Neutrophil Elastase Modulates Cytokine Expression

CONTRIBUTION TO HOST DEFENSE AGAINST PSEUDOMONAS AERUGINOSA-INDUCED PNEUMONIA

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Background: Neutrophil elastase (NE) is a potent serine protease with bactericidal activity against Pseudomonas aeruginosa.

Results: We now provide evidence that NE modulates inflammatory cytokine expression in response to this pathogen.

Conclusion: In addition to its bactericidal action, NE promotes cytokine response that contributes to host antibacterial defense.

Significance: This finding reveals that NE plays a multifaceted role in protecting against bacterial infections.

There is accumulating evidence that following bacterial infection, the massive recruitment and activation of the phagocytes, neutrophils, is accompanied with the extracellular release of active neutrophil elastase (NE), a potent serine protease. Using NE-deficient mice in a clinically relevant model of Pseudomonas aeruginosa-induced pneumonia, we provide compelling in vivo evidence that the absence of NE was associated with decreased protein and transcript levels of the proinflammatory cytokines TNF-α, MIP-2, and IL-6 in the lungs, coinciding with increased mortality of mutant mice to infection. The implication of NE in the induction of cytokine expression involved at least in part Toll-like receptor 4 (TLR-4). These findings were further confirmed following exposure of cultured macrophages to purified NE. Together, our data suggest strongly for the first time that NE not only plays a direct antibacterial role as it has been previously reported, but released active enzyme can also modulate cytokine expression, which contributes to host protection against P. aeruginosa. In light of our findings, the long held view that considers NE as a prime suspect in P. aeruginosa-associated diseases will need to be carefully reassessed. Also, therapeutic strategies aiming at NE inhibition should take into account the physiologic roles of the enzyme.

In the setting of respiratory bacterial infections (e.g. bacterial pneumonia), the host innate immune response is characterized by the initial recognition of invading microbes by host “sentinel” cells via Toll-like receptors (TLRs) or other pattern recognition molecules (1). Subsequently, this results in the production of an array of inflammatory mediators including early responsive cytokines. Another hallmark of innate host lung defense, especially when the first lines of defense: epithelial barrier and resident macrophages, are breached is the massive recruitment of polymorphonuclear neutrophils (PMN) to the infected site (2). PMNs are efficient phagocytes whose main function upon activation is thought to be the clearance of infecting bacteria. To do so, these cells are equipped with a myriad of antimicrobial molecules grouped into oxidative and nonoxidative systems (3, 4).

Regarding the nonoxidative system, we and others have identified the PMN-specific serine protease, neutrophil elastase (NE), as a key antimicrobial enzyme (5). NE catalytic activity relies on the His77-Asp102-Ser195 triad (chymotrypsin number) (6). NE also has the capacity to attenuate the pathogenicity of invading bacteria by targeting their virulence factors (11). In determining NE-mediated defense against Gram-negative bacteria, it was shown that active enzyme degrades the major outer membrane protein (Omp), structural proteins localized on the cell wall (9, 10). NE also has the capacity to attenuate the pathogenicity of invading microbes by targeting their virulence factors (11). In recent years, it was shown that NE binds to PMN-derived chro-

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The abbreviations used are: TLR, Toll-like receptor; PMN, polymorphonuclear neutrophil; NE, neutrophil elastase; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; SLPI, secretory leukocyte proteinase inhibitor; BAL, bronchoalveolar lavage; MyDB8, myeloid differentiation primary response protein 88; IRAK, interleukin-1 receptor-associated kinase; TRAF6, TNF receptor associated factor 6; NF-κB, nuclear factor-κ-light-chain-enhancer of activated B cells; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein-2.
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matin structures, termed neutrophil extracellular traps, and exerts its antimicrobial function (12).

Although NE has been always regarded as pathogenic in Pseudomonas aeruginosa-associated tissue inflammatory and destructive diseases, we have recently provided compelling in vivo evidence that the enzyme contributes considerably to PMN-mediated host protection in a mouse model of P. aeruginosa-induced pneumonia (10). There is also accumulating evidence that during P. aeruginosa infections, active NE is released in the extracellular milieu by recruited PMNs (13, 14). A number of in vitro studies have suggested that NE has the potential to change biologic activities of various inflammatory mediators (15). Altogether, these observations prompted us to hypothesize that the NE role in host defense against P. aeruginosa lung infection may not be only limited to just killing bacteria. Here, we report that in vivo, extracellular active NE has the capacity to induce mRNA expression of the early responsive proinflammatory cytokines, tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-2 (MIP-2), and interleukin-6 (IL-6); an induction that is mediated at least in part through TLR-4. These findings were further confirmed following exposure of cultured macrophages to purified NE. Our studies reveal for the first time that extracellularly released NE can have physiologic inflammatory properties that contribute to host defense against P. aeruginosa.

