



Research paper

Gain-of-function Tibetan PHD2^{D4E;C127S} variant suppresses monocyte function: A lesson in inflammatory response to inspired hypoxia

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ABSTRACT

Background: We have previously described an evolutionarily selected Tibetan prolyl hydroxylase-2 (PHD2^{D4E;C127S}) variant that degrades the hypoxia-inducible factor (HIF α) more efficiently and protects these highlanders from hypoxia-triggered elevation in haemoglobin concentration. High altitude is known to cause acute mountain sickness (AMS) and high-altitude pulmonary edema (HAPE) in a section of rapidly ascending non-acclimatised lowlanders. These morbidities are often accompanied by inflammatory response and exposure to hypobaric hypoxia is presumed to be the principal causative agent. We have investigated whether PHD2^{D4E;C127S} variant is associated with prevention of hypoxia-mediated inflammatory milieu in Tibetan highlanders and therefore identify a potential target to regulate inflammation.

Methods: We genotyped the Tibetans using DNA isolated from whole blood. Thereafter immunophenotyping was performed on PBMCs from homozygous PHD2^{D4E;C127S} and PHD2^{WT} individuals using flow cytometry. RNA isolated from these individuals was used to evaluate the peripheral level of important transcripts associated with immune as well as hypoxia response employing the nCounter technology. The ex-vivo findings were validated by generating monocytic cell lines (U937 cell line) expressing PHD2^{D4E;C127S} and PHD2^{WT} variants post depletion of endogenous PHD2. We had also collected whole blood samples from healthy travellers and travellers afflicted with AMS and HAPE to evaluate the significance of our ex-vivo and in vitro findings. Hereafter, we also attempted to resolve hypoxia-triggered inflammation in vitro as well as in vivo by augmenting the function of PHD2 using alpha-ketoglutarate (α KG), a co-factor of PHD2.

Findings: We report that homozygous PHD2^{D4E;C127S} highlanders harbour less inflammatory and patrolling monocytes in circulation as compared to Tibetan PHD2^{WT} highlanders. In response to in vitro hypoxia, secretion of IL6 and IL1 β from PHD2^{D4E;C127S} monocytes, and their chemotactic response compared to the PHD2^{WT} are compromised, corresponding to the down-modulated expression of related signalling molecules *RELA*, *JUN*, *STAT1*, *ATF2* and *CXCR4*. We verified these functional outcomes in monocytic U937 cell line engineered to express PHD2^{D4E;C127S} and confirmed the down-modulation of the signalling molecules at protein level under hypoxia. In contrast, non-Tibetan sojourners with AMS and HAPE at high altitude (3,600 m above sea level) displayed significant increase in these inflammatory parameters. Our data henceforth underline the role of gain-of-function of PHD2 as the rate limiting factor to harness hyper-activation of monocytes in hypoxic environment. Therefore upon pre-treatment with α KG, we observed diminished inflammatory response of monocytes in vitro and reduction in leukocyte infiltration to the lungs in mice exposed to normobaric hypoxia.

Interpretation: Our report suggests that gain-of-function PHD2^{D4E;C127S} variant can therefore protect against inflammation elicited by hypobaric hypoxia. Augmentation of PHD2 activity therefore may be an important method to alleviate inflammatory response to inspired hypoxia.

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Research in Context

Evidence before this study

High altitude is characterized by low oxygen tension, increased insolation and lower temperatures. Sojourners to mountains therefore are often confronted with myriad of health problems, referred to as AMS and HAPE. Of all environmental features, the primary causative agent however is hypobaric hypoxia. One of the associated features of AMS and HAPE is inflammation, which is thought to play a role in exacerbation of these high-altitude complications. Ethnic groups dwelling in high altitudes however are genetically and physiologically adapted to this hypoxic condition. One such genetic adaptation is observed in Tibetans in the form of a unique genetic variant of *EGLN1* gene which codes for PHD2. This variant PHD2^{D4E;C127S} has higher affinity for oxygen and therefore remain active even under hypoxia [10]. This variant maintains the red blood cell (RBC) count within normal range in Tibetan highlanders. However, as hypobaric hypoxia is known to modulate inflammation, we investigated if PHD2^{D4E;C127S} is associated with prevention of hypoxia-driven inflammatory response in Tibetan highlanders.

Added value of this study

This study sheds light upon the fact that the gain-of-function PHD2^{D4E;C127S} homozygous variant which has evolved as a result of selection pressure of hypoxia can prevent hyper-activation of monocytes in Tibetans and in vitro. Thus, possibly putting a rein on the development of hypobaric hypoxia-related complications including inflammation in Tibetan highlanders. The observation in Tibetans serves as a model for us to appreciate the crucial role PHD2 plays in modulation of hypoxia-mediated inflammatory response. Hereafter, we metabolically enhanced the activity of PHD2 using α -ketoglutarate in vitro and in mice model of inspired hypoxia. We could confirm that augmented-PHD2 activity is a rate limiting factor in prevention of inflammatory response to hypoxia including reduction in leukocyte infiltration to the lungs in mice.

Implications of all the available evidence

Our study highlights PHD2 as a key regulator of inflammatory immune response to hypoxia. The evidences gathered suggest that calibrated modulation of PHD2 activity may help us in tuning immune response beneficially. The metabolic approach to augment PHD2 activity has been adopted earlier in cell systems. For the first time, we translate this approach to limit exaggerated inflammatory response. α -Ketoglutarate being a physiological metabolite, without any discernible adverse effects in the purview of our mice study, may serve as a modulator of hypoxia-driven inflammatory responses.

1. Introduction

Hypobaric hypoxia is an environmental feature of the high altitudes. Exposure to hypobaric hypoxia leads to proliferation of RBCs i.e. polycythaemia. Some of the sojourners to high altitude develop acute mountain sickness (AMS) and high-altitude pulmonary edema (HAPE), which are characterized by nausea, headache, tachycardia, tightness of the chest, shortness of breath, hypertension and hypoxemia [1,2]. Alongside these, sterile inflammation induced by inspired hypoxia is also reported in AMS and

HAPE patients [3,4]. Inflammation is manifested as elevated pro-inflammatory cytokines and leukocytes in alveolar lavage fluid as well as in circulation in HAPE [5–7] and AMS [4,8]. The rapid ascent of non-acclimatized lowlanders to high altitudes results in the above complications more severely. Although reports suggest the association of inflammation with AMS and HAPE, but the mechanism and severity of the event is still unclear. Several reports suggest that HAPE may be an inflammation-independent condition [9]. But inflammation is thought to play a role in exacerbation of these high-altitude complications and may even result in fatality.

In contrast, some high-altitude populations such as Tibetans, Ethiopians and Andeans have developed evolutionary adaptation to environmental hypoxia and are protected from AMS, HAPE and polycythaemia [10–17]. A report however does suggest that some sections of Andeans, depending on their genetic background, may be more susceptible to chronic mountain sickness [18]. In Tibetans, genetic adaptation to high altitude has been primarily associated with the genes *EPAS1* [encodes hypoxia inducible factor (HIF)2 α] and *EGLN1* (encodes PHD2), which are under the strongest evolutionary genetic selection [11–13]. PHD2 variant alone or in combination with HIF2 α variant are associated with some degree of protection against elevated haemoglobin concentration in Tibetans [10,11,14,16].

PHD2 is an oxygen dependent hydroxylase that degrades HIF1 α and HIF2 α , which primarily regulate oxygen homeostasis [19,20] and modulate a large number of genes associated with various cellular functions [21]. In normoxia, PHD2 hydroxylates proline residues (Pro402 and Pro564) of HIF1 α , and Pro-405 and Pro-531 of HIF2 α in a reaction decarboxylating α -ketoglutarate (α KG). The von Hippel-Lindau tumour suppressor protein (pVHL) then binds to hydroxylated HIF1 α and HIF2 α , and initiates their ubiquitination. This leads to their degradation via ubiquitin mediated proteasomal pathway. However, in hypoxia, PHD2 displays limited hydroxylation activity upon its substrates. This leads to HIF α stabilization and dimerization with HIF1 β [22–24]. We have earlier reported that two missense *EGLN1* mutations that encode Asp4Glu and Cys127Ser in PHD2 (PHD2^{D4E;C127S}) have about ~85% gene frequency in Tibetans and are associated with their protection from polycythaemia in hypoxic environment of high altitude [10]. While another study has suggested impaired down-regulation of HIF pathway by this haplotype [25], we, in contrast have shown that the protein of PHD2^{D4E;C127S} variant has significantly higher affinity for oxygen than that of wild type (PHD2^{WT}) and thus degrades HIF1 α and HIF2 α more efficiently. Thus, inhibition of HIF stabilization in a hypoxic environment due to the enhanced activity of PHD2^{D4E;C127S} variant is one of the facilitators of Tibetan adaptation to high altitude [10]. Data from other reports were also consistent with gain-of-function properties of PHD2^{D4E;C127S} [13,26].

Among the molecular mechanisms, the impact of hypoxia on inflammatory response of immune cells can vary depending on the microenvironment. Increased oxygen consumption by immune cells increases rates of glycolysis and in turn alters the metabolic environment, which counter-regulates their inflammatory responses through activation of hypoxia inducible factor (HIF) [27]. Hypoxia-induced inflammatory response is orchestrated by HIFs and NF κ B [28], and also by crosstalk between both the transcription factors [29]. There is also evidence that PHD2 may directly regulate NF- κ B/p65 to influence inflammatory outcomes and cytokine secretion [30]. PHD2 also interacts directly with several other signalling molecules including Akt [31], protein phosphatase B55 α [32] and co-chaperone p23 [25], and regulates cellular functions. *Phd2*^{+/-} mice displayed an altered phenotype and function of peritoneal M2 macrophages in promoting angiogenesis [33].

In this study, we have investigated the possible role of gain-of-function PHD2^{D4E;C127S} variant in favourably modulating the in-

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flammatory immune response amongst Tibetan highlanders. Also, we have examined if PHD2 can be considered a target in regulation of inflammation induced by inspired hypoxia. Our findings show that PHD2^{D4E:C127S} suppresses pro-inflammatory functions of monocytes in response to hypoxia by down-modulation of signalling molecules p65, ATF2, STAT1, cjun and CXCR4. In contrast, these parameters were upregulated in sojourners with AMS or HAPE at same high altitudes. Further, augmentation of PHD2 with α KG in vitro and in mice led to recapitulation of our observations in PHD2^{D4E:C127S} monocytes. Thus, identifying PHD2 as a crucial regulator of hypoxia-driven inflammatory responses.

2. Methods

2.1. Ethics

The Institutional Ethics Committee of Regional Centre of Biotechnology and Sher-i-Kashmir Institute of Medical Sciences approved the human study, reference numbers RCB-IEC-H-01 and RCB-IEC-H-22. Written informed consent was received from all participants.

The experimental protocol for using mice was approved by Institutional Animal Ethics Committee of Regional Centre of Biotechnology reference number RCB/IAEC/2020/068, and the experiments were conducted in the Small Animal Facility (SAF) of our Institute.

