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Neutrophil Elastase Mediates Innate Host Protection against *Pseudomonas aeruginosa*[1]

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According to the widely accepted view, neutrophil elastase (NE), a neutrophil-specific serine protease, is a major contributor to *Pseudomonas aeruginosa* infection-associated host tissue inflammation and damage, which in severe cases can lead to death. Herein, we provide for the first time compelling evidence that the host rather employs NE to protect itself against *P. aeruginosa* infection. Using a clinically relevant model of pneumonia, targeted deficiency in NE increased the susceptibility of mice to *P. aeruginosa*. We found that NE was required for maximal intracellular killing of *P. aeruginosa* by neutrophils. In investigating the mechanism of NE-mediated killing of *P. aeruginosa*, we found that NE degraded the major outer membrane protein F, a protein with important functions, including porin activity, maintenance of structural integrity, and sensing of host immune system activation. Consistent with this, the use of an isogenic mutant deficient in outer membrane protein F negated the role of NE in host defense against *P. aeruginosa* infection. ***The Journal of Immunology,*** **2008, 181: 4945–4954.**

When the first lines of defense against invading microorganisms are breached, neutrophils rapidly accumulate at the site of infection. The primary purpose of this neutrophilic infiltration is the killing of pathogens and resolution of the associated inflammation (1). The importance of neutrophils in protection against microbes is demonstrated in patients with neutropenia or neutrophil dysfunctions who commonly experience recurrent and/or life-threatening infections (2–4). Various antimicrobial molecules have been identified in neutrophil granules and grouped into oxygen-dependent (5) and -independent systems (6). The latter system comprises the readily active serine proteinases, neutrophil elastase (NE),[5] cathepsin G (CG), and proteinase 3, among other polypeptides known to alter the integrity of bacteria. Like its family members, NE catalytic activity relies on the His^{57}-Asp^{102}-Ser^{195} triad (chymotrypsin numbering system) where serine represents the active residue (7). Mature NE, a cationic glycoprotein, is stored in neutrophil primary granules at concentrations in the millimolar range, making it a major component of neutrophils (8). The human and mouse NE genes are highly related, and the codons of His-Asp-Ser catalytic triad residues are well conserved (9). In recent years, gene targeting of NE in mice revealed that the enzyme contributes considerably to host defense against microbial infections (10–15).

Pseudomonas aeruginosa is an obligately aerobic, rod-shaped bacterium that inhabits a variety of environmental niches, including soil, water, and opportunistically the human host. Due to its genetic flexibility and large genome, *P. aeruginosa* is biochemically versatile (16). Such features confer on this Gram-negative pathogen the ability to grow in many habitats and thrive in diverse circumstances. *P. aeruginosa* is virtually nonpathogenic in healthy people, but individuals with defective function of the cystic fibrosis transmembrane conductance regulator or impaired defense systems such as neutropenic or immunocompromised patients are commonly subjects to *Pseudomonas* infection (17, 18). *P. aeruginosa* is thus an opportunistic pathogen that causes both acute and chronic infections. The pathogen is notable for its high intrinsic resistance to antibiotics, and it is frequently isolated in various infectious settings involving the eye, skin, and respiratory tract. Regarding lung infections, *P. aeruginosa* is a leading pathogen causing nosocomial pneumonia, and among the most vulnerable hosts are the mechanically ventilated patients (19). Of note, mortality rates of patients with *P. aeruginosa* pneumonia have been shown to be higher than those of patients with pneumonia caused by other pathogens (20–22).

Following *P. aeruginosa* lung infection, neutrophils represent the earliest phagocytic cells that are attracted to the infected site (23). As a result of its capacity to target a variety of host cells and both soluble and insoluble substrates, NE has been always regarded as major contributor to *P. aeruginosa* infection-associated tissue inflammation and damage, which in severe cases can lead to death (24, 25). This view is supported by the long-held dogma that activated neutrophils excrete NE, which, because of the number of

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[5] Abbreviations used in this paper: NE, neutrophil elastase; α1-AT, α1-antitrypsin; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; BAL, bronchoalveolar lavage; CG, cathepsin G; CI, competitive killing index; IEF, isoelectric focusing; i.n., intranasal; MMP, metalloproteinase; MPO, myeloperoxidase; NPN, 1-*N*-phenylnaphthylamine; Om, outer membrane; Omps, outer membrane proteins; OprF, outer membrane protein F; SEM, scanning electron microscopy; SLPI, secretory leukocyte protease inhibitor; TEM, transmission electron microscopy; WT, wild type.

neutrophils present, overwhelms the lung's ability to neutralize it (26, 27). Consequently, while much interest has focused on the pathogenic effects of this enzyme, it remains unclear whether NE could actually combat *P. aeruginosa*.

Using genetically engineered mice deficient in NE, we demonstrate that the enzyme plays an important role in innate defense against *P. aeruginosa* infection. The importance of the protective role of NE was revealed in a clinically relevant model of *Pseudomonas* pneumonia. Our data indicate that neutrophils employ NE to kill *P. aeruginosa*. In investigating the mechanism of NE-mediated killing of *P. aeruginosa*, we identified the major outer membrane protein F (OprF) as a critical molecule degraded by NE. Significantly, the use of an isogenic mutant of *P. aeruginosa* deficient in OprF negated NE antibacterial role both in vitro and in the pneumonia model.

Materials and Methods

Mice, neutrophils, and bacteria

Animal handling and procedures were approved by the Animal Studies Committee at our institution. NE-deficient ($NE^{-/-}$) mice were generated by targeted mutagenesis (10). $NE^{-/-}$ mice and their wild-type (WT) littermates had 129/SvEv or C57BL/6 genetic background and were sex and age (8–10 wk) matched.

Neutrophils were isolated from the mouse bone marrow or following i.p. injection of mice with glycogen as previously described (10, 28).

P. aeruginosa H103 and its isogenic mutant H636 (streptomycin resistant) deficient in the major OprF ($OprF^{-}$) were used in this work (29). The mutant H636 was generated by insertion mutagenesis. Two clinical *P. aeruginosa* strains, *P.a.* 11881 and *P.a.* 12749, isolated from patients with pneumonia were obtained from the Bacteriology and Virology Division at Reims Hospital. *Escherichia coli* was from our laboratory collection of bacterial strains. Bacteria were washed twice with PBS (pH 7.4), and the OD of the cultures was determined at 600 nm (1 $OD_{600\ nm} \cong 1 \times 10^9$ bacteria/ml) (15).

P. aeruginosa *intranasal infection and survival of mice*

Bacteria were passaged twice in mouse lungs before use (13). Mice were anesthetized and intranasally (i.n.) challenged with bacteria (23). At least 30 WT mice were i.n. injected with varying amounts of *P. aeruginosa* H103 bacteria, and their survival was followed over time. From these experiments, the concentration of *P. aeruginosa* H103 that killed ~50% of WT mice was determined by interpolation (LD_{50}, $\sim 9 \times 10^6$ CFUs/mouse in 50 μl PBS). Next, $NE^{-/-}$ and WT mice ($n = 12$/genotype) were i.n. infected with the LD_{50} and their survival was monitored during 72 h.

In subsequent experiments, groups of $NE^{-/-}$ and WT mice ($n = 12$/genotype) were i.n. infected with *P. aeruginosa* H103 or its isogenic mutant $OprF^{-}$, and their survival was followed during 72 h. Bacteria were used at a dose of $\sim 9 \times 10^6$ CFUs and 5-fold higher ($\sim 5 \times 10^7$ CFUs).

