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MEHANIZMI GIBANJA MITOHONDRIJEV OB AKTIVACIJI NEVTROFILCEV

MITOCHONDRIA IN NEUTROPHILS: REGULATION OF THEIR LOCALIZATION

Ljubljana, 2013

Diplomsko delo sem opravljala na Institute of Pharmacology, University of Bern, v sodelovanju s Fakulteto za farmacijo, Univerza v Ljubljani, pod mentorstvom prof. dr. Irene Mlinarič-Raščan in somentorstvom prof. dr. dr. Hans-Uwe Simonom.

Izjava

Izjavljam, da sem diplomsko nalogo izdelala samostojno pod mentorstvom prof. dr. Irene Mlinarič-Raščan in somentorstvom prof. dr. dr. Hans-Uwe Simonom.

Ljubljana, april 2013 Evelin Krajnc

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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. I worked under the supervision of Prof. Dr. Irena Mlinarič-Raščan and Prof. Dr. Dr. Hans-Uwe Simon.

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Statement

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, April 2013 Student signature: Evelin Krajnc

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ABSTRACT

Protection from pathogens is one of the basic functions that immune system has evolved to. It is fundamentally important for survival to have a highly discriminatory immune system.

Neutrophils are one of the main types of effector cells in the innate immune system and were shown to effectively kill microorganisms by phagocytosis, more than 100 years ago. In 2004 was found that stimulated neutrophils can also produce extracellular structures called *neutrophil extracellular traps (NETs)* that capture and kill microorganisms.

The aim of the project was to investigate the regulation mitochondrial localization in neutrophils upon activation. Mature neutrophils are short-lived cells that cannot be genetically manipulated; therefore we also used immortalized, genetically modified immature mouse neutrophils. We worked mainly on six different genetically modified Hoxb8 cell lines: wild-type, Parvalbumin knockout, RhoH knockout, Wiskott-Aldrich syndrome protein knockout, Optic atrophy gene 1 knockout, and Big Potassium channels knockout.

The main method used was confocal laser scanning microscopy. Confocal images with selectively labelled functional regions provided useful information about mitochondria, nucleus, and plasma membrane in cells. Neutrophils were either fixed with 4 % paraformaldehyde or observed in live cell microscopy experiments. For activation, we used the physiological stimulators, such as granulocyte/ macrophage colony-stimulating factor (GM-CSF) and complement factor 5a (C5a). Several pharmacological inhibitors affecting microtubules and microfilaments, such as nocodazole, lantrunculin B, and FIPI, were used to study the role of the cytoskeleton in mitochondrial movement and NET formation. The same functional endpoints were tested in genetically modified Hoxb8 mouse neutrophils.

The results of our study point to the importance of microfilaments, microtubules, intracellular calcium and mitochondrial fusion processes for mitochondrial movement and NET formation.

Taken together, our study provides new insights into molecular mechanisms of innate immune responses and possibly provides new strategies for treating and preventing infectious diseases.

Keywords: mitochondria, neutrophils, estrogen-regulated Hoxb8 mouse neutrophil, mitochondrial DNA, cytoskeleton.

POVZETEK

Imunski sistem se je razvil, da nas zaščiti pred patogeni. Sposobnost ločevanja imunskega sistema med telesu lastnimi in tujimi makromolekulami je bistvenega pomena za preživetje. Nevtrofilci so najštevilčnejše in najbolj pomembne celice prirojenega imunskega sistema. Že pred več kot 100 leti so bili prikazani kot učinkoviti uničevalci mikrobov s fagocitozo. V letu 2004 je bilo ugotovljeno, da lahko stimulirani nevtrofilci proizvajajo tudi zunajcelične strukture, imenovane nevtrofilne zunajcelične pasti, ki zajemajo in uničijo mikroorganizme.

Cilj diplomske naloge je bila raziskava regulacije mitohondrijske lokalizacije v nevtrofilcih ob njihovi aktivaciji. Zreli nevtrofilci so kratkožive celice, ki jih ni mogoče gensko spremeniti. Zato smo v tej raziskavi uporabili tudi nesmrtne, gensko spremenjene mišje nevtrofilce. Teste smo izvajali na petih različnih gensko spremenjenih celičnih linijah Hoxb8, ki so imeli odstranjen gen za Parvalbumin, RhoH, beljakovino Wiskott-Aldrichovega sindroma, Optično atrofijo 1 in gen za velike kalijeve kanalčke. Kot kontrolo smo uporabili mišje nevtrofilce divjega tipa Hoxb8.

Glavna metoda dela je bila konfokalna laserska mikroskopija. Konfokalne slike s selektivno obarvanimi funkcionalnimi regijami nam zagotavljajo uporabne informacije o mitohondrijih, jedru in plazemski membrani v celicah. Nevtrofilce smo bodisi fiksirali s 4 % paraformaldehidom ali opazovali v živo celične procese s fluorescenčno mikroskopijo. Za aktivacijo smo uporabili fiziološke stimulatorje: granulocitno makrofagne kolonije spodbujajoči faktor (GM-CSF) in fragment komplementa C5a. Za preučevanje vloge citoskeleta pri mitohondrijskem gibanju in nastajanju nevtrofilnih ekstracelularnih pasti smo uporabili več farmakoloških inhibitorjev, ki vplivajo na mikrotubule in mikrofilamente, kot so nokodazol, lantrunkulin B in FIPI. Iste funkcionalne končne točke so bile testirane v gensko spremenjenih Hoxb8 mišjih nevtrofilcih.

Rezultati naše raziskave kažejo na pomen mikrofilamentov, mikrotubulov, pomen znotrajceličnega kalcija in mitohondrijskih fuzijskih procesov na gibanje mitohondrijev in nastanek nevtrofilnih ekstracelularnih pasti.

Naša študija prinaša nove vpoglede v molekularne mehanizme prirojenega imunskega odziva in potencialno ponuja nove strategije za zdravljenje in preprečevanje infekcijskih bolezni.

Ključne besede: mitohondriji, nevtrofilci, estrogensko regulirani Hoxb8 mišji nevtrofilci, mitohondrijski DNA, citoskelet.

GLOSSARY

BK	Calcium dependent potassium channel
BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscope
C5a	Complement component 5a
DNase	Deoxyribonuclease
EGTA	Ethylene glycol tetraacetic acid
ER-Hoxb8	Estrogen-regulated Hoxb8
ET	Extracellular traps
FCS	Fetal calf serum
FIPI	5-fluoro-2-indolyl-des-chlorohalopemide
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/ macrophage colony-stimulating factor
LSM	Laser scanning microscope
MFs	Microfilaments
mtDNA	Mitochondrial DNA
MTs	Microtubules
N	Neutrophils
NETs	Neutrophil extracellular traps
OPA	Optic atrophy
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
RhoH	Ras homolog gene family, member H
PMN	Polymorphonuclear leukocyte
PV	Parvalbumin
RT	Room temperature
SFM	Serum-free hematopoietic cell medium
SCF	Stem cell factor
WASp	Wiskott-Aldrich syndrome protein
WT	Wild-type

1 INTRODUCTION

Protection from pathogens is one of the basic functions that immune system has evolved to. It is fundamentally important for survival to have a highly discriminatory immune system (1). Infectious diseases are one of the major death causes in the world. They represent one-third of all deaths, which is more than all forms of cancer together. Besides continuing severe forms of ancient diseases, such as tuberculosis and malaria, new forms of infectious diseases are constantly emerging, including pandemic of AIDS (acquired immune deficiency syndrome) (2). Disorder of the immune system falls into two main categories: *immunodeficiency*, in which the immune system is missing one or more of its parts, and *autoimmunity*, in which the immune system mistakenly attacks its own body cells and tissues. Other immune system disorders include different *hypersensitivities*, in which the body responses to harmless substances with an exaggerated immune response (3, 4). The burden of infectious disease has been substantially reduced by medical interventions, such as vaccinations, antimicrobial drugs, and routine testing of blood before using it for transfusion for many humans. Direct transmission can be diminished by adequate hygiene.

A human organism not only maintains constant temperature and renews itself, but is also a nutrient-rich, warm and moist environment. Many microorganisms have developed the ability to survive and reproduce in this desirable niche. The immune system has evolved a powerful collection of defence mechanisms to protect against potential invaders willing to take advantage from it. Like other organisms, pathogen is simply fulfilling its biological imperative to live and procreate. Barriers against infection that keep out microorganisms are skin, the secretion of mucus, ciliary action, the action of bacterial fluids (e.g. tears), gastric acid, and microbial antagonism (2, 5).

Understanding that pathogens infect host cells through exploitation of their biological properties can give us a new aspect into biology of the cell as well as principles and strategies for treating and preventing infectious diseases. Vertebrates use two types of immune defence which are performed by specialized proteins and cells to fight against particularly powerful pathogens that breach these barriers: **innate** immune responses take action immediately after an infection begins, while strong **adaptive** immune responses act later in an infection and are highly specific to the pathogen that induced them (2). B cells and T cells initiate adaptive immune responses and are responsible for the specific

recognition of antigens. All lymphocytes are derived from bone marrow stem cells, but T lymphocytes (T cells) then develop in thymus, while B lymphocytes (B cells) develop in the bone marrow (1).

Various molecules are involved in the development of immune responses, including complement proteins and cytokines. The role of *complement proteins* is to mediate phagocytosis, control inflammation, and interact with antibodies in immune defence. Microbes are engulfed by phagocytic cells which are attracted by complement system. C3 is the most abundant component. C5a is a small peptide that is produced after activation of component C5. C5a increases capillary permeability and acts as a potent chemotactic agent for neutrophils. Both C3a and C5a affect mast cells which results in the further release of mediators such as leukotriene B4, histamine and tumour necrosis factor (TNF) with effects on capillary permeability, adhesiveness, and neutrophil chemotaxis; they also activate neutrophils (1, 6).

Cytokines are proteins or glycoproteins that transmit signals between lymphocytes, phagocytes and other cells of the body. Interleukins and interferons represent a group of cytokines. Interleukins have a variety of functions. The function of many interleukins is to cause other cells to divide and differentiate. Spreading of certain viral infections is limited by interferons. *Colony stimulating factors* are also very important because they regulate the division and differentiation of bone marrow stem cells as well as precursors of blood leukocytes. Movement of leukocytes in the body is managed by chemokines (1).

Function of phagocytes is to engulf particles, including infectious agents, internalize them and destroy them. Long-lived monocytes and macrophages belong to the group of mononuclear phagocytes. On the other hand, polymorphonuclear neutrophils (often called just neutrophils or PMNs) are short-lived phagocytes. The inflammation of tissue causes neutrophil invasion and migration into it. They ingest or ″phagocytose″ material, destroy it and then die. The phagocytic cells use their pattern recognition receptors (PRRs) to recognize and adhere to pathogen-associated molecular patterns (PAMPs) on the microbe surface (1). In this work, we have investigated intracellular mechanisms regulating neutrophil functions within innate immune responses.

1.1 NEUTROPHILS

Neutrophils are unique in their capacity both to destroy and to help healing any tissue in the body. Understanding the circuits that confer and control such behaviour is as challenging a problem as any other in cell biology (10).

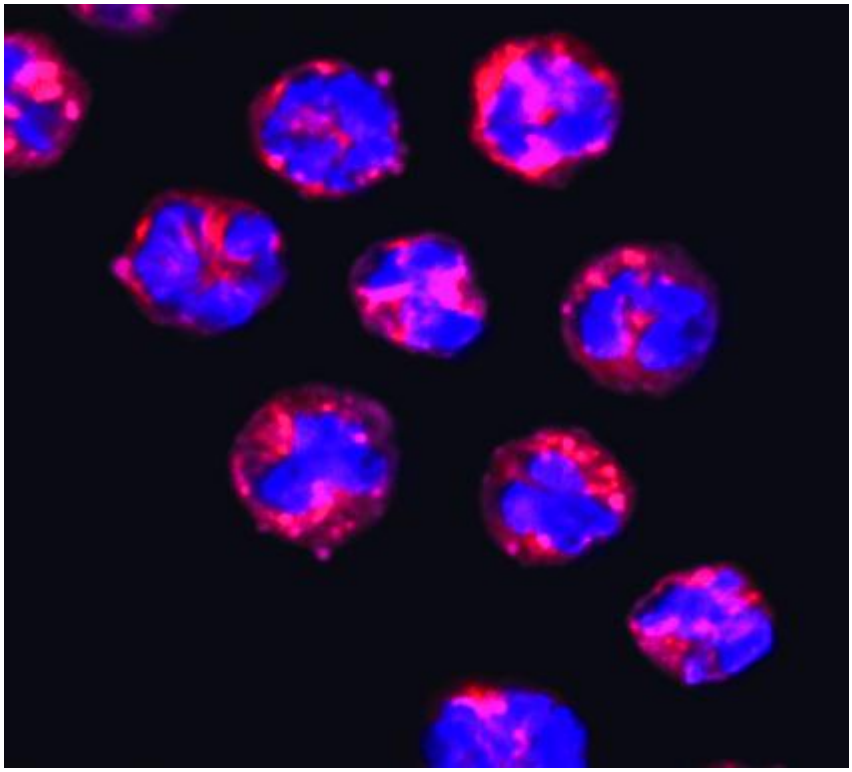


Fig. 1: Neutrophils.
Neutrophils have been fixed and mitochondria was stained with Mitotracker Orange (red), nucleus with Hoechst (blue) and imaged by scanning confocal microscopy.

Neutrophils (Fig. 1) represent the body`s primary line of defence against invading pathogens such as bacteria. Under normal conditions, neutrophils constitute around 60 % of all the white blood cells in humans and mediate the earliest phases of inflammatory reactions. The nucleus of a neutrophil is segmented into three to five connected lobules, hence the synonym polymorphonuclear leukocyte. This composition might make the nucleus more pliable resulting in possible movement of neutrophils through gaps in the endothelium. The cytoplasm contains granules of two types. The majority, called specific granules, are filled with enzymes, such as lysozyme, collagenase, and elastase. These granules do not stain strongly with either basic or acidic dyes (hematoxylin and eosin respectively), which distinguishes neutrophil granules from those of two other types of circulating granulocytes, called basophils and eosinophils (8, 9)

Neutrophils in the initial perception are playing a passive role and are merely responding to external signals. It has now been found out that activated **neutrophils in inflammation** can perform most of the functions of macrophages. Neutrophils are essential effector cells of the innate immune system. They mature in the bone marrow and arise from a common lineage with mononuclear phagocytes. After terminal differentiation, they are released into the bloodstream where their half-life lasts only a few hours. An adult human produces more than 1×10^{11} neutrophils per day. It is important that neutrophils exist in a resting state in the circulation of healthy adults which protects host tissue from their toxic intracellular contents (8).

Neutrophils become activated via a two-stage process. Agents that include bacterial products and cytokines or chemokines, e.g. TNF- α, GM-CSF, IL-8 and IFN-γ, can prime resting neutrophils. After that, primed neutrophils enter the site of infection or inflammation where they meet activation signals to trigger bacterial killing. Appropriately activated neutrophils secrete a variety of pro-inflammatory cytokines. They also express major histocompatibility complex class II (MHCII) in a way that enables presentation of antigen and activation of T cells. Supplementary to their ability of presenting antigen, primed neutrophils actively synthesize and secrete cytokines, chemokines, leukotrienes and prostaglandins, and may contribute to local production of inflammatory mediators (8).

Neutrophils are one of the first cell-responders of inflammatory cells and migrate towards inflammatory sites where they kill ingested microorganisms by producing superoxide through a rapid, NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase-mediated, respiratory burst, in combination with an arsenal of proteolytic enzymes and antimicrobial peptides. These compartments synergize and effectively kill microorganisms. Neutrophils derive most of the energy required for these and other functions from a high rate of glycolysis. This ensures that neutrophils can function in an inflammatory environment where oxygen tension may be low or even absent. If a circulating neutrophil is not recruited into the site of inflammation within a few hours after the entry of microbes, it undergoes apoptosis. Macrophages remove apoptotic neutrophils into the liver, spleen or bone marrow, and this removal protects tissue from damage of releasing neutrophil proteins (2, 8 and 9).

Neutrophils are involved in the activation, regulation and effector functions of innate and adaptive immune cells. Accordingly, neutrophils have a crucial role in the **pathogenesis of a broad range of diseases**, including infections caused by intracellular

pathogens, autoimmunity, chronic inflammation, and cancer (12, 11). The usual goal of neutrophil-targeted pharmacology is not to increase inflammation but instead to suppress it, for example in rheumatoid arthritis, osteo-arthritis, or chronic obstructive pulmonary disease (12).

1.1.1 Neutrophil extracellular traps

Stimulated neutrophils can produce extracellular structures called neutrophil extracellular traps (NETs) that capture and kill microorganisms (Fig. 2). This phenomenon is not restricted to neutrophils but shared by other specialized leukocytes (9, 12 and 13).

