

1 **Inducible Lung Epithelial Resistance Requires Multisource Reactive Oxygen Species**  
2 **Generation to Protect against Bacterial Infections**

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23 Short title: Multisource ROS in inducible epithelial antibacterial resistance

24 **ABSTRACT**

25 Pneumonia remains a global health threat, in part due to expanding categories of susceptible  
26 individuals and increasing prevalence of antibiotic resistant pathogens. However, therapeutic  
27 stimulation of the lungs' mucosal defenses by inhaled exposure to a synergistic combination of  
28 Toll-like receptor (TLR) agonists known as Pam2-ODN promotes mouse survival of pneumonia  
29 caused by a wide array of pathogens. This inducible resistance to pneumonia relies on intact  
30 lung epithelial TLR signaling, and inducible protection against viral pathogens has recently been  
31 shown to require increased production of epithelial reactive oxygen species (ROS) from multiple  
32 epithelial ROS generators. To determine whether similar mechanisms contribute to inducible  
33 antibacterial responses, the current work investigates the role of ROS in therapeutically-  
34 stimulated protection against *Pseudomonas aeruginosa* challenges. Inhaled Pam2-ODN  
35 treatment one day before infection prevented hemorrhagic lung cytotoxicity and mouse death in  
36 a manner that correlated with reduction in bacterial burden. The bacterial killing effect of Pam2-  
37 ODN was recapitulated in isolated mouse and human lung epithelial cells, and the protection  
38 correlated with inducible epithelial generation of ROS. Scavenging or targeted blockade of ROS  
39 production from either dual oxidase or mitochondrial sources resulted in near complete loss of  
40 Pam2-ODN-induced bacterial killing, whereas deficiency of induced antimicrobial peptides had  
41 little effect. These findings support a central role for multisource epithelial ROS in inducible  
42 resistance against bacterial pathogens and provide mechanistic insights into means to protect  
43 vulnerable patients against lethal infections.

44

45 **INTRODUCTION**

46 Lower respiratory tract infections remain the leading cause of premature death and disability  
47 among both otherwise healthy and immunosuppressed people worldwide (1-5). In an era of  
48 increasing antimicrobial resistance, human global hypermobility, proliferation of emerging and  
49 weaponized pathogens, aging populations, and ever-expanding categories of

50 immunocompromised patients, the acute complications of pneumonia exact a staggering toll,  
51 killing an estimated 2.7 million people per year (6-10). The 1943 introduction of penicillin for  
52 pneumonia management was a medical triumph (11), but the intervening decades have  
53 witnessed escalating age-adjusted pneumonia hospitalization rates (12-14) without survival rate  
54 improvements of corresponding magnitude (15). In an effort to address the persisting threat of  
55 pneumonia to vulnerable populations, our laboratory has developed a program focused on  
56 manipulating the intrinsic antimicrobial capacity of the host to prevent pneumonia.

57

58 Based on this program, we have reported that the mucosal defenses of the lungs can be  
59 stimulated to protect mice against a wide array of otherwise lethal pneumonias, including those  
60 caused by antibiotic-resistant bacteria (16-19). This *inducible resistance* is achieved following a  
61 single inhaled treatment comprised of a synergistic combination of Toll-like receptor (TLR)  
62 agonists: a diacylated lipopeptide ligand for TLR2/6, Pam2CSK4, and a class C unmethylated  
63 2'-deoxyribocytidine-phosphate-guanosine (CpG) ligand for TLR9, ODN M362 (hereafter,  
64 Pam2-ODN) (16, 17, 20).

65

66 Inducible resistance against pneumonia requires intact lung epithelial TLR signaling  
67 mechanisms, whereas no individual leukocyte populations have been identified as essential to  
68 Pam2-ODN-enhanced pneumonia survival (16, 21). Given the epithelial requirement for  
69 inducible resistance *in vivo* (16, 22), we sought to determine whether epithelial cells were  
70 sufficient to act as autonomous antibacterial effector cells of therapeutically inducible protection.  
71 We recently reported that Pam2-ODN-induced antiviral protection requires therapeutic induction  
72 of reactive oxygen species (ROS) via a novel multisource mechanism (23), but it is unknown  
73 whether similar processes are required for inducible antibacterial defense.

74

75 We report here that Pam2-ODN induces active antibacterial responses from intact lungs and  
76 isolated lung epithelial cells that reduce pathogen burden, attenuate infectivity, and enhance  
77 survival. Moreover, we find that the protection requires epithelial generation of ROS via dual  
78 mechanisms, providing meaningful insights into the mechanisms underlying the novel  
79 synergistic interactions observed between the TLR ligands.

80

81 **RESULTS**

82

83 **Pam2-ODN treatment reduces pathogen burden and inflammatory injury in bacterial**  
84 **pneumonia.** We have previously reported that a single nebulized treatment with Pam2-ODN  
85 results in improved survival of otherwise lethal pneumonias, including those caused by *P.*  
86 *aeruginosa* (16, 17, 20, 21). Here, we found that the protection afforded by Pam2-ODN (Figure  
87 1A) is associated with reduced bacterial burden immediately after infection, whether culturing  
88 lung homogenates or bronchoalveolar lavage (BAL) fluid (Figure 1B), suggesting that a Pam2-  
89 ODN-induced antimicrobial environment existed at the time of infection. No significant  
90 differences were noted in the performance of the two culture methods, in terms of precision or  
91 magnitude of induced bacterial reductions by Pam2-ODN, though the absolute bacteria per ml  
92 tended to be higher in the BAL-obtained samples than in the lung homogenates. Proportionally  
93 similar inducible reductions in pathogen burden were observed both immediately after infection  
94 and 24 h after infection when mice were infected with fluorescent *P. aeruginosa*, then their lungs  
95 were examined by fluorescence microscopy (Figure S1).

96

97 **Figure 1. Pam2-ODN protects against bacterial pneumonia.** (A) Survival of wildtype mice  
98 treated with Pam2-ODN or PBS (sham) by aerosol 24 h before challenge with *P. aeruginosa*.  
99 (B) Pathogen burden of mice in A immediately after challenge, as assessed by serial dilution  
100 culture of lung homogenates (*left* panel) or BAL fluid (*right* panel). (C) Gross appearance of

101 mouse lungs 24 h after *P. aeruginosa* challenge following treatment with Pam2-ODN or sham.  
102 (D) Hematoxylin-eosin stained histology of lungs in C. Scale bar = 400  $\mu$ m *left* panels, 100  $\mu$ m  
103 *right* panels. Each panels is representative of at least three independent experiments. N = 8  
104 mice/group for survival, N = 4 mice/group for pathogen burden. \* p < 0.0002 vs. PBS-treated; \*\*  
105 p < 0.002 vs PBS-treated.

