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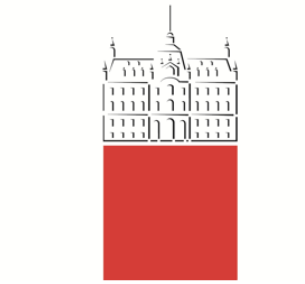

LUKA ŠRAJNER

ŠTUDIJ NASTANKA IZVENCELIČNIH DNA MREŽ PRI NEVTROFILCIH

INVESTIGATION OF NEUTROPHIL EXTRACELLULAR DNA TRAPS

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJE

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The present master thesis was realized at the Institute of Pharmacology at the University of Bern, with collaboration of the Faculty of Pharmacy, University of Ljubljana, under mentorship of Prof. Dr. Irena Mlinarič Raščan and co-mentorship of 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 guidance of my supervisors: Prof. Dr. Irena Mlinarič-Raščan and Prof. Dr. Dr. Hans-Uwe Simon.

Ljubljana, 2017

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ABSTRACT

Neutrophils play a crucial role in immunity against pathogens and represent the front line defence of our immune system. Their response is immediate and non-specific; therefore, they are classified as a part of the innate immune system. Pathogen removal occurs by phagocytosis or in the extracellular space by toxic granule proteins, which are highly concentrated within a DNA scaffold called neutrophil extracellular traps (NETs). NETs are a complex web of extracellular fibres, containing DNA from neutrophils, possessing the capacity to bind and kill pathogens.

It is commonly known that activating pathogen recognition receptors, namely Toll-like receptors (TLRs), e.g. TLR4 receptors with lipopolysaccharide, can lead to NET formations (1). The aim of the thesis was to investigate if stimulation of another family of pathogen recognition receptors, namely NOD receptors, can trigger NET formations. We stimulated NOD receptors on freshly isolated human neutrophils and differentiated Hoxb8 neutrophils with NOD1 or NOD2 agonists to release neutrophil extracellular traps. For the evaluation of NETs, confocal laser scanning microscopy and quantification of released dsDNA were used. The NOD agonists used were commercially available as well as newly developed and synthesized NOD1-specific and NOD2-specific agonists. We compared the effect of synthesized NOD1 agonist (SZZ-41) to that of compound C12-iE-DAP on NET formation, as well as the effect of synthesized NOD2 agonist (ZJ-237) to that of muramyl dipeptide (MDP). Viability assays were performed to exclude the possible cytotoxic effect on the results of the NET formations. As supplementary data, we also investigated NOD expression in human and mouse Hoxb8 neutrophils to support the results of our findings and for laying the foundations for future experiments.

In conclusion, our results show that NOD1 and NOD2 agonists can stimulate mouse Hoxb8 neutrophils to form NETs. We have also shown that NOD2 agonists can stimulate human neutrophils to form NETs. Our data is in agreement with the NOD expression data from scientific literature, indicating high expression of Nod1 and Nod2 in mouse Hoxb8 cells and NOD2 in human neutrophils.

Key words: neutrophils, NETs, Hoxb8 mouse neutrophils, NOD receptors, NOD agonists

RAZŠIRJENI POVZETEK

Nevtrofilci igrajo ključno vlogo pri odpornosti proti patogenom in predstavljajo prvo bojno linijo obrambe našega imunskega sistema. Njihov odziv je takojšen in nespecifičen, zato jih uvrščamo k prirojenemu imunskemu sistemu. Patogene lahko uničijo s fagocitozo, strupenimi granulami ali z zunajceličnimi pastmi nevtrofilcev.

Zunajcelične pasti nevtrofilcev so kompleksen preplet zunajceličnih vlaken, ki vsebujejo DNA ter granule iz nevtrofilcev in imajo sposobnost, da se vežejo na patogene in jih ubijejo. Proces tvorbe teh pasti se sproži že v nekaj minutah po aktivaciji nevtrofilcev. DNA se nato v izvencelični prostor izstreli v delcu sekunde, kar jim potencialno daje tudi sposobnost lovljenja hitreje pomikajočih se patogenov. Tvorbe imajo mreži podobno strukturo, ki se lahko raztezajo v velikosti do nekaj sto nanometrov in okupirajo območja, ki so do 15-krat večja od celic. Tvorjene izvencelične mreže DNA so po svoji naravi za patogene »lepljive«, večinoma zaradi ionskih interakcij. Med vezavo so patogeni izpostavljeni granulam in njihovimi toksini (kot so na primer proteaza katepsin G, mieloperoksidaze, elastaze, laktoferin ter reaktivne kisikove zvrsti). Omenjena kombinacija zunajceličnih mrež in strupenih granul tako predstavlja obrambo proti širokemu spektru patogenov, kot so po Gramu negativne in po Gramu pozitivne bakterije, glive (npr. *Candida albicans),* paraziti in virusi. Poleg naštetih toksičnih lastnosti pa predstavljajo mreže DNA tudi mehanske bariere, ki onemogočajo patogenom, da bi se širili globlje v tkiva.

Splošno je znano, da lahko aktiviranje receptorjev, ki prepoznavajo vzorce patogenov, privede do formacij zunajceličnih pasti v nevtrofilcih. Primer takšnega pojava je aktivacija Tollu podobnih receptorjev tipa 4 z lipopolisaharidom. V družino receptorjev, ki prepoznavajo patogene, sodijo tudi receptorji nukleotidno vezavnih oligomerizacijskih domen (NOD). Receptorji NOD imajo po aktivaciji podobne signalne poti kot Tollu podobni receptorji, ki močno pripomorejo k uspešnosti nespecifičnega imunskega odziva. Najpomembnejša predstavnika receptorjev NOD sta receptorja nukleotid vezavnih oligomerizacijskih domen 1 (NOD1) in NOD2.

Primarni cilj naloge je bil raziskati, ali lahko z aktivacijo receptorjev NOD1 in NOD2 sprožimo formacije izvenceličnih mrež DNA. Za oceno formiranih mrež smo uporabili dve

metodi; (i) metodo skeniranja s konfokalnim laserskim mikroskopom, kjer smo aktivirane nevtrofilce fiksirali s 4 odstotno raztopino paraformaldehida na krovna stekelca ter obarvali z barvili MitoSOX Red in Hoechst33342 ter slikali nastale tvorbe in (ii) metodo kvantifikacije sproščene dvojnovijačne DNA (dsDNA) v celičnih supernatantih, ki je omogočila kvantifikacijo sproščenih mrež z uporabo spektrofluorimetra in kompleta Quant-iTTM PicoGreen Assay Kit. Kot dodatek k rezultatom in za osnove prihajajočih poskusov smo s kvantitativno verižno reakcijo s polimerazo v realnem času analizirali ekspresije receptorjev NOD v uporabljenih celicah.

Za naše poskuse smo izbrali celično linijo mišjih nevtrofilcev Hoxb8 in sveže izolirane človeške nevtrofilce, saj so bili že predhodno uporabljeni v različnih poskusih pri preiskovanju zunajceličnih formacij pri nevtrofilcih. Veliki prednosti mišjih nevtrofilcev Hoxb8 sta stabilna proliferacija in kontrolirana diferenciacija. Obe metodi sta se izkazali kot občutljivi in sta omogočili dobro oceno formiranih mrež, induciranih z agonisti receptorjev NOD. Metodi sta po našem mnenju zato primerni za primerjavo različnih modulatorjev oblikovanja izvenceličnih mrež DNA v prihajajočih študijah.

Skladno z znanimi študijami o ekspresijah receptorjev smo s pomočjo kompleta iQ SYBR Green Supermix analizirali izražanje informacijske RNA Nod1 in Nod2 receptorjev v mišjih nevtrofilcih Hoxb8. Prav tako smo ugotovili, da je v človeških nevtrofilcih receptor NOD2 mnogo bolj izražen kot receptor NOD1. Za referenčne gene smo pri obeh tipih celic uporabili vzdrževalni gen 18S.

Na podlagi dejstev o ekspresijah smo diferencirane nevtrofilce Hoxb8 stimulirali z agonisti receptorjev NOD1 ali NOD2, sveže izolirane človeške nevtrofilce pa z agonisti receptorjev NOD2. Pri aktivacijah smo uporabili tudi dejavnik rasti makrofagov in granulocitov (GM-CSF), kot pozitivno kontrolo pa spojino lipopolisaharid (LPS). S spojinami smo stimulirali tako nevtrofilce, ki so bili predhodno aktivirani z GM-CSF, kot tudi mirujoče nevtrofilce. Agonisti NOD, ki smo jih uporabili v preizkusih, so bili za NOD1 specifični agonist SZZ-41, za NOD2 specifični agonist ZJ-237 ter uveljavljena agonista receptorjev NOD1 (spojina C12-iE-DAP) in NOD2 (muramil dipeptid – MDP). Učinkovini SZZ-41 in ZJ-237 so sintetizirali na Fakulteti za farmacijo v Ljubljani, njune modulacijske sposobnosti pa so ocenili bodisi na celični liniji Ramos-blue bodisi na celični liniji HEK-blue.

Ovrednotili smo razsežnosti oblikovanih zunajceličnih mrež DNA vseh uporabljenih agonistov in primerjali učinek sintetiziranega agonista SZZ-41 z učinkom spojine C12-IE-DAP ter sintetiziranega agonista ZJ-237 z učinkom MDP. Skladno z našimi pričakovanji smo dokazali, da lahko agonisti receptorjev NOD1 in NOD2 inducirajo tvorbo mrež DNA v mišjih nevtrofilcih Hoxb8. Za agoniste NOD1 in NOD2 smo dokazali, da lahko to inducirajo tudi na človeških nevtrofilcih. Uporabljeni spojini SZZ-41 in ZJ-237 sta imeli primerljive sposobnosti tvorjenja zunajceličnih mrež kot njuni referenčni spojini C12-iE-DAP in MDP. Vsi učinki preiskovanih spojin so bili manjši od učinka pozitivne kontrole,lipopolisaharida. Zanimivo je, da so imele vse preiskovane spojine zelo primerljive sposobnosti tvorjenja mrež, saj imajo različne tarčne receptorje NOD1 in NOD2. Prav tako smo ugotovili, da lahko spojine izzovejo mreže v diferenciranih mišjih nevtrofilcih Hoxb8, ne glede na predhodno stimulacijo z dejavnik rasti makrofagov in granulocitov, kar pa ne velja za preizkuse na človeških nevtrofilcih.

Pod konfokalnim laserskim mikroskopom smo opazili, da so stimulirane celice ostale ustreznih oblik, kljub formiranju zunajceličnih mrež. Njihova jedra so bila obarvana z barvilom Hoechst33342, sproščene mreže DNA pa so se obarvale z barvilom MitoSOX Red in ne z barvilom Hoechst33342, kar skupaj nakazuje, da so formirane mreže DNA mitohondrijskega izvora ter da nevtrofilci pri samem procesu ne podležejo apoptozi. Morebiten pojav apoptoze smo preverili tudi tako, da smo celicam ob koncu preizkusov dodali etidijev bromid in izmerili absorbanco. Etidijev bromid je interkalirajoča molekula, ki se veže na DNA in RNA ter v specifičnem območju absorbira in oddaja UV svetlobo. Ker barvilo ne prehaja celičnih membran, je tako možno določiti odstotek apoptotičnih celic glede na mrtve. Ugotovili smo, da nevtrofilci ob stimulaciji z uporabljenimi spojinami ne podležejo apoptozi in da odstotek živih celic na koncu preizkusov znaša okoli 97 %.

Ključne besede: nevtrofilci, mišji nevtrofilci Hoxb8, receptorji NOD, agonisti receptorjev NOD

GLOSSARY

ALRs	Absent-in-melanoma like receptors
BMDM cellc	Bone marrow-derived macrophage cells
bp	Base pair
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
CSF	Colony-stimulating factor
CTLs	C-type lectins
C5a	Complement component 5a
C12-iE-DAP	Lauroyl-γ-D-glutamyl-meso-diaminopimelic acid
DAMPs	Damage-associated molecular patterns
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	Double-stranded Deoxyribonucleic acid
ER-Hoxb8	Estrogen-regulated Hoxb8
ET	Extracellular traps
FCS	Fetal calf serum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/ macrophage colony-stimulating factor
Hoxb8	Homeobox protein Hox-b8
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat

LSM	Laser scanning microscope
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
mtDNA	Mitochondrial DNA
NETs	Neutrophil extracellular traps
NF- κB	Nuclear factor-κB
NLR	Nucleotide-binding oligomerization domain-like receptors
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocyte
PRR	Pathogen recognition receptors
PV	Parvalbumin
qPCR	quantitative polymerase chain reaction
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Standard error of mean
SFM	Serum-free hematopoietic cell medium
SCF	Stem cell factor
TLRs	Toll-like receptors
U	Units
WT	Wild-type

1. INTRODUCTION

1.1 Immune system

The immune system's most prominent physiological role is to maintain the homeostasis of the body by protecting it from pathogens and exogenous substances, while it also targets and removes body's own cells in case they are infected or become malignant. In order to achieve constant protection, a network of organs, tissues and cells comprising the body's immune system have to act in harmony and synergy. The immune system includes thymus, tonsils, lymph nodes and vessels, spleen, liver, bone marrow, white blood cells, antibodies and proteins that attack and destroy or disable bacteria, viruses, fungi, etc.

