Video Article

Characterization of Inflammatory Responses During Intranasal Colonization with *Streptococcus pneumoniae*

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URL: https://www.jove.com/video/50490
DOI: doi:10.3791/50490

Keywords: Immunology, Issue 83, *Streptococcus pneumoniae*, Nasal lavage, nasopharynx, murine, flow cytometry, RNA, Quantitative PCR, recruited macrophages, neutrophils, T-cells, effector cells, intranasal colonization

Date Published: 1/17/2014

Citation: Puchta, A., Verschoor, C.P., Thurn, T., Bowdish, D.M. Characterization of Inflammatory Responses During Intranasal Colonization with *Streptococcus pneumoniae*. *J. Vis. Exp.* (83), e50490, doi:10.3791/50490 (2014).

Abstract

Nasopharyngeal colonization by *Streptococcus pneumoniae* is a prerequisite to invasion to the lungs or bloodstream[1]. This organism is capable of colonizing the mucosal surface of the nasopharynx, where it can reside, multiply and eventually overcome host defences to invade to other tissues of the host. Establishment of an infection in the normally lower respiratory tract results in pneumonia. Alternatively, the bacteria can disseminate into the bloodstream causing bacteraemia, which is associated with high mortality rates[2], or else lead directly to the development of pneumococcal meningitis. Understanding the kinetics of, and immune responses to, nasopharyngeal colonization is an important aspect of *S. pneumoniae* infection models.

Our mouse model of intranasal colonization is adapted from human models[3] and has been used by multiple research groups in the study of host-pathogen responses in the nasopharynx[4-7]. In the first part of the model, we use a clinical isolate of *S. pneumoniae* to establish a self-limiting bacterial colonization that is similar to carriage events in human adults. The procedure detailed herein involves preparation of a bacterial inoculum, followed by the establishment of a colonization event through delivery of the inoculum via an intranasal route of administration. Resident macrophages are the predominant cell type in the nasopharynx during the steady state. Typically, there are few lymphocytes present in uninfected mice[8], however mucosal colonization will lead to low- to high-grade inflammation (depending on the virulence of the bacterial species and strain) that will result in an immune response and the subsequent recruitment of host immune cells. These cells can be isolated by a lavage of the tracheal contents through the nares, and correlated to the density of colonization bacteria to better understand the kinetics of the infection.

Video Link

The video component of this article can be found at https://www.jove.com/video/50490/

Protocol

Before you begin: all steps are done in a Biohazard Level 2 (BSL2) Biological Safety Cabinet (BSC) unless otherwise stated. Please ensure that you have obtained the appropriate Biohazard Approval for use of infectious bacterial pathogens as per institutional guidelines prior to initiation of the experiments. Additionally, please ensure that you have all the materials and reagents necessary to conduct the procedure prepared beforehand. Mice used in these experiments have included female C57BL/6 mice from Jackson Laboratories, Charles River or Taconic and were 10-14 weeks of age (although we have not found any gender-dependent significant differences in kinetics of nasal colonization clearance or infection). All mice used in these experiments were bred and maintained under specific-pathogen free conditions, and were free of common viruses, (LCMV, MNV,MPV, reovirus ECTV, and other) bacteria (*e.g. H. pylori*) and parasites (*e.g.* pinworm, ectoparasites) by fecal sample testing as well as frequent anatomical assessment of sentinel mice cohoused within their facility rooms. When conducting these experiments, we recommend using control mice no younger than 10-12 weeks of age and no older than 6 months of age. Mice younger or older than this age range are more susceptible to longer nasopharyngeal carriage duration and increased likelihood of disseminating infection. Mouse background is another important consideration that may impact the outcomes of a colonization experiment, as several groups have demonstrated that mice of different genetic backgrounds have different susceptibilities to the *S. pneumoniae* D39 (serotype 2) strain[9,10]. *S. pneumoniae* is not a naturally occurring murine pathogen and its only natural reservoir is the human nasopharynx. Transmission occurs via respiratory droplets, and as mice do not produce respiratory secretions, individual mice cannot transmit the bacterium to other mice, so there is no concern for mouse-to-mouse transmission[11]. For a visual overview of the procedures described within this manuscript, please refer to **Figure 1.**

1. Preparation of *S. pneumoniae* Culture

1. Inoculate 5 ml of tryptic soy agar for the suspension growth of *Streptococcus pneumoniae*.
2. Culture under static conditions at 37 °C in 5% CO_2 until the bacterial inoculum reaches log phase growth with a corresponding liquid density of 10^8 CFU/ml as determined by an odometer set to 600 nm. The exact reading corresponding to this CFU will differ depending

on the specific selected bacterial strain; for most strains of *S. pneumoniae* this corresponds to an OD_{600} range of 0.45-0.55. Typically, *S. pneumoniae* strains in liquid culture will grow to this density within 1.5-2.5 hr under the recommended conditions, with no need for subculturing. The culture should not be allowed to overgrow (beyond an OD reading of 0.75) as this represents the point at which the bacteria are no longer in log phase growth and are undergoing extensive autolysis.

3. Each mouse will be inoculated with approximately 10^7 bacteria. Therefore, for every 9 mice to be colonized, pipette 1 ml of inoculum into an Eppendorf tube and spin at 15,000 x g for 1 min. A whitish pellet should be visible. Remove the supernatant, being careful not to disturb the pellet and resuspend the bacteria in 100 µl of phosphate buffered saline (PBS), thus increasing the concentration to 10^9 CFU/ml. At this stage, the bacteria should remain viable, but will not readily replicate.
4. If using multiple aliquots, combine into one tube to control for slight inter-sample variations in bacterial density.
5. Keep bacteria on ice until ready for inoculation, for a maximum of 1 hr.
6. To obtain an exact bacterial count, perform log-wise serial dilution series starting with neat bacterial inoculum. Dilute serially 10-fold, adding 10 µl of to 90 µl of sterile PBS.
7. Plate out 3 drops of 10 µl samples of dilutions 10^{-5} - 10^{-9}, plus a PBS-only contamination control, onto separately labeled sections of tryptic soy agar (TSA) plate supplemented with 5% sheep's blood (**Figure 2**). Ensure that pipette tips are changed for each step diluting from a higher CFU concentration to a lower CFU concentration to avoid carrying over excess bacteria and increasing variability of results. Human blood agar (HBA) plates may also be used in lieu of TSA. Since many strains of *S. pneumoniae* are resistant to neomycin (from 5-20 µg/ml), this antibiotic may also be added to the agar medium of choice during the plate preparation phase. This facilitates enumeration as it eliminates nonresistant bacteria. The antibiotic susceptibility of each strain must be tested in advance to determine the optimal concentration of antibiotic to use for each bacterial strain.
8. Allow to dry for 15-30 min uncovered, then cover plates and place upside down in bacterial incubator set to 37 °C and 5% CO_2. Grow up bacterial colonies on plate for 24 hr.
9. Determine number of colony forming units and their corresponding concentration. Based on determinations of the OD_{600} value, concentration should be within the range of 1-4 x 10^9 CFU/ml. *S. pneumoniae* colonies should appear as small, circular colonies a yellowish-beige in color, with a small depression at the center giving them a donut-like appearance (**Figure 3**).

