Glutaredoxin-1 Attenuates S-Glutathionylation of the Death Receptor Fas and Decreases Resolution of *Pseudomonas aeruginosa* Pneumonia

Vikas Anathy^{1*}, Scott W. Aesif^{1*†}, Sidra M. Hoffman^{1*}, Jenna L. Bement², Amy S. Guala¹, Karolyn G. Lahue¹, Laurie W. Leclair², Benjamin T. Suratt², Carlyne D. Cool⁴, Matthew J. Wargo³, and Yvonne M. W. Janssen-Heininger¹

¹Department of Pathology, ²Department of Medicine, and ³Department of Microbiology and Molecular Genetics, University of Vermont College of Medicine, Burlington, Vermont; and ⁴University of Colorado Anschutz Medical Campus, Aurora, Colorado

Abstract

Rationale: The death receptor Fas is critical for bacterial clearance and survival of mice after *Pseudomonas aeruginosa* infection.

Objectives: Fas ligand (FasL)-induced apoptosis is augmented by S-glutathionylation of Fas (Fas-SG), which can be reversed by glutaredoxin-1 (Grx1). Therefore, the objective of this study was to investigate the interplay between Grx1 and Fas in regulating the clearance of *P. aeruginosa* infection.

Methods: Lung samples from patients with bronchopneumonia were analyzed by immunofluorescence. Primary tracheal epithelial cells, mice lacking the gene for Grx1 (*Grx1*^{-/-}), *Grx1*^{-/-} mice treated with caspase inhibitor, or transgenic mice overexpressing Grx1 in the airway epithelium were analyzed after infection with *P. aeruginosa*.

Measurements and Main Results: Patient lung samples positive for *P. aeruginosa* infection demonstrated increased Fas-SG compared with normal lung samples. Compared with wild-type primary lung epithelial cells, infection of *Grx1*^{-/-} cells with *P. aeruginosa* showed enhanced caspase 8 and 3 activities and cell death in association with increases in Fas-SG. Infection of *Grx1*^{-/-} mice with *P. aeruginosa* resulted in enhanced caspase activity and increased Fas-SG as compared with wild-type littermates. Absence of *Grx1* significantly enhanced bacterial clearance, and decreased mortality postinfection with *P. aeruginosa*. Inhibition of caspases significantly decreased bacterial clearance postinfection with *P. aeruginosa*, in association with decreased Fas-SG. In contrast, transgenic mice that overexpress Grx1 in lung epithelial cells

had significantly higher lung bacterial loads, enhanced mortality, decreased caspase activation, and Fas-SG in the lung after infection with *P. aeruginosa*, compared with wild-type control animals.

Conclusions: These results suggest that S-glutathionylation of Fas within the lung epithelium enhances epithelial apoptosis and promotes clearance of *P. aeruginosa* and that glutaredoxin-1 impairs bacterial clearance and increases the severity of pneumonia in association with deglutathionylation of Fas.

Keywords: *Pseudomonas*; glutaredoxin-1; protein S-glutathionylation; Fas; apoptosis

At a Glance Summary

Scientific Knowledge on the Subject: *Pseudomonas aeruginosa* is an opportunistic bacterial pathogen that can cause life-threatening infections in the lungs.

What This Study Adds to the Field: We have identified a novel role for redox-based control of the Fas death receptor pathway by glutaredoxin-1 in the pathogenesis of *P. aeruginosa* pneumonia, and our work suggests that glutaredoxin-1 and Fas S-glutathionylation are potential targets for therapeutic intervention during *P. aeruginosa* pneumonia.

(Received in original form October 27, 2013; accepted in final form December 5, 2013)

*These authors contributed equally to the work.

†Present address: Department of Pathology, The George Washington University, 2100-W Pennsylvania Avenue NW, Washington, DC 20037.

Supported by National Institutes of Health R01 HL060014, HL079331, R03 HL095404 (Y.M.W.J.-H.); American Thoracic Society unrestricted grant; Parker B. Francis Fellowship; Department of Pathology and internal grant program College of Medicine (UVM) (V.A.); and P20 GM103496 (M.J.W.). S.W.A. was supported by T32 ES07122.

Author Contributions: V.A., S.W.A., M.J.W., and Y.M.W.J.-H. conceived and designed the study. V.A., S.W.A., S.M.H., J.L.B., A.S.G., K.G.L., and M.J.W. performed experiments. L.W.L., B.T.S., and C.D.C. provided human lung samples. V.A., S.W.A., S.M.H., M.J.W., and Y.M.W.J.-H. analyzed data and wrote the paper.

Correspondence and requests for reprints should be addressed to Yvonne M. W. Janssen-Heininger, Ph.D., University of Vermont College of Medicine, Department of Pathology, 89 Beaumont Avenue, Burlington, VT 05405. E-mail: yjanssen@uvm.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 189, Iss 4, pp 463–474, Feb 15, 2014

Copyright © 2014 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201310-1905OC on December 10, 2013

Internet address: www.atsjournals.org

Pseudomonas aeruginosa is a well-studied opportunistic bacterial pathogen that can cause life-threatening infections in the lungs of patients with cystic fibrosis, chronic obstructive pulmonary disease, and who are immunocompromised (1, 2). *P. aeruginosa* also causes significant morbidity and mortality in critically ill ventilated patients (3). The airway epithelium contributes significantly to lung immunity and clearance after *P. aeruginosa* infection in several ways, including activation of the death receptor Fas (4, 5). Absence of the Fas-FasL system results in the rapid development of sepsis and death of animals (4).

S-glutathionylation is an oxidative modification of protein cysteines that regulates structure and function (6). We have demonstrated that oxidative modification of Fas in the form of S-glutathionylation (Fas-SSG) enhances its ability to form the death-inducing signaling complex and accelerates apoptosis in epithelial cells. Furthermore, we also showed that overexpression of glutaredoxin-1 (Grx1) resulted in deglutathionylation of Fas and decreased FasL-induced apoptosis in lung epithelial cells (7), highlighting the importance of Fas-SSG in promoting apoptosis and the importance of Grx1 in restricting Fas-dependent apoptosis.

