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RESEARCH ARTICLE

REVISED Differential roles for the oxygen sensing enzymes PHD1 and PHD3 in the regulation of neutrophil metabolism and function [version 2; peer review: 2 approved, 2 approved with reservations]

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Abstract

Background

Neutrophils are essential in the early innate immune response to pathogens. Harnessing their antimicrobial powers, without driving excessive and damaging inflammatory responses, represents an attractive therapeutic possibility. The neutrophil population is increasingly recognised to be more diverse and malleable than was previously appreciated. Hypoxic signalling pathways are known to regulate important neutrophil behaviours and, as such, are potential

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therapeutic targets for regulating neutrophil antimicrobial and inflammatory responses.

Methods

We used a combination of *in vivo* and *ex vivo* models, utilising neutrophil and myeloid specific PHD1 or PHD3 deficient mouse lines to investigate the roles of oxygen sensing prolyl hydroxylase enzymes in the regulation of neutrophilic inflammation and immunity. Mass spectrometry and Seahorse metabolic flux assays were used to analyse the role of metabolic shifts in driving the downstream phenotypes.

Results

We found that PHD1 deficiency drives alterations in neutrophil metabolism and recruitment, in an oxygen dependent fashion. Despite this, PHD1 deficiency did not significantly alter *ex vivo* neutrophil phenotypes or *in vivo* outcomes in mouse models of inflammation. Conversely, PHD3 deficiency was found to enhance neutrophil antibacterial properties without excessive inflammatory responses. This was not linked to changes in the abundance of core metabolites but was associated with increased oxygen consumption and increased mitochondrial reactive oxygen species (mROS) production.

Conclusions

PHD3 deficiency drives a favourable neutrophil phenotype in infection and, as such, is an important potential therapeutic target.

Keywords
Neutrophil, Hypoxia, Prolyl hydroxylase, PHD1, PHD3, Inflammation

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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED **Amendments from Version 1**

We are providing an updated version of this manuscript in order to address the very helpful reviewer comments we have received. This updated version contains some limited new data demonstrating normal baseline leucocyte populations in the MRP8Cre mouse lines and confirming neutrophil expression of the MRP8Cre recombinase (Figure 1A&B and Figure 6A&B).

The majority of the comments raised have been addressed by revision of the text in particular with regards to the limitations of our study and to the conclusions drawn. We are grateful to the reviewers for their recognition of clarity and accuracy of our work and hope that in this revised version they will agree that the conclusions drawn are supported by the data.

This updated version also includes additional methodological detail, specifically regarding isolation of pure populations of neutrophils.

Any further responses from the reviewers can be found at the end of the article

Introduction

In the era of multi-drug resistant organisms, limited development of novel antimicrobials and health threats from emerging pathogens, harnessing our own antimicrobial machinery to better fight infections is an attractive therapeutic option. Neutrophils are essential first responders in the immune system with an arsenal of antimicrobial factors. However, without tight regulation of the neutrophil response, inappropriate or excessive inflammation can lead to significant pathology. Neutrophilic inflammation is implicated in a number of acute and chronic inflammatory disorders, exemplified by acute respiratory distress syndrome (ARDS)[1] and chronic obstructive pulmonary disease (COPD)[2] in the lung. A more comprehensive understanding of the factors which drive these harmful neutrophil phenotypes, as well as neutrophil antimicrobial capacity, is an essential step in the development of novel therapies for both inflammation and infection.

An important feature of the neutrophil population is their resilience in the face of hostile inflammatory environments. They are highly adapted to not only survive, but to thrive in oxygen and nutrient deplete inflammatory environments. Indeed, hypoxia results in prolonged neutrophil survival[3] and enhanced inflammatory responses[4].

Cellular adaptations to hypoxia are driven predominantly by the transcription factor hypoxia inducible factor (HIF). HIF activity is regulated by the oxygen sensing prolyl hydroxylase (PHD) enzymes, PHD1, PHD2 and PHD3. In the absence of oxygen, HIFα subunits accumulate, translocate to the nucleus, and form a functional heterodimer with the constitutively expressed HIFβ subunit, leading to transcription of a wide range of genes essential in cellular adaptations to hypoxia[5]. In the presence of oxygen, the PHDs hydroxylate the α subunit of HIF, targeting it for ubiquitin mediated degradation, thus preventing downstream transcriptional activity. PHDs and HIFα subunits demonstrate significant isoform and context specific activity, affording additional complexity in cellular responses to hypoxia[6,7].

The development of chemical modulators of hypoxic response pathways is a major research focus in this field. A number of pan-hydroxylase inhibitors are either approved for clinical use or currently in clinical trials for treatment of renal anaemia (due to the role of HIF in erythropoietin production) and for inflammatory disorders[8,9]. However, the development of truly isoform specific reagents has not yet been achieved. The non-equivalent roles of different HIFα and PHD isoforms is therefore of pathophysiological importance; the outcome of pan-hydroxylase inhibition will depend on the inflammatory context including the dominant isoform and cell type involved.

We have previously shown that PHD2 is the dominant regulator of HIFα in neutrophils under normoxic conditions[10]. Loss of PHD2 resulted in unchecked HIF activity, enhanced glycolysis and uncontrolled inflammation. Importantly, despite this increased inflammatory capacity, PHD2 deficient neutrophils did not acquire improved bactericidal capacity. In contrast, PHD3 is upregulated in hypoxic neutrophils (consistent with other cell types[6]) and specifically regulates neutrophil hypoxic survival. Whole animal PHD3 deficiency leads to improved inflammation resolution in hypoxic acute lung injury due to increased neutrophil apoptosis[11]. The effect of this pro-resolution phenotype on neutrophil antimicrobial responses has not previously been studied and is an important consideration when considering therapeutic targeting of PHD3.

PHD1 has not previously been studied in neutrophils. Knockout of PHD1 has been found to be beneficial in animal models of ischaemic or hypoxic challenge: PHD1 deficiency was protective in models of skeletal muscle ischaemia[12], liver ischaemic/reperfusion injury[13] and ischaemic stroke[14]. In each case, the protection was conferred by metabolic alterations, but the specific metabolic adaptations varied with tissue type.

We sought to further delineate the isoform-specific roles of both PHD3 and PHD1 in neutrophils. Hypoxia drives a hyperinflammatory neutrophil phenotype[15,16]. Given the evidence from other tissue types that PHD1 deficiency may be protective in hypoxic/ischaemic injuries we investigated whether PHD1 deficiency in the neutrophil population altered inflammation outcomes in hypoxia. We show that, in contrast to other tissues, neutrophil specific PHD1 deficiency is not protective in hypoxic sterile inflammation. Alterations in recruitment dynamics and metabolic flux are seen but are not associated with any identifiable functional differences in this model.

We then investigated the consequences of neutrophil PHD3 deficiency. We expanded our previous findings to include infection models to better understand the global consequences of PHD3 loss. We show that myeloid specific PHD3 deficiency confers enhanced bactericidal capacity in neutrophils with improved *in vivo* outcomes in infection models. Importantly, this

is not associated with detrimental hyperinflammatory responses in sterile inflammation. We investigated the potential mechanisms underlying this phenotype and identified an increase in mROS production (with associated increased oxygen consumption in response to bacteria) as a potential driver of enhanced bactericidal capacity.

Methods

Generation of mouse lines

Whole animal PHD1 deficient mice and PHD1 floxed mice have been described previously and display no significant health problems[12]. PHD1$^{fl/fl}$ animals were crossed with MRP8-Cre-ires/GFP animals purchased from the Jackson lab to generate neutrophil specific[17] PHD1 deficient mice (PHD1$^{fl/fl}$MRP8Cre$^{+/-}$) and Cre negative litter mates (PHD1$^{fl/fl}$MRP8Cre$^{-/-}$). The mice were backcrossed for at least eight generations and maintained on a C57BL/6 background. This line bred normally with no underlying health issues.

Our previous work has utilised whole animal PHD3 deficient mice. We sought to clarify the specific role of neutrophil populations by using myeloid specific and neutrophil specific PHD3 deficient mice. PHD3 floxed mice are previously described[18]. Myeloid specific deletion was achieved through a lysozyme M-driven (LysM) Cre recombinase[19] to generate myeloid specific PHD3 deficient mice (PHD3$^{fl/fl}$LysMCre$^{+/-}$) and Cre negative littermates (PHD3$^{fl/fl}$LysMCre$^{-/-}$), as previously described[20]. Neutrophil specific PHD3 knockout mice were also used for some *ex vivo* experiments and were generated through crossing PHD3$^{fl/fl}$ animals with MRP8-Cre-ires/GFP animals, as above to give neutrophil specific PHD3 deficient mice (PHD3$^{fl/fl}$MRP8Cre$^{+/-}$) and Cre negative litter mates (PHD3$^{fl/fl}$MRP8Cre$^{-/-}$). In both these PHD3 deficient lines, the mice were backcrossed for at least eight generations and maintained on a C57BL/6 background and the mice bred normally with no underlying health issues.

Mice were housed in individually ventilated cages under 12 h light/darkness cycles and controlled temperature (20–23°C) in accordance with UK Home Office guidance. All mice used for experiments were healthy with quarterly and annual testing carried out in accordance with FELASA 2014 Guidelines, using a mixture of environmental, random colony samples and sentinel testing by serology and PCR. Mice had free access to food (Special diets service rat and mouse number 1 maintenance food RMI (P) 801151) and water. For all experiments, both male and female mice were used at age 8–12 weeks. All animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 with local ethics approval.

Blood samples were collected from the inferior vena cava following overdose of intraperitoneal (IP) pentobarbital (250mg/kg) and immediately mixed with 0.5M sterile EDTA at a ratio of 1:9, EDTA:whole blood). For flow cytometry analysis of leucocyte differentials two red cell lysis steps were carried out using RBC lysis buffer (Biolegend) added at a ratio of 1ml per 100µL blood followed by topping up with PBS without magnesium and calcium (Gibco) and centrifugation at 300G for 5 minutes. The resulting cell pellet was resuspended in PBS and cells counted using a BioRad cell counter. They were resuspended at 1x10^6/ml and 200µL aliquots were used for staining. They underwent FC block with TruStain FcX (Biolegend) for 15 minutes on ice then stained with the antibody panel as follows for 30 minutes on ice: Ly6G-Pacific blue (Biolegend 127612), CD3/CD19-PE (Biolegend 100206/152407), SiglecF-PECF594 (BD 562757), CD115-APC (Biolegend 135510), CD45-AF700 (Biolegend 103128). Samples were then washed 3 times with FACS buffer. Appropriate unstained and FMO (fluorescence minus one) controls were run in parallel. Samples were run on a 5 laser LSR Fortessa flow cytometer (Becton Dickinson) and analysed on FlowJo version 10.

In vivo mouse models

LPS lung injury. Mice were treated with lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* (10) (1mg/mL, Sigma Aldrich) for 10 minutes using oxygen driven nebulisation to generate an acute lung injury. Following LPS treatment, animals were housed in standard conditions or were exposed to hypoxia (10% inspired O_2) in an InVivo Hypoxic Cabinet System (Coy Labs, USA). This sealed system allows animals to be housed in 10% oxygen at room temperature with excess CO_2 scavenged using Sofnolime soda lime chips (Molecular Products, UK) with colour indicator.

Mice were culled using an overdose of IP pentobarbital (250mg/kg) at 24 or 48 hours post-LPS. Bronchoalveolar lavage (BAL) samples were collected post-mortem through cannulation of the trachea and lavage with 5 aliquots of 0.8mL ice cold 0.9% NaCl.

Staphylococcus aureus skin abscess model. Mice were shaved on the right flank and left to recover for 24 hours in standard housing conditions prior to the experiment. On the day of injection, mice were administered 5×10^7 colony forming units (CFU) of SH1000 *Staphylococcus aureus* in 50µL PBS subcutaneously into the right flank. Mice were scored on sickness based on gross external appearance, including assessment of fur ruffling, activity, peri-optic exudate and dehydration.

Mice were culled on day 7 (or at day 2 or 4 for the CFU counts and myeloperoxidase (MPO) measurement) via an overdose of pentobarbital administered intraperitoneally, and the abscess (overlying scab and pustule) excised with a scalpel and weighed. Abscesses were then dissected into three equal pieces, and the pieces weighed and snap frozen and stored for bacterial counts and MPO assay.

In vivo model of fulminant Streptococcal pneumonia. Mice were instilled intra-tracheally (IT) with a high dose (10^7 CFU) of *Streptococcus pneumoniae* (type 2, D39) under anaesthesia as previously described[21]. Following recovery, mice were housed in standard conditions and were culled at 14 hours by overdose of intraperitoneal anaesthetic. Blood samples were

collected from the inferior vena cava and BAL was recovered by cannulation of the trachea and lavage with 5 aliquots of 0.8mL ice cold 0.9% NaCl as above.

Neutrophil isolation

Bone marrow: Both hind limbs were dissected out and stored in a clean bijou container on ice until processed. Each bone was flushed using 10ml of 1xHBSS with 0.2% BSA. The cell suspension was resuspended, passed through a 70μm filter into a fresh 50ml polystyrene falcon tube and then centrifuged at 450G for 10 minutes. 90% vol/vol percoll stock was made using 10X HBSS with $NaHCO_3$ and this stock was used to make 81%, 62% and 55% percoll with 1x HBSS. All three percoll preparations were layered in a 15ml falcon tube and the cells were resuspended in 3ml of 1xHBSS with 0.2% BSA which was layered on top of the 52% percoll layer. The gradient was centrifuged at 2000G for 30 minutes (acceleration 1, deceleration 0). Bone marrow monocyte precursors were removed from the top layer with a pastette. Neutrophils were removed from the middle layer and washed in 30ml of 1xHBSS with 0.2%BSA. Hypotonic saline red blood cell lysis was carried out. Following the percoll purification above, neutrophil purity was found to be approximately 90%. In order to generate highly pure bone marrow neutrophils samples, cells from the neutrophil layer were resuspended in 1xHBSS with 0.2% BSA and purified by fluorescence-activated cell sorting (FACS) based on the forward/side scatter profile and auto-fluorescence as previously described[22]

Bronchoalveolar lavage: Where highly pure BAL neutrophils were required, we used a previously optimized discontinuous percoll gradient coupled with a red cell lysis step in order to gain purities of >98%. BAL cells were first pelleted by centrifugation at 350G for 10 minutes. BAL samples from 1–3 mice (depending on the final cell number required) were pooled onto a single percoll gradient. 90% vol/vol percoll was made using 10X PBS and further percoll solutions were made using this stock 90% percoll mixed with 1X PBS. 3ml of 78% percoll was pipetted into the bottom of a 15ml falcon tube, followed by 3ml of 69% percoll. The cell pellet(s) were resuspended in 3ml of 52% percoll which was then layered onto the 69% layer. The gradient(s) were centrifuged at 1200G for 30mins with an acceleration of 1 and deceleration of 0. Following centrifugation, the macrophages accumulated on the top of the 52% layer and were removed using a sterile pastette. The neutrophils (within the 69% layer) were then removed using a fresh sterile pastette and placed into a 50ml polystyrene falcon tube. This was topped up to 50ml with sterile PBS to wash off the percoll and the purified neutrophils pelleted at 350G for 8 mins. Residual red cells were lysed by hypotonic saline lysis. A cytospin was made prior to the final centrifugation step to assess purity by morphology. During optimisation of this protocol, purity was also confirmed by flow cytometry and the neutrophils were found to be >98% pure.

Assessing bacterial load/CFU counts

For the *Staphylococcus aureus* skin abscess model, abscess tissue was placed in a sterile dish and cut into small pieces with a sterile scalpel blade. Contents were placed into a sterile bijou container and forceps and scalpel blade rinsed with 1mL PBS into the petri dish. The plate was then washed with a further 1mL PBS and the liquid transferred to the bijou container. The mixture was vortexed for 1 minute and left on ice for 1 hour to allow the scab to dissolve, before vortexing again for 1 minute. 1:10 serial dilutions were made of the homogenate and three 10μL aliquots of each dilution plated onto Columbia Blood Agar plates (VWR). Plates were incubated at 37°C overnight before counting resultant colonies to determine CFUs per lesion and per gram of lesion.

For the *Streptococcus pneumoniae* model, ten-fold serial dilutions were performed on whole blood and BAL aliquots. 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.

Assessment of MPO activity in *Staphylococcus aureus* skin abscess model

The abscess was transferred to a sterile screw-cap tube with six homogenisation beads and 0.5mL cold hexadecyltrimethylammonium bromide (HTAB) buffer and the abscess homogenised in a bullet blender for 5 minutes. The homogenate was sonicated for 10 minutes in 30 second bursts then freeze-thawed on dry-ice once to ensure cell lysis. The solution was centrifuged at 4°C and the supernatant transferred to a new sterile tube. 100μL of supernatant was added to 1.9mL of O-dianisidine solution (0.167mg/mL O-dianisidine hydrochloride (Sigma-Aldrich Company Ltd., UK) and the change in absorbance at 450nm from 30 seconds to 90 seconds after addition of supernatant was read on a spectrophotometer (Jenway 6310, Barloworld Scientific, UK) to give relative MPO activity.

Additional analysis of BAL parameters

BAL cell counts were performed using a haemocytometer and differential counts were assessed by morphology on cytospin preparations. BAL albumin (Abcam Ab108792), IgM (Abcam Ab133047) and elastase concentration (Ab252356) were measured using commercially available ELISA kits. Elastase activity in the PHD1 BAL experiments was measured using the EnzCheck Elastase assay (Invitrogen E12056). MPO activity was analysed using the EnzCheck MPO assay (E33856). BAL inflammatory cytokines were measured using a BD Cytometric Bead Array multiplexed bead-based immunoassay (BD Biosciences): BAL supernatant was collected from BAL following *Streptococcus pneumoniae* infection and analysed for cytokine concentrations by flow cytometry as per manufacturer instructions.

Bacterial killing assay

Murine neutrophils were isolated from BAL 24 hours following LPS induced lung injury as described above. 1×10^6 neutrophils were resuspended in 200μL glucose free media and plated in a 48-multiwell TC plate. Live SH1000 *Staphylococcus aureus* was opsonised by incubation in 500μL dialysed FBS at 37°C with shaking for 30 minutes. Opsonised SH1000 (multiplicity of infection (MOI) 1:10) was mixed with the neutrophils for a 60-minute co-incubation period. After

killing of extracellular bacteria through the addition of gentamycin (2μg/mL) and vancomycin (7μg/mL) for 30 minutes, neutrophils were harvested (T0) or cultured for a further one hour (T1) prior to cell lysis and measurement of internalised bacteria. CFUs were counted and % bacterial killing after 1 hour was calculated.

Phagocytosis (PHD3 deficient neutrophils)

CFSE labelling of Streptococcus pneumoniae. Aliquots of D39 *Streptococcus pneumoniae* bacteria were centrifuged at 4000G for 5 minutes and then resuspended in 2mL of 10μM Carboxyfluorescein succinimidyl ester (CFSE). The cells were incubated at room temperature for 1 hour in the dark while gently rocking to mix.

After 1 hour, the culture was centrifuged, and the pellet washed three times in PBS and culture adjusted to OD600. Bacteria were heated at 65°C for 10 minutes to heat-kill and stored at 4°C for up to 1 month before use. Heat-killed CFSE labelled bacteria were vortexed to separate clumps. 50μL FBS were added to bacterial suspensions, vortexed and incubated at 37°C for 1 hour to opsonize, then washed twice with 1mL ice-cold PBS. Concentration of bacterial particles was determined through counting in a haemocytometer and concentration of bacteria adjusted to 1×10^9.

In vitro phagocytosis assay. Neutrophils were isolated from murine BAL and resuspended in RPMI +10% FBS at a concentration of 2×10^6/ml. For phagocytosis of D39 *Streptococcus pneumoniae*, 2μL of 1×10^9 CFU/mL bacterial suspension (described above) was then added to cells for an MOI of 10:1. For analysis of staphylococcal phagocytosis, FITC labelled *Staphylococcus aureus* (Wood strain without protein A) Bioparticles were purchased from Invitrogen (S2851) and added at an MOI of 1:1 cells were incubated for a further 30 minutes at 37°C before being aspirated and transferred into 1.5mL Eppendorf tubes, washed in ice-cold PBS once, and then resuspended in ice-cold PBS. Cells were analysed via flow cytometry on the Attune NxT cytometer (Thermo Fisher Scientific).

Seahorse extracellular flux assays

BAL neutrophils were recovered from $PHD3^{fl/fl}MRP8Cre^{-/-}$ and $PHD3^{fl/fl}MRP8Cre^{+/-}$ mice 24 hours after LPS induced ALI (as described above). BAL neutrophils were plated in a Seahorse experiment plate which was prepared and equilibrated the day before: A Seahorse XFe24 cartridge was hydrated using 1mL Sodium bicarbonate (0.1M) and stored overnight at 37°C in an incubator without CO_2. On the day of the experiment, all the wells of an XFe24 assay plate were coated with Cell-Tak (Corning 354240). Neutrophils were left incubating at 37°C for one hour in Seahorse media without glucose and with 2mM L-glutamine (DMEM without phenol red). Heat-killed *Staphylococcus aureus* (SH1000) was opsonised using dialysed FBS and used to infect neutrophils (MOI 1:25) during the assay. The experiment plate was taken out from the incubator 30 minutes prior to the assay and joined with the cartridge plate containing all the compounds for the standard Glycolysis Stress Test which includes the addition of Oligomycin and 2-deoxy-glucose and run with the Seahorse XFe24.

