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Synergistic Interactions of TLR2/6 and TLR9 Induce a High Level of Resistance to Lung Infection in Mice

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Infectious pneumonias exact an unacceptable mortality burden worldwide. Efforts to protect populations from pneumonia have focused historically on antibiotic development and vaccine-enhanced adaptive immunity. However, we have reported recently that the lungs' innate defenses can be induced therapeutically by inhalation of a bacterial lysate that protects mice against otherwise lethal pneumonia. In this study, we tested in mice the hypothesis that TLRs are required for this antimicrobial phenomenon and found that resistance could not be induced in the absence of the TLR signaling adaptor protein MyD88. We then attempted to recapitulate the protection afforded by the bacterial lysate by stimulating the lung epithelium with aerosolized synthetic TLR ligands. Although most single or combination treatments yielded no protection, simultaneous treatment with ligands for TLR2/6 and TLR9 conferred robust, synergistic protection against virulent Gram-positive and Gram-negative pathogens. Protection was associated with rapid pathogen killing in the lungs, and pathogen killing could be induced from lung epithelial cells in isolation. Taken together, these data demonstrate the requirement for TLRs in inducible resistance against pneumonia, reveal a remarkable, unanticipated synergistic interaction of TLR2/6 and TLR9, reinforce the emerging evidence supporting the antimicrobial capacity of the lung epithelium, and may provide the basis for a novel clinical therapeutic that can protect patients against pneumonia during periods of peak vulnerability. *The Journal of Immunology*, 2011, 186: 5916–5926.

Despite decades of antibiotic development and hygiene programs, pneumonia continues to affect hundreds of millions of people annually and remains the leading cause of infectious mortality worldwide (1–4). In the course of normal ventilation, the sterile lower respiratory tract is recurrently exposed to inhaled pathogens, often resulting in serious infections (5–7). Although the lungs have been regarded traditionally as passive gas exchange barriers, it is now apparent that they have robust intrinsic defense mechanisms that prevent the incidence of lower respiratory tract infections from being much higher (6–13).

Epithelial surfaces that are constantly in contact with microbes, such as the colonic mucosa, constitutively generate antimicrobial effectors to modulate their local microbiome (14). Because lung

epithelial cells are exposed only intermittently to pathogens and because chronic immune activation would negatively impact gas exchange, their baseline innate immune activity is relatively low. However, when exposed to pathogens, the lung epithelium rapidly responds by enhancing barrier function, recruiting leukocytes, and expressing antimicrobial products (13).

Viewing this epithelial plasticity as an opportunity for intervention, we recently demonstrated that the lungs' antimicrobial defenses are therapeutically inducible. The inhalational pretreatment of mice with an aerosolized lysate of nontypeable *Haemophilus influenzae* (NTHi) protected against otherwise lethal pneumonia from a variety of pathogens (15–18). Supporting an innate immune mechanism underlying this inducible resistance was the observation of protection against pathogens noncognate with the stimulus, protecting against all of the tested bacterial, fungal, and viral organisms (15, 17, 18). Furthermore, induction of resistance occurred too rapidly for an adaptive immune response (onset within 2 h of treatment, maximal by 24 h) and did not rely upon innate immune leukocytes (neutrophils, macrophages, or mast cells) (15, 17).

In contrast to the highly refined epitope sensing of T and B cell-expressed adaptive immune receptors, innate immune signaling depends upon recognition of conserved pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs). TLRs remain the best characterized of the PRRs (19, 20). They are highly conserved transmembrane proteins, consisting of an ectodomain with multiple leucine-rich repeats for pattern recognition, a membrane-spanning α helix, and a Toll/IL-1 receptor (TIR) domain for intracellular signaling. At least 13 mammalian TLRs have been identified, each specifically localizing to either the plasma membrane or the endosomal membranes and each detecting unique complements of PAMPs (20–22). Upon PAMP recognition, signal transduction occurs via TLR-specific recruitment of cytosolic TIR adaptor protein combinations. The TIR

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Abbreviations used in this article: BAL, bronchoalveolar lavage; CYC, cyclophosphamide; [DC_{max}], ligand concentration resulting in maximal dendritic cell cytokine expression; IRF, IFN regulatory factor; MLPA, monophosphoryl lipid A; mTEC, mouse tracheal epithelial cell; NTHi, nontypeable *Haemophilus influenzae*; ODN, oligodeoxynucleotide; Pam2CSK4, S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine; Pam3CSK4, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TIR, Toll/IL-1 receptor; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN- β ; TSA, tryptic soy agar.

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adaptor protein MyD88 is required for signaling from most TLRs. The MyD88-independent signaling events observed from TLR3 and TLR4 require TIR domain-containing adaptor inducing IFN- β (TRIF, also known as TICAM-1), with or without the participation of TRAM (23). The TLR-specific TIR adaptor signaling cascades activate receptor-specific transcription factors, such as NF- κ B, AP-1, and IFN regulatory factors (IRFs), leading to the expression of inflammatory and antimicrobial genes (21, 24, 25).

To test our hypothesis that lysate-induced resistance is at least partially dependent upon TLR signaling (15, 17), mice deficient in TIR adaptors were challenged with virulent pathogens with or without NTHi lysate pretreatment. In this study we present our observation that lysate-inducible resistance is MyD88-dependent but not TRIF-dependent. Moreover, we report the robust and unanticipated synergistic protection afforded by simultaneous stimulation of two MyD88-dependent TLRs. These findings may provide the basis for the clinical use of synthetic TLR ligand combinations to prevent respiratory infections in high-risk human populations.

Materials and Methods

Animals and reagents

All of the general reagents were obtained from Sigma-Aldrich (St. Louis, MO), except as indicated. All of the mice were handled in accordance with the policies of the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. Wild-type 5- to 8-wk-old female Swiss-Webster mice (Charles River, Wilmington, MA) were used for most protection and cell count experiments. As indicated, 5- to 8-wk-old female *Myd88*^{-/-} mice provided by Shizuo Akira (26), *Trif*^{-/-} (The Jackson Laboratory, Bar Harbor, ME), *Il1r*^{-/-} (The Jackson Laboratory), and *Tlr2*^{-/-} mice (The Jackson Laboratory), all backcrossed >10 generations to C57BL/6J, were used in comparison with wild-type mice C57BL/6J (The Jackson Laboratory).

Bronchoalveolar lavage fluid analysis

As described previously (15–17), bronchoalveolar lavage (BAL) fluid was obtained by instilling and collecting two aliquots of 1 ml each of PBS through a luer stub adapter cannula (BD Biosciences) inserted through rings of the exposed trachea at the indicated time points. Total leukocyte count was determined with a hemocytometer (Hauser Scientific, Horsham, PA), and differential count by cytocentrifugation of 300 μ l of BAL fluid at 500 \times g for 5 min, followed by Wright-Giemsa staining.

Aerosolized treatments

Frozen stock of NTHi was grown on chocolate agar (Remel, Lenexa, KS), expanded in brain-heart infusion broth (Acumedia, Baltimore, MD) supplemented with 3.5 μ g/ml NAD, and disrupted with an EmulsiFlex C5 (Avestin, Mannheim, Germany), as described (15, 17, 27). The protein concentration was adjusted to 2.5 mg/ml in saline by bicinchoninic acid assay (Pierce, Rockford, IL), and the lysate was frozen in 10-ml aliquots at -80°C . For treatment, a thawed aliquot was placed in an Aerotech II nebulizer (Biodex Medical Systems, Shirley, NY) driven by 10 l/min air supplemented with 5% CO₂ (to promote deep breathing) for 20 min. The nebulizer was connected by polyethylene tubing (30 cm \times 22 mm) to a 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.

N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(*R*)-cysteinyll-(lysyl)3-lysine (Pam3CSK4), *S*-[2,3-bis(palmitoyloxy)-propyl]-(*R*)-cysteinyll-(lysyl)3-lysine (Pam2CSK4), poly(I:C), monophosphoryl lipid A (MPLA), synthetic flagellin, imiquimod, and oligodeoxynucleotide (ODN)2395 were purchased from InvivoGen (San Diego, CA). To treat the animals, all of the synthetic TLR agonists except flagellin were reconstituted in endotoxin-free water, suspended in 8 ml sterile PBS at indicated concentrations, and aerosolized to the animals for 20 min using the same technique as used for NTHi lysate treatment. Flagellin was reconstituted in endotoxin-free PBS and administered intranasally to mice anesthetized with isoflurane.