Fluorescence imaging

To ensure that mouse lungs were infected, $NE^{-/-}$ and WT mice were challenged with fluorescent-labeled *P. aeruginosa* H103 and examined using fluorescence imaging (30). Specifically, bacteria were labeled with the near-infrared fluorophore bispropylcarboxymethylindocyanine (cypate). Cypate was synthesized as described previously and dissolved in DMSO (10 mg/ml). Twenty microliters of this solution was added to bacteria (1×10^9) in a total volume of 1 ml PBS at room temperature. Following 30 min incubation on a nutator, bacteria were extensively washed to remove unbound fluorophore and resuspended in 1 ml PBS. Of note, cypate and DMSO treatments affected neither the viability nor the growth of bacteria as determined by the plating method (data not shown). Next, groups of mice were challenged i.n. with 50 μl of labeled bacteria (3.5×10^7 CFUs/mouse) (four per genotype) or saline alone (two per genotype). Four hours later, all mice were sacrificed as above and their lungs, liver, spleen, and stomach excised (23). Imaging of fluorescence ($\lambda_{max,abs}$ 786 nm; $\lambda_{max,em}$ 811 nm) in each organ was performed using a Photometrix CoolSNAP HQ scanner and WinView software (Princeton Instruments). The near-infrared light permits the study of the distribution of the fluorophore in deep tissues since absorption and scattering of emitted light by endogenous biomolecules are minimal. Also, the fluorophore penetrates tissue barriers without the accompanying autofluorescence that occurs in the UV/visible spectrum.

Lung histology and immunostaining

$NE^{-/-}$ and WT mice were subjected to i.n. instillation of *P. aeruginosa* (LD_{50}) or sterile HBSS (pH 7.4) ($n = 4$ mice/genotype and condition) and sacrificed 16 h postchallenge (23). Next, mice were processed for lung histology and immunohistochemistry using mouse myeloperoxidase Ab (MPO, dilution, 1/3000) (31).

Differential cell counts and bacterial viability

Experiments were performed as previously described (13, 23). Blood samples were collected from the inferior vena cava. Next, mouse lungs were perfused and lavaged in situ with HBSS (pH 7.4) and aseptically removed. Both blood and bronchoalveolar lavages (BALs) were normalized to the returned volumes. Total and differential counts and viability of inflammatory cells in lavage fluids were immediately determined. Equal aliquots of blood, BAL fluids, or homogenized lungs were serially diluted and plated to determine the number of viable bacteria.

Bactericidal activity of neutrophils and purified NE

Neutrophil antibacterial activity was assessed as previously described, with the following modification (10). Briefly, the number of WT neutrophils that killed ~50% of *P. aeruginosa* (10^7 bacteria) was first predetermined (10^6 cells). Next, bacteria were prepared as described above, resuspended in DMEM supplemented with 10% mouse serum, and added onto the monolayer of $NE^{-/-}$ or WT neutrophils at a ratio of 1:10 (cell/bacterium). Neutrophil-mediated killing was assessed as the decrease in the number of viable bacteria relative to the total number of input bacteria. Experiments were performed four times.

NE bactericidal assay was conducted as previously described, with the following modifications (13). Bacteria were exposed to varying NE doses and their viability was determined. From these experiments, the dose of NE that killed ~50% of bacteria (10^7 bacteria) was determined (2 μg). Next, freshly grown *P. aeruginosa* (1×10^7 bacteria) were incubated alone or with NE alone or preincubated with the specific serine proteinase inhibitors AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) (1 mM) or α1-AT (α1-antitrypsin) (5 μg) (Sigma-Aldrich). Neither AEBSF nor α1-AT affected bacterial growth at the indicated concentrations (data not shown). Following incubation for 60 min, the numbers of CFUs were determined by plating method. In parallel experiments, NE activity in the reaction was examined using its specific chromogenic substrate, Meo-Suc-Al-Al-Pro-Val-pNA (Sigma-Aldrich), in the absence or presence of the above inhibitors (31).

Unless otherwise stated, this NE dose was used in all subsequent experiments. Of note, the purity and activity of NE (Elastin Products) were confirmed by SDS-PAGE and zymography and/or spectrophotometrically using NE-specific chromogenic substrate.

Fluorescence microscopy

The viability of bacteria following exposure to NE was further assessed by fluorescence microscopy using two-color visualization of dead and live cells (13).

Transmission and scanning electron microscopy

After incubation with or without NE, bacteria were processed for scanning electron microscopy (SEM) or transmission electron microscopy (TEM) as previously described (10, 13).

Isolation and incubation with NE of P. aeruginosa *outer membrane proteins (Omps)*

An overnight culture of *P. aeruginosa* (H103 and clinical strains) diluted 1/100 was grown in Luria-Bertani medium (500 ml) aerobically under gentle shaking conditions at 37°C to late exponential phase. Cultures were centrifuged ($6000 \times g$, 15 min) and pellets washed with Tris-HCl (50 mM (pH 7.3)). Next, bacteria fractionation was performed (32). Briefly, bacterial pellets were resuspended in 5 ml of Tris-HCl (50 mM (pH 7.3)) and lysed by sonication. Following endonuclease treatment, the mixture was centrifuged ($8000 \times g$, 10 min). The supernatant was diluted up to 60 ml in ice-cold sodium carbonate (0.1 M (pH 11)), stirred for 1 h at 4°C, and subjected to ultracentrifugation ($100{,}000 \times g$, 1 h at 4°C). Next, the pellet was resuspended in 5 ml of Tris-HCl (50 mM, pH 7.3) for one more additional wash. The pellet, which contains Omps fraction, was collected by ultracentrifugation and resuspended in 2 ml of Tris solution.

Omps and purified flagellin protein were incubated without or with NE and migrated on protein gels. Next, the reactions were Coomassie blue stained or processed for Western blotting using an Ab specific for the OprF (dilution 1/20,000) (13, 15, 33).

Two-dimensional electrophoresis, gel image analysis, and mass spectrometry

Five hundred micrograms of Omps was incubated without or with NE (10 μg) for 1 h at 37°C (300 μl reaction volume) as described above. An aliquot of the reaction (5 μl) was resolved by SDS-PAGE and Coomassie blue stained to ensure a similar degradation profile as above. Next, the reactions were lyophilized and resuspended in 300 μl of a solution suitable for isoelectric focusing (IEF, first dimension) (7 M urea, 2 M thiourea, 1% (w/v) tetradecanoylamido-propyl-dimethyl amido-sulfobetaine-14 (ASB-14), 40 mM Tris, 2 mM tributyl phosphine (TBP), and 0.5% (v/v) Biolytes 3-10). Following the manufacturer's protocol, 17-cm IPG strips (pH 3–10) were rehydrated with IEF solution containing untreated or NE-treated Omps under active mode using the Protean IEF Cell (Bio-Rad) at 50 V for 12–16 h. Next, IEF was conducted using the above apparatus for 40,000 V-hours at a maximum of 10,000 V. Before the second dimension, IPG strips were equilibrated for 10 min in 6 M urea, 0.38 M Tris (pH 8.8), 2% SDS, 20% glycerol, and 2% (w/v) DTT, and then in the same solution in which DTT was replaced with iodoacetamide for an additional 10 min. Next, the IPG strips were embedded onto a 12.5% SDS-PAGE gel (two-dimension) with 1% (w/v) low melting agarose in SDS-PAGE buffer. Gels were run at 25 V for 2 h at 4°C, then 85 V overnight. The reactions were performed in duplicate. One set of gels was processed for immunoblotting as described above using anti-OprF Ab. The other set of gels was stained using a fluorescent dye-based stain, SYPRO Ruby (Bio-Rad), according to the manufacturer's recommendations. Stained gels were scanned using Fluor-S MAX MultiImager System (Bio-Rad) and their images processed for comparative analysis using PDQuest software (Bio-Rad). Protein spots that were similar in control (untreated Omps) and sample (NE-treated Omps) gels were used as landmarks for matching and to facilitate better comparison of gels. Experiments were performed in triplicate to ensure the reproducibility of the protein pattern. The protein spot detected by immunoblotting with anti-OprF Ab was localized on the stained gel, cut, and in-gel digested with trypsin and the peptides were recovered as described elsewhere (32, 34). The peptide mass fingerprints of tryptic peptides were generated by MALDI-TOF mass spectroscopy and subsequently compared with database containing virtual fingerprints obtained by theoretical tryptic cleavage of *Pseudomonas* proteins (http://www.pseudomonas.com). The identity of the selected protein spot from the major band was further confirmed by amino acid sequencing.

