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A SerpinB1 Regulatory Mechanism Is Essential for Restricting Neutrophil Extracellular Trap Generation

Kalamo Farley,*† J. Michael Stolley,* Picheng Zhao,*† Jessica Cooley,* and Eileen Remold-O’Donnell*†‡

NETosis (neutrophil extracellular trap [NET] generation), a programmed death pathway initiated in mature neutrophils by pathogens and inflammatory mediators, can be a protective process that sequesters microbes and prevents spread of infection, but it can also be a pathological process that causes inflammation and serious tissue injury. Little is known about the regulatory mechanism. Previously, we demonstrated that serpinb1-deficient mice are highly susceptible to pulmonary bacterial and viral infections due to inflammation and tissue injury associated with increased neutrophilic death. In this study, we used in vitro and in vivo approaches to investigate whether SerpinB1 regulates NETosis. We found that serpinb1-deficient bone marrow and lung neutrophils are hypersusceptible to NETosis induced by multiple mediators in both an NADPH-dependent and -independent manner, indicating a deeply rooted regulatory role in NETosis. This role is further supported by increased nuclear expansion (representing chromatin decondensation) of PMA-treated serpinb1-deficient neutrophils compared with wild-type, by migration of SerpinB1 from the cytoplasm to the nucleus of human neutrophils that is coincident with or preceding early conversion of lobulated (segmented) nuclei to delobulated (spherical) morphology, as well as by the finding that exogenous human recombinant SerpinB1 abrogates NET production. NETosis of serpinb1-deficient neutrophils is also increased in vivo during Pseudomonas aeruginosa lung infection. The findings identify a previously unrecognized regulatory mechanism involving SerpinB1 that restricts the production of NETs. The Journal of Immunology. 2012, 189: 000–000.

SerpinB1 (previously called MNEI) is an ancient protein that is abundant in neutrophil cytoplast. Its best-known function is inhibition of the neutrophil serine proteases, elastase, cathepsin G, and proteinase-3, major agents of human inflammatory injury (1, 2). Mice with germline ablation of serpinb1 are viable and are grossly normal. On infection with bacterial and viral pathogens, their immune responses are initially normal, but they transition over time to tissue injury, protracted inflammation, and early and excess mortality (2, 3). While studying the highly pro-inflammatory IL-17-skewed lung pathology of influenza virus-infected serpinb1−/− mice, we noted excess death of infiltrating leukocytes (3). The magnitude of this pathology raised the question of whether serpinb1−/− neutrophils, when present in an inflammatory environment, preferentially die by a mechanism other that apoptosis or spontaneous necrosis. This led us to examine the possible link between SerpinB1 and NETosis (neutrophil extracellular trap [NET] generation).

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Abbreviations used in this article: AAT, α₁-antitrypsin; BALF, clarified bronchoalveolar lavage fluid; DFP, diisopropyl fluorophosphate; DPI, diphenylene iodonium; FOV, field of view; KO, knockout; LBR, lamin B receptor; MPO, myeloperoxidase, NET, neutrophil extracellular trap; PAD-4, peptidylarginine deiminase-4; PAF, platelet activating factor; ROS, reactive oxygen species; rSerpinB1, human recombinant SerpinB1; WT, wild-type; Z-VAD-fmk, carbenoxoxy-Val-Ala-Asp-fluoromethylketone.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12 $16.00 NETosis is a programmed death pathway involving complex intracellular signaling induced in terminally differentiated neutrophils on activation by pathogens and inflammatory mediators and in vitro by PMA (4). Late events of NETosis include chromatin decondensation, nuclear expansion, and extrusion of linearized DNA decorated with elastase, cathepsin G, and proteinase-3 (the neutrophil serine proteases) and other antimicrobial proteins, including histones and myeloperoxidase (MPO). NETs can ensnare and kill pathogens; at the time of its discovery, NETosis was considered protective, a final act by dying neutrophils to prevent or delay systemic infection. However, mounting evidence shows that NETs, or more specifically excess NETs, can inflict serious local inflammatory damage, such as endothelial cell death, as seen in a wide range of diseases, including cystic fibrosis, thrombosis, and, most recently, fatal murine influenza (5–7). Excess NETs also exacerbate the pathology of autoinflammatory and autoimmune diseases: rheumatoid arthritis, systemic lupus erythematosus, and small-vessel vasculitis (8). Because NETs consist of DNA and RNA decorated with self-proteins, they can be targeted by self-Ab and endocytosed as immune complexes by plasmacytoid dendritic cells to signal via TLR-7/9, as well as induce type I IFN responses that propagate autoimmunity (9, 10).

