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Neutrophil Chemotaxis and Respiratory Burst in Term and Preterm Newborn Infants

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Abstract
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Neutrophil activation is the most important initial immune defense against invading microbes in newborn infants. The reduced neutrophil migration and uncontrolled regulation of reactive oxygen species (ROS) production observed in neonates, could result in a diminished infectious response or in tissue damage. The aims were to study neutrophil chemotactic response towards IL-8 and fMLP in term neonates; to examine neutrophil receptor expression involved in adhesion, migration, phagocytosis and complement after stimulation with IL-8 and fMLP in term neonates; and to investigate neutrophil production of ROS, induced by PMA and E.coli, after preincubation with IL-8 and fMLP in term and preterm newborn infants. Comparisons were made to neutrophils from healthy adults.

Chemotaxis was distinguished from randomly migrating neutrophils, and the neutrophil migration distance and the number of migrating neutrophils per distance was evaluated. Neutrophils were labeled with antibodies to cell surface antigens (CD11b, CD18, CD65, CD15S, CD162, CD44, CD35, CD88, CD181, CD182 and CD64) after stimulation with IL-8 and fMLP. After preincubation of neutrophils with fMLP or IL-8 and stimulation with PMA or E.coli, respiratory burst was detected. The same analyses were also made in preterm infants (median 25+3weeks GA; range 23+0–29+2) within 3 days postnatal age.

Neutrophils from neonates exhibited different migratory and receptor responses to IL-8 and fMLP, with a diminished response towards IL-8 in term newborn infants in terms of reduced chemotaxis and modulation of receptors involved in adhesion, chemotaxis, complement and phagocytosis as compared to adults. fMLP reduced PMA- and E.coli-induced respiratory burst in neutrophils from term neonates and adults. The reduced respiratory burst by fMLP may be a mechanism for reducing the detrimental effects of uncontrolled inflammation. Although a similar burst reduction was observed in preterm infants born >25 weeks GA with fMLP, a diminished neutrophil respiratory burst modulation in very preterm infants cannot be excluded and requires further studies at different gestational and postnatal ages.

Keywords: innate immunity, neutrophil, term newborn infants, preterm newborn infants, chemotaxis, respiratory burst, reactive oxygen species, cell surface receptor, IL-8, bacterial N-formyl peptide

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To all the newborn infants who
participated in this study

And to all newborn infants who
may benefit from this work

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Stålhammar, M.E.**, Douhan Håkansson, L., Jonzon, A., Sindelar, R. Differential Neutrophil Chemotactic Response towards IL-8 and Bacterial N-formyl Peptides in Term Newborn Infants. *Upsala Journal of Medical Sciences*. 2016 Oct 3:1-8.

II **Stålhammar, M.E.**, Sindelar, R., Douhan Håkansson, L. (2016) Neutrophil Receptor Response to Bacterial N-formyl Peptides is Similar in Term Newborn Infants and Adults in Contrast to IL-8. *Scandinavian Journal of Immunology*. Sep 8. doi: 10.1111/sji12477

III **Stålhammar, M.E.**, Douhan Håkansson, L., Sindelar, R. Bacterial N-formyl Peptides Reduce PMA- and *E.coli*-induced Neutrophil Respiratory Burst in Term Newborn Infants and Adults. *Submitted.*

IV **Stålhammar, M.E.**, Douhan Håkansson, L., Sindelar, R. Compromised Ability to Modulate Neutrophil Respiratory Burst in Very Preterm Newborn Infants - A Pilot Study. *In manuscript.*

Abbreviations

BAL	bronchoalveolar lavage
BPD	bronchopulmonary dysplasia
CXCL	chemokine (C-X-C motif) ligand
DAG	diacylglycerol
DHR	dihydrorhodamine
E.coli	*Escherichia coli*
fMLP	formyl-methionyl-leucyl-phenylalanine
GA	gestational age
GM-CSF	granulocyte monocyte-colony stimulating factor
GPCR	G-protein coupled receptor
IFN	interferon
LPS	lipopolysaccharides
LTB_4	leukotriene b_4
MC	monocytic cells
MFI	median fluorescence intensity
NADPH	nicotinamide adenine dinucleotide phosphate
NEC	necrotizing enterocolitis
NK cells	natural killer cells
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PNA	postnatal age
PVL	periventricular leukomalacia
ROP	retinopathy of prematurity
ROS	reactive oxygen species
TNF	tumor necrosis factor
WBC	white blood cells

Contents

Introduction

Newborn infants are vulnerable to infections because of an immature immune system (PrabhuDas et al, 2011). Consequently, neonates are at risk of developing sepsis and other diseases, which in turn can lead to increased morbidity and mortality. In preterm newborn infants, i.e. infants born <37 weeks of gestational age (GA), prenatal pro inflammatory factors consist of leukocyte accumulation in the immature lung after initial infection of chorion, amnion and fetal fluid i.e. chorioamnionitis. The inflammation is characterized by an initial activation and accumulation of neutrophils and then of monocytic cells (MC) in the lung. These cells could have a prolonged level of activity, stimulated by endotoxin and increased cytokine activity (Jobe et al., 2001, Jobe, 1999, Speer, 2001, Kramer et al, 2002, Sindelar and Jobe, 2007, Kramer et al, 2007), which could be harmful to the lung and other organs (Leviton et al, 2012, Neu and Walker, 2011, Speer, 2003). Neutrophil chemotaxis and respiratory burst which is the production of reactive oxygen species (ROS) can be compromised in preterm newborn infants in whom neutrophil accumulation, insufficient stores and diminished synthesis of antioxidants and uncontrolled burst might be present. This can cause tissue and organ injuries such as bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC) and periventricular leukomalacia (PVL) (Speer 2003, Neu et al, 2011, Leviton et al, 2013). Inflammation is strongly linked to the pathogenesis of the chronic lung disease BPD in premature infants. The preterm infants poorly developed antioxidant capacity also renders them vulnerable to high levels of oxygen, a chemoattractant factor in itself, with subsequent lung injury and pathological development of the lung. Lung injury could be further potentiated by mechanical stretch of the immature lung, deficient in the surface tension-reducing factor surfactant (Sugiura et al. 1994, Coalson et al., 1999). Cellular lung injury triggers local inflammatory reactions that develop into BPD (Kotecha et al, 1995, Watterberg et al, 1996, Yoon et al, 1997), but may lead to systemic inflammation with effects on other organs such as the gastrointestinal tract and CNS (Neu and Walker, 2011, Leviton et al, 2013). Although steroids are often used in the treatment of BPD, they are not recommended outside the framework of controlled studies due to the adverse effects on neurological and somatic development (Halliday and Ehrenkranz, 2001, Comittee on Fetus and Newborn, 2001). A more specific modulation of the inflammatory mechanisms could overcome these obstacles. Exploration of the inflammatory mechanisms that initiate

and propagate lung injury, with subsequent impaired lung development, could provide opportunities for controlling and inhibiting these tissue damaging mechanisms and for developing more individualized treatment for infants at risk of developing chronic organ injuries.The immune system can be classified into subsystems, the innate and the adaptive (acquired) immune system, or humoral and cell-mediated immunity. The immune system can detect a variety of pathogens, from viruses to parasitic worms and distinguish them from the healthy tissue. Pathogens rapidly evolve and adapt, and thereby avoid detection and neutralization by the immune system; on the other hand, multiple defense mechanisms have also evolved to recognize and neutralize pathogens. The adaptive immunity has the ability to create immunological memory after an initial response to a pathogen, leading to an enhanced response to ensuing invaders of the same pathogen. (Ley, 2001)

The protective response of the innate immune system against microbial or other agents, is characterized by the accumulation of leukocytes in the affected tissue. The first responders of activated leukocytes are neutrophils, and their accumulation is preceded by endothelial cell activation, neutrophil to endothelial adherence interaction, and transmigration through the endothelium. These events are followed by chemotactic migration along increased concentrations of chemoattractants into the tissue, and finally the release of reactive oxidants and other antimicrobial agents and phagocytosis of the invading bacteria at the site of infection/inflammation. (Ley, 2001)

Phagocytes are “professional" or "non-professional" depending on how effective they are at phagocytosis (Ernst et al, 2006). The professional phagocytes include neutrophils, monocytes, macrophages, mast cells and dendritic cells (Robinson et al, 1998). Professional phagocytes have cell surface receptors that can detect harmful objects, such as bacteria, in contrast to non-professional phagocytes (Ernst et al, 2006). Phagocytes are crucial in fighting infections and in maintaining healthy tissues through the removal of dead and dying cells.

Leukocytes

Leukocytes or white blood cells consist of 60-70% granulocytes in adults and 70-80% in newborn infants, 0.5-1% basophils, 2-4% eosinophils, 20-25% lymphocytes and 3-8% monocytes. These different types of leukocytes are distinguished by their physical and functional characteristics. Granulocytes have granules and segmented nuclei in their cytoplasm. In neutrophils, the contents of the granules have antimicrobial properties that are released by a process called degranulation. Lymphocytes and monocytes are mononuclear leukocytes without granule and lobular nuclei. Lymphocytes consist of natural killer cells (NK cells) (cytotoxic), T-cells (cytotoxic), and B-cells (antibody-driven). An increased number of leukocytes in the blood is often

an indicator of disease and is leukocytosis, whereas, a decreased number of leukocytes is called leukopenia. The capacity of chemokines and their receptors to control the migration of immune cells makes them good targets for therapeutic interventions for modifying outcome (Luster et al, 2001).