Amino acid sequencing

N-terminal sequences of OprF or its degradation products were identified by automated Edman degradation with an Applied Biosystems sequenator (35).

Purification and exposure to proteases of OprF

OprF was purified from the crude *P. aeruginosa* outer membrane (Om) fractions by reversed-phase HPLC (System Gold analyzer, Beckman Coulter) using a previously described method (36). Purified OprF was incubated alone or in the presence of the indicated concentrations of active NE, CG, lysozyme, or metalloproteinase-7 (MMP-7) for varying times (13). The reactions were resolved by SDS-PAGE and Coomassie blue stained or subjected to Western blotting using anti-OprF Ab. Of note, human neutrophil CG (Elastin Products) and lysozyme (Sigma-Aldrich) and recombinant active human matrix MMP-7 (kindly provided by Dr. W. Parks, University of Washington, Seattle, WA) were tested for their purity and activity by SDS-PAGE and zymography and/or spectrophotometrically using specific substrates according to the manufacturer's recommendations.

Exposure of intact bacteria to NE and Omps to neutrophils

Bacteria were cultured in the absence or presence of NE for varying times and the reactions were processed for Western blotting using anti-OprF Ab (10).

Aliquots of WT or $NE^{-/-}$ neutrophils (2×10^6 cells in 200 μl HBSS (pH 7.4)) were primed and stimulated as previously described (35). Next, Omps (5 μg) were added to the reactions, which were incubated overnight at 37°C (pH 7.4). In some experiments, neutrophils were preincubated with the serine proteinase inhibitors AEBSF (1 mM) or secretory leukocyte protease inhibitor (SLPI) (10 μg) (R&D Systems) for 15 min at 37°C before addition of Omps. Controls included Omps incubated alone or with purified NE (2 μg) and neutrophils alone. Following incubation, samples were acetone-concentrated and subjected to SDS-PAGE and Western blotting (35). Under these experimental conditions, 60% of the cells were dead following overnight incubation, as judged by trypan blue dye exclusion. Levels of released active NE from WT neutrophils were determined by comparison to purified NE using colorimetric enzymatic assay as described above (data not shown).

Competition assay

Comparison of the susceptibility of WT *P. aeruginosa* and its isogenic mutant $OprF^-$ to NE attack was conducted using the competition assay. In vitro competition studies were performed following modifications of a previously described method (37). First, WT *P. aeruginosa* and its isogenic mutant $OprF^-$ were freshly grown to mid-log phase and adjusted to the same OD. Then, an OD value of WT *P. aeruginosa* that resulted in ~50% bacterial killing in the presence of NE (2 μg) was predetermined. Subsequently, bacterial suspensions comprising a 50:50 mixture of WT and mutant bacteria were cultured in the absence or presence of NE as described above. Next, serial dilutions of the reactions were plated onto Luria-Bertani agar plates without or with streptomycin to determine the numbers of CFUs for WT and $OprF^-$ strains. The competitive killing index (CI) is calculated by dividing the ratio of mutant CFUs/WT CFUs obtained in the presence of NE by the ratio of mutant CFUs/WT CFUs obtained in the absence of NE. If the CI is <1, this indicates that the susceptibility of isogenic mutant $OprF^-$ to NE was at least similar to that of WT *P. aeruginosa*. If the CI is >1, this indicates that isogenic mutant $OprF^-$ was more resistant than WT *P. aeruginosa* to NE.

1-N-phenylnaphthylamine (NPN) assay

NPN is a fluorescent probe commonly used to study membrane integrity (38, 39). NPN fluoresces weakly in aqueous solution, but becomes strongly fluorescent when taken up by altered membranes (hydrophobic environment). Bacteria were cultured with or without addition of NE as described above. Following 30 min incubation, NPN was added and the fluorescence intensity was measured 5 min later ($\lambda_{max,abs}$ 350 nm; $\lambda_{max,em}$ 420 nm) using the SpectraMax Gemini spectrofluorometer (Molecular Devices). Control experiments without or with NE alone were included. Fluorescence corresponding to cell-bound NPN was corrected by subtracting the values obtained in control experiments. The fold increase of NPN uptake was expressed as the ratio of fluorescence values recorded in NE-treated bacteria over untreated bacteria.

Statistical analysis

Kaplan-Meier survival curves were analyzed by the log-rank test. Data of other experiments are expressed as the means ± SEM. Where appropriate, statistical differences between groups were tested using Student's unpaired *t* test. For samples that were not normally distributed, the Mann-Whitney *U* test was applied. Statistical significance was assumed at a predefined level of $p < 0.05$.

Results

NE deficiency increases mouse mortality in P. aeruginosa *i.n. infection model*

To determine the role of NE in combating *P. aeruginosa* infection, groups of $NE^{-/-}$ and WT control mice were exposed via i.n. instillation to 9×10^6 CFUs of *P. aeruginosa* H103 (representing the LD_{50} for WT mice) and their survival was monitored during 72 h. Initially, $NE^{-/-}$ mice exhibited signs of severe infection (ruffled fur, lethargy, diarrhea) that were markedly less striking than in control mice. At 24 h postchallenge, 50% of $NE^{-/-}$ mice but none of the WT mice succumbed to infection. By 56 h, >80% of $NE^{-/-}$ mice died, whereas, as expected, only 50% of WT mice died (Fig. 1*A*). None of the survivors died subsequently. Of note, the NE immune role was bacterial dose dependent. For example, a 2.5-fold lesser dose of bacteria than LD_{50} resulted in pronounced sign of distress in $NE^{-/-}$ mice a few hours postinfection, but no death was recorded in both WT and $NE^{-/-}$ mice (Refs. 15, 23 and data not shown). However, 100% of $NE^{-/-}$ mice vs 83% of WT mice died when challenged with a 3-fold higher dose than the LD_{50} (data not shown). In separate experiments, groups of $NE^{-/-}$ and WT control mice were i.n. challenged with near-infrared fluorescent-labeled *P. aeruginosa* H103. Fluorescence imaging analysis demonstrated that the main site of infection was the lung, suggesting that i.n. instillation of bacteria is a reliable and reproducible lung infection