2.2. Study subjects

We collected limited volume (total 5 ml) of whole blood in EDTA and acid citrate dextrose anticoagulant vials from each healthy volunteers (18–40 yrs) of Tibetan and non-Tibetan ancestries. Healthy Tibetan volunteers were recruited on the basis of two major criteria: 1) healthy (without any chronic disease, drug use or a history of any infective illness in the one month preceding the recruitment, outlined in Table S1), and 2) living for at least a generation in the same area. The sample size estimation was performed using genotype frequency data from our previous publications [10,16]. We calculated the sample number at 95% confidence interval, 80% chance of detection and ratio of wild type to homozygous haplotype carrying individuals being set at 0.2 for high altitude given 80% of the Tibetans carry this haplotype at high altitude and 50% of Tibetans carry this haplotype at sea level [10,16]. Based on available literature [34,35] addressing WBC and monocyte percentage alteration as a result of hypobaric hypoxia exposure we set a difference of 5% between means of the two genotypes and a standard deviation of 5. The calculation was done using CDC's statistics tool (http://www.openepi.com/Menu/OE_Menu.htm) The prediction of the sample size calculation was to collect 48 PHD2^{D4E:C127S} homozygous individuals and 10 PHD2^{WT} individuals from high altitude and 16 PHD2^{D4E:C127S} homozygous and 16 PHD2^{WT} individuals from sea-level. Sample of 55 Tibetans (20 homozygous, 21 heterozygous and 14 WT) and 17 non-Tibetans were collected from Tibetan settlements in Leh and Srinagar (3600 and 1700 m above the sea level). 44 Tibetan (13 homozygous, 22 heterozygous and 9 WT) and 6 non-Tibetan samples were collected from the Tibetan settlement at Delhi. However, we could not achieve the predicted number due to strict Governmental regulations and COVID-19 related travel restrictions during the study period.

Inclusion and exclusion criteria are described in Table S1. Matched case-control study could not be performed. The median age and gender distribution of sample collected is mentioned according to genotype and altitude in supplementary Table S1. The experimental protocol is outlined in Fig. S1a.

5 ml of whole blood was collected from AMS (n=9) and HAPE (n=7) patient at a local clinic in Leh under the same protocol. AMS

diagnosis was based on Lake Louise (LL) score >5 and HAPE was confirmed by chest X-ray as well as Lake Louise (LL) score >5. Patients with co-morbidities were excluded from the study, outlined in Table S3. We could recruit a small number of AMS and HAPE patients due to limitation and restriction. The median ages of healthy traveller and AMS were comparable but that of HAPE was slightly higher (31±1.9, 30±2.54 and 41±3.7 yrs respectively). It is also noticeable that most patients were male and therefore the preliminary data obtained is more representative of the male population. Another limitation of the study was the recruitment of a smaller number of healthy travellers (n=6), mainly accompanying the patients at hospital. Whole blood was used for isolation of plasma and PBMCs and the studies outlined in the text.

2.3. Genotyping of SNPs

Genomic DNA was isolated using Qiagen flexigene kit. The targeted EGLN1 SNPs (NCBI RefSeq NG_015865), present in exon 1, were PCR amplified using the primers-EGLN1-X1F: CCCCTATCTCTCCCCG and EGLN1-X1R: CTGTCCAGCACAAACCC and Phusion Polymerase Master Mix (New England Biolabs, Thermo Fisher Scientific, USA). Amplification was carried out at 98°C for 5 mins and 40 cycles of 98°C for 120 secs, 60.1°C for 60 secs and 72°C for 120 secs. This was followed by final extension at 72°C for 5 mins and a 4°C hold, generating the 1025 bps product size. The amplified product was then purified and sequenced to identify the SNPs c.12C<G and c.380G<C as outlined in our previous works [10].

2.4. Isolation of PBMCs and immunophenotyping of leukocytes

PBMCs were isolated from whole blood using the Ficoll-Hypaque (Lymphoprep) density gradient centrifugation method [36]. Flow cytometry based immunophenotyping of monocytes was performed using a mixture of appropriate concentration of monoclonal antibodies directed against CD45 V500, CD11c V450, CD14 FITC, CD16 APCCy7 and HLA-DR APC (BD Biosciences, USA) and CXCR4 PECy7 (eBioscience, USA). The detailed information of antibodies is mentioned in Table S5. PBMCs were first assessed on forward and side scatter plot. CD45⁺ cells were gated to identify the leukocytes as outlined [36,37]. 50,000 events were acquired for immunophenotyping using PBMCs and 30,000 events were recorded while phenotyping enriched monocytes. The acquired data was analysed using the Flowjo software (Tree Star, USA). For detailed gating strategy see Fig. S1c.

2.5. mRNA expression analysis using nCounter technology

Total RNA was isolated from PBMCs of native Tibetans of high altitude and sea level. The mRNA expression was assessed using nanostring nCounter technology as per manufacturer's instructions (nanoString, Seattle, USA). Total RNA from each sample was hybridized with the pre-designed reporter code set and then capture probe set for 16 hrs at 65°C. The sample-probe mixture volume was made up to 30 μ L using RNase-free water and loaded into the nCounter SPRINT Cartridge. The loading ports were sealed and placed onto the cartridge of the nCounter® SPRINT Profiler, located at the CSIR-IGIB, New Delhi. Differential expression between genotypes was analysed using the nSolver 4.0 software. Wald test was used to analyse the data and significance was adjudged using adjusted p-value. Data analysis involved counting of 47 transcripts in each sample, normalized with housekeeping genes, described in Table S2.

2.6. Real time PCR

Total RNA was isolated as described above and cDNA synthesis was performed using random hexamers and reverse transcriptase in a one-step PCR method (Bio-Rad, USA). Real time PCR was performed using the Sybr green method (Solis) on Quant studio 6 (ABI). The primers used are described in Table S4. Data was calculated using the δC_T using the threshold cycle (C_T) of TUBB as the housekeeping control. Relative gene expression was calculated using $2^{-\delta C_T}$.

2.7. Generation of monocytic cell line expressing PHD2^{D4E:C127S} or PHD2^{WT} variant

Human U937 cell line (ATCC, USA) expressing PHD2^{D4E:C127S} or PHD2^{WT} variant was generated following the protocol described in our previous publication [10]. Briefly, endogenous PHD2 expression was suppressed using shRNA targeting the 3' UTR of endogenous EGLN1 using a liposome mediated delivery (Life Technologies, Thermo Fisher Scientific, USA) and then PHD2^{D4E:C127S} or PHD2^{WT} variant expressing plasmids were introduced using lentiviral particles. Detailed method is mentioned in Fig. S8a-b. The original cell line is validated and was also Mycoplasma contamination free.

2.8. Cytokine secretion assay

The cytokine secretion assay was performed from secretome of primary monocytes as well as the cell lines. Primary CD14 positive monocytes were either kept in hypoxia (1% oxygen) or normoxia (21% oxygen) for 24 hrs; and thereafter the culture supernatant was collected. The viability of primary monocytes exposed to 1% oxygen was 85%. The supernatant was then assessed for the cytokines IL6, IL8, IL1 β , TNF α , IL10, TGF β and VEGF using the cytometric bead array (BD Biosciences, USA), as described in our previous work [36].

2.9. Chemotaxis assay

The treatment of primary monocytes for each sample and the cell lines was performed in a similar way as described in the above section. The Boyden chamber (Corning, USA) system was used for this assay. After treatment, 100 μ L of cell suspension in PBS with 5×10^4 cells was then carefully pipetted on to the 5 μ m size filter of the insert. In the 24-wells plate, the inserts were placed and each well was filled with 200 μ L of PBS containing either 4 pM of Ubiquitin or CXCL12 (PeproTech, NJ, USA). The migration was allowed to take place for 2 hrs at 37°C, 5% CO₂ under either 21% or 1% oxygen in accordance with preceding experiments described above. The number of cells migrated to the lower chamber was counted using flow cytometry (BD FACS verse).

2.10. Immunoblotting

The whole cell (primary monocyte or monocytic cell line) lysate was prepared using RIPA lysis buffer and protease inhibitor (Sigma, USA). SDS-PAGE gel was followed by immunoblotting using primary antibodies against phospho and non-phospho STAT1 and p65, PHD2 and HIF1 α (Cell Signaling Tech, USA), c-Jun, phospho and non-phospho ATF2, HIF2 α and α -tubulin (Thermo Fisher scientific) as described in detail in our previous work [38]. The detailed information of antibodies is mentioned in Table S5. All the antibodies are commercially validated and application is amply cited in published literature.

2.11. Octyl α -ketoglutarate treatment

U937 cells were pre-treated with 1mM octyl α -ketoglutarate (Sigma, USA) for 4 hrs. Fresh media was replaced and cells were exposed to either hypoxia (1% oxygen) or normoxia (21% oxygen) for 24 hrs. Cell supernatant was used for assessing cytokines using the cytometry-based array. Cells were used for performing chemotaxis assay as described above. Protein lysate prepared from these cells were used for immunoblotting of signalling molecules.

2.12. Dietary α -ketoglutarate administration to mice

The sample size calculation was performed after doing a pilot study with 4 C57BL/6 mice per group. Based on the outcome of this pilot study, we calculated number of mice per group at confidence interval of 95% and power of 80% to get a p-value less than 0.01 (<http://www.lasec.cuhk.edu.hk/sample-size-calculation.html>). The predicted number of mice to be kept in each group was calculated, n=6. mice in each group: 1) normoxia (21% oxygen) and 2) normobaric hypoxia (11% oxygen) 3) with and 4) without 1% of dietary α -ketoglutarate (SRL, Mumbai, India) in drinking water. α -Ketoglutarate (α KG) was administered 12 hrs before exposing mice to hypoxia in a chamber for 48 hrs. Initially we exposed the mice to normobaric hypoxia and assessed an elevation in inflammatory response mainly in lungs. We therefore continued with normobaric hypoxia exposure to mice with and without dietary supplement of α KG. The experimental design is mentioned as schematic Fig. S14a. Thereafter the animals were anaesthetized using Ketamine and Xylazine injection, and bronchoalveolar lavage fluid (BALF) was collected. Whole blood was collected through cardiac puncture and used for immunophenotyping using Nihon Kohden's CelltacF haematology analyser and also flow cytometry using surface markers for monocytes and neutrophils [CD45.2 APCCy7, Ly6C V450, Ly6G FITC (Biolegend, USA) and CD11b Percp (Invitrogen)], PBMCs were used for RNA isolation and cDNA synthesis for qPCR analysis of several genes. Whole cell lysates from PBMCs were also used for immunoblot analysis of HIF2 α , P-p65 and p65. Plasma was used for evaluation of cytokines using flow cytometry-based array and estimation of α KG level. Mice lung lobes were fixed with 4% paraformaldehyde and embedded in paraffin blocks to prepare longitudinal sections. The Haematoxylin and Eosin staining was performed and image was captured using 60x oil objective in a Nikon eclipse Tie2 series microscope.

2.13. Single metabolite measurement

Steady-state level of α KG was measured in plasma and PBMC-granulocytes (10^6 cell) lysate using colorimetry based assay kits (Sigma Aldrich, USA, catalog No. MAK054 and MAK335) according to the manufacturers' protocol.

2.14. Statistical analysis

Statistical analysis was performed using Kruskal Wallis test followed by Dunn's multiple comparison post-test or unpaired t-test as per requirement. Skewness and Kurtosis analysis and frequency distribution curves were used to check for normal distribution of data. Graph Pad Prism 7.0 software was used for data analysis and p values <0.05 were considered to be statistically significant.

3. Role of funding source

This study is supported by the Department of Biotechnology (DBT), Govt. of India. DBT promotes basic as well as applied research in the area of biotechnology. The funder had no role in study design, data collection, data analysis, interpretation, or writing the manuscript.

