Development of a Novel Preclinical Model of Pneumococcal Pneumonia in Nonhuman Primates

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Abstract

Pneumococcal pneumonia is a leading cause of bacterial infection and death worldwide. Current diagnostic tests for detecting *Streptococcus pneumoniae* can be unreliable and can mislead clinical decision-making and treatment. To address this concern, we developed a preclinical model of pneumococcal pneumonia in nonhuman primates useful for identifying novel biomarkers, diagnostic tests, and therapies for human *S. pneumoniae* infection. Adult colony-bred baboons ($n = 15$) were infected with escalating doses of *S. pneumoniae* (Serotype 19A-7). We characterized the pathophysiological and serological profiles of healthy and infected animals over 7 days. Pneumonia was prospectively defined by the presence of three criteria: (1) change in white blood cell count, (2) isolation of *S. pneumoniae* from bronchoalveolar lavage fluid (BALF) or blood, and (3) concurrent signs/symptoms of infection. Animals given 10^9 CFU consistently met our definition and developed a phenotype of tachypnea, tachycardia, fever, hypoxemia, and radiographic lobar infiltrates at 48 hours. BALF and plasma cytokines, including granulocyte colony-stimulating factor, IL-6, IL-10, and IL-1ra, peaked at 24 to 48 hours. At necropsy, there was lobar consolidation with frequent pleural involvement. Lung histopathology showed alveolar edema and macrophage influx in areas of organizing pneumonia. Hierarchical clustering of peripheral blood RNA data at 48 hours correctly

identified animals with and without pneumonia. Dose-dependent inoculation of baboons with *S. pneumoniae* produces a host response ranging from spontaneous clearance (10^6 CFU) to severe pneumonia (10^9 CFU). Selected BALF and plasma cytokine levels and RNA profiles were associated with severe pneumonia and may provide clinically useful parameters after validation.

Keywords: cytokines; biological markers; gene expression; *Streptococcus pneumoniae*; sepsis

Clinical Relevance

Previous work in *Streptococcus pneumoniae* pneumonia in laboratory animals has had limited applicability to humans, and current diagnostic tests for this disease lack sensitivity and specificity. A new model of *S. pneumoniae* pneumonia in baboons is reported that closely resembles the human disease. Animals with lobar pneumonia display a unique combination of pathophysiological, cytokine, and genomic responses compared with control animals that may provide improved diagnostic accuracy in patients with pneumococcal pneumonia.

Streptococcus pneumoniae is a bacterial pathogen that worldwide causes up to 11% of the deaths in children under 5 years of

age (1). In the United States, hospitalizations for pneumococcal pneumonia are expected to double by 2040,

increasing healthcare service cost by \$2.5 billion annually (2). The early diagnosis of pneumococcal pneumonia is paramount to

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treatment success and has improved with pneumococcal urinary antigen testing. Despite this, up to 30% of cases are missed (3). Urinary antigen testing can also be misleading in children due to high false-positive rates from nasopharyngeal carriage or recent exposure to pneumococcal vaccination (4).

The promise of proteomic and genomic technologies for the development of more accurate and reliable tests to diagnose infections, as exemplified by influenza, has not been realized for pneumococcal pneumonia (5–7). To this end, we sought to develop a nonhuman primate model of pneumococcal pneumonia to begin to characterize potential clinically relevant molecular profiles. Early work in nonhuman primates on pneumococcal pneumonia (8, 9) has been limited by small numbers and by variable clinical and microbiological data. Here, we chose to study *S. pneumoniae* inoculation in baboons, which are susceptible to human respiratory pathogens, such as *Mycobacterium tuberculosis* (10, 11), respond to bacterial infection like the human (12–16) and unlike the rodent (17), and whose lungs closely resemble the human lung anatomically and physiologically (18). Baboons and other nonhuman primates also exhibit immune responses to bacterial infections similar to the human and have a long history in vaccine development, including for *S. pneumoniae* (19–21).

Given the rising burden of *S. pneumoniae* disease and the suboptimal performance of current diagnostic tests, we have investigated the feasibility of developing molecular biomarker profiles unique to pneumococcal pneumonia in adult colony-bred baboons. Some of our results have been reported in abstract form (22).

