Mechanisms of sterile inflammation and modulation of TLR4 activity

Inaugural-Dissertation
zur
Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für
Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Chiranjeevi Chebrolu
Geboren in Repalle

Essen, Februar 2017

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Institut für Fakultät für Medizinische Mikrobiologie der Universität Duisburg-Essen durchgeführt.

1. Gutachter: Prof. Dr. Carsten Kirschning
2. Gutachter: PD Dr. Bernd Giebel

Vorsitzender des Prüfungsausschusses: Prof. Dr. Matthias Gunzer

Tag der mündlichen Prüfung: 28. Juni. 2017

Table of Contents

List of abbreviations

-	untreated
-/-	knock-out
aa	amino acid
ATP	adenosine triphosphate
BM	bone marrow
BMMs	bone marrow derived macrophages
BMT	bone marrow transplant
bp	base pair(s)
CpG	cytosine-guanosine Oligonucleotide
Da	Dalton
DAMP	danger associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
ds	double stranded
ER	endoplasmic reticulum
Gy	Gray
hiSa	heat inactivated *Staphylococcus aureus*
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	interferon
IIR	ionizing irradiation
IL	interleukin
kb	kilo base
k.o.	knock out
LPS	lipopolysaccharide
LRR	leucine-rich repeats
LTA	lipoteichoic acid
Mb	megabase(s)
NO	nitric oxide
n.d.	not detected
n.p.	not performed
ODN	oligodeoxynucleotide
ORN	oligo ribonucleotide
P_3C	Pam_3CSK_4
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PGN	peptidoglycan
PRR	pattern recognition receptor
r	ribosomal
ROS	reactive oxygen species
rpm	revolutions per minute
ss	single stranded
TLR	Toll-like receptor
TNF	tumor necrosis factor
tRNA	transfer RNA
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
wt	wild type

1. Introduction

1.1 The immune system

The immune system comprises cells, tissues, and organs that protect from microbial and endogenous sterile insults, thus not always distinguishing between non-self and self. The immune system branches into two subsystems, namely the evolutionary old innate immune system which provides immediate defense, and the advanced adaptive immune system which upon by random somatic mutation and resultant clonal individuality provides increased specificity potentially. Innate immunity thus encompasses phagocytosis and antimicrobial peptide such as defensins production (Fearon and Locksley, 1996).

1.1.1 Innate immune system

The innate immune system is the first line of defense which provides an immediate immune response and is microbial clade rather than species specific. Physical barricades including skin, fluids such as saliva, gastric fluids, mucosal endo and exothelia and mucus serve to trap and entangle microorganisms (Boyton and Openshaw, 2002) such as bacteria, viruses and parasites as well as toxins thronging potential hosts. If pathogens breach these primary barriers, innate immune second line of defense is surveilling macrophages, scavenger neutrophil granulocytes, and dendritic cells (DCs) present in tissue and blood. They sense and mediate pathogen presence. DCs and other antigen presenting cells link the innate and the adaptive immune systems by presenting antigens to T cells of which specific clones get activated to support B cell function and perform effector activity also in concert with natural killer cells. The complement system is a group of serum proteins which digest themselves consecutively to opsonize and create pores in the invading pathogens and attract immune cells towards the area of infection (Rus et al., 2005). NK cells identify virally infected host tumor cells by monitoring changes in cell surface constitution such as MHC I expression.

1.1.2 Adaptive immune system

Pathogens rapidly evolve and adapt and thereby avoid detection by the innate immune system. Four to seven days post infection thus a second layer of protection namely adaptive immunity becomes operative. The adaptive immune system

mediates a pathogen and even antigen specific immune response which is present in jawed vertebrates. A major characteristic feature of the adaptive immune system is it´s remembrance and generation of memory cells to be wakened upon secondary challenge. Its activation relies on recognition of specific microbial specific and thus “non-self” antigens such as upon antigen presentation or soluble antigen recognition by B cells. Antigen specificity underlies immune responses that are pathogen or pathogen infected cell specific (Kasamatsu, 2013; Litman et al., 2010).

The adaptive immune system includes B cells and T cells derived from hematopoietic stem cells. Both B and T cells carry alterable receptors molecules that recognize specific “non-self” epitopes such as small fragments of pathogen that have been processed and presented on major histocompatibility complex (MHC) molecules. Antibodies on the surface of B cells or insoluble form bind to a specific soluble antigen. Antibody antigen complexes are taken up by the cell, processed upon proteolysis into peptides and presented on surface MHC class II molecules. MHC antigen complexes attract matching helper T cells and activate them. Activated B cells convert into antibody producing plasma cells. Killer T cells are activated when T cell receptor (TCR) binds to a specific antigen in a complex with MHC class I on the target cell, whereas helper T cells such as $T_h1$and T_h2 recognize antigens presented on MHC class II molecules (Litman et al., 2010).

1.2 Pattern recognition receptors

Pattern recognition receptors (PRRs) are germ line encoded receptors expressed mainly by immune cells such as macrophages, epithelial cells, DCs, neutrophils, B cells, and possibly T cells to sense the presence of microorganisms by binding conserved structures, namely immune stimulatory and xenobiotic pathogen associated molecular patterns (PAMPs). PRRs sense also endogenous danger such as damaged cell compounds termed danger associated molecular patterns (DAMPs). Four different classes of PRRs have been established. They encompass cell or endosomal membrane bound transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as well as cytoplasmic Retinoic acid-inducible gene -I-like receptors (RLRs) and NOD-like receptors (NLRs) (Greaves and Gordon, 2009; Hawlisch and Kohl, 2006; Hoving et al., 2014; Jiang et al., 2014; Netea et al., 2004; Takeuchi and Akira, 2010; Underhill and Ozinsky, 2002).

1.2.1 Toll- like receptors

TLRs are conserved germ line encoded PRRs expressed primarily by innate immune cells towards sensing of invading foreign pathogens. TLRs are PRRs that recognize microbial PAMPs or damage/danger associated molecular patterns (DAMPs) from damaged tissue. The concept of innate PAMP receptors dates back to 1989 (Medzhitov and Janeway, 1997). A first candidate molecule considered was IL-1 R type 1 (IL-1R1)-like randomly sequenced cDNA (RSC) 786, later named TLR1 (Mitcham et al., 1996). In 1985, mutations of the *Drosophila melanogaster* Toll gene had been described and were later cloned (Hansson and Edfeldt, 2005; Hashimoto et al., 1988).

In 1991 IL-1R1 domain was shown to be homologous to the cytosolic domain of a *D. melanogaster* protein termed “Toll” (Gay and Keith, 1991). In *D. melanogaster* Toll mediates embryogenesis in respect to dorsoventral polarization. It was later found that PRRs are expressed not only in invertebrates but also in mammals (Kirschning et al., 1998; Lemaitre et al., 1996; Mitcham et al., 1996; Poltorak et al., 1998; Qureshi et al., 1999; Yang et al., 1998). Thirteen human and murine TLRs have been identified out of which mice lack the expression of TLR10 and humans lack the expression of TLR11, -12, and -13. TLRs mediate specific PAMP and possibly DAMP recognition (Fig. 1).

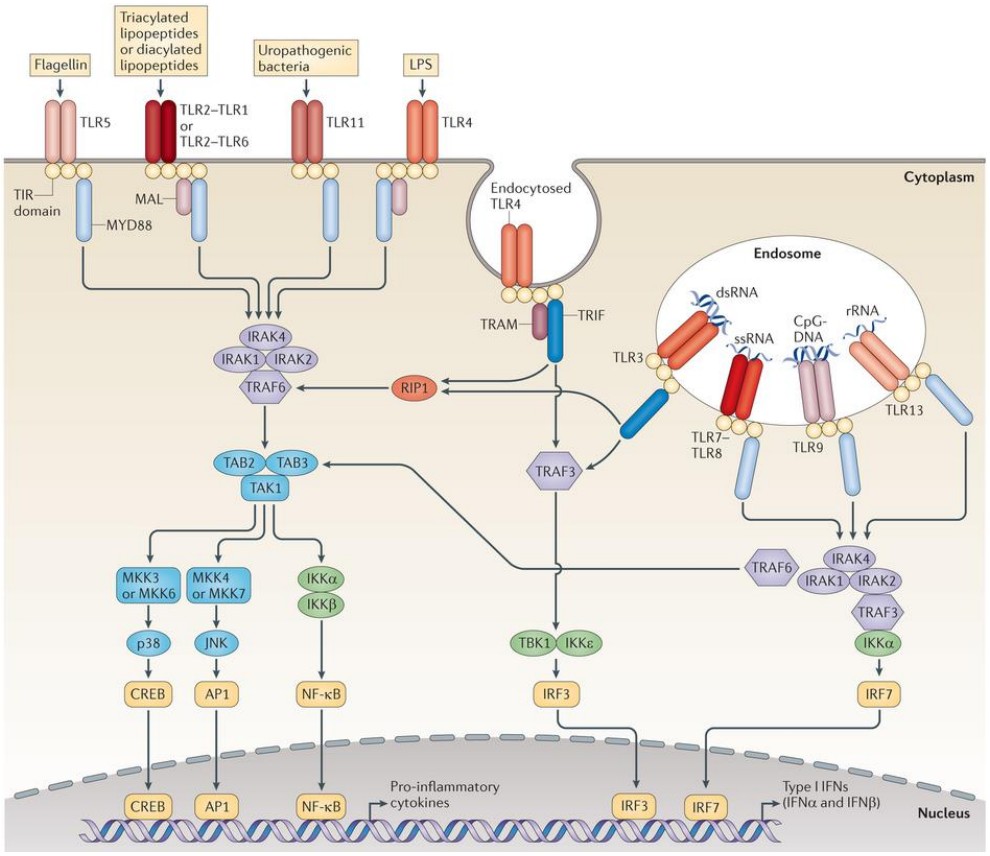


Figure 1: TLR signaling pathways. TLRs are localized in cytoplasmic or endosomal membranes. Upon activation, TLRs mediate pro-inflammatory cytokine, chemokine and type-I IFN production. TLRs recruit common adaptor molecules. Upon activation, TLR5, 7, 8, 9, 10, 11, 12 and 13 as well as IL-1 receptors interact with MyD88. TLR2 and TLR4 additionally employ the adaptors MAL and TRAM, while TRIF is recruited by TLR3 and TLR4 (MYD88, myeloid differentiation factor protein 88; TRIF, toll interleukin-1 protein containing interferon beta; TRAM, TRIF- related adaptor molecule; TRAF, TNF receptor associated factor; IRAKs, interleukin-1 receptor-associated kinases; IRF, interferon regulatory transcription factor; IKK, IκB kinase) (Figure from (O'Neill et al., 2013)).

TLRs forms a characteristic horse-shoe like extra cellular domain (ECD) structure coupled to the transmembrane domain (TM) to which a Toll/interleukin-1 receptor (TIR) intracellular domain (ICD) is attached that is carried also by interleukin-1 receptors (IL-1Rs). The extracellular domains are a string of tandem leucine-rich repeats (LRRs; LXXLXXLXLXN, L: leucine, X: arbitrary amino acid, N: asparagine). Single LLRs are stabilized by conserved hydrophobic residues (XØXXØX4FXXLX motif; X: arbitrary, Ø: hydrophobic amino acid, F: phenylalanine) facing inwards. The internal and the external domains of a TLR are connected by a membrane spanning α-helix consisting of 20 nonpolar amino acids. The arrangement of LRRs determines the ligand specificity for each TLR (Botos et al., 2011). TLRs are localized either at/in the cell surface such as TLR1, TLR2, TLR4, TLR5 and TLR6 or in the endosomal

membrane such as TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13. TLRs on the cell surface are largely involved in the detection of bacterial cell wall products in the extracellular space, while most endosomal TLRs detect nucleic acids of viral and bacterial origin according to the current view (Kawai and Akira, 2010) (Table 1).

Table 1: TLR specific ligands and their origin

TLR	**Ligand**	**Origin**
TLR1/2	triacylated proteins	bacteria
TLR2	lipoproteins,peptidoglycan, LTA, LPS, zymosan	bacteria, fungi
TLR2/6	diacylated proteins	Mycoplasma
TLR3	dsRNA, Poly I:C	viruses, bacteria
TLR4	LPS	Gram-negative bacteria
TLR5	flagellin	flagellated bacteria
TLR7	ssRNA, R848, Loxoribine, Imiquimod RNA	viruses, bacteria
TLR8	ssRNA, R848, Loxoribine RNA	viruses, bacteria
TLR9	DNA, unmethylated CpG DNA	viruses, bacteria
TLR10	un known	viruses, *Plasmodium*
TLR11	profilin, flagellin	*Toxoplasma gondii*
TLR12	profilin	*Toxoplasma gondii*
TLR13	Bacterial 23S rRNA segment "Sa19"	bacteria

Binding of PAMPs or DAMPs to TLRs results in dimerization of two TLR receptor molecules wherein TLR2 forms either heterodimers with TLR1 or TLR6 or homodimers (Ozinsky et al., 2000). Allosteric conformational change results from dimerization (Colonna, 2007) of the TIR domain to recruit adaptor molecules. Five such adaptor molecules are known, namely myeloid differentiation factor 88 (MyD88), Myd88-adaptor-like protein (MAL), TIR domain-containing adaptor inducing interferon-β (TRIF/Ticam)-1, TRIF related adaptor protein (TRAM/Ticam)-2 and sterile-α and HEAT/armadillo motifs-containing protein (SARM) (Takeda and Akira, 2004). A sixth adaptor molecule BCAP (B cell adaptor for phosphoinositid-3-kinase (PI3K)) has been identified (Troutman et al., 2012). The first interactions are classified in to two different signaling cascades such as Myd88-dependent and TRIF-dependent pathways (Brikos and O'Neill, 2008).

All TLRs except for TLR3 recruit MyD88 to mediate the phosphorylation such as of interleukin-1 receptor-associated kinases (IRAKs). They mediate ubiquitination of TNF receptor associated factor 6 (TRAF6) which further mediates phosphorylation of mitogen-activated kinases (MAPKs) through the TAK-complex, resulting in NF-κB activation via phosphorylation and dissociation of the IκB kinase (IKK)-complex which consists of IKK-α, IKK-β and IKK-γ and mediates the dissociation of the inhibitor IκB. Dephosphorylated IκB is bound to the NF-κB subunits p50, p52 and p56 to retain

them in the cytosol (Hacker et al., 2011). TLR3 and TLR4 utilize the TIR domain containing adaptor inducing interferon β (TRIF) wherein TLR3 does not employ MyD88 (Fig. 1). Recruitment of specific adaptor proteins MyD88, MyD88 adaptor like (MAL) also known as TIR domain containing adaptor protein (TIRAP) (Fitzgerald et al., 2001), and TRIF- related adaptor molecule (TRAM) function as bridging molecules between TLR and MyD88 or TRIF. MAL is involved in Myd88 driven induction of NF-κB downstream of TLR2 and TLR4 (Horng et al., 2002). MAL also inhibits TLR3 signaling (Brikos and O'Neill, 2008; Kenny et al., 2009; O'Neill and Bowie, 2007). On the other hand, TRAM acts as a switch for the internalized TLR4 and TRIF to induce type-1 IFNs such as IFNα/β in an IRF3 dependent manner. Endocytosed TLR4 mediates activation of a TRAM-TRIF pathway (Kagan et al., 2008; Tanimura et al., 2008; Zanoni et al., 2011). Endoplasmic reticulum (ER) - endosome translocation is mediated by the chaperon Unc93B1. It is a highly conserved protein consisting of 12 trans-membrane domains and two N-terminal glycosylation motifs (Brinkmann et al., 2007; Kashuba et al., 2002). Endosomal TLR localization thus depends on Unc93B1 for the endosomal TLR translocation (Itoh et al., 2011; Lee et al., 2013). Accordingly, a missense point mutation in Unc93B1 protein, called 3D (H412R), abrogates endosomal TLR translocation and thereby sensing of endosomal nucleic acids (Tabeta et al., 2006).

1.2.2 C-type lectins

The superfamily of proteins containing C-type lectin-like domain (CTLD) is a large group of extracellular surface metazoan proteins. The CTLD carries a characteristic double-loop (loop-in-a-loop) stabilized by two highly conserved disulfide bridges located at the bases of the loops, as well as conserved hydrophobic and polar interactors. They are sub divided into Ca^{2+} dependent and independent carbohydrate binding proteins. They are involved in cell adhesion, tissue regeneration and remodeling, as well as platelet and complement activation and pathogen recognition mediating endo- and phagocytosis. CLRs are expressed by dendritic cells. CLRs signal towards expression of specific cytokines which determine T cell polarization fates. On the basis of their molecular structures CLRs are divided into Type I consisting of DEC-205 and the macrophage mannose receptor (MMR) and Type II consisting of Dectin-1, Dectin-2, macrophage inducible C-type lectin (Mincle), the dendritic cell specific ICAM3-grabbing non-integrin (DC-SIGN), DC NK lectin group receptor-1 (DNGR-1), and soluble CLR, namely mannose binding lectin (MBL), which

forms oligomers upon binding to ligands in the blood serum and activates the complement system (Dambuza and Brown, 2015; Gal and Ambrus, 2001; Kerrigan and Brown, 2009). It is also an oligomeric protein that binds to an array of carbohydrate patterns on the surface of pathogens.

1.2.3 NOD-like receptors and NLRPs

NOD-like receptors (NLRs) are cytoplasmic pathogen sensors (Inohara et al., 2005). They carry three distinctive domains, variable N-terminal protein-binding domain, a central nucleotide-binding domain (NACHT), and a C-terminal leucine-rich repeat (LRR) domain (Lamkanfi and Dixit, 2012; Ting et al., 2008). N-terminal portions consist of caspase recruitment domain (CARD), pyrin domain (PYD), and/or baculovirus inhibitor of apoptosis protein repeat (BIR) domain (Kanneganti et al., 2006). 22 murine and 34 human NLRs operative are divided into five subfamilies. NOD1 and NOD2 are the first known NLRs, except for NODs other NLRs assemble in the cytosol to form inflammasomes and mediate recognition of bacterial peptidoglycans, g-D-glutamyl-mesodiaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively and activate transcription factors leading to the induction of pro-inflammatory mediators through receptor-interacting protein 2 (RIP2) (Philpott et al., 2014).