2. Murine Intranasal Colonization

1. Restrain mice by placing them into a mouse restrainer apparatus (a modified 50 ml Falcon tube with the tip cut off to create an aperture) securing them by the base of their body with thumb so that their noses just emerge out of tapered end of the restrainer apparatus (**Figure 4**). Use of this apparatus allows for immobilization of the mouse's head and segregation of its nares in a manner that minimizes movement as well as impedes attempts from the animal to masticate the pipette tip, allowing for complete delivery of the inoculum. Alternatively, mice can be immobilized via scuffing at the neck and manual restraint. We do not recommend anesthesia of the animals prior to intranasal inoculation. Administrating the inoculum to animals under anesthesia results in some of the inoculum spreading to the lungs[12,13].
2. Using a P10 or P20 pipette, inoculate each mouse by depositing 10 µl of the prepared culture, distributing it evenly between both nares (allow inoculum to drip into nose by pulsing the inoculation gradually, taking time for mice to inhale inoculum). To achieve complete delivery of the inoculum, pause administration at any point the mouse begins to move its nose excessively. The entire inoculum may not be injected into the nares as the mice may expel some through the nose during exhalation; however, as the expelled amount tends to be minuscule, and the inoculum contains an extremely high amount of bacteria, this does not significantly affect the colonizing bacterial load. Additionally, the surface area available for colonization in the nasopharyngeal mucosa is limited and consequently we and others have found that the recommended 10^7 dose is sufficient to obtain consistent levels of bacteria in all mice, resulting in minimal variability in initial amounts of colonizing bacteria[14,15].
3. Weigh mice if utilizing weight indicators as part of your end-point monitoring. Monitor mice every 12-24 hr for clinical symptoms, including lethargy, ruffled fur and weight loss. Mice typically will not show symptoms of illness until 3-5 days post-colonization, and these will be preceded by weight loss which may average around 5% of total body weight a day. As the mice become increasingly ill they will assume hunched postures and show decreased activity and decreased responsiveness to stimulation, including handling. At this stage, illness is typically indicative of sepsis and/or pneumonia and will likely be terminal, although mice may be treated with 1 ml of subcutaneous saline daily to improve outcomes. Surviving mice should start showing improvement after day 7 post-colonization, as evidenced by stabilization of weight followed by weight gain, although different strains of *S. pneumoniae* may induce illness more quickly and result in progression of clinical symptoms along a different time-line. Please see **Figure 5** for a representative result of weight tracked in mice colonized with the P1547 strain.

3. Nasal Lavage Sample Collection

Before beginning: prepare cannulated needles using 1 ml syringes capped with 26 3/8 G beveled needles. Cut 2.5 cm pieces of PE20 polyethylene tubing with an inner diameter 0.38 mm, ensuring that each end has a beveled tip. Using forceps, slide a 2.5 cm long piece of PE20 polyethylene tubing (inner diameter 0.38 mm) on to the needle tip, avoiding puncturing the tubing side. Cannulated needles can be kept in 70% ethanol until needed.

1. Euthanize experimental mice. As cervical dislocation can potentially damage the trachea, this method of euthanasia must be avoided. Our preferred method is isoflurane anesthesia followed by exsanguination, however, ensure you follow institutional guidelines when selecting mode of euthanasia.
2. Using 70% aqueous ethanol, sterilize the superoanterior fur of the animal, particularly the neck, taking care to prevent ethanol from accessing the nares.
3. Make a single longitudinal cut along the midline of the neck of the animal, and two horizontal cuts on either end, creating an opening to envision the trachea.
4. Carefully peel back skin to either side, revealing neck tissue beneath.
5. Trachea should be visible, surrounded by longitudinal muscles on either side. Carefully snip these to provide a clear view of the trachea itself, taking care not to sever the surrounding vasculature.

6. If the vasculature was cut and blood is present, prior to proceeding, allow bleeding to stop, and then cleanse the area several times by dispensing sterile PBS and using sterile gauze to gently soak up excess moisture in the area.
7. Once trachea is properly exposed, make a transverse, semilunar cut in the trachea about half-way up (**Figure 6**).
8. Draw up 1,000 µl of sterilized PBS into previously prepared cannulated needle.
9. Insert cannula into the trachea towards the nose, keeping the beveled edge pointing downwards for ease of insertion (**Figure 7**). Once needle is in place, rotate it 180°, and gently probe upwards until you feel light resistance.
10. Place Eppendorf designated for sample collection just beneath the nose of the mouse.
11. Test correct placement of needle by dispensing a minimal (~20 µl) amount of PBS lavage fluid - drop of fluid should form around the nares of the mouse; if this is the case, proceed to step 3.13).
12. If test PBS emerges directly out the mouth of the animal, pull the cannula back, and reposition by again gently probing forward until very slight resistance is felt - take care not to push cannula too far past this resistance, as you will move it past the nasal palate and through to the oral cavity.
13. Dispense contents of needle rapidly to help displace and collect maximal amount of cells - contents should flow out through the nares of the mouse and into collection tube. Place sample immediately on ice.
14. To collect samples for RNA analysis, repeat steps 3.8-3.13) using a cannulated needle containing 500 µl of RNA lysis buffer on the same mouse. This will allow the collection of lysate sample from the remaining cell populations, largely composed of the nasopharyngeal mucosal epithelium, as nonadherent cells should have been removed following the initial PBS lavage. Please note that the RNA lysis buffer will denude the epithelium and destroy surrounding tissue, so care must be taken to avoid contact with organs such as the lungs, if retention of these tissues is desired. Once collected, place sample in RNA lysis buffer directly on dry ice to snap-freeze. Once in lysis buffer, samples can be stored as per the manufacturer's instructions, and are stable typically at -70 °C for several months.

4. Determination of Bacterial Load in the Nasopharynx

1. Quantitate bacteria by preparing a serial dilution series for each murine nasal lavage sample. In general, bacterial load can be expected to be between 0-10^4 CFU, therefore conduct three 10-fold serial dilutions. Add 10 µl of the neat nasal lavage sample (100 CFU/ml) to the first tube to a concentration of 10^{-1} CFU/ml. Vortex thoroughly.
2. Divide bacteriological plate into quadrants, and label quadrants each with a member of the dilution series (10^0-10^{-3} CFU/ml). Plate out 3 drops of 10 µl samples of the 3 dilutions and the neat sample on tryptic soy agar plates supplemented with 5% sheep's blood, as in **Figure 2**.
3. Allow to dry for 15-30 min uncovered, then cover plates and place upside down in bacterial incubator with optimal conditions for bacterial growth (typically 37 °C and 5% CO_2).
4. Grow up bacterial colonies on plate for 18-24 hr.
5. Determine number of colonizing bacteria by averaging the colonies formed on plate for each dilution (**Figure 3**). **Figure 8** demonstrates bacterial density during different timepoints, as determined by culture of nasal lavages, in mice colonized with 3 different strains of *S. pneumoniae* for up to 21 days.

