

Opposing regulation and roles for PHD3 in lung dendritic cells and alveolar macrophages

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ABSTRACT

The prolyl hydroxylase domain-containing enzymes (PHDs) are important metabolic sensors of the cell and its environment, which might be employed to alert cells of the immune system. These enzymes regulate the expression of the hypoxia inducible factor (HIF) isoforms and NF- κ B, crucial transcription factors controlling cellular metabolism and inflammation. PHD/HIF signaling is activated in the allergic lung and is proposed as a potential druggable pathway. Here, we investigated the regulation and role of the PHD isoforms in CD11c-expressing dendritic cells (DCs) and macrophages (M ϕ), sensors of the environment and crucial antigen-presenting cells in the pathogenesis of asthma. Although PHD2 and PHD3 were expressed in baseline, stimulation with house dust mite (HDM) allergen, hypoxia, and TLR4 ligands induced the expression of PHD3 in DCs. Conditional deletion or overexpression of PHD3 in CD11c^{hi} cells had minor effects on DCs and alveolar M ϕ biology in steady state. However, when put into competition with wild-type counterparts in mixed chimeric mice, alveolar M ϕ uniquely required PHD3 for optimal reconstitution of the alveolar space. Using genetic and chemical approaches, we were unable to find a clear role for PHD3 or the other PHD isoforms in DCs in asthma development. These data show cell-specific competitive advantage of PHD3 expression in antigen-presenting cells, but question whether therapeutic manipulation of PHDs in DCs would offer therapeutic benefit in asthma. *J. Leukoc. Biol.* 102: 000-000; 2017.

Abbreviations: AAI = allergic airway inflammation, BALF = bronchoalveolar lavage fluid, β TUB = β -tubulin, BM = bone marrow, DC = dendritic cell, DMOG = dimethyloxoglutamate, HDM = house dust mite, HIF = hypoxia inducible factor, HK2 = hexokinase 2, HRE = hypoxia-responsive element, Hx = hypoxia, i.n. = intranasal, i.t. = intratracheal, KO = knockout, MLN = mediastinal lymph node, OE = overexpression, PHD = prolyl hydroxylase domain-containing enzyme, qRT-PCR = quantitative real-time PCR, WT = wild-type

The online version of this paper, found at www.jleukbio.org, contains supplemental information.

Introduction

The PHDs are important metabolic sensors of the cell. In the presence of oxygen, Fe²⁺, 2-oxoglutarate, and ascorbic acid, these dioxygenases catalyze the hydroxylation of proline residues on proteins [1]. PHDs modulate the availability and function of critical transcription factors, such as the HIFs and NF- κ B [2]. In the case of HIF1 α and HIF2 α , the Von Hippel Lindau E3-ligase recognizes and ubiquitinates the prolyl hydroxylated HIFs and targets them for proteasomal degradation [3, 4]. Because the hydroxylation reaction requires oxygen, the reduced levels of oxygen during Hx reduce the enzymatic efficiency of the PHD enzymes and stabilize HIF1 α and HIF2 α , allowing the dimerization with HIF1 β . The HIF1 α /HIF1 β and HIF2 α /HIF1 β complexes drive the transcription of a diverse array of genes containing HREs. Collectively, these genes activate the Hx response, which consists of cellular metabolic adaptations (enhanced glucose uptake and glycolysis), stimulation of blood vessel growth, and erythropoiesis [5].

Inflammation and PHD/HIF signaling are closely intertwined [6]. HIF accumulation is observed in inflammatory diseases and mediates the TLR-driven metabolic reprogramming during immune cell activation [7]. This conversion from mitochondrial respiration to glycolysis is crucial for cell function in the inhospitable inflammatory microenvironment [7, 8]. Mice lacking HIF1 in the myeloid lineage exhibited impaired inflammatory responses, reduced phagocytosis of pathogens, and were resistant to LPS-induced sepsis [9–11]. The PHD/HIF pathway also directly contributes to inflammation. HREs have been identified in a number of inflammatory genes [12]. Moreover, Hx activates NF- κ B and induces sterile inflammation [13, 14].

Allergic asthma is characterized by recurrent episodes of bronchial hyperreactivity and airway obstruction caused by inflammation, goblet cell metaplasia, and airway remodeling

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[15]. DCs initiate and perpetuate asthmatic allergic inflammation [16–18]. DCs endocytose and process inhaled allergens (e.g., HDMs), migrate to the MLN, and present these allergens to cognate T cells, driving Th2/Th17 polarization [19, 20]. Subsequent repeated exposure to the allergen induces Th2/Th17 lymphocyte accumulation in the lung mucosa, orchestrating a complex inflammatory disease dominated by eosinophils [18, 19, 21]. The asthmatic lung parenchyma bears a HIF gene signature. Moreover, both HIF1 α and HIF2 α proteins and their target genes have been detected in lung tissue of murine models of asthma and endobronchial biopsies of human patients [22, 23]. What drives HIF stabilization in the asthmatic lung? Data suggest that exposure to HDM reduces the expression of the HIF inhibitors PHD1 and 3 [24], whereas TGF- β and GM-CSF, cytokines contributing to disease development, promote HIF1 α transcription [25–28]. As asthma progresses, the resulting accumulation of inflammatory cells inside the lung parenchyma and the enhanced vascular leakage can potentially form a hypoxic nidus, driving further HIF1 accumulation. Genetic and chemical inhibition of HIF signaling confers protection in murine models of airway inflammation [23]. These preclinical studies suggest that the PHD/HIF signaling pathway is a tangible pharmaceutical target.

In contrast to the HIFs, the contribution of the upstream PHD isoform regulators to asthma and allergic airway development has not been characterized. In this study, we investigated the role of the PHD enzymes in allergic airway inflammation. Because DCs are crucial to the initiation and maintenance of disease and function as sensors of the microenvironment, we focused on the role of oxygen sensing in DCs. We found in DCs that PHD3 is regulated by TLR4 and HIF signaling and more notable than the allergen HDM. Genetic deletion or overexpression of PHD3 did not alter survival and maturation of DCs in vitro or in the lung DC network in vivo, and the genetic manipulation did not alter the course of HDM-induced AAI (HDM-AAI). Adoptive transfer of HDM-pulsed DCs successfully sensitized mice to the allergen, but treatment with the pan-PHD inhibitor DMOG did not influence asthma development, arguing against the PHD isoforms as viable candidates for antiasthma therapy. Unexpectedly, we noted a differential expression of the PHD isoforms between alveolar M ϕ s and DCs. Whereas PHD3 expression was dominant in DCs, alveolar M ϕ s only expressed low levels of PHD3 mRNA. By careful analysis of mixed BM chimeric mice, we found that PHD3 levels affect the potential of DCs and M ϕ s to successfully occupy their niches in the lung, spleen, and alveolar space when faced with WT competing cells.

MATERIALS AND METHODS

Mice and generation of BM chimeras

Phd1^{fl/fl}, *Phd2*^{fl/fl}, and *Phd3*^{fl/fl} and *Rosa26LsLmPhd1*, *Rosa26LsLmPhd2*, and *Rosa26LsLmPhd3* mice were crossed to B6.Tg^{ltagax-cre1-1Reiz} mice (CD11c^{Cre} mice) and, subsequently, backcrossed 10 times to C57BL/6. All experiments were performed with control littermates. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred at Ghent University in specific pathogen-free conditions. To generate irradiation

chimeras in C57BL/6 CD45.1/CD45.2 hosts, the BM of CD45.1/2 WT mice and PHD3^{CD11c-KO} or PHD3^{CD11c-OE} mice was used. After obtaining a single-cell suspension of BM cells, the CD45.1/2 BM cells were mixed into a 1:1 ratio with BM cells derived from PHD3^{CD11c-KO} or PHD3^{CD11c-OE} mice. Next, 12 h after lethal irradiation (8 Gray), CD45.1/2 mice were injected i.v. with 2×10^6 BM cells of this mixture of BM cells. To correct for variation occurring during the engraftment of WT and transgenic hematopoietic stem cells, immune cell populations (neutrophils and monocytes), minimally targeted by the Cre recombinase were used as internal controls. Mice were used for experiments at ≥ 4 wk after reconstitution. All animal experiments were performed in accordance with institutional guidelines for animal care of the VIB site Ghent–Ghent University Faculty of Sciences. Litters with mice of both sexes at 6–14 wk of age were used for experiments.