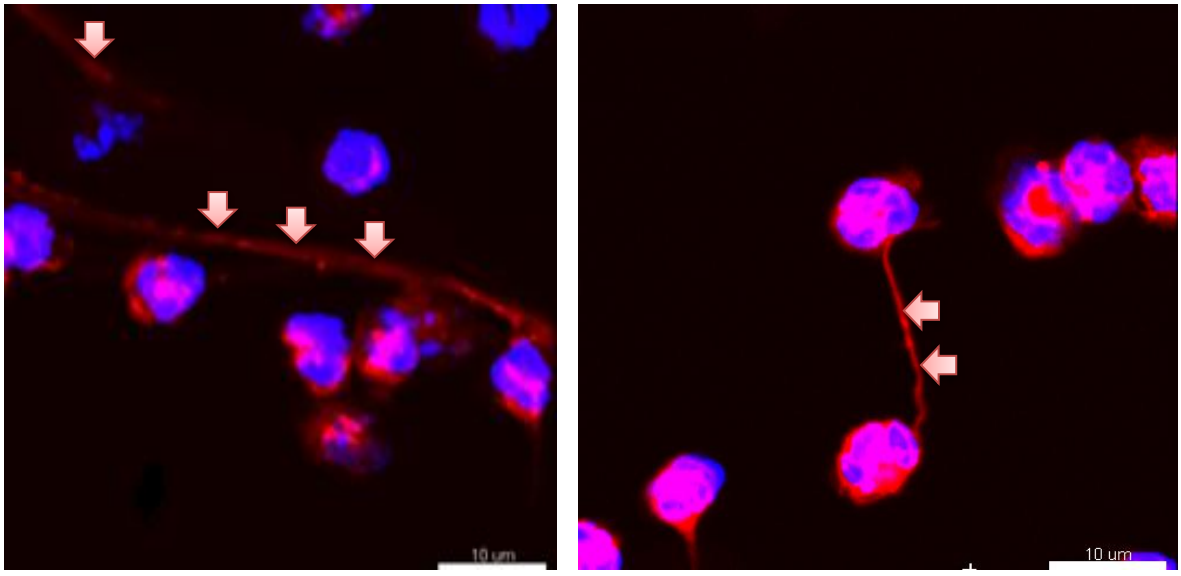


Fig. 2: Neutrophil extracellular traps.
NETs appear to be flexible and surround the cell from which they originated, as visualized by the red mtDNA dye MitoSox and blue nuclear DNA dye Hoechst. Arrows are pointed to NETs. Scale bars: 10 μm.

NETs are complex structures, represented by unfolded chromatin nucleosomes chain. DNAses unlike proteases can degrade NETs structures (9). Different research groups showed mechanisms of microbes how to avoid entrapment in extracellular traps. Example of evasion strategy is degradation NETs by DNase, which resulted in the disappearance of extracellular DNA structures (12, 14).

The discovery of DNA-based antimicrobial extracellular traps (ETs) elaborated by neutrophils (NETs) has important implications for our understanding of the innate immune system and the pathophysiology of infectious and inflammatory diseases (12). Microscopic observations have shown that Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumonia* and *Listeria monocytogenes*), Gram-

negative bacteria (*Escherichia coli*, *Salmomella enterica*, *Shigella flexenari*, *Haemophilus influenza*, *Pseudomonas luminescens* and *Salmonella tryphimurium*), mycobacteria (*Mycobacterium tuberculosis*) as well as fungi (*Candida albicans*) and parasites (*Leishmania amazonesis*) bind to NETs. It remains uncertain whether viruses are entrapped by ETs (9, 12).

It seems plausible that NET formation is a common mechanism of the innate immune system in vertebrates, as it has been observed in humans, mice, rabbits, horses, cows, and fish. In an animal model of sepsis, it has been shown that activation of neutrophils by platelets leads to rapid NET formation that might be more effective than phagocytosis (9).

Upon *in vitro* activation with various agonists, including lipid mediators (lipopolysaccharide), bacteria, fungi, activated platelets, or combined GM-CSF and complement factor C5a neutrophil stimulation start the formation of NETs (9). Strikingly, NETs formed by living cells contain **mitochondrial** (mtDNA) but not nuclear **DNA** (14).

1.2 MITOCHONDRIA

Mitochondria are cell organelles that participate in a wide range of cellular processes, such as ATP generation, programmed cell death and calcium homeostasis. They are also involved in amino acids, lipids, nucleotides and haem biosynthesis (15). Mitochondria are usually depicted as stiff, elongated cylinders with diameter of 0.5-1 μm, resembling bacteria (2).

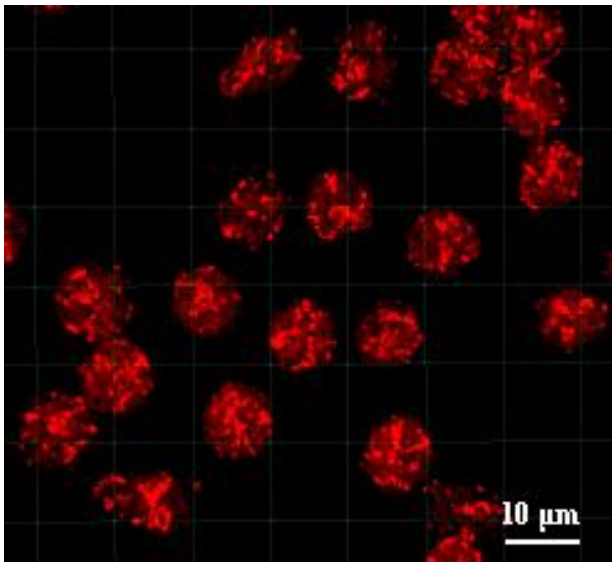


Fig. 3: Mitochondria.
Neutrophils have been fixed and stained with Mitotracker Orange for mitochondria (red) and imaged by scanning confocal microscopy. Scale bar 10 μm.

A recent research suggests that mitochondria are involved in a wide range of innate immune pathways, which features as a signalling basis and contributes to effector responses. Studies have shown that mitochondria facilitate antibacterial immunity by generating reactive oxygen species and assist in innate immune activation followed by cellular damage and stress (15). Neutrophils contain a complex network from relatively small

amount of mitochondria (Fig. 3) inside the cell. This complex is involved in chemotaxis and regulating apoptosis and is not important for initiation of rapid respiratory burst or phagocytosis. It has been assumed that neutrophil mitochondria depend mainly on glycolysis for their energy provision. These organelles hardly contribute to ATP levels (8, 17).

The motility of mitochondria is based on interactions with cytoskeleton. Movement over shorter distances is based on interactions with actin while for long distances faster microtubules-based interactions are predominant (16). Mitochondria are dynamic structures. Morphology of mitochondria is changed through processes called fission (splitting into smaller pieces) and fusion (combining pieces). These changes in morphology are essential for the normal operation of mitochondria (39).

1.3 CYTOSKELETON

The role of cytoskeletal filaments is spatial arrangement on cell and mechanical properties. Mechanical strength is provided by intermediate filaments. While microtubules are responsible for positioning of organelles and intracellular transport, actin filaments determine the shape of cell surface and are necessary for whole-cell locomotion (19).

1.3.1 Microfilaments

Microfilaments (MFs) or actin filaments are two-stranded helical polymers of the protein actin. They appear as flexible structures, with a diameter of 5 to 9 nm, and they are organized into a variety of linear bundles, two-dimensional networks, and three-dimensional gels. Although actin filaments are dispersed throughout the cell, they are most highly concentrated in the cortex, just beneath the plasma membrane (Fig. 4) (19).

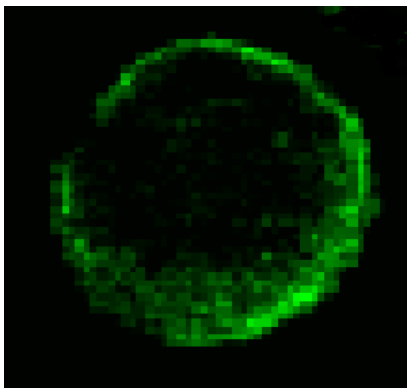


Fig. 4: Actin filaments.
Fixed neutrophil stained with Phalloidine for actin (green) and imaged by CLSM.

1.3.2 Microtubules

Microtubules (MTs) are long, hollow cylinders made of the protein tubulin. With outer diameter of 25 nm, they are much more rigid than actin filaments. Microtubules are long and straight, and typically have one end attached to a single microtubule-organizing centre called a centrosome (19).

1.4 MODIFICATION OF FILAMENT POLYMERISATION

Cytoskeletal filaments formed from actin and tubulin, whose assembly is crucial for survival of eukaryotic cells, are often *targets of natural toxins*. Plants, fungi, or sponges do not wish to be eaten but cannot run away from predators; consequently, they produce toxins in self-defence, which generally disrupt the filament polymerization. Lantrunculin is an example of preventing actin monomers to bind and assembly into filaments. It is extracted from sea sponge Lantrunculia magnifica. In addition to lantrunculin, FIPI and nocodazole can also be used to probe the roles of actin and microtubules during cell processes. These reagents are commonly used by cell biologists to manipulate cytoskeleton. Some of them are also used to treat cancer. Both microtubule-depolymerizing drugs, such as nocodazole, and microtubule-polymerizing drugs, such as taxol, preferentially kill dividing cells, since both microtubule assembly and disassembly are crucial for correct function of mitosis. These drugs efficiently kill certain types of tumour cells in a human patient, but have also side effects on rapidly dividing normal cells, including those in bone marrow, intestine, and hair follicles (18).

1.4.1 FIPI

FIPI is phospholipase D inhibitor. The signalling enzyme phospholipase D (PLD) and the lipid second messenger it generates, phosphatidic acid (PA), are implicated in many cell biological processes, including Ras activation, cell spreading, stress fibre formation, chemotaxis, and membrane vesicle trafficking. Potent specific small-molecule phospholipase D inhibitors 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) has recently been shown to rapidly block in vivo phosphatidic acid production with subnanomolar potency. FIPI inhibits phospholipase D *regulation of F-actin cytoskeleton reorganization*, cell spreading, and chemotaxis, indicating potent utility for it as a therapeutic for autoimmunity and cancer metastasis (21).

1.4.2 Lantrunculin

Lantrunculin is actin-specific drug which binds subunits and prevents their polymerization. It binds one-to-one with monomeric G-actin but has no effect on microtubular structure. Lantrunculin B is a marine toxin (22-24).

1.4.3 Nocodazole

Nocodazole is microtubule-specific drug which binds with high affinity to tubulin and inhibits microtubule assembly. It prevents their polymerization. As already mentioned, nocodazole is an anticancer drug (25, 26).

1.5 MODELS OF HOXB8 MOUSE NEUTROPHILS

Mature neutrophils are short-lived cells that cannot be genetically manipulated. Analysis of gene function therefore requires genetically modified animals, which are expensive, time-consuming, and costly in animal life. Pre-neutrophil of Hoxb8 cell lines had to be isolated lineage-negative progenitors cells from mouse bone. Hox oncoproteins enforce self-renewal of factor-dependent myeloid progenitors. This ability to conditionally immortalize estrogen-regulated Hoxb8 (ER-Hoxb8), granulocyte-macrophage precursor cells not only offers the possibility to study complex genetic experiments but also provides the ability for high-throughput screening of specific functional pathways. Both the SCF and GM-CSF in ER-Hoxb8 progenitors execute normal differentiation programs and normal innate immune function upon ER-Hoxb8 inactivation. The system relies on the activity of an estrogen receptor binding domain - Hoxb8 fusion protein. *In vitro*- derived mouse neutrophils have equal physiological and functional characteristic as primary mouse neutrophils which makes them appropriate for further analysis (27, 28).

In presented research, we have mainly worked on six different genetically modified Hoxb8 cell lines: wild-type (WT), Parvalbumin knockout ($PV^{-/-}$), RhoH knockout ($RhoH^{-/-}$), Wiskott-Aldrich Syndrome protein knockout ($WASp^{-/-}$), Optic atrophy 1 knockout ($Opa1^{-/-}$) and BK knockout ($BK^{-/-}$). Wild-type refers to standard, normal cell as it occurs in nature. Knockouts are used to understand the role of a specific gene or DNA region in specific biological processes by comparing the knockout cells to the wild-type under similar conditions.

1.5.1 $PV^{-/-}$

Parvalbumin (PV) is a small (typically 9-11 kDa) protein with two EF-hand type Ca^{2+}-binding sites. PV plays a role in many physiological processes, namely cell-cycle regulation, second messenger production, muscle contraction, *organization of microtubules*, and vision (29). Calcium-binding proteins have been implicated in many

serious disease states, such as Alzheimer`s disease, nervous system disorders, age-related cognitive defects, and various forms of cancer (30).

1.5.2 RhoH$^{-/-}$

RhoH is a small (~21 kDa) signalling GTPase, Ras homolog gene family, member H. It is Rac subfamily member of the Rho family of GTPases. Rho family monomeric GTPases *regulate both actin and microtubule cytoskeletons*, controlling cell shape, polarity, motility, and adhesion; they also regulate cell-cycle progression, gene transcription, and membrane transport. Rho GTPases usually cycle between an active, GTP-bound, and an inactive, GDP-bound, state. In contrast, RhoH lacks intrinsic GTPase activity and always resides in the active form. RhoH protein is not expressed under physiological conditions in neutrophils and monocytes but in blood T and B cells. Increased RhoH gene expression has been reported in neutrophils following GM-CSF stimulation, as assessed by a differential gene display and techniques (31, 32 and 33).

1.5.3 WASp$^{-/-}$

Human patients deficient in WASp suffer from the Wiskott-Aldrich syndrome, a rare, inherited, recessive disease. It is a severe form of immunodeficiency where immune system cells have *abnormal actin-based motility* and platelets do not form normally (thrombocytopenia). The Wiskott-Aldrich syndrome family of proteins share similar domain structure, and are involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton. The WAS gene product is cytoplasmic protein (WASp), expressed exclusively in hematopoietic cells. WASp proteins can exist in an inactive folded confirmation and an activated open confirmation. Association with Cdc42-GTP stabilizes the open form of WASp, enabling it to bind to the ARP complex and strongly enhancing the nucleation activity of this complex actin. In this way, activation of Cdc42 increases actin nucleation. After all, the main function of WASp is to activate actin polymerization (34, 35).

1.5.4 OPA1$^{-/-}$

Optic atrophy 1 (OPA1) gene product is a mitochondrial protein with similarity to dynamin-related GTPases. Within cells, protein Opa1 is located in the inner membrane of mitochondria. It helps to regulate the morphology of mitochondria by playing a key role in the fusion process. Mutations in this gene have been associated with optic atrophy type 1

(OPA1). OPA1 is a dominantly inherited optic neuropathy that results in progressive loss of visual acuity, which can lead to blindness (36, 37, 38 and 39).

1.5.5 $BK^{-/-}$

BK channels (Big Potassium) are ion channels characterized by their large conductance of potassium ions (K^+) through cell membranes. These channels are activated (opened) by changes in membrane electrical potential and/or increased in concentration of intracellular calcium ion (Ca^{2+}). Several human genetic diseases, such as pathologies involving cardiac arrhythmias, deafness, epilepsy, diabetes, and misregulation of blood pressure, are caused by disruption of K^+ channel genes (37, 38, 40 and 41).

1.6 CONFOCAL LASER-SCANNING MICROSCOPY

The principle of confocal laser-scanning microscopy (CLSM) was originally patented by Marvin Minsky in 1957. Since then it took approximately thirty years that CLSM became a standard technique (42).

