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Analysis of Toll-like receptors and retinoic-acid-inducible gene-I (RIG-I)-like helicases in human neutrophils and HL60 cells

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This thesis is dedicated to my parents. They have encouraged me throughout my young medical career in the most wonderful and loving way.

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1. INTRODUCTION

1.1. Introduction to the Immune System

1.1.1. Innate Immunity

A functioning immune system is an essential characteristic for the integrity of any living organism. In the case of the human body, the immune system is the collection and meaningful interaction of tissues, cells and molecules that participate in the various defense mechanisms. It can be divided in 2 parts, the innate immunity and the adaptive immunity. The innate immunity, like the word ”innate” means, is a natural defense against microbial infections, which is always present, ready to recognize and eliminate microbes. The adaptive immunity, unlike the innate immunity, learns to recognize microbes as pathogens only after having had contact with them. Epithelial barriers, phagocytes (neutrophils, monocytes, and macrophages), natural killer cells, the complement system, and some cytokines and plasma proteins build up the innate immunity. One shared characteristic of innate immunity is that they can create an immune response to microbes and then give an adequate protection until the adaptive immunity develops its full potential.

It was believed for many years that innate immunity is rather nonspecific, weak, uncomplicated, and is not very effective against most infections. Intense research in the field of innate immunity over the last years has shown that innate immunity, like the adaptive immunity, shows specificity

toward microbes and is a powerful early defense mechanism which can keep the infections under control or even eliminate them before activation of adaptive immunity occurs. Moreover, it is now understood that interactions in innate immunity areas complicated as the adaptive immune response, or even more complicated.

As a part of innate immunity, the neutrophils circulate in the human blood along with monocytes and other immune cells and are recruited to sites of infection. There they recognize microbes and eliminate them by phagocytosis and other mechanisms. Neutrophils, also called polymorphonuclear leukocytes or PMNs, are abundant leukocytes in the blood with the amount of 4,000 to 10,000 per μL blood in a healthy adult male. During an immune response, cytokines are produced to stimulate proliferation and maturation of neutrophils. Neutrophils are the first cell type to be activated in response to most infections, especially infections caused by a bacterial or fungal origin. After having been recruited to extravascular sites of infection and inflammation, neutrophil activation leads to two important steps in the creation of a meaningful immune response. On the one hand, phagocytosis leads to immediate control of inflammation; on the other hand, secretion of cytokines leads to activation of the adaptive immune response and therefore has important regulatory functions.

1.1.2. Communication between Innate and Adaptive Immunity

As stated, not only is there early defense by innate immunity, but also is there a close communication between innate and adaptive immunity. Regulatory cytokines are one major tool of such communication. Innate immunity enhances the effectiveness of adaptive immunity in response to microbes while combating with them. Also, the adaptive immune response often uses similar mechanisms in order to eliminate microbes. The most basic mechanism to do so is through the major histocompatibility complex (MHC) molecules, also referred to as human leukocyte antigen (HLA) molecules in humans. These molecules have a vital role in the complex immunological dialogue that occurs between T cells and other cells of the body. MHC molecules, which are anchored in the cell membrane, constantly display short polypeptides to T cells. Via the T cell receptors (TCR) T-cells can recognize these short peptides, and depending on the presents or abstinence of co-stimulatory signals the T cells differentiate between "self"- and "non-self"- protein. The MHC-TCR interaction enables that T cells should ignore self-peptides while reacting appropriately to the foreign peptides, which are then considered antigens. If recognized as an antigen by CD4+ T cells, also called T_H cells, these cells will activate the B cells and macrophages, and if recognized by CD8+ T cells, direct killing will follow from activation of CD8+ T cells.

The subtype of T_H cells, T_H1 cell, helps macrophages to enhance the microbial killing, and another subtype, T_H2 cells, helps eosinophils to

combat with helminthes, which are generally too large to be engulfed by macrophages. Furthermore, both subtypes of T_H cells induce the B cells to release antibodies, which help macrophages to recognize microbes. Conversely, the antigen-presenting cells (APC) of innate immunity play an essential role in proliferation and maturation for T cells and B cells.

A full-force immune response usually requires the activation of B cells via the B cell receptor (BCR) and T cells via the MHC-TCR interaction. This duality creates a system of "checks and balances". Without some limitation to the activation cascade, the immune system has the potential to run amok and to cause harm to the body, such as a state generally known as autoimmune disease. The balance of immune system can be achieved by so called regulatory T cells, which, if activated appropriately, suppress the immune response to a meaningful extent(Schwartz 2005).

1.1.3. Pattern Recognition Receptors

Microbial molecules recognized by innate immunity share highly conserved molecular structures, called molecular patterns. These patterns are essential for the microbes' integrity and are therefore expressed despite millions of years of evolutionary pressure. The receptors of innate immunity which are able to recognize these molecular patterns are called pattern recognition receptors, or PRRs.

Molecular patterns are found in bacteria, fungi, and viruses, but essentially not in mammalian cells. For instance, phagocytes express receptors for bacterial liposaccharide, which is present in many bacterial species, but is not produced by mammalian cells. Other receptors of phagocytes recognize terminal mannose residues on glycoproteins; many bacterial glycoproteins have terminal mannose, but mammalian glycoproteins end with sialic acid or *N*-acetylgalactosamine. Phagocytes recognize and respond to double- stranded RNA (dsRNA), which is found in many viruses but not in mammalian cells. A common structure found in bacterial DNA is unmethylated CpG motives. These are generally not found in mammalian cells. Therefore, such molecular patterns are also called pathogen-associated molecular patterns, or PAMPs.

The PRRs consist of several main families depending on their structure. These include the large families of membrane-bound Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs) as well as endocytic PRRs which promote the attachment, engulfment and destruction of

microorganisms by phagocytes, without relaying an intracellular signal. Some PRRs recognize carbohydrates, such as mannose receptors of macrophages, glucan receptors on all phagocytes; other PRRs, like scavenger receptors which recognize charged ligands, are found on all phagocytes and mediate removal of apoptotic cells(Platt, da Silva et al. 1999).

PRRs can be separated by localization in three different types: membrane-bound, cytoplasmic, and secreted. The mannose receptor, one of the membrane-bound PRRs, is a PRR primarily present on the surface of macrophages and dendritic cells. The mannose receptor belongs to the multi-lectin receptor protein group and, provides a link between innate and adaptive immunity(Apostolopoulos and McKenzie 2001). It recognizes and binds to repeated mannose units on the surfaces of infectious agents and its activation triggers endocytosis and phagocytosis of the microbes via the complement system. The TLRs, like the mannose receptor, are also membrane-bound and mediate the recognition of extracellular or endosomal PAMPs (Beutler, Jiang et al. 2006). TLRs were first discovered in the Drosophila fruit fly and are known to trigger a series of mechanisms leading to the synthesis and secretion of cytokines and activation of other host defense programs that are crucial to the development of innate or adaptive immune responses. At present, TLRs have been found in many species. In mammals, these receptors have been assigned numbers 1 to 11 (TLR1-TLR11). Interaction of TLRs with their specific PAMP induces NFκB signaling and

mitogen-activated protein kinase (MAPK)pathway in most cases and therefore the secretion of pro-inflammatory cytokines and co-stimulatory molecules. Molecules that are released following TLR activation give signals to other cells of the immune system. Therefore, TLRs are a key element of both innate immunity and adaptive immunity (Doyle and O'Neill 2006). Table 1 shows an overview of the different TLRs and their ligands.

Together with the NOD-like receptors, which are cytoplasmic proteins that may have a variety of functions in the regulation of inflammatory and apoptotic responses and have been found in the mammalian genome, some RNA helicases (RHs) mediate intracellular recognition of viral double-stranded and single-stranded RNA and in turn recruit factors via twin N-terminal caspase recruitment domains, or CARD domains, to activate antiviral gene programs. Three such helicases have been described in mammals: retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene-5 (MDA5) (recognizing 5’-triphosphate RNA (3pRNA) and double-stranded RNA (dsRNA), respectively), which activate antiviral signaling, and LGP2, which appears to act as a dominant-negative inhibitor(Komuro and Horvath 2006). In addition to NLRs and RHs, absent in melanoma-2 (AIM2) is estimated to be a 39 kDa protein encoded by the AIM2 gene and is a newly identified gene that is not expressed in a human melanoma cell line and is localized primarily in the cytoplasm (Choubey, Walter et al. 2000). Cytoplasmic double-stranded DNA (dsDNA) triggers cell death

and secretion of the pro-inflammatory cytokine IL-1β in macrophages. Recent reports now describe the mechanism underlying this observation(Fernandes-Alnemri, Yu et al. 2009). Upon sensing of DNA, the HIN-200(hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats) family member,AIM2, triggers the assembly of the inflammasome, culminating in caspase-1 activation, IL-1β maturation and pyroptotic cell death (Schroder, Muruve et al. 2009).

A number of PRRs do not remain associated with the cell that produces them. Complement receptors, collectins, pentraxin proteins such as serum amyloid and C-reactive protein, lipid transferases, peptidoglycan recognition proteins (PGRs) and the leucine-rich repeat (LRR), Xa21D(Wang, Ruan et al. 1998) are all secreted proteins. One very important collectin is mannan-binding lectin (MBL), a major PRR of the innate immune system that binds to a wide range of bacteria, viruses, fungi and protozoa. MBL predominantly recognizes certain sugar groups on the surface of microorganisms but also binds phospholipids, nucleic acids and non-glycosylated proteins(Dommett, Klein et al. 2006).

TLR	PAMP	Origin	Reference
TLR1	Cooperate with TLR2		
TLR2	Lipoproteins	gram-positive bacteria	(Takeuchi, Hoshino et al. 1999)
	Zymosan	yeast	(Ozinsky, Smith et al. 2000; Ozinsky, Underhill et al. 2000)
	Macrophage-activating lipopeptide 2 (MALP-2)	mycoplasma	(Takeuchi, Hoshino et al. 2000)
	Lipoarabinomannan (LAM)	mycobacteria	(Underhill, Ozinsky et al. 1999)
TLR3	dsRNA	virus	(Alexopoulou, Holt et al. 2001)
TLR4	LPS	gram-negative bacteria	(Poltorak, Smirnova et al. 1998)
	Protein F	RSV	(Kurt-Jones, Popova et al. 2000)
	Hsp 60	host	(Ohashi, Burkart et al. 2000)
	Hyaluronan	host	(Termeer, Benedix et al. 2002)
	Fibronectin	host	(Okamura, Watari et al. 2001)
TLR5	Flagellin	different kinds of gram-negative bacteria	(Hayashi, Smith et al. 2001)
TLR6	Cooperate with TLR2		(Takeuchi, Kawai et al. 1999)
TLR7	ssRNA, dsRNA, and guanosine analog	virus	(Hemmi, Kaisho et al. 2002)
TLR8	ssRNA, dsRNA, and guanosine analog	virus	(Jurk, Heil et al. 2002)
TLR9	CpG-motive	Bacteria,virus	(Hemmi, Takeuchi et al. 2000)
TLR10	unknown	unknown	
TLR11	Profilin and profilin-like protein	Toxoplasma gondii	(Yarovinsky, Zhang et al. 2005)

Table 1 Human TLRs and their ligands

1.1.4. Immunostimulation via RNA and DNA molecules

Immunostimulatory RNAs (isRNAs) are known to generate a potent immune response and are currently under intense investigation for new antiviral and anticancer treatment strategies. They were originally identified due to their immunostimulatory sequences as ligands for TLR7 and TLR8. It has recently become apparent that in addition to stimulation of TLRs, certain chemical modifications on the 5'-ends of small interfering RNA (siRNA) can induce similar effects as isRNAs via receptors in the cytosol, such as RIG-I. RIG-I (also known as DDX58) was identified as a candidate for cytoplasmic viral detection. RIG-I is compromised of two N terminal caspase-recruitment domains (CARDs), followed by a DExD/H box RNA helicase domain. Together with MDA5 (also known as helicard or IFIH1) and LGP2, RIG-I forms the RLH family based on their high similarities among their helicase domains. RLHs interact with dsRNAs through their helicase domain, and dsRNA stimulation induces their ATP catalytic activity. The N terminal CARDs are responsible for activating downstream signaling pathways that mediate dsRNA-induced type I interferon (IFN) production(Hornung, Ellegast et al. 2006). 3pRNA is one ligand for RIG-I(Hornung, Ellegast et al. 2006; Pichlmair, Schulz et al. 2006). RNAs from some viruses are 5' triphosporylated and uncapped and are therefore recognized by RIG-I. MDA5, in contrast, is thought to response to uncapped, 5' unmodified dsRNA such as polyinosinic:polycytidylic acid (poly(I:C))(Kato, Takeuchi et al. 2006).Through this mechanism, both RIG-I and MDA5 were

observed to induce the production of interferon (IFN-β1) (Figure 1, (Baccala, Hoebe et al. 2007)).

