase is used to eliminate oxygen to allow the efficient growth of *S. pneumoniae* in liquid culture[27].

12. Inoculate the bacteria from the plate into fresh CDM + oxyrase by washing the bacteria off the plate by adding 1 mL of the CDM + oxyrase and gently lifting the bacterial colonies using the side of a 1 mL pipette tip, being careful to not scrape the agar. Alternatively, use an inoculating loop to lift the bacteria and inoculate them into a tube containing 1 mL of the CDM + oxyrase.
13. Dilute the bacteria in CDM + oxyrase to a starting OD_{600} of 0.05.
14. Grow the bacteria in a loosely capped 50 mL conical tube standing at 37 °C/5% CO_2 until an OD_{600} of 0.2 is reached (this will take anywhere between 2-5 h). Check the OD_{600} every hour to ensure the OD does not exceed 0.2.
15. Once the OD has reached 0.2, vortex the bacterial culture tube. Seed 0.5 mL of the bacteria on the fixed H292 cells and add another 0.5 mL of CDM + oxyrase medium per well. Add 1 mL of CDM + oxyrase to the control wells with no bacteria. Incubate the plate for 48 h at 34 °C/5% CO_2.
    NOTE: Growth at 34 °C is used to more closely mimic the lower temperature in the nasopharynx[21].
16. Every 12 h following the initial seeding, gently remove 0.5 mL of the medium and replenish with 0.5 mL of fresh CDM + oxyrase. Be careful not to disrupt the forming biofilm. Check the bottom of the plate for biofilm and look for increasing cloudiness as time goes on due to the growth of the biofilm. To control for contamination, check the wells without bacteria to ensure that the control wells remain clear.
17. At 48 h post inoculation, remove the supernatant and wash 2x very gently with 1 mL of PBS. Resuspend in 1 mL of fresh CDM and pipette up and down vigorously to lift the biofilm. For each bacterial strain, pool the bacteria from all the wells into a 50 mL conical tube. Mix well by gently tilting the tightly capped tube up and down several times.
18. To the 50 mL conical tube, add 40% glycerol in CDM at equal volumes to achieve a bacterial suspension with a final concentration of 20% glycerol. Aliquot 1 mL into microcentrifuge tubes, flash-freeze on dry ice, and save at −80 °C.
19. Prior to use, enumerate the bacteria by thawing one aliquot on ice, spinning the tube at 1,700 × *g* for 5 min, removing the supernatant, resuspending the pellet in 1 mL of PBS, and plating serial dilutions on blood agar plates[28].
20. Grow the agar plates overnight at 37 °C/5% CO_2 and count the colonies at relevant dilutions to obtain the bacterial concentration in colony-forming units (CFU)/mL.
    NOTE: It is recommended to enumerate the bacteria in the stocks at least a day after freezing or later, as there is a drop in bacterial viability within the first 24 h. The stored frozen aliquots can be used for subsequent infection of mice for a maximum of 2 months.

3. Intranasal inoculation of mice with biofilm-grown *S. pneumoniae*

1. Purchase mice and use at the desired age.
    NOTE: Mice aged 3-4 months old are preferred to model young hosts, and mice aged 21-24 months old can be

used to model elderly individuals >65 years of age[29]. The data presented here are with C57BL/6 male mice.

2. Thaw the biofilm-grown bacterial aliquots on ice and spin at 1,700 × *g* for 5 min. Carefully remove and discard the supernatant without disrupting the pellet, wash the bacteria by resuspending the pellet in 1 mL of PBS, and spin again at 1,700 × *g* for 5 min. Remove the supernatant and resuspend the pellet in the volume needed to reach the desired concentration (aim for 5 × 10^6 CFU/10 µL for intranasal inoculation). Confirm the amounts of bacteria administered by plating the prepared inoculum on blood agar plates as in step 2.19.

3. Inoculate the mice intranasally with 5 × 10^6 CFU by pipetting 5 µL of the diluted inoculum into each naris. Make sure to hold the mice firmly, stabilizing the head, until the volume is inhaled (typically within seconds of pipetting the volume into the nares). Perform this step in the absence of anesthesia to prevent pulmonary aspiration of the inoculum.

4. Viral infection with influenza A virus (IAV)

1. At 48 h following intranasal inoculation with *S. pneumoniae*, thaw the IAV strain of interest on ice.
   NOTE: The data presented here are with a mouse-adapted strain of influenza A virus A/PR/8/34 H1N1 that was obtained *via* collaborative exchange[30].

2. Once the virus has thawed, dilute the virus in PBS to the desired concentration; aim for 20 plaque-forming units (PFU)/50 µL for intratracheal infection and 200 PFU/10 µL for intranasal infection. For mock-infected and bacteria-only groups, use PBS to inoculate the mice.

3. Place ophthalmic lubricant on the eyes of the mice prior to anesthesia. Anesthetize the mice using 5% isoflurane and confirm anesthesia by a firm toe pinch.

4. Once the animal is anesthetized, remove it from the isoflurane chamber and immediately infect the anesthetized mice with 50 µL (20 PFU) of IAV intratracheally using blunt tweezers to pull the tongue out of the mouth and pipetting the volume of liquid down the trachea.

5. Place the mice in a separate cage and monitor until complete recovery (they are able to maintain sternal recumbency [able to lay upright on the chest]).

6. Following recovery, immediately intranasally inoculate the mice with 10 µL (200 PFU) of IAV using the inoculation method in step 3.3.

7. House mice that have undergone single or dual bacterial and viral infection with the same infection group and separate them from the other groups.

5. Monitoring the mice for disease symptoms

1. Monitor the mice daily for at least 10 days and blindly score for signs of sickness as follows:
   1. Score as follows for weight loss: 0 = 5% or less; 1 = 5%-10%; 2 = 10%-15%; 3 = 20% or more. Euthanize the mice using CO_2 inhalation when the weight loss score is at 3.
   2. Score as follows for activity: 0 = normal/active; 1 = moving but slightly diminished; 2 = diminished; 3 = severely diminished/lethargic (only moves if touched), 4 = coma/immobile. Euthanize the mice when the activity score is at 3.