Neutrophils

Neutrophils are the first line of defense against invading microorganisms and the most abundant cells of the innate immune system. These cells are distributed in different compartments of the body, and bone marrow contains the neutrophil storage pool (Speer et al, 1998). The total blood granulocyte pool consists of two sub compartments: the circulating (50%) and the marginating neutrophil pools (50%). The marginating pool are those granulocytes adhering to the endothelium of vessel walls. Neutrophils are recruited from the bone marrow and migrate through the endothelial lining, into the circulation where they normally spend 6-8 hours (Ley, 2001, Fjaertoft, 2005). Most neutrophils circulating in the blood are mature but the cells newly released from the bone marrow are less mature with a non-segmented nucleus.

Although most bacterial infections stimulate an increase in neutrophil count, some infections cause reduction of neutrophils and neutropeni. Many viral infections, including hepatitis, influenza and rubella, decrease neutrophil count. Antibiotics and tricyclic antidepressants can induce neutropeni. White matter brain injury in preterm newborn infants is often accompanied by high levels of the inflammatory factor IL-8 and other circulating cytokines (Leviton et al, 2013). IL-8 has been implicated as an early marker for preterm newborn infants at risk of developing BPD, a lung disease where perinatal infection or/and inflammation and preterm delivery in combination with exposure to high oxygen levels and prolonged ventilation causes inflammation of the lung and consequent deranged lung development (Karlsson et al, 2002, Gessler et al, 2003). Ischemia (lack of blood flow) and reperfusion injury resulting in endothelial cell activation leading to massive neutrophil recruitment (Hernandez et al, 1987). Leukocytes are essential for healing but the initial recruitment can result in exacerbation and expansion of the tissue damage and destruction of the microcirculation and lead to necrosis.

Chemoattractants

Chemokines are chemoattractants inducing chemotaxis. Chemokines control the homeostasis and direct leukocytes during development (Ley, 2001). Immune cells continuously move through the blood, tissues and the lymphatic

system. Some chemokines are proinflammatory, such as TNF, IL-1, IL-6 and IL-8 (Kilpatrick et al, 1998) and can be induced during immune responses for recruiting immune cells to the site of infection or inflammation: they are highly expressed in inflammatory diseases (Luster et al, 2001). Chemokines also have a role in development and homeostasis i.e. controlling the migration of cells during normal tissue processes. Chemokines are classified into four main subfamilies: CXC, CC, CX3C, and C. All these proteins exert their biological effects through interaction with G protein-linked transmembrane receptors (GPCR), which are selectively found on the surfaces of their target cells. Chemoattractants and their receptors are targets for therapeutic interventions because of their capacity for controlling the movement of immune cells.

In small amounts, proinflammatory chemoattractants benefit the host, however, at higher levels, they may have deleterious effects (Giacoia, 1993, Pennington et al, 1993), and in neonates, they contribute to the inflammatory response in sepsis, meningitis and NEC. A general indicator of severe bacterial infection is TNF (Giardin, 1990). According to Shimoya et al (1992), IL-8 concentrations are elevated in preterm fetuses with chorioamnionitis and are suppressed by maternal steroid therapy to promote fetal lung maturation. In infants with bacterial sepsis, IL-8 levels are also elevated (Kaufman et al, 1996). Chemoattractants can be divided into subgroups: initiating (LPS, TNF-α); intermediate (IL-8, LTB_4), modulating (IFN-γ); and, end target chemoattractants (fMLP and PMA). Both intermediate and end target chemoattractants are used in this study with dose response experiments on agarose plates (Heit et al, 2002, Foxman et al 1997, Nelson et al 1975, Luster, 1998). Many chemoattractants derive from endothelial cells, epithelial cells, bacteria-generated N-formyl peptides, and lipopolysaccharides (LPS), which exhibit both chemotaxis and random migration (Gallin, 1993).

IL-8

The intermediate, pro inflammatory cytokine IL-8 is produced by local inflammatory cells, such as the endothelium, during immune and inflammatory responses to pathogens (Ley, 2001). IL-8 mediates transmigration by reducing adherence between leukocytes and the endothelium (Kilpatrick et al, 1998). Neutrophils are regulated primarily by CXC chemokines, and CXCL8 or IL-8 is an example of a chemoattractant for neutrophils that also activates their degranulation. Il-8 concentration increases by oxidative stress and during early infection in newborn infants (Mehr, 2001, Vlahopoulos et al, 1999). In preterm neonates, the IL-8 concentration did not decrease in bronchoalveolar lavage (BAL) during their first 96 h of life in contrast to term neonates (Craig, 1996).

fMLP

The end target, chemotactic bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP) is generated by microorganisms such as *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (Marasco et al, 1984, Atkinson et al, 1988). fMLP activates the GPCR which induces signal amplification (Jacobs et al, 1995, Wenzel-Seifert et al, 1998). fMLP attracts the migration of neutrophils to the site of bacterial invasion for their subsequent elimination (Marasco et al, 1984). When the neutrophils arrive at the site of infection, the fMLP-concentration increases and ROS production and lysosomal enzyme secretion are activated for destruction of the bacteria (Dillon et al, 1988).

Chemotaxis

The ability of cells to perform chemotaxis is essential for life (Foxman et al, 1999). Chemotaxis is the primary mechanism by which cell movements are directed towards gradients of chemoattractant in the environment (Foxman et al, 1999, Heit et al, 2003). Chemotaxis can be distinguished from chemokinesis, which is random or non-directional motility. In multi-cellular organisms, chemotaxis is a major component of immune responses and critical to cell migration in embryogenesis and subsequent phases of development (Foxman et al, 1999, Rajagopal et al 2010).

Chemotaxis involves a cascade of events: formation of signaling pathways, receptor polarization, adhesion receptor activation, cytoskeletal reorganization, and transendothelial migration (Rajagopal et al, 2010). Upon activation by chemoattractants blood neutrophils start rolling on endothelial cells, followed by firm adhesion and transendothelial migration, through the endothelium and into the tissue, and finally to the site of invading bacteria, where the neutrophils phagocytize the invading microbes (Ley, 2001). Neutrophil firm adhesion, a function distinct from transmigration, implies that the cells stop by adhering strong enough to resist blood flow, in contrast to rolling. The oxidative state of the neutrophils changes from resting to primed and then to activated as they move towards the site of infection in response to chemotactic agents.

Chemotaxis in newborn infants is generally studied through filter methods that simulate transmigration from blood through endothelium (Krause et al, 1986, Pahwa et al 1977, Urlichs and Speer, 2004). With a filter assay, the distribution of a population of cells after exposure to a chemoattractant can be established in a steep gradient across a thin porous filter.

With the under-agarose assay, the number of chemotactic cells can easily be separated from cells moving randomly. This assay is a method for studying chemotactic leukocytes and has been used for determining the effects of

disease in leukocyte chemotaxis (Campbell et al, 1997, Heit et al, 2002), the role of various signaling pathways, and for the role of adhesion molecules in neutrophil chemotaxis (Rajagopal et al, 2010, Zigmond et al, 2001).

In this study, the under-agarose cell migration assay gave us the possibility to assess graded chemotactic response with respect to the number of migrating neutrophils in relation to the migration distance, with different concentrations of chemoattractants i.e. dose-response.

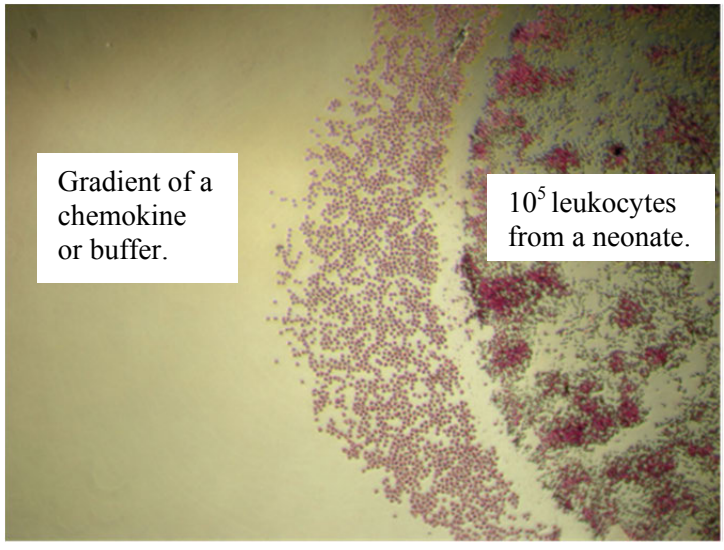

Figure 1. The under-agarose assay. An example of neutrophils from a newborn infant, migrating towards a chemoattractant. The gel was removed and cells stained with Wright's stain.