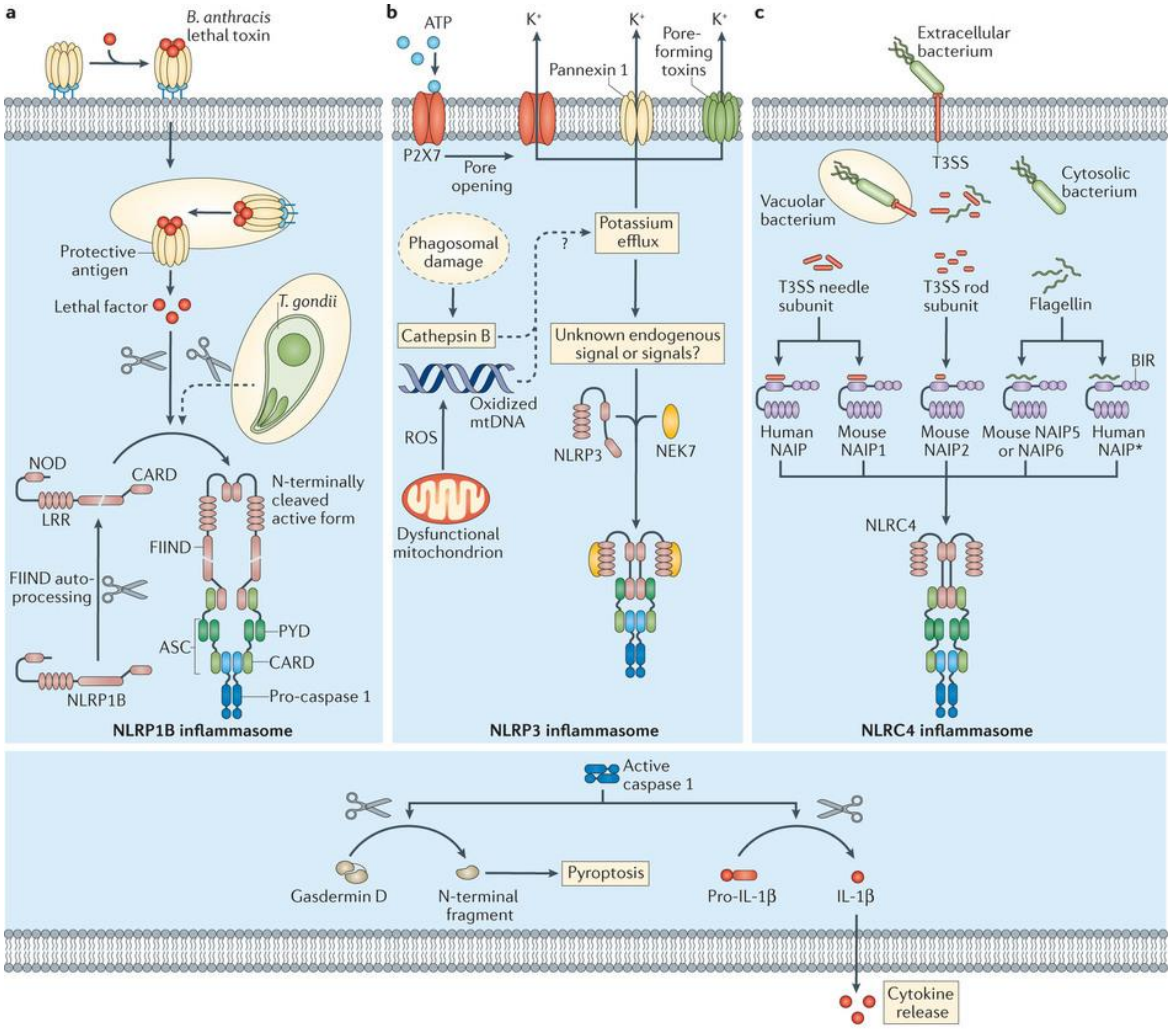


Figure 2: The NLRP inflammasomes. Mouse NLRP1B and rat NLRP1 bind *Bacillus anthracis* lethal factor and *Toxoplasma gondii* infection (a); Potassium efflux is associated with activity elicited by a diverse array of NLRP3 stimuli (b); NLR apoptosis inhibitory proteins (NAIP) NLRC4 binds bacterial flagellin and the type 3 secretion system (T3SS) needle and rod subunits (c) (Figure from (Broz and Dixit, 2016)).

Adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (Schroder and Tschopp, 2010) is involved in inflammasomes formed by NLRP1, NLRP3, NLRC4, NLRP6 and AIM-2. Inflammasomes are multiprotein complexes which govern the activation of caspase-1 (Lamkanfi and Dixit, 2014). Caspase 1 is the IL-1-converting enzyme and catalyzes cleavage of pro-IL-1β, -IL-18, and -IL-33 into their respective released and thus active forms (Walker et al., 1994).

1.2.4 RIG-I like helicases

The retinoic acid-inducible gene I (RIG-I) like receptor (RLR) family is composed of RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Takeuchi and Akira, 2009; Yoneyama and Fujita, 2008). They are localized within the cytoplasm to recognize ss- and ds-RNA. The expression of RLRs is enhanced in response to type IIFN stimulation or virus infection. RIG-I and MDA5 consist of two N-terminal CARDs, a central RNA helicase DEAD-box and a regulatory C-terminal domain (CTD). In contrary, LGP2 lacks a CARD. The helicase domain mediates the recognition of dsRNA, whereas CARD domains are required to relay the signal.

Although RIG-I and MDA5 recognize different ligands, they share common signaling features. Upon recognition of ds and ss viral RNA, both recruit adaptor protein IPS-1 (also known as MAVS, CARDIF or VISA) to the outer membrane of mitochondria towards activation of transcription factors such as IRF3, IRF7 (interferon regulatory factor) and NF-κB to drive type-I interferon and pro-inflammatory cytokine production (Freaney et al., 2013; Kawai et al., 2005; Liu et al., 2013; Takeuchi and Akira, 2008). Recognition of dsRNA by RIG-I/MDA-5 and TLR3 is cell type dependent. Conventional dendritic cells (cDCs), macrophages and fibroblasts isolated from *RIG-I*$^{-/-}$ and *MDA-5*$^{-/-}$ mice IFN induction after viral RNA infection was impaired, while plasmacytoid dendritic cells (pDCs) IFN production was normal (Kato et al., 2005).

1.3 Therapeutic modulation of pattern recognition receptor activity exemplified by TLR4 blockade or activation.

Lipopolysaccharide (LPS, also known as endotoxin) is a prototypic immune stimulatory PAMP and a component of the outer membrane of the cell envelope of Gram-negative bacteria (Raetz and Whitfield, 2002). TLR4 has been implicated as an exquisite immune stimulatory signal transducing cellular LPS receptor (Beutler and Rietschel, 2003). Besides TLR4, a co-receptor namely myeloid differentiation factor (MD) -2, other receptors such as TLR2 or caspase-4 mediate the recognition of LPS (Hagar et al., 2013; Kayagaki et al., 2013; Shimazu et al., 1999; Spiller et al., 2007; Tan and Kagan, 2014; Werts et al., 2001).

Moreover, numerous other immune stimulatory PAMPs and DAMPs of endogenous origin such as HMGB1, ATP as well as allergens have been associated with TLR4

(Bald et al., 2014; Biragyn et al., 2002; Hagar et al., 2013; Kayagaki et al., 2013; Kurt-Jones et al., 2000; Malley et al., 2003; Park and Lee, 2013; Qiang et al., 2013; Ryan et al., 2011; Schmidt et al., 2010; Termeer et al., 2002; Trompette et al., 2009; Vogl et al., 2007). Accordingly, TLR4 confers immunity to microbes of different classes and mediates immune activation upon sterile host insults. However, the TLR4/MD-2 complex together with the LPS binding protein (LBP) and cluster of differentiation (CD) 14 through which a monomeric LPS molecule is being recruited to MD-2 is most likely the first line cellular sensor of Gram-negative bacteria (Beutler and Rietschel, 2003; Schumann, 2011; Spiller et al., 2008; Tan and Kagan, 2014).

The lipid-containing moiety of LPS, namely hexaacylated bisphosphorylated lipid A is recognized by the TLR4/MD-2 complex and is attached by the hydrophobic binding pocket of MD-2. While five out of six lipid chains are accommodated within the deep binding groove of the co-receptor protein, the sixth acyl chain is exposed on the surface of MD-2 at the secondary dimerization interface, which drives the homodimerization of two TLR4/MD-2/LPS complexes thus leading to cell activation as has been shown for *E. coli* LPS (Park and Lee, 2013; Park et al., 2009).

In contrast, all four lipid chains of the tetraacylated bisphosphorylated lipid A structures such as biosynthetic precursor of *E. coli* lipid A, lipid IVa, or synthetic drug candidate Eritoran, are fully inserted in the hydrophobic cavity of hMD-2 which prevents the dimerization of two receptor complexes and claim these compounds as hTLR4/hMD-2 antagonists (Kim et al., 2007; Ohto et al., 2007; Peri and Piazza, 2012; Thieblemont et al., 1998). Though lipid IVa possessing antagonistic properties on hTLR4, it was shown to exhibit agonistic activity on mTLR4 the structural basis of the so called “m”-shaped 2:2:2 complex of which has been proposed and subsequently demonstrated (DeMarco and Woods, 2011; Ohto et al., 2012).

1.4 TLRs as nucleic acid sensors.

Pattern recognition encompasses ligand binding and activation of the host cell to mount a host defense against invading causatives. Prototype immune stimulatory PAMPs largely lack similarity with host products. Examples are LPS and the bacterial 23S rRNA segment that is the binding site of macrolide lyncosamin streptomycin (MLS) antibiotics activating TLR2 and TLR4 as well as TLR13, respectively. Its chemical structure distinguishes Gram-negative bacterial LPS while the sequence

rather than the principal chemical structure of unmodified bacterial 23S rRNA segment activating TLR13 differs from abundant host RNA.

Recently, sensing of homeostasis perturbance such as upon toxic or sterile tissue injury triggering release and binding of inorganic toxins and host borne constituents has been observed for otherwise PAMP binding PRRs. TLR4 recognizes nickel and TLR2 and/or -4 bind for instance extracellular matrix compounds such as hyaluronan and biglycan as well as various cytoplasmic proteins such as high mobility group box protein B1 (HMGB1) and myeloid-related protein (MRP8/MRP14) (Table 2). Moreover, host cellular nucleic acids are known to activate host cells such as through TLR7 (Raghavan et al., 2012).

However, whether RNA recognition depends on specific motifs and thus on a limited total RNA portion or whether its recognition is largely independent upon its sequence such as exemplified by DNA the sugar backbone of which is a major TLR9 sensing determinant is largely unexplored. Notwithstanding, the high frequency of infection free clinical conditions such as trauma, auto immune and graft versus host disease eliciting inflammation like sepsis mandates understanding of the underlying pathogeneses and thus comprehensive identification of its triggers such as DAMPs.

Mitochondria are mediators of pattern recognition and possibly due to their evolutionary kinship with bacteria might be particularly rich DAMP sources. Indeed, mitochondrial constituents such as unknown agent and DNA have been implicated as TLR4 and TLR9 activators, respectively (Hauser et al., 2010; Nicholas et al., 2011). However, specific mitochondria ribosomal constituents have not yet been explored for their immune stimulatory property. We considered mitochondrial RNA as good tissue injury signal and thus immune stimulatory DAMP candidate. It is known that various antibiotics such as specific ones inhibiting bacterial protein synthesis by ribosome binding interfere also with mitochondrial protein biosynthesis and thus induce mitochondriopathy resulting in irreversible hearing loss. However, structural features such as the size of mtRNAs differ to a substantial degree as compared to bacterial rRNAs. Thus bacterial ribosome targeting antibiotics do not affect host homeostasis principally. Upon observing a relatively strong immune stimulatory capacity of 16S mtRNA as compared to that of 12S mtRNA, we focused here on specific segments of the former RNA molecule that resembles 23S bacterial rRNA and predictably shares functional and/or sequence characteristics with the latter.

TLR13 specificity for a unique 23S rRNA segment depends on the identity of individual bases contained by it indicating TLR13´s exceptionally high sequence dependency.

1.5 Danger/damage signals in immunology

In the year 1994 immune stimulatory and self-derived DAMP recognition by organisms which constitute a lack of the innate immune system was brought up and challenged discrimination between self and non-self as a major function of the immune system (Matzinger, 1994). Accordingly, danger/injury released DAMPs bind to innate receptors (Land; Seong and Matzinger, 2004). Uric acid was identified as “the first” DAMP in 2003 (Chen et al., 2006; Shi et al., 2003). DAMPs released during hostile conditions such as cell stress and tissue injury are sensed by specific PRRs present on the cell surface or in the cytosolic compartments. DAMPs can be classified based on the origin and sources such as extracellular matrix or plasma membrane. A large variety of DAMPs from various sources are generated and released upon trauma (Land et al., 2016) (Table 2).

DAMPs released from renal allografts post Ischemia/reperfusion-injury (I/RI) (Kawai and Akira, 2010; O'Neill et al., 2014; Tang et al., 2011; Vanaja et al., 2015) trigger sterile I/RI-mediated inflammation upon recognition by PRRs such as NLRs and AIM2 like receptors (ALRs) that form inflammasomes (Di Virgilio and Vuerich, 2015; Man and Kanneganti, 2015), but also TLRs. However, numerous DAMP-PRR assignments are a matter of discussion and ongoing investigations have been challenged by publications previously (Gao and Tsan, 2003).

Table 2: Prominent DAMPs associated with cell stress/cell death, and/or tissue injury and proposed cognate receptors.

DAMP	Mode of emission	Receptor
Adenosine triphosphate (ATP)	Mostly passively released; sometimes actively secreted	P2Y2; P2X7; (indirectly: NLRP3)
Biglycan (BGN)	Extracellular matrix	TLR2, TLR4, P2X4, P2X7
Calreticulin (CALR)	Mostly surface exposed; sometimes passively released	CD91
Fibrinogen	Extracellular matrix	TLR4
Fibronectin extra domain A	Extracellular matrix	TLR4
Heat shock proteins HSP70/72, HSP90, HSP60	Surface exposed; actively secreted; passively released	TLR2, TLR4, CD91, SREC-1, FEEL-1
Heparan sulfate fragments	Extracellular matrix	TLR4
High-mobility group box 1 (HMGB1)	Mostly passively released; sometimes actively secreted	TLR2, TLR4, RAGE, TIM3
Hyaluronan fragments (fHA)	Extracellular matrix	TLR2, TLR4, NLRP3?
MHC class I chain-related proteins (MICs)	Surface exposed	NKG2D
Monosodium urate (MSU) or uric acid	Passively released	Purinergic receptors (indirect: NLRP3)
Nonmuscle myosin II-A heavy chain (NMHC-II)	Surface exposed	Pre-existing natural IgM antibodies
Self-nucleic acids (RNA, cytDNA, mtDNA)	Passively released (e.g. from nucleus, mitochondria, cytosol)	TLR3, TLR7, TLR9, RIG-I, AIM2, cGAS,
Oxidation-specific molecules/epitopes	Passively released	CD36, SR-A, TLR2/4, CD14, natural IgM antibodies
S100 proteins (e.g. S100 A1/8/9/12)	Passively released	RAGE, TLR4
Tenascin	Extracellular matrix	TLR4
UL16 binding proteins (ULBPs)	Surface exposed	NKG2D
Histone- DNA complex	Passively released (from nucleus)	TLR9

AIM2, absent in melanoma 2; CD, cluster of differentiation; cGAS, cyclic GMP-AMP synthase; cytDNA, cytosolic DNA; FEEL-1, fasciclin epidermal growth factor–like/common lymphatic endothelial and vascular endothelial receptor-1; mtDNA mitochondrial DNA; NKG2D, natural-killer group 2, member D; NLRP3, NLR family, pyrin domain-containing protein 3; P2X4/7, purinergic P2X4/7 receptor; P2Y2, purinergic P2Y2 receptor; RAGE, receptor for advanced glycation end products; RIG-I, retinoic acid inducible gene I; SREC-1, scavenger receptor class f member 1; TIM, transmembrane immunoglobulin and mucin domain; TLR, toll-like receptor. (Table adapted from (Land et al., 2016)).

1.5.1.1 High mobility group box protein 1

HMGB1 is a DNA-binding nuclear protein that acts as a transcription factor (Muller et al., 2001). It was later identified as a pro-inflammatory cytokine in sepsis and endotoxemia upon its release after early cytokine production (Wang et al., 1999). It thus emerged as a key player in sterile injury, such as hemorrhagic shock and I/RI (Tsung et al., 2005). It is ubiquitously expressed by all cell types within the nucleus. HMGB1 is released predominantly from macrophages and monocytes but also from

other cell types that are exposed to pathogenic insult (Lotze and Tracey, 2005). Complement activation product C5a also triggers its release (Rittirsch et al., 2008). HMGB1 is released passively from necrotic or disrupted cells while it was initially believed that it is not released from apoptotic cells (Muller et al., 2001). HMGB1 has also shown to exert pleiotropic pro-inflammatory effects which include activation of phagocytic and endothelial cells which is collectively referred to as sickness syndrome (Tracey, 2010; Wang et al., 2004). HMGB1 blockade might complement anti-inflammatory therapy.

1.5.1.2 Interleukin-1 cytokines

The family of interleukin 1 like cytokines consists of 11 members (Dinarello, 2009), which have both pro-inflammatory as well as anti-inflammatory properties. In general, IL-1 is known to activate lymphocytes to enhance the defensive activity of monocytes and act as co-stimulant on natural killer cells (Dinarello, 1991). IL-1β is most often studied as it is been involved in autoimmune disease such as asbestosis characterized by lung and pleural fibrosis and by immune system dysregulation. IL-1α can also exert juxtacrine functions in a receptor-independent manner (Kaplanski et al., 1994). Recently it has been shown that IL-1α acts as DNA damage sensor which links stress signaling to sterile inflammation. Since it carries a nuclear localizing signal, it might contribute to DNA damage sensing which is regulated by histone deacetylases (HDAC) and IL-1α acetylation (Cohen et al., 2016).

1.5.1.3 Interleukin-33

IL-33 is the latest member of the IL-1 cytokine family, which is mainly expressed in structural and lining cells, including endothelial cells and epithelial cells of the tissues exposed to the environment (Moussion et al., 2008). IL-33 is active as a transcription factor (Gautier et al., 2016) and as a cytokine, but unlike IL-α and HMGB1, IL-33 features anti-inflammatory properties (Kunes et al., 2010). On mast cells, IL-33 triggers the production and release of pro-inflammatory cytokines such as IL-1β, IL-6 which promotes maturation and induces degranulation (Ali et al., 2007; Liew et al., 2010; Moulin et al., 2007).