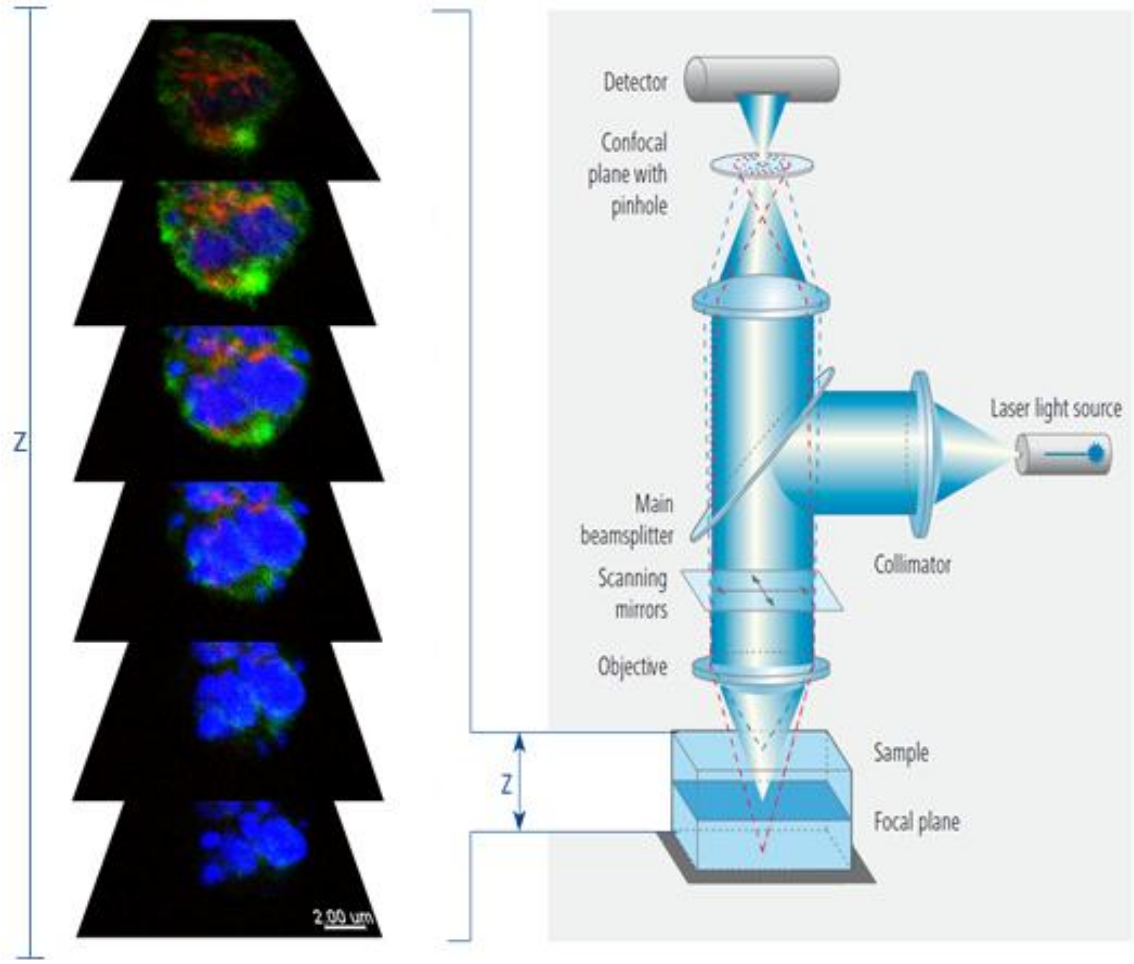


Fig. 5: Confocal laser-scanning microscope.
Beam path in the confocal LSM 5 Exciter Laser Scanning Microscope (schematic) (43). On the left part of the figure are z stacks of a human neutrophil.

The advantage of laser scanning microscope (LSM) 5 Exciter is close monitoring of physiological experiments. Its image acquisition and processing functions are ideally suited to the investigation of fast processes in *live cells*. We can capture processes with correct timing. Confocal microscopy is a system that scans a specimen with laser light, point by point and line by line, in order to acquire an optical section. Many optical sections are collected from different Z planes form 3D image stack (Fig. 5). We gain new insights from 3D image stacks into the complex structures and interrelations on the cellular level. Confocal image with selectively labelled functional regions provides us information about mitochondria, nucleus, membrane and cells in our case (43).

The special advantage of confocal laser scanning microscopy results from the use of a *pinhole diaphragm* located conjugate to the focal plane. The pinhole only admits light coming from the focal plane, while emissions from planes above or below it are rejected (43).

Fluorescence is an emission of light released when electrons go from electronically excited state to a lower energy level. The excited state is the result of the absorption of light. Mostly, the emitted light has a longer wavelength than the absorbed. A fluorochrome can be excited by different wavelengths within its excitation spectrum (43).

Detection is made by highly sensitive photomultipliers. To have the complex functionality under control, we can *reuse the settings* made in earlier experiments and thus exactly reproduce experimental conditions (43).

Cells have been visualized using a LSM 5 Exciter confocal microscope, which has three lasers: an Argon (Ar laser 458/ 488/ 514 nm), Helium-Neon (HeNe laser 543/ 633 nm) and Diode (405 nm) laser. To analyse triple staining, all three lasers have been needed because the fluorescence dyes Mitotracker Orange (red) is excited by 543 nm, Alexa Fluor 488 Phalloidin, and Hoechst dye is excited by 405 nm.

1.6.1.1 Mitotracker Orange (red)

To label mitochondria, cells are incubated with MitoTracker probes, which passively diffuse across the plasma membrane and accumulate in active mitochondria (44).

Excitation / emission: 554 nm / 576 nm.

1.6.1.2 Rhod-2 (red)

Rhod-2 is a selective indicator for mitochondrial Ca^{2+}.

Pluronic acid is a non-ionic, surfactant polyol that has been found to facilitate the solubilisation of water-insoluble dyes. The Pluronic acid is used with Rhod-2 for live analysis (45).

Excitation / emission: 552 nm / 581 nm.

1.6.1.3 Alexa Fluor 488 Phalloidin (green)

Fluorescently- labelled phalloidine is a high affinity filamentous actin (F-actin) probe. It is bicyclic peptide belonging to a family of toxins isolated from the deadly Amanita phalloides “death cap” mushroom (47, 52).

Excitation / emission: 518 nm / 540 nm.

1.6.1.4 Hoechst 33342 (blue)

Hoechst is fluorescence stain high quality solution for fixed and live-cell fluorescent staining for DNA and nuclei in cellular imaging techniques.

Excitation / emission: 350 nm / 461 nm.

1.6.1.5 CellMask Deep Red plasma membrane stain (violet)

It stains cellular plasma membranes for standard fluorescence microscopy (46).

Excitation / emission: 590 nm / 617 nm.

2 OBJECTIVES

This study is part of the project: »Molecular mechanisms of granulocyte activation«. Microbial killing does not occur just after phagocytosis but also by formation of neutrophil extracellular traps (NETs). NETs contain mitochondrial DNA. In this research, we want to investigate how mitochondria are moving upon activation of neutrophils toward cell plasma membrane and release mitochondrial DNA.

The aim of the project is the investigation of mitochondrial movement in human neutrophils and mouse Hoxb8 neutrophils. We would like to see the difference in localization of mitochondria in neutrophils before and after stimulation. Therefore neutrophils will be primed with 100 ng/ml granulocyte/ macrophage colony-stimulating factor (GM-CSF) and subsequently stimulated with 10^{-8} M complement factor 5a (C5a).

Cells will be visualised by confocal laser scanning microscopy. Neutrophils will be either fixed with 4 % paraformaldehyde or observed in live-cell microscopy experiments. The percentages of fixed cells with central mitochondria will be counted in ten different fields in at least three independent experiments. Human neutrophils will be incubated with different inhibitors of cytoskeleton and wild-type mouse neutrophils will be compared with different genetically modified cells. Mitochondrial movements will be followed by computer-aided video analysis after staining the organelle with Rhod-2. We will add C5a during live analysis to show difference in localization of mitochondria upon stimulation.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment

Device	Type	Company
CELLSTAR	6 Well Cell Culture Plate sterile	Greiner Bio One
	24 Well Cell Culture Plate sterile	
Cell Counter	KX-21	Sysmex Digitana SA
Centrifuge	5415 D	Eppendorf
	5417 R	
Centrifuge	Multifuge 3 S-R	Thermo Fisher scientic, Heraeus AG
Centrifuge	Shandon Cytospin III Centrifuge	DAKO Diagnostics AG
Centrifuge	Megafuge 40R	Thermoscientific, Heraeus
Centrifuge	Biofuge pico	Huber & Co. Ag
Cytoslide	Microscope Slides for Shandon Cytospin (Non-Coated, Circle on Back)	Thermo scientific
Falcon Tubes	BD Falcon	BD Biosciences
Freezer (-20°C)	MI 1207 A	Miostar
Freezer (-80°C)	V 535 Vacum Insulation Panel	New Brunswick Scientific-ultra low temperature freezer
Glass cover slips	12 mm	BD Biosciences

Glass pipettes	Pipette sterile ind. Wrapped 1 ml, 2 ml, 5 ml, 10 ml and 25 ml	VWR Supplier Partnerships for Customer Solutions
Incubator	HERAcell 150i CO2 incubator	Thermo scientific
LSM 510	Confocal laser scanning microscope	Carl Zeiss
Lasers	Ar laser (458, 488, 514 nm) 25 mW	Lasos
	HeNe laser (543 nm) 1 mW, HeNe laser (633 nm) 5 mW	Lasos
	Diode laser (405 nm) 25 mW	
Neubauer chamber	Hemocytometer for cell counting	Oscar Bastidas
Pipette Boy	Accu-jet pro	Brand
Pipettes	Research (different volumes)	Eppendorf
Refrigerator	Different models	Miostar
Shaker	MS2 Minishaker	IKA
Automated Hematology Analyzer	KX-21N	Sysmex
Vortrex Mixer or shaker	Vortex-Genie 2	Scientific Industries
X-Cite Series 120	powerful 120W lamp	EXFO

3.1.2 Software

Imaris Cell software	Scientific software module for data visualization, analysis, segmentation, fluorescence intensity graphs and interpretation of 3D and 4D microscopy datasets (48).
Zen lite	Imaging software for acquire images and video sequences. For measuring distances and making profile intensity graphs.
LSM Image Browser	Application for managing all images generated with Carl Zeiss Laser Scanning Systems LSM 5.
GraphPad Prism 5	Combination of biostatistics and scientific graphing in one comprehensive programme. Originally designed for experimental biologists in medical schools and drug companies, especially those in pharmacology.
Vegas Pro 11.0	Video editing software, used to make movies with live cells images.

3.1.3 Chemicals

Substance	**Description**	**Company**
BSA (7,5%)	Bovine serum albumin	Sigma-Aldrich
C5a human	Human Complement factor C5a	Calbiochem-Novabiochem Corp.
C5a mouse	Mouse Complement factor C5a	Calbiochem-Novabiochem Corp.
EGTA-AM	Ethylene glycol tetraacetic acid	Sigma-Aldrich
Ficoll solution	Biocoll Separating Solution	Biochrom
FIPI	Phospholipase D inhibitor	R&D
FCS	Fetal calf serum	Invitrogen

GM-CSF human	Human GM-CSF	Sigma-Aldrich
GM-CSF mouse	Mouse GM-CSF	Sigma-Aldrich
Hoechst 33342 in water	Nucleic acid dye	Molecular Probes
Immersion oil	Immersol 518 F flurescence free	Zeiss
Lantrunculin	Disrupt microfilaments	Enzo
RPMI Medium 1640	Gibco	Invitrogen
Mitotracker Orange	Mitochondrion-selective dye	Molecular Probes
Nocodazole	Microtubule specific drug	Sigma
PAF	Paraformaldehid extra pur	Riedel-de-Häen
PBS	Phosphate buffered saline without Ca& Mg sterile	PAA Laboratories GmbH
Penicillin	Antibiotic drug	Life Technologies, Basel
Phalloidin	Alexa Fluor 488 Phalloidin	Invitrogen, Molecular probes
Pluronic F127	20% solution in DMSO	Molecular probes, life technologies
ProLong Gold	Antifade reagent supress photobleaching	Life technologies
Dow corning	Silicon high vacuum grease	Ideal Vacuum Products, LLC
Gibco	DMEM (1x)+ GlutaMAX^{TM}-1	Life technologies
PBS	Phosphate buffered saline pH 7,4	PAA Laboratories

RPMI 1640	Gibco, Medium to culture cells	Life technologies
Saponin	Used in permeabilization of cell membranes	Sigma Aldrich
SCF human	Recombinant human protein	Pepro Tech EC
SCF mouse	Recobinant mouse protein	R&D Systems
Streptomycin	Antibiotic drug	Life Technologies, Basel
TRITON X-100	Triton X-100	Sigma Aldrich
Trypan blue	Vital stain to selectively colour cells blue	Sigma
X-VIVO 15 (SFM)	Chemically Defined, Serum-free Hematopoietic Cell Medium	Lonza

3.1.4 Solutions

Complete medium	100 µl RPMI+ 10 % FCS
Lysis solution	12,45 g NH_4Cl, 1,5 g $KHCO_3$, 53,7 mg EDTA Disodiumsalt Dihydrate, dissolve in 150 ml ddH2O; sterilize by filtration through 0,2 um filter
4% Paraformaldehyde solution	100 ml PIPES buffer (0,1 M, pH 6,8) at 50-55°C add 4 g paraformaldehyde. Filter the solution.
PBS+	0,4% BSA in PBS
Ringer solution	Na pyruvate 5 mM, KCl 5 mM, NaCl 125 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1,2 mM, Na_2HPO_4 2 mM, HEPES 32 mM, Glucose 5 mM
RPMI 1640	50ml FCS+ 5 ml Penicillin/ Streptomycin solution

3.2 METHODS

3.2.1 Neutrophil isolation

Mature blood neutrophils were isolated from human peripheral blood of healthy donors by Ficoll-Hypaque gradient centrifugation. Briefly, peripheral blood mononuclear cells (PBMC) were separated by centrifugation on Ficoll-Hypaque. The lower phase, consisting mainly of granulocytes and erythrocytes, was treated with erythrocyte lysis solution for 10 minutes on ice. The next step was centrifuging at 1400 rpm, 7 minutes at 4 °C. After this, the supernatant was removed, as much as possible by aspiration, and 1 ml cold PBS+ was added to the cell pellet. We resuspended them carefully, filled them up with PBS+ and centrifuged them again at 1400 rpm for 7 minutes at 4 °C. The resulting cell populations contained greater than 95 % mature neutrophils as assessed by staining with Diff-Quik and light microscopy analysis (14).

3.2.1.1 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 in the absence or presence of the indicated concentrations of GM-CSF, C5a for the indicated time periods, using complete culture medium at 37 °C in 5 % CO_2 in a humidified atmosphere. If not indicated, we used C5a at 10^{-8}M, GM-CSF (100 ng/ml), G-CSF (100 ng/ml).

3.2.2 Hoxb8 mouse models

For immortalization of pre-neutrophil of Hoxb8 cell lines, we had to isolate lineage-negative progenitor cells from the mouse bone; an additional transfection with a lentivirus which contained the 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 90 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; this later leads to an up-regulation of Hox proteins. Since Hox oncoproteins enforce self-renewal of factor-dependent myeloid progenitors, we utilized estrogen-

regulated Hoxb8 (ER-Hoxb8) to immortalize neutrophil progenitors that would execute normal differentiation and normal innate immune function upon ER-Hoxb8 inactivation. In addition, 8 μg/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 cells were able to survive since they had puromycin resistance.

We also isolated primary mature mouse neutrophils from bone marrow of wild-type and different knockout mice.

3.2.2.1 Cell passaging

The Hoxb8 cells were cultured in the following medium: RPMI 1640, 10 % FCS, 1 % penicillin/streptomycin, 500 μl β-mercaptoethanol, 5 % SCF (added freshly), 0,1 μM 4-hydroxytamoxifene (added freshly).

Every Monday and Friday, we diluted to approximately 50 000 cells/ml.

3.2.2.2 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 the 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 % CO_2.

Granulocyte-Colony Stimulating Factor (G-CSF) was added (1 μl/ml) to the differentiating cells because of two reasons: G-CSF accelerates the differentiation and improves the viability of the cells. From previous experiments it had been known that the best benefit was provided when the factor was added on day 3 (data not shown).

3.2.2.3 Cell counting

1. Automated Hematology Analyzer

We took 100 μl cell solution to Eppendorf tube and measured the number of cells with Sysmex.

2. Neubauer chamber

The Neubauer Chamber was taken and the glass slide was fixed onto it. 10 µl cell medium solution was pipetted at the glass slide edge. The solution got pulled inside and four squares containing nine little squares were counted under the microscope. To get the exact cell number the count was divided by four and then multiplied by $50*10^4$. It was divided by four because 4 squares were counted and multiplied by 50 because the tube contained 50 ml of substrate-cell solution. The term 10^4 was multiplied because in four squares a volume of 0.1 µl was counted and we wanted the volume in millilitres. Therefore, it was multiplied by 10^4.

3.2.3 Fixed cells for localization analysis

Fixed cells: to first immobilize, kill and preserve the cells, they must be treated with a fixative. Common fixatives include formaldehyde, which forms covalent bonds with the free amino groups of proteins (cross-linking them) so they are stabilized and locked into position (2).

3.2.3.1 Human neutrophils staining procedure

Firstly, $1x10^6$ cells per condition were spun down for 2 minutes 2000 rpm and resuspended in 1 ml complete medium. One reagent was added per one condition and no reagent for control experiment. Working concentration used for FIPI was 500 nM, for Nocodazole 5 µM, and for Lantrunculin B 1,25 µM. Reagents were diluted from stock concentration in PBS to get right working concentration. Cells were mixed gently after adding inhibitors. Eppendorf tubes with open caps were incubated at 37 °C, 5% CO_2 for 30 min.

After treating cells with inhibitors, cells were spun down at 2000 rpm for 2,5 min and resuspended in 210 µl SFM. Autoclaved glass cover slips were placed in 8 wells of 24 well plates. 500,000 neutrophils per 100 µl SFM were seeded on 12-mm glass cover slips and the stimulated ones were primed with 100 ng/ml GM-CSF for 25 min at 37 °C, 5 % CO_2. The light was turned off because Mitotracker Orange is light sensitive. During this time dilutions were prepared for unstimulated conditions: in 1 ml SFM 1 µl Mitotracker Orange was added, and for stimulated conditions, 1 µl C5a was added as well. 200 µl of these dilutions was added on the side of the wells and incubated at 37 °C, 5% CO_2 for 20 min. The fluid was aspirated and cells were fixed with 200 µl 4 % paraformaldehyde, which

was added on the side of the wells protected from the light for 4 min at RT. This step should be fast. After 4 min fixed cells were washed 2-3 times with 200 µl PBS on side of the wells. Saponin was diluted to working concentration 0,05 % with PBS. 100 µl of prediluted Saponin was added to the middle of the cover slip for 5 min. Dilution of Saponin (working concentration 0,05 %) with PBS, Phalloindin 488 (working concentration 165 nM) and 1 % BSA were prepared. After prediluted Saponin was removed, 100 µl dilution of Saponin was added to the middle of the cover slip for 20 min. Firstly, Hoechst was diluted to have working concentration 1 µg/ml and then 100 µl diluted Hoechst was added. Fixed cells were washed two times with 200 µl PBS. 3 µl anti-fading reagent ProLong Gold was added on the slides which were covered by coverslips and analysed by confocal scanning microscopy. Slides were stored at 4 °C protected from the light, but for the first night at RT.