Respiratory burst

Respiratory burst or oxidative burst is the production of reactive oxygen species (ROS) by immune cells for destroying microorganisms. The NADPH system generates ROS in phagocytes that eliminate microbes (Seifert et al, 1991, Jones et al 1996). The phagocytes release ROS when they encounter microbes. The endocytic vesicle (phagosome) that is formed during phagocytosis fuses with a lysosome and digests the content. After phagocytosing or internalization of the bacteria, they are degraded with ROS. These chemicals are also released from the fertilized ovum. A lack of NADPH oxidase prevents the formation of ROS and results in chronic granulomatous disease.

Neutrophil migration and respiratory burst have been studied with respect to priming, in which preincubation with proinflammatory agents such as IL-8, tumor necrosis factor (TNF), lipopolysaccharide (LPS), interferon (IFN),

and granulocyte/granulocyte-macrophage colony stimulating factor (G/GM-CSF), followed by the stimulation with the chemotactic agent fMLP results in increased ROS production (Kuijpers et al, 1991, Elbim et al, 1994, Foxman et al, 1997, Karlsson et al, 2002, Gessler et al, 2003, Kuijpers et al 1991, Heit et al, 2002). TNF and IL-1 prime leukocytes for enhanced phagocytosis, antibody-dependent cytotoxicity, oxygen radical production, and degranulation triggered in response to a second stimulus such as fMLP. Less is known about priming of respiratory burst with fMLP as a primary neutrophil stimulator and in newborn infants where the immune system might be immature.

PMA and *E.coli*.

Phorbol 12-myristate 13-acetate (PMA), also known as 12-*O*- Phorbol 12-myristate 13-acetate (PMA), also known as 12-*O*-tetradecanoylphorbol-13-acetate, is a diester of phorbol and a potent tumor promoter often used in biomedical research to activate the kinase signal transduction enzyme protein kinase C (PKC) (Castagna et al 1982, Niedel et al, 1983). The effects of PMA on PKC result from its similarity to diacylglycerol (DAG), a natural activator of PKC. PMA is routinely used as an inducer for endogenous ROS production and as a soluble and intracellular stimulus, whereas, *Escherichia coli* is a Gram-negative, facultative anaerobic bacterium used as a particulate stimulus for ROS production.

The complement system

The complement system is a part of the innate immune system and complements the ability of phagocytic cells to clear pathogens from an organism. Phagocytosis and clearance are possible through opsonization of bacteria and cells. Antibodies bound to microbes and other structures are effectively recognized by phagocytes and the complement system (Morgan, 2001). Complement enzymes perforate the surface of the antibody bound structure and degrade it by lysis. Three parallel complement cascades, the classical pathway, the alterative pathway, and the mannose-binding-lectin (MBL) pathway, are activated by different stimuli and converge in a common pathway that induces local inflammation, recruits phagocytes, and lyses cells. The classical pathway primarily lyses cells and bacteria already recognized and opsonized by plasma antibodies and target these cells for immunological clearance. The alternative pathway acts independently of plasma antibodies and binds to cells and bacteria that do not express complement decay accelerating factor. The MBL pathway recognizes common bacterial surface proteins, and acts primarily to lyse bacteria and for immunological clearance.

Receptors

GPCR or 7-transmembrane domain receptors (7TMR) are a large protein family of receptors that sense molecules outside the cell and upon binding initiates an intracellular transduction cascade that activates different functions of the cell. GPCR are involved in many diseases and are the target of approximately 40% of all modern medicinal drugs. Despite major structural differences between different chemoattractants, GPCR mediate an almost identical intracellular reaction chain that leads to a rapid rearrangement of the cytoskeleton and thus chemotaxis Schraufstatter et al, 2001. There are two different routes for the G-protein coupled intracellular re-action chain and two important molecules: PI3K (phosphoinositol-3-kinase) and p38 MAPK, which can be inhibited (Hirsch et al. and Nick et al, 1997). The ligand binds to the receptor, which changes the receptor shape. G protein activates/inactivates enzymes by causing the enzymes to change shape. Kinase enzymes activate other enzymes that trigger various cell functions. The receptors involved in adhesion, migration, complement and phagocytosis (**Paper II)** are listed in Table 1.

Table 1. Neutrophil functions of the 11 different receptors.

Cell surface antigen	**Type of receptor**	**Function**
CD11b	C3b-receptor, CR3, α-chain of the CD11b/CD18 integrin heterodimer, ligand to ICAM-1	Adhesion, phagocytosis, migration
CD15s	Selectin ligand	Adhesion, binds to E-, P-, and L-selectin
CD18	C3b-receptor, CR3, β-chain of the CD11b/CD18 integrin heterodimer, ligand to ICAM-1	Adhesion, migration, phagocytosis
CD35	C3b-and C4b-receptor, CR1	Adhesion, granula exocytosis, migration, regulates classical and alternative complement pathways, G-protein coupled
CD44	Receptor for hyaluronan	Adhesion, migration, binds hyaluronan
CD64	Fcγ-receptor I	Binds monomeric IgG and IgG-immune complexes, phagocytosis
CD65	Selectin ligand	Adhesion, CD65s binds to E-and P-selectin
CD88	C5a-receptor	Binds C5a, G-protein coupled, chemotaxis
CD162	P-selectin ligand	Binds to P-selectin (SELPLG), adhesion
CD181	CXCRα	Binds IL-8, G-protein coupled, migration
CD182	CXCRβ	Binds IL-8, G-protein coupled

Aims

The general aim of this thesis was to investigate neutrophil chemotaxis; neutrophil expression of receptors involved in adhesion, transmigration and complement; and interaction between neutrophil migration and respiratory burst in full term and preterm newborn infants compared to adults, with special respect to the intermediary and end-target chemoattractants IL-8 and fMLP.

The specific aims were:

I To observe neutrophil chemotactic response towards the pro-inflammatory chemoattractant IL-8 and the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP) in term newborn infants and adults.

II To examine neutrophil receptor expression, involved in adhesion and migration, in term newborn infants and adults, after stimulation with IL-8 and fMLP.

III To investigate neutrophil production of ROS by preincubation with IL-8 and fMLP in response to PMA and E.coli in term newborn infants and adults.

IV To investigate neutrophil production of ROS by preincubation with IL-8 and fMLP in response to PMA and E.coli in very preterm newborn infants.

Materials and Methods

Subjects and blood sampling.

Paper I, II and III: Blood was collected from the placental side of the umbilical cord from 8, 16 and 17 healthy, term infants (Paper I, II and III respectively) immediately after elective caesarean section was performed with spinal anesthesia, at the Uppsala University Children's Hospital, Uppsala, Sweden. The infants were born at 38–39 weeks gestational age, weighed between 2800–4500 g, and had a mean Apgar score of 8 at one minute of age, 10 at 5 minutes, and 10 at 10 minutes.

Peripheral blood was collected from healthy adults (n=8, n=17, n=29 in **Paper I, II and III** respectively) aged between 18-65 years old. One newborn infant and one adult participated simultaneously in Paper I, II and III.

In **Paper IV**: Eight preterm newborn infants born at 23-29 weeks of gestational age (GA) at the Uppsala University Children's Hospital were included in Paper IV. Five of the infants were delivered normally and three of them by elective caesarean section. No selection of patients was performed besides having arterial catheter and being less than 3 days of age. Blood was collected from the catheter of all infants within three days of age. The infants weighed between 460-1400 g, and had a median Apgar score of 6 at one minute of age, 6 at 5 minutes, and 8 at 10 minutes 8.

All blood samples from term and preterm newborn infants and adults **(Paper I, II, III and IV**) were collected in Sodium Heparin tubes (Vacuette, Hettich Labinstrument AB, Sollentuna, Sweden).

Preparation of chemoattractans (Paper I, II, III and IV).

fMLP and IL-8 (Sigma-Aldrich, St Louis, MO, USA) were serially diluted in a buffer consisting of 53% RPMI 1640 (Gibco by Life Technologies, Carlsbad, CA, USA), 13% HyClone defined FBS (Nordic Biolabs, Täby, Sweden) and sodium bicarbonate (Sigma-Aldrich) mixed with 33% HBSS (Gibco by Life Technologies).

Preparation of leukocytes.

Paper I: Leukocytes from newborn infants and adults were isolated from heparinized blood by means of dextran sedimentation (20), and contained 65-75% neutrophils with no difference in distribution between neonates and adults. Leukocytes were then suspended in buffer to the remaining concentration of 1 x 10^7 cells/mL. The final number of leukocytes in each cell well consisted of 1 x 10^5 cells.

Paper II: One mL samples of heparinized blood treated with IL-8, fMLP or negative control were mixed with 1 mL fixation buffer (0.4 % (w/v) paraformaldehyde in PBS). The samples were incubated for exactly 4 minutes at 37°C, and then 40 mL lysis buffer (155 mM NH_4Cl, 10 mM Tris (hydroxymetyl)-aminomethan, pH 7.4) was added and incubation at 37°C was continued for 15 minutes. Finally the cells were washed three times with 5 mL PBS-0.1% HSA and then suspended in 600 µL PBS-0.1% HSA and kept at 4°C.

The under-agarose cell migration assay (Paper I).