Given the impact of Grx1 on the regulation of Fas-SSG, and the critical role of Fas in the clearance of *P. aeruginosa*, the goal of the present study was to use *Grx1*^{-/-} mice (8) and transgenic mice that overexpress Grx1 specifically in the lung epithelium, to examine the interplay between the Grx1 and Fas-SSG in the pathogenesis of *P. aeruginosa*-induced pneumonia. Our results strongly indicate that epithelial Grx1 plays a critical role in limiting clearance of *P. aeruginosa* and enhancing mortality in part by deglutathionylation of Fas, decreased caspase activities, and attenuation of epithelial cell death.

Methods

Mice

For all experiments, 2-month-old male *Grx1*^{-/-} mice and littermate wild-type (WT) C57BL/6 control mice were used. Transgenic *CC10rtTA/TetO-FLAG Grx1* mice were maintained on doxycycline-

containing chow (6 g/kg, Purina Diet Tech, St. Louis, MO) for 1 week before infection. Mice were housed in a barrier facility. All studies were approved by the Institutional Animal Care and Use Committee at the University of Vermont. Additional information about the source of mice and generation of *CC10-rtTA/TetO-FLAG Grx1* transgenic mice is described in the online supplement.

Human Samples

Human lung autopsy tissue samples from patients with bronchopneumonia were obtained from Dr. Cool (University of Denver). The samples were coded by Dr. Cool. The uninfected (normal) human lung autopsy tissue samples were obtained from the National Institutes of Health lung tissue research consortium. Studies were approved by institutional review boards of the University of Denver and the University of Vermont, and recognized as nonhuman subject research.

In Situ Detection of S-Glutathionylated Fas in Human Tissue after Grx1 Catalyzed Cysteine Derivatization

S-glutathionylated Fas was detected as described elsewhere (9), with slight modification. After dewaxing tissue samples, free thiol groups and endogenous biotin were blocked using a buffer that contained 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 0.1 mmol/L ethylenediaminetetraacetic acid, pH 8.0, 0.01 mmol/L neocuproine, 40 mmol/L *N*-ethylmaleimide (Sigma, St. Louis, MO), 200 ng/ml streptavidin, and 1% Triton (Sigma) for 1 hour. After three washes with phosphate-buffered saline (PBS), S-glutathionylated cysteine groups were reduced by incubation with 13.5 μg/ml human Grx1 (Lab Frontiers, Seoul, South Korea), 35 μg/ml GSSG reductase (Roche, Madison, WI), 1 mmol/L GSH (Sigma), 1 mmol/L NADPH (Sigma), 18 μmol ethylenediaminetetraacetic acid, and 137 mmol/L Tris HCl (pH 8.0) for 20 minutes. After three washes with PBS, newly reduced cysteine residues were labeled with 1 mmol/L *N*-(3-maleimidylpropionyl) biocytin (Roche) for 1 hour. Slides were washed with PBS three times. Next, tissue samples were incubated with 0.5 μg/ml streptavidin-conjugated Alexa Fluor 647 for 1 hour. Tissue were blocked using 5% bovine serum

albumin in PBS for 1 hour. They were then incubated with anti-Fas antibody (5 μg/ml) (BD Biosciences, San Jose, CA) overnight at 4°C. Next, tissues were incubated with 2.5 μg/ml of an antimouse antibody, conjugated with Alexa Fluor 488 for 1 hour. Nuclei were stained using 25 nmol/L of DAPI (Molecular Probes, Grand Island, NY). As negative controls, Grx1 or anti-Fas antibody was omitted from the reaction mix. Tissues were analyzed using a confocal laser scanning microscope (Zeiss LSM 510, Thornwood, NY).

Statistics

Statistical analyses of all data were performed using the Graph Pad Prism software (Graphpad Inc., San Diego, CA) by the Gehan-Breslow-Wilcoxon test, two-way analysis of variance, or the Student *t* test, where appropriate. Data from each experiment are presented as $\pm$ of the SEM. Analyses with resultant *P* values of less than 0.05 were identified as significant. For the cell culture experiments, data are expressed as mean ($\pm$ SEM) of three experiments in triplicate, reflecting a total of nine measurements. Additional information is available in the online supplement.

Results

Patients Infected with *Pseudomonas aeruginosa* Show Increased Fas-SSG

Pseudomonas aeruginosa is known to induce Fas-mediated apoptosis in mouse lung epithelial cells (4). We have demonstrated that formation of Fas-SSG enhances epithelial apoptosis (7, 10). Therefore, to test whether Fas-SSG is increased in patients positive for *P. aeruginosa* infection, lung samples from patients diagnosed with bronchopneumonia (*n* = 3) and patients without underlying pneumonia (normal, *n* = 3) were stained for Fas and S-glutathionylated proteins (PSSG), and imaged using confocal laser scanning microscopy. Patients with bronchopneumonia clearly showed an increase in protein S-glutathionylation (PSSG, red). Colocalization with Fas (green) indicated an overlap of Fas and PSSG (yellow in merge image), suggesting formation of Fas-SSG (merge, yellow), predominantly within the bronchial epithelium (Figure 1A), which was confirmed using Mander coefficient

Figure 1. Fas S-glutathionylation is increased in humans infected with *Pseudomonas aeruginosa*. (A) Normal lung tissue sections and sections from patients diagnosed with bronchopneumonia were stained using an antibody for Fas (green), or subjected to glutaredoxin-1 (Grx1)-mediated derivatization of S-glutathionylated proteins (PSSG, red). (B–D) Measurement of colocalization and intensity of Fas and PSSG in normal lungs and patients with bronchopneumonia. Data are expressed as mean ($\pm$ SEM) of three random measurements per patient, $*P < 0.05$ (t test) as compared with normal subjects. (E) Confirmation of *P. aeruginosa* infection in tissues stained in A, using a *P. aeruginosa*-specific antibody (red). Insets show high magnification images ($\times 400$). (F) Confirmation of *P. aeruginosa* (red) in mouse lungs, as a positive control. PBS = phosphate-buffered saline.

Figure 1. (Continued).

