Innate Immune Collectin Surfactant Protein D Simultaneously Binds Both Neutrophil Extracellular Traps and Carbohydrate Ligands and Promotes Bacterial Trapping

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*J Immunol* published online 1 July 2011
http://www.jimmunol.org/content/early/2011/07/01/jimmunol.1004201

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/07/05/jimmunol.1004201.DC1

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Neutrophils release DNA-based extracellular traps to capture and kill bacteria. The mechanism(s) and proteins that promote neutrophil extracellular trap (NET)-mediated bacterial trapping are not clearly established. Surfactant protein D (SP-D) is an innate immune collectin present in many mucosal surfaces. We hypothesized that SP-D can bind both the pathogens and NETs to augment NET-mediated bacterial trapping. To test this hypothesis, we used LPS and Pseudomonas aeruginosa pneumonia mouse models and performed in vivo and ex vivo assays. In this study, we show that NETs are produced by the neutrophils recruited to the airways in response to the bacterial ligand. Notably, NETs are detected as short fragments of DNA–protein complexes in the airways as opposed to the long stringlike structures seen in ex vivo cultures. SP-D recognizes both the short NET fragments and the long NET DNA structures. SP-D–NET copurification studies further show that SP-D can simultaneously recognize NETs and carbohydrate ligands in vivo. Similar to the LPS model, soluble DNA–protein complexes and increased amounts of SP-D are detected in the murine model of P. aeruginosa pneumonia. We then tested the effect of SP-D on NET-mediated trapping of P. aeruginosa by means of Western blots, fluorescence microscopy, and scanning electron microscopy. Results of these experiments show that SP-D microagglutinates P. aeruginosa and allows an efficient bacterial trapping by NETs. Collectively, these findings provide a unique biological relevance for SP-D–DNA interactions and places SP-D as an important innate immune protein that promotes bacterial trapping by NETs during neutrophil-mediated host defense. The Journal of Immunology, 2011, 187: 000–000.
signal intensity versus kilobase.

In this study, we hypothesized that SP-D can bind both the pathogens and NETs to augment NET-mediated bacterial trapping. To test this hypothesis, we characterized NET formation in a murine LPS-induced neutrophilic airway inflammation model and corroborated its relevance to a *Pseudomonas aeruginosa* infection model. Our studies show that NETs are cast by pulmonary neutrophils, which are recruited to the airways in response to LPS. We also show that SP-D binds to NETs and carbohydrate ligands simultaneously, induces microaggregate formation of *P. aeruginosa*, and increases bacterial trapping. Thus, our results demonstrate that SP-D plays a unique role in neutrophil-mediated host defense and places SP-D as an important innate immune protein that promotes NET function.

Materials and Methods

Reagents

All buffer salts and reagents were obtained from Sigma-Aldrich unless otherwise stated. Human SP-D from bronchoalveolar lavage (BAL) fluid of a patient with alveolar proteinosis was purified as previously described (24, 31–33).

Mice and BAL

Male BALB/c mice between 8 and 12 wk old were obtained from the Toronto Centre for Phenogenomics (Toronto, ON, Canada) in-house breeding facility. For neutrophil recruitment, mice were sedated with a ketamine (150 mg/kg; Bioniche Animal Health, Bellville, ON, Canada)/xylazine (5 mg/kg; Bayer Health Care, Toronto, ON, Canada) mix and were instilled intratracheally with 5 μL PBS from *Escherichia coli* 0111:B4 in 50 μL PBS volume. For the BAL, mice were sacrificed with 0.1 mL Euthanyl (Bimeda-MTC, Cambridge, ON, Canada). BAL was then performed with 1 mL cold HBSS (Invitrogen, Carlsbad, CA) with three washes for each milliliter of lavage to a total of 5 mL. BAL fluid was then centrifuged for 10 min at 400 × g to pellet cells. Cell-free supernatant was analyzed for the presence of DNA by using the Quant-IT PICoGreen dsDNA quantification kit (Invitrogen). Pelleted cells were treated with RBC lysis buffer (0.83% [w/v] NH4Cl, 1.0 mM KHCO3, and 1 mM EDTA) for 2 min at room temperature and centrifuged again for 10 min at 400 × g. Cells were then resuspended in HBSS buffer. Total cell count was performed by manual counting using a hemocytometer, and cytospin preparations were made for differential cell counts. For differential cell count, cells were stained with a Hemacolor histology staining kit (EMD Chemicals, Gibbstown, NJ). Cell types were quantified from randomly taken images of cytospin preparations. At least 100 cells were counted for each condition. The animal care facility’s ethics committee approved all protocols involving mice.

Instillation of *P. aeruginosa*-laden agar beads

*P. aeruginosa*-laden agar beads were prepared by using a previously described protocol (34). For each mouse, 50 μL bead suspension containing 2 × 108 CFU/mL was instilled into the trachea under direct vision. Three days postinfection, BAL was performed, and the lavage fluid was centrifuged at 700 × g to obtain supernatant.

Analysis of NET-derived DNA in vivo

BAL fluid taken from mice treated with either PBS control or LPS (2.5 μg) at 2, 4, 6, or 25 h was analyzed by agarose gel electrophoresis. Each sample was either run without treatment or after treatment with proteinase K (PK) (0.2 mg/mL). To confirm whether DNA was present in the cell-free supernatant, 25-h LPS BAL fluid was treated first with PK only (0.2 mg/ml) for 30 min or PK first, then with DNase (50 μg/mL) and/or RNase (250 μg/mL) for 1 h, followed by PK (0.2 mg/ml) for 30 min at 37°C in the presence of 3 mM CaCl2. The samples were subjected to agarose gel electrophoresis to visualize NET-derived DNA. The image of the agarose gel was then analyzed for pixel intensity using Adobe Photoshop CS3 software (Adobe Systems), and the signals were plotted on the graph of signal intensity versus kilobase.

