Prolyl hydroxylase 2 inactivation enhances glycogen storage and promotes excessive neutrophilic responses

Pranvera Sadiku,1,3 Joseph A. Willson,1 Rebecca S. Dickinson,1 Fiona Murphy,1 Alison J. Harris,1 Amy Lewis,2 David Sammut,3 Ananda S. Mirchandani,1 Eilise Ryan,1 Emily R. Watts,1 A.A. Roger Thompson,1 Helen M. Marriott,4 David H. Dockrell,4 Cormac T. Taylor,3 Martin Schneider,1 Patrick H. Maxwell,7 Edwin R. Chilvers,7 Massimilliano Mazzone,8 Veronica Moral,2 Chris W. Pugh,9 Peter J. Ratcliffe,8 Christopher J. Schofield,10 Bart Ghersièvre,7 Peter Carmeliet,2 Moira K.B. Whyte,1 and Sarah R. Walmsley1

1MRC/University of Edinburgh Centre for Inflammation Research, Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom. 2Laboratory of Angiogenesis and Vascular Metabolism, Vascular Research Center, Leuven, Belgium. 3Academic Unit of Respiratory Medicine and 4Academic Unit of Immunology and Infectious Diseases, Department of Infection, Immunity and Cardiovascular Disease, The Medical School, University of Sheffield, Sheffield, United Kingdom. 5UCD School of Medicine and Medical Science, Conway Institute, University College Dublin, Dublin, Ireland. 6General, Visceral and Transplantation Surgery, University of Heidelberg, Heidelberg, Germany. 7Department of Medicine, University of Cambridge, Cambridge, United Kingdom. 8Laboratory of Tumour Inflammation and Angiogenesis, Department of Oncology, Leuven, Belgium. 9Nuffield Department of Medicine and 10The Department of Chemistry, University of Oxford, Oxford, United Kingdom.

Fully activated innate immune cells are required for effective responses to infection, but their prompt deactivation and removal are essential for limiting tissue damage. Here, we have identified a critical role for the prolyl hydroxylase enzyme Phd2 in maintaining the balance between appropriate, predominantly neutrophil-mediated pathogen clearance and resolution of the innate immune response. We demonstrate that myeloid-specific loss of Phd2 resulted in an exaggerated inflammatory response to Streptococcus pneumoniae, with increases in neutrophil motility, functional capacity, and survival. These enhanced neutrophil responses were dependent upon increases in glycolytic flux and glycogen stores. Systemic administration of a HIF–prolyl hydroxylase inhibitor replicated the Phd2-deficient phenotype of delayed inflammation resolution. Together, these data identify Phd2 as the dominant HIF–hydroxylase in neutrophils under normoxic conditions and link intrinsic regulation of glycolysis and glycogen stores to the resolution of neutrophil-mediated inflammatory responses. These results demonstrate the therapeutic potential of targeting metabolic pathways in the treatment of inflammatory disease.

Introduction

Inappropriate or persistent neutrophilic inflammation is implicated in a number of disease states exemplified by acute lung injury responses and chronic obstructive pulmonary disease. There are, to date, no effective therapeutic strategies for targeting neutrophilic inflammation, a reflection in part of the fine balance that exists between maintaining effective host pathogen responses and limiting host-mediated tissue damage. Local and systemic hypoxia represent a critical component of the inflammatory response, with oxygen-sensing pathways implicated in the regulation of neutrophil survival. The coordination of cellular responses to differing oxygen tensions is tightly controlled and involves hydroxylation of the HIF subunits, with prolyl hydroxylation targeting HIFs for ubiquitylation and proteosomal degradation (1, 2). In humans, 3 prolyl hydroxylase enzymes are described (PHD1–3), with PHD2 the most abundant isoform and important in setting basal levels of HIF-1α in the majority of cells cultured in normoxia (3–6). While there is emerging evidence that genetically observed PHD2 variations result in distinct clinical phenotypes (7–9), the overall physiological roles of PHD2 are only now emerging. An important example is the critical role of PHD2 in macrophage polarization, with haplodeficiency of Phd2 resulting in expansion of tissue-resident M2 macrophages with consequences for arteriogenesis (10).

We have previously observed that neutrophils express all 3 PHDs. PHD2 is both expressed constitutively and upregulated in sterile inflammation, where PHD2 expression follows HIF activity. In marked contrast to PHD3 expression, however, only modest changes in PHD2 transcript are observed in response to hypoxic cell culture (11). HIF-1α, HIF-2α, and PHD3 have all been shown to regulate neutrophil survival and function, with implications for neutrophilic inflammation (11–13). In vivo inactivation of both Hif1α and Phd3 results in abolished hypoxic survival and loss of functional capacity. Inactivation of Hif2α is also associated with a reduction in neutrophilic inflammation and tissue damage, as a consequence of increased neutrophil apoptosis. This suggests PHD2 is likely to also play a role in mechanisms regulating neutrophil apoptosis. Conceptually, it is also likely that individual PHD family members play distinct roles in regulating key neutrophil functional and survival responses under differing oxygen tensions (11, 12, 14).