HDM model of allergic airway inflammation

To induce allergic airway inflammation, mice were sensitized i.t. with 1 μ g HDM extracts (*Dermatophagoides pteronyssinus* extracts XPB82D3A2.5; Greer Laboratories, Lenoir, NC, USA) on d 1 or with BM-DCs cultured with 20 μ g/ml HDM and/or 1 mM DMOG (71210; Cayman Chemical, Ann Arbor, MI, USA) were subsequently challenged with 10 μ g HDM i.n. on d 7–11. On d 14, BAL was performed with 3×1 ml of PBS-containing EDTA (51234; Lonza, Basel, Switzerland). MLNs were homogenized, and cells were plated at a density of 200,000 cells/well. Cells were restimulated for 3 d with 30 μ g HDM extracts. Cytokine production was measured by specific ELISA processes, following manufacturer's guidelines (eBioscience, San Diego, CA, USA).

Preparation of cell suspensions

In the case of lung and spleen, organs were digested at 37°C for 30–45 min in RPMI 1640 (61870036; Thermo Fisher Scientific, Waltham, MA, USA) containing Liberase TM (0.02 mg/ml; Roche, Basel, Switzerland) and DNase I recombinant (0.01 U/ μ l; Roche) after mincing. The obtained cell suspension was cleared of red cells by osmotic lysis.

Flow cytometry

Cells (5×10^6) from the organs of interest were stained with mixture of mAbs. MAb labeled with fluorochromes recognizing the following surface markers were used: CD3 (145-2C11; Tonbo Biosciences, San Diego, CA, USA), CD19 (1D3; Tonbo), CD45R (RA3-6B2; BD Biosciences, San Diego, CA, USA), TER119 (TER119; eBioscience), Ly-6G (1A8; BD Biosciences), CD64 (X54-5/7.1; BD Biosciences), CD11c (N418; eBioscience), MHCII (M5/114.15.2; eBioscience), CD103 (2E7; eBioscience), CD86 (PO3; BioLegend, San Diego, CA, USA), CD40 (3/23; BD Biosciences), CD45 (30-F11; eBioscience), CD45.1 (A20; eBioscience), CD45.2 (104; eBioscience), Ly-6C (HK1.4; eBioscience), CD11b (M1/70; BD Biosciences), CD24 (M1/69; BD Biosciences), mPDCA1 (120g8; in-house manufactured), CD4 (RM4-5; eBioscience), CD8a (53-6.7; eBioscience), and SiglecF (E50-2440; BD Biosciences). Viable cells were discriminated by the use of DAPI (D3571; Thermo Fisher Scientific) or Fixable viability dye eFluor 506 (eBioscience). Acquisition of labeled cell suspensions was performed with an LSRFortessa (BD Biosciences), and subsequent analysis of data used FlowJo10 software (FlowJo, Ashland, OR, USA).

GM-CSF-supplemented BM cultures

Tibia and femurs were collected from mice. Bones were crushed in mortar with a pestle and were subsequently filtered and spun down. The resulting pellet was cleared of RBCs by osmotic lysis. BM cells (2.5×10^6) were cultured in RPMI 1640 medium supplemented with GlutaMAX, 2-mercaptoethanol, gentamycin (all from Thermo Fisher Scientific), 5% FCS, and 20 ng/ml recombinant GM-CSF for 8 d. Nonadherent cells were collected for subsequent analysis. During experiments, cells were plated at a density of 1×10^6 cells/ml and exposed to 100 ng/ml LPS (tlr-3pelps; InvivoGen, San Diego, CA, USA), 1 mM DMOG (71210; Cayman Chemical), 100 μ g/ml HDM, 2 mg/ml chetomin (C9623; Sigma-Aldrich, St. Louis, MO, USA), or reduced oxygen concentrations (2–5% O₂).

RNA isolation, qRT-PCR, and primers

Cells were lysed in 1 ml TriPure (11667165001; Roche) and stored at -80°C until further processing. RNA was obtained following manufacturer's instructions. Purity and concentration of RNA were assessed with a Nanodrop 8000 (Thermo Fisher Scientific). Complementary DNA was generated from 1 μg total RNA using iScript advanced cDNA synthesis kit (172-5038; Bio-Rad Systems), according to manufacturer's guidelines. mRNA transcripts were quantified using SensiFAST SYBR no-ROX mix (BIO-98020; Biorline, Eveleigh, New South Wales, Australia) during quantitative real-time PCR (LightCycler 480; Roche). Primers for quantitative RT-PCR used in this study were enlisted as follows: *Phd1* forward, 5'-GGCAACTACGTCATCAATGGG-3'; *Phd1* reverse, 3'-TGCGG-ATTGTCAACATGCCTC-5'; *Phd2* forward, 5'-TTGTTACCCAGGCAACG-GAAC-3'; *Phd2* reverse, 3'-CCTTGGCGTCCCAGTCTTT-5'; *Phd3* forward, 5'-GGCTGGGCAAATACTATGTCAA-3'; *Phd3* reverse, 3'-GGTTGTCCAC-ATGGCGAACA-5'; *Nos2* forward, 5'-ACCCTAAGAGTCACCAAAATGG-3'; *Nos2* reverse, 3'-ATGTCACATGCAGCTTGTC-5'; *HK2* reverse, 3'-AACC-GCCTAGAAATCTCCAGA-5'; *Pgk* forward, 5'-ATGTCGCTTTCCAACAA-GCTG-3'; *Pgk* reverse, 3'-GCTCCATTGTCCAAGCAGAAT-5'; *Pfkfb* forward, 5'-GAAACATGAGGCGTTCTGTGT-3'; *Pfkfb* reverse, 5'-GAAACATGAGGC-GTTCTGTGT-3'; *L27* forward, 5'-TGAAGGTTAGCGGAAGTGC-3'; *L27* reverse, 3'-CATGAACCTGCCATCTCG-5'; *Hprt* forward, 5'-TGAAGAGC-TACTGTAATGATCAGTCAAC-3'; and *Hprt* reverse, 3'-AGCAAGCTTGCA-ACCTTAACCA-5'. mRNA expression data was analyzed using qbase+ software version 2.6 (Biogazelle, Gent, Belgium).

Immunoblotting

Cells (4×10^5 – 1×10^6) were lysed in 20–30 μl of E1A buffer (1% NP40, 20 mM HEPES, pH 7.9, 250 mM NaCl, 1 mM EDTA), complemented with Complete-ULTRA (05892970001; Roche) and PhosSTOP (04 906 837 001; Roche). Lysates were snap-frozen in liquid nitrogen and stored at -80°C until further use. Before SDS-PAGE, samples were spun at 12,000 g, at 4°C , to

remove insoluble material in the case of PHD3. In the case of HIF1 and HIF2, the complete lysate was run. Protein lysates were resuspended in a 4 $\times$ loading dye and loaded on a polyacrylamide gel. After wet transfer to nitrocellulose (Hybond-ECL; GE Healthcare, Waukesha, WI, USA) or polyvinylidene difluoride membrane (Immobilon; EMD Millipore, Billerica, MA, USA), proteins were analyzed by immunoblotting and visualized by chemiluminescence (NEL 104001EA; PerkinElmer, Waltham, MA, USA). Antibodies used recognized NOS2 (sc-650; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIF1 (NB100-449SS; Novus Biologicals, Littleton, CO, USA), HIF2 (NB100-122; Novus Biologicals), PHD3 (ab184714; Abcam, Cambridge, United Kingdom) and β -TUBULIN (ab21058; Abcam).

Statistical analysis

Two-sided statistics were used throughout this study. Results with a P -value ≤ 0.05 were considered significant. Mean values, SEM, and statistics were calculated with Prism6 (GraphPad Software, La Jolla, CA, USA). No criteria of inclusion or exclusion of data were used in this study. Experiments were performed without prior randomization of the animals and without blinding.