Materials and Methods

Animal Model

Fifteen adult, male, colony-bred baboons (*Papio cynocephalus*) purchased from Texas Biomedical Research Institute (San Antonio, TX) were housed in the Duke University Vivarium (Durham, NC) and handled in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. The experimental protocol was approved by the Duke

University Institutional Animal Care and Use Committee. On Day 1, the animals were sedated, intubated, and ventilated mechanically. A baseline bronchoalveolar lavage (BAL) was performed in the left lower lobe with 20 ml 0.9% NaCl using a fiberoptic bronchoscope (Pentax, Montvale, NJ). The baboons were then randomly assigned to receive saline vehicle ($n = 4$) or *Streptococcus pneumoniae* (Serotype 19A-7; ATCC, Manassas, VA) in log increments of 10^6 ($n = 1$), 10^7 ($n = 1$), 10^8 ($n = 3$), or 10^9 ($n = 6$) CFU. The bacterial suspension was divided and instilled equally between the left lower lobe and lingula. After 6 hours, the animals were recovered, extubated, and placed in isolation.

At 24 hours, the animals were sedated briefly to collect blood samples. At 48 hours, the animals were again sedated, intubated, and ventilated. A repeat BAL was performed, and the animals were extubated and returned to isolation. After the 48-hour samples were obtained, ceftriaxone (Hospira Inc., Lake Forest, IL) was administered once daily for 3 days. At 168 hours, the animals were deeply sedated and killed with 20 ml intravenous saturated KCl solution, followed by immediate necropsy.

Sample Collection

Blood sample, nasopharyngeal swab (NPS), heart rate (HR), temperature, and blood pressure (MAP) data were collected at 0, 6, 24, 48, and 168 hours. Urine was collected at the same times except at 24 hours. Ventrodorsal chest X-ray (CXR) and BAL were obtained at 0, 48, and 168 hours. We also recorded respiratory rate (RR), cough, rhinorrhea, oral intake, and activity level before sedation at 0, 24, 48, 72, 96, and 168 hours.

Definition of Pneumonia

The diagnosis of pneumonia was established by three criteria: (1) white blood cell (WBC) $> 15,000/\mu\text{l}$ (“leukocytosis”), WBC $< 4,000/\mu\text{l}$ (“leukopenia”), WBC ≥ 2 -fold change from baseline, or $\geq 90\%$ neutrophils on differential; (2) positive cultures for *S. pneumoniae* in blood or BAL fluid (BALF) at 48 hours; and (3) at least one of the following at 24 or 48 hours: fever $> 38.2^\circ\text{C}$, HR > 100 beats per minute (bpm), RR $> 25\%$ above baseline, cough, rhinorrhea, decreased oral intake or activity, or infiltrate on CXR. All three of

the above criteria were required to establish pneumonia.

Additional details about the animal model; microbiology and laboratory measurements; RNA sample collection, sequencing, and analysis; tissue collection and preparation; and statistical analysis are provided in the online supplement.

Statistical Analysis

Grouped data were expressed as means $\pm$ SE. Data analysis was by Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA) using ANOVA with appropriate *post hoc* testing. Only the data from the control, 10^8 , and 10^9 groups were included in the statistical analysis. $P < 0.05$ was accepted as statistically significant.

Results

Characterization of Pneumonia

Escalating *S. pneumoniae* inocula produced a dose-dependent increase in signs and symptoms of pneumonia. One animal each from the 10^6 and 10^8 groups developed a culture-negative, self-limited infection that resolved after 24 to 48 hours, whereas animals given 10^7 ($n = 1$), 10^8 ($n = 2$), and 10^9 ($n = 6$) CFU met diagnostic criteria for pneumonia by 48 hours (see Table E1 in the online supplement). Animals in all groups had decreased activity and/or oral intake, which resolved by 24 hours in the control, 10^6 , and 10^7 groups. At higher inocula, animals remained symptomatic at 48 hours and exhibited decreased activity ($10^8 = 2$; $10^9 = 3$), rhinorrhea ($10^8 = 1$), and cough ($10^9 = 1$) (Figure E1). These animals responded rapidly to antibiotics; their signs resolved, and they returned to normal activity by 96 hours.