1.5.1.4 Mitochondrial DAMPs

According to the danger theory, traumatic cells release otherwise internal contents that are hidden under normal conditions. In search for endogenous agents that

provoke immune activation and owing to the endosymbiont theory according to which mitochondria originate from bacteria with the ability to conduct respiration were incorporated by eukaryotic cell by endocytosis (Wallin, 1922), mitochondria have been considered as a source of DAMPs. Mitochondrial DNA concentration is elevated in severely injured patients (Zhang et al., 2010b). Mitochondrial DNA released during shock directly activates neutrophils by binding to TLR9 (West et al., 2006; Zhang et al., 2010a). Accordingly, systemic administration of mtDNA in mice resulted in inflammation and lung injury (Zhang et al., 2010b). Moreover, mitochondria were reported to mediate induction of apoptosis during systemic inflammatory response syndrome (SIRS) in trauma and shock (Hubbard et al., 2004; Power et al., 2002). Besides their functions in ATP-generation and calcium homeostasis, mitochondria are now known to activate innate immunity (Bonawitz et al., 2006).

1.5.2 PRRs involved in ionizing irradiation induced inflammation.

Ionizing irradiation (IIR) releases hydroxyl radicals (˙OH), which are most reactive and damaging free radicals. All three endoplasmic reticulum stress sensors, namely protein kinase (PK) R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1α), and activating transcription factor 6 (ATF6) activate signaling events to re-establish homeostasis in the ER and promote cell survival. Two prominent members of the inflammasome forming family of NOD-like receptors namely pyrin domain containing 3 (NLRP3) and AIM2, mediate caspase-1-dependent death of intestinal epithelial and bone marrow cells in response to double- strand DNA breaks caused by ionizing radiation and chemotherapy (Hu et al., 2016). However, their activation is unclear in that a typical dual signal mode of activation is not obviously operative upon IIR.

Patients carrying a *Tlr4* loss of function allele relapse quickly after radiotherapy and chemotherapy due to a failure to mount an IIR-TLR4 driven anti-tumor immune response (Apetoh et al., 2007). Moreover, administration of TLR ligands protects against IIR toxicity (Vijay-Kumar et al., 2008). For instance, the TLR5 agonist, CBLB502 protects mice and primates from IIR induced pathology (Burdelya et al., 2008). Also, pre and post TLR9 agonist treatment protected mice from IIR induced gastrointestinal syndrome (Saha et al., 2012). Otherwise, tumors lacking TLR3 or IFNAR expression failed to respond to anthracycline based chemotherapy (Sistigu et

al., 2014). TLR3 blockade protected mice from IIR-induced gastrointestinal syndrome (Takemura et al., 2014). TLR7/8 activation in combination with radiotherapy induced strong immune response against gastrointestinal tumors (Scholch et al., 2015). Radiation exposure induces inflammasome activity (Stoecklein et al., 2015). Specifically, AIM2 mediates the caspase-1-dependent death of intestinal epithelial and bone marrow cells in response to double-stranded DNA breaks caused by IIR (Hu et al., 2016). IL-13 has been shown to mediate radiation induced pulmonary fibrosis. Hence, *Il-13*$^{-/-}$ mice failed to recruit alternatively activated macrophages to the site of irradiation, which qualifies IL-13 as a novel therapeutic target in IIR induced lung injury (Chung et al., 2016). Whether TLRs mediate sterile inflammation upon IIR suggested by current literature is an open question.

1.5.3 PRRs involved in sensing of DAMPs released upon liver Ischemia/reperfusion injury.

The danger/damage hypothesis was brought up in the early 2000s (Land, 2002; Land, 2004, 2005) and is relevant such as to the field of organ transplantation. Oxidative injury is a prominent type of injury prevalent post ischemic reperfusion in the host that induces various classes of DAMPs activating PRR expressing cells of both the donor and recipient immune system. The graft typically has to undergo a painstaking storage post withdrawal from the donor upon which the blood supply is blocked and organ transportation follows. Upon its engraftment in the recipient reperfusion, injury is established by blood re-circulation post-surgery. Consequently, both donor organ immune cells and the recipient´s immune system are activated by the sterile inflammatory response. A primary parameter of severe injury to an allograft is reactive oxygen species (ROS). Significant ROS-mediated oxidative stress is typically involved in brain-death of organ donors.

1.5.3.1 ROS is a post-ischemic reperfusion driven insult.

ROS are chemically reactive chemical species containing oxygen. Vascular cells are considered to be the first line source of ROS in the first few minutes of reperfusion (Land, 2012). Secondary source of ROS in the course of I/RI originate from phagocytic cells such as polymorphonuclear neutrophils (PMNs) and macrophages. ROS include peroxides, superoxide ($^{\cdot}O_2^-$), hydroxyl radicals (OH) and singlet oxygen ($^1O_2$). Current data suggest that there are several sources of ROS in post ischemic tissues, the most prominent molecular source of ROS are enzymes, namely

mitochondrial electron transport chain–associated enzymes (mainly through complex I and III), xanthine oxidase, and the reduced form of nicotinamide adenine dinucleotide phosphate oxidases as well as uncoupled nitric oxide synthase capable of reducing molecular oxygen to form superoxide ($^{\cdot}O_2^-$) and/or hydrogen peroxide. Endogenous ROS are produced intracellularly in a cell type specific manner by NADPH oxidase (NOX) complexes in cell membranes of mitochondria, peroxisomes, and the endoplasmic reticulum (Han et al., 2001; Muller, 2000). ROS production also occurs during normal physiological process such as oxidative phosphorylation in mitochondria converting glucose into adenosine triphosphate (ATP). Cytochrome c oxidase sometimes referred to as complex IV, transfers electrons to oxygen which in turn causes the mitochondrial matrix to pump protons out to create a proton gradient towards synthesis of ATP.

Under normal circumstances oxygen requires four electrons for the complete conversion to water. Failure to receive the four electrons precipitates free radical generation. If oxygen receives only one electron, it forms superoxide anion ($^{\cdot}O_2^-$) (Li et al., 2013). On receiving two electrons, it is converted into hydrogen peroxide (H_2O_2), while three electrons mediate hydroxyl radical ($^{\cdot}OH$) formation. If the cell fails to quench ROS, the damage induced triggers apoptosis. Bcl-2 proteins present on the mitochondrial membrane detect damage, activate Bax protein to leak out the membrane associated with a release of the cytochrome C to the cytoplasm to bind to apoptotic protease activating factor-1 (Apaf-1) towards formation of apoptosomes. Apoptosomes activate caspase-9, which cleaves mitochondrial proteins towards disintegration and apoptosis (Czabotar et al., 2014).

Another mode of damage caused by free radicals is inhibition of transferrin, a protein mainly aiding in iron transport in the serum, resulting in accumulation of excess iron which is converted to free radicals in the process of Fenton´s reaction. ROS are known to induce host defense gene activity in platelets which recruit additional platelets to the site of injury during wound repair. In case of infection, neutrophils recruited to the site of injury curb the invading pathogens by oxygen dependent oxidative burst, a process in which NADPH oxidase converts oxygen to superoxide which is further converted to bleach invading pathogens. TLRs are known to be involved in rejection and tolerance, I/RI, and infections post transplantation. In liver transplantation, TLRs might mediate sensing of DAMPs that are released by stressed

and dying cells. I/RI elicit an inflammatory response which poses a potentially lethal threat to the host upon organ allograft transplantation. Inflammation might have profound influence on acute as well as long-term graft function (Tsung et al., 2007).

1.6 Aims of the thesis

The central aim of this thesis was to characterize and modulate potential TLR or other PRR driven immune activation upon sterile or septic challenge. The severe sepsis syndrome upon acute infection results in organ dysfunction upon a dysregulated systemic inflammatory hyper-response. LPS or endotoxin present in the outer membrane of Gram-negative bacteria is a potent stimulator of such inflammatory response mediated by TLR4 largely. To inhibit TLR4-mediated pro-inflammatory signaling, we analyzed tetraacylated lipid A mimetics antagonists and agonists that had been synthesized by our collaborator (Assoc. Prof. Dr. Alla Zamyatina, University of Natural Resources and Life Sciences, Vienna) based on the synthetic βGlcN(1↔1)αGlcN scaffold. The aim of the first part of this thesis was to evaluate the effectiveness of six antagonists as potential anti-inflammatory therapeutic agents. Moreover, a set of seven Lipid A like, compounds were also analyzed for their adjuvant properties by challenging primary mouse immune cells.

Former studies from our group revealed murine TLR13 as bacterial 23S rRNA sensor. The ligand motif consists of a 19 nucleotide sequence from virtually all bacteria (Sa19) with a core sequence of “GGAAAGA”. Considering the endosymbiont theory, mitochondrial ribosomal RNA was compared with bacterial ribosomal RNA for its immune stimulatory capacity. Therein, human PBMCs, despite lacking TLR13 expression, were activated by bacterial ssRNA and mitochondrial RNA challenge. The aim of the second part of this study was to identify the human Sa19 receptor and its specificity. Gain and loss of function analyses were applied to bring up receptor candidate molecules and identify the receptor.

Trauma elicits inflammatory responses which are clinically relevant since they raise pathology in various diseases. The aim of the third part of this study was to identify DAMP and its receptor mediating the pathology of IIR induced tissue damage driven inflammation. Wild-type and specific PRR- or signaling molecule-deficient and interferon (IFN-γ) primed mice will be exposed to high dose IIR. According to the literature, TLRs might be involved in the innate immune response to DAMPs released by stressed and dying cells. I/RI injury elicit an inflammatory response that poses a potentially lethal threat to the host post solid organ allograft transplantation. Hypothermia associated with reperfusion induces cell death which is a major cause

of primary graft non-function following liver transplantation. We therefore evaluated the stimulatory potential of homogenates of cold stored and/or heat shocked primary hepatocytes by challenging macrophages of various genotypes. The aim of the fourth part of this thesis was to identify endogenous DAMP and its receptor mediating I/RI driven immune activation.

2. Materials and Methods

2.1 Materials

2.1.1 Equipment

Agarose gel cast stand (Bio Rad)
Agarose gel electrophoresis chamber, Sub Cell (Bio Rad)
Cell incubator, HERA cell (Thermo Scientific)
Centrifuge, Heraeus Fresco 17 (Thermo Scientific)
Chemiluminescence imaging, Fusion Fx7 (Vilber)
Electrophoresis chamber, Mighty Small SE250/260 Vertikal (Hoefer)
Exhaust pump (Knf lab)
FastPrep®-24 Instrument (MP Biomedicals)
Luminometer, Orion II (Berthold)
Magnetic stirrer, SB 162 (Stuart)
Microplate-reader, Epoch (BioTek)
Microplate-washer, 12-well (BioTek)
Microscope, Axiovert 40C (Zeiss)
Microwave MW 800 (Continent)
Mini centrifuge (Biozym)
Multifuge X3R Haereus (Thermo Scientific)
Photometer (Eppendorf)
Pipettor (Hirschmann, Brandt)
Pipetts (Eppendorf, Gilsen)
Power supply (Bio Rad, Hoefer)
Refrigerator/freezer (Liebherr)
Scale, Acculab (Sartorius group)
Semi-dry Trans-Blot® TurboTM (Bio Rad)
Shaker, Polymax 1020/1040 (Heidolph)
Shaking incubator, KS 400i (IKA)
Sterile work bench, HERA safe (Thermo Scientific)
Thermocycler (Eppendorf)
Thermomixer (HCL Toledo)
Tube roller, RM5 (CAT)
Ultrasonic bath Ultrasonic Clean (VWR)
Ultrasonic probe, Sonoplus HD 2070 (Bandetin)
UV-gel documentation system (Intas UV-Systeme)
Vortex mixer, VTX-3000L (LLG)
Water bath (GFL)
Water ultra-purification system, Easypure II (Werner Reinstwassersysteme)

2.1.2 Kits and enzymes

Kit / Enzyme	Application	Supplier
BCA Protein Assay Kit	Protein quantification	Pierce
ELISA DuoSet	cytokine concentration	R&D Systems
ELISA hIFNα	cytokine concentration	eBioscience
OptEIA TMB Substrate Reagent Set	substrate for ELISA detection	BD Biosciences
HRP		R&D
LDH cytotoxicity assay kit	LDH	Pierce

2.1.3 Stimulants and inhibitors

Stimulant	Description	Receptor	Supplier
CL075 (3M002)	thiazoloquinolone derivative	TLR7 & TLR8	Invivogen
CpG-DNA (1668)	synthetic ODN containing unmethylated CpG	TLR9	MWG Eurofins
Flagellin	isolated from bacteia	TLR5	Invivogen
Lipopolysaccharide, *E. coli* 0111:B4	isolated from bacteria	TLR4 (& TLR2)	Sigma Aldrich
Loxoribine	guanosine analog derivatized at N7 and C8	TLR7 & TLR8	Invivogen
Pam_3CSK_4	synthetic triacylated lipopeptide	TLR2/TLR1	Invivogen
Polyinosinic:Polycytidylic acid (poly I:C) LMW	synthetic dsRNA analog (average size 0.2-1 kb)	RIG-I, MDA5	Sigma Aldrich
Resiquimod (R848)	imidazoquinoline compound	TLR8, TLR7	Invivogen
ssRNA40/LyoVec	20-mer phosphorothioate protected ss ORN	TLR8, TLR7	Invivogen
79i	TLR7 and TLR9 inhibitory ODN		TIB MolBiol
lipid A mimetics antagonists and agonists	TLR4 inhibitors (6 compounds) and activators (7 compounds)	TLR4	Prof. Dr. Alla Zamyatina, University of Natural Resources and Life Sciences, Vienna

Transfection reagent	Description	Supplier
DOTAP Liposomal	cationic liposome-forming compound for transfection of negatively charged molecules formulation for transfection of nucleic	Roche
Lipofectamine® 2000		Invitrogen

	acids	
poly-L-arginine (pLA)	cationic liposome-forming polymer	Sigma Aldrich

2.1.4 Chemicals and solutions

Solution/Reagent	Supplier
β-Mercaptoethanol	Gibco
Ammouniumperoxysulfate (APS)	Sigma
BSA	Sigma
Coelenterazin	PJK
Coenzyme A	PJK
CpG1668	TIB MolBiol
Disodium EDTA ($Na_2EDTA \times 2H_2O$)	Sigma
D-Luciferin	PJK
DMSO	Sigma
DNA-Ladder 1 Kb	Invitrogen
EDTA	Sigma
Interferon γ, recombinant human	Peprotech
Interferon γ, recombinant murine	Peprotech
LPS *E. coli* O111:B4	Sigma
LPS *S.minnesota* Re595 (rough strain)	Sigma
Lucigenin	Enzo Life Science
Methanol	Roth
Milk powder	Roth
Normal goat serum (NGS)	Invitrogen
Pam_3CSK_4 (Tri-palmitoyl-cysteinyl-seryl-tetralysine)	EMC Microcollections
PBS, Dulbecco, powder	Biochrom
Poly I:C	Invivogen
RNase Away Spray	Sigma Aldrich
RNase Inhibitor, RiboLock	Thermo Fisher (Fermentas)
Roti Store Cryo tubes	Roth
Rotiphorese® NF-Acrylamid/Bis-solution 30% (29:1)	Roth
Sodium chloride	Roth
Streptavidin-HRP (horse raddish peroxidase)	R&D Systems
SYBR® Safe DNA Gel Stain	Invitrogen
TEMED	Sigma
TMB ELISA Substrate Reagent Set	BD
Tri Reagent	Sigma
TriFast, peqGold	Peqlab
TRIS	Roth

TRIS hydro chloride	Roth
Tween20	Sigma

2.1.5 Buffers

Buffer	Supplier
DNA loading dye (5X)	Thermo Scientific
DNA ladder 1 kb	Thermo Scientific
Reporter Lysis Buffer (5X)	Promega
RiboRuler High Range RNA Ladder	Thermo Scientific

2.2. Methods

2.2.1 Murine bone marrow derived macrophage generation

Mouse bone marrow was harvested from femurs and hind leg bones. In brief, bones were flushed with sterile 20 ml of DMEM supplemented with 10% FBS, Penicillin-Streptomycin (100 U/ml, Gibco), Antibiotic-Antimicotic (amount as indicated by supplier Gibco). Cells were re-suspended and spun at 1100 rpm for 5 minutes at room temperature. Cell pellet was re-suspended in 100 ml DMEM supplemented with 10% FBS, 15% L929 conditioned medium, Penicillin-Streptomycin (100 U/ml, Gibco), Antibiotic-Antimicotic (amount as indicated by supplier Gibco) and 50 µM of β-mercapto ethanol (Sigma-Aldrich). Cells were incubated for one week under regular cell culture conditions on petri dishes (145mm). Medium containing un-differentiated and dead cells were aspirated from the dishes, Adhered differentiated cells were washed with PBS and detached with 5 ml accutase, a mixture of proteolytic and collagenolytic enzymes addition of 5 ml BMM medium neutralized the enzymatic activity. BMMs were dislodged from the surface, centrifuged (1100 rpm, RT, 5'), re-suspended the cell pellet in fresh DMEM supplemented with 10% FBS, Penicillin-Streptomycin (100 U/ml, Gibco), Antibiotic-Antimicotic (amount as indicated by supplier Gibco), counted and plated ($2x10^5$ cells per well in 200 µl medium) to reach the desired density.

2.2.2 Isolation of splenocytes

Mice were sacrificed by cervical dislocation and the site of operation was disinfected with 70% EtoH. Harvested spleen was carefully placed on top of the 70µM cell strainer placed in a petri dish containing 5ml of DMEM supplemented with 10% FBS, Penicillin-Streptomycin (100 U/ml, Gibco), Antibiotic-Antimicotic (amount as indicated by supplier Gibco) spleen was smashed using sterile rubber syringe plung. Cell suspension was spun at 1200 rpm for 5 minutes at RT. Pellet was resuspended in 5 ml of ACK lysis buffer and incubated for 5 minutes at RT. To neutralize lysis 10 ml of DMEM was added and resuspended, suspension was spun at 1200 rpm for 5 minutes at RT. Pellet was resuspended in 5 ml of DMEM supplemented with 10% FBS, Penicillin-Streptomycin (100 U/ml, Gibco), Antibiotic-Antimicotic (amount as indicated by supplier Gibco)

ACK lysis buffer

NH_4Cl	150 mM
$KHCO_3$	10 mM
Na_2EDTA	0.1 mM

2.2.3 Protein biochemistry.

2.2.3.1 Cell lysis.

Cell lysis was carried out on ice. After stimulation cells were washed twice with ice-cold 1x PBS and then lysed with 100 µl lysis buffer per $1x10^6$ cells for 30 min. The lysate was then collected and centrifuged at 13000 rpm for 15 min in a tabletop centrifuge to remove nuclei and debris. The supernatants were collected in a 1.5 ml tube, stored at -80°C or were directly subjected to electrophoresis. Protein concentration was determined using the BCA Protein Assay Reagent Kit according to manufacturer's guidelines.