EXPERIMENTAL PROCEDURES

Reagents—Purified NE and elastin were obtained from Elastin Products Co. (Owensville, MO). NE activity was determined spectrophotometrically using the specific chromogenic substrate N-methoxyxuccinyl-Ala-Ala-Pro-Val-pNA (Elastin Products Co.) according to the manufacturer’s recommendations. Pefabloc SC®, 4-(2-aminoethyl)-benzenesulfonyl-fluoride (AEBSF) and secretory leukocyte proteinase inhibitor (SLPI) were from Roche Applied Science and R&D Systems, respectively. RPMI 1640 medium, DMEM, fetal bovine serum (FBS), penicillin, streptomycin, and PBS were obtained from Invitrogen. Ketamine hydrochloride and medotomidine hydrochloride were obtained from CEVA Santé Animale (Libourne, France). Primers for semiquantitative and real time RT-PCR were purchased from Operon Biotechnologies (Cologne, Germany). SYBR Green for real time RT-PCR was obtained from Invitrogen. Polyclonal anti-murine NE antibody was produced in rabbit (16). Antibody raised against amino acids 198–395 of mouse TLR-4 (sc-293072) were purified from rabbit sera. Antibody raised against amino acids 198–395 of mouse TLR-4 (sc-293072) were purified from rabbit sera. Antibody raised against amino acids 198–395 of mouse TLR-4 (sc-293072) were purified from rabbit sera. Antibody raised against amino acids 198–395 of mouse TLR-4 (sc-293072) were purified from rabbit sera. Antibody raised against amino acids 198–395 of mouse TLR-4 (sc-293072) were purified from rabbit sera. Antibody raised against amino acids 198–395 of mouse TLR-4 (sc-293072) were purified from rabbit sera. Antibody raised against amino acids 198–395 of mouse TLR-4 (sc-293072) were purified from rabbit sera.
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Cell lysates were prepared using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.4) (Sigma) and centrifuged at 600 × g for 30 min at 4 °C to remove cell debris. Next, lysates were exposed to a fixed NE concentration (500 nM) for the defined time periods (0.25, 0.5, 1, and 5 min). In parallel experiments, NE was preincubated with SLPI (10 μg) for 5 min at 37 °C or heat inactivated for 10 min at 65 °C prior to addition to cell lysates.

Assessment of Cytokine Levels—Cell-free WT and NE−/− BAL fluids of the 24-h time point post-infection were processed to assess the levels of various cytokines using the Raybio® Mouse Cytokine Antibody Array III according to the manufacturer’s instructions (RayBiotech, Tebu-Bio, Le Perray-en-Yvelines, France) (22). Briefly, equal volumes of cell-free BAL fluids (400 μl) were added to antibody-coated membranes and detection of immunoreactive cytokines was carried out following sequential incubations of the membranes with biotinylated anti-cytokine antibodies and streptavidin-horseradish peroxidase and visualization by enhanced chemiluminescence. Images were obtained with a ChemiDoc XRS imaging system (Bio-Rad). Densitometric analysis was performed on captured images using Quantity One one-dimensional analysis software (version 4.5.2) (Bio-Rad). Spots of interest were normalized to an internal control after subtraction of the representative background sample. Next, to determine the concentration of cytokines of interest (TNF-α, MIP-2, and IL-6), equal volumes of WT and NE−/− cell-free BAL fluids (50 μl) were processed using a multiplex bead-based immunoassay kit (Bio-Rad). Cytokine assays were performed as described by the manufacturer’s protocol. Each reaction in the kit was performed in triplicate. Both cytokine antibody array and multiplex assays were performed on all BAL pools.

