HIF2α is a Direct Regulator of Neutrophil Motility

Sundary Sormendi[1], Mathieu Deygas[2,3], Anupam Sinha[1], Anja Krüger[1], Ioannis Kourtzelis[1], Gregoire Le Lay[2,3], Mathilde Bernard[2,3], Pablo J. Sáez[2,3], Michael Gerlach[4], Kristin Franke[1], Ana Meneses[1], Martin Kräter[5], Alessandra Palladini[6], Jochen Guck[5], Ünal Coskun[6], Triantafyllos Chavakis[1], Pablo Vargas[2,3], Ben Wielockx[1]

[1]Institute of Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, 01307 Dresden, Germany. [2] Institut Curie, PSL Research University, CNRS, UMR 144, Paris, France, [3]Institut Pierre-Gilles de Gennes, PSL Research University, Paris, France, [4]Core Facility Cellular Imaging (CFCI) at the Faculty of Medicine Carl Gustav Carus, Fetscherstraße 74, 01307 Dresden, Germany. [5]Biotechnology Center, Center for Molecular and Cellular Bioengineering, Technische Universität Dresden, 01307 Dresden, Germany. [6]Paul Langerhans Institute Dresden of the Helmholtz Zentrum Munich at the University Hospital and Faculty of Medicine Carl Gustav Carus of TU Dresden, Germany.

Authorship note: P.V. and B.W. jointly supervised this work.

Correspondence: Pablo Vargas, Systems Biology of Cell Polarity and Cell Division, Institut Curie (UMR144) & Institut Pierre Gilles de Gennes (IPGG), 6 rue Jean Calvin, 75005 Paris, France., e-mail: Pablo.Vargas@curie.fr , Phone: +33.1.40795895 and Ben Wielockx, Institute of Clinical Chemistry and Laboratory Medicine, Technical University Dresden, Fetscherstrasse 74, 01307 Dresden, Germany, e-mail: Ben.Wielockx@tu-dresden.de, Phone: +49.351.45816260

Abstract

Orchestrated recruitment of neutrophils to inflamed tissue is essential during initiation of inflammation. Inflamed areas are usually hypoxic, and adaptation to reduced oxygen pressure is typically mediated by hypoxia pathway proteins. However, it is still unclear how these factors influence the migration of neutrophils to and at the site of inflammation either during their transmigration through the blood-endothelial cell barrier, or their motility in the interstitial space. Here, we reveal that activation of the Hypoxia Inducible Factor-2 (HIF2α) due to deficiency of HIF-prolyl hydroxylase domain protein-2 (PHD2) boosts neutrophil migration specifically through highly confined microenvironments. *In vivo*, the increased migratory capacity of PHD2-deficient neutrophils resulted in massive tissue accumulation in models of acute local inflammation. Using systematic RNAseq analyses and mechanistic approaches, we identified RhoA, a cytoskeleton organizer, as the central downstream factor that mediates HIF2α-dependent neutrophil motility. Thus, we propose that the here identified novel PHD2-HIF2α-RhoA axis is vital to the initial stages of inflammation as it promotes neutrophil movement through highly confined tissue landscapes.

Introduction

In the innate immune response, neutrophils represent the first line of protection against infections, extravasating quickly from circulation to inflamed tissues for fast pathogen elimination. This process necessitates transit from an oxygen-rich circulatory system to the inflammation site, which is typically hypoxic due to vasculature damage and/or high metabolic demand of pathogens and host cells.[1] Thus, neutrophil adaptation to low oxygen levels is crucial during the early phases of the inflammatory response.

Under hypoxic conditions, the transcription factors Hypoxia Inducible Factor-1 (HIF1α) and its isoform HIF2α are key elements that control immune cell metabolism and function,[2-7] and importantly, HIF activity is controlled by a class of oxygen sensors known as the HIF prolyl-hydroxylase domain enzymes (PHD1-3) (reviewed in [8,9]). When oxygen levels decrease, PHDs get inactivated, which results in HIFα stabilization and transcription of relevant target genes. Interestingly, HIF1α-deficiency results in subdued inflammation[2,3] while, inversely, PHD inactivation and/or HIFα stabilization leads to enhanced neutrophil survival,[4,10] chemotaxis and degranulation (reviewed in[11]). Although both HIFα subunits have overlapping activities, unique roles for HIF2α, including in neutrophil function, have been reported.[4-6]

Over the past decade, several mechanisms have been shown to participate in the multi-step recruitment of neutrophils from circulation to sites of infection or inflammation.[12-14] The recruitment process requires cell plasticity because cells deform as they move through the blood-endothelial cell barrier and the confined areas of interstitial tissues. Leucocyte migration through these microenvironments is orchestrated by actin polymerization regulators, such as the rho GTPases RhoA, Cdc42 and Rac1.[15-18] In this context, HIF1α expression has been suggested to modulate both functional changes in the cytoskeleton and metabolic reprograming [19-22]. Importantly, disruption of mechanisms that control neutrophil infiltration in tissues is

74 associated with sepsis, a life-threatening condition with multi-organ failure and one of the
75 leading causes of death in the intensive care unit (ICU).[23] Conversely, till date, no effective
76 therapeutic strategies are available for mitigating an uncontrolled neutrophilic inflammatory
77 response.

78 In this study, we address the effects of PHD2-deficiency on the motility of neutrophils,
79 including their recruitment during localized inflammation. Using *ex vivo* and *in vivo* imaging
80 in a variety of highly confined microenvironments, we demonstrate for the first time that HIF2α
81 over-activation enhances the migratory capacity of neutrophils in a chemotaxis-independent
82 manner. Through whole transcriptome analysis and combined migratory regulation, we
83 describe a role for the PHD2-HIF2α-RhoA axis in the prompt initiation of the innate immune
84 response.
85

86 **Materials and methods**

87 **Mice**

88 All mouse strains were housed in our local mouse facility under specific pathogen-free
89 conditions. Experiments were performed with male and female mice at the age of 8 to 12 weeks.
90 Vav:cre-PHD2$^{f/f}$ (cKO P2) and Vav:cre-PHD2/HIF2$^{ff/ff}$ (cKO P2H2) mouse lines were created
91 in our laboratory, using PHD2$^{f/f}$,[24] Vav:cre[25] (generous gift from Dr. Graf, Spain) and/or
92 HIF2α$^{f/f}$.[26] All offspring were born in normal Mendelian ratios and individual floxed lines have
93 been previously backcrossed to C57BL/6J for at least 9 times. WT controls in all experiments
94 were Cre-negative littermates without any chimerism (partial deletion of floxed genes in early
95 blastomeres).[25] Mice were genotyped using primers described in supplemental Table 1 and
96 knock-down efficiency confirmed via qRT-PCR on isolated neutrophils (supplemental Figure
97 1A, C) and/or genomic PCR on ear biopsies.[27] KRN TCR transgenic mice were inter-crossed

with NOD Shilt/J mice (Charles River, Italy) to generate K/BxN mice as described previously.[28] A detailed description of the inflammation models can be found as supplemental data. Breeding of all mouse lines and animal experiments were in accordance with the local guidelines on animal welfare and were approved by the Landesdirektion Sachsen, Germany.

Histological analysis

5µm thick cryo-sections from 24-hour PMA-treated ears or 7µm thick cryo-sections of knees from 5-day K/BxN-treated mice were were incubated for 1 hour at 37 °C with primary antibodies to detect Gr1+ neutrophils or $cCas3^+$ apoptotic cells. Imaging was performed using an epi-fluorescence microscope with Zeiss EC Plan-Neofluar objectives. Number of $Gr1^+$ cells per tissue area and percentage of $cCas3^+$ in $Gr1^+$ cells were quantified using *Zen* software Version 3.1 (see supplemental Table 2 for more information on antibodies).

Flow cytometry

Immune cell profile of synovial fluid from 5-day K/BxN-treated arthritic knee joints was assessed via FACS performed on LSRII (Becton Dickinson), and cell numbers were counted on MACS quant (Miltenyi). After knee isolation, digestion to extract the cellular compartment of the synovial cavity was performed using collagenase D, Dispase II, and DNAse I in DMEM. Knees were incubated for 30 minutes at 37 °C the supernatant was centrifuged, washed and single cells stained for specific myeloid cell markers using the following fluorophore-conjugated antibodies for 30 minutes at +4 °C (see supplemental Table 2 for more information on antibodies).

121 **Bone Marrow-Derived neutrophils (BMDN)**

122 BMDNs were obtained by crushing long bones the bones in 5% FCS using a mortar and either
123 isolated by negative selection using the EasySep Mouse Neutrophil Enrichment Kit (Stemcell
124 Technologies) or by positive selection using biotinylated antibodies (see supplemental data for
125 more details).

126

127 **1-D and 2-D confined migration in micro-channels**

128 Customized polydimethylsiloxane (PDMS) micro devices containing micro-channel areas and
129 2D-free areas were used to study cell migration in highly confined environments as described
130 previously.[29] PDMS micro-chips were coated with fibronectin (10µg/ml), their nuclei pre-
131 labelled with Hoechst for 30 minutes at 37°C and 10^5 neutrophils (in 5µl) loaded in 3µm-
132 diameter wells. Migrating neutrophils were imaged by video-microscopy (Leica DMI8).
133 Images were analysed using *Fiji* software[30] and a customised script was used to create
134 kymographs for individual migrating cells within the micro-channels. Cell speed was
135 calculated using *MATLAB* software (The MathWorks, Inc). 2D-confined random migration
136 was analysed using *Imaris* (Bitplane) cell tracking software. Where indicated and prior to their
137 loading in the PDMS micro-device, cells were resuspended in media containing 1 µM of CCG-
138 100602 (from Sigma), 1 µM of ML141 (from Sigma) or 1 µM of the Cell permeant C3
139 transferase (from Cytoskeleton).

