Effects of hypoxic and inflammatory priming on HIF activation in human leukocytes

Dissertation

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Contents

Declaration

I hereby declare that I wrote this dissertation without sources other than indicated in the main text and without help from third parties. I have designed and conducted all the experiments described in this thesis. Data presented in section 3.3 was generated as part of the master thesis of Alexandra Heinrich, designed and supervised by the author of this thesis.

Data that was collected as part of human subject studies (see sections 3.4 and 3.5) was processed, analysed, and visualised by the author of this thesis if not mentioned otherwise.

According to the common practice in English scientific writing, this dissertation is written using the first-person plural narrator.

Abstract

Leukocytes need to adapt to different oxygen conditions and hypoxia in short time, especially under pathophysiological conditions when the cells migrate from the bloodstream into inflamed tissue. Hypoxia-inducible factors (HIF) are important transcription factors that mediate cellular adaption to low oxygen supply in all nucleated cells. Moreover, they are essential for effective immune response and leukocyte function. Although inflammation is often associated with hypoxia due to high energy and oxygen demand of inflammatory cells, the interplay of hypoxia and inflammation regarding the HIF pathway remains unresolved. For a more in-depth understanding of immune cell function it is necessary to study the mutual influence of both conditions. To this aim we challenged monocytic *THP-1* cells and primary peripheral blood mononuclear cells (PBMCs) *in vitro* with different concentrations of lipopolysaccharide (*E. coli* LPS) with or without different combinations of hypoxia or the addition of the HIF-stabilising drug *Roxadustat*. Additionally, we exposed human subjects *in vivo* to hypoxia (normobaric, 10.5 % and 15 % oxygen) and simulated acute inflammation using a human endotoxemia protocol with a single intravenous LPS injection (0.4 ng/kg). Our analysis focussed mainly on HIF-1α protein and gene expression changes of specific HIF target genes and inflammatory mediators using Western blotting, immunofluorescent staining, qRT-PCR, and flow cytometry. Our investigations showed differently altered gene expression patterns of HIF and target genes in distinct immune cell subsets (neutrophils, monocytes, T cells) upon *in vivo* treatment with LPS and hypoxia. Further, we found evidence for highly cell-specific effects of hypoxic priming upon inflammation in combination with immunomodulatory effects in leukocytes *in vivo*. In cell culture, we observed an increased inflammatory response to LPS after a hypoxic phase in PBMCs and *THP-1* cells. Moreover, treatment with the drug and PHD-inhibitor *Roxadustat* led to increased HIF-1α protein and HIF target gene expression in human leukocytes and promoted the acute inflammatory response to *in vitro* LPS treatment. In summary, our studies provide valuable insights into the cellular adaption processes of leukocytes during inflammation and hypoxia and highlight the multi-layered interplay between both stimuli.

Zusammenfassung

Leukozyten sind, vor allem unter pathologischen Bedingungen unterschiedlichsten Sauerstoffkonzentrationen und Hypoxie ausgesetzt und müssen ihren Metabolismus innerhalb kürzester Zeit anpassen, um eine wirkungsstarke Immunreaktion bei Entzündungen gewährleisten zu können. Die Hypoxie-induzierbaren Faktoren (HIF) sind wichtige Transkriptionsfaktoren, die die zelluläre Anpassung an eine geringe Sauerstoffversorgung in allen kernhaltigen Zellen vermitteln. Darüber hinaus sind diese Hauptregulatoren für eine wirksame Immunantwort und die Funktion von Leukozyten unerlässlich. Obwohl Entzündungen aufgrund des hohen Energie- und Sauerstoffbedarfs der betroffenen Zellen häufig mit Hypoxie in Verbindung gebracht werden, ist das Zusammenspiel von Sauerstoffmangel und Entzündungen in Bezug auf den HIF-Signalweg noch immer Gegenstand von Untersuchungen. Um die zellulären Anpassungsprozesse in zirkulierenden menschlichen Leukozyten besser zu verstehen, haben wir monozytäre *THP-1*-Zellen und primäre periphere mononukleäre Blutzellen (PBMCs) *in vitro* mit verschiedenen Kombinationen mit dem immun-stimulierenden Lipopolysaccharid (*E. coli* LPS), Hypoxie oder dem Medikament *Roxadustat* behandelt. Zusätzlich wurden freiwillige Probanden *in vivo*, vor oder nach der Behandlung mit intravenös verabreichtem LPS (0,4 ng/kg) in einem Höhentrainingszentrum niedrigen Sauerstoffkonzentrationen ausgesetzt (normobar, 10,5 % O_2) um eine akute Entzündung und Hypoxie zu simulieren. Unsere Forschung konzentrierte sich hauptsächlich auf die Untersuchung der Akkumulation des HIF-1α-Proteins und die Veränderungen der Genexpression spezifischer HIF-Zielgene in Kombination mit Expression von entzündungsfördernden Zytokinen. Dazu nutzen wir die Methoden Western Blotting, Immunfluoreszenzfärbung, qRT-PCR und Durchflusszytometrie. Wir konnten zeigen, dass verschiedene Untergruppen von Immunzellen (Neutrophile, Monozyten, T-Zellen) nach einer *in vivo* Behandlung mit LPS und Hypoxie unterschiedlich veränderte Genexpressionsmuster von HIF und dessen Zielgenen aufweisen. Darüber hinaus fanden wir Hinweise darauf, dass Leukozyten durch vorangegangene hypoxische Bedingungen hochgradig zellspezifisch reagieren und ihre Genexpression und Immunantwort in Kombination mit simulierter Entzündung verändern. *In vitro* konnten wir in PBMCs und *THP-1*-Zellen eine verstärkte Entzündungsreaktion auf LPS nach einer hypoxischen Phase beobachten. Darüber hinaus führte die Behandlung mit dem Medikament und PHD-Inhibitor *Roxadustat* in Leukozyten zu einem erhöhten HIF-1α-Proteingehalt in Kombination mit einer verstärkten Expression von HIF-Zielgenen und verstärkte die akute Immunreaktion auf LPS. Zusammenfassend lässt sich sagen, dass unsere Studien wertvolle Einblicke in die zellulären Anpassungsprozesse von Leukozyten bei Entzündungen und Hypoxie liefern und das vielschichtige Zusammenspiel von beiden betonen.

1. Introduction

1.1 The human immune system

The human immune system is a complex interactive network of lymphoid organs, cells, and molecules with specific functions that act together to maintain host defence and fight against infections. Important cellular components of the immune defence system are circulating cells of innate and adaptive immunity also known as white blood cells or leukocytes. All blood cells are derived from hematopoietic stem cells (HSCs) in the bone marrow (Schofield, 1978). These progenitor cells give rise to myeloid and lymphoid progenitor cells, which further differentiate in either myeloid cells of the innate immune response, including monocytes, macrophages, granulocytes (neutrophils, eosinophils, and basophils), and dendritic cells or in lymphoid cells of the innate immunity, including natural killer (NK) cells and the adaptive T and B lymphocytes that circulate in the bloodstream (Parkin & Cohen, 2001), (Beutler, 2004).

As indicated in figure 1, immunity is divided into two parts: The fast innate immune response acts non-specific and widespread and consists of phagocytic cells like monocytes and macrophages and of granulocytes like neutrophils, basophils, and eosinophils that release inflammatory mediators as well as of mast cells and natural killer cells that can induce apoptosis. Cells of innate immunity are quick-acting and can recognize various exogenous molecules as “foreign”.

Cells of the myeloid lineage like granulocytes and monocytes or macrophages are one of the main components of innate immunity (figure 1). Upon inflammation, around 95% of recruited cells are of the myeloid lineage, which need to migrate against an oxygen and nutrition gradient (Lewis et al., 1999), (Taylor & Colgan, 2017), (Chen & Gaber, 2021). Granulocytes and more specifically neutrophils are the most common type of phagocytes and present around 40 – 60 % of circulating immune cells. Once attracted by specific chemokines they migrate to the side of inflammation. Thereby cells of this short-lived type are specialised to act fast in the elimination of pathogens via phagocytosis, production of ROS, the formation of neutrophil extracellular DNA traps (NETs), or recruitment of other immune cells via secretion of cytokines like tumour necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) (Moynagh, 2003), (Amulic et al., 2012). Eosinophils and Basophils are specialised in virus and parasite detection and elimination and are known to play a key role in allergies. The other part of the innate myeloid lineage are monocytes that differentiate into macrophages or dendritic cells (DCs) once they are activated. They are antigen-presenting cells (APC), which mediate an important link between innate and adaptive immunity as they phagocytose pathogens, attract neutrophils and present their antigen fragments on the major histocompatibility complex 2 (MHC2) surface marker to T cells. Despite their relatively small number dendritic cells (around 1 % of leukocytes) are also essential players that lead to T cell activation and differentiation. Besides

neutralizing potential harmful pathogens cells of the innate immunity send out signals or act as antigen-presenting cells to recruit other immune cells to the side of action and thus initiate the second part of the immune system – the adaptive response (Parkin & Cohen, 2001), (Beutler, 2004) (figure 1).

The adaptive or acquired immunity is slower but specifically directed against antigens (e.g., proteins of pathogenic organisms). The defence system and its cells adapt to antigens with the help of their "immunological memory", which allows long-lasting protection in form of memory cells. The cells of the acquired immunity are lymphocytes, consisting of thymus-derived T lymphocytes and bone marrow-derived B lymphocytes. T cells make up the largest proportion of lymphocytes (45 – 70 %) and undergo a process of cell priming followed by activation and differentiation in the thymus upon antigen presentation of pathogens (Bonilla & Oettgen, 2010). With the help of costimulatory signals, T cells get activated and differentiate via clonal expansion into different T cell subclasses of helper T cells (Th1, Th2, Th17) effector T cells, or regulatory T cells (T regs), each with different functions and cytokine secretion patterns. Depending on the subpopulation, T cells play roles in killing infected host cells, enhancing the adaptive cell response, activating mononuclear phagocytes, or regulating the inflammatory response to limit tissue damage. In turn, B cells differentiate into plasma cells once they are stimulated by T helper cells and secrete antigen-specific antibodies to act as effectors of the humoral immune system. In addition, they guide T cells to the side of infection (Parkin & Cohen, 2001), (Bonilla & Oettgen, 2010).

The majority of leukocytes involved in the immune response are challenged with many different environmental stimuli and oxygen concentrations as they pass through different organs while transported via the bloodstream to fulfil their purpose at the side of infection (Beutler, 2004), (Chen & Gaber, 2021). Within a short time, leukocytes need to adapt to those diverse conditions to fight pathogens and maintain physiological health. Physiological hypoxia significantly affects leukocyte function and controls the innate and adaptive immune response mainly through transcriptional gene regulation via the hypoxia-inducible factors (HIFs). Under pathophysiological conditions, such as those arising from infections, injuries, and inflammation, a severe form of hypoxia can significantly alter leukocyte function and thus lead to a dysfunctional immune response that can cause tissue damage or autoimmunity (Watts & Walmsley, 2019), (Chen & Gaber, 2021).

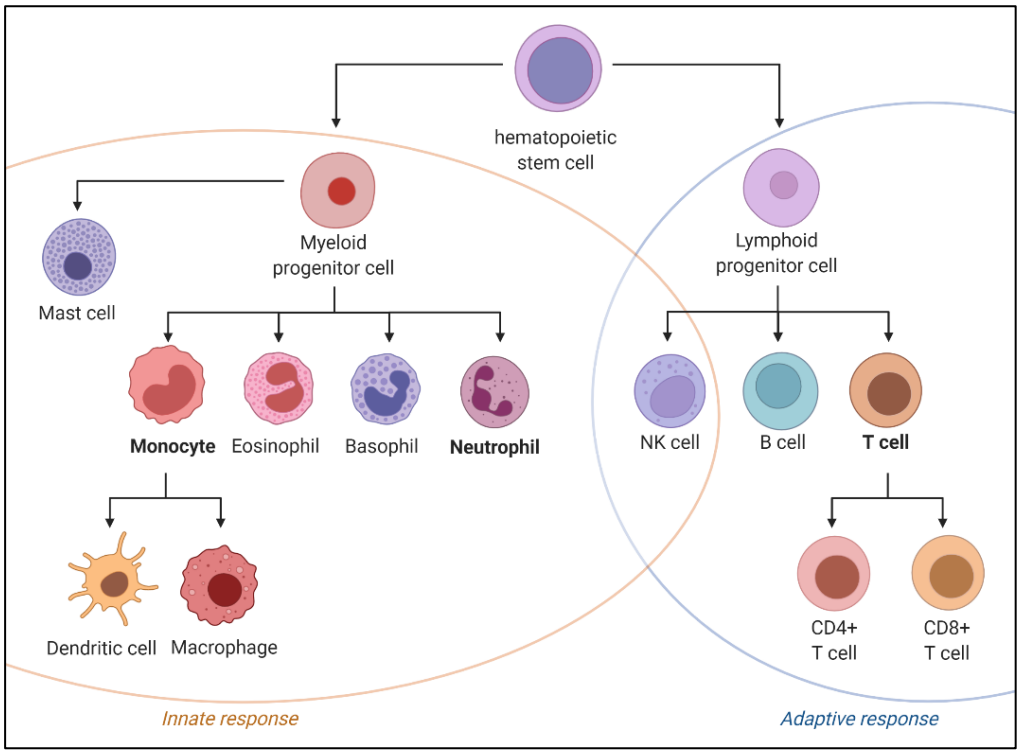


Figure 1: Representation of the immune cell lineage tree

Hematopoietic stem cells differentiate into myeloid or lymphoid progenitor cells, which in turn differentiate into the different effector cells of the innate and adaptive immune response. Myeloid cells include mast cells, monocytes, granulocytes (neutrophils, eosinophils, and basophils), dendritic cells, and macrophages. Natural killer (NK) cells, B cells, and T cells are lymphoid cells that mediate the adaptive immune response. In bold are the cells marked, that were studied upon *in vivo* immune stimulation on gene expression level as part of this thesis (section 3.5). Graphic was created using biorender.com.

1.2 The hypoxia-inducible factors (HIFs)

When cells are challenged with insufficient oxygen supply (hypoxia) a rapid mode of action is essential for the survival of cells, tissues, and whole organisms. The reasons for hypoxia can be diverse: ranking from environmental conditions like reduced oxygen partial pressure in the air to multiple pathologies including cancer, inflammation, and infections. To counteract deadly consequences due to hypoxia multiple adaption processes have evolved that are transcriptionally regulated via the master regulatory pathway of the hypoxia-inducible factors (HIFs) (Wang & Semenza, 1993), (Greijer et al., 2005). HIFs regulate a majority of oxygen-dependent genes inducing enhanced erythropoiesis, iron transport, vascular growth, nitric oxide synthesis, phosphate metabolism, glycolysis, and gluconeogenesis (Goldberg et al., 1991), (Shweiki et al., 1992), (Kietzmann et al., 1993), (Firth et al., 1994), (Semenza et al., 1994), (Melillo et al., 1995), (Ebert et al., 1996), (Rolfs et al., 1997).

In 2019, Gregg L. Semenza, Sir Peter J. Ratcliffe, and William G. Kaelin Jr. were awarded the Nobel Prize in Physiology or Medicine “for their discoveries of how cells sense and adapt to oxygen availability” (Institutet, 2019), (Fandrey et al., 2019). This awarding highlights the importance of the identification of the molecular machinery behind the hypoxia-inducible factor pathway and the regulation of important target genes for fundamental physiological processes in health and disease.

1.2.1 Structure and regulation of HIFs

The master regulatory pathway for cellular adaption to low oxygen concentrations is mainly mediated by hypoxia-inducible factors, which regulate, as mentioned before, the majority of oxygen-dependent genes (Wang & Semenza, 1993), (Greijer et al., 2005). The dimeric transcription factor HIF is composed of one of 3 oxygen-regulated α-subunits (HIF-1α, HIF-2α, or HIF-3α) and a constitutively expressed β-subunit, named HIF-1β or ARNT (Aryl hydrocarbon Nuclear Translocator) (Semenza & Wang, 1992), (Reyes et al., 1992), (Wang et al., 1995). Whereas HIF-1α can be found in all nucleated mammalian cells, HIF-2α is characterised by a tissue-specific expression (Ema et al., 1997), (Tian et al., 1997). Much less is known about HIF-3α, which has a high variety of isoforms that are present in different tissues and developmental stages and differ in regulation (Duan, 2016). Depending on the variant, HIF-3α can regulate specific target genes or is discussed to negatively regulate the transcriptional activity of HIF-1α (Gu et al., 1998), (Hara et al., 2001), (Makino et al., 2001) (Maynard et al., 2005).

The HIF-α subunits have an almost identical domain structure. They consist of a basic helix-loop-helix (bHLH) domain that binds to the hypoxia-responsive elements (HREs) on the DNA of target genes (figure 2). The PER-ARNT-SIM (PAS) domain serves as the binding domain for the dimerisation of the α and β subunits (Wang et al., 1995), (Carrero et al., 2000). The N-terminal transactivation domain (N-TAD), which is responsible for stability and target gene specificity, overlaps with the oxygen-dependent degradation domain (Pugh et al., 1997), (Huang et al., 1998), (Dodd et al., 2015). The oxygen-dependent degradation domain consists of two proline residues that are hydroxylated in the presence of oxygen, thus inducing degradation of the α-subunit (Dodd et al., 2015). In addition to the N-TAD, HIF-1α and HIF-2α also have a C-terminal transactivation domain containing an asparagine that serves for the binding of transcriptional coactivators such as SRC-1, TIF2, and CBP/p300 (Carrero et al., 2000), (Schofield & Ratcliffe, 2004) (figure 2).

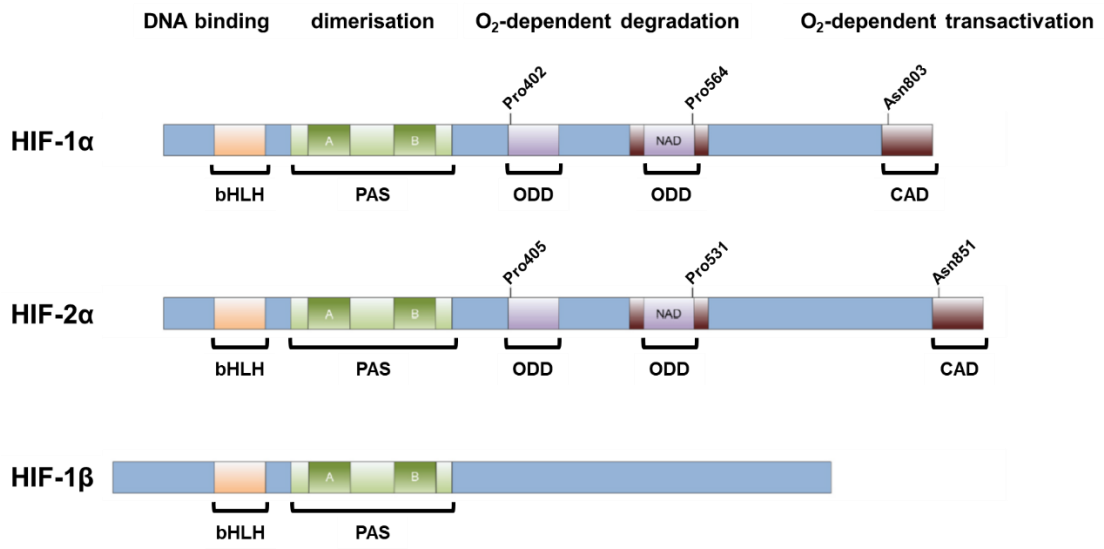


Figure 2: Domain structures of the Hypoxia-inducible factors

HIF-1α and HIF-2α subunits possess a basic helix-loop-helix domain (bHLH) that mediates DNA binding and a PER-ARNT-SIM (PAS) domain that mediates HIF-α and HIF-β heterodimerisation (Carrero et al., 2000). The N-terminal and the C-terminal transactivation domain serve to activate gene expression of the HIF target genes. Within the oxygen-dependent degradation domain (ODD) (Dodd et al., 2015); two proline residues are hydroxylated under normoxia to mark the HIF-1α subunit for von Hippel-Lindau tumour suppressor protein (pVHL)-mediated proteasomal degradation. Pro: Proline, Asn: Asparagine. Illustration by Tristan Leu.

All α-subunits are regulated in a post-transcriptional manner in the cytosol via protein stability (Pugh et al., 1997), (Huang et al., 1998): Under normoxic conditions with sufficient oxygen supply, two proline residues of the HIF-α-subunit get hydroxylated by Prolyl-4-Hydroxylase-Domain (PHD) proteins and thereby marked for proteasomal degradation (Huang et al., 1998), (Fandrey et al., 2006) (figure 2 and 3). Because the activity of the 3 homolous hydroxylases PHD1, PHD2, and PHD3 is strictly dependent on oxygen concentrations they are considered the oxygen sensors of the cells (Bruick & McKnight, 2001), (Epstein et al., 2001). Overall, the regulation of the HIF-α and PHD isoforms is highly specialised and partly tissue-specific, which shows the need to gain greater knowledge about different hypoxic settings and the specific cellular adaption processes (Semenza, 2001), (Watts & Walmsley, 2019).

Under normoxia, oxygen binds to one Fe atom in the active center of the PHD enzymes and leads, in combination with 2-oxoglutarate, ascorbate, and iron as co-factors, to hydroxylation of the proline residues of the HIF protein. This enables recognition and binding to the von-Hippel-Lindau tumour suppressor protein (pVHL) of an E3 ubiquitin ligase complex (Maxwell et al., 1999), (Cockman et al., 2000). The residues undergo ubiquitination, which finally labels HIF-α for proteasomal degradation (Fandrey et al., 2006). An additional cytosolic oxygen-dependent sensor hydroxylating an asparagine residue in the N-terminal activation domain of HIF-α is the factor-inhibiting HIF (FIH), which prevents binding to transcriptional coactivators (CBP/p300) that are needed for DNA binding of the transcription factor (Mahon et al., 2001), (Lando et al., 2002) (figure 3).

Under hypoxic conditions, the PHDs and FIH are inhibited due to absent oxygen supply and initiation of the HIF-α degradation is blocked. The HIF-α-subunits accumulate in the cytosol of the cells and translocate via a core localisation sequence into the nucleus (Chachami et al., 2009). After dimerisation of the α-subunits with the constitutively expressed β-subunit, binding to hypoxia response elements located in the promoter region of different HIF target genes is facilitated by the cofactors SRC-1, TIF2, and CBP/p300 (Carrero et al., 2000), (Schofield & Ratcliffe, 2004), (Fandrey et al., 2006), (Schito & Semenza, 2016). With this mode of action, the HIF transcription factors regulate a variety of genes, not only to restore sufficient energy and oxygen supply but also to regulate cell cycle, dell death, or pH regulation in affected cells and tissues (Wenger, 2002), (Schito & Semenza, 2016) (figure 3).

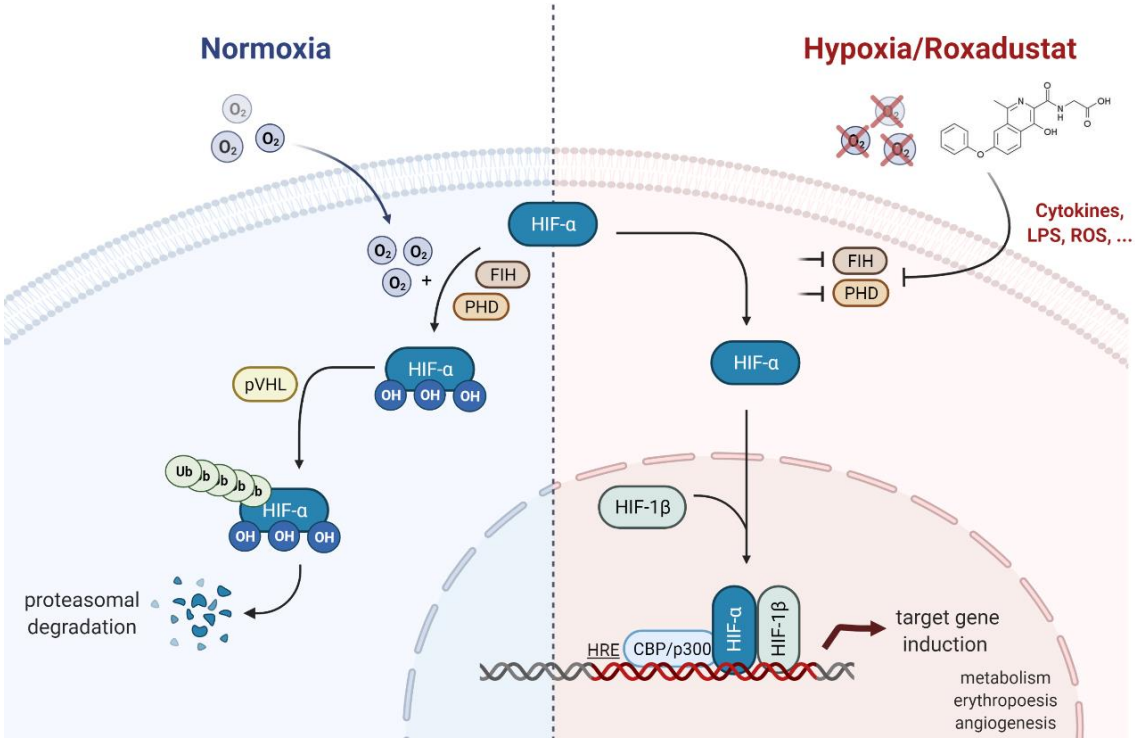


Figure 3: Hypoxia-inducible factor pathway

In the presence of O_2 (Normoxia, left), the proline and asparagine residues of the HIF-α subunits are hydroxylated (OH) by prolyl hydroxylases (PHD) and factor-inhibiting HIF (FIH). This leads to von-Hippel-Lindau protein (pVHL)-mediated ubiquitination (Ub) and proteasomal degradation. Under oxygen deprivation (Hypoxia, right), inflammation, or *Roxadustat* treatment the hydroxylases are inactive, leading to accumulation and nuclear translocation of HIF-α and subsequent dimerisation with HIF-β in the nucleus. The HIF complex in the nucleus binds to hypoxia-responsive elements (HREs) and the co-activator p300/CBP and enables the transcription of HIF target genes. Graphic was created using biorender.com.

Probably most prominent examples of genes regulated by HIF are the genes for the glycoprotein hormone erythropoietin (*EPO*), the vascular endothelial growth factor (*VEGF*) protein, the metabolic mediators' glucose transporter 1 (*GLUT-1*), pyruvate dehydrogenase kinase 1 (*PDK1*) and adrenomedullin (*ADM*) (Semenza et al., 1994), (Wenger, 2000), (Greijer et al., 2005) (figure 3). One important part of the survival strategy to counteract insufficient oxygen supply is boosting oxygen availability, transport, and supply on different levels. EPO stimulates the production of erythrocytes to increase the oxygen capacity of the blood and thus counteract anaemia (Goldberg et al., 1991), (Fandrey et al., 1994), (Fandrey, 2004). Another step in this machinery is the transcriptional upregulation of *VEGF*, which stimulates angiogenesis and vascular density to enable an improved oxygen supply in hypoxic regions (Shweiki et al., 1992), (Forsythe et al., 1996). The multi-functional peptide ADM is known to promote vasodilation, angiogenesis, and cell proliferation (Cormier-Regard et al., 1998), (Ribatti et al., 2005), (Chen et al., 2012), (Zhang et al., 2017). Cellular adaption to ATP shortage is regulated via enhanced anaerobic glycolysis and glucose uptake mediated by increased expression of glucose transporter 1. In addition, HIF increases the key metabolic enzyme pyruvate dehydrogenase kinase 1, which inhibits the mitochondrial pyruvate dehydrogenase and thereby decreases oxidative phosphorylation in mitochondria (Semenza et al., 1994), (Ebert et al., 1996).

Even under physiological settings, the oxygen tension differs extremely throughout the human body, from around 100 mmHg in arterial blood, over 30 – 70 mmHg in tissues within to a cellular range of 10 -19 mmHg, which corresponds to 1 - 2 % oxygen (Chen & Gaber, 2021). These great differences in oxygen availability argue for highly sensitive adaption mechanisms on all organisation levels and are even more important under extreme conditions in infection or inflammation where oxygen tensions can be lower than 1 %. Thereby, HIF enables the immune cells to tolerate the hypoxic environment in inflammation and modulates the immune response through metabolism and cross-talk with other immune-relevant signalling pathways in parallel (Palazon et al., 2014).

1.2.2 Oxygen-independent HIF regulation

Apart from the regulation of the HIF-1/2 pathway via changed oxygen concentrations, several other oxygen-independent mechanisms can lead to inhibition of the proteasomal HIF degradation and stimulation of target gene transcription (figure 3).

Especially under inflammatory conditions, multiple factors can lead to HIF-dependent gene regulation. Inflammation is often associated with a hypoxic or anoxic and acidic environment, hypoglycaemia (low blood glucose), and reactive oxygen species (ROS) from immune cells. As diverse as the inflammatory microenvironment can be – so are the possibilities within this environment for HIF-1 activation: The HIF response can be activated directly by pathogens, by cytokines or chemokines and immune cells, or indirect upon environmental changes in nutrient and oxygen availability and presence of ROS or nitric oxide (NO) (Devraj et al., 2017), (figure 4). The presence of inflammatory cytokines alone, such as tumour necrosis factor-alpha (TNF-α) or interleukin-1β or chemokines, secreted by immune cells can lead to an increase in HIF-dependent gene expression (Hellwig-Bürgel et al., 1999), (Haddad & Harb, 2005). TNF-α dependent HIF activation can be mediated by multiple pathways, in particular through phosphatidylinositol 3-kinase (PI3K) and transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Nitric oxide was shown to dampen HIF-1α ubiquitination and to decrease PHD activity under normoxia, probably by blocking enzyme activity via direct coordination of ferrous iron. However under hypoxia NO seems to reduce protein accumulation (Brüne & Zhou, 2007). ROS are known to influence HIF stability via the NF-κB side and mediate the activity of PHDs (Görlach et al., 2003), (Acker et al., 2006), (Bonello et al., 2007), (Niecknig et al., 2012) (figure 4).
Furthermore, exogenous substances such as lipopolysaccharides (LPS), which are components of the outer cell membrane of gram-negative bacteria, can cause induction of cellular HIF response and are used to study inflammatory conditions *in vitro*. In contrast to the oxygen-dependent activation, the *in vitro* LPS treatment results in an increased expression of *HIF1A* mRNA, also activated by NF-κB-dependent signalling in which p44/42 MAPK (mitogen-activated protein kinase) is involved (Frede et al., 2006). The crosstalk between NF-κB and HIF is mutual: The *HIF1A* gene harbours binding sites for NF-κB and NF-κB is important for basal HIF-1 transcription, linking those two master regulatory pathways of innate immunity and adaption to hypoxia (Van Uden et al., 2008). In more detail, upon inflammation NF-κB is mainly controlled by IkappaB kinase-beta, which promotes degradation of IkappaB inhibitors via phosphorylation and activation of HIF-1 (Cummins et al., 2006) (figure 4).

Additionally, regulation of the cellular hypoxic response can also be influenced post-transcriptionally due to hypoxia-induced microRNAs, mainly hypoxamiR miR-210 that are known to influence T cell differentiation and activation (Wang et al., 2014), (Wu et al., 2018).

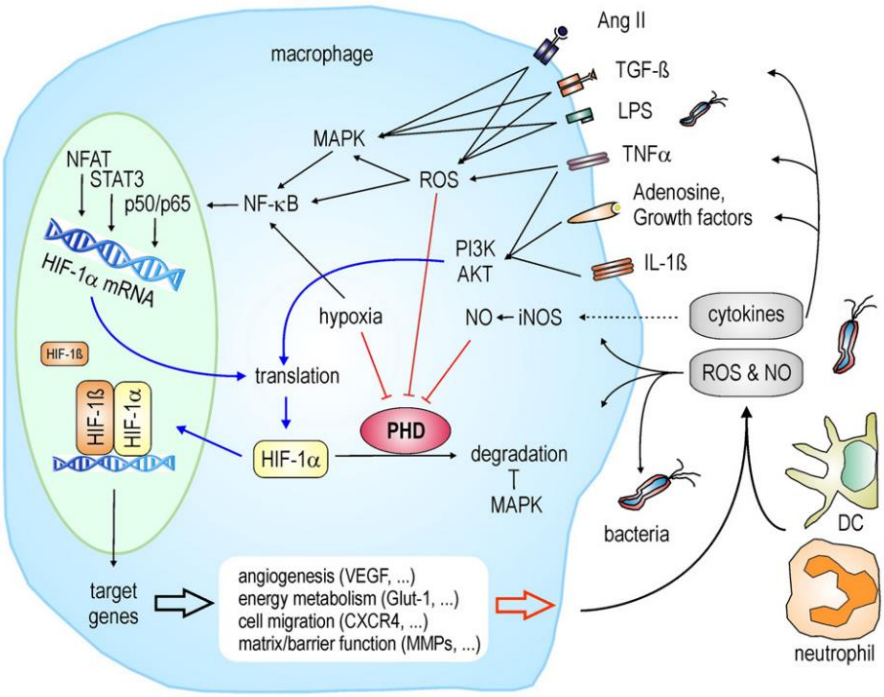


Figure 4: HIF regulation under inflammation

Schematic illustration of factors that influence HIF-1 regulation and therefore HIF-mediated cellular response in leukocytes under inflammatory conditions. Red lines indicate blockage of prolyl hydroxylases (PHD), pathways that lead to direct HIF dimerisation and target gene transcription are marked in blue. Arrows in general indicate stimulation. The abbreviations can be found in the list of abbreviations. Graphic from Dehne & Brüne, 2009.

1.3 Immune cells and HIF regulation

The interplay of HIF regulation via oxygen-dependent and oxygen-independent mechanisms is especially meaningful in inflammatory settings, where immune cells are challenged by both (Dehne & Brüne, 2009).

Beginning at the start of an infection and the innate immune response, myeloid cells recognise invading pathogens with their pattern recognition receptors (PRR), which include the Toll-like receptors (TLR). These receptors further activate the master regulator NF-κB, which induces HIF-1α accumulation and therefore the cellular HIF response for adaptation to oxygen and glucose shortage and e.g. for the survival of neutrophils (Sarah R Walmsley et al., 2005), (Rius et al., 2008). Granulocytes, but more in detail their subgroup the neutrophils are specialised for recruitment of other immune cells via secretion of TNF-α and IL-1β, which in turn both also can activate the HIF-1 pathway in multiple immune cells by increase of HIF-1α protein and HIF-1 DNA binding to target genes (Hellwig-Bürgel et al., 1999), (Amulic et al., 2012). Upon stimulation, monocytes differentiate into macrophages or dendritic cells, whereby the activation via HIF-1α is essential for both, activation and infiltration as well as for regulation of glycolytic capacity and effectiveness of the immune response (Cramer et al., 2003), (Naldini et al., 2012), (Flück et al., 2016). Although pro-inflammatory cytokines (TNF-α and IL-1β) or LPS do activate

HIF under inflammatory conditions, some typical HIF target genes like erythropoietin are not induced. In contrast, *ADM* expression is induced in inflammation as well, suggesting independent regulatory mechanisms via HIF-1 and NF-κB through IL-1β (Zaks-Zilberman et al., 1998), (Hellwig-Bürgel et al., 1999), (Hofbauer et al., 2002), (Frede et al., 2005). Antigen-presenting cells, once activated by bacterial infections or LPS treatment, upregulate *HIF1A* mRNA via the NF-κB signalling pathway (Blouin et al., 2004), (Sarah R. Walmsley et al., 2005), (Frede et al., 2006), (Jantsch et al., 2008), (Van Uden et al., 2008). Later, HIF activation also plays a major role in the mediation of the adaptive immune response. T-cell receptor stimulation in T lymphocytes, for example via LPS, leads to HIF-1 pathway activation and PHD inhibition via the PI3K/mTOR pathway (Jiang et al., 2001), (Nakamura et al., 2005), (Bernardi et al., 2006). Further, it is known that T cells display strong HIF activation in inflammatory lesions, probably due to the presence of transforming growth factor-β and Interleukin-6 that increase *HIF1A* mRNA expression via activator of transcription protein 3 (STAT3) (Vink et al., 2007), (Dang et al., 2011) (figure 4).

While HIF-dependent oxygen regulation occurs primarily at the post-transcriptional level, HIF immune regulation occurs at both the transcriptional and post-transcriptional levels. Besides the activation, via PI3K or NF-κB, another example is HIF-1α stabilisation due to regulation of the cytokine activated JAK (Janus tyrosine kinase)-STAT3 signalling transduction. Thereby, the binding of STAT3 to the HIF-α CAD domain prevents sterically the degradation of HIF-1α (Jung et al., 2008). In addition, the transcription factors induce further expression of *HIF1A* RNA (Dodd et al., 2015). Another example is the signal transduction of the mammalian target of rapamycin complex 1 and 2 (mTORC1/2), which also induces the transcription of *HIF1A* in immune cells (Dodd et al., 2015) (figure 4).

1.4 Pharmacological interventions and Roxadustat

The wide-ranging effects of the HIF pathway regulation can have a great impact on several processes in health and disease. Not surprisingly, the adaption to hypoxia is found to be dysregulated in many disease processes such as inflammation, autoimmune diseases, or cancer. Within the last years, more and more pharmacological approaches with drugs aiming to reduce or increase HIF-1α and/or HIF-2α transcription activity have been developed (Chen & Gaber, 2021), (Schönberger et al., 2021). Regarding cancer treatment, the research focuses on inhibition of HIF, because HIF activity mostly aggravates the disease. With the reverse mode of action, the development of new small-molecule prolyl hydroxylase (PHD) inhibitors, which lead to HIF activation and initiate an increase in erythropoietin expression, are currently receiving particular attention for the treatment of anaemia in patients with chronic kidney disease (Sugahara et al., 2017), (Sanghani & Haase, 2019), (Haase, 2021) (figure 3).

The PHD inhibitor *Roxadustat* (FG-4592/ Evrenzo) is the first drug of its class to receive official approval in 2019 in China and since 2021 in Europe for the treatment of anaemia associated with chronic kidney disease (CKD) (Dhillon, 2019), (Chen et al., 2019), (Fishbane et al., 2021), (Shutov et al., 2021). Treatment with *Roxadustat* leads to transient inhibition of the special prolyl hydroxylases, which initiate the degradation of the HIF-1α protein by hydroxylation under normoxia (figure 3). The orally administered drug thereby reversibly competes with 2-oxoglutarate for the active site of PHDs and inhibits the enzymes upon binding (Groenendaal-van de Meent et al., 2022). The consequence of this mode of action is an increase in HIF-1α protein and thus the expression of HIF target genes like *EPO*, which promotes erythropoiesis (Su et al., 2020), (Li et al., 2020) (figure 3). Further preclinical studies could show that the use of PHD inhibitors has the potential to protect organs from severe ischemic injuries such as stroke and myocardial infarction (Chen et al., 2014), (Vogler et al., 2015), (Sanghani & Haase, 2019). On the other hand, although already used in the clinic, the potential HIF stabilising effects of *Roxadustat* on distinct cell types are hardly studied. As the drug is administered orally it has potential systemic effects on the whole body and especially on blood circulating immune cells. Recent clinical studies showed that *Roxadustat* treated patients seem to be more prone to urinary tract and upper respiratory tract infections, which highlights the need for further research on immune cell specific effects (Chen et al., 2019), (Shutov et al., 2021).

Aim of the study

Multiple diseases are associated with inflammation and hypoxia, and both have a high potential to facilitate and influence each other. The hypoxia-inducible transcription factors (HIFs) are master regulators of cellular adaption to low oxygen levels and mediate target gene transcription of a variety of genes involved in erythropoiesis, iron transport, vascular growth, nitric oxide synthesis, phosphate metabolism, glycolysis, and gluconeogenesis (Wang & Semenza, 1993), (Greijer et al., 2005). Although it is known that the HIF pathway is integrated with major inflammatory signalling cascades like the PI3K/mTOR pathway or NF-κB, the interplay in inflammatory settings and immune reactions is still under investigation (Dehne & Brüne, 2009). To unveil the distinct effects on immune cells that are challenged with inflammation in combination with hypoxia or pharmacological HIF stabilisation, could lead to new implementations for clinical research of acute infections or help patients with dysfunctional or deregulated immune responses, like as autoimmune diseases, sepsis, or cancer.

We aim to study the interplay of hypoxia and inflammation regarding HIF pathway activation *in vitro* in immortalised human monocytes (*THP-1* cells) and primary peripheral blood mononuclear cells. Therefore, cells will be treated with different combinations and concentrations of immune-stimulating *E. coli* lipopolysaccharide or exposure to hypoxia (1 % O_2) to study hypoxic or inflammatory priming effects on the HIF pathway on protein and gene expression levels using Western blotting and qRT-PCR. Further, we want to understand the effects of the first approved HIF-stabilising drug *Roxadustat* on primary leukocytes concerning toxicity, HIF-1α protein stabilisation, and expression of HIF target genes and inflammatory mediators under physiological and inflammatory conditions. In addition, we seek to investigate HIF activation *in vivo* in human subjects that will be exposed to simulated high-altitude hypoxia before or after injection of immune-stimulating lipopolysaccharide.

In summary, the work displayed in this thesis aims to elucidate the complex interplay of hypoxic and inflammatory HIF regulation in human immune cells and offer new perspectives for further clinical research.

2. Materials and methods

2.1 Material

2.1.1 Human subjects

Some data presented in this thesis was obtained from human subject studies that were conducted at the German Aerospace Center in Cologne, Germany, or the University Hospital Essen, Germany. Information about the study protocol and involved parties can be found in section 2.2.1.

In both studies, the pool of volunteers consisted solely of non-smoking, healthy volunteers, aged 18 – 30 years without any pre-existing pathological condition. Further regular medication intake, a Body Mass Index (BMI) over 30 or a foregone confirmed COVID-19 infection were exclusion criteria.

The “ChronOx” study conducted at the German Aerospace Center in Cologne included 12 women and 10 men with a mean age of 25.2 ± 2.7 years that received the same treatment with *in vivo* hypoxia (see section 2.2.1.1).

The human subject study conducted at the University Hospital Essen included 36 male participants with a mean age of 25.9 ± 3.1 years. The study involved three experimental branches, which included either lipopolysaccharide (LPS) treatment only or combined LPS treatment (*Escherichia coli*, HOK364, 0.4 ng/kg) before or after a stay in a hypoxia chamber (see section 2.2.1.2).

Written and informed consent was obtained from each enrolled individual human subject. Further official approval was received from the responsible Ethics Committee of the study site, respectively.

2.1.2 Cell lines and primary cells

The cells used for this work are shown in table 1, and corresponding culture conditions are described in section 2.2.4.

Table 1: Used cell types and origin

Cell type	Classification	Origin
THP-1 (ACC16) cells	Human acute monocytic leukaemia (AML) cell line, established in 1978	purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Culture GmbH
Human peripheral blood mononuclear cells (PBMCs)	Human primary leukocytes, freshly isolated one day after blood donation from leucocyte concentrates (buffy coats)	Institute of Transfusion Medicine (ITM) under Prof. Dr. Peter Horn of the University Hospital Essen, Essen, Germany

The buffy coats for isolation of peripheral blood mononuclear cells were kindly provided on-site from the Institute of Transfusion Medicine (ITM), headed by Prof. Dr. Peter Horn of the University Hospital Essen, Germany. The used leukocyte concentrates were produced as a by-product of the purification of blood donations of healthy volunteers. According to the data protection guidelines, no personal information (e.g., gender or age) of the blood donors was shared. The use of the human material was approved by the ethics committee of the University Hospital Essen, Germany, headed by Prof. Dr. U. Schara-Schmidt (21-9917-BO).

2.1.3 Laboratory equipment

Table 2: Name and manufacturer of the used laboratory equipment

Equipment	Company
analytical balance	Ohaus Europa
Mastercycler	Eppendorf
table centrifuge	Biozym
Exhaust pump	Vacuumbrand GmbH & Co. KG
Invivo2 400 Hypoxia Workstation	Baker Ruskinn
Culture microscope CK40	Olympus
Incubator Hera cell	Heraeus Instruments
Cell Counter R1	Olympus
CFX 96 Touch Real-Time-System	Bio-Rad Laboratories, Inc.
Epoch™ Microplate Spectrophotometer	BioTek
FACSCantoTM II flow cytometer	BD Biosciences
Fusion-FX 7	Peqlab Biotechnologie GmbH
Axiovert 200M microscope	Carl Zeiss Microscopy GmbH
Freezer	Liebherr-International GmbH
Heat block HTM 130 / HTM 130-6	HCL Haep Labor Consult
Camera system DS Ri1	Nikon Corporation
Tuberoller RS-TR 05	Phoenix Instrument
refrigerator	Liebherr-Internationalm GmbH
Laser lamp ebq 100 isolated	LEJ GmbH
magnetic stirrer	Carl Roth GmbH + Co. KG / Peter Oehmen GmbH
Mastercycler®	Eppendorf AG
Mini-Protean® Tetra System	Bio-Rad Laboratories, Inc.
pH-Meter	Precisa Gravimetrics AG
PowerPac Basic	Bio-Rad Laboratories, Inc.
shaker Titramax 101	Heidolph Instruments
Clean bench HERA Safe	Heraeus Instruments
Thermocycler, Techne® Prime	Techne
Transblot® Turbo	Bio-Rad Laboratories, Inc.
Vortex	Scientific Industries Inc.
Water bath	Julabo GmbH / GFL
Rotary Microtome Microm HM 340E	Thermo Scientific
UV desk (BioDoc-ItTM Imaging System)	UVP
Centrifuges	Eppendorf / Heraeus / Thermo Scientific
MACSxpress Separator	MACS Miltenyi Biotec
The Big Easy & *Easy 50* EasySep™ Magnet	Stemcell Technologies
TCSPC module PicoHarp 300	PicoQuant

2.1.4 Consumables and plastic ware

Table 3: Used consumables and producers

Consumable	Company
PCR vascular strips, 0.2 ml	STARLAB International GmbH
98-Well PCR Plate (Clear), skirted	4titude® Ltd
Cell culture flask (T25 & T75)	SARSTEDT AG & Co.KG
Cell plate (96-well skirted, protein assay)	4titude® Ltd
Conical centrifuge tube (15 / 50 ml)	SARSTEDT AG & Co.KG
Rotilabo®-filter, PVDF	Carl Roth GmbH + Co. KG
Beakers	Schott AG
Biosphere® LowPet FilTips (2.5 -1000 µl)	SARSTEDT AG & Co.KG
Cover slips Ø 10 mm	Marienfeld GmbH & Co. KG
Gel-blotting paper, GB003	Whatman®
Microscope slide	R.Langenbrinck GmbH
Cell plate (96-well with skirt, qPCR)	SARSTEDT AG & Co.KG
Adhesive PCR Plate Seal	4titude® Ltd
tweezers	Peter Oehmen GmbH
Pipette tip PCR Performance Tested (20 - 1000 µl)	SARSTEDT AG & Co.KG
Plastic pipettes, steril (5 ml, 10 ml, 25 ml)	Greiner Bio-One International
PVDF-Transfer membrane, 0.2 µm Porengröße	Schleicher & Schuell
Reaction tubes (0.5 ml, 1.5 ml, 2 ml)	SARSTEDT AG & Co.KG
SafeSeal reaction tube	SARSTEDT AG & Co.KG
TC-Plate 24 Well, Standard, F	SARSTEDT AG & Co.KG
TC-Plate 6 Well, Standard, F	SARSTEDT AG & Co.KG
TC-flask (T25, T75, T150)	SARSTEDT AG & Co.KG
Flow cytometry tube (75 x 12 mm)	SARSTEDT AG & Co.KG
S-Monovette® Plasma Lithium-Heparin 7.5 ml	SARSTEDT AG & Co.KG
S-Monovette® K3 EDTA, 3.5 ml	SARSTEDT AG & Co.KG
PAXgene® blood RNA collection tubes	Qiagen, Mississauga, Canada
Superfrost™ microscope slides	Thermo Scientific

2.1.5 Chemicals and reagents

Table 4: Used chemicals and reagents, listed with the respective producers

Chemicals	Company
Roxadustat	Cayman Chemicals
Agarose	AppliChem
Acetic acid	AppliChem
2-[4-(Aminoiminomethyl)phenyl]-1H-Indole-6-carboximidamide hydrochloride (DAPI)	Sigma-Aldrich, Inc.
Tissue-Tek® O.C.T.™ Compound	Sakura Finetek, Netherlands
Ethidium bromide	Sigma-Aldrich
GoTaq G2 polymerase	Promega
GoTaq reaction buffer (5X)	Promega
DNA ladder (100 bp)	Invitrogen
D-sorbitol	Sigma-Aldrich, Inc.
Methanol	Sigma-Aldrich, Inc.
Paraformaldehyde (PFA)	Sigma-Aldrich, Inc.
Acrylamid/Bisacrylamid (30 %)	Carl Roth GmbH + Co. KG
Ammonium persulfate (APS)	Bio-Rad Laboratories, Inc.
Blue S´Green qPCR 2x Mix	Biozym Scientific GmbH
Bovine serum albumin (BSA)	SERVA Electroph. GmbH
Bromphenol blue	Sigma-Aldrich, Inc.
Diethylpyrocarbonat (DEPC)	Sigma-Aldrich, Inc.
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Inc.
Dimethyloxaloylglycine (DMOG)	Biomol
Ethanol	Honeywell International, Inc.
Ethylenediaminetetraacetic acid (EDTA)	Merck KGaA
Fetal calf serum (FCS)	Biochrom AG
Glycine	AppliChem GmbH
Glycerine	Sigma-Aldrich, Inc.
Guanidinium thiocyanate (GTC)	Carl Roth GmbH + Co. KG
Immersol™ 518 F	Carl Zeiss Microscopy GmbH
Isopropanol	AppliChem GmbH
Potassium chloride (KCl)	AppliChem GmbH
Luminol	Carl Roth GmbH + Co. KG
Lymphoprep™	Stemcell Technologies
skimmed milk powder	TSI GmbH & Co. KG
M-MLV Reverse Transcriptase (RT)	Promega Corporation
M-MLV RT 5x reaction buffer	Promega Corporation
Mowiol	Calbiochem, Merck
N, N, N′, N′-Tetramethylethylendiamine (TEMED)	Sigma-Aldrich, Inc.
Sodium acid (HCl)	AppliChem GmbH
Sodium chloride (NaCl)	Sigma-Aldrich, Inc.
Sodiumdihydrogenphosphat (Na_2HPO_4)	Carl Roth GmbH + Co. KG
Sodium dodecyl sulfate (SDS) pellets	Carl Roth GmbH + Co. KG
Nonidet® P40 (NP40)	AppliChem GmbH
Nucleoside triphosphate (NTP)	Promega Corporation
Oligo Desoxythymidin (Oligo-dT) primers	Invitrogen AG
PAGE Ruler Prestained Protein Ladder	Thermo Fisher Scientific
p-Cumarinacid	Sigma-Aldrich, Inc.
Penicillin/streptavidin (10000 U/ml)	Thermo Fisher Scientific
Proteinase inhibitor (PI) (Complete Mini EDTA-free)	Sigma-Aldrich, Inc.

Poly-L-Ornithin	Sigma-Aldrich, Inc.
Protease-Inhibitor	F. Hoffmann-La Roche AG
Protein Assay Reagent A	Bio-Rad Laboratories, Inc.
Protein Assay Reagent B	Bio-Rad Laboratories, Inc.
RPMI Media 1640	Gibco
hydrochloric acid (HCl)	Merck KGaA
ß-mercaptoethanol	Merck KGaA
Tris(hydroxymethyl)-aminomethane	AppliChem GmbH
Triton-X-100	Sigma-Aldrich, Inc
Trypan blue	Merck KGaA
Tween 20	Carl Roth GmbH + Co. KG
Hydrogen peroxide (H_2O_2)	Merck KGaA
Sucrose	Merck KGaA
E. coli O111:B4 lipopolysaccharide (LPS)	Sigma-Aldrich
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	AppliChem
Brilliant Stain Buffer (FACS)	BD Biosciences
Protein Assay Reagent A (Reagenz A)	Bio-Rad
Protein Assay Reagent B (Reagenz B)	Bio-Rad
Donkey serum	Sigma-Aldrich

2.1.6 Solutions, Media, and Kits

Table 5: Formula of used solutions and reagents

Solution	**formula**
10x running buffer (SDS-PAGE)	25 mM Tris 192 mM Glycin 3.5 mM SDS
10x PBS, pH 7.2	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4
10x TBS (Western Blot), pH 7.6	137 mM NaCl 20 mM Tris
10x TBS (Immunohistochemistry), pH 7.6	301.4 mM NaCl 50 mM Tris
4x SDS buffer	200 mM Tris pH 6.8 8 % SDS 10 % ß-Mercaptoethanol 0.02 % bromphenol blue 40 % Glycin
Blocking solution (Western Blot)	5 % milk powder 1 x TBS-T
DEPC-H_2O	H_2O 0.1 % DEPC stir 1 h at RT, autoclave
Flow cytometry buffer	1 x PBS 0.1 % FCS 0.02 % Sodium azide
Lower Buffer (Western Blot), pH 8.8	1.5 M Tris 13.9 mM SDS

Protein lysis buffer	1 % Nonidet P40 150 mM NaCl 20 mM Tris pH 7.9 5 mM EDTA Add 10 % Protease Inhibitor before usage
Cell culture Media (THP-1, PBMC)	RPMI 1640 Gibco 10 % FCS (100 U/ml) 1 % Penicillin/Streptomycin (100 µg/ml)
(HRP)-Luminol detection solution (Western Blot)	100 mM TRIS pH 8.5 250 mM Luminol 90 mM p-Cumarin acid 30 % H_2O_2
Mowiol	5 g Mowiol 20 ml PBS 10 ml Glyzerin
PBT	1 x PBS Triton X-100 (0.5 % / 0.1 %)
1xTBS-T (Western Blot)	1x PBS 0.05 % Tween 20
1xTBS-T (Immunohistochemistry)	1 x PBS 0.1 % Tween 20
GTC solution (4M)	4 M GTC 250 mM NaOAc 0.75 % β-mercaptoethanol in DEPC water
Upper Buffer (Western Blot), pH 6.8	251 mM Tris 6.9 mM SDS
TAE buffer (PCR), pH 8.0	40 mM Tris 0.11 % Acetic acid 1 mM EDTA

Table 6: Used Kits and producers

Kit	**Company**
PAXgene® Blood RNA Kit	Qiagen, Mississauga, Canada
NucleoSpin® RNA Isolation Kit	Macherey-Nagel GmbH
PE Annexin V Apoptosis Detection Kit I	BD Biosciences
Super Signal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific
Trans-Blot Turbo RTA Transfer Kit, LF PVDF	Bio-Rad Laboratories, Inc.
MACSxpress® Whole Blood Neutrophil Isolation Kit, human	MASC Miltenyi Biotec
EasySep™ Direct Human Monocyte Isolation Kit	Stemcell Technologies
EasySep™ Direct Human T Cell Isolation Kit	Stemcell Technologies

2.1.7 Antibodies

All antibodies used were directed against human antigen structures.

Table 7: Antibodies used for Western Blotting

Target	Species	Dilution	Company
Primary Antibodies			
β-actin	rabbit	1:2000	Sigma-Aldrich, Inc
Hif-1α	mouse	1:500	BD Transduction Laboratories™
Secondary Antibodies			
rabbit IgG (H+L)	goat	1:10.000	Sigma-Aldrich, Inc
Mouse IgG (H+L)	goat	1:10.000	Sigma-Aldrich, Inc

Table 8: Antibodies used for Immunohistochemistry

Target	species	Dilution	Company
Primary Antibody			
Hif-1α	mouse	1:200	BD Transduction Laboratories™
Secondary Antibodies			
Alexa Fluor® 488 anti-mouse IgG	goat	1:200	Life Technologies
Alexa Fluor® 568 anti-mouse IgG	goat	1:200	Invitrogen AG

Table 9: Antibodies used for flow cytometry analysis

Target	fluorophore	species	Dilution	Company
CD4	PE	mouse	1:20	BioLegend, Inc.
CD14	APC-Cyanine7	mouse	1:20	BioLegend, Inc.
CD8	BV650 Mouse	mouse	1:20	BD Biosciences
CD19	APC Mouse	mouse	1:10	BD Biosciences
CD3	FITC Mouse	mouse	1:10	BD Biosciences
CD45	PerCP Mouse	mouse	1:16	BD Biosciences
CD284 (TLR4)	Brilliant Violet™ 421	mouse	1:20	BD Biosciences
CD86	BV786 2331 (FUN-1)	mouse	1:20	BD Biosciences
CD16	APC	mouse	1:20	BioLegend, Inc
HLA-DR	PE	mouse	1:20	BioLegend, Inc.

2.1.8 Oligonucleotides

Table 10: Primer sequences used for PCR and qRT-PCR

Target gene	sequence	Product length
3´ *ACTB*	CAGCGGAACCGCTCATTGCCAATGG	295 bp
5´ *ACTB*	TCACCCACACTGTGCCCATCTACGA	
3´ *HIF1A*	CTCCATTACCCACCGCTGAA	202 bp
5´ *HIF1A*	TCACTGGGACTATTAGGCTCAGGT	
3´ *HIF2A*	CGGAGGTGTTCTATGAGCTGG	115 bp
5´ *HIF2A*	AGCTTGTGTGTTCGCAGGAA	
3´ *HIF3A*	ATCTGCATTTGCCGTTGGAC	259 bp
5´ *HIF3A*	CCAGCCCTTACCTGAGCTAT	
3´ *GLUT-1*	CTAGCGCGATGGTCATGAGT	214 bp
5´ *GLUT-1*	TCTGGCATCAACGCTGTCTT	
3´ *ADM*	AGTCGTGGGAAGAGGGAACT	141 bp
5´ *ADM*	ATCCGGACTGCTGTCTTCGG	
3´ *PDK1*	TGAACGGATGGTGTCCTGAG	194bp
5´ *PDK1*	GGCCAGGTGGACTTCTACG	
3´ *VEGF-A*	CCGCCTCGGCTTGTCACA	165 bp
5´ *VEGF-A*	GCAAGACAAGAAAATCCCTGTGGGCC	
3´ *PHD1*	TGGCCCTGGACTATATCGTG	236 bp
5´ *PHD1*	GGCACCAATGCTTCGACAG	
3´ *PHD2*	GCACGACACCGGGAAGTT	176 bp
5´ *PHD2*	CCAGCTTCCCGTTACAGT	
3´ *PHD3*	CACAGCGAGGGAATGAACCT	129 bp
5´ *PHD3*	TCCTGCTGTTAAGGCTTCCG	
3´ *IL6*	CCACTCACCTCTTCAGAACGAA	354 bp
5´ *IL6*	TGCATCTAGATTCTTTGCCTTTTT	
3´ *TNFA*	CTCAGCTTGAGGGTTTGCTAC	146 bp
5´ *TNFA*	TGCACTTTGGAGTGATCGGC	
3´ *TLR2*	GGGTCATCATCAGCCTCTCC	181 bp
5´ *TLR2*	AGGTCACTGTTGCTAATGTAGGTG	
3´ *TLR4*	CAGAGTTTCCTGCAATGGATCA	86 bp
5´ *TLR4*	TGCTTATCTGAAGGTGTTGCACAT	

2.1.9 Software

Table 11: Used software for data analysis and visualisation

Software	Company
CFX Manager 3.1	Bio-Rad Laboratories
FACS Diva Software	BD Bioscience
ImageJ 1.53q (Fiji)	National Institutes of Health (NIH), USA
GraphPad Prism Version 8.4.3	GraphPad Software
ZEN Imaging	Carl Zeiss Microscopy

2.2 Methods

2.2.1 Human subject studies

As a part of this thesis, data obtained from two human subject studies were used. The conduction of these studies was based on collaborative, interdisciplinary work and the here presented data do represent only a subset of the studies. If not marked otherwise, the results used in this thesis were obtained and analysed personally by the author of this thesis. Some results were achieved in a collaboration with other researchers and parts can be mentioned in this work for comprehensiveness. These results are marked as collaborative work and involved researchers are mentioned in the respective sections.

2.2.1.1 The "ChronOx" study, German Aerospace Center Cologne, Germany

The human subject "ChronOx" study was organised and conducted by Titiaan Post, Dr. Riccardo De Gioannis, and Prof. Dr. Daniel Aeschbach of the Institute of Aerospace Medicine, Sleep and Human Factors Research of the German Aerospace Center in Cologne Germany. The study aimed to investigate the effects of moderate hypoxia (15 % O_2), *in vivo* on the circadian melatonin rhythm in humans.

One part of the study was the analysis of whole blood gene expression, focussing on the hypoxia-inducible factor (HIF) pathway. Blood samples for gene expression analysis were provided by the study team of the German Aerospace Center, Cologne, and were further processed and analysed by the author of this thesis at the University Hospital Essen (see sections 2.2.2 and 2.2.5).

