Online Data Supplement

Hemolytic Phospholipase C Inhibition Protects Lung Function During *Pseudomonas Aeruginosa* Infection

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Complementation of PlcHR:

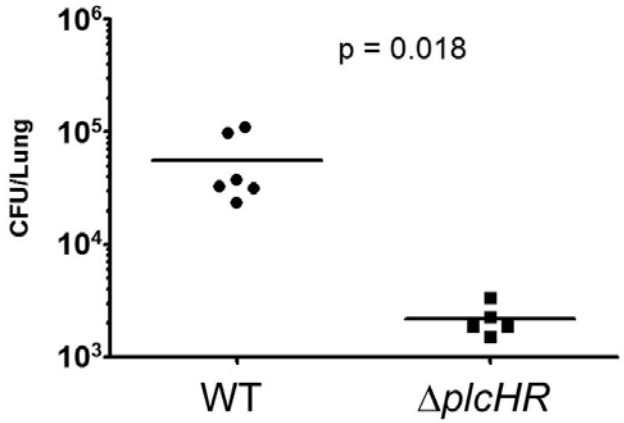
The *plcHR* deletion mutant was complemented using two separate methods, and *att*::*plcHR* complementation and reconstruction of the wild-type locus at the original location. To generate the *att*::*plcHR* complementation strain, we amplified the *plcHR* locus from PAO1 using the primers plcH-promF1 5'-CGCTCCTTCGAGGTGCTC-3' and the reverse primer used to generate the original deletion plcH-GOI-R 5'-CAGCAGTTGTTCGTCGACAT-3'. This product was cloned into the pCR Blunt vector using the ZeroBlunt kit (Invitrogen). The product was cloned into the pUC18miniTn7TGm plasmid (1) and verified by sequencing. This resultant plasmid was co-electroporated into the Δ*plcHR* strain with pTNS2 and integrants recovered by growth on PIA with 50 µg/ml gentamicin. The integration event was verified by PCR as described by Schweizer et al. (1) and choline-inducible NPPC hydrolysis activity was measured as described in the main text. The regeneration of the WT *plcHR* locus in the Δ*plcHR* mutant was done by amplifying the *plcHR* locus from WT using the following primers: plcHR-F-EcoR1 5'-cctaaccgaattcCGACGATACTGTCCCAACCT-3' and plcHR-R-EcoR1 5'-cctaaccgaattcCAGCAGTTGTTCGTCGACAT-3'. This PCR product was digested, ligated into

the pMQ30 plasmid, sequenced, and transformed into S17λpir. Regeneration of the wild-type plcHR genes at the Δ*plcHR* deletion locus was done by two-step recombination as described in the main text, yielding double recombinants where ~40% were wild-type *plcHR* and ~60% remained Δ*plcHR*. Infection and lung function was performed as described in the main text.

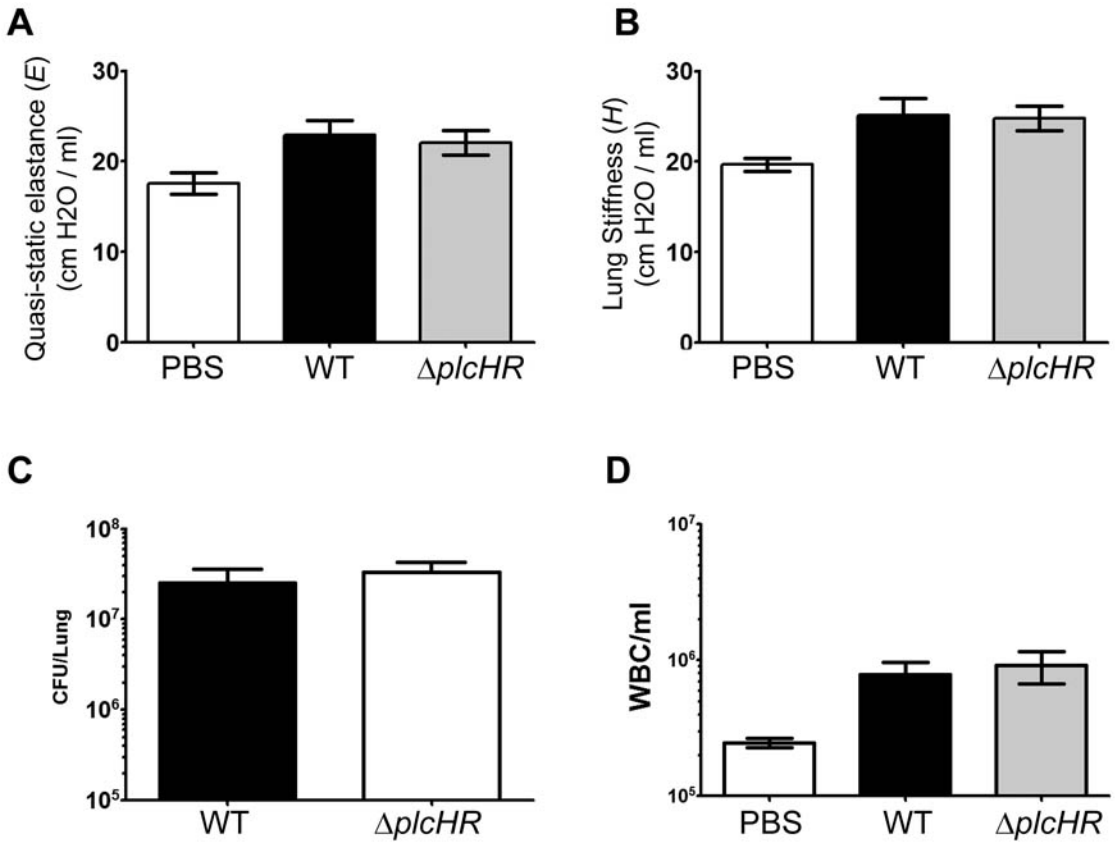
Mouse Ventilation:

Specifics of mouse ventilation are detailed here. The mouse tracheas were cannulated with an 18 gauge metal cannula, and connected to the flexiVent ventilator. Mice received 0.25 mL tidal volumes (*Vt*) at 180 breaths/min with a positive end-expiratory pressure (PEEP) of 3 cm H_2O. Following a 5 minute stabilization, two 1.0 mL, constant flow deep inflations were delivered over 4 seconds (pressure limit 30 cmH_2O) in order to standardize volume history (i.e. "most open" starting point). The mice were then returned to quasi-sinusoidal ventilation at 180 breaths per min. Respiratory impedance (*Zrs*) was measured every 20 seconds for 8 minutes, as determined via Fourier transform of ventilator piston volume displacement and cylinder pressure signals, obtained during 2-sec oscillatory volume perturbations. These perturbations were composed of 13 superimposed sine waves with frequencies between 1 and 20.5 Hz, all mutually primed to reduce harmonic distortion (2, 3). *Zrs* itself was interpreted by being fit to the constant phase model of the viscoelastic lung, from which we derived the parameter *H*, which characterizes the elastic properties of the respiratory system (4). This same protocol was repeated at PEEP of 1 cmH_2O. The value of *H* obtained from the end of the measurement period at PEEP 1 cm H_2O was used for comparison between groups. At the end of the measurement period at

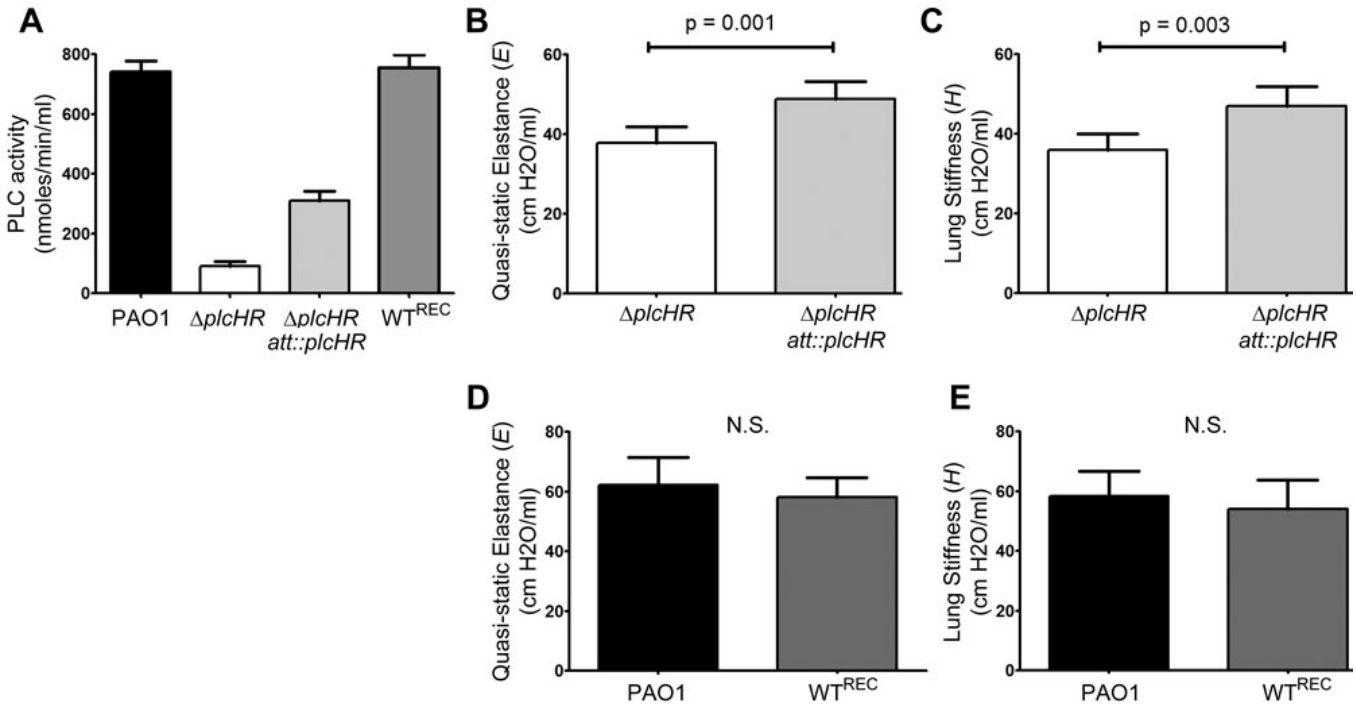
PEEP 1 cm H_2O, a quasi-static pressure-volume curve was obtained from resting residual lung volume by dropping PEEP to 0 cm H_2O and immediately delivering seven 0.1 mL steps of inspiratory volume to a total volume of 0.7 mL, followed by 7 equal expiratory steps, pausing at each step for one second. Plateau cylinder pressure measured during each pause was plotted against piston displacement volume, the latter corrected for gas compression.