In vivo infectious challenges

As described previously (15–17), mice were challenged inhalationally with bacterial inocula targeted to LD₈₀–LD₁₀₀. *Pseudomonas aeruginosa* strain

PA103 was obtained from the American Type Culture Collection and stored as a frozen stock (1×10^8 CFU/ml) in 20% glycerol in Luria-Bertani medium (Bio 101 Systems). One milliliter of stock was incubated for 16 h in 100 ml Luria-Bertani medium at 37°C in 5% CO₂, then diluted in 1 l fresh broth, and grown at 37°C for 6–7 h to an OD₆₀₀ of 0.3, yielding $1\text{--}4 \times 10^{10}$ CFU/ml. *Streptococcus pneumoniae* serotype 4 was stored as frozen stock (1×10^9 CFU) in 20% glycerol in Todd-Hewett broth (Becton Dickinson). One milliliter of thawed stock was incubated for 16 h in 150 ml Todd-Hewett broth at 37°C in 5% CO₂, then diluted in 1.5 l fresh broth, and grown in logarithmic phase for 6–7 h to an OD₆₀₀ of 0.3, yielding $2\text{--}6 \times 10^{11}$ CFU/ml. The bacterial suspensions were centrifuged, washed, resuspended in 10 ml PBS, and aerosolized over a period of 60 min using a system identical to that used for the treatments. Bacterial concentrations were determined by plating serial dilutions onto tryptic soy agar (TSA) plates (Becton Dickinson). To deplete neutrophils in wild-type mice, 150 mg/kg cyclophosphamide was injected i.p. on days -4 and -1 prior to infection. BAL analysis of identically cyclophosphamide-treated mice confirmed the depletion of lung neutrophils, even after treatment with Pam2-ODN.

Quantification of lung pathogen burden

As described previously (15–17), immediately postinfection with bacterial pathogens, mice were anesthetized, and their lungs were harvested and homogenized in 1 ml PBS utilizing a 2-ml tissue grinder (Kontes, Vine-land, NJ). Serial dilutions of the homogenates were plated on TSA plates, incubated at 37°C for 16 h, and bacterial colonies were counted.

In vitro killing assay

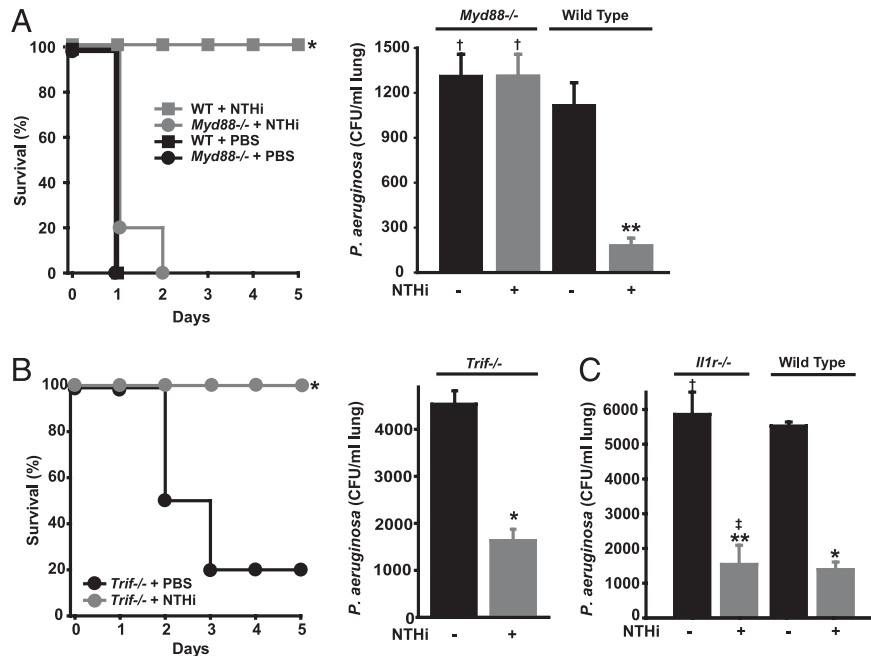
As described previously (15, 17), MLE-15 cells and A549 cells were cultured on six-well plates in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (Invitrogen). When grown to $\sim 80\%$ confluence, cells were washed with PBS, supplied with fresh antibiotic-free medium with 10% heat-inactivated FCS, and treated with 20 μ l PBS or a 20 μ l volume of ODN2395 (20 μ g/ml), Pam2CSK4 (10 μ g/ml), or both in RPMI 1640 medium containing 10% heat-inactivated FCS. After 4 h, 1000 spores of *Bacillus anthracis* Sterne strain or 2000 CFU *P. aeruginosa* strain PA103 then were added to all of the wells. Four hours postinfection, 20 μ l of the supernatant from each well was aspirated, serially diluted, plated on a TSA agar plate, and incubated for 16 h at 37°C, and CFUs were counted.

To test killing by primary epithelial cells, mouse tracheal epithelial cells (mTECs) were isolated as described previously (28). In brief, tracheas from wild-type Swiss Webster mice were isolated and digested overnight at 4°C in Ham's F-12 media (Mediatech) containing 25 U penicillin/streptomycin (Invitrogen) and 0.15% pronase (Roche). Disaggregated cells were incubated in Ham's F-12/DMEM (Invitrogen) containing 4 mM glutamine (Invitrogen), 10 U penicillin/streptomycin, 0.25 μ g/ml amphotericin B (Fisher Scientific), and 50 μ g/ml gentamicin (Fisher Scientific) at 37°C in 5% CO₂ for 5 h. After incubation, floating cells were collected, and 3.8×10^5 cells per well were plated on collagen I/III (Sigma-Aldrich)-coated 24-well tissue culture plates in Ham's F-12/DMEM containing 5% heat-inactivated FCS, 4 mM glutamine, 10 U penicillin/streptomycin, 0.25 μ g/ml amphotericin B, 50 μ g/ml gentamicin, 10 μ g/ml insulin (Sigma-Aldrich), 30 μ g/ml bovine pituitary extract (Lonza), 5 μ g/ml human transferrin (Fisher Scientific), 0.1 μ g/ml cholera toxin (Sigma-Aldrich), 25 ng/ml epidermal growth factor (Fisher Scientific), and 10^{-8} M retinoic acid (Sigma-Aldrich). After incubation at 37°C in 5% CO₂ for 3–4 d, confluent cells were washed and treated with 20 μ l PBS or a 20 μ l volume of ODN2395 (20 μ g/ml) and Pam2CSK4 (10 μ g/ml) in antibiotic-free mTEC culture media. Four hours later, the wells were infected with 150 CFU luminescent *S. pneumoniae* (a gift from Dr. Jon McCullers, St. Jude Children's Research Hospital). Culture luminescence was measured postinfection by a Biotek Synergy 2 plate reader. Culture luminescence correlated with serial dilution CFUs with an $R^2 > 0.998$. Similar experiments were performed with primary alveolar macrophages harvested from wild-type Swiss Webster mice. Alveolar macrophages were collected by BAL, as described above. The cells were resuspended in RPMI 1640 medium with 10% heat-inactivated FCS supplemented with penicillin/streptomycin, then plated on 24-well plates. Once adherent to the wells, the wells were washed, and antibiotic-free RPMI 1640 medium was added. Treatment and infection then proceeded as described for the mTECs.

Microarray gene expression analysis

MLE-15 cells were treated with PBS, ODN2395 (20 μ g/ml), Pam2CSK4 (10 μ g/ml), or both ligands for 4 h. Total RNA from MLE-15 cells was isolated using RNeasy Mini kit (Qiagen). cRNA then was synthesized and

FIGURE 1. MyD88, but not TRIF, signaling is required for bacterial lysate-induced resistance to pneumonia. **A**, *Myd88*^{-/-} and wild-type mice were challenged inhalationally with *P. aeruginosa* with or without pretreatment 24 h earlier with an aerosolized lysate of NTHi. **Left panel**, Survival ($n = 10$ mice per group; $*p < 0.0001$). **Right panel**, Bacterial lung burden immediately postinfection ($n = 3$ mice per group; $**p < 0.004$, $^{\dagger}p = 0.39$ versus wild-type control). **B**, *P. aeruginosa* challenge of *Trif*^{-/-} mice with or without pretreatment with the bacterial lysate. **Left panel**, Survival ($n = 10$ mice per group; $*p < 0.0001$). **Right panel**, Bacterial lung burden immediately postinfection ($n = 3$ mice per group; $*p < 0.0001$). **C**, *Il1r*^{-/-} and wild-type mice were treated with aerosolized PBS or the lysate 24 h before challenge with *P. aeruginosa*. Shown is the bacterial burden of lung homogenates immediately postinfection ($n = 3$ mice per group; $*p = 0.001$ versus wild-type mice treated with PBS, $**p = 0.01$ versus *Il1r*^{-/-} mice treated with PBS, $^{\dagger}p = 0.66$ versus wild-type mice treated with PBS, $^{\ddagger}p = 0.89$ versus wild-type mice treated with NTHi).



amplified using an Illumina TotalPrep RNA amplification kit (Ambion), labeled and hybridized onto Sentrix Mouse-6 Expression BeadChips, and then scanned by a BeadStation 500 (Illumina). Consistent with minimum information about a microarray experiment standards, all of the primary microarray data were deposited at the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE26864.

