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The effect of anoxia on functions of neonatal monocytes and neutrophils

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My wife and son

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1 List of Abbreviations

7-AAD 7-aminoactionomycin D.

APC Allophycocyanin Conjugates

APS Ammonium Persulfate

Ax Anoxia

BCA Bicinchoninic acid assay

BPD bronchopulmonary dysplasia

$CaCl_2 \cdot 2H_2O$ Calcium chloride dehydrate

CBMC Cord blood mononuclear cell (mononuclear cells from umbilical cord blood)

CB-PMN Cord blood polymorphonuclear neutrophil

CD Cluster of Differentiation

CR3 Complement receptor 3

DHR Dihydrorhodamine 123

DFO Deferoxamine

E. coli Escherichia coli

EDTA ethylenediaminetetraacetic acid

EOS early-onset sepsis

FACS Fluorescent activated cell sorting

FCS Fetal Calf Serum (fetal calf serum)

FITC fluorescein isothiocyanate

FSC Foward Scatter (forward scattered light)

GBS group B streptococci

GFP green fluorescent protein

Hx Hypoxia

HIF 1 Hypoxia-inducible factor 1

ICAM-1 and 2 Intercellular adhesion molecule 1 and 2

IL-6 interleukin-6

IL-8 interleukin-8

IPTG isopropyl- β- D- thiogalactopyranoside

LB medium Lennox-L-Broth medium

LOS late-onset sepsis

MFI Mean Fluorescence Intensity (mean fluorescence intensity)

MOI Multiplicy of infection (multiplicity of infection)

NaN_3 Sodium azide

NADPH nicotinamide adenine dinucleotide phosphate

NETs Neutrophil extracellular traps

NH_4Cl ammonium chloride

NSB umbilical cord blood

Nx Normoxia

OD optical density

PBMC Peripheral blood mononuclear cell (mononuclear cells from adults)

PB-PMN Cord blood polymorphonuclear neutrophil

PBS Phosphate buffered saline

PE phycoerythrin

PerCP peridinin chlorophyll protein

PHDs Prolyl hydroxylases

pi. post infectionem

PIC Protease Inhibitor Cocktail

PMA phorbol-12-myristate-13-acetate

PMN polymorphonuclear neutrophil

PMSF Phenylmethylsulfonyl fluoride

P/S Penicillin-Streptomycin

PRR Pathogen Recognition Receptors (pathogen-recognizing receptors)

PLV Periventricular leucomalacia

ROS Reactive oxygen species (reactive oxygen species)

rpm rounds per minute

RPMI Roswell Park Memorial Institute = cell culture medium

SDS Sodium dodecyl sulphate

SSC Side Scatter (side scattered light)

SIRS Systemic inflammatory response syndrome

TNF- α Tumor Necrosis Factor- α

VCAM 1 Vascular cell adhesion molecule 1

2 Introduction

2.1 Neonatal infection

Neonatal sepsis is a systemic inflammatory response syndrome (SIRS) caused by bacterial, viral or fungal infection [1]. According to the age of onset, neonatal sepsis has been classified to two groups: early-onset sepsis (EOS) and late-onset sepsis (LOS). Pathogens causing EOS commonly representing vertical mother-to-child transmission and are obtained before or during delivery from the mothers´ rectovaginal flora, while LOS is acquired after delivery and attributed to organisms which descend from the hospital environment [1-2]. The most common causes of neonatal sepsis are bacterial infections caused by beta-hemolytic group B streptococci (GBS) or Escherichia coli (*E. coli*) [3].

Neonatal sepsis is a leading cause of neonatal morbidity and mortality [4]. Severe infections lead to more than 1 million neonatal deaths globally each year as estimated by the World Health Organization [5]. The incidence of neonatal sepsis ranges between 1 to 5 out of 1000 live births in developed countries and 49 to 170 out of 1000 live births in developing countries [6].

While in term neonates the incidence of neonatal sepsis is relatively low (0.05%) it increases to up to 36% among very low birth weight infants (VLBWI) [7].

The consequences of neonatal sepsis are severe; in addition to a high mortality rate of 10-20% [8], neonatal sepsis increases the risk of post-inflammatory secondary diseases such as bronchopulmonary dysplasia (BPD) and periventricular leucomalacia (PVL) thereby influencing the long-term outcome of the affected infants [9-12]. For example, premature babies with neonatal sepsis are about twice as likely to develop infantile cerebral palsy compared to babies without sepsis [8].

2.2 The neonatal immune system

The immune system consists of two essential components – the innate immune system and the adaptive immune system. The former responds quickly and non-specifically to pathogens, while the latter responds slowly but has antigenic specificity and a long-lived immunological memory [13]. During an infection, the innate immunity is triggered immediately, and the time for complete activation does not exceed a few minutes to a few hours [14]. Adaptive immunity, which takes 1-2 weeks to establish, is very important for host defense in the later phases of an

infection and secondary infection due to its capacity to respond more effectively to re-stimulation [13].

The innate immune system consists of soluble elements (such as complement proteins) and a diversity of cellular constituents including granulocytes (eosinophils, basophils and neutrophils), monocytes, mast cells, dendritic cells, macrophages and natural killer cells. The adaptive immunity is comprised of $CD4^+$ and $CD8^+$ T lymphocytes, antibodies and B cells, which respond slowly and show augmented immune memory and antigenic specificity[15].

The increased susceptibility of the newborn and especially the preterm infant to infections is mainly explained by the “neonatal” state of the immune system [16]. After birth, the immune system has to adapt within a very short time to the conditions outside the womb from the fetal state, in which the induction of tolerance towards the maternal organism is crucial, to the neonatal state, in which it has to “learn” to fight off a wide variety of pathogens and to distinguish pathogens from commensals [17]. Many immune cells react differently to infectious agents during this phase compared to the adult organism. Especially in the first days of life, innate immunity is essential for the survival of newborns, since adaptive immunity needs some time to develop and shows functional differences to that of adults [18-21]. However, also innate immune functions differ between neonates and adults. For example, known differences include a reduced antimicrobial activity of neonatal granulocytes [18-19], a reduced costimulatory ability of antigen-presenting cells [20-21] and an altered cytokine production [22]. This functional "immaturity" of the neonatal immune system compared to the immune system of adults may explain the increased risk for generalization of infections and for development of post-inflammatory diseases like BPD or PVL [23].

Despite significant scientific progress, the mechanisms that contribute to the “neonatal” state of the innate immunity and how it comes to immune adaptation to the adult state are poorly understood.

2.3 The Role of Monocytes and Neutrophils in Host Defense

Monocytes are a part of innate immunity and produced by the bone-marrow and mononuclear phagocyte system (MPS) members. Monocytes play a pivotal role in innate immunity. They can perform phagocytosis, antigen presentation, secretion of chemokines and cytokines, and proliferation as a reaction to infection and injury and can differentiate into macrophages and dendritic cells when they are recruited to tissues [24].

The most abundant leukocytes circulating in the body are neutrophils, which are the first defense against infections. Neutrophils are recruited into infected tissues from the circulation. Main neutrophil functions are migration, phagocytosis, killing of intracellular microorganisms by oxidative and non-oxidative cytotoxic mechanisms, release of antimicrobials, formation of neutrophil extracellular traps (NETs) and release of pro-and anti- inflammatory cytokines, chemokines and other mediators [25-26].

Migration of monocytes and neutrophils to inflamed tissue requires the expression of so called integrins on leucocytes and their corresponding ligands on endothelial cells. One of the most important integrins for trans-endothelial migration of monocytes and neutrophils is the integrin $\alpha_M\beta_2$/complement receptor 3 (CR3) or Macrophage-1 antigen (Mac-1) consisting of CD11b (integrin α_M) and CD18 (integrin β_2) [27]. Integrin $\alpha_M\beta_2$ is also involved in phagocytosis [28].

Phagocytosis is the active uptake of particles (> 0.5 µm), which in higher organisms is mainly performed by specialized cells called professional phagocytes (macrophages, dendritic cells, monocytes and neutrophilic granulocytes) [29]. After binding of opsonic receptors (e.g.C-type lectins, Fcγ receptors or complement receptors), the phagocytosis of opsonized microbes is performed by complicated interactions of cytoskeletal rearrangements and membrane lipids. Afterwards, the so-called phagosome fuses with the lysosome leading to degradation of its content [30].

Degradation and thereby elimination of phagocytized pathogens is mediated by bactericidal peptides, proteases, and reactive oxygen species (ROS) [31]. In the presence of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, molecular oxygen (O2) is converted by NADPH to generate superoxide anion (O2-), Which is a precursor of most other ROS [32]. The mechanisms by which ROS kill bacteria are incompletely understood [33]. The rapid release of ROS which requires a strong increase in oxygen consumption is called “respiratory burst” [34].

Neutrophil extracellular traps (NETs) are extracellular reticular structures formed by granule and cytoplasmic proteins which are congregated on a scaffold of depolymerized chromatin [35]. NETs can capture, neutralize and kill pathogens and are considered to prevent the spread of bacteria and fungi [36]. The release of NETs mainly happens via a cell death process known as NETosis [37-38].

Apoptosis is a programmed cell death used for the controlled breakdown of cells. It can occur via at least two signaling pathways, a caspase-dependent and a caspase-independent pathway [39]. During infection, apoptosis is essential to control the growth of invading

microorganisms. Apoptosis functionally separates phagocytes from their pro-inflammatory environment and prevents the release of destructive intracellular components, which may exacerbate the resolution of inflammation [40].

Cytokines are proteins that are released from various cell types to their environment. They are involved in cell signaling and immune modulation. Cytokines produced by neutrophils can be classified into members of the TNF family (comprising TRAIL, FasL and BAFF), colony-stimulating and angiogenic factors (comprising G-CSF and VEGF), growth factors (HB-EGF), chemokines (comprising IL-8, CCL2) and anti-/pro- inflammatory factors (comprising IL-1, IL-6 and TNFα) [41-43]. The most important cytokines produced by neutrophils are pro- and anti-inflammatory cytokines and chemokines. Interleukin-8 (IL-8) is a pro-inflammatory chemokine, whose secretion results in neutrophil activation and migration from peripheral blood to the infected area. [44]. Interleukin 6 (IL-6) is one of the multi-functional cytokines, displaying pro- and anti- inflammatory activity. IL-6 participates in inflammatory response by controlling differentiation, activation, proliferation, migration, and apoptosis of target cells [45]. Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine engaged in a variety of physiological procedures, for example inflammation, cell growth and apoptosis [46]. In neonates and adults with sepsis, serum concentrations of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α) have been reported to be increased [47].

2.4 Hypoxia and HIF during infammation

The function of immune cells strongly depends on environmental conditions. Decreased oxygen partial pressure (hypoxia), as it can be found in inflamed tissue and tumor microenvironment is an important regulatory signal for adult immune cells. Immune functions like phagocytosis, cytokine secretion, adhesion, migration and survival are stimulated by hypoxia in adult innate immune cells [48-49]. On adaptive immune cells, hypoxia mainly acts inhibitory, for example T-cell proliferation may be suppressed [50], the function of the T-cell receptor is reduced [51] and in B-cells cell cycle arrest may occur [52]. The transcription factor hypoxia-inducible factor 1 (HIF-1) is as one of the major modulators of the cellular response to hypoxia[53]. HIF-1 is a heterodimer complex formed by an alpha subunit (HIF-1α) which is sensitive to oxygen tension and a beta subunit (HIF-1β) that is constituently expressed. After transcription, the stability of HIF-1α subunit is regulated by the iron-dependent enzymes prolyl hydroxylases (PHDs). Under the condition of oxygen presence, HIF-1α is hydroxylated by PHDs and degraded in a procedure which is regulated by Von Hippel–Lindau (VHL) (Figure 1). Under hypoxia, PHDs are inactivated leading to HIF-1α accumulation. The HIF-1

transcription complex is formed by the binding of HIF1α to HIF1β, then it translocates to the nucleus and triggers the gene expression related to metabolism, angiogenesis, invasion and cell survival. [54]. Besides hypoxia, HIF-1α can also be activated by inflammatory pathways like NF-κB [55].

The fetus develops in an environment with low oxygen pressure (10-35mmHg) comparable to the oxygen pressure in inflamed tissue [56-57]. Little is known until now about the effects of hypoxia on the innate immune function of the newborn and about the effect of the chronic hypoxia in utero on the expression of HIF-1α in neonatal immune cells.

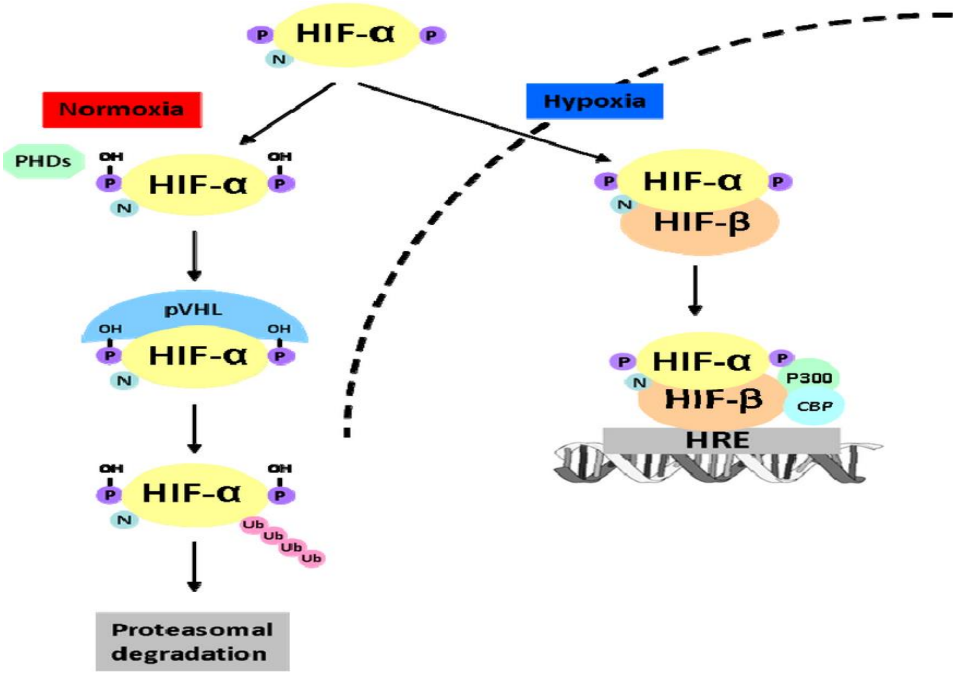


Figure 1: HIF-1α pathway [58]. In the presence of oxygen, HIF-1α is hydroxylated by PHDs and degraded under the regulation of Von Hippel-Lindau (VHL), however, under hypoxia, PHDs are inactivated, leading to the accumulation of HIF-1α.

2.5 Hypothesis

In a previous doctoral thesis in our working group it was found that expression of HIF-1α can be stimulated by anoxia in adult peripheral blood mononuclear cells (PBMC) but not in cord blood mononuclear cells (CBMC) leading to the hypothesis that chronic exposure to hypoxia leads to inactivation of HIF-1α in neonatal immune cells.

Therefore, the purpose of our study was to investigate the effect of anoxia on main functions of neonatal monocytes and neutrophils in comparison to adult monocytes and neutrophils with the hypothesis that anoxia stimulates inflammatory immune functions in adult but not in cord blood cells.

3 Material

3.1 Patients

The Ethics Committee of the Medical Faculty of the University of Tübingen approved this study (458/2019BO1). Umbilical cord blood was collected from healthy term newborns (≥37+0 gestational weeks) immediately after Caesarean section or vaginal delivery. Parents gave their written informed consent. Children with intra-amniotic infection were excluded. Peripheral blood from healthy adults was collected from adult volunteers.

3.2 Materials of biological origin

Brefeldin A	Sigma Company, Taufkirchen
E. coli DH5α (GFP) mut2	Tübingen University
E. coli DH5α - non- fluorescent	Tübingen University
Gentamycin	Sigma Company, Taufkirchen
Fetal Bovine Serum (FCS)	Fa. Biochrom, Berlin
Kanamycin	Sigma Company, Taufkirchen
Penicillin-Streptomycin	Biochrom AG, Berlin

3.3 Materials of non-biological origin

Acrylamid	Carl Roth GMBH, Karlsruhe
Ammonium chloride	University Pharmacy, Tübingen
Ammonium persulfate APS	Sigma-Aldrich,Taufkirchen
Ampuwa	Fresenius Kabi Deutschland GmbH, Bad Homburg
BD Fix / Perm Buffer	BD Biosciences, Heidelberg
BD Perm / Wash Buffer	BD Biosciences, Heidelberg
Bicinchoninic acid assay (BCA Protein assay)	Thermo Fisher Scientific,USA
Biocoll Separating Solution (density 1.077 g / ml)	Pan Biotech, Aidenbach
Bromphenolblau	Sigma-Aldrich,Taufkirchen
Calcium chloride dihydrate	Sigma-Aldrich,Taufkirchen
Deferoxamine	Sigma-Aldrich,Taufkirchen

Dihydrorhodamine 123	Life Technologies GmbH,Darmstadt
ECL Western Blotting Analysis System	GE Healthcare
FACS flow	Messrs.BectonDickinson, Heidelberg
Fixation buffer	Bio Legend,USA
Glutamine	Pan Biotech, Aidenbach
Glycerol	Carl Roth GMBH,Karlsruhe
Glycin	Carl Roth GMBH,Karlsruhe
Heparin sodium (5000 IU / 0.5 ml)	B. Braun, Melsungen
Hepes	Biochrom AG, Berlin
Low-fat milk powder	Carl Roth GMBH,Karlsruhe
Isopropyl- β -D- thiogalactopyranoside (IPTG)	Sigma company, Taufkirchen
Lennox-L-Broth-medium	Fa Invitrogen, Karlsruhe
Mercaptoethanol	Carl Roth GMBH,Karlsruhe
Methanol	Carl Roth GMBH,Karlsruhe
Nacl	Carl Roth GMBH,Karlsruhe
Page Ruler pre-stained protein ladders	Thermo Fisher Scientific,USA
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich,Taufkirchen
Phosphate-buffered saline solution (PBS-Dulbecco)	Pan Biotech, Aidenbach
Phorbol myristate acetate (PMA)	Sigma company, Taufkirchen
ProLong Diamond Antifade mounting solution	Thermo Fisher Scientific,USA
Protease Inhibitor Cocktail	Roche Diagnostics Gmbh, Mannheim
RPMI 1640 medium	Pan Biotech, Aidenbach
Running buffer	University pharmacy, Tübingen
Sodium azide	Fa. AppliChem GmbH, Darmstadt
Sodium dodecyl sulphate (SDS)	Carl Roth GMBH,Karlsruhe
TEMED	Carl Roth GMBH,Karlsruhe
Tris Base	Carl Roth GMBH,Karlsruhe
Triton X-100	Sigma-Aldrich,Taufkirchen
Trypan blue	Fa. Biochrom, Berlin
Tween 20	Carl Roth GMBH,Karlsruhe
12 M HCl Solution	Carl Roth GMBH,Karlsruhe

3.4 Antibodies

Alexa Fluor 647 / Allophycocyanin Conjugate (APC) - marked:

Mouse Anti-Human CD66b REA306	Miltenyi Biotec,Bergisch Gladbach

Fluorescein isothiocyanate (FITC) labeled:

Mouse Anti-Human CD18 TS1/18	Miltenyi Biotec,Bergisch Gladbach
Annexin -V	BD Biosciences, Heidelberg

Phycoerythrin (PE) labeled:

Mouse Anti-Human CD14 M5E2	BD Bioscience, Heidelberg
Mouse Anti-Human CD11b REA713	Miltenyi Biotec,Bergisch Gladbach
IL-6 MQ2-6A3	BD Bioscience, Heidelberg
IL-8 G265-8	BD Bioscience, Heidelberg
TNF-a 6401.1111	BD Bioscience, Heidelberg
Zap-70 1E7.2	Life Technologies GmbH,Darmstadt

Peridinin- chlorophyll-protein Complex Conjugate (PerCP) labeled

7-aminoactinomycin (7-AAD)	BD Biosciences, Heidelberg
Mouse Anti-Human CD14 TÜK4	Miltenyi Biotec,Bergisch Gladbach

Western blot labeled

Mouse-anti-human HIF-1alpha	BD Biosciences, Heidelberg
Mouse-monoclonal-IgG GAPDH	Santa Cruz, Heidelberg
M-IgGκ BP -HRP	Santa Cruz, Heidelberg

Confocal microscopy labeled

Hoechst 33342	Sigma-Aldrich,Taufkirchen

3.5 Glass and Plastic Materials

15 ml and 50 ml centrifuge tubes	Greiner Bio- One GmbH, Frickenhausen
6,12, 24 and 48 well cell culture cluster, f bottom	Costar company, Bodenheim
5 ml Polystyrene Round-Bottom Tubes (“FACS-Tubes ”)	BD Biosciences, Heidelberg
96 well cell culture plate, f and u bottom	Greiner Bio- One GmbH, Frickenhausen
AnaeroGen 2.5L	Thermo Scientific, UK
Colorless pipette tips (10 µl)	Viozym Scientific, Oldendorf
Cover glass	VWR International
Disposable syringes, Plastipack (30 ml, 50 ml)	BectonDickinson, Heidelberg
Eppendorf Cup Reaction Tubes	Greiner Bio- One GmbH, Frickenhausen
GasPak	BD BBL, USA
Microscope slide	R. Langenbrinck, Emmendingen
Mini Protean System	Bio-Rad,USA
Mini Protean Tetra Cell	Bio-Rad,USA
Neubauer counting chamber	Karl Hecht GmbH & Co. KG "Assistant", Sondheim / Rhön
Parafilm M	Parafilm USA
Poly-L-Lysine coated coverslips	Corning USA
Pipette tips blue (500-1000 µl) and yellow (20-200 µl)	Greiner Bio- One GmbH, Frickenhausen
Safety multifly set	Sarstedt AG & Co., Nümbrecht
Whatman paper	Sigma-Aldrich,Taufkirchen

3.6 Devices and Software

8-channel transfer pipette (20-200 µl)	Brand GmbH & Co. KG, Wertheim
Bosch economic refrigerator	Bosch, Stuttgart
CellQuest Pro	BD Biosciences, Heidelberg

Centrifuge Rotanta 460 RS and 46 RS	Messrs. Hettich, Tuttlingen
Certomat H and MO II	B.Braun Biotech international, Melsungen
Clean bench	Messrs. Heraeus Instruments, Stuttgart
Confocal microscope-cLSM 710 NLO	Zeiss, Oberkochen
Consort E831	Consort bvba;Belgium
Cytospin 16A centrifuge	Messrs. Hettich, Tuttlingen
Duomax 1030	Heidolph Instruments GmbH,Schwabach
ELISA plate reader	Sunrise Tecan GMBH, Germany
Eppendorf ThermoMixer	Eppendorf AG, Hamburg
FACSDiva softerware	BD Biosciences, USA
Flow cytometer FACS Canto II	BD Biosciences, Heidelberg
Flow cytometer FACS Calibur	BD Biosciences, Heidelberg
FlowJo Software	Becton, Dickinson and Company,USA
Freezer (-80 ° C)	Telstar Igloo GreenLine 570L
GFL water bath	GFL Labortechnik mbH, Burgwede
GraphPad Prism 8.4	GraphPad Software, La Jolla, California, USA
iBright CL1000	Thermo Fisher Scientific, United States
ImageJ software	National institues of health, USA
Immobilon P Transfer Membrane	Millipore corporation,USA
Incubator (5% CO2, 37 ° C, saturated with water vapor)	Messrs. Heraeus Instruments, Stuttgart
Light microscope	Olympus, Hamburg
Microsoft Excel Microsoft professional plus 2010	Microsoft Corporation, Unterschleißheim,
Microsoft Word Microsoft professional plus 2010	Microsoft Corporation, Unterschleißheim,
Photometer	Eppendorf, Hamburg

Pipettes (2, 10, 20, 100, 200, 1000 µl)	Fa. Abimed, Langenfeld
Ruskinn InVivo2 Hypoxia workstation	Biotrace I.B.U, UK
Schüttelmixer Certomat H	B. Braun Biotech International, Göttingen
SYSMEX-KX21	Sysmex GmbH, Norderstedt
Thermo Heraeus Herasafe HS12 Safety Cabinet	Heraeus Instruments, Stuttgart
Titramax 1000	Heidolph Instruments GmbH;Schwabach
Vortex	Bender & Hobein AG, USA

4 Methods

4.1 Isolation of mononuclear cells and polymorphonuclear neutrophils (PMNs) from umbilical cord blood or adult peripheral blood

Mononuclear cells from cord blood (cord blood mononuclear cells, CBMC) and polymorphonuclear neutrophils from cord blood (cord blood polymorphonuclear neutrophils CB-PMNs) as well as mononuclear cells from adult peripheral blood (peripheral blood mononuclear cells, PBMC) and polymorphonuclear neutrophils from adult peripheral blood (adult peripheral blood polymorphonuclear neutrophils PB-PMNs) were isolated by density gradient centrifugation according to Boyum [59-60]. 15 ml of whole blood were diluted 1: 2 with PBS and then carefully layered onto 15 ml of Biocoll separating solution in 50 ml tubes without mixing. This was followed by a centrifugation at 400 × *g*, 20 ° C for 25 minutes without brake. After this step, CBMC or PBMC separated in the cell ring in the intermediate layer above the Biocoll solution, the cord blood or adult blood PMNs in the cell pellet.

For isolation of PBMC and CBMC, the cell ring of the interphase was suctioned off and taken up in 50 ml PBS. The cell suspension was centrifuged at 500 × *g* for 10 minutes at 10 ° C. The supernatant was carefully removed and the cell pellet was resuspended in 10 ml of cold PBS. The cell count was determined using the Sysmex KX-21 and a further centrifugation step followed with 400 × *g* at 4 ° C for 10 minutes. After discarding the supernatant, the cell pellet was adjusted to 2×10^6 cells/ml in RPMI medium with a 10% FCS.

For isolation of PMN the remaining liquid (after removing the cell ring) was sucked off except for the polymorphonuclear neutrophils and erythrocytes in the sediment. The pellet was taken up in 50 ml of ammonium chloride and the cell suspension was incubated on ice for a maximum of 30 minutes until the erythrocytes were completely lysed. Cell suspension was then centrifuged at 300 x g for 5 minutes at 10 ° C, the supernatant was poured off and the sample was re-suspended in 35 ml of cold PBS. The sample was centrifuged again at 300 × g for 5 minutes at 10 ° C, the supernatant was poured off and the sample was re-suspended in 20 ml of cold PBS. The cell count was determined using the Sysmex KX-21 and a further centrifugation step followed with 400 × *g* at 4 ° C for 10 minutes. After discarding the supernatant, the cell pellet was adjusted to 2×10^6 cells/ml in RPMI medium with 10% FCS.

4.2 Cell counting

Before culturing cells, the cell count was determined in order to ensure a setting of 2×10^6 cells / ml. The measurement was made by an automatic hemocytometer (Sysmex KX-21). The measuring accuracy was validated by manual counting in the Neubauer counting chamber.

4.3 Cultivation of bacteria

For the experiments, two different *E. coli* laboratory strains were used: A clinical isolate of *E. coli* K1, carrying the green fluorescence protein (gfp)-mut2 encoding plasmid pCD353, expressing a prokaryotic variant of GFP under control of a lac promoter (*E.coli*^{GFP}) and the laboratory strain *E. coli* DH5α [61]. Bacteria were stored at -80°C. They were grown in 5 ml Lennox-L-Broth (LB) medium with shaking overnight at 37 ° C at 200 rounds per minute (rpm). For *E.coli*^{GFP} , 50 µg/ml kanamycin and 1 mmol/l IPTG were added for *GFP* induction. The next morning 100 µl of the bacterial suspension were pipetted into 5 ml of new LB medium with or without additives (see above) and incubated for another 2-4 hours in the shaker until the early logarithmic growth phase was reached. The number of bacteria was determined in a spectrophotometer. An OD of 600nm corresponds to approximately $2x10^6$ bacteria / µl. The bacterial culture was then placed on ice to prevent further multiplication. Bacteria were used in a multiplicity of infection (MOI, ratio of stimulated cells: bacteria) of 1:50.

4.4 Cell culture

Cells were cultured at a concentration of 2×10^6 cells/ml in microtiter plates (6-well (4×10^7 cells / well), 12-well w(4×10^6 cells / well), 24-well(2×10^6 cells / well), 48-well (1×10^6 cells / well) and 96-well (1×10^5 cells / well) format) at 37 ° C with 5% CO_2 and an atmosphere saturated with water vapor (normoxic condition) or at 37 ° C in a sealed jar with an AnaeroGen sachet (Oxoid) to maintain conditions with < 0.1% O_2 and 7-15% CO_2 (anoxic condition). For hypoxic incubation at 1% O_2 cells were cultured in a Ruskinn InVivo2 Hypoxia workstation. For Deferroxamine (DFO) treatment, cells were treated with 10 µM DFO. RPMI-1640 with 10% addition of fetal calf serum (FCS) and 1% glutamine was used as the culture medium.

4.4.1 *in-vitro* infection model with *E. coli* ^{GFP}

After 4h incubation in normoxia, anoxia or hypoxia, PMNs and mononuclear cells were infected with *E. coli* ^{GFP} (MOI 1:50) for one hour at 37 ° C and 5% CO_2 in 48-well plates.

Uninfected cells served as controls. After one hour, the cells were removed and adherent cells were rinsed from the bottom of the plate. Cells were centrifuged at 300 × *g* at 4 ° C for 5 minutes, the supernatant was removed and the cells were layered on an FCS cushion (1.5 ml) in a 15 ml Falcon tube and centrifuged at 300 × *g* at 4 ° C for 5 minutes in order to remove remaining extracellular bacteria. After centrifugation, the supernatant was removed and the cells were taken up in the original amount in new RPMI medium with 10% FCS. In addition, 200 µg / ml gentamycin were added to the suspension in order to eliminate remaining extracellular bacteria. The cells were analyzed directly by flow cytometry.

4.5 Flow cytometry

Flow cytometry (fluorescence activated cell sorting, FACS) is an established method for the evaluation of cells in size, granularity and expression of fluorescence stained antigens [62]. Flow cytometers used for analyses in this thesis were the FACS Calibur and the FACS Canto, both from Becton Dickinson (Heidelberg). The data was recorded and further processed on a HP computer with the help of FlowJo Software (Becton, Dickinson & Company).

4.5.1 Immunophenotyping

For staining of extracellular antigens, 2×10^5 cells / 100µl PMNs and 2×10^5 cells / 100µl mononuclear cells from the culture were transferred to FACS tubes and incubated with the appropriate amount of antibodies for 10 minutes at 4 ° C in the dark. For antibody details, (see Table 1). The suspension was then diluted with 4 ml FACS-Flow and centrifuged at 330 × *g* at 4 ° C for 5 minutes. Cell analysis was performed in a flow cytometer after discarding the supernatant.

Antibody (Mouse anti-human)	Conjugate	Amount
CD66b- REA306	APC	0.125 µg / 10^6cells
CD14- TÜK4	PerCP	0.5 µg / 10^6cells
CD14- M5E2	PE	1.0 µg / 10^6cells
Annexin-V	FITC	0.25 µg / 10^6cells
7-AAD	PerCP	0.5 µg / 10^6cells
CD18- TS1/18	FITC	0.5 µg / 10^6cells
CD11b- REA713	PE	0.5 µg / 10^6cells
IL-6 MQ2-6A3	PE	0.125 µg / 10^6cells
IL-8 G265-8	PE	0.125 µg / 10^6cells
TNF-a 6401.1111	PE	0.25 µg / 10^6cells

Table 1: Antibody concentrations used in this study

4.5.2 Quantification of phagocytic activity

After incubation with *E. coli* ^{GFP} (see 4.4.1), the cells were further treated strictly on ice water. The staining against CD66b or CD14 was carried out as described in Section 4.5.2 using anti-CD66b-APC or anti-CD14-PerCP. Fifty thousand CD66b or CD14 positive cells were analyzed by flow cytometry. The phagocytic activity was determined as the percentage of CD66b-GFP or CD14-GFP double-positive cells from all CD66b-positive or CD14-positive cells and the mean fluorescent intensity (MFI) of the CD66b-GFP-double-positive or CD14-GFP-double-positive population.

4.5.3 Detection of apoptosis using Annexin-V / 7-AAD staining

The detection of apoptosis in PMNs and mononuclear cells was done by staining with Annexin-V and 7-AAD. Annexin-V is an early marker of apoptosis and binds to the membrane phospholipid phosphatidylserine. During apoptosis, phosphatidylserine translocates from the inside of the membrane to the outside and can then be bound by Annexin-V [63, 64]. 7-AAD binds directly to the DNA between cytosine and guanine. It can only get into the cell when the cell membrane is destroyed and serves as a late marker for apoptosis [65]. Thus, living cells were defined as Annexin-V negative and 7-AAD negative, early apoptotic cells were defined as Annexin -V positive and 7-AAD negative and late apoptotic cells were defined as Annexin-V positive and 7-AAD positive cells [66]. For apoptosis staining, cells were cultured under

normoxia or anoxia for 4h. Afterwards, cell suspension was washed with 2 ml of annexin buffer (see Table 2) by centrifugation at 300 × *g* at 4 ° C for 5 minutes, followed by tipping off the supernatant and re-suspending the sample. Then, staining with Annexin V-FITC, anti-CD66b-APC or anti-CD14-PE was performed (see Table 1) by adding antibodies and incubating for 15 minutes at room temperature. Afterwards, 1 ml of annexin buffer was added and the previous wash cycle was repeated. In the last step, 7-AAD-PerCP was added to the sample and incubated again for 10 minutes at room temperature, followed by flow cytometric analysis.

Buffer	Composition	Amount
10 x Annexin buffer	Sodium azide	2g
	Nacl	81.816g
	Calcium chloride dihydrate	3.676g
	Hepes	23.821g
	Distilled water	1000ml
1 x Annexin buffer	10 x Annexin buffer	50ml
	Distilled water	450ml

Table 2:Annexin Buffer

4.5.4 Measurement of ROS production with DHR

Reactive oxygen species (ROS; e.g. H_2O_2, O_3, etc.) are byproducts of the normal oxygen metabolism. They serve as intercellular signaling molecules, have antimicrobial activity but also can cause damage of the host DNA [67]. DHR (Dihydrorhodamine123) is a non-fluorescent ROS indicator. DHR penetrates into the cell, binds to ROS and gets oxidized to rhodamine 123. Finally, the rhodamine has a green fluorescence and can be detected by FACS [68].

For ROS staining, cells were cultured in normoxia and anoxia for 4h and the cell count was set to 5×10^5/ml in RPMI. 10µl DHR (100µM) were added to 1 ml of the cell suspension and cells were incubated for 5 minutes at 37°C in a water bath. Next, cell suspension was stimulated with or without 100 µM phorbol myristate acetate (PMA) and incubated for another 15 minutes at 37°C in a water bath. Tubes were then transferred to and kept on ice after adding 1ml ice cold PBS. The supernatant was removed after centrifugation of cells at 300g for 5 minutes at 4°C. Cells were stained with extracellular antibodies (CD14-PerCP or CD66b-APC)

for 10minutes at 4 ° C in the dark. After washing and centrifugation (300 × *g* at 4 ° C for 5 minutes), cells were analyzed by flow cytometry.

4.5.5 Expression of CD11b and CD18

CD11b/CD18 (Macrophage antigen-1 (Mac-1), complement receptor 3 (CR3) a heterodimer of the αM (CD11b) and β2 (CD18)) subunits, is a pathogen recognition receptor, which has key functions in phagocytosis of bacteria and migration of activated leucocytes [69] For measuring of CD11b and CD18 expression on PMN and mononuclear cells, cells were cultured in normoxia, anoxia or hypoxia for 4h, extracellularly stained with CD11b and CD18 as described under 4.5.2 and analyzed by flow cytometry.

4.5.6 Intracellular cytokine staining

For intracellular staining of cytokines, PMNs from cord blood or adult blood were cultured over night at a concentration of 4×10^6/ml in RPMI-1640 medium supplemented with 10% FCS, 1% glutamine and 1% P/S in a 24-well plate under normoxia, anoxia or hypoxia. During the last 4 hours of culture, Brefeldin A (10µg/ml) was added to block cytokine secretion. Afterwards, cell suspension was transferred into a 15 ml Falcon tube (100ul of the cell suspension were directly used for FACS as negative control). Extracellular staining for CD66b was performed as described under 4.5.1. Following centrifugation at 330 × g for 5 minutes at 4°C, the pellet was resuspended after removing the supernatant. 200µl respectively 20 µl (for the control) of Fix/Perm buffer were added for 20 min at 4°C to permeabilize and fix the cells. Cells were then washed with Perm/Wash buffer (original stock diluted with Ampuwa at 1:10 ratio) and pelleted by centrifugation at 330 × *g* at 4 ° C for 5 minutes. The pellets were resuspended in 250µl Perm/Wash buffer after removing the supernatant. 50 µl cell suspension(8×10^5cells) were used per staining. Intracellular antibodies and their concentrations used for the experiments are shown in Table 1. Intracellular antibodies were added and incubated at 4°C for 30 min in the dark. The tubes were pelleted at 330 × *g* at 4 ° C for 5 minutes, then the cells were analyzed on a flow cytometer after removing the supernatant.

4.6 Western blot

Western blotting is an analytical technique for detecting specific proteins. In short, the sample undergoes gel electrophoresis after protein to separate proteins according to size. A primary antibody (synthetic or animal-derived antibody) is then used to detect a particular target

protein and a secondary antibody is used to visualize the primary antibody and thereby the protein of interest by various visualization methods such as chemiluminescence or radioactivity.