Immune system encompasses two major arms, namely the non-specific (innate) immune system and the specific (adaptive) immune system. For the pathogen to be able to enter an organism, it must first overcome the physical barriers such as skin, body hair, respiratory tract, the gastrointestinal tract, the nasopharynx, etc., and defensive mechanisms such as saliva, gastric acid, tears, bile, mucus and sweat. These constitute the first layer of defence of innate immunity. Following a successful penetration of the physical barriers and defensive mechanisms, the pathogen encounters other elements of innate immunity like complement system, inflammation and non-specific cellular responses. Cells, which are predominantly involved in innate immune response are macrophages, mast cells, neutrophils, eosinophils, basophils, natural killer cells and dendritic cells.

In the case when the innate immunity is not able to clear the pathogens on its own, it activates the adaptive immune system, which unlike the innate immune system, responds strongly and specifically as well as provides the body with long-term protection, based on immunological memory. The latter enables a faster and stronger response in the future when confronted with the same pathogen (2, 3). The ability to distinguish foreign tissues, cells and chemicals from body's own components is yet another important quality of our immune system.

1.2 Neutrophils

Neutrophils (Figure 1) play a crucial role as part of the non-specific innate immune response against pathogens. Their nucleus is segmented, usually into 2 to 5 lobes, on account of which they have been classified, together with basophils and eosinophils, as polymorphonuclear cells (PMNs) (4, 5).

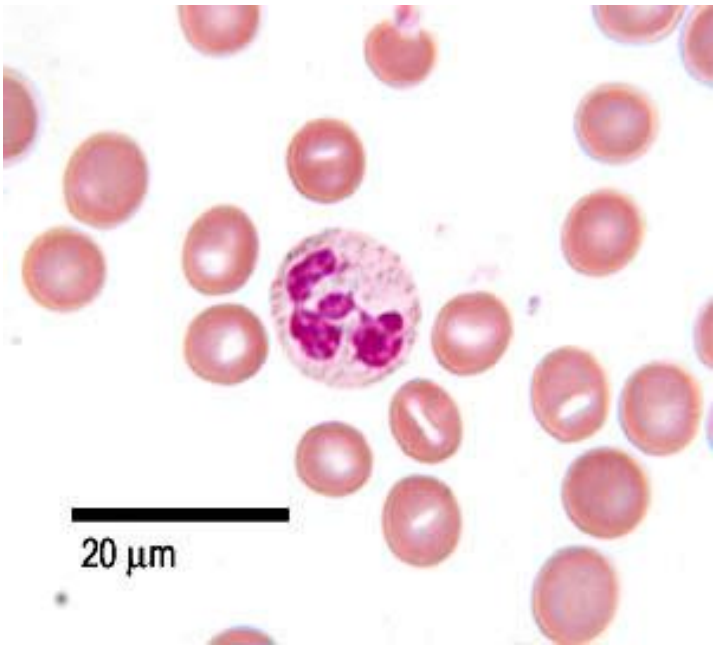

Figure 1: ***Neutrophils in human blood smear (6).***

Neutrophils are short-lived leukocytes and are generated in bone marrow from stem cells during haematopoiesis in large numbers of approximately 10^{11} per day (7). After haematopoiesis, they are "released" into the blood, where they spend in average up to 10 hours, before they are activated and able to migrate into the inflamed tissue. During the production and differentiation period, neutrophils are highly influenced by colony-stimulating factors (CSFs), including granulocyte (G-CSF) and granulocyte-macrophage (GM-CSF) colony stimulating factors, which increase the production and boost their activation (8). At the site of inflammation, the neutrophils become "activated", their key

mission being to immediately kill as many pathogens as possible, or at least, inhibit their proliferation (5).

The reasons behind their efficient elimination of pathogens are the following:

(i) firstly, they are the most abundant of the immune cells (representing up to 60% of all immune cells).

(ii) secondly, if called into action during the acute stage of inflammation, they can leave the blood vessels and move towards the site of infection, following a chemotactic gradient produced by microbial or endogenous signals (such as interleukin-8 (IL-8), C5a, fMLP, leukotriene B4 and H_2O_2) (9).

(iii) thirdly, they can be recruited to the infected tissue within minutes and with being one of the first-responders of inflammatory cells, they represent the front-line defence of our immune system.

(iv) lastly, they can kill pathogens through different mechanisms (10).

The mechanisms underlying their ability to kill pathogens include phagocytosis, toxic granule proteins and neutrophil extracellular traps (NETs). During phagocytosis they engulf microbes into phagosomes and fuse them with granules, consequently exposing them to toxins, such as proteases, lysozymes, reactive oxygen species, phospholipases, bactericidal permeability-increasing protein (BPI), cathelicidins and defensins (11, 12). A more recently discovered anti-bacterial mechanism of neutrophils, so-called NETs, represents networks of extracellular fibres, primarily composed of DNA and granule proteins. Unlike phagocytosis, the formation of NETs allows for the elimination of not only pathogens that are in direct contact with neutrophils, but also those that are distant (13).

1.2.1 Hoxb8 neutrophils

Mature neutrophils are short-lived and cannot be genetically manipulated. Hoxb8 neutrophils are created during proliferation of genetically engineered precursor cells. The cell line has fused Hoxb8 as chimeric protein to oestrogen receptor binding domain and can be immortalized, when oestrogen is bound on the receptor. They have identical functional and physiological characteristics as mature neutrophils and are therefore adequate for in vitro experiments (14).

1.3 PRRs

A milestone for a better understanding of the immune system is the discovery of pattern-recognition-receptors (PRRs). PRRs act as a sensor, recognising and perceiving pathogen-associated molecular patterns (PAMPs), and facilitate the generation of a strong immune answer against pathogens (15). In addition, evolution of PRRs took one step further, expanding their range of detection with ability to sense non-microbial danger signals (DAMPs) (16). When encountering pathogens, different PRRs usually act in synergy, thus achieving an even stronger response (17).

Since then, five subgroups of PRRs have been identified: toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectins (CTLs), absent-in-melanoma -like receptors (ALRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) (18, 19). While NLRs, RLRs and ALRs are intracellular, the CTLs and TLRs are located on the plasma membrane and on the membrane of endosomes.

1.3.1 NODs

Nucleotide-binding oligomerization domain (NOD) receptors are a subgroup of pattern recognition receptors (PRRs) and play an essential role in the innate immune response with recognition PAMPS and DAMPs (20, 21). They are a subfamily consisting of 22 known

nucleotide-binding oligomerization domain-like receptors (NLRs). The most known representatives of NODs are NOD1 and NOD2 (22).

Their structure is composed of three domains: (i) C-terminal leucine-rich repeat (LRR) domain, whose function is to sense and consequently bind appropriate ligands; (ii) nucleotide-binding NACHT (NOD) domain, which connects the two terminal domains and is responsible for mediating ATP-dependent self-oligomerization; (iii) N-terminal caspase activation and recruitment domain (CARD), whose function is to form protein-protein (CARD-CARD) interactions. The structure of NOD1 mostly differs from NOD2 in the N-terminal domain since it contains one CARD domain, while NOD2 incorporates two CARD domains (23).

Following ligand recognition, they develop suitable inflammatory or apoptotic response and thus have the ability to regulate the innate immunity. It is well known that when activated NODs transduce signal via nuclear factor κB (NF-κB) pathway and also mitogen-activated protein kinase (MAPK) pathway, which results in an increased secretion of pro-inflammatory cytokines (24). The activation of NODs also provokes autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry, thus facilitating the removal of pathogens (25). However, this pathway has not been fully clarified, and further studies are needed to fully understand the connection between NODs and autophagy.

Different cells develop different NOD responses, due to the variations of NOD expressions. There are numerous available data regarding the expression of receptors NOD 1 and NOD2 in human and murine cells. In humans, both receptors are expressed equally in PBMCs, eosinophils, dendritic cells, BMDM cells, T cells and B cells (26, 27, 28, 29). Interestingly, human neutrophils express up to thousand times more NOD2 than NOD1, while this expression pattern has also been observed in platelets, THP-1 cells, granulocytes, monocytes, lymphocytes and primary hematopoietic cells (CD19, CD3, CD15, CD14, and CD40/CD86) (30, 31). In the cells of mouse origin, however, Nod1 and Nod2 have been found expressed in hepatocytes, macrophages, PBMCs, platelets and neutrophils (32, 33, 34). Apart from the cells, the expression of both receptors is increased in brain, colon, intestine, liver, skin, lung, fat, spleen and kidney.

1.4 NOD agonists

NOD agonists are compounds that bind to and stimulate a NOD receptor. They have been shown to trigger NOD-related pathways, such as NF-κB and MAPK, leading to pro-inflammatory cytokine release (35, 36, 37, 38, 39). The mostly researched agonists are that of NOD1 and NOD2 receptors, with first studies dating back to 1970s and 80s (40, 41).

1.4.1 NOD1 agonists

The smallest peptidoglycan motif, recognized specifically by NOD1, is called γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP; Figure 2). It is found primarily in the cell wall of Gram-positive bacteria and has been shown to be a crucial fragment needed to bind with Nod1 receptor (42, 43, 44).

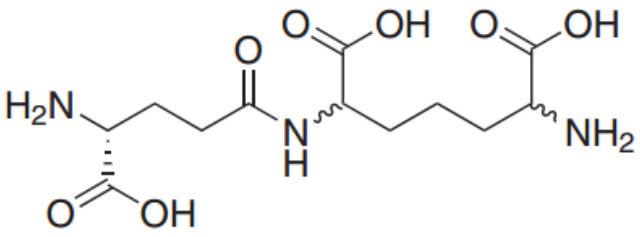

Figure 2: γ-D-Glu-mDAP (iE-DAP)

Since the discovery of iE-DAP, scientists have tried to enhance its potency. Interesting results were achieved with N-acylglutamyl derivates as a result of introducing a lipophilic substituent on the D-Glu portion of the molecule (45). Commercially available lauroyl derivate, for example, can stimulate NOD1 hundredfold stronger that the original iE-DAP and is widely used in research as a reference. A group of scientist from the Faculty of Pharmacy, University of Ljubljana synthesized numerous N-acylglutamyl analogues of iE-

DAP and evaluated their effects (38). The strongest agonistic effects were achieved with NOD1 specific agonist – compound SZZ-41 (Figure 3).

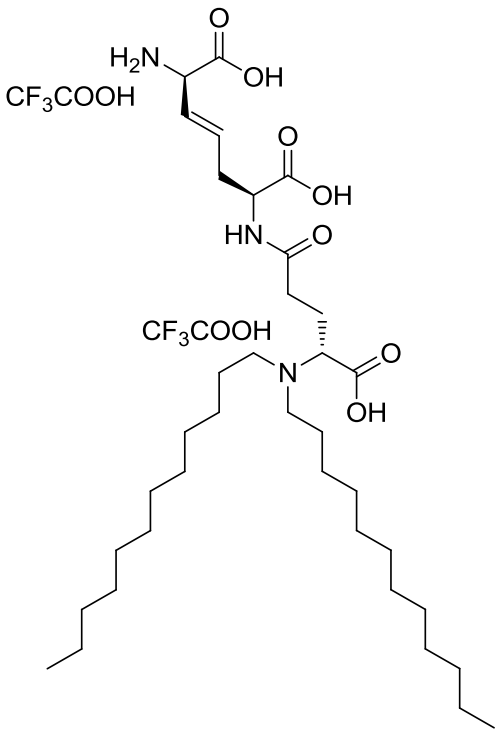

Figure 3: SZZ-41 – N-acylglutamyl analogue of iE-DAP; NOD1 specific agonist

In general, there is still a lot of unknown regarding the effects of NOD1 agonists on variety of cells. However, the majority of NOD1 agonists (including SZZ-41) up-regulate the release of pro-inflammatory cytokines (IL-6, IL-8, IL-10 and TNFα) in human bone marrow-derived macrophages and PBMCs, and increase the NF-κB activity in Ramos-blue cells. Furthermore, C12-iE-DAP has even been found to provoke NF-κB pathway in bovine neutrophils (46). In theory, the stimulation of NOD1 receptors leads to an enhanced host inflammatory answer against pathogens.

1.4.2 NOD2 agonists

The most renowned NOD2 agonist, Muramyl dipeptide (MDP; Figure 4), originates from peptidoglycan of both Gram-positive and Gram-negative bacteria and constitutes the smallest functional agonist of a NOD2 receptor (42, 47, 48).