**SP/D-NET copurification assay**

NET DNA and SP-D were copurified from LPS-treated (overnight) mouse BAL fluid using maltose-agarose beads using a batch method. The beads were mixed with BAL fluid with 10 mM CaCl2 added for overnight at 4°C. Beads were then washed three times with a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, and 10 mM CaCl2. A high-salt buffer containing 20 mM Tris (pH 7.4), 1 M NaCl, and 10 mM CaCl2 (H-TSCa) was used to disassociate DNA from SP-D. To elute SP-D from the maltose agarose beads, a glycin-EDTA buffer (pH 10.5) containing 50 mM glycine, 45 mM NaOH, and 5 mM EDTA (Gly-EDTA) was used. For each sample preparation, half of the sample was treated with H-TSCa first and then treated with the Gly-EDTA solution and vice versa for the other half of the sample to determine whether there was nonspecific binding.

**Western blot**

For Western blotting of SP-D, rabbit anti-mouse SP-D antiserum was used for all Western blots. Rabbit polyclonal Ab to histone H3 (critulline 2 + 8 + 17, ab1507; Abcam, Cambridge, MA) was used for citrullinated histone H3 (Cit-H3) Western blotting. Densitometry on the scanned blots was performed using Adobe Photoshop (Adobe Systems). Mean signal intensity was taken and multiplied by the number of pixels from each band. The fold increase in SP-D protein expression was determined by comparing the control sample versus *P. aeruginosa*-infected BAL fluid sample.

**Liquid chromatography-coupled tandem mass spectrometry**

For mass spectrometry, the Gly-EDTA elution from the SP-D/NET copurification assay of PBS-instilled mice was used. As a negative control, Gly-EDTA eluate from PBS instilled mice was used, and identified peptides were considered positive only if not found in the PBS-instilled negative control BAL fluid. For liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS), the pH of the eluates was adjusted to pH 7 to 8 using 5% NH4HCO3. The samples were then digested with trypsin (Roche Diagnostics, Laval, QC, Canada) overnight at 37°C in a digestion buffer containing 50 mM NH4HCO3. The resulting peptides were separated by nano HPLC 1100 (Agilent Technologies Canada, Mississauga, ON, Canada) and analyzed using an LTQ ion trap mass spectrometer (Thermo Scientific, Mississauga, ON, Canada). The NCBI/Inr database on the Mascot server was used for protein identification.

**Cell culture**

Cells were isolated from BAL fluid with centrifugation at 400 × g and cultured with DMEM with 10 mM HEPES buffer in a 96-well special optics plate (Coming, Lowell, MA). For monoculture assay, adherent alveolar macrophages were removed by a brief incubation to allow for their adhesion, and nonadherent cells floating in the media were then cultured in the 96-well special optics plates. In most studies, NETs were allowed to form for 4 h, unless stated otherwise. To quantify NET formation and follow its kinetics over time, 5 μM Sytox Green (Invitrogen) extracellular DNA dye was added, and total fluorescence was measured using a Gemini EM fluorescence microplate reader ( Molecular Devices, Sunnydale, CA). For DNase assay, 0.4 mg/mL DNase was added to NETs culture to confirm the presence of extracellular DNA-based NETs. For NET clearance assay, cells were cultured in macrophage serum-free medium (Invitrogen).

**Fluorescence imaging**

Samples were fixed with 4% (v/v) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) and stained with Sytox Orange DNA stain (Invitrogen). To immunostain for SP-D, samples were blocked with PBS containing 5% (w/v) BSA for 1 h, washed with PBS, and then incubated with rabbit anti-SP-D Abs directly conjugated with Alexa Fluor 647 (Invitrogen) in PBS containing 1% (w/v) BSA for 1 h. For immunostain for elastase, rabbit anti-elastase Ab (ab21595; Abcam) was used. For cathelicidin stain, rabbit anti-cathelicidin Ab (ab94848; Abcam) was used. Alexa Fluor 555 F(ab')2 fragment of goat anti-rabbit IgG (H+L) (A21340; Invitrogen) was used as secondary Ab for elastase and cathelicidin. Images were taken using the following two microscopes: 1) an LSM510 META Laser Scanning Confocal Microscope using a 63×/1.4 oil immersion objective and operated by LSM510 software (Carl Zeiss Canada, Toronto, ON, Canada); and 2) a Zeiss Axiovert 200 with spinning disk confocal scan head (Carl Zeiss Canada), equipped with a Hamamatsu C9100-13 EM-CCD (Hamamatsu) and a 40×/0.95 water immersion objective and operated by Velocity software (version 5.3.2; PerkinElmer, Waltham, MA). Images taken using the spinning disk confocal microscope were deconvolved by iterative restoration using Velocity (PerkinElmer) with the confidence limit set to 95% and the iteration limit set to 15.

SP-D promotes bacterial trapping by NETs
processed images reached the confidence limit before reaching the iteration limit. Images taken using laser scanning confocal microscopy were exported to Velocity software (PerkinElmer) for image analysis and to adjust contrast. The contrast of the images taken with the spinning disk confocal microscope was also adjusted using Velocity software (PerkinElmer).