Immunofluorescence microscopy

A549 cells were cultured on Lab-Tek II chamber slides (Nunc, Rochester, NY) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (Invitrogen) for 48 h, then treated with a 20 μ l volume of Texas Red-labeled ODN2395 (20 μ g/ml; Invivogen), FITC-labeled Pam2CSK4 (10 μ g/ml; Invivogen), or both in RPMI 1640 medium containing 10% heat-inactivated FCS. After 2 h, the media was suctioned, the chambers were detached, and the cells were washed three times with iced PBS. The cells then were fixed with 4% paraformaldehyde, quenched with glycine, washed three times with PBS, nuclear-counterstained with DAPI (0.1 μ g/ml), and examined with fluorescence microscopy (BX-60 microscope; Olympus, Melville, NY) using appropriate optics (Texas Red, excitation = 540 nm, emission = 620 nm; FITC, excitation = 495 nm, emission = 520 nm; DAPI, excitation = 360 nm, emission = 460 nm). Images were collected sequentially with a computer-regulated Spot RT Camera (Diagnostic Instruments, Sterling Heights, MI) and assembled in Photoshop CS3 (Adobe, San Jose, CA). Overlapping red and green fluorescence appeared yellow.

Statistical analysis

Statistical analysis was performed using SPSS, version 19 (SAS Institute). Student *t* test was used to compare the lung bacterial burdens between groups. Percentage of mice surviving pathogen challenges was compared using Fisher's exact test, and the log-rank test was used to compare the survival distribution estimated by the Kaplan-Meier method. One-way ANOVA with Dunnett's post hoc test was used to compare the BAL fluid differential counts between treated and untreated animals.

Gene expression data were background-corrected using the robust microarray averaging method, then transformed by taking the base-two logarithm, and quantile-normalized. Analysis of the microarray output was performed using a gene-by-gene class comparison ANOVA to identify treatment-induced changes. To identify genes with synergistic effects of the TLR ligand treatments, we fit a model of $Y = \beta_0 + \beta_1 \times \text{ODN} + \beta_2 \times \text{Pam2} + \beta_3 \times \text{ODN} \times \text{Pam2}$, where Y is the expression value of a gene and the β terms are the coefficients for main effects of ODN (β_1), Pam2 (β_2), and the interaction between ODN and Pam2 (β_3). Then, to test whether the interaction term is significant, we compared this linear model with one lacking an interaction term. To identify genes with additive effects, we fit

the ANOVA model without the interaction term and tested whether both main effects (ODN and Pam2) are significantly nonzero. To adjust for multiple testing, we used a β uniform mixture model to estimate the false discovery rate. Tukey's honestly significant difference test is used to compare the differences between each pair of means with an appropriate adjustment for multiple testing on the level of the individual gene.

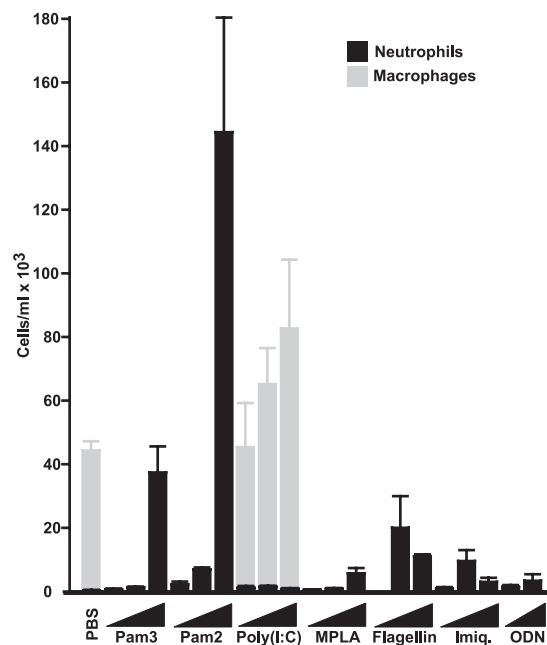


FIGURE 2. Leucocyte counts in BAL fluid after treatment with single synthetic TLR ligands. Mice were submitted to BAL 24 h after treatment with PBS or one of the following TLR ligands: Pam3CSK4 (TLR2/1 agonist; 1, 3, and 10 μ g/ml), Pam2CSK4 (TLR2/6 agonist; 1, 3, and 10 μ g/ml), poly(I:C) (TLR3 agonist; 1, 10, and 100 μ g/ml), synthetic lipid A (MPLA; TLR4 agonist; 1, 10, and 100 μ g/ml), flagellin (TLR5 agonist; 40, 400, and 4000 ng), imiquimod (TLR7 and TLR8 agonist; 100, 300, and 1000 μ g/ml), or ODN2395 (TLR9 agonist; 2 and 20 μ g/ml). All of the ligands delivered as 8-ml suspensions nebulized over 20 min, except flagellin which was delivered intranasally in 40 μ l. Shown are neutrophil (black bars) and macrophage (gray bars) counts in BAL fluid.

Results

MyD88, but not TRIF, is required for the induction of resistance to pneumonia by an aerosolized bacterial lysate

We have shown previously that stimulation of the lung epithelium by an aerosolized bacterial lysate induces a high level of resistance to a broad array of microbial pathogens (15–18). To test whether TLR signaling is required for lysate-induced protection, mice deficient in TIR adaptors were challenged inhalationally with *P. aeruginosa*. Wild-type and *Trif*^{-/-} mice were protected fully against lethal *P. aeruginosa* challenges by pretreatment with the aerosolized bacterial lysate, whereas resistance could not be induced in *Myd88*^{-/-} mice (Fig. 1A, 1B, left panels). Consistent with our prior observations (15–17), protection closely correlated with the induction of rapid pathogen killing in the lungs (Fig. 1A, 1B, right panels). The IL-1 receptor also signals through MyD88 (26, 29) but responds to host cytokine signaling rather than directly to microbial products. Pathogen killing was preserved fully in *Il1r*^{-/-} mice (Fig. 1C) stimulated by the aerosolized bacterial lysate. This finding indicates that not all of the receptors signaling through MyD88 are required for lysate-induced protection and suggests that direct microbial signaling through TLRs is more important than indirect signaling through host cytokines for inducible epithelial resistance.

Individual TLR agonists fail to induce a high level of resistance to pneumonia

In view of the requirement for MyD88 signaling, we tested whether any individual synthetic TLR agonists could induce resistance similar to that afforded by the aerosolized bacterial lysate. Because TLR1 and TLR6 are expressed as heterodimers with TLR2 and because TLR7 and TLR8 both recognize imiquimod, mouse TLR1–9 could be stimulated with the following seven synthetic ligands: Pam3CSK4 (TLR2/1 agonist), Pam2CSK4 (TLR2/6 agonist), poly(I:C) (TLR3 agonist), synthetic lipid A (MPLA, TLR4 agonist), synthetic flagellin (TLR5 agonist), imiquimod (TLR7 and TLR8 agonist), or ODN2395 (TLR9 agonist).

The appropriate airway doses of these agonists are not known, so a strategy was formulated to ensure that an adequate dose was delivered to the lungs to avoid a type II (β) error. Each of the synthetic TLR agonists that we used has a reported concentration at which maximal cytokine secretion is stimulated from dendritic cells ($[DC_{max}]$) (23, 30–35). On the basis of our calculations of effective airway delivery of aerosolized compounds (16, 36), we determined the nebulizer fluid concentrations required to achieve $[DC_{max}]$ at the airway epithelial surface. Although aerosolized lysate-induced resistance does not depend upon leukocyte influx, we have reported previously that the protective phenomenon is correlated tightly with the timing and magnitude of induced lung neutrophilia (15). Therefore, to identify TLR agonist doses sufficient for testing, we began at the reported $[DC_{max}]$ for each ligand and increased the starting concentrations logarithmically until leukocyte infiltration was achieved as a biomarker of ligand activity in the lungs.

