Non-Typeable *Haemophilus influenzae* Infection of the *Junbo* Mouse

Michael T. Cheeseman¹ and Derek W. Hood²

¹Developmental Biology Division, The Roslin Institute and Royal (Dick) School of Veterinary Studies University of Edinburgh, Easter Bush, United Kingdom
²Molecular Genetics Unit, MRC Harwell Institute, Harwell Science and Innovation Campus, Oxfordshire, United Kingdom

Acute otitis media, inflammation of the middle ear bulla, is the most common bacterial infection in children. For one of the principal otopathogens, non-typeable *Haemophilus influenzae* (NTHi), animal models allow us to investigate host-microbial interactions relevant to the onset and progression of infection and to study treatment of middle ear disease. We have established a robust model of NTHi middle ear infection in the *Junbo* mouse. Intranasal inoculation with NTHi produces high rates of bulla infection and high bacterial titers in bulla fluids; bacteria can also spread down the respiratory tract to the mouse lung. An innate immune response is detected in the bulla of *Junbo* mice following NTHi infection, and bacteria are maintained in some ears at least up to day 56 post-inoculation. The *Junbo*/NTHi infection model facilitates studies on bacterial pathogenesis and antimicrobial intervention regimens and vaccines for better treatment and prevention of NTHi middle ear infection. © 2017 by John Wiley & Sons, Inc.

Keywords: *Junbo* mouse • lung infection • middle ear infection • non-typeable *Haemophilus influenzae* (NTHi) • otitis media

**How to cite this article:**

Cheeseman, M.T. and Hood, D.W. 2017. Non-typeable *Haemophilus influenzae* infection of the *Junbo* mouse. *Curr. Protoc. Mouse Biol.* 7:29-46.
doi: 10.1002/cpmo.24

**INTRODUCTION**

Acute otitis media (AOM), inflammation of the middle ear bulla, is caused by bacterial infection. It is the most common bacterial infection in children and the major reason for antibiotic prescription in this age group. Otitis media with effusion (OME), otherwise known as glue ear, can be a sequel to AOM, and chronic OME is the most common cause of hearing impairment in children; grommet surgery to alleviate this condition is the most frequent pediatric surgery in Western countries. In the U.K., on average, at least one episode of AOM occurs in every child by the age of five. Of the three major otopathogens, non-typeable *Haemophilus influenzae* (NTHi) is emerging as the predominant bacterium associated with AOM. NTHi is part of the normal commensal flora present in the human nasopharynx (NP); no natural animal hosts other than man have been identified for NTHi and there are no inanimate reservoirs. The molecular basis of the processes that lead to contiguous spread of NTHi within the respiratory tract and subsequent disease in the middle ear has been difficult to establish, largely because animal models enabling study of all aspects of pathogenesis are not readily available.

Although other animal models have been reported for studies of AOM, mice have been utilized historically to elucidate virulence factors of otopathogens, mechanisms of
adherence and invasion, and induction and specificity of immune responses to pathogens such as NTHi. When considering cost, litter size, availability of immunological reagents, and control of host genetics through inbred and mutant host lines, the mouse presents substantial potential advantage over other animals for otitis media (OM) studies. Several genes associated with increased susceptibility to OM in the mouse have now been shown to be relevant to human disease through candidate gene studies performed using allelic association analyses in family-based cohorts (Rye et al., 2011). Genetic predisposition can be investigated by studying disease in multiple mouse lines that are each mutated in relevant genes that increase susceptibility to spontaneous or experimental infection of the middle ear. Mouse models can facilitate investigations of the molecular basis and pathophysiology of NTHi infection and provide a means to realize improved treatment and prevention of disease in humans. Here we describe a robust model of NTHi infection using the Junbo mouse; this focuses upon the study of AOM, but bacterial carriage and lung clearance can also be investigated.

Basic Protocol 1 describes the basis for establishing and monitoring middle ear infection in the Junbo mouse following intranasal inoculation of NTHi. Basic Protocol 2 describes the adaptation of Basic Protocol 1 to study protection against infection following immunization of the animals, and Basic Protocol 3 describes the use of antibiotic to clear NTHi. Basic Protocol 4 describes a modified infection procedure that favors residence of bacteria in the mouse lung.

NOTE: All protocols using live animals must first be reviewed and approved by the appropriate ethics process such as the Animal Welfare and Ethical Review Body (AWERB) in the U.K. or the Institutional Animal Care and Use Committee (IACUC) in the U.S., and must conform to local or national governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL 1

JUNBO MOUSE NTHi MIDDLE EAR INFECTION

Junbo mouse

Junbo is a mutant mouse line that spontaneously develops chronic middle ear (ME) inflammation under specific-pathogen-free (SPF) conditions at 4 to 5 weeks of age. The heterozygote Junbo mouse (Jbo/+), hereafter termed Junbo) bears an Asn763Ile mutation in the gene encoding the Evi1 transcription factor, otherwise known as Mecom (Parkinson et al., 2006). One mechanism that may underlie the predisposition to OM in Junbo mice is that Evi1 is a negative regulator of NFκB; the loss-of-function Evi1 Junbo mutation exacerbates NTHi-induced inflammation in the lung (Xu et al., 2012). The pre-existing ME inflammation in Junbo mice is critical for bacterial ME infection following intranasal inoculation; the healthy air-filled ME of wild-type mice and the small proportion of Junbo mice (5% to 10%) with no fluid present do not sustain infection by NTHi.

NTHi strains

Wild-type NTHi strains used by us for ME infection studies are all isolates from children with OM (Cody et al., 2003) and are phylogenetically distinct (De Chiara et al., 2014). Following infection in the mouse, indigenous commensal bacterial flora, particularly Proteus spp., render a proportion of bacterial count plates unreadable through overgrowth. For this reason, NTHi strains expressing spontaneous resistance to streptomycin (sr) or engineered resistance to kanamycin (kan) were generated (Hood et al., 2016). These strains enable antibiotic selection of NTHi during culture from mouse samples and counter-selection against any indigenous bacteria present; streptomycin selection proved particularly useful at maximizing quantitative NTHi culture data from mouse samples.
**Materials**

NTHi [stored at –80°C in BHI (see below)/20% (v/v) glycerol]: human OM disease isolates (162, 176, 375, 486, 1124 and 1158; available from MRC Harwell Institute) are used by us for mouse infection studies (Cody et al., 2003); streptomycin-resistant NTHi strains are designated sr, e.g., strain 162sr

Supplemented brain-heart infusion (sBHI) broth and agar plates (see recipe)

Mice (8 to 11 weeks of age): *Junbo* mice are congenic on a C3H/HeH background (Parkinson et al., 2006); mice are housed under SPF conditions and are mostly used at 8 to 11 typically used by us at 8 ± 1 or 11 ± 1 weeks of age with a similar experimental outcome [available from the European Mouse Mutant Archive (EM:00091) via MRC Harwell Institute; for non-academic groups, available through MRC Technology, U.K.]; for some studies, germ-free (GF) mice are used (details of the mouse husbandry and microbiological surveillance are found in Hood et al., 2016)

Sterile PBS/2% (w/v) gelatin (Fluka, cat. no. 48723) for inoculum preparation

Barbiturate solution (50% Euthatal) for intraperitoneal injection (delivered at 3.3 ml/kg body weight); alternatively a rising CO₂ concentration (Donovan and Brown, 2006b) may be used to euthanize mice

PBS for collecting and diluting in vivo samples

70% ethanol to sterilize the instruments between the sampling of ears

37°C, 5% CO₂ incubator

1-µl inoculating loop

Spectrophotometer for determining OD₄₉₀

Scissors for removal of the head

Binocular dissecting microscope with 10× magnification and LED stage lighting.