An increasing body of work has focused on the potential roles of prolyl hydroxylases as metabolic sensors, in addition to their...
role as oxygen sensors, both indirectly through their regulation of HIF-mediated signaling and directly through their dependence on the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (α-KG) and their ability to interact with the glycolytic enzyme phosphoketolase (PK) (1, 15–20). This linking of metabolism to cell survival and function has important implications for the role of PHD enzymes in regulating myeloid cell functional responses, particularly in light of recent macrophage studies linking metabolic status to macrophage polarization and immune signaling (21–23). In neutrophils, we observed that mutations in succinate dehydrogenase B result in enhanced neutrophil survival responses, despite evidence of a dysfunctional TCA cycle (24), directly linking metabolism with neutrophil survival. The importance of changes in the metabolic capacity of neutrophils and, more specifically, flux between individual metabolic pathways under resting and activated states remains to be fully explored, as does the role PHD enzymes may play in coordinating these responses.

In this work, we address the consequences of myeloid-specific loss of Phd2 for host-pathogen interactions and link the metabolic state of neutrophils to survival and functional status. Our findings reveal that loss of Phd2 results in increases in neutrophil activation and persistence, resulting in increased tissue injury following challenge with intratracheal Streptococcus pneumoniae. We demonstrate that enhanced survival and function of Phd2-deficient neutrophils is directly linked to their metabolic capacity and that modulating neutrophil metabolism can alter outcomes of infection and inflammation.

Results

Myeloid-specific deletion of Phd2 results in enhanced neutrophilic inflammation and lung injury. To investigate the consequences of Phd2 loss for host pathogen responses, we challenged Phd2fl/fl LysM-Cre positive and negative (hereafter myeloid-specific Phd2–/– and WT, respectively) littermate controls with serotype 4 S. pneumoniae (TIGR4) in a model of fulminant bacterial pneumonia with pathogen clearance (25), we proceeded to define the importance of myeloid-specific expression of Phd2 in another murine model of acute lung injury, with a neutrophil-intrinsic response, Phd2-deficient airway neutrophils expressed higher levels of CXCR2 and CD11a (Figure 1, G and H), with no effect of Phd2 loss on BMDM cytokine expression (Supplemental Figure 2, E–H), as previously reported (26). Importantly, maximal neutrophil recruitment reached equivalence between genotypes at 24 hours, suggesting Phd2–/– mice had more rapid neutrophil recruitment rather than recruiting greater cell numbers (Figure 1I). In keeping with the phenotype observed following pulmonary infection with S. pneumoniae, we found delayed inflammation resolution (Figure 1J) and neutrophil persistence (Figure 1, K and L) in Phd2–/– animals and a significant reduction in detectable neutrophil apoptosis (Figure 1M), with a parallel increase in BAL IgM release (Figure 1N). In a model of more chronic inflammation, dextran sulfate sodium (DSS) diet-induced colitis, greater neutrophil persistence was seen in heterozygous Phd2+/− than in WT mice (Figure 1O). Taken together, these data implicate Phd2 in both the recruitment and resolution of neutrophil-mediated inflammatory responses, with potentially important consequences for resultant tissue damage.

Phd2-deficient neutrophils display enhanced function and survival ex vivo. The importance of Phd2 expression in regulating key inflammatory neutrophil functions was investigated ex vivo in neutrophils recruited to the airways at 24 hours following in vivo challenge with LPS. Freshly isolated BAL neutrophils lacking Phd2 displayed increased basal motility in the chemokines control (KC/Kc) and enhanced chemotaxis to the murine IL-8 homologue KC at the point of isolation (Figure 2A). Ex vivo stimulation with the N-formylated bacterial chemotactic peptide N-formylmethylionyl-leucyl-phenylalanine (fMLF) was unable to induce an increase in respiratory burst activity in either genotype (Figure 2B), and no difference in inner mitochondrial membrane potential was seen (Figure 2C). Following aging for an additional 6 hours, to investigate the functional reserve capacity of the inflammatory cells, the differences in chemotactic behavior were measured and were even more apparent (Figure 2D), while respiratory burst (Figure 2E) and inner mitochondrial membrane potential (Figure 2F) remained unchanged, despite a detectable increase in mitochondrial ROS production measured by Mitosox (Figure 2G). In keeping with the equivalent lung CFU counts observed in vivo (Figure 1D) and in contrast with BMDMs (Supplemental Figure 3A), Phd2-deficient neutrophils demonstrated equivalent bacterial phagocytic capacity ex vivo (Figure 2H). At both an early (Figure 2I) and late (Figure 2J) time point, peripheral blood neutrophils from Phd2–/– animals displayed reduced constitutive rates of apoptosis and preserved survival responses to both LPS (Figure 2I) and hypoxic culture (WT 34.5% ± 7.39%, Phd2–/– 18.0% ± 3.74% apoptotic) (Figure 2J), in keeping with the observed differences in apoptosis rates in vivo (Figure 1L). There was no effect of Phd2 loss on macrophage effecotocysis (Supplemental Figure 3B). Thus, neutrophils show both increased activation and increased longevity in the absence of Phd2. The further augmentation of Phd2-deficient neutrophil survival by hypoxia implies both PHD2-dependent and -independent regulation of neutrophil apoptosis responses. To address the neutrophil specificity of the phenotype in vivo, we challenged Phd2fl/fl LysM-Cre positive and negative mice with LPS and with serotype 2 (D39) S. pneumoniae following alveolar macrophage