106  
107 Although inhaled treatment with Pam2-ODN induces transient lung neutrophilia (16), we found  
108 here that the antimicrobial environment associated with Pam2-ODN-induced resistance also  
109 protected against inflammatory lung injury. Lungs harvested 24 h after *P. aeruginosa* challenge  
110 demonstrate severe hemorrhagic pneumonia in sham-treated mice, but there is almost no  
111 evidence of such injury in Pam2-ODN-treated mice (Figure 1C). Similarly, histologic inspection  
112 of Pam2-ODN-treated lungs 24 h after infection demonstrate substantially less inflammatory cell  
113 infiltration and notably fewer bacteria. This is congruent with earlier studies (16) suggesting that  
114 the difference in *P. aeruginosa* continues to increase between active and sham treated groups  
115 as time elapses, indicating that the antimicrobial environment persists beyond the period of the  
116 initial challenge.

117  
118 **Pam2-ODN induces bacterial killing by isolated lung epithelial cells across a broad**  
119 **concentration and temporal range.** Congruent with the *in vivo* observations, we have found  
120 that treatment of isolated human or mouse lung epithelial cells results in significant reductions in  
121 culture bacterial burdens (16, 17, 21, 22). Based on empiric *in vivo* efficacy optimization, Pam2-  
122 ODN is administered in a fixed 4:1 molar ratio (18, 20). Figure 2A-B shows that, when delivered  
123 in this ratio, the antibacterial effect is inducible across treatment concentrations that extend to at  
124 least a 2 log<sub>10</sub> range (Pam2 0.124-12.4  $\mu$ M; ODN 0.031-3.1  $\mu$ M). Higher Pam2-ODN treatment  
125 concentrations are expected to induce even greater bacterial killing than that shown, but when  
126 calculating the estimated deposition of the ligands in 20  $\mu$ l mouse airway lining fluid (24) or in

127 10-30 ml of human airway lining fluid (25) after nebulization, it is unlikely that such high  
128 concentrations can be achieved in vivo. To avoid presenting responses that are easily  
129 detectable but not physiologically relevant to the *in vivo* model, all subsequent figures include  
130 data achieved with a lower Pam2-ODN dose (2.23 uM Pam2 and 0.56 uM ODN) that we  
131 calculate to be achievable by nebulization, except when labelled as dose response plots.

132

133 In this model, bacteria are inoculated into the epithelial cultures in log phase growth and there  
134 are no antibacterial leukocyte contributions. So, the antimicrobial epithelial responses must be  
135 active very early in the course of infection. To determine how quickly these responses could be  
136 induced, we tested treatment intervals prior to challenge and found that the most profound  
137 antibacterial responses seemed to be achieved with six or more hours of treatment, but  
138 significant bacterial burden reductions were achieved in a much shorter period (Figure 2C-D) in  
139 both mouse and human cells. In fact, the antibacterial effect was even observed when Pam2-  
140 ODN is administered simultaneous to the infectious challenge.

141

142 **Figure 2. Pam2-ODN induces antibacterial responses in isolated lung epithelial cells.**

143 HBEC3kt (**A**) or MLE15 (**B**) cells were treated for 6 h with PBS or escalating doses of Pam2-  
144 ODN (range: Pam2 0.12-12.4 uM, ODN 0.03-3.10 uM), then challenged with *P. aeruginosa*.  
145 Shown are culture bacterial burdens 6 h after challenge. HBEC3kt (**C**) or MLE15 (**D**) cells were  
146 treated for 6 h with PBS or Pam2-ODN (middle dose used in **A** and **B**, 2.23 uM Pam2 and 0.56  
147 uM ODN) for the indicated interval prior to challenge with *P. aeruginosa*. Shown are culture  
148 bacterial burdens 6 h after challenge. HBEC3kt (**E**) or MLE15 (**F**) cells were treated for 6 h with  
149 the indicated treatments, then challenged with *P. aeruginosa*. Shown are culture bacterial  
150 burdens 6 h after challenge. HBEC3kt (**G**) or MLE15 (**H**) cells were treated with PBS or Pam2-  
151 ODN for 6 h prior to *P. aeruginosa* challenge. Cell survival determined by Trypan blue exclusion  
152 is shown at the indicated time points. Each panels is representative of at least three

153 independent experiments. \* p < 0.05 vs. PBS-treated; \*\* p < 0.005 vs. PBS-treated; † p < 0.05  
154 vs. either single ligand treatment.

155

156 ***Pam2-ODN interact synergistically to induce bacterial killing.*** Further substantiating the *in*  
157 *vitro* model as relevant to study of the *in vivo* pneumonia protection associated with Pam2-ODN  
158 treatment, we found that the antibacterial effect of the combined Pam2-ODN treatment was  
159 supra-additive to the effects of equimolar ligands delivered individually. Pam2 alone induced a  
160 modest reduction in bacterial burdens in human epithelial cells (Figure 2E). The magnitude of  
161 this effect is similar to the degree of protection we have observed *in vivo* with Pam2 alone (17).  
162 ODN alone did not induce any significant reductions in bacterial burden in either human or  
163 mouse epithelial cultures (Figure 2F). However, in both models, the combination of Pam2 and  
164 ODN resulted in greater anti-pseudomonal effects than the combined effects of the two ligands  
165 delivered alone.

166

167 ***Pam2-ODN extends epithelial survival of Pseudomonas infections.*** While Pam2-ODN  
168 induces a robust antibacterial effect, it has not been previously established whether the  
169 antimicrobial response was associated with a fitness cost to the cells themselves. For instance,  
170 it is conceivable that the microbicidal response might also be toxic to the host cells or it is  
171 possible that programmed cell death pathways contribute to bacteriostatic effects. Indeed, we  
172 have previously reported that inducible epithelial resistance is correlated with transient but  
173 profound induction of inflammatory mediators (16, 17, 20), and here find significant induction of  
174 genes for both proinflammatory cytokines and antimicrobial peptides from lung epithelial cells  
175 treated with Pam2-ODN (Figure S1A). However, we have not found reduced survival of lung  
176 epithelial cells following Pam2-ODN treatment in the absence of infection and see dramatically  
177 improved cell survival of viral infections when the cells received Pam2-ODN pretreatment (23).  
178 To investigate the effect of Pam2-ODN treatment on epithelial cell survival of bacterial

179 infections, Trypan blue exclusion was used to determine cell viability following *P. aeruginosa*  
180 infection. While all epithelial cells were dead by 36 h after the infectious challenge, regardless  
181 of pretreatment, both mouse and human epithelial cells lived longer on average and had a  
182 greater percentage of cells alive at every intermediate time point if pretreated with Pam2-ODN  
183 (Figure 2G-H). These findings support that the antibacterial effect of the epithelial response to  
184 Pam2-ODN is more beneficial than any potential fitness cost.