4.6.1 Preparation of Buffers for Western blotting

Buffer/Gel	Material	Amount
Lysis buffer	Ampuwa	2.15ml
	Tris/Hcl PH 7.4	125 µl
	5M Nacl	50 µl
	10% Triton x 100	25 µl
	Protease Inhibitor Cocktail	100 µl
	Phenylmethylsulfonyl fluoride	25µl
1× Running buffer	Tris base	30.3 g
	Glycine	144.0 g
	SDS	10.0 g
	H_2O	1000 ml
Loading buffer	250 mM Tris/HCI pH 6.8	1.25ml
	10 % SDS	4ml
	0.5 % Bromphenolblau	1mg
	10 % Glycerol	2ml
	Ampuwa	1.75ml
	5 % Mercaptoethanol	100µl
Transfer buffer	6g Tris Base 25mM	6g
	28.8g Glycine	28.8g
	400 ml Methanol	400 ml
	Distilled water	2000
20× TBST buffer	1M Tris/base ph 7,4	100ml
	5M NaCl	150ml
	0.5M EDTA	20ml

	Tween 20	10ml
	Distilled water	500ml
0.1% TBSTween buffer	20 x TBST-buffer	25ml
	Distilled water	500ml
10% Separating gel	Ampuwa	4ml
	Acrylamid	3.3ml
	1.5M Tris/HCL PH 8,8	2.5ml
	10% SDS	100 µl
	APS	50 µl
	TEMED	5 µl
5% Stacking gel	Ampuwa	2.93ml
	Acrylamid	495 µl
	1M Tris/HCl pH 6.8	225 µl
	Ammonium persulfate	16.3 µl
	TEMED	3.8 µl

Table 3 Buffers and Gels used in this study

4.6.2 Preparation of cell lysates for Western blotting

For Western blotting, cells were cultured for four hours under normoxia and stimulation with *E.coli* or under anoxia. Afterwards, cells were immediately transferred to 20 ml of ice-cold PBS in a 50ml Falcon on ice. Cells were washed twice with ice-cold PBS and centrifugation at 400 *g* for 10 minutes at 4°C. The Falcon tube was inverted and placed on a piece of paper to make sure as few liquid as possible after the supernatant was removed. 100µl of lysis buffer (Composition see table 3) were added to 2×10^7-8×10^7cells. Then cells were transferred to an Eppendorf safe-lock 1.5ml tube and snap-frozen in liquid nitrogen. After thawing on ice, the suspension was centrifuged at 13,000 rpm for 10 min at 4°C. 15 µl of the supernatant was diluted with 15 µl lysis buffer in a new 1.5ml tube (for later protein quantification), the remaining 85 µl supernatant were transferred into another 1.5ml tube (for later western blotting). Samples were stored at −80 °C until further processing.

4.6.3 Protein quantification for Western blotting

Protein quantification for western blotting was done by bicinchoninic acid assay (BCA-Assay) (from Thermo Fisher Scientific). The BCA protein assay is employed with the aim of quantifying the total protein in a sample. The principle of this method is that Cu^{2+} is reduced to Cu^{+1} by proteins in an alkaline solution (the biuret reaction) leading to bicinchoninic acid forming a purple color.

The BCA working reagent was prepared by mixing an appropriate amount of BCA reagent A with BCA reagent B at a ratio of 50:1 (190µl per well were required). Samples were pipetted into a 96 well plate according the pipetting scheme seen in Figure 1. Afterwards, 190µl BCA working reagent were added to each well. The plate was then put on a plate shaker and incubated at 37 °C for 30 min. Afterwards, the plate was analyzed at a wavelength of 550 nm (absorbance, OD550 nm) in an ELISA plate reader (Sunrise, Tecan GMBH, Germany). Based on the standard concentrations, a standard curve was plotted and OD values were calculated accordingly.

BSA-Standard + Lysis-Buffer											
	0,5 39,5 10µl	2,5 37,5 je 10µl	5 35 10µl	10 30 10µl	15 25 10µl	20 20 10µl	25 15 10µl	30 10 10µl		2 8	2 8
10									10	5 5	5 5
10									10	7 3	7 3
10									10	10 0	10 0
2 8	2 8	2 8	2 8	2 8	2 8	2 8	2 8	2 8	2 8	2 8	2 8
5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5
7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 3
10 0	10 0	10 0	10 0	10 0	10 0	10 0	10 0	10 0	10 0	10 0	10 0
Protein-Lysate + Lysis-Buffer											

Figure 2: Pipetting worksheet

4.6.4 Gel electrophoresis

4.6.4.1 Gel preparation

Gels for gel electrophoresis were prepared by mixing the components shown in Table 3. The separating gel was immediately put between two glass plates up to a mark in a Mini Protean Tetra Cell and 1ml H_2O was added on it to get a smooth surface. Gel was allowed to polymerize for 20-30 min. H_2O was removed by paperboard. The stacking gel was added by a pipette, a comb was inserted immediately without any air bubbles and gel was again allowed to polymerize for 20-30 mins.

4.6.4.2 Sample preparation

The desired amount of protein (after quantification using the BCA-Assay) was mixed with lysis buffer to get a total volume of 32µl and 8µl loading buffer (composition see Table 3) were added. Samples were boiled at 95 ° C for 5 minutes.

4.6.4.3 SDS polyacrylamide gel electrophoresis

Electrophoresis was performed in a Mini Protean Tetra Cell. The gels were transferred to the electrophoresis chamber and 1× running buffer (composition see Table 3) was added. Protein samples were loaded into the wells of the gel. 15ul Page Ruler pre-stained protein ladders (Thermo Fisher Scientific) was used as molecular weight marker. Electrophoresis was run at 15mA per gel for stacking gel and 20mA per gel for separating gel for about 3 hours. Voltage limit was set on 300V (Consort E831).

4.6.4.4 Immunoblotting

Transfer buffer (composition see Table 3) was prepared and precooled. A PVDF (polyvinylidene difluoride) membrane (Millipore Immobilon P) was cut appropriately and transferred to transfer buffer (composition see Table3) after activating in methanol for 5 seconds. The gel was then taken out of the electrophoresis apparatus. The black side of the cassette was put in the tray with the transfer buffer facing downwards. The items were arranged according to the following order on the black side of the cassette (Figure 2): sponge, whatman paper, gel, PVDF membrane, whatman paper, and an extra sponge. The cassette was closed tightly and put in the electrode chamber after the air bubbles have been removed. Transfer was run with 40V, 200mA and 150W for about 16 hours.

Figure 3: Western blot transfer assembly

4.6.4.5 Immunostaining

When the transfer was complete, the PVDF membrane was taken out after dismantling of the blot sandwich. The membrane was blocked with 5% milk in 0.1% Tween (2.5g low-fat milk powder in 50ml 0.1% Tween) for 1h by rocking at room temperature. Afterwards, the membrane was cut into two strips according to the red marker from Page Ruler, incubated with mouse-anti-human HIF-1α (1:500 in 5% milk) or mouse-monoclonal-IgG GAPDH (1:1500 in 5% milk) as a loading control overnight at 4°C on a shaker. The next day, membranes were washed 4 times with 0.1% TBSTween (Tris-buffered saline with 0.1% Tween 20 detergent; composition see Table 3) for 15min each time. Then, the secondary antibody (mouse IgG kappa binding protein (m-IgGκ BP) conjugated to Horseradish Peroxidase (HRP), 1:1000 in 5% milk) was added and incubated at room temperature for 1h by shaking. The membrane was washed again 4 times with 0.1% TBSTween for 15min each time.

4.6.4.6 Imaging and analysis

Detection of proteins was performed using the ECL Western Blotting Analysis System (RPN2109) from GE Healthcare and visualization using iBright CL1000. Western Blot results were quantified with the ImageJ software.

4.7 Neutrophil extracellular traps (NET) detection

Neutrophil extracellular traps (NETs) are reticular constructions that are released by neutrophils into the extracellular space under the stimulation of specific microorganisms or sterile inflammation. They are formed by nuclear material and neutrophil granule proteins. NET

formation of PMN was analyzed by confocal microscopy (CLSM) [70]. By capturing different focal planes and generating stacked images, it is possible to depict a three-dimensionality.

4.7.1 Preparation of samples

For analysis of NET formation, 0.8 × 10^6 PMN/ 500 µl were seeded on Poly-L-Lysine coated coverslips (positively charged, Corning) in a 24 well plate either under normoxia or under anoxia for four hours. Normoxic cultured cells stimulated with PMA (600nM) served as positive control. After four hours the medium was carefully removed by pipetting and NETs were fixed by adding 250 µl of fixation buffer (Bio Legend) and incubating 10 minutes at room temperature in the dark. The fixation buffer was removed, the plates were sealed with parafilm and stored at 4°C containing PBS in each well (at least 500 µl-1 ml per well) prior to stain for a maximum of one week.

4.7.2 Fluorescence microscopy of fixed neutrophils

Coverslips were washed 3 times for 3-5 minutes with PBS on an Eppi rack and, put on a tissue by using a very fine forceps. The coverslips were stained with Hoechst 33342 1:10 000 in PBS (50 µl per coverslip) for 5 mins at room temperature in the dark. Afterwards, the coverslips were washed again 3 times with PBS. The coverslips were mounted by pipetting 5 µl ProLong Diamond Antifade mounting solution on a normal microscopy slide (non-charged) by orienting the side of the coverslip which contains the cells facing down on the slide. Slides were dried overnight at room temperature in the dark. The next day, slides were stored at 4°C until analysis. The measurements were conducted with a Nikon Ti2 eclipse (×100 magnification).

4.8 Statistical Analysis

Statistical analysis was done using GraphPad Prism 8.4.0 for Windows. For comparisons between two groups the Wilcoxon matched pairs signed rank test (paired samples, not normally distributed) or the Students t-test (paired samples, normally distributed) was used. When comparing more than two groups, a repeated measures one way ANOVA was used. A p-value less than 0.05 was considered significant. The values given in the text are mean values ± standard deviation.

4.9 Flow chart of the work steps

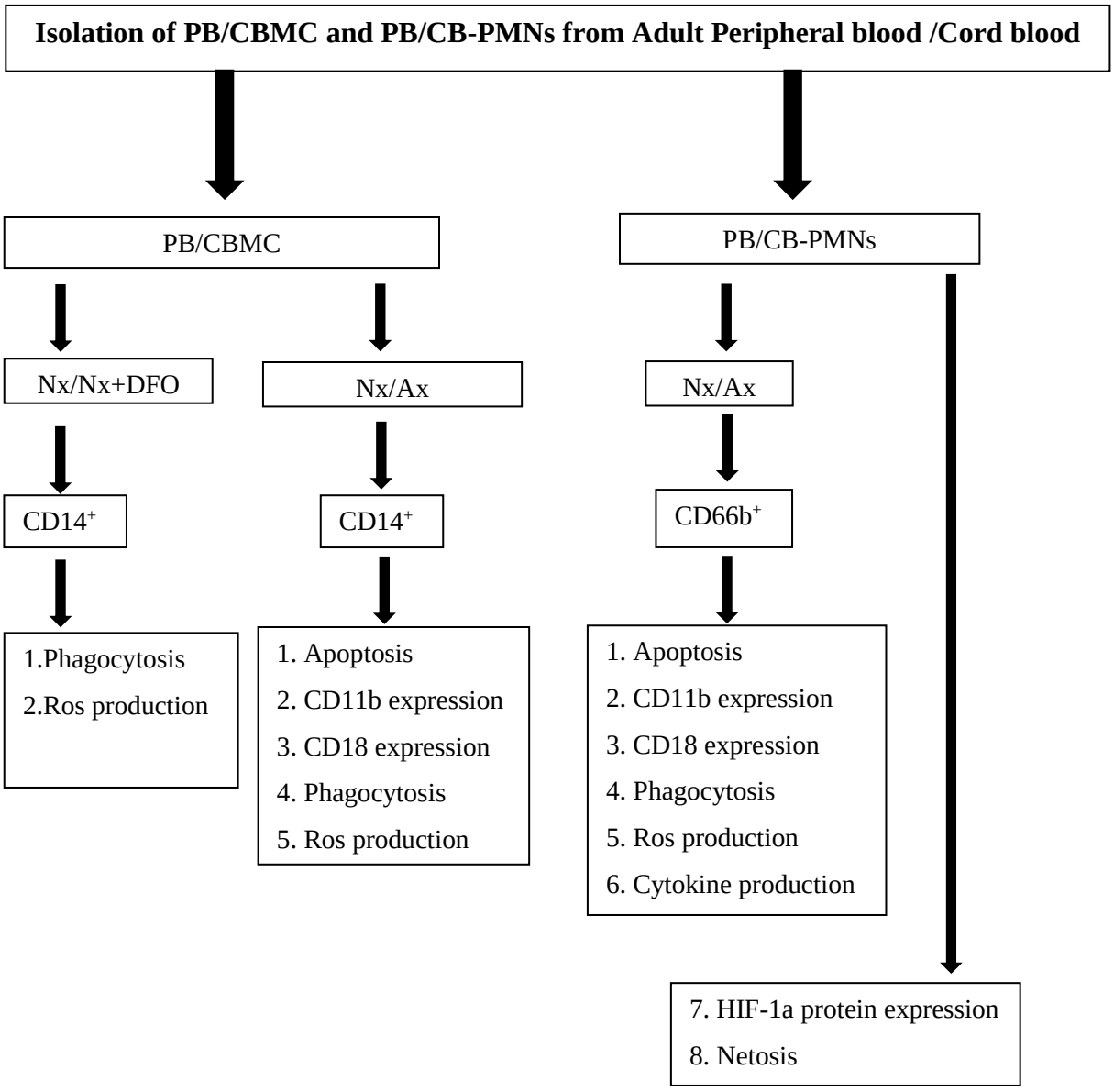


Figure 4: Flow chart of the individual work steps

5 Results

5.1 Impact of Anoxia on adult and cord blood monocyte function

In a recent doctoral thesis from our group (Christiane Schlegel, "Hypoxia and HIF-1α as factors influencing the susceptibility of neonatal sepsis") it could have been shown that anoxia or bacterial stimulation with *E.coli* led to a strong induction of the expression of the hypoxia-inducible factor HIF-1α in adult PBMC but not in CBMC and that this effect was mainly observed in monocytes but not in other cells in the mononuclear cell fraction. Thus, in the first part of this study, we aimed to investigate the impact of anoxia on adult and cord blood monocyte functions.

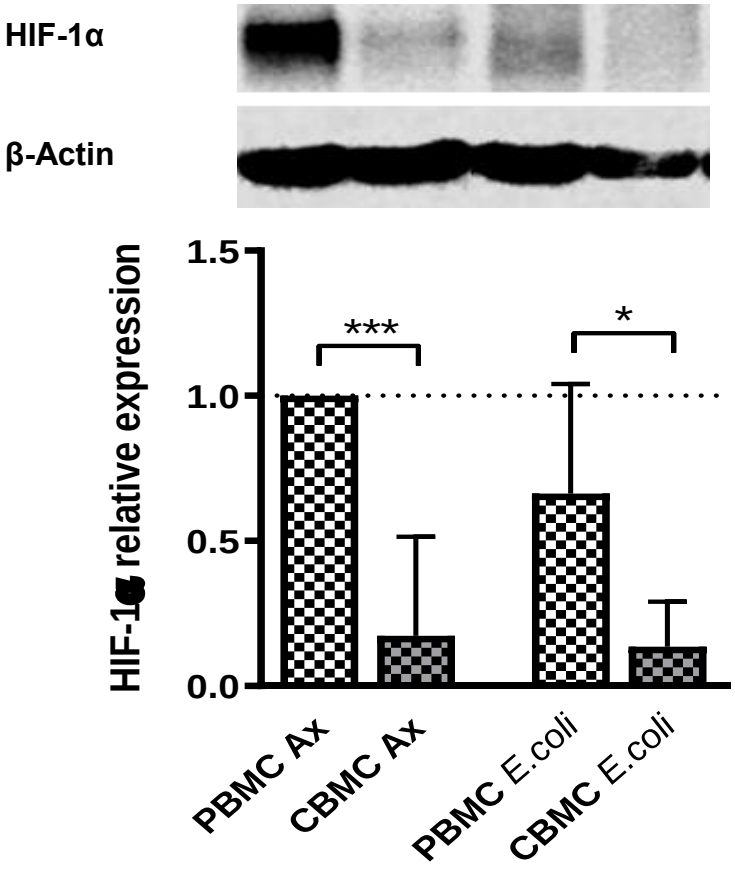
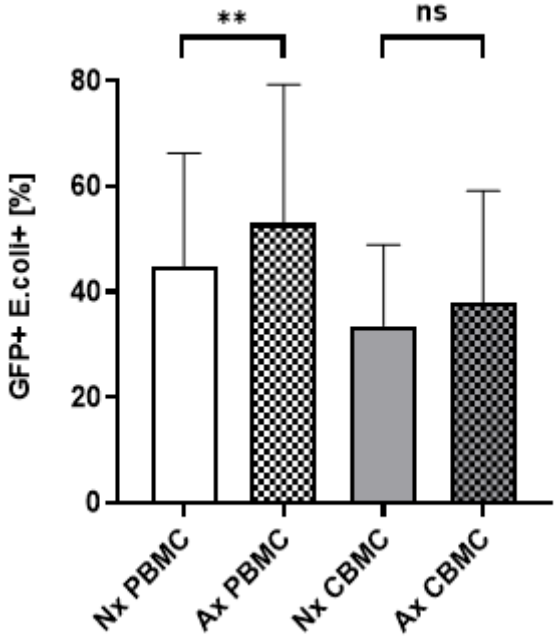


Figure 5: Expression of HIF-1α in PBMC and CBMC under anoxia or after stimulation with *E.coli*. PBMC and CBMC were cultivated for 4h either under anoxia or stimulation with *E.coli* under normoxia, Cells were lysed and HIF-1a protein expression was analyzed. n = 4, ** $p<0.01$, *** $p<0.001$, Wilcoxon matched pairs signed rank test.

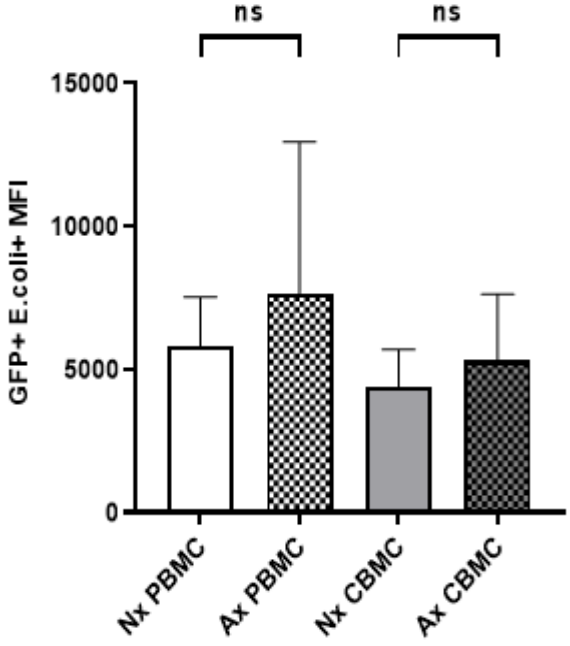
5.1.1 Phagocytic activity of adult and cord blood monocytes under normoxia and anoxia

To investigate phagocytic activity of PB monocytes and CB monocytes under normoxia and anoxia, PBMC and CBMC were incubated for four hours in normoxia or anoxia and then stimulated with *E. coli*-GFP for another 1h. Phagocytosis rate of $CD14^+$ monocytes was then analyzed by flow cytometry. We found that phagocytic activity of PB monocytes was upregulated by anoxia (44.92% ± 21.36% vs. 52.99% ± 26.34%, n = 9, $p< 0.05$, Fig. 6A), while phagocytic activity of CB monocytes remained unchanged (33.6% ± 15.36% vs. 38.07% ± 21.10%, n = 9, $p> 0.05$, Fig. 6A). Figure 6B shows the MFI for GFP of GFP^+ monocytes. For the MFI of GFP-expressing monocytes in PBMC and CBMC under normoxia and anoxia, there were no significant differences among both groups (5785 ± 1733 vs.7695 ± 5291 for PB and 4400 ± 1288 vs.5311 ± 2314 for CB, n = 9, $p> 0.05$, Fig. 6B).