The study was conducted as a randomised, single-blind crossover study. Participants volunteered for two 4-day laboratory visits, where they were one time exposed to a lower oxygen concentration of 15 % for a duration of 6.5 h, corresponding to a high-altitude of 2438 meters. During the second visit, no changes in oxygen concentrations were made. A total of twenty-two healthy adults, consisting of 12 women and 10 men participated in this study approach. The mean age of participants was 25.2 ± 2.7 years. Blood oxygenation was measured with a finger pulse oximeter throughout the night of exposure. Transcription levels of HIFs (*HIF1A*, *HIF2A, HIF3A*) and HIF target genes (*GLUT-1*, *PDK1*, *ADM*, *VEGF*, *PHD1*, *PHD2*, *PHD3*) were determined in whole blood samples before and during 3:15 hours and 6:25 hours of hypoxic exposure (normobaric, 15 % O_2 in the air) compared to normoxia (21 % O_2) and are included in this thesis.

2.2.1.2 The “HOX-LPS” Study, University Hospital Essen, Germany

The human subject study “Hypoxia-induced gene expression in human leukocytes under simulated altitude conditions with and without administration of lipopolysaccharide (LPS)” was organised by Dr. med. Bastian Tebbe of the Department of Nephrology of the University Hospital Essen, Germany. This translational cooperation project united researchers from the Institute of Physiology under Prof. Dr. med. Joachim Fandrey, the Institute of Medical Psychology and Behavioral Immunobiology under Prof. Dr. rer. Biol. Hum. Dipl.-Psych. Manfred Schedlowski and the Department of Nephrology under Prof. Dr. med. Benjamin Wilde and Prof. Dr. med. Oliver Witzke, all situated on the site of the University Hospital Essen and part of the University of Duisburg-Essen in Essen, Germany. The study was approved by the ethics committee of the University Hospital Essen, Germany (18-8258-BO).

The study included a total of 30 male healthy participants (mean age: 25.9 ± 3.1) to research immune reactions following lipopolysaccharide injection (*E. coli*, HOK364, 0.4 ng LPS per kg bodyweight) before or after hypoxia a 4-hour stay in a hypoxia chamber, simulating a high altitude of 4500 meters with normobaric oxygen concentrations of 10.5 % O_2. For control purposes, 6 participants were solely treated with a single LPS injection and followed up. In all cases, participants were examined and donated blood right before the start of the study protocol to determine the “baseline” levels of all collected data. After the first stimulus (LPS injection or exposure to hypoxia), blood was collected every 2 hours for 8 hours and additionally 24 hours after the start of the study program (time points: +2 h, +4 h, +6 h, +8 h, +24 h). For more details, we refer to the result section 3.5. Blood sampling was followed by direct processing of respective material for flow cytometry analysis (2.2.8), immune cell isolation (2.2.3), and RNA isolation (2.2.2 and 2.2.5). The blood oxygenation was measured with a finger pulse oximeter during the duration of the study. Besides that, other parameters and analyses were included in the study but are not part of this thesis.

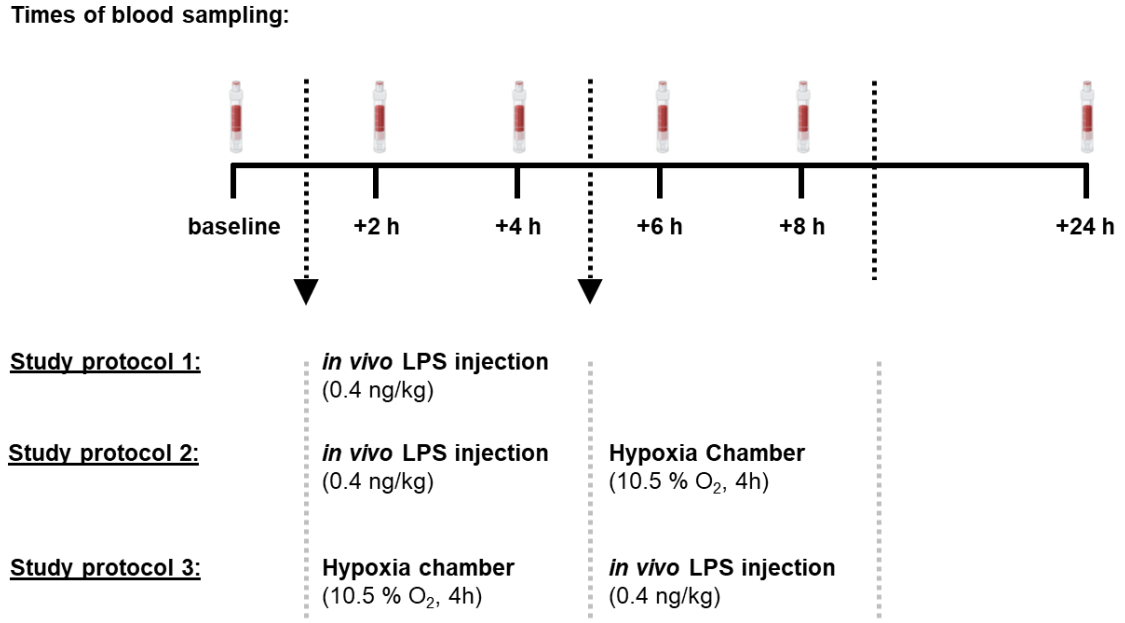


Figure 5: Study design and blood sampling times

Schematic illustration of the 3 different study protocols and corresponding blood sampling times in the human subject study conducted at the University Hospital Essen, Germany. Blood samples were taken at 6 different time points (baseline, +2 h, +4 h, +6 h, +8 h, and +24 h). The study included 3 different study protocols with human subjects being treated with a single lipopolysaccharide (LPS) injection only (1) or in combination with 4 hours of 10.5 % O_2 hypoxia before (3) or after the injection (2).

2.2.2 Collection of subjects' whole blood samples

For direct stabilisation of RNA of whole blood cells, the PAXgene® blood RNA collection system (Qiagen, Mississauga, Canada) was used. 2.5 ml of whole blood was drawn into PAXgene® blood RNA tubes (Qiagen, Mississauga, Canada) and was transferred to -20 or -80 °C after storage at RT for 24 h, according to the manufacturer´s protocol (table 6).

2.2.3 Immune cell isolation from human whole blood

To access gene transcription of distinct immune cell subsets within the circulating blood of study subjects, a negative selection of immune cells with magnetic beads was performed. With the help of magnetic labelled antibody beads, which bind to the unwanted cell populations and the application of magnetic forces neutrophils, T cells, and monocytes were isolated from whole blood samples. The negative selection method guaranteed untouched target cells and minimized side effects due to the isolation procedure. The isolation was done according to the instructions of the respectively used Kit within 1 hour of blood sampling (table 6). The purity of isolated cell populations was verified using flow cytometry analysis.

For isolation of neutrophils 1.5 ml of whole blood drawn into an EDTA-Monovette was processed using the “MACSxpress® Whole Blood Neutrophil Isolation Kit” and the “MACSxpress Separator” Magnet (both MASC Miltenyi Biotec), according to the instructions. Depending on the human subject, processing of 1.5 ml whole blood resulted in 1 x 10^6 to 1x 10^7 gained cells.

Isolation of T cells was generally performed with 2 ml of whole blood drawn into a Heparin-Monovette using the “EasySep™ Direct Human T Cell Isolation Kit” and the "The Big Easy EasySep™” Magnet (both Stemcell Technologies). At blood sampling times two hours past LPS injection, where reduced circulating T cells in the bloodstream were expected 10 ml of whole blood were processed. Depending on the human subject, the T cell isolation resulted in 1 x 10^6 to 1x 10^7 gained cells.

Isolation of monocytes was performed with 5 ml of whole blood drawn into an EDTA-Monovette using the “EasySep™ Direct Human Monocyte Isolation Kit” (Stemcell Technologies) and the “Easy 50 EasySep™” Magnet (both Stemcell Technologies). Depending on the human subject, processing of 5 ml whole blood resulted in 1 x 10^6 to 1x 10^7 gained cells.

The obtained cell pellets of all fractions were each lysed in 350 µl GTC (4 M) and frozen at -20°C until following RNA isolation.

2.2.4 Cell culture

2.2.4.1 THP-1 monocytic cell line culture

Cells from the human monocytic cell line *THP-1* (ACC16, DSMZ, Leibniz Institute) were thawed from frozen stocks that were stored in liquid nitrogen (1 x 10^6 cells/stock in 1 % DMSO). Cells were diluted and centrifuged for 5 min at 300 x g in RPMI Media with 10 % FCS and 1 % PS (table 5). The cell pellet was suspended in fresh media and the culture was maintained in a cell incubator at 37 °C with 21 % O_2, and 5 % CO_2. Cells were supplied with fresh media every two to three days and allowed to grow to a density of 2 x 10^6 cells/ml. For the determination of the cell concentration, the cell counter "Cell Counter R1" from Olympus was used. For this purpose, 10 µl cell suspension was mixed with 10 µl trypan blue in a ratio of 1:1 and placed on a disposable slide and counted using the cell counter (settings: cell size: 3 - 40 µm, 20 % roundness, 5 % background minimization, dilution factor 2). For experiments, 1 x 10^6 cells/sample were used in 2 ml volume per well in a 6-well cell culture plate.

2.2.4.2 Isolation and culture of PBMCs from buffy coats

Human peripheral blood mononuclear cells (PBMCs) were isolated and cultured from leukocyte concentrates (buffy coats) of healthy blood donors. The Buffy Coats were obtained from the Institute of Transfusion Medicine (ITM), under the direction of Prof. Dr. Peter Horn of the University Hospital Essen, Germany. At this site enrichment of leukocyte, concentrates took place directly after blood sampling and were stored until usage. For *in vitro* experiments PBMCs were isolated from buffy coats one day after the original blood donation.

To obtain PBMCs from the leukocyte concentrate, the blood mixture was first transferred to two 50 ml Falcon centrifuge tube tubes. Two 50 ml centrifuge tubes were filled with 20 ml of the density gradient medium *Lymphoprep* (1.077 g/ml) per Buffy Coat. In each of these tubes, 30 ml of blood were then carefully pipetted onto the layer of *Lymphoprep* without piercing its surface, so that the blood could accumulate above the density gradient medium. This was followed by density gradient centrifugation to separate the individual components of the concentrate according to their density. For this purpose, the tubes were centrifuged for 25 min at 1200 x g and room temperature (RT) without the break switched on. After suction of most of the plasma layer, the PBMCs were carefully picked up with a pipette and transferred to a new 50 ml centrifuge tube and mixed with 1 x PBS. This was followed by four washing steps with 1 x PBS to keep contamination by erythrocytes, platelets, and granulocytes as low as possible. The samples were centrifuged three times for 8 min at 300 x g and then again for 10 min at 200 x g at RT with the brake switched on. After each washing step, the supernatant was extracted, and the pellet was resuspended in 1 x PBS (1st-3rd washing step) and finally in RPMI medium (after the 4th washing step) for cell culture.

After isolation from buffy coats, PBMCs were directly counted using the “Cell counter R1” from Olympus (settings: cell size: 3 - 40 µm, 20 % roundness, 5 % background minimization, dilution factor 1) and diluted to the desired experimental concentration of 4 x 10^6 cells/well in 2 ml media into a 6-well cell culture plate. Cells were cultured at 37 °C with 21 % O_2, and 5 % CO_2 in the air in RPMI media supplemented with 10 % FCS and 1 % PS until the experiment was started (table 5).

2.2.4.3 Experimental cell culture conditions

Unless otherwise described, all cell culture experiments were started after seeding the cells in the desired cell concentration the following day without changing the medium. The hypoxic incubation of the cells took place in the Invivo2 400 Hypoxia Workstation (Baker Ruskinn) at 37 °C and a water vapor saturated gas mixture of 5 % CO_2, 1 % O_2, and 94 % N_2. The normoxic incubation (control conditions) took place in the cell incubator in a gas mixture of air (21 % O_2) and 5 % CO_2 at 37 °C. All cell culture experiments were carried out in a 6-well plate format.

To investigate the effects of the hypoxic environment *THP-1* cells and PBMCs were exposed to hypoxia of 1 % O_2 for different periods (30 min – 24 h). Cells were further challenged with different concentrations (5 ng/ml – 1000 ng/ml) of *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) for different periods (1 h – 24 h) to examine the cellular reactions to an inflammatory stimulus on HIF-1α protein accumulation and gene expression. Different experiments were carried out with combined stimuli of hypoxia and LPS to study the effects of hypoxic incubation before or after an LPS stimulus on HIF-1α protein or gene expression levels.

PBMCs were further treated with different concentrations of the HIF-1α stabilising drug *Roxadustat* (sock in DMSO, diluted to 10 µM, 25 µM, 50 µM final concentration) over different periods (1 h – 24 h) under normoxic conditions to assess the effects of the approved compound on circulating immune cells regarding the HIF-1α protein levels and inflammatory and HIF-related target gene transcription (experiments were part of the master thesis project by Alexandra Heinrich under the supervision of the author of this thesis).

After the respective experiment cells were collected by centrifugation (5 min, 350 x g, RT), the supernatant was discarded and the cell pellet was processed either for flow cytometry analysis, protein analysis or lysed in 350 µl GTC (4 M) and stored at -20 °C for following RNA isolation.

2.2.5 Gene expression analysis

2.2.5.1 RNA isolation of human whole blood samples

For RNA isolation of whole blood samples from human subjects, the PAXgene® Blood RNA Kit (Qiagen, Mississauga, Canada) was used according to the manufacturer´s protocol. Total RNA was eluted in 40 µl elution buffer (BR5) and stored at -80 °C.

2.2.5.2 RNA isolation of cell culture samples

The cells were transferred to a 2 ml reaction vessel and centrifuged (5 min, 350 x g, RT). The supernatant was carefully waned, and cells were lysed via the addition of 350 µl GTC (4 M). Isolation of the RNA was performed according to the protocol of the NucleoSpin® RNA kit from Macherey-Nagel. The RNA was last eluted in 40 µl RNase-free water and stored at -80 °C until further use.

RNA concentration and purity were measured with the Epoch™ Microplate Spectrophotometer on a Take3 plate using 1.5 µl of undiluted RNA sample. As purity control, the 260/280 nm absorption ratio was calculated and showed values between 1.8 to 2.2.

2.2.5.3 Complementary DNA synthesis

For complementary DNA (cDNA) synthesis for each sample, 100 - 200 ng of total RNA was used and rewritten. The corresponding amount of RNA was filled up with DEPC-H_2O to 19 µl total volume. After the addition of 5 µl oligo-dT, the mixture was incubated in the master cycler® (Eppendorf) at 68 °C for 10 min and with subsequent storage of the samples on ice (4 °C) for 5 minutes. Following, 10 µl M-MLV RT 5-fold buffer (Promega, US), 10 µl dNTPs, 1 µl Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega, Walldorf, Germany), and 5 µl DEPC-H_2O were added to each sample. The cDNA synthesis was then carried out in the master cycler® for 90 min at 45 °C (accumulation of the oligo dTs) and 52 °C for 30 min (synthesis of the cDNA). In the last synthesis step, the samples were denatured at 95 °C for 15 min. The synthesized cDNA was stored at 4 °C until further use. To verify the purity of the reaction components, for each cDNA synthesis procedure, one negative control with deionized water instead of an RNA sample was added to the samples. For every set of samples, the synthesized cDNA was checked by a standard polymerase chain reaction for the housekeeping gene β-actin (*ACTB*).

2.2.5.4 Quantitative real-time polymerase chain reaction

The quantification of gene expression at the transcription level was performed by quantitative real-time (qRT) polymerase chain reaction (PCR). For this purpose, a reaction mixture consisting of 12.5 µl Blue S´ Green qPCR 2 x mix (Eurogentec, Verviers, Belgium), 11 µl dH_2O water, and 0.5 µl per primer were prepared. For the double determination, 9.5 µl of this approach were pipetted into an optical 96-well plate, afterwards, 0.5 µl of cDNA was added to the reaction mixture. The negative control was an approach with demineralized water instead of the cDNA. The real-time PCR took place in a CFX96™ Real-Time System (Bio-Rad), using the following heating protocol: 95 °C for 2 min (initial phase), 95 °C for 5 sec (denaturation), 20 sec for 60 °C (annealing/elongation). The steps of denaturation, annealing, and elongation were repeated 40 times. To verify the purity of the used primers, a melting curve of the synthesized PCR product was created upon every reaction to exclude the presence of unwanted by-products or errors. The cycler detected the amount of PCR product based on the fluorescence of the S' Green dye and presents the CT value (cycle threshold) with an automatically calculated threshold value. The evaluation of the qPCR was carried out using the $2^{-(\Delta CT)}$ and $2^{-(\Delta\Delta CT)}$ calculation methods. The CT values of every sample were normalised to those of the constitutively expressed house-keeping gene β-actin (*ACTB*). As a result, a change in gene expression of the investigated genes could be calculated as an n-fold change compared to the control condition ($2^{-\Delta\Delta CT}$). The specificity of the used primers was checked via the “Primer BLAST” online tool (NIH) and the size of the PCR amplicons was confirmed via PCR (Spandidos et al., 2010).

2.2.6 Protein extraction, determination, and analysis

2.2.6.1 Cell lysis and protein determination

After carrying out the respective experiment, the cell suspension of each sample was transferred into a 2 ml reaction tube and cells were pelleted via centrifugation (5 min, 350 x g, RT). After removal of the supernatant, the cell pellet was resuspended in 1 ml of cold 1 x PBS and centrifuged again. The cell pellet was suspended in 50 µl protein lysis buffer and incubated on ice for 20 minutes. Afterward, the lysates were centrifuged for 5 min at 800 x g at 4 °C. The proteins in the supernatant were removed and stored at -20 °C until further analysis. 5 µl of the removed supernatant were used for protein concentration determination.

To determine the amount of protein in each sample the method of Lowrey et al. was used (Lowry et al., 1951). Therefore 5 µl of the protein lysate, as well as a standard series consisting of BSA concentrations of 25, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 mg/ml BSA, were diluted 1:10 with distilled water. 20 µl of each mixture was pipetted into a 96-well plate, 10 µl reagent A (Bio-Rad) and 75 µl reagent B (Bio-Rad) was added to each sample and incubated for 5 min at RT. The extinction was measured at 700 nm with the Epoch™ Microplate spectrophotometer and the protein concentrations were calculated based on the BSA standard series.

2.2.6.2 SDS-PAGE and immunoblotting

For the detection, proteins were first separated according to their molecular weight using the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For electrophoresis, 1.5 mm thick gels consisting of a broad, dense separating gel and an upper less dense part were prepared between two glass panes according to table 12. A sample comb with 10 pockets was placed into the liquid stacking gel to create slots for the following sample administration. Depending on the test conditions, 30 to 100 µg of protein were used for each sample. After the hardening of the gel, the sample comb was removed. Before separation protein samples were diluted 1:4 with 4x sodium dodecyl sulfate (SDS) sample buffer (table 5) and denatured for 5 min at 95 °C in a heating block. The protein samples and a standard protein ladder were filled in the sample slots on the gel and electrophoresis was executed at 80 Volts (V) for about 120 min in a chamber filled with running buffer (table 5).

Table 12: Composition of gels used for SDS-PAGE

Component	**Stacking gel (5 %)**	**Separating gel (7.5 %)**
Bisacrylamide (30 %)	0.83 ml	2.5 ml
4x Upper buffer, pH 8.8	1.25 ml	-
4x Lower buffer, pH 6.8	-	2.5 ml
Distilled water (Aqua dest.)	2.92 ml	5 ml
Ammonium persulfate (APS), 10 %	50 µl	100 µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	5 µl	10 µl

After the electrophoresis proteins were transferred to a Microporous polyvinylidene difluoride (PVDF) membrane using an electric field. The transfer was carried out according to the protocol of the *Trans-Blot Turbo RTA Transfer Kit* (Bio-Rad) and using the Transblot® Turbo Transfer Device (Bio-Rad). After activation of the PVDF membrane for 1 min in Ethanol, a stack of three layers of Turboblot buffer-soaked filter papers, the PVDF membrane, the SDS gel, and again three layers of soaked filter papers were placed in the charging cassette of the transfer device. The protein transfer was carried out for 10 min at 1.3 V, followed by a 20-minute cooling phase at 4 °C and a further transfer phase at 1.3 V for 5 min. Thereafter the PVDF membrane with the transferred proteins was incubated for one hour at RT in 5 % skimmed milk in TBS-T (blocking solution, table 5) to minimize non-specific bonds by the primary antibody. For immunological detection, the membrane was cut at the level of the 70 kDa band of the marker to be able to incubate the different parts of the membrane with different primary antibodies. The upper membrane (≥ 70 kDa) was incubated with a HIF-1α antibody (BD, dilution 1:500) and the lower membrane (0 - 70 kDa) with an β-actin antibody (Sigma-Aldrich, dilution 1:2000) at 4°C overnight (table 7). All antibodies were diluted in 5 % skimmed milk in TBS-T. On the following day, the individual membrane parts were washed three times in TBS-T for 5 min each. Subsequent incubation with the secondary antibodies (1:10.000) was carried out in 5 % skimmed milk in TBS-T for one hour at RT (table 7). After washing the membrane parts again in TBS-T three times for 10 minutes, the protein bands were detected either using the ECL reagent of the Thermo Scientific reaction kit (table 6) or using the HRP luminol detection solution (table 5). In both cases, the membrane was incubated in the respective detection solution for one minute and then placed in the Fusion-FX7 for detection of the chemiluminescent signal.

The quantification of the protein band intensities was carried out with the help of the software "Fusion" and "ImageJ" (table 11). The obtained intensity values were used for the calculation of the HIF-1α/β-actin protein ratios.

2.2.7 Immunofluorescence staining of PBMCs

To further examine the intracellular accumulation of the HIF-1α protein in PBMCs upon *Roxadustat* treatment immunofluorescence staining was performed. This method is based on the specific binding of antibodies to antigenic proteins on the surface of the cells. Species-specific second antibodies coupled to fluorescent dyes bind to primary antibodies and thus enable the detection of specific proteins. The detection is achieved by the excitation of the coupled fluorochrome utilizing different light wavelengths. For microscopical analysis, PBMCs were first fixed on poly-L-ornithine (PORN)-coated coverslips (coating: 45 min, 37 °C). For each experiment, 400.000 cells were seeded on top of the coverslips in a 24-well plate under common media conditions. After the experiment coverslips were washed twice with 1 x PBS, followed by a fixation step with 4 % PFA for 15 min at RT. Afterward, cells were washed twice with 1 x PBS before permeabilization with 0.5 % Triton-X in 1 x PBS (PBT) for 10 min at RT to make the membrane consistent for antibodies. After another three washing steps with 0.1 % PBT, non-specific binding sites were blocked with 10 % donkey serum in 0.1 % PBT for 45 min at RT. The step was followed by incubation with the primary HIF-1α antibody (table 8) diluted in 0.1 % PBT, and 1 % donkey serum overnight at 4 °C. On the following day, the remaining antibody solution was removed, and the cells were washed three times with 0.1 % PBT. Thereafter, the secondary fluorescent-labelled antibody (Alexa Fluor® 488, table 8) was diluted in 1 % donkey serum in 0.1 % PBT and administered for one hour in the dark at RT. After that, the cells were washed three times with 1 x PBS and were then covered with 2-[4-(Aminoiminomethyl)phenyl]-1H-Indole-6-carboximidamide hydrochloride (DAPI)- added Mowiol. The slides were stored at 4 °C in the dark, after at least 12 hours of light-protected drying time at RT. At least 18 cells per condition, from 3 individual blood donors were quantified.

Immunofluorescent stained PMBCs were visualized using the Axiovert 200M fluorescence microscope from Zeiss with a 100 x oil immersion objective and recorded using Zeiss "ZEN Imaging" software. The exposure time of the cells in the corresponding colour channels was kept constant (exposure per channel: green "HIF-1α protein" - 3000 ms, red “background" - 3000 ms, blue "DAPI/cell nucleus" - 200 ms). For quantification (HIF-1α pixel count) and visualisation "ImageJ" of the National Institute of Health (NIH), USA was used.

2.2.8 Flow cytometry

To assess the immune cell composition, activation of immune cells as well as apoptosis and necrosis rates of collected human whole blood and specific cell samples flow cytometry with fluorescent analysis with special fluorescent labelled antibodies and dyes was used. All data were measured and analysed via the FACSCanto™ II flow cytometer (BD Biosciences) and displayed via the GraphPad prism software (table 11).

2.2.8.1 Phenotype staining of human blood cells

To determine the different cell types within the obtained whole blood, PBMCs, and isolated immune cell samples, an antibody phenotype staining was performed using the FACSCanto™ II flow cytometer. For determination of the immune cell populations within the samples, 50 µl of whole blood samples or 0.5 x 10^6 of suspension cells were transferred to 15 ml tubes and incubated for 20 min in the dark with 50 µl of an antibody mixture of fluorescent-labelled antibodies targeting human CD45 for detection of leukocytes, CD3 for lymphocytes, CD14 for monocytes, CD4 for helper T cells, CD8 for cytotoxic T cells, and CD19 for B cells diluted in Brilliant Stain Buffer (table 9). After incubation, the cells were centrifuged for 5 min at 400 x g at 4 °C and afterward resuspended in 2 ml “flow cytometry” buffer (table 5). This was followed by an additional washing step under the conditions already described. After removing the supernatant, the stained cells were diluted in 300 µl buffer and measured within 1 h. For short-term storage samples were kept at 4 °C in the dark. 50.000 events per condition were detected. The gating strategy is shown in the supplements (figure 61 – 63).

2.2.8.2 Macrophage discrimination and activation in human blood samples

For investigation of the subgroups and activation of circulating monocytes in whole blood of study participants upon *in vivo* exposure to an LPS injection and/or hypoxia, we performed a specific antibody staining targeting the activation markers CD86, HLA-DR (MHC II), and the Toll-like receptor 4 (TLR4) within immune cells. Therefore 50 µl of whole blood were incubated with 50 µl of antibody mixture, containing Brilliant Stain Buffer and antibodies against CD45 for determination of leukocytes, CD14 and CD16 for determination of monocytes, and CD86, HLA-DR, and TLR4 as activation markers for the immune cells of interest in the dark at RT for 20 min (table 9). After incubation, the cells were washed twice by centrifugation (5 min, 400 x g, 4 °C) with 2 ml flow cytometry buffer (table 5). Finally, the stained cells were diluted in 300 µl FACS buffer and measured within 1 h. For short-term storage samples were kept at 4 °C in the dark. The gating strategy included discrimination of monocytes by CD45 and CD16/CD14 antibody staining according to Marimuthu et. al. (Marimuthu et al., 2018). 50.000 events per condition were detected. The gating strategy is shown in the supplements (figure 60).

2.2.8.3 Determination of Apoptosis and Necrosis

The apoptosis rate of isolated PBMCs was measured using the PE Annexin V Apoptosis Detection Kit I by BD Biosciences according to the manufacturer's specifications. The cellular fluorescence was measured by detecting 30.000 events per condition at a FACSCanto™ II flow cytometer. The gating strategy was done according to the manufacturer's instructions.

2.2.9 Statistics

For the statistical analysis of the results, the GraphPad PRISM® software, version 9.0.0 (GraphPad Software, Inc.) was used. If not marked otherwise, all data sets were checked for statistical outliers using the ROUT test (Q = 1 %), and significant outliers were subsequently excluded from the statistical evaluation. For the results of cell culture experiments, the ordinary one-way ANOVA test with multiple comparisons was applied. For analysing gene expression data from study participants, the mixed-effects analysis with Tukey´s test multiple comparisons test or RM one-way ANOVA for repeated measurements was used, as participants received subsequent treatments. Statistical significance is displayed as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ or **** = $p < 0.0001$. Illustrated data bars represent mean values with error bars representing the standard derivation, as a measure of dispersion in all figures (mean ± SD). For human subject data, each data point is illustrated as a single circle or square within the bars.

3. Results

The work presented in this thesis is focused on studies regarding the Hypoxia-inducible factor pathway in human leukocytes. To investigate the impact of different conditions on the HIF pathway and immune reactions in leukocytes, we combined *in vitro* and *in vivo* studies, starting with research on an immortalized cell line (section 3.1), then on human primary leukocytes (sections 3.2 and 3.3), and finally, on human volunteers (sections 3.4 and 3.5) The workflow of the studies is presented in figure 6.

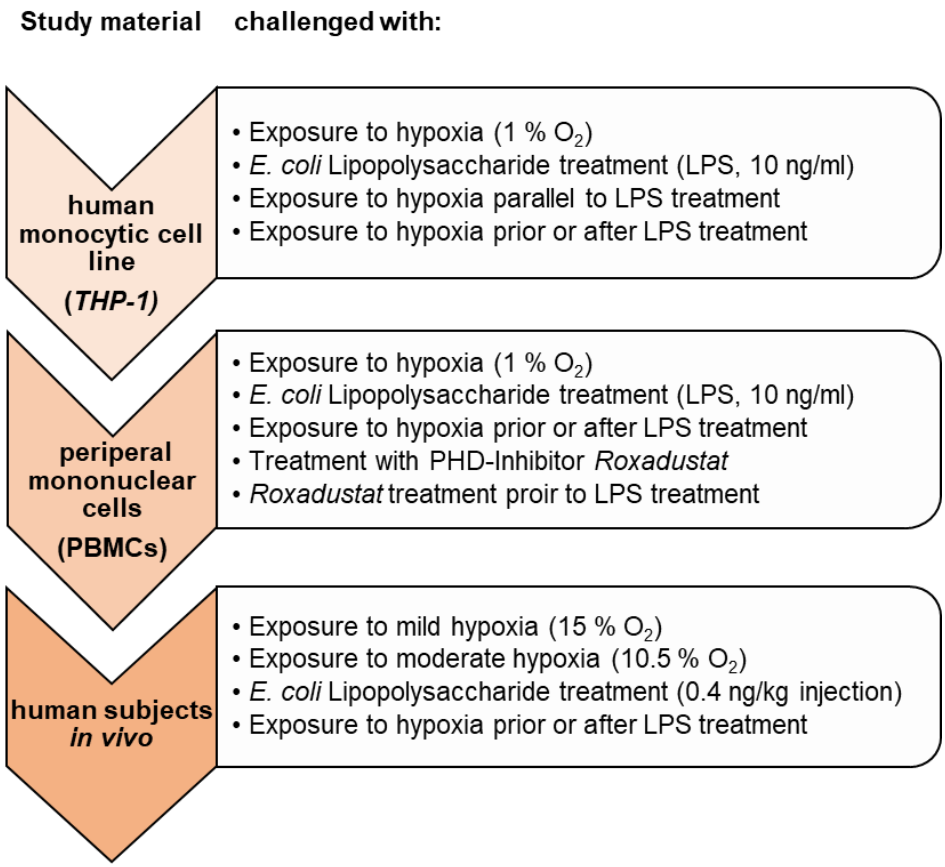


Figure 6: Schematic illustration of the study workflow

Research presented in this thesis started with investigations on the monocytic cell line *THP-1*, followed by *in vitro* studies on primary human peripheral mononuclear cells (PBMCs) and *in vivo* studies on healthy volunteers. Arrows indicate the timeline and structure of investigations, and respective treatment conditions are mentioned in the right boxes aside.

3.1 HIF pathway regulation in the human *THP-1* cells under hypoxia and inflammation

To study the interplay between inflammation and hypoxia-induced gene expression patterns regarding the HIF pathway in human immune cells, the human monocytic *THP-1* cell line was used as a cell culture model. For studying gene regulation of HIFs (*HIF1A*, *HIF2A*) and distinct HIF target genes (*GLUT-1*, *ADM*, *PDK1*, *PHD3*) and the inflammatory mediators IL-6 and TNF-α (*IL6*, *TNFA*), *THP-1* cells were stimulated with a low dose of 10 ng/ml *Escherichia coli* lipopolysaccharide (LPS) or exposed to 1 % O_2 normobaric hypoxia in different combinations. In general, cells were treated with the single stimulus or the last acute condition for 4 hours before lysis and RNA extraction (see section 2.2.5). Gene expression changes were analysed using the qRT-PCR method with all data normalised to the housekeeping gene β-actin (*ACTB*). Transcriptional changes are displayed as relative fold changes compared to the gene expression level of the untreated, normoxic control cells ($2^{-\Delta\Delta CT}$). For each condition 3 - 6 independent experiments, each with technical duplicates were performed. Statistical analysis was done by using the ordinary one-way ANOVA test with multiple comparisons between all groups.

3.1.1 HIF-dependent gene regulation under hypoxia and inflammation

First, we investigated the effects of oxygen shortage and inflammation and the combination of both on *THP-1* cells on gene expression level (see section 2.2.5). Therefore, cells were either challenged with 4 hours of hypoxia (1 % O_2, 5 % CO_2), 4 hours of inflammation (10 ng/ml *E. coli* LPS), or the combination of both (4 h of LPS treatment under acute hypoxia).

Low dose LPS treatment and 4 hours of hypoxia alone showed a slight tendency to upregulate *HIF1A* mRNA to the same amount (mean ± SD: 1.23 ± 0.46-fold and 1.38 ± 0.63-fold), while the combination of both significantly upregulates *HIF1A* in *THP-1* cells (2.18 ± 0.99-fold) (figure 7, A). Interestingly the same treatment had opposite effects on *HIF2A* mRNA levels. *HIF2A* decreased by more than half after 4 hours of combined treatment (0.36 ± 0.16-fold), but the LPS or hypoxic stimuli alone also led to a significant decrease compared to the normoxic control. Thereby LPS induced a stronger downregulation of *HIF2A* mRNA (0.52 ± 0.22-fold) than treatment with hypoxia (0.72 ± 0.23-fold) (figure 7, B). Although we could detect clear changes in gene expression of the transcription factors themselves the important HIF-1α target gene *GLUT-1* showed only minor changes in gene expression after LPS treatment. While LPS slightly decreased the expression (0.81 ± 0.17-fold), hypoxia tended to upregulate *GLUT-1* (1.21 ± 0.37-fold), while no changes were seen in the combined treatment (figure 7, C). *PDK1* displayed similar to *GLUT-1* a downregulation of mRNA after 4 hours of LPS treatment (0.73 ± 0.24-fold), while hypoxia alone or the combination of LPS and hypoxia did not result in significant mRNA changes (figure 7, D). Strikingly, the HIF target gene *ADM* showed a completely different picture: The acute LPS stimulus alone led to a 6.82-times (± 4.15) mRNA upregulation, and when combined with hypoxia to a 15.3-times (± 6.62) upregulation of *ADM* compared to the normoxic control. However, the hypoxic stimulus alone had a tendency for upregulation but without any statistical significance (figure 7, E). *THP-1* cells challenged with hypoxia displayed a 2.41-fold (± 1.68) mRNA expression increase of the HIF regulator and target gene *PHD3* compared to untreated cells. In comparison LPS treatment did not influence *PHD3* gene expression (1.06 ± 0.42-fold) and the combined treatment did not reach a significant increase (1.99 ± 1.10-fold) (figure 7, F). Having a look at the gene expression of important inflammatory mediators it becomes clear that hypoxia alone did not affect either *IL6* or *TNFA* in *THP-1* cells. As expected already a low dose of LPS triggered a highly significant increase in both genes (*IL6*: 62.4 ± 59.2-fold, *TNFA*: 3.44 ± 1.36-fold). In contrast, the combined treatment showed only a tendency for gene upregulation but without any significant changes (*IL6*: 24.3 ± 20.23-fold, *TNFA*: 1.21 ± 1.05-fold) (figure 7, G, H).

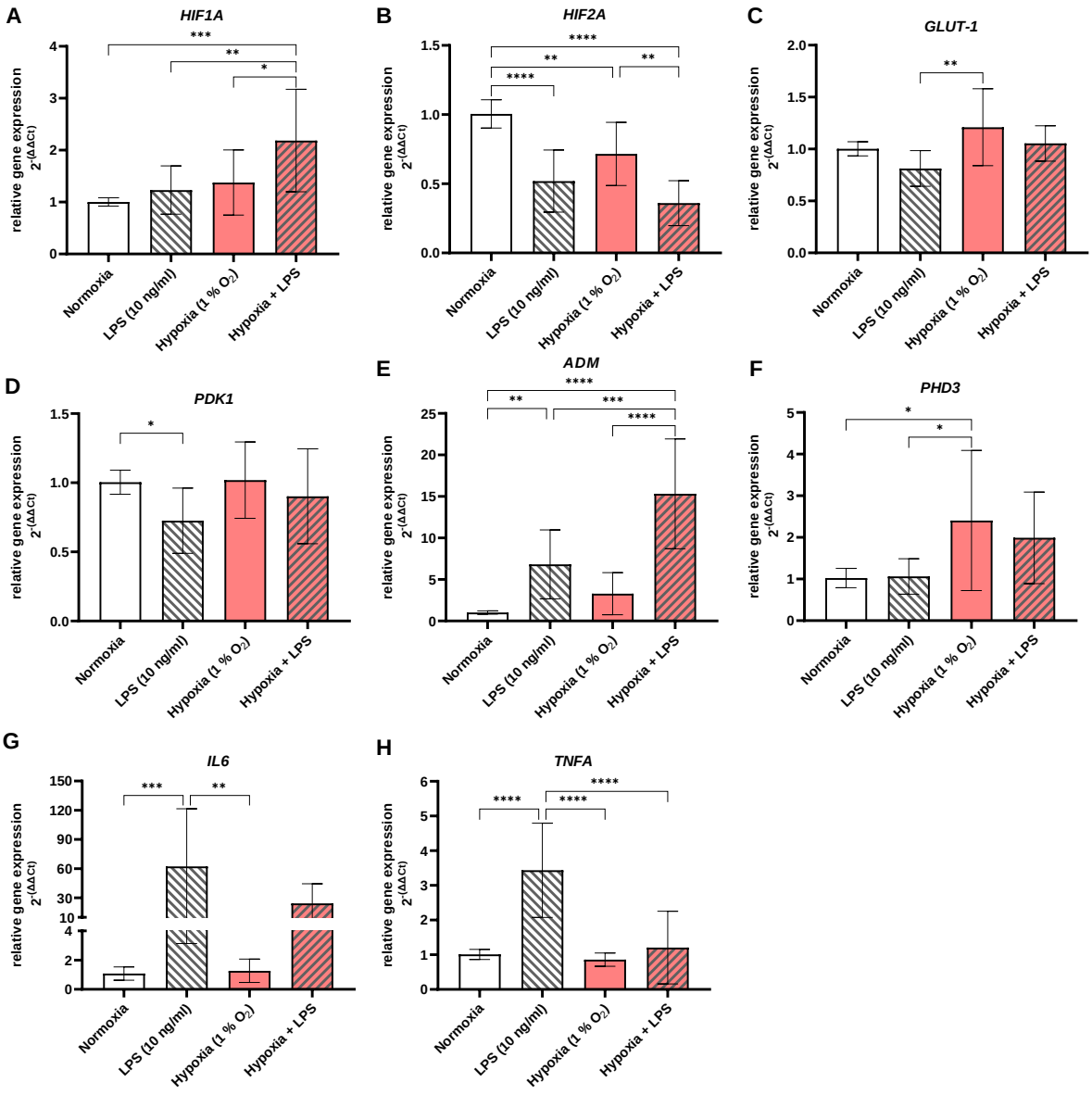


Figure 7: Gene expression of hypoxic and LPS stimulated *THP-1* cells

THP-1 cells were either not treated, treated with 10 ng/ml *E. coli* LPS for 4 hours, exposed to 1 % O2 hypoxia for 4 hours, or treated with LPS under hypoxic conditions for 4 hours. Displayed are n-fold expression changes of genes normalised to *ACTB* (mean ± SD, ordinary one-way ANOVA test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n= 6 - 12).

3.1.2 Effects of hypoxic priming on acute inflammation

Second, to further address the question if hypoxia does shape the acute inflammatory LPS response, *THP-1* cells were kept under hypoxic conditions for 4 or 24 hours before LPS treatment in normoxic oxygen conditions of 21% O_2 (figure 8). The gene expression analysis showed that 4 h of hypoxic priming further increased *HIF1A* compared to the LPS stimulus alone (1.76 ± 0.70-fold compared to 1.23 ± 0.46-fold) but 24 h of hypoxic preconditioning had no effect compared to the untreated and LPS only control (0.99 ± 0.29-fold) (figure 8, A). While LPS treatment led to a slight increase of *HIF1A* mRNA, *HIF2A* was shown to be decreased (0.52 ± 0.22-fold) (figure 8, B). Interestingly, preconditioning under hypoxia for 4 hours before the LPS stimulation diminished that effect, and *HIF2A* levels changed significantly compared to the LPS control (0.92-fold ± 0.35 compared to normoxic control). Fitting to the first analysis (figure 8), there were no effects on the *GLUT-1* gene upon LPS treatment after the short hypoxic stimulus, but a 24 h hypoxic phase before the LPS treatment induced a decrease of *GLUT-1* mRNA to half the levels of the other groups (0.46-fold ± 0.13) (figure 8, C). In terms of the target gene *PDK1,* 4 h of hypoxic priming before the LPS treatment did not induce any changes in gene expression (0.87-fold ± 0.24) while 24 h of hypoxic incubation induced a significant increase (1.34-fold ± 0.34) compared to the LPS treated group alone (figure 8, D). The analysis of the HIF target gene *ADM* revealed that the short and long-term hypoxic preincubation dampened the pure LPS-induced *ADM* mRNA increase from 6.82-fold (± 4.15) to 1.73 (± 1.35) and 4.47-fold (± 1.11) respectively (figure 8, E). The analysed treatments affected *PHD3* expression only slightly (LPS: 1.06 ± 0.42-fold, 4 h hypoxia prior LPS: 0.80 ± 0.35-fold, 24 h hypoxia prior LPS: 1.33 ± 0.99-fold) (figure 8, F). *THP-1* cells display a great increase in gene expression levels of *IL6* and *TNFA* after LPS treatment compared to the untreated control (*IL6*: 62.4 ± 59.2-fold, *TNFA*: 3.44 ± 1.36-fold). 4 and 24 hours of hypoxic priming before the LPS treatment dampened the inflammatory response in terms of *IL6* expression (33.0-fold ± 25.6 and 44.0-fold ± 20.9), while 24h of hypoxia had the opposite effect on *TNFA* (figure 8, G, H). Hereby 24 h of hypoxic preincubation led to a 5.04-fold (± 3.18) increase compared to the 3.44-fold (± 1.36) increase in the LPS control alone. In conclusion, in *THP-1* cells short-term (4 h) hypoxic priming before an acute inflammatory stimulus significantly increased *HIF2A* mRNA levels and decreased HIF-2α target gene *ADM* mRNA levels (figure, B, E). However, long-term hypoxic priming over 24 hours led to a significant downregulation of *GLUT-1* expression and significant upregulation of *PDK1* expression compared to LPS treatment alone (figure 8, C, D).

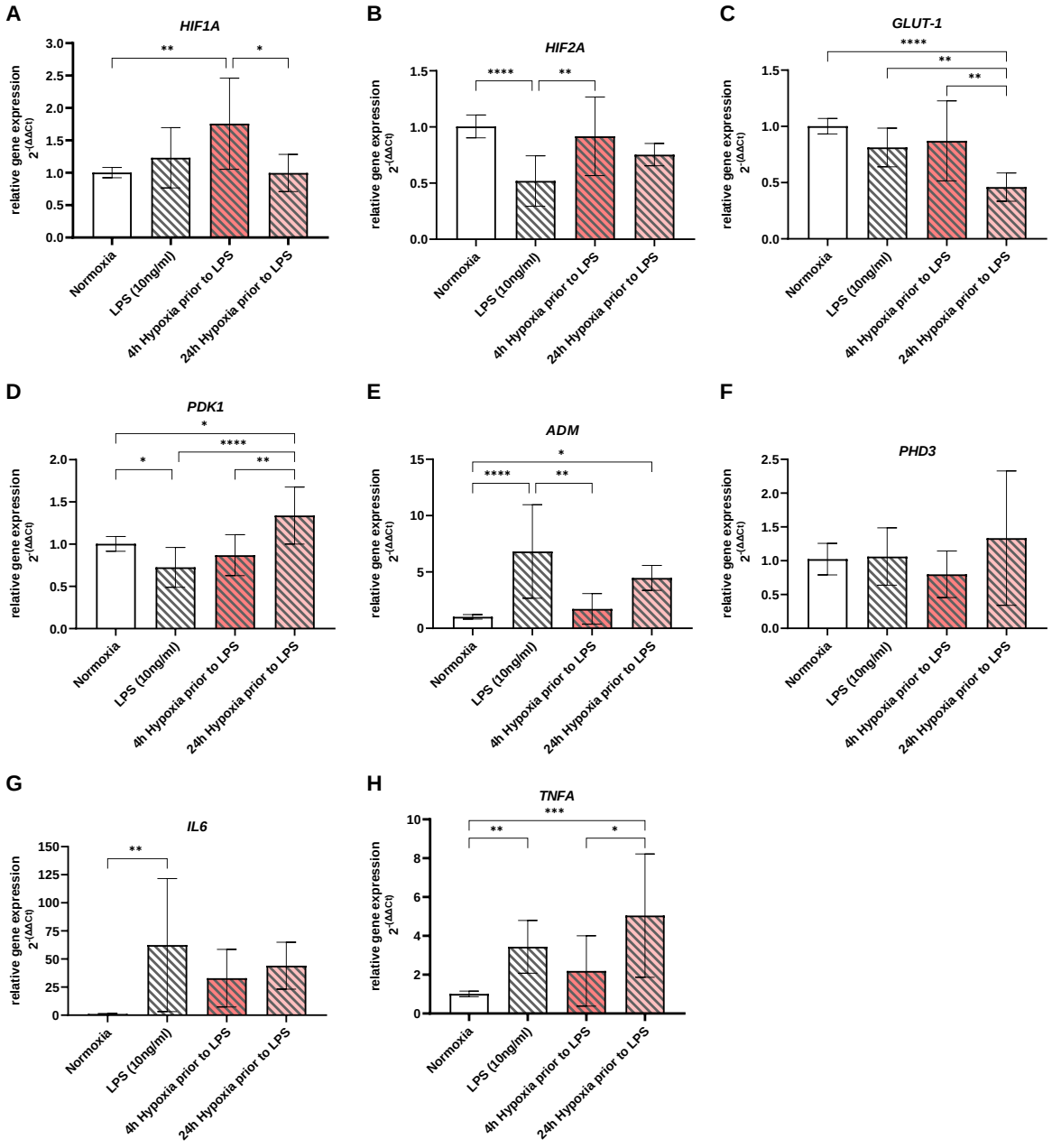


Figure 8: Gene expression of hypoxic primed and stimulated *THP-1* cells

Analysis of relative gene expression levels of *THP-1* cells that were primed for 4 or 24 hours in 1 % oxygen hypoxia before an acute *E. coli* LPS stimulus (10 ng/ml). As control served cells, which were either not treated or treated with 10 ng/ml LPS for 4 hours. Presented are n-fold expression changes of genes normalised to *ACTB* (mean ± SD, ordinary one-way ANOVA test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n= 6 - 12).

3.1.3 Effects of underlying inflammation on the acute hypoxic response

In a third experimental approach, we addressed the question how *THP-1* cells adjust their HIF-related gene expression to hypoxia when already challenged with underlying inflammation. To Investigate gene regulatory effects of occurring oxygen shortage upon inflammation cells were stimulated with low dose lipopolysaccharide (LPS, 10 ng/ml) for 4 hours in normoxia and afterward either kept in normoxia as control or in hypoxic conditions (1% O_2) for additional 4 hours before lysis and RNA isolation. While *HIF1A* displayed a 1.66-fold (± 0.65) increase upon LPS treatment under normoxia, this effect was diminished under hypoxic conditions (1.43 ± 0.55-fold) (figure 9, A). As already seen in the first experiment *HIF2A* levels decreased upon LPS but did - similar to *HIF1A* levels - not differ when the LPS stimulus was followed by a normoxic or hypoxic phase (0.75 ± 0.29-fold vs. 0.76 ± 0.22-fold) (figure 9, B). Interestingly, all tested HIF target genes acted in the same manner: Relative expression of the genes was significantly upregulated when inflamed cells were further challenged with hypoxia (*GLUT-1*: 1.83 ± 0.84-fold, *PKD1*: 1.70 ± 0.57-fold, *ADM*: 30.22 ± 23.0-fold, *PHD3*: 3.79 ± 3.46-fold) but apart from *PDK1* (0.45 ± 0.19-fold) no changes were found when cells were incubated under normoxic conditions (figure 9, C - F). The two analysed inflammatory mediators displayed the same regulatory pattern, with the hypoxia as an additional stimulus boosting their gene expression to some extent. However, while *IL6* mRNA was still significantly increased to 3.02-fold (± 1.42) under normoxia 8 hours after the LPS stimulus and 4.54-fold (± 1.68) under hypoxia, the *TNFA* gene expression levels were still slightly higher under hypoxia (1.68 ± 1.53-fold) but almost back to the levels of unstimulated control cells (figure 9, G, H). In summary, all tested HIF target genes, including those for glucose transporter-1, pyruvate dehydrogenase kinase 1, adrenomedullin, and prolyl-hydroxylase 3 were significantly upregulated in *THP-1* cells, which were exposed to hypoxia 4 hours after the LPS treatment in comparison to cells that were exposed to 21 % O_2 normoxia after the treatment.

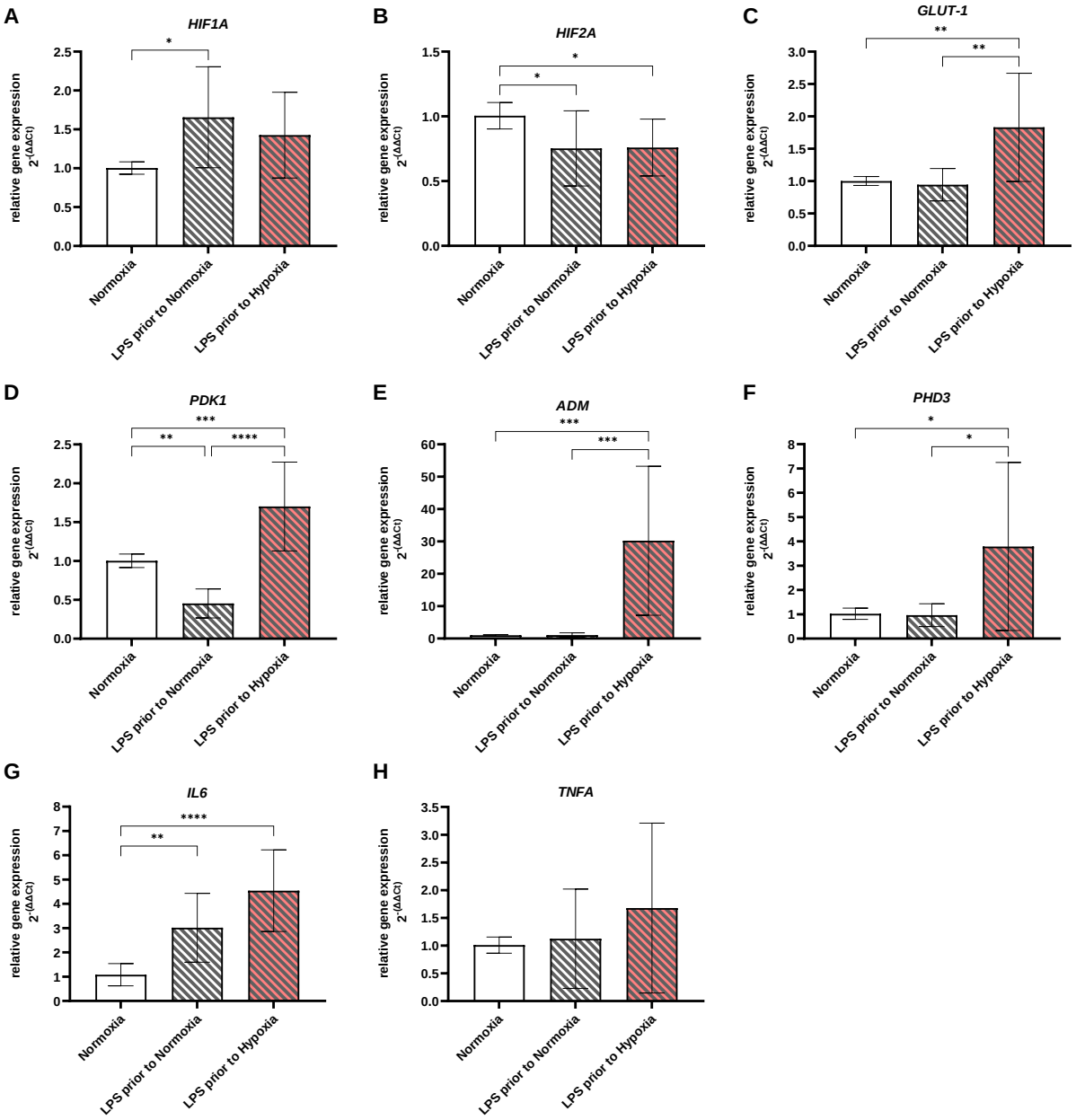


Figure 9: Gene expression of *THP-1* cells, challenged with LPS before hypoxia

Relative gene expression changes of *THP-1* cells that were primed for 4 hours with an *E. coli* LPS stimulus (10 ng/ml) before there were incubated in normoxic (21 % O_2) or hypoxic conditions (1 % O_2) for 4 additional hours. Control cells were untreated and cultured under normoxic conditions. Illustrated are n-fold expression changes of genes normalised to *ACTB* (mean ± SD, ordinary one-way ANOVA test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n= 6 - 12).

3.2 HIF regulation in primary human PBMCs under hypoxia and inflammation

Our experiments using the monocytic *THP-1* cell line revealed interesting insights into HIF-dependent gene regulation when inflammation and hypoxia act together. Although the human monocytes are widely used to study immune reactions, we wanted to study those interactions more in detail. To this aim we used primary human immune cells to mimic the *in vivo* situation for human immune cells upon inflammatory hypoxia and to exclude possible artefacts from the immortalized cell line.

To unveil the possible effects of hypoxia and inflammation on blood circulating immune cells we conducted our experiments with primary human peripheral blood mononuclear cells (PBMCs) isolated from buffy coats of blood donors (see section 2.2.4.2). For every experiment, isolated PBMCs were seeded in cell culture dishes at least in technical duplicates for each blood donor. PBMCs were cultured under the same media and oxygen conditions as *THP-1* cells overnight before usage (see section 2.2.4.3). For experiments, PBMCs were challenged with 10 ng/ml *E. coli* LPS or exposed to normobaric hypoxia (1 % O_2, 5 % CO_2) or a combination of both. At the end of the experiment, cells were lysed and either prepared for protein or gene expression analysis following the $2^{-(\Delta\Delta Ct)}$ method (see sections 2.2.5 and 2.2.6).

3.2.1 Composition of isolated human PBMCs

Before using human PBMCs as an *in vitro* model system for further experiments we first determined the cellular composition of the isolated cells using antibody phenotype staining and flow cytometry (see section 2.2.8.1). The gating strategy is shown in the supplements (figure 61 - 63). Flow cytometry analysis revealed that isolated PBMCs from buffy coats consisted of 83.5 % $CD45^+$ leukocytes and a leftover of 0.3 % granulocytes (figure 10). The low percentage of granulocytes is due to the isolation protocol. Subsequent classification of the $CD45^+$ leukocyte population into different subgroups showed that the cells consisted mainly of T cells (43.4 % $CD3^+CD4^+$ T helper cells and 34.2 % $CD3^+CD8^+$ cytotoxic T cells), $CD14^+$ monocytes (25.3 %) and a small portion (5.3 %) of $CD19^+$ B cells (figure 10).

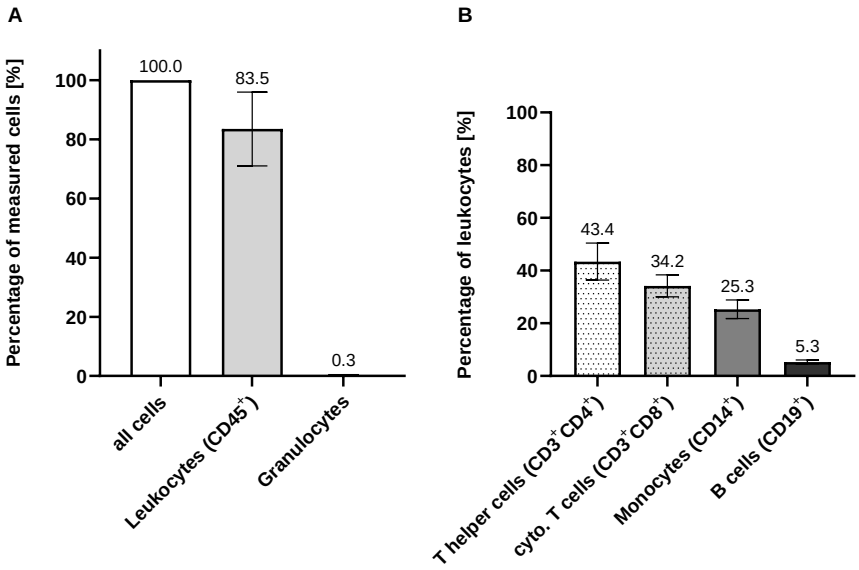


Figure 10: Cellular composition of isolated PBMCs

Percentages of cell types included in the isolated peripheral blood mononuclear cells (PBMCs). The isolated PBMCs were classified into cell types (A) and specific leukocyte subpopulations (B) using antibody-mediated detection of the specifically expressed surface antigens of cells and flow cytometry analysis. Data evaluation: Mean values ± SD, n = 3 with 30.000 detected events each.

3.2.2 Adaption of PBMCs to different durations of hypoxia

Blood circulating immune cells need to adapt to hypoxic conditions within the shortest times, as they migrate e.g., from the bloodstream into tissues, and are already under physiological conditions exposed to a high variety of oxygen concentrations (Chen & Gaber, 2021). To better understand the time course of hypoxia-inducible factor accumulation and hypoxia-induced gene regulation in human immune cells we exposed PBMCs of at least 3 different blood donors for different durations to 1 % O_2 hypoxia *in vitro*.

HIF-1α protein expression in PBMCs under hypoxia

The major regulatory mechanism behind the HIF pathway is via HIF protein stabilisation and accumulation in the cytosol of cells, which results in enhanced dimerisation of HIF subunits and finally target gene transcription. Therefore, we analysed HIF-1α protein accumulation in isolated human PBMCs of three different blood donors, which were challenged with increasing times of 1 % O_2 hypoxia *in vitro* for times from 30 minutes up to 24 hours. After incubation in the hypoxic chamber cells were lysed and prepared for protein analysis via Western Blotting (see section 2.2.6).

Visualisation of the protein bands revealed that HIF-1α protein was gradually increased when PBMCs were exposed for longer times to hypoxia *in vitro* (figure 11, A). Evaluation of Western blot protein bands unveiled significantly upregulated HIF-1α protein levels to 3.9 to 4.6-fold when cells were kept for 4, 6, and 24 hours in 1% O_2 hypoxia (figure 11, B). Thereby, HIF-1α protein bands were normalised to the housekeeping protein bands of β-actin, and significance was statistically calculated using the ordinary one-way ANOVA test on all protein levels compared to the normoxic control.

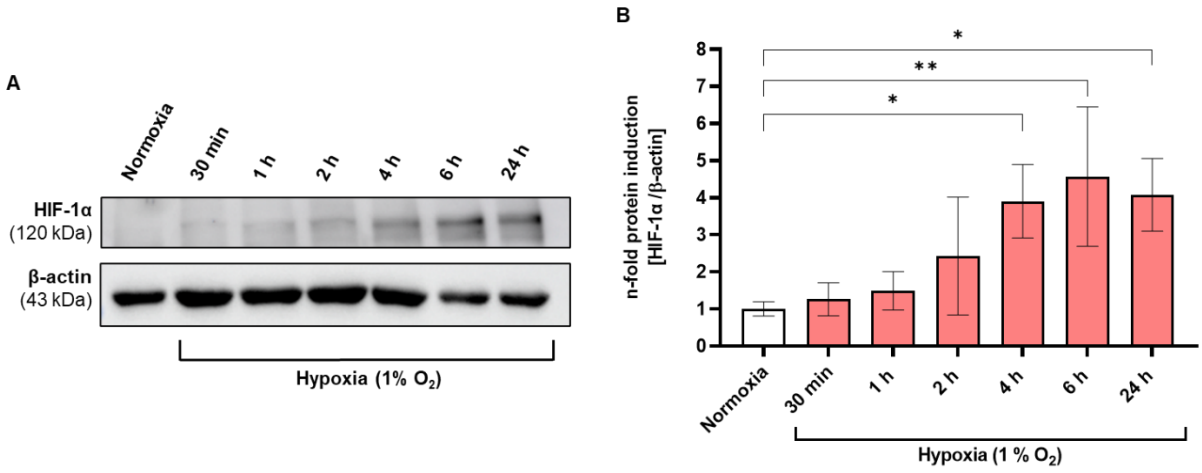


Figure 11: HIF-1α protein accumulation in PBMCs under hypoxia

Western blot analysis of PBMCs, which were exposed to increasing times of 1 % O_2 hypoxia *in vitro*. A: Representative image of HIF-1α and β-actin protein bands of PBMCs cultured from 30 min to 24 hours in hypoxia. B: Calculated n-fold HIF-1α protein induction, normalised to β-actin and compared to normoxic control cells (mean ± SD, ordinary one-way ANOVA, * = $p < 0.005$, ** = $p < 0.0005$, n = 3).