Neutrophil RNA extraction and TaqMan analysis of gene expression

A minimum of 1×10^6 neutrophils were used to generate RNA samples. The mirVana miRNA isolation kit (Invitrogen) was used to isolate mRNA from highly pure neutrophil samples. The samples then underwent a DNase step using the Invitrogen TURBO DNA free kit. The quantity and purity of the RNA was assessed using a Nanodrop 100 spectrophotometer. cDNA was made using AMV reverse transcriptase and random primers (Promega) with the following settings: 23°C for 5 minutes, 42°C for 2 hours, 99°C for 2 minutes. Gene expression was analysed using predesigned qPCR primer/probe assays and Prime Time Gene Expression Mastermix (IDT, Leuven). For all assays, samples were run in triplicate and the gene of interest expressed relative to expression of a housekeeping gene (β-actin). Assays were run on a 7900HT Fast real-time PCR system (Applied Biosystems) with the following thermal cycling: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes. Data was analysed using SDS 2.0 software (Thermo Scientific).

Details of primer/probes:

Egln1 (PHD2): Thermo Fisher Scientific Mm00459770_m1 (exon 3–4)

Egln2 (PHD1): Thermo Fisher Scientific Mm00519067_m1 (exon 2–3)

Egln3 (PHD3): Thermo Fisher Scientific Mm00472200_m1 (exon 1–2)

Actb: Integrated DNA technologies Mm.PT.39a.22214843.g (exon 5–6)

HPLC-MS analysis of metabolites

Highly pure BAL neutrophils were analysed for metabolite abundance. Following purification on a discontinuous percoll gradient, cells were washed in ice cold 0.9% NaCl prior to being pelleted (300G for 5 minutes). The pellet was lysed by resuspension in 80% Methanol which had been stored overnight at -80°C and kept on dry ice during resuspension. Prior to analysis, the lysed sample was centrifuged at 20,000G for 10 minutes at 4°C and the supernatant removed into a fresh Eppendorf tube. The pellet was retained, and protein content measured by Pierce BCA assay (Thermo Scientific). Supernatant samples were analysed at the VIB Centre for Cancer Biology in Leuven using a Dionex UltiMate 3000 LC System (Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) operated in negative mode. Data collection was performed using Xcalibur software (Thermo Scientific). All data values were subsequently corrected for protein content based on the BCA assay of the retained cell pellets.

Ex-vivo functional assays of PHD1 deficient neutrophils

Chemotaxis. Whole BAL was counted and resuspended at 2×10^6cells/mL. The assay measured movement of cells across a filter towards a chemoattractant (KC, also called CXCL1, for murine neutrophils) using the ChemoTx plate system with 5μm pores (Neuro Probe). The negative control is media with no chemoattractant added. The positive control has cells pipetted into the well at the start (instead of onto the membrane). The percentage of positive control is calculated as (cell count in well for experimental condition/cell count in positive control well)x100. The chemoattractant or control was pipetted into the bottom well of the plate, the filter was then placed on top and the cell suspension pipetted onto the filter. The plate was incubated at 37°C for 1 hour with a lid on. Residual cell suspension was removed from the top of the filter using a cotton bud and the plate centrifuged (without the lid) for 10 minutes at 300G. The filter was removed and the cellular concentration (*i.e.* the number of cells which have migrated across the filter) was measured in each well using a haemocytometer count of the resuspended well contents. This number was adjusted for the volume in each well to give an absolute cell count.

Phagocytosis. Phagocytosis was assessed by uptake of fluorescently labelled, heat killed *E. Coli* (Invitrogen). BAL neutrophils were resuspended at 1×10^6 cells/mL and incubated at 37°C for 1 hour in RPMI with 10% FCS with the bacteria (MOI 1:1). All samples were washed 3 times with ice cold PBS prior to being resuspended in FACS buffer and analysed by flow cytometry.

Respiratory burst assay (PHD1 deficient neutrophils). *Ex vivo* BAL neutrophils from whole animal PHD1 deficient mice were used for this assay. Total reactive oxygen species (ROS) was measured using a chloromethyl derivative of 2',7'-Dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Invitrogen). CM-H2DCFDA was resuspended at 50μg/5μL in DMSO. This was then diluted 1:100 with PBS to make up the stock solution. 3μl of this stock solution was added to 100μl of BAL neutrophils at 1×10^6/mL and cells were incubated at 37°C for 45 minutes. To measure respiratory burst capacity, the formylated peptide f-met-leu-phe (fMLF, Sigma Aldrich), was added after 45 minutes for a further 45 minutes at a final concentration of 10μM. The samples were analysed by flow cytometry.

Additional functional assays in GFP positive PHD3 deficient neutrophils

A number of assays were adapted due to the green fluorescence of the MRP8Cre positive cells in the neutrophil specific knockout mouse lines.

CellROX assay for total ROS. Total ROS in the PHD3 cells was measured using CellROX red rather than the previously described DCF assay. Briefly, BAL neutrophils were isolated 24 hours post-nebulised LPS as previously described. They were resuspended at 1×10^6/mL in glucose free RPMI (consistent with the hypoglycaemic lung microenvironment). CellROX Deep Red Reagent (Invitrogen) was added at a final concentration of 5mM for 30 minutes at 37°C prior to washing and running samples on a 6 laser LSR Fortessa flow cytometer (Becton Dickinson).

MitoSOX assay for mROS production. Cells were isolated and plated as above. MitoSOX red reagent (Invitrogen) was added to samples at a final concentration of 2.5μM for 30 minutes at 37°C prior to washing and running samples on a 6 laser LSR Fortessa flow cytometer (Becton Dickinson).

SyTox Red assay for NET formation. Cells were isolated as above. These activated BAL cells have a high degree of baseline NETosis and NETosis was measured without the addition of a further stimulus. Cells (at a density of 1×10^6/mL) were fixed in 4% PFA for 15 mins at room temperature and then washed in PBS. SyTox Red reagent (Invitrogen) was then added at a final concentration of 0.1mM for 30 minutes at room temperature. Samples were washed again prior to running on the flow cytometer.

All flow cytometry data was analysed using FlowJo V10 and statistical analysis carried out on GraphPad Prism V9.4.

Results

Conservation of core neutrophil phenotype and functions in PHD1 deficiency

Neutrophil specific PHD1 knockout mice ($PHD1^{fl/fl}MRP8Cre^{+/-}$) did not display any overt phenotype at baseline and demonstrated normal circulating leucocyte differentials counts when compared to their Cre negative litter mates (Figure 1A). A GFP tag is co-expressed with the MRP8Cre and we found that 93.7% of blood neutrophils from $PHD1^{fl/fl}MRP8Cre^{+/-}$ were GFP positive compared to 0.44% in $PHD1^{fl/fl}MRP8Cre^{-/-}$ controls (Figure 1B). $PHD1^{fl/fl}MRP8Cre^{+/-}$ were compared with their Cre negative litter mates ($PHD1^{fl/fl}MRP8Cre^{-/-}$) in an LPS induced acute lung injury (ALI) model. Given the role of PHD1 in other models of hypoxia and ischaemia, and the detrimental effect of hypoxia in the model, we used animals housed in both normoxic (21% inspired oxygen (FiO_2)) and hypoxic (10% FiO_2) conditions to replicate the marked systemic hypoxia associated with ALI in patients.

PHD1 deficient bone marrow neutrophils did not show any compensatory upregulation in PHD2 or PHD3 transcripts (Figure 1C&D). Nor was PHD1 expression increased in hypoxic conditions (Figure 1E). Following nebulised LPS, animals housed in normoxia displayed mild hypothermia at 4 and 8 hours which fully recovered by 24 hours with no difference noted between genotypes (Figure 1F). As we have shown previously, concurrent hypoxia was associated with significant hypothermia in this model[16] but neutrophil specific PHD1 deficiency did not confer any protection against this (Figure 1F). In normoxia, $PHD1^{fl/fl}MRP8Cre^{+/-}$ animals displayed equivalent bronchoalveolar lavage (BAL) total cell, neutrophil and macrophage numbers (Figure 1G-H), 24 hours following LPS. In hypoxic animals, PHD1 deficiency was associated with a significant increase in total cell counts at

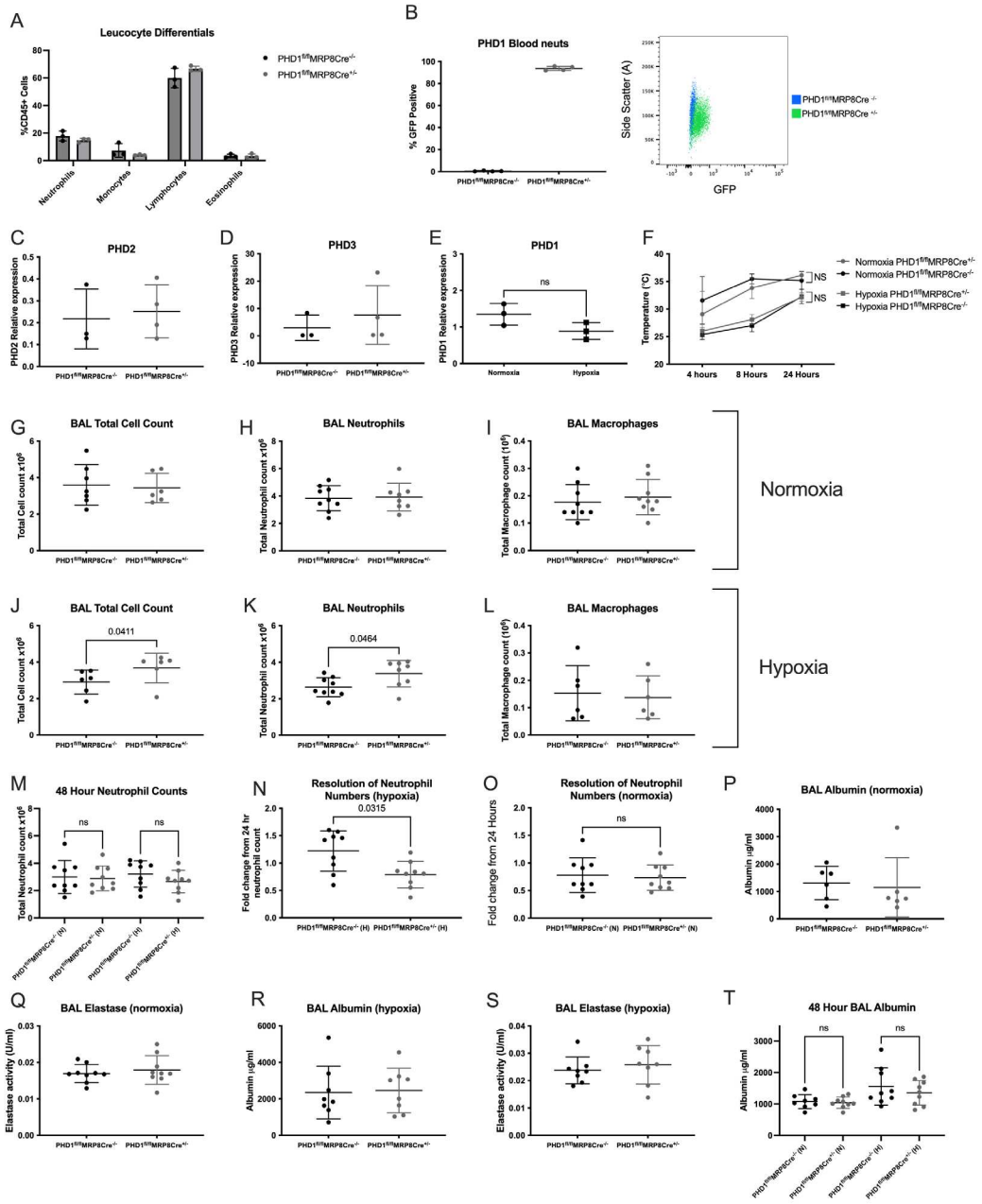

Figure 1. Loss of PHD1 results in oxygen dependent alterations in neutrophil recruitment. Blood leucocyte differentials were determined by flow cytometry (**A**) and Ly6G positive neutrophils were assessed for GFP positivity (**B**). Bone marrow neutrophils were isolated from naïve PHD1 $^{fl/fl}$MRP8Cre $^{-/-}$ and PHD1 $^{fl/fl}$MRP8Cre $^{+/-}$ mice by percoll gradient followed by fluorescence activated cell sorting to ensure sufficient purity. Taqman analysis of PHD2 (egln1) (**C**) and PHD3 (egln3) (**D**) gene expression was carried out (N=3 PHD1 $^{fl/fl}$MRP8Cre $^{-/-}$, 4 PHD1 $^{fl/fl}$MRP8Cre $^{+/-}$). (**E**) Highly pure BAL neutrophils were isolated from normoxic and hypoxic C57BL/6 wildtype mice 24 hours after LPS induced ALI and taqman analysis of PHD1 (egln2) gene expression was carried out (N=3). (**C**)–(**E**) expressed as gene of interest relative to β-actin, analysed by unpaired t-test. (**F**) Core body temperatures of PHD1 $^{fl/fl}$MRP8Cre $^{-/-}$ and PHD1 $^{fl/fl}$MRP8Cre $^{+/-}$ mice housed in normoxia or hypoxia for 24 hours following LPS induced ALI. N=6, 2-way ANOVA with multiple comparisons (corrected for multiple comparisons by Tukey test). Total BAL cell counts 24 hours after LPS induced ALI of mice housed in normoxia (**G**) or hypoxia (**J**), N=6-7. BAL neutrophils counts in (**H**) normoxia and (**K**) hypoxia, N=8-9. BAL macrophage counts in **(I)** normoxia (N=9) and (**L**) hypoxia (N=6). (**G**)–(**L**) analysed by Mann-Whitney test (unpaired). (**M**) BAL neutrophil counts were measured 48 hours following LPS induced ALI in PHD1 $^{fl/fl}$MRP8Cre $^{-/-}$ and PHD1 $^{fl/fl}$MRP8Cre $^{+/-}$ mice housed in normoxia or hypoxia, N=9, ordinary one-way ANOVA with multiple comparisons (corrected for multiple comparisons by Tukey test). (**N&O**) Resolution of neutrophil infiltration was then calculated from fold change of 48 hour neutrophil counts from the corresponding mean 24 hour neutrophil count, N=9. BAL supernatant collected 24 hours following LPS induced lung injury was analysed by ELISA for albumin (**P**)&(**R**) and elastase (**Q**)&(**S**) concentrations (N=6-9). (**N**)–(**S**) analysed by Mann-Whitney test (unpaired). Albumin concentration was measured by ELISA in BAL supernatant collected 48 hours post LPS induced lung injury in hypoxia **(T)**, N=8-9, analysed by ordinary one-way ANOVA with multiple comparisons (corrected for multiple comparisons by Tukey test). All data expressed as mean±SD.

24 hours (Figure 1J) which was due to significantly higher neutrophil recruitment (Figure 1K&L). This difference had resolved by 48 hours where there is no difference between genotypes, irrespective of oxygenation (Figure 1M). Thus, in hypoxia, PHD1 deficiency was associated with increased but more rapidly resolving neutrophil recruitment (Figure 1N) with the higher resolution driven by slightly higher 24 hour neutrophils counts in the PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ coupled with slightly lower numbers at 48 hours. This is consistent with our previous work showing that hypoxia results in slightly fewer neutrophils being recruited[16], a phenotype which is abrogated in the PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice. Resolution in neutrophil numbers was not different between genotypes in normoxic mice (Figure 1O). Despite this increase in neutrophil recruitment at 24 hours in hypoxia, there was no difference between genotypes in lung damage as measured by BAL albumin leak in either normoxia (Figure 1P) or hypoxia (Figure 1R). Neutrophil degranulation was also equivalent with no difference between PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ and PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ in BAL neutrophil elastase in either normoxic or hypoxic conditions (Figure 1Q&S), despite the higher neutrophil numbers in the PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice. Thus, the PHD1 deficient neutrophils may be producing slightly less elastase but there is no net effect of neutrophil specific PHD1 deficiency on either elastase abundance or lung damage (as measured by albumin leak). The more rapid resolution of neutrophil numbers observed in hypoxic PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice did not alter lung damage at 48 hours with no difference in albumin leak (Figure 1T).

We then investigated whether PHD1 deficiency altered neutrophil metabolic profiles. Inflammatory BAL neutrophils were isolated from mice housed in either normoxia or hypoxia 24 hours after LPS induced ALI. In keeping with data from other cell types, PHD1 deficiency did lead to changes in metabolite abundance. Glycolytic metabolites were equivalent between genotypes in both normoxia and hypoxia (Figure 2A&D). In normoxia, PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ BAL neutrophils had reduced oxidative pentose phosphate pathway (OxPPP) metabolites but preserved TCA cycle metabolites (Figure 2B&C) whereas in hypoxic neutrophils, OxPPP metabolites were unchanged between genotypes but PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ neutrophils showed significantly higher TCA cycle metabolites (Figure 2E&F). In post-hoc multiple comparison tests of those pathways showing significant differences, no individual metabolite reached statistical significance, thus there is a small effect across the whole pathway, rather than a significant effect on a select group of metabolites. Despite these changes, energy status (measured by the ATP:ADP ratio from the HPLC-MS) was equivalent between genotypes in both oxygen tensions (Figure 2G&H), thus increased TCA cycle activity did not lead to an improved energetic profile in hypoxic PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ neutrophils. This is consistent with our previous finding that oxidative phosphorylation contributes minimally to neutrophil ATP stores[23]. Additionally, despite reduced OxPPP flux in normoxic PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ neutrophils when compared with PHD1$^{fl/fl}$MRP8Cre$^{-/-}$, a corresponding decrease in ROS production was not observed (Figure 2I). Further analysis of *ex vivo* PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ neutrophils did not identify any significant functional differences when compared with PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ with normal chemotaxis and phagocytosis (Figure 2J&K).

Thus, despite the important role which PHD1 plays in responding to hypoxic/ischaemic injuries in other tissue types, it does not appear to have a role in the regulation of harmful hypoxic neutrophil responses. Alterations in metabolic profiles do not translate to functional differences. Although the added physiological stress of hypoxia does appear to alter neutrophil recruitment dynamics in PHD1 deficient mice with increased neutrophil recruitment at 24 hours, this is not sufficient to confer any identifiable changes to *in vivo* inflammatory outcomes.

PHD3 deficiency results in reduced neutrophil recruitment in sterile inflammation

In contrast to PHD1 and PHD2 expression and consistent with previous findings[6], PHD3 was significantly upregulated in the BAL neutrophils of hypoxic WT mice following LPS induced lung injury (Figure 3A&B). We have previously shown that in sterile lung inflammation whole animal PHD3 deletion may be associated with enhanced inflammation resolution in hypoxia[11]. We have now extended these findings in a LysMCre driven, myeloid specific PHD3 deficient mouse to understand whether PHD3 deficiency alone, without the additional physiological stress of hypoxia, altered inflammation outcomes. In the context of LPS induced ALI in normoxic conditions, PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice showed signs of a suppressed neutrophil response, with fewer total cells recruited to the airways at 24 hours post-LPS (Figure 3C), due to reduced neutrophil numbers (mean neutrophil number in PHD3$^{fl/fl}$LysMCre$^{-/-}$ 7.26 x10^6 Vs 4.6 x10^6 in PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice, Figure 3D&E). This is in contrast to neutrophil PHD2 deficiency in which we have previously reported an exaggerated and persistent neutrophil response following LPS challenge[10] and with neutrophil PHD1 loss, as detailed above. Lung damage, evidenced by albumin leak into the BAL supernatant, was equivalent between genotypes (Figure 3F), as was degranulation with equivalent BAL supernatant neutrophil elastase (Figure 3G) and MPO (Figure 3H).

Myeloid specific PHD3 deficiency results in improved infection outcomes

We then investigated how myeloid specific PHD3 deficiency might impact acute responses to infection. We used an established model of *Staphylococcus aureus* skin abscess[15] in myeloid specific PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice and their Cre negative littermates (PHD3$^{fl/fl}$LysMCre$^{-/-}$) and measured sickness scores, weight, body temperature and abscess size on days 1–7 to monitor the health of the animals and progression of infection. Neither genotype showed evidence of systemic illness at any point during the experiment (sickness scores consistently zero in both groups, data not shown), even with the development of large abscesses extending to an area greater than 1cm^2 (typical abscess appearance shown in Figure 4A). There was no difference in baseline body weight between genotypes.

