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Neutrophil Serine Proteinases Cleave Bacterial Flagellin, Abrogating Its Host Response-Inducing Activity

Yolanda S. López-Boado,† Marcia Espinola,‡ Scott Bahr,‡ and Abderrazzaq Belaaouaj§‡

After bacterial infection, neutrophils dominate the cellular infiltrate. Their main function is assumed to be killing invading pathogens and resolving the inflammation they cause. Activated neutrophils are also known to release a variety of molecules, including the neutrophil serine proteinases, extracellularly. The release of these proteinases during inflammation creates a proteolytic environment where degradation of different molecules modulates the inflammatory response. Flagellin, the structural component of flagella on many bacterial species, is a virulence factor with a strong proinflammatory activity on epithelial cells and other cell types. In this study we show that both human and mouse neutrophil serine proteinases cleave flagellin from Pseudomonas aeruginosa and other bacterial species. More important, cleavage of P. aeruginosa flagellin by the neutrophil serine proteinases neutrophil elastase and cathepsin G resulted in loss of the biological activity of this virulence factor, as evidenced by the lack of innate host defense gene expression in human epithelial cells. The finding that flagellin is susceptible to cleavage by neutrophil serine proteinases suggests a novel role for these enzymes in the inflammatory response to infection. Not only can these enzymes kill bacteria, but they also degrade their virulence factors to halt the inflammatory response they trigger. The Journal of Immunology, 2004, 172: 509–515.

Neutrophils represent the earliest phagocytic cells that dominate the sites of bacterial infection. Their main function is assumed to be the killing of invading pathogens. Various antimicrobial molecules have been identified in neutrophil granules and grouped into oxygen-dependent and -independent systems (1). This latter system comprises the readily active serine proteinases (NSPs),3 neutrophil elastase (NE), cathepsin G (CG), and proteinase 3 (PR3), among other polypeptides known to alter the bacterial structural integrity. NSPs are structurally related and share the conserved charge-relay triad, His57-Asp102-Ser195, where Ser is the active residue (chymotrypsinogen numbering) (2). Previously, we have shown that NE is required for host defense against Gram-negative bacteria (3, 4) and demonstrated that NE kills Gram-negative Escherichia coli by degrading its major outer membrane protein A (5). Together, these findings demonstrate that NSPs serve a physiological role in the intracellular killing of microbes.

Activated neutrophils are also known to release a variety of molecules, including NSPs, during their egress from the vasculature, when their defensive functions (e.g., phagocytosis) are impaired or the bacterial dose is overwhelming. The release of these proteinases during inflammation creates a proteolytic environment where degradation of different molecules modulates the inflammatory response. For example, NE is capable of cleaving a papain of substrates in vitro. These include extracellular matrix proteins, coagulation factors, and Igs (6). NE degrades proinflammatory mediators such as TNF-α and IL-1β (7) and hence down-regulates inflammation. NE cleaves ICAM-1, indicating that the enzyme might regulate neutrophil adhesion and migration (8, 9). NE induces the secretion of GM-CSF and IL-8 (10), which could amplify the inflammation. This substrate repertoire, which continues to expand, has already led to the incrimination of NE in the pathogenesis of various tissue-destructive diseases, including pneumonia-mediated acute lung injury and cystic fibrosis (CF) (6).

At sites of infection, bacteria and/or their components modulate the inflammatory response as well. In recent years, flagellin, the structural component of flagella in many bacterial species (11), has been demonstrated to have a strong proinflammatory activity on epithelial cells and other cell types (12–14). With respect to epithelial cells, flagellin induces IL-8 secretion, inducible NO synthetase expression, and NF-κB activation in several models of cells (15–19). In addition, the secretion of flagellin is necessary to induce the activation of proinflammatory signaling pathways and neutrophil trans-epithelial migration (20). We and others have recently demonstrated that flagellin up-regulates the expression of the innate host defense genes, matrilysin and human β-defensin-2 (hBD-2), in epithelial cells (21, 22). This bacterial protein can also activate other cell types, including monocytes, to produce proinflammatory cytokines (23, 24). Finally, flagellin can trigger adaptive immune responses both by stimulating chemokine secretion by epithelial cells and subsequent migration and maturation of dendritic cells (25) and by modulating T cell activation in vivo (26). Although the presence and role of flagellin in mounting an inflammatory response are documented, mechanisms that control its inflammation-inducing activities are still unknown.

