The Apoptotic Response to Pneumolysin Is Toll-Like Receptor 4 Dependent and Protects against Pneumococcal Disease

Amit Srivastava,¹ Philipp Henneke,² Alberto Visintin,³ Sarah C. Morse,¹ Victoria Martin,¹ Claire Watkins,¹ James C. Paton,⁴ Michael R. Wessels,¹ Douglas T. Golenbock,³ and Richard Malley^{1*}

Division of Infectious Diseases, Department of Medicine, Children's Hospital, Boston, Massachusetts¹; Children's Hospital, Freiburg University, Freiburg, Germany²; Division of Infectious Diseases, Department of Medicine, University of Massachusetts, Worcester, Massachusetts³; and School of Molecular and Biomedical Sciences, University of Adelaide, Adelaide 5005, Australia⁴

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Pneumolysin, the cholesterol-dependent cytolysin of *Streptococcus pneumoniae*, induces inflammatory and apoptotic events in mammalian cells. Toll-like receptor 4 (TLR4) confers resistance to pneumococcal infection via its interaction with pneumolysin, but the underlying mechanisms remain to be identified. In the present study, we found that pneumolysin-induced apoptosis is also mediated by TLR4 and confers protection against invasive disease. The interaction between TLR4 and pneumolysin is direct and specific; ligand-binding studies demonstrated that pneumolysin binds to TLR4 but not to TLR2. Involvement of TLR4 in pneumolysin-induced apoptosis was demonstrated in several complementary experiments. First, macrophages from wild-type mice were significantly more prone to pneumolysin-induced apoptosis than cells from TLR4-defective mice. In gain-of-function experiments, we found that epithelial cells expressing TLR4 and stimulated with pneumolysin were more likely to undergo apoptosis than cells expressing TLR2. A specific TLR4 antagonist, B1287, reduced pneumolysin-mediated apoptosis in wild-type cells. This apoptotic response was also partially caspase dependent as preincubation of cells with the pan-caspase inhibitor zVAD-fmk reduced pneumolysin-induced apoptosis. Finally, in a mouse model of pneumococcal infection, pneumolysin-producing pneumococci elicited significantly more upper respiratory tract cell apoptosis in wild-type mice than in TLR4-defective mice, and blocking apoptosis by administration of zVAD-fmk to wild-type mice resulted in a significant increase in mortality following nasopharyngeal pneumococcal exposure. Overall, our results strongly suggest that protection against pneumococcal disease is dependent on the TLR4-mediated enhancement of pneumolysin-induced apoptosis.

Within the last decade, *Streptococcus pneumoniae* and *Neisseria meningitidis* have become the most common causes of bacterial meningitis in adults and children in countries that have implemented immunization with *Haemophilus influenzae* type b conjugate vaccines (50). Of the two pathogens, *S. pneumoniae* has the highest fatality and morbidity rate; in addition to a mortality rate approaching 30%, about 30 to 50% of survivors have various degrees of neurologic compromise (3, 4, 17). These high rates of morbidity and mortality have been ascribed, at least in part, to the ability of pneumococci to induce neuronal cell death in the central nervous system (57, 58). Previously, investigators have shown that programmed cell death, or apoptosis, played an important role in the neuronal damage due to pneumococcal infection and that the administration of a caspase inhibitor prevents hippocampal cell death (9). More specifically, the following two toxins produced by *S. pneumoniae* have been shown to be associated with neuronal apoptosis in meningitis: hydrogen peroxide and pneumolysin, a protein produced by virtually all clinical isolates of pneumococci (10).

Pneumolysin is an important virulence factor of *S. pneumoniae* and has numerous effects on eukaryotic cells. This 53-kDa member of the cholesterol-dependent cytolysin family binds to cholesterol in cell membranes and creates transmembrane pores (24, 27). At sublytic concentrations, pneumolysin interferes with various aspects of the immune system, such as the respiratory burst, chemotaxis, and bactericidal activity of polymorphonuclear leukocytes (27, 44). Pneumolysin activates the classical pathway of complement by binding to the Fc region of immunoglobulin G (46), and the toxin's complement binding activity has been implicated in recruitment of T cells during pneumococcal infection (28–30). Pneumolysin is a potent inflammatory stimulus, inducing the release of various cytokines, such as tumor necrosis factor alpha, interleukin-6, and interleukin-8, from macrophages and monocytes (12, 26). In a previous study, we showed that the inflammatory response to pneumolysin is independent of the cytolytic properties of the toxins, as a noncytolytic mutant of pneumolysin, PdT, is also highly inflammatory (37). In contrast, the apoptotic responses to pneumolysin are critically dependent on the cytolytic properties of the molecule (10).