4. Results

4.1. Healthy homozygous PHD2^{D4E;C127S} Tibetans of high altitudes maintain sea-level comparable monocyte counts unlike elevated numbers in PHD2^{WT} or heterozygous counterparts of same altitude

In order to explore the possible association of PHD2^{D4E;C127S} variant with immune response in Tibetans residing at high altitude, we undertook analysis of total leukocytes (experimental protocol is outlined in Fig. S1a). The healthy PHD2^{WT} high-altitude Tibetans (for inclusion criteria and genotyping data see Table S1) had elevated total monocytes (Fig. 1a and S2a) including higher inflammatory and patrolling (Fig. 1b-c) but lower classical subsets (Fig. S2d). In contrast, PHD2^{D4E;C127S} Tibetan highlanders maintain sea-level comparable percentage and total number of monocytes (Fig. 1a and S2a). In Tibetans residing at sea level, no such difference was observed between PHD2^{D4E;C127S} and PHD2^{WT} (Fig. 1a and S2a). Although the total leukocyte count was higher in PHD2^{WT} than PHD2^{D4E;C127S} at high altitudes (Fig. S2b) no significant difference in lymphocyte counts were detected (Fig. S2c). Our data thus provide first evidence of association of this evolutionarily selected Tibetan-specific PHD2^{D4E;C127S} variant with a distinct distribution of monocyte subsets in hypoxic environment.

While we expected similar changes in heterozygotes PHD2^{D4E/WT;C127S/C127S} and PHD2^{D4E/WT;C127S/WT} to PHD2^{D4E;C127S} homozygotes; their leukocyte and monocyte counts were similar to those of PHD2^{WT} at both high altitude and sea level (Fig. S2a-b).

4.2. Reduced secretion of pro-inflammatory cytokines by PHD2^{D4E;C127S} monocytes upon exposure to hypoxia in vitro

The monocytes of Tibetans carrying PHD2^{D4E;C127S} variant secreted less pro-inflammatory cytokines IL6 and IL1 β in response to hypoxia at 1% oxygen for 24 hrs, compared to PHD2^{WT} counterparts (Fig. 1d-e). No significant difference was observed in the levels of TNF α , TGF β and IL10 in both genotypes (Fig. S3a-c). Heterozygous PHD2^{D4E/WT;C127S/C127S} or PHD2^{D4E/WT;C127S/WT} monocytes exhibited no significant change in levels of IL6 and IL1 β compared to PHD2^{WT} (Fig. S3d-e). Also under normoxic condition, minimal secretion of cytokines was observed without any significant difference between the genotypes.

When the same experiment was conducted in vitro with monocytes of Tibetans from sea level under hypoxia, a similar pattern in secretion of these cytokines was observed as with those of PHD2^{D4E;C127S} monocytes collected from high altitude (Fig. S4a-e). Thus down-modulation of pro-inflammatory phenotype of PHD2^{D4E;C127S} monocytes occur only in response to hypoxia. We also measured IL6, IL1 β , TNF α , IL10, TGF β , VEGF and IL8 in the plasma of high altitude resident Tibetans and observed a significantly less IL1 β level in PHD2^{D4E;C127S} than PHD2^{WT} counterparts (Fig. S5a-g).

We then assessed the expression of hypoxia-responsive factors associated with immune response in leukocytes of both PHD2^{D4E;C127S} and PHD2^{WT} Tibetans residing at sea level and high altitude.

4.3. Down-modulation of immune response related genes including RELA, JUN, ATF2, STAT1 and CXCR4 in healthy PHD2^{D4E;C127S} Tibetans compared to PHD2^{WT} at high altitude

We used the nCounter technology from Nanostring to measure the expression of 47 transcripts at the interface of hypoxia and immune response of PBMCs in PHD2^{D4E;C127S} and PHD2^{WT} Tibetans (n=10 each) from high altitude and sea level. The PHD2^{D4E;C127S} Tibetan of high altitude had significantly down-modulated expression of several transcripts including *RELA*, *ATF2*, *STAT1*, *JUN* and

CXCR4, known for their role in cytokine secretion and chemotaxis, than PHD2^{WT} (Fig. 2a). Total transcript data are depicted in Table S2. However, this downmodulated expression was not observed in the genotypically identical healthy Tibetans living at sea level (Fig. 2b). This finding indicates that the suppressive effect of PHD2^{D4E;C127S} on the above mentioned molecules is manifested only in the presence of a hypoxic environment.

In order to validate these findings, monocytes from Tibetans of sea level with either PHD2^{D4E;C127S} or PHD2^{WT} were exposed in vitro to hypoxia at 1% oxygen for 24 hrs. The decreased expression of *RELA*, *STAT1*, *ATF2* and *JUN* genes in these hypoxia-exposed PHD2^{D4E;C127S} monocytes (Fig. 2c-f) was consistent with our observation in Tibetans living at high altitudes (Fig. 2a), which confirmed the crosstalk between this variant and hypoxic environment in down-modulating monocyte response. Thus, epigenetic changes due to acclimatization by prolonged residence at high altitude since birth may not be the primary or sole mode of modulation of these genes.

In the same experiments, we did not observe decreased expression of *RELA*, *STAT1*, *ATF2* and *JUN* genes in monocytes from heterozygous individuals carrying PHD2^{D4E/WT;C127S/C127S} or PHD2^{D4E/WT;C127S/WT} in response to hypoxia and also observed no significant change with respect to the PHD2^{WT} monocytes (Fig. S6a-d). These observations suggest that hypoxia suppresses the inflammatory pathway demonstrably in homozygous but not measurably in heterozygous individuals. However, more detailed evaluation will be needed to explore the role of the PHD2^{D4E;C127S} heterozygosity in immune functions.

4.4. Reduced chemotactic ability of PHD2^{D4E;C127S} monocytes in response to ubiquitin and CXCL12 upon exposure to hypoxia in vitro

In addition to cytokine secretion data, we investigated the chemotactic ability of PHD2^{D4E;C127S} monocytes. We found significant suppression of transcript (Fig. 3a) as well as surface expression (Fig. 3b) of CXCR4, known for its crucial role in cell migration in PHD2^{D4E;C127S} Tibetan monocytes, while the expression pattern of the molecule in heterozygotes (PHD2^{D4E/WT;C127S/C127S} or PHD2^{D4E/WT;C127S/WT}) was not measurably decreased and was found to be similar to that of PHD2^{WT} (Fig. S7a-b).

We then tested chemotactic ability of PHD2^{D4E;C127S} monocytes under hypoxia at 1% oxygen for 24 hrs. In response to ubiquitin as well as CXCL12 (cognate chemoattractants for CXCR4), the chemotactic ability of hypoxia-exposed PHD2^{D4E;C127S} monocytes was significantly reduced (Fig. 3c-d). Our data indicate that the PHD2^{D4E;C127S} monocytes have limited migration in hypoxia likely to be due to the down-modulated expression of CXCR4.

4.5. U937 monocytic cell line engineered to express PHD2^{D4E;C127S} variant display diminished secretion of inflammatory cytokines and reduced chemotactic ability upon exposure to hypoxia

To confirm the above ex vivo observations we generated monocytic cell line engineered to express PHD2^{D4E;C127S} variant (Fig. S8a-b). U937 cells expressing PHD2^{D4E;C127S} suppressed hypoxia-triggered elevation of pro-inflammatory cytokines IL6 and IL1 β (Fig. 4a-b) along with expression of genes *RELA*, *STAT1*, *ATF2* and *JUN* (Fig. S8f-i) as well as phosphorylated and total protein of p65, ATF2 and STAT1, and cJun (Fig. 4c) compared to PHD2^{WT} cells upon exposure to hypoxia at 1% oxygen for 24 hrs. Also the nuclear extract of the PHD2^{D4E;C127S} cells displayed less p65 compared to PHD2^{WT} cells (Fig. 4e). However, a detailed mechanism remains to be established how PHD2 regulates the expression of these molecules. As HIF α -subunits are the substrate of PHD2, we examined the expression of HIF2 α . The expression of HIF2 α in total cell extract (Fig. 4d) as well as nuclear extract (Fig. 4e) was sig-