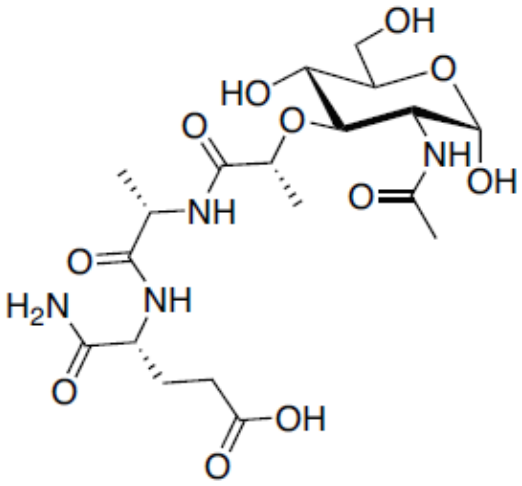

Figure 4: ***Muramyl dipeptide (MDP)***

In 1974, it was discovered that MDP is responsible for the efficacy of Freund's complete adjuvant (49). It has already been in use as adjuvant in veterinary medicine (50, 51). It cannot be used for the treatment of humans, however, due to its pyrogenicity. On the other hand, it created a great opportunity for the discovery of MDP derivatives that would not have a pyrogenic effect, but would keep the immunomodulatory effects of MDP. Numerous MDP derivatives were synthesized, e.g. mifamurtide, which has already entered clinical trials as a component of a potential influenza vaccine (52). A group of scientist on the Faculty of Pharmacy, University of Ljubljana, synthesized numerous MDP derivatives and evaluated their effects (53). Among numerous synthesized NOD2 specific agonists, ZJ-237 (Figure 5) has shown comparable agonistic capabilities to MDP.

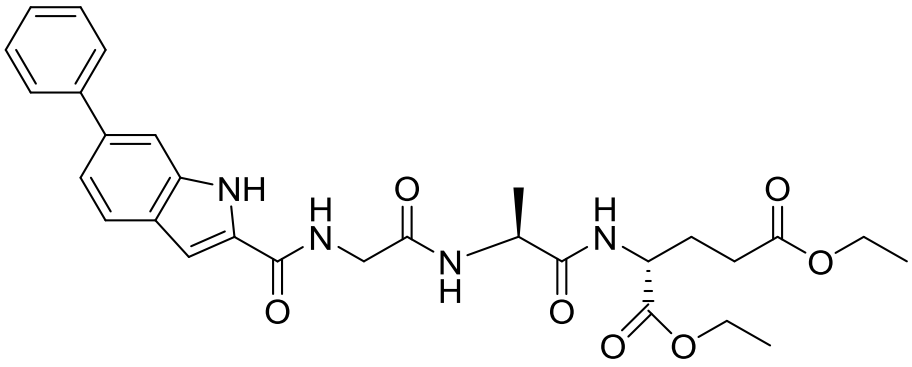

Figure 5: ZJ-237 – NOD2 specific agonist

Effects of MDP and NOD2 agonists such as ZJ-237 are increased pro-inflammatory cytokine release in PBMCs (IL-1β, IL-6), NF-kB activity in HEK-Blue cells and increased NF-kB activity in HEK-Blue Nod2-specific cells (53). In the experiments with WT mice, injected MDP even upregulated the secretion of IL-6, TNF-α, IL-10, IL-12p70 and CCL2 (54).

1.5 Neutrophil extracellular traps

Extracellular traps have been found to be released in vertebrates and even plants, specifically by granulocytes, such as eosinophils, mast cells, basophils, macrophages and neutrophils (55, 56, 57, 58, 59). Neutrophil extracellular traps (NETs; Figure 7) are a complex web of extracellular fibres, containing mainly DNA specifically from neutrophils, possessing the capacity to bind and kill pathogens. This phenomenon occurs when, upon activation, neutrophils release chromatin with granules into extracellular place (13).

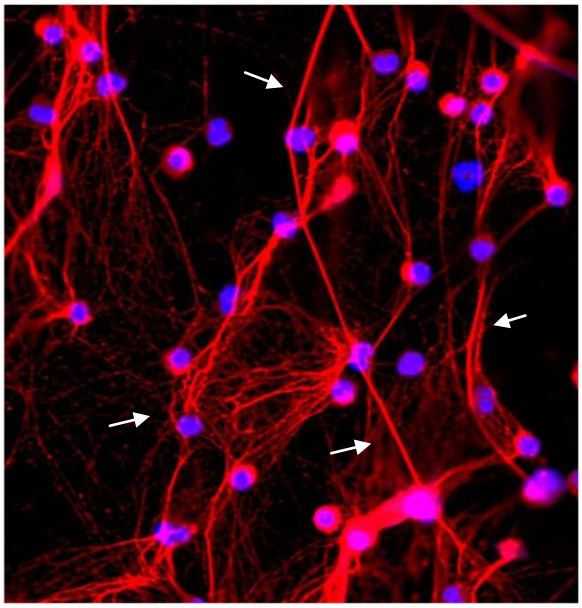

Figure 6: ***Neutrophil extracellular traps;*** *white arrows point to the released dsDNA*

The mechanism of NETs is triggered in minutes after activation of neutrophils and the DNA is fired within a second in a catapult-like manner, which could potentially catch even fast moving pathogens. The formed extracellular fibres alone are by nature very "sticky" towards pathogens, having the ability to bind and disarm them, mostly due to charge interactions (60, 61). The most potent effect is achieved when combined with granules, forcing the fusion of granular interior with pathogens and exposing the bound pathogens to very high locally concentrated toxins, consisting mostly of pathogen-killing proteins, such as catepsin G, myeloperoxidase, neutrophil elastase, lactoferrin and gelatinase (62). The above-listed toxins provide NETs with an enormous killing spectrum. They are well-known for killing Gram-positive as well Gram-negative bacteria and even fungi, such as *Candida albicans* and yeast-form cells. Recent in vitro studies suggest that they are very important when fighting parasites and even viruses (63, 64, 65).

Furthermore, NETs provide highly interesting web-like shaped physical barriers to prevent further spreading of the pathogens, which could potentially damage the tissue (66). Their shape is determined by the location of intertwined extracellular fibres. Electron microscopy with high-resolution scanning provided us with information that NETs can expand to a size of 17 nm to 50 nm dimensions, in flow conditions even up to few hundreds of nanometres in dimensions, occupying an area that is up to 15 times greater than that of the cell alone (67). On these fibres, azurophilic, specific and tertiary granules are located which consist of deadly toxins. In addition, they prevent further distribution of these granular toxins, keeping them at the site of inflammation and prevent the potential dispersal into neighbouring tissue and injuring it.

At the beginning of the understanding the complex mechanisms behind the formation of NETs, it was thought that in order for neutrophils to release NETs, apoptosis is unavoidable. Fuchs et al. even created the expression "NETosis" due to the sudden and impressive morphological modifications, happening to the cell during NET expression (68). However, Yousefi et al. discovered that NETosis is not essential for the formation of NETs (1, 55). For neutrophils to be able to induce NET formation, they must be activated with either microbial components or other stimuli (such as Phorbol 12-myristate 13-acetate (PMA) and C5a). A number of structurally different inducers has been proposed, mostly PAMPs, such as lipopolysaccharide (LPS) or lipophosphoglycans (69, 70, 71). Anti-HIV-1 activity was observed as a direct result of NET formation, triggered by single-stranded RNA of the human immunodeficiency virus 1 (HIV-1) genome (activating TLR-7 and TLR-8). Among various inducers, playing important role in the formation of NETs, are also DAMPs, for example histones, complement component 5a (C5a) and reactive oxygen species, especially hydrogen peroxide (68, 72). According to Garcia-Romo et al., Lande et al. and Kessenbrock et al, even antibodies and antibody-antigen complexes can trigger NET formation although not very efficaceously (73, 74, 75).

To control NETs and prevent unwanted NET-induced chain reactions, the organisms have deoxyribonuclease enzymes, such as DNase 1, which are able to cleave single as well as double stranded DNA and chromatin. These enzymes are therefore primarily responsible for the removal of NETs (76, 77).

2. OBJECTIVES

- The primary goal of this thesis will be to find out whether NOD1 and NOD2 agonists stimulate mouse Hoxb8 to form neutrophil extracellular traps (NETs).

- We will further investigate whether NOD2 agonists could stimulate human neutrophils to form neutrophil extracellular traps (NETs).

- We will develop and optimize cell-based assays using primary human neutrophils and *in vitro* differentiated mouse Hoxb8 neutrophils to screen for modulators of NET formation.

- We will compare the ability to induce NETs of native and synthesized NOD1 and NOD2 ligands to that of the positive control LPS.

- We will evaluate the viability of the neutrophils, upon activation and exposure to NOD agonists.

- We will determine the mRNA expression levels of NOD receptors in human and mouse neutrophils.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Device	Type	Company, cat. -No.
CELLSTAR	24 Well Cell Culture Plate sterile	Greiner Bio One, # 662 160
Cell Counter	KX-21	Sysmex Digitana SA
Centrifuge	5415 D 5417R	Eppendorf
Centrifuge	Multifuge 3 S-R	Thermo Fisher scientic, Heraeus AG
Centrifuge	Shandon Cytospin III Centrifuge	DAKO Diagnostics AG
Centrifuge	Biofuge pico	Huber & Co. Ag
Cytoslide	Microscope slides for Shandon Cytospin (Non-Coated, Circle on Back)	Thermo scientific
Falcon Tubes 15 mL	CELLSTAR® TUBES, 15 mL	BD Biosciences, #188 271
Falcon Tubes 50 mL	CELLSTAR® TUBES, 50 mL	BD Biosciences, #227 261
Flow cytometer	BD FACS Calibur	BD Biosciences
Freezer (-20°C)	MI 1207 A	Miostar
Freezer (-80°C)	V 535 Vacum Instalation Panel	New Brunswick Scientific- ultra low temperature freezer
Glass cover slips	12 mm	BD Biosciences
Glass pippettes	Pipette sterile ind. Wrapper 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	HeNe laser (543 nm) 1 mW, HeNe Laser (633 nm) 5 mW Diode laser (405 nm) 25 mW	Lasos
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 Haematology Analyser	KX-21N	Sysmex
Vortex Mixer or shaker	Vortex-Genie 2	Scientific Industries
X-Cite Series 120	powerful 120W lamp	EXFO

Software

Imaris Cell software (77)	Scientific software module for data visualization, analysis, segmentation and interpretation of 3D and 4D microscopy datasets.
Zen lite (78)	Imaging software for acquire images and video sequences. For measuring distances and making profile intensity graphs.
GraphPad Prism 6 (79)	Combines scientific graphing, comprehensive curve fitting (nonlinear regression), understandable statistics, and data organization.

3.1.2 Chemicals

Substance	Description	Company, cat. N° #
BCA Protein Assay kit	Kit for measuring protein concentrations	Thermo Scientific, #23227
C5a human	Human Complement factor C5a	Calbiochem – Novabiochem Corp., # HC1101
C5a mouse	Mouse Complement factor C5a	Calbiochem – Novabiochem Corp., # HC2101
C12-iE-DAP	NOD1 specific agonist	Invivogen, # tlrl-c12dap
DNAse	Recombinant DNase 1, RNase & Protease Free	Worthington, # LS006353
DPBS	Dulbecco's Phosphate buffered saline without Ca and MG	Lonza, #BE17-512F
EDTA	EDTA 0.5 M pH 8.0	Invitrogen, # 15575-038
FCS	Fetal calf serum	Invitrogen
G-CSF murine	Mouse Granulocyte-colony stimulating factor	Peprotech, #250-05
GM-CSF (Leukomax 300)	Human GM-CSF	Novartis
GM-CSF (mouse recombinant)	Mouse GM-CSF	Peprotech, # 315-03
Hoechst 33342 in water	Nucleic acid dye	Molecular Probes, # H-3570
Hydroxytamoxifen	(Z)-4- Hydroxytamoxifen	Sigma – Aldrich #H7904
Immersion oil	Immersol 518 F flurescence free	Zeiss, batch no.: #140327
LPS	Lipopolysaccharide	
MitoSOX™	Red mitochondrial superoxide indicator	Molecular Probes,#M36008
MDP	Muramyl dipeptide, NOD2 specific agonist	Invivogen, # tlrl-mdp
PAF	Paraformaldehid extra pur	Riedel-de-Häen, # 16005
Pancol human	Biocoll Separating Solution	Pan Biotech, # P04-60500
Penicillin/Streptomycin	Antibiotic drug	Roche, # 1429868

PicoGreen	dsDNA Quant-iT PicoGreen	Invitrogen, #P11496
ProLong Gold	Antifade reagent supress photobleaching	Life technologies, # P36930
RPMI 1640	Medium to culture cells	Life technologies, ref: 61870-010
SCF human	Recombinant human protein	Pepro Tech EC, # 300-07
SCF mouse	Recombinant mouse protein	Pepro Tech EC, # 250-03
SZZ-41	NOD1 specific agonist	Designed and produced on the Faculty of Pharmacy, University of Ljubljana.
X-VIVO™ 15 (SFM)	Chemically Defined, Serum-free Hematopoietic Cell Medium	Lonza, #04-418Q
ZJ-237	NOD2 specific agonist	Designed and produced on the Faculty of Pharmacy, University of Ljubljana.

Solutions

Lysis solution	12,45 g NH_4Cl, 1,5 g $KHCO_3$ 53,7 mg EDTA Disodium salt Dihydrate, dissolve in 150 mL ddH_2O; 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+	2% FCS in PBS
RPMI 1640	50 mL FCS+ 5 mL Penicillin / Streptomycin solution
Complete medium	RPMI 1640 + 10 % FCS

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 (PBMCs) were separated by centrifugation on Ficoll-Hypaque at 800 rpm for 20 minutes. The lower phase, consisting mainly of granulocytes and erythrocytes, was treated with erythrocyte lysis solution for 10 minutes on ice. The next step was centrifugation at 1400 rpm for 7 minutes at 4°C. After this, the supernatant was removed, as much as possible by aspiration, and 1 mL of cold PBS+ was added to the cell pellet. We resuspended the cells carefully, filled them up with PBS+ and centrifuged them again at 1400 rpm for 7 minutes at 4°C. The resulting cell populations contained more than 95% mature neutrophils as assessed by staining with Diff-Quik and light microscopy analysis.