140

141 **Cell migration in 3D collagen matrices**

142 Neutrophil migration in a 3D environment was evaluated in customized PDMS chambers filled
143 with varying concentrations of a collagen matrix (3-5 mg/ml) containing $2x10^6$ neutrophils/ml.

After polymerization of the collagen matrix, phase imaging was performed using video-microscopy (DMI8). For the chemotaxis assay, CXCL2 (20ng/ml) was added locally to the matrix.

Rho-GTPase activity assays

The activity of Activated RhoA, Rac and Cdc42 was measured in lysates from negatively sorted neutrophils from all genotypes by performing respective activation G-LISA assays (RhoA, Rac1,2,3 and Cdc42 G-LISA Activation Assays; Cytoskeleton) on fibronectin-coated plates as per manufacturer's protocol.

Statistics

Data and graphs represent mean ± SEM of representative experiments. Statistical significance was calculated using the Mann Whitney U test (unpaired) or the Wilcoxson matched-pairs signed rank test (paired) using GraphPad Prism (v7.02 or higher); *$p<0.05$ was considered statistically significant.

Data Sharing Statement

RNAseq data are available at GEO (**GSE151703**).

Additional data may be found in a data supplement available with the online version of this article.

For original data, please contact Pablo.Vargas@curie.fr or Ben.Wielockx@tu-dresden.de

Results

PHD2-deficient neutrophils display enhanced migration in highly confined environments in a HIF-2α-dependent manner

Although changes in the hypoxia pathway are involved in multiple stages of the inflammatory response, details on how the PHD/HIF axis governs neutrophil migration remain elusive. Given that PHD2 is a central regulator of the hypoxia response, we studied the motility of PHD2-deficient BMDNs isolated from vav:cre-PHD2$^{f/f}$ mice (henceforth denoted as cKO P2; supplementary Figure 1A). Initially, *1D migration assays* in polydimethylsiloxane (PDMS) micro-channel devices of different levels of constriction (channel widths of 3, 4 or 5μm) were used to characterize the migratory capacity of individual neutrophils (Figure 1A).[17,18,31-34] Interestingly, cKO P2 neutrophils moved significantly faster than their WT counterparts, but only in the most confined channels (Figure 1B and Supplemental Figure 1B). To identify downstream effectors of this phenotype, we evaluated the contributions of HIF2α, a PHD2 target and a central factor in inflammation[4,35], in cKO P2H2 neutrophils compared to their littermate controls (Supplemental Figure 1C). Interestingly, there were no differences in speed at any of the degrees of confinement tested (Figure 1C). These data strongly suggest that enhanced HIF2α activation regulates neutrophil motion in very confined microenvironments.

We extended our analysis to evaluate neutrophil migration in a *2D confined microenvironment* (4.5 μm height) (Figure 1D). Similar to the results obtained in the 1D migration assay, neutrophils from cKO P2 mice showed increased motility compared to their WT counterparts, as evidenced by longer trajectories of equivalent durations (Figure 1E), as well as greater speed (Figure 1F), along with higher mean square displacement (MSD) values (Figure 1G). On the other hand, under identical conditions, cKO P2H2 neutrophils did not show any difference in speed or MSD compared to their WT counterparts (Figure 1H and 1I). Interestingly, cell

191 migration in a non-confining 2D chamber (12µm height) showed no difference in speed,
192 trajectories, or MSD (Supplemental Figure 1D-F). Thus, these data indicate that the PHD2-
193 HIF2α pathway regulates cell migration by facilitating mobility strictly in confined spaces.

194

195 **PHD2-deficient neutrophils display enhanced non-directed motility in complex**
196 **environments**

197 We used *3D-collagen matrices* to confirm the role of PHD2 in neutrophil migration in a
198 microenvironment of fibers and different pore sizes, adequately mimicking the tissue
199 complexity *in vivo*. Therefore, migration of freshly-isolated BMDNs from cKO P2 mice and
200 WT littermates was compared in dense 3D collagen gels (4mg/ml) (Figure 2A) during which
201 cKO P2 neutrophils showed greater motility, as evidenced by a higher displacement radius
202 (Figure 2A). Detailed analysis of their random trajectories showed that cKO P2 neutrophils
203 displayed greater speed and MSD values compared to WT cells (Figure 2B, C). Interestingly,
204 this difference was completely lost in less dense collagen gels (2mg/ml) (Figure 2B and
205 Supplemental Figure 2A).

206

207 As it has been suggested that silencing of PHD2 in neutrophils leads to their enhanced
208 chemotaxis,[36] we assessed this effect in our complex 3D collagen matrix setup using CXCL2
209 as a classical neutrophil chemokine. Neutrophil trajectory analysis in dense collagen gels
210 (4mg/ml) showed that absence of PHD2 did not affect neutrophil chemokine sensing because
211 their directionality towards CXCL2 remained unaltered (Figure 2D) and a similar strong
212 increase in cell speed was found in both PHD2-deficient and WT neutrophils (Figure 2E).
213 Thus, these results show that PHD2-deficient neutrophils display an enhanced migratory
214 capacity in dense 3D collagen gels and that PHD2 loss does not affect CXCL2-induced

chemotactic capacity. In other words, the faster migration of cKO P2 neutrophils is independent of chemotaxis induction and is rather linked to enhanced undirected motility or chemokinesis.

The migratory capacity of several cell types in complex microenvironments is highly dependent on their capacity to deform when encountering narrow pores.[37,38] Therefore, we evaluated whether cKO P2 neutrophils can overcome severely constricted spaces of only 1µm width (Figure 2F). [17,39] Remarkably, PHD2-deficient neutrophils showed an enhanced preference to pass through these constrictions (Figure 2G) and were also faster compared to WT neutrophils (Figure 2H). Interestingly, under these conditions, cKO P2H2 neutrophils showed reduced migration; and, similar migration kinetics than WT cells (Figure 2I, J), again suggesting a PHD2/HIF2α-dependent axis in migration through extreme narrow constrictions.

Next, we studied whether the ability of cKO P2 neutrophils to pass through small confinements is related to changes in their deformability when an external force is applied. For this, we first analyzed neutrophil deformability using r*eal time fluorescence and deformability cytometry* (RT-FDC) (see supplemental data), which can extract the stiffness of cells (Young´s Modulus) in high-throughput, without contact at ms-timescales.[40,41] We used steady-state BMDNs, Phorbol 12-Myristate 13-Acetate (PMA)-activated BMDNs, and peripheral blood neutrophils isolated at 6h after thioglycolate-induced peritonitis. However, no differences were observed between cKO P2 and WT neutrophils under any of the conditions tested (supplemental Figure 2B). Likewise, a *microcapillary microcirculation mimetic* (MMM) assay[42,43] using peritonitis neutrophils showed no difference in their ability to passively navigate through multiple constrictions at high speed (supplemental Figure 2C). Taken together, these assays strongly suggest that loss of PHD2 does not affect neutrophil deformability under externally applied stress without confinement.

PHD2-deficient neutrophils extravasate faster *in vivo* and accumulate in inflamed tissue

Based on the enhanced ability of PHD2-deficient neutrophils to overcome very small constrictions, we decided to study the behavior of these cells *in vivo*; specifically, in a more complex setting of sterile skin inflammation. Ear lobes from cKO P2 and WT littermate mice that displayed no difference in total numbers of hematopoietic stem cells, myeloid progenitors or mature neutrophils (supplemental Figure 3A), were ectopically treated with PMA and the recruitment of $Ly6G^+$ cells was visualized using intra-vital 2-photon microscopy (Figure 3A). In line with our previous experiments, we found that PHD2-deficient neutrophils were able to extravasate about 30% faster from the vessel into the ear tissue than their WT counterparts (Figure 3B-C). Furthermore, the cumulative effect of faster neutrophil extravasation time resulted in an anticipated increase in $Gr1^+$ cells in the inflamed cKO P2 ear compared to that in WT littermates at 24 hours after PMA-treatment (Figure 3D). Conversely but consistently, this difference in migration was abolished in cKO P2H2 mice (Figure 3E), further confirming a role for HIF2α activity in driving increased migration capacity of these neutrophils.

As previous studies have described PHD2-related improved survival of neutrophils during inflammation,[4,44] we evaluated the level of apoptotic cells in 24 hour PMA-treated ears, but found no difference in cleaved caspase-3^+ cell numbers ($cCas3^+$) between the different genotypes (Figure 3F, G; supplemental Figure 3B, C). Additionally, as recent work has associated PHD2 with enhanced neutrophil glycolysis and their recruitment to sites of inflammation,[36] we assessed the glycolytic capacity of BMDNs from cKO P2, cKO P2H2, and their respective WT counterparts by measuring extracellular acidification rate (ECAR). In line with previous reports, PHD2-deficient neutrophils appeared to be significantly more glycolytic than their respective WT counterparts (Figure 3H). However, neutrophils lacking both PHD2

and HIF2α also showed significantly higher glycolysis (Figure 3I). Taken together, although HIF2α directly controls the migration speed of neutrophils in confined spaces and inflamed tissues, this effect is independent of their survival or glycolytic activity.