5. Preparation of Samples for Flow Cytometery

Before beginning: Prepare mix of antibodies. For quantification of leukocyte populations, we recommend the following mix at the specified dilutions: PE-Ly6G (clone 1A8, 1 µg/ml), FITC-Ly6C (clone AL-21,1 µg/ml), eFluor 450-CD45 (clone 30-F11, 2.67 µg/ml), APC-F4/80 (clone PM8 RUO, 0.67 µg/ml), PerCP-Cy5.5-CD11c (clone N418 RUO, 0.5 µg/ml), PE-Cy7-CD11b (clone M1/70, 0.33 µg/ml), Alexa Fluor 700-CD3 (clone 1782, 4 µg/ml), eFluor 605NC-CD4 (clone GK1.5, 6.67 µg/ml). Please note that this mix is 2x concentration (see step 5.5). All antibodies should be diluted in FACs Wash buffer (0.5% fetal calf serum, 2mM EDTA, 0.1% sodium azide in PBS) which should also be prepared beforehand. In a mixture of isotype matched control antibodies, ideally from the same supplier as the labeled antibodies and at the same concentrations as the specific antibodies, should be prepared. The samples treated with the isotype control antibodies will function as the negative control. Any fluorescence observed in the samples treated with the isotype control antibodies should be considered background.

1. Prechill a centrifuge capable of spinning 1.5 ml Eppendorf tubes to 4 °C.
2. Centrifuge nasal lavage samples at 2,000 x g for 10 min at 4 °C. Carefully pipette out supernatant and reserve. Note: due to the small amount of cells within the nasopharynx, the cell pellet will not be visible unless there is undesired red blood cell contamination, which will bright red. If this is seen, sample should be discarded.
3. Resuspend sample in 50 µl of Fc?RIIb/CD16-2 (2.4G2) antibody (which binds Fc receptors and reduces nonspecific antibody binding) in FACs Wash Buffer at a concentration of 4 µg/ml.
4. Incubate sample on ice for 30 min.
5. Add 50 µl of preprepared 2x concentrated fluorescent antibody mix to sample. Set aside a representative sample from each experimental group to act as an isotype control. Add the isotype antibody mix to this sample in lieu of stain mix.
6. Incubate sample on ice for 1 hr.
7. Centrifuge samples at 2,000 x g for 10 min at 4 °C. Discard supernatant and resuspend in 200 µl of PBS.
8. Repeat step 5.7.
9. After the second wash, centrifuge samples again at 2,000 x g for 10 min at 4 °C.
10. Resuspend in either in PBS (if running sample immediately) or 2% paraformaldehyde (if running samples 1-3 days post-staining).
11. When conducting flow cytometry collect the maximum amount of events per sample or until the entire sample has been aspirated. In uninfected, healthy, young mice, this will be as little as 1,000-2,000 total events; During a bacterial colonization event, this number may increase more than 2-to-5-fold dependeing on disease status in animal and factors such as age and genetic background. **Figure 9** shows representative flow cytometry results collected from a 3-laser Becton Dickenson LSRII flow cytometer using a Forward Scatter of 450 and Side Scatter of 300, although we recommend optimizing parameters for the specific flow cytometer you intend to use prior to sample collection. Note: if a sample contains even trace amounts of blood contamination, the total events collected will be significantly higher than expected and the sample should be discounted from analysis.

6. Quantitative PCR (qPCR) Analysis of Nasal Lavages

1. Thaw cell lysates from step 3.14 room temperature.
2. Follow the recommended protocol provided with the preferred RNA extraction of choice.
3. After completing RNA extraction procedure as instructed, quantify the amount of RNA using a spectrophotometer or electrophoresis based method (**Figure 10**). We routinely obtain between 975 and 3,250 ng total RNA per sample with a 260/280 nm ratio of >1.7 or an RNA integrity number (RIN) around, 8.1±0.13.
4. Transcribe cDNA using the M-MULV Reverse Transcriptase according to the manufacturer's protocol with 1,000 ng of RNA (maximum 13 µl).
5. Dilute resulting cDNA samples 8x, and aliquot equally into 4 separate tubes for longterm storage at -20 or -80 °C.
6. To measure gene expression by qPCR, prepare 25 µl samples reactions in triplicate on ice or cold block containing: 12.5 µl of 2x qPCR master mix from qPCR kit of your choice, 0.25 µl reference dye, 2 µl of diluted cDNA (step 7.5), 1 µl of mixed forward and reverse primers (400 nM final), 9.25 µl of RNAse-DNAse free water. This protocol is an adaptation of previously published methods[16].
7. In general we find that a two-step qPCR amplification (95 °C for 10 min followed by up to 40 cycles x [95 °C x 15 sec, 60 °C x 1 min]) is effective (**Figure 11a**); however, each primer pair must be optimized. Dissociation (melting) curves must be performed following amplification to ensure that no nonspecific amplification occurred. amplification (**Figure 11b**)
8. We routinely run standard curves for each gene analyzed as well as a standard calibrator (derived from lung or spleen homogenate) for each 96-well plate analyzed. Relative transcript amounts are obtained by first normalizing raw cycle threshold (Ct) values by the reference dye and transforming the resultant values through the respective standard curve. These relative amounts are subsequently normalized to the standard calibrator and a housekeeping gene, as applicable.

Representative Results

Figure 1 represents an overview schematic summarizing the main steps of the protocol. **Figures 2-3** provide visualization of the microbiological methodology inherent to the protocols described herein. **Figure 4** represents proper positioning of a mouse to perform an intranasal colonization, while **Figure 5** depicts typically changes in weight of mice colonized with *S. pneumoniae* strain P1547. **Figures 6-7** represent specific stages of the nasal lavage portion of the process, for assisted visualization of these two techniques. **Figures 8-11** consist of representative results of analyses conducted on samples collected from the nasopharynx of a mouse following nasal lavage. Specifically, **Figure 8** is a representative result of bacterial load in the nasopharynx, as determined through culturing of nasal lavages obtained from mice colonized either with *S. pneumoniae* strain P1121, P1547 or P1542. **Figure 9** represents cell phenotyping of isolated nasopharyngeal immune cells using flow cytometric techniques. **Figures 10-11** display representative results pertaining to expressional analysis of nasopharyngeal mRNA via quantitative PCR.

