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THE ROLE OF CA^{2+} AND ROS IN ACTIVATION OF NEUTROPHILS WITH PHYSIOLOGICAL AGONISTS

VLOGA CA^{2+} IN REAKTIVNIH KISIKOVIH ZVRSTI V AKTIVACIJI NEVTROFILCEV S FIZIOLOŠKIMI AGONISTI

Diplomsko delo

Ljubljana, 2012

The present graduation thesis was realized at the Institute of Pharmacology at the University of Bern, with collaboration of the Faculty of Pharmacy, University of Ljubljana, Slovenia. I worked under the supervision of Prof. Dr. Irena Mlinarič-Raščan and Prof. Dr. Dr. Hans-Uwe Simon.

Acknowledgment

First of all, I would like to thank Prof. Dr. Dr. Hans-Uwe Simon for giving me the possibility to be part of his team and motivating and advising me when planning the project.

I also wish to thank Prof. Dr. Shida Yousefi for all the instructions, guidance and utter support she gave me.My work at the Institute of Pharmacology in Bern was also supported by my supervisor in Slovenia, Prof. Dr. Irena Mlinarič-Raščan. I am grateful to her for giving me the possibility to perform my graduation thesis at this Institute and her incredible support during my stay in Bern and after that.

This project was done in collaboration with Morshed Mahbubul, Poorya Amini, Inès Schmid, Kevin Oberson and Saša Rožman. Some of their results were used to fully detail my thesis. I thank them and all the members at the Institute of Pharmacology at the University of Bern. You are a great team and it was a pleasure to work with you. Also, I want to thank Ivana Duckinoska for reading the paper and correcting my English.

I would like to thank Kristina for always being by my side. Her support and patience helped me complete this work in due course.

And, finally, I would like to thank my family and family Petrović for always supporting me.

Statementment

Hereby, I testify having performed the experiments to the best of my knowledge and having written this thesis independently under the guidance of my supervisors: Prof. Dr. Irena Mlinarič-Raščan and Prof. Dr. Dr. Hans-Uwe Simon.

Ljubljana, September, 2012 Darko Stojkov

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Glossary

APC	allophycocyanin
ATG	autophagy-related gene
BAPTA	1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid
BK	Calcium dependent potassium channel
BSA	Bovine serum albumin
CAD	lentivirus used for the ATG5 overexpression
CGD	chronic granulomatous disease
CMI	cell-mediated immunity
C5a	complement component
DAG	diacylglycerol
DHR	dihydrorhodamine 123
DPI	diphenyleneiodonium chloride
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
F	fluorescence
FACS	fluorescent activated cell sorter
FCS	fetal bovine serum
FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionine-leucine-phenylalanine
FURA-2AM	fura-2-acetoxymethyl ester
G-CSF	granulocyte colony-stimulating factor
HSC	Hematopoietic stem cell
IL	Interleukin
IP3	inositoltrisphosphate
IP3R	inositoltrisphosphate receptor
LPS	Lipopolysaccharide
MCS	lentivirus used for the ATG5 overexpression
mTOR	mammalian target of rapamycin
NET	neutrophil extracellular traps
PAF	plated activated factor
PE	phycoerythrin

PerCP	peridin-chlorophyll
PMN	polymorphonuclear leukocyte
PLC	phosphatidylinositol-specific phospholipase C
PV	parvalbumin
R	ratio
ROS	reactive oxygen species
RT	room temperature
RyR	ryanodine receptor
SERCA	sarco - endoplasmic reticulum Ca^{2+} ATPase
SOD	superoxide dismutase
SCF	stem cell factor
WT	wild type
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Ca^{2+}]_c$	cytosolic calcium concentration

Abstract

In order to fight invading pathogens, neutrophils possess a potent machinery to produce reactive oxygen species (ROS), the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. ROS is also crucial in the execution of most neutrophil cell death mechanisms. We have studied the involvement of Ca^{2+} as a key second messenger in the activation of various processes and its role as a signal for oxidase activity leading to the production of reactive oxygen species in stimulated polymorphonuclear leukocytes (PMNs). In order to establish whether increased cytosolic Ca^{2+} concentration is an important signal for oxidase activity, after stimulation with complement component C5a, we measured intracellular Ca^{2+} using Fura-2AM as Ca^{2+} indicator. We studied the rapid elevation of intracellular Ca^{2+} concentration due to the depletion of its intracellular stores induced by inositol 1,4,5 – trisphosphate (IP3) that subsequently leads to Ca^{2+} influx from the extracellular space.

Evidence for the requirement of extracellular Ca^{2+} entry to activate neutrophil oxidases is based on our analysis showing significant decrease of superoxide anion production when extracellular Ca^{2+} was inhibited or chelated with EGTA treatment followed by C5a stimulation. BAPTA also caused a significant inhibition of ROS activity due to reduction in cytosolic Ca^{2+} concentration.

Using estrogen-regulated Hoxb8 mouse neutrophils that lacks $gp91^{phox}$ gene, an important component for oxidase activity, we concluded that lack of ROS activity leads to sustained high level of $[Ca^{2+}]i$ due to Ca^{2+} influx from the extracellular space. This Ca^{2+} entry is often referred to as capacitative or store operated Ca^{2+} influx. We also observed that DPI (diphenyleneiodonium chloride) treated human neutrophils or $gp91^{phox}$ knockout mature mouse neutrophils have an increase of $[Ca^{2+}]i$ despite the lack of ROS activity. This suggests that ROS is not required for initial increase of intracellular Ca^{2+}, and it is indeed Ca^{2+} the one that activates different pathways of ROS production.

Taken together, our results show that physiological $[Ca^{2+}]i$ is required for regulation of ROS activity, acting in a synergy with Ca^{2+} influx from the extracellular space.

Keywords: *neutrophils, NADPH oxidase, reactive oxygen species, estrogen-regulated Hoxb8 mouse neutrophils*.

Povzetek

Za obrambo pred napadajočimi patogeni imajo nevtrofilci visoko sposobnost proizvajanja reaktivnih kisikovih zvrsti (ROS), in sicer preko fagocitnih izoform nikotinamid adenin dinukleotid fosfata (NADPH) oksidaze. ROS igra tudi ključno vlogo pri izvršitvi večine nevtrofilnih mehanizmov, ki vodijo v celično smrt. Preučevali smo vlogo Ca^{2+} kot ključnega sekundarnega prenašalca pri aktiviranju različnih procesov in njegovo vlogo kot aktivatorja oksidazne aktivnosti, ki vodi do proizvajanja reaktivnih kisikovih zvrsti v stimuliranih polimorfonuklearnih levkocitih (PMN-jih). Da bi ugotovili, ali je po stimulaciji s podenoto komplementa C5a povečana koncentracija citozolnega Ca^{2+} pomemben signal za aktivnost oksidaze, smo izmerili znotrajcelični Ca^{2+}, za kar smo uporabili Fura-2AM kot Ca^{2+} indikator. Raziskovali smo hiter dvig znotrajcelične koncentracije Ca^{2+}, ki je nastala zaradi izčrpanja znotrajceličnih zalog, kar je povzročil inositol 1,4,5 – trifosfat (IP3), ki posledično vodi do pritoka Ca^{2+} iz zunajceličnega prostora.

Dokaz za potrebo vstopa zunajceličnega Ca^{2+} za aktivacijo nevtrofilnih-oksidaz, je zasnovan na naši analizi, ki je pokazala bistveno zmanjšanje produkcije superoksidnega aniona, kadar smo inhibirali Ca^{2+}, ali ga naprej kelirali z EGTA, in nato stimulirali s C5a. Tudi BAPTA je povzročila bistveno inhibicijo ROS aktivnosti zaradi zmanjšanja citozolne koncentracije Ca^{2+}.

Z uporabo estrogensko reguliranih Hoxb8 mišjih nevtrofilcev brez gena za $gp91^{phox}$, ki je pomemben element oksidazne aktivnosti, smo ugotovili, da vodi pomanjkanje ROS aktivnosti v trajno povišane koncentracije $[Ca^{2+}]_i$ zaradi pritoka Ca^{2+} iz zunajceličnega prostora. V tem primeru govorimo o kapacitativno vodenemu vstopu Ca^{2+}. Ugotovili smo, da imajo humani nevtrofilci obravnavani z DPI (difenilenjodonijev klorid), ali mišji nevtrofilci brez gena $gp91^{phox}$, povečan $[Ca^{2+}]$i in kljub temu pomanjkanje ROS aktivnosti. Iz tega sklepamo, da ROS ni potreben za začetno povečanje znotrajceličnega Ca^{2+} in je pravzaprav Ca^{2+} tisti, ki aktivira različne procese za proizvodnjo ROSa. Naši rezultati kažejo, da je fiziološki $[Ca^{2+}]$i potreben za regulacijo ROS aktivnosti v sinergiji s pritokom Ca^{2+} iz zunajceličnega prostora.

Ključne besede: *nevtrofilci, NADPH oksidaza, reaktivne kisikove zvrsti, estrogensko regulirani Hoxb8 mišji nevtrofilci.*

1. Introduction

Human immune system is a complex network of interacting cells, tissues, organs and humoral factors that work together to defend the body. The human body provides an ideal environment for many microbes and this is why the immune system is necessary for keeping them out, or, when failing to do that, to seek out and destroy them **(1).**

The immune system has a series of dual natures, the most important of which is self/non-self recognition. Self/non-self recognition is achieved by having every cell display a marker based on the major histocompatibility complex (MHC) which is encoded by a large cluster of genes with related function found on the short arm of chromosome 6. The importance of the MHC in the immune system is to allow T lymphocytes to detect cells, such as macrophages, that have ingested microorganisms. Proteins inside the cell are broken into fragments and displayed as peptide antigens by MHC proteins on the surface. Here, the immune system is able to discriminate between normal (self) antigens and foreign (non-self) that are potentially dangerous. When the process breaks down due to defects in certain MHC genes, the body fails to recognize self-antigens and this lead to autoimmune disorders **(2).**

The immune system relies on many mechanisms, and the main division between them is between the innate immunity and the acquired immunity system which are closely related to each other **(3).** The innate immunity is a less specific component and provides the first line of defense against infections. This system which consists of genetically programmed defence mechanisms reacts very quickly against molecular components found only in microorganisms. The cells involved in innate immunity, like neutrophils and dendritic cells recognize foreign substances with toll-like receptors (TLR), the receptors for innate immunity.

On the contrary, the acquired immune system is activated only when antigens deepest level get past thru the innate immune system. Although this response is delayed, it provides a more specific, answer to the deeper attack against the pathogens. In acquired immunity, the dominant forces are antigen-antibody reactions and lymphocytes such as T-cells and B-cells play important roles **(4).**

1.1 Neutrophils

Neutrophils provide the first line of defence of the innate immune system. They constitute 50-70% of the circulating white blood cells, are much more numerous than eosinophils (1-3%) or basophils (1%), and are, in fact, much more likely than macrophages to kill ingested microorganisms. Neutrophils are produced at the rate of 10^{11} cells per day in the bone marrow from stem cells that proliferate and differentiate in to mature neutrophils during hematopoiesis. The process of hematopoiesis generally maintains a steady state in which the production of mature blood cells equals their loss. Various mechanisms are included in regulating the hematopoiesis and a failure in one or a combination of those can have serious consequences on the expression of the hematopoietic cytokines, or could lead to unregulated cellular proliferation. Early in hematopoiesis, a multipotent stem cell differentiates along one of two pathways, giving rise to either a common lymphoid progenitor cell or a common myeloid progenitor cell. Its differentiation is controlled by several types and amounts of growth factors in the microenvironment of a particular stem cell or progenitor cell. Presence of the appropriate factors and cytokines allow the progenitor cells to proliferate and differentiate into the corresponding cell type **(4).** Figure 1 displays the differentiation pathways of the blood cells.