3. Score as follows for posture: 0 = no hunch (normal); 1 = slightly hunched posture; 2 = severe hunch. Euthanize the mice when the posture score is at 2.
4. Score as follows for the eyes: 0 = normal; 1 = protruding; 1 = sunken; 1 = closed; 1 = discharge. It can be a combination. Add the totals for the final eye score.
5. Score as follows for breathing: 0 = Normal breathing; 1 = irregular or altered (higher/lower rate); 2 = labored (exaggerated effort or gasping). Euthanize the mice when the breathing score is at 2.

2. Based on the above criteria, add the individual scores for a total clinical score of healthy (0) to extremely sick (15). Consider any mouse displaying a total score above 2 to be sick. Humanely euthanize any mice displaying a total score above 9 or the indicated scores for each criterion and mark them on the survival curve.

6. Processing of infected tissues for bacterial enumeration

1. At 48 h following IAV infection, euthanize the mice.
2. Place the mouse in a supine position. Using 70% ethanol, spray the chest and abdomen of the mouse to clean the fur. Using forceps, pinch the fur and skin in the middle of the mouse and cut the fur with 4.5 in dissection scissors to expose the area from the liver up to the chest.
3. Blood collection
    1. Using dissection scissors, gently cut into the peritoneal cavity to expose the liver. Using forceps, expose the hepatic portal vein at the top of the liver near the diaphragm. Cut the hepatic portal vein using the dissection scissors. Once the blood starts to pool in the peritoneal cavity, collect 10 µL of blood using a micropipette and place into 90 µL of anticoagulant solution (50 mM EDTA solution in PBS) in a microcentrifuge tube for plating for bacterial burden.
    2. Use a P-1000 micropipette to collect the rest of the blood, place it in a blood collection tube, and centrifuge at 7,600 × *g* for 2 min to collect the serum. Save the sera in microcentrifuge tubes at −80 °C for subsequent analysis of any desired cytokine or metabolite.
4. Lung collection
    1. Using dissection scissors, make a cut up the sides of the exposed rib cage and gently pull the ribs up toward the head of the mouse to expose the heart. Insert a 25 G needle attached to a 10 mL syringe prefilled with PBS into the right ventricle and begin slowly perfusing. Look for bleaching of the lungs as an indicator of successful perfusion. Flush slowly to avoid breaking the pulmonary tissue.
    2. Lift the heart with the forceps and make a cut to separate the lungs and heart. Once separated, pick up all lobes of the lung with the forceps and rinse in a dish with sterile PBS to remove any residual blood. In a Petri dish, mince the lungs into small pieces and mix well. Remove half of the lung mix for determination of the bacterial CFU or viral PFU and place it in a round bottom 15 mL tube prefilled with 0.5 mL of PBS for homogenization.

        NOTE: It is important not to take different lobes of the same lung for the various assessments. Instead, all the lobes should be minced, mixed well together, and parsed out equally for the different assessments.

3. Remove the other half of the lung for flow cytometry (section 7 below) and place it in a non-tissue culture-treated 24-well plate with each well prefilled with 0.5 mL of RP-10. Leave at room temperature until processing.

5. Nasopharynx collection

1. At the neck, use the dissection scissors to cut away the fur, and then cut away the muscle and expose the trachea.
NOTE: The trachea is a tube-like structure located under the muscle.

2. Place small forceps under the trachea at a distance of 1 cm from the mouse's jaw to stabilize it. Using dissection scissors, gently make a 0.1 cm slit on the anterior portion of the trachea, avoiding cutting the trachea completely.

3. Prepare a 1 mL syringe filled with 0.5 mL of PBS with 0.58 mm tubing attached to a 25 G needle. Collect the nasal wash by inserting the tubing into the trachea going upward toward the nasopharynx. Once resistance is felt entering the nasal cavity, place a microcentrifuge tube at the nose and slowly flush the PBS through the trachea to collect the nasal lavage.

4. Place the mouse in a prone position. Spray the head of the mouse with ethanol. Use dissection scissors to cut the fur and mystacial pad to expose the head bone of the mouse.

5. Using the dissection scissors, make a 1 cm cut down the sides of the mandible and between the eyes. Using forceps, slowly pull the facial bones away from the body to expose the nasal cavity.

6. Use forceps to gently remove the nasal tissue and place it into a round bottom tube prefilled with 0.5 mL of PBS for homogenization.

6. To homogenize the collected tissue, first clean the homogenizer probe by putting it in 70% ethanol and turning on the homogenizer at 60% power for 30 s. Repeat the step in sterile water for 10 s. Homogenize each tissue for 1 min. Clean the homogenizer probe in sterile water between each sample and in a fresh tube of 70% ethanol between each organ and sample group.

7. Enumeration of bacterial numbers

1. Once all the organs have been harvested and homogenized, plate serial dilutions on blood agar plates. To calculate the total CFU, use 10 µL to plate and note down the final volume in mL for each sample. Plate the nasopharynx samples on blood agar plates supplemented with 3 µg/mL gentamicin to select for the growth of *S. pneumoniae* while inhibiting the growth of other microorganisms that colonize that tissue. Incubate overnight at 37 °C/5% CO_2.

2. To enumerate the bacterial CFU for the lung and nasopharynx, first count the colonies on the blood agar plates. Then, use equation (**1**) and equation (**2**) to calculate the amount per mL and total number.
Amount per mL = number of colonies × dilution factor × 100 (**1**)
Total number = amount per mL × total volume per sample (**2**)
NOTE: In equation (**1**), 100 is used to multiply since 10 µL is plated, which is a 100-fold dilution of 1 mL. The total volume per sample in equation (**2**) is from

step 6.7.1, which results in the limit of detection of 100 per organ.

3. To enumerate the bacterial CFU For bacteremia, first count the colonies on the blood agar plates. Then, use equation (**3**) to determine the amount per mL of blood.

   Amount per mL of blood = number of colonies × dilution factor × 100 × 10 (**3**)

   NOTE: In equation (**3**), 100 is used as 10 µL is plated, which is a 100-fold dilution of 1 mL, and 10 indicates a 1:10 dilution of the blood in anticoagulant. This results in the limit of detection of 1,000/mL.