(Figure 1B). We did not observe any significant increases in Fas itself in bronchopneumonia samples as compared with normal lungs (Figure 1C). However, quantitation of PSSG showed a significant increase in bronchopneumonia samples over normal lungs (Figure 1D). Evaluation for the presence of *P. aeruginosa* by immunohistochemistry confirmed infection of *P. aeruginosa* in patients (red) with bronchopneumonia, and in mice infected with PAO1 (Figures 1E and 1F). All together these findings indicate an

association between bronchopneumonia and increases in Fas-SSG in lung epithelial cells.

Infection of Primary Mouse Tracheal Epithelial Cells with *Pseudomonas aeruginosa* Induces Fas-SSG and Increases Caspase Activity

Pseudomonas aeruginosa is known to activate the Fas pathway in lung epithelial cells (4). To confirm that *P. aeruginosa* induces epithelial apoptosis in a Fas- and caspase- dependent manner, we infected

primary mouse tracheal epithelial (MTE) cells (11) from WT mice, mice lacking functional Fas (*lpr*), and mice lacking caspase 3 (*Casp3*^{-/-}) with the clinical *P. aeruginosa* isolate, PA14. Our results showed that CFUs were similar in WT, *lpr*, and *Casp3*^{-/-} MTE cells (Figure 2A). However, activation of caspases 8 and 3 and subsequent cell death was significantly lower in *lpr* MTE cells as compared with WT (Figures 2B–2D). Similarly, PA14-mediated cell death was significantly decreased in *Casp3*^{-/-} MTE cells compared

Figure 2. PA14 increases Fas S-glutathionylation and activity of caspases in mouse tracheal epithelial (MTE) cells. (A) MTE cells isolated from wild-type (WT), *Glrx1*^{-/-}, Fas deficient (*lpr*), or *Caspase3*^{-/-} (*Casp3*^{-/-}) genotypes were infected with *Pseudomonas aeruginosa* (PA14 strain) for the indicated times, and supernatants were assessed for CFUs. (B and C) Measurement of caspases in PA14-infected MTE cells. (D) Analysis of cell death using the dead cell protease assay. Data are expressed as mean (±SEM) of three experiments each conducted in triplicate, reflecting data averaged from a total of nine measurements. **P* < 0.05 (analysis of variance) compared with uninfected controls of the same genotype, #*P* < 0.05 (analysis of variance) compared with WT groups at the same time point, †*P* < 0.05 (analysis of variance) compared with the *lpr* and *Casp3*^{-/-} genotype at the same time point. (E) Representative Western blots of Fas S-glutathionylation, and content of FasL in the supernatants (SN) of PA14-infected MTE cells. WCL = whole cell lysates to confirm expression of Fas, caspase 3, glutaredoxin-1 (Grx1), and β-actin as a loading control. RLU = relative light units.

with WT cells (Figures 2B–2D). Collectively, these results confirm the importance of Fas in mediating increases in caspase activities and the importance of caspase 3 in apoptosis of lung epithelial cells after infection with *P. aeruginosa*. Given our previous findings demonstrating that FasL-Fas-mediated death is amplified by its S-glutathionylation (Fas-SSG), and enhanced death of cells lacking the deglutathionylating enzyme, Grx1 (7), we next evaluated the impact of Grx1 status on Fas-SSG, caspase activation, and apoptosis after infection with PA14. Results in

Figure 2A show that CFUs were similar in *Glrx1*^{-/-} MTE cells as compared with the other genotypes. However, *Glrx1*^{-/-} MTE cells showed more robust increases in caspase 8 and 3 activities and enhanced death after infection with PA14, compared with WT cells. These results contrast the findings in *lpr* or *Casp3*^{-/-} MTE cells. As expected, Fas-SSG was markedly increased in *Glrx1*^{-/-} MTE cells compared with WT cells infected with PA14, whereas *Casp3*^{-/-} MTE cells show decreased Fas-SSG content compared with WT cells (Figure 2E), consistent with our previous

observations (7, 10). We have previously shown that stimulation of lung epithelial cells with FasL leads to increases in Fas-SSG (7). To determine whether increases in Fas-SSG were associated with increases in FasL in *Glrx1*^{-/-} MTE cells, we subjected culture supernatants to Western blot analysis, using a FasL antibody. The Western blots clearly demonstrated a similar increase in secretion of the 26-kD soluble form of FasL (SN FasL) from all genotypes (Figure 2E), indicating that increases in Fas-SSG in PA14-infected *Glrx1*^{-/-} MTE cells compared with

WT cells were not caused by increases in secretion of FasL *per se*.

***Glrx1*^{-/-} Mice Show Protection from *Pseudomonas aeruginosa* Pneumonia**

Pseudomonas aeruginosa has been shown to trigger apoptosis of bronchial epithelial cells, which has been associated with bacterial clearance and survival (4). Given that *P. aeruginosa* infection of *Glrx1*^{-/-} MTE cells resulted in increased Fas-SSG formation, caspase activity, and cell death (Figure 2), we next sought to determine the impact of genetic ablation of *Glrx1* in *P. aeruginosa*-induced pneumonia. Compared with WT mice, *Glrx1*^{-/-} mice showed significantly decreased bacterial loads at 24 and 48 hours in the lung, bronchoalveolar lavage fluid (BALF), blood, spleen, and liver; the latter three organs are measures of bacteremia and dissemination (Figures 3A and 3B). Importantly, *Glrx1*^{-/-} mice infected with *P. aeruginosa* strain PAO1 show a significant survival advantage (67%) as compared with WT mice (34%) (Figure 3C). In contrast, infection with *Escherichia coli* resulted in no difference in lung bacterial loads

between WT and *Glrx1*^{-/-} mice (see Figure E1 in the online supplement), demonstrating that the impact of Grx1 on clearance of bacterial infections is species dependent.

Assessment of Inflammatory Cytokines and Markers of Lung Damage after PAO1 Pneumonia

To determine whether altered inflammatory responses corresponded to the enhanced bacterial clearance and survival of *Glrx1*^{-/-} mice compared with WT littermates, we assessed total cell counts in BALF. Variable fluctuations in BAL cell counts were observed between WT and *Glrx1*^{-/-} mice infected with PAO1. Following 24 hours after PAO1-infection, *Glrx1*^{-/-} showed significant decreases in airway inflammation. However, at 48 hours BALF cell counts were enhanced in *Glrx1*^{-/-} mice compared with littermate (WT) mice. At 96 hours postinfection, BALF inflammatory cell counts were comparable between the two strains infected with PAO1 (see Figure E2). In contrast, infection with *E. coli* resulted in similar BALF cell counts in WT and *Glrx1*^{-/-} mice (see Figure E3).