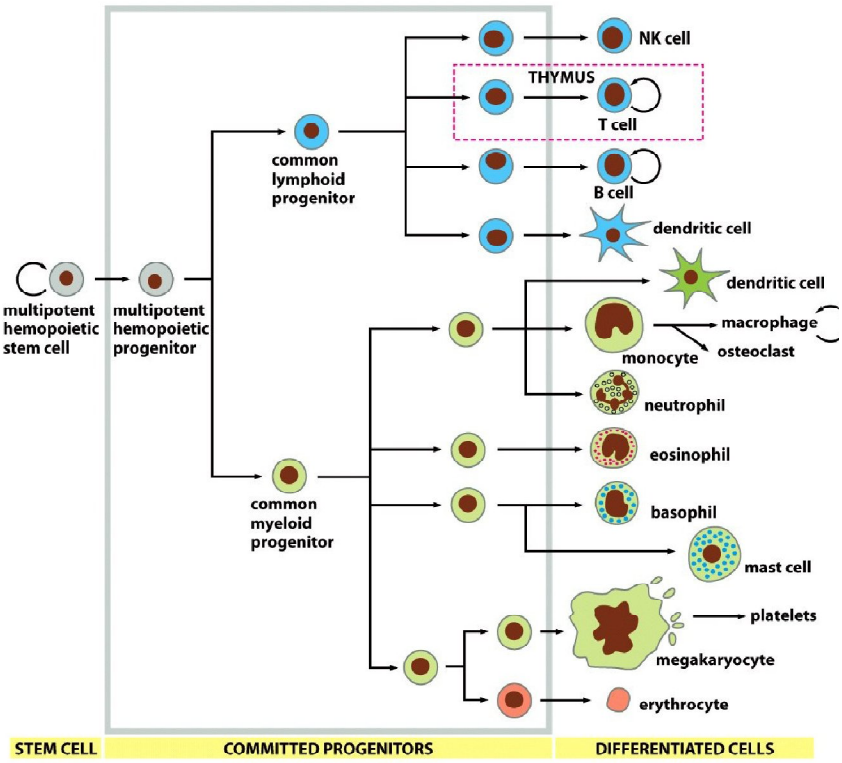

Fig. 1 Blood cell differentiation pathways (Albers B, Johnson A, Lewis J et al./ *Molecular Biology of THE CELL. 2008,5thEdition*.pp.1450 on Media DVD-ROM**)**

After they have been produced by hematopoiesis, neutrophils, from the bone marrow are released into the peripheral blood and circulate for 7-10 hours before migrating into the tissues, where they have a life span of only a few days. The two colony-stimulating factors (CSFs), granulocyte (G-CSF) and granulocyte-macrophage (GM-CSF) factor are directly involved in the production and differentiation of neutrophils, and can amplify the activation of various neutrophil functions **(6).** Polymorphonuclear leukocyte (PMN), which often refers specifically to neutrophil granulocytes, has a multilobulated chromataindense nucleus with no nucleolus and granulated cytoplasm that stains with both acid and basic dyes **(4).** Although neutrophils play important roles in host defence against all classes of infectious agents, they are also, paradoxically, involved in the pathology of various inflammatory conditions. To terminate that, a process of apoptosis is triggered, a mechanism where these cells shut down and are removed safely by macrophages. PMNs directly recognize surface-bound or freely secreted molecules produced by bacteria and, under normal conditions, they bind and ingest invading microorganisms by a process known as phagocytosis **(7).** These pathogen-derived molecules are recognized by complement surface receptors on PMNs, such as Toll-like receptors (TLRs), CD14, CD35 (CR1), CD11b/CD18 (CR3) and CD11c/CD18 crucial in the pathogen-mediated process of neutrophils activation. In this mechanism which is required for effective host defence and leading to further activation of neutrophils, complement C5a fragment is mostly involved and, furthermore, it amplifies neutrophil proinflammatory responses **(8).**

Neutrophils microbicidal activity includes oxidative and oxygen-independent processes that are activated simultaneously upon phagocytosis. At the same time with phagocytosis, two microbicidal mechanisms are activated: the oxidative burst, so called because of the increase in O_2 consumption which results in production of reactive oxygen species, and degranulation, which corresponds to the release of contents of azurophilic and specific granules in the phagosom to form a phagolysosome **(6).**

Neutrophils, indicating a larger respiratory burst than macrophages, are able to produce more reactive oxygen and reactive nitrogen intermediates and in that way they generally express higher levels of defence than macrophages do **(4).** These mechanisms involve initiative of contractile proteins, triggered by changes in the level of free cytosolic calcium required for production of reactive oxygen species and for granule secretion, more important, for granular fusion with phagosomes in neutrophils **(9).**

1.1.1 Models of Hoxb8 mouse neutrophils

Mature human neutrophils have short life span and cannot be genetically manipulated. For this reason, analyses of neutrophils functions are performing on in vitro-generated mouse neutrophils, derived from conditional Hoxb8-immortalized precursors. This system relies on the activity of an estrogen receptor binding domain-Hoxb8 fusion. These in vitro-derived mouse neutrophils have equal physiological and functional characteristics as human neutrophils what makes them appropriate for further analyses **(10).**

In the present study, we worked with four different genetically modified Hoxb8 cell lines: wild type (WT), BK knock-out ($BK^{-/-}$), Parvalbumin knock-out ($PV^{-/-}$) and gp91 knock-out ($gp91^{-/}$).

Parvalbumin is known as a very small (9-11 kDa) Ca^{2+} binding protein. It contains three EF hand helix-loop-helix motif which bind Ca^{2+} with high affinity. It is involved in calcium signaling and has an important role in many physiological processes, namely cell-cycle regulation, second messenger production and the organization of microtubules and vision **(11).**

BK channel or also known as calcium-activated potassium channel is one of the group of BK channels that are characterized by their large conductance of potassium ions (K^+) through cell membranes, also activated by intracellular Ca^{2+}. Changes in the membrane electrical potential and the increase in concentration of intracellular Ca^{2+} ions activate these channels. Depolarization of the membrane voltage and the increased intracellular Ca^{2+} levels both cause BK channels to be opened, which hyperpolarizes the membrane and opens voltage-dependent channels, including Ca^{2+} channels leading to Ca^{2+} influx into the cell **(12, 13).**

$Gp91^{phox}$ is a glycosylated heme binding subunit of the superoxidegenerating NADPH oxidase. Together with the nonglycosylated subunit $p22^{phox}$, they are part of the phagocyte NADPH oxidase flavocytochrome *b*558, a membrane-bound heterodimer. The NADPH oxidase flavocytochrome b558 mediates the terminal steps in electron transfer, resulting in the generation of O_2^- during the phagocyte respiratory burst. Thus, $gp91^{phox}$ is crucial heme binding subunit of flavocytochrome b558 since there is no requirement for a shared heme. The lack of $gp91^{phox}$ leads to the inability of O_2^- production although all cells are properly activated **(14).**

1.2 Role of reactive oxygen species in neutrophils

Neutrophils as "a first line of defence" against infections agents employ both oxygen-dependent and oxygen-independent pathways to generate antimicrobial substances. During the inflammatory processes, reactive oxygen species (ROS), like superoxide radical ($O_2^{-\bullet}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$), hypochlorous acid (HOCl), nitrogen reactive species (RNS), namely nitric oxide ($NO^\bullet$) and peroxynitrite anion ($ONOO^-$) are generally formed and implicated in its pathophysiology **(15).** Cellular production of ROS arises from both enzymatic and nonenzymatic sources **(16).**

Several different mechanisms are included in formation of reactive oxygen species:

- interaction of ionizing radiation with biological molecules
- synthesis by dedicated enzymes in phagocytic cells like neutrophils and macrophages
    - NADPH oxidase (in both type of phagocytes)
    - Myeloperoxidase (in neutrophils only).
- as an unavoidable byproduct of cellular respiration. Some electrons passing "down" the electron transport chain may leak away from reduced sites in the respiratory chain (especially as they pass through ubiquinone) and go directly to reduce oxygen molecules to the superoxide anion **(17).**

Reactive oxygen species as a phrase is used to describe a variety of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen **(18).**

The reduction of oxygen by one electron at a time produces relatively stable intermediates. Superoxide anion ($O_2^{-\bullet}$), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of $O_2^{-\bullet}$ (either spontaneously or through a reaction catalysed by superoxide dismutases) produces hydrogen peroxide (H_2O_2), which then may be fully reduced to water or partially reduced to hydroxyl radical ($OH^\bullet$), one of the strongest oxidants in nature. The formation of $OH^\bullet$ is catalyzed by reduced transition metals, which in turn may be re-reduced by $O_2^{-\bullet}$ expending this process **(19).** In addition, $O_2^{-\bullet}$ can also react with other radicals such as nitric oxide ($NO^\bullet$) in a reaction controlled by the rate of diffusion of the both radicals **(20).**

Under normal metabolic conditions, each cell in our body is exposed to about 10^{10} molecules of superoxide each day. Once formed, superoxide is converted to other ROS **(21).** Reactive oxygen species play either harmful or beneficial role in the biological systems.

Beneficial effects of ROS include physiological roles in cellular responses against infectious agents and in cell signaling, including: apoptosis, gene expression, and the activation of cell signaling cascades. It should be noted that ROS can serve as both intra- and inter-cellular messenger **(22).**

Specificity in signaling is achieved through the non-covalent binding of a ligand to its cognate receptor. By contrast, ROS operate in signaling through chemical reactions with specific atoms of target proteins that lead to covalent protein modifications. Therefore, ROS molecular recognition occurs at the atomic and not at the macromolecular level. This, in turn, necessarily expands the potential number of ROS-specific receptors because the atomic targets of ROS are the amino-acid building blocks of numerous proteins **(23).** These pathways generally make use of ROS sensors that "measure" the intracellular concentration of ROS by a redox-based mechanism, and, in the other case, the expression of ROS-specific scavengers is set in a way to maintain the concentration of ROS below a toxic level. These pathways can regulate the physiological response that is fitted to the ROS signal in which it has a role of agonist and the sensor is a ROS-specific receptor **(22, 23).**

ROS performs important functions in neutrophils. They must generate ROS in order to kill some types of bacteria they engulf by phagocytosis. The reactive oxygen that appears during phagocytosis is produced on the membranes of the endosome of the phagocytosing cells with the involvement of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the cellular membranes **(24).** In unstimulated neutrophils, components of the NADPH oxidase complex are separated in cytosol (p40*phox*, p47*phox*, p67*phox*, Rac2) and membrane compartments (flavocytochrome *b558*, Rap1A). During phagocytosis, the cytosolic components translocate to the plasma or phagosome membrane and associate with flavocytochrome *b558*, a transmembrane heterodimer comprised of $gp91^{phox}$ (Nox2) and $p22^{phox}$, thus forming the active oxidase **(7).**

Superoxide dismutase (SOD) has an important role in converting the large amount of oxygen, produced during the respiratory burst, into hydrogen peroxide, which kills off the engulfed bacteria. Neutrophils also kill off engulfed pathogens by using the enzyme myeloperoxidase which catalyzes the reaction of hydrogen peroxide (made from superoxide anions) with chloride ions to produce the strongly antiseptic hypochlorite ion (OCl^-).

Paradoxically, the roles of ROS as essential biomolecules in the regulation of cellular functions and as toxic by-products of metabolism are related to differences in the concentrations of produced ROS **(16).** Harmful effects are due to high concentrations of ROS, which can damage biomolecules, including lipids, proteins and nucleic acids. The harmful effects of ROS are balanced by the antioxidant action of both antioxidant enzymes and non-enzymatic antioxidants. However, despite the presence of the cell's antioxidant system, oxidative damage accumulates during the life cycle and has been proposed to play a key role in the development of age-dependent diseases, such as atherosclerosis, arthritis, neurodegenerative disorders and cancer **(22).** Under normal conditions, there is a balance existing between moderate ROS production to modulate physiological signaling and overproduction of ROS that leads to oxidative stress. Beside this balance in some pathological scenarios, the antioxidant defense becomes insufficient resulting in oxidative stress, often leading to apoptosis and cell death **(25).**

ROS detoxification pathways exist to minimize oxidative damage. The key metabolic steps are SOD catalysis of the dismutation of superoxide to hydrogen peroxide and oxygen, and the conversion of H_2O_2 to $2H_2O$ by glutathione peroxidase or to $O_2 + H_2O$ by catalase **(26).** Finally, radical scavenging antioxidants protect us because they can scavenge ROS before they cause damage to the various biological molecules or prevent oxidative damage from spreading. **(21, 26)** The antioxidant defence systems in the human body are extensive and consist of multiple layers, which protect us at different sites and against different types of reactive oxygen species. In addition to the antioxidant enzymes that are playing an important role of the antioxidant defence system inside cells, there are several other small-molecule antioxidants also playing important roles in these defence systems. These small-molecule antioxidants are particularly important in the blood and fluids present in the extracellular space where antioxidant enzymes are absent or present only in small quantities.

The small-molecule antioxidants include lipid-soluble and water-soluble antioxidants. Alpha-tocopherol, the biologically and chemically most active form of vitamin E, is, by far, the most abundant lipid-soluble antioxidant in humans. Vitamin C, the prominent water-soluble antioxidant, is also very effective in scavenging a wide range of ROS and free radicals **(21).**

1.3 Role of Ca^{2+} in neutrophils

Despite the enormous variety in its expression, cellular activities of all cell types are regulated by common intracellular signaling systems, and calcium is one important intracellular messenger, controlling a diverse range of cellular processes. In response to adequate stimuli, $[Ca^{2+}]_i$ (Intracellular Ca^{2+} concentration) increases, oscillates and decreases, leading to the activation, modulation and termination of cell function **(27).** The Ca^{2+} signal of neutrophils mainly consists of two components:

- a rapid, transient increase of $[Ca^{2+}]_i$ due to Ca^{2+} release from the internal stores induced by inositol 1,4,5-trisphosphate
- a sustained elevation of $[Ca^{2+}]_i$ due to Ca^{2+} influx from the extracellular space **(28).**

Variations in $[Ca^{2+}]_i$ have also been linked with neutrophils function, such as the production of ROS, chemotaxis, phagolysosome formation, degranulation, adherence, and integrin recycling **(29).** Many channels and pumps are responsible for Ca^{2+} entering and exiting cells, and moving between the cytosol and intracellular stores. Ca^{2+} signaling system includes:

- the RyR (Ryanodine Receptor Channels) that is the SR (Sarcoplasmic Reticulum) Ca^{2+} release channel
- the Troponin protein complex that mediates the Ca^{2+} effect to the myofibrillar structures leading to contraction
- the Ca^{2+} pump responsible for Ca^{2+} reuptake into the SR
- Calsequestrin, the Ca^{2+} storage protein in the SR

In addition, numerous Ca^{2+} binding proteins, such as parvalbumin, calmodulin, S100 proteins and others have a role in mediating Ca^{2+} signaling **(30).**

