






RESEARCH ARTICLE

Pseudomonas expression of an oxygen sensing prolyl hydroxylase homologue regulates neutrophil host responses *in vitro* and *in vivo* [version 1; peer review: 3 approved, 1 approved with reservations]

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Abstract





Background: *Pseudomonas* species are adapted to evade innate immune responses and can persist at sites of relative tissue hypoxia, including the mucus-plugged airways of patients with cystic fibrosis and bronchiectasis. The ability of these bacteria to directly sense and respond to changes in local oxygen availability is in part consequent upon expression of the 2-oxoglutarate oxygenase, *Pseudomonas* prolyl hydroxylase (PPHD), which acts on elongation factor Tu (EF-Tu), and is homologous with the human hypoxia inducible factor (HIF) prolyl hydroxylases. We report that PPHD expression regulates the neutrophil response to acute pseudomonal infection.




Methods: *In vitro* co-culture experiments were performed with human neutrophils and PPHD-deficient and wild-type bacteria and supernatants, with viable neutrophil counts determined by flow cytometry. *In vivo* consequences of infection with PPHD deficient *P. aeruginosa* were determined in an acute pneumonia mouse model following intra-tracheal challenge.

Results: Supernatants of PPHD-deficient bacterial cultures contained higher concentrations of the phenazine exotoxin pyocyanin and induced greater acceleration of neutrophil apoptosis than wild-type PAO1 supernatants *in vitro*. *In vivo* infection with PPHD mutants compared to wild-type PAO1 controls resulted in increased levels of neutrophil apoptosis and impaired control of infection, with higher numbers of *P. aeruginosa* recovered from the lungs of mice infected with the PPHD-deficient strain. This resulted in an overall increase in

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mortality in mice infected with the PPHD-deficient strain.

Conclusions: Our data show that *Pseudomonas* expression of its prolyl hydroxylase influences the outcome of host-pathogen interactions *in vitro* and *in vivo*, demonstrating the importance of considering how both host and pathogen adaptations to hypoxia together define outcomes of infection. Given that inhibitors for the HIF prolyl hydroxylases are in late stage trials for the treatment of anaemia and that the active sites of PPHD and human HIF prolyl hydroxylases are closely related, the results are of current clinical interest.

Keywords

Pseudomonas, neutrophil, hypoxia, *Pseudomonas* prolyl hydroxylase (PPHD), pyocyanin, apoptosis.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen, which colonizes the airways of patients with chronic inflammatory lung diseases including cystic fibrosis (CF) and bronchiectasis^{1,2}, and is an important pathogen in the setting of acute ventilation-associated pneumonia³. In the 2004 US CF patient registry, 57% of patients were found to be colonized with *P. aeruginosa*⁴, whilst children with CF who have sputum positive for *P. aeruginosa* experience more frequent hospitalisation and higher mortality⁵. These patients have chronic sputum production, with areas of mucus 'plugging', resulting in local hypoxia, a condition in which bacteria thrive⁶. Despite high levels of neutrophilic inflammation, *P. aeruginosa* continues to survive in these patients, evidence that the bacteria employ a number of effective immune-evasion strategies.

A key mechanism by which *P. aeruginosa* impairs host neutrophil function is by generation of phenazine metabolites, particularly pyocyanin, which contributes to the characteristic blue-green colour of infected sputum⁷. Pyocyanin has previously been shown to accelerate neutrophil apoptosis through activation of the lysosomal death pathway, a process dependent upon the generation of reactive oxygen intermediaries within the neutrophil⁸⁻¹⁰ and thus on the availability of molecular oxygen¹¹. More recently, the possibility *Pseudomonas* itself can directly sense and respond to changes in local oxygen availability was raised by the observation that *Pseudomonas spp* contain a 2-oxoglutarate (2OG)-dependent *Pseudomonas* prolyl hydroxylase (PPHD), which acts on the abundant translation elongation factor Tu (EF-Tu) and is homologous to the oxygen sensing hypoxia inducible transcription factor (HIF) prolyl hydroxylase (PHD) enzymes described in eukaryotes¹².

Importantly, an insertional mutant strain of *P. aeruginosa* lacking PPHD manifests increased production of pyocyanin under normoxic (room oxygen) standardized broth culture conditions. Moreover, growth of *P. aeruginosa* under conditions of hypoxia has been observed to reduce the pathogenicity of *P. aeruginosa* through repression of production of the siderophores pyoverdine and pyochelin and the secreted virulence factor Exotoxin A^{13,14}. Thus, the outcomes of host-pathogen interactions may be in part defined by adaptation of both the host and the pathogen to local oxygen availability. In this context, we hypothesised that (1) a PPHD-deficient *P. aeruginosa sp* might demonstrate a survival advantage *in vivo* as a consequence of increased pyocyanin production, leading to accelerated neutrophil apoptosis and impaired neutrophil mediated bacterial killing, and (2) that these effects would be influenced by oxygen availability.