HIF2α stabilization upon loss of PHD2 affects cytoskeletal gene expression profiles

It is well-accepted that the functionality of innate immune cells varies depending on the lipid-type composition of its cytoplasmic membrane.[45,46] Therefore, we evaluated if altered membrane lipid composition of the cKO P2 neutrophils could account for their different migratory ability, by performing high-throughput lipidomic analysis of freshly isolated BMDNs (see supplemental data). However, as there were no significant alterations between the cKO P2 and WT BMDNs (supplemental Figure 2D and Table 3), it appears unlikely that differences in the lipid composition are directly responsible for the dramatic difference in the migratory capacity of the cKOP2 neutrophils.

Next, to further characterize the molecular underpinnings of the HIF2α-driven neutrophil migration phenotype, we used *next generation sequencing* (NGS) wherein the steady state transcriptome of BMDNs derived from cKO P2 and cKO P2H2 mice were analyzed and compared to that from their respective WT counterparts (Figure 4A). Gene signatures of various lineages were evaluated using gene set enrichment analyses (GSEA) as described previously.[47-49] In line with our *in vivo* cCas3+ results, we detected no significant apoptosis signatures among any of the genotypes (Figure 4B) and NGS confirmed a significant enrichment of glycolysis/gluconeogenesis related genes in both cKO P2 and cKO P2H2 BMDNs (Figure 4C). Strikingly, steady state BMDNs lacking PHD2, with or without HIF2α, displayed a significant reduction in genes related to the innate immune response but not the

chemokine signaling pathway (Figure 4D). Together, these observations suggest that significant HIF2α-independent changes in glycolytic capacity and immune response of PHD2-deficient neutrophils can be likely linked to HIF1α activity, as previously suggested.[2,36] Conversely, a number of HIF2α-dependent gene signatures associated with PHD2 deficiency related to function and structure of the neutrophil cytoskeleton, including Rho GTPase activity (Figure 4E). Additionally, using an integrative method, we identified a number of HIF2α-associated *master regulators* that could potentially control cellular cytoskeletal rearrangements through transcriptional or protein regulation (supplemental Figure 4A).

Diminished RhoA GTPase activity underlies the faster HIF2α-dependent migration of PHD2-deficient neutrophils

Small Rho GTPases (RhoA, cdc42 and Rac) are the final molecular effectors that steer cytoskeletal dynamics. In line with this, we identified numerous potential associations (direct and/or indirect) among 49 genes/proteins and with RhoA and/or Cdc42 (bold lines), but not Rac GTPase (Figure 5A). Notably, 7 of these genes have been previously identified as being associated with HIF2α binding sites (supplemental Figure 4B).[50]

To substantiate this link between PHD2/HIF2α and Rho GTPases, we used an *ex vivo* enzymatic assay to quantify the activity of these Rho GTPases in untreated freshly-isolated BMDNs from cKO P2 and P2H2 mice. Interestingly, cKO P2 neutrophils exhibited diminished RhoA and Cdc42 GTPase activity (Figure 5B, C), while Rac GTPase activity was comparable with WT neutrophils (Figure 5D). Further, cKO P2H2 neutrophils displayed no significant reduction in either RhoA, Cdc42 or Rac GTPase activity, suggesting that regulation of RhoA and/or Cdc42 is dependent on the PHD2/HIF2α-axis (Figure 5B-D).

Given this reduction in RhoA and Cdc42 GTPase activity in PHD2-deficient neutrophils, we examined whether their direct inhibition in WT neutrophils can mimic the motility phenotype

displayed by cKO P2 neutrophils. Therefore, we performed a series of *ex vivo* 1D-migration assays using the RhoA inhibitor CCG100602 (CCG) or the Rho inhibitor Exoenzyme C3 Transferase (C3) and found that while the use of low doses of CCG or C3 enhanced the speed of migrating neutrophils in 3µm micro-channels (Figure 5E), treatment of cells with a Cdc42 inhibitor (ML141) did not have any effect on the velocity of BMDNs (Figure 5F). Taken together, our data strongly argue for a PHD2/HIF2α–orchestrated regulatory loop in RhoA GTPase activity-dependent motility of BMDNs.

The PHD2/HIF2α-axis controls neutrophil accumulation in joints during severe inflammatory arthritis

To test the biological effects of the enhanced migratory capacity of PHD2-deficient neutrophils, we subjected the different mouse strains to an autoantibody-induced inflammatory arthritis model (K/BxN), which has been shown to be myeloid dependent (Figure 6A).[51,52] cKO P2 mice displayed enhanced swelling of the hind limbs compared to their WT littermates (Figure 6B) and this effect was sustained throughout the first 2 weeks of the experiment. In line with our previous results, cKO P2H2 and their WT littermates displayed no difference in swelling (Figure 6C). To characterize the myeloid composition of the inflamed knee joints, we performed flow cytometry analysis of the synovial fluid drawn on day 5, which revealed much higher accumulation of neutrophils in cKO P2 knee joints (>3-fold increase versus WT), along with slightly enhanced macrophages (Figure 6D); immunofluorescence for Gr1 on knee joints further confirmed this observation (Figure 6E). Conversely, although no differences were observed in joint swelling between cKO P2H2 mice and their WT littermates, their synovial fluid showed a slight but significant reduction in neutrophil numbers at day 5 compared to cKO P2 mice (Figure 6F). Thus, also in arthritic joints PHD2/HIF2α is a central axis during the initial stages of the inflammation.

Discussion

In the current work we have explored if hypoxia pathway proteins can directly regulate neutrophil motility, and reveal that activation of HIF2α in mouse neutrophils due to constitutive PHD2 loss enhances neutrophil migration through very confined environments independent of chemotactic -, glycolytic- or apoptotic-activity. Using a combination of *in vivo*, *ex vivo* and deep sequencing approaches, we provide evidence that these neutrophils have the capacity to migrate faster than their WT counterparts, and that this phenotype may be directly related to changes in their cytoskeleton mediated by a substantial reduction in RhoA GTPase activity.

Although it is generally accepted that neutrophils are the first immune cells to arrive in the tissue during inflammation, the molecular basis of neutrophil recruitment, which encompasses extravasation and interstitial migration, remain elusive. Further, neutrophil recruitment has been evaluated using a variety of migration assays in multiple studies related to the innate immune response,[53-56] including in the context of hypoxia pathway proteins,[2,4] but these studies call into debate the role of adhesion molecules.[57,58] Here, we consistently show in 1D, 2D and 3D assays that neutrophils lacking PHD2 alone, and not both PHD2 and HIF2α, display enhanced cell motility and that only in severely confined environments. This difference in chemokinesis between cKO P2 and WT remained in a comparable setup using a chemokine as attractant (chemotaxis), demonstrating that the enhanced migratory capacity regulated by the PHD2-HIF2α axis is probably a cell intrinsic characteristic.

An important process during neutrophil recruitment is the final and time-limiting step of trans-endothelial migration (TEM), which is, in part, mediated by mechanical forces generated by the migrating neutrophil itself.[59-61] We reveal a central role for HIF2α in this process. Indeed, considering the narrow pores between neighboring endothelial cells during the early phase of

neutrophil diapedesis,[60] our results from multiple approaches reiterate two main observations, viz., that greater numbers of cKO P2 neutrophils pass through small constrictions with enhanced speed. Intuitively, these observations account for the shorter TEM-time in a local ear inflammation model. The cumulative effects of such enhanced transmigration into inflamed tissues that were observable even at later time points in two completely independent *in vivo* models, i.e., inflammatory skin lesions and sterile arthritis. Indeed, it is possible that the enormous increase in cKO P2 neutrophils is positively affected by the fact that once a pore is opened, successive neutrophils are more likely to extravasate at this spot, enabling more neutrophils to enter the interstitium (skin) or the synovium (joint) of the inflamed tissue.[60]

Previous studies in a model of acute lung injury have reported enhanced glycolytic capacity of PHD2-deficient neutrophils, potentially due to HIF1α stabilization, which also enhanced neutrophil recruitment to the inflammatory site.[36] Here, we confirm enhanced glycolysis in cKO P2 neutrophils and show that it is HIF2α-independent, strongly suggesting that glycolytic metabolism does not underlie the chemokinesis phenotype described here. The absence of differences in neutrophil apoptosis *in vivo* was corroborated by the RNAseq data from both single and double knock-out neutrophils, implying that the prolonged inflammation phenotype in cKO P2 mice was probably not due to persistence of the neutrophils. This is in contrast to results obtained using *in vitro* approaches that describe reduced apoptosis in HIF2α over-expressing neutrophils, which then resulted in delayed resolution of the inflammatory response.[4] This group also reported delayed apoptosis in PHD2-deficient neutrophils and connected this to persistent inflammation.[36] We believe these discrepancies are related to differences in the experimental models used.