A



B



C

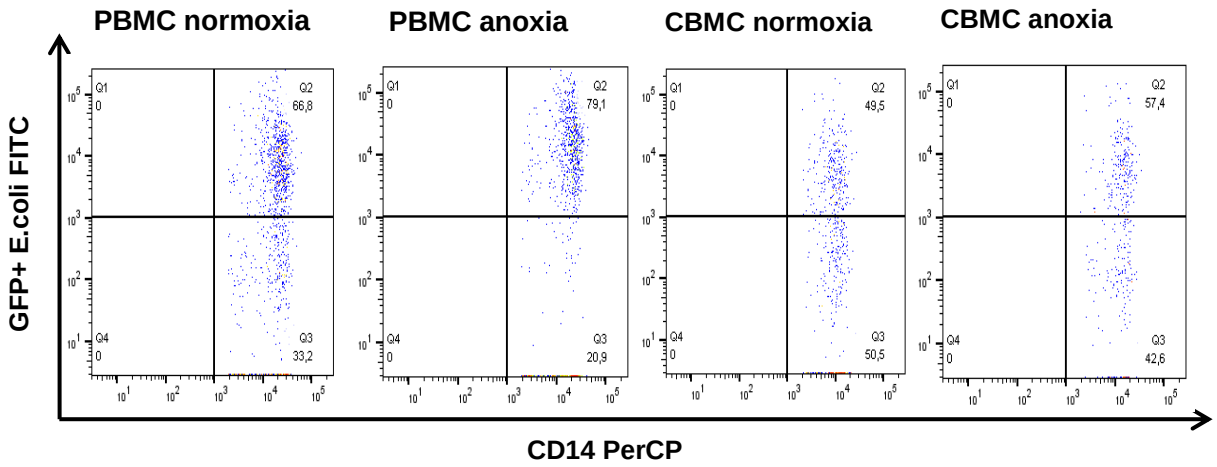
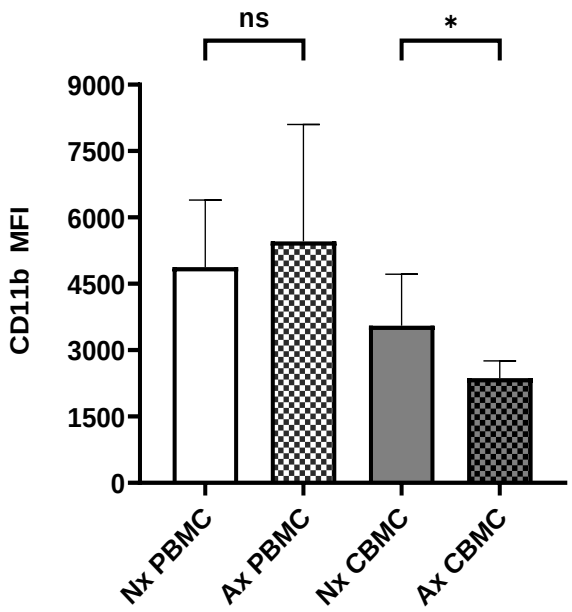


Figure 6 : Phagocytic activity of PB monocytes and CB monocytes after 4h incubation under normoxia and anoxia. PBMC and CBMC were cultivated for 4hs either under normoxia or under anoxia and stimulated for 60mins with *E. coli*-GFP. Subsequently flow cytometric analysis of the percentage (A) and the mean fluorescence (B) of GFP-expressing $CD14^+$ monocytes were carried out. Representative dot plots of data from flow cytometric analysis are shown (C). n = 9, ** $p<0.01$, ns not significant; Wilcoxon matched pairs signed rank test.

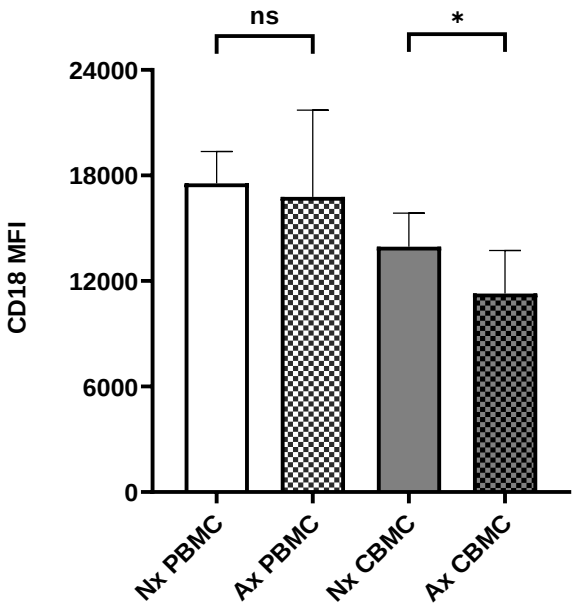
5.1.2 Expression of CD11b and CD18 on adult and cord blood monocytes under normoxia and anoxia

Since anoxia induced phagocytosis of adult monocytes but not cord blood monocytes, we next analyzed expression of phagocytosis receptors CD11b and CD18 on PB and CB monocytes after four hours culture under normoxia or anoxia. We found that anoxia downregulated CD11b and CD18 expression in CB monocytes (MFI 3350.67 ± 1166.49 vs. 2365.00 ± 389.54 for CD11b and MFI 13947.80 ± 1908.04 vs.11284.8 ± 2450.53 for CD18, both n = 6, $p< 0.05$), but had no impact on CD11b (MFI 4876.33 ± 1515.0 vs. 5455.67 ± 2645.44, n = 6, $p> 0.05$, Fig. 7A) and CD18 (17549.80 ± 1812.58 vs. 16778.00 ± 4940.85, n = 6, $p> 0.05$, Fig. 5B) expression on PB monocytes (Figure 7 A+B).

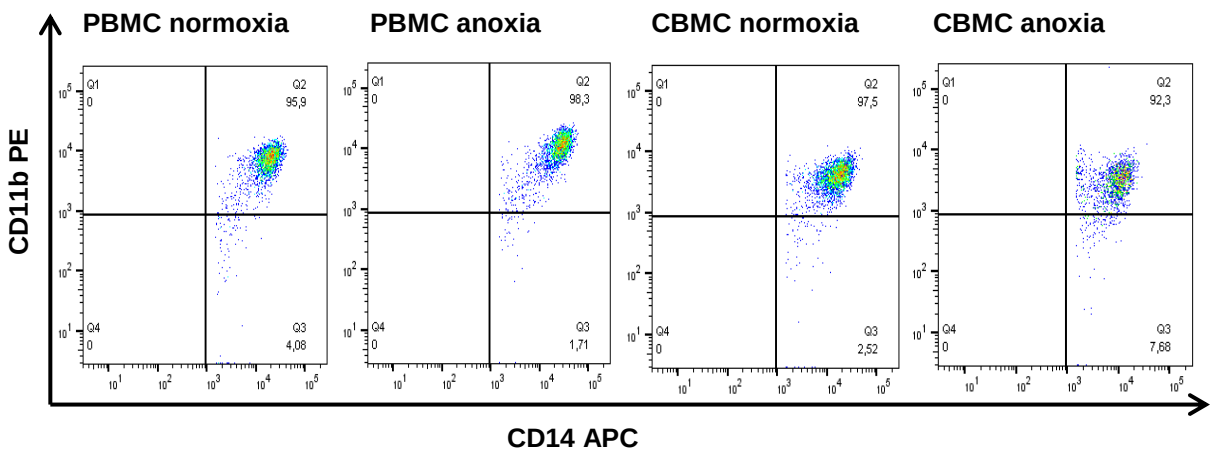
A


ns
*
9000
7500
6000
4500
3000
1500
0
CD11b MFI
Nx PBMC
Ax PBMC
Nx CBMC
Ax CBMC

B


ns
*
24000
18000
12000
6000
0
CD18 MFI
Nx PBMC
Ax PBMC
Nx CBMC
Ax CBMC

C


PBMC normoxia
PBMC anoxia
CBMC normoxia
CBMC anoxia
CD11b PE
CD14 APC
Q2 95,9
Q3 4,08
Q2 98,3
Q3 1,71
Q2 97,5
Q3 2,52
Q2 92,3
Q3 7,68

D

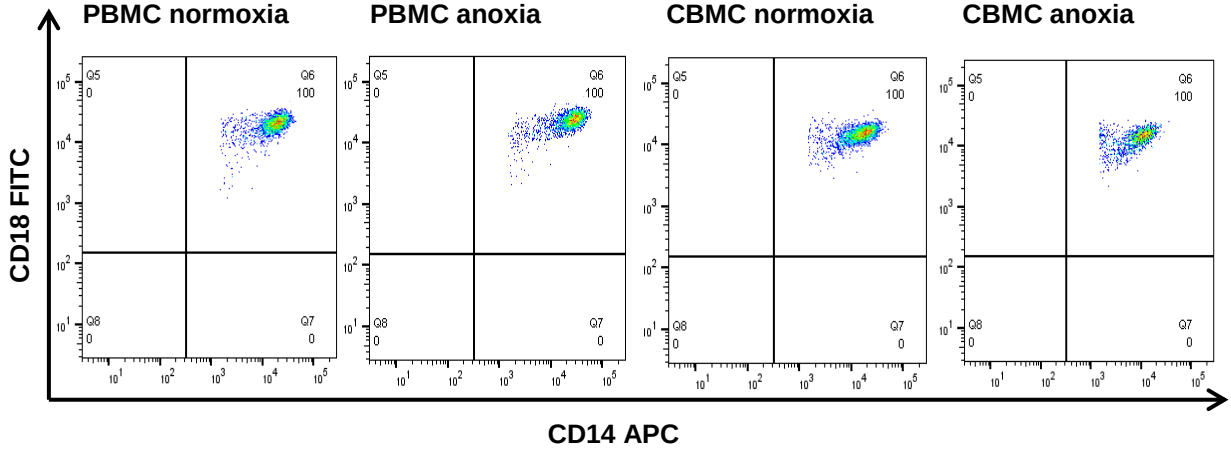
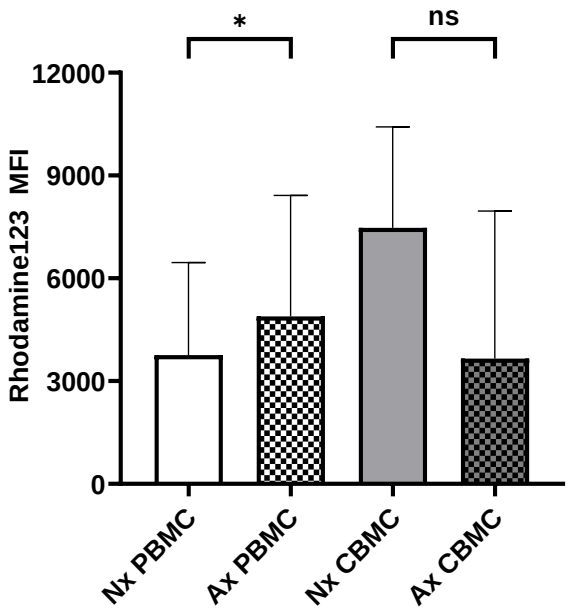

PBMC normoxia
PBMC anoxia
CBMC normoxia
CBMC anoxia
CD18 FITC
CD14 APC
Q6 100
Q6 100
Q6 100
Q6 100

Figure 7: **Expression of CD11b and CD18 on PB monocytes and CB monocytes under anoxia and normoxia.** PBMC and CBMC were cultivated for 4h either under normoxia or under anoxia. Expression of surface markers CD11b (A) and CD18 (B) on $CD14^+$ monocytes was analyzed by flow cytometry. Bar graphs show the mean fluorescent intensity (MFI) of CD11b (A) and CD18 (B) on monocytes. Representative dot plots of data from flow cytometric analysis are shown (C+D). n= 6, *p<0.05, ns not significant, Wilcoxon matched pairs signed rank test.

5.1.3 ROS production of adult and cord blood monocytes under normoxia and anoxia

To assess ROS production, PBMC and CBMC were incubated for four hours under normoxia or anoxia and then labeled with DHR 123 (Dihydrorhodamine 123) and stimulated with PMA. ROS-production was quantified by measuring Rhodamine fluorescence intensity on CD14+ monocytes by flow cytometry. We found that anoxia upregulated ROS production of PB monocytes (MFI 3759.00 ± 2702.99 vs. 4887.33± 3529.58; n = 6, p< 0.05), but no significant effect was observed in CB monocytes (MFI 7471.00± 2947.93 vs. 3657.83± 4301.54, n = 6, p> 0.05) (Figure 8A).

A



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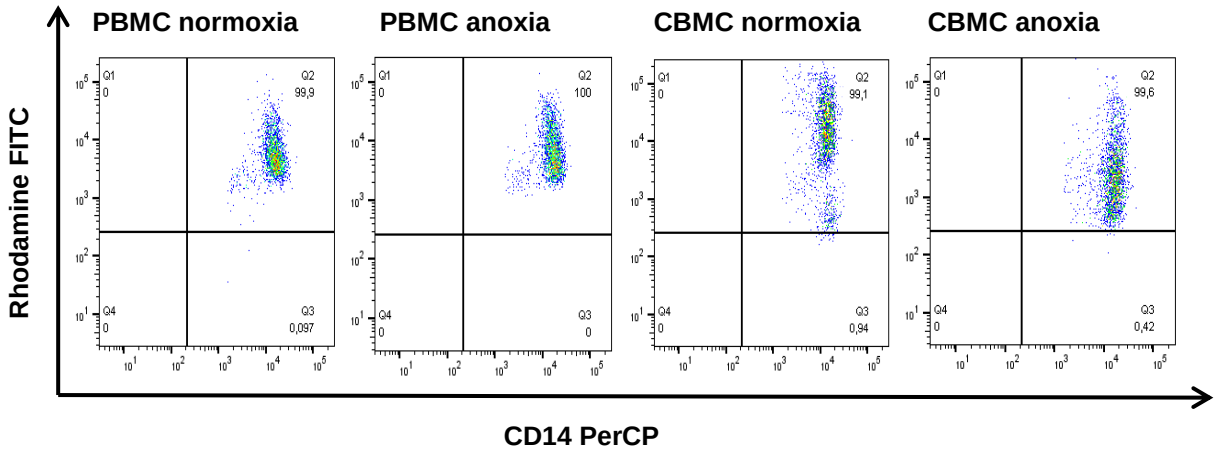
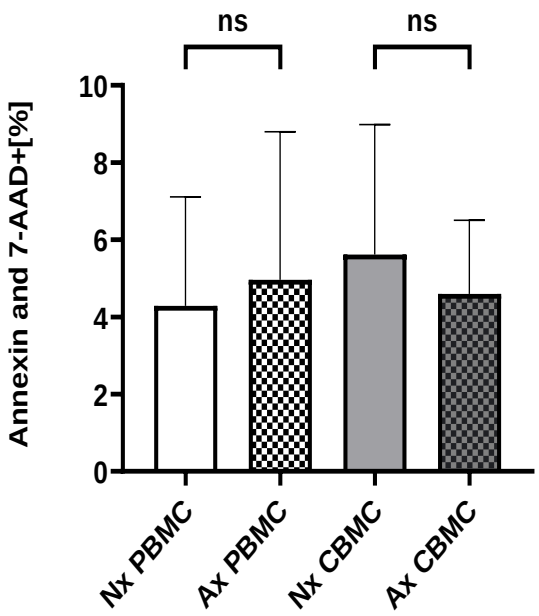


Figure 8:ROS production of PB monocytes and CB monocytes after 4h incubation under normoxia and anoxia. PBMC and CBMC were cultured for 4hs either under normoxia or under anoxia. Cells were then incubated with DHR for 5mins and stimulated with PMA for 15mins. ROS production by CD14+ monocytes was determined by measurement of Rhodamine intensity by flow cytometriy (A). Representative dot plots of data from flow cytometric analysis are shown (B). n = 6, *p<0.05, ns not significant, Wilcoxon matched pairs signed rank test.

5.1.4 Apoptosis of adult and cord blood monocytes under normoxia and anoxia

Lastly, we aimed to analyze differences in monocyte apoptosis between PB and CB after culture in normoxia or anoxia. Therefore, PBMC and CBMC were cultured under normoxia or anoxia for four hours. Apoptosis rate of CD14+ monocytes was assessed by AnnexinV/7AAD staining and flow cytometry. Overall, apoptosis rates were very low both in PB and CB monocytes (about 4%). No differences were observed between PB monocytes under normoxia and anoxia (4.28 ± 2.82 vs.4.96 ± 3.83, n = 5, p> 0.05) as well as between CB monocytes under normoxia and anoxia (5.61 ± 3.36 vs.4.6 ± 1.9, n = 5, p> 0.05) (Figure 9A).

A



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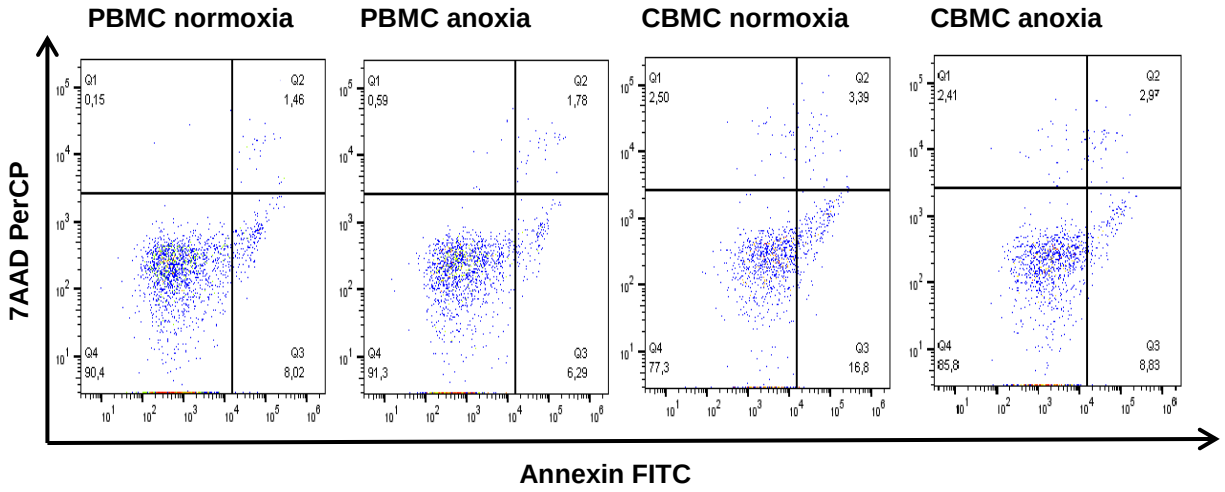


Figure 9: Apoptosis rate of PB monocytes and CB monocytes after 4h incubation under normoxia or anoxia. PBMC and CBMC were cultured for 4hs under normoxia or anoxia. Apoptosis rates of CD14+ monocytes were assessed by Annexin-V and 7AAD-staining and analyzed by flow cytometry (A). Representative dot plots of data from flow cytometric analysis are shown (B). n = 5, ns not significant, Wilcoxon matched pairs signed rank test.

Taken together, we could show that anoxia led to an increase in phagocytosis rate and ROS production in PB monocytes but not CB monocytes, while expression of phagocytosis receptors

CD11b and CD18 remained unchanged in PB and even decreased in CB. Apoptosis rates of monocytes did not differ between PB or CB between normoxia or anoxia.

5.2 Impact of deferoxamine treatment on adult and cord blood monocyte function

As our group previously found that Anoxia led to an increased expression of the hypoxia-regulated transcription factor HIF-1α in PB monocytes but not in CB monocytes, we next asked whether the observed effects of anoxia on phagocytosis and ROS production in PB and CB monocytes could be mediated by HIF-1α. Deferoxamine (DFO) is a substance that binds iron and aluminum. Furthermore, DFO is known to induce expression of HIF-1. DFO can stabilize HIF-1α levels by blocking PHD activity even in normoxic conditions [71]. Thus, we analyzed the effect of DFO treatment on phagocytic activity and ROS production of PB and CB monocytes.