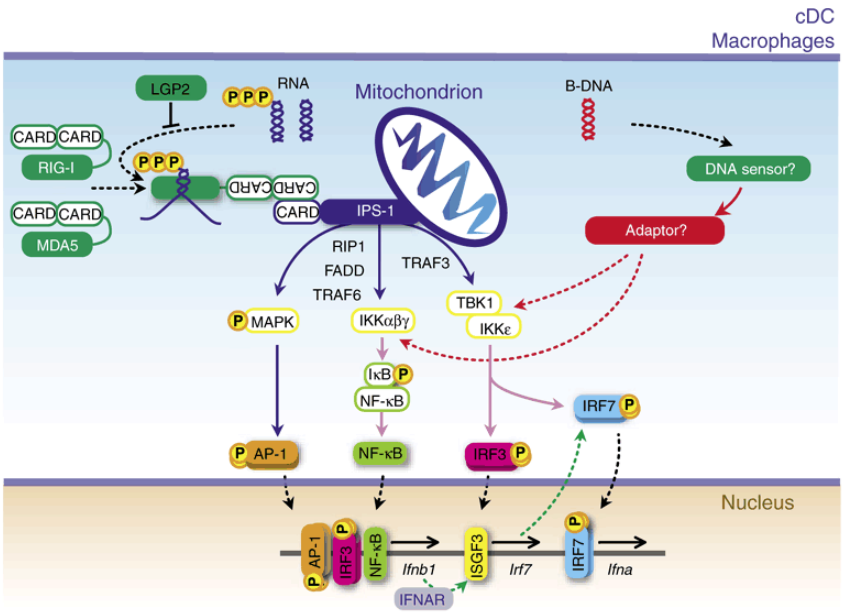


Figure 1. RIG-I and MDA5 induce the production of IFN-β1

Engagement of the cytosolic RNA helicases RIG-I (for example, by 3pRNA) and MDA5 (for example, by poly(I:C)) induces conformational changes that allow CARD-mediated homotypic interaction between RIG-I/MDA5 and the mitochondrial adaptor IPS-1. The ensuing TBK1/IKKε, IKKαβγ and MAPK signaling induces IRF3, AP-1 and NF-κB translocation, and Ifnb1 transcription.(Baccala, Hoebe et al. 2007)(TANK-binding kinase 1(TBK1), IkappaB-related kinase (IKK))

Recent research showed that AIM2 is a sensor for cytoplasmic DNA in macrophages that trigger antiviral and/or inflammatory responses, which forms an inflammasome with the ligand and apoptosis-associated speck-like protein (ASC) to activate caspase-1.(Hornung, Ablasser et al. 2009)The HIN200 domain of AIM2 binds to DNA, whereas the pyrin domain (but not that of the other PYHIN family members) associates with

the adaptor molecule ASC (containing a caspase activation and recruitment domain) to activate both NF-κB and caspase-1. Knockdown of AIM2 abrogates caspase-1 activation in response to cytoplasmic dsDNA and the dsDNA vaccinia virus.

1.2. Objectives

There is reasonable hope that siRNAs with immunostimulatory potential will lead to jet more advanced antiviral and anticancer therapy. One interesting approach is to stimulate RIG-I with modified siRNA in order to cause simultaneous specific gene knock-down via RNA interference (RNAi) and immunostimulation. E. g., Poeck et al. showed simultaneous knockdown of Bcl 2 and IFN induction to be superior to either therapy alone in an in vivo melanoma mouse model (Poeck, Besch et al. 2008). However, little is known about possible side effects of such therapies. For example, stimulation of off target cells such as human neutrophils may limit the therapeutic potential of isRNAs. Human neutrophils are a substantial part of the innate immune system and recognize pathogen through PRRs. They are usually the first cell population to be involved in an immune response and modulate subsequent immune response not only due to their potential to directly eliminate pathogens by phagocytosis but also by induction of regulatory cytokines such as interleukin 8 (IL-8). It is known that human neutrophils express TLR8 but not TLR3 and TLR7. In a recent publication, Ekman and Cardell showed that neutrophils express functional active NLRs(Ekman and Cardell

2010). NLRs are PRRs and have been implicated in several immunological diseases such as Crohn's disease and asthma, but the knowledge of their role in neutrophils is still limited. It is entirely unknown if human neutrophils express functional active RIG-I or MDA5 and if this results in a limitation of 3pRNA-based therapeutic approaches. The aim of this study was therefore to analyze the expression of RIG-I and MDA5 in human neutrophils and human promyelocytic leukemia cells (HL60cells), a tumor cell line with similar characteristics as neutrophils and to compare this to the expression of TLRs. Further we want to investigate the potential of human neutrophils to be activated by 3pRNA. Since AIM2can trigger antiviral and inflammatory responses by binding to cytoplasmic DNA in macrophages similar to PRRs, another objective was to analyze the expression of AIM2 in neutrophils and whether they can be activated by dsDNA mimicking cytoplasmic DNA.

2. MATERIALS AND METHODS

2.1. Material

2.1.1. Isolated human neutrophils

Due to the short ex vivo lifetime of human neutrophils, the neutrophils were freshly isolated prior to each experiment. Neutrophils from the peripheral blood of healthy volunteers were chosen and kept under 4°C in phosphate buffered saline (PBS) or Roswell Park Memorial Institute(RPMI) solution for further use to slow down the apoptotic process of isolated neutrophils. No additional cytokines were given to the culture medium.

2.1.2. Cell lines

The HL-60 (also called neutrophil-like cell line) cell line is a leukemic cell line, which was originally derived from a 36-year old woman with promyelocytic leukemia. HL-60 proliferates continuously in culture medium supplemented with fetal bovine serum (FBS), L-glutamine, HEPES and antibiotic chemicals. The doubling time is about 36–48 hours.HL-60 cells are predominantly neutrophilic promyelocytes (Gallagher, Collins et al. 1979).

When treated with dimethyl sulfoxide (DMSO) or retinoic acid, these cells differentiate to a cell type with strong similarities to mature human granulocytes(Jacob, Leport et al. 2002). In our study, we exclusively used DMSO for differentiation of HL60 cells. These differentiated,

mature HL60 cells resemble human neutrophils and have been a resource for continuous granulocyte research.

As stated above, human neutrophils go into apoptosis quickly after isolation and therefore cultured HL-60 cell line provides a continuous source of granulocyte-like cells, which serve excellently for studying the physiologic and immunologic characters of human neutrophils.

2.1.3. Chemicals

General use for isolation of human neutrophils:

Name	Detail
Ficoll-Paque® Plus	by Amersham Biosciences
Dulbecco's PBS (1x)	without Ca & Mg, by PAA
Erylysis Buffer	ice-cold, with 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4

Chemicals for quantitative Real-time PCR:

Name	Detail
TRI Reagent®	ice-cold, by Ambion
Ampuwa	RNase-free, by Abnova
UltraPure	RNase-free and DNase-free, by Invitrogen
Ethanol	70% and 100% bySigma Aldrich
Isoprorpanol	by Sigma-Aldrich
Chloroform	by Sigma-Aldrich
β-Mercaptoethanol	by Sigma-Aldrich
RNeasy Mini Kit	with RLT-Buffer, RPE-Buffer, RNase-free Water, RW1-Buffer, by QIAGEN
RNase-Free DNase Set	with 1500 units RNase-free DNase I, RNase-free Buffer RDD, and RNase-free water by QIAGEN

Random Hexamer	by Abgene
5x1st strand buffer	by Invitrogen
0,1µM DTT	by Invitrogen
10mM dNTPs	by Invitrogen
RNase H	by Invitrogen
SuperScript II	by Invitrogen
SYBR ® GREEN	by Abgene, Thermo Scientific

Chemicals for Confocal microscopy and 3pRNA labeling:

Name	**Detail**
NHS-Fluorescein	5-(and 6-)carboxyfluorescein, succinimidyl ester by Thermo Scientific
DMSO	Dimethyl sulfoxide by Sigma-Aldrich
Borate buffer	BupH™ Borate Buffer Packs, 50mM, pH 8.5 by Thermo Scientific
Dye Removal Column	Fluorescent Dye Removal Columns by Thermo Scientific
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride by Invitrogen
NGS	Normal goat serum by SouthernBiotech

Miscellaneous:

Name	**Detail**
FACS Flow	BD FACSFlow™ Sheath Fluid from BD Biosciences
FACS Rinse	BD FACS Rinse Solution from BD Biosciences
FACS Clean	BD FACS Clean Solution from BD Biosciences
MACS Running Buffer	autoMACS™ Running Buffer.by Miltenyi Biotec

2.1.4. Antibodies

Name	Specificity	Clone	Host	Target	Conjugation	Manufacturer
Anti-AIM2	AIM2	polyclonal	rabbit	human	none	Abcam
Anti-CD11b	CD11b	ICRF44	mouse	human	APC	BioLegend
Anti-MDA5	MDA5	polyclonal	rabbit	human	none	Abcam
Anti-RIG-I	RIG-I	polyclonal	rabbit	human	none	SantaCruz
Anti-TLR8	TLR8	44C143	mouse	human	PE	Imegenex
Anti-TLR9	TLR9	eB72-1665	rat	human	PE	eBioscience
Anti-CD16	CD16	3G8	mouse	human	APC	BioLegend
Anti-CD62L	CD62L	DREG-56	mouse	human	FITC	BioLegend
Goat anti Rabbit IgG 2ndAb*	rabbit IgG	polyclonal	goat	rabbit	FITC	Abcam
Alexa Fluor® 555 Goat anti Rabbit 2ndAb	rabbit IgG	polyclonal	goat	rabbit	Alexa Fluor® 555	Invitrogen
CD16 MicroBeads	CD16					Miltenyi Biotec
Human Interferon-β ELISA Kit	Interferon-β					TFB
Human TNF-α Immunoassay Kit	TNF-α					Invitrogen

* The goat anti rabbit IgG 2nd antibody was served as secondary antibody for the primary antibodies, like anti-AIM2, anti-MDA5, and anti-RIG-I antibodies in FACS analysis.

** The Alexa Fluor®555 Goat anti Rabbit 2ndantibody was served as secondary antibody foranti-RIG-I antibody in confocal microscopy.

2.1.5. Stimulants

The following stimulants were used in the stimulation assays on neutrophils and the HL60 cells.

Miscellaneous Stimulants:

Stimulant	Target	Detail
E.coli K12 LPS	TLR4	Gram-negative bacteria
Poly(I:C)	TLR3/MDA5	Synthetic analog of dsRNA
ODN2006(type B)	TLR9	Synthetic oligonucleotides containing unmethylated CpG dinucleotides, 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (Phosphorothioate bases)
ssRNA40	TLR8	20-mer phosphorothioate protected single-stranded RNA oilgonucleotide containing a GU-rich sequence, complexed with LyoVecTM 5'-GCCCGUCUGUUGUGUGACUC-3' (Phosphorothioate bases)
R848	TLR7/8	an imidazoquinoline compound

Stimulants synthesized by Eurogentec:

Stimulant	Target	Detail
Poly-A-Control		Sequence: 5'-AAAAAAAAAAAAAAAAAAA-3'
isRNA9.2s-unmodified	TLR7/8	Sequence: 5'-AGCUUAACCUGUCCUUCA-3'

Stimulants synthesized by Ambion:

Stimulant	Target	Detail
siRNA(OHBcl2_2)	RIG-I	Sequence(Sense): 5'-GCAUGCGACCUCUGUUUGAUU-3' Sequence(Antisense): 5'-UCAAACAGAGGUCGCAUGCUU-3'
siRNA-Mismatch (Control)		Sequence(Sense): 5'-UUCUCCGAACGUGUCACGUUU-3' Sequence(Antisense): 5'-ACGUGACACGUUCGGAGAAUU-3'

Self-synthesized stimulants:

Stimulant	Target	Detail
3pRNA	RIG-I	Sequence(Sense): 5'-pppGCAUGCGACCUCUGUUUGAUU-3' Sequence(Antisense): 5'-pppUCAAACAGAGGUCGCAUGCUU-3'
3pRNA (Control)		Sequence(Sense): 5'-pppUUCUCCGAACGUGUCACGUUU-3' Sequence(Antisense): 5'-pppACGUGACACGUUCGGAGAAUU-3'

Stimulant produced by Sigma-Aldrich:

Stimulant	Target receptor	Detail
Poly(dA-dT) • Poly(dA-dT)	RIG-I/AIM2	Double-stranded DNA

2.1.6. Materials for the HL60 Cells Culture

As medium, RPMI 1640 READY MIX with L-Glutamine was mixed with 10% FBS but without antibiotics for the culture of HL60 cells.

2.1.7. Primer for Real-time-PCR

Oligo-name	Sequence	
	Forward	**Reverse**
TLR1	CTGGTATCTCAGGATGGTGTGC	TTGGAGTTCTTCTAAGGGTATGTTCC
TLR2	GCCTCTCCAAGGAAGAATCC	TCCTGTTGTTGGACAGGTCA
TLR3	TCCCAAGCCTTCAACGACTG	TGGGTGAAGGAGAGCTATCCACA
TLR4	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC
TLR5	TCGAGCCCCTACAAGGGAA	CACTGAGACTCTGCTATACAAGCTA
TLR6	CTATTGTTAAAAGCTTCCATTTTGT	ACCTGAAGCTCAGCGATGTAGTTC
TLR7	TTACCTGGATGGAAACCAGCTAC	TCAAGGCTGAGAAGCTGTAAGCTA
TLR8	GAGAGCCGAGACAAAAACGTTC	TGTCGATGATGGCCAATCC
TLR9	TGGTGTTGAAGGACAGTTCTCTC	CACTCGGAGGTTTCCCAGC
TLR10	GAAAGGTTCCCGCAGACTTG	TGGAGTTGAAAAAGGAGGTTATAG
MDA5	ATGTGGCAGCAAGAGCATCC	GGTAAGGCCTGAGCTGGAGTT
AIM2	GCTTTTGGCAAAACGTCTTCA	TCAGCTTGACTTAGTGGCTTTGG
RIG-I	TGACTGGACGTGGCAAAACA	TGCCGGGAGGGTCATTC

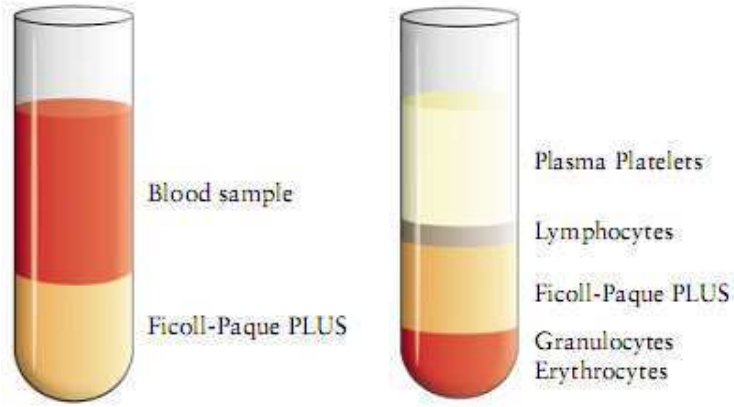
2.2. Cellular Immunology Methods

2.2.1. Isolation of Human Neutrophils

Ficoll-Paque® Plus Method:

Human neutrophils were isolated by Ficoll-Paque density gradient centrifugation from the EDTA treated blood of healthy volunteers. As showed in the figure 2, the phase of the erythrocytes and the granulocytes on the bottom was kept after centrifugation. Then the erythrocytes were lysed by the Erylysis buffer and removed by further centrifugations.