Immunoblotting—NE or TLR-4 antigens were detected by Western blotting (18). Briefly, samples (cell-free BAL fluids (20 μl) and lung or cell lysates (35 μg)) were resolved by SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). The membranes were sequentially incubated with primary antibodies to mouse NE (dilution, 1:2,000) or TLR-4 (dilution, 1:500) followed by their respective horseradish peroxidase-conjugated secondary antibodies. When indicated, the membranes were stripped (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50 °C) and immunoblotted using primary rabbit polyclonal anti-mouse albumin antibody (Rockland, Gilbertsville, PA) and its corresponding horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Elastin Zymography—NE activity was determined by α-elastin zymography (23). Briefly, cell-free BAL fluids (20 μl) were electrophoresed under nonreducing conditions at 4 °C on 12% SDS-PAGE gels containing 1 mg/ml of elastin. Following electrophoresis, gels were soaked in 2% Triton X-100 for 30 min (twice), rinsed briefly, and incubated at 37 °C for 72 h in 50 mM Tris-HCl (pH 8.2), containing 5 mM CaCl₂. The gels were then stained with Coomassie Blue and destained in 5% acetic acid and 10% methanol. Active NE appears as a transparent lysis.
band at ~29 kDa. NE activity in cell-free BAL fluids was further confirmed using conventional chromogenic peptide assays as previously described (21).

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA isolation was performed using MasterPure™ RNA Purification kit as described by the manufacturer’s protocol (Epicenter, Biotechnologies, Madison, WI) (19). Briefly, mouse lung tissues (half-lobe) or cultured macrophage cell pellets were lysed and RNA was extracted. Purified RNA was resuspended in RNase inhibitor-containing TE Buffer. RNA concentration and purity were determined by spectrophotometry (A_260/A_280 ratio). Integrity of RNA samples was verified by electrophoresis on 2% agarose gels and visualization under UV light. RNA aliquots of individual mice were pooled in the same manner as for BAL fluids at equal concentrations.

Total RNA samples (1 μg) were reverse-transcribed using the SuperScript First Strand Synthesis System (Invitrogen). Next, cDNAs were amplified by PCR using specific primers (40 cycles starting with DNA denaturation for 2 min at 94 °C; each cycle corresponded to denaturation for 15 s at 94 °C, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s). RT-PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualization under UV light. Quantification of RNA content was performed using Quantity One Software (Bio-Rad). Levels of cytokine mRNA transcripts were normalized to the internal control 28 S mRNA. RT-PCR was performed in quadruplicate within each pool.

Forward and reverse primers for TNF-α, MIP-2, IL-6, and 28 S were designed as follows: TNF-α forward, 5′-GGG-ACA-GTG-ACC-TGG-ACT-GT-3′; TNF-α reverse, 5′-CTC-CCT-TTG-CAG-AAC-TCA-GC-3′; MIP-2 forward, 5′-CCA-CTC-TCA-AGG-GGC-GTC-AA-3′; MIP-2 reverse, 5′-CCC-CTT-ATC-CCC-AGT-CTT-TTT-CAC-3′; IL-6 forward, 5′-GAT-GCT-ACC-AAA-CTG-GAG-ATA-AAT-C-3′; IL-6 reverse, 5′-GTT-CCT-TAG-CCA-CTC-CTT-CTG-TG-3′; 28 S forward, 5′-CGG-AAT-TCG-CCA-CCA-GCC-GCC-TG-3′; 28 S reverse, 5′-CGT-CTA-GAC-TTT-CTC-GCT-TTA-CTT-GC-3′.

Real Time RT-PCR—Briefly, total RNA of cultured cells was reverse-transcribed into cDNA using the SuperScript First Strand Synthesis System (Invitrogen). Next, real-time PCR amplification was performed on the ABI PRISM 7500 Strand Synthesis System (Invitrogen). Next, real-time PCR was performed in triplicate and repeated at least 3 times in each experimental condition for data reproducibility.

Forward and reverse primers for TNF-α, MIP-2, IL-6, and GAPDH were designed as follows: TNF-α forward, 5′-GGC-AGG-TTT-TGG-CCC-TTT-CA-3′; TNF-α reverse, 5′-CTG-TGC-TCA-TGG-TTT-CTT-TG-3′; MIP-2 forward, 5′-GTG-AAC-TGC-GCT-GTC-AA-3′; MIP-2 reverse, 5′-ACT-AGT-GCT-CTG-GAT-GCA-3′; IL-6 forward, 5′-AAA-CTA-CGG-GCT-TCC-CTA-CTA-3′; IL-6 reverse, 5′-GTT-GGG-AGT-GGT-ATC-CTG-TGT-GA-3′; GAPDH forward, 5′-CAG-CCT-CTG-CCT-GTA-GAC-AA-3′; GAPDH reverse, 5′-CCC-AAT-ACG-GCC-AAA-TTC-G-3′.

Statistical Analysis—Unless specified, data are expressed as mean ± S.E. Where appropriate, statistical differences between groups were assessed via Student’s unpaired t test. Differences were considered significant at p ≤ 0.05.