5.2.1 Phagocytic activity of adult and cord blood monocytes with or without DFO treatment

To investigate phagocytic activity of PB monocytes and CB monocytes with or without DFO treatment, PBMC and CBMC were incubated for four hours with or without DFO treatment under normoxia and then stimulated with *E. coli*-GFP for another 1h. Phagocytosis rate of $CD14^+$ monocytes was then analyzed by flow cytometry. We found that phagocytic activity of PB monocytes was upregulated by DFO treatment (87.5% ± 9.99% vs. 90.7% ± 7.79%, n = 6, $p< 0.05$, Fig. 10A and MFI 20043 ± 4317 vs 25491± 6637, n = 6, $p< 0.05$, Fig. 10C), while phagocytic activity of CB monocytes remained unchanged (78.2% ± 11.8% vs. 76.7% ± 10.6%, n = 6, $p> 0.05$, Fig. 10B and MFI 17407± 3507 vs.16864± 6560, n = 6, $p> 0.05$, Fig. 10C).

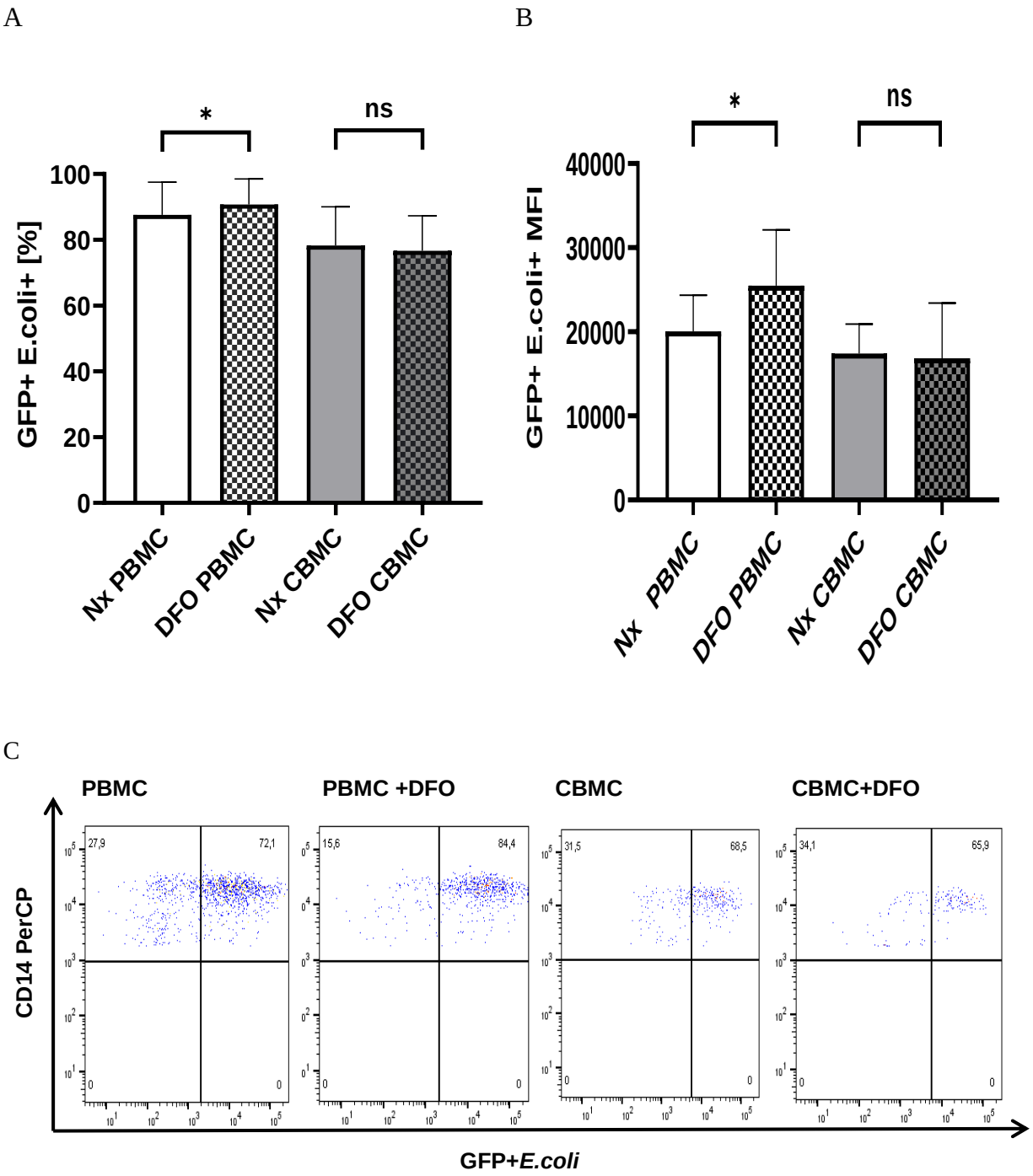
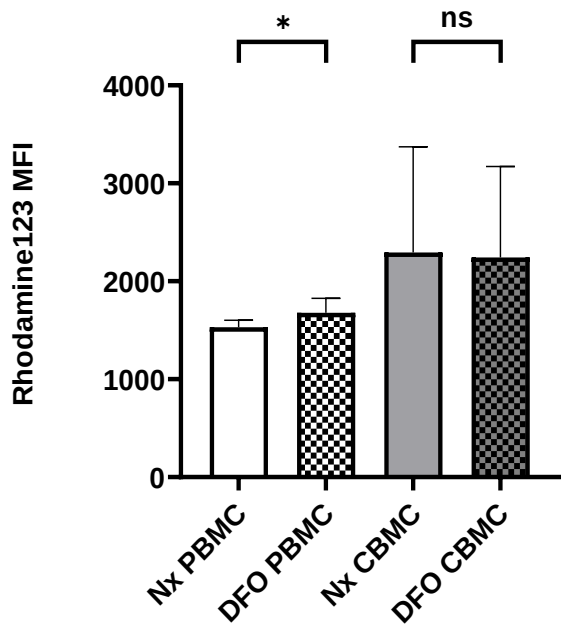


Figure 10: Phagocytic activity of PB monocytes and CB monocytes after 4h incubation with or without DFO treatment. PBMC and CBMC were cultivated for 4hs either with DFO treatment or without DFO treatment and then stimulated for 60mins with *E. coli*-GFP. Subsequently flow cytometric analysis of the percentage (A) and the mean fluorescence (B) of GFP-expressing $CD14^+$ monocytes were carried out. Representative dot plots of data from flow cytometric analysis are shown (C). n = 6, * $p<0.05$, ns not significant; Wilcoxon matched pairs signed rank test.

5.2.2 ROS production of adult and cord blood monocytes with or without DFO treatment

To assess ROS production under DFO stimulation, PBMC and CBMC were incubated for four hours with or without DFO treatment and then labeled with DHR 123 (Dihydrorhodamine123) and stimulated with PMA. ROS-production was quantified by measuring Rhodamine fluorescence intensity on CD14+ monocytes by flow cytometry. We found that DFO treatment upregulated ROS production of PB monocytes (1529± 72.47 vs.1677± 148.5, n = 6, p< 0.05), but no effect was observed in CB monocytes (2293.83± 1078.30 vs.2241.83± 929, n = 6, p> 0.05).

A



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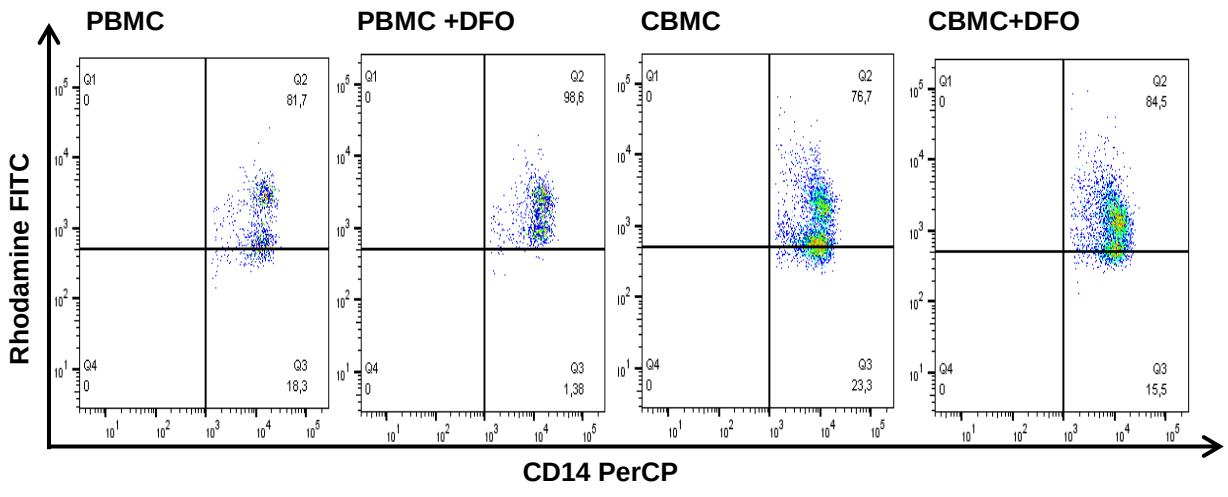


Figure 11: ROS production of PB monocytes and CB monocytes after 4h incubation with or without DFO treatment. PBMC and CBMC were cultured for 4hs either with or without DFO treatment. Cells were then incubated with DHR for 5mins and stimulated with PMA for 15mins. ROS production of CD14+ monocytes was determined by measurement of Rhodamine intensity by flow cytometry (A). Representative dot plots of data from flow cytometric analysis are shown (B). n = 6, *p<0.05, ns not significant, Wilcoxon matched pairs signed rank test.

Taken together, we could show that DFO treatment led to an increase in phagocytosis rate and ROS production in PB monocytes but not CB monocytes.

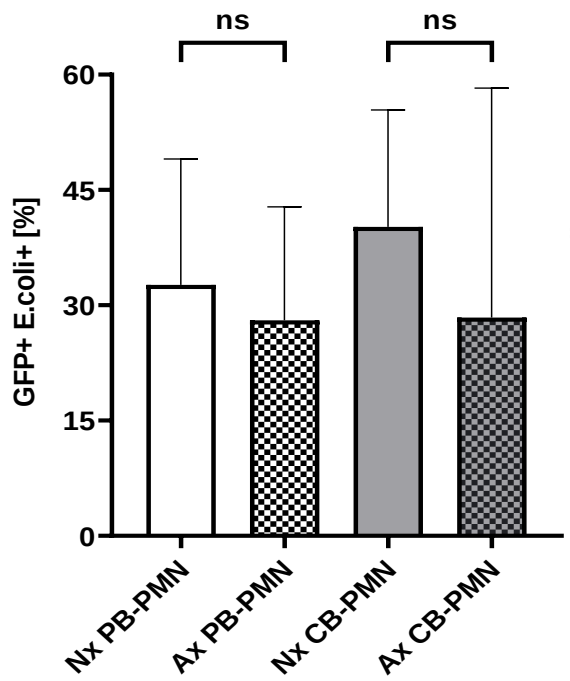
5.3 Impact of Anoxia on adult and cord blood neutrophil function

Since we found differences in monocyte functions between adult and cord blood monocytes caused by anoxia, we next asked whether there are also differences in the response to anoxia between adult and cord blood neutrophils.

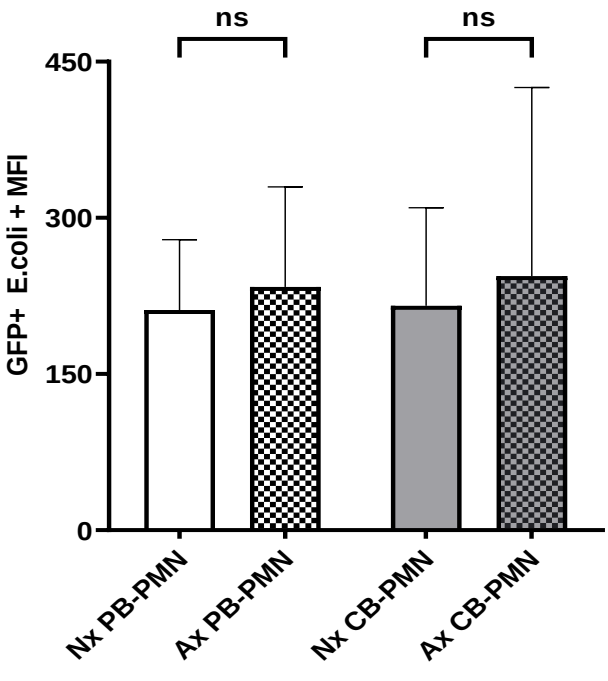
5.3.1 Phagocytic activity of adult and cord blood neutrophils under normoxia and anoxia

To investigate phagocytic activity of PB neutrophils and CB neutrophils under normoxia and anoxia, PB-PMN and CB-PMN were cultured for four hours under normoxia or anoxia and then stimulated with *E. coli*-GFP for another 1h. Phagocytosis rate of $CD66b^+$ neutrophils was analyzed by flow cytometry. We found that anoxia had no impact on phagocytic activity of both PB neutrophils. (32.67% ± 16.38% vs. 28.05% ± 14.75%, n = 7, p>0.05, Fig. 12A) and CB neutrophils (40.2% ± 15.2% vs. 28.39% ± 29.84%, n = 7, p> 0.05, Fig. 12A). For the MFI of GFP-expressing neutrophils in PB-PMN and CB-PMN under normoxia and anoxia, there were also no significant differences among both groups (211.57 ± 67.49 vs.233.71 ± 96.01 for PB and 215.41 ± 94.21 vs.244.13±181.05 for CB, n = 7, p> 0.05, Fig. 12B)

A



B



C

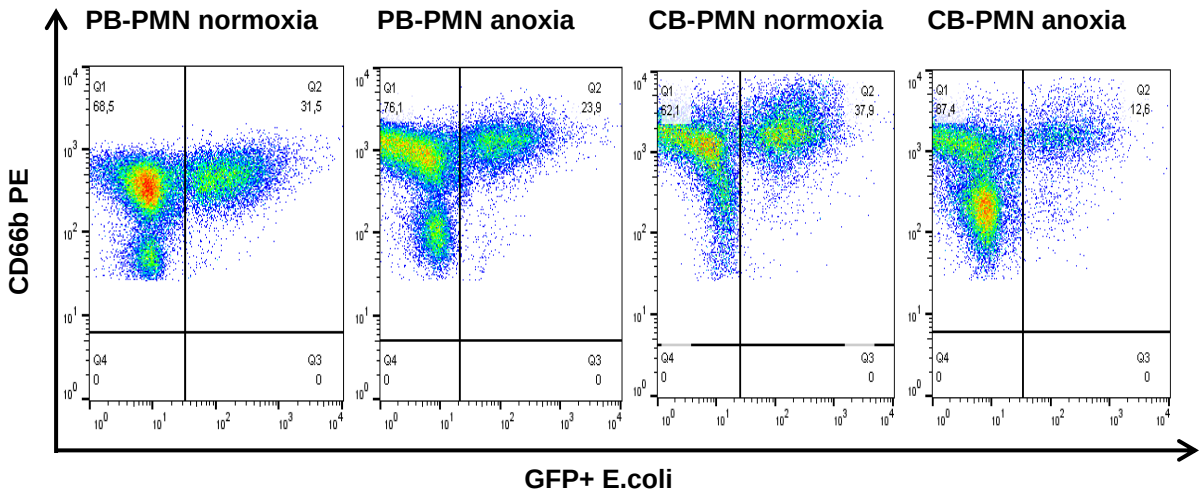
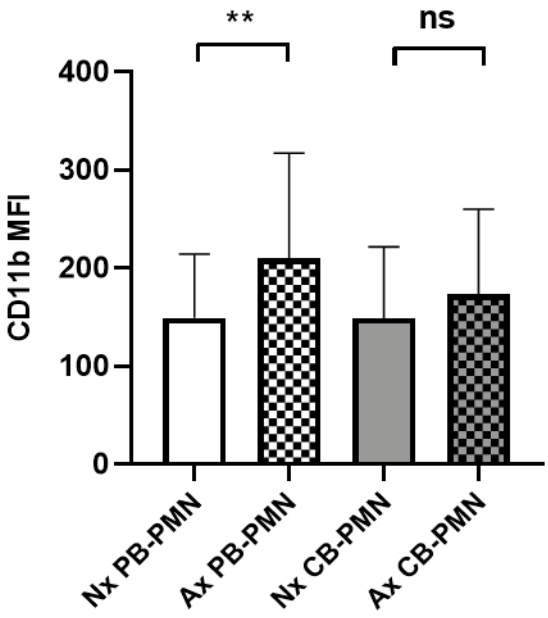


Figure 12: Phagocytic activity of PB neutrophils and CB neutrophils after 4h incubation under normoxia and anoxia. PB-PMN and CB-PMN were cultivated for 4hs either under normoxia or under anoxia and stimulated for 60mins with *E. coli*-GFP. Subsequently flow cytometric was performed and the percentage (A) and the mean fluorescence intensity (MFI) (B) of GFP-expressing CD66b+ neutrophils were analyzed. Representative dot plots of data from flow cytometric analysis are shown (C). n = 7, ns not significant; Wilcoxon matched pairs signed rank test.

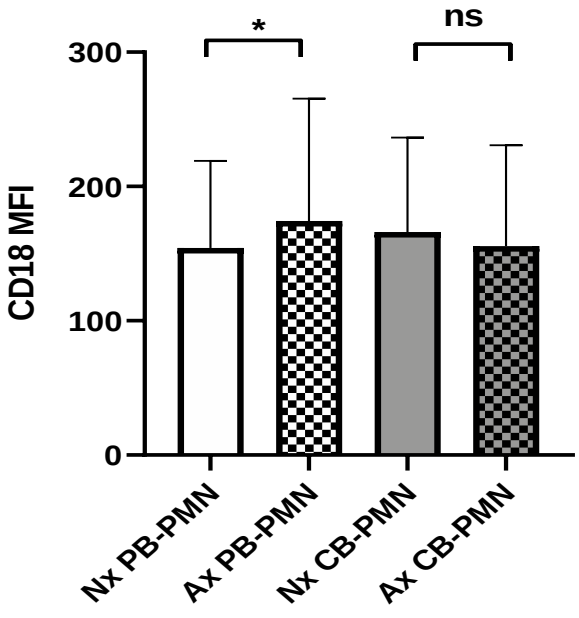
5.3.2 Expression of CD11b and CD18 on adult and cord blood neutrophils under normoxia and anoxia

We next analyzed expression of CD11b and CD18 on PB and CB neutrophils after four hours culture under normoxia or anoxia. We found that anoxia upregulated CD11b and CD18 expression in PB neutrophils (MFI 149.03 ± 65.29 vs.209.90 ± 107.15 for CD11b and MFI 154.11 ± 64.98 vs.174.22 ± 91.27 for CD18, both n = 17, $p < 0.05$), but had no impact on CD11b and CD18 expression on CB neutrophils (MFI 148.80 ± 72.64 vs.172.69 ± 87.29 for CD11b and 166.17 ± 70.31 vs.155.64± 75.07 for CD18, n = 17, $p > 0.05$) (Figure 13 A+B).