Methods

Ethical approval

All participants gave written informed consent in accordance with the Declaration of Helsinki principles, with AMREC approval for the study of healthy human volunteers through the MRC/University of Edinburgh Centre for Inflammation Research blood resource (15-HV-013). Human peripheral blood neutrophils were isolated from whole blood using dextran sedimentation and discontinuous Percoll gradients¹⁵.

Bacterial growth curves

A Columbia blood agar culture plate (VWR International, UK) was inoculated with a single bead from a thawed master stock vial of either wild-type (PA01) or *PA0310* insertional knockout mutant strain (PPHD knockout) *Pseudomonas* and then incubated overnight at 37°C. The following day, ten colonies were taken from the plate using a sterile inoculating loop and used to inoculate 15 ml of sterile Luria-Bertani (LB) broth (Sigma, UK) in a 50ml Falcon tube. The tube was then incubated at 37°C on a shaking platform with the lid loosened. Optical density at 595 nm was measured regularly until plateau.

Intratracheal pneumonia model

All animal experiments were conducted under an Home Office approved project license in accordance with the Home Office Animals (Scientific Procedures) Act 1986 and University of Edinburgh guidelines in line with the NC3Rs. Six to eight week male C57Bl6J mice were group-housed under standard 12hr light/dark cycles with access to food and water *ad libitum*. All efforts were made to ameliorate any suffering of the animals. Mice were closely monitored over the course of the experiments and humanely culled once threshold of severity was reached.

Mice were anaesthetised with ketamine (76mg/kg, Willows Francis Veterinary, UK) and medetomidine (1mg/kg, Orion Pharma, UK) intraperitoneally. Once adequately anaesthetised, the animals were suspended from a frame by the upper incisors and a blunt needle was passed into the trachea via the orotracheal route. Each mouse then had 1×10^7 cfu of either PA01 (wild-type) or PPHD knockout (mutant) *Pseudomonas* instilled in 50µl PBS via the endotracheal. Twenty minutes after anaesthesia, the mice were given atipamezole (2mg/kg, Orion Pharma, UK), an anaesthetic reversal agent, and recovered for six hours. At indicated time points (6, 12, 24, 36 and 48h after instillation) mice were assessed and tissues harvested. For the Kaplan-Meier plots, mice were culled once the threshold of sickness was reached.

Assessment of lung injury

Bronchoalveolar lavage (BAL) was obtained by cannulation of the trachea. Total cell counts were calculated using haemocytometer counts and differential cell counts assessed on cytocentrifugation slides. IgM levels were quantified using commercially available kits (Mouse IgM ELISA quantitation set, Bethyl Laboratories Inc, Montgomery, USA; EnzChek Elastase Assay Kit, Molecular Probes Europe BV, Leiden, The Netherlands).

For histological analysis, lungs were fixed with 10% buffered formalin and embedded into paraffin blocks. Tissues slices were fixed and stained with haematoxylin and eosin.

Flow cytometry for BAL neutrophil apoptosis

BAL cells were counted and 1×10^6 cells were centrifuged at 300g for 5 minutes at 4°C. Cell pellets were resuspended in 50µL of FC block (1:100 anti-CD16/32 Ab, **RRID:AB_312801**; Biolegend) and 1:10 mouse serum in FACS buffer (PBS with 0.5% BSA and 0.02mM EDTA) and incubated on ice for 15 minutes. Subsequently, cells were stained with 50µl anti-Ly6G Ab (**RRID:AB_1236494**; BioLegend) at 1:200 final concentration and incubated on

ice for 30 minutes in the dark. Following a wash with FACS buffer and centrifugation at 300g for 5 minutes at 4°C, cells pellets were resuspended in Annexin-binding buffer and Annexin-V PE stain (Becton Dickinson) for 15 minutes at room temperature in the dark. Prior to flow cytometry acquisition, cells were stained with Topro3 APC (Molecular Probes). Neutrophils were gated based on Ly6G expression and Annexin-V and Topro3 expression was quantified.

Cells were acquired using a BD Calibur machine and analysed using FlowJo version 10 software (Tree Star).

Quantification of viable bacterial counts

10-fold serial dilutions were performed on whole blood aliquots and lungs homogenized in sterile tubes following collection of BAL fluid. Three 10µl drops from each of 6 dilutions were then plated onto blood agar plates and cultured overnight in 37°C to calculate viable bacterial counts, which were normalized to count per ml of blood or per pair of lungs.

Production of bacterial supernatants

Ten colonies were taken from the blood agar culture plate and used to inoculate plates containing 20ml of pseudomonas isolation agar (Difco). These plates were incubated overnight at 37°C and then placed in direct sunlight for 48h to allow pigment to develop. Each plate was then flooded with 6ml RPMI media (Sigma, UK) and left at room temperature for 2 hours. The RPMI was removed, spun at 4000g for 15 min, twice, and filter sterilised through a 0.22µm filter to remove any bacteria. To ensure sterility, 100µl of each supernatant was used to inoculate a blood agar plate and cultured for 48h at 37°C. Supernatants were stored at -80°C.