Figure 2. Ficoll-Paque® Plus Centrifugation(GE Healthcare Life Sciences)



Further Isolation with MACS® cell separation:

In some experiments, neutrophils were additionally positive-selected with anti-CD16 microbeads (Magnetic Cell Sorting, Miltenyi Biotec). With the magnetic labeling on target cells, they were kept in the column within a strong magnetic field of the separator while the sample was passing

through the column. After the sample passed through the column, the column was removed from the magnetic separator. The labeled target cells were then eluted from the column. Evaluation of the neutrophil population with fluorescence activated cell sorting (FACS) showed purity of >85-88% (SEM 2.5%) for isolation with Ficoll-Hypaque density gradient centrifugation alone and 93-95% (SEM 2%) for additional cell sorting with microbeads.

2.2.1. Cell culture of HL60 cells

The HL60 cells were kept in 37°C laminar air flow cabinet within 25 mL total volume of RPMI (10%FBS) medium and were split every 3 days. In the interest of the physiological functions of HL60 cells and due to the similarity between DMSO-treated HL60 and human neutrophils, the cells were separated into 2 cell lines. One of the cell lines was treated with 1.25% DMSO, and the other one not. After 5 days treatment with DMSO the treated cells and the untreated cells underwent the same FACS analysis of the TLR8, TLR9, MDA5, AIM2, and RIG-I and also the quantitative PCR.

2.2.2. Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single cells as they flow in a fluid stream through a beam of light. The properties measured include a

particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. Several detectors are used for that purpose. One is directed in line with the light beam, the forward scatter, which measures the size of the cell. Several detectors are perpendicular to the light beam. The side scatter measures the intracellular granularity. Separate fluorescence channels detect fluorescent light associated with the particle. The surface antigens are marked with the correspondent labeled antibody to a fluorescence dye (direct immunofluorescence) or a first unlabeled antibody would be coupled with a second fluorescence-labeled antibody (indirect immunofluorescence).

List mode data are collected on each particle or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. This data can be analyzed to provide information about subpopulations within the sample (Figure 3, Introduction to flow cytometry Manual NO.11-11032-01April, 2000 BD Biosciences).

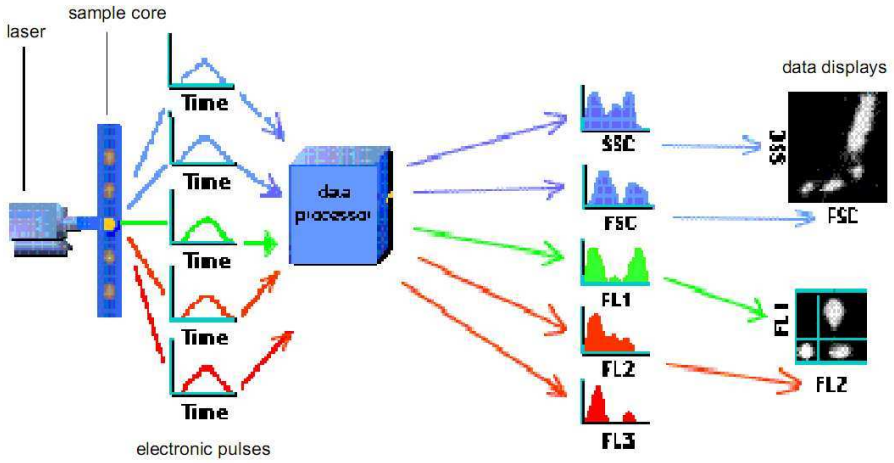


Figure 3. The detection of fluorescence turns into computer signals

Scattered and emitted light signals are converted to electronic pulses that can be processed by the computer. Further detail is described in text.

Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers. It provides a great way to analyze human neutrophils. In our experiments, flow cytometric data were obtained on a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences). Staining was performed following standard procedures to detect the proteins in the cytoplasm and on the cell surface of human neutrophils. The anti-CD11bantibody which is conjugated with allophycocyanin (APC) was used in a membranous staining to distinguish the neutrophils from the other white blood cells. Mouse anti human TLR8 and rat anti human TLR9 antibodies which are conjugated with phycoerythrin (PE) were used for staining TLR8, TLR9.Rabbit anti human MDA5, rabbit anti human AIM2, and rabbitanti human RIG-I antibodies were with the assistance of 2nd antibodies, goat anti-rabbit 2nd

antibody to complete the staining of MDA5, AIM2, and RIG-I.The 2nd antibody was conjugated with fluorescein isothiocyanate (FITC).

For the cytoplasmic detection of the receptors, the IntraPrep Kit (Beckman Coulter) was used to pre-incubate the cells with Reagent 1 of this kit, which contains formaldehyde for the fixation of the cells. Then the cells were permeabilized with Reagent 2 of the kit, which contains saponine, and stained to detect the receptors in the cells. For the membranous staining of the receptors, cells were only stained with the antibodies and then fixed with 2% paraformaldehyde for the flow cytometric analysis.

In the flow cytometric analysis, the antibody labeled sample was always measured alone with its isotype control labeled sample to obtain the mean fluorescence intensity ratio (MFI ratio), which will be explained under the section on statistical analysis.

2.2.3. Confocal Microscopy

Compared to conventional wide field optical microscopy,confocal microscopy is a relatively new optical imaging technique, which is able to control the depth of detected field and reduction of background information by using a spatial filtering technique to eliminate out-of-focus light. The laser-induced fluorescence light will pass through a pinhole and be screened before reaching the detector (Figure 4). The information of a focus plane can be re-constructed into 3D Information.

Because of its better resolution than conventional fluorescence microscopy, it is now widely used in cell immunology for live or fixed cells.

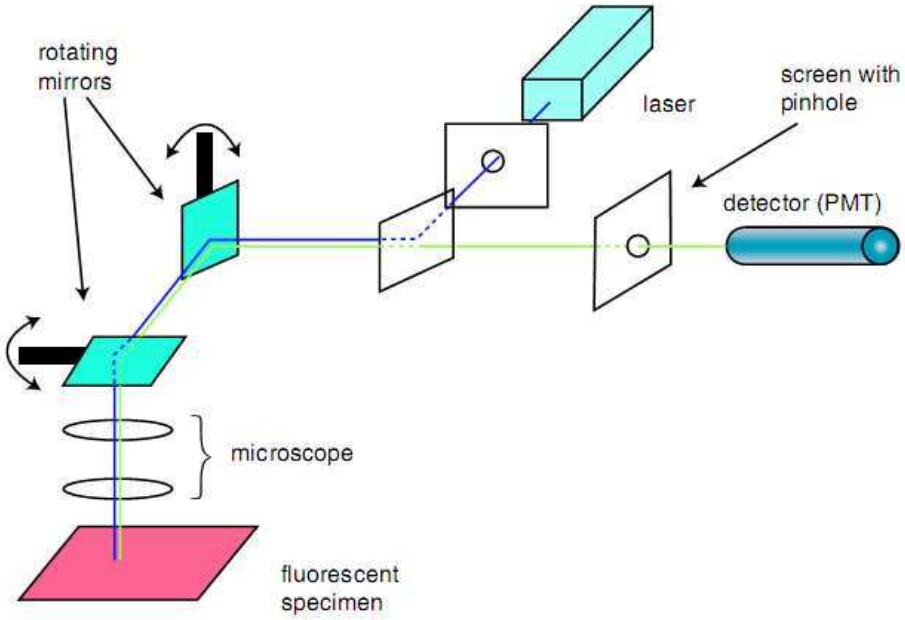


Figure 4. Basic setup of a confocal microscope

Light from the laser is scanned across the specimen by the scanning mirrors. Optical sectioning occurs as the light passes through a pinhole on its way to the detector (Confocal Microscopy, D Semwogerere, USA).

The isolated neutrophils were first fixed with 4% paraformaldehyde and blocked with 10% normal goat serum (NGS) in diluent. Then the rabbit anti human RIG-I antibody as the primary antibody was used to localize RIG-I in neutrophils together with secondary antibody of goat anti rabbit antibody which was labeled with Alexa Fluoro® 555. For labeling cell nuclei in human neutrophils, we chose 4,6-diamidino-2-phenylindole

(DAPI) which can diffuse easily through the cell membrane and be used both in live cells and fixed cells.

2.3. Molecular Methods

2.3.1. RNA Isolation

The isolated polymorphonuclear leukocytes were lysed and homogenized in the *Tri Reagent* at room temperature. The suspension was layer separated with chloroform. The upper clear phase of solution, in which the RNA was dissolved, was precipitated and cleaned with 100% Isopropanol and 70% Ethanol, and the RNA was again dissolved in the Ampuwa (RNase-free water) at 55°C.

2.3.2. cDNA Synthesis

In our experiments, we used complementary DNA (cDNA) synthesis to analyze the relative amount and the existence of mRNA for each protein that we are interested in, the TLRs, MDA5, AIM2, and RIG-I.

For cDNA synthesis, to each 2µg isolated RNA sample from human neutrophils of each healthy volunteer random hexamer primers were added, which contain every possible 6 bases single strand of DNA and can therefore hybridize anywhere on the RNA, and the sample was incubated at 70°C for the denaturation of the RNAs and the primers. Then the strand buffer, 0.1µM DTT, and 10mM dNTP were added into the samples, and by adding the reverse transcriptase, superscript II cDNA was produced. Finally, the mRNA templates were hydrolyzed by RHase H.

2.3.3. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technique in molecular biology capable to produce a great amount of a particular DNA copies using only a single or few copies of a piece of DNA by serial changes of temperature and several repeated cycles of DNA amplification. In each cycle, DNA will be heated to break its double-stranded structure and be cooled down again, and primers with sequences complementary to the target region can bind. Together with assistance of DNA polymerase the selected DNA sequences will be amplified. This process is repeated in a thermocycle several times creating exponential amplification of the desired DNA sequences. In our study, we used this technique in order to quantify the mRNA expression in human neutrophils by producing the cDNA of mRNA and analyzing cDNA in quantitative Real-time PCR.

With the Mastercycler® ep realplex (Eppendorf) the Real-time quantitative PCR (qPCR) was set to a specific amount of DNA product at which the amplification of each sample should be broken down. This process is called kinetic detection (Figure 5). As a reference gene (housekeeping gene) we chose the gene of TATA-Box binding protein (TBP), which is abundant in eukaryotic cells and shows good stability by qPCR (Nygard, Jorgensen et al. 2007). We used previously described primer sequences of TLR1 to TLR10, MDA5, AIM2, and RIG-I(Hayashi, Means et al. 2003). The cDNA was quantitatively analyzed referring to the housekeeping gene. The statistical analysis will be described in a later section.

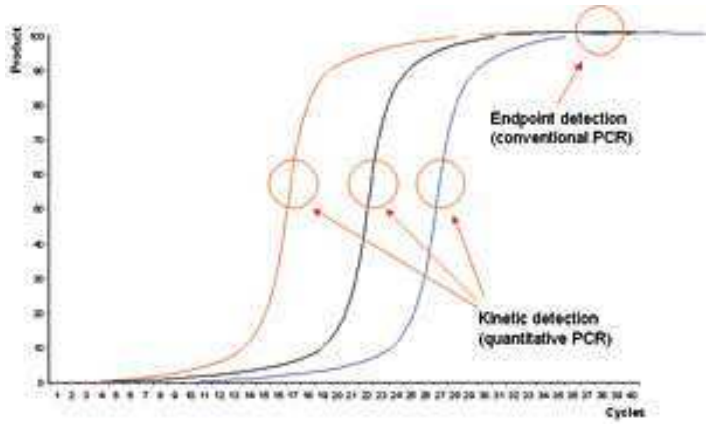


Figure 5. PCR: kinetic vs. endpoint detection (Eppendorf)

A plot of the quantity of amplicon DNA over time; in real-time PCR we are only concerned with amplification during the exponential phase of amplification, as accurate quantification of DNA is not possible at the plateau.

2.4. Stimulation of cells

2.4.1. Stimulation of Toll-like receptors

All TLR-ligands were titrated to defined optimal stimulating concentrations and underwent 40 minutes of stimulation with 37°C of incubation in the different cell types analyzed in the study. Cells were generally stimulated with LPS from Escherichia coli. Poly(I:C) was used for stimulation of TLR3, and for stimulation of TLR7 and TLR8, and we used R-848and CpG 2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') to stimulate the TLR9.

In order to have a further functional view on TLR7 and TLR8 in our cells, we stimulated the cells with our previously described isRNA 9.2 antisense (5'-UUGAAGGACAGGUUAAGCUdTdT-3'), which was synthesized by Eurogentec Germany GmbH. For better transfection into

the cells, we have complexed nucleic acid by using the poly-cationic polypeptide, poly-L-arginine (P7762 by Sigma-Aldrich). As a control, we used non-stimulatory poly-A-RNA repeats, also purchased at Eurogentec Germany GmbH.

2.4.2. Stimulation of RIG-I

Similar to the stimulation with TLR-ligands, 3pRNA and small interfering RNA used as ligands for RIG-I, as well as dsDNA with poly (AT) repeat used as a ligand for AIM2 and poly(I:C) as a ligand for MDA5 were all titrated to defined optimal stimulating conditions in the stimulation assay.

For generation of in vitro transcribed dsRNA, the DNA templates of the sense and antisense strands were transcribed for 6 hours in separate reactions using PCR. An extra guanosine was present at the 5' end to both the sense and the antisense strands in order to transcribe with T7 RNA polymerase. The reactions were then mixed and incubated overnight at 37°C to anneal the transcribed RNA strands. The DNA template was digested using DNase-I (Ambion) and subsequently RNAs were purified by phenol:chloroform extraction and alcohol precipitation. Excess salts and NTPs were removed by passing the RNAs through a Mini Quick SpinTM Oligo Column (Roche). In order to test the immunostimulatory potential of 3pRNA on human neutrophils, 3pRNA was first complexed with lipofectamine and then given into cultures of human neutrophils to incubate at 37°Cfor 40 minutes.