RESULTS

PHD enzymes are regulated by TLR4 and HIF1 in DCs

PHD isoform distribution is regulated across tissues and cell subsets [29]. Using qRT-PCR, we measured the relative expression of the different PHD isoforms in BM-DCs. We found that *Phd2* and *Phd3* mRNA was dominantly expressed, whereas the transcripts for *Phd1* were scarce (Fig. 1A). The metabolic requirements of TLR-activated DCs are significantly different from the quiescent, immature DC population. To match the synthetic capacity and ATP production with the activation state,

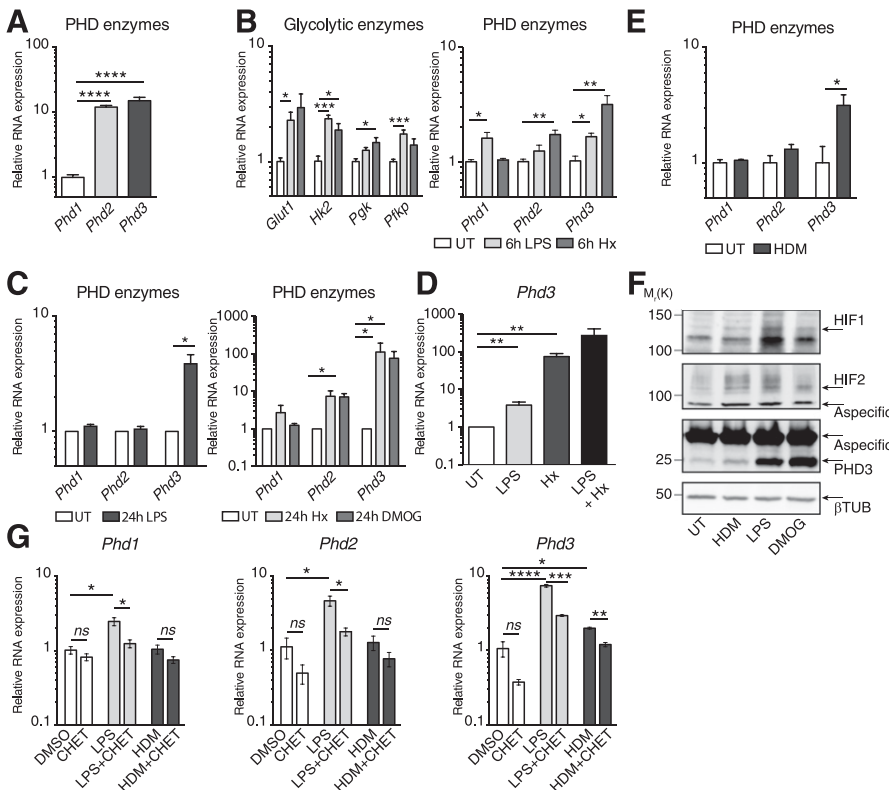


Figure 1. PHD enzymes in DCs are regulated by TLR4 and HIF1 and the allergen HDM. (A) qRT-PCR of *Phd1-3* transcripts in BM-DCs. Results are normalized to the housekeeping gene *L27* ($n = 3$). (B) qRT-PCR of transcripts from enzymes regulating the glycolytic pathway (*Glut1*, *HK2*, *Pgk*, and *Pfkfb*) and *Phd1-3* transcripts in BM-DCs, exposed to 100 ng/ml LPS or Hx for 6 h. Results are normalized to the housekeeping genes *L27* and *Hprt* ($n = 6$). (C) qRT-PCR of *Phd1-3* transcripts from LPS, 1 mM DMOG, or Hx-treated BM-DCs. BM-DCs were treated for 24 h. Results are normalized to the housekeeping genes *L27* and *Hprt* ($n = 3$). (D) qRT-PCR of *Phd3* transcripts of BM-DCs exposed to LPS and/or Hx. Results are normalized to the housekeeping gene *L27* ($n = 3$). (E) qRT-PCR of *Phd1-3* transcripts from 100 $\mu\text{g}/\text{ml}$ HDM-treated BM-DCs. Results are normalized to the housekeeping genes *L27* and *Hprt* ($n = 4$). (F) Western blot analysis of HIF1, HIF2, and PHD3 in BM-DCs exposed to HDM, LPS, or DMOG. β -tubulin (β TUB) was used as loading control. (G) qRT-PCR of *Phd1-3* transcripts in BM-DCs treated with LPS, HDM, or 2 mg/ml chetomin (CHET). Results are normalized to the housekeeping genes *L27* and *Hprt* ($n = 3$). All bar graphs represent means $\pm$ SEM. Unpaired Student's t test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

DCs increase aerobic glycolysis at the expense of mitochondrial respiration, a metabolic switch reminiscent of the Warburg effect observed in tumor cells [30, 31]. Indeed, both exposure to the TLR4 ligand LPS or reduced oxygen levels in hypoxic culture chambers induced mRNA transcripts of the glucose transporter 1 (*Glut1*) and the crucial glycolytic enzymes hexokinase (*Hk2*), phosphoglycerate kinase (*Pgk*), and phosphofructokinase platelet (*Pfkfb*) (Fig. 1B). Because the PHD/HIF signaling pathway is crucial in this metabolic switch to glycolysis during hypoxic conditions and HREs have been characterized in the promoter region of *Phd2* and *Phd3*, we set out to examine PHD regulation during DC activation [12, 32]. As observed in neutrophils [33], *Phd1* was only minimally regulated upon exposure to LPS or Hx. In contrast, both *Phd2* and *Phd3* were strongly up-regulated after 6 h, whereas only increased *Phd3* mRNA levels were preserved after 24 h of exposure to LPS (Fig. 1B and C). Induction of HIF with the pan-PHD inhibitor DMOG mirrored the Hx-induced expression of *Phd2* and *Phd3*, suggesting that HIF regulates PHD expression in DCs during Hx (Fig. 1C). HIF and TLR signaling displayed an additive effect on *Phd3* mRNA expression in DCs (Fig. 1D), presumably through enhanced *Hif1 α* mRNA transcription by NF- κ B [7]. In summary, PHD2 and PHD3 are the main expressed PHD isoforms in DCs, whereas PHD3 is strongly regulated upon DC activation and exposure to Hx.

HDM regulates PHD3 in DCs

The lung is continuously exposed to many antigens, bacteria, viruses, spores, and particles present in ambient air. Among these are allergens like HDMs, which can induce the activation of lung DCs, leading to allergen uptake, and subsequent allergic sensitization. To explore the regulation of the PHD enzymes upon exposition to HDM extracts, we cultured BM-DCs with a crude HDM extract, known to contain TLR4 ligands and numerous allergens, including cysteine proteinases. Only *Phd3* transcripts were significantly induced after HDM exposure (Fig. 1E). Next, we set out to confirm these qRT-PCR data on protein level. BM-DCs were treated with HDM, LPS, or DMOG and PHD3 and both transcription factors HIF1 and HIF2 were probed using Western blotting. Although LPS and DMOG readily induced PHD3, we also detected a modest increase upon HDM exposure. This was accompanied by induction of both HIF1 and HIF2 in all conditions (Fig. 1F). Finally, using both chemical and genetic approaches, we found that loss of HIF1 or inhibition of HIF transcriptional activity by the compound chetomin was sufficient to block the LPS-mediated activation of *Phd2*, *Phd3*, and the HIF1 target gene nitric oxide synthase 2 (*Nos2*). A similar trend was observed for HDM treatment (Fig. 1F and Supplemental Fig. 1A and B). In conclusion, HDM activates PHD3 in a HIF-dependent manner in DCs.

Genetic manipulation of PHD3 levels does not alter the DC network in the steady state

To further investigate the role of PHD3 in DCs, we crossed *Phd3^{fl/fl}* and *Rosa26LmPhd3* mice with mice harboring a transgene expressing the Cre recombinase under the control of the CD11c promoter (CD11c-Cre mice) [34]. Examining both *Phd3* transcripts and PHD3 protein in BM-DCs derived from the

Phd3^{fl/fl} $\times$ CD11cCre (PHD3^{CD11c-KO}) and *Rosa26LmPhd3* $\times$ CD11cCre (PHD3^{CD11c-OE}) mice, we confirmed the efficient deletion and overexpression of PHD3 in DCs (Fig. 2A and B). The transgene in PHD3^{CD11c-OE} mice contains an IRES-GFP that allowed us to monitor the overexpression of PHD3 and validate the Cre recombinase targeting specificity using flow cytometry. GFP was readily expressed in lung CD103⁺ and CD11b⁺ DCs. Furthermore, we also distinguished GFP fluorescence in the CD11c⁺ alveolar macrophage (M ϕ). In line with earlier reports [34], we only detected GFP expression in a subset of T cells, whereas Cre-mediated recombination was absent in neutrophils (Fig. 2C). Deficiency or overexpression of PHD3 did not cause defects in DC differentiation or survival in vivo. Indeed, characterization of the DC network and alveolar M ϕ s in the lung and the DC subsets in the spleen did not reveal significant deficiencies (Fig. 2D–I). Altogether, PHD3 was efficiently deleted or overexpressed using the Cre recombinase system. Modulating PHD3 levels in DCs did not alter differentiation or survival in the steady-state lung or spleen.

