

Stimulated Innate Resistance of Lung Epithelium Protects Mice Broadly against Bacteria and Fungi

Scott E. Evans^{1,5}, Brenton L. Scott¹, Cecilia G. Clement¹, Derek T. Larson¹, Dimitrios Kontoyiannis³, Russell E. Lewis³, P. Rocco LaSala², Jennifer Pawlik⁴, Johnny W. Peterson⁴, Ashok K. Chopra⁴, Gary Klimpel⁴, Gabriela Bowden⁵, Magnus Höök⁵, Yi Xu⁵, Michael J. Tuvim^{1,5}, and Burton F. Dickey^{1,5}

¹Department of Pulmonary Medicine, ²Department of Molecular Pathology, and ³Department of Infectious Diseases, University of Texas, M. D. Anderson Cancer Center, Houston, Texas; ⁴Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas; ⁵Center for Lung Inflammation and Infection, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, Texas

Pneumonia is a serious problem worldwide. We recently demonstrated that innate defense mechanisms of the lung are highly inducible against pneumococcal pneumonia. To determine the breadth of protection conferred by stimulation of lung mucosal innate immunity, and to identify cells and signaling pathways activated by this treatment, mice were treated with an aerosolized bacterial lysate, then challenged with lethal doses of bacterial and fungal pathogens. Mice were highly protected against a broad array of Gram-positive, Gram-negative, and class A bioterror bacterial pathogens, and the fungal pathogen, *Aspergillus fumigatus*. Protection was associated with rapid pathogen killing within the lungs, and this effect was recapitulated *in vitro* using a respiratory epithelial cell line. Gene expression analysis of lung tissue showed marked activation of NF- κ B, type I and II IFN, and antifungal *Card9-Bcl10-Malt1* pathways. Cytokines were the most strongly induced genes, but the inflammatory cytokines TNF and IL-6 were not required for protection. Lung-expressed antimicrobial peptides were also highly up-regulated. Taken together, stimulated innate resistance appears to occur through the activation of multiple host defense signaling pathways in lung epithelial cells, inducing rapid pathogen killing, and conferring broad protection against virulent bacterial and fungal pathogens. Augmentation of innate antimicrobial defenses of the lungs might have therapeutic value for protection of patients with neutropenia or impaired adaptive immunity against opportunistic pneumonia, and for defense of immunocompetent subjects against a bioterror threat or epidemic respiratory infection.

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

Among both healthy and immunosuppressed people, pneumonia is the leading cause of premature death in the world (1–3). Nosocomial pneumonias result in more deaths than any other hospital infection (4), and impose a particular burden on

CLINICAL RELEVANCE

Pneumonia results in significant worldwide mortality. Stimulating antimicrobial signaling to induce broad protection against respiratory pathogens has novel scientific and clinical implications for protection against opportunistic, bioterror, and epidemic infections.

immunocompromised patients, such as those with hematologic malignancies and others undergoing cytotoxic therapies (5–7). The lower respiratory tract is also a frequent locus of emerging infections, such as severe acute respiratory syndrome (8) or epidemic influenza (9), and of difficult to manage pathogens, such as multiple drug resistant *Pseudomonas aeruginosa* or extensively drug resistant *Mycobacterium tuberculosis*. Furthermore, the respiratory route is the most likely portal of entry for bioterror agents, and the only means by which such pathogens have been delivered successfully in the modern era (i.e., inhalation anthrax) (10, 11).

Although the need for gas exchange leaves the lungs structurally vulnerable to infection, they successfully contain and eliminate most pathogens. The airway and alveolar epithelia, in particular, are critical to the prevention of infection through their capacity to recruit leukocytes and to initiate antimicrobial innate immune responses (12–15). We have recently found that stimulation of lung innate immune mechanisms with an aerosolized bacterial lysate confers a high level of protection against challenge with otherwise lethal inocula of *Streptococcus pneumoniae* (16). Pretreatment with the lysate induces complete protection against challenge if given 4 to 24 hours before challenge. Lesser levels of protection occur at shorter and longer intervals between treatment and challenge, and some protection occurs even when treatment follows challenge. Protection does not depend on recruited neutrophils or resident mast cells and alveolar macrophages, and is specific to the airway route of infection. The survival benefit correlates in magnitude and time with rapid bacterial killing within the lungs, and is associated with increased concentrations of numerous antimicrobial polypeptides in lung lining fluid. We have termed the protective phenomenon, stimulated innate resistance (StIR).

In contrast to the highly refined epitope sensing of adaptive immune responses, innate immunity relies on rapid recognition of pathogen-associated molecular patterns (PAMPs) shared across many pathogen species (17–19). Suppression of adaptive immunity and hematopoiesis, as observed in hematologic malignancy and/or cytotoxic chemotherapy, does not obviate the lung's innate immune response to PAMPs. Rather, profoundly immunosuppressed patients generate remarkable cytokine responses to pneumonia (20), and we have shown that

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Correspondence and requests for reprints should be addressed to Scott E. Evans, M.D., Department of Pulmonary Medicine, Unit 403, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. E-mail: seevans@mdanderson.org

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innate lung defenses are highly inducible in mice receiving myeloablative chemotherapy (16). Because of the characteristic breadth of innate immunity, and the complexity of the lysate used to stimulate protection against *S. pneumoniae*, the survival effect induced by the aerosolized bacterial lysate might be expected to extend to other bacterial pathogens, or even to other microbial classes. Here, we tested this hypothesis by treating mice with the lysate before challenge with numerous respiratory pathogens, including nosocomial pathogens and National Institute of Allergy and Infectious Diseases class A bioterror agents. We find dramatic protection against diverse bacterial and fungal pathogens, and that this effect appears to arise from the simultaneous induction of multiple antimicrobial pathways in the epithelium. These findings could provide a basis for novel clinical strategies to protect immunosuppressed patients from fatal infectious complications, and to safeguard broader populations from emerging infections or bioterror assaults.

MATERIALS AND METHODS

Bacterial Lysate Aerosol Treatment

Nontypeable *Haemophilus influenzae* (NTHi) was stored, grown, and harvested as previously described (16, 21). In the current work, the cell pellet was washed and resuspended in 20 ml 0.9% sodium chloride solution. This suspension was passed three times through an Emulsi-Flex C5 cell disruptor (Avestin, Ottawa, ON, Canada) at greater than 10,000 psi, then diluted to 4–5 mg/ml in 0.9% sodium chloride solution by bicinchoninic assay (Pierce, Rockford, IL), and centrifuged at $15,000 \times g$ for 10 minutes. The supernatant was collected, the protein concentration was adjusted to 2.5 mg/ml, and the lysate was sterilized by passage through a 0.2- μ m filter and frozen in 8 ml aliquots at -80°C . Treatment of mice with aerosolized lysate was performed as previously described (16).

Animals

Experiments with *S. pneumoniae*, *P. aeruginosa*, *Staphylococcus aureus*, and *Aspergillus fumigatus* were conducted at M. D. Anderson Cancer Center using specific pathogen-free, 5- to 9-week-old female BALB/c mice (Harlan, Indianapolis, IN), except for the experiments with IL-6-deficient C57BL/6 *il6*^{-/-} and littermate C57BL/6 *il6*^{+/+} mice of either sex that were bred within our colony and used at 5 to 8 weeks of age. Experiments with *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* were conducted at the University of Texas Medical Branch in Galveston using 6- to 10-week-old female Swiss-Webster mice (Taconic, Hudson, NY). Mice were handled in accordance with the institutional animal care and use committees of each institution, and were killed if distressed.