3.2.2 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 µg/mL), G-CSF (100 ng/mL).

3.2.3 Cell passaging

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

Every Monday and Friday, we diluted the cells to approximately 100,000 cells/mL.

3.2.4 Differentiation of Hoxb8 neutrophils

A differentiation medium had the same composition as a culture medium but without tamoxifene. Its absence allows the 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 re-suspended in 10 mL of PBS. The supernatant was washed twice with PBS. The cells were re-suspended in 5 mL of differentiation medium. Their concentration was counted with Sysmex and diluted to a final concentration of 25,000 cells/mL in the total volume. Cells were stored for three days at 37°C and 5% CO_2. After three days, G-CSF was added to the cells at the concentration of 5 ng/1 mL cell suspension. On day 5, the cells were ready to be used in assays.

3.2.5 Cell counting

Automated Hematology Analyzer

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

3.2.5.1 Neubauer chamber

The Neubauer Chamber was taken and the glass slide was fixed onto it. 10 µl cell medium solutions were 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. The term 10^4 was used for multiplication because in four squares a volume of 0.1 µl was counted.

3.2.6 Confocal laser scanning microscopy

Freshly isolated human neutrophils or differentiated mouse Hoxb8 neutrophils were re-suspended in X-VIVO™ 15 medium (2.5×10^6/mL). One hundred microliters of cell suspension was primed with 25 ng/mL GM-CSF for 20 min on untreated glass coverslips, which had previously been washed with acetone, ethanol, ddH2O and baked in an oven. Cells were subsequently stimulated with 10^{-5} M of desirable NOD agonist or 0.3 μg/mL LPS for 15 minutes. NOD agonists that were used were: C12-iE-DAP, SZZ-41, MDP and ZJ-237. Cells were then fixed with 4% paraformaldehyde for 5 min, subsequently washed three times in PBS (pH 7.4), and mounted in ProLong Gold mounting medium. For extracellular DNA detection, cells were stained with 5 μM MitoSOX Red and 1 μg/mL Hoechst 33342. Extracellular DNA was analysed by indirect immunofluorescence (90). Briefly, cells were fixed with 4% paraformaldehyde. Slides were examined and images acquired by LSM 700 (Carl Zeiss Micro Imaging, Jena, Germany) using 63×/1.40 Oil DIC objective and followed by analysis with IMARIS software (78, 90).

3.2.7 Quantification of released dsDNA in culture supernatants

Briefly, 2×10^6 of either freshly isolated human neutrophils or differentiated mouse Hoxb8 neutrophils were diluted in 500 μL of X-VIVO™. Neutrophils were primed with 25 ng/mL GM-CSF for 20 min and later stimulated for 15 minutes either with 10^{-7} M of the selected NOD agonist or 0.3 μg/mL LPS. NOD agonists that were used were: C12-iE-DAP, SZZ-41, MDP and ZJ-237. At the end of the incubation time, a low concentration of DNase I (2.5 U/mL; Worthington) was added for additional 10 min. Reactions were stopped by the addition of 2.5 mM EDTA, pH 8.0. Cells were centrifuged at 1400 g for 5 min at 4°C. One hundred microliters of supernatant were transferred to black, glass-bottom 96-well plates (Greiner Bio-One GmbH), and the fluorescent activity of PicoGreen dye bound to dsDNA was excited at 502 nm and the fluorescence emission intensity was measured at 523 nm using a spectrofluorimeter (SpectraMax M2, Molecular Devices, Biberach an der Riß, Germany), according to the instructions described in the Quant-iTTM PicoGreen Assay Kit (91).

3.2.8 Viability assay

Cell death was assessed by an uptake of 1 μM ethidium bromide and flow cytometric analysis (FACS-Calibur). 2×106 cells/mL were taken and ethidium bromide was added before measurement.

3.2.9 Quantitative real-time polymerase chain reaction (qPCR)

RNA was isolated using the SV Total RNA Isolation System (Promega, Duebendorf, Switzerland) according to the technical manual provided (81). RNA was reverse transcribed to cDNA, and real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad, Reinach BL, Switzerland) with a real-time PCR machine (iQ5 Multicolor Real-Time PCR Detection System, Bio-Rad).

The primers used were:

Human:

NOD1 (82)	Forward: 5′-GTG GAC AAC TTG CTG AAG AAT GAC-3′
	Reverse: 5′-CTG TAC CAG GTC CAG AAT TTT GC-3′
NOD2 (28)	Forward: 5′-AGCCATTGTCAGGAGGCTC-3′
	Reverse: 5′-CGTCTCTGCTCCATCATAGG-3′

Mouse primers:

NOD1 (83)	Forward: 5′-TCC CTT GCC TGT GAG CAG AAA GTA-3′
	Reverse: 5′-GTG GGT ATG TGC CAT GCT TTG CTT-3′
NOD2 (83)	Forward: 5′-CAC ACA TGG CCT TTG GTT TCC AGT-3′
	Reverse: 5′-AAA GAG CTG CAG TTG AGG GAG GAA-3′

Primers were ordered via Microsynth, Balgach, Switzerland (84).

4. RESULTS

4.1 NET formation

Interestingly, to date the link between extracellular traps formation in neutrophils and NOD agonists has not been addressed and properly investigated. In our experiments, we stimulated the selected cells or cell lines with NOD1 and NOD2 agonists and observed whether this results in the formation of NETs. The NET formation was observed under the confocal scanning microscope and evaluated with the quantification of the released dsDNA with a spectrofluorometer.

4.1.1 The effects of NOD1 agonists on NET formations in murine neutrophils

The experiments were conducted on a differentiated Hoxb8 murine neutrophil cell line. These cells have to be suitably mature in order to form extracellular traps. According to previously established conditions, Hoxb8 murine neutrophils proved to be the most suitable when used on the fifth day of differentiation. The cells were treated either with an active compound or a combination of GM-CSF priming and an active compound. A combination of GM-CSF and LPS was used as a positive control (1). For negative controls, supernatant of the resting neutrophils and neutrophils with GM-CSF priming were used.

The analysis of mtDNA release with the dsDNA quantification method (Figure 7) demonstrated that NOD1 agonists can stimulate differentiated murine Hoxb8 neutrophils to form NETs. NETs were formed on GM-CSF primed and resting neutrophils with the NOD1 specific agonists SZZ-41 and C12-iE-DAP. SZZ-41 significantly triggered NETs, as opposed to the resting neutrophils. When used in experiments with untreated cells, the mean RFU measurements were around 400, which is significantly higher ($p=0.0105$) than in unprimed neutrophils (mean RFU=190). Furthermore, if neutrophils were primed before stimulation with SZZ-41, the RFU measurements were around 410, which is also significantly higher than the control ($p=0.0038$). C12-iE-DAP, a commercially available NOD1 specific agonist, which was used as a reference, showed similar results to SZZ-41.

It had a significant effect on both GM-CSF primed neutrophils with a mean RFU=400 (p=0.0066) and on the resting neutrophils with a mean RFU=360 (p=0.0285). There is no significant difference between the effects of the synthetic NOD1 agonist SZZ-41 and analog of native NOD1 agonist C12-iE-DAP, neither on the primed (p=0.6002) nor the resting (p=0.4931) neutrophils. This can be ascribed to the similar molecular structure of both compounds, as they have both attached lipophilic groups (lauroyl or didodecyl) to the glutamic residue of DAP.

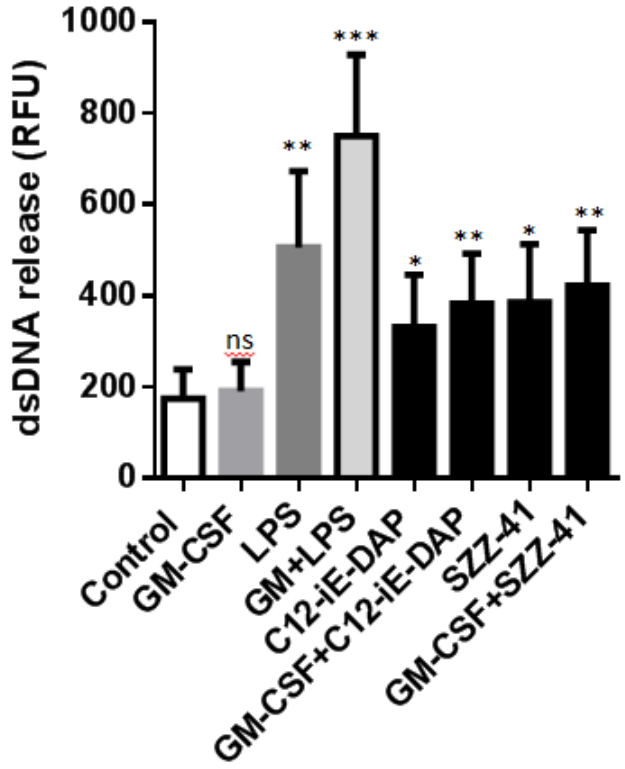

Figure 7: ***dsDNA release in murine HoxB8 neutrophils,*** *treated with LPS (0.33 µg/mL) and NOD1 agonists (10 µM). For positive control, cells (2×10^6) were primed with GM-CSF and stimulated with the LPS. Neutrophils have been stimulated with the NOD1 agonists (10 µM), either with or without priming with GM-CSF (25 ng/mL). The data is shown as a mean ± SEM of the indicated number of five (n=5) independent experiments. *p<0.05; **p0.01; ***p<0.001; t-test.*

The strongest stimulation was achieved using LPS on the GM-CSF primed neutrophils. It showed a RFU signal of around 770 (p=0.0001). In the case of using LPS on unprimed cells, the mean RFU was 500 (p=0.0032). We also primed the cells solely with GM-CSF and the data clearly showed that GM-CSF priming alone does not stimulate the neutrophils to form NETs (p=0.7027) because of the similar value obtained, compared to that of the unprimed cells.

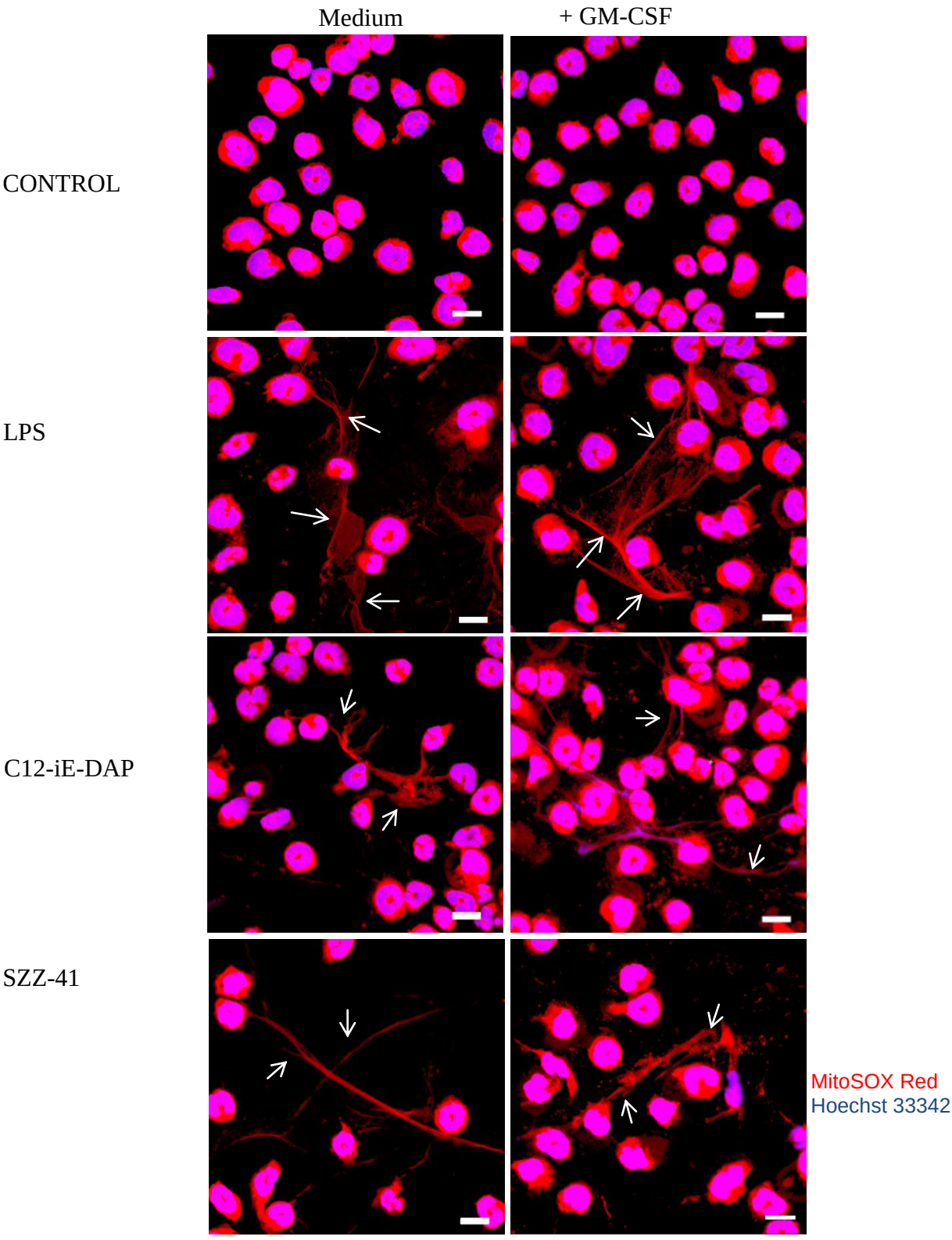

Figure 8: ***dsDNA release in murine HoxB8 neutrophils****, treated with LPS and NOD1 agonists on GM-CSF primed or resting neutrophils. The confocal microscopy was made with objective field 63 x. Scale bars, 10 µm. Nuclear DNA has been stained with Hoechst 33342 (blue) and MitoSOX Red has been used to stain mitochondrial DNA. White arrows point to the neutrophil extracellular dsDNA.*

Confocal images (Figure 8) depict representative examples of groups of cells, which were analysed by the Imaris software. The images showing the effect of NOD1 agonists on NET formations are in good agreement with the findings of dsDNA quantification (Figure 7). The most significant release of NETs was seen when stimulating the GM-CSF primed neutrophils with LPS. Furthermore, clear signs of NET formations were seen in the stimulation of the cells either with C12-iE-DAP or SZZ-41. No difference in the extent of NET formations could be observed between C12-iE-DAP and SZZ-41.