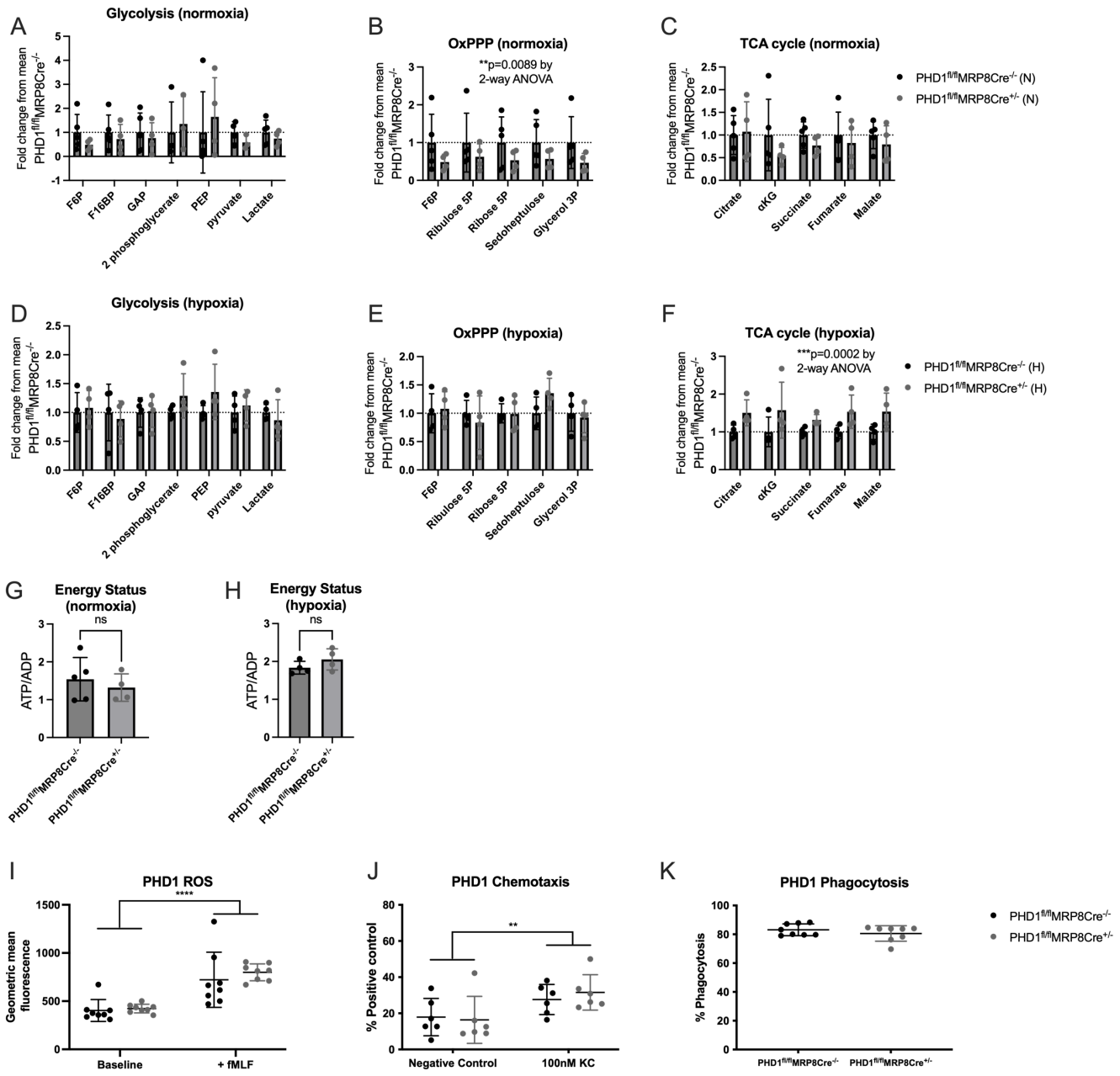

Figure 2. PHD1 deficiency alters metabolic profiles in an oxygen dependent fashion. HPLC-MS analysis of neutrophil metabolic profiles were carried out on *ex vivo* BAL neutrophils harvested 24 hours following LPS induced lung injury in normoxia (**A**)–(**C**) and hypoxia (**D**)–(**F**). (**A**)–(**F**) N=4–5, data expressed as fold change from mean PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ and analysed by 2-way ANOVA. Energy status (**G**) and (**H**) expressed as the ratio of ATP:ADP (also measured by HPLC-MS), analysed by Mann-Whitney test (unpaired). *Ex vivo* BAL neutrophils (24 hours post LPS induced ALI) from PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ and PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice were analysed for ROS production by flow cytometry following addition of DCF (**I**), chemotaxis (**J**) and phagocytosis (**K**). (**I**)–(**K**) N=6–8, analysed by 2-way ANOVA. All data expressed as mean±SD. F6P, fructose-6-phosphate; F16BP, fructose-1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; αKG, α-ketoglutarate.

Both genotypes lost a small but significant amount of weight in the first 1–2 days (Figure 4B) but returned to baseline weight by day 2 (PHD3$^{fl/fl}$LysMCre$^{+/-}$) and day 3 (PHD3$^{fl/fl}$LysMCre$^{-/-}$). Core temperatures were equivalent between genotypes over the course of the 7 days of infection (Figure 4C). However, in PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice, abscess development was significantly reduced compared to PHD3$^{fl/fl}$LysMCre$^{-/-}$ controls (Figure 4D). Bacterial burden was assessed by carrying out CFU counts on excised and homogenised abscesses. We found that abscess size was proportionate to bacterial burden in both genotypes (Figure 4E). PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice had lower total CFU counts at day 2

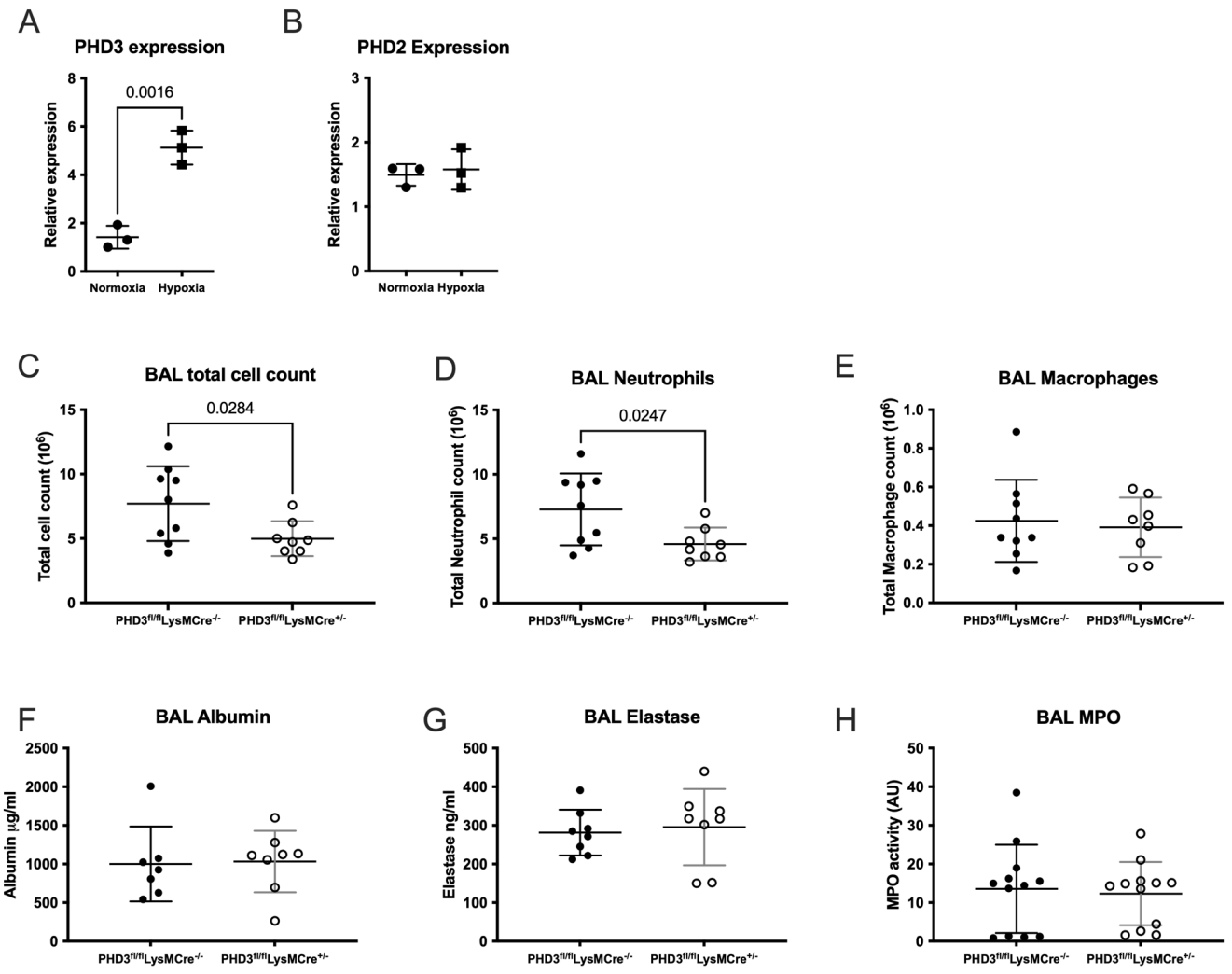

Figure 3. Myeloid specific PHD3 deficiency results in reduced neutrophil recruitment. Highly pure BAL neutrophils were isolated from mice housed in normoxia or hypoxia 24 hours following LPS induced lung injury and taqman analysis of (**A**) PHD3 (*egln3*) and (**B**) PHD2 (*egln1*) gene expression was carried out. Data expressed relative to β-actin. N=3, unpaired t-test. BAL cells were isolated from PHD3 $^{fl/fl}$LysMCre $^{-/-}$ and PHD3 $^{fl/fl}$LysMCre $^{+/-}$ mice housed in normoxia 24 hours following nebulised LPS and total cells counts (**C**), neutrophil counts (**D**) and macrophage counts (**E**) were analysed. N=8-9. BAL supernatant was subsequently analysed by ELISA for albumin (**F**) and elastase (**G**), N=7-8. MPO activity was analysed by an EnzCheck assay (**H**), N=12. (**C**)–(**H**) analysed by Mann-Whitney test (unpaired). All data expressed as mean±SD.

(Figure 4F) but this difference was not statistically significance by days 4 and 7 (Figure 4G&H). Measurement of MPO activity within the abscess did not show a significant difference between genotypes (Figure 4I), suggesting that the improved bacterial control is not due to enhanced neutrophil degranulation.

We next expanded our findings to include a *Streptococcus pneumoniae* model. Again, PHD3$^{fl/fl}$LysMCre$^{+/-}$mice demonstrated reduced local bacterial burden with significantly lower CFU counts in the BAL in comparison to PHD3$^{fl/fl}$LysMCre$^{-/-}$ controls, 14 hours following intra-tracheal (IT) inoculation (Figure 5A) although there was not a statistically significant reduction in blood CFU counts (Figure 5B). This enhanced local bacterial control was not associated with increased total cell (Figure 5C) or neutrophil (Figure 5D) recruitment to the airways. Nor was it associated with increased lung damage (as measured by airway IgM leak (Figure 5E) or increased concentrations of inflammatory mediators in the airways (Figure 5F–M), suggesting that enhanced bacterial control is not achieved at the expense of increased tissue damage.

PHD3 loss in the neutrophil compartment, therefore, confers a potentially advantageous phenotype with reduced local bacterial burden without excessive tissue damage or persistent sterile inflammation.

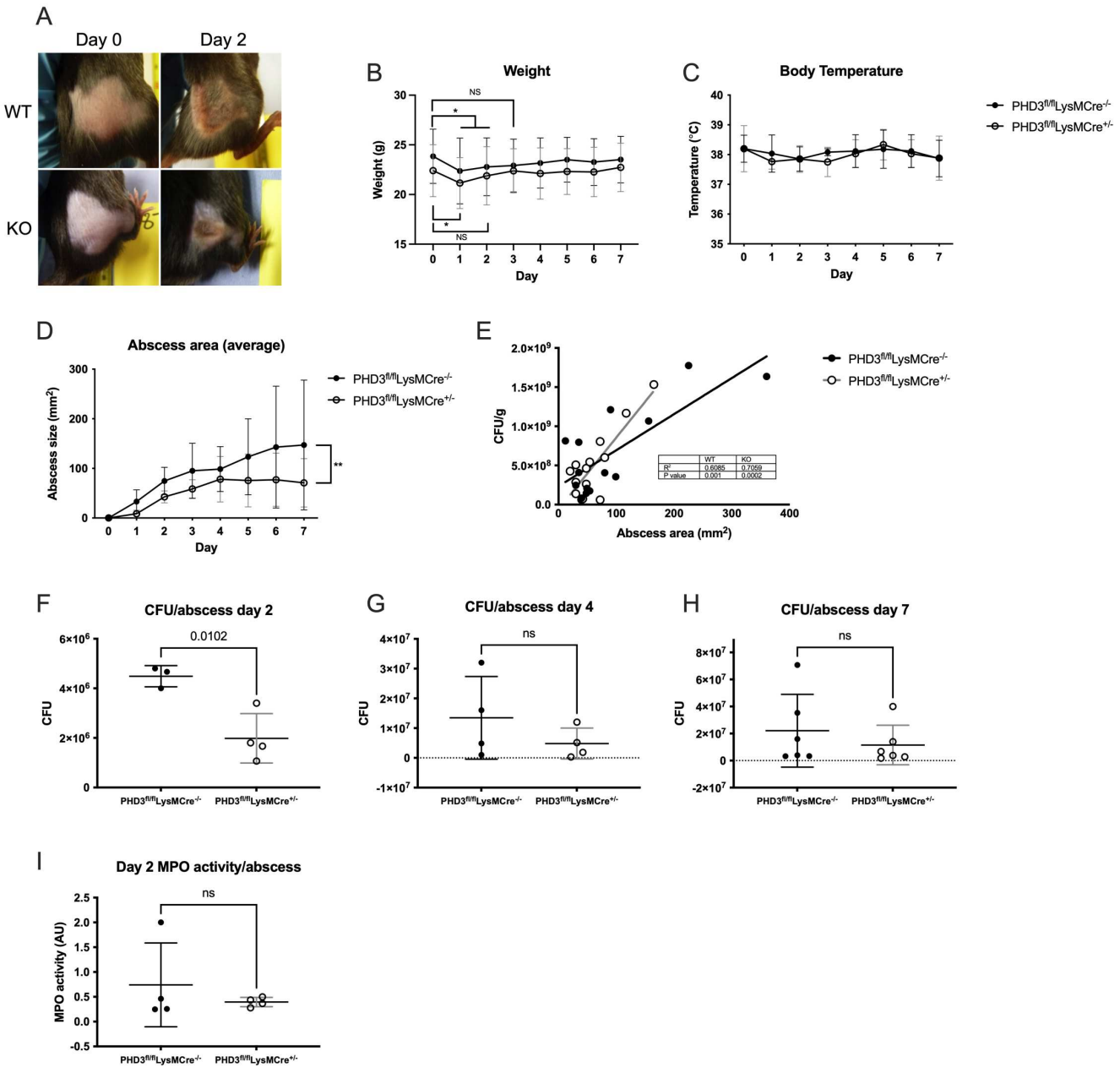

Figure 4. Improved staphylococcal infection outcomes in PHD3 deficiency. PHD3 $^{fl/fl}$LysMCre $^{-/-}$ and PHD3 $^{fl/fl}$LysMCre $^{+/-}$ Mice were inoculated with *Staphylococcus aureus* subcutaneously into the right flank and abscesses developed over the course of 7 days, with noticeable abscesses by day 2 (typical appearances shown in (**A**)). Weight (**B**), rectal temperature (**C**) and abscess size (**D**) were measured daily. (**B**)–(**D**) N=6, analysed by 2-way ANOVA with multiple comparisons (corrected for multiple comparisons by Tukey test). CFU/g and abscess area are significantly correlated (measured by Pearson's rank coefficient (**E**), N = 14. Mice were culled at day 2 (**F**), 4 (**G**) and 7 (**H**), abscesses homogenised and viable bacteria counted. N=3 for PHD3 $^{fl/fl}$LysMCre $^{-/-}$ day 2 due to exclusion of an outlier (see Figshare data upload for details), N=4 for days 2 and 4 and N=6 on day 7. MPO activity was measured in homogenised abscess tissue (**I**), N=4. (**F**)–(**I**) analysed by unpaired t-test. (**B**)–(**D**) and (**F**)–(**I**) expressed as mean±SD.

Improved bacterial killing is associated with increased oxygen consumption and mROS production in PHD3 deficient neutrophils

In addition to the myeloid specific PHD3 knock out mice described above, we have developed an MRP8Cre driven neutrophil specific knock out mouse line. We found that circulating neutrophil and other leucocyte numbers were normal in naïve mice in this line (Figure 6A) and that neutrophils effectively expressed the MRP8 driven Cre, as measured by the GFP positivity (as above for the PHD1 MRP8 Cre mice)

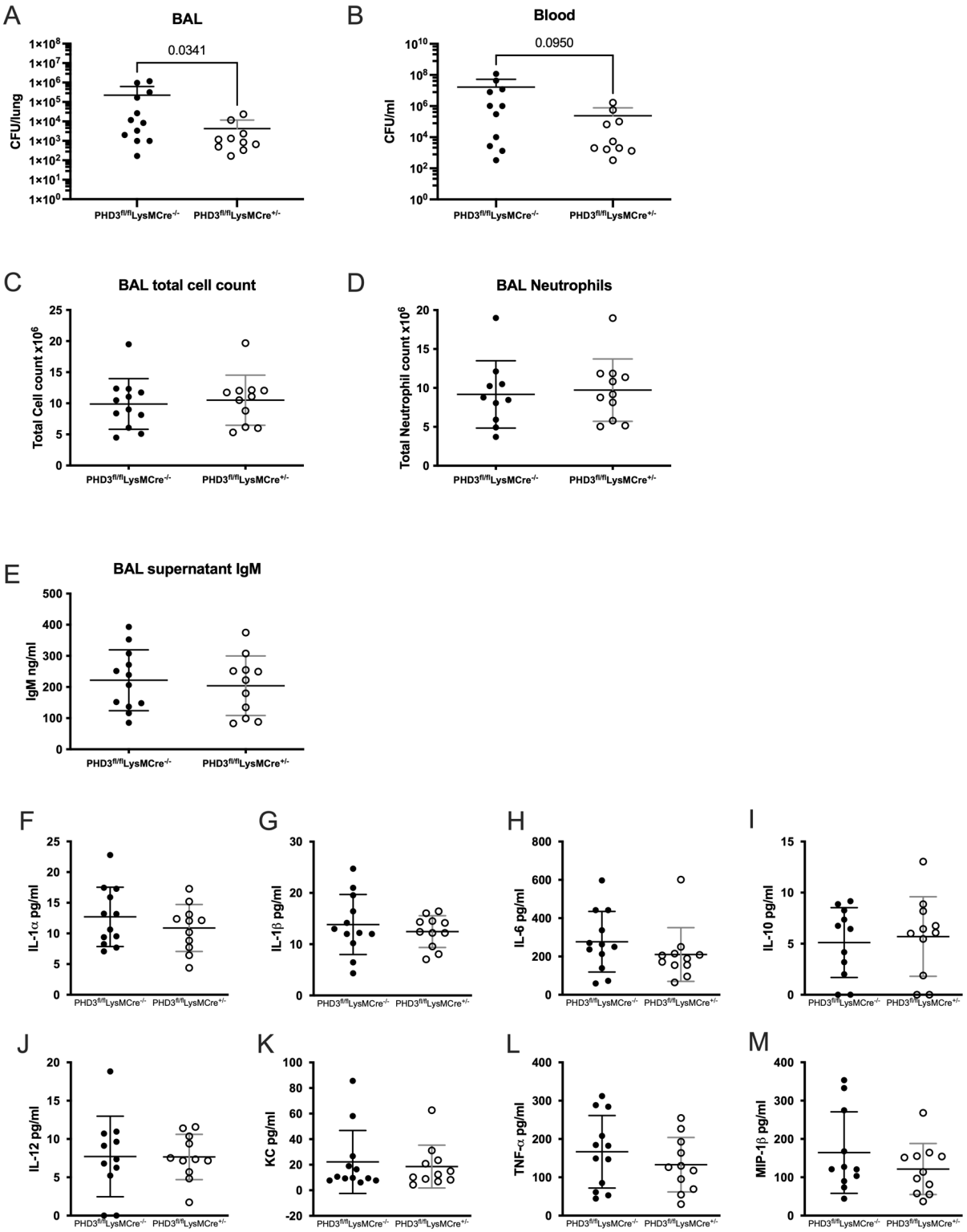

Figure 5. PHD3 deficiency results in reduced bacterial load in pneumococcal infection. PHD3 $^{fl/fl}$LysMCre $^{+/-}$ and Cre negative littermates (PHD3 $^{fl/fl}$LysMCre $^{-/-}$) were inoculated with *Streptococcus pneumoniae* intratracheally and culled after 14 hours. Bacteria were grown from BAL (**A**) and blood (**B**) samples, and BAL cell counts (**C**) and neutrophil differentials (**D**) calculated. BAL supernatant was analysed by ELISA for IgM concentration (**E**). N=10-12, all analysed by Mann-Whitney test (unpaired). (**F**)–(**M**) BAL supernatant from PHD3 $^{fl/fl}$LysMCre $^{+/-}$ and PHD3 $^{fl/fl}$LysMCre $^{-/-}$ mice subjected to IT *Streptococcal pneumoniae* infection was isolated after 14 hours and analysed by cytokine bead array for abundance of chemokines and cytokines. N=13-14, analysed by Mann-Whitney test (unpaired). (**A**) and (**B**) downward error bars not shown due to logarithmic scale. All data expressed as mean±SD.