As shown in Fig. 2, in PBS-treated mice, the number of neutrophils in BAL fluid is low ($0.1 \times 10^3 \pm 0.2$ cells per milliliter). Only Pam2CSK4 demonstrated a significant increase in neutrophils at $[DC_{max}]$, though all but poly(I:C) showed significant increases in neutrophil levels at concentrations one to two logs above $[DC_{max}]$. Alternately, poly(I:C) induced a significant influx of macrophages in BAL fluid 24 h after treatment. Flagellin and imiquimod each had a ligand concentration above which there was a reduction in neutrophil infiltration. Pam2CSK4 induced a level of neutrophilia nearly 5-fold higher than that of Pam3CSK4 and 15-fold higher than that of any other ligand.

The concentration chosen for each ligand was the lowest dose to induce a 10-fold increase in neutrophils per milliliter or to induce doubling of the macrophages (none did both). Although some of the ligands induced robust cellular infiltration, none of the synthetic agonists provided significant protection against lethal *P. aeruginosa* pneumonia (Fig. 3). There was a modest trend toward protection with Pam2CSK4 and imiquimod, though these did not reach statistical significance for individual experiments or in the mean of multiple experiments. MPLA-treated mice showed

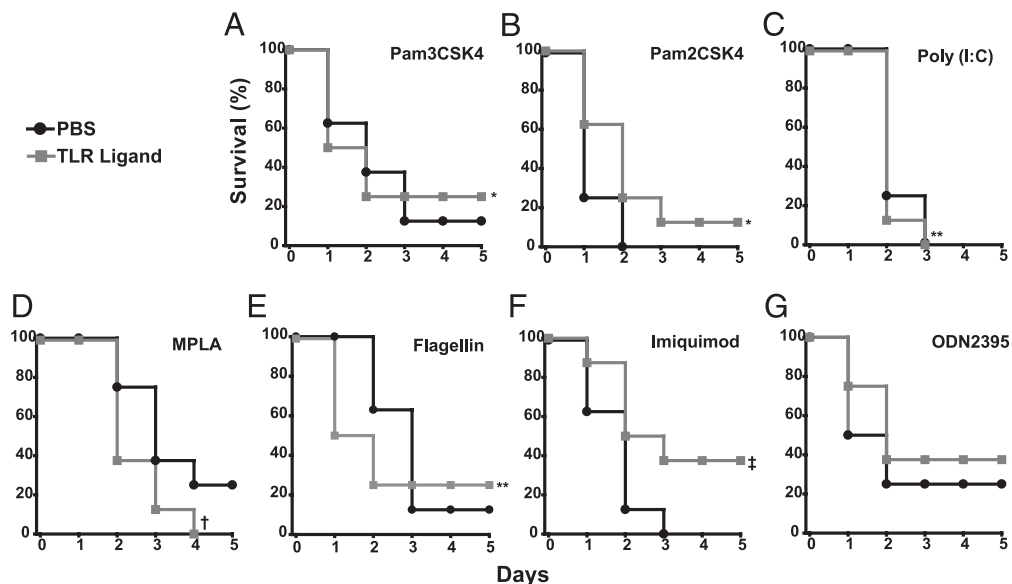


FIGURE 3. Aerosolized treatment with individual synthetic TLR ligands does not induce a high level of resistance against pneumonia. Wild-type mice were challenged with *P. aeruginosa* after treatment with PBS or the following synthetic TLR ligands 24 h prior: (A) TLR2/1 agonist Pam3CSK4 (100 μ g/ml nebulized for 20 min), (B) TLR2/6 agonist Pam2CSK4 (10 μ g/ml nebulized for 20 min), (C) TLR3 agonist poly(I:C) (100 μ g/ml nebulized for 20 min), (D) TLR4 agonist MPLA (100 μ g/ml nebulized for 20 min), (E) TLR5 agonist flagellin (400 ng intranasal), (F) TLR7 and TLR8 agonist imiquimod (1 mg/ml nebulized for 20 min), or (G) TLR9 agonist ODN2395 (20 μ g/ml nebulized for 20 min). Survival curves are representative examples of at least three distinct experiments for treated and untreated mice ($n = 8$ mice per group; * $p = 0.5$, ** $p = 1.0$, † $p = 0.47$, ‡ $p = 0.2$).

a nonsignificant trend toward increased mortality after pathogen challenge.

A combination of TLR2/6 and TLR9 agonists induces a high level of resistance against pneumonia

Although we did not observe significant protection after treatment with single synthetic TLR agonists, we and others have postulated that simultaneous stimulation of multiple PRRs is required to induce a high level of resistance (13, 15, 17). To determine whether combinations of TLR agonists could induce resistance, we tested

the 21 nonredundant pairwise permutations of the seven synthetic ligands.

Remarkably, the first tested combination, simultaneous treatment with Pam2CSK4 and ODN2395 (Pam2-ODN), resulted in survival of 100% of mice from an otherwise lethal challenge with Gram-negative *P. aeruginosa* (Fig. 4A, left panel) and survival of 80% from a lethal challenge with Gram-positive *S. pneumoniae* (Fig. 4B, left panel). Doubling the concentration of both ligands in the aerosol treatment resulted in 90% survival of the challenge with *S. pneumoniae* (Fig. 4B).