RESULTS

Increased Levels of Active NE in Cell-free BALs Parallel Neutrophil Influx during P. aeruginosa-induced Pneumonia—Neutrophil recruitment of infected WT and NE/−/− mice that survived infection were sacrificed at the designated time points (4, 12, and 24 h). Aliquots with equal volumes of cell-free NE/−/− and WT BAL fluids were subjected to elastin zymography. As reflected by the intensity of band transparency, Fig. 1A (upper panel) shows the presence of active NE in cell-free WT, but not NE/−/− BAL fluids as early as 12 h that were markedly enhanced at the 24-h time point. Detection of active NE was further confirmed by incubation of cell-free BAL fluids with synthetic NE specific substrate (data not shown). Of relevance, BAL pools within each genotype showed similar NE profile. Moreover, the identity of the active band was confirmed by Western blotting using anti-mouse NE antibody (Fig. 1A, lower panel). Of note, there was no evidence of active NE in BAL fluids derived from saline-treated mice (data not shown).

With respect to immune cell recruitment in response to P. aeruginosa, there was a sharp increase of inflammatory cells in infected WT and NE/−/− BAL fluids in function of time. However, total cell counts were comparable in both genotypes at all periods of infections (4, 12, and 24 h) (Fig. 1B). By comparison to time points 4 and 12 h, the influx of recruited cells was consensually high at 24 h. Morphologic analyses and differential counts of BAL cytospins revealed the predominance of PMNs in both types of BAL fluids (Fig. 1C). There was no detectable defect in the recruitment of other immune cells (macrophages and T cells) in NE/−/− mice (data not shown). In accordance with our previous work (10, 23), H&E-stained lung sections of both NE/−/− and WT mice showed similar patchy neutrophilic infiltrates, typical of pneumonia and there was a decrease in the recruitment of PMN at 48 h (data not shown). Regardless host defense against P. aeruginosa, no death was recorded in both types of mice at the 4-h time point. At 12- and 24-h time points, of the 24 NE/−/− mice 3 and 8 mice died, respectively, and no WT mice succumbed to infection. The number of viable bacteria in NE/−/− lung tissues was 2-fold greater than that seen in WT tissues at the 24-h time point (data not shown). These findings further reinforce the relative importance of NE for maximal PMN killing of P. aeruginosa.

It must be emphasized that, unlike our previous studies, this is the first study where we focused on the protective role of extracellularly released NE in the setting of lung infection. Curiously, the contribution of free active NE to antibacterial defense of the lungs has not been clarified. Because of the relatively high level of active NE by 24 h post-challenge, all subsequent studies were carried out with mouse BAL fluids and lung tissues corresponding to this time point.
Decreased Levels of Secreted TNF-α, MIP-2, and IL-6 in Infected Cell-free BALs in the Absence of NE—To explore in vivo, the impact of released NE on cytokine production, we assessed the levels of various cytokines in equal aliquots of infected WT and NE−/− cell-free BAL fluids using cytokine antibody array approach. Our data revealed changes in the levels of a number of mediators in the absence of NE (supplemental Fig. S1). In this study, we focused on TNF-α, MIP-2, and IL-6, part of the early responsive cytokine network of infection. Remarkably, whereas their levels increased as expected in infected WT and NE−/− BAL fluids when compared with those of NE−/− BAL fluids (Fig. 2A). Such an increase coincided with enhanced NE activity (Fig. 1A). Of note, cytokine levels in BALs of mice that received PBS were insignificant regardless of mouse genotype and their values were subtracted from those of infected BALs. Next, we employed the multiplex approach to quantitatively compare the levels of TNF-α, MIP-2, and IL-6. As shown in Fig. 2B, protein concentrations of these mediators were significantly low in NE−/− cell-free BALs by comparison to WT cell-free BALs confirming further the cytokine antibody array data. Of importance, these cytokine profiles were similar among BAL pools within each genotype.

Decreased TNF-α, MIP-2, and IL-6 mRNA Transcripts in Infected Lungs in the Absence of NE—The decreased levels of TNF-α, MIP-2, and IL-6 in cell-free NE−/− BALs could be attributed to protein degradation, which is unlikely or changes in transcript expression of these mediators involving NE. To test the latter hypothesis, mRNA expression of TNF-α, MIP-2,
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A

TNF-α

WT

NE−/−

Optical density (arbitrary unit)