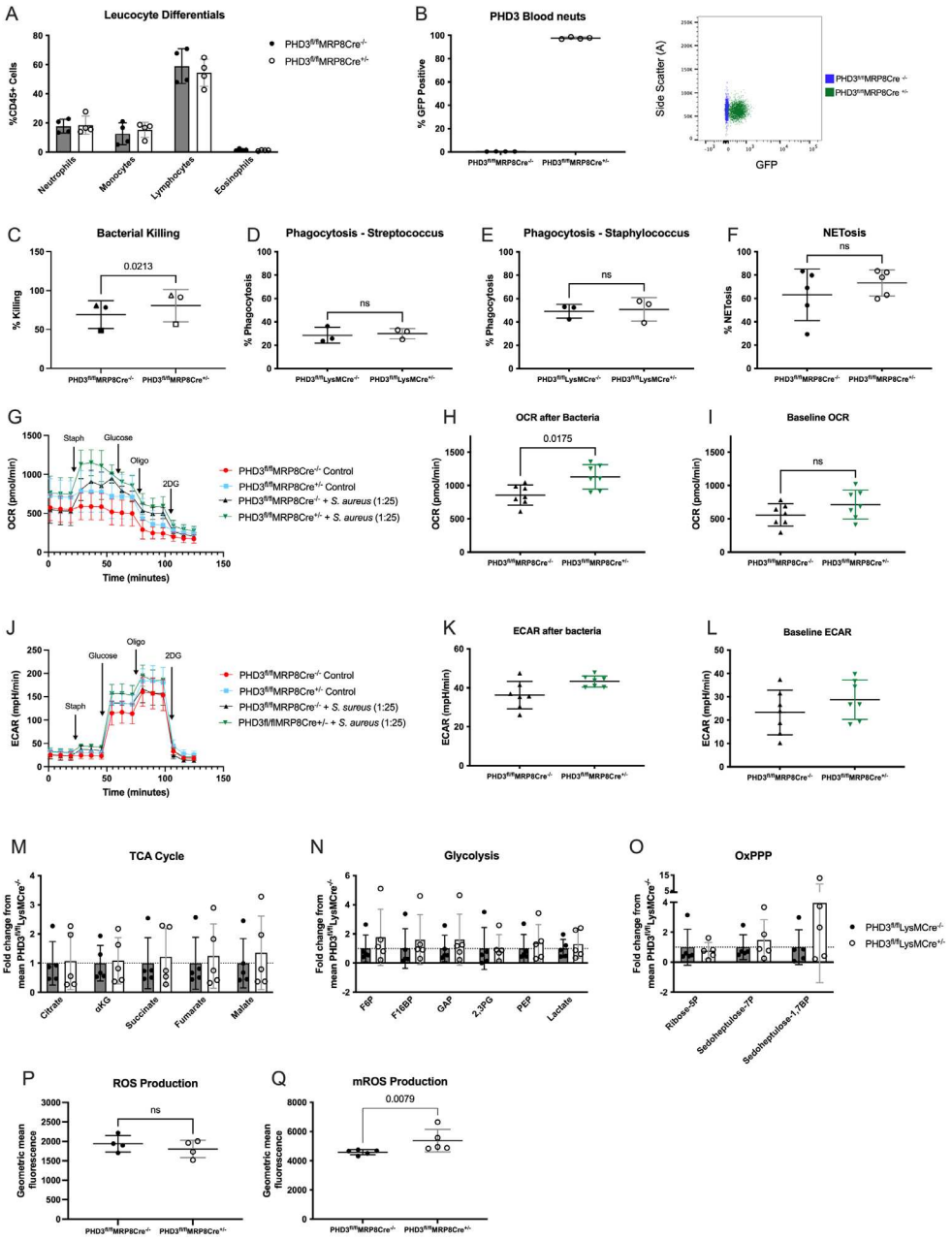

Figure 6. PHD3 deficient neutrophils demonstrate increased oxygen consumption and mROS production. Blood leucocyte differentials were determined by flow cytometry (**A**) and Ly6G positive neutrophils were assessed for GFP positivity (**B**). *Ex vivo* BAL neutrophils were isolated from PHD3$^{fl/fl}$MRP8Cre$^{+/-}$ and PHD3$^{fl/fl}$MRP8Cre$^{-/-}$ (**C**, **F**, **G**-**L**, **P**-**Q**) or PHD3$^{fl/fl}$LysMCre$^{-/-}$ and PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice (**D**-**E** & **M**-**O**) 24 hours following LPS induced ALI. (**A**) Bacterial killing was measured using *ex vivo* BAL neutrophils (24 hours post-LPS induced ALI) cultured with opsonised *Staphylococcus aureus* bacteria. Cells were either lysed following washing (T0) or after a further one-hour incubation (T1) and % killing calculated. N=3 over 3 separate experiments, paired t-test (with each experiment designated by a different symbol). Phagocytosis of fluorescently labelled Streptococcus (**D**) and Staphylococcus (**E**) was measured by flow cytometry. N=3, analysed by unpaired t-test. (**F**) NETosis was measured using SytoxRed reagent and analysed by flow cytometry (N=5), analysed by Mann-Whitney test (unpaired). Metabolic flux was measured using Seahorse technology. OCR (**G**-**I**) and ECAR (**J**-**L**) were analysed before and after the addition of heat killed *Staphylococcus aureus.* N=7 per condition, **H**-**I**&**K**-**L** analysed by Mann-Whitney test (unpaired). (**M**)–(**O**) HPLC-MS analysis of neutrophil metabolic profiles were carried out on *ex vivo* BAL neutrophils harvested 24 hours following LPS induced lung injury. N=5, data expressed as fold change from mean PHD3$^{fl/fl}$LysMCre$^{-/-}$ and analysed by 2-way ANOVA. (**P**) ROS production was measured by incubation of *ex vivo* BAL cells with CellRox red and measured by flow cytometry, data expressed as geometric mean fluorescence. N=4. (**Q**) mROS production was measured by incubating *ex vivo* BAL neutrophils with mitoSOX reagent and analysed by flow cytometry, data expressed as geometric mean fluorescence, N=5. (**P**)&(**Q**) analysed by Mann-Whitney test (unpaired). All data expressed as mean±SD. F6P, fructose-6-phosphate; F16BP, fructose-1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; 2,3PG, 2,3 biphosphoglycerate; PEP, phosphoenolpyruvate; αKG, α-ketoglutarate.

(Figure 6B). To delineate the mechanisms by which PHD3 deficient neutrophils promote antimicrobial control, we cultured *ex vivo* BAL cells from the neutrophil specific PHD3$^{fl/fl}$MRP8Cre$^{+/-}$ mouse lines with *Staphylococcus aureus*. We used glucose deplete media to culture these airway cells to represent the hypoglycaemic airway[16,24,25]. We confirmed a significant increase in bacterial killing by PHD3$^{fl/fl}$MRP8Cre$^{+/-}$ neutrophils (Figure 6C). *In vitro* we observe this enhanced bacterial killing to be independent of phagocytosis (Figure 6D&E) and NETosis (Figure 6F)). However, we did observe a more marked increase in local oxygen consumption rate (OCR), measured by Seahorse, in response to bacteria (Figure 6G–I)). This uplift in OCR was observed in the context of conserved ECAR (a surrogate for glycolysis, Figure 6J–L), and equivalent abundance of TCA cycle, glycolysis and OxPPP metabolic intermediaries, as measured by HPLC-MS (Figure 6M–O). As in Figure 2A–F, we also performed post-hoc multiple comparison tests and found that no individual metabolite was significantly different between genotypes. We therefore considered whether this increase in oxygen consumption rates reflected increased ROS production. Total ROS production was equivalent between genotypes (Figure 6P) but mROS production was significantly higher in PHD3 deficient neutrophils in comparison to Cre negative cells (Figure 6Q).

Mitochondrial ROS (mROS) are produced by complexes I and III of the electron transport chain and are an important effector of antimicrobial responses[26]. They have been shown to be induced by infection and to contribute to cytokine production in response to infection in macrophages[27,28]. Thus, an increase in mROS production represents a potential mechanism whereby PHD3 deficient neutrophils achieve enhanced bacterial killing associated with increased oxygen consumption upon exposure to bacteria.

Discussion

Cellular adaptations to hypoxia are critical in maintaining homeostasis and responding to pathogenic environments. Complexity within this system is conferred through the presence of multiple isoforms of the key HIF and PHD proteins which in turn respond differently in different cell types and environments[7]. We investigated the role of PHD1 and PHD3 in neutrophil responses, having previously demonstrated the critical role which PHD2 plays in neutrophilic inflammation[10].

In other cell types PHD1 has been shown to be important in responding to hypoxic and ischaemic insults[12,13]. These responses are mediated by metabolic rewiring in PHD1 deplete cells, although the precise changes are cell type dependent. We have shown that PHD1 deficient neutrophils also demonstrate metabolic changes with increased OxPPP metabolites in normoxia and increased TCA cycle metabolites in hypoxia. Further work will be required to elucidate reasons for these differences. In other cell types, the mechanisms underlying PHD1 dependent metabolic shifts include alterations in HIF-1α target expression such as pyruvate dehydrogenase kinase 1 (PDK1) in skeletal muscle[12] and increased expression of TP53-inducible glycolysis and apoptosis regulator (TIGAR) in neurons[14]. We have not further dissected the mechanisms in PHD1 deficient neutrophils but such cell type dependent metabolic rewiring will be of interest in the future. In a hypoxic acute lung injury model, neutrophil specific PHD1 deficiency was associated with increased neutrophil recruitment at 24 hours but more rapid resolution, restoring equivalent neutrophil numbers by 48 hours post-challenge. Alterations in neutrophil metabolic profiles did not lead to changes in inflammatory outcomes, irrespective of oxygenation. Thus, unlike skeletal myocytes and hepatocytes, neutrophil specific PHD1 deficiency does not confer protection against the hyperinflammatory phenotype associated with hypoxia in acute lung injury. Further work is required to interrogate the mechanisms which underly the metabolic shifts observed in PHD1 deficient neutrophils and whether there are any downstream functional consequences. In light of the findings described in PHD3 deficient neutrophils, it will also be of interest to investigate whether neutrophil PHD1 deficiency alters outcomes in infection models.

The protective phenotype in PHD1 deficient muscle fibres and hepatocytes relates to reduced oxygen consumption and consequent reduced oxidative stress. Unlike these highly aerobic cells, neutrophils utilise glycolysis to generate ATP at baseline as well as in hypoxic environments[21]. Additionally, the generation of ROS, via the NADPH oxidase associated with the phagolysosome, is critical to the neutrophil's antimicrobial response[29]. Neutrophils are likely to have different mechanisms of protection against oxidative stress, with compartmentalisation of ROS and effective redox buffering. These fundamental differences in metabolic phenotype may explain why neutrophil PHD1 deficiency does not confer the same protection against a hypoxic insult as is observed in other cell types.

PHD3 deficiency did have a significant impact on inflammation outcomes. Myeloid specific PHD3 deficiency led to a potentially highly advantageous phenotype with improved outcomes in infection without any evidence of excessive sterile inflammation. Using a neutrophil specific mouse line, we further examined PHD3 deficient neutrophils *ex vivo*. We showed a small but significant improvement in bacterial killing in the PHD3$^{fl/fl}$MRP8Cre$^{+/-}$ neutrophils, consistent with *in vivo* findings in the myeloid specific mice. This was not related to increases in NETosis or total ROS production. Using Seahorse technology, we identified enhanced oxygen consumption in PHD3 deficient neutrophils upon challenge with bacteria which we have demonstrated is associated with increased mROS production in inflammatory BAL neutrophils. We therefore suggest that loss of PHD3 confers a beneficial neutrophil phenotype with enhanced mROS production resulting in improved bacterial control, without any identifiable hyperinflammatory phenotype in the context of sterile inflammation. It will be critical to confirm that the *ex vivo* enhanced bacterial killing observed in the neutrophil specific mouse model does translate to improved *in vivo* outcomes, as were seen in the myeloid specific mouse line. In light of this important phenotype, future

work should also include investigation of the interface between mitochondrial metabolism, hypoxic signalling pathways and host antimicrobial capacity. It will also be of interest to assess how PHD3 deficiency impacts the activity of different HIF isoforms. These data suggest that PHD3 represents a potential therapeutic target. In the era of multi-drug resistant infections, strategies which target host responses to regulate pathogen control are of huge importance. Fine tuning the immune response via the closely linked hypoxic response system is one potential route to this. In the absence of isoform specific pharmacological PHD inhibitors, the impact of pan hydroxylase inhibition will be dependent on the dominant cell types involved and context of any inflammatory or infective response. It will be important in future work to confirm the role of PHD3 in human, as well as mouse neutrophils. This may be addressed using human neutrophil like cells lines which are amenable to genetic manipulation but ultimately will require specific inhibitors of PHD3. An important future challenge therefore is the need to achieve isoform specific targeting.

Limitations

Our Study identifies an important neutrophil phenotype but we acknowledge there are some limitations.

We have used both neutrophil specific and myeloid specific mouse lines in these studies and this does limit our capacity to directly compare PHD3 and PHD1 deficiency in neutrophil populations. Our aim in this paper was not necessarily to directly compare PHD1 with PHD3 deficiency, rather to present the data from both genotypes for interest and completeness. Our in vitro data using the PHD3$^{fl/fl}$MRP8Cre line is consistent with our *in vivo* data which was carried out in the LysMCre line however, a priority for future studies will be to ensure that the *in vivo* phenotypes do hold true in a neutrophil specific mouse model. Whilst the MRP8 model is ideal to identify the specific impact of neutrophil PHD3 loss, the use of the myeloid specific LysMCre line is also of value. When considering the effect of pharmacological inhibition of PHD proteins, the overall effect on inflammation outcomes will be determined by the sum effect on all infiltrating cells, including monocyte/macrophage cells. The *in vivo* findings of enhanced bacterial control in the LysMCre mouse line are consistent with the *ex vivo* increased bactericidal capacity (caried out on MRP8Cre, neutrophil specific knock out cells) therefore demonstrating that targeting PHD3 *in vivo* may have a net beneficial effect. In order to strengthen these findings further, we have included both lung and skin infection models to ensure the phenotype is broadly relevant. We also acknowledge that we do not have parallel unfloxed but Cre$^{+/-}$ controls for the mice described here. This would have required us to breed and maintain additional mouse lines. We have previously utilised both LysM[10,30] and MRP8[23,31] driven Cre and do not identify a Cre specific phenotype. We therefore determined that using these additional controls was not justified when working within the 3Rs framework[32].

At present our findings demonstrate an association between bactericidal capacity and enhanced mROS production. Further work will be required to establish the causative role of mROS in the *in vivo* and *ex vivo* phenotypes we have described here. We do not see significant changes in neutrophil recruitment in the pneumonia model in PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice but have not been able to exclude this in the skin abscess model. In future work, intravital imaging may provide a route to measure neutrophil numbers and dynamics in this setting. Additionally, although the loss of PHD1 does not appear to alter neutrophil behaviour, it will be important to confirm this in infection models in addition to the LPS induced ALI model described here and to investigate other potential consequences of the metabolic shifts which are observed.

Data availability

Underlying data

Figshare: Differential roles for the oxygen sensing enzymes PHD1 and PHD3 in the regulation of neutrophil metabolism and function, https://doi.org/10.6084/m9.figshare.24106686.v4[33].

This project contains the following underlying data:

- PHD1 Normoxia mass spec raw data
- PHD1 Hypoxia mass spec raw data
- PHD1 Normoxia mass spec corrected for BCA data
- PHD1 Hypoxia mass spec corrected for BCA data
- PHD3 mass spec raw data
- PHD3 mass spec corrected for BCA data
- Fig1A–Fig6Q Data underlying corresponding figures

Reporting guidelines

Figshare: ARRIVE checklist for ' *Differential roles for the oxygen sensing enzymes PHD1 and PHD3 in the regulation of neutrophil metabolism and function*', https://doi.org/10.6084/m9.figshare.24106686.v4[33].

Acknowledgements

We are very grateful to Prof Sir Peter Ratcliffe (University of Oxford) for provision of the floxed PHD1 and 3 mice and for helpful discussions of this work.

Flow cytometry data were generated with support from the QMRI Flow Cytometry and Cell Sorting Facility, University of Edinburgh. We are grateful to the staff in the Bioresearch and Veterinary Services department at the Little France site, University of Edinburgh, for their assistance with the animal work in this manuscript.

For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

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http://www.doi.org/10.6084/m9.figshare.24106686.v4

Open Peer Review

Current Peer Review Status: ? ? ✓ ✓

Version 2

Reviewer Report 10 September 2024

https://doi.org/10.21956/wellcomeopenres.25257.r96744

✓ **Eileen Uribe-Querol**
Universidad Nacional Autónoma de México, Mexico City, Mexico

I confirm that I have reviewed this submission and, find it suitable for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neutrophil functions, developmental biology

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.

Reviewer Report 09 September 2024

https://doi.org/10.21956/wellcomeopenres.25257.r96742

✓ **Frederik Denorme**
Emergency Medicine, Washington University in St Louis School of Medicine, St. Louis, Missouri, USA

I have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neutrophil biology in the context of (sterile) inflammation.

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.

Version 1

Reviewer Report 27 March 2024

https://doi.org/10.21956/wellcomeopenres.22052.r74197

?

Eileen Uribe-Querol
[1] Universidad Nacional Autónoma de México, Mexico City, Mexico
[2] Universidad Nacional Autónoma de México, Mexico City, Mexico

In the manuscript “Differential roles for the oxygen sensing enzymes PHD1 and PHD3 in the regulation of neutrophil metabolism and function, Watts and colleagues investigated the consequences of neutrophils oxygen sensing prolyl hydroxylase (PHD) enzymes, PHD1 and PHD3 deficiency in normoxia and hypoxia. While the authors addressed a clinically relevant issue and the data, character count, and number of figures make this manuscript a good candidate to be published, there are some limitations to their study and the findings need a more elaborated and clear mechanism of action. This topic is important because of the therapeutical use it can provide. Authors used a combination of in vivo and ex vivo models, utilizing neutrophil and myeloid specific PHD1 or PHD3 deficient mouse lines to investigate the roles of oxygen sensing prolyl hydroxylase enzymes in the regulation of neutrophilic inflammation and immunity and conclude that PHD1 deficiency drives alterations in neutrophil metabolism and recruitment, in an oxygen dependent fashion. On the other hand, PHD3 deficiency was found to enhance neutrophil antibacterial properties without excessive inflammatory responses.

Mayor concerns:

1) Methods section is not clear and incomplete. Each method should have a reference or a brief detailed description to allow replication. It is confusing were did neutrophils came from in some experiments. Please explain how blood and bone marrow neutrophils did were obtained. More information about the kits used is also needed.

2) The papes has some interesting results that are not explained.

3) Discussion section should be improved including a more elaborated description and analysis of the findings.

Minor changes>

1) All abbreviations need to be defined the first time mentioned on the paper.
The paper must be proofread to correct typographic errors. Chemical formulas nomenclature and names should be reviewed. (O2), doble parenthesis, of some words that are not in context. In section, In vivo mouse models ..."Pseudomonas aeruginosa 10". What does the 10 stands for?

2) Figure labels are two small and, in some cases, incomplete. All figures must be reviewed.

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

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

Are sufficient details of methods and analysis provided to allow replication by others?
No

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

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

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neutrophil functions, developmental biology

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 05 Aug 2024

Emily Watts

We would like to thank the reviewer for their time and their review of this manuscript. We have submitted these data to Wellcome Open Research as we wanted to share both the interesting positive findings, but also the important negative data, with the scientific community in a timely fashion. We agree that some of the findings are preliminary and that we have not fully dissected the mechanisms underlying the phenotypes which we have observed. Nevertheless, we felt that the phenotype observed in the PHD3 deficient mice was of sufficient interest to publish the data in its current form. Further studies will of course be required to confirm the mechanisms underpinning these findings. We are providing some limited new experimental data in this revised version but the majority of the comments raised have been addressed in the attached rebuttal and by revision of the text

and conclusions drawn. We are grateful to the reviewer for their recognition of clinical relevance and of our work and hope that in this revised version they will agree that the conclusions drawn are supported by the data.

In the manuscript "Differential roles for the oxygen sensing enzymes PHD1 and PHD3 in the regulation of neutrophil metabolism and function, Watts and colleagues investigated the consequences of neutrophils oxygen sensing prolyl hydroxylase (PHD) enzymes, PHD1 and PHD3 deficiency in normoxia and hypoxia. While the authors addressed a clinically relevant issue and the data, character count, and number of figures make this manuscript a good candidate to be published, there are some limitations to their study and the findings need a more elaborated and clear mechanism of action. This topic is important because of the therapeutical use it can provide.
Authors used a combination of in vivo and ex vivo models, utilizing neutrophil and myeloid specific PHD1 or PHD3 deficient mouse lines to investigate the roles of oxygen sensing prolyl hydroxylase enzymes in the regulation of neutrophilic inflammation and immunity and conclude that PHD1 deficiency drives alterations in neutrophil metabolism and recruitment, in an oxygen dependent fashion. On the other hand, PHD3 deficiency was found to enhance neutrophil antibacterial properties without excessive inflammatory responses.