Fine forceps for puncture of the tympanic membrane

< 2-µl-volume pipet with sterile filtered micro-tip to collect the small volume of ME fluids

Additional reagents and equipment for assessing titer of bacteria (Elbing and Brent, 2002), isoflurane anesthesia of mice (Phoon and Turnbull, 2016), injection of mice (Donovan and Brown, 2006a), and euthanasia of mice by CO₂ asphyxiation (Donovan and Brown, 2006b)

**Preparation of mouse NTHi inoculum**

1. Grow NTHi overnight at 37°C in a 5% CO₂ atmosphere on sBHI agar. From this plate, inoculate sBHI broth with a 1-µl loop full of colony growth (to give an initial OD₄₉₀ of ~0.03 to 0.05) and grow bacteria to log phase (OD₄₉₀ 0.3 to 0.6) at 37°C in a shaking incubator.

2. Calculate the size of inoculum for the mouse from optical density (OD₄₉₀) using a conversion factor whereby an absorbance of 0.4 is equivalent to ~1 × 10⁹ cfu/ml.

3. Pellet bacteria by centrifugation for 3 min at 13,000 × g, room temperature, remove supernatant, and resuspend pellet in PBS/2% (w/v) gelatin to achieve 10¹⁰ cfu/ml.

4. Assess the titer of the inoculum by plating dilutions of each suspension onto sBHI agar plates (Elbing and Brent, 2002) prior to, and after, inoculation of the mice.

**Intranasal challenge**

5. Anesthetize mice aged either 8 ± 1 week or 11 ± 1 week with isoflurane (mixed with O₂ in anesthetic chamber; Phoon and Turnbull, 2016) and inoculate by applying 5 µl of bacterial suspension to each nostril via a pipet tip (Hood et al., 2016).

A typical inoculum is 10⁶ cfu, but a range of inoculum sizes from 10³ to 10⁶ cfu NTHi bacteria have been used in individual experiments with cohort sizes of n = 11 to 15 mice.
Using bacteria that emit light (expressing lux genes), we have shown that NTHi are distributed along the entire length of the NP within minutes of intranasal inoculation (Hood et al., 2016).

Terminal sampling of nasopharynx and bulla fluids

6. Euthanize the mouse (typically at 7 days post-inoculation) by intraperitoneal injection (Donovan and Brown, 2006a) of 50% Euthatal to deliver the drug at 3.3 ml/kg body weight (alternatively, use CO$_2$ asphyxiation as described in Donovan and Brown, 2006b), use scissors to remove and skin the head (Fig. 1), then remove the mandible. Sample the NP by washing with 200 µl PBS introduced into the NP opening on the palate and collecting the wash fluid from the nares into a microcentrifuge tube (Fig. 2).

Sampling the NP either before or after the ME has no statistically significant effect on the NP bacterial titers attained.

Avoid euthanasia by cervical dislocation of the mouse, as it causes hemorrhage in the upper airway that will compromise sample collection.

A more quantitative approach to determine NP NTHi titers is to dissect out a part (e.g., soft palate) and homogenize the tissue in PBS prior to dilution and plating. Although this typically yields higher bacterial titers than the wash alone, we find that there is a general positive correlation between the data from the two methods.

7. Under 10× binocular magnification, sample fluids from the ME bulla following perforation of the TM and removal of the ME conductive bones using sterile forceps (Fig. 2). Collect bulla fluids and estimate the volume using a 0- to 2-µl filtered pipet tip (average sampled volume, ~0.75 µl; range, 0.1 to 1.50 µl; Fig. 2). Transfer fluids into 500 µl of PBS in a microcentrifuge tube.

In a small proportion of Junbo mice with clear TM, there is no detectable bulla fluid. These bullae can be washed twice with 2 µl of sterile PBS and the washings added back to 500 µl of PBS for culture to monitor infection.

If required for gene- or protein-expression analysis, the primary bulla fluid preparation (suspended in PBS) is centrifuged 3 min at 13,000 × g, then the pellet and supernatant are frozen on dry ice prior to storage at −80°C.

Infection rate and bacterial titer

8. Disperse bulla fluid in PBS or NP washes by three 10-sec bursts on a vortex mixer, then make 10-fold dilutions ($10^{-1}$, $10^{-2}$, etc.) in PBS. Spread 50 µl of each ME dilution or undiluted NP wash on an sBHI agar plate (also see Elbing and Brent, 2002). Incubate agar plates overnight at 37°C and count viable colonies the next day (also described in Elbing and Brent, 2002).
The detection limit is 10 cfu/µl for the initial bulla fluid suspension and 100 cfu/µl for the 10^{-1} dilution. In experiments using antibiotic-resistant NTHi, samples are plated on sBHI agar plates supplemented with the appropriate antibiotic (e.g., 300 µg/ml streptomycin or 20 µg/ml kanamycin). If required, non-selective agar plates can be used in parallel to monitor the presence of commensal bacteria.

9. Calculate an infection rate for the middle ear:

Infection rate = number of NTHi positive bulla fluids/number of bulla fluids sampled.

As an example, if from a cohort of 12 mice (24 ears) there are two ears with no fluid and 22 bulla fluids that have been sampled, 18 of which give a positive NTHi culture on plates, then the ME infection rate is calculated as 18/22 or 81.8%.

10. Calculate a bacterial titer (cfu/µl ME fluid) from the number of colonies obtained on culture plates, adjusted for sample dilution and bulla fluid volume.

As an example, if 85 bacterial colonies were obtained on a culture plate spread with 50 µl of a 10^{-2} dilution made from 1 µl ME fluid dispersed into 500 µl PBS, this would correspond to an ME bacterial titer of 8.5 × 10^4 cfu/µl fluid.
NTHi has also been shown to infect the bullae of other mutant mouse lines that exhibit ME inflammation and OM (Hood et al., 2016); these include the Jeff (Hardisty-Hughes et al., 2006) and Tgif (Tateossian et al., 2013) mouse mutants. The protocol will similarly work for Streptococcus pneumoniae (pneumococcus) infection, although this has been tested by us only in the Junbo mouse. We note that Streptococcus pneumoniae strain D39 is highly virulent in Junbo mice. All new infection models should be established with less virulent bacterial strains, and pilot studies with small numbers of mice should be conducted first to establish whether there are unexpected adverse effects.

**SUPPORT PROTOCOL 1**

**JUNBO MOUSE NTHi MIDDLE EAR INFECTION: HISTOLOGY AND DISTRIBUTION OF NTHi**

Following infection of the *Junbo* mouse, the histology and the relative distribution of NTHi of the infected ME bullae can be examined by a number of techniques that include histology, immunohistochemistry (IHC), in situ hybridization (ISH), and lesion profiling. Typically, this is carried out using 11–week-old *Junbo* mice inoculated intranasally with 10^6 cfu NTHi. Heads are collected 7 days post-inoculation as described in Basic Protocol 1. Heads from non-NTHi challenged *Junbo* mice collected at the same time serve as negative controls.