The assay was performed according to the principles outlined by Heit and Kubes (Heit and Kubes, 2003). Agarose gels were cast on tissue culture plates (Sigma-Aldrich, St Louis, MO, USA) and stored overnight at 4°C to make the gels firm and to facilitate punching of the wells. Wells were punched the following day at a standardized distance of 2.5 mm from each other. Leukocytes from newborn infants and adults were added simultaneously to different tissue culture plates, for each experiment. Leukocytes (1 x 10^5 per well) were added to each of the two outer wells, in order to demonstrate that the migration was directed towards the gradient of the central well. Chemoattractant or buffer was added to central wells. IL-8 was used at concentrations of 0.1 and 1 µM and fMLP at concentrations of 0.01, 0.1 and 1 µM. Gels were incubated for 2 hours at 37°C , 5% CO_2, whereby only neutrophils migrated outside the wells. Methanol (100%) was added to each well to terminate the migration and fix the cells. The gels were stored overnight at 4°C.

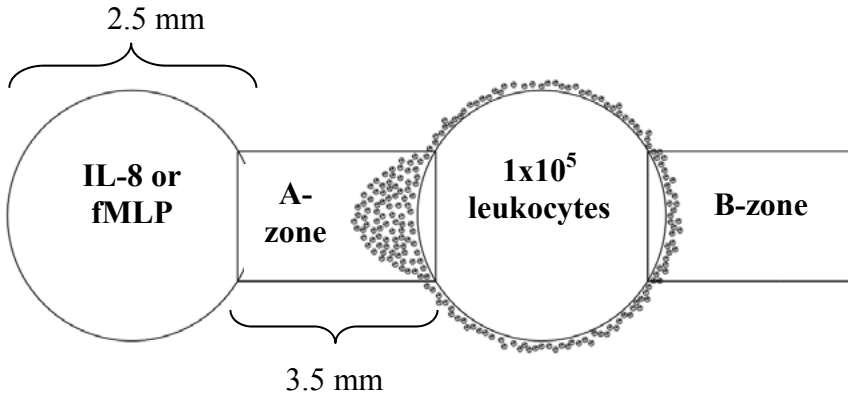

Figure 2. A schematic representation of the under-agarose assay. The wells were loaded with $1x10^5$ leukocytes/well, from a newborn infant or an adult. The central wells were loaded with IL-8 or fMLP or buffer (controls). By subtracting the number of neutrophils that migrated into the B-zone from the number in the A-zone, the number of neutrophils undergoing chemotaxis could be determined.

Analysis of neutrophil chemotactic response (Paper I).

The gels were removed and the cells were stained with Wright's stain (Sigma-Aldrich, St Louis, MO, USA). Photos of the migration were taken with an inverted phase contrast microscope (Nikon Diaphot 300). Chemotactic neutrophils were distinguished histologically by their size and segmented nucleus. Only neutrophils were detected outside the wells, and they were counted in the predefined target zones A and B with a width of 0.5 mm. Randomly migrating neutrophils appear in equal numbers in target zone A and B, while both chemotactic and randomly migrating cells are found in the A-zone. The number of chemotactic neutrophils was thereby calculated by subtracting the number of cells in the B-zone from cells in the A-zone. All experiments were made simultaneously in adults and neonates, with 3 adults and 3 neonates during testing with IL-8, and 5 adults and 5 neonates during testing with fMLP. No patients or experiments were excluded.

Migration distance and correlation to the number of migrating cells (Paper I).

The distance the neutrophils migrated under the agarose was measured from the edge of the well to the edge of the leading front (inside the A-zone). The correlation between the distance the cells migrated to gradients of IL-8 or

fMLP and the number of migrating neutrophils in the A-zone was measured in order to define the pattern of migration of the neutrophil population.

Preincubation with fMLP and IL-8.

Paper II: Heparinized whole blood, 1 mL, was mixed with IL-8, fMLP, or negative control (buffer), to the final concentration of 1 µM IL-8 and 0.1 µM fMLP and incubated (37°C) for 15 minutes. The concentrations of the chemoattractants were previously defined by maximum chemotaxis.

Paper III: Heparinized whole blood, 500 µL, was mixed with 5 µL fMLP, 5 µL IL-8 or 5 µL negative control (53% RPMI 1640, 13% HyClone defined FBS and sodium bicarbonate mixed with 33% HBSS). The final concentration of both fMLP and IL-8 was 0.01 µM. Testing 15 to 30 minutes of preincubation, revealed that the optimal time was 15 minutes. After preincubation for 15 min at 37°C, the samples were put on ice and placed in the dark for 10 min.

Paper IV: Heparinized whole blood, 200 µL, was mixed with 2 µL fMLP, 2 µL IL-8 or 2 µL negative control. After incubation for 15 min at 37°C, the samples were put on ice and placed in the dark for 10 min.

Labeling of leukocytes with antibodies to cell surface antigens.

Fifty µL samples of leukocyte suspension were incubated with FITC-labelled antibodies to CD11b, CD18, CD35, CD64, CD65, CD181, CD182, PE-labeled anti-CD44 or unlabelled antibodies to CD15s, CD88, and CD162, respectively, combined with PE- or FITC-labelled anti-CD14, for 30 minutes in 4°C. After incubation all samples were washed with 2 mL PBS. Samples treated with anti-CD15s, anti-CD88 and anti-CD162 were incubated with 1µL FITC-conjugated rabbit-anti-mouse Ig G for 30 minutes in 4°C, washed, incubated with anti-CD14-PE and finally washed with 2 mL PBS. After the last centrifugation the leukocytes were diluted in 300 µL PBS-0.1% HSA. The tubes were placed in dark at 4°C until flow cytometry analysis was performed of neutrophils.

Dose response.

Paper III: To study the modulation of burst induced by preincubation with fMLP and IL-8, respectively, heparinized blood was preincubated with fMLP and IL-8 at concentrations between 1 x 10^{-5} – 1 x 10^{-16} M followed by analysis of respiratory burst induced by E.coli or PMA as described above. Dose response curves for PMA defined the maximal respiratory burst to be induced by 1.35 µM for PMA, as in the instructions from the manufacturer (Phagoburst™, Glycotope Biotechnology, Heidelberg, Germany). Dose responses of granulocytes preincubated with a fixed concentration of fMLP (0.01 µM) and subsequently activated by increasing PMA concentrations (0.0135 µM, 0.135 µM and 1.35 µM) were also tested. The optimal concentration of *E.coli* was 2.5 x 10^8 bacteria/mL, in accordance with the manufacturer's recommendation. We also confirmed the weak burst response of granulocytes to fMLP compared to that of PMA and E.coli, by measuring burst response to 0.01 µM, 0.1 µM and 1.0 µM fMLP, separately and according to the manufacturer's instructions. The amounts of positive neutrophils were 2 ± 0.5%, 3 ± 1.6%, and 11 ± 9.5% respectively, compared with the negative control of 1.0 ± 0.1%, and the MFI of the positive neutrophils were 0.69±0.14, 0.55±0.10 and 0.76±0.11 respectively compared to the negative control of 0.99±0.16.

Paper IV: Dose response curves of fMLP (n=8) and IL-8 (n=5) at 15 minutes preincubation (37°C), followed by stimulation with PMA or *E.coli* (Glycotope Biotechnology, Heidelberg, Germany), defined the maximal burst reduction. The optimal concentration of PMA of respiratory burst was 1.35 µM and 2.5 x 10^8 *E.coli* bacteria/mL (Phagoburst™, Glycotope Biotechnology, Heidelberg, Germany).

Respiratory burst assay (Paper III and IV).

The Phagoburst™ Glycotope assay kit was purchased from Glycotope Biotechnology (GmbH, Heidelberg, Germany). PMA was diluted to 8.1 µmol/L in Wash solution, and the dihydrorhodamine 123 fluorogenic substrate (DHR-123; Glycotope Biotechnology) was prepared according to the manufacturer's instructions. The assay was performed according to the principles outlined in the manufacturer's instructions. In short, whole blood was preincubated for 15 min with either fMLP, IL-8 or HBSS/RPMI-buffer and put on ice. Then 50 µL of the preincubated blood was added to each tube with 10 µL PMA (duplicate), 10 µL *E.coli* (duplicate) or 10 µL Wash solution (negative control). The final concentration of PMA was 1.35 µM PMA and of *E.coli* 2.5 x 10^8 /mL. The tubes were incubated in a water bath at 37°C for 10 min. The substrate (10 µL; DHR 123) was added to each tube and then

incubated for an additional 10 min. The reaction was terminated by the addition of preheated hemolysing solution: 1 mL in newborn infants and 750 µL in adults. The tubes were incubated at room temperature for 30 minutes with whole blood from term neonates and adults, and 60 minutes with blood from preterm infants and then centrifuged for 5 minutes at 240 x g and 4°C, after which, the supernatants were discarded. The cells were washed twice with 1000 µL Wash solution, and finally 500 µL of Wash solution was added. To assure reduced substrate activity did not affect performance, the cells were kept in the dark on ice until analysis by flow cytometry, which was within 45 minutes. All experiments were completed within 5 h of obtaining the blood samples, to assure durability and validity.