Major concerns:

1) Methods section is not clear and incomplete. Each method should have a reference or a brief detailed description to allow replication. It is confusing were did neutrophils came from in some experiments. Please explain how blood and bone marrow neutrophils did were obtained. More information about the kits used is also needed.
Response: Thank you for this feedback. We have added additional details to the methods section to describe in detail the neutrophil isolation protocols. For convenience these are also detailed here: Bone marrow: Both hind limbs were dissected out and stored in a clean bijou container on ice until processed. Each bone was flushed using 10ml of 1xHBSS with 0.2% BSA. The cell suspension was resuspended, passed through a 70mm filter into a fresh 50ml polystyrene falcon tube and then centrifuged at 450G for 10 minutes. 90% vol/vol percoll stock was made using 10X HBSS with $NaHCO_3$ and this stock was used to make 81%, 62% and 55% percoll with 1x HBSS. All three percoll preparations were layered in a 15ml falcon tube and the cells were resuspended in 3ml of 1xHBSS with 0.2% BSA which was layered on top of the 52% percoll layer. The gradient was centrifuged at 2000G for 30 minutes (acceleration 1, deceleration 0). Bone marrow monocyte precursors were removed from the top layer with a pastette. Neutrophils were removed from the middle layer and washed in 30ml of 1xHBSS with 0.2% BSA. Hypotonic saline red blood cell lysis was carried out. Following the percoll purification above, neutrophil purity was found to be approximately 90%. In order to generate highly pure bone marrow neutrophils samples, cells from the neutrophil layer were resuspended in 1xHBSS with 0.2% BSA and purified by fluorescence-activated cell sorting (FACS) based on the forward/side scatter profile and auto-fluorescence as previously described[1]. Bronchoalveolar lavage: Where highly pure BAL neutrophils were required, we used a previously optimized discontinuous percoll gradient coupled with a red cell lysis step in order to gain purities of >98%. BAL cells were first

pelleted by centrifugation at 350G for 10 minutes. BAL samples from 1-3 mice (depending on the final cell number required) were pooled onto a single percoll gradient. 90% vol/vol percoll was made using 10X PBS and further percoll solutions were made using this stock 90% percoll mixed with 1X PBS. 3ml of 78% percoll was pipetted into the bottom of a 15ml falcon tube, followed by 3ml of 69% percoll. The cell pellet(s) were resuspended in 3ml of 52% percoll which was then layered onto the 69% layer. The gradient(s) were centrifuged at 1200G for 30mins with an acceleration of 1 and deceleration of 0. Following centrifugation, the macrophages accumulated on the top of the 52% layer and were removed using a sterile pastette. The neutrophils (within the 69% layer) were then removed using a fresh sterile pastette and placed into a 50ml polystyrene falcon tube. This was topped up to 50ml with sterile PBS to wash off the percoll and the purified neutrophils pelleted at 350G for 8 mins. Residual red cells were lysed by hypotonic saline lysis. A cytospin was made prior to the final centrifugation step to assess purity by morphology. During optimisation of this protocol, purity was also confirmed by flow cytometry and the neutrophils were found to be >98% pure.

2) The paper has some interesting results that are not explained.
Response: We agree that this paper does not fully dissect the mechanisms underlying the observed phenotypes and have provided a more detailed explanation in response to reviewers comments (please see separate responses). We have published these results (including relevant negative data) in Wellcome Open Reports to ensure timely availability of these data to the wider community. We have expanded the discussion and limitations sections to acknowledge clearly that these data are preliminary findings rather than definitive mechanistic data and hope that by sharing the data, further work can be done to interrogate the interesting findings in more detail.

3) Discussion section should be improved including a more elaborated description and analysis of the findings.
Response: As above, taking in account comments from the reviewers we have now expanded the discussion and limitations sections to better describe the findings and to address specific queries.

Minor changes

1) All abbreviations need to be defined the first time mentioned on the paper.
The paper must be proofread to correct typographic errors. Chemical formulas nomenclature and names should be reviewed. (O2), doble parenthesis, of some words that are not in context. In section, In vivo mouse models ...”Pseudomonas aeruginosa 10”. What does the 10 stands for?
Response: Thank you. We have corrected the errors detailed. The LPS is sourced from *Pseudomonas aeruginosa* (10) (https://www.sigmaaldrich.com/GB/en/product/sigma/l9143). We apologise that the omission of the brackets may have made this unclear and have updated the manuscript for clarity.

2) Figure labels are two small and, in some cases, incomplete. All figures must be reviewed.
Response: Thank you. We have increased the size of the figure labels. All figures were

submitted at equal resolution and this technical issue has been highlighted to the editorial team and will be resolved in the final version.

1. Dorward, D. A. *et al.* Technical advance: autofluorescence-based sorting: rapid and nonperturbing isolation of ultrapure neutrophils to determine cytokine production. *Journal of Leukocyte Biology* **94,** 193–202 (2013).

Competing Interests: No competing interests were disclosed.

Reviewer Report 12 March 2024

https://doi.org/10.21956/wellcomeopenres.22052.r74200

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Frederik Denorme
[1] Emergency Medicine, Washington University in St Louis School of Medicine, St. Louis, Missouri, USA
[2] Emergency Medicine, Washington University in St Louis School of Medicine, St. Louis, Missouri, USA

In this article, Watss and colleagues investigate the role of neutrophil PHD1 and 3 in murine models of inflammation. The study is interesting, however, some findings are rather preliminary due to low N. Additionally, the authors tend to over-interpret their data, drawing somewhat strong conclusions from a rather minor phenotype.

- For the in vitro assays, neutrophils were isolated. However, it is nowhere specified how they were isolated and how purity was validated.

- NET formation assays are also not specified. What agonist was used to induce NET formation and for how long?

- Since these are new mice that have been generated, I am surprised no more baseline characterisation was performed. I.e. on neutrophils isolated in the absence of an inflammatory trigger.

- How was sample size determined for the experiments? It seems to vary a lot based on Figure and doesn't seem to make much sense. Was there a power analysis? For example Fig; 1C seems to be trending or close to significance, yet there are only 3 samples per group and no P-value is provide. While some other assays show significance with smaller differences, however, bigger sample size. This becomes somewhat concerning if the authors try to draw mechanistic conclusions from these results.

- Another way to interpret Figure 1H-I, is that there are less neutrophils being recruited under hypoxia, and that this reduction is abrogated in the KO mice.

- What about neutrophil resolution in normoxia in both groups?

- What do the authors hypothesise is the reason for similar NE but different neutrophil numbers in mice. If there are more neutrophils but they don't get activated as well, wouldn't the netto impact be no difference.

- Figure 3: reduced neutrophil numbers are rather minor, as half of the WT mice are in the same range of KO animals. Similar for Figure 4, the differences are rather minor, the conclusions need to be toned down a little bit. The biggest difference seems to be with CFU at day 2, however this difference disappears over time.

- Figure 5 fits with the rest of the manuscript as there are some small differences. The title reduced bacterial load seems a little over-reaching since the difference is only in BAL and only really 4/10 WT mice have more bacteria than the KO mice.

- Figure 6A could be an explanation for a lot of the previous data, however only an N of 3 is done. This would need to be increased. It is also unclear if a paired t-test is the best statistical test for this type of assay.

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?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neutrophil biology in the context of (sterile) inflammation.

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 05 Aug 2024

Emily Watts

We would like to thank the reviewer for their time and their detailed review of this manuscript. We have submitted these data to Wellcome Open Research as we wanted to share both the interesting positive findings, but also the important negative data, with the scientific community in a timely fashion. We agree that some of the findings are preliminary and that we have not fully dissected the mechanisms underlying the phenotypes which we have observed. Nevertheless, we felt that the phenotype observed in the PHD3 deficient mice was of sufficient interest to publish the data in its current form. Further studies will of course be required to confirm the mechanisms underpinning these findings. We are providing some limited new experimental data in this revised version but the majority of the comments raised have been addressed in the attached rebuttal and by revision of the text and conclusions drawn. We are grateful to the reviewer for their recognition of the interesting nature of our work and hope that in this revised version they will agree that the conclusions drawn are supported by the data.

In this article, Watts and colleagues investigate the role of neutrophil PHD1 and 3 in murine models of inflammation. The study is interesting, however, some findings are rather preliminary due to low N. Additionally, the authors tend to over-interpret their data, drawing somewhat strong conclusions from a rather minor phenotype. - For the in vitro assays, neutrophils were isolated. However, it is nowhere specified how they were isolated and how purity was validated.

Response: We apologise for this oversight. We have now updated the methods section to include the detailed protocols for neutrophil isolation and have included them below for convenience: Bone marrow: Both hind limbs were dissected out and stored in a clean bijou container on ice until processed. Each bone was flushed using 10ml of 1xHBSS with 0.2% BSA. The cell suspension was resuspended, passed through a 70mm filter into a fresh 50ml polystyrene falcon tube and then centrifuged at 450G for 10 minutes. 90% vol/vol percoll stock was made using 10X HBSS with $NaHCO_3$ and this stock was used to make 81%, 62% and 55% percoll with 1x HBSS. All three percoll preparations were layered in a 15ml falcon tube and the cells were resuspended in 3ml of 1xHBSS with 0.2% BSA which was layered on top of the 52% percoll layer. The gradient was centrifuged at 2000G for 30 minutes (acceleration 1, deceleration 0). Bone marrow monocyte precursors were removed from the top layer with a pastette. Neutrophils were removed from the middle layer and washed in 30ml of 1xHBSS with 0.2%BSA. Hypotonic saline red blood cell lysis was carried out. Following the percoll purification above, neutrophil purity was found to be approximately 90%. In order to generate highly pure bone marrow neutrophils samples, cells from the neutrophil layer were resuspended in 1xHBSS with 0.2% BSA and purified by fluorescence-activated cell sorting (FACS) based on the forward/side scatter profile and auto-fluorescence as previously described[1] Bronchoalveolar lavage: Where highly pure BAL neutrophils were required, we used a previously optimized discontinuous percoll gradient coupled with a red cell lysis step in order to gain purities of >98%. BAL cells were first pelleted by centrifugation at 350G for 10 minutes. BAL samples from 1-3 mice (depending on the final cell number required) were pooled onto a single percoll gradient. 90% vol/vol percoll was made using 10X PBS and further percoll solutions were made using this stock 90% percoll mixed with 1X PBS. 3ml of 78% percoll was pipetted into the bottom of a 15ml falcon tube,

followed by 3ml of 69% percoll. The cell pellet(s) were resuspended in 3ml of 52% percoll which was then layered onto the 69% layer. The gradient(s) were centrifuged at 1200G for 30mins with an acceleration of 1 and deceleration of 0. Following centrifugation, the macrophages accumulated on the top of the 52% layer and were removed using a sterile pastette. The neutrophils (within the 69% layer) were then removed using a fresh sterile pastette and placed into a 50ml polystyrene falcon tube. This was topped up to 50ml with sterile PBS to wash off the percoll and the purified neutrophils pelleted at 350G for 8 mins. Residual red cells were lysed by hypotonic saline lysis. A cytospin was made prior to the final centrifugation step to assess purity by morphology. During optimisation of this protocol, purity was also confirmed by flow cytometry and the neutrophils were found to be >98% pure.

NET formation assays are also not specified. What agonist was used to induce NET formation and for how long?
Response: The NET assay was carried out on *ex-vivo* BAL neutrophils following LPS induced lung injury, therefore highly activated cells. We did not further stimulate them (for example with PMA) but instead measured baseline NETosis using SyTox Red. We apologise for the lack of clarity in the description of this assay and have reworded it to improve this.

Since these are new mice that have been generated, I am surprised no more baseline characterisation was performed. I.e. on neutrophils isolated in the absence of an inflammatory trigger.
Response: The PHD3 $^{fl/fl}$LysMCre $^{+/-}$ mouse line has been previously published[2]. MRP8 driven Cre is established as a method of inducing neutrophil specific knock down[3-6]. In this revised version we provide additional data to show normal circulating leucocyte populations in naïve mice from both PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ PHD3$^{fl/fl}$MRP8Cre$^{+/-}$ mice. We also demonstrate effective expression of the Cre recombinase using it's GFP tag in circulating neutrophils.

How was sample size determined for the experiments? It seems to vary a lot based on Figure and doesn't seem to make much sense. Was there a power analysis? For example Fig; 1C seems to be trending or close to significance, yet there are only 3 samples per group and no P-value is provide. While some other assays show significance with smaller differences, however, bigger sample size. This becomes somewhat concerning if the authors try to draw mechanistic conclusions from these results.
Response: Sample size was determined by a number of factors including our previous experience using these models and the degree of biological variability which is seen. For example, in our experience, there is significant variability in response to the streptococcal pneumonia model and so we planned to use a higher sample size in these experiments. In some assays, such as the metabolite mass spec data sets and in the PHD3 *in vitro* bacterial killing, each sample represents cells pooled from up to 3 mice and so sample size was kept to a minimum to avoid excessive use of animals. We agree that there is a trend towards reduced PHD1 expression in the hypoxic BAL neutrophils and it may be informative to repeat this to see if it does reach significance, the p value here is 0.1010 with n=3. In light of the predominantly negative data we found in the PHD1 mice, we have elected not to repeat these experiments at present as it is unlikely to alter our immediate future work but accept

the reviewer's comments regarding this figure.

Another way to interpret Figure 1H-I, is that there are less neutrophils being recruited under hypoxia, and that this reduction is abrogated in the KO mice.
Response: Thank you for this interesting comment. We do consistently see a small reduction in neutrophil recruitment to the airways in hypoxia (see Watts *et al.*[7] Fig 6D). We suggest that our data here shows that the increased rate of resolution seen in the hypoxic PHD1 mice is a result of both higher numbers at 24 hours and lower numbers at 48 hours when compared to wild type animals. We have updated the text in the manuscript to better describe this phenotype.

What about neutrophil resolution in normoxia in both groups?
Response: Resolution is not altered in the PHD1 deficient mice in normoxia, we have now included this data in the manuscript for completeness (Fig 1M).

What do the authors hypothesise is the reason for similar NE but different neutrophil numbers in mice. If there are more neutrophils but they don't get activated as well, wouldn't the net impact be no difference.
Response: Thank you for this interesting point. We would have to conclude that the PHD1 deficient neutrophils are producing less elastase than their wild-type counter parts. Indeed, if one calculates the "elastase per neutrophil" and then the fold change from WT to KO, this is ~0.85. Nevertheless, the overall impact of these changes is not significant as we don't see any difference in lung injury (i.e. albumin leak) or in physiological parameters such a temperature. These data are in keeping with our overarching conclusion that, although PHD1 deficiency does have some effects on neutrophil function, it is not of sufficient magnitude to result in a significant phenotype overall. We have expanded on the existing text to better describe these findings.

Figure 3: reduced neutrophil numbers are rather minor, as half of the WT mice are in the same range of KO animals. Similar for Figure 4, the differences are rather minor, the conclusions need to be toned down a little bit. The biggest difference seems to be with CFU at day 2, however this difference disappears over time.
Response: With regards to figure 3D, there is overlap between the $PHD3^{fl/fl}LysMCre^{-/-}$ and $PHD3^{fl/fl}LysMCre^{+/-}$ groups, in keeping with biological variability between animals in response to the LPS induced lung injury. However, the mean neutrophil number for $PHD3^{fl/fl}LysMCre^{-/-}$ is 7.28 million versus 4.60 million in the $PHD3^{fl/fl}LysMCre^{-/+}$, a fold change of 0.63 which we consider to be both a biologically and statistically significant reduction in neutrophil cell number. We accept that the amplitude of the changes in Figure 4 are small in some cases. The significant difference in CFU counts at day 2 is important as it likely leads to the overall smaller abscess size over the course of the 7 days course. We have reworded the conclusions as suggested in order to avoid overstating the size of the effect.

Figure 5 fits with the rest of the manuscript as there are some small differences. The title reduced bacterial load seems a little over-reaching since the difference is only in BAL and only really 4/10 WT mice have more bacteria than the KO mice.
Response: The scale on this graph is logarithmic which does make it difficult to appreciate the difference in the groups. As before, there is significant variability between mice in both

groups but the fold change is from PHD3$^{fl/fl}$LysMCre$^{-/-}$ to PHD3$^{fl/fl}$LysMCre$^{+/-}$ groups is 0.02 and reaches statistical significance. These data are in keeping with our other *in vivo* and *in vitro* findings so we do feel that they are valid and important results.

Figure 6A could be an explanation for a lot of the previous data, however only an N of 3 is done. This would need to be increased. It is also unclear if a paired t-test is the best statistical test for this type of assay.
Response: We accept that this is a small N number. However, each N is generated by pooling the BAL cells from 3-4 mice (per genotype) in order to generate enough cells to run in duplicate and at multiple dilutions for CFU counts. Given that we are able to see a small but significant difference with the data we have generated we didn't feel it was justifiable within the 3Rs framework to repeat this assay simply to increase numbers. Within our group we have carried out this assay in multiple mouse lines and in human peripheral blood neutrophils and found the data to be normally distributed, justifying the use of a t-test here. There is a degree of variability between runs e.g. due to differences in aliquots of bacteria used and so we have used a paired t-test. We could also have presented this data as fold change from WT in each run but felt that the raw data was a more complete and transparent way to present the findings.

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2. Swain, L. *et al.* Prolyl-4-hydroxylase domain 3 (PHD3) is a critical terminator for cell survival of macrophages under stress conditions. *Journal of Leukocyte Biology* **96,** 365–375 (2014).
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4. Patel, R. B. *et al.* Targeting Neutrophil α9 Improves Functional Outcomes After Stroke in Mice With Obesity-Induced Hyperglycemia. *Stroke* **54,** 2409–2419 (2023).
5. Idol, R. A. *et al.* Neutrophil and Macrophage NADPH Oxidase 2 Differentially Control Responses to Inflammation and to Aspergillus fumigatus in Mice. *J. Immunol.* **209,** 1960–1972 (2022).
6. Elliott, E. R. *et al.* Deletion of Syk in Neutrophils Prevents Immune Complex Arthritis. *The Journal of Immunology* **187,** 4319–4330 (2011). 7. Watts, E. R. *et al.* Hypoxia drives murine neutrophil protein scavenging to maintain central carbon metabolism. *J. Clin. Invest.* **131,** (2021).

Competing Interests: No competing interests were disclosed.

Reviewer Report 04 March 2024

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In this study by Watts et al., the researchers have expanded upon their previous work wherein they investigated the expressions and roles of three isoforms of prolyl hydroxylase domain (PHD) enzymes - namely, PHD1, PHD2, and PHD3 - within neutrophils. Previously, their focus centered on PHD2, revealing its constitutive expression in neutrophils with a potential for upregulation during sterile inflammation. The deletion of PHD2 specifically within the myeloid population was found to potentially exacerbate inflammatory response to bacterial infection by augmenting glycolytic flux, thereby enhancing neutrophil functional capacity. However, myeloid-specific PHD2 deficiency did not amplify antibacterial properties. In this current study, the authors further examined the significance of the other two isoforms of PHD enzymes in neutrophils, elucidating their distinct functions. Although PHD1 deficiency in neutrophils was found to alter oxygen-dependent metabolic profiles and the recruitment of neutrophils, it did not affect neutrophil functional capacity either *ex vivo* or *in vivo*. On the other hand, the depletion of PHD3 in myeloid cells resulted in enhanced bactericidal capacity, potentially through increased oxygen consumption and mitochondrial reactive oxygen species generation.

While the study reveals intriguing findings, it is imperative to address specific comments and concerns to bolster the robustness of the results and improve clarity. The primary concern lies in the lack of a clear rationale and coherence between the studies, making it challenging to comprehend the authors' transitions from one experiment to another. For instance:

- The rationale behind the switch in Cre lines from MRP8-Cre for PHD1 deletion to LysM-Cre for PHD3 deletion remains unclear, leading to difficulties in comparing the outcomes of gene deficiency, particularly considering the discrepancy in patterns of expression of these two promoter-driven deletions. While the MRP8-Cre is by far the most neutrophil-specific Cre line, the LysM-Cre targets several myeloid cell types, posing challenges in isolating the effects solely on neutrophils. Although the authors acknowledged in the Limitations that both neutrophil-specific and myeloid-specific mouse lines in the PHD3 were used, only myeloid-specific deletion data were presented in this study. Moreover, the mice used in this study were newly generated and involved the crossing of different lines together (e.g., crossing PHD1fl/fl animals with MRP8-Cre-ires/GFP mice). The evidence showing the successful deletion of the interested gene only in anticipated specific cell types such as neutrophils, but not other related immune cells is required. In particular, it is crucial to ascertain whether the expression of PHD3 gene was absent only in neutrophils or if it was also affected in other myeloid cells such as alveolar macrophages in $PHD3^{fl/fl}LysMCre^{+/-}$ mice. And how would the authors explain all the results if the expression of the PHD3 gene was altered in other cell types as well? Figure 3A shows the upregulation of PHD3 expression in BAL neutrophils after LPS-induced lung injury in hypoxia conditions, but were there upregulation in other non-neutrophil myeloid cells as well? Additionally, assessing HIF level/activity or its downstream of PHD/HIF axis in the immune cells isolated from these transgenic animals could provide insight into the association of the results with the oxygen sensing pathway, thereby strengthening the findings.
- It is unclear why the authors mainly utilized the LPS+normoxia/hypoxia model (but no infection model at all) in studying the role of PHD1, whereas the LPS+normoxia model (without hypoxia condition) was only briefly used prior to shifting focus to primarily investigate infection models for PHD3 study.