3.2.3.2 Mouse neutrophil staining procedure

Wild-type and different genetically modified Hoxb8 mouse neutrophils were not treated with inhibitors such as FIPI, Lantrunculin and Nocodazole. After that step the staining procedure was the same as for human neutrophils. Human neutrophils were centrifuged for 2 min at 2000 rpm whereas Hoxb8 mouse neutrophils for 3 min at 2000 rpm.

3.2.4 Live-cell microscopy

3.2.4.1 Human neutrophils

2x 10^6 cells per one condition were spun down for 2 min at 2000 rpm and resuspended in 1 ml complete medium. Inhibitors FIPI, Lantrunculin and Nocodazole were used in the same working concentration as for fixed cells. Cells in Eppendorf tubes with open caps were also incubated at 37 °C, 5 % CO_2 for 30 min. During that time Rhod-2 and Pluronic acid were diluted to working concentration. Rhod-2, selective dye for Ca^{2+} in mitochondria and Pluronic acid, non-ionic acid were incubated at 37 °C, 5 % CO_2 for 15 min. The light was turned off, because Rhod-2 is light sensitive. After that cells were spun down for 2 min at 2000 rpm and resuspended in 0,3 ml warm complete medium. 0,3 µl CellMask Deep Red plasma membrane stain was added for 1 min at 37 °C, 5 % CO_2. Cells were washed 3 times in 1 ml cold PBS. After the washing step, cells were resuspended in 1 ml complete medium. Working concentration 1 µg/ml of Hoechst was added for 5 min in RT. Cells were spun down for 2 min at 2000 rpm and resuspended in 300 µl Ringer solution

and loaded in chamber prepared for live-cell analysis. Software Auto time Macro in LSM 5 Exciter was opened. Confocal microscope started to make live images. At the beginning, approximately 5 images has been made without any reagent, after that human C5a was added.

3.2.4.2 Hoxb8 mouse neutrophils

Wild-type and different genetically modified Hoxb8 mouse neutrophils were not treated with inhibitors such as FIPI, Lantrunculin and Nocodazole. After that step live-cell analysis was the same as for human neutrophils. Hoxb8 mouse neutrophils were spun down for 3 min at 2400 rpm for the first time. After adding Rhod-2, CellMask Deep Red plasma membrane stain and Hoechst were spun down the same as human neutrophils live-cell analysis.

3.2.5 Statistical analysis

For statistical evaluation of mitochondrial localization at least three experiments in each condition were made. We made 10 images with one slide, objective 100 and counted in how many cells mitochondria accumulated in the centre of the cell. The percentages of cells with central mitochondria were imported in GraphPad Prism 5. Graphs were drawn and statistical analysis was performed with GraphPad Prism 5 Software. Paired groups were analysed with the non-parametric, unpaired t test. P-values of $p < 0.05$ were considered as *- significant, $p < 0.01$ as **- significant, and $p < 0.001$ as ***- significant. Error bars in graphs represented SEM (standard error of mean); results were displayed as Mean ± SEM. Images were processed in Imaris, Zen and LSM Image Browser.

4 RESULTS AND DISCUSSION

4.1 FIXED CELLS

4.1.1 Human neutrophils

Interdependence between microtubule and F-actin dynamics following neutrophil priming and activation remains ill-defined. Here, confocal microscopy has been used to investigate the localization of mitochondria dependent on F-actin and microtubules, and to achieve analysis of mitochondrial localization. In our study, human neutrophils have been cultured in a continuous presence of drug preventing actin polymerization (lantrunculin B) or disrupting actin microfilaments due to phospholipase D inhibition (FIPI). Inhibitor of microtubule dynamics such as nocodazole has also been used. The used physiological stimulators are: Granulocyte/ macrophage colony-stimulating factor GM-CSF for priming effects and complement factor-activator C5a for activation. Upon activation neutrophils release extracellular traps (NETs) which contain mitochondrial DNA. The results investigate the connection between movement of mitochondria close to plasma membrane (Fig. 6 A, B), and mitochondrial DNA release (Fig. 6 C).

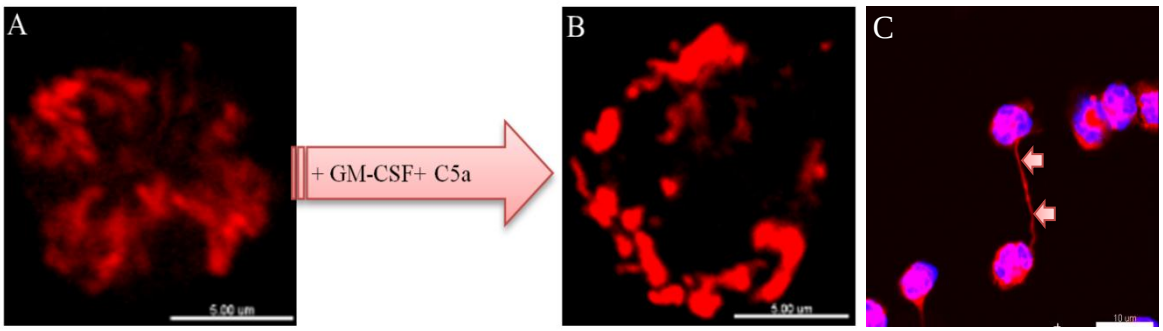


Fig. 6: Mitochondrial movements and release of their DNA. Resting (A) neutrophil and primed with GM-CSF and subsequently activated with C5a (B). Mitochondria (red) have been stained with Mitotracker Orange (A and B) or with MitoSox (C). Nuclei (C) have been stained with Hoechst (blue). Arrows show NETs (C). Scale bars 5 µm (A and B) and 10 µm (C).

First statistical evaluation of results of human neutrophils is presented (Fig. 7), treated with reagents which alter cytoskeleton organization. Evaluation of mitochondrial localization has been performed by microscopic analysis which is further demonstrated in Fig. 9 and Fig. 10.

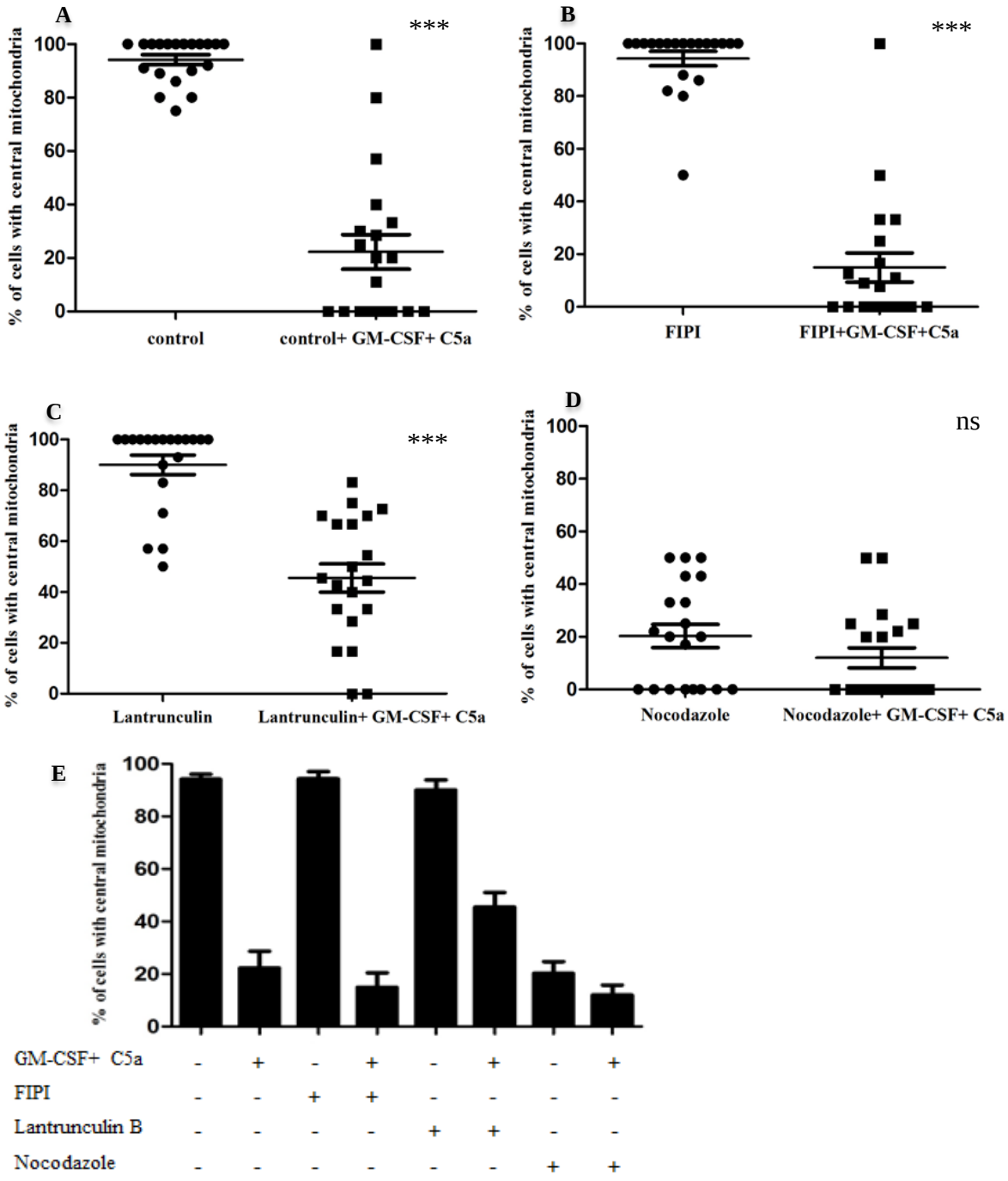


Fig. 7: Localization of mitochondria in human neutrophils. For control, purified blood neutrophils have been incubated directly with and without GM-CSF and C5a, cultured in absence of pharmacological inhibitors of cytoskeleton (A). Firstly, neutrophils ($1x10^6$ cells/ml) have been incubated at 37 °C, 5 % CO_2 for 30 minutes in the presence of various microfilaments and microtubules inhibitors such as FIPI (500 nM, B), lantrunculin B (1.25 µM, C) and nocodazole (1 nM, D). After 30 minutes of pre-incubation with inhibitors, cells have been incubated with (■) and without (●) GM-CSF (100 ng/ ml) for priming effect and for activation C5a (10^{-8} M). Afterwards, the cells have been stained with the fluorescent dyes (as described in Fig. 8). Each dot in graphs (A, B, C and D) represents the percentage of cells with central mitochondria on one image that has been made for statistical evaluation by confocal microscopy with objective field 100 x. Ten images of at least three experiments have been analysed by GraphPad Prism 5 software. Graph E represents the percentage of cells with central mitochondria as the mean ± SD. ***, $P< 0.05$.

Graph A (Fig. 7) represents control experiment showing mitochondrial localization in neutrophils, treated without inhibitors for cytoskeleton. It is evident that activation of cells leads to mitochondrial redistribution toward the cell membrane. Resting cells have mitochondria accumulated in the centre (Fig. 6 A) of the cell and activated cells have mitochondria close to the plasma membrane (Fig. 6 B). In Fig. 7 graphs B and C represent effect of microfilament inhibitor on mitochondrial localization. Cells treated with FIPI (Fig. 7 B) show movement of mitochondria upon activation like control experiment (Fig. 7 A). Whereas in cells treated with lantrunculin (Fig. 7 C) inhibition of movement can be seen. There are fewer activated cells with mitochondria close to the plasma membrane. Lantrunculin is F-actin polymerization inhibitor. Disruption of F-actin polymerization affects localization of mitochondria upon physiological activation. Cells treated with nocodazole are insignificantly moving toward plasma membrane, due to disruption of microtubules and location of the mitochondria close to the plasma membrane already before activation (Fig. 7 D). Thus mitochondrial movement within cells treated by nocodazole cannot be detected.

Confocal images show representative examples of single cells (Fig.), which has been analysed by Imaris software. Results of Imaris software are represented by red dots-position of mitochondria in the cell and a cross-sectional distance profiles. There are unactivated cells (A1-D1) on the left side and on the right side there are cells activated with GM-CSF and C5a (A2-D2). Fluorescence intensity graphs show cross-sectional line of the cell in order to prove different localization of mitochondria, which has been statistically evaluated and presented in Fig. 7. Fluorescence intensity graphs show that unactivated cell mitochondria stay in the middle of the cell resulting in a red peak (as indicated by the arrows A1, B1 and C1 in Fig. 8). In activated cells, mitochondria move to the membrane, therefore we can see red colour on the edge (as indicated by the arrow A2, B2 and D2 in Fig. 8). Despite the activation, cells treated with lantrunculin B look as if they have not activated. Mitochondria have stayed in the middle of the cell (Fig. 8 C2). Upon pharmacological disruption of microtubules with nocodazole (Fig. 8 D) untreated mitochondria are already close to the plasma membrane in the cell before activation (Fig. 8 D1) and after it (Fig. 8 D2). Mitochondria are bigger in cells cultured with nocodazole.

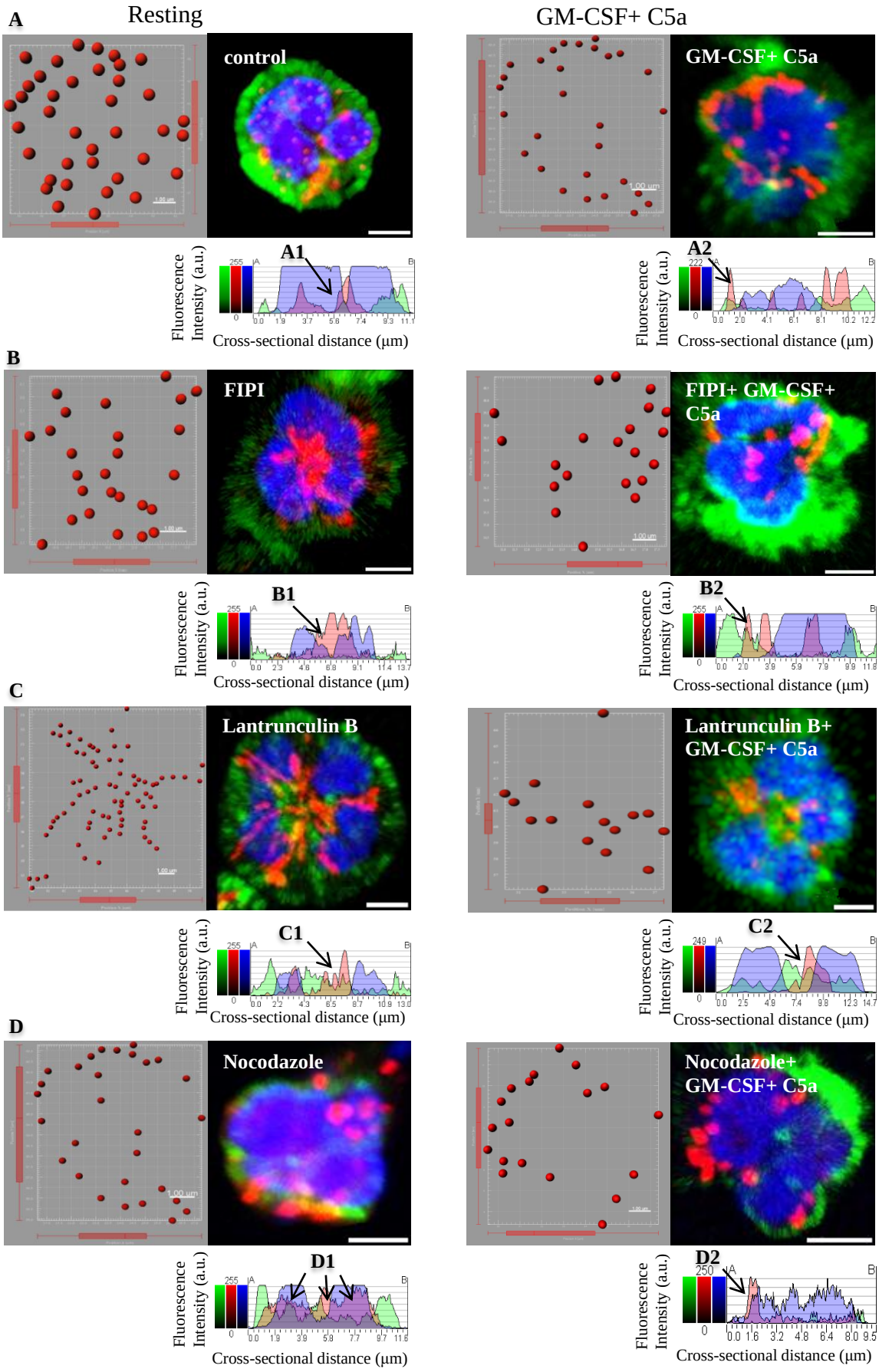


Fig. 8: Representative cells show localization of mitochondria in either resting or GM-CSF and C5a activated human neutrophils.

