Plasma gelsolin improves lung host defense against pneumonia by enhancing macrophage NOS3 function

Zhiping Yang,1 Terry Ting-Yu Chiou,2,3,4 Thomas P. Stossel,2 and Lester Kobzik1

1Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, Massachusetts; 2Department of Medicine, Brigham and Women’s Hospital, Boston, Massachusetts; 3Department of Medicine, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung City, Taiwan; and 4Chang-Gung University College of Medicine, Tao-Yuan City, Taiwan

Submitted 31 March 2015; accepted in final form 7 May 2015

Bacterial pneumonia remains a major cause of morbidity and mortality (21). The secondary pneumonias that occur after influenza or other severe injuries are especially problematic clinically (19, 23, 26). One approach to the problem might be to enhance innate immunity and host resistance to infection. The resident lung macrophage is a “first-responder” phagocyte, ingesting and killing inhaled bacteria, an important function confirmed by greater susceptibility to infection and diminished bacterial clearance after their depletion experimentally (8, 11) or by their impairment after relevant clinical risk factors such as recent influenza infection (27).

We have investigated whether a normal human blood protein, plasma gelsolin (pGSN), can enhance innate defenses against pneumonia. pGSN is secreted into extracellular fluids; it differs from the ubiquitous cytoplasmic isoform by an additional NH2-terminal sequence of 25 amino acids. Blood levels of pGSN are 200–300 μg/ml, placing it among the most abundant single proteins in plasma. pGSN functions as part of an “extracellular actin-scavenging system,” binding and facilitating removal of potentially inflammatory actin released by injured cells (17). The discovery that pGSN binds and inactivates lipid, peptide, and small molecule mediators of inflammation suggests another role (3, 12, 22). pGSN depletion due to actin exposure at local sites of injury could enable mediators generated there to modulate host defense and repair. However, the large reservoir of circulating pGSN would bind and inhibit mediators escaping into the circulation, preventing them from exerting organ damage. Loss of such systemic protection would explain the correlation between critical pGSN depletion and adverse outcomes of severe primary injuries, such as acute respiratory distress syndrome, sepsis, major trauma, and prolonged hyperoxia. This inflammation localization hypothesis may explain why pGSN repletion in a variety of animal injury models has consistently reduced inflammatory complications and, in some cases, promoted survival (6, 16, 24). The mechanisms for these beneficial effects of pGSN are likely multiple, but they indicate its potential as a modulator of acute, innate immune responses.

We report here the new finding that pGSN also has direct benefits on macrophage-mediated antimicrobial and host defense functions. We found that pGSN enhances the ability of lung macrophages to kill bacteria in vitro, in large part through a nitric oxide (NO) synthase type III (NOS3)-dependent mechanism (28). We also used a model of primary pneumococcal infection that approximates the frequent challenge to lung defenses of small numbers by bacteria and then studied a model of postviral susceptibility to bacterial pneumonia. We found that pGSN improved resistance in mouse models of both primary infection and postinfluenza secondary pneumonia. Since pGSN depletion occurs in acute pneumonia in humans (18), pGSN repletion may offer a therapeutic approach to enhance resistance to common and serious lung infections.

Materials and Methods

Mouse models of pneumococcal pneumonia. Normal 8- to 12-wk-old male and female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) or Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice genetically deficient in NOS3 (25) were obtained from Jackson Laboratories. All animals were housed in sterile microisolator cages in a barrier facility and showed no evidence of spontaneous infection. Prior approval for all experimentation was obtained from the Harvard Medical Area Standing Committee on Animals.