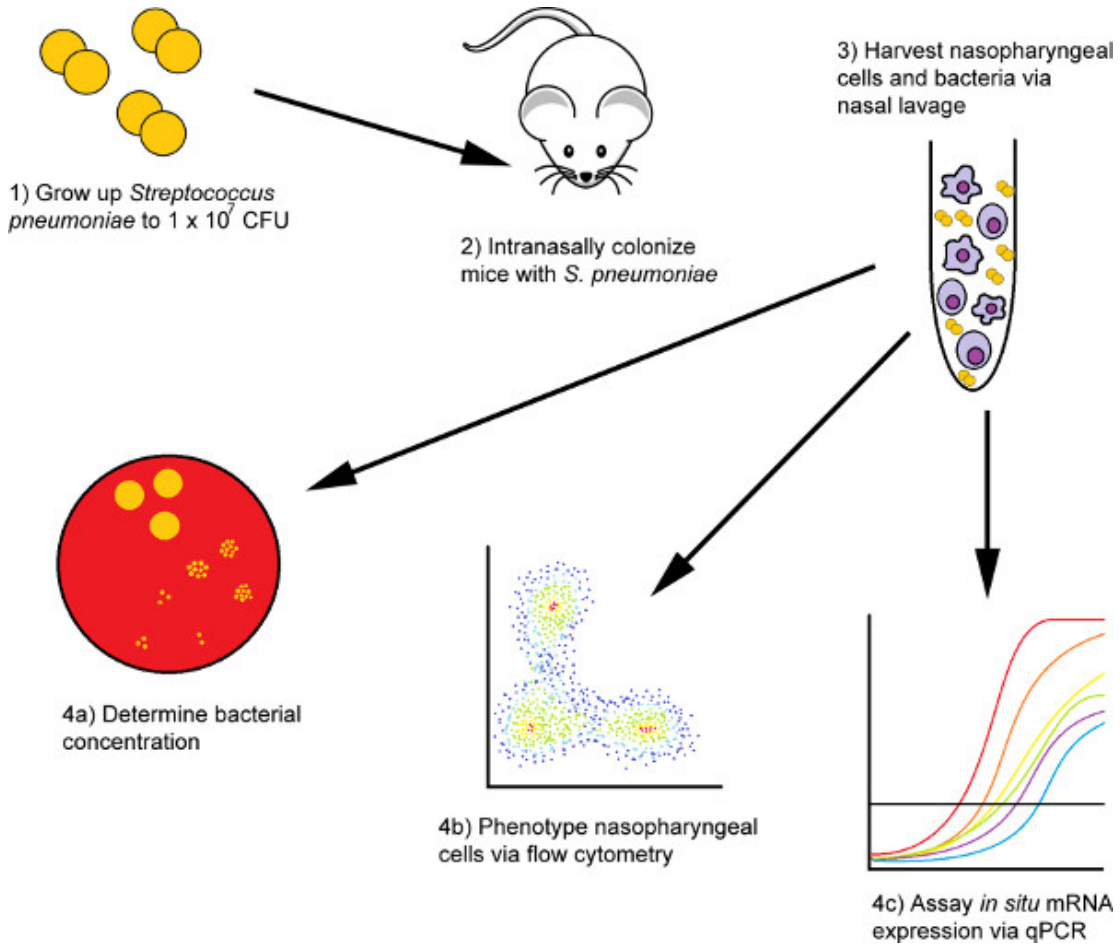

Figure 1. Flow chart of the intranasal inoculation and nasal lavage cell isolation procedures using a mouse model. First, the bacteria are prepared for inoculation, and then given to murine subjects intranasally. After the desired length of time elapses, mice are euthanized via terminal bleed, and their nasopharyngeal cells are isolated via two nasal lavage steps: a PBS wash step followed by a secondary wash in RNA lysis buffer. The cells from the preliminary PBS wash are isolated and analyzed using flow cytometry techniques, while RNA isolated from the second sample can be used to investigate the relative abundances of molecules of interest at the transcriptional level.

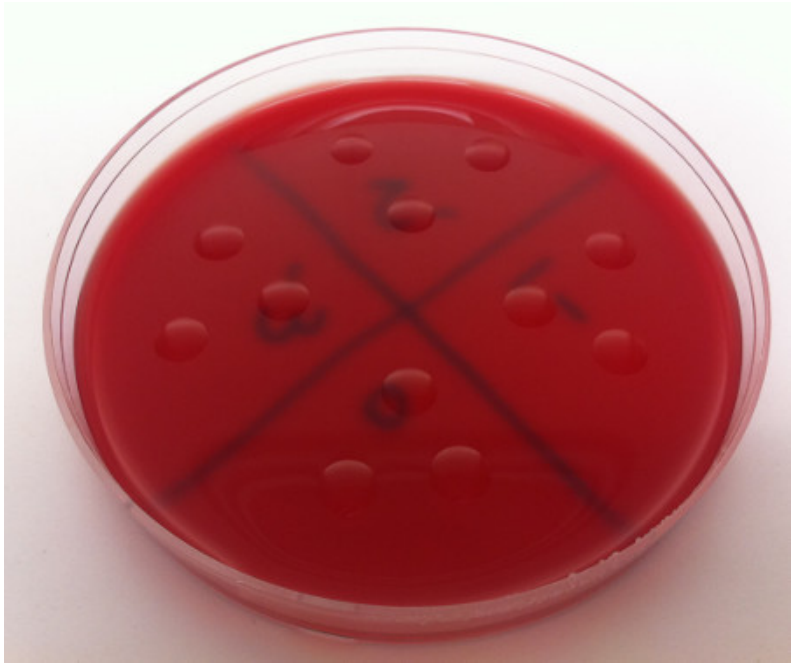

Figure 2. To determine bacterial concentration, 10 µl drops are plated in triplicate on a plate divided into sections representing a different serial dilution. These drops are then allowed to dry and the plates are incubated overnight at 37 °C, 5% CO_2.

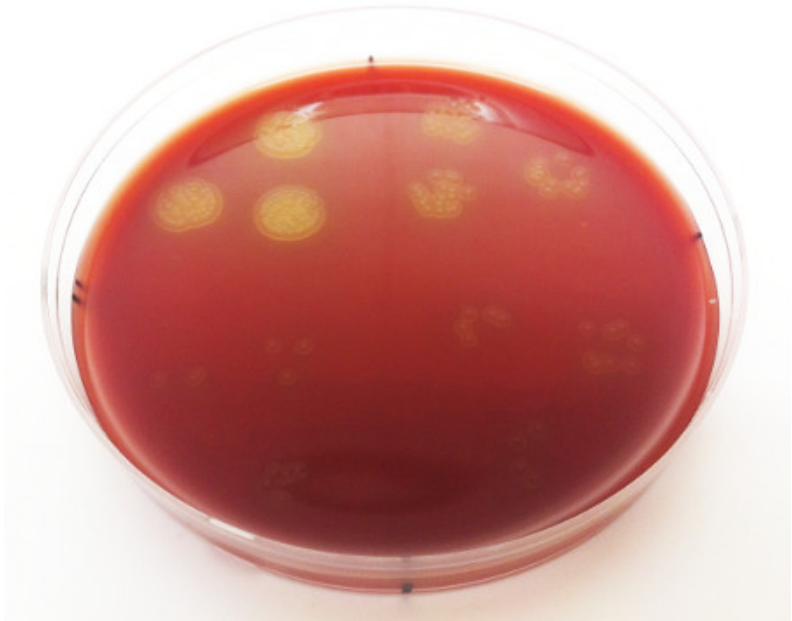

Figure 3. Concentration of *Streptoccous pneumoniae* isolated from the nasopharynx of a representative animal. Each discrete colony represents one colony forming unit, each collection of colonies represents one 10 µl drop (plated in triplicates) and each quadrant on the plate represents a separate serial dilution. Bacterial concentration is determined in CFU/ml by averaging the number of countable, fully formed colonies within, and then between, qualifying quadrants.