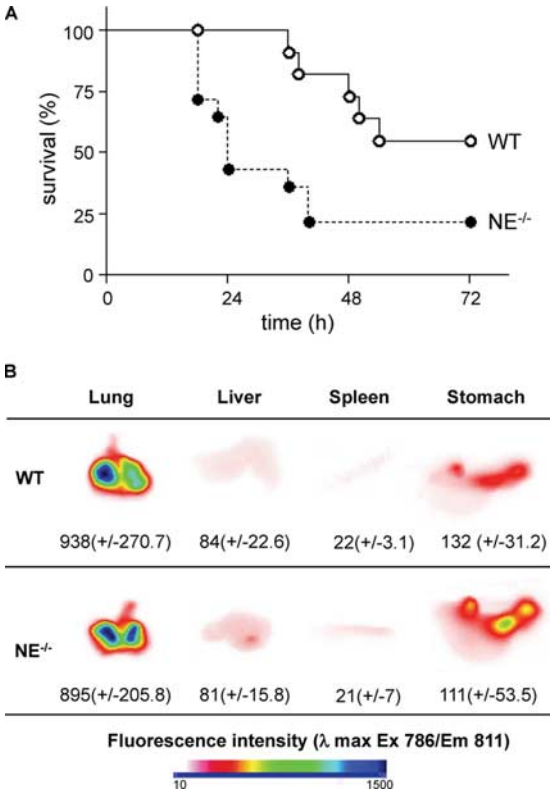

FIGURE 1. Intranasal infection of $NE^{-/-}$ and WT mice. *A*, Kaplan-Meier survival curves of $NE^{-/-}$ and WT C57BL/6 mice following i.n. challenge with *P. aeruginosa* (9×10^6 CFUs/mouse, which represents the LD_{50} for WT mice). Increased mortality of $NE^{-/-}$ mice was statistically significant ($p = 0.0169$). Similar data were obtained using mice with 129/SvEv genetic background (data not shown). *B*, Spreading of *P. aeruginosa* monitored by fluorescence imaging. Shown are representative images of the lungs and other organs 4 h following i.n. challenge of $NE^{-/-}$ and WT mice with cypate-labeled *P. aeruginosa*. Quantified data of fluorescence intensity are indicated in which each datum represents the mean ± SEM of four mice. No fluorescence staining was detected in the organs of mice challenged with saline (data not shown). Note that most bacteria were localized in the lungs ($p > 0.05$ for differences between genotypes).

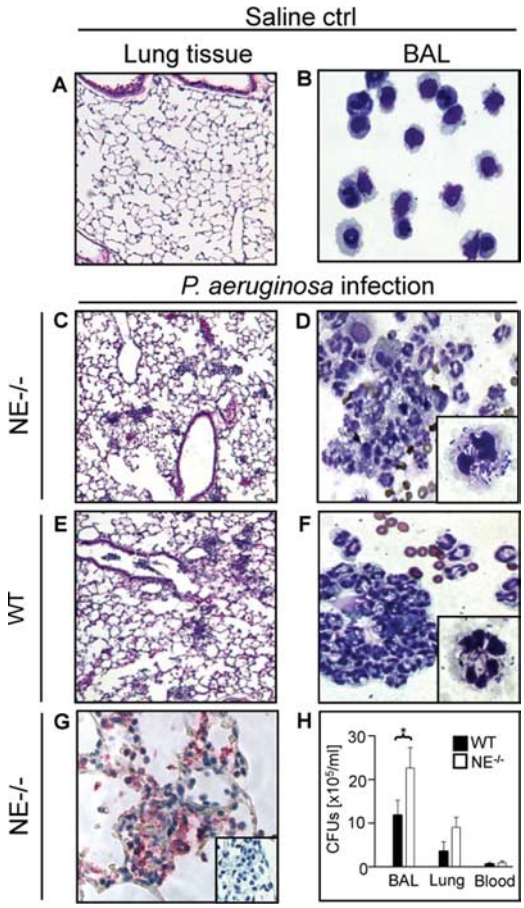

FIGURE 2. Mouse lung histology and bacterial clearance. *A* and *B*, Representative H&E-stained lung tissues and micrograph of Wright's-stained cytospins from $NE^{-/-}$ mice 16 h after i.n. instillation of sterile saline (magnification, ×40 and ×400 respectively). Similar histology was observed in WT lung tissues. *C*, *D* and *E*, *F*, Representative H&E-stained lung tissues and micrograph of Wright's-stained cytospins from $NE^{-/-}$ and WT mice 16 h after i.n. infection with *P. aeruginosa* (magnification, ×40 and ×400, respectively). *Inset*, Representative neutrophil surrounding and/or engulfing bacteria (magnification, ×1000). Of note, the patterns of patchy cellular infiltrates were similar in $NE^{-/-}$ and WT mice and morphologic analysis found the predominance of neutrophils as confirmed by immunostaining for MPO in *G*. *G*, Representative immunostaining of *P. aeruginosa*-infected $NE^{-/-}$ lung tissues using anti-MPO Ab, a specific marker for nuetrophils (red color, alkaline phosphatase staining) (magnification, ×200). No immunostaining was observed in infected lungs using rabbit preimmune serum (*inset*). Note that a similar immunostaining pattern was observed in WT lung tissues. *H*, Representative data of bacterial clearance from BAL fluids, lavaged lungs, and blood of $NE^{-/-}$ and WT mice 16 h postinfection. As controls, no bacterial growth was detected in lungs of $NE^{-/-}$ mice that were i.n. instilled with sterile saline. Note that while CFU counts of lavaged lungs were different between genotypes, they did not reach statistical significance. Data are presented as mean CFU counts ± SEM; differences between genotypes were analyzed by the Mann-Whitney *U* test (*, $p = 0.0278$ for BALs).

model and that *P. aeruginosa* did not disseminate (Fig. 1*B*). Importantly, survival data demonstrate that NE contributes substantially to host protection from morbidity and mortality associated with *Pseudomonas* lung infection.

NE mediates P. aeruginosa *killing by neutrophils*

To explore the underlying basis for the vulnerability of $NE^{-/-}$ mice to *P. aeruginosa* infection, we examined the capacity of $NE^{-/-}$ and WT mice to clear bacteria from the lungs. First, lung tissues and BAL fluids obtained from saline-instilled WT and $NE^{-/-}$ mice appeared to have normal histology and resident macrophages (Fig. 2, *A* and *B*). Analyses of lung tissue sections from *P. aeruginosa*-challenged $NE^{-/-}$ and WT mice revealed similar patchiness of cellular infiltrates (Fig. 2, *C* and *E*). In accordance with our previously published results (10, 23, 31), total cell and differential counts of BAL fluids from both types of mice showed comparable leukocyte numbers and confirmed neutrophils as the dominant cells (data not shown; Fig. 2, *D* and *F*). Imunostaining for MPO confirmed that these infiltrates are indeed predominated by neutrophils (Fig. 2*G*). As judged by cytospin analyses, there were no significant differences in the numbers of other immune cells including macrophages and lymphocytes. Furthermore, the absence of NE did not impair the ability of neutrophils to interact with bacteria (Fig. 2, *D* and *F*, *inset*). However, the number of bacteria in $NE^{-/-}$ lung tissues (BALs and lavaged lungs) was much greater than that seen in WT lung tissues (Fig. 2*H*). Indeed, the inability of $NE^{-/-}$ mice to clear *Pseudomonas* as efficiently as WT mice was clearly observable early in infection and persisted for up to 72 h (data not shown). Of note, the bacterial burden was similar but insignificant in the bloodstream as well as in spleen, liver, and kidneys of both groups of mice, further confirming the

absence of *P. aeruginosa* dissemination (Fig. 2*H* and data not shown).

Next, we compared the ability of $NE^{-/-}$ and WT neutrophils to kill *P. aeruginosa* in vitro. To assess their bactericidal activity, $NE^{-/-}$ and WT bone marrow neutrophils were exposed to bacteria. After a 60-min coincubation, $NE^{-/-}$ neutrophils were less able to kill bacteria when compared with WT neutrophils (<10% and >40% reduction in bacteria from $3.3 \pm 0.41 \times 10^5$ CFUs in controls to $3 \pm 0.49 \times 10^5$ in $NE^{-/-}$ neutrophils and to $2.03 \pm 0.2 \times 10^5$ in WT neutrophils). Of note, we confirmed that the respiratory burst and extent of phagocytosis were not impaired in $NE^{-/-}$ neutrophils (data not shown). Taken together, these results strongly suggest an important role for NE in neutrophil-mediated clearance of *P. aeruginosa*.