Bacterial trapping assay

For the bacterial trapping assays, neutrophils from LPS BAL fluid (6 h) were cultured first for 4 h to allow for the formation of NETs and centrifuged at 300 × g for 5 min. P. aeruginosa was washed three times with HBSS containing 5 mM CaCl2 and resuspended in HSC buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, and 1 mM CaCl2). P. aeruginosa was then inoculated to the culture (multiplicity of infection: 50) in the presence or absence of SP-D (5 µg/ml). Where appropriate, bacterial culture was preincubated with SP-D for 60 min prior to addition to NET culture. The plates were then centrifuged at 700 × g for 10 min and cultured for 30 min prior to fixation for scanning electron microscopy (SEM). For SEM, previously described protocols were used with some modifications (35, 36). NETs culture containing P. aeruginosa ± SP-D were fixed overnight at 4°C with 2% (v/v) paraformaldehyde and 4% (v/v) glutaraldehyde (Electron Microscopy Sciences) with a buffer containing 5 mM HEPES (pH 7.4), 10 mM NaCl, and 1 mM CaCl2. Samples were then postfixed with 0.5% (v/v) OsO4 (Electron Microscopy Sciences) and 1% (v/v) tannic acid (Electron Microscopy Sciences) for 1 h at room temperature and washed with distilled H2O. The samples were dehydrated with increasing concentrations of ethanol (20–100%) and critical point-dried prior to gold coating. Samples were imaged with JEOL JSM-820 or JSM6700 SEM (Jeol).

Statistical analysis

To test for statistical significance, Student t test or one-way ANOVA with Tukey’s multiple comparison test was used where appropriate. A p value was set at 0.05, 0.01, or 0.001 for statistical significance. All statistical analysis was performed using GraphPad Prism statistical analysis software (version 5.0a for Mac OS X; GraphPad).

Results

Airway neutrophils form NETs

To show that neutrophils form NETs in the airways in response to microbial ligands, we first used an LPS-instillation mouse model. We obtained BAL fluid 24 h after intratracheal LPS (5 µg) instillation, isolated the immune cells by low-speed centrifugation, placed them in imagable culture plates, and visualized the DNA using Sytox Orange nucleic acid stain (Invitrogen). Long string-like extracellular DNA structures were visible under fluorescence microscopy (Fig. 1A). These extracellular DNA structures were only seen with the cells isolated from BAL fluid of LPS-treated, but not PBS-instilled, mice (Supplemental Fig. 1). To determine NET formation kinetics, the cultures were maintained for various time periods. After 4 h in culture, a large number of elaborate extracellular DNA structures were visible using Sytox Orange (Invitrogen) (Fig. 1B–G). For the quantitative kinetic analysis (Fig. 1G), NET formation was measured by the increase in the fluorescence intensity (F.I.) in the presence of Sytox Green extracellular DNA dye (5 µM; Invitrogen) and was confirmed by the decrease in F.I. after DNase treatment. Moreover, DNase (0.4 mg/ml) treatment of NET cultures and visual inspection of these cultures by fluorescence microscopy confirmed that these structures were made of DNA (Fig. 1H). We further verified that these structures were NEts by staining for neutrophil elastase (Fig. 1I), which is a well-known NET-associated protein (1, 3, 11, 13). We also identified another neutrophil granule protein, cathelicidin, on the mouse NEts (Fig. 1J). The distribution of cathelicidin on the NEts differed from that of neutrophil elastase, in that cathelicidin was distributed in specific patches as granules, whereas neutrophil elastase was distributed more uniformly on the NEts. Overall, our data illustrate two major points. First, mouse neutrophils recruited to the airways following LPS instillation form DNA-based extracellular structures within 1–4 h. Second, these extracellular structures are in fact NETs because they contain cathelicidin and neutrophil elastases, which are the known components of human NETs (1, 37, 38).

Establishing a 6-d model: concentrations of SP-D and NET DNA in mouse airways follow similar kinetics

Because the kinetics of NET formation in the airways and their relevance to SP-D were unknown, we sought to develop a mouse model to study NET formation in the airways. To do so, we first determined the optimal LPS dose and time point for neutrophil recruitment to the mouse airways (Supplemental Fig. 2). The results showed that 2.5–5 µg LPS for 6–25 h was optimal for neutrophil recruitment to the airways of BALB/c mice. We next followed the population dynamics of alveolar macrophages and neutrophils in the BAL fluid over a 6-d period. At baseline, there were mostly (>95%) alveolar macrophages. By 1 to 2 d post-LPS (5 µg) instillation, the dynamics reversed; there were mostly (>95%) neutrophils present in these airways (Fig. 2A, 2B). By 6 d after the LPS instillation, the population was restored to baseline. Quantifying absolute cell numbers revealed that the neutrophil population reached its peak at 2 d post-LPS instillation with 7.27 ± 0.23 × 106 total cells in BAL fluid from each mouse, and the cell number steadily declined thereafter. After day 5, no neutrophils were detected in the airways. In contrast, the alveolar macrophage population reached its peak at ~5 d post-LPS instillation with 1.95 ± 0.12 × 106 total cells in the BAL fluid from each mouse. There was no change in the number of the alveolar macrophages over the 6-d period in PBS-treated mice (Fig. 2A, 2B).

After carefully characterizing this LPS-induced model of neutrophil inflammation, we next determined whether LPS stimulation also resulted in a change in the amount of extracellular DNA in the airways. To that end, the amount of cell-free soluble DNA present in the supernatant of BAL fluid centrifuged at 400 × g was quantified. The amount of extracellular DNA steadily increased over time and peaked at ~3 d with 9.08 ± 0.98 µg total DNA present in BAL fluid supernatant (Fig. 2C). The pattern of increase in DNA concentration closely followed that of neutrophil population increase, suggesting that this DNA was a product of neutrophils; however, the time point for the DNA peak shifted and was delayed by 1 d (Fig. 2B, 2C). We next determined the SP-D profile over time and found that the change in the soluble SP-D levels followed the same kinetics as that of DNA levels (Fig. 2C, 2D). There was no detectable amount of DNA or any change in SP-D level in PBS-treated mice (data not shown). These results illustrate that, in this LPS model of acute inflammation, neutrophils release extracellular DNA and that changes in SP-D levels correlate with the changes in the amount of extracellular DNA present in the airways.