Bacterial Pneumonia Challenges

For the clinical pathogens, the challenges were targeted to inocula that induced 80–100% mortality (LD₈₀–LD₁₀₀). *S. pneumoniae* serotype 4, *P. aeruginosa* strain PA103, and *Klebsiella pneumoniae* serotype 2 were suspended in PBS and delivered by aerosol using an AeroMist nebulizer driven by 10 L/min of 5% CO₂ in air for 60 minutes. This was similar to the NTHi lysate exposure, except that an additional 5 ml of the bacterial suspensions were added to the nebulizer after 30 minutes, in addition to the 10 ml placed at the beginning, with a total of 10 ml delivered. Pantone-Valentine leukocidin (PVL) toxin-producing methicillin-resistant *S. aureus* USA300 was suspended in PBS, and 20 μ l was instilled into the left naris of mice anesthetized with an intraperitoneal injection of 2.5% tribromoethanol (16 μ l/g). For the bioterror pathogens, the dose was targeted to $5 \times \text{LD}_{50}$. Ames strain *B. anthracis* spores, *Y. pestis* CO92, and *F. tularensis* Schu 4 were delivered by nasal instillation (22, 23). Mice were anesthetized by intraperitoneal injection of ketamine (48 mg/kg) and xylazine (9.6 mg/kg), then suspended vertically by their upper incisors, and 20 μ l of pathogen suspension was instilled into each naris, followed by 10 μ l of PBS into

each naris. All survival studies were performed using groups of 10 mice per condition.

A. *fumigatus* Challenge

Conidia of strain Af293 were stored as frozen stock (1×10^9 conidia/ml) in 20% glycerol in PBS (24). A 1-ml aliquot of stock was plated on yeast extract agar plates at 37°C in 5% CO₂ for 3 days, then harvested by gentle scraping in PBS containing 0.1% Tween-20. The suspension was filtered through 40- μ m filters, centrifuged at $2,500 \times g$ for 10 minutes, washed, resuspended in PBS, and aerosolized as for *S. pneumoniae* challenge. Conidia counts in the nebulizer suspension were determined using a hemacytometer. Mice were immunosuppressed by intraperitoneal injection of cyclophosphamide (75 mg/gm) and cortisone acetate (300 mg/kg) 4 days and 1 day before challenge with *A. fumigatus* (24). For assessment of lung fungal burden 24 hours after challenge, real-time quantitative PCR for *A. fumigatus* 18S rRNA was performed as previously described (24), and *Aspergillus* galactomannan was measured by a single-incubation sandwich ELISA, as detailed in the online supplement.

Gene Expression Analysis

C57BL/6 mice were treated, or not, with the aerosolized NTHi lysate, then killed after 2 or 4 hours. To reduce leukocytes, the lung vasculature was perfused and the airways lavaged with PBS. The lungs were homogenized, total RNA was isolated, and cRNA was synthesized, amplified, labeled, and hybridized on Sentrix Mouse-6 Expression BeadChips (Illumina, San Diego, CA). Primary microarray data were deposited at the National Center for Biotechnology Information Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>, accession number GSE13685) consistent with minimum information about a microarray experiment standards, and were analyzed using an ANOVA-based scheme after quantile normalization between samples and conditions, followed by pathway analysis using multiple strategies described in the MATERIALS AND METHODS in the online supplement.

Host Response to Pathogen Challenge

Lung lavage, cell counts, and bronchoalveolar lavage (BAL) cytokine ELISAs were performed as previously described (16, 21). For histological analysis, the aortas of anesthetized mice were transected, and 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 10–15 cm H₂O pressure at 21°C . The lungs were removed from the thoracic cavity and further fixed overnight at 4°C , then serially dehydrated and embedded in paraffin, sectioned, and stained for light microscopy with hematoxylin and eosin or Gomori methanamine silver. For all pathogens, bacterial concentrations in nebulizers, nasal instillate, and lung tissue were determined by plating serial dilutions. For experiments involving anti-TNF antibody, mice were injected with 1 mg of rat/mouse chimeric monoclonal antibody cV1q (Centocor, Horsham, PA) in 100 μ l PBS 5 or 24 hours before NTHi lysate treatment, as indicated. TNF blocking efficacy was confirmed by prevention of TNF-induced neutrophil infiltration after intranasal instillation of recombinant mouse TNF (PeproTech, Rocky Hill, NJ), as previously described (21).

Epithelial Cell Response to Treatment

MLE-15 murine lung epithelial cells were cultured in six-well plates ($\sim 3 \times 10^6$ cells/well) in RPMI supplemented with 10% FCS and penicillin/streptomycin. When grown to confluence, media were changed to serum- and antibiotic-free RPMI, and the cells were treated (or not) with NTHi lysate at a concentration of 100 μ g/ml for 4 or 24 hours before inoculation with ~ 100 CFU *S. pneumoniae* serotype 4 or 1,200 *B. anthracis* Sterne spores. Pathogen burden was assessed 4 hours after infection by serial dilution culture of the media. Alternate wells without MLE-15 cells were infected with or without addition of NTHi lysate to demonstrate the absence of intrinsic antibiotic effects of the lysate. To further characterize the response to treatment, we investigated differential gene expression in isolated epithelial cells. The analysis was performed identically to the whole-lung studies, harvesting total RNA from MLE-15 cells 2 hours after treatment with 100 μ g/ml

NTHi lysate or equal volume of PBS. As with the whole-lung studies, the primary data were deposited at the National Center for Biotechnology Information GEO (accession number GSE13740).

Statistical Analysis

Summary statistics for bacterial counts in lung tissue were compared using Student's *t* test. Proportions of mice surviving pathogen challenges were compared using Fisher's exact test, and log-rank comparisons of survival distribution were performed using Kaplan-Meier estimation. All data shown are representative of at least two independent experiments, and were not combined for analysis, because of differences in microbial challenge doses. Analyses were performed using SAS/STAT (SAS Institute, Cary, NC).

RESULTS

Broad Protection against Bacterial Clinical Pathogens

Given the striking protection conferred against *S. pneumoniae* by NTHi lysate, and the characteristic breadth of innate immune responses, we tested whether stimulation with the aerosolized NTHi lysate protected against additional bacterial pathogens. The gram-negative bacilli, *P. aeruginosa* and *K. pneumoniae*, are important causes of pneumonia in hospitalized and immunocompromised patients (3), and gram-positive methicillin-resistant *S. aureus* expressing Pantone-Valentine leukocidin is a highly virulent bacterium that causes severe pneumonia in both the community and the hospital (25). Aerosolization of the NTHi lysate 18 to 24 hours before bacterial challenge provided nearly complete protection against lethality

from aerosolized *P. aeruginosa* and *K. pneumoniae*, or intranasally instilled *S. aureus* (Figures 1A–1C, left). Pretreatment with the NTHi lysate resulted in rapid killing of all three bacterial species within the lungs (Figures 1A–1C, right), similar to our prior observations with *S. pneumoniae*. NTHi lysate-induced reductions in lung pathogen burden immediately after infection were associated with clearance of pathogens from the lungs and survival, whereas sham-treated mice experienced progressive lung infections and sepsis (see Table E1 in the online supplement).

Broad Protection against Bacterial Bioterror Pathogens

Our observation of protection against highly pathogenic gram-positive and gram-negative clinical organisms suggests that protection might also extend to agents of bioterror. *B. anthracis* is the most likely agent to be used in a bioterror attack, because it is highly virulent, available from infected soil worldwide, and its spores are stable and readily aerosolized (22, 23). Intranasal instillation of $5 \times \text{LD}_{50}$ *B. anthracis* Ames strain spores caused 100% mortality in mice that did not receive NTHi lysate pretreatment, but <20% mortality in pretreated mice (Figure 1D, left). Protection was associated with a significant reduction in lung bacterial CFUs, even though *B. anthracis* was delivered as a spore; however, the degree of reduction was less than with the other bacteria (Figure 1D, right). Pretreatment with the aerosolized NTHi lysate similarly reduced mortality and lung bacterial burden from intranasal instillation of $5 \times \text{LD}_{50}$ *Y. pestis* (Figure 1E). NTHi lysate pretreatment also increased the time to median mortality, and reduced the lung bacterial burden