Mitotracker Orange: mitochondria
Phalloidine: actin
Hoechst: nucleus

Fig.8: Representative cells show localization of mitochondria in either resting or GM-CSF and C5a activated human neutrophils. Purified blood neutrophils have been fixed on slides for confocal microscopy and stained with Mitotracker Orange for mitochondria (red), with Hoechst for nucleus (blue) and with Phalloidine for actin (green). Distribution of mitochondria is represented in resting and stimulated conditions of neutrophils. Upon stimulation with GM-CSF (100 ng/ml) for 25 min and C5a (10^{-7} M) for another 20 min isolated neutrophils have been analysed by CLSM and Imaris software (2). Neutrophils are pre-treated with drugs to eliminate MFs or MTs: (A) control, (B) FIPI, (C) lantrunculin B, and (D) nocodazole. Position of mitochondria is indicated with red dots and with cross-sectional intensity profiles (red colour) by arrows (1, 2). Cross-sectional intensity profiles for mitochondria (red), nucleus (blue) and actin (green) are shown on the right side of cells (1,2). Mitochondria have been analysed for localization in the cell using confocal microscopy (cells in figure) and Imaris software (red dots and cross-sectional intensity profiles). Data shown are representative of at least three independent experiments. Scale bars on images with cells are 3 μm.

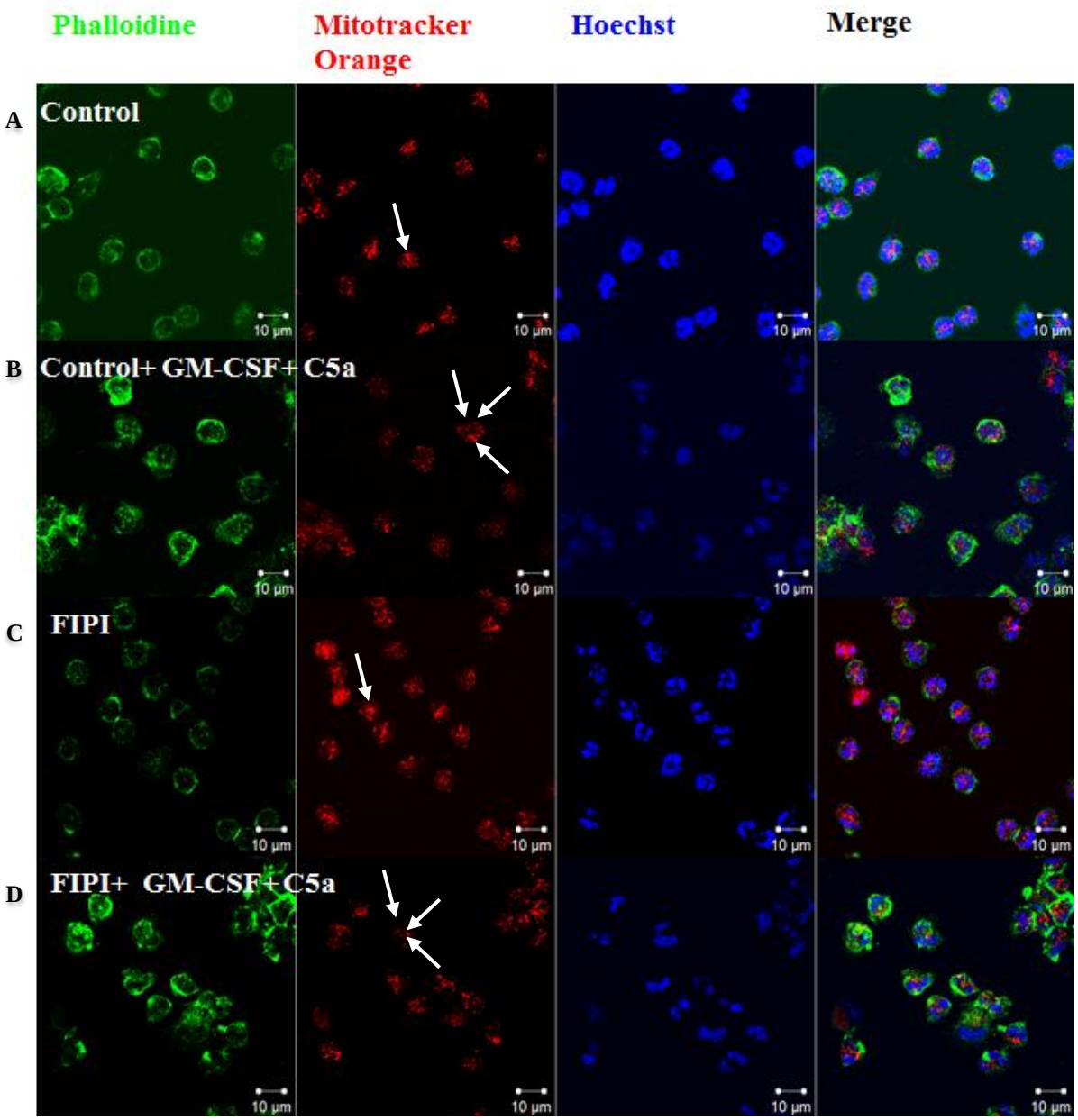


Fig. 9: Localization of mitochondria in human neutrophils control and treated with FIPI. Confocal microscopy made with objective field 63 x. Cells have either been resting or primed with GM-CSF and activated with C5a. DNA has been stained with Hoechst (blue), mitochondria with Mitotracker Orange (red) and actin with Phalloidine (green). The white arrows show the position of mitochondria. Scale bars: 10 µm.

After fixation of cells on slides, the experiments begin with CLSM. Firstly, images on confocal microscope have been made; here cell population representative for our statistic can be seen (Fig. 9). These images depict neutrophil population. White arrows point to neutrophils with mitochondria accumulated in the centres of the cells (A, C) and activated neutrophils where mitochondria move close to the plasma membrane (B, D).

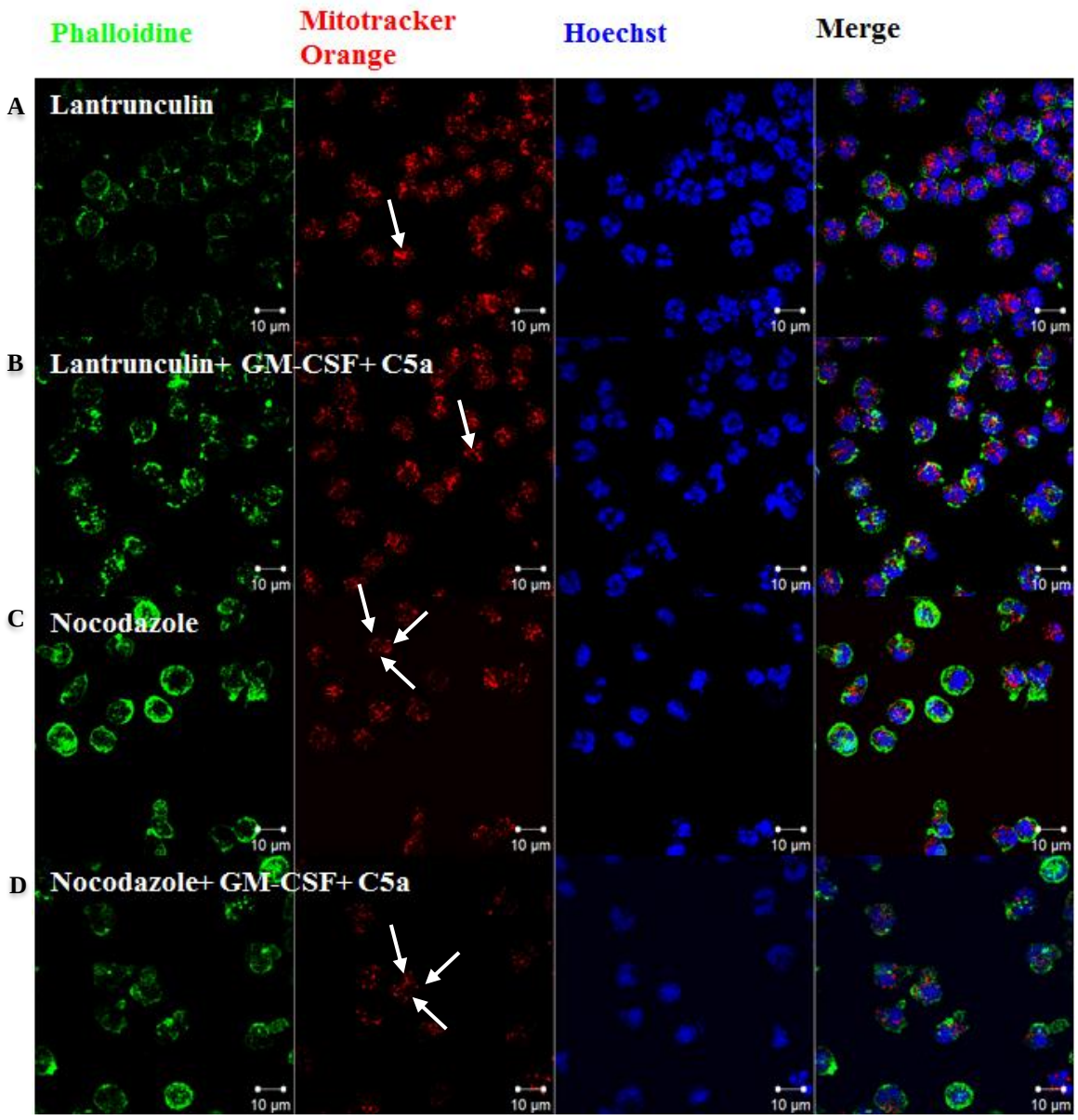


Fig. 10: Localization of mitochondria in human neutrophils treated with lantrunculin B and nocodazole. Confocal microscopy made with objective field 63 x. Cells have either been resting or primed with GM-CSF and activated with C5a. DNA has been stained with Hoechst (blue), mitochondria with Mitotracker Orange (red) and actin with Phalloidine (green). Mitochondria (red) are pointed out with white arrows. Scale bars: 10 µm.

Cells treated with lantrunculin B have mitochondria accumulated in the middle of the cell (Fig. 10 A), upon activation of cells mitochondria move toward plasma membrane (B) but not as markedly as this is the case in control experiment (Fig. 9 B) or in activated cells treated with FIPI (Fig. 9 D). Unactivated cells treated with Nocodazole (Fig. 10 C) have mitochondria already beneath the plasma membrane like activated cells (Fig. 10 D). Cells treated with nocodazole are used to depolymerize the microtubule network.

The role of cytoskeleton in mitochondrial localization is complex. In one interpretation, mitochondrial interactions with cytoskeleton are simply trapped and partially aligned within this cytoskeletal array of microtubules (MTs) and are further dispersed by their own intrinsic motility (shape changes, fission, and fusion). In other interpretation, it has been argued that mitochondrial movement along microtubules is promoted by the interaction of an outer membrane protein, Miro, with kinesin, through an adaptor protein (16, 20).

Movement of mitochondria in axons can serve as a general model for how all organelles move: mitochondria are easy to identify, they move along both microtubule and actin tracks, they pause and change direction, and their transport is modulated in response to physiological signals (52).

In addition, experiments on axons show that average mitochondrial velocity has increased in both directions lacking only microfilaments (MFs), but net directional transport has decreased. In axons with MFs but no MTs, mitochondria have also moved in both directions at a reduced average velocity and excursion length; net retrograde transport has been favoured under these conditions. No movement has been observed when both MTs and MFs have been eliminated. The studies clearly support the terms of actin-based motility of mitochondria over shorter distances and a faster microtubules-based motility over long distances (16).

Neutrophil extracellular traps (NETs) are formed after cell activation which results in mitochondrial localization towards plasma membrane and subsequent DNA release. This has also been the case in our central experiment (Fig. 7). However, no NETs have been visualized in the experiments using inhibitors of cytoskeleton organization (Confocal microscopy images, which show NETs release results, Fig. 21 to Fig. 23 are in Appendix). Our results have shown that mitochondrial movement in neutrophils treated with lantrunculin B is inhibited. Accordingly, there is no DNA release, suggesting that mitochondrial DNA release is F-actin polymerization dependent. These results confirm our theory that movement of mitochondria is necessary for NETs. When we used microtubule inhibitor nocodazole there was no detected movement at all and also no NETs. When cells were treated with nocodazole, mitochondria were localized beneath plasma membrane before and after activation. When cells were treated with inhibitor FIPI, no inhibition of mitochondrial movement was detected (Fig. 7 E), similar to control. However, no NETs were detected. Control, lantrunculin B and nocodazole confirmed our theory that

movement of mitochondria is necessary for mtDNA release. When function of cytoskeleton is inhibited, there are no NETs. It has been recorded that there are no NETs if inhibitors of MTs or MFs are used to block neutrophils. For instance, incubation of human neutrophils with nocodazole, a tubulin polymerization inhibitor, or cytochalasin D, an inhibitor of actin filamentation, has severely diminished the ability of neutrophils to respond to LPS by releasing chromatin from the cells. Their data indicate that NET formation requires functional tubulin and actin filaments (53).

It is possible that actin filaments play a role in allowing permeabilization of the plasma membrane and subsequent NET formation. In addition, the actin network may function to push the chromatin through the breach in the plasma membrane.

The coordinated interplay between the microtubule and actin filaments may be required in order to ensure the proper temporal and spatial control of mitochondrial deployment.

Further studies are necessary to define the molecular and functional interactions between actin cytoskeleton and microtubules in neutrophils. The use of the confocal-based method described here should help in undertaking these studies.

4.1.2 Hoxb8 mouse neutrophils

While experiments on human neutrophils have proved the role of microfilaments and microtubules in mitochondrial localization, we wished to further determine the role of other key molecules in this process. Therefore, we have used mouse models for PV, RhoH, WASp, BK and OPA1 deficiencies.

We have made experiments on Hoxb8 mouse neutrophils. Neutrophils have to be sufficiently mature in order to express the required machinery for transmembrane signalling events. Accordingly to that finding, we always used Hoxb8 mouse neutrophils on fifth day of differentiation.

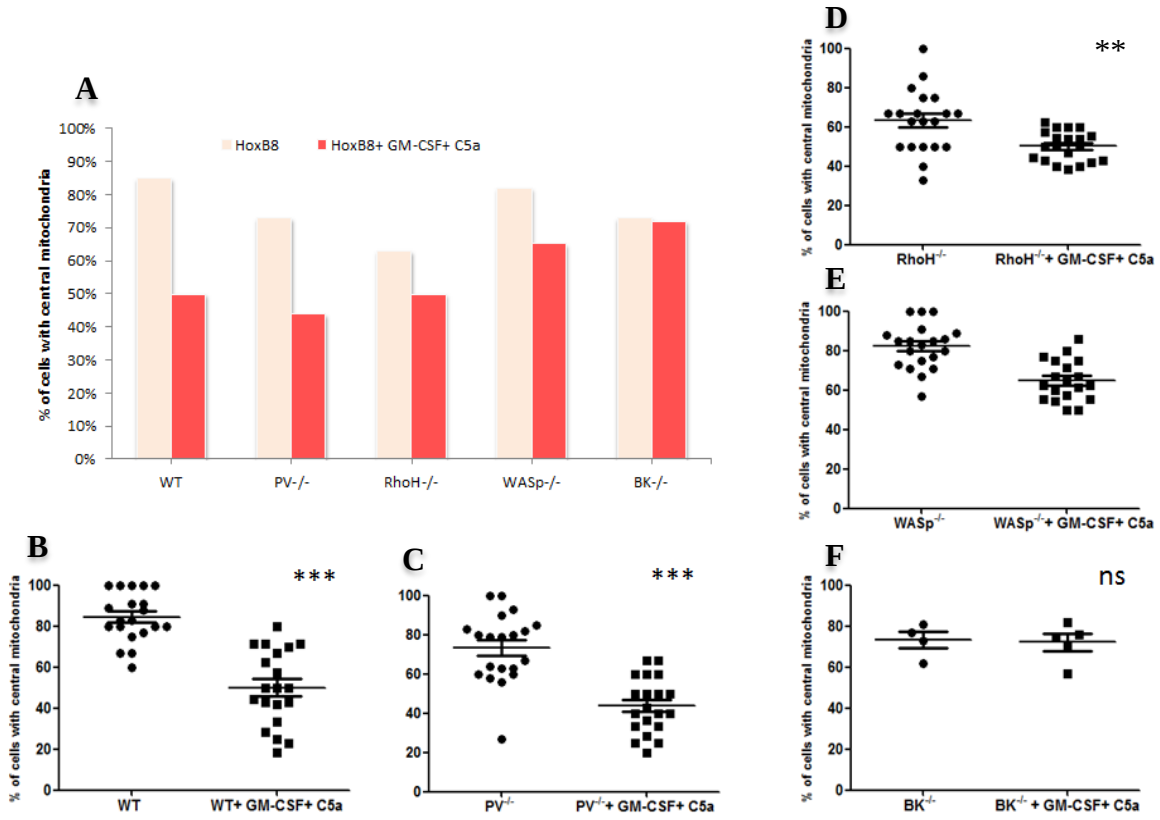


Fig. 11: Localization of mitochondria in Hoxb8 mouse neutrophils. Hoxb8 cells have been differentiated to neutrophil-like cells in 5 days. Hoxb8 mouse neutrophils have been incubated at 37 °C, 5 % CO_2 with (■) and without (●) GM-CSF (100 ng/ml) and C5a (10^{-8} M). Subsequently the cells have been stained with fluorescent dyes (as describe in Fig.). Each dot in graphs (B, C, D, E and F) represents the percentage of cells with central mitochondria in confocal microscopy images (objective field 100 x). Ten images per condition have been analysed in at least three independent experiments. Results have been analysed by GraphPad Prism 5 software. Graph A summarizes above experiments. Ns, P> 0.05; ***, P< 0.05.