WT

NE−/−

B

MIP-2

28 S

WT

NE−/−

C

IL-6

28 S

WT

NE−/−

FIGURE 3. Expression of TNF-α, MIP-2, and IL-6 mRNA in infected WT and NE−/− lungs. A–C, expression of TNF-α, MIP-2, and IL-6 in whole lung tissues at 24 h post-infection was analyzed by RT-PCR using 28 S as an internal control. Left side, representative gel images of amplified cDNAs corresponding to individual mediators within a pool. The size of the amplified products (on the left) for TNF-α, MIP-2, IL-6, and 28 S were 110, 248, 268, and 212 bp, respectively. Note the decreased expression level of the mediators in infected NE−/− lungs by comparison to infected WT lung tissues. Right side, densitometric analysis confirms decreased levels of the mediators of interest. Shown are representative histograms of individual mediators. Data represent the mean ± S.E. of four values within a pool. *, p < 0.05 for differences between genotypes. Of note, mRNA expression profile was similar in all pools and reproducible in repeated infection experiments.

and IL-6 was examined by RT-PCR. Gel micrographs and densitometry histogram analyses demonstrate that levels of mRNAs encoding for TNF-α, MIP-2, and IL-6 were lower in infected NE−/− lungs when compared with WT lungs (Fig. 3, A–C). Of note, detected levels of TNF-α, MIP-2, and IL-6 transcripts in saline-instilled WT and NE−/− mice were insignificant (data not shown) and were subtracted from those of infected samples for densitometric analyses. Taken together, these data strongly suggest a role for NE in inducing the expression of TNF-α, MIP-2, and IL-6.

NE Increases mRNA Expression of TNF-α, MIP-2, and IL-6 in Macrophages—Macrophages are known producers of TNF-α, MIP-2, and IL-6. To begin to understand the role of NE in inducing TNF-α, MIP-2, and IL-6 expression, mouse J774 macrophages were exposed to purified NE and mRNA expression of TNF-α, MIP-2, and IL-6 was assessed by real time RT-PCR as described under “Experimental Procedures.” First, in dose-response and time course experiments, incubation of the cells with NE at 500 nM for 1 h followed by a 4-h incubation after removal of NE led to maximal mRNA expression of TNF-α (Fig. 4A). Using these optimized experimental conditions and by comparison to untreated cells, exposure of J774 cells to NE led to 2.2-, 3-, and 3.1-fold increases of TNF-α, MIP-2, and IL-6 mRNA transcripts, respectively (Fig. 4B). Of importance, NE affects neither the integrity nor the viability of cell monolayers (data not show). These results support in vivo findings and indicate that NE can mediate the induction of TNF-α, MIP-2, and IL-6 expression in macrophages.

NE Employs Its Catalytic Activity to Induce TNF-α, MIP-2, and IL-6 mRNA Expression by Primary Macrophages Involving in Part TLR4—To further ensure that NE has a similar effect on primary macrophages, we employed thioglycollate-elicited peritoneal macrophages. Using similar experimental conditions as above, incubation of macrophages with purified NE resulted in a significant increase of TNF-α, MIP-2, and IL-6 mRNA expression by comparison to untreated cells (Fig. 5, A and B). Importantly, preincubation of NE with the AEBSF, a relatively stable serine protease inhibitor abrogated the inducing activity of the enzyme (Fig. 5A). Of relevance, cell monolayers were not disrupted in the presence of NE (supplemental Fig. S2). These data clearly indicate that NE utilizes its catalytic activity to induce mRNA expression of TNF-α, MIP-2, and IL-6 in primary macrophages.

In recent years, in vitro studies have reported the implication of TLR4 in NE-induced expression of IL-8 in human bronchial
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FIGURE 5. NE effect on TNF-α, MIP-2, and IL-6 expression by murine primary WT and TLR4−/− macrophages. A, representative gel micrograph of RT-PCR on unexposed or NE-exposed primary WT macrophages. Confluent peritoneal macrophages derived from WT C57BL/6 mice were left untreated or treated with purified NE at 500 nM for 1 h and cultured for 4 h. In parallel experiments, NE was preincubated with AEBSF prior to addition to cells. Cells were processed for RT-PCR. Note the increased expression of TNF-α following exposure of cells to NE, which was prevented when the enzyme was inactivated with its inhibitor. Expected size of amplicons is shown on the left. B, in separate experiments, confluent peritoneal macrophages derived from WT C3H/HeN and C3H/HeJ (TLR4−/−) mice were left untreated or treated with purified NE at 500 nM for 1 h and cultured for 4 h as described under “Experimental Procedures.” Cells were processed for RNA extraction and real time RT-PCR. Shown are representative histograms corresponding to data of TNF-α, MIP-2, and IL-6 expression. Note the increased expression of TNF-α, MIP-2, and IL-6 following exposure of WT cells to NE, whereas macrophages with nonfunctional TLR-4 remained unresponsive. Data represent the mean ± S.E. of three values. *, p < 0.05 for differences between NE-treated and untreated samples. Results are expressed as fold-increase of expression of the mediators in NE-treated cells by comparison to untreated cells. Similar data were obtained in repeated experiments.