Assessment of IL-1 β , IL-6, IL-10, and tumor necrosis factor- α in BALF showed similar fluctuating profiles, demonstrating decreases in these inflammatory markers in *Glrx1*^{-/-} mice compared with WT mice 24 hours after PAO1 infection. In contrast, after 48 hours of infection, *Glrx1*^{-/-} mice BALF cytokine levels were increased compared with WT mice with the exception of IL-10, which was virtually undetectable (see Figure E4). In addition, after 24 hours of PAO1-induced pneumonia, *Glrx1*^{-/-} mice had decreases in protein and lactate dehydrogenase (LDH) content in BALF compared with WT mice, suggestive of decreases in cellular injury (see Figure E5).

Pulmonary Infection with PAO1 Significantly Enhances Caspase Activation and Fas-SSG in *Glrx1*^{-/-} Mice

A previous report investigating the role of apoptosis in lung epithelial cells identified the critical role of the Fas/caspase axis in bacterial clearance and survival after *P. aeruginosa* infection (4). We therefore determined whether activation of caspases

Figure 3. Ablation of *Glrx1* protects mice from PAO1-induced pneumonia. (A and B) Clearance and dissemination of PAO1 in wild-type and *Glrx1*^{-/-} mice as measured by CFU at 24 (A) and 48 (B) hours postinfection. Data are expressed as mean ($\pm$ SEM) total CFU ($n = 8$ mice/group/time point), $*P < 0.05$ (analysis of variance) compared with C57BL/6 mice. (C) Survival study comparing the wild-type and *Glrx1*^{-/-} mice after pulmonary infection with PAO1. Data are expressed as percent survival of total population infected ($n = 7$ –8 mice per group), $P < 0.05$ (Gehan-Breslow-Wilcoxon test) as compared with C57BL/6 mice. BALF = bronchoalveolar lavage fluid.

Figure 4. Assessment of caspase activation and Fas S-glutathionylation in lung tissue of wild-type and *Glrx1*^{-/-} mice after infection with PAO1. (A and B) Wild-type mice were infected with PAO1, 24 hours later lungs were dissected and mechanically homogenized. As control subjects, mice were instilled with sterile phosphate-buffered saline (PBS). CD45 positive (+) and negative (-) cells were separated by antibody-conjugated magnetic beads, and assayed for caspase 8 and 3 activity. Data are expressed as mean ($\pm$ SEM) relative light units (RLU) and normalized to protein concentration ($n = 4$ mice per group), * $P < 0.05$ (analysis of variance) compared with respective PBS control animals, # $P < 0.05$ (analysis of variance) compared with the respective group in the CD45⁺ fraction. (C) Wild-type mice were infected with PAO1 and 24 hours later, lungs were dissected and paraffin-embedded sections were stained for active caspase 3 (red). Blue represents the DAPI nuclear stain. (D) Wild-type and *Glrx1*^{-/-} mice were infected with PAO1 and 24 hours later, lungs were dissected and whole-lung homogenates assayed for caspase 8 and 3. Data are expressed as mean ($\pm$ SEM) RLU ($n = 8$ mice per group), * $P < 0.05$ (analysis of variance) compared with the corresponding C57BL/6 group. (E) Wild-type and *Glrx1*^{-/-} mice were infected with PAO1 and 24 hours later lungs were dissected and mechanically homogenized. Lung lysates were immunoprecipitated (IP) using an anti-glutathione (GSH) antibody. Precipitated proteins were resolved by reducing gel electrophoresis and probed using an anti-Fas antibody. As control animals, *Glrx1*^{-/-} sample was incubated with dithiothreitol (+DTT) before electrophoresis. WLL = whole-lung lysates confirming expression of Fas, FasL, glutaredoxin-1 (Grx1), and β -actin as a loading control.

in response to infection of WT and *Glrx1*^{-/-} mice with *P. aeruginosa* occurred in hematopoietic cells (CD45⁺) or non hematopoietic cells (CD45⁻). After 24 hours of PAO1-induced pneumonia, WT mice showed a significant induction of caspase 8 and 3 activation in both CD45⁺ and CD45⁻ cells, with the highest increases occurring in CD45⁻ cells (Figures 4A and 4B). Increases in active caspase 3 were apparent in bronchiolar

epithelial cells (Figure 4C). After 24 hours of PAO1-induced pneumonia, *Glrx1*^{-/-} mice showed significantly higher caspase 8 and 3 activities in homogenized lung tissue compared with WT mice (Figure 4D). Recent studies from our laboratory have linked Grx1 as being critical to limiting FasL-induced caspase activation through the reversal of Fas-SSG (7, 10). We therefore investigated the extent of Fas-SSG after infection with PAO1. Compared with WT animals, *Glrx1*^{-/-} mice

showed significantly higher levels of Fas-SSG in lung homogenates without apparent differences in soluble (26 kD) or membrane bound (46 kD) FasL in either genotype (Figure 4E).

Activation of Caspases Is Required for *Pseudomonas aeruginosa* Clearance from the Lung

To evaluate the requirement of caspases in clearance of *P. aeruginosa* from the lung, we treated WT and *Glrx1*^{-/-} mice with pan

caspase inhibitor zVAD-FMK, or the vehicle control dimethyl sulfoxide (DMSO), 16 hours before and simultaneously with PAO1 infection. As expected, zVAD led to strong decreases in caspase 3 activities in lung tissues from WT and *Glrx1*^{-/-} mice, compared with DMSO control animals, and

increases in caspase 3 activity in lung tissue from *Glrx1*^{-/-} mice were almost completely attenuated to levels observed WT mice in response to zVAD (Figure 5A). CFUs were significantly lower in lung tissues from DMSO-treated *Glrx1*^{-/-} mice compared with WT littermates, in

agreement with data in Figure 3A. Treatment with zVAD significantly increased lung CFUs to comparable levels in both genotypes, as compared with vehicle control animals (Figure 5B). Total cell counts, reflective of neutrophils, in BALF were significantly increased in *Glrx1*^{-/-} mice as compared with WT mice infected with *P. aeruginosa* (see Figure E6). These findings contrasted results in Figure E2, likely caused by administration of DMSO. BALF macrophages were decreased in zVAD-treated WT animals, and in vehicle-exposed *Glrx1*^{-/-} mice (see Figure S6). Lastly, increases in Fas-SSG observed in PAO1-infected *Glrx1*^{-/-} mice compared with WT groups were attenuated to almost undetectable levels after treatment with zVAD (Figure 5C). These data collectively demonstrate the importance of caspase activation in promoting Fas-SSG and enhancing bacterial clearance from lungs of *Glrx1*^{-/-} mice.