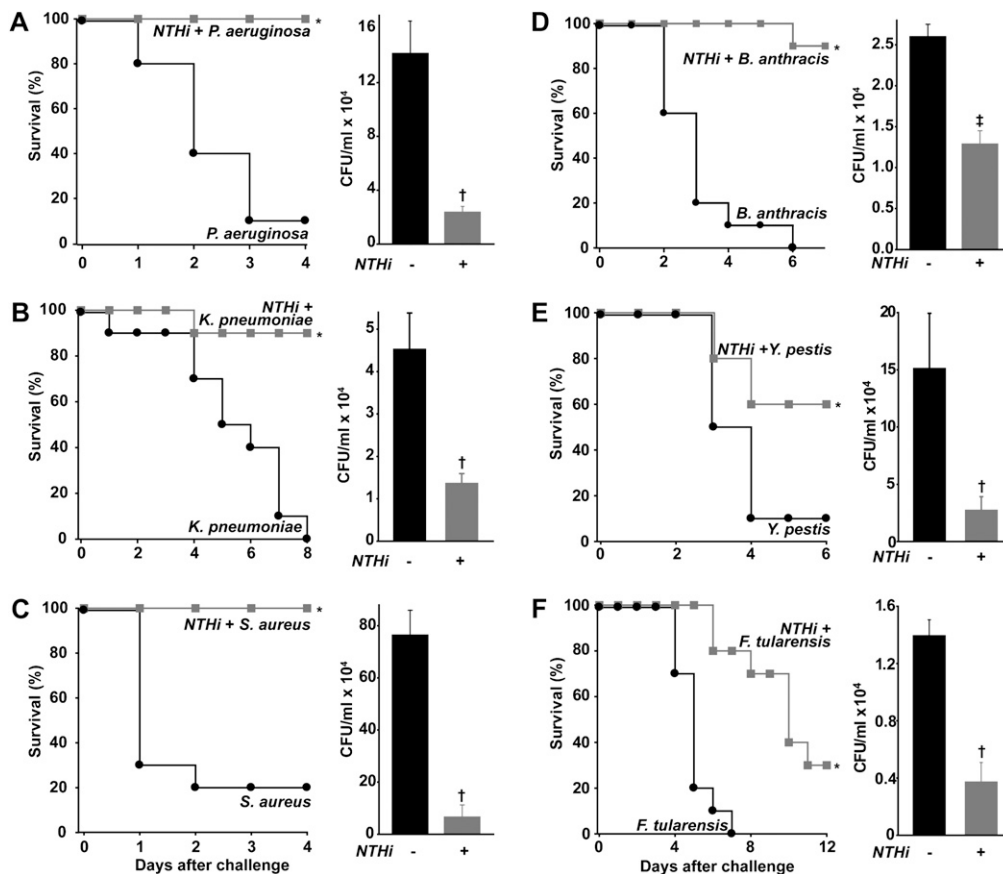


Figure 1. Aerosolized nontypeable *Haemophilus influenzae* (NTHi) lysate protects broadly against bacterial pathogens. Mice, with or without NTHi lysate pretreatment 24 hours earlier, were challenged with clinical pathogens, aerosolized *Pseudomonas aeruginosa* (3×10^{10} CFU/ml) (A), aerosolized *Klebsiella pneumoniae* (1×10^{10} CFU/ml) (B), or intranasal Pantone-Valentine leukocidin-producing methicillin-resistant *Staphylococcus aureus* (5×10^7 CFU) (C), or with the bioterror pathogens, intranasal *Bacillus anthracis* Ames spores (5×10^4 CFU) (D), intranasal *Yersinia pestis* (1.7×10^3 CFU) (E), or intranasal *Francisella tularensis* (2.2×10^3 CFU) (F). Shown for each pathogen are mouse survival (left) and lung pathogen burden 1 hour after challenge (right), except for (E) and (F), where pathogen burden was measured 24 hours after challenge due to very small inocula. All mice were observed for at least 3 days beyond the period shown, and no additional mortality was observed. In all experiments, survival was significantly better for NTHi lysate-treated mice on the last day of observed mortality ($P < 0.001$; $n = 10$ mice per group for survival studies), except with *F. tularensis* ($P = 0.1$). NTHi lysate-treated mice in all experiments, including *F. tularensis*, showed greater Kaplan-Meier-estimated median survival ($*P < 0.001$ compared with sham-treated mice). Lung pathogen burden is reported as the mean (\pm SEM) ($†P < 0.01$, $‡P < 0.05$; $n = 4$ –5 mice per group for bacterial counts).

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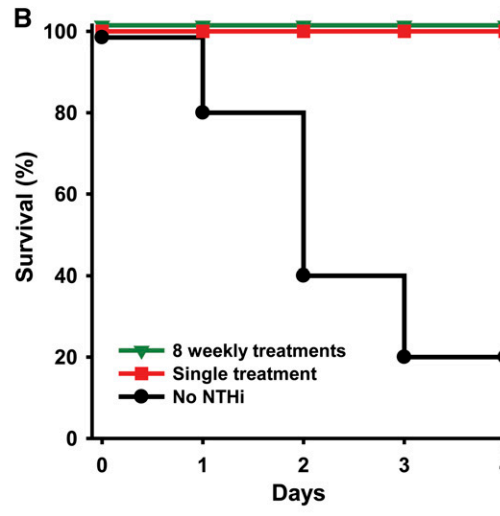
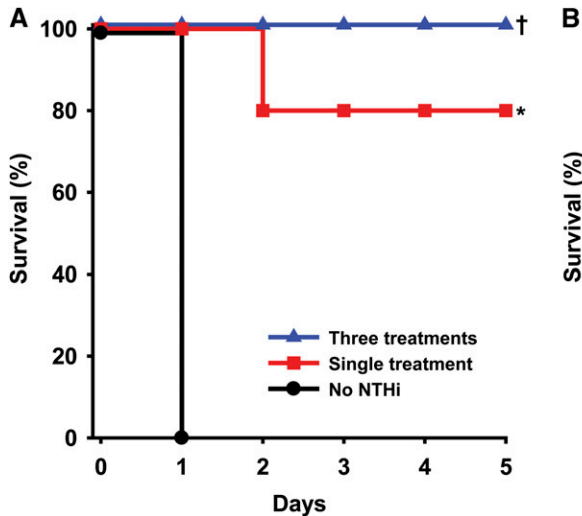


Figure 2. Stimulated innate resistance (StIR) is not associated with tachyphylaxis. (A) Before challenge with *P. aeruginosa* (3×10^{10} CFU/ml), Swiss-Webster mice were treated with NTHi lysate 7, 4, and 1 day before the infection, or only once 1 day before infection, or with PBS alone. (B) C57BL/6 mice were treated with eight weekly NTHi lysate treatments, only one treatment, or PBS before challenge with *P. aeruginosa* (3.3×10^{10} CFU/ml) ($n = 10$ mice/group; $*P < 0.001$, $\dagger P = 0.0007$ compared with no NTHi).

after intranasal instillation of $5 \times LD_{50}$ *F. tularensis*, although it did not significantly reduce overall mortality from this intracellular pathogen (Figure 1F).

Bacterial Protection without Tachyphylaxis

Depending on the length of potential exposure to an infectious threat, the time course of bacterial protection conferred by StIR may necessitate repetitive treatments of an at-risk population (16). Consistent with our recently reported findings of stimulated resistance to influenza virus after multiple treatments (26), no tachyphylaxis of the bacterial-protective effect was observed with repetitive treatments. Whether mice were exposed to multiple treatments in 1 week, or to weekly treatments for 8 weeks, we observed robust protection against bacterial pathogens (Figure 2).

Protection against a Fungal Pathogen

We next tested whether NTHi lysate-induced protection would extend to a nonbacterial pathogen. Pneumonia due to *A.*

fumigatus is an important cause of mortality in immunocompromised patients (3), so protection against this fungus was tested. Mice that were not immunosuppressed experienced no mortality from an aerosolized inoculum of 3×10^8 conidia/ml (data not shown), but mice immunosuppressed with cyclophosphamide and cortisol experienced approximately 70% mortality (Figure 3A). Pretreatment with the NTHi lysate reduced mortality to less than 10% (Figure 3A). Using a quantitative galactomannan assay (Figure 3B) and a quantitative RT-PCR assay for *Aspergillus* 18S rRNA (Figure 3C), the lung pathogen burden was found to be reduced approximately 10-fold 1 day after infection. Histopathologic evaluation of unpretreated immunosuppressed mice demonstrated abundant hyphal elements with extensive tissue invasion by 24 hours after challenge, in contrast to NTHi lysate-pretreated mice that demonstrated few fungal forms and little inflammation (Figure 3D). This suggests that, similar to protection against bacteria, NTHi lysate-stimulated protection against this fungus arises primarily from the induction of a microbicidal environment rather than the generation of cellular inflammation.