7. Processing of the lung samples for flow cytometry

1. Prepare the required media as follows:
   1. Prepare RP-10 as described in step 2.1.
   2. Prepare digestion buffer by mixing RP-10 with 2 mg/mL collagenase and 30 µL/mL DNase I.
   3. Prepare lysis buffer by dissolving 8.29 g of NH_4Cl, 1 g of $NaHCO_3$, and 0.038 g of EDTA in 1 L of H_2O.
   4. Prepare 10x FACS buffer by mixing 450 mL of HBSS with 50 mL of heat-inactivated FBS and 5 g of sodium azide.
   5. Prepare 1x FACS buffer by diluting 50 mL of 10x FACS buffer in 450 mL of HBSS.
2. Take the lung samples from step 6.4.3 and place in a 24-well plate. Add 500 µL of digestion buffer to each well. Incubate for 45 min up to 1 h at 37 °C/5% CO_2.
3. Prefill 50 mL conical tubes for each sample with 5 mL of RP-10. When the incubation is over, place a 100 µm filter at the top of the 50 mL conical tube and wet it with 1 mL of RP-10.
4. Using a P-1000 micropipette, move the digested lungs and place them on the filter. Use the plunger of a 3 mL syringe to mash the organ. Rinse 2x with 1 mL of RP-10 each time.
5. Spin the samples at 4 °C and 327 × *g* for 5 min. Aspirate the supernatant and resuspend the pellet in 1 mL of lysis buffer. Leave for 3 min to allow lysis of the red blood cells. Neutralize with 5 mL of RP-10.
6. Spin the samples at 4 °C and 327 × *g* for 5 min. Aspirate the supernatant, resuspend the pellet in 1 mL of RP-10, and take 10 µL for counting the samples.
7. Spin the samples at 4 °C and 327 × *g* for 5 min. Aspirate the supernatant and resuspend the pellet in RP-10 at 2 × 10^6-4 × 10^6 cells/mL. Add 60 µL of each sample into a 96-well plate to stain for the desired cell types[23] listed in step 7.9, **Table 5**, and **Table 6**.
8. Spin the plate at 4 °C and 327 × *g* for 5 min.
9. Meanwhile, prepare the antibody master mixes, florescent minus one (FMOs), and single-stain controls with the desired antibodies. To stain for polymorphonuclear leukocytes (PMNs), macrophages, monocytes, dendritic cells, and T cells, use the antibodies and final dilutions listed in **Table 5** and **Table 6**. Use a total volume of 100 µL/well of the antibody mix. Follow the dilutions listed in the tables for determining the appropriate volume of the master mix and the individual antibodies required.
10. When the spin is done (step 7.8), decant the supernatant, resuspend the pellets in 100 µL of the antibody mixes,

FMOs, or single-stain controls, and incubate on ice for 30 min in the dark.

11. Wash the cells 2x by adding 150 µL of FACS buffer to the wells and spinning the plate at 4 °C and 327 × *g* for 5 min.
12. When the spin is done, decant the supernatant, resuspend the pellets in 100 µL of fixation buffer, and incubate on ice for 20 min.
13. Wash the cells 2x by adding 150 µL of FACS buffer to the wells and spinning the plate at 4 °C and 327 × *g* for 5 min.
14. Prepare labeled FACS tubes with 200 µL of FACS buffer. Resuspend the pellets in 150 µL of FACS buffer. Individually filter each sample into their corresponding FACS tube using a 100 µm filter. Keep on ice or at 4 °C and protected from the light until ready to analyze.
15. Analyze the cells using a flow cytometer.

8. Plaque assay for enumerating IAV

1. Prepare the required media as follows:
    1. Prepare infection medium by dissolving 2.5 g of bovine serum albumin (BSA) into 40 mL of DMEM while stirring at 37 °C for 10-20 min until dissolved. Filter-sterilize into 460 mL of DMEM.
    2. Prepare 2.4% microcrystalline cellulose by dissolving 1.2 mg of microcrystalline cellulose into 50 mL of H_2O. Autoclave on the liquid setting and store at room temperature.
    3. Prepare 5% of BSA DMEM by dissolving 2.5 g of BSA into 40 mL of DMEM while stirring at 37°C for 10-20 min. Add the remaining 10 mL of DMEM for a final volume of 50 mL. Filter-sterilize and store at 4 °C.
    4. Prepare 2x MEM/0.5% BSA by mixing 1 mL of 5% BSA DMEM with 9 mL of 2x MEM.
    5. Prepare low-viscosity overlay medium by mixing a 1:1 ratio of 2.4% microcrystalline cellulose and 2x MEM/0.5% BSA with 1 mg/mL TPCK (inhibitor of chymotrypsin) trypsin.
    6. Prepare EMEM/10% FBS by mixing 450 mL of Eagle's Minimum Essential Medium (EMEM) with 50 mL of heat-inactivated FBS.
2. Grow the Madin-Darby canine kidney (MDCK) cell line. Add the cells from one purchased vial to 5 mL of EMEM/10% FBS in a T-25 tissue culture-treated flask. Incubate for 3-5 days at 37 °C/5% CO_2 until the cells reach 100% confluence. Check for confluency as in step 2.3.
3. Remove and discard the culture medium, and rinse 2x with 5 mL of room-temperature PBS. Add 1 mL of trypsin-EDTA to the flask and incubate at 37 °C/ 5% CO_2 for 10-15 min until the cells detach. Once lifted, neutralize with 4mL of EMEM/10% FBS to obtain a cell suspension at 2 × 10^5 cells/mL.
4. Seed the MDCK cells in a 12-well tissue culture-treated plate by adding 1 mL of resuspended cells per well (at 2 × 10^5 cells/well) 1 day before beginning the plaque assay. NOTE: Make sure the cells reach 100% confluency prior to use and incubate for longer if needed to reach confluency.
5. For use as standards, make 10-fold serial dilutions (10^6-10^1) of IAV stock (of a known titer) in the infection medium listed in step 8.1.1. Make 1.2 mL of each dilution to test in triplicate.