epithelial cells (24). Given that macrophages also express TLR4, we assessed the role of this receptor in NE-mediated induction of TNF-α, MIP-2, and IL-6 expression. Equivalent numbers of peritoneal WT macrophages and macrophages with nonfunctional TLR4 (TLR4−/−) were incubated with NE as described above. Analyses of real time RT-PCR data found that mRNA expression of TNF-α, MIP-2, and IL-6 increased by 2.2-, 2.6-, and 1.6-fold, respectively, in NE-treated WT cells when compared with untreated cells supporting further data of Fig. 4B. Of note, IL-6 expression was less impressive in primary WT C3H/HeN macrophages than cell-line-derived macrophages, a finding that was reproducible with WT C57BL/6 cells. Although we are unable to explain such an observation, one plausible explanation could be related to differences in intracellular signaling events of IL-6 between the J774 cell line and primary macrophages. More importantly, there were no significant changes in mRNA expression of TNF-α, MIP-2, and IL-6 by TLR4−/− macrophages when compared with untreated WT or TLR4−/− cells (Fig. 5B). These findings indicate that NE-mediated induction of TNF-α, MIP-2, and IL-6 expression by macrophages involves, at least in part, TLR4.

NE Cleaves TLR4 in Intact Macrophages and Cell Protein Extracts—Next, we sought to determine the fate of TLR4 following exposure of macrophages to NE. Using an antibody against the N-terminal part of TLR4, Western blot analysis revealed the presence of intact TLR4 at the expected size in control cells, which disappeared in NE-treated cells (Fig. 6A). Next, immunoblotting analysis following incubation of WT macrophage lysates with NE found progressive loss of TLR4 antigen in function of exposure time to NE (Fig. 6B). Interestingly, pretreatment of NE with its physiologic inhibitor, SLPI, or its denaturation by heat prevented degradation of TLR4 (Fig. 6B and data not shown). Noteworthy, discrete TLR4 cleavage products were detected by immunoblotting following migration of ×10 concentrated cell-free culture media on 16% SDS-PAGE gel (supplemental Fig. S3).

In Vivo Detection of Degraded TLR4 Coincides with Free Active NE—To determine the in vivo significance of our findings, we looked for evidence of TLR4 degradation in cell-free BAL fluids and lung lysates of infected WT mice by immunoblotting. Fig. 7A shows the presence of distinct degradation fragments in cell-free WT BALs and only intact TLR4 in lung lysates. To assess the contribution of NE to TLR4 cleavage, we first observed the absence of immunoreactive TLR4-derived fragments in cell-free BALs of both NE−/− and WT control mice (Fig. 7C, left panel, and data not shown). Next, Western blotting analysis of infected cell-free NE−/− BALs revealed a TLR4 cleavage pattern that appears different from that seen in infected WT BALs. Also, protein levels of intact TLR4 in infected lavaged NE−/− lungs was higher than that detected in infected lavaged WT lungs (supplemental Fig. S4), a finding that was corroborated by cell culture data in this work and previously reported studies (Fig. 6 and Ref. 24). Altogether, these data suggest the contribution of NE and potentially other enzymes to TLR4 proteolysis. Of note, all immunoblotting...
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![Image of gel electrophoresis](image_url)

**FIGURE 7.** Cleaved TLR4 and NE co-exist in *P. aeruginosa*-infected lungs. Pooled BAL fluids and lung tissues from control mice or sacrificed mice at 24 h post-challenge were processed for immunoblotting using TLR-4 antibody. A, all the pools of cell-free BALs and lavaged lungs of infected WT mice were processed for Western blotting. B, the membranes in A were stripped and blotted against albumin. C, next, the pools of cell-free BALs of unchallenged mice and infected NE−/− mice were subjected to immunoblotting. D, the membranes in C were stripped and blotted against albumin. Note: in A, intact TLR-4 and NE in lavaged lungs (diamond); in A and right panel of C, detection of different TLR-4 degradation profiles in cell-free BALs of both infected WT and NE−/− mice, respectively (asterisk); in left panel of C, absence of immunoreactive TLR-4 fragments in unchallenged NE−/− mice as well as unchallenged WT mice (data not shown); in the presence of unspecific bands in cell-free BAL and lung tissue samples of both unchallenged and *P. aeruginosa*-challenged WT and NE−/− mice. M, molecular mass standards in kDa.