Lysis buffer:

50 mM	HEPES pH7.6
50-150	mM NaCl
1 mM	DTT
1 mM	EDTA
1 mM	EGTA
0.5-1.5% (v/v)	Nonident P-40
10% (v/v)	Glycerol
20 mM	β-Glycerophosphate
1 mM	Na_3VO_4
0.4 mM	PMSF

1 Tab/ml Protease Inhibitor Cocktail Tablet (Roche)

2.2.3.2 SDS-polyacrylamide gel electrophoresis.

SDS-PAGE was carried out using glass slides stacked creating a space of 1.5 mm thickness. First the resolving gel (10 or 12 %) was added slowly into the space, gently 500µl of isopropanol was added to cover the resolving gel. As soon as the resolving gel was polymerized isoproponal was removed by tilting it side wards and the residual isoproponal was wiped off with paper towel. Stacking gel (4 %) was gently added on top of the resolving gel comb was inserted. Upon complete polymerization the gel was installed, overlayed with Laemmli buffer and the combs were removed. Wells were flushed with Laemmli buffer to remove residual acrylamid. The cell lysates were diluted with 6x sample buffer and incubated for 5 min at 95°C. 100µg of protein was loaded on to the wells. 5 µl of standard (Fermentas) were loaded on the gel for the determination of protein size. Gel electrophoresis was performed at 80 V till the samples reach resolving gel, subsequently the voltage was increased to 120V to separate proteins based on the molecular weight.

Buffers for SDS-polyacrylamide gel electrophoresis

Resolving gel buffer: 1.5 M Tris-HCl pH 8.8

Stacking gel buffer: 0.5 M Tris-HCl pH 6.8

10 % SDS: 10% SDS in ddH_2O

10 % APS: 10% Ammonium persulfate in ddH_2O

6x sample buffer:

7 ml	stacking gel buffer
1 g	SDS
3 ml	Glycerol
0.9 g	DTT
0.06%	Bromphenol blue

Laemmli buffer:

30.28g	Tris
208.2g	Glycine
50ml	10 % SDS
Ad 10 l	ddH_2O

	Resolving gel		Stacking gel
	10%	12%	4%
Acrylamide solution	3.3 ml	4 ml	0.66 ml
4x Resolving gel buffer	2.5 ml	2.5 ml	-
4x Stacking gel buffer	-	-	0.3 ml
10 % SDS	0.1 ml	0.1 ml	0.2 ml
ddH2O	4 ml	3.3 ml	4 ml
TEMED	5 µl	5 µl	5 µl
10 % APS	50 µl	50 µl	25 µl

2.2.3.3 Western blot analysis.

Proteins were transferred from polyacrylamide gels to a nitrocellulose membrane using semi dry blot method. In brief, after SDS gel electrophoresis gel was carefully transferred from the gel-chamber and the stacking gel was removed. Four Whatman filter papers in the size of the gel, the nitrocellulose membrane and the gel were incubated in blotting buffer for 5 min. Nitrocellulose membrane was placed on top of the filter papers then gel was placed on top of the membrane and finally filter papers were placed on top of the gel. Assembled filter papers including gel and membrane were transferred onto the semi-dry blotting apparatus. Air bubbles were removed by gently rolling the assembled set up with a blotting roller. Blotting procedure was performed at 1mA/cm^2 for 60 min. To avoid unspecific binding the membrane was blocked for 1 h in 5 ml of blocking buffer. Incubation with the primary antibody (diluted in 1x blocking buffer) took place at 4°C overnight. On the next day the membrane was washed three times for 5 min in PBT and then incubated with the secondary HRP-conjugated antibody (diluted in PBT) for 2 h at RT on the shaker. After three washing steps with PBT for 10 min each, proteins were detected using Western Lightning Chemiluminescence Reagent. All steps were performed on a shaker. The membrane was then blocked again in blocking buffer for 1 h. Antibodies toward IκBα(44D4), phosphor-ERK and -p65(Ser536)(93H1) were from Cell Signaling Technology and applied according to manufacturer's instructions.

Buffers for Western Blot

Blotting buffer:

1.94 g	Tris
8,656 g	Gylcine
200 ml	Methanol
	ad 1000 ml ddH_2O

PBT:

1x PBS

0.05% Tween20

Blocking buffer:

1x PBT

3% NGS

50 g/l Milk powder

2.2.4 Reporter gene assay

The luciferase reporter gene system was used to measure the activation of the NF-κB transcription factor and it's binding to a promotor ligated to the luciferase coding sequence (CDS) activity of which was thereby induced in a transfected receptor dependent manner. A *Renilla reniformis* luciferase construct was monitored as a control for transfection efficiency and normalization. The luciferase enzyme activity correlates with the activation of the promotor ligated to luciferase CDS. HEK293 cells were seeded on a 96-well plate (2×10^6) and incubated at 37°C for at least 4 h. The receptors, of which the activation to be quantified, were transfected into the cells via calcium-phosphate transfection. NF-κB driven reporter gene activation was analyzed in HEK293 (human embryonic kidney fibroblast) cells transfected with mouse TLR4 and mouse MD2 either together with 6xNF-κB or ELAM promotor encoding luciferase DNA plasmids and subsequently challenged with LPS.

Transfection mix

660 µl	H_2O
90 µl	2 M CaCl2
0.2 µg	NfκB 2 ng/well
2-50 ng	Receptor
pRK5	
(empty vector)	ad. 50 ng

1:1 with 2x HBS, vortex

The transfection mix was incubated at RT for 20'. 15 µl of transfection mix were added to the cells of each well for transfection o/n. Subsequently, medium was replaced with 2% FCS and the cells were challenged with stimuli for 16 h. Supernatants were discarded and 40 µl of 1x Reporter Lysis Buffer (Promega) was added to each well. Lysis was carried out by rocking (800 rpm) for one hour at RT. 20 µl of lysates were transferred to a white opaque microtiter plate. Conversion of the substrate D-luciferin by luciferase produced chemi-luminescence was measured in an Orion II luminometer (Berthold).

Luciferase substrate

470 µM	D-Luciferin
270 µM	Coenzyme A
33.3 mM	DTT
530 µM	ATP
1.07 mM	$(MgCO_3)_4Mg(OH)_2$
2.67 mM	$MgSO_4$
20 mM	Tricine pH 5.0
0.1 mM	Na_2EDTA
pH 7.8	

Renilla substrate

1.1 M	NaCl
2.2 M	Na_2EDTA
220 mM	$KHPO_4$
1.3 M	NaN_3
440 µg/ml	BSA
1.43 µM	Coelenterazine
pH 5.0	

2.2.5 Human PBMCs isolation.

Total PBMCs were isolated from buffy coats from mixed and anonymous blood donations kindly provided by the local blood donation center (P. Horn, Director, Institute of Transfusion Medicine, University hospital Essen) upon approval by the responsible ethics committee. 13 ml of blood was pipetted gently to the side of tubes

filled with 20 ml of Ficoll (GE Health care). Tubes were spun 400xg (Sorvall ST16 cell centrifuge) with accelerator set to 1 and decelerator set to 0 for 30 min, resulting in formation of three phases, namely a clear upper serum, a middle cloudy PBMC, and the lower debris and erythrocytes phase. Serum was collected into a fresh 50 ml falcon tube for later use. The cloudy PBMC phase was re-suspended in PBS. The resulting suspension was spun at 400xg for 10 min at room temperature, the supernatant was discarded and the pellet was re-suspended in 20 ml of PBS. Suspensions were spun again at 400xg for 10 min. Pellets were re-suspended in 10 ml of RPMI medium supplemented with 10% of heat inactivated autologous serum.

2.2.6 Cell culture

Regular cell culture comprising 37°C, 5%CO_2 humidified atmosphere was performed using DMEM or RPMI1640 supplemented with 10% heat inactivated FBS supplemented with 10% of autologous serum for hPBMCs. Cells were plated at 18 x 10^4 per well on 96-well plates. Cells were confronted with endotoxin mimetics at concentrations of 10 µg/ml and 1 µg/ml. Cells were incubated for 30 min and subsequently challenged with TLR ligands (identities and concentrations of which are being described above) for 16 h.

2.2.6.1 THP-1 Cells

THP-1 is a human monocytic cell line, derived from an acute leukemia patient. THP-1 cells were cultured in 175 cm^2 cell culture flasks. Cells were split when 60-70% confluency is attained by transferring 2 ml of cell suspension to fresh medium. THP-1 monocytic cells were differentiated to macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) (1x10^7 cells per 96-well plate or per well of 6-well plate) for either 24 h followed by 3 days culture in PMA-free medium (3ddi), or 72 h followed by 5 days culture in PMA-free medium (8ddi). Cells were challenged in medium containing 2% FCS for 16 h.

THP-1 medium

500 ml RPMI 1640

50 ml FCS

5 ml Penicillin-Streptomycin solution (Gibco)

5 ml Antibiotic-Antimitotic 100X solution (Gibco)

2.2.6.2 Culturing and differentiation of Hoxb8 cells

ER-Hoxb8 neutrophil granulocytes cells used in this paper are from the lab of Prof. Georg Häcker (Institute for Medical Microbiology and Hygiene, Freiburg) (Wang et al. 2006). Hoxb is expressed under the control of estrogen which was achieved through retroviral transduction. Cells were cultured in 3 ml Hoxb8 precursor medium in a 6-well plate at 37°C, 5% CO_2 and 95% humidity. 10% of the cell suspension form 60-70% confluent plate is seeded in a fresh 6 well the following day. For differentiation cells were spun at 800 rpm for 5 min and washed twice with 5 ml PBS. Cells were re-suspended in Hoxb8 differentiation medium and seeded at 3x 10^5 cells/ well (6- well plate) for 4 days.

Hoxb8 Precursor medium:

Opti-MEM, Glutamax

10% FBS

1% (v/v) Penicillin-Streptomycin

I µM β-Estradiol

1% CHO- cell supernatant

(Contains Stem cell factor (CSF))

Hoxb8 Differentiation medium:

Hoxb8 precursor medium with out

β-Estradiol

2.2.6.3 HEK293 cells

The human embryonic kidney cell line HEK293 was applied for ectopic protein overexpression and functional analysis. HEK293 cells were cultured as adherent monolayer. The cells were grown to 70% confluence, Cells were washed with PBS and detached with 5 ml (per 10 cm dish) of 1% (w/v) trypsin-EDTA (Gibco) for 5 min. Trypsin activity was neutralized by addition of 1 volume of medium, cells were then thoroughly re-suspended. 1/10 of the suspension was transferred to a new dish with fresh medium.

HEK293 medium

500 ml DMEM

50 ml FCS

5 ml Penicillin-Streptomycin solution (Gibco)

5 ml Antibiotic-Antimycotic 100X solution (Gibco)

2.2.6.4 Primary rat hepatocytes

Primary rat hepatocytes were isolated from wistar rat liver. Isolated hepatocytes were provided in organ preservation medium containing histidine, tryptophan, and ketoglutarate (HTK) by the group of Prof. Rauen (Institute for Physiological Chemistry, University Hospital Essen).

2.2.7 Nitrite measurement (Griess assay)

Cell culture supernatants were harvested. 100 µl of supernatant were mixed 100 µl of griess reagent consisting of equal portions of solution A (0.2% N-(1- naphthyl) ethylenediamine dihydrochloride) and solution B (2% sulphanilamide, 5% phosphoric acid). Plates were incubated for 10 min in the dark at room temperature and absorption was analyzed at 540 nm in an ELISA reader (Epoch BioTek). Concentrations of the unknown samples were determined by relation of absorption values to a standard curve prepared by analysis of a serial dilution of sodium nitrite with 300 µM as highest and 0.3 µM as the lowest concentration.

2.2.8 Enzyme linked immuno sorbent assay (ELISA)

Cytokine concentrations in cell culture supernatants were analyzed by enzyme linked immunosorbent assay with enzyme-mediated colorimetry. All proteins were detected by DuoSet ELISA Development System (R&D Systems) or IFNα ELISA (eBioscience) following the manufacturer's protocols. Briefly, ELISA plates were coated with capture antibody (diluted in PBS, 50 µl/well), placed on plate shaker o/n at RT (over the weekend at 4°C). Plates were washed (ELISA-washer, 3x 300 µl PBT/well). Blocking buffer was applied to prevent unspecific binding to the plastic surface for 1h (150 µl /well, at RT). The plates were washed, samples and standard curve dilutions of the respective cytokine was applied (5; 2.5; 1.25; 0.63; 0.31; 0.16; 0.08 ng recombinant cytokine; H2O as blank; samples were diluted in RD if applicable). Plates were incubated on plate shaker (65 rpm) for 90' at 37°C (or 2h at RT). Plates were washed. Biotin-coupled detection Ab was added (diluted in RD, 50 µl /well) followed by 90' of incubation at 37°C with shaking (or 2h at RT). Plates were washed. Streptavidin-coupled horse radish peroxidase (HRP) was added (1:200, RD, 50 µl /well, 5 µl Streptavidin in 1 ml RD). The plates were shaken for 20' at 37°C in the dark. After washing, freshly prepared TMB substrate (reagent A: reagent B equally mixed, BD) was applied to the plates (50 µl /well). Plates were developed by incubating in dark at room temperature (blue colored product). The reaction was

terminated by 1M H_2SO_4 (25 µl /well, color turns to yellow). Cytokine concentrations were measured with a micro plate spectrophotometer at an absorption of 450nm (Epoch BioTek) and analyzed with Gene5 software.

Blocking buffer	**Reagent Diluent (RD)**
25 g Sucrose	10 g BSA
5 g BSA	ad 1 l PBS
250 mg NaN3	
ad. 1 l PBS	

Blocking buffer and Reagent Diluent were sterile filtered and stored at 4°C.

Stop solution	**PBT (washing buffer)**
2N (1M) H_2SO_4	0.05 % (v/v) Tween 20
ad. 1 l water	ad. 1 l PBS

2.2.9 Lactate dehydrogenase assay

The activity of lactate dehydrogenase (LDH) was determined with a fully automated clinical chemistry analyzer (Respons920; DiaSys Greiner GmbH) using commercially available reagents. Alternatively, Pierce LDH cytotoxicity assay kit (Pierce) was used following manufacturer's instructions. To determine LDH activity absorbance at 680 nm (background signal) was subtracted from the absorbance at 490 nm.

2.2.10 ROS Chemiluminescence assay

A chemiluminescent agent Lucigenin is implemented in the measurement of reactive oxygen species which is specific to superoxide radicals. Lucigenin reacts in its univalently reduced form with superoxide anion results in the formation of unstable endoperoxide, which decomposes to an electronically exited product. This product release a photon as it falls to the ground state. Light signal is measured at 529nm (luminescence). 1-2x 10^5/well (96 well plate) Hoxb8 neutrophils were seeded in 2% medium containing 50 µM lucigenin, cells were then challenged with 100µl of the necrotic homogenates or the TLR ligands generated ROS reacts with Lucigenin generates luminescence. Luminescence was measured with luminometer, Berthold

2.2.11 Mitochondria and subsequent mtRNA preparation

Liver was harvested from Wistar rats. Tissues as well as harvested cultured HepG2 cells were homogenized in ice-cold isolation buffer (2 mM Hepes, 220 mM mannitol, and 70 mM sucrose, pH 7.4). Homogenized suspension was spun at 494xg (Sorvall ST16 cell centrifuge) for 3 min at 4°C. Supernatant was transferred to a fresh tube and spun. This washing step was repeated twice. Clear supernatant was spun at 26,000xg for 10 min at 4°C. Pellet was re-suspended in ice-cold isolation buffer and the centrifugation step was repeated twice which resulted in a pure mitochondria preparation. Pellets were re-suspended in TRI reagent (Sigma) for RNA isolation (as described above). 0.25, 0.5, 1, or 2 µg of mtRNA per 200 µl was applied to PBMCs and 0.5, 1, or 2 µg per 200 µl to THP-1 cells.

2.2.12 Agarose gel electrophoresis

Agarose gels (1 %) supplemented with SYBR Safe (Thermo Fisher Scientific) was used to separate RNA fragments variable in size. 1x MOPS was used as electrophoresis and gel buffer. The RNA and the RNA ladder were mixed with equal volumes of gel loading buffer were then denatured at 75°C for 5' before loading onto the gel (1% in 1x MOPS). The electrophoresis was carried out at 75 V. For size determination of the fragments, 5 µl of a 1 kb ladder were separated next to the samples. DNA bands were visualized with UV light (254 nm).

MOPS buffer (10x)

0.2 M	MOPS
50 mM	NaOAc
10 mM	EDTA
ad 1 l	DEPC water
	pH 7 (NaOH)

2.2.13 Transfection of ORNs, bacterial and mtRNA fractions

RNA40 was transfected principally using LyoVec (Invivogen) which was also used for ORNs, bacterial and mitochondrial RNAs challenge of BMs. RAW264.7 cells were transfected with Sa19 by LyoVec and with BtmtD3_4 by pLA (Sigma). HEK293 cells were transfected with ORNs by Lipofectamine 2000 (Life Technologies). All bacterial and mitochondrial RNA preparations were transfected with Lipofectamine 2000 in THP-1 cells and PBMCs except for BtmtD3_4 and *mtPTL for the

transfection of which pLA was used. ORNs were transfected at a concentration of 100 pmol per 200 µl of medium.

2.2.14 Total body irradiation

Mice were placed at 50 cm from the radiation source (FSD) and exposed to 9 or 30 Gy. X-rays radiation source X-Strahl 320 machine was operated at 300 kV,10 mA at a dose rate of 161.55 cGy/min. X-ray beam filter consisting of 1.65 mm Al was used. Alternatively, Cobalt 60 (^{60}Co) at a dose rate of 12.5 cGy/min was used for total body irradiation. Both irradiation sources are from the group of Prof. Stuschke (Radiation and Tumor Clinic). Mice were housed in micro isolator cages, maintained on a 12/12 h light–dark cycle fed with standard rodent diet ad libitum. 8 to 10 weeks old unless otherwise mentioned were used for the entire set of experiments. All experimental procedures of animal were in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent experiment provided by the Animal Care and Use Committee of NRW. At the end of the experiment mice were sacrificed by cervical dislocation.