Direct killing of P. aeruginosa *by NE*

We incubated purified NE directly with *P. aeruginosa* H103 to assess the enzyme's antibacterial activity against this pathogen. Addition of NE markedly decreased *P. aeruginosa* growth (Fig. 3*A*). Preincubation of NE with both synthetic and physiologic inhibitor resulted in normal bacterial growth, indicating that NE employs its catalytic activity for bactericidal function. In other experiments, NE-mediated decrease of *P. aeruginosa* growth was investigated as a function of various parameters, including those of the phagolysosomal environment (e.g., ionic strength and pH). Overall, NE killing of *P. aeruginosa* was dependent on the ratio of enzyme concentration to inoculum size, the pH of the reaction, and incubation time, but was independent of the growth phase and salt concentration under our experimental conditions (data not shown). Interestingly, using similar experimental conditions as with *P. aeruginosa* H103, the growth of two clinical strains isolated from patients with pneumonia was also markedly decreased in the presence of NE (Fig. 3*B*).

To investigate the impact of NE on *P. aeruginosa*, a series of experiments were conducted under conditions where NE killed ~50% of bacteria. The killing ability of NE was examined by fluorescence staining to distinguish live from dead bacteria. The results demonstrated that in the presence of NE, DNA of dead bacteria with damaged membranes fluoresced bright green, whereas DNA of live bacteria with intact membranes stained red (Fig. 3*C*). Next, NE-mediated damage to *P. aeruginosa* was assessed by examining the bacterial membrane permeability (Fig. 3*D*). Using NPN assay, we observed that incubation of *P. aeruginosa* with NE was followed by an increased fluorescence intensity of the reaction, reflecting increased NPN binding to altered membranes. Both SEM and TEM further confirmed these observations. SEM revealed that NE-exposed *P. aeruginosa* lost their typical rod-shaped morphology with the appearance of nodules and collapsed architecture (Fig. 3*E*). TEM demonstrated that in the presence of NE, the inner and outer membranes of *P. aeruginosa* were no longer discernable, resulting in a distorted structural integrity (Fig. 3*F*).

NE degrades P. aeruginosa *major OprF*

We hypothesized that proteins from *P. aeruginosa* Om would be susceptible to degradation by NE, resulting in bacterial death. To determine the bacterial target(s) of NE, we grew *P. aeruginosa* and subjected it to fractionation to selectively extract Omps. Unlike *E. coli*, which comprises two major Om bands, Om fractions of *P. aeruginosa* H103 and clinical strains have a single predominant band (Fig. 4*A* and data not shown). Remarkably, this major Om band was completely degraded by NE (Fig. 4*A*). Western blot analysis found that the polyclonal Ab raised against major OprF immunoreacted specifically with this band and did not crossreact with other *P. aeruginosa* cell surface proteins (e.g., flagellin) (Fig. 4*B*).

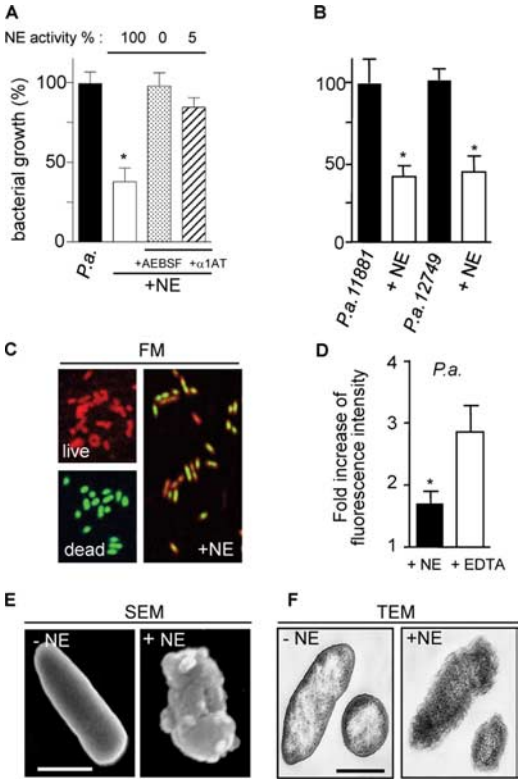

FIGURE 3. NE effect on bacteria. *A* and *B*, NE kills *P. aeruginosa* using its catalytic activity. *A*, *P. aeruginosa* H103 (1×10^7) was incubated alone or with NE (2 μg). In duplicate experiments, NE was preincubated with its chemical (AEBSF, 1 mM) or physiologic (α1-AT, 5 μg) inhibitors for 5 min before addition of bacteria. Bacterial viability was expressed as percentage of bacterial of growth. Aliquots of the reactions were tested for NE activity using the colorimetric assay as previously described (23) (*top*). AEBSF and α1-AT inhibited NE activity (>96%) but did not affect *P. aeruginosa* growth. *B*, As with *P. aeruginosa* H103, NE had comparable antibacterial effect on *P. aeruginosa* clinical isolates. Means ± SEM of four independent experiments are shown, and data were analyzed by Student's unpaired *t* test (*, $p < 0.05$ for differences between NE-treated vs untreated conditions). *C*, Fluorescence microscopy of *P. aeruginosa*. Bacteria were incubated without (live) or with NE (+NE) as described above. As control for death (dead), bacteria were heat-killed. Next, the reactions were stained with a mixture of SYTO59 and SYTOX and viewed using two separate bandpass optical filters. Captured images were merged. DNA of live (intact cell membranes) and dead (disrupted membranes) bacteria fluoresce red with SYTO59 and bright green with SYTOX, respectively (magnification, ×2000). *D*, Following incubation of *P. aeruginosa* H103 with or without NE, NPN was added and the fluorescence, indicator of bacterial membrane permeability, was measured. Shown are data for the fold increase in fluorescence intensity in NE-treated *P. aeruginosa* H103 as defined in *Materials and Methods*. EDTA (50 mM final) was used as control for perturbed membrane integrity. Means ± SEM of three independent experiments are shown, and data were analyzed by the unpaired *t* test (*, $p < 0.05$ for differences between NE-treated vs untreated conditions). *E* and *F*, Electron micrographs of *P. aeruginosa* incubated without (−NE) or with NE (+NE). Bacteria were incubated without or with NE as described above and the reactions were processed for SEM or TEM. *E*, Shown are electron micrographs of single cells (magnification, ×40,000; scale bar, 1 μm). *F*, Shown are electron micrographs of longitudinal and transverse cross-sections of single cells (magnification, ×40,000; scale bar, 1 μm). Micrographs are illustrative of four independent experiments.

To ensure the identity of the major Om band, *P. aeruginosa* Om fraction was incubated alone or with NE and the reactions were processed for two-dimensional gel electrophoresis as described in *Materials and Methods*. As shown in Fig. 4*C*, *P. aeruginosa* Om