There have been several recent studies that have evaluated the interaction between pneumococci and the innate immune system. Pneumococcal components have been found to be recognized by the innate immune receptor Toll-like receptor 2

* Corresponding author. Mailing address: Children's Hospital, Division of Infectious Diseases, 300 Longwood Avenue, Boston, MA 02115. Phone: (617) 919-2902. Fax: (617) 730-0255. E-mail: richard.malley@childrens.harvard.edu.

(TLR2), and the presence of TLR2 appears to influence pneumococcal disease progression (18, 31, 34). We reported that the inflammatory activity of pneumolysin was mediated by TLR4. Upon intranasal pneumococcal challenge, mice lacking functional TLR4 are significantly more susceptible to death than wild-type mice following intranasal challenge with a type 3 strain; furthermore, this increased susceptibility of TLR4-defective mice is absent when pneumolysin-deficient pneumococci are used as the challenge organisms (37). However, the precise mechanism by which TLR4 confers protection against pneumococcal infection remains to be determined.

Dockrell et al. have reported that macrophage apoptosis regulates clearance of bacteria in a murine model of pneumococcal pneumonia (14). Apoptotic properties have been associated with inflammatory molecules that interact with TLRs, such as bacterial lipoproteins that interact with TLR2 (2) and lethal toxin of *Bacillus anthracis*, a TLR4 ligand that triggers apoptosis in macrophages (45). Thus, we decided to test the hypothesis that pneumolysin-induced apoptosis in the upper respiratory tree is mediated by TLR4 and confers protection against pneumococcal disease.

MATERIALS AND METHODS

Reagents. Phosphate-buffered saline (PBS), Dulbecco modified Eagle medium (DMEM), and trypsin-EDTA were obtained from BioWhittaker (Walkersville, MD). Low-endotoxin fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, CT). G418 was obtained from Gibco BRL (Gaithersburg, MD). The selective TLR4 antagonist B1287 (11) was a gift from the Eisai Research Institute (Andover, MA). Pneumolysin was expressed in *Escherichia coli* as a 6xHis-tagged fusion using the QIAExpress expression system according to the manufacturer's instructions (QIAGEN, Valencia, CA). The gene for pneumolysin was cloned into the pQE30 expression vector that generates N-terminal 6xHis-tagged fusion proteins. The recombinant plasmid was transformed into an *E. coli* *msbB* strain; the *msbB* mutation results in production of a lipid A whose ability to activate TLR4 is substantially reduced (23, 60). Endotoxin-free plasticware and glassware were used; endotoxin-free water (Braun, Irvine, CA) was used to make all solutions. Briefly, bacterial lysates were prepared from 500-ml cultures by sonication. Lysate was mixed with Ni-agarose at 4°C overnight to allow for binding with 6xHis-tagged pneumolysin. Columns were prepared and washed extensively, and tagged proteins were eluted with buffer containing 250 mM imidazole. In order to remove as much lipopolysaccharide (LPS) as possible, fractions containing the tagged proteins were treated with End-X endotoxin affinity resin (Associates of Cape Cod, East Falmouth, MA) with end-over-end rotation overnight at 4°C. The protein solution was centrifuged at 2,000 rpm for 2 min, and the supernatant was carefully transferred to a fresh tube. An equal volume of glycerol (RNase and DNase free; Sigma-Aldrich, St. Louis, MO) was added to the pure protein fraction, and aliquots were stored at -20°C until they were used.

Heat-treated LPS and pneumolysin were prepared by heating aqueous stock suspensions at 100°C for 1 h. Protein-free LPS from *E. coli* K235 was a gift from S. Vogel (University of Maryland, Baltimore).

Cell lines. HEK293 cell lines that are stably transfected with TLR2 and TLR4 have been described previously (35). Murine RAW246.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM with 10% FBS and 10 µg/ml ciprofloxacin. RAW macrophages were plated at a concentration of 2×10^5 cells/well in 24-well plates and stimulated within 24 h of plating. The HEK293-TLR2-YFP and HEK293-TLR4-YFP cell lines, which were stably transfected with TLR2-yellow fluorescent protein (YFP) and TLR4-YFP fusions, were maintained in DMEM with 10% FBS and 1 mg/ml G418 (36, 61) and were plated at a concentration of 2×10^5 cells/well in 24-well plates and stimulated within 24 h of plating.

