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The Peptidoglycan-Degrading Property of Lysozyme Is Not Required for Bactericidal Activity In Vivo

James A. Nash, Tiffany Nicole S. Ballard, Timothy E. Weaver, and Henry T. Akinbi

Lysozyme is an abundant, cationic antimicrobial protein that plays an important role in pulmonary host defense. Increased concentration of lysozyme in the airspaces of transgenic mice enhanced bacterial killing whereas lysozyme deficiency resulted in increased bacterial burden and morbidity. Lysozyme degrades peptidoglycan in the bacterial cell wall leading to rapid killing of Gram-positive organisms; however, this mechanism cannot account for the protective effect of lysozyme against Gram-negative bacteria. The current study was therefore designed to test the hypothesis that the catalytic activity (muramidase activity) of lysozyme is not required for bacterial killing in vivo. Substitution of serine for aspartic acid at position 53 (D53S) in mouse lysozyme M completely ablated muramidase activity. Muramidase-deficient recombinant lysozyme (LysM\textsuperscript{D53S}) killed both Gram-positive and Gram-negative bacteria in vitro. Targeted expression of LysM\textsuperscript{D53S} in the respiratory epithelium of wild-type (LysM\textsuperscript{+/-}/LysM\textsuperscript{D53S}) or lysozyme Mnull mice (LysM\textsuperscript{-/-}/LysM\textsuperscript{D53S}) resulted in significantly elevated lysozyme protein in the airspaces without any increase in muramidase activity. Intratracheal challenge of transgenic mice with Gram-positive or Gram-negative bacteria resulted in a significant increase in bacterial burden in LysM\textsuperscript{-/-} mice that was completely reversed by targeted expression of LysM\textsuperscript{D53S}. These results indicate that the muramidase activity of lysozyme is not required for bacterial killing in vitro or in vivo. 


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3 Abbreviations used in this paper: SP, surfactant protein; WT, wild type; BALF, bronchoalveolar lavage fluid.
vivo, a mouse lysozyme M<sup>D53S</sup> construct was generated and expressed in type II cells of WT and LysM<sup>−/−</sup> mice.

**Materials and Methods**

**Recombinant WT lysozyme and D53S protein**

Lysozyme M was amplified from mouse type II cell cDNA using specific primers that discriminate between lysozyme M and P. Sequences encoding a polyhistidine tag were added to the 5′ end of the downstream primer. Muramidase-deficient lysozyme M (LysM<sup>D53S</sup>) was generated by site-directed mutagenesis (QuickChange kit; Stratagene) which altered the codon for aspartic acid at position 53 to encode serine. Both LysM and LysM<sup>D53S</sup> cDNAs were cloned into pVL1393 (BD Pharmingen). Recombinant baculovirus was generated by homologous recombination in Spodoptera frugiperda 9 cells. For expression of recombinant protein, fresh monolayers of 10<sup>4</sup> Trichoplusia ni cells (Invitrogen Life Technologies) were infected with plaque-purified recombinant virus at a multiplicity of infection of 2 and cultured in serum-free medium (EU72 h). Recombinant WT lysozyme M and D53S proteins were purified from the medium by nickel-ni-triloacetic acid (Ni NTA) chromatography and characterized by Western blotting and silver staining as previously described (15). Alternatively, the LysM and LysM<sup>D53S</sup> constructs were cloned into pE21-a (EMD Biosciences) for expression in BL21(DES) bacterial cells (EMD Biosciences). Recombinant lysozyme was recovered from bacterial lysates under denaturing conditions, purified by Ni NTA chromatography, and refolded by passage over a buffer exchange column (PD-10; Amershams Biosciences) into 10 mM potassium phosphate (pH 7.4)/10% glycerol. The muramidase activities of WT lysozyme M and D53S proteins were compared as described below (Lysozyme enzyme activity in bronchoalveolar lavage fluid (BALF)). The hexahistidine sequence was not removed from the recombinant proteins as we have previously shown that both muramidase and micробidal activities are unaffected by the presence of the tag (15).