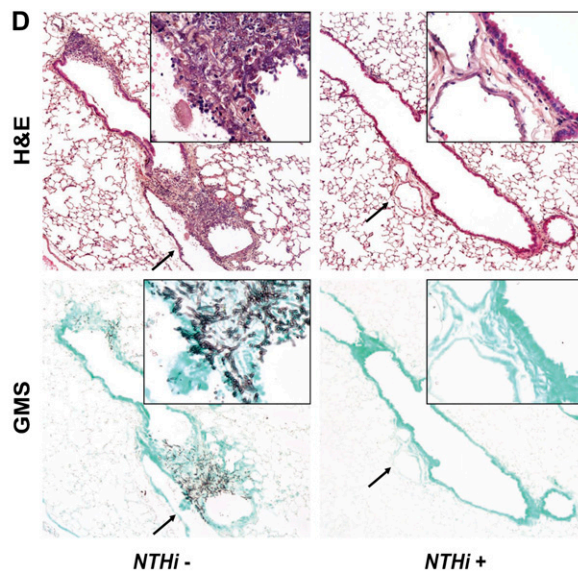
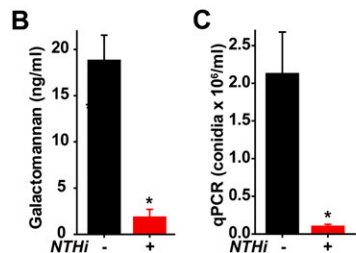
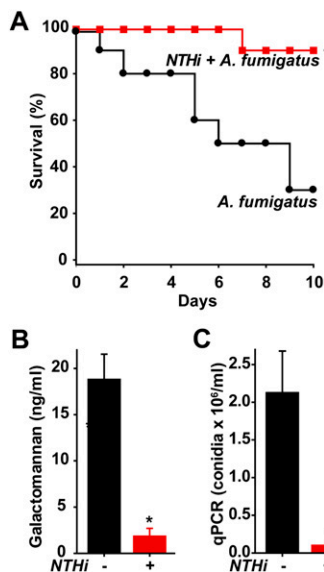


Figure 3. Aerosolized NTHi lysate protects against fungal pneumonia in an immunocompromised host. (A) Neutrophils were suppressed with cyclophosphamide and lymphocytes with cortisol. Mice were then challenged with aerosolized *Aspergillus fumigatus* (3×10^8 conidia/ml) with or without NTHi lysate pretreatment 24 hours earlier, and the time course of survival is shown ($*P < 0.01$ compared with sham-treated mice). (B) ELISA for galactomannan concentration in lung homogenates 24 hours after infection (mean \pm SEM) ($*P = 0.00002$). (C) Quantitative RT-PCR for *A. fumigatus* 18S rRNA in lung homogenates 24 hours after infection (mean \pm SEM) ($*P = 0.0008$). (D) Lung sections from mice challenged 1 day earlier with *A. fumigatus*, with or without NTHi lysate pretreatment. Hematoxylin and eosin staining (top panels) or Gomori methanamine silver staining (bottom panels); arrows indicate the lower left corner of the inset.

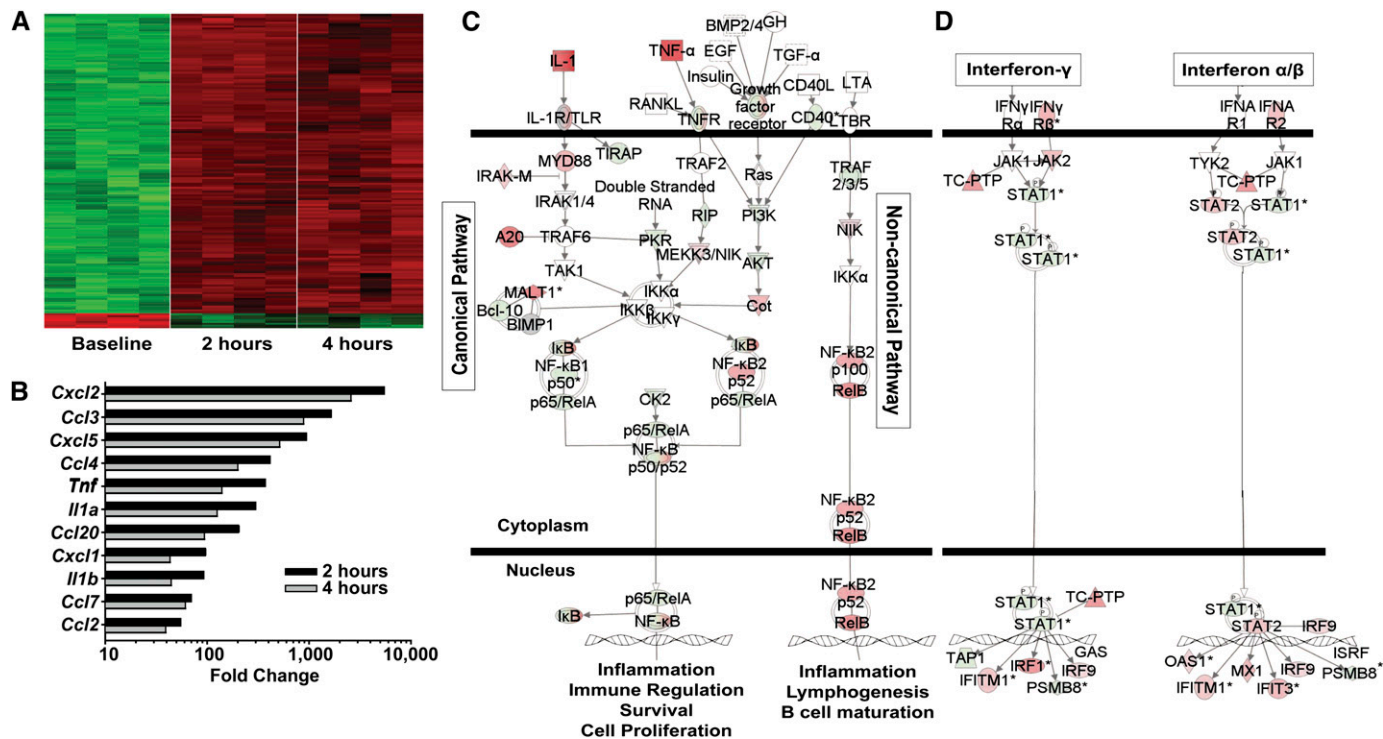


Figure 4. StIR is associated with broad changes in host defense gene expression. (A) Aerosolized NTHi lysate induced differential expression of 164 genes in mouse lungs at 2 hours, with a false discovery rate less than 0.0001; most of these remained differentially expressed at 4 hours. (B) The 11 most strongly up-regulated genes are identified, with fold increase relative to baseline indicated. (C) Pathway analysis revealed NF- κ B activation to be the single most enriched cellular process 2 hours after NTHi lysate treatment ($P < 10^{-13}$). Red, increased gene expression; green, decreased gene expression. (D) Strong induction of γ - and α/β -IFN signaling ($P < 10^{-11}$) predicts antibacterial and antiviral host responses to NTHi lysate treatment.