A



B



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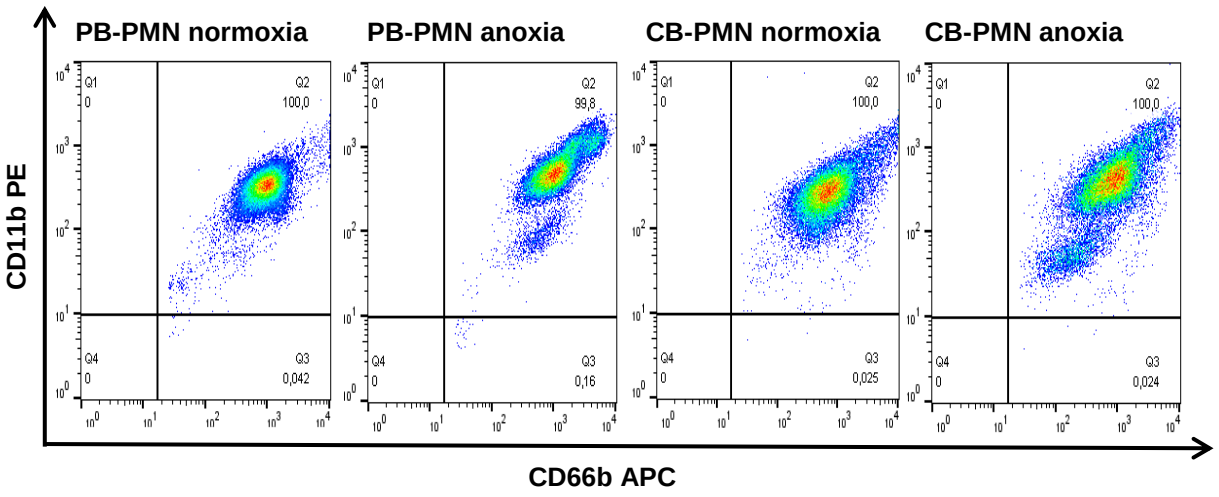

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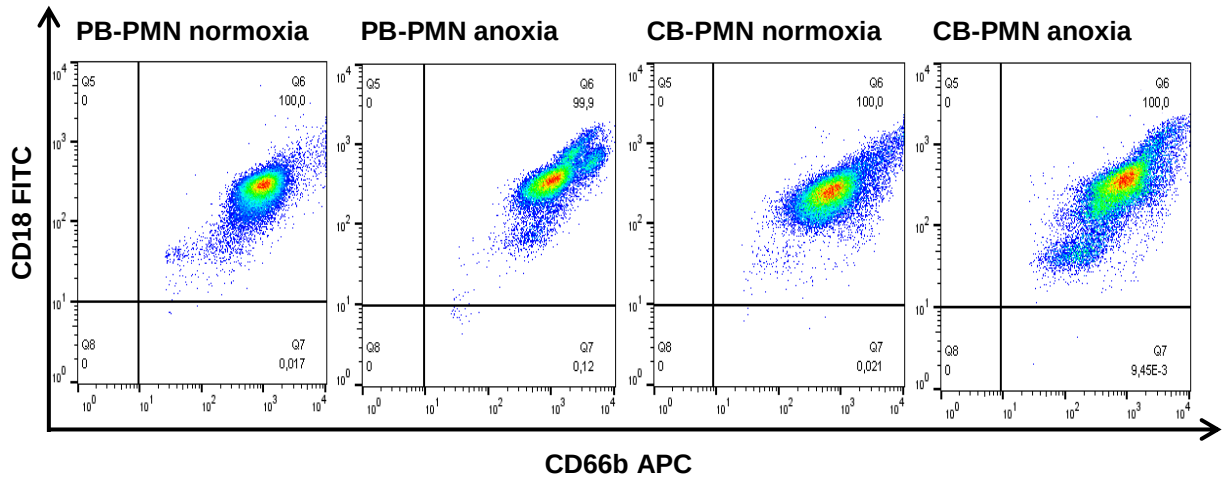
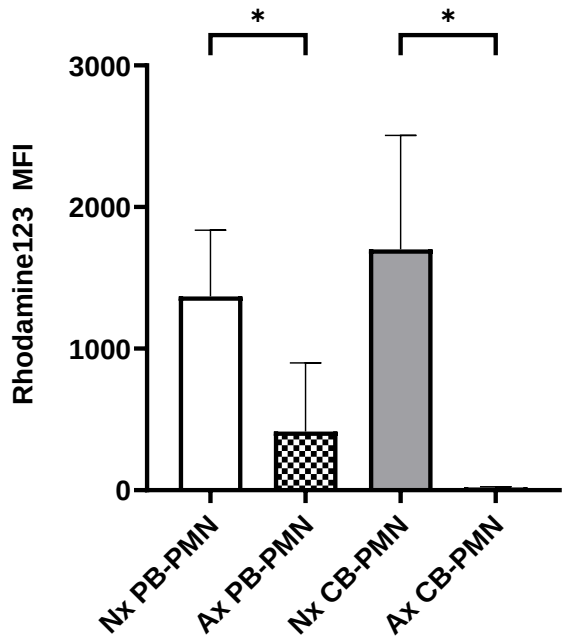


Figure 13: Expression of CD11b and CD18 on PB neutrophils and CB neutrophils under normoxia and anoxia. PB-PMN and CB-PMN were cultivated for 4hs either under normoxia or under anoxia. Expression of surface markers CD11b (A) and CD18 (B) on $CD66b^+$ neutrophils was analyzed by flow cytometry. Bar graphs show the mean fluorescent intensity (MFI) of CD11b (A) and CD18 (B) on neutrophils. Representative dot plots of data from flow cytometric analysis are shown (C+D). n = 17, **p<0.01, *p<0.05, ns not significant, Wilcoxon matched pairs signed rank test.

5.3.3 ROS production of adult and cord blood neutrophils under normoxia and anoxia

To assess ROS production, PB-PMN and CB-PMN were incubated for four hours under normoxia or anoxia and then labeled with DHR 123 (Dihydrorhodamine 123) and stimulated with PMA. ROS-production was quantified by measuring Rhodamine fluorescence intensity on CD66b+ neutrophils by flow cytometry. We found that anoxia downregulated ROS production of both PB neutrophils (MFI 1369.00 ± 467.03 vs. 414.5± 484.7, n = 6, p< 0.05) and CB neutrophils (1701.00 ± 805.3 vs. 20.15± 4.08, n = 6, p< 0.05) (Figure 14A).

A



B

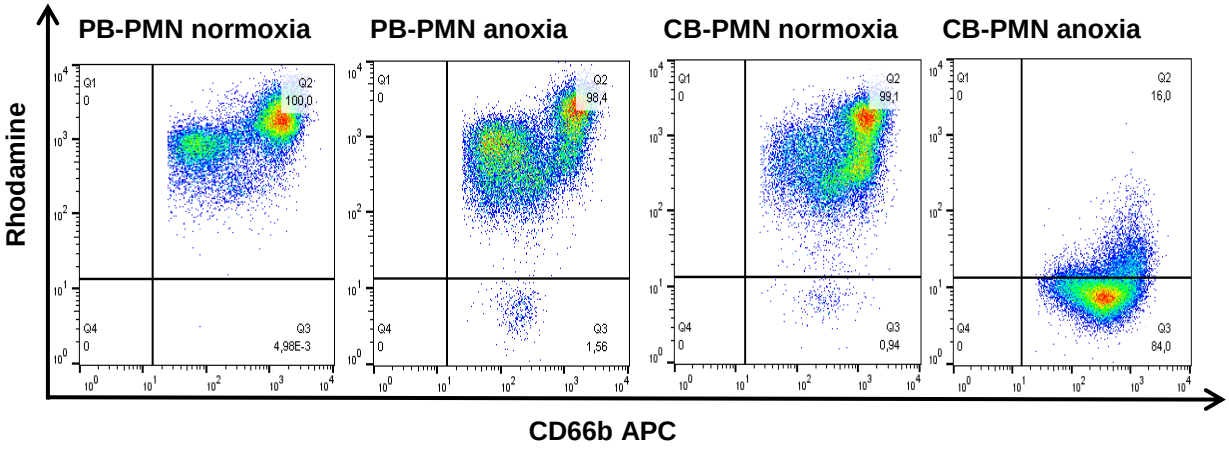
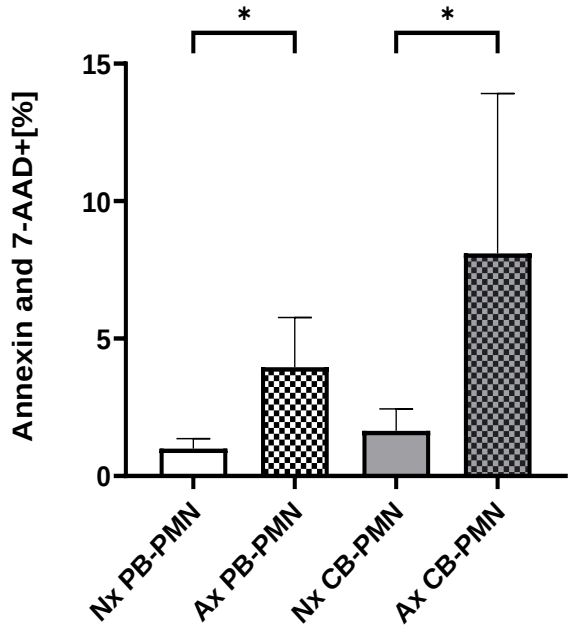


Figure 14: ROS production of PB neutrophils and CB neutrophils after 4h incubation under normoxia and anoxia. PB-PMN and CB-PMN were cultured for 4hs either under normoxia or under anoxia. Cells were then incubated with DHR for 5mins and stimulated with PMA for 15mins. ROS production of $CD66b^{+}$ neutrophils was determined by measurement of Rhodamine intensity by flow cytometry. Representative dot plots of data from flow cytometric analysis are shown (B). n = 6, *$p<0.05$, ns not significant, Wilcoxon matched pairs signed rank test.

5.3.4 Apoptosis of adult and cord blood monocytes under normoxia and anoxia

We next aimed to analyze differences in neutrophil apoptosis between PB and CB after culture in normoxia or anoxia. Therefore, PB-PMN and CB-PMN were cultured under normoxia or anoxia for four hours. Apoptosis rate of $CD66b^+$ neutrophils was assessed by AnnexinV/7AAD staining and flow cytometry. Overall, apoptosis rates were upregulated by anoxia both in PB and CB neutrophils (0.99 ± 0.37 vs. 3.96 ± 1.80 for PB and 1.65 ± 0.80 vs. 8.10 ± 5.81 for CB, n = 5, $p < 0.05$, Fig. 15A).

A



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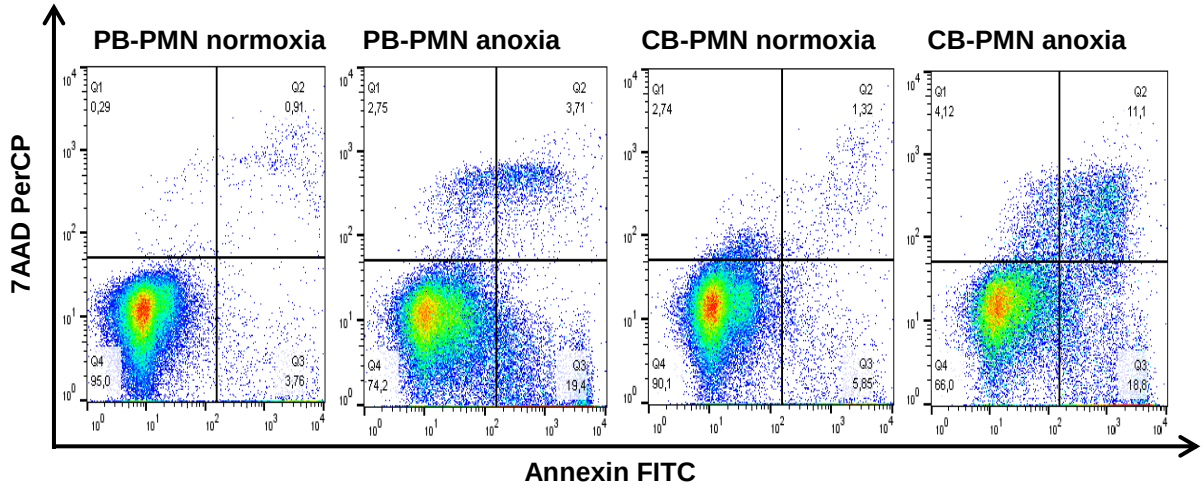


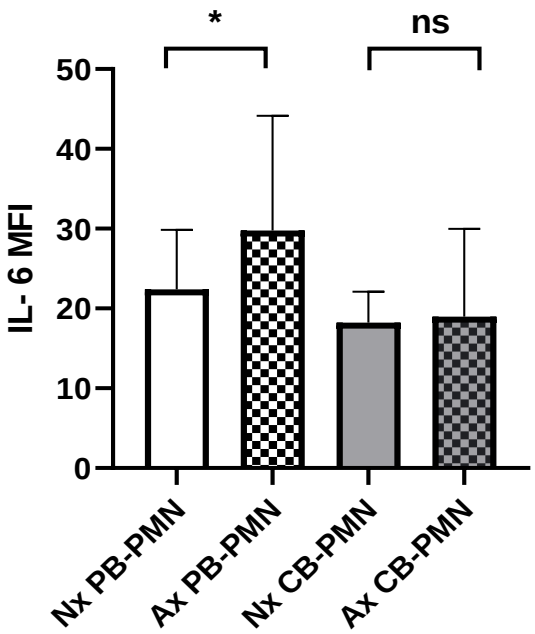
Figure 15: Apoptosis rate of PB neutrophils and CB neutrophils after 4h incubation under normoxia or anoxia. PB-PMN and CB-PMN were cultured for 4hs under normoxia or anoxia.

Apoptosis rate of CD66b+ neutrophils was assessed by Annexin-V and 7AAD-staining and analyzed by flow cytometry (A). Representative dot plots of data from flow cytometric analysis are shown (B). n = 5, *p<0.05, ns not significant, Paired t-test.

5.3.5 Cytokine production of adult and cord blood neutrophils under normoxia and anoxia

For neutrophils, we further aimed to investigate the impact of anoxia on intracellular cytokine production. Therefore, PB-PMN and CB-PMN were cultured for four hours under normoxia or anoxia and intracellular cytokine staining was performed. The results show that anoxia upregulated IL-6 expression in PB neutrophils (22.41 ± 7.42 vs. 29.79± 14.37, n = 8, p< 0.05, Fig. 16A) but not CB neutrophils (CB 18.21± 3.90 vs.18.99± 11.00, n = 8, p< 0.05, Fig. 16A), while IL-8 production was unaffected in both (23.31 ± 11.72 vs.24.18± 15.10 for PB and 16.61± 4.53 vs.20.76± 10.62 for CB, n = 8, p> 0.05, Fig. 16B). TNF-a was expressed in very low levels in both PB and CB under normoxia and anoxia making it impossible to detect relevant differences between groups.

A



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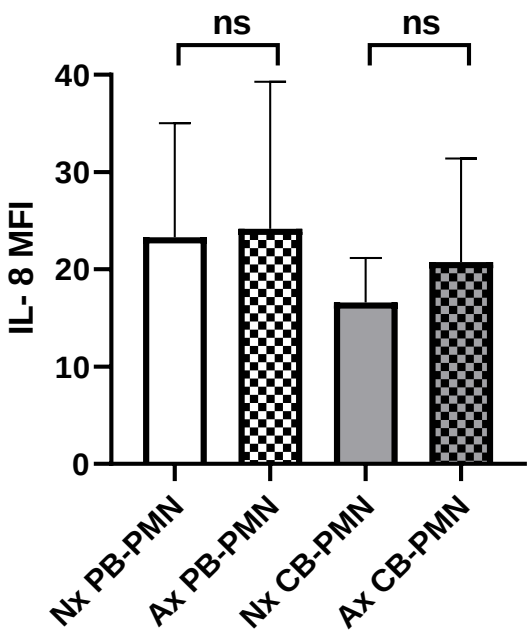

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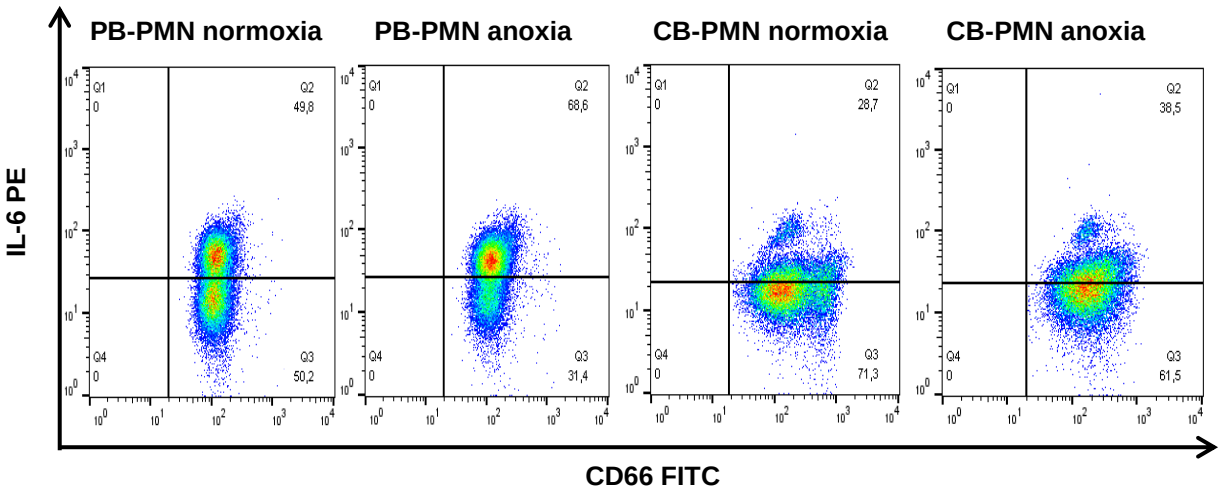

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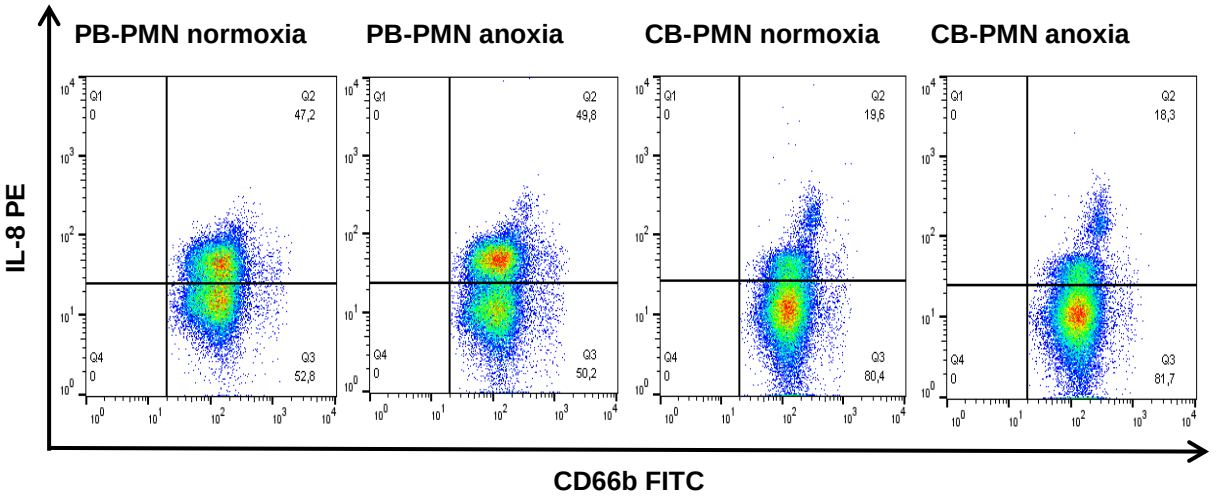

Figure 16: Cytokine expression in PB neutrophils and CB neutrophils after 4h incubation under normoxia or anoxia. PB-PMN and CB-PMN were cultured for 4hs either under normoxia or anoxia. Expression of IL-6 and IL-8 in CD66b+ neutrophils was assessed by intracellular staining and flow cytometry (A+B). Representative dot plots of data from flow cytometric analysis are shown (C+D). n = 8, *p<0.05, ns not significant, Wilcoxon matched pairs signed rank test.