Although several studies have linked the hypoxia pathway to the migratory capacity of a cell, only a few have suggested a role for the PHD/HIF axis in regulating cell migration through changes in cytoskeletal function.[20-22] In migrating neutrophils *in vivo*, dynamic polymerized

387 actin converges at the leading-edge of pseudopods, while stable actin with high acto-myosin
388 contractility assemble at the rear. Both polarization and maintenance of this cytoskeletal
389 asymmetry strongly rely on Rho GTPase activity.[62,63] Using deep sequencing data from
390 neutrophils of single and double transgenic lines, we show that a vast number of genes
391 associated with Rho GTPase signaling are either directly or indirectly regulated by HIF2α.
392 Interestingly, cKO P2 neutrophils displayed a significant downregulation of RhoA GTPase and
393 we show this to be directly associated with enhanced motility because RhoA-inhibitor treated
394 WT neutrophils behaved similarly in confined environments. These findings are similar to
395 those reported earlier, i.e., increased flux of RhoA-deficient neutrophils and aggravated tissue
396 injury in LPS-induced acute lung injury.[64] A potential explanation is that the partial RhoA
397 inhibition would primarily decrease dynamic cell protrusions, known to restrict cell migration
398 by competing with stable actin cables at the cell rear.[18] Alternatively, the HIF2α axis could be
399 directly involved in the induction of cell contractility, which promotes neutrophil and DCs
400 migration under strong confinement.[17,34] However, further efforts are required to identify the
401 specific molecular mechanism.

402 In conclusion, our results demonstrate that HIF2α-activation, due to constitutive loss of PHD-
403 2, enhances the motility of neutrophils in highly confined surroundings, also during
404 inflammation. Importantly, this phenotype is independent of chemotaxis signaling, glycolysis
405 or apoptosis. Mechanistically, it is the reduction of RhoA GTPase activity that enhances the
406 motility of PHD2 deficient neutrophils through very confined microenvironments. These
407 findings highlight the potential deleterious effects of sustained HIF2α activity and may have
408 important implications for the uncontrolled use of hypoxia mimetic agents that are currently
409 licensed or are in phase II and III clinical trials.

410

411 **Conflict-of-interest**

412 The authors have declared that no conflict of interest exists.

413

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428

429 **Authorship**

430 S.S. designed the study, performed the majority of experiments, analysed data, and contributed
431 in writing the manuscript. M.D., I.K., K.F. and M.K. designed and performed experiments,
432 analysed data and contributed to the discussions. P.J.S., GLL and MB analysed data and
433 contributed to the discussions. A.S. performed deep sequencing analysis and A.P. performed
434 lipidomic analysis. M.G. performed intravital microscopy and contributed to the discussions.

435 A.K. and A.M. performed experiments and analysed data. J.G., Ü.C. provided tools and
436 contributed to the discussions. T.C. provided tools, contributed to the discussions and edited
437 the manuscript. P.V. designed and supervised the ex vivo migration studies, performed
438 experiments, analysed data, and contributed in writing the manuscript. B.W. designed and
439 supervised the overall study, analysed data, and wrote the manuscript.

440

441 **Current affiliation:** I.K.: Hull York Medical School, York Biomedical Research Institute,
442 University of York, York, United Kingdom. M.K. and J.G.: Max Planck Institute for the
443 Science of Light & Max-Planck-Zentrum für Physik und Medizin, 91058 Erlangen, Germany.

References

1. Taylor CT, Colgan SP. Regulation of immunity and inflammation by hypoxia in immunological niches. *Nat Rev Immunol*. 2017;17(12):774-785.

2. Cramer T, Yamanishi Y, Clausen BE, et al. HIF-1α is essential for myeloid cell-mediated inflammation. *Cell*. 2003;112(5):645-657.

3. Peyssonnaux C, Datta V, Cramer T, et al. HIF-1alpha expression regulates the bactericidal capacity of phagocytes. *J Clin Invest*. 2005;115(7):1806-1815.

4. Thompson AA, Elks PM, Marriott HM, et al. Hypoxia-inducible factor 2alpha regulates key neutrophil functions in humans, mice, and zebrafish. *Blood*. 2014;123(3):366-376.

5. Dai Z, Li M, Wharton J, Zhu MM, Zhao YY. Prolyl-4 Hydroxylase 2 (PHD2) Deficiency in Endothelial Cells and Hematopoietic Cells Induces Obliterative Vascular Remodeling and Severe Pulmonary Arterial Hypertension in Mice and Humans Through Hypoxia-Inducible Factor-2alpha. *Circulation*. 2016;133(24):2447-2458.

6. Imtiyaz HZ, Williams EP, Hickey MM, et al. Hypoxia-inducible factor 2alpha regulates macrophage function in mouse models of acute and tumor inflammation. *J Clin Invest*. 2010;120(8):2699-2714.

7. Clever D, Roychoudhuri R, Constantinides MG, et al. Oxygen Sensing by T Cells Establishes an Immunologically Tolerant Metastatic Niche. *Cell*. 2016;166(5):1117-1131 e1114.

8. Sormendi S, Wielockx B. Hypoxia Pathway Proteins As Central Mediators of Metabolism in the Tumor Cells and Their Microenvironment. *Front Immunol*. 2018;9:40.

9. Watts ER, Walmsley SR. Inflammation and Hypoxia: HIF and PHD Isoform Selectivity. *Trends Mol Med*. 2019;25(1):33-46.

10. Walmsley SR, Chilvers ER, Thompson AA, et al. Prolyl hydroxylase 3 (PHD3) is essential for hypoxic regulation of neutrophilic inflammation in humans and mice. *Journal of Clinical Investigation*. 2011;121(3):1053-1063.

11. Lodge KM, Cowburn AS, Li W, Condliffe AM. The Impact of Hypoxia on Neutrophil Degranulation and Consequences for the Host. *Int J Mol Sci*. 2020;21(4).

12. Hajishengallis G, Chavakis T. Endogenous modulators of inflammatory cell recruitment. *Trends Immunol*. 2013;34(1):1-6.

13. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13(3):159-175.

14. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7(9):678-689.

15. Cernuda-Morollon E, Ridley AJ. Rho GTPases and leukocyte adhesion receptor expression and function in endothelial cells. *Circ Res*. 2006;98(6):757-767.

16. Salvermoser M, Pick R, Weckbach LT, et al. Myosin 1f is specifically required for neutrophil migration in 3D environments during acute inflammation. *Blood*. 2018;131(17):1887-1898.

17. Thiam HR, Vargas P, Carpi N, et al. Perinuclear Arp2/3-driven actin polymerization enables nuclear deformation to facilitate cell migration through complex environments. *Nat Commun*. 2016;7:10997.

18. Vargas P, Maiuri P, Bretou M, et al. Innate control of actin nucleation determines two distinct migration behaviours in dendritic cells. *Nat Cell Biol*. 2016;18(1):43-53.

19. Semba H, Takeda N, Isagawa T, et al. HIF-1alpha-PDK1 axis-induced active glycolysis plays an essential role in macrophage migratory capacity. *Nat Commun*. 2016;7:11635.

490 20. Vogel S, Wottawa M, Farhat K, et al. Prolyl Hydroxylase Domain (PHD) 2 affects cell
491 migration and F-actin formation via RhoA/ROCK-dependent cofilin phosphorylation. *Journal*
492 *of Biological Chemistry*. 2010:jbc. M110. 132985.
493 21. Choi HJ, Sanders TA, Tormos KV, et al. ECM-dependent HIF induction directs
494 trophoblast stem cell fate via LIMK1-mediated cytoskeletal rearrangement. *PloS one*.
495 2013;8(2):e56949.
496 22. Huang C, Qian SL, Sun LY, Cheng B. Light-Emitting Diode Irradiation (640 nm)
497 Regulates Keratinocyte Migration and Cytoskeletal Reorganization Via Hypoxia-Inducible
498 Factor-1α. *Photomedicine and laser surgery*. 2016;34(8):313-320.
499 23. Angus DC, van der Poll T. Severe sepsis and septic shock. *N Engl J Med*.
500 2013;369(21):2063.
501 24. Singh RP, Franke K, Kalucka J, et al. HIF prolyl hydroxylase 2 (PHD2) is a critical
502 regulator of hematopoietic stem cell maintenance during steady-state and stress. *Blood*.
503 2013;121(26):5158-5166.
504 25. Stadtfeld M, Graf T. Assessing the role of hematopoietic plasticity for endothelial and
505 hepatocyte development by non-invasive lineage tracing. *Development*. 2005;132(1):203-213.
506 26. Gruber M, Hu C-J, Johnson RS, Brown EJ, Keith B, Simon MC. Acute postnatal
507 ablation of Hif-2alpha results in anemia. *Proc Natl Acad Sci U S A*. 2007;104(7):2301-2306.
508 27. Kiers D, Wielockx B, Peters E, et al. Short-Term Hypoxia Dampens Inflammation in
509 vivo via Enhanced Adenosine Release and Adenosine 2B Receptor Stimulation. *EBioMedicine*.
510 2018;33:144-156.
511 28. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-
512 specific disease provoked by systemic autoimmunity. *Cell*. 1996;87(5):811-822.
513 29. Vargas P, Terriac E, Lennon-Dumenil AM, Piel M. Study of cell migration in
514 microfabricated channels. *J Vis Exp*. 2014(84):e51099.