Primary pneumococcal pneumonia was modeled as previously reported (1, 28). Pneumonia was induced by intranasal instillation of ~10⁶ colony-forming units (CFU) of Streptococcus pneumoniae type 3 into mice under anesthesia with ketamine (120 mg/kg ip) plus xylazine (16 mg/kg ip); actual CFU administered were quantitated by CFU assay and used subsequently to calculate percent clearance. For analysis at 4 or 24 h of lung inflammation and bacterial clearance,
Macrophage binding, internalization, and killing of internalized bacteria were measured quantified elsewhere (1, 28). To model postinfluenza secondary pneumonia, mice were instilled intranasally on day 0 with a single 25- or 50-μl suspension containing influenza virus [H1N1 A/PR8/34; 1 plaque-forming unit (PFU)], under general anesthesia with ketamine (120 mg/kg ip) plus xylazine (16 mg/kg ip). Influenza-treated animals routinely lost weight and then recovered by day 7, when the secondary infection was administered. On day 7 after initial influenza infection, mice were anesthetized and then instilled intranasally with ~500 CFU of S. pneumoniae serotype 3. pGSN was administered at 400 mg/kg sc daily starting 1 day before pneumococcal challenge. Subsequent analyses were performed as described for the primary pneumonia model described above.

**Bacteria.** S. pneumoniae serotype 3 and *Escherichia coli* (catalog nos. 6303 and 19138, respectively, American Type Culture Collection) was used as a positive control for (“endothelial”) NOS3. Antibodies for NOS3, Akt, and phosphorylation-state-specific isoforms include NOS3 rabbit polyclonal antibody (C-20, Santa Cruz Biotechnology) and phosphorylated (Ser1177) endothelial NOS (C9C3) rabbit monoclonal antibody, phosphorylated (Ser1173) Akt (D9E) rabbit monoclonal antibody, Akt rabbit polyclonal antibody, and β-actin (13E5) rabbit monoclonal antibody, all from Cell Signaling Technologies. Polyclonal antibodies to pGSN were developed as previously reported (15, 16). After incubation with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Pierce), labeling was detected using chemiluminescence.

**Statistical analysis.** Data were analyzed using Prism software (GraphPad Software). Differences in Kaplan-Meier survival curves were considered significant at *P* < 0.05.

**RESULTS**

**pGSN improves macrophage bacterial killing in vitro.** Since lung macrophages mediate initial innate resistance to bacteria, we investigated pGSN effects on their antimicrobial function. We used a murine lung macrophage-derived cell line (29) in an assay that measures bacterial binding, internalization, and killing (30). Phagocytosis and bacterial killing assay. Macrophage binding, internalization, and killing of internalized bacteria were measured using CFU assays of cell samples lysed by 10-fold excess H2O (pH 10, 3 min) (1, 28). The B6 cell line was created by retroviral immortalization of alveolar macrophages from normal male C57BL/6 mice, as previously reported (29); a similar cell line derived by immortalization of alveolar macrophages from NOS3-deficient (NOS3−/−) mice was also used. Effects of pGSN were tested by including agents or their vehicle solutions in the assay buffers.

**Western blot analysis and ELISA.** Western blot analysis was performed on macrophage lysates lysis buffer 1% NP-40 with protease and phosphatase inhibitors using the protocols described elsewhere (13). The endothelial cell line bEnd.3 (CRL-2299, American Type Culture Collection) was used as a positive control for (“endothelial”) NOS3. Antibodies for NOS3, Akt, and phosphorylation-state-specific isoforms include NOS3 rabbit polyclonal antibody (C-20, Santa Cruz Biotechnology) and phosphorylated (Ser1177) endothelial NOS (C9C3) rabbit monoclonal antibody, phosphorylated (Ser1173) Akt (D9E) rabbit monoclonal antibody, Akt rabbit polyclonal antibody, and β-actin (13E5) rabbit monoclonal antibody, all from Cell Signaling Technologies. Polyclonal antibodies to pGSN were developed as previously reported (15, 16). After incubation with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Pierce), labeling was detected using chemiluminescence.

**Statistical analysis.** Data were analyzed using Prism software (GraphPad Software). Differences in Kaplan-Meier survival curves were considered significant at *P* < 0.05.