HIF-dependent gene regulation in PBMCs under hypoxia

To examine if the observed HIF-1α protein accumulation had downstream effects on gene expression patterns of the transcription factors HIF-1α and HIF-2α themselves and typical HIF target genes, RNA of PBMCs was isolated to perform qRT-PCR (see section 2.2.5). Relative transcription changes were investigated for *HIF1A*, *HIF2A,* and genes encoding for important mediators of the energy metabolism (*GLUT-1*, *PDK1*) as well as differentiation and vasodilation (*ADM*) and the HIF regulating hydroxylase PHD3 (figure 12).

Analysis of the hypoxia time course in PBMCs revealed that *HIF1A* mRNA levels were significantly decreased to 0.52 ± 0.2-fold after 4 hours of hypoxic incubation and decreased further upon longer incubation times of 6 h (0.30 ± 0.2-fold) and 24 h (0.37 ± 0.2-fold) (figure 12, A). *HIF2A* gene expression did not change over the time of hypoxic exposure from 30 minutes to 24 hours (figure 12, B). All tested HIF target genes displayed the same hypoxia-duration-depended pattern: they began to show the first upregulation of mRNA levels beginning 2 hours after hypoxic incubation. After 4 hours of hypoxia *GLUT-1* and *ADM* were significantly upregulated to 3.2 ± 2.8-fold, and 14.1 ± 10.3-fold), respectively (figure 12, C, E). *ADM* mRNA levels did not further increase upon a prolonged duration of hypoxia to 24 hours (15.7 ± 13.2-fold) and presented with high values of standard deviation. In contrast to that, *GLUT-1* mRNA levels increased further after 6 h (4.0 ± 3.1-fold) and 24 h (6.7 ± 4.9-fold) of 1 % O_2 hypoxia (figure 12, C). Hypoxia-induced upregulation of *PDK1* mRNA stayed similar after 2 and 4 hours at around 2.1 ± 1.2-fold on average. Statistically significant changes were detected after 6 hours of hypoxia with a 3.9-fold (± 3.0) increase and at 24 hours with a 4.2 ± 2.0-fold increase compared to the normoxic control (figure 12, D). *PHD3* gene expression was also substantially upregulated already after 2 h and 4 h to 4.4 ± 5.1-fold and 9.5 ± 6.6-fold but displayed a high standard deviation (figure 12, F). 6 hours of hypoxia increased mRNA levels of *PHD3* up to 104.3 ± 101.9-fold and 24 h of hypoxia to 574.5 ± 569.6-fold. Again, the values of individual blood donors displayed high variations (figure 12, F). Gene expression of all groups was normalised to *ACTB* mRNA expression and statistical significance was calculated using the ordinary one-way ANOVA test with multiple comparisons with all groups being compared to the normoxic control. In total PBMCs of 4 different blood donors were analysed at least in technical duplicates.

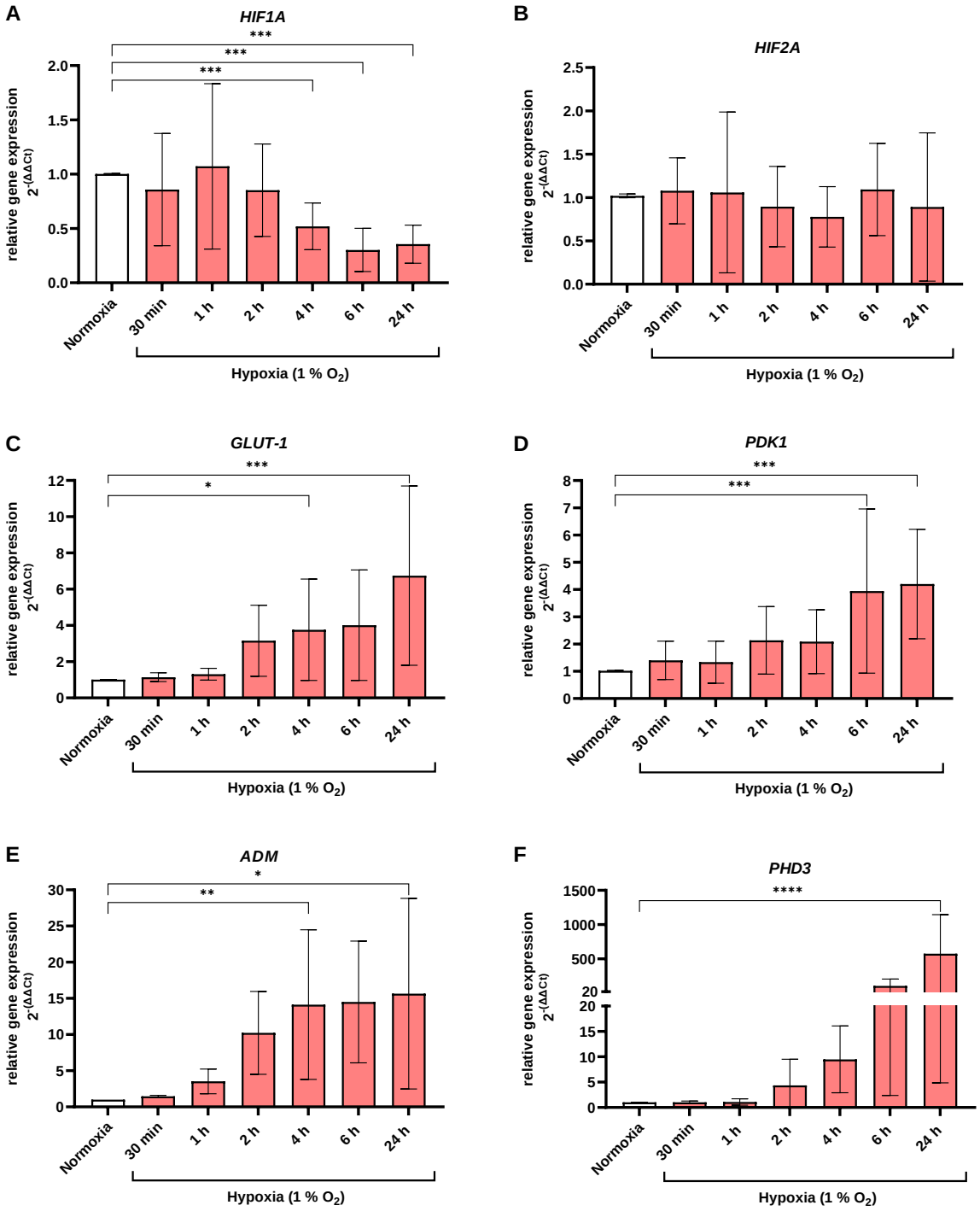


Figure 12: Hypoxia-induced gene expression changes in PBMCs

Relative gene expression of PBMC, which were exposed to different times to 1 % O_2 hypoxia *in vitro*. Times in hypoxia of a duration of 30 min,1 h, 2 h, 4 h, 6 h and 24 h were statistically compared to PBMCs under normoxic conditions (21 % O_2). Significant increase of mRNA upon exposure to hypoxia was found in *HIF1A* (A), *GLUT-1* (C), *PDK1* (D), *ADM* (E) and *PHD3* (F). Bars represent mean $2^{-(\Delta\Delta CT)}$ values with standard deviation (mean ± SD). Data was normalised to *ACTB* expression and to the normoxic control (Ordinary one-way ANOVA test with multiple comparisons, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 3).

3.2.3 Adaption of PBMCs to increasing LPS-induced inflammation

In addition to a fast adaptation to different oxygen concentrations immune cells are specialised to detect and react to already very small amounts of pathogens. In this work lipopolysaccharides (LPS) from the outer bacterial cell wall of *Escherichia coli* were used to stimulate and activate human PBMCs *in vitro*. We classified the effects of different LPS concentrations from 5 ng/ml to 1000 ng/ml after 4 hours of *in vitro* stimulation on HIF-1α protein and HIF-related gene expression levels.

HIF-1α protein expression in PBMCs under inflammatory conditions

In a second approach, we were interested, whether the administration of different doses of lipopolysaccharide would influence HIF-1α protein levels in PBMCs *in vitro* similar to exposure to hypoxia. To test the effect of LPS on PBMCs *in vitro* we treated cells with different *E. coli* LPS concentrations from 5 ng/ml to 1000 ng/ml for 4 hours under normoxic conditions with 21 % oxygen. Western blot analysis showed stabilisation of HIF-1α protein upon LPS treatment in all tested concentrations (figure 13). Although evaluation of the intensity of protein bands revealed a 3.4 to 4.7-fold increase of HIF-1α protein compared to untreated PBMCs, results did not reach significance, probably due to high fluctuations between the samples (figure 13, B). Western blots prepared out of PBMCs from 3 different blood donors were analysed. No statistical significance was found when protein intensities were calculated using the ordinary one-way ANOVA test on all normalised HIF-1α protein levels compared to the untreated control levels.

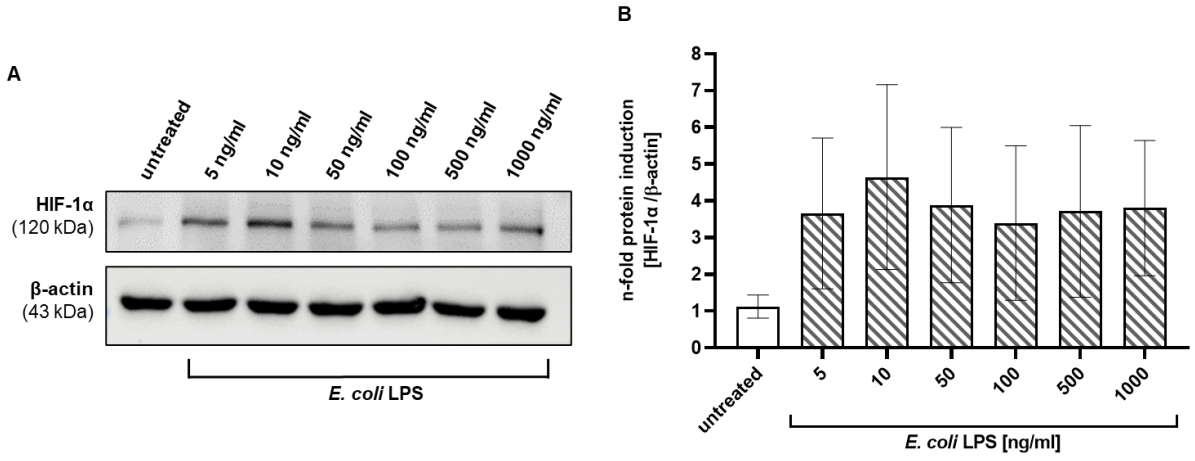


Figure 13: HIF-1α protein accumulation of PBMCs treated with LPS

Western blot analysis of HIF-1α in PBMCs showed a protein increase when cells were treated with increasing concentrations of *E. coli* lipopolysaccharide (LPS) for 4 hours *in vitro*. A: Representative image of HIF-1α and β-actin protein bands of PBMCs treated with 5, 10, 50, 100, 500, or 1000 ng/ml LPS. B: Calculated n-fold HIF-1α protein induction, normalised to β-actin and compared to untreated control cells (mean ± SD, ordinary one-way ANOVA, n = 3).

HIF-dependent gene regulation under LPS treatment

Even though HIF-1α protein expression was not significantly changed there seemed to be a strong tendency of upregulation HIF-1α (figure 13). To further analyse inflammation-dependent effects on the HIF pathway gene expression changes of HIFs and specific target genes were examined by qRT-PCR. Besides the already mentioned target genes *GLUT-1*, *PDK1*, *ADM,* and *PHD3*, we further analysed the mRNA expression of the two important inflammatory mediators' interleukin-6 and tumour necrosis factor-alpha in PBMCs challenged with LPS concentrations from 5 ng/ml to 1000 ng/ml.

HIF1A gene expression was upregulated upon all 6 tested LPS doses. 5 ng/ml induced a 3.3 ± 0.8-fold increase in *HIF1A* mRNA that reached its maximum at a dose of 100 ng/ml LPS inducing an 8.3 ± 6.1-fold upregulation (figure 14, A). *HIF2A* expression was regulated in the opposite way to *HIF1A* when PBMCs were challenged with LPS (figure 14, B). Nearly all tested LPS concentrations, besides the highest concentration, led to a similar downregulation of *HIF2A* mRNA around 0.40 to 0.57-fold (± 0.16 – 0.42). The use of 1000 ng/ml LPS did not alter *HIF2A* gene expression (1.02 ± 0.5-fold), which could also be an artefact due to the high standard deviation within the samples (figure 14, B). The expression of the HIF target genes *GLUT-1* and *PHD3* hardly changed, when challenged with LPS. *GLUT-1* mRNA levels ranked from 0.81 ± 0.58-fold to 1.1 ± 0.70-fold, while *PHD3* mRNA levels varied from 0.71 ± 0.58-fold to 1.19 ± 0.68-fold change compared to the untreated control (figure 14, C, F). In contrast *ADM* gene expression was significantly upregulated already at a LPS dose of 5 ng/ml to 11.8 ± 6.1-fold, which decreased slightly again to 9.0-10.7-fold under the LPS concentrations of 50 - 500 ng/ml and peaked at 1000 ng/ml LPS with a 13.3 ± 2.4-fold increase compared to control conditions (figure 14, E). *PDK1* mRNA levels followed a similar dose-dependent increase starting with a 2.1 ± 1.0-fold upregulation at a treatment of 5 ng/ml LPS. With exception of the 100 ng/ml LPS concentration, *PDK1* mRNA increased further up to 4.2 ± 2.7-fold when PBMCs were challenged with 1000 ng/ml LPS (figure 14, D). Already the lowest LPS dose led to an upregulation of *IL6* mRNA to 3761 ± 3474-fold which further increased in a dose-dependent manner up to 15504 ± 19973-fold. Although the standard deviation between the PBMCs was high, the lowest measured *IL6* mRNA increase of one individual donor already was 237.8-fold, which points to the extensive upregulation following inflammation in general (figure 14, G). The second inflammatory cytokine TNF-α showed a clearer dose-dependency. Similar to *IL6*, *TNFA* mRNA was increased already after an LPS dose of 5 ng/ml to 32.8 ± 19.5-fold which further increased with higher concentrations. A concentration of 500 ng/ml LPS induced an of 44.9 ± 28.5-fold, the highest dose of 1000 ng/ml an upregulation of 71.2 ± 16.7-fold *TNFA* mRNA compared to the untreated control PBMCs (figure 14, H). The statistical significance of all gene expression changes was calculated using the ordinary One-way ANOVA analysis with multiple comparisons, with all groups being compared to the untreated control mRNA levels. In total

the experiment revealed that already small amounts of LPS are sufficient to massively upregulate gene expression of inflammatory cytokines like IL-6 and TNF-α in human PBMCs, with a dose-dependent increase in gene activation. Further *HIF1A* and *HIF2A* were shown to be inversely regulated during the LPS treatment. While *PDK1* and especially *ADM* mRNA were upregulated, no clear effects were found for *GLUT-1* and *PHD3* gene expression when PBMCs were challenged with LPS (figure 14).

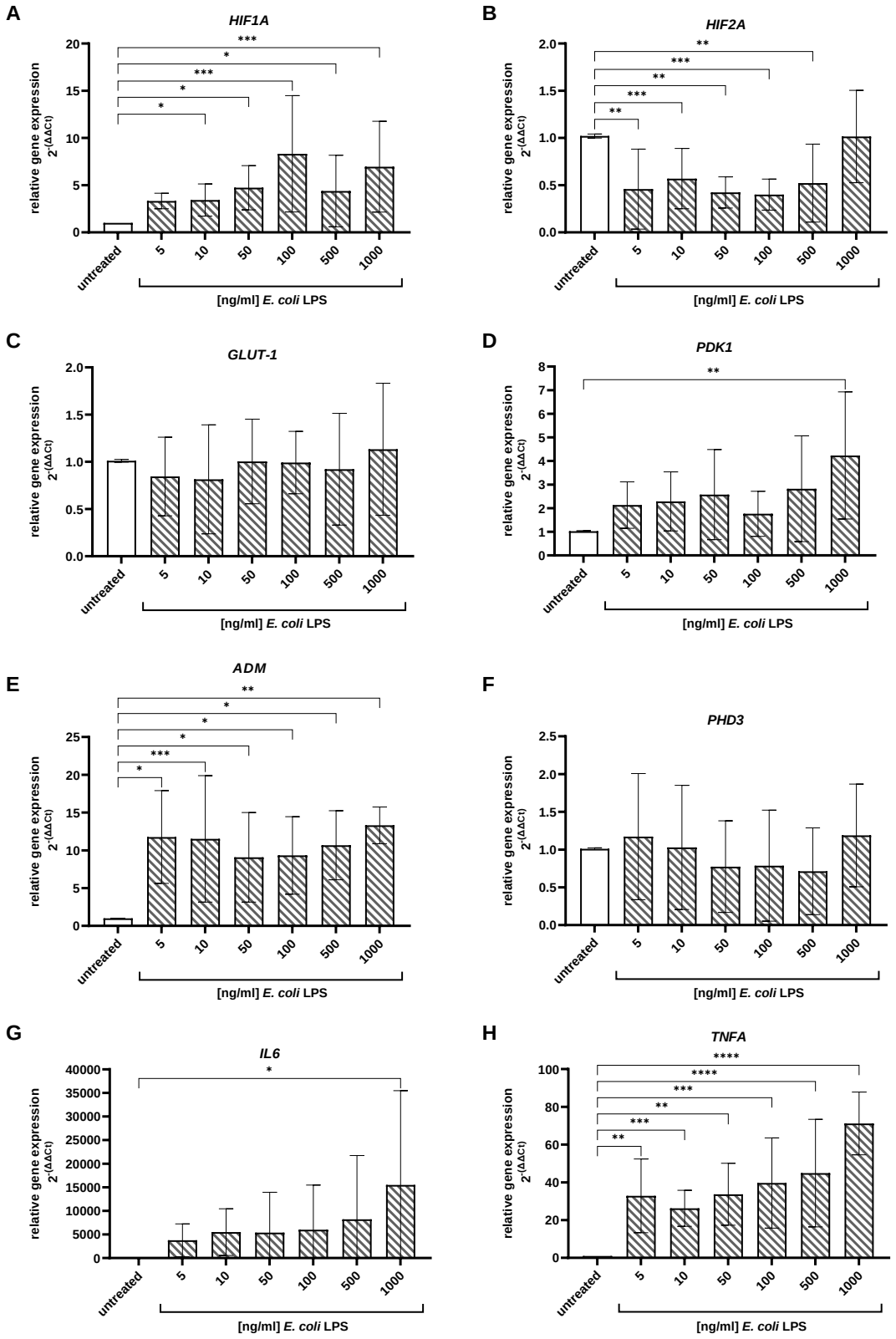


Figure 14: LPS-induced gene expression changes in PBMCs

Relative gene expression of PBMC, which were exposed to different concentrations of *E. coli* LPS for 4 hours *in vitro* showed significant upregulation of *HIF1A* (A), *HIF2A* (B), *PDK1* (D), *ADM* (E), *IL6* (G), and *TNFA* (H) for different LPS concentrations from 5 - 1000 ng/ml *E. coli* LPS. Bars represent mean $2^{-(\Delta\Delta CT)}$ values with standard deviation (mean ± SD). Data was normalised to *ACTB* expression and analysed using the ordinary one-way ANOVA test with multiple comparisons (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 3).

3.2.4 Effects of hypoxic vs. inflammatory priming in PBMCs

Our data showed that the adaption of protein and gene expression levels of human immune cells was shaped by different oxygen tensions as well as different LPS concentrations regarding the HIF pathway (see sections 3.2.2, 3.2.3). Next, we aimed to gain a greater insight into the HIF-dependent adaptational changes of PBMCs when hypoxic and inflammatory conditions are combined. Therefore, we first focused on the direct comparison between an acute low-dose LPS stimulus (10 ng/ml) and 4 hours of hypoxia (normobaric, 1 % O_2, 5 % CO_2) in PBMCs. Secondly, possible effects of 4 hours of hypoxic priming prior to the acute inflammation were investigated. Last, we focused on the effects of inflammatory priming on acute hypoxia in PBMCs, meaning we applied an LPS stimulus (10 ng/ml) prior to 4 h hypoxia on PBMCs *in vitro*.

HIF-1α protein expression under combined hypoxic and inflammatory conditions in PBMCs

To approach possible priming effects in PBMCs we primarily investigated HIF-1α protein accumulation in the different conditions of treatment via Western blotting. For this purpose, PBMCs were treated either untreated, treated for 4 hours with 10 ng/ml *E. coli* LPS, for 4 hours in 1 % O_2 hypoxia, or a combination of LPS treatment 4 hours before exposure to hypoxia (4 h) or vice versa (figure 15). All cells were lysed and prepared for protein extraction 4 hours after the last administered stimulus (see section 2.2.6). The strongest stabilisation of HIF-1α protein in Western blotting was in PBMCs, which were treated with 10 ng/ml LPS 4 hours before the 4-hours hypoxic phase (figure 15). Quantification of the protein bands showed that this 2.6 ± 0.2-fold protein increase was statistically significant when compared to HIF-1α levels of normoxic cells (0.97 ± 0.2), cells treated with LPS but incubated under 4 hours of normoxia afterward, and the reversed treatment order (figure 15, B). While hypoxic incubated PBMCs demonstrated HIF-1α protein upregulation to 1.6 ± 0.3-fold, 4 hours of LPS treatment before 4 hours of normoxia and 4 hours of hypoxic priming prior to LPS (10 ng/ml, 4 h) displayed no differences compared to the normoxic control cells (figure 15, B). Statistical significance of the protein band intensities of 3 different blood donors was calculated using the ordinary one-way ANOVA test with multiple comparisons between all groups.

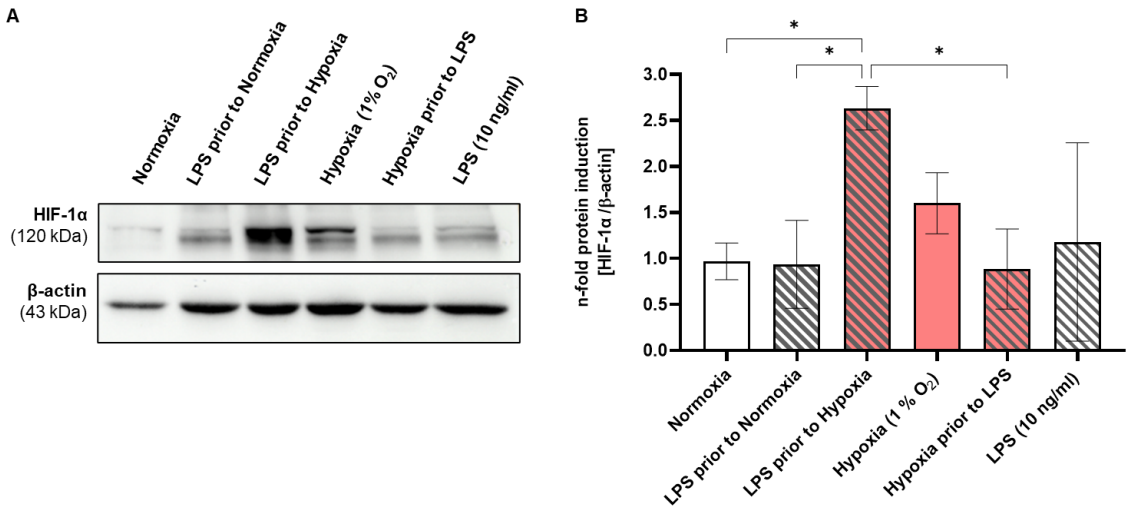


Figure 15: HIF-1α protein accumulation under inflammatory hypoxia in PBMCs

Western blot analysis of PBMCs, which were treated with either *E. coli* LPS (10 ng/ml, 4 h), hypoxia (1 % O_2, 4 h), or a sequence of both. HIF-1α protein accumulation was found to be the highest when PBMCs were treated with LPS and following hypoxia. A: Representative image of HIF-1α and β-actin protein bands of PBMCs. B: Calculated n-fold HIF-1α protein induction, normalised to β-actin and compared to untreated control cells (mean ± SD, ordinary one-way ANOVA with multiple comparisons, * = $p < 0.05$, n = 3).

HIF-dependent gene regulation in PBMCs under inflammatory vs hypoxic conditions

In the following experiments, we treated PBMCs with either a single *E. coli* LPS dose of 10 ng/ml for 4 hours or with 4 hours of 1 % O_2 hypoxia to create a direct comparison (figure 16). Gene expression analysis revealed that in terms of *HIF1A* and *HIF2A* PBMCs showed a similar regulation to *THP-1* cells (see section 3.1.1, figure 7): While LPS increased *HIF1A* mRNA levels 4.3-fold, hypoxia reduced the levels to about 40 % (mean: 0.58) compared to the untreated control cells (figure 16, A). *HIF2A* mRNA showed a 30 % decrease upon hypoxia, which was even more significant when cells were treated with LPS (48 %) (figure 16, B). The HIF target genes *GLUT-1* and *PHD3* were regulated contrary to *HIF1A*. LPS treatment decreased *GLUT-1* to 0.46 and *PHD3* to 0.70 compared to the gene expression levels of the untreated control cells. Under hypoxia *GLUT-1* mRNA levels increased 3.5-fold and *PHD3* levels 9.5-fold (figure 16, C, F). The genes *PDK1* and *ADM* were also significantly increased under hypoxic conditions to 2.1-fold and 12.5-fold respectively. Thereby, *ADM* mRNA levels displayed high fluctuations, spanning from 2.5-fold up to 32.7-fold mRNA increase under hypoxic and inflammatory conditions, which probably origins from the different blood donors (figure 16, E). *PDK1* and *ADM* both were upregulated upon 4 hours of LPS treatment with (*PDK1* 1.7-fold, *ADM*: 12.3-fold) (figure 16, D, E). The PBMCs were further analysed in terms of immunologically relevant cytokines, namely IL-6 and TNF-α. As expected, gene expression levels of both did not change when cells were challenged with hypoxia only. However, under inflammatory conditions *IL6* mRNA levels were 5652.8-fold increased and *TNFA* levels were

significantly upregulated up to 26.6-fold on average (figure 16, G, H). It should be noticed that the examined PBMCs display partially a very high fluctuation in measured gene expression data, with *IL6* mRNA levels spreading from a 14.6 to 15734.8-fold increase in the LPS treated group (figure 16, G). Gene expression levels of PBMCs out of 11 blood donors were normalised to housekeeping gene *ACTB* and the normoxic control. Statistical significance was tested using the ordinary one-way ANOVA analysis with a comparison of multiple groups.

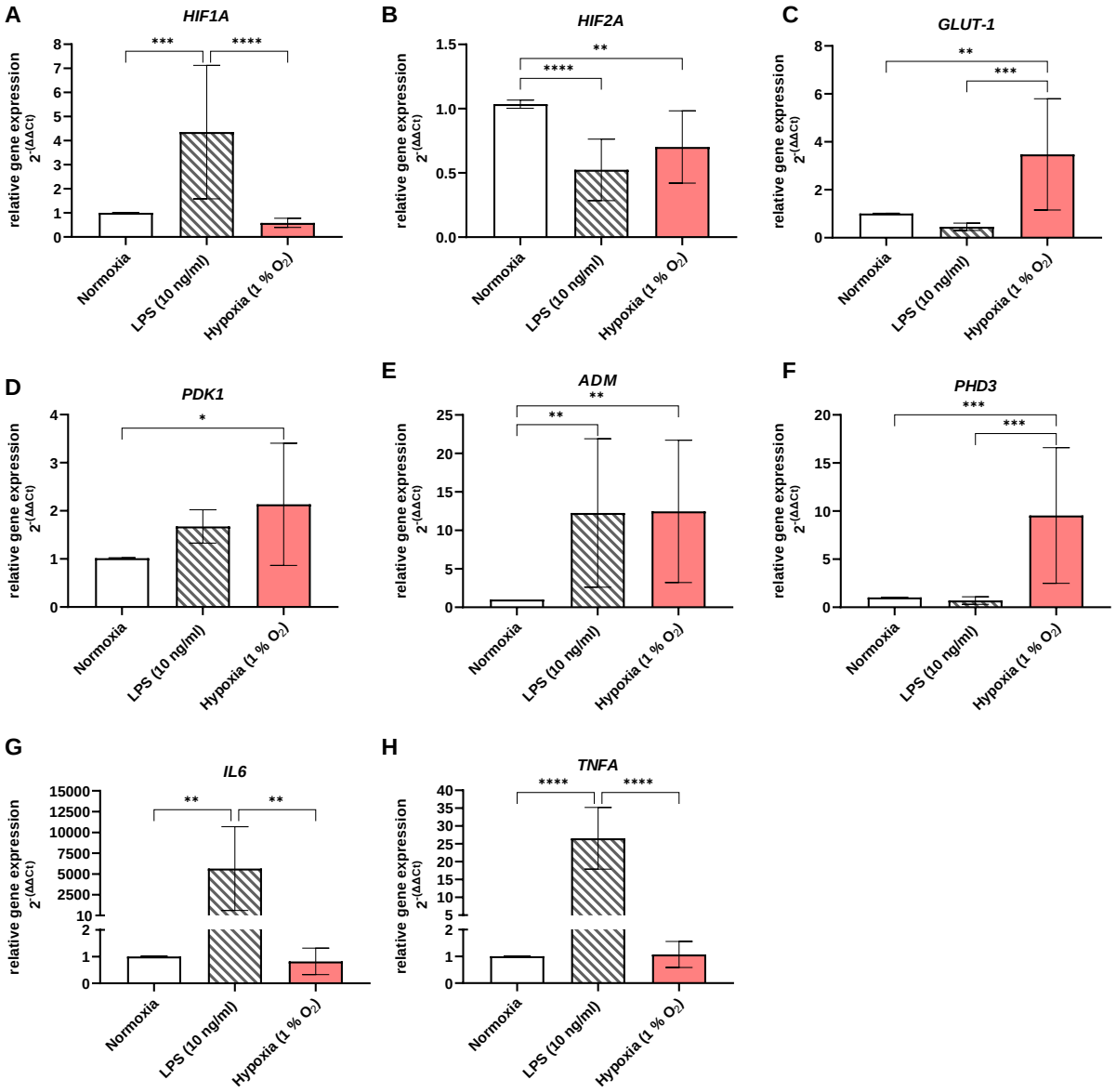


Figure 16: Hypoxia and LPS-induced gene expression changes in PBMCs

Gene expression analysis from PBMCs that were either not treated, treated with 10 ng/ml *E. coli* LPS for 4 hours or exposed to 1 % O_2 hypoxia for 4 hours. *HIF1A* (A), *IL6* (G) and *TNFA* (H) were significantly highest expressed in LPS-treated PBMCs, while hypoxia increased *GLUT-1* (C), *PDK1* (D) and *PHD3* (F) the most. Displayed are relative expression changes (n-fold induction) of genes normalised to *ACTB* (mean ± SD, ordinary one-way ANOVA test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 11).

Effects of hypoxic priming in immune-stimulated PBMCs

Moreover, we wanted to investigate if an oxygen-lacking environment shapes the inflammation-mediated response in PBMCs. The preceding Western blot analysis did not show changed HIF-1α protein levels when PBMCs were primed by hypoxia before the LPS stimulus (figure 15). Nevertheless, we decided to additionally conduct gene expression analysis of PBMCs challenged with the mentioned treatment. Therefore, PBMCs were primarily exposed to 1% O_2 hypoxia for 4 hours before they were stimulated with 10 ng/ml *E. coli* LPS under normoxic conditions for additional 4 hours. Gene expression of PBMCs treated in this combined condition were compared to cells that were kept untreated or solely treated with LPS for 4 hours (figure 17). No matter if treated with hypoxia before or not – all tested genes maintained the same tendency of being up- or downregulated upon LPS treatment. Further, there was no statistical difference between the group treated with hypoxia prior to the LPS stimulus and the group treated with LPS alone in all analysed genes (figure 17). As seen before *HIF1A* was upregulated upon LPS treatment but to a smaller degree when exposed to hypoxia before (3.8-fold), while *HIF2A* was even more downregulated in combination with hypoxia (64%) (figure 17, A, B). When treated with hypoxia prior to the LPS stimulus, the PBMCs displayed nearly the same gene expression changes as if they were not treated with hypoxia before in the tested HIF target genes: *GLUT-1* and *PHD3* mRNA levels decreased to 0.54 and 0.58-fold on average, while *ADM* and *PDK1* increased to 12.6 and 1.5-fold, respectively (figure 17, C, F, E, D). In terms of inflammation, Interleukin 6 and TNF-α were slightly more upregulated in the “hypoxia prior to LPS” group than in the solely LPS treated group. *IL6* mRNA levels increased from 5652.8-fold to 7166-fold and *TNFA* mRNA levels from 26.6-fold to 32.7-fold compared to mRNA levels of the unstimulated normoxic control cells (figure 17, G, H). However, a high standard deviation between the individual subjects prevented statistical significance between the groups. For analysis PBMCs isolated out of blood from 10 - 11 different donors were normalised to housekeeping gene *ACTB* and the untreated normoxic control. Statistical significance was tested using the ordinary one-way ANOVA analysis with a comparison of all groups.

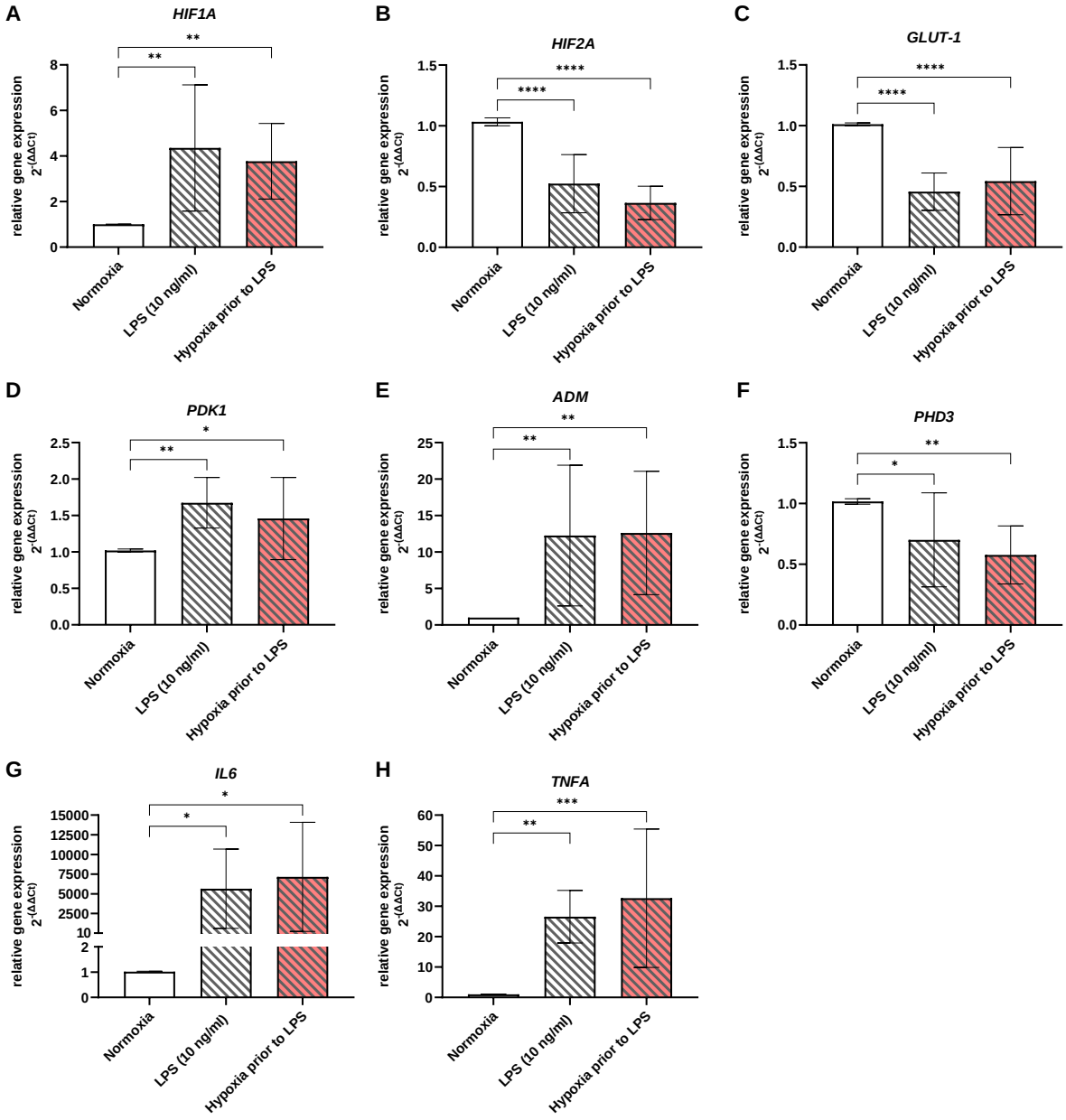


Figure 17: Gene regulation of LPS-treated and hypoxic primed PBMCs

Gene expression analysis from PBMCs that were either not treated, treated with 10 ng/ml *E. coli* LPS for 4 hours or primed with 1 % O_2 hypoxia for 4 hours before the LPS stimulus. LPS treatment led to similar significant expression changes in both LPS-treated groups in all tested genes. Illustrated are relative expression changes (n-fold induction) of genes normalised to *ACTB* (mean ± SD, ordinary one-way ANOVA test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 10 - 11).

Effects of inflammatory priming on hypoxia exposed PBMCs

On the other hand, we were, like in *THP-1* cells, interested in the effects of hypoxia on top of an underlying inflammation on gene expression level. Therefore, PBMCs were treated with a low dose of 10 ng/ml *E. coli* LPS for 4 hours in normoxia (21 % O_2, 5 % CO_2), following exposure to either 4 h of normoxia or hypoxia (1 % O_2, 5 % CO_2), before lysis and gene expression analysis of cells (see section 2.2.5). The two major regulators *HIF1A* and *HIF2A* were significantly downregulated when challenged with low oxygen concentration after an LPS stimulus compared to the LPS treated group alone (figure 18, A). *HIF1A* mRNA levels of PBMCs exposed to normoxia were 4.5 ± 2.7-fold increased while *HIF1A* mRNA levels of PBMCs exposed to hypoxia increased only 2.5 ± 1.3-fold (figure 18, A). *HIF2A* mRNA levels showed the reversed tendency downregulation of 0.55 ± 0.43-fold upon normoxia and 0.19 ± 0.11-fold under hypoxia (figure 18, B). All HIF-related target genes presented a significant upregulation when PBMCs were incubated in a hypoxic environment after the LPS stimulus compared to normoxia and did partly even exceed mRNA levels of the PBMCs that were only treated with hypoxia alone (figure 18, C-F). *GLUT-1* gene expression levels increased 2.3 ± 0.6-fold, *ADM* mRNA levels 31.6 ± 14.1-fold, *PDK1* mRNA levels 3.7 ± 2.0-fold, and *PHD3* gene expression levels 6.6 ± 2.4-fold compared to the untreated control. *IL6* gene expression levels significantly increased in both LPS treated groups but, there was only a tendency of hypoxia increasing the inflammatory *IL6* levels (850.8 ± 650-fold increase) over the normoxic *IL6* levels (691.2 ± 624-fold increase) (figure 18, G). *TNFA* mRNA levels followed the same pattern, with both LPS treated groups were significantly upregulated compared to the untreated control and tended to slightly higher upregulation after hypoxic exposure (4.8 ± 1.9-fold) compared to normoxia (3.5 ± 1.8-fold) (figure 18, H). In summary, gene expression of all hypoxia-related genes differed significantly between PBMCs kept in normoxia vs. hypoxia after the LPS stimulus and showed a tendency for increased expression of inflammatory cytokines under hypoxia (figure 18). Experiments included PBMCs from 9 – 10 blood donors. Gene expression levels were normalised to housekeeping gene *ACTB* and the untreated normoxic control, displaying n-fold induction. Statistical significance was calculated using the ordinary One-way ANOVA analysis with multiple comparisons between all groups.

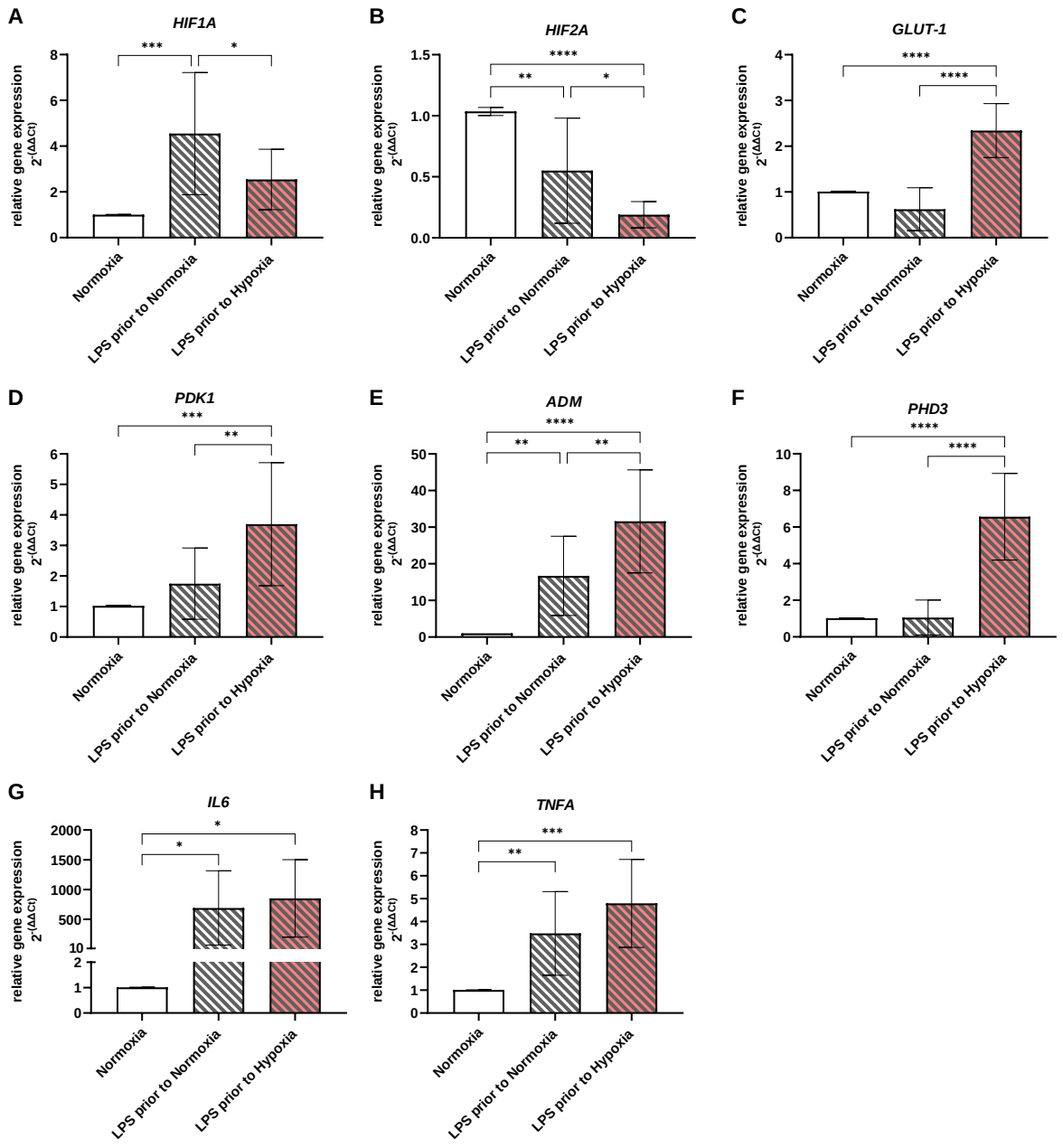


Figure 18: Gene expression in inflammatory primed PBMCs, exposed to hypoxia

Relative gene expression levels from PBMCs that were either untreated or treated with LPS (10 ng/ml) 4 hours prior to normoxia (21 % O_2, 4 h) or prior to hypoxia (1 % O_2, 4 h). Gene expression levels differed significantly between following normoxic vs. hypoxic exposure in all genes of the HIF pathway: *HIF1A* (A), *HIF2A* (B), *GLUT-1* (C), *PDK1* (D), *ADM* (E), and *PHD3* (F) but inflammatory cytokines showed only a tendency of being upregulated under hypoxia (G: *IL6*, H: *TNFA*). Shown are relative expression changes (n-fold induction) of genes normalised to *ACTB* (mean ± SD, ordinary one-way ANOVA test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 9 - 10).

3.3 Effects of the PHD-Inhibitor *Roxadustat* on the HIF pathway in PBMCs

In combination with our studies on physiologically occurring hypoxia, we were further interested in the effects of pharmacological relevant substances, which are supposed to activate the HIF pathway. One of those is the clinically used prolyl-hydroxylase inhibitor *Roxadustat*, which is known to inhibit PHD activity and thereby prevent HIF protein degradation, inducing a rise in HIF protein levels to treat patients with chronic kidney disease (Dhillon, 2019), (Chen et al., 2019), (Shutov et al., 2021). Although the general mode of action is known, it is still not clear how the orally administered drug *Roxadustat* acts on distinct cell types like immune cells. To gain a more in-depth understanding of the drug-specific effects on circulating immune cells of treated patients, we analysed the effects of different *Roxadustat* concentrations on cell survival (section 3.3.1), HIF-1α protein expression (section 3.3.2), and HIF-dependent gene expression (section 3.3.3) in human PBMCs *in vitro*. Additionally, we investigated the impact of *Roxadustat* treatment on the cellular reaction to acute inflammation, induced by LPS, focusing on the HIF pathway (section 3.3.4).

3.3.1 Vitality of PBMCs upon *Roxadustat* treatment

To assess potential toxic effects of *Roxadustat* on human immune cells, we determined the viability of PBMCs, which were treated for 24 hours with different *Roxadustat* concentrations. Therefore, we used a special apoptosis staining kit combined with flow cytometry analysis (see section 2.2.8). None of the tested drug concentrations (10 µM, 25 µM, 50 µM, 100 µM) had an impact on apoptosis or necrosis in PBMCs, compared to the untreated cells (figure 19). Overall, greater than 96 % of all treated cells were viable in all tested groups. Moreover, the percentages of necrotic and apoptotic cells were almost identical in all *Roxadustat*-treated cells (0.2 - 0.3 %) to those of the untreated control (0.1 %). Of note, the rate of early apoptotic cells even decreased slightly upon *Roxadustat* treatment in a dose-dependent manner from 3.1 % (untreated, 10 µM) to 2.3 % (100 µM) (figure, 19). However, statistical testing of the viability status revealed no significant changes compared to the untreated control data (2-way ANOVA).

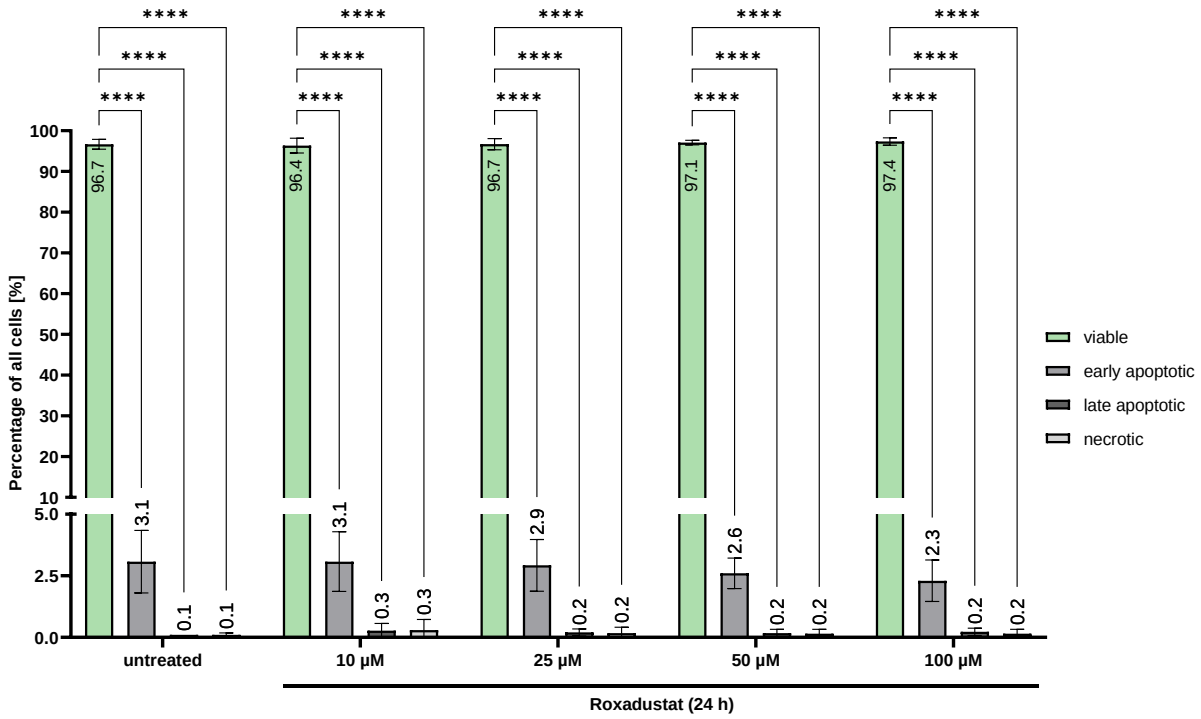


Figure 19: Vitality of *Roxadustat*-treated PBMCs

Roxadustat treatment for 24 hours did not affect the survival of PBMCs *in vitro*. PBMCs were untreated or treated with 10 µM, 25 µM, 50 µM, or 100 µM *Roxadustat* for 24 h under normoxic conditions, and rates of viable, necrotic, early, and late apoptotic cells were determined using the "PE Annexin V Apoptosis Detection Kit I" (BD Bioscience) in combination with flow cytometry analysis according to the manufacturers' instructions (FACSCanto™ II). The gating. 50.000 events per condition were detected. Data are shown as means ± SD (two-way ANOVA with multiple comparisons, **** = $p < 0.0001$, n = 4).

3.3.2 Effects of *Roxadustat* on HIF-1α protein accumulation in PBMCs

Due to the fact that *Roxadustat* is a PHD-Inhibitor, which is supposed to stabilise HIF-1α protein and thereby induce Erythropoietin expression in patients, we were curious about how the drug influences HIF-1α protein levels in circulating immune cells. Therefore, we treated human isolated PBMCs with different concentrations of *Roxadustat* for increasing durations. For the following investigation of HIF-1α protein accumulation upon treatment, we used Western blotting as well as immunofluorescent antibody staining.

HIF-1α Western blot analysis of *Roxadustat*-treated PBMCs

For Western blotting, isolated PBMCs from four different blood donors were treated with 10, 25, or 50 µM *Roxadustat* over 1, 2, 4, 6, 16, and 24 hours prior to protein isolation (figure 20). Western blot analysis demonstrated that all tested *Roxadustat* concentrations tended to increase HIF-1α protein levels in PBMCs compared to untreated control cells. Even though treatment with 10 µM *Roxadustat* induced a 5.0 ± 3.5-fold increase after 4 hours, the high standard deviation between the samples probably prevented statistical significance (figure 20,

A). However, treatment of PBMCs with 25 µM *Roxadustat* for 2, 4, and 6 hours did significantly increase HIF-1α protein levels up to 11.8 ± 5.3-fold compared to untreated control levels but slightly decreased again upon 16 and 24 hours of treatment to 7.1 and 8.4-fold (figure 20, B). PBMCs treated with 50 µM *Roxadustat* showed significant upregulation of HIF-1α protein to around 9-fold upon 2 h and again upon 24 hours of treatment *in vitro*, but not in between (figure 20, C).

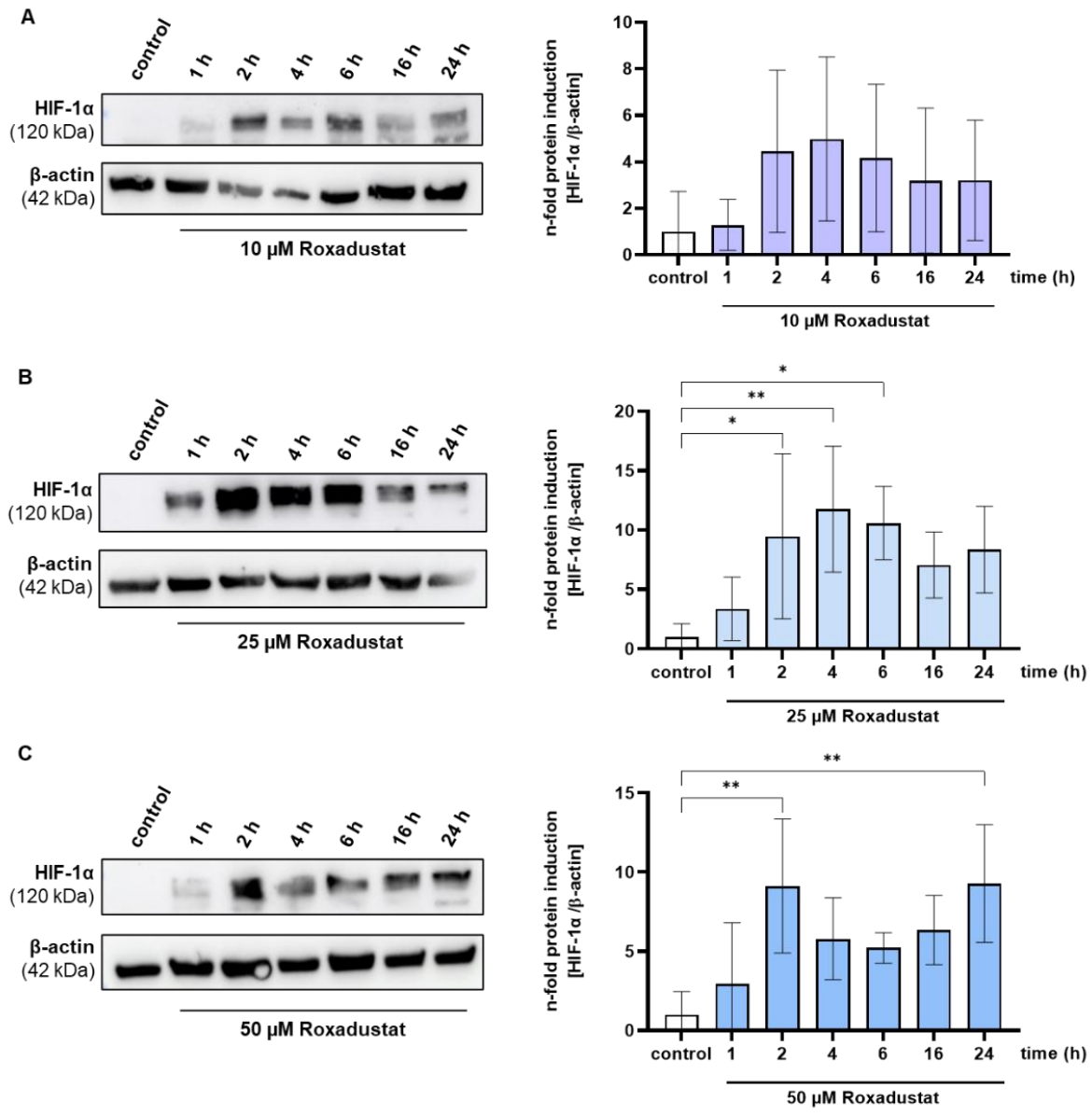


Figure 20: Effect of *Roxadustat* treatment on HIF-1α protein expression in PBMCs

Western blot analysis of PBMCs, which were exposed for increasing times (1 - 24 h) with *Roxadustat* concentrations of 10 µM (A), 25 µM (B), and 50 µM (C) *in vitro* showed a significant accumulation of HIF-1α protein upon 2 to 6 hours with 25 µM *Roxadustat* and upon 2 and 24 h with 50 µM *Roxadustat*. Left: representative image, right: densitometric evaluation and calculation of n-fold induction of HIF-1α protein, normalised to β-actin and compared to normoxic control cells (mean ± SD, ordinary one-way ANOVA, * = $p < 0.005$, ** = $p < 0.0005$, n = 4).

HIF-1α immunofluorescent staining of *Roxadustat*-treated PBMCs

In addition to the analysis of HIF-1α protein levels using Western blotting, HIF-1α immunofluorescent antibody staining was performed to get additional insights into protein localisation and accumulation within the immune cells. PBMCs of three blood donors were treated with 10, 25, or 50 µM *Roxadustat* for 2, 4, 6, and 24 h, under normoxic conditions (21 % O_2 and afterward fixed with 4 % PFA for staining. HIF-1α protein was first labelled with a HIF-1α antibody (BD) and secondly stained with a green fluorescent antibody (Alexa Fluor® 488), while nuclei were stained blue with DAPI (see section 2.2.7). PBMCs treated with *Roxadustat* did display higher HIF-1α fluorescence intensities than untreated cells (figure 21, A). Quantification of HIF-1α staining intensities among cells by pixel count proved that *Roxadustat* induced a significant increase of HIF-1α protein levels in leukocytes (figure 21, B, C, D). Besides drug treatment of 4 hours with 50 µM *Roxadustat* (figure 21, D), all tested concentrations and treatment durations significantly increased HIF-1α compared to the untreated control cells (figure 21, B, C, D).

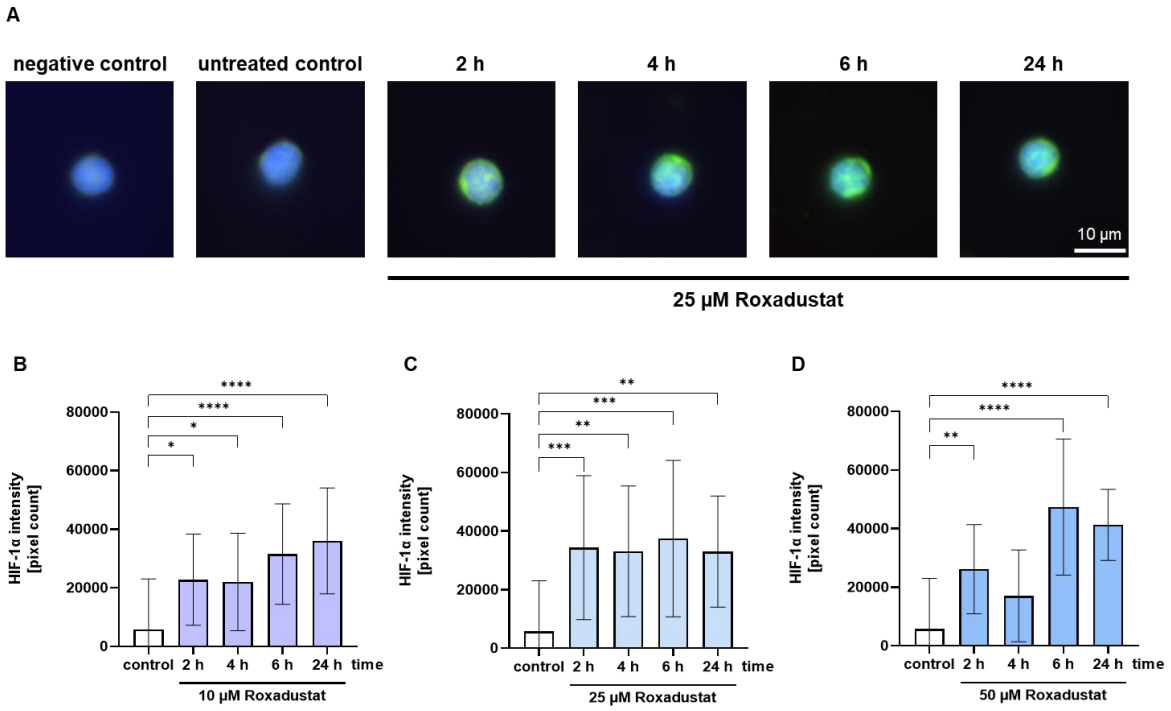


Figure 21: immunofluorescent staining of HIF-1a in *Roxadustat*-treated PBMCs

Analysis of immunofluorescent antibody staining of PBMCs treated with 10, 25, or 50 µM of *Roxadustat* for 2 to 24 hours. A: representative images of PBMCs treated with or without 25 µM of *Roxadustat* shows increasing HIF-1α intensity over time. Nuclei: blue (DAPI), HIF-1α protein: green (Alexa Fluor® 488), negative control with second antibody staining only (scale: 10 µm). B - D: Quantification of intracellular HIF-1α after treatment with 10 µM (B), 25 µM (C), and 50 µM (D) *Roxadustat* for 2, 4, 6, and 24 h. Fluorescence intensity was determined by HIF-1α fluorescent pixel count using "ImageJ" software. All *Roxadustat* concentrations led to a significant HIF-1α increase. The untreated normoxia sample served as a control (Data evaluation B - D: mean values ± SD, ordinary one-way ANOVA, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 18 cells per condition, 3 blood donors).