- Several inconsistencies in experimental designs across different studies are noticed without providing adequate reasons. For example, although metabolic profiles were obtained in both studies, the Seahorse experiments were exclusively conducted in PHD3 studies, while energy status measurement (ATP/ADP level) was only conducted in the PHD1 study. Moreover, certain assays such as MPO or NETosis were not performed in the PHD1 *ex vivo* study, whereas chemotaxis was not observed in the PHD3 *ex vivo* study. Additionally, while neutrophil degranulation (e.g., MPO and elastase concentration) was evaluated mainly in *in vivo* studies, it was not assessed in *ex vivo* studies at all. The BAL albumin was used to assess vascular leakage in the lung for sterile inflammation in Fig. 3, but the BAL IgM was obtained to evaluate vascular leakage in the lung for bacterial infection in Fig. 5.

Throughout the manuscripts, the authors neglected to include control groups in most experiments to assess whether the deficiency of the gene impacted baseline parameters. For example, it remains unclear whether PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice exhibited neutrophil numbers at the baseline comparable to PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ control animals in Fig. 1, or whether the absence of PHD1 in neutrophils altered the oxygen consumption rate or any metabolic state of neutrophils at the baseline (without LPS treatment) in Fig. 2?

Figure 1 is somewhat ambiguous. The sole difference observed between PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ vs PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ was a slight increase in neutrophils recruitment in PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice after LPS-induced lung injury in hypoxia. However, this conclusion was drawn without knowledge of the basal lung neutrophil levels of these two strains. The title of Fig. 1L, specified as the "resolution" of neutrophils numbers, was confusing as it was uncertain if the value indeed reflected the resolution (PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice appear to have better resolution, yet the value displayed was lower). The issue could be raised from the finding that the levels of BAL neutrophils in PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ mice remained unchanged between 24 hours and 48 hours (Fig. 1I and 1K), with no identified basal level of lung neutrophil number. This could be attributed to the possibility that the neutrophils were not recruited to the lungs after all the challenges in these PHD1$^{fl/fl}$ MRP8Cre$^{-/-}$ mice, thus obviating the need for resolution.

Additionally, concerning Fig. 1, it is unclear why the authors opted to segregate the graphs displaying the results of normoxia (Fig. 1E-1G, Fig. 1M-1N) vs hypoxia (Fig. 1H-1J, Fig. 1O-1P) at 24 hours while merging both normoxia and hypoxia conditions for the 48-hours studies (Fig. 1K and Fig. 1Q). The concerns were raised considering the different statistical analysis used for 24 hours (Mann-Whitney test) vs 48 hours (ordinary one-way ANOVA).

Although the authors illustrated that the expression of PHD3, but not PHD1 and PHD2, was significantly upregulated in neutrophils isolated from animals maintained in hypoxia (Figure 1C, 3A), none of the experiments in this manuscript explore the role of PHD3 in hypoxic conditions. Despite investigating the effects of PHD1 deficiency in neutrophils under both normoxic and hypoxic conditions, it is unexpected that all subsequent experiments regarding PHD3 functions were conducted using PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice solely in normoxia.

Regarding the pneumococcal infection model depicted in Fig.5, clarification is needed regarding why the authors examined colony-forming units (CFU) only 14 hours after infection, a time point that may be too early to observe differences in terms of neutrophil function/recruitment (again, no baseline level of neutrophil number is provided, thus it is difficult to assess recruitment). At this early stage, it is worth considering whether the difference in CFU could potentially be attributed to the depletion of PHD3 in the other resident myeloid cells such as alveolar macrophages?

A number of aspects of the Methods section and experimental design require careful attention. Detailed protocols for several parts are notably absent.

- Most *in vitro* studies in the manuscript relied on either isolated bone marrow neutrophils or isolated BAL neutrophils. However, the Methods section lacks comprehensive information on how to isolate and purify these cells. While it was mentioned that “Bone marrow neutrophils were isolated by percoll gradient followed by fluorescence activated cell sorting to ensure sufficient purity.” in the legend of Fig. 1, specific details such as how to perform percoll gradient, whether BAL samples underwent percoll gradient enrichment, or the FACS sorting process, including the markers used to label neutrophils, were not provided in the Methods section. The authors should also mention if the purity of the neutrophils post-purification was confirmed.
- In the “LPS lung injury” section: details regarding the conditions/treatments used as control, such as nebulizing the animals with PBS, or the method of inducing normoxia compared to hypoxia, are lacking. Also, in the statement: “Mice were treated with lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* **10**”, not sure what “10” means?
- The Methods in “Phagocytosis” section lack details of how flow cytometry was employed to assess phagocytosis and obtain **%**phagocytosis (in Fig. 2K, 6B, 6C). The term "ice control" shown in the X-axis of Fig. 2K requires explanation. Including representative flow cytometry analysis figures of phagocytosis could enhance clarity. Moreover, while the method section specifies the use of “fluorescently labelled, heat-killed *E. Coli* (Invitrogen)” for the study which I believe shown in Fig. 2K, it did not mention the Staphylococcus and Streptococcus strains used in the phagocytosis assay in Fig. 6B and 6C. These should be mentioned in the Methods section as well.
- For NETosis assay, more details are needed on how Sytox Red could be used to assess NET formation and NETosis by flow cytometry. It is unclear what percentage of NETosis means. What was the control of this NETosis study? Were isolated neutrophils challenged with any bacteria/insults to stimulate the release of NETs before fixing the cells with PFA?
- Regarding the respiratory burst assay, the authors used 10 uM fMLP to induce a respiratory burst. However, considering the authors’ previous work [Fig. 2, Sadiku P, et. al. 2017 (Ref 1)], 10uM of fMLP did not activate respiratory burst signal in BAL neutrophils isolated from WT animals. An explanation for this discrepancy between the two studies is warranted. Also, the abbreviation "fMLF" in the Methods section should be changed to "fMLP" for consistency with the abbreviation used in Fig. 2.
- The specification of negative control and positive control for the chemotaxis assay shown in Fig. 2J, along with clarification on how to obtain %positive control, is necessary.
- The authors should provide details on how they analyzed qPCR results to determine relative gene expression. Were they reported as 2^-(ddCT) or just normalized to *Actb* expression? If analyzed using the 2^-(ddCT) method, it is unclear what sample was used as a reference for normalization, as none of the values in Fig. 1A were close to 1. Conversely, if the results were relative to *Actb* expression, it is difficult to believe that PHD3 expression was upregulated to a level 20-fold higher than *Actb* expression. Confirmation of these findings and the methodology used for analysis is necessary.
- Further elaboration on Seahorse experiments (Fig. 6E-6J) should be included in the Methods section. This includes information on when compounds (glucose, oligo, 2DG) were added to the cells, and at what concentrations, as well as the time point after bacterial infection shown in Fig. 6F and 6I (before or after adding other compounds). Also, did these compound treatments facilitate the explanation of any findings of the story?

- Please verify why the authors used 2-way ANOVA for metabolic profile analysis depicted in Fig. 2A-2F.

Considering the findings on metabolic activity, it would be beneficial to provide an explanation or discussion on why metabolites in the OxPPP flux were reduced in PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice specifically under normoxic conditions (Fig. 2B), while metabolites in TCA cycles were increased in these mice only under hypoxia condition (Fig. 2F).

Furthermore, in Fig. 6G, there appears to be a trend toward a higher oxygen consumption rate (OCR) in PHD3$^{fl/fl}$MRP8Cre$^{+/-}$ mice even at baseline before bacterial infection. It would be valuable for the authors to address this observation. Additionally, although there was an increase in OCR in these PHD3-depleted neutrophils, no changes were observed in any metabolite intermediates, including those in the OxPPP pathway (Fig. 6M). It would be helpful if the authors could provide an explanation for this discrepancy.

Minor concerns:

- Please ensure to define all the abbreviations the first time mentioned in the manuscript e.g., IVC, BAL, IT, CFU, MPO, 2DG, all metabolites (e.g., F6P, F16BP, GAO, PEP, etc.)
- Although the interested gene was intact in these animals, I suggest avoiding to use of the term “wildtype” for all the floxed control animals.
- Methods in “Assessing bacterial load/CFU counts” section: Suggest changing “bijou” to “Bijou container”
- Methods in “Assessment of MPO activity in Staphylococcus aureus skin abscess model” section: Please make sure to italicize *Staphylococcus aureus*
- Methods in “CFSE labelling of *Streptococus pneumoniae*” section: should be “*Streptococcus*”
- Please make sure to write the correct format for all mRNA/protein nomenclature with consistency throughout the manuscript. For example, RNA level (mouse) should be written italicized as “*Egln1*”
- Figure 1A: please make sure the title is labeled as “PHD2” (looks like PI-D2 in the pdf file), and also label the Y-axis with the gene name in Fig. 1C to be consistent with Fig. 1A, 1B
- Please change the Y-axis label in Fig.2A, 2D to be similar to Fig. 2B-2C or Fig. 2E-2F
- Figure 2I-2K needs labels for what black vs gray dots represent. Also, it should be DCF (“**I”**), chemotaxis (“**J”**), and phagocytosis (“**K”**) in the figure legend
- The authors stated that “ex-vivo BAL cells from the neutrophil specific PHD3fl/flMRP8Cre+/- mouse lines with *Staphylococcus aureus*. We used glucose**-depleted** media to represent the hypoglycaemic airway”. This is confusing since the authors used *Staphylococcus aureus* for the skin infection model, not the airway infection model.
- Figure 4A requires better-quality images
- Figure 4E needs labels for what black vs white dots represent. What was the time-point after infection of this finding? And what did the p-value mean here?
- Fig. 4F-4H: why did the authors normalize the CFU per abscess instead of per gram as shown in Fig. 4E? And it is surprising to see that CFU was increased over time from day 2 to day 7. Does this normally occur in this staphylococcal skin infection model?
- Fig. 5E: what is “SN” in the title of the figure?
- Fig. 6A: more N should be added to confirm the findings.
- Fig. 6E, 6H: please ensure species names were written in the correct format (*S. aureus*)

References
1. Sadiku P, Willson JA, Dickinson RS, Murphy F, et al.: Prolyl hydroxylase 2 inactivation enhances glycogen storage and promotes excessive neutrophilic responses.*J Clin Invest*. 2017; **127** (9): 3407-3420 PubMed Abstract | Publisher Full Text

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?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
No

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

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

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Innate immunity, Allergy, Immunology, Infectious diseases, Pulmonary diseases, MRGPR, Mast cells

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 05 Aug 2024

Emily Watts

We would like to thank the reviewer for their time and their detailed review of this manuscript. We have submitted these data to Wellcome Open Research as we wanted to share both the interesting positive findings, but also the important negative data, with the scientific community in a timely fashion. We agree that some of the findings are preliminary and that we have not fully dissected the mechanisms underlying the phenotypes which we have observed. Nevertheless, we felt that the phenotype observed in the PHD3 deficient mice was of sufficient interest to publish the data in its current form. Further studies will of course be required to confirm the mechanisms underpinning these findings. We are providing some limited new experimental data in this revised version but the majority of the comments raised have been addressed in the attached rebuttal and by revision of the text

and conclusions drawn. We are grateful to the reviewer for their recognition of the interesting nature of our findings and hope that in this revised version they will agree that the conclusions drawn are supported by the data.

In this study by Watts et al., the researchers have expanded upon their previous work wherein they investigated the expressions and roles of three isoforms of prolyl hydroxylase domain (PHD) enzymes - namely, PHD1, PHD2, and PHD3 - within neutrophils. Previously, their focus centered on PHD2, revealing its constitutive expression in neutrophils with a potential for upregulation during sterile inflammation. The deletion of PHD2 specifically within the myeloid population was found to potentially exacerbate inflammatory response to bacterial infection by augmenting glycolytic flux, thereby enhancing neutrophil functional capacity. However, myeloid-specific PHD2 deficiency did not amplify antibacterial properties. In this current study, the authors further examined the significance of the other two isoforms of PHD enzymes in neutrophils, elucidating their distinct functions. Although PHD1 deficiency in neutrophils was found to alter oxygen-dependent metabolic profiles and the recruitment of neutrophils, it did not affect neutrophil functional capacity either *ex vivo* or *in vivo*. On the other hand, the depletion of PHD3 in myeloid cells resulted in enhanced bactericidal capacity, potentially through increased oxygen consumption and mitochondrial reactive oxygen species generation.

While the study reveals intriguing findings, it is imperative to address specific comments and concerns to bolster the robustness of the results and improve clarity. The primary concern lies in the lack of a clear rationale and coherence between the studies, making it challenging to comprehend the authors' transitions from one experiment to another. For instance: -The rationale behind the switch in Cre lines from MRP8-Cre for PHD1 deletion to LysM-Cre for PHD3 deletion remains unclear, leading to difficulties in comparing the outcomes of gene deficiency, particularly considering the discrepancy in patterns of expression of these two promoter-driven deletions. While the MRP8-Cre is by far the most neutrophil-specific Cre line, the LysM-Cre targets several myeloid cell types, posing challenges in isolating the effects solely on neutrophils. Although the authors acknowledged in the Limitations that both neutrophil-specific and myeloid-specific mouse lines in the PHD3 were used, only myeloid-specific deletion data were presented in this study.
Response: We have presented both myeloid and neutrophil specific PHD3 data in this study, a number of the figures in Figure 6 are carried out in the MRP8, neutrophil specific line, as per the axis labels. We accept that there are challenges in comparing the data between these different Cre lines due to their different specificities but felt that the PHD1 data was important negative data which we wished to make available to other researchers working in the field of hypoxia. This work was carried out separately from the PHD3 work and our aim in this paper was not necessarily to directly compare PHD1 with PHD3 deficiency, rather to present the data from both genotypes for interest and completeness. We have reworded the conclusions and limitations sections in order to improve clarity and to better explain the use of the different mouse lines.

Moreover, the mice used in this study were newly generated and involved the crossing of different lines together (e.g., crossing PHD1fl/fl animals with MRP8-Cre-ires/GFP

mice). The evidence showing the successful deletion of the interested gene only in anticipated specific cell types such as neutrophils, but not other related immune cells is required. In particular, it is crucial to ascertain whether the expression of PHD3 gene was absent only in neutrophils or if it was also affected in other myeloid cells such as alveolar macrophages in PHD3$^{fl/fl}$LysMCre$^{+/-}$ mice.
Response: MRP8 driven Cre is established as a method of inducing neutrophil specific knock down[1-4]. We now provide additional data to demonstrate effective expression of the Cre recombinase using it's GFP tag in circulating neutrophils (Figure 1B and 6B in the revised manuscript).

And how would the authors explain all the results if the expression of the PHD3 gene was altered in other cell types as well? Figure 3A shows the upregulation of PHD3 expression in BAL neutrophils after LPS-induced lung injury in hypoxia conditions, but were there upregulation in other non-neutrophil myeloid cells as well?
Response: PHD3 is likely to be regulated in other cells types in hypoxia. The PHD3 data in this manuscript only relates to normoxic conditions. We have previously published on PHD3 deficiency in hypoxic neutrophils[5] and further characterisation of the impact of PHD3 loss on hypoxic conditions is of course of great interest but beyond the scope of this article. We have not looked at regulation of PHD3 by hypoxia in other immune cells but others have shown that PHD3 is upregulated in response to hypoxia in a wide range of cell lines and primary cells, including in the macrophage/monocytes compartment[6,7]

Additionally, assessing HIF level/activity or its downstream of PHD/HIF axis in the immune cells isolated from these transgenic animals could provide insight into the association of the results with the oxygen sensing pathway, thereby strengthening the findings.
Response: We agree this would be very interesting to include in future work and thank the reviewer for this suggestion.

It is unclear why the authors mainly utilized the LPS+normoxia/hypoxia model (but no infection model at all) in studying the role of PHD1, whereas the LPS+normoxia model (without hypoxia condition) was only briefly used prior to shifting focus to primarily investigate infection models for PHD3 study.
Response: We agree it will be of interest to investigate the role of neutrophil PHD1 expression in infection responses and hope to carry out this work in the future but elected to make available our current data rather than delay to include future work on infection models. We have prioritised the PHD3 work due to the interesting phenotypes we have observed and were keen to publish this in a timely fashion in order that the data are available to the wider hypoxia research community.

Several inconsistencies in experimental designs across different studies are noticed without providing adequate reasons. For example, although metabolic profiles were obtained in both studies, the Seahorse experiments were exclusively conducted in PHD3 studies, while energy status measurement (ATP/ADP level) was only conducted in the PHD1 study.
Response: We have not yet carried out seahorse assays on the PHD1 deficient neutrophil for the same reasons described above. These assays require a high number of cells to be used

and therefore high mouse numbers which we did feel able to justify given the subtle changes noted in the data presented here.

Moreover, certain assays such as MPO or NETosis were not performed in the PHD1 *ex vivo* study, whereas chemotaxis was not observed in the PHD3 *ex vivo* study. Additionally, while neutrophil degranulation (e.g., MPO and elastase concentration) was evaluated mainly in *in vivo* studies, it was not assessed in *ex vivo* studies at all. The BAL albumin was used to assess vascular leakage in the lung for sterile inflammation in Fig. 3, but the BAL IgM was obtained to evaluate vascular leakage in the lung for bacterial infection in Fig. 5. Throughout the manuscripts, the authors neglected to include control groups in most experiments to assess whether the deficiency of the gene impacted baseline parameters. For example, it remains unclear whether PHD1 $^{fl/fl}$MRP8Cre$^{+/-}$ mice exhibited neutrophil numbers at the baseline comparable to PHD1 $^{fl/fl}$MRP8Cre$^{-/-}$ control animals in Fig. 1, or whether the absence of PHD1 in neutrophils altered the oxygen consumption rate or any metabolic state of neutrophils at the baseline (without LPS treatment) in Fig. 2?
Response: We thank the reviewer for highlighting these limitations and apologise for any confusion due to the use of different assays between experiments. It is important to note that in healthy, naïve mice, there are no neutrophils found in the BAL[8-10] and therefore it is impossible to compare baseline tissue neutrophils in this system. The isolation of neutrophils from the blood of mice is possible but the numbers are small and so it is challenging to carry out e.g. LC-MS metabolic studies on these cell populations whilst operating within the 3Rs principles. Bone marrow neutrophils are more numerous but the degree to which they are representative of mature transmigrated neutrophils is at best uncertain and therefore we did not feel that detailed phenotyping of bone marrow neutrophils would provide additional relevant data. We have not carried out *ex-vivo* degranulation assays as, 24 hours following LPS induced lung injury (or indeed 14 hours post-pneumonia induction), the neutrophils will have already undergone their maximal degranulation and so we instead measure the granule proteins in the BAL supernatant as a surrogate of neutrophil degranulation.

Figure 1 is somewhat ambiguous. The sole difference observed between PHD1$^{fl/fl}$ MRP8Cre$^{+/-}$ vs PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ was a slight increase in neutrophils recruitment in PHD1$^{fl/fl}$MRP8Cre$^{+/-}$ mice after LPS-induced lung injury in hypoxia. However, this conclusion was drawn without knowledge of the basal lung neutrophil levels of these two strains. The title of Fig. 1L, specified as the "resolution" of neutrophils numbers, was confusing as it was uncertain if the value indeed reflected the resolution (PHD1$^{fl/fl}$ MRP8Cre$^{+/-}$ mice appear to have better resolution, yet the value displayed was lower). The issue could be raised from the finding that the levels of BAL neutrophils in PHD1 $^{fl/fl}$MRP8Cre$^{-/-}$ mice remained unchanged between 24 hours and 48 hours (Fig. 1I and 1K), with no identified basal level of lung neutrophil number. This could be attributed to the possibility that the neutrophils were not recruited to the lungs after all the challenges in these PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ mice, thus obviating the need for resolution.
Response: As noted above, at baseline there are no BAL neutrophils and so we don't think that baseline numbers are an explanation for the differences seen. In figure 1N in the revised manuscript we have tried to demonstrate that the rate at which neutrophils are lost from the airways (which we term resolution of neutrophil numbers) is higher and therefore

the ratio of the 48 hour counts to the 24 hour counts is lower, the value which is shown in the graph. We apologise that this was not clear. In response to another reviewer's comments, we have now also included the resolution data for the normoxic animals as well and hope this will improve clarity (Figure 1O in the revised manuscript). The mean BAL neutrophil numbers for the PHD1$^{fl/fl}$MRP8Cre$^{-/-}$ in hypoxia are 2.63 million at 24 hours and 3.21 million at 48 hours. In contrast, in the PHD1$^{fl/fl}$MRP8Cre$^{-/+}$ animals the mean values were 3.37 million at 24 hours and 2.66 million at 48 hours. Hypoxia is a well-known survival stimulus for neutrophils and the persistence of neutrophils in the airways of hypoxic mice is consistent with this and with our previously published data.

Additionally, concerning Fig. 1, it is unclear why the authors opted to segregate the graphs displaying the results of normoxia (Fig. 1E-1G, Fig. 1M-1N) vs hypoxia (Fig. 1H-1J, Fig. 1O-1P) at 24 hours while merging both normoxia and hypoxia conditions for the 48-hours studies (Fig. 1K and Fig. 1Q). The concerns were raised considering the different statistical analysis used for 24 hours (Mann-Whitney test) vs 48 hours (ordinary one-way ANOVA).
Response: We apologise for this confusion regarding the presentation of the data. When the 48 hour data is analysed by Mann-Whitney (as per the 24 hour data), it remains non-significant. Given that no significant differences were observed, we have compressed the 48 hour data into single graphs to make the data more reader friendly.