The best-characterized NETosis model is activation of isolated neutrophils by PMA. This is an active process that is mechanically distinct from apoptosis and necrosis; it requires reactive oxygen species (ROS) generated by NADPH oxidase, the protease elastase but not caspases, and it includes decondensation of nuclear chromatin (11–13). NET generation also requires peptidylarginine deiminase-4 (PAD-4), a nuclear enzyme that deaminates histone tail arginine residues (i.e., converts positively charged arginines to uncharged citrullines and, thereby, mediates chromatin decondensation) (14–16). Currently, there is only minimal understanding of how NETosis is regulated.

To examine a role for SerpinB1 in NETosis, we studied lung and bone marrow neutrophils of serpinb1−/− and wild-type (WT) mice
stimulated in vitro with PMA and physiological mediators. We evaluated NET production in vivo in infection, and we examined the effect of exogenous human recombinant SerpinB1 (rSerpinB1) on NETosis. The findings, some of which were reported in an abstract (17), identify SerpinB1 as a nonredundant regulator of NETosis. The findings indicate that SerpinB1, which migrates to the nucleus early in NETosis, functions downstream of NADPH oxidase and ROS to regulate NETosis by restricting chromatin decondensation.

Materials and Methods

Reagents
rSerpinB1 was expressed in insect cells, purified, and stored in aliquots (2 mg/ml) in PBS at −80°C (18).

Mice
Serpinb1-deficient mice (serpinb1−/−) generated on a 129Sv/SvEvTac (129S6) background were studied; alternatively, where indicated, serpinb1+/− mice were backcrossed to C57BL/6 for 10 generations (2). WT 129S6 and C57BL/6 mice from Taconic Labs or The Jackson Laboratory, respectively, were maintained with serpinb1+/− mice in the animal facility of the Immune Disease Institute or Boston Children’s Hospital for at least several weeks or were bred in-house. Animal studies were approved by the Institutional Animal Care and Use Committee of the Immune Disease Institute and/or Boston Children’s Hospital.

Neutrophil isolation
Lung neutrophils for in vitro study of NET production were recruited by intranasal delivery of 30 µg LPS (Pseudomonas aeruginosa) (Sigma-Aldrich, St. Louis, MO). The mice were sacrificed 24 h later, and the lungs were lavaged with PBS. The cells, which were pelleted, suspended in RPMI 1640, and counted using a Hemavet 950FS (Drew Scientific, Waterbury, CT), were ≥90% neutrophils by flow cytometry. Following an established protocol for isolating morphologically mature bone marrow neutrophils, cells were flushed from femurs and fractionated by Percoll gradient (78, 69, 52%) centrifugation (19). Washed interface cells were 80–90% neutrophils.

Neutrophils were purified from whole blood of healthy donors obtained with informed written consent and approval of the Institutional Review Board of the Immune Disease Institute. The blood was collected in EDTA-coated vacutainers (BD Biosciences, San Diego, CA) and purified (>95% neutrophils) as described (4).

Production of NETs
Murine lung neutrophils (1–2 × 10^6 in 200 µl RPMI 1640) were allowed to adhere to 96-well culture plates coated with 0.001% poly-L-lysine for 1 h at 37°C. PMA (100 nM) was added, and cells were cultured for 12 h (20). Bone marrow neutrophils (2.5 × 10^6 in 200 µl) in RPMI 1640 with 10 mM HEPES and 50 µM 2-ME were allowed to adhere to eight-well (uncoated) glass chamber slides (Thermo Scientific, Waltham, MA) for 1 h at 37°C. The following activating agents were added: PMA (50 nM) for 4 h; platelet activating factor (PAF; 10 nM; Sigma-Aldrich) for 30 min; MIP-2 (10 nM; R&D Systems, Minneapolis, MN) for 45 min; MIP-2 (50 nM; P. aeruginosa) (10 µg/ml) for 4 h. Human neutrophils (5 × 10^6 in 200 µl) in RPMI 1640 with 10 mM HEPES, 50 µM 2-ME, and 2% FCS (heated at 70°C for 30 min to inactivate DNase) (21) were allowed to adhere to eight-well glass chamber slides for 1 h at 37°C and were stimulated with 50 nM PMA for 4 h or as indicated. When neutrophils were treated with diphénylene iodonium (DPI; 10 µM), carbobenzoxy-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) (50 µM), aminooxybenzene sulfonylfluoride (PMSF; 2 mM), diisopropyl fluorophosphate (DFP; 2 mM), or rSerpinB1 (10 µg/ml), the agents were added at the beginning of the 1-h preincubation/adherence step, unless otherwise indicated.