185

186 ***Pam2-ODN-induced antibacterial effects require DUOX-dependent ROS production.***

187 Antimicrobial peptides are well established contributors to lung epithelium-mediated  
188 antibacterial defense (22), and genes encoding antimicrobial peptides such as lipocalin 2 and  
189 acute phase serum albumin A proteins are some of the most strongly upregulated transcripts  
190 following Pam2-ODN treatments of lungs or isolated lung epithelial cells (16, 17). However, *P.*  
191 *aeruginosa* challenge of mice deficient in these antibacterial molecules revealed little defect in  
192 Pam2-ODN-inducible protection, even when more than one antimicrobial peptide gene was  
193 knocked out (Figure S2 B-E). These data suggest that, although they are robustly induced, these  
194 individual antimicrobial peptides are not essential effectors of inducible resistance, and  
195 prompted investigations into alternate effector mechanisms.

196

197 ROS are increasingly recognized to function as direct antimicrobial effector molecules, most  
198 likely through lipid peroxidation of microbial membranes and DNA damage, in addition to their  
199 well-established roles as signaling molecules (26). We previously hypothesized that ROS  
200 contribute to Pam2-ODN-induced epithelial antibacterial effects (16, 18, 19). More recently, we  
201 confirmed that ROS are essential to Pam2-ODN-induced antiviral responses and have  
202 published a comprehensive characterization of the epithelial ROS species induced by Pam2-  
203 ODN treatment (23). Figures 3A-B confirm that Pam2-ODN induces dose-dependent  
204 production of ROS from both human and mouse lung epithelial cells, as measured by

205 fluorescence signal from cell permeant carboxy-H<sub>2</sub>DCFDA. Our previous findings indicate that  
206 only hydrogen peroxide and superoxide are demonstrably increased by epithelial treatment (23),  
207 and there is no reason to suspect that induction of alternate species is reflected by the carboxy-  
208 H<sub>2</sub>DCFDA signal in the current studies using identical culture and treatment models.

209

210 **Figure 3. Pam2-ODN induces antibacterial ROS from isolated lung epithelial cells.**

211 HBEC3kt (**A**) or MLE15 (**B**) cells were exposed to CO-H<sub>2</sub>DCFDA, treated with the indicated  
212 doses of Pam2-ODN, then fluorescence intensity was measured every 5 min. HBEC3kt (**C**) or  
213 MLE15 (**D**) cells were pretreated with PEG-HCC or PBS, exposed to CO-H<sub>2</sub>DCFDA, then  
214 treated with the indicated dose of Pam2-ODN. Shown are fluorescence intensity 100 min after  
215 treatment. HBEC3kt (**E**) or MLE15 (**F**) cells were pretreated with PEG-HCC or PBS, treated for  
216 6 h with PBS or Pam2-ODN (Pam2 2.23 uM, ODN 0.56 uM), then challenged with *P.*  
217 *aeruginosa*. Shown are culture bacterial burdens 6 h after challenge. (**G**) HBEC3kt cells were  
218 stably transfected with scrambled (control) shRNA or shRNA targeting *DUOX1* or *DUOX2*, then  
219 treated with PBS or Pam2-ODN for 6 h prior to *P. aeruginosa* challenge. Shown are culture  
220 bacterial burdens 6 h after challenge. Each panels is representative of at least three  
221 independent experiments. \* p < 0.005 vs no Pam2-ODN treatment; † p < 0.005 vs no PEG-  
222 HCC, same Pam2-ODN; †† p < 0.02 vs scrambled shRNA + Pan2-ODN; ‡ p < 0.003 vs *DUOX1*  
223 knockdown + Pam2-ODN.

224

225 Acting by superoxide dismutation and radical annihilation (27, 28), application of poly(ethylene  
226 glycolated) hydrophilic carbon clusters (PEG-HCCs) (28, 29) to the culture media significantly  
227 reduced epithelial ROS, as demonstrated by CO-H<sub>2</sub>DCFDA fluorescence, at all Pam2-ODN  
228 doses (Figure 3C-D). Notably, by reducing epithelial ROS, PEG-HCC treatment also  
229 significantly impaired the Pam2-ODN-induced epithelial antibacterial effect (Figure 3E-F),

230 supporting a ROS requirement for inducing the protective response from both human and  
231 mouse lung epithelial cells.

232

233 While all NADPH oxidase (NOX) isoforms are reported to be expressed by lung epithelia, the  
234 primary source of lung epithelial ROS are the dual oxidases DUOX1 and DUOX2 (sometimes  
235 called NOX6 and NOX7) (30-32). To test the specific requirement for DUOX-derived ROS in  
236 Pam2-ODN-induced antibacterial defense, we used shRNA to stably knockdown *DUOX1* and  
237 *DUOX2* in HBEC3kt cells, then assessed the effect on Pam2-ODN-induced reductions in  
238 influenza burden. Figure 3G shows that knocking down *DUOX1* moderately impairs the Pam2-  
239 ODN-induced epithelial antimicrobial response and knocking down *DUOX2* severely impairs the  
240 inducible antibacterial effect. This is congruent with prior reports that *DUOX1* produces a  
241 relatively consistent amount of ROS, though this production can be moderately enhanced by IL-  
242 4 and IL-13 exposure(33), whereas *DUOX2*-dependent ROS production can be profoundly  
243 increased by activating existing *DUOX2* and increasing *DUOX2* and *DUOXA2* transcription  
244 following exposure to cytokines such as IFN $\gamma$ (33). Interestingly, while the *DUOX1* requirement  
245 for inducible antipseudomonal defense appears to be less substantial than the *DUOX2*  
246 requirement, the inducible protection defect observed in *DUOX1* knockdown cells is more  
247 profound than that observed in virus challenged *DUOX1* knockdown cells (23).

