Stimulation of Lung Innate Immunity Protects against Lethal Pneumococcal Pneumonia in Mice

Cecilia G. Clement[1], Scott E. Evans[1], Christopher M. Evans[1,2], David Hawke[3], Ryuji Kobayashi[3], Paul R. Reynolds[4], Seyed J. Moghaddam[1], Brenton L. Scott[1], Ernestina Melicoff[1], Roberto Adachi[1,2], Burton F. Dickey[1,2], and Michael J. Tuvim[1,2]

[1]Department of Pulmonary Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; [2]Center for Lung Inflammation and Infection, Institute of Biosciences and Technology, Houston, Texas [3]Department of Molecular Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas and [4]Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania

***Rationale*: The lungs are a common site of serious infection in both healthy and immunocompromised subjects, and the most likely route of delivery of a bioterror agent. Since the airway epithelium shows great structural plasticity in response to inflammatory stimuli, we hypothesized it might also show functional plasticity.**

***Objectives*: To test the inducibility of lung defenses against bacterial challenge.**

***Methods*: Mice were treated with an aerosolized lysate of ultraviolet-killed nontypeable (unencapsulated) *Haemophilus influenzae* (NTHi), then challenged with a lethal dose of live *Streptococcus pneumoniae* (Spn) delivered by aerosol.**

***Measurements and Main Results*: Treatment with the NTHi lysate induced complete protection against challenge with a lethal dose of Spn if treatment preceded challenge by 4 to 24 hours. Lesser levels of protection occurred at shorter (83% at 2 h) and longer (83% at 48–72 h) intervals between treatment and challenge. There was also some protection when treatment was given 2 hours after challenge (survival increased from 14 to 57%), but not 24 hours after challenge. Protection did not depend on recruited neutrophils or resident mast cells and alveolar macrophages. Protection was specific to the airway route of infection, correlated in magnitude and time with rapid bacterial killing within the lungs, and was associated with increases of multiple antimicrobial polypeptides in lung lining fluid.**

***Conclusions*: We infer that protection derives from stimulation of local innate immune mechanisms, and that activated lung epithelium is the most likely cellular effector of this response. Augmentation of innate antimicrobial defenses of the lungs might have therapeutic value.**

Keywords: innate immunity; pneumonia; immunocompromised host; lung epithelium

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Antimicrobial proteins promote bacterial clearance from the lungs and are inducible in lung cells. However, the efficacy of stimulation of innate immunity in protection against lethal pneumonia is unknown.

What This Study Adds to the Field

Aerosolized treatment with a lysate from nontypeable *Haemophilus influenzae* induced protection against subsequent challenge with *Streptococcus pneumoniae*. These results indicate that augmentation of innate antimicrobial defenses of the lungs may have therapeutic benefit.

Pneumonia is the leading cause of death due to infection worldwide, and affects both healthy persons and those who are immunocompromised (1–3). The susceptibility of the lungs to infection derives from the architectural requirements of gas exchange, resulting in continuous exposure of a large surface area to the outside environment while imposing a minimal barrier to gas diffusion. This precludes protective strategies, such as encasement of the alveolar gas exchange surface in an impermeable barrier, as in the skin, or continuous generation of a heavy blanket of mucus, as in the gastrointestinal tract.

Despite their structural vulnerability, the lungs generally defend themselves successfully against infection through a variety of mechanical, humoral, and cellular mechanisms (4–8). First, most inhaled microbial pathogens fail to penetrate to the alveoli because of impaction or sedimentation against the walls of the conducting airways, where they are entrapped by mucus, then cleared by sneezing, coughing, or mucociliary action. Next, the airway lining fluid contains antibodies and antimicrobial peptides that limit the growth of pathogens that succeed in penetrating the mucus gel layer. Finally, alveolar macrophages that reside in the distal airspaces of the lungs ingest organisms that penetrate to that depth. When necessary, the parenchymal and resident inflammatory cells of the lungs release signaling molecules that result in exudation of plasma proteins and recruitment of leukocytes, although this impairs gas exchange and can be viewed as a defensive strategy of last resort (9).

In addition to defense mechanisms that function at baseline, the secretory cells of the airway epithelium are capable of a remarkable change in structure termed "inflammatory metaplasia." In response to viral, fungal, or allergic inflammation, these cells rapidly increase their height in association with filling of the apical cytoplasm with electron lucent secretory granules and conversion of apical smooth endoplasmic reticulum to rough endoplasmic reticulum (10, 11). Many of these structural changes can be ascribed to increased synthesis of the gel-forming mucin Muc5ac, a component of the innate immune system, although other molecular changes also occur (5, 11–14). The adaptive value of this structural and molecular plasticity of the airway epithelium is presumed to be augmented defense against microbial pathogens. This is supported by the inducible production of antimicrobial proteins by epithelial cells (15–20), which have been shown to contribute to bacterial clearance (21–25).

(*Received in original form July 27, 2006; accepted in final form March 31, 2008*)

Supported by the George and Barbara Bush Endowment for Innovative Cancer Research and the Odyssey Fellowship Program from the University of Texas M.D. Anderson Cancer Center, and grants HL072984, CA105352, and CA016672 from the National Institutes of Health.

Correspondence and requests for reprints should be addressed to Burton F. Dickey, M.D., Department of Pulmonary Medicine, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. E-mail: bdickey@mdanderson.org

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 177. pp 1322–1330, 2008
Originally Published in Press as DOI: 10.1164/rccm.200607-1038OC on April 3, 2008
Internet address: www.atsjournals.org

To assess the functional plasticity of the lungs in pathogen defense *in vivo*, we stimulated mice with an aerosolized bacterial lysate to activate multiple pathogen-associated molecular pattern recognition pathways simultaneously. We found that mice rapidly acquired a high level of resistance to lethal pneumonia from a virulent noncognate bacterium, indicating that innate immune defenses of the lungs are highly inducible. This finding could provide a basis for the development of novel clinical therapies. Some of the results of these studies were reported in abstract form (26, 27).

METHODS

Animals

For all experiments except those with mast cell–deficient mice, specific pathogen–free, 5- to 6-week-old female BALB/c mice were purchased from Harlan and used within 4 weeks. Mast cell–deficient C57BL/6 $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ and littermate mast cell–sufficient C57BL/6 Kit^{+}/Kit^{+} mice were bred within our colony and used at 12 to 20 weeks of age. Mice were examined twice daily and killed if distressed with an intraperitoneal injection (5 ml/kg) of a mixture of ketamine (37.5 mg/ml), xylazine (1.9 mg/ml), and acepromazine (0.37 mg/ml). Mice were handled in accordance with the Institutional Animal Care and Use Committee of M.D. Anderson Cancer Center.

Aerosol Lysate Treatment

Nontypeable *Haemophilus influenzae* (NTHi) was stored as frozen stock (1×10^7 cfu/ml) in 20% glycerol in brain–heart broth (Acumedia, Lansing, MI) (28). Thawed stock was grown on chocolate agar at 300 μl per 10-cm plate (Remel, Lenexa, KS) for 24 hours at 37°C in 5% CO_2, then harvested and incubated for 16 hours in 1 L brain–heart infusion broth (Acumedia) supplemented with 3.5 μg/ml nicotinamide adenine dinucleotide (Sigma-Aldrich, St. Louis, MO). The culture was centrifuged at 2,500 × *g* for 10 minutes at 4°C, washed and resuspended in 20 ml phosphate-buffered saline (PBS), ultraviolet (UV) irradiated in a 100-mm Petri dish at 3,000 $\mu J/cm^2$, then sonicated three times for 30 seconds each in a 50-ml conical plastic tube (Sonic Dismembrator 50; Fisher Scientific, Waltham, MA). Protein concentration was adjusted to 2.5 mg/ml in PBS by bicinchoninic assay (Pierce, Rockford, IL), and the lysate was frozen in 10-ml aliquots at −80°C. Sterility was confirmed by culture on chocolate agar. For treatment, a thawed aliquot was placed in an AeroMist CA-209 nebulizer (CIS-US, Bedford, MA) driven by 10 L/minute 5% CO_2 in air for 20 minutes to promote deep ventilation, resulting in aerosolization of 4 ml of lysate, with the protein concentration in residual lysate confirmed at 2.5 mg/ml. The nebulizer was connected by polyethylene tubing (30 cm × 22 mm) to a 1–10-L polyethylene exposure chamber, with an identical efflux tube with a low-resistance microbial filter (BB50T; Pall, East Hills, NY) at its end vented to a biosafety hood.

Pneumococcal Pneumonia

Streptococcus pneumoniae (Spn) serotype 4 isolated from the blood of a patient with pneumonia was serially injected four times into the peritoneal cavity of mice and harvested after 24 hours from the spleens of sick animals to select for virulence towards mice, then stored as frozen stock (1×10^9 cfu) in 20% glycerol in Todd-Hewett broth (Becton Dickinson, Franklin Lakes, NJ). One milliliter of thawed stock was incubated for 16 hours in 150 ml Todd-Hewett broth at 37°C in 5% CO_2, then diluted in 1.5 L of fresh broth and grown in logarithmic phase for 6 to 7 hours to an OD_{600} of 0.3, yielding approximately 6×10^{11} cfu. The suspension was centrifuged at 2,500 × *g* for 10 minutes at 4°C, then washed and resuspended in PBS, and bacterial concentration was determined by plating serial dilutions onto blood agar (Remel). For aerosolization, 10 ml of the suspension was placed in an AeroMist CA-209 nebulizer driven by 10 L/minute of 5% CO_2 in air with an identical exposure chamber to that for the NTHi lysate. After 30 minutes, another 5 ml was added, with a total of 10 ml of the suspension aerosolized during the full 60-minute exposure.