Quantification of pyocyanin concentration

PPHD mutant and wildtype colonies were inoculated into 10 ml of LB broth and incubated overnight at 37°C in a shaking incubator. 1ml of the overnight cultures were then inoculated into 9ml of LB broth and incubated for 2 hours at 37°C in a shaking incubator. 100 µl of each strain was then pipetted onto Pseudomonas Isolation agar (Difco) plates and incubated overnight under conditions of normoxia (21% O₂) and hypoxia (3% O₂) and supernatants produced as detailed above. 4.5ml of chloroform was added to 7.5ml of sterile bacterial supernatant and vortexed. Samples were centrifuged at 2000g for 10 minutes. 3ml of the chloroform layer was transferred to a clean tube and 1.5ml 0.2M hydrochloric acid was added. Tubes were vortexed and spun at 2000g for 2 minutes. 1ml of the top layer was removed, absorbance at 520nm measured and pyocyanin concentrations determined¹⁶.

Isolation and culture of human neutrophils

Human peripheral blood neutrophils were isolated from whole blood using dextran sedimentation and discontinuous Percoll gradients. Neutrophils were resuspended in RPMI with 20% fetal calf serum (Lifetechn, Paisley, UK) at 10x10⁶/ml. 75µl of this suspension was cultured with 75µl of either wild-type (PAO1) or mutant (PPHD knockout) pseudomonas supernatant for five hours in either normoxia (room air) or hypoxia (1% oxygen, *in vivo* 400 hypoxia workstation, Ruskin). After 5 hours, cells were removed from the culture plate and pelleted at 400g for 5 minutes. The pellets were resuspended in 95µl annexin binding buffer and

5µl annexin V/PE (Becton-Dickinson) and incubated on ice for 20 minutes. 100µl of Topro3/APC (Molecular Probes) and 5x10⁴ Countbright™ absolute counting beads (ThermoFisher, UK) were added to each sample, and samples run using a BD FACSCalibur (BD Biosciences, UK).

Statistical analysis

Data were analysed using Prism 7.0 software (GraphPad Software Inc., San Diego, CA). Unpaired t-tests were used for comparisons between wild-type and knockout sample means. Two-way ANOVA with Bonferroni's post-test comparisons was performed if multiple time points were used. For comparison of viable bacterial counts, Mann-Whitney test was performed. Survival was analysed using log-rank test. Statistical significance was accepted when p<0.05.

Results

Neutrophil co-culture with PPHD mutant bacterial supernatants induced cell loss, which was reversed with hypoxic culture. To directly address whether expression of the hypoxia sensing prolyl hydroxylase PPHD by *P. aeruginosa* would affect rates of neutrophil apoptosis, freshly isolated human peripheral blood neutrophils were cultured for 5h with sterile supernatants harvested from wild type PAO1 and PPHD-deficient bacterial cultures *in vitro*, a time-point at which pyocyanin markedly accelerates neutrophil apoptosis⁸. Total neutrophil numbers and neutrophil viability were assessed by flow cytometry (Figure 1A). In normoxia, both PAO1 and PPHD-deficient supernatants caused significant loss of neutrophil numbers (Figure 1B) and a reduction in Annexin V- /Topro 3- (viable) neutrophils recovered. Greater reductions in viable cell numbers (Annexin V-/Topro 3-) were observed when neutrophils were cultured in the presence of PPHD-deficient supernatants (Figure 1C). Hypoxic cell culture reversed the increases in both cell loss and apoptosis observed with PAO1 and PPHD-deficient supernatants (Figure 1A-C), in keeping with the dependence of pyocyanin-induced apoptosis on the availability of oxygen¹¹.

To address whether the observed differences in neutrophil loss reflected altered pyocyanin production, we measured pyocyanin production over a 48 hour inoculation of blood agar following recovery into RPMI media. Under conditions of normoxia (21% O₂), the PPHD-deficient strain produced significantly higher levels of pyocyanin than the wild-type strain (Figure 1D), in keeping with the greater loss of viable neutrophil numbers (Figure 1C). Elevated pyocyanin production by the PPHD-deficient strain was abrogated entirely by use of a hypoxic (1% O₂) cell culture (Figure 1D). This was not a consequence of differential bacterial growth rates, with equivalent 595 nm absorbance and bacterial counts being observed for PAO1 and PPHD mutants at each oxygen tension studied (Figure 1E, F). Hypoxia (1% O₂), whilst impairing bacterial growth, had no differential growth effects on PAO1 compared with the PPHD-deficient strain.