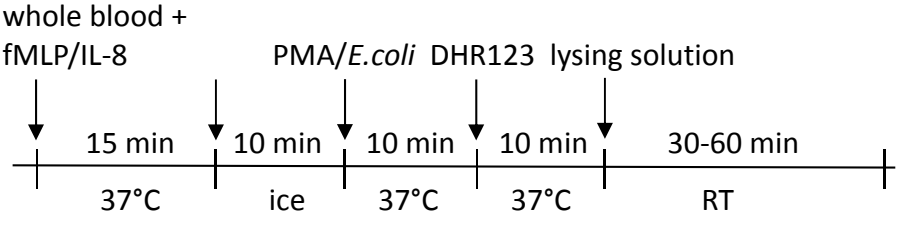

Figure 3. Procedure of respiratory burst *in vitro*. In short, whole blood was preincubated with fMLP, IL-8 or buffer before activating the blood with PMA or *E.*coli. After incubation with DHR123, lysing solution was added and the incubation time was 30 minutes with whole blood from term neonates and 60 minutes with blood from preterm infants.

Flow cytometry (Paper II, III and IV).

Flow cytometry analysis was performed with the flow cytometer EPICS-XL (**Paper II and III**) and by a Navios flow cytometer (**Paper IV**, Beckman Coulter, Bromma, Sweden for both). Granulocytes were identified from their forward and side scatter dot-plot profiles. A gate was set around the granulocyte population and the formation of ROS was measured as median fluorescence at 525 nm. In (**Paper II and III**) a minimum of 2000 events in the granulocyte gate were analyzed. Median fluorescence intensity (MFI) was analyzed in all comparisons. Ten thousand cells in the granulocyte gate were analyzed and mean fluorescence intensity (MFI) was analyzed in all comparisons (**Paper IV**). The relative number (%) of positive neutrophils were defined as neutrophils showing a fluorescence higher than that of non-stimulated neutrophils.

Statistical analyses.

All statistical tests were two-sided and analyzed with IBM SPSS Statistics 20. All comparisons in **Paper I**; between newborn infants and adults, between different concentrations of IL-8, fMLP and random migration and the distance the cells migrated were made with Mann-Whitney U-test for Independent samples. A p-value ≤ 0.05 was considered significant.

All comparisons of receptor expression before and after stimulation (**Paper II**) were analyzed by related-samples Wilcoxon Signed Rank test, and Mann-Whitney U test for Independent samples was used to detect differences between newborn infants and adults (**Paper II and III**). All results for detecting differences between control, preincubation and stimulation (**Paper III and IV**) were analyzed with Wilcoxon Signed Rank test.

Ethical approval.

Ethical approval for **Paper I, II and III** (Ups 03-692) and for **Paper IV (**2011/335) by the Central Ethical Review Board at Uppsala University, Uppsala, Sweden. Written informed consent was obtained from the parents of all term and preterm newborn infants, as well as from the adults, before recruitment.

Summary of results

The under-agarose cell migration assay (Paper I).

Dose response.

A reduced number of neutrophils from newborn infants migrated chemotactically towards IL-8 in comparison to adults ($p<0.05$; Figure 4AB). The number of chemotactic neutrophils migrating towards different concentrations of fMLP, did not differ in neonates and adults. In both neonates and adults, a chemotactic response was detected towards concentrations of ≥ 0.1 μM IL-8 or fMLP (Figure 4AB). In neonates and adults, the number of randomly moving neutrophils was the same and did not change with increasing concentrations of the chemoattractants (Figure 4AB).

Migration distance

Neutrophils from neonates migrated a shorter distance towards both concentrations of IL-8 in comparison to neutrophils from adults (Table 2). In contrast, migration towards all concentrations of fMLP, was equidistant for neutrophils from neonates and adults (Table 2).

Correlation between number of neutrophils and migration distance

There was a strong positive linear correlation between the number of migrating neutrophils and the distance they migrated towards IL-8 and fMLP in both neonates and adults, thus neutrophils were migrating homogenously in all instances. The populations of neutrophils from newborn infants and adults were distinct in response to IL-8 with few cells migrating a shorter distance in neonates (Table 2). No such distinction could be made between neonates and adults with respect to the cells migrating towards fMLP.

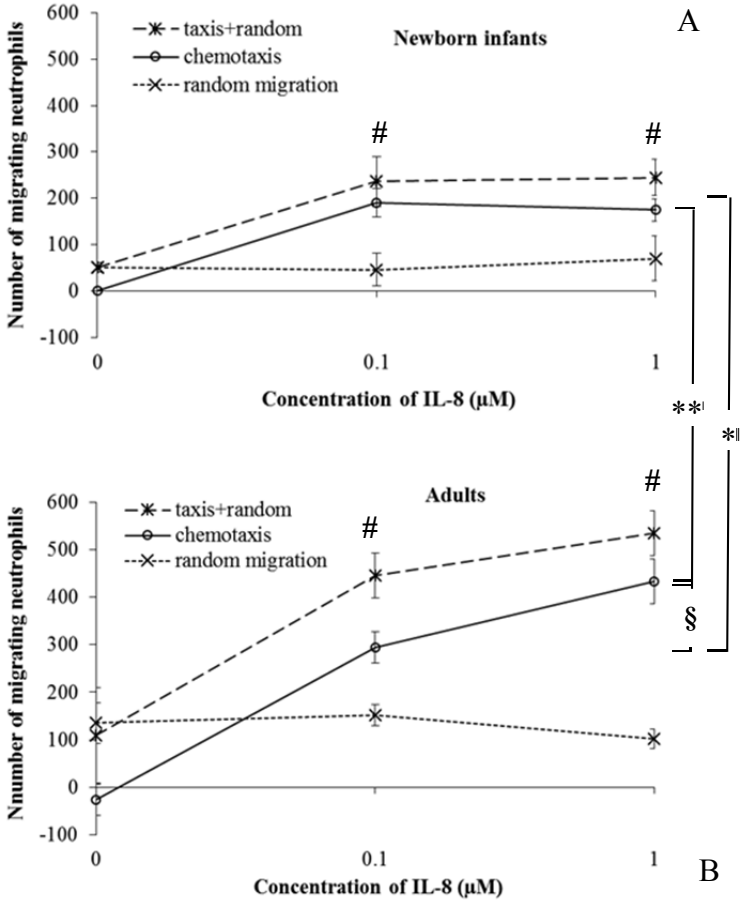

Figure 4AB. Dose response curves of migrating neutrophils to gradients of IL-8. Leukocytes, from (**A**) newborn infants and (**B**) adults were exposed to different concentrations of IL-8 (controls, 0.1 µM and 1 µM) and buffer. Results are presented as mean ± SEM, and chemotaxis (taxis) was distinguished from random migration (random). *p<0.05 indicates significant difference between neonates and adults at 0.1 µM IL-8, and **p<0.001 at 1.0 µM IL-8. §p<0.05 indicates significant difference between 0.1 µM and 1.0 µM IL-8 in adults. At all concentrations of IL-8, a significantly higher number of neutrophils from both neonates and adults migrated towards IL-8 than to buffer (#p<0.001).

Table 2. Neutrophil migration distance (mean ± SEM) towards different gradients of IL-8 and fMLP.

Chemo-attractant	**Concen-tration**	**Migration distance (µm)** Newborn infants	**Migration distance (µm)** Adults	**p-value**
IL-8	0	145 ± 15	223 ± 18	NS
IL-8	0.1 µM	221 ± 30	724 ± 155	<0.01
IL-8	1.0 µM	412 ± 61	890 ± 130	<0.001
fMLP	0	167 ± 13	215 ± 24	NS
fMLP	0.01 µM	186 ± 10	189 ± 16	NS
fMLP	0.1 µM	339 ± 42	359 ± 59	NS
fMLP	1.0 µM	270 ± 20	373 ± 82	NS

Neutrophil receptor expression (Paper II).

Resting neutrophils from newborn infants displayed a higher expression of CD35, CD64 and CD65 than resting neutrophils from adults, ($p<0.05$, $p\leq0.001$ and $p\leq0.001$ respectively). In contrast, a lower expression of CD15S and CD44 ($p<0.05$ and $p<0.01$ respectively) was found on resting neutrophils from the newborn infants when compared with neutrophils from adults.

Neutrophil receptor expression after stimulation with IL-8.

IL-8 up regulated the expression of CD11b, CD18 and CD35 on neutrophils from both neonates and adults. On the contrary, IL-8 reduced neutrophil expression of CD162, CD44, CD181 and CD182 in both neonates and adults. IL-8 also decreased the expression of CD15S and CD88 on neutrophils from adults ($p\leq0.05$ for both). The increase in neutrophil expression of CD11b and the suppression in expression of CD88 and CD182 induced by IL-8, demonstrated a lower magnitude in neonates than in adults ($p<0.05$, $p<0.05$ and $p\leq0.001$ respectively, Table 3A).

Neutrophil receptor expression after stimulation with fMLP.

The alterations in neutrophil receptor expression induced by fMLP displayed comparable magnitudes in neonates and adults (Table 3B). fMLP up regulated the neutrophil expression of CD11b and CD35 in both newborn infants and adults. In addition, an increased expression of CD18 on neutrophils from neonates and of CD65 on neutrophils from adults ($p\leq0.01$ for both) was demonstrated. Moreover, neutrophil expression of CD181 and CD182 was reduced by fMLP in both newborn infants and adults. fMLP also decreased

neutrophil expression of CD162 and CD44 in newborn infants and of CD15S in adults.