Host Response to Spn Challenge and NTHi Treatment

Bronchoalveolar lavage fluid (BALF) was obtained by sequentially instilling and collecting two 1-ml aliquots of PBS through a Luer stub adapter cannula (Becton Dickinson) inserted through rings of the exposed trachea of mice that had been killed. Total leukocyte count was determined with a hemocytometer, and differential count by cytocentrifugation of 300 μl of BALF at 450 *g* for 5 minutes followed by Wright-Giemsa staining. For histologic analysis, the aortas of anesthetized mice were transected, the lungs perfused *in situ* with PBS via the right cardiac ventricle, then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) infused through a tracheal cannula at 10 to 15 cm pressure at 21°C. The lungs were removed from the thoracic cavity and further fixed overnight at 4°C, then embedded in paraffin, sectioned and stained for light microscopy with hematoxylin and eosin to examine cellular elements, and periodic acid fluorescent Schiff's reagent to examine intracellular mucin (10). Quantitative real-time reverse transcriptase–polymerase chain reaction for airway mucin transcripts was performed as described (13). For measurement of bacterial counts, tissues were harvested from dead mice, homogenized in 1 ml of PBS using a 2-ml tissue grinder (Kontes Glass Co., Vineland, NJ), then serially diluted onto blood agar.

Leukocyte Depletion and Proteomic Analyses

See the online supplement for details.

Statistical Methods

Summary statistics for bacterial counts in lung tissue after Spn exposure were computed within time groups, and analysis of variance (ANOVA) with adjustment for multiple comparisons using Dunnett's test was performed to examine the differences between the mean cell counts of the control group and each of the NTHi treatment groups. For leukocyte depletion studies, two-way ANOVA of bacterial counts was performed according to NTHi treatment and leukocyte status. Proportions of mice surviving Spn challenge were compared using Fisher's exact text. Analyses were performed using SAS/STAT software (Version 8.2; SAS Institute, Cary, NC).

RESULTS

Mouse Model of Pneumonia

Exposure to increasing concentrations of aerosolized Spn was associated with increasing mortality (Figure 1). During the first day after aerosol challenge, none of the mice showed adverse effects. During the second and third days, some became lethargic, huddled together, showed ruffled fur and arched backs, or

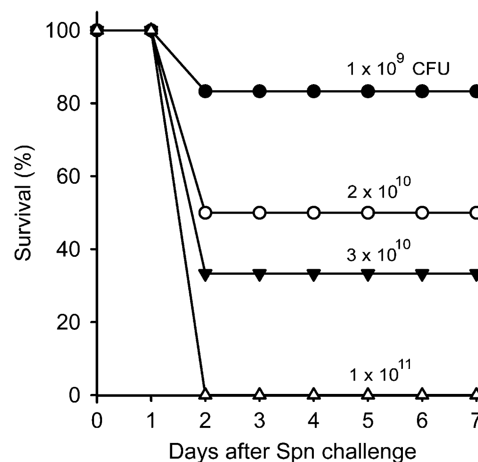


Figure 1. Survival after *Streptococcus pneumoniae* (Spn) aerosol challenge. Groups of six mice were exposed for 1 hour to aerosols containing increasing concentrations of Spn, and surviving mice were counted daily.

were found dead in their cages. Infection progressed in two distinct patterns depending on whether Spn was used at low (<4 × 10^9 cfu/ml) or high (>4 × 10^{10} cfu/ml) doses. With low doses, there was a small increase in neutrophils in BALF during the first day that resolved by the second day (Figure 2A), there was no discernable infiltration of lung tissue by inflammatory cells (not shown), and viable bacteria were cleared from the lungs by 48 hours with no evidence of bacterial escape from the lungs (Table 1). With high doses of Spn, neutrophils increased in BALF throughout the first day, but measurements were not possible on the second day because so few mice remained alive (Figure 2B). Histologically, the numbers of neutrophils in edematous peribronchial and perivascular connective tissue increased during the course of the first day, and neutrophils were also seen in alveoli on the second day (Figure E1 in the online supplement). The numbers of viable bacteria in the lungs increased during the first 48 hours, and large numbers of bacteria were cultured from blood and the spleen after 48 hours (Table 1).

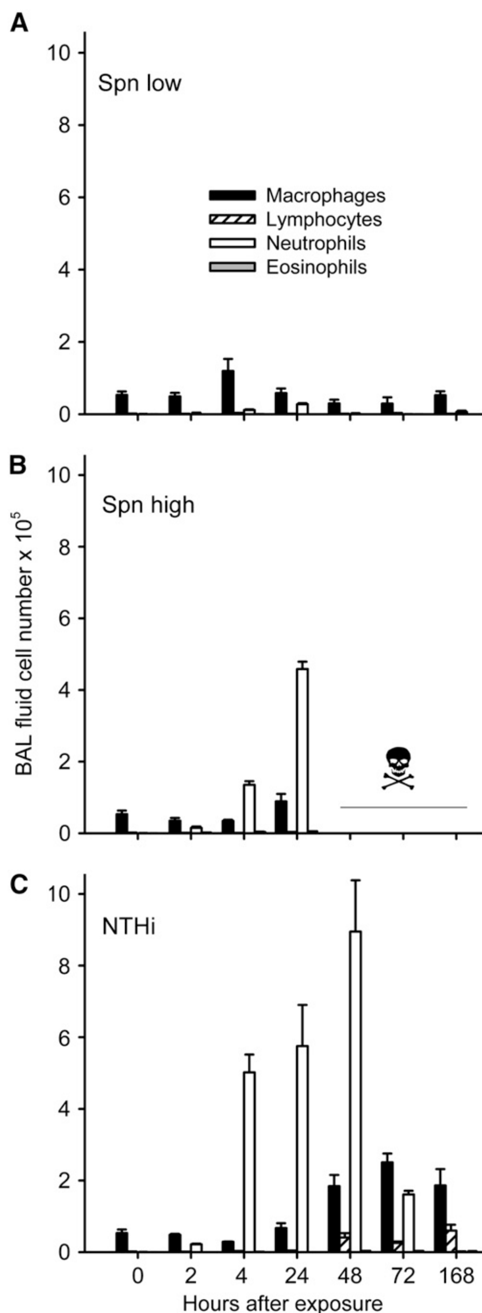


Figure 2. Inflammatory cell counts in bronchoalveolar lavage (BAL) fluid after *Streptococcus pneumoniae* (Spn) challenge or nontypeable *Haemophilus influenzae* (NTHi) treatment. Mice were exposed to aerosols containing low-dose Spn (1.0 × 10^9 cfu/ml) (*A*), high-dose Spn (6.1 × 10^{10} cfu/ml) (*B*), or NTHi lysate (*C*). Inflammatory cells were measured in BAL fluid of groups of five mice at the indicated time points (mean ± SEM).

TABLE 1. BACTERIAL COUNTS AFTER *STREPTOCOCCUS PNEUMONIAE* CHALLENGE

	Hours after Challenge					
	Lungs		Blood		Spleen	
	0	48	0	48	0	48
Low-dose Spn	1.5 × 10^5	0	0	0	0	0
High-dose Spn	1.5 × 10^6	1 × 10^8	0	>10^8	0	2.6 × 10^8

Definition of abbreviation: Spn = *Streptococcus pneumoniae*.
Low-dose Spn challenge was with 2.2 × 10^9 cfu/ml, and high-dose Spn challenge was with 4.5 × 10^{10} cfu/ml. Blood and tissue bacterial counts are expressed as cfu/ml of whole blood or tissue homogenates.

Thus, low-dose Spn challenge resulted in falling numbers of viable Spn in the lungs despite minimal leukocyte infiltration, and low rates of bacteremia and host death, suggesting that successful containment of low-level bacterial infection of the lungs depends only minimally on leukocytes recruited from the circulation. In contrast, high-dose Spn challenge resulted in rising neutrophilic inflammation, rising numbers of viable Spn in the lungs, and high rates of bacteremia and host death, suggesting that leukocyte recruitment occurs too sluggishly to contain lung infection with a large inoculum of virulent bacteria. High-dose Spn challenge was used in all subsequent experiments except those in neutropenic mice.

Stimulation of Lung Innate Immunity Protects against Lethal Pneumonia

To stimulate lung innate immunity, mice were exposed to increasing concentrations of NTHi lysate, with BALF neutrophils used as a marker of stimulus intensity, and a goal of identifying a stimulus that caused more BALF neutrophils than high-dose Spn infection. Exposure to NTHi lysate at 2.5 mg/ml for 20 minutes resulted in a brisk response, with BALF neutrophils 4 hours after treatment comparable to those 24 hours after high-dose *Spn* challenge, and maximal at 48 hours when they were accompanied by a small number of lymphocytes and an increase in macrophages (Figure 2C). Light microscopy showed neutrophils in peribronchial and perivascular connective tissue and in alveolar airspaces during the first 3 days (Figure E1), and these rapidly cleared over the next 3 days (data not shown). No increase in airway epithelial mucin was seen by histochemical staining or quantitative reverse transcriptase–polymerase chain reaction at any time (data not shown), with lungs from allergen-sensitized and -challenged mice serving as a positive control (10, 12, 29).

Mice treated with the NTHi lysate were then challenged with high-dose Spn. Pretreatment from 4 to 24 hours before challenge resulted in full protection from mortality (Figure 3), and lesser or greater intervals between treatment and challenge resulted in partial protection. When mice were treated 4 hours before challenge, even the maximal concentration of Spn deliverable by aerosol during a 2-hour period (5 × 10^{11} cfu/ml) caused no mortality (data not shown). Some protection was also seen when the NTHi treatment was given soon after Spn challenge, with an increase in survival from 14% with no treat-

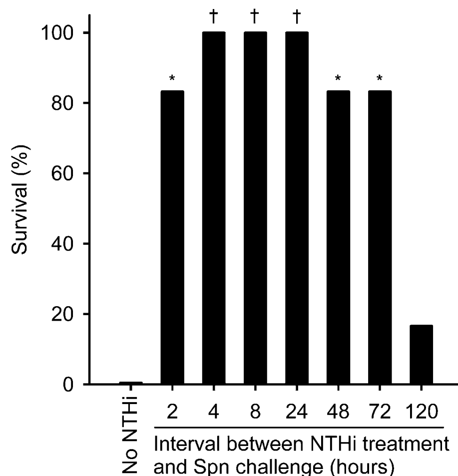


Figure 3. Survival after *Streptococcus pneumoniae* (Spn) aerosol challenge following treatment with nontypeable *Haemophilus influenzae* (NTHi) lysate. Mice were pretreated in groups of six with NTHi lysate, then challenged as a single group with high-dose Spn (6.1×10^{10} cfu/ml). Survival at 7 days is shown as a function of the interval between treatment and challenge ($^*P = 0.015$, $^†P = 0.002$, treated vs. untreated).

ment to 57% when treatment was given 2 hours after challenge, but no increase in survival when treatment was given 24 hours after challenge (Figure 4).