Protection Is Associated with Increased Expression of Host Defense Genes

To gain insight into the mechanism of protection conferred by StIR, we compared the gene expression profiles of treated and untreated lungs. Maximal protection against *S. pneumoniae* was achieved by 4 hours after treatment (16), so gene expression patterns were examined within this interval. Mice were exposed to the aerosolized NTHi lysate for 20 minutes, then killed at 2 or 4 hours. Because we have previously shown that alveolar macrophages and recruited neutrophils are dispensable for StIR, we sought to maximize our ability to detect gene expression changes in the lung parenchyma. As such, the lungs were lavaged to remove alveolar macrophages and other leukocytes in the airway lumen, and the pulmonary circulation was perfused with PBS to remove intravascular leukocytes. Transcripts isolated from the lungs were then analyzed by whole-genome microarray.

We identified 3,011 differentially expressed genes at 2 hours after treatment using a false discovery rate (FDR) of less than 0.05 (not shown), and 164 genes when applying an FDR of less than 0.0001 (Figure 4A and Table E2). Most differentially expressed genes were up-regulated relative to baseline, most remained differentially expressed at 4 hours, and very few genes were differentially expressed at 4 hours, but not at 2 hours. The differentially expressed genes encode a broad array of proteins involved in host defense, including cytokines and chemokines, pattern recognition receptors, intracellular signaling proteins, antimicrobial polypeptides, reactive oxygen species generators, and numerous other modifiers of the local host environment (Figure 4B, Table E2, and the GEO database).

Pathway analysis demonstrated NF- κ B signaling to be the most enriched process, followed closely by IFN- α/β and γ , IL-

10, IL-6, and Toll-like receptor signaling (Figures 4C–4D). Other engaged networks included the *Card9-Bcl10-Malt1* pathway that is important for antifungal defense (27), phosphatidylinositol 3-kinase (PI3K)/Akt, Janus kinase (JAK)/signal transducers and activators of transcription (STAT) (JAK2, SOCS3, STAT2, STAT3, STAT5a) (also reported in the IFN network), p38 mitogen-activated protein kinase and phosphatase and tensin homolog (PTEN). Together, these results indicate rapid and broad activation of antimicrobial and anti-apoptotic pathways within the lungs.

BAL Inflammatory Cytokines Correlate with Protection, but neither TNF nor IL-6 Is Required

Analysis showed cytokine-encoding genes to be the most highly up-regulated individual transcripts after NTHi lysate treatment (Figure 4B and Table E2). Protein-level inflammatory cytokine concentrations also increased in BAL fluid after treatment, with increases of 1,569-fold for TNF and 186-fold for IL-6 (Figures 5A and 5D) after 4 hours. As protective responses to PAMPs have been shown to be IL-6- and TNF-dependent in other settings (28), we sought to determine whether NTHi lysate-induced resistance was dependent on these inflammatory cytokines. Using an anti-TNF antibody that we have previously shown sufficient to block inflammatory cell infiltration after intratracheal TNF instillation (21), we found no difference in the protection induced by NTHi lysate treatment, nor in induced rapid pathogen killing (Figures 5B and 5C). Although IL-6 $^{-/-}$ mice have an increased baseline susceptibility to bacterial infections relative to wild-type C57BL/6 mice (29), we were still able to induce protection and rapid pathogen killing with NTHi lysate in the mutant mice (Figures 5E and 5F).

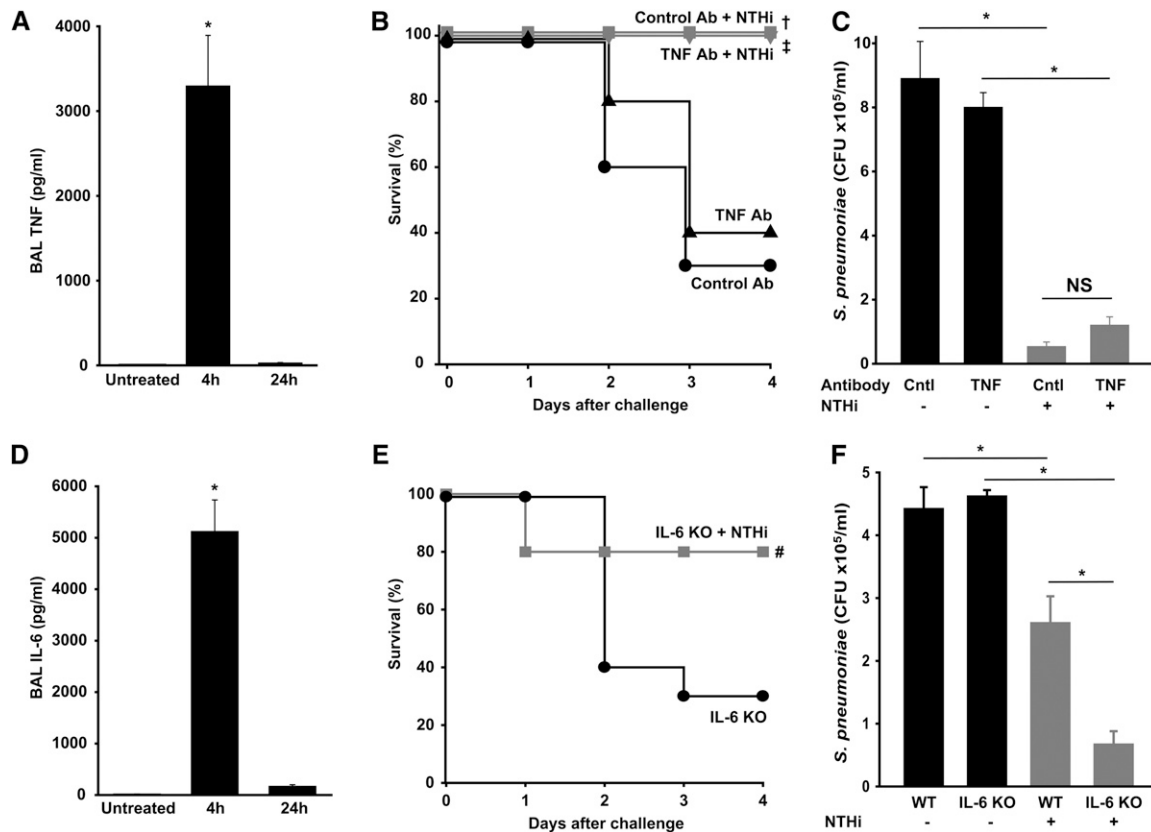


Figure 5. NTHi lysate-induced protection correlates with bronchoalveolar lavage (BAL) cytokine levels, but does not require them. Consistent with our gene expression data, cytokine levels of TNF (A) and IL-6 (D) in BAL fluid dramatically increase after NTHi lysate treatment. (B) Intraperitoneal administration of blocking anti-TNF antibody 24 hours before NTHi treatment did not abrogate protection against *Streptococcus pneumoniae* (3×10^{10} CFU/ml challenge) ($^{\dagger}P < 0.003$ compared with irrelevant IgG, sham-treated; $^{\ddagger}P < 0.005$ compared with anti-TNF antibody, sham-treated). (C) Similarly, anti-TNF antibody given 5 hours before NTHi treatment resulted in no loss of the immediate bacterial killing compared with irrelevant IgG. (E) NTHi lysate-induced protection persisted against *S. pneumoniae* (3×10^{10} CFU/ml challenge) ($^{\#}P < 0.01$ compared with sham treated) in IL-6-deficient mice. (F) There was no loss of rapid pathogen killing compared with wild-type controls. Lung pathogen burden is reported as mean (\pm SEM) ($^*P \leq 0.002$).

Therefore, although inflammatory cytokines rise dramatically after stimulation, they may not be required. These results are consistent with our prior findings that neutrophil influx parallels induction of protection, but is not required (16).

Protection Is Associated with Increased Expression of Lung-Expressed Antimicrobial Genes

We previously postulated the respiratory epithelium to be the most likely effector of StIR in the lung (16), so we investigated whether lung-expressed antimicrobial genes were induced by exposure to NTHi lysate. Table 1 lists 30 transcripts with products known to have antimicrobial activity that are highly up-regulated after NTHi treatment, and that have previously been shown to be expressed by lung cells. As shown, these include a broad array of products with diverse antimicrobial mechanisms, ranging from direct pathogen killing to pathogen opsonization to nutrient sequestration. Many of the product proteins were previously noted to be up-regulated on our proteomic analyses of BAL fluid after NTHi lysate treatment.