Animals given 10^9 CFU consistently developed fever, tachypnea, and tachycardia, which were variable at the lower inoculations. The baseline RR was normal for this species (24 ± 1). At 24 hours, the RR in the 10^9 group increased dramatically (51 ± 4) and remained elevated through Day 7 ($P < 0.01$) (Figure 1A), whereas the RRs of the control and lower dose groups were unchanged. Preinoculation body temperature was normal ($36.9 \pm 0.2^\circ\text{C}$) (Figure 1B). All groups except the 10^6 dose experienced fever, which peaked at 24 hours after inoculation and was highest at 10^9 CFU. At

Figure 1. Vital sign trends in control, 10^8 , and 10^9 baboon groups (groups receiving *Streptococcus pneumoniae* in log increments of 10^8 or 10^9 CFU, respectively) at 0 to 168 hours after inoculation. (A) Respiratory rate increased dramatically in the 10^9 group as early as 24 hours after inoculation and remained elevated through 168 hours relative to the other groups. (B) Body temperature rose in all animals at 24 hours but remained elevated in only the 10^9 animals at 48 hours. (C) Heart rate was increased at 24 hours in the 10^8 and 10^9 animals and at 48 hours in the 10^9 animals alone relative to controls. Values are mean $\pm$ SEM. * $P < 0.01$ compared with controls; ** $P < 0.01$ compared with control and 10^8 groups; analyzed by two-factor ANOVA.

48 hours, only the animals in the 10^9 group remained febrile ($38.2 \pm 0.4^\circ\text{C}$; $P < 0.01$). The baseline HR was 77 ± 3 bpm. At 24 hours, the HR increased modestly in the control and 10^6 groups (range, 85–107) and considerably more in the 10^7 (124 bpm), 10^8 (128 ± 24 bpm; $P < 0.01$), and 10^9 groups (124 ± 7 bpm; $P < 0.01$) (Figure 1C). Tachycardia persisted at 48 hours in the 10^7 animals (115 bpm) and in the 10^9 animals (108 ± 8 bpm; $P < 0.01$). The baseline MAP in all animals was 72 ± 2 mm Hg. Every animal experienced an increase in MAP at 6 hours (mean change, 22 ± 3 mm Hg), which gradually returned to baseline by Day 7. This rise in MAP was significantly blunted in the 10^9 group at 6 and 24 hours ($P < 0.01$). All fever, tachycardia, and deviations in MAP resolved after antibiotic therapy.

Laboratory Measurements

S. pneumoniae infection caused hypoxemia. The baseline $\text{PaO}_2/\text{PAO}_2$ ratio was normal (0.9 ± 0.05) and remained normal in the control animals throughout the experiment

(Figure 2A). The 10^8 and 10^9 animals, however, developed hypoxemia as early as 6 hours after inoculation; this persisted in both groups at 48 hours but was most pronounced in the 10^9 animals and continued to be depressed in this group at 168 hours ($P < 0.05$).

The inoculations caused leukocytosis or leukopenia (at the highest dose) relative to baseline. Before inoculation, the mean WBC count was $8.9 \pm 0.8 \times 10^3/\mu\text{l}$ (Figure 2B). The control animals exhibited a transient, postbronchoscopy rise in WBC count that resolved by 48 hours. The lowest *S. pneumoniae* dose caused a leukocytosis ($22.2 \times 10^3/\mu\text{l}$) at 24 hours that also resolved by 48 hours. The 10^7 and 10^8 doses, however, produced a leukocytosis (26.6×10^3 and $27.6 \times 10^3/\mu\text{l}$, respectively) that remained 2-fold above baseline at 48 hours. The 10^9 dose produced a milder leukocytosis at 6 to 24 hours in four animals and leukopenia by 48 hours in one animal (Figure 2C). Leukopenia developed in another animal by necropsy (absolute neutrophil count,

669 cells/ μl), whereas the leukocyte derangements in the others resolved with antibiotics. Overall, all of the inoculated animals and one of the control animals met one criterion for change in WBC count, with $> 90\%$ neutrophils on differential predicting the presence of pneumonia.

S. pneumoniae infection also caused late-onset thrombocytosis. Platelet counts were normal at baseline and remained normal until 168 hours, when all inoculated animals except the 10^7 animal (platelet count 301,000/ μl) displayed a dose-dependent thrombocytosis (Figure 2D). At 168 hours, platelet counts in the 10^8 group were significantly higher than the control baboons ($P < 0.01$), and platelet counts in the 10^9 group were higher than in either group ($P < 0.01$).