Bacterial strains. *S. pneumoniae* type 3 strains WU2 and A66.1 Xen 10 (both pneumolysin positive), as well as WU2-PLA (a pneumolysin-deficient isogenic mutant of WU2), were used for animal experiments, as described previously (21, 37).

Preparation of HEK293-TLR2-YFP and HEK293-TLR4-YFP protein extracts. The HEK293-TLR2-YFP and HEK293-TLR4-YFP cell lines were grown to confluence in Falcon T-175 flasks (Becton-Dickinson, Franklin Lakes, NJ). Each monolayer was washed with chilled, sterile, LPS-free PBS. One milliliter of lysis

buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) was added to each flask, and the flask was incubated on ice for 20 min to allow gentle lysis. Lysed cells were then collected with a cell scraper and transferred to a chilled tube. Typically, lysates were pooled from at least five T-175 flasks in this manner. The protein concentration was estimated, and aliquots of the lysate were frozen at -80°C. Before use, the lysate was centrifuged at 10,000 rpm for 1 min at 4°C, and the clarified supernatant was transferred to a fresh tube.

Pneumolysin-TLR4 interaction assay. Nunc Maxisorp enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 µl/well of pneumolysin at a concentration of 0.17 µg/well (with or without treatment at 100°C for 1 h) or with bovine serum albumin (BSA) at a concentration of 0.2 µg/well in coating buffer (50 mM Na₂CO₃/NaHCO₃, pH 9.6) overnight at 4°C. Wells were blocked with 0.025% casein in coating buffer (300 µl/well) for 1 h at room temperature. The wells were washed four times with PBS-Tween 20 between steps. After blocking, 100 µl of HEK293-TLR2-YFP or HEK293-TLR4-YFP protein extract per well was added in an eight-point curve using twofold serial dilutions in PBS starting at a concentration of 11 µg/well protein. The plates were incubated at 37°C for 1 h in a humid chamber. After washing, primary immunodetection was carried out using polyclonal rabbit anti-green fluorescent protein (GFP) (1/2,000 dilution; Molecular Probes, Eugene, OR). Secondary immunodetection was carried out using biotinylated goat anti-rabbit antibody (1/10,000 dilution; Santa Cruz Biotechnology, California). Finally, streptavidin-horseradish peroxidase (1/200 dilution) was added. All antibodies were diluted in 1% fetal calf serum in PBS-Tween 20. The plates were developed using SureBlue peroxidase substrate (KPL, Gaithersburg, MD). The color reaction was terminated by addition of 2 N H₂SO₄, and the absorbance at 450 nm was determined. All interaction assays were performed in duplicate, and the results shown below are representative of three or more experiments.

Isolation of peritoneal macrophages. Five- to 8-week-old C3H/HeOuJ and C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). C3H/HeJ mice possess a missense mutation of the TLR4 gene (P712H), which renders them hyporesponsive to LPS (47) and hypersusceptible to pneumococcal infection following intranasal colonization with a type 3 pneumococcal strain, WU2 (37); C3H/HeOuJ mice respond to LPS and pneumolysin. Mice were injected intraperitoneally with 2.5 ml 3% thioglycolate (Remel, Lenexa, KS). After 3 days, mice were euthanized, and cells were recovered by peritoneal lavage with 10 ml DMEM containing 10% FBS and 10 µg/ml of ciprofloxacin. Cells were washed and plated to obtain the appropriate density in tissue culture dishes (1×10^5 cells/well for 96-well plates, 9×10^5 cells/well for 24-well plates). After 24 h, nonadherent cells were removed by washing with medium, and adherent cells were stimulated as described below.

Apoptosis assays. For TUNEL staining, cells were lifted by gentle scraping with a rubber policeman 18 h after stimulation, harvested by centrifugation at $800 \times g$ for 5 min, fixed with 70% ethanol for 15 min at 4°C, permeabilized, and labeled according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Fluorescein; Roche, Indianapolis, IN). For hypodiploid DNA staining, cells were harvested and fixed as described above and then resuspended in PBS with propidium iodide (50 µg/ml) and RNase A (500 µg/ml) for 20 min at room temperature. For evaluation of apoptosis in HEK293 epithelial cells stably expressing TLR2-YFP and TLR4-YFP, histone-associated DNA was measured per the manufacturer's instructions (Roche) since the YFP fluorophore interferes with detection by TUNEL. In each case, cells were finally washed in PBS and examined by flow cytometry using a FACScan instrument (Becton-Dickinson), and data were analyzed using the CellQuest software (BD Biosciences, San Jose, CA).