Micrographs of *in vivo* studies showed two unspecific bands that were prominent in infectious conditions despite the stringent membrane washing conditions. Also, high molecular bands above the intact TLR-4 band could be seen in some instances. These could be either unspecific immunoreactive fragments or high molecular bands resulting from binding of TLR-4 or its degraded fragments with BAL proteins. At any rate, detection of cleaved TLR-4 and active NE (Fig. 1A) suggest that both proteins co-localize in infected lungs and support the hypothesis that NE could target TLR-4 *in vivo*.

**DISCUSSION**

A number of *in vitro* studies have suggested that proteolytic modifications of cytokines by neutrophil serine proteases including NE change their biologic activities (15, 25). In the present work, we show that in the setting of *P. aeruginosa* lung infection, NE deficiency resulted in changes of protein levels of a variety of cytokines coinciding with increased mortality of mutant mice to infection. The increased or decreased levels of cytokine levels (supplemental Fig. S1) suggest the NE pleiotropic effect in infected situations. Probably, the purpose of these NE-mediated effects is to bring into harmony the levels of cytokines for host lung protection against *P. aeruginosa*. Focusing on TNF-α, MIP-2, and IL-6, we went on to provide compelling *in vivo* evidence that released active NE has the capacity to induce mRNA expression of these inflammatory mediators. Cell culture experiments were crucial in that not only did they allow us to circumvent the confounding stimulatory effects of bacteria, but clearly support the role of NE in inducing cytokine expression. It must be emphasized that this is the first report that shows both in *in vivo* and cell culture studies that active NE induces mRNA expression of these pro-inflammatory mediators.

In our study, we focused on the 24-h time point post-infection because it corresponds to a sharp increase of neutrophil numbers and enhanced NE activity by comparison to other time points (23). But, the associated changes in cytokine expression could be seen at any time as long as significant amounts of free active NE are available and the microenvironment allows interaction of the enzyme and its target cells. Of interest, activated PMNs also release two other members of the neutrophil serine protease family, cathepsin G (CG) and proteinase 3 (PR3). As for NE, the relative importance of CG and PR3 in modulating cytokine expression (*e.g.* induction of TNF-α, MIP-2, and IL-6 transcript expression) will be best defined using mice that are deficient in all these proteases. Regarding cell culture studies, the concentration of NE used in this study is relevant because it can be reached or even exceeded in the lungs in diseased conditions (26). In fact, these suggested estimates do not even take into account possible evanescent quantum bursts of pericellular NE activity, local microenvironment, or the half-life of NE in tissues, which might increase the effective concentration of the enzyme (7). Importantly, enzyme inhibition experiments revealed that NE must be catalytically active to induce mRNA expression of TNF-α, MIP-2, and IL-6. In support of this observation, sivelestat, a selective synthetic neutrophil elastase inhibitor, has been reported to suppress the production of TNF-α and MIP-2 by LPS and/or NE-stimulated leukocytes in whole blood culture (27, 28). We have purposely used the synthetic inhibitor AEBSF instead of physiologic inhibitors such as SLPI because these latter have been reported to down-regulate the expression of inflammatory mediators (29, 30). Recently, NE has been shown to cleave SLPI (31).

TNF-α, MIP-2, and IL-6 are expressed following host recognition of pathogens and triggering of NF-κB signaling path-
ways. They have been reported to participate in the recruitment and/or activation of immune cells, particularly neutrophils in the setting of bacterial infections (32, 33). Surprisingly, in vivo changes in the levels of these mediators in the absence of NE has no statistically significant bearing on the recruitment of immune cells in response to P. aeruginosa suggesting the presence of a multitude of host and pathogen-derived factors that call in inflammatory cells.