**Materials**

- Dissected mouse heads (Basic Protocol 1)
- Xylene
- 10% neutral buffered formalin
- 100% ethanol
- Tris-buffered saline (TBS: see recipe)
- Dako REAL peroxidase blocker (code no. S2023)
- Dako proteinase K (code no. S3020)
- Primary antibody [e.g., rat monoclonal anti-F4/80 (Serotec, cat. no. MCA497G); rabbit polyclonal anti-caspase 3 (Abcam, cat. no. ab2302); rabbit polyclonal anti-histone 3 (Abcam, cat. no. ab61251)]
- ImmPress HRP anti-rat kit (Vector Labs, cat. no. MP-744-15)
- Dako antibody diluent (code no. S0809)
- Dako REAL peroxidase blocker (code no. S2023)
- Dako liquid DAB+ substrate chromogen system (code no. K3468)
- Harris hematoxylin (Sigma-Aldrich, cat. no. HH516)
- Dako Envision+ System HRP anti-rabbit antibody (code no. K4011)
- HRP Visualization Kit (Advanced Cell Diagnostics)
- ClearVue mountant (Thermo Fisher Scientific)
- Probe B-HInfluenzae-NTHi375-16SrRNA (Advanced Cell Diagnostics)
- Positive control for RNA integrity (e.g., PpiB; Advanced Cell Diagnostics)
- Negative hybridization control (e.g., DapB; Advanced Cell Diagnostics)
- Electrostatically charged slides (e.g., SuperFrost Plus; VWR Scientific)
- 60°C drying oven
- Slide scanner: e.g., Hamamatsu NanoZoomer
- Software for morphometric measurements (e.g., Hamamatsu NanoZoomer)

**Histology**

*To prepare hematoxylin and eosin–stained sections*

ME histology on infected and non-infected mice is assessed using 4-μm wax sections (Parkinson et al., 2006).

1. Fix skinned mouse heads for 48 hr in 10% neutral buffered formalin. Decalcify bone using EDTA, embed decalcified tissues in paraffin wax, then cut 4-μm dorsal plane sections before staining with hematoxylin and eosin, or special stains. Carry
out morphometric analysis and lesion profiling on standard 1000 µm lengths of ME mucosa as described below.

We note that Gram staining fails to identify NTHi in tissue sections, so IHC and ISH (see steps below) may be the best approaches to identify and localize bacteria.

Immunohistochemistry and in situ hybridization

2. Decalcify formalin-fixed tissues using EDTA dissected mouse heads can be decalcified in 5 to 7 days.

   To maximize RNA integrity for any in situ work, a band saw can be used to isolate the bullae from heads. This is done by first removing the jaw and the brain, then making cross-section cuts in the skull base, 2 mm rostral to the pituitary gland and 2 mm caudal to the opening of the external ear canal, to isolate the bullae. Skull base blocks are then fixed for 24 hr, and EDTA decalcification can be achieved in 48 hr.

3. Cut 4-µm thick wax sections onto electrostatically charged slides and dry overnight at 37°C before a final drying at 60°C for 25 min. De-wax sections in xylene, then hydrate by placing in 100% ethanol for three 2-min periods, then in distilled water for 2 min. Next, wash three times in Tris-buffered saline (TBS).

4. Detect target cells in the mouse ME fluid using antibodies against appropriate antigen markers.

   An illustrative example is using rat monoclonal anti-F4/80 (Serotec MCA497G) to detect mouse macrophages.

   a. Perform antigen retrieval using Dako proteinase K (S3020) for 20 min at room temperature as per the suppliers instructions, then wash in TBS for three 2-min periods.

   b. Incubate the primary antibody (diluted in Dako antibody diluent according to the suppliers instructions) with sections for 30 min at room temperature, then block endogenous peroxidase using Dako REAL peroxidase blocker for 10 min. Next, wash in TBS for three 2-min periods.

   c. Carry out secondary antibody detection using the Vector Labs ImmPress HRP anti-rat kit; include negative controls using the antibody diluent alone.

   d. Visualize the target using the Dako liquid DAB+ substrate chromogen system (K3468).

   e. Counterstain using Harris hematoxylin prior to dehydration with 100% ethanol for three 2-min periods, clearing in xylene, and mounting in ClearVue mountant.

5. Perform in situ hybridization on representative NTHi-challenged and non-challenged Junbo mice prepared as described above. As an illustrative example we use a probe that targets the 16SrRNA of the bacteria. Hybridize 4-µm wax sections with probe B-HInfluenzae-NTHi375-16SrRNA as per the manufacturer’s instructions using the HRP visualization reagents from Advanced Cell Diagnostics. Use a positive control for RNA integrity (e.g., PpiB) and a negative hybridization control (e.g., DapB) in experiments.

   ISH is a useful approach when antibodies against bacteria produce high background or suitable antibodies are not commercially available. We have found that mouse antisera from NTHi immunization and protection studies did not make useful reagents to localize bacteria in bulla sections. Another advantage of ISH is that there are a wide range of mouse probes available, making it potentially possible to obtain hybridization signals for bacteria and host cells in the same section.

   NTHi bacteria can also be visualized directly following infection of the Junbo mouse. NTHi strains expressing the lux genes emit bioluminescent signals that can be used to
monitor bacterial distribution in situ in the skinned heads of infected Junbo mice; bacteria are imaged using an IVIS Lumina II system (Perkin Elmer) (Hood et al., 2016). NTHi strains expressing green fluorescent protein (GFP) can be detected following infection by confocal microscopy on mouse bulla fluid and soft palate samples.

**Lesion profiling**

6. Acquire bright-field images of hematoxylin and eosin–stained sections using a slide scanner such as a Hamamatsu NanoZoomer and make the morphometric measurements using the associated software.

7. Assess the mucosal thickness and the numbers and size of capillary and lymphatic vessels present as required (Cheeseman et al., 2011).

8. Calculate the average thickness of the mucosa lining the medial surface of the bulla (avoiding the cochlea and the region close to the Eustachian tube) by dividing the area of mucosa overlying a delineated ~1000 µm length of supporting bulla bone.

9. Calculate the proportion of bulla space occupied by exudate by dividing the exudate area by the area bounded by bulla mucosa surface and the TM.

If comparing infected and non-infected ME then analyze slides in a blinded manner.

**SUPPORT PROTOCOL 2**

**IMMUNE RESPONSE MEASUREMENT BY REAL-TIME QUANTITATIVE PCR (RT-qPCR) OF BULLA FLUIDS**

Following NTHi infection of the Junbo mouse as described in Basic Protocol 1, the degree of the host immune response dependent upon microbial infection in the ME can be estimated by RT-qPCR determination of cytokine and chemokine mRNA levels.

**Additional Materials (also see Basic Protocol 1)**

- Germ-free Junbo mice (MRC Harwell Institute)
- RNase-free H₂O
- Nucleospin RNA/protein isolation kits (Macherey-Nagel)
- AB 7500 software v2.0.1
- Additional reagents and equipment for quantitative PCR (qPCR; Bookout et al., 2006; Cheeseman et al., 2011)

1. Inoculate SPF Junbo mice with 10⁶ cfu NTHi. Sample cohorts terminally at day 1, 3, 7, 10, or 14 post-inoculation; collect and process ME fluids as described in Basic Protocol 1.