Fluorescence microscopy
To quantify NETs, the cells at the termination of NET induction were washed once with warm PBS, and Sytox orange or Sytox green (0.1 µM) was added. Where indicated, the cells were stained prior to Sytox addition with Alexa Fluor 488-labeled Gr-1 Ab for 30 min at ambient temperature. The unfixed NETs were imaged with a fluorescence microscope, Zeiss Axiovert (Carl Zeiss, Thornwood, NY), or Nikon Eclipse TS100 (Nikon Instruments, Melville NY), and nonoverlapping random images were obtained with a 20× and a 40× objective. NETs were manually identified on digitized images as Sytox-positive structures emanating from cells with an overall length at least twice as long as the cell diameter and were counted for at least three fields of view (FOV) per variable using ImageJ software (National Institutes of Health, Bethesda, MD). Sytox-positive cells were similarly counted. Total cells were quantified by analysis of bright field images or Gr-1–stained images using ImageJ software. Results are expressed as the percentage of NETs/total number of cells. Based on an independent blinded enumeration of WT and serpinb1−/− NETs and Sytox-positive cells by a second evaluator, the intra-assay coefficient of variability was <10%.

For Ab staining, cytopins and slides of neutrophils and NETs were fixed with paraformaldehyde (4% for cell staining, 2% for NET staining); treated with 0.5% Triton-X for 10 min (this step was omitted for staining of NETs); blocked with 2% normal goat serum in PBS-0.1% Tween-20 for 1 h; stained with 1 µg/ml primary rabbit Abs to human neutrophil elastase (Calbiochem, Rockland, MA; Abcam, Cambridge, MA), human SerpinB1 (in-house), or guinea pig Abs to lamin B receptor (LBR) amino acids 1–228 (provided by Monika Zwerger, German Cancer Research Center, Heidelberg, Germany) for 1 h; washed; and incubated for 1 h with 2 µg/ml secondary Abs (Invitrogen). For MPO, cells were stained with FITC-labeled anti-mouse MPO (Hycult Biotech, Plymouth Meeting, PA). The slides were washed and mounted with DAPI-labeled mounting medium (Vector Laboratories, Burlingame, CA) and evaluated by fluorescent microscopy on a Zeiss Axiovert microscope with Zeiss Axiovision LE software or an Olympus Fluoview 1000 confocal microscope (Olympus America, Center Valley, PA) with Olympus software.

DNA fluorimetry assays for NETs
NETs were induced in lung neutrophils in 96-well black plates (Thermo Fisher Scientific). Sytox green (5 µM; Invitrogen) was added, and the fluorescence was detected (485 nm excitation, 535 nm emission) by a Fluoroskan Ascent microplate fluorometer (Thermo Labsystems, Helsinki, Finland). Alternatively, NETs induced in chamber slides were quantitated as released DNA by adding micrococcocal nuclease (Worthington Biochemical, Lakewood, NJ) to 1 µM, incubating at 37°C for 10 min, stopping the reaction with EDTA, and determining the fluorescent supernatant (15,000 rpm for 5 min at 4°C) prior to Sytox addition.

P. aeruginosa lung infection
Groups of weight-matched female WT and serpinb1−/− mice were sedated (100 mg/kg ketamine and 10 mg/kg xylazine) and intratracheally inoculated with 3 × 10^5 CFU P. aeruginosa PA01, as described (2). Mice were sacrificed 24 h later, and the lungs were lavaged once with 1 ml cold sterile PBS. Aliquots of the fluid were centrifuged to produce clarified bronchoalveolar lavage fluid (BALF), which was stored at −80°C for DNA and elastase assays or held briefly on ice for MPO assay. To quantify NETs produced in vivo, NETs and cells in noncentrifuged lavage fluid were allowed to adhere to eight-well glass chamber slides (200 µl/well) at 37°C for 3 h in the presence of DFP (10 µM) to inhibit ex vivo NETosis. The slides were stained with Sytox and evaluated for content of NETs, as described above. Aliquots of the fluid were also incubated without additive to allow additional NET generation ex vivo. The number of NETs produced ex vivo was calculated as the difference between total NETs without DFP and NETs in DFP-treated incubations.

Reactive oxygen species
Neutrophils (2 × 10^6 in 200 µl) were allowed to adhere in 24-well plates at 37°C for 1 h. Dihydrorhodamine-123 (1 µM; Sigma-Aldrich) was added for 5 min at 37°C. Supernatant was removed, and the cells were washed once. PMA (100 nM), MIP-2 (10 nM), or PAF (10 µM) was added in 1 ml medium, and the cells were incubated for 15 min at 37°C. After one wash, the cells were removed from the plate by cell scrapers and resuspended in PBS with 1% FCS for detection of ROS-induced rhodamine-123 on a FACS Calibur (BD Biosciences). Neutrophils were identified on the basis of forward and side scatter properties.