**Mice**

The four groups of mice used in the current study included mice deficient in lysozyme M (LysM<sup>−/−</sup>), transgenic mice expressing the muramidase-deficient lysozyme M transgene (D53S) in the distal respiratory epithelium (LysM<sup>+/+</sup>/LysM<sup>D53S</sup>), lysozyme M-deficient mice expressing the D53S transgene in the distal respiratory epithelium (LysM<sup>−/−</sup>/LysM<sup>D53S</sup>) and WT mice. Generation and characterization of LysM<sup>−/−</sup> mice was described previously (11, 17). Mice LysM<sup>+/+</sup>/LysM<sup>D53S</sup> transgenic mice were generated by microinjection of fertilized oocytes with a “mammalianized” transgene construct (21) consisting of the 3.7-kb human SP-C promoter, rabbit β-globin intron I and exon II, a cDNA fragment encoding the D53S transgene in the distal respiratory epithelium (LysM<sup>−/−</sup>/LysM<sup>D53S</sup>) and WT mice. Generation and characterization of LysM<sup>−/−</sup> mice was described previously (11, 17). Mice LysM<sup>+/+</sup>/LysM<sup>D53S</sup> transgenic mice were generated by microinjection of fertilized oocytes with a “mammalianized” transgene construct (21) consisting of the 3.7-kb human SP-C promoter, rabbit β-globin intron I and exon II, a cDNA fragment encoding the D53S transgene in the distal respiratory epithelium (LysM<sup>−/−</sup>/LysM<sup>D53S</sup>). Sequence encoding LysM<sup>+/+</sup>/LysM<sup>D53S</sup> and LysM<sup>−/−</sup>/LysM<sup>D53S</sup> mice were crossed to LysM<sup>−/−</sup>/LysM<sup>D53S</sup> mice to generate LysM<sup>+/+</sup>/LysM<sup>D53S</sup> mice. LysM<sup>+/+</sup>/LysM<sup>D53S</sup> mice were mated to produce LysM<sup>+/+</sup>/LysM<sup>D53S</sup> mice. LysM<sup>−/−</sup>/LysM<sup>D53S</sup> and LysM<sup>+/+</sup>/LysM<sup>D53S</sup> mice were inbred from WT mice with respect to longevity, fecundity, and somatic growth. All four groups of mice were maintained in the FVB/N genetic background. Mice were handled according to the Institutional Animal Care and Use Committee guidelines at Cincinnati Children’s Hospital Medical Center, and were maintained in a barrier containment facility. Serologies were periodically checked for common murine pathogens. Five- to 7-wk-old mice from each group were used in all experiments.

**Analysis of protein expression in BALF**

An aliquot of BALF containing 1 µg of protein was analyzed by SDS-PAGE followed by Western blotting with polyclonal rabbit Ab, directed against human lysozyme (Accurate Chemicals and Scientific), as previously described (15). Relative levels of lysozyme proteins in lung BALF were assessed by scanning densitometry using Image-Quant software (Molecular Dynamics).

**Spatial expression of lysozyme in the lungs**

To assess gross lung structure and expression of lysozyme in the lungs of transgenic mice, lungs from 5-wk-old LysM<sup>+/+</sup>/LysM<sup>D53S</sup> transgenic mice and WT littermates (n = 4 for each genotype) were inflation-fixed for immunohistochemistry and light microscopy, as previously described (11). Immunostaining for lysozyme was performed using antiserum directed against human lysozyme (Accurate Chemicals and Scientific). Parallel lung sections were incubated with preimmune rabbit serum to verify the specificity of immunostaining.

**Lysozyme enzyme activity assay in BALF**

To assess muramidase activity in BALF, an aliquot containing 1 µg of protein was incubated with 1 ml of killed Micrococcus luteus suspended in 0.4 M phosphate buffer (pH 6.7) at an OD of 1 (450 nM) at 37°C. Changes in OD during the 30-min incubation were plotted against time using a recording spectrophotometer. Purified chicken lysozyme was used to generate a standard curve (1 U of enzyme activity = 0.001 change in OD).