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MRP8-Cre<sup>+/–</sup> mice displayed increased total BAL cell counts and BAL neutrophil numbers when compared with WT mice at 48 hours after LPS challenge (Figure 2K).

Inflammatory neutrophils deficient in Phd2 display enhanced glycolytic capacity with parallel increases in both ATP and intracellular glycogen stores. To investigate whether Phd2-deficient phenotypes could be, at least in part, due to compensatory overexpression of genes encoding for other PHD family members, relative quantification of Phd<sup>–/–</sup> mRNA was undertaken on freshly isolated BAL neutrophils 24 hours after LPS challenge. No differences in expression of either Phd1 or Phd3 were observed at an mRNA level (Figure 3A).

Figure 1. Myeloid-specific Phd2 deficiency results in aberrant neutrophilic inflammation. WT (white bars) and myeloid-specific Phd2<sup>–/–</sup> (black bars) mice were studied in parallel. (A–E) Mice were infected via the trachea with 1 × 10<sup>7</sup> CFU of TIGR4. Cells were harvested by BAL at 14 hours and total cell counts (A) and neutrophil differential counts (B) obtained. Total IgM release into bronchoalveolar fluid (C) and viable bacterial counts recovered from homogenized lung (D) or whole blood (E) were performed in parallel (n = 15). (F–L) Acute lung injury. Intratracheal LPS (0.3 mg) was instilled in anesthetized mice. Mice were sacrificed at 6 hours after challenge and cells harvested by BAL for total cell counts (F) and surface expression of CXCR2 (G) and CD11a (H). At 24 (I), 48, and 72 hours after challenge, cells/supernatants were harvested by BAL for total cell counts (I, J, L), neutrophil differential counts (I, K, L), morphological counts of apoptosis (M), and measures of total IgM release (N) (n = 7). (O) DSS colitis. Six days following DSS diet–induced colitis, colonic sections were harvested, paraffin-fixed, and anti-MPO antibody stained. ALI, acute lung injury. P values obtained via unpaired t test.
Figure 2. Phd2-deficient neutrophils display augmented inflammatory responses. (A–G) Murine inflammatory BAL neutrophils were isolated from WT (white bars) and myeloid-specific Phd2−/− (black bars) mice 24 hours after in vivo LPS (3 mg) challenge and studied either at point of isolation (A–C) or following aging in culture for 6 hours (D–G). (A and D) Chemotaxis. Chemotaxis to KC (100 nM) was determined using neuroprobe chambers. (B and E) Respiratory burst. Change in DCF emission, an indicator of cellular oxidative stress, was quantified by flow following 30 minutes stimulation with fMLP (10 μM). (C and F) Inner mitochondrial membrane potential. FL2 geometric mean fluorescence with tetramethylrhodamine, methyl ester (TMRM) uptake was quantified by flow cytometry in the presence or absence of CCCP (10 μM). CCCP was used as a negative control for the TMRM measure of inner mitochondrial membrane potential and works by collapsing the proton gradient. TMRM is a cell-permeant cationic fluorescent dye that accumulates in active mitochondria and is distributed throughout the cytosol when mitochondrial membrane potential collapses. (G) Mitochondrial ROS. MitoSOX fluorescent emission was quantified by flow cytometry in the presence or absence of CCCP (10 μM). (H) Inflammatory BAL neutrophil bacterial phagocytosis. Alexa Fluor 488–conjugated E. coli BioParticles (MOI 5:1) were administered to inflammatory BAL neutrophil for 1 hour. (I and J) Apoptosis. Peripheral blood neutrophils were isolated from WT (white bars) and knockout Phd2−/− (black bars) mice and cultured in the presence/absence of LPS (100 ng/ml) in normoxia (19 kPa, 21% O₂) or hypoxia (3 kPa, 3% O₂) for 5 (I) or 9 (J) hours and apoptosis assessed by morphology. (K) Phd2−/− MRP8-Cre−/− (MRP8;Phd2−/−) and WT control mice were nebulized with LPS (3 mg) and cells harvested by BAL for total cell counts and neutrophil differential counts. Data represent mean ± SEM, n = 4. MFI, mean fluorescence intensity. P values obtained via 2-way ANOVA (A, D, I, J) and unpaired t test (G and K).
No changes in either Hif1a or Hif2a mRNA were observed (Figure 3A), but key regulators of glycolysis and known HIF target genes Pkm2, Pgk, Tpi1, and Gapdh were increased (Figure 3B), with a parallel and sustained increase in tissue myeloid cell expression of HIF-1α protein in Phd2−/− mice (Figure 3C). In light of the observed increase in glycolytic enzymes, we questioned whether Phd2-deficient neutrophils would display either a basal increase in glycolysis or an exaggerated metabolic response to proinflammatory media-
Figure 4. Inflammatory neutrophils deficient in Phd2 display enhanced glycolytic capacity and glycogen storage with parallel increases in ATP production and utilization. Murine inflammatory BAL neutrophils from WT (white bars) and Phd2−/− (black bars) mice were studied following isolation 24 hours after in vivo challenge with nebulized LPS (3 mg). (A) Glycolytic metabolites. Murine inflammatory BAL neutrophils were cultured in the presence of U-13C glucose for 6 hours under conditions of normoxia (21% O2) and hypoxia (3% O2). Incorporation into G6P and redistribution of 13C carbons derived from U-13C glucose were measured using LC-MS. Data are presented as relative metabolite abundance and show mean ± SEM, n = 3. A diagram of the glycolytic and gluconeogenesis pathways depicting 2 possible outcomes resulting in the generation of G6P m3 isotopomer by recycling of 13C carbons is included. Total G6P is a measure of conversion of nonglucose substrates into glucose in Phd2-deficient BAL neutrophils, an observation further validated by a parallel increase in intracellular glycogen stores (Figure 4B). This functional increase in glucose uptake occurred in the context of unchanged Glut1 (Supplemental Figure 6A) and Glut3 (Supplemental Figure 6B) mRNA expression. The global consequences of enhanced glycolytic flux and glycogen stores for neutrophil energetics, ATP production, and utilization were therefore determined. Compared with WT cells, Phd2-deficient neutrophils contained higher relative levels of ATP (Figure 4C) and equivalent levels of ADP (Figure 4D) and AMP (Figure 4E), reflecting increased nucleotide production, with an overall increase in cellular energy charge also observed (Figure 4F).