After stimulation, the expression of CD62L and CD11b on human neutrophils was analyzed in FACS to compare stimulation intensity of each stimulant. For the cell staining in FACS, we used mouse anti human CD11b antibodies (conjugated with APC) and mouse anti human CD62L antibodies (conjugated with FITC). For instance, siRNA with the same nucleotide sequence like 3pRNA was used to compare the stimulation intensity with 3pRNA.Additionally, TNF-α production was measured with ELISA.

2.5. Statistical Analysis

Quantitative Real-time PCR with normalization with TBP genes

In the quantitative Real-time PCR, the TBP genes were chosen to normalize the values of our proteins. With the kinetic detection of Mastercycler® ep realplex (Eppendorf) we set a stop point of amplification of the polymerase chain reaction which is supposed to be “X” copies of DNA fragments. Each primer of our protein underwent amplification until reaching this end point, and the hardware counted the cycles of reactions each sample needed to reach this end point. For instance, the TBP genes need 20 cycles to reach the end point, and the sample needs 18 cycles. With this information, one can obtain the relative amount of genes of this sample on cDNA of our volunteer with the following equation referring to the TBP:

$$\text{The relative amount of genes} = 2^{-(\text{cycles of sample gene} - \text{cycles of TBP gene})}$$

In this case, the sample gene in the cDNA sample is present fourfold compared to the TBP gene. With this equation we obtained the relative quantitative gene expression of our proteins in cDNA sample which are related to the mRNA expression.

Mean Fluorescence Intensity Ratio was used for FACS analysis:

In order to avoid the interference of antibody staining with unspecific isotype control staining, we chose the mean fluorescence intensity ratio(MFI ratio), an established immunological analysis method(Chwae, Lee et al. 2007; Buhtoiarov, Neal et al. 2011)to analyze the values of our FACS analysis with the following equation.

$$\text{MFI ratio} = \frac{\text{(MFI of the antibody)}}{\text{(MFI of the isotype control)}}$$

Student's paired two-tailed t test was used to analyze data

Data are presented as means± S.E.. The statistical significance of differences was determined by Student's paired two-tailed t test. In all tests, differences were considered significant at $p < 0.05$.

3. RESULTS

3.1. Expression of Toll-like Receptors, MDA5, AIM2 and RIG-I in human neutrophils

3.1.1. Expression of mRNA

It is known that human neutrophils express TLRs (Hayashi, Means et al. 2003). Recently, it was shown that neutrophils express NOD-like receptors and can be activated by these receptors (Ekman and Cardell 2009). However, in human neutrophils, there are hardly any detailed reports on the expression of RIG-I, AIM2 and MDA5. Therefore, we aimed to establish a profile for these receptors in neutrophils by analyzing both messenger RNA (mRNA) and protein expression.

In order to analyze mRNA expression of TLRs, MDA5, AIM2 and RIG-I we performed quantitative real-time PCR. In quantitative real-time PCR analysis, all the values showed are relative to gene expression of a typical housekeeping gene, in our case TATA-binding protein (TBP). In this analysis, TBP and all genes that code for protein investigated here were amplified to specific an amount, which is called kinetic detection.

Analysis of TLRs-coding mRNA showed significant expression for all TLRs except TLR3 and TLR7.The mRNA of TLR1 and TLR2 were expressed most. With respect to the mRNA of other TLRs capable of detecting nucleic acids, TLR8 was expressed most (20-fold compared to TLR9, which was low but significant, and 3-fold compared to LPS-binding-receptor TLR4, Figure 6).The mRNA coding for RIG-I, AIM2, and MDA5showed low but significant expression in human neutrophils,

and expression for all was significantly higher when compared to TLR3 and TLR7, higher when compared to TLR9 but significantly lower than expression for TLR8.

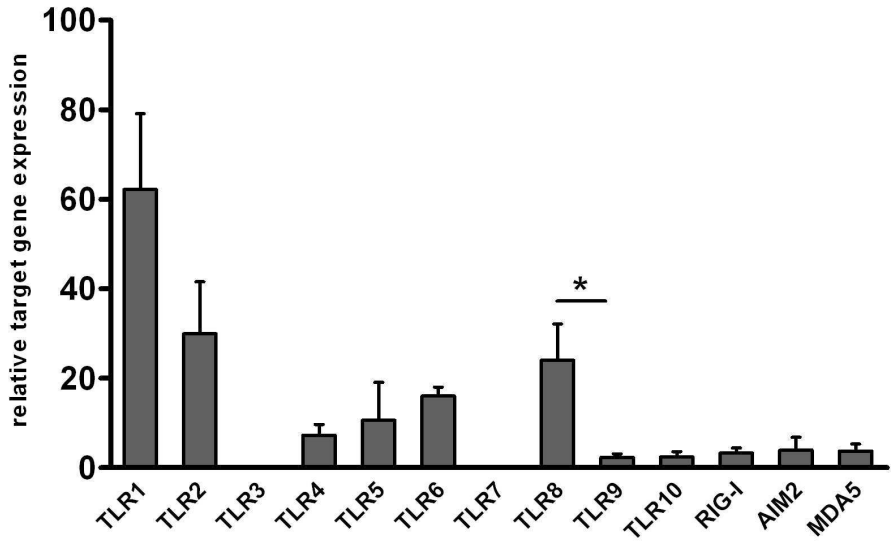


Figure 6. Expression of mRNA for TLRs, RIG-I and MDA5 in granulocytes

Isolated granulocytes were obtained from healthy volunteers and analyzed by real-time PCR for the expression profile of TLRs, RIG-I, AIM2 and MDA5. TBP was used as a reference for expression. As mentioned in the text, a kinetic detection was used to measure the relative quantitative expression of each protein. The mean ± S.E. of 6 donors is shown. *, $p < 0.05$.

3.1.2. FACS Analysis of Protein Expression

After having established an mRNA expression profile of neutrophils by real-time PCR, in the next step the actual protein expression was analyzed with FACS and compared to mRNA expression. After isolation of neutrophils, cells were stained with specific antibodies for both intracellular and membranous expression of TLR8, TLR9, MDA5, AIM2 and RIG-I.

We found that TLR8, MDA5, AIM2, and RIG-I but not TLR9 were expressed both intracellularly and on the cell surface in human neutrophils. This is in contrast to data from qPCR because here we observed low but relevant expression of mRNA for TLR9 (see Figure 7).

When comparing intracellular expression to expression on the cell surface, we found similar expression for TLR8. MDA5was mostly expressed intracellularly, but significant expression of MDA5 was observed additionally on the cell surface. Expression of AIM2 was observed on the cell surface and intracellularly to a lower degree, which was not statistically significant. Interestingly, expression on the cell surface for RIG-I was 6-fold higher compared to the intracellular expression.

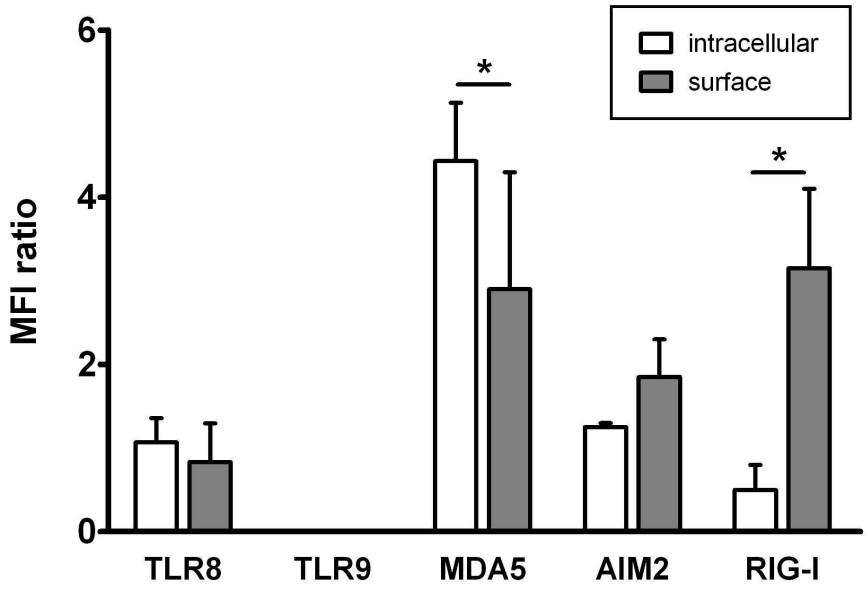


Figure 7. Protein Expression of TLR8, TLR9, AIM2, RIG-I and MDA5 in granulocytes

Expression of TLR8, TLR9, RIG-I and MDA5in human neutrophils from healthy volunteers was analyzed by FACS without any type of stimulation. The mean fluorescence intensity ratio was used to show the fluorescence strength of each protein relative to its control sample. In order to see the distribution of each protein, within human neutrophils, cells were stained intracellularly and for cell surface expression. The mean ± S.E. of 3 donors is shown. *, $p < 0.05$.

3.1.3. Subcellular Localization

Because our findings in FACS analysis showed significantly more expression of RIG-I on the cell surface in comparison to intracellular expression, we analyzed the subcellular localization of TLR8 and RIG-I with confocal microscopy (Figure 8). When analyzing the subcellular localization of TLR8, we found mostly intracellular expression. In the subcellular localization of RIG-I we found expression both intracellularly and on the cell surface which confirmed our findings from FACS analysis.

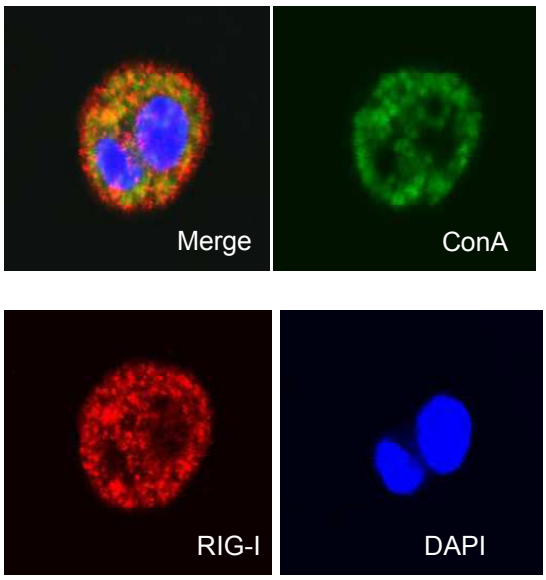


Figure 8. RIG-I shows both intracellular and membranous expression in confocal microscopy

Human neutrophils were isolated from healthy volunteers. The isolated neutrophils were fixed with 4% paraformaldehyde and blocked with 10% NGS in diluent. Then the RIG-Iwas treated with antibodies and labeled with Alexa Fluoro® 555 leading to the red stain in the pictures. Cell's nuclei in human neutrophils were labeled with DAPI(blue color), and FITC-Con A (green) was used for staining the cytoplasm.

3.1.4. Expression of RIG-I in murine neutrophils

The high expression of RIG-I on the cell surface on human neutrophils was further analyzed by comparing these findings to the expression of RIG-I in murine neutrophils. Murine neutrophils were isolated from bone marrow by ficoll isolation and stained with specific antibodies against RIG-I as described for human neutrophils.

Compared to human neutrophils no expression was observed on the cell surface of murine neutrophils, but high expression was seen in the cytoplasm (see figure 9).

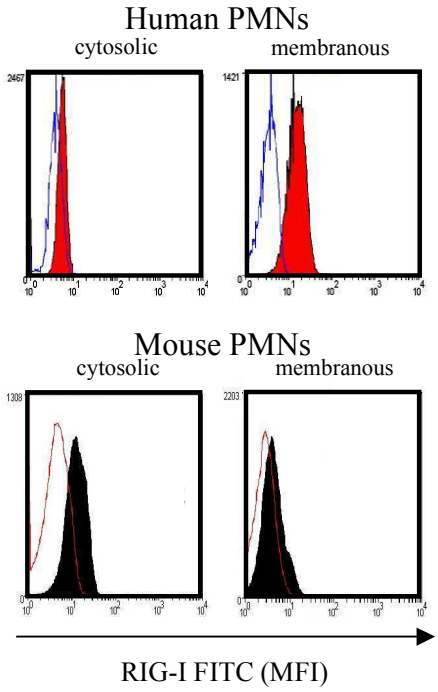


Figure 9. Expression of RIG-I in murine PMNs

The expression of RIG-I in human (above) and murine (below) PMNs. Histograms are show for both intracellular and cell surface expression. Isotype control is shown as in blue for human cells and in red for murine cells. MFI = mean fluorescence intensity.

3.2. Expression of Toll-like Receptors, MDA5, AIM2 and RIG-I in HL60 cells

3.2.1. Expression of mRNA

PMNs are challenging to cultivate and have very short survival ex vivo. Alternative strategies have recently been described in order to investigate this important cell population (Klinker, Wenzel-Seifert et al. 1996). The neutrophil-like cell line (HL-60) is a powerful alternative to analyze neutrophilic granulocytes(Collins, Ruscetti et al. 1979). This cancer cell line was originally isolated from a patient with acute myelogenic leukemia and has similar markers and characteristics as human granulocytes, especially after further differentiation with DMSO (Gallagher, Collins et al. 1979; Jacob, Leport et al. 2002).

With the same primers used to analyze the mRNA expression of TLRs, RIG-I, MDA5 and AIM2of human neutrophils, both untreated HL60 cells and HL60 cells pretreated with 5 days of DMSO maturation were analyzed with PCR. When compared to the mRNA profile of human neutrophils, the profile ofHL60 cells showed many similarities but also differences. Similarities were, especially seen in comparison to the DMSO-treated HL60 cells.