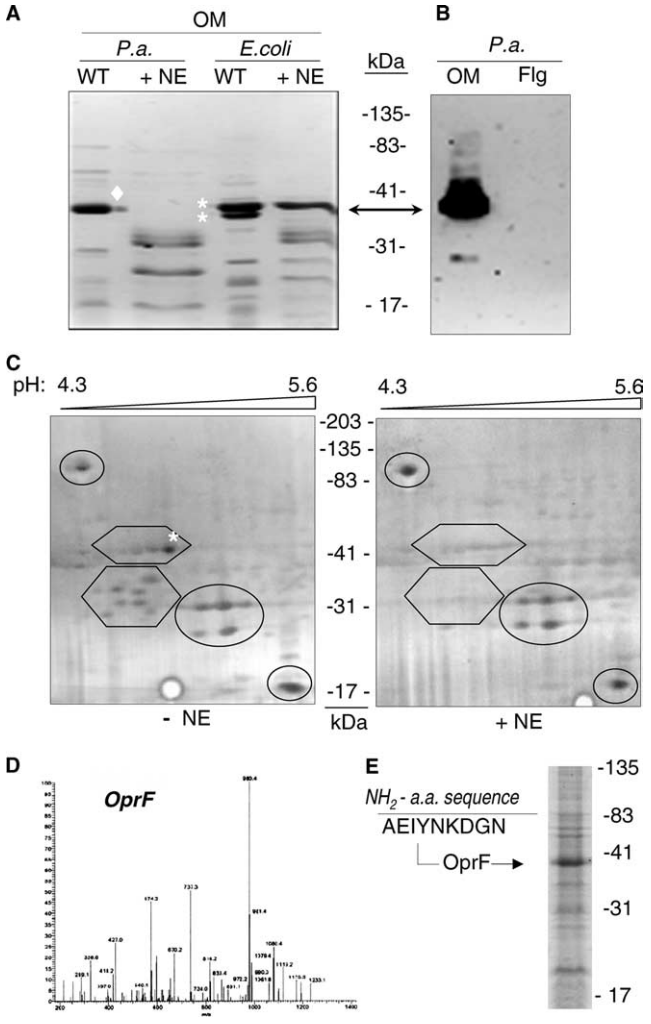

FIGURE 4. Exposure of *P. aeruginosa* Om protein fraction to NE. *P. aeruginosa* or *E. coli* were grown and subjected to fractionation. *A*, Both *P. aeruginosa* H103 and *E. coli* Om fractions (5 μg) were incubated with or without NE (2 μg) and the reactions were resolved by SDS-PAGE under reducing conditions and visualized by Coomassie blue staining. Unlike *E. coli*, which contains two major Omps (*), *P. aeruginosa* contains one predominant Om protein (♦), which is completely degraded following incubation with NE. *B*, Aliquots of *P. aeruginosa* Om fraction and flagellin proteins were resolved by SDS-PAGE and processed for Western blotting using a specific Ab against major outer memprane protein OprF. *C*, *P. aeruginosa* Om fraction (500 μg) was incubated alone or in the presence of NE (10 μg) and the reactions were separated using two-dimensional PAGE (2-D). The reactions were performed in duplicate. One set of reactions (*C*) was SYPRO-stained. The other set was processed for immunoblotting using anti-OprF Ab (data not shown). Shown are representative two-dimensional gel images. Ovals depict proteins that are resistant to NE proteolytic activity, whereas hexagons depict proteins that are degraded by NE. Data are illustrative of at least three independent experiments per condition. *D*, The protein spot (asterisk in *C*), which immunoreacted specifically with anti-OprF Ab (data not shown) was processed for mass spectrometry and identification in the database as described in methods. *E*, N-terminal amino acid sequence of OprF. Arrowhead points to sequenced band.

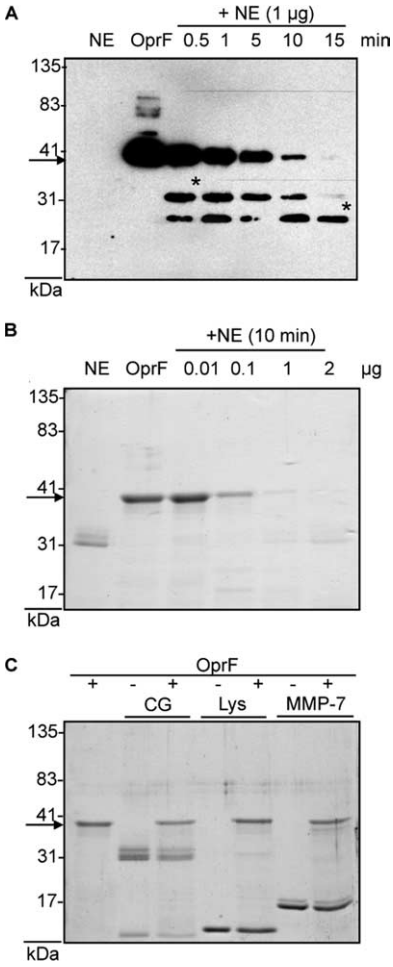

FIGURE 5. Analysis of proteolytic degradation of purified OprF. *A*, Purified OprF (1 μg) from *P. aeruginosa* Om protein fraction was incubated alone or with NE for the indicated times and the reactions were processed for immunoblotting using anti-OprF-specific Ab. Note that degradation of OprF by NE began with the appearance of intermediate fragments (up to 15 min) and is complete by 30 min (data not shown). OprF Ab did not cross-react with NE. *B*, OprF was incubated with increasing concentrations of NE for 10 min and the protein integrity was assessed by Coomassie blue staining. Note that complete degradation of OprF is NE concentration-dependent. *C*, Purified OprF protein was incubated with 2 μg of active CG, lysozyme (Lys), or MMP-7 for 60 min and the reactions were processed for Coomassie blue staining. None of these enzymes degraded OprF like NE. Arrow indicates the position of intact OprF. Experiments were repeated three times with similar results.

fraction is complex, and despite NE potent activity, the enzyme degraded some (hexagons) but not all Omps (ovals). Immunoblotting of two-dimensional transfer membranes using OprF Ab detected specifically one protein spot (data not shown). Overlapping of Western and stained-gel images allowed the localization of this protein spot (Fig. 4*C*, asterisk). Mass spectrometry analysis and database searching identified the spot as the major Om protein (OprF) (Fig. 4*D*). This finding was further confirmed by N-terminal amino acid sequencing (Fig. 4*E*).

Degradation of OprF is NE-specific and dependent on incubation time and enzyme concentration

To characterize its cleavage by NE, OprF was purified from *P. aeruginosa* Om fraction. Incubation of the protein with NE for the indicated times resulted in stepwise degradation of OprF with the generation of distinct intermediate fragments (Fig. 5*A*, asterisk). To localize NE cleavage sites, the two major degradation products were identified by Coomassie blue staining and subjected to NH_2-terminal amino acid sequencing. Analysis of these results indicated a similar amino acid sequence for both fragments corresponding to OprF N-terminal sequence indicating a C-terminal processing of OprF by NE (data not shown). Next, NE-mediated degradation

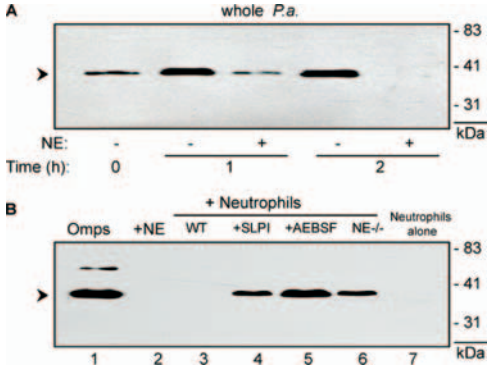

FIGURE 6. Characterization of OprF degradation by NE. *A*, Mid-log grown bacteria (1×10^7) were incubated with or without NE (2 μg) for varying times and the reactions processed for Western blotting using anti-OprF-specific Ab. Arrowhead indicates the expected position of OprF. *B*, *P. aeruginosa* Om proteins (Omps, 5 μg) were incubated with purified NE (2 μg) or isolated murine neutrophils (2×10^6 cells) alone or preincubated with NE physiologic (SLPI, 2.5 μg) or chemical (AEBSF, 1 mM) inhibitors overnight, and the reactions were processed for immunoblotting using anti-OprF-specific Ab. Note that anti-OprF Ab did not crossreact with neutrophil proteins (*lane 7*). Arrowhead indicates the expected position of OprF. The findings are illustrative of three independent experiments.

was monitored as a function of enzyme concentration. Fig. 5*B* shows that treatment of OprF with increasing concentration of NE led to progressive degradation of the protein. Conversely, preincubation of NE with the serine proteinase inhibitors AEBSF or α1-AT prevented OprF degradation (data not shown). To assess the relative importance of NE-mediated degradation of OprF, other enzymes known to be involved in host antibacterial defense mechanisms were examined. Using experimental conditions comparable to those of NE, the proteases cathepsin G and lysozyme did not degrade OprF and MMP-7 cleaved this protein very poorly (Fig. 5*C*).