**RESULTS**

**pGSN improves macrophage bacterial killing in vitro.** Since lung macrophages mediate initial innate resistance to bacteria, we investigated pGSN effects on their antimicrobial function. We used a murine lung macrophage-derived cell line (29) in an assay that measures bacterial binding, internalization, and killing (30). Phagocytosis and bacterial killing assay. Macrophage binding, internalization, and killing of internalized bacteria were measured using CFU assays of cell samples lysed by 10-fold excess H2O (pH 10, 3 min) (1, 28). The B6 cell line was created by retroviral immortalization of alveolar macrophages from normal male C57BL/6 mice, as previously reported (29); a similar cell line derived by immortalization of alveolar macrophages from NOS3-deficient (NOS3−/−) mice was also used. Effects of pGSN were tested by including agents or their vehicle solutions in the assay buffers.

**Western blot analysis and ELISA.** Western blot analysis was performed on macrophage lysates lysis buffer 1% NP-40 with protease and phosphatase inhibitors using the protocols described elsewhere (13). The endothelial cell line bEnd.3 (CRL-2299, American Type Culture Collection) was used as a positive control for (“endothelial”) NOS3. Antibodies for NOS3, Akt, and phosphorylation-state-specific isoforms include NOS3 rabbit polyclonal antibody (C-20, Santa Cruz Biotechnology) and phosphorylated (Ser1177) endothelial NOS (C9C3) rabbit monoclonal antibody, phosphorylated (Ser1173) Akt (D9E) rabbit monoclonal antibody, Akt rabbit polyclonal antibody, and β-actin (13E5) rabbit monoclonal antibody, all from Cell Signaling Technologies. Polyclonal antibodies to pGSN were developed as previously reported (15, 16). After incubation with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Pierce), labeling was detected using chemiluminescence.

**Statistical analysis.** Data were analyzed using Prism software (GraphPad Software). Differences in Kaplan-Meier survival curves were considered significant at *P* < 0.05.
killing of internalized bacteria by quantitation of CFU at each step (1). Using this assay under serumless conditions to approximate normal plasma levels for 1 h prior to challenge with S. pneumoniae serotype 3 at a 10:1 bacteria-to-macrophage ratio. We found no effect on bacterial binding (data not shown). In contrast, pGSN enhanced internalization of bound bacteria and also markedly increased the rate of killing of internalized bacteria (Figs. 1, A and B). The enhanced killing was seen even when the pGSN was washed away prior to bacterial challenge, consistent with a direct effect on the alveolar macrophage, rather than opsonization of bacteria. Similar results were seen when E. coli and F. tularensis bacteria were used (Fig. 1, C–F).

pGSN improves lung host defense against pneumococcal pneumonia in vivo. We pretreated mice with subcutaneous injections of rhu-pGSN, which gradually (over 6 h) raises pGSN levels (16). After 2 h, animals were challenged with intranasal instillation of S. pneumoniae (≈10^5 CFU). After 24 h, we measured bacterial clearance (CFU assay) and lung inflammation (cell yield and differential) in bronchoalveolar lavage samples. pGSN-treated animals showed substantially better clearance of bacteria (survival of fewer live bacteria) and less inflammation than vehicle-treated controls (Fig. 2, A and B). To test the effect of lung-only delivery, we exposed mice to aerosolized pGSN followed by bacterial challenge. We observed persistence of rhu-pGSN in the lungs for several hours, an increase in clearance of bacteria from lungs, and diminished lung neutrophilia (Fig. 2, C–E). Even without antibiotic treatment, mice receiving pGSN showed markedly greater survival in this model of primary pneumococcal pneumonia (Fig. 2F).