The mechanism of Ca^{2+} entry in non-excitable cells is established through a process known as capacitative Ca^{2+} entry or store-operated Ca^{2+} entry. Here, the depletion of intracellular stores due to the action of 1,4,5-inositoltrisphosphate (IP3) or other Ca^{2+} releasing signals initiates a signaling pathway leading to the opening of plasma membrane Ca^{2+} channels **(31).** In excitable cells, the major pathway for Ca^{2+} influx is via highly Ca^{2+} selective VGCC (Voltage-Gated Ca^{2+} Channels). Store Operated Channels open in response to the emptying of intracellular stores through the interaction with IP3 and its 1,4,5-inositoltrisphosphate receptor channel (IP3R). CRAC (Ca^{2+} Release-Activated Ca^{2+} channel) is found in cells of the blood lineage and is highly selective for Ca^{2+} **(32, 33).** Voltage-independent pathways are generally activated by signaling cascades and the most common pathway involves activation of phosphatidylinositol-specific phospholipase C (PLC) and generation of IP3 and diacylglycerol (DAG) from PIP2 (Phosphatidylinositol 4, 5-Bisphosphate). Ca^{2+} release from the intracellular stores is mediated by RyR and IP3R channels, while RyR are activated by a rise in intracellular CICR (Ca^{2+}-Ca^{2+} Induced Ca^{2+} Release). Extracellular Ca^{2+} influx is generally limited to two families of proteins: the PMCA (Plasma Membrane Ca^{2+} ATPase) and the NCX (Na^{+}/Ca^{2+} Exchanger). In general, intracellular Ca^{2+} is reduced by Ca^{2+} uptake into cellular organelles via a variety of organelle-specific pumps and transporters. The uptake into the ER is regulated by the SERCA (Sarco - Endoplasmic Reticulum Ca^{2+} ATPase) family, while the uptake into mitochondria is mediated by the mitochondrial Ca^{2+}-Uniporter and an uptake into Golgi is mediated by the PMR1/ATP2C1 (P-type Ca^{2+}-transport ATPase) **(33).**

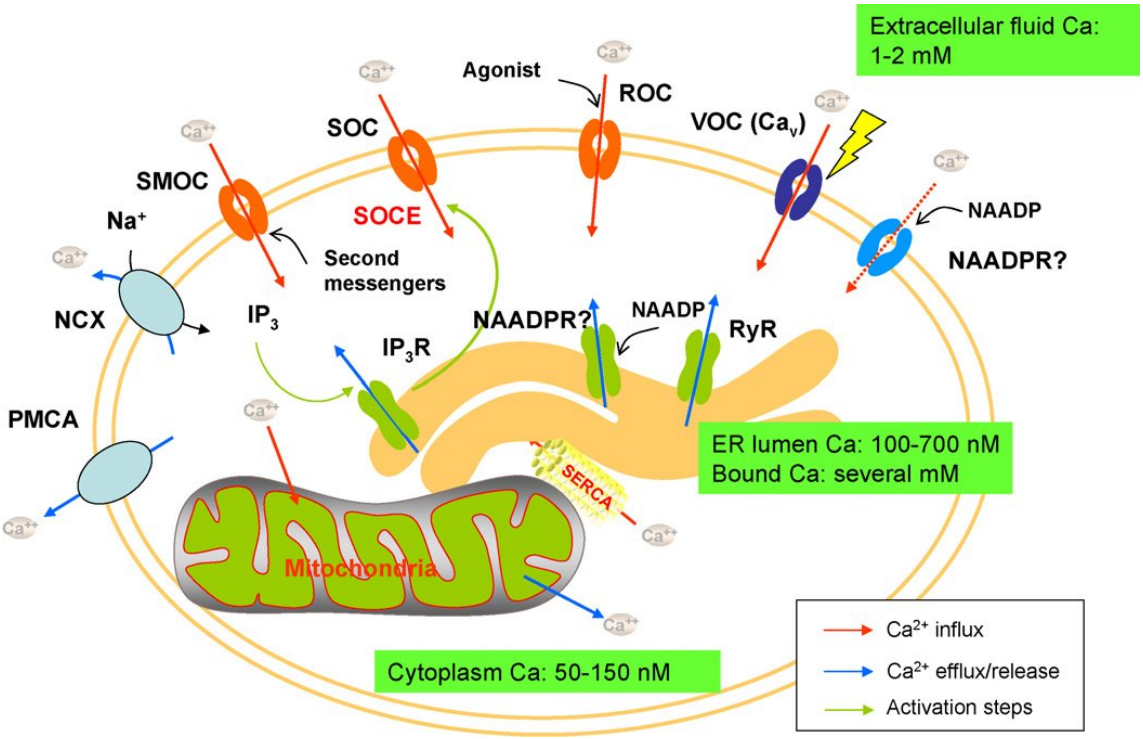

Fig. 2 Modes of $[Ca^{2+}]_c$ regulation in a cell. (Y. Suzuki et al. / Molecular Immunology 2010, 47: 640-648)

In resting cells, the cytosolic Ca^{2+} concentration $[Ca^{2+}]_c$ is maintained at a low level (50-150nM). However, agonist including hormones, growth factors and antigens induce changes in the intracellular Ca^{2+} dynamics, including the elevation of $[Ca^{2+}]_c$ **(34).** Neutrophil activation is initiated by the binding of a chemotactic agonist to its receptor, and then, this induces the activation of PLC which generates two second messengers: (IP3) and DAG. IP3 binds to IP3 receptors located on the surface of the ER (endoplasmic reticulum) and activates the release of Ca^{2+}. In addition to IP3, two other intracellular Ca^{2+} mobilizing agents, cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), are involved in the release of Ca^{2+} from intracellular stores by binding to ryanodine receptors and nicotinic acid adenine dinucleotide phosphate receptors (NAADPRs), respectively. Upon Ca^{2+} store depletion, STIM1 (stromal interacting molecule 1), which is on the surface of ER, interacts with SOC (store-operated channels), and extracellular Ca^{2+} entry occurs, which might allow the direct reloading in Ca^{2+} of ER through the SERCA pump. This results in elevation in the cytosolic Ca^{2+}, and as such is a prerequisite to regulate the assembly of all NADPH oxidase components at the plasma membrane or granular membrane upon opsonized particle stimulation **(35, 36).** Changes in

Ca^{2+} flux, in turn, affect the activity of various oxidases when Ca^{2+} mediated kinase activation causes phosphorylation of their subunits **(37).** The correlation between an increased production of reactive oxygen species and an enhanced calcium entry in primed neutrophils stimulated with C5a indicate that ROS activity could serve as an agonist to enhance calcium signaling by positive feedback **(38).**

1.3.1 Calcium channel blockers

In order to understand the importance of Ca^{2+} as a signaling molecule in many cell types and its complex mechanism of entry via different channels, many specific and nonspecific Ca^{2+} channel blockers such as Verapamil, Nifedipine, Diltiazem or Amlodipin, can directly or indirectly influence to the changes of cytosolic Ca^{2+} concentration.

In the present, study we focused mainly on Verapamil and Nifedipin as L-type calcium channel blockers. Although Nifedipine as a dihidropyridine calcium channel blocker is known as a more specific inhibitor than Verapamil itself (which represents the phenylalkylamine class of calcium channel blockers), they both inhibit the movement of calcium ions across cell membrane.

Paxilline indole alkaloid, produced by Penicillium paxilli, which is a specific inhibitor of large conductance calcium dependent potassium channels, was also used in our study. Its activity is indirectly connected with the voltage dependent Ca^{2+} channels. In fact, by blocking potassium channels, a depolarization of the cell membrane occurs and keeps the voltage dependent Ca^{2+} channels closed.

2. Objectives

During our previous research, we have been able to correlate changes in cytosolic Ca^{2+} concentration with the neutrophils activation by several different stimulants: the complement fragment C5a, which interacts with specific receptors on the neutrophil surface, N-formyl-methionine-leucine-phenylalanine (fMLP) that elevates $[Ca^{2+}]_i$ by releasing Ca^{2+} from the intracellular stores, then, platelet-activating factor (PAF) that induces a rise in $[Ca^{2+}]_i$ from Ca^{2+} influx and the hydrophobic inophore ionomycin, which selectively carries Ca^{2+} across membranes **(39, 40).**

Further in our research, we focused mainly on the correlation between cytosolic Ca^{2+} concentration and its role in the activation of neutrophil responses under conditions that are, as closely as possible, to the physiological activation of neutrophils. In particular, we have used the physiological stimulus provided by the complement-derived polypeptide C5a to activate production of reactive oxygen species in neutrophils and search for the correlation between Ca^{2+} mobilization from the extracellular space and its role in the regulation of ROS production.

3. Materials and methods

3.1 Materials

3.1.1 Equipment

Name	**Type**	**Company**
Balance	Excellence Plus	Mettler Toledo
Cell Counter	KX-21	Sysmex Digitana AG
Centrifuge	Multifuge 3 S-R	Heraeus AG
Centrifuge	Megafuge 40R	Heraeus AG
Centrifuge	Biofuge primo R	Heraeus AG
Cytospin	Cytospin 3	Thermo Scientific
Falcon Tubes	BD Falcon	BD Biosciences
Filter (for syringes)	Millipore GV 0.22 um	Millex
Flow Cytometer	BD FACSCalibur	BD Biosciences
Fluorescence Spectrometer	Fluorimeter LS 50	BD Biosciences
Freezer (-20°C)	mediline	Fors Liebherr
Freezer (-80°C)	U535 -86	New Brunswick Scientific
Glass pipettes	serological pipette	Greiner Bio-One
Incubator	cell 150	Hera
Pipette Boy	Accu-jet® pro	Brand
Pipettes	Research (different volumes)	Eppendorf
Refrigerator	different models	Miostar
Shaker	MS2 Minishaker	IKA
Syringe	BD Plastipak 20 ml	BD Biosciences
Tubes	PP tubes, conical bottom	Greiner Bio-One
Vortex	Vortex-Genie 2	Scientific Industries

3.1.2 Chemicals and solutions

Name	Description	Company
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid	Sigma-Aldrich
BSA	Bovine serum albumin	Sigma-Aldrich
C5a	Complement factor 5a	Calbiochem-Novabiochem Corp.
DHR	ethylene glycol tetraacetic acid	Invitrogen
DPI	diphenyleneiodonium chloride	Sigma-Aldrich
EDTA-AM	ethylenediaminetetraacetic acid	GIBCO
EGTA-AM	ethylene glycol tetraacetic acid	Sigma-Aldrich
fMLP	N-formyl-methionine-leucine-phenylalanine	Sigma-Aldrich
Fura-2AM	fura-2-acetoxymethyl ester	Boehringer Mannheim
FSC	Fetal calf serum	Invitrogen
GM-CSF	Human GM-CSF	Sigma-Aldrich
Ionomycin	ionomycin	Boehringer Mannheim
Medium	RPMI 1640	GIBCO
Nifedipine	1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester	Sigma-Aldrich
Paxilline	paxilline	Calbiochem-Novabiochem Corp.
PBS	Phosphate-buffered saline	PAA Laboratories
PMA	Phorbol 12-myristate 13-acetate	Calbiochem-Novabiochem Corp.
TRITON X-100	Triton X-100	Sigma-Aldrich
Verapamil	(*RS*)-2-(3,4-dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl]-(methyl)amino}-2-prop-2-ylpentanenitrile	Sigma-Aldrich

3.2 Methods

3.2.1 Neutrophil isolation

Mature blood neutrophils were isolated from the peripheral blood of healthy donors by Ficoll-Hypaque centrifugation. Briefly, peripheral blood mononuclear cells (PBMC) were separated by centrifugation on Ficoll-Hypaque (Seromed-Fakola AG, Basel, Switzerland). The lower phase, consisting mainly of granulocytes and erythrocytes, was treated with erythrocyte lysis solution (155 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA, (pH 7.3)) for 10 min on ice. The next step was a centrifuging at 1400rpm, 7 min at 4^0C. After this, the supernatant was removed as much as possible by aspiration, and 1 ml cold PBS+ (7.5% BSA and PBS) was added to the cell pellet. We resuspended them carefully, filled them up with PBS+ and centrifuged them again at 1400rpm for 7 min at 4^0C. The resulting cell populations contained more than 95% mature neutrophils as assessed by staining with Diff-Quik (fixation 1 min, the cytosol-staining 1min 20 sec, the nucleus-staining 1 min) and light microscopy analysis.

Cell cultures

Neutrophils were cultured at 1×10^6/ml in RPMI-1640 containing 5% fetal calf serum (FCS) and 200 IU/ml penicillin/100 μg/ml streptomycin (all from Life Technologies, Basel, Switzerland) in the absence or presence of the indicated concentrations of GM-CSF, PMA, C5a for the indicated time periods, using complete culture medium at 37°C in 5% CO_2 in a humidified atmosphere. If not indicated, PMA was used at 10^{-7}M, C5a at 10^{-8}M, GM-CSF (25ng/ml), G-CSF (25ng/ml).

3.2.2. Hoxb8 mouse models

To get immortal Hoxb8 cell lines, firstly, we had to isolate lineage negative-progenitors cells (HSC) from the mouse bone marrow and an additional transfection with a lentivirus which contained a so-called GEV-element was necessary to be performed. GEV16 is a composed transcriptional factor consisting of a DNA-binding site (G-domain, Gal14), an activating site (E-domain, estrogen receptor) and a variable site (V-domain). The resulting protein is localized in the cytosol where it is bound to heat shock proteins in the absence of estrogen. These Hoxb8 cells exhibit estrogen-dependent transcriptional activation, and since tamoxifene binds to the estrogen receptor, it also leads to a Hoxb8 over expression in our cells. If tamoxifene is present, the heat shock proteins are replaced and the GEV-

complex translocates into the nucleus where the Gal14 domain binds to UAS elements on the DNA which later leads to an upregulation of Hox proteins. Since Hox oncoproteins enforce self-renewal of factor-dependent myeloid progenitors, we utilized estrogen-regulated (ER-) Hoxb8 to immortalize neutrophil progenitors that would execute normal differentiation and normal innate immune function upon ER-Hoxb8 inactivation. In addition, 8ug/ml of polybrene was used to enhance the transfection, and puromycin had to be added to every cell line to help in the selection because only the transfected cell were able to survive since they had puromycin resistance.

Cell passaging

The Hoxb8 cells were cultured in the following medium:

RPMI 1640, 10 % FCS HIA, 1 % penicillin/streptomycin, 50μM β-mercaptoethanol, 5 % SCF (added freshly), 0.1μM 4-hydroxytamoxifene (added freshly)

Every Monday and Friday, they were diluted to approximately 50'000 cells/ml.