Pneumolysin-induced caspase 3 activation was examined by using an activity assay kit according to the manufacturer's instructions (ApoTarget; Biosource, Camarillo, CA). Briefly, RAW macrophages were exposed to increasing doses of purified pneumolysin for times ranging from 20 min to 8 h. Cytosolic extracts were prepared from exposed cells and then incubated with chromogenic substrate conjugates. Caspase 3 activity was measured colorimetrically at 405 nm.

In vivo apoptosis and animal models of pneumococcal sepsis. For assessment of in vivo apoptosis in cells from the upper respiratory tract, groups of 5- to 8-week-old male C3H/HeOuJ and C3H/HeJ mice were gently restrained without anesthesia and inoculated intranasally with 10 µl containing 2×10^8 CFU of either WU2 (pneumolysin-producing) or WU2-PLA (pneumolysin-deficient) bacteria (five mice per group). Eighteen hours later, mice were sacrificed, and tracheal washes were collected. Cells were harvested from the tracheal washes by centrifugation, fixed with fresh 2% paraformaldehyde in PBS for 1 h at room temperature, and subsequently permeabilized and labeled according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Fluorescein; Roche, Indianapolis, IN). An aliquot of cells from each sample was analyzed by fluo-

rescence microscopy (emission wavelength, 515 to 565 nm), and the rest of the cells were examined by flow cytometry as described above.

For sepsis experiments, wild-type mice (12 mice per group) were randomized per cage to receive intranasal doses of either the pan-caspase inhibitor zVAD-fmk in dimethyl sulfoxide (DMSO) or DMSO alone. At the time of challenge, mice were gently restrained without anesthesia and inoculated intranasally with 10 μ l of strain A66.1 Xen 10 (type 3, pneumolysin-producing strain) (21) containing 5×10^8 CFU. Immediately following this inoculation, mice were intranasally inoculated with 20 μ l of either zVAD-fmk (20 μ g) in 6% DMSO-PBS or 6% DMSO-PBS alone depending on the group assignment. Intranasal administration of zVAD-fmk or DMSO alone was repeated every 12 h for a total of nine doses. The mice were then carefully observed daily for 7 days. Animals that appeared to be ill prior to day 7 were euthanized, and blood obtained by cardiac puncture was cultured for pneumococci. In all such cases, culturing confirmed the presence of pneumococcal bacteremia.

Statistical analysis. Differences between two groups were evaluated by Student's *t* test or the Mann-Whitney U test, depending on whether the data were normally distributed. For comparison of three or more groups, the Kruskal-Wallis test was used, with Dunn's adjustment for multiple comparisons. Survival curves for mice given the apoptosis inhibitor or the diluent alone were compared using Kaplan-Meier's test. For all comparisons, a *P* value of ≤ 0.05 was considered significant.

RESULTS

Pneumolysin interacts with TLR4 but not with TLR2 in a solid-phase binding assay. We began our studies by investigating whether a physical interaction between pneumolysin and TLR4 could be demonstrated. We designed an ELISA-based solid-phase interaction assay using pure preparations of pneumolysin (after passage over endotoxin-neutralizing protein beads to remove contaminating LPS). Lysates prepared from HEK293 cell lines expressing TLR4-YFP and TLR2-YFP fusion proteins were employed as a source of the TLR proteins (36, 61). Pneumolysin was coated onto 96-well ELISA plates. After blocking with casein, equal amounts of the TLR-YFP extracts were overlaid to allow interaction. Unbound protein was washed off, and bound TLRs were detected by a polyclonal anti-GFP antiserum, followed by a biotinylated secondary antibody and peroxidase-coupled streptavidin (Fig. 1A). Pneumolysin exhibited strong physical association with TLR4-YFP. This interaction was dose dependent and reproducible. Pneumolysin did not show any interaction with TLR2-YFP, demonstrating that this assay is able to detect specific interaction between pneumolysin and TLR4 (Fig. 1B and C). When an unrelated protein, BSA, was used as the coating antigen instead of pneumolysin, no interaction was observed with either TLR2 or TLR4 even with five times (50 μ g) the maximum amount of extract used with pneumolysin (11 μ g) (Fig. 1C). Furthermore, when pneumolysin was denatured by heat treatment (100°C for 1 h), the interaction with TLR4 was abrogated, ruling out any residual LPS contamination as an explanation for our findings (data not shown), since LPS is not heat labile. Finally, the addition of soluble pneumolysin to the TLR4-YFP lysates resulted in $>50\%$ inhibition of the solid-phase interaction between pneumolysin and TLR4 (data not shown). Taken together, these results strongly indicate that pneumolysin is able to specifically and stably interact with TLR4.