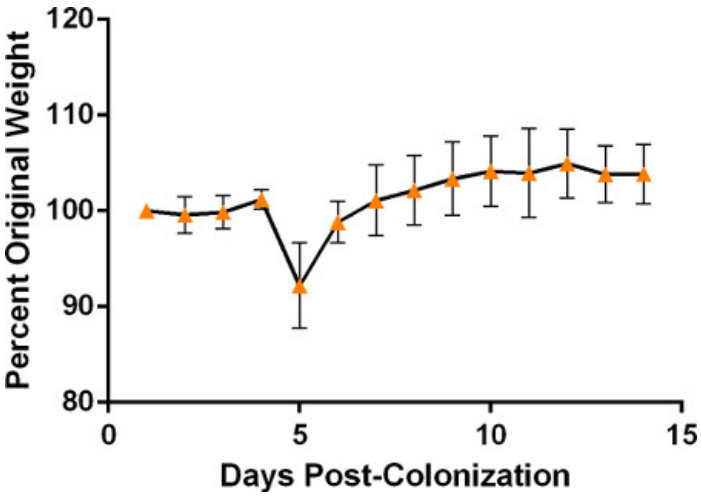

Figure 4. The movement of any mouse to be inoculated must be minimized, particularly at the neck, to allow for proper delivery of bacterial inoculum. To accomplish this, subject mouse is restrained in a modified restriction apparatus consisting of a 50 ml Falcon tube with an aperture at its tapered end. The mouse is then positioned so that its nose emerges from the aperture, where it can be accessed by the researcher, allowing for intranasal inoculation to be performed.

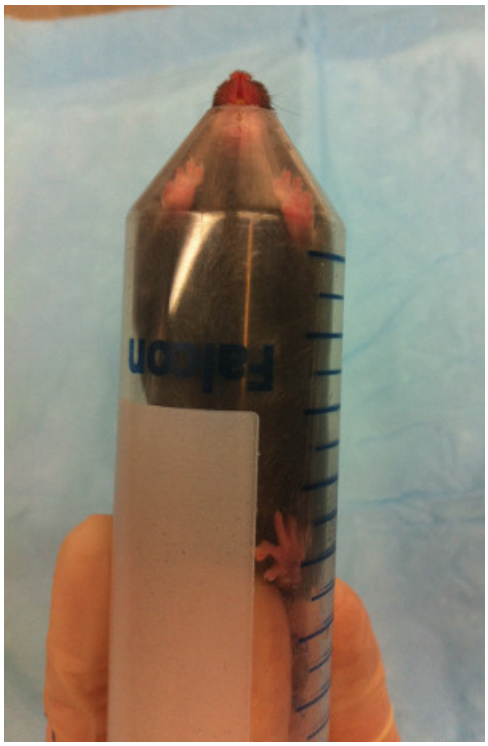

Figure 5. Weight of mice colonized with strain P1547 from a minimum of 2 representative experiments tracked daily following initial inoculation (n=6) to depict typical changes in weight expected following nasopharyngeal colonization. Weight is shown as a percent change of initial weight. Please note the expected sharp initial weight loss seen between days 3-5, followed by stabilization and gradual increase in weight in surviving mice.

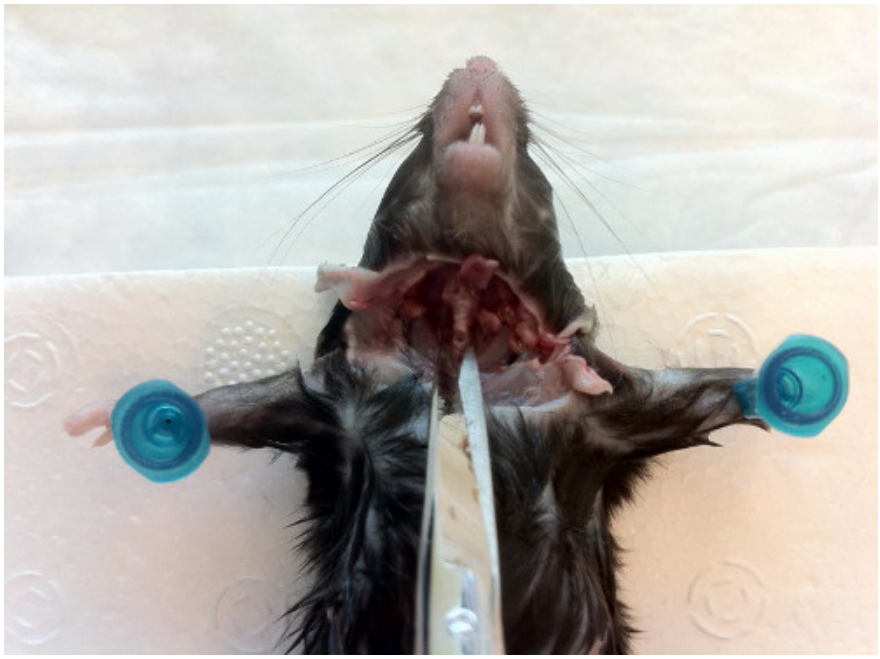

Figure 6. Upon tracheal exposure, flanking longitudinal muscles are removed carefully prior to tracheal incision in a manner that does not severe the surrounding blood vessels. A small, semilunar incision is then made halfway up the trachea using fine surgical scissors. It is important to cut through the diameter of the trachea only partially, leaving it intact posteriorly.

Figure 7. Insertion of the cannulated needle into the tracheal aperture upwards towards the nose. Once cannula is in place, probe gently until resistance is met, then flush contents out through the nares.