The two highest inoculations produced exudative and acute inflammatory changes in BALF. The baseline BALF cell count was $16 \pm 3 \times 10^4$ cells/ml (Figure E2A). At 48 hours after inoculation, total BALF cell counts remained stable in the uninfected and lower-dose groups but rose 20-fold in

Figure 2. Laboratory trends in the control, 10^8 , and 10^9 groups at 0 to 168 hours after inoculation. (A) PaO_2/PAO_2 over time. PaO_2/PAO_2 declined in the 10^8 and 10^9 animals as early as 6 hours after inoculation and remained depressed in the 10^9 animals at 48 and 168 hours. (B) Trends in mean white blood cell (WBC) count over time. The control WBC count ($10^3/\mu L$) peaked at 6 hours after bronchoscopy and normalized by 48 hours. WBC counts in the 10^8 and 10^9 groups peaked at 24 hours after inoculation; counts at this time point were higher in the 10^8 group and variable in the 10^9 group (evidenced by the wide error bars). At 48 hours, the 10^9 group's WBC count was similar to controls, whereas the 10^8 group's WBC count, which had declined, was still elevated relative to the other groups. (C) WBC counts of individual 10^9 animals ($n = 6$) plotted with mean control values for comparison. At 24 hours, four animals given 10^9 CFU developed leukocytosis, one had no change in WBC count, and one developed leukopenia. At 48 hours, five animals displayed a relative ($n = 4$) or absolute ($n = 1$) leukopenia, which persisted in one animal to 168 hours. (D) Platelet count trends over time. There was a dose-dependent rise in platelet count ($10^3/\mu L$) at 168 hours after inoculation (* $P < 0.05$ or $\phi P < 0.01$ compared with controls; ** $P < 0.05$ or $\phi\phi P < 0.01$ compared with control and 10^8 groups; analyzed by two-factor ANOVA). Values are mean $\pm$ SEM unless noted otherwise.

the 10^8 and 10^9 groups ($P = 0.02$). Percent neutrophils similarly rose at 48 hours in the 10^8 ($62 \pm 20\%$; $P =$ not significant) and 10^9 ($75 \pm 7\%$; $P = 0.02$) groups compared with controls ($19 \pm 10\%$) (Figure E3A). At necropsy, the BALF leukocyte phenotype in the 10^9 group had shifted to $87 \pm 3\%$ mononuclear cells as total cell counts declined (Figure E3B). BALF total protein in the 10^9 group was elevated 40-fold ($6,976 \mu g/ml$) at 48 hours and 15-fold ($2,845.8 \mu g/ml$) at 168 hours, compared with little change in the other groups at these times ($P = 0.02$) (Figure E2B). Similarly, BALF LDH rose at 48 hours to 216.8 ± 94.9 units/l in the 10^9 group and remained 4-fold higher (73 ± 32.5 units/l) than in the other groups at 168 hours ($P = 0.06$) (Figure E2C).

The lung wet/dry ratio was not significantly different among groups (range, 4.2–5.2; the 10^9 group was 5.2 ± 0.1). The small bowel wet/dry ratio was higher

after the 10^9 inoculation (5.0 ± 0.5) compared with the lower doses (range, 4.2–4.5) and the uninfected (4.0 ± 0.2) animals, but this was not statistically significant. Conversely, the change in myeloperoxidase activity in whole lung homogenates was roughly dose dependent and was significantly higher in the baboons given 10^9 (9.43 ± 2.24 ; $P = 0.02$) and 10^8 CFU (6.61 ± 0.56 ; $P = 0.03$) compared with the lower doses (1.6–3.52) and controls (3.65 ± 0.73).

Plasma and BALF cytokines increased substantially in the 10^9 group and correlated with severe pneumonia. There were marked spikes at 24 hours in plasma IL-1ra, IL-6, IL-10, and G-CSF and in CCL2 (MCP-1) at 48 hours (all $P \leq 0.01$) (Figure 3). These cytokines were also significantly elevated in BALF at 48 hours (Figure 4). IL-1 β , CXCL8 (IL-8), CCL3 (MIP-1 α), CCL4 (MIP-1 β), and TNF- α increased similarly in BALF at 48 hours (all $P < 0.05$ except IL-1 β) but were unchanged

in the plasma. Generally, cytokine levels in plasma and BALF had returned to baseline at 168 hours. Other plasma and BALF cytokines that did not change significantly during the experiment are shown in Tables E2 and E3.