The goals of these studies were, then, 2-fold. We wanted to determine whether NSPs cleave flagellin and alter its ability to induce host responses. We have found that NE and CG degrade flagellin, rendering it inactive. Indeed, our data demonstrate that

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3 Abbreviations used in this paper: NSP, neutrophil serine proteinase; BAL, bronchoalveolar lavage; CT, cystic fibrosis; CG, cathepsin G; hBD, human β-defensin; i.n., intranasally; MMP, matrix metalloproteinase; NS, neutrophil elastase; PR3, proteinase 3; TLR, Toll-like receptor; TSB, tryptic-soy broth; WT, wild type.

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flagellin, when degraded to completion, failed to induce the expression of the innate host defense genes, matrilysin and hBD-2. Taken together, these results point to flagellin as a novel substrate of NE and CG and highlight a new mechanism by which NSPs modulate the inflammatory response in the setting of bacterial infection.

Materials and Methods
Reagents

Puriﬁed NSPs, NE, CG, and PR3 and their speciﬁc peptide substrates were obtained from Elastin Products (Owensville, MO). Puriﬁed matrix metalloproteinases (MMPs), MMP-1, MMP-7, and MMP-8 were obtained from Calbiochem (La Jolla, CA). Activation of MMP proenzymes was achieved by exposure to 1 mM 4-aminophenylmercuric acetate at 37°C for 1 h or by a combination of 4-aminophenylmercuric acetate and trypsin as recommended by the manufacturer. The purity and activity of each enzyme were conﬁrmed by a spectrophotometric method using speciﬁc substrates and/or SDS-PAGE analysis. Regarding the NSP preparations, the proteinases were also incubated with the broad serine proteinase inhibitor PMSF (100 μM) or the cysteine protease inhibitor E64 (10 μM) for 15 min at 37°C before addition of their peptide substrates to verify their speciﬁc activities. The anti-ﬂagellin polyclonal Ab was provided by Dr. A. Prince (Columbia University, New York, NY). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cells, mice, and bacteria

The human lung carcinoma cell line Calu-3 was obtained from American Type Culture Collection (Manassas, VA) and was maintained in RPMI 1640 medium supplemented with 10% FBS without antibiotics or in the presence of 50 μg/ml gentamicin. To obtain neutrophils, wild-type mice and mice deﬁcient in NE or CG, or both were injected i.v. with 15% glycogen (1 ml/mouse) to elicit the cells to the peritoneum. Mice deﬁcient in NE or CG were generated by gene-targeting technique and interbred to obtain mice doubly deﬁcient in NE and CG (3, 4). All mice are in the C57/Sv129 background. Four hours after i.p. injection, mice were sacriﬁced by CO2 narcosis according to the animal studies committee of Washington University School of Medicine (St. Louis, MO). Next, the peritoneum was lavaged with 5 ml of HBSS (1× HBSS). The lavage ﬂuid was spun and resuspended in a hypotonic lysis solution to remove red cell contamination. Neutrophils represented >95% of the cell population, and >98% were viable as judged by differential counting and trypan blue dye exclusion. Aliquots of cells (2 × 106 cells) were prepared in a total volume of 0.2 ml of HBSS and were kept on ice until use. Pseudomonas aeruginosa and Salmonella typhimurium strains were also obtained from American Type Culture Collection (Manassas, VA) and routinely grown at 37°C in 3% tryptic-soy broth (TSB).

Puriﬁcation of ﬂagellin

P. aeruginosa and S. typhimurium ﬂagellin were puriﬁed as described previously (21) with some modiﬁcations. A 400-μl sample of an overnight culture was centrifuged at 10,000 × g for 15 min. Ammonium sulfate was added to the bacterial supernatant to reach 40% saturation over a period of 2 h at room temperature. After centrifugation at 20,000 × g for 40 min, the pellets were resuspended in 6 ml of 50 mM Tris-HCl, pH 9.5, containing 0.5 mM DTT and dialyzed extensively against the same buffer. Flagellin was collected by centrifugation at 27,000 × g for 40 min and was resuspended in 2 ml of 50 mM Tris-HCl, pH 7.5, containing 0.5 mM DTT. To obtain a preparation of completely detached monomeric ﬂagellin, the pH of the protein solution was adjusted to 3.5 by the addition of 0.25 M HCl for 5 min at room temperature and was immediately restored to a neutral pH by the addition of 0.25 M NaOH. Finally, ﬂagellin was analyzed by SDS-PAGE and native gel electrophoresis on 12% acrylamide gels.