5.3.6 NETosis of adult and cord blood neutrophils under normoxia and anoxia

Since results from another group showed that CB-PMN have a decreased capacity to form NETs compared to PB-PMN [20], we asked whether anoxia would induce NET formation in neutrophils and if yes there may be differences between PB-PMN and CB-PMN. Therefore, we cultured PB-PMN and CB-PBM for four hours under normoxia or anoxia. For positive control we used stimulation with PMA. Cells were then stained by Hoechst 33342 and NET formation

was assessed by confocal microscopy. However, while we found a clear NET formation of PB-PMN and CB-PMN after stimulation with PMA, there was no NET formation in both PB-PMN and CB-PMN after culture under anoxia (Figure 17).

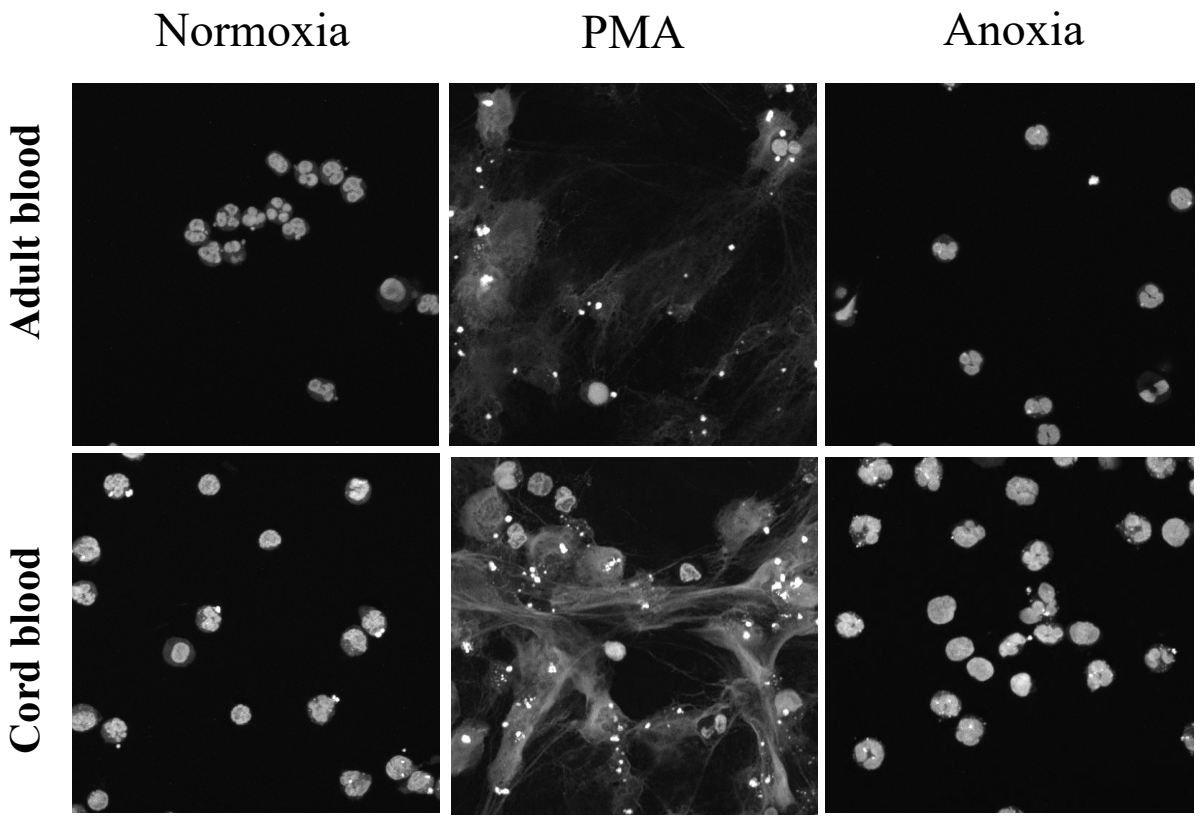

Figure 17: NETosis of PB neutrophils and CB neutrophils after 4h incubation under normoxia or anoxia. PB-PMN and CB-PMN were cultured for 4hs either under normoxia or anoxia. Stimulation with PMA served as positive control. Cell were stained and NET formation was assessed by confocal microscopy.

5.3.7 HIF-1α protein expression of adult and cord blood neutrophils under anoxia

For monocytes, we found that anoxia stimulated expression of HIF-1α in PB but not in CB. Therefore, we aimed to examine if this is also is true for neutrophils. Thus, we performed western blot analysis of PB-PMN and CB-PMN after four hours of culture under anoxia or after stimulation with *E.coli* for four hours. As positive control, we used PBMC cultured in anoxia or stimulated with *E.coli*. As expected, HIF-1α expression could be detected in PBMC after four hours of anoxia or after stimulation with *E.coli*.(Figure 18 ,PBMC 4h). However, in

neutrophils we could not detect HIF-1α expression after culture in anoxia and after stimulation with *E.coli* neither after four hours nor after shorter stimulation periods of one and two hours.

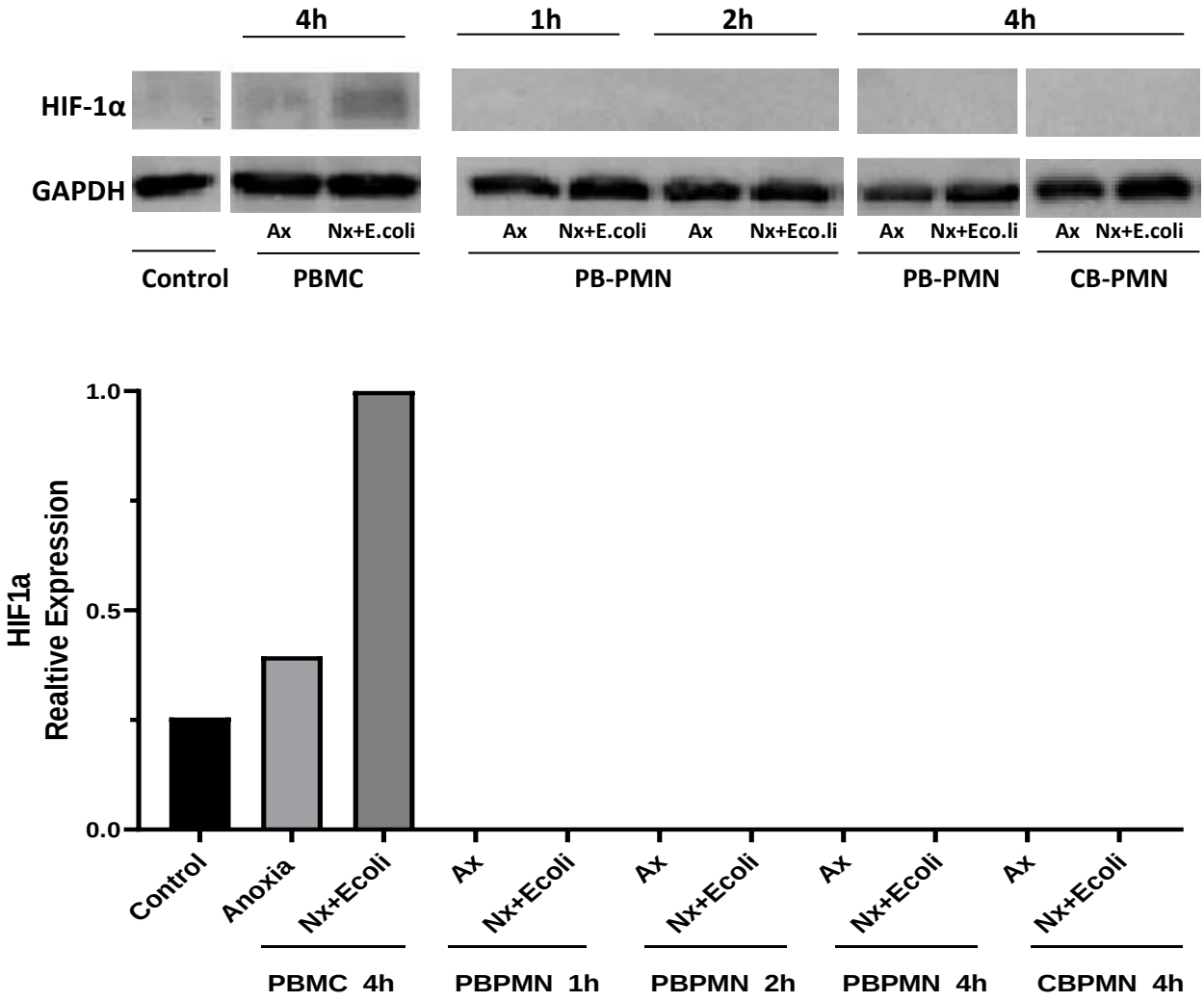

Figure 18: Expression of HIF-1α in PB-PMN and CB-PMN under anoxia and after stimulation with *E.coli*. PBMC and PB-PMN were isolated from healthy human donors. CB-PMN were isolated from umbilical cord blood. PBMC, PB-PMN and CB-PMN were exposed to anoxia or stimulated with *E.coli* for 4hs. Additionally, PB-PMN were exposed to anoxia or stimulated with *E.coli* for 1h and 2hrs. Cells were lysed and HIF-1α protein expression was analyzed by western blot. n=4.

Taken together, we could show that anoxia led to an increase in apoptosis in both PB and CB neutrophils, while ROS production was downregulated under anoxia in both PB and CB neutrophils. Expression of phagocytosis receptors CD11b/CD18 and production of IL-6 were upregulated by anoxia in PB neutrophils but not in CB neutrophils. Phagocytosis rates, and NETosis and production of IL-8 did not differ between PB neutrophils or CB neutrophils between normoxia or anoxia. HIF-1α expression could not be detected neither in PB-PMN nor in CB-PMN.

5.4 Summary of Results

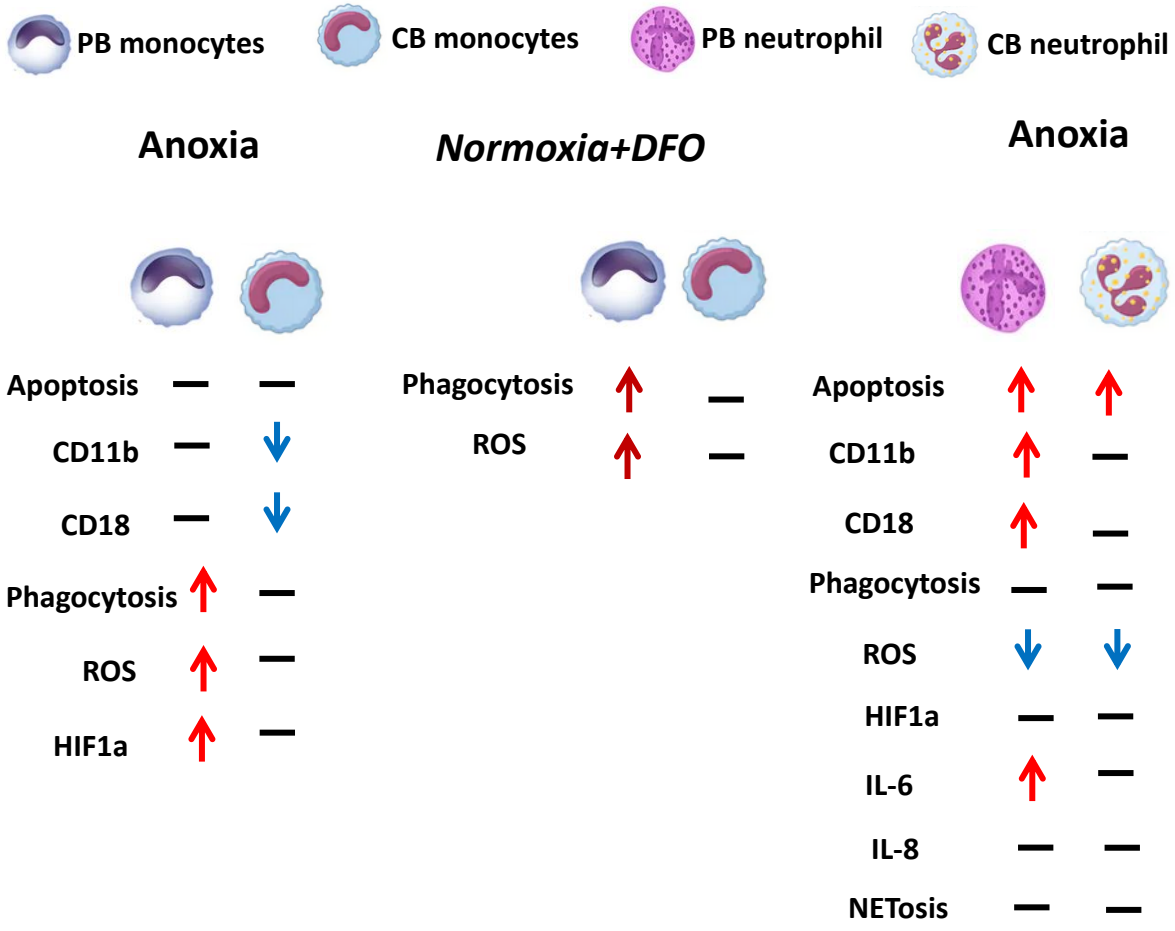

Figure 19: Graphic summary of the results. PB monocytes and CB monocytes functions under anoxia, PB monocytes and CB monocytes functions with DFO treatment, and PB-PMN and CB-PMN functions under anoxia.

6 Discussion

In a previous doctoral thesis in our group, it was found that expression of HIF-1α can be stimulated by anoxia in adult peripheral blood mononuclear cells (PBMC), especially in adult monocytes, but not in cord blood mononuclear cells (CBMC). Based on these results, we aimed to investigate, whether anoxia has differential impact on functionality of adult and cord blood monocytes. We demonstrated that (1) anoxia led to an increase in phagocytosis rate and ROS production in PB monocytes but not in CB monocytes, while expression of phagocytosis receptors CD11b/ CD18 remained unchanged in PB and even decreased in CB monocytes. Apoptosis rates of monocytes did not differ between PB and CB under normoxia or anoxia. (2) Treatment with the HIF-1α stabilizer DFO also led to an increase in phagocytosis rate and ROS production in PB monocytes but not in CB monocytes.

Furthermore, we asked whether anoxia may also differentially impact adult and cord blood neutrophil function and HIF-1α expression corresponding to our results in monocytes. Here we found that (3) anoxia led to an upregulation of phagocytosis receptors CD11b/CD18 and production of IL-6 in PB neutrophils but not in CB neutrophils and to an increase in apoptosis and a decrease in ROS production in both PB and CB neutrophils. Phagocytosis rate, production of IL-8 and NETosis did not differ between PB neutrophils or CB neutrophils under normoxia or anoxia. (4) Surprisingly, we were not able to detect HIF-1α protein expression in western blot.

Our results will be discussed in the following sections

PB and CB monocytes under anoxia

Phagocytosis rates are increased in PB monocytes but not CB monocytes upon stimulation with anoxia

We showed that phagocytic activity of PB monocytes was upregulated by anoxia, while phagocytic activity of CB monocytes remained unchanged. Treatment with DFO, a potent HIF-1α activator, provided similar results. These results are in keeping with previous results from other groups and results from our group concerning HIF-1α expression in PB and CB monocytes. In adult cells it has been shown that hypoxia enhanced phagocytosis of bacteria, an effect that was abrogated in the presence of HIF1α-directed siRNA [73]. Furthermore, it was shown that overexpression of HIF-1α enhanced phagocytosis strongly indicating that the expression of HIF-1α plays an important role in this process [73].

HIF-1α is known to be required for key monocytes/macrophage functions, such as cytokine production, macrophage glycolysis, ATP generation and bactericidal activity [74]. Human monocytes and macrophages isolated from adult individuals upregulate HIF-1α levels when they are exposed to hypoxia *in vitro* and *in vivo* [75-79]. Our group found that anoxia or bacterial stimulation with *E.coli* led to a strong expression of the hypoxia-inducible factor HIF-1α in adult PBMC but not in cord blood, and that this effect was mainly observed in monocytes but not in other cells in the mononuclear cell fraction (unpublished data). The reason for these differences remain unclear but can be regarded from three aspects.

On the transcription level, HIF is the crucial molecule regulating gene expression in response to hypoxia, but many other transcription factors also play a role during hypoxic conditions. For instance, transcription factor NF-κB activation is also mediated by hypoxia [80,81] and expression of HIF-1α in monocytes can be induced by the binding of NF-κB to its promoter region [82]. Furthermore, it was shown that the mRNA and protein levels of HIF-1α are increased by activation of NF-κB in several cell lines [83,84] and expression of HIF-1α signaling is defective in bacteria-infected macrophages and mice exposed to hypoxia after deleting the gene encoding IKKβ which is necessary for NK-kB activation [85]. Interestingly, it was shown that after stimulation with LPS, NF-κB activation in monocytes from preterm and term newborns is reduced [86]. Therefore, the reduced HIF-1α expression under anoxia in CB monocytes might be explained by reduced NF-κB activation.

On the translation level, mammalian target of rapamycin complex 1(mTORC1) has been shown to regulate HIF-1α synthesis through the co-regulation of the initiation factor 4E-binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase 1 (S6K1), whereas the degradation of HIF-1α is not affected [87]. Comparing to PB macrophages, mTOR activation is reduced in CB macrophages [88]. Decreased mTOR activation in CB monocytes under anoxia may result in low HIF-1α expression.

On the post-translation level, prolyl hydroxylases (PHDs) hydroxylate HIF-1α under normoxia resulting in subsequent proteasomal degradation by the von Hippel Lindau (VHL) protein ubiquitin ligase complex [89-92]. PHD activity is inhibited under hypoxia which results in HIF-1α stabilization and translocation to the nucleus for activating downstream genes and cellular adaptation to hypoxia [89-92]. Therefore, PHDs and VHL have been recognized as the major regulators for the protein stability of HIF-1α. It is evident that different factors affect hypoxia signaling via regulation of PHDs and VHL [93-98]. For instance, elevated ROS levels lead to enzyme inactivation of PHD resulting in HIF-1α stabilization [99]. We here found that anoxia upregulated ROS production in PB monocytes, but not in CB monocytes suggesting a

positive feedback loop for HIF-1α stabilization in PB but not in CB monocytes under anoxia. Another important point is that the fetus develops under chronic hypoxic conditions [100]and that chronic hypoxia has been shown to desensitize HIF-1α by overactivation of PHDs [101]. This may cause that cord blood monocytes are "resistant" to HIF-1α activation by anoxia.

Further studies are needed to determine whether NF-κB pathway, mTOR pathway or factors regulating PHDs and VHL are involved in HIF-1α stabilization during neonatal time resulting in different HIF-1α expression between PB monocytes and CB monocytes.