2. Obtain cell pellets by centrifugation for 5 min at 13,000 × g, 4°C, of bulla fluids (n = 4 to 6) collected in PBS that yielded NTHi monocultures from the 10⁻¹ dilution when cultured; this is equivalent to <100 cfu/µl commensal bacteria present. Place pellets on dry ice and store at −80°C prior to RNA isolation. Obtain at least three biological replicate pools for each time point.

3. As a baseline control, collect bulla fluids (n = 4 to 6) from 8- to 10-week-old non-infected germ-free (GF) Junbo mice into 20 µl of RNase-free water for RNA isolation. Each GF pool of bulla fluids comprises at least three biological replicates as controls for each time point.

As an alternative to GF mice, measure the baseline control expression level using bulla fluids obtained from age-matched non-NTHi infected SPF Junbo mice.

4. Extract RNA from the re-suspended bulla fluid cell pellets using the Nucleospin RNA/protein isolation kit and carry out cDNA synthesis and RT-qPCR TaqMan as described by Cheeseman et al. (2011).
Typical immune-molecule genes to be monitored are those activated by bacterial ligands binding to TLR receptors that are expressed by macrophages, neutrophils, and monocytes, and that are known to be relevant to OM and AOM in man (Juhn et al., 2008; Kaur et al., 2015). Genes that we routinely monitor include Ccl3, Ccl4, Ccl5, IL-1b, IL-6, IL-12a, IL-17a, TNF-α, and VEGF-a (Hood et al., 2016). Perform RT-qPCR in technical triplicates. Normalize data using Hrpt1 and β-actin as the endogenous control and calculate fold changes of expression (ddCts) of NTHi-infected bulla fluid cells over non-infected bulla fluid cells using AB 7500 software v2.0.1 and express as mean relative quantification (RQ) ± min/max error bars representing 95% CI.

MOUSE IMMUNIZATION AND PROTECTION AGAINST NTHi INFECTION

In this procedure, mice are subcutaneously immunized with either whole killed bacteria or with derived and purified cell-wall fractions or selected antigens (Ercoli et al., 2015). Following a three-step immunization procedure, the mice are inoculated intranasally with live NTHi bacteria to assess if antibody raised in the mouse elicits protection against infection in the ME or carriage in the NP. Significant levels of protection when found compared to control animals can act as a surrogate that predicts the efficacy of an antigen combination for use as a vaccine in humans.

Materials

- 5-week-old SPF Junbo mice (MRC Harwell Institute; see Basic Protocol 1 materials list for more information)
- Live NTHi bacteria (Basic Protocol 1, steps 1 to 4) for intranasal challenge post-immunization
- Supplemented brain-heart infusion (sBHI) broth and agar plates (see recipe)
- Phosphate-buffered saline (PBS; see recipe) containing 0.08% (w/v) paraformaldehyde
- Phosphate-buffered saline (PBS; see recipe)
- Adjuvant (Adjuplex from Sigma)
- 1.1-ml Z-Gel spin columns (Sarstedt)
- Microscope slides
- Microscope with phase-contrast optics

Additional reagents and equipment for blood collection from mice (Donovan and Brown, 2006c), injection of mice (Donovan and Brown, 2006a), and intranasal inoculation and determination of ME infection rate and bacterial titer by terminal sampling (Basic Protocol 1)

1. Obtain pre-immune serum as a base-line control for antibody levels prior to the first immunization being carried out. To do this, obtain blood via tail-vein bleed (Donovan and Brown, 2006c). Prepare small volumes of sera using 1.1-ml Z-Gel spin columns according to the supplier’s instructions. Store serum at −80°C until required.

2. When using whole NTHi bacteria for mouse immunization, culture NTHi to mid-log phase (OD490 0.2 to 0.6) in sBHI broth. Pellet 1 ml of culture for 3 min at 13,000 × g, room temperature, then resuspend the pellet in 1 ml PBS, centrifuge 3 min at 13,000 × g, room temperature, and resuspend pellet in 1 ml PBS.

3. To kill the bacteria, pellet the washed suspension 3 min at 13,000 × g, room temperature, and remove the supernatant. Resuspend pellet in 500 µl of PBS/0.08% paraformaldehyde (PFA). Incubate the suspension for 1 hr at 37°C, then overnight at 4°C before culturing 20 µl of the neat bacterial suspension on sBHI agar (Elbing and Brent, 2002) to confirm loss of viability of bacteria.
4. Store killed NTHi in PBS/ PFA for up to 8 weeks at 4°C. Confirm bacterial integrity before each use by spreading 5 µl of the bacterial suspension on a microscope slide and examining by phase-contrast microscopy.

5. Prior to immunization, pellet the killed bacteria by centrifugation for 3 min at 13,000 × g, room temperature, remove the supernatant, and then re-suspend in PBS to achieve 10^10 cfu/ml before mixing with adjuvant according to the suppliers instructions.

   We typically use Adjuplex (Sigma) adjuvant at a 1:4 ratio with the bacterial suspension.

6. Immunize each Junbo mouse with three subcutaneous injections (Donovan and Brown, 2006a) in intra-scalpular skin of a mixture of 10^8 cfu killed bacteria and adjuvant in a 50-µl final volume.

   The first immunizations are at the age of 5 weeks, then subsequently at 8 weeks and 10 weeks of age.

7. Take a second intermediate blood sample by tail vein bleed (Donovan and Brown, 2006c) prior to the second immunization when the mice are at 8 weeks of age.

8. Inoculate immunized mice intranasally (see Basic Protocol 1, step 5) at 12 weeks with 10^6 cfu NTHi bacteria. Determine the ME infection rate and bacterial titer by terminal sampling at 7 days post-inoculation (mice at 13 weeks of age), as described above in Basic Protocol 1.

9. Obtain terminal blood samples at the end of the experiment by euthanizing the mouse (Basic Protocol 1, step 6) and performing a retro-orbital bleed (Donovan and Brown, 2006c) immediately after euthanasia. Incubate blood samples at room temperature for 2 hr, then obtain serum using 1.1-ml Z-Gel spin columns according to the supplier’s instructions. Store serum at −80ºC until required.

   In a typical protection experiment, cohorts of 12 to 15 mice are immunized with alternative NTHi strains, and then infected with either the homologous or heterologous bacteria. Control mice are immunized with PBS and adjuvant. When comparing the bulla infection rate and bacterial titers in PBS-immunized control mice with those of NTHi-immunized mice, any significant reduction in NTHi ME infection rate or titer in the NTHi-immunized mice will indicate protection dependent upon antibody produced in those vaccinated mice (Hood et al., 2016).

### ANTIMICROBIAL TREATMENT OF NTHI INFECTION

Antibiotic resistance is on the increase for otopathogens such as NTHi, and in the clinic this could soon have a major impact on treatment regimens for disease. The Junbo/NTHi infection model can be used to study antimicrobial treatment for AOM; as an example, we describe the procedure for oral administration of the clinically relevant antibiotic, azithromycin, for systemic treatment of NTHi-infected mice.