Pretreatment with an aerosolized lysate of *Escherichia coli* or *Staphylococcus aureus* provided similar protection against Spn challenge (data not shown), indicating that stimulation of lung innate immunity is not specific to components of NTHi. The level of endotoxin in the NTHi lysate was measured at 1,085 U/ml, but aerosolized *E. coli* endotoxin at this concentration did not significantly protect against Spn challenge (Figure E2). Even at 10-fold higher concentration, endotoxin protected only 63% as well as NTHi lysate, indicating that endotoxin alone cannot fully account for the stimulation of protection by NTHi lysate. Protection was equally effective when the microbial challenge was by nasal instillation or intratracheal injection (data not shown), indicating that protection was not due simply to impedance to entry of Spn into distal airspaces by airway lumen obstruction resulting from the aerosolized NTHi lysate.

Protection Is Compartment Specific

To determine whether stimulation of lung innate immunity results in local or systemic protection against bacterial pathogens, mice pretreated with the aerosolized NTHi lysate were also challenged with intravenous or intraperitoneal Spn. In pilot studies, the mortality dose–response relationship to Spn injection was determined so a minimal lethal dose could be used to maximize the chance of identifying a protective systemic effect of the aerosolized lysate. As few as 1–10 cfu of Spn by either route killed most mice on the first or second day after injection (data not shown), so fewer than 10 cfu were used. Although the aerosolized NTHi lysate provided complete protection against Spn aerosol challenge, it provided no protection against intravenous or intraperitoneal Spn challenge (Figure 5). Thus, protection against bacterial infection induced by the aerosolized NTHi lysate is localized to the lungs and is not systemic.

Protection Is Associated in Magnitude and Time with a Microbicidal Environment in the Lungs

To elucidate the mechanism of protection, we tested whether the aerosolized NTHi lysate induced bacterial killing. The lungs of mice pretreated with the NTHi lysate were excised immediately after Spn challenge, homogenized, and plated for bacterial culture. The numbers of live bacteria correlated inversely with protection against lethal pneumonia, such that 1.7×10^6 cfu were present in the lungs of naive mice immediately after Spn challenge, but only 1.0×10^5 cfu were present in the lungs of mice pretreated 24 hours earlier with NTHi lysate, which were fully protected (Figure 6). Intermediate numbers of viable bacteria were present in the lungs of mice with intermediate levels of protection during the rising and falling limbs of the time-dependent survival curve (Figures 3 and 6). From these data and those in Table 1, we inferred that the mechanism of protection is local killing of bacteria before they cross lung mucosal barriers, because access of even small numbers of Spn to the vascular space or internal compartments leads rapidly to death

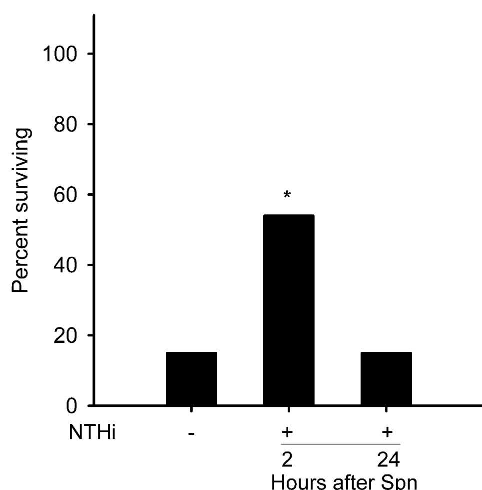


Figure 4. Survival after *Streptococcus pneumoniae* (Spn) aerosol challenge followed by treatment with nontypeable *Haemophilus influenzae* (NTHi) lysate. Mice were challenged as a single group with high-dose Spn (3.5×10^{10} cfu/ml), then treated in groups of 14 with NTHi lysate 2 or 24 hours after Spn challenge ($^*P = 0.046$).

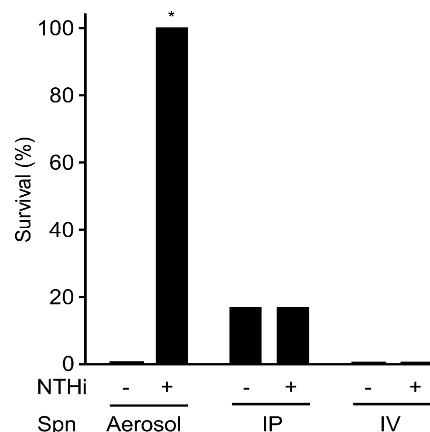


Figure 5. Survival after aerosol, intraperitoneal or intravenous *Streptococcus pneumoniae* (Spn) challenge. Mice were pretreated with nontypeable *Haemophilus influenzae* (NTHi) lysate 4 hours before Spn challenge, or left untreated. Groups of six treated and six untreated mice were then challenged with Spn delivered by aerosol (6.1×10^{10} cfu/ml) intraperitoneal (IP) injection (5–10 cfu), or intravenous (IV) tail vein injection (5–10 cfu), and survival at 7 days is illustrated ($^*P = 0.002$, treated vs. untreated).

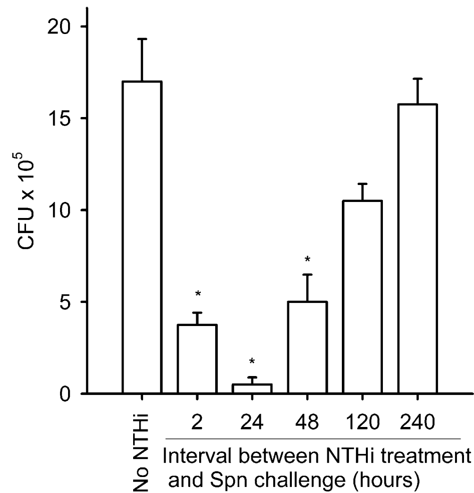


Figure 6. Bacterial counts in the lungs of mice after treatment with nontypeable *Haemophilus influenzae* (NTHi) lysate. Mice were pretreated in groups of 4 with NTHi lysate at various time points, then challenged as a single group with high-dose *Streptococcus pneumoniae* (Spn) (2.1×10^{10} cfu/ml). Lungs were removed immediately after the aerosol challenge, homogenized, and plated for bacterial culture (mean ± SEM, *$P < 0.05$ for treated vs. untreated).

of mice (Figure 5). Therefore, we sought the mechanism of bacterial killing.

Protection against Lethality Does Not Depend on Neutrophil Recruitment to the Lungs

We had initially titrated the strength of the aerosolized NTHi lysate treatment to neutrophil recruitment to the lungs, and the time course of BALF neutrophilia roughly parallels that of protection (Figures 2 and 3). Therefore, we tested whether neutrophil recruitment is required for protection. Intravenous antibody RB6-8C5 against neutrophil Ly6G reduced BALF neutrophil numbers 24 hours after NTHi treatment by 96% from 2.5×10^5 to 1.0×10^4 (Figure E3). In addition, alveolar macrophage numbers were reduced by 70% using liposomal clodronate delivered through the airway. To prevent excessive mortality in these immunocompromised mice, which would obscure differences among treatment groups, an intermediate dose of Spn was used for the challenge.

All mice pretreated with NTHi lysate survived Spn challenge whether or not they were depleted of alveolar macrophages and neutrophils (Figure 7A). Death among leukocyte-depleted mice continued to occur after 3 days (Figure E4), which was different from all experiments in leukocyte-replete mice. Lung bacterial counts correlated inversely with mouse survival, and depended on both leukocytes and NTHi treatment (Figure 7B). Similar results were obtained in mice deficient in mast cells (Figures E5 and E6). These results suggest that protection from lethality by treatment with the aerosolized NTHi lysate does not depend on recruited neutrophils or resident alveolar macrophages and mast cells, but that rapid bacterial killing in the lungs of treated mice and late mortality in untreated mice depend partially on these leukocytes of the innate immune system.

Because it was possible that the antineutrophil antibodies and clodronate induced protection through inflammation resulting from leukocyte lysis despite a reduction in leukocyte number, we also tested the role of neutrophil recruitment by suppressing hematopoiesis with the nucleoside analog cytosine arabinoside. Using a high-dose, short-term regimen that prevented any detectable rise in BALF neutrophils in response to the NTHi lysate

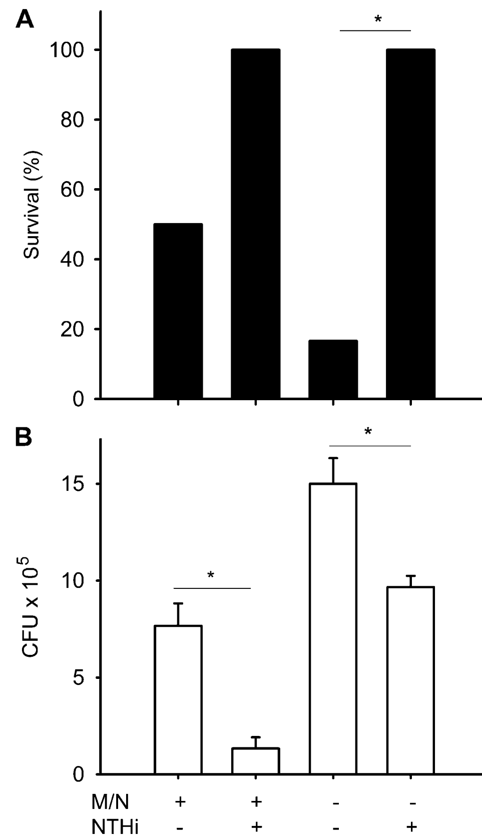


Figure 7. Host survival and lung bacterial counts in mice deficient in alveolar macrophages and neutrophils. Half the mice were given intranasal clodronate to deplete alveolar macrophages and intravenous antibody RB6-8C5 to deplete neutrophils (M/N−). Half each of the M/N+ and M/N− groups were then treated with nontypeable *Haemophilus influenzae* (NTHi) lysate. All mice were challenged as a single group 4 hours later with *Streptococcus pneumoniae* (Spn) (1.5×10^{10} cfu/ml). Shown is survival at 7 days for six mice from each group (*A*) and bacterial culture from the lungs of three mice immediately after aerosol challenge (*B*) (mean ± SEM, *$P < 0.05$ for comparisons indicated by *horizontal lines*).