515 30. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for
516 biological-image analysis. *Nat Methods*. 2012;9(7):676-682.
517 31. Vargas P, Chabaud M, Thiam H-R, Lankar D, Piel M, Lennon-Dumenil A-M. Study of
518 dendritic cell migration using micro-fabrication. *Journal of immunological methods*.
519 2016;432:30-34.
520 32. Ufer F, Vargas P, Engler JB, et al. Arc/Arg3. 1 governs inflammatory dendritic cell
521 migration from the skin and thereby controls T cell activation. *Science immunology*.
522 2016;1(3):eaaf8665-eaaf8665.
523 33. Sáez PJ, Vargas P, Shoji KF, Harcha PA, Lennon-Duménil A-M, Sáez JC. ATP
524 promotes the fast migration of dendritic cells through the activity of pannexin 1 channels and
525 P2X7 receptors. *Sci Signal*. 2017;10(506):eaah7107.
526 34. Barbier L, Saez PJ, Attia R, et al. Myosin II Activity Is Selectively Needed for
527 Migration in Highly Confined Microenvironments in Mature Dendritic Cells. *Front Immunol*.
528 2019;10:747.
529 35. Dai Z, Li M, Wharton J, Zhu MM, Zhao Y-Y. PHD2 deficiency in endothelial cells and
530 hematopoietic cells induces obliterative vascular remodeling and severe pulmonary arterial
531 hypertension in mice and humans through HIF-2α. *Circulation*. 2016;133(24):2447.
532 36. Sadiku P, Willson JA, Dickinson RS, et al. Prolyl hydroxylase 2 inactivation enhances
533 glycogen storage and promotes excessive neutrophilic responses. *J Clin Invest*.
534 2017;127(9):3407-3420.
535 37. Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. *The Journal*
536 *of cell biology*. 2009:jcb. 200909003.
537 38. Cramer LP. Forming the cell rear first: breaking cell symmetry to trigger directed cell
538 migration. *Nature cell biology*. 2010;12(7):628.

539 39. Saez PJ, Barbier L, Attia R, Thiam HR, Piel M, Vargas P. Leukocyte Migration and
540 Deformation in Collagen Gels and Microfabricated Constrictions. *Methods Mol Biol*.
541 2018;1749:361-373.
542 40. Mietke A, Otto O, Girardo S, et al. Extracting Cell Stiffness from Real-Time
543 Deformability Cytometry: Theory and Experiment. *Biophys J*. 2015;109(10):2023-2036.
544 41. Mokbel M, Mokbel D, Mietke A, et al. Numerical Simulation of Real-Time
545 Deformability Cytometry To Extract Cell Mechanical Properties. *ACS Biomaterials Science &*
546 *Engineering*. 2017;3(11):2962-2973.
547 42. Prathivadhi-Bhayankaram SV, Ning J, Mimlitz M, et al. Chemotherapy impedes in
548 vitro microcirculation and promotes migration of leukemic cells with impact on metastasis.
549 *Biochemical and biophysical research communications*. 2016;479(4):841-846.
550 43. Ekpenyong AE, Toepfner N, Fiddler C, et al. Mechanical deformation induces
551 depolarization of neutrophils. *Science advances*. 2017;3(6):e1602536.
552 44. Elks PM, van Eeden FJ, Dixon G, et al. Activation of hypoxia-inducible factor-1α (Hif-
553 1α) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in
554 a zebrafish inflammation model. *Blood*. 2011;118(3):712-722.
555 45. Alarcon-Barrera JC, von Hegedus JH, Brouwers H, et al. Lipid metabolism of
556 leukocytes in the unstimulated and activated states. *Anal Bioanal Chem*. 2020;412(10):2353-
557 2363.
558 46. Tzeng HT, Chyuan IT, Chen WY. Shaping of Innate Immune Response by Fatty Acid
559 Metabolite Palmitate. *Cells*. 2019;8(12).
560 47. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a
561 knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad*
562 *Sci U S A*. 2005;102(43):15545-15550.

563 48. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1alpha-responsive genes involved
564 in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*.
565 2003;34(3):267-273.
566 49. Singh RP, Grinenko T, Ramasz B, et al. Hematopoietic Stem Cells but Not Multipotent
567 Progenitors Drive Erythropoiesis during Chronic Erythroid Stress in EPO Transgenic Mice.
568 *Stem Cell Reports*. 2018;10(6):1908-1919.
569 50. Schodel J, Oikonomopoulos S, Ragoussis J, Pugh CW, Ratcliffe PJ, Mole DR. High-
570 resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood*.
571 2011;117(23):e207-217.
572 51. Ditzel HJ. The K/BxN mouse: a model of human inflammatory arthritis. *Trends in*
573 *Molecular Medicine*. 2004;10(1):40-45.
574 52. Monach PA, Mathis D, Benoist C. The K/BxN arthritis model. *Curr Protoc Immunol*.
575 2008;Chapter 15:Unit 15 22.
576 53. Lämmermann T, Bader BL, Monkley SJ, et al. Rapid leukocyte migration by integrin-
577 independent flowing and squeezing. *Nature*. 2008;453(7191):51.
578 54. Afonso PV, Janka-Junttila M, Lee YJ, et al. LTB4 is a signal-relay molecule during
579 neutrophil chemotaxis. *Developmental cell*. 2012;22(5):1079-1091.
580 55. Lämmermann T, Afonso PV, Angermann BR, et al. Neutrophil swarms require LTB4
581 and integrins at sites of cell death in vivo. *Nature*. 2013;498(7454):371.
582 56. Reátegui E, Jalali F, Khankhel AH, et al. Microscale arrays for the profiling of start and
583 stop signals coordinating human-neutrophil swarming. *Nature biomedical engineering*.
584 2017;1(7):0094.
585 57. Renkawitz J, Sixt M. Mechanisms of force generation and force transmission during
586 interstitial leukocyte migration. *EMBO Rep*. 2010;11(10):744-750.

587 58. Yamada KM, Sixt M. Mechanisms of 3D cell migration. *Nat Rev Mol Cell Biol*.
588 2019;20(12):738-752.
589 59. Toyjanova J, Flores-Cortez E, Reichner JS, Franck C. Matrix confinement plays a
590 pivotal role in regulating neutrophil-generated tractions, speed, and integrin utilization. *J Biol*
591 *Chem*. 2015;290(6):3752-3763.
592 60. Heemskerk N, Schimmel L, Oort C, et al. F-actin-rich contractile endothelial pores
593 prevent vascular leakage during leukocyte diapedesis through local RhoA signalling. *Nat*
594 *Commun*. 2016;7:10493.
595 61. Filippi MD. Neutrophil transendothelial migration: updates and new perspectives.
596 *Blood*. 2019;133(20):2149-2158.
597 62. Hind LE, Vincent WJ, Huttenlocher A. Leading from the Back: The Role of the Uropod
598 in Neutrophil Polarization and Migration. *Dev Cell*. 2016;38(2):161-169.
599 63. Sit ST, Manser E. Rho GTPases and their role in organizing the actin cytoskeleton. *J*
600 *Cell Sci*. 2011;124(Pt 5):679-683.
601 64. Jennings RT, Strengert M, Hayes P, et al. RhoA determines disease progression by
602 controlling neutrophil motility and restricting hyperresponsiveness. *Blood*.
603 2014;123(23):3635-3645.