MPO and elastase
MPO activity was measured by spectrophotometric assay in 50 mM potassium phosphate (pH 6) with 0.17 mg/ml o-dianisidine and 0.0005% H₂O₂, using human leukocyte MPO (Sigma-Aldrich) as standard. Elastase was measured by hydrolysis of N-methoxy-succinyl-Ala-Ala-Pro-Ala-nitroanilide (2 mM; Sigma-Aldrich) in 20 mM Trit-HCl (pH 7.4), 500 mM NaCl, 0.05% Tween, and 4 mM DTT as change of OD₄₁₀ nm over time at 22°C, with human neutrophil elastase (Elastin Products, Owensville, MO) as standard.

Statistical analysis
Means ± SEM are presented except for bacterial CFU, for which log-transformed individual data points are shown, and medians are indicated.
Results

Increased NET generation by serpinb1−/− neutrophils

Upon culture of lung neutrophils with PMA, NETs were generated that could be detected in fluorescence micrographs as DNA-based structures emanating from cells (Fig. 1A). On counting, we found increased numbers of NETs in serpinb1−/− photomicrographs compared with WT (Fig. 1B). A similar increase in serpinb1−/− NETs compared with WT was found when quantitation was based on fluorimetry of Sytox-stained DNA (Fig. 1C) or by treatment with micrococcal nuclease to release DNA (Supplemental Fig. 1C). The increase in serpinb1−/− NETs was not associated with an increase in adherent cells remaining at the end of PMA culture (WT: 173 ± 23; knockout [KO]: 146 ± 23 cells/FOV, n = 6) or an increase in total dead cells (plasma membrane-permeable cells) (WT: 45 ± 6%; KO: 57 ± 4%). This eliminates genotype differences in adherence and overall cell death as explanations for the extra increment of serpinb1−/− NETs. PMA-induced NET production by serpinb1−/− and WT neutrophils was abrogated by the NADPH oxidase inhibitor DPI, consistent with the findings for human NETs (11). Other known features of NETosis that were not qualitatively different between serpinb1−/− and WT include staining of the NETs for elastase and MPO, sensitivity of the NETs to DNase, and insensitivity of the NETosis process to the pan-caspase inhibitor Z-VAD-fmk (Supplemental Fig. 1A, 1B).

Because NET generation requires chromatin decondensation (15), we tested whether nuclear expansion was altered. Although the range was broad, the distribution of nuclear size of PMA-treated serpinb1−/− lung neutrophils was highly skewed toward larger values compared with WT neutrophils (Fig. 1D). By using “50% of cell area” to define an expanded nucleus (Fig. 1D), we found that the frequency of serpinb1−/− neutrophils with expanded nuclei was twice that of WT neutrophils (Fig. 1E).

To study lung neutrophils (as in the above experiments), mice must be instilled with chemokines or LPS, which initiates the process of recruitment (extravasation) that substantially alters neutrophil properties (22, 23). To test whether the effects of SerpinB1 on NETosis are limited to neutrophils that have undergone this process, we examined bone marrow neutrophils of both genotypes. Indeed, increased NETs were induced in serpinb1−/− bone marrow neutrophils compared with WT in response to PMA, as well as in response to the phospholipid mediator PAF, the chemokine MIP-2/cxcl2 (Fig. 2A), and LPS (Supplemental Fig. 2), indicating that the enhancing effect of serpinb1 deletion extends to bone marrow neutrophils and to multiple NETosis-inducing mediators. Treatment with DPI to inhibit NADPH oxidase, the major neutrophil ROS-generating system, blocked PMA-induced NET production by bone marrow neutrophils of both genotypes as anticipated and also blocked PAF-induced NET production; however, MIP-2–induced NET production was not inhibited by DPI (Fig. 2A). The findings indicate that the MIP-2–induced NETosis pathway differs from the PMA and PAF pathways and, at least for MIP-2, the additional increment of NETs produced by serpinb1−/− neutrophils is not due to increased ROS. Consistent with there being different pathways for different inducers, the time for optimal NET production also differed: 4 h for PMA, 30 min for PAF, and 45 min for MIP-2.

To further evaluate the role of ROS, we measured oxidation of the intracellular dye dihydrorhodamine. PMA caused DPI-sensitive

* \( p < 0.05 \), ** \( p < 0.001 \), *** \( p < 0.0001 \).