Differentiation of neutrophil precursors

A differentiation medium had the same composition as a culture medium but without tamoxifene. Its absence allows cells to differentiate. To set up a differentiation, the following procedure had to be performed:

Cultured cells were transferred to a 14 ml tube and spun down at 1400 rpm at RT for 7 min. The supernatant was aspirated and the cells were resuspended in 10 ml of PBS. This washing step with PBS had to be done twice. The cells were resuspended in 5 ml of differentiation medium and their concentration was counted with Sysmex. Cells had to have a final concentration of 25'000 cells/ml in the total volume. Until the cells had reached the desired age for an experiment, they were stored at 37°C and 5 % CO2.

Surface antigen staining

One of the many functions of a flow cytometer is to analyze surface antigens of cells. For every marker, an isotype control was tested as well, just to see if the signal was specific. Since the antigens are not fluorescent, the emission was generated by fluorochromes which were attached to their antibodies. Typical fluorochromes are allophycocyanin (APC, excitation at 650 nm, emission at 660 nm), peridin-chlorophyll (PerCP, excitation at 470

nm, emission at 680 nm) or phycoerythrin (PE, excitations at 498, 540 and 565 nm, emission at 573 nm). The outcome was a digital signal that corresponds to the quantity of surface antigens.

To characterize the maturation status of the cells, usually 50'000-200'000 cells were used per FACS staining, and cells were spun down (2000 rpm, 4°C, 2 min). After washing with 0.4% BSA in PBS the supernatant was removed and cells were resuspend in 50µl blocking buffer (10 % FCS, 5 % mouse IgG1 negative control in PBS) and incubated on ice, protected from light for 25 minutes. Antibodies (APC anti-mouse CD11b, BioLegend, San Diego) and isotype (APC-conjugated Rat IgG 2b, Kappa, BioLegend, San Diego) controls were then added (between 1: 50 and 1: 1000 end concentration) and the solutions were incubated for another 45 minutes at 37^0 C, 5% CO_2 or on ice protected from light. After two washings (centrifuging, removing supernatant, resuspending in washing solution, another centrifugation and supernatant removal), the cells were resuspended in 300µl of washing solution and quickly measured by FACS-Calibur.

The acquisition and analysis were always performed with FlowJo software (Version 10.0.3).

Oxidative burst measurements

MasterMix 1 (1uM DHR): 20 µl dihydrorhodamine 123 (DHR; 1:50 pre-diluted in PBS) in 1000 µl of ex vivo medium (10 % FCS HIA in 1640 RPMI).

MasterMix 2 (DHR and 10uM C5a): same as MasterMix 1 plus 1µl of (1:10 pre-diluted) C5a.

About 250'000 cells were spun down, resuspended in 100 µl of ex vivo medium and distributed on 3 eppendorf tubes. Then the following substances were added:

- 100 µl of MasterMix 1
- 100 µl of MasterMix 1 and 1 µl of (25 ng/ml) GM-CSF
- 100 µl of MasterMix 2 and 1 µl of (25 ng/ml) GM-CSF

Cells were incubated in open tubes for 15 minutes at 37°C, 5% CO_2. 200 µl of ice-cold PBS was added and the measurements were performed by FACS-Calibur.

DHR 123 is an oxidant-sensitive probe, which is cell permeable non fluorescent molecule. During interaction with free radicals, DHR is oxidized, resulting in the liberation of rhodamine123, a highly fluorescent marker.

If the cells produced ROS, DHR was oxidized and this reaction translated into a shift of the signal on the FL-1 histogram.

Phagocytosis assay

Working suspension: 1µl of beads (Fluoresbrite®) in 182 µl of ex vivo medium.

To obtain an opsonization of the beads, 5 µl of mouse serum was added to 20 µl of working suspension and this mixture was then incubated in a shaker for 30 minutes at 37°C in a non-pyrogenic polypropylene capped tube. About 750'000 cells were harvested, spun down, resuspended in 300 µl of ex vivo medium and distributed to 2 eppendorf tubes. The following was added to each 150 µl of cell suspension:

• 25 µl of opsonized beads

• 25 µl of opsonized beads and LPS (100 nM end concentration; positive control)

The tubes were incubated at 37°C for 45-60 minutes in a shaker. Phagocytosis was stopped with 200 µl of ice-cold PBS with 0.02 % EDTA. Cells were kept on ice and protected from light. Cells were immediately analyzed with (FACS-Calibur). If the cells phagocyted the beads, they were detectable.

Determination of cell death

Cell death was assessed by an uptake of 1 µM ethidium bromide and flow cytometric analysis (FACS-Calibur). Therefore, ($1x10^6$ cell/ml) were taken and ethidium bromide was added before measurement. This fluorescent substance changes its emission spectrum characteristically if it intercalates into DNA. Since ethidium bromide cannot permeate the cell membrane, it only intercalates into the DNA of dead cells. The varying emission spectrum allows the measurement.

Flow cytometric analysis - Theoretical background

Flow cytometry is a very convenient device for characterizing cells. It does so by directing a laser beam onto a cell suspension and then detecting the reflecting and emitting light by

different detectors. FACS Calibur is equipped with a blue (488 nm) and red laser (635 nm). Basic detectors are the forward scatter (FSC), located towards the light source and giving information about the size of the cells, and the side scatter (SSC), located vertically to the laser beam and correlating with the granularity of the cells. Moreover, there are four additional detectors (FL 1-4) used for detecting fluorescent emissions at different wavelengths.

3.2.3 Intracellular calcium measurements

Intracellular ionized calcium concentrations $[Ca^{2+}]_i$ were assayed with a bulk spectrofluorometric assay using a Perkin Elmer LS 50B spectrofluorimeter. We used, for each condition, 5×10^6 cells, spun them down at 1400 rpm for 5 min and resuspended in 1 ml of complete culture medium of RPMI-1640 + 5% FCS . We added 2.5 ul of a 1 mM stock solution of the acetoxymethylester derivative of Fura-2AM with a final concentration of 2.5 uM and incubated them at 37^0C for 20 min. The dye was then removed by washing and cells were resuspended in a completely warm culture medium and incubated for an additional 30 min at 37^oC 5% CO_2 until measurement. If the cells need to be pre-treated with inhibitors, we add the proper dilution of inhibitors to 1 ml of the labeled cells in an eppendrof tube after 20 min incubation time with Fura-2AM. Before measurement cell suspension was spun down at 2000 rpm for 4 min, the pellet resuspended in 1 ml of warm Ringer medium (125 mM NaCl, 5 mM KCl, 2 mM Na_2HPO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 EGTA, 5 mM pyruvate, 5 mM glucose, 32 mM Hepes pH 7.4). If necessary, a proper dilution of inhibitor was added and the cell suspension was placed in quartz cuvette (Hellma, Basel, Switzerland) before measurement. Excitation and emission wavelengths were appropriate for the dye of interest (eg. 340 nm and 380 nm for the bound and free forms of Fura-2AM, respectively). Each analysis was calibrated by addition of 1 μM Ionomycin and 0.02% Triton X-100 followed by 15 mM EGTA. The calcium concentration corresponding to fluorescence (F) emitted by trapped Fura-2AM was calculated by the equation: $[Ca^{2+}]_i = 227 \text{ nM } (R - R_{min})/(R_{max} - R)$. F_{max} was obtained after dye release with 0.02% Triton X-100. R_{min} was determined by setting Ca^{2+} to 1 nM by addition of 15 mM EGTA **(41).**

Fluorescence spectroscopy - Theoretical background for LS50 Fluorescence Spectrometer

Fluorescence spectroscopy is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. It involves a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light, typically but not necessarily, visible light. Devices that measure fluorescence are called fluorimeters. Our LS50 Spectrometer contains rapidly rotating filter (fast filter) with wavelengths appropriate for our dye Fura-2AM where the excitation peak shifts from 380 nm in the unbound form to 340 nm when bound to calcium. The fast filter gives data interval down to 40 milliseconds and is used to isolate the incident light and fluorescent light. The instrument uses a pulsed xenon source with a frequency of 20 milliseconds as excitation source. The detector gain should be carefully selected so that the dynamic range of the instrument can accommodate the largest intensity. Obtained data was processed with special software FLWINLAB.

3.2.4 Morphological analysis

We used the so-called Cytospin for this method. The following procedure had to be performed: about 400'000 cells were harvested and centrifuged (2000 rpm, 2 min, RT). The medium was removed, cells were resuspended in 1 ml of PBS and then counted. 200'000 to 300'000 cells were washed with 1 ml of PBS and resuspended in 100 µl of PBS. This suspension was injected into the plastic attachment on the slide. Cytospin was run for 2 minutes at 2000 rpm. At the end, we stained the slides with Diff-Quik (fixation 1 min, the cytosol-staining 1 min 20 sec, the nucleus-staining 1 min). The nucleus-staining fluid was removed just after staining by washing it away with water.

4. Results

4.1 General proof of Hoxb8 differentiation

Before claiming any significant differences concerning the differentiation between all four different cell lines that we have used for our experiments, the differentiation process had to be demonstrated and we had to show on which day these cell lines are completely differentiated and most mature. We established from our experiments that day 5 of differentiation is the day when all of the cell lines are well differentiated, most mature and their viability is on the highest level.

4.1.1 Morphology

Morphological changes during differentiation can be seen quite clearly. Undifferentiated Hoxb8 WT mouse neutrophils are bigger compared to mature cells, and, in general, very dense and have a circular-shaped nucleus (3a). The first indicator of differentiation is the donut-shape of the nucleus, on day 3 of differentiation of Hoxb8 WT mouse neutrophils (b). Morphologically mature Hoxb8 WT (3c), Hoxb8 $BK^{-/-}$ (3d), Hoxb8 $PV^{-/-}$ (3e) and Hoxb8 $gp91^{-/-}$ (3f) neutrophils on day 5 of differentiation are smaller and have a segmented, granulated nucleus.

4.1.2 Determination of cell surface markers' expression

To investigate maturity of our neutrophils, we had to detect the change in surface antigen expression. Whereas stem cell-typical proteins (e.g. c-kit/CD117) are downregulated, Gr-1, CD11b and IL-8R are upregulated in neutrophils.

At first we evaluated CD11b 170 kD glycoprotein, expressed in neutrophils and thus upregulated throughout differentiation (Fig. 4). Functionally, CD11b regulates leukocyte adhesion and migration to mediate the inflammatory response.

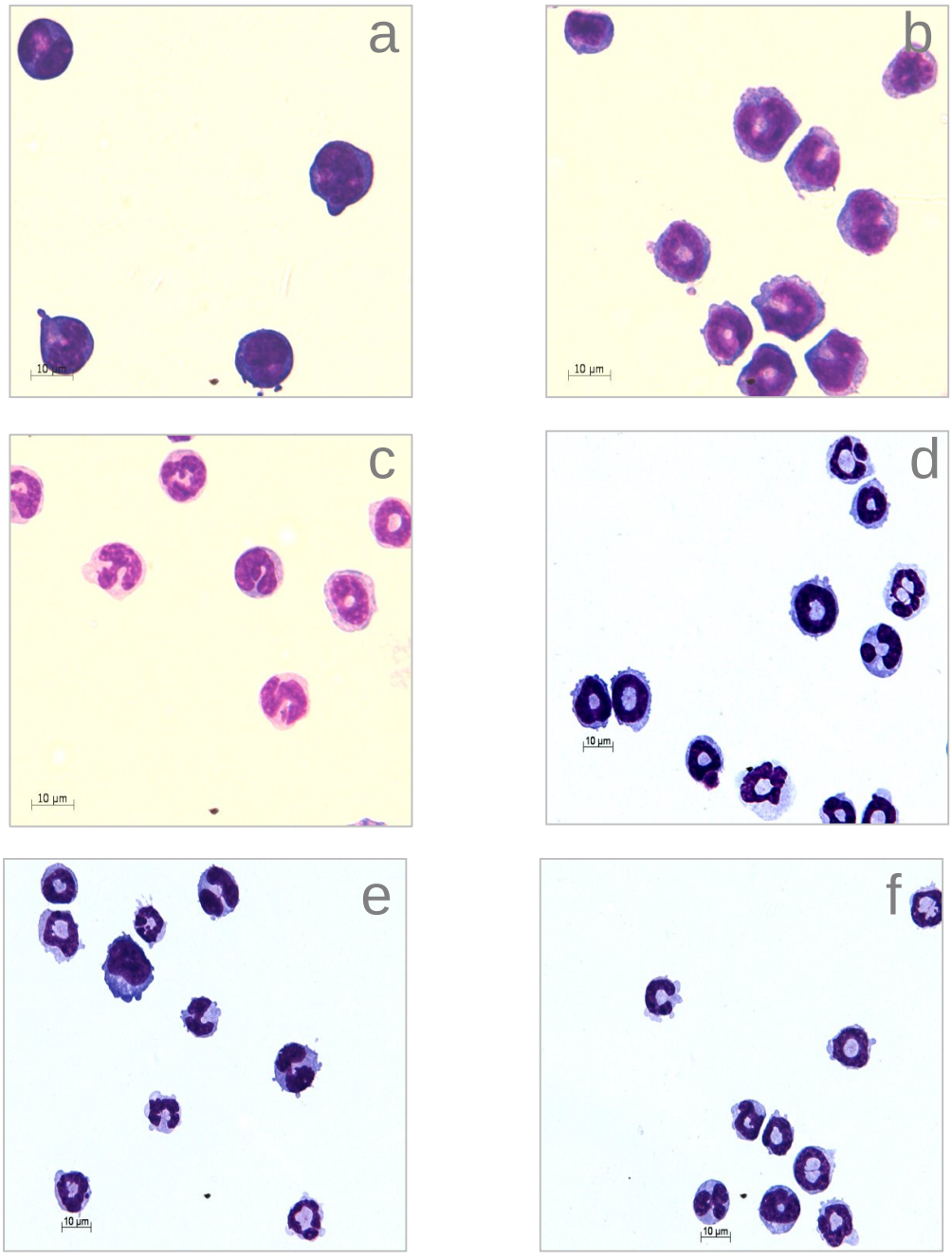

Fig. 3 Morphological changes during differentiation. Hoxb8 mouse neutrophils ($2x10^6$ cells/ml) were cytospinned and stained with Diff-Quik (Baxter, Düdingen, Switzerland). Axiovert 35 microscope equipped with a 63X/1.4 oil objective lens was used (Carl Zeiss, Jena, Germany). Scale bars, 10 µm. Images were processed with Adobe Photoshop 5.0 software (Adobe Systems, San Jose, CA). (a) Undifferentiated Hoxb8 WT mouse neutrophils. (b) Hoxb8 WT mouse neutrophils on day 3 of differentiation. Hoxb8 WT (c), Hoxb8 $BK^{-/-}$ (d), Hoxb8 $PV^{-/-}$ (e), Hoxb8 $gp91^{-/-}$ (f) mouse neutrophils on day 5 of differentiation.