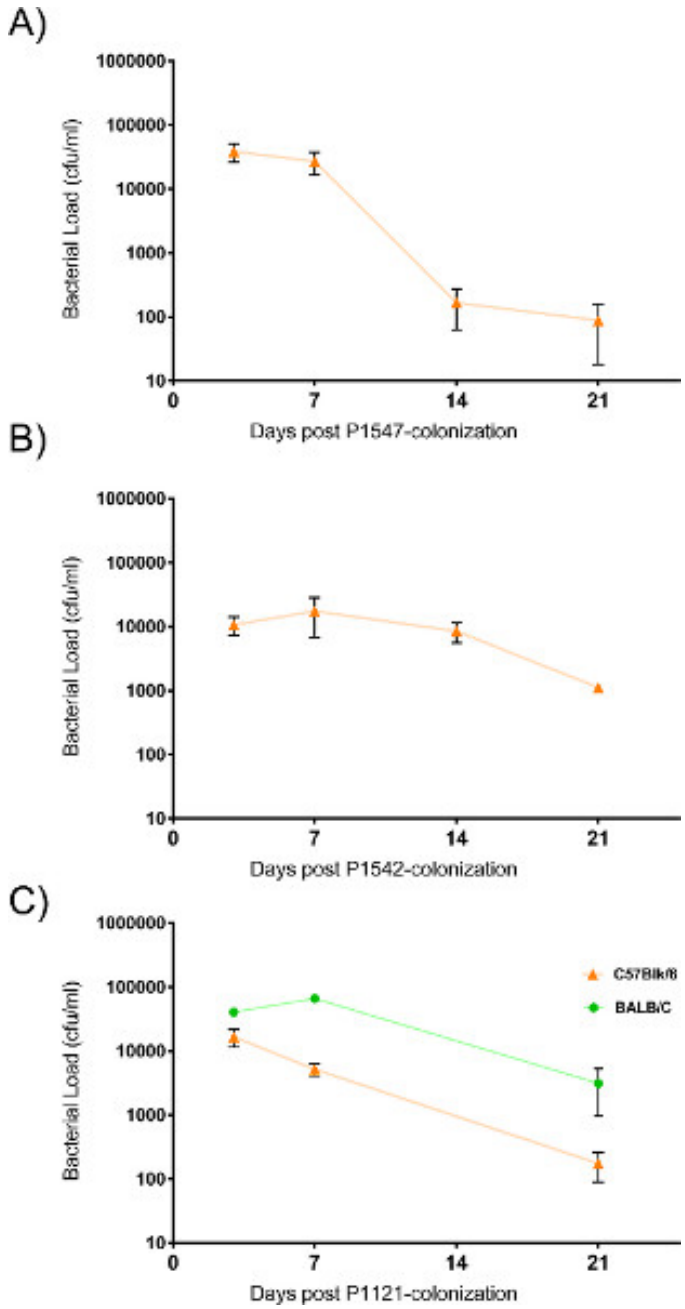

Figure 8. A representative series of bacterial load isolated from the nasopharynx using the nasal lavage procedure described following colonization of C57BL/6 mice (triangles) with *S. pneumoniae* strain P1547 (A), P1542 (B) or P1121 (C). A comparative colonization of BALB/C mice (circles) following P1121 colonization is also displayed in (**C**). Different time points are shown throughout the course of colonization, including days 3, 7, 14, and 21. Generally, a high initial load is expected at day 3, with little diminishment at day 7. Clearance is typically initiated by day 14, with full or near-full clearance evidenced by day 21 following colonization with most strains. Click here to view larger figure.

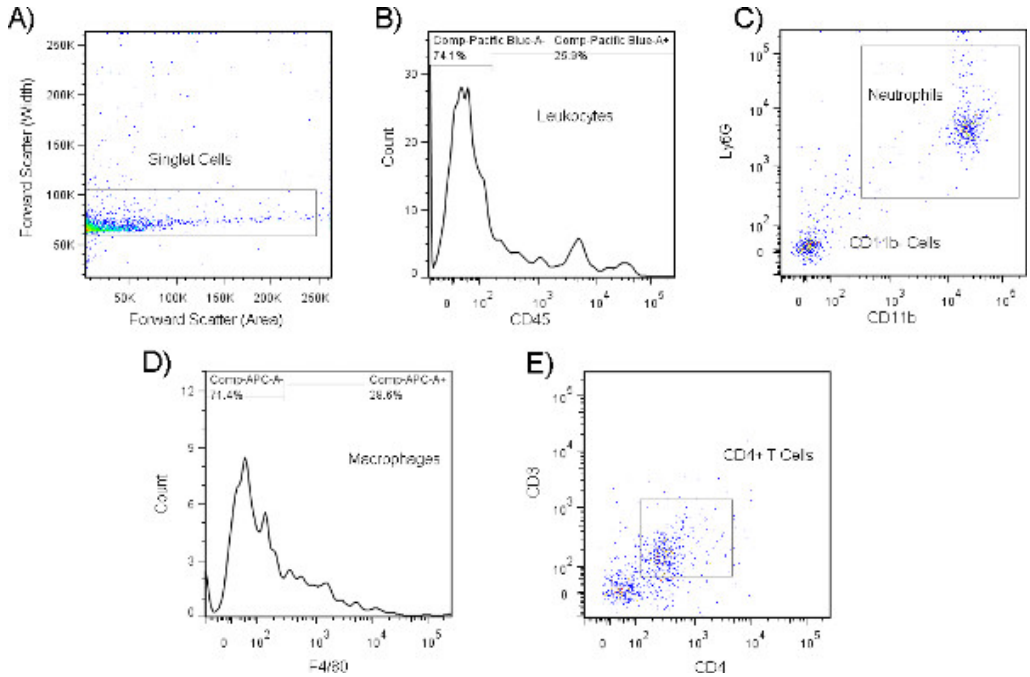

Figure 9. Representative histogram (A) and dot plot (B) of total cells isolated from murine nasal lavages as analyzed by flow cytometry. The differential expression of markers on cell populations allows for the identification of leukocyte subsets through the use of fluorescent antibodies directed against these proteins. As shown here, leukocyte populations are selected by first gating on singlet cells using a Forward Scatter (area) versus Forward Scatter (width) gate (**A**), and then enriching for CD45+ cells within that subset (**B**). This population can be further subdivided into specific cell types by gating for CD11b and Ly6G double positive neutrophils (**C**). Analysis of the CD11b- population can be conducted to reveal F4/80+, CD11b-macrophages (**D**) or CD11b-, CD3 and CD4 double positive CD4 T cells (**E**). Cell populations can be phenotyped as long as they express either one, or a combination of several unique surface receptors that can be used to distinguish them from other cell types. Click here to view larger figure.