Chest Radiographs

Baseline CXRs were normal, but lobar infiltrates were seen consistently at the two highest inocula. At 48 hours, dose-dependent alveolar opacities were seen at the installation sites in only the 10^8 ($n = 2$) and 10^9 ($n = 6$) baboons (Figure 5). All infiltrates were present at 168 hours, although they had improved significantly in three of the animals ($10^8 = 2$; $10^9 = 1$).

Microbiology

S. pneumoniae pneumonia and bacteremia were seen consistently in the 10^9 group. In the 10^8 ($n = 1$) and 10^9 ($n = 6$) groups, BALF Gram stain demonstrated

Figure 3. Comparison of plasma cytokine levels in the control, 10^6 , 10^7 , 10^8 , and 10^9 baboon groups at 0, 6, 24, 48, and 168 hours after inoculation. There were significant elevations in plasma cytokine levels (pg/ml) in the 10^9 animals at 24 and 48 hours, with little or no change in cytokine levels in the lower dose and control animals (* $P < 0.01$ compared with control and 10^8 animals by two-factor ANOVA). Values are mean $\pm$ SEM for control ($n = 3$), 10^6 ($n = 1$), 10^7 ($n = 1$), 10^8 ($n = 3$), and 10^9 ($n = 4$) groups.

gram-positive cocci in pairs and chains at 48 hours (Figure E3A), and *S. pneumoniae* was isolated from BALF culture in the 10^7 , 10^8 ($n = 2$), and 10^9 ($n = 6$) animals (Figure 6A). After antibiotics, BALF from these groups was sterile. Baseline BALF cultures were sterile except for one 10^9 animal that unexpectedly grew 120 CFU of *S. pneumoniae*. This isolate tested negative for the experimental 19A serotype. This animal was healthy, with a normal baseline CXR, but after inoculation with the experimental 19A strain this animal met pneumonia criteria by 48 hours. Blood and BALF cultures from this animal grew only the experimental 19A *S. pneumoniae* strain, not the endogenous strain. *S. pneumoniae* grew from blood cultures at 24 hours in the 10^8 ($n = 1$) and 10^9 groups ($n = 4$) and at 48 hours in the 10^9 ($n = 4$) group but were sterile in all baboons at necropsy (Figure 6B).

NPS cultures did not correlate with blood or BALF cultures, but they did identify additional animals colonized with non-19A *S. pneumoniae* and *Staphylococcus aureus* at baseline. Normal microbiota was isolated at each time point from 12 of the 15 animals (control = 3, 10^6 = 1, 10^7 = 1, 10^8 = 2, and 10^9 = 5). NPS cultures from a 10^8 animal grew a non-19A *S. pneumoniae* at 0, 6, 24, and 48 hours after

inoculation, consistent with a carrier state. This 10^8 animal had positive blood cultures at 24 hours and positive BALF cultures at 48 hours for the 19A serotype and met our criteria for pneumonia. *S. aureus* was isolated from the NPS cultures of two animals: a control animal at baseline and a 10^9 animal at baseline and at 6, 24, and 48 hours after inoculation. *S. aureus* was not isolated in blood or BALF in either animal, and these were considered endogenous isolates.

All baseline pneumococcal urinary antigen assays were negative, including those from the two animals colonized with non-19A *S. pneumoniae*, but turned positive as early as 6 hours after inoculation in two 10^8 animals (one did not develop pneumonia) and in all of the 10^9 animals (Figure 6C). At 48 hours, all animals inoculated with $\geq 10^7$ CFU had detectable pneumococcal antigen. Pneumococcal urinary antigen remained detectable in those animals at necropsy and remained undetectable in the 10^6 and control animals.

Hierarchical Clustering of RNA Profiles

Hierarchical clustering of RNA profiles identified two major groups of animals (Figure E4). One cluster included all the

animals that met pneumonia criteria at 48 hours, and the other included all the animals that did not meet the pneumonia criteria. Table E4 is a preliminary and unvalidated list of the top 12 enriched gene ontologies in the up-regulated and down-regulated genes. Notable enriched gene ontologies for up-regulated genes in the 10^9 group include “immune response,” “inflammatory response,” and “I- κ B kinase/NF- κ B cascade.”

Pathology

Post mortem examination of the lungs revealed extensive consolidation of the left lung in the baboons given 10^8 and 10^9 CFU (10^9 = 4; 10^8 = 1) (Figure 7A) with pleural effusions or adhesions (10^9 = 3). The control and remaining inoculated animals had normal post mortem examinations.