#### Materials

- 8-week old SPF Junbo mice (MRC Harwell Institute; see Basic Protocol 1 materials list for more information)
- NTHi bacteria for intranasal challenge as described in Basic Protocol 1
- Azithromycin (Sigma-Aldrich, cat. no. 57947) in 2% methoxycellulose (or other antibiotic in solution as appropriate)
- 2% methoxycellulose (Sigma-Aldrich, cat. no. M7140)
- Gavage needle

Additional reagents and equipment for intranasal inoculation and determination of ME infection rate and bacterial titer by terminal sampling (Basic Protocol 1)
1. Inoculate 8-week old *Junbo* mice intranasally with $10^6$ cfu of NTHi bacteria (Basic Protocol 1, steps 1 to 5).

2. At day 4 post intranasal inoculation, give mice a 3-day course of an antimicrobial; for example, 100 mg/kg of the antibiotic azithromycin in a 2% methoxycellulose solution delivered once a day by oral gavage.

3. As a control, use mice inoculated with NTHi at the same time as the treatment group of animals but gavaged with 2% methoxycellulose solution alone for 3 days.

4. Sample bulla fluids terminally on day 7 post intranasal inoculation and ascertain the number of bacteria by dilution and plating as described in Basic Protocol 1. If required, also determine the number of bacteria in the NP by sampling through a terminal NP wash (Basic Protocol 1).

5. Determine the efficacy of antibiotic treatment by the difference in ME bulla infection rates and bulla NTHi titers between the antimicrobial-treated and control mice.

*Importantly, in addition to its use for oral administration of antimicrobials, the Junbo/NTHi infection model has added value through its utility for investigating potential new application strategies specifically targeted at the ME; antimicrobials can potentially be applied directly on the TM and subsequently transferred into the ME bulla.*

*The outcome of the antimicrobial treatment can be ascertained for NTHi alone by plating mouse samples on the appropriate selective growth plate for the respective NTHi strain, or can include an indication of the effect on other general bacterial flora present by also plating samples on non-selective growth medium.*

**NTHi MOUSE PULMONARY INFECTION MODEL**

NTHi pulmonary infection has been used to investigate the host immune response in the *Junbo* mouse (Xu et al., 2012). To achieve lung infection, the mouse can be inoculated via the intratracheal route, but here we describe the more straightforward intranasal method adapted from that of Morey et al. (2013).

**Materials**

- NTHi bacteria for intranasal challenge (see Basic Protocol 1)
- 8-week-old SPF Junbo mice (see Basic Protocol 1 materials list for more information)
- Homogenizer (we use an IKA Ultra-Turax T25 operated in a Class II microbiological safety cabinet)
- Additional reagents and equipment for intranasal inoculation and determination of ME infection rate and bacterial titer by terminal sampling (Basic Protocol 1) and fixation, paraffin embedding, sectioning, and staining (Support Protocol 1)

1. Prior to infection, grow NTHi overnight on sBHI agar, then use to inoculate sBHI broth as described in Basic Protocol 1, step 1. Grow bacteria to log phase ($OD_{490}$ 0.3 to 0.6), pellet by centrifugation for 3 min at 13,000 × *g*, room temperature, remove the supernatant, and then resuspend to achieve $5 \times 10^9$ cfu/ml in PBS/2% gelatin.

2. Anesthetize mice aged 8 weeks with isoflurane (Phoon and Turnbull, 2016) and inoculate intranasally by applying 10 μl of bacterial suspension to each nostril ($10^8$ cfu in 20-μl total volume; see Basic Protocol 1, step 5).

3. Euthanize the mouse as described in Basic Protocol 1, typically at time points either 24 or 48 hr post-inoculation. Remove lungs aseptically, weigh individually, then homogenize in PBS to release bacteria from the tissue; we typically homogenize the tissue at a ratio of 1:10 (w/v) in PBS.
4. Plate serial 10-fold dilutions of the lung homogenate in PBS on sBHI agar plates, incubate overnight at 37°C, then determine the number of bacteria in the lung from the colony counts on plates, the dilution factor, and the lung homogenate volume (detection limit <10 cfu/ml) as described in Basic Protocol 1, steps 8 and 10.

5. To obtain a histopathology and lesion score, fix trachea and lungs overnight in 10% buffered formalin, embed in paraffin, obtain 4- to 6-µm thick sections, and stain with hematoxylin and eosin prior to examination by microscope. See Support Protocol 1 for these procedures.

These experiments are typically used to compare bacterial counts following infection between different wild-type strains or paired isogenic wild-type and mutant NTHi strains; the difference in bacterial count serves as an indicator of altered propensity for bacterial clearance in the lung.

**REAGENTS AND SOLUTIONS**

*Use sterile deionized, distilled water in all recipes.*

**Brain-heart infusion broth and agar plates, supplemented (sBHI)**

*For supplemented brain-heart infusion (sBHI) broth:*

Make up 37 g brain-heart infusion broth powder (Merck) to 1 liter with distilled water. Autoclave in glass bottles and store at room temperature for 2 to 3 months. Before use, supplement BHI by adding 2 µg/ml nicotinamide adenine dinucleotide (NAD) (from a 1 mg/ml stock solution) and 10 µg/ml hemin (from a 10 mg/ml stock solution).

*NAD and hemin are both growth requirements for NTHi. For antibiotic-resistant NTHi strains, antibiotics [e.g., streptomycin (300 µg/ml) or kanamycin (20 µg/ml)] are added to the growth medium as appropriate. Use sBHI within 24 hr.***

*For supplemented brain-heart infusion (sBHI) plates:*

Combine 37 g brain-heart infusion broth powder and water to 1 liter, then add agar (Oxoid) to 1% (w/v) before autoclaving. Cool medium to 50°C, add supplements as described above, then pour into sterile 9 cm petri dishes and allow to set; store plates at 4°C for up to 2 weeks.

**Phosphate-buffered saline (PBS)**

Purchase PBS powder (available from most laboratory suppliers) and prepare as per supplier’s instructions (a typical solution contains 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4). Sterilize and store at room temperature for up to 6 months.

**Tris-buffered saline (TBS)**

50 mM Tris·Cl, pH 7.6
150 mM sodium chloride
Store at room temperature for up to 6 months

**COMMENTARY**

**Background Information**

*Haemophilus influenzae* is a Gram-negative bacterium that is part of the normal flora present in the human NP but is also a frequent etiological agent of disease in humans. *H. influenzae* is divided into six typeable (a through f) or non-typeable (NTHi) forms based on the presence or absence of a polysaccharide capsule, respectively. A majority of healthy adults have upper airway colonization with *H. influenzae*, and the predominant strains (>98%) are NTHi. Residence in the NP enables *H. influenzae* to colonize and initiate infections in both the upper and lower respiratory tracts.
through contiguous spread; diseases caused by NTHi are of significant public health importance and include AOM and acute pneumonia in young children, and bronchopneumonia in patients with chronic pulmonary diseases such as cystic fibrosis and chronic obstructive pulmonary disease (COPD; Murphy, 2003). In the U.K., on average, at least one episode of AOM occurs in every child by the age of five, making it one of the most common reasons for antibiotic prescription in general practice. In the developing world, acute lower respiratory tract infections top the list of causes of death in young infants, of which about 20% are attributable to NTHi (Shann et al., 1984). For adults, in 2013, COPD was the third leading cause of death in the U.S.A. and considered to be the third leading cause worldwide. Following the implementation of type b capsular conjugate vaccines, invasive (bacteremic) H. influenzae infections have declined in frequency, although NTHi is an emerging and significant cause of bacteremia and meningitis.