Degradation of ﬂagellin by proteolytic enzymes

All enzymes tested were used at equimolar concentrations. Flagellin (5 μM) was incubated with each enzyme in a 20-μl reaction volume at 37°C for designated times. In separate experiments, NE and CG were incubated with PMSF or E64 as described above before addition of flagellin. When using NSPs, the reactions were conducted in PBS, pH 7.4. The reactions with MMPs were, however, performed in a buffer containing 20 mM Tris-HCl, pH 7.5, supplemented with 10 mM CaCl2, 150 mM NaCl, and 1 μM ZnCl2. Unless indicated, the reactions were performed in duplicate. After designated times of incubation, they were subjected to protein electrophoresis under reducing conditions. The gels were stained with Coomassie Brilliant Blue and photographed. Duplicate gels were processed for ECL Western blotting (NE, Boston, MA) using speciﬁc Abs. In other experiments, degradation of ﬂagellin by NE was assessed directly in intact bacteria. Brieﬂy, P. aeruginosa bacteria were freshly grown in TSB medium at 37°C and washed twice with and resuspended in PBS, and the OD of the culture was determined at 600 nm (1 OD = 108 bacterial/ml). Mid-log grown bacteria (108 bacteria) were incubated in the presence or the absence of NE (2.5 μM) in a total volume of 100 μl of PBS containing 1% (v/v) TSB for varying times (0, 2, and 4 h) (5). Next, the reactions were processed for ECL Western blotting (NE) using ﬂagellin-speciﬁc Abs as described below.

Degradation of ﬂagellin by neutrophils

Aliquots of peritoneum-isolated neutrophils (2 × 106) were primed with LPS for 15 min and stimulated with N-MLP for an additional 15 min to allow activation and release of lysosomal enzymes (27). Next, ﬂagellin was incubated alone and with or without activated cell aliquots at 37°C. After overnight incubation, levels of ﬂagellin degradation were determined by Western blotting using an Ab speciﬁc to ﬂagellin. To ensure the release of active proteinases, including NE, from neutrophils, NE activity (Aaab-sorbance/min at 410 nm) was determined by the spectrophotometric method using NE-speciﬁc chromogenic substrate Meo-Suc-Al-Al-Pro-Val-pNA (3), as recommended by the manufacturer (Elastin Products). As controls, this peptide substrate was degraded by puriﬁed NE, but not CG or PR3.

Immunoblotting

In general, samples were separated on 12% SDS-polyacrylamide gels and transferred by semidyed electrophoretic transfer at 15 V for 20 min to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Little Chalfont, U.K.) in 48 mM Tris, 30 mM glycine, 20% methanol, and 0.0375% SDS. Nonspeciﬁc binding sites were blocked by soaking the membranes in 3% nonfat dry milk in TBS at 4°C overnight. Blots were incubated with either a 1/10,000 dilution of anti-Pseudomonas ﬂagellin polyclonal antisem (12) or a 1/1,000 dilution of an Ab against E. coli ﬂagellin (Igen International, Gaithersburg, MD), which cross-reacts with Salmonella ﬂagellin. In some experiments, blots were incubated with TBS containing 0.1% Tween 20 for 10 min. Membranes were subsequently incubated with an appropriate dilution of peroxidase-linked secondary IgG (Amersham Pharmacia Biotech) in blocking buffer for 1 h, washed twice, and developed with the ECL system according to the manufacturer’s instructions.

N-terminal sequence analysis of ﬂagellin degradation products

Flagellin (10 μM) was incubated with increasing concentrations of NE or CG at 37°C for 30 min. The samples were then subjected to SDS-PAGE, and protein bands were transferred to PolyBlot membranes (PE Applied Biosystems, Foster City, CA) as described by the manufacturer. Bands were visualized by staining with 0.1% Coomassie Brilliant Blue, and major degradation products were excised and sequenced by automated Edman degradation using a PE Applied Biosystems 492 sequence.