248

249 **Pam2-ODN-induced antibacterial effects require mitochondrial ROS production.** Although  
250 we confirmed that DUOX-dependent ROS production is required for the inducible bacterial  
251 killing, there is accumulating evidence that mitochondria-derived ROS can also participate in  
252 antimicrobial responses of nonphagocytes (23, 34-37). To test whether mitochondrial ROS also  
253 contribute to the inducible antibacterial effect of Pam2-ODN, mitochondrial ROS were  
254 selectively scavenged with mitoTEMPO prior to *P. aeruginosa* challenge with or without Pam2-  
255 ODN pretreatment. Figure 4 A-B shows that mitochondrial ROS scavenging profoundly impaired

256 the Pam2-ODN induced bacterial killing by mouse and human epithelial cells. To address  
257 potential off-target effects or nonspecific ROS scavenging of mitoTEMPO, we tested whether  
258 we could reduce inducible mitochondrial ROS production, rather than scavenging produced  
259 ROS. Figure 4C shows that combination treatment with a mitochondrial complex II inhibitor and  
260 a respiratory chain uncoupler reduces mitochondrial ROS production at every tested dose of  
261 Pam2-ODN. This impaired Pam2-ODN-induced mitochondrial ROS production resulted in  
262 bacterial killing defects that mirrored the mitochondrial ROS scavenging experiments (Figure 4  
263 D-E), revealing a requirement for mitochondrial ROS in Pam2-ODN-induced antibacterial  
264 responses.

265

266 **Figure 4. Mitochondrial ROS are required for Pam2-ODN-induced antibacterial epithelial**  
267 **responses.** HBEC3kt (**A**) or MLE15 (**B**) cells were pretreated with MitoTEMPO or PBS, treated  
268 for 6 h with PBS or Pam2-ODN (Pam2 2.23 uM, ODN 0.56 uM), then challenged with *P.*  
269 *aeruginosa*. Shown are culture bacterial burdens 6 h after challenge. (**C**) HBEC3kt cells were  
270 pretreated with FCCP-TTFA or PBS, exposed to MitoSOX, then treated with PBS or Pam2-ODN  
271 at the indicated doses. Shown are culture fluorescence intensities at 100 min after treatment.  
272 HBEC3kt (**D**) or MLE15 (**E**) cells were pretreated with FCCP-TTFA or PBS, treated for 6 h with  
273 PBS or Pam2-ODN (Pam2 2.23 uM, ODN 0.56 uM), then challenged with *P. aeruginosa*. Shown  
274 are culture bacterial burdens 6 h after challenge. Each panels is representative of at least three  
275 independent experiments. N= 4-5 samples/condition for all experiments. \* p < 0.01 vs. PBS-  
276 treated without inhibitor/scavenger; † p < 0.02 vs. Pam2-ODN-treated without  
277 inhibitor/scavenger.

278

279 **DISCUSSION**

280 Although the airway and alveolar epithelia have historically been considered inert barriers,  
281 accumulating evidence now clearly supports their essential contributions to antimicrobial

282 defense. In addition to their established capacity to recruit and activate leukocyte-mediated  
283 defenses in the lower respiratory tract, epithelial cells can exert directly antimicrobial effects on  
284 invading pathogens (22). Indeed, we have found that lung epithelial cells function as primary  
285 effector cells of inducible resistance to pneumonia, and we have reported the epithelial  
286 expression of numerous antimicrobial molecules following in vitro or in vivo exposure to Pam2-  
287 ODN (16, 17, 21, 23). The current work demonstrates that a single epithelial Pam2-ODN  
288 treatment promotes mouse survival of bacterial challenges by reducing pathogen burden and  
289 attenuating associated immunopathology. Similar in vivo Pam2-ODN-induced reductions in  
290 pathogen burden have been demonstrated by every investigated quantification technique,  
291 suggesting that the induced antimicrobial environment results in elimination of bacteria from all  
292 anatomic and cellular compartments of the lungs. This same inducible pathogen killing effect is  
293 observed from Pam2-ODN-treated isolated lung epithelial cells, where the reduced pathogen  
294 burden enhances cellular survival of bacterial challenges, even in the absence of leukocyte  
295 contributions.

296

297 Although numerous transcriptionally-regulated antimicrobial peptide species are induced by  
298 Pam2-ODN treatment of epithelial cells, no individual peptides have been demonstrated to be  
299 required for the Pam2-ODN-enhanced mouse survival of infectious challenge by any pathogen  
300 nor for the inducible intrapulmonary pathogen killing that uniformly correlates with inducible  
301 resistance. While it was, perhaps, unanticipated that none of these highly enriched peptides  
302 would prove essential to the protection, there are a number of plausible explanations for this  
303 observation. Foremost among these is the possibility that the extreme multiplicity of induced  
304 antimicrobial molecules renders the loss of only one or two peptides largely irrelevant, since the  
305 remaining species are sufficient to protect. Another important alternate consideration is that  
306 different peptide combinations may be required to protect against different challenges. So, we  
307 might eventually recategorize certain peptides that we have previously deemed to be non-

308 essential for protection as required for protection against specific (as yet untested) alternate  
309 pathogens.

310

311 Regardless of explanation, the inability to detect any essential antimicrobial peptides, however,  
312 serves to underscore the robustness of the Pam2-ODN-induced protection against otherwise  
313 lethal pneumonias and profoundly emphasize the importance of our recent finding that epithelial  
314 ROS production is required for inducible antiviral defense. Unlike the peptide studies, we have  
315 clearly established that inducible ROS are essential to protecting against *in vivo* and *in vitro*  
316 challenges by orthomyxoviruses and paramyxoviruses (23). The present study similarly finds  
317 that inducible ROS production is essential to Pam2-ODN-induced epithelial bacterial killing.  
318 Strikingly, we find that ROS derived from both DUOX-dependent and mitochondrial sources are  
319 required for protection.

320

321 ROS have been long recognized to contribute to antibacterial defenses, particularly in the  
322 context of NADPH oxidase-dependent killing of bacteria in phagolysosomes of myeloid cells  
323 (26). However, the broadly microbicidal capacity of ROS generated by a wide range of cells has  
324 been demonstrated in recent years with evidence that ROS can exert antimicrobial actions  
325 against Gram-negative, Gram-positive, viral and fungal pathogens, even in the setting of  
326 established biofilms or antibiotic resistance (38-40). This vigorous protection may rely in part on  
327 the capacity of ROS-dependent strategies to synergize with other antimicrobial treatments to  
328 overcome antibiotic resistance (38, 41). Similarly, ROS can also promote bacterial clearance by  
329 reducing the minimum inhibitory concentrations of host effector molecules, such as neutrophil  
330 proteases (26). Consequently, it has been proposed that therapeutic induction of microbial ROS  
331 may potentiate the microbicidal activity of other antibiotics (42) and that eliciting ROS  
332 production is an essential component of the pathogen killing mechanisms of some antimicrobial  
333 pharmaceutical agents (43, 44).