Lack of PHD3 cripples alveolar M ϕ s to repopulate an empty alveolar niche

Our observations are in contrast with earlier reports showing that loss of PHD3 dramatically reduces the life span of neutrophils [33]. Furthermore, PHD3 deficiency rendered BM-derived M ϕ s resistant against proapoptotic stimuli, indicating a complex role for PHD3 in immune cell survival [35]. To address possible subtle defects in the DC or M ϕ compartment caused by deregulated expression of PHD3, we generated BM chimeric mice by injecting lethally irradiated CD45.1/2 mice with a mixture of 50% WT CD45.1/2 and 50% CD45.2 BM cells from PHD3^{CD11c-KO} or PHD3^{CD11c-OE} origin (Fig. 3A). Repopulation of the irradiated BM by the competing hematopoietic stem cells can be variable (Fig. 3A). To address that issue, neutrophils and monocytes were used as internal controls during the experiments using chimeric mice because these cells do not express CD11c and are not targeted by the Cre recombinase (Fig. 2C). Using this approach, we found that both overexpression and elimination of PHD3 slightly reduced the ability of conventional DCs to compete with their CD45.1/2 counterparts to colonize the spleen and lung (Fig. 3B and C). In contrast, modulation of PHD3 levels did not significantly alter plasmacytoid DC ratios in lung and spleen (Fig. 3B and C). Strikingly, however, deletion of PHD3 markedly reduced the potential of CD45.2 alveolar M ϕ s to occupy the alveolar space (Fig. 3A and B). Intrigued by these findings, we evaluated the levels of the various PHD isoforms in alveolar M ϕ s by qRT-PCR. In contrast to DCs, alveolar M ϕ s only expressed low levels of *Phd3*. (Fig. 3D) Furthermore, alveolar M ϕ s induced *Phd1*, but not *Phd3*, transcripts upon LPS and HDM exposure. This lack of PHD3 expression in alveolar M ϕ s was not due to a general repression of PHD3 because *Phd3* transcripts could be readily detected upon DMOG treatment. In the steady state, alveolar M ϕ s are derived from fetal monocytes that occupy the alveolar space and self-renew throughout life [36]. Upon depletion induced by irradiation, we have previously shown that alveolar M ϕ s are rapidly

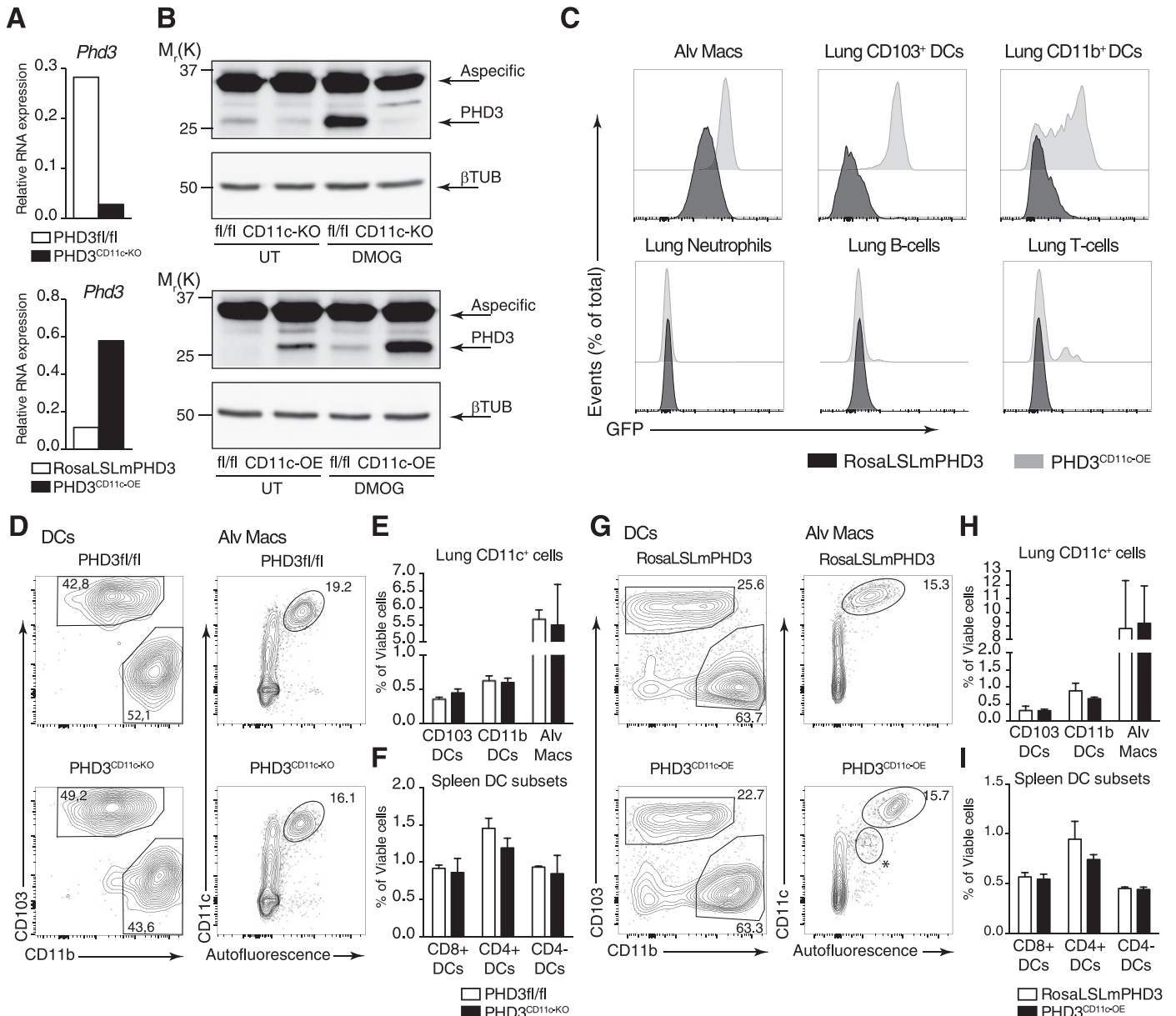
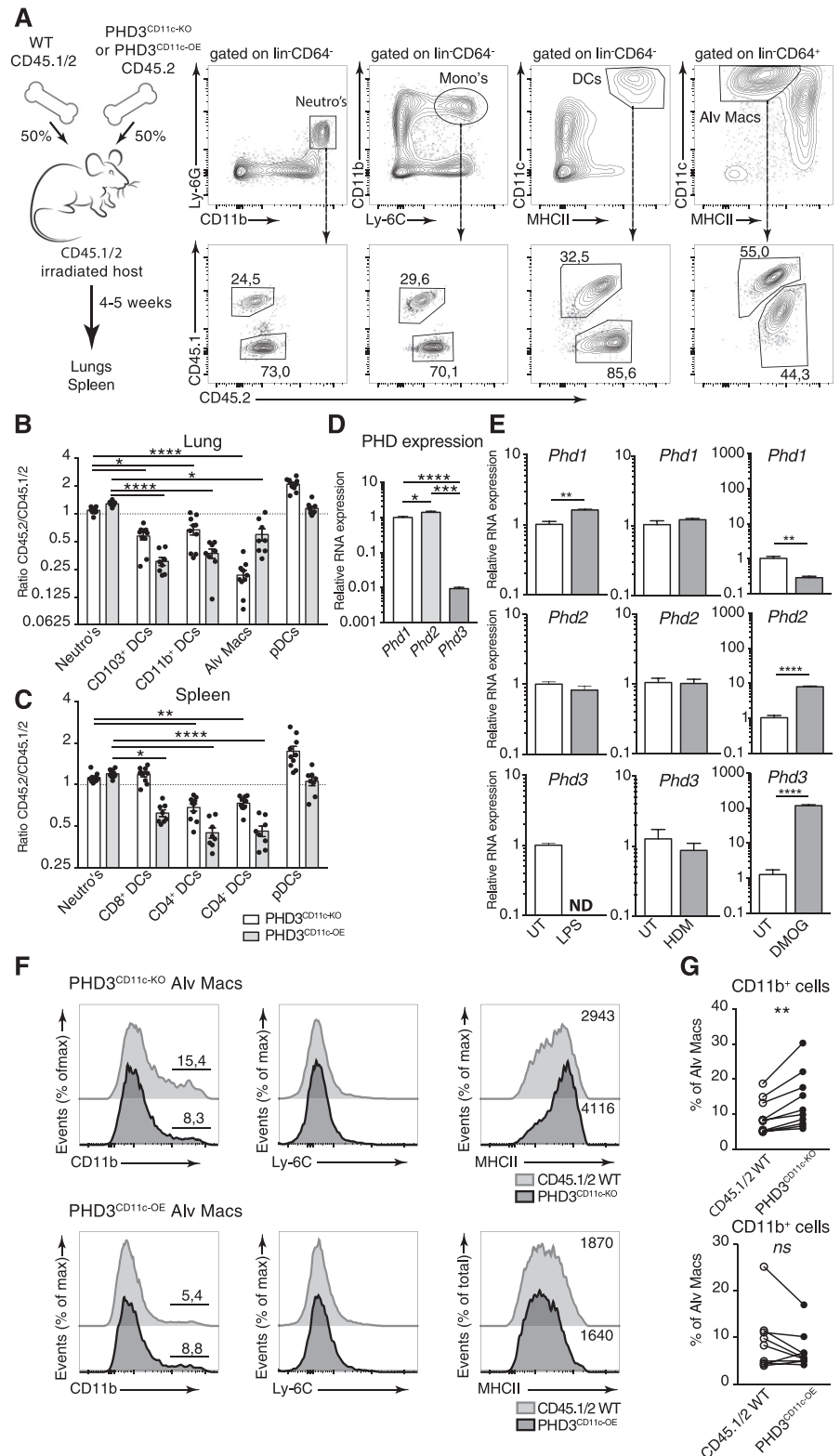