Northern blotting and RT-PCR

Calu-3 human lung epithelial cells were seeded onto six-well plates and grown to ~80% conﬂuence. Puriﬁed ﬂagellin alone and treated with NE or CG was added to epithelial cells at a concentration of 10 nM in 1 ml of RPMI 1640 medium supplemented with 10% FBS and 50 μg/ml gentamicin and was incubated at 37°C for 90 min. Epithelial monolayers were then washed extensively with PBS, and the cultures were further incubated in fresh RPMI 1640 medium supplemented with 10% FBS and 50 μg/ml gentamicin. After 6 h, total RNA from the cells was prepared with RNAzol (Tel-Test, Friendswood, TX), separated by electrophoresis in 1.2% agarose-formaldehyde gels, and blotted onto Hybond nylon ﬁlters (Amersham Pharmacia Biotech). The integrity of the RNA in the different samples was ascertained by direct visualization of the gels under UV light. Northern hybridization for matrilysin and GAPDH mRNAs was performed as previously described (21). For analysis of the expression of human β-defensins, total RNA samples were reverse transcribed using random hexamer primers (PerkinElmer, Branchburg, NJ). cDNAs were then ampliﬁed by PCR using speciﬁc primers: H-BD-1: forward, 5′-GGA TTC TGA GTG TTG CCT GCC-3′; and reverse, 5′-GGA TTC TCA ATG ATG ATG ATG CCT GCA GCA CCT GCC CCT CC-3′; and hBD-2: forward primer, 5′-GGA TCC CCT CCC AGC CAT CAG-3′; and the reverse primer, 5′-GGA TCC TCA ATG GTG ATG ATG TTG CTT TTT GCA GCA TTT TG-3′.
Reactions were conducted for 25 cycles at an annealing temperature of 57°C and analyzed on 3% agarose gels.

**Mouse pneumonia model**

Mice (n = 6) were intranasally (i.n.) challenged with *P. aeruginosa* as previously described (28). Briefly, mice were anesthetized by i.p. injection of ketamine hydrochloride (75 mg/kg) and medetomidine hydrochloride (1 mg/kg), followed by i.n. administration of *P. aeruginosa* bacteria (4 × 10^6 CFU/mouse). At this dose, mice exhibit signs of infection, including ruffled fur and lethargy, but the majority recover by 48 h postinfection (Y. S. Lopez-Boado, M. Espinola, S. Bahr, and A. Bélaouaj, manuscript in preparation). As a control, mice (n = 6) were instilled i.n. with *P. aeruginosa* LPS (10 μg/mouse) in 50 μl of PBS to incite a neutrophilic response (29). At designated time points (24 h postchallenge), mice were sacrificed, and their lungs were gently perfused with saline via the right ventricle. The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge catheter (BD Biosciences, Mountain View, CA). The lungs were lavaged in situ (bronchoalveolar lavage (BAL)) with a total of 2 ml of HBSS (pH 7.4). The BAL fluids were centrifuged for 10 min at 4°C, and the supernatants were aliquoted and stored at ~80°C until use.

**Elastin zymography**

The elastolytic activity in cell-free BAL fluids was assessed by elastin zymography as previously described (3). BAL supernatants (20 μl) were migrated under nonreducing conditions at 4°C on SDS-PAGE gels (12%), containing 1 mg/ml elastin. Purified NE (0.1 μg) was used as a control. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min and incubated at 37°C for 48 h in 50 mM Tris-HCl (pH 8.2) containing 5 mM CaCl2. The gels were then stained with Coomassie Brilliant Blue and destained in 5% acetic acid and 10% methanol. Active NE appears as a transparent lysis band at ~29 kDa.

**Results**

**Neutrophil serine proteinases cleave *P. aeruginosa* flagellin**

The ability of NSPs to cleave the virulence factor flagellin was examined using purified flagellin from *P. aeruginosa*. As shown in Fig. 1A, flagellin was effectively degraded by NE and CG, suggesting the high susceptibility of this protein to proteolysis by these enzymes. However, degradation of flagellin by proteinase 3 was very limited and was not further characterized. Preincubation of NE and CG with the serine proteinase inhibitor PMSF, but not with the cysteine protease-specific inhibitor E64, prevented flagellin degradation (Fig. 1, B and C). This serine proteinase-specific inhibition was further confirmed using the secretory leukocyte proteinase inhibitor (data not shown). In contrast, using experimental conditions comparable to those of NSPs, none of the MMPs tested cleaved flagellin (Fig. 1A). These included neutrophil collagenase (MMP-8), interstitial collagenase (MMP-1), and matrixin (MMP-7). Of note, we have previously shown that flagellin can up-regulate the expression of MMP-7, an MMP involved in host defense mechanisms (21, 30).