**Bacteria**

GFP-expressing P. aeruginosa, PA01 (provided by Dr. T. Machen, University of California, Berkeley, CA), K. pneumoniae strain K2 (from Dr. Korfhagen, Cincinnati Children’s Hospital, Cincinnati, OH) and a clinical isolate of S. aureus were used in the current study. To minimize variability in virulence, all bacteria were selected from aliquots of the same passage that had been frozen at –70°C in 20% glycerol/PBS. For each experiment, an aliquot of bacteria was thawed and plated on tryptic soy/5% sheep blood agar. A colony was inoculated in 4 ml of Luria Bertani medium (P. aeruginosa, K. pneumoniae) or brain-heart-infusion medium (S. aureus) and grown to late log phase. Bacteria were pelleted from the medium, washed in sterile PBS, and resuspended in 4 ml of HBSS supplemented with 4.5 mM glucose. Bacteria were enumerated as CFU on blood agar plates from serial dilutions in PBS. For each experiment, the inoculum was confirmed by plating dilutions of the aliquot used for intratracheal injection.

**Bacterial killing in vitro**

One thousand CFU of S. aureus or 1 × 10<sup>6</sup> CFU of P. aeruginosa were suspended in 100 µl of 10 mM potassium phosphate (pH 7.4) and incubated for 3 h at 37°C with increasing amounts of purified recombinant lysozyme M, D53S, or buffer alone in quadruplicate in a 96-well plate. Quantitative cultures were performed and viable pathogen counts determined.

**Bacterial killing in vivo**

The doses of bacteria selected for the current study were based on previous experiments in the FVB/N mouse strain (11, 22). To assess bacterial killing, 1 × 10<sup>5</sup> CFU P. aeruginosa, 1 × 10<sup>6</sup> CFU of K. pneumoniae, or 1 × 10<sup>5</sup> CFU of S. aureus in 100 µl of PBS were administered by intratracheal instillation as previously described (10). Mice were sacrificed 12 h (S. aureus), 24 h (S. aureus, P. aeruginosa, K. pneumonia), or 48 h (S. aureus) postinfection, the lungs were weighed, homogenized, and dilutions were plated for quantitative culture. The numbers of colonies were expressed as CFU per gram of lung tissue. To assess systemic dissemination of infection, splenic homogenates were plated and incubated overnight at 37°C and the number of colonies counted. For each experiment, 8–10 mice from each group were infected. Studies were conducted twice for each pathogen and results pooled.

**Lung histopathology**

Twenty-four hours following intratracheal challenge with P. aeruginosa, lungs from 5-wk-old LysM<sup>−/−</sup> mice (n = 4) or LysM<sup>+/+</sup>/LysM<sup>D53S</sup> littermates (n = 4) were inflation-fixed and 5-µm paraffin-embedded sections were stained with Brown and Brenn reagents (Poly Scientific). Bacteria were visualized at ×250 magnification with a Zeiss microscope.

**Survival studies**

LysM<sup>−/−</sup> and LysM<sup>+/+</sup>/LysM<sup>D53S</sup> mice (n = 20 for each group) were infected by intratracheal instillation with either 1 × 10<sup>5</sup> CFU of P. aeruginosa or 1 × 10<sup>6</sup> CFU of K. pneumoniae suspended in 100 µl of PBS. Water and food were provided ad libitum during the period of observation. The number of surviving mice was documented every 12 h for up to 120 h, at which time surviving mice were sacrificed.

**Statistical analyses**

Data are expressed as mean ± SEM. For bacterial clearance, data are reported as CFU per gram of lung tissue. Differences between groups were assessed by one-way ANOVA, and differences between means were assessed by contrast comparisons and the Student-Newman-Keuls test (Statview; SAS Institute). Nonparametric survival distributions were estimated to examine differences in survival between LysM<sup>−/−</sup> and LysM<sup>+/+</sup>/LysM<sup>D53S</sup> mice. Differences between the groups were analyzed from the Kaplan-Meier curve by log-rank test.