6. Thaw the organ homogenates on ice. Spin down on a tabletop centrifuge at 2,000 × *g* and collect the clear supernatant.
7. Repeat step 8.5 but with the supernatant from the samples in step 8.6.
8. Aspirate the medium from the cells and wash 2x with 1 mL of PBS to remove all the FBS.
9. Add 300 µL of each standard dilution or serially diluted sample gently along the side of each well, beginning at the highest dilution to the lowest, and do this in triplicate.
10. Place the plates in the incubator at 37 °C/5% CO_2, shaking the plate every 10 min for a total of 50 min. Make sure to place them flat in the incubator and do not stack them.
11. After the 50 min, wash the cells 2x with 1 mL of PBS.
12. Add 2 mL of the low-viscosity overlay medium into each well except the lowest-dilution and no-virus wells; to those, add infection medium and trypsin.
13. Place the plate back into the incubator at 37 °C/5% CO_2 for 2-4 days to achieve plaques that can be visualized by the naked eye.
14. Wash the plates by adding 2 mL of PBS into each well fast from the side and gently shake to suspend the settled low-viscosity overlay medium.
15. Discard the whole liquid volume in the well by gently pipetting the medium off.
16. Repeat the wash one more time with 2 mL of PBS in each well, and then discard the whole liquid volume by gentle pipetting.
17. To fix the plaques, add 500 µL of 4% paraformaldehyde into each well, shake, and let sit for 30 min.
18. Wash slowly down the side with 1 mL of PBS; then, gently discard the liquid.
19. Add 500 µL of 1% crystal violet (diluted in water) to each well to cover the cell monolayer. Incubate for 5 min.
20. Wash with 1 mL of tap water. Make sure to discard all the liquid in the well by gentle pipetting. Place the plate upside down on a diaper pad to dry overnight.
21. Count the plaques visually and save the images on any available imager.

Representative Results

Biofilm-grown S. *pneumoniae* (**Figure 1A**) were used to infect mice (**Figure 1B**) using a small 10 µL inoculum delivered intranasally to unanesthetized mice. This small-volume inoculum results in consistent pneumococcal carriage restricted to the nasopharynx (**Figure 2A**, +sp groups) while avoiding systemic spread (**Figure 2B,C**, +sp groups). Two days following intranasal inoculation, the mice were infected with a murine-adapted H1N1 influenza A virus A/PR/8/34 (IAV)[22,30] delivered both intranasally and intratracheally to achieve consistent delivery of specific amounts to the nasopharynx and the lungs[23].

Here, the model was used to compare the course of disease following viral infection in mice intranasally challenged with different strains of *S. pneumoniae,* including TIGR4 and D39, which are invasive strains that result in pneumonia that progresses to bacteremia, and EF3030, which is an otitis media strain[21,24,25,26,31]. The disease presentation in *S. pneumoniae*/IAV co-infected mice was dependent on the bacterial strain (**Figure 2**). While there was no significant difference in bacterial numbers of the nasopharynx (**Figure 2A**) among any of the strains, *S. pneumoniae* TIGR4 and D39, but not EF3030, disseminated to the lungs by 48 h

post IAV infection (**Figure 2B**). Forty percent of the mice intranasally infected with *S. pneumoniae* TIGR4 displayed bacterial dissemination to the lungs, and of those, half of them became bacteremic (**Figure 2C**), consistent with prior findings[23].

Mice intranasally infected with *S. pneumoniae* D39 showed more efficient dissemination, because spread to the lungs was observed in 100% of the co-infected mice (**Figure 2B**). Similar to *S. pneumoniae* TIGR4, half of those experienced bacteremia (**Figure 2C**). In tracking the overall survival, regardless of the bacterial strain, the rate of survival of co-infected mice was significantly lower than the mice singly challenged with *S. pneumoniae* alone for all the strains tested (**Figure 2D**). Compared to the control mice challenged with IAV alone, the mice intranasally infected with *S. pneumoniae* TIGR4 and D39, but not EF3030, displayed accelerated rates of disease. By day 2 post IAV infection, 30% (D39) and 20% (TIGR4) of mice had succumbed, while the IAV-only control groups did not start to succumb until day 5 post challenge (**Figure 2D**). The mice co-infected with *S. pneumoniae* EF3030 and IAV had delayed symptoms, more similar to the IAV-only controls (**Figure 2D**). These findings demonstrate that the co-infection model results in disease in young healthy mice that is bacterial strain-dependent, which makes it ideal for exploring the bacterial factors required at each step of disease progression.

This model was used to assess the presence of various immune cells in the lungs (cell types and gating strategy in **Figure 3**) following IAV infection in mice intranasally inoculated with different strains of *S. pneumoniae.* The bacterial strains D39 and TIGR4, which dispersed into the lungs following IAV infection, elicited a significant increase above baseline (uninfected) in the influx of inflammatory immune cells from the circulation, such as neutrophils (PMNs) and monocytes, while EF3030 did not (**Figure 4A-C**). IAV infection alone elicited a significant increase above baseline in the influx of immune cells important for host defense against viral infection, such as NK cells and gamma-delta T cells (**Figure 4A-C**). These antiviral responses were significantly blunted in mice intranasally infected with *S. pneumoniae* prior to viral challenge (**Figure 4A-C**). This is consistent with prior studies assessing cytokine responses that found that *S. pneumoniae* carriage blunted the production of type I interferons and impaired the ability of the host to control IAV loads in the lungs[23]. These findings demonstrate that the co-infection model can be used to study how immune responses change in mono versus polymicrobial infections.

This model was also used to assess the effect of aging on the course of disease following IAV infection in mice intranasally infected with *S. pneumoniae* TIGR4. In singly infected mice, the viral titers did not vary between the young and aged cohorts (**Figure 5A**)[23]. As in prior studies[23], old mice displayed earlier and significantly more severe signs of disease compared to their young counterparts, as demonstrated by the higher clinical scores (**Figure 5B**). Consistent with the disease symptoms, old mice inoculated with *S. pneumoniae* started dying faster within 24 h post IAV infection, and all of them succumbed to the disease, whereas the young controls survived the infection at a significantly higher (33%) rate (**Figure 5C**). These findings demonstrate that the co-infection model can be used to detect more severe disease in vulnerable hosts, making it ideal for exploring host factors that confer resistance or susceptibility to co-infection.