Overexpression of Grx1 in the Lung Epithelium Decreases Fas-SSG and Caspase Activation and Increases *Pseudomonas aeruginosa* Infection

Given previously published data implicating the lung epithelium as being critical to *P. aeruginosa* clearance after lung infection (4), we next determined the direct impact of Grx1 overexpression in airway epithelial cells. We generated a transgenic mouse overexpressing FLAG-Grx1 in a doxycycline-dependent manner, specifically in lung epithelial cells by crossing *TetO-FLAG Grx1* mice with *CC10rtTA* transgenic animals (12). Results in Figures 6A and 6B demonstrate the lung-specific overexpression of the FLAG-Grx1 protein in a doxycycline-dependent manner in bitransgenic mice. Addition of the FLAG tag (~1 kD) resulted in impeded migration of the low-molecular-weight Grx1 (12 kD) through a 15% polyacrylamide gel resulting in two bands when analyzed by Western blot. Immunohistochemistry using an anti-FLAG antibody also confirmed the expression of the transgene specifically within lung epithelial cells, both within the bronchiolar epithelium and in type II pneumocytes (Figure 6C), consistent with expression patterns observed previously using the same transgene (12). To directly assess the impact of epithelial Grx1 expression on PAO1 clearance, doxycycline

Figure 5. Inhibition of caspase increases CFUs, and decreases Fas S-glutathionylation in the lung after infection with PAO1. (A) Assessment of caspase 3 activity in lung tissue, 24 hours after infection with PAO1 in wild-type (WT) and *Glrx1*^{-/-} mice treated with dimethyl sulfoxide (DMSO) or zVAD-FMK. Data are expressed as mean ($\pm$ SEM) relative luminescence units (RLU) and normalized to protein concentration ($n = 9$ – 10 mice per group), $*P < 0.05$ (analysis of variance) compared with respective DMSO groups. $^{\#}P < 0.05$ (analysis of variance) compared with the corresponding WT groups. (B) Quantitation of CFU in homogenized lung tissue 24 hours after infection with PAO1 in WT and *Glrx1*^{-/-} mice treated with either vehicle (DMSO) or zVAD-FMK (zVAD). Data are expressed as mean ($\pm$ SEM) CFU ($n = 9$ – 10 mice per group), $*P < 0.05$ compared with respective DMSO groups, $^{\#}P < 0.05$ (analysis of variance) as compared with the corresponding WT group. (C) Impact of zVAD on Fas S-glutathionylation. WT and *Glrx1*^{-/-} mice were infected with PAO1 and 24 hours later lungs were dissected and homogenized. Lung lysates were immunoprecipitated (IP) using an anti-glutathione (GSH) antibody. Precipitated proteins were resolved by reducing gel electrophoresis and probed using an anti-Fas antibody. As control animals a sample from a *Glrx1*^{-/-} mouse was incubated with dithiothreitol (+DTT) before electrophoresis. Whole-lung lysates (WLL) were resolved by reducing electrophoresis and probed to evaluate total Fas and glutaredoxin-1 (Grx1) protein levels by Western blot analysis.

Figure 6. Impact of enhanced glutaredoxin-1 overexpression in lung epithelial cells for PAO1-induced pneumonia. (A–C) Characterization of a transgenic mouse that overexpresses a FLAG tagged version of glutaredoxin-1 (Grx1) protein, specifically within the lung epithelium (A), in a doxycycline (Dox)-dependent manner (B). After 1 week of Dox feeding, lungs or other organs were homogenized for assessment of Grx1 and FLAG-Grx1 by Western blotting. *CC10rtTA* single transgenic animals were run as a control. (C) Assessment of the FLAG tagged version of Grx1 (FG) in *CC10rtTA* and *CC10rtTA/TetOFlagGrx1* mice. Lung tissues were analyzed using an anti-FLAG antibody (brown). (D) Assessment of CFUs counts in lungs or spleens from *CC10rtTA* and *CC10rtTA/TetOFlagGrx1* mice 24 hours after infection with PAO1. Data are expressed as mean ($\pm$ SEM) CFU ($n = 8$ mice per group), $*P < 0.05$ (analysis of variance) compared with respective *CC10-rtTA* groups. (E) Assessment of inflammatory infiltrates in bronchoalveolar lavage fluid (BALF) of *CC10rtTA* and *CC10rtTA/TetOFlagGrx1* mice 24 hours after infection with PAO1. Data are expressed as mean ($\pm$ SEM) total cell counts ($n = 8$ mice per group). (F) Assessment of survival of *CC10rtTA* and *CC10rtTA/TetOFlagGrx1* mice after pulmonary infection with PAO1. Data are expressed as percent survival of total population infected ($n = 8$ mice per group), $*P < 0.05$ (Gehan-Breslow-Wilcoxon test) compared with respective *CC10-rtTA* mice. (G) Assessment of caspase 8 and 3 activation in *CC10rtTA* and *CC10rtTA/TetOFlagGrx1* mice 24 hours after infection with PAO1. Data are expressed as mean ($\pm$ SEM) relative luminescence units (RLU) and normalized to protein ($n = 8$ mice per group), $*P < 0.05$ (analysis of variance) compared with respective *CC10-rtTA* groups. (H) Impact of overexpression of Grx1 in lung epithelium for S-glutathionylation of Fas. *CC10rtTA* and *CC10rtTA/TetOFlagGrx1* mice were infected with PAO1 and 24 hours later lungs were dissected and homogenized. Lung lysates were immunoprecipitated (IP) using an anti-glutathione (GSH) antibody. Precipitated proteins were resolved by reducing gel electrophoresis and probed using an anti-Fas antibody. As control animals a sample from a *CC10rtTA* mouse was incubated with dithiothreitol (DTT) before electrophoresis. WLL = whole-lung lysates confirming expression of Fas and FasL.