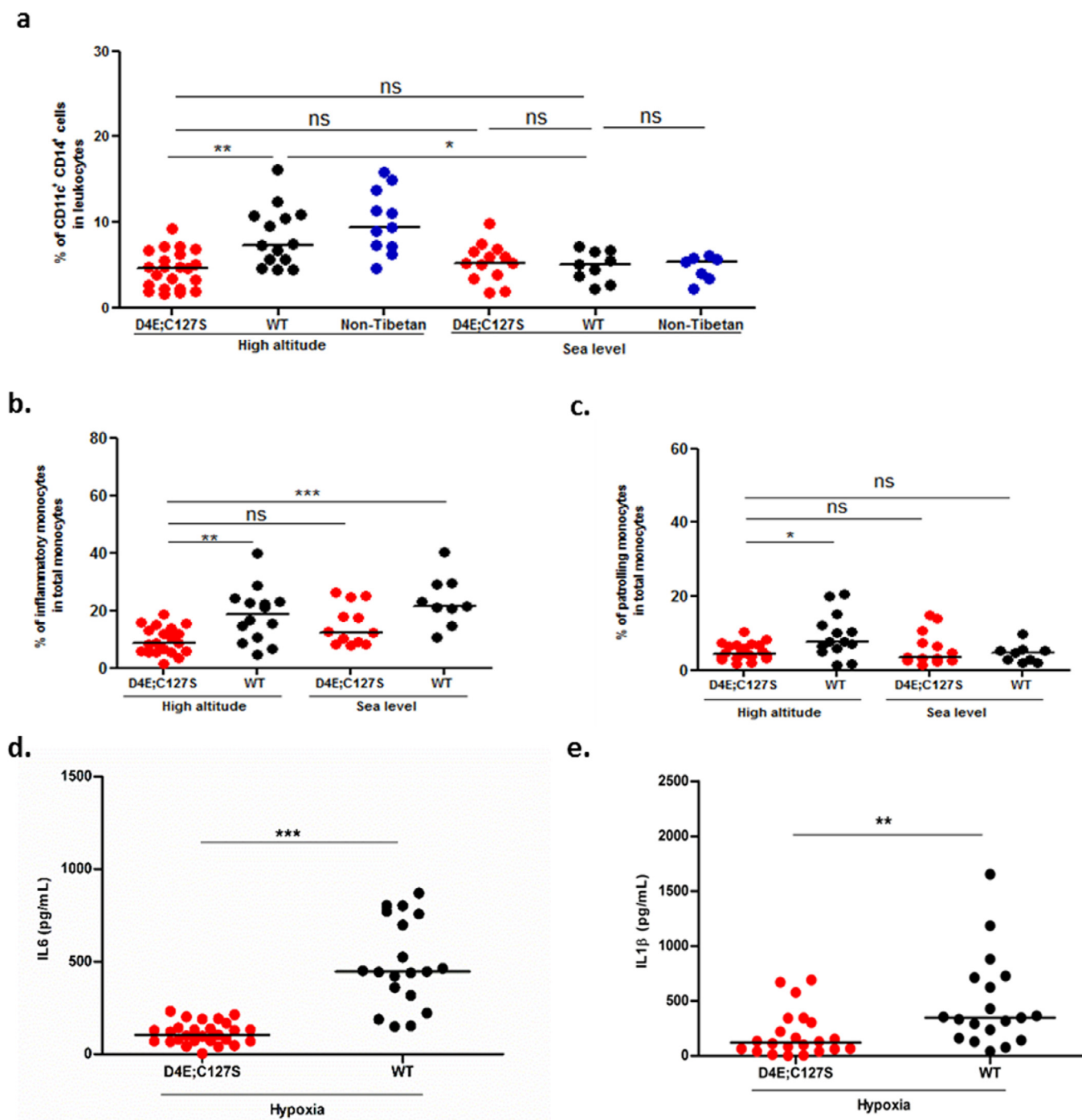


Fig. 1. Elevated monocyte counts in PHD2^{WT} Tibetans than PHD2^{D4E;C127S} at high altitude. Monocyte count was measured in PBMCs of healthy Tibetans carrying either homozygous PHD2^{D4E;C127S} or PHD2^{WT} from high altitude and sea level using flow cytometry. (a) Percentage of monocytes in total leukocyte, (b) inflammatory and (c) patrolling subsets in total monocytes were measured. The detail immunophenotyping gating strategy is described in Fig. S1. Each dot represents the percentage of cells in each individual. Kruskal Wallis test was used on median values for comparison between genotypes followed by Dunn's multiple comparison post test, ****p* < 0.001, ***p* < 0.01, **p* < 0.05 and ns = non-significant. Classical monocyte subtype count is mentioned in Fig. S2d. Also the monocyte count was analysed from whole blood using haematology analyser; these data show similar trend, depicted in Fig. S2a. A similar count of monocytes was observed in Tibetans between PHD2 heterozygous and wild-type, is shown in Fig. S2a. **Reduced secretion of pro-inflammatory cytokines by PHD2^{D4E;C127S} monocytes than PHD2^{WT}.** The CD14⁺ monocytes isolated from PBMCs were exposed to 1% oxygen for 24 hrs. CBA assay was used to assess supernatants for the levels of (d) IL6 and (e) IL1β, to compare between PHD2^{D4E;C127S} and PHD2^{WT} Tibetans. Unpaired *t*-test was used for comparison between genotypes, ****p* < 0.001 and ***p* < 0.01. Levels of other cytokines as well as comparison of values amongst homozygous, heterozygous and wild type are mentioned in Fig. S3.

nificantly down-modulated in PHD2^{D4E;C127S} U937 cells under hypoxia treatment compared to U937 cells with either PHD2^{WT} or empty vector (Fig. S8k). We also noted a marginal increase in total protein of p65, ATF-2 and HIF2α in the PHD2^{D4E;C127S} U937 cells maintained under normoxia. This increase was however not sig-

nificant and we couldn't associate it with any of the phenotypic outcomes. Hence it needs further investigation.

The PHD2^{D4E;C127S} U937 cells also had decreased expression of CXCR4 transcript (Fig. S8j) as well as its surface expression (Fig. 4f) in hypoxia, compared to the PHD2^{WT}. This led to observation of

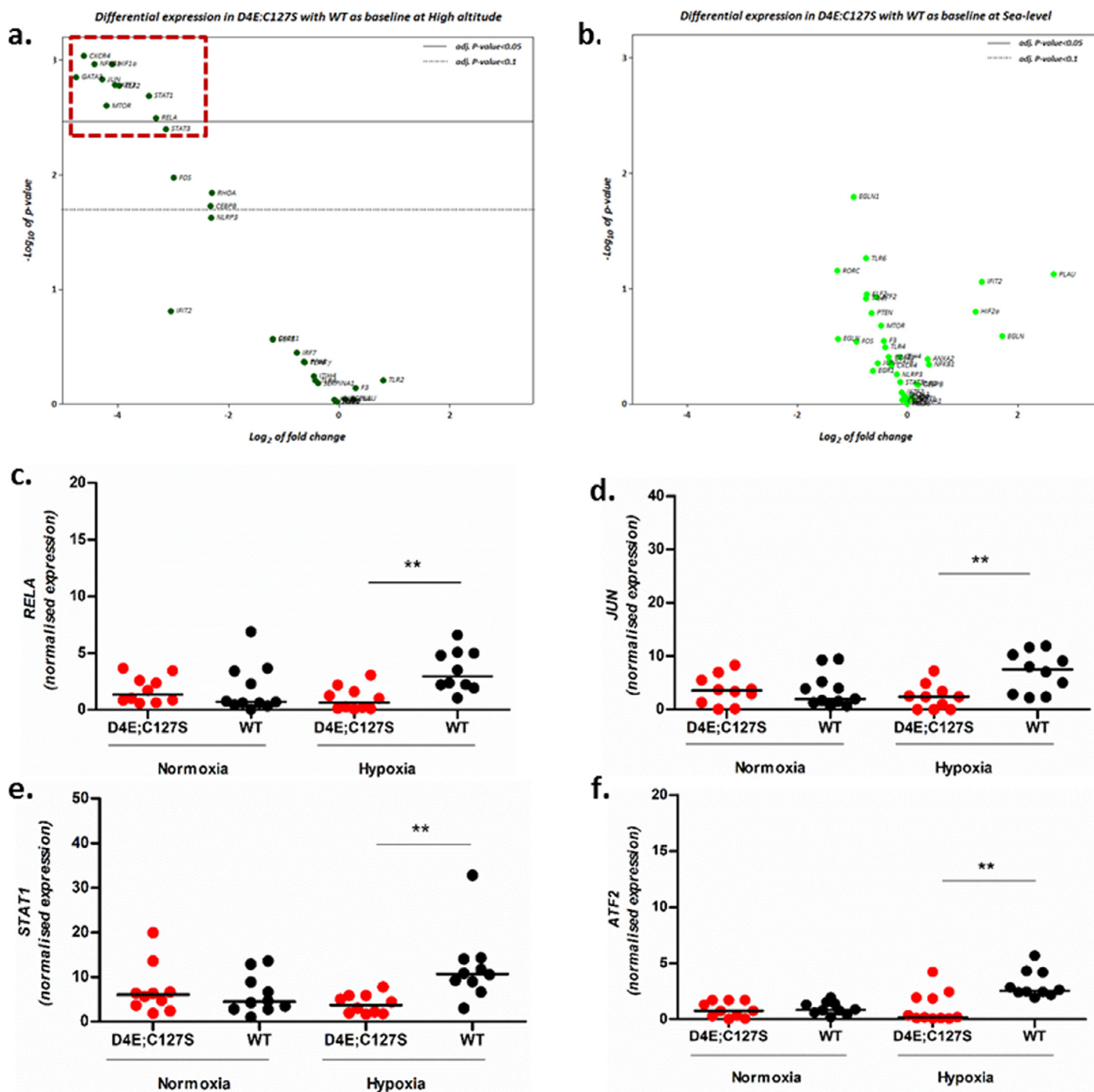


Fig. 2. Differential expression of hypoxia and immune response genes in Tibetans. Total RNA isolated from PBMCs was probed for expression of 47 genes at the interface of hypoxia and immune response. The differential expression of the genes is shown as volcano plot for (a) high altitude and (b) sea level Tibetans. The nCounter technology was used to measure gene expression. Differential expression between PHD2^{D4E:C127S} and PHD2^{WT} (n=10 each) Tibetans was calculated using the advanced analysis module of the nSolver software. It uses the Wald test with adjusted p-value. Data in detail are described in Table S2. The differentially expressed genes in nCounter data were validated in CD14⁺ monocytes of PHD2^{D4E:C127S} and PHD2^{WT} individuals residing at sea level. The monocytes were exposed to 21% or 1% oxygen for 24 hrs and gene expression of (c-f) *RELA*, *JUN*, *ATF2* and *STAT1* was evaluated using real-time PCR. Each dot represents the relative quantification for each individual. Unpaired t-test was used for comparison between genotypes, **p<0.01. The expression of above genes between homozygous, heterozygous and wild type is mentioned in Fig. S6.

compromised chemotactic ability PHD2^{D4E:C127S} cells in response to ubiquitin and CXCL12 in hypoxia, compared PHD2^{WT} (Fig. 4g-h), recapitulating our primary monocyte data (Fig. 3). Therefore, the above observations comprehensively eliminate the possibility of difference in cell proportion between homozygotes and wild-type Tibetans ensuing into difference in transcript level of above mentioned genes in leukocytes and primary monocytes (Fig. 2 and 3).

4.6. Elevation of pro-inflammatory parameters alongside increased *RELA*, *JUN*, *ATF2*, *STAT1* and *CXCR4* was observed in sojourners with AMS and HAPE at same high altitude

We then investigated the role of the above identified molecules in hypoxia-induced inflammations in non-Tibetan sojourners to same high altitude who had developed AMS and HAPE. Our data show that in comparison to healthy sojourners, HAPE patients dis-