The role of TNF-α in mediating host defense against bacterial infection has been reported in different studies using gene targeting and ligand or receptor blocking antibody approaches (34, 35). Also, this cytokine stimulates inflammatory mediator production such as MIP-2 and IL-6 that contribute to the recruitment and activation of inflammatory cells (36–38), strengthens the bactericidal capacity of phagocytic cells by an as yet undefined mechanism (39), and behaves as a secretagogue for neutrophils to secrete their granule content (32). Although MIP-2 is known to selectively attract PMNs, it is considered as a potent inducer of degranulation and enhancer of bacterial killing by phagocytic cells including PMNs themselves (40–42). In vivo studies using anti-MIP-2 serum in an infection model with Klebsiella pneumoniae revealed that this chemo-kine is an important mediator for effective bacterial clearance. Like TNF-α, IL-6 can be detected readily in stressed conditions as in infection. Regarding host defense against invading pathogens, genetically engineered mice deficient in IL-6 displayed impaired antibacterial protection (43). Collectively, these reported studies show the relative importance of TNF-α, MIP-2, and IL-6 in host protection against bacterial infections inferring that the observed decrease in their levels in our study should contribute to the increased susceptibility of NE-deficient mice to P. aeruginosa infection. It would be of interest to determine the role of NE in expression of the remaining cytokines and how this might relate to host susceptibility to infection.

The capacity of NE to induce gene expression of TNF-α, MIP-2, and IL-6 suggests that the enzyme interacts with TLRs among others pattern recognition receptors, we narrowed our focus in this study to TLR-4 whose implication in cytokine expression. Although cytokine expression is mediated by TLRs under stress conditions, pattern recognition receptors, we narrowed our focus in this study to TLR-4 whose implication in NF-κB activation and induction of cytokine expression. Although we have no clear evidence about the underlying mechanism of the NE-triggered function of TLR-4, among the possibilities by which the enzyme could act are: it could engage TLR-4 directly, especially since the protease has been reported to trigger TLR-4 signal transduction in the absence of the accessory protein MD2 (24). A simple cleavage in the extracellular domain might change the conformation of and activate the receptor. TLR-4 cleavage products could be biologically active and serve as a ligand for other receptor(s) to signal cytokine expression. NE might be involved in the generation of a ligand
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that signals through TLR-4 (56). The possibility that cleavage of TLR-4 corresponds to an epiphenomenon is plausible as well in that NE may interact with the receptor “rapidly” to generate an intracellular signaling event prior to its proteolysis. In this regard, NE has been shown to bind to the membrane of monocytes/macrophages (58–60). Alternatively, NE could act indirectly on TLR-4 function. In recent years, it was reported that NE activates the metalloprotease, meprin-α, which in turn releases TGF-α, a ligand for the epidermal growth factor receptor. EGF receptor co-localizes with TLR4 initiating a signal transduction cascade triggering activation of NF-κB and increased cytokine expression (61). Studies investigating the interaction mechanism(s) of NE and TLR4 are needed to better understand our findings, the long held view that considers NE as “a prime proinflammatory mediator” (53, 64, 65). In fact, these cells represent the most potent sources for TNF-α, MIP-2, and IL-6 (3). Collectively, the phenotype of human metapneumonia is chemoattractant for neutrophils and recognizing microbial membrane products (62). Consistent with our previous findings, NE can accumulate in close spatial proximity to local cells at sites of active inflammation (18). This suggests that unchecked NE could interact and activate host cells. In support of this hypothesis, our culture study (this work) in addition to a series of published reports indicates that NE is capable of activating various types of host immune (e.g. dendritic cells) and nonimmune (e.g. epithelial cells) cells in vivo (54, 63). The rationale of investigating macrophages is that this cell population plays a prominent role in lung immunity by orchestrating inflammatory and immune responses (53, 64, 65). In fact, these cells represent the most potent sources for TNF-α, MIP-2, and IL-6 in the setting of bacterial infection. Also, TLR-4 is not presented at the apical surface of lung epithelial cells and does not function in signaling responses to P. aeruginosa (54).

Previously, we have reported that NE is required for maximal PMN intracellular killing of P. aeruginosa. Collectively, the present findings reveal a protective role for extracellular NE against the pathogen. They clearly demonstrate that NE, an endogenous effector, could also participate in the orchestration of lung inflammatory response against P. aeruginosa infection by modulating the expression of cytokines (e.g. induction of the expression of the proinflammatory TNF-α, MIP-2, and IL-6). The enzyme might be required to drive a robust NF-κB pathway activation and subsequent cytokine expression. Thus, the net effect of NE (intra- and extracellularly) on P. aeruginosa and perhaps other Gram-negative bacteria is an “astute” response for better lung protection. In conclusion, unopposed active NE is anticipated in any PMN-rich inflammatory milieu. In light of our findings, the long held view that considers NE as “a prime suspect” in inflammatory and tissue-destructive diseases as in acute or chronic pulmonary diseases will need to be carefully reassessed. More importantly, therapeutic strategies aiming at NE inhibition should take into account the physiologic contribution of the enzyme to host inflammatory response against pathogenic agents.

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