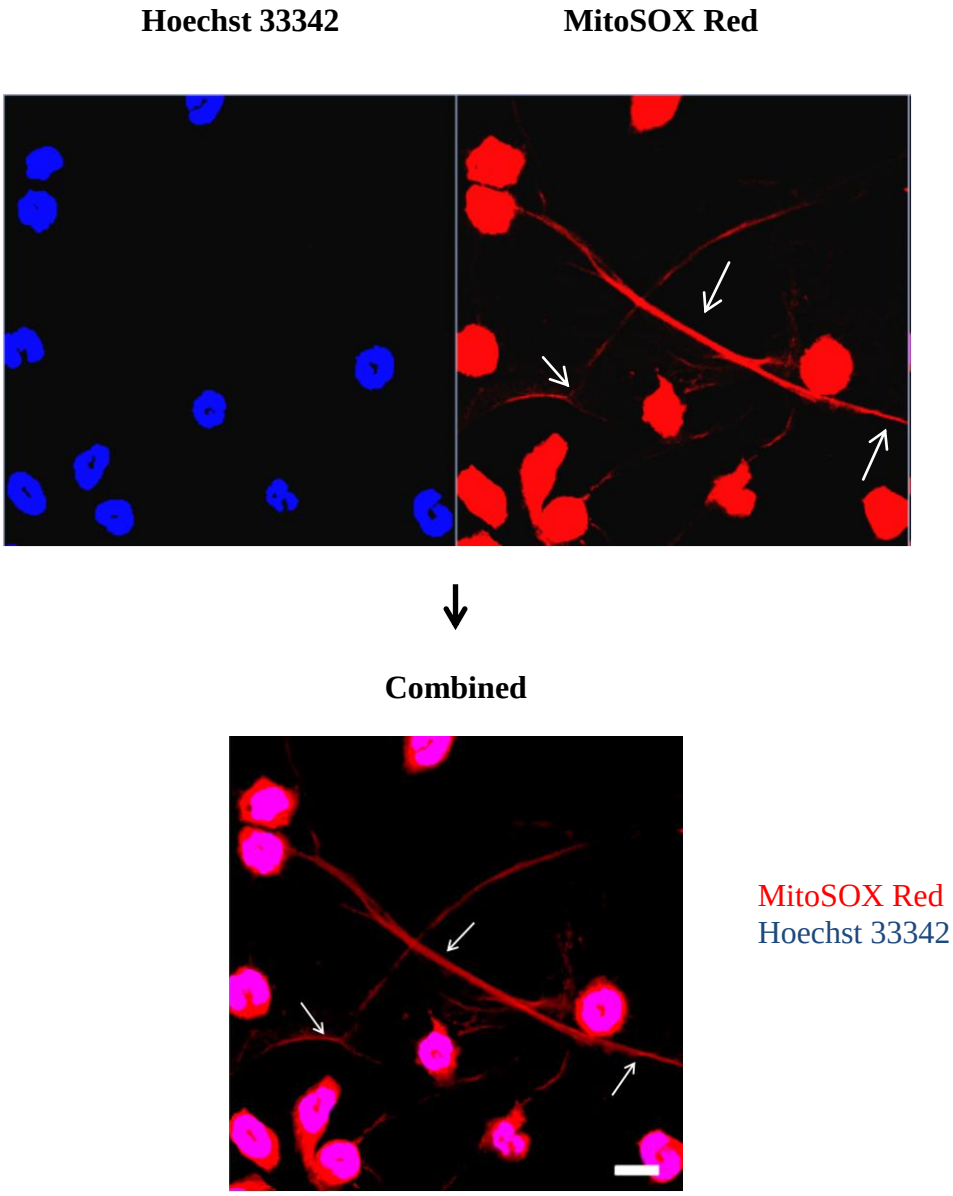

Figure 9: ***dsDNA release in murine Hoxb8 neutrophils*** *treated with a SZZ-41 agonist. The confocal microscopy was obtained with objective field 63×. Scale bars, 10 µm. Nuclear DNA has been stained with Hoechst 33342 (blue) and MitoSOX Red has been used to stain mitochondria DNA. White arrows point to the neutrophil extracellular dsDNA.*

On the confocal microscopy image in Figure 9, the MitoSOX Red staining of mtDNA in NETs is clearly seen. Hoechst dye stained selectively DNA in nucleus. When the image is combined, the nucleus is seen as pink and the NETs are seen as red. This was observed in all NET formations triggered by NOD1 and NOD2 agonists.

4.1.2 The effects of NOD2 agonists on NET formations in murine neutrophils

We also tested the ability to trigger NET formations in murine Hoxb8 neutrophils with NOD2 agonists. dsDNA quantification method was used to analyse mtDNA release. Results (Figure 10) demonstrate that utilized NOD2 agonists can induce NET formation in both the GM-CSF primed and resting neutrophils.

As a reference and positive control, LPS on GM-CSF primed and resting neutrophils was used. It showed significant formation of neutrophil extracellular traps on primed neutrophils with mean RFU around 770 ($p=0.0003$). We also used LPS on the resting murine Hoxb8 neutrophils and the result was significant ($p=0.0044$), when compared to the results of the resting cells. In one situation, we only primed the cells with GM-CSF and the obtained data show that GM-CSF priming alone does not significantly stimulate the neutrophils to form NETs ($p=0.7007$), with the mean RFU being around 200.

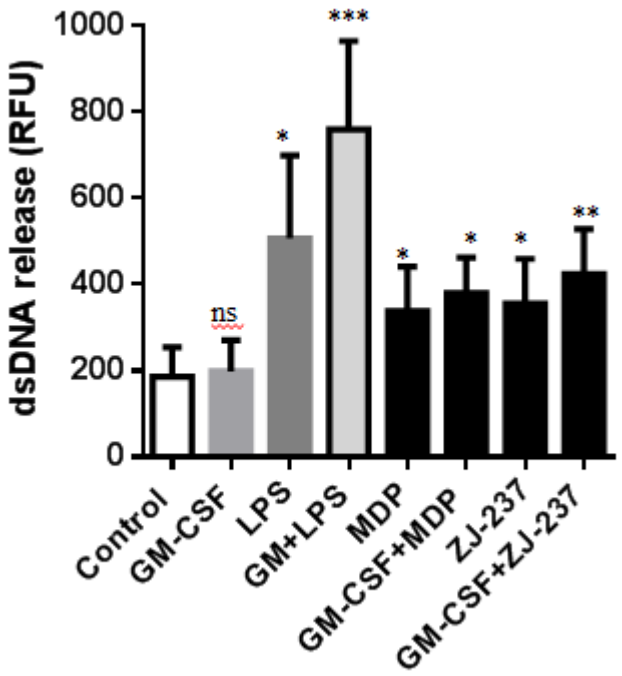

Figure 10: ***dsDNA release in murine Hoxb8 neutrophils,*** *treated with LPS (0.33 µg/mL) and NOD2 agonists. For positive control, cells (2×10^6) were primed with GM-CSF and stimulated with LPS. Neutrophils have been stimulated with compounds of interest (10 µM), either with or without priming with GM-CSF (25 ng/mL). Data are shown as mean ± SEM of the indicated number of four (n=4) independent experiments. *p<0.05; **p<0.01; ***p<0.001; t-test.*

MDP is a NOD2-specific native agonist, which was used as a reference to the synthesized NOD2 agonist ZJ-237 and showed significant effect on the GM-CSF primed neutrophils (p=0.0112) with a RFU of around 380, and on the resting neutrophils (p=0.0483) with the mean RFU at 339. The compound ZJ-237 is a NOD2-specific agonist and it showed a significant effect on the GM-CSF primed neutrophils with a RFU signal of 423 (p=0.0091). When stimulating the resting neutrophils with ZJ-237, the mean RFU was around 355, indicating significantly increased NET formations (p=0.035). When making a comparison, the results of both NOD2 agonists indicate a non-significant difference between the effects of the synthetic ZJ-237 and MDP, neither on the primed (p=0.5335) nor the resting neutrophils (p=0.8287). The reason for this could lie in the very similar molecular structure of both compounds.

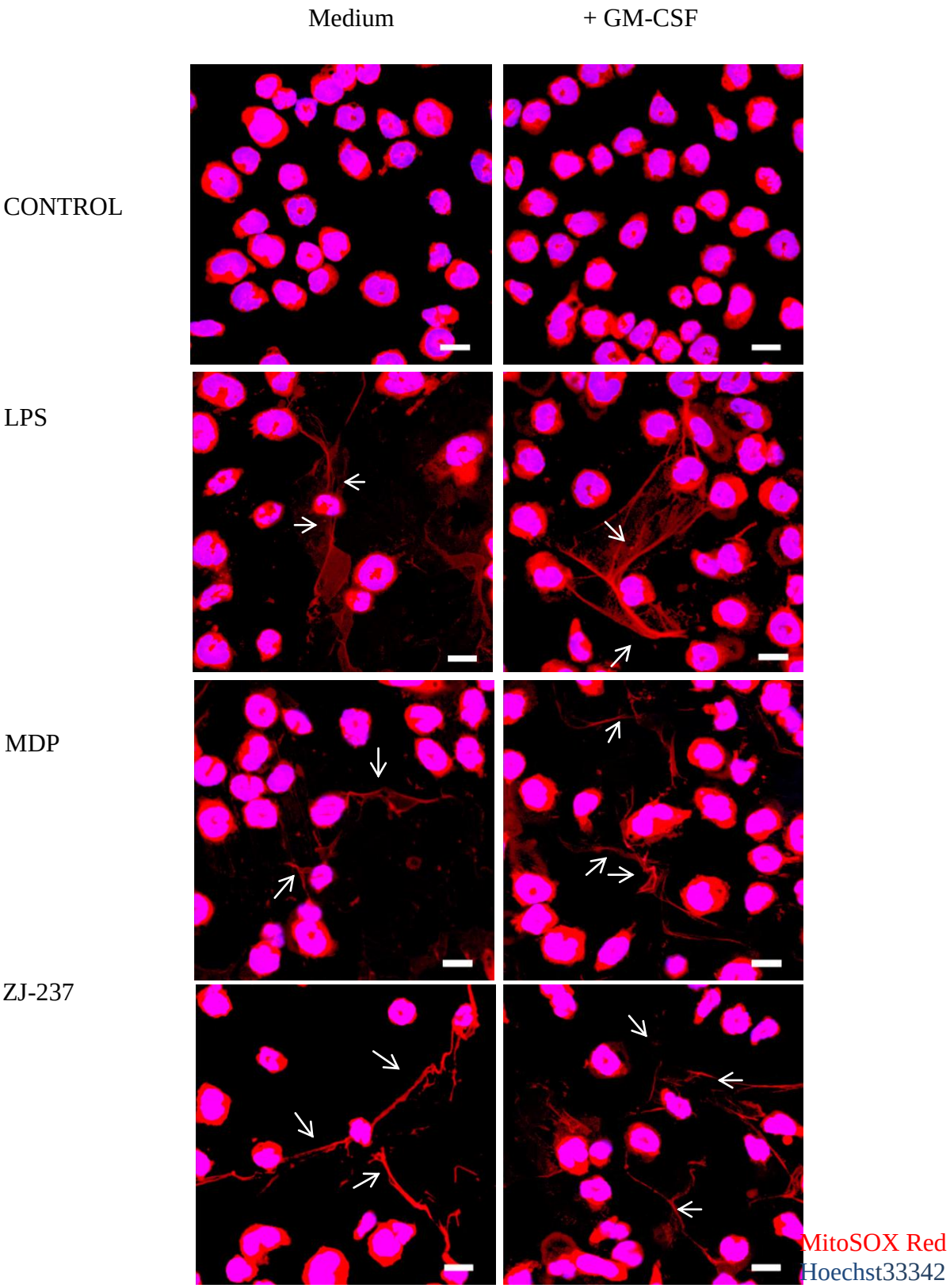

Figure 11: ***dsDNA release in murine Hoxb8 neutrophils****, treated with LPS and NOD2 agonists either on GM-CSF primed or resting neutrophils. Confocal microscopy was made with objective field 63×. Scale bars, 10 µm. Nuclear DNA has been stained with Hoechst 33342 (blue) and MitoSOX Red has been used to stain mitochondria DNA. White arrows point to the neutrophil extracellular dsDNA.*

The confocal images (Figure 11) show the representative groups of cells. These images depicting the effect of NOD2 agonists on NET formations in Figure 4 are in good agreement with the findings of the dsDNA quantification (Figure 10). As was the case with the NOD1 agonists, the most significant release of NETs was seen when stimulating GM-CSF primed neutrophils with LPS. Also, clear signs of intensified NET formations were seen following the cell stimulation either with MDP or ZJ-237. However, there was no difference in the NET formation capacity between those two compounds.