As evident in Fig. 11, mitochondria move close to plasma membrane upon stimulation in WT and $PV^{-/-}$ knockout cells (A, B and C), but no significant movement of mitochondria in $RhoH^{-/-}$ (D), $WASp^{-/-}$ (E) and $BK^{-/-}$ (F) Hoxb8 mouse neutrophils has been detected.

These results are consistent with previous data on mtDNA release: WT and $PV^{-/-}$ knockout are able to release mtDNA (confocal microscopy images showed in Appendix Fig. 24 and Fig. 25) in contrast to RhoH-/-, WASp-/-, BK-/- and OPA1-/- knockout, which are unable to release mtDNA (Fig. 27). Accordingly, the release of extracellular DNA traps depends on the movement of mitochondria towards plasma membrane.

Calcium-binding protein PV plays a role in organization of microtubules. In our case, in $PV^{-/-}$ knockout movement of mitochondria has not been disturbed. Mitochondria have moved to the plasma membrane of the cell and released their DNA.

RhoH regulates both actin and microtubule-cytoskeletons. The movement of mitochondria towards plasma membrane is inhibited in absence of RhoH, so the movement is dependent on regulation of both actin and microtubule together. Consequently, there are no NETs.

The main function of WASp is to activate actin polymerization. $WASp^{-/-}$ knockout mouse neutrophils have shown inhibition of the mitochondrial movement, so this is another proof that movement is actin polymerization-dependent.

Statistically evaluated results of different genetically modified Hoxb8 mouse neutrophils are further presented by confocal microscopy (Fig. 13- Fig. 15).

To confirm the results obtained by in vitro model on immortalized Hoxb8 mouse neutrophils, we have obtained results from primary bone marrow-derived mature neutrophils (Fig. 12).

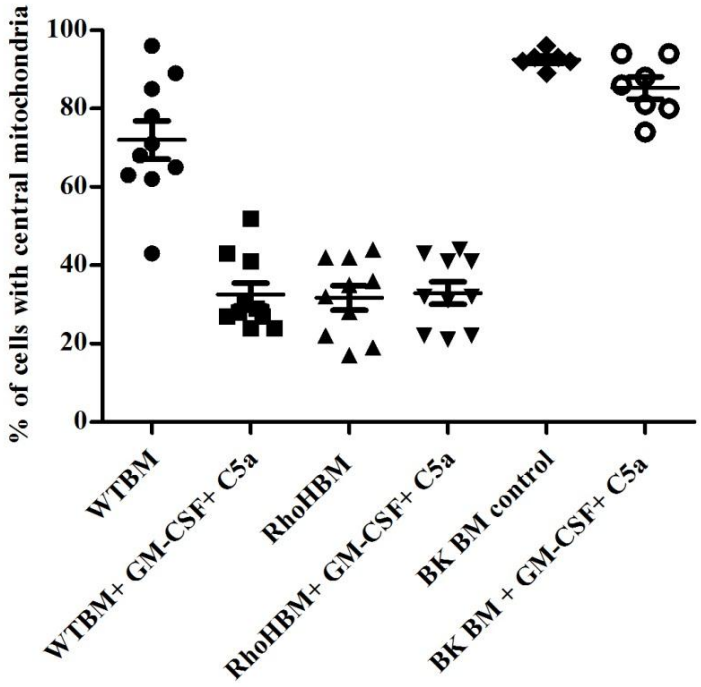


Fig. 12: Localization of mitochondria in primary bone marrow-derived mature neutrophils. Bone marrow-derived mature neutrophils have been primed with GM-CSF and stimulated with C5a. Images have been made by confocal microscopy (data not shown) and analysed by GraphPad Prism 5. Wild type, $RhoH^{-/-}$ bone marrow-isolated and $BK^{-/-}$ bone marrow-isolated mature neutrophils are shown on the graph.

Results gathered from bone marrow-based experiment are in agreement with *in vitro* immortalized Hoxb8 mouse neutrophils. It has been confirmed that in the absence of RhoH (A) and BK (B) there is no significant movement of mitochondria, while in WT bone marrow-isolated mature neutrophils the movement of mitochondria from the centre of the cell to the plasma membrane is clearly evident.

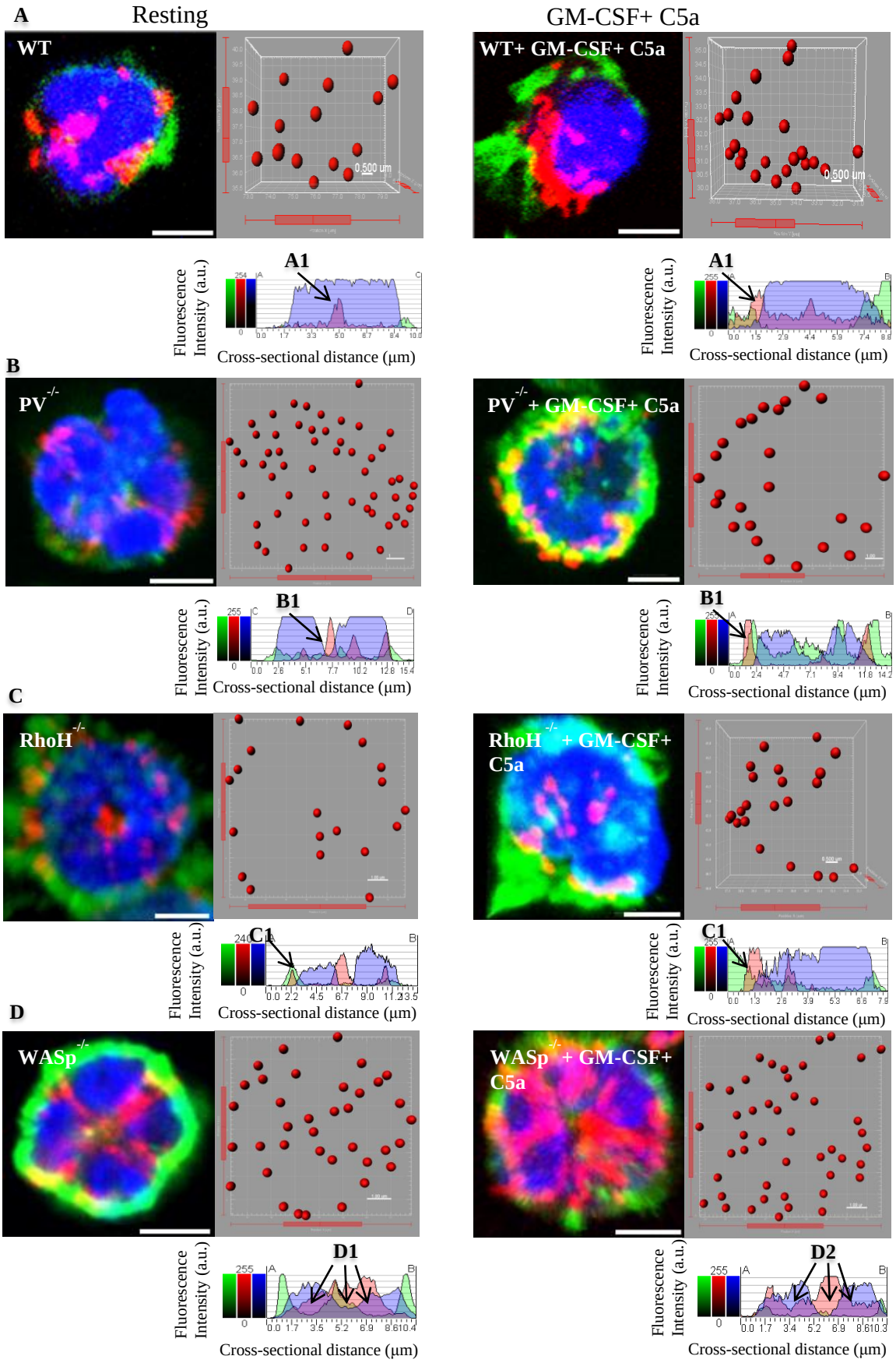


Fig. 13: Representative cells show localization of mitochondria in either resting or GM-CSF/C5a activated Hoxb8 mouse neutrophils.

Mitotracker Orange: mitochondria
Phalloidine: actin
Hoechst: nucleus

Fig. 13: Representative cells show localization of mitochondria in either resting or GM-CSF/C5a activated Hoxb8 mouse neutrophils. Hoxb8 mouse neutrophils have been fixed on slides for confocal microscopy and stained with Mitotracker Orange for mitochondria (red), with Hoechst for nucleus (blue) and with Phalloidine for actin (green). Mitochondria have been analysed for localization in the cell using confocal microscopy (cells) and Imaris software (red dots and cross-sectional intensity profiles). Position of mitochondria is represented in resting and stimulated conditions of neutrophils, which have been treated as described in Fig. 11. (A) Hoxb8 WT, (B) Hoxb8 $PV^{-/-}$, (C) Hoxb8 $RhoH^{-/-}$, and (D) Hoxb8 $WASp^{-/-}$ mouse neutrophils are shown on fifth day of differentiation. Position of mitochondria is indicated with red dots and pointed out on cross-sectional intensity profiles (red colour) with arrows (1, 2). Data shown are representative of at least three independent experiments. Scale bars on images with cells are 3 μm.

Single cell-based analysis of resting cells demonstrates the presence of mitochondria in the middle of the cell, while strong peripheral localization is evident in activated cells (Fig. 13). In fluorescence intensity graph for control cell we can see red peaks (A1) in the middle of the cell. And in the activated cell we can see red peaks, which show position of mitochondria close to the plasma membrane (A2). Red dots represent mitochondria, spread over the resting cell and in activated cell we can see red mitochondria close to plasma membrane (A, B). Described movement is characteristic for WT and $PV^{-/-}$ Hoxb8 mouse neutrophils. But $RhoH^{-/-}$ Hoxb8 mouse neutrophil mitochondria (C) had already been on the membrane before activation (C1) and a prominent difference after activation does not appear (C2). Nevertheless, movement of mitochondria in $WASp^{-/-}$ Hoxb8 mouse neutrophil is not evident either before or after activation (D).

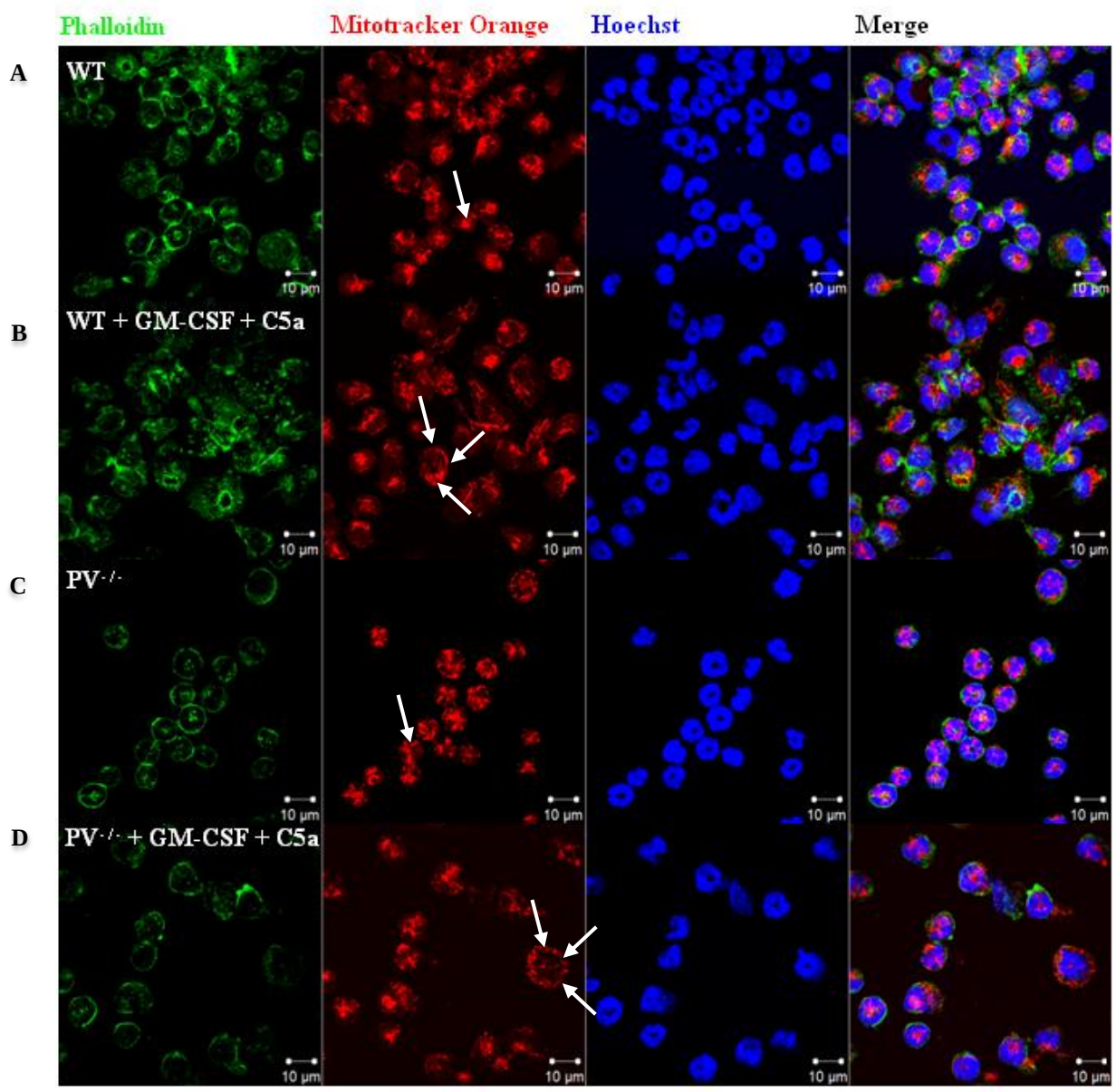


Fig. 14: Localization of mitochondria in Hoxb8 mouse neutrophils WT and PV $^{-/-}$ cells. Confocal microscopy made with objective field 63 x. Hoxb8 mouse neutrophils have either been resting or primed with GM-CSF and activated with C5a. DNA has been stained with Hoechst (blue), mitochondria with Mitotracker Orange (red) and actin with Phalloidine (green). (A) Hoxb8 WT mouse neutrophils, (B) Hoxb8 WT primed with GM-CSF and subsequently stimulated with C5a, (C) Hoxb8 PV$^{-/-}$and (D) Hoxb8 PV$^{-/-}$ mouse neutrophils primed with GM-CSF and stimulated with C5a. The white arrows show the position of mitochondria. Scale bars: 10 µm.

Representative image of the study of mitochondrial localization is presented in Fig. 14. Mitochondria are accumulated (A, C) in the centre and after activation of cells (B, D) they move towards plasma membrane.