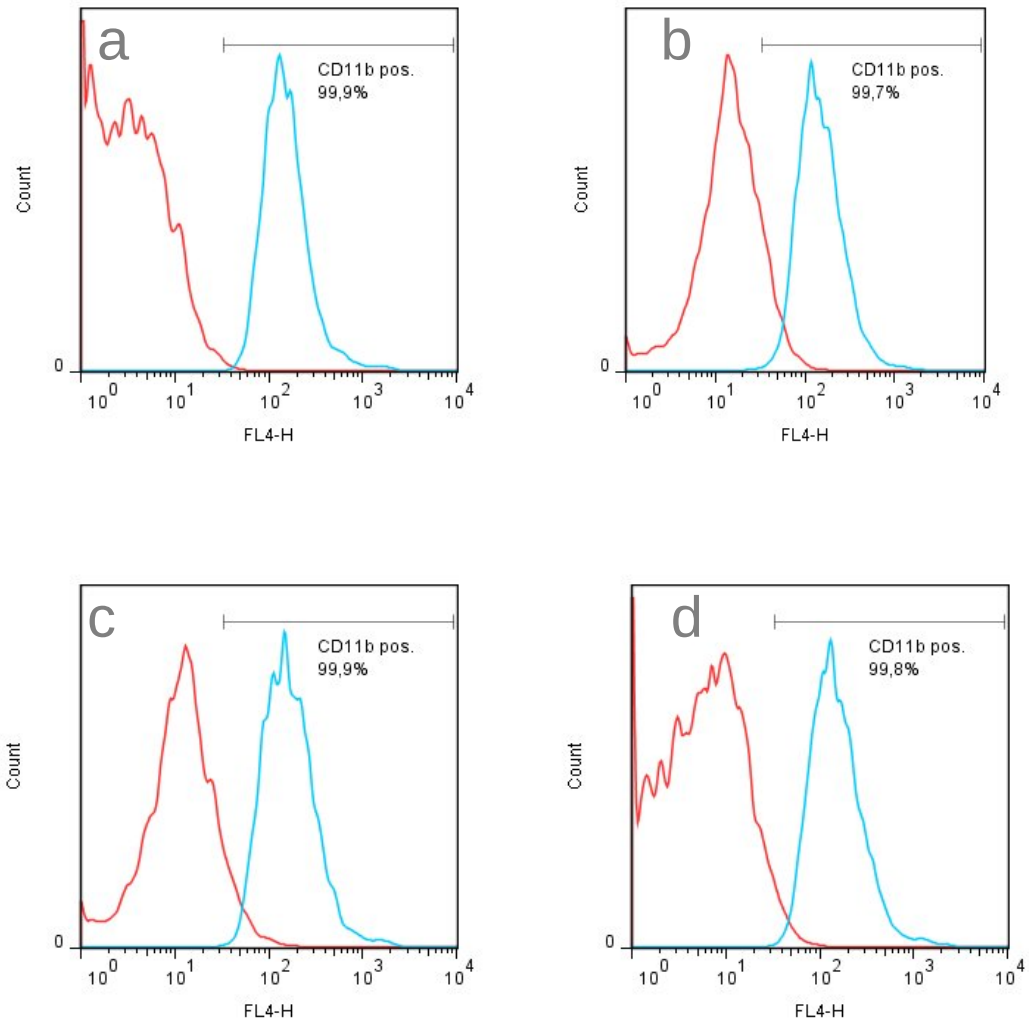

Fig.4 CD11b expression in Hoxb8 mouse neutrophils. Hoxb8 mouse neutrophils ($2x10^6$ cells/ml) were stained with APC anti-mouse CD11b Antibody. Analysis were performed with a FlowJo software (Version 10.0.3). CD11b expression of Hoxb8 WT (a), Hoxb8 $BK^{-/-}$ (b), Hoxb8 $PV^{-/-}$ (c) and Hoxb8 $gp91^{-/-}$ (d) mouse neutrophils on day 5 of differentiation. The red line represents the isotype control APC-conjugated Rat IgG 2b, Kappa (BioLegend, San Diego).

Then, we evaluated Gr-1 or Ly-6G/C 21-25 kD protein, expressed on mature granulocytes in bone marrow and peripheral tissues. It is also expressed transiently during monocyte differentiation in the bone marrow, and, at low levels, on plasmacytoid dendritic cells in lymphoid tissues. It is a very characteristic marker for neutrophils and it is upregulated during differentiation (Fig. 5).

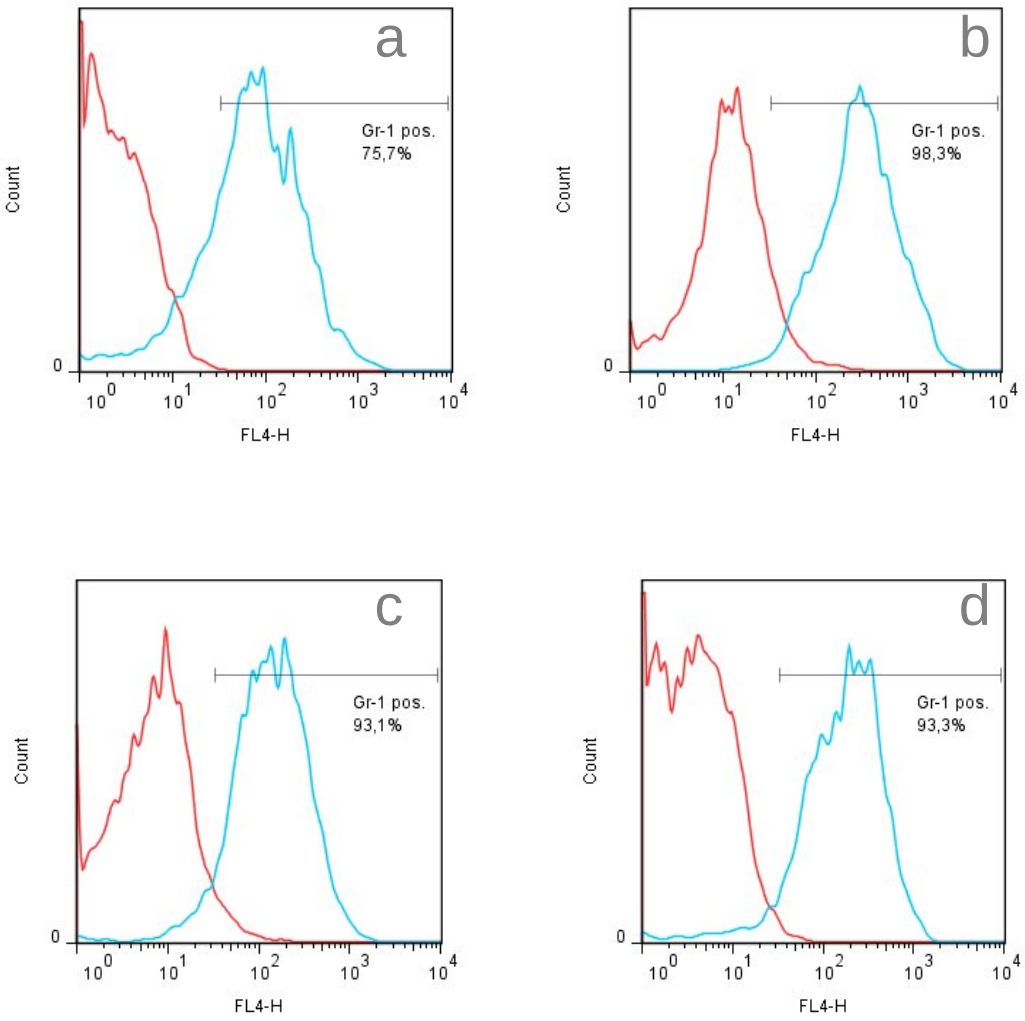

Fig.5 Gr-1 expression in Hoxb8 mouse neutrophils. Hoxb8 mouse neutrophils ($2x10^6$ cells/ml) were stained with APC anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody. Analysis were performed with a FlowJo software (Version 10.0.3). Gr-1 expression of Hoxb8 WT (a), Hoxb8 $BK^{-/-}$ (b), Hoxb8 $PV^{-/-}$ (c) and Hoxb8 $gp91^{-/-}$ (d) mouse neutrophils on day 5 of differentiation. The red line represents the isotype control APC-conjugated Rat IgG 2b, Kappa (BioLegend, San Diego).

4.1.3 Functional assays for Hoxb8 mouse neutrophils

Phagocytosis assay

In order to additionally confirm that our Hoxb8 mouse neutrophils are mature, a phagocytosis assay was performed. The ability of phagocytosis is the main function of fully mature neutrophils, and it increases proportionally during differentiation process.

Undifferentiated cells are mainly incapable of phagocytosis. Only 4.72% phagocytic cells were detected (Fig. 6a). After 3 days of differentiation, cells are more active, namely 13.66% phagocytic cells were detected (Fig. 6b). It is clearly shown that after 5 days of differentiation, the functional competence of our Hoxb8 mouse neutrophils is comparable to the competence of primary bone marrow-derived mature neutrophils, 45.47% versus 42.89% (Fig. 6c and 6d).

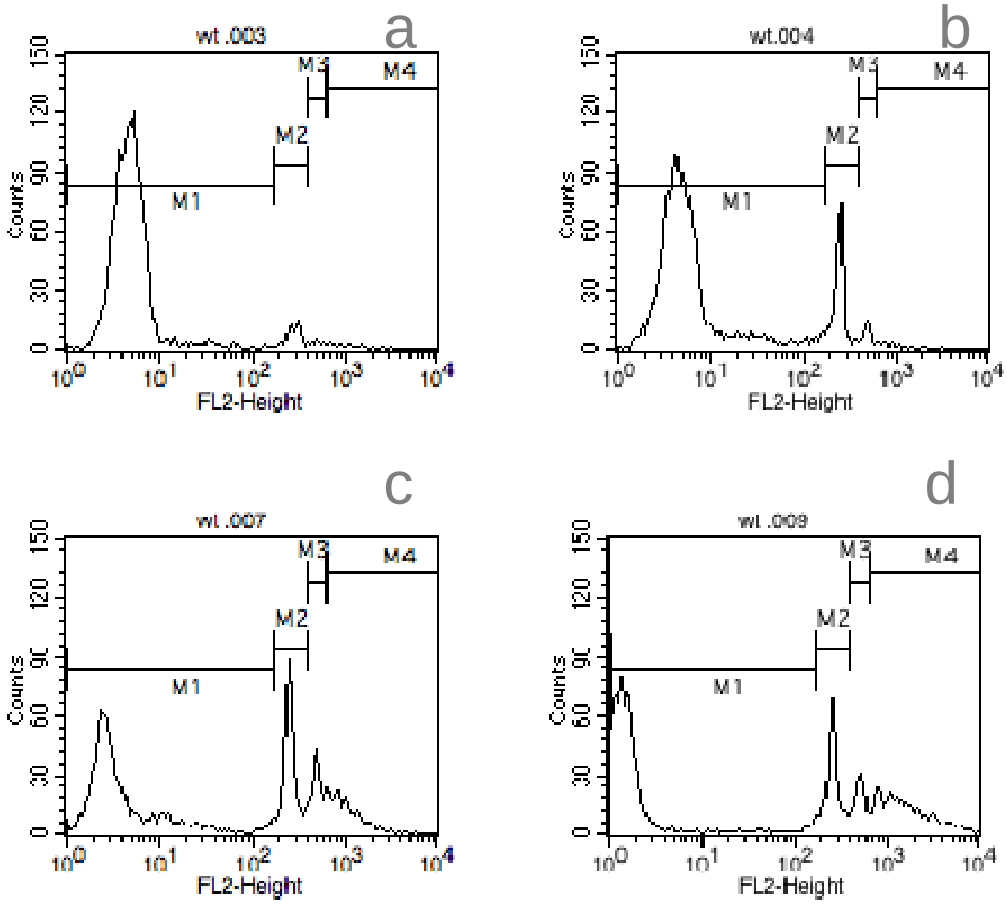

Fig. 6 Phagocytosis assay of Hoxb8 mouse neutrophils. Hoxb8 mouse neutrophils ($7.5x10^6$ cells/ml) were treated with Fluoresbrite opsonized beads (Miltenyi Biotec, Germany). Cells were immediately analyzed by FACS. Phagocytosis assay of Hoxb8 WT (a), Hoxb8 $BK^{-/-}$ (b) and Hoxb8 $PV^{-/-}$ (c) mouse neutrophils on day 5 of differentiation. Phagocytosis assay of primary bone marrow-derived mature neutrophils (d). M1 represents the inactive cells, while M2-M4 the ones with phagocytic activity. The additional peaks (M3 and M4) on mature neutrophils represent the phagocytosis of several beads.