334

335 Superoxide and hydrogen peroxide are the predominant species produced by lung epithelial  
336 cells (26, 34, 45). We have recently presented a comprehensive assessment of ROS production  
337 following Pam2-ODN treatment of isolated lung epithelial cells. Congruent with the findings in  
338 the current work, we found superoxide and hydrogen peroxide to be the only species detectably  
339 induced by Pam2-ODN (23).

340

341 The principal sources of hydrogen peroxide from the lung epithelium are DUOX1 and DUOX2  
342 (30, 31, 46, 47), and therapeutic induction of DUOX2 has been proposed as a potential  
343 antimicrobial therapeutic strategy (48). Indeed, we have reported that DUOX-related genes are  
344 enriched following epithelial exposure to Pam2-ODN, both in vitro and in vivo (17, 23). The  
345 current study confirms that DUOX-dependent ROS are required for inducible *P. aeruginosa*  
346 killing, as this protective effect is profoundly attenuated when cellular ROS are annihilated by  
347 PEG-HCC or either DUOX is selectively knocked down. It is well established that the DUOX-  
348 dependent product of the lactoperoxidase/hydrogen peroxide/thiocyanate system,  
349 hypothiocyanate, exerts antimicrobial effects (46, 47, 49), however it is unlikely that the ROS  
350 dependency of Pam2-ODN-induced protection simply reflects hydrogen peroxide-mediated  
351 hypothiocyanate production, as our in vivo models lack tracheobronchial seromucus glands as a  
352 lactoperoxidase source (50) and our in vitro models lack a source of thiocyanate (33). This  
353 suggested that the DUOX-dependent ROS effects are likely achieved either through direct  
354 pathogen toxicity or through host signaling events.

355

356 In addition to DUOX-dependent hydrogen peroxide production, mtROS have been increasingly  
357 reported to contribute to wide ranging aspects of both innate and adaptive immunity (34, 51),  
358 and increased production of mitochondria-derived species likely explains the Pam2-ODN-  
359 increased superoxide we have detected here and in previous work (23).

360

361 mtROS are generated via leakage from the electron transport chain (45), resulting in production  
362 of superoxide that diffuses through mitochondrial membranes following dismutation to hydrogen  
363 peroxide (52). This process is exquisitely tightly regulated by changes in scavenging,  
364 production, and localization (34), so the substantial induction of mtROS by Pam2-ODN  
365 represents an important homeostatic perturbation. A reported eleven different mitochondrial  
366 sites can be perturbed to result in mtROS production and release (53), and we are presently  
367 investigating the signaling events that promote Pam2-ODN-induced mtROS production.  
368 However, it has been reported that TLR manipulation can promote generation of both  
369 antibacterial mtROS (37) and NADPH oxidase-generated ROS (54) in macrophages, so it is  
370 highly plausible that TLR ligands could induce ROS from both mitochondrial and DUOX sources  
371 in epithelial cells. Interestingly, although coordinated regulation of NADPH oxidase ROS and  
372 mtROS production has been reported (52, 55), the current study and our recent antiviral work  
373 (23) remain the only reports of concurrent induction of ROS from mitochondrial and DUOX  
374 sources in any cell type.

375

376 The precise mechanisms underlying the unanticipated requirement for dual sources of ROS  
377 have yet to be elucidated. However, this may be explained by dependence on the phenomenon  
378 of ROS-induced ROS to promote high ROS concentrations (52, 56, 57). Alternate potential  
379 explanations also include the hypothesis that multiple sources are required to achieve  
380 sufficiently high aggregate ROS concentrations to exert microbicidal actions (additive effect) or  
381 that the different sources play different roles, such as one source directly causing pathogen  
382 membrane damage while the other facilitates ROS-dependent signaling events. It is even  
383 possible that the mitochondrial function serves to regulate DUOX functions (58). This remains  
384 an area of active investigation.

385

386 These data indicate that multisource ROS are required for Pam2-ODN-induced bacterial killing,  
387 extending the range of pathogens that are known to be susceptible to inducible epithelial ROS  
388 and highlighting the centrality of ROS generation to the protective phenomenon of inducible  
389 epithelial resistance. By advancing understanding of the mechanisms of Pam2-ODN-induced  
390 resistance, these data may facilitate development of even more efficacious resistance-inducing  
391 therapeutics and offer hope that pneumonia can be prevented in vulnerable populations.

392

## 393 **MATERIALS AND METHODS**

### 394 ***Animals, cells, and reagents***

395 All general reagents were obtained from Sigma-Aldrich (St Louis, MO), except as indicated. All  
396 mouse experiments were performed with 5–8 week-old C57BL/6J (The Jackson Laboratory, Bar  
397 Harbor, ME) of a single gender in accordance with the Institutional Animal Care and Use  
398 Committee of The University of Texas MD Anderson Cancer Center, protocol 00000907-RN01.  
399 Immortalized human bronchial epithelial (HBEC3kt) cells were kindly provided by Dr. John  
400 Minna. Murine lung epithelial (MLE-15) cells were kindly provided by Dr. Jeffrey Whitsett. The  
401 cell lines used were authenticated by the MD Anderson Characterized Cell Line Core Facility.

402

### 403 ***Cell Culture***

404 HBEC3kt cells were cultured in keratinocyte serum-free media (KSFM, ThermoFisher Scientific,  
405 Grand Island, NY) supplemented with human epidermal growth factor and bovine pituitary  
406 extract. MLE-15 cells were cultured in RPMI supplemented with 10% fetal bovine serum.  
407 Cultures were maintained in the presence of penicillin and streptomycin.

408

### 409 ***TLR treatments***

410 For *in vivo* studies, S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl) 3-lysine (Pam2CSK<sub>4</sub>)  
411 and ODN M362 (InvivoGen, San Diego, CA) were reconstituted in endotoxin-free water, then

412 diluted to the desired concentration in sterile PBS. As previously described (16), the Pam2-  
413 ODN was placed in an Aerotech II nebulizer (Biodex Medical Systems, Shirley, NY) driven by 10  
414 L min<sup>-1</sup> air supplemented with 5% CO<sub>2</sub> for 20 min. The nebulizer was connected by polyethylene  
415 tubing to a polyethylene exposure chamber. 24 h prior to infections, 8 ml of Pam2 (4 $\mu$ M) -ODN  
416 (1 $\mu$ M) was delivered via nebulization to unrestrained mice for 20 minutes, and then mice were  
417 returned to normal housing. For *in vitro* studies, Pam2-ODN was added to the culture media 4 h  
418 prior to inoculation with bacteria or at the indicated time point. Pam2-ODN was given in fixed  
419 ratio, but at varying doses as indicated.