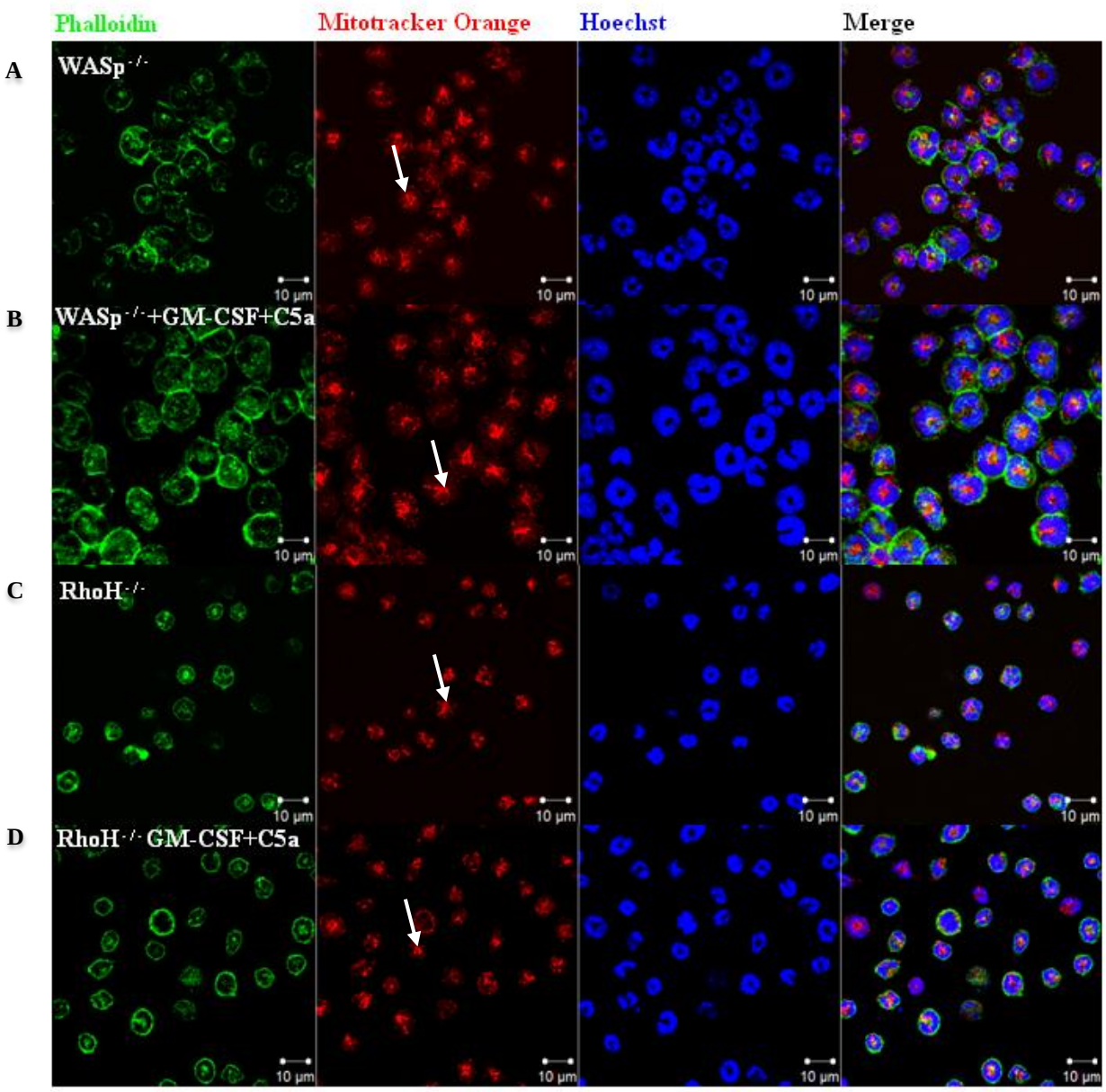


Fig. 15: Localization of mitochondria in Hoxb8 mouse neutrophils WASp $^{-/-}$ and RhoH$^{-/-}$ cells. Confocal microscopy made with objective field 63 x. Cells have either been resting or primed with GM-CSF and activated with C5a. DNA has been stained with Hoechst (blue), mitochondria with Mitotracker Orange (red) and actin with Phalloidine (green). (A) Hoxb8 WASp$^{-/-}$, (B) Hoxb8 WASp$^{-/-}$ mouse neutrophils primed with GM-CSF and subsequently stimulated with C5a, (C) Hoxb8 PV$^{-/-}$and (D) Hoxb8 PV$^{-/-}$ mouse neutrophils primed with GM-CSF and stimulated with C5a. The white arrows show the position of mitochondria. Scale bars: 10 µm.

Mitochondria in WASp$^{-/-}$ (A, B) stay accumulated in the centre of the cell before and after activation. The similarity between WASp$^{-/-}$ and RhoH$^{-/-}$ (C, D) is that there is no difference in mitochondrial movement before and after activation. But RhoH$^{-/-}$ (C, D) mitochondria are not accumulated in the centre so prominently before activation (C).

Furthermore, we investigate the role of Opa1 in mitochondrial localization. For this we have performed experiments on genetically modified cells Opa1 (Fig. 16) to see the influence of mitochondrial fusion on their movement upon neutrophil activation.

Three mammalian proteins are required for mitochondrial fusion two (Mfn1 and Mfn2) for outer membrane fusion, and OPA1 for inner membrane fusion. In the absence of fusion, the mitochondrial population fragments and a subset show ultra-structural defects and dysfunction. Defects in fusion have been shown to decrease mitochondrial movement. The mitochondria secondarily have transport defects that prevent proper distribution to periphery (49).

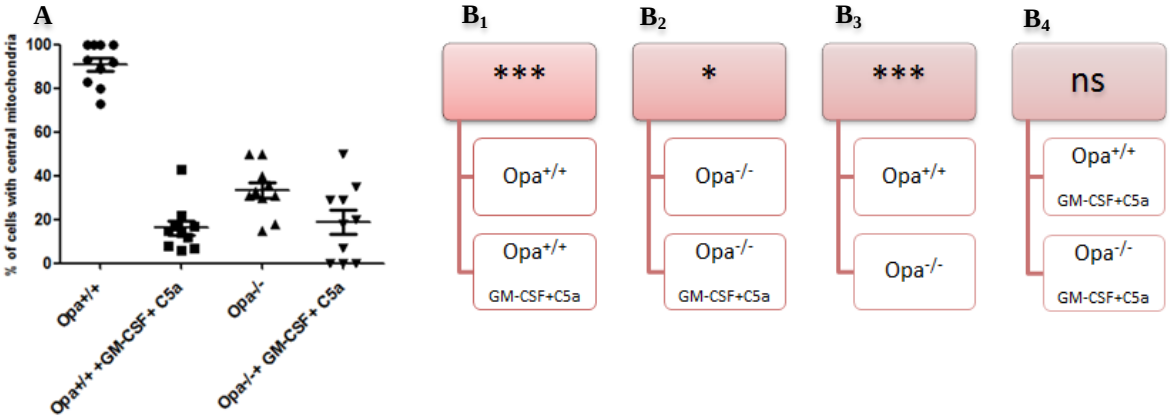


Fig. 16: Localization of mitohondria in Hoxb8 mouse neutrophils OPA1$^{+/+}$ and OPA1$^{-/-}$. Hoxb8 mouse neutrophils OPA1$^{+/+}$ and OPA1$^{-/-}$ have been fixed on slides for confocal microscopy and stained with Mitotracker Orange for mitochondria (red), with Hoechst for nucleus (blue) and with Phalloidine for actin (green). Cells have been incubated with (■, ▼) and without (●, ▲) GM-CSF (100 ng/ ml) for priming effect and for activation C5a (10^{-8} M). Mitochondria have been analysed for localization using confocal microscopy. Each dot in graph A represents the percentage of cells with central mitochondria in figure, that has been made for statistical evaluation by confocal microscopy with objective 100 x. 10 images per one condition have been made. Part B represents results analysed by GraphPad Prism 5 software. Ns, P> 0.05; *, P< 0.05; ***, P< 0.0001.

Fig. 16, graph A, shows localization of mitochondria in Opa1$^{+/+}$ as control and Opa1$^{-/-}$ mouse neutrophils. Significant difference of mitochondrial localization before and after activation in Opa1$^{+/+}$ (B_1) is detected, whereas for Opa1$^{-/-}$ neutrophils (B_2) no significant difference is detected. Significant difference is also seen between Opa1$^{+/+}$ control and Opa1$^{-/-}$ inactivated (B_3). While no significant difference is detected between Opa1$^{-/-}$ and Opa1$^{+/+}$ activated cell (B_4).

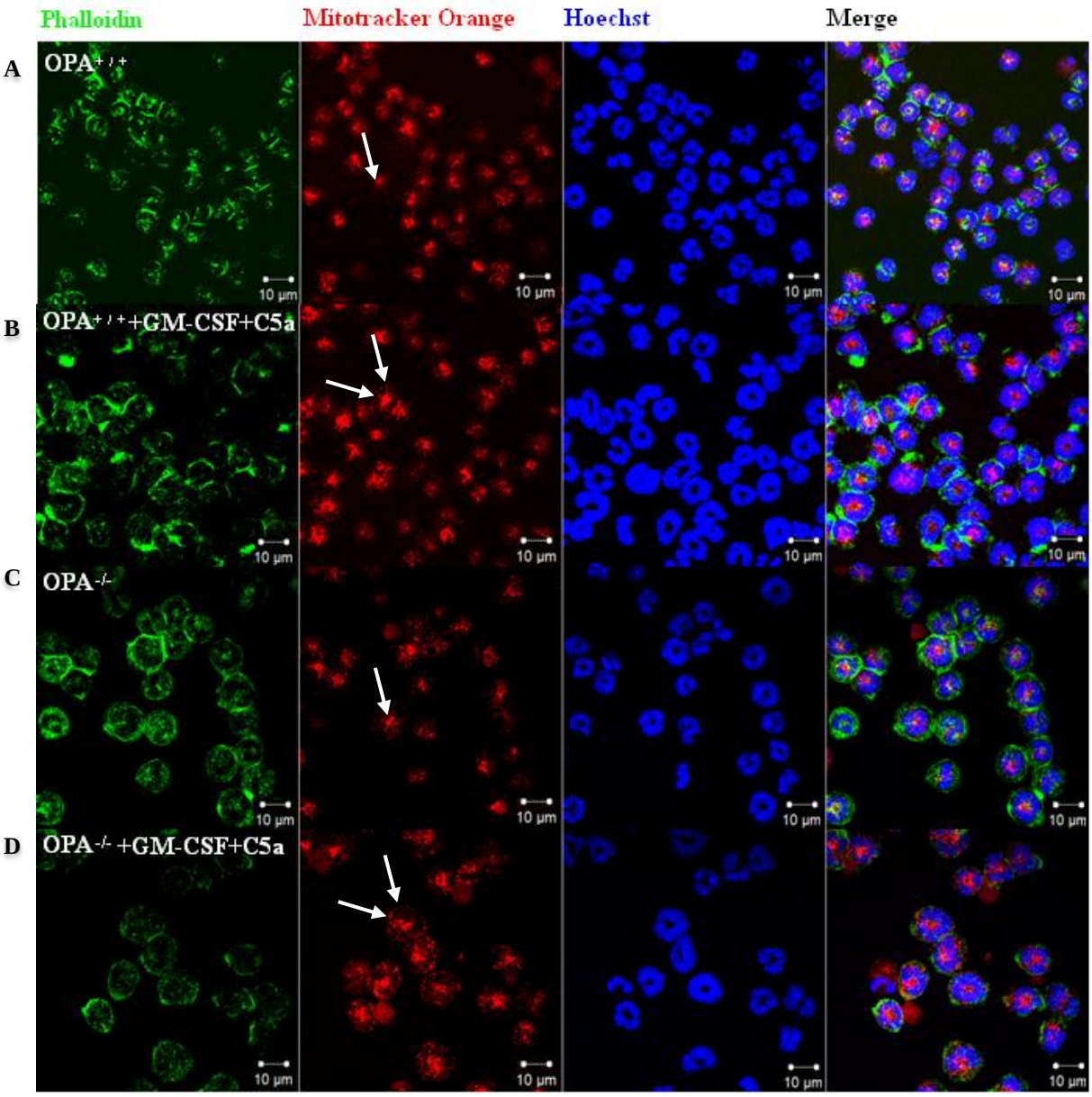


Fig. 17: Localization of mitochondria in mouse neutrophils Opa1$^{+/+}$ and Opa1$^{-/-}$. Confocal microscopy made with objective field 63 x. Mouse neutrophils Opa1 and Opa1$^{-/-}$ have either been resting or primed with GM-CSF and activated with C5a. DNA has been stained with Hoechst (blue), mitochondria with Mitotracker Orange (red) and actin with Phalloidine (green). The white arrows show the position of mitochondria. Scale bars: 10 µm.

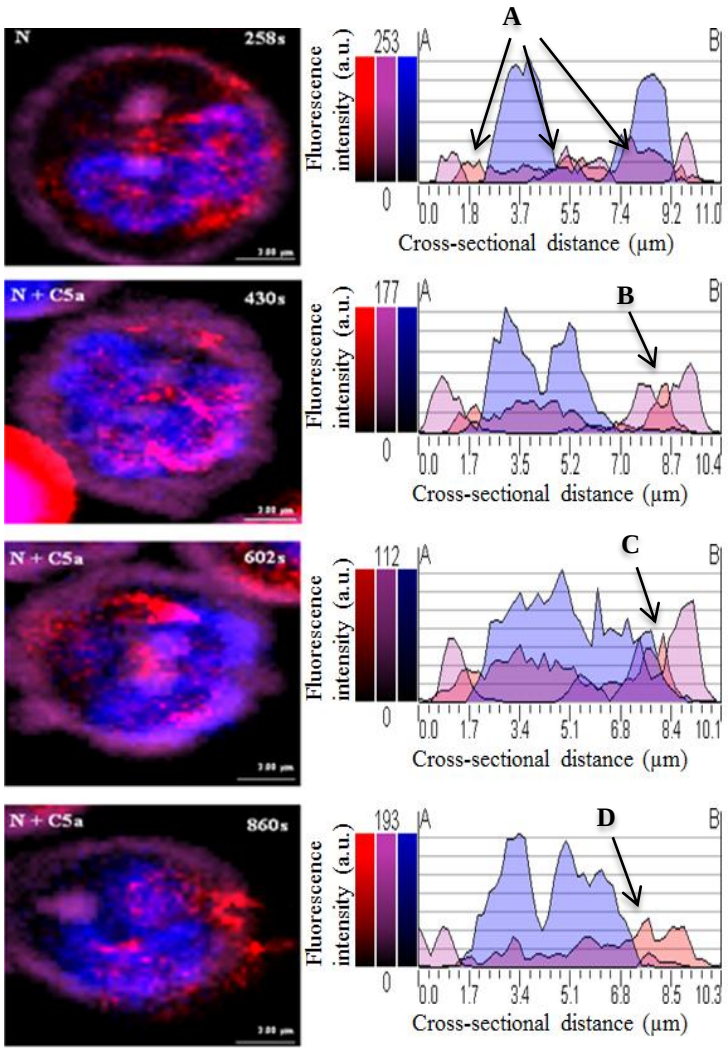
Fig. 17 represents confocal images of representative population, which represent our results of Opa1$^{-/-}$ mitochondria prior (A, B) and post (C, D) activation are already more on the plasma membrane than control (A), where they are accumulated closer to the centre of the cell. We can see the activated Opa1$^{-/-}$ cells (D) very nicely, where mitochondria are really bigger and close to the plasma membrane.

On the basis of these results we have concluded that Opa1 plays an important role in mitochondrial movement. Hence, in $Opa1^{-/-}$ neutrophils mitochondria have been localized close to plasma membrane but these cells exhibit no DNA release. The mitochondrial movement is inhibited compared to the control experiment. To conclude with, mitochondrial fusion is an important part of their movement close to the plasma membrane. Without gene OPA1, which is obligatory for fusion, there is inhibition of movement and consequently no NETs.

4.2 LIVE CELL ANALYSIS

4.2.1 Human neutrophils

Mitochondrial movements have been followed by computer-aided video analysis (Fig. 18) after staining neutrophils with fluorescence dyes. We have used Time-lapse acquisition mode. The images have been made every few minutes.



CellMask Deep Red Plasma membrane stain
Rhod-2: mitochondrial calcium
Hoechst: nucleus

Fig. 18: Live cell imaging with C5a activated human neutrophils. Purified human neutrophils ($2x10^6$ cells/ml) have been labelled using fluorescence dyes Rhod-2 (red) for mitochondria, Hoechst (blue) for nucleus and CellMask Deep Red plasma membrane stain (violet) for plasma membrane. Measurements have been performed by CLSM. Time elapsed is indicated on right upper side of figures with cell. In between the microscopy of labelled cells we have added C5a to activate cell. Cells and cross-sectional fluorescence intensity profiles (A, B, C and D) have been made by Imaris software. Arrows show position of mitochondria (red) in cell in cross-sectional intensity profiles. Scale bars on confocal microscopy images: 2 µm.

Cells have been incubated with three dyes. At beginning CellMask Deep Red stained the whole cell instead of staining just plasma membrane. After trying different conditions, the best developed possibility was culturing for 1 min at 37 °C and subsequently washing three times with ice-cold PBS.

The fluorescence intensity of red dye is changing the position (Fig. 18) after stimulation with C5a. At the beginning it is evenly distributed within the cell (A). After stimulation it can be seen that mitochondria translocate to plasma membrane (B, C and D). On the right side of cells there are cross-sectional intensity profiles before activation (A) and after C5a is added (B, C, D).

4.2.2 Hoxb8 mouse neutrophils

In time-lapse fluorescence microscopy of Hoxb8 mouse neutrophils (Fig. 19), mitochondria have changed the position upon activation. The video microscopy method, that allows the simultaneous monitoring of mitochondrial localization, has demonstrated the movement of mitochondria in cell with a ring-like plasma membrane and labelled nucleus by Hoechst. The confocal microscopy live images were made every few minutes.