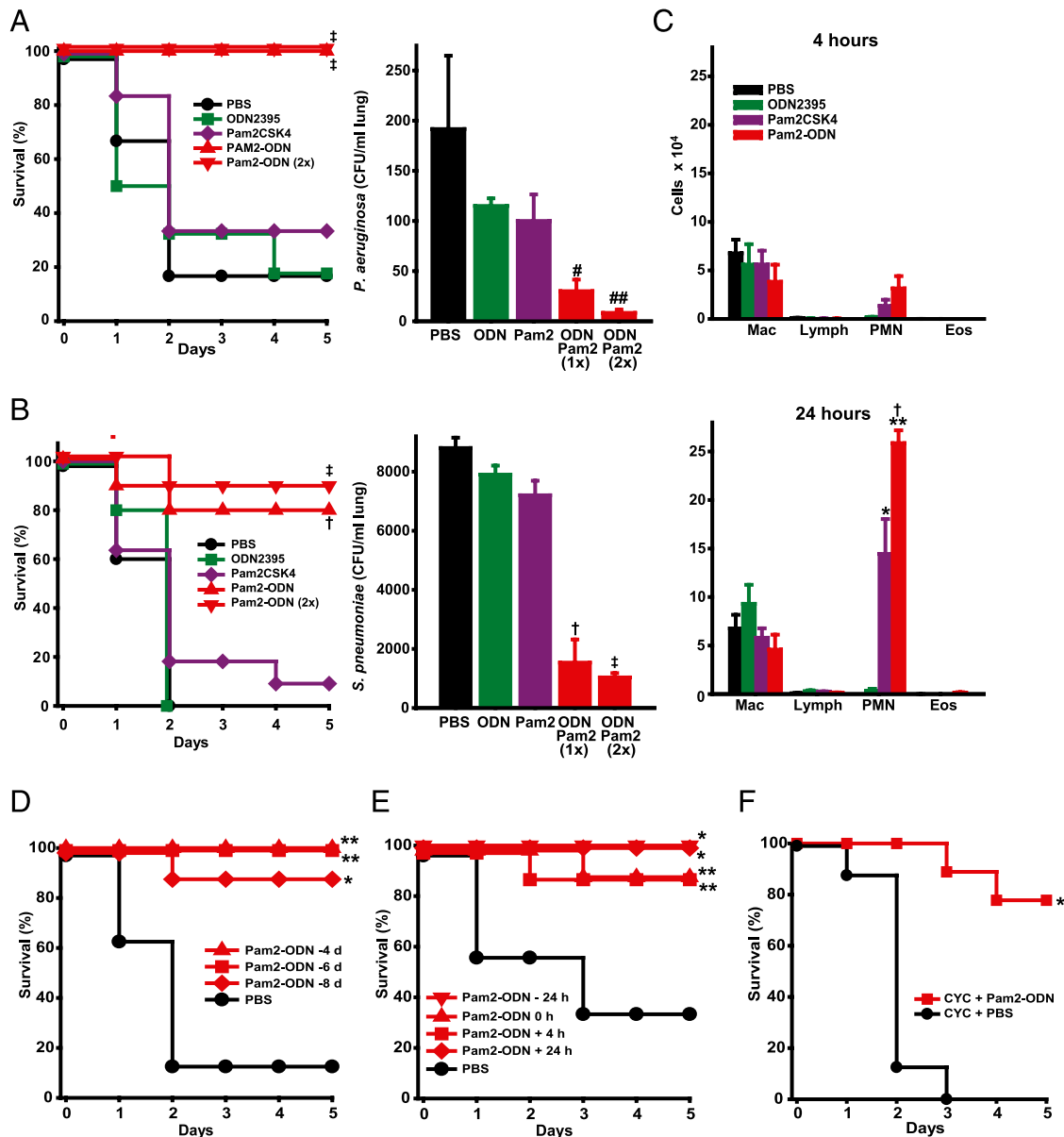


FIGURE 4. TLR2/6 and TLR9 agonists cooperate to induce resistance against bacterial pneumonia. *A, Left panel*, Survival of mice challenged with *P. aeruginosa* 24 h after treatment with PBS, Pam2CSK4 (10 μ g/ml), ODN2395 (20 μ g/ml), the combination, or the combination at double dose ($n = 6$ mice per group; $\ddagger p = 0.008$ versus PBS). *Right panel*, Bacterial burden of lung homogenates immediately postinfection with *P. aeruginosa* ($n = 3$ mice per group; $\# p = 0.045$ versus PBS, $\#\# p = 0.030$ versus PBS). *B, Left panel*, Survival of mice challenged with *S. pneumoniae* 24 h after treatment with PBS, Pam2CSK4 (10 μ g/ml), ODN2395 (20 μ g/ml), the combination, or the combination at double dose ($n = 10$ mice per group; $\dagger p < 0.001$ versus PBS, $\ddagger p < 0.0001$ versus PBS treated). *Right panel*, Bacterial burden of lung homogenates immediately after *S. pneumoniae* infection ($n = 3$ mice per group; $\dagger p < 0.001$, $\ddagger p < 0.0001$). *C*, BAL cell counts from mice 4 or 24 h after treatment with PBS, Pam2CSK4 (10 μ g/ml), ODN2395 (20 μ g/ml), or the combination ($n = 3$ mice per group; $* p = 0.02$ versus PBS, $** p < 0.0001$ versus PBS, $\dagger p = 0.04$ versus Pam2 alone). *D*, Survival of mice challenged with *P. aeruginosa* after treatment with Pam2-ODN 8, 6, or 4 d prior to infection. ($n = 8$ mice per group; $* p = 0.01$ versus PBS, $** p = 0.001$ versus PBS). *E*, Survival of *P. aeruginosa* challenge in mice receiving Pam2-ODN treatment 24 h before infection, at the time of infection, or postinfection (PBS group $n = 9$ mice, other groups $n = 8$ mice; $* p = 0.01$, $** p = 0.05$). *F*, Survival of cyclophosphamide (CYC) neutrophil-depleted mice challenged with *P. aeruginosa* 24 h after treatment with Pam2-ODN or PBS ($n = 9$ CYC + Pam2-ODN, 8 CYC + PBS; $* p = 0.002$).

Table I. Temporal dissemination of bacteria after inhalational challenge with *P. aeruginosa*

Organ	Treatment	0 h Postinfection	24 h Postinfection	48 h Postinfection
Lung	PBS	16,000 ± 2121	39,000 ± 30,405	–
	Pam2-ODN	1500 ± 1322*	1100 ± 1678	23 ± 22
Blood	PBS	0	5 ± 2	–
	Pam2-ODN	0	0**	0
Spleen	PBS	0.5 ± 0.1	60.25 ± 23	–
	Pam2-ODN	0	0**	0.3 ± 0.5
Liver	PBS	0	365 ± 262	–
	Pam2-ODN	0	0**	0.6 ± 1.2

There were $n = 3$ mice per group at each time point, except the PBS group 24 h postinfection in which one mouse did not survive to the predesignated time point. For cells that include only a dash, all of the mice in the PBS group died prior to the 48-h time point.

* $p < 0.001$ versus PBS, ** $p = 0.01$ versus PBS.

infectious challenges was associated with synergistic killing of the pathogens within the lungs (Fig. 4A, 4B, right panels), and doubling the concentration of the ligands was associated with a trend toward greater pathogen killing. Synergistic interactions between Pam2CSK4 and ODN2395 also were observed in leukocyte recruitment to the lungs at 4 and 24 h (Fig. 4C). Together, these results indicate that ligands for TLR2/6 and TLR9 induce synergistic activation of antimicrobial effector responses, including those for pathogen killing and leukocyte recruitment, which result in a synergistic level of protection against pneumonia. Similar to the kinetics of the aerosolized bacterial lysate-induced resistance, protection was present by 4 h after treatment (data not shown). This protection persisted for up to 8 d after a single inhaled treatment (Fig. 4D). Moderate protection also was observed when the treatment was delivered up to 24 h postinfection (Fig. 4E). Later postinfection time points cannot be assessed in our model because too many untreated/sham-treated mice meet euthanasia criteria prior to receiving planned treatments (15). Similar to our findings with lysate-induced protection (15, 16), although the induction of lung neutrophilia correlated with the induction of resistance, depletion of neutrophils did not obviate the protection conferred by Pam2-ODN treatment (Fig. 4F). Also similar to lysate-induced resistance, Pam2-ODN-enhanced survival rates also correlate with containment of the infection within the lungs.

Table I shows that Pam2-ODN treatment induces pathogen killing at the time of infection, promotes ongoing pathogen clearance, and prevents hematogenous dissemination of infection relative to PBS treatment. Thus, we observe evidence of direct intrapulmonary pathogen killing and enhanced containment of pathogens within the lungs.

Not all of the TLR agonist combinations protect against infection

As shown in representative examples of other TLR combinations with Pam2CSK4 (Fig. 5A–C) and ODN2395 (Fig. 5D–F), few other treatments induced protection that was statistically superior to PBS treatment. These results indicate that not all of the TLR agonist combinations confer the same immune stimulation as Pam2-ODN, even when both ligands signal via MyD88, suggesting that a degree of specificity must account for the immune activation.

TLR2 is required to promote protective Pam2CSK4 and ODN2395 synergy but not required for induced resistance by the bacterial lysate

The detection of synergistic effects of TLR ligands Pam2CSK4 and ODN2395 with well-defined receptor specificities provides presumptive evidence of the participation of TLR2/6 and TLR9. We

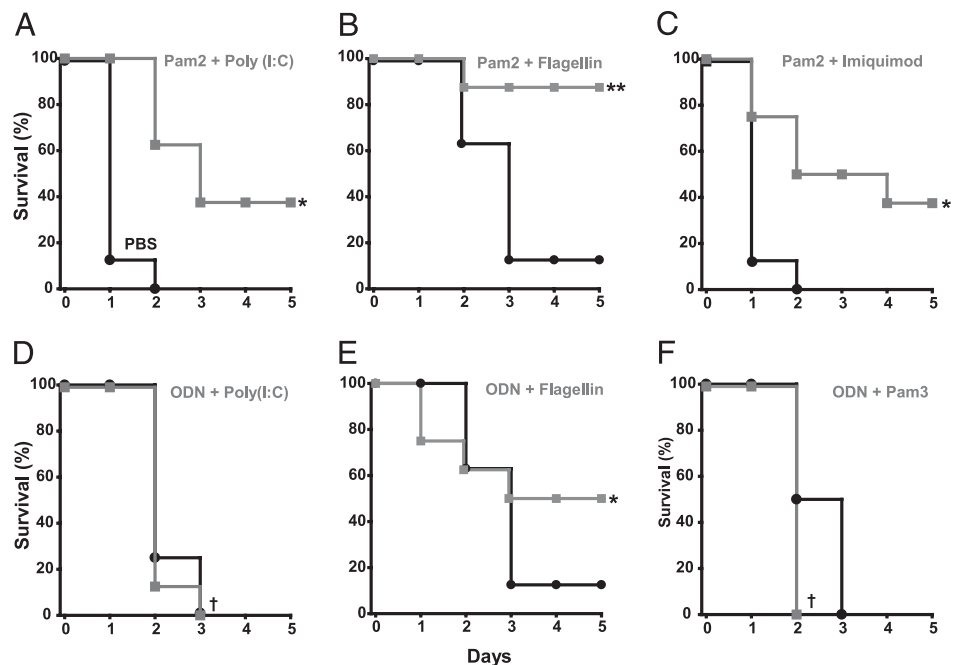


FIGURE 5. Not all of the TLR agonist combinations protect against pneumonia. Wild-type mice were challenged with *P. aeruginosa* after treatment with PBS or the following TLR agonist combinations 24 h prior: (A) Pam2CSK4 and poly(I:C), (B) Pam2CSK4 and flagellin, (C) Pam2CSK4 and imiquimod, (D) ODN2395 and poly(I:C), (E) ODN2395 and flagellin, and (F) ODN2395 and Pam3CSK4. ($n = 8$ mice per group; * $p = 0.20$, ** $p = 0.01$, † $p = 1.0$).

