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Bc. Eliška Miková

Proportional and functional characteristics of particular neutrophil subpopulations in umbilical cord and peripheral blood

Proporční zastoupení jednotlivých subpopulací neutrofilů a jejich funkční vlastnosti v pupečníkové a periferní krvi

Diploma thesis

Supervisor: doc. RNDr. Jiří Hrdý, Ph.D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

Acknowledgment

Abstract

Early postnatal period is characterised by generally immature phenotype of the newborn's immune system. The maturation of the immune system including setting appropriate regulatory responses is occurring during this period and encountering pioneering bacteria colonizing neonate plays an important role. In the early days after birth, the immune system of a newborn is very limited, and the adaptive part is mostly represented by antibodies transferred from the mother by cord blood (CB) in the womb and then by colostrum and mother's milk after labour. Therefore, innate immunity plays a key role in defence (against pathogens) in neonates and is largely represented by neutrophils.

This study aims to better understand neutrophil biology and phenotype in umbilical CB, compared to neutrophils from peripheral blood (PB) of mothers and healthy non pregnant women (referred to as HC). The assessment of neutrophil phenotype based on surface markers was performed using flow cytometry. Expression of genes linked to antimicrobial function was measured using quantitative PCR. Functional properties of neutrophils, metabolic activity during activation and phagocytosis, and suppressive properties were assessed using the SeaHorse machine and flow cytometry, respectively.

Here we confirm the presence of immature $CD16^{low}CD64^{high}$ and $CD16^{low}CD62L^{+}$ neutrophil subpopulations in CB of neonates, which could be responsible for the increased expression of genes associated with antimicrobial response. Upon challenge with inflammatory stimulus (e.g. *Escherichia coli* or lipopolysaccharide), neutrophils from both CB and maternal PB exerted normal activation and oxidative burst.

Key words: neutrophils, cytokines, phagocytosis, myeloperoxidase, defensins

Abstrakt

Časné postnatální období se vyznačuje obecně nezralým fenotypem imunitního systému novorozence. Během tohoto období dochází k vývoji imunitního systému včetně nastavování imunitních reakcí, kde důležitou roli hraje setkávání imunitních buněk s prvotními bakteriemi kolonizujícími novorozence. V prvních dnech po narození je imunitní systém novorozence velmi omezený a adaptivní část je z velké části reprezentována protilátkami přenášenými z matky pupečníkovou krví v děloze, dále kolostrem a mateřským mlékem po porodu. Vrozená imunita, do značné míry tvořena neutrofily, proto hraje klíčovou roli v obranyschopnosti novorozenců.

Cílem této studie je lepší pochopení biologie a fenotypu neutrofilů v pupečníkové krvi, ve srovnání s neutrofily z periferní krve matek a periferní krve zdravých netěhotných žen. Hodnocení fenotypu neutrofilů na základě exprese povrchových markerů bylo provedeno pomocí průtokové cytometrie. Exprese genů spojených s antimikrobiální funkcí byla měřena pomocí kvantitativní PCR. Funkční vlastnosti neutrofilů, metabolická aktivita během aktivace a fagocytózy a supresivní vlastnosti byli hodnoceny pomocí technologie SeaHorse a průtokové cytometrie.

Výsledky předložené v této diplomové práci potvrzují přítomnost nezralých $CD16^{low}CD64^{high}$ a $CD16^{low}CD62L^{+}$ subpopulací neutrofilů v pupečníkové krvi novorozenců, které by mohly být případně zodpovědné za zvýšenou expresi genů spojených s antimikrobiální odpovědí. Při setkání se zánětlivým podnětem (např. *Escherichia coli* nebo lipopolysacharid) jsou neutrofily z pupečníkové krve i periferní krve matek schopny normální aktivace a oxidačního vzplanutí.

Klíčová slova: neutrofily, cytokiny, fagocytóza, myeloperoxidáza, defensiny

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List of abbreviations

APC	antigen-presenting cell
Arg-1	arginase-1
BM	breast milk
CBMC	cord blood mononuclear cells
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CRP	C-reactive protein
CS	caesarean section
DC	dendritic cell
DHR	dihydrorhodamine
ECAR	extracellular acidification rate
fMLP	N-formylmethionine-leucylphenylalanine
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDN	high-density neutrophils
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
IL	interleukin
Ig	immunoglobulin
iNOS	inducible nitric oxide synthase
LDG	low-density granulocytes
LDN	low-density neutrophils
LOX-1	lectin-type oxidized LDL receptor 1
LPS	lipopolysaccharide
Mac-1	macrophage-1 antigen

MDSC	myeloid-derived suppressor cells
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MPO	myeloperoxidase
ELANE	neutrophil elastase
NETs	neutrophil extracellular traps
NK	natural killer
nNIF	neonatal NET-inhibitory factor
OCR	oxygen consumption rate
OxPhos	oxidative phosphorylation
PADI4	peptidyl arginine deiminase IV
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PD-L1	programmed death-ligand 1
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophil
PPP	pentose phosphate pathway
RA	rheumatoid arthritis
ROS	reactive oxygen species
RT	room temperature
SLE	systemic lupus erythematosus
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α
Treg	regulatory T lymphocyte
VD	vaginal delivery

1. Introduction

Pregnancy largely influences the mother's immune system. With tight regulation needed to prevent harmful immune responses against the semi-allogenic foetus, many suppressive processes occur during the span of pregnancy. According to the literature, one of these regulatory cell populations also being expanded are suppressive neutrophils termed either low-density neutrophils (LDN) or polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC). As a part of innate immunity, the activity of neutrophils has to be regulated as well to prevent harmful inflammation in the placenta. Extensive neutrophil activation during pregnancy can lead to pregnancy complications that can influence both the foetus (e.g. preterm birth) and the mother (e.g. preeclampsia). Immune processes taking place during the pregnancy and during labour (caesarean section or vaginal delivery) can influence the neonate and the setting of its immune system.

Early after delivery, the newborn is mostly protected by antibodies transferred from the mother (first transplacentally, later by breast milk), given that its adaptive system is not fully developed yet. Consequently, the primary protection is mainly represented by innate immunity, where neutrophils present a major part. The functionality and phenotype of neutrophils in neonates have been studied extensively. However, in recent years, new technology enabled the recognition of new neutrophil subpopulations (e.g. PMN-MDSC) and functions (e.g. NETosis), which could play a role in the neonatal immune milieu.

This thesis focuses on the phenotype and functionality of neonatal neutrophils early after birth. Utilising cord blood (CB) as the sample source enables obtaining larger numbers of cells and therefore, possible downstream functional analysis, which would not be realizable with the sample volume (microliters) that would be taken from newborns peripheral blood (PB). Obtained data were compared with PB neutrophils from mothers and nonpregnant healthy woman volunteers.

2. Literature review

2.1. Neonatal immune system

Haematopoiesis of the foetus is provided by the yolk sack early in the gestation (around 5th week), then the fetal liver (from around the 5th to 23rd week) and ultimately, haematopoiesis is transferred mainly to bone marrow (from the 15th week and on) (Zhang *et al.*, 2017).

As depicted by Olin *et al.* (2018), neonatal immune system is highly dynamic and can differ significantly even 1 week after the birth. They also showed that interaction with microbes early after the birth is vital for proper immune system development (Olin *et al.*, 2018). Early after the birth and in the first few months of life, neonates are highly susceptible to infection since their adaptive immune system has not fully developed yet.

The question of sterility of the foetus in the womb remains to be fully elucidated. It was proposed that placenta harbours its own microbiome (Aagaard *et al.*, 2014), but as other articles opposed, the results could be due to the contamination during sample collection as well as from DNA isolation kits and other reagents used during the sequencing procedure (de Goffau *et al.*, 2019; Theis *et al.*, 2019).

Prenatally, antibodies (mostly immunoglobulin (Ig) G) from mothers are transferred to foetus through neonatal FcRn expressed on the trophoblast (Lozano *et al*., 2018). In the early days after birth, newborns are protected by antibodies from their mothers through colostrum and later breast milk. Colostrum (first secretion from mammary glands after birth) is rich in antibodies (secretory IgA), lactose, lipids, human milk oligosaccharides, and other essential molecules providing nutrition and immune defence to the newborn. Notably, some maternal milk compounds (e.g. oligosaccharides) represent the energy source for microorganisms contributing to neonatal gut microbiome development (Bardanzellu *et al.*, 2017; Plaza-Díaz *et al.*, 2018). Since neonate's adaptive immune responses are not fully developed, the importance of innate immunity is pivotal. As a consequence of the relative immaturity of the neonatal immune system, newborns are at a higher risk of sepsis, with elevation in incidence in preterm infants (Lukacs and Schrag *et al*., 2012). Sepsis is characterized by rapid recruitment of immature neutrophils to blood leading to increment of the ratio of immature neutrophils to total neutrophil count (I/T) (Mare *et al.*, 2015, Saboohi *et al*., 2019).

Neutrophils present the first line of defence against pathogens. Sundqvist and her team showed that neonatal neutrophils exist in a pre-primed state (as shown by reactive oxygen species (ROS) production in response to galectin-3) in comparison to adult neutrophils and that the mode of delivery, caesarean section (CS) or vaginal delivery (VD), can alter this state (Sundqvist *et al.*, 2013). Birle *et al.* (2015) also focused on CB neutrophils and the impact of the mode of delivery on neutrophil functions. In neutrophils treated with phosphate-buffered saline (PBS), migration was enhanced in CB compared to adult PB. Nevertheless, when neutrophils migrated towards N-formylmethionine-leucyl-phenylalanine (fMLP), neutrophils from children delivered by VD exhibited decreased migratory properties compared to adult PB neutrophils. Migration towards fMLP was further decreased in CB neutrophils of newborns delivered by CS (Birle *et al.*, 2015).

2.2. Immune system during pregnancy

There is a tight immune regulation at the fetal-maternal interface carried out by many cells and inhibitory molecules, such as unique natural killer (NK) cells, called uterine NK cells, which do not possess traditional NK cell cytotoxicity (Kopcow *et al.*, 2005) and participate in the vascular remodelling during placentation (Robson *et al.*, 2012). As shown in murine model, special type of uterine dendritic cells (DC) also contributes to proper placentation by producing growth factors essential for angiogenesis, namely soluble fms-like tyrosine kinase 1 and transforming growth factor β (TGF-β) (Plaks *et al.*, 2008). Regulatory T lymphocytes (Treg) represent another important cell subset, which suppresses potentially harmful response of cytotoxic cells (CD8$^+$ T cells, NK cells) against the foetus (Somerset *et al.*, 2004; Tsuda *et al.*, 2019). Another tolerance mechanism includes the expression of non-classical human leukocyte antigen G (HLA-G) by the trophoblast. HLA-G is present at the fetal-maternal interface as a cell-bound molecule but can also be shed into maternal circulation in a soluble form (Hunt *et al.*, 2000). Both forms are thought to function on immune cells, non-soluble HLA-G suppresses uNK cells, soluble form alters cytokine production by mononuclear cells (stimulates production of anti-inflammatory cytokines) and suppresses CD8$^+$ cytotoxic T cells (Hunt *et al.*, 2006). Among other regulatory immune cell subpopulations, a suppressive subpopulation of myeloid cells with phenotype resembling neutrophils expands during pregnancy; this topic will be discussed below in paragraph *2.3.1.4 (Polymorphonuclear myeloid-derived suppressor cells in pregnancy)*.

Along with local immune suppressive mechanisms described above, systemic immunity is also altered in pregnant women. Overt immune suppression could lead to severe infections and danger to both mother and her child. Therefore a certain balance has to be set between suppression of inappropriate immune responses potentially damaging the foetus and proper responses against pathogens. In an extensive review, Abu-Raya *et al.* (2020) described fluctuations in almost all immune cell populations during pregnancy. However, as suggested by the authors, the results of individual research groups sometimes varied aplenty (Abu-Raya *et al.*, 2020).

Focusing on neutrophils, their numbers are gradually elevated during the course of pregnancy, following the increase in growth factors (granulocyte (G-CSF) and granulocyte-macrophage colony-stimulating factors (GM-CSF)) stimulating neutrophil production from the bone marrow (Belo *et al.*, 2005). As shown in studies using experimental animal models, human hormones estrogene and progesterone regulate the infiltration of neutrophils into female reproductive tracks by modulation of permeability of the reproductive tract lining (Salinas-Muñoz *et al.*, 2018).

2.3. Neutrophils

Neutrophils were considered a rather homogenous population with inflammatory phenotype and limited functions in the immune response for a long time. Recently, this view has been changed with the discovery of novel neutrophil functions and distinct phenotypes, which neutrophils acquire under specific conditions, or in certain environments, such as during cancer development, chronic infection or during pregnancy.

The perception of neutrophils as short-time living cells has been changed as well. Pillay *et al.* (2010) demonstrated using *in vivo* ^{2}H$_2$O labelling and tracking that human neutrophils can survive up to 5 days (Pillay *et al.*, 2010). It has been documented that all neutrophils do not necessarily have to undergo apoptosis when they reach inflamed tissue and that, under certain circumstances, neutrophils can travel back to the circulation in a process called reverse migration. The contribution of neutrophil reverse migration to the resolution of inflammation was shown in detail using zebrafish as an experimental model (Mathias *et al.,* 2006). Also, Buckley *et al.* (2006) observed, in patients with chronic inflammation, a characteristic phenotype of neutrophils that have reverse migrated and were distinct from naive circulatory and tissue-resident neutrophils (Buckley *et al.,* 2006). This evidence suggests that neutrophils do not only function as an extensive arm of the

innate immune system but could potentially influence the functions and polarization of the adaptive immune response.

Neutrophil identification in humans is based on several markers used in various combinations. Names and functions of the most commonly used neutrophil markers are listed in **Table 1**.

Table 1. The function and expression of neutrophil cell surface markers

Marker	Alternative name	Function on neutrophils	Expression on neutrophils
CD11b	Mac-1 (with CD18)	β2 integrin, co-receptor of CD18, involved in neutrophil transmigration (Hyun *et al*., 2019)	consistent in steady state (Lakschevitz *et al*., 2016) elevated after stimulation with IL-2 (Abdel-Salam *et al*., 2014)
CD14		LPS receptor, production of TNF-α after CD14 stimulation (Haziot *et al*., 1993)	low in steady state, induced during sepsis (Wagner *et al*., 2003)
CD15	Sialyl-Lewis X	involved in cell-to-cell contact, phagocytosis, stimulation of degranulation and respiratory burst	enhances during maturation, elevated on low density neutrophils (Ssemaganda *et al*., 2014)
CD16	FcγRIII	involved mainly in degranulation (Fossati *et al*., 2002)	consistent (Lakschevitz *et al*., 2016), high on mature neutrophils, lost from surface after cultivation with LPS (Wagner *et al*., 2003)
CD33	Siglec-3	inhibitory molecule, adhesion molecule	decreases during maturation, high on immature neutrophils (Ssemaganda *et al*., 2014)
CD62L	L-selectin	mediates the adhesion to epithelium at the sites of inflammation (Kamp *et al*., 2012)	decreased in aging neutrophils (Casanova-Acebes *et al*., 2013)
CD64	FcγRI	high affinity receptor for IgG	elevated in primed or activated cells (Dai *et al*., 2017)
CD66b	CAECAM-8	adhesion molecule, involved in respiratory burst	consistent expression (Lakschevitz *et al*., 2016), elevated after activation (Schmidt *et al*., 2012)

Abbreviations: *CAECAM-8 – carcinoembryonic antigen-related cell adhesion molecule 8, IgG – immunoglobulin G, IL – interleukin, LPS – lipopolysaccharide, TNF-α – tumor necrosis factor α*

It was shown that neutrophils could shape adaptive immunity through the induction of DC maturation and activation. In mouse model of *Toxoplasma gondii* infection, neutrophils activated upon infection were able to activate DC through the production of tumor necrosis factor α (TNF-α) (Bennouna *et al.*, 2003). Another described mechanism of DC priming was through cell-to-cell contact mediated by Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and Macrophage-1 antigen (Mac-1 integrin) (van Gisbergen *et al.*, 2005).

There is also *in vitro* evidence for the capability of neutrophils to function as antigen-presenting cells (APC) expressing major histocompatibility complex (MHC) type II and co-stimulatory molecules, such as CD80 and CD40, after phagocytosis of opsonised red blood cells and to induce activation of CD4$^+$ T cells and polarize them towards Th1 phenotype (Meinderts *et al.*, 2019). In mice, neutrophils can also activate CD8$^+$ T cells during viral infection, for instance, by cross-presentation of influenza antigens on MHC-I (Hufford *et al.*, 2012) or the induction of memory CD8$^+$ T lymphocytes by transporting viral antigens from skin to the bone marrow (Duffy *et al.*, 2012).

2.3.1. Neutrophil heterogeneity

The main distinction among neutrophil subpopulations described in this thesis is based on different neutrophil localisation after density-gradient centrifugation. Density-gradient centrifugation utilises the distinct density of particular subsets of cells for the separation of blood into several parts. After centrifugation, plasma localises on the top followed by the ring of mononuclear cells, then density gradient reagent part and erythrocyte sediment at the bottom of the tube. Mononuclear ring, or called PB/CB mononuclear cells (PBMC/CBMC) consist of lymphocytes, monocytes, and neutrophils with low-density (LDN). Neutrophils, which co-purify with the erythrocyte fraction, are referred to as high-density neutrophils (HDN).

In the following paragraphs, designated neutrophil subpopulations will be introduced, and their implications in pregnancy and neonatal immune system, with special emphasis on their properties in umbilical CB, will be discussed.

2.3.1.1. Low-density neutrophils

Although LDN were originally described in 1986 in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and acute rheumatic fever as a contamination in PBMC (Hacbarth and Kajdacsy-Balla, 1986), LDN are mainly studied in the context of cancer progression and tumor microenvironment. In 2015, Sagiv *et al.* defined neutrophil population, which expands transiently in self-resolving inflammation in tumor bearing mice as well as in patients suffering from cancer. They stated that this LDN population consists of immature and mature neutrophils with anti-tumor properties. They reported that separated LDN had decreased phagocytic activity and impaired production of ROS. They also showed that the pro-tumor HDN population is capable of switching to LDN phenotype in the presence of anti-inflammatory cytokine TGF-β (Sagiv *et al.*, 2015).

Hsu *et al.* (Hsu *et al.*, 2019) performed RNA-sequencing of neutrophils from PB of tumor-bearing mice and revealed transcriptional diversity among immature LDN, mature HDN, and naive bone-marrow neutrophils from healthy mice. Further, they showed that immature LDN exhibit considerable metabolic flexibility, with a 2.6-fold increase in bioenergetic capacity (total adenosine triphosphate (ATP) production from glycolysis and oxidative phosphorylation) in comparison to HDN. Basal neutrophil metabolism was the same, but upon glucose addition, LDN exhibited an increased ECAR relative to HDN. Mitochondrial respiration of LDN was significantly higher but was lost after the addition of glucose. In co-culture experiments, Hsu *et al.* were not able to prove LDN function as MDSC since LDN were unable to suppress neither $CD4^+$ nor $CD8^+$ T cell proliferation. Authors suggest that immature LDN may be able to perform metabolically demanding functions (such as NETosis) under conditions of nutrient deprivation (Hsu *et al.*, 2019).

Some studies proposed that the expansion of LDN could be caused by a decrease in neutrophil buoyancy due to activation seen in patients with chronic inflammation (Hacbarth and Kajdacsy-Balla, 1986; Hassani *et al.,* 2020), such as cancer. *In vitro* activation (using fMLP or platelet activation factor) of isolated neutrophils from healthy donors also led to a decrease in density and increase of LDN numbers depending on the concentration of stimulant used. They also showed that after LPS introduction, LDN consisted of a high percentage of $CD16^{dim}CD62L^{high}$ neutrophils (Hassani *et al.*, 2020). Induction of systemic inflammation by LPS in healthy human volunteers led to the release of $CD16^{dim}CD62L^{bright}$ and $CD16^{bright}CD62L^{dim}$ neutrophils, with banded and hypersegmented morphology, respectively, into circulation. $CD16^{dim}CD62L^{bright}$ neutrophils showed prolonged survival in cell culture but were not able to suppress T cell proliferation in contrast to $CD16^{bright}CD62L^{dim}$ neutrophils, which potently inhibited T cell proliferation. As shown by real-time imaging, $CD16^{bright}CD62L^{dim}$ neutrophils suppressed T lymphocytes by local release of H_2O_2, mediated through Mac-1 integrin (Pillay *et al.*, 2012).