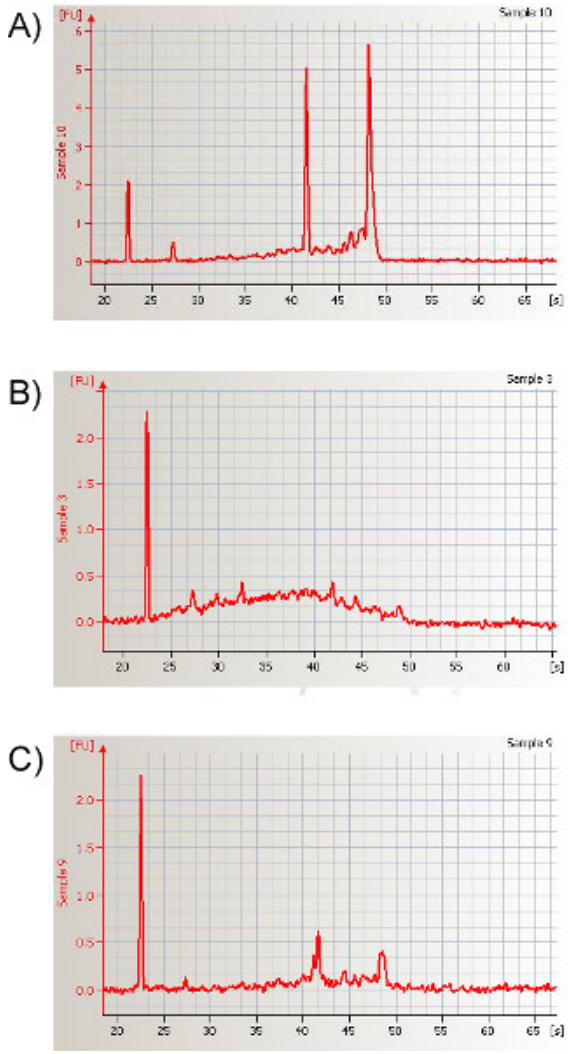

Figure 10. Representative electropherogram following electrophoresis automatic sequencing of a sample isolated from murine nasal lavages. The resulting electropherogram shows the quantitation data and the characteristic signature of a high quality total RNA sample derived from the nasopharyngeal region. When conducting analyses of total RNA, the areas under the RNA peaks for the two major ribosomal RNA, 18S and 28S, are used to calculate their corresponding ratio. Significant changes in the ratios of peaks attributable to 18S and 28S are typically indicative of degraded RNA. The degree of degradation can be summarized by RNA integrity number (RIN); the RIN for this representative sample is 8.1. An example of highly degraded RNA is shown in (**B**) and (**C**), and the subsequent RIN is 1.9 and 4.6, respectively. Click here to view larger figure.

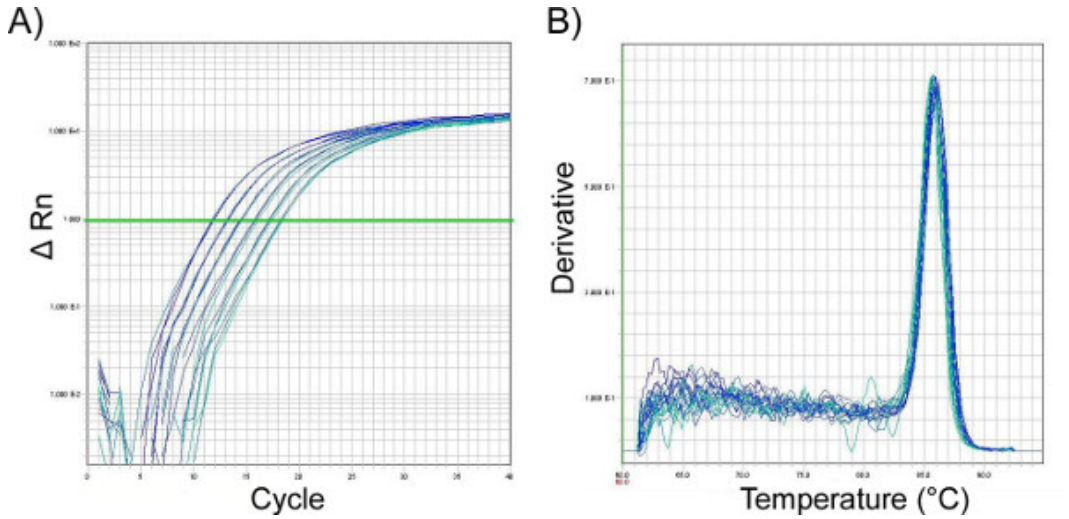

Figure 11. Amplification plot (A) and dissociation (melting) curve (B) from qPCR analysis of nasal wash cell lysates, providing an example of how these two readouts should typically look following an efficient and correctly detected amplification of mRNA products isolated from the murine nasopharynx. Represented is a standard curve for the housekeeping gene 18S. The results displayed in (**A**) show the desired PCR product following amplification using primers against GAPDH. The line represents the cycle threshold (Ct). The point at which the amplification plots corresponding to different samples cross this threshold allows for comparison across samples, with lower values corresponding to higher amounts of RNA of interest contained therein. The plot in (**B**) shows that the maximum melting temperature of the qPCR product is 85 °C and that there are no contaminating products present in this reaction, which would show up as an additional peak separate from the desired product peak. Click here to view larger figure.

Strain Name	Serotype	Virulence	Mortality in Mice	Expected Colonization Duration
P1121	23F	Asymptomatic	0%	21-28 days
P1542	4	Low	0-20%	21-28 days
P1547	6A	Mid	20-50%	14-21 days
D39	2	High	70-100%	14-21 days

Table 1. A tabular overview of 4 commonly employed *S. pneumoniae* clinical isolate strains, their corresponding serotype number, associated degree of virulence, expected proportion of in invasiveness within a colonized subset of mice and typical duration of a nasopharyngeal colonization.

Discussion

In this study we presented detailed methods for the intranasal colonization of mice using a clinical isolate strain of *Streptococcus pneumoniae* and the subsequent isolation and characterization of the immune cells recruited to nasopharynx in response to the bacteria. We demonstrated how a bacterial inoculum can be cultured in nutrient-rich media and used to establish a colonization event in mice, which is initially restricted to the nasopharynx. We then showed how responding immune cell types that are recruited to nasopharynx can be isolated following tracheal exposure, incision and a nasal lavage through the use of a cannulated needle. Nasal lavage samples can be collected in PBS to isolate intact, lightly adherent cells; the RNA from more tightly adherent cells and surrounding epithelial mucosal layer can be isolated by applying a secondary wash consisting of RNA lysis buffer. The former of these samples can then be used to phenotype the specific cells recruited in the context of the colonization via flow cytometry techniques, while the latter can be applied to Q-PCR analysis, to determine the effector functions of these recruited cells by looking at the transcriptional expression of immune regulators of interest. Nasal lavage samples can additionally be used to determine the kinetics of clearance of a bacterial colonization event comparing different experimental groups to address specific research questions.

Utilization of this method of intranasal colonization allows for the establishment of a colonization event that is initially limited to the nasopharynx of the animal. Any subsequent dissemination of the bacteria to the blood or organs therefore occurs secondary to breaches in the immune defences localized within the nasopharyngeal mucosa. The stepwise progression achieved through this model reflects more accurately the process of pneumoccocal invasion in humans, allowing one to study the dynamics between the colonizing bacteria and the host nasal mucosa - and perhaps better understand shifts in bacterial pathogenicity and/or host immunity that allow for the development of disseminating disease. This is in contrast to models that forgo the establishment of an initial colonization event and elect to study invasive disease in isolation through direct delivery of the bacterial inoculum to the lungs via intratrachael instillation, to the blood via vascular injection or to the peritoneum via peritoneal injection.