The difference between human neutrophils and DMSO-treated as well as untreated HL60 cells was that the HL60 cells express mRNA of TLR3 but not TLR5,and only marginal levels of TLR10. However, when comparing TLR3 expression between untreated and DMSO-treated HL60 cells, we observed significant reduction of expression for

DMSO-treated cells. Like in the human neutrophils, TLR7 is not expressed in the HL60 cell lines. Likewise, in human granulocytes expression of TLR1 and TLR2 was the highest. Expression of TLR6, expressed in a robust manner in mature human granulocytes, was expressed high solely in DMSO-stimulated HL60 cells. Surprisingly, and in sharp contrast to mature granulocytes, TLR8 was expressed low in untreated HL60 cells and marginally in DMSO-treated cells. TLR9, expressed marginally in mature granulocytes, showed significant expression in both DMSO-treated and untreated HL60 cells.

The untreated HL60 cells further showed a very low expression of RIG-I and MDA5, but their expression was increased after treatment with DMSO and was then higher than TLR9 comparable to mature human neutrophils.

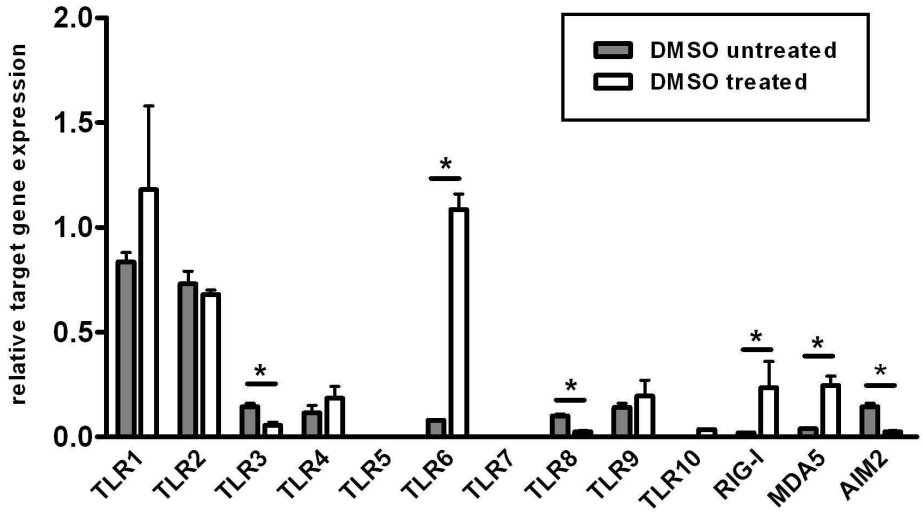


Figure 10. Expression of mRNA for TLRs, AIM2, RIG-I and MDA5 on HL-60 cells

HL60 cells were either cultured untreated or treated with DMSO for 5 days and analyzed by real-time PCR for the expression profile of TLRs, RIG-I, AIM2 and MDA5. TBP was used as a reference for expression. As mentioned before, a kinetic detection was used to measure the relative quantitative expression of each protein. *, $p < 0.05$.

3.2.2. Protein Expression of HL60 cells

Similar to our approach in granulocytes, we additionally analyzed protein expression of TLR8, TLR9, RIG-I, MDA5 and AIM2 for both DMSO-treated and untreated HL60 cells. We stained these proteins by specific antibodies both for intracellular expression and expression on the cell surface and analyzed the cells by FACS.

HL60 cells, similar to human neutrophils, express TLR8, MDA5, AIM2, and RIG-I but not TLR9.TLR8 was expressed both in the cytoplasm and on the surface of cells in untreated HL60 cells express, and there was no significant difference between these two.

Untreated HL60 cells showed expression of MDA5 in both compartments, but there was significantly more expression on the cell surface, which is in contrast to the results found in human granulocytes. However, after the maturation with DMSO, expression of surface MDA5wasalmost entirely suppressed. High expression of intracellular MDA5 was part of the characteristic profile observed for human granulocytes.

This suppression observed for expression of MDA5 after maturation of HL60 cells was observed in a similar way for the expression of AIM2 and RIG-I, even though these two proteins where expressed to a significantly lower degree in HL60 cells that were not treated with DMSO.

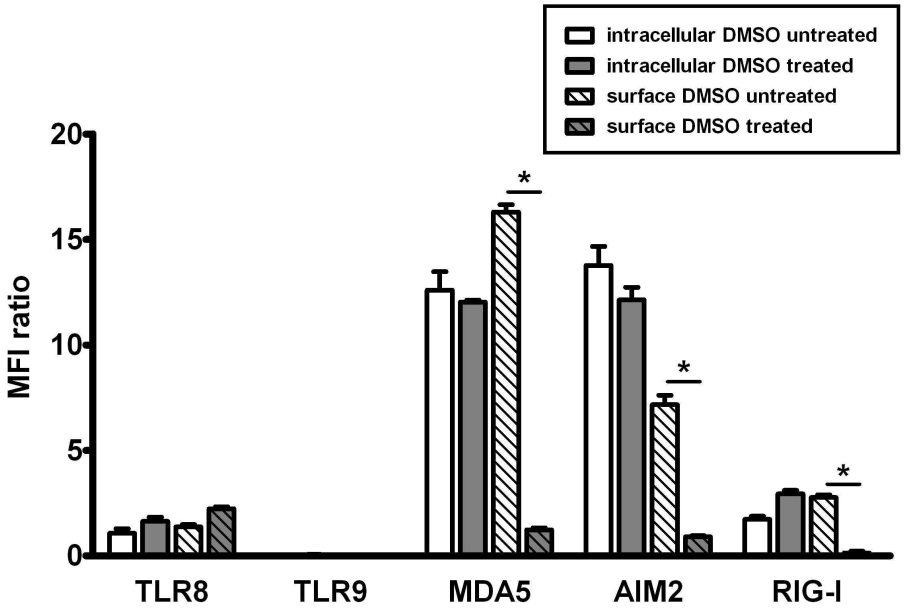


Figure 11. Protein Expression of TLRs, AIM2, RIG-I and MDA5 on HL-60 cells

TLR8, TLR9, MDA5, AIM2, and RIG-I were analyzed in untreated and DMSO-treated HL-60 cells with FACS. The mean fluorescence intensity ratio was used to show the fluorescence strength of each protein relative to their control sample. In order to appreciate the subcellular distribution of each protein, similar to the experiments performed in human neutrophils, HL60 cells were stained intracellularly and for cell surface expression.*, $p < 0.05$.

3.3. Stimulation of human neutrophils

3.3.1. Stimulation with immunostimulatory RNA

In our previous results we observed expression of TLR8, MDA5, AIM2, and RIG-I in human neutrophils. In the next step, we now stimulated these receptors with their respective ligands in order to analyze their functionality. Human neutrophils were isolated as described above and stimulated with CpG 2006 (TLR9), dsDNA (AIM2), LPS (TLR4), R848 (TLR7/8), isRNA 9.2 sense complexed with Poly-L-Arginine (TLR8), Poly-L-Arginine alone (control), 3pRNA complexed with lipofectamine (intracellular RIG-I), 3pRNA (extracellular RIG-I) and lipofectamine alone (control), as well as siRNA (control) in different concentration.

After 40 minutes of stimulation at 37°C human neutrophils were analyzed regarding their expression of CD62L and CD11b on the cell surface. Up-regulation of CD11b and simultaneous down-regulation of CD62L are well established markers of neutrophil activation(Wittmann, Rothe et al. 2004).

LPS, which is known to bind TLR4, showed strong activation of neutrophils characterized by both up-regulation of CD11b and down-regulation of CD62L at the lowest concentration used (0,1µg/mL) and served as a positive control. Activation observed by R848, which activates TLR8, was of similar characteristic in that it caused up-regulation of CD11b, down-regulation of CD62L and showed activity at low concentration, although it was somewhat weaker compared to LPS. Also, while LPS showed its maximal activating effect on human

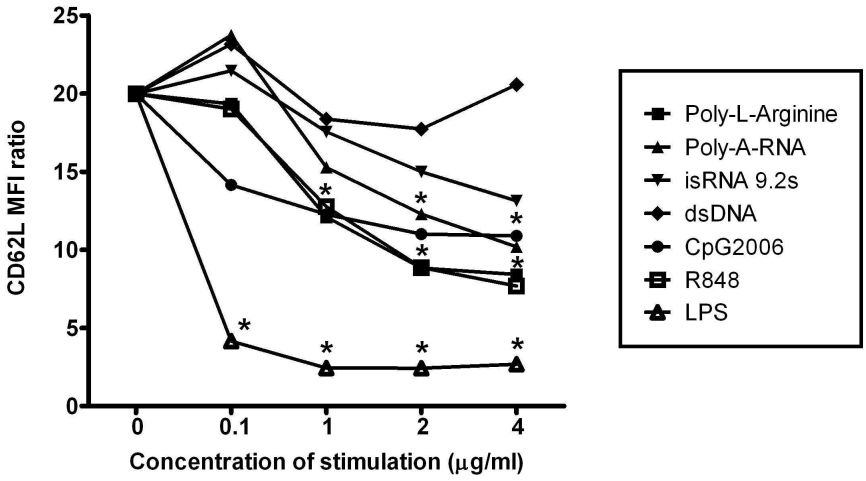
neutrophils with a concentration of0,1µg/mL, activation caused by R848 could be increased with higher concentrations.

The other ligand used for stimulation of TLR8 was isRNA 9.2sensecomplexed with poly-L-Arginine. We saw no up-regulation of CD11b for this ligand but down-regulation of CD62L for both the complex and the poly-L-Arginine alone. The maximal activation caused by isRNA 9.2s complexed with poly-L-Arginine at a concentration of 4µg/mL was even weaker than the activation caused by poly-L-Arginine alone (Figure 12 (A) and (B)).

For the stimulation of the low but reproducible and constant expressed RIG-I in human neutrophils, we designed 3pRNA,which contains a triphosphate chemical modification at the 5'-end of both stands (see material and methods for details). For controls, we used double-stranded siRNA with the exact same sequence but without the triphosphate modification. Both siRNA and 3pRNA were complexed with lipofectamine for intracellular activation. The stimulation curve of lipofectamine only represented its amount in each titrated concentration of complexed solution with stimulants. We saw no significant activation of human neutrophils by this ligand in comparing to the stimulation curve of to lipofectamine alone (Figure 13 (A) and (B)).

The dsDNA and CpG, which binds to AIM2 and TLR9, respectively, showed no significant activation of human neutrophils. Even though we observed some down-regulation of CD62L on the cell surface, we observed no up-regulation of CD11b for these stimuli.

(A)



(B)

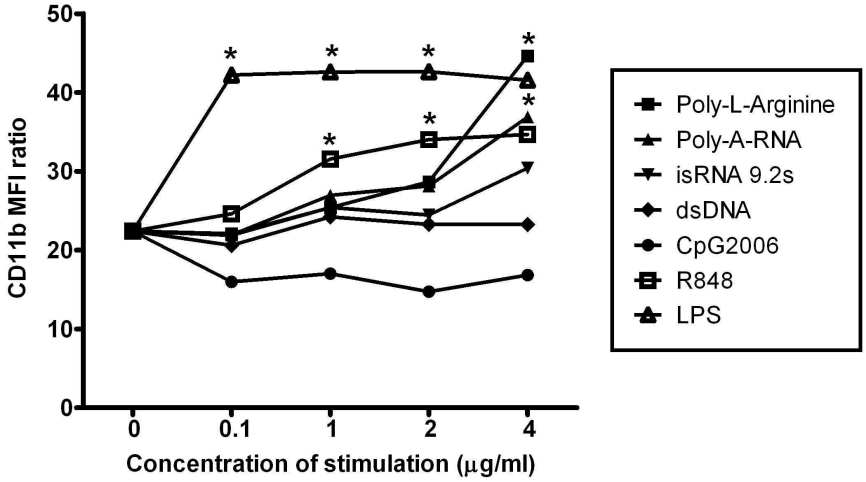
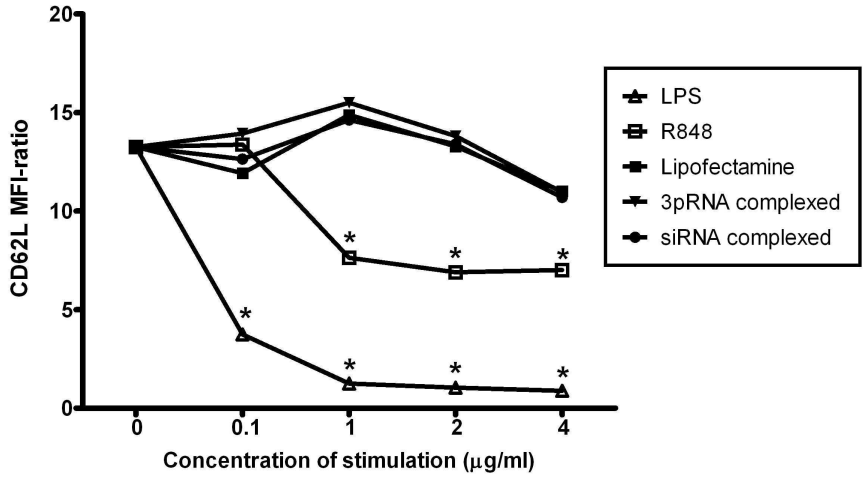


Figure 12. Stimulation assay of human neutrophils

PMNs were isolated and stimulated with LPS, R848, CpG-2006, dsDNA, isRNA9.2s (complexed with Poly-L-Arginine), poly-A-RNA (complexed with Poly-L-Arginine), and poly-L-Arginine complexion solution in different stimulation concentrations.40 minutes after stimulation, expression of both CD62L (A) and CD11b (B) were examined by FACS. The mean of 3 donors is shown. *, $p < 0.05$.