3.3.3 Effects of *Roxadustat* on gene regulation of PBMCs

To verify whether the HIF-1α accumulation shown in the preceding protein analysis was transcription-dependent, the effects of *Roxadustat* treatment were further analysed on the gene expression level of PBMCs. For this purpose, PBMCs were treated in normoxia in the same conditions as mentioned before, for 4, 6, and 24 h with 10 µM, 25 µM, and 50 µM *Roxadustat*, respectively. The mRNA expression of the two transcription factors HIF-1α (*HIF1A*) and HIF-2α (*HIF2A*) was analysed in combination with gene expression levels of the HIF target genes glucose transporter-1 (*GLUT-1*), adrenomedullin (*ADM*), prolyl-hydroxylase 2 (*PHD2*) and 3 (*PHD3*). To determine a possible immune activation in leukocytes after *Roxadustat* treatment, gene expression of the inflammatory cytokines TNF-α (*TNFA*) and IL-6 (*IL6*) was assessed using qRT-PCR (see section 2.2.5).

It was shown that treatment with all concentrations of *Roxadustat* (10, 25, 50 µM) did not lead to altered gene expression of either HIF-α isoform compared to the untreated normoxia control (figure 22, A, B). However, all tested HIF target genes presented a time-dependent mRNA upregulation upon *Roxadustat* treatment to some extent (figure, C, D, E, F). Relative gene expression of *GLUT-1* was significantly increased over 5-fold in PBMCs treated with 25 µM Roxadustat for 6 hours (5.0 ± 3.1-fold) or 50 µM for 6 h (5.8 ± 4.1-fold) and 24 h (5.7 ± 2.4-fold, figure 22, C). *ADM* mRNA was upregulated at the same conditions up to 12.7 ± 8.4-fold (6h, 50 µM) and additionally significantly upregulated after 6 hours of 10 µM (7.4 ± 5.4-fold) and 4 hours of 50 µM (6.8 ± 3.0-fold) drug treatment (figure 22, D). In all conditions of treatment, *PHD2* was highly elevated between 4.4 - 8.5-fold compared to the control (1.01 ± 0.005, figure 22, E). *PHD3* expression reached even higher mRNA levels after 6 and more prominently after 24 hours after treatment with *Roxadustat* (10µM: 6 h: 6.4, 24 h: 35.2; 25 µM: 6 h: 13.9, 24 h: 52.5; 50 µM: 6 h: 11.8, 24 h: 49.9 - fold) but displayed quite high standard deviation in the 24-hour values (figure 22, F). The treatment did not induce notable changes in *IL6* mRNA expression, with one exception at the 24-hour time point with 10 µM, where *IL6* levels were significantly upregulated to a comparably small, yet significant 2.9 ± 2.4-fold (figure 22, G). Besides that, the picture was mostly consistent with *TNFA* mRNA (highest 1.5-fold increase at 24 h, 10 µM) with both genes displaying a slight time dependency (figure 22, G, H). Gene expression of all groups was normalised to *ACTB* and the untreated normoxia control levels (n-fold induction, $2^{-(\Delta\Delta CT)}$). Statistical significance was calculated using the ordinary one-way ANOVA test with multiple comparisons with all groups being compared to the untreated, normoxic control. In total PBMCs of 10 different blood donors were used at least in technical duplicates.

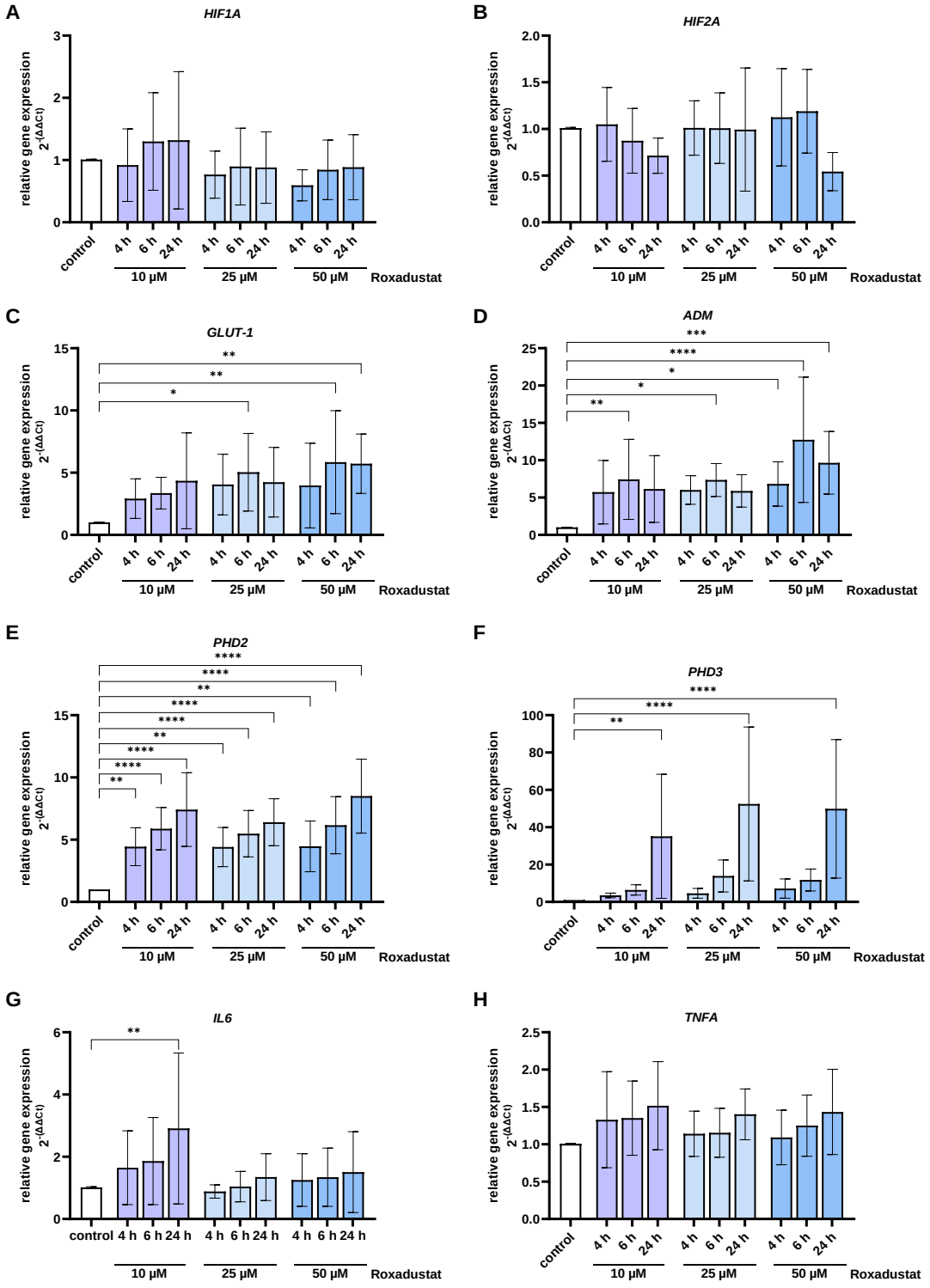


Figure 22: Gene expression changes in PBMCs treated with *Roxadustat*

PBMCs were treated for 4, 6, and 24 h with 10 µM, 25 µM and 50 µM *Roxadustat*, respectively. Relative gene expression ($2^{-(\Delta\Delta Ct)}$) of *HIF1A* (A) and *HIF2A* (B) was unchanged, but all tested HIF target genes *GLUT-1* (C), *ADM* (D), *PHD2* (E), and *PHD3* (F) showed upregulation of mRNA upon *Roxadustat* treatment to some extent. The expression of the inflammatory mediators *TNFA* (G), and *IL6* (H) was hardly changed. Gene expression was normalised to *ACTB* and is displayed as n-fold increase compared to the untreated normoxic control (Data evaluation: mean values ± SD, ordinary one-way ANOVA with multiple comparisons, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 10).

3.3.4 Effects of *Roxadustat* treatment in acute inflammation in PBMCs

Even though the orally administered drug *Roxadustat* is approved for clinical use in several countries, precise effects on the HIF pathway of immune cells are still unknown. Some clinical observations point to a higher risk for bacterial infections in patients that are treated with *Roxadustat* (Chen et al., 2019), (Shutov et al., 2021). To mimic an occurring bacterial infection in *Roxadustat*-treated patients, isolated PBMCs were pre-treated with 25 µM *Roxadustat* for 20 hours under normoxic conditions (21 % O_2), followed by supplementation with 10 ng/ml *E. coli* lipopolysaccharide for additional 4 hours *in vitro*. After 24 hours in total, PBMCs were lysed and analysed on protein and gene expression levels, focussing on the HIF pathway activation and inflammatory mediators (see sections 2.2.5 and 2.2.6). In this series of experiments untreated PBMCs, PBMCs treated solely with 10 ng/ml LPS for 4 hours and PBMCs treated solely with 25 µM *Roxadustat* for 24 hours served as controls. In total isolated PBMCs of 6 different blood donors were used at least in duplicates.

HIF-1α protein accumulation in *Roxadustat*-treated PBMCs upon inflammation

HIF-1α protein levels in *Roxadustat* and LPS-treated PBMCs were analysed via Western blotting (see section 2.2.6). PBMCs that were treated with the combination of *Roxadustat* and LPS demonstrated a significant 10.9-fold increase of HIF-1α protein compared to HIF-1α protein levels of the untreated normoxia sample (1.0 ± 0.6, figure 23). Furthermore, this upregulation of HIF-1α was also significantly elevated compared to the protein levels of PBMCs solely treated with LPS (4 h, 10 ng/ml: 1.3 ± 0.2-fold) or solely treated with *Roxadustat* (24 h, 25 µM: 4.6 ± 4.1-fold, figure 23, B). In this series of experiments, *Roxadustat* and low-dose LPS treatment alone did not notably upregulate HIF-1α protein compared to untreated cells (figure 23). For analysis, the intensity of HIF-1α protein bands was normalised to the β-actin bands and related to control levels (n-fold induction, figure 23, B).

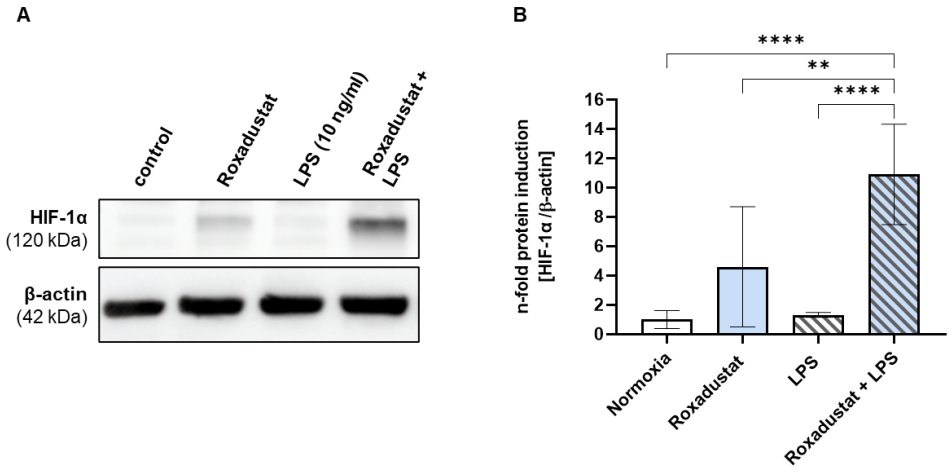


Figure 23: HIF-1α protein accumulation in PBMCs treated with *Roxadustat* and LPS

PBMCs which were pre-treated with 25 µM *Roxadustat* (20 h) before the acute LPS stimulation (10 ng/ml, 4 h) did show increased HIF-1α protein levels compared to cells that were treated solely with LPS (4 h) or solely with *Roxadustat* (24 h). A: representative Western blot image (80 µg protein per sample), B: densitometric evaluation of protein bands normalised to β-actin and the untreated normoxic control levels: mean values ± SD (ordinary one-way ANOVA with multiple comparisons, ** = $p < 0.01$, **** = $p < 0.0001$, n = 6).

Effects of *Roxadustat* in acute inflammation on gene expression of PBMCs

To check whether the HIF-1α protein accumulation shown in the Western blot was transcription-dependent, relative mRNA expression of HIFs themselves (*HIF1A*, *HIF2A*), of specific target genes (*GLUT-1*, *ADM*, *PHD2*, *PHD3*) and inflammatory cytokines (*TNFA*, *IL6*) were determined by quantitative real-time PCR (n-fold induction, see section 2.2.5). PBMCs treated with LPS, presented a significant increase in *HIF1A* transcript levels compared to the non-LPS groups (control and *Roxadustat*-treated, figure 24, A). LPS treatment alone led to a 4.1 ± 1.7-fold, combination of *Roxadustat* (Rox) and LPS to a 3.4 ± 1.7-fold upregulation with no difference between those two groups (figure 24, A). *HIF2A* mRNA had a tendency of upregulation in PBMCs stimulated with the combination treatment (2.3 ± 1.8-fold) but displayed no changes upon the single treatments (figure 24, B). Target gene *GLUT-1* was significantly increased in PBMCs treated with *Roxadustat* (2.6 ± 1.0-fold) but even more in the combined treatment with LPS, when compared to all other groups (4.0 ± 1.3-fold, figure 24, C). Besides high standard deviation *ADM* mRNA was upregulated significantly in the combined treatment (35.0 ± 32.8-fold), compared to the untreated and "*Roxadustat* only"-group but not to cells that were treated solely with LPS (figure 24, D). Gene regulation of *PHD2* and *PHD3* illustrated a very similar pattern, with the highest significant increase of mRNA after *Roxadustat* treatment alone (*PHD2*: 4.4 ± 3.4-fold, *PHD3*: 13.1 ± 4.9-fold, figure 24, E, F). Regarding *PHD3*, the upregulation was also significantly lower than the increase in PBMCs treated with both stimuli (7.2 ± 2.4-fold, figure, F). Treatment with both did also induce significantly higher mRNA levels of *TNFA* and *IL6*, compared to untreated and solely *Roxadustat*-treated cells, although the

standard deviation between the blood donors was high (*TNFA*: 60.5 ± 72.3-fold, *IL6*: 22139 ± 27078-fold, figure 24, E, F).

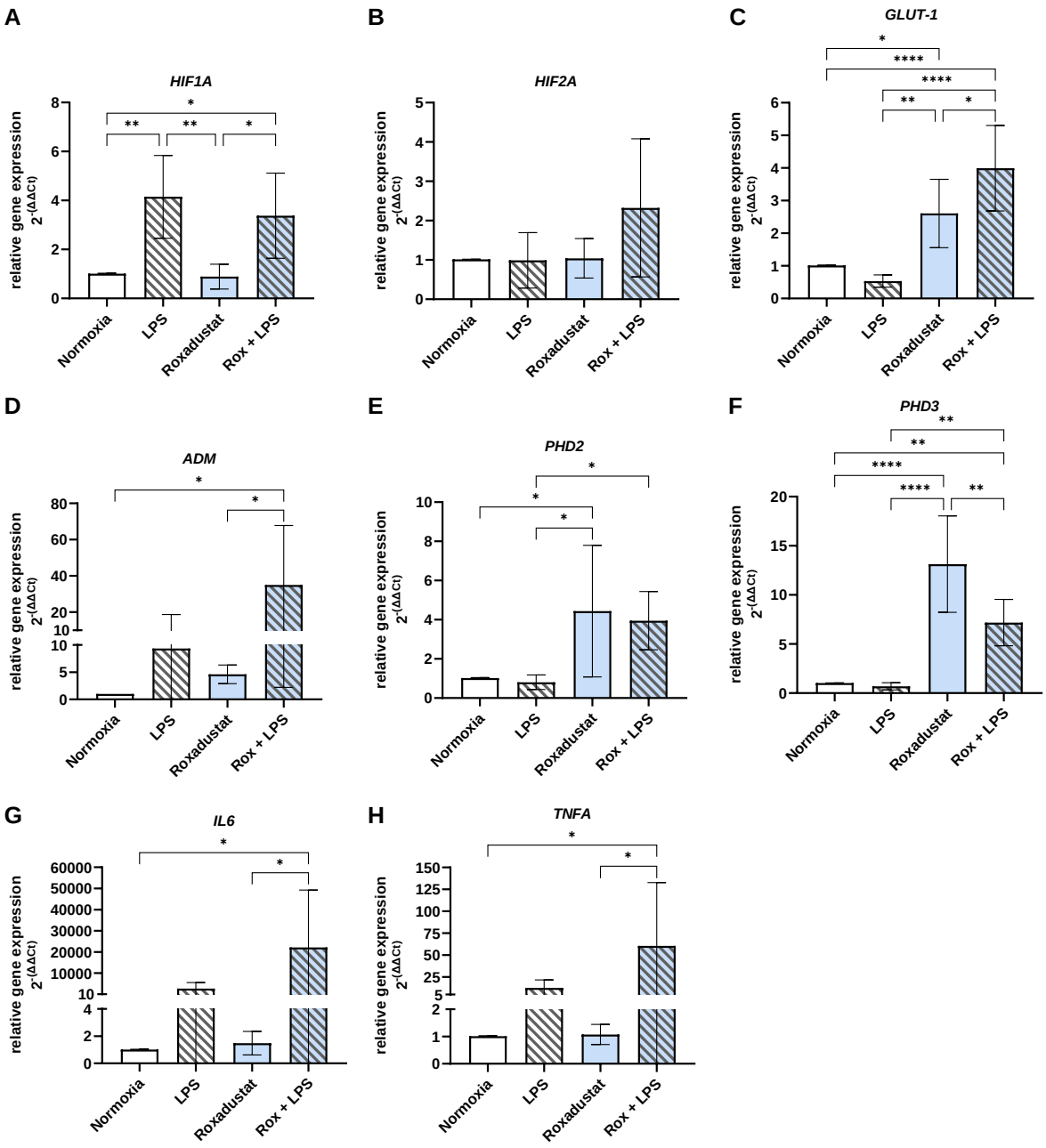


Figure 24: Gene expression of PBMCs treated with *Roxadustat* and LPS

PBMCs were kept untreated or treated with 10 ng/ml LPS (4 h), 25 µM *Roxadustat* (24 h), or a combination of both (20 h *Roxadustat* followed by 4 h of LPS) before analysis of mRNA levels using qRT-PCR. Relative gene expression ($2^{-(\Delta\Delta Ct)}$) of *HIF1A* (A) and *HIF2A* (B) and HIF target genes *GLUT-1* (C), *ADM* (D), *PHD2* (E), and *PHD3* (F) were analysed in combination to those of the inflammatory mediators *IL6* (G) and *TNFA* (H). Gene expression was normalised to housekeeping gene *ACTB* and is displayed as n-fold increase compared to the untreated normoxic control (Rox = *Roxadustat*, Data evaluation: mean values ± SD, ordinary one-way ANOVA with multiple comparisons, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 6).

3.4 Effects of mild *in vivo* hypoxia on whole blood gene expression in humans

In addition to our *in vitro* studies on the influence of hypoxia on circulating immune cells, pursuing investigations on primary human immune cells from participants exposed to mild *in vivo* hypoxia were conducted. As part of the so-called “ChronOx” study, 22 participants (12 females, 10 males, age 25.2 ± 2.7) were exposed to 6.5 hours of 15 % O_2 normobaric hypoxia, simulating a high-altitude of 2438 meters above sea level (see section 2.2.1.1). Within this setting, we analysed whole blood samples on gene expression level using the PAXgene RNA tubes followed by qRT-PCR (see sections 2.2.2 and 2.2.5). Relative gene expression of specific genes of interest was normalised to *ACTB* mRNA levels and calculated with the $2^{-(\Delta CT)}$ method, without normalisation to the baseline gene levels. Relative gene expression levels of *HIF1A*, *HIF2A*, *GLUT-1*, *PDK1*, *ADM*, *VEGF*, *PHD1*, and *PHD3* were determined in circulating blood cells in two different rounds of the study, each time for baseline control levels before the study and after 3:15 h and 6:25 h of exposure to 15 % O_2 hypoxia or 21 % O_2 normoxia respectively (figure 25).

Within the study measurements of blood oxygen concentrations showed that oxygen levels (SpO_2) decreased from around 98 % to a mean of 86.4 % (95 % CI: 85.2 - 87.7 %) during the last three hours of hypoxia when participants were asleep (corresponding to the 6:25 h time point, data not shown).

Gene expression analysis revealed that none of the examined genes of the HIF pathway displayed significant changes between the laboratory stay in mild hypoxia versus normoxia (figure 25, A - I). Transcription levels of the hypoxia-inducible factor 1 (*HIF1A*: mean values: 0.0022 – 0.0026) were much higher expressed than those of hypoxia-inducible factor 2 (*HIF2A*: mean values: 0.00006 – 0.0001, figure 25, B). With regard to HIF target genes *GLUT-1* (mean: 0.0039 – 0.0045, figure 25, C), *PHD1* (mean: 0.0045 – 0.0067, figure 25, G) and *PHD2* (mean: 0.0048 – 0.0063, figure 25, H) were expressed in all circulating blood cells much higher than *ADM* (mean: 0.0019 – 0.0022, figure 25, E) and *PDK1* (mean: 0.00058 – 0.00074, figure 25, D). The lowest relative mRNA levels were found in the HIF target genes *PHD3* (mean: 0.00032 – 0.00057, figure 25, I) and *VEGF* (mean: 0.00013 – 0.00018, figure 25, F). Data of *HIF3A* and *PHD2* levels are not shown but did also display no changes.

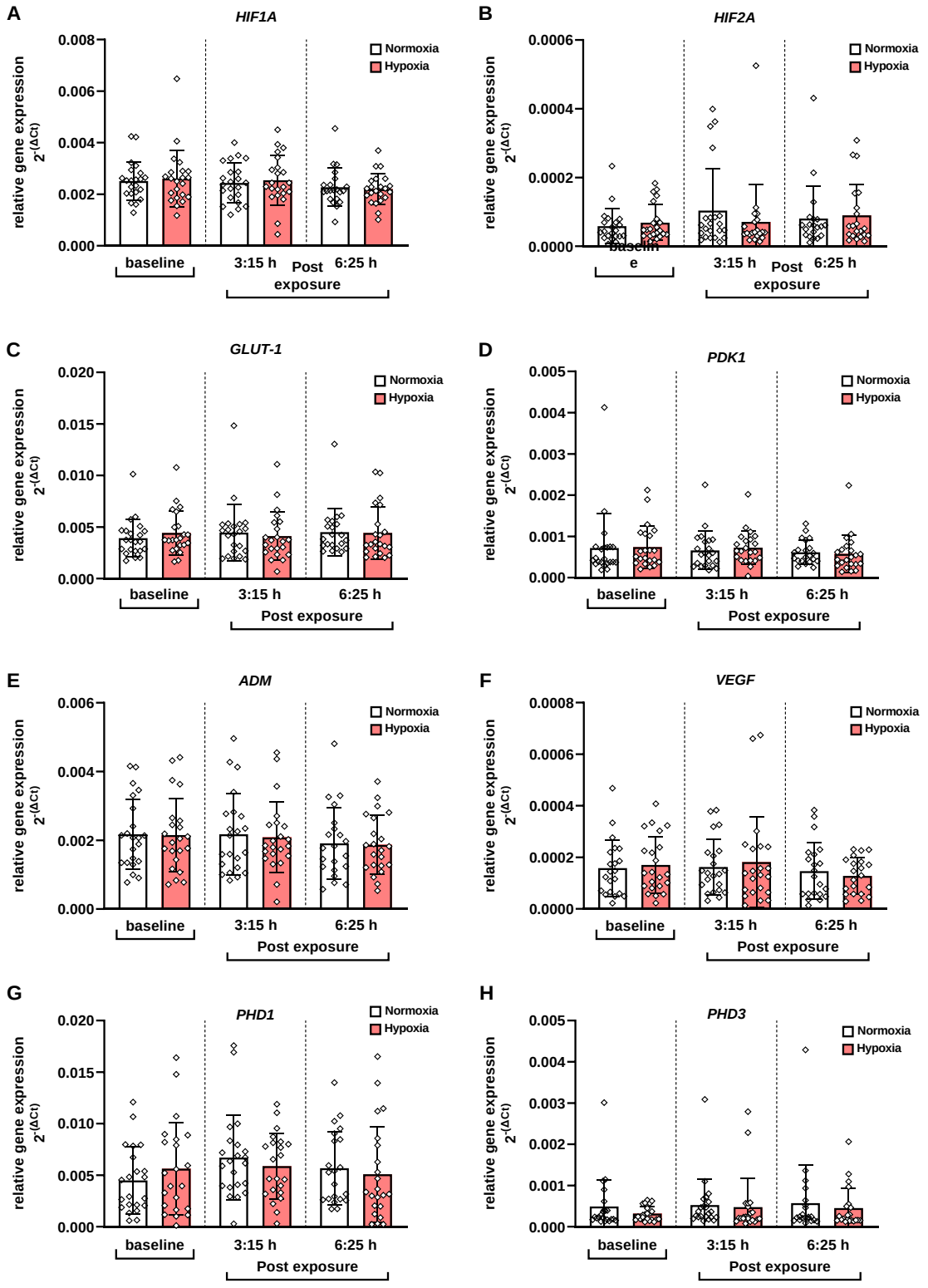


Figure 25: Gene expression in human blood cells, upon *in vivo* hypoxia

Gene expression in blood cells sampled from human subjects that were exposed to 6.5 hours of mild *in vivo* hypoxia (normobaric, 15 % O_2) did not display any effects of adaption upon low oxygen levels among the investigated genes *HIF1A* (A), *HIF2A* (B), *GLUT-1* (C), *PDK1* (D), *ADM* (E), *VEGF* (F), *PHD1* (G) and *PHD3* (H). Expression levels of genes were normalised to *ACTB* and presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test), n = 22 subjects (12 female, 10 male, age 25.2 ± 2.7).

3.5 Effects of combined *in vivo* LPS and hypoxia in human subjects

To bring our investigations on the interplay of hypoxia and inflammation in immune cells from the *in vitro* laboratory work one step further to possible implementations for *in vivo* situations we conducted a human subject study investigating the effects of hypoxia and inflammation. In total 30 male healthy participants were included in the study (mean age: 25.9 ± 3.1) to research immune reactions following lipopolysaccharide injection (0.4 ng LPS per kg bodyweight, *E. coli* HOK364) with or without the combination of low oxygen supply *in vivo*. First, we challenged participants solely with a single LPS injection to analyse acute LPS-dependent immune reactions from 2 to 24 hours after the stimulus (section 3.5.1). Another group of participants was treated with an LPS injection followed by a stay in a hypoxia chamber for 4 hours at 10.5 % normobaric O_2, simulating a high altitude of 4500 meters (section 3.5.2) to investigate if and how hypoxia shapes the response to underlying inflammation. In a third study branch, volunteers were exposed to 10.5 % O_2 hypoxia for 4 hours prior to the LPS injection, focussing on the possible priming effects of hypoxia on immune cells (section 3.5.3).

For analysis, whole blood samples were used. The blood was directly processed for flow cytometry analysis, immune cell isolation, and RNA isolation. Flow cytometry included the determination of the cellular fractions in whole blood of participants by so-called “phenotype” staining and determination of monocyte subsets and activation by special “monocyte” staining (see sections 2.2.8). Blood samples were additionally collected for gene expression analysis of all circulating immune cells by PAXgene RNA tubes (see section 2.2.2) and in parallel processed for isolation of neutrophils, T cells, and monocytes for gene expression analysis via qRT-PCR (see section 2.2.5).

3.5.1 Effects of *in vivo* LPS treatment of human subjects

To start with, 6 male participants were challenged with a single *E. coli* lipopolysaccharide injection of 0.4 ng LPS per kg bodyweight in normoxic conditions. Participants were examined and the first blood sample was taken immediately before the start of the study protocol to determine the “baseline” levels of all collected data. After the LPS injection, blood was collected every 2 hours for 8 hours in total and additionally 24 hours after the LPS injection (time points: +2 h, +4 h, +6 h, +8 h, +24 h, figure 26). Blood sampling was followed by direct processing of material for flow cytometry analysis, immune cell isolation, and RNA isolation.

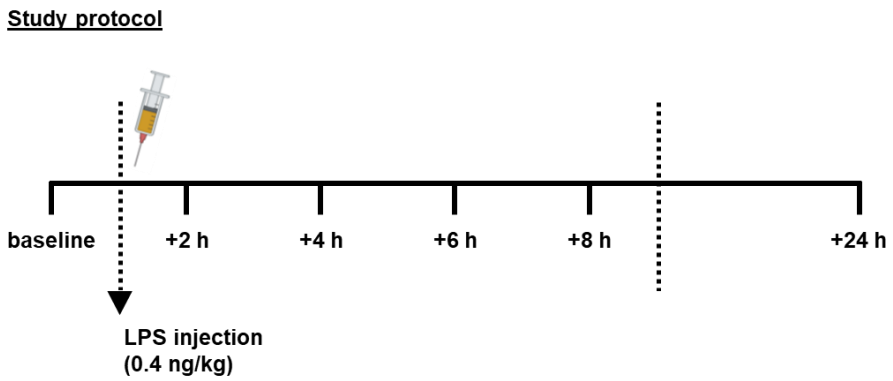


Figure 26: Study protocol of LPS administration in human subjects

Schematic representation of the study protocol of human subjects that were only treated with a single *E. coli* lipopolysaccharide injection (0.4 ng LPS / kg body weight). Times of blood sampling for further analysis were "baseline" levels, shortly before the start of the treatment and indicated time points 2 h, 4 h, 6 h, 8 h, and 24 hours after the treatment. 6 participants passed the study branch.

3.5.1.1 Flow cytometry analysis of circulating leukocytes upon *in vivo* challenge with LPS

To investigate the effects of acute inflammation on the composition and activation of circulating immune cells we conducted fluorescent antibody staining combined with flow cytometry analysis using the blood samples of 6 subjects (see section 2.2.8). The general composition of cells was determined via the so-called "phenotype staining" (section 2.2.8.1), which was carried out in this study by Marie Jakobs from the Institute of Medical Psychology and Behavioral Immunobiology at the University Hospital Essen, Germany. In addition, the circulating monocyte population was characterised in terms of immune activation and monocyte subgroups (section 2.2.8.2, gating shown in supplements, figures 60 - 63).

Composition of circulating immune cells in humans challenged with *in vivo* LPS

An *in vivo* injection of 0.4 ng/kg *E. coli* lipopolysaccharide did lead to a time-dependent inflammatory response in terms of distribution of circulating immune cells. Following LPS injection the number of circulating granulocytes increased from 61.8 to a peak of 87.3 % of all leukocytes ($CD45^+$ cells) after 4 h and stayed elevated at 75.5 % after 8 hours but decreased back to baseline levels in the long term (figure 27, A). All other measured immune cells presented the opposed pattern and were significantly downregulated 2 or 4 hours after the LPS injection (figure 27). T lymphocytes ($CD3^+$) decreased upon the inflammatory stimulus from 21.2 to 5.1 %, with no changes in the trend in T helper cells (C: 3.5 %) or cytotoxic T cells (D: 1.3 %) after 4 hours (figure 27, B - D). B lymphocytes similarly decreased to a minimum of 0.5 percent of circulating leukocytes, compared to the baseline level of 1.3 % (figure 27, F). The number of monocytes already decreased to its minimum earlier than the other cells and was downregulated 2 hours after the LPS injection from 10.5 to 3.3 % among all measured leukocytes but were back to baseline levels 6 hours afterward (figure 27, E). None of the

investigated cell types showed any long-term changes upon the treatment, meaning cell percentages were back to baseline levels 24 hours after the start of the study (figure 27).

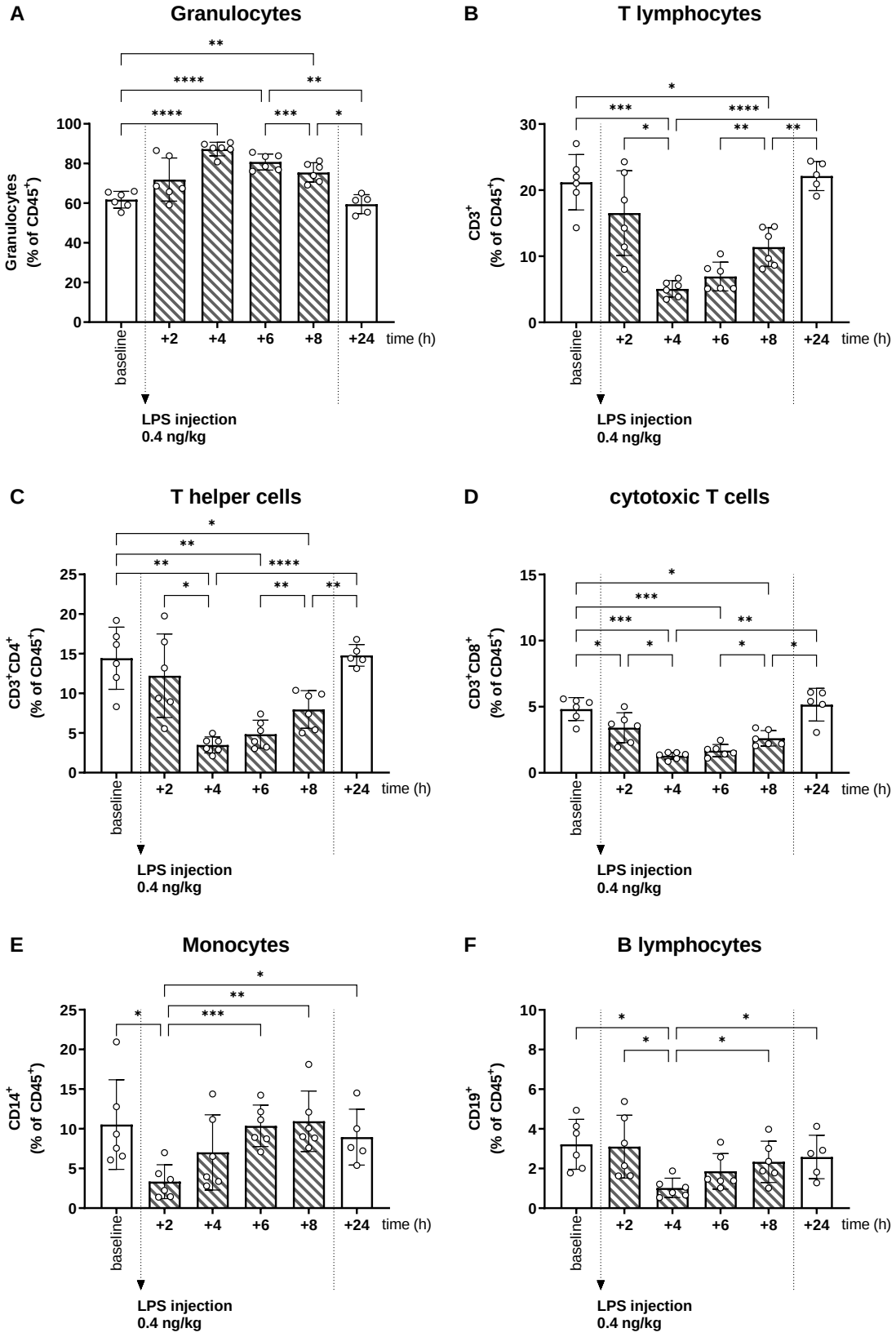


Figure 27: Composition of blood cells upon *in vivo* LPS treatment

Flow cytometry analysis of cellular composition in whole blood from participants challenged with *an in vivo* lipopolysaccharide (LPS) injection (0.4 ng/kg). Investigated were the percentages of granulocytes (A, negative gating), lymphocytes ($CD3^+$, B), T helper cells ($CD3^+CD4^+$, (C), cytotoxic T cells ($CD3^+CD8^+$, D), monocytes ($CD14^+$, E) and B lymphocytes ($CD19^+$, F) among all measured circulating blood cells via flow cytometry and specific gating (see supplements, figure 61-63). Displayed are mean percentage ± SD (mixed-effects analysis with repeated measures, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, with not all significances being illustrated in the graphic, n = 6, measurements by Marie Jakobs).

Characterisation and activation of circulating monocytes in humans challenged with *in vivo* LPS

In addition to the determination of the cellular composition of whole blood, we further characterised the subgroups of circulating monocytes throughout the progression of the study via fluorescent antibody staining and flow cytometry analysis (see section 2.2.8). Furthermore, activation of monocytes was assessed by expression of Toll-like receptor 4 (TLR4) and the combination of CD86 and HLA-DR (major histocompatibility complex, class II) expression. *In vivo* LPS treatment led to a significant increase of classical monocytes from 77.9 % (baseline) to a peak value of 96.8 % (+6 h) within all detected monocytes (figure 28, A). 24 hours after LPS treatment classical monocytes were significantly decreased compared to the 8-hour time point to 60.3 % (figure 28, A). Intermediate and non-classical monocytes populations followed an opposed pattern and were both decreased upon LPS injection (figure B, C). The “intermediate” monocytes decreased from 8.5 % (baseline) down to 1.9 % (+4 h) but significantly increased to a mean of 25.2 % after 24 hours again (figure 28, B). Non-classical monocytes decreased also from 12.4 % to 1.2 % (+6 h) but had a delayed response to the LPS injection compared to the other fractions with the minimum of cells present at the 6 and not 4-hour time point (figure 28, C). In the unseparated monocyte fraction, only 1.1 % of monocytes displayed TLR4 expression at baseline levels, which slightly increased after 2 hours but decreased again after 6 hours down to 0.45 % (figure 28, D). Expression of the monocyte activation markers CD86 and HLA-DR were present on 48.0 % of monocytes at control levels and decreased significantly to 17.6 % 4 hours after the LPS injection (figure 28, D). 6 and 8 hours after LPS treatment the amount of CD86/HLA-DR-positive cells were back to baseline and even higher expressed 24 hours after the study on 67.3% of circulating monocytes, compared to control (figure 28, D). *In vivo* LPS treatment led in total, to an increase of classical monocytes while the small “intermediate” and “non-classical” monocyte fractions decreases upon stimulation. Summarized in all fractions TLR4 and CD86/HLA-DR seemed rather decreased following the stimulus (figure 28).

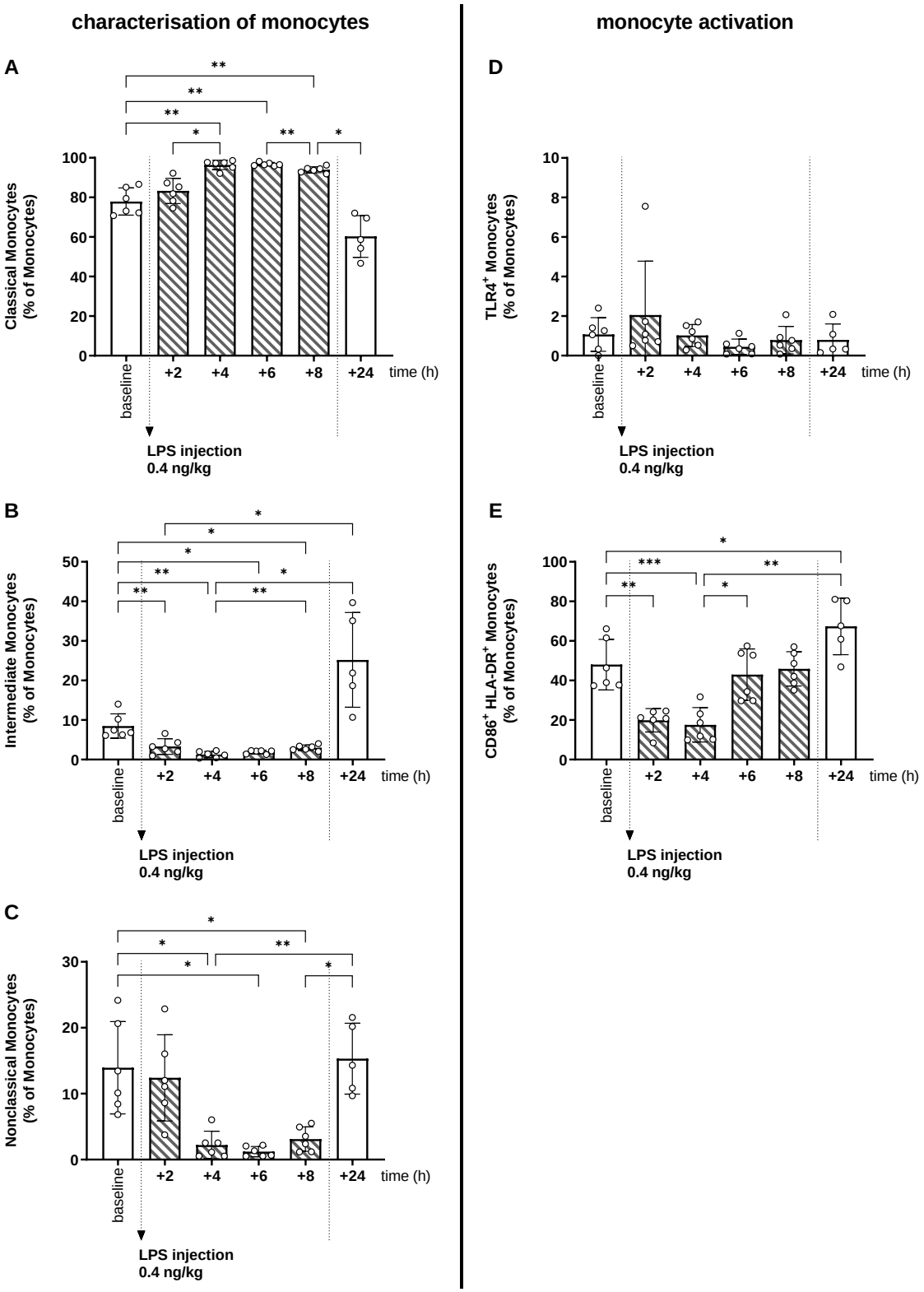


Figure 28: Characterisation of monocytes of LPS-treated humans

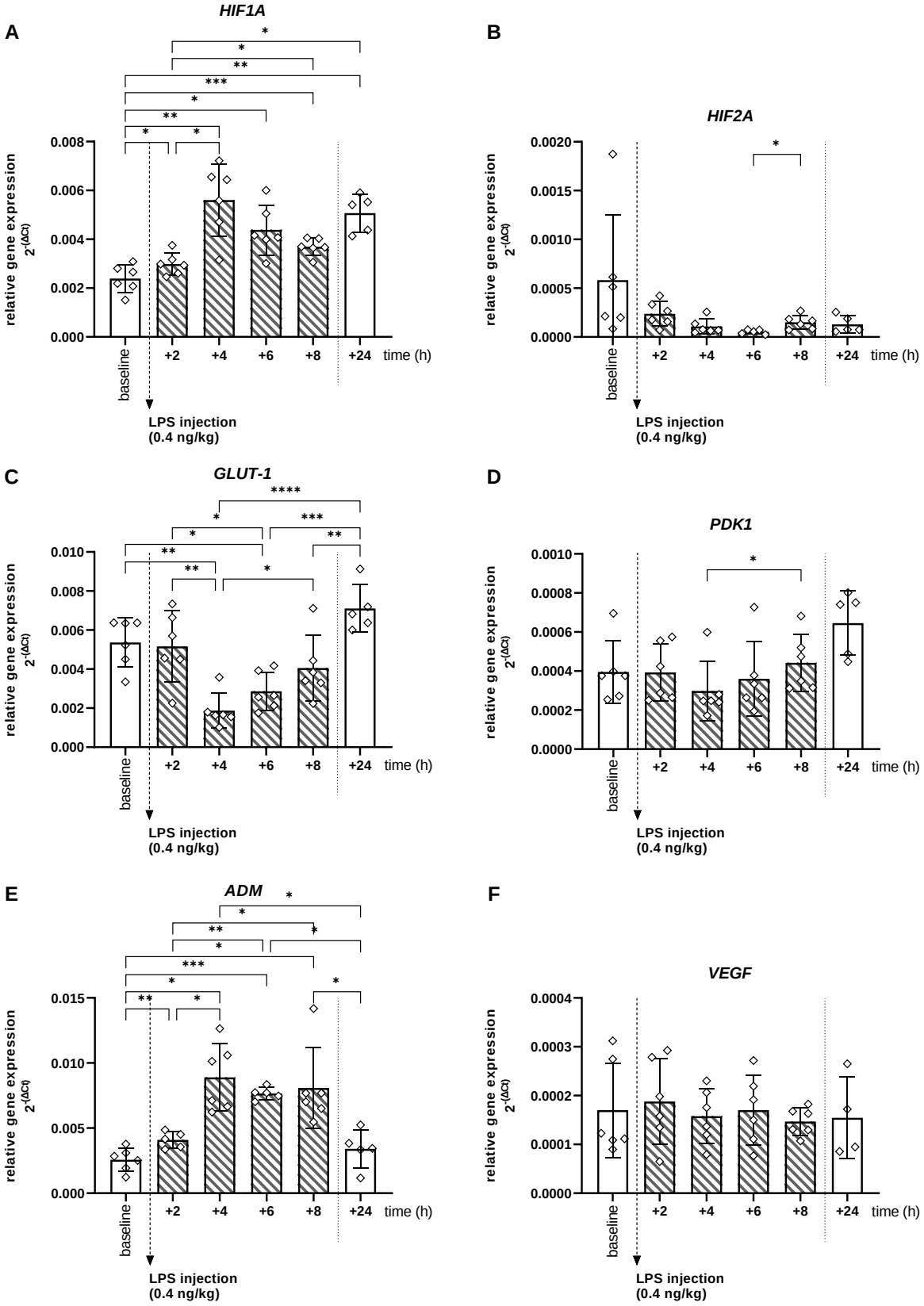
Percentages of monocyte subsets, isolated from blood samples of human subjects that were treated with a single *E. coli* LPS injection (0.4 ng/kg) and followed up for 24 hours. Investigated was the distribution of classical (A), intermediate (B), and non-classical (C) monocytes among all measured circulating monocytes via flow cytometry and specific gating of CD14/CD16 positive cells (see supplements, figure 60). Activated monocytes were identified via detection of Toll-like receptor 4 (TLR4, D) or CD86/HLA-DR expression (E). mean percentage ± SD (mixed-effects analysis with repeated measures, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 6).

3.5.1.2 Effects of *in vivo* LPS treatment on gene expression of whole blood cells

Whole blood samples of 6 study participants were analysed in terms of gene expression changes, following the single LPS injection. The investigation included genes encoding for the HIF transcription factors HIF-1α (*HIF1A*), HIF-2α (*HIF2A*), distinct target genes encoding for GLUT-1, PDK1, ADM, VEGF, and the three important HIF hydroxylases and degradation enzymes PHD1, PHD2, and PHD3 as well as for the inflammatory cytokines IL-6 and TNF-α. Blood samples were taken every 2 hours during the study protocol and whole blood RNA was directly stabilised using the PAXgene RNA tubes (see section 2.2.2). Data are shown as relative gene expression compared to *ACTB* calculated as $2^{-(\Delta Ct)}$ values, displaying values of each human subject (mean ± standard deviation).

The gene expression of the transcription factor HIF-1α was significantly increased already 2 hours after LPS injection, peaked 4 hours after the injection and stayed elevated during all time points, including after 24 hours (figure 29, A: *HIF1A* baseline: 0.0024 ± 0.0006, +4 h: 0.0056 ± 0.0014, +24 h: 0.0051 ± 0.0008). *HIF2A* mRNA levels were much lower in general and had the tendency to be downregulated upon LPS injection at all measured time points (figure 29, B: *HIF2A* baseline: 0.00058 ± 0.00066, +24 h: 0.00013 ± 0.000087). The mRNA levels of HIF target genes involved in energy metabolism *GLUT-1* and *PDK1* displayed both a similar expression pattern upon LPS treatment: Both genes did not change 2 hours after LPS injection, but decreased 4 hours later (figure 29, C: *GLUT-1* baseline: 0.0054 ± 0.0013, +4 h: 0.0019 ± 0.0009, D: *PDK1* baseline: 0.00040 ± 0.00016, +4 h: 0.00030 ± 0.00015). The mRNA decrease measured at +4 h increased again after 6 and 8 hours and even exceeded baseline levels 24 hours after LPS injection (+24 h: C: *GLUT-1*: 0.0071 ± 0.0012, D: *PDK1*: 0.00065 ± 0.00016). It is to be noted that changes in *GLUT-1* mRNA were mostly significant while *PDK1* levels did only reach significance between +4 and +8 h. Interestingly gene expression of HIF target gene *ADM* acted opposed to *GLUT-1* expression by a gradual significant increase 2 and 4 hours after injection, which stayed upregulated until 8 hours after infection but decreased back to baseline level after 24 hours again (figure 29, E: baseline: 0.0026 ± 0.0009, +2 h: 0.0041 ± 0.0006, +4 h: 0.0089 ± 0.0026). *VEGF* mRNA levels were not affected by the LPS treatment and stayed quite stable with mean values from 0.00015 (+8 h) to 0.00019 (+2 h, figure 29, F). Gene expression of the HIF hydroxylases *PHD1* and *PHD2* followed a similar pattern than it was observed in the target genes *GLUT-1* and *PKD1*. Both genes did not change 2 hours after LPS injection but slightly decreased 4 hours after the stimulus (figure 29, G: *PHD1*: baseline and +2 h: 0.011 ± 0.004, +4 h: 0.0090 ± 0.0017, H: *PHD2*: baseline and +2 h: 0.0062 ± 0.0030, +4 h: 0.0054 ± 0.0021). Both genes peaked 8 hours after the injection to a mean value of 0.012 and decreased back to baseline levels after 24 hours. *PHD2* mRNA expression was in comparison to *PHD1* confirmed to display significant changes compared to baseline after 8 hours (figure 29, G, F). The regulation of PHD3 seemed to follow a different pattern: mRNA levels tended to decrease upon LPS injection and stayed that way even in the long term after

24 hours (figure 29, I: baseline 0.00068 ± 0.00039, +24 h: 0.00029 ± 0.00011). Gene expression of Interleukin-6 (*IL6*) seemed to be increased after 2 and 4 hours of LPS injection but back to even lower levels after 6 to 24 hours (figure 29, I: baseline 0.000015 ± 0.00016, +4 h: 0.000032 ± 0.0000026, +6 h - +24 h: 0.000003 – 0.000006). It has to be mentioned that *IL6* levels of some samples were below the detection limit, reducing the sample size of the analysis to 3 to 5 human subjects, depending on the time point, which reduces the validity of the data. Analysis of the second cytokine TNF-α revealed a gene expression increase 2 hours after the immune activation, which gradually decreased already after 4 hours back to baseline mRNA levels (figure 29, K: baseline 0.00081 ± 0.00028, +2 h: 0.0017 ± 0.00075, +4 h: 0.0011 ± 0.00054, +6 h - +24 h: 0.00073 – 0.00083).


A
HIF1A
relative gene expression
2-(ΔCt)
0.008
0.006
0.004
0.002
0.000
baseline
+2
+4
+6
+8
+24
time (h)
LPS injection
(0.4 ng/kg)
B
HIF2A
0.0020
0.0015
0.0010
0.0005
0.0000
C
GLUT-1
0.010
0.008
0.006
0.004
0.002
0.000
D
PDK1
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
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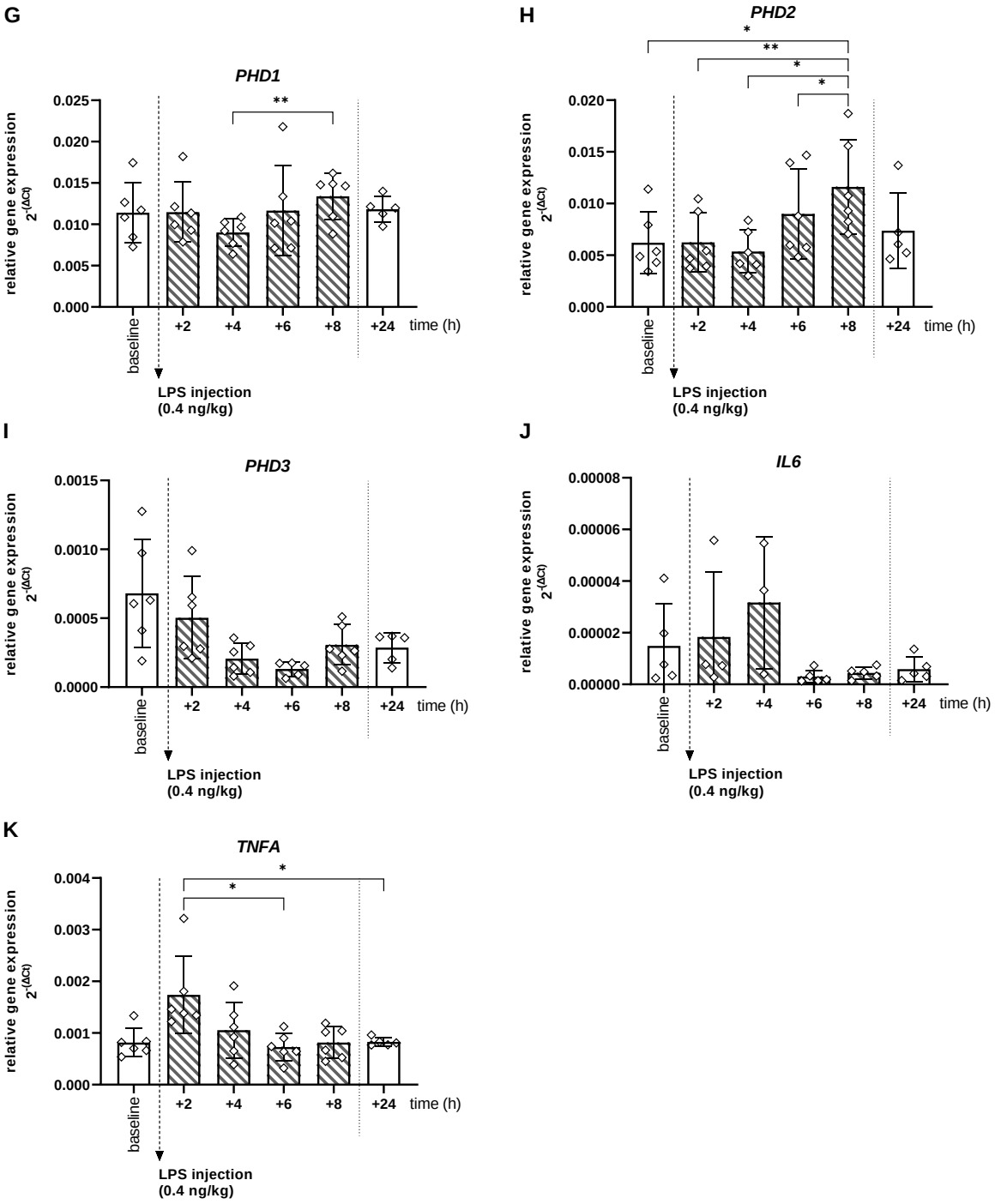


Figure 29: Gene expression of whole blood upon *in vivo* LPS treatment

Gene expression of whole blood cells, from blood, sampled from human subjects that were treated with a single *E. coli* lipopolysaccharide injection (0.4 ng/kg) and followed up for 24 hours. Investigated genes included *HIF1A* (A), *HIF2A* (B), *GLUT-1* (C), *PDK1* (D), *ADM* (E), *VEGF* (F), *PHD1* (G), *PHD2* (H), and *PHD3* (I), *IL6* (J), *TNFA* (K). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 6).

3.5.1.3 Effects of *in vivo* LPS treatment on gene expression of distinct circulating immune cell types

For complementation of whole blood analysis, gene expression analysis of specific genes was done in isolated human neutrophils, T cells, and monocytes (see section 2.2.3 and 2.2.5. The analysis included the genes from the prior analysis (*HIF1A*, *HIF2A*, *GLUT-1*, *PDK1*, *ADM*, *VEGF*, *PHD1*, *PHD2*, *PHD3*, *TNFA*, *IL6*) and as an add-on the less-studied hypoxia-inducible factor-3α (*HIF3A)* and the Toll-like receptors 2 and 4 (*TLR2*, *TLR4*) for determination of immune activation of neutrophils and monocytes. Gene expression changes were analysed via qRT-PCR and are presented as $2^{-(\Delta Ct)}$ values normalized to *ACTB* mRNA.

Gene expression of hypoxia-inducible factors 1 and 2 differed between the analysed immune cell types (figure 30). In neutrophils, *HIF1A* levels gradually increased with time upon the LPS injection, with significant upregulation of the 8-hour time point compared to 2, 4, and 6 h after the LPS injection (figure 30, A: baseline: 0.014 ± 0.005, + 24 h: 0.018 ± 0.004). *HIF1A* levels in T cells and monocytes were much lower and did not change significantly, although T cells seem to upregulate *HIF1A* mRNA upon the stimulus (figure 30, B: T cell baseline: 0.0042 ± 0.001, C: monocyte baseline: 0.0037 ± 0.0012). *HIF2A* gene expression levels were not significantly changed upon the LPS treatment, but neutrophils and T cells showed a slight upregulation of *HIF2A* 8 hours after the injection (figure 30, D: 0.00017 ± 0.00012, C: 0.00025 ± 0.00013).

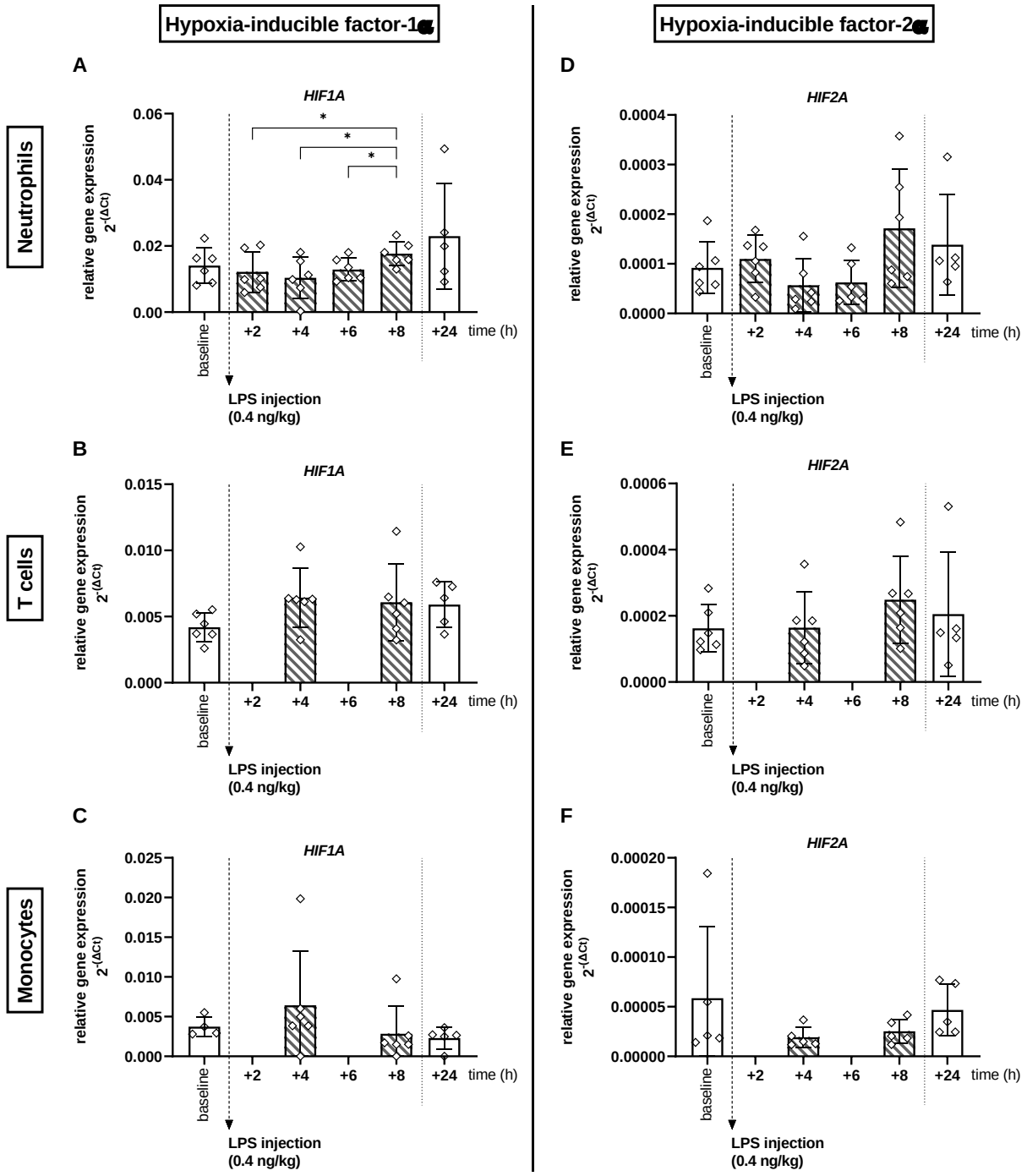


Figure 30: HIF1A and HI2A gene expression upon *in vivo* LPS treatment

Relative gene expression of hypoxia-inducible factor -1 and -2 alpha (*HIF1A* (A-C), *HIF2A* (D-F)), of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) and followed up for 24 hours. Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 6).