Conducting a PBS nasal lavage following a colonization event allows for isolation of non- or mildly-adherent cells recruited to the nasopharynx, as well as any mucosally-associated bacteria. It should be noted, however, that this technique is limited as it will not release cells or bacteria that have travelled between or beneath the epithelium, nor will it allow for the harvesting of cells or bacteria that have localized to the nasal-associated lymphoid tissue (NALT), a lymphoid organ that has been reported to be a potential site of infection following a pneumococcal

colonization[17,18]. If further study of the NALT is desired, we recommend microdissection and removal of this tissue wholesale for study following PBS nasal lavage; as these two techniques are not mutually exclusive, they may be conducted on the same animal. However, due to the lytic and destructive nature of the RNA harvesting step (the secondary lavage using RNA lysis buffer), this step should be omitted if intending to harvest the NALT. Although the nasal lavage is a less technically challenging procedure, for groups wishing to obtain a more comprehensive assessment of bacterial load that includes not only mucosally-associated bacteria, but also those that have invaded the nasopharyngeal tissue, we suggest harvesting the nasopharyngeal tissue following removal of the upper skull bone of colonized mice and dissection of the tissue within the nasal conchae, as described by others[19].

The nature of an elicited immune response is dependent on the interaction between host and pathogen. Over 90 serotypes of *S. pneumoniae* have been characterized to date, all with differing levels of pathogenicity and virulence factor expression, resulting in differential prevalence in the human population[20-23]. Similarly, in mice, it has been reported that the extent of, and kinetics associated with, the immune response elicited in response to a nasopharyngeal colonization is dependent on the colonizing strain itself[24]. Thus, selection of an appropriate strain to utilize for the establishment of a nasopharyngeal colonization is not a trivial matter, nor is selection of mouse genetic background. **Figure 8** provides sample data that depicts the kinetics of clearance of a nasopharyngeal colonization from 3 different *S. pneumoniae* strains following intranasal colonization of female mice on a C57BL/6 background. **Table 1** provides an overview of the degree of virulence and length of colonization time expected (when utilized on the C57BL/6 background) with 4 *S. pneumoniae* clinical isolate strains described in the literature and known to be capable of establishing a nasopharyngeal coloninization[25]: the avirulent P1121 (serotype 23F)[26,27] the low-virulence P1542 (serotype 4)[28], the mid-virulence P1547 (serotype 6A)[29-31], and the widely-used, well-characterized, highly virulent D39 (serotype 2)[32-36]. If the experimental goal is to strictly study an asymptomatic nasal colonization event with no accompanying bacterial dissemination to other tissues, we recommend use of the avirulent P1121 strain, which is characterized as a potent colonizer, as longer colonization events (up to 28 days prior to observed clearance) are a hallmark of this strain. Typically mice colonized with P1121 will run no risk of invasive disease and will display no clinical indicators of illness (with the exception of temporary weight loss). The remainder of the strains should be employed depending on desired degree of virulence and associated mortality, with virulence taken to mean not the degree of infection that develops within an individual mouse, but rather the proportion of mice that display clinical signs of illness. It should also be noted that typically, degree of virulence correlates inversely with length of colonization duration, with more virulent strains colonizing for a shorter period of time. All 3 of the described virulent strains lead to mortality in mice due to, most commonly, sepsis, with fulminant pneumonia, or concurrent pneumonia and sepsis developing in a subset of mice. The differences in localization of invasive bacteria may be strain-specific, as it has been previously reported that certain strains show tropisms for particular organs[37]. In a small percentage of animals, spontaneous meningitis may also develop following colonization. Determination of cause of death, as well as degree of invasiveness, can be accomplished via collection of associated tissues (lungs, spleen and/or brain) from animals at endpoint. Homogenization of these tissues and subsequent plating can indicate presence of invasive bacteria and corresponding titres.

An example of a bacterial culture density quantification is shown in **Figure 3**. If the culture is too concentrated, colonies grow too densely to be individually counted, however colonies derived from single cells can be distinguished if a log-wise dilution series is plated. Plating three technical replicates per dilution minimizes variability. Please note that when quantifying bacteria retrieved from a nasal colonization event, one may encounter cocultured contaminants, representing other bacterial species concurrently isolated from the murine nasopharynx. If the bacterial strain of interest has any known antibiotic resistances (for example, many strains of *S. pneumoniae* are resistant to gentamycin or neomycin up to 5 µg/ml), one can minimize the incidence of contaminants by supplementing the growth media with the antibiotic at an appropriate concentration, thereby limiting contaminant growth.

Flow cytometry can be used to analyze cell surface markers on nasal lavage samples. For example, for the analysis of cell types recruited in the context of an infection, a mix of antibodies specific for the gross differentiation of leukocytes, including macrophages ($F4/80^+$), neutrophils ($CD11b^+$ and $Ly6G^+$), and T-cells ($CD3^+$ and $CD4^+$ or $CD8^+$), can be used as previously published . Furthermore, these analyses can be combined with flow cytometric analysis conducted on different tissues or blood, to better understand immune cell trafficking during the course of an infection. Due to the limiting number of cells (typically numbering in the low thousands) that can be isolated from the nasopharynx, identifying rare subsets is typically challenging, although researchers wishing to accomplish this should consider pooling samples from multiple mice to achieve desired cell counts. Furthermore, because a finite number of cells can be extracted from this region, we recommend analyzing this data with regards to total cell numbers.

Although protein expression levels are typically low in the nasopharynx limiting the possibility of assaying protein production, it is possible to analyze the production of host molecules in response to the colonizing bacteria at the RNA level. To accomplish this, nasal lavages can be conducted using RNA lysis buffer in lieu of PBS, which allows for analysis of gene expression. For qPCR amplification detection, it is important to run a corresponding dissociation curve (**Figure 11**) to ensure the correct and desired product was detected. This is due to the fact that the assay will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer.

We hope the methods described here will encourage you to apply an intranasal colonization model to study host responses to pathogens important in the context of this understudied region. For certain human pathogens, such as *S. pneumoniae*, a preceding nasopharyngeal colonization event acts as an important precursor to ensuing bacterial dissemination and the fatal sequelae that may follow, including propagation into the lungs, which may lead to pneumonia, or else to the blood, and resultant bacteremia and septic shock. Thus, by studying bacterial colonization in this region, we may understand better how to control it and prevent more serious pathology from occurring altogether.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to thank Dr. Jeffery Weiser of the University of Pennsylvania for his gift of the clinical strains of *Streptococcus pneumoniae*. This work was funded by the Canadian Institutes for Health Research. CV was funded by a M. G. DeGroote fellowship and a fellowship from the Canadian Thoracic Society. This work was funded by the Ontario Lung Association and Canadian Institutes of Health

Research (CIHR). Work in the Bowdish laboratory is supported in part by he Michael G. DeGroote Centre for Infectious Disease Research and the McMaster Immunology Research Centre.

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