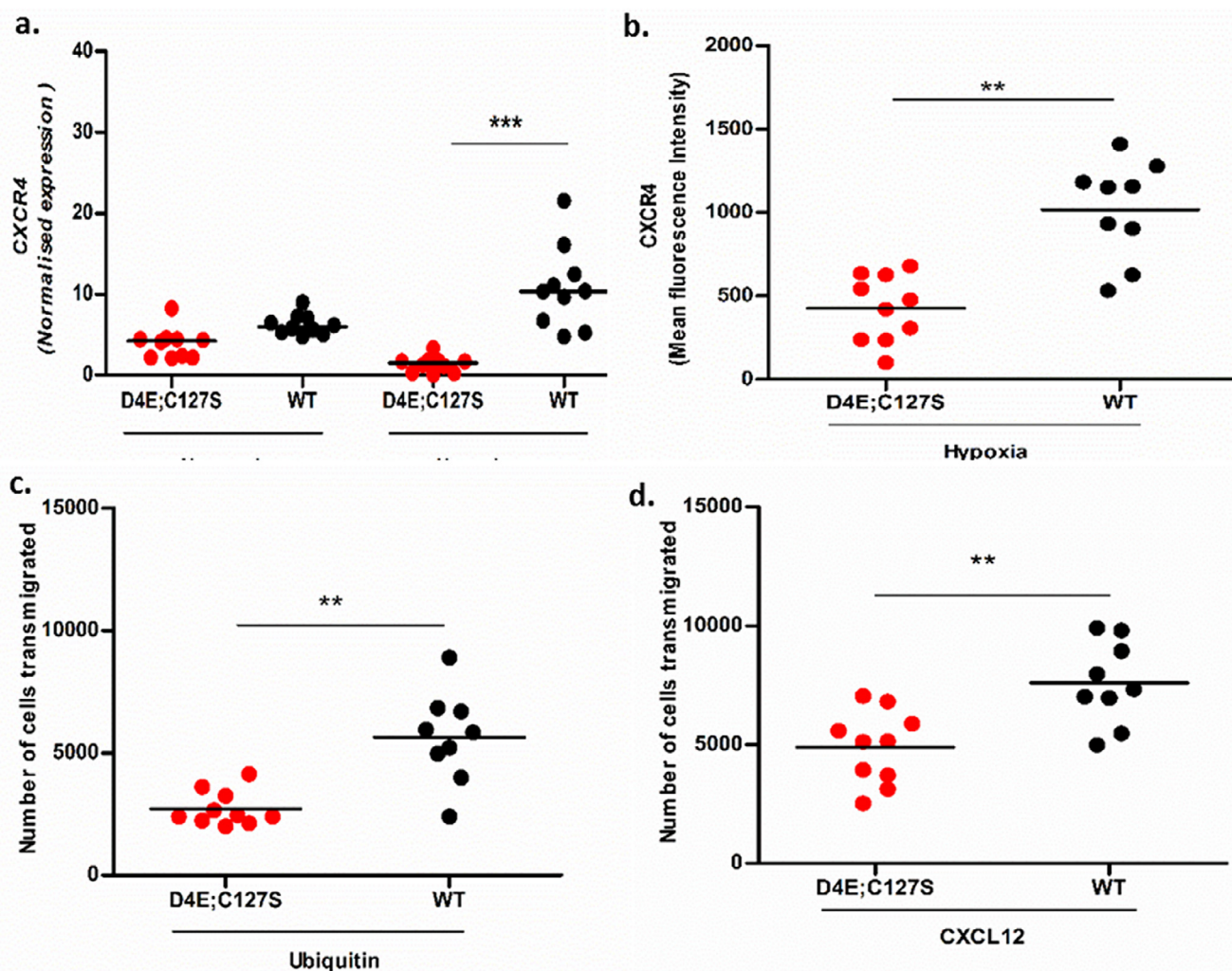


Fig. 3. Reduced chemotactic ability of PHD2^{D4E;C127S} monocytes. CXCR4 level was measured in CD14⁺ monocytes isolated from PBMCs of PHD2^{D4E;C127S} or PHD2^{WT} individuals residing at sea level. The monocytes were exposed to 21% O₂ or 1% O₂ and evaluated for (a) gene expression and (b) cell surface expression of CXCR4. Unpaired *t*-test was used for comparison between genotypes, ****p* < 0.001 and ***p* < 0.01. Chemotactic ability of these cells was measured using (c) ubiquitin and (d) CXCL12 in the lower chamber of the transwell plate. Each dot represents data from a single individual. Unpaired *t*-test was used for comparison between genotypes, ***p* < 0.01. The comparative expression of CXCR4 amongst homozygous, heterozygous and wild type is depicted in Fig. S7.

played increased monocyte count alongside increased percentage of patrolling subsets, and AMS patients displayed increased percentage of inflammatory monocytes in peripheral blood (Fig. 5a-c). The clinical data of AMS and HAPE patients are outlined in Table S3. The plasma levels of inflammatory cytokines including IL6, IL1 β (Fig. 5d-e) and TNF α (Fig. S9f) were significantly increased in these patients. Leukocytes obtained from AMS patients displayed significant upregulation in expression of *RELA*, *ATF2*, *JUN* (Fig. 5f-i) and *CXCR4* (Fig. S9g) in comparison to healthy sojourners. Significant upregulated expression of *STAT1* however was observed solely in HAPE patients. Both HAPE and AMS patients' monocytes had elevated levels of CXCR4 surface expression (Fig. 5j), suggestive of their augmented chemotactic ability.

Thus, our above data suggest that gain-of-function PHD2^{D4E;C127S} variant prevents exaggerated inflammatory response of monocytes in hypoxia, suggesting that augmentation of PHD2 activity may possibly abrogate the hypoxia-triggered immune response. As a proof to our above observations, we employed a strategy to inhibit hypoxia-triggered inflammation *in vitro* by augmentation of PHD2 activity. We used α -ketoglutarate,

a cofactor of PHD2, to augment PHD2 enzymatic activity of monocytes *in vitro*.

4.7. α -ketoglutarate mediated augmentation of PHD2 activity and in turn suppression of hypoxia-induced inflammatory response of monocytes *in vitro*

A pre-treatment of U937 monocytic cells for 4 hrs with octyl α -ketoglutarate (1mM, as described in [39]) significantly suppressed the hypoxia-induced elevation of cytokines IL6 and IL1 β (Fig. 6a-b) and CXCR4-mediated chemotactic response upon exposure to hypoxia at 1% oxygen for 24 hrs (Fig. 6c-e). Augmented PHD2 activity was confirmed by observing significant suppression of both HIF1 α and HIF2 α expression under hypoxia (Fig. 6f). As observed in the PHD2^{D4E;C127S} expressing U937 cells, a significant decrease in phosphorylated p65, ATF2 and STAT1, and total protein of cJun was observed in α KG-treated U937 cells under hypoxia (Fig. 6g). However, unlike PHD2^{D4E;C127S} U937 cells (Fig. 4c), the expression of total protein of the above transcription factors was found unaltered in α KG-treated U937 cells in hypoxia (Fig. 6g). This may be because PHD2^{D4E;C127S} also has alteration in functions distinct from hydroxylation, which needs to be further investigated. In the

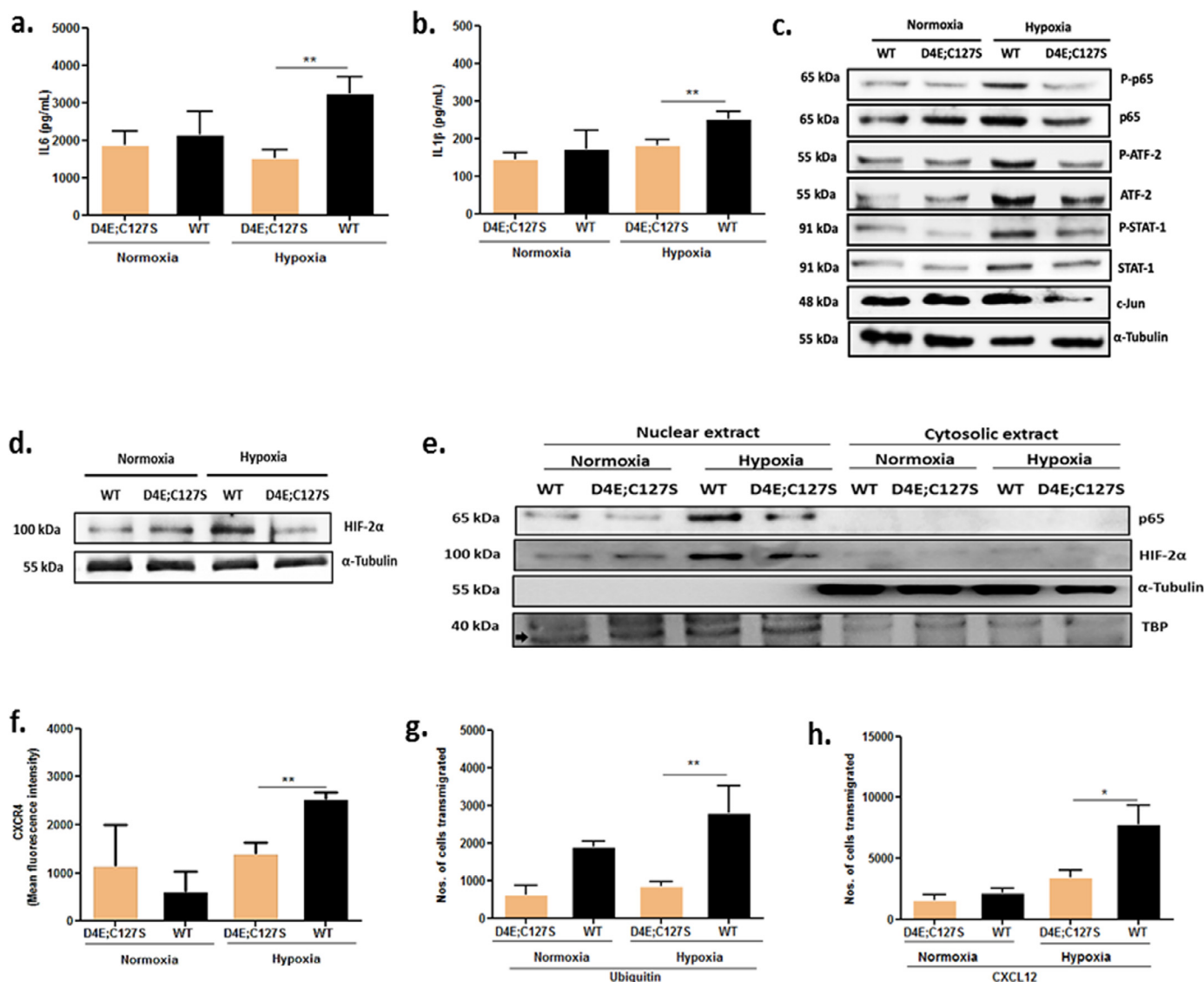


Fig. 4. U937 cell line expressing PHD2^{D4E;C127S} displays reduced cytokine secretion. Endogenous PHD2 was depleted using shRNA targeting the 3' UTR of endogenous *EGLN1*. Then, cells were transduced with constructs expressing either PHD2^{D4E;C127S} or PHD2^{WT} using lentiviral particles, detail in Fig. S8a-b. U937 cells expressing either PHD2^{D4E;C127S} or PHD2^{WT} were exposed to 21% or 1% oxygen for 24 hrs and cytometry based assay was used to assess supernatants for the level of (a) IL6 and (b) IL1β. Each bar represents mean ± SEM from three independent experiments in duplicates. Unpaired *t*-test was used for comparison, ***p* < 0.01. Other cytokine data are mentioned in Fig. S8c-d. **Down-modulated expression of signalling molecules in PHD2^{D4E;C127S} cells.** The cells from above experiments were assessed for genes, *RELA*, *JUN*, *ATF2* and *STAT1* (Fig. S8f-i) and (c) proteins- P-p65, P-ATF2, P-STAT1 and c-Jun, and (d) HIF2α. (e) Nuclear localisation of p65 and HIF2α was evaluated with respect to TBP. Protein expression was evaluated by western blot from three independent experiments. Densitometry data are shown in Fig. S15a-h. **Reduced chemotactic ability of PHD2^{D4E;C127S} cells.** The above cells assessed for gene (Fig. S8j) and (e) cell surface expression of CXCR4. Chemotactic ability of these cells was assessed using (f) ubiquitin and (g) CXCL12 in the lower chamber of the transwell plate. Data represented as mean ± SEM from three independent experiments. Unpaired *t*-test was used for comparison, ****p* < 0.001, ***p* < 0.01 and **p* < 0.05.

absence of hypoxic stimulus, we observed a marginal elevation in the levels of phosphorylated p65, ATF2 and STAT1, and also total HIF1α and HIF2α in αKG-treated cells (Fig. 6f-g) without any alteration in functions such as cytokine secretion and cell migration (Fig. 6a-e). We could not assign any significant functional relevance to this phenomenon within the purview of this report and therefore require further investigation. We hereafter attempted to verify that if the αKG mediated phenotype, under hypoxia, is indeed a result of increased activity of PHD2. The enzymatic activity of PHD2 was inhibited using ethyl-3-4-dihydroxybenzoic acid (DHB) in the presence of αKG. The experimental system was confirmed by observing that hypoxia-induced HIF2α expression was elevated upon treatment with DHB in the presence of αKG. We observed a partial reversal of the suppressive effect of αKG on hypoxia-triggered inflammatory cytokine secretion (Fig. S10e-g). Thus, showing that

augmentation of PHD2 activity is definitely one of the ways by which αKG exerts its protective effect in hypoxia. We also observed similar effects of αKG on primary monocytes of healthy individuals in vitro (Fig. S11a-c). Thus, the αKG supplementation to U937 cells/primary monocytes endogenously expressing PHD2^{WT} simulated the gain-of-function phenotype of PHD2 by inhibiting the hypoxia-triggered inflammatory response of these cells, similar to the phenotype of PHD2^{D4E;C127S} monocytes (Fig. 4). By performing survival assay with octyl αKG, attribution of down-modulation of monocyte function to cell death was ruled out as we observed no impairment in survival of these cells (Fig. S12).