Similarly, *HIF3A* mRNA levels were found to be highest 8 hours after LPS injection in neutrophils and monocytes, while T cells showed upregulation already at the 4-hour time point (figure 31, A: +8 h: 0.00022 ± 0.00017, B: +4 h: 0.00007 ± 0.000062, C: +8 h: 0.000023 ± 0.000026). Target gene *VEGF* was slightly downregulated 6 hours after LPS injection, but the other cell types did not present any major changes upon the stimulation (figure 31, D -F).

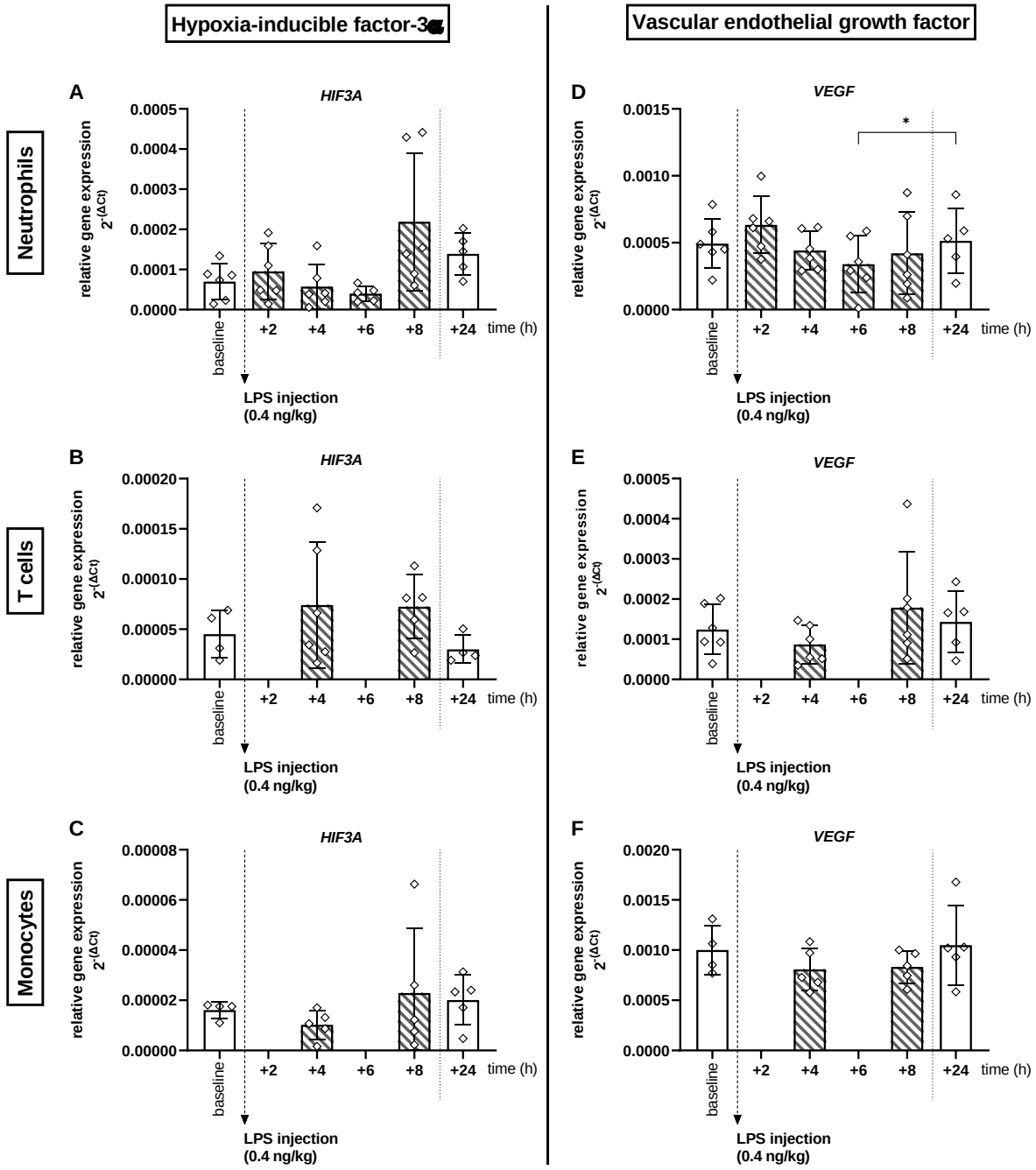


Figure 31: HIF3A and VEGF gene expression upon *in vivo* LPS treatment

Relative gene expression of hypoxia-inducible factor-3 alpha (*HIF3A,* A-C), and vascular endothelial growth factor (*VEGF*, D-F), of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) and followed up for 24 hours. Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 6).

Gene expression of HIF target genes *GLUT-1* and *PDK1* mostly reacted similar to the LPS stimulus in the immune cell types and decreased upon injection of LPS (figure 32, A, B, C, D, F). The exception was *PDK1* expression in T cells, which rose significantly after 4 hours compared to the control values (figure 32, E: baseline 0.0056 ± 0.0022, +4 h: 0.0089 ± 0.0034) and was calculated as significantly changed compared to the baseline and 24 h mRNA levels, like the reduction in monocytes 4 hours after LPS injection (figure 32, F).

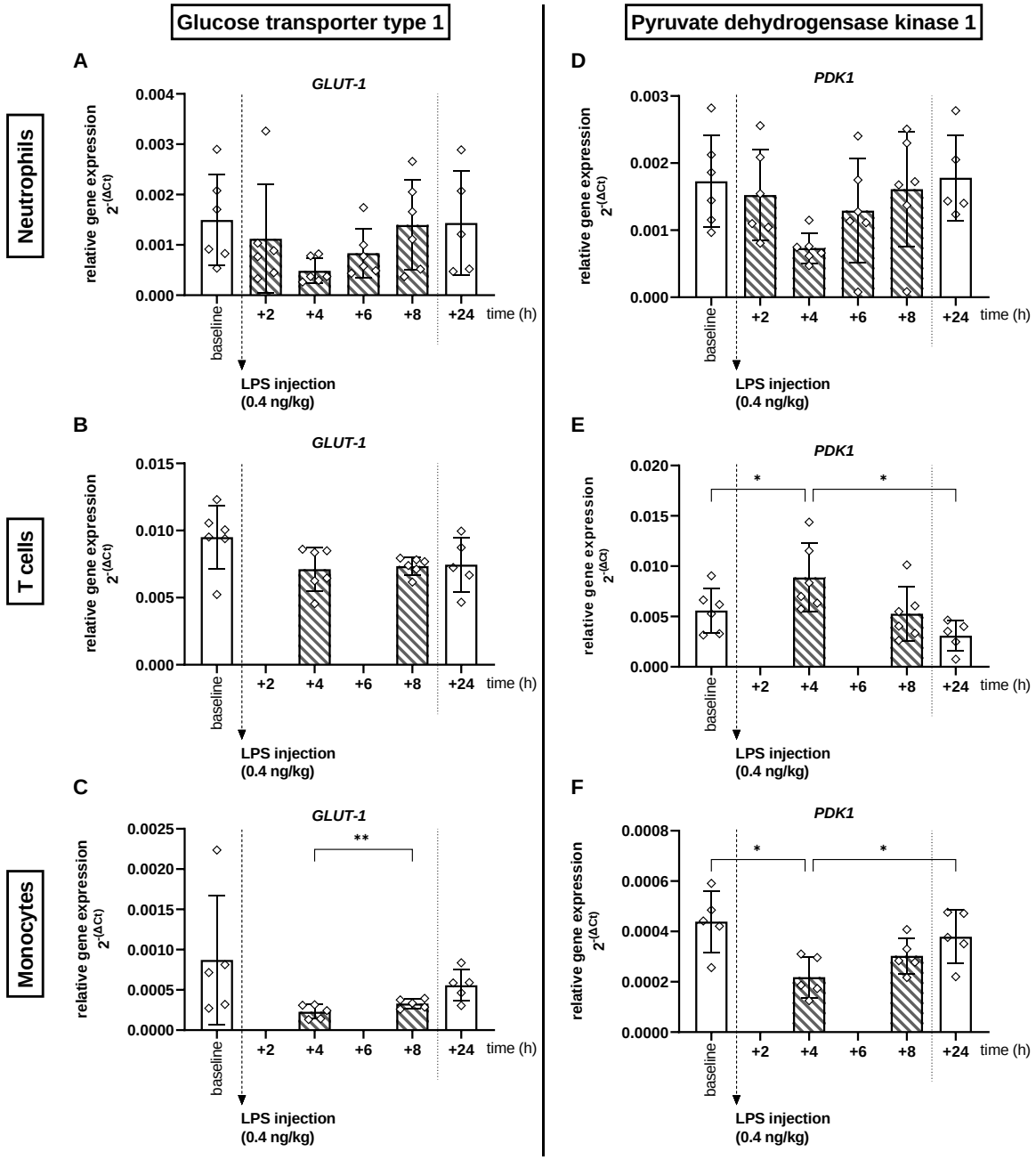


Figure 32: GLUT-1 and PDK1 gene expression upon *in vivo* LPS treatment

Relative gene expression of glucose transporter type 1 (*GLUT-1,* A-C), and pyruvate dehydrogenase kinase 1 (*PDK1*, D-F) of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) and followed up for 24 hours. Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, n = 6).

The levels of the HIF target gene *ADM* increased in all tested immune cell populations upon *in vivo* LPS stimulation but did only reach significance in monocytes between 4 and 8 hours after stimulation (figure 33, C: +4 h: 0.0028 ± 0.0011, +8 h: 0.00083 ± 0.0002, +24 h: 0.00066 ± 0.00017) and was much higher expressed in neutrophils compared to monocytes and least in T cells (figure 33, mean baseline *ADM* levels: A: 0.024, B: 0.000035, C: 0.00085). *PHD1* mRNA was not significantly affected by the LPS treatment but was slightly downregulated in neutrophils and monocytes (figure 33, A: baseline: 0.021 ± 0.008, +4 h: 0.013 ± 0.004, F: baseline: 0.000037 ± 0.000012, +4 h: 0.000016 ± 0.000005).

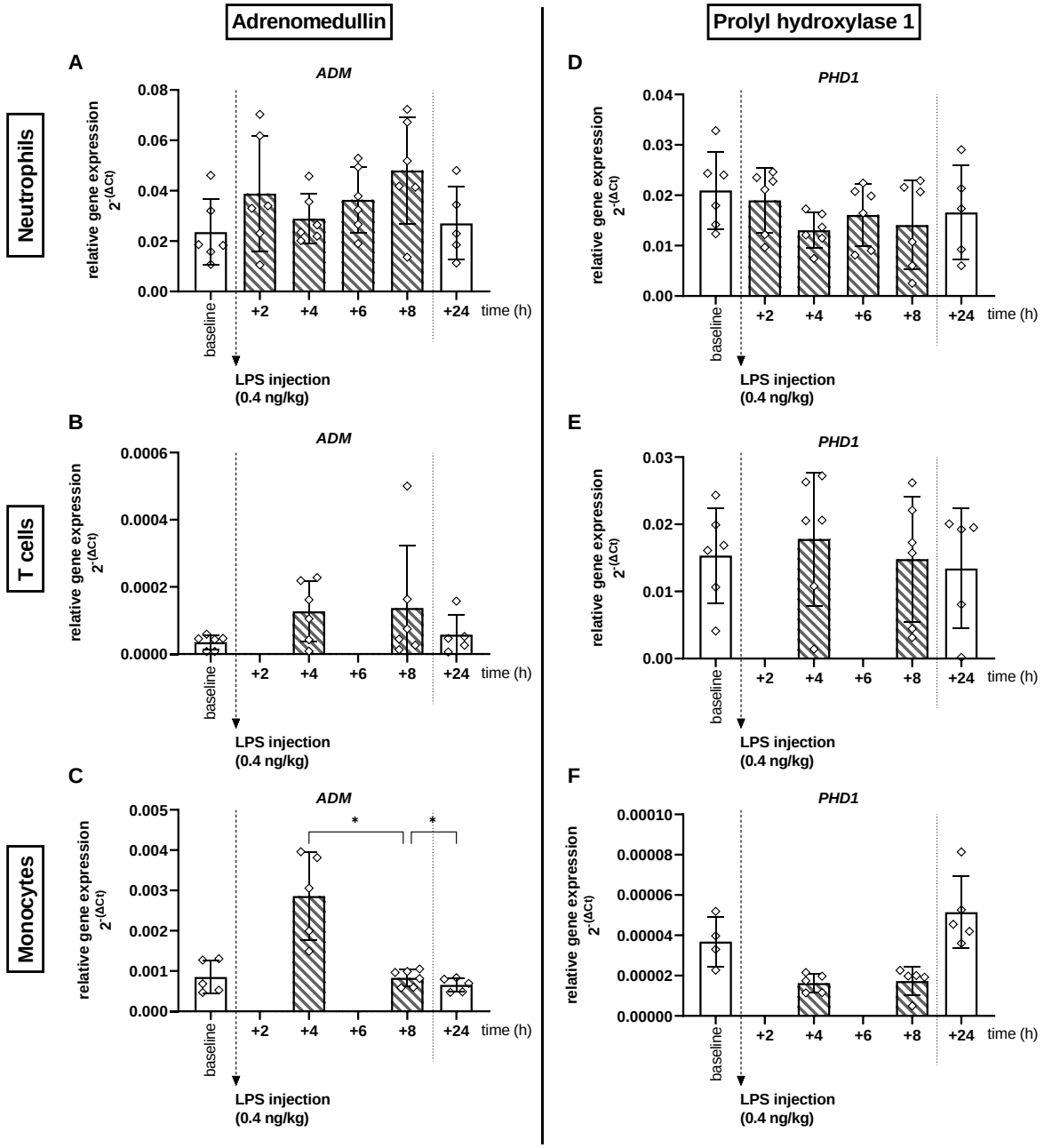


Figure 33: ADM and PHD1 gene expression upon *in vivo* LPS treatment

Relative gene expression of adrenomedullin (*ADM,* A-C), and prolyl hydroxylase 1 (*PHD1*, D-F) of isolated neutrophils, T cells and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) and followed up for 24 hours. Expression levels of mRNA were normalised to *ACTB* and

are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, n = 6).

PHD2 gene expression decreased 4 hours after the LPS injection, but then increased above baseline levels after 8 hours in neutrophils (figure 34, A: baseline: 0.019 ± 0.012, +4 h: 0.0069 ± 0.0040, +8 h: 0.029 ± 0.016). T cells and monocytes displayed hardly any changes in *PHD2* mRNA levels but had their highest mean mRNA levels also at the 8-hour time point (figure 34, B, C). *PHD3* levels were significantly downregulated 4 hours after injection in neutrophils but not in the other cell types (figure 34, D: baseline: 0.00011 ± 0.00006, +4 h: 0.000027 ± 0.000021).

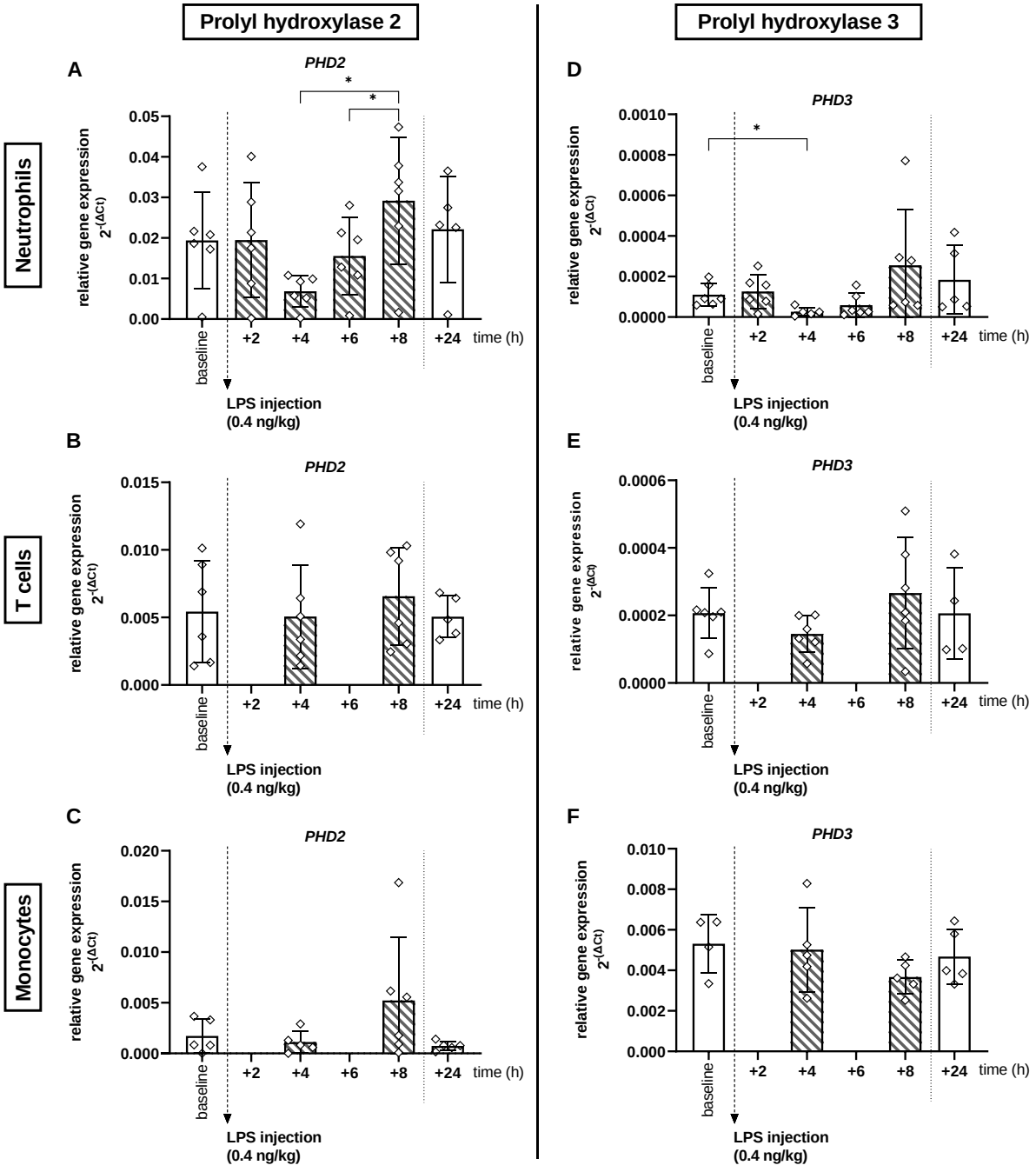


Figure 34: PHD2 and PHD2 gene expression upon *in vivo* LPS treatment

Relative gene expression of prolyl hydroxylase 2 (*PHD2,* A-C), and prolyl hydroxylase 3 (*PHD3*, D-F) of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) and followed up for 24 hours. Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 6).

Strongest inflammatory response to the *in vivo* LPS treatment was seen in neutrophils by upregulated *TNFA* mRNA levels 2 hours after the injection (figure 35, A: baseline: 0.0025 ± 0.0012, +2 h: 0.012 ± 0.006) and in monocytes 4 hours after application of the LPS dose (figure 35, C: baseline: 0.019 ± 0.0007, +4 h: 0.028 ± 0.007, +8 h: 0.012 ± 0.003, +24 h: 0.016 ± 0.005). T cells showed no clear changes upon stimulation regarding *TNFA* expression (figure 35, B). The mRNA levels of the cytokine Interleukin 6 seemed to be upregulated as well after 4 hours in neutrophils and monocytes and after 8 hours in T cells, but due to experimental outliers and values staying below the detection limit sample size was partly decreased down to 2 participants, which does not allow to draw clear conclusions (figure 35, D - F).

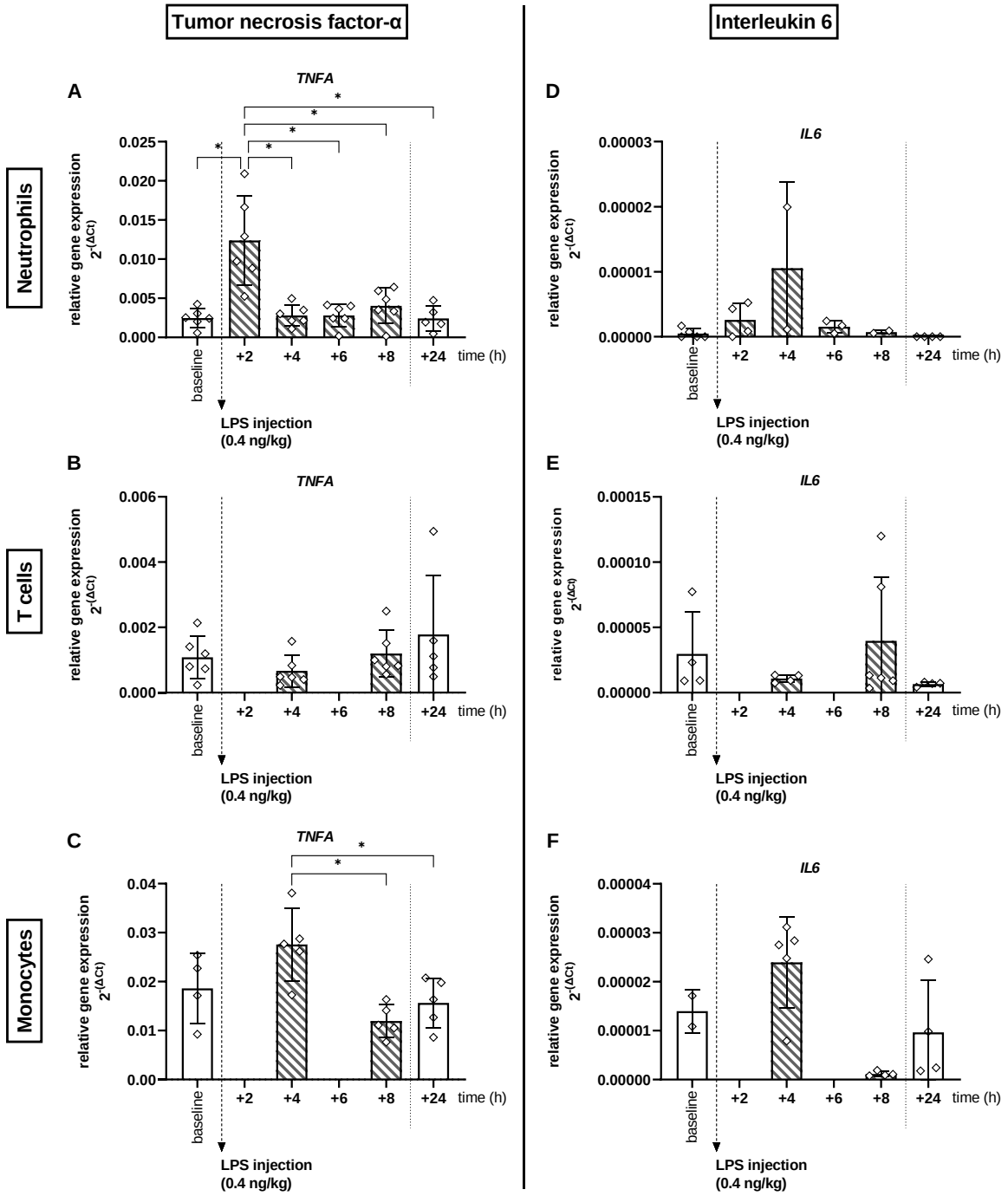


Figure 35: TNFA and IL6 gene expression upon *in vivo* LPS treatment

Relative gene expression of tumour necrosis factor-alpha (*TNFA,* A-C), and Interleukin 6 (*IL6*, D-F), of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) and followed up for 24 hours. Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 6).

Analysis of the mRNA expression of Toll-like receptors in neutrophils and monocytes revealed that *TLR2* was downregulated in both cell types 4 hours after LPS injection but back to baseline levels within the next 2 or 4 hours, respectively (figure 36, A: baseline: 0.058 ± 0.022, +4 h: 0.036 ± 0.002, +6 h: 0.052 ± 0.028, B: baseline: 0.0013 ± 0.0005, +4 h: 0.00094 ± 0.00036, +8 h: 0.0014 ± 0.00047). *TLR4* gene expression was gradually upregulated from 0.016 (± 0.0048, baseline) to 0.035 (± 0.011) 8 hours after LPS application (figure 36, C) in neutrophils, while *TLR4* expression in monocytes followed the same pattern as *TLR2* (figure, B, D: baseline: 0.0045 ± 0.0021, +4 h: 0.0027 ± 0.0011).

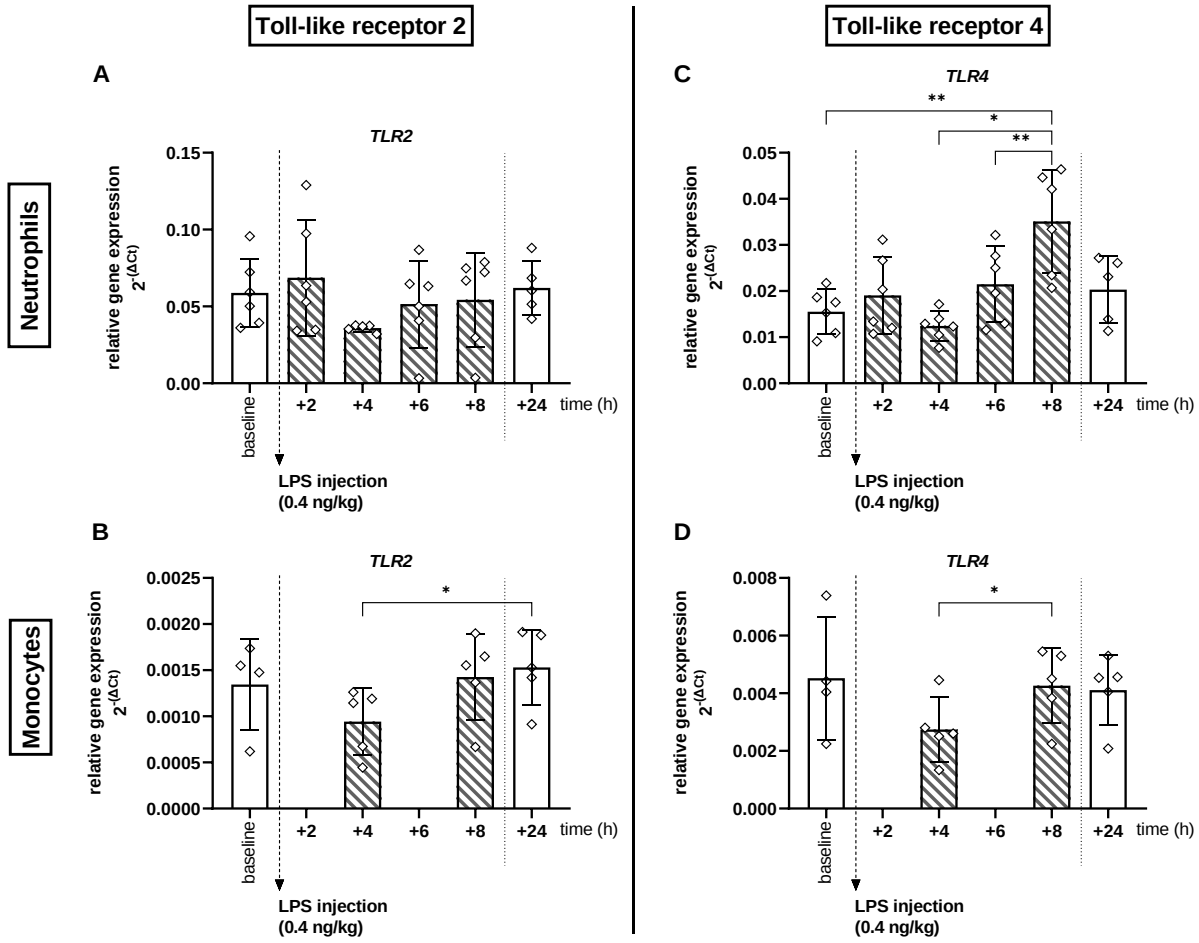


Figure 36: TLR2 and TLR4 gene expression upon *in vivo* LPS treatment

Relative gene expression of the Toll-like receptors 2 (*TLR2,* A, B), and 4 (*TLR4*, C, D), of isolated neutrophils and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) and followed up for 24 hours. Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, n = 6).

3.5.1.4 Validation of purity of isolated immune cells

To correctly classify our results, we verified our method of choice for the isolation of distinct immune cell types (section 2.2.3). According to the manufacturer's manuals of the used immune cell isolation kits, the isolation of neutrophils, T cells, and monocytes via negative selection and magnetic beads should have been highly accurate. To be sure about the composition of the isolated human cells we used antibody phenotype staining and flow cytometry analysis (see section 2.2.7). The gating strategies are shown in the supplements (figures 61 - 63). Flow cytometry analysis proved that isolated cells from whole blood samples fulfilled the purity specifications of the manufacturers (figure 37, A). Using the respective kits yielded either 98.9 % of neutrophils, 95.0 % of T cells ($CD45^+$ $CD3^+$), or 82.7 % of monocytes ($CD\ 45^+$ $CD14^+$) out of a whole blood sample on average (figure 37, A). To interpret our data more reliably we further analysed the cellular composition of isolated T cells in our sample by flow cytometry. Classification of T cells ($CD45^+$ $CD3^+$) showed that 55.1 % of cells could be assigned clearly as helper T cells ($CD3^+$ $CD4^+$) and 36.5 % as cytotoxic T cells ($CD3^+$ $CD8^+$) with a significant difference between the subgroups (figure 37, B).

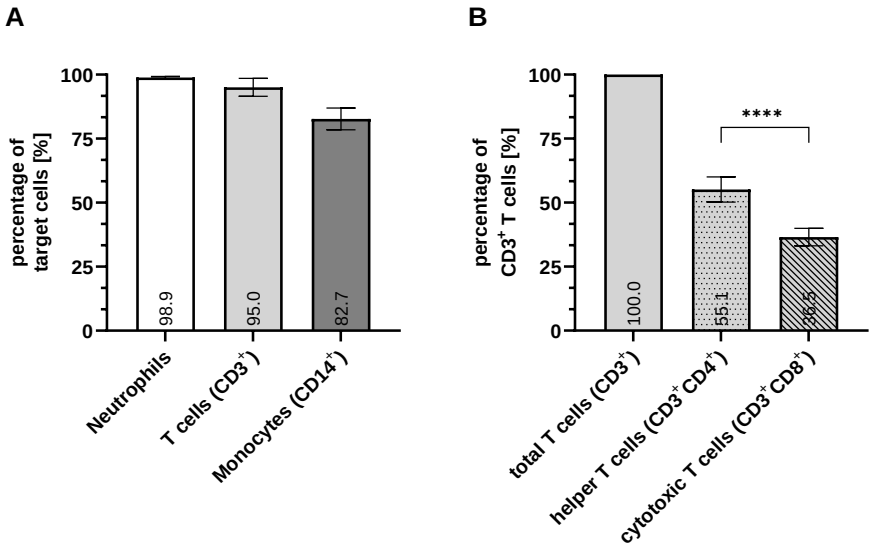


Figure 37: Classification of isolated human immune cells

Flow cytometry analysis showed that the magnetic bead isolation method yielded purities of isolated cells from 98.9 % (neutrophils) to 82.7 % (monocytes) (A). Further classification of isolated T cells (CD45+ CD3+) showed that 55.1 % of cells were helper T cells (CD3+ CD4+) and 36.5 % cytotoxic T cells (CD3+ CD8+) (B). CD: cluster of differentiation. Data evaluation: Mean values ± SD, ordinary one-way ANOVA (**** = $p < 0.0001$), n = 3 with 30.000 detected events each.

3.5.2 Effects of *in vivo* LPS treatment followed by exposure to hypoxia

In a second branch of the human subject study, we were keen to investigate if and how hypoxia could shape the immune response to an underlying inflammation *in vivo* (see section 2.2.1.2). For this purpose, we treated 15 volunteers with a single *E. coli* LPS injection (0.4 ng/kg) after 4 hours in normoxia with a 4-hour lasting exposure to hypoxia with 10.5 % normobaric O_2, simulating a high altitude of 4500 meters. As mentioned before, participants were examined and donated blood before the study and at the time points of 2 h, 4 h, 6 h, 8 h, and 24 h after the LPS injection (figure 38). Blood sampling was followed by direct processing of material for flow cytometry analysis, immune cell isolation, and RNA isolation.

Blood oxygen concentrations (SpO_2) decreased from around 98 % to a mean of 83.5 % after 4 hours of hypoxia (time point "+8 h", figure 38).

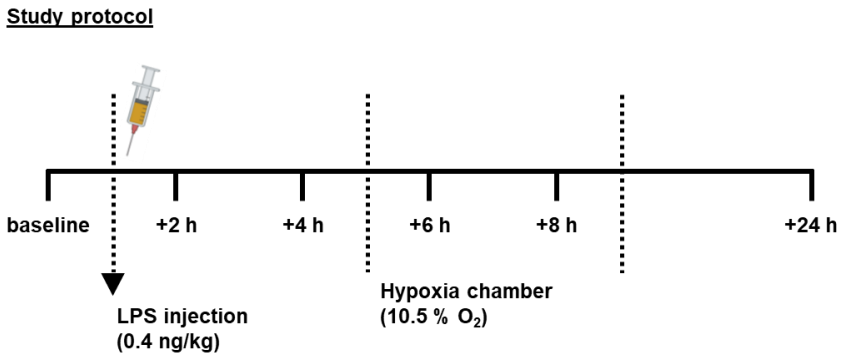


Figure 38: Study protocol of *in vivo* LPS treatment followed hypoxia

Schematic representation of the study protocol of human subjects that were treated with a single *E. coli* lipopolysaccharide injection (0.4 ng/kg) followed by 4 hours of hypoxic exposure (normobaric, 10.5 % O_2). Times of blood sampling for further analysis included "baseline" levels, shortly before the start of the treatment and indicated time points at 2, 4, 6, 8, and 24 hours after the treatment.

3.5.2.1 Flow cytometry analysis of immune cell populations upon *in vivo* challenge with LPS followed by hypoxia

To investigate the effects of acute inflammation followed by exposure to hypoxia on the composition and activation of circulating immune cells we conducted fluorescent antibody staining combined with flow cytometry analysis using the blood samples of subjects (see section 2.2.8). The general composition of cells was determined via the so-called "phenotype staining" (section 2.2.8.1), which was carried out in this study by Marie Jakobs from the Institute of Medical Psychology and Behavioral Immunobiology at the University Hospital Essen, Germany. In addition, the circulating monocyte population was characterised in terms of immune activation and monocyte subgroups (section 2.2.8.2).

Composition of circulating immune cells in humans challenged with *in vivo* LPS prior to hypoxia

Flow cytometry analysis of blood cells showed that *in vivo* LPS injection led to a fast and significant increase of granulocytes from 58.2 to 77.7 % of leukocytes ($CD45^+$ cells) already 2 hours after injection (figure 39, A). In line with that, T lymphocytes decreased significantly upon the inflammatory stimulus after the injection (figure 39, B – D, F). The same was seen in monocytes, which decreased from 9.8 to 1.9 % among all measured leukocytes 2 hours after LPS treatment (E) and in B lymphocytes 4 hours after treatment (3.9 % to 1.1 % (F), figure, 39). Upon the following exposure to 10.5 % O_2 hypoxia levels of circulating granulocytes decreased significantly while levels of T cells increased significantly. In both cell types there seemed to be a slight time-dependent return to baseline levels, but percentages of circulating cells were still significantly changed after 8 hours compared to baseline levels (figure 39, A-D). T helper cells and cytotoxic T cells showed the same pattern during the study protocol, with the difference of T helper cells being more present at all time points than cytotoxic T cells among all leukocytes in the bloodstream (baseline percentages: 14.4 % vs. 8.1 %, figure 39, C, D). Monocytes were upregulated back to baseline levels 6 hours after the LPS injection, corresponding to 2 hours upon hypoxia, and stayed at similar levels (+6 h: 11.1 %, figure 39, E). B cells returned also closer to baseline levels upon exposure to hypoxia for 2 and 4 hours (figure 39, F). None of the investigated cell populations showed any long-term changes upon the treatment, meaning cell percentages were back to baseline levels 24 hours after the start of the study (figure 39).

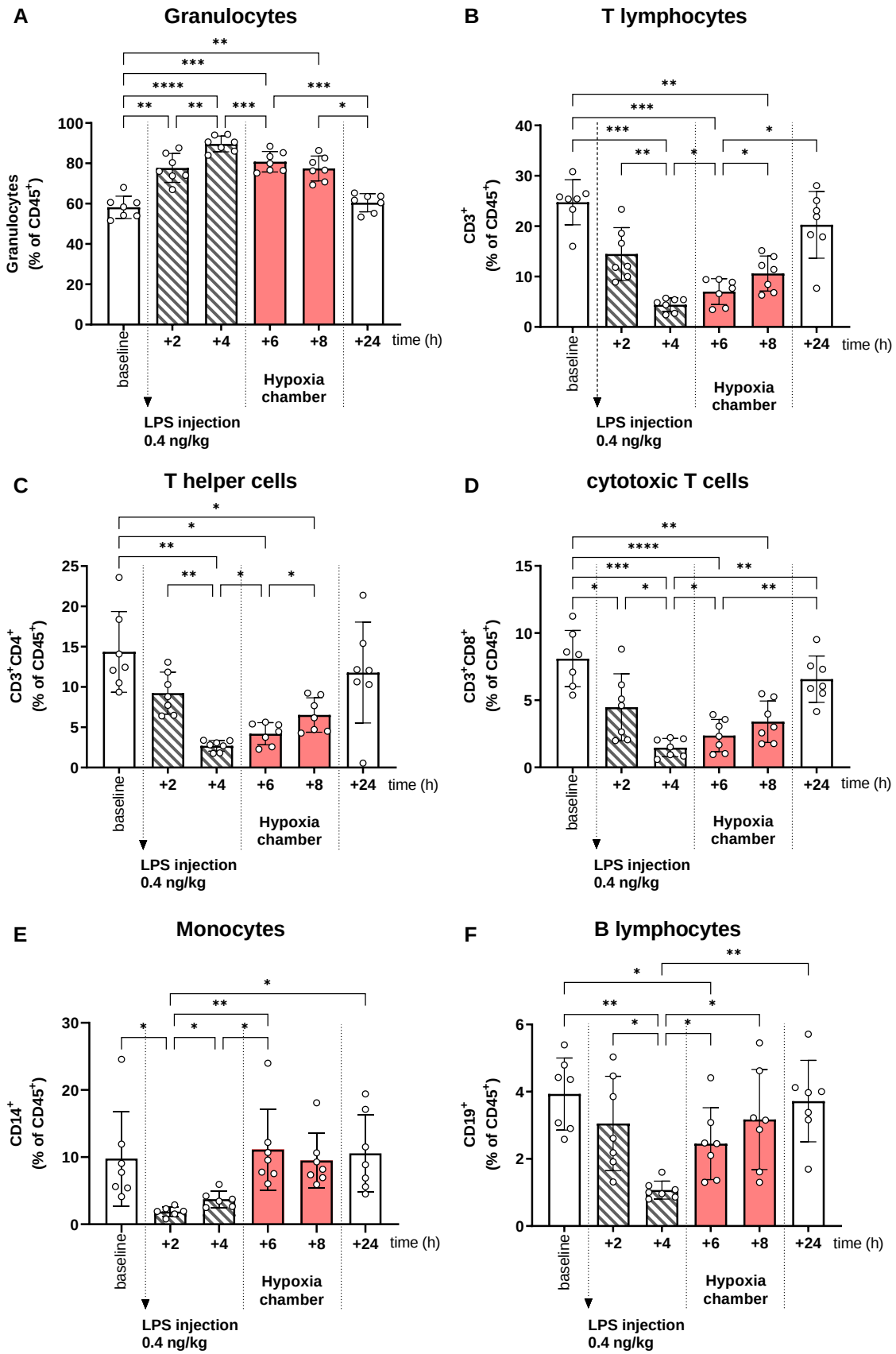


Figure 39: Composition of blood cells upon *in vivo* LPS treatment prior to hypoxia

Flow cytometry analysis of cellular composition in whole blood from participants challenged with *an in vivo* lipopolysaccharide (LPS) injection (0.4 ng/kg) 4h before exposure to hypoxia (4h, 10.5 % O_2) Investigated were the

percentages of granulocytes (A, negative gating), lymphocytes ($CD3^+$, B), T helper cells ($CD3^+CD4^+$, (C), cytotoxyic T cells ($CD3^+CD8^+$, D), monocytes ($CD14^+$, E) and B lymphocytes ($CD19^+$, F) among all measured circulating blood cells via flow cytometry and specific gating (see supplements, figure 61-63). Displayed are mean percentage ± SD (mixed-effects analysis with repeated measures, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, with not all significances being illultrated in the graphic, n = 7, measurements by Marie Jakobs).

Characterisation and activation of monocytes of humans challenged with LPS treatment prior to exposure to hypoxia

We further characterised the subgroups of circulating monocytes throughout the progression of the study via fluorescent antibody staining and flow cytometry analysis (see section 2.2.8). Activation of monocytes was assessed by the determination of Toll-like receptor 4 (TLR4) and the combination of CD86 and HLA-DR expression. *In vivo* LPS treatment induced a significant increase of classical monocytes from 77.8 % to 94.2 % 4 hours after the LPS injection. The following 4-hour long hypoxic phase did not change those levels and 24 hours after the start of the study classical monocytes dropped down to 62.5 % of all measured monocytes (figure 40, A). Intermediate and nonclassical monocytes displayed the same picture as seen in participants that were solely stimulated with LPS: Both monocyte subgroups displayed a decrease in cell count 4 hours after the stimulation but were back to baseline levels (nonclassical) or even upregulated (intermediate) after 24 hours (figure 40, B, C). There was a tendency of upregulation of TLR4 expressing cells 2 hours after the LPS injection (1.4 %) but apart from that percentages varied only slightly around 0.59 to 1.0 % (figure 40, D). Cells expressing the monocyte activation markers CD86, and HLA-DR were significantly downregulated upon LPS treatment for over 6 hours (from 55.4 % to a minimum of 31.4 % after 4 hours of LPS injection) and again upregulated to baseline levels 8 hours after the LPS injection or upon exposure to hypoxia for 4 h (53.0 %, figure 40, E).

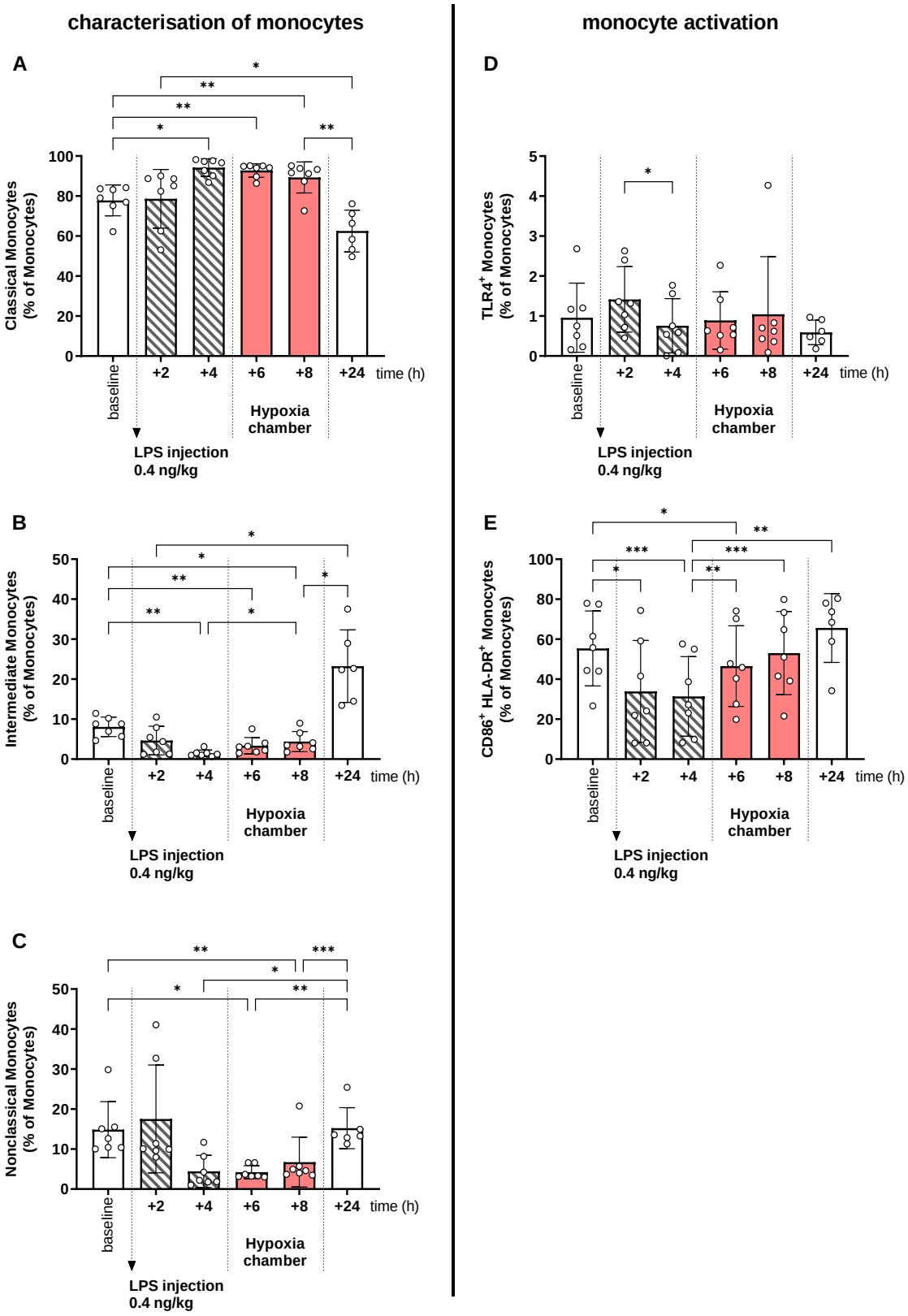


Figure 40: Characterisation of monocytes treated with *in vivo* LPS prior to hypoxia

Percentages of monocyte subsets, isolated from blood samples of human subjects that were treated with a single *E. coli* LPS injection (0.4 ng/kg) and were exposed to 10.5 % oxygen hypoxia 4 hours later. Investigated was the distribution of classical (A), intermediate (B), and non-classical (C) monocytes among all measured circulating monocytes via flow cytometry and specific gating of CD14/CD16 positive cells (see supplements, figure 60).

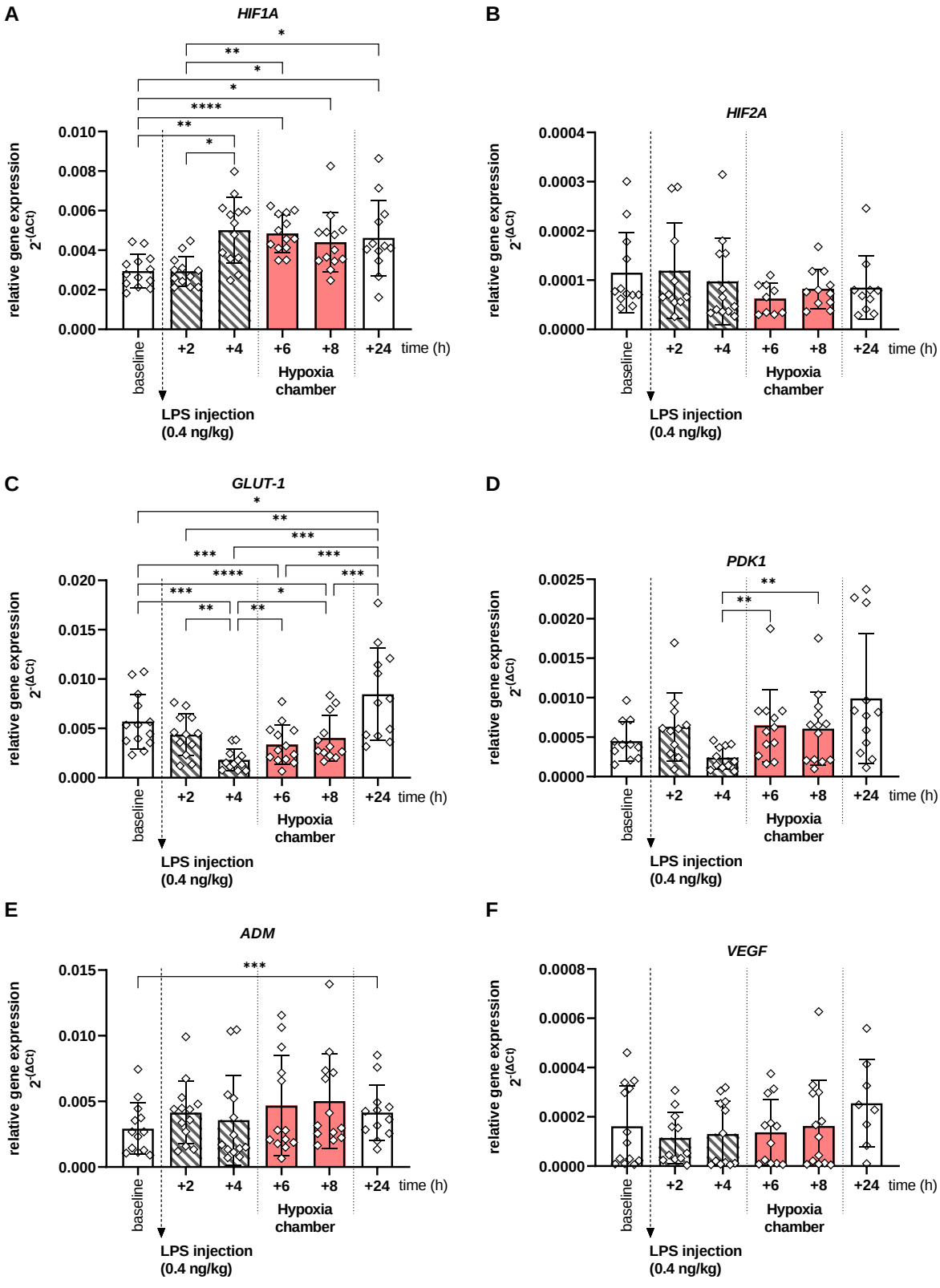
Activated monocytes were identified via detection of Toll-like receptor 4 (TLR4, D) or CD86/HLA-DR expression (E). mean percentage ± SD ((mixed-effects analysis with repeated measures, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 6).

3.5.2.2 Effects of *in vivo* LPS treatment followed by exposure hypoxia on gene expression of whole blood cells

We were further interested in gene expression changes induced by the combined *in vivo* treatment of the LPS injection 4 hours prior to the 4-hour exposure to hypoxia (10.5 % oxygen) in this branch of the subject study. To investigate if and how acute hypoxia shapes the inflammatory response on gene expression level we conducted qRT-PCR of whole blood samples from 13 participants (see sections 2.2.1.2 and 2.2.5). Thereby we focussed on the already mentioned genes (*HIF1A*, *HIF2A*, *GLUT-1*, *PDK1*, *ADM*, *VEGF*, *PHD1*, *PHD2*, *PHD3*, *TNFA*, *IL6*). Data are shown as relative gene expression compared to *ACTB* calculated as $2^{-(\Delta Ct)}$ values, displaying values of each human subject (mean ± standard deviation, n = 13).

HIF1A mRNA was first significantly elevated 4 hours after the LPS injection and stayed upregulated during the whole study procedure until 24 hours later compared to the baseline levels (figure 41, A: baseline: 0.0029 ± 0.00083, +4 h: 0.0050 ± 0.0017, +24 h: 0.0046 ± 0.0019). Gene expression of *HIF2A* was hardly affected during the study procedure but decreased slightly at the 6- and 8-hour time point and stayed low until the last measurement (figure 41, B: baseline: 0.00012 ± 0.00008, +6 h: 0.000063 ± 0.00003, +8 h: 0.000082 ± 0.00004). Target gene *GLUT-1* showed, like in the LPS control group (figure 29, A) significant expression change 4 hours after the LPS injection compared to baseline levels (figure 41, C: baseline: 0.0057 ± 0.0028, +4 h: 0.0018 ± 0.0011). Afterward, *GLUT-1* mRNA levels increased again significantly 2 h and 4 h after the participants entered the hypoxic chamber but were always below baseline levels (figure 41, C). 24 hours after the treatment *GLUT-1* gene expression was even increased compared to the control values (figure 41, C: +24 h: 0.0085 ± 0.0047). *PKD1* mRNA displayed some fluctuations with the tendency to increase 2 hours after the *in vivo* LPS stimulus but decreased again even below baseline levels after additional two hours (figure, D: baseline: 0.00045 ± 0.00025, +2 h: 0.00063 ± 0.00043, +4 h: 0.00024 ± 0.00014). Upon the following 4 hours of hypoxic exposure, *PDK1* mRNA levels were again elevated to a similar amount than at the 2-hour time point, with a tendency of long-term upregulation (figure 41, D: +6 h: 0.00065 ± 0.00046, +8 h: 0.00061 ± 0.00046, +24 h: 0.00099 ± 0.00082). In contrast to the reversed version of the study *ADM* mRNA levels were only significantly upregulated at the 24-hour time point compared to baseline levels (figure 41, E: baseline: 0.0029 ± 0.0020, +24 h: 0.0041 ± 0.0021). The LPS injection and exposure to hypoxia seemes to create a slight increase in *ADM*, but without statistical significance (figure 41, E). Expression of the HIF target gene *VEGF* did also not change during the study and ranked

around mean $2^{-(\Delta Ct)}$ values of 0.00011 to 0.00025 including high standard deviations between the participants (figure 41, F). *PHD1* gene expression decreased down significantly 2 hours after the LPS injection and 4 hours upon hypoxic exposure (figure 41, +2 h: 0.0014 ± 0.0009, +8 h: 0.0023 ± 0.0018). Interestingly *PHD2* mRNA did not change upon LPS treatment but displayed a significant increase when participants were situated in the hypoxic chamber (figure 41, H: baseline: 0.0053 ± 0.0025, +6 h: 0.011 ± 0.009). The last examined hydroxylase PHD3 showed a significant decrease of mRNA 4 hours after LPS injection, while the following hypoxia seemed to increase gene expression levels again (figure 41, I: baseline: 0.00037 ± 0.00018, +4 h: 0.00021 ± 0.00014, +6 h: 0.00048 ± 0.00036). Gene expression levels of the inflammatory cytokine TNF-a were elevated upon LPS treatment but did not reach significant levels and decreased again in the second part of hypoxic exposure (figure 41, J: baseline: 0.00093 ± 0.00060, +2 h: 0.0020 ± 0.0014, +6 h: 0.00076 ± 0.00045). *IL6* mRNA increased upon LPS treatment significantly and stayed elevated in some participants through the hypoxic treatment, while it decreased again in others creating a high deviation between the samples (figure 41, K: baseline: 0.00039 ± 0.00046, +2 h: 0.0017 ±0.0012, +8 h: 0.0018 ± 0.0016).


A
HIF1A
relative gene expression
2-(ΔCt)
0.010
0.008
0.006
0.004
0.002
0.000
baseline
+2
+4
+6
+8
+24
time (h)
Hypoxia chamber
LPS injection (0.4 ng/kg)
B
HIF2A
0.0004
0.0003
0.0002
0.0001
0.0000
C
GLUT-1
0.020
0.015
0.010
0.005
0.000
D
PDK1
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
E
ADM
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VEGF
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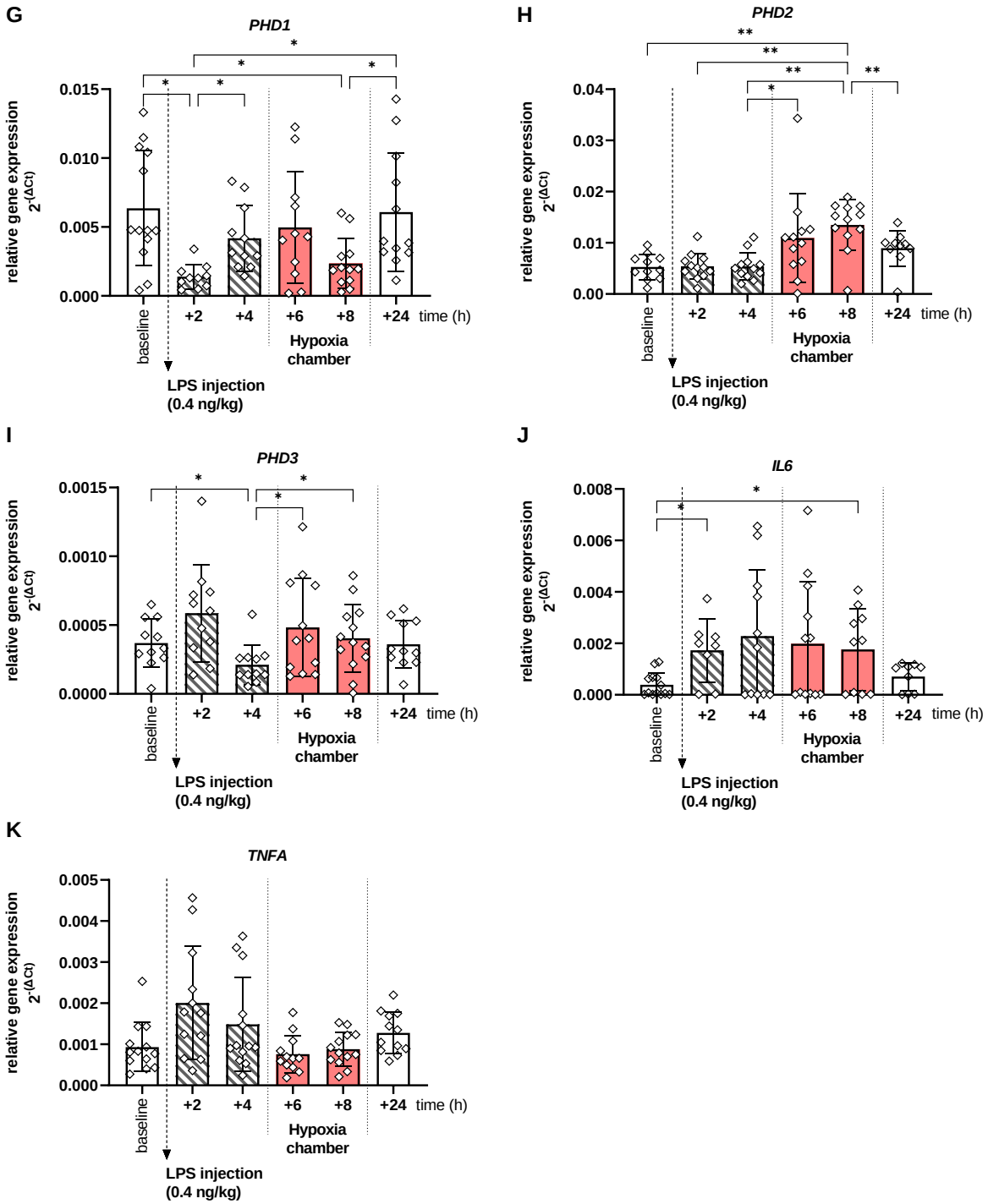


Figure 41: Gene expression of whole blood upon LPS treatment prior to hypoxia

Gene expression of whole blood cells, from blood samples of human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) followed by a 4-hour phase of exposure to hypoxia (normobaric, 10.5 % O_2). Investigated genes included *HIF1A* (A), *HIF2A* (B), *GLUT-1* (C), *PDK1* (D), *ADM* (E), *VEGF* (F), *PHD1* (G), *PHD2* (H), and *PHD3* (I), *IL6* (J), *TNFA* (K). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, n = 13).

3.5.2.3 Effects of *in vivo* LPS treatment followed by hypoxia in distinct immune cell subsets

To specify the adaption of circulating leukocytes to *in vivo* LPS treatment (0.4 ng/kg) followed by hypoxia (10.5 % O_2) neutrophils, T cells, and monocytes were directly isolated from blood samples at the respective time points and prepared for RNA isolation (see sections 2.2.3 and 2.2.5). Relative gene expression changes were analysed for *HIF1A*, *HIF2A*, *HIF3A*, *GLUT-1*, *PDK1*, *ADM*, *VEGF*, *PHD1*, *PHD2*, *PHD3*, *TNFA*, *IL6, TLR2,* and *TLR4* via qRT-PCR. Data of 9 participants were included in the analysis and are presented as $2^{-(\Delta Ct)}$ values normalized to *ACTB* mRNA.