was administered for 1 week to *CC10-rtTA* or *CC10-rtTA/FLAG-Grx1* transgenic mice before infection. PAO1 infection of *CC10-rtTA/FLAG-Grx1* mice resulted in a significant increase in bacterial loads and enhanced dissemination compared with the *CC10-rtTA* control group (Figure 6D). Comparable increases in cellular infiltrates in the airways were observed in both groups of mice after 24 hours of PAO1-induced pneumonia (Figure 6E). Significantly, *CC10-rtTA/FLAG-Grx1* transgenic mice showed enhanced mortality after infection with PAO1, compared with *CC10-rtTA* animals (Figure 6F). The lack of mortality in *CC10-rtTA* transgenic mice infected with PAO1 compared with C57BL/6 mice observed earlier (Figures 3C and 3D) is unclear, but could be the result of the doxycycline administration regimen or expression of the *CC10-rtTA* transgene. We next analyzed the impact of overexpression of FLAG-Grx1 on caspase activation and formation of Fas-SSG. Compared with *CC10-rtTA*, *CC10-rtTA/FLAG-Grx1* mice showed a significant decrease in caspase 8 and 3 activation and a dramatic decrease in Fas-SSG in lung homogenates (Figures 6G and 6H). These findings collectively suggest that Grx1 overexpression in lung epithelium attenuates host defense to *P. aeruginosa* by deglutathionylation of Fas, attenuated caspase 8 and 3 activities, resulting in decreased epithelial apoptosis.

Discussion

Oxidants have traditionally been considered as molecules that evoke cellular and tissue damage. However, a recent body of evidence has emerged in support of a critical regulatory role of oxidants in biologic processes, exerted in part through controlled oxidations of regulatory cysteines within proteins. Protein S-glutathionylation is an example of such cysteine oxidation that under physiologic conditions can be reversed by the protein thiol oxidoreductase, Grx (6). To date, virtually no information exists with regard to the role of Grx1 in the pathogenesis of infectious lung diseases. Similarly, the role of protein S-glutathionylation in the pathogenesis of lung disease also remains unknown. Most reports thus far have documented increases in oxidation of glutathione to the disulfide form as an indicator of oxidative stress, without

reporting alterations in protein S-glutathionylation. In the present study we demonstrate increases in protein S-glutathionylation that overlap with immunoreactive Fas in patients with *P. aeruginosa* pneumonia. We also demonstrate that Grx1 contributed to acute *P. aeruginosa* dissemination and mortality in mice in association with lowering S-glutathionylation of Fas, and dampened apoptosis in epithelial cells, indicating the importance of the Grx1/Fas S-glutathionylation axis in pneumonia. The role of Grx1 has been explored in other disease conditions. In patients with chronic obstructive pulmonary disease, the number of Grx1-positive macrophages correlated positively with disease progression and diminished lung function (13). Similarly, Grx1 levels in induced sputum of people with asthma also correlated strongly with decreases in FEV₁ (14). Conversely, in patients with sarcoidosis and allergic alveolitis, Grx1 immunostaining in alveolar macrophages was decreased compared with healthy control subjects (15). We previously documented enhanced Grx1 expression within the conducting airways of mice after the induction of allergic airways disease (16), and that ablation of *Grx1* resulted in enhanced resolution of airways hyperresponsiveness (17). Collectively these and other reports (9, 16) demonstrate that Grx1 and S-glutathionylation are modulated in diverse settings, in response to multiple agonists, and that their role in disease pathogenesis may be complex.

Our laboratory has investigated the role of the apoptosis-inducing Fas/FasL pathway, and documented that FasL-induced signaling and apoptosis is enhanced after S-glutathionylation of its receptor Fas, which results in a more robust death-inducing signaling complex (7, 10). Previously published studies investigating the role of the FasL/Fas pair in the clearance of *P. aeruginosa* pneumonia and keratitis have documented a pivotal role for Fas-induced apoptosis for efficient clearance of *P. aeruginosa* (4, 18). These studies demonstrated that abrogation of either Fas or FasL resulted in a significant reduction in survival of mice after *P. aeruginosa*-induced pneumonia and exacerbation of keratitis (18). Furthermore, airway epithelial cells represented the primary site of apoptosis after *P. aeruginosa*-induced pneumonia. It was speculated that Fas/FasL-induced apoptosis

result in pathogen confinement, allowing for more efficient clearance by phagocytic and infiltrating granular cells (4). Our data partially support the above hypothesis, in that after genetic ablation of *Grx1*, elevated S-glutathionylation of Fas occurred, which was demonstrated to accelerate apoptosis (9). The lack of an impact on bacterial clearance in cultures of epithelial cells isolated from *Grx1*^{-/-} mice, compared with WT groups, despite markedly enhanced Fas-SSG, caspase activation, and cell death in *Grx1*^{-/-} epithelial cells, is supportive of a requirement of phagocytic cells, such as macrophages or neutrophils, to engulf apoptotic bodies and degrade *P. aeruginosa* *in vivo* (19–21).

Systemic ablation of *Grx1* precludes determination of the critical cell type or tissue compartment in conferring the protective phenotype of *Grx1*^{-/-} mice after infection with *P. aeruginosa*. Nonetheless, the demonstration that activation of caspases occurred predominantly in structural cells, as compared with myeloid cells, and were markedly elevated in *Grx1*^{-/-} mice compared with WT groups (Figure 4), collectively support a role for structural cells in regulating cell death after infection with *P. aeruginosa*. Furthermore, overexpression of Grx1 specifically within the lung epithelium resulted in significantly elevated bacterial loads in the lungs and increased mortality compared with Flag-Grx1-negative littermates (Figure 6). Collectively these data suggest a significant negative role for epithelial Grx1 in regulating the clearance of *P. aeruginosa* after lung infection.