2.2.15 Cytokine response

Quantification of cytokines and chemokines in plasma were quantified using bead based luminex assay performed according the manufacture’s recommendations. Assay was run with a Luminex2000 instrument using Luminex IS software (Luminex corporation, Austin,Tx).

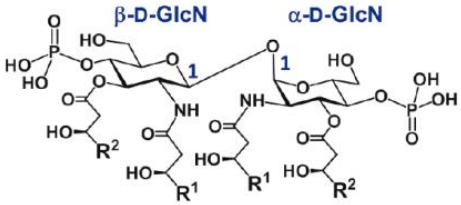
2.2.16 Statistical analysis

Results were analyzed from at least three biological replicates (n = 3), unless indicated otherwise. Each illustrated column in the graph represents mean ± SD (error bars) of triplicate data points of one out of at least three independent experiments. The level of significance was calculated using the Student's t-test for unconnected samples, with p-values indicated as "*" ($p \leq 0.05$), "**" ($p \leq 0.01$), "***" ($p \leq 0.001$).

3. Results

3.1.1 Analyzing the antagonist activity of synthetic tetra-acylated lipid-A mimetics.

Mice lacking TLR4 expression were protected from high dose LPS induced shock which indicates TLR4 as a major sensor of LPS produced by Gram-negative bacteria (Beutler and Rietschel, 2003; Merlin et al., 2001). Moreover, blockade of TLR4 protected mice from the lethal pathology of influenza infection indicating TLR4 also as a major sensor of the negatively polar and single stranded RNA flu virus (Shirey et al., 2013). TLR4 has been implicated in sterile inflammation and metastasis as well (Bald et al., 2014; Vogl et al., 2007). To inhibit TLR4-mediated pro-inflammatory signaling and the underlying pathologies of the respective syndromes, we analyzed tetra-acylated conformationally restricted lipid A mimetics based on the synthetic βGlcN(1↔1)αGlcN scaffold (Fig. 3). Each of the six compounds analyzed carries two pairs of different (*R*)-3-hydroxyacyl chains linked to the β,α-(1↔1)-connected glucosamine backbone in positions C-2, C-2′ (R^1), and C-3, C-3′ (R^2) respectively.



	R^1	R^2
DA 193	$C_{11}H_{23}$	C_9H_{19}
DA 253	$C_{11}H_{23}$	C_7H_{15}
DA 256	C_9H_{19}	C_9H_{19}
DA 255	C_7H_{15}	C_9H_{19}
DA 254	C_9H_{19}	C_7H_{15}
DA 257	C_7H_{15}	C_7H_{15}

Figure 3: Chemical structures of the DA-compounds. Tetra-acylated conformationally constrained βGlcN (1↔1) αGlcN-based lipid A mimetics are assembled such that the two GlcN moieties are stereo-specifically (α/β) glycosidically (1↔1) connected. Thus, instead of a three-bond flexible (1→6) linkage as in natural lipid A, DA-compounds possess the two-bond rigid (1↔1) linkage. The disaccharide scaffold bears variable acylations at C-2/C-2′ and C-3/C-3′ as indicated by R^1 and R^2, respectively.

Previously, it was shown that the DA-compounds, dissimilarly to lipid IVa, inhibited cell activation by both human (h-) and mouse (m-) TLR4 (Artner et al., 2013). Specifically, when applying the DA-compounds for the inhibition of either *E. coli* LPS-mediated signaling in the hTLR4/hMD-2/hCD14 overexpressing HEK293 cells or *E. coli* lipid A-mediated signaling in mTLR4/mMD-2 overexpressing HEK293 cells, no significant species-specific difference of the inhibitory capacities was observed. We have also reported the dependence of TLR specificity of lipopeptides upon the length of the acylations carried by the latter (Spiller et al., 2007).

Herein, we comparatively assessed the anti-inflammatory activity of the DA-compounds in both human and mouse primary immune cells, namely hPBMCs and mBMMs. First, we demonstrated canonical TLR4 driven signaling to be operative by challenging wild type, *Myd88*$^{-/-}$ and *Tlr4*$^{-/-}$ mBMMs. IL-6 and NO release were both absent in the supernatants from *Myd88*$^{-/-}$ and *Tlr4*$^{-/-}$ mBMMs challenged with LPS (Fig. 4) indicating strict dependence of cell activity.

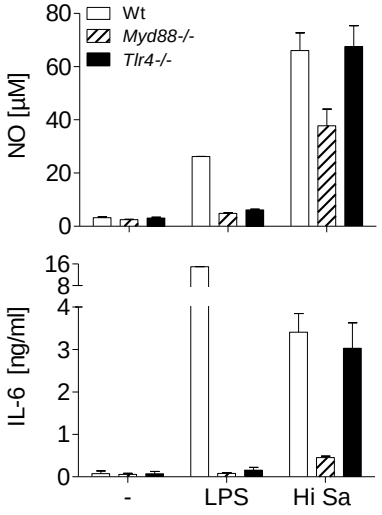


Figure 4: Canonical LPS driven signaling is MyD88 and TLR4 dependent. Murine (m) BMMs of the genotypes indicated were challenged with 10 ng/ml LPS or heat inactivated *Staphylococcus aureus* (Hi Sa) for 16 h upon which supernatants were analyzed by Griess assay or ELISA for nitrite (NO) or cytokine content (- unstimulated). The graphs show mean ± SD of duplicates. A representative result of two independent experiments performed at different times - at which results were similar.

Moreover, transfection of specific TLR4 expression plasmids into HEK293 cells indicated TLR4 drove the NF-κB activity upon challenge with the LPS applied (Fig. 5)(experiment requested by reviewer).

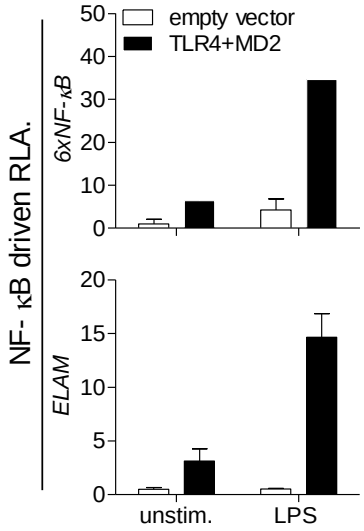


Figure 5: TLR4 and MD2 over expression in HEK293 cells conferred responsiveness to LPS. Two different reporter gene constructs specifically mediating NF-κB driven luciferase gene transcription through ELAM or a sextet lineup of NF-κB recognition elements in front of a minimal promoter, respectively, were transfected into HEK293 cells for 8 h. Subsequently, cells were challenged with 10 ng/ml of LPS for 16 h upon which cells were lysed and analyzed for relative luciferase activity (RLA) by luciferase reporter gene assay. The graphs show mean ± SD of triplicates. A representative result of two independent experiments performed at different times - at which results were similar.

LPS mimetic DA 253 representing six compounds analyzed was applied to mBMMs for one hour and subsequently challenged with LPS (Fig. 6). DA 253 clearly impaired NF-κB/p65 and extracellular signal regulated kinase (ERK) phosphorylation, as well as IκBα degradation. These results indicate TLR4 as the target of the LPS applied alone. Consequently, we titrated the LPS mimetics to identify the concentration at which a maximal inhibition of smooth *E*. *coli* O111:B4 LPS driven cell activation was operative. At a concentration of 1 μg/ml the DA-compounds were effective in both mBMMs and hPBMCs as compared to other doses tested (not shown) and thus 1 μg/ml and a tenfold higher concentration (10 μg/ml) were applied for all subsequent experiments to cover effects at the most appropriate range of concentrations.

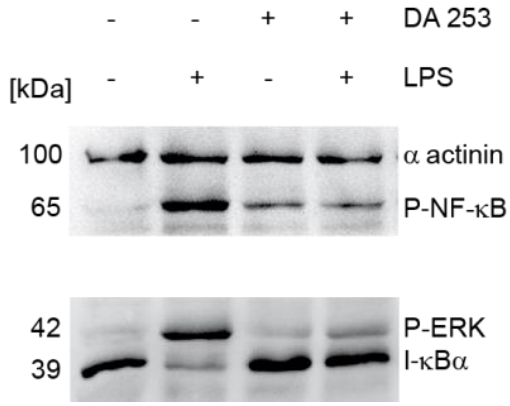


Figure 6: Lipid a mimetic impaired NF- κB/p65 and ERK phosphorylation.

Lysates of mBMMs treated as indicated for 30 min were analyzed by immunoblot analysis (DA 253, specific LPS mimetic; P, phospho). A representative result of two independent experiments performed at different times - at which results were similar is shown.

While DA254, -255, and -256 at a concentration 1 μg/ml effectively inhibited MIP-2 and KC release by mBMMs upon LPS challenge, the compounds DA193, -253, and -257 did not (Fig. 7A and B). TNF release inhibition in mBMMs was not significant upon application of the whole series of DA-compounds at the lower dose tested (1 μg/ml) whereas the application of the higher dose (10 μg/ml) resulted in efficient suppression of the expression of TNF by all compounds except for the long-chain DA193 and the short-chain DA257 (Fig. 7C). In contrast, the release of IL-6 was abrogated to a substantial degree (50-70%) by all DA-compounds applied to the cells 30 min prior to challenge with LPS, at a concentration of 1 μg/ml, except for DA257. 10-Fold higher concentration of the DA-compounds (10 μg/ml) inhibited IL-6

production to the background level (again, except for DA257) (Fig. 7D). The release of nitric oxide (NO) was effectively inhibited by five DA-antagonists already at the lower dose (1 μg/ml) (Fig. 7E). Thus, DA-compounds possessing shorter β-hydroxy-fatty acids (C_{12}-C_{10}), namely DA256, DA255 and DA254 exhibited the most pronounced antagonistic effects in LPS-challenged mBMMs by inhibiting at a concentration of 1 μg/ml the release of four immune mediators namely MIP-2, KC, IL-6, and NO.

In contrast, DA193 and -253 did not impede MIP-2 and KC release while inhibiting IL-6 and NO release. Different TLR4 epitopes might initiate different portions of TLR4 driven cell activation and some compounds block only one epitope while the bulk of them binds both of them of which one drives MIP-2 and KC on the one hand and the other drives IL-6 and NO production and release on the other hand. The complete abrogation of the expression of TNF required higher doses of the antagonists (10 μg/ml). The TLR4 inhibitory effect was largely specific given that TLR2 and TLR7 driven cell activation were not impaired by any of the six DA-compounds applied. LDH release of specifically those cells to which 10 μg/ml DA-compounds was applied might indicate impaired integrity of the cells analyzed (Fig. 7F). However, TLR7 driven cell activation was not at all impaired by the high dose (10 μg/ml) DA-compounds applied which strongly indicates a lack of cytotoxicity of the DA-compounds even at high concentration of 10 μg/ml, as well as TLR4 specificity of the agonists applied.

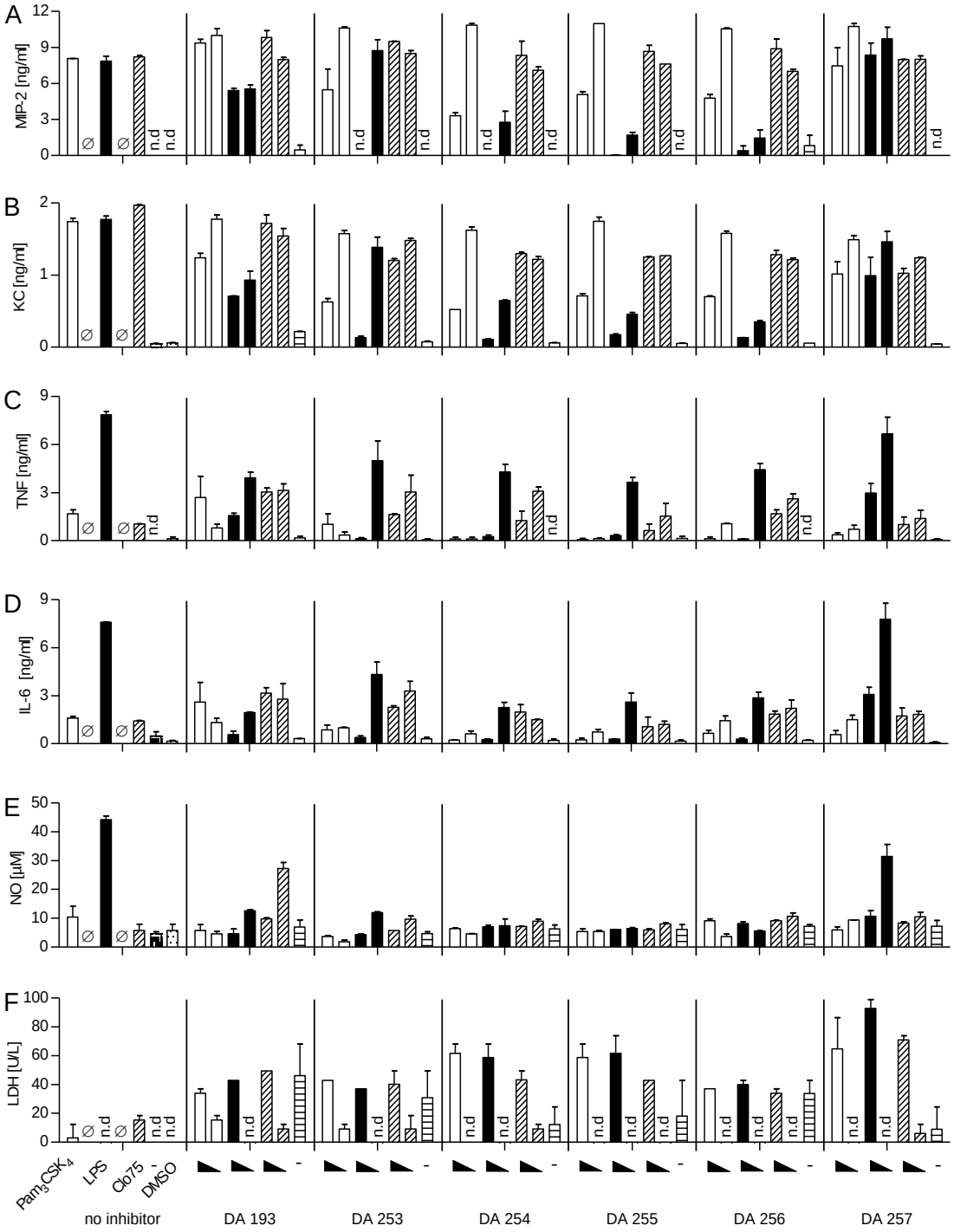


Figure 7: Responsiveness of mBMMs treated with DA-compounds prior to TLR specific challenge. 16 h supernatants of mBMMs challenged with 10 ng/ml of a tripalmytoylated hexapeptide (Pam_3CSK_4, white columns), 10 ng/ml of LPS O111:B4 (black columns), or 10 µg/ml of Clo75 (column with slanting striped patterns) and pretreated with DA-compounds where indicated for 30 min were analyzed for chemokine (A, B) or cytokine content (C, D) by ELISA. Their nitrite (NO) content was analyzed by Griess assay (E) and their lactate dehydrogenase (LDH) was as indicated (F). DA-compounds were applied at 10 µg/ml and 1 µg/ml (black triangles), samples for which high

dose (10 μg/ml) DA-compound only (column with horizontal striped patterns) or no compound (columns black with white pattern) was applied are marked by (-); mock control (DMSO, column white sprinkled); n.d, not detectable. The graphs show mean ± SD. A representative result of each experiment performed three different times - at which results were similar - and in triplicates is shown in each case.

In contrast to mouse cells, the most comprehensive and thus effective inhibition of the LPS-induced pro-inflammatory activity of hPBMC was achieved by the longer-chain mimetics DA193, DA253, and DA256 in that they inhibited the release of TNF to the background levels at a concentration of 1 μg/ml, whereas three shorter-chain DA-antagonists achieved a similar degree of inhibition only at a 10-fold higher dose (10 μg/ml, Fig. 8A and B). The expression of IL-6 in hPBMC was efficiently suppressed by DA-compounds bearing longer chain acyl residues (DA193, -253, -256) at a dose of 1 μg/ml, whereas DA255 was slightly active and DA257 was not at all active (Fig. 8A and B).

The DA-compound effects observed were specific in that the compounds did not impair Clo75 driven cell activation (data not shown). The latter was true for the cases in which a low dose (1 μg/ml) or a high dose (10 μg/ml) was applied indicating the absence of cytotoxicity even from a higher dose of DA-compounds. However, LDH was released by hPBMCs that were challenged with high dose DA-compounds in the absence of TLR agonists to a substantial degree but not by cells that were sequentially confronted with the DA-compounds and TLR agonists such that TLR activation blocked LDH release by the DA-compound challenged cells (Fig. 8C). Possibly does the mitogenic capacity of TLR agonists correlate with their LDH release inhibitory activity on DA-compound challenged cells.