On the other hand, as shown in patients with autoimmune disease like SLE or RA, LDN can also exert a proinflammatory phenotype. These proinflammatory low-density granulocytes (LDG) do not exert immunosuppressive functions typical for LDN but express cell surface markers (lectin-type oxidized LDL receptor 1 (LOX-1) and programmed death-ligand 1 (PD-L1)) similar to PMN-MDSC (Rahman *et al.*, 2019). In SLE patients, it was demonstrated that neutrophils release increased number of neutrophil

extracellular traps (NETs) and that the NET clearing mechanism (function of DNase I) is impaired, contributing to the pathogenesis of SLE (Hakkim *et al.*, 2010). It was proposed that LDG could be the cells responsible for defective activation and enhanced NETosis (Silvestre-Roig *et al.*, 2019) since they vastly expand in PBMC of SLE patients (Denny *et al.*, 2010). It was shown that LDG isolated from SLE patients express an excessive amount of enzymes and proteins linked to antimicrobial activity (e.g. *MPO*, *ELANE*, *DEFA4*) and are prone to release significant quantities of NETs (Villanueva *et al.*, 2011).

2.3.1.2. Low-density neutrophils in pregnancy and the neonatal immune system

Ssemaganda and his team (2014) observed the presence of both LDN and HDN subpopulations in umbilical CB and PB of pregnant women but were only able to obtain LDN fraction from term placentas. Based on the expression profiles of cell surface molecules, the authors suggested that LDN consist of both immature and mature neutrophils and display higher activation state in comparison to HDN. CB LDN represented the largest part of $CD15^+$ neutrophils in comparison to PB of mothers and placentas, where the percentages of LDN did not differ. LDN express lower amounts of CD16 in comparison to HDN in all three studied compartments indicating their immaturity. In the placentas, LDN were shown to regulate T cell responsiveness through the expression of arginase 1 (Arg-1) (Ssemaganda *et al.*, 2014).

In a recent study published by Weinhage *et al.* (2020), the research team focused on the role of LDN in neonatal sepsis. LDN were shown to express low levels of CD16 and high levels of CD64, pointing to their immaturity, supporting observation by Ssemaganda (Ssemaganda *et al.*, 2014). They suggested that CB LDN exhibit a distinct phenotype, different from LDN phenotype in adults. In their experimental setting, CB neutrophils had impaired functions (e.g. reduced phagocytosis and NETosis), but LDN exhibited prolonged survival in comparison to HDN. They were not able to demonstrate an inhibitory effect of LDN on T cell proliferation (Weinhage *et al.*, 2020).

Information regarding LDN in pregnancy and CB is scarce because more scientific groups focus preferably on polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC). The role of PMN-MDSC in pregnancy and in the neonatal immune system will be discussed in detail bellow.

2.3.1.3. Polymorphonuclear myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC) were mostly studied in the context of cancer and tumor microenvironment. During homeostasis, MDSC are almost undetectable in PB. Their levels rise in the presence of chronic inflammation such as cancer or obesity. The name MDSC defines all their major properties. As reviewed in Millrud *et al.* (2017) MDSC stem out from the myeloid cell lineage and are considered immature cells which were released from bone marrow under pathological conditions (persistent low level stimulating signal present in chronic inflammation) in the process of emergency granulopoiesis and later activated by inflammatory stimuli (e.g. LPS or TNF-α). Experimental evidence also suggests that MDSC may arise from neutrophils and monocytes, which have lost activation markers upon activation and developed immunosuppressive properties (Millrud *et al.*, 2017). MDSC exert suppressive functions mediated by the production of anti-inflammatory cytokines (e.g. IL-10, TGF-β), expression of immunomodulatory molecules, namely Arg-1, inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), and ROS, and direct suppression of T cells by cell-to-cell contact (Tamadaho *et al.*, 2017). Based on their phenotypic and morphological characteristics, MDSC can be distinguished into two distinct subpopulations, monocytic and granulocytic. In mice, MDSC are defined as $CD11b^+Gr-1^+$ cells, with $Ly6C^+$ characterising monocytic-MDSC (M-MDSC) and MDSC expressing $Ly6G^+$ are considered as polymorphonuclear-MDSC (PMN-MDSC). In humans, multiple attempts to define MDSC were made (as specified in the paragraph bellow), but specific phenotypical and transcriptional signatures remain to be ascertained.

Bronte *et al.* (Bronte *et al.*, 2016) proposed certain criteria, including phenotypic, functional, and molecular characteristics, which should be applied for proper MDSC description and definition. According to Bronte, due to the lack of specific cell surface markers, the only way to separate human PMN-MDSC from mature neutrophils is density gradient centrifugation, which enriches PMN-MDSC in mononuclear low–density cell fraction. PMN-MDSC are defined as $CD11b^+CD14^-CD15^+/CD66^+$ whereas M-MDSC are $CD11b^+CD14^+HLA-DR^{-/low}CD15^-$ (Bronte *et al.*, 2016). Some studies focused on PMN-MDSC in pregnancy and neonatal immune system define PMN-MDSC as $CD66b^+CD33^+CD14^-HLA-DR^{low/-}$ (Köstlin *et al.*, 2014; Schwarz *et al.*, 2018). A novel molecule described as a characteristic PMN-MDSC marker, able to differentiate PMN-MDSC from mature PMN without the need to use density gradient centrifugation, is

called LOX-1 and is expressed in reaction to increased endoplasmatic stress (Condamine *et al.*, 2016). However, LOX-1 expression was only reported in cancer patients and so far was never used to differentiate PMN-MDSC in CB.

2.3.1.4. Polymorphonuclear myeloid-derived suppressor cells in pregnancy

Pregnancy is characterised by a certain level of immunosuppression which is considered to be essential for the survival of the semi-allogenic foetus in the womb, as described above. MDSC, with their potent immunomodulatory functions, were illustrated as one of the effector cells driving the immune tolerance at the fetal-maternal interface. Multiple studies confirmed elevated levels of PMN-MDSC during pregnancy in PB as well as in the placenta (Köstlin *et al.*, 2014, Veglia *et al.*, 2018). After delivery, the numbers of PMN-MDSC normalize to the ones found in nonpregnant healthy women (Köstlin *et al.*, 2014). As illustrated in mouse and human studies, the inability to achieve the appropriate levels of PMN-MDSC was associated with pregnancy complications, such as pregnancy loss, miscarriage, or preeclampsia (Nair *et al.*, 2015; Ren *et al.*, 2019; Wang *et al.*, 2018).

Using mouse model, Kang *et al.* (2016) demonstrated PMN-MDSC recruitment to the fetal-maternal interface through CXCR2/CXCL1 axis, which also regulates the suppressive activity (represented in the study by Arg-1 expression). As suggested by the authors, CXCL1 in mice is analogous to interleukin (IL) 8, one of the main chemoattractants for neutrophils in humans, which is elevated during healthy pregnancy (Kang *et al.*, 2016). In tumor environment, elevated IL-8 (along with IL-6) was shown to drive the accumulation of PMN-MDSC (Tobin *et al.*, 2019), suggesting that IL-8 could present one of the triggers driving PMN-MDSC accumulation in pregnancy.

PMN-MDSC expanded in pregnancy and suppressed T cell activation through the expression of Arg-1 and iNOS. PMN-MDSC produced greater amounts of ROS, as shown by the oxidation of dihydrorhodamine (DHR) 123 after activation with phorbol myristate acetate (PMA) when compared to PMN-MDSC from nonpregnant controls (Köstlin *et al.*, 2014). Increased numbers of PMN-MDSC were also seen in breast milk (BM). These so-called BM-MDSC possess strong immunomodulatory functions in connection to T lymphocytes (suppression of both CD4^{+} and CD8^{+} T cells) as well as monocytes (suppression of TLR4 expression on monocytes). The authors suggest that BM-MDSC could contribute to immune regulation in the gut of neonates (Köstlin *et al.*, 2018).

2.3.1.5. Polymorphonuclear myeloid-derived suppressor cells in cord blood and neonatal immune system

Multiple studies reported that MDSC are significantly expanded in CB of neonates and that PMN-MDSC are the prevailing subtype of CB MDSC (Rieber *et al.*, 2013; Gervassi *et al.*, 2014; He *et al.*, 2018). Accumulation of PMN-MDSC in CB that did not differ in preterm and term infants was also described by Schwarz and his team (Schwarz *et al.*, 2018). In addition to that, Schwarz *et al.* (2018) showed slightly lower levels of PMN-MDSC in CB of infants born by CS in comparison to infants born spontaneously. Elevated levels of PMN-MDSC in infants remain throughout the neonatal period and are thought to help to regulate the potentially harmful inflammatory responses (He *et al.*, 2018; Schwarz *et al.*, 2018). This view would correlate with the observation that PMN-MDSC further expand during neonatal sepsis (Schwarz *et al.*, 2018), potentially reducing the collateral damage caused as a side effect of immune responses primarily directed against the infectious agents.

CB PMN-MDSC were able to suppress both $CD4^+$ and $CD8^+$ T cell proliferation as well as production of interferon γ, IL-5, and IL-17 by T cells and the cytotoxicity of NK cells (Rieber *et al.*, 2013). *In vivo* experiments showed the ability of CB PMN-MDSC to polarize T cells towards Th2 phenotype and induce regulatory $FoxP3^+$ Treg, alongside Th1 cell inhibition mediated by cell-to-cell contact (Köstlin *et al.*, 2017). Another way of immune modulation by MDSC from CB was described by Dietz *et al.* (Dietz *et al.*, 2019) using *in vitro Escherichia coli* (*E. coli*) infection model focusing on the role of monocytes. CB MDSC were able, through secreted molecules (as demonstrated by trans-well experiments), to decrease the expression of MHC-II molecules and increase the expression of co-inhibitory molecules (e.g. PD-L1, PD-L2) on monocytes, thus decreasing their ability to stimulate T cell proliferation. The aforementioned effect was only seen when CB MDSC were added to the whole PBMC fraction. When CD14-enriched monocytes were used for the co-culture with CB MDSC the effect was lost. Therefore, as the authors themselves suggested, the immune suppressive effect could be mediated through another cell type, not merely by MDSC alone (Dietz *et al.*, 2019). Although the authors did not specify the MDSC subset, the use of anti-CD66b for separation of MDSC from CBMC would suggest that the studied population indeed was PMN-MDSC.

The ability to suppress T cell responses was also demonstrated in an experiment using MDSC generated *in vitro* from CB $CD34^+$ cells, using a combination of GM-CSF

and stem cell factor (SCF). *In vitro* derived MDSC, defined as HLA-DR^{low}CD11b$^+$CD33$^+$, expressed immune regulatory molecules, like Arg-1, iNOS, or IDO and secreted IL-10, TGF-β, and vascular endothelial growth factor similar to MDSC obtained *in vivo*, showing the therapeutic potential, for example in graft versus host disease (Park *et al.*, 2019).

He *et al.* (He *et al.*, 2018) reported expanded CD15$^+$LOX-1$^+$ PMN-MDSC in CB in comparison to CD15$^+$LOX-1$^+$ neutrophils in PB of healthy volunteers. When comparing LOX-1$^+$ and LOX-1$^-$ neutrophils in CB, only LOX-1$^+$ cells were able to suppress T cell proliferation. He and her team performed RNA-sequencing on isolated PMN-MDSC from newborn mouse and found that the genes linked to antimicrobial response were considerably up-regulated. The proteins identified as the effectors in suppressive functions of PMN-MDSC in newborn mice were S100A8, S100A9, and prostaglandin E2 (He *et al.*, 2018).

2.3.2. Neutrophil metabolism

Neutrophil activation and functional performance are tightly linked to the metabolic program employed in a given situation. Similarly, to the idea of neutrophil homogeneity and limited functionality, it was long considered that neutrophils generated ATP preferably from glycolysis while repressing other metabolic pathways, like oxidative phosphorylation (OxPhos) and pentose phosphate pathway (PPP). In recent years, scientific investigation has unravelled the utilization of a wide range of metabolic pathways for distinct neutrophil functions (Kumar and Dikshit, 2019). However glycolysis still represents the major energetic source (Chacko *et al.*, 2013).

A complex study focusing on the metabolism of particular cellular populations in blood performed by Chacko and his team (2013) showed that neutrophils are generally metabolically less active than other blood cell types (lymphocytes, monocytes, platelets). OxPhos is very limited in circulating neutrophils since the mitochondrial inhibitors (oligomycin, antimycin A) had little to no effect on neutrophil oxygen consumption rate (OCR) (Chacko *et al.*, 2013). Tricarboxylic acid cycle and OxPhos utilization is limited to neutrophil differentiation, in which the process of autophagy (and generation of free fatty acids) plays a major role (Riffelmacher *et al.*, 2017). After the addition of PMA, neutrophil OCR is significantly elevated, along with the increase in proton production rate, which is mediated by the activity of NADPH oxidase (Chacko *et al.*, 2013).

NADPH oxidase is essential for proper neutrophil function since its activation leads to the production of ROS, which can be directly used for microbe killing or can eventually contribute to the release of NETs. Azevedo *et al.* (2015) demonstrated that PPP is necessary for the induction of NETosis since the intermediates of PPP fuel NADPH oxidase. Glucose-6-phosphate, oxidized in the PPP reducing $NADP^+$ to NADPH, is provided by the initial step of glycolysis, which is essential for NETosis (Azevedo *et al.*, 2015). According to Rodríguez-Espinosa *et al.* (2015), NETosis could be divided into two separate phases; chromatin decondensation and the release of NETs alone. In the presence of glycolysis inhibitor, the formation of NETs was almost completely abolished, but the cells lost their characteristic nuclear morphology suggesting chromatin decondensation took place. PMA stimulation increased neutrophil glucose uptake along with glycolytic rate. The high demand for glucose was compensated by an increased expression of Glut-1 transporters on neutrophil membrane (Rodríguez-Espinosa *et al.*, 2015).

As discussed by Injarabian *et al.* (2019), neutrophil metabolic pathways and metabolic microenvironment in which neutrophil occurs could possibly influence its differentiation and phenotype. As demonstrated in tumor-bearing mice, mitochondrial metabolism engagement could help immature neutrophils maintain their suppressive functions (Rice *et al.*, 2018).

Data on neutrophil metabolism in CB are very limited and contradictory. In 2001, a study focusing on CB neutrophil metabolism in relation to superoxide anion (O_2^-) production illustrated decreased O_2^- production in PMA stimulated CB neutrophils in comparison to adult neutrophils. When stimulated with fMLP, only CB neutrophils of preterm infants showed decreased production of O_2^- when compared to term CB and adult neutrophils (Komatsu *et al.,* 2001). On the contrary, Chudgar *et al.* (2005) showed slightly increased respiratory burst in CB neutrophils, which they ascribed to the alterations in oxidase components, such as higher content of cytochrome b_{558} in the membrane of CB neutrophils (Chudgar *et al.,* 2005). This is in agreement with a study conducted in 1983, where authors also observed increased O_2^- production in CB neutrophils (Strauss and Snyder, 1983), which, as stated by the authors themselves, does not explain the data showing decreased oxidative metabolism in CB neutrophils.

Metabolism of neutrophils during the course of pregnancy is a long-studied subject, but the results differ substantially. Early work suggested decreased production of ROS by neutrophils in PB of pregnant women (Cotton *et al.*, 1983; Crouch *et al.*, 1995).

Kindzelskii and his colleagues (Kindzelskii *et al*., 2002 and 2006) published few papers dedicated to this topic. According to their observations, unstimulated maternal neutrophils exhibited an enhanced state of activation *in vitro* in comparison to neutrophils from nonpregnant healthy women, but during activation, neutrophils from pregnant women did not reach the same level of ROS production as activated neutrophils from nonpregnant women (Kindzelskii *et al*., 2002). They showed that this higher basal activation could be caused by MPO, which accumulates on the surface of neutrophils isolated from PB of pregnant women (Kindzelskii *et al*., 2006).

2.3.3. Antimicrobial response

During neutrophil development, the expression of genes is progressively decreased. With the condensation of neutrophil nucleus, transcription is mostly silenced. Genes coding the antimicrobial peptides, proteases, and proteins involved in the antimicrobial response are activated primarily in the early stages of neutrophil development, and the final products are stored in neutrophil granules for further use. The formation of neutrophil granules is well studied, with special focus on protein trafficking into the four distinct granule types (Yin and Heit, 2018). The formation of neutrophil granules during neutrophil development and their content is depicted in **Figure 1**.

Neutrophil granules can fuse with the phagosome to release their content and dismantle the engulfed pathogen (as discussed below). The exocytosis of neutrophil granule content into the extracellular space can also take place when neutrophils are faced with pathogens too large to be phagocytized. Granules are released sequentially (from first to last – secretory, specific, gelatinase, and azurophilic) to enable neutrophils the trafficking from the blood into inflamed tissue and the subsequent inflammatory response against invading pathogens (Ramadass and Catz, 2016; Yin and Heit, 2018).
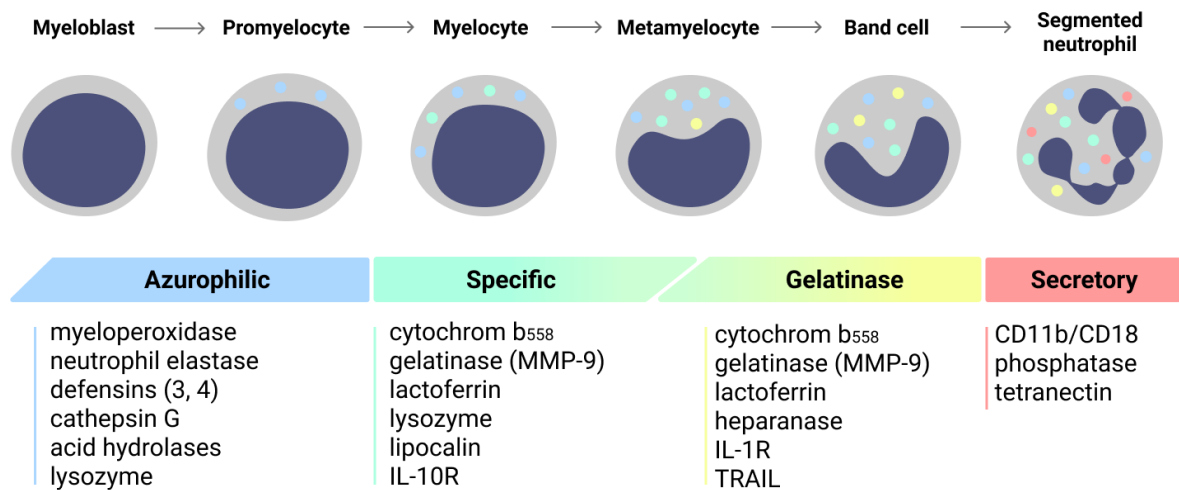
Azurophilic	Specific	Gelatinase	Secretory
myeloperoxidase neutrophil elastase defensins (3, 4) cathepsin G acid hydrolases lysozyme	cytochrom b$_{558}$ gelatinase (MMP-9) lactoferrin lysozyme lipocalin IL-10R	cytochrom b$_{558}$ gelatinase (MMP-9) lactoferrin heparanase IL-1R TRAIL	CD11b/CD18 phosphatase tetranectin