ROS assay

Functionality of the Hoxb8 mouse neutrophils was also confirmed by performing ROS assay. The ability of ROS production is increased proportionally to the maturity of the neutrophils. Undifferentiated cell are not capable of producing reactive oxygen species (Fig.7a), while after three days of differentiation, they have significantly higher capacity of ROS production (Fig.7b). Compared to the primary bone marrow-derived neutrophils (Fig.7d), after 5 days of differentiation, our cells show almost identical ability of ROS production (Fig.7c).

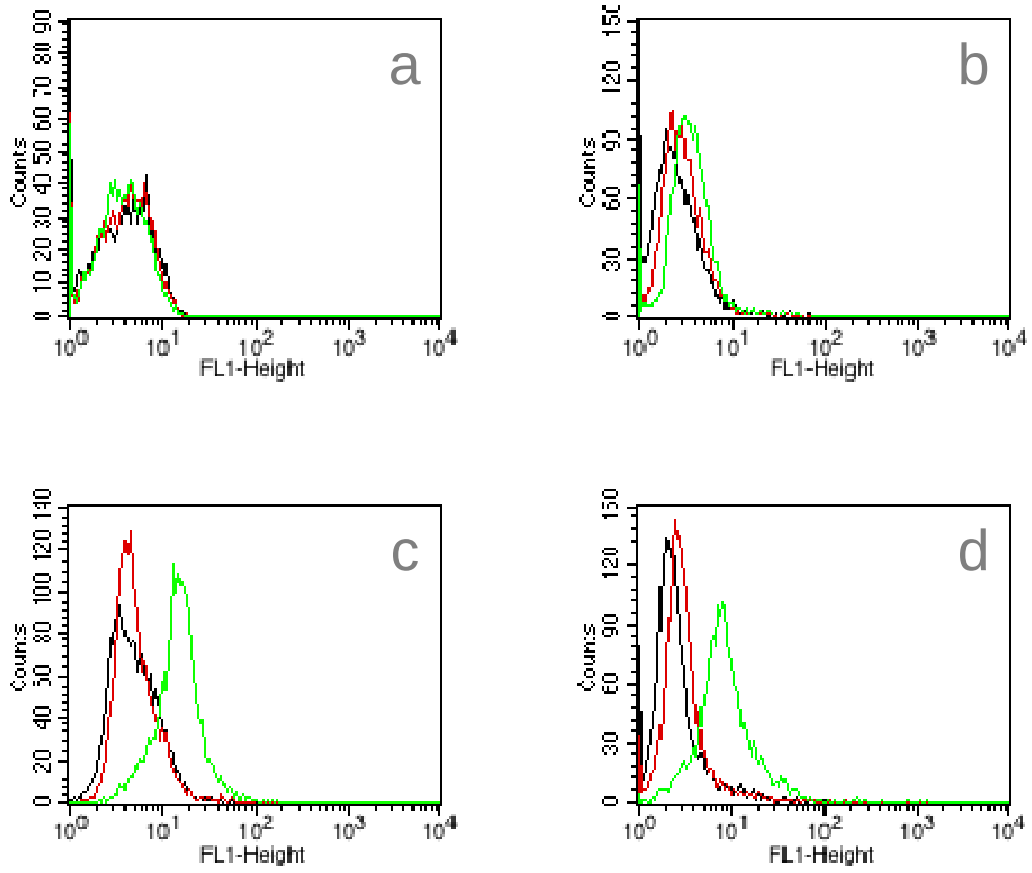

Fig. 7 Generation of ROS in Hoxb8 mouse neutrophils. Hoxb8 mouse neutrophils ($2.5x10^6$ cells/ml) were primed with GM-CSF or stimulated with C5a and measured by FACS using dihydrorhodamine (DHR) 123 fluorescent probe (Invitrogen, Switzerland). ROS production of undifferentiated Hoxb8 WT mouse neutrophils (a), Hoxb8 WT mouse neutrophils on day 3 (b), day 5 of differentiation (c) and ROS production of primary bone marrow-isolated mature neutrophils (d). The black curve represents the control without stimulation. The red curve represents priming with GM-CSF, while the green curve the ability of the cells for ROS production after stimulation with GM-SCF and C5a.

4.1.4 Viability assay

To further confirm our theory that our differentiated cells are mature enough, in good shape and most prepared for further experiments, precisely on day 5 of differentiation (also evident from Fig.8 where taken together for all cell lines), we can see that their viability is at the highest level on day 5 of differentiation, and then it reduces with time when the cells begin to die gradually.

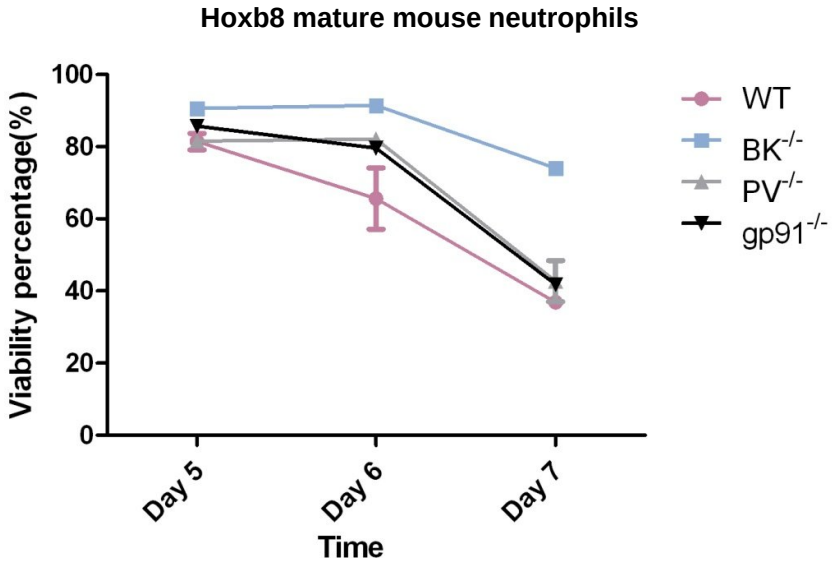

Fig. 8 Determination of cell death. Hoxb8 mature mouse neutrophils ($1x10^6$ cells/ml) were primed with GM-CSF and cultured for the indicated periods of time. Cell death was assessed by an uptake of ethidium bromide and flow cytometric analysis.

4.2 Generation of ROS and response of Ca^{2+} in Hoxb8 mature mouse neutrophils

Current data is showing differences in ROS activity between all four cell lines. Our Hoxb8 WT is producing more reactive oxygen species than Hoxb8 $BK^{-/-}$ but the difference is not significant. As expected, the ROS production of Hoxb8 WT is a lot more prominent then the ROS production of Hoxb8 $gp91^{-/-}$ which is directly connected to the function of gp91 in NADPH oxidase activity, mentioned earlier. The Hoxb8 $PV^{-/-}$ has slightly lower ROS activity in comparison to the Hoxb8 WT. (Fig. 9**)**

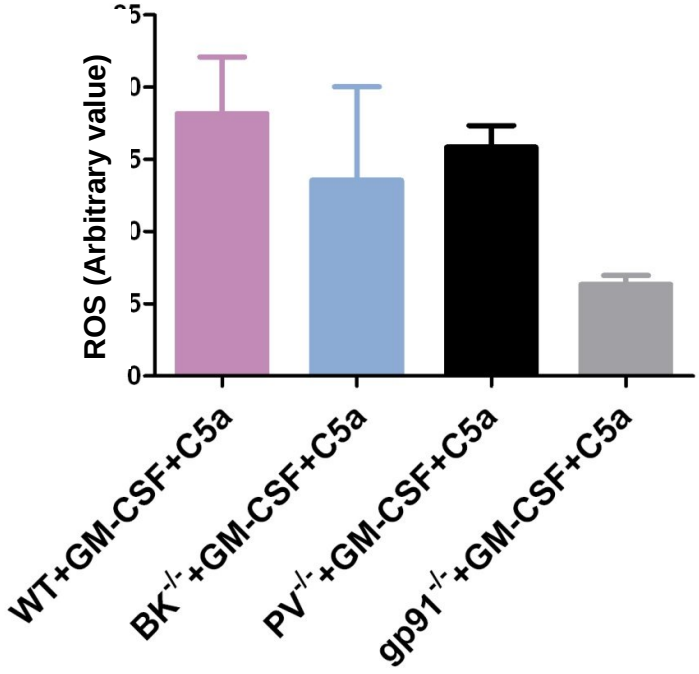

Fig. 9 Generation of ROS in Hoxb8 mouse neutrophils. Hoxb8 mature mouse neutrophils ($2x10^6$ cells/ml) on day 5 of differentiation were cultured in the presence of GM-CSF, for 30 min and subsequently stimulated with C5a in a time-dependent manner. Measurements were performed by FACS-Calibur using DHR 123 fluorescent probe (Invitrogen, Switzerland).

The response of calcium in our genetically modified Hoxb8 mature mouse neutrophils is presented in Figure 10 where we can see the differences between Hoxb8 WT, $BK^{-/-}$, $gp91^{-/-}$ and Hoxb8 $PV^{-/-}$. Lower response to calcium after stimulation with C5a refers to the knock out mature mouse neutrophils compared with Hoxb8 WT, which after its response returns to the initial state. This is contrary to the others where the lack of ROS activity leads to a sustained high level of [Ca2+]i due to Ca^{2+} influx from the extracellular space. This Ca^{2+} entry is often referred to as capacitative or store operated Ca^{2+} influx.

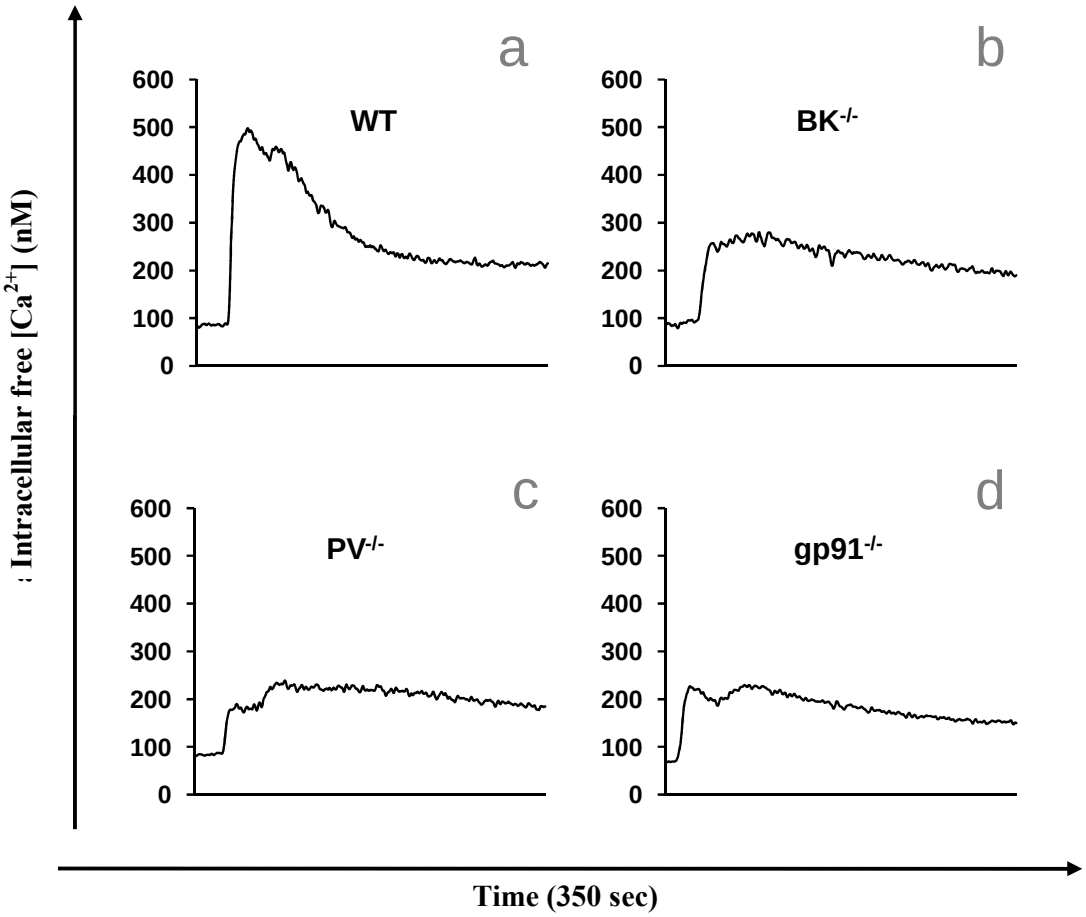

Fig. 10 Calcium response in C5a stimulated genetically modified Hoxb8 mature mouse neutrophils. Changes in $[Ca^{2+}]_i$ were analyzed in Hoxb8 WT (a), Hoxb8 $BK^{-/-}$ (b), Hoxb8 $PV^{-/-}$ (c) and Hoxb8 $gp91^{-/-}$ (d) on day 5 of differentiation. Cells ($5x10^6$ cells/ml) were incubated for 20 min with Fura-2AM (Boehringer Mannheim, Germany) and subsequently stimulated with C5a. Measurements were performed by Perkin Elmer 50B spectrofluorimeter and analyzed with FLWINLAB software.

4.3 Generation of ROS and response of Ca^{2+} in human neutrophils.

The current data indicates a lower ROS production in purified human blood neutrophils pre-treated with specific inhibitors of NADPH oxidase, such as DPI and known permeable free calcium chelators including BAPTA-AM and EGTA-AM, but not with Nifedipine a specific, or Verapamil non specific inhibitor of L-type voltage-dependent calcium channels.