Table 3. Comparison of neutrophil receptor expression (mean fluorescence intensity) in newborn infants and adults after incubation with 0.01 μM IL-8 and 0.001 μM fMLP. Median change (Δ) of neutrophil receptor expression.

IL-8	**Newborn infants**		**Adults**		**p-value**
receptor	Median (Δ)	n	Median (Δ)	n	
CD11b	4.2	16	10.1	17	<0.05
CD18	1.9	16	4.0	17	NS
CD65	1.8	16	0.8	17	NS
CD15S	-1.1	16	-3.7	14	NS
CD162	-1.3	16	-1.6	17	NS
CD44	-6.1	16	-9.9	16	NS
CD35	1.2	16	2.2	17	NS
CD88	-1.7	16	-6.3	15	<0.05
CD181	-4.2	16	-4.6	17	NS
CD182	-0.9	15	-1.8	17	≤0.001
fMLP	**Newborn infants**		**Adults**		**p-value**
receptor	Median (Δ)	n	Median (Δ)	n	
CD11b	4.8	11	3.6	11	NS
CD18	1.8	11	1.7	11	NS
CD65	-0.4	10	1.5	11	NS
CD15S	-1.2	11	-4.6	11	NS
CD162	-1.2	11	-0.7	10	NS
CD44	-6.8	11	-5.0	11	NS
CD35	1.7	11	0.9	11	NS
CD88	-4.2	10	-3.3	10	NS
CD181	-4.5	11	-2.1	11	NS
CD182	-1.3	11	-1.1	11	NS

Neutrophil respiratory burst in full term newborn infants (Paper III).

PMA induced a markedly increased neutrophil respiratory burst ($p<0.01$) compared with negative controls both in newborn infants and adults. *E.coli* also induced an increased respiratory burst ($p<0.01$) in both neonates and

adults. In general, stimulation with PMA or *E.coli* only, displayed similar levels of burst in neutrophils from newborn infants and adults.

Dose response

Dose response curves with varying concentrations of fMLP and IL-8 were generated in order to define a concentration dependent modulation of respiratory burst by fMLP and IL-8, induced by PMA or *E.coli* in neutrophils from adults. There was a gradual decrease of the production of ROS, with increasing concentrations of fMLP, induced by PMA or *E.coli*. At the concentration of 0.01 μM fMLP, the maximal reduction with fMLP in response to PMA or *E.coli* was observed. Preincubation with fMLP at the concentration of 0.01 μM markedly reduced respiratory burst of neutrophils from adults activated by PMA at concentrations of 0.135 and 1.35 μM. No modulation of the production of ROS induced by PMA or E.coli could be detected with increasing concentrations of IL-8.

Burst after preincubation with fMLP and IL-8

fMLP reduced respiratory burst induced by PMA ($p<0.05$ in both) and *E.*coli ($p<0.01$ and $p<0.05$ respectively) in neonates and adults Newborn infants and adults displayed a reduction of the same magnitude (Δ) in PMA- (NS, Figure 5) and *E.coli*-induced (NS, Figure 6) production of ROS after preincubation with fMLP (Table 1). In all experiments with fixed concentrations of PMA and *E.coli*, and after preincubation with different concentrations of fMLP, ROS was detected in >97% of the neutrophils with no hypo- or hyper-responsive subpopulations. Preincubation with IL-8 did not modify burst in neutrophils, induced by either PMA or *E.coli*, neither in newborn infants nor in adults.

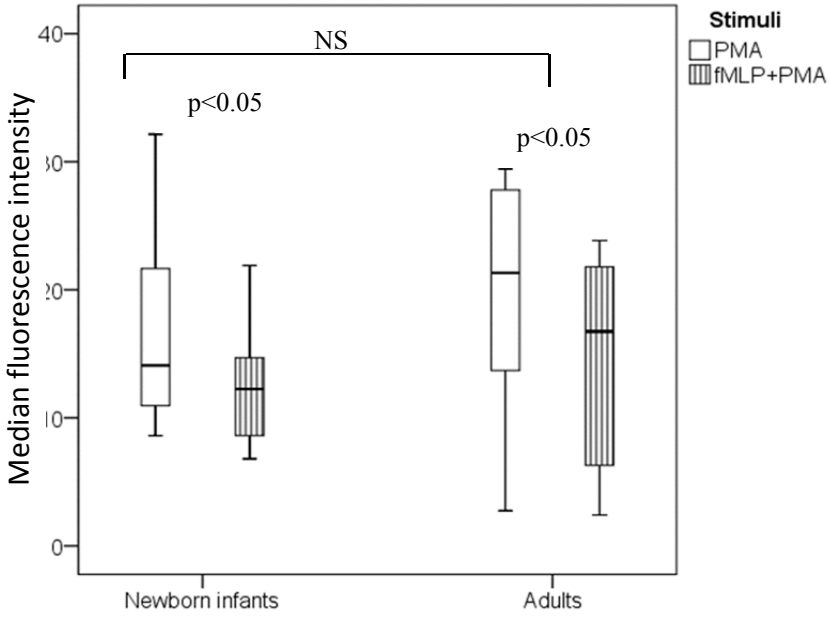

Figure 5. The influence of preincubation with fMLP on neutrophil respiratory burst in response to PMA in newborn infants and adults. Preincubation with fMLP (0.01 μM) on neutrophil respiratory burst in response to PMA (1.35 μM) was compared with respiratory burst induced by PMA only, in newborn infants (n = 10) and adults (n = 10). Results are presented as median fluorescence intensity. The reductions of respiratory burst (MFI) are presented with median (bold line within the box, 25th percentile (lower limit of the box), 75th percentile (upper limit of the box), 10th, and 90th percentiles (whiskers) are represented. Significant differences ($p<0.05$) between the response to PMA alone and the combination of fMLP and PMA are indicated.

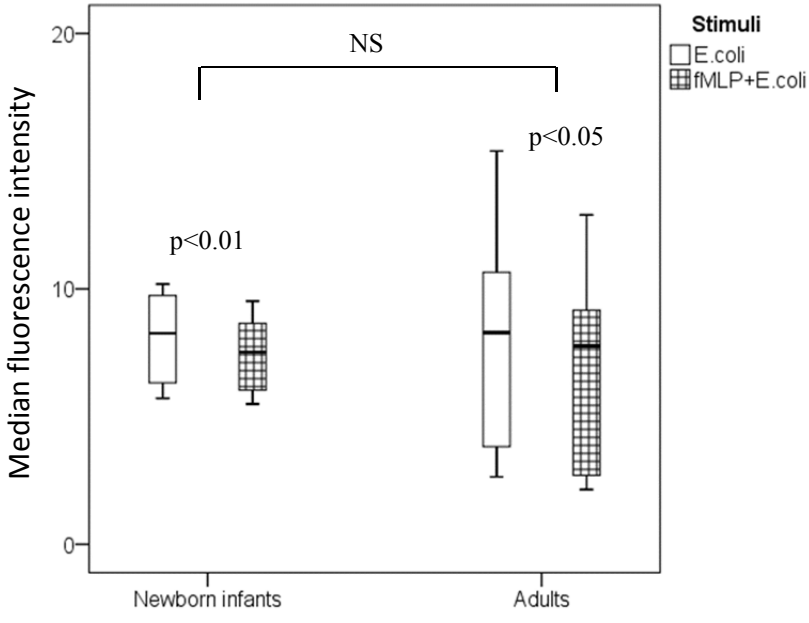

Figure 6. The influence of preincubation with fMLP on neutrophil respiratory burst in response to *E.coli*. Preincubation with fMLP (0.01 μM) on neutrophil respiratory burst in response to *E.coli* bacteria (1-2 x 10^7), compared with respiratory burst induced by *E.coli* only, in newborn infants (n = 10) and adults (n = 10). Results are presented as median fluorescence intensity. The reductions of respiratory burst (MFI) are presented with median (bold line within the box), 25th percentile (lower limit of the box), 75th percentile (upper limit of the box), 10th, and 90th percentiles (whiskers) are represented. Significant differences ($p<0.05$ and $p<0.01$) between the response to E. coli alone and the combination of fMLP and *E.coli* are indicated.

Neutrophil respiratory burst in preterm newborn infants (Paper IV).

Characteristics of the mothers.

Five of the mothers were treated with tocolytica to suppress premature labor. One mother received labetalol, a treatment for high blood pressure. Antenatal steroids were administered to all mothers, but in one case less than 8 hours before delivery. If suspected infection (choriamnionitis), mothers received antibiotics prior to delivery.

Characteristics of the infants.

Postnatal characteristics of the infants are found in Table 4. Seven of eight infants were born extremely preterm (<28 GA weeks, Table 4). Even though four of eight mothers were considered to have clinical signs of chorioamni-

onitis, none of the infants had positive blood cultures or other signs of infections at the time of blood sampling, and were considered ventilatory and circulatory stable (Table 3.) Since infection could not be excluded as a reason for preterm delivery, seven infants received antibiotics (gentamicin and benzyl penicillin), all infants received prophylactic treatment for apnea (caffeine), and four received prophylactic antifungal treatment (fluconazole) according to standard clinical procedure. Long term outcomes for all infants are presented in Table 5, where none of the infants had signs of NEC at the time of blood sampling (less than 3 days of age).