Figure 1. Neutrophil granule formation and their content

*Neutrophil granules are formed sequentially during the neutrophil development (granulopoiesis) from azurophilic, through specific and gelatinase to secretory. Their content is mainly represented by antimicrobial peptides, proteases, and diverse enzymes. Secretory granules contain high amounts of CD11b/CD18, molecules that mediate neutrophil adhesion. Prepared based on Ramadass and Catz, 2016 and Yin and Heit, 2018. **Abbreviations:** MMP9 – matrix metalloproteinase 9, TRAIL – TNF-related apoptosis-inducing ligand.*

2.3.4. Phagocytic capacity of neutrophils

Phagocytosis represents one of the first-line defence mechanisms of neutrophils against pathogens. It has been extensively studied in both health and disease. It is apparent that the composition of ingested material has a crucial effect on subsequent neutrophil activation (e.g. microbial components, necrotic cells) or possible unresponsiveness (e.g. apoptotic cells). The process of phagocytosis is tightly regulated. Surface molecules mediating the target recognition are mostly Fc receptors for IgG or receptors for fragments of the complement. Other receptors mediating the activation and priming of neutrophils for phagocytosis include toll-like receptors (e.g. TLR 2/6), chemokine receptors (e.g. CXCR1 recognizing IL-8), or receptor for TNF (van Kessel *et al.*, 2014). Principal Fc receptors expressed on circulating neutrophils are FcγRIIIB (CD16) and FcγRIIA (CD32). FcγRI (CD64) expression on neutrophils is linked to their activation status. For recognition of C3b and iC3b complement fragments, neutrophils express complement receptor 3 (CR3/Mac-1), a dimer consisting of α_M integrin (CD11b) and β_2 integrin (CD18), and CR1 (CD35). After target recognition, the actin cytoskeleton is reorganized to mediate the engulfment, leading to the creation of phagosome vesicle. Subsequently, neutrophil granules fuse with phagosome, releasing potent antimicrobial peptides (defensins), proteases (e.g. neutrophils elastase), and enzymes (e.g. myeloperoxidase (MPO)) capable

of disintegrating the engulfed cargo. Granule content is summarized in **Figure 1**. Concurrently, the NADPH oxidase complex is assembled *de novo* on the phagosome membrane or is enriched after phagosome fusion with granules. NADPH oxidase is responsible for the production of ROS (oxidative burst), namely the O_2^-, along with free electrons. Superoxide dismutase produces hydrogen peroxide (H_2O_2), which can in turn, be enzymatically modified into hypochlorous acid (HOCl) by MPO as shown in **Figure 2**.
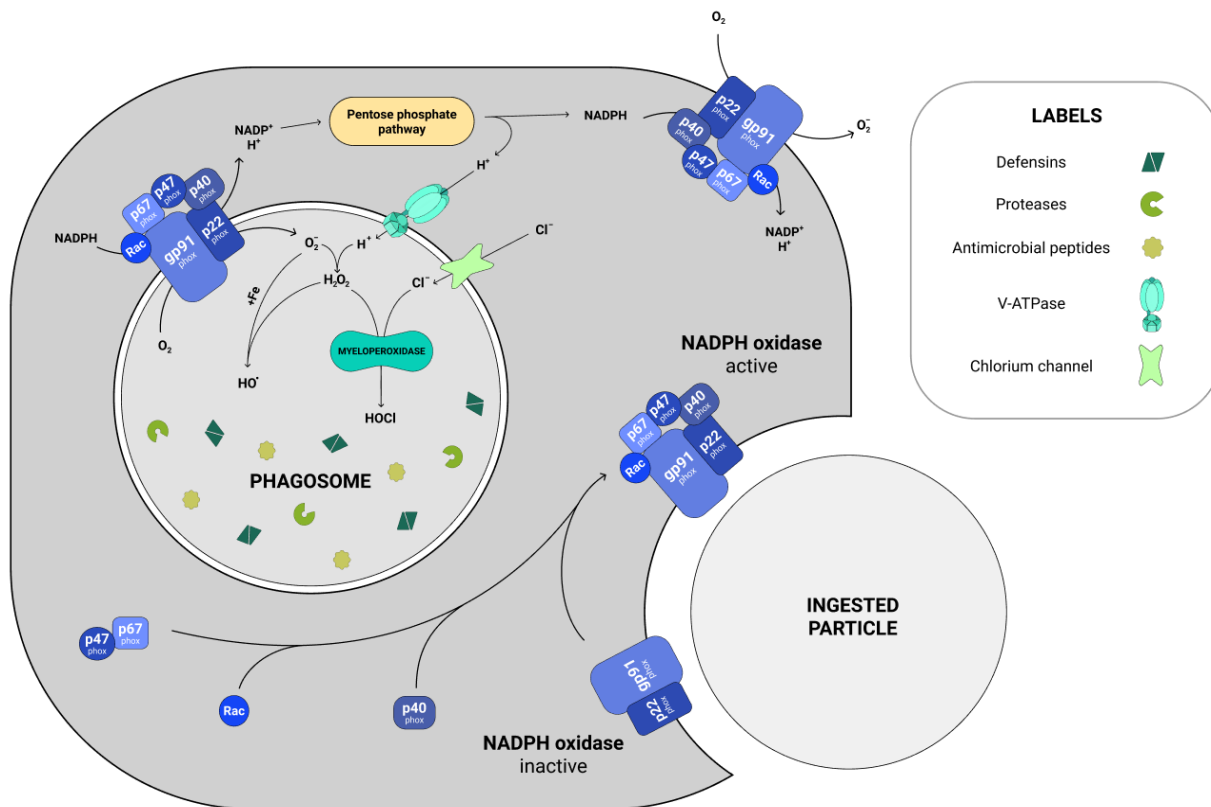

Figure 2. General scheme of NADPH activation and phagosome formation

NADPH oxidase is formed from parts and assembled both on the neutrophil membrane and on the phagosome. Reactive oxygen species are generated by the conjoint function of NADPH oxidase and myeloperoxidase. Granule fusion releases the potent antimicrobial molecules (defensins, proteases, antimicrobial peptides) in the phagosome to mediate the elimination of ingested pathogens. Prepared based on Dahlgren et al., 2019 and Nordenfelt and Tapper, 2011.

The literature regarding the phagocytic capacity of neutrophils in pregnancy varies substantially. Older studies suggest that the phagocytic potential of neutrophils is not affected by pregnancy (when using inert latex beads) or is even higher (when using *Candida albicans*) in comparison to the nonpregnant state. The authors suggested that this increase could be a compensation for general immunosuppression in pregnancy (Barriga *et al.*, 1994). This contradicts a study conducted in 1999, where Spanish research group

showed unaffected phagocytic activity in the first two trimesters and decreased phagocytic activity of neutrophils from women in the third trimester (Guerra-Infante *et al.*, 1999).

Recent studies remain inconsistent as well. It was shown that the phagocytic activity of neutrophils is increased in the 2nd and 3rd trimesters, also suggesting compensatory effect for general immune suppression during pregnancy (Pramanik *et al.*, 2007). One of the most recent studies conducted by Lampé *et al.* (Lampé *et al.,* 2015), stands a complete opposite view, with the general immunosuppression in pregnancy also affecting the phagocytic capacity of neutrophils. They showed that phagocytic capacity, illustrated as the phagocytic index (the number of ingested particles per cell), is decreased in neutrophils isolated from PB of pregnant women compared to neutrophils from nonpregnant women (Lampé *et al.*, 2015). The authors suggest that this decrease could affect the ability of neutrophils to clear microbial pathogens and increase the susceptibility of pregnant women to infection. Subsequently, the same team proposed that the decrease in phagocytic capacity could be caused by factors present in the maternal circulation (Lampé *et al.*, 2017). What are these factors still remains to be elucidated.

The phagocytic potential of neutrophils from CB was studied mainly as a possible contributor to increased susceptibility of neonates to bacterial infection and high rates of neonatal sepsis. Using the Phagotest flow cytometry method (incubation of whole blood with opsonised fluorescein isothiocyanate-labelled *E. coli*), Filias *et al.* (2011) showed decreased phagocytic ability of neutrophils from CB, which reached levels seen in adult PB by 3rd day after birth. Though the data concerning the phagocytic activity of neutrophils in CB have large distribution (55.8-81.7%) in comparison to phagocytic activity of neutrophils in adult PB (74.8-81.2%), thus the significant decrease could be caused by one deviating sample. No significant difference was seen when comparing term and preterm neonates (Filias *et al.,* 2011). Using a similar approach (incubation of whole blood with pHrodo labelled bacteria), Prosser and her team (2013) compared the phagocytic capacity of PB (withdrawn 24h post-birth) neutrophils from term and preterm neonates. They observed an increased number of phagocytic neutrophils in PB of term neonates (higher % of pHrodo$^+$ cells). Interestingly, the overall phagocytic capacity (median fluorescence intensity (MFI) of pHrodo$^+$ cells) was higher in neutrophils from PB of preterm neonates. Data acquired from PB of 24h old neonates showed a good correlation with data acquired from CB of neonates proving the suitability of CB for neonatal immune system investigation (Prosser *et al.,* 2013).

2.3.5.   NETosis

As discussed thoroughly by Manfredi *et al.* (2018), upon encounter with a pathogen, neutrophils can either employ phagocytosis or release neutrophil extracellular traps (NETs). This decision is based on pathogen size and number. The particular function utilised has to be tightly regulated to prevent collateral damage (Manfredi *et al.,* 2018). Although original experiments showed that NETs are capable of killing entrapped microbes (Brinkmann *et al.*, 2004), newer studies are suggesting that NETs are only capable of microbe immobilization without direct killing, despite the potent antimicrobial content of NETs (e.g. proteases, antimicrobial peptides, MPO). Nevertheless, their importance is not less since NETs release prevents the spreading and dissemination of bacteria throughout the body (Menegazzi *et al.*, 2012; Ortmann and Kolaczkowska, 2018).

As suggested by Brinkmann (2004), the release of NETs could represent an early event preceding the programmed cell death. Later, researchers described the formation of NETs as a novel type of cell death, differing from apoptosis (programmed cell death), necrosis (immunogenic cell death), and pyroptosis (inflammatory programmed cell death) called NETosis (Fuchs *et al.,* 2007). Since its discovery, NETosis was studied thoroughly solely as an immune defence mechanism and also for its implication in many inflammatory and noninflammatory diseases. NETs were described as a source of citrullinated autoantigens (mainly histones, which are citrullinated by peptidyl arginine deiminase IV (PADI4) as described in detail below) in SLE (Villanueva *et al.,* 2011; Pruchniak *et al.,* 2019) or in RA (Pratesi *et al.,* 2014). Exaggerated NETosis was associated with cancer progression (Cools-Lartigue *et al.,* 2013, Yang *et al.,* 2020), thrombus formation in cardiovascular diseases (Fuchs *et al.,* 2010, Franck *et al.,* 2018), or even pregnancy complications (e.g. preeclampsia) (Hu *et al*., 2018).

Due to immediate neutrophil death after the release of NETs, the process was termed suicidal NETosis. In 2010, Pilsczek and his team (2010) discovered a novel mechanism of NETs expulsion, during which neutrophils survive and are capable of further phagocytosis. In response to *Staphylococcus aureus*, neutrophil nucleus underwent budding of vesicles containing DNA, which were transferred to the plasmatic membrane and finally ruptured in the extracellular space, creating web-like structures typical for NETs. Using transmission electron microscopy, they also demonstrated that vesicles containing DNA mix with the content of neutrophil granules in the extracellular space (Pilsczek *et al.,* 2010). This process was later termed vital NETosis (Yipp and Kubes, 2013).

NETs are released following neutrophil stimulation with diverse pathogens and chemical compounds. The most potent inducer of *in vitro* NETosis is PMA (a chemical compound that directly activates the protein kinase C – NF-κB pathway). Other commonly used NETosis inducers include LPS, IL-8, living or dead bacteria (e.g. *E. coli* or *S. aureus*), activated platelets, ionomycin, or glucose. The activity of NADPH oxidase is essential in the process of NETosis since ROS play a crucial role in the formation of NETs (Fuchs *et al.,* 2007). Critical proteins involved in the process of NETosis are PADI4 and neutrophil elastase (ELANE). PADI4 is responsible for citrullination (conversion of the amino acid residue arginine into citrulline) of histones and subsequent chromatin decondensation (Wang *et al.,* 2009; Thiam *et al.,* 2020). Histone cleavage is also done by protease ELANE, which drives further chromatin disengagement and extracellular DNA expulsion (Papayannopoulos *et al.,* 2010).

2.3.5.1. Neutrophil extracellular traps in pregnancy and neonatal immune system

During pregnancy, tight immunoregulation at the fetal-maternal interface is essential and thus also NETosis has to be regulated, and possibly suppressed to prevent fetal damage or maternal pregnancy complications.

Giaglis and his team (2016) showed evidence for hormonal regulation of NETosis during pregnancy. G-CSF, along with the hormone estradiol (E2), promotes the formation of NETs later in pregnancy (third trimester) while progesterone (P4) acts as an antagonist and inhibits NETs release by restraining the translocation of NE to the nucleus (Giaglis *et al.,* 2016). Described mechanism of hormonal regulation of NETosis would correlate with P4 supplementation in the prevention of preterm birth. Evidence that increased presence of NETs was associated with recurrent pregnancy loss and other pregnancy complications, such as preeclampsia (Gupta *et al.,* 2005; Giaglis *et al.,* 2016), suggests that regulation of NETosis is essential for the proper course of pregnancy.

In 2009, it was observed that PMN isolated from CB (of both preterm and term infants) failed to release NETs (Yost *et al.*, 2009). Following this observation, Yost and his team (2016) discovered a factor inhibiting the formation of NETs in CB, which they termed neonatal NET-inhibitory factor (nNIF). Besides nNIF, they also identified other neonatal inhibitory factors, termed nNIF-related peptides (NRP). Both nNIF and NRP were potent inhibitors of NETosis both in *in vitro* (isolated adult and neonatal neutrophils) and *in vivo* (mouse model) studies (Yost *et al.*, 2016).

3. Materials and methods

3.1. Material

3.1.1. Biological material

Approximately 20-30 ml of umbilical CB was withdrawn from the umbilical vein with a sterile needle into a flask containing sodium heparin (10 U/ml). PB of mothers was withdrawn during the standard pre-labour examination and collected into K_2EDTA, or sodium heparin treated 2-5 ml tubes. CB and PB were stored in a dark box at room temperature until pick-up. Blood was processed immediately. Peripheral blood of healthy nonpregnant age-matched woman volunteers was withdrawn into sodium heparin, K_2EDTA, or sodium citrate treated 2-5 ml tubes and processed immediately. All samples were collected after signed written informed consent from the volunteers participating in the study. The study was approved by the Ethical Committee at the Institute for the Care of Mother and Child in Podolí, Prague.

3.1.2. Reagents and chemicals

Antimycin A: Agilent, USA

BD FACS^{TM} Lysing Solution: BD Biosciences, USA

CFSE: Sigma-Aldrich, USA

Corning® Cell-tak^{TM} cell and tissue adhesive: Baria, CZ

EDTA: Sigma-Aldrich, USA

Ethanol (96%): Dr. Kulich Pharma, CZ

FBS: Cambrex, USA

Ficoll-Paque^{TM} PLUS: GE Healthcare, Sweden

Gentamicin: Pharmaceuticals dd, Slovenia

Glucose: Agilent, USA

Heparin: Zentiva, Netherlands

HEPES: Sigma-Aldrich, USA

KCl: Penta, CZ

KHCO$_3$: Penta, CZ

KH$_2$PO$_4$: Penta, CZ

L-glutamine: Agilent, USA

LPS: Sigma-Aldrich, USA

Lysis Buffer: QIAGEN, Germany

2-Mercaptoethanol: Sigma-Aldrich, USA

NH$_4$Cl: Penta, CZ

NaCl: Penta, CZ

Na$_2$EDTA: Penta, CZ

Na$_2$HPO$_4$: Penta, CZ

Pyruvate: Agilent, USA

RMPI 1640: Sigma-Aldrich, USA

Rotenone: Agilent, USA

Türk′s solution: Vakos, CZ

XF RMPI Assay Media: Agilent, USA

3.1.3. Solutions and buffers

1X Phosphate-buffered saline (PBS), pH 7.4:

Distilled water containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4.

EasySep recommended medium:

PBS containing 1 mM EDTA free of Ca^{2+} and Mg^{2+} (for the Direct Human Neutrophil Isolation Kit).

PBS containing 2% FBS and 1 mM EDTA free of Ca^{2+} and Mg^{2+} (for Human CD15 Positive Selection Kit).

Cell culture medium for cocultivations:

RMPI-1640 Medium containing 10% FBS, 10 mg/ml of Gentamycin, 2 mM HEPES.

Ammonium-Chloride-Potassium (ACK) Lysing Buffer, pH 7.4:

Distilled water containing 150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA.

XF RPMI Assay Medium, pH 7.4:

RMPI Medium containing 2mM L-glutamine, 1mM pyruvate and 10mM glucose.

3.1.4. Kits and Sets

BD^{TM} Cytometry Setup and Tracking Beads (BD Biosciences, USA, cat. number 642412)

BD Trucount^{TM} Absolute Counting Tubes (BD Biosciences, USA, cat. number 340334)

EasySep^{TM} Direct Human Neutrophil Isolation Kit (STEMCELL, Canada, cat. number 19666)

EasySep^{TM} Human CD15 Positive Selection Kit (STEMCELL, Canada, cat. number 18651)

FagoFlowExKit (EXBIO, CZ, cat. number ED7042)

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA, cat. number 4368814)

Luna$^®$ Universal qPCR Master Mix (New England BioLabs, UK, cat. number M3003)

MACS CD4$^+$ T Cell Isolation Kit (Miltenyi Biotec, USA, cat. number 130-096-533)

RNeasy$^®$ Mini Kit (QIAGEN, Germany, cat. number 74106)

SeaHorse XFe96 FluxPak (Agilent, USA, cat. number 102416-100)

3.1.5. Antibodies and probes

Table 2. Antibodies used for flow cytometry analysis

Antibody	Conjugate	Clone	Cat. number	Manufacturer
CD3	FITC	SK7	342416	BD Biosciences, USA
CD3	PerCP	MEM-57	PC-202-T100	EXBIO, CZ
CD4	APC	MEM-241	1A-359-T100	EXBIO, CZ
CD8	FITC	SK1	345772	BD Biosciences, USA
CD14	PerCP-Cy5.5	61D3	45-0149-42	Invitrogen, USA
CD15	FITC	HI98	301904	BioLegend, USA
CD15	PE-Cy7	HI98	560827	BD Biosciences, USA
CD16	APC	CB16	17-0168-42	Invitrogen, USA
CD45	PerCP	2D1	342416	BD Biosciences, USA
CD62L	PE	DREG-56	304806	BioLegend, USA
CD64	PE-Cy7	10.6	305022	BioLegend, USA

Table 3. Probes used for gene expression analysis

Gene symbol	Assay ID	Cat. number	Manufacturer
ACTB	Hs99999903_m1	4331182	Applied Biosystems USA
ARG1	Hs00163660_m1	4331182	Applied Biosystems USA
CD274	Hs00204257_m1	4331182	Applied Biosystems USA
DEFA3D	Hs00414018_m1	4331182	Applied Biosystems USA
DEFA4	Hs00157252_m1	4331182	Applied Biosystems USA
ELANE	Hs00357734_m1	4331182	Applied Biosystems USA
IDO1	Hs00984148_m1	4331182	Applied Biosystems USA
IL10	Hs00174086_m1	4331182	Applied Biosystems USA
LTF	Hs00914334_m1	4331182	Applied Biosystems USA
MMP9	Hs00957562_m1	4331182	Applied Biosystems USA
MPO	Hs00165162_m1	4331182	Applied Biosystems USA
PDCD1	Hs00228839_m1	4331182	Applied Biosystems USA
TNF	Hs00174128_m1	4331182	Applied Biosystems USA