NETs are present as soluble protein–DNA complexes in the airways

To further characterize the nature of NETs that accumulate in airways in response to LPS instillation, we examined the NEts isolated by the BAL procedure using agarose gel electrophoresis. Large amounts of high m.w. nucleic acids were detected at the 25-h time point (Fig. 3A). PicoGreen DNA quantification assay (Invitrogen) on each of the samples showed that by 25 h, there was a significant increase in DNA concentration in the airways of mice treated with LPS compared with PBS controls (0.463 ± 0.014 versus 0.065 ± 0.001 µg/ml; p < 0.01; Fig. 3B). We also detected the presence of CitH3, a well-described marker of NEts (10, 11), from the BAL fluid of LPS-treated mice (Fig. 3C). PK treatment of the BAL fluid.
FIGURE 1. Neutrophils from mouse airways form NETs after LPS instillation. A, Sytox orange DNA stain (Invitrogen) showing that neutrophils isolated from BAL fluid formed stringlike NETs after 4 h in culture. Note that nuclear DNA of intact cells also stained red. In this experiment, Sytox Orange staining (Invitrogen) was performed after the cells were fixed with 4% (v/v) paraformaldehyde. Scale bar, 50 μm. Time-course analysis for NET formation at 0 min (B), 60 min (C), 120 min (D), 180 min (E), and 240 min (F). By 240 min, long strands of DNA are visible (arrowhead). Scale bars, 50 μm. Images presented above are representative images taken from independent experiments using at least three mice per time point. G, Kinetic analysis of NET formation from 0–240 min as described by F.I. Presence of NETs was confirmed by the decrease in F.I. upon treatment with DNase. DNase was added at 240-min time point, and the F.I. was measured at 30 and 60 min after DNase treatment (270- and 300-min time points). F.I. at 0 min was considered background and subtracted from each time point. H, Extracellular structure is abolished after DNase treatment. Neutrophil culture was treated with or without DNase. Data presented are representative images taken from three independent experiments. Scale bars, 100 μm. I and J, Colocalization of elastase (I) and cathelicidin (J) to mouse pulmonary NETs. NETs were allowed to form for 4 h, fixed, and stained for elastase or cathelicidin. Confocal immunofluorescence images were taken, and elastase and cathelicidin were pseudocolored red and purple, respectively. Lower panels represent magnified images of region in the white box in the upper panels. Images shown are representative of images taken from three independent experiments. Scale bars, 15 μm.
samples resulted in a shift of high m.w. bands predominantly to ~250–500 bp, suggesting that small NET-DNA fragments were held together by proteins in vivo (Fig. 3A,3D). To determine whether the nucleic acid bands on the agarose gel were in fact DNA, the samples were treated with RNase, DNase, or RNase plus DNase and subsequently treated with PK. These experiments confirmed that the visible bands indeed contained primarily DNA (Fig. 3C). A signal intensity plot of the above agarose gel image confirmed that DNA was the primary form of nucleic acid present in these BAL fluid samples (Fig. 3E). These results show that the soluble DNA is present as DNA–protein complexes. Taken collectively (Fig. 3), these results demonstrate that pulmonary neutrophils release DNA fragments in response to LPS in vivo.

**SP-D binds simultaneously to carbohydrate ligands and NET DNA fragments**

Because we were able to show that SP-D and NET-DNA fragments were present in the airways (Figs. 2, 3), we next sought to determine whether SP-D bound to these NET fragments in vivo. To do so, we set out to copurify these two molecules by maltose-agarose affinity

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**FIGURE 2.** Airway NET formation kinetics. A, Time-course analysis of percent neutrophils and alveolar macrophages in the LPS (5 μg) instillation model over 6-d time period in mice instilled with LPS and PBS as negative control. Data are presented as mean ± SEM from three to five mice in each condition. B, Absolute cell count for neutrophils and alveolar macrophages present in BAL fluid described in A. C, Amount of extracellular DNA and relative increases in the amounts of SP-D. For SP-D, densitometry analysis for pixel count was performed on Western blot (D), and pixel counts were normalized for day 0 (baseline) as 1. Data are presented as mean ± SEM from three to five mice for DNA quantification and three mice for SP-D densitometry in each condition. D, Western blot analysis for SP-D. Data shown are representative of one of the three independent experiments. No DNA was detected (C), and no change in SP-D level was found (D) in PBS-treated control mice (data not shown).

**FIGURE 3.** Presence of NET-derived DNA–protein complex in vivo. A, A faint high m.w. band appears on an agarose gel at 4 and 6 h after LPS instillation. Control mice were treated with PBS (labeled PBS) for 6 h. By 25 h, the high m.w. band is clearly visible. With PK treatment (lanes with + sign), the high m.w. band shifts to a lower m.w., suggesting the presence of a complex that includes nucleic acid and protein (n = 4 mice). B, Compared to PBS instillation, LPS significantly increases the amount of extracellular DNA in the mouse airways (p < 0.01). Data are presented as mean ± SEM from three mice for PBS instillation and five mice for LPS instillation. C, Western blot analysis revealed the presence of CitH3 in the BAL fluid supernatant of LPS- but not PBS-treated mice. D, BAL fluid samples from LPS-instilled mice (25 h) were treated with nucleases followed by PK. DNase treatment confirmed that the visible bands are mostly DNA. RNase treatment removed some of the higher m.w. smear, suggesting the presence of RNA in these samples. Image of the gel shown is a representative of four independent experiments. E, A representative density plot showing signal intensity of the agarose gel in D. The plot shown is representative of four independent experiments. Student t test, *p < 0.01.
purification followed by differential elution (Fig. 4A). We captured SP-D and SP-D–NET fragment complexes present in the cell-free BAL supernatant (25 h post-PBS or LPS instillation) by maltose agarose affinity beads, washed away unbound components, and selectively eluted either DNA or SP-D. High-salt buffer (H-TSCa) and EDTA buffer (Gly-EDTA) were used for the elution of DNA and SP-D, respectively. These buffers were chosen because SP-D binds DNA mainly via charge interactions (24), whereas it binds carbohydrate ligands in a calcium-dependent manner (39). In this copurification assay, treating the samples with high-salt buffer first and then with EDTA buffer should yield DNA and SP-D in a stepwise manner (Fig. 4A, Method A). Conversely, treating the samples with Gly-EDTA buffer first and then with H-TSCa should coelute SP-D and DNA in the EDTA eluate because SP-D and DNA should remain bound to each other in the presence of EDTA (24) (Fig. 4A, Method B).