MURAMIDASE-DEFICIENT LYSOZYME KILLS BACTERIA
Results

Recombinant WT and mutant lysozyme M

Glutamic acid 35 and aspartic acid 53 are both essential for the muramidase activity of lysozyme M. In the current study, the codon for aspartic acid 53 was mutated to encode serine (D53S). The mutation was confirmed by sequence analysis and cDNA constructs encoding mutant and WT lysozyme M were cloned into baculovirus vectors for expression in insect cells. Recombinant WT and D53S lysozyme, \( M_r = 14 \text{k} \), were readily detected and purified from medium, indicating that the D53S substitution did not perturb folding and secretion of mouse lysozyme M (Fig. 1, A and B). To confirm that muramidase activity was ablated in the D53S recombinant protein, lysozyme enzyme activity was assessed using a well-characterized turbidimetric assay. Muramidase activity for the WT protein was 50 U/ng whereas no activity was detected with up to 10-fold more D53S protein (Fig. 1C). Collectively, these results indicate that the D53S substitution completely abrogated muramidase activity without affecting the immunoreactivity or secretion of the protein.

The antimicrobial activity of recombinant WT and D53S lysozyme was tested against a Gram-negative bacterium (\( P. \) aeruginosa) and a Gram-positive bacterium (\( S. \) aureus). The bactericidal activity of muramidase-deficient lysozyme was not significantly different from that of the recombinant WT enzyme (Fig. 2). As previously reported (15), the dose required to kill 50% of \( P. \) aeruginosa (70 pM) was lower than that required to kill 50% of \( S. \) aureus (110 pM).

Generation and characterization of transgenic mice

The ability of muramidase-deficient lysozyme to kill airway pathogens in vivo was tested by generating mice that expressed the D53S protein in the distal airway epithelium. Five independent \( LysM^{+/-} / LysM^{D53S} \) transgenic lines were generated and screened...
by Western blotting to determine the concentration of lysozyme in BALF (Fig. 3). Lysozyme concentration was elevated ~8-fold in transgenic line 5.7 and this line was subsequently crossed into the lysozyme M<sup>−/−</sup> background (LysM<sup>−/−</sup>/LysoM<sup>D53S</sup>). S1 nuclease analyses indicated that the level of lysozyme M mRNA (which included both WT and D53S transcripts) was significantly increased in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice compared with WT mice (Fig. 4A). The level of lysozyme M mRNA was similarly elevated in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice, suggesting that expression of the D53S transgene (rather than increased expression of endogenous (WT) gene) accounted for elevated LysM mRNA in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice. Lysozyme P mRNA was detected at relatively low abundance in both WT and LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice; however, expression of lysozyme P was increased in LysM<sup>−/−</sup> mice and this increase was maintained when the transgene was expressed in the null background (LysM<sup>−/−</sup>/LysoM<sup>D53S</sup>).

Lysozyme M and P proteins appeared to be present in similar concentrations in BALF of FVB/N mice (Fig. 4B); however, lysozyme P was previously shown to be more immunoreactive than lysozyme M in Western blots, indicating that lysozyme M was in fact the predominant isof orm (15). Lysozyme P protein was increased to the same extent in BALF from LysM<sup>−/−</sup> and LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice consistent with S1 nuclease analyses (Fig. 4B). The concentration of D53S protein in BALF was similar in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> and LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice, confirming that the mutant protein was secreted into the airspaces where it comprised the majority of lysozyme protein.

Immunohistochemical analyses detected lysozyme in alveolar type II cells and in alveolar macrophages of WT mice (Fig. 4C). Strong immunoreactivity was also detected in nonciliated bronchiolar epithelial cells (Clara cells) of LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice (Fig. 4C) and LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice (data not shown), suggesting that these cells were a major source of secreted lysozyme protein in the lungs of transgenic mice. Lung architecture was normal and cellular infiltration of airspaces was not detected in transgenic mice of both genotypes. Muramidase activity in BALF from LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> was not significantly different from that in WT mice (Fig. 4D). Muramidase activity was also decreased to the same extent (~50%) in LysM<sup>−/−</sup> mice and LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice confirming that the D53S protein was enzymatically inactive and did not alter LysP expression. Overall, expression of the D53S transgene significantly increased the level of lysozyme protein in the airspaces without increasing muramidase activity.