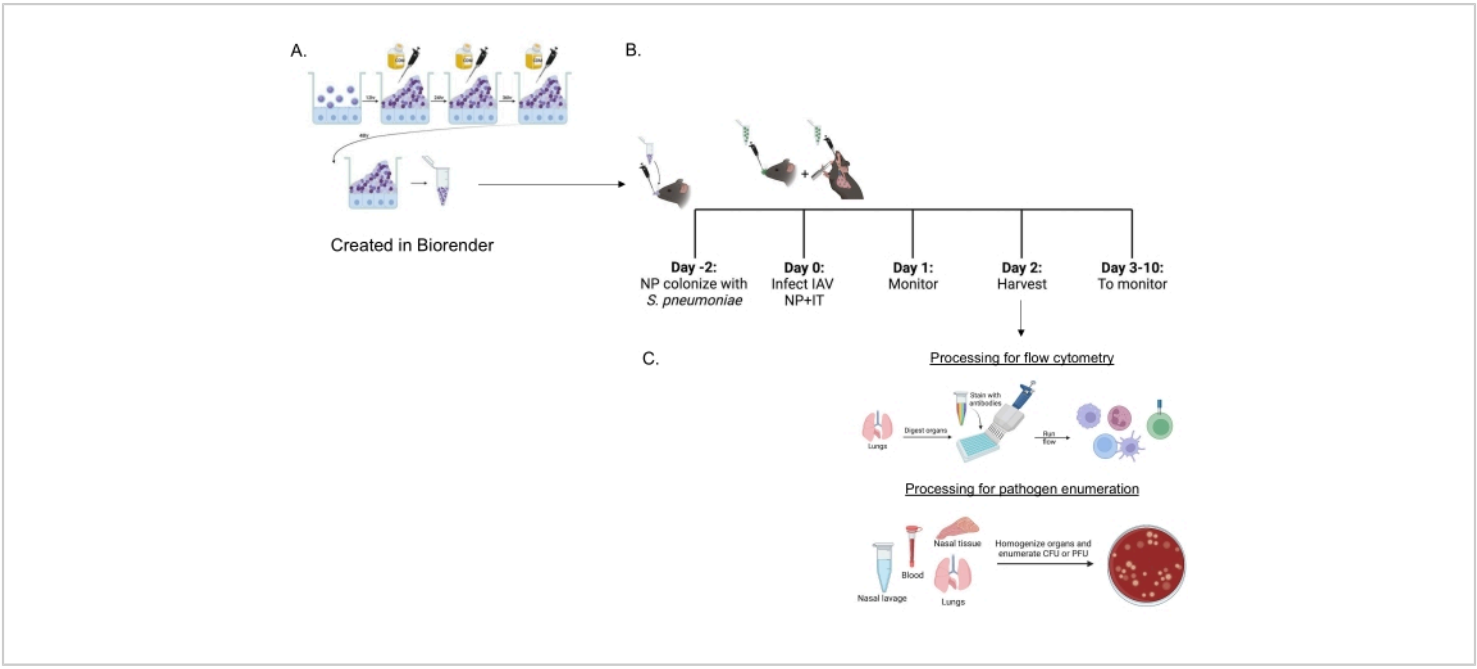

Figure 1: Timeline of co-infection and organ processing for the assessment of immune cell influx and pathogen burden. (**A**) *Streptococcus pneumoniae* are grown in biofilms. (**B**) Mice are inoculated intranasally with 5 × 10^6 CFU of the indicated biofilm-grown *S. pneumoniae* strain to establish nasopharyngeal carriage or left untreated. Forty-eight hours later, the mice are either mock treated with PBS or receive 200 PFU of influenza A virus PR8 intranasally and 20 PFU intratracheally. Mice are monitored over time for clinical disease scores and survival. (**C**) At 48 h post IAV infection, bacterial CFU or viral PFU in the different organs or immune cell influx in the lungs are assessed. Abbreviations: CFU = colony-forming units; PFU = plaque-forming units; IAV = influenza A virus PR8; IT = intratracheally; NP = nasopharyngeally. Please click here to view a larger version of this figure.

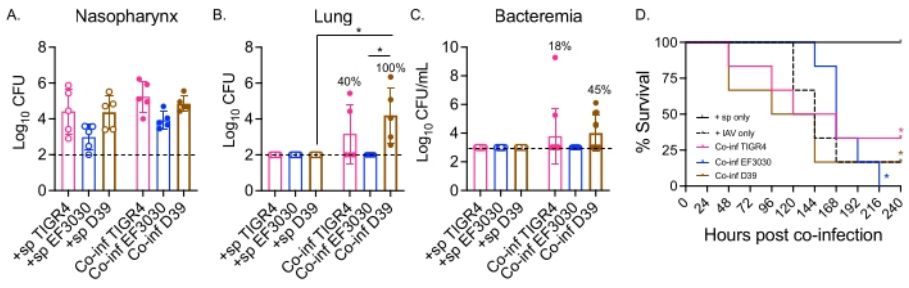

Figure 2: Dual intranasal/intratracheal IAV infection of *S. pneumoniae*-inoculated mice leads to bacterial spread and disease that is dependent on the bacterial strain. Young (10-12 weeks old) male C57BL/6 (B6) mice were infected as in **Figure 1**. Bacterial numbers in the (**A**) nasopharynx, (**B**) lungs, and (**C**) blood were all determined at 48 h post IAV infection. (**B**,**C**) Percentages denote the fraction of mice that exhibited spread. (**D**) Survival was monitored for 10 days post IAV infection. Pooled data from (**A**,**B**) n = 5, (**C**) n = 11, and (**D**) n = 6 mice per group are shown. Each circle corresponds to one mouse, and the dashed lines indicate the limit of detection. (**A-C**) *, indicates a significant difference ($p < 0.05$) between the indicated groups as determined by the Kruskal-Wallis test. (**D**) *, indicates a significant difference ($p < 0.05$) between +sp and Co-inf mice per bacterial strain as determined by the log-rank (Mantel-Cox) test. Abbreviations: +sp = mice infected intranasally with bacteria only using the indicated strain; Co-inf = bacterial-infected mice that were infected with IAV; IAV = mice that received the influenza A virus; CFU = colony-forming units. Please click here to view a larger version of this figure.