Respiratory Epithelial Cells Are Sufficient to Promote an NTHi Lysate-Induced Antimicrobial Environment

As inducible protection is consistently associated with rapid, substantial reductions in pathogen burden, we have inferred that StIR is achieved primarily through promotion of an antimicrobial environment. Epithelial cells are the most likely

mediators of this effect, based on the large epithelial surface area of the lungs, the proximity of the epithelium to pathogens, and the induction of epithelium-expressed antimicrobial genes (Table 1) and proteins (16). Figure 6 shows that pretreatment of MLE-15 respiratory epithelial cells with NTHi lysate for 24 hours substantially reduces the pathogen burden in the culture media when inoculated with *S. pneumoniae*, *B. anthracis*, or *P. aeruginosa* (Figures 6A–6C, respectively). This observation was not restricted to MLE-15 cells. Rather, we consistently observed inducible bacterial killing by a variety of murine and human respiratory epithelial cells (Figure E1). Additional media-only wells (i.e., no MLE-15 cells) confirmed that the antibacterial effect results from stimulation of the epithelial cells, rather than an intrinsic antibiotic effect of the lysate. In fact, the lysate appeared to promote pathogen growth in the absence of stimulated cells (Figures 6D–6F). In contrast to our findings with respiratory epithelial cells, we did not observe similar inducible bacterial killing after treating dendritic cell or macrophage cell lines with NTHi lysate (Figures 6G–6I).

Induction of Inflammatory Cytokines and Broad Gene Expression Changes after NTHi Lysate Treatment of Respiratory Epithelial Cells

Having found that the bacterial killing effect of StIR could be recapitulated by isolated respiratory epithelial cells, we assessed whether this occurred in association with similar changes in

TABLE 1. WHOLE-LUNG NONTYPEABLE *Haemophilus influenzae* LYSATE-INDUCED ANTIMICROBIAL GENE EXPRESSION

Symbol	Definition	2-h Fold Change	4-h Fold Change
<i>Saa1</i>	Serum amyloid A 1 (NM_009117.1)	232.92	491.63
<i>Orm2</i>	Orosomucoid 2 (NM_011016.1)	96.22	200.66
<i>Saa3</i>	Serum amyloid A 3 (NM_011315)	51.66	72.00
<i>Saa2</i>	Serum amyloid A 2 (NM_011314.1)	32.89	61.55
<i>Clec4e</i>	C-type lectin domain family 4, member e (NM_019948.1)	13.56	12.96
<i>Clec4d</i>	C-type lectin domain family 4, member d (NM_010819.1)	8.55	6.34
<i>Serpina3g</i>	Serine proteinase inhibitor clade A member 3G (XM_354694.1)	8.34	10.01
<i>S100a8</i>	Calgranulin A (NM_013650.1)	7.72	2.41
<i>Lcn2</i>	Lipocalin 2 (NM_008491.1)*	7.55	6.95
<i>S100a9</i>	Calgranulin B (NM_009114.1)*	6.50	3.05
<i>C1sb</i>	Complement component 1 s subcomponent (NM_173864.2)	5.41	3.77
<i>Orm1</i>	Orosomucoid 1 (NM_008768.1)	5.00	7.67
<i>Selp</i>	P-selectin (NM_011347.1)	4.89	6.13
<i>Hpxn</i>	Hemopexin (NM_017371.1)*	4.71	3.96
<i>Ltf</i>	Lactotransferrin (NM_008522.2)*	3.73	2.91
<i>Defb5</i>	Defensin beta 5 (NM_030734.1)	3.61	2.70
<i>Clec7a</i>	C-type lectin domain family 7, member a (NM_030734.1)	3.29	3.07
<i>Adm</i>	Adrenomedullin (NM_009627.1)	3.08	2.13
<i>C1rl</i>	Complement component 1 r subcomponent-like (NM_181344.2)	2.02	1.44
<i>Selpg</i>	P-selectin ligand (AK089188)	1.94	0.83
<i>Camp</i>	Cathelicidin antimicrobial peptide (NM_009921.1)	1.88	0.72
<i>Serpib1a</i>	Serine proteinase inhibitor clade B member 1a (NM_025429.1)*	1.79	0.71
<i>Hp</i>	Haptoglobin (NM_017370.1)*	1.75	1.67
<i>Defcr-rs2</i>	Defensin related cryptdin related sequence 2 (NM_007847.1)	1.74	1.35
<i>Defb34</i>	Defensin beta 34 (NM_183035.1)	1.65	1.21
<i>Defa1</i>	Defensin alpha 1 (NM_010031.2)	1.63	1.31
<i>Colec12</i>	Collectin subfamily member 12 (NM_130449.1)	1.63	0.08
<i>Defb39</i>	Defensin beta 39 (NM_183038)	1.61	2.27
<i>C3</i>	Complement component 3 (NM_009778.1)*	1.54	2.03
<i>Chi3l1</i>	Chitinase 3-like 1 (NM_007695.1)*	1.52	1.74

Nontypeable *Haemophilus influenzae* (NTHi) lysate up-regulated antimicrobial genes were checked against expression sequence-tagged National Center for Biotechnology Information databases to identify those expressed in the lung. The top 30 lung-expressed antimicrobial genes are shown, ranked by fold-change over baseline at 2 hours after NTHi lysate treatment.

* Protein products previously shown to be induced by NTHi lysate exposure (16).

gene expression. After treatment of MLE-15 cells with NTHi lysate or PBS (sham), we again found induction of broad gene expression changes in the NTHi lysate-treated cells, including many of the same inflammatory cytokines induced in the whole-lung model (Figures 7A and 7B). The induction of inflammatory cytokines by treatment was also confirmed at the protein level (Figures 7C and 7D). Using an FDR of 0.01, we found 207 differentially expressed transcripts after NTHi lysate treatment. In the interval between the *in vivo* and *in vitro* analyses, the commercially available Illumina Sentrix Mouse-6 BeadChip was changed to a new version (v1.0b to v1.1). To compare the whole-lung gene expression patterns to those of the *in vitro* studies, while accounting for minor changes in the probe sets, we decided to compare only the best-described transcripts. As such, we compared the RefSeq-annotated transcripts induced by NTHi lysate treatment with an FDR of less than 0.05. This yielded 379 common differentially expressed transcripts, of

which 318 (83.9%) changed in a similar manner (i.e., were either both up- or down-regulated) in both models. Considering the potential effectors of the bacterial killing effect, we looked specifically at the antimicrobial peptides in Table 1. A total of 18 of the 30 (60.0%) antimicrobial peptide transcripts identified in whole-lung model were also increased when the epithelial cells were stimulated in isolation (Table 2). Of these, five transcripts were identified to be increased in all three analyses performed to date (proteomic analysis of mouse BAL fluid after treatment [16], gene expression analysis of whole mouse lungs after treatment, and gene expression analysis of epithelial cells *in vitro*). These were chitinase 3-like 1, lipocalin 2, hemopexin, haptoglobin, and calgranulin B.

DISCUSSION

We show here that stimulation of the lungs with a complex mixture of bacterial products results in rapid activation of multiple antimicrobial pathways. This activation is associated with broad and powerful protection against respiratory challenge by gram-negative bacterial, gram-positive bacterial, and fungal pathogens. Mechanistically, we support the hypothesis that the principal means of protection is through elaboration of antimicrobial products by the lung epithelium.

StIR

The activation of multiple pattern recognition receptors, which our gene expression data suggest occurs after NTHi lysate treatment, can result in synergizing, tolerizing, or priming interactions at the cellular level (28, 30–32). However, for the whole organism, survival of a microbial challenge relies on only two host strategies: tolerance (the ability to limit the damage caused by the pathogen); and resistance (the ability to limit the pathogen burden) (33, 34). Although previous studies, including our own studies with endotoxin, have shown limited resistance stimulated with single Toll-like receptor ligands (16, 35–37), treatment with NTHi lysate results in profound protection from otherwise lethal pneumonias caused by disparate classes of organisms. For all investigated pathogens, protection is associated with significant and rapid reductions in pathogen burden. We have applied to this phenomenon the descriptor, StIR.