PPHD-deficient *P. aeruginosa* infection results in increased mortality and lung injury during acute pneumonia and greater impairment of neutrophil-mediated host defense compared with wild-type PAO1 infection

To define whether PPHD deficiency results in an altered course of acute *P. aeruginosa* infection *in vivo*, mice were challenged

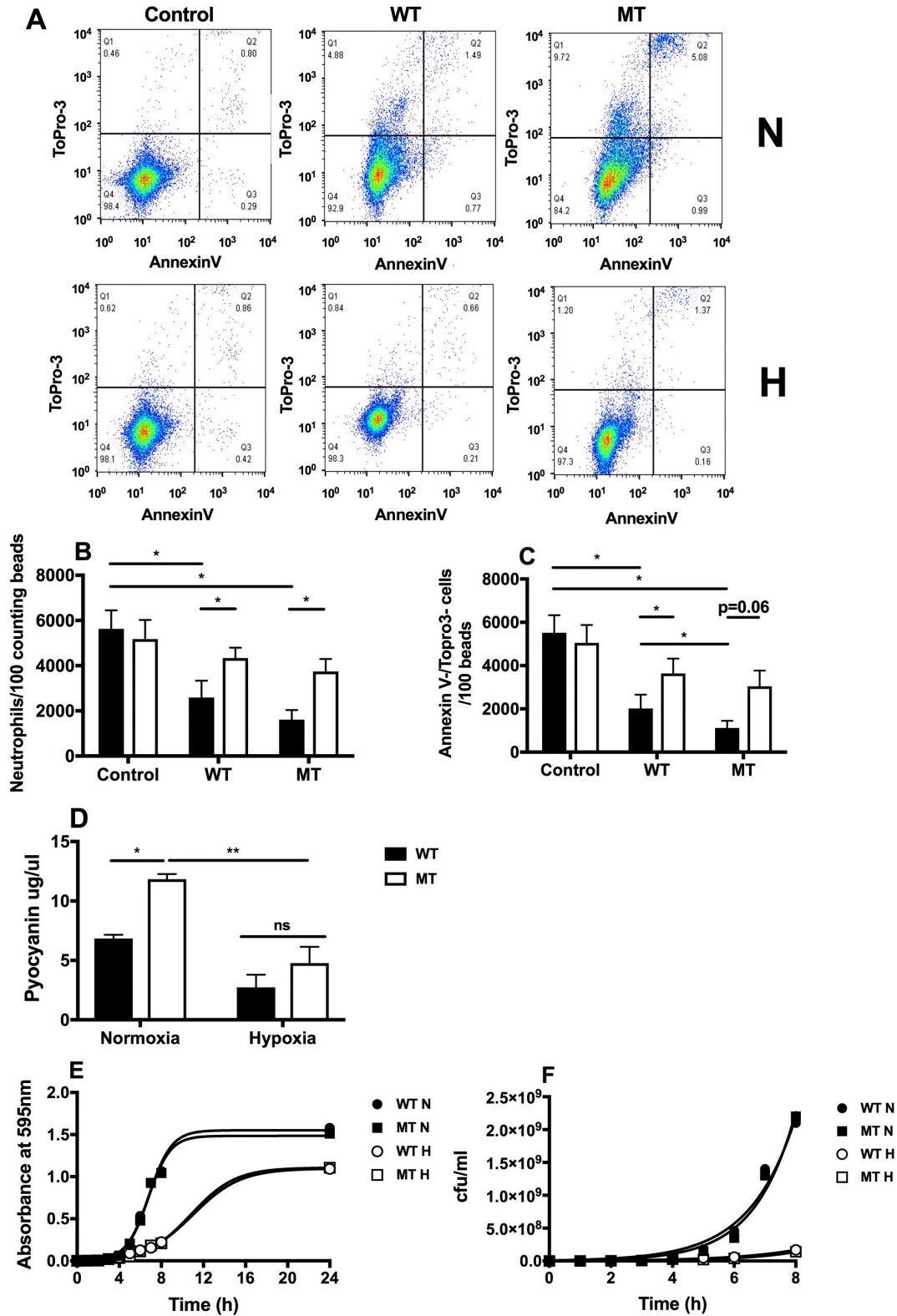


Figure 1. Supernatants from *Pseudomonas prolyl hydroxylase* (PPHD) knockout *P. aeruginosa* induce neutrophil death via increased production of pyocyanin. Human neutrophils were cultured with PA01 wildtype (WT) or PPHD knockout (MT) bacterial supernatant for 5 hours in normoxia (N; filled bars) or hypoxia (H; open bars). Flow cytometry (A) was performed to calculate total (B) and viable neutrophil numbers (C). n= 5 *p<0.05. (D) Pyocyanin concentrations in supernatants from wildtype (WT) and PPHD knockout *P. aeruginosa* (MT) in normoxia and hypoxia were measured. n=3, *p<0.05, **p<0.01. Wildtype (WT) and PPHD knockout *P. aeruginosa* (MT) were grown in normoxia (N, 21% oxygen) and hypoxia (H, 1% oxygen). Absorbance at 595nm (E) and viable bacterial count (F) were recorded to plot growth curves.