Neutrophil-derived NE degrades OprF in intact P. aeruginosa

To determine whether NE degrades OprF in intact *P. aeruginosa*, we incubated viable bacteria with NE. Our immunoblotting results using anti-OprF-specific Ab showed that in the absence of NE, OprF remained intact and that its expression increased considerably over time (0 vs 1 h) (Fig. 6*A*). However, incubation of bacteria with NE was accompanied by substantial degradation of OprF as early as 1 h (Fig. 6*A*), coinciding with loss of bacterial structure and morphology and death (Fig. 3, *A* and *C–F*, and data not shown). These results indicate that NE is capable of targeting OprF localized in the Om of intact *P. aeruginosa* bacteria.

Next, we sought to determine whether neutrophils (containing NE) generate similar cleavage of OprF as did purified NE. Using chromogenic substrate-based enzymatic assay, the number of activated neutrophils added per reaction was estimated to yield a concentration of NE comparable to that used in Fig. 4*A*. Incubation with activated WT neutrophils resulted in complete degradation of OprF similar to that obtained with purified NE (Fig. 6*B*, *lanes 2* and *3*). Significant inhibition of OprF degradation was observed with SLPI, the physiologic inhibitor known to block NE and cathepsin G, but not proteinase 3, catalytic activities (Fig. 6*B*, *lane 4*). OprF cleavage by WT neutrophils was substantially abrogated when incubation was conducted in the presence of AEBSF, a relatively stable broad-spectrum serine protease inhibitor (Fig. 6*B*, *lane 5*). Incubations in the presence of EDTA gave no appreciable inhibition, suggesting the inability of neutrophil-derived metalloproteinases to cleave OprF (data not shown). To assess the specific

Table I. *Effect of NE on the viability of P. aeruginosa H103 (WT) and its isogenic mutant deficient in OprF (OprF$^-$)*

Bacteria	Number of CFUs −NE	Number of CFUs +NE
Mix of WT *P. aeruginosa* and OprF$^-$	90.8 ± 2.3	60.3 ± 3.9
Mix of WT *P. aeruginosa* and OprF$^-$ (+streptomycin)	43.0 ± 5.2	36.7 ± 2.5
WT *P. aeruginosa* (deduced)	47.8 ± 4.5	23.6 ± 4.1
Ratio of OprF$^-$ CFUs/WT CFUs	0.89	1.55
CI	1.74[a]	

[a] CI is calculated by dividing the ratio of OprF$^-$ CFUs and WT CFUs obtained in the presence of NE (+NE) over the ratio of OprF$^-$ CFUs and WT CFUs obtained in the absence of NE (−NE).

role of NE in mediating OprF degradation by neutrophils, we incubated OprF with neutrophils isolated from mice deficient in NE. Equivalent numbers of NE-deficient neutrophils showed less degradation of OprF as compared with WT neutrophils (Fig. 6*B*, *lanes 3* and *6*). Of note, overnight incubation of Omps alone did not cause a spontaneous degradation of OprF (Fig. 6*B*, *lane 1*). Thus, these findings demonstrate that NE contributes to the efficient degradation of OprF by neutrophils.

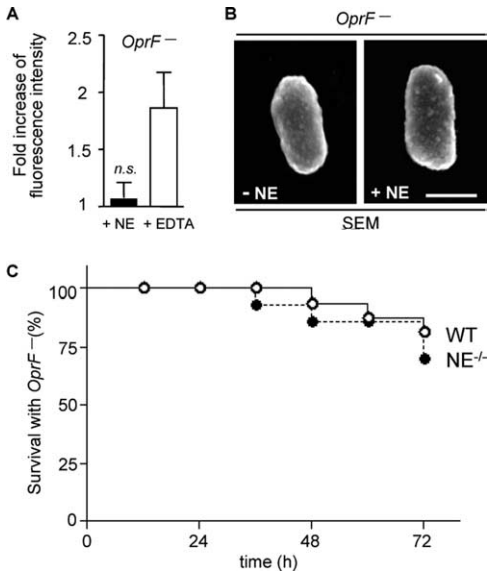

FIGURE 7. Roles of OprF in maintenance of bacterial integrity and mouse susceptibility to *P. aeruginosa* lung infection. *A*, Following incubation of mutant (OprF$^-$) bacteria with or without NE, NPN was added and the fluorescence was measured. Shown are data of fold increase in fluorescence intensity in NE-treated OprF$^-$ reaction. EDTA (50 mM final) was used as control for perturbed membrane integrity. Means ± SEM of three independent experiments are shown, and data were analyzed by Student's unpaired *t* test (*, $p < 0.05$ for differences between NE-treated vs untreated conditions). *B*, Electron micrographs of OprF-deficient *P. aeruginosa* incubated without (−NE) or with NE (+NE). Bacteria were incubated without or with NE and their viability and morphology were examined as described in *Materials and Methods*. In the absence of OprF, mutant strains showed no statistically significant decrease in their growth (Table I, data not shown) and no altered morphology in the presence of NE (magnification, ×40,000; scale bar, 1 μm). Micrographs are illustrative of four independent experiments. *C*, Kaplan-Meier survival curves for NE$^{-/-}$ and WT mice in response to intranasal challenge with OprF$^-$ (5×10^7 CFUs/mouse). Note that survival rates were not significantly different between WT and NE$^{-/-}$ mice ($p = 0.4896$).

Deficiency in OprF protects P. aeruginosa *from NE attack*

To assess the contribution of OprF to NE-mediated killing of *P. aeruginosa*, we performed the competition assay as described in *Materials and Methods*. As shown in Table I, the number of CFUs of the bacterial mix of WT *P. aeruginosa* and streptomycin-resistant isogenic mutant $OprF^-$ decreased considerably in the presence of NE (Table I, *row 1*). However, the numbers of CFUs in the presence of streptomycin were insignificantly different in the presence or absence of NE (Table I, *row 2*). As expected, the deduced numbers of CFUs of WT *P. aeruginosa* indicate ~50% killing of bacteria in the presence of NE (Table I, *row 3*). As reflected by the competition killing index (CI > 1.74), NE had no apparent effect on the viability of bacteria in the absence of OprF. To confirm that the presence of OprF was required for NE bacterial killing, we incubated WT *P. aeruginosa* and $OprF^-$ without or with NE and processed the reactions for NPN membrane permeability assay and SEM. In the presence of NE, strikingly altered morphology and increased Om permeability were observed with WT *P. aeruginosa* (Fig. 3, *D* and *E*, and data not shown), but not with the $OprF^-$ mutant (Fig. 7, *A* and *B*).

The absence of OprF negates the role of NE in host defense against P. aeruginosa

To determine the relevance of this mechanism in vivo, WT and $NE^{-/-}$ mice were i.n. challenged with $\sim 9 \times 10^6$ CFUs of $OprF^-$ and their survival was monitored over time. While at this bacterial dose, WT *P. aeruginosa* killed ~50% of WT mice and >80% of $NE^{-/-}$ mice (Fig. 1), no death was recorded with $OprF^-$ in either $NE^{-/-}$ or WT mice. Also, analysis of BAL fluids found similar total cell and differential counts (data not shown). Interestingly, a 5-fold increase of $OprF^-$ inoculum size (5×10^7 CFUs) still did not result in significant death either, whereas WT *P. aeruginosa* at this dose killed 100% of both types of mice (Fig. 7*C* and data not shown). Thus, the presence of OprF was required for NE-mediated host defense against *P. aeruginosa* lung infection.