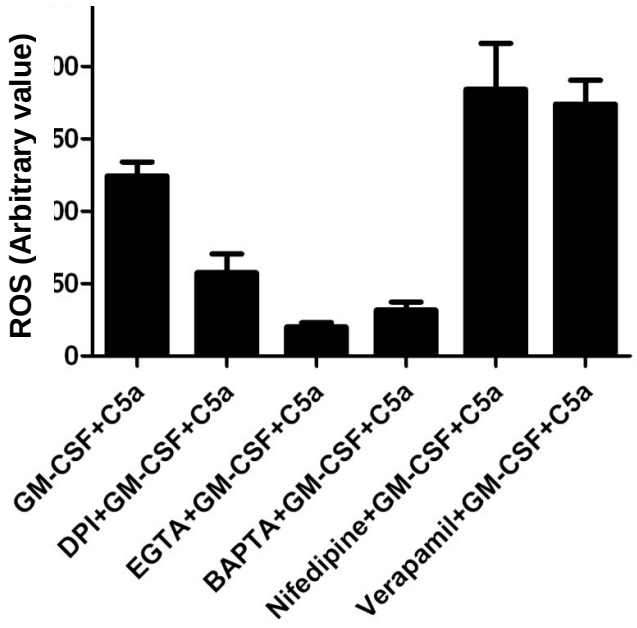

Fig. 11 Generation of ROS in purified human blood neutrophils. GM-CSF-primed neutrophils ($2x10^6$ cells/ml) were cultured in the presence of pharmacological inhibitors and different chelators. After 15 min of stimulation with C5a in combination with DHR 123 fluorescent probe, measurements were performed and analyzed by FACS-Calibur.

In order to link ROS activity measured in presence of BAPTA, DPI, EGTA, Nifedipine and Verapamil with the response of calcium in purified blood human neutrophils, the cells were pre-treated with above mentioned reagents and stimulated with C5a before measurement.

BAPTA-AM prevent elevation of $[Ca^{2+}]_i$ in response to C5a added before measurement (Fig.12b). In the same case when neutrophils were pre-treated with DPI, we observed small difference compared with the control (Fig.12e), while the voltage-dependent Ca^{2+} channel blockers Nifedipine (Fig.12d) and Verapamil (Fig.12c) did not have any effect on the entry of Ca^{2+} into the cells. EGTA-AM treatment also prevent elevation of $[Ca^{2+}]_i$ in response to C5a compared with the control (Fig.12f).

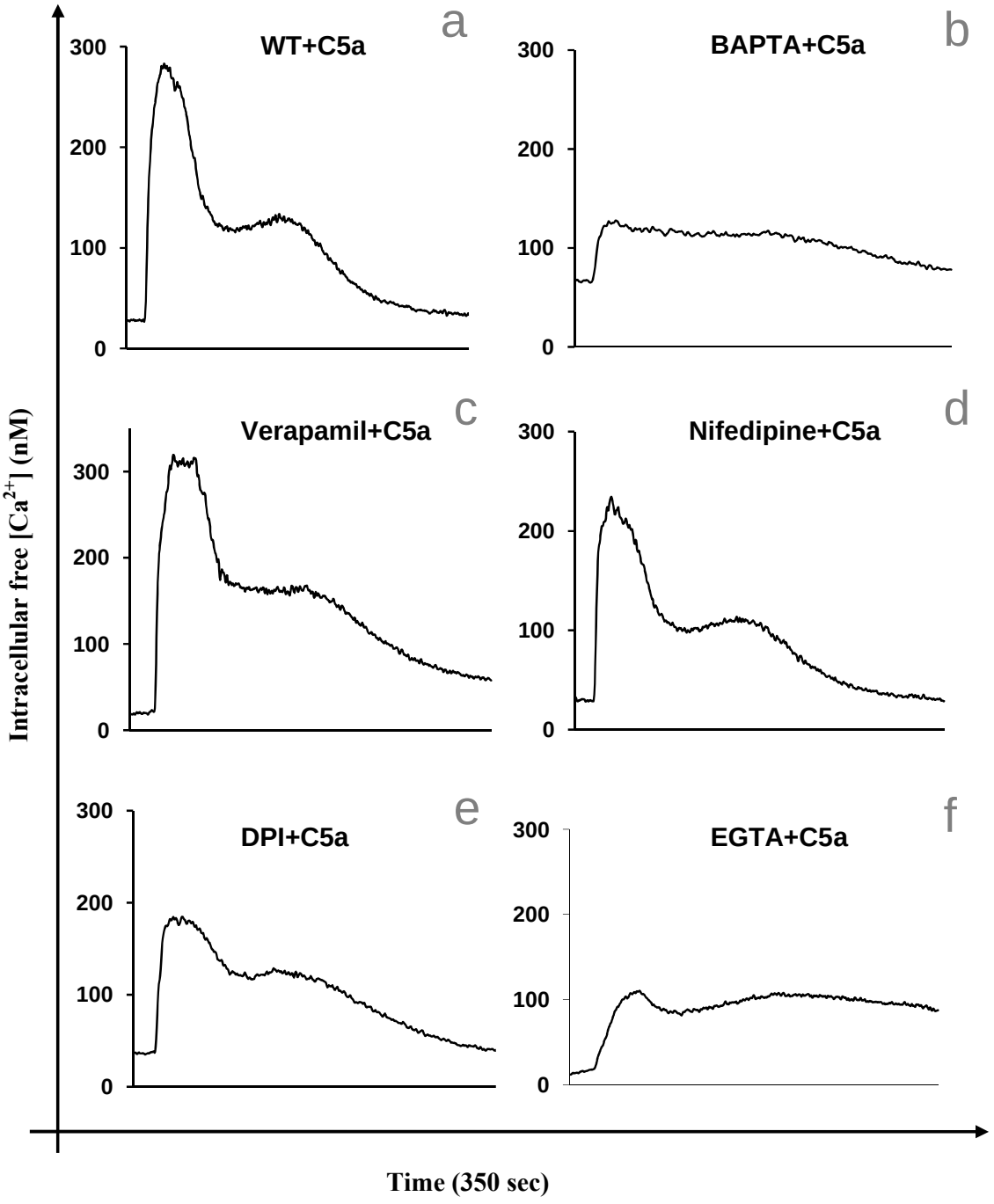

Fig. 12 Response of Ca^{2+} in purified blood human neutrophils. Neutrophils ($5x10^6$ cells/ml) were incubated for 20 min with Fura-2AM (Boehringer Mannheim, Germany), and cultured with BAPTA (b), Verapamil (c), Nifedipine (d), DIPI (e), EGTA (f). After stimulation with C5a measurements were performed by Perkin Elmer 50B spectrofluorimeter and analyzed with FLWINLAB software. For positive control, purified blood neutrophils were incubated for 20 min with Fura-2AM, cultured in absence of pharmacological inhibitors or Ca^{2+} chelators and subsequently stimulated with C5a (a).

4.4 Role of Paxilline in generation of ROS and Ca^{2+} response in human and Hoxb8 mouse neutrophils.

Paxilline, known as an inhibitor of the large-conductance Ca^{2+} activated K^+ channel, helped us to confirm and understand the involvement of others channel in Ca^{2+} entry and the importance of this signaling molecule. Purified human blood neutrophils treated with Paxilline, followed by GM-CSF and a C5a stimulation shown in Figure 13a, indicate decreased ROS production. Additionally, treatment with Paxilline, followed by a C5a stimulation also caused a decrease of $[Ca^{2+}]_i$ (Fig. 14b). These events indicate the involvement of other channels in the regulation of Ca^{2+} entry in to the cell. Reduced ROS activity, after treatment with recombinant Parvalbumin already known as Ca^{2+}-binding protein is shown in Figure 13b. When Hoxb8 $PV^{-/-}$ mature mouse neutrophils were treated with Paxilline, followed by a C5a stimulation, a decrease in $[Ca^{2+}]_c$ was observed (Fig.15b). This can be attributed to lack of influx of Ca^{2+} from the extracellular space. Hoxb8 $PV^{-/-}$ mature mouse neutrophils, treated only with C5a in each attempt, had an additional second peak referring to the influx of extracellular Ca^{2+} (Fig. 15a).

This confirms our hypothesis that reduced levels of $[Ca^{2+}]_i$ lead to reduced ROS activity. However, NADPH activity requires a second signal, provided by the extracelllular Ca^{2+}, which acts in synergy with $[Ca^{2+}]_i$.

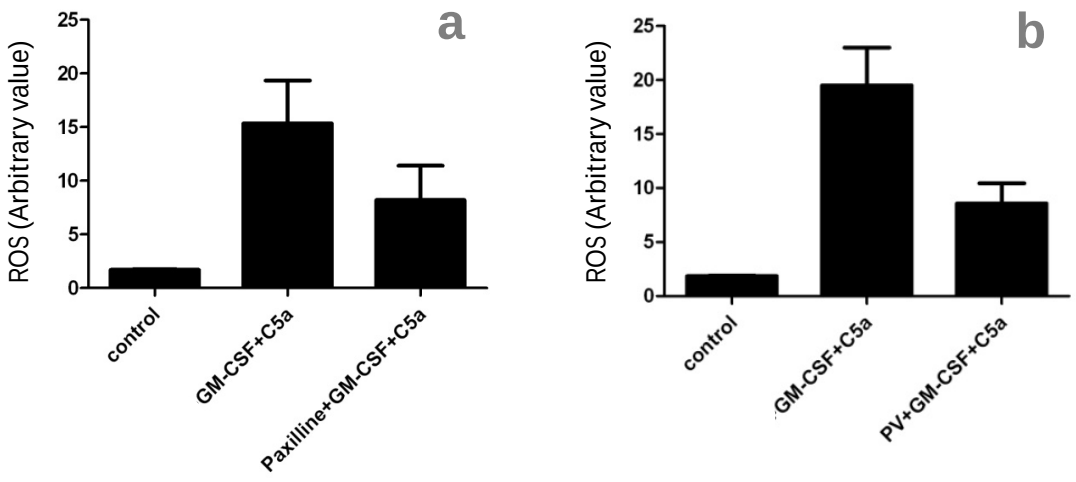

Fig. 13 Generation of ROS in purified human blood neutrophils. GM-CSF-primed neutrophils ($2x10^6$ cells/ml) were cultured in presence of Paxilline (a) or recombinant Parvalbumin (b) for 30

min. After 15 min of stimulation with C5a in combination with DHR 123 fluorescent probe, measurements were performed and analyzed by FACS-Calibur. For positive control, cells were primed with GM-CSF, followed by C5a stimulation.

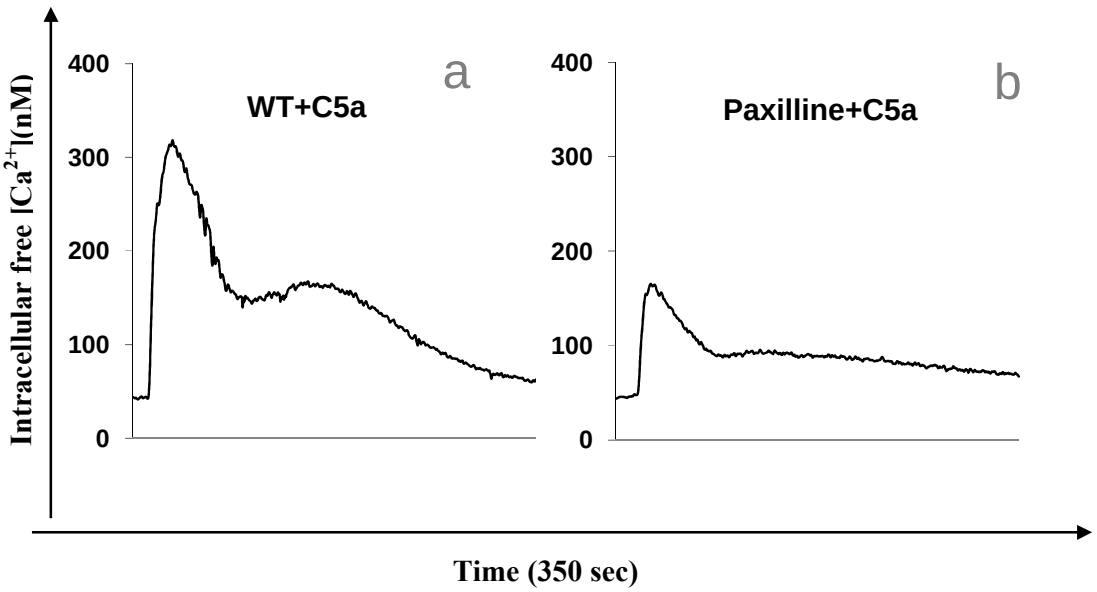

Fig. 14 Response of Ca^{2+} in purified human blood neutrophils. Neutrophils ($5x10^6$ cells/ml) were incubated for 20 min with Fura-2AM (Boehringer Mannheim, Germany). Changes in $[Ca^{2+}]_i$ were analyzed in purified blood neutrophils treated with Paxilline, followed by C5a stimulation (b). For positive control, purified blood neutrophils were cultured in absence of Paxilline and subsequently stimulated with C5a (a). Measurements were performed by Perkin Elmer 50B spectrofluorimeter and analyzed with FLWINLAB software.