420

421 ***Infection models***

422 As previously described (16), frozen stock of *Pseudomonas aeruginosa* strain PA103 (American  
423 Type Culture Collection, Manassas, VA) was incubated overnight in tryptic soy broth, then  
424 expanded in Luria-Bertini media to OD<sub>600</sub> 0.35. Bacterial suspensions were centrifuged, washed,  
425 re-suspended in PBS, and aerosolized over 60 min. For all bacterial challenges, a nebulized  
426 inoculum of 10 ml of ~2 x 10<sup>10</sup> CFU/ml were delivered. Immediately after bacterial challenges,  
427 some mice were anesthetized and their lungs were harvested and homogenized (16) using a  
428 Mini-Beadbeater-1 (Biospec, Bartlesville, OK). Serial dilutions of the nebulizer inoculum and  
429 lung homogenates were plated on tryptic soy agar plates (Becton Dickinson). The remaining  
430 mice were observed for 12 d to determine whether their clinical conditions met euthanasia  
431 criteria. Following infection, lab personnel coordinated with staff of the MD Anderson  
432 Department of Veterinary Medicine to ensure that the mice were evaluated a minimum of three  
433 times daily to determine whether euthanasia criteria were met. As specified in the above noted  
434 animal protocol, mice that were not submitted to anesthetic excess followed by thoracotomy  
435 with bilateral pneumonectomy for pathogen burden assessments were humanely sacrificed by  
436 inhalational exposure to approved concentrations of carbon dioxide until respiratory efforts  
437 ceased, followed by cervical dislocation as a secondary method of euthanasia, when they either

438 achieved the end of the observation period or met the predesignated euthanasia criteria. The  
439 relevant euthanasia-triggering criteria include any evidence hypothermia, impaired mobility,  
440 respiratory distress, inability to access food or water, or any evidence of distressed behavior.  
441 Weight loss is also among the approved indications for euthanasia, but the mice that met  
442 euthanasia criteria in this model became ill or distressed within 1-2 d (before losing > 25% body  
443 weight), so no mice were euthanized due to weight loss in the current study. Despite the close  
444 observation, this same rapidity of illness resulted in up to 3 of the 56 infected mice dying  
445 spontaneously from pneumonia before being euthanized in some experimental replicates.  
446 Although meeting euthanasia criteria is the primary endpoint, the presented “Survival (%)” in  
447 Figure 1 formally indicates mice that had not either met euthanasia criteria or spontaneously  
448 died. When mice were identified to meet criteria, they were submitted to euthanasia within 30  
449 minutes by either lab personnel or Department of Veterinary Medicine staff. All lab personnel  
450 and Department of Veterinary Medicine staff receive formal instruction in methods to minimize  
451 stress and discomfort to experimental animals and analgesia is provided to animals that  
452 demonstrate any evidence of discomfort but do not meet euthanasia criteria.

453  
454 For the in vitro challenges, after the indicated treatments, confluent mouse or human epithelial  
455 cell cultures were inoculated with *P. aeruginosa* (20  $\mu$ l x 1x10<sup>5</sup> CFUs/ml), incubated for 6 h, then  
456 harvested and submitted to serial dilution culture.

457

#### 458 ***Lentiviral shRNA knockdown of DUOX1 and DUOX2***

459 GIPZ human *DUOX1* and *DUOX2* lentiviral shRNA clones were purchased from GE  
460 Dharmarcon (Lafayette, CO). Lentiviruses bearing human *DUOX1* and *DUOX2* shRNA were  
461 produced by transfection in 293T cells per manufacturer’s instructions. Infection efficiency was  
462 enhanced by addition of 8  $\mu$ g/ml Polybrene into the culture media and centrifuging the cells at  
463 2,000 rpm for 60 min at 32 °C. Lentiviral-infected HBEC3kt cells were selected by cell sorting

464 based on GFP expression. shRNA knockdown efficiency was determined by immunoblot  
465 analysis, as previously shown (23).

466

467 ***ROS detection, scavenging and inhibition***

468 To assess ROS generation, cells were treated with 5 $\mu$ M of each indicated detector for 1 h  
469 before exposure to Pam2-ODN or sham, as previously reported (23). Fluorescence was  
470 continuously measured on a BioTek Synergy2 for 250 min after treatment. Excitation/emission  
471 wavelengths for ROS-detecting agents are: Carboxy-2',7'-dichlorodihydrofluorescein diacetate  
472 (CO-H<sub>2</sub>DCFDA, ThermoFisher), 490nm/525nm; and MitoSOX<sup>TM</sup> Red (ThermoFisher),  
473 510nm/580nm.

474

475 Cellular ROS were scavenged by 1 h exposure to PEGylated hydrophilic carbon clusters (PEG-  
476 HCC, 5 $\mu$ g/mL) prior to application of Pam2-ODN or PBS (23). Mitochondrial ROS were  
477 scavenged by 1 h exposure to (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)  
478 triphenylphosphonium chloride monohydrate (MitoTEMPO, 30nM, ThermoFisher) prior to  
479 treatment with Pam2-ODN or PBS (23). Disruption of *in vitro* mitochondrial ROS production  
480 was achieved through concurrent application of trifluoromethoxy carbonylcyanide  
481 phenylhydrazone (FCCP, 400 nM, Cayman Chemical, Ann Arbor, MI), and 2-  
482 thenoyltrifluoroacetone (TTFA, 200  $\mu$ M, Sigma) (23).

483

484 ***Statistical analysis***

485 Statistical analysis was performed using SPSS v19 (SAS Institute, Cary, NC). Student's t-test  
486 was used to compare the lung pathogen burdens between the groups. Error bars shown in all  
487 the figures represent technical replicates within the displayed experiment, rather than  
488 aggregation of experimental replicates. Percentage of mice surviving pathogen challenges was

489 compared using Fisher's exact test on the final day of observation, and the log-rank test was  
490 used to compare the survival distribution estimated by the Kaplan–Meier method.