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**FIGURE 1.** Increased production of NETs and expansion of nuclei of serpinb1−/− lung neutrophils. (A–C) NET production. WT (+/+ and serpinb1−/−) lung neutrophils were cultured in poly-L-lysine–coated tissue culture wells with no additive, PMA, or PMA plus DPI. Staining with Sytox green, a plasma membrane-impermeable DNA dye, revealed NETs as Sytox-positive structures emanating from cells with lengths greater than twice the cell diameter. (A) Representative fluorescence images of PMA-treated neutrophils; examples of NETs are indicated by arrows (original magnification ×40). (B) NETs enumerated in micrographs. (C) NETs quantified by DNA fluorimetry. (D and E) Nuclear size. (D) Distribution of PMA-treated Sytox-stained WT and serpinb1−/− neutrophils on the basis of nuclear area (as percentage of cell area). (E) Frequency of neutrophils with expanded nuclei (>50% of cell area). Means ± SEM for three or four experiments (each with two or three mice/group).

For pair-wise comparisons, differences between means (or medians) were analyzed by the Student t test; differences among multiple means were analyzed by one-way ANOVA, followed by the Tukey post hoc test. Analyses were performed using Prism software (GraphPad, La Jolla, CA). A \( p \) value < 0.05 was considered statistically significant.
production of ROS by serpinb1−/− and WT neutrophils as anticipated, but there was no difference between the genotypes (Fig. 2B), indicating that the NETosis-enhancing effect of serpinb1 deletion in the PMA pathway is not due to increased ROS. Neither PAF nor MIP-2 induced ROS as measured by dihydrorhodamine (Fig. 2B) and by luminol-based chemiluminescence (data not shown). This finding for PAF is consistent with reports that PAF primes neutrophils but does not activate NADPH oxidase (24). The DPI sensitivity of PAF-induced NETosis in the absence of NADPH oxidase activation suggests involvement of a different DPI-sensitive enzyme that still needs to be determined; DPI is known to inhibit a range of flavoenzymes, including mitochondrial oxidase and NO synthase (25, 26). NET production was also increased for serpinb1−/− neutrophils compared with WT upon treatment with H2O2, a direct source of ROS (Fig. 2C, 2D); again, there was no genotype difference with regard to adherent neutrophil number (WT: 517 ± 27; KO: 457 ± 27 cells/FOV, n = 6; p = 0.16) or total neutrophil death (WT: 31.0 ± 3.2%; KO: 39.5 ±

**FIGURE 2.** Production of NETs and ROS by WT and serpinb1−/− bone marrow neutrophils. (A) Neutrophils were cultured in uncoated glass chamber slides with the indicated agents, and the resulting NETs were quantified in fluorescence images after Sytox staining. (B) ROS. Neutrophils prelabeled with dihydrorhodamine were stimulated for 15 min, and accumulated ROS was measured as mean fluorescence intensity (MFI). (C and D) NETs produced by neutrophils treated with H2O2. Representative WT and serpinb1−/− neutrophils stained with Gr-1 Ab (green) and Sytox orange (original magnification ×20). Examples of NETs are indicated by arrows. (D) Quantitation of NETs. Data in (A), (B), and (D) are means ± SEM for two experiments (each with three mice/genotype). *p < 0.05, **p < 0.001, ***p < 0.0001; two symbols show the results for the WT versus WT comparison, followed by the KO versus KO comparison.

**FIGURE 3.** Translocation of SerpinB1 to the nucleus during NETosis of human neutrophils. Resting neutrophils (A) and neutrophils treated with PMA for 1 h (B, C) or 4 h (E) or with PMA for 1 h after DFP pretreatment (D) were stained for SerpinB1 (SB1), elastase (ELA), LBR, or DAPI. Cells were examined by fluorescence microscopy (A, B, original magnification ×40) or confocal microscopy (C–E, original magnification ×63). Scale bar, 10 μm.
SerpinB1 migrates to the nucleus during NETosis

During granulopoiesis, postmitotic progenitor neutrophils progressively acquire the abundance of heterochromatin and the segmented (lobular) nuclear shape that characterize the mature cells (28). We used fluorescence imaging to examine terminally differentiated human neutrophils during NETosis. In the resting state, SerpinB1 was exclusively in the cytoplasm (Fig. 3A), consistent with the current understanding of clade B serpins. Clade B serpins, an ancient group also known as OVA serpins, are found in the nucleus, as well as the cytoplasm (nucleocytoplasmic location), despite their lack of classical nuclear-localization signals; a few, including SerpinB1, are also found extracellularly (29–31).