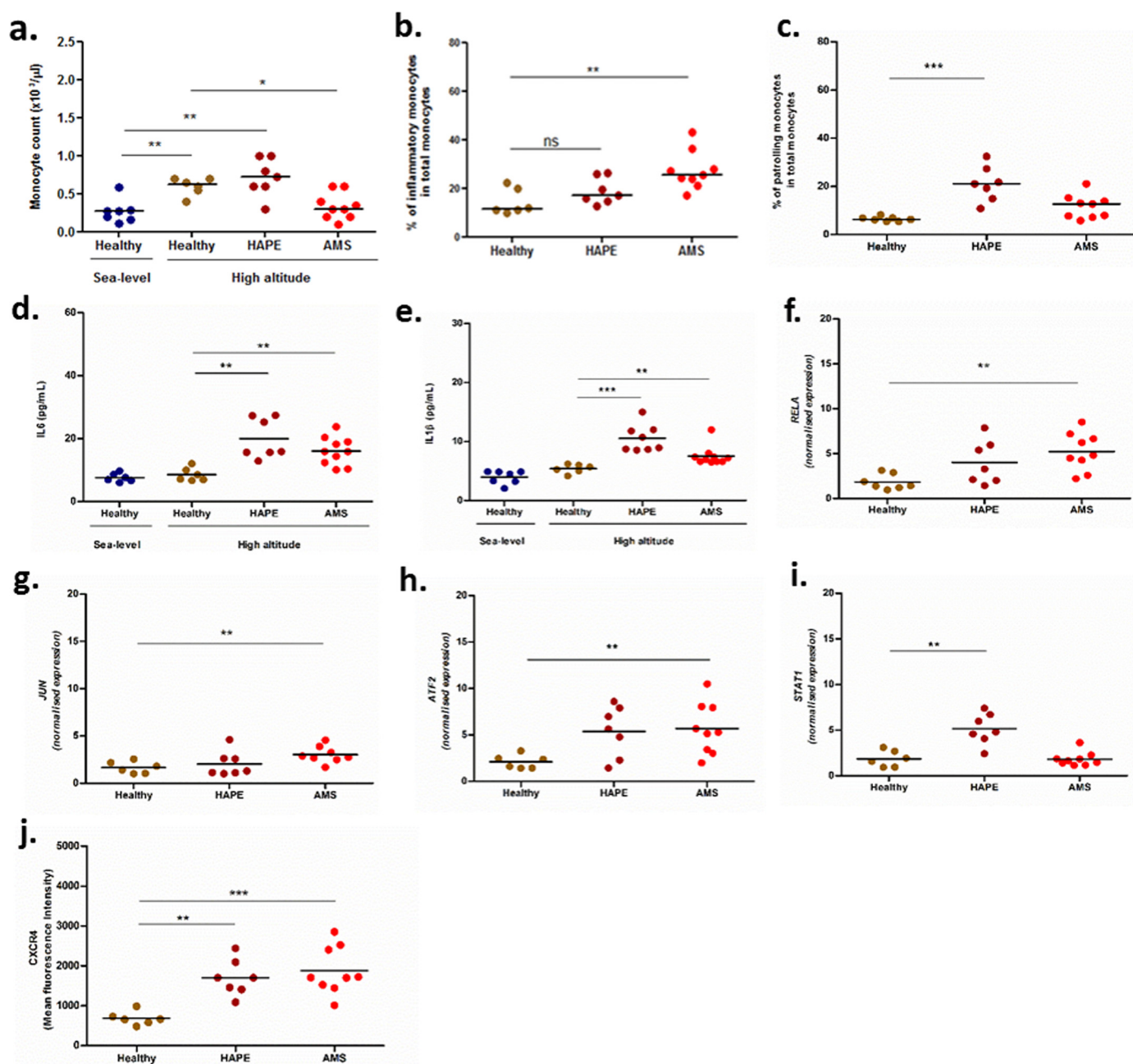


Fig. 5. Elevated inflammatory response of monocytes in AMS and HAPE patients. PBMCs were isolated from whole blood of non-Tibetan sojourners with AMS/HAPE or healthy individuals from high altitude. Healthy individuals from sea level were used as reference. **(a)** Total monocytes were counted using haematology analyser. **(b)** Inflammatory and **(c)** patrolling monocyte subsets were measured using flow cytometry as described in Fig. 1. Each dot represents a single individual. Kruskal Wallis test was used on median values for comparison between patients and healthy individuals followed by Dunn's multiple comparison post test, *** $p < 0.001$ and ** $p < 0.01$. Plasma level of cytokines **(d)** IL6 and **(e)** IL1 β was measured, alongside TNF α and TGF β (Fig. S9e-f) using CBA assay. Data were recorded in duplicates and calculated as mentioned above, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. **(f-i)** The gene expression of *RELA*, *JUN*, *ATF2* and *STAT1* was measured using real-time PCR and data were normalized using expression of housekeeping gene *TUBB*. Data were calculated as mentioned above, ** $p < 0.01$. The *CXCR4* gene expression (Fig. S9g) was measured in these individuals as mentioned above. **(j)**: Alongside the surface expression of CXCR4 on CD14⁺ monocytes were assessed as mentioned in Fig. 3. Data were calculated as mentioned above, ** $p < 0.01$ and * $p < 0.05$.

4.8. Dietary α KG administration to mice suppresses hypoxia-induced elevation in pro-inflammatory responses

In support of our above observation we tested further if α KG (1% in drinking water) supplementation can alleviate hypoxia-induced inflammatory response in mice. A significant elevation of α KG level in plasma (Fig. 7a) as well as in PBMCs (Fig. 7b) was observed. The elevated intracellular α KG in turn augmented PHD2 activity (defined by a decrease in HIF2 α expression) in PBMCs (Fig. 7l) was measured. α KG supplementation attenuated the el-

evation of peripheral monocyte and neutrophil count (Fig. 7c-d), and IL6 and IL1 β levels (Fig. 7e-f). At molecular level, alongside HIF2 α we also observed down-modulation of phosphorylated p65, a crucial orchestrator of inflammation, in this group (Fig. 7i). This treatment also reduced the transmigration of monocytes and neutrophils to the bronchoalveolar regions as well as airway spaces of the lung (Fig. 7g-j and S14g) alongside down-modulated CXCR4 expression (Fig. 7k) in leukocytes. The above experiment also showed that even the withdrawal of α KG administration at 24th hr (a total of 48 hrs of hypoxia exposure) had a nearly similar effect. Impor-

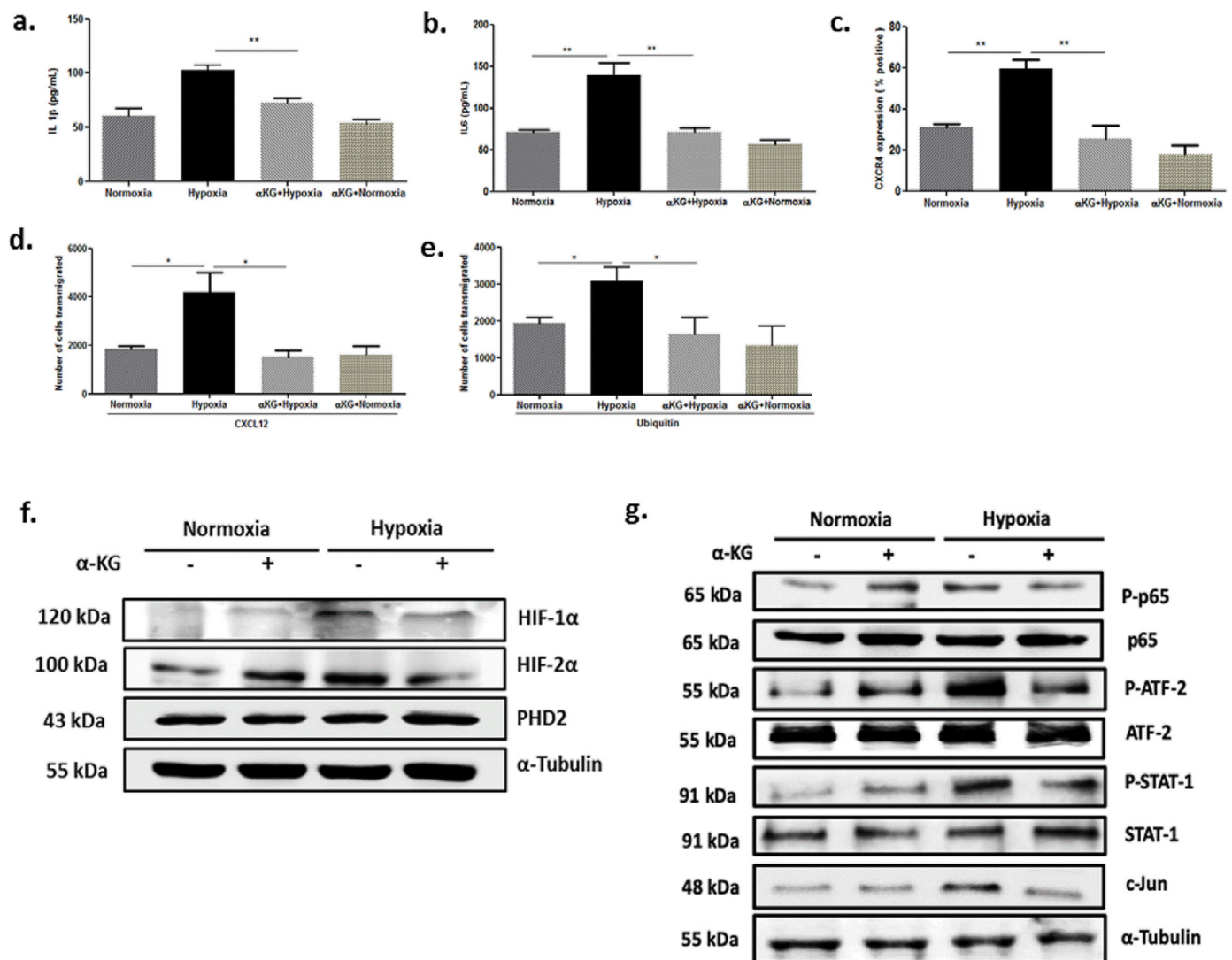


Fig. 6. α KG-mediated augmentation of PHD2 and inhibition of hypoxia-mediated inflammatory response in U937 cell line. U937 cells were pre-treated with either octyl α -ketoglutarate (1 mM) or vehicle for 4 hrs and kept under either 21% or 1% oxygen for 24 hrs. Cytokines were measured in the supernatant using CBA assay as described in Fig. 1. (a) IL1 β and (b) IL6 levels were elevated in hypoxia but reduced after α KG treatment. Data are mean \pm SEM of three independent experiments. Unpaired t -test was used for comparison, ** $p < 0.01$. Other cytokines levels are mentioned in Fig. S10a-c. Cell surface expression of (c) CXCR4 and chemotactic potential of the cells in response to (d) CXCL12 and (e) Ubiquitin was evaluated and data analyzed from three independent experiments, ** $p < 0.01$, and * $p < 0.05$. Expression of (f) HIF1 α , HIF2 α and PHD2, and (g) P-p65, P-ATF2, P-STAT1 and c-Jun was evaluated by western blot to ascertain the effect of enhanced enzymatic function of PHD2 in monocytes after octyl α KG treatment under normoxia and hypoxia. Detail densitometry data are described in Fig. S151-t.

tantly, dietary supplementation of α KG for 24 or 48 hrs did not alter the above mentioned immune parameters and also RBC and platelet counts in mice under normoxia, suggesting a safe implementation of this metabolite. These *in vivo* observations thus underline the role of augmented-PHD2 activity as the rate limiting factor in prevention of hyper-activation of immune cells under hypoxic environment.