The three major human otopathogens, NTHi, Streptococcus pneumoniae (pneumococcus), and Moraxella catarrhalis, are all commensal bacteria commonly found in the human NP. The NP serves as a reservoir for respiratory tract infection. Each bacterium can spread contiguously. In addition to being responsible for OM, they are also commonly found in the sputum of COPD patients with acute and recurrent exacerbations (Sethi and Murphy, 2001). Thus, the increasing use of pneumococcal vaccines in children is resulting in NTHi becoming the predominant cause of bacterial respiratory infections, including AOM.

Some modifications used for NTHi infection in other mouse models

The presence of fluid in the Junho mouse ME facilitates translocation of NTHi to the ear via a natural route (Eustachian tube) following intranasal inoculation. In other NTHi mouse AOM models, live or heat-killed NTHi bacteria are introduced directly into the ME bulla; this can be achieved via direct injection through the tympanic membrane (Woo et al., 2014); alternatively, an incision is made in the mouse neck to expose the bulla bone, through which the inoculum is injected (Yao et al., 2014). The size of the NTHi inoculum can be adjusted depending upon the mouse line used and the nature of the investigation undertaken. Direct-inoculation mouse OM models have been used to investigate potential treatment for the disease; an example is human β-defensin 2, expressed in the ME following introduction via an adenoviral vector (Woo et al., 2015). In a modified co-infection AOM model, mice can be inoculated intranasally with influenza A virus, then 3 days later challenged intranasally with NTHi; this results in significant bacterial infection of both the mouse ME and NP (Langereis et al., 2012).

Following intranasal inoculation, the distribution of NTHi in the mouse NP can be monitored; in a typical mouse line this can only be achieved reliably for the first 24 hr before bacteria are cleared. The carriage of NTHi in the mouse NP following intranasal inoculation has been used to investigate the competitive index for colonization between two NTHi strains, and has also been used to study the efficacy of mouse immunization procedures.

NTHi are commonly introduced into the mouse lung to investigate host and bacterial factors relevant to the pathogenesis associated with COPD; this model is an important resource to study in vivo the immune mechanisms and regulation that respond to NTHi infection. Several methods can be used to alter the pathophysiology of the mouse lung to be more like that found in COPD patients. These include pre-exposure of mice to cigarette smoke (Roos et al., 2015), a treatment carried out for 4 to 30 weeks prior to introduction of NTHi, and treatment of the lung with modifying molecules such as the enzyme elastase (Pang et al., 2008). Intra-tracheal inoculation is a less commonly used route to deliver NTHi than the intranasal route, but can provide higher bacterial doses more directly to the target tissue. Bacterial numbers, lung immunopathology, and the host response over time (typically up to 48 hr post-inoculation) can be studied by utilizing mutant mouse lines that are altered in specific immune genes; this allows host-microbial interactions important for the progression and persistence of lung disease to be teased out (Roos et al., 2015). The expression pattern of both bacterial and host genes in response to NTHi infection of the lung can be studied from broncho-alveolar lavage (BAL) fluids and homogenized lung material. The lung infection model can also be used to test potential antimicrobial regimens to alleviate disease (Euba et al., 2015a) and the effectiveness of vaccination to prevent it (Lugade et al., 2014); again, the focus is usually upon treatment of NTHi-associated exacerbations of COPD. NTHi clearance in the mouse lung can be delayed when mice are first infected by rhinovirus and then subsequently...
superinfected with NTHi (Unger et al., 2012). A modified mouse model has also been used to investigate the relationship between NTHi infection, COPD, and lung cancer (Chang et al., 2014).

**NTHi infection in other animal OM models**

Other than the mouse, several animal models have been reported for studies on OM including the chinchilla (*Chinchilla lanigera*; Bakaletz, 2009) and the rat (Clark et al., 2000). The chinchilla has taken a lead in studies of NTHi pathogenesis because of the ease of access to the middle ear bullae for infection and sampling. However, translocation to the ear from the NP is difficult to achieve in this model unless barotrauma or concomitant viral infection procedures are employed. When considering cost, litter size, availability of immunological reagents, and control of host genetics through inbred and mutant host lines, the mouse presents substantial potential advantages for OM studies.

**Critical Parameters and Troubleshooting**

**Middle ear infection**

Following intranasal inoculation, NTHi bacteria transfer rapidly along the NP and can access the ME space of the *Junbo* mouse within 1 hr (Hood et al., 2016). After day 4 post-inoculation, there is a strong positive correlation between the presence of NTHi in both the ME and the NP of the *Junbo* mouse (Hood et al., 2016); this suggests that the ME can act as a reservoir for NP re-infection, or vice versa. The hypoxic inflamed ME in *Junbo* mice (Cheeseman et al., 2011) may favor the growth of microaerophilic bacteria such as NTHi.

Normal mouse microbial flora are also present in bulla fluid of the *Junbo* mouse after 5 weeks of age. In experiments using non-antibiotic-resistant NTHi strains, bulla cultures giving *Proteus* overgrowth on the culture plate can prevent detection of NTHi colonies; these are not included in the calculations for infection rates and titers.

**Protection experiments**

Mice can be immunized with whole bacteria, bacterial lysates, outer membrane vesicles (OMVs), single purified antigens, or any combination thereof. To test the effectiveness of the antibody response in the mouse following immunization, serum obtained from immunized mice can be utilized in in vitro assays of bacterial killing to ascertain and compare the effectiveness of immunization between individual animals. The serum bactericidal (Ercoli et al., 2015) and opsonophagocytosis assays measure the effectiveness of the antibody raised in bacterial killing by complement and phagocytes respectively. These assays are key for determining the effectiveness and functional outcome of an immunization regime in the mouse and provide useful data that can be extrapolated to predict the effectiveness of vaccination in man with the same antigens.

**Pulmonary infection**

Intra-tracheal rather than intranasal inoculation of NTHi can be used to maximize the lung infection dose achieved. Intra-tracheal inoculation has been used to establish chronic NTHi lung infection by repeated dosing (e.g., twice a week for 8 weeks; Lugade et al., 2014). As an alternative to homogenizing lung tissue, BAL fluids collected from the NTHi-infected mouse lungs can be used to investigate bacterial numbers and the mouse lung inflammatory response to infection. Immune cells present in the BAL can be isolated and examined, and RNA prepared for differential gene expression analysis.

**Anticipated Results**

**Junbo mouse infection**

Typical infection rates for NTHi OM strains in the *Junbo* mouse range from 40% to 90% ME infection, with titers of $10^4$ to $10^8$ cfu/µl attained at 7 days post-inoculation. At the same time point, semi-quantitative recovery of NTHi in NP washes typically yields bacterial titers of $10^1$ to $10^2$ cfu in a 200-µl wash volume. During longitudinal studies with our most tested NTHi strain, 162sr, ME infection rates peak to 80% to 90% at day 7 to 14 post-inoculation and decrease to around 20% by day 35, then are maintained at this rate up to the maximum length of time testing, 56 days (Hood et al., 2016). The ME infection rates and titers are not significantly different when inoculum doses in the range of $10^4$ to $10^8$ cfu are used for NTHi strain 162 (Hood et al., 2016).