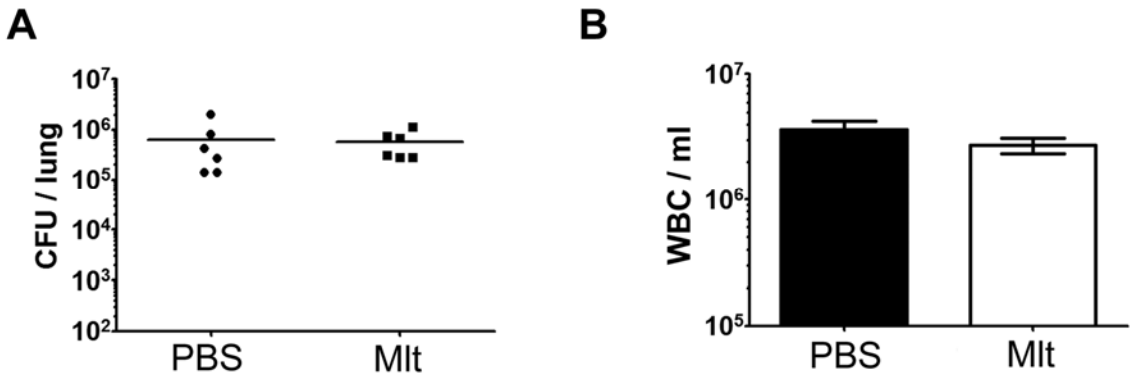
Supplemental Figure E1. The *plcHR* deletion mutant shows a defect in lung survival at low inoculum. Mice were inoculated as described in the methods section, except with $1x10^6$ viable bacteria (considered a low inoculum). Shown here is the CFU in lung homogenates at 24h post-infection. This data is representative of two independent experiments with at least 5 mice per group. Significance determined using a two-tailed t-test.



Supplemental Figure E2. There are no significant differences in lung physiology, bacterial burden, or inflammation between WT and Δ*plcHR* at 6h post-infection. Infection conducted identically to those shown in Figure 3&4 with lung mechanics and harvest done at 6 hours post-infection. Shown here are the quasi-static elastance (**A**), lung stiffness (**B**), CFU/lung (**C**) and WBC/mL BALF (**D**). This data is representative of two independent experiments with at least 5 mice per group. Significance determined using a two-tailed t-test (C) or one-way ANOVA with a Bonferroni's Multiple Comparison Test (A,B,D).



Supplemental Figure E3. Complementation of plcHR restores PlcH production and associated loss of lung function. A) NPPC hydrolysis assay comparing wild type and Δ*plcHR* to the strains complemented for *plcHR* at the *att*Tn7 site and a Δ*plcHR* in which the wild-type *plcHR* locus was reconstructed by recombination (WT^{REC}). B-C) Insertion of the plcHR genes at the att site partially restored the loss of lung physiology as measured by elastance (B) and lung stiffness (C). D-E) Reconstruction of the wild-type *plcHR* locus in a Δ*plcHR* strain resulted in indistinguishable lung physiology from PAO1 wild type. Error bars represent standard deviation. The measurements in (A) are from biological triplicates. Data from (B-E) are each from a single experiment of 6 animals per group and are representative of two independent experiments of 6 animals per group.



Supplemental Figure E4. Miltefosine treatment does not alter inflammation or bacterial burden. (**A**) Bacterial burden from whole homogenized lung from wild-type PAO1 infected animals. CFU counts from individual mice plotted with mean noted by the horizontal lines. Means are not significantly different. (**B**) WBC infiltration into the BALF of wild-type PAO1 infected animals as measured by automated counter (Advia). Mean +/- SEM plotted for 6 mice/group, and are not significantly different. Three independent experiments with at least 5 animals per infected group show similar results. Test for significance done using a two-tailed student t-test.

Supplemental References

1. Choi KH, Schweizer HP. Mini-tn7 insertion in bacteria with single atttn7 sites: Example *pseudomonas aeruginosa*. *Nature protocols* 2006;1:153-161.
2. Allen GB, Suratt BT, Rinaldi L, Petty JM, Bates JH. Choosing the frequency of deep inflation in mice: Balancing recruitment against ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L710-717.
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