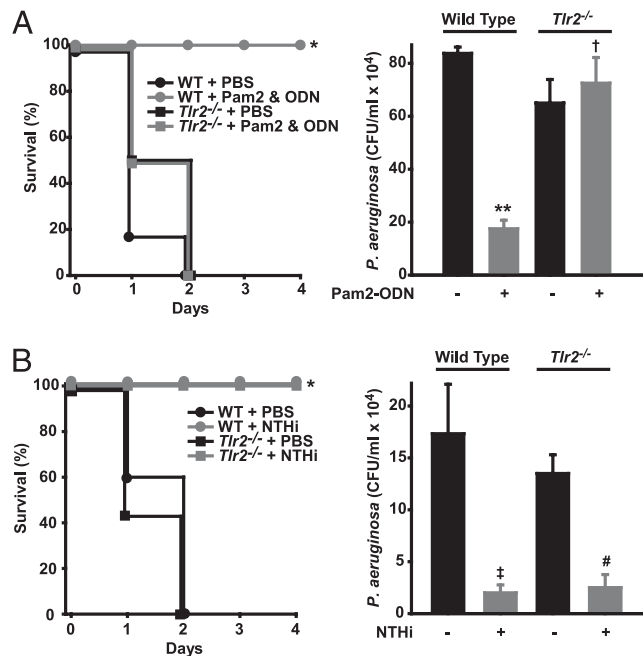
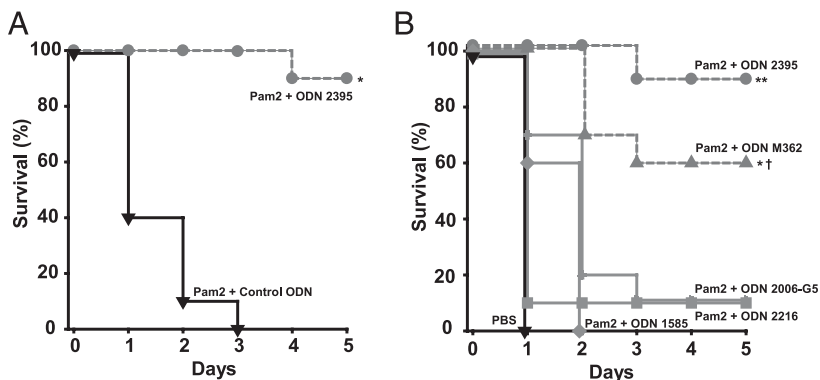


FIGURE 6. TLR2 is sufficient to promote protective Pam2CSK4 and ODN2395 synergy but not required for induced resistance. *A, Left panel*, Survival of *Tlr2*^{-/-} and wild-type mice challenged with *P. aeruginosa* with or without ODN2395 and Pam2CSK4 treatment 24 h prior ($n = 8$ mice per group; $*p < 0.0002$). *Right panel*, Bacterial burden of lung homogenates immediately postinfection with *P. aeruginosa* ($n = 4$ mice per group; $**p < 0.0001$ versus wild-type mice treated with PBS, $†p = 0.59$ versus *Tlr2*^{-/-} mice treated with PBS). *B, Left panel*, Survival of *Tlr2*^{-/-} and wild-type mice challenged with *P. aeruginosa* with or without treatment 24 h prior with an aerosolized lysate of NTHi ($n = 10$ mice per group; $*p < 0.0002$). *Right panel*, Bacterial burden of lung homogenates immediately postinfection with *P. aeruginosa* ($n = 3$ mice per group; $*p = 0.03$ versus wild-type mice treated with PBS, $#p = 0.002$ versus *Tlr2*^{-/-} mice treated with PBS).

sought further evidence using knockout mice and additional ligands.

First, we compared the survival of wild-type and *Tlr2*^{-/-} mice pretreated with Pam2-ODN or PBS prior to challenge with *P. aeruginosa*. Although the wild-type mice were protected fully by Pam2-ODN, there was no survival in the sham-treated wild-type group or either *Tlr2*^{-/-} group (Fig. 6A, left panel), confirming the requirement for TLR2 in Pam2-ODN-induced protection. Consistent with our prior findings, the loss of protection in the *Tlr2*^{-/-} mice correlated tightly with the loss of Pam2-ODN-induced intrapulmonary pathogen killing (Fig. 6A, right panel).

FIGURE 7. TLR9-binding class C, but not class A or B, CpG ODNs interact synergistically with Pam2CSK4 to induce resistance to bacterial pneumonia. *A*, Survival of wild-type mice treated with Pam2CSK4 and ODN2395 or Pam2CSK4 and a scrambled control ODN 24 h prior to *P. aeruginosa* challenge ($n = 10$ mice per group; $*p < 0.0001$). *B*, Survival of wild-type mice challenged with *P. aeruginosa* 24 h after treatment with PBS or Pam2CSK4 combined with a class A CpG ODN (ODN1585 or ODN2216), a class B CpG ODN (ODN2006-G5), or a class C CpG ODN (M362 or ODN2395, dashed lines) ($n = 10$ mice per group; $*p = 0.01$ versus PBS, $**p = 0.0001$ versus PBS; $†p = 0.3$ versus Pam2 + ODN2395).



Because Pam2CSK4 and Pam3CSK4 discriminate between TLR2/6 and TLR2/1 and Pam2CSK4 but not Pam3CSK4 interacts synergistically with ODN2395, this suggested a requirement for TLR2/6 heterodimers in inducing lung epithelial resistance. However, we also challenged *Tlr2*^{-/-} and wild-type mice after treatment with the aerosolized bacterial lysate and found neither loss of protection (Fig. 6B, left panel) nor a defect in lysate-induced bacterial killing (Fig. 6B, right panel). Taken together, we found that TLR2/6 is sufficient to synergistically interact with TLR9 but not required for all of the induced resistance.

Class C CpG ODNs, but not class A or B CpG ODNs, interact synergistically with Pam2CSK4 to induce resistance to bacterial pneumonia

We next sought to confirm that TLR9 is required for the synergistic interaction of Pam2-ODN. *Tlr9*^{-/-} mice were not available to us, so we further tested TLR9 involvement using a scrambled ODN known to not bind TLR9. Whereas pretreatment with Pam2-ODN resulted in 90% survival of *P. aeruginosa*-challenged mice, none survived when pretreated with Pam2CSK4 and the control ODN (Fig. 7A), indicating that TLR9 binding by the ODN is required for the synergistic protection.

To further explore the specificity of the Pam2-ODN interaction, we treated wild-type mice with Pam2CSK4 and different classes of TLR9-binding CpG ODNs prior to challenge with *P. aeruginosa*. The combination of a class A ODN (ODN1585 or ODN2216) or a class B ODN (ODN2006-G5) with Pam2CSK4 conferred no protection, whereas equimolar combinations of Pam2CSK4 with a class C ODN (ODNM362 or ODN2395) promoted significant resistance against otherwise lethal pneumonia (Fig. 7B). These results indicate that, not only do TLR2/6 and TLR9 ligands synergize, but there are specific TLR9 ligands that interact more favorably than others.