NOS3 mediates pGSN beneficial effects in macrophages. Mechanisms for killing phagocytosed bacteria include production of reactive oxygen species (e.g., superoxide produced by NADPH oxidase) and reactive nitrogen intermediates (e.g., NO produced by NOS). We recently reported that NOS3, constitutively expressed in murine and human alveolar macrophages, functions in lung macrophage host defense against bacterial pneumonia (28). We tested the role of NOS3 in the ability of pGSN to enhance macrophage antibacterial function. We compared effects of pGSN in vitro on wild-type or NOS3−/− macrophages. Absence of NOS3 eliminated the beneficial enhancement of bacterial killing caused by addition of pGSN (Fig. 3A). Studies of NOS3 in endothelial cell biology have identified rapid activation via phosphorylation of NOS3, e.g., at Ser1177 (13), and this activation occurs via specific signaling cascades, i.e., via phosphorylated inositol-3 kinase and protein kinase B, also known as Akt (4, 9). To investigate the pathway in macrophages, we exposed macrophages to pGSN and performed Western blot analysis on lysates after 1–4 h.

![Fig. 2. pGSN enhances resistance to pneumococcal pneumonia. A and B: 24 h after intranasal inoculation of S. pneumoniae (≈10^5 colony-forming units (CFU)), lung samples from mice treated with pGSN (10 mg·mouse⁻¹·dose⁻¹ sc) contain fewer live bacteria (bacterial survival) and show less acute inflammation (bronchoalveolar lavage (BAL) neutrophils) than lung samples from vehicle-treated (control) mice. Values are means ± SD from ≥3 independent experiments (n ≥ 12). *P < 0.01 vs. control. C: After aerosol exposure to rhu-pGSN, Western blot analysis of lung lavage samples shows detectable levels of rhu-pGSN for up to 7 h (2 µg protein loaded/lane); normal levels of endogenous murine pGSN (mu-pGSN) were also detected but were unchanged. D and E: Aerosol exposure to pGSN followed by S. pneumoniae inoculation causes enhanced bacterial clearance, as measured by diminished bacterial survival in lungs at 24 h (D, 30-min exposure, n ≥ 12) and reflected in decreased neutrophilia in lavage samples (E, 30-min exposure, n ≥ 12, summary of 3 independent experiments; data from 15-min (15') exposure are from a single trial using 3 replicates). F: Systemic (subcutaneous) treatment with pGSN results in substantially better survival from pneumonia. Values are means ± SD of 3 independent experiments (n ≥ 12). *P < 0.01.](http://ajplung.physiology.org/)
analysis showed rapid phosphorylation of Akt and NOS3 in response to pGSN (Fig. 3B). Using the primary pneumococcal pneumonia model, we found that NOS3−/− mice failed to show the enhanced bacterial clearance seen in wild-type mice (Fig. 3C).

pGSN improves outcomes in secondary postinfluenza bacterial pneumonia. Secondary bacterial pneumonia is a frequent complication of seasonal or epidemic viral influenza. We tested pGSN for potential benefit in a murine model of enhanced susceptibility to secondary pneumococcal pneumonia after nonlethal influenza challenge. In mice infected with low-dose inocula of influenza (PR8, 1 PFU) or S. pneumoniae alone (100 or 500 CFU), survival was 100%. In contrast, mortality was 100% in mice receiving similarly small bacterial challenges on postinfluenza day 7. The magnitude and timing of enhanced susceptibility are similar to those reported previously (11, 27) but are, nonetheless, remarkable: for comparison, in normal mice (without prior influenza), Arredouani et al. (1) reported 100% survival with 105 CFU (data not shown) and only partial mortality at higher inocula, e.g., ∼50% with 106 CFU.

With this model, bacterial clearance measurements at 24 h show extraordinary bacterial growth in the postinfluenza lung, consistent with marked impairment of innate host defenses (19, 20). pGSN treatment markedly improved bacterial clearance and reduced acute inflammation at 24 h after bacterial inoculation (Fig. 4, A and B). Mice treated with a lower dose of pGSN showed ∼50% survival and recovered from initial weight loss (Fig. 4, C and D), while a higher-dose trial showed 100% survival (Figs. 4, D and E). In both cases, the improved outcome took place without antibiotic treatment.