Inhibition of glycolysis and glycogen availability rescues the enhanced neutrophilic responses observed in myeloid-specific Phd2−/− mice. Clinically useful strategies for selectively targeting neutrophil-mediated inflammatory responses are currently lacking. We hypothesized that the uplift in glycolytic capacity seen in Phd2-deficient neutrophils was directly responsible, or at least critical for, the exaggerated neutrophil responses seen following challenge with live bacteria or bacterial products and thus represents a therapeutic target. Mice were initially challenged with intratracheal LPS for 24 hours prior to i.p. administration of the glycolytic inhibitor 2 deoxy-d-glucose (2DG). The dosing regimen for 2DG was validated by the observed suppression of bone marrow neutrophil ECAR by in vivo treatment with 2DG (Figure 5A), and the consequences for neutrophil apoptosis and inflammation resolution were then determined. The enhanced survival of peripheral blood neutrophils lacking Phd2 expression was completely reversed by treatment with 2DG (Figure 5B). Moreover, in vivo administration of 2DG abrogated the persistent increase in BAL total cell counts (Figure 5C) and neutrophil differential counts (Figure 5D). BAL neutrophils isolated from mice treated with 2DG also revealed a reduction in their chemotactic capacity to KC (Figure 5E). To assess whether neutrophils have the capacity to utilize glycogen for survival responses, we first questioned whether they express glycogen synthase, the key enzyme required for glycogen synthesis. Relative quantification of Gys1 mRNA was undertaken on bone marrow neutrophils and revealed expression of the transcript in both WT and Phd2−/− cells (Figure 5F). In vitro, inhibition of glycogen breakdown in Phd2-deficient inflammatory BAL neutrophils with CP-91149 (a glycogen phosphorylase inhibitor) resulted in significantly increased apoptosis rates (Figure 5G). Together, these data support the concept that changes in the metabolic status of neutrophils critically regulate their function and survival. To investigate the importance of glycolytic capacity for inflammation resolution in the context of hypoxia, human peripheral blood neutrophils were cultured ex vivo and apoptosis determined both in deplete culture conditions and with the glycolytic inhibitor 2DG. Hypoxic survival was abrogated by the absence of glucose (Figure 6A) and by glycolytic inhibition (Figure 6B). In vivo treatment with 2DG during the resolution phase of the inflammatory response resulted in rescue of the persistent inflammation seen in the presence of hypoxia (Figure 6, C–E, G) with return of neutrophil apoptosis rates to normoxic levels (Figure 6F).