HIF1A mRNA gradually increased in neutrophils following the two treatment steps of LPS and hypoxia but did not reach significant differences to baseline levels (figure 42, A). In T cells *HIF1A* gene expression was elevated significantly between the 4 and 8-hour time point and was even higher 24 hours after the study started (figure 42, B, +4 h: 0.0037 ± 0.0012, + 8h: 0.0050 ± 0.0008, +24 h: 0.0064 ± 0.0024). Monocytes did not adapt *HIF1A* gene expression upon LPS or hypoxia (figure 42, C). Transcripts of the *HIF2A* gene were not changed significantly in any of the tested cell populations either (figure 42, D - F).

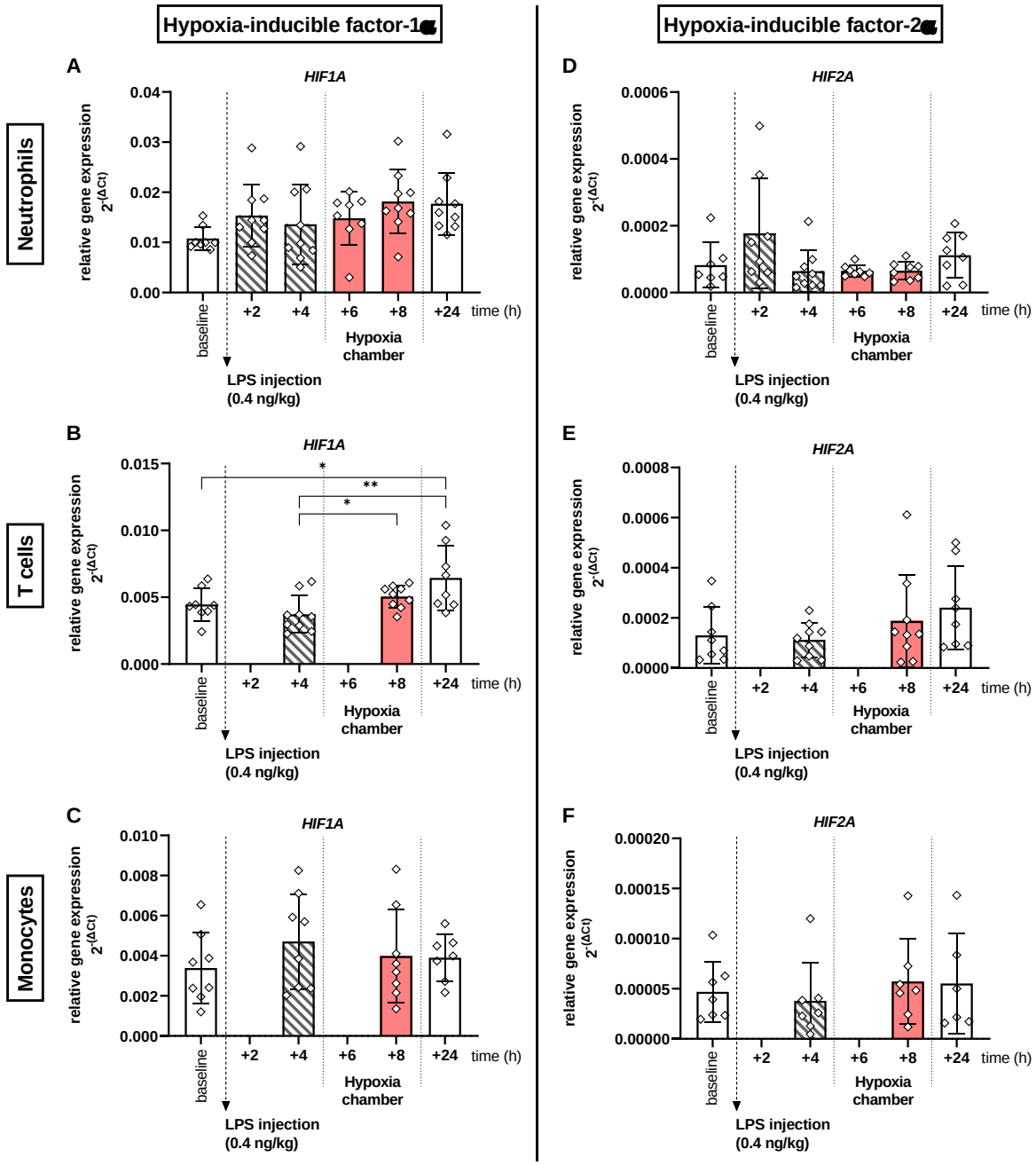


Figure 42: HIF1A and HI2A gene expression upon *in vivo* LPS and hypoxia

Relative gene expression of hypoxia-inducible factor -1 and -2 alpha (*HIF1A* (A-C), *HIF2A* (D-F)), of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) followed by exposure to hypoxia (normobaric, 10.5 % O_2, 4h). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, n = 9).

A similar gene expression pattern with no clear changes upon stimulation was found in terms of *HIF3A* mRNA, where levels increased 2 hours after the LPS injection in neutrophils but went back to baseline levels afterward and were not notably changed in the other cell types (figure 43, A-C). Furthermore, the only significant change in terms of *VEGF* expression was found in neutrophils between 4 and 6 hours into the study procedure (figure 42, D: + 4h: 0.00052 ±

0.000044, +6 h: 0.00028 ± 0.00020). T cells and monocytes displayed a slight tendency to upregulate *VEGF* expression after 4 hours of hypoxia and in the long term, but the changes did not reach significant levels (figure 43, E, F).

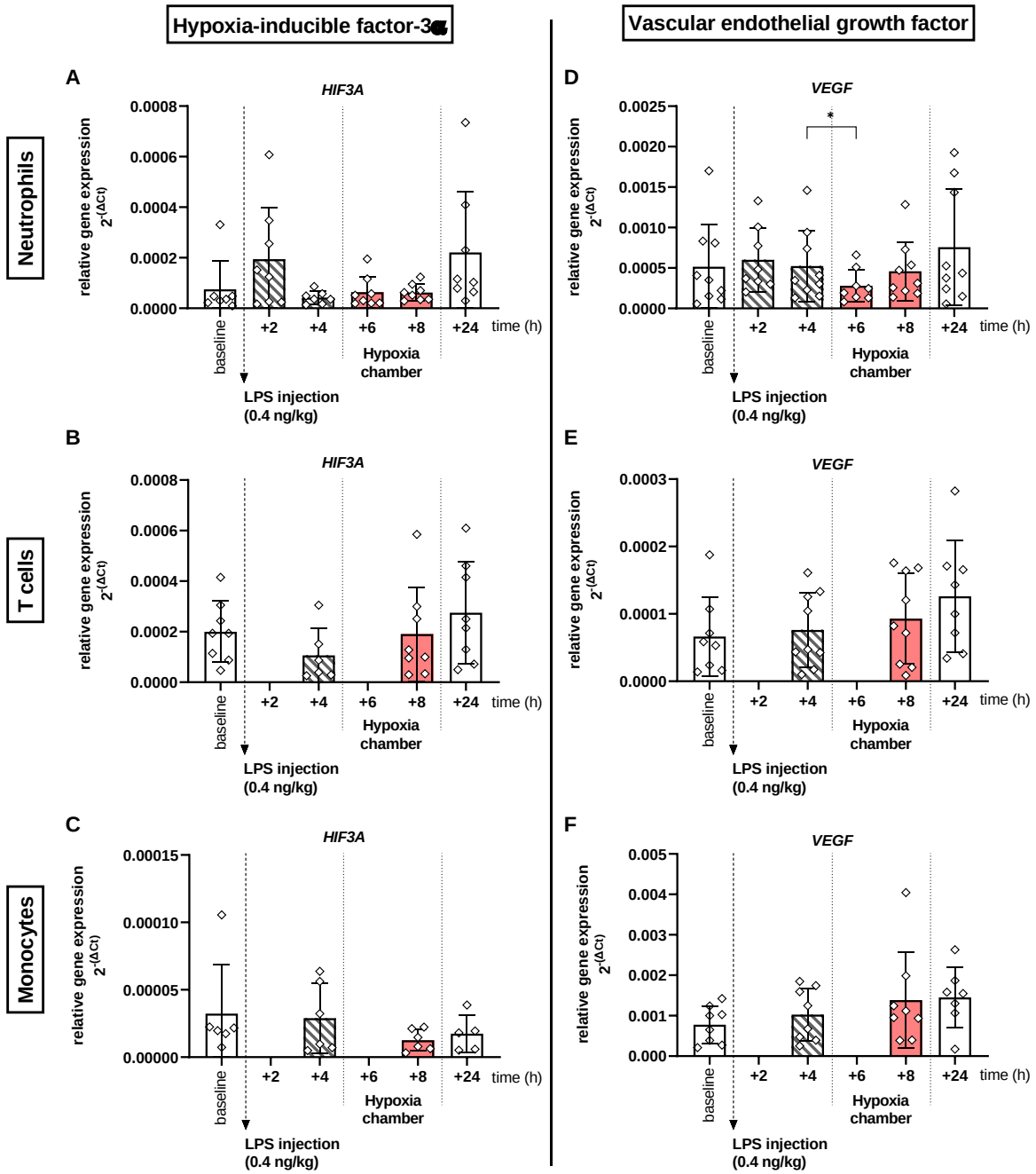


Figure 43: HIF3A and VEGF gene expression upon *in vivo* LPS and hypoxia

Relative gene expression of hypoxia-inducible factor-3 alpha (*HIF3A,* A-C), and vascular endothelial growth factor (*VEGF*, D-F), of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) followed by exposure to hypoxia (normobaric, 10.5 % O_2, 4h). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 9).

Gene expression levels of *GLUT-1* were significantly decreased compared to baseline levels in all analysed cell types 4 hours after the *in vivo* LPS injection but again increased back to control levels after 4 hours in hypoxia (figure 44, A: baseline: 0.0011 ± 0.00007, +4 h: 0.00037 ± 0.00018, B: baseline: 0.0094 ± 0.0037, +4 h: 0.0056 ± 0.0020, C: baseline: 0.00049 ± 0.00034, +4 h: 0.00017 ± 0.00008). *PDK1* gene expression followed a similar expression pattern to *GLUT-1* in neutrophils and monocytes, but *PDK1* downregulation at the 4-hour time point in neutrophils was proven to be significant only compared to the 24-hour time point not to baseline levels (figure 44, D, F). Further, *PDK1* mRNA levels were significantly upregulated from 2 to 4 hours under hypoxic exposure in neutrophils (figure 44, D: +6 h: 0.0012 ± 0.0004, +8 h: 0.0023 ± 0.0010). No clear changes were seen in terms of *PDK1* expression in circulating T cells (figure 44, E).

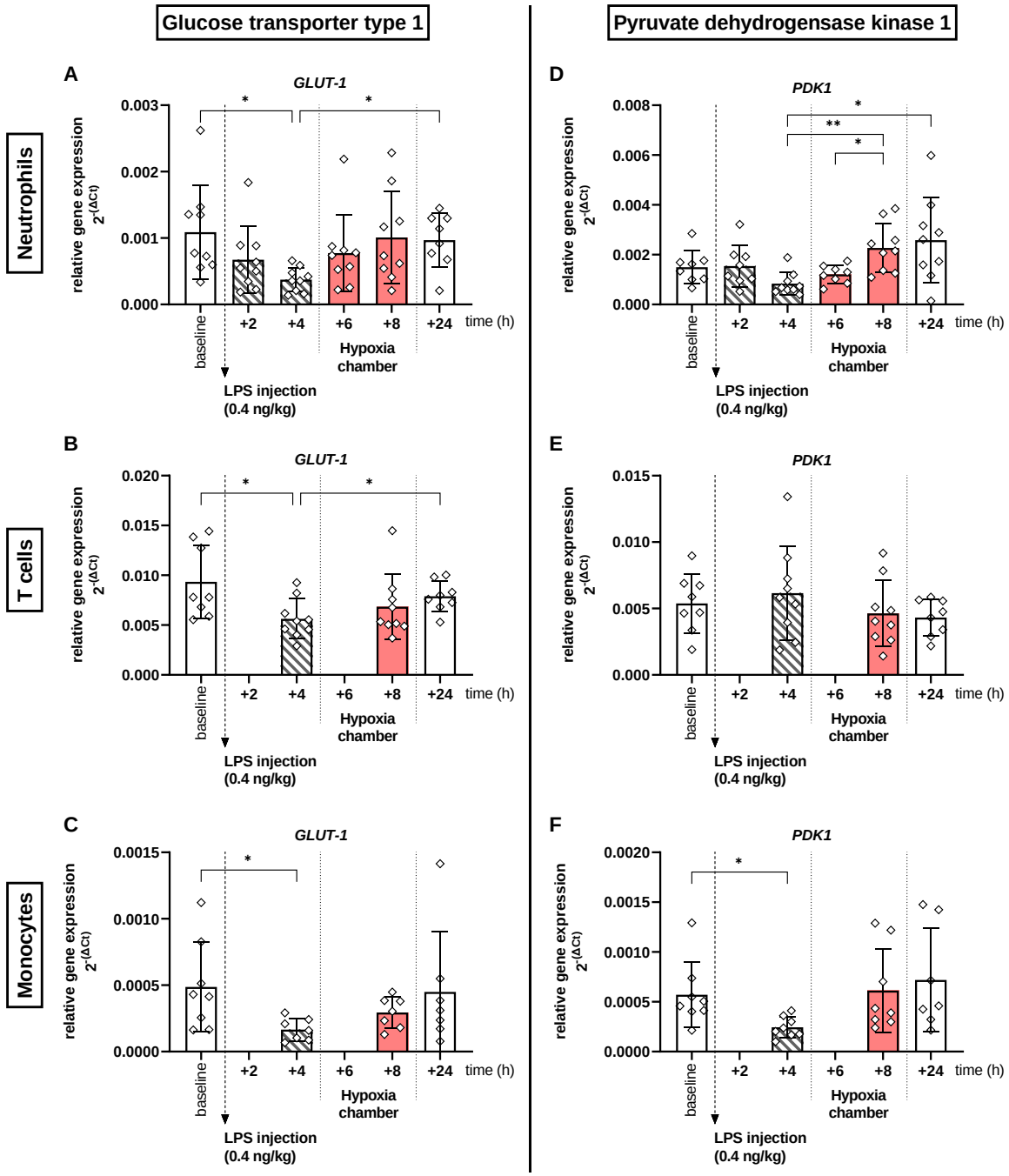


Figure 44: GLUT-1 and PDK1 gene expression upon *in vivo* LPS and hypoxia

Relative gene expression of glucose transporter type 1 (*GLUT-1,* A-C), and pyruvate dehydrogenase kinase 1 (*PDK1*, D-F) of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) followed by exposure to hypoxia (normobaric, 10.5 % O_2, 4h). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, n = 9).

HIF target gene *ADM* increased 4 hours after LPS treatment in all cell types but only reached significance in neutrophils (figure 45, A: baseline: 0.0080 ± 0.0044, +4 h: 0.014 ± 0.009, B, C). Additionally, *ADM* stayed significantly evaluated after the first 2 hours of exposure to hypoxia in neutrophils (figure 45, A: +6 h: 0.021 ± 0.015). Monocytes but not T cells showed also upregulated *ADM* mRNA levels under 4 hours of hypoxia (figure 45, B, C: +8 h: 0.00061 ± 0.00042). *PHD1* expression was differently regulated between the distinct immune cell types (figure 45, D-F). Neutrophils showed a tendency of downregulation after the LPS injection and the following hypoxia, but a significant *PHD1* mRNA decrease only when compared to the 24-hour time point (figure 45, D, +2 h: 0.0084 ± 0.0051, +24 h: 0.0015 ± 0.0014). T cells also displayed downregulation of *PHD1* after 8 hours under hypoxic conditions (figure 45, E: +8 h: 0.0084 ± 0.0051) However, *PHD1* gene expression in monocytes was only affected by LPS (+4 h: 0.000036 ± 0.000022), not by hypoxia or on the long term (figure 45, F).

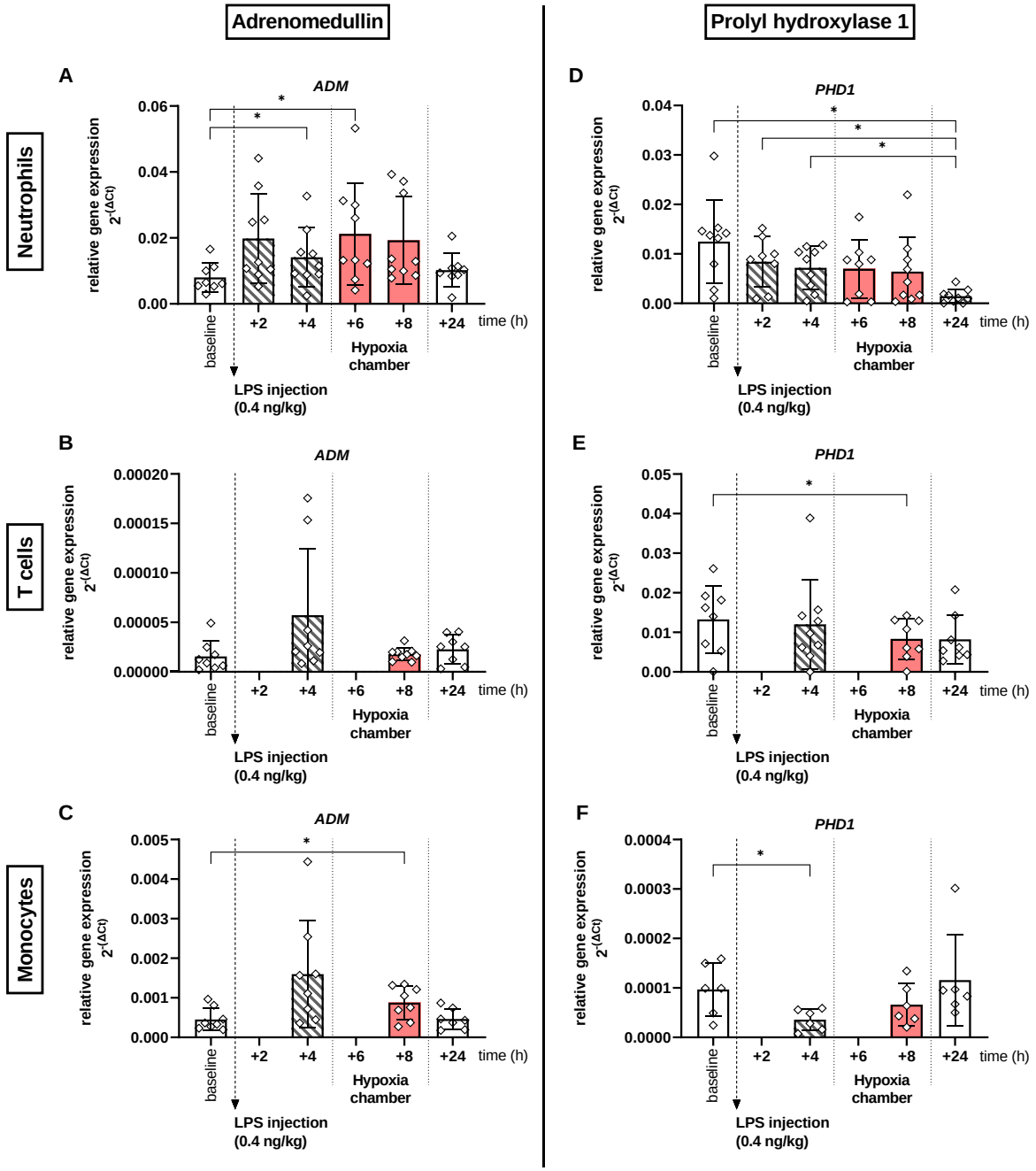


Figure 45: ADM and PHD1 gene expression upon *in vivo* LPS and hypoxia

Relative gene expression of adrenomedullin (*ADM,* A-C), and prolyl hydroxylase 1 (*PHD1*, D-F) of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) followed by exposure to hypoxia (normobaric, 10.5 % O_2, 4h). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 9).

Analysis of *PHD2* mRNA levels revealed that LPS treatment slightly decreased *PHD2* levels, while hypoxia seemed to increase expression again in isolated neutrophils (figure 46, A: +4 h: 0.013 ± 0.0063, +8 h: 0.017 ± 0.010). The same tendency could be interpreted in the data of *PHD2* expression in T cells and monocytes but without significance (figure 46, B, C). *PHD3* expression displayed some small fluctuations in neutrophils and T cells when challenged with LPS, followed by exposure to hypoxia but only in monocytes, 4 hours of hypoxia did create a significant change in *PHD3* mRNA (figure 46, D, E, F: baseline: 0.0090 ± 0.0064, +8 h: 0.0070 ± 0.0051).

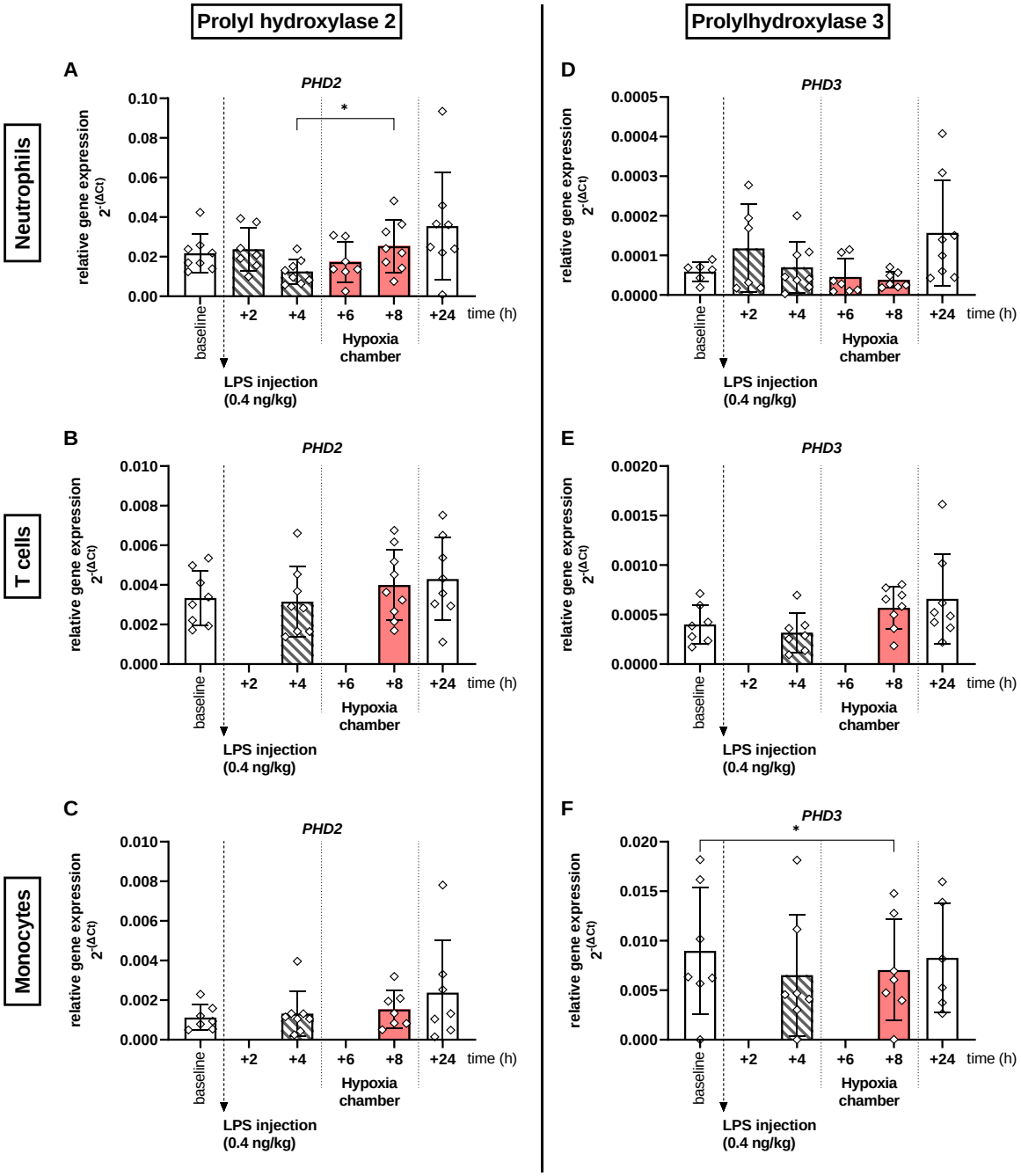


Figure 46: PHD2 and PHD2 gene expression upon *in vivo* LPS and hypoxia

Relative gene expression of prolyl hydroxylase 2 (*PHD2,* A-C), and prolyl hydroxylase 3 (*PHD3*, D-F) of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide

(LPS) injection (0.4 ng/kg) followed by exposure to hypoxia (normobaric, 10.5 % O_2, 4 h). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 9).

Among the isolated cells a tendency of *TNFA* upregulation was only seen in neutrophils 2 hours after the LPS injection, followed by a fast decrease back to baseline mRNA levels (figure 47, A: +2 h: 0.0082 ± 0.0069, +4 h: 0.0031 ± 0.0022). In T cells *TNFA* mRNA was decreased 4 hours after LPS treatment compared to the 24-hour time point (figure 47, B: +4 h: 0.00038 ± 0.00035, + 24 h: 0.00064 ± 0.00033). *TNFA* gene expression did not change in monocytes (figure 47, C). Gene expression levels of the second inflammatory cytokine IL-6 tended to be upregulated 4 hours after the LPS injection in T cells and monocytes but not in neutrophils (figure 47, D - F). The hypoxic treatment 4 hours after the LPS stimulus did not influence any of the cells in terms of gene expression of the inflammatory cytokines (figure 47, A-F).

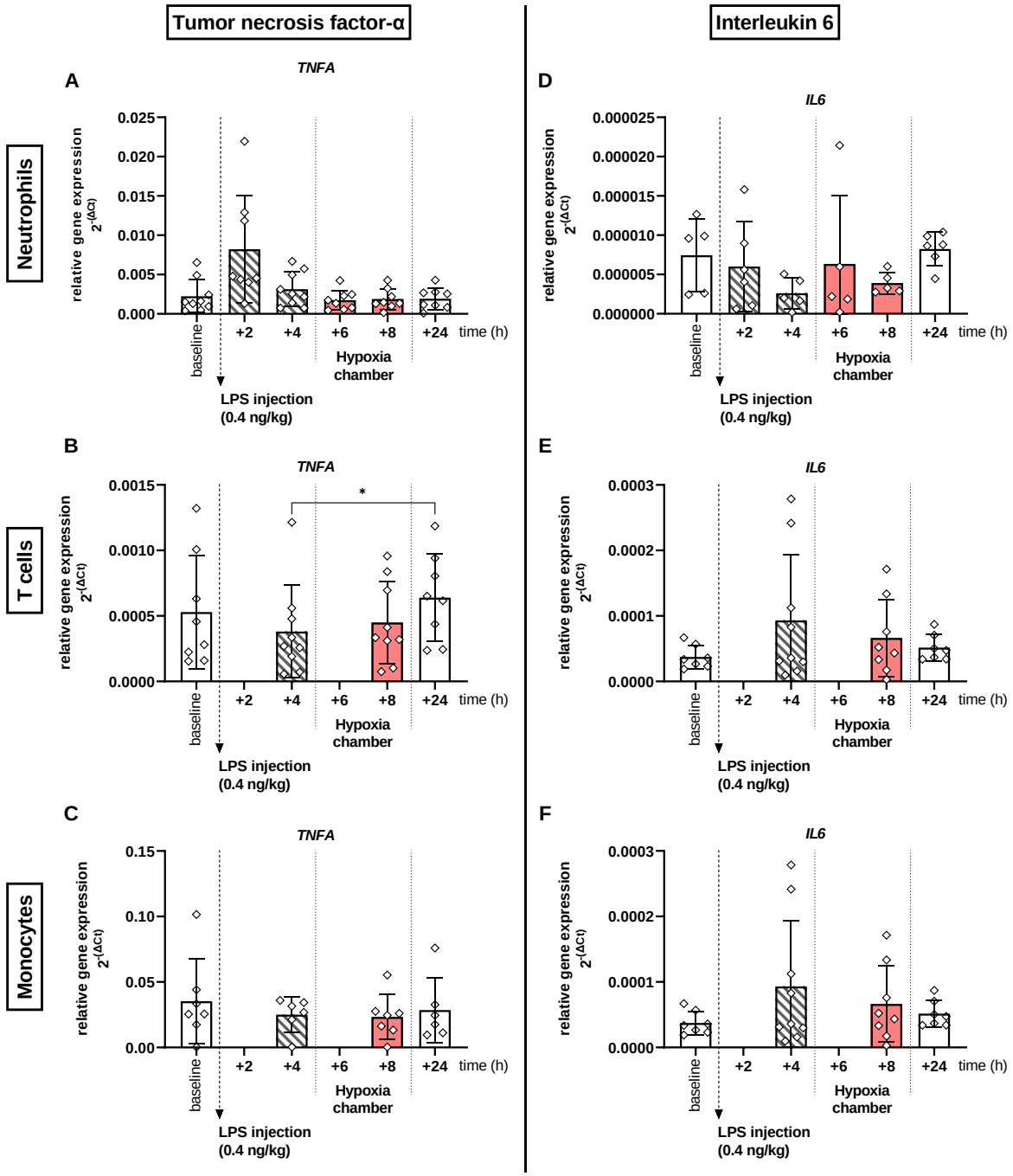


Figure 47: TNFA and IL6 gene expression upon *in vivo* LPS and hypoxia

Relative gene expression of tumour necrosis factor-alpha (*TNFA,* A-C), and Interleukin 6 (*IL6*, D-F), of isolated neutrophils, T cells, and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) followed by exposure to hypoxia (normobaric, 10.5 % O_2, 4h). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, n = 9).

The expression of the Toll-like receptors 2 and 4 was not changed during the first 4 hours after the LPS administration in neutrophils and monocytes (figure 48, A- D). However, the following exposure to hypoxia triggered a significant increase in *TLR2* mRNA levels in monocytes (+8 h: 0.0026 ± 0.0015) and *TLR4* mRNA levels in neutrophils (figure 48, C: +8 h: 0.038 ± 0.021).

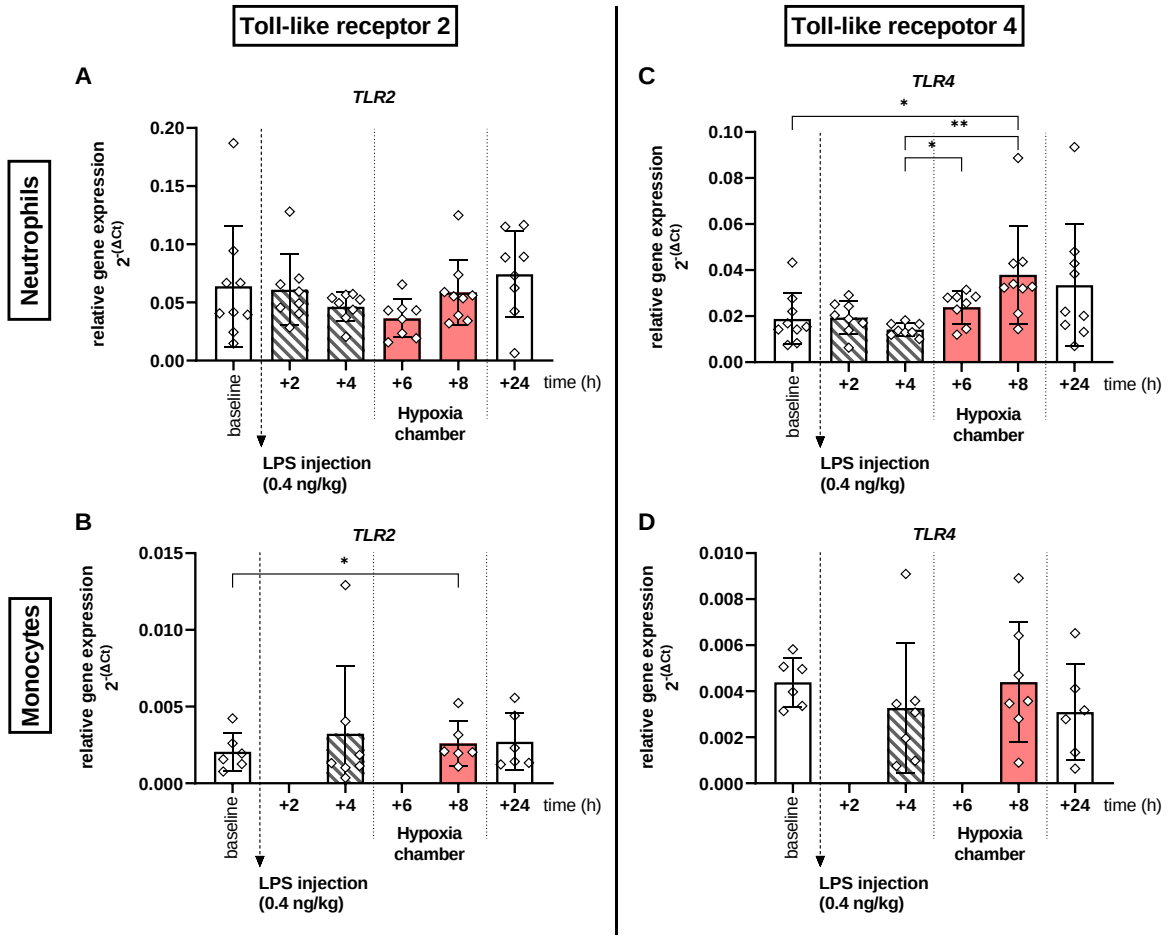


Figure 48: TLR2 and TLR4 gene expression upon *in vivo* LPS and hypoxia

Relative gene expression of the Toll-like receptors 2 (*TLR2,* A, B), and 4 (*TLR4*, C, D), of isolated neutrophils and monocytes, from human subjects that were treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg) followed by exposure to hypoxia (normobaric, 10.5 % O_2, 4h). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, n = 9).

3.5.3 Effects of hypoxic priming prior to LPS treatment *in vivo*

In a third study branch, volunteers were exposed to 10.5 % O_2 normobaric hypoxia for 4 hours prior to the LPS injection (*E. coli*, 0.4 ng/kg) to investigate possible priming effects of hypoxia on acute inflammatory response, focussing on distribution and gene expression of human immune cells (section 2.2.1.2). 15 participants were examined and donated blood before the study (baseline) and at the time points of 2 h, 4 h, 6 h, 8 h, and 24 h after the beginning of the hypoxic exposure (figure 49). Blood sampling was followed by direct processing of material for flow cytometry analysis, and immune cell and RNA isolation.

Blood oxygen concentrations (SpO_2) decreased from around 98 % to a mean of 84 % after 4 hours of hypoxia (time point "+4 h", figure 49).

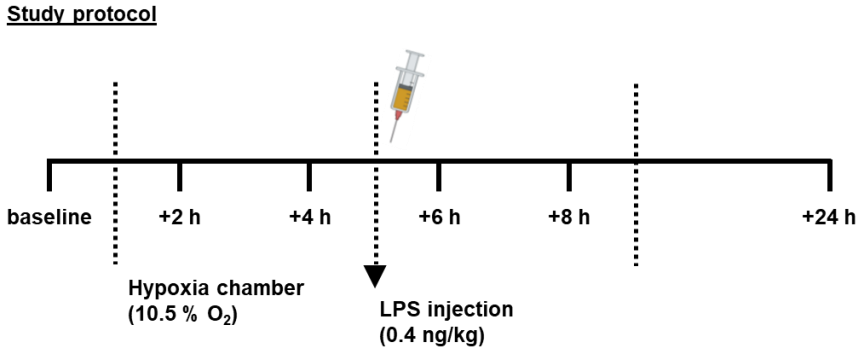


Figure 49: Study protocol of human subjects exposed to hypoxia prior to LPS

Schematic representation of the study protocol of human subjects that were first exposed to normobaric hypoxia of 10.5 % O_2 in a hypoxic chamber and after 4 hours immune stimulated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Times of blood sampling for further analysis included "baseline" levels, shortly before the start of the treatment and indicated time points at 2, 4, 6, 8, and 24 hours after the treatment.

3.5.3.1 Flow cytometry analysis of immune cell populations upon *in vivo* hypoxic priming prior to LPS treatment

To investigate the effects of *in vivo* hypoxic priming prior on acute LPS-induced inflammation on the composition and activation of circulating immune cells we conducted fluorescent antibody staining combined with flow cytometry analysis using the blood samples of subjects (see section 2.2.8). The general composition of cells was determined via the so-called "phenotype staining" (section 2.2.8.1), which was carried out in this study by Marie Jakobs from the Institute of Medical Psychology and Behavioral Immunobiology at the University Hospital Essen, Germany. In addition, the circulating monocyte population was characterised in terms of immune activation and monocyte subgroups (section 2.2.8.2).

Composition of circulating immune cells in humans challenged with *in vivo* hypoxia prior to LPS treatment

Flow cytometry analysis revealed that *in vivo* exposure to 10.5 % hypoxia for 4 hours did not affect the composition of immune cells in the bloodstream, with the small exception of T helper cells being upregulated between 2 and 4 hours of hypoxia from 12.1 % to 13.5 %, but not compared to control levels (figure 50, C). However, the injection of *E. coli* LPS after 4 hours of hypoxia led to significant changes in the cellular composition of all circulating immune cells. 4 hours after the LPS administration, granulocytes increased to 87.2 % of leukocytes ($CD45^+$ cells), while T lymphocytes decreased to 5.2 % and B lymphocytes to 1.5 % (+8 h time points, figure 50, A, B, F). Monocytes displayed the biggest decrease already after 2 hours of LPS treatment from 6.6 to 1.5 % (figure 50, E: +6 h). As seen in the analysis of the other study branches T helper cells and cytotoxic T cells displayed the same dynamic of downregulation upon LPS treatment, with the difference that cytotoxic T cells were already significantly downregulated at the 6 -hour time point (from 5.9 to 3.1 %, figure 50, C, D). Similar to the reversed study protocol none of the cell populations showed any long-term changes upon the combined treatment after 24 hours (figure 50).

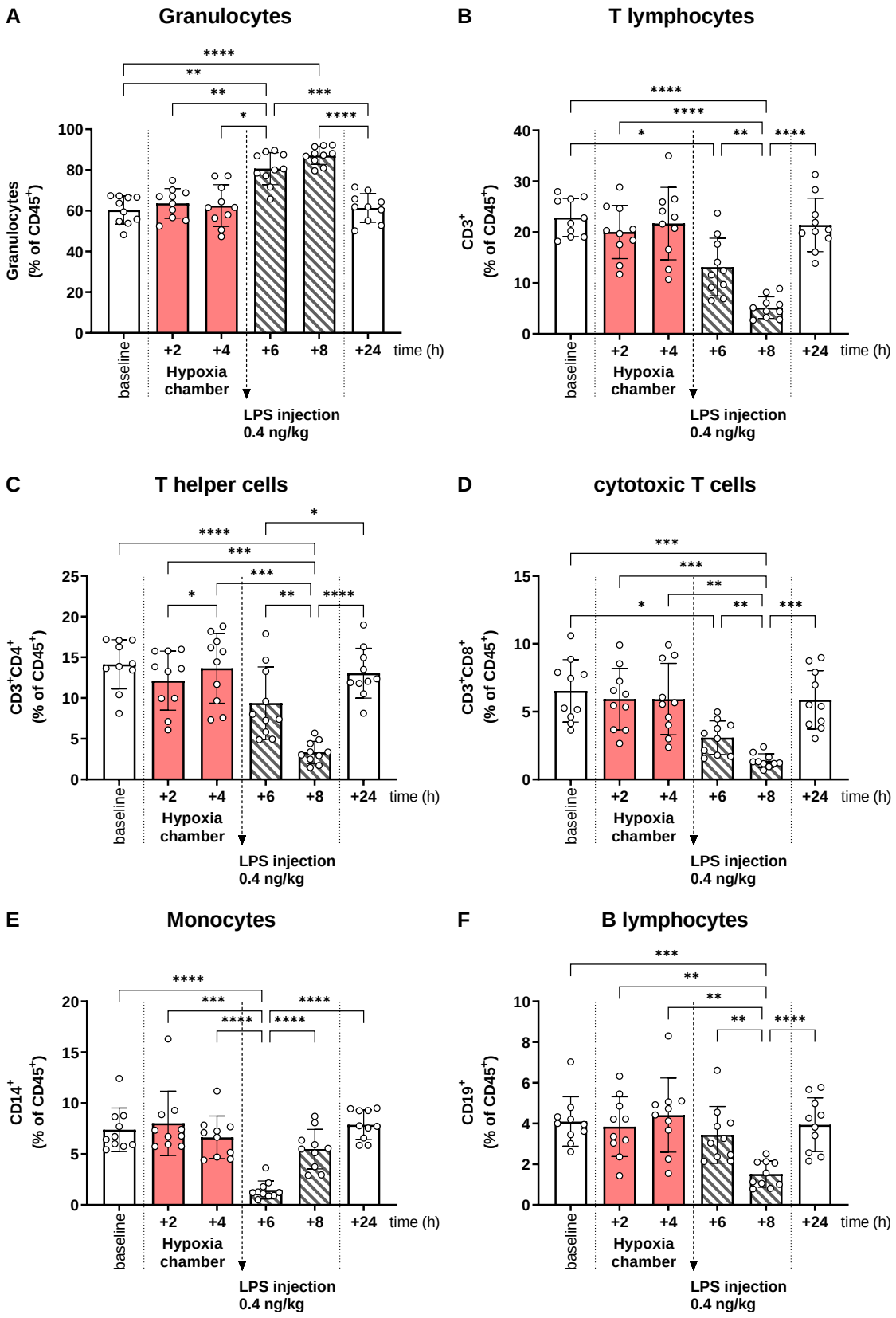


Figure 50: Composition of circulating blood cells upon *in vivo* hypoxia prior to LPS

Flow cytometry analysis of cellular composition in whole blood from participants challenged with hypoxia (4 h, 10.5 % O_2) and LPS treatment (0.4 ng/kg) afterward. Investigated were the percentages of granulocytes (A, negative gating), lymphocytes ($CD3^+$, B), T helper cells ($CD3^+CD4^+$, (C), cytotoxic T cells ($CD3^+CD8^+$, D), monocytes ($CD14^+$,

E) and B lymphocytes ($CD19^+$, F) among all measured circulating blood cells via flow cytometry and specific gating (see supplements, figure 61-63). Displayed are mean percentage ± SD (mixed-effects analysis with repeated measures, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, with not all significances being illustrated in the graphic, measurements by Marie Jakobs, n = 10).

Characterisation and activation of monocytes from humans challenged with exposure to *in vivo* hypoxia prior to LPS treatment

As mentioned before, subgroups of circulating monocytes were characterised throughout the progression of the study via fluorescent antibody staining and flow cytometry analysis (see section 2.2.8). In addition, activation of monocytes was assessed by the determination of Toll-like receptor 4 (TLR4) and the combination of CD86 and HLA-DR expression. Compared to cellular distribution at baseline levels, 4 hours of exposure to hypoxia did not induce any changes in the percentages of classical and nonclassical monocytes but increased the cell counts of intermediate monocytes from 10.2 to 13.9 % (figure 51, B). Other monocyte subpopulations showed only a tendency of upregulation in nonclassical and downregulation in classical monocytes upon exposure to hypoxia (figure 51, A, C) *In vivo* LPS treatment, however, led to significant changes in all groups at the 8-hour time point, compared to baseline distribution (figure 51, A-C). 4 hours after the LPS injection the fraction of classical monocytes increased from 65.3 % (+6 h) to 90.6 % and back to the lowest levels of 56.5 % 24 hours after the start of the study (figure 51, A). As seen before in the other study protocols the intermediate and nonclassical monocytes were both opposed regulated to the classical monocytes. Both subpopulations decreased significantly 4 hours after LPS injection in the circulating blood flow to 2.1 % (B) and 7.6 % (C), respectively. The number of TLR4-expressing monocytes did not change throughout the study with values between 1.8 (+4 h) and 2.8 % (+6 h) of total monocytes (figure 51, D). In contrast, CD86/HLA-DR-positive monocytes significantly increased upon *in vivo* hypoxia (+4 h: 60.5 %), while the following LPS injection had the opposite effect and led to a strong significant decrease to 25.9 % positive cells among all monocytes after 8 hours of the study procedure (figure 51, E). Further, it should be noted that the amount of classical, intermediate, and CD86/HLA-DR-activated monocytes stayed on significantly changed levels 24 hours after the study (figure 51, A, B, F).

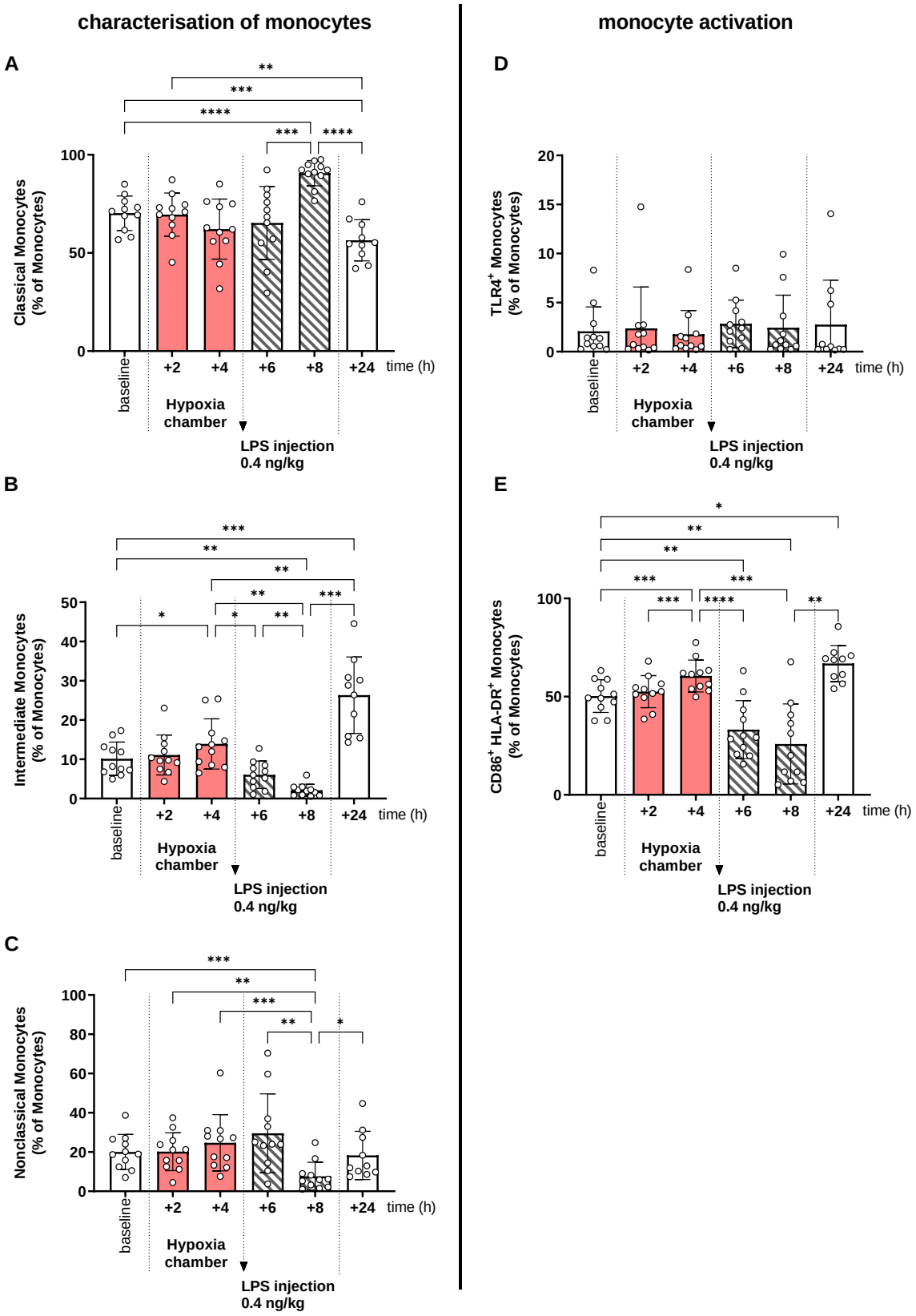


Figure 51: Characterisation of monocytes upon *in vivo* hypoxia prior to LPS

Percentages of monocyte subsets, isolated from blood samples of human subjects that were exposed for 4 hours of 10.5% oxygen hypoxia and afterwards treated with a single *E. coli* LPS injection (0.4 ng/kg). Investigated was the distribution of classical (A), intermediate (B), and non-classical (C) monocytes among all measured circulating monocytes via flow cytometry and specific gating of CD14/CD16 positive cells (see supplements, figure 60).

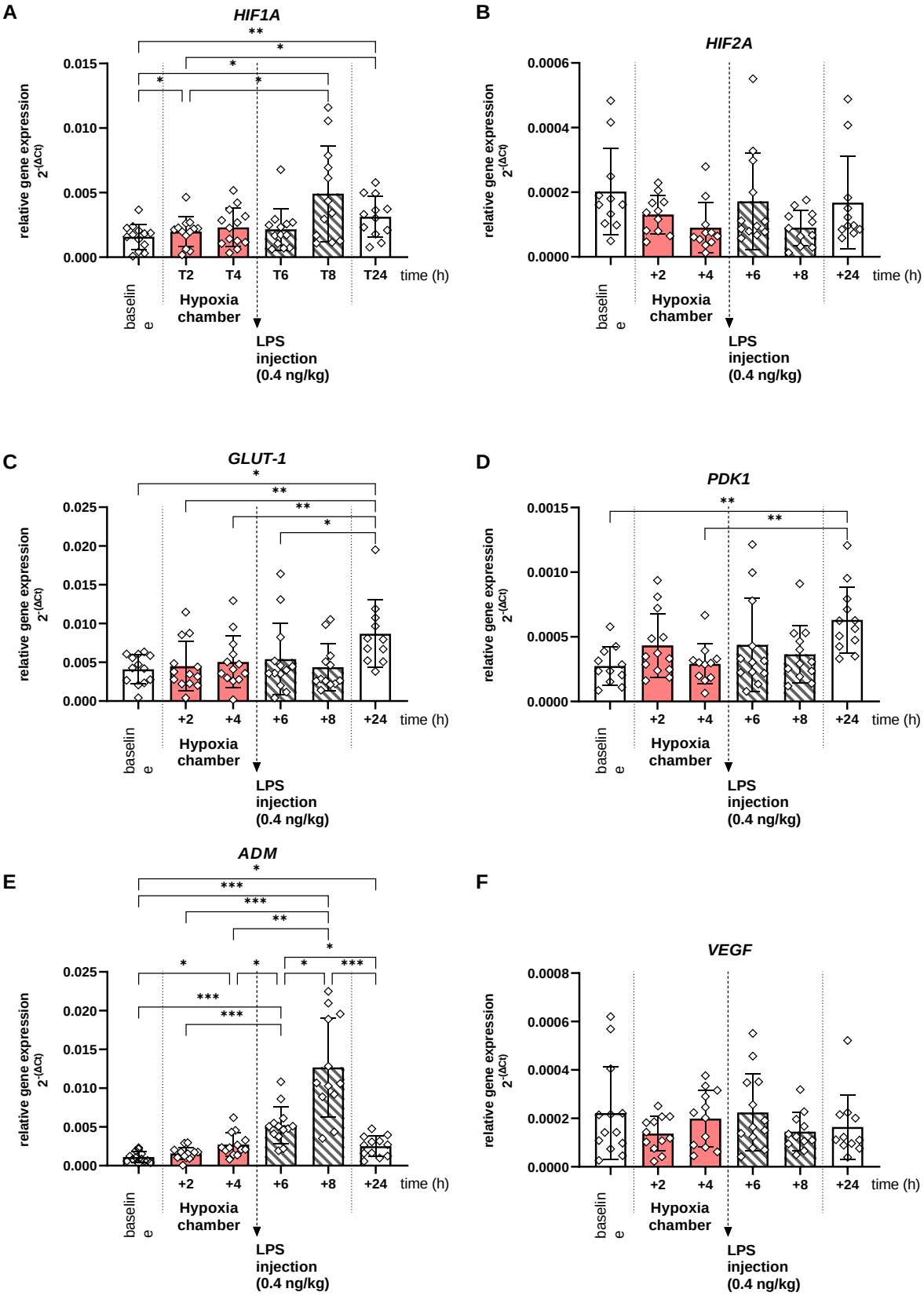
Activated monocytes were identified via detection of Toll-like receptor 4 (TLR4, D) or CD86/HLA-DR expression (E). mean percentage ± SD (mixed-effects analysis with repeated measures, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 11).

3.5.3.2 Effects of *in vivo* hypoxic priming prior to LPS treatment on gene expression of whole blood cells

One part of the human subject study was to unveil the role of hypoxic priming before an acute infection *in vivo*. Therefore, healthy male volunteers were exposed to 10.5 % O_2 hypoxia in a special hypoxic training side for 4 hours prior to an intravenous *E. coli* lipopolysaccharide (LPS) injection of 0.4 ng/kg body weight (see section 2.2.1.2). To analyse gene expression changes of HIF and target genes and inflammatory cytokines blood was sampled every 2 hours during the study and 24 hours after the start of the study as an additional control (figure 49). Data are shown as relative gene expression compared to *ACTB* calculated as $2^{-(\Delta Ct)}$ values, displaying values of each human subject (mean ± standard deviation, n = 13).

After 2 hours of moderate hypoxia of 10.5 % oxygen *in vivo*, *HIF1A* mRNA levels were significantly upregulated compared to the baseline level (baseline: 0.0016 ± 0.00098 vs. +2 h: 0.002 ± 0.001). 4 hours after the LPS injection *HIF1A* levels increased further to 0.00492 (± 0.0037) being significantly increased compared to baseline and 2 hours of hypoxia levels. 24 hours afterward *HIF1A* mRNA levels were still significantly increased compared to the baseline levels (0.0031 ± 0.0016) (figure 52, A). *HIF2A* mRNA levels were much lower than *HIF1A* levels and had the tendency to decrease after 4 hours of hypoxia (9.008e-005 ± 7.723e-005) and 4 hours after LPS injection (9.046e-005 ± 5.454e-005) compared to baseline conditions (0.0002 ± 0.0001) (figure 52, B). In line with *HIF1A* mRNA data, the HIF target genes *GLUT-1*, *PDK1*, *ADM,* and *PHD2* were all significantly upregulated 24 hours after the study procedure compared to baseline gene expression levels (figure 52, C, D, E, H). However, exposure to hypoxia or LPS treatment hardly affected *GLUT-1* mRNA levels of whole blood samples upon the acute stimuli but in comparison to the mRNA levels at 24 hours (baseline: 0.0041 ± 0.0019, +24 h: 0.0087 ± 0.0044) (figure 52, C). *PDK1* expression was already slightly upregulated upon 2 hours of hypoxia (+2 h) and 2 hours after the LPS injection (+6 h) but without any significance (*PDK1* baseline: 0.00027 ± 0.00015, +2 h: 0.00043 ± 0.00025, +6 h: 0.00044 ± 0.00036) (figure 52, D). In contrast, adrenomedullin mRNA (*ADM*) displayed a clear upregulation 4 hours after hypoxia (4+ h: 0.0027 ± 0.0015), 2 and 4 hours after LPS injection (+6 h: 0.0052 ± 0.0024), and on the long term, compared to baseline mRNA levels (figure 52, E). *VEGF* gene expression showed no signs of adaption to the given challenges and ranked around mean values of 0.00023 to 0.00014 (figure 52, F). Interestingly the three prolyl-hydroxylases showed distinct expression patterns upon *in vivo* challenge with low oxygen levels or LPS stimulation (figure 52, G, H, I). *PHD1* mRNA levels seem to react to hypoxia and LPS quite sensitively:

Compared to baseline levels, 2 hours of hypoxia led to a highly significant decrease in gene expression (0.0024 ± 0.0016), which was - despite ongoing hypoxia - back to starting levels 2 hours later (0.0048 ± 0.0024). The LPS injection after the 4 hours in the hypoxic chamber first led to a small increase of *PHD1* mRNA after 2 hours (+ 6h: 0.0055 ± 0.0040) but decreased significantly 4 hours after the LPS injection (+8h: 0.0021 ± 0.0019) (figure 52, G). *PHD2* gene expression showed no changes during the 4 hours in hypoxia or after LPS treatment (means: 0.0050 – 0.0063) compared to control but a long-term upregulation after 24 hours (0.0087± 0.0060) (figure 52, H). In contrast, mRNA of HIF regulator and target gene *PHD3* was not affected at any time during the study procedure and ranked around low mean values between 0.00028 (+8 h) to 0.00053 (+2 h) (figure 52, I). Gene expression analysis of the two inflammatory cytokines revealed different expression patterns upon stimulation. Exposure to 10.5 % oxygen in the air did not affect gene expression of either *IL6* or *TNFA* mRNA (figure 52, J, K). However, *IL6* mRNA levels were surprisingly unchanged upon LPS injection (baseline: 2.844e-005 ± 4.947e-005, +6 h: 1.182e-005 ± 1.021e-005, +8 h: 1.433e-005 ± 1.460e-005) (figure 52, J) while *TNFA* mRNA was significantly upregulated 2 hours after LPS injection (+6 h: 0.0021 ± 0.0014) compared to baseline (0.0008 ± 0.0004) and hypoxia levels (+2 h: 0.0008 ± 0.0003, +4 h: 0.0005 ± 0.0002) (figure 52, K).


A
HIF1A
relative gene expression
2-(ΔCt)
0.015
0.010
0.005
0.000
T2
T4
T6
T8
T24
time (h)
baseline
Hypoxia chamber
LPS injection (0.4 ng/kg)
B
HIF2A
0.0006
0.0004
0.0002
0.0000
+2
+4
+6
+8
+24
C
GLUT-1
0.025
0.020
0.015
0.010
0.005
0.000
D
PDK1
0.0015
0.0010
0.0005
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E
ADM
F
VEGF
0.0008
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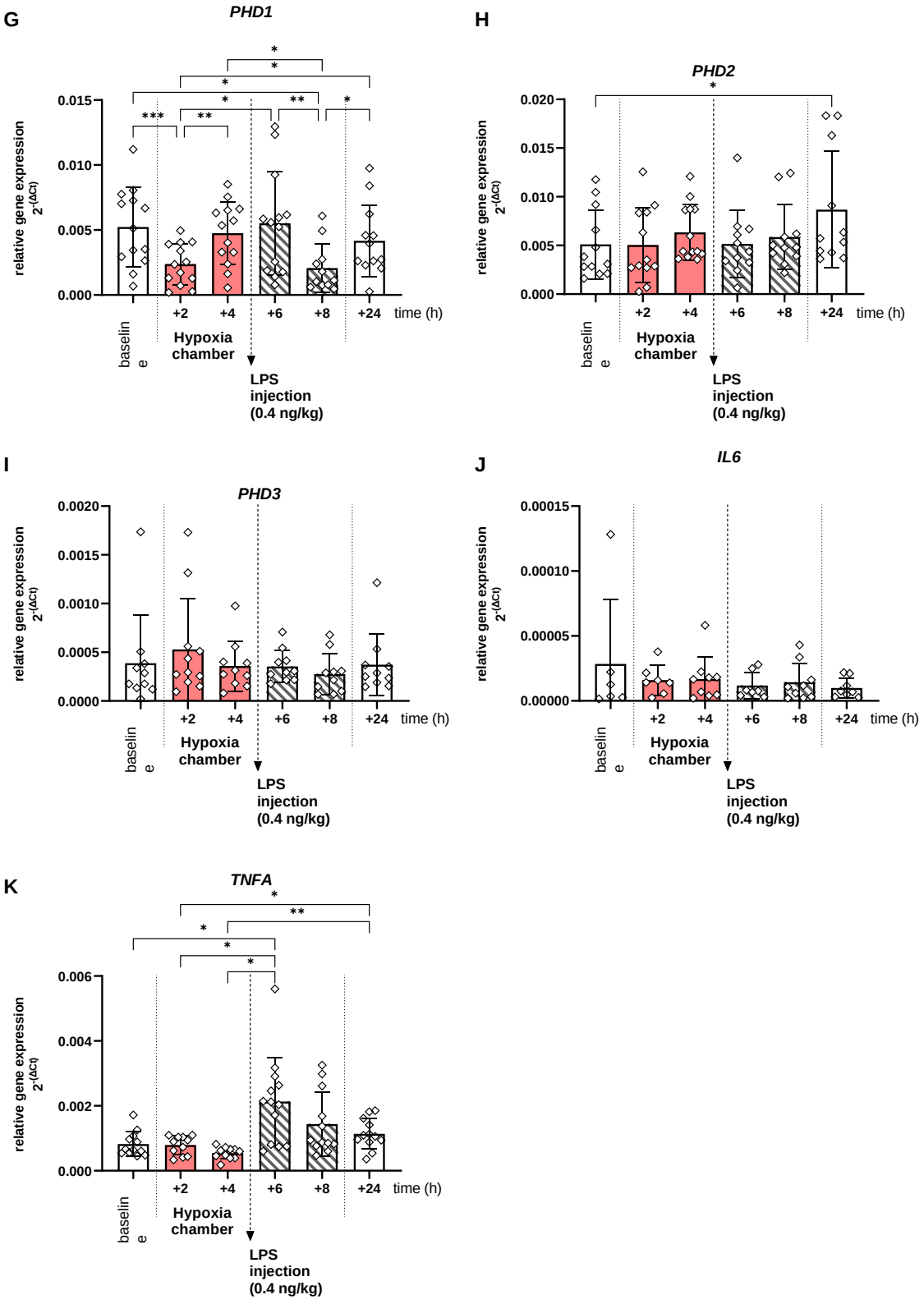

Figure 52: Gene expression of whole blood upon *in vivo* hypoxia prior to LPS

Gene expression of whole blood cells, from blood samples of human subjects that were first exposed to normobaric, 10.5 % O_2 hypoxia and 4 hours later treated with a single *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Investigated genes included *HIF1A* (A), *HIF2A* (B), *GLUT-1* (C), *PDK1* (D), *ADM* (E), *VEGF* (F), *PHD1* (G), *PHD2* (H), and *PHD3* (I), *IL6* (J), *TNFA* (K). Expression levels of mRNA were normalised to *ACTB* and are presented as

$2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, n = 13).