604

Figure 1

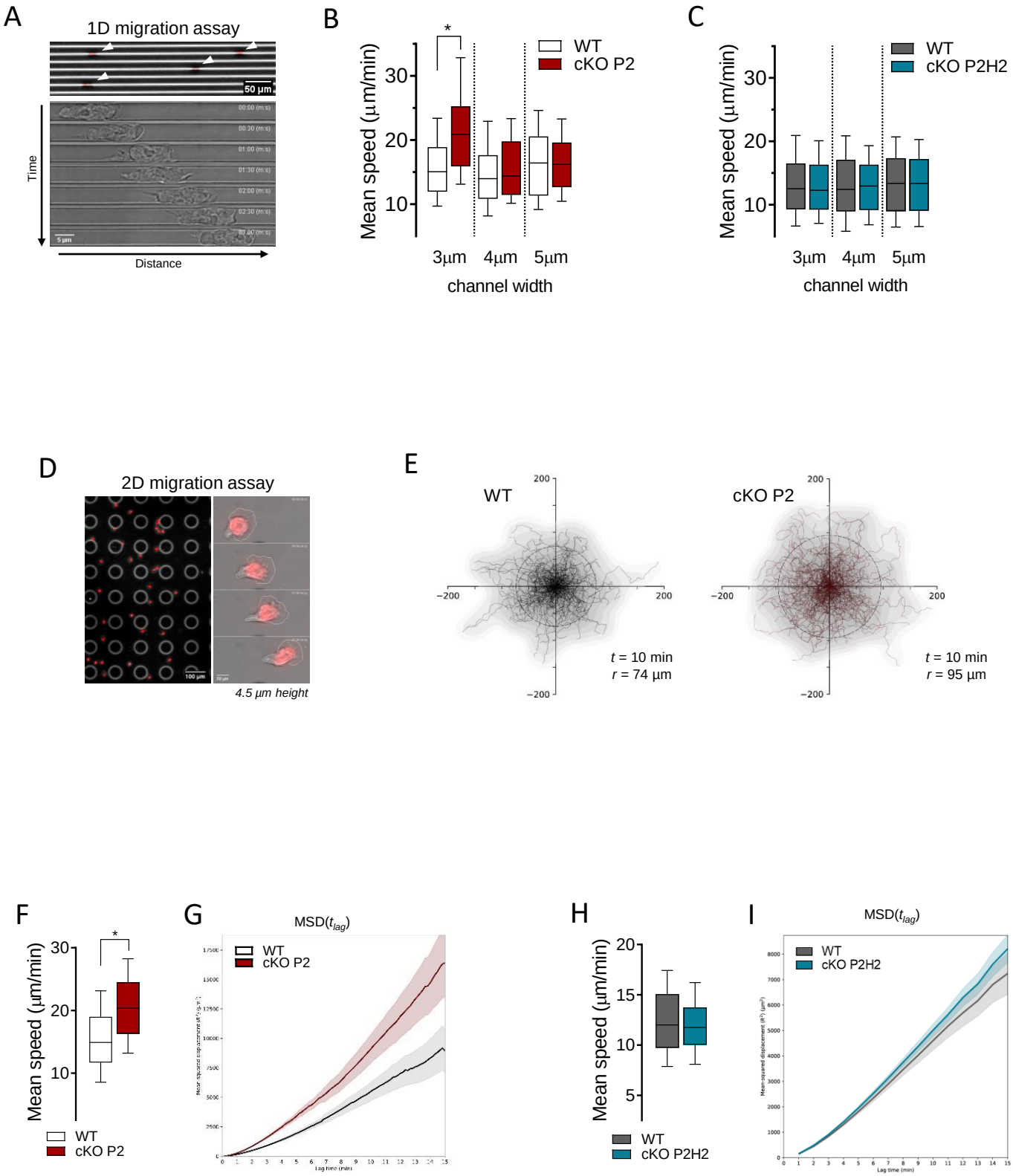
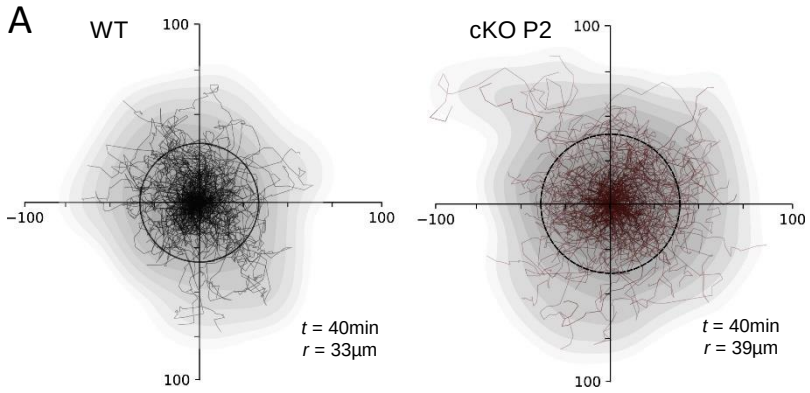
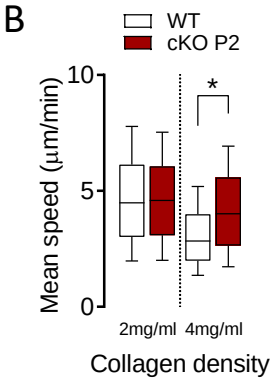
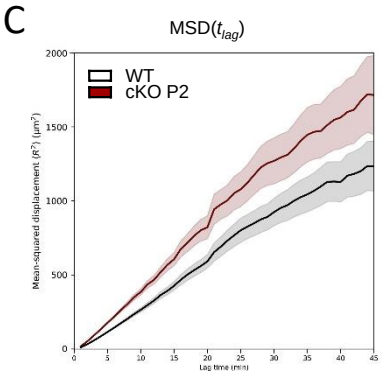
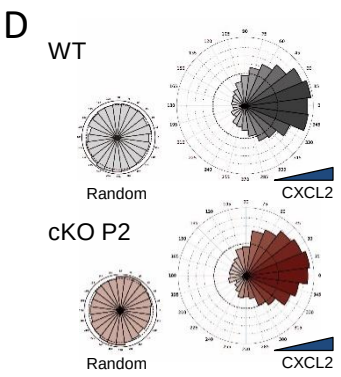

A
1D migration assay
50 µm
Time
Distance
B
Mean speed (µm/min)
WT
cKO P2
3µm
4µm
5µm
channel width
C
Mean speed (µm/min)
WT
cKO P2H2
3µm
4µm
5µm
channel width
D
2D migration assay
4.5 µm height
E
WT
cKO P2
t = 10 min
r = 74 µm
t = 10 min
r = 95 µm
F
Mean speed (µm/min)
WT
cKO P2
G
MSD(t_lag)
WT
cKO P2
H
Mean speed (µm/min)
WT
cKO P2H2
I
MSD(t_lag)
WT
cKO P2H2

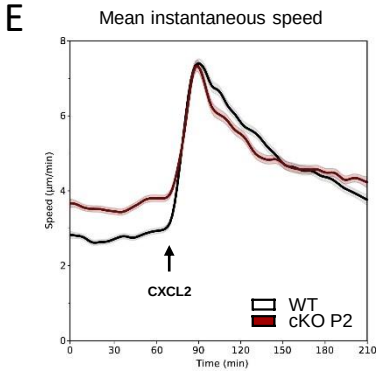
Figure 2

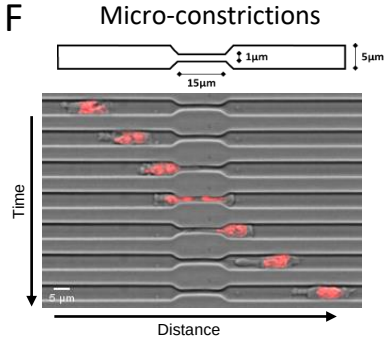

A
WT
cKO P2
100
−100
t = 40min
r = 33µm
t = 40min
r = 39µm

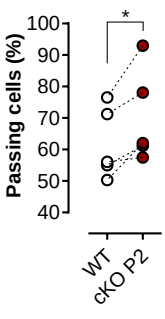

B
WT
cKO P2
Mean speed (µm/min)
10
5
0
*
2mg/ml 4mg/ml
Collagen density

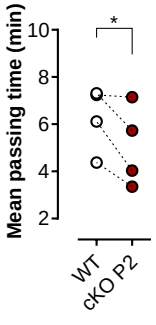

C
MSD(t_{lag})
WT
cKO P2
Mean-squared displacement (R^2) (µm^2)
Lag time (min)

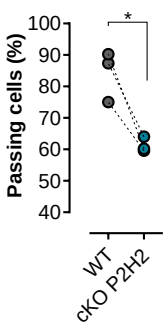

D
WT
Random
CXCL2
cKO P2
Random
CXCL2


E
Mean instantaneous speed
Speed (µm/min)
CXCL2
WT
cKO P2
Time (min)


F
Micro-constrictions
1µm
5µm
15µm
Time
Distance


G
Passing cells (%)
*
WT
cKO P2


H
Mean passing time (min)
*
WT
cKO P2


I
Passing cells (%)
*
WT
cKO P2H2

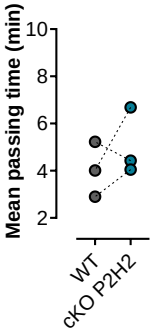
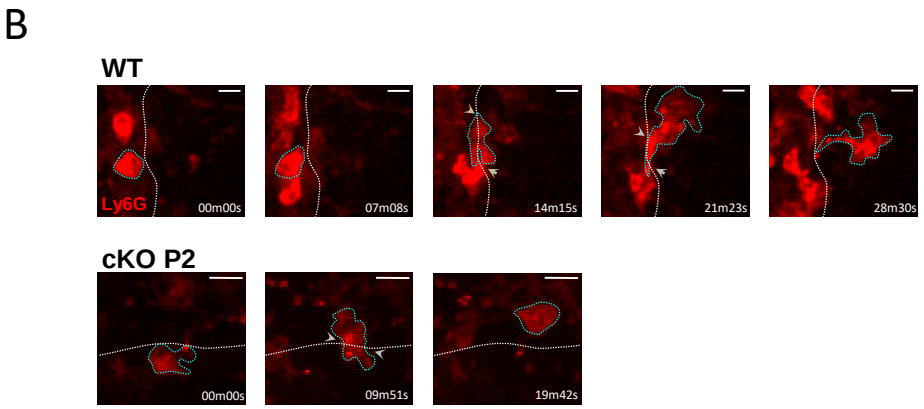
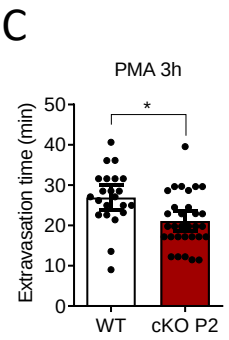
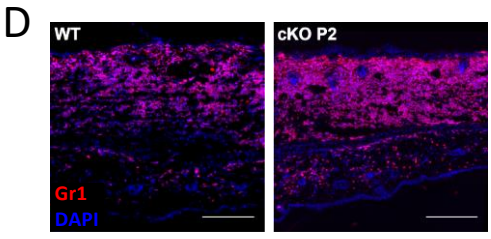