Bulla fluids and dissected soft palate tissue obtained from the NP of mice 7 days post-inoculation with NTHi 375gfp reveal the presence of individual and small aggregates of bacteria, but no evidence of significant microcolony growth or large bacterial aggregates consistent with a mature NTHi biofilm being present in the ME of these mice (at this time point).
The histology of the ME in 12-week-old Junbo mice is generally similar between NTHi-challenged and non-challenged animals at day 7 post intranasal inoculation (Hood et al., 2016). 60% of the bulla is occupied by neutrophils and foamy macrophages, and the average thickness of middle ear mucosa is 100 to 111 µm in NTHi-challenged and non-challenged mice, respectively. Typically in the ME bulla, a necrotic caseous core of neutrophils is surrounded by viable and apoptotic neutrophils (cleaved caspase 3 positive) and an outer, variably thick, band of foamy macrophages (F 4/80 positive). Variable amounts of amorphous extracellular chro-matin exist within the caseous areas.

When localizing NTHi bacteria in the bulla using in situ hybridization targeting the 16S rRNA of NTHi, we found strong signals in the bulla exudate, but not elsewhere in head tissues, from Junbo mouse challenged with NTHi, but not in non-challenged mice. NTHi hybridization signals comprise punctate or larger aggregates scattered throughout the ME bulla exudate, but less frequently in the caseous core (Hood et al., 2016). Again, evidence consistent with the presence of significant or mature biofilm was not found.

Following infection intranasally with 10⁵ cfu NTHi bacteria, we found that chemokine/cytokine levels were generally enhanced in the Junbo mouse ME over the period tested of 1 to 14 days post-inoculation, when compared to GF control animals; IL-17α, TNF-α, Ccl3, and Ccl14 demonstrated the highest relative up-regulation (Hood et al., 2016).

Using the Junbo infection model, we have shown, by comparing isogenic wild-type and mutant bacterial strains, that the major NTHi membrane lipoprotein P4 is important for maintaining high bacterial loads during middle ear infection of the mouse (Su et al., 2016).

**Protection experiments**

When Junbo mice are immunized with one of three NTHi strains (162, 176, 375) and then are each infected with NTHi 162sr, significant protection was found (15% infection rate for immunized versus 81% infection rate for control) for mice immunized with the homologous, but not with the heterologous, NTHi strains (Hood et al., 2016). For mice that were immunized with heterologous NTHi strains (176, 375), bacterial titers attained in the middle ear were approximately one log₁₀ lower than those found in control (PBS)–immunized animals. Thus, there is discrimination in the model against homologous and heterologous NTHi challenge.

**Antibiotic treatment**

NTHi-infected mice treated with a 3-day course of azithromycin starting at day 4 post-inoculation had eliminated all NTHi from their middle ears when sampled post treatment (0% ME infection rate and NTHi titer beneath detection limit of < 10² cfu/µl in treated mice), whereas > 80% of non-antibiotic control treated ears were infected at titers of 10⁴ to 10⁵ cfu/µl bulla fluid.

**Lung infection**

Typical lung infection titers achieved for NTHi by this method are ~10⁴ cfu/lung at 24 hr and 10² cfu/lung at 48 hr post-inoculation. When the lung of the NTHi-infected Junbo mouse is monitored at up to 24 hr post-inoculation, mRNA levels of NF-κB regulated pro-inflammatory cytokines, such as TNF-α, IL-1β, and MIP-2, are markedly increased compared to the lung of the wild-type littermate mouse (Xu et al., 2012). Correspondingly, the histopathology of the NTHi-infected Junbo mouse lung shows enhanced leukocyte infiltration and neutrophil activity when compared to the wild-type mouse. Lesions in the lungs can be scored subjectively depending upon the observed percentage of tissue affected, the epithelial changes noted, the degree of inflammatory cell infiltration, and the nature of the exudate present—for example, a score of 0 to 3 where 0 = absent, 1 = mild, 2 = moderate, and 3 = severe (Morey et al., 2013).

A mouse pulmonary infection model has been used to demonstrate in vivo efficacy of host-directed antimicrobial drugs against NTHi lung infection (Euba et al., 2015a) and the role of NTHi membrane proteins P5 and Hap in NTHi virulence (Euba et al., 2015b).

**Time Considerations**

**NTHi infection of Junbo mouse**

The time for the combined bacterial preparation and inoculation procedures, relevant to a cohort of 12 mice being utilized in Basic Protocols 1 to 4, is between 4 and 5 hr, having previously cultured the NTHi strain overnight on plates. Typical times taken are 2.5 to 3 hr for bacterial culture in liquid, 30 min to prepare the inoculum, and 60 min to inoculate the mice. Our standard infection period for NTHi in the Junbo mouse is 7 days post-inoculation. Terminal sampling of the mouse NP and ME and plating of bacteria from a cohort of 12 mice
takes ~3 hr; culture plates must then be incubated overnight before counting colonies to ascertain bacterial titers.

**Mouse immunization and protection experiment**

A typical three-step immunization procedure covers a 7-week period, followed immediately by a 7-day infection period with the test bacteria, making a total of 8 weeks for each experiment. At each step, immunization of a cohort of 12 mice takes up to 30 min depending upon experience. Obtaining blood samples by retro-orbital bleed will add up to 30 min to the time taken for terminal sampling following bacterial infection. The processing of blood to obtain serum samples takes a further 2.5 hr.

**Antibiotic treatment of NTHi infection**

The protocol for NTHi infection of the Junbo mouse, followed by a three-dose oral administration of antibiotic, spans a 7-day period. Oral gavage takes ~30 min for a cohort of 12 mice.

**NTHi mouse pulmonary infection**

Following the standard NTHi intranasal inoculation procedure, the infection experiment is typically run for a 24- or 48-hr time period prior to terminal sampling of bacteria from the animals. Terminal sampling of bacteria from mouse lung homogenate takes ~3 hr.

**Acknowledgments**

This work was supported by the Medical Research Council U.K. (MC_EX_MR/K014986/1 and MC_U142684175). M.T.C. is supported by a BBSRC Institute Strategic Programme Grant (BB/J004227/1) to the Roslin Institute.

**Conflicts of Interest**

The authors have no conflicts of interest to report.
lower airways. *PLoS ONE* 10:e0123154. doi: 10.1371/journal.pone.0123154.

Hardisty-Hughes, R.E., Tateossian, H., Morse, S.A., Romero, M.R., Middleton, A., Tymowska-Lalanne, Z., Hunter, A.J., Cheeseman, M., and Brown, S.D. 2006. A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. *Hum. Mol. Genet.* 15:3273-3279. doi: 10.1093/hmg/ddi403.

Hood, D., Moxon, R., Purnell, T., Richter, C., Williams, D., Azar, A., Crompton, M., Wells, S., Fray, M., Brown, S.D., and Cheeseman, M.T. 2016. A new model for non-typeable Haemophilus influenzae middle ear infection in the Junbo mutant mouse. *Dis. Model. Mech.* 9:69-79. doi: 10.1242/dmm.021659.