Pan hydroxylase inhibition replicates the Phd2-deficient phenotype in vivo. In order to investigate the consequences of in vivo
administration of the potent HIF-prolyl hydroxylase inhibitor molidustat (30, 31) for inflammation resolution, molidustat was administered in vivo by gavage 2 hours (T–2) prior to challenge with nebulized LPS (3 mg) and, 24 hours after challenge, further treated with either i.p. 2DG (500 mg/kg) or PBS control. Forty-eight hours after LPS challenge, mice were sacrificed and bone marrow neutrophils harvested for Seahorse quantification of ECARs, with additional FMLP stimulation ex vivo. (B) Apoptosis. Peripheral blood neutrophils were isolated from WT (white bars) and myeloid-specific Phd2–/– (black bars) mice and apoptosis rates assessed at 5 hours in the presence or absence of 2DG (100 μM). (C) In vivo inflammation resolution. WT (white bars) and Phd2–/– cre+ (black bars) mice were challenged with nebulized LPS (3 mg) and 24 hours after challenge further treated with either i.p. 2DG (500 mg/kg) or PBS control. Forty-eight hours after LPS challenge, mice were sacrificed and cells harvested by BAL for total cell counts (C) and neutrophil differential counts (D). Data represent mean ± SEM, n = 5. (E) WT mice were challenged with nebulized LPS (T0) (3 mg), and i.p. installation with 2DG (500 mg/kg) or PBS vehicle control was carried out at T10. Mice were sacrificed at 24 hours (T24) and cells harvested by BAL. Chemotaxis of the freshly isolated BAL neutrophils to KC was determined ex vivo using neuroprobe chambers. Data represent mean ± SEM, n = 6. (F) Glycogen synthase 1 (Gys1) expression in bone marrow neutrophils. FACS-sorted bone marrow neutrophils of LPS-nebulized WT and Phd2-deficient mice were lysed and TaqMan analysis of cDNA performed with data normalized to β-actin expression. Data represent mean ± SEM, n = 4. (G) Phd2-deficient inflammatory BAL neutrophils were incubated for 20 hours in the presence or absence (Unt) of DMSO vehicle control or the glycogen phosphorylase inhibitor CP-91149. Effects on neutrophil apoptosis were assessed by morphology. Data represent mean ± SEM (n = 4). P values obtained via unpaired t test (A–E) and 1-way ANOVA (G).

**Discussion**

A tightly coordinated innate immune response is critical for effective pathogen clearance with limited collateral tissue damage. There is a clinical need for therapies that selectively target excessive or inappropriate inflammation while maintaining effective antimicrobial host responses. In this work, we identify an important role for PHD2 in the regulation of neutrophilic inflammation. We observe that myeloid-specific loss of Phd2 results in a delayed inflammatory response that is detrimental to the host and imparts no measured benefit for bacterial killing or clearance. In marked contrast to the HIF-independent phenotype of abrogated hypoxic survival in Phd3-deficient neutrophils (11), loss of Phd2 results in a delay in both neutrophil apoptosis and inflammation resolution. In parallel with enhanced neutrophil survival, we also observed an intrinsic neutrophil phenotype of augmented recruitment, enhanced chemotaxis, and an increase in the functional reserve capacity of aged neutrophils. Given neutrophil migration is a highly energy-requiring process (33), that neutrophils are unusu-
al in their capacity to predominantly utilize glycolysis for ATP production (34), and that glycolysis enables much more rapid access to ATP than other metabolic processes (35), the glycolysis-driven augmented function is energetically favorable. The mechanisms that drive this glycolytic response and its functional consequences do, however, require further discussion. The increase in energy charge in Phd2-deficient neutrophils suggests that an increased demand for and utilization of ATP, as reflected in the enhanced chemotactic capacity of these cells, is fully matched by the augmented flux through glycolysis. Interestingly, the increase in glycolytic flux occurs in the setting of increased G6P production. We observed an increase in both m6 fully labeled and m5–m0 partially labeled and unlabeled G6P fractions, respectively, following culture with U13-C. This identifies first that Phd2-deficient neutrophils are capable of increased glucose uptake and utilization where glucose is freely available, and second, that neutrophils are capable of the conversion of nonglucose substrates into glucose. This has particular relevance to the in vivo state, and inflamed sites in particular, given the limited glucose availability. While previous electron microscopy (EM) studies have detailed the presence of glycogen bodies within quiescent neutrophils (36, 37), our work provides direct evidence for regulation of neutrophil apoptosis through glycogen availability. The wider implications of the ability of neutrophils to maintain intracellular glucose and glycogen stores also require consideration, given the previous descriptions of neutrophil dysfunction in association with G6PT deficiency in humans (38) and G6Pase-b inactivation in mice (39). Furthermore, this leads us to speculate that neutrophils can themselves undergo gluconeogenesis and that PHD2 is an important regulator of this process. It is of particular interest, given the recent observation that both liver-specific knockdown of Phd2 and oral administration of a nonisoform-selective PHD inhibitor GSK360A enhanced
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nebulized LPS and systemic hypoxia, given we have previously described the activation of HIF-1α in neutrophils in this setting (11) and would predict this to occur in the setting of suppressed PHD2 activity. Administration of the glycolytic inhibitor 2DG during the resolution phase of the lung injury response completely abrogated both hypoxic neutrophil survival and the sustained inflammatory response. This observation suggests that the therapeutic targeting of neutrophil glycolysis may represent a novel approach to limiting inappropriate or persistent neutrophilic inflammation in situations where increased HIF-1α activity is observed.