3.1.6. Plastic

CELLSTAR® 48 well Cultivation Plate: Greiner Bio-One, Austria

Centrifuge Tubes 15 ml: Jet Biofil, China

Centrifuge Tubes 50 ml: Jet Biofil, China

FALCON Tubes 5 ml BD Pharmigen™: BioSciences, USA

JET BIOFIL® Serological Pipet 10 ml: Jet Biofil, China

Microcentrifuge Tubes 2 ml: SSIbio, USA

PCR 96 and 384 well Reaction Plates: Applied Biosystems, USA

3.1.7. Instruments and software

Centrifuges: Micro 22 R, Universal 30 RF, Universal 320R (Hettich, Germany)

Incubators: ESCO CellCulture Incubator CO_2 (ESCO, Singapore)

Memmert Incubator IF30 Plus (Memmert, Germany)

Cyclers: PCR Thermal Cycler 7300 Real-Time PCR System (Applied Biosystems, Singapore)

Reverse Transcription Thermal Cycler Peltier Cycler 200 (MJ Research, Singapore)

LightCycler® 480 II Real-Time PCR System (Roche Molecular Systems, USA)

Flow Cytometer: BD FACS Canto™ II Flow Cytometer (BD Biosciences, USA)

Microscope: Olympus BX41 (Olympus, USA)

Magnets: EasySep™ Magnet (STEMCELL, Canada)

MidiMACS™ Separator (Miltenyi Biotec, USA)

Nanodrop: NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA)

SeaHorse: SeaHorse XFe96 Analyzer (Agilent, USA)

Software: BD FACS Diva Software v6.1.2 (BD Biosciences, USA)

FlowJo 7.6.5 (FlowJo LLC, USA)

GraphPad Prism 8 (GraphPad Software, USA)

Vortex: Vortex-Genie 2 (Scientific Industries, CZ)

3.2. Methods

3.2.1. Cell isolation

3.2.1.1. Whole neutrophil population isolation

Blood was collected as described above (*3.1.1. Biological material*). EasySep^{TM} Direct Human Neutrophil Isolation Kit was used for the isolation of a whole neutrophil population according to the manufacturer's instructions with *slight modifications*. 2 ml of whole blood was transferred into 5 ml FALCON Tubes. 100 µl of Isolation Cocktail, *100 µl of 0.5 M EDTA*, and 100 µl of RapidSpheres^{TM} (vortexed for 30s) was added. Blood was mixed briefly with a pipette and incubated at room temperature (RT) for *6* min. The recommended medium was added up to total volume of 4 ml and mixed gently by pippeting up and down 3 times. The tube was placed into EasySep^{TM} Magnet and incubated at RT for 5 min (without cap). The magnet with the tube was picked up and in one continuous motion inverted, pouring the enriched cell suspension into a new 5 ml FALCON tube. 100 µl of RapidSpheres^{TM} was added to the enriched suspension, mixed and incubated at RT for *6* min. The tube was placed into EasySep^{TM} Magnet and incubated at RT for 5 min (without cap). The magnet with the tube was picked up and in one continuous motion inverted, pouring the enriched cell suspension into a new 5 ml FALCON tube. For third and final separation, the tube was placed into EasySep^{TM} Magnet and incubated at RT for 5 min (without cap). The magnet with tube was picked up and in one continuous motion inverted, pouring the isolated cell suspension into a new 5 ml FALCON tube. Isolated cells were centrifuged for 10 min, 300g, 20°C, resuspended in 1 ml of PBS, counted in a Bürker chamber, and used for subsequent analysis. Neutrophil viability was determined using Trypan blue staining solution at 0.5%, and purity of isolated cells was >96% as determined by flow cytometry.

3.2.1.2. Density-gradient centrifugation

5 ml of heparinised umbilical CB was diluted 1:1 with PBS containing 100 µl of 0.5 M EDTA. 3 ml of Ficoll-Paque^{TM} PLUS (density 1.077 g/l) was placed into 15 ml centrifugation tube. Carefully, 10 ml of diluted blood was layered over Ficoll, without disturbing the interface. Tubes were centrifuged for 25 min at 400g, 20°C, with slow acceleration and no brakes. The ring of CBMC was transferred using Pasteur pipette into 15 ml tubes and filled up to 15 ml PBS. Cells were centrifuged for 10 min at 600 g, 20°C.

Cells were washed once with PBS. 2 ml of ACK lysing solution was added to the cells. Cells were lysed for 5 min with gentle mixing every minute. The tube was toped up with PBS, and cells were centrifuged for 10 min, 300 g, 20°C. Cells were counted in a Bürker chamber and prepared for further use.

3.2.1.3. Isolation of low- and high-density neutrophils

Low-density neutrophils (LDN) were isolated using EasySep^{TM} Human CD15 Positive Selection Kit from CBMC according to the manufacturer's instructions. High-density neutrophils (HDN) were isolated from the erythrocyte fraction left at the bottom after density gradient centrifugation. Pelleted cells were diluted 1:1 with PBS prior to separation. Neutrophils were isolated using the method described above (*3.2.1.1. Whole neutrophil population isolation using negative separation*).

3.2.1.4. Isolation of CD4+ cells and CFSE staining

CBMC were isolated using density-gradient centrifugation as described above (*3.2.1.2 Density-gradient centrifugation*). CD4^{+} cells were isolated using Magnetic Activated Cell Sorting (MACS) CD4^{+} T Cell Isolation Kit according to manufacturer's instructions. Isolated cells were centrifuged for 10 min, 300g, 20°C, resuspended in 1 ml of PBS, and counted in a Bürker chamber. The cell pellet was resuspended in PBS (1 ml of PBS per $1^{*}10^{6}$ of cells) in a 50 ml tube. CFSE diluted to 5 µM working concentration was added to cells (10 µl per $1^{*}10^{6}$ of cells). Cells were stained for 15 min at 37°C in an incubator (5.7% CO_2). Subsequently, 5x volume of cold PBS with 10% FBS was added to the sample, incubated on ice for 5 min, and centrifuged for 10 min, 300g, 4°C. Stained cells were washed twice with cold PBS and used in the proliferation assay (*3.2.3.4. Cocultivation assay*).

3.2.2.   Reverse   Transcription   and   Quantitative Real-Time PCR

RNA from separated neutrophils (whole neutrophil population and subpopulations - HDN, LDN; respective isolation protocols are stated above in *3.2.1.1.* and *3.2.1.3*) was isolated using RNeasy$^{®}$ Mini Kit according to the manufacturer's instructions with the addition of RNase-Free DNase I incubation step (to prevent genomic DNA contamination). Isolated RNA was stored at -80°C until further analysis. The concentration of yielded RNA

was measured spectrophotometrically using Nanodrop. RNA was calculated to 0.5 μg per one PCR reaction. RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (exact measurements are listed in **Table 4**) according to manufacturer's instructions using Thermal Cycler 200. Quantification of mRNA was done using Luna Universal qPCR Master Mix (exact measurements are listed in **Table 4**) and performed on 7300 Real-Time PCR Systems or LightCycler$^®$ 480 II Real-Time PCR System. TaqMan probes used in the thesis are listed in **Table 3**.

Table 4. Individual components of the RT-PCR and qPCR reactions

RT-PCR		qPCR	
Component	*Volume (μl)*	*Component*	*Volume (μl)*
RT buffer	2	Master-mix	5
DNT mix	0.8	Probe	0.5
Random primers	2	Rnase free H_2O	2.5
Reverse Transcriptase	1	cDNA	2
RNase inhibitor	1		
RNA (5μg) + RNase free H_2O	13.2		

3.2.3. Flow cytometry

Antibodies used for individual flow cytometry experiments performed in this thesis are listed in **Table 2**. Data were acquired using BD FACSCanto II Flow Cytometer and BD FACS Diva Software v6.1.2. CST Setup Beads were used for the Cytometry Setup. Final analysis was done using FlowJo 7.6.5 Software.

3.2.3.1. Neutrophil phenotype analysis – whole blood staining

50 μl of whole blood (CB, PB, or HC) was added into a 5 ml Falcon tube. 1 μl of every monoclonal antibody (anti-CD14-PerCP, anti-CD15-FITC, anti-CD16-APC, anti-CD62L-PE, anti-CD64-PE-Cy7 – exact clones are listed in **Table 2**) was added into the tube and incubated at RT for 15 min in the dark. Subsequently, 2 ml of BD Lysing Solution (diluted 1:10 with PBS) was added into the tube and incubated in RT for 10 min (for PB) in the dark to lyse red blood cells. After lysis, cells were centrifuged for 5 min, 300g, 20°C and washed twice with PBS. Cells were kept on ice and promptly analysed by flow cytometry.

3.2.3.2. Absolute cell number determination

The absolute number of cells was assessed using BD Trucount™ Absolute Counting Tubes according to manufacturer instructions. Briefly, 50 µl of whole blood (CB or maternal PB) was added into the Trucount Tube along with 1 µl of anti-CD45 mAb in PerCP, 1 µl of anti-CD3 mAb in FITC and 1 µl of anti-CD15 mAb in PE-Cy7 (exact clones are listed in **Table 2**). The tube was capped; blood was vortexed gently and incubated for 15 min in the dark in RT. Subsequently, 450 µl of BD Lysing Solution (diluted 1:10 with PBS) was added, and lysis was carried out for 15 min in the dark in RT. The sample was analysed immediately to prevent further lysis. To assess the percentages of CD4$^+$ and CD8$^+$ T cells, 50 µl of whole blood was stained with 5 µl of CD3, 1 µl of CD4 and 5 µl of CD8 monoclonal antibodies (exact clones and conjugated fluorochromes are listed in **Table 2**) and staining was carried out the same way as described in *3.2.3.1 (Neutrophil phenotype analysis – whole blood staining)*.

3.2.3.3. Phagocytic assay

The phagocytic capacity of neutrophils was determined using FagoFlowExKit with slight *modifications*. Briefly, 50 µl of whole blood was added into three individual 5 ml Falcon tubes. *1 µl of anti-CD15 mAb conjugated to PE-Cy7 was added to each sample*. Subsequently, 10 µl of PMA diluted solution (1 vial containing 40 µg/ml of PMA is intended for 25 tests) was added (stimulation control), 10 µl of *E. coli* (1 vial containing 0,775-0,862*10^9 bacteria is intended for 25 tests) was added, and nothing else was added into the third tube (negative control). Samples were gently mixed. 10 µl of diluted solution of dihydrorhodamine (DHR) 123 was added to each sample and gently mixed. Samples were incubated for 30 min in the thermostat (37°C, 5.7 CO$_2$). After incubation, cells were lysed with 50 µl of undiluted lysis solution provided in the kit for 5 min and with 1 ml of ddH$_2$O for 8 min. The prepared samples were kept on ice, and fluorescence of rhodamine 123 was measured immediately using Flow Cytometer. Acquired data were analysed using FlowJo 7.1.1.

3.2.3.4. Cocultivation assay

CD4$^+$ T cells were isolated and stained with CFSE as described above (*3.2.1.4 Isolation of CD4$^+$ cells and CFSE staining*). Neutrophils were isolated as described above (*3.2.1.1 Whole neutrophil population isolation using negative separation*) and added in a 1:1 ratio (precisely 250 000 : 250 000 cells in one well with 0,5 ml of medium) to CFSE

stained CD4$^+$ T cells from the same umbilical CB sample and stimulated with LPS. As a control, only fresh medium was added to the CFSE-stained CD4$^+$ cells. The layout of the experiment is depicted bellow (**Figure 3**). Cells were incubated for 72 h in the thermostat (set to 37°C, 5.7% CO_2). Afterward, cells were harvested from the wells, centrifuged for 10 min, 300g, 20°C. The supernatant was aspired and stored at -80°C. Cells were stained with anti-CD4-APC antibody (exact clone listed in **Table 2**). CFSE fluorescence intensity was assessed by flow cytometry to determine the proliferation capacity of CD4$^+$ T cells.
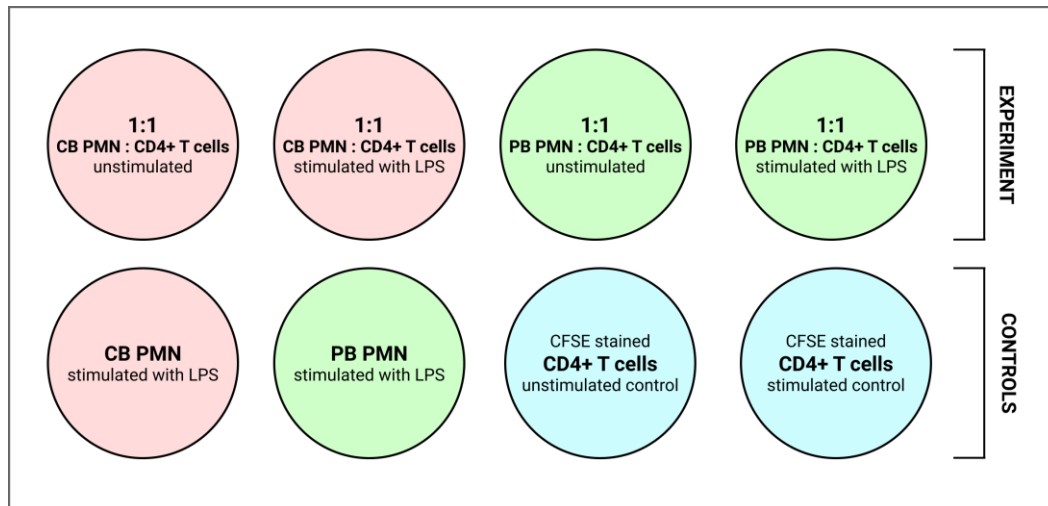

Figure 3. Layout of the cocultivation experiment

*CD4$^+$ T cells were isolated from CBMC fraction and stained with CSFE. Neutrophils were isolated from whole blood. Cells were seeded in a 1:1 ratio in the presence or absence of LPS as a stimulant for neutrophils. CD4$^+$ T cell proliferation was induced by the addition of anti-CD3/anti-CD28 monoclonal antibodies. **Abbreviations:** CB - cord blood, LPS - lipopolysaccharide, PB - peripheral blood, PMN - polymorphonuclear neutrophils*

3.2.4.   SeaHorse neutrophil metabolism analysis

To determine glycolysis rate (extracellular acidification rate, ECAR) and oxidative burst (oxygen consumption rate, OCR) during neutrophil activation with LPS Agilent SeaHorse Extracellular Flux Analysis was utilised, and the assay was run according to the Neutrophil Activation Application Note from Agilent. Whole neutrophil population was isolated as described in *3.2.1.1* (*Whole neutrophil population isolation using negative separation*) as gently as possible to avoid any unwanted activation of the cells. Before centrifugation, 2 ml of XF RPMI Assay Medium was added, and cells were centrifuged for 10 min, 300g, 20°C. Afterward, supernatant was discarded, cells were gently resuspended in 1 ml of XF RPMI Assay Medium and counted in Bürker chamber, and cells density was adjusted to $1*10^6$ cells/1 ml of XF RPMI Assay Medium. Cells were seeded in XFe96 well

culture microplate (previously coated with Cell-Tak^{TM} adhesive) at $8*10^4$ cells per well (80 µl of prepared cell suspension). Even distribution of cells in the wells was checked in a light microscope. Cells were attached to the bottom of the plate by centrifugation for 1 min, 40 g (with no brake to prevent disturbing cell attachment) at 20°C. Cells adhesion was checked in the light microscope, and subsequently, 100 µl of XF RPMI Assay Medium was added into each well (to achieve a total volume of 180 µl). Cells were checked one more time to see if the addition of medium did not disturb the cell adhesion. Plated cells were incubated for 45 min at 37°C without CO_2. During the incubation, calibration of detection cartridge (where the ports were previously loaded with the mitochondrial inhibitors rotenone and antimycin A (port A) and stimulant LPS (port B)) was performed for optimal measurement. After incubation plate was placed in the SeaHorse machine and protocol was executed. The assay was run for 36 sequent measurement cycles (3 cycles to determine the baseline, 3 cycles after the addition of inhibitors, and 30 cycles after the addition of LPS) as described in the Neutrophil Activation Application Note. Experimental workflow is depicted in **Figure 4**.
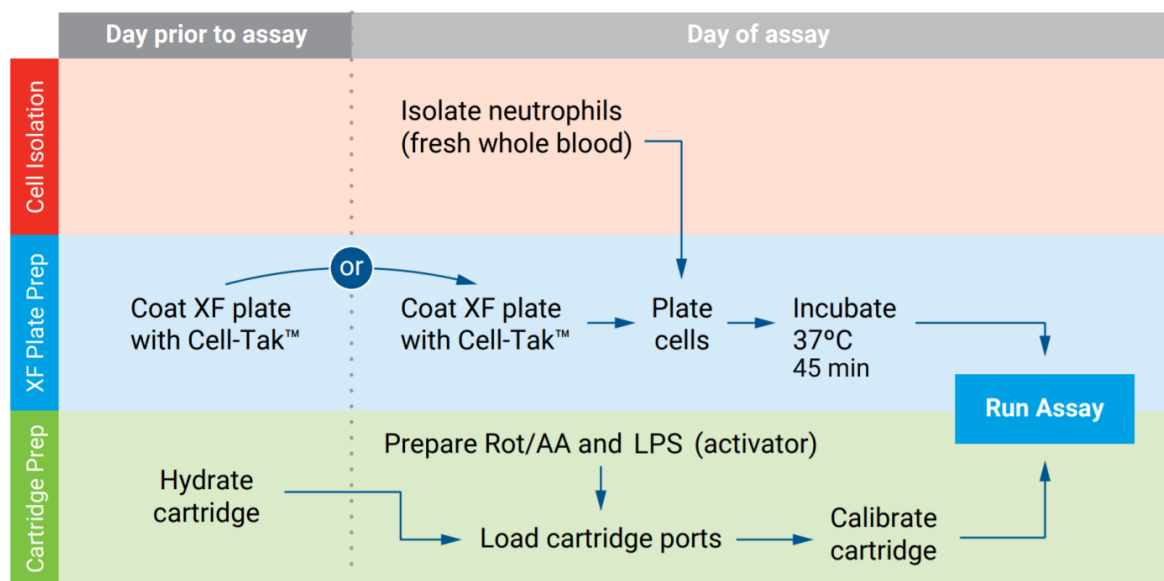

Figure 4. SeaHorse neutrophil activation analysis

Neutrophil metabolic response to LPS stimulation was measured using the Agilent SeaHorse XF Technology. The experimental workflow is depicted. Neutrophils were isolated from CB, maternal PB or PB of healthy volunteers. Plates were covered with Cell-Tak^{TM} adhesive, and cells were seeded at $8*10^4$ cells per well. Rot/AA were used to inhibit mitochondrial respiration and LPS was used as stimulants. Modified from Neutrophil Activation Application Note (Agilent). **Abbreviations:** *AA – antimycin A, CB - cord blood, LPS – lipopolysaccharide, PB - peripheral blood, Rot - rotenone*

3.2.5.   Statistical analysis

Flow cytometry results were standardized (CST beads setting), and analysed using FlowJo software using suitable compensation controls (single staining, compensation beads, isotype control), and fluorescence minus one (FMO) controls to set proper gating strategy. Transcript expression of the target gene was quantified relative to β-actin (*ACTB*) mRNA levels using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (Livak and Schmittgen, 2001). For flow cytometry analysis and qPCR analysis, parametric statistical tests (unpaired t-test, ANOVA for multiparameter comparison) were utilised. Acquired data were statistically evaluated and graphically processed using GraphPad Prism 8 software. Summary of P values will be indicated by asterisks as shown in **Table 5**.