4.1.3 The effect of NOD2 agonists on human neutrophils

While the experiments on murine neutrophils were conducted on cultivated *in vitro* differentiated Hoxb8 mouse neutrophils, freshly isolated neutrophils from blood were used for the human neutrophil experiments. In these experiments, we focused on the effect of NOD2 agonists. This is due to available data showing that human neutrophils do not express NOD1 receptors (82).

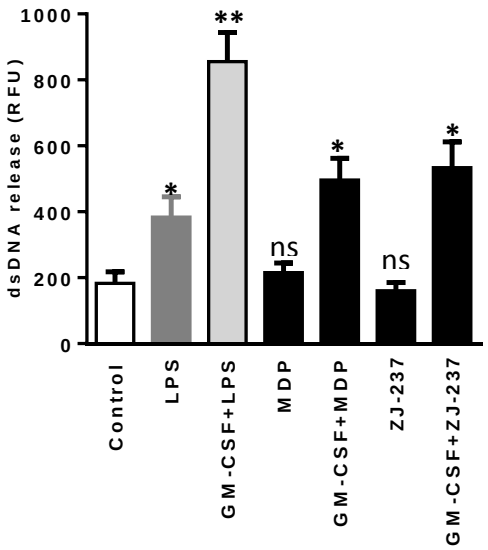

Figure 12: ***dsDNA release in human neutrophils****, treated with LPS (0.33 µg/mL) and NOD2 agonists. As in previous experiments, cells (2×10^6) were primed with GM-CSF and stimulated with LPS. Neutrophils have been stimulated with compounds of interest (10 µM), either with or without priming with GM-CSF (25 ng/mL). Data are shown as mean ± SEM of the indicated number of three (n=3) independent experiments. *p<0.05; **p<0.01; ***p<0.001; t-test.*

The analysis of the mtDNA release with dsDNA quantification method (Figure 12) demonstrated that NOD2 agonists (ZJ-237) can induce NET formation in GM-CSF primed freshly isolated human neutrophils. As in the experiments on murine Hoxb8 neutrophils, LPS was used as a positive control in GM-CSF primed neutrophils and showed the highest response in terms of NET formations, with a RFU of around 860. When stimulating resting neutrophils with LPS, the RFU was around 380.

MDP was used as a reference NOD2 agonist and showed a significant increase of NET formation (RFU=496; p=0.014) in GM-CSF primed neutrophils, while the resting neutrophils were not affected by the stimulation with MDP (RFU=215; p=0.517). Similar results were obtained for the compound ZJ-237, which also displayed the most prominent induction of NET formation on the GM-CSF primed neutrophils (RFU=534; p=0.015), while having no effect in resting neutrophils (RFU=160; p=0.635). In comparison, the results of both NOD2 agonists indicate a non-significant difference between the effects of the synthetic ZJ-237 and MDP, neither on the primed (p=0.728) nor the resting neutrophils (p=0.528). The reason for this could lie in the very similar molecular structure of both compounds.

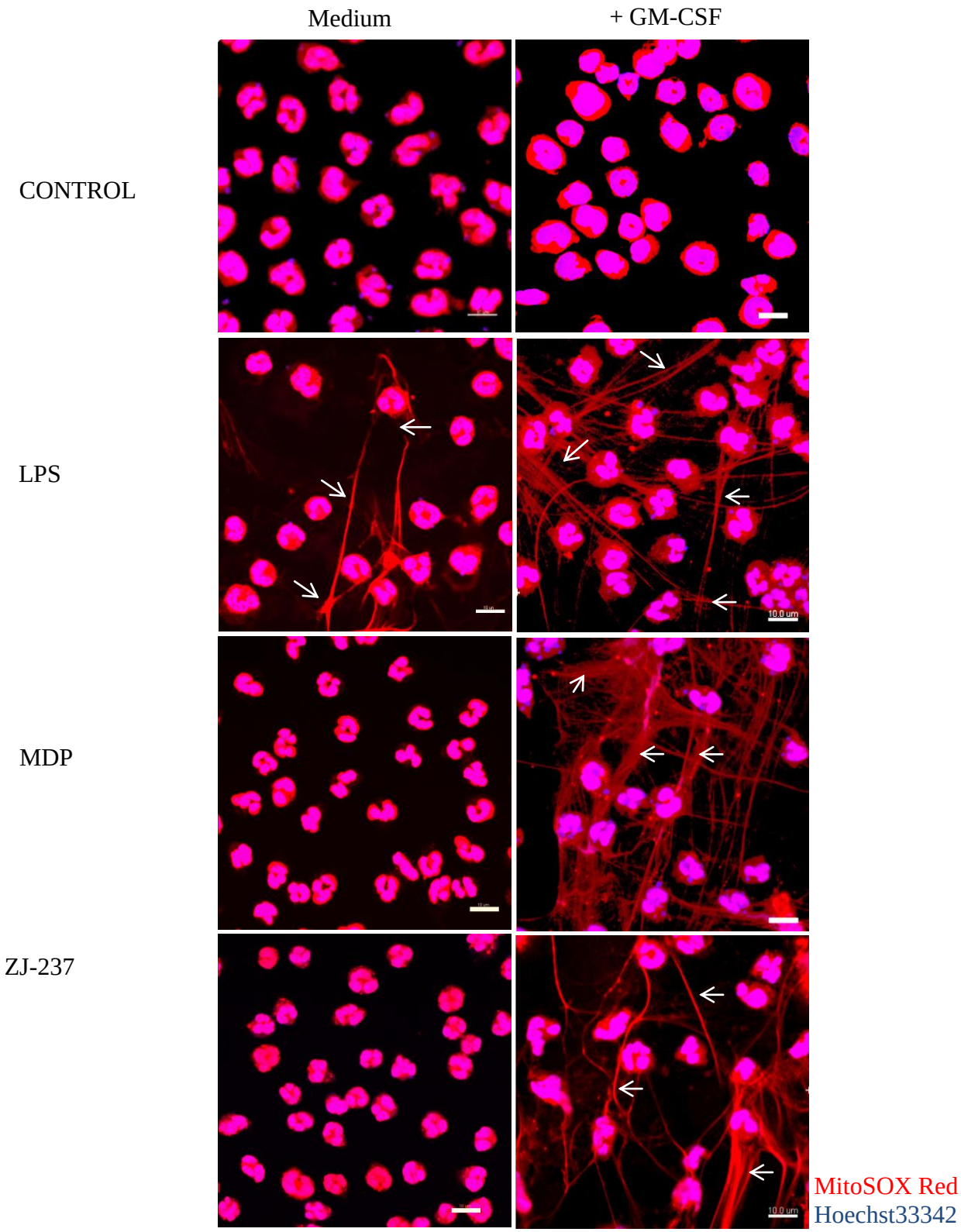

Figure 13: ***dsDNA release in human neutrophils***, *treated with LPS and NOD2 agonists. Purified human blood neutrophils were stimulated with indicated reagents and analysed by confocal microscopy with objective field 63×. Cells were either resting or primed with GM-CSF. DNA was stained with Hoechst 33342 (blue) and mitochondria were stained with MitoSOX Red. Scale bars 10 μM. White arrows point to neutrophil extracellular dsDNA.*

Confocal images in Figure 13 show representative groups of cells. These images depicting the effect of NOD2 agonists on NET formations in Figure 13 are in good agreement with the findings of dsDNA quantification (Figure 12). The most significant release of NETs was observed when stimulating GM-CSF primed neutrophils with LPS. Additionally, clear signs of intensified NET formations were seen following the cell stimulation either with MDP or ZJ-237. However, there was hardly any difference in the NET formation capacity between those two compounds. Interestingly, as opposed to the results obtained in murine neutrophils, the resting human neutrophils do not form NETs on stimulation with MDP or ZJ-237.

4.2 Viability assay

Cell death could potentially affect the results of our experiments due to the released DNA from nuclei. To ensure our chosen conditions were not cytotoxic for the cells, we performed viability assays as described in the Method section. Primed and resting cells were treated with different agonists as described in previous experiments (DNA quantification, qualification).

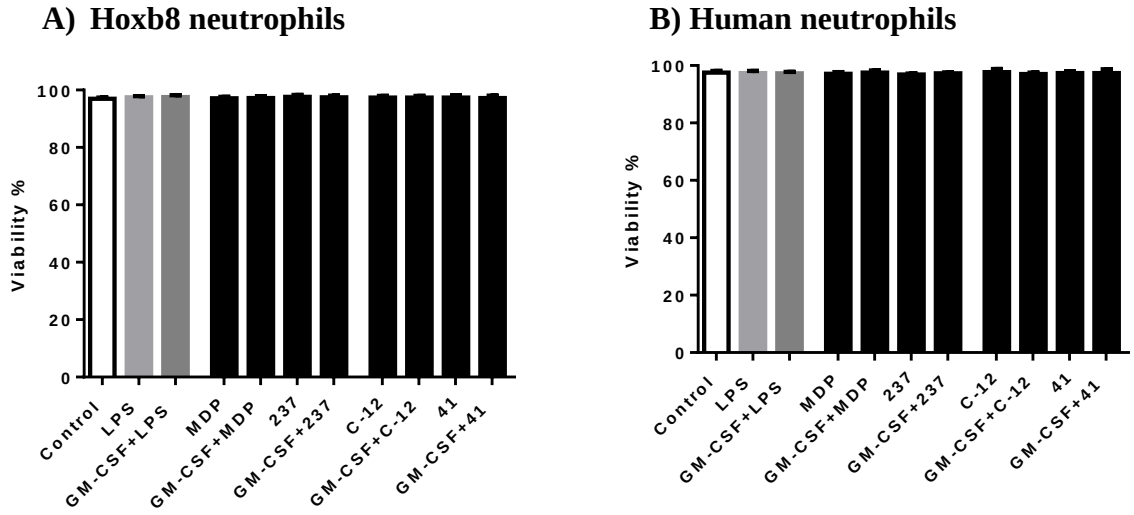

Figure 14: ***Viability of Hoxb8 (A) and human (B) neutrophils, after stimulation of NOD agonist;*** *the cells were stimulated with the indicated agonists. An uptake of ethidium bromide and a flow cytometry were used to assess cell death. Values are means ± SEM of three independent experiments (n=3).*

Resting neutrophils of either murine Hoxb8 neutrophils or freshly isolated human neutrophils were used as a negative control. As seen on the graphs in Figure 14, the compounds did not affect the viability of the cells. The cells exhibited 97% of viability in all conditions. Furthermore, the additional indicators of viable cells are clearly seen in the confocal images in Figures 2, 4 and 6, which display intact cells with accurate shapes. Those two sets of data additionally confirm that the NOD agonists are not cytotoxic to the cells at the 10 μM concentration.

4.3 Expression of NOD1 and NOD2 mRNA

The expression of NOD1 and NOD2 mRNA in murine and human neutrophils is still poorly investigated and the obtained information from previous published reports often contradicts each other findings. Here, reverse transcriptase and subsequent qPCR was employed and the product of the PCR reaction was separated by size using gel electrophoresis and observed on agarose gel to investigate the expression of *Nod1* and *Nod2* receptor mRNA in murine as well as human neutrophils.