(Table E1 and Figure E3), but without clodronate to kill alveolar macrophages, comparable results were obtained, even though these mice all died from bone marrow failure on the fourth and fifth days after Spn challenge, similar to mice treated with cytosine arabinoside without Spn challenge (data not shown). Comparable results were also obtained with the alkylating agent cyclophosphamide (data not shown). Thus, protection by the NTHi lysate from lethal Spn pneumonia does not depend on neutrophil recruitment to the lungs.

Protection Is Associated with an Increase of Multiple Antimicrobial Polypeptides in Lung Lining Fluid

Because bacterial killing in lungs stimulated with the NTHi lysate was rapid and only partially dependent on leukocytes, we suspected that the lysate stimulates production of antimicrobial polypeptides by lung parenchymal cells. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of BALF supernatants showed multiple increased or new Coomassie blue–stained bands, particularly at 10,000–18,000 kDa beginning 2 hours after NTHi treatment and reaching a maximum at 48 hours (data not shown). BALF from mice treated 48 hours earlier with NTHi lysate was then analyzed by semiquantitative proteomic techniques. HPLC revealed several absorbance

peaks that were new or markedly increased (Figure 8). By mass spectrometry, these peaks contained multiple antimicrobial polypeptides including lysozyme, lactoferrin, haptoglobin, calgranulin, and surfactant apoprotein D. Differential gel electrophoresis (Figure E7) and isobaric stable isotope labeling (Figure E8) also identified increased antimicrobial polypeptides in the treated samples, with overlap among techniques (Table 2). Some polypeptides are known to be expressed primarily by lung epithelial cells, such as chitinase-3-like-1 and surfactant protein D (29, 30); others are expressed by leukocytes, such as lymphocyte cytosolic protein 1 (31); and some are expressed by both epithelial cells and leukocytes, such as lysozyme, lactoferrin, and calgranulin (24, 32). Thus, augmented host protection and bacterial killing are associated with increased amounts of antimicrobial polypeptides in lung lining fluid.

DISCUSSION

Inspired by the structural plasticity of the airway epithelium in response to inflammatory stimuli (10), we tested its functional plasticity in defense against microbial pathogens. Our results show that stimulation with a complex mixture of bacterial products induces a high level of resistance against a virulent bacterial pathogen introduced by the respiratory route. We infer that the acquired protection is due to innate immune mechanisms because it occurs too rapidly for an adaptive immune response, and the challenge is not cognate to the stimulus (33). Protection is localized to the lungs (Figure 5), and is associated with rapid bacterial killing within the lungs (Figure 6). It does not depend on neutrophil recruitment to the lungs or on resident mast cells and macrophages (Figure 7). Rather, protection is associated with increased levels of multiple antimicrobial proteins in lung lining fluid (Table 2), suggesting that protection is due to the coordinated induction of these and other local innate immune defenses by resident lung cells.

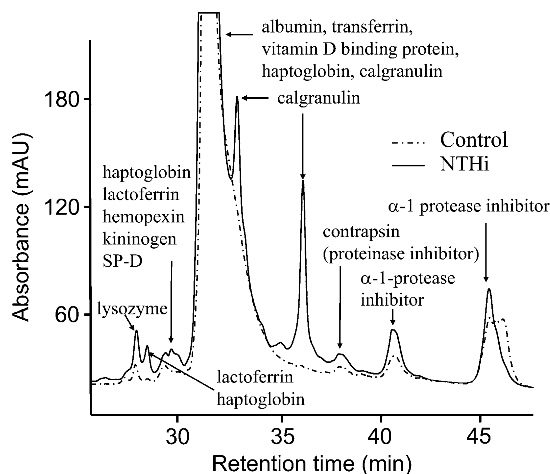


Figure 8. Reversed phase HPLC analysis of proteins in bronchoalveolar lavage fluid (BALF) after nontypeable *Haemophilus influenzae* (NTHi) treatment. BALF supernatants were collected from the lungs of mice that were untreated (*dashed line*) or treated 48 hours previously with NTHi lysate (*solid line*), desalted by acetone precipitation, then fractionated on a C-18 column eluted with an acetonitrile gradient. Representative elution profiles with ultraviolet absorbance measured at 214 nm are shown. Proteins from individual fractions were digested with trypsin, then analyzed by liquid chromatography followed by electrospray ionization quadropole time-of-flight mass spectrometry and ion trap mass spectrometry (LC-MS/MS) and identified by database searching (*see* METHODS in the online supplement for additional details).

TABLE 2. PROTEOMIC ANALYSIS OF NONTYPEABLE *HAEMOPHILUS INFLUENZAE*–TREATED LUNG LAVAGE FLUID

	Proteomic Technique		
Identified Protein (GenBank Accession No.)	HPLC	iTRAQ	DIGE
Pulmonary surfactant-associated protein D (NP_033186)	•	•	•
Haptoglobin-2 (NP_059066)	•	•	•
Calgranulin B (P31725)	•	•	
Kininogen (AAH18158)	•	•	
Chitinase-3-like protein 1 (NP_031721)		•	•
Complement C3 (AAH43338)	•		•
Transferrin (NP_598738)	•		
Lactoferrin (NP_032548)	•		•
Lysozyme (NP_059068)	•		
Alpha-1-protease inhibitor (P22599)	•		
Hemopexin (NP_059067)	•		
Contrapsin (CAA38948)	•		
Vitamin D binding protein (AAA37669)	•		
Hemoglobin alpha chain (P01942)		•	
Hemoblogin beta chain (P02088)		•	
Alpha-1-acid glycoprotein 1 (NP_032794)		•	
Inter-alpha-trypsin inhibitor heavy chain H4 (NP_061216)		•	
Transketolase (NP_033414)			•
Serpin 1 A protein (NP_079705)			•
Glucose phosphate isomerase (NP_032181)			•
Rho GDI alpha (NP_598557)			•
Polymeric immunoglobulin receptor (NP_035212)			•
Leukotriene E4 hydrolase (NP_032543)			•
Enolase 1 (NP_075608)			•
Lymphocyte cytosolic protein 1 (AAH22943)			•
Lipocalin 2 (NP_032517)			•
WD repeat domain protein 1 (NP_035845)			•

Definition of abbreviations: DIGE = difference gel electrophoresis (*see* METHODS in online supplement for details); iTRAQ = isobaric stable isotope tag.

Proteins listed were found at a higher level in lavage fluid of nontypeable *Haemophilus influenzae* lysate–treated mice than in untreated mice by one or more of the three proteomic techniques.

The airway epithelium likely plays a central role in induced protection in view of the large surface area it covers (100 m^2 in an adult human) and its close apposition to deposited pathogens. Although this makes the epithelium susceptible to being breached, it also ideally positions it for microbial killing when activated. In addition, epithelium activated by innate immune stimulation becomes resistant to invasion (34). Such a change in functional capability is consistent with our findings of increased epithelium-specific antimicrobial proteins in lung lining fluid proteomic analyses (Table 2), and the marked structural plasticity of the epithelium in response to inflammatory stimuli (10, 11). It is also supported by work of others showing an essential role for lung parenchymal cell activation in defense against bacterial and viral pneumonia (8, 35–37), and the dependencies of bacterial killing and host defense on epithelial expression of antimicrobial polypeptides (21–25, 38). Indeed, mechanisms for suppression of antimicrobial protein expression contribute importantly to microbial pathogenicity (39, 40). Further work will be required to establish whether lung epithelial cells sense the NTHi stimulus autonomously, or require costimulation by bone marrow–derived cells such as dendritic cells to fully express a protective response (41).

Death of mice in our Spn pneumonia model is likely related to escape of bacteria from the lungs into the systemic circulation since the time of death correlates with the presence of bacteria in blood and distant organs (Table 1). Indeed, less than 10 Spn introduced directly into the bloodstream results in death (Figure 5), and the lungs of mice dying of overwhelming infection do not show severe lung injury (Figure E1). Bacterial invasion appears to be a stochastic event, related directly to the dose of bacteria delivered to the lungs, and inversely to the rate

of bacterial killing within the lungs. With delivery of low numbers of Spn, only small numbers of mice die despite minimal lung inflammation, presumably because innate antimicrobial mechanisms active at baseline clear bacteria before they invade. Alveolar macrophages likely participate in rapid bacterial killing because lung bacterial counts were higher in mice given clodronate (Figure 7), at an early time point before recruited neutrophils appear (Figure 2). With delivery of high numbers of Spn, large numbers of mice die despite a vigorous late inflammatory response, presumably because bacteria have already invaded or bacterial growth outstrips the microbicidal capacity of the lungs and recruited inflammatory cells. By greatly increasing the rate of bacterial killing within the lungs and the resistance of the lung epithelium to bacterial invasion, treatment with the NTHi lysate could protect against lethality.