A study of human neutrophils undergoing PM-induced NETosis characterized the step-wise changes in the cells. The first event, observed in one third of neutrophils after 15 min, was the transformation from lobulated to delobulated nuclei, a change accompanied by minimal alteration of nuclear area (32). After treating human neutrophils with PMA, we found a mixture of neutrophils with fully delobulated and partially delobulated nuclei at 1 h, at which time nuclear expansion was minimal. Importantly, SerpinB1 had entered the nucleus of almost all of the neutrophils (Fig. 3B). Localization within the nucleus was verified in confocal micrographs using LBR as the nuclear envelope marker (Fig. 3C).

Migration of SerpinB1 into the nucleus coincident with or prior to the first detectable morphological change indicates that it may regulate events in the nucleus.

Previous studies showed that elastase also enters the nucleus during PM-induced NETosis (12). Thus, we questioned whether SerpinB1 translocation requires formation of a complex with elastase, as occurs in protease inhibition (1). The same study showed that small molecule inhibitors of elastase abrogate PM-induced elastase migration to the nucleus and NET production (12). Consistent with this report, we found that PM-induced NET production was abrogated when neutrophils were treated with potent inhibitors of elastase: DFP, PMSF, and aminoethylbenzene sulfonylfluoride (data not shown). Evaluation of DFP-pretreated neutrophils after 1 h with PM showed that elastase remained confined to the cytoplasmic region as predicted, but SerpinB1 entered the nucleus of almost all cells (Fig. 3D), indicating that complex formation with elastase is not required for SerpinB1 translocation. Finally, after 4 h with PM, SerpinB1 was localized to NETs along with DNA and elastase (Fig. 3E).

Increased NET production in vivo in serpinb1−/− mice

We used the previously characterized P. aeruginosa lung infection model to determine whether serpinb1−/− NET production is increased in vivo. Findings generated with this model strongly indicate that SerpinB1 protects pulmonary antibacterial capacity and prevents lung inflammation and injury (2). After intranasal infection with P. aeruginosa, the immune response of serpinb1−/− mice was initially normal (i.e., normal levels of cytokines/chemokines were generated, normal numbers of neutrophils were recruited, and bacteria were restricted normally at 6 h); however, by 24 h, when WT mice had largely cleared the infection and restored cytokines toward baseline levels, serpinb1−/− mice had logs-higher bacterial numbers, increased levels of inflammatory cytokines, proteolytic injury, and unexplained excess neutrophil death (2). To replicate the model, groups of mice were inoculated with P. aeruginosa strain PAO1 and sacrificed 24 h later. As anticipated, WT mice largely cleared the infection, but bacterial numbers were increased in serpinb1−/− mice (Fig. 4A). The specific neutrophil proteins MPO and elastase, which were measured in cell-free BALF, were increased as anticipated in serpinb1−/− mice compared with WT mice (Fig. 4B, 4C). The increase in free elastase and MPO indicates increased death of serpinb1−/− neutrophils. Free DNA, which is frequently used as a measure of NETosis, was assayed in cell-free lavage fluid and was increased in serpinb1−/− mice compared with WT mice and was not detected in the absence of infection (Fig. 4D). These findings are consistent with increased NETosis in infected serpinb1−/− mice; however, there are other possible sources of free DNA in the lungs.
DNA in lungs of infected mice. Therefore, to directly quantify in vivo-generated NETs, we incubated noncentrifuged lavage fluid in chamber slides to allow NETs to adhere and be subsequently quantified by Sytox staining and microscopy. DFP was present during incubation to block ex vivo NET generation. The number of NETs in DFP-treated lavage fluid (i.e., in vivo generated NETs), was increased in infected serpinb1<sup>-/-</sup> mice compared with WT mice (Fig. 4E, 4F). The increase did not reflect a corresponding increase in neutrophils. Rather, the number of cells (primarily neutrophils) recovered from infected serpinb1<sup>-/-</sup> mice was decreased, although not significantly, compared with WT mice (WT: 3.2 ± 0.9 × 10<sup>6</sup> cells/mouse; KO: 2.0 ± 0.5 × 10<sup>6</sup> cells/mouse), consistent with previous reports (2). We also incubated (noncentrifuged) lavage fluid without DFP and without further additive and found that the neutrophils continued to generate additional NETs. This ex vivo NET production by neutrophils in lung fluid of infected mice was also increased in serpinb1<sup>-/-</sup> mice compared with WT mice (Fig. 4G). These findings demonstrate that NET production occurs in vivo in the setting of infection and that both in vivo NETosis and ex vivo NETosis are substantially increased in infected serpinb1<sup>-/-</sup> mice.