(A)



(B)

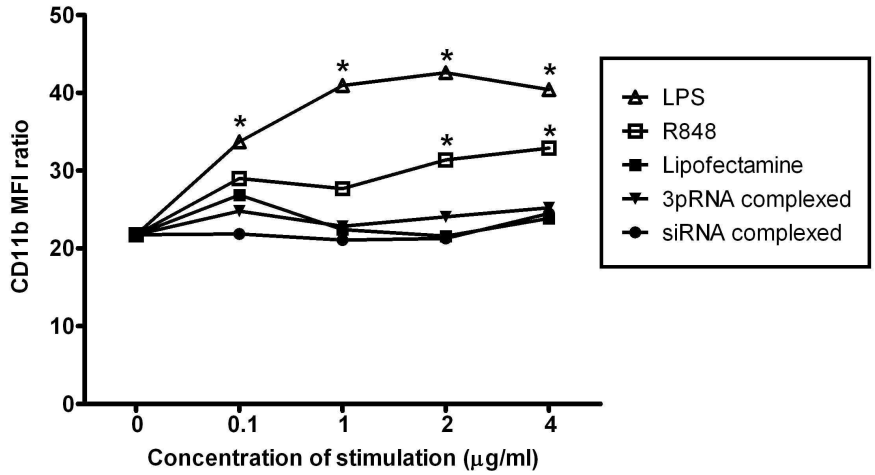


Figure 13. Stimulation assay of human neutrophils

Granulocytes were isolated and stimulated with LPS, R848, siRNA (complexed with lipofectamine) or 3pRNA (complexed with lipofectamine), and lipofectamine complexion solution in different stimulation concentrations.40 minutes after stimulation, expression of both CD62L (A) and CD11b (B) were examined by FACS. The mean of 3 donors is shown. *, $p < 0.05$.

3.3.2. Cytokine production of neutrophils after stimulation

Having observed both the expression and down-regulation of certain cell surface markers characterizing the activation of human neutrophils after stimulation of PRRs, we wanted to analyze the cytokine production as an additional marker of activation. We isolated neutrophils and stimulated neutrophils as described. Cells were stimulated for 40 minutes at 37°C, and enzyme-linked immunosorbent assay (ELISA) was performed for detection of tumor necrosis factor-alpha (TNF-α) in the supernatants. TNF-α is a well-established marker of activation in human neutrophils.

We did not observe cytokine production of untreated neutrophils or stimulation with isRNA, 3pRNA alone or lipofectamine alone, similar to the results found in the FACS analysis. There was very low activation with 3pRNA complexed with lipofectamine (31pg/mL). The highest activation was observed for stimulation with R848 activating TLR8.Stimulation with LPS activating TLR4 likewise showed significant activation.

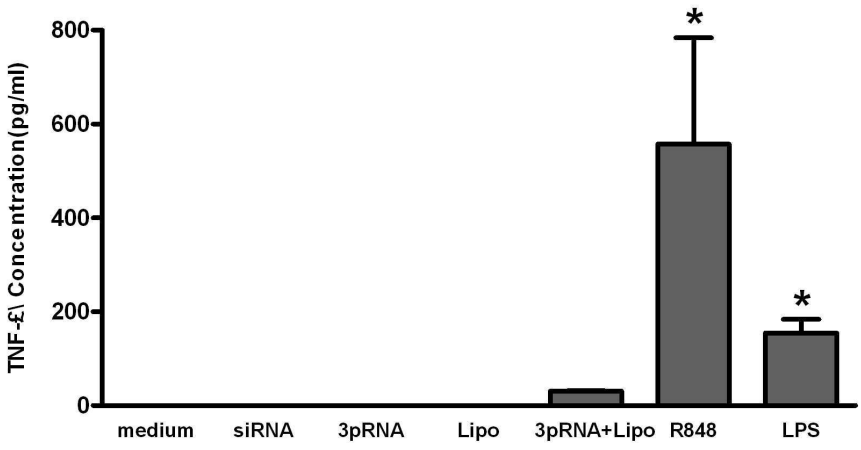


Figure 14. TNF-α production in human neutrophils after stimulation

After human neutrophils were isolated and stimulated for 40 minutes as described, ELISA was used in order to assess cytokine levels in the supernatants. The mean ± S.E. of 3 donors is shown. *, $p < 0.05$. Lipo = Lipofectamine

3.3.3. Change of Receptor Profile after Stimulation

It is known that certain PRRs alter their expression after stimulation with their respective ligands (Poltorak, Smirnova et al. 1998; Muzio, Bosisio et al. 2000; Nomura, Akashi et al. 2000; Visintin, Mazzoni et al. 2001). For example, both the expression of TLR7 and TLR9 may be altered after stimulation with their ligands (Hornung, Rothenfusser et al. 2002). Whether this is true for PRRs in neutrophils is entirely unknown. Therefore, in the next step, we assessed the expression profile of TLR8, TLR9, AIM2, MDA5, and RIG-I in human neutrophils after activation. Neutrophils were isolated as described and stimulated with phorbol-myristate-acetate (PMA), a known stimulant of human neutrophils (Nishihira and O'Flaherty 1985), at a concentration of 40 ng/mL at 37°C for 10 minutes. Then, using FACS stain and analysis as described above, the expression profile after activation was analyzed.

The protein expression of TLR8 showed no change in the expression profile or the overall level of expression. As in unstimulated human neutrophils, we found no expression of TLR9 after stimulation of these cells.

For MDA5 and AIM2 there was no change in the expression profile for both the subcellular distribution and the overall level of expression. The same holds true for RIG-I, which again was found predominantly on the cell surface when compared to intracellular expression.

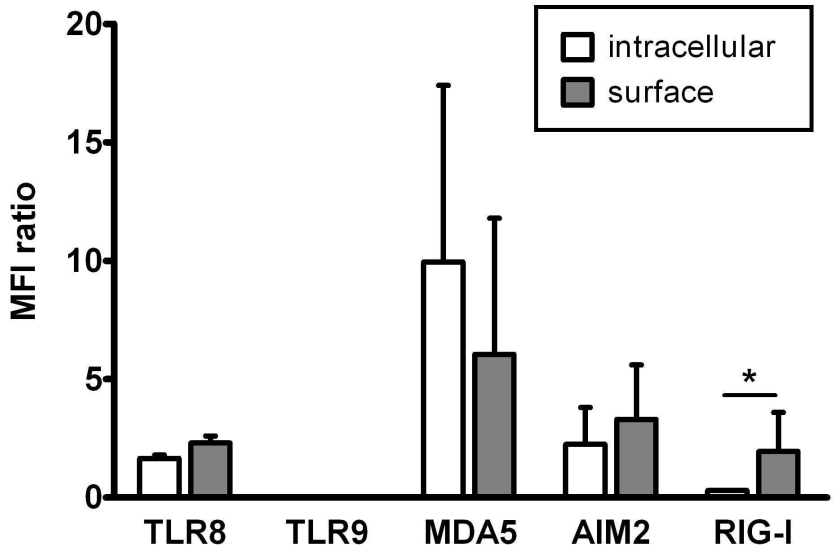


Figure 15. Protein expression after 10 mins stimulation with PMA in human neutrophils

Similar to earlier experiments in unstimulated human neutrophils, protein expression of TLR8, TLR9,MDA5, AIM2, and RIG-I was now analyzed with FACS for human neutrophils after stimulation for 10 minutes with 40ng/mL of PMA at37°C. Mean fluorescence intensity ratio was used to show the fluorescence strength of each protein relative to their control sample. Both intracellular stains as well as cell surface expression were analyzed. The mean ± S.E. of six different healthy donors is shown. *, $p < 0.05$.

3.3.4. Colocalization of 3pRNA and RIG-I

In our previous experiments, we observed significant expression of RIG-I in human neutrophils but no activation upon stimulation with its natural ligand 3pRNA. We now wanted to investigate the colocalization of the receptor and the ligand on both the cell surface and intracellularly in order to exclude degradation of the ligand or lack of binding. We isolated human neutrophils as described and stimulated them with pre-stained 3pRNA with NHS-Fluorescein. Then we stained cells as described above and analyzed them with confocal microscopy (Figure 16).

We found that the expression of RIG-I colocalized with the 3pRNAboth on the cell surface and intracellularly. Controls using TLR8 ligand R848 and siRNA without the triphosphate compound showed no colocalization with RIG-I(data not shown).

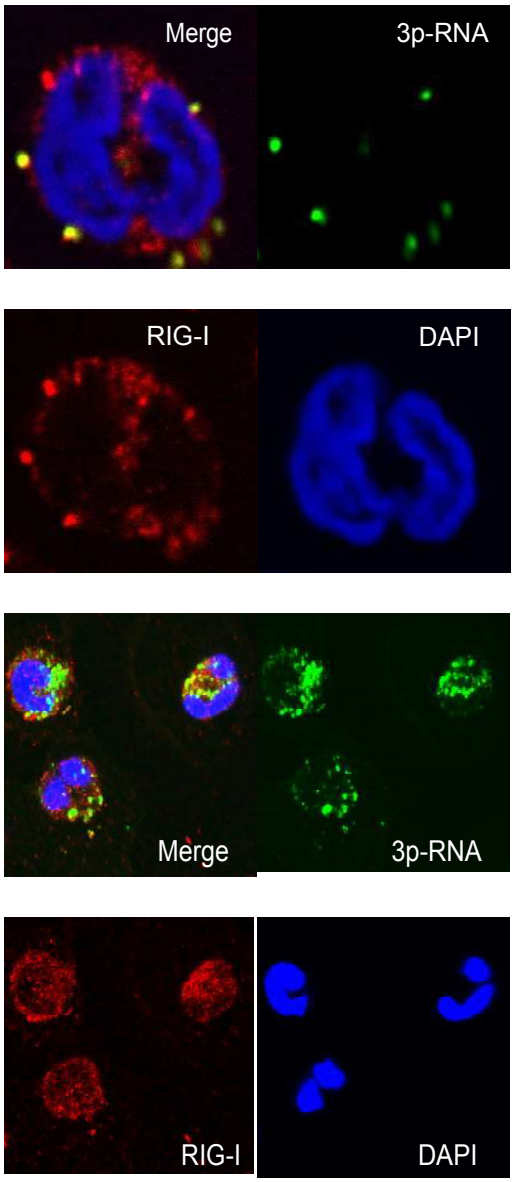


Figure 16. Colocalization of 3pRNA with RIG-I in human neutrophils

In confocal microscopy, 3pRNA was labeled with NHF-Fluorescein to have green color. RIG-I was again stained with Alexa Fluoro® 555 resulting in rot points in the pictures. Cell nuclei in human neutrophils were labeled with DAPI to have blue color.

4. DISCUSSION

4.1. Overview of our Experimental Findings

In this study we established the mRNA expression profiles of TLRs (from TLR1 to TLR10), MDA5, AIM2, and RIG-I in human neutrophils and HL60 cells by using quantitative real-time PCR. Additionally, experimental studies were performed on the level of protein expression, using both FACS analysis and confocal microscopy.

With respect to the mRNA level of TLR1 through TLR10, we found no expression of TLR3 and TLR7 in human neutrophils, regardless of their state of activation. TLR3 was expressed in untreated HL-60 cells but not in DMSO-treated cells. As in human neutrophils, TLR7 was not expressed in HL-60 cells. We found a high expression of TLR8 in human neutrophils, low expression in untreated HL-60 cells and no expression of TLR8 in DMSO-treated cells. TLR9 was expressed marginally on human neutrophils and low in both untreated and DMSO-treated HL-60cells. Therefore, despite the general theory that DMSO-treated HL-60 cells resemble many of the characteristics of human neutrophils, we found many differences for those PRRs that are capable to be activated by nucleic acids such as DNA and RNA.

There was no protein expression for TLR9 but significant expression of TLR8 in human neutrophils and HL60 cells. In addition, TLR8 was expressed not only in the cytoplasm but also on the cell surface in these

cells and showed no significant difference between cytoplasmic and surface expression.

Along with the TLRs, MDA5, AIM2, and RIG-I were analyzed on both the levels of mRNA and protein expression. We saw significant expression of MDA5, RIG-I andAIM2 in human neutrophils. We saw no expression of RIG-I and MDA5 in untreated HL-60 but significant expression of these receptors in DMSO-treated, mature HL-60 cells. Conversely,AIM2 was expressed on untreated HL60 cells but not in mature HL-60 cells MDA5 was found primarily intracellularly for untreated and DMSO-treated cells, but expression was found on the cell surface only of untreated HL-60 cells. The same held true for the expression of RIG-I and AIM2. Confocal microscopy confirmed the expression of RIG-I on the cell surface of human neutrophils, and colocalization studies with the confocal microscope confirmed the binding of RIG-I with its ligand, 3pRNA.

However, despite binding of 3pRNA to its receptor, stimulation of human neutrophils with the known RIG-I ligand (3pRNA) did not result in activation of human neutrophils. Stimulation with known activators of neutrophils such as TLR4 ligand (LPS) and TLR8 ligand (R848) resulted in strong up-regulation of CD11b, down-regulation of CD62-L and induction of TNF-α.

4.2. Interpretation of Data with Respect to the Current Literature

4.2.1. TLRs, MDA5, AIM2, and RIG-I exist in human neutrophils

Of the 10 known TLRs to date, only TLR3, TLR7, TLR8 and TLR9 are capable of recognizing nucleic acid. The expression profile of mRNAs for TLRs on human neutrophils has been described by Hayashi et al. and Janke et al. Similar to their results; we did not see TLR3 but significant TLR8 expression in PMNs. However, in contrast to Hayashi et al. and in accordance to Janke et. al., we did not see expression of TLR7 and only marginal expression of TLR9. The expression of TLR1, TLR2, TLR4, TLR5, and TLR6 showed similar results between our study and the study of Hayashi et al. Strong expression of TLR2 and TLR4 was observed by Janke et al. These differences may be influenced by the use of different primers as well as different reference genes. Instead of TBP, both cited investigators choseβ_2-microglobulin and β-actin as reference genes, respectively. β_2-microglobulin and β-actin are abundant genes in nucleated cells and expressed far more than TBP. They are both useful and frequently used reference gene for quantitative real-time PCR. However, as was observed by Nygard et al., TBP is good reference genes especially for low abundant transcripts in expression studies.

In human neutrophils, essentially nothing is known about the expression of MDA5, RIG-Ior AIM2. In other immune cells, like macrophages and dendritic cells, AIM2 was reported to be expressed predominantly as a cytosolic sensor for dsDNA and was essential for inflammasome activation in response to *Francisella tularensis*, vaccinia virus and mouse

cytomegalovirus and had a partial role in the sensing of *Listeria monocytogenes*(Fernandes-Alnemri, Yu et al. 2010; Rathinam, Jiang et al. 2010). In this study, we demonstrated the existence of AIM2 in human neutrophils by using qPCR, FACS analysis and confocal microscopy.