J
Mean passing time (min)
WT
cKO P2H2

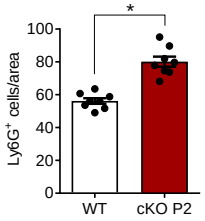
Figure 3

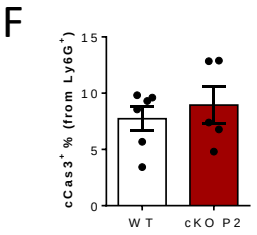

A
0h
3h
24h
10µg PMA
Intra-vital microscopy
Histological analysis

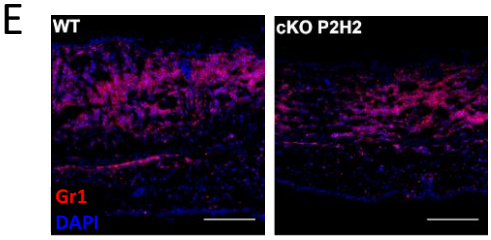

B
WT
Ly6G
00m00s
07m08s
14m15s
21m23s
28m30s
cKO P2
00m00s
09m51s
19m42s

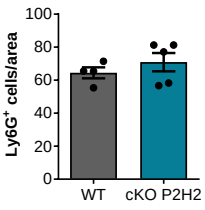

C
PMA 3h
Extravasation time (min)
50
40
30
20
10
0
*
WT
cKO P2

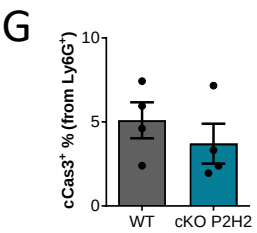

D
WT
cKO P2
Gr1
DAPI

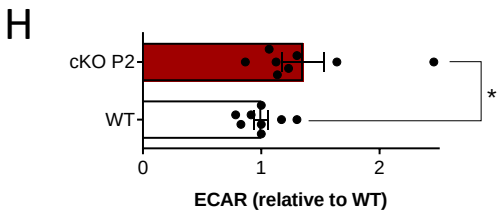

Ly6G+ cells/area
100
80
60
40
20
0
*
WT
cKO P2


F
cCas3+ % (from Ly6G+)
15
10
5
0
WT
cKO P2


E
WT
cKO P2H2
Gr1
DAPI


Ly6G+ cells/area
100
80
60
40
20
0
WT
cKO P2H2


G
cCas3+ % (from Ly6G+)
10
5
0
WT
cKO P2H2


H
cKO P2
WT
*
0
1
2
ECAR (relative to WT)

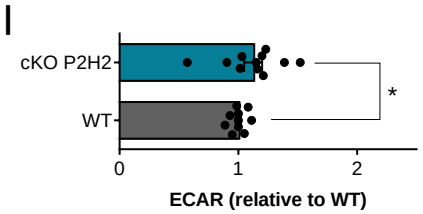
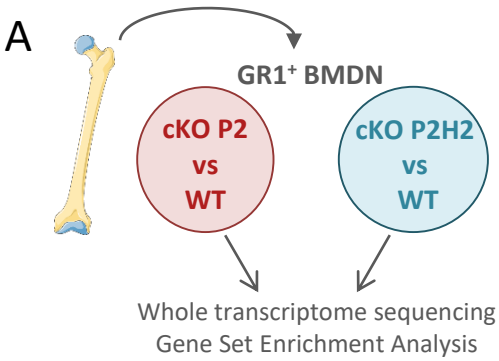
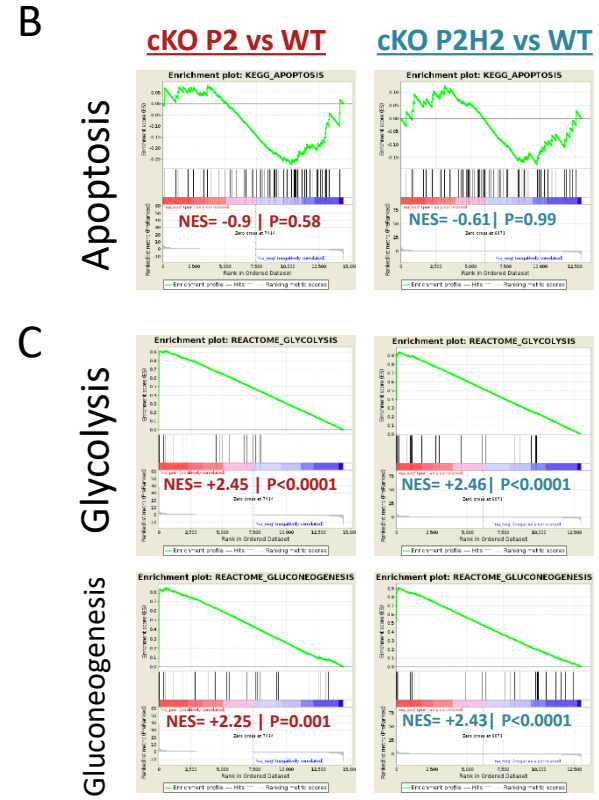
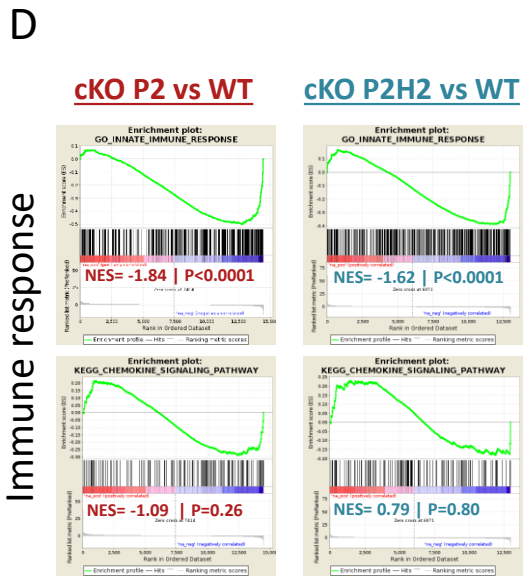

I
cKO P2H2
WT
*
0
1
2
ECAR (relative to WT)

Figure 4


A
GR1+ BMDN
cKO P2 vs WT
cKO P2H2 vs WT
Whole transcriptome sequencing
Gene Set Enrichment Analysis


B
cKO P2 vs WT
cKO P2H2 vs WT
Apoptosis
NES= -0.9 | P=0.58
NES= -0.61| P=0.99
C
Glycolysis
NES= +2.45 | P<0.0001
NES= +2.46| P<0.0001
Gluconeogenesis
NES= +2.25 | P=0.001
NES= +2.43| P<0.0001


D
cKO P2 vs WT
cKO P2H2 vs WT
Immune response
NES= -1.84 | P<0.0001
NES= -1.62 | P<0.0001
NES= -1.09 | P=0.26
NES= 0.79 | P=0.80

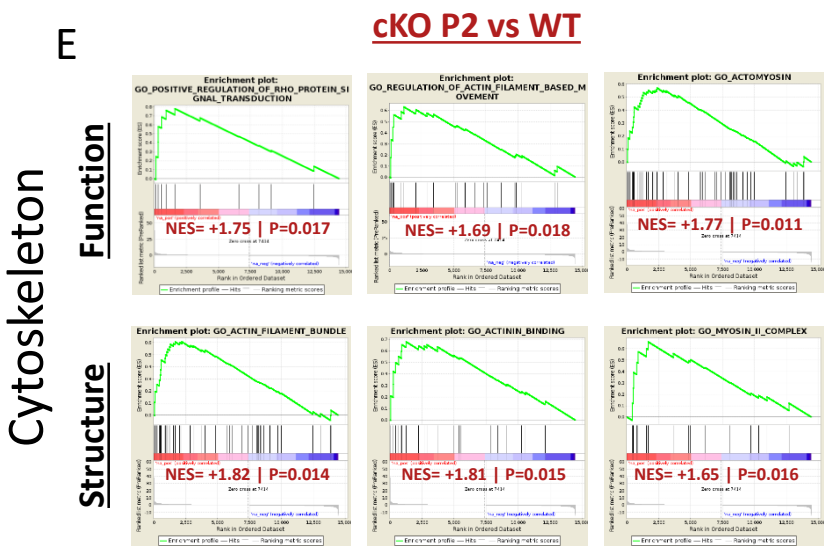
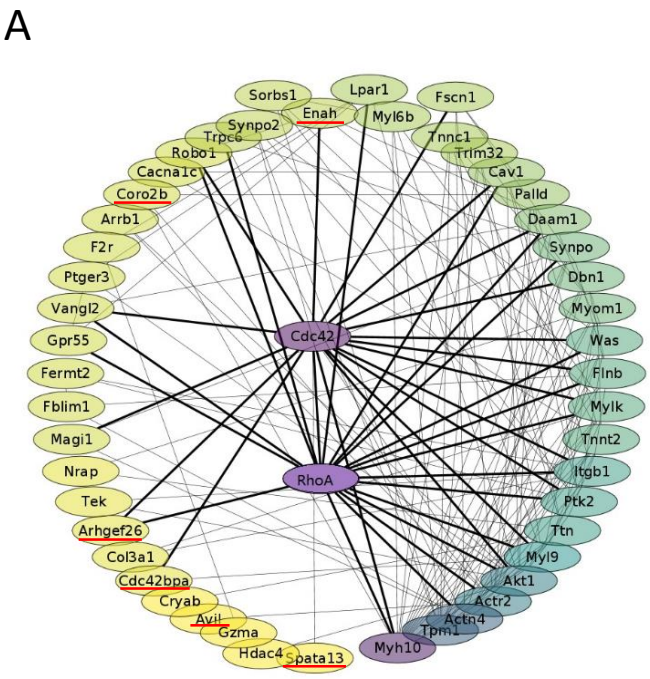
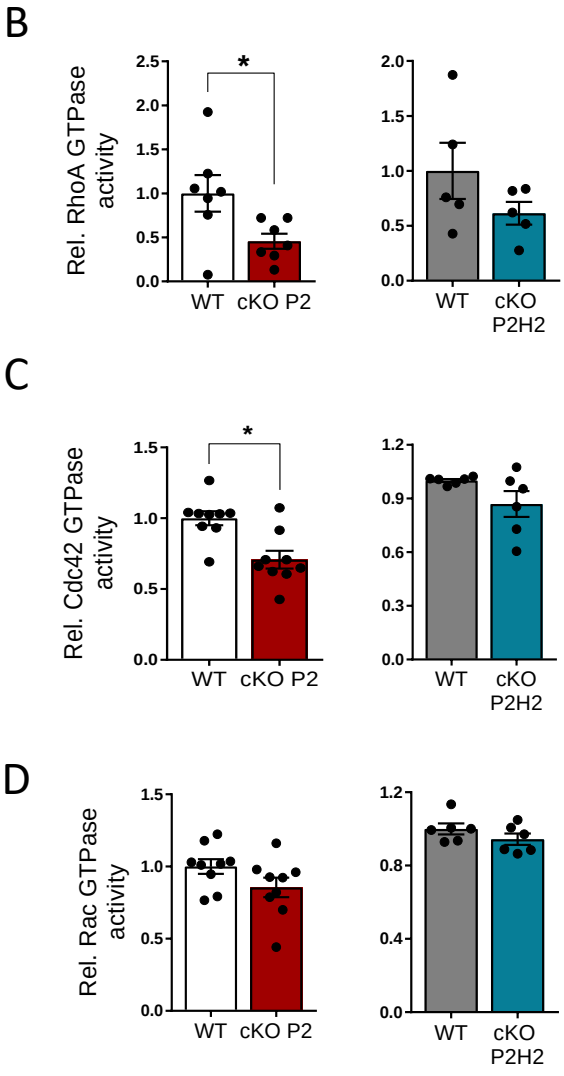
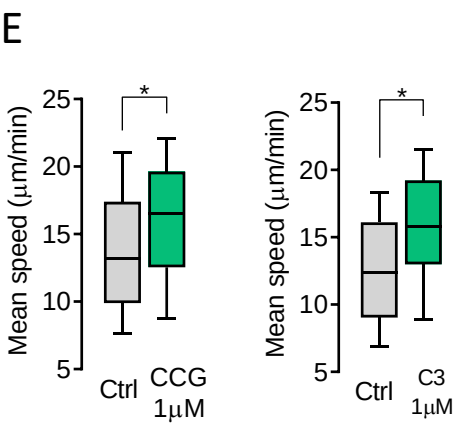