4.3.1 Expression pattern of *Nod* receptors in cells of murine origin

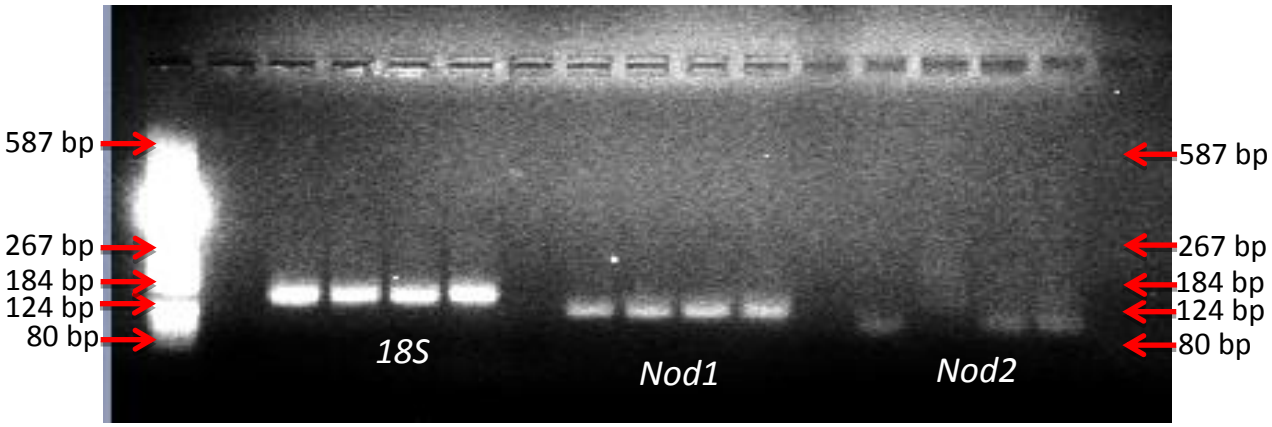

Figure 15: ***Final Nod1/2 qPCR visualization on 1% agarose gel.*** *Left to right: white blood cells (WBC), fibroblasts, undifferentiated Hoxb8 and differentiated Hoxb8 cells. Amplification from WBC served as positive control. 18S served as reference gene. Red arrows point to suitable lanes of base pairs.*

In the quantitative PCR (qPCR) analysis we were able to observe expressions of *Nod1* and *Nod2* receptor mRNA in all murine cells (Figures 8-10). The qPCR products on the 1% agarose gel (Figure 15) vary in brightness, which could be explained because of the difference in amplicon size (117 bp for *Nod1* and 86 bp for *Nod2*) since the dye SYBRGreen intercorporates itself mostly in bigger amplicons, causing them to be brighter. As positive control, we used white blood cells and we assumed fibroblasts to act as negative control. There are no previous reports shedding light on the expression of *Nods* in murine fibroblasts, however, some published papers suggest that human fibroblasts express NOD1 and NOD2 (85).

Our preliminary result suggests that murine fibroblasts either do not express *Nod2* or it is expressed in very small quantities. Since white blood cells, Hoxb8 undifferentiated and differentiated neutrophils all have the amplicon of the expected size, according to the observed data; we can only assume that they express more *Nod2* than fibroblasts.

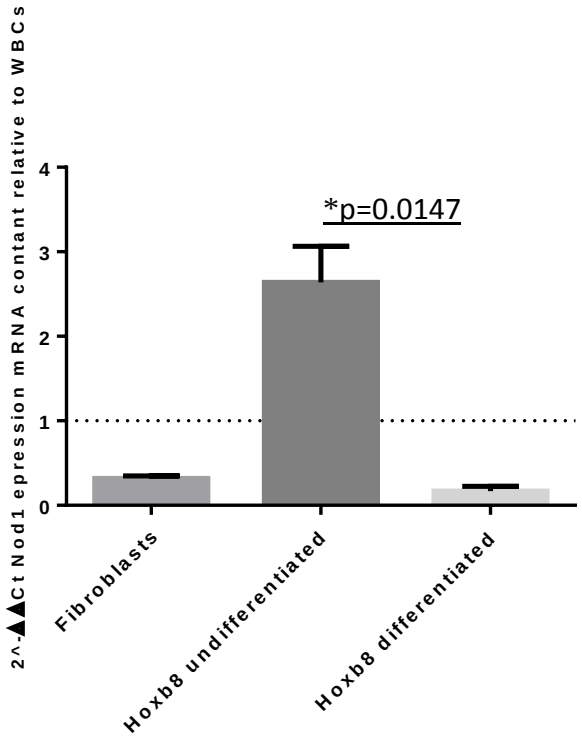

Figure 16: ***Nod1 is down-regulated in murine Hoxb8 neutrophils upon differentiation.*** *Quantification of messenger RNA by qPCR. The 18S gene was used as a reference. Data is shown as mean ± SEM of two independent experiments. The 2^-ΔΔCq method was used to calculate the mRNA content normalized to WBCs with the 2^-ΔΔCt values shown above. Data is shown as mean ± 95% CI from two independent experiments. Statistical differences were calculated using an unpaired t-test in the GraphPad v6.0 software. *p<0.05; **p<0.01; ***p<0.001; t-test.*

The chart Figure 16 represents 2^-ΔΔCt values of *Nod1* mRNA expression of fibroblasts, undifferentiated Hoxb8 and differentiated Hoxb8 cells relative to white blood cells. Positive values represent higher expression. The undifferentiated Hoxb8 cells express significantly more *Nod1* mRNA than differentiated Hoxb8 cells (p=0.0147). Similarly, the undifferentiated Hoxb8 cells also express significantly more *Nod2* mRNA than differentiated Hoxb8 cells (p=0.0222; Figure 17). These preliminary data suggest that undifferentiated Hoxb8 cells express more of both mRNAs of interest, than after differentiation.

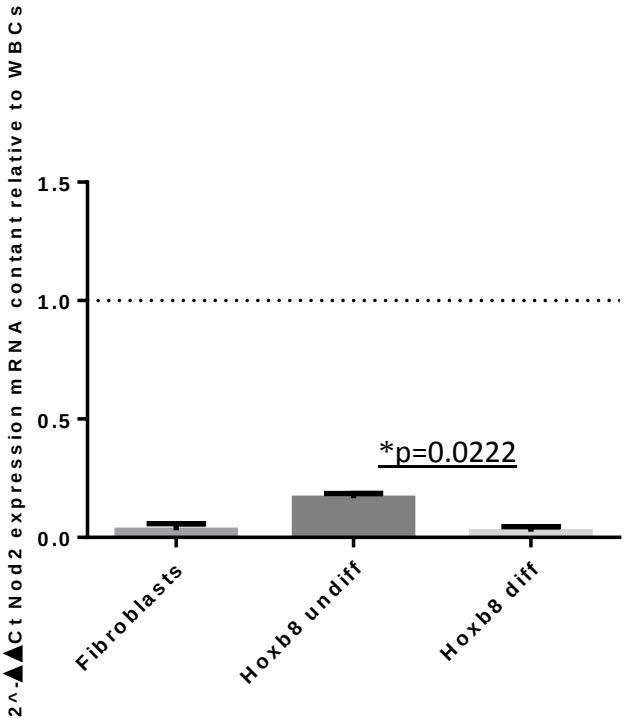

Figure 17: ***Nod2 is down-regulated in murine Hoxb8 neutrophils upon differentiation.*** *Quantification of messenger RNA by qPCR. The 18S gene was used as a reference. Data is shown as mean ± SEM of two independent experiments. The 2^-ΔΔCq method was used to calculate the mRNA content normalized to WBCs with the 2^-ΔΔCt values shown above. Data is shown as mean ± 95 % CI from two independent experiments. Statistical differences were calculated using an unpaired t-test in the GraphPad v6.0 software. *p<0.05; **p<0.01; ***p<0.001; t-test.*

4.3.2 Expression pattern of NOD receptors in cells of human origin

NOD2 messenger RNA is relatively abundant in several cell types like PBMCs, THP-1 and neutrophils; however, little is known about the *NOD1* mRNA levels in these cell types (30). Most of the data available suggest that *NOD2* transcript amount by far exceeds those of *NOD1*; therefore, we decided to investigate the expression pattern.

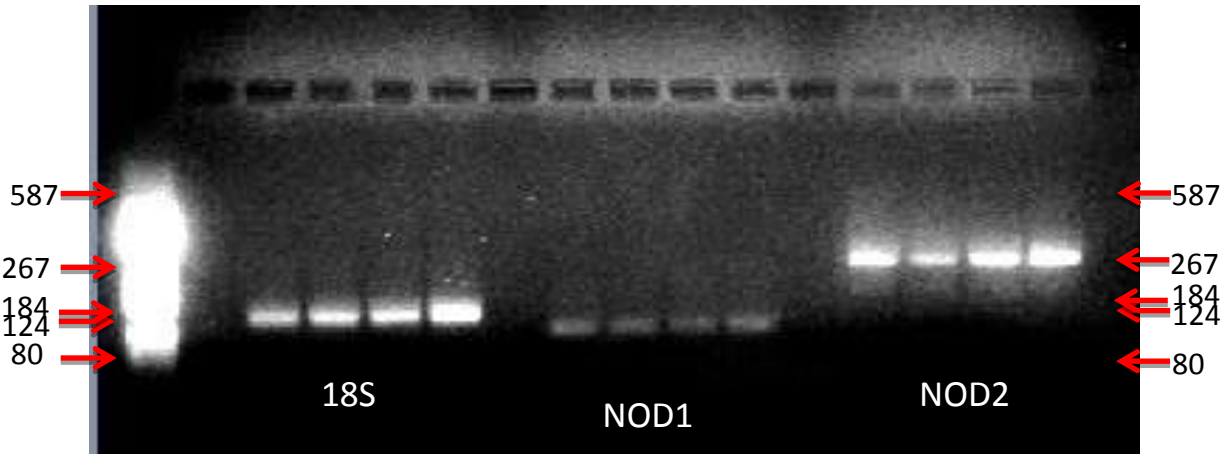

Figure 18: ***Final NOD1/2 qPCR product visualization in 1% agarose gel.*** *Left to right; PBMCs, THP-1, resting neutrophils and stimulated neutrophils. Amplification from PBMCs and THP-1 cell line served as positive controls and 18S as reference gene. Red arrows point to suitable lanes of base pairs.*

Analysis by qPCR demonstrated that both *NOD1* and *NOD2* are expressed in all of the studied cell lines (Figures 18-20). Interestingly, when the final product of the qPCR was visualized on 1% agarose gel, the signal intensities greatly vary between both NOD genes (Figure 18). This difference in intensities could be explained due to unequal amplicon size (319 for *NOD2* and 108 for *NOD1*), since larger amplicons incorporate more SYBRGreen dye, which results in more intense bands. In our setup, we used PBMCs and THP-1 as positive controls for *NOD1* and *NOD2* expression, and investigated their levels in both resting and stimulated neutrophils. To recreate the stimulation process, we added GM-CSF (25 ng/mL) to freshly isolated human neutrophils and incubated them at 37°C during 4 hours, followed by RNA isolation and qPCR analysis.

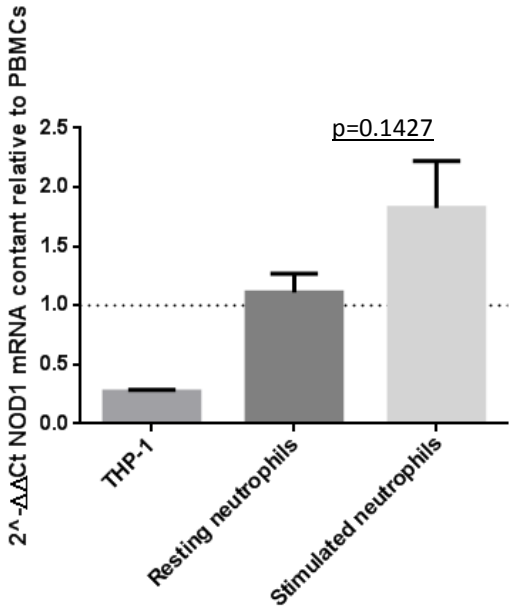

Figure 19: ***NOD1 is up regulated in neutrophils upon GM-CSF stimulation.*** *Quantification of messenger RNA by qPCR. The 18S gene was used as a reference. Data is shown as mean ± SEM of two independent experiments. The 2^-ΔΔCq method was used to calculate the mRNA content normalized to PBMCs with the 2^-ΔΔCt values shown above. Data is shown as mean ± 95%CI from two independent experiments. Statistical differences were calculated using an unpaired t-test in the GraphPad v6.0 software. *p<0.05; **p<0.01; ***p<0.001; t-test.*

The 2^–ΔΔCt values in figure 19 represent *NOD1* mRNA expression levels in THP-1 cell line, resting and stimulated human neutrophils, relative to PBMCs. Positive 2^-ΔΔCt values represent higher expression; therefore, the stimulated neutrophils exhibited a higher content of *NOD1* compared to the resting neutrophils, despite this difference not being statistically significant (p=0.1427).

Similar analysis was also performed to evaluate *NOD2* expression in resting and stimulated neutrophils (shown in figure 19). As opposed to *NOD1*, the transition from the resting to activated neutrophils was accompanied by a robust up-regulation of *NOD2* levels (p=0.0218; Figure 20).

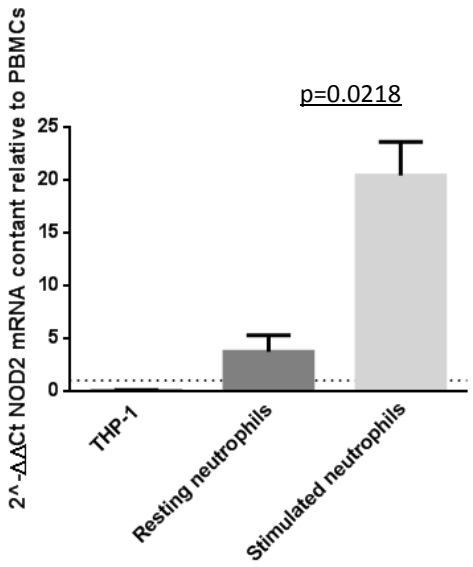

Figure 20: ***NOD2 is up-regulated in neutrophils upon GM-CSF stimulation***. *Quantification of messenger RNA by qPCR. The 18S gene was used as a reference. Data is shown as mean ± SEM of two independent experiments. The 2^-ΔΔCq method was used to calculate the mRNA content normalized to PBMCs with the 2^-ΔΔCt values shown above. Data is shown as mean ± 95%CI from two independent experiments. Statistical differences were calculated using an unpaired t-test in the GraphPad v6.0 software. *p<0.05; **p<0.01; ***p<0.001; t-test.*

5. DISCUSSION

In this study, we investigated how NOD agonists influence the formation of NETs in neutrophils. It is commonly known that activating TLR4 receptors with LPS in neutrophils leads to NET formations (1). However, there have been no reports of the effect of NOD1 or NOD2 receptors stimulation in neutrophils and NET formation. NODs and TLRs are both members of the PRR family and part of the innate immune system. Therefore, we expected that NOD agonists stimulate neutrophils to form NETs. Furthermore, we hypothesized that the NOD1 specific agonist SZZ-41 would have a similar effect on NET formation compared to that of the reference NOD1 agonist C12-iE-DAP due to their similar molecular structure. Based on this similarity in structure, we also tested NOD2 specific agonist ZJ-237 to have a similar effect on NETs as its NOD2 reference, MDP.