via the trachea with 1×10^7 cfu PAO1 (wildtype) or PPHD-deficient bacteria. 50% of animals receiving PPHD-deficient *P. aeruginosa* reached sickness thresholds requiring the animals to be culled by day 5 (Figure 2A). In contrast, all PAO1 infected

mice were viable up to 5 days following infection challenge (Figure 2A, $*p < 0.05$). Importantly, this increase in mortality was associated with a 2.5-fold greater bacterial burden in the lungs of PPHD mutant infected (11.1×10^4 CFU/lung $\pm 5.99 \times 10^4$)

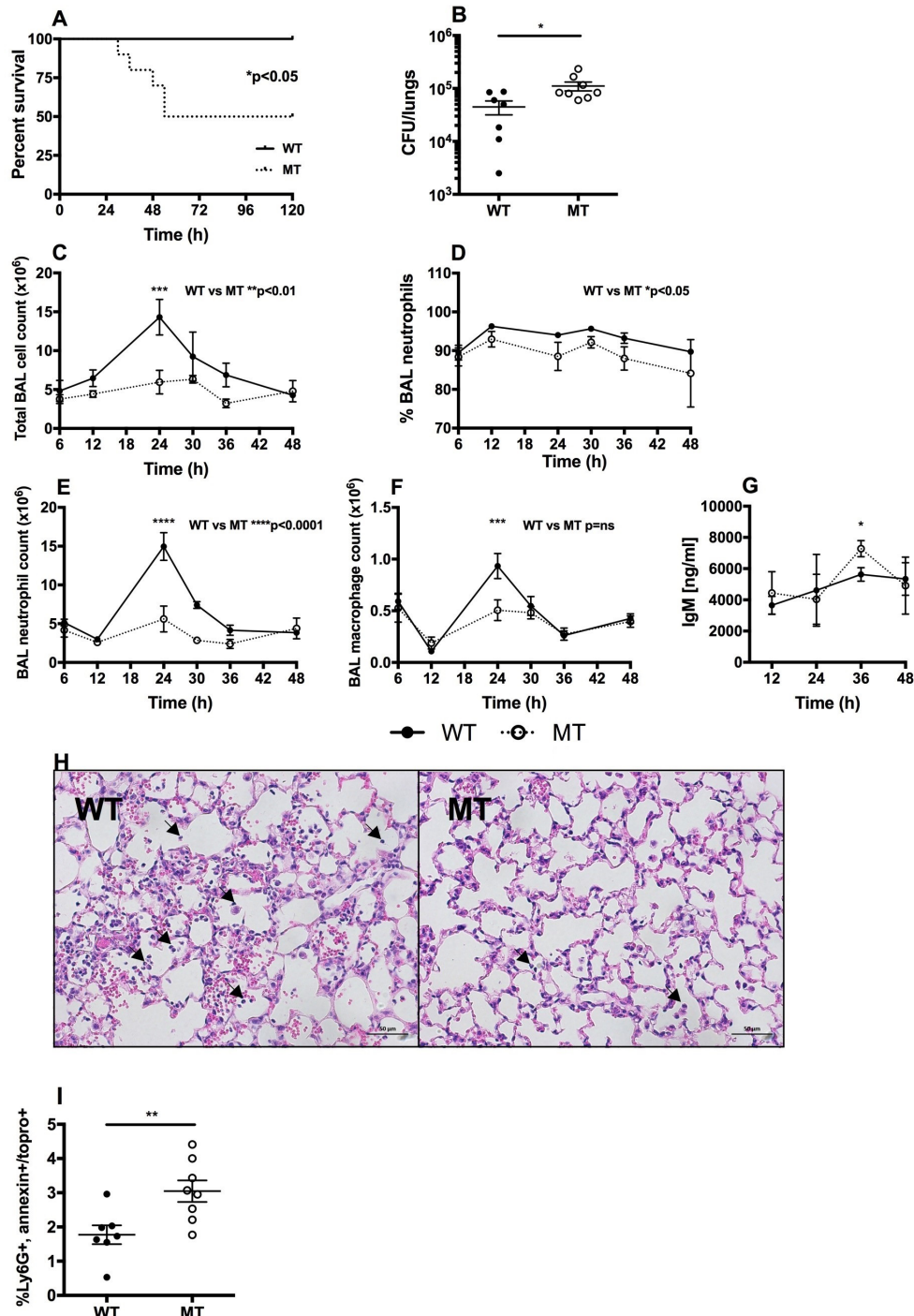


Figure 2. Infection with *Pseudomonas prolyl hydroxylase* (PPHD) knockout *P. aeruginosa* carries higher mortality. C57/BL6 mice were instilled intratracheally with 1×10^7 cfu PAO1 wildtype (WT) or PPHD knockout (MT) *P. aeruginosa*. (A) Survival was recorded for 5 days post infection, $n=10$ mice per group. (B) At 12 hours post infection lungs were harvested and viable bacteria count calculated, $n=8$ mice per group $*p < 0.05$. Bronchoalveolar lavage (BAL) total cell count (C), % neutrophils (D), neutrophil count (E), macrophage count (F) and BAL supernatant immunoglobulin M (IgM) (G) were measured at timepoints from 12–48 hours post infection, $n= 4-7$ mice per group, $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$. (H) H+E staining of lung tissue taken at 36h after infection (arrows point to neutrophils, $\times 20$ magnification). Images representative for $n=2$ mice per group. (I) BAL was harvested and the cell pellets analysed by flow cytometry for apoptosis at 12 hours post infection, $n=8$ mice per group $**p < 0.01$.

compared to wildtype infected mice (4.48×10^4 CFU/lung $\pm 3.48 \times 10^4$, Figure 2B, $p < 0.05$).

In light of the equivalent growth of wild-type and PPHD mutant strains we had observed *in vitro*, we questioned whether the increase in bacterial numbers in PPHD mutants was a consequence of an impaired host response. Whilst the initial recruitment of inflammatory cells to the lungs (6h and 12h) was similar between wildtype and PPHD-deficient *P. aeruginosa* infected mice (Figure 2C–F), significantly fewer cells were recovered from the airways of PPHD-deficient infected mice by 24h after infection (Figure 2C, $**p < 0.01$), as a consequence of reductions in both the percentage (Figure 2D, $*p < 0.05$) and total number of airway neutrophils (Figure 2E and F, $**** p < 0.0001$). In keeping with a more severe infection in PPHD-deficient infected mice, higher levels of IgM, an indirect marker of vascular leak and lung injury, were detected in mice infected with mutant PPHD (Figure 2G, $*p < 0.05$). Histological analysis of lungs of mice infected with *P. aeruginosa* supported the observed differences in BAL, with fewer neutrophils in the lungs of mice infected with mutant PPHD (Figure 2H).