Finally, the broader consequences of long-term non-PHD isoform-selective inhibition for outcomes of the innate immune response require consideration. Our in vivo data would suggest that pan hydroxylase inhibition with molidustat results not only in enhanced neutrophil functional responses with increased basal motility and chemotaxis, but also in delayed inflammation resolution. This raises important questions as to the consequence of unchecked HIF activity in the setting of PHD inactivation and lactate-glucose recycling between the muscle and the liver and improved survival in an endotoxic shock model (40). Since the myeloid cell response to an endotoxin challenge predominates in the acute response, this raises important questions as to the potential for neutrophils to undergo gluconeogenesis and the role of gluconeogenesis in the systemic response to infection.

To address whether the exaggerated inflammatory response observed in Phd2-deficient mice was modulated by the observed increase in glycolytic capacity and amenable to therapeutic manipulation in vivo, animals were treated with i.p. 2DG 24 hours after challenge with nebulized LPS. Despite the potential global consequences for the host of glycolytic inhibition, in vivo blockade of glycolysis resulted in abrogation of exaggerated neutrophil inflammatory responses and survival in the myeloid-specific Phd2-deficient mice. This led us to question whether there were other situations in which neutrophils demonstrate suppressed PHD activity and in which the innate immune response could be modified by inhibiting glycolysis. We used the combination of nebulized LPS and systemic hypoxia, given we have previously described the activation of HIF-1α in neutrophils in this setting (11) and would predict this to occur in the setting of suppressed PHD2 activity. Administration of the glycolytic inhibitor 2DG during the resolution phase of the lung injury response completely abrogated both hypoxic neutrophil survival and the sustained inflammatory response. This observation suggests that the therapeutic targeting of neutrophil glycolysis may represent a novel approach to limiting inappropriate or persistent neutrophilic inflammation in situations where increased HIF-1α activity is observed.

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represents an important divergence from the normal physiological state in which PHD2 expression follows HIF transcriptional activation. Our observation, while providing important supportive evidence for the dominance of Phd2 in neutrophils in normoxia, also highlights the potential for disordered neutrophil-mediated inflammatory responses following treatment with non-PHD isoform selective inhibitors and suggests a degree of caution when considering the long-term clinical use of pan hydroxylase inhibitors (30) in the treatment of chronic anemia. Our data are also further demonstration of the need for the development of more selective targeting of individual PHD enzymes in the clinical arena.

Methods

Animals

Lysozyme M-driven Cre (LysM-Cre) targeted Phd2 deletions to myeloid lineage cells with animals backcrossed to a C57BL/6 background (10). Phd2Δ/Δ LysM-Cre−/− littermates were used as WT controls. Mice with MRFP8-driven Cre-targeted Phd2 deletion (Phd2Δ/Δ MRFP8-Cre−/−) were generated by crossing Phd2Δ/Δ animals with the previously reported neutrophil-specific Cre driver MRFP8 (27). For DSS colitis, C57BL/6 WT and previously described whole animal heterozygous Phd2Δ/Δ animals were studied (41). Mice with conditional tamoxifen-induced deletion of Phd2 (Phd2Rosa26CreERT2;fl/fl/CreERT2) were used in the study of neutrophil energy states and glycolytic capacity (10). Alveolar macrophage depletion was performed using 48 hours pretreatment with clodronate, as previously described (25).

Intratracheal pneumonia model

WT C57BL/6 mice were anesthetized with ketamine (100 mg/kg i.p.; Vetalar V, Pfizer) and acepromazine (5 mg/kg i.p.; Calmivet Solution, Pfizer) and acetylsalicylic acid (20 mg/kg i.p.; Smiths Medical International Ltd.). Each mouse then had 1 × 107 CFU M. avium (CGMCC 1.1052) targeted to the lungs instilled with 3.5 ml of ice-cold PBS in 0.5 ml aliquots. Hemocytometer counts were performed on the recovered BAL samples, which were then pelleted (1000 g, 5 minutes, 4°C) and resuspended in FCS prior to cytocentrifugation for differential cell counts and morphologic scoring of apoptosis. Chemokine/cytokine concentrations in lavage samples were determined by BD cyometric bead array using BD FACSArray acquisition software. Limits of detection were 10–2500 pg/ml.