491

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495

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643

#### 644 **SUPPORTING INFORMATION FIGURE LEGENDS**

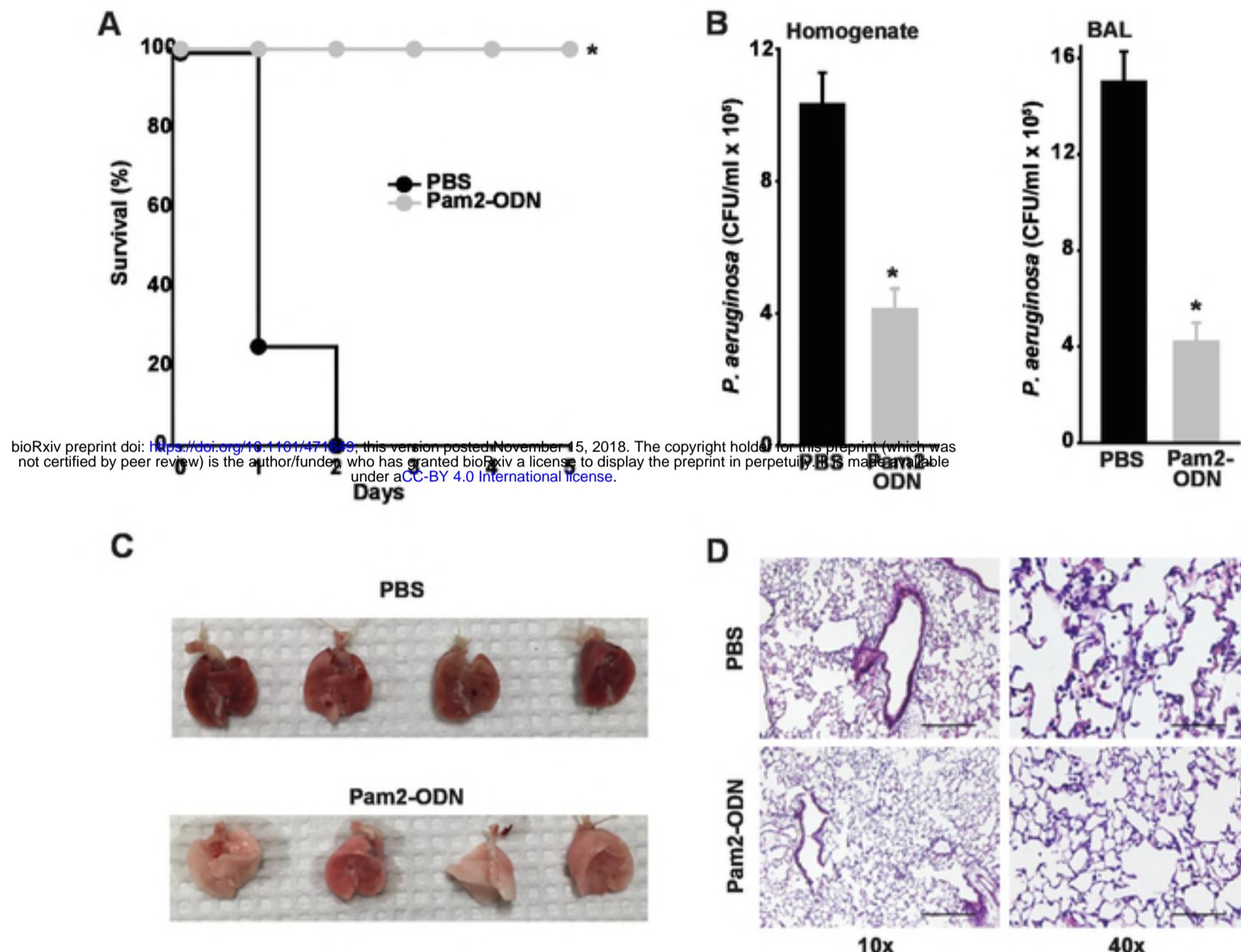
645 **Figure S1. Early and late bacterial burden reduction by Pam2-ODN treatment.** Wild type  
646 C57BL/6J mice were treated with Pam2-ODN or PBS (sham) 24 h prior to challenge with  
647 fluorescently-labeled *P. aeruginosa*. (A) Representative micrograph overlays of lung sections  
648 immediately after infection and 24 h after infection. Blue = DAPI, Green = bacteria. (B)  
649 Quantification of GFP signal in the indicated conditions. N = 3 mice per condition, 10 high power  
650 fields measured per mouse. \* p = 0.002 vs. PBS treated 0 h after challenge. p = 0.00006 vs.  
651 PBS treated 24 h after challenge.

652

653 **Figure S2. Cytokine and antimicrobial peptide induction in Pam2-ODN-induced**  
654 **resistance.** (A) HBEC3kt cells were treated with PBS (sham) or Pam2-ODN for 2 h, then  
655 submitted to RT-qPCR for the indicated transcripts. Shown are RQ values for the target  
656 transcript relative to 18s gene. Each panel is representative of at least three independent  
657 experiments. N=4-5 samples/condition for all experiments. Wild type or mice deficient in (B)  
658 *Lcn2*, (C) *Cramp*, (D) *Lcn2* and *Cramp*, or (E) the indicated acute phase SAA genes were  
659 treated with PBS (sham) or Pam2-ODN by aerosol 24 h prior to challenge with *P. aeruginosa*.  
660 Shown are survival plots for each challenge. Each panel is representative of at least three  
661 independent experiments. N=8-10 mice/condition. \* p < 0.001 vs PBS treated. \*\* p < 0.007 vs.  
662 syngeneic PBS treated. † p < 0.05 vs. syngeneic PBS treated.