In light of the increased production of pyocyanin by PPHD mutant *P. aeruginosa* and the observed increase in neutrophil loss with PPHD supernatants, we hypothesised the reduction in neutrophil numbers observed at 24 hours to be a consequence of increased levels of neutrophil apoptosis. Ly6G+ airway recovered neutrophils were therefore dual stained with Annexin V/Topro3 to directly quantify the number of apoptotic cells following *Pseudomonas* infection. Infection with mutant PPHD *P. aeruginosa* resulted in higher detectable levels of apoptosis than infection with the PAO1 wild type strain (Figure 2I, $p < 0.01$).

Discussion

A significant focus of research from our group and others has centered round defining the mechanisms by which hypoxia directly regulates immune cell function^{17–22}. Innate responses to bacterial challenges are critically regulated by oxygen availability, with neutrophils in particular being adapted to survive in hypoxic tissues where they phagocytose and kill bacteria^{11,21,23}. Until recently, the possibility that oxygen may also regulate the behavior of bacterial pathogens has not been considered. This is of particular relevance to *Pseudomonas spp* that persist in chronically inflamed tissues characterized by limited oxygen availability²⁴ and induce oxidant-dependent cell death via the production of the phenazine, pyocyanin^{10,25}. The results described here reveal the importance of the *Pseudomonas* prolyl hydroxylase, PPHD, in regulating the effectiveness of neutrophil mediated host defenses *in vivo*, likely, at least in part, as mediated by variations in the levels of the toxic metabolite pyocyanin.

Suppression of PHD activity is described in eukaryotic systems in the context of hypoxia – indeed is central to regulation of the hypoxic response^{26–30}. Diminished *Pseudomonas aeruginosa* pathogenicity in hypoxia has recently been described as a consequence of reduced expression of the virulence factors pyoverdine and exotoxin A¹⁴, with our work extending this to include production of pyocyanin. This is of interest, given that neutrophil respiratory burst activity, a key anti-microbial defence, is associated with promotion of a hypoxic niche²². It is also of relevance to the oxygen requiring process by which the pyocyanin induces ROI-mediated lysosomal dysfunction and neutrophil apoptosis¹⁰,