Figure 2. Genetic manipulation of PHD3 levels does not alter the DC network. (A) qRT-PCR of *Phd3* transcripts from BM-DCs of PHD3^{CD11c-KO} and PHD3^{CD11c-OE} mice. Results are normalized to the housekeeping gene *L27* ($n = 1$). (B) Western blot analysis of PHD3 in PHD3^{CD11c-KO} and PHD3^{CD11c-OE} BM-DCs exposed to DMOG. β -tubulin (β TUB) was used as the loading control. (C) Histogram of GFP fluorescence from PHD3^{CD11c-OE} lung derived alveolar M ϕ (Alv Macs), B-cells, T-cells, neutrophils, and CD103⁺ and CD11b⁺ DCs. *Rosa26LsLmPhd3* littermates were used as control mice. (D) Characterization of lung-derived DC subsets and alveolar M ϕ in the *Phd3*^{fl/fl} and PHD3^{CD11c-KO} animals. Dot plots and percentages of a representative animal are depicted. (E) Percentages of DCs and alveolar M ϕ relative to all viable lung cells in *Phd3*^{fl/fl} and PHD3^{CD11c-KO} mice. Means $\pm$ SEM are given ($n = 3$). (F) Percentage of splenic DCs relative to all viable cells in *Phd3*^{fl/fl} and PHD3^{CD11c-KO} mice. Bar graphs represent means $\pm$ SEM ($n = 3$). (G) Characterization of lung DC network and alveolar M ϕ in *Rosa26LsLmPhd3* and PHD3^{CD11c-OE} mice. Data from a representative animal are provided. (H) Relative distribution of DCs and alveolar M ϕ in *Rosa26LsLmPhd3* and PHD3^{CD11c-OE} mice. Means $\pm$ SEM are shown ($n = 3$). The asterisk (*) depicts GFP⁺ DCs present in the channel measuring autofluorescence. (I) Splenic DC architecture in *Rosa26LsLmPhd3* and PHD3^{CD11c-OE}. Percentages are shown as means $\pm$ SEM ($n = 3$).

repopulated from circulating Ly-6C⁺ blood monocytes, which gradually lose expression of Ly-6C and CD11b, and gain expression of CD11c, SiglecF, and F4/80 [36, 37]. We, therefore, further characterized these alveolar M ϕ in mixed BM chimeric mice and found that, although Ly-6C expression was efficiently lost in both WT and PHD3^{CD11c-KO} alveolar M ϕ ,

some of the alveolar M ϕ s still retained CD11b expression, reminiscent of their monocyte origin [36]. This fraction was greater in the PHD3^{CD11c-KO} alveolar M ϕ s (Fig. 3F and G), suggesting a greater turnover of the PHD3^{CD11c-KO} population or delayed maturation. Furthermore, the alveolar M ϕ s with a PHD3^{CD11c-KO} origin also expressed less MHCII. In the

Figure 3. BM chimeras reveal opposing effects of PHD3 on DCs and alveolar Mφs. (A) Graphical representation of the PHD3^{CD11c-KO} and PHD3^{CD11c-OE} BM chimera setup and gating strategy. Representative sample of PHD3^{CD11c-KO} BM chimeric mice is given (B) Ratios of lung-derived CD45.2 (PHD3^{CD11c-KO} and PHD3^{CD11c-OE}) neutrophils, alveolar Mφ, pDCs, and CD103⁺ or CD11b⁺ DCs over CD45.1/2 WT counterparts in irradiated CD45.1/2 WT hosts. (C) Ratios of CD45.2 (PHD3^{CD11c-KO} or PHD3^{CD11c-OE}) splenic neutrophils and DC subsets over CD45.1 WT counterparts in irradiated CD45.1/2 WT hosts. Ratios shown are relative to the ratios of CD11c⁺ neutrophils and monocytes. Bar graphs (means ± SEM) and individual data points are shown (*n* = 8). Kruskal-Wallis test corrected for multiple comparisons (Dunn's test). **P* < 0.05; ***P* < 0.01; ****P* < 0.0001. (D) qRT-PCR of *Phd1-3* transcripts in alveolar Mφs. Results are normalized to the housekeeping gene *L27* (*n* = 3). (E) qRT-PCR of *Phd1-3* transcripts in LPS-, HDM-, or DMOG-treated alveolar Mφs. Results are normalized to the housekeeping genes *L27* and *Hprt* (*n* = 3). Bar graphs represent means ± SEM. Unpaired Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. (F) Flow cytometric analysis of CD11b, Ly-6C and MHCII expression in PHD3^{CD11c-KO} and PHD3^{CD11c-OE} alveolar Mφ (Alv Mφs). Histograms of a representative sample are given. Figures denote percentage of positive cells or geometrical mean fluorescence. (G) Before-and-after graphs representing the paired data for CD11b⁺ alveolar Mφ in PHD3^{CD11c-KO} and PHD3^{CD11c-OE} Mφs. Wilcoxon test. ***P* < 0.01.



PHD3^{CD11c-OE} BM chimeric mice, we observed a trend for fewer CD11b⁺ cells in the transgenic population, although that was not statistically significant. Similarly, there was a tendency for greater MHCII expression in the PHD3^{CD11c-OE} alveolar Mφs

(Fig. 3F and G). Altogether, our results indicate that loss or overexpression of PHD3 largely preserves the splenic and lung DC network, whereas alveolar Mφs seem to rely uniquely on PHD3 for efficient population of the alveolar space.

Genetic regulation of PHD3 levels does not affect DC metabolism, maturation, and survival

Next, we examined the functional consequences of loss or overexpression of PHD3 in DCs. In Mφs, PHD3 is considered a defining marker for M1-polarized Mφs, and the absence of PHD3 limits the proinflammatory cytokine production [38, 39]. Using escalating doses of LPS as an activator of DCs, we observed increasing expression of the costimulatory surface markers CD40 and CD86. However, compared with littermate controls, we were unable to detect differences in the PHD3^{CD11c-KO} and PHD3^{CD11c-OE} DCs (Fig. 4A and B). Furthermore, secretion of the cytokines IL-6, IL-12-p70, and TNF was significantly induced upon LPS stimulation but was largely unaltered among the

different genotypes (Fig. 4C and D). Keeping the role of PHD3 in mind in the differentiation and survival of immune cells, we checked the survival of DCs under conditions of LPS stimulation or Hx. Using the uptake of DAPI as a discriminator between viable and dead cells, we found that Hx significantly decreased the percentage of viable cells in culture. Although cell death during hypoxic conditions was reversed by the coculture of BM-DCs with LPS, PHD3 expression did not alter the survival of DCs under those conditions (Fig. 4E and F). Finally, we analyzed HIF1 protein levels in PHD3-deficient or -overexpressing DCs during escalating exposures to LPS. Although we observed decreased HIF1 levels after 24 h of LPS treatment, that difference was only temporal and could not be observed after 36 h (Supplemental