DSS-induced acute colitis model

Colitis was induced using a previously described method (44) and mice sacrificed at day 6. Sections of intestine were stained with anti-MPO antibody following deparaffinization, and total neutrophils per high-power field (hpf) averaged from 5 fields per section.

Neutrophil isolation and culture

Murine inflammatory BAL neutrophils were isolated 24 hours after challenge with LPS (0.3 mg), with peripheral blood neutrophils isolated using negative magnetic selection and bone marrow neutrophils isolated by discontinuous Percoll gradients. Cells were cultured for 5–20 hours in normoxia (19 kPa) or hypoxia (3 kPa) at 5% CO2, as previously described (11).

Neutrophil functional assays

Chemotaxis. S × 10^5 neutrophils were plated on a semipermeable membrane (Chemotx Chemotaxis System, pore size 5 μm, Neuroprobe) in the presence or absence of KC at Tc and following 6 hours of aging, incubated for 1 hour at 37°C, spun at 300 g for 10 minutes, and hemocytometer counts performed. The number of cells in each well was expressed as a percentage of the total number of cells loaded into each well.

Inner mitochondrial membrane potential. Neutrophils (1 × 10^6/ml) were suspended in 3 nM TMRM (Life Technologies) dissolved in 1× HBSS, with and without 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich), and FL1 geometric mean fluorescence determined by flow cytometry.

Mitochondrial ROS production. Neutrophils (1 × 10^6/ml) were resuspended in 5 μM MitoSOX (Life Technologies) dissolved in 1× DPBS, with and without 10 μM CCCP (Sigma-Aldrich), and FL2 geometric mean fluorescence determined by flow cytometry.

Flow cytometry

Mouse BAL cells were treated with α-CD16/32 Fc block (eBioscience) and mouse serum (Thermo Fisher Scientific) prior to staining with antibodies. Relevant full minus one (FMO) samples for each group were used as controls. Antibodies used were as follows: Ly6G (IA8, BioLegend), CD11a (M17/4, BioLegend), CD11b (M1/70, BioLegend), CXCR2 (SA044G4, BioLegend), and CXCR1 (FAB8628A-025, RnD).
Live cells were gated following staining with DAPI (Invitrogen) prior to acquisition. BAL neutrophils were gated according to Ly6G^− and forward scatter (FSC)/side scatter (SSC) properties. Cells were acquired on an LSRFortessa (BD). Compensation was performed using BD FACSDiva software and data analyzed with FlowJo version 10.

**Isolation, culture, and functional assays of BMDM**

Red blood cell lysis was carried out on whole bone marrow cells from naive WT and Phd2-deficient mice. Cells were cultured in Glutamax DMEM supplemented with 1% penicillin/streptomycin, 10% FBS, and 20% L929 medium. Successful differentiation following 7 days of culture was determined by FACS staining for the macrophage marker F4/80.

**Phagocytosis.** Alexa Fluor 488-conjugated E. coli BioParticles (MOI 1:1) were administered to cells for 1 hour. Following vigorous washing with PBS, phagocytosis of E. coli was measured using flow cytometry. Data analysis was carried out with FlowJo version 10.

**Efferocytosis.** Differentiated BMDM cells were incubated with PKH26-stained apoptotic human neutrophils (cultured for 20 hours in normoxic conditions) at a ratio of 5:1 for 1 hour. Following vigorous washing with PBS, uptake of apoptotic human neutrophils was assessed by flow cytometry. Data analysis was carried out with FlowJo version 10.

**RNA isolation and relative quantification**

Murine BAL leukocytes (1 × 10^6/condition) were lysed and RNA extracted using the mirVana Total RNA Isolation Protocol (Ambion). Samples were treated with DNase (Ambion) and random hexamer cDNA synthesized by reverse transcription. Assays-on-Demand Gene Expression TaqMan MGB 6FAM dye-labeled products (Applied Biosystems) were used for relative quantification of cDNA.

**Validation of Phd2 transcript knockdown**

For Phd2^+^/LysM-Cre^+/−_mice, Percoll-purified bone marrow neutrophils from Phd2^−^ and WT littermates were FACS sorted based on FSC/SSC. For Phd2^+^/MRP8-Cre^+/−_mice, following BAL neutrophil isolation from mice challenged with nebulized LPS (3 mg) sacrificed at 48 hours after challenge, cells were subjected to Percoll density centrifugation. Purified cells were lysed, and RNA was extracted and reverse transcribed. Assays-on-Demand Gene Expression TaqMan MGB 6FAM dye-labeled products (Applied Biosystems) were used for relative quantification of cDNA.