as evidenced by the reduction in cell loss we observed when neutrophils were challenged with *P. aeruginosa* conditioned media in the context of hypoxia. Thus in clinical scenarios in which tissue oxygen availability is severely limited, oxygen dependent regulation of the balance between innate immune responses and bacterial virulence and replicative capacity may be critical in defining the outcomes of infection and host morbidity and mortality. This is, however, further complicated by the observations that differential expression of oxygen sensing prolyl hydroxylase enzymes either by immune cells^{20,31,32}, or bacterial pathogens¹² can also directly regulate cellular function and bacterial virulence when oxygen is not a limiting factor. For example, neutrophil loss of PHD2 under physiological normoxia promotes a phenotype of excessive neutrophilic inflammation³³, whilst deletion of PPHD is associated with increased production of pyocyanin¹². *In vivo* therefore, the dominant phenotype is likely to be in part determined by the physiological K_m , in which both PPHDs and PHDs function in both innate immune and bacterial cells. Of interest, kinetic analysis of the isolated PPHD enzyme has identified a lower apparent K_m for O_2 than PHD2, but a higher K_m for Fe(II), suggesting that iron regulation may also be of critical importance in defining the activity of PPHD enzyme activity in a physiological setting¹². This is of particularly relevance to *Pseudomonas spp* where enhanced iron redox states enable competitive outgrowth from other bacterial species³⁴.

In this work, we provide *in vivo* data, describing the clinical outcomes when mice are challenged with acute *P. aeruginosa* infection in the context of normal lung architecture and therefore relatively preserved local tissue oxygenation. In this setting we observe increased mortality with PPHD-deficient strains as a consequence of insufficient neutrophil host defense and failure to control bacterial replication. Thus, we can now extend the concept that immune cell loss of PHD2 in the context of preserved tissue oxygenation promotes a detrimental immune response to also include detrimental consequences of prokaryotic loss of PPHD expression. This has potentially important ramifications in light of the current development of relatively non-selective PHD inhibitors (which may well inhibit PPHD), as well as the use of iron chelators in the clinical arena, and how they may impact more widely on the host pathogen response with consequence both for the host and the pathogen.

Data availability

Raw data counts available via Figshare: <https://doi.org/10.6084/m9.figshare.5484178.v1>³⁵

Competing interests

No competing interests were disclosed.

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Stilla Frede

Department of Anesthesiology and Intensive Care Medicine, University Hospital Bonn, Bonn, Germany

In this manuscript the authors clearly describe the fatal effects of PPHD knockout for the clinical outcome of mice challenged with this bacterial strain. The data are convincing and point to the problems possibly occurring with the use of small molecule inhibitors of PHDs.

I have a question regarding the signalling pathway(s) underlying the increased Pyocyanin production in PPHD knockout bacteria. If the classical inhibition of PHD activity under hypoxic conditions is involved why is the production of Pyocyanin not increased in the wt strain under these conditions?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Inflammatory hypoxia, sepsis, pathway analysis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 12 Dec 2017

Sarah Walmsley, University of Edinburgh, Edinburgh, UK

We thank Dr Frede for her review of our manuscript. In regard to the question raised, the pyocyanin concentrations reported in Figure 1D are expressed as ug/ul and do not take into account differences in bacterial number under different conditions. In Figure 1F we show the viable bacterial count is greatly reduced for both the wt and mutant PSA strains when incubated in hypoxia compared to normoxia. If we correct for pyocyanin concentration per cfu we see an increased level of pyocyanin produced in the wt bacteria in hypoxia.

Competing Interests: No competing interests were disclosed.

Reviewer Report 22 November 2017

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Benoît S. Marteyn

Pasteur Institute, Molecular Microbial Pathogenesis, Paris, France

In this manuscript, Dickinson and colleagues reveal the impact of *P. aeruginosa* PPHD on the bacteria virulence using in vitro and in vivo models. In particular, the PPHD mutation-dependent increased concentration of pyocyanin in bacterial supernatants was characterized and associated with increased neutrophil apoptosis. The manuscript is clear, well written and the presented data support the conclusions.

In order to better appreciate the contribution of pyocyanin on *P. aeruginosa* mediated neutrophil apoptosis, the following points should be addressed:

1. The authors studied the impact of pyocyanin on neutrophil survival using bacterial supernatants from WT and PPHD-deficient mutant. In order to evaluate the contribution of pyocyanin on *P. aeruginosa*-dependent neutrophil apoptosis induction, the authors should additionally infect neutrophils with WT and PPHD-deficient mutant strains and assess neutrophil viability (w/wo oxygen). The comparison with neutrophil apoptosis levels induced with bacterial supernatant should be then discussed.
2. It would be informative to assess the ability of neutrophils to kill WT and MT strains (w/wo

oxygen) and the results should be discussed to better interpret in vivo results.

3. In the discussion, the authors say that "the possibility that oxygen may also regulate the behavior of bacterial pathogens has not been considered". This statement is not true, additional references should be included regarding the O₂-modulation of bacteria virulence, adhesion, secretion, etc.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Nov 2017

Sarah Walmsley, University of Edinburgh, Edinburgh, UK

We thank Dr Marteyn for the review of our manuscript.