Lung histology in the 10^9 group and in one of the 10^8 animals with pneumonia showed intense consolidation with intraalveolar edema, activated macrophages, and fibrin on hematoxylin and eosin staining (Figures 7C and 7D). There were also areas of organizing pneumonia (10^8 = 1; 10^9 = 5) (Figure 7E) by Masson’s trichrome staining. Hematoxylin and eosin-stained lung from the two additional

Figure 4. Comparison of bronchoalveolar lavage fluid (BALF) cytokines in the control, 10^6 , 10^7 , 10^8 , and 10^9 groups at 0, 48, and 168 hours after inoculation. There were significant elevations in the BALF cytokine levels (pg/mL) at 48 hours in the 10^9 animals relative to the other animals ($*P < 0.05$ compared with controls; $**P < 0.05$ or $\phi P < 0.01$ compared with control and 10^8 animals; analyzed by two-factor ANOVA). Values are mean $\pm$ SEM for control ($n = 3$), 10^6 ($n = 1$), 10^7 ($n = 1$), 10^8 ($n = 3$), and 10^9 ($n = 4$) groups.

animals that met criteria for pneumonia ($10^8 = 1$; $10^7 = 1$) showed minimal or no acute cellular inflammation and no intraalveolar fibrin, indicating resolution of the pneumonia. Lung histology in the 10^8 animal that did not meet criteria for pneumonia demonstrated only rare intraalveolar fibrin. In contrast, lung histology in the remaining control ($n = 4$) and 10^6 ($n = 1$) animals demonstrated delicate alveolar septae with inflated alveoli (Figure 7B). Lung

injury scores were significantly higher in the 10^9 group compared with the other groups ($P < 0.01$) (Figure 7F). Additional histopathological findings are reported in the online supplement.

Discussion

Inoculation with a human pathogenic strain of *S. pneumoniae* produces a dose-

dependent host inflammatory response in baboons, ranging from spontaneous clearance of infection at 10^6 CFU to severe lobar pneumonia with bacteremia at 10^9 CFU. Generally, animals that met criteria for pneumonia but received a lower dose of *S. pneumoniae* (10^7 – 10^8 CFU) had less severe disease that had usually resolved by Day 7. The 10^9 dose of *S. pneumoniae* consistently met preestablished pneumonia criteria and produced a phenotype resembling human pneumonia characterized by tachypnea, tachycardia, fever, malaise, cough, hypoxemia, and lobar consolidation. Selected pro- and antiinflammatory cytokines in BALF and plasma were significantly elevated at 24 to 48 hours after inoculation, suggesting a potentially useful future approach for biomarker development for pneumonia. The feasibility of using peripheral blood RNA sequencing and hierarchical clustering to distinguish animals with pneumonia from those without, regardless of inoculum size, suggests this approach for the future development of diagnostic assays for *S. pneumoniae* pneumonia.

Figure 5. Representative ventrodorsal chest X-ray (CXR) at 0, 48, and 168 hours after inoculation with 10^9 CFU *S. pneumoniae*. (A) Normal baseline CXR. (B) CXR demonstrating dense consolidation in the lingula and left lower lobe at 48 hours. (C) Persistent left lung consolidation at 168 hours.

Figure 6. Microbiology data for the control, 10^6 , 10^7 , 10^8 , and 10^9 groups. (A) BALF cultures at 0, 48, and 168 hours after inoculation. Baseline BALF cultures (log CFU/mL) were negative in all but one animal (in the 10^9 group), which grew non-19A *S. pneumoniae*. BALF cultures at 48 hours reflected the dose of inocula and by 168 hours had sterilized. (B) Blood cultures at 24, 48, and 168 hours. *S. pneumoniae* was isolated from blood cultures (CFU/mL) at 24 hours in the 10^8 ($n = 1$) and 10^9 ($n = 4$) groups and at 48 hours in the 10^9 ($n = 4$) group. (C) BinaxNOW *S. pneumoniae* urinary antigen at 0, 6, 48, and 168 hours after inoculation. *S. pneumoniae* urinary antigen was detected in the 10^8 ($n = 2$) and 10^9 ($n = 6$) groups 6 hours after inoculation and in all animals given $\geq 10^7$ CFU at 48 and 168 hours ($P < 0.01$ compared with controls by Mann-Whitney U test). abx, antibiotics.