**NSPs degrade flagellin present in intact bacteria**

To determine whether NSPs degrade flagellin in intact *P. aeruginosa* bacteria, we incubated whole bacteria with NE and analyzed the reaction by Western blotting using a flagellin-specific Ab. Our data demonstrate that in the absence of NE, flagellin remained intact and that its expression increased slightly over time (0 vs 2 h) as judged by immunoblotting (Fig. 2A). However, incubation of...
bacteria with NE was accompanied by complete flagellin degradation as early as 2 h. These results indicate that NSPs, including NE and CG are capable of cleaving not only soluble flagellin that is secreted or leaked by the bacteria, but also when the protein is assembled at the cell surface.

**Relative importance of neutrophil serine proteinases in flagellin degradation**

To further characterize flagellin degradation by NSPs, we incubated flagellin with wild-type (WT) mouse neutrophils directly. In parallel experiments the cells were treated with LPS followed by fMLP to maximize the release of proteinases and then added to flagellin. As shown in Fig. 2B, flagellin was entirely degraded in the presence of unstimulated or stimulated neutrophils. These data suggest that flagellin was degraded by released proteases, including NE and CG. To address the relative importance of NSPs to degrade flagellin, we performed similar ex vivo experiments, this time using neutrophils that were isolated from WT and NE-, CG-, or NE- and CG-deficient mice and activated with LPS/fMLP (Fig. 2C). Although flagellin was completely degraded in the presence of WT and NE-deficient neutrophils, incubation of this protein with neutrophils derived from CG- or NE/CG-deficient mice resulted in incomplete degradation of the protein, suggesting that NSPs, in particular CG, play an important role in flagellin cleavage by neutrophils. Of note, overnight incubation of flagellin alone did not cause a spontaneous degradation of the protein (Fig. 2, B and C, lane a), and flagellin Ab did not immunoreact with neutrophil proteins (Fig. 2, B and C, lane b).

**Relative contributions of NSPs to degrade bacterial flagellin**

To compare the relative contributions of NSPs to cleave flagellin, *P. aeruginosa* flagellin was incubated with NE or CG, and degradation was monitored as a function of enzyme concentration and time. Fig. 3A shows that flagellin was degraded by both NE and CG. However, at similar concentrations the rates of catalysis were different for NE and CG. CG catalyzed rapid and complete cleavage of flagellin (100% of flagellin was completely degraded by 0.25 μM CG within 20 min). In contrast, degradation of flagellin by NE occurred at a somewhat slower rate (0.25 μM of NE generated multiple flagellin fragments within 60 min). Next, to determine whether NSPs could cleave flagellin from other bacterial species, we purified flagellin from *S. typhimurium* cultures and exposed it to NE and CG. Similarly to *P. aeruginosa*, *S. typhimurium* flagellin was degraded by NE in an enzyme concentration- and time-dependent manner (Fig. 3B). Like NE, CG degraded *S. typhimurium* flagellin, but rapidly (data not shown). These data suggest that although NSPs effectively cleave flagellin from different pathogens, their degradation rates are different.

**Cleavage sites of flagellin**

To attempt to identify the cleavage sites of NSPs in flagellin, we incubated both *P. aeruginosa*- and *S. typhimurium*-purified flagellin with low concentrations of NE and CG for 30 min (Fig. 4, A and B). Major cleavage products were identified by Coomassie Blue staining and were processed for N-terminal sequencing. Analysis of our results showed that NE catalyzed an initial cleavage in *S. typhimurium* flagellin at Ile-Ala, 50 residues from the N terminus of the protein, and within the D₉ N-terminal domain (Fig. 4C) (31). Of note, two bands of NE-cleaved *S. typhimurium* flagellin migrating at ~40 and 45 kDa had the same N terminus. Notably, we identified a cleavage site in *P. aeruginosa* flagellin 59 residues from the N terminus of the protein, which by simulation of the proposed structure of *Salmonella* flagellin could be located within the D₉ N-terminal domain (31) (Fig. 4D). Interestingly, CG cleaved *S. typhimurium* flagellin at Phe-Thr and *P. aeruginosa* flagellin at Asn-Ala, 54 and 63 residues from the N terminus, respectively. This suggests that CG, like NE, targets flagellin within the D₉ N-terminal domain.