Juhn, S.K., Jung, M.K., Hoffman, M.D., Drew, B.R., Preciado, D.A., Sausen, N.J., Jung, T.T., Kim, B.H., Park, S.Y., Lin, J., Ondrey, F.G., Mains, D.R., and Huang, T. 2008. The role of inflammatory mediators in the pathogenesis of otitis media and sequelae. *Clin. Exp. Otorhinolaryngol.* 1:117-138. doi: 10.3342/ceo.2008.1.3.117.

Kaur, R., Casey, J., and Pichichero, M. 2015. Cytokine, chemokine, and Toll-like receptor expression in middle ear fluids of children with acute otitis media. *The Laryngoscope* 125:E394-44. doi: 10.1002/lary.24920.

Langereis, J.D., Stol, K., Schweda, E.K., Tweldeer, B., Bootsma, H.J., de Vries, S.P., Burghout, P., Diavatopoulos, D.A., and Hermans, P.W. 2012. Modified lipooligosaccharide structure protects nontypeable Haemophilus influenzae from IgM-mediated complement killing in experimental otitis media. *mbio* 3:e00079-00012. doi: 10.1128/mBio.00079-12.

Lugade, A.A., Bogner, P.N., Thatcher, T.H., Sime, P.J., Phipps, R.P., and Thanavala, Y. 2014. Cigarette smoke exposure exacerbates lung inflammation and compromises immunity to bacterial infection. *J. Immunol.* 192:5226-5235. doi: 10.4049/jimmunol.1302584.

Morey, P., Viadas, C., Euba, B., Hood, D.W., Barberan, M., Gil, C., Grillo, M.J., Bengoechea, J.A., and Garmentia, J. 2013. Relative contributions of lipooligosaccharide inner and outer core modifications to nontypeable Haemophilus influenzae pathogenesis. * Infect. Immun.* 81:4100-4111. doi: 10.1128/IAI.00492-13.

Murphy, T.F. 2003. Respiratory infections caused by non-typeable Haemophilus influenzae. *Curr. Opin. Infect. Dis.* 16:129-134. doi: 10.1097/00001432-200304000-00009.

Pang, B., Hong, W., West-Barnett, S.L., Kock, N.D., and Swords, W.E. 2008. Diminished ICAM-1 expression and impaired pulmonary clearance of nontypeable Haemophilus influenzae in a mouse model of chronic obstructive pulmonary disease/emphysema. * Infect. Immun.* 76:4959-4967. doi: 10.1128/IAI.00664-08.

Parker, A., Chessum, L., Mburu, P., Sanderson, J., and Bowl, M.R. 2016. Light and electron microscopy methods for examination of cochlear morphology in mouse models of deafness. *Curr. Protoc. Mouse Biol.* 6:272-306. doi: 10.1002/cpmo.10.

Parkinson, N., Hardisty-Hughes, R.E., Tateossian, H., Tsai, H.T., Brooker, D., Morse, S., Lalane, Z., MacKenzie, F., Fray, M., Gレン, S., Pooley, S., Barbaric, I., Dear, N., Hough, T.A., Hunter, A.J., Cheeseman, M.T., and Brown, S.D. 2006. Mutation at the Evi1 locus in Junbo mice causes susceptibility to otitis media. *PLoS Genet.* 2:e149. doi: 10.1371/journal.pgen.0020149.

Phoon, C.K.L. and Turnbull, D.H. 2016. Cardiovascular imaging in mice. *Curr. Protoc. Mouse Biol.* 6:15-38. doi: 10.1002/9780470942390.mo150122

Roos, A.B., Sethi, S., Nikota, J., Wrona, C.T., Dorrington, M.G., Sanden, C., Bauer, C.M., Shen, P., Bowdish, D., Stevenson, C.S., Erjefalt, J.S., and Stampfl, M.R. 2015. IL-17A and the promotion of neutrophilia in acute exacerbation of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care. Med.* 192:428-437. doi: 10.1164/rccm.201409-1689OC.

Rye, M.S., Bhutta, M.F., Cheeseman, M.T., Burgner, D., Blackwell, J.M., Brown, S.D., and Jamieson, S.E. 2011. Unraveling the genetics of otitis media: From mouse to human and back again. *Mamm. Genome.* 22:66-82. doi: 10.1007/s00335-010-9295-1.

Sethi, S. and Murphy, T.F. 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: A state-of-the-art review. *Clin. Microbiol Rev.* 14:336-363. doi: 10.1128/CMR.14.2.336-363.2001.

Shann, F., Hart, K., and Thomas, D. 1984. Acute lower respiratory tract infections in children: Possible criteria for selection of patients for antibiotic therapy and hospital admission. *Bull. World Health Organ.* 62:749-753.

Su, Y.C., Mukherjee, O., Singh, B., Hallgren, O., Westergren-Thorsson, G., Hood, D., and Riesbeck, K. 2016. Haemophilus influenzae P4 interacts with extracellular matrix proteins promoting adhesion and serum resistance. *J. Infect. Dis.* 213:314-323. doi: 10.1093/infdis/jiv374.

Tateossian, H., Morse, S., Parker, A., Mburu, P., Warr, N., Acevedo-Arozena, A., Cheeseman, M., Wells, S., and Brown, S.D. 2013. Otitis media in the Tgif knockout mouse implicates TGF-beta signalling in chronic middle ear inflammation. *Hum. Mol. Genet.* 22:2553-2565. doi: 10.1093/hmg/ddt103.

Unger, B.L., Faris, A.N., Ganesan, S., Comstock, A.T., Hershenson, M.B., and Sajjan, U.S. 2012. Rhinovirus attenuates non-typeable Hemophilus influenzae–stimulated IL-8 responses via TLR2-dependent degradation of IRAK-1. *PLoS Pathog.* 8:e1002969. doi: 10.1371/journal.ppat.1002969.

Woo, J.I., Oh, S., Webster, P., Lee, Y.J., Lim, D.J., and Moon, S.K. 2014. NOD2/RICK-dependent beta-defensin 2 regulation is protective for nontypeable Haemophilus influenzae-induced middle ear infection. *PLoS ONE* 9:e90933. doi: 10.1371/journal.pone.0090933.
Woo, J.I., Kil, S.H., Brough, D.E., Lee, Y.J., Lim, D.J., and Moon, S.K. 2015. Therapeutic potential of adenovirus-mediated delivery of beta-defensin 2 for experimental otitis media. *Innate Immun.* 21:215-224. doi: 10.1177/1753425914534002.

Xu, X., Woo, C.H., Steere, R.R., Lee, B.C., Huang, Y., Wu, J., Pang, J., Lim, J.H., Xu, H., Zhang, W., Konduru, A.S., Yan, C., Cheeseman, M.T., Brown, S.D., and Li, J.D. 2012. EVI1 acts as an inducible negative-feedback regulator of NF-kappaB by inhibiting p65 acetylation. *J. Immunol.* 188:6371-6380. doi: 10.4049/jimmunol.1103527.

Yao, W., Frie, M., Pan, J., Pak, K., Webster, N., Wasserman, S.I., and Ryan, A.F. 2014. C-Jun N-terminal kinase (JNK) isoforms play differing roles in otitis media. *BMC Immunol.* 15:46. doi: 10.1186/s12865-014-0046-z.