No DNA was detectable in PBS-instilled control BAL samples, and only SP-D was eluted by both methods in these PBS-instilled samples (Fig. 4B, 4C). In the BAL isolated from LPS-instilled mice, DNA was eluted when the beads were washed first with H-TSCa buffer; SP-D was subsequently eluted with Gly-EDTA buffer (Fig. 4A, Method A, 4B, 4C). Conversely, DNA coeluted with SP-D when the beads were washed with Gly-EDTA buffer first and then with H-TSCa buffer (Fig. 4A, Method B, 4B, 4C). Quantifying the amount of DNA by PicoGreen assay (Invitrogen) confirmed that a significant amount of DNA was present in the BAL isolated from the mice instilled with LPS and that the DNA was bound to SP-D and eluted at expected washes ($p < 0.01$ compared with PBS-instilled control; Fig. 4D). Taken together (Fig. 4), these results indicate that SP-D binds DNA in vivo and that the same SP-D can also bind carbohydrate targets while interacting with DNA.

To confirm that these were NET DNA fragments (Fig. 3), we analyzed the proteins present in Gly-EDTA eluate using LC-MS/MS, which identified the presence of histones, neutrophilic granule protein, cathelicidin, and SP-D (Supplemental Table I). Cathelicidin has previously been shown to associate with extracellular traps (38, 40). These proteins, therefore, would not be present in the DNA originating from sources other than NETs confirming that the DNA fragments are indeed NET DNA fragments. These mass spectrometry data further confirmed that SP-D copurifies with NET-derived DNA and that SP-D binds NETs in vivo.

**SP-D binds NETs**

We have previously shown that SP-D binds DNA in vitro (24). Building on our previous study (24) and the data obtained from the copurification experiments (Fig. 4), we sought to directly show that SP-D binds to NETs. We isolated neutrophils from the BAL fluid of LPS-instilled mice (24 h, 5 µg LPS), allowed these neutrophils to form NETs for 3 h, incubated the culture for another 1 h in the presence of SP-D (5 µg/ml), and analyzed the specimen by fluorescence microscopy. Sytox orange stain (Invitrogen) for DNA and immunolabeling for SP-D revealed that SP-D did bind along the DNA strands of NETs (Fig. 5, arrows). Furthermore, SP-D also bound to some of the neutrophils (Fig. 5, arrowheads). Therefore, these data demonstrate that SP-D not only binds to neutrophils but also interacts with the NETs.

**DNA and SP-D levels increase in the airways of mice infected with *P. aeruginosa***

We next tested whether the responses observed in our LPS model can be validated with a *P. aeruginosa* model of pulmonary infection. To test this, a *P. aeruginosa*-laden agar bead instillation model of chronic lung infection (41–43) was used. Similar to the LPS model (Figs. 2, 3), SP-D concentration increased in the BAL fluid 3 d after instillation of *P. aeruginosa*-laden agar beads.

**FIGURE 4.** SP-D binds to NET-derived DNA in vivo while retaining the ability to bind carbohydrate ligands. BAL was performed on mice at 25 h after LPS instillation. A. A general scheme for SP-D–DNA affinity copurification from BAL fluid. Gly-EDTA buffer was used to elute SP-D off from the maltose-agarose affinity beads, and a high-salt (H-TSCa) buffer was used to elute DNA off SP-D. In Method A, H-TSCa wash was performed first followed by Gly-EDTA wash. In Method B, Gly-EDTA wash was performed first followed by H-TSCa wash. The affinity beads were washed with H-TSCa buffer to determine nonspecific interaction between DNA and the beads. Western blots (B) and agarose gels (C) show stepwise elutions of SP-D and DNA. In stepwise elution (Method A), DNA eluted first with H-TSCa buffer and SP-D subsequently eluted with Gly-EDTA buffer. In the coelution (Method B), SP-D and DNA coeluted with Gly-EDTA buffer, whereas neither SP-D or DNA eluted with a subsequent wash with H-TSCa buffer. D, PicoGreen assay (Invitrogen) shows that a majority of DNA was bound to SP-D. Data are presented as mean ± SEM from four independent experiments. Student t test, *$p < 0.05$, **$p < 0.01$. FT, flow through.
Furthermore, we also detected the presence of CitH3 in *P. aeruginosa* bead-instilled mouse BAL fluids (Fig. 6A). We then determined whether the amount of soluble DNA was also increased in these mouse airways by agarose gel electrophoresis. Along with the increase in SP-D (Fig. 6B, top panel; *p*, 0.01), a large amount of soluble DNA was present during *P. aeruginosa* infection (Fig. 6B, middle and bottom panels). Similar to the LPS acute model, this high m.w. DNA (Fig. 6B, middle panel) shifted to become a lower m.w. species following PK treatment (Fig. 6B, lower panel). Quantifying DNA by PicoGreen assay (Invitrogen) confirmed that increased amounts of DNA were present in the *P. aeruginosa*-infected BAL fluid (2.97 ± 0.19 μg DNA) compared with the control BAL (0.24 ± 0.02 μg DNA; *p* < 0.001; Fig. 6D). *P. aeruginosa* has previously been shown to induce NET formation in human neutrophils (44, 45). These results suggest that NETs are also formed in response to *P. aeruginosa* infection ex vivo and in vivo.