E
cKO P2 vs WT
Cytoskeleton
Function
NES= +1.75 | P=0.017
NES= +1.69 | P=0.018
NES= +1.77 | P=0.011
Structure
NES= +1.82 | P=0.014
NES= +1.81 | P=0.015
NES= +1.65 | P=0.016

Figure 5


A
Sorbs1
Lpar1
Fscn1
Enah
Myl6b
Synpo2
Tnnc1
Robo1
Trim32
Cacna1c
Cav1
Coro2b
Palld
Arrb1
Daam1
F2r
Synpo
Ptger3
Dbn1
Vangl2
Myom1
Cdc42
Gpr55
Was
Fermt2
Flnb
Fblim1
Mylk
Magi1
Tnnt2
Nrap
Itgb1
RhoA
Tek
Ptk2
Arhgef26
Ttn
Col3a1
Myl9
Cdc42bpa
Akt1
Cryab
Actr2
Avil
Actn4
Gzma
Tpm1
Hdac4
Spata13
Myh10


B
Rel. RhoA GTPase activity
WT
cKO P2
cKO P2H2
C
Rel. Cdc42 GTPase activity
D
Rel. Rac GTPase activity


E
Mean speed (µm/min)
Ctrl
CCG 1µM
C3 1µM

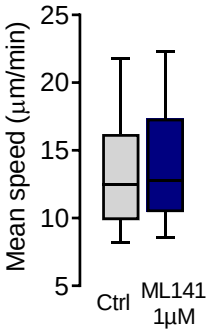
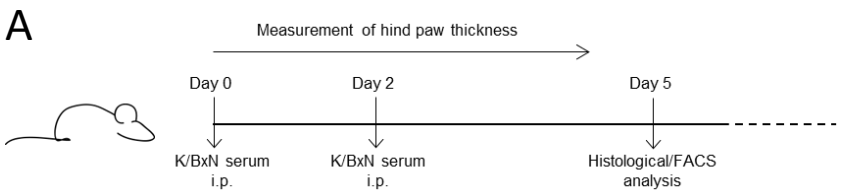
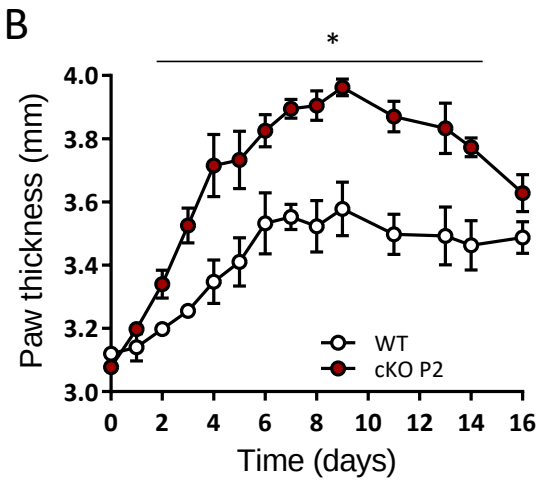
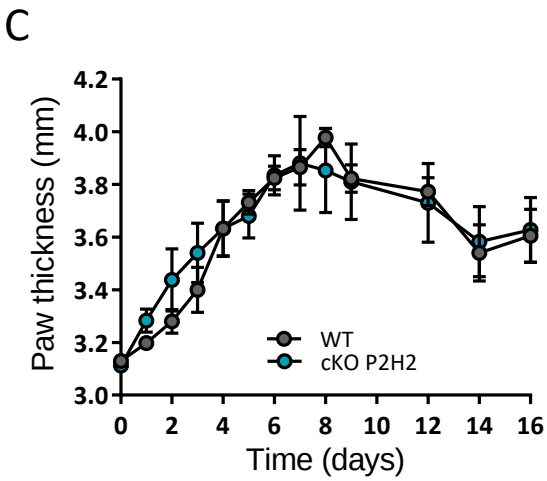
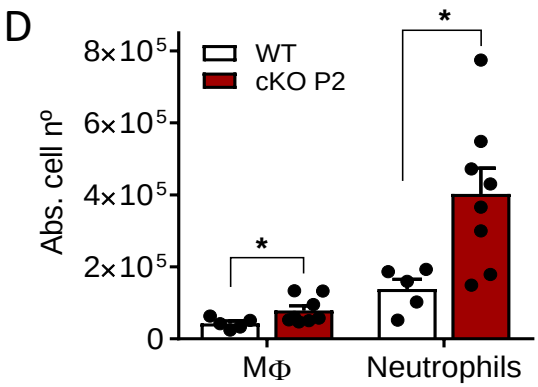

F
Mean speed (µm/min)
Ctrl
ML141 1µM

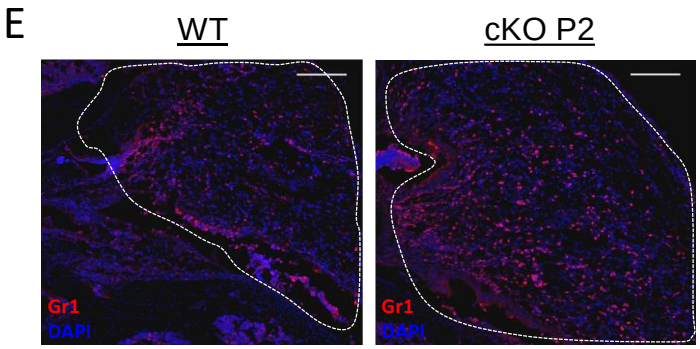
Figure 6

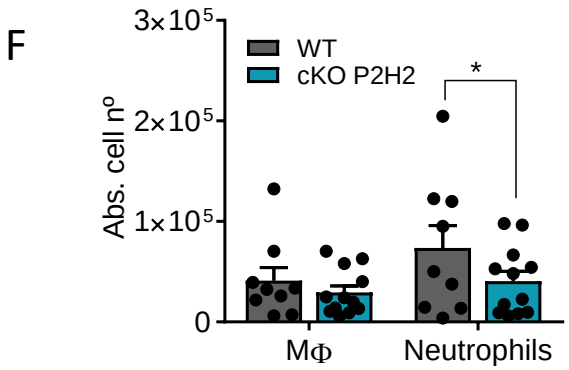

A
Measurement of hind paw thickness
Day 0
Day 2
Day 5
K/BxN serum i.p.
K/BxN serum i.p.
Histological/FACS analysis


B
*
Paw thickness (mm)
4.0
3.8
3.6
3.4
3.2
3.0
0 2 4 6 8 10 12 14 16
Time (days)
WT
cKO P2


C
Paw thickness (mm)
4.2
4.0
3.8
3.6
3.4
3.2
3.0
0 2 4 6 8 10 12 14 16
Time (days)
WT
cKO P2H2


D
Abs. cell nº
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0
WT
cKO P2
*
*
MΦ
Neutrophils


E
WT
cKO P2
Gr1
DAPI
Gr1
DAPI


F
Abs. cell nº
3×10⁵
2×10⁵
1×10⁵
0
WT
cKO P2H2
*
MΦ
Neutrophils