Discussion

According to the widely accepted view, NE, a neutrophil-specific serine protease, is deleterious to the host in *P. aeruginosa* infectious diseases. In the present study, we provide for the first time compelling evidence that NE has rather a protective role against *P. aeruginosa* infection. Using a clinically relevant model of pneumonia, targeted deficiency in NE increased the susceptibility of mice to *P. aeruginosa*. When the capacity of mice to clear *P. aeruginosa* was examined, $NE^{-/-}$ mice were less able to contain infection or reduce bacterial burden. Thus, NE plays a relatively important role in host defense against *P. aeruginosa* since apparently other host "weapons" cannot substitute for it in vivo.

The acute inflammatory response to *P. aeruginosa* infection is reportedly characterized by the migration of neutrophils from the pulmonary capillaries into the airspaces (40). In contrast to a series of studies proposing a role for NE in neutrophil transmigration (41–43), the absence of the enzyme did not hamper neutrophil accumulation at the infected site under our experimental conditions. Indeed, our data are in accordance with published reports showing the inconsequential impact of NE on neutrophil migration in infection settings using different pathogens and/or routes of challenge (10, 11, 23). Similarly, the density of alveolar macrophages, another immune cell important for bacterial lung clearance (44), was not affected by NE deficiency. The primary purpose of neutrophil recruitment to infected sites is the rapid clearance of invading bacteria. Comparison of the antibacterial functions of $NE^{-/-}$ and WT neutrophils against *P. aeruginosa* indicated that phagocytosis and respiratory burst functions were not compromised in $NE^{-/-}$ neutrophils. However, while the numbers of viable bacteria decreased in the presence of both cell types, $NE^{-/-}$ neutrophils exhibited substantially less bactericidal activity than did WT neutrophils. This defect was attributed to the absence of NE, which proved to be directly bactericidal against *P. aeruginosa*.

In determining the mechanism of NE-mediated killing of *P. aeruginosa*, we observed that exposure of bacteria to NE resulted in the loss of their rodlike morphology concomitant with membrane disruption and increased permeability, indicating that the enzyme targets proteins of essential importance to *P. aeruginosa* integrity. *P. aeruginosa*, like most Gram-negative bacteria, contains a thin peptidoglycan overlaid with an Om in which proteins are embedded within a phospholipid bilayer. The outer member protein fraction, although complex, was predominated by a protein that we identified as OprF. OprF represents ~15% of *P. aeruginosa* outer member protein fraction ($\sim 2 \times 10^5$ copies/cell), making it a major protein of the pathogen (45). Strikingly, NE degraded OprF, coinciding with death of bacteria.

OprF is associated with the underlying peptidoglycan and contributes to the stabilization of the Om as well as exhibiting physiologically relevant porin activity (45–47). Degradation of OprF by NE would therefore explain the observed phenotypic alterations and ensuing death of *P. aeruginosa*. Recently, it was reported that *P. aeruginosa* binds host IFN-γ through OprF, resulting in the quorum-sensing system activation and virulence gene expression (48). This finding suggests that by degrading OprF, NE dampens the ability of *P. aeruginosa* to sense the host immune system activation and to mount an effective virulence response. NE has been previously shown to target directly the virulence factor flagellin (15) and repress its transcription in *P. aeruginosa* by an as yet undefined mechanism (49). Taken together, these observations highlight the capacity of NE to not only kill *P. aeruginosa*, but also to abrogate its virulence. Degradation of OprF by NE may also accentuate *P. aeruginosa* death by other alternative pathways. OprF degradation products could be toxic to *P. aeruginosa*. By cleaving OprF, NE might diffuse inside and degrade proteins important to bacterial survival. Lastly, NE-mediated OprF degradation contributes to efficient host defense against *P. aeruginosa* by altering the pathogen's membrane permeability, paving the way to other neutrophil antibacterial molecules for further damage and death.

A model of OprF arrangement in *P. aeruginosa* Om has been proposed wherein β-sheets cross the membrane and are interconnected in part by conserved surface-exposed loops (33). Interestingly, inspection of OprF primary structure revealed various preferred NE cleavage peptide bonds, some of which were likely to be cell surface-localized based on the proposed OprF secondary structure model (50). That NE degrades OprF was further confirmed following incubation of neutrophils with Om containing OprF or live bacteria with NE. Taken together, these findings suggest that NE could encounter and degrade OprF in vivo. Importantly, in this study NE was used at concentrations below its physiological levels, which have been reported to exceed the millimolar range (8, 51).

In vivo, NE could target OprF inside the phagocytic vacuole where bacteria are trapped. In support of this possibility, NE has been shown to surround bacteria engulfed inside the phagolysosome and degrade their virulence factors (13, 14). Alternatively, degradation of OprF could occur extracellularly through NE released by degranulation. There is accumulating evidence that microbial challenge is associated with not only extracellular release of free active NE (23, 35), but also with the production of chromatin-based structures containing NE. These

structures, termed neutrophil extracellular traps, capture and target invading pathogens and/or their virulence factors (52). While phagocytosis represents the main antibacterial function of neutrophils, the relative importance of free or neutrophil extracellular trap-associated NE in host defense against *P. aeruginosa* remains to be determined. Based on our NE inhibition data, the extracellular degradation of OprF in vivo will be determined by the balance of active NE and its physiologic inhibitors (specific and nonspecific).

Incomplete inhibition of neutrophil-mediated degradation of OprF suggests some contributions of other proteases. With respect to neutrophil serine protease family, it seems likely that proteinase 3 would contribute to OprF degradation since SLPI does not inhibit this enzyme and cathepsin G does not degrade OprF. Coincidentally, a recent study showed that cathepsin G was not required for host defense against *P. aeruginosa* in a mouse model of *Pseudomonas* endobronchitis (53). Whether proteinase 3 kills *P. aeruginosa* and contributes to host protection, however, is unknown. Generation of mice deficient in all three serine proteases is underway and should enhance our understanding about the relative contributions of individual enzymes in protecting the host from *P. aeruginosa*.

The finding that NE degrades OprF to kill *P. aeruginosa*, including the two pneumonia isolates, is of importance. It reinforces the hypothesis that NE mediates maximal neutrophil killing of Gram-negative bacteria by targeting these latter's major Omps and cleaving perhaps common surface-exposed epitopes (13, 54). The major Om protein OprF may then represent a target in the design of therapeutic strategies against *P. aeruginosa*. However, since *P. aeruginosa* is known to change its phenotype, further studies using clinical strains isolated from various infection settings are awaited to determine whether a correlation between the susceptibility of these strains to NE attack and the expression of OprF could be established. In recent years, several studies proposed that NE has the capacity to modulate biologic activities of various inflammatory mediators (55). It would be of interest to investigate the relative importance of this aspect in host defense against *P. aeruginosa*.

As mentioned earlier, NE is a potent enzyme and is regarded as the prime suspect in the destructive activities in *P. aeruginosa* infections, casting doubt on the enzyme's beneficial role. Collectively, the findings of this work clearly demonstrate that NE mediates innate host protection against *P. aeruginosa*, at least in mice. As such, strategies aimed at controlling excessive neutrophil recruitment and/or activation (e.g., NE release) in response to *P. aeruginosa* infection should be considered. In the same token, the inhibition of NE to treat *P. aeruginosa*-associated tissue destructive diseases should be reconsidered taking into account the enzyme's antibacterial function.

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Disclosures

The authors have no financial conflicts of interest.

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