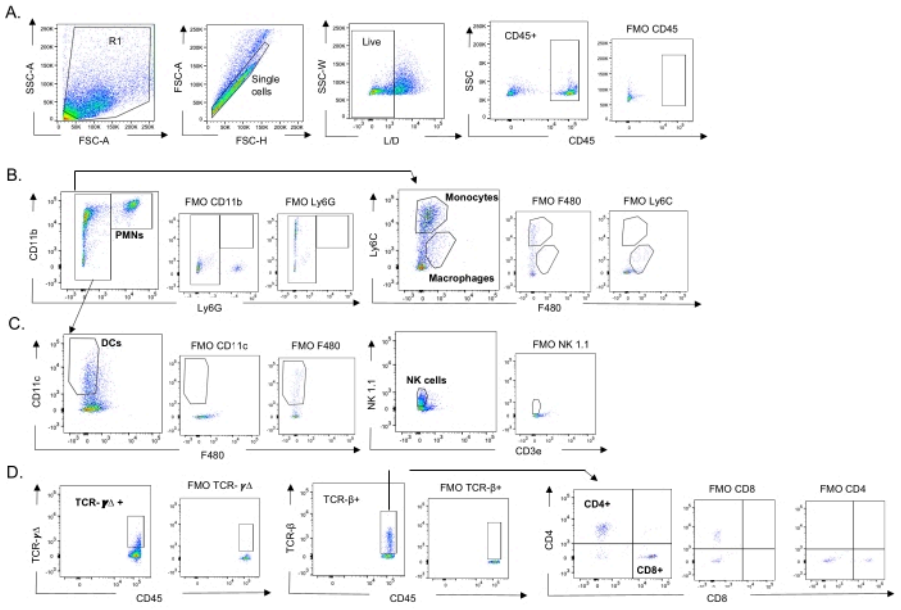

Figure 3: Immune cell gating strategy. The lungs were harvested, and the immune cell influx was determined by flow cytometry. The representative gating strategy of the different cell types is shown. (**A**) $CD45^+$, live single cells were gated on and the percentages of (**B**) PMNs ($Ly6G^+$, $CD11b^+$), macrophages ($Ly6G^-$, $Ly6C^-$, $F480^+$), and monocytes ($Ly6G^-$, $Ly6C^+$), (**C**) DCs ($Ly6G^-$, $CD11c^+$) and NK cells ($NK1.1^+$, $CD3^-$), (**D**) TCR^- γΔ and CD8 ($CD8^+$, $TCR\beta^+$) and CD4 ($CD4^+$, $TCR\beta^+$) T cells were determined. Abbreviations: SSC-A = side scatter-peak area; FSC-A = forward scatter-peak area; FSC-H = forward scatter-peak height; SSC-W = side scatter-peak width; L/D = live/dead; FMO = fluorescent minus one; NK = natural killer; PMN = polymorphonuclear leukocyte; DC = dendritic cell; TCR = T cell receptor. Please click here to view a larger version of this figure.

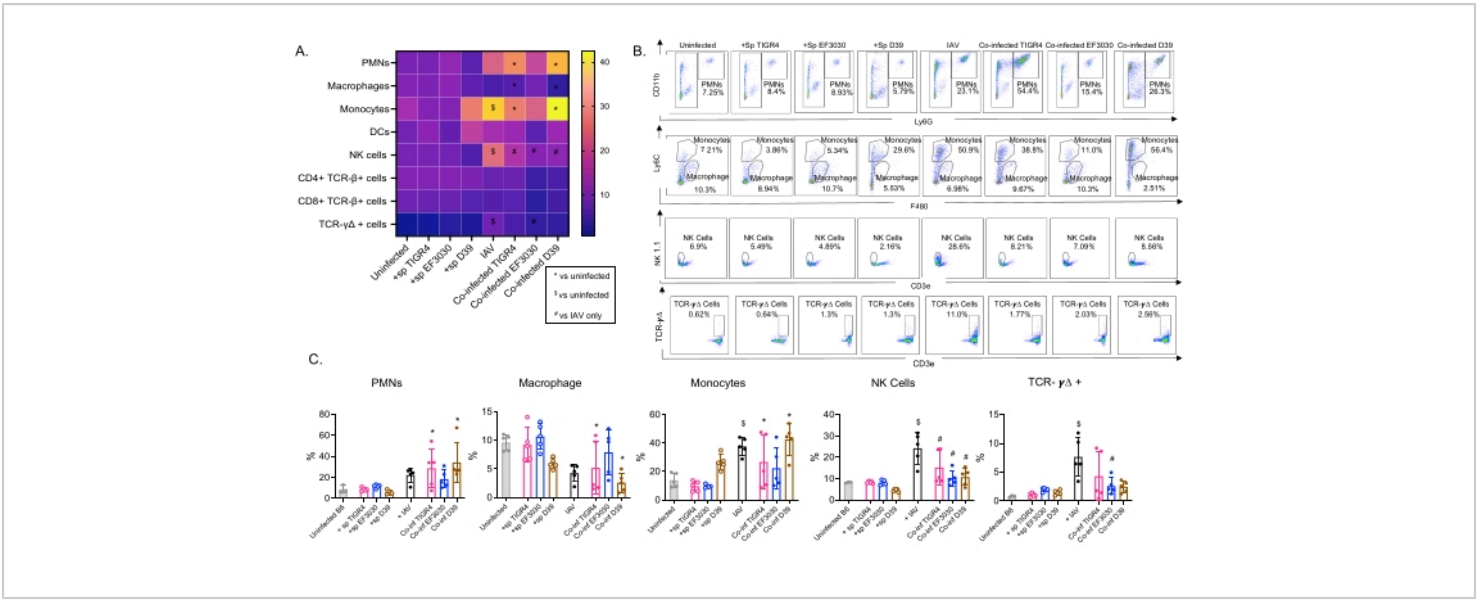

Figure 4: Pulmonary immune responses are bacterial strain-dependent. Young (10-12 weeks old) C57BL/6 male mice were either uninfected, singly inoculated with the indicated *Streptococcus pneumoniae* strain (+sp), singly challenged with IAV (IAV), or co-infected with *S. pneumoniae* and IAV (Co-inf). Forty-eight hours following IAV infection (see the experimental design in **Figure 1**), the lungs were harvested, and the immune cell influx was determined by flow cytometry following the gating strategy in **Figure 3**. (**A**) The average percentages of each indicated cell type within the CD45 gate are displayed for all the treatment groups on the heat map. (**B**) Representative dot plots of cell types that displayed significant differences between treatments are shown for each mouse group. (**C**) The percentages of the indicated immune cell types are shown. Each circle corresponds to one mouse. (**A**,**C**) Pooled data from n = 5 mice per group are shown. *, indicates a significant difference ($p < 0.05$) between Co-inf and uninfected; $^{\$}$, indicates a significant between IAV and uninfected; #, indicates a significant difference between Co-inf and IAV alone. Significant differences between the challenge groups for each cell type were determined by ANOVA followed by the Tukey's test. Abbreviations: NK = natural killer; PMN = polymorphonuclear leukocyte; DC = dendritic cell; TCR = T cell receptor; IAV = influenza A virus. Please click here to view a larger version of this figure.