Pam2CSK4 and ODN2395 induce bacterial killing by epithelial cells in vitro

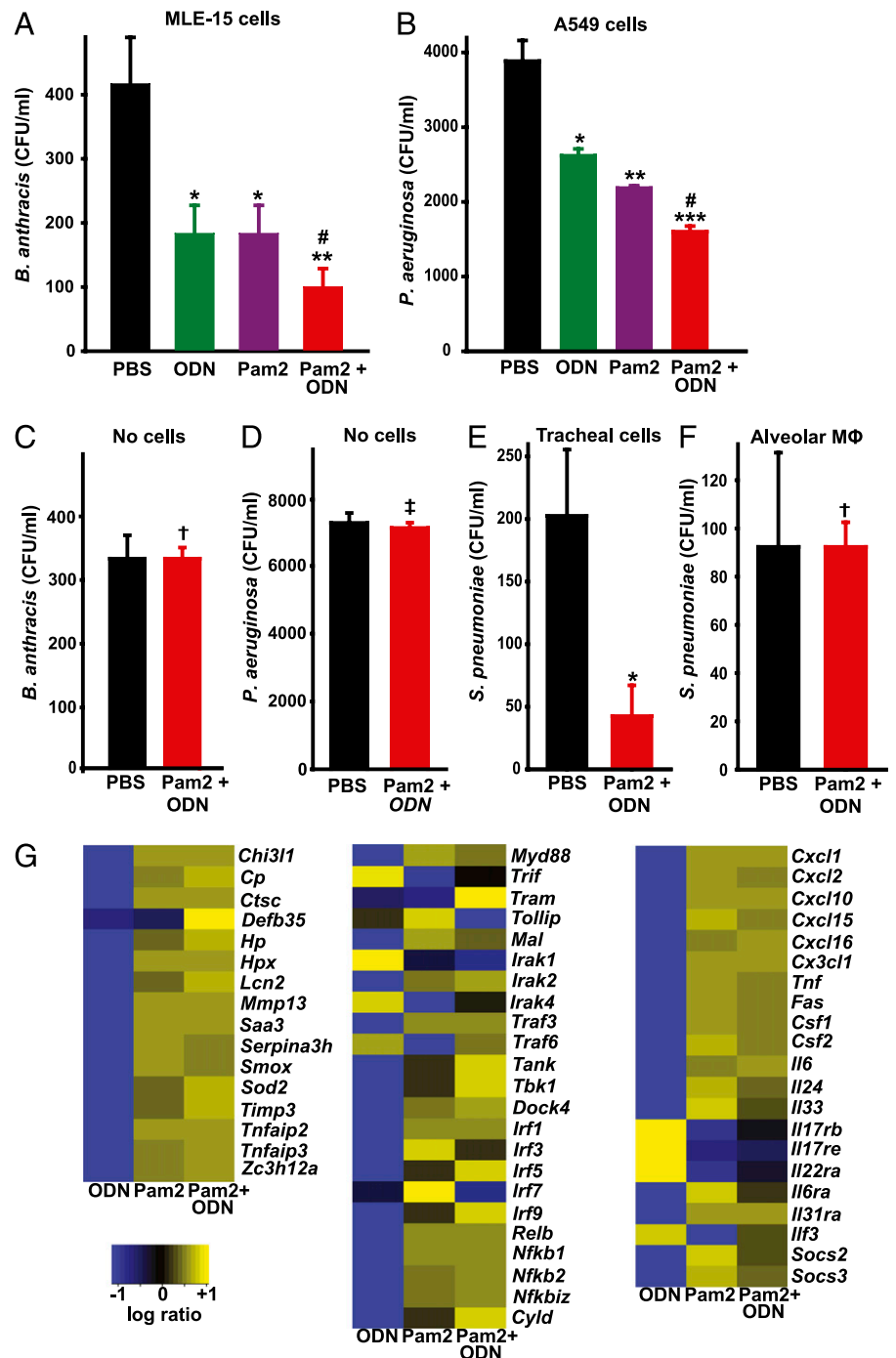
We have reported previously the induction of bacterial killing by lung epithelial cells in vitro when stimulated with bacterial lysate (16, 17). Because Pam2-ODN recapitulates the immunostimulatory effect of the bacterial lysate in vivo, we tested whether the combination could induce pathogen killing by isolated lung epithelial cells in vitro as well. Pretreatment of murine MLE-15 respiratory epithelial cells for 4 h with Pam2-ODN significantly reduced bacterial CFUs in cell culture media after inoculation with the Gram-positive organism *B. anthracis* (Fig. 8A). Similarly, treatment of human A549 cells with Pam2-ODN resulted in significant reductions in the Gram-negative organism *P. aeruginosa* CFUs 4 h postinfection (Fig. 8B). Demonstrating that the observed pathogen killing occurs through the stimulation of epithelial cells

rather than through direct antibiotic effects of Pam2-ODN, bacteria grew to equal numbers in wells containing no cells, whether the wells were treated with Pam2-ODN or PBS (Fig. 8C, 8D). Demonstrating that this was not an artifact of immortalized cells, we found that *S. pneumoniae* was killed by Pam2-ODN-treated primary mTECs (Fig. 7C). However, freshly isolated alveolar macrophages could not be induced to kill *S. pneumoniae* by Pam2-ODN in vitro (Fig. 8F).

Thus, the antimicrobial effect of Pam2-ODN is induced in both murine and human epithelial cells and results in killing of both Gram-positive and Gram-negative bacteria pathogens. These data mimic the bacterial killing seen in vivo after Pam2-ODN treatment. Serial increases in Pam2-ODN dosing up to 32-fold higher than indicated in this study did not significantly increase pathogen killing (data not shown).

To further explore the mechanisms by which the epithelial cells are induced to kill pathogens, we performed a microarray analysis of MLE-15 cells after treatment with PBS (sham), ODN2395, Pam2CSK4, or both. Compared to PBS-treated samples, 1129 genes were differentially expressed 2 h after ODN2395 and/or Pam2CSK4 treatment, with a false discovery rate of <0.05. Of these differentially expressed genes, 338 demonstrated additive or synergistic effects of the two ligands. To emphasize treatment-specific gene expression differences, Fig. 8G shows fold-change log ratios of the three ligand treatments (Pam2, ODN, or both) for selected differentially expressed genes. These include antimicrobial effectors, TLR signaling genes, and inflammatory mediators that are differentially expressed after Pam2-ODN treatment (compared with PBS). To reveal detail, the scale is truncated at log ratio ± 1 , so differences >2.7-fold are shown as saturated blue or

FIGURE 8. TLR2/6 and TLR9 agonists cooperate to induce bacterial killing by murine and human respiratory epithelial cells in vitro. **A**, MLE-15 cells were treated with Pam2CSK4 (10 μ g/ml) and/or ODN2395 (20 μ g/ml) for 4 h prior to infection with *B. anthracis* (1000 spores). Shown are bacterial CFUs 4 h postinfection (* p = 0.05 versus PBS, ** p = 0.016 versus PBS, # p > 0.05 versus either single agonist). **B**, A549 cells were treated with ODN2395 and Pam2CSK4 for 4 h prior to infection with *P. aeruginosa* (2700 CFU). Shown are bacterial CFUs 4 h postinfection (* p = 0.01 versus PBS, ** p = 0.003 versus PBS, *** p = 0.001 versus PBS, # p > 0.05 versus either single agonist). **C**, A549 culture media (without cells) was treated with ODN2395 and Pam2CSK4, infected with *B. anthracis* (1000 spores), and cultured after 4 h ($\dagger p$ = 1.0). **D**, MLE-15 culture media (without cells) was treated with ODN2395 and Pam2CSK4, infected with *P. aeruginosa* (4000 CFU), and cultured after 4 h ($\ddagger p$ = 0.58). **E**, Primary mouse tracheal epithelial cells were grown at the air/liquid interface, then treated with PBS or Pam2CSK4 (10 μ g/ml) and ODN2395 (20 μ g/ml) for 4 h prior to infection with *S. pneumoniae* (150 CFU). Shown are bacterial CFUs 19 h postinfection (* p = 0.03 versus PBS). **F**, Freshly isolated mouse alveolar epithelial cells were treated with Pam2CSK4 (10 μ g/ml) and ODN2395 (20 μ g/ml) for 4 h prior to infection with *S. pneumoniae* (150 CFU). Shown are bacterial CFUs 7 h postinfection ($\dagger p$ = 1.0 versus PBS). **G**, Microarray analysis was performed on MLE-15 cells treated with PBS, Pam2CSK4 (10 μ g/ml), ODN2395 (20 μ g/ml), or both for 2 h. Shown are heat maps reporting relative enrichment by TLR ligand treatments for differentially expressed antimicrobial effectors (left panel), TLR signaling elements (middle panel), and differentially expressed cytokine signaling (right panel) (n = 6 replicates per condition).



yellow. There is high concordance between these induced changes and the gene expression enrichment demonstrated 2 h after treatment of MLE-15 cells with the NTHi lysate. In fact, more than half of the Pam2-ODN-induced effector genes were upregulated by treatment with the lysate, and almost all of the enriched cytokines were induced by the lysate (17). These results not only further substantiate that the lung epithelia have the necessary machinery to generate antimicrobial responses but also demonstrate that combined treatment with TLR agonists is sufficient to induce effective responses.