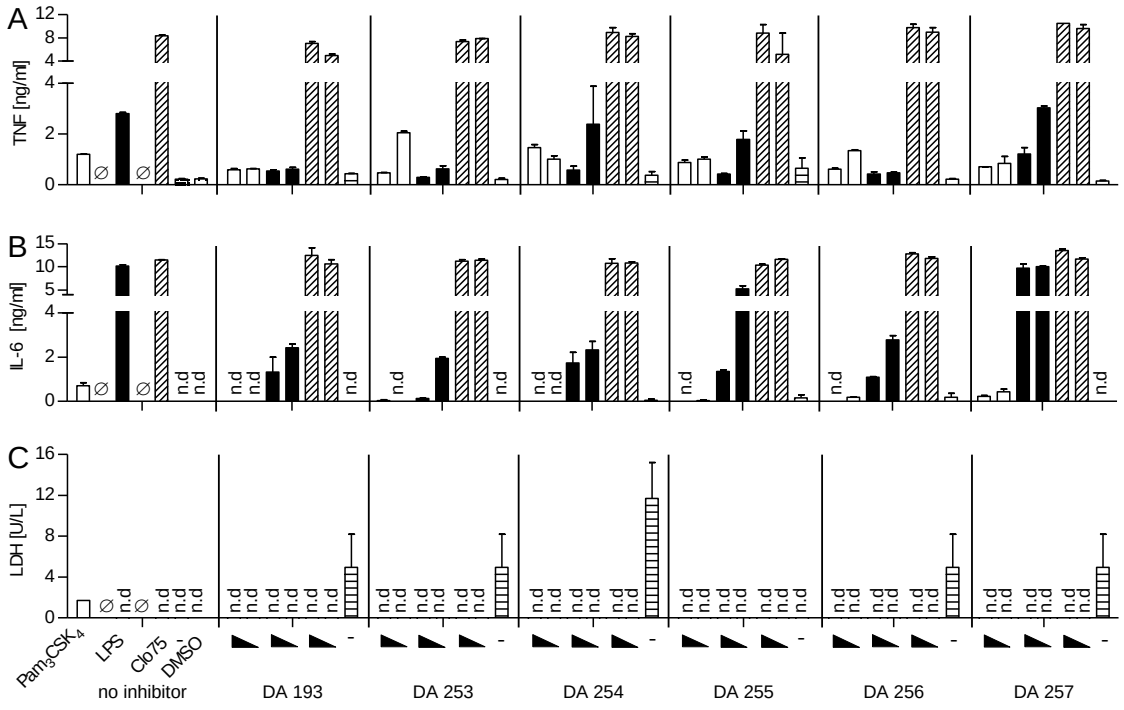


Figure 8: Responsiveness of hPBMCs treated with DA-compounds prior to TLR specific challenge.

hPBMCs pretreated with indicated DA-compounds for 30 min were challenged with 10 ng/ml of a tripalmytoylated hexapeptide (Pam_3CSK_4, white columns), 10 ng/ml of LPS O111:B4 (black columns), or 10 μg/ml of Clo75 (column with slanting striped patterns) Supernatants were analyzed 16 h post challenge for cytokine (A, B) content by ELISA and their lactate dehydrogenase (LDH) release (C). DA-compounds were applied at 10 μg/ml and 1 μg/ml (black triangles), samples for which high dose (10 μg/ml) DA-compound only (column with horizontal striped patterns) or no compound (columns black with white pattern) was applied are marked by (-); mock control (DMSO, column white sprinkled); n.d: not detectable. The graphs show mean ± SD. A representative result of each experiment performed three different times - at which results were similar - and in triplicates is shown in each case.

3.1.2 Analyzing the agonistic activity of synthetic tetra-acylated lipid-A mimetics.

The immune system protects the host against progressive growth of cancer and influences the immunogenicity of tumors, a concept known as immune surveillance (Smyth et al., 2006). Therefore studies intend to identify effective immune therapeutic approaches to eradicate outgrowth of cancers. Monophosphoryl lipid A (MPL) a potent vaccine adjuvant derived from *Salmonella minnesota* ligates TLR4. We analyzed seven synthetic TLR4 agonists namely FA 99, 125, 146, 147, 148, 151, and 161. To analyze these agonists, mBMMs from wt, *Myd88*$^{-/-}$, *Tlr2/4*$^{-/-}$, *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice were challenged with 1 μg/ml of the indicated synthetic agonists. 16 hours later supernatants were analyzed for cytokine and nitric oxide contents. All agonists induced cytokine (IL-6) and chemokine (CXCL1/KC) release in a TLR4 dependent manner (Fig. 9).

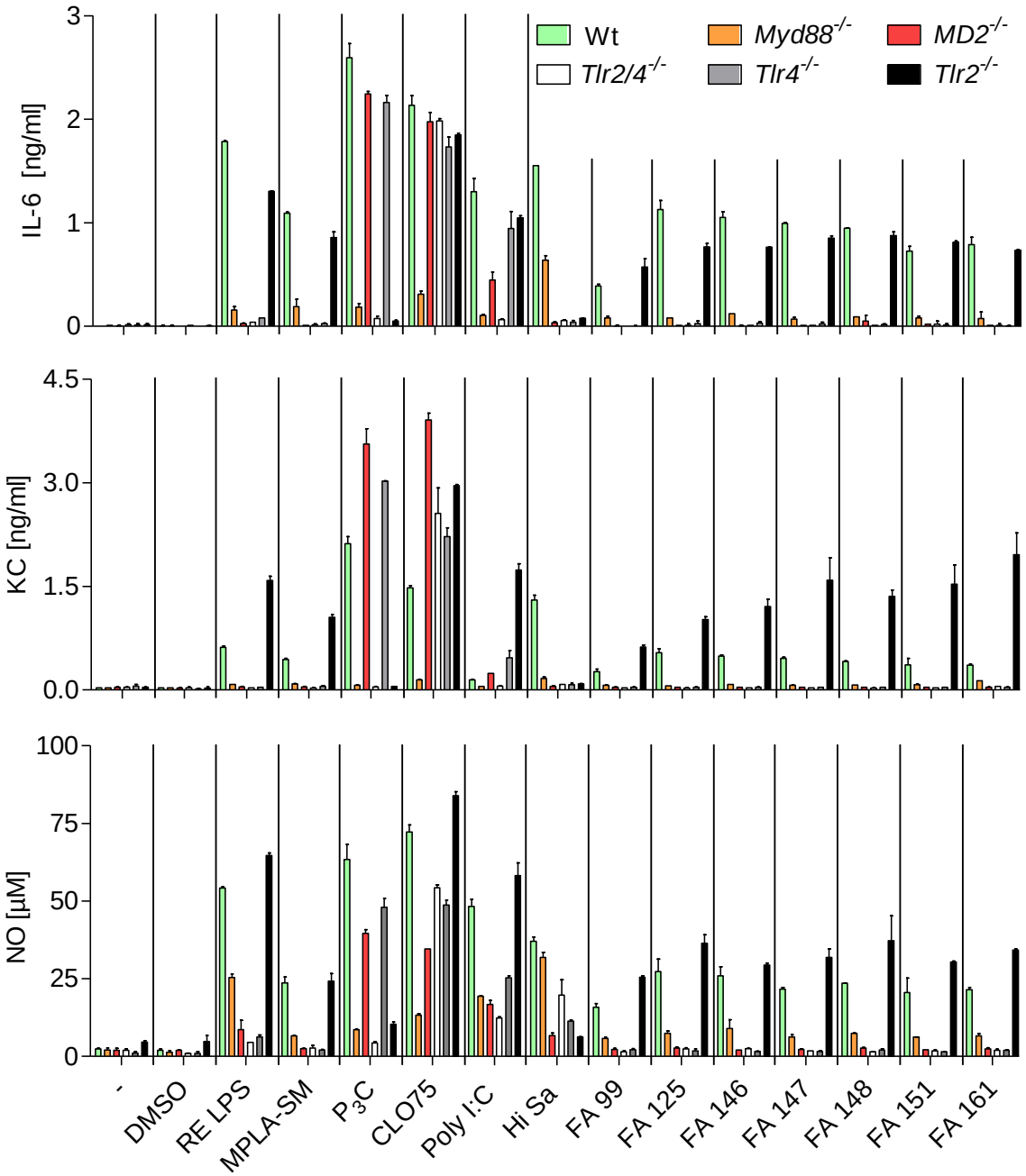


Figure 9: Synthetic agonists are TLR4 specific. Wild type (wt), adaptor protein Myd88, accessory protein MD2 deficient and other TLR deficient BMMS were primed with 20 ng/ml of murine IFN-γ and subsequently challenged with TLR specific stimuli (LPS O111:B4 1 µg/ml; MPLA-SM 1 µg/ml; RE LPS 1 µg/ml and agonists 1 µg/ml) for 16hours. Supernatants were analyzed for cytokine IL-6, chemokine KC/CXCL1 and nitric oxide release. The graphs show mean ± SD of duplicates. A representative result of two independent experiments performed at different times - at which results were similar.

3.2 Mitochondrial RNA and Sa19 like ORNs activate human TLR8.

In the year 2010, mitochondrial DNA (mtDNA) was implicated as an immune stimulatory DAMP operative in trauma (Zhang et al., 2010a). Mitochondria evolved from prokaryotes according to the endosymbiotic theory. Prokaryotic archaea phagocytosed prokaryotic bacterial Rickettsiales which evolved towards mitochondria (Thrash et al., 2011; Williams et al., 2013). Mitochondria carry an own genome and specific ribosomes. In mice, upon Gram-positive bacterial infection innate immune activity is elicited through binding of a specific large (23S) ribosomal (r) RNA borne segment to TLR13 (Kruger et al., 2015). Precisely, the segment called Sa19 is located in the 23S r RNA peptidyl transferase loop (PTL). Therefore, we compared PTL of bacterial 23S r RNA with large mitochondrial (mt) rRNA, namely 16S rRNA. Strikingly, the otherwise highly conserved PTL sequence carries a G↔A switch within the Sa19 homologous segment (Fig.10)

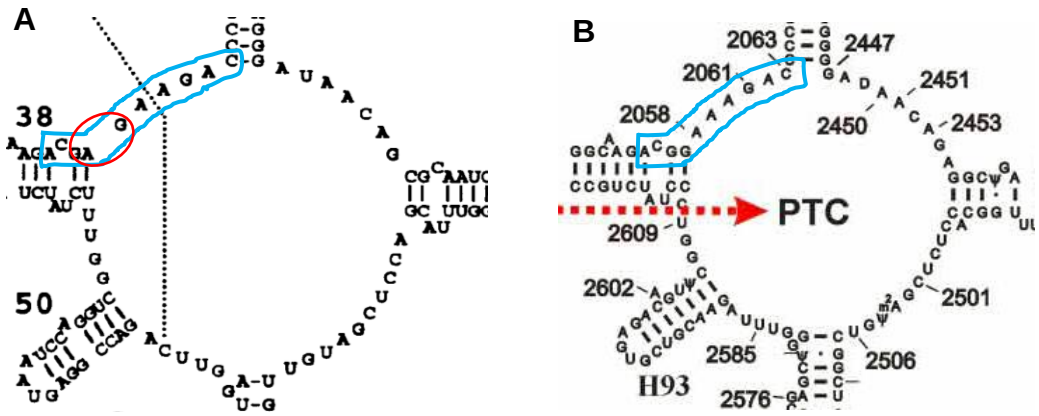


Figure 10: Comparison of Peptidyl transferase centers of eukaryotic mitochondrial and prokaryotic bacterial large ribosomal RNAs. *Bos Taurus* (A) and *E.coli* (B) highlighted MLS antibiotic and TLR13 ligand binding motif in blue and the difference in red art forms.

Cellular RNA-antimicrobial peptide complexes were reported to activate human dendritic cells (DCs) via TLR7 (Ganguly et al., 2009). Asking whether mitochondrial RNA might also bear an immune stimulatory capacity and considering Sa19-like motif as good candidate, we searched for Sa19 like motifs in the 16S mt rRNA. We selected motifs from human, cattle, mouse and rat 16S mt rRNA to design specific 19-mer Sa19 “like” oligoribonucleotides (ORNs) (Table 3, Table 4).

Table 3: Alignment of Sa19 and mitochondrial 16S rRNA Sa19 "like" segments. (mt, mitochondrial; PTL, peptidyl transferase loop; D, domain; _, transition region) sequence with common core motif (blue) and uracils (red U); *conserved in human (Hs), cattle (Bt), mouse and rat; G/A underlined, mutated core motif; ma, 6N methylation of adenosine 7.

Sa19	5′- GGACGGAAAGACCCCGUGG -3′
Sa19mA7	5′- GGACGG(^{m}A)AAGACCCCGUGG -3′
Sa19A7G	5′- GGACGGGAAGACCCCGUGG -3′
*mtPTL	5′- AGACGAGAAGACCCUAUGG -3′
BtmtD3_4	5′- AUCUAAGGAAAGAUUAAAA -3′
HsmtD3_4	5′- UCAUAAGGAAAGGUUAAAA -3′
HsmtD1	5′- CGCAAGGGAAAGAUGAAAA -3′

Table 4: Sequence information of Sa19 like segments. abbr., abbreviation; no., number accessible at "http://www.ncbi.nlm.nih.gov/nuccore/"; term., terminal residue; b, base/s; *, sequence identical in 16S mtrRNA of all four species considered; Hs, *Homo sapiens*; Bt, *Bos taurus*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*.

No	species	abbr.	16S rRNA affiliation nr.	length [b]	ORN name	5´-term. [residue]	3´-term. [residue]
1	human	Hs	NC_012920.1	1557	*mtPTL	1045	1063
2	"	"	"	"	HsmtD3_4	772	790
3	"	"	"	"	HsmtD1	116	134
4	cattle	Bt	AF492351.1	1570	*mtPTL	1057	1075
5	"	"	"	"	BtmtD3_4	783	801
6	mouse	Mm	NC_005089.1	1582	*mtPTL	1059	1077
7	rat	Rn	KF011917.1	1559	*mtPTL	1042	1060

The mitochondria contain large 16S and small 12S rRNAs in contrast to respective bacterial 23S and 16S rRNAs. We have chosen a segment within mitochondrial 16S rRNA of *Bos Taurus* and named it as BtmtD3_4 as it is localized between domain 3 and 4 (Fig. 11). It shares with Sa19 the core sequence ”GGAAAGA”.

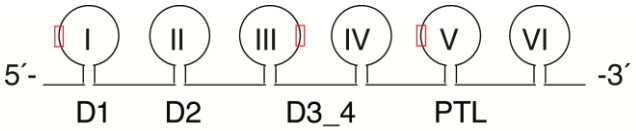


Figure 11: Schematic representation of both, bacterial 23S and mitochondrial 16S rRNAs. The six subdomains (I-VI) are represented as circles. Red rectangles indicate relative positions of the ORNs listed in table 3. D, domain; PTL, peptidyl transferase loop.

Since BtmtD3_4 carries a Sa19-core motif, we considered it as a likely TLR13 ligand. Despite this property, BtmtD3_4 failed to activate mouse splenocytes while Sa19 induced substantial IL-6 release (Fig. 12).

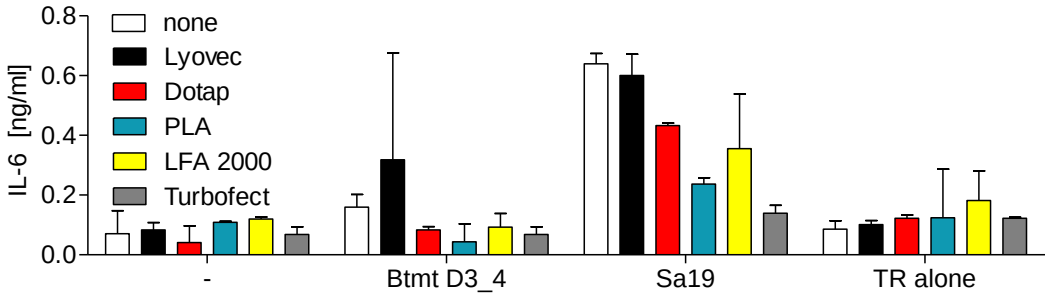


Figure 12: Wt splenocytes challenged with ORN BtmtD3_4 failed to release cytokine. Cytokine release into the supernatants from splenocytes transfected for 16 h with ORNs with various transfection reagents (pLA, poly-L-arginine; LFA, Lipofectamine 2000; TR, transfection reagent). The graph show mean ± SD, n=3 and in duplicates is shown in each case.

Next, we sought to analyze a systemic response to BtmtD3_4 in mice. Mice were sensitized with IFN-γ for 45 min. Subsequently, phosphorothioate-stabilized ORNs such as BtmtD3_4 and Sa19 were injected intravenously. Mice were bled at the indicated time point’s to determine plasma IL-6 concentrations (Fig.13).

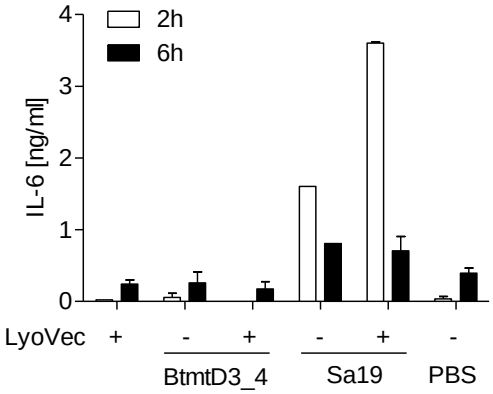


Figure 13: Systemic challenge with ORN BtmtD3_4 failed to induce an immune response. Plasma IL-6 concentrations of mice sensitized with IFN-γ (*i.v.*; 50 μg/kg; Peprotech*)* for 45 min upon which 4 mg/kg of phosphorothioate-stabilized ORNs indicated were injected *i.v*. Subsequently, blood was collected by puncturing the retro orbital sinus vein at the indicated time points. The graph show mean ± SD, n=3 mice per group.

3.2.1 *mtPTL activates mouse macrophages in a TLR7 dependent manner.

The 16S mtrRNA Sa19 orthologous segment termed *mtPTL, like Sa19 located within the domain V of the large rRNA, is conserved in human, cattle, mouse and rat, yet mutated toward “GAGAAGA” (Table 4, Sa19 “GGAAAGA”). Moreover, Sa19 motifs are present in the other regions of 16SmtrRNAs (Greber et al., 2014). To narrow down the PRR recognizing the *mtPTL ORN and considering RNA sensing TLRs namely TLR3 and TLR7 as good candidate molecules, I employed BMMs from TLR deficient mice. Comparative analysis indicates BMMs lacking TLR7 expression were unresponsive upon *mtPTL challenge. Accordingly, TLR7 was responsible for cell activation, certainly due to *mtPTL carriage of a TLR7-prone “UAU” motif (Fig. 14).