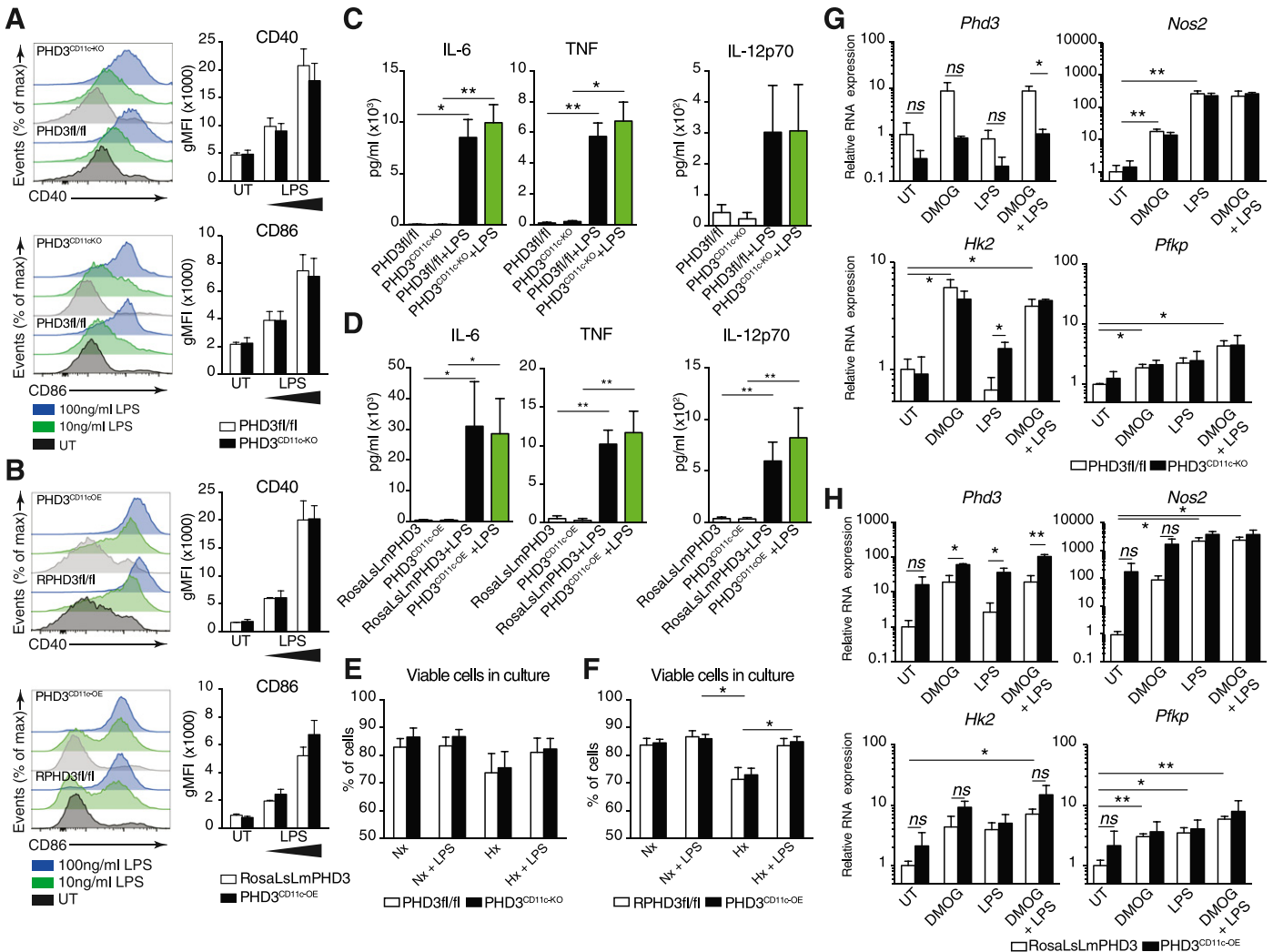


Figure 4. Genetic regulation of PHD3 levels does not affect DC metabolism, maturation, and survival. (A and B) CD40 and CD86 surface expression in BM-DCs derived from *Phd3*^{fl/fl}, *PHD3*^{CD11c-KO}, *Rosa26LmPhd3*, and *PHD3*^{CD11c-OE} animals. BM-DCs were subjected to 10 or 100 ng/ml LPS. Bar graphs represent means ± SEM. (*n* = 3). (C and D) Production of IL-6, IL-12p70, and TNF-α cytokines by *Phd3*^{fl/fl}, *PHD3*^{CD11c-KO}, *Rosa26LmPhd3*, and *PHD3*^{CD11c-OE} BM-DCs after stimulation with LPS, as measured by ELISA. Bar graphs represent means ± SEM. (*n* ≥ 4) Kruskal-Wallis test with Dunn's test correcting for multiple comparisons. **P* < 0.05; ***P* < 0.01. (E and F) Percentage of DAPI⁺ BM-DCs of *Phd3*^{fl/fl}, *PHD3*^{CD11c-KO}, *Rosa26LmPhd3*, and *PHD3*^{CD11c-OE} mice after exposure to normoxia (Nx), LPS and/or Hx. Bar graphs represent means ± SEM. (*n* ≥ 6). Kruskal-Wallis test with Dunn's test correcting for multiple comparisons. **P* < 0.05. (G and H) qRT-PCR of *Phd3*, *Nos2*, *Hk2*, and *Pfkfb3* transcripts in LPS- and/or DMOG-treated *PHD3*^{CD11c-KO} and *PHD3*^{CD11c-OE} BM-DCs. Results are normalized to the housekeeping genes *L27* and *Hprt* (*n* = 3). Bar graphs represent means ± SEM. Unpaired Student *t*-test. **P* < 0.05; ***P* < 0.01.

Fig. 2A and B). These findings were validated by analyzing the regulation of the HIF1-dependent gene *Nos2* and the glycolytic enzymes *Hk2* and *Pfkfb*. DCs increased *Nos2*, *Hk2*, and *Pfkfb* transcripts in presence of LPS and DMOG, but neither the deletion nor overexpression of PHD3 significantly affected expression of those genes (Fig. 4G and H).

Genetic manipulation of PHD3 does not modulate HDM-driven asthma

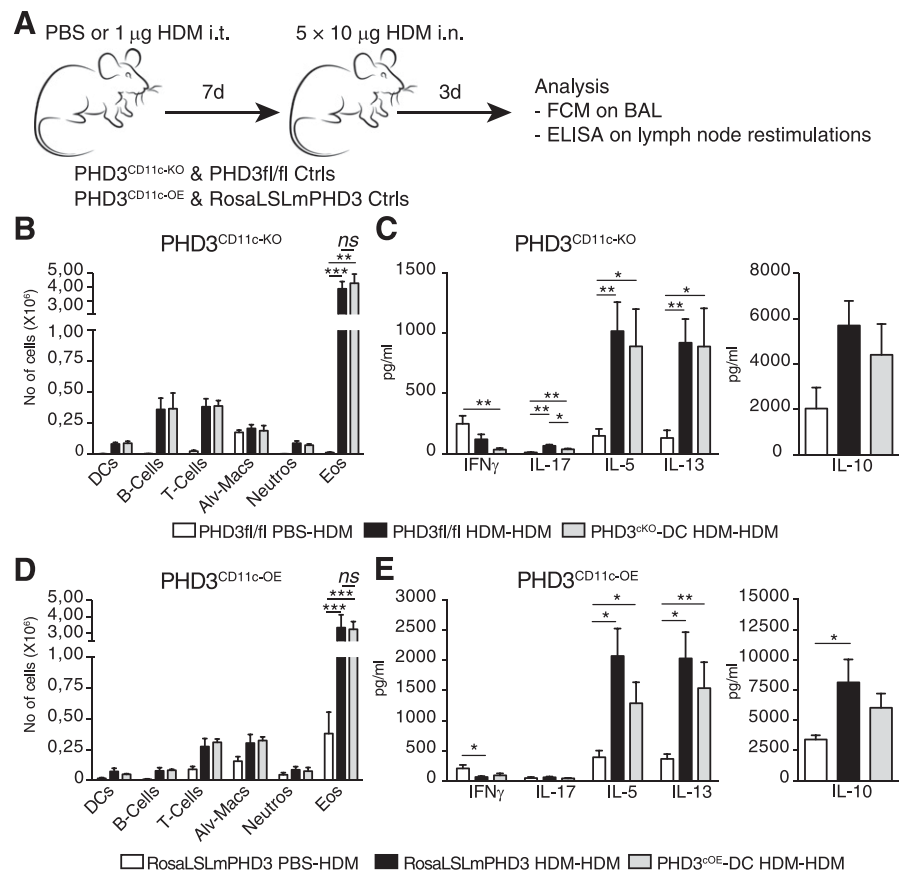
Having found that HDM induces PHD3 in DCs and having described the effects of PHD3 deficiency or overexpression in CD11c^{hi} cells in vivo and DCs in vitro, we next investigated the importance of the PHD3 isoform in CD11c^{hi} cells in asthma development. To test that, we sensitized PHD3^{CD11c-KO} or PHD3^{CD11c-OE} mice and their respective littermate controls i.t. to 1 µg of HDM, and after 1 wk, challenged them for a consecutive 5 d with i.n. application of 10 µg of HDM. Inflammation was assessed by flow cytometry analysis of the immune cell infiltration of the BALF and by quantification of the cytokine production in the HDM-restimulated cells of the MLN (Fig. 5A). This model results in a strong Th2-driven inflammatory response, as observed by Th2 cytokine production and eosinophilia in the BALF. Loss or overexpression of PHD3 in CD11c^{hi} cells did not significantly alter lung eosinophilia or Th2 cytokine production in the MLN, although we could observe a minor, but significant, decrease in Th1 and Th17 cytokines in the absence of PHD3 (Fig. 5B–E). We next hypothesized that, rather than PHD3,

the other PHD isoforms could be involved in the pathogenesis of asthma. Applying an identical experimental approach as outlined above, we generated the PHD1^{CD11c-KO}, PHD1^{CD11c-OE}, PHD2^{CD11c-KO}, and PHD2^{CD11c-OE} mouse strains. Both the DC network in the lung interstitium and the alveolar Mφ population were unaffected by the genetic deletion or overexpression of PHD1 or PHD2 (Supplemental Fig. 3A–D). Similar to PHD3, the genetic modulation of PHD isoform levels in CD11c^{hi} cells did not alter asthma development (Supplemental Fig. 3A–D).