Table 5. Summary of P values and their indications used in the thesis

Asterisk	P value	Result
ns	≥ 0.05	Not significant
*	$0.01 - 0.05$	Significant
**	$0.001 - 0.01$	Very significant
***	< 0.001	Extremely significant

4. Aims of the thesis

- To characterize the phenotype of neutrophils in umbilical cord blood and peripheral blood of mothers and healthy nonpregnant woman volunteers
- Compare functional properties (suppressive function, metabolic activity, and phagocytic potential) of neutrophils in umbilical cord and peripheral blood
- Quantify the expression of genes associated with antimicrobial response and suppressive functions in the whole neutrophil population as well as distinct neutrophil subpopulations in umbilical cord blood

5. Results

5.1. Phenotype analysis of neutrophils in umbilical cord and maternal peripheral blood

Neutrophil phenotype in umbilical CB, maternal PB and, PB of healthy nonpregnant woman volunteers (referred to as HC in the figures) was assessed based on the presence of cell surface markers using flow cytometry approach. Markers used were described previously in neutrophil subpopulation characterization (Ssemaganda *et al.*, 2014, Pillay *et al.*, 2012), and their function is described in **Table 1** in Chapter *2.3.* (*Neutrophils*). The representative gating strategy is depicted in **Supplementary Figure 1**. Granulocytes were identified based on forward scatter (FSC), and side scatter (SSC), and neutrophils were gated as $CD14^-CD15^+$ cells (**Figure 5A**). $CD14^+CD15^+$ cells were considered monocytes and did not significantly differ among studied groups (**Figure 5B**). An interesting population expressing lower amounts of CD15 was found in higher percentages in CB (**Figure 5C**). $CD14^-CD15+$ neutrophils were further gated based on the expression of CD16 and CD64 (**Figure 5D**) or CD62L (**Figure 5E**) to identify the activation and maturity status.

The same analysis was applied to cells from the mononuclear fraction after density gradient centrifugation. Granulocytes from CB and maternal PB mononuclear cell (CBMC and PBMC respectively) compartment were compared (**Figure 6**).
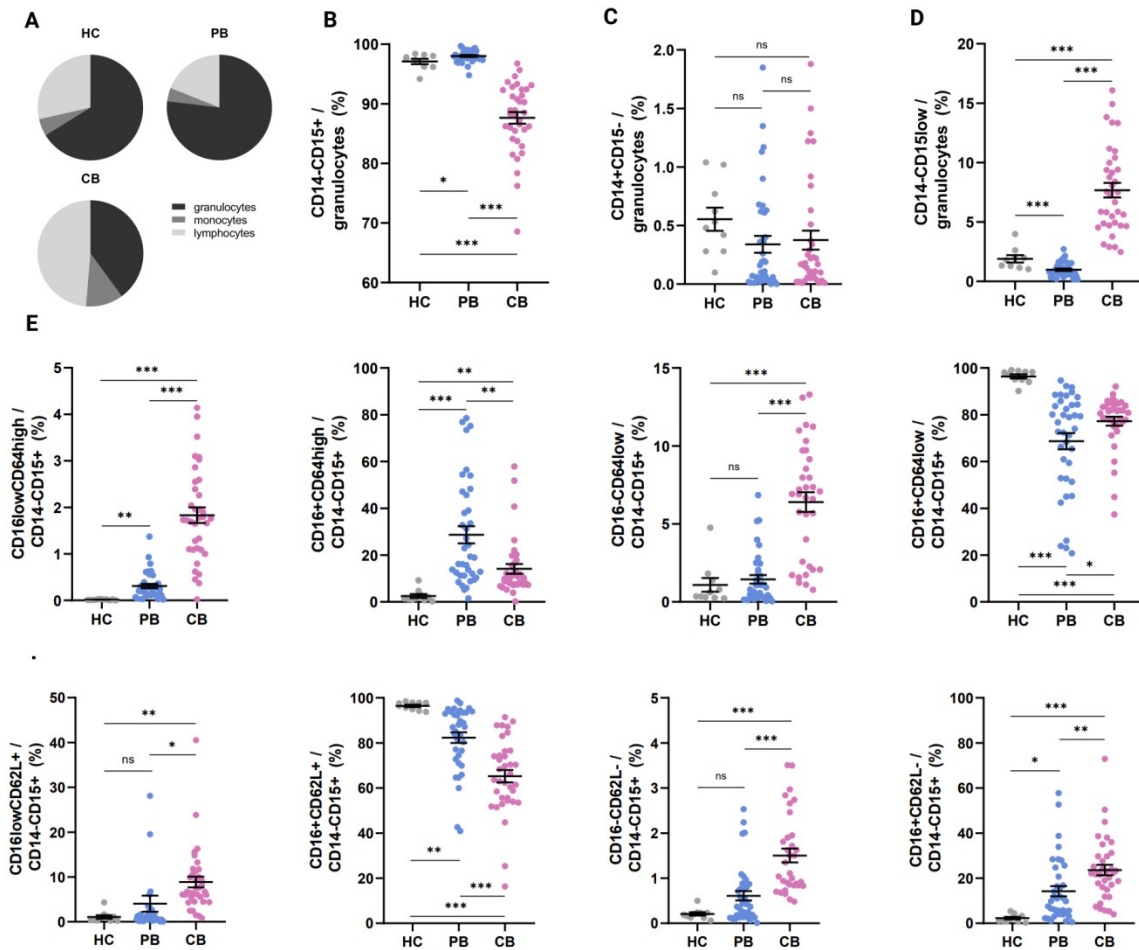
Figure 5. Phenotype analysis of neutrophils from whole umbilical cord blood and peripheral blood

*Granulocytes represented around 70-80% of cells in peripheral blood of healthy nonpregnant women (HC) and mothers (PB) and around 40% of cells in cord blood (CB) (**A**). Phenotype analysis was performed using flow cytometry based on markers CD14, CD15, CD16, CD62L, and CD64. Granulocytes were gated based on FSC, and SSC, and neutrophils were identified as CD14$^-$CD15$^+$ cells (**B**). Monocytes were considered CD14$^+$CD15$^-$ cells (**C**). A distinct population was identified in CB expressing lower amounts of CD15 (**D**). CD14$^-$CD15$^+$ neutrophils were further gated based on the expression of CD16/CD64 (**E**) and CD16/CD62L (**F**). For all analyses n=10 for HC, n=37 for PB, n=35 for CB. **Abbreviations:** CB – cord blood, FSC – forward scatter, HC – healthy control peripheral blood, PB – maternal peripheral blood, SSC – side scatter*
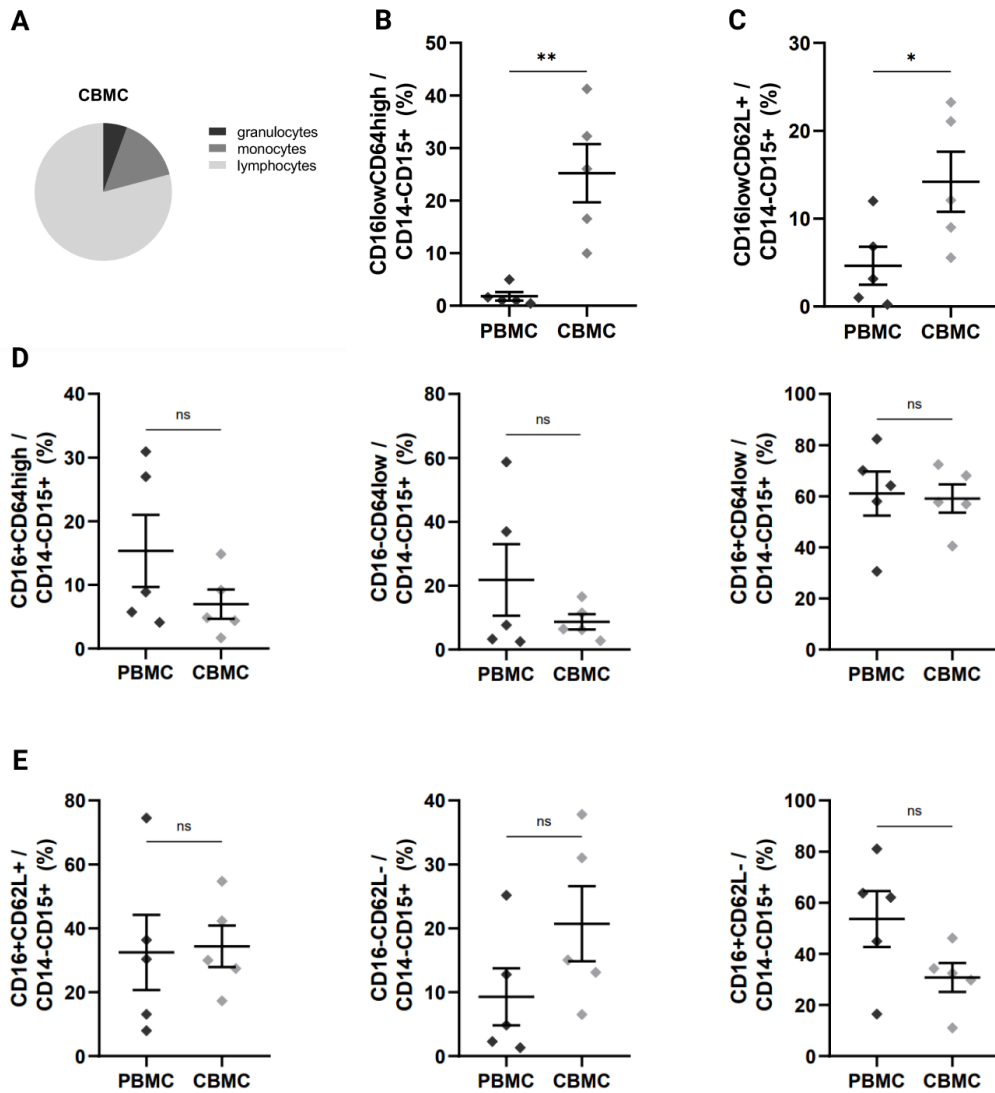
Figure 6. Phenotype analysis of neutrophils from umbilical cord and peripheral blood mononuclear cells

*Phenotypic analysis was performed using flow cytometry based on markers CD14, CD15, CD16, CD62L, and CD64. Granulocytes were gated based on FSC, and SSC, and neutrophils were identified as CD14⁻CD15⁺ cells. Granulocytes represented around 2% of the CBMC compartment. (**A**). CD14⁻CD15⁺ neutrophils from CBMC were gated based on the expression of CD16/CD64 (**B, D**) and CD16/CD62L (**C, E**). For all analyses n=5 for PBMC, n=5 for CBMC. **Abbreviations:** CBMC – cord blood mononuclear cells, FSC – forward scatter, PB – peripheral blood mononuclear cells, SSC – side scatter*

5.2. *In vitro* cocultivation assay

Based on previous reports of the capability of PMN subpopulations to suppress T cell proliferation, cocultivation assay of isolated neutrophils with CD4$^+$ T cells was performed. Firstly, whole neutrophil population was isolated from both CB and PB and cocultivated with CD4$^+$ T cells separated from CBMC. Following the evidence that activation might lead to enhancement of the LDN compartment and thus the suppressive properties of PMN, cocultivation was performed both in the presence and absence of LPS. CD4$^+$ T cell proliferation was stimulated by the addition of anti-CD3/anti-CD28 mAb. **Figure 7A** shows representative histograms of unstimulated and stimulated CD4$^+$ T cells as well as the proliferation of CD4$^+$ T cells when cocultured with CB and PB PMN, which were left unstimulated or were stimulated by LPS. Neither PMN from CB nor PB were able to suppress CD4$^+$ T cell proliferation (**Figure 7B**).
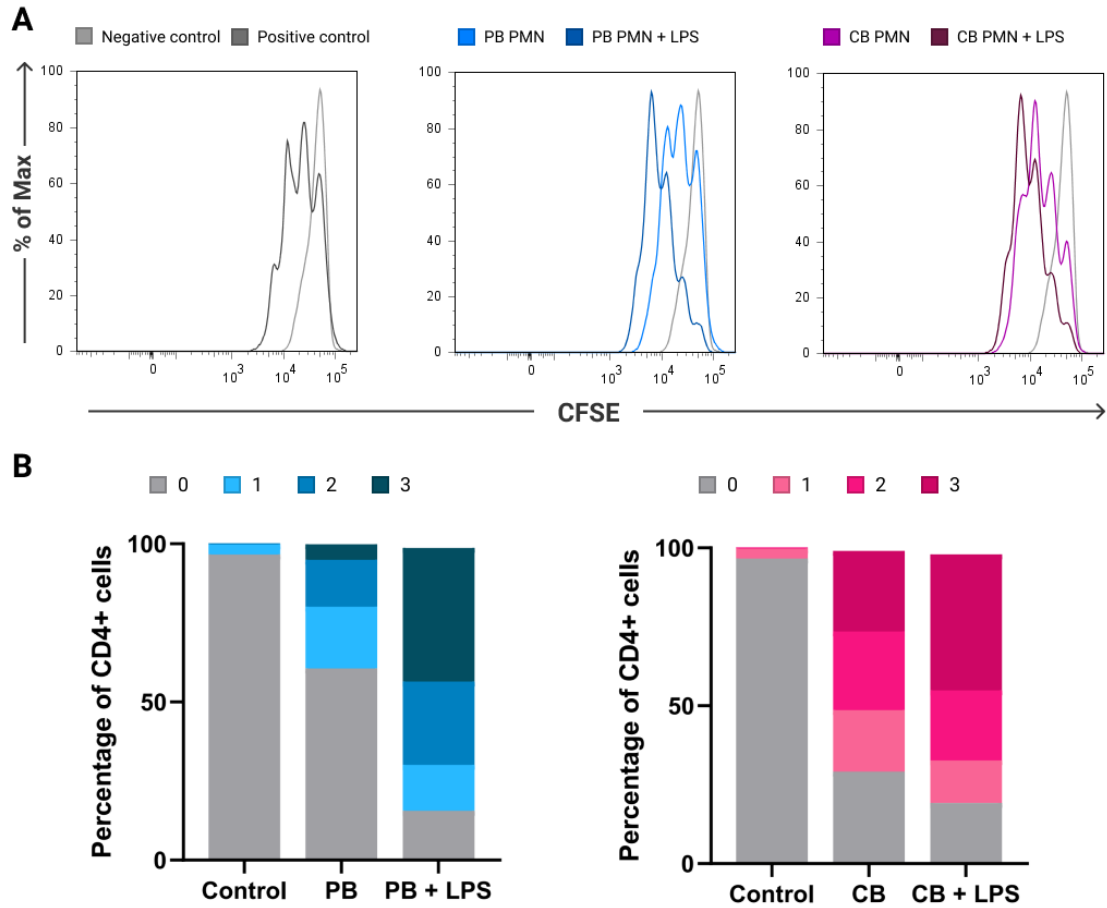
Figure 7. The effect of neutrophils on CD4⁺ T cell proliferation

*Cocultivation assay was performed with CD4⁺ T cells isolated from CBMC stimulated with anti-CD3/anti-CD28 mAbs. CD4⁺ T cells were cocultured in 1:1 ratio with PMN isolated from CB and PB in the presence and absence of LPS. Representative histograms showing the positive control (CD4⁺ T cells stimulated with anti-CD3/anti-CD28 mAbs) and negative control (unstimulated CD4⁺ T cells) and the proliferation of stimulated CD4⁺ T cells after the addition of isolated PMN (left unstimulated or stimulated with LPS) (**A**). Graphs showing the number of CD4+ T cell divisions when cocultured in the presence of isolated PMN (data from 3 experiments) (**B**). **Abbreviations:** CB – cord blood, LPS – lipopolysaccharide, PB – peripheral blood, PMN – polymorphonuclear neutrophils*

5.3. Gene expression analysis in isolated neutrophils and neutrophil subpopulations

Based on observation from the cocultivation experiment (**Figure 7**) and the inability to prove the suppressive capacity of CB neutrophils, our aim was to compare the antimicrobial potential of CB neutrophils in comparison to neutrophils from both maternal and healthy control PB by measuring the expression of genes linked to antimicrobial response. Neutrophils were isolated from CB, maternal PB (marked as PB), and PB of healthy nonpregnant woman volunteers (marked as HC), and gene expression of particular genes was quantified using qPCR. Genes linked to antimicrobial activity were analysed (**Figure 8**). Further analysis focused on the expression of cytokines and genes linked to suppressive properties (**Figure 9**).
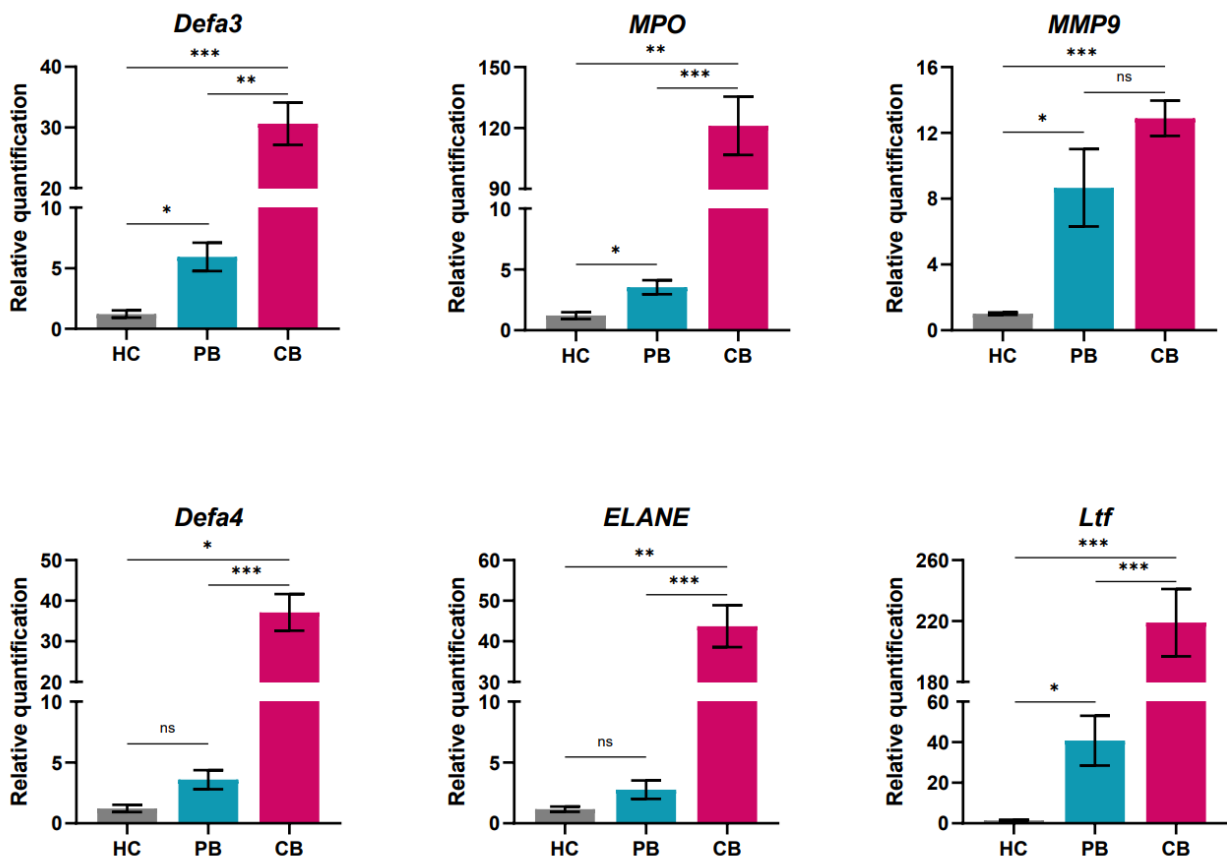