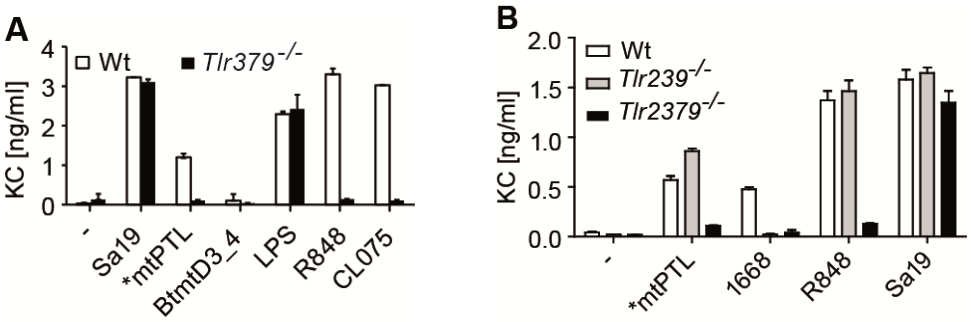


Figure 14: ORN mtPTL activates mBMMs in a TLR7 dependent manner. Wild type (Wt) and the indicated TLR deficient mBMMs were challenged with TLR specific stimuli (CLO75, 2.5 µg/ml; R848, 0.2 µg/ml; LPS, 0.1 µg/ml, 1668, 10 µM), and transfected ORNs (BtmtD3_4; *mtPTL, 100 pmol per 200 µl). Supernatants were analyzed for cytokine content by ELISA 16 h post challenge. The graphs show mean ± SD, n=3.

In order to validate synthetic ORN analyses, we isolated mtRNA from rat liver and human liver cancer cell line (Hep G2) (Fig. 15).

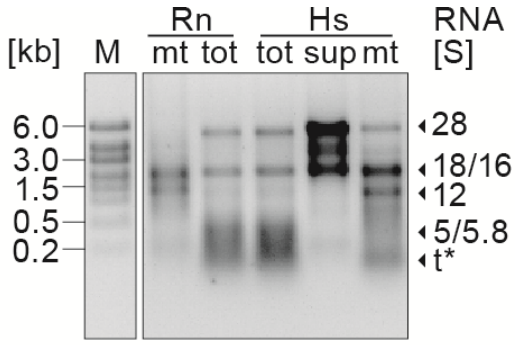


Figure 15: Total (tot) and mitochondrial (mt) RNA fractions. Agarose electrophoresis gels carrying purified mammalian mtRNA. (kb, kilobases; M, RNA size marker; tot, total; S, Svedberg; r, ribosomal; t, transfer; mt, mitochondrial RNA; sup, supernatant at initial mitochondria separation step; Rn, rattus norvegicus; Hs, homo sapiens).

Human TLR7 mediates bacterial tRNA driven type I interferon production from DCs (Gehrig et al., 2012; Jockel et al., 2012). Therefore, we analyzed IFNα production from human PBMCs upon challenge with the total mtRNA. Both, Sa19 like ORN BtmtD3_4 and mtRNA failed to induce IFNα production. Cognate TLR7 ligands such as RNA40 induced type I interferon indicating TLR7 as operative while IL-6 production indicated principal cell activating capacity of all RNAs (Fig. 16).

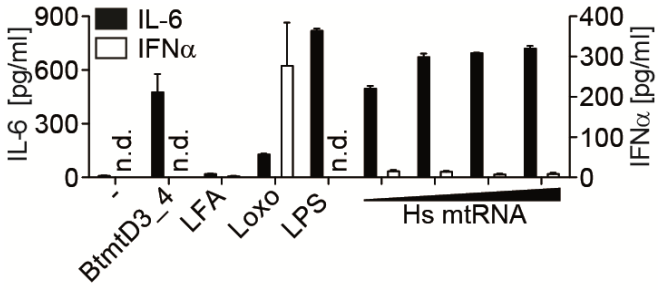


Figure 16: Mitochondrial RNA and BtmtD3_4 induced pro inflammatory cytokine release from hPBMCs, yet failed to induce IFNα. hPBMCs were transfected with 0.25, 0.5, 1 and 2 µg of Hs mtRNA, BtmtD3_4 (100 pmol per 200 µl) or challenged with LPS (0.1 µg/ml). Supernatants were analyzed for their cytokine content by ELISA 16 h post challenge. (LFA, Lipofectamine 2000; Loxo, Loxoribine; Hs, homo sapiens; mt, mitochondria) The graphs show mean ± SD, n=3.

Also, rattus norvegicus (Rn) mtRNA failed to induce IFN-α from human PBMCs (Fig 17).

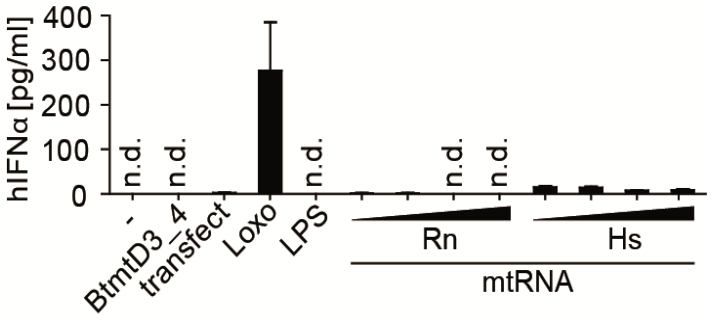


Figure 17: Mitochondrial RNA and BtmtD3_4 failed to induce IFNα release from hPBMCs. PBMCs were challenged with TLR specific stimuli (LPS, 0.1μg/ml, Loxo, 0.6 mM) transfected with ORN (BtmtD3_4 100 pmol per 200 μl) transfected 0.25, 0.5, 1 and 2 μg of mtRNA with L2K. Supernatants were analyzed for their cytokine content by ELISA 16 h post challenge.(L2K, Lipofectamine 2000; Loxo, Loxoribine; Rn, rattus norvegicus; Hs, homo sapiens; mt, mitochondria) The graph show mean ± SD, n=3.

TLR7 and 8 bind to U/G or U/A rich viral-, Si-, and self RNA sequences (Barrat et al., 2005; Forsbach et al., 2008; Heil et al., 2004; Hornung et al., 2005; Lund et al., 2004). RNA specificity of TLR8 is known to depend on the U and G content of ssRNA. According to structural analysis, two binding sites are employed by degraded products of ssRNA in a synergistic manner. The first site binds uridine mononucleoside, whereas the second site binds UG/UGG ssRNA segments (Tanji et al., 2015). Our analysis of different ORNs indicated also UA/UAA/UAG/UGA motifs as operative (Kruger et al., 2015). Therefore, UR/URR likely represents the TLR8 ligand consensus motif in a more comprehensive manner.

We next analyzed human PBMCs for their responsiveness to all Sa19 like ORNs designed of which only *mtPTL was murine immune stimulatory (Fig. 14 and data not shown). While all ORNs applied including TLR13-silent, point mutated Sa19A7G, and a methylated Sa19 variant (Sa19mA7) induced robust TNF release from PBMCs, none triggered IFNα (Fig.18) indicating absence of TLR7 involvement in their cellular recognition. The stimulatory capacity of a low U content ORNs such as Sa19, Sa197AG or Sa19mA7 was slightly lower as compared to BtmtD3_4.

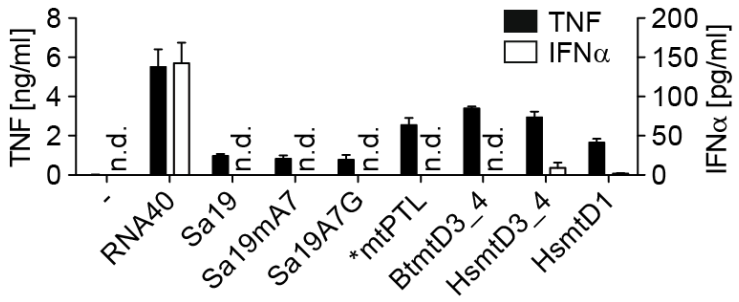


Figure 18: ORNs effectively induced TNF but not IFNα release from hPBMCs upon any of the modifications applied. PBMCs were transfected with RNA40 (5 μg/ml transfected with LyoVec). ORNs indicated were transfected with LyoVec (each with 100 pmol per 200 μl). Supernatants were analyzed for their cytokine content by ELISA 16 h post challenge. (Hs, homo sapiens; Bt, bos taurus; mt, mitochondria). The graphs show mean ± SD, n=3.

We next sought to narrow down the identity of the obviously non-TLR7 sensor of the ORNs applied. First, PBMCs of a human individual expressing a non-functional Glu53Δ MyD88 mutant (Alsina et al., 2014) were analyzed for their responsiveness to Sa19 “like” ORNs (Kruger et al., 2015). PBMCs represent numerous cell populations such as B-, T-, NK-cells, monocytes and DCs. Focusing next on monocyte/macrophage activity, we employed monocytic THP-1 cells differentiated with phorbol 12-myristate 13-acetate (PMA) treatment for 24 h, followed by culture in fresh medium for 72 h (3ddi). Medium was exchaged with fresh medium proir to challenge for 16 h. Functional Unc93B1 defective human PBMCs failed to respond to the challenge while they released IL-8 upon LPS challenge, indicating viability and functionality of the cells and non-involvement of Myd88 in LPS driven human IL-8 production (data not shown, Kruger et al., 2015). To advance narrowing down the respective PRR, we employed Unc93B1 deficient THP-1 cells besides wt counterparts. Unc93B1 shuttles endosomal TLRs from the endoplasmic reticulum (ER) to the endosome. THP-1 cells lacking Unc93B1 expression (*Unc93B1*$^{-/-}$

provided by V.Hornung, Bonn) had been prepared by introduction of a frameshift mutation into the *Unc93B1* orf applying the CRISPR/Cas9 technique (Schmid-Burgk et al., 2014). When challenged with the ORNs, *Unc93B1*$^{-/-}$ 3ddiTHP-1 cells were refractory while wt controls released substantial amounts of TNF (Fig.19). These results indicate mediation of Sa19-like ORN driven immune activation by an endosomal TLR.

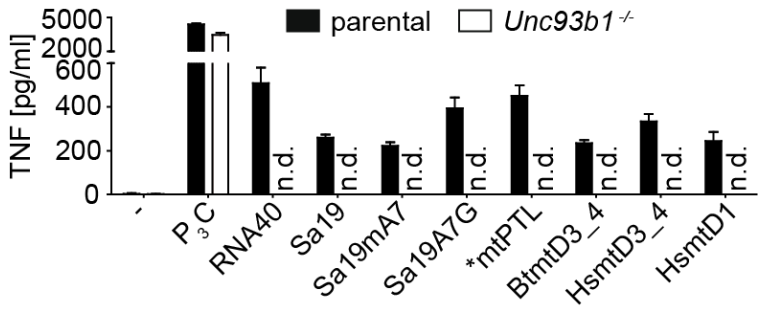


Figure 19: Stimulatory capacity of Sa19-like ORNs depends upon an endosomal TLR. Parental and *Unc93B1*$^{-/-}$ 3ddi-THP1 cells were challenged with P_3C (1 μg/ml), RNA40 (5 μg/ml transfected with LyoVec), Sa19 or Sa19-like ORNs indicated (100 pmol per 200 μl each transfected with L2K or pLA respectively). Supernatants were analyzed for the cytokine content by ELISA 16 h post challenge. (L2K, Lipofectamine 2000; pLA, Poly-L-arginine; Hs, homo sapiens; Bt, bos taurus; mt, mitochondria) The graph show mean ± SD, n=3.

Aiming at the identification of the PRR involved, we performed a comparitive transcriptome analysis of 3ddiTHP-1 cells challenged with Sa19-like ORNs. Analyzing the result, we focused at transmembrane receptor encoding mRNAs. Since Sa19-like ORN responsiveness correlated with expression of mRNA encoding the TLR13-like TLR8 (Kruger et al., 2015) we considered TLR8 as a good candidate molecule. Consequently, we analyzed TLR8 deficient THP-1 cells (*Tlr8*$^{-/-}$). Indeed, *Tlr8*$^{-/-}$-3ddiTHP-1 cells were unresponsive to ORN challenge (Fig. 20).

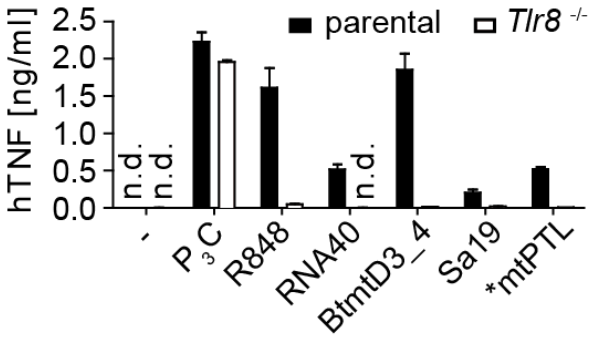


Figure 20: Stimulatory capacity of Sa19-like mtRNA representing ORNs is TLR8 dependent. Parental and *Tlr8*$^{-/-}$-3ddiTHP1 cells were challenged with P_3C (1 μg/ml), R848 (10 μg/ml), RNA40 (5 μg/ml transfected with LyoVec), Sa19, BtmtD3_4, and *mtPTL (100 pmol per 200 μl transfected with L2K, pLA or LyoVec respectively). Supernatants were analyzed for the cytokine content by ELISA 16 h post challenge. (L2K, Lipofectamine 2000; pLA, Poly-L-arginine; Bt, bos taurus; mt, mitochondria). The graph show mean ± SD, n=3.

We next transfected Hep G2 cell line derived mtRNA into parental, *Unc93b1*$^{-/-}$-, and *Tlr8*$^{-/-}$-3ddi-THP1 cells. MtRNA-driven activation of 3ddiTHP1 cells was absent from *Unc93b1*$^{-/-}$-, *Tlr8*$^{-/-}$-3ddiTHP1 cells (Fig. 21). In contrast to mTLR13, hTLR8 carries a Z-loop mediating extracellular domain (ECD) cleavage towards formation of a ligand binding site is likely nonfunctional/mutated mTLR8 (Kruger et al., 2015; Tanji et al., 2015) which might explain the inability of the murine immune system to respond to all Sa19-like ORNs except for *mtPTL.

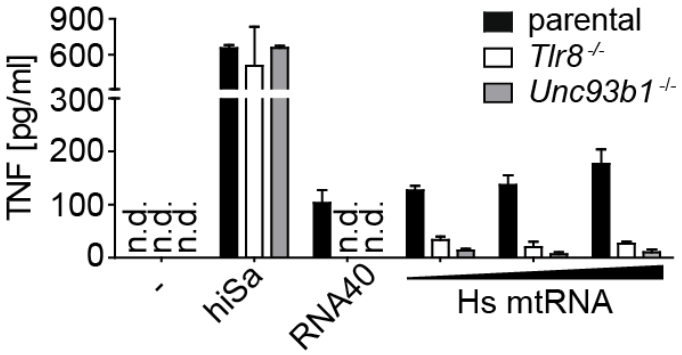


Figure 21: Mitochondrial RNA activates endosomal TLR8. Parental, *Unc93b1*$^{-/-}$, *Tlr8*$^{-/-}$ 3ddi-THP1 cells were challenged with RNA40 (5 μg/ml transfected with LyoVec), hiSa (10^8 cfu/ml) and transfected 0.25, 0.5, 1 and 2 μg of Hs mtRNA with L2K. (L2K, Lipofectamine 2000; Hs, homo sapiens; mt, mitochondria). Supernatants were analyzed for cytokine content by ELISA 16 h post challenge. The graph show mean ± SD, n=3.

To further validate our implication of TLR8 we performed a gain of function analysis. Specifically, we applied all Sa19-like ORNs to hTLR8 overexpressing HEK293 cells. In contrast to receptor controls, TLR8^{+} HEK293 cells activated NF-κB upon Sa19-like mtRNA derived ORN challenge (Fig.22).

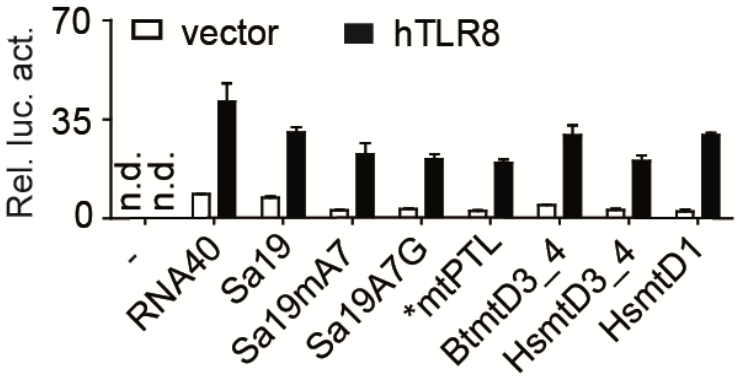


Figure 22: BtmtD3_4 activates TLR8. HEK293 cells were transfected with an empty vector (vector) or hTLR8 expression plasmid (30 ng/well) and a plasmid encoding NF-κB promotor luciferase reporter construct. 16 h later, cells were challenged with RNA40 (5 μg/ml transfected with LyoVec), Sa19 and BtmtD3_4 (100 pmol per 200 μl) transfected with L2K and pLA respectively. Other indicated ORNs were transfected with LyoVec for 16 h cells were lysed for luciferase activity measurement. (L2K, Lipofectamine 2000; pLA, Poly-L-arginine; Bt, bos taurus; Hs, homo sapiens; mt, mitochondria; Rel. luc. act., relative luciferase activity). The graph show mean ± SD, n=3.

Our results implicate TLR8 as the major self-mtRNA sensor. Moreover, uridine is a major ligand element of ORN HsmtD1, like Sa19 containing only one U (UGA) which strongly activated HEK293 cells overexpressing TLR8. Also *mtPTL which contains two Us (UA and UGG) was active. BtmtD3_4 and HsmtD3_4 containing two Us (UA and UAA) activated human PBMCs. Thus, UR/R rather than only U- content of the ORNs was essential for hTLR8 binding. Both UG/UGG and UA/UAA or U followed by combination of different purines (R) activated TLR8. Accordingly, we propose UR/URR as the human TLR8 ligand consensus motif.

3.3 Radiation induced sterile inflammatory response.

TLR activity has been implicated as being operative upon interruptions of homeostasis such as by tissue damage in trauma eliciting acute inflammation pathology (Muller et al., 2001; Tsung et al., 2005; Wang et al., 1999). IIR is also a sterile insult and applied for preconditioning towards bone marrow transplant (BMT) alone/or in combination with chemotherapy. IIR is also employed for antitumor radiation therapy (Thariat et al., 2013). High dose IIR elicits acute radiation sickness. Conceptually, tissue damage upon exposure to IIR might release DAMPs activating the immune system and elicit inflammation. Danger signals released by damaged cells alarm the neighboring cells and trigger adaptive immune responses (McBride et al., 2004).