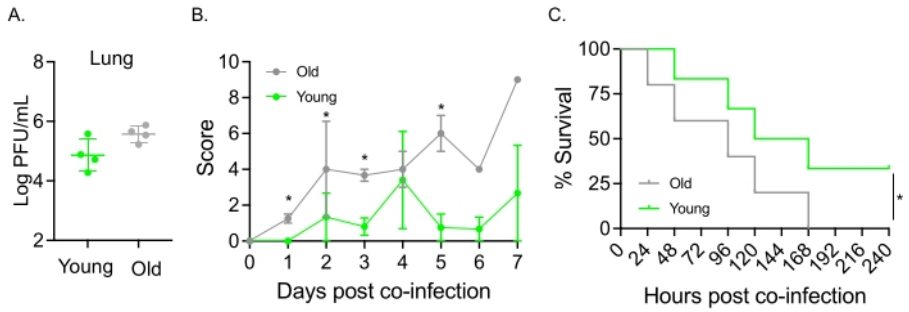

Figure 5: Aging and increased host susceptibility to IAV/*Streptococcus pneumoniae* co-infection. Young (10-12 weeks) and aged (21-22 months) C57BL/6 male mice were co-infected with *S. pneumoniae* TIGR4 i.n. and IAV i.n. and i.t. (as in **Figure 1**) or singly challenged with IAV alone. (**A**) Viral titers were determined 48 h later. Asterisks indicate statistical significance ($p < 0.05$) as determined by the Student's *t*-test. Data are pooled from n = 4 mice per group. (**B**) Clinical score and (**C**) survival were monitored over time. (**B**) The mean ± SEM pooled from n = 6 mice per group are shown. Asterisks indicate statistical significance ($p < 0.05$) between the young versus old mice at the indicated timepoint as determined by the Mann-Whitney test. (**C**) Data are pooled from n = 6 mice per group. Asterisks indicate statistical significance ($p < 0.05$) between the young versus old mice as determined by the log-rank (Mantel-Cox) test. Abbreviations: IAV = influenza A virus; i.n. = intranasally; i.t. = intratracheally; SEM = standard error of the mean. **Figure 5A** is reprinted with permission from Joma et al.[23]. Please click here to view a larger version of this figure.

Mix I stock for CDM	
Adenine	0.1 g
D-Alanine	0.25 g
$CaCl_2$ Anhydrous	0.025 g
Manganese Sulfate	0.03 g
Cyanocobalamin	100 µL of 10 mg/mL stock
Para-Aminobenzoic Acid	400 µL of 5 mg/mL stock
Pyridoxamine 2HCl	100 µL of 10 mg/mL stock
Mix II stock for CDM	
Guanine	0.05 g
Uracil	0.05 g
Mix III stock for CDM	
Ferric Nitrate $9H_2O$	50 mg/mL
Ferric Sulfate $7H_2O$	10 mg/mL
Mix IV stock for CDM	
Beta-Nicotinamide adenine dinucleotide	25 mg/mL

Table 1: Mix I, II, III, and IV stocks for CDM. Abbreviation: CDM = chemically defined media.

Vitamin Mix Stock for CDM	
Pyridoxal Hydrochloride	0.8 g
Thiamine Cl_2	0.4 g
Riboflavin	0.4 g
Ca-pantothenate	0.4 g
Biotin	0.04 g
Folic Acid	0.4 g
Niacinamide	0.4 g

Table 2: Vitamin Mix Stock for CDM. Abbreviation: CDM = chemically defined media.

Amino Acid Stock for CDM	
L-Alanine	0.480 g
L-Arginine	0.250 g
L-Asparagine	0.700 g
L-Aspartic Acid	0.600 g
L-Cysteine	1.000 g
L-Cystine	0.100 g
L-Glutamic Acid	0.200 g
L-Glutamine	0.780 g
L-Glycine	0.350 g
L-Histidine	0.300 g
L-Isoleucine	0.430 g
L-Leucine	0.950 g
L-Lysine	0.880 g
L-Methionine	0.250 g
L-Phenylalanine	0.550 g
L-Proline	1.350 g
L-Serine	0.680 g
L-Threonine	0.450 g
L-Tryptophan	0.100 g
L-Valine	0.650 g

Table 3: Amino Acid Stock for CDM. Abbreviation: CDM = chemically defined media.

Starter Stock for CDM	
Dextrose	1.0 g
Magnesium Sulfate-7-Hydrate	0.070 g
Potassium Phosphate Dibasic	0.02 g
Potassium Phosphate Monobasic	0.1 g
Sodium Acetate Anhydrous	0.45 g
Sodium Bicarbonate	0.25 g
Sodium Phosphate Dibasic	0.735 g
Sodium Phosphate Monobasic	0.32 g
Final Supplements for CDM	
Choline Chloride	0.1 g
L-Cysteine HCl	0.075 g
Sodium Bicarbonate	0.25 g

Table 4: Starter stock and final supplements for CDM. Abbreviation: CDM = chemically defined media.

Antibody/Fluorophore	Clone	Dilution Factor
L/D for UV excitation	N/A	0.38888889
Ly6G AF 488	1A8	0.25
CD11b APC	M1/70	0.25
CD11c PE	N418	0.18055556
Mouse Fc Block	2.4G2	0.11111111
F4/80 PE Cy7	BM8	0.18055556
Ly6C BV605	AL-21	0.25
CD103 BV 421	M290	0.18055556
CD45 APC-eF-780	30-F11	0.18055556

Table 5: Antibody panel 1.