Stimulated Resistance against Clinical Pathogens

Pneumonia with gram-negative, gram-positive, and fungal pathogens is a common and often fatal complication among immunocompromised patients (3, 38, 39). The use of prophylactic antibiotics to prevent infection cultivates microbial antibiotic resistance and causes host drug toxicity. We have previously demonstrated that protection against bacterial pneumonia by stimulation of lung innate immunity does not depend upon recruited neutrophils. Here, we similarly find that the aerosolized lysate protects against fungal pneumonia, despite neutrophil suppression with cyclophosphamide and cortisol (Figure 3). Therefore, patients undergoing myeloablative chemotherapy as part of cancer treatment, or in preparation for hematopoietic stem cell transplantation, might benefit from stimulation of lung innate immunity during vulnerable periods of transient, severe myelosuppression. Because the augmentation of innate immune defenses is independent of adaptive immune function, the protective response can be induced even in the setting of T-cell suppression by fludarabine, or immunosuppression with calcineurin inhibitors or alkylating agents.

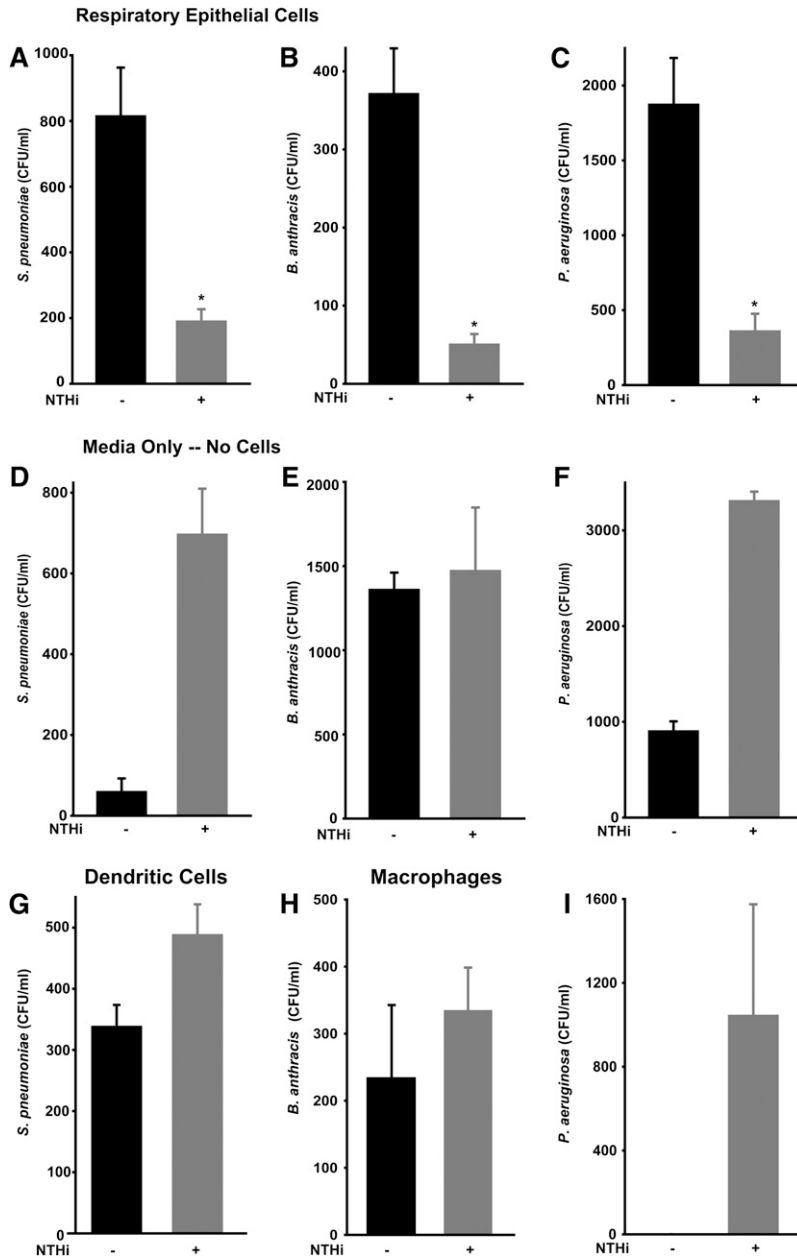


Figure 6. Respiratory epithelial cells are sufficient to generate an antimicrobial response to NTHi lysate. MLE-15 murine lung epithelial cells were treated with 100 μ g/ml NTHi lysate for 24 hours, then inoculated with (A) *S. pneumoniae* (110 CFU), (B) *B. anthracis* (1,500 spores), or (C) *P. aeruginosa* (13 CFU). Six-well plates containing 500 μ l MLE-15 growth media, but no cells, were treated (or not) for 4 hours with NTHi lysate at 100 μ g/ml, then infected with (D) *S. pneumoniae* (67 CFU), (E) *B. anthracis* (1,500 spores), or (F) *P. aeruginosa* (115 CFU). (G) Approximately 1×10^6 JAWSII dendritic cells cultured in six-well plates were treated for 4 hours with PBS or NTHi lysate at 100 μ g/ml, then infected with *S. pneumoniae* (200 CFU). Approximately 3×10^6 RAW 264.7 macrophage cells were cultured in six-well plates and treated for 4 hours with PBS or NTHi lysate at 100 μ g/ml, then infected with (H) *B. anthracis* (2,000 spores) or (I) *P. aeruginosa* (100 CFU). Values reported as mean (\pm SEM) 4 hours after inoculation (* $P < 0.005$).

Stimulated Resistance against Bioterror Pathogens

The 2001 anthrax attacks in the United States highlighted the challenges of defending populations against bioterror agents (40, 41). Presently, only military personnel and first responders have access to anthrax vaccination in the United States. Even if an adequate vaccine stockpile could be maintained, as anti-anthrax acquired immunity requires months to develop, such vaccination would not protect the general population acutely (40). Furthermore, vaccines are not available for all potential bioterror agents (42). Perhaps most important, early in an attack, the identity of a pathogen may not be immediately evident, delaying the determination of appropriate preventative and/or postexposure therapies (40). Therefore, the broad protection conferred by stimulation of innate immunity is attractive for management of large populations in the event of a bioterror attack. We have demonstrated increased mean survival for every bioterror pathogen against which we have tested.

Mechanisms of StIR

The current studies provide our first insight into the cellular effectors of the resistant phenotype in the lung. Whereas neutrophils, mast cells, and alveolar macrophages are expendable in NTHi lysate-induced protection, we show here *in vitro* that respiratory epithelial cells are sufficient to sense the treatment and to generate an antimicrobial response. Having direct contact with the treatment, close apposition to deposited pathogens, and established capacity to generate antimicrobial products, the respiratory epithelium seems well suited to serve the StIR effector function *in vivo*. Although the 11 most strongly up-regulated genes after treatment encode cytokines, common mediators of inflammation, such as TNF and IL-6, are not required for induced resistance. This is consistent with our previous finding that neutrophil infiltration is not required for protection. On the other hand, our proteomic (16) and genomic (Table 1) analyses indicate that the respiratory epithelium

TABLE 2. *IN VITRO* NONTYPEABLE *Haemophilus influenzae* LYSATE-INDUCED ANTIMICROBIAL GENE EXPRESSION