The present study also demonstrates that *caspase 3*^{-/-} MTE cells had strong decreases in Fas-SSG and apoptosis on infection with *P. aeruginosa*. Furthermore, treatment of WT or *Grx1*^{-/-} mice with a pan caspase inhibitor decreased Fas-SSG, active caspase 3, and impaired clearance of bacteria from the lung comparably in both genotypes. These results, along with previous observations (4), demonstrate that Fas-mediated activation of downstream caspases is critical in clearance of *P. aeruginosa* from the lung, and that the enhanced bacterial clearance in *Grx1*^{-/-} mice was strongly associated with enhanced S-glutathionylation of Fas, subsequent activation of caspases, and apoptosis.

The Fas pathway is known to be critical in acute lung injury (22, 23), and promotion

of Fas activation via S-glutathionylation to enhance clearance of *P. aeruginosa* could represent a potential double-edged sword. Further studies are required to address the impact of Fas-SSG for promoting acute lung injury in settings of infection with *P. aeruginosa*, which were beyond the scope of the present study. Despite the significantly elevated caspase activities in *P. aeruginosa*-infected *Grx1*^{-/-} mice compared with WT groups, protein and LDH in BALF were decreased 24 hours postinfection with *P. aeruginosa* in *Grx1*^{-/-} animals (see Figure E5). It is known that apoptosis and necrosis occur during *P. aeruginosa* infection (24–27). One reason for the decreases in LDH and protein in BALF from *Grx1*^{-/-} mice could be that absence of *Grx1* may shift the dominant form of cell death to caspase-mediated apoptosis, rather than necrosis. Additional studies are required to determine whether the Grx1/S-glutathionylation axis controls the mode of epithelial cell death, and subsequent implications for *P. aeruginosa*-induced pneumonia, inflammatory responses, and associated lung injury.

The impact of the Grx1/S-glutathionylation/Fas axis for the regulation of inflammatory responses, and subsequent impact for clearance of *P. aeruginosa* also requires further investigation, given our observations that inflammatory cells and proinflammatory mediators were modestly affected in *Grx1*^{-/-} mice compared with WT groups after infection with *P. aeruginosa* (see Figures E2 and E4). Previous work in our laboratory demonstrated that S-glutathionylation inhibits the proinflammatory transcription factor, nuclear factor- κ B (NF- κ B), and that absence of *Grx1* further dampens the activation of NF- κ B (28–30). Because of the known role of NF- κ B in regulating clearance of *P. aeruginosa* (5, 31), it is possible that modulation of NF- κ B through S-glutathionylation could have accounted for some of the effects observed herein. Formal assessment of this scenario requires additional studies that were beyond the scope of the present manuscript.

In summary, we have identified a novel role for redox-based control of the Fas pathway by Grx1 in the pathogenesis of

P. aeruginosa pneumonia, and suggest that Grx1 and Fas S-glutathionylation are potential targets for therapeutic intervention. The implications of the present findings may be broad, given that inhibition of Grx1 may represent a novel avenue for therapeutic intervention of *P. aeruginosa*-induced pneumonia. It remains to be determined, however, what impact of *Grx1* deficiency may have in preventing colonization by *P. aeruginosa*, because this is the primary pathology seen in cystic fibrosis-associated *P. aeruginosa* infection, and has been associated with decreases in Fas expression and apoptosis of epithelial cells (32). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Jessica Reiss, for technical assistance, Dr. Ralph Budd for *Caspase3*^{-/-} and *lpr* strains of mice, and University of Vermont Microscopy Imaging Facility (supported by NCCR 1S10RR019246). They thank Dr. Jeffrey Whitsett, University of Cincinnati, for generously providing the CC10-*rtTA* transgenic mice.