Although the authors illustrated that the expression of PHD3, but not PHD1 and PHD2, was significantly upregulated in neutrophils isolated from animals maintained in hypoxia (Figure 1C, 3A), none of the experiments in this manuscript explore the role of PHD3 in hypoxic conditions. Despite investigating the effects of PHD1 deficiency in neutrophils under both normoxic and hypoxic conditions, it is unexpected that all subsequent experiments regarding PHD3 functions were conducted using PHD3$^{fl/fl}$ LysMCre$^{+/-}$ mice solely in normoxia.
Response: We have previously published on PHD3 deficiency in hypoxic neutrophils[5] and further characterisation of the impact of PHD3 loss on hypoxic conditions is of course of great interest but beyond the scope of this article. Given the existing literature regarding PHD1 deficiency which consistently demonstrates a protective effect in hypoxic or ischaemic injury, we felt it was important to assess the role of PHD1 in both normoxic and hypoxic neutrophils and to report our overall negative findings in this cell type.

Regarding the pneumococcal infection model depicted in Fig.5, clarification is needed regarding why the authors examined colony-forming units (CFU) only 14 hours after infection, a time point that may be too early to observe differences in terms of neutrophil function/recruitment (again, no baseline level of neutrophil number is provided, thus it is difficult to assess recruitment). At this early stage, it is worth considering whether the difference in CFU could potentially be attributed to the depletion of PHD3 in the other resident myeloid cells such as alveolar macrophages?
Response: Extensive and detailed characterisation has been performed on pneumococcal challenge models and we have based our experimental protocol on the standard recognised in this field[11,12]. There is significant mortality by day 2 following inoculation in the high dose pneumococcal model (>50%[12]) which is the reason for culling the mice at the 14 hour time point. There is already significant neutrophil recruitment to the airways in this

model by 14 hours (as shown in Figure 5D). It is of course important to consider the impact of other myeloid cells, including alveolar macrophages, in this model. Although the literature would suggest that their role is in the resolution of pneumonia rather than the acute phase with depletion of the alveolar macrophage pool resulting in increased mortality 72 hours after infection but no difference in lung or blood CFU counts at 20 hours post-infection[13]. In future work it will of course be important to repeat the *in vivo* infection models in the neutrophil specific lines, in order to confirm that PHD3 loss specifically in the recruited neutrophils is responsible for the observed phenotypes. We have now addressed this in the discussion and limitations section.

A number of aspects of the Methods section and experimental design require careful attention. Detailed protocols for several parts are notably absent.

- **Most *in vitro* studies in the manuscript relied on either isolated bone marrow neutrophils or isolated BAL neutrophils. However, the Methods section lacks comprehensive information on how to isolate and purify these cells. While it was mentioned that "Bone marrow neutrophils were isolated by percoll gradient followed by fluorescence activated cell sorting to ensure sufficient purity." in the legend of Fig. 1, specific details such as how to perform percoll gradient, whether BAL samples underwent percoll gradient enrichment, or the FACS sorting process, including the markers used to label neutrophils, were not provided in the Methods section. The authors should also mention if the purity of the neutrophils post-purification was confirmed.**

Response: We apologise that these details were not in the original manuscript and have updated the methods section to provide more comprehensive information about neutrophil isolation protocols. We have included it below for convenience: Bone marrow: Both hind limbs were dissected out and stored in a clean bijou container on ice until processed. Each bone was flushed using 10ml of 1xHBSS with 0.2% BSA. The cell suspension was resuspended, passed through a 70mm filter into a fresh 50ml polystyrene falcon tube and then centrifuged at 450G for 10 minutes. 90% vol/vol percoll stock was made using 10X HBSS with $NaHCO_3$ and this stock was used to make 81%, 62% and 55% percoll with 1x HBSS. All three percoll preparations were layered in a 15ml falcon tube and the cells were resuspended in 3ml of 1xHBSS with 0.2% BSA which was layered on top of the 52% percoll layer. The gradient was centrifuged at 2000G for 30 minutes (acceleration 1, deceleration 0). Bone marrow monocyte precursors were removed from the top layer with a pastette. Neutrophils were removed from the middle layer and washed in 30ml of 1xHBSS with 0.2%BSA. Hypotonic saline red blood cell lysis was carried out. Following the percoll purification above, neutrophil purity was found to be approximately 90%. In order to generate highly pure bone marrow neutrophils samples, cells from the neutrophil layer were resuspended in 1xHBSS with 0.2% BSA and purified by fluorescence-activated cell sorting (FACS) based on the forward/side scatter profile and auto-fluorescence as previously described[14] Bronchoalveolar lavage: Where highly pure BAL neutrophils were required, we used a previously optimized discontinuous percoll gradient coupled with a red cell lysis step in order to gain purities of >98%. BAL cells were first pelleted by centrifugation at 350G for 10 minutes. BAL samples from 1-3 mice (depending on the final cell number required) were pooled onto a single percoll gradient. 90% vol/vol percoll was made using 10X PBS and further percoll solutions were made using this stock 90% percoll mixed with 1X PBS. 3ml of 78% percoll was pipetted into the bottom of a 15ml falcon tube, followed by 3ml of 69%

percoll. The cell pellet(s) were resuspended in 3ml of 52% percoll which was then layered onto the 69% layer. The gradient(s) were centrifuged at 1200G for 30mins with an acceleration of 1 and deceleration of 0. Following centrifugation, the macrophages accumulated on the top of the 52% layer and were removed using a sterile pastette. The neutrophils (within the 69% layer) were then removed using a fresh sterile pastette and placed into a 50ml polystyrene falcon tube. This was topped up to 50ml with sterile PBS to wash off the percoll and the purified neutrophils pelleted at 350G for 8 mins. Residual red cells were lysed by hypotonic saline lysis. A cytospin was made prior to the final centrifugation step to assess purity by morphology. During optimisation of this protocol, purity was also confirmed by flow cytometry and the neutrophils were found to be >98% pure.

- **In the "LPS lung injury" section: details regarding the conditions/treatments used as control, such as nebulizing the animals with PBS, or the method of inducing normoxia compared to hypoxia, are lacking. Also, in the statement: "Mice were treated with lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* 10", not sure what "10" means?**

Response: We did not utilise a PBS nebulisation control as this does not result in neutrophil recruitment to the airways and so does not provide useful control data. The normoxic mice were simply housed in standard conditions, breathing room air via a standard individually ventilated cage. In contrast, as per the methods, the hypoxic mice were housed in a hypoxic cabinet with an inspired oxygen concentration of 10%. The LPS is sourced from *Pseudomonas aeruginosa* (10) (https://www.sigmaaldrich.com/GB/en/product/sigma/l9143). We apologise that the omission of the brackets may have made this unclear and have updated the manuscript for clarity.

- **The Methods in "Phagocytosis" section lack details of how flow cytometry was employed to assess phagocytosis and obtain %phagocytosis (in Fig. 2K, 6B, 6C). The term "ice control" shown in the X-axis of Fig. 2K requires explanation. Including representative flow cytometry analysis figures of phagocytosis could enhance clarity. Moreover, while the method section specifies the use of "fluorescently labelled, heat-killed *E. Coli* (Invitrogen)" for the study which I believe shown in Fig. 2K, it did not mention the Staphylococcus and Streptococcus strains used in the phagocytosis assay in Fig. 6B and 6C. These should be mentioned in the Methods section as well.**

Response: We apologise for the lack of clarity here. The ice control is a standard control in this assay to distinguish non-specific binding of the fluorescent bacteria to the outside of the cell from true uptake by phagocytosis. In this data it does not provide useful experimental data and so, to avoid confusion and for consistency with other phagocytosis data in the manuscript we have replotted the data without the ice controls. The species used for the PHD3 phagocytosis assay were D39 *S. pneumonia* and Wood strain without protein A *S. aureus*. We apologise that these were not included in the methods and have now added them to the relevant section.

- **For NETosis assay, more details are needed on how Sytox Red could be used to assess NET formation and NETosis by flow cytometry. It is unclear what percentage of NETosis means. What was the control of this NETosis study? Were isolated neutrophils challenged with any bacteria/insults to stimulate the release of NETs before fixing the cells with PFA?**

Response: SyTox red is a cell impermeable dye which binds to nucleic acids. We agree that it

is not specific for NETosis but is an accepted surrogate marker for this. Percentage NETosis is the percentage of cells which were positive for red fluorescence. It is true that this will also include some cells which have died by necrosis. In order to fully assess NETosis it would be important to use an additional assay such as microscopy or an ELISA to confirm the presence of both DNA and e.g. MPO. Given the lack of difference between genotypes on this initial assay we did not pursue this avenue further. We felt that it was important to include this negative data, accepting the limitations of the assay used. When optimising this assay we trialled both BAL neutrophils with an additional stimulus (PMA) and freshly isolated BAL cells. We found that the addition of PMA did not result in a clearer NETosis signal but did result in really significant cell loss in the assay and so the data presented here are for freshly isolated BAL neutrophils without any further stimulus.

- **Regarding the respiratory burst assay, the authors used 10 uM fMLP to induce a respiratory burst. However, considering the authors' previous work [Fig. 2, Sadiku P, et. al. 2017 (Ref 1)], 10uM of fMLP did not activate respiratory burst signal in BAL neutrophils isolated from WT animals. An explanation for this discrepancy between the two studies is warranted. Also, the abbreviation "fMLF" in the Methods section should be changed to "fMLP" for consistency with the abbreviation used in Fig. 2.**

Response: In order to allow the relevant comparison to be made in the referenced paper investigating PHD2, the BAL neutrophils in this context were aged for 6 hours in media prior to being stimulated with fMLF. Whereas in the current study, freshly isolated neutrophils were used. We suggest that this difference in experimental protocol is the cause of the discrepancy in response to fMLF. We apologise for the different abbreviations used and have changed them all to fMLF.

- **The specification of negative control and positive control for the chemotaxis assay shown in Fig. 2J, along with clarification on how to obtain %positive control, is necessary.**

Response: The negative control is media with no chemoattractant added. The positive control has cell pipetted into the well at the start (instead of onto the membrane). The percentage of positive control is calculated as (cell count in well for experimental condition/cell count in positive control well)*100. Additional details regarding the controls have been added to the methods section.

- **The authors should provide details on how they analyzed qPCR results to determine relative gene expression. Were they reported as 2^-(ddCT) or just normalized to *Actb* expression? If analyzed using the 2^-(ddCT) method, it is unclear what sample was used as a reference for normalization, as none of the values in Fig. 1A were close to 1. Conversely, if the results were relative to *Actb* expression, it is difficult to believe that PHD3 expression was upregulated to a level 20-fold higher than *Actb* expression. Confirmation of these findings and the methodology used for analysis is necessary.**

Response: The raw data for all qPCR experiments is available on the Figshare associated with this paper. The raw CT values were normalised to b-actin expression. The CT value will of course depend not only on the abundance of the gene of interest but also on the efficiency of the primers and other technical factors, thus we would not try to draw conclusions on the relative abundance of the *Egln* transcripts in comparison to *ActnB* and are using this data only to compare *Egln* transcript expression between treatment groups.

- **Further elaboration on Seahorse experiments (Fig. 6E-6J) should be included in**

the Methods section. This includes information on when compounds (glucose, oligo, 2DG) were added to the cells, and at what concentrations, as well as the time point after bacterial infection shown in Fig. 6F and 6I (before or after adding other compounds). Also, did these compound treatments facilitate the explanation of any findings of the story?

Response: We carried out a modified glycolysis stress test on these cells which involved the addition of bacteria prior to running the standard stress test (Agilent Technologies Kit 103020-100) using the compounds oligomycin and 2-deoxy-glucose at the time points indicated on figures 6E&H. The compound treatments were not relevant to the other findings – they were added to allow calculation of additional factors in the ECAR test but were not significantly different between genotypes. They are not relevant to the OCR measurements but for clarity and transparency have been noted on the figure.

- **Please verify why the authors used 2-way ANOVA for metabolic profile analysis depicted in Fig. 2A-2F.**

Response: A two way ANOVA allowed us to ascertain whether there was an overall effect of genotype on metabolite abundance and is the test of choice for this type of grouped data with two variables (genotype and metabolite). In response to other reviewer comments, we have additionally carried out post-hoc multiple comparison tests within the ANOVA and found no metabolite reaches significance on its own in these experiments.

Considering the findings on metabolic activity, it would be beneficial to provide an explanation or discussion on why metabolites in the OxPPP flux were reduced in PHD1 $^{fl/fl}$MRP8Cre$^{+/-}$ mice specifically under normoxic conditions (Fig. 2B), while metabolites in TCA cycles were increased in these mice only under hypoxia condition (Fig. 2F).

Response: Thank you for this suggestion. We have now expanded the discussion section to try to address these findings in more detail.

Furthermore, in Fig. 6G, there appears to be a trend toward a higher oxygen consumption rate (OCR) in PHD3$^{fl/fl}$MRP8Cre$^{+/-}$ mice even at baseline before bacterial infection. It would be valuable for the authors to address this observation. Additionally, although there was an increase in OCR in these PHD3-depleted neutrophils, no changes were observed in any metabolite intermediates, including those in the OxPPP pathway (Fig. 6M). It would be helpful if the authors could provide an explanation for this discrepancy.

Response: There is a trend towards higher OCR at baseline in the PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice, although this does not reach statistical significance (as shown in Figure 6I). Both genotypes demonstrate an uplift in OCR in response to bacteria. The fold change in OCR is not significantly different between genotypes when calculated as (OCR immediately post-bacteria injection)/(mean OCR pre-bacteria injection) – 1.6 for WT Vs 1.55 for KO. However, when absolute increase in OCR is calculated, i.e. (OCR immediately post-bacteria injection) - (mean OCR pre-bacteria injection), this is significantly different – 267 for WT V 375 for KO, p=0.04, analysed by unpaired t-test. We would suggest that whilst both increase their OCR, the higher level (Figure 6H) and higher absolute increase in the PHD3fl/fl-MRP8Cre+/- (as described above) are indicative of overall increased oxygen consumption in the PHD3 deficient neutrophils which becomes significant with the addition of a bacterial stimulus. We do not see a significant difference in overall ROS production which is in keeping with the unchanged OxPPP metabolites seen here. The production of

mROS is not dependent on the oxidative pentose phosphate pathway (as shown in papers which block OxPPP and demonstrate enhanced mROS production[15] and so this does not represent a discrepancy.

Minor concerns:

- **Please ensure to define all the abbreviations the first time mentioned in the manuscript e.g., IVC, BAL, IT, CFU, MPO, 2DG, all metabolites (e.g., F6P, F16BP, GAO, PEP, etc.)**

Response: Thank you, these have now been added

- **Although the interested gene was intact in these animals, I suggest avoiding to use of the term "wildtype" for all the floxed control animals.**

Response: Thank you, we have removed the term and used the term cre negative and the complete genotype in each case for clarity

- **Methods in "Assessing bacterial load/CFU counts" section: Suggest changing "bijou" to "Bijou container"**

Response: This has been changed.

- **Methods in "Assessment of MPO activity in Staphylococcus aureus skin abscess model" section: Please make sure to italicize *Staphylococcus aureus*** - done
- **Methods in "CFSE labelling of *Streptococus pneumoniae*" section: should be " *Streptococcus*"**-done
- **Please make sure to write the correct format for all mRNA/protein nomenclature with consistency throughout the manuscript. For example, RNA level (mouse) should be written italicized as "*Egln1*"**-done
- **Figure 1A: please make sure the title is labeled as "PHD2" (looks like PI-D2 in the pdf file), and also label the Y-axis with the gene name in Fig. 1C to be consistent with Fig. 1A, 1B** - done
- **Please change the Y-axis label in Fig.2A, 2D to be similar to Fig. 2B-2C or Fig. 2E-2F**-done
- **Figure 2I-2K needs labels for what black vs gray dots represent. Also, it should be DCF ("I"), chemotaxis ("J"), and phagocytosis ("K") in the figure legend**-done
- **The authors stated that "ex-vivo BAL cells from the neutrophil specific PHD3fl/flMRP8Cre+/- mouse lines with *Staphylococcus aureus*. We used glucose-depleted media to represent the hypoglycaemic airway". This is confusing since the authors used *Staphylococcus aureus* for the skin infection model, not the airway infection model.**

Response: The cells used in this assay were *ex-vivo* BAL cells, thus from the airways which are known to be hypoglycaemic. For this reason we assessed phagocytosis in glucose deplete media to recapitulate the environment where the cells originated, rather than to recapitulate the staphylococcal skin model.

- **Figure 4A requires better-quality images –** done
- **Figure 4E needs labels for what black vs white dots represent. What was the time-point after infection of this finding? And what did the p-value mean here?**

Response: The labels have now been added. This data set includes all three time points. The p-value reflects that the slope of the line is "significantly non-zero".

- **Fig. 4F-4H: why did the authors normalize the CFU per abscess instead of per gram as shown in Fig. 4E? And it is surprising to see that CFU was increased over**

time from day 2 to day 7. Does this normally occur in this staphylococcal skin infection model?

Response: The total CFU count was used in order to fully reflect the extent of (or lack thereof) bacterial control in each genotype. Given that the size of the abscess increases over time (as per Figure 4D) it is not surprising that the CFU count per abscess increases over the same time period. These data are in keeping with that of others showing a peak in CFU counts around day 4-6 and enlarging abscesses up to day 5-7[16]

- **Fig. 5E: what is "SN" in the title of the figure?**

Response: Thank you for highlighting this, it has been changed to supernatant.

- **Fig. 6A: more N should be added to confirm the findings.**

Response: We accept that this is a small N number. However, each N is generated by pooling the BAL cells from 3-4 mice (per genotype) in order to generate enough cells to run in duplicate and at multiple dilutions for CFU counts. Given that we are able to see a small but significant difference with the data we have generated we didn't feel it was justifiable within the 3Rs framework to repeat this assay simply to increase numbers.

- **Fig. 6E, 6H: please ensure species names were written in the correct format (*S. aureus*)**

Response: Done.

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Competing Interests: No competing interests were disclosed.

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The manuscript from Watts et al. reports the differential roles of the oxygen-sensing enzymes PHD1 and PHD3 in regulating neutrophil metabolism and function using a combination of in vivo and ex vivo models. On the one hand, the authors report that PHD1 deficiency drives alterations in neutrophil metabolism and recruitment in an oxygen-dependent fashion. Despite this, PHD1 deficiency did not significantly alter *ex vivo* neutrophil phenotypes or *in vivo* outcomes in mouse models of inflammation. On the other hand, PHD3 deficiency was found to enhance neutrophil antibacterial properties without excessive inflammatory responses. This effect was not linked to changes in the abundance of core metabolites but was associated with increased oxygen consumption and increased mitochondrial reactive oxygen species production. The authors conclude that PHD3 deficiency drives a favourable neutrophil phenotype in infection and, as such, is an important potential therapeutic target.

The work is clearly and accurately presented and cites the current literature. Concerns were raised regarding the study design, the methodological details, the statistical analyses, and the conclusions drawn, as detailed below, and they should be addressed to make the article scientifically sound.

One of the biggest criticisms is using the two Cre-Lox strategies (LysM- and MRP8-driven Cre-Lox recombination) in the study. Although the authors recognize this as a limitation and justify the use of these two expression models with the fact that they previously utilized both and did not identify a Cre-specific phenotype and therefore determined that using these additional controls was not justified when working within the 3Rs framework, it makes it complex, or almost virtually impossible, to compare the results obtained for PHD1 and PHD3 if the mouse strains, the Cre expression and promoter/enhancer elements of each technology are different. In Fig. 6, mice with both Cre-Lox strategies were used for different experiments and a global conclusion was drawn. In fact, the authors would need to do a set of identical experiments, such as bacterial killing (Fig. 6A) and mROS (Fig. 6O), where significant differences were observed between the PHD3fl/fl-MRP8Cre-/- and PHD3fl/fl-MRP8Cre+/- mice, with both the MRP8 Cre- and LysM Cre-mediated recombinations to prove that the results are the same regardless of the Cre-Lox technology used. In its current format, it is hard to state that the conclusions drawn adequately are supported by the results since the PHD1 and PHD3 experiments were not performed with the same myeloid cell-specific targeted mutants.

Some methodological details are lacking. The authors state that highly pure neutrophil preparations were used for gene expression and HPLC-MS analysis of metabolites (Seahorse extracellular flux assays also require highly purified preparations, as it is impossible to distinguish the metabolic parameters of different cell populations). The methodology used to obtain these highly pure neutrophil preparations from BALs is not detailed other than using a discontinuous Percoll gradient (mentioned in the HPLC-MS analysis of metabolites section). The same can be said with bone marrow neutrophils used for gene expression (such as Fig. 1A-B), where fluorescence-activated cell sorting was used to ensure sufficient purity. What markers were used, and what purity level qualifies as “sufficient”? The authors must provide the method and techniques to quantify this high purity and the percentage of purity obtained with their preparations. In addition, the authors should provide details regarding the filter used for the chemotaxis assays (i.e., the size of the pores) as well as how were ATP and ADP levels determined (Fig. 2G-H; data may have been generated by HPLC-MS, but it is not clear and should be detailed in the Mat & Met) or the genotypes of the mice used for Fig. 3A-B.

Most of the statistical analyses seem adequate. However, the 2-way ANOVA performed in Fig. 2A-F and Fig. 6K-M does not provide detailed information on differences among the various study groups. To fully understand group differences in an ANOVA, the authors should conduct post hoc tests (called multiple comparison analysis) of the differences between particular pairs of experimental and control groups. Moreover, the conclusion of a “non-significant positive association” (page 10) for the result presented in Fig. 5B is inadequate. If no statistical difference is found between the groups then the authors should not conclude that there is a positive association.