**NETs trap bacteria that are agglutinated by SP-D**

The effect of SP-D-mediated bacterial agglutination in NET-mediated bacterial trapping is unknown. Because SP-D binds to NETs as well as microbes, we next asked whether SP-D could alter bacterial trapping by NETs. To answer this question, we first tested whether SP-D can bind to our laboratory strain of *P. aeruginosa*, mPAO1 (46). We incubated *P. aeruginosa* or *E. coli* with SP-D (5 μg/ml) for various time periods and monitored the changes in absorbance at 600 nm. Although the absorbance of SP-D and *E. coli* together dropped sharply over time, *P. aeruginosa* incubated with SP-D had a slower kinetic, and the absorbance (Fig. 6A; *p* < 0.01). Furthermore, we also detected the presence of CitH3 in *P. aeruginosa* bead-instilled mouse BAL fluids (Fig. 6A). We then determined whether the amount of soluble DNA was also increased in these mouse airways by agarose gel electrophoresis. Along with the increase in SP-D (Fig. 6B, top panel; *p* < 0.01), a large amount of soluble DNA was present during *P. aeruginosa* infection (Fig. 6B, middle and bottom panels). Similar to the LPS acute model, this high m.w. DNA (Fig. 6B, middle panel) shifted to become a lower m.w. species following PK treatment (Fig. 6B, lower panel). Quantifying DNA by PicoGreen assay (Invitrogen) confirmed that increased amounts of DNA were present in the *P. aeruginosa*-infected BAL fluid (2.97 ± 0.19 μg DNA) compared with the control BAL (0.24 ± 0.02 μg DNA; *p* < 0.001; Fig. 6D). *P. aeruginosa* has previously been shown to induce NET formation in human neutrophils (44, 45). These results suggest that NETs are also formed in response to *P. aeruginosa* infection ex vivo and in vivo.

**FIGURE 5.** SP-D binds to NETs. NETs were allowed to form for 3 h. SP-D was then added, and the culture was incubated at 37°C for 1 h prior to fixation. Immunofluorescence imaging reveals that SP-D (blue) not only binds to dead neutrophils (arrowheads), but also decorates the NET DNA (arrows). Note that nuclear DNA stained with Sytox Orange (Invitrogen) because the staining was performed after cells were fixed with 4% (v/v) paraformaldehyde. Images shown are representative of three independent experiments. Scale bars, 10 μm.

**FIGURE 6.** SP-D and DNA levels are increased in mice infected with *P. aeruginosa*. BAL fluid was collected from mice 3 d after instillation with *P. aeruginosa*-laden agar beads. A, Western blot shows that there is an increase in SP-D level (blot and densitometry; one-way ANOVA, **p** < 0.01), and there is a presence of CitH3 only when *P. aeruginosa*-laden beads are instilled. B, Top panel, Western blot shows that SP-D level increases postinfection with *P. aeruginosa* (*n* = 4 per group). B, Middle and bottom panels, Agarose gel shows that a high m.w. DNA band (top panel), similar to the one seen with LPS instillation (Fig. 2), is present in the BAL fluid after *P. aeruginosa* infection and shifts to a lower m.w. band after treatment of BAL fluid with PK (bottom panel). C, A representative signal density plot of the agarose gel for BAL fluid (BALF) and PK-treated BAL fluid (+PK) shown in B (middle and bottom panels). The plot is a representative of the four samples shown in B, D. Densitometry analysis was performed on the blot shown in A (top panel) to determine the fold increase in SP-D protein level and PicoGreen assay (Invitrogen) for quantifying the concentration of soluble DNA in BAL fluid. In response to *P. aeruginosa* infection, SP-D level significantly increased 2-fold. Data are presented as mean ± SEM from four mice in each condition. The amount of DNA was significantly higher than the negative control sample. Student *t* test, **p** < 0.01, ***p*** < 0.001.
dropped gradually over time (Supplemental Fig. 3A, left panel). Visual inspection of the cuvettes used for the agglutination assay showed that although *E. coli* plus SP-D culture became very clear, *P. aeruginosa* plus SP-D culture remained slightly turbid (Supplemental Fig. 3A, right panel). At the end of a 1-h incubation period, we separated the bacteria and soluble proteins by centrifugation and conducted Western blot analysis of each fraction. SP-D was found in the bacterial pellet fraction, indicating that SP-D was binding to the bacteria (Fig. 7A). To determine the nature of SP-D–bacterial complexes, we examined a 1-h culture by fluorescence imaging. These images revealed that SP-D microagglutinated *P. aeruginosa* (Fig. 7B, 7C).