**NSPs cleave flagellin, altering its biologic activity**

We have determined by size exclusion chromatography that our purification procedure of flagellin usually results in a mix of monomers and aggregates of higher m.w. (data not shown). As reported previously (32), this could be due to the autopolymerization of the protein that occurs at high concentrations of flagellin or in the presence of salt (e.g., ammonium sulfate) during the purification process. When these protein preparations were subjected to a transitory acidification or heat treatment, flagellin became essentially monomeric, as shown by native PAGE (data not shown). Of importance, these treatments did not affect flagellin activity, as illustrated by the similar induction of expression of both matrilysin and hBD-2 (data not shown). These data also indicate that monomeric and/or polymerized flagellin have similar biological activity as bacteria-associated flagellin.

To determine whether the biologic activity of flagellin is altered after its degradation by NSPs, we incubated the protein with different concentrations of NE and CG and examined the ability of the degradation products to induce the expression of innate host defense genes in human lung epithelial cells. In Fig. 3, we showed that treatment of flagellin with increasing concentrations of NE and CG led to progressive degradation of the protein. This cleavage of flagellin resulted in a parallel loss in its ability to induce the expression of host defense genes in epithelial cell lines, Calu-3 and A549 (Fig. 5 and data not shown). Indeed, degradation of flagellin by NE or CG resulted in the loss of induction of matrilysin and hBD-2 gene expression (Fig. 5, A, B, and D). Furthermore, when the catalytic activity of NE or CG was blocked with PMSF and the secretory leukocyte protease inhibitor, flagellin was not degraded and induced the expression of both matrilysin and hBD-2 (Fig. 5, A and B, and data not shown). The cysteine protease inhibitor E64,

![FIGURE 3](http://www.jimmunol.org/Downloadedfrom/images/Figure3.jpg)
the level of hBD-1 expression, which does not change in response to infection of two NE-preferred by the enzymes (34). Also, the identifications of characteristic domains of flagellin (adapted from Ref. 41) and localization of the initial cleavage site in the D0 N-terminal domain. The arrow on the left indicates the location of the NE cleavage site; the arrow on the right points to characteristic domains of flagellin protein.

FIGURE 4. Cleavage sites of NE in P. aeruginosa and S. typhimurium flagellin. Purified flagellin from P. aeruginosa (A) and S. typhimurium (B) was incubated with low concentrations of NE or CG (data not shown for CG) for 30 min. Samples were resolved in 12% SDS-PAGE and transferred to ProBlott membranes, and distinct bands were excised and processed for sequencing by automated Edman degradation. C, N-terminus sequences of NE-cleaved P. aeruginosa or S. typhimurium flagellin fragments. Arrowheads point to sequenced fragments. D, Schematic structure of flagellin (adapted from Ref. 41) and localization of the initial cleavage site in the D0 N-terminal domain. The arrow on the left indicates the location of the NE cleavage site; the arrow on the right points to characteristic domains of flagellin protein.

however, did not protect flagellin from cleavage, and therefore its ability to induce the expression of host defense genes was lost (Fig. 5, A and B). This effect of flagellin on host gene expression was dependent on NSP concentration. For example, the capacity to induce the expression of matrilysin and hBD-2 genes was retained in partially degraded flagellin (e.g., NE at 0.25 μM) and was lost in totally degraded flagellin (e.g., NE at 2.5 μM; Fig. 5, C and D). Similar results were observed for CG (data not shown). Of note, the level of hBD-1 expression, which does not change in response to bacterial infection (33) or flagellin, was not altered in the presence of NE-degraded flagellin (Fig. 5E) or CG-degraded flagellin (data not shown).