3.5.4.3 Effects of *in vivo* hypoxic priming prior to LPS treatment in distinct immune cell subsets

To understand hypoxic priming effects in immune cells more in detail we further examined gene expression changes of distinct target genes in isolated neutrophils, T cells, and monocytes in parallel to the whole blood RNA analysis (see sections 2.2.3 and 2.2.5). Isolated cells out of 14 participants were used for the analysis of target genes of the HIF pathway in combination with genes that are connected to inflammation using qRT-PCR. Data are presented as $2^{-(\Delta Ct)}$ values normalized to *ACTB* mRNA.

HIF1A mRNA was significantly upregulated in neutrophils from participants that were exposed to 4 hours of hypoxia and again 4 hours after the following LPS injection (figure 53, A: baseline: 0.0090 ± 0.0037, +4 h: 0.013 ± 0.0059, +8 h: 0.023 ± 0.015). However, in T cells and monocytes, *HIF1A* mRNA levels increased just slightly after the treatments and were only significantly changed in T cells at the 24-hour time point (figure 53, B: baseline: 0.0047 ± 0.0020, +4 h: 0.0052 ± 0.0028, +8 h: 0.0067 ± 0.0041, C: baseline: 0.0036 ± 0.0021, +4 h: 0.0047 ± 0.0032, +8 h: 0.0042 ± 0.0025). Similar to that, gene expression of *HIF2A* was also not affected by the study procedure in T cells and monocytes but led to significant changes in neutrophils (figure 53, D – F). While 4 hours of hypoxia alone did not trigger *HIF2A* gene expression changes, the following LPS treatment led to a clear reduction of mRNA levels 2 hours afterward (figure 53, D: +4 h: 0.000062 ± 0.00005, +6 h: 0.000028 ± 0.000014, +8 h: 0.000056 ± 0.000040).

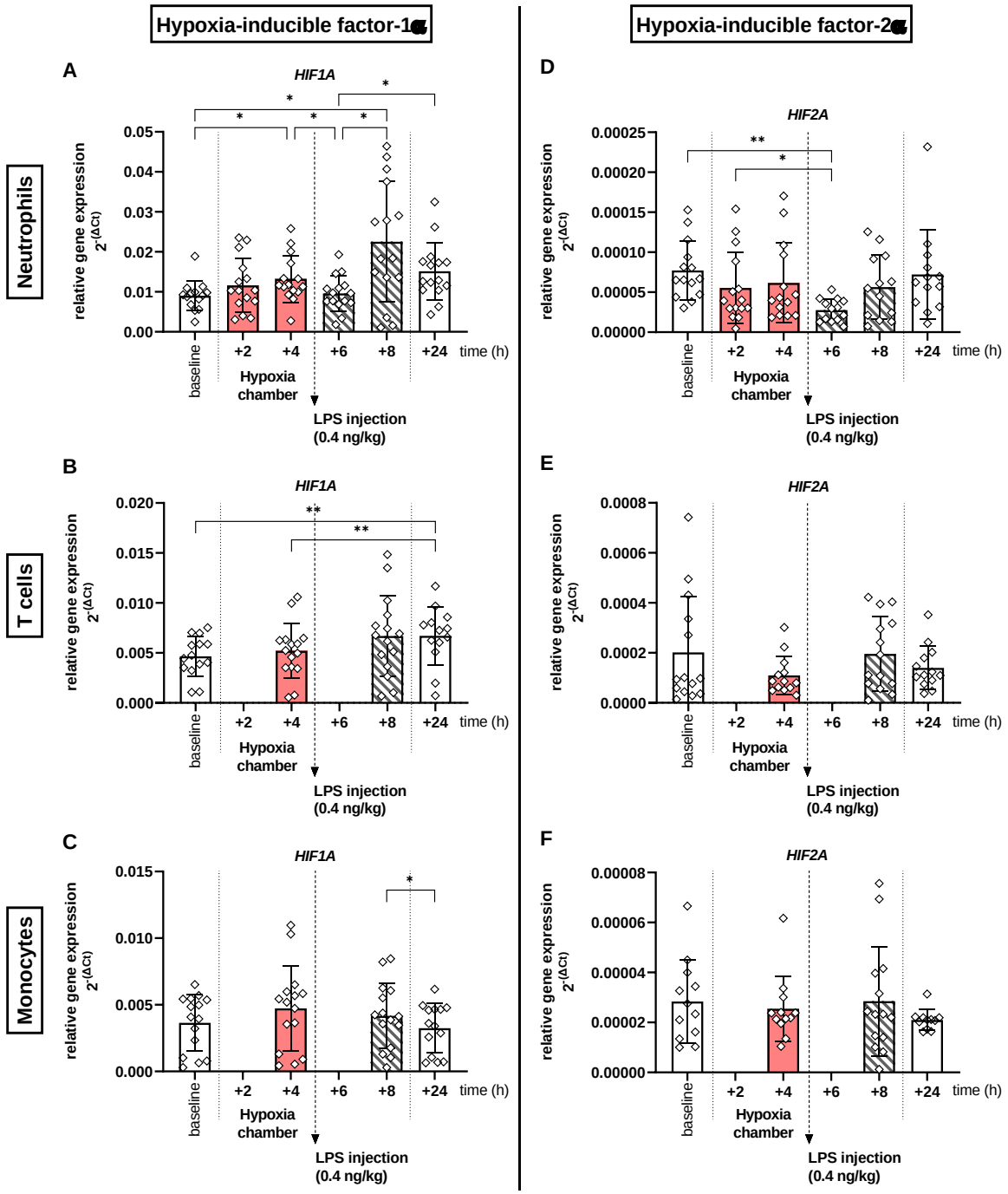

Figure 53: HIF1A and HI2A gene expression upon *in vivo* hypoxia prior to LPS

Relative gene expression of hypoxia-inducible factor -1 and -2 alpha (*HIF1A* (A-C), *HIF2A* (D-F)), of isolated neutrophils, T cells, and monocytes, from human subjects that were exposed to hypoxia (normobaric, 10.5 % O_2, 4 h) followed by an *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, n = 14).

Gene regulation of the transcription factor HIF-3α presented an interesting pattern in neutrophils: 2 hours in hypoxia (+2 h) and 2 hours (+6 h) after the inflammatory LPS stimulus, *HIF3A* levels decreased and were back to baseline levels 2 hours later, respectively. To note, *HIF3A* displayed high variation between the participants (figure 54, A: baseline: 0.000063 ± 0.000065, +2 h: 0.000018 ± 0.00002, +4 h: 0.000067 ± 0.000074, +6 h: 0.000031 ± 0.000029, +8 h: 0.000052 ± 0.000062). *HIF3A* levels in T cells significantly decreased after 4 hours of hypoxia (figure 54, B: +4 h: 0.000081 ± 0.000055), but monocytes were not affected by hypoxia or the following LPS injection and had the lowest mRNA levels among the tested immune populations (figure 54, C: baseline: 0.000005 ± 0.000004). Gene expression levels of the HIF target gene *VEGF* were only affected in monocytes by hypoxic exposure, where mRNA levels significantly increased compared to all other measured time points (figure 54, F: +4 h: 0.00045 ± 0.00024). *VEGF* expression in T cells stayed around similar mean mRNA levels between 0.00004 (+ 4) and 0.00006 (+8 h) and was much lower than in the other immune cells (figure 54, E). Neutrophils did not adapt *VEGF* expression within the 4 hours of hypoxic treatment but showed a tendency to upregulate mRNA levels upon LPS injection (figure 54, D: baseline: 0.00021 ± 0.00020, + 4 h: 0.00017 ± 0.00011, +6 h: 0.00024 ± 0.00015, + 8h: 0.00024 ± 0.00015).

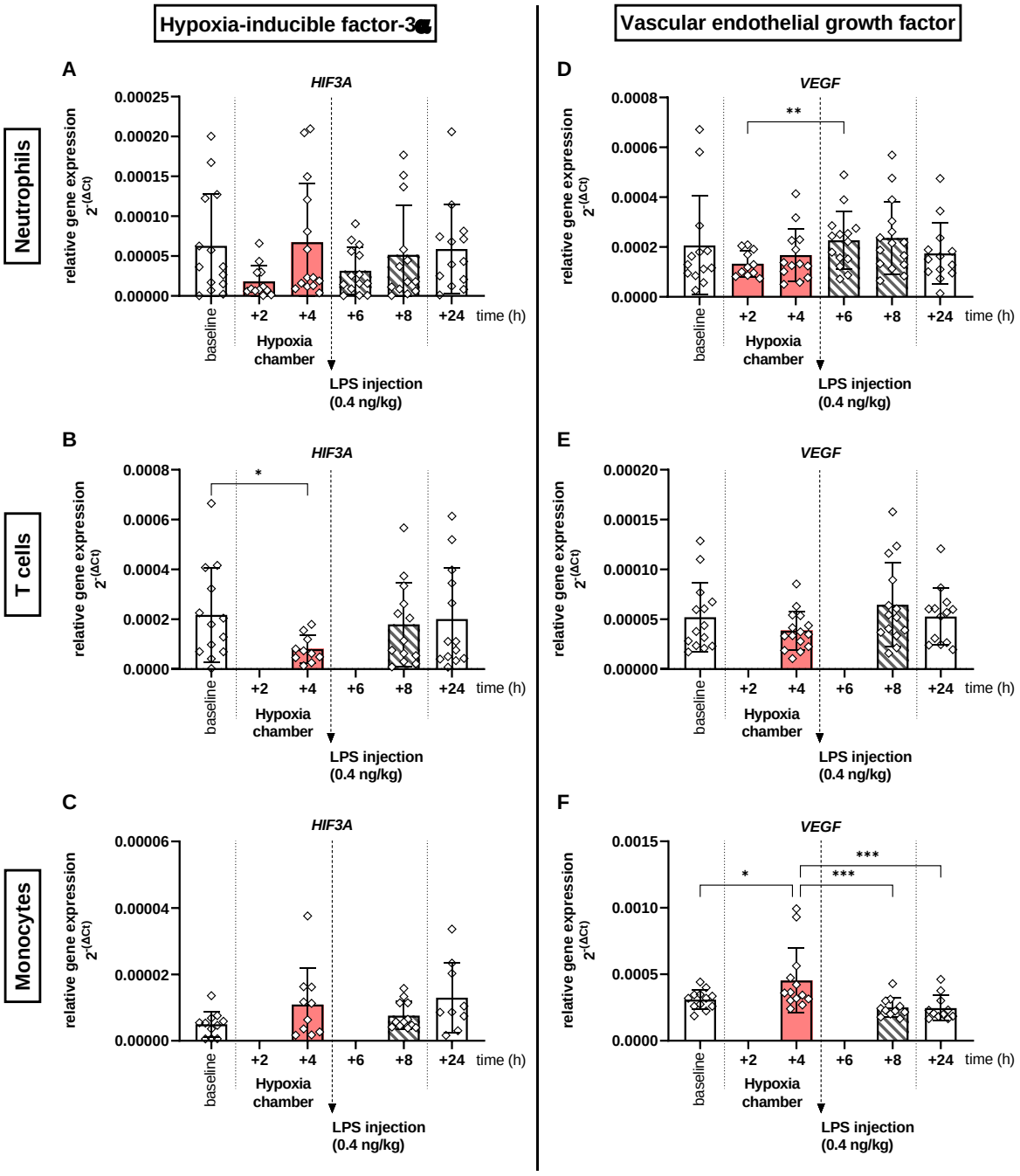

Figure 54: HIF3A and VEGF gene expression upon *in vivo* hypoxia prior to LPS

Relative gene expression of hypoxia-inducible factor-3 alpha (*HIF3A,* A-C), and vascular endothelial growth factor (*VEGF*, D-F), of isolated neutrophils, T cells, and monocytes, from human subjects that were exposed to hypoxia (normobaric, 10.5 % O_2, 4 h) followed by an *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n = 14).

Similar to *VEGF* gene expression, *GLUT-1* mRNA was affected by the hypoxic treatment alone only in monocytes, where *GLUT-1* levels were significantly downregulated compared to baseline levels upon 4 hours of hypoxia and even more after 4 hours of the additional LPS injection (figure 55, C: baseline: 0.00043 ± 0.00018, +4 h: 0.00030 ± 0.00011, +8 h: 0.00018 ± 0.00008). Further, *GLUT-1* mRNA expression levels were significantly downregulated in monocytes even on the long term after 24 hours of treatment (figure 55, C: +24 h: 0.00033 ± 0.00012). Contrary to *GLUT-1* regulation in monocytes were gene expression changes in neutrophils. Exposure to hypoxia caused a slight upregulation of *GLUT-1* mRNA, which was diminished upon LPS injection but again significantly upregulated 24 hours after the study start (figure 55, A: baseline: 0.00036 ± 0.00013, +4 h: 0.00054 ± 0.00022, +6 h: 0.00030 ± 0.00014, +24 h: 0.00070 ± 0.00016). In T cells hypoxia alone did not influence *GLUT-1* expression but 4 hours after the LPS injection mRNA was significantly downregulated compared to all other time points (figure 55, B: baseline: 0.0074 ± 0.0019, +4 h: 0.0068 ± 0.0027, +8 h: 0.0049 ± 0.0012, +24 h: 0.0074 ± 0.0015). *PDK1*, which is also involved in energy metabolism showed the reverse picture in T cells: Hypoxia alone did not affect *PDK1* mRNA levels, while the LPS treatment triggered a strong significant upregulation of the gene (figure 55, E: baseline: 0.0028 ± 0.0011, +4 h: 0.0029 ± 0.0013, +8 h: 0.0065 ± 0.0033, +24 h: 0.0038 ± 0.0018). In line with that, no changes were seen in *PDK1* mRNA after 2 or 4 hours of hypoxia in neutrophils and monocytes. However, in contrast to T cells, *PDK1* mRNA was downregulated after the LPS injection in neutrophils and monocytes (figure 55, D: baseline: 0.0012 ± 0.0006, +4 h: 0.0010 ± 0.0004, +6 h: 0.00068 ± 0.00027, +24 h: 0.0013 ± 0.0005; F: baseline: 0.00025 ± 0.00007, +4 h: 0.00028 ± 0.00007, +8 h: 0.00020 ± 0.00013, +24 h: 0.00028 ± 0.0001).

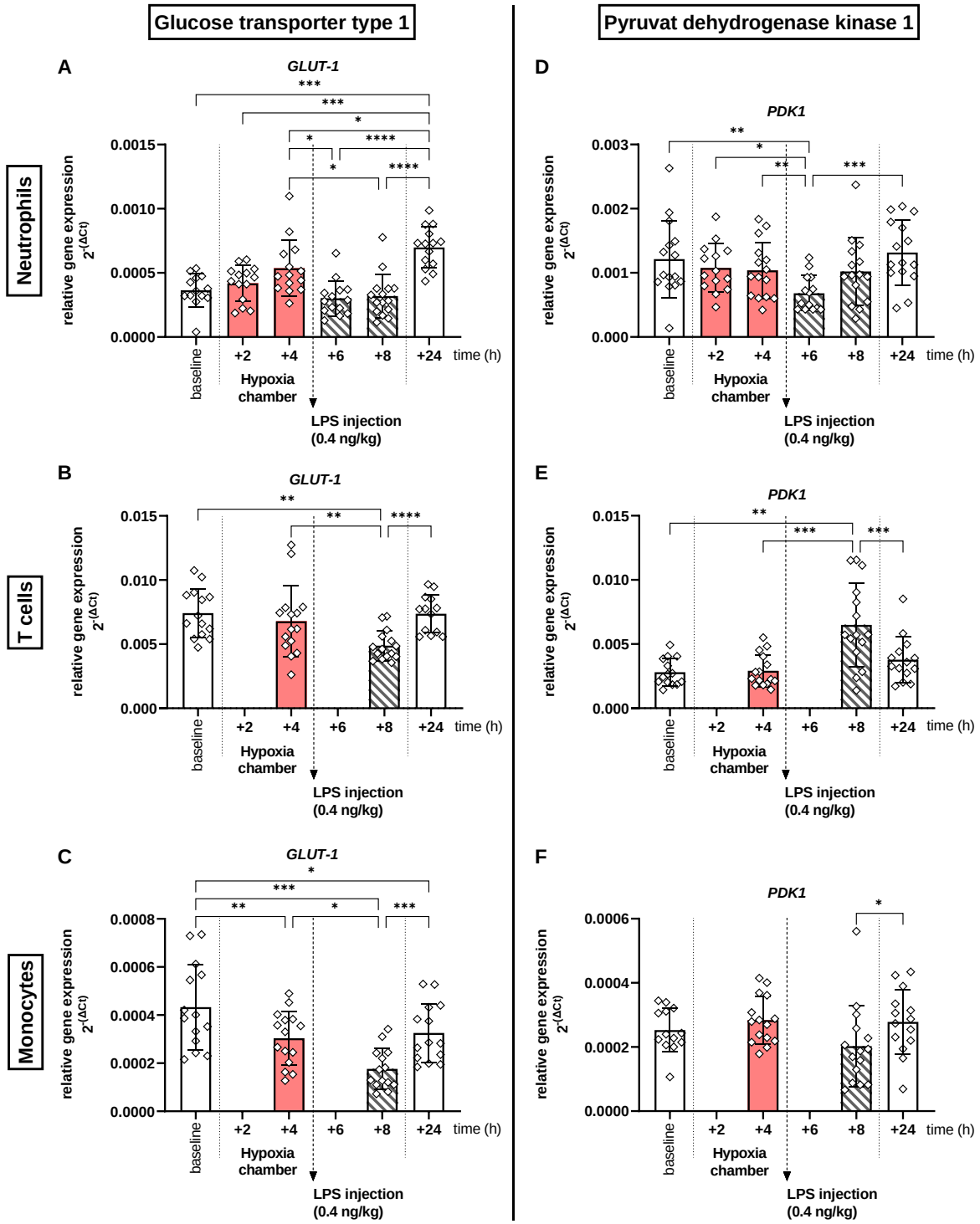

Figure 55: GLUT-1 and PDK1 gene expression upon *in vivo* hypoxia prior to LPS

Relative gene expression of glucose transporter type 1 (*GLUT-1,* A-C), and pyruvate dehydrogenase kinase 1 (*PDK1*, D-F) of isolated neutrophils, T cells, and monocytes from human subjects that were exposed to hypoxia (normobaric, 10.5 % O_2, 4 h) followed by an *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 14).

Gene expression analysis of *ADM* revealed that neutrophils were much more sensitive to hypoxic stimulation than T cells or monocytes. *ADM* mRNA levels were significantly upregulated after 2 and 4 hours in hypoxia and increased even further 4 hours after LPS injection (figure 56, A: baseline: 0.0052 ± 0.0034, +2 h: 0.0095 ± 0.0059, +4 h: 0.0093 ±0.0047, +8 h: 0.021 ±0.007). In T cells and monocytes *ADM* levels did not change upon hypoxia but were significantly upregulated 4 hours after the LPS stimulus (figure 56, B: baseline: 0.000031 ±0.000020, +8 h: 0.00016 ± 0.00014; C: baseline: 0.00030 ± 0.00012, +8 h: 0.0020 ± 0.0010). However, common to all cell types, *ADM* levels were not elevated anymore after 24 hours (figure 56, A-C). Expression of the prolyl hydroxylase 1 barely changed in neutrophils through the study procedure but decreased significantly 24 hours after the start of the study (figure 56, D: baseline: 0.0085 ± 0.0058, +24 h: 0.0028 ± 0.0027). In T cells, *PHD1* mRNA was downregulated upon hypoxia and tended to be upregulated after LPS treatment (figure 56, E: baseline: 0.0064 ± 0.0050, +4 h: 0.0046 ±0.0040, +8 h: 0.0061 ± 0.0054). Monocytes did not adapt *PHD1* mRNA levels 4 hours upon hypoxia but 4 hours after the LPS injection gene expression decreased significantly compared to baseline levels (figure 56, F: baseline: 0.0027 ± 0.0015, +8 h: 0.0017 ± 0.0008).

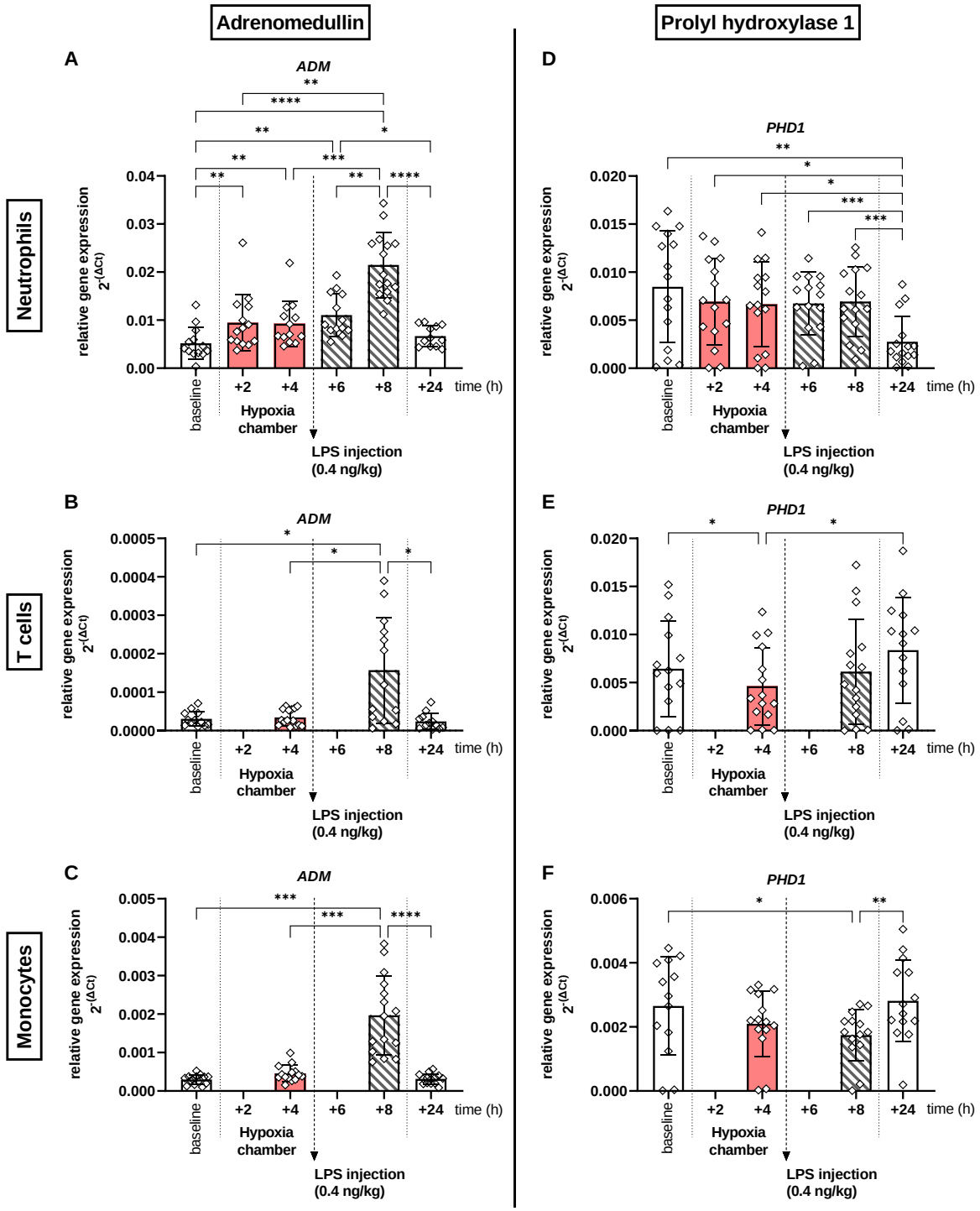

Figure 56: ADM and PHD1 gene expression upon *in vivo* hypoxia prior to LPS

Relative gene expression of adrenomedullin (*ADM,* A-C), and prolyl hydroxylase 1 (*PHD1*, D-F) of isolated neutrophils, T cells, and monocytes, from human subjects that were exposed to hypoxia (normobaric, 10.5 % O_2, 4 h) followed by an *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, n = 14).

In neutrophils, *PHD2* mRNA levels stayed stable during the hypoxic phase but significantly decreased upon LPS injection (figure 57, A: +8 h: 0.013 ± 0.005). *PHD3* expression tended to decrease under hypoxia but reached significant levels only with the following inflammatory stimulus (figure 57, D: +2 h: 0.000037, +8 h: 0.000023). In monocytes, *PHD2* and *PHD3* mRNA expression followed a similar pattern: Hypoxia alone had only minor effects, while the following LPS injection triggered a decrease after 4 hours (figure 57, C: *PHD2*: +8 h: 0.0012 ± 0.0005, F: *PHD3*: +8h: 0.00005 ± 0.00003). In T cells *PHD2* expression was significantly elevated after 4 hours of hypoxia and stayed upregulated after the LPS injection for up to 24 hours (figure 57, C: +4 h: 0.0044 ± 0.0027). *PHD3* mRNA was only slightly upregulated by hypoxia and LPS treatment but displayed also a significant long-term increase after 24 hours (figure 57, E: +24 h: 0.00049 ± 0.00046).

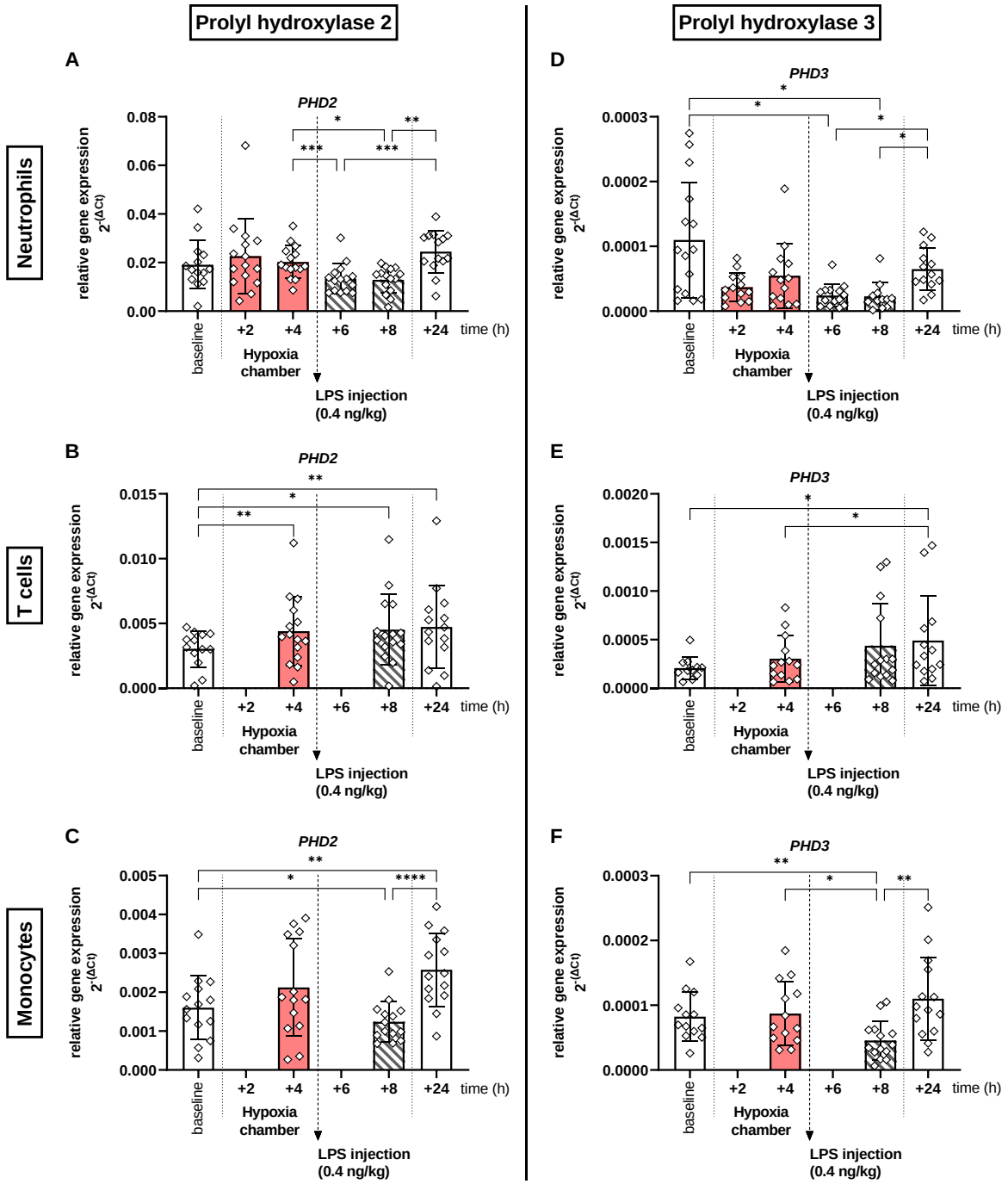

Figure 57: PHD2 and PHD2 gene expression upon *in vivo* hypoxia prior to LPS

Relative gene expression of prolyl hydroxylase 2 (*PHD2,* A-C), and prolyl hydroxylase 3 (*PHD3*, D-F) of isolated neutrophils, T cells, and monocytes, from human subjects that were exposed to hypoxia (normobaric, 10.5 % O_2, 4 h) followed by an *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n = 14).

Analysis of mRNA levels of the inflammatory cytokines showed, that *TNFA* was strongly upregulated at the 6-hour time point, and still slightly elevated at 8 hours in neutrophils compared to baseline levels (figure 58, A: baseline: 0.00064 ± 0.00048, +6 h: 0.0056 ± 0.0037, +8 h: 0.0011 ± 0.0009). Regulation of *TNFA* in T cells followed a different pattern: 4 hours of hypoxia and 4 hours after the LPS injection *TNFA* mRNA levels were significantly decreased compared to the baseline levels (figure 58, B: +4 h: 0.00022 ± 0.00012). Monocytes displayed no acute gene regulatory changes but significant upregulation of *TNFA* after 24 hours (figure 58, C: +24 h: 0.00079 ± 0.0005). Gene expression of Interleukin 6 was significantly upregulated in monocytes, 4 hours after the LPS injection (F: +8 h: 0.000059), but levels were still extremely low compared to other measured *IL6* levels (see sections 3.5.1.3 and 3.5.2.3). No differences were shown in T cells (figure 58, E). In neutrophils, *IL6* levels tended to decrease at the 4 to 8-hour time points but the standard deviation between the participants prevents a clear conclusion (figure 58, D).

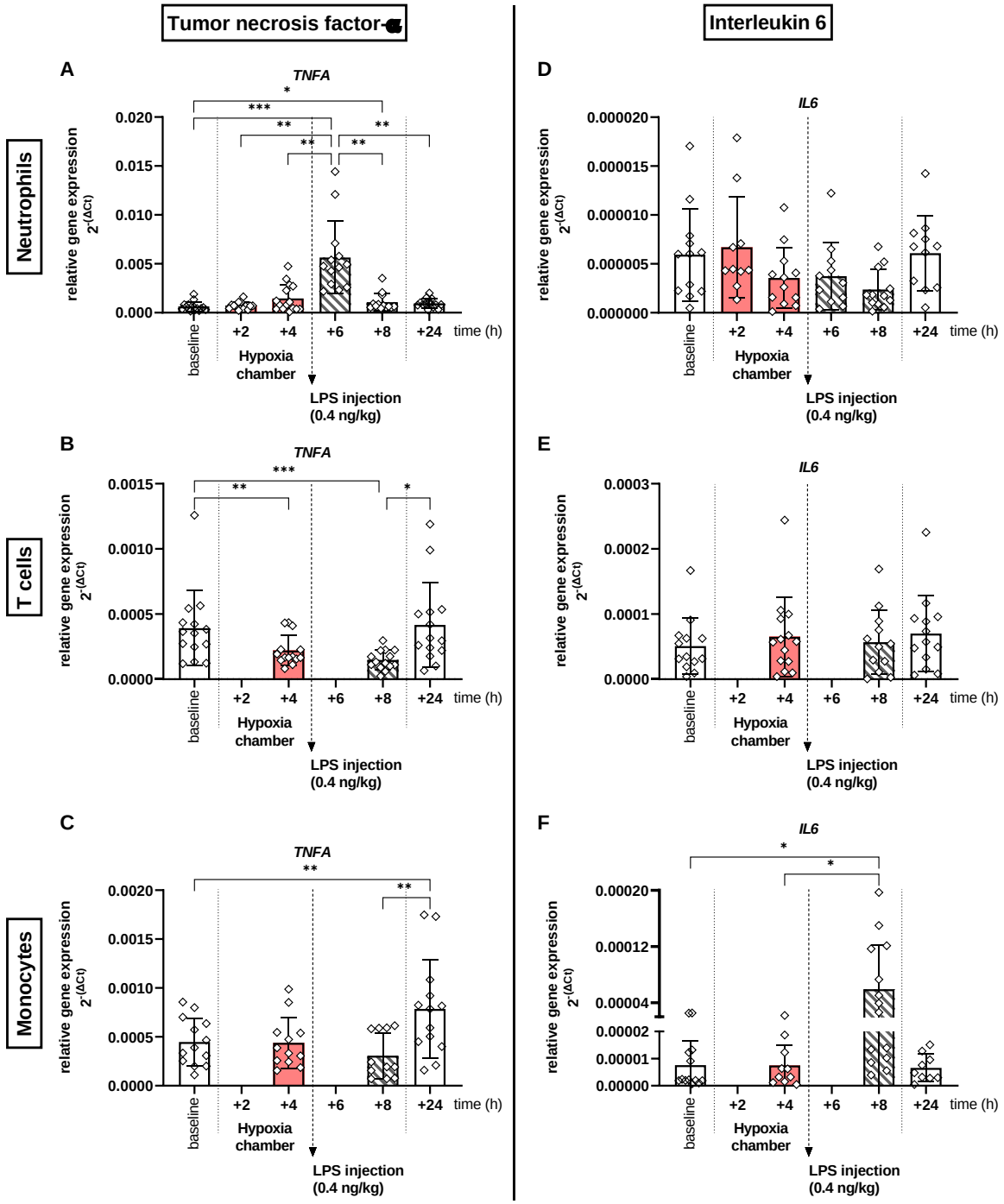

Figure 58: TNFA and IL6 gene expression upon *in vivo* hypoxia prior to LPS

Relative gene expression of tumour necrosis factor-alpha (*TNFA,* A-C), and Interleukin 6 (*IL6*, D-F), of isolated neutrophils, T cells, and monocytes, from human subjects that were exposed to hypoxia (normobaric, 10.5 % O_2, 4 h) followed by an *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, n = 14).

Both *TLR2* and *TLR4* mRNA were not affected by exposure to hypoxia alone in neutrophils and monocytes. However, LPS treatment led to a clear upregulation of *TLR2* and *TLR4* in neutrophils (figure 59, A: +8 h: 0.079 ± 0.051, C: +8 h: 0.026 ± 0.016). Monocytes increased *TLR2* but not *TLR4* mRNA levels 4 hours after LPS injection (figure 59, B: +8 h: 0.025 ± 0.015, D).

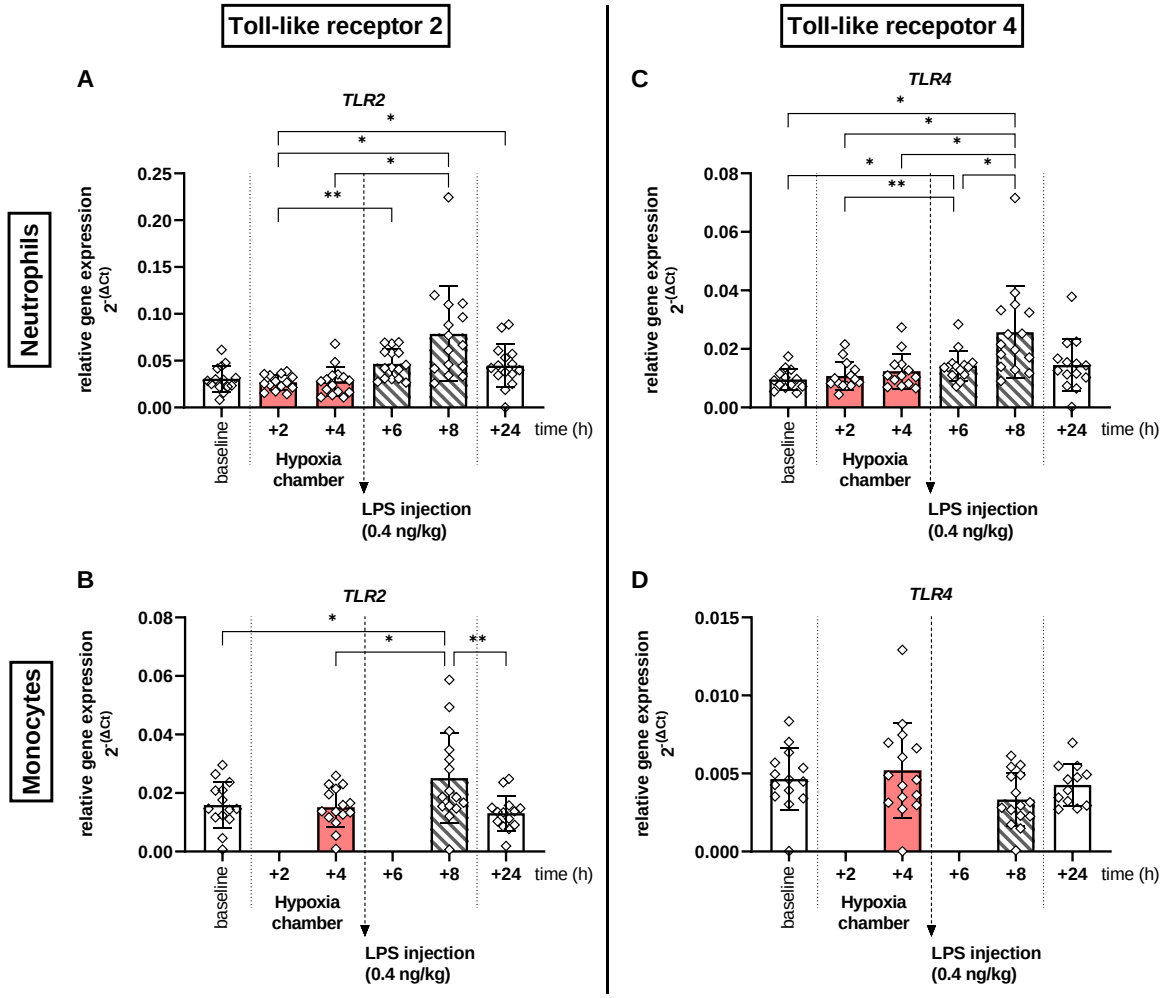

Figure 59: TLR2 and TLR4 gene expression upon *in vivo* hypoxia prior to LPS

Relative gene expression of the Toll-like receptors 2 (*TLR2,* A, B), and 4 (*TLR4*, C, D), of isolated neutrophils and monocytes from human subjects that were exposed to hypoxia (normobaric, 10.5 % O_2, 4 h) followed by an *E. coli* lipopolysaccharide (LPS) injection (0.4 ng/kg). Expression levels of mRNA were normalised to *ACTB* and are presented as $2^{-(\Delta CT)}$ values (mean ± SD, mixed-effects analysis with repeated measures, and Tukey´s multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$, n = 14).

4. Discussion

4.1 Effects of hypoxia and inflammation on HIF pathway activation in THP-1 cells

Gene expression analysis of the monocytic cell line *THP-1* voided a basic understanding of the interplay between hypoxic and inflammatory HIF regulation in human immune cells (see section 3.1). Depending on the species (e.g., murine or human) HIF mRNA itself is often found to be hardly affected or even downregulated by hypoxic stimuli. In the examined human monocytic *THP-1* cells *HIF1A* mRNA was slightly upregulated upon 4 hours of hypoxia (1 % O_2) and treatment with low dose LPS (10 ng/ml) but the combination of both triggered a significant over 2-fold increase of gene transcripts (section 3.1.1 and 4.1, table 13). Thus, in the combined setting of inflammatory hypoxia cells undergo HIF pathway activation on multiple levels, including the fundamental hypoxic HIF protein stabilisation due to environmental shortage of oxygen supply and increased consumption of oxygen by activated immune cells (Parkin & Cohen, 2001), (McGettrick & O'Neill, 2020). Beyond that, stimulation with LPS induces NF-κB-dependent signalling pathways that were shown to increase *HIF1A* mRNA in monocytes earlier, which is in line with our data (Frede et al., 2006), (Rius et al., 2008). Interestingly, *HIF2A* mRNA followed a quite opposed regulation pattern compared to *HIF1A*: The *HIF2A* gene transcripts were significantly decreased in all tested conditions (hypoxia, LPS) and lowest when *THP-1* cells were treated with both stimuli simultaneously (table 13). This reduction of *HIF2A* upon stimulation is most likely due to negative feedback regulation via the highly accumulated HIF-2α protein (Wang & Semenza, 1993), (Wenger, 2000). This interpretation is supported by the effects on the HIF-2α target gene *ADM*, encoding for adrenomedullin. *ADM* was significantly increased up to 15-fold in the combined treatment of LPS and hypoxia, where *HIF2A* mRNA was the lowest but HIF-2α protein probably high (section 3.1.1). However, this needs to be experimentally proven by the determination of HIF protein levels, e.g., using Western blot.

Exposure to hypoxia (1 % O_2) alone for 4 hours was sufficient to only upregulate gene transcripts of the enzyme Prolyl hydroxylase 3 (*PHD3*), which acts as an oxygen sensor of cells and mediates HIF protein degradation under normoxia (Schofield & Ratcliffe, 2004), (Fandrey et al., 2006). Other HIF target genes were not changed. From prior experiments from our working group, it is known that most HIF target genes are transcriptionally activated 6 hours upon hypoxia, which could explain the observed slight but not yet significant upregulation of the other tested HIF target genes *GLUT-1* and *ADM* (section 3.1.1). In turn, it makes good sense that *PHD3* levels increase faster upon acute hypoxia than those of other HIF target genes to buffer extensive HIF protein accumulation and to prevent a potential cellular overreaction to the hypoxic stimulus (Bruick & McKnight, 2001), (Schofield & Ratcliffe, 2004), (Fandrey et al., 2006). The different reaction to LPS or hypoxia alone and the unchanged gene expression levels upon the combined treatment of well-characterized HIF target genes (*GLUT-*

1, *ADM*, *PKD1*) speak once more for different modes of action in terms of regulation. It seems that in *THP-1* cells HIF-1 target genes, which play a role in the glucose and energy metabolism like *GLUT-1* and *PDK1* are less affected by the acute inflammatory and hypoxic stimuli than *ADM*, a proposed target gene of HIF-2α (table 13). This is supported by prior findings of our and other groups, suggesting a HIF independent gene regulation via NF-κB under inflammatory LPS conditions (Zaks-Zilberman et al., 1998), (Hofbauer et al., 2002), (Frede et al., 2005).

To our surprise, in the combined treatment of hypoxia and LPS the inflammatory response in *THP-1* cells seems to be decelerated compared to the solely LPS-treated group, where inflammatory cytokines were highly induced (*IL6*: 62.4-fold, *TNFA*: 3.44-fold, section 3.1.1). The hypoxic incubation in combination with the acute immune stimulus inhibited the fast upregulation of inflammatory cytokines in the monocytic cell culture model to some extent (table 13). This could be beneficial for patients suffering from overwhelming immune reactions like in the acute phase of sepsis or allergic reactions. To investigate the effects of hypoxic priming we performed additional *in vitro* experiments where *THP-1* cells were primed either for 4 or 24 hours in 1 % oxygen hypoxia prior to the acute LPS treatment (10 ng/ml) (section 3.1.2). In total, 4 hours of hypoxic priming did not show any effects on *GLUT-1*, *PDK1,* and *PHD3*, while *HIF2A* levels were significantly increased and mRNA levels of HIF-2α target *ADM* significantly decreased compared to the LPS-only treated monocytes (table 13). This result is quite stirring as both genes were regulated in an opposite manner upon simultaneous stimulation with hypoxia and LPS, which speaks for a tightly regulated HIF system within monocytes and non-negligible importance for the order of applied stimuli. In line with our prior findings 4 hours and 24 hours of hypoxic priming seemed to also dampen the inflammatory response of *IL6* and similar to the simultaneous stimulation in the experiment before (sections 3.1.1, 3.1.2). The same was true for *TNFA* regulation 4 hours after hypoxic priming, but 24 hours of hypoxia before the LPS stimulus increased mRNA levels to 2-fold higher than in the LPS treatment alone. Long-term hypoxic priming over 24 hours further led to a significant downregulation of *GLUT-1* expression and significant upregulation of *PDK1* expression compared to LPS treatment alone. This could indicate a switch in energy metabolism to anaerobic glycolysis (Hu et al., 2003), (Greijer et al., 2005), (Kim et al., 2006), (Schito & Semenza, 2016) (section 3.1.2).

In the reversed protocol, *THP-1* cells were treated with low dose LPS under normoxic conditions (4 h, 21 % O_2) followed by incubation in either again normoxia or hypoxia (1 % O_2) for additional 4 hours to investigate gene regulatory effects of occurring oxygen shortage upon inflammation (section 3.1.3). No differences between the groups were found regarding *HIF1A* or *HIF2A* regulation but all tested HIF target genes were significantly higher expressed in the cells exposed to hypoxia after LPS treatment (table 13). This is most likely due to the fact, that the hypoxic stimulus is the last acute treatment before analysis (section 3.1.3). Nevertheless,

if all combined settings of inflammatory hypoxia are compared the last tested combination of LPS prior to 4 h of hypoxia tended to boost HIF-dependent gene transcription beyond all other combinations and hypoxia treatment alone, while expression of cytokines was comparably low 8 hours after LPS treatment (see sections 3.1.1 - 3.1.3). It would be reasonable, that immune-stimulated monocytes, which are already high in demand for energy and oxygen undergo extreme HIF pathway activation, beyond the normal measure, to ensure sufficient supply of glucose and oxygen upon additionally occurring hypoxia (Cramer et al., 2003), (Frede et al., 2006).

The experiments in human monocytes show that even small changes in time or order of immune and hypoxic stimulation can have notable effects on HIF-dependent gene expression. However, it is difficult to extrapolate from the observed differences to *in vivo* situations as hypoxia and inflammation are mostly integrated without a clear order and adaption of monocytes to both is a complex yet fast reaction (Frede et al., 2007), (Dehne & Brüne, 2009).

Table 13: Summary of gene expression changes in *THP-1* cells treated with hypoxia and LPS

Listed are relative gene expression changes compared to the untreated normoxic control cells. ↑ represents significant upregulation, ↓ represents significant downregulation of the gene, brackets ( ) indicate tendencies of gene regulation without significance.

	***in vitro* treatment**					
gene	**LPS (10 ng/ml, 4h)**	**Hypoxia (1 % O_2, 4h)**	**Hypoxia + LPS (4 h)**	**4 h Hypoxia prior to LPS**	**LPS prior to 4 h Normoxia**	**LPS prior to 4 h Hypoxia**
HIF1A	(↑)	(↑)	↑↑	↑	↑	(↑)
HIF2A	↓↓	↓	↓↓	–	↓	↓
GLUT-1	–	(↑)	–	–	–	↑↑
PDK1	↓	–	–	–	↓	↑↑
ADM	↑	(↑)	↑↑	–	–	↑↑
PHD3	–	↑	(↑)	–	–	↑
IL6	↑↑	–	(↑)	(↑)	↑↑	↑↑↑
TNFA	↑↑	–	(↑)	(↑)	(↑)	(↑)

4.2 Effects of hypoxia and inflammation on the HIF pathway in human leukocytes

Our experiments using the monocytic *THP-1* cell line revealed some interesting insights into HIF-dependent gene regulation upon simultaneous stimulation by inflammation and hypoxia *in vitro*. Although the immortalized human monocytes are widely used to study immune reactions, we wanted to investigate this simultaneous stimulation in primary human immune cells. To that aim, we used human primary peripheral blood mononuclear cells (PBMCs) isolated from buffy coats of blood donations (see section 2.2.4.2). The chosen isolation method via density gradient centrifugation eliminates red blood cells and granulocytes from the blood samples, leaving a typical cellular composition of mainly T lymphocytes, monocytes, and B lymphocytes (section 3.2.1). PBMCs offer the possibility to examine HIF-dependent gene expression closer to reality with multiple immune cell types being activated and acting together in the bloodstream. Nevertheless, for interpreting the results it must be considered, that isolated PBMCs lack the biggest immune cell fraction of neutrophils, which are key mediators of fast innate immune response and certainly have a big impact on HIF-dependent adaption and immune activation *in vivo* (Parkin & Cohen, 2001), (Sarah R. Walmsley et al., 2005), (Amulic et al., 2012). On the other hand, granulocytes are very delicate and known to be easily activated already by regular cell culture handling steps or centrifugation, which could lead to unwanted immune or HIF pathway activation of other leukocytes e.g., by secretion of TNF-α or IL-1β (Hellwig-Bürgel et al., 1999), (Haddad & Harb, 2005), (Amulic et al., 2012).

4.2.1 Effects of increasing durations of hypoxia on HIF activation in leukocytes

First, we classified the time-dependent HIF-response to increasing durations of hypoxic incubation at the standardised levels of 1 % O_2, 5 % CO_2 at 37 °C (section 3.2.2). In line with common knowledge, both, HIF-1α protein and HIF target gene transcription levels were significantly elevated upon longer durations in hypoxia (4 – 24 hours), with some variations depending on the respective target gene (Wang & Semenza, 1993), (Semenza et al., 1994), (Greijer et al., 2005). To note, *ADM* gene upregulation seemed to reach its maximum in PBMCs already after 4 hours of hypoxia, as mRNA levels stayed up high around 14.1 to 15.7-fold increased from 4 to 24 hours. However, this does not exclude that lower oxygen concentrations, which can be physiologically found in organs or inflamed tissues would lead to even higher *ADM* mRNA levels (Chen & Gaber, 2021). *HIF1A* mRNA was downregulated upon prolonged hypoxia and was therefore reversely changed to the accumulating HIF-1α protein, indicating the negative feedback loop following protein accumulation (Cockman et al., 2000), (Mahon et al., 2001), (Wenger, 2002). In line with the proposed mechanism of HIF-1α regulation, HIF-1α protein accumulation peaked after 6 hours of hypoxia but decreased again slightly after 24 hours. Besides high standard deviation, this observation could be explained by the strong upregulation of HIF target gene *PHD3* (574.5-fold), encoding for the prolyl hydroxylase 3, which mediates cellular HIF-1α protein degradation forming a negative

feedback loop to counteract extensive HIF activation and accumulation in the nucleus (Bruick & McKnight, 2001), (Epstein et al., 2001), (Schofield & Ratcliffe, 2004), (Fandrey et al., 2006). In summary, these data convincingly show the regulatory mechanisms behind HIF pathway activation upon hypoxia in PBMCs.

4.2.2 Effects of increasing LPS concentrations on HIF activation in leukocytes

In the second series of experiments even low doses of *E. coli* lipopolysaccharides (LPS, 5 -10 ng/ml) were shown to be sufficient to induce HIF-1α protein accumulation after 4 hours of treatment to around 4 - 5-fold, similar to all other tested concentrations from 50 to 1000 ng/ml LPS (section 3.2.3). Likewise, *HIF1A* mRNA levels were significantly upregulated in an LPS dose-dependent manner, while *HIF2A* was significantly decreased (section 3.2.3). Again, these findings are in line with the literature, suggesting a role of NF-κB pathway activation leading to *HIF1A* mRNA upregulation in immune-stimulated PBMCs (Frede et al., 2006), (Rius et al., 2008), (Devraj et al., 2017). Interestingly, HIF target genes *GLUT-1*, *PDK1,* and *PHD3* were hardly affected by different LPS concentrations, which could point to different temporal gene activation, as all expression levels were solely monitored 4 hours after the LPS treatment. It seems noteworthy, that even with accumulated HIF-1α protein, hydroxylase PHD3 seems not to be involved in the regulation and recovery of HIF-1α levels in terms of acute LPS-induced inflammation in PBMCs.

However, our data from PBMCs is, even containing only 25 % of monocytes, in line with our observations from the monocytic *THP-1* cell line, where the respective target genes were also not changed or even slightly decreased upon inflammation (see sections 3.2.3 and 3.1.1). In contrast, *ADM* was significantly upregulated upon all tested LPS concentrations in PBMCs, which points once more, like in *THP-1* cells, to more complex regulation, most likely via NF-κB, compared to the induction of the other tested HIF target genes upon inflammation (Hellwig-Bürgel et al., 1999), (Frede et al., 2005), (Frede et al., 2006).

Regarding the results, it should be noted, that experiments with PBMCs tended to display quite high fluctuations between the biological repetitions – this is most likely due to the fact, that PBMCs were isolated from blood donations from anonymised donors, with no information about gender, age, origin, or underlying health conditions. Considering the observations in this study it is further not unlikely that also recently overcome diseases or allergies could have priming effects on leukocytes of donors *in vivo* that lead to different reactions in the here presented *in vitro* experiments.

4.2.3 Effects of combined hypoxia and LPS-induced inflammation in leukocytes

After determination of some basic adaptational changes in PBMCs, we were curious about the direct comparison of 4 hours upon hypoxia (1 % O_2) and low dose LPS treatment (10 ng/ml) and the effects of the treatment order in a combined setting, regarding HIF pathway activation (section 3.3.2). Western Blot analysis showed significantly enhanced HIF-1α protein accumulation when PBMCs were treated with LPS prior to a 4-hour hypoxic phase to 2.6-fold, compared to baseline levels or combined LPS and normoxic treatment. The hypoxic stimulus alone tended to induce higher HIF-1α protein levels than low-dose LPS treatment but without significant differences and quite high fluctuations between the blood donors (section 3.2.4). Gene expression analysis revealed that, like *THP-1* cells, PBMCs reacted by HIF target gene upregulation of *GLUT-1*, *PDK1, ADM*, and *PHD3* to 4 hours of hypoxia, while inflammatory cytokines *IL6* and *TNFA* were not affected (section 3.2.4 and 4.2.3, table 14). *HIF1A* mRNA did not change in line with target genes being regulated via HIF protein accumulation and not HIF mRNA, but *HIF2A* was shown to be decreased under hypoxia, which was a similar result to *THP-1* cells as well (Wang & Semenza, 1993), (Ratcliffe et al., 1998). LPS treatment triggered also a significant increase of *HIF1A* and *ADM* mRNA in human primary PBMCs, as described for *THP-1* cells (section 3.1) and other cells in the past (Zaks-Zilberman et al., 1998), (Zhou et al., 1999), (Frede et al., 2005). Besides that, also *PDK1* levels tended to be increased upon LPS treatment in PBMCs, which was not seen before (table 14). The strong upregulation of *IL6* and *TNFA* proved that even low levels of *E. coli* LPS are sufficient to induce a powerful immune activation in human PBMCs *in vitro*. At this point it should be mentioned that to our knowledge most *in vitro* experiments dealing with LPS induction are conducted with much higher concentrations starting at 100 ng/ml LPS (Zaks-Zilberman et al., 1998), (Blouin et al., 2004), (Frede et al., 2006), (Peyssonnaux et al., 2007). The relatively low concentration of 10 ng/ml LPS was chosen on purpose to mimic bacterial immune stimulation *in vivo* in the bloodstream more precisely and to minimize the risk of an 'overshooting' immune reaction, which could overlay and hide underlying regulatory effects.

In the next approach, the effects of hypoxic priming on immune reaction in PBMCs were investigated (section 3.2.4). HIF-1α protein was not notably upregulated in primed or non-primed PBMCs, suggesting no strong effects on the HIF pathway due to hypoxic priming (section 3.2.4). Completely in line with that, there were also no differences on gene expression levels of any of the tested genes (*HIF1A*, *HIF2A*, *GLUT-1*, *PDK1, ADM*, *PHD3*, *IL6,* and *TNFA*) between hypoxic priming and non-primig of PBMCs, leaving solely the LPS-dependent changes as cause for the observed mRNA changes (section 3.2.4 and table 14). These observations differ from those in the monocytic *THP-1* cells, which could indicate a difference between the model systems of the immortalized cell line and primary human cells or for general regulatory differences between the monocytes (*THP-1*) and other distinct immune cell types in the PBMCs, which contain mainly T cells (see section 3.1.2 and 3.2.1).

To investigate gene regulatory effects of hypoxia upon an ongoing inflammation, PBMCs were treated with LPS first followed by exposure to either normoxia or hypoxia for additional 4 hours before lysis (section 3.2.4). In summary, gene expression of all hypoxia-related genes significantly differed between PBMCs kept in normoxia vs. hypoxia after the LPS stimulus (table 14). In addition, PBMCs showed a tendency for increased expression of the inflammatory cytokines *IL6* and *TNFA* in the hypoxia-treated group. *HIF1A* and *HIF2A* were significantly downregulated in the 'LPS-hypoxia' treated group compared to the 'LPS-normoxia' group, which was again different from observations in monocytes. Apart from that, all HIF target genes were significantly increased upon the addition of hypoxia fitting to the observations in monocytes. The slight upregulation of inflammatory cytokines under hypoxia indicates a prolonged immune activation, leading to higher overall secretion levels of inflammatory cytokines, which in turn could further boost or prolong the pro-inflammatory phase in other immune cells or the whole organism. To prove this hypothesis, future analysis should include measurements of cytokine levels in the supernatant of cultured cells, e.g., via ELISA. It is known that HIF activation arms innate immune cells for severe environmental conditions in immune defence e.g. by prolonging neutrophil survival and enforcing aggregation, migration, and invasion abilities of macrophages (Cramer et al., 2003), (Sarah R Walmsley et al., 2005). On the other hand, hypoxia can also lead to enhanced bacterial invasion and antibiotic resistance in inflammatory infections (Schaible et al., 2012), (Zeitouni et al., 2016). Hence, it is not clear exactly how hypoxia alters acute inflammatory response and under what conditions hypoxia can be beneficial or harmful.

Table 14: Summary of gene expression changes in PBMCs treated with hypoxia and LPS

Listed are relative gene expression changes compared to the untreated normoxic control cells. ↑ represents significant upregulation, ↓ represents significant downregulation of the gene, brackets ( ) indicate tendencies of gene regulation without significance.

	***in vitro* treatment**				
gene	**LPS (10 ng/ml, 4 h)**	**Hypoxia (1% O_2, 4 h)**	**4 h Hypoxia prior to LPS**	**LPS prior to 4 h Normoxia**	**LPS prior to 4 h Hypoxia**
HIF1A	↑	(↓)	↑	↑	(↑)
HIF2A	↓	↓	↓	↓	↓
GLUT-1	↓	↑	↓	−	↑↑
PDK1	(↑)	↑	↑	(↑)	↑↑
ADM	↑	↑	↑	↑	↑↑
PHD3	(↓)	↑↑	↓	−	↑↑
IL6	↑	−	↑	↑	↑
TNFA	↑	−	↑	↑	↑

4.3 Effects of *Roxadustat* on HIF pathway activation and immune response in leukocytes

HIF pathway regulation plays a major role in health and disease. Not surprisingly, numerous pathological conditions are associated with dysregulated HIF response and research is focusing to a greater extent on pharmacological interventions to reduce or increase HIF pathway activation (Chen & Gaber, 2021), (Schönberger et al., 2021). To complement our investigations on the interplay of hypoxia and inflammation in human leukocytes, we were further interested in the effects of non-hypoxic but pharmacological HIF stabilisation and its impact on acute LPS-mediated inflammation (see section 3.3). The PHD Inhibitor *Roxadustat* (FG-4592/ Evrenzo) is the first drug of the class of PHD inhibitors, which received clinical approval in 2019 in China and since 2021 in Europe for the treatment of anaemia associated with chronic kidney disease (CKD) (Dhillon, 2019), (Chen et al., 2019), (Fishbane et al., 2021), (Shutov et al., 2021). Thereby, treatment with *Roxadustat* leads to transient inhibition of the prolyl hydroxylases (PHDs), which initiate the degradation of the HIF-1α protein by hydroxylation under normoxia. Although *Roxadustat* is already in use in the clinic the effects on distinct cell types, like immune cells are hardly studied. Due to oral administration of the drug, it carries the risk for potential systemic HIF stabilising effects in other organs and especially in blood circulating leukocytes. Moreover, recent clinical studies showed that *Roxadustat*-treated patients seem to be more prone to urinary and upper respiratory tract infections, which highlights the need for further research on immune cell-specific effects (Chen et al., 2019), (Shutov et al., 2021).