Figure 8. Expression of genes associated with antimicrobial response

*Neutrophils were isolated from CB (n=54), maternal PB (n=16) and PB of healthy nonpregnant woman volunteers (n=6) and the expression of particular genes was measured using qPCR. **Abbreviations:** CB – cord blood, Defa3 – defensin 3, Defa4 – defensin 4, ELANE – neutrophil elastase, HC - healthy control peripheral blood, PB – maternal peripheral blood, MMP9 – matrix metalloproteinase-9, MPO – myeloperoxidase, Ltf – lactoferrin*
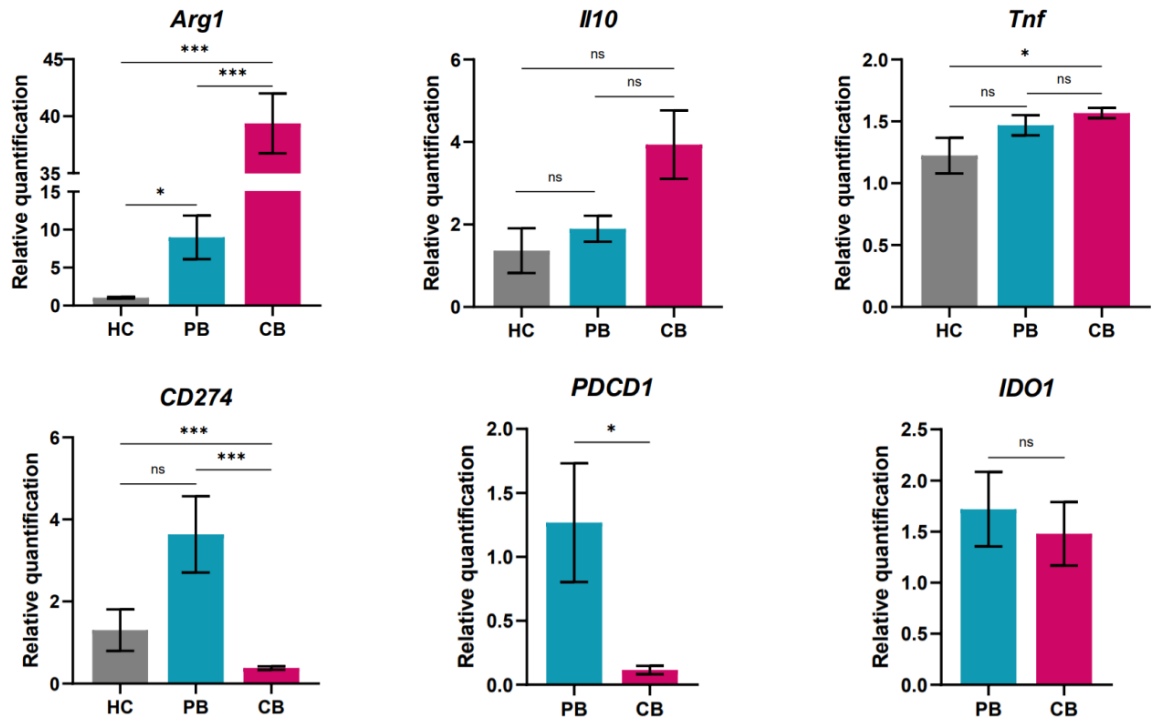
46

Figure 9. Expression of cytokines and genes associated with suppressive properties
Neutrophils were isolated from CB (n=17), maternal PB (n=9) and PB of healthy nonpregnant woman volunteers (n=6) and the expression of particular genes coding for suppressive molecules and cytokines was measured. **Abbreviations:** *Arg1 – arginase 1, CB – cord blood, CD274 – programmed death ligand 1 (PD-L1), HC - healthy control peripheral blood, IDO1 – indoleamine 2,3 dioxygenase, Il10 – interleukin 10, PB – maternal peripheral blood, Tnf – tumor necrosis factor*

To elucidate if the LDN compartment is responsible for the enhancement in gene expression, LDN and HDN were isolated from CB, and the level of gene expression was determined (**Figure 9**). Although the analysis did not reach significance, a certain trend was seen. Previous results (Ssemaganda *et al.*, 2014, Weinhage *et al.*, 2020) implicated that $CD16^{low}CD64^{high}$ neutrophils could represent the LDN compartment, the percentage of $CD16^{low}CD64^{high}$ cells from $CD14^-CD15^+$ cells (**Figure 5D**) was correlated with the expression of particular genes from the whole neutrophil population isolated from CB (**Figure 10C, D**).

Based on previous evidence that the mode of delivery could have an impact on the functional properties of neutrophils in CB, the expression of particular genes of interest was compared based on the mode of delivery (VD vs. CS) (**Figure 11**).
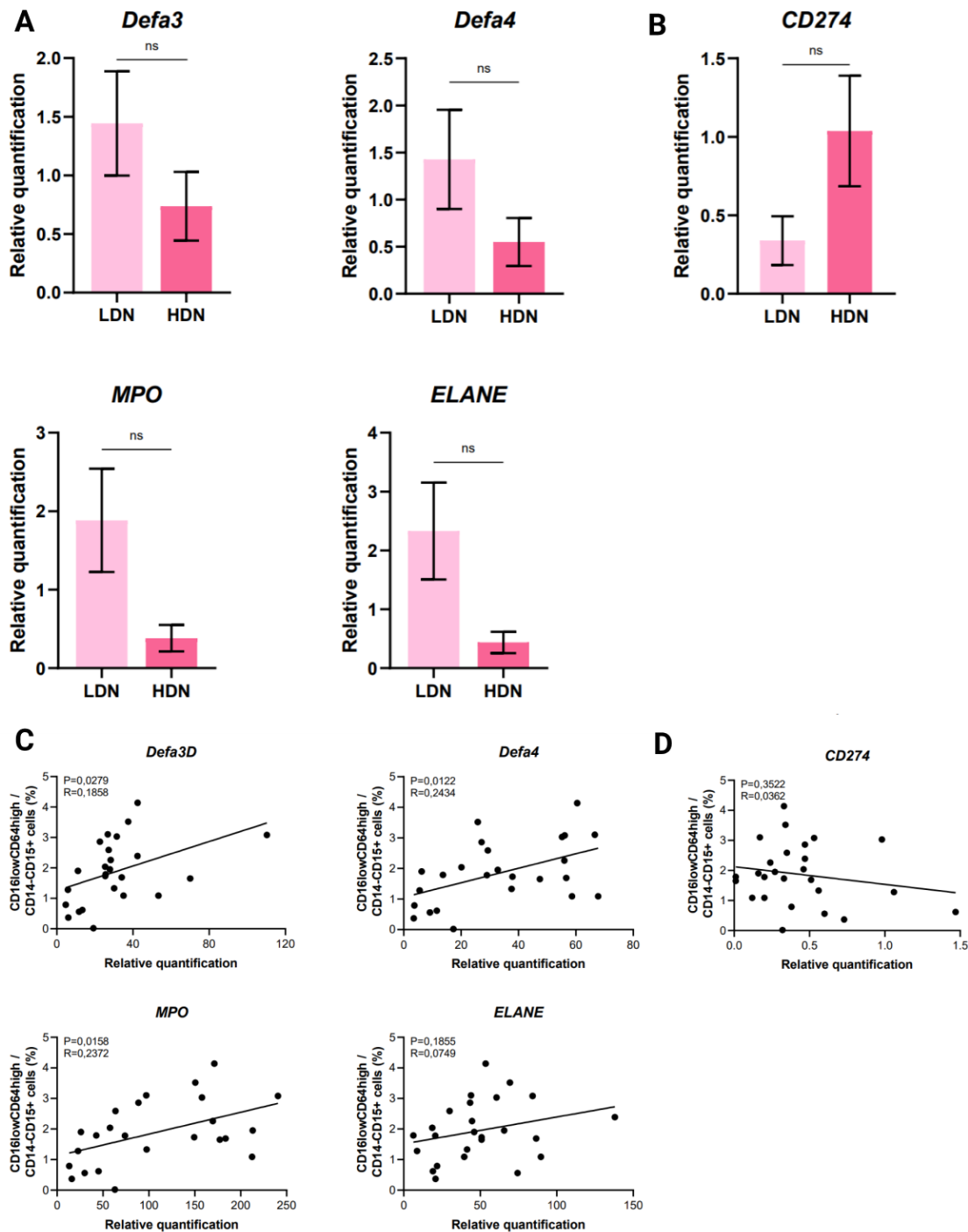
Figure 10. Expression of particular genes in neutrophil subpopulations

*Neutrophil subpopulations (LDN, HDN) were isolated from CB and gene expression of genes linked to antimicrobial response (**A**) and genes linked to suppressive properties (**B**) were measured using qPCR. The relative expression of particular genes in whole neutrophil population isolated from cord blood was correlated with the percentages of $CD14^-CD15^+CD16^{low}CD64^{high}$ cells in cord blood (**C**, **D**). For gene expression analysis n=10 for LDN and n=7 for HDN. For correlations n=25 in all analyses. **Abbreviations:** CB – cord blood, CD274 – programmed death ligand 1 (PD-L1), Defa3 – defensin 3, Defa4 – defensin 4, ELANE – neutrophil elastase, HDN – high-density neutrophils, LDN – low-density neutrophils, MPO – myeloperoxidase*
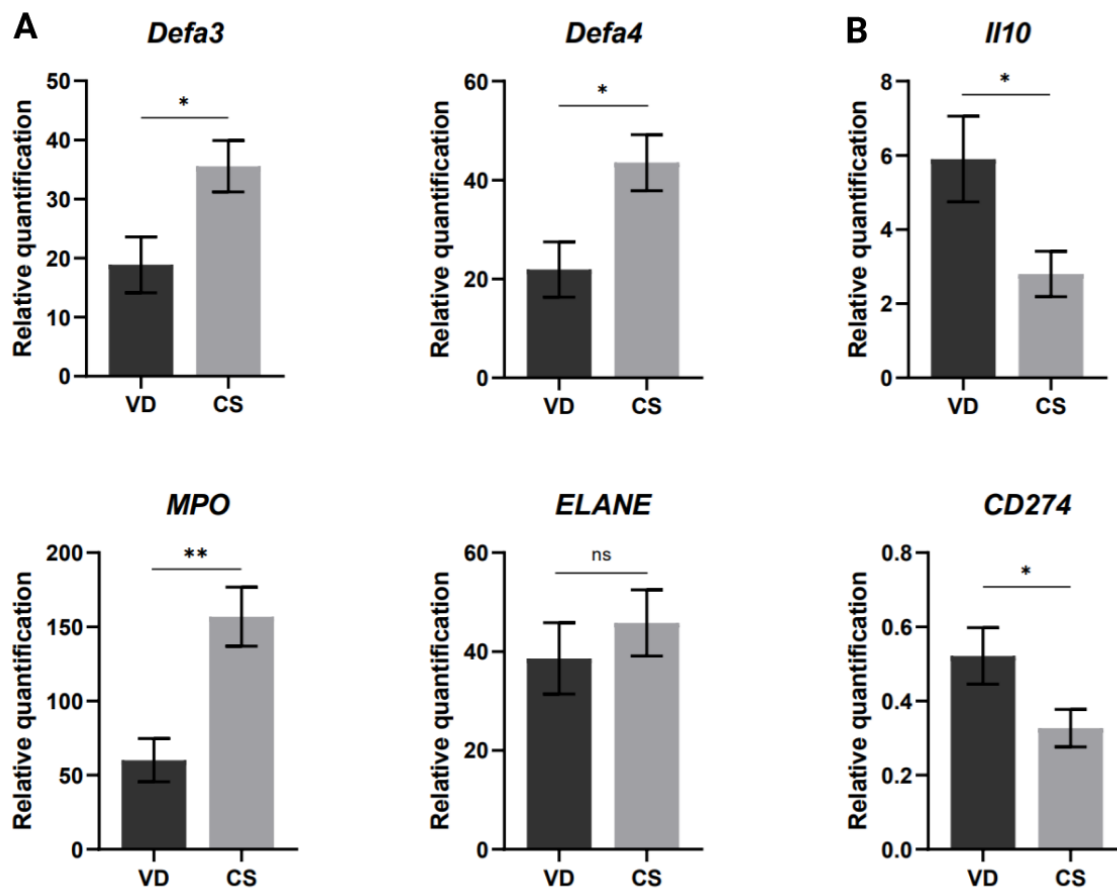
Figure 11. The impact of mode of delivery on cord blood neutrophil gene expression

*Neutrophils were isolated from CB and gene expression of genes linked to antimicrobial response (**A**) and genes linked to suppressive properties (**B**) were measured using qPCR and compared based on the mode of delivery. For all analyses n=16 for VD and n=38 for CS.* **Abbreviations:** *CB – cord blood, CD274 – programmed death ligand 1 (PD-L1), CS – caesarean section, Defa3 – defensin 3, Defa4 – defensin 4, ELANE – neutrophil elastase, Il10 – interleukin 10, MPO – myeloperoxidase, VD – vaginal delivery*

5.4. Activation during phagocytosis

Since we were not able to see any suppressive properties in the whole neutrophil population isolated from both CB and PB but observed the enhancement in antimicrobial gene expression, therefore we focused on antimicrobial response upon neutrophil activation. One of the main functions of neutrophils is phagocytosis of pathogens in the periphery, and their elimination is carried out by the production of ROS and the fusion of phagosome with granules containing potent antimicrobial peptides and enzymes. To carry out the analysis of neutrophil activation during phagocytosis, the PhagoFlowExKit (EXBIO) was used. The method is based on the oxidation of Dihydrorhodamine123 (DHR123) to Rhodamine123 by MPO, following activation of cells either by PMA (positive control) or by phagocytosis of *E. coli*. The procedure was optimized slightly to be able to gate neutrophils from the granulocyte gate (addition of anti-CD15 mAb in PE-Cy7 in the first step as indicated in **Chapter 3.2.3.3** *Phagocytic assay*). The addition of anti-CD15 mAb did not affect the results of the measurement both in PB (compared in samples collected to EDTA treated tubes, thus the low numbers) and CB (**Figure 12C**), following the previous observation (Jeraiby *et al*., 2017). Collection of PB in EDTA tubes caused a severe decline in the effectiveness of the phagocytosis as seen in the representative histograms (**Figure 12A** and **B**) and in the graph (**Figure 12D**), showing that collection of blood into heparinised tubes was essential. As depicted in **Figure 12E**, the activation of neutrophils following phagocytosis of *E. coli* did not differ significantly between neonatal CB and maternal PB.

As depicted in **Supplementary Figure 2A** a substantial increase in CD15 expression was seen when whole blood was stimulated with *E. coli* among all studied groups. The highest increase was seen in PB of healthy donors in cells activated both by PMA (positive control – PC) and by *E. coli* (EC) (**Supplementary Figure 2B**). In all groups, the increase of CD15 expression correlated with the level of activation (shown as MFI of Rhodamine123) (**Supplementary Figure 2C**).
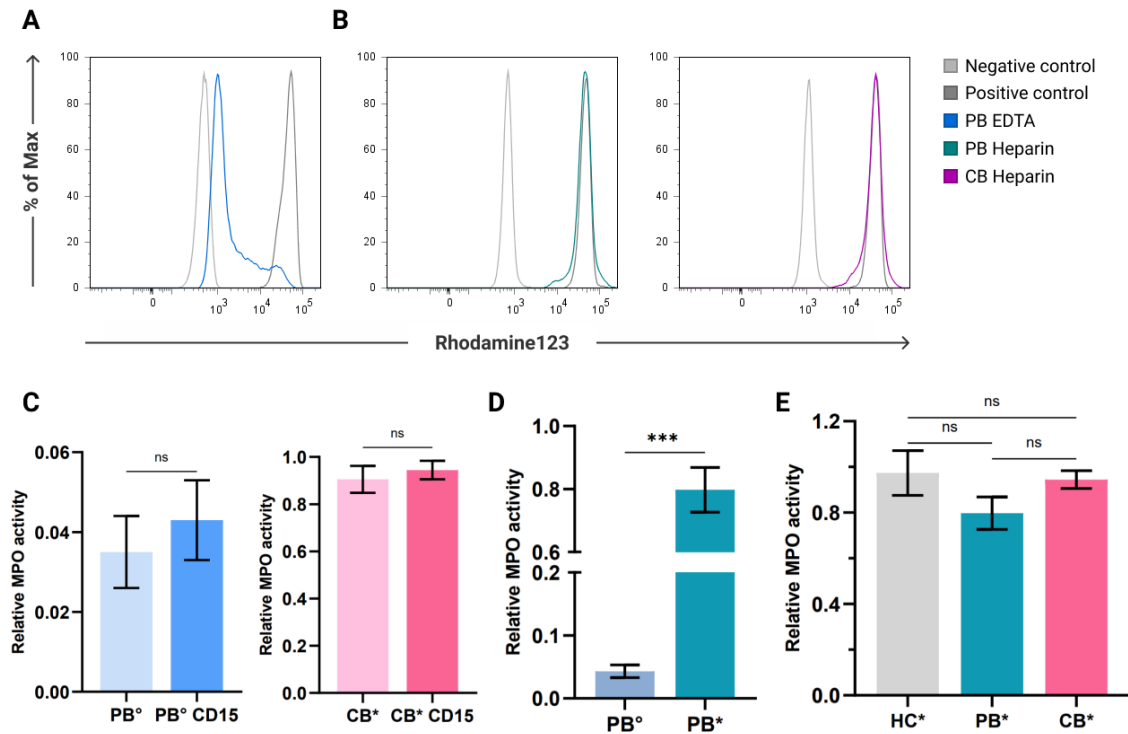
Figure 12. Activation of neutrophils following phagocytosis of Escherichia coli

*Analysis of neutrophil activation represented by the oxidation of Dihydrorhodamine123 to fluorescent Rhodamine123, following phagocytosis of E. coli. Analysis was measured using the FagoFlowExKit (EXBIO). Representative histograms of PB (n=10) collected into EDTA (**A**) and heparin (**B**) tubes. Addition of anti-CD15 mAb conjugated to PE-Cy7 did not affect the measurement (n=10 for both) (**C**). EDTA inhibited the effectiveness of E. coli phagocytosis (n=10 for both) (**D**). The activation of neutrophils did not differ among individual groups (n=10 for all) (**E**). Relative MPO activity was counted as shown:*

$$Relative\ MPO\ activity = \frac{MFI_S * MFI_N}{MFI_P * MFI_N}$$

*S stands for E. coli stimulated sample, N negative control and P positive control. ° represents samples collected to EDTA tubes and * represents samples collected to tubes containing heparin. **Abbreviations:** CB – cord blood, HC – healthy control, MFI – median fluorescence intensity, PB – peripheral blood*

5.5. Metabolic response upon neutrophil activation

Following the metabolic response during phagocytosis of *E. coli*, our aim was to observe and compare the metabolic response during activation of neutrophils from both CB and maternal PB. In mouse studies, it was proposed that LDN could exert higher metabolic flexibility after activation (Hsu *et al.*, 2019) compared to HDN and that CB neutrophils showed increased O_2^- production (Chudgar *et al.*, 2005), which could be possibly measured as higher demand for oxygen, represented as OCR.

The principal source of energy for neutrophils comes from glycolysis, which is proffered source of energy to oxidative respiration, as described above in *2.3.2* (Neutrophil metabolism). The consumed oxygen is, therefore, mostly utilized in the production of ROS, which is essential for the proper antimicrobial function of neutrophils. The higher OCR in stimulated neutrophils represents the heightened demand for oxygen to generate ROS. Elevation in ECAR reflects a higher rate of glycolysis, which compensates for the elevated ATP demand during neutrophil activation. Along with the fact that glycolysis intermediates are essential for PPP, which in turn generates NADPH to fuel NADPH oxidase, an enzyme crucial for ROS generation.

First, we wanted to make sure that the anticoagulants used for the blood collection and short-term storage of blood will not interfere with the measurement. On that account, we compared PB from healthy donor collected to three different anticoagulants (sodium heparin, K_2EDTA, sodium citrate) and measured neutrophil activation following LPS stimulation right after blood collection and 4 hours post collection. As seen in **Supplementary Figure 3**, the most consistent measurement was seen when blood was collected to heparin. 4h storage did not attenuate oxidative burst of neutrophils. Also, using $8*10^4$ cells per well (instead of $4*10^4$ cells per well), we were able to obtain more signal for OCR since neutrophils utilize such small amounts of oxygen when unstimulated.