Table 4. Postnatal characteristics. Gestational age (GA) and body weight (BW). Apgar score at one minute, 5 minutes and 10 minutes of age. CRP at time of blood sampling.

Patient No.	GA	BW (g)	Apgar 1	Apgar 5	Apgar 10	No of doses of surfactant
1.	23+0	460	3	4	7	1
2.	23+0	720	2	6	7	1
3.	23+0	588	8	9	9	1
4.	24+4	813	4	6	7	1
5.	26+2	940	7	9	9	1
6.	26+4	735	6	5	8	1
7.	27+1	1040	8	9	9	0
8.	29+2	1365	5	6	8	1

Table 5. Long term outcome. Bronchopulmonary dysplasia (BPD) graded none, mild, moderate and severe; necrotizing enterocolitis (NEC) needing operation; retinopathy of prematurity (ROP) needing operation; ligated patent ductus arteriosus (PDA): intraventricular hemorrhage (IVH) graded I-IV; periventricular leukomalacia (PVL).

Patient No.	BPD	NEC	ROP	PDA	IVH	PVL	Died
1.	No	Yes	No	No	0	Yes	Yes
2.	No	Yes	No	No	0	Yes	Yes
3.	Moderate	No	Yes	No	II	No	No
4.	Mild	No	No	No	0	No	No
5.	No	No	No	No	0	No	No
6.	No	No	No	No	0	No	No
7.	No	No	No	No	0	No	No
8.	No	No	No	No	0	No	No

Neutrophil respiratory burst.

Stimulation by PMA induced a markedly increased neutrophil respiratory burst (median 19.5: $p<0.05$) compared to negative controls in preterm newborn infants (median 0.3). Activation by *E.coli* also induced an increased respiratory burst ($p<0.05$) in preterm infants (median 11.9; compared to controls, median 0.3). PMA generated a higher neutrophil respiratory burst than *E.coli* in these infants ($p<0.05$). Neutrophils from the two youngest patients (Patients 1 and 2), later diagnosed with NEC, displayed an atypical pattern of respiratory burst (Figures 2). About one-third of the neutrophils formed a population with a markedly reduced respiratory burst. This pattern was most pronounced when neutrophils were stimulated with PMA.

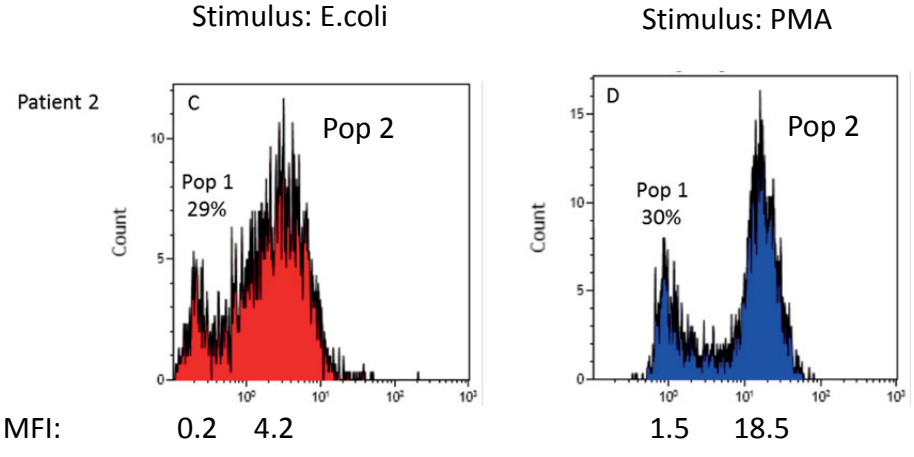

Figure 7. Respiratory burst response to E.coli and PMA of neutrophils from patient 2. The estimated proportion (%) of neutrophils with a low response and the mean fluorescence intensity (MFI) of the two populations are depicted.

Dose response.

In order to define a concentration dependent modulation of respiratory burst by fMLP (n=8) and IL-8 induced by PMA or *E.coli* in neutrophils from preterm neonates, dose response curves with varying concentrations of fMLP and IL-8 (n=5) were generated. With increasing concentrations of fMLP, there was a tendency to decrease of respiratory burst induced by PMA in preterm newborn infants born at >25 weeks of gestational age (NS). The maximal reduction with fMLP in response to PMA was observed at a concentration of 0.1 μM fMLP.

Burst after preincubation with fMLP and IL-8

In neutrophils from preterm neonates, fMLP reduced burst in response to PMA by 25% compared to single stimulus with PMA (n=8, NS, Figure 8). No modification of neutrophil respiratory burst was observed with fMLP in

response to *E.coli* (Figure 9). Preincubation with IL-8 did not modify burst (n=5) in neutrophils in response to either PMA or *E.coli*. ROS was detected in all neutrophils studied in response to PMA and *E.coli*, and after preincubation with fMLP or IL-8. Hypo- and hyperresponsive subpopulations were detected in preterm infants born 23 weeks of gestational age (Patients 1 and 2).

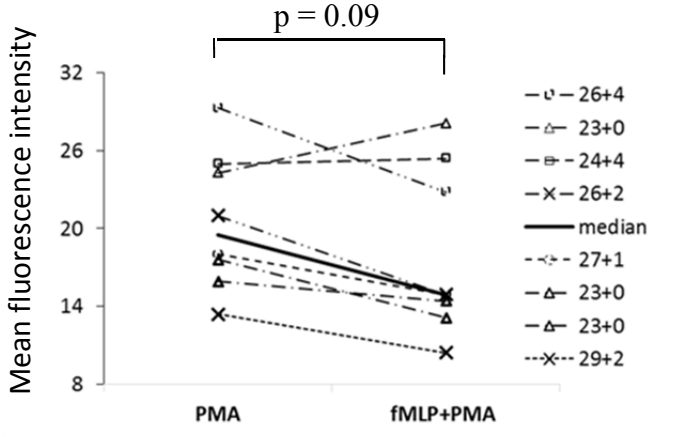

Figure 8. The influence of preincubation with fMLP on neutrophil respiratory burst induced by PMA (n=8) in preterm newborn infants. Preincubation with fMLP (0.01 µM) induced by PMA (1.35 µM), compared with respiratory burst induced by PMA only. Results are presented as mean fluorescence intensity.

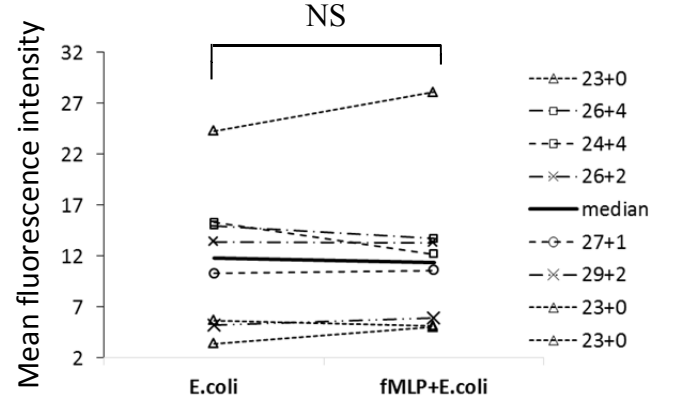

Figure 9. The influence of preincubation with fMLP on neutrophil respiratory burst induced by *E.coli* (n=8) in preterm newborn infants. Preincubation with fMLP (0.01 µM) induced by *E.coli* bacteria (1-2 x 10^7), compared with respiratory burst induced by *E.coli* only. Results are presented as mean fluorescence intensity.

Discussion

A reduced number of chemotactic neutrophils from term newborn infants, migrated a shorter distance towards IL-8, in comparison to neutrophils from adults (Figure 2, Table 2). We also observed a reduced modulation of receptor expression involved in adhesion and chemotaxis to IL-8, in neonates (Table 3A). Urlichs et al also observed a compromised response to intermediate chemoattractants (Urlichs, 2004). In contrast, the number of migrating neutrophils and the distance they migrated towards fMLP was the same in neonates and adults (Paper I). This normal response to fMLP, also observed by Birle et al (Birle et al, 2015) was supported by the same neutrophil receptor response in neonates and adults; for all the receptors involved in adhesion, chemotaxis, granule activation and phagocytosis (Table 3B).

This differential response towards intermediate and end-target chemoattractants could result in reduced accumulation of neutrophils at the site of infection. And in turn a delayed and reduced protective immune response against microbes. Increased bacterial growth may then lead to a progressive infection which may deplete the bone marrow neutrophil pool in newborn infants (Polin and Fox, 1998). Since neutrophils are the first immune cells accumulating at the site of infection, the reduced immune response in neonates could be detrimental. IL-8 induces the transmigration from the blood into the tissue, which is preceded by endothelial cell activation and neutrophil adherence to the endothelium. Chemotactic neutrophils are guided through the tissue into the site of infection, by increasing concentrations of different chemoattractants. When finally reaching the microbes, the neutrophils start to produce and release ROS to destroy them. (Ley, 2001)

Previous studies reveal that neutrophils from neonates interact less with endothelial cells, compared to neutrophils from adults (Pahwa et al, 1977, Shuster et al, 1995) which results in reduced transmigration towards chemoattractants like IL-8 (Krause et al 1986), and diminished antimicrobial defense (Fox et al, 2005). We observed in Paper II, in accordance with several other studies, that the number and affinity of receptors for fMLP are the same in neutrophils from neonates and adults (Birle et al, 2015, Anderson et al, 1981, Sacchi et al, 1984).