The expression of RIG-I has been reported for dendritic cells and certain mesothelial cells (Hornung, Ellegast et al. 2006; Takeuchi and Akira 2008; Wornle, Sauter et al. 2009). The presents of RLHs in PMNs and the PMN responses upon their activation have not previously been described. The typically described location for this receptor is the cytosol. In our experiments, in addition to intracellular expression, surprisingly we repeatedly observed protein expression on the cell surface.

One limitation of this study is that the experiments presented here largely depend on the specificity of the antibodies used for flow cytometry. Therefore, every key result of the presented work was carried out using two different specific antibodies with two different binding sites. Nevertheless, even though highly unlikely, one can make the legitimate argument that our results may possibly be biased or influenced by unspecific cross-staining. Therefore, as a continuation of this dissertation, we used additional experimental methods including subcellular fractionation to further clarify this important aspect. The subcellular components were stained for RIG-I or MDA-5 and analyzed separately by Western blotting(Berger, Hsieh et al. 2012). As a positive control, autologous PBMCs were utilized, which are well known to express cytosolic RIG-I and MDA-5. These experiments demonstrated

that human neutrophils expressed both RIG-I and MDA-5 with a similar molecular mass compared with PBMCs. Remarkably, RIG-I and MDA-5 in neutrophils were detectable in fractions characteristic for secretory vesicles and plasma membranes, whereas the RLH proteins were absent in primary/azurophilic, secondary/specific, or tertiary/gelatinase granule fractions. Besides secretory vesicles, the proteins were detectable in cytosolic fractions. In immune-transmission electron microscopy, RIG-I proteins were frequently associated with membranous/vesicle-like structures (Berger, Hsieh et al. 2012).

One of the most interesting findings we made in our study was the fact that while RIG-I seems to indeed be expressed primarily on the cell surface of human neutrophils, this is not the case for murine neutrophils. One possible explanation for cell surface expression of RIG-I in human neutrophils could be that this receptor is functioning as a scavenger receptor, especially in the light of no observed downstream activation after stimulation with the ligand 3pRNA. For example, previous studies have shown that innate surface receptors on neutrophils, such as C-C chemokine receptor type 5 (CCR5), can act as scavenging receptors (Ariel, Fredman et al. 2006). Therefore, surface RIG-I on neutrophils could serve as RNA binding and inactivation receptor at sites of neutrophilic inflammation. It would not be surprising that such a function of RIG-I is not present in the murine system. Despite the close genetic similarity between the murine and the human immune system, many dissimilarities are describes especially for innate immunity. For example,

while detection of bacterial DNA by TLR9 in the human system induces large amount of IFN-α, no IL-12 is induced by this pathway, leading to a succumbed, insufficient T_H-1 immune response. In contrast, in the murine system, stimulation of TLR9 leads to full blown, competent T_H-1 immune response including both the induction of IL-12 and IFN-α.

4.2.2. R848 stimulates human neutrophils

Off-target effects of RNA-based immunostimulatory ligands for TLR7 and TLR8 on neutrophils has recently been investigated thoroughly by Janke et al (Janke, Poth et al. 2009). They stimulated isolated human neutrophils with isRNA and R848 and saw activation of neutrophils but not eosinophils via TLR8. However, this effect was abolished when the RNA-backbone of isRNA consisted not of nuclease-stable phosphothioate but of regular phosphodiester bindings. In our experiments, we used nuclease unstable isRNA with phoshodiester backbone as a control and saw no activation of neutrophils, compared to high activation by TLR8ligand (R848). Our findings are therefore in accordance with Janke et al. for stimulation of TLR8. Therefore, as recognized by Janke et al., small-molecule TLR8 ligands and nuclease-stable RNA bear limitations for clinical utility. Similar to the results of Janke et al., we did not see any change in the expression profile of TLR7 and TLR8, even though the modes of stimulation were different. Janke et al. activated cells with IFN-β and then assed TLR expression compared to unstimulated cells; we used the known

neutrophil activator PMA. Both molecules are known to cause unspecific, general activation in neutrophils. Therefore, results are comparable.

4.2.3. TLR9 activates neutrophils only with pretreatment of GM-CSF

In the study of Hayashi et al., the authors reported an increased TLR9 expression in quantitative real-time PCR in human neutrophils and a significant response to CpG DNA with induction of IL-8 expression only after treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF). They also found that CpG DNA was not able to trigger the L-Selectin (CD62L) shedding without pre-treatment, which is interpreted as one form of activation of neutrophils. In our study, we saw no response to CpG2006 if human neutrophils were directly stimulated with CpG2006 without prestimulation. These findings suggest that human neutrophils need to first be activated to increase expression of TLR9, and with higher concentration of translated TLR9 protein human neutrophils can finally react to CpG DNA. We pre-treated human neutrophils with PMA. However, we did not see any change of protein expression of TLR9 in FACS analysis. This is in contrast to Hayashi et al. and could be explained by the different stimuli used for pretreatment. PMA is thought to cause general activation of neutrophils towards respiratory burst, while GM-CSF causes specific induction of the maturation process of granulocytes.

4.2.4. 3pRNA exhibited no activation of human neutrophils

In the study of Hornung et al, 3pRNAwas reported as a potent inducer of IFN-α in human monocytes. They used in vitro transcription to generate a dsRNA oligonucleotide with an overhang of one nucleotide at the 5' position. The two single-stranded oligonucleotides and the double-stranded oligonucleotide induced comparable levels of IFN-α in monocytes. Cleavage of the 5'-overhang (including the 5'-triphosphate) of the dsRNA or dephosphorylation of the 5' end completely abrogated the IFN response. They also observed that plasmacytoid dendritic cells (PDCs), however, showed no decrease in IFN production when oligonucleotides were dephosphorylated. Together, the results of Hornung et al. suggested that the 5'-triphosphate is at least one well-defined structural feature responsible for IFN-α–inducing activity of in vitro–transcribed RNA in monocytes and that a 5'-triphosphate confers IFN-α–inducing activity to both single-stranded RNA (ssRNA) and dsRNA. In addition, they demonstrated a direct binding on RIG-I by 3pRNA in human embryonic kidney (HEK) 293 cells.

In the light of these findings, the expression of RIG-I in human neutrophils and the functional response of RIG-I on human neutrophils were investigated by treating isolated human neutrophils with 3pRNA, and expression of surface markers CD62L and CD11b were used as markers of neutrophil activation. Even though we found low but significant expression of RIG-I on both the protein and mRNA level, we observed no activation of human neutrophils by 3pRNA. In addition, we

also stimulated human neutrophils with 3pRNA and assessed the production of TNF-α by human neutrophils after stimulation with different ligands, including 3pRNA. Compared to LPS and R848, which are known ligands to cause activation of human neutrophils, 3pRNA showed only marginal effect on the induction of TNF-α in human neutrophils (Figure 14).

In addition to stimulation of neutrophils with the complexed RIG-I ligand, we treated human neutrophils directly with 3pRNA in absence of complexion with lipofectamine in order to declare that the low existence of RIG-I in the cytoplasm of human neutrophils didn't result in the disability of activation by 3pRNA. However, we saw no activation of human neutrophils with the treatment of 3pRNA alone (Figure 16). Importantly, we confirmed colocalization of RIG-I on the cell surface and its ligand 3pRNA by confocal microscopy, excluding the possibility of early degradation of the RNA.

4.2.5. Poly(I:C) and dsDNA had no effect on human neutrophils

In the murine model, MDA5has been reported to be activated by poly(I:C) resulting in IFN production(Kato, Takeuchi et al. 2006). In our study, we stimulated human neutrophils with poly(I:C) and saw no activation. We did not compare these results to stimulation in the murine system, however, due to our findings in human cells and the findings of Kato et al.

in murine cells it is possible that neutrophils react differently to poly(I:C) for murine and human neutrophils.

4.3. Clinical Significance of our Findings

There is reasonable hope that the detection of small molecules capable of manipulating the human immune system will lead to some improvement of current adjuvant cancer therapies. This is especially true for such molecules that can simultaneously stimulate the immune system and cause genetic knock-down by RNA interference. A proof of principle of these molecules has been accomplished by Poeck et al. (Poeck, Besch et al. 2008). For many years it seemed that DNA based nucleotides stimulating TLR9 possessed sufficient potential for efficient anticancer vaccines; however the very encouraging results observed when treating murine tumors could unfortunately not be translated to human species. One of the reasons believed to be responsible for the poor efficiency of TLR9-ligands in the human species compared to the mouse is that while CpG-oligonucletoides can create a full blown T_H1 immune response in mice solely by stimulating TLR9; this is not the case in humans. For full T_H1 immune response in humans including the induction of both key cytokines, IFN-αand IL12, additional stimulation of TLR7 or TLR8 on myeloid dendritic cells is necessary. Much hope arose when it was discovered that RNA based oligonucleotides possessed this potential by sequence specific activation of TLRs, including TLR7 and other cytosolic PRRs, such as RIG-I. The discovery that these molecules

may simultaneously be used not only for specific immune activation but also gene silencing by RNA interfering make these molecules a promising addition to future anticancer strategies. Obviously, despite their enormous potential, off-target effects must be avoided at all times in order to prevent uncontrolled overstimulation of the immune system and potential harm to the patient. In this study it was shown that 3pRNA did not lead to any off-target effects when intended to stimulate RIG-I on human neutrophils on a molecular and cellular level. In order to investigate if these encouraging results hold true in a more advanced and complex *in vivo* model and small molecules such as 3pRNA are safe to be use as therapeutics is to be established by further and intense investigation.

5. SUMMARY

Neutrophils are typically the cells of the immune system to arrive earliest at the site of inflammation and infection. Due to their great number in peripheral blood and because these cells are equipped with a large variety of both regulatory and effective immune functions, they can significantly alter the course of an immune reaction. For example, human neutrophils are well known to express certain PRRs, and one of the PRRs is RIG-I. 3pRNA was recently described to be one physiologic ligand of cytosolic RIG-I. Activation of RIG-I by 3pRNA results in a direct induction of interferons (IFNs)and apoptosis in tumor cells.RNA based RIG-I ligands are currently under intense investigation for anticancer treatment strategies because it was discovered that such molecules could simultaneously cause specific gene knockdown of any desired gene, which creates huge potential for innovative therapeutic strategies toward a variety of infectious as well as inflammatory and tumorous diseases.

How human neutrophils are affected by these new therapeutic approaches and whether there are potential off target effects is currently under investigation. Due to their central role during any immune response, activation of neutrophils during an artificial, therapeutic immune response would significantly limit the potential of RNA-based anticancer molecules.

Very little is known about the expression of PRRs in neutrophils, especially the newly discovered cytosolic PRRs, such as RIG-I, MDA5, and AIM2.The aim of this study was therefore to answer the following questions in order to help to clarify the role of neutrophils during immunostimulation with RNA based immunotherapeutics.

1. If TLRs, MDA5, AIM2, and RIG-I are expressed in human neutrophils? If so, are they expressed on the cell surface or in the cytosol?

2. Whether or no these important receptors in human neutrophils can be activated by immunostimulatory molecules?

3. DMSO-treated HL60 cells have been reported to have similar characteristics with human neutrophils. Does this hold true for PRR expression profile, and do they serve as a convenient model for studying innate immune responses in human neutrophils?

In this dissertation, we were able to obtain the following results:

Human neutrophils showed expression of several TLRs, MDA5, AIM2, and RIG-I. However, the localization of RIG-I did not correspond with that of other cell types. While RIG-I has been described to be expressed in the cytosol, here we showed that in human but not murine neutrophils it is additionally expressed on the cell surface. We did not see any immunostimulatory effect of 3pRNA on human neutrophils, even though confocal microscopy confirmed binding of the ligand to RIG-I both at the cell surface and in the cytosol.

There are several significant differences in the expression profile of human neutrophils and untreated as well as DMSO-treated HL60 cells. Therefore, HL60 cells might not be an ideal model to study neutrophil innate immune mechanisms.

Even though these results give some insight into how neutrophils react to certain ligands for PRR and their expression profile on these cells, further research is needed in order to determine the full spectrum of underlying mechanisms that are responsible for the complex interaction of neutrophils with RNA molecules, both in a physiologic and a therapeutic setting.

Summary in German (Zusammenfassung)

Neutrophile Granulozyten sind Immunzellen, die vor allem dadurch charakterisiert sind, schnellstmöglich an einen Entzündungs- und Infektionsort zu gelangen. Auf Grund ihrer großen Anzahl im peripheren Blut und ihrer vielfältigen regulatorischen und effektiven Immunfunktionen können sie den Ablauf der Immunreaktion stark beeinflussen. Zum Beispielist bekannt, dass verschiedene PRRs auch in neutrophilen Granulozyten exprimiert werden. Mit RIG-I wurde kürzlich ein weiterer, wichtiger PRR beschrieben. Dieser Rezeptor befindet sich gewöhnlich im Zytosol einer Zelle und bindet dort virale RNA. 3pRNA konnte als ein spezifischer Ligand dieses Rezeptors identifiziert werden. Durch die Bindung der 3pRNA an RIG-I wird dieser Rezeptor aktiviert, und dieser Binding folgt eine direkte Induktion von Interferonen (IFNs). Auf RNA basierte Liganden von RIG-I und anderen PRRs stellen für zukünftige, moderne Tumortherapien einen interessanten Ansatz dar und werden derzeit intensiv beforscht.3pRNA Moleküle sind zusätzlich deshalb interessant, weil solche Moleküle neben immunstimulatorischen Eigenschaften zusätzlich ein spezifisches Gen-Knockdown induzieren können. Vor allem diese Kombination beinhaltet enormes Potential für innovative Therapiestrategien gegen verschiedene Infektionen, entzündlichen Erkrankungen, und Tumorerkrankungen.