Preliminary data from CB and maternal PB are depicted in **Figure 13**. As observed, both CB and maternal PB were able to undergo oxidative burst upon stimulation with LPS (**Figure 13A**), with a slight increase in CB neutrophils. As seen in **Figure 13B**, neutrophils from CB had a slightly increased rate of glycolysis, suggesting their relatively higher demand for glycolysis to perform their antimicrobial functions.
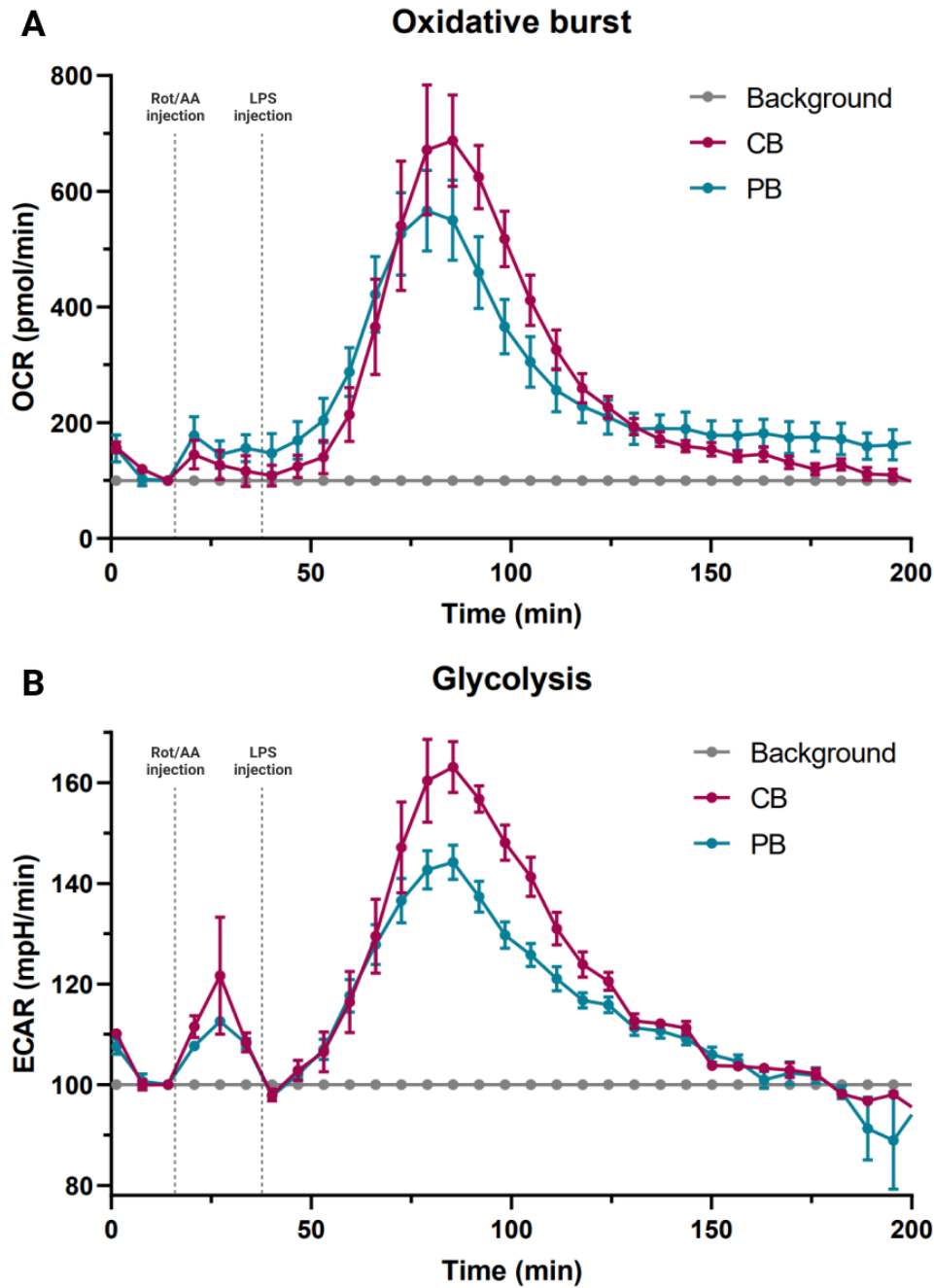
A

Oxidative burst

B

Glycolysis

Figure 13. Oxidative burst and increased glycolysis rate in CB and maternal PB neutrophils following stimulation with LPS

*Neutrophils isolated from umbilical CB and maternal PB, which was collected to tubes containing sodium heparin were subjected to SeaHorse analysis of oxidative burst (measured as oxygen consumption rate) (**A**) and rate of glycolysis (extracellular acidification rate)(**B**). **Abbreviations:** AA – animycin A, CB – cord blood, LPS – lipopolysaccharide, PB – peripheral blood, Rot - rotenone*

6. Discussion

Pregnancy poses a significant challenge for the maternal immune system to balance the immune suppression to prevent harmful reactions towards the paternal antigens coming from the fetus and to maintain the immune system's readiness to fight against infection (like bacteria, viruses, etc.). Neutrophils play an important role during the whole span of pregnancy, from the initial placentation (Nadkarni *et al*., 2016), angiogenesis promotion (Amsalem *et al*., 2014) to the placental tissue breakdown and remodelling during and after the labour (Osman *et al*., 2003; Shynlova *et al*., 2013). Contrarily, excessive neutrophil activation can lead to pregnancy complications ranging from preeclampsia to pregnancy loss (Giaglis *et al*., 2016).

The course of pregnancy and even the mode of delivery can modulate the offspring's future immune response. As discussed by many articles, the mode of delivery can drastically influence the composition of neonatal gut microbiota (Rutayisire *et al*., 2016) and therefore, the initial development of tolerance and the setting of the proper immune reactions towards both harmless microorganisms and pathogens. Neonatal immune system develops rapidly in the early days after birth (Olin *et al*., 2018). However, newborns can be more susceptible to infection since their adaptive immune system does not respond sufficiently in the first few weeks after birth. Innate immunity, largely represented by neutrophils, is therefore responsible for the clearance of infectious pathogens. Nevertheless, since the neonatal immune system is relatively immature, the immune response regulation has to be very tight to prevent detrimental activation and overt inflammation. Research focusing on the immune setting during labour and its influence on the neonatal immune response is essential for the recognition of early events following the delivery and their implication for future immune development.

In the light of the presented importance of pregnancy and labour on the neonatal immune system, this thesis focused on the analysis of neutrophil phenotype and functionality in CB compared to maternal PB and PB of healthy nonpregnant woman volunteers (referred to as HC). Obtained data from neutrophil analysis in whole blood showed that most of neutrophils from both maternal PB and PB of healthy controls are CD14⁻CD15⁺ cells, whereas in CB the number of cells expressing high amounts of CD15 was lower, mainly due to a specific enhancement of CD14⁻CD15^{low} subpopulation of cells. CD14⁻CD15^{low} was almost undetectable in maternal PB and slightly higher in PB of healthy controls (mostly due to one sample). Out of CD14⁻CD15⁺ neutrophils we were able

to see a large enhancement in the number of cells expressing lower amounts of CD16 with higher expression of both CD64 and CD62L in CB compared to both maternal PB and HC (where these subpopulations were almost undetectable). As expected, the CD16^{low} neutrophil subpopulations were enriched in the mononuclear fraction after density gradient centrifugation in CB compared to PB. In our experimental setting, whole neutrophil population did not suppress the proliferation of CD4$^+$ T cells, even when stimulated with LPS (as it was suggested that LPS could induce suppressive subpopulations of neutrophil).

The most striking differences between CB and PB is represented by increased expression of genes associated with antimicrobial response (mostly proteins usually present in azurophilic granules – MPO, defensins, ELANE), which was seen in neutrophils isolated from CB and slightly in neutrophil isolated from maternal PB in comparison to HC. The qPCR data from isolated CB LDN and HDN suggest this phenomenon could be carried out by LDN, although the differences did not reach statistical significance. Relative expression of certain genes (defensins, MPO) also positively correlated with the percentages of CD16^{low}CD64^{high} cells in the CD14$^-$CD15$^+$ compartment, again suggesting that the CD16^{low} subpopulations could be responsible for the enhancement in expression of antimicrobial genes. An increase in expression of genes associated with antimicrobial response was also seen in neutrophils, which were isolated from cord blood of children delivered by caesarean section, but genes responsible for the regulation of immune response were higher in neutrophils isolated from cord blood of neonates born by vaginal delivery.

Numerous articles suggested that neutrophils from cord blood have impaired phagocytic activity, oxidative burst or do not generate ROS as effectively as neutrophils from PB. Phagocytic capacity was compared between CB, PB and HC neutrophils by measuring the oxidation of Dihydrorhodamine to Rhodamine123 (a process carried out by MPO, which is a key component in the generation of ROS). We did not see any impairment of neutrophil activation after phagocytosis of *E. coli*. Our preliminary data suggest that neutrophils isolated from CB are capable of oxidative burst to the same degree as neutrophils from PB.

For the phenotype analysis five markers, namely CD14, CD15, CD16, CD62L and CD64 (their function and expression on neutrophils is stated in **Table 1**), were used for the identification of neutrophils and certain neutrophil subpopulations. According to the literature, classical and intermediate monocytes express high amounts of the TLR

co-receptor CD14 (Merah-Mourah et al., 2020), therefore CD14 was used to eliminate monocytes from the analysis. The non-classical monocyte subsets expressing lower amounts of CD14 (Merah-Mourah et al., 2020) and could eventually gate with CD14$^-$ neutrophils, express high amounts of CD16, so it should not be responsible for the enhancement of CD15$^+$CD16^{low}CD64^{high} cells in cord blood. Neutrophils were therefore considered CD14$^-$CD15$^+$ granulocytes as described many times in the literature (e.g. Pillay et al., 2013; Gustafson et al., 2015) and further gated based on the expression of CD16, CD62L and CD64.

The overall number of granulocytes in cord blood (CB) was lower, around 40-50% than in peripheral blood of healthy volunteers (HC) and mothers (PB), where the granulocyte numbers reached around 70-80% of all cells (although the absolute numbers of granulocytes did not differ, see **Supplementary Table 1**). This was an interesting phenomenon because lymphocytes in cord blood reached almost half of the acquired cells (the absolute number of lymphocytes was also increased). Articles focusing on overall numbers of leukocyte in cord blood described a similar increase in cord blood lymphocytes (D'Arena et al., 1998, Chirumbolo et al., 2011) and as the authors suggested these cells were phenotypically immature (the majority of CD3$^+$ T lymphocytes being naive CD45RA$^+$ cells) (D'Arena et al., 1998). The decreased number of neutrophils (identified as CD14$^-$CD15$^+$ granulocytes) was mainly caused by an increase in CD14$^-$CD15^{low} distinct subpopulation seen in cord blood. The expression of CD15 elevates during maturation, which could mean that this distinct CD14$^-$CD15^{low} cell population is a kind of immature cell population, different from LDN, which were described to express high amounts of CD15 (Ssemaganda et al., 2014). In further analysis CD14$^-$CD15^{low} cells were mostly CD16$^-$ (**Supplementary Figure 4**), again pointing to their relative immaturity. Based on a literature search, no information is available about the functions of this population.

Similar to Weinhage et al. (2020), who studied LDN in umbilical CB, we were able to observe a higher number of CD15$^+$CD16^{low}CD64^{high} neutrophils in CB in comparison to PB and HC. Further, there was a slight increase in the number of CD15$^+$CD16^{low}CD64^{high} neutrophils in PB in comparison to HC, where ascribed cells were almost undetectable. Also, there was a slight increase in CD15$^+$CD16^{low}CD62L$^+$ neutrophils in CB compared to both PB and HC. A higher amount of CD16^{low}CD62L$^+$ cells in the LDN compartment was seen by Hassani et al. (2020) in patients with chronic inflammation and a similar increase in CD16^{low}CD62L$^+$ in circulation was observed in volunteers introduced with LPS to

induce inflammation (Pillay *et al*., 2012). Suggesting that described cells are either released into the circulation *de novo* or their density (increase in the LDN compartment) and phenotype changes upon inflammatory stimulus.

For example, Jimenez *et al*. (2019) showed that the accumulation of C-reactive protein (CRP) can drive the release of MDSC (from 80% represented by PMN-MDSC) from mouse bone marrow. CRP also heightened the production of ROS by MDSC and their ability to suppress T cell proliferation, which they also demonstrated with isolated human neutrophils (Jimenez *et al*., 2019). CRP is a widely used biomarker for acute inflammation, although its diagnostic potential in neonatal sepsis did not prove to be so sensitive in oppose to neutrophil CD64 expression, which was shown to be very sensitive (Hashem *et al*., 2020). As discussed by Injarabian *et al*. (2019), neutrophil metabolic pathways and metabolic microenvironment in which neutrophil occurs could influence its differentiation and phenotype (Injarabian *et al*., 2019). The metabolic shift occurring during neutrophil activation, for example during inflammation, could affect its buoyancy and its possible transition to LDN.

To prove if $CD15^+CD16^{low}CD64^{high}$ and $CD15^+CD16^{low}CD62L^+$ are enriched in the low-density compartment, the same phenotype analysis was performed on CBMC/PBMC. After density gradient centrifugation granulocytes represented around 2% of cells in the mononuclear compartment. As expected, the numbers of $CD15^+CD16^{low}CD64^{high}$ and $CD15^+CD16^{low}CD62L^+$ neutrophils were higher in CBMC compared to PBMC (from mothers) despite the low number of samples analysed. The high expression of CD64 on immature $CD16^{low}$ neutrophil subpopulation in CB and their further recruitment during infection, for example through increased levels of CRP, could be one of the driving forces of the high sepsis rates seen in neonates (Lukacs and Schrag, 2012; Saboohi *et al*., 2019).

Including further markers and extending the panel for neutrophil subpopulation identification would probably be beneficial. In our experimental setting, we were limited by the number of flow cytometer channels. This could be overcome by using multiple panels in various tubes, although for the sake of this thesis we mainly focused on neutrophil phenotypes previously described to be changed in neonatal cord blood (e.g. LDN) (Ssemaganda *et al*., 2014; Weinhage *et al*., 2020). The extension of characterising distinct neutrophil populations (e.g. CD11b, CD33, CD66b, LOX-1) and functional capacity might bring more insight into neutrophil biology in neonates and would be a good suggestion for further detailed research.

To test the potential neutrophil suppressive capacity on T cell proliferation, cocultivation assays were performed. Isolated cord blood neutrophils did not inhibit the proliferation of stimulated CD4^{+} T cells, following the results of Weinhage *et al.* (2020), where even isolated LDN did not suppress stimulated T cell proliferation. Neutrophils were also stimulated with LPS, to possibly increase the number of cells with suppressive phenotype, as was described earlier (Pillay *et al.*, 2012). Nevertheless, the addition of LPS, led to a similar result as seen in cocultivation without LPS in our experimental settings. In conclusion, we were not able to prove cord blood neutrophil suppressive capacity function on CD4^{+} T cell proliferation.

Since in our experimental setting we could not prove CB neutrophil suppressive properties, we focused on their inflammatory functions. Gene expression analysis was performed first from whole and later from isolated CB LDN and HDN. CB neutrophils had markedly increased expression of genes related to antimicrobial functions in comparison to both maternal PB (except *MMP9*) and PB of healthy volunteers. In some genes (*Defa3*, *MPO*, *MMP9*, *Ltf*), maternal PB neutrophils had slightly increased expression when compared to PB of healthy volunteers. MMP-2 and MMP-9 are present in higher quantities in placental tissue and fetal membranes during labour (Xu *et al.*, 2002). A phenomenon to which neutrophils are likely contributing since it was shown that neutrophils travel to the cervix to help dissolve the fetal membranes via the production of MMPs. This may explain the enhancement in *MMP9* expression seen in maternal PB.

Although in different settings, other studies have shown upregulation in genes connected to antimicrobial response. For instance, He *et al.* (2018) have shown such increase in CB PMN-MDSC of mice. Identified genes included S100 proteins and lactoferrin. Whereas, Villanueva *et al.* (2011) described an increase in genes associated with antimicrobial response in LDG isolated from SLE patients. Similar to results obtained in this thesis, isolated lupus LDG had elevated levels of genes coding for proteins found in neutrophil azurophilic granules, such as MPO, ELANE, DEFA4. By qPCR, they also showed increased expression of *MMP8*. This work would support the idea that CB LDN could be more transcriptionally active due to their immature phenotype in comparison to CB HDN and neutrophils from PB.

The expression of suppressive molecules, which were associated with either LDN or PMN-MDSC regulatory function in CB, was also compared. Both CB and maternal PB neutrophils had elevated levels of the *Arg1* gene. The production of Arg-1, IL-10 along

with PD-L1 expression is mainly associated with the suppressive mechanism of PMN-MDSC (Tamadaho *et al*., 2017). Interestingly, *Il10* expression did not differ among studied groups, but the expression of *CD274* (gene coding for PD-L1) was highly elevated in maternal PB, suggesting that PD-L1 expression could be associated with immune suppressive mechanisms seen in pregnancy, but differ from regulatory mechanisms taking place in CB. The upregulation of PD-L1 on neutrophils was described in multiple scenarios, like sepsis-induced immunosuppression (Wang *et al*., 2015) or chronic HIV infection (Bowers *et al*., 2014). It is important to mention that in the first study PD-L1$^+$ neutrophils expressed also higher amounts of CD16 and CD64 (phenotype associated with mature cells), whereas in the seconds study the expression of PD-L1 correlated with the percentages of LDN with PMN-MDSC phenotype. Furthermore, maternal PB neutrophils also showed increase expression of the *Pdcd1* gene (coding for the PD-L1 receptor PD-1), which could mean that neutrophils regulate their own numbers by expression of both receptor and ligand. *CD274* was also slightly increased in CB LDN in comparison to HDN and its expression in CB neutrophils correlated negatively with CD16^{low}CD64^{high} neutrophil numbers, suggesting similar autoregulatory mechanisms for mature neutrophils. It should also be pointed out that the gene expression of both *CD274* and *Pdcd1* does not have to correlate with the number of molecules expressed on the neutrophil surface. Thus this observation would need to be subjected to more detailed examination to be able to draw a clear conclusion. Like the cocultivation of isolated PB and CB LDN with CD4$^+$ T cells in the presence and absence of PD-L1 inhibitor.

To test a hypothesis, that the mode of delivery could affect neutrophil functional properties (Birle *et al*., 2015) the expression of distinct genes was compared. Remarkably, the expression of genes associated with antimicrobial response (except *ELANE*) was increased in CB of children delivered by CS and oppositely in children delivered by VD genes coding for suppressive molecules were upregulated. In line with the evidence, that children born by CS are at a higher risk of longer hospitalization due to infection (Christensen *et al*., 2018; Wainstock *et al*., 2019), this might suggest that the higher expression genes coding antimicrobial proteins correlates with the immaturity status of CB neutrophils and that the mode of delivery influences the maturation of neutrophils and their functional capability. And that the delivery by CS could lead to an additional increase in the I/T (immature to total neutrophil) ratio increasing the predisposition for neonatal sepsis development (Saboohi *et al*., 2019).

As was already mentioned above in the literature review, the studies focusing on CB neutrophil phagocytosis remain inconsistent. Our results show that neutrophils from CB can employ MPO for the production of ROS to the same extent as neutrophils from PB of healthy volunteers same as neutrophils from maternal PB. Although we did not measure the phagocytic potential of neutrophils (e.g. phagocytic index) in the true meaning, the activity of MPO is a direct consequence of phagocytosis (Arnhold *et al.*, 2020). Our preliminary data measured on the SeaHorse extracellular flux analyzer confirm the ability of CB neutrophils to perform the oxidative burst. A slight increase in CB oxidative burst was seen, consistent with the observation of Chudgar *et al.* (2005) and Strauss and Snyder (1983). On the other hand, opposite results were seen by Komatsu *et al.* (2001), where PMA stimulated neutrophils from CB produced lower amounts of O_2^- upon stimulation with PMA, but not with fMLP. This would suggest that stimulants used for neutrophil activation can also play a major role. The decrease in the respiratory burst of CB neutrophils, observed in some research articles (Bu *et al.*, 2015) could be due to the application of anaesthetics during labour (Billert *et al.*, 2016). But as described by Bu *et al.* (2015) the diminished ROS production was rescued when CB neutrophils were stimulated with PB plasma, suggesting that the suppression of ROS production could be mediated by factors present in CB plasma. When taken from the other side, the slight increase in CB neutrophil oxidative burst could also mean a decrease in maternal PB neutrophil activation, which was described by the team of Andrei Kindzelskii (2002 and 2006). As shown by Hsu *et al.* (2019) in mice, LDN exerted up to 2.6 fold higher metabolic flexibility, which could correlate with the increased glucose rate seen in CB, where the CD16^{low} immature subpopulations are enriched.