**Immunohistochemistry**

For histological sections, unlavaged lungs were fixed via the trachea with 10% buffered formalin at 20 cm H_2O. Paraffin-embedded blocks were prepared and sections stained with anti–HIF-1α (polyclonal; Novus Biologicals), anti–HIF-2α (clone ep 190b; Novus Biologicals), or isotype control following deparaffinization.

**Seahorse**

Neutrophils were resuspended in XF assay media at a concentration of 3 × 10^6/ml. Three million cells per condition were plated onto a XF24 cell plate precoated with Cell-Tak (Corning). Cells were stimulated with fMLP (10 μM) or fMLP plus glucose (1 mg/ml). The oxygen consumption rate (OCR) and ECAR were measured at intervals of 7 minutes over a 90-minute cycle using a Seahorse XF24 (Seahorse Bioscience USA).

**Glycolytic flux**

Murine BAL leukocytes (0.5 × 10^6) were incubated for 6 hours in RPMI 1640 medium (supplemented with 5.5 mM unlabeled glucose, 10% FCS, and 1% penicillin/streptomycin) containing 0.4 μCi/ml [5-^3H]-d-glucose (PerkinElmer). Cells were pelleted (420 g for 10 minutes) and supernatant transferred into glass vials containing 12% perchloric acid sealed with rubber stoppers. ^3H_2O was captured in hanging wells containing a piece of Whatman paper soaked with H_2O over a period of 48 hours at 37°C to reach saturation. Radioactivity in the paper was determined by liquid scintillation counting.

**Intracellular glycogen stores**

Murine BAL leukocytes (1 × 10^6/condition) were lysed with 200 μl ice-cold H_2O and boiled for 10 minutes at 95 degrees; lysates were centrifuged at 18,000 g at 4°C for 10 minutes to remove cell debris and snap frozen. Glycogen concentration was measured by colorimetric assay (BioVision).

**Energy status**

A total of 1 × 10^6 murine BAL leukocytes were harvested in 100 μl of ice-cold 5% PCA supplemented with 1 mM EDTA. ATP, ADP, and AMP levels were measured using ion-pair RP-HPLC. The energy charge was expressed as ([ATP] + 1/2 [ADP])/[ATP] + [ADP] + [AMP]), and the energy status of the cells as the ratio of ATP to ADP content.

**LC-MS**

Murine BAL leukocytes (2 × 10^6/condition) were harvested in 100 μl of 80% methanol. Measurements of relative levels of analyte abundance and 13C incorporation into glycolytic intermediates were performed using a Dionex UltiMate 3000 LC System (Thermo Scientific) coupled to a Q Exactive Orbitrap Mass Spectrometer (Thermo Scientific) operated in negative mode. Practically, 25 μl of sample was injected on a SeQuant ZIC/PHILIC Polymeric column (Merck Millipore). The gradient started with 10% solvent B (10 mM NH4-acetate in mQH_2O, pH 9.3) and 90% solvent A (acetonitrile) and remained at 10% B until 2 minutes after injection. Next, a linear gradient to 80% B was carried out until 29 minutes. At 38 minutes, the gradient returned to 40% B, followed by a decrease to 10% B at 42 minutes. The chromatography was stopped at 58 minutes. The flow was kept constant at 100 μl/min, and the column was kept at 25°C throughout the analysis. The MS operated in full scan–SIM mode using a spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas at 10.0, auxiliary gas at 5.0. AGC target was set at 1e6 using a resolution of 140,000, with a maximum IT of 500 ms. Data collection and analysis were performed using Xcalibur Software (Thermo Scientific). Isotope correction was carried out as previously described (45) using an in-house software tool.

**Reducing equivalents**

Ratios of oxidized to reduced NADP/NADPH were calculated in murine BAL leukocyte lysates (1 × 10^6/condition) following quantification by fluorimetric enzyme cycling assay (AbCam).

**Statistics**

Significance was determined by unpaired 2-tailed t tests unless otherwise stated. Data are expressed as mean ± SEM and are representative of at least 3 independent experiments. *P* < 0.05 was considered significant.
Study approval
Animal experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986. All animal studies were approved by The University of Edinburgh Animal Welfare and Ethical Review Board.

Author contributions
PS, JAW, HMM, DHD, PC, MKBW, and SRW designed the experiments. PS, JAW, RSD, FM, AJH, AL, AART, HMM, and BG performed the experiments. DHD, CTT, MS, CP, PJJ, CJS, PHM, ERC, MM, BG, PC, MKBW, and SRW provided technical expertise and performed data analysis. All authors contributed to writing the manuscript.

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Address correspondence to: Sarah R. Walmsley, MRC/University of Edinburgh Centre for Inflammation Research, Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, EH16 4TJ UK, United Kingdom. Phone: 0044.131.2422426785; Email: sarah.walmsley@ed.ac.uk.