Baboons are well suited for modeling human respiratory infections because their airway and lung anatomy closely approximate the human (18). Like the human and unlike mice, baboons develop lobar pneumonia and, due to their large size and upright posture, display dependent alveolar edema and heterogeneous gas exchange. The use of a larger animal in this study allowed us to document the time course of pneumonia with serial blood and BALF collections, which rodents do not tolerate well. The disadvantages of the baboon over rodents (i.e., baboons being more sentient species and the much higher cost and intensity of labor/per animal) are offset by the relevance to human disease and by the inability of rodent experiments, regardless of number of mice, to provide the translational data collected in this experiment.

S. pneumoniae pneumonia was previously studied by Philipp and colleagues (8) in another nonhuman primate species, rhesus macaques. Similar to our model, those animals were given

4×10^8 to 1×10^9 CFU ($n = 3$), but this resulted in variable postinoculation fever ($n = 1$), tachypnea ($n = 2$), tachycardia ($n = 0$), and radiographic infiltrates ($n = 2$). Those results contrast considerably with our model, likely due to differences in *S. pneumoniae* strain and inoculation methods. In squirrel monkeys inoculated with *S. pneumoniae* (10^3 to 6×10^8 CFU; $n = 4$), Berendt and colleagues (9) produced similar signs and symptoms to those in our 10^9 group, but the wide range of inocula and the limitations of smaller laboratory animals make it hard to draw direct comparisons. In our experiment, the 10^9 dose also produced hypoxemia, a marker of severe infection, and blunted the increase in MAP experienced by the other animals.

Leukocyte responses were variable within and among groups, as we observed both leukocytosis and absolute leukopenia postinoculation in the 10^9 group. Leukopenia in patients with community-acquired *S. pneumoniae* pneumonia is well described (23–26), particularly in the

setting of bacteremia. In experimental *S. pneumoniae* infection and bacteremia in rabbits, leukopenia was associated with pulmonary capillary leukostasis (27, 28). Such pulmonary leukostasis and/or decreased WBC release by the bone marrow might help explain the peripheral leukopenia we observed in our 10^9 group.

We also observed a dose-dependent, late-onset thrombocytosis in our baboons with pneumonia. In humans, particularly in young children, thrombocytosis after severe bacterial pneumonia is well known and generally peaks in the second or third week of illness (29–31). In fact, maximum platelet count may be proportional to the severity of pneumonia (29, 30, 32) as well as a marker of pleural disease (29, 30). This triad of severe pneumonia, pleural involvement, and thrombocytosis was reproduced by our 10^9 experiments and may be a clinically useful syndrome.

Cytokine levels in plasma and BALF were dramatically higher in the 10^9 animals compared with the lower-dose and

Figure 7. Gross and histopathology. (A) Representative necropsy photograph of the lingula (Li) and left lower lobe (LL) of a baboon inoculated with 10^9 CFU demonstrating extensive congestion and consolidation. (B–E) Representative photomicrographs of sections of lung tissue taken at necropsy. The top left is hematoxylin and eosin–stained tissue from a control lung (B; original magnification: $\times 100$). The top right and bottom left photomicrographs are hematoxylin and eosin–stained tissue from a 10^9 animal lung demonstrating alveolar septal thickening with intraalveolar mononuclear cells (C) and alveolar edema with intraalveolar foamy macrophages (D; original magnification: $\times 400$). (E) Masson's trichrome stained tissue from a 10^9 animal demonstrating intraalveolar collagen (stained green; original magnification: $\times 200$). (F) Modified lung injury score as a function of inoculum dose. The lung injury score was significantly higher in the 10^9 group than in the lower-dose and control groups ($*P < 0.01$ by one-way ANOVA). Values are mean $\pm$ SEM.

control animals. Circulating and BALF cytokine levels corresponded to peak of illness. BALF cytokines in patients with community-acquired lobar pneumonia have been found to be significantly higher with greater IL-6, TNF- α , IL-1 β (33–35), and CXCL8 levels (36, 37) in the involved lung compared with the contralateral lung and compared with healthy subjects. These BALF cytokines, perhaps together with CCL3, CCL4, IL-1ra, IL-10, and/or G-CSF, may be useful for biomarkers development in *S. pneumoniae* pneumonia. Our findings also implicate IL-1 β , CXCL8, TNF- α , CCL3, and CCL4 in the host response to *S. pneumoniae* pneumonia; these were elevated exclusively in BALF, suggesting they are produced locally during the infection. Animals that developed pneumonia at lower inoculations had little

to no rise in cytokine levels, suggesting the 10^9 dose produces the best approximation of severe pneumonia in humans.