DISCUSSION

We sought to identify new approaches to enhancing innate immunity to bacterial pneumonia by investigating whether pGSN could act to augment lung host defense. We conclude that pGSN can mediate greater host resistance to pneumonia, in large part, via effects on the constitutively expressed NOS3 in lung macrophages. Pharmacological administration of pGSN led to improved bacterial clearance and improved survival from secondary pneumococcal pneumonia. These observations identify a novel host defense mechanism and a potential therapeutic benefit for pGSN, an endogenous protein that has undergone phase I clinical testing in limited human studies (5).

Our findings complement previous work demonstrating that pGSN administration improves the survival of animals subjected to peritoneal bacterial infection (7, 16). However, those outcomes were ascribed solely to protective effects of pGSN against systemic inflammation. These prior results suggest that some modulation of inflammatory responses might also contribute to the enhanced survival observed with pGSN treatment, along with effects on improved bacterial clearance. Indeed, the ability of pGSN to augment phagocytosis uptake suggests additional effects on signaling or receptor(s) function.

The mechanism by which pGSN may act includes priming or activating macrophage NOS3 (28), which can then contribute to improved killing of ingested bacteria by direct bactericidal mechanisms, either alone or in combination with superoxide (10); pGSN might also act to mediate salutary intracellular signaling events (14). Additional pathways are also suggested by the observation that pGSN caused enhanced internalization of bacteria, an effect not observed in the presence of other activators of macrophage NOS3 (28). Although our understanding of the mechanisms by which pGSN stimulates macrophage function is rudimentary, our finding that pGSN results in the phosphorylation of NOS3 to activate it for bactericidal action is consistent with pGSN’s enhancement of bacterial uptake. Membrane polyphosphoinositides are important intermediates for phagocytosis and protein kinase B activation, which is upstream from NOS3 phosphorylation (2). Although externally disposed membrane receptors for pGSN have not been identified, pGSN has been shown to act as a carrier of ligands that are released by and/or interact with macrophage receptors (3, 12, 22).

We consider that the translational potential of these findings may be most relevant for the problem of secondary pneumonia,
specifically the increased susceptibility to bacterial lung infections that follows influenza. Treatment before the bacterial challenge is useful for proof-of-principle in the primary pneumonia model, but this clearly does not mirror likely clinical usage. In contrast, pretreatment, as used in the secondary pneumonia model, represents a realistic scenario for short-term, prophylactic immunomodulatory therapy. This might benefit patients identified to be at high risk of secondary pneumonia, e.g., hospitalized individuals with severe flu, after seasonal or pandemic influenza. Such translation is more than a theoretical possibility, since recombinant pGSN administration has been shown to be therapeutically beneficial in animals (present study) and feasible and safe in humans (5).

REFERENCES

GRANTS
This work was supported by National Institutes of Health Grants HL-11578 and ES-00002 (to L. Kobzik) and HL-19429 (to T. P. Stossel).

DISCLOSURES
T. P. Stossel’s employer, Brigham and Women’s Hospital, has licensed pending and issued patent applications regarding pGSN to BioAegis Therapeutics. T. P. Stossel is a consultant for BioAegis Therapeutics, from which he has been granted stock options.

AUTHOR CONTRIBUTIONS
Z.Y. and T.T.-Y.C. performed the experiments; Z.Y., T.T.-Y.C., T.P.S., and L.K. analyzed the data; Z.Y., T.T.-Y.C., T.P.S., and L.K. interpreted the results of the experiments; Z.Y. and L.K. prepared the figures; Z.Y., T.T.-Y.C., T.P.S., and L.K. edited and revised the manuscript; Z.Y., T.T.-Y.C., T.P.S., and L.K. approved the final version of the manuscript; L.K. developed the concept and designed the research; L.K. drafted the manuscript.