**Abrogation of NET generation by exogenous rSerpinB1**

To further probe the role of SerpinB1 in NET generation, we tested whether exogenous SerpinB1 affects the process. Inclusion of rSerpinB1 abrogated or substantially decreased NET production in response to PMA, as well as PAF, MIP-2 (Fig. 5A), and LPS (Supplemental Fig. 2). Abrogation of NETosis was found for both WT and serpinb1<sup>-/-</sup> neutrophils (Fig. 5A), and inclusion of rSerpinB1 did not alter neutrophil recovery or total neutrophil production by human neutrophils treated with PMA, and inhibition by rSerpinB1 but not by AAT or OVA. For (A)–(E), data are mean ± SEM for two to four experiments (each with three mice/group). Data in (A) and (B) are from the same series of experiments; data in (E) and (F) are from another series. Two symbols in (A) and (D) show the results for the WT versus WT comparison, followed by the KO versus KO comparison. Data in (F) are the mean ± SEM for cells from three or four normal healthy donors. *<i>p</i> < 0.05, **<i>p</i> < 0.001, ***<i>p</i> < 0.0001.

**FIGURE 5.** Abrogation of NET generation by exogenous rSerpinB1. Bone marrow neutrophils of WT<sup>+/+</sup> and serpinb1<sup>-/-</sup> mice (A–E) or human blood neutrophils (F) were preincubated or not with rSerpinB1 (rSB1; 10 μg/ml) for 1 h (A–C, F) or for the indicated times (D, E) and stimulated with PMA, PAF, or MIP-2 (A) or with PMA (B–F). NETs were quantified by microscopy after Sytox staining. (A) NET generation by both genotypes of murine neutrophils stimulated with PMA, PAF, or MIP-2 and its inhibition by rSerpinB1. (B) Lack of inhibition of PMA-stimulated NET generation by AAT (SerpinA1), which largely replicates the protease inhibitory specificity of SerpinB1 (1), and OVA (serpinb14), a nonprotease inhibitor serpin of the same clade (30). AAT, OVA (Fig. 5B), and BSA (data not shown) did not alter PMA-induced NET production. In contrast to its effect on NET production, exogenous rSerpinB1 did not alter PMA-induced ROS production (Fig. 5C). To determine whether delaying the addition of rSerpinB1 alters its effect, we varied the time of addition. Compared with addition at the start of preincubation (<60 min), the inhibitory effect was only partial for rSerpinB1 added at the same time as, or 15 min after, PMA (Fig. 5D), indicating that rSerpinB1 is more efficient at inhibiting NETosis if it is present during the preincubation and adherence steps prior to NETosis induction. There was no inhibitory effect when rSerpinB1 was present only during the final 1 h with PMA (Fig. 5E), verifying that the recombinant protein acts by blocking NET generation rather than inducing NET degradation. Finally, NETs were induced as anticipated when human neutrophils were treated with PMA, and human NET production was inhibited by rSerpinB1 and not by AAT or OVA (Fig. 5F).

**Discussion**

In this study, we identified a novel NETosis regulatory mechanism involving SerpinB1, an intracellular clade B serpin. A number of lines of evidence support this conclusion. First, isolated serpinb1<sup>-/-</sup> neutrophils generated substantially more NETs than did WT neutrophils. Second, the increase in serpinb1<sup>-/-</sup> NETs was induced by unrelated activating agents (PMA, PAF, MIP-2, LPS) and was noted...
for bone marrow neutrophils and recruited lung neutrophils. Third, increased \textit{serpinb1} \textsuperscript{-/-} NET production in response to PMA, PAF, and MIP-2 was found in two genetic backgrounds, 129S6 (Figs. 1, 2, 4, 5) and C57BL/6 (Supplemental Fig. 3), indicating that the defect of NETosis regulation is highly penetrant. Fourth, the frequency of expanded nuclei, indicative of chromatin decondensation, was increased in \textit{serpinb1} \textsuperscript{-/-} neutrophils. Fifth, exogenous rSerpinB1 abrogated NET production. Lastly, increased NETs were found in vivo in \textit{P. aeruginosa}-infected \textit{serpinb1} \textsuperscript{-/-} mice.