Symbol	Definition	Fold Change
<i>Saa3</i>	Serum amyloid A 3 (NM_011315)	9.54
<i>Chi311</i>	Chitinase 3-like 1 (NM_007695.1)*	5.21
<i>Lcn2</i>	Lipocalin 2 (NM_008491.1)*	4.25
<i>Defa1</i>	Defensin alpha 1 (NM_010031.2)	2.79
<i>Defb34</i>	Defensin beta 34 (NM_183035.1)	2.77
<i>Hpxn</i>	Hemopexin (NM_017371.1)*	2.69
<i>Serpina3g</i>	Serine proteinase inhibitor clade A member 3G (XM_354694.1)	2.38
<i>Selp</i>	P-selectin (NM_011347.1)	1.60
<i>Colec12</i>	Collectin subfamily member 12 (NM_130449.1)	1.49
<i>Hp</i>	Haptoglobin (NM_017370.1)*	1.44
<i>Adm</i>	Adrenomedullin (NM_009627.1)	1.41
<i>C1rl</i>	Complement component 1 r subcomponent-like (NM_181344.2)	1.38
<i>Clec4d</i>	C-type lectin domain family 4, member d (NM_010819.1)	1.38
<i>Clec4e</i>	C-type lectin domain family 4, member e (NM_019948.1)	1.26
<i>Defcr-rs2</i>	Defensin related cryptdin related sequence 2 (NM_007847.1)	1.21
<i>S100a9</i>	Calgranulin B (NM_009114.1)*	1.17
<i>Camp</i>	Cathelicidin antimicrobial peptide (NM_009921.1)	1.12
<i>Saa2</i>	Serum amyloid A 2 (NM_011314.1)	1.10

The antimicrobial transcripts up-regulated in Table 1 were evaluated in MLE-15 murine lung epithelial cells 2 hours after treatment with 100 μ g/ml nontypeable *Haemophilus influenzae* lysate. The 18 up-regulated transcripts are shown.

* Candidate effectors identified on proteomic screens of BAL fluid, whole-lung gene expression analysis, and *in vitro* gene expression analysis.

expresses numerous antimicrobial products in response to NTHi lysate treatment.

Our gene expression analyses identified several intriguing StIR candidate mediators. The three known mouse acute-phase serum amyloid A (SAA) genes (43) comprise three of the four most highly up-regulated lung antimicrobial genes after *in vivo* NTHi lysate treatment. These enigmatic apolipoproteins promote host defense through widely varied mechanisms, including cathelicidin-like direct killing, pathogen opsonization, and induction of leukocyte chemotaxis (44–46). Although only *Saa3* was significantly up-regulated on the *in vitro* array, it is notable that the other two transcripts were higher at the 4-hour than the 2-hour time point in the *in vivo* studies, so it is possible that we had not observed the maximal expression at 2 hours. Also up-regulated are the lipocalins, orosomucoid 1, orosomucoid 2, and lipocalin 2, that have recently been shown critical in host defenses against bacterial pathogens (47–49). Their protective functions seem to result mainly from iron sequestration (48, 49), although lipocalin 2 also appears key to IL-22-induced defenses in the respiratory epithelium (47). Lipocalin 2 is one of five antimicrobial effectors that have been found to be up-regulated on all of our proteomic and gene expression screens after NTHi lysate treatment, making it an interesting candidate. However, we have not seen similarly broad or robust protection in previous studies addressing the antimicrobial effects of lipocalin 2. Therefore, we suspect that, if lipocalin 2 does participate in NTHi lysate-induced StIR, it is probably one of several antimicrobial effectors. The SAA proteins, the lipocalins, and the other antimicrobial products that we identified on these analyses will each be carefully explored as potential effectors, as they may play different roles with different pathogens. Investigations to identify novel NTHi-induced antimicrobial effectors are also underway, as well as exploration of other possible effector mechanisms, such as augmented barrier function.

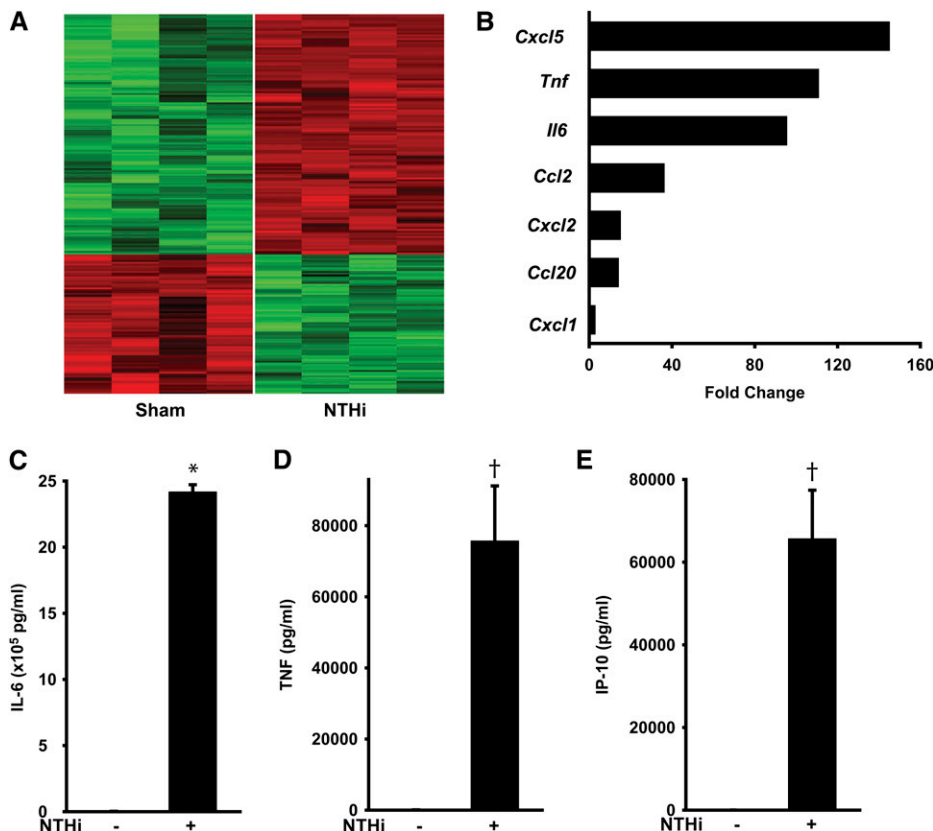


Figure 7. Broad induction of gene expression changes in NTHi lysate-treated respiratory epithelial cells. MLE-15 cells were treated with 100 μ g/ml NTHi lysate for 2 hours, then RNA was harvested to analyze treatment-induced gene expression changes. A total of 207 differentially expressed transcripts were identified (A). Red, increased gene expression; green, decreased gene expression. These included many of the same inflammatory cytokines found to be up-regulated in the whole-lung model (B). These changes were confirmed at the protein level (C–E). Cytokine levels are reported as mean (\pm SEM) (* P < 0.0001, $\dagger P$ < 0.01).

Whether StIR is a purely epithelial phenomenon in the lung remains to be seen. We found that epithelial cells in isolation can be stimulated to kill bacteria, and that this is associated with gene expression patterns and cytokine elaboration that is very similar to that noted in the whole lung system, supporting our hypothesis that the epithelium is critical to StIR. In striking contrast, macrophages and dendritic cells could not be stimulated by the lysate to directly kill extracellular bacteria *in vitro* (Figure 6), consistent with our prior findings that macrophages are not required for *in vivo* StIR (16). Nonetheless, dendritic cells may sense the stimulus *in vivo* and reinforce the epithelial effector response through local signaling via IL-22/STAT3-dependent mechanisms (47, 50, 51).

In summary, we have demonstrated that stimulation of lung innate immunity provides protection of remarkable breadth against respiratory pathogens. Therapeutic manipulation of StIR may be possible to reduce the burden of respiratory infections in immunosuppressed patients, and to reduce the risk of infection of the general public during a bioterror attack with a pathogen introduced via the respiratory route.

Conflict of Interest Statement: M.H. is a cofounder of Pulmotect, Inc., owns shares in the company, and has received a \$50,000 research grant from the company. D.K. has been reimbursed by Merck, Pfizer, Enzon, and Astellas (2008–2009, <\$10,000 total) for participating as a speaker and/or lecturer in various meetings, symposiums, and conferences, and advisory board meetings. He has also received industry grants from Astellas and Merck in excess of \$20,000 each. C.G.C., M.J.T., and B.F.D. are 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. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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