Augmentation of lung innate immune defenses may have therapeutic value. Patients with transient neutropenia, such as those receiving myeloablative cancer chemotherapy or conditioning for hematopoietic stem cell transplantation, are at high risk for fatal pneumonia (2). Stimulation of innate immune lung defenses could provide protection during this vulnerable period. Similarly, patients with transiently impaired adaptive immunity might benefit, such as those receiving immunosuppressive therapy for autoimmune diseases or organ transplantation, and patients with cancer receiving agents that induce T-cell dysfunction, such as fludarabine. Augmentation of lung defenses could also be useful in a bioterror attack with a virulent respiratory pathogen. The lungs are the most likely route of delivery of a bioterror agent, protection by vaccination or antibiotics is not yet feasible for all pathogens, and stimulation of lung innate immunity could provide rapid broad protection during the early period after an attack when the identity of the pathogen is not yet known (42).

Identifying the molecular species in the NTHi lysate that induce protection should allow improvement in the therapeutic ratio by using only the purified molecules with benefit or by substituting them with synthetic analogs that stimulate the same targets. The lysate has the potential to stimulate lung defenses through multiple innate immune mechanisms simultaneously, such as formyl peptide receptors, complement receptors, lectin pathways, and Toll-like receptors (TLRs). Stimulation of individual pathways may not induce a comparable level or spectrum of protection because individual pathways may either differentially activate pathogen-specific effector mechanisms or synergistically activate a single mechanism (4, 43).

Others have stimulated lung innate immune mechanisms with varying effects. Lung instillation of endotoxin promoted bacterial clearance and protected against mortality in a rat model of *Pseudomonas aeruginosa* challenge (44), and lung instillation of endotoxin mimetics protected against influenza pneumonia in mice in a TLR4-dependent manner (45). In contrast, TLR4 was found to mediate inflammation but not bacterial elimination in a mouse model of *E. coli* pneumonia (46). CpG oligonucleotides (47), di-GMP (48), heat-killed *E. coli* (49), live *Klebsiella pneumoniae* (50), and a modified heat-labile *E. coli* toxin (51) have all conferred varying degrees of protection against subsequent pathogen challenge. Together, these results suggest that stimulation of lung innate immunity could be a useful therapeutic strategy in patients.

Alternatively, identification of the molecular species in the BALF that confer protection might allow their therapeutic delivery to the lungs, or the delivery of synthetic analogs with similar activity. Proof of this principle is provided by transgenic overexpression of antimicrobial proteins in the lungs of mice to enhance microbial killing and host defense (21, 22). We identified several candidate proteins that might contribute to protection, including lysozyme, lactoferrin, haptoglobin, calgranulin, and surfactant apoprotein D. However, their importance relative to other proteins that may have eluded our proteomic analysis (7), or to nonprotein antimicrobial mechanisms such as induction of an oxidizing milieu in the lung lining fluid (52, 53), will require further analysis. Somewhat surprisingly, we did not identify defensins in our proteomic analyses (Table 2), despite a marked increase in proteins of the appropriate mobility by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Furthermore, only a minority of proteins were found to be increased by all three proteomic techniques, indicating that each technique identified only a subset of up-regulated proteins. Together, these data suggest we have not yet identified all possible effector mechanisms. The lack of induction of the airway mucin gene, Muc5ac, is surprising in view of its increased expression from exposure of cells to NTHi lysate or neutrophil elastase *in vitro* (54, 55), but indicates that a protective antimicrobial response can occur without inducing harmful mucin hypersecretion that often accompanies airway inflammation (11, 28).

In summary, our studies reveal great functional plasticity of the lungs of mice in defense against a virulent bacterial pathogen through activation of innate immune mechanisms. Therapeutic manipulation of this functional plasticity of the lungs may be possible. However, a caveat in translating the results of our studies into human subjects is that innate immune responses of mice differ in detail from those of humans. This is true both at the level of stimulus sensing (e.g., the mouse TLR11 ortholog in humans is a pseudogene) (56), and at the level of effector responses (e.g., stimulation of mycobacterial killing within macrophages by bacterial lipopeptides depends primarily on nitric oxide in mice but on cathelicidin in humans) (52). Thus, the therapeutic efficacy of stimuli identified in mice will require confirmation in human subjects.

Conflict of Interest Statement: C.G.C., and B.F.D., and M.J.T. are the inventors of the subject matter disclosed in the patent application "Compositions and Methods for Stimulation of Lung Innate Immunity" filed by the Board of Regents of the University of Texas System. S.E.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.M.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.R.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.L.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Blaga Iankova for her technical assistance, Marcy Johnson and L. Todd Weiss for assistance with statistical analysis, Daniel M. Musher (Baylor College of Medicine, Houston, TX) for helpful discussions and provision of Spn and NTHi bacterial strains, and Jian-Dong Li (University of Rochester, Rochester, NY) for provision of an NTHi bacterial strain.

References

1. File TM. Community-acquired pneumonia. *Lancet* 2003;362:1991–2001.
2. Joos L, Tamm M. Breakdown of pulmonary host defense in the immunocompromised host: cancer chemotherapy. *Proc Am Thorac Soc* 2005; 2:445–448.
3. World Health Organization. The world health report: 2004—changing history [Internet]. Geneva; The Organization; 2004. Available from: http://www.who.int/whr/2004/en/index.html
4. Martin TR, Frevert CW. Innate immunity in the lungs. *Proc Am Thorac Soc* 2005;2:403–411.
5. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest* 2002;109:571–577.
6. Travis SM, Singh PK, Welsh MJ. Antimicrobial peptides and proteins in the innate defense of the airway surface. *Curr Opin Immunol* 2001;13: 89–95.