In welcher Weise humane neutrophile Granulozyten durch diese neuen therapeutischen Methoden beeinflusst werden und ob es potentielle Off-Target-Effekte durch Aktivierung dieser Zellen gibt, wird derzeit

durchdringend erforscht. Auf Grund ihrer zentralen Rolle in jeder Immunreaktion würde das Potential von RNA-basierten Anti-Tumor Molekülen signifikant begrenzt werden, wenn neben den eigentlichen Targetzellen auch Granulozyten während einer künstlichen therapeutischen Immunreaktion aktiviert werden würden.

Noch ist wenig über die genaue Expression von PRRs in neutrophilen Granulozyten bekannt. Dies gilt besonders für die neu entdeckten intrazellulären PRRs, RIG-I, MDA5, und AIM2. Das Ziel dieser Doktorarbeit war es daher, die folgenden Fragen zu beantworten.

Werden TLRs, MDA5, AIM2, und RIG-I in humanen neutrophilen Granulozyten exprimiert? Welcher subzellulären Lokalisation unterliegen sie?

Im Fall einer signifikanten Expression erhebt sich zusätzlich die Fragestellung, ob diese Rezeptoren durch immunstimulatorische Moleküle aktiviert werden können.

Für mit DMSO behandelte HL60 Zellen wurden beschrieben, das diese ähnliche Eigenschaften wie humane neutrophile Granulozyten besitzen. Daher war ferner die Fragestellung interessant, ob eine Ähnlichkeit des Profils der Expressionen von PRRs in dieser Zellreihe besteht und ob diese Zelle gegebenenfalls für Untersuchungen dieser Rezeptoren herangezogen werden kann.

In dieser Dissertation haben wir die folgenden Ergebnisse erarbeiten können:

Humane neutrophile Granulozyten zeigten Expression von TLRs, MDA5, AIM2, und RIG-I.

Interessanterweise entsprach die Lokalisation von RIG-I in humanen neutrophilen Granulozyten nicht der von anderen beschriebenen Zelltypen. Während RIG-I gewöhnlicherweise intrazellulär exprimiert wird, konnten wir hier zeigen, dass RIG-I in humanen neutrophilen Granulozyten aber nicht in murinen Granulozyten auf der Zellmembran exprimiert wird.

Von besonderer Wichtigkeit ist, dass wir keinen immunstimulatorischen Effekt von 3pRNA auf humanen neutrophilen Granulozyten sahen, obwohl die intrazelluläre und membranöse Bindung von 3pRNA an RIG-I durch konfokale Mikroskopie bestätigt werden konnte.

Darüber hinaus konnten wir zeigen, dass es klare Unterschiede zwischen der Expression von humanen neutrophilen Granulozyten, nicht behandelten und DMSO-behandelten HL60 Zellen gibt. Deshalb ist es eher unwahrscheinlich, dass HL60 Zellen als einwandfreie Zell-linie für die Erforschung von humanen PRRs zumindest in Granulozyten geeignet sind.

Obwohl diese Ergebnisse einen guten Überblick darüber geben, wie neutrophile Granulozyten auf PRR-Ligande reagieren und wie deren Expressionsprofil bezüglich dieser Rezeptoren ist, sind weitere Untersuchung notwendig, um das komplette Spektrum unterliegender Mechanismen zu bestimmen, die für die komplexe Interaktion zwischen

neutrophilen Granulozyten und RNA Molekülen zuständig sind, sowohl im physiologischen als auch im therapeutischen Bereich.

References

Alexopoulou, L., A. C. Holt, et al. (2001). "Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3." Nature**413**(6857): 732-738.

Apostolopoulos, V. and I. F. McKenzie (2001). "Role of the mannose receptor in the immune response." Curr Mol Med**1**(4): 469-474.

Ariel, A., G. Fredman, et al. (2006). "Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression." Nat Immunol**7**(11): 1209-1216.

Baccala, R., K. Hoebe, et al. (2007). "TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity." Nat Med**13**(5): 543-551.

Berger, M., C. Y. Hsieh, et al. (2012). "Neutrophils express distinct RNA receptors in a non-canonical way." J Biol Chem**287**(23): 19409-19417.

Beutler, B., Z. Jiang, et al. (2006). "Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large." Annu Rev Immunol**24**: 353-389.

Buhtoiarov, I. N., Z. C. Neal, et al. (2011). "Differential internalization of hu14.18-IL2 immunocytokine by NK and tumor cell: impact on conjugation, cytotoxicity, and targeting." J Leukoc Biol**89**(4): 625-638.

Choubey, D., S. Walter, et al. (2000). "Cytoplasmic localization of the interferon-inducible protein that is encoded by the AIM2 (absent in melanoma) gene from the 200-gene family." FEBS Lett**474**(1): 38-42.

Chwae, Y. J., J. M. Lee, et al. (2007). "Activation-induced upregulation of inhibitory killer Ig-like receptors is regulated by protein kinase C." Immunol Cell Biol**85**(3): 220-228.

Collins, S. J., F. W. Ruscetti, et al. (1979). "Normal functional characteristics of cultured human promyelocytic leukemia cells

(HL-60) after induction of differentiation by dimethylsulfoxide." J Exp Med**149**(4): 969-974.

Dommett, R. M., N. Klein, et al. (2006). "Mannose-binding lectin in innate immunity: past, present and future." Tissue Antigens**68**(3): 193-209.

Doyle, S. L. and L. A. O'Neill (2006). "Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity." Biochem Pharmacol**72**(9): 1102-1113.

Ekman, A. K. and L. O. Cardell (2009). "The expression and function of Nod-like receptors in neutrophils." Immunology.

Ekman, A. K. and L. O. Cardell (2010). "The expression and function of Nod-like receptors in neutrophils." Immunology**130**(1): 55-63.

Fernandes-Alnemri, T., J. W. Yu, et al. (2009). "AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA." Nature**458**(7237): 509-513.

Fernandes-Alnemri, T., J. W. Yu, et al. (2010). "The AIM2 inflammasome is critical for innate immunity to Francisella tularensis." Nat Immunol**11**(5): 385-393.

Gallagher, R., S. Collins, et al. (1979). "Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia." Blood**54**(3): 713-733.

Hayashi, F., T. K. Means, et al. (2003). "Toll-like receptors stimulate human neutrophil function." Blood**102**(7): 2660-2669.

Hayashi, F., K. D. Smith, et al. (2001). "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." Nature**410**(6832): 1099-1103.

Hemmi, H., T. Kaisho, et al. (2002). "Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway." Nat Immunol**3**(2): 196-200.

Hemmi, H., O. Takeuchi, et al. (2000). "A Toll-like receptor recognizes bacterial DNA." Nature**408**(6813): 740-745.

Hornung, V., A. Ablasser, et al. (2009). "AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC." Nature**458**(7237): 514-518.

Hornung, V., J. Ellegast, et al. (2006). "5'-Triphosphate RNA is the ligand for RIG-I." Science**314**(5801): 994-997.

Hornung, V., S. Rothenfusser, et al. (2002). "Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides." J Immunol**168**(9): 4531-4537.

Jacob, C., M. Leport, et al. (2002). "DMSO-treated HL60 cells: a model of neutrophil-like cells mainly expressing PDE4B subtype." Int Immunopharmacol**2**(12): 1647-1656.

Janke, M., J. Poth, et al. (2009). "Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8." J Allergy Clin Immunol**123**(5): 1026-1033.

Jurk, M., F. Heil, et al. (2002). "Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848." Nat Immunol**3**(6): 499.

Kato, H., O. Takeuchi, et al. (2006). "Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses." Nature**441**(7089): 101-105.

Klinker, J. F., K. Wenzel-Seifert, et al. (1996). "G-protein-coupled receptors in HL-60 human leukemia cells." Gen Pharmacol**27**(1): 33-54.

Komuro, A. and C. M. Horvath (2006). "RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2." J Virol**80**(24): 12332-12342.

Kurt-Jones, E. A., L. Popova, et al. (2000). "Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus." Nat Immunol**1**(5): 398-401.

Muzio, M., D. Bosisio, et al. (2000). "Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells." J Immunol**164**(11): 5998-6004.

Nishihira, J. and J. T. O'Flaherty (1985). "Phorbol myristate acetate receptors in human polymorphonuclear neutrophils." J Immunol**135**(5): 3439-3447.

Nomura, F., S. Akashi, et al. (2000). "Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression." J Immunol**164**(7): 3476-3479.

Nygard, A. B., C. B. Jorgensen, et al. (2007). "Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR." BMC Mol Biol**8**: 67.

Ohashi, K., V. Burkart, et al. (2000). "Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex." J Immunol**164**(2): 558-561.

Okamura, Y., M. Watari, et al. (2001). "The extra domain A of fibronectin activates Toll-like receptor 4." J Biol Chem**276**(13): 10229-10233.

Ozinsky, A., K. D. Smith, et al. (2000). "Co-operative induction of pro-inflammatory signaling by Toll-like receptors." J Endotoxin Res**6**(5): 393-396.

Ozinsky, A., D. M. Underhill, et al. (2000). "The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors." Proc Natl Acad Sci U S A**97**(25): 13766-13771.

Pichlmair, A., O. Schulz, et al. (2006). "RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates." Science**314**(5801): 997-1001.

Platt, N., R. P. da Silva, et al. (1999). "Class A scavenger receptors and the phagocytosis of apoptotic cells." Immunol Lett**65**(1-2): 15-19.

Poeck, H., R. Besch, et al. (2008). "5'-Triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma." Nat Med**14**(11): 1256-1263.

Poltorak, A., I. Smirnova, et al. (1998). "Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as

a candidate gene in the critical region." Blood Cells Mol Dis**24**(3): 340-355.

Rathinam, V. A., Z. Jiang, et al. (2010). "The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses." Nat Immunol**11**(5): 395-402.

Schroder, K., D. A. Muruve, et al. (2009). "Innate immunity: cytoplasmic DNA sensing by the AIM2 inflammasome." Curr Biol**19**(6): R262-265.

Schwartz, R. H. (2005). "Natural regulatory T cells and self-tolerance." Nat Immunol**6**(4): 327-330.

Takeuchi, O. and S. Akira (2008). "MDA5/RIG-I and virus recognition." Curr Opin Immunol**20**(1): 17-22.

Takeuchi, O., K. Hoshino, et al. (2000). "Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection." J Immunol**165**(10): 5392-5396.

Takeuchi, O., K. Hoshino, et al. (1999). "Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components." Immunity**11**(4): 443-451.

Takeuchi, O., T. Kawai, et al. (1999). "TLR6: A novel member of an expanding toll-like receptor family." Gene**231**(1-2): 59-65.

Termeer, C., F. Benedix, et al. (2002). "Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4." J Exp Med**195**(1): 99-111.

Underhill, D. M., A. Ozinsky, et al. (1999). "Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages." Proc Natl Acad Sci U S A**96**(25): 14459-14463.

Visintin, A., A. Mazzoni, et al. (2001). "Regulation of Toll-like receptors in human monocytes and dendritic cells." J Immunol**166**(1): 249-255.

Wang, G. L., D. L. Ruan, et al. (1998). "Xa21D encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution." Plant Cell**10**(5): 765-779.

Wittmann, S., G. Rothe, et al. (2004). "Cytokine upregulation of surface antigens correlates to the priming of the neutrophil oxidative burst response." Cytometry A**57**(1): 53-62.

Wornle, M., M. Sauter, et al. (2009). "Novel role of toll-like receptor 3, RIG-I and MDA5 in poly (I:C) RNA-induced mesothelial inflammation." Mol Cell Biochem**322**(1-2): 193-206.

Yarovinsky, F., D. Zhang, et al. (2005). "TLR11 activation of dendritic cells by a protozoan profilin-like protein." Science**308**(5728): 1626-1629.

Abbreviations and Acronyms

3pRNA : triphosphate RNA

AIM2 : absent in melanoma-2

APC: allophycocyanin

APC : antigen-presenting cells

ASC : apoptosis- associated speck-like protein

BCR : B cell receptor

CARD : caspase recruitment domains

CCR5 : C-C chemokine receptor type 5

cDNA : complementary DNA

DAPI : 4´,6-diamidino-2-phenylindole, dihydrochloride

DMSO: dimethyl sulfoxide

dNTPs : deoxyribonucleotide triphosphate

dsDNA : double-stranded DNA

dsRNA : double-stranded RNA

DTT: dithiothreitol

EDTA : ethylenediaminetetraacetic acid

ELISA : enzyme-linked immunosorbent assay

FACS : fluorescence activated cell sorting

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

GM-CSF : granulocyte-macrophage colony-stimulating factor

HEK : human embryonic kidney

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIN-200 : hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats

HL60 cells : human promyelocytic leukemia cells

HLA : human leukocyte antigen

Hsp 60 : heat shock protein 60

IFN : inteferon

IKK : IkappaB-related kinase

IL-8 : interleukin 8

isRNAs : immunstimulatory RNAs

LAM : lipoarabinomannan

LPS : lipopolysaccharides

MALP-2 : macrophage-activating lipopeptide 2

MAPK : mitogen-activated protein kinase

MBL : mannan-binding lectin

MDA5 : melanoma differentiation associated gene-5

MFI ratio: mean fluorescence intensity ratio

MHC : major histocompatibility complex

mRNA : messenger RNA

NGS : normal goat serum

NHS-Fluorescein : fluorescein N-hydroxy-succinimidyl-ester

NLRs : NOD-like receptors

PAMPs : pathogen-associated molecular patterns

PBS : phosphate buffered saline

PCR: polymerase chain reaction

PDCs : plasmacytoid dendritic cells

PE: phycoerythrin

PGRs : peptidoglycan recognition proteins

PMA : phorbol-myristate-acetate

Poly(dA-dT) • Poly(dA-dT): poly(deoxyadenylic-thymidylic) acid sodium salt

poly(I:C) : polyinosinic:polycytidylic acid

PRRs : pattern recognition receptors

qPCR : quantitative PCR

RHs : RNA helicases

RIG-I : retinoic acid inducible gene I

RNAi : RNA Interference

RPMI : Roswell Park Memorial Institute

siRNA : small interfering RNA

ssRNA : single-stranded RNA

TBK1 : TANK-binding kinase 1

TBP: TATA-binding protein

TCR : T cell receptor

TLRs : Toll-like receptors

TNF-α : tumor necrosis factor-alpha

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