The sole use of beta-actin as a reference gene is questionable, as one assumes that the expression of this gene is constitutively high and that a given treatment will have little effect on the expression level. The MIQE guidelines (Bustin SA. et al., 2009 [Ref 1]) report that “Normalization against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described. The optimal number and choice of reference genes must be experimentally determined, and the method reported”. Indeed, it is often difficult to identify even a single gene that meets this criterion (Glare EM. et al., 2002 [ref 2]; Schmittgen TD et. al., 2000 [Ref 3]; Thellin O.

et al., 1999 [Ref 4]). The authors should consider the more accurate strategy for normalization that was proposed by Vandesompele and collaborators (Vandesompele J. et al., 2002 [Ref 5]), and detailed in the MIQE guidelines, to carefully select a set of genes that display minimal variation across the treatment, determine the geometric mean of these genes, and normalize the gene(s) of interest to the geometric mean.

The strategy used for the sequential injections of drugs in the Seahorse assays should be better explained. What is the rationale for injecting the bacteria, then glucose, oligomycin and 2DG? Why not also report the OCR and ECAR following the injection of glucose? What can be concluded following the injection of oligomycin? Also, the OCR after the bacteria injection is indeed increased in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice (Fig. 6F). However, the OCR before the bacteria injection is also increased in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice (Fig. 6E). To account for this basal difference between the two groups, the authors need to baseline the data before the injection of the bacteria and then report the increase after the injection.

The authors report that the OCR is increased after bacteria injection in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice (Fig. 6F). After confirming that this uplift in OCR was observed in the context of conserved ECAR (Fig. 6H-J) and equivalent abundance of TCA cycle, glycolysis and OxPPP metabolic intermediaries (Fig. 6K-M; done with the LysM-Cre mice), the authors considered whether this increase in OCR reflected increased ROS production. However, total ROS (Fig. 6N) and mROS production (Fig. 6O), the latter one being increased in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/-, mice were examined in unstimulated cells. How can the authors explain that a similar increase was not observed in baseline OCR (Fig. 6G) in their Seahorse assays? In their discussion, the authors state: “Using Seahorse technology, we identified enhanced oxygen consumption in PHD3 deficient neutrophils upon challenge with bacteria which we have demonstrated is associated with increased mROS production in inflammatory BAL neutrophils.” This is actually an overstatement, as this association was never demonstrated. For this, the whole set of experiments should have been performed with a single genotype (MRP8-Cre mice) and the total and mROS production examined following the activation of cells in the presence of bacteria, as done in the Seahorse assays.

The authors are most likely aware of the multiple differences between mouse and human neutrophils, and this limitation should be addressed in the discussion, especially when it is concluded that PDH3 represents a potential therapeutic target. Experiments are most likely required with human cells to prove that PDH3 represents, indeed, a potential therapeutic target.

Corrections are needed regarding the figures and the text:
1) The manuscript should include only high-resolution figures. For example, the quality of Fig. 1N, 1O and 1P are currently inadequate, as the axis labels are fuzzy.
2) The figures should clearly mention the experimental group. For example, Fig. 1E and 1H have the same title and axis labels. It is therefore critical to read the figure legend to understand that Fig. 1E refers to normoxia while Fig. 1H refers to hypoxia. The same can be said for Fig. 1F-1I, Fig. 1G-1J, Fig. 1M-1O and Fig. 1N-1P. The authors should combine these results in a single graph, such as in Fig. 1C, 1D or 1Q, or clearly state the experimental group in the figure title.
3) PMN is used in the axis title of some figures but has not been defined in the text or the figure legend.
4) The Greek letter Mu should be used instead of the letter “u” for micro.
5) b-actin should be replaced by beta-actin (using the Greek letter) in the legend of Fig. 1.

6) FMLP should be replaced by fMLF in Fig. 2I, as this is the abbreviation used in the text.
7) In the legend of Fig. 5, the text “BAL supernatant from PHD3fl/flLysMCre+/- and PHD3fl/flLysMCre-/- mice subject to IT…” should be corrected to “BAL supernatant from PHD3fl/flLysMCre+/- and PHD3fl/flLysMCre-/- mice subject**ed** to IT…”.
8) IT should be defined in the text.
9) In Fig. 6E-G, the axis label states OCR (mpH/min). In Seahorse assays, OCR is actually provided as nmoles/min. The axis label needs to be corrected.

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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?
Partly

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

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

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

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neutrophils, immunometabolism, inflammation

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 05 Aug 2024

Emily Watts

We would like to thank the reviewer for their time and their detailed review of this manuscript. We have submitted these data to Wellcome Open Research as we wanted to share both the interesting positive findings, but also the important negative data, with the scientific community in a timely fashion. We agree that some of the findings are preliminary and that we have not fully dissected the mechanisms underlying the phenotypes which we have observed. Nevertheless, we felt that the phenotype observed in the PHD3 deficient mice was of sufficient interest to publish the data in its current form. Further studies will of course be required to confirm the mechanisms underpinning these findings. We are providing some limited new experimental data in this revised version but the majority of the comments raised have been addressed in the attached rebuttal and by revision of the text and conclusions drawn. We are grateful to the reviewer for their recognition of clarity and accuracy of our work and hope that in this revised version they will agree that the conclusions drawn are supported by the data.

The manuscript from Watts et al. reports the differential roles of the oxygen-sensing enzymes PHD1 and PHD3 in regulating neutrophil metabolism and function using a combination of in vivo and ex vivo models. On the one hand, the authors report that PHD1 deficiency drives alterations in neutrophil metabolism and recruitment in an oxygen-dependent fashion. Despite this, PHD1 deficiency did not significantly alter *ex vivo* neutrophil phenotypes or *in vivo* outcomes in mouse models of inflammation. On the other hand, PHD3 deficiency was found to enhance neutrophil antibacterial properties without excessive inflammatory responses. This effect was not linked to changes in the abundance of core metabolites but was associated with increased oxygen consumption and increased mitochondrial reactive oxygen species production. The authors conclude that PHD3 deficiency drives a favourable neutrophil phenotype in infection and, as such, is an important potential therapeutic target.

The work is clearly and accurately presented and cites the current literature. Concerns were raised regarding the study design, the methodological details, the statistical analyses, and the conclusions drawn, as detailed below, and they should be addressed to make the article scientifically sound.

One of the biggest criticisms is using the two Cre-Lox strategies (LysM- and MRP8-driven Cre-Lox recombination) in the study. Although the authors recognize this as a limitation and justify the use of these two expression models with the fact that they previously utilized both and did not identify a Cre-specific phenotype and therefore determined that using these additional controls was not justified when working within the 3Rs framework, it makes it complex, or almost virtually impossible, to compare the results obtained for PHD1 and PHD3 if the mouse strains, the Cre expression and promoter/enhancer elements of each technology are different. In Fig. 6, mice with both Cre-Lox strategies were used for different experiments and a global

conclusion was drawn. In fact, the authors would need to do a set of identical experiments, such as bacterial killing (Fig. 6A) and mROS (Fig. 6O), where significant differences were observed between the PHD3fl/fl-MRP8Cre-/- and PHD3fl/fl-MRP8Cre+/- mice, with both the MRP8 Cre- and LysM Cre-mediated recombinations to prove that the results are the same regardless of the Cre-Lox technology used. In its current format, it is hard to state that the conclusions drawn adequately are supported by the results since the PHD1 and PHD3 experiments were not performed with the same myeloid cell-specific targeted mutants.
Response: We agree that there are challenges in comparing the data between these different Cre lines due to their different specificities but felt that the PHD1 data was important negative data which we wished to make available to other researchers working in the field of hypoxia. This work was carried out separately from the PHD3 work and our aim in this paper was not necessarily to directly compare PHD1 with PHD3 deficiency, rather to present the data from both genotypes for interest and completeness. The data from the LysMCre line in figure 6 demonstrate no difference in phagocytosis between genotypes. We have reworded the conclusions and limitations sections in order to improve clarity and to better explain the use of the different mouse lines.

Some methodological details are lacking. The authors state that highly pure neutrophil preparations were used for gene expression and HPLC-MS analysis of metabolites (Seahorse extracellular flux assays also require highly purified preparations, as it is impossible to distinguish the metabolic parameters of different cell populations). The methodology used to obtain these highly pure neutrophil preparations from BALs is not detailed other than using a discontinuous Percoll gradient (mentioned in the HPLC-MS analysis of metabolites section). The same can be said with bone marrow neutrophils used for gene expression (such as Fig. 1A-B), where fluorescence-activated cell sorting was used to ensure sufficient purity. What markers were used, and what purity level qualifies as "sufficient"? The authors must provide the method and techniques to quantify this high purity and the percentage of purity obtained with their preparations.
Response: We apologise for this omission. These details have now been included in the manuscript and, for convenience are also detailed here: Bone marrow: Both hind limbs were dissected out and stored in a clean bijou container on ice until processed. Each bone was flushed using 10ml of 1xHBSS with 0.2% BSA. The cell suspension was resuspended, passed through a 70mm filter into a fresh 50ml polystyrene falcon tube and then centrifuged at 450G for 10 minutes. 90% vol/vol percoll stock was made using 10X HBSS with $NaHCO_3$ and this stock was used to make 81%, 62% and 55% percoll with 1x HBSS. All three percoll preparations were layered in a 15ml falcon tube and the cells were resuspended in 3ml of 1xHBSS with 0.2% BSA which was layered on top of the 52% percoll layer. The gradient was centrifuged at 2000G for 30 minutes (acceleration 1, deceleration 0). Bone marrow monocyte precursors were removed from the top layer with a pastette. Neutrophils were removed from the middle layer and washed in 30ml of 1xHBSS with 0.2% BSA. Hypotonic saline red blood cell lysis was carried out. Following the percoll purification above, neutrophil purity was found to be approximately 90%. In order to generate highly pure bone marrow neutrophils samples, cells from the neutrophil layer were resuspended in 1xHBSS with 0.2% BSA and purified by fluorescence-activated cell sorting (FACS) based on the forward/side scatter profile and auto-fluorescence as previously described[1].

Bronchoalveolar lavage: Where highly pure BAL neutrophils were required, we used a previously optimized discontinuous percoll gradient coupled with a red cell lysis step in order to gain purities of >98%. BAL cells were first pelleted by centrifugation at 350G for 10 minutes. BAL samples from 1-3 mice (depending on the final cell number required) were pooled onto a single percoll gradient. 90% vol/vol percoll was made using 10X PBS and further percoll solutions were made using this stock 90% percoll mixed with 1X PBS. 3ml of 78% percoll was pipetted into the bottom of a 15ml falcon tube, followed by 3ml of 69% percoll. The cell pellet(s) were resuspended in 3ml of 52% percoll which was then layered onto the 69% layer. The gradient(s) were centrifuged at 1200G for 30mins with an acceleration of 1 and deceleration of 0. Following centrifugation, the macrophages accumulated on the top of the 52% layer and were removed using a sterile pastette. The neutrophils (within the 69% layer) were then removed using a fresh sterile pastette and placed into a 50ml polystyrene falcon tube. This was topped up to 50ml with sterile PBS to wash off the percoll and the purified neutrophils pelleted at 350G for 8 mins. Residual red cells were lysed by hypotonic saline lysis. A cytospin was made prior to the final centrifugation step to assess purity by morphology. During optimisation of this protocol, purity was also confirmed by flow cytometry and the neutrophils were found to be >98% pure.

In addition, the authors should provide details regarding the filter used for the chemotaxis assays (i.e., the size of the pores) as well as how were ATP and ADP levels determined (Fig. 2G-H; data may have been generated by HPLC-MS, but it is not clear and should be detailed in the Mat & Met) or the genotypes of the mice used for Fig. 3A-B.
Response: We apologise for these omissions in methodological details. The pore size was 5mm and this detail has been added. The ATP and ADP levels were indeed measured as part of the HPLC-MS experiments and this has now been clarified.

Most of the statistical analyses seem adequate. However, the 2-way ANOVA performed in Fig. 2A-F and Fig. 6K-M does not provide detailed information on differences among the various study groups. To fully understand group differences in an ANOVA, the authors should conduct post hoc tests (called multiple comparison analysis) of the differences between particular pairs of experimental and control groups.
Response: We have performed these post-hoc, multiple comparison analyses and found that, despite there being a significant difference overall between the genotypes in the metabolic pathways detailed, no individual metabolite is significantly different between genotypes. The manuscript has been updated to include this additional information.

Moreover, the conclusion of a "non-significant positive association" (page 10) for the result presented in Fig. 5B is inadequate. If no statistical difference is found between the groups then the authors should not conclude that there is a positive association.
Response: Thank you for this comment, we apologise for this and this conclusion has been removed.

The sole use of beta-actin as a reference gene is questionable, as one assumes that the expression of this gene is constitutively high and that a given treatment will have

little effect on the expression level. The MIQE guidelines (Bustin SA. et al., 2009 [Ref 1]) report that "Normalization against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described. The optimal number and choice of reference genes must be experimentally determined, and the method reported". Indeed, it is often difficult to identify even a single gene that meets this criterion (Glare EM. et al., 2002 [ref 2]; Schmittgen TD et. al., 2000 [Ref 3]; Thellin O. et al., 1999 [Ref 4]). The authors should consider the more accurate strategy for normalization that was proposed by Vandesompele and collaborators (Vandesompele J. et al., 2002 [Ref 5]), and detailed in the MIQE guidelines, to carefully select a set of genes that display minimal variation across the treatment, determine the geometric mean of these genes, and normalize the gene(s) of interest to the geometric mean.
Response: Thank you for this helpful information regarding the qPCR data which we will certainly take into consideration in future work. In order to increase confidence that the data we present here is valid we have reviewed our own and others' proteomic datasets (immpres.co.uk) and found no significant variation in b-actin expression in different neutrophil populations in these data sets. Additionally, Zhang and colleagues[2] and Ledderose and colleagues[3] have also investigated this important question in neutrophil populations and concluded that b-actin is a suitable reference gene in neutrophils.

The strategy used for the sequential injections of drugs in the Seahorse assays should be better explained. What is the rationale for injecting the bacteria, then glucose, oligomycin and 2DG? Why not also report the OCR and ECAR following the injection of glucose? What can be concluded following the injection of oligomycin?
Response: We carried out a modified glycolysis stress test on these cells which involved the addition of bacteria prior to running the standard stress test (Agilent Technologies Kit 103020-100) using the compounds oligomycin and 2-deoxy-glucose at the time points indicated on figures 6E&H. The compound treatments were not relevant to the findings regarding oxygen consumption which are detailed in Figure 6 – they were added to allow calculation of additional factors in the ECAR. The addition of glucose allows calculation of baseline glycolysis, oligomycin allows calculation of glycolytic capacity and finally, the addition of 2-DG allows glycolytic reserve to be calculated. None of these parameters were different between genotypes or with the addition of bacteria and so have not been plotted in this paper. They are not relevant to the OCR measurements of interest but for clarity and transparency have been noted on the figure.

Also, the OCR after the bacteria injection is indeed increased in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice (Fig. 6F). However, the OCR before the bacteria injection is also increased in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice (Fig. 6E). To account for this basal difference between the two groups, the authors need to baseline the data before the injection of the bacteria and then report the increase after the injection.
Response: There is a trend towards higher OCR at baseline in the PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice, although this does not reach statistical significance (as shown in Figure 6I). Both genotypes demonstrate an uplift in OCR in response to bacteria. The fold change in OCR is not significantly different between genotypes when calculated as (OCR immediately post-bacteria injection)/(mean OCR pre-bacteria injection) – 1.6 for WT Vs 1.55

for KO. However, when absolute increase in OCR is calculated, i.e. (OCR immediately post-bacteria injection) - (mean OCR pre-bacteria injection), this is significantly different – 267 for WT V 375 for KO, p=0.04, analysed by unpaired t-test. We would suggest that whilst both increase their OCR, the higher level (Figure 6H) and higher absolute increase in the PHD3fl/fl-MRP8Cre+/- (as described above) are indicative of overall increased oxygen consumption in the PHD3 deficient neutrophils.

The authors report that the OCR is increased after bacteria injection in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/- mice (Fig. 6F). After confirming that this uplift in OCR was observed in the context of conserved ECAR (Fig. 6H-J) and equivalent abundance of TCA cycle, glycolysis and OxPPP metabolic intermediaries (Fig. 6K-M; done with the LysM-Cre mice), the authors considered whether this increase in OCR reflected increased ROS production. However, total ROS (Fig. 6N) and mROS production (Fig. 6O), the latter one being increased in PHD3fl/fl-MRP8Cre+/- vs PHD3fl/fl-MRP8Cre+/-, mice were examined in unstimulated cells. How can the authors explain that a similar increase was not observed in baseline OCR (Fig. 6G) in their Seahorse assays?
Response: This is an interesting observation and we thank the reviewer for highlighting this potential discrepancy. As noted above, although the trend in higher OCR is present at baseline, it is only when neutrophils are further stimulated with bacteria that the difference becomes significant. We would hypothesise that the total OCR will comprise not only mROS but also cytosolic ROS production, fatty acid oxidation and other oxygen requiring metabolic processes. The small but significant increase in mROS production at baseline may therefore be masked by these other oxygen consuming processes which may not be different between genotypes. It would be very interesting to further test this hypothesis by repeating the mROS experiment with the addition of bacteria and this will be addressed in future work. In addition, although the neutrophils in the *ex-vivo* ROS and mROS studies were not stimulated with bacteria, they were harvested from the lungs following an LPS induced lung injury and so were activated rather than unstimulated cells.

In their discussion, the authors state: "Using Seahorse technology, we identified enhanced oxygen consumption in PHD3 deficient neutrophils upon challenge with bacteria which we have demonstrated is associated with increased mROS production in inflammatory BAL neutrophils." This is actually an overstatement, as this association was never demonstrated. For this, the whole set of experiments should have been performed with a single genotype (MRP8-Cre mice) and the total and mROS production examined following the activation of cells in the presence of bacteria, as done in the Seahorse assays.
Response: Thank you for this feedback, we have now rewritten these sections of the discussion and expanded the limitations sections to avoid overstating the associations which we have observed.

The authors are most likely aware of the multiple differences between mouse and human neutrophils, and this limitation should be addressed in the discussion, especially when it is concluded that PDH3 represents a potential therapeutic target. Experiments are most likely required with human cells to prove that PDH3 represents, indeed, a potential therapeutic target.

Response: Thank you for this helpful suggestion, we have now included these caveats in the limitations section of the paper.

Corrections are needed regarding the figures and the text:
1) The manuscript should include only high-resolution figures. For example, the quality of Fig. 1N, 1O and 1P are currently inadequate, as the axis labels are fuzzy.
Response: This issue has now been corrected and we apologise for the variable quality of the images.

2) The figures should clearly mention the experimental group. For example, Fig. 1E and 1H have the same title and axis labels. It is therefore critical to read the figure legend to understand that Fig. 1E refers to normoxia while Fig. 1H refers to hypoxia. The same can be said for Fig. 1F-1I, Fig. 1G-1J, Fig. 1M-1O and Fig. 1N-1P. The authors should combine these results in a single graph, such as in Fig. 1C, 1D or 1Q, or clearly state the experimental group in the figure title.
Response: Thank you for this feedback, we have now clearly labelled Fig 1E-F as relating to normoxic data and Fig 1H-J as relating to hypoxic data and hope this will make the data easier to view.

3) PMN is used in the axis title of some figures but has not been defined in the text or the figure legend.
Response: Thank you, PMN has been replaced with neutrophil in all figures.

4) The Greek letter Mu should be used instead of the letter "u" for micro.
Response: Thank you, this has been corrected.

5) b-actin should be replaced by beta-actin (using the Greek letter) in the legend of Fig. 1.
Response: Thank you, this has been corrected.

6) FMLP should be replaced by fMLF in Fig. 2I, as this is the abbreviation used in the text.
Response: Thank you, this has been corrected.

7) In the legend of Fig. 5, the text "BAL supernatant from PHD3fl/flLysMCre+/- and PHD3fl/flLysMCre-/- mice subject to IT…" should be corrected to "BAL supernatant from PHD3fl/flLysMCre+/- and PHD3fl/flLysMCre-/- mice subjected to IT…".
Response: Thank you, this has been corrected.

8) IT should be defined in the text.
Response: Thank you, this has been corrected in the methods section.

9) In Fig. 6E-G, the axis label states OCR (mpH/min). In Seahorse assays, OCR is actually provided as nmoles/min. The axis label needs to be corrected.
Response: We apologise for this error and thank the reviewer for noting it. This has been corrected to pmol/min to best display our data.

References
1. Dorward, D. A. *et al.* Technical advance: autofluorescence-based sorting: rapid and nonperturbing isolation of ultrapure neutrophils to determine cytokine production. *Journal of Leukocyte Biology* **94,** 193–202 (2013).
2. Zhang, X., Ding, L. & Sandford, A. J. Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. *BMC Mol Biol* **6,** 4–7 (2005).
3. Ledderose, C., Heyn, J., Limbeck, E. & Kreth, S. Selection of reliable reference genes for quantitative real-time PCR in human T cells and neutrophils. *BMC Res Notes* **4,** 427–11 (2011).

Competing Interests: No competing interests were disclosed.