Pam2CSK4 and ODN2395 colocalize intracellularly in vitro

The mechanism by which Pam2CSK4 and ODN2395 interact to induce synergy remains unresolved. Because TLR2/6 is reported to localize to the plasma membrane and TLR9 is reported to localize to endosomes (37, 38), we did not anticipate physical interaction of their cognate ligands. However, in light of recent reports that TLR4 may require internalization to signal (39), we investigated whether the two ligands were internalized by epithelial cells. A549 cells were treated for 2 h with FITC-labeled Pam2CSK4 and Texas Red-labeled ODN2395 at the same concentrations used in the pathogen killing experiments, then submitted to fluorescence microscopy. As shown in Fig. 9, both Pam2CSK4 (green) and ODN2395 (red) were internalized by the epithelial cells. Further, they colocalize (yellow) in the cytoplasmic compartment, presumably within endosomes. Although this does not prove that the receptors interact in that compartment, it supports that possibility. To assess whether they physically interact will require additional testing.

Discussion

We describe in this study an unanticipated, synergistic combination of TLR agonists that broadly protects mice against bacterial pneumonia, recapitulating the survival advantage previously reported after inhalation of a crude bacterial lysate. The combination of Pam2CSK4 and ODN2395 promoted intrapulmonary pathogen killing and survival of infectious challenges to an extent that far surpassed most other tested TLR ligand doublets, suggesting that the mechanisms underlying these observations are more specific than anticipated when we hypothesized that the bacterial lysate induced resistance by simultaneous stimulation of multiple PRRs. Similar to our earlier reports of inducible resistance, Pam2-ODN-induced protection appears to involve the elaboration of antimicrobial effectors from the lung epithelium and containment of infection within the lungs.

We demonstrate in this study the requirement for TLR signaling in lysate-induced resistance in the discovery of MyD88 dependence. That the deficiency of a single gene could result in such

a profound loss of function was remarkable given the numerous PRRs potentially engaged by the lysate. Given the importance of MyD88 in bacterial defense (24, 29), the susceptibility of *Myd88*^{-/-} mice to infection was not surprising. However the *Myd88*^{-/-} mice did not die more rapidly than the PBS-treated wild-type mice, nor did they exhibit higher lung bacterial CFUs after challenge than PBS-treated wild-type mice, suggesting that the central defect was an inability to induce resistance rather than a diminution of baseline resistance, although both likely contribute to some extent. Further, although the MyD88 studies refined our focus from PRRs in general to TLRs, the lack of a defect in the *Trif*^{-/-} mice revealed that only a subset of TLRs were required for induced resistance.

Single TLR ligands have been described previously to induce cytokine responses from leukocytes and to confer survival benefits in some models of infection (40–43), but their ability to therapeutically induce lung mucosal defenses is incompletely characterized and likely inferior to the stimulation of multiple PRRs (6, 15, 44–46). To more precisely define the receptors involved in inducible resistance than is possible with the crude lysate, we investigated the use of synthetic TLR ligands. Despite our aggressive ligand dosing strategy, we did not observe high-level protection from any single TLR ligand against *P. aeruginosa* infections. Because we and others (15, 40, 43) have observed protection with similar doses of single TLR ligands in other models, we believe the lack of protection reflects the extreme lethality of our cytotoxic *Pseudomonas* model, underscoring the robustness of Pam2-ODN-induced resistance.

To investigate whether simultaneous TLR stimulation could induce antibacterial resistance, we combined TLR ligands into doublets and failed to induce a high level of protection with most combinations but achieved protection that was similar to that induced by the lysate when treating mice with the synergistic combination of TLR2/6 and TLR9 agonists.

In recent years, a number of TLR ligands have been investigated therapeutically for infectious and noninfectious conditions, with a handful presently in clinical use (46–48). However, to our knowledge, this is the first description of the therapeutic combination of TLR2/6 and TLR9 agonists and the first TLR treatment reported to confer broad protection against pneumonia. Although we did not anticipate such exceptional performance of this specific doublet, retrospective review of the literature provides some clues that this protective effect is consistent with a more broadly observed biologic phenomenon.

TLR2 and TLR9 cooperate in the detection and control of several pathogens, including *Mycobacterium tuberculosis* and *Trypanosoma cruzi* (49, 50), HSV (51, 52), *Helicobacter pylori* (53, 54), and possibly *S. pneumoniae* (55), by unresolved mechanisms. Sato et al. (51) claimed that the dual recognition of HSVs required serial detection by TLR2 then TLR9, though Sørensen et al. (52) subsequently found no evidence for sequential signaling. Certainly, signal amplification of one TLR by another in series theoretically could result in such an effect. However, a potentially more appealing explanation than serial signaling is synergistic coincident detection of dual TLR-dependent signals. Such convergence could occur either in the process of intracellular secondary signaling or by extracellular interactions of elaborated antimicrobial products. Our gene expression data indicate that at least some of the synergy arises from intracellular signaling convergence, because the two ligands synergistically enrich TLR signaling pathways. However, extracellular synergy also has been shown in humans with submicrobicidal concentrations of lysozyme and lactoferrin enhancing bacterial killing by cathelicidin and β -defensin 2 (9, 56); combinations of lactoferrin, secretory

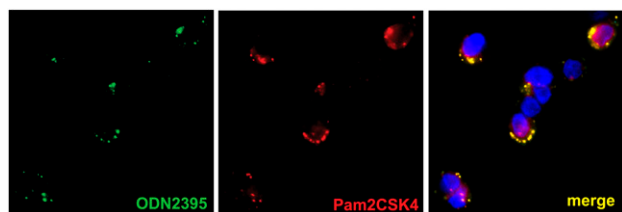


FIGURE 9. TLR2/6 and TLR9 agonists colocalize when applied in vitro. FITC-labeled ODN2395 (20 μ g/ml, green) and Texas Red-labeled Pam2CSK4 (10 μ g/ml, red) were added to A549 cells in monolayer for 2 h; the cells then were washed, stained with DAPI (blue), and subjected to fluorescence microscopy (original magnification $\times 40$). Overlapping red and green fluorescence is shown in yellow. Microscopy experiments were performed four times.

leucocyte protease inhibitor, and lysozyme showing synergistic killing; and antimicrobial proteins interacting with reactive oxygen species (13, 57). Because we have found orthologs of several of these effector molecules to be inducible in mouse epithelial cells by the lysate (17, 18) and by Pam2-ODN (Fig. 8) but have infrequently seen synergistic upregulation of individual effectors by combination treatment, such an extracellular interaction seems likely to contribute, as well.

Potentially lending some insight into the mechanism of synergistic TLR interaction is our finding that class C CpG ODNs combine with Pam2CSK4 to yield greater protection than that observed when class A or class B ODNs are delivered with Pam2CSK4. Synthetic CpG ODN classes can be defined both functionally and structurally (58–61). Class A ODNs have phosphodiester backbones with palindromic sequences and characteristically induce IFN- α and IFN- γ secretion from leukocytes. Class B ODNs have phosphorothioate backbones with 6-mer linear sequences that induce B cell proliferation and IL-6 and IL-10 responses. Class C ODNs have characteristics of both A and B classes (60, 62). These class-specific cytokine responses appear to arise from differential endosomal compartmentalization and signaling, with class A ODNs predominantly triggering IRF-7-mediated signaling from early endosomes, whereas class B ODNs primarily induce NF- κ B activation from late endosomes (60). As such, we hypothesize that class C ODN-induced concurrent signaling by both IRF-7 and NF- κ B pathways may be required for the robust Pam2-ODN effect, perhaps resulting in elaboration of distinct sets of effector molecules that positively interact. This hypothesis is particularly intriguing given our observation of apparent colocalization of the ligands in endosomes and the recent demonstrations of endolysosomal IRF-7 signaling induction by a TLR2/6 agonist (63) and of endosomal trafficking of TLR9 (64).

Given the worldwide mortality burden of pneumonia, therapeutics to broadly induce lung mucosal immunity could be of tremendous potential benefit, particularly for susceptible populations during self-limited periods of peak vulnerability, such as cancer patients undergoing myeloablative therapies, healthy people during emerging epidemics, or individuals at risk for exposure to weaponized bioterror agents. Building on our discovery that MyD88 is required for inducible resistance, our finding of robust protection synergistically induced by synthetic TLR ligands may provide the basis for a clinical intervention to protect broadly against pneumonia.

Disclosures

M.J.T., B.F.D., and S.E.E. are authors on a related United States patent application entitled “Stimulation of Innate Resistance of the Lungs to Infection with Synthetic Ligands”, and M.J.T. and B.F.D. are authors on related patent US60/833,857 entitled “Compositions and Methods for Stimulation of Lung Innate Immunity”. M.J.T., B.F.D., and S.E.E. own stock in Pulmotect, Inc., which holds the commercial options on these patent disclosures. The other authors have no financial conflicts of interest.

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