Finally, we asked whether SP-D was able to alter the mode of bacterial trapping by NETs. To determine this, the airway neutrophils were first allowed to form NETs for 4 h using a similar protocol to the SP-D binding assay (Fig. 5A). *P. aeruginosa* was then incubated in the presence or absence of SP-D (5 μg/ml) for 1 h, and this bacterial culture was added to NETs and incubated for 30 min. SEM analyses of these specimens revealed that elaborate NETs were seen when neutrophils were cultured for 4 h (Supplemental Fig. 3B–F). Conversely, no NET was observed in samples with live neutrophils fixed shortly after seeding in culture (Supplemental Fig. 3B). Similar to our fluorescence microscopy (Fig. 7B, 7C), *P. aeruginosa* became microagglutinated in the presence of SP-D (Fig. 7D, 7E). Furthermore, SEM imaging made it apparent that nonagglutinated *P. aeruginosa* were trapped by NETs, but in a dispersed manner (Fig. 7F). The presence of SP-D microagglutinated *P. aeruginosa* and promoted the binding of these microaggregates to NETs (Fig. 7G). These results suggest that SP-D alters the mode of bacterial trapping by NETs.

**Discussion**

In this study, we have established a model to study NET functions in mouse airways, and characterization of this model revealed the following four findings: 1) the NET formation profile peaks 1 d after neutrophil influx peaks; 2) the concentration profiles of NET-DNA and SP-D in the airways follow similar kinetics; 3) SP-D binds to NETs in vivo; and 4) SP-D simultaneously recognizes NETs and carbohydrate ligands. Most importantly, we tested the ability for SP-D to enhance NET function using our LPS model of airway NET formation and demonstrated the following: 1) SP-D induces microaggregate formation of *P. aeruginosa*; and 2) SP-D facilitates the interaction of these immune complexes with NETs. Taken together, our results lead us to propose a model for SP-D–mediated enhancement of NET function and clearance (Fig. 7H). Our findings provide a biological relevance to our previous finding that SP-D binds DNA (24) and places SP-D as an important protein that promotes bacterial trapping function of the NETs.

Several recent studies show that NET formation is a key aspect of neutrophil-mediated extracellular microbial trapping (4, 5, 47); however, NET formation in the airways and the factors involved in promoting NET functions have not been systematically investigated. In this study, we characterized the kinetics of NET formation and the relevance of SP-D to NET function in the mouse airways. We present in this paper two models of pulmonary infection and inflammation: bacterial infection and LPS-mediated infection-related inflammation. The use of two biological targets of SP-D (LPS and *P. aeruginosa*) strengthened the relevance of our findings to acute pneumonia. Our LPS model is ideal to study NET formation and NET clearance with limited confounding effects compared with an infection by living bacteria. This model shows that LPS is sufficient to induce neutrophils to generate NET-DNA and SP-D, NETs trap a few dispersed bacteria (*P. aeruginosa*), but in the presence of SP-D, NETs trap the bacterial aggregates (*E. coli*). Images shown are representative of three independent experiments. Scale bar, 5 μm. SEM of *P. aeruginosa* in the absence (D) or presence of SP-D (E). The images show that SP-D microagglutinates *P. aeruginosa*. Images shown are representative of three independent experiments. Scale bar, 5 μm. F and G. SEM showing that SP-D microagglutinates bacteria and facilitates the interaction of these aggregates to NETs. In the absence of SP-D, NETs trap a few dispersed bacteria (F), but in the presence of SP-D, NETs trap the bacterial aggregates (G). Images shown are representative of four independent experiments. Scale bar, 5 μm. H, NETs and NET fragments produced in response to bacterial infection can trap bacteria. SP-D can bind to NET-DNA and its bacterial target. In addition, NETs can trap the bacteria that have been agglutinated by SP-D.
NETs in the airways (Figs. 1–3), and the inflammatory environment created by LPS in the lungs alone is enough to cause NETosis. This may suggest that direct association between bacteria and neutrophils is not necessary for NET formation. The results from the LPS experiments also imply that there may be a potential role for NETs during sterile inflammation. In fact, it has been shown that non–infection-related inflammation such as small vessel vasculitis also leads to NET formation (38).

Pulmonary NETs that we describe in this study are stringlike structures when formed ex vivo (Figs. 1, 5A), which are different from the meshlike morphology described for human NETs induced with pharmacological agents such as PMA and glucose oxidase (14) (Supplemental Fig. 3). Furthermore, results obtained from agarose gel electrophoresis of BAL fluid supernatant suggest that pulmonary NETs exist as short fragments (Figs. 3, 6). Our studies further show that these pulmonary NETs contain neutrophil elastase and cathelicidin (Fig. 1). Neutrophil elastase functions as an antimicrobial protein (48), and a recent finding suggests that it is also essential for NET formation (13). Cathelicidin is an antimicrobial neutrophil defense peptide (49). Cathelicidins found in the mouse NETs (Fig. 1) are likely to participate in the extracellular microbial killing as well. There is a growing list of antimicrobial proteins that are found on NETs (3) including MPO (1), peptidoglycan recognition protein S (50), and calprotectin (3). SP-D also has direct antimicrobial activity (51), and our finding that SP-D binds to NETs (Figs. 2–4) demonstrates that SP-D is also an integral component of the antimicrobial defense mechanism of NETs formed in the lung mucosa.

SP-D is also known to recognize various microbial pathogens by binding to the carbohydrate moieties on bacterial surfaces, including that of P. aeruginosa, and enhances phagocytic clearance of these microbes by macrophages and neutrophils (25, 52–55). SP-D is known to bind various strains of P. aeruginosa but can only agglutinate certain strains of this pathogen (30, 56). In this study, we show that SP-D binds and agglutinates the P. aeruginosa laboratory strain mPAO1 (Fig. 7). Our data also show that SP-D binds to both carbohydrate ligands and NETs simultaneously (Fig. 4). This is an ideal setup for SP-D to have a potent biological function. Our copurification study suggests that SP-D is bound to NET fragments via ionic interaction because it was disrupted with high-salt–containing buffer (Fig. 4). Our previous studies showed that SP-D binds DNA via ionic interaction, and the collagen-like region is involved in these bindings (24). This provides a plausible explanation for the ability of SP-D to bind both carbohydrate and NET at the same time through NET DNA. A recent report, however, showed that surface-bound MPO acts as a ligand for SP-D in apoptotic neutrophils (57). Therefore, SP-D could also bind to NETs through other NET components such as MPO.