7. Rogan MP, Geraghty P, Greene CM, O'Neill SJ, Taggart CC, McElvaney NG. Antimicrobial proteins and polypeptides in pulmonary innate defence. *Respir Res* 2006;7:29.
8. Diamond G, Legarda D, Ryan LK. The innate immune response of the respiratory epithelium. *Immunol Rev* 2000;173:27–38.
9. Bernard GR. Acute respiratory distress syndrome: a historical perspective. *Am J Respir Crit Care Med* 2005;172:798–806.
10. Evans CM, Williams OW, Tuvim MJ, Nigam R, Mixides GP, Blackburn MR, DeMayo FJ, Burns AR, Smith C, Reynolds SD, *et al.* Mucin is produced by clara cells in the proximal airways of antigen-challenged mice. *Am J Respir Cell Mol Biol* 2004;31:382–394.
11. Williams OW, Sharafkhaneh A, Kim V, Dickey BF, Evans CM. Airway mucus: from production to secretion. *Am J Respir Cell Mol Biol* 2006; 34:527–536.
12. Young HW, Sun CX, Evans CM, Dickey BF, Blackburn MR. A3 adenosine receptor signaling contributes to airway mucin secretion after allergen challenge. *Am J Respir Cell Mol Biol* 2006;35:549–558.
13. Young HW, Williams OW, Chandra D, Bellinghausen LK, Perez G, Suarez A, Tuvim MJ, Roy MG, Alexander SN, Moghaddam SJ, *et al.* Central role of Muc5ac expression in mucous metaplasia and its regulation by conserved 5' elements. *Am J Respir Cell Mol Biol* 2007; 37:273–290.
14. Zhen G, Park SW, Nguyenvu LT, Rodriguez MW, Barbeau R, Paquet AC, Erle DJ. IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am J Respir Cell Mol Biol* 2007;36:244–253.
15. Diamond G, Russell JP, Bevins CL. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proc Natl Acad Sci USA* 1996;93:5156–5160.
16. Hertz CJ, Wu Q, Porter EM, Zhang YJ, Weismuller KH, Godowski PJ, Ganz T, Randell SH, Modlin RL. Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol* 2003;171:6820–6826.
17. Bals R, Wang X, Meegalla RL, Wattler S, Weiner DJ, Nehls MC, Wilson JM. Mouse beta-defensin 3 is an inducible antimicrobial peptide expressed in the epithelia of multiple organs. *Infect Immun* 1999;67: 3542–3547.
18. Becker MN, Diamond G, Verghese MW, Randell SH. CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. *J Biol Chem* 2000;275:29731–29736.
19. Singh PK, Jia HP, Wiles K, Hesselberth J, Liu L, Conway BA, Greenberg EP, Valore EV, Welsh MJ, Ganz T, *et al.* Production of beta-defensins by human airway epithelia. *Proc Natl Acad Sci USA* 1998; 95:14961–14966.
20. Wang X, Zhang Z, Louboutin JP, Moser C, Weiner DJ, Wilson JM. Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. *FASEB J* 2003;17:1727–1729.
21. Akinbi HT, Epaud R, Bhatt H, Weaver TE. Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice. *J Immunol* 2000;165:5760–5766.
22. Bals R, Weiner DJ, Moscioni AD, Meegalla RL, Wilson JM. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect Immun* 1999;67:6084–6089.
23. Cole AM, Liao HI, Stuchlik O, Tilan J, Pohl J, Ganz T. Cationic polypeptides are required for antibacterial activity of human airway fluid. *J Immunol* 2002;169:6985–6991.
24. Cole AM, Thapa DR, Gabayan V, Liao HI, Liu L, Ganz T. Decreased clearance of Pseudomonas aeruginosa from airways of mice deficient in lysozyme M. *J Leukoc Biol* 2005;78:1081–1085.
25. Moser C, Weiner DJ, Lysenko E, Bals R, Weiser JN, Wilson JM. beta-Defensin 1 contributes to pulmonary innate immunity in mice. *Infect Immun* 2002;70:3068–3072.
26. Moron CG, Tuvim MJ, Evans CM, Dickey BF. Stimulation of innate immune defense protects against *S. pneumoniae* infection [abstract]. *Proc Am Thorac Soc* 2005;2:A452.
27. Scott BL, Clement C, Evans SE, Gilbert BE, Peterson JW, Tuvim MJ, Dickey BF. Stimulation of lung innate immunity protects against a broad range of infectious microbes [abstract]. *Mol Biol Cell* 2007;18:A1336.
28. Moghaddam SJ, Clement CG, De la Garza MM, Zou X, Travis EL, Young HW, Evans CM, Tuvim MJ, Dickey BF. *Haemophilus influenzae* lysate induces aspects of the chronic obstructive pulmonary disease phenotype. *Am J Respir Cell Mol Biol* 2007.
29. Kingma PS, Whitsett JA. In defense of the lung: surfactant protein A and surfactant protein D. *Curr Opin Pharmacol* 2006;6:277–283.
30. Homer RJ, Zhu Z, Cohn L, Lee CG, White WI, Chen S, Elias JA. Differential expression of chitinases identify subsets of murine airway epithelial cells in allergic inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L502–L511.
31. Lin CS, Chang CH, Huynh T. The murine L-plastin gene promoter: identification and comparison with the human L-plastin gene promoter. *DNA Cell Biol* 1997;16:9–16.
32. Klempt M, Melkonyan H, Hofmann HA, Sorg C. Identification of epithelial and myeloid-specific DNA elements regulating MRP14 gene transcription. *J Cell Biochem* 1999;73:49–55.
33. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 2006;124: 849–863.
34. Song J, Bishop BL, Li G, Duncan MJ, Abraham SN. TLR4 initiated and cAMP mediated abrogation of bacterial invasion of the bladder. *Cell Host Microbe* 2007;1:287–298.
35. Hajjar AM, Harowicz H, Liggitt HD, Fink PJ, Wilson CB, Skerrett SJ. An essential role for non-bone marrow-derived cells in control of *Pseudomonas aeruginosa* pneumonia. *Am J Respir Cell Mol Biol* 2005;33:470–475.
36. Holtzman MJ, Morton JD, Shornick LP, Tyner JW, O'Sullivan MP, Antao A, Lo M, Castro M, Walter MJ. Immunity, inflammation, and remodeling in the airway epithelial barrier: epithelial-viral-allergic paradigm. *Physiol Rev* 2002;82:19–46.
37. Sadikot RT, Zeng H, Joo M, Everhart MB, Sherrill TP, Li B, Cheng DS, Yull FE, Christman JW, Blackwell TS. Targeted immunomodulation of the NF-kappaB pathway in airway epithelium impacts host defense against Pseudomonas aeruginosa. *J Immunol* 2006;176:4923–4930.
38. Pastva AM, Wright JR, Williams KL. Immunomodulatory roles of surfactant proteins A and D: implications in lung disease. *Proc Am Thorac Soc* 2007;4:252–257.
39. Legarda D, Klein-Patel ME, Yim S, Yuk MH, Diamond G. Suppression of NF-kappaB-mediated beta-defensin gene expression in the mammalian airway by the Bordetella type III secretion system. *Cell Microbiol* 2005;7:489–497.
40. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, Davis RW, Rahme LG. Profiling early infection responses: Pseudomonas aeruginosa eludes host defenses by suppressing antimicrobial peptide gene expression. *Proc Natl Acad Sci USA* 2005;102:2573–2578.
41. Iwasaki A. Mucosal dendritic cells. *Annu Rev Immunol* 2007;25:381–418.
42. Hackett CJ. Innate immune activation as a broad-spectrum biodefense strategy: prospects and research challenges. *J Allergy Clin Immunol* 2003;112:686–694.
43. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 2007;7:179–190.
44. Jean D, Rezaiguia-Delclaux S, Delacourt C, Leclercq R, Lafuma C, Brun-Buisson C, Harf A, Delclaux C. Protective effect of endotoxin instillation on subsequent bacteria-induced acute lung injury in rats. *Am J Respir Crit Care Med* 1998;158:1702–1708.
45. Cluff CW, Baldridge JR, Stover AG, Evans JT, Johnson DA, Lacy MJ, Clawson VG, Yorgensen VM, Johnson CL, Livesay MT, *et al.* Synthetic Toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. *Infect Immun* 2005;73:3044–3052.
46. Lee JS, Frevert CW, Matute-Bello G, Wurfel MM, Wong VA, Lin SM, Ruzinski J, Mongovin S, Goodman RB, Martin TR. TLR-4 pathway mediates the inflammatory response but not bacterial elimination in E. coli pneumonia. *Am J Physiol Lung Cell Mol Physiol* 2005;289: L731–L738.
47. Deng JC, Moore TA, Newstead MW, Zeng X, Krieg AM, Standiford TJ. CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary Klebsiella infection. *J Immunol* 2004;173:5148–5155.
48. Karaolis DK, Newstead MW, Zeng X, Hyodo M, Hayakawa Y, Bhan U, Liang H, Standiford TJ. Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. *Infect Immun* 2007;75:4942–4950.
49. Empey KM, Hollifield M, Garvy BA. Exogenous heat-killed Escherichia coli improves alveolar macrophage activity and reduces Pneumocystis carinii lung burden in infant mice. *Infect Immun* 2007;75:3382–3393.
50. Ben David I, Price SE, Bortz DM, Greineder CF, Cohen SE, Bauer AL, Jackson TL, Younger JG. Dynamics of intrapulmonary bacterial growth in a murine model of repeated microaspiration. *Am J Respir Cell Mol Biol* 2005;33:476–482.
51. Williams AE, Edwards L, Humphreys IR, Snelgrove R, Rae A, Rappuoli R, Hussell T. Innate imprinting by the modified heat-labile toxin of Escherichia coli (LTK63) provides generic protection against lung infectious disease. *J Immunol* 2004;173:7435–7443.
52. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C, *et al.* Toll-like receptor triggering of

a vitamin D-mediated human antimicrobial response. *Science* 2006; 311:1770–1773.

53. Forteza R, Salathe M, Miot F, Forteza R, Conner GE. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2005;32:462–469.
54. Fischer BM, Voynow JA. Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. *Am J Respir Cell Mol Biol* 2002;26:447–452.
55. Wang B, Lim DJ, Han J, Kim YS, Basbaum CB, Li JD. Novel cytoplasmic proteins of nontypeable Haemophilus influenzae up-regulate human MUC5AC mucin transcription via a positive p38 mitogen-activated protein kinase pathway and a negative phosphoinositide 3-kinase-Akt pathway. *J Biol Chem* 2002;277:949–957.
56. Lauw FN, Caffrey DR, Golenbock DT. Of mice and man: TLR11 (finally) finds profilin. *Trends Immunol* 2005;26:509–511.

Stimulation of Lung Innate Immunity Protects Against Lethal Pneumococcal Pneumonia in Mice

Cecilia G. Clement, Scott E. Evans, Christopher M. Evans, David Hawke, Ryuji Kobayashi, Paul R. Reynolds, Seyed Javad Moghaddam, Brenton L. Scott, Ernestina Melicoff, Roberto Adachi, Burton F. Dickey, and Michael J. Tuvim

ONLINE DATA SUPPLEMENT

SUPPLEMENTAL METHODS

Endotoxin Measurement and Administration. Endotoxin levels in the *NTHi* lysate were measured using the PyroGene Assay kit, and purified *E. coli* endotoxin for aerosol treatment was dissolved in PBS (both from Cambrex).

Depletion of Neutrophils and Alveolar Macrophages. Rat monoclonal antibody RB6-8C5 against mouse Ly6G (Becton Dickinson), cytosine arabinoside, busulfan, 5-fluorouracil, and cyclophosphamide (Sigma-Aldrich) were tested in the dose regimens shown in Table E1, and their efficacies in depleting neutrophil influx into *NTHi*-stimulated BALF are shown in Fig. E3. To deplete alveolar macrophages, 100 μl of liposome-encapsulated clodronate (Sigma-Aldrich) or liposome-encapsulated PBS as control was delivered intranasally to sedated mice 1, 2 and 3 days prior to infection with *Spn*, as described (Reference E1).

Proteomic Analyses. BALF was centrifuged at 15,000 x *g* for 5 min, supernatants were lyophilized and resuspended in 150 μl H_20, then samples were precipitated with 600 μl acetone at -20°C and spun at 15,000 x *g* for 15 min. For preliminary analysis, pellets were resuspended in 100 μl H_20, bringing the protein concentration to ~1 mg/ml, then SDS-PAGE was performed using 4-15% gradient Tris-HCL Ready Gels (Bio-Rad) loaded with 20 μg of protein per lane, run at 100 V for 2 h, then stained with Coomassie Blue-R250 (Bio-Rad).

HPLC Analysis of BALF. HPLC was performed on a Hewlett-Packard 1090 binary gradient machine (Agilent) using a 1 mm x 25 cm C18 column (Vydac). BALF pellets were resuspended in 100 μl H_20 and loaded on the column, then eluted with a water/acetonitrile gradient containing 0.1% trifluoroacetic acid run at 120 μl/min, and monitored by UV absorbance at both 214 nm and 295 nm. Fractions were manually collected, reduced in volume by vacuum centrifugation, and digested with 200 ng sequencing-grade modified trypsin (Promega) in 30 mM ammonium bicarbonate overnight at 37°C. The resulting peptides were analyzed by LC-MS/MS using electrospray ionization (ESI) quadrupole time-of-flight (TOF) mass spectrometry (QSTAR, Applied Biosystems) and ESI ion trap mass spectrometry (LTQ, Thermo-Finnigan). Proteins were identified by database searching against the non-redundant NCBI protein database using Mascot (Matrix Science).