For our experiments, we chose to use the in vitro differentiated murine Hoxb8 neutrophil cells and freshly isolated human neutrophils, as they were previously used successfully for the NET formation. A big advantage of murine Hoxb8 neutrophils is that they can be immortalized on request and can be rapidly differentiated in a large quantity. Both cell systems proved to be sensitive and thus allowed for a good evaluation of NOD agonists on the NET formation. The used methods are therefore suitable for screening modulators of NET formations in future experiments.

Based on the quantification assays of the released dsDNA in culture supernatants and images of confocal laser scanning microscopy, we can draw a conclusion that NOD agonists are indeed able to trigger NETs (Figures 7–13). NETs were successfully formed by differentiated Hoxb8 neutrophils after incubation with both the NOD1 as well as NOD2 agonists. Similarly NET formation by human neutrophils was observed

When activated with NOD2 agonists. Interestingly, experiments on the Hoxb8 cells demonstrated that GM-CSF priming prior to cell activation is not necessary for a successful NET formation. However, this was not the case in experiments on human neutrophils, where GM-CSF priming is required. This can be explained not only by the fact that murine neutrophils differ from human neutrophils, but also because sometimes neutrophils act differently when freshly isolated from blood compared to those cultivated and kept under laboratory conditions. Another possible explanation would be that human

neutrophils express fewer NOD receptors; therefore, experiments must include the priming phase to enable formations of NETs. One possible explanation could be that the G–CSF that is added to the differentiated Hoxb8 neutrophils two days before they are used in experiments potentially stimulates the expression of receptors. To better understand the difference between the resting mature human neutrophils and resting murine Hoxb8 neutrophils, additional experiments have to be conducted and further detailed research is necessary. As expected and also in good agreement with many studies on this issue previously conducted at the Institute of Pharmacology at the University of Bern, GM–CSF alone did not alter the formation of NETs in Hoxb8 neutrophils. It is also well-known that GM-CSF alone is not sufficient for stimulating human neutrophils.

In all experiments, LPS has shown the ability to form NETs regardless of priming in both the human and murine neutrophils, which is in agreement with previous studies (8). Based on recent studies, LPS is well known to have better activation capabilities than C12-iE-DAP and MDP (86, 87, 88). The conducted dsDNA quantification assays (Figures 7–13) revealed that LPS possesses the best ability to form NETs out of all the stimulants used in our experiments.

SZZ-41 has similar NET formation capabilities as its reference C12-iE-DAP and ZJ-237 have similar NET formation capabilities as MDP. Interestingly, no significant differences were observed between the capability of NET formations between NOD1 and NOD2 activations and can be concluded that iE-DAP and MDP analogues form qualitatively equivalent interactions with the binding sites of NOD receptors. This can be explained by a very similar structure of NOD1 and NOD2 receptors and the fact that they have the same activation pathways when activated.

To exclude the possible cytotoxic effect on the results of the NET formations, viability assays (Figure 14) were performed. They were carried out after stimulating the cells with agonists. Ethidium bromide, which has the capability of intercalating into DNA and emitting light, but cannot penetrate the cell membrane, was used to quantify number of dead cells using flow cytometry. The result indicates that neutrophils do not die when exposed to 10 μM concentrations of the NOD1 or NOD2 agonists. This data is in agreement with other studies, where the same or even higher concentrations of these agonists were used in absence of any cytotoxicity. Additionally, it was also clearly seen in

images that stimulated neutrophils that formed NETs were round in shape and their nucleus was intact. Furthermore, it can be seen that NETs are mainly stained by mtDNA dye MitoSOX Red and not Hoechst 33342, which is a weaker dye for the released DNA (Figure 7). The fact that NOD agonists trigger NETs independent of apoptosis is very important and could potentially mean that the NET release mechanism behaves in a catapult-like manner, as reported in Yousefi et al., where they stimulated IL 5 primed eosinophils with C5a or LPS and were able to trigger extracellular traps, independent of cell death (55).

The expression of NOD receptors differs between different cells. The available data is often contradictory. To lay the groundwork for this study, we also investigated the Nod expression pattern in murine Hoxb8 neutrophils and mature human neutrophils using qPCR. Based on our preliminary data, we observed *Nod1* and *Nod2* mRNA expression in murine Hoxb8 neutrophils (Figures 15–20). The data correlates with the results of the NET formations, as we successfully stimulated differentiated murine Hoxb8 neutrophils with both the NOD1 and NOD2 agonists. Interestingly, we observed greater *Nod1* and *Nod2* mRNA expression in undifferentiated than differentiated urine Hoxb8 neutrophils. This could be explained, because differentiated neutrophils express in general lower protein levels compared to the progenitors, and the reason could be that they start shutting down and dying (92).

Under ideal conditions, the sizes of the amplicons must be similar for achieving the best possible comparison between different messenger RNA expressions. Because specific primers are very difficult to find, we had to break the rules about amplicon size (*Nod1* amplicon = 108 bp, *Nod2* amplicon = 319 bp). Unfortunately, different amplicon sizes prevent us from successfully qualitatively comparing *Nod1* with *Nod2* expressions in the chosen cells. We cannot assume the dimensions of the receptor expression based on our data, as some mRNA may be vastly degraded by the degradation mechanisms (regulators), which are present in cells. Such an example are BOK cells, which tend to express a lot of mRNA, but have a low receptor expression due to their degradation mechanisms (89). Another reason for high mRNA expression could lie in the vast possibilities of receptor polymorphisms. In this case, the cells have to express more mRNA to reach the desired concentrations of a sufficient receptor. The results could also have been affected by double melting peaks (data in appendix – Figure 21) of the primers in fibroblast and differentiated

Hoxb8 neutrophils. In order to ensure preliminary results, some experiments might be repeated with different murine *Nod2* primer.

In our experiments with mRNA NOD expressions in human neutrophils, we determined that human neutrophils express NOD2 mRNA in far higher concentrations than NOD1 mRNA, which can also be seen in other studies (82). Interestingly, we observed significantly higher NOD mRNA expression when the neutrophils were stimulated with GM-CSF. This could also explain why NOD2 agonists successfully stimulated GM-CSF primed human neutrophils to form NETs but could not stimulate the resting neutrophils.

6. CONCLUSION

Neutrophils play a crucial role as part of the non-specific innate immune response against pathogens. One of their mechanisms to kill pathogens is formation of NETs.

- We determined that NOD1 and NOD2 agonists can stimulate mouse Hoxb8 to form NETs and that NOD2 agonists can stimulate human neutrophils to form NETs. In our experiments, iE-DAP and MDP analogues SZZ-41 and ZJ-237 possessed similar NET-inducing capacity as the reference compounds C12-iE-DAP and MDP with the binding sites of NOD receptors The capacities of NOD1 and NOD2 agonists to trigger NETs are, however, lower than those of LPS.

- The studied compounds were not cytotoxic to the cells at concentrations of 10 μM.

- Our cell-based assays with primary human and mouse Hoxb8 neutrophils proved to be suitable to screen for modulators of NET formations and can therefore be used in future experiments.

- In experiments with mRNA NOD expressions in human neutrophils, we determined that human neutrophils express NOD2 mRNA in higher concentrations than NOD1 mRNA. This expression is also in good agreement with our studies, where we stimulated the neutrophils with NOD2 agonists to form NETs.

- Based on the established capabilities of NOD agonists to form NETs, it would also be interesting to evaluate in future studies their capacity to induce bacteria – killing and degranulation in neutrophils.

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8. APPENDIX

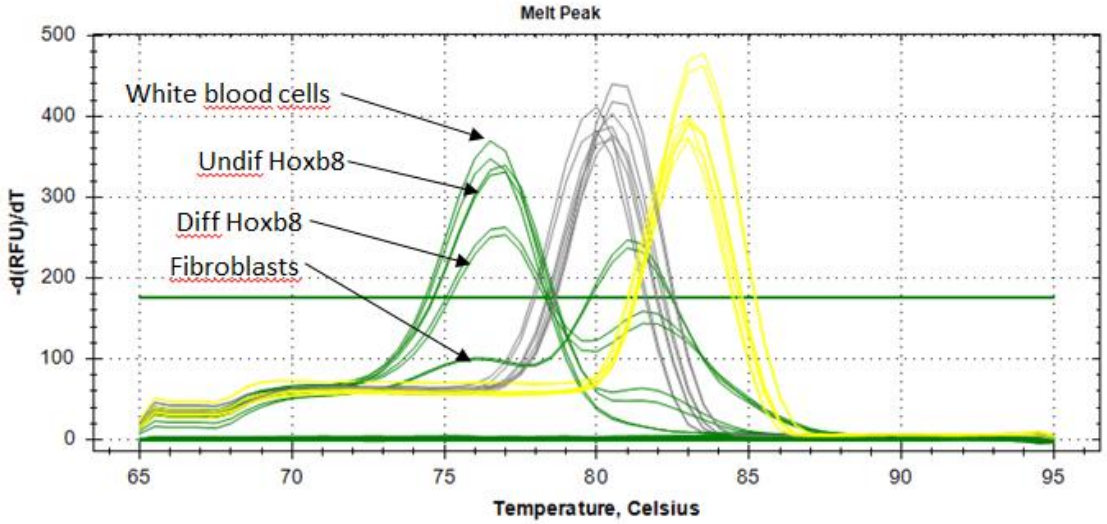

Figure 21: Melting peaks of the primers in mouse Hoxb8 neutrophils. Grey curves represent melting peaks of *Nod1* primers, green curves those of *Nod2* primers and yellow curves those of 18S primers. The arrows are labelling the corresponding graphs to the *Nod2* primer pair in the suitable cells.

During our investigation, we observed the melting peaks of expected primers (at roughly 76.5°C) and melting peak of unexpected product (at roughly 81.5°C). Figure 14 depicts that in fibroblasts primers tend to amplify unexpected amplicon in greater quantities.

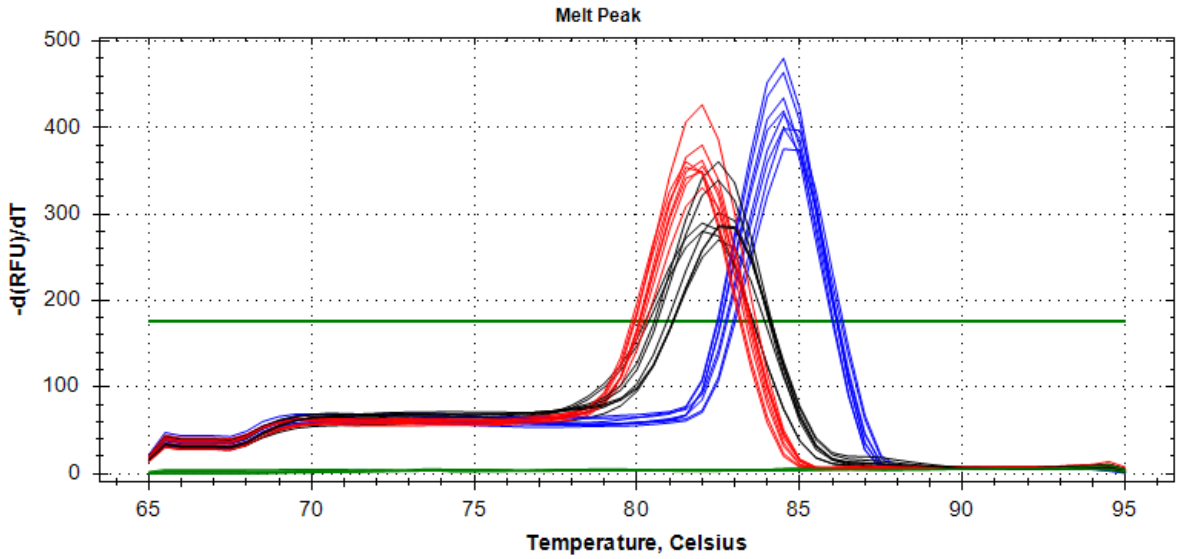

Figure 22: Melting peaks of the primers in qPCR experiments with human cell lines. Black curves represent melting peaks of NOD1 primers, blue curves those of NOD2 primers and red curves those of 18S primers. The arrows are labelling the corresponding graphs to the NOD2 primer pair in the suitable cells.