References

- Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* 2002;15:194–222.
- Kong F, Young L, Chen Y, Ran H, Meyers M, Joseph P, Cho YH, Hassett DJ, Lau GW. *Pseudomonas aeruginosa* pyocyanin inactivates lung epithelial vacuolar ATPase-dependent cystic fibrosis transmembrane conductance regulator expression and localization. *Cell Microbiol* 2006;8:1121–1133.
- Chastre J, Fagon JY. Ventilator-associated pneumonia. *Am J Respir Crit Care Med* 2002;165:867–903.
- Grassmé H, Kirschnek S, Riethmueller J, Riehle A, von Kürthy G, Lang F, Weller M, Gulbins E. CD95/CD95 ligand interactions on epithelial cells in host defense to *Pseudomonas aeruginosa*. *Science* 2000;290:527–530.
- Sadikot RT, Zeng H, Joo M, Everhart MB, Sherrill TP, Li B, Cheng DS, Yull FE, Christman JW, Blackwell TS. Targeted immunomodulation of the NF- κ B pathway in airway epithelium impacts host defense against *Pseudomonas aeruginosa*. *J Immunol* 2006;176:4923–4930.
- Anathy V, Roberson EC, Guala AS, Godburn KE, Budd RC, Janssen-Heininger YM. Redox-based regulation of apoptosis: S-glutathionylation as a regulatory mechanism to control cell death. *Antioxid Redox Signal* 2012;16:496–505.
- Anathy V, Aesif SW, Guala AS, Havermans M, Reynaert NL, Ho YS, Budd RC, Janssen-Heininger YM. Redox amplification of apoptosis by caspase-dependent cleavage of glutaredoxin 1 and S-glutathionylation of Fas. *J Cell Biol* 2009;184:241–252.
- Ho YS, Xiong Y, Ho DS, Gao J, Chua BH, Pai H, Mielay JJ. Targeted disruption of the glutaredoxin 1 gene does not sensitize adult mice to tissue injury induced by ischemia/reperfusion and hyperoxia. *Free Radic Biol Med* 2007;43:1299–1312.
- Aesif SW, Anathy V, Havermans M, Guala AS, Ckless K, Taatjes DJ, Janssen-Heininger YM. In situ analysis of protein S-glutathionylation in lung tissue using glutaredoxin-1-catalyzed cysteine derivatization. *Am J Pathol* 2009;175:36–45.
- Anathy V, Roberson E, Cunniff B, Nolin JD, Hoffman S, Spiess P, Guala AS, Lahue KG, Goldman D, Flemer S, et al. Oxidative processing of latent Fas in the endoplasmic reticulum controls the strength of apoptosis. *Mol Cell Biol* 2012;32:3464–3478.
- Alcorn JF, Guala AS, van der Velden J, McElhinney B, Irvin CG, Davis RJ, Janssen-Heininger YM. Jun N-terminal kinase 1 regulates epithelial-to-mesenchymal transition induced by TGF- β 1. *J Cell Sci* 2008;121:1036–1045.
- Glasser SW, Korfhagen TR, Wert SE, Whitsett JA. Transgenic models for study of pulmonary development and disease. *Am J Physiol* 1994;267:L489–L497.
- Peltoniemi MJ, Ryttilä PH, Harju TH, Soini YM, Salmenkivi KM, Ruddock LW, Kinnula VL. Modulation of glutaredoxin in the lung and sputum of cigarette smokers and chronic obstructive pulmonary disease. *Respir Res* 2006;7:133.
- Kuipers I, Louis R, Manise M, Dentener MA, Irvin CG, Janssen-Heininger YM, Brightling CE, Wouters EF, Reynaert NL. Increased glutaredoxin-1 and decreased protein S-glutathionylation in sputum of asthmatics. *Eur Respir J* 2013;41:469–472.
- Peltoniemi M, Kaarteenaho-Wiik R, Säily M, Sormunen R, Pääkkö P, Holmgren A, Soini Y, Kinnula VL. Expression of glutaredoxin is highly cell specific in human lung and is decreased by transforming growth factor- β in vitro and in interstitial lung diseases in vivo. *Hum Pathol* 2004;35:1000–1007.
- Reynaert NL, Wouters EF, Janssen-Heininger YM. Modulation of glutaredoxin-1 expression in a mouse model of allergic airway disease. *Am J Respir Cell Mol Biol* 2007;36:147–151.
- Hoffman SM, Tully JE, Lahue KG, Anathy V, Nolin JD, Guala AS, van der Velden JL, Ho YS, Aliyeva M, Daphtary N, et al. Genetic ablation of glutaredoxin-1 causes enhanced resolution of airways hyperresponsiveness and mucus metaplasia in mice with allergic airways disease. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L528–L538.
- Zhou Z, Wu M, Barrett RP, McClellan SA, Zhang Y, Hazlett LD. Role of the Fas pathway in *Pseudomonas aeruginosa* keratitis. *Invest Ophthalmol Vis Sci* 2010;51:2537–2547.

19. Van de Weert-van Leeuwen PB, Van Meegen MA, Speirs JJ, Pals DJ, Rooijackers SH, Van der Ent CK, Terheggen-Lagro SW, Arets HG, Beekman JM. Optimal complement-mediated phagocytosis of *Pseudomonas aeruginosa* by monocytes is cystic fibrosis transmembrane conductance regulator-dependent. *Am J Respir Cell Mol Biol* 2013;49:463–470.
20. Amiel E, Lovewell RR, O'Toole GA, Hogan DA, Berwin B. *Pseudomonas aeruginosa* evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression. *Infect Immun* 2010;78:2937–2945.
21. Mishra M, Byrd MS, Sergeant S, Azad AK, Parsek MR, McPhail L, Schlesinger LS, Wozniak DJ. *Pseudomonas aeruginosa* Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cell Microbiol* 2012;14:95–106.
22. Herrero R, Kajikawa O, Matute-Bello G, Wang Y, Hagimoto N, Mongovin S, Wong V, Park DR, Brot N, Heinecke JW, et al. The biological activity of FasL in human and mouse lungs is determined by the structure of its stalk region. *J Clin Invest* 2011;121:1174–1190.
23. Glavan BJ, Holden TD, Goss CH, Black RA, Neff MJ, Nathens AB, Martin TR, Wurfel MM; ARDSnet Investigators. Genetic variation in the FAS gene and associations with acute lung injury. *Am J Respir Crit Care Med* 2011;183:356–363.
24. Jensen PO, Bjarnsholt T, Phipps R, Rasmussen TB, Calum H, Christoffersen L, Moser C, Williams P, Pressler T, Givskov M, et al. Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology* 2007;153:1329–1338.
25. Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, Brain JD, Accurso FJ, Henson PM. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest* 2002;109:661–670.
26. Watt AP, Courtney J, Moore J, Ennis M, Elborn JS. Neutrophil cell death, activation and bacterial infection in cystic fibrosis. *Thorax* 2005;60:659–664.
27. Silva MT. Bacteria-induced phagocyte secondary necrosis as a pathogenicity mechanism. *J Leukoc Biol* 2010;88:885–896.
28. Pineda-Molina E, Klatt P, Vázquez J, Marina A, García de Lacoba M, Pérez-Sala D, Lamas S. Glutathionylation of the p50 subunit of NF- κ B: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry* 2001;40:14134–14142.
29. Reynaert NL, van der Vliet A, Guala AS, McGovern T, Hristova M, Pantano C, Heintz NH, Heim J, Ho YS, Matthews DE, et al. Dynamic redox control of NF- κ B through glutaredoxin-regulated S-glutathionylation of inhibitory κ B kinase beta. *Proc Natl Acad Sci USA* 2006;103:13086–13091.
30. Shelton MD, Kern TS, Mieyal JJ. Glutaredoxin regulates nuclear factor κ B and intercellular adhesion molecule in Müller cells: model of diabetic retinopathy. *J Biol Chem* 2007;282:12467–12474.
31. Chen SM, Cheng DS, Williams BJ, Sherrill TP, Han W, Chont M, Saint-Jean L, Christman JW, Sadikot RT, Yull FE, et al. The nuclear factor κ B pathway in airway epithelium regulates neutrophil recruitment and host defense following *Pseudomonas aeruginosa* infection. *Clin Exp Immunol* 2008;153:420–428.
32. Cannon CL, Kowalski MP, Stopak KS, Pier GB. *Pseudomonas aeruginosa*-induced apoptosis is defective in respiratory epithelial cells expressing mutant cystic fibrosis transmembrane conductance regulator. *Am J Respir Cell Mol Biol* 2003;29:188–197.