**Bacterial killing**

To assess the effect of secreted D53S protein on bacterial killing in vivo, mice from all four genotypes (WT, LysM<sup>−/−</sup>/LysoM<sup>D53S</sup>, LysM<sup>−/−</sup>, and LysM<sup>−/−</sup>/LysoM<sup>D53S</sup>) were intratracheally infected with <i>P. aeruginosa</i> and bacterial burden was assessed after 24 h (Fig. 5A). Bacterial burden was increased 3.5-fold in LysM<sup>−/−</sup> mice compared with WT mice. Expression of D53S enhanced bacterial killing 5-fold in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice compared with WT mice and 17.5-fold compared with LysM<sup>−/−</sup> mice. Importantly, D53S protein completely restored killing of <i>P. aeruginosa</i> in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice. Muramidase activity was similarly elevated in LysM<sup>−/−</sup> and LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice following infection, indicating that lysozyme P compensation did not account for restoration of pathogen killing. To determine whether D53S conferred protection against infection with another clinically important Gram-negative bacteria, mice were challenged with Klebsiella pneumoniae. Consistent with results of a previous study (11), bacterial burden in the lungs of LysM<sup>−/−</sup> mice was increased 3- to 4-fold compared with WT mice (Fig. 5B). Expression of D53S protein completely restored killing of <i>K. pneumoniae</i> in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice and enhanced bacterial killing in WT mice, similar to results for <i>P. aeruginosa</i> (Fig. 5A). Decreased susceptibility of LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice to infection with <i>P. aeruginosa</i> was reflected in histopathology at 24 h postinfection (Fig. 5C). Lung sections showed lobar pneumonia in all four groups of mice with the most severe consolidation in LysM<sup>−/−</sup> mice. <i>P. aeruginosa</i> was detected in all alveoli of LysM<sup>−/−</sup> mice whereas only occasional alveoli were infected in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice. Intratracheal challenge with a larger dose of <i>P. aeruginosa</i> (1 × 10<sup>6</sup> CFU) resulted in death of 40% of LysM<sup>−/−</sup> mice 84 h after infection (Fig. 6). At this time point, survival was modestly (80 vs 60%) but significantly (<i>p</i> < 0.04) increased for infected LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> mice. A similar pattern was observed when mice were challenged with <i>K. pneumoniae</i> (data not shown). These results indicate that muramidase-deficient lysozyme protects against airway infection by clinically important Gram-negative organisms.

The ability of D53S to enhance killing of Gram-positive bacteria was assessed after intratracheal installation of <i>S. aureus</i>. Bacterial burden was significantly increased in LysM<sup>−/−</sup> mice compared with WT mice at 12, 24, and 48 h after infection, although relatively few bacteria remained in the airspaces after 48 h (Fig. 7). Expression of D53S completely restored killing of <i>S. aureus</i> in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice at all three time points. These results indicate that muramidase-deficient lysozyme confers protection against a clinically important Gram-positive organism in vivo.

In a separate experiment, mice were intratracheally inoculated with <i>S. aureus</i> and BALF were collected at time points up to 120 h postinfection for analyses of cellular composition (Fig. 8). In WT mice, neutrophil influx peaked 12–24 h postinfection and returned to preinfection levels by 72 h. Neutrophil influx in LysM<sup>−/−</sup> mice was dramatically elevated at 12–24 h (<i>p</i> < 0.01) and persisted to 120 h postinfection. Despite enhanced bacterial killing conferred by the D53S protein, inflammation in LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice was elevated and not significantly different from that in LysM<sup>−/−</sup> mice. Surprisingly, the percentage of neutrophils was also significantly elevated in BALF from LysM<sup>−/−</sup>/LysoM<sup>D53S</sup> transgenic mice (<i>p</i> < 0.02 at 12 and 24 h). This result likely
reflects the fact that the D53S protein can bind but not hydrolyze substrate and thus may act as a competitive inhibitor of endogenous lysozyme M in these mice.