Cellular DAMP challenge triggers cytokine and reactive oxygen species (ROS) release. PRRs are candidate sensors of DAMPs according to specific literature. For instance, TLR4 has been implicated as a sensor of IIR induced tissue damage by sensing HMGB1 released from destroyed cells (Apetoh et al., 2007). Implicating TLR activity in IIR driven host immune activation rather indirectly, application of ligands of numerous TLRs such as TLR2, TLR3, TLR4, TLR5 or TLR9 protects mice and primates from IIR induced pathology towards gastrointestinal syndrome and lethality (Burdelya et al., 2008; Saha et al., 2012; Sistigu et al., 2014; Takemura et al., 2014; Vijay-Kumar et al., 2008). Moreover, application of TLR7/8 ligands prior, concurrently or after radiotherapy has been shown to induce a systemic antitumor immune response (Scholch et al., 2015).

Comprehensively, the literature suggests an involvement of TLRs in sterile tissue damage such as trauma, tumor expansion, and IIR driven immune activation towards eliciting both protective and curative response. However, numerous TLR implications in sterile immune activity have been challenged and the identity of the mechanisms underlying the protective and curative responses remains largely unknown. We chose IIR as both sterile and clinically relevant insult to mice and cell culture. Our hypothesis was that IIR drives inflammatory immune activity upon BMT preconditioning. Accordingly, IIR would set the stage for graft versus host disease (GvHD) by triggering establishment of an inflammatory milieu in the recipient host-to-be organism. At the time (2009) parameters of an IIR induced inflammation were largely unknown. The first step was to illustrate an inflammatory response towards

IIR. Specifically, IFN-γ priming sensitizes *Tlr4*$^{-/-}$ mice for lethal LPS challenge (Spiller et al., 2007) and thus enhances antigen presenting cell (APC) activity (Marchi et al., 2014). Moreover, D-GalN treatment sensitizes mice for LPS treatment (Galanos et al., 1979). To potentially enhance sensitivity for irradiation induced DAMP challenge, we applied IFN-γ and D-galactosamine (D-GalN) either alone or in combination (data not shown).

Based on our expertise on mouse immune activation towards an extreme degree (Spiller et al., 2007; Werts et al., 2001) and expecting rather mild immune stimulation as compared to that upon PAMP challenge, we chose ^{60}Co as the source of IIR and exposed mice with high dose (30 Gy) in which mice were either untreated, IFN-γ primed, D-GalN challenged or treated in combination of both. As the time of exposure is approximately 4 h, we chose the 6 h time point thus as an early time point at which blood can be withdrawn for cytokine content analysis from plasma. Since D-GalN treatment turned out to be less effective (not shown), we performed only IFN-γ priming. Moreover, the 24 h and 48 h post IIR were considered in our early set of experiments (Fig. 23).

3.3.1 Short term immune stimulatory response by IIR.

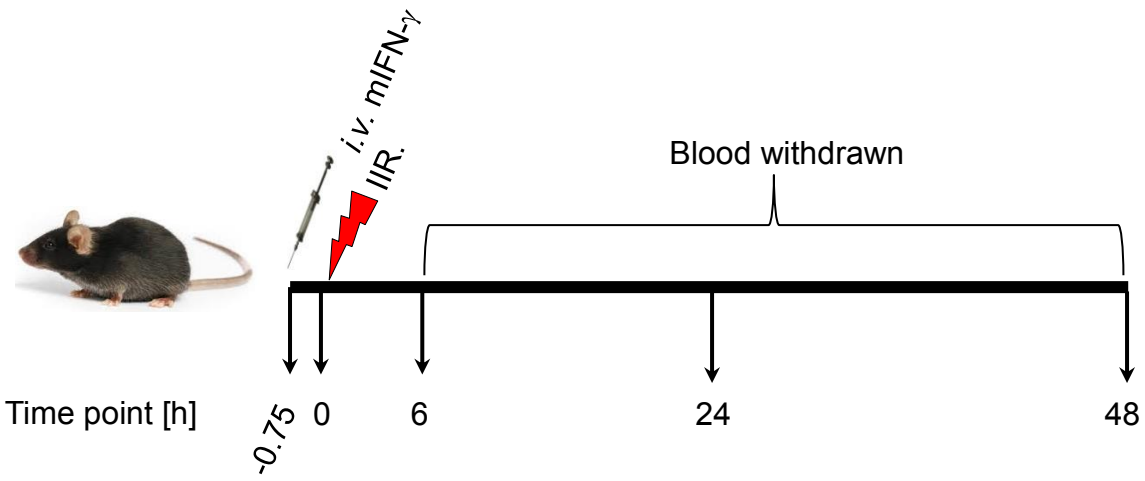


Figure 23: Schematic representation of short term irradiation model. Mice were sensitized with murine IFN-γ i.v. injection (50 μg/kg body weight) for 45 minutes. Subsequently, mice were exposed to 30 Gy of total body irradiation. Blood was harvested 6 h, 24 h, and 48 h post irradiation. Plasma cytokine (IL-6) and chemokine (KC) concentrations were determined by luminex assay.

Systemic cytokine and chemokine levels of INF-γ primed mice were significantly increased as compared to un-primed mice post LPS challenge (Fig. 24).

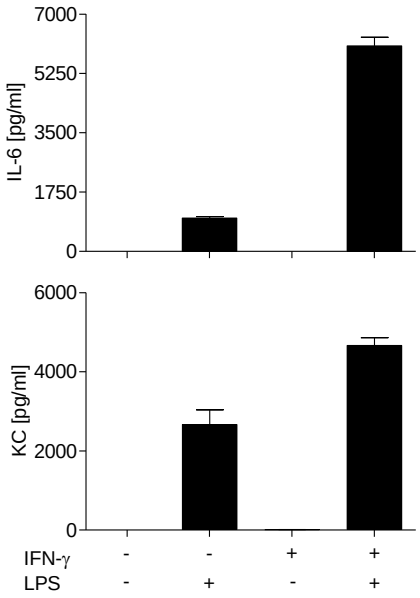


Figure 24: LPS induced plasma cytokine and chemokine release is significantly enhanced by IFN-γ priming. Wt mice were left untreated or primed by intravenous injection of murine IFN-γ (50 μg/kg body weight). 45 min later mice were left untreated or LPS O111:B4 (4 mg/kg body weight) was injected intraperitoneally. Blood was harvested 6 h post LPS challenge. Plasma cytokine (IL-6) and chemokine (KC) contents were analyzed by luminex assay. The graphs show mean ± SD of two mice per group. Experiment was performed once.

IFN-γ primed mice exposed to 30 Gy of total body irradiation (TBI) released more chemokine (CXCL1/KC) to their serum as compared to unprimed counterparts. At time points later than 6 h post IIR, immune activity was hardly detectable (Fig. 25).

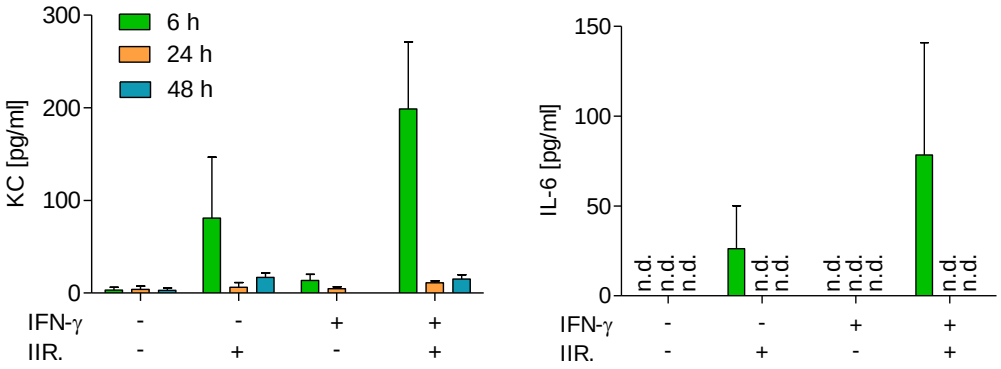


Figure 25: Cytokine release post ionizing irradiation peaked at the 6 hour time point was substantially enhanced by IFNγ priming. Wild type (Wt) mice were intravenously injected with murine IFN-γ (50 μg/kg body weight). 45 min later, mice were exposed to 30 Gy of total body IIR. Subsequently, blood was harvested at the indicated time points. Plasma chemokine (KC) and cytokine (IL-6) concentrations were determined by luminex assay. The graphs show mean ± SD of at least 3 mice per group. A representative result of two independent experiments performed at different times - at which results were similar. n.d., not detected.

Upon induction of IL-1 type cytokine production, nucleotide-binding oligomerization domain like receptors (NLRs) mediate activation of caspase-1 cleaving pro IL-1 type cytokines towards their maturation and biological activity (Martinon et al., 2002). The NLR multi-molecular complexes were termed “inflammasomes”. Besides numerous compounds such as uric acid and DNA, also radiation exposure activates inflammasomes accordingly to a recent report (Stoecklein et al., 2015). Therein, DNA driven AIM2 mediates caspase-1-dependent IL-1β release and death of intestinal epithelial and bone marrow cells in response to release of DNA to the cytosol possibly involving double-stranded DNA breaks induced by IIR (Hu et al., 2016). However, IL-1α or IL-1β contents were substantially lower at times and not detected in sera of irradiated mice (not shown).

According to a common view, an immune response upon IIR is due to an increase of the permeability of the gut lining toward the circulation. Specifically, microbial product transgression from the intestinal lumen towards the blood “must” be responsible for a fast immune response to the challenge. Since such assumption puts our “truly nonspecific insult –IIR” statement into question, in this context we next analyzed germ free Balb/c received from an axenic breeding facility (A. Bleich, MH Hannover) in sterile containers ventilated via sterile hepafilters. Upon receipt, mice were transferred to sterilized irradiation cages out of which mice were then transferred

back to transport containers under a sterile hood. 6 h upon IIR, plasma and organs were harvested. As a control, specific pathogen-free (SPF)-Balb/c mice were investigated. Fur and feces smear plated indicated sterility of the mice. Our results indicate independence of the systemic response detected from commensal microbiota (Fig. 26).

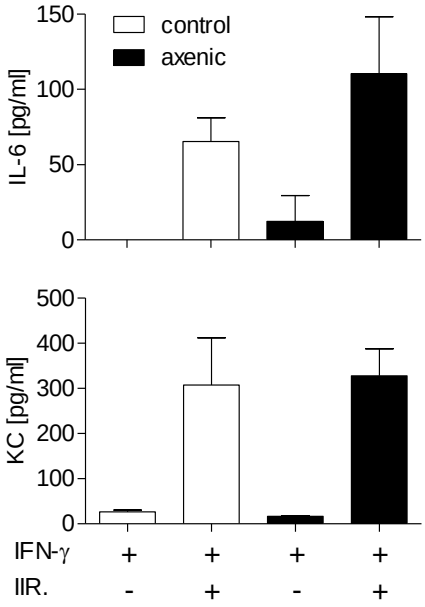


Figure 26: Cytokine release 6 h post ionizing irradiation is independent of gut microbiota. Wt SPF (control) and germ free (axenic) mice were untreated or intravenously injected with murine IFN-γ (50 μg/Kg body weight). 45 min later mice were exposed to 30 Gy total body irradiation. Blood was withdrawn 6 h post irradiation. Plasma cytokine (IL-6) and chemokine (KC) contents were determined by luminex assay. The graphs show mean ± SD of at least 3 mice per group. A representative result of two independent experiments performed at different times - at which results were similar.

Neutrophils and macrophages are considered as major responders that are recruited to the site of damage/infection. Macrophages perform both injury inducing and repair–promoting tasks in different mouse models of inflammation. Accordingly, blocking macrophage infiltration mitigates radiation skin injury and radiation lung injury (Thanasegaran et al., 2015). To explore the role of macrophages and neutrophils in IIR immune activation, we depleted macrophages by injecting intraperitoneally and intravenously clodronate liposomes, as well as anti-Ly6G (1A8) antibody to also deplete neutrophils. Reduced, yet not abrogated cytokine release post IIR (Fig. 27) supported attribution of a central role to both innate immune cell populations in IIR driven immune activation.

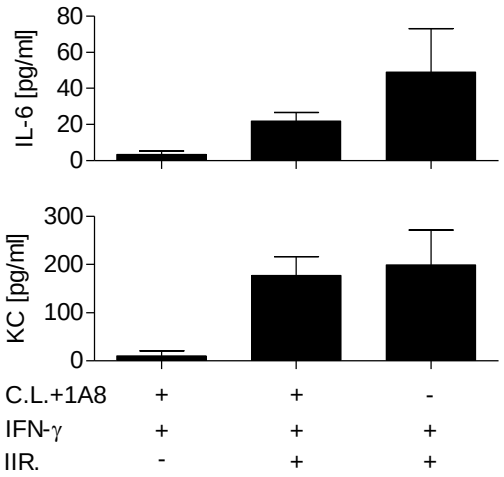


Figure 27: Macrophages and/or neutrophils are involved in immune response post IIR. Mice were injected intravenously and intraperitoneally with clodronate liposome (C.L.) (100 μl/kg body weight) and intraperitoneally with monoclonal antibody 1A8 (50 mg/kg body weight). One day later, mice were injected intravenously with murine IFN-γ (50 μg/kg body weight). 45 min later mice were exposed to 30 Gy total body irradiation. Subsequently blood was withdrawn 6 h post irradiation. Plasma cytokine (IL-6) and chemokine (KC) contents were determined by luminex assay. The graphs show mean ± SD of at least 3 mice per group. Experiment was performed once.

TLR3 blockade protected mice from radiation-induced gastrointestinal syndrome (Takemura et al., 2014). TLR7 and TLR8 induce systemic IIR driven antitumor immune response (Scholch et al., 2015). In order to evaluate the potential of TLR7 and TLR9 contribution to IIR driven immune activation, we applied the inhibitory oligo deoxynucleotide “79i” (Barrat et al., 2005) intraperitoneally prior to irradiation. Application of 79i significantly reduced cytokine release post irradiation (Fig. 28). However, 79i blocks also TLR13 activation and might be considered as a “master endosomal TLR inhibitor” which might put TLR7 and -9 specificity into relation. It might thus be considered that rather than TLR7 and/or TLR9 a broader mechanism of unknown identity might be implicated by this result (Fig. 28)

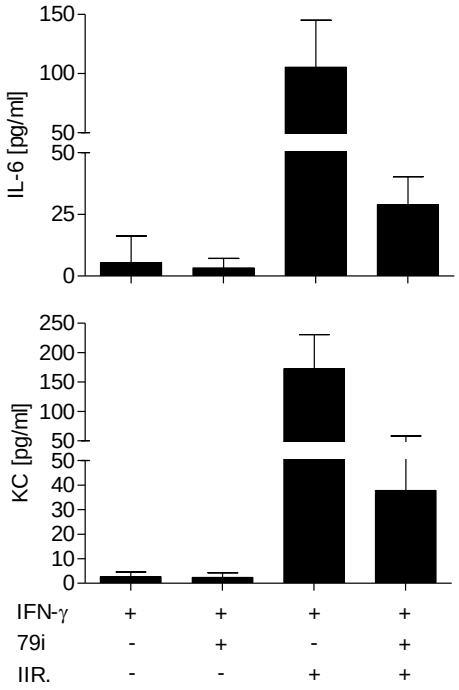


Figure 28: Application of TLR7/9 inhibitory oligodeoxyribonucleotide significantly reduced cytokine release post IIR. IFN-γ (50 μg/kg body weight) primed mice were challenged with 79i (8 mg/kg body weight) 45 min upon which mice were exposed to 30 Gy total body irradiation. Blood was withdrawn 6 h post irradiation. Plasma cytokine (IL-6) and chemokine (KC) concentrations were determined by bead based luminex assay. The graphs show mean ± SD of at least 3 mice per group. Experiment was performed once.

MyD88 and TRIF are the major adaptor proteins and mediators of TLR and IL-1 receptor (IL1R) signal transduction (Kawai and Akira, 2010; Takeda and Akira, 2004). According to substantial portion of literature, tissue injury upon IIR leads to release of DAMPs acting as ligands of TLRs to elicit substantial immune activity (Galluzzi et al., 2017; Zitvogel et al., 2010). Having observed strong immune response in wt mice, we sought to evaluate TLRs for their involvement in IIR driven immune activity. Accordingly, mice lacking expression of adaptor molecules MyD88 and TRIF were analyzed next. *Myd88/Trif* $^{-/-}$ mice failed to mount an immune response observed in wt controls which indicated involvement of yet unspecified TLRs and/or IL-1 cytokine receptors in IIR driven immune activation (Fig. 29).

Analysis of mice lacking IL-1 receptor associated kinase (IRAK) 4 corroborated the previous statement in that *Irak4*$^{-/-}$ mice like *Myd88*$^{-/-}$ mice were refractory towards IIR in respect to the parameters analyzed by us (Fig. 29). Our results thus implicate

MyD88 and IRAK4 but not TRIF in IIR driven cytokine release to the serum. Since inflammasomes were reported to be IIR driven (Stoecklein et al., 2015), we accessed adopter protein apoptosis-associated speck like protein (ASC) deficient *Asc*$^{-/-}$ mice lacking IL-1β, IL-18, and IL-33 processing upon ASC-inflammasome specific challenge. Also their responsiveness was unremarkable in that no reduction of the cytokine plasma cytokine levels was detectable (Fig. 29), as if maturation of these cytokines through cleavage by caspase-1 is not important for IIR driven cytokine release to the serum of mice. Moreover, mice lacking TLR2, -3, -4, -7, and -9 expression, namely *Tlr23479*$^{-/-}$ mice responded strongly to IIR as if TLRs frequently implicated in sterile immune activates of numerous kinds were not involved in the model of IIR dependent tissue damage applied by us here.

To evaluate a potential endosomal TLRs involved in IIR driven cytokine production, we challenged *Tlr8*$^{-/-}$, *Tlr11*$^{-/-}$, *Tlr12*$^{-/-}$, *Tlr13*$^{-/-}$, as well as *3d* mice carrying a missense point mutation in Unc93B1 (3D) a chaperone, lacking ER-endosome TLR trafficking and thus endosomal TLR function (Brinkmann et al., 2007; Tabeta et al., 2006). However, not even *3d/Tlr245*$^{-/-}$ mice lacking literally all, namely cell superficial and endosomal TLR activity displayed a phenotype according to the results of their comparative analyses in each case encompassing the results (Fig. 29). We conclude an absence of involvement of both TLRs and ASC-inflammasomes in the IIR driven 6 h- cytokine “storm” observed by us in IFN-γ primed mice. However, our results imply an essential role of yet undefined IL-1 type cytokine and its receptor in IIR driven immune response.