Antibody/Fluorophore	Clone	Dilution Factor
L/D for UV excitation	N/A	0.388888889
TCR-β APC Cy7	H57-597	0.180555556
CD4 V450 (Pacific Blue)	RM4-5	0.25
CD8 BV650	53-6.7	0.180555556
Mouse Fc Block	2.4G2	0.111111111
CD45 PE	30-F11	0.180555556
CD3 AF488	145-2C11	0.180555556
TCR- γΔ APC	GL-3	0.180555556
NK1.1 AF 700	PK136	0.180555556

Table 6: Antibody panel 2.

Discussion

Most of the existing *S. pneumoniae*/IAV co-infection experimental studies rely on bacterial delivery into the lungs of mice pre-infected with IAV. These models have helped identify changes in the pulmonary environment and systemic immune response that render the host susceptible to secondary bacterial infection[15,16,17,32,33,34,35,36,37]. However, these models have failed to mimic the transition of *S. pneumoniae* from an asymptomatic colonizer to a pathogen capable of causing serious lung and systemic infections. Further, these models are not suitable for studying the host factors and host-pathogen interactions in the upper respiratory tract that contribute to susceptibility to infection. A prior model for the movement of pneumococci from the nasopharynx to the lung after IAV infection relied on bacterial infection of the nasopharynx followed by viral infection. However, it failed to reproduce the severe signs of disease observed in human patients[21]. The modified murine infection model described here recapitulates the transition of *S. pneumoniae* from asymptomatic carriage to a pathogen that causes severe clinical disease.

A critical step of this model is establishing *S. pneumoniae* infection in the nasopharynx. *Streptococcus pneumoniae* form biofilms and colonize the nasopharynx at different efficiencies[21,38]. To establish consistent infection, at least 5 × 10^6 CFU of the biofilm-grown bacterial strains tested so far are required[23]. It is recommended that any new bacterial strain be tested for stable infection of the nasopharynx prior to viral infection. For viral co-infection, previous studies have found that intranasal infection with IAV is required for the dispersion of the bacteria from the nasopharynx[21,22,23]. In those prior studies, 500 PFU of IAV for intranasal delivery were used, while in this study, 200 PFU were sufficient to increase bacterial numbers in the nasopharynx. IAV infection is not limited to the upper airways and can spread to the lungs[39,40], which is key for rendering the pulmonary environment more permissive for bacterial infection[15,16,41]. The delivery of IAV to the lungs can be achieved by either

intranasal delivery or intratracheal installation of anesthetized mice. Prior work with BALB/cByJ mice found that intranasal delivery results in viral pneumonia[21]; however, access of the inoculum to the lungs following intranasal inoculation is more restricted in C57BL/6 mice. In C57BL/6 mice, intratracheal installation is required for consistent delivery of the virus[23]. In this model, prior bacterial colonization accelerates the presentation of disease symptoms after viral infection[23]. As viral infection can itself cause disease symptoms with potential variation in kinetics, it is recommended to first test a range of doses for any new viral strain tested and choose a dose that reveals accelerated kinetics in co-infected hosts.

The lungs provide another critical readout for disease evaluation in this model. For the assessment of pathogen burden and immune cell influx, a lung from the same mouse can be used. However, as infection and inflammation severity can differ between lobes, it is recommended to not take different lobes of the same lung for the various assessments. Rather, all the lobes can be minced into small pieces, mixed well together, and then parsed out equally for the different assessments. Similarly, the nasopharynx can be used for the enumeration of bacterial CFU or viral PFU and immune response. However, the number of cells obtained from the washes and tissue is too low to perform flow cytometry without pooling the samples from mice within the same group. Alternatively, inflammation in the nasopharynx can be assessed histologically[23].

A critical feature of this model is that it recapitulates the clinical disease seen in patients. In humans, secondary pneumococcal pneumonia following IAV infection often results in obvious signs of disease, including cough, dyspnea, fever, and muscle aches that can lead to hospitalizations, respiratory failure, and even death[8,15,42,43]. This model recapitulates the severe signs of clinical disease observed in humans in terms of difficulty in breathing (reflected in the breathing score) and overall malaise (reflected in posture and movement scores) displayed by the mice, as well as death in some of the healthy young controls. The exacerbated disease symptoms in co-infected mice are likely a result of both bacterial dissemination to the lungs and impaired viral clearance in mice with pneumococcal carriage[23]. A limitation of the model is that the incidence of clinical disease and bacterial dissemination from the nasopharynx varies between mice and is influenced by bacterial strain, host age, and genotype[21,22,23]. Reflecting this, for invasive strains, the progression from localized infection (with no detectable bacteremia) to death can occur within 24 h. Therefore, for a true assessment of systemic spread, bacteremia should be followed over shorter intervals (every 6-12 h). Similarly, the disease score can change rapidly, particularly in the first 72 h following co-infection. Therefore, to closely track the disease symptoms, it is advisable to monitor mice three times per day for days 1-3 post IAV infection.

In summary, this model replicates the movement of *S. pneumoniae* from an asymptomatic colonizer of the nasopharynx to a pathogen capable of causing pulmonary and systemic disease upon IAV infection. In this model, IAV triggers the transition of *S. pneumoniae via* modifying the bacterial behavior in the nasopharynx, increasing bacterial spread to the lung, and altering antibacterial immunity[23]. Similarly, bacterial carriage blunts the antiviral immune responses and impairs IAV clearance from the lungs[23]. This renders this model ideal for parsing out changes in immune responses in single versus polymicrobial infections. Additionally, the course of disease following co-infection is, in part, dependent on the strain of pneumococci present in the nasopharynx. Therefore, the model is suited to dissecting

the bacterial factors required for asymptomatic colonization versus pathogenic transition of *S. pneumoniae.* Lastly, this model reproduces the susceptibility of aging to co-infections, and although this was not tested here, it can be easily used to assess the impact of host background on the disease course. In conclusion, separating carriage and disease into distinct steps provides the opportunity to analyze the genetic variants of both the pathogens and the host, allowing the detailed examination of the interactions of an important pathobiont with the host at different phases of disease progression. Moving forward, this model can be used to tailor treatment options for vulnerable hosts.

Disclosures

The authors have no conflicts of interest to disclose.

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