We found that SP-D agglutinates P. aeruginosa mPAO1 and interconnects the bacterial agglutinate to NETs (Fig. 7). Consistent with this notion, we found that the increase in the concentrations of SP-D and NETs following LPS instillation or P. aeruginosa infection correlated with one another (Figs. 2, 6). Our data for the increase in the amounts of SP-D and NETs in response to infection/infection related inflammation are correlative. However, these findings do demonstrate that NETs and SP-D could play important roles in antimicrobial defense during airway infection.

Collectively, these results suggest that a clear understanding in NET biology, and the importance of SP-D in regulating NET functions should open up the possibilities for new therapeutic targets for effectively treating lung infections. For example, patients with cystic fibrosis (CF) often suffer from chronic neutrophilic inflammation and infection with P. aeruginosa. Notably, pulmonary infection is the leading cause of morbidity and mortality in these patients (58). A recent study shows that NETs are present in the airways of patients with CF (15), and P. aeruginosa can induce NET formation in neutrophils from both normal human and from patients with CF. We also confirmed the ability of P. aeruginosa to induce NET formation by neutrophils isolated from patients with CF (data not shown). Previous studies have shown that patients with CF lack functional SP-D (59, 60). Our finding (Figs. 1–7) shows that SP-D can enhance P. aeruginosa trapping by NETs; hence, SP-D can participate in multiple ways to promote the maintenance of healthy lungs in these patients. A previous study showed that NETs are formed primarily in the pulmonary capillaries during sepsis (47), whereas other microscopy-based studies showed that NETs were also present in the large airways (3). Our study shows that NETs are present as short fragments and are able to interact with SP-D in the airways. Thus, it is reasonable to consider that SP-D and NETs are integral parts of mucosal innate immune defense system.

Acknowledgments

We thank the following for technical assistance: Yew Meng Heng of the electron microscopy facility of the Pathology Laboratory Services at Sick-Kids for SEM studies, Hailu Huang for P. aeruginosa-infected mouse model studies, Hayley Craig-Barnes for LPS mouse model studies, Li Zhang of Advanced Protein Technology Centre at SickKids for LC-MS/MS analysis, and the Toronto Centre for Phenogenomics and Lab Animal Services staff for maintaining mouse colonies.

Disclosures

The authors have no financial conflicts of interest.

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Supplemental data

**Figure S1. BAL cells isolated from LPS-instilled mice form NETs.** Co-culture of AMφ and neutrophils isolated from the BAL fluids of PBS or LPS treated mice. DNA was stained with Sytox Orange nucleic acid stain. NETs (arrow) appeared only after LPS instillation into the airways. Alexa 488 Phalloidin signal was present on intact AMφ with round nuclei; however, the Phalloidin signal was absent in NETs producing cells, suggesting the break down of cell membrane. Note that nuclear DNA in intact cells is also visible since the staining was performed after cells were fixed with 4% (v/v) paraformaldehyde. Bar: 50 μm
Figure S2. Determination of optimal LPS dose and time. (A) Dose response to pulmonary instillation of LPS. Total number of cells present in BAL fluid 6 h after the instillation of LPS. Data are presented as mean ± SEM from two mice in each condition. (B) Time course analysis for total cell present in BAL fluid after LPS instillation (2.5 μg). Significant numbers of cells were present 25 h after LPS instillation compared to baseline (t = 0). Data are presented as mean ± SEM from two mice in each condition. One-Way ANOVA; **: p < 0.01. (C) Differential cell count of cells present in BAL fluid at different times after LPS instillation (2.5 μg). By 6 and 25 h after LPS instillation, the number of neutrophils present in the airways is significantly higher than at 2 h, and the number of alveolar macrophages in the airways is significantly lower than at 2 h. Data are presented as mean ± SEM from two mice in each condition. Two-Way ANOVA; ***: p < 0.001. (D) Differential cell count of cells present in BAL fluid at 25 h after 2.5 or 5 μg of SP-D/mouse. Data are presented as mean ± SEM from three mice in each condition. Two-Way ANOVA; ***: p < 0.001.
Figure S3. Determination of SP-D mediated *P. aeruginosa* agglutination, and SEM of live neutrophils and NETs. (*A*, left panel) Time course analysis of change in optical density (A$_{600}$) of *E. coli* and *P. aeruginosa* cultures in the presence and absence of SP-D. Bacteria were incubated in the presence or absence of SP-D in microcuvettes. If SP-D can agglutinate the bacteria into large clumps, the agglutination, or “clumping” will result in decrease in the optical density of the sample. Data are presented as mean ± SEM of 2 independent experiments for *E. coli* and 3 independent experiments for *P. aeruginosa*. The absorbance in *E. coli* culture dropped sharply in the presence of SP-D (0.5 μg/ml) by 15 min, while that of *P. aeruginosa* in the presence of SP-D (0.5 μg/ml) dropped steadily over the 90 min. (*A*, right panel) Representative images of each cuvette at the end of the experiment. While *E. coli* + SP-D became clear, *P. aeruginosa* + SP-D was still slightly turbid. Two-Way ANOVA showed a significant difference between all samples by 90 min (p < 0.001). (*B – F*) Scanning electron micrograph of live neutrophil (*B*) and mouse pulmonary neutrophil extracellular traps (*C – F*). Image shown are representative of four independent experiments. *C – F*, Bar, 5 μm.
Table S1. Summary of LC-MS/MS data

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