Difference Gel Electrophoresis (DIGE) Analysis of BALF. DIGE analysis was performed mostly as described (Reference E2). In brief, precipitated BALF proteins were dissolved in denaturing lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% Triton X-100, 10 mM DTT, and 10 mM HEPES, pH 8.0, all from Sigma-Adrich). Particulate matter was removed by centrifugation at 12,000 x *g* for 15 min at 4°C, then 100 μg of each of two protein samples to be compared were labeled on lysine residues with either Cy3 or Cy5 fluorescent dyes (Integrated Core Facility, University of Pittsburgh). The labeled samples were loaded into 24 cm Immobiline pH 3-10 isoelectric focusing strips (Amersham Biosciences), and the first dimension gels were focused to 70,000 volt-h using an Ettan IPGphor power source (Amersham). Second dimension gels were 10% acrylamide, and were run on an EttanDalt Six apparatus (Amersham). Images were acquired

on a Typhoon scanner (Amersham), and downloaded into ImageJ, a freeware program available at rsb.info.nih.gov/ij/. Cy3 and Cy5 images were stacked, and a two-frame movie was evaluated visually for changes in spot intensity. At least two independent comparisons were performed to identify repeatable differences. Gels were post-stained with colloidal Coomassie Blue (BioRad), and proteins differentially expressed were excised from the gel as 1.5 mm diameter plugs with a OneTouch manual spot picker (The Gel Company, San Francisco, CA). Tryptic digestion was performed on the gel pieces and the peptide solutions were evaporated and dried. Matrix-assisted laser desorption/ionization (MALDI) TOF mass spectrometry was performed on a Voyager-DE Pro workstation (Applied Biosystems), the filtered peak list was analyzed using Mascot, and ambiguous protein identifications were established on a 4700 MALDI-TOF-TOF instrument (Applied Biosystems).

Quantitative Isobaric Stable Isotope Tag (iTRAQ) Analysis. iTRAQ analysis was performed mostly as described (Reference E3). In brief, BALF precipitates were resuspended in 20 μl of reaction buffer (0.1% SDS, 20 mM PBS, pH 8), reduced with 2 mM Tris(2-carboxyethyl) phosphine, alkylated with 10 mM S-methyl methanethiosulfonate, and digested overnight with trypsin. The control digest was then derivatized with the iTRAQ114 isobaric reagent, and the *NTHi*-stimulated digest with iTRAQ117 (Applied Biosystems). The derivatized digests were combined and analyzed by LC-MS/MS on the QSTAR-Pulsar-i instrument. Data were analyzed either manually by database search and inspection of the spectra, or using ProQuant software (Applied Biosystems).

SUPPLEMENTAL RESULTS

Figure E1.

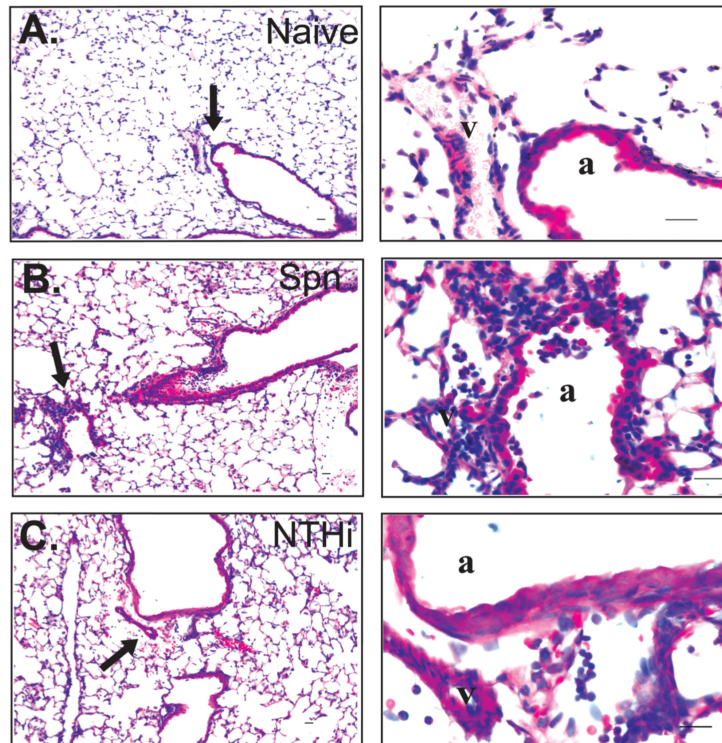


Figure E1. Histopathology of the Lungs of Mice after Challenge with *Spn* or Treatment with *NTHi* Lysate. Photomicrographs of hematoxylin and eosin stained lungs of naïve mice (A), mice 24 h after challenge with high dose *Spn* (B), or mice 24 h after treatment with *NTHi* lysate (C). Areas indicated by arrows in the low magnification images in the left column are also shown at higher magnification in the right column, with scale bars indicating 20 μm. Structures in the high magnification images are labeled "a" for airway and "v" for blood vessel.

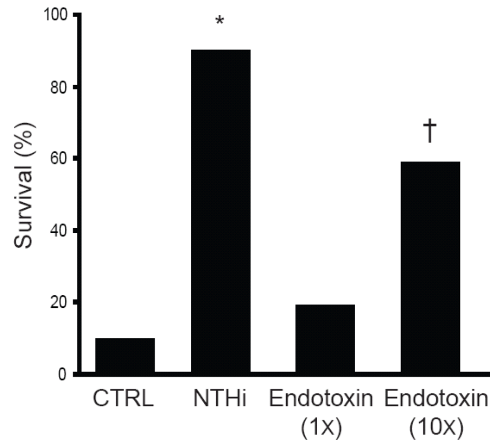


Figure E2. Protection Against Spn Challenge by Endotoxin. Mice were either left untreated (CTRL), or treated with aerosolized *NTHi* lysate for 20 min (NTHi), aerosolized endotoxin at a concentration equal to that in the *NTHi* lysate for 20 min (Endotoxin 1x), or aerosolized endotoxin at a concentration ten times that in the *NTHi* lysate for 20 min (Endotoxin 10x) (* $p = 0.001$, † $p = 0.057$, treated vs. untreated).

Agent	Dose	Timing (days)	Route	Mortality
RB6-8C5	50 μg	-1, 0	IV	0/6
Ara-C (1)	300 mg/kg	-8, -5, -2, -1	IP IP	0/6
Ara-C (2)	600 mg/kg	-5, -2, -1, 0	IP	1/6
Busulfan (1)	125 mg/kg	-8, -5, -2, -1	IP	2/6
Busulfan (2)	125 mg/kg	-11, -8, -6, -4, -1	IP	6/6
5-Fluorouracil (1)	150 mg/kg	-8, -5, -2, -1	IP	4/6
5-Fluorouracil (2)	150 mg/kg	-8, -3	IP	2/6
Cyclophosphamide	200 mg/kg	-5, -2, -1	IP	2/6

Table E1. Neutrophil Depletion Regimens. Mice were pretreated with various regimens to reduce neutrophil recruitment to the lungs. Abbreviations are: RB6-8C5 – rat monoclonal antibody against mouse neutrophils, Ara-C – cytosine arabinoside, IV – intravenously, IP - intraperitoneally. The timing of doses is listed as the number of days prior to *NTHi* treatment (day 0). Mortality was assessed at 48 h, and surviving mice were then sacrificed.

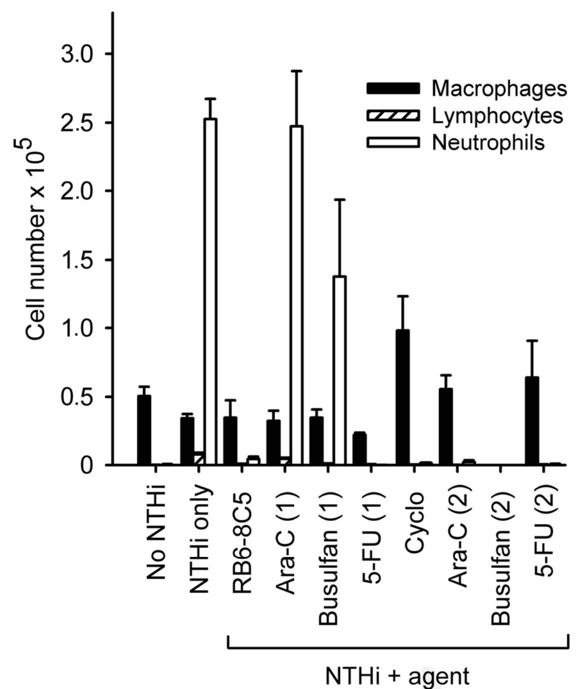
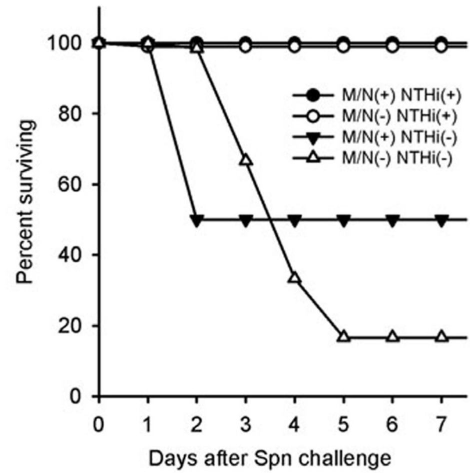
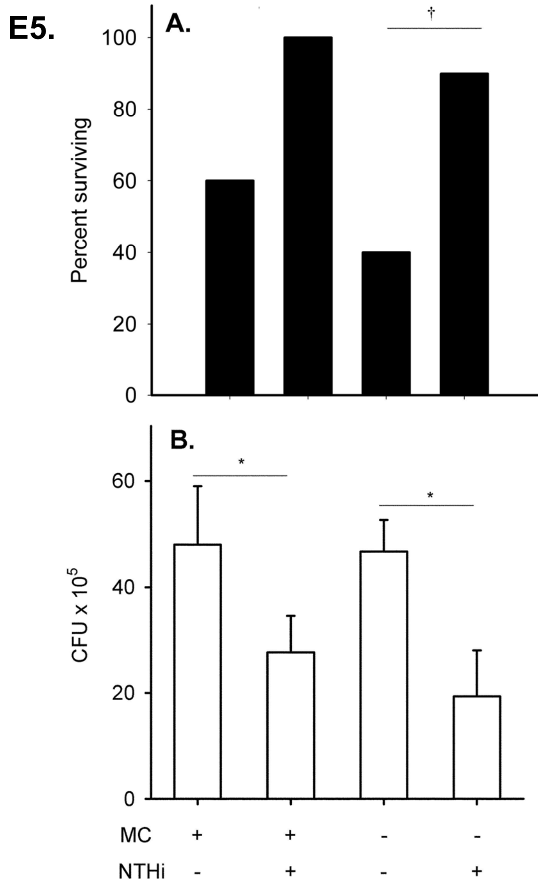


Figure E3. Inflammatory Cell Counts in BALF of Mice Pretreated to Reduce Neutrophils, then Treated with *NTHi* Lysate. Mice in groups of 6 each were pretreated with regimens listed in Supplemental Table E1 to reduce neutrophil recruitment to the lungs. They were then exposed for 20 min to the aerosolized *NTHi* lysate, and 24 h later BALF was recovered and inflammatory cells counted (mean ± SEM).