The findings that excess NETs are produced in vitro by highly purified \textit{serpinb1} \textsuperscript{-/-} neutrophils and in vivo in infected lungs of \textit{serpinb1} \textsuperscript{-/-} mice strongly suggest that the in vitro and in vivo outcomes have their basis in the same NETosis regulatory mechanism. This nonredundant restriction of NET production by SerpinB1 occurs despite the expression in neutrophils of other clade B serpin inhibitors (33) and the clade A serpin AAT (34), which shares the SerpinB1 inhibitory specificity. Factors eliminated as a cause of the enhanced NET production associated with SerpinB1 deletion include altered neutrophil adherence and overall cell death. A mechanism involving caspases is unlikely, because NET production by neutrophils of both genotypes is insensitive to the pan-caspase inhibitor Z-VAD-fmk (Supplemental Fig. 1). ROS was found to produce NETs when the activating agent was PMA, but ROS was not different in \textit{serpinb1} \textsuperscript{-/-} and WT neutrophils; MIP-2, PAF, and LPS (Supplemental Fig. 2) induced increased \textit{serpinb1} \textsuperscript{-/-} NETs without requiring ROS. Thus, the NETosis-enhancing effect of \textit{serpinb1} deletion is ROS independent. Three NET-generating pathways, each regulated by SerpinB1, were distinguished: PMA; MIP-2, which is NADPH/ROS independent; and PAF, which is NADPH oxidase/ROS independent but requires a DPI-sensitive enzyme. Thus, SerpinB1 acts at a signaling step common to the PMA, MIP-2, and PAF pathways and located downstream of ROS in the PMA pathway.

We can only speculate about the mechanism of NETosis inhibition by added rSerpinB1; however, the current findings suffice to place endogenous SerpinB1 within a schematic of NETosis regulation (Fig. 6). NETosis occurs in two stages: an initial predominantly cell surface/cytoplasmic stage and a subsequent nuclear stage. Initial events include signaling cascades specific to the inducing agent, which, in the case of PMA, involves the Raf–MEK–ERK pathway and leads to NADPH oxidase activation and ROS (32). Multiple other agents induce NETosis, including bacteria, fungi, protozoa, TNF-\(\alpha\), LPS, MIP-2, and PAF (4, 13, 27, 32, 35, 36); these are represented in Fig. 6 by PMA, MIP-2, and PAF (4, 13, 27, 32, 35, 36); all of which are ROS independent (27) and one of which, PAF, requires a DPI-sensitive enzyme. Elastase, which can be released from damaged granules, was reported to participate in the PMA pathway (12); thus, SerpinB1 might restrict NETosis by inhibiting cytoplasmic elastase. Importantly, SerpinB1 translocates independently to the nucleus coincident with or prior to the early PMA-induced transformation of the nucleus from a segmented to a spherical shape, strongly suggesting that SerpinB1 has a regulatory role in the nucleus. A rapid conversion of the polymorphonuclear nucleus to a condensed sphere also occurs in \textit{S. aureus}-induced NETosis (27), suggesting that this transformation of the nucleus may be a common feature of NETosis.

The dominant subsequent nuclear event is decondensation of chromatin, which requires that histone tail arginines be deiminated (converted to citrulline) by PAD-4, a change that involves loss of positive charges. Histone citrullination is required for NET production in response to PMA, LPS, TNF-\(\alpha\), and IL-8 plus \textit{Shigella flexneri} (14–16), strongly indicating that multiple pathways have converged at or before this step. The responsible enzyme, PAD-4, is expressed at high levels in neutrophils and localized to the nucleus (37); its mechanism of activation in vivo has not been studied. We hypothesize that SerpinB1 regulates events at the level of chromatin decondensation, possibly by restricting histone citrullination. Putative mechanisms might include blocking PAD-4 access to histone tails or preventing dissociation or destruction of a PAD-4 inhibitory protein (Fig. 6).

A role for SerpinB1 in regulating chromatin decondensation was also suggested by an independent study, which showed that SerpinB1 associates tightly with condensed chromatin of human neutrophils prepared so that SerpinB1 was localized in the nucleus (38). That study also showed the unusual composition of mature neutrophil nuclei, in particular the presence, in heterochromatin regions, of the repressive marker H3K9, but depletion of heterochromatin protein-1\(\alpha\), -1\(\beta\), and -1\(\gamma\), which normally bind H3K9 and maintain heterochromatin. Nuclear envelope-stabilizing proteins, lamins A/C, B1, and B2, LAP2\(\beta\), and emerin, are also depleted in mature neutrophils; the resulting nucleus, although malleable, is also fragile (39), perhaps increasingly fragile after conversion to a spherical shape. These findings led Olins et al. (39) to speculate that chromatin binding of SerpinB1 contributes to maintaining the fragile nuclear shape. Importantly, the proposed role of SerpinB1 in regulating chromatin decondensation of mammalian neutrophils is not without precedent. The closely related protein serpinb10b, also known as MENT (mature erythrocyte nuclear termination-stage specific protein), a specialized clade B serpin in avian spe-

![Figure 6](http://www.jimmunol.org/)
SERPINB1 REGULATES NETosis