HDM sensitization by DCs is not influenced by pan-PHD inhibition

The enzymatic redundancies between PHD3 and PHD2 possibly interfere in the outcome of the aforementioned model [40]. Conversely, the absence of pro- or anti-inflammatory effect of PHD3 modulation in CD11c^{hi} might be due to opposing effects on immunostimulatory DCs or immunoregulatory alveolar Mφs. Consequently, we turned our attention to an alternative strategy. We have previously shown that the adoptive transfer of minute numbers of activated Ag-loaded DCs into the lungs of mice is sufficient to break tolerance and induce airway inflammation [41, 42]. By instilling 10⁴ HDM-treated DCs i.t., we were able to sensitize mice to HDM challenge, as reflected by induction of airway eosinophilia upon HDM challenge (Fig. 6A). DMOG, a 2-oxoglutarate analog, is a competitive inhibitor of the PHD hydroxylation capacity, and in vitro incubation of DCs with DMOG effectively inhibited PHD function and stabilized HIF1α

Figure 5. Genetic manipulation of PHD3 does not modulate HDM driven asthma. (A) Graphical representation of HDM-AAI model used in the characterization of the PHD3^{CD11c-KO} and PHD3^{CD11c-OE} mice. (B) Flow cytometry (FCM) analysis of BALF cellularity in HDM- or PBS-sensitized/HDM challenged *Phd3*^{fl/fl} or PHD3^{CD11c-KO} mice. Bar graph depicts absolute cell counts of distinct immune cell population (means ± SEM; n = 6–8). (C) Secreted cytokines in cultures of MLNs of *Phd3*^{fl/fl} or PHD3^{CD11c-KO} mice, restimulated for 3 d with HDM and, subsequently, measured by ELISA (n = 6–8). (D). Absolute cell counts of immune cell populations in BALF of *Rosa26LmPhd3* and PHD3^{CD11c-OE} mice, subjected to HDM-AAI protocol. Bar graph shows means ± SEM. Mann-Whitney U test. **P < 0.01; ***P < 0.01 (n = 7–8). (E) Cytokine production in MLN restimulation cultures of *Rosa26LmPhd3* and PHD3^{CD11c-OE} mice. MLNs were restimulated for 3 d with HDM, and cell supernatant was analyzed by ELISA (n = 6–7). Means ± SEM are given. Mann-Whitney U test. *P < 0.05; **P < 0.01.



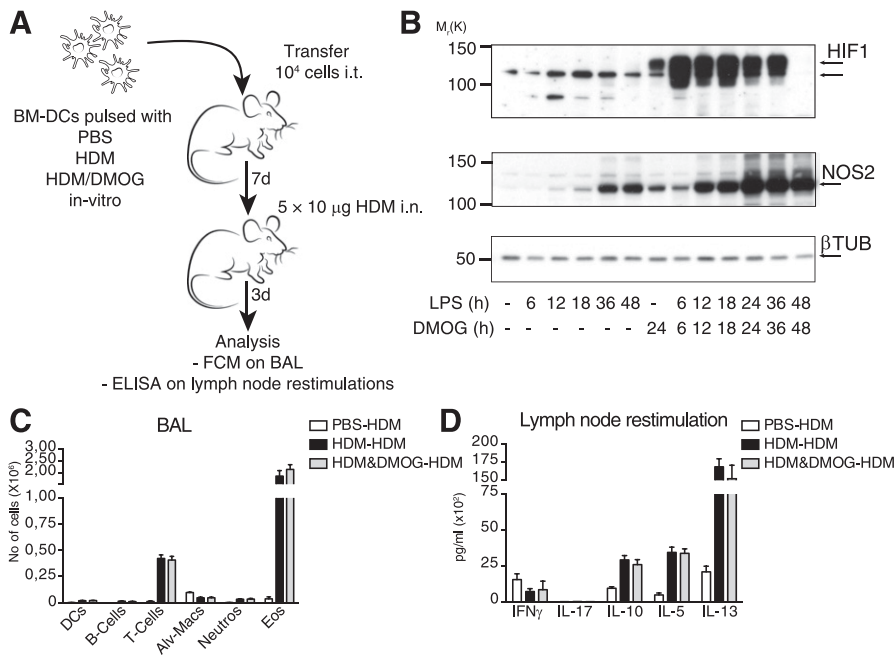


Figure 6. HDM sensitization by DCs is not influenced by PHD inhibition. (A) Graphical representation of the adoptive transfer of HDM- or HDM/DMOG-treated BM-DCs, HDM challenge and analysis of HDM-AAI model. (B) Western blot analysis of NOS2 and HIF1 protein levels in BM-DCs, exposed to LPS and/or DMOG for varying times. β -tubulin (β TUB) served as the loading control. (C) Flow cytometry analysis of BALF obtained from mice sensitized by the adoptive transfer of HDM- or HDM/DMOG-exposed BM-DCs. Bar graph shows means $\pm$ SEM ($n = 5-6$). (D) Cytokine profile of MLN restimulation cultures of mice as treated in C. MLNs were restimulated for 3 d with HDM, and cell supernatants were analyzed by ELISA ($n = 5-6$). Means $\pm$ SEM are given. Mann-Whitney U test. $*P < 0.05$; $**P < 0.01$.

protein. NF- κ B-dependent mRNA transcription in LPS-activated DCs similarly increased HIF1 α protein levels. Interestingly, both stimuli had a synergistic effect on HIF1 α and its target gene *NOS2* (Fig. 6B). To test whether inhibition of all PHDs in DCs altered the course of HDM-induced asthma, we injected mice i.t. with DCs cultured in medium, HDM, or HDM and DMOG and, subsequently, challenged the mice i.n. with HDM. Inhibiting function of all PHD isoforms in DCs during the sensitization did not change inflammatory parameters, as quantified by similar levels of eosinophilia in the BALF and by Th2 cytokine production in the draining MLN (Fig. 6C and D). These data show that even pan-inhibition of all 3 PHD isoforms in DCs does not suppress the capacity of these cells to induce Th2 immunity that leads to asthma.

DISCUSSION

At the current stage, our therapeutic armamentarium is insufficient to control disease in a many patients with asthma. This unmet clinical need has fueled the search for novel treatments and revealed the PHD/HIF signaling pathway as a potential target. Collectively, there is good evidence that implicates PHD/HIF signaling in the pathogenesis of allergic asthma. An unbiased proteomics study by Fajardo et al. [22] revealed the accumulation of Hx-inducible glycolytic proteins in the allergic lung, and both HIF1 α and HIF2 α were identified in the BALF of allergen-challenged patients with asthma [23]. Furthermore, the prototypic HIF target gene, VEGF α , is highly expressed in patients with asthma and is involved in peribronchial angiogenesis and enhanced vascular permeability during allergic airway remodeling [43]. Pre-clinical models of asthmatic disease have confirmed the anti-inflammatory properties of HIF blockade because both genetic ablation and chemical inhibition of HIF reduces airway inflammation [23, 24, 44, 45].