5. Discussion

Our study describes that the healthy Tibetans carrying evolutionarily selected homozygous PHD2^{D4E;C127S} variant are protected from the elevated total monocyte count as a response to inspired hypoxia at high altitude. Individuals with this variant exhibit a reduced percentage of non-classical monocytes, indicating a limited skewing of monocyte subsets towards inflammatory phenotype as compared to their PHD2^{WT} counterparts. The monocytes of PHD2^{D4E;C127S} Tibetan highlanders display less secretion of pro-inflammatory cytokines IL1 β and IL6, and a re-

duced CXCR4-mediated chemotactic ability in conjunction with downmodulation of the related transcription factors and signaling molecules p65, ATF2, STAT1, cJun and HIF2 α . Unexpectedly the TNF α secretion by PHD2^{D4E;C127S} monocytes was not altered significantly though these cells displayed down-modulation in phosphorylated p65 compared to WT monocytes, suggesting a need of further detailed investigation in this axis. The above observations thus indicate a crucial immunosuppressive role of the variant in response to low oxygen tension. The exclusivity of the above mentioned outcomes only under hypoxia insinuates that homozygous PHD2^{D4E;C127S} variant manifests its downstream effects as a function of partial pressure of oxygen. In contrast, several reports suggest that low oxygen tension may increase inflammatory responses in sojourners at high altitudes [4,6–8]. We studied the afore-mentioned immune parameters in non-Tibetan sojourners who ascended to the same high altitude by air and developed AMS and HAPE. These patients displayed elevated counts of inflammatory and patrolling monocytes and increased IL6 and TNF α in circulation alongside higher expression of the above-

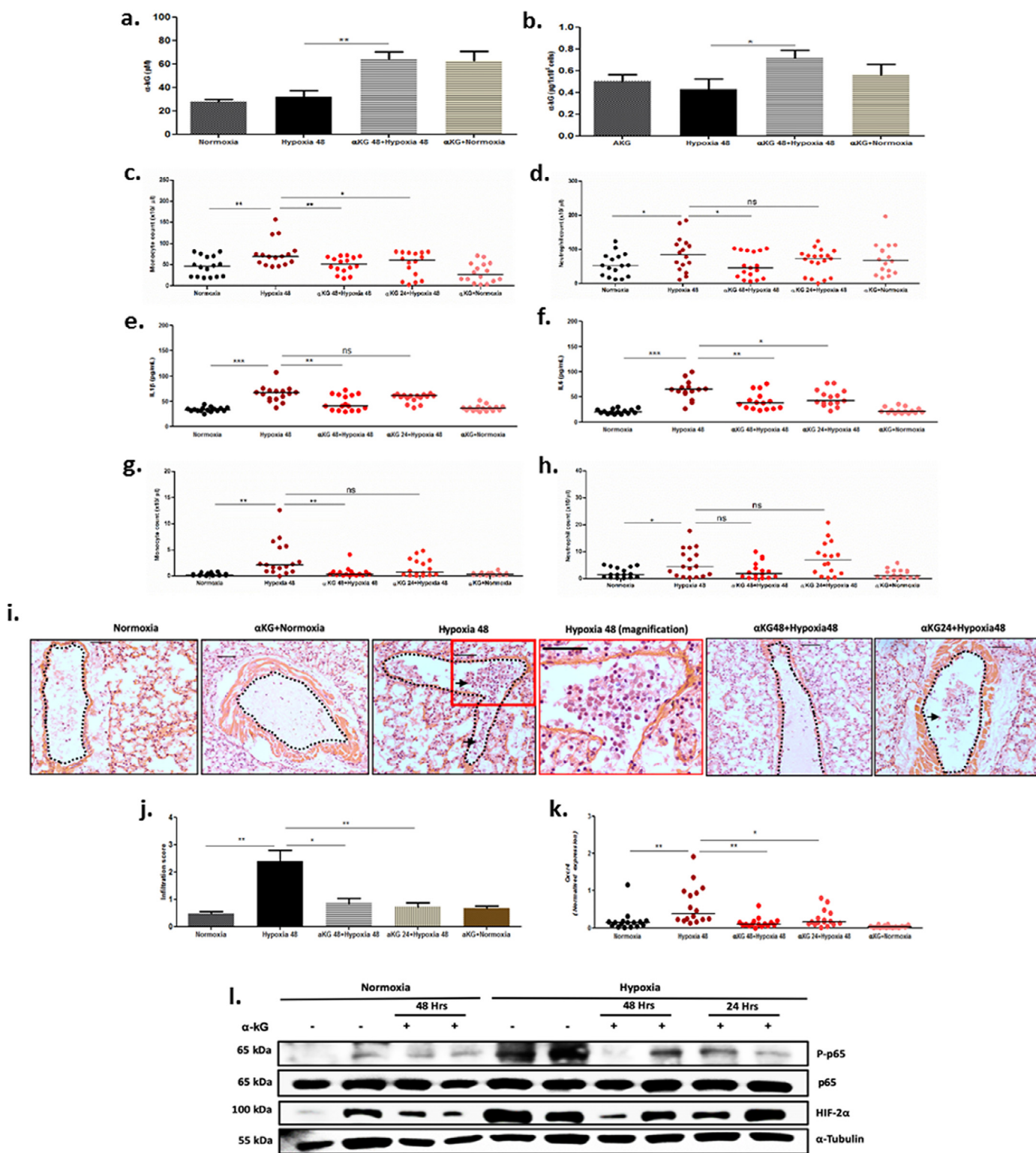


Fig. 7. Effect of dietary α KG on hypoxia-immune response in mice. The C57/BL6 mice (5-6 weeks old) were administered with 1% α KG via drinking water, 12 hrs prior to hypoxia exposure (11% oxygen for 48 hrs), experimental design is presented as a schematic in Fig. S14a. (a-b) After treatment, plasma and PBMC-granulocyte cell pellet were used for measuring α KG in (a) plasma and (b) PBMCs (1×10^6 cells) lysate using colorimetry based assay. Unpaired *t*-test was used for comparison between groups ($n=7$ in each group), ** $p<0.01$, * $p<0.05$ and ns=non-significant. (c-d) Monocytes and neutrophils counts were measured from whole blood using hematology analyzer. (e-f) Plasma level of cytokines IL1 β and IL6 were assessed using CBA assay. (g-h) Monocyte and neutrophil counts were measured from bronchoalveolar lavage fluid (BALF) using hematology analyzer. The counts of both cell types in whole blood and BALF were confirmed using staining with CD45, CD11b, Ly6C and Ly6G and analyzed using flow cytometry analysis, mentioned in Fig. S13. Each dot represents a mouse from three independent experiments. Unpaired *t*-test was used for comparison between the groups as in Fig. 7c-h, *** $p<0.001$ ** $p<0.01$, and * $p<0.05$. (i) The H & E staining of lung section of mice showed the infiltration of immune cells into airway space under hypoxia treatment (magnification view shows large numbers of leukocytes inside airway space), which was rescued by 48 hrs α KG supplementation; arrows indicate the cell infiltration, bars in all images $\sim 50 \mu$ m. (j) The infiltration score was calculated in case of each treatment group based on H & E lung sections from independent experiment. Additional images for quantification has been depicted in Fig. S14g. (k) The expression of *Cxcr4* gene in leukocytes was assessed using real-time PCR and normalized with housekeeping gene *Hprt*. Unpaired *t*-test was used for comparison between groups, ** $p<0.01$, and * $p<0.05$. (l) Expression of P-p65 and HIF2 α in leukocytes pellet was evaluated by western blot to ascertain the enzymatic function of PHD2 after α KG administration under hypoxia. Representative blot shows data of 2 mice from each group. Densitometry showed data from 5 mice for P-p65/p65 and 8 mice for HIF2 α from independent experiments as mentioned in Fig. S15u-w. The α KG treatment for 48 hrs rescued more effectively than 24 hrs treatment for all above parameters in mice exposed for 48 hrs under 11% hypoxia.

mentioned transcription factors. This was in line with reports suggesting a significant elevation in signaling molecules and transcription factors *RELA*, *MAP2K*, *JAK*, *TNF* [40], *FOS*, *MAP3K* and *NFKB1* [41] in the acute phase of HAPE in mountain travelers. These observations therefore suggest the possible modes by which homozygous *PHD2*^{D4E:C127S} variant is part of the umbrella of mechanisms that protect the Tibetan highlanders against high-altitude hypoxia associated inflammatory complications.

Besides, our data also suggest that *PHD2*^{WT} or heterozygous Tibetan highlanders may be predisposed to high-altitude associated inflammatory events, but are protected from these clinical consequences probably by the dint of long term acclimatization. We however speculate that the homozygous *PHD2*^{D4E:C127S} variant, which exists in half of the Tibetan population, may not be solely responsible for the protection against hypoxia-induced elevation in inflammatory phenotype. As suggested by others the physiological adaptations such as favourable hypoxic ventilatory response and pulmonary arterial pressure also support the high-altitude survivability of the Tibetan highlanders [42] protecting them from AMS and HAPE [43]. Studies including our previous work also suggest that other evolutionarily selected genes including *EPAS1* are associated with some degree of protection against the high-altitude complications in Tibetans [11,14,16]. Therefore, the mechanisms independent of *PHD2*^{D4E:C127S} variant involved in the high-altitude adaptability of Tibetans require further investigations.

We then extended the knowledge of gain-of-function of homozygous *PHD2*^{D4E:C127S} variant and tested if α KG supplementation can ensue into a rescue from the hypoxia-driven inflammatory events by augmenting *PHD2* activity. *PHD2* facilitates degradation of its substrates like HIF α through catalysis of proline hydroxylation by decarboxylating α KG. A study describes that the elevated intracellular α KG increased the hydroxylase activity of *PHD2* and promoted degradation of HIF α in hypoxia [44]. α KG has been used extensively for in vivo experimental therapies for manipulating multiple cellular processes related to organ development and viability of organisms [45,46], restriction of tumour growth and extending survival [47], and preventing obesity [48]. Supplementation of octyl α KG to U937 cells endogenously expressing *PHD2*^{WT} augmented *PHD2* activity and suppressed the hypoxia-driven increase in secretion of cytokines and chemotactic ability of the cells in conjunction with down-modulation of phosphorylated p65, ATF2 and STAT1, and cJun. This phenotype simulated the *EGLN1* gain-of-function phenotype of monocytes expressing *PHD2*^{D4E:C127S} and serves as foundation to further our concept of augmenting *PHD2* activity for beneficial effects under inspired hypoxia. Further, our in vivo data show that the dietary supplementation of α KG to mice significantly augmented *PHD2* activity and suppressed expression of HIF2 α and phosphorylated p65, and prevented the inflammatory events such as elevation of monocyte and neutrophil counts, and cytokine levels in peripheral blood, and also the transmigration of leukocytes to the bronchoalveolar region of the lungs in mice exposed to hypoxia. In our work we have for the first time identified that *PHD2* regulates the key molecules that orchestrate monocyte response to hypoxia and that evolution has used this connection to protect the Tibetans from high-altitude complications including inflammation fomented by inspired hypoxia. It however remains to be understood the signalling molecules which form a connection between *PHD2* and these molecules.