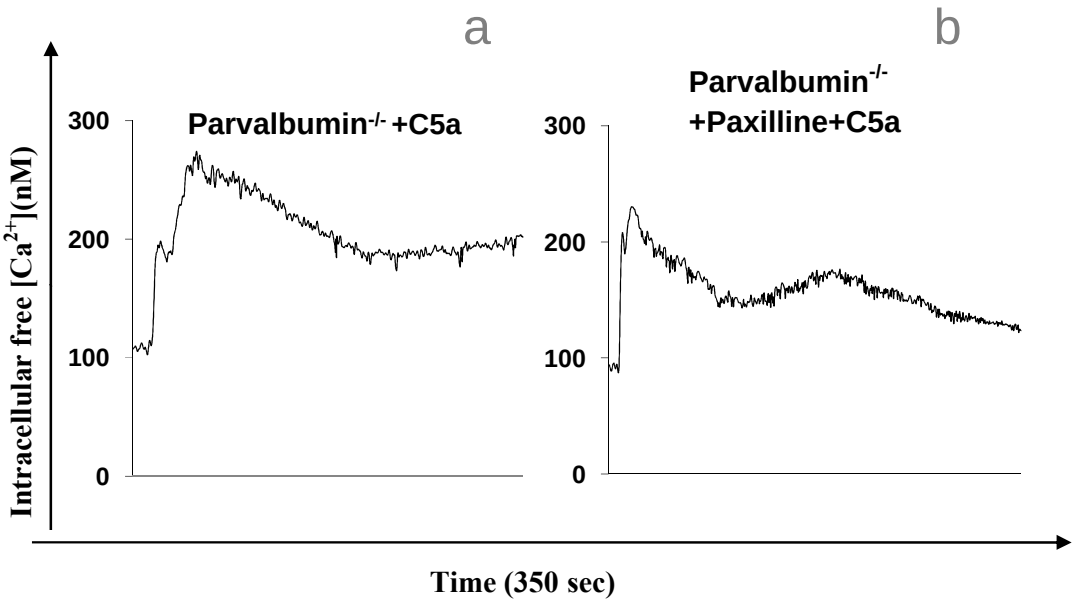

Fig. 15 Response of Ca^{2+} in Hoxb8 $PV^{-/-}$ mature mouse neutrophils. Changes in $[Ca^{2+}]_i$ were analyzed in Hoxb8 $PV^{-/-}$ mouse neutrophils on day 5 of differentiation in presence (b) or absences (a) of Paxilline. Cells ($5x10^6$ cells/ml) were incubated for 20 min with Fura-2AM (Boehringer Mannheim, Germany) and subsequently stimulated with C5a. Measurements were performed by Perkin Elmer 50B spectrofluorimeter and analyzed with FLWINLAB software.

5. Discussion

This work has studied the involvement of Ca^{2+} as a key second messenger in the activation of various processes and its role as a signal for oxidase activity, leading to the production of reactive oxygen species in stimulated polymorphonuclear leukocytes.

The Hoxb8 genetically modified mature neutrophils used for this cause is a good model since the morphology and functionality of the cells were changed after the removal of tamoxifene, indicating a successful differentiation. The morphological studies have enabled us to determine differences in morphological maturity between the cell lines on different days. Figure 3 in present study clearly reveal a difference in morphological maturity between the cell lines, but, taken together, we have concluded that on day 5 of differentiation they are ready for further studies as mature neutrophils. This hypothesis was also confirmed by showing the expression of specific neutrophil surface proteins as Gr-1 and CD11b. The results show that Gr-1 on day 5 of differentiation was regulated as expected for all cell lines and CD11b as well.

In order to confirm that our Hoxb8 system works, phagocytosis and ROS assay were performed on our Hoxb8 WT. From the obtained results we can conclude that our Hoxb8 cell lines after five days of differentiation are more capable of phagocytosis compared with the Hoxb8 cell lines after three days of differentiation. After this number of days of differentiation, the ability of phagocytosis for these Hoxb8 cells lines was on the same level as primary bone marrow-derived mature neutrophils. It was also clearly visible that after five days of differentiation they were able to produce ROS as primary bone marrow-derived mature neutrophils and much more than after three days of differentiation.

Immunoblot for the Hoxb8 $PV^{-/-}$ and qPCR methods for the Hoxb8 $BK^{-/-}$ mature mouse neutrophils which were previously performed at the Institute for Pharmacology at the University of Bern, helped us to characterize these cell lines and to confirm their genetic modification. Presented data confirmed presence of parvalbumin in human neutrophils and absence in our Hoxb8 $PV^{-/-}$ mature mouse neutrophils. We could also conclude that our $Hoxb8^{-/-}$ mouse mature neutrophils do not express BK mRNA, whereas Hoxb8 WT cells do. Presence of BK channel in human neutrophils was also confirmed by qPCR method (data not shown).

During our study, we revealed that extracellular Ca^{2+}, $[Ca^{2+}]_i$ and intracellular stores of Ca^{2+} in PMNs are in a rapid and dynamic equilibrium in activated and inactivated cells. C5a stimulated PMN deplete their intracellular stores as a result of the activation of $InsP_3$ by phospholipase Cβ (PLCβ), which activates the release of Ca^{2+} from intracellular stores through its channel receptor ($InsP_3$-R). This depletion, in turn, causes an increase of plasma membrane Ca^{2+} permeability allowing SOC channels the direct reloading of intracellular Ca^{2+} stores from the extracellular medium.

By using different inhibitors, we saw a significant decrease of ROS production caused by BAPTA, DPI and EGTA, but not with Nifedipine and Verapamil which are known as L-type voltage-depended Ca^{2+} channel blockers. Nifedipine and Verapamil also did not prevent the entry of Ca^{2+} in Ca^{2+}-depleted PMNs in contrast to their effect on this well described Ca^{2+} channel. We did not investigate thoroughly the mechanisms why the Ca^{2+} release is insensitive to Nifedipine or Verapamil, but there are studies that do not exclude the possibility that L-type Ca^{2+} channels are not present in the PMN plasma membrane. Also, they are more responsible for the exchange of Ca^{2+} between the cytosol and intracellular stores **(42).** Preincubation with Nifedipine or Verapamil, followed by a C5a stimulation, demonstrated increase in $[Ca^{2+}]_c$ due to Ca^{2+} influx from the extracellular space. These changes in Ca^{2+} flux, in turn, affect the activity of various enzymes such as protein kinase C (PKC) when Ca^{2+} mediated kinase activation causes phosphorylation of their subunits. This leads to the production of reactive oxygen species. It is clearly shown in our study that neutrophils treated with Nifedipine or Verapamil had high ROS production in correlation with an increased $[Ca^{2+}]_c$.

Evidence for the requirement of extracellular Ca^{2+} entry as to activate neutrophil oxidases is based on our analysis showing a significant decrease of superoxide anion production when extracellular Ca^{2+} was inhibited or chelated with EGTA treatment followed by C5a. Similarly, BAPTA-AM also caused a significant inhibition of ROS activity due to reduction in cytosolic Ca^{2+} concentration.

Another interesting observation on the inhibition of the C5a pathway with BAPTA and EGTA was the difference in magnitude: EGTA treatment had more impact than BAPTA treatment in inhibiting C5a–mediated stimulation of ROS activity, which is compatible with the requirement for a high local Ca^{2+} concentration. DPI treated neutrophils also

decreased NADPH activity, but we observed partial and not very significant $[Ca^{2+}]_i$ decrease. This suggests that ROS is not required for initial increase of intracellular Ca^{2+} and it is calcium the one that is responsible for activating different pathways leading to the production of reactive oxygen species.

We have seen that C5a elevates the cytosolic Ca^{2+} concentration of neutrophils from about 20-80nM to 300-400nM. This C5a-dependent increase in intracellular free Ca^{2+} mainly involves mobilization of Ca^{2+} from the intracellular stores shown in our graphs as a first peak and the Ca^{2+} influx peak comes later. The rapid increase in cytosolic Ca^{2+} levels is a transient response to C5a receptor interaction and persists for several minutes. In fact, there is, upon attainment of maximal values, a slow decrease of free Ca^{2+} in the cytosol down to resting values.

However, IP_3 may possibly cause Ca^{2+} release from intracellular stores by more than one mechanism. It is possible that an IP_3 independent mechanism for Ca^{2+} release from intracellular stores exists in PMNs since, in some cells, the Ca^{2+} release from intracellular stores can occur independently of inositol phosphate formation or other mechanisms, and independently of intracellular Ca^{2+} signaling pathways, required for the priming of NADPH oxidase **(42).**

As described earlier how complex the mechanism of Ca^{2+} entry is, we tried to determine the role of other channels that might be involved in Ca^{2+} signaling pathways. The preliminary results are relatively interesting and indicate the presence of calcium dependent potassium channel (BK channel) in human neutrophils that might play an important role in the cross talk between Ca^{2+} and ROS activity.

In order to find the answer, we used Paxilline indole alkaloid, known as specific BK channel blocker, in both cases of ROS production and measurements of intracellular calcium concentration. Paxilline treated neutrophils, followed by a C5a stimulation, demonstrate a lower production of reactive oxygen species compared with the control and significant decrease of $[Ca^{2+}]_c$. In addition, when Hoxb8 $PV^{-/-}$ mature mouse neutrophils were treated with Paxilline, followed by a C5a stimulation, we observed a decrease in $[Ca^{2+}]_c$ regarding the influx of Ca^{2+} from the extracellular space. Hoxb8 $PV^{-/-}$ mature mouse neutrophils, treated only with C5a in each attempt, had an additional second peak

which refers to the influx of Ca^{2+}, but not when they were treated with Paxilline, which is obliviously capable of blocking the influx of Ca^{2+} from the extracellular space.

This results enhances the participation theory of various channels in Ca^{2+} entry and also our hypothesis that an increase of $[Ca^{2+}]_c$ is apparently not sufficient to initiate NADPH oxidase activation, but an additional signal in combination with Ca^{2+} influx is almost certainly required for NADPH oxidase activation.

Many studies related to this issue are ongoing by the Institute of Pharmacology at the University of Bern, and very interesting results and conclusions will be presented by this Institute in the near future.

The contribution of transient $[Ca^{2+}]_c$ elevation in NADPH oxidase activation was also tested by using genetically modified estrogen-regulated Hoxb8 mouse neutrophils, previously described in our study. Genetically modified Hoxb8 mouse neutrophils that lack $gp91^{phox}$ important subunit of NADPH oxidase and Hoxb8 $BK^{-/-}$ mouse neutrophils demonstrate lower ROS activity after a C5a stimulation compared with the Hoxb8 WT.

When recombinant parvalbumin (PV), a calcium binding protein, was added to human neutrophils followed by a C5a stimulation, we saw a decrease in production of reactive oxygen species, and our Hoxb8 $PV^{-/-}$ mouse neutrophils also demonstrated lower ROS production compared with the Hoxb8 WT mature mouse neutrophils.

These results once again confirmed the role of Ca^{2+} as an important signaling molecule in the control of the NADPH oxidase activity. In respect of $[Ca^{2+}]_{i,}$ our genetically modified Hoxb8 mouse neutrophils that lack $gp91^{phox}$, BK or parvalbumin demonstrate interesting results because they all had smaller first initial responses to C5a when compared with the Hoxb8 WT, but a sustained level of $[Ca^{2+}]_i$ which holds constantly and does not return to the initial level. This led us to conclude that the lack of ROS activity leads to a sustained high level of $[Ca^{2+}]_i$ due to a Ca^{2+} influx from the extracellular space.

This suggests that NADPH oxidase regulation requires a second signal, a Ca^{2+} independent pathway, acting in a synergy with a Ca^{2+} influx from SOCE.

However, there is one point in our study, still not clear and in the process of research at the Institute of Pharmacology, with respect to Hoxb8 $PV^{-/-}$ mouse neutrophils. It was

previously known that $PV^{-/-}$ cells have larger mitochondrial mass (40% increase) **(43),** and, therefore, our further study was focused to determine the concentration of calcium in the mitochondria. Our preliminary data confirmed our hypothesis that lower but sustained concentration of Ca^{2+} in the cytosol of genetically modified Hoxb8 $PV^{-/-}$ mature mouse neutrophils is a result of a high accumulation of Ca^{2+} in the mitochondria (data not shown). This mechanism is probably activated in order to efficiently reduce $[Ca^{2+}]_i$ below its toxic level. In addition, we are planning to perform the localization of the mitochondria in Hoxb8 $PV^{-/-}$ mouse neutrophils. Measurements of mitochondrial Ca^{2+} in Hoxb8 $PV^{-/-}$ cells are already in the process as are the measurements of mitochondrial Ca^{2+} in Hoxb8 $BK^{-/-}$ cells to see whether the mitochondria in these cells has any role in the accumulation of excessive calcium. By using Thapsigargin endomembrane ATPase inhibitor that can deplete the intracellular Ca^{2+} stores, we need to find out to what extent the extracellular Ca^{2+} is contributing to the ROS activity. Measurements of Ca^{2+} free medium need also to be performed in order to determine the difference in the intensity of generation reactive oxygen species when cells are suspended in Ca^{2+} medium and medium not containing Ca^{2+}. This can help us understand the role of extracellular Ca^{2+} required for ROS activity under different conditions.

6. Conclusion

In PMNs, extracellular Ca^{2+}, $[Ca^{2+}]_i$ and intracellular stores of Ca^{2+} are in a rapid and dynamic equilibrium in activated and inactivated cells. Activation of neutrophil oxidases, including NADPH oxidase, is Ca^{2+}-dependent. An increase of $[Ca^{2+}]_i$ is crucial for the regulation of ROS activity, acting in a synergy with a Ca^{2+} influx from the extracellular space and certainly required for NADPH oxidase activation.

Evidence for the requirement of extracellular Ca^{2+} entry to activate neutrophil oxidases is based on our analysis showing a significant decrease of superoxide anion production when extracellular Ca^{2+} was inhibited or chelated with EGTA treatment followed by C5a. BAPTA also caused a significant inhibition of ROS activity due to the reduction in cytosolic Ca^{2+} concentration.

An increase of $[Ca^{2+}]_i$ despite the lack of ROS activity in DPI-treated neutrophils, suggest that ROS is not required for initial increase of intracellular Ca^{2+}, and it is indeed Ca^{2+} the one that activates different pathways of ROS production.

The lack of ROS activity leads to sustained high level of $[Ca^{2+}]_i$ due to Ca^{2+} influx from the extracellular space.

It is evident that both intra- and extracellular Ca^{2+} are required for full oxidase activation. Another important point is that the Ca^{2+} influx from the extracellular space plays an important role in both the generation of reactive oxygen species and the activation of other independent pathwys leading to NADPH oxidase activation.

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