Figure E4. Host Survival and Lung Bacterial Counts in Mice Deficient in Alveolar Macrophages and Neutrophils. The same data as those illustrated in Figure 6 are shown here as a function of time to illustrate the delayed time to death in M/N deficient mice not protected by *NTHi* treatment.





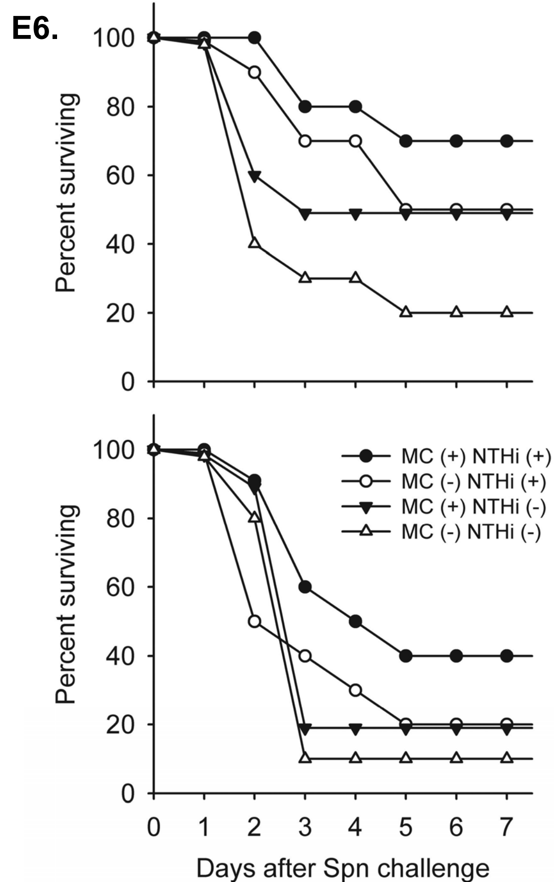


Figure E5. Host Survival and Lung Bacterial Counts in Mast Cell Deficient Mice. Mast cell deficient C57BL/6 $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (MC-) and littermate mast cell sufficient C57BL/6 Kit^{+}/Kit^{+} (MC+) mice were treated with *NTHi* lysate (NTHi+) or PBS (NTHi-). Mice were then challenged 6 h later with *Spn* 4.5×10^{10} CFU/ml. Shown is survival at 48 h for 10 mice/group (A), and bacterial culture from the lungs of 3 mice/group immediately after *Spn* aerosol challenge (B) (mean ± SEM; * $p < 0.05$, and † $p = 0.057$ for comparisons indicated by the lines).

Figure E6. Host Survival and Lung Bacterial Counts in Mast Cell Deficient Mice. The same data as those illustrated in Figure E5 are shown here as a function of time (top), and a second experiment with an *Spn* challenge 1.0×10^{11} CFU/ml is also shown (bottom).

Figure E7. Two Dimensional Difference Gel Electrophoresis (DIGE) Analysis of Proteins Present in BALF after Treatment with *NTHi* Lysate. BALF supernatants of mice that were untreated (A) or pretreated 48 h previously with *NTHi* lysate (B) were labeled on lysine residues with Cy3 or Cy5 fluorescent dyes, combined, electrofocused in pH 3-10 isoelectric focusing strips, electrophoresed in 10% acrylamide gels, and Cy3 and Cy5 images acquired separately. Numbers in the representative illustration identify proteins elevated in the treated mice as follows: (1) polymeric immunoglobulin receptor, (2) lymphocyte cytosolic protein 1, (3) haptoglobin, (4) Rho GDI alpha (arghdia), (5) serpin 1A, (6) complement C3, (7) leukotriene E4 hydrolase, (8) enolase 1, (9) pulmonary surfactant-associated protein D, (10) WD repeat domain protein 1, (11) transketolase, (12) glucose phosphate isomerase, (13) chitinase 3-like protein 1, (14) lipocalin 2, (15) lactoferrin. GenBank accession numbers are given in Table 2.

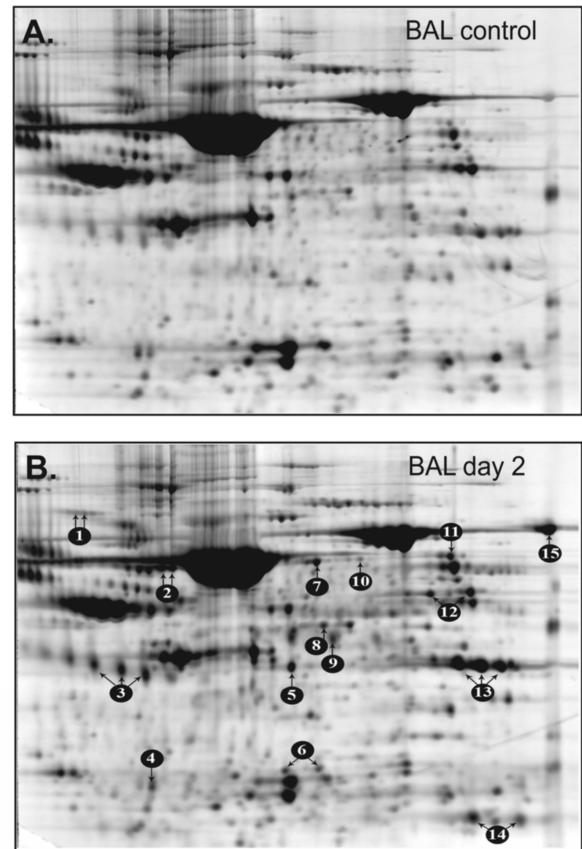
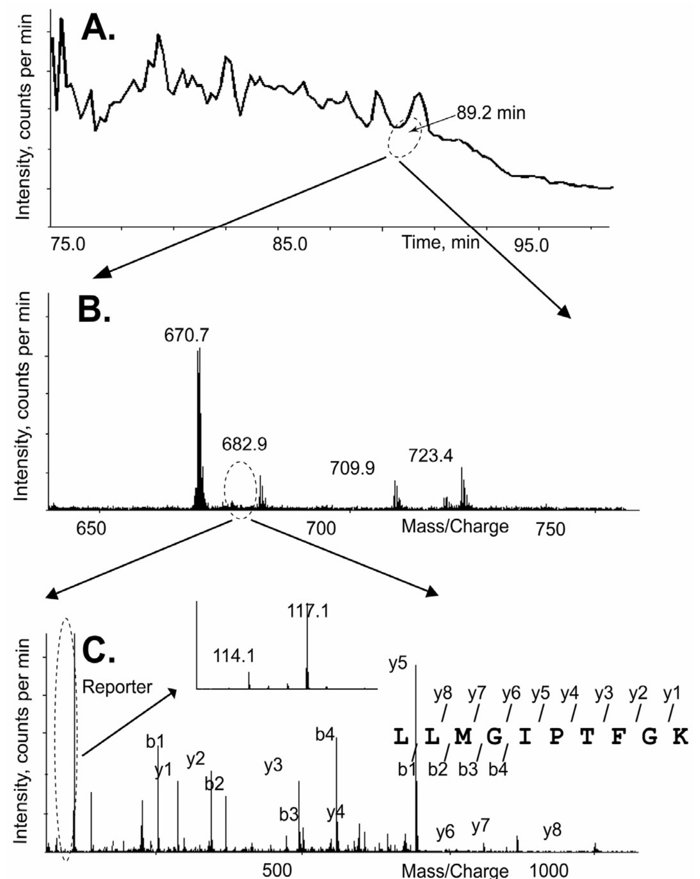


Figure E8. Identification and Relative Quantification of a Peptide from Chitinase-3-Like Protein Using Isobaric Stable Isotope Tag (iTRAQ) Analysis of Proteins Present in BALF after Treatment with *NTHi* Lysate. BALF supernatants were precipitated with acetone, alkykated with methyl methanethiosulfonate, digested with trypsin, and separately derivatized with iTRAQ114 (BAL control) or iTRAQ117 (BAL day 2). The derivatized digests were then combined and analyzed by nano-LC-MS/MS, and proteins identified by database searching. Shown is a representative total ion chromatogram from 74-96 min displaying the sum of the ion-current at each time point (A), the mass spectrum at 89.2 min (B), and a high resolution image of the mass spectrum of an ion with a mass/charge ratio of 682.9 (C). The “y” ions are those that include the C-terminus, the “b” ions are those that include the N-terminus, the inset at the right shows a match with the sequence of chitinase-3-like protein, and the inset above shows the intensity of the 117 reporter peak was 4.6 times that of the 114 peak. This and other proteins found to be elevated in the *NTHi* treated mice using iTRAQ are listed in Table 2.



SUPPLEMENTAL REFERENCES

E1. Cote, C. K., N. Van Rooijen, and S. L. Welkos. 2006. Roles of macrophages and neutrophils in the early host response to Bacillus anthracis spores in a mouse model of infection. *Infect.Immun.* 74:469-480.

E2. Unlu, M., M. E. Morgan, and J. S. Minden. 1997. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 18:2071-2077.

E3. Ross, P. L., Y. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson, and D. J. Pappin. 2004. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol.Cell Proteomics.* 3:1154-1169.