663

Ware, Figure 1



Ware, Figure 2

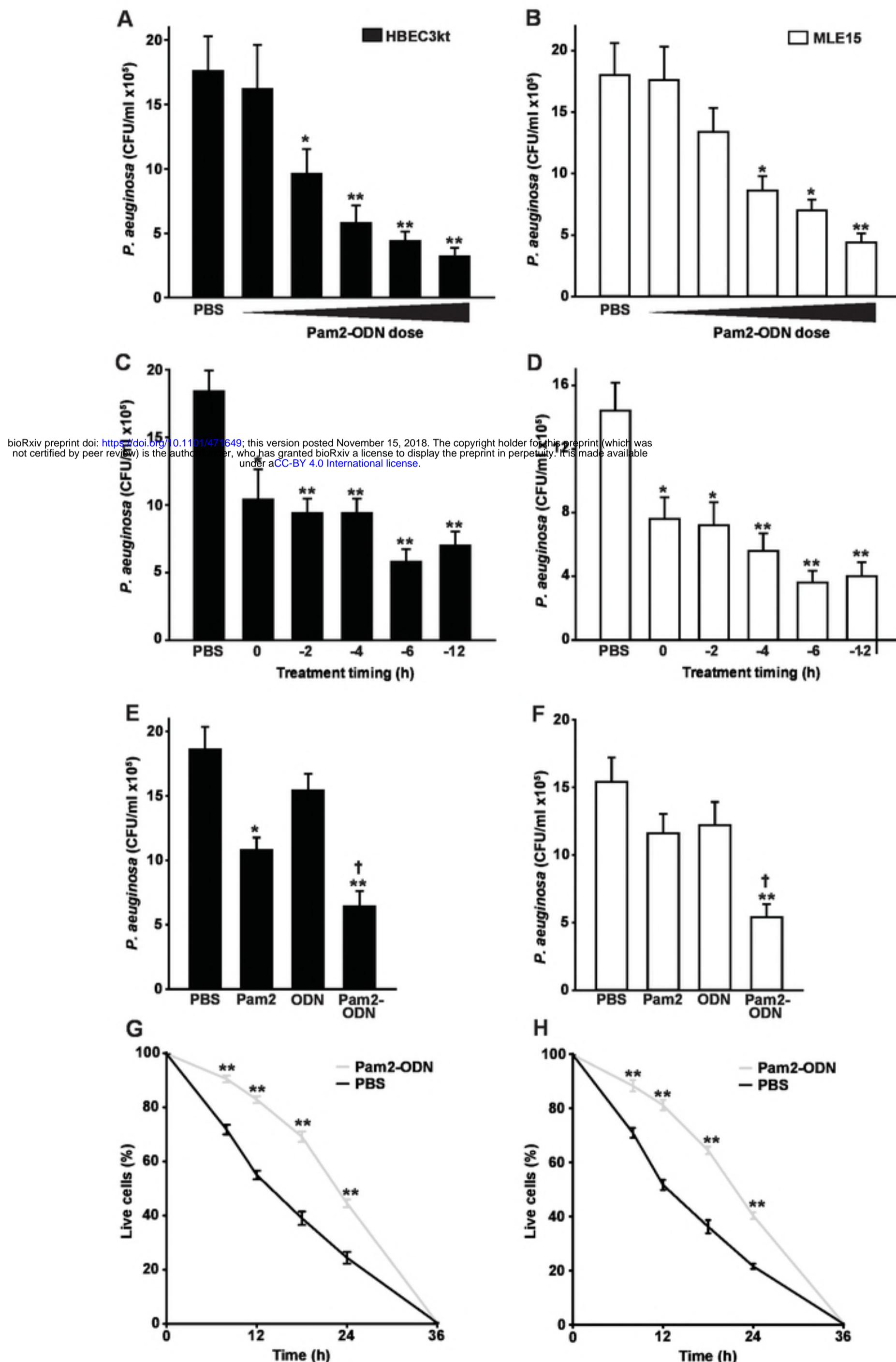
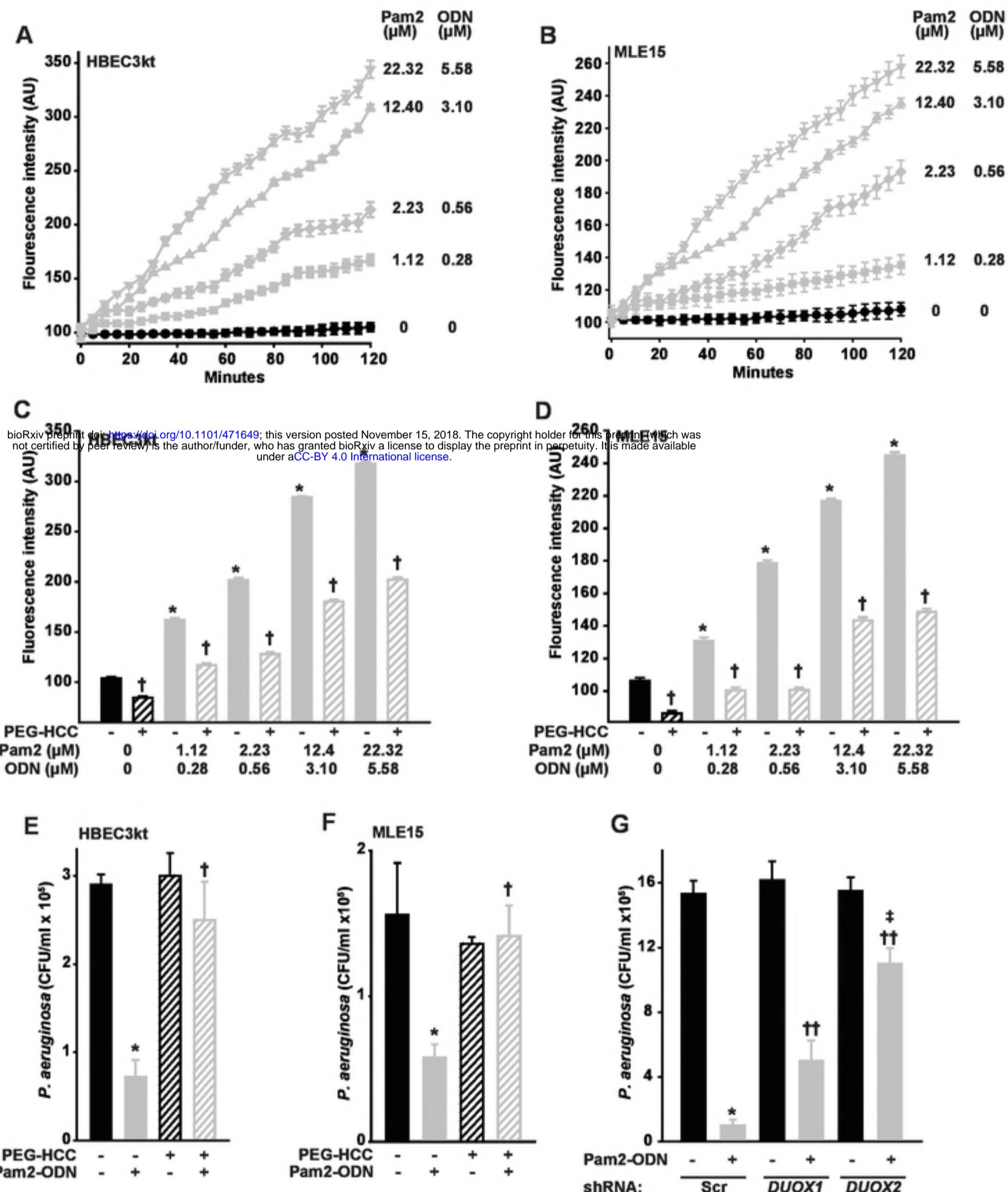


Fig 2

Ware, Figure 3



Ware, Figure 4