CR3 (CD11b/CD18) expression

Surprisingly, although we found increased phagocytosis rates in adult monocytes under anoxia, we did not detect differences in expression of phagocytosis receptors CD11b and CD18 between monocytes cultured under normoxia and anoxia. Previous studies showed that hypoxia induces the expression of IFNγ, which upregulates the surface level of the CD11b/CD18 receptor to promote cell phagocytosis [102] and that the expression of CD11b strongly correlates with HIF-1α expression [103]. Furthermore, it was shown that hypoxia induces CD18 expression on myeloid cells due to binding of HIF-1α in the CD18 gene [104]. However, the findings of our study do not support the previous reports. The majority of existing literature focuses on hypoxic conditions and we found no reports in the literature on monocyte CD11b and CD18 expression under anoxia. Due to the different experimental conditions, it is difficult to compare our results directly with previous reports.

Several reports have shown that the integrin-type CR3 was functional activated by the GTPase Rap1 [105] and CD11b upregulation was associated with increased levels of serum TNF-α under hypoxia [106]. Therefore, further investigations for example adressing leukocyte trans-endothelial migration pathways, chemokine signaling pathways or the role of the cytoskeleton in activation of CD11b/CD18 under anoxia are required.

ROS production

We demonstrated that anoxia upregulated ROS production of PB monocytes, but no effect was observed in CB monocytes. These results are in line with those of previous studies who also showed that ROS levels in general increase during hypoxia [107,108] and that macrophages produce ROS in response to hypoxia [109]. Furthermore, experiments with ROS synthesis inhibitors have shown that hypoxia-induced ROS production in human monocytes is primarily caused by the activity of nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase[110]. As mentioned above, vice versa, ROS stabilizes HIF-1α by preventing its hydroxylation[111].

Interestingly, decreased antimicrobial activity of neonatal neutrophils [112] has been demonstrated as a consequence of alterations in the NADPH oxidase system [113]. In comparison to adults, production of superoxide anions (O2−) is increased while the generation of hydroxyl radical (OH) is decreased in neonatal neutrophils [114]. It is possible that an altered NADPH oxidase system also exists in CB monocytes, which leads to decreased ROS production under anoxia.

Furthermore, it has been shown that increased proportions of immature leucocytes are found in cord blood in comparison to adult blood leading to altered expression of surface molecules [115] but also may lead to altered ROS-production.

Further research should be undertaken to investigate the differences in the NADPH oxidase system between PB and CB monocytes under anoxia.

Apoptosis

In our experiments, apoptosis rates after four hours of culture were very low in both PB and CB monocytes under normoxia and under anoxia (about 4%). Anoxia had no effect on apoptosis of both PB and CB monocytes. Human monocytes survival under hypoxia can be prolonged by increasing glucose uptake and inducing the expression of glycolytic genes [116]. Furthermore, it was shown that HIF-1α up-regulates key components of the glycolytic pathway in macrophages [117]. Thus, one could expect that anoxia may enhance survival of PB monocyte but not of CB monocytes due to the different expression of HIF-1α protein. However, the aim of our apoptosis experiments was not to determine differences in apoptosis of adult and CB monocytes due to anoxia but rather to ensure that the observed differences in phagocytosis and ROS production are not consequence of altered apoptosis of the cell types analyzed. To figure out the real effect of anoxia on PB and CB monocyte apoptosis experiments with longer anoxia exposure and/or additional stimulation should be performed.

PB and CB neutrophils function under anoxia

Phagocytosis

Unlike in monocytes, we found that anoxia had no impact on phagocytic activity of both PB neutrophils and CB neutrophils. This is in contrast with the literature, showing that systemic hypoxia caused increased phagocytosis in neutrophils [118,119]. However these studies have

been performed under hypoxic condition and not anoxic conditions like our study making a direct comparison difficult.

It was shown that phagocytosis of Streptococcus pneumoniae by neutrophils from patients who have a heterozygous mutation of VHL protein was enhanced under normoxic condition. [120]. This supports the assumption that increased phagocytosis under hypoxia is mediated by HIF-1α. Interestingly, we also could not detect HIF-1α in neutrophils under so that it can be guessed that under our culture conditions HIF-1α was not activated and in consequence no increase in phagocytosis was seen.

CR3 (CD11b/CD18) expression

We found that anoxia upregulated CD11b and CD18 expression in PB neutrophils but had no impact on CD11b and CD18 expression in CB neutrophils contrasting our results upon phagocytosis under anoxia as CR3 (CD11b/CD18, MAC-1) is known to enhance neutrophil phagocytosis [121]. Our results are in line with other studies also describing that hypoxia induced CD11b expression [104] and that HIF-1 upregulated leukocyte CD18 mRNA and surface protein expression. We observed an upregulation of CD11b and CD18 under anoxia although we did not detect HIF-1α. The induction of integrin CD11b expression also requires NF-kB p65 [122,123] which could explain our results.

For CB neutrophils, McEvoy et al. [124] showed that cell membrane quantities of CD11b/CD18 were decreased in neonatal cells. Also upon activation, CD11b/CD18 levels of neonatal neutrophils remained decreased in comparison to adult cells [124]. Other studies showed that CD11b/CD18 expression remained low during the postnatal period and reached adult levels at an age of 11 months [125]. These results are in correspondence with our results of a missing upregulation of CD11b/CD18 under anoxia in CB neutrophils. Further studies are needed to examine the mechanisms underlying the differential regulation of CD11b/CD18 under anoxia in CB and B neutrophils.

ROS production

Corresponding to the literature showing that hypoxia reduces neutrophil ROS production due to the lack of available molecular oxygen [126], we found that anoxia downregulated ROS production of both PB neutrophils and CB neutrophils.

Gabig et al. showed that neutrophil $O2^-$ production at 1% oxygen was 75% of the control group, however, the O2- production decreased rapidly when the oxygen concentration fell below 1% [127]. As mentioned before, hypoxia had no effect on the neutrophil NADPH oxidase

composition, suggesting that the decline in O2- was moderated by the absence of available substrate, i.e. molecular oxygen [126]. Support for this interpretation is provided by the fact that hypoxia abrogates Pyocyanin-Induced ROS Production (Independent of NADPH Oxidase) and ROS production is fully restored by re-exposing hypoxic neutrophils to ambient oxygen [126]Thus, as well as under hypoxic condition, neutrophil ROS production under anoxia may be oxygen-dependent and does not depend on the HIF-1α [128,129].

Apoptosis

Our results showed that apoptosis rates were increased under anoxia in both PB and CB neutrophils. In contrast to our results, other groups showed that hypoxia inhibited apoptosis in neutrophils in a HIF-1α-dependent manner [130] and that a loss-of-function mutation in the von Hippel Lindau (VHL) protein which stabilized HIF led to delayed apoptosis in human neutrophils [120]. Similarly, apoptosis of TNF-a-treated human neutrophils was delayed by intermittent hypoxia [131]. Walmsley et al. described a marked reduction in neutrophil survival in a murine HIF-1α conditional knockout model under anoxic conditions [132]. These observations indicate that neutrophil survival under hypoxic conditions is mediated by HIF-1α.

Hence, it could conceivably be hypothesised that the lack of HIF-1α protein expression under our conditions led to the increased apoptosis rates in PB and CB neutrophils.

Cytokine production

Concerning neutrophil cytokine production we found an upregulation of IL-6 in PB neutrophils but not CB neutrophils under anoxia, while IL-8 production was unchanged.

IL-6, IL-8 and TNF- α are the main cytokines released under hypoxic stress and macrophages and vascular endothelial cells are the major sources of these cytokines [133-135]. Corresponding to our results, it was shown that in mice subjected to 5% O2 for 60 minutes IL-6 protein expression in isolated macrophages was significantly increased [133]. Furthermore, HIF-1α was shown to be involved in IL-6 production in macrophages [138] and in mast cells [137,138]. However, most of the literature concerning IL-& production under low oxygen conditions come from macrophages or endothelial cells under hypoxia. Due to different oxygen concentration and cell type, it is difficult to compare these results directly with ours.

Furthermore, Contradictory results have also been published concerning the human neutrophils' ability to express IL-6 [41]. Tamassia et al, [139] showed that human neutrophils generate IL6 in vitro only under experimental conditions(stimulate with TLR8 specific ligand or very elevated concentrations of LPS) in which they are capable of re-organizing the

chromatin of the IL6 locus. Chromatin remodeling has been shown to be time-dependent, and the best measurement time for neutrophil-derived IL-6 is after overnight cell incubation, but not after 4 hours incubation [139]. On the other hands, IL-6 secretion from LPS stimulated neonatal and adult neutrophils was similar [140].

Concerning IL-8, Feng et al. showed that induction of HIF-1α by hypoxia promoted IL-8 expression in hepatocellular carcinoma cells [141]. In neonatal cells, it was shown that neonatal neutrophils stimulated with LPS produced higher levels of IL-8 compared to adult neutrophils [142]. However similar to our results, human neutrophils cultured under normoxic, hypoxic, or anoxic conditions showed no difference in IL-8 levels at 6 or 20 hours [143].

A limitation of our experiments is that we analyzed cytokine production only at the time-point 4 hours. Since cytokine production is time-dependent, longer or shorter incubation periods may produce different results. Therefore, experiments with longer anoxia exposure and/or additional stimuli should be performed. For example, Studies are needed to investigate whether hypoxia or HIF-1α is involved in chromatin remodeling of the IL6 locus in PB and CB neutrophils

NETosis

Since Yost et al. found impaired neutrophil extracellular trap (NET) formation in CB neutrophils in comparison to adult neutrophils, we asked whether anoxia may impact NET formation[20]. Interestingly, we could not confirm the results of Yost et al. since in our hands, CB-PMN showed no defects in NET formation under stimulation with PMA. We do not have a clear explanation for these conflicting results. Differential stimulation conditions like Lipopolysaccharide (LPS) and platelet activating factor (PAF) may be responsible for the disparate results.

Although HIF-1 was identified as a central component to the release NETs, since pharmacological and genetic HIF-1α knockdown decreased NET production [144], we found no NET formation in both PB-PMN and CB-PMN after culture under anoxia. NADPH and ROS production are the main drivers of NETosis [37], and therefore NET formation is dependent on the presence of molecular oxygen. An explanation for the missing NET formation under anoxia in our experiments may be caused by lack of oxygen and decreased ROS levels.

HIF-1α protein expression

Surprisingly, we could not detect HIF-1α protein in western blot after four hours culture of neutrophils under anoxia. Subsequent work of our group showed that also in whole proteome analyses of anoxa stimulated neutrophils HIF-1α could not be detected. It has been described several times that neutrophils express both HIF-1α and HIF-2α [145] and that its activation can be induced by hypoxia [128,136,146,147]. HIF pathways have been shown to play an important role in the regulation of hypoxia signaling in neutrophils [128,136,147].

One possible explanation for our results may be the time of culture under anoxia; as discussed before, activation of NF-κB elevates mRNA and protein levels of HIF-1α Walmsley, et al. showed that expression of NF-κB did not change between normoxia and hypoxia or anoxia in neutrophils after 3 h culture. However, after prolonged culture (6 h), NF-κB in neutrophils showed a significant increase in expression in both hypoxia and anoxia compared with normoxia. Additionally, progressive and near complete loss of constitutive NF-κB expression was observed in neutrophils under hypoxia or anoxia at early time points (3–12 h), while later (after 12–20 h) a recovery in NF-κB was found [130]. Therefore, temporal changes in NF-κB expression may influence HIF-1α expression in human neutrophils under anoxia.

Another explanation may be the decreased ROS levels after 4h of anoxia leading to overexpression of PHDs resulting in HIF-1α degradation (see Discussion, HIF-1α expression in monocytes under anoxia). Possibly, a transient inhibition of HIF-activation occurs via this way before other mechanisms are engaged leading to HIF-expression.

Thus, further investigations are needed to identify HIF-1α mRNA and protein expression as well as NF-κB expression in PB and CB neutrophils under anoxia at different timepoints. However, it has to be concluded that the observed effects of upregulated CD11b/CD18 expression and increased IL-6 expression in adult neutrophils but not CB neutrophils not seem to be regulated via HIF-1α.

7 Summary

The effect of anoxia on functions of neonatal monocytes and neutrophils

Background and hypothesis:

Neonatal sepsis is a major cause of neonatal morbidity and mortality. The consequences of neonatal sepsis are severe; in addition to high mortality rates, neonatal sepsis increases the risk of post-inflammatory secondary diseases such as bronchopulmonary dysplasia (BPD) and periventricular leucomalacia (PVL). A functional "immaturity" of the neonatal immune system compared to the adult immune system may explain the increased risk for generalization of infections. In adult immune cells, hypoxia is an important activator of inflammatory responses. The fetus develops in a hypoxic environment. However, little is known about the impact of hypoxia on neonatal immune functions.

The aim of the present study was to investigate the effect of anoxia on functions of neonatal monocytes and neutrophils in comparison to adult monocytes and neutrophils with the hypothesis that anoxia stimulates inflammatory immune functions in adult but not in cord blood cells.

Methods:

Neonatal and adult cells were incubated for four hours under normoxia or anoxia. Phagocytic activity, expression of phagocytosis receptors CD11b and CD18, production of ROS and apoptosis rates and cytokine production was analyzed by flow cytometry. Net formation was investigated by confocal microscopy and the expression of HIF-1α protein was assessed by western blot.

Results:

Anoxia led to a significant increase in phagocytosis rates and ROS-production in adult monocytes but not cord blood monocytes and to a downregulation of CD11b and CD18 expression in cord blood but not adult monocytes. In neutrophils, the phagocytosis rate did not change upon anoxia, neither in cord blood nor in adult cells, whereas anoxia upregulated CD11b and CD18 expression in adult neutrophils but not in cord blood neutrophils. Unlike in monocytes, anoxia downregulated ROS production and increased apoptosis in both adult and cord blood cells. Expression of the cytokine IL-6 was upregulated by anoxia in adult neutrophils, while no change was observed in cord blood neutrophils. No effect of anoxia was observed on IL-8 expression. Both adult and cord blood neutrophils were able to form NETS

upon stimulation with PMA, but anoxia did not lead to NETosis in both cell types. Finally, No HIF-1α expression was found neither in adult nor in cord blood neutrophils after culture in anoxia.

Conclusion:

It might be due to different HIF-1α protein expression, anoxia increases phagocytosis rate, ROS production and CD11b and CD18 expression in adult peripheral blood monocytes but not in cord blood monocytes. For neutrophils, anoxia stimulates CD11b and CD18 and IL-6 expression in adult peripheral blood but not in cord blood whereas phagocytosis rates, production of IL-8 and NETosis did not differ between PB neutrophils or CB neutrophils under normoxia or anoxia. HIF-1α could not be detected in adult and cord blood PMNs.

Taken together, anoxia seems to stimulate inflammatory response of adult innate immunity, but diminishes cord blood innate immune functions.

German summary

Die Wirkung von Anoxie auf die Funktionen von neonatalen Monozyten und Neutrophilen

Hintergrund und Hypothese:

Die neonatale Sepsis ist gekennzeichnet durch eine hohe Morbidität und Mortalität. Die Folgen der neonatalen Sepsis sind schwerwiegend; neben der hohen Sterblichkeitsrate erhöht sie das Risiko für postinflammatorische Folgeerkrankungen wie bronchopulmonale Dysplasie (BPD) und periventrikuläre Leukomalazie (PVL). Eine funktionelle "Unreife" des neonatalen Immunsystems im Vergleich zum Immunsystem von Erwachsenen könnte das erhöhte Risiko von Infektionen erklären. Bei Immunzellen von Erwachsenen ist Hypoxie ein wichtiger Aktivator von Entzündungsreaktionen. Der Fötus entwickelt sich in einer hypoxischen Umgebung. Es ist jedoch wenig über den Einfluss von Hypoxie auf die neonatalen Immunfunktionen bekannt.

Das Ziel der vorliegenden Studie war es, die Wirkung von Anoxie auf die Funktionen von Monozyten und Neutrophilen Granulozyten (PMN, *engl: Polymorph nuclear cells*)von Neugeborenen und Erwachsenen zu vergleichen. Die Hypothese lautet, dass Anoxie entzündliche Immunreaktionen in adulten, aber nicht in Nabelschnurblut-Zellen induziert.

Methoden:

Leukozyten wurden aus Nabelschnurblut (CB, *engl: Cord blood*) und aus dem Blut von Erwachsenen (PB, *engl: Peripheral blood*) isoliert und anschließend für vier Stunden unter Normoxie oder Anoxie inkubiert. Die phagozytische Aktivität, die Expression der Phagozytose-Rezeptoren CD11b und CD18, die Produktion von ROS und die Apoptoserate sowie die Produktion von proinflammatorischen Zytokinen wurden mittels Durchflusszytometrie analysiert. Die (DNA) Netzbildung von PMNs wurde mit Hilfe von konfokaler Mikroskopie untersucht und die Expression des HIF-1a-Proteins wurde durch Western Blot bestimmt.

Ergebnisse:

Anoxie führte zu einem signifikanten Anstieg der Phagozytoserate und der ROS-Produktion in PB Monozyten, aber nicht in CB-Monozyten und zu einer Downregulation der CD11b- und CD18-Expression in Nabelschnurblut-, aber nicht in adulten Monozyten. Bei PMNs änderte sich die Phagozytoserate nach Anoxie in beiden Gruppen nicht. Dennoch wurde

die Expression von CD11b und CD18 in PB PMNs, aber nicht in CB PMNs nach Anoxie hochreguliert. Im Gegensatz zu Monozyten wurde in PMNs in beiden Gruppen durch Anoxie die ROS-Produktion herunterreguliert und die Apoptose erhöht. Die Expression des Zytokins IL-6 wurde durch Anoxie in PB PMNs hochreguliert, während in CB PMNs keine Veränderung beobachtet wurde. Auf die IL-8-Expression hatte Anoxie in beiden Gruppen keinen Effekt. Hingegen verhinderte eine Anoxie in beiden Gruppen die Fähigkeit von PMNs infolge einer Stimulation DNA Netze zu bilden. Letztlich wurde weder in adulten noch in Nabelschnurblut-PMNs nach Inkubation in Anoxie eine HIF-1a-Expression gefunden.

Schlussfolgerung:

Anoxie erhöht die Phagozytoserate, die ROS-Produktion und die CD11b- und CD18-Expression in adulten peripheren Blutmonozyten, aber nicht in Nabelschnurblutmonozyten, was auf eine unterschiedliche HIF-1a-Proteinexpression zurückzuführen sein könnte. Bei den PMNs stimuliert Anoxie die CD11b-, CD18-und IL-6-Expression im adulten peripheren Blut, aber nicht im Nabelschnurblut, während sich die Phagozytoseraten, die Produktion von IL-8 und die NETosis nicht zwischen PB- und CB- PMNs unter Normoxie oder Anoxie unterschieden. HIF-1alpha konnte in adulten und Nabelschnurblut- PMNs nicht nachgewiesen werden.

Insgesamt scheint die Anoxie die Entzündungsreaktion der adulten angeborenen Immunität zu stimulieren, aber die angeborenen Immunfunktionen des Nabelschnurblutes zu vermindern.

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9. Declaration of Contribution

The work was performed in the Department of Pediatrics Division IV Neonatology under the supervision of PD Dr. med. Christian Gille.

The conception of the study was done in collaboration with the doctoral supervisor PD Dr. med. Christian Gille.

All experiments were performed by myself (after training by laboratory members: Dr. med. Natascha Köstlin, Dr. rer. nat. Bärbel Spring and Stefanie Dietz). The statistical analysis was performed independently by me.

I assure that I have written the manuscript independently and that I have not used any sources other than those indicated by me.

The submitted work has not been the subject of any other examination procedure, neither in its entirety nor in essential parts.

Tübingen 2021

Signature

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11.Curriculum Vitae

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