**Discussion**

Based on the results of in vitro studies, lysozyme is widely acknowledged to be bactericidal for some Gram-positive bacteria with relatively little activity against Gram-negative organisms (23, 24). Resistance to the bactericidal effects of lysozyme has been attributed to the presence of an outer membrane in Gram-negative bacteria that limits access of the enzyme to the underlying peptidoglycan substrate. In contrast, peptidoglycan in the cell wall of Gram-positive bacteria is readily accessible to lysozyme and is consequently rapidly degraded, ultimately leading to cell lysis. These observations in vitro, coupled with the fact that lysozyme is one of the most abundant antimicrobial proteins in the lungs, have led to predictions that 1) lysozyme plays an important role in killing Gram-positive airway pathogens in vivo and 2) the peptidoglycan substrate. In contrast, peptidoglycan in the cell wall of bacteria that limits access of the enzyme to the underlying peptidoglycan is readily accessible to lysozyme and is consequently rapidly degraded, ultimately leading to cell lysis.

To test the importance of muramidase activity for bacterial killing in vivo, a catalytically inactive form of lysozyme was generated. Substitution of serine for aspartic acid in the active site of mouse lysozyme M resulted in complete loss of muramidase activity, as previously reported for lysozyme from other species (20, 25, 26). Muramidase-deficient recombinant lysozyme readily killed *S. aureus* in vitro, confirming the findings of Ibrahim et al. (20). Recombinant D53S protein also killed two Gram-negative bacteria, *P. aeruginosa* (Fig. 2) and *K. pneumoniae* (data not shown). Interestingly, the ability of recombinant lysozyme to kill Gram-positive or Gram-negative bacteria was not significantly affected by the loss of muramidase activity. Targeted expression of muramidase-deficient lysozyme in respiratory epithelial cells of *LysM<sup>-/-</sup>* mice significantly increased lysozyme protein concentration in the airspaces without increasing muramidase activity. Consistent with the results of in vitro assays, elevated levels of catalytically inactive lysozyme in BALF were associated with enhanced bactericidal activity against clinically important Gram-positive and Gram-negative bacteria. Intratracheal infection of *LysM<sup>-/-</sup>* mice with *S. aureus* resulted in a significant increase in bacterial burden. Importantly, expression of the D53S transgene completely restored killing of *S. aureus* in *LysM<sup>-/-</sup>* mice. Similarly, increased susceptibility of *LysM<sup>-/-</sup>* mice to the Gram-negative bacteria *P. aeruginosa* and *K. pneumoniae* was completely reversed by targeted expression of catalytically inactive lysozyme. Further, expression of the D53S transgene modestly but significantly enhanced survival of *LysM<sup>-/-</sup>* (*LysM<sup>-/-</sup>/LysM<sup>D53S</sup>) mice and significantly enhanced bacterial clearance in WT (*LysM<sup>+/+</sup>*/
**FIGURE 5.** Muramidase-deficient lysozyme M enhances killing of Gram-negative bacteria in transgenic mice. Five-week-old *LysM<sup>D53S</sup>* / *LysM<sup>D53S</sup>* mice. Neutrophil influx was similarly increased in *LysM<sup>-/-</sup>* and *LysM<sup>-/-</sup>/ *LysM<sup>D53S</sup>* mice suggesting that the D53S protein, rather than neutrophil-derived antimicrobial peptides, accounted for the correction of bacterial killing in *LysM<sup>-/-</sup>* mice. Collectively, these results provide the first evidence that the muramidase-independent bactericidal activity of lysozyme plays an important role in airway host defense.