In vivo colocalization of flagellin and NSPs
To determine the in vivo significance of our findings, we examined whether flagellin and free active NE colocalize in infected milieu. We challenged mice i.n. with P. aeruginosa bacteria and checked for the presence of flagellin and free active NE in the cell-free BAL fluids of infected mice 24 h postchallenge (Fig. 6A). Immunoblotting data showed the presence of flagellin in cell-free BAL fluids of P. aeruginosa-infected mice. Different flagellin bands were detected, including intact (band migrating similar to control), degraded (lower bands), and high m.w., complex (upper bands) bands. These latter could result from autopolymerization of flagellin or flagellin that interacted with bacterial or other proteins in the BAL. As expected no signal for flagellin was detected in the samples from LPS-instilled mice, demonstrating the high specificity of flagellin Ab. With respect to NE, there was a single lysis band in cell-free BAL of P. aeruginosa-infected mice that migrated similarly to control NE (Fig. 6B). These findings demonstrate that flagellin (free or bacteria-associated) and NSPs, including NE and CG, colocalize in the lung in the setting of infection and support the hypothesis that NSPs could encounter and degrade flagellin in vivo.

Discussion
Using different biochemical approaches, we demonstrated that NSPs cleave free or P. aeruginosa-associated flagellin, a cleavage that was prevented when the catalytic activity of the enzymes was specifically inhibited. We also found that NE and CG degrade S. typhimurium flagellin, a very potent cytokine inducer (24), suggesting that NSPs could target flagellin from other microbial species. Characterization of degraded flagellin of both P. aeruginosa and S. typhimurium found that flagellin cleavage was catalyzed by NE and CG at different rates and occurred in peptide bonds that are preferred by the enzymes (34). Also, the identification of two NE-degraded fragments in S. typhimurium flagellin with the same N-terminus, but different molecular masses, suggests that additional C-terminal processing of flagellin by NSPs may occur. Interestingly, both NE and CG generated a cleavage site within the D0 domain for the presence of NSPs cleave free or bacteria-associated flagellin and free active NE in the cell-free BAL fluids of infected mice 24 h postchallenge (Fig. 6A). Immunoblotting data showed the presence of flagellin in cell-free BAL fluids of P. aeruginosa-infected mice. Different flagellin bands were detected, including intact (band migrating similar to control), degraded (lower bands), and high m.w., complex (upper bands) bands. These latter could result from autopolymerization of flagellin or flagellin that interacted with bacterial or other proteins in the BAL. As expected no signal for flagellin was detected in the samples from LPS-instilled mice, demonstrating the high specificity of flagellin Ab. With respect to NE, there was a single lysis band in cell-free BAL of P. aeruginosa-infected mice that migrated similarly to control NE (Fig. 6B). These findings demonstrate that flagellin (free or bacteria-associated) and NSPs, including NE and CG, colocalize in the lung in the setting of infection and support the hypothesis that NSPs could encounter and degrade flagellin in vivo.

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nsaP. -infected mice. Mice (six per group) were challenged i.n. with FNSPs have a common cleavage mechanism of N-terminal domain of fl agellin. Together, these results suggest that NSPs have a common cleavage mechanism of flagellin, but degrade the protein at different rates within discrete and specific areas. More physiologically relevant, flagellin was completely degraded when incubated with neutrophils, indicating that NSPs could contribute to neutrophil-mediated flagellin cleavage.

Flagellin is a member of the pathogen-associated molecular patterns group, a collection of bacterial products that activate various inflammatory molecules in various cell types (13, 16, 36). The purpose of the flagellin-TLR5 interaction is to reinforce the host antimicrobial systems. If uncontrolled, this interaction could also lead to excessive inflammatory response and injury of the host. In view of our findings, degradation of flagellin by NSPs could be regarded as beneficial to the host. In other words, neutrophils are called in when the first line of host defense is breached (e.g., epithelial lining). This neutrophil recruitment represents one of the means by which the host attempts to curb not only bacterial infection, but also the associated inflammation. However, under overwhelming conditions (e.g., uncontrolled infection), neutrophils and their secreted products could damage the surrounding tissues as well. In sum, our present work points to the virulence factor, flagellin, as a novel substrate targeted by NSPs. It also highlights the potential role of NSPs in modulating the host inflammatory response to bacterial infection. Taken altogether, these findings add new insights to the role of NSPs during the complex host-pathogen interactions. Future studies using mice deficient in NSPs will allow us to determine the relative importance of these proteases in the resolution of flagellin-induced inflammation.

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References