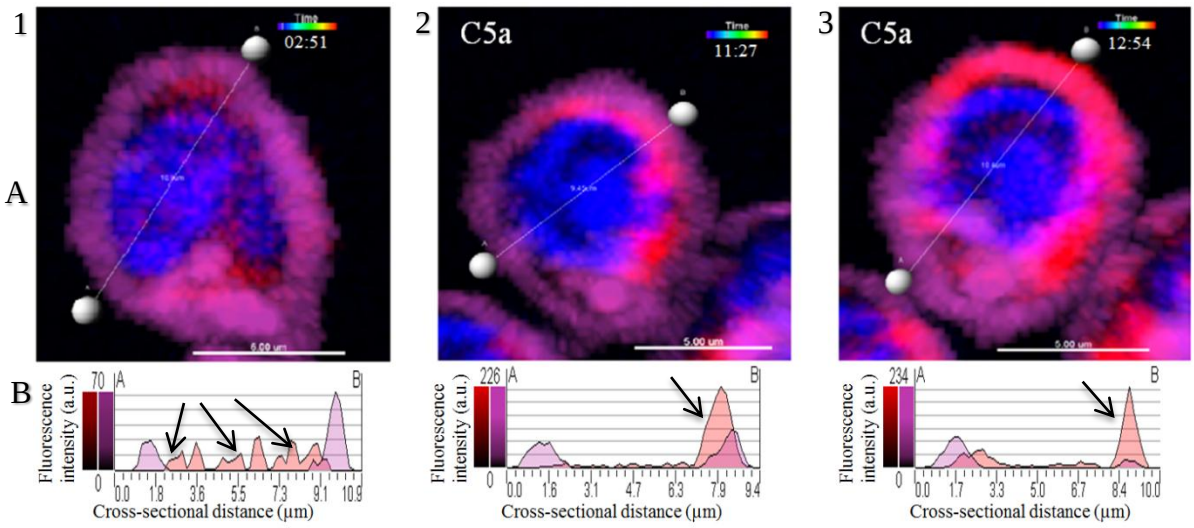


Fig. 19: Live cell imaging of Hoxb8 mouse neutrophil. Hoxb8 mouse neutrophils have been labelled using fluorescence dyes Rhod-2 (red) for mitochondria, Hoechst (blue) for nucleus and CellMask Deep Red plasma membrane stain (violet) on day 5 of differentiation. Time elapsed is indicated on right upper side of figure. In between the live analysis microscopy we have added C5a to activate cell. Cross-sectional intensity profiles of cell represent mitochondria (red) and plasma membrane (violet) in part B. Arrows indicate position of mitochondria in cell by cross-sectional fluorescence intensity profiles. Scale bar: 6 µm (A1), 5 µm (A2, A3).

CellMask Deep Red Plasma membrane stain
Rhod-2: mitochondrial calcium
Hoechst: nucleus

Similarly to the mitochondrial movement in human neutrophils it is presented in Hoxb8 mouse neutrophils. Fig. 19 A depicts how mitochondria are distributed in the cell (A 1), in contrast to the second (A 2) and third (A 3) figures, where localization of mitochondria in periphery is demonstrated. In fluorescence intensity graphs, the arrows indicate presence of mitochondria localization throughout the cell cytoplasm (B1). Upon activation mitochondria move towards plasma membrane of the cell (B, 2 and 3). These steps are leading to NETs formation.

Time-lapse micro cinematography of living cells, however, shows that mitochondria are remarkably mobile and plastic organelles, constantly changing their shape and even fusing with one another and then separating again. As they move about in the cytoplasm, they often seem to be associated with microtubules (MTs), which can determine the unique orientation and distribution of mitochondria in different types of cells. Thus, the mitochondria in some cells form long moving filaments or chains. In others they remain fixed in one position where they provide ATP directly to the site of unusually high ATP consumption (2).

In neutrophils, mitochondria are mostly distributed throughout the cytoplasm. Mitochondria slightly change the position every few minutes, when a new image is made by CLSM live analysis.

5 CONCLUSION

NETs formed by neutrophils constitute an important component of innate immunity. In our study, the detailed molecular mechanism of mitochondrial DNA release has been investigated. The following conclusions refer to experimental model studied in the present work, the regulation of mitochondrial movement toward neutrophil surface upon their activation.

Upon neutrophil activation, mitochondria have moved toward the plasma membrane, seen both in human neutrophils and Hoxb8 WT mouse neutrophils.

Mitochondrial localization at the plasma membrane is essential for mtDNA release which can be proved with the results of lantrunculin B, $WASp^{-/-}$, $RhoH^{-/-}$, $BK^{-/-}$, $OPA1^{-/-}$, and $PV^{-/-}$.

F-actin polymerization inhibitor lantrunculin-B has disrupted the movement of mitochondria toward plasma membrane. The results suggest that localization of mitochondria is F-actin polymerization-dependent.

Interference with depolymerisation of microtubules (nocodazole) has destroyed cell organization.

$WASp^{-/-}$, $OPA1^{-/-}$ and $RhoH^{-/-}$ have had less mitochondrial mobility upon activation. A movement of mitochondria in $BK^{-/-}$ mouse neutrophils is also not evident.

The method for live cell imaging by confocal laser-scanning microscopy has been newly established.

Taken together, mitochondrial DNA-containing NETs seem to require the movement of mitochondria toward the plasma membrane of neutrophils. However, further studies are required to understand the detailed molecular mechanisms of mitochondrial DNA release.

It will be challenging to unravel more aspects of this mitochondrial DNA release by neutrophils and to use the knowledge gained to design new therapeutic strategies.

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7 APPENDIX

Images of human neutrophils and Hoxb8 mouse neutrophils have been made by confocal microscopy (Fig. 20-Fig. 27). Human neutrophils have been treated with FIPI, lantrunculin B and nocodazole. Cells have been loaded with two flurescence probes: Hoechst 33342, which stains nucleus, and MitoSox Red, which specifically targets micohondria. White arrows show NETs.

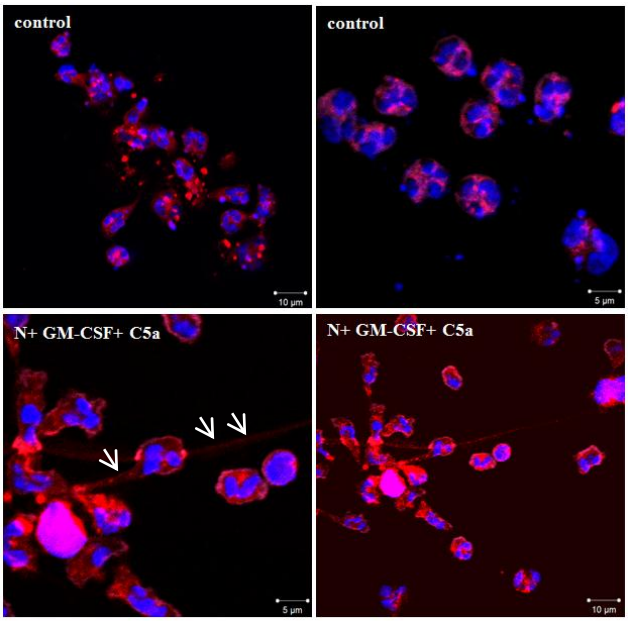

Fig. 20: mitochondrial DNA release: human neutrophils for control

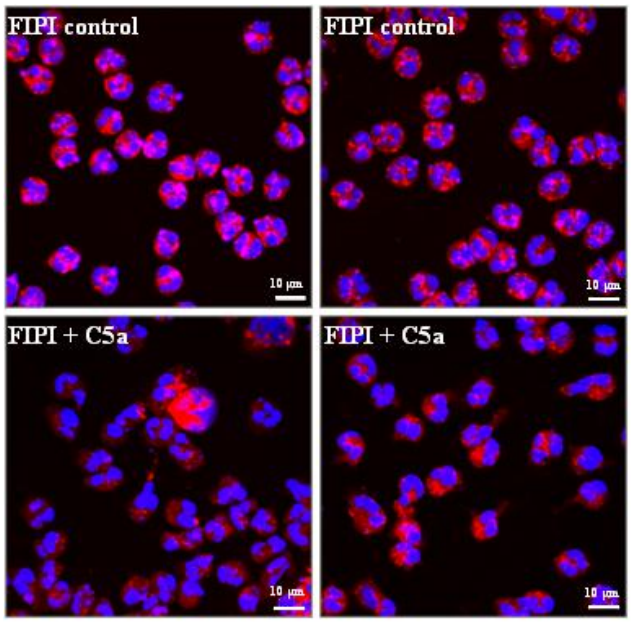

Fig. 21: no DNA release: human neutrophils treated with FIPI

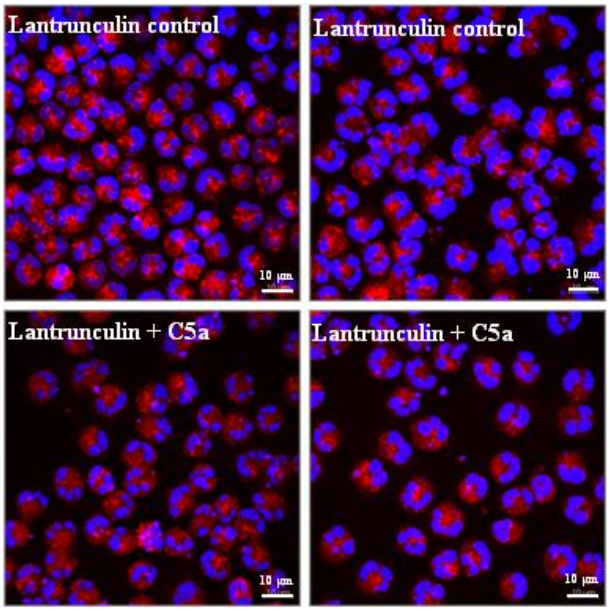

Fig. 22: no DNA release: human neutrophils treated with Lantrunculin

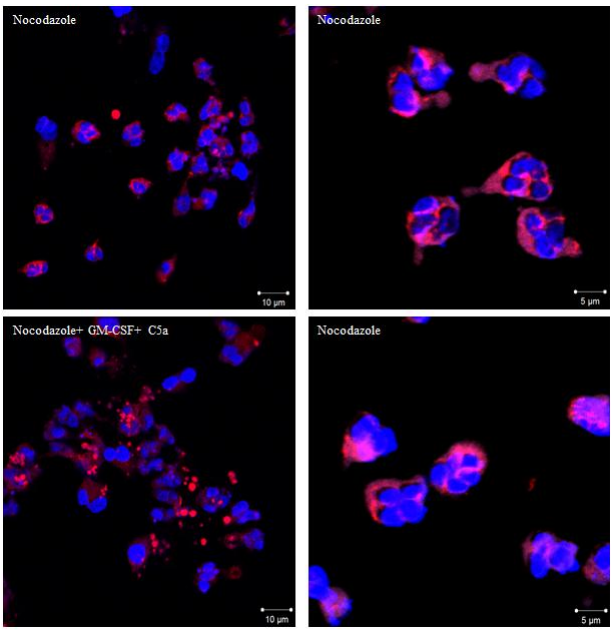

Fig. 23: no DNA release: human neutrophils treated with Nocodazole

Images of Hoxb8 mouse neutrophils made with confocal microscopy. Different genetically modified mouse neutrophils have been loaded with two fluorescence probes: Hoechst 33342, which stains nucleus, and MitoSox Red, which specifically targets mitochondria. White arrows show NETs.

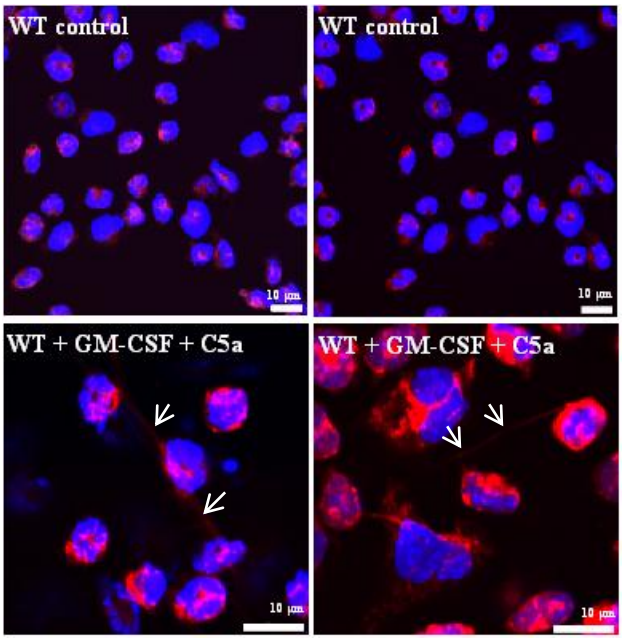

Fig. 24: mtDNA release WT mouse neutrophils

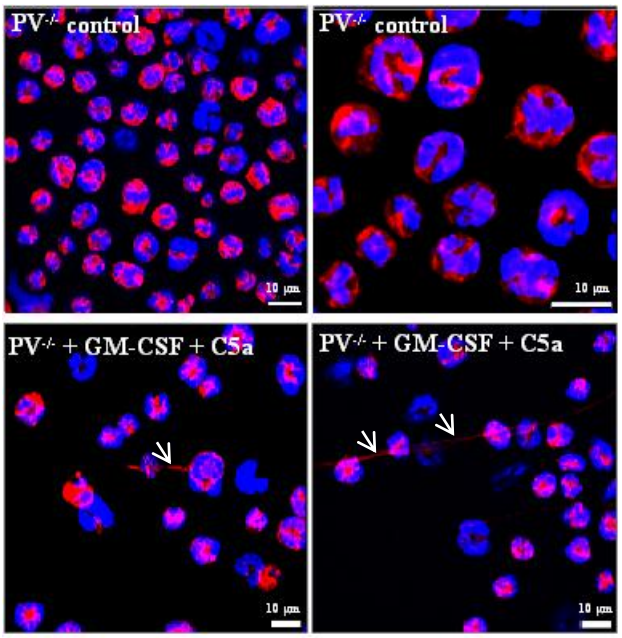

Fig. 25: mtDNA release $PV^{-/-}$ mouse neutrophils

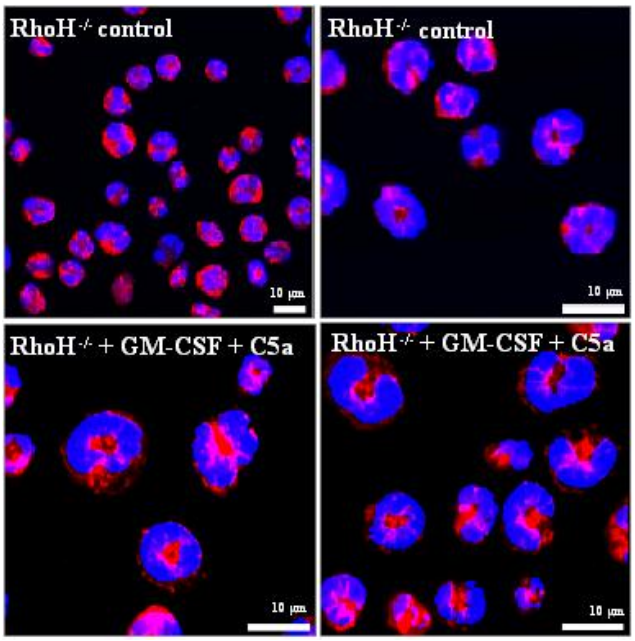

Fig. 26: no mtDNA release RhoH$^{-/-}$ mouse neutrophils

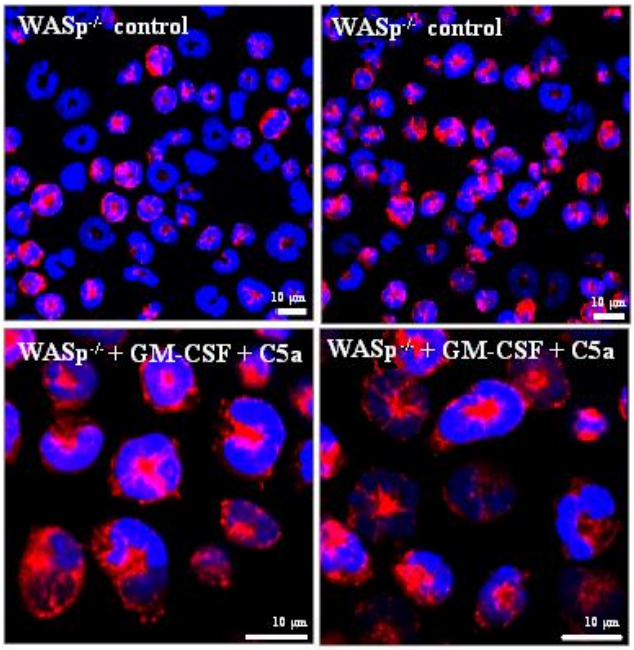

Fig. 27: no mtDNA release WASp$^{-/-}$ mouse neutrophils