Although the concept of muramidase-independent killing of bacteria continues to be debated (27), there is increasing evidence in support of this hypothesis (20, 22, 28, 29). Recent studies identified a bactericidal domain that mapped to the C-terminal region of chicken and human lysozyme (30). Antimicrobial peptides in general are cationic at physiologic pH and associate with microbial membranes through electrostatic interactions that are often facilitated by an amphipathic helical structure. Lysozyme contains a conserved, cationic helix-loop-helix (residues 87–115 of lysozyme) motif, which is responsible for bactericidal activity against both Gram-positive and Gram-negative bacteria (24, 30). It is conceivable that the helix-loop-helix motif is responsible for part or all of the bactericidal activity of catalytically inactive lysozyme in transgenic mice.

The present study cannot exclude a role for muramidase activity in bacterial killing. Constitutively elevated concentration of the D53S transgene protein may partially compensate for the loss of muramidase activity by increasing the content of catalytically inactive but bactericidal lysozyme protein in the airspaces. It is also possible that muramidase activity may be acquired for killing of pathogens not tested in the current study. Although the role of muramidase activity in bacterial killing remains unclear, there is increasing evidence that muramidase activity may play an important role in modulating inflammation at the site of infection by rapidly degrading peptidoglycan. Peptidoglycan fragments derived from autolysis of Gram-positive cell walls induce a strong inflammatory response (31). Ganz et al. (18) demonstrated that inefficient hydrolysis of peptidoglycan in *LysM<sup>-/-</sup>* mice was associated with a prolonged and intense inflammatory response following s.c. injection of *Micrococcus luteus*, consistent with the hypothesis that muramidase activity limits inflammation associated with infection by Gram-positive organisms. This hypothesis is supported by the results of the present study which demonstrated that expression of the D53S protein completely restored killing of *S. aureus* without correcting the persistent, elevated inflammatory response in *LysM<sup>-/-</sup>* mice. The abundant muramidase activity in mouse

**FIGURE 6.** Muramidase-deficient lysozyme enhances survival following infection with Gram-negative bacteria. Twenty 5-wk-old *LysM<sup>-/-</sup>* or *LysM<sup>-/-</sup>/ *LysM<sup>D53S</sup>* mice were infected with 10<sup>7</sup> CFU of *P. aeruginosa* by intratracheal instillation. Survival was monitored for 120 h at which time surviving mice were sacrificed; *p* < 0.01 by log-rank test; *n* = 20 mice/group; *p* < 0.04.
BALF, arising from lysozyme secreted by macrophages and alveolar type II epithelial cells, as well as a nonlysozyme muramidase activity of unknown cellular origin (15), also supports an important role for peptidoglycan degradation in innate host defense of the airspaces.

Disruption of the LysM locus by insertion of GFP (17) resulted in elevated expression of lysozyme P protein in type II epithelial cells (15) and macrophages (18) but not neutrophils. Despite compensatory expression of LysP, muramidase activity in LysM+/– mice was only partially restored resulting in increased susceptibility to infection by K. pneumoniae (11) and M. luteus (18). Interestingly, lysozyme P mRNA and protein remained elevated in LysM+/–/LysMD53S transgenic mice despite abundant LysMD53S protein in type II epithelial cells and in the airspaces. Further, transcription from the LysM locus was maintained in both LysM+/– mice (15, 18) and LysM+/–/LysMD53S transgenic mice (data not shown), as indicated by detection of GFP in type II cells and macrophages. These results suggest that muramidase activity may also be an important regulator of LysP gene expression, although the pathway through which such signaling is transduced is not known.

The results of current studies in transgenic mice and previous experiments with catalytically inactive, recombinant enzyme suggest that lysozyme kills bacteria independent of its muramidase activity. This conclusion is supported by the observation that peptides derived from lysozyme and lacking muramidase activity effectively killed both Gram-positive and Gram-negative bacteria. It remains to be determined whether the muramidase-independent bactericidal property of lysozyme is generalizable to other common respiratory pathogens.

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Disclosures

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