24 hours of i*n vitro* treatment of PBMCs with different concentrations of *Roxadustat* (10 - 100 µM) did not affect cell viability or apoptosis and necrosis rates, which is in line with the approval of the drug and our expectations, proving the safety of the compound (section 3.3.1) (Dhillon, 2019), (Li et al., 2020). The concentrations used in this study reflect the plasma concentrations expected during treatment. To simulate *in vivo* effects in leukocytes from *Roxadustat*-treated patients *in vitro*, we selected the corresponding drug concentrations (Besarab et al., 2015), (Chen et al., 2017). With these *Roxadustat* concentrations we could show via Western blotting and immunofluorescent staining for the first time, that HIF-1α protein levels were significantly upregulated in human PBMCs. This indicates direct PHD-inhibition and HIF pathway activation in leukocytes, beginning already 1-2 hours after administration of the drug in a concentration range from 10 – 50 µM (section 3.3.2). This was in line with a recent publication, where T. Eleftheriadis and colleagues reported strong HIF-1α protein accumulation upon a 10-day *Roxadustat* treatment of human helper T cells (Eleftheriadis et al., 2020). To verify whether the HIF-1α protein accumulation was transcription-dependent, the effects of *Roxadustat* treatment were further analysed on gene expression levels in PBMCs (section 3.3.3). In line with the proposed mode of action of HIF-1α protein stabilisation via PHD inhibition, *HIF1A,* and *HIF2A* mRNA were not changed upon *in vitro* treatment, while all HIF target genes were significantly

upregulated (*GLUT-1*, *PDK1, ADM*, *PHD2, PHD3*, table 15). Interestingly, the inhibited prolyl hydroxylase 2 and 3 displayed substantial upregulation of mRNA. Both hydroxylases are known to be responsible for HIF protein degradation to avoid excessive accumulation of HIF-1α (Metzen et al., 2003), (Fandrey et al., 2006). PHD upregulation might be responsible for degradation of HIF-1α protein around 4 hours of *Roxadustat* treatment (section 3.3.2, see Western blots), pointing to incomplete PHD inhibition and an activated negative feedback loop in terms of HIF protein regulation (Bruick & McKnight, 2001), (Metzen et al., 2003), (Fandrey et al., 2006). In general, HIF target gene transcription upon *Roxadustat* treatment followed more a time-dependent than a dose-dependent manner. Almost no effects were observed on cytokine gene expression indicating no effects on immune activation of cells. It should be mentioned, that *IL6* mRNA was calculated as significantly changed after 24 hours of 10 µM *Roxadustat* treatment, displaying a 2.9 ± 2.4-fold increase. Besides high standard deviation, prior results demonstrated, that *IL6* induction easily reaches 500-fold higher mRNA levels, which relativises the presumed expression changes (see section 3.2.3, 3.2.4).

Additionally, to our investigations of drug-specific effects on the HIF pathway, we focussed on the tolerability *of Roxadustat* in combination with inflammatory conditions (section 3.3.4). Supplementation of low dose *E. coli* LPS (10 ng/ml) to *Roxadustat* pre-treated PBMCs induced HIF-1α protein accumulation significantly stronger than LPS or *Roxadustat* alone and even to a much greater extent than the combination of physiological hypoxia and LPS, which was investigated before (section 3.3.4 and 3.2.2). This observation might be explained by the effectiveness of PHD inhibition by *Roxadustat*, that only becomes apparent when an additional stimulus, such as LPS in this case, leads to HIF accumulation beyond the normal level. Gene expression analysis demonstrated upregulation of *HIF1A* mRNA under inflammatory LPS conditions to the same extent with or without *Roxadustat* pre-treatment, which further indicates that the drug does not affect the transcription of *HIF1A* (section 3.3.4 and table 15). HIF target genes *GLUT-1* and *ADM* were expressed to higher levels in the '*Roxadustat* + LPS' group than in the other fractions, which is in line with priorly observed protein accumulation. In contrast, *PHD2* and *PHD3* expression was decreased in the combined treatment compared to cells with *Roxadustat*-only treatment, meaning LPS treatment buffered the *PHD* feedback regulation upon increasing HIF-1α accumulation to some extent (table 15). Importantly, *IL6* and *TNFA* cytokine gene transcription tended to be further upregulated upon the combination of *Roxadustat* and LPS treatment, compared to LPS treatment alone. Besides high fluctuations within the samples, this could be an indication of a faster or enhanced release of pro-inflammatory cytokines from activated monocytes and T cells, modulating immune reaction in a pro-inflammatory direction (Watts & Walmsley, 2019). This however stands in contrast to findings from other groups. To our knowledge, only two comparable studies are published, both investigating the effects of *Roxadustat* on human T cells exclusively. In one of the studies *in vitro Roxadustat* treatment was shown to present immunosuppressive properties in $CD4^+$ T

cells in mixed-lymphocyte reaction. The drug treatment induced suppression of clonal expansion of helper T cells, induced T-cell apoptosis, and turned T-cell differentiation towards an immunosuppressive phenotype (Eleftheriadis et al., 2020). In addition, the treatment dampened humoral immunity, which could altogether create massive side effects for *Roxadustat*-treated patients upon infections. Another study demonstrated that treatment with *Roxadustat* activated myeloid HIF-1α, which led to NO production and further had an impact on the ability of myeloid cells to inhibit activation and division of cytotoxic $CD8^+$ T cells *in vitro* (Gojkovic et al., 2021). As shown before, the used isolated PBMCs in our experiments also consisted mainly of T cells (43.4 % $CD3^+CD4^+$, 34.2 % $CD3^+CD8^+$ T cells, section 3.2.1), which makes it most likely, that increased HIF-1α accumulation and transcription can be attributed to LPS-mediated activation of monocytes via Toll-like receptor binding and subsequent stimulation of T cells (Blouin et al., 2004), (Nakamura et al., 2005). Apart from that, it is of particular importance to also include analysis of the biggest fraction of cells, meaning neutrophils, which are mainly mediating fast innate response and have the potential to play a major role in observed side-effects in *Roxadustat* treated anaemia patients. Mentioned side effects of *Roxadustat* treatment included acute pancreatitis, slightly increased infections as well as increased rates of thromboembolism pointing to dysregulated immune response and systemic effects on HIF pathway regulation (Sanghani & Haase, 2019). Further studies are needed to ensure patient safety upon *Roxadustat* treatment and to unveil distinct effects on human leukocytes more profoundly.

Table 15: Summary of gene expression changes in PBMCs treated with *Roxadustat*

Listed are relative gene expression changes compared to the untreated normoxic control cells. ↑ represents significant upregulation, ↓ represents significant downregulation of the gene, brackets ( ) indicate tendencies of gene regulation without significance.

	***in vitro* treatment**			
gene	**Hypoxia (1% O_2, 4 h)**	***Roxadustat* (10 – 50 µM)**	**LPS (10 ng/ml)**	***Roxadustat* prior to LPS**
HIF1A	(↓)	−	↑	↑
HIF2A	↓	−	↓	(↑)
GLUT-1	↑	↑	↓	↑↑
PDK1	↑	/	(↑)	/
ADM	↑	↑	↑	↑
PHD2	/	↑↑	/	(↑)
PHD3	↑↑	↑↑	(↓)	↑
IL6	−	−	↑	↑↑
TNFA	−	−	↑	↑↑

4.5 Effects of mild hypoxia on HIF-mediated gene expression *in vivo*

In addition to investigations of human leukocytes *in vitro* we analysed whole blood RNA expression from human subjects exposed to mild *in vivo* hypoxia of 15 % normobaric O_2, for 6.5 hours (sections 2.2.1.1 and 3.4). After 3 hours of hypoxia, blood oxygen concentrations (SpO_2) were decreased to an average of 86.4 %, which is much lower than the optimal oxygen saturation of 94 – 98 % and is equivalent to about 55 mmHg or 7.35 kPa (Collins et al., 2015). However, none of the investigated HIF target genes were affected by the hypoxic exposure on gene expression level (*HIF1A*, *HIF2A*, *GLUT-1*, *PDK1, ADM*, *VEGF*, *PHD1*, *PHD2, PHD3*, section 3.4). This seems surprising at first, but considering that even under physiological settings, the oxygen tension differs extremely throughout the human body, from around 80 - 100 mmHg in arterial blood, to a cellular range of 9 -19 mmHg (1 - 2 % PO_2) it is most likely that acute hypoxic response by HIF activation starts much lower in leukocytes in venous blood than at PO_2 values of around 55 mmHg (Collins et al., 2015), (Chen & Gaber, 2021).

4.6 Effects of inflammation under normoxia or hypoxia on leukocytes *in vivo*

To also bring our investigations on the interplay of hypoxia and inflammation in immune cells one step further to possible implementations for *in vivo* situations, we conducted a human subject study investigating the effects of hypoxia and inflammation on circulating leukocytes using an experimental human endotoxemia model (Andreasen et al., 2008). Thereby, we challenged healthy male human volunteers with a single, intravenous lipopolysaccharide (LPS *E. coli* HOK364, 0.4 ng/kg) injection before or after exposure to *in vivo* hypoxia at a simulated altitude of 4500 meters (normobaric,10.5 % O_2, 4 h) and collected blood samples to analyse immune cell changes upon the treatment (sections 2.2.1.2 and 3.5). Typical and well-studied symptoms with onset two hours after the *in vivo* LPS injection can include flu-like symptoms with fever, myalgia, and headache (Andreasen et al., 2008), (Lichte et al., 2013), (Labrenz et al., 2016), (Lasselin et al., 2020). The used concentration of 0.4 ng per kilogram of bodyweight LPS corresponds approximately to 5 ng/ml LPS usage in cell culture, which is close to the concentration we used *in vitro*.

4.6.1 Effects of acute LPS-induced inflammation on leukocytes *in vivo*

To create a baseline for our investigations, the first 6 subjects were challenged solely with an LPS injection (section 3.5.1). Completely in line with previous studies using the human endotoxemia model, LPS administration alone led to a transient systemic inflammatory response. Flow cytometry analysis from whole blood samples demonstrated a time-dependent increase of circulating granulocytes (from 61.8 to 87.3 %) in combination with decreasing lymphocyte counts (from 21.2 to 5.1 %) after LPS injection. Monocytes were found to be strongly decreased 2 hours after LPS injection but returned to baseline levels already after 6 hours, which was also in line with prior studies (Andreasen et al., 2008), (Lichte et al., 2013), (Labrenz et al., 2016). The analysis proved, that the human endotoxemia model worked as

expected and induced the desired state of systemic inflammation (section 3.5.1.1). In addition, we characterised circulating monocyte fractions more in detail, because these fractions can differ in their distribution in inflammatory settings (Wildgruber et al., 2016). In line with literature, we found that the “classical” monocyte type accounted for 77.9 % of all detected monocytes, which significantly increased upon LPS treatment to 96.8 %. The “intermediate” and “nonclassical” monocytes followed an opposite pattern and decreased upon treatment, which could be due to enhanced emigration of those cells from the blood into the tissue or general redistribution of populations (Ziegler-Heitbrock et al., 2010), (Wildgruber et al., 2016).

Furthermore, we focussed on activation marker expression of the whole monocyte fraction in terms of Toll-like receptor 4 (TLR4) and $CD86^+$ $HLA\text{-}DR^+$, which are mediators of the inflammatory response (section 3.5.1.1). Confirming a prior *in vivo* human endotoxemia study, we did not observe changes in TLR4 expressing cells upon LPS treatment (Lichte et al., 2013). The high relevance of Toll-like receptor activation for mediation of an adequate innate and adaptive immune response is undisputed, therefore the authors of the study hypnotized, that TLR expression on monocytes is already high enough at an unstimulated level to guarantee a fast response with no need for further upregulation upon immune stimulation, at least for low LPS concentrations (Sarah R Walmsley et al., 2005), (Bonilla & Oettgen, 2010), (Lichte et al., 2013). CD86/HLA-DR expression is crucial for activation of T cells, more in detail for antigen presentation (HLA-DR = MHC II) and ligand binding of monocytes to T cells (CD86) (Bonilla & Oettgen, 2010), (Mokart et al., 2011). *In vivo*, in LPS stimulated human subjects CD86/HLA-DR expressing monocytes were significantly decreased in the short term but increased in the long term. This finding is in line with published literature, which also showed a decrease of activated monocytes in patients suffering from acute or chronic sepsis, whereby it seems to be essential when the measurement was taken (Nockher & Scherberich, 1998), (Mokart et al., 2011).

Up to date, no other study focused on the effects of LPS-induced immune activation regarding the Hypoxia-inducible factor pathway. To close that gap, we analysed gene expression levels of HIFs and distinct target genes as well as inflammatory cytokines in whole blood samples via qRT-PCR upon LPS injection (section 3.5.1.2). *HIF1A* mRNA levels of leukocytes were significantly increased upon LPS injection and presented the opposed picture compared to downregulated or unchanged HIF target genes (*GLUT-1*, *PDK1*, *PHD1,* and *PHD3)*, which speaks for HIF-1α-driven regulation of those genes upon inflammation (Rius et al., 2008), (Palazon et al., 2014). To note *HIF1A* was the only examined gene, that stayed significantly elevated up to 24 hours after the LPS treatment, indicating possible long-term effects on leukocytes and HIF activation. *HIF2A* and *VEGF* were affected at all, in contrast to *ADM* displaying a strong upregulation after 4 hours of LPS injection. That difference in ADM gene regulation compared to the other genes was consistent with our observation in *THP-1* cells and isolated PBMCs. In contrast to *PHD1* and *PHD3*, expression of *PHD2* was significantly

upregulated 6 and 8 hours after LPS injection, which therefore seems to be the predominant hydroxylase for mediating HIF protein degradation via the already mentioned negative feedback mechanism (Bruick & McKnight, 2001), (Metzen et al., 2003), (Fandrey et al., 2006). The expression of the inflammatory cytokines (*IL6* and *TNFA*) was both upregulated 2 to 4 hours after acute LPS treatment and back to baseline levels already at the 6-hour time point. This acute and fast immune activation was also in line with data from human studies using the endotoxemia model, indicating the systemic inflammatory response to LPS (Andreasen et al., 2008), (Lichte et al., 2013), (Labrenz et al., 2016).

Gene expression analysis of whole blood samples provides a relatively straightforward approach to access regulation of circulating immune cells *in vivo*. Usage of the so-called PAXgene RNA tubes enabled direct stabilisation of RNA and prevented alterations due to handling of samples (section 2.2.2). Despite this, analysis of whole blood can only present a general picture of underlying gene expression changes without the opportunity to classify the observed changes into distinct cell types. Therefore, we performed isolation and gene expression analysis of the largest immune cell populations in blood, namely neutrophils, T cells, and monocytes (sections 2.2.3 and 3.5.1.3, table 16).

By comparison of the selected immune cell types, it became clear, that the same genes can be quite differently regulated, depending on the distinct leukocyte type, even under the same environmental conditions (section 3.5.1.3). Under LPS treatment only, *HIF1A* mRNA was significantly upregulated only in neutrophils, but with a tendency in T cells and monocytes as well, suggesting that the high *HIF1A* levels in whole blood presented a combination of all cells (table 16). *HIF2A* displayed a small increase in neutrophils and T cells 8 hours after LPS administration, which was also visible in the whole blood RNA analysis. Interestingly, *HIF3A* levels were regulated very similarly to *HIF2A* mRNA, which could indicate similar mechanisms of induction upon LPS (table 16). None of the leukocyte subsets showed notable changes in *VEGF* mRNA, but *GLUT-1* and *PDK1*, the important players involved in shifting the energy metabolism were in parts differently regulated within the leukocyte's fractions (Hu et al., 2003), (Greijer et al., 2005), (Kim et al., 2006), (Schito & Semenza, 2016). While levels of both genes were downregulated upon LPS treatment in neutrophils and monocytes, T cells did not seem to be affected (*GLUT-1*) or even displayed upregulation (*PDK1*), a piece of information that would have been lost in whole blood samples (table 16). The highly significant *ADM* expression changes that were seen in whole blood samples could not be directly accounted for one of the tested immune cell types, but only monocytes displayed significant upregulations upon 4 and 8 hours of LPS treatment (table 16). The three hydroxylases and mediators of HIF-1α protein stability PHD1, PHD2, and PHD3 did also show some differences among the cell types with the highest mRNA upregulation of *PHD2* in neutrophils, 8 hours after LPS, representing the pattern from the foregone whole blood analysis. One should note, that in general *PHD1*

transcription levels were much higher in neutrophils and T cells than in monocytes. In line, *PHD2* also were found to be highly expressed in neutrophils and lowest in monocytes. *PHD3* in turn had, without doubt, the lowest levels of total transcripts in neutrophils and T cells (0.0001 – 0.0002, $2^{-(\Delta CT)}$) and was reverse to *PHD1* the highest expressed in monocytes, and lower in T cells and neutrophils. As discussed elsewhere before, PHD regulation and selectivity are far from being completely understood and our findings argue once more for distinct PHD regulation in different immune cell types (Semenza, 2001), (Metzen et al., 2003), (Watts & Walmsley, 2019). Not surprisingly, mRNA expression of the inflammatory cytokines Interleukin 6 and TNF-α was driven by neutrophils and monocytes, a finding that fits with our *in vitro* data. It should be mentioned, that *IL6* data in neutrophils in some cases were below the detection limit of qRT-PCR, making these results in part less reliable. Fitting to the flow cytometry data, no upregulation of Toll-like receptors was seen on gene expression level of monocytes. However, neutrophils upregulated *TLR4* mRNA 8 hours after the LPS administration (table 16). This could argue again for the fact that TLRs are already expressed to a high extend on monocytes, sufficient to fast-forward inflammatory signalling upon acute infections but are maybe regulated more sensitively in neutrophils (Lichte et al., 2013). As mode of action, lipopolysaccharides bind to CD14 surface receptors and Toll-like receptor 4 of monocytes, which induces the transcription factor NF-kB (Moynagh, 2003). As mentioned before, NF-kB is also crucial for constitutive expression of *HIF1A* mRNA (Frede et al., 2006). Fitting to our data, this close connection between NF-kB and HIF-1α was also demonstrated in neutrophils, suggesting that both regulators are part of a positively regulated enhancer loop, which is activated under inflammation and hypoxia (Sarah R Walmsley et al., 2005).

Table 16: Summary of gene expression changes in leukocytes after *in vivo* LPS injection

Listed are relative gene expression changes compared to mRNA baseline levels. ↑ represents significant upregulation, ↓ represents significant downregulation of the gene, brackets ( ) indicate tendencies of gene regulation without significance. Differences between the cell types are highlighted in colour.

		after *in vivo* LPS injection		
gene	**cell type**	**4 h**	**8 h**	**24 h**
HIF1A	Neutrophils	–	(↑)	(↑)
	T cells	–	–	–
	Monocytes	–	–	–
HIF2A	Neutrophils	(↓)	(↑)	–
	T cells	–	(↑)	–
	Monocytes	–	–	–
HIF3A	Neutrophils	–	(↑)	–
	T cells	–	–	–
	Monocytes	–	–	–
VEGF	Neutrophils	–	–	–
	T cells	–	–	–
	Monocytes	–	–	–
GLUT-1	Neutrophils	(↓)	–	–
	T cells	–	–	–
	Monocytes	(↓)	(↓)	–
PDK1	Neutrophils	–	(↓)	–
	T cells	–	↑	–
	Monocytes	–	↓	–
ADM	Neutrophils	–	(↑)	–
	T cells	–	(↑)	–
	Monocytes	(↑)	–	–
PHD1	Neutrophils	–	–	–
	T cells	–	–	–
	Monocytes	–	–	–
PHD2	Neutrophils	(↓)	(↑)	–
	T cells	–	–	–
	Monocytes	–	(↑)	–
PHD3	Neutrophils	↓	(↑)	–
	T cells	–	–	–
	Monocytes	–	–	–
TNFA	Neutrophils	(↑) (2 h)		
	T cells	–	–	–
	Monocytes	(↑)	–	–
IL6	Neutrophils	(↑)	–	–
	T cells	–	–	–
	Monocytes	(↑)	–	–
TLR2	Neutrophils	–	–	–
	Monocytes	–	–	–
TLR4	Neutrophils	–	↑	–
	Monocytes	–	–	–

4.6.2 Effects of acute LPS-induced inflammation prior to *in vivo* hypoxia on leukocytes

Bringing our investigations further we analysed the same parameters in human subjects, that were exposed to a 4-hour hypoxic phase (10.5 % O_2) 4 hours after the *E. coli* LPS injection (section 3.5.2). After 4 hours of hypoxia, blood oxygen concentrations (SpO_2) were decreased to an average of 83.5 %, which is much lower than the optimal oxygen saturation of 94 – 98 % and equivalent to about 50 mmHg or 6.7 kPa (Collins et al., 2015). Further the oxygen saturation was lower than in the values measured within the 'mild hypoxia' of 15 % O_2 before (86.4 %).

Circulating leukocyte populations, including granulocytes, T lymphocytes, monocytes, and B lymphocytes demonstrated in flow cytometry analysis the same typical pattern of lymphopenia and upregulation of granulocytes as in the "LPS-only" treated group (section 3.5.2.1). This represents the typical features of systemic immune activation (Andreasen et al., 2008), (Lichte et al., 2013), (Labrenz et al., 2016). In line with that, there were no changes in the distribution of monocyte populations in circulating blood with or without the additional hypoxic stimulus after the LPS injection. In both settings, around 78 % of monocytes were grouped as "classical" monocytes which increased in reaction to the *in vivo* LPS injection up to 94 - 98 % after 4 hours and stayed at those significantly higher levels within the next hours. 24 hours after the study the percentages of classical monocytes returned to 60 - 63 %. The percentages of intermediate and non-classical monocytes did also not differ between the study branches. Acute short-term hypoxia did therefore not affect the distribution of already immune-stimulated monocytes in circulating blood. Toll-like receptor expression was found to be at very low levels among the monocyte population between 0.5 - 1.4 % but did not change among the treatments (Lichte et al., 2013). Similar, the number of cells expressing the monocyte activation markers CD86 and HLA-DR, did not differently change with or without the additional stimulus of *in vivo* hypoxia (Nockher & Scherberich, 1998), (Mokart et al., 2011).

In accordance with unchanged cell populations between LPS injection followed by normoxia vs. hypoxia seen in the flow cytometry analysis, also gene expression levels of the tested genes were hardly affected by the additional exposure to low oxygen concentrations. They mostly displayed the same adaptational changes in whole blood samples and isolated immune cell populations as seen before (section 3.5.2.2). However, there is some room for interpretation as in whole blood *PDK1* mRNA was found slightly increased upon the following hypoxic treatment at 6 and 8 hours compared to levels in normoxia, which could be due to hypoxic and HIF-dependent gene activation. The target gene *ADM* did not display any changes during the 8 hours of treatment in this second study group, which was in strict contrast to the "LPS-only" group, where LPS administration led to a significant increase in *ADM* mRNA levels. The absent *ADM* increase in the first 4 hours after LPS treatment can be most likely explained by biological differences of human subjects and standard deviation within the samples. In addition, *ADM* levels were significantly upregulated only in subjects with the combined

treatment of the LPS injection followed by *in vivo* exposure to hypoxia, which points towards a long-term effect on gene expression levels of circulating blood cells by hypoxia. Gene expression levels of *PHD1* in whole blood cells were stable in the solely LPS-treated group but decreased upon 4 hours in hypoxia in this study branch, while *PHD3* expression was significantly increased by hypoxic treatment in comparison to normoxia-treated subjects. These findings could indicate a cellular regulatory shift in PHD regulation upon the change of solely inflammatory conditions to combined conditions with additional hypoxia *in vivo*. Interestingly, mRNA levels of the cytokine *IL6* were upregulated consistently during the 8 hours of the study in some human subjects, while others seemed to be back to baseline levels faster. However, besides the individual differences in human subjects, a steady *IL6* upregulation was only found in subjects treated with hypoxia after the LPS injection, indicating a possible prolonged immune activation of leukocytes.

In the isolated immune cell fractions, the hypoxic phase 4 hours after LPS administration did not induce prominent changes compared to the observed adaptions in neutrophils, T cells, and monocytes from subjects that were solely treated with LPS (section 3.5.2.3, and table 17). Apart from the general gene expression pattern, some genes in distinct cell types seemed to be affected by the additional hypoxic phase *in vivo*. In T lymphocytes *HIF1A* mRNA was significantly upregulated 24 hours after the start of the study procedure, speaking for long-term activation of the HIF system and possible implementations for T cell function e.g., in patients at sides of infection. T lymphocytes undergo a process of cell priming followed by activation and differentiation in the thymus upon antigen presentation of pathogens, which results in the formation of specialised T cell subgroups (helper T cells, regulatory T cells, cytotoxic T cells) (Bonilla & Oettgen, 2010). Depending on the stimuli and therefore the specific need for functional adaptive immunity, T cell subgroups are highly specialised, which in turn means that small changes in stimulation and HIF activation have the potential to make a big difference in directing immune responses (Parkin & Cohen, 2001), (Chen & Gaber, 2021). Some observed differences between cell-specific gene regulation with or without subsequent *in vivo* hypoxia after LPS injection can be most likely accounted to individual fluctuations within the pool of participants and are not solid for interpretation (see absent *HIF3A* upregulation at 8 hours under hypoxia). The typical HIF target genes were not changed in any immune cell population between the two treatments, while mRNA of the hydroxylase *PHD1* was significantly downregulated in neutrophils at the 24-hour time point in all tested subjects that received additional hypoxic treatment. Furthermore, T cells also tended to decrease *PHD1* levels on the long-term upon hypoxia *in vivo* (table 17). No changes were visible in *PHD1* regulation in monocytes and generally not in any of the other hydroxylases, strengthening the idea of high PHD isoform selectivity in regulation of different leukocyte subsets (Semenza, 2001), (Metzen et al., 2003), (Niecknig et al., 2012), (Watts & Walmsley, 2019).

Table 17: Summary of gene expression changes in leukocytes treated with LPS prior to hypoxia

Listed are relative gene expression changes compared to mRNA baseline levels. ↑ represents significant upregulation, ↓ represents significant downregulation of the gene, brackets ( ) indicate tendencies of gene regulation without significance. Differences between the cell types are highlighted in colour.

		LPS injection prior to hypoxia		
		after LPS injection	after 4 h in hypoxia	
gene	**cell type**	**4 h**	**8 h**	**24 h**
HIF1A	Neutrophils	–	(↑)	(↑)
	T cells	–	–	↑
	Monocytes	–	–	–
HIF2A	Neutrophils	–	–	–
	T cells	–	–	–
	Monocytes	–	–	–
HIF3A	Neutrophils	–	–	–
	T cells	–	–	–
	Monocytes	–	–	–
VEGF	Neutrophils	–	–	–
	T cells	–	–	–
	Monocytes	–	–	–
GLUT-1	Neutrophils	↓	–	–
	T cells	↓	–	–
	Monocytes	↓	–	–
PDK1	Neutrophils	–	(↑)	(↑)
	T cells	–	–	–
	Monocytes	↓	–	–
ADM	Neutrophils	(↑)	↑	–
	T cells	(↑)	–	–
	Monocytes	(↑)	–	–
PHD1	Neutrophils	–	–	↓
	T cells	–	↓	(↓)
	Monocytes	↓	–	–
PHD2	Neutrophils	–	–	(↑)
	T cells	–	–	–
	Monocytes	–	–	–
PHD3	Neutrophils	–	–	(↑)
	T cells	–	–	–
	Monocytes	–	↓	–
TNFA	Neutrophils	(↑) (2 h)	–	–
	T cells	–	–	–
	Monocytes	–	–	–
IL6	Neutrophils	–	–	–
	T cells	(↑)	–	–
	Monocytes	(↑)	–	–
TLR2	Neutrophils	–	–	–
	Monocytes	–	↑	–
TLR4	Neutrophils	–	↑	–
	Monocytes	–	–	–

4.6.3 Effects of hypoxic priming prior to LPS treatment on leukocytes *in vivo*

In the third study branch, we aimed to investigate the possible priming effects of hypoxia on acute inflammatory response focusing on the distribution and gene expression of human immune cells (sections 2.2.1.2 and 3.5.3). Participants were exposed first for 4 hours to normobaric hypoxia (10.5 % O_2), followed by an LPS injection of 0.4 ng/kg. After 4 hours of hypoxia, blood oxygen concentrations (SpO_2) were decreased to an average of 84 %, which was already seen in the study branch before (section 3.5.2). These low oxygen saturation levels around 50 mmHg (6.7 kPa) were sufficient to induce gene expression changes in circulating leukocytes of human subjects. This shows that already small changes in SpO_2 can have considerable effects on the HIF pathway activation (Collins et al., 2015).

Using flow cytometry analysis, none of the investigated cell populations were notably changed compared to control levels upon the first 4 hours of hypoxic exposure (section 3.5.3.1). There was a small tendency of upregulation in B lymphocytes and downregulation in monocytes after 4 hours of oxygen shortage, but not to a significant level. However, the subsequent LPS injection shifted immune cell populations in whole blood to the typical values of systemic inflammation, which were seen in the other groups upon LPS treatment before (Andreasen et al., 2008), (Lichte et al., 2013), (Labrenz et al., 2016). One explanation for the strong response to LPS and only tendential changes upon exposure to hypoxia is the underlying importance of fast immune cell response. While entering pathogens, like the used lipopolysaccharides demand a fast host response, including redistribution of immune cells to the side of infection and activation of the adaptive system by antigen presentation of circulating cells, the host response to moderate oxygen shortage in the air does not urge for instant cellular regulation (Parkin & Cohen, 2001), (Beutler, 2004), (Chen & Gaber, 2021). When focussing more precisely on monocyte regulation we found changes in composition.

The fraction of classical monocytes tended to downregulation after 4 hours of hypoxia, while intermediate monocytes were significantly upregulated at the same time point. This is interesting as the subclass of intermediate monocytes does express the highest amounts of genes around processing and presentation of major histocompatibility complex II (MHC II), important for stimulation and antigen presentation for T lymphocytes (Ziegler-Heitbrock et al., 2010), (Wong et al., 2011). Strikingly, the population of intermediate monocytes was also found to be increased in severe asthmatic patients, which supports the findings in our *in vivo* data (Moniuszko et al., 2009). Fitting to the literature, also HLA-DR expression was highest in intermediate monocytes compared to the other monocyte subsets, which could explain the strong significant increase in CD86/HLA-DR expressing cells in our subjects upon challenge of *in vivo* hypoxia (see section 3.5.4.1.2) (Wong et al., 2011). The combination of these observations proposes possible priming effects on monocyte function and activation due to acute hypoxia, which would be beneficial in human pathologies, when monocytes and macrophages migrate to their operation site in inflamed tissues. At the same time TLR4 surface

expression in monocytes was not affected by hypoxia or the following LPS injection, which supports prior data (Lichte et al., 2013).

Gene expression analysis showed that *HIF1A* mRNA in whole blood samples was upregulated after 2 hours of exposure to hypoxia *in vivo* (section 3.5.3.2), which was consistent with *THP-1* cell culture and PBMC culture *in vitro*. In line with our observations from the other study branches, 4 hours after LPS injection *HIF1A* levels were again significantly increased, speaking for inflammatory induction via NF-κB (Sarah R Walmsley et al., 2005), (Frede et al., 2006), (Van Uden et al., 2008). Besides *HIF1A* also the genes *GLUT-1*, *PDK1*, *ADM,* and *PHD2* were significantly upregulated 24 hours after the study with the combined inflammatory and hypoxic stimulus, which was not seen in the whole blood of participants that received LPS treatment only. Further, acute hypoxia in parts also shaped the transcriptional response to infection, e.g., in terms of *GLUT-1* regulation. Hypoxic priming prevented downregulation of *GLUT-1* levels in reaction to the acute LPS stimulation, as seen in the other study branches before, probably with effects on glucose uptake and energy metabolism in circulating immune cells. This indicates a clear influence of *in vivo* short-term hypoxia on the metabolism of circulating immune cells upon combined inflammatory conditions. When interpreting such data with longer-lasting effects on leukocyte gene expression profiles, it should be considered that especially immune cells highly interact with surrounding tissues and mediate inflammatory responses between each other (Parkin & Cohen, 2001), (Beutler, 2004), (Bonilla & Oettgen, 2010). Immune cell priming by hypoxia does even get higher implications if we consider, that cells normally start to migrate from the well-oxygenised bloodstream (80 – 100 mmHg) into potential hypoxic tissue under 10 mmHg oxygen partial pressure (Beutler, 2004), (Chen & Gaber, 2021). It can only be assumed that already immune cells slightly primed by hypoxia react differently to repetitive exposure to severe hypoxia e.g., at the site of infection. *PHD1* expression was again highly sensitive to the different stimuli and was strongly downregulated 2 hours after hypoxia but returned to baseline levels 4 hours afterward. LPS injection also decreased *PHD1* significantly after 4 hours, which was in complete contrast to the observations in the other two study branches. Similarly, *PHD2* expression was only affected on the long-term and upregulated after 24 hours, which was again different to the other groups in the whole blood cells. *PHD3* mRNA levels did not change upon hypoxia or the following LPS injection, again displaying another gene expression pattern than the other two hydroxylases and implying cell and isotype-specific roles of the different PHDs (Semenza, 2001), (Metzen et al., 2003), (Niecknig et al., 2012), (Watts & Walmsley, 2019). Expression of *TNFA* was typical, with a fast upregulation 2 hours after the LPS injection but no change under hypoxic conditions. *IL6* levels stayed at the same low levels even after the immune stimulation. It seems that hypoxia dampened or, more in line with literature delayed inflammatory IL-6 expression (see discussion below). When focussing on the distinct immune cell subsets the reversed study

protocol provided some interesting insights into the cellular response to *in vivo* hypoxia and into effects of hypoxic priming in inflammatory conditions (section 3.5.4.3).

In neutrophils, 4 hours of acute hypoxia led to a significant mRNA increase of *HIF1A* and *ADM* and a tendency of upregulation of *GLUT-1* and *HIF3A* levels (table 18). Similar tendencies were found in the reversed study branch, where participants received the LPS injection prior to hypoxia but gene expression levels did not change significantly in this setting. T cells also displayed significant *HIF1A* upregulation upon hypoxia and LPS after 24 hours as seen in the reserved setting. In neutrophils treated with *in vivo* hypoxia first, the following inflammatory stimulus further induced a significant upregulation of *HIF1A* mRNA after 4 hours, which was a lot faster than in the LPS-only treated group after 8 hours. However, only HIF target gene *ADM* displayed a similar highly significant upregulation after the additional LPS treatment, suggesting a potential priming effect due to the foregone hypoxic stimulation (table 18). However, *HIF2A* levels in neutrophils surprisingly dropped after 6 hours, which was also in contrast to the other participants that were treated with LPS only or LPS prior to hypoxia. This leads to the hypothesis that - even if not visible in qRT-PCR - the hypoxic stimulus must have some underlying effects on transcription of *HIF2A*. In line with the previous results, monocytes did not present any changes in terms of HIF gene transcription upon the treatment. However, *VEGF* mRNA was significantly upregulated in contrast to *GLUT-1* being downregulated upon 4 hours of hypoxic exposure to participants, which speaks for activation of the HIF pathway in monocytes at concentrations of 10.5 % oxygen *in vivo* (table 18). ADM gene expression was significantly increased in all leukocyte subsets after the LPS injection after the hypoxic stimulus, which was not seen to this extent in the LPS-treated groups before. Again, this can be interpreted as a confirmation of the hypoxic priming effects on inflammation-induced gene expression changes in leukocytes, highlighting the importance to investigate the interplay of both conditions more in detail (Bosco et al., 2006), (Chen & Gaber, 2021).

Gene expression of all three PHDs was downregulated in neutrophils and monocytes upon LPS treatment at 6 and 8 hours and returned to baseline levels after 24 hours, except for *PHD1* mRNA. *PHD1* expression in neutrophils was exactly in line with previously discussed data in the reversed “LPS-Hypoxia” and did not change during the 8 hours of the study but was significantly decreased 24 hours afterward. As this change was only seen in participants that were additionally exposed to *in vivo* hypoxia and not LPS only, we propose PHD1 downregulation as a neutrophil-specific response to hypoxia. In T cells, PHD regulation again demonstrated a different isoform-specific pattern, with *PHD1* being significantly decreased and *PHD2* being significantly increased after 4 hours of *in vivo* hypoxia (table 18). In monocytes, none of the PHDs was affected by hypoxic exposure but all PHDs were significantly downregulated similarly 4 hours after the LPS injection, which was only seen tendential for *PHD1* upon LPS treatment alone. This demonstrates once more that PHD regulation via the negative feedback loop is a highly adjusted system with very cell-specific features in

leukocytes. As outlined elsewhere before, it seems to be cell-specific how and if the different PHD isoforms regulate HIF activation with some redundancy or exclusively upon different challenges like hypoxia or inflammation (Semenza, 2001), (Metzen et al., 2003), (Niecknig et al., 2012), (Watts & Walmsley, 2019).

Expression of inflammatory cytokine IL-6 did not differ from prior observations and only partly reacted to immune activation, while *TNFA* mRNA displayed interesting changes upon hypoxic priming in T cells and monocytes (table 18). In T cells *TNFA* was significantly downregulated upon 4 hours of exposure to hypoxia and even more 4 hours after the LPS injection, which was in contrast to our prior findings, with no change in *TNFA* levels, suggesting an immunosuppression or delaying effect of hypoxia in T cells. In monocytes *TNFA* levels were not changed during the treatment but significantly upregulated 24 hours afterward, which was also not observed in the study branches before and can be accounted to the influence of hypoxic priming. Completely in line with that were our gene expression data for the Toll-like receptors, which increased significantly in neutrophils (TLR2 and TLR4) and monocytes (TLR2) 2 hours after the LPS injection when treated with 4 hours of *in vivo* hypoxia before but not without the hypoxic treatment (table 18). Taken together, these observations indicate that *in vivo* hypoxia with 10.5 % oxygen is sufficient to have an impact of inflammatory induced gene expression and potentially modulates immune reaction of leukocytes.

In line with our observations, other studies also demonstrated decreased T cell counts upon *in vivo* hypoxia and reduced T cell-mediated immune function *in vitro* after a stay at high altitude (Facco et al., 2005), (Mishra & Ganju, 2010), (Oliver et al., 2013). There is evidence that hypoxia has long-lasting effects on the redistribution of circulating T cells and their function, but not on B lymphocytes (Facco et al., 2005), (Mishra & Ganju, 2010), (Oliver et al., 2013). Further, some studies showed a tendency of decreased cytokine levels upon long-term high-altitude hypoxia (after days – weeks) under unstimulated baseline conditions (Facco et al., 2005), (Mishra & Ganju, 2010). Controversially, other studies showed strong evidence of increased Interleukin 6 levels upon acute and chronic hypoxia in human blood serum. It was hypnotised, that increased IL-6 levels could play a physiological role in promoting angiogenesis via VEGF and EPO induction (Klausen et al., 1997), (Hartmann et al., 2000), (Mazzeo et al., 2001). When comparing our *in vivo* data to human high-altitude studies one should note that our study was designed to investigate acute short-term effects with a maximum duration of 24 hours, which makes our observations not strictly comparable with those of the mentioned weeks-lasting studies in hypobaric hypoxia. In consequence, in our study protocol, using 4 hours of acute hypoxia, we could not see gene expression changes in terms of *IL6* or *VEGF*, nor did we detect a significant decrease in circulating T cells by hypoxia alone. However, in T cells of study participants gene expression of the inflammatory cytokine TNF-α was significantly decreased upon 4 hours of exposure to hypoxia and even more 4 hours after the additional inflammatory LPS stimulus, pointing into the same directions as the prior mentioned *in vivo* studies. In contrast to T cells monocytes were shown to upregulate mRNA levels of the cytokine TNF-α on the long term after challenge with hypoxia and LPS. Further monocytes and neutrophils showed increased Toll-like receptor mRNA levels (table 18). These results suggest a highly cell-specific effect of hypoxic priming upon inflammation, maybe differentiating between cells of the acute innate immunity (monocytes, neutrophils) and the adaptive system (T cells).

Table 18: Summary of gene expression changes in leukocytes treated with hypoxia prior to LPS

Listed are relative gene expression changes compared to mRNA baseline levels. ↑ represents significant upregulation, ↓ represents significant downregulation of the gene, brackets ( ) indicate tendencies of gene regulation without significance. Differences between the cell types are highlighted in colour.

		Hypoxia prior to LPS		
		after hypoxia	4 h after LPS injection	
gene	**cell type**	**4 h**	**8 h**	**24 h**
HIF1A	Neutrophils	↑	↑	–
	T cells	–	–	↑
	Monocytes	(↑)	–	–
HIF2A	Neutrophils	–	↓ (6 h)	–
	T cells	–	–	–
	Monocytes	–	–	–
HIF3A	Neutrophils	(↓) (2 h)	–	–
	T cells	↑	–	–
	Monocytes	–	–	–
VEGF	Neutrophils	–	–	–
	T cells	–	–	–
	Monocytes	–	↑	–
GLUT-1	Neutrophils	(↑)	–	↑
	T cells	–	↓	–
	Monocytes	↓	↓	↓
PDK1	Neutrophils	–	↓ (6 h)	–
	T cells	–	↑	–
	Monocytes	–	–	–
ADM	Neutrophils	↑	↑↑	–
	T cells	–	↑	–
	Monocytes	–	↑	–
PHD1	Neutrophils	–	–	↓
	T cells	↓	–	–
	Monocytes	–	↓	–
PHD2	Neutrophils	–	(↓)	–
	T cells	↑	↑	↑
	Monocytes	–	↓	–
PHD3	Neutrophils	–	↓	–
	T cells	–	–	↑
	Monocytes	–	↓	–
TNFA	Neutrophils	–	↑	–
	T cells	↓	↓	–
	Monocytes	–	–	↑
IL6	Neutrophils	–	(↓)	–
	T cells	–	–	–
	Monocytes	–	↑	–
TLR2	Neutrophils	–	(↑)	–
	Monocytes	–	↑	–
TLR4	Neutrophils	–	↑	–
	Monocytes	–	–	–

4.7 Conclusion and outlook

In summary, our data present interesting insights into the interplay of hypoxia and inflammation-induced HIF regulation *in vitro* and *in vivo*. Regulation and activation of the HIF pathway were shown by both hypoxia and inflammation, but with different implementations for HIF gene regulation. Our results from *THP-1* cells and PMBCs *in vitro* convincingly showed, that there is a strong time-dependency in HIF regulation in the combination of hypoxia and LPS-induced inflammation. Moreover, we provide evidence, that immune cells are highly sensitive regarding the order in which inflammation in combination with hypoxia occurs.

Our investigations on the first in-clinic used HIF-stabiliser *Roxadustat* unveil the potential of the drug to act on and strongly influence the HIF pathway in circulating leukocytes in a context-dependent manner with inflammation, which was not shown before.

For the first time, we report that moderate *in vivo* hypoxia leads to immune cell-specific gene expression changes regarding the HIF pathway. Using the experimental human endotoxemia model we further provide new insights into the regulation of HIF and its target genes in distinct immune cell subsets upon acute inflammation *in vivo*. It becomes clear, that there are strong cell-specific differences in the mediation of HIF activation by distinct PHD isotypes, especially under combined hypoxic and inflammatory conditions. Further, we could show, that hypoxic priming plays an important role in shaping both, the HIF and immune response of circulating leukocytes with the potential for long-term effects in gene regulation.

Taken together, hypoxia and inflammation are integrated and present a multi-layered interplay, which depends highly on the order of occurring stimuli. Acute hypoxia but also HIF-interfering medication has the potential to shape immune reactions and immune cell-specific responses. Further investigations are needed to elucidate this interplay in more detail on a molecular basis. This might improve patient outcome in clinical pathologies dealing with hypoxic inflammation. Moreover, additional studies are needed to examine the safety, long-term effects but also new application fields of drugs, interfering with the HIF system regarding systemic and cell-specific effects.

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6. Appendix

6.1 Supplementary figures

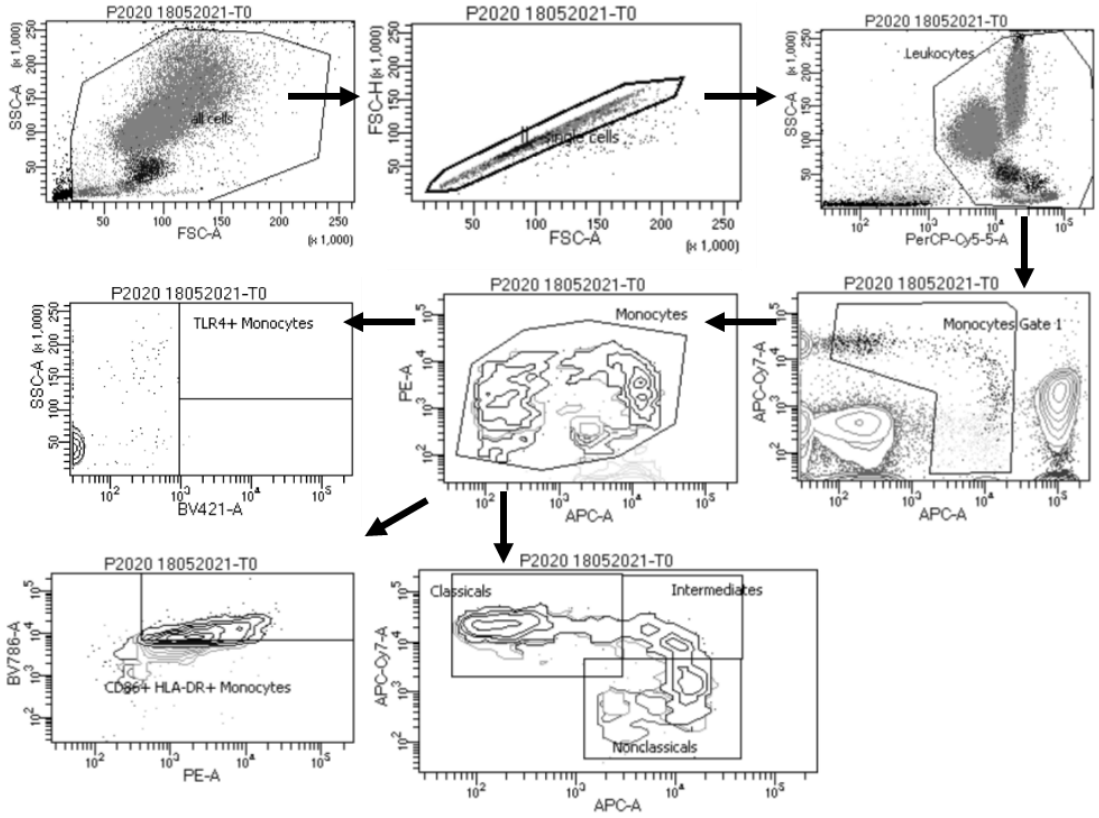

Figure 60: Flow cytometry gating strategy for monocyte characterisation and activation

After discrimination of single cells, gating monocytes from whole blood samples of human subjects included the following steps: discrimination of leukocytes ($CD45^+$), monocytes ($CD16^+CD14^+$), exclusion of outliers, gating for Toll-like receptor 4 (TLR4) positive monocytes, $CD86^+HLA\text{-}DR^+$ monocytes, and differentiation into classical ($CD16^+CD14^{++}$), intermediate ($CD16^{++}CD14^{++}$) and nonclassical ($CD16^{++}CD14^+$) monocytes. Arrows indicate sequent gating steps, representative example.

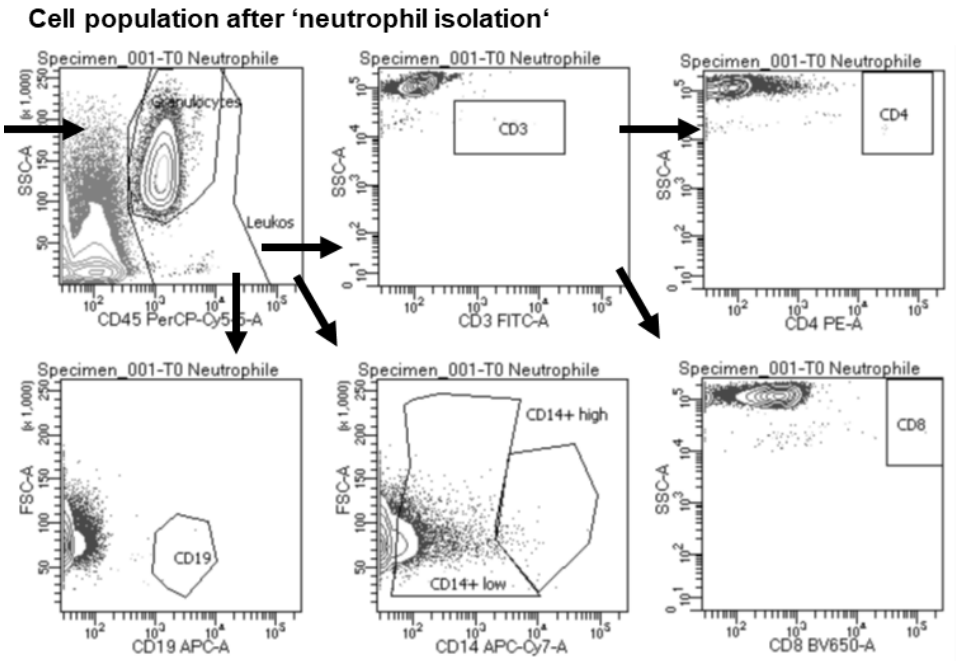

Figure 61: Flow cytometry gating strategy for isolated neutrophils

Purity control of neutrophil isolation from whole blood of human subjects. 'Phenotype' staining: After gating for single cells, the leukocyte population was discriminated via $CD45^+$ staining and further divided into the respective leukocyte subgroups. $CD3^+$: T lymphocytes, $CD3^+CD4^+$: helper T cells, $CD3^+CD8^+$: cytotoxic T cells, $CD19^+$: B lymphocytes, $CD14^{++}$: monocytes, representative example.

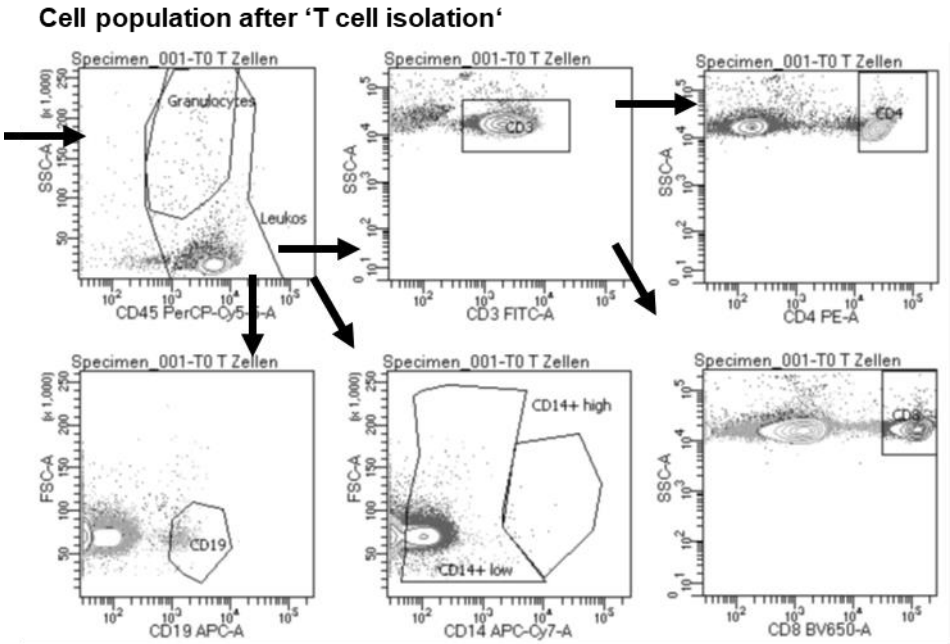

Figure 62: Flow cytometry gating strategy for isolated T cells

Purity control of T cell (CD3) isolation from whole blood of human subjects. 'Phenotype' staining: After gating for single cells, the leukocyte population was discriminated via $CD45^+$ staining and further divided into the respective leukocyte subgroups. $CD3^+$: T lymphocytes, $CD3^+CD4^+$: helper T cells, $CD3^+CD8^+$: cytotoxic T cells, $CD19^+$: B lymphocytes, $CD14^{++}$: monocytes, representative example

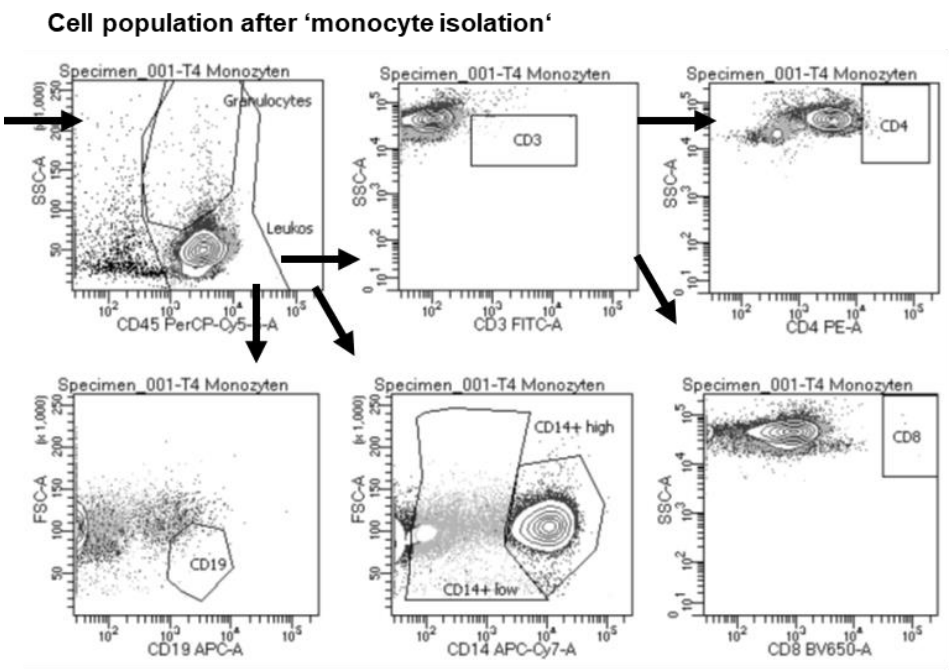

Figure 63: Flow cytometry gating strategy for isolated monocytes

Purity control of monocytes isolation from whole blood of human subjects. 'Phenotype' staining: After gating for single cells, the leukocyte population was discriminated via $CD45^+$ staining and further divided into the respective leukocyte subgroups. $CD3^+$: T lymphocytes, $CD3^+CD4^+$: helper T cells, $CD3^+CD8^+$: cytotoxic T cells, $CD19^+$: B lymphocytes, $CD14^{++}$: monocytes, representative example.

6.2 List of figures

6.3 List of tables

6.4 List of abbreviations

2-OG	2-oxoglutaric acid
ACTB	beta-actin
ADM	adrenomedullin
AKT	protein kinase B
AngII	Angiotensin II
ANOVA	analysis of variance
APCs	antigen-presenting cells
ARNT	aryl hydrocarbon nuclear translocator
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
CD	Cluster of differentiation
cDNA	complementary DNA
CI	Confidence interval
CT	cycle of threshold
C-TAD	C-terminal transactivation domain
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked Immunosorbent Assay
EPO	erythropoietin
FACS	flow cytometry
FCS	Fetal Calf Serum
Fe	iron
FIH	factor-inhibiting HIF
GLUT1	glucose transporter 1
h	hour
HIF	hypoxia-inducible factor
HLA-DR	human leukocyte antigene DR isotype
HREs	hypoxia-responsive elements
HSCs	hematopoietic stem cells
IKK	IkappaB kinase
IL	Interleukin
ILCs	lymphoid cells
iNOS	inducible NO synthase
IRAKM	Interleukin-1 receptor-associated kinase
JAK	Janus tyrosine kinase
kDa	kilodalton
kPa	kilo pascal
LPS	lipopolysaccharide
m	meter
MAPK	mitogen-activated protein kinase
MHCII	major histocompatibility complex 2
min	minute

mmHg	millimetre of mercury
mRNA	messenger ribonucleic acid
mTORC1/2	mammalian target of rapamycin complex 1 and 2
NET	neutrophil extracellular trap
NF-κB	transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cells
NO	nitric oxide
N-TAD	N-terminal transactivation domain
NTP	nucleotide triphosphate
O_2	oxygen
ODD	oxygen-dependent degradation domain
ODD	oxygen-dependent degradation domain
p44/42 MAPK	mitogen-activated protein kinase
PAS	Per-Arnt-Sim
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	pyruvate dehydrogenase kinase 1
PFA	paraformaldehyde
PHD	prolyl-4-hydroxylase-domain
PI3K	phosphatidylinositol 3-kinase
PO_2	partial pressure of oxygen
PRR	pattern recognition receptors
PS	penicillin streptomycin,
PVDF	polyvinylidene difluoride
pVHL	von-Hippel-Lindau protein
qRT-PCR	quantitative reverse transcription PCR
ROS	reactive oxygen species
ROUT	robust regression and outlier removal
Rox	Roxadustat
RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SpO_2	peripheral capillary oxygen saturation
STAT3	transcription protein 3
T regs	regulatory T cells
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th	T helper cells
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-alpha
UV	ultraviolet
V	Volt
VEGF	vascular endothelial growth factor

6.5 List of publications and congress contributions

Papers

1. **Schönberger, T.**, Fandrey, J., Prost-Fingerle, K. Ways into Understanding HIF Inhibition. Cancers. 2021; 13 (1):159. https://doi.org/10.3390/cancers13010159

2. Mafakheri, S., Flörke, R. R., Kanngießer, S., Hartwig, S., Espelage, L., De Wendt, C., **Schönberger, T**., Hamker, N., Lehr, S., Chadt, A., & Al-Hasani, H. AKT and AMP-activated protein kinase regulate TBC1D1 through phosphorylation and its interaction with the cytosolic tail of insulin-regulated aminopeptidase IRAP. The Journal of biological chemistry. 2018; 293 (46): 17853 – 17862. https://doi.org/10.1074/jbc.RA118.005040

Talks

1. **Schönberger, T.,** Tebbe, B., Hörbelt-Grünheidt, T., Jakobs, M., Witzke, O., Schedlowski, M., Fandrey, J. Adaption of immune cells to Hypoxia & Inflammation. 11th Young Physiologist Symposium, Essen, Germany. 2022

2. **Schönberger, T**., Tebbe, B., Hörbelt-Grünheidt, T., Jakobs, M., Witzke, O., Schedlowski, M., Fandrey, J. Inflammatory hypoxia in human leukocytes. Human Physiology Workshop, German Aerospace Center (DLR), Cologne, Germany. 2021

3. **Schönberger, T**., Tebbe, B., Fandrey, J. Effects on inflammatory hypoxia on human leukocytes. 20th Day of Science at the Medical Faculty, University of Duisburg-Essen, Essen, Germany. 2021

4. **Schönberger, T**., Tebbe, B., Hörbelt-Grünheidt, T., Jakobs, M., Witzke, O., Schedlowski, M., Fandrey, J. How do human leukocytes respond to inflammatory hypoxia? The 100th Annual Meeting of the German Physiological Society, Frankfurt, Germany. 2021

5. **Schönberger, T**., Fandrey, J. Crosstalk of Hypoxia and Lipid Signalling. 14th winter seminar of the medical faculty of the University of Duisburg-Essen, Pichl bei Schladming, Austria. 2020

6. **Schönberger, T**., Fandrey, J. Crosstalk of Hypoxia and Lipid Signalling. Retreat Research Training Group 2098, Essen, Germany. 2019

Posters

1. **Schönberger, T**., Glaser, U., Meyer zu Heringdorf, D., Gulbins, E., Fandrey, J. Crosstalk of Hypoxia and Lipid Signalling. 98th Meeting of the German Physiological Society (DPG), Ulm, Germany. 2019

2. **Schönberger, T**., Glaser, U., Meyer zu Heringdorf, D., Gulbins, E., Fandrey, J. Crosstalk of Hypoxia and Lipid Signalling. 42nd Annual Scientific Metting of the European Lipoprotein Club (ECL), Tutzing, Germany. 2019

3. **Schönberger, T**., Glaser, U., Meyer zu Heringdorf, D., Gulbins, E., Fandrey, J. Crosstalk of Hypoxia and Lipid Signalling.18th Day of Science at the Medical Faculty, University of Duisburg-Essen, Essen, Germany. 2019

4. **Schönberger, T**., Glaser, U., Meyer zu Heringdorf, D., Gulbins, E., Fandrey, J. Crosstalk of Hypoxia and Lipid Signalling. Joined Retreat GRK 2098, Rauischholzhausen, Ebsdorfergrund, Germany. 2019

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