1. In vitro co-culture of neutrophils directly with pseudomonas species rapidly results in neutrophil loss. This is why we firstly explored the consequence of neutrophil culture with bacterial supernatants before undertaking in vivo experiments in which we were able to directly address the consequence of infection with the different strains of *P. aeruginosa* on neutrophil survival in a biological setting.
2. Due to the ability of pseudomonas to induce neutrophil apoptosis, this is a difficult question to directly address. We would however argue that it is the ability of *Pseudomonas* to evade the host response that is critical in defining the outcome of the infection challenge, a concept supported by the published literature (Usher et al. JI 2002; Allen et al. JI 2005; Prince et al. JI 2008) and our in vivo observations.

3. We are sorry for any confusion caused, in the discussion we do actually state that “until recently, the possibility that oxygen may also regulate the bacterial pathogens has not been considered” and provide a number of references specific to *Pseudomonas* throughout the text that reference that capacity of hypoxia to alter pathogenicity (Scotti et al. PNAS 2014; Schaible et al. PLOS One 2013; Schaible et al. J Infect Dis 2017).

Competing Interests: No competing interests were disclosed.

Reviewer Report 22 November 2017

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Andrew S. Cowburn 

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² Department of Medicine, University of Cambridge, Cambridge, UK

The authors very elegantly describe a key role for *Pseudomonas* prolyl hydroxylase (PPH) in the sensing of oxygen tension and the release of the phenazine exotoxin pyocyanin, this enhances the rate of neutrophil apoptosis and compromises the host innate immune response. The authors competently used two model systems, human neutrophil incubated in the presence of supernatants from wt *Pseudomonas* or mutant lacking PPH cultured in either normoxia or hypoxia, and a murine pneumonia model. Both systems clearly show that infection with *Pseudomonas* deficient in PPH results with increased neutrophil cell death and compromised host response to the infection resulting in increased mortality in the murine model.

I have one minor question regarding the murine model used. Fig 2E shows a significant drop in BAL neutrophil number at 24hrs, however in Fig 2I the authors analysed BAL from the 12hr time point showing a small but significant shift in neutrophil cell death. The description in the results refers to BAL 24hr data set. If the data is available from 24hr BAL I believe this would enhance Fig 2 and benefit the readers understanding.

This manuscript further highlights the importance of understanding how the new generation of small molecular inhibitors that interact with the oxygen sensing pathway need to be comprehensively investigated not only at the cellular/tissues level but also at the point of host pathogen interaction.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 22 Nov 2017

Sarah Walmsley, University of Edinburgh, Edinburgh, UK

We thank Dr Cowburn for highlighting the importance of the observations detailed in the manuscript. With respect to the time points studied, given we were already detecting a marked difference in BAL neutrophil counts at 24 hours, and that apoptotic cells are rapidly cleared in the in vivo setting, we chose to study a time point preceding one associated with significant cell loss (12 hours), to enable us to measure changes in surface apoptosis markers.

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 November 2017

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Susanne Schlisio 

Department of Cell and Molecular Biology, Ludwig Institute for Cancer Research, Karolinska

Institute, Stockholm, Sweden

The authors provide convincing *in vivo* evidence that prolyl hydroxylase-deficient *P. aeruginosa* infection results in increased levels of neutrophil apoptosis, impaired control of infection, and consequently increased mortality in mice. The authors conclude that the expression of the oxygen sensing prolyl hydroxylase homologue (PPHD) in *Pseudomonas* regulates neutrophil host responses *in vivo*. The importance of this finding is in light of current development of relatively non-selective PHD inhibitors that most likely inhibit both, the host and *Pseudomonas* prolyl hydroxylase and thus impact both, host and pathogen.

The *in vivo* experiments and analyses presented in this work appear robust and of high quality.

I only have a minor comment regarding *in vitro* studies in Fig 1B:

It seems that under normoxia, there was no significant change in neutrophil numbers cultured with WT or PPHD mutant supernatant (Fig 1B normoxia -black bar- WT versus MT). Since pyocyanin was significantly increased in the MT under normoxia (Fig 1D), why was that not reflected in significant decrease of neutrophil counts under normoxic conditions in MT versus WT (FIG 1B)? Please provide comments. What is the p value: WT versus MT under normoxia in Fig 1B? Is the mild reduction of neutrophil counts significant under normoxia (WT vs MT)?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 16 Nov 2017

Sarah Walmsley, University of Edinburgh, Edinburgh, UK

We thank Dr Schlisio for her careful review of our manuscript and comments. Although the mean number of neutrophils following co-culture with mutant supernatants is lower than wildtype (1610 ± 426 MT vs 2591 ± 749) this does not reach statistical significance ($P=0.89$ by two way ANOVA). We attribute this to significant cell loss with both wildtype and mutant supernatants under normoxic culture conditions given both strains of *P. aeruginosa* produce toxic levels of pyocyanin in normoxia.

Competing Interests: No competing interests were disclosed.