Recently, our notion of PHD/HIF signaling at the mucosal barrier has become more refined. Epithelial expression of HIF has important anti-inflammatory features, regulating epithelial cell survival and preserving mucosal integrity and inhibiting inflammatory cell recruitment [46–48]. In M ϕ s, polarization is associated with specific HIF and PHD isoform expression, and defective PHD/HIF signaling perturbs both proinflammatory and regulatory properties [9, 38, 39, 49, 50]. Relevant to asthma, loss of HIF1 in interstitial M ϕ s reduces IL-10 production and exacerbates airway eosinophilia [51]. Furthermore, HIF-mediated metabolic reprogramming is inherent to T cell activation, skewing, and memory control [52]. HIF1 not only orchestrates the balance of Th17 and FoxP3 $^{+}$ T $_{reg}$ cells but also stabilizes T $_{reg}$ identity and limits Th1 responses [53]. These pleiotropic functions of the PHD/HIF pathway indicate that, although the net result of PHD/HIF blockade in allergic airway inflammation is clear, the molecular mechanisms and the cellular targets are not.

DCs are critical sensors of the extracellular environment and inducers of the allergic response in the lung via PHD enzymes, and the HIF pathway in those cells has not been characterized in detail. In this study, we found that PHD2 and PHD3 are the main PHD isoforms expressed in DCs. Stimulation with the allergen HDM, Hx, or LPS strongly enhanced the expression of PHD3. This was associated with the stabilization of HIF1 α and the activation of the glycolytic pathway. PHD3 not only targets HIF1 α for proteasomal degradation but also acts as a coregulator of HIF1 α gene expression [54]. Furthermore, a recent, unbiased proteomics screen identified several new substrates of the PHD3 hydroxylase, broadening its role as a regulator of the cell signaling machinery. Of interest, PHD3 substrates were enriched for members of the NF- κ B and Wnt signaling pathways, suggesting that PHD3 could regulate the activation of DCs in an oxygen-dependent manner [55]. To elucidate the role of PHD3 in DC function, we opted for

conditional deletion or overexpression by crossing the *Phd3^{fl/fl}* and *Rosa26^{LSLmPhd3}* mice with the Cd11c-Cre mice. Deficiency or overexpression of PHD3 did not alter the maturation of DCs at basal levels or after activation with LPS. Furthermore, cytokine production was largely unaltered and Ag uptake and processing did not seem to be affected [unpublished results]. This is in contrast with the reduced maturation and IFN production observed in the HIF1 α -deficient DCs [7, 56]. Similar to what is observed in PHD3-deficient neutrophils and M ϕ s, HIF target genes were unaltered in DCs lacking or overexpressing PHD3, suggesting that PHD3 in DCs is not the main regulator of HIF1 α [33, 35].

Research in the cancer field has demonstrated an unexpected role for PHD3 in cell survival [57]. In immune cells, its function is pleiotropic because PHD3 is critical for neutrophil survival during Hx but sensitizes M ϕ s for apoptosis [33, 35]. In our hands, PHD3 was largely redundant for DC survival in vivo, although overexpression of PHD3 resulted in a minor disadvantage toward repopulating the peripheral tissue after whole-body irradiation when cells had to compete with WT cells. Importantly, this can also be attributed to deficiencies in other biologic processes, such as altered migration or differentiation. In that respect, DC development of PHD3^{CD11c-KO} and PHD3^{OE}-DC BM in vitro was indistinguishable from their WT littermates, and PHD3 levels did not influence the reduced survival of DCs in hypoxic conditions.

An unexpected finding from our study was the role that PHD3 seems to play in development of alveolar M ϕ . After whole-body irradiation that causes genotoxic stress to self-repopulating and long-lived, embryo-derived, resident alveolar M ϕ s, this pool of cells can be replenished by circulating monocytes [36, 37, 58]. Loss of PHD3 reduced the capacity of repopulating monocytes to reconstitute the alveolar M ϕ compartment after whole-body irradiation. Considering that the CD11c⁺ monocytes repopulating the alveolar space only acquire CD11c upon differentiation into alveolar M ϕ s, these observations hint to a possible defect in the terminal differentiation of these cells in the absence of PHD3 [37]. In support of that, we found, indeed, that cells lacking PHD3 had less down-regulation of CD11b, a hallmark of differentiated alveolar M ϕ s [36, 37] and had less expression of MHCII molecules. We can only speculate as to why loss of PHD3 would disfavor alveolar M ϕ development. In their exposed position, alveolar M ϕ s are exposed to high oxygen tensions, leading to high demands on the reducing power in the cell. This is reflected by the fact that alveolar M ϕ s are one of the most autofluorescent cells of the body. Autofluorescence is caused by the presence of reduced NADH, NADPH, and oxidized flavin adenine dinucleotide [59]. We speculate that adequate PHD3 levels in alveolar M ϕ s are needed to deal with this oxygen-rich environment and its associated risk of reactive oxygen species production or needed for tuning the right metabolism (glycolysis vs. mitochondrial respiration) in the harsh environment of the alveolus. Our findings in alveolar M ϕ are in marked contrast with earlier studies demonstrating a proapoptotic effect of PHD3 in cultured human M ϕ s [35], which might again be due to tissue-specific hard wiring of the PHD molecules and metabolism of M ϕ s.

Ultimately, inducing AAI in the PHD3^{CD11c-KO} and PHD3^{CD11c-OE} mice failed to reveal a role for PHD3 in asthma development.

Because other PHD isoforms in CD11c^{hi} cells could be involved in asthma pathogenesis, we also tested the importance of PHD1 and PHD2 but failed to observe differences in airway inflammation. Furthermore, the opposing effects of PHD3 on DCs and alveolar M ϕ s could obscure a role for PHD3 in DCs in asthma development because DCs promote AAI, whereas alveolar M ϕ s suppress it [60]. Using an adoptive-transfer model of HDM-AAI, we tested whether the absence of differences in inflammation could be attributed to redundancy between PHD isoforms or diverging effects on DCs and alveolar M ϕ s. Preincubation of DCs with HDM and the pan-hydroxylase inhibitor DMOG before adoptive transfer into naïve, recipient mice failed to modulate airway inflammation. Endotoxin-activated myeloid cells rapidly enhance glycolysis [30], shunt metabolic intermediates of the Krebs cycle to the cytosol [61], and enhance mitochondrial reactive oxygen species production [62]. These various cellular changes inhibit PHD function, stabilize HIF proteins, and ultimately enhance inflammatory responses [63]. Although promoting PHD enzymatic activity to limit allergic lung inflammation represents an attractive therapy, this strategy currently lies beyond our possibilities.

In conclusion, we found clear evidence for regulated expression of PHD3 in 2 types of APCs. Although loss of PHD3 affected the homeostasis of alveolar M ϕ s, it did not alter asthma development driven by DCs. Given the complex nature of PHD/HIF signaling in the allergic lung, and the many cells that have a role in this disease [15], more research will be needed to elucidate the cellular and molecular mechanism of HIF inhibition and its potential in asthma treatment.

AUTHORSHIP

S.J.T., N.V., S.J., and B.N.L. designed the research. S.J.T., N.V., and J.V. performed the experiments. S.J.T. and N.V. analyzed the results. P.C. provided essential reagents for the study. S.J.T., S.J., and B.N.L. wrote the manuscript.

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DISCLOSURE

The authors declare no conflicts of interest.

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Opposing regulation and roles for PHD3 in lung dendritic cells and alveolar macrophages

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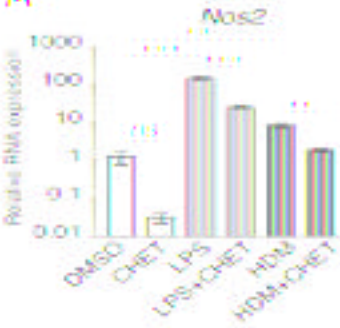
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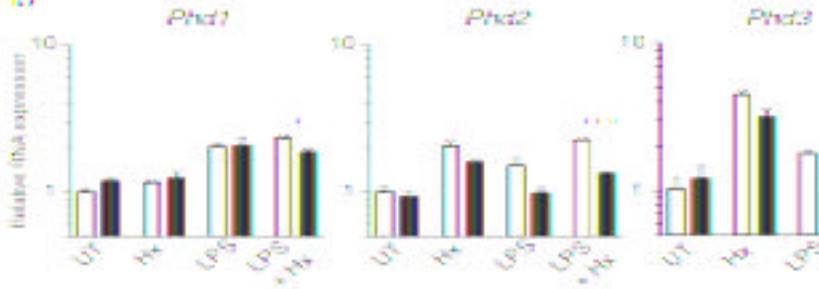
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Supplemental Figure 1

A



B

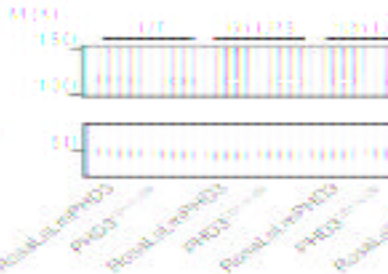


Supplemental Figure 2

A



B



Supplemental Figure 3