Pneumolysin-induced apoptosis is TLR4 dependent. To test the hypothesis that TLR4 mediates pneumolysin-induced apoptosis, murine RAW264.7 macrophages were exposed to pneumolysin in the presence and absence of a selective TLR4 antagonist, B1287 (11). As shown in Fig. 2A, increasing concentrations of pneumolysin caused an increase in the number

FIG. 1. Pneumolysin interacts with TLR4 but not with TLR2 in a solid-phase binding assay. (A) Ninety-six-well ELISA plates were coated with pneumolysin, blocked, and then overlaid with increasing amounts of lysates from HEK293-TLR2-YFP or HEK293-TLR4-YFP cells. After extensive washing, TLR-YFP bound to the coating protein was detected by an anti-GFP antibody. (Inset) Immunoblot with anti-GFP antibody with equal amount of lysates used in the interaction assay. (B) Pneumolysin coated at a concentration of 0.17 μ g/well and maximum HEK293-TLR lysate concentration of ~ 11 μ g/well. (C) BSA coated at concentration of 0.2 μ g/well and maximum HEK293-TLR lysate concentration of 50 μ g/well. The results shown are representative of three or more experiments. HRP, horseradish peroxidase; IgG, immunoglobulin G; Ply, pneumolysin.

FIG. 2. Pneumolysin-induced apoptosis is TLR4 dependent. (A) Selective TLR4 antagonist B1287 blocks pneumolysin-induced apoptosis. TLR4 wild-type (C3H/HeOuJ) macrophages were stimulated with increasing concentrations of pneumolysin in the presence or absence of B1287. Apoptosis was assessed by enumerating cells with hypodiploid DNA by flow cytometry; the assay was repeated twice, and the results of a representative experiment are shown. (B) Macrophages with functional TLR4 (C3H/HeOuJ) exhibit significantly more pneumolysin-induced apoptosis than TLR4-defective (C3H/HeJ) macrophages. Macrophages derived from wild-type and TLR4-defective mice were exposed to increasing concentrations of pneumolysin and assayed for apoptosis by enumerating cells with hypodiploid DNA as described above. An asterisk indicates that the *P* value is 0.037 as determined by a *t* test. (Inset) Human epithelial cells transfected with TLR4 (HEK-TLR4) are more susceptible to apoptosis than cells transfected with TLR2 (HEK-TLR2). Stably transfected cells were exposed to increasing concentrations of pneumolysin, and apoptosis was assessed by measurement of histone-associated DNA by flow cytometry. The assay was repeated more than three times, and the results of a representative experiment are shown.

of apoptotic cells, whereas in the presence of B1287, the number of apoptotic cells was reduced. When another proapoptotic agent (doxorubicin) was used, no effect of B1287 could be demonstrated (data not shown). We evaluated the involvement of TLR4 in pneumolysin-induced apoptosis more directly in a loss-of-function assay using macrophages derived from TLR4 wild-type (C3H/HeOuJ) and TLR4-defective (C3H/HeJ) mice. Upon exposure to increasing concentrations of pneumolysin, the macrophages from the wild-type mice showed a signifi-

FIG. 3. Pneumolysin-induced apoptosis is caspase dependent. The apoptotic response to pneumolysin is inhibited by the pan-caspase inhibitor zVAD-fmk. RAW264.7 macrophages were exposed to increasing concentrations of pneumolysin in the presence or absence of the pan-caspase inhibitor (20 µg/ml for 18 h), and apoptosis was assessed by TUNEL staining followed by flow cytometry as described in Materials and Methods. Asterisks indicate that the *P* value is 0.047. The results shown are representative of three or more experiments. Ply, pneumolysin.

cantly greater percentage of apoptotic cells than the macrophages from the TLR4-defective mice (comparison of the percentages of apoptotic cells in the TLR4 wild-type and defective macrophages exposed to the highest concentration of pneumolysin, *P* = 0.037) (Fig. 2B). Similarly, in gain-of-function experiments, human embryonic kidney epithelial cells (HEK293) expressing TLR4 also underwent more apoptosis following pneumolysin exposure than cells expressing TLR2 (Fig. 2B, inset). These cell lines do not express MD-2 and therefore are not responsive to the effects of LPS, the canonical TLR4 ligand (35, 49). Taken together, these data indicate that in both murine macrophages and human epithelial cells, the apoptotic effect of pneumolysin is, at least in part, TLR4 dependent.