Although our study has several limitations altogether our data show for the first time a link between the gain-of-function *PHD2*^{D4E:C127S} variant and a suppressed inflammatory phenotype amongst the Tibetan highlanders in response to inspired hypoxia of the high altitude. Gene and protein expression study revealed that downmodulation of several signalling molecules including p65, ATF2, STAT1, cJun, CXCR4 and HIF2 α play crucial role in imparting this phenotype, which probably provides protection to the genet-

ically adapted Tibetans from high-altitude hypoxia. On the other hand, elevation of these signalling molecules in AMS and HAPE patients indicated their key association with inflammatory phenotype. Thus, suggesting that augmentation of *PHD2* activity may be a potential method to alleviate high-altitude hypoxia associated inflammatory complications in sojourners. To verify our above assumption, we employed a strategy to inhibit hypoxia-triggered inflammation in monocytes in vitro as well as in mice by α -ketoglutarate-mediated augmentation of *PHD2* activity. We have depicted a summary of our data in schematic Fig. 8. Our study henceforth opens up new avenues of understanding in the fields of inflammation biology and high altitude medicine.

6. Some of the Limitations of our study

- Due to the regulatory restrictions, the study could not recruit the predicted sample size from the Tibetan community. The small volume, 5 mL of the sample, obtained from each individual, also limited our immunophenotyping study to monocytes only.
- We could collect only 5 mL of blood samples from a small number of AMS and HAPE patients due to most patients being too sick to provide samples and also due to other restrictions. It is also noticeable that most patients were male and therefore the preliminary data obtained is more representative of the male population. The COVID-19 pandemic has added limitations too in the form of travel restrictions and recruitment of additional human samples.
- We observed that 1) Tibetan specific variant *PHD2*^{D4E:C127S} suppresses the factors including p65, c-Jun, STAT1, ATF-2 and CXCR4 under hypoxia. However, we also observed that some of these transcription factors were marginally elevated in *PHD2*^{D4E:C127S} monocytes in normoxia. 2) TNF α level was not significantly altered in D4E; C127S monocytes though phosphorylation of p-65 was greatly reduced compared to wild type. However, we could not assign any significant functional relevance to the above observations within the purview of this study and therefore further investigation is needed.
- We speculate that the homozygous *PHD2*^{D4E:C127S} variant, which exists in half of the Tibetan population, may not be solely responsible for the protection against hypoxia-induced elevation in inflammatory phenotype. Therefore, the mechanisms independent of *PHD2*^{D4E:C127S} variant involved in the high-altitude adaptability of Tibetans require further investigations.
- We tested the effect of dietary α KG supplement in mice exposed to normobaric hypoxia (11% O₂) for 48 hrs. Normobaric hypoxia treatment was able to successfully induce lung inflammation in mice. We thus continued with this treatment rather than trying other treatment conditions under hypobaric hypoxia and also for longer duration. We could not measure arterial oxygen pressure.

7. Contributors

SB designed and performed all experiments, analyzed data, and wrote the manuscript. NS performed transgenic cell line and mice experiments, and immunoblot assay. GM, PK, SB and NS collected human samples and analyzed clinical data. PK supervised recruitment of study participants and critically read and revised the manuscript. JTP provided crucial conceptual inputs and wrote manuscript. PG designed and supervised the study, conceptualized the approach, designed the experiments, analyzed the data, and wrote the manuscript. All authors read, edited and approved the final manuscript.

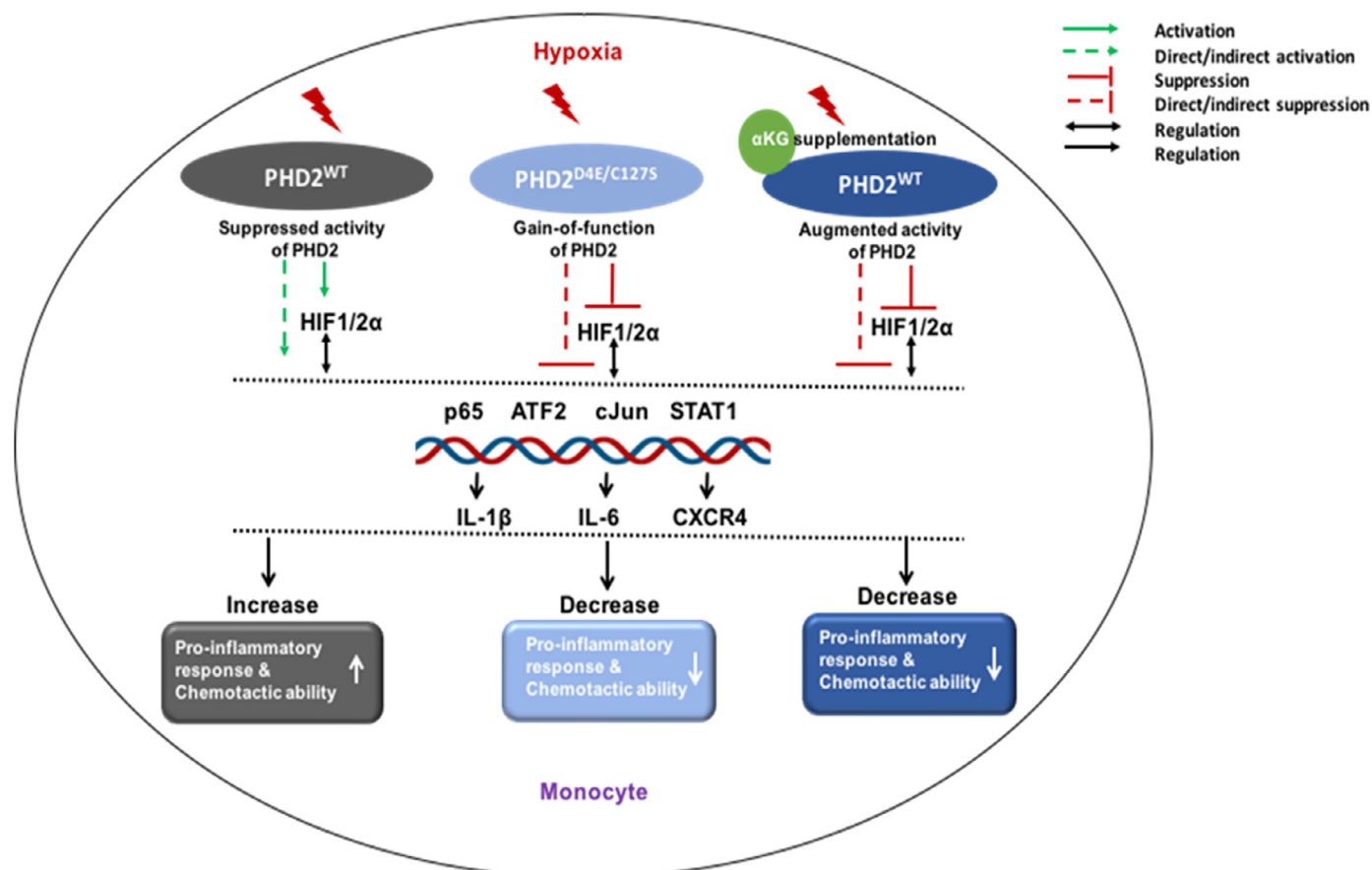


Fig. 8. PHD2 regulates inflammatory response of monocyte. (Left panel) The monocytes of PHD2^{D4E/C127S} Tibetans highlanders exhibit protection against hypoxia-triggered elevation of inflammatory cytokines including IL6 and IL1 β , as well as CXCR4-mediated chemotactic ability by down-modulating transcription factors such as p53, ATF2, c-Jun, STAT1 and HIF α compared to their wild type counterparts, suggesting the gain-of-function for PHD2^{D4E/C127S} variant. (Mid panel) The non-Tibetans sojourners who traveled to high altitudes and developed AMS and HAPE display high level of pro-inflammatory cytokines alongside elevation of above-mentioned transcription factors. (Right panel) The dietary supplementation of α -ketoglutarate (α KG, co-factor of PHD2) to mice exposed to hypoxia (11% oxygen) suppressed the hypoxia-induced elevation of inflammatory cytokines and transmigration of immune cells in lungs in conjunction with augmented PHD2 activity.

8. Data sharing

The materials described in the manuscript, including all relevant raw data will be freely available from the corresponding author

Online supplemental data

Fig. S1 shows the sample collection and stratification strategy as well as gating strategy of CBC and immunophenotyping. Fig. S2 demonstrates the immune cell counts of homozygous PHD2^{D4E/C127S}, heterozygous PHD2^{D4E/WT;C127S} and PHD2^{D4E/WT;C127S/WT} and PHD2^{WT} Tibetans of high altitude and sea level. Fig. S3 shows the cytokine secretion by monocytes of above Tibetan variants of high altitudes in vitro under hypoxia. Fig. S4 demonstrates the cytokine secretion by monocytes of PHD2^{D4E/C127S} or PHD2^{WT} variant from high altitude or sea level in vitro under hypoxia. Fig. S5 shows the plasma cytokine levels of PHD2^{D4E/C127S} or PHD2^{WT} Tibetans from high altitude or sea level. Fig. S6 and S7 demonstrate expression of various signalling genes in homozygous PHD2^{D4E/C127S}, heterozygous PHD2^{D4E/WT;C127S} and PHD2^{D4E/WT;C127S/WT} and PHD2^{WT} monocytes. Fig. S8 shows protocol for generation of PHD2^{D4E/C127S} engineered cells. More cytokine and signalling gene data related to Fig. 4. Fig. S9 demonstrates more cytokine and signalling gene data of AMS or HAPE patients related to Fig. 5. Fig. S10 shows the rescue effect of ethyl-3-4-dihydroxybenzoic acid (DHB), an inhibitor to PHD2, on cytokine secretion and HIF2 α expression by α KG-treated U937 cells under

hypoxia. Fig. S11 demonstrates the effect of α KG supplementation to primary monocytes isolated from healthy individuals, a similar study as mentioned in Fig. 6, using cell line. Fig. S13 shows the gating strategy of counting of mouse immune cells. Fig. S14 shows more data of cells count, cytokine level, lung inflammation data from mouse, as mentioned in Fig. 7. Fig. S15 demonstrates the densitometry analysis of all western blot data.

Table S1 demonstrates genotype, gender and age of the volunteers and inclusion/exclusion criteria of Tibetan individuals. Table S2 demonstrates the data of all 47 genes related to hypoxia-immune response in PHD2^{D4E/C127S} or PHD2^{WT} Tibetans from high altitude and sea level. Table S3 demonstrates the inclusion/exclusion criteria of AMS/HAPE patients and healthy individuals at high altitude, and their related clinical data. Table S4 shows the primer list and Table S5 shows list of antibody used in our study.

Declaration of Competing Interest

The authors declare no competing interests.

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Supplementary materials

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