IL-8 and fMLP belong to the GPCR family, but stimulate different intracellular pathways, leading to an activation of cell functions differently. To regulate the signal transduction from GPCRs, the signaling can be interrupted, terminated or down regulated by internalization or recycling of the recep-

tors (Rajagopal et al, 2010). The general pattern of up and down regulation of receptors belonging to the GPCRs, by IL-8 and fMLP, was the same in neonates and adults.

The up regulation of the complement receptors CD11b, CD18 and CD35 by IL-8 or fMLP, on neutrophils from neonates and adults, have also been observed in adults during infection, possibly to clear pathogens (Furebring et al, 2006). The lack of down regulation of CD88 expression by IL-8, in term neonates might lead to a differential regulation of chemotaxis towards C5a compared with the regulation in adults (Furebring et al, 2006). During later stages of sepsis, C5a can contribute to multi organ failure which could be more emphasized in neonates because of less internalization of the C5a receptor (Kaufman, 1996, Giacioa, 1993). The IL-8 receptors CD181 and CD182 were down regulated by both IL-8 and fMLP. Both of the IL-8Rs mediate chemotaxis but only CD181 mediate production of ROS (Richardson et al, 1998). We observed a more pronounced down regulation of CD182 as compared to CD181 induced by either IL-8 or fMLP in accordance with the findings by Richardson et al, that receptor internalization induced by IL-8 is more rapid and need lower concentrations to internalize CD182 than CD181 (Richardson et al, 1998).

The chemotactic factor fMLP reduced the PMA- and *E.coli*-induced production of ROS in neutrophils from both neonates and adults. These results are in accordance with our under-agarose migration study, that neutrophil chemotactic response towards fMLP is the same in newborn infants and adults. Indicating that neonatal neutrophil response to end target attractants produced by bacteria is as developed as in adults.

With increasing concentrations of fMLP, there was a gradual decrease of the production of ROS, induced by PMA or *E.coli*, indicating that neutrophils is responsive to increasing concentrations of chemotactic stimuli. Previous studies demonstrate that neutrophils possess memory for chemotactic agents; during competitive migration and priming with proinflammatory agents like TNF-α before stimulus with fMLP (Foxman et al, 1997, Heit et al, 2002, 1993). In comparison to single stimulus with fMLP, priming with for example TNF-α, granulocyte/granulocyte-macrophage colony stimulating factor and IL-8 increased respiratory burst induced by 2nd stimulus with fMLP (Elbim et al, 1994, Kuijpers et al, 1991). Instead, we preincubated whole blood with fMLP, and subsequently stimulated with PMA and *E.coli.* It is still unclear at what stage neutrophils are exposed to different cytokines, why cautiousness is needed when interpreting the sequential design of in vitro experiments. Preincubation with IL-8 did not modulate the neutrophil production of ROS induced by PMA or *E.coli*, neither in preterm or term neonates, nor in adults (Paper III, IV). This could be depend on the fact that fMLP is a stronger chemotactic factor than IL-8 (Foxman et al, 1997, Heit et al, 2002). fMLP is released by bacteria, directing neutrophils to the site of bacterial infection for subsequent elimination by phagocytosis and ROS.

Since fMLP have the capacity to regulate and reduce the release and the detrimental effects of ROS, we speculate that this migratory factor could even divert immune cells away from the site of infection in both neonates and adults. This could in turn delimit the detrimental effects of uncontrolled inflammation, but further studies are needed to elucidate the clinical application.

The same concentrations for preincubation with IL-8 and fMLP (0.01 μM) were used in the study on preterm neonates and in the previous burst study on term neonates and adults (Paper III, IV). Therefore, conclusions can be drawn when comparing these studies.

In preterm newborn infants born at ≤29 weeks of gestational age, the production of ROS attended to decrease with increasing concentrations of fMLP induced by PMA ($p=0.09$, Figure 5).

In preterm newborn infants born at >25 weeks of gestational age, there was a reduction of the production of ROS in all patients (n=4/4: Figure 4). In preterm neonates born at <25 weeks of gestational age, fMLP did not reduce PMA-induced production of ROS. This regulation of the production of ROS appears to be absent in extremely preterm newborn infants, thus, uncontrolled burst causing oxidative stress and morbidity because of tissue injury in terms of BPD, NEC, ROP, and PVL (Speer 2003, Neu et al, 2011, Leviton et al, 2013). No modification of neutrophil burst in preterm neonates was observed with fMLP in response to *E.coli* (Figure 6), in contrast to the modulation previously observed in term neonates and adults. This is also an absent regulation of the release of ROS by fMLP induced by *E.coli*, eventually causing oxidative stress and tissue injury.

Two of the youngest patients, born 23 weeks of gestational age, suffered from NEC and PVL and died at 5-6 days PNA. These two preterm neonates had a sub population of neutrophils with a markedly reduced production of ROS in response to PMA. This implied that extremely preterm neonates had a diminished ability to produce ROS resulting in a reduced capacity to eliminate microbes. In contrast, no hypo- or hyper-responsive subpopulations were detected in term neonates or in adults, with fixed concentrations of PMA and *E.coli*, and after preincubation with different concentrations of fMLP, instead ROS was detected in >97% of the neutrophils.

Conclusions

A diminished response was observed in newborn infants towards the intermediate chemoattractant IL-8, in terms of reduced chemotaxis and modulation of receptors involved in adhesion, chemotaxis and phagocytosis compared to adults.

The chemotactic factor fMLP reduced the PMA- and *E.coli*-induced production of ROS in neutrophils from both neonates and adults. These results are in accordance with our under-agarose migration study, where neutrophil chemotactic response towards fMLP is the same in newborn infants and adults. In addition with a similar modulation of receptor expression involved in adhesion, migration, granule activation and phagocytosis after stimulus with fMLP indicates that neonatal neutrophil response to end target attractants (fMLP) produced by bacteria is as developed as in adults.

Since fMLP have the capacity to regulate and reduce the release of ROS in full term neonates, this could delimit the detrimental effects of uncontrolled inflammation, but further studies are needed to elucidate the clinical application.

Although a tendency to respiratory reduction by fMLP was observed in preterm infants born >25 GA, a compromised modulation of neutrophil production of ROS in extremely preterm newborn infants could not be excluded, and further studies at different gestational and postnatal ages are required.

Summary in Swedish

Nyfödda barn är känsliga för infektioner på grund av ett omoget immunförsvar och detta kan leda till ökad sjuklighet och dödlighet. För tidigt födda barn, dvs barn födda <37 fulla gestationsveckor, föds ofta pga chorioamnionit som är en infektion av fosterhinnorna och fostervätskan, vilket kan leda till en ansamling av vita blodkroppar och inflammation i fostrets omogna lungor. Inflammationen kännetecknas av en initial neutrofil och därefter monocytär ansamling i lungan. Dessa cellers utdragna aktivitet och bildning av fria radikaler, stimulerad av bl a endotoxin och cytokiner, kan vara skadlig för lungan och leda till den kroniska lungsjukdomen bronchopulmonell dysplasi (BPD), och även påverka andra organ i form av nekrotiserande enterokolit (NEC) och periventrikulär leukomalaci (PVL). Det för tidigt födda barnets känslighet för höga nivåer av syrgas, som i sig är en kemoattraktant, beror bl a på ökad fri radikalbildning och låga nivåer av antioxidanter.

Vi observerade att kemoattraktanten fMLP kan reglera och minska frisättningen av fria radikaler från neutrofiler hos fullgångna nyfödda barn och vuxna, vilket skulle kunna begränsa de skadliga effekterna av okontrollerad inflammation. Vi såg även att nyfödda barns och vuxnas neutrofiler migrerade lika långt och i samma mängd mot fMLP, till skillnad mot IL-8, där både sträckan och antalet neutrofiler var reducerade hos nyfödda barn i jämförelse med vuxna. Dessutom var neutrofilers receptoruttryck för adhesion, kemotaxis, komplement och fagocytos efter stimulering med IL-8 lägre hos nyfödda barn jämfört med vuxna. Detta skulle kunna leda till en reducerad neutrofil aktivitet vid infektion och sänkt immunförsvar hos nyfödda barn.

Den modulerade neutrofila produktionen av fria radikaler med fMLP kunde påvisas hos för tidigt födda barn födda >25 fulla gestationsveckor, men inte hos mer omogna barn, vilket innebär att de mycket för tidigt födda barnen inte kan reglera sin produktion av fria radikaler. Ytterligare studier på för tidigt födda barn med olika gestations- och postnatal ålder krävs för att fastställa dessa fakta.

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