Pneumolysin-induced apoptosis is caspase dependent. Using different cell types, other investigators have reported that pneumococci induce apoptosis by a caspase-dependent mechanism, but in these studies it appeared that the effect was not dependent on pneumolysin (13, 48). In light of our finding that pneumolysin can induce apoptosis in a TLR4-dependent fashion and previous studies showing that certain Toll ligands activate caspases (2, 13), we reexamined the issue of caspase involvement in pneumolysin-induced apoptosis. RAW264.7 macrophages were exposed to increasing amounts of pneumolysin in the absence and presence of the pan-caspase inhibitor zVAD-fmk. In the presence of this inhibitor, apoptosis was significantly reduced (comparison of the percentages of apoptotic cells in the absence and presence of zVAD-fmk, *P* = 0.047) (Fig. 3). Therefore, zVAD-fmk inhibits pneumolysin-induced apoptosis, and this indicates that caspases are involved. The effect is only partial, however; at higher doses of pneumolysin zVAD-fmk does not inhibit the apoptotic response. Upon examining pneumolysin-induced caspase 3 activity, we found that there was induction within 30 min of exposure to pure pneumolysin and that there was up to a 40% increase at 2 h compared with the uninduced controls (data not

shown). Taken together, our results suggest that pneumolysin induces apoptosis in macrophages by a pathway that involves, at least in part, caspases.

TLR4 enhances apoptosis in nasopharyngeal tissue of mice in response to pneumolysin-producing pneumococci. Wild-type mice are significantly more resistant to invasive disease following intranasal inoculation with a type 3 virulent pneumococcus than mice that lack functional TLR4 (37), but the underlying mechanisms of resistance are unknown. We hypothesized that induction of apoptosis of cells in the upper respiratory tree may limit the ability of the bacterium to invade the host and thus explain the increased resistance to pneumococcal disease of TLR4-bearing mice. To test this hypothesis, we first evaluated whether the presence of TLR4 enhances apoptosis of cells in the upper respiratory tree, as suggested by our in vitro experiments. TLR4 wild-type and TLR4-defective mice (four or five mice per group) were intranasally inoculated with 2×10^8 CFU of pneumococcal strain WU2 or its isogenic pneumolysin-deficient mutant, WU2-PLA. Eighteen hours later, tracheal and nasopharyngeal secretions were collected from euthanized animals by retrograde tracheal washing (38). Cells recovered in the tracheal washes were analyzed for apoptosis by TUNEL staining, followed by fluorescence microscopy and flow cytometry.

As shown in Fig. 4A, cells showing bright fluorescent TUNEL staining indicative of DNA fragmentation also displayed other features characteristic of apoptosis visible upon bright-field examination, notably cell shrinkage due to condensation of the cytoplasm with tightly packed organelles and the formation of micronuclei and apoptotic bodies along with blebbing from the cell surface. In contrast, cells that did not stain with TUNEL were much bigger, had an intact membrane, and showed none of the stress features exhibited by the apoptotic cells (Fig. 4B). The cells recovered from tracheal washes were examined by flow cytometry to determine the percentage of apoptotic cells. Based on the apoptotic phenotype observed by microscopy, we focused on the population of cells showing bright TUNEL staining (high FL-1) and reduced size (low forward scatter). As shown in Fig. 4C, wild-type and TLR4-defective mice exhibited similar levels of apoptosis in response to infection with pneumolysin-deficient strain WU2-PLA (comparison of the percentages of apoptotic cells recovered from tracheal washes of C3H/HeOuj and C3H/HeJ mice, $P = 0.34$, as determined by a Mann-Whitney U test). Cells recovered from TLR4-defective mice exhibited similar levels of apoptosis when they were infected with either strain WU2 or its isogenic pneumolysin-deficient mutant, WU2-PLA ($P = 0.29$, as determined by a Mann-Whitney U test). In contrast, with pneumolysin-producing strain WU2, significant differences were observed between wild-type and TLR4-defective mice. Wild-type mice challenged with WU2 exhibited the greatest level of apoptosis overall, which was significantly higher than the level seen in TLR4-defective mice ($P = 0.016$, as determined by a Mann-Whitney U test). Thus, the results of the in vivo apoptosis experiments are consistent with our in vitro observations linking pneumolysin-induced apoptosis and TLR4. Furthermore, it is noteworthy that when wild-type or TLR4-defective mice were infected with pneumolysin-deficient strains, no significant differences in apoptosis were noted, indicating that this effect is critically dependent on pneumolysin.