Additional research addressing neutrophil metabolism in pregnancy and neonates would be essential and could bring new insights into the anti-inflammatory properties of innate immune cells, which are so important for a proper course of pregnancy and neonatal prosperity.

7. Conclusion

Based on the results obtained during solving of this thesis, we can confirm that neutrophils in CB possess diverse phenotypes with enrichment in subpopulations expressing low amounts of CD16 with high expression of CD64 and CD62L previously termed LDN due to their increased presence in mononuclear cell fraction after density gradient centrifugation.

Further, we observed notable elevation in the expression of genes associated with antimicrobial response in neutrophils isolated from CB. Although the differences did not reach statistical significance, the qPCR and correlation data suggest that the LDN compartment could be responsible for this enhancement.

Lastly, we did not observe any impairment in both CB and maternal PB neutrophil activation in response to stimulation with both *Escherichia coli* and LPS. On the contrary, our preliminary data show that CB neutrophils might even exert greater metabolic response upon activation.

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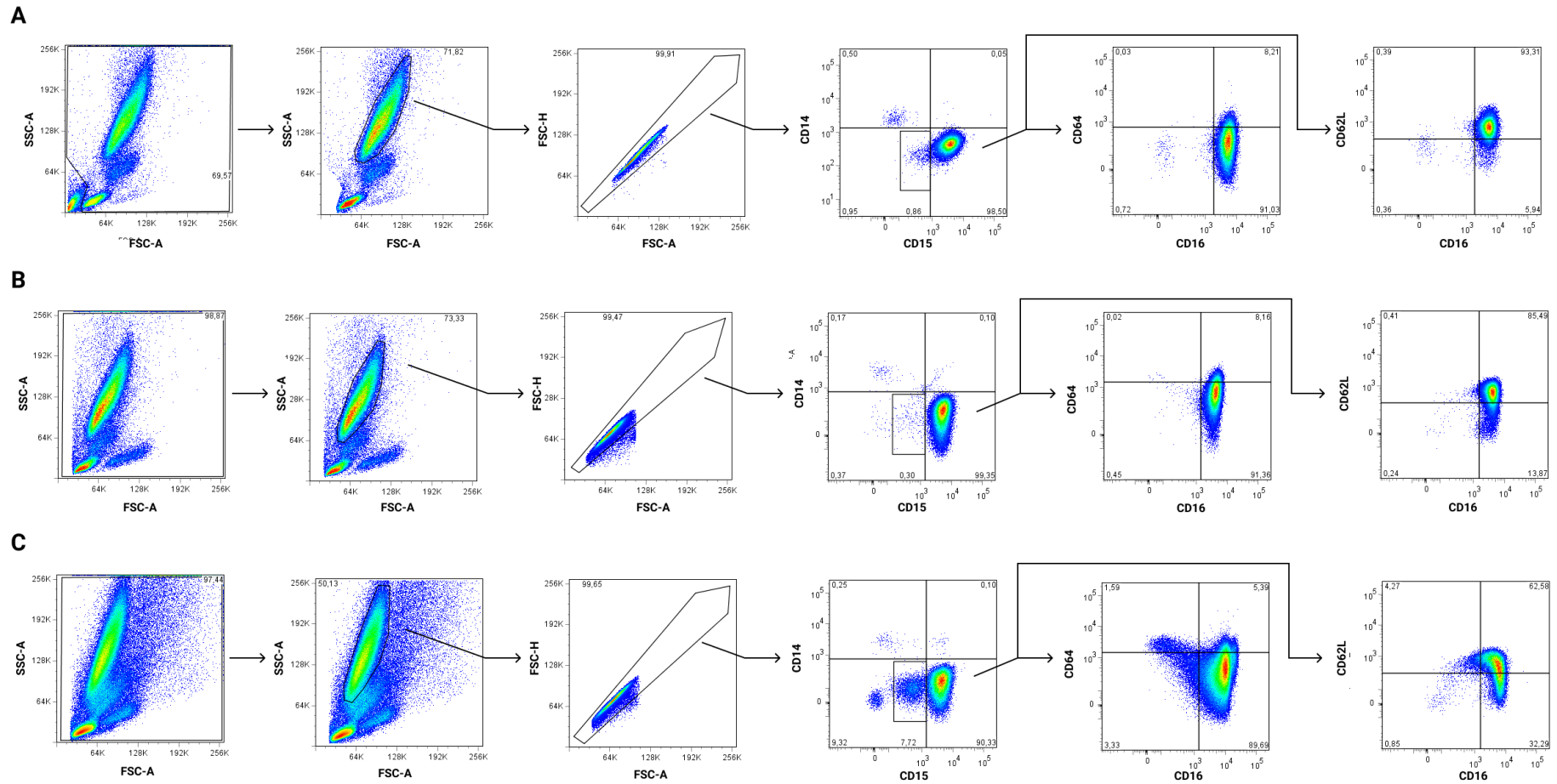
Supplementary Figure 1. Gating strategy for flow cytometry analysis of neutrophils

*Neutrophils from PB of healthy nonpregnant women (**A**), maternal PB (**B**) and CB(**C**) were stained with monoclonal antibodies against cell surface markers CD14 (PerCP), CD15 (FITC), CD16 (APC), CD62L (PE) and CD64 (PE-Cy7). First cells were identified, granulocytes were gated based on FSC-A and SSC-A and only single cells were analysed. Neutrophils were characterised as $CD15^+CD14^-$ cells and a distinct population of $CD15^{low}CD14^-$ cells was also gated on. $CD15^+CD14^-$ were further analysed for their expression of CD16, CD64 and CD62L.*
Abbreviations: *CB – cord blood, FCS-A/H – forward scatter area/height, PB – peripheral blood, SSC-A – side scatter area*
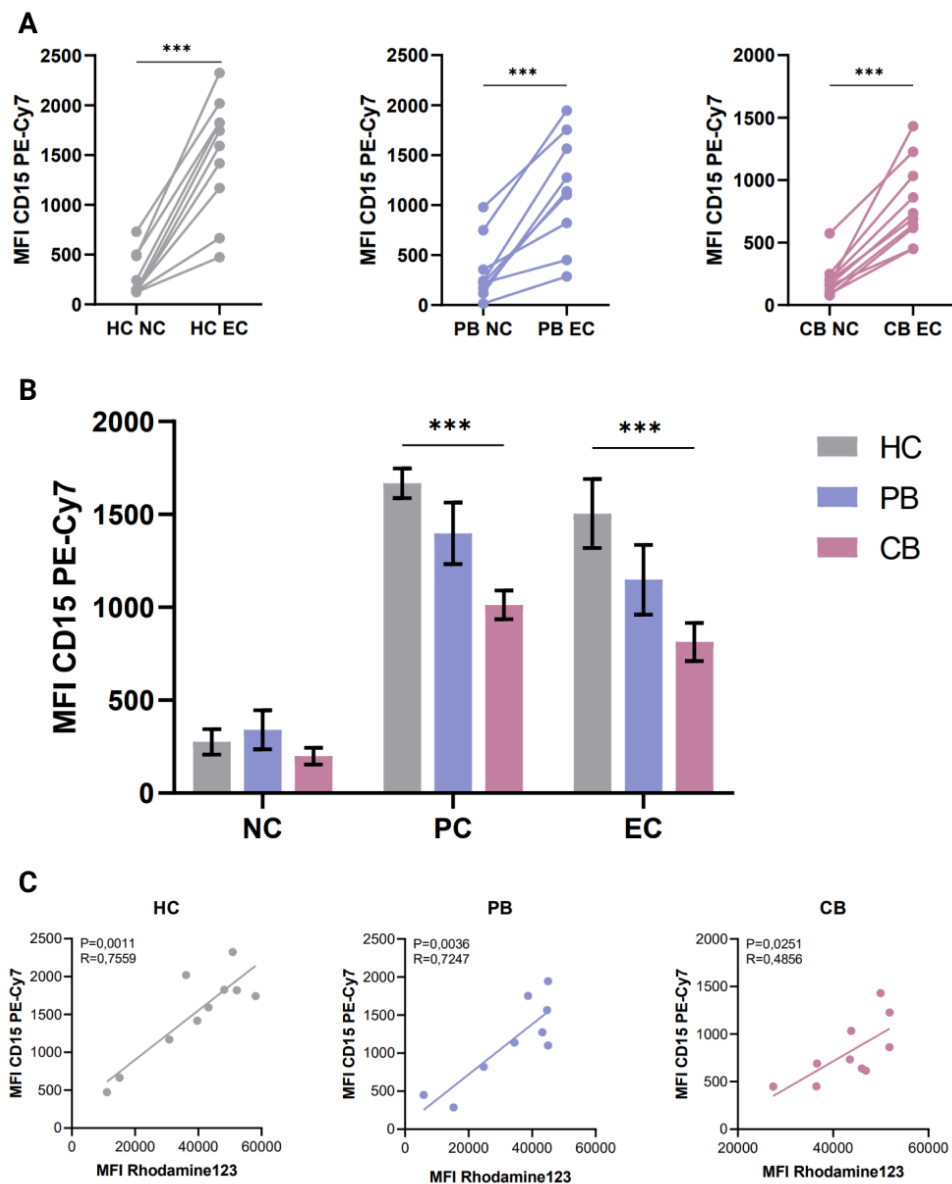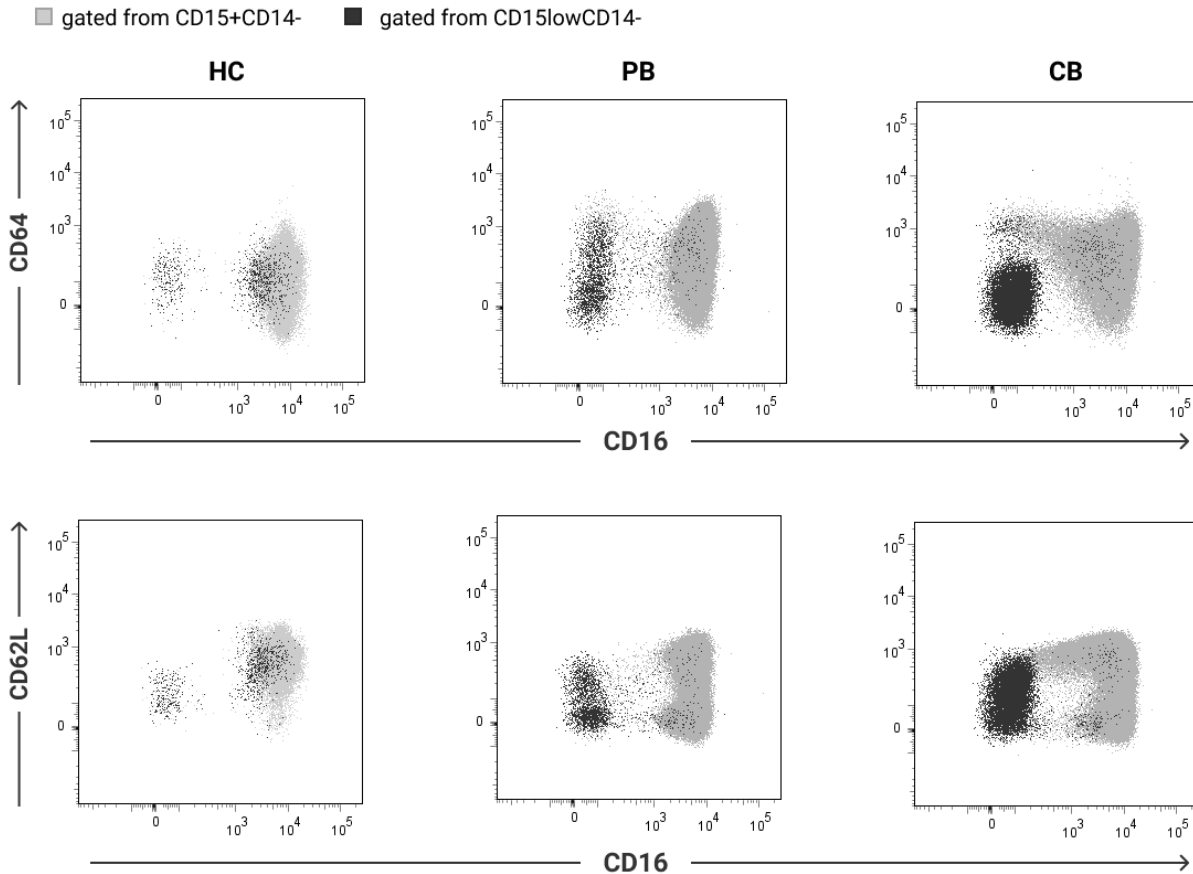
Supplementary Figure 2. CD15 expression during activation and phagocytosis

*Analysis of CD15 expression on neutrophils from cord blood, maternal peripheral blood and peripheral blood of healthy volunteers during activation with both PMA (positive control) and during phagocytosis of Escherichia coli. The enhancement of CD15 expression after phagocytosis of E. coli (**A**). Comparison of CD15 expression among all studied groups in nonstimulated samples (NC), samples stimulated with PMA (PC) and after the phagocytosis of E. coli (EC) (**B**). Correlation of CD15 expression (expressed as MFI of CD15 in PE-Cy7) with the level of activation after phagocytosis (MFI of Rhodamine123) (**C**). For all analysed groups n=10. **Abbreviations:** CB – cord blood, EC – Escherichia coli, HC – healthy control, MFI – median fluorescence intensity, NC – negative control, PB – maternal peripheral blood, PC – positive control*
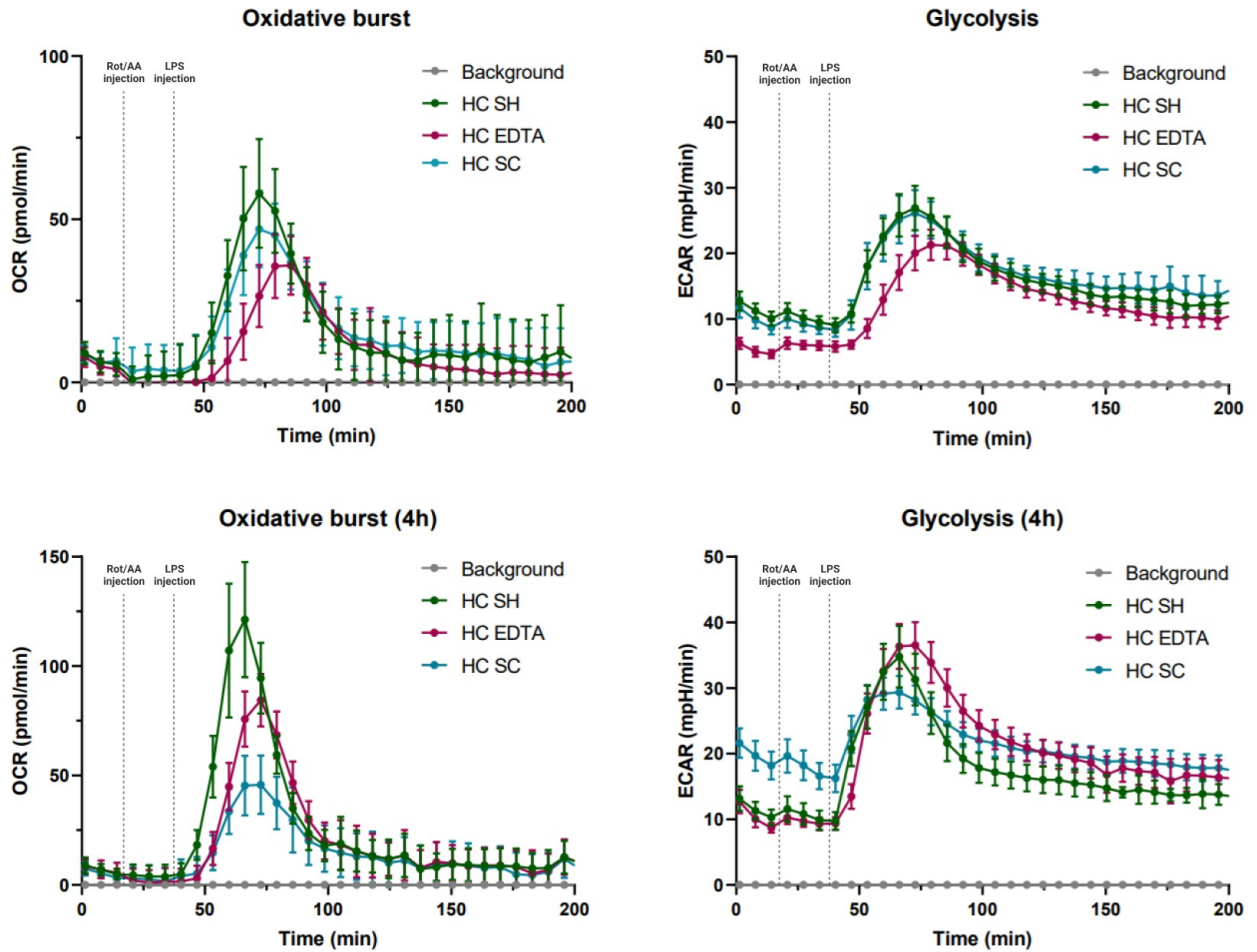
Supplementary Figure 3. The effect of anticoagulants and storage time on neutrophil activation (oxidative burst) following stimulation with LPS

*Neutrophils isolated from PB of healthy donors, which was collected to tubes with diverse anticoagulants - sodium heparin, K_2EDTA and sodium citrate were subjected to SeaHorse analysis of oxidative burst (measured as oxygen consumption rate) and rate of glycolysis (extracellular acidification rate) immediately after blood collection (**A**, **B**) or 4 hours post collection (**C**, **D**). **Abbreviations:** AA – antimycin A, EDTA - K_2EDTA, HC – healthy control peripheral blood, LPS – lipopolysaccharide, Rot – rotenone, SC – sodium citrate, SH – sodium heparin*

Supplementary Figure 4. Comparison of CD16, CD64 and CD62L expression on CD15$^+$CD14$^-$ and CD15^{low}CD14$^-$ granulocytes

Phenotype analysis was performed using flow cytometry based on markers CD14, CD15, CD16, CD62L and CD64. Granulocytes were gated based on FSC and SSC and two distinct populations were identified – CD15$^+$CD14$^-$ and CD15^{low}CD14$^-$ cells, which were further analysed for their expression of CD16, CD64 and CD62L. CD15^{low}CD14$^-$ cells were mostly CD16$^-$ with intermediate expression of both CD64 (top) and CD62L (bottom) or resembled the expression pattern of CD15$^+$CD14$^-$ cells. Representative images are shown. **Abbreviations:** *CB – cord blood, HC – healthy control peripheral blood, PB – maternal peripheral blood*

Supplementary Table 1. Absolute cell numbers in cord and maternal peripheral blood

	Absolute cell counts			
	PB		CB	
	Mean (cells/µl)	*SD*	*Mean (cells/µl)*	*SD*
Granulocytes	3447	±1845	4606	±1516
Monocytes	912	±541	735	±1206
Lymphocytes	3572	±1722	3555	±1748
CD3$^-$ lymphocytes	2830	±1605	2927	±343
CD3$^+$ lymphocytes	606	±332	1717	±346
CD4$^+$ T cells	1689	±1021	2099	±272
CD8$^+$ T cells	656	±406	744	±133
CD4$^-$CD8$^-$ T cells	473	±621	68	±16
CD4$^+$CD8$^+$ T cells	12	±6	14	±4
		n=7		*n=7*

Abbreviations: CB – cord blood, PB – maternal peripheral blood, SD – standard deviation