Certain circulating cytokines have been shown to be elevated in patients with community-acquired pneumonia, including IL-1 β , IL-1ra, IL-6, IL-10, and TNF- α (38). Studies have associated elevations in serum (or plasma) IL-6 and IL-10 with increased disease severity and risk of mortality (39–41). Our data confirm the importance of increased plasma IL-1ra, IL-6, and IL-10 and implicate roles for increased CCL2 and G-CSF as markers of pneumonia severity.

We observed a shift in the BALF leukocyte differential across the experiment from neutrophil rich at 48 hours to mononuclear rich at 168 hours. The significance of mononuclear predominance

in the resolution phase of pneumonia is not clear, although it implies that mononuclear cells are critical to this phase. Positive BALF cultures predicted the diagnosis of pneumonia and reflected the level of *S. pneumoniae* inocula. *S. pneumoniae* was not recovered from BALF 48 hours after inoculation in the 10^6 animal or in one animal given 10^8 CFU. This implies that the lung has a great capacity to neutralize certain bacterial pathogens, although this process varies among individuals. Blood cultures were positive in only one of three animals given 10^8 CFU and in four of six animals given 10^9 CFU, also suggesting that bacteremia is not only pathogen- and host-dependent but is inoculum-dependent as well.

We found baseline colonization of four of the animals with *S. pneumoniae* or

S. aureus by NPS or BALF culture. These animals were clearly healthy during quarantine, suggesting the isolation of these common human pathogens represents natural colonization of this species. This affirms the suitability of baboons as a model for human bacterial lung infections and suggests a similar host immune response during such infections.

At necropsy, gross inspection of the lungs of animals with severe pneumonia demonstrated consolidation and pleuritis. Histologically, we identified two major adjacent processes: early-phase alveolar consolidation and later-phase organizing pneumonia. The acute findings, quantified using the lung injury score, were significantly more severe in the 10⁹ group. Organizing pneumonia is generally considered pathologic in nonresolving pneumonia (42), but it may also be a form of nonspecific healing after an acute injury and has been found in patients dying from *S. pneumoniae* pneumonia (42, 43).

Despite the histologic finding of alveolar edema in animals with severe pneumonia, the lung wet/dry weight did not differ significantly among the groups. This may be explained by the use of a conservative fluid strategy,

overnight fasting before procedures, and dehydration in the sickest animals. The wet/dry weights were also measured during the resolution rather than the acute phase of pneumonia, which may have obscured differences among groups.

Finally, based on preliminary RNA sequencing studies, hierarchical clustering of the animals perfectly matched our criteria for pneumonia and suggests future diagnostic applications of RNA sequencing in pneumonia. RNA sequencing may also provide insight into disease pathogenesis because gene ontologies such as “I- κ B kinase/NF- κ B cascade” were enriched in genes up-regulated at 48 hours. The NF- κ B cascade is well known to induce early proinflammatory cytokine expression and is internally consistent with the elevated NF- κ B-dependent cytokines (e.g., TNF- α , IL-1 β , IL-6) we found in the 10⁹ group. Although these results are encouraging, they need to be independently validated. We are in the process of developing and validating classifiers that more fully describe the genomic response to *S. pneumoniae* pneumonia. Ultimately, the diagnosis of pneumococcal infection may be based on a well characterized host genomic

response, as described for viral infection (6).

In summary, we report a novel, clinically relevant experimental model of *S. pneumoniae* pneumonia in baboons that closely resembles the human disease. This model is characterized by pro- and antiinflammatory cytokine production, both at the site of infection and peripherally, which if confirmed may be useful to establish the early diagnosis of pneumococcal pneumonia and identify patients with severe pneumonia. We were also able to correctly classify the animals according to preestablished pneumonia criteria using RNA sequencing of peripheral blood, which holds future promise in the diagnosis of pneumococcal pneumonia. Moreover, our work may be applicable to a number of clinical venues, including drug discovery, vaccine testing, and the advancement of our general understanding of adult and pediatric pneumococcal infection. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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