Inhibition of apoptosis by topical administration of zVAD-fmk favors progression of invasive pneumococcal disease in wild-type mice. So far, we have shown that following exposure to pneumolysin-producing pneumococci, cells from the upper respiratory tree of wild-type mice are significantly more likely to undergo apoptosis than cells from TLR4-defective mice. To evaluate whether apoptosis may be protective in this setting, we used a mouse model in which intranasal exposure to pneumococci leads to sepsis and death in TLR4-defective mice but not in wild-type mice following challenge with the WU2 strain (37) and evaluated whether pharmacological inhibition of apoptosis in wild-type mice by topical administration of zVAD-fmk increased their susceptibility to invasive disease and death. TLR4 wild-type mice were challenged intranasally with pneumolysin-producing pneumococci at a dose of 10^8 CFU. In addition to the bacteria, the mice were given intranasal doses of the pan-caspase inhibitor zVAD-fmk or the vehicle (6% DMSO-PBS), both prior to pneumococcal nasopharyngeal challenge and after the challenge at 12-h intervals for a total of 5 days (Fig. 5). The intranasal inocula containing bacteria, inhibitor, or the vehicle control (all in 10 μ l) were always administered to unanesthetized mice, so that aspiration into the lungs did not occur (38). As shown in Fig. 5, mice that received zVAD-fmk died steadily in greater numbers and had a significantly shorter time to death than mice that did not receive the pan-caspase inhibitor ($P = 0.043$, as determined by a Kaplan-Meier test). Thus, pharmacological inhibition of apoptosis by topical administration in the nasopharynx significantly increased the susceptibility of wild-type mice to pneumococcal sepsis, demonstrating that apoptosis in the upper airways is a mechanism of resistance to pneumococcal invasive disease.

DISCUSSION

Recognition of bacterial components by the innate immune system has been recognized as an effective method for protecting the host against various pathogens (25, 42). More specifically, the interaction of bacterial components with TLRs has been shown to confer protection against viral and bacterial diseases (16, 20, 37, 39, 51, 53, 55). The mechanisms by which this protection occurs, however, have not been fully elucidated yet. Activation of phagocytes by inflammatory cytokines is likely to play an important role in enhancing killing of the pathogen, as shown by the susceptibility of tumor necrosis factor alpha-depleted or -deficient mice and other cytokine-deficient mice to viral and bacterial infections (7, 19, 52). Another postulated mechanism for clearance of pathogens is by apoptosis of infected phagocytes and other cells, as seen in the case of pneumococci, *Streptococcus pyogenes*, and uropathogenic *E. coli* (1, 14, 43, 56).

Our findings confirm the importance of apoptosis as an innate mechanism of protection from invasive disease and extend the recent finding that apoptosis in pulmonary cells enhances clearance of pneumococci in the lung (14). Furthermore, we (37, 62) and other workers (32, 33) have previously demonstrated the importance of TLR2 and TLR4 as critical components of the innate immune protection against pneumococcus. In the present report, we show that TLR4- and caspase-dependent apoptosis following nasopharyngeal chal-

FIG. 4. TLR4 mediates apoptosis by pneumolysin-producing pneumococci in the upper respiratory tree of mice. Wild-type (C3H/HeOuJ) and TLR4-defective (C3H/HeJ) mice were intranasally inoculated with strain WU2 (five mice per group) or its isogenic, pneumolysin-negative derivative WU2-PLA (four mice per group); 18 h after challenge, the mice were sacrificed, and cells recovered from retrograde tracheal washes were assessed for apoptosis by TUNEL staining. Cells were first examined by fluorescence (detection at 515 to 565 nm [green]) and bright-field microscopy, which was followed by enumeration by flow cytometry. (A and B) Collages of micrographs showing phenotypes of recovered cells. Cells exhibiting bright TUNEL staining (A), indicative of DNA fragmentation associated with apoptosis, were characteristically shrunken and were much smaller than cells that remained unstained and possessed numerous apoptotic bodies in the cytoplasm. Cells that did not show any TUNEL staining due to the absence of DNA fragmentation (B) were much larger and possessed an intact cell membrane. Photographs were taken at the same magnification and are representative of the cell populations observed. (C) Flow cytometry analysis revealed that wild-type mice challenged with WU2 have a significantly larger proportion of apoptotic cells than TLR4-defective mice ($P = 0.016$); no differences were noted when pneumolysin-deficient strain WU2-PLA was used ($P = 0.29$).

FIG. 5. In vivo inhibition of apoptosis in the upper respiratory tract favors progression of invasive pneumococcal disease. Wild-type (C3H/HeOuJ) mice received either the pan-caspase inhibitor zVAD-fmk ($n = 12$) or vehicle alone ($n = 12$) intranasally every 12 h prior to and following intranasal inoculation with strain WU2, a pneumolysin-producing type 3 pneumococcus. Survival was monitored twice daily for 8 days. The difference in survival between zVAD-fmk- and vehicle-treated mice was significant ($P = 0.043$, as determined by a Kaplan-Meier test).

challenge with pneumolysin-producing pneumococci is likely to represent a mechanism by which TLRs contribute to protection against bacterial challenge. Our results, therefore, implicate TLR4-mediated apoptosis as a potent protective immune response against pneumococcal infection and provide a mechanistic explanation for the increased susceptibility of TLR4-deficient mice to pneumococcal disease.

Induction of the apoptotic pathway has been shown to be critically dependent on the presence of pneumolysin (8, 10, 13), a toxin whose release in most but not all strains (5) is tightly regulated by the pathogen. Our results are consistent with two previous studies by Braun and colleagues which showed the importance of pneumolysin as a mediator of microglial and neuronal apoptosis (10) and demonstrated that treatment with a caspase inhibitor prevents hippocampal neuronal cell death in experimental pneumococcal meningitis (8). Two different mechanisms of apoptosis in bone marrow-derived dendritic cells exposed to whole pneumococci in vitro have been described: a rapid, caspase-independent response due to pneumolysin and a more delayed, caspase-dependent mechanism associated with pneumococcal subcapsular components (13). Using a model for pneumococcal meningitis in mice with defined genetic lesions in a caspase-dependent apoptotic pathway, two phases of neuronal cell death due to bacterial infection have been described: an initial caspase 3-independent program that is elicited by pneumolysin and hydrogen peroxide and a second phase that is caspase 3 dependent caused by release of pneumococcal cell wall components (41). Most recently, Bermopohl et al. showed that the induction of apoptosis by pneumococci in transfected epithelial cells was dependent on TLR2 but independent of TLR4 (6). In contrast, using purified pneumolysin and peritoneal macrophages, we demon-

strated that induction of apoptosis by pneumolysin is in fact TLR4 and caspase dependent and can be diminished by treatment with a pan-caspase inhibitor. It is certainly possible that the cell type and the nature of the stimulus (whole organism versus purified toxin) could account for the differences in our findings. Additionally, the pneumolysin-positive pneumococcal strains used in the experiments (D39 in the experiments of Colino and Snapper and WU2 or A66 in our experiments) have previously been shown to have different mechanisms of pneumolysin release (5), which may also explain the contrasting results. Nevertheless, all these studies buttress the observation that apoptosis plays an important role in pneumococcal pathogenesis.

Our studies have several important implications. Our previous demonstration that pneumolysin is a TLR4 ligand is supported and further extended in the present report. We demonstrated not only the role of TLR4 in the apoptotic response but also that there is a physical interaction between this receptor and pneumolysin. Whereas some workers have questioned whether small amounts of contaminating LPS may contribute to TLR4 activation by putative TLR4 ligands, such as heat shock proteins (22), such a concern is less relevant in binding studies or apoptosis assays, in which LPS alone does not induce apoptosis. Second, results obtained with our mouse model clearly demonstrated the importance of apoptosis as a protective response to exposure to a pathogen. In this regard, the propensity of certain viruses, such as respiratory syncytial virus, to induce an anti-apoptotic program in mammalian cells (15, 40, 54) may explain the association between certain respiratory viral infections and pneumococcal disease in humans. Finally, our results raise the possibility that interference with the action of certain TLRs or the inhibition of apoptosis by specific caspase inhibitors may increase susceptibility to specific bacterial infections and thus have deleterious effects on the host (59).

Based on our results, we submit that TLR4-mediated apoptosis in host cells in the upper respiratory tract in response to pneumolysin constitutes an important mechanism of host defense against pneumococci. Our results are largely in agreement with the previous study that established a mouse model for pneumococcal infection in the lung and demonstrated that apoptosis in alveolar macrophages in response to pneumococcal infection helped clear the infection (14). Our data suggest a possible mechanistic explanation for this effect: the physical interaction between TLR4 and pneumolysin enhances the apoptotic response to the toxin, by a caspase-dependent pathway, and thus may induce resistance to pneumococcal disease.

In conclusion, we show here that TLR4 mediates resistance to pneumococcal infection via enhancement of the apoptotic effects of pneumolysin. While this apoptotic effect has clearly been shown to be deleterious in animal models of central nervous system infection, we suggest that it can be viewed as a mechanism of host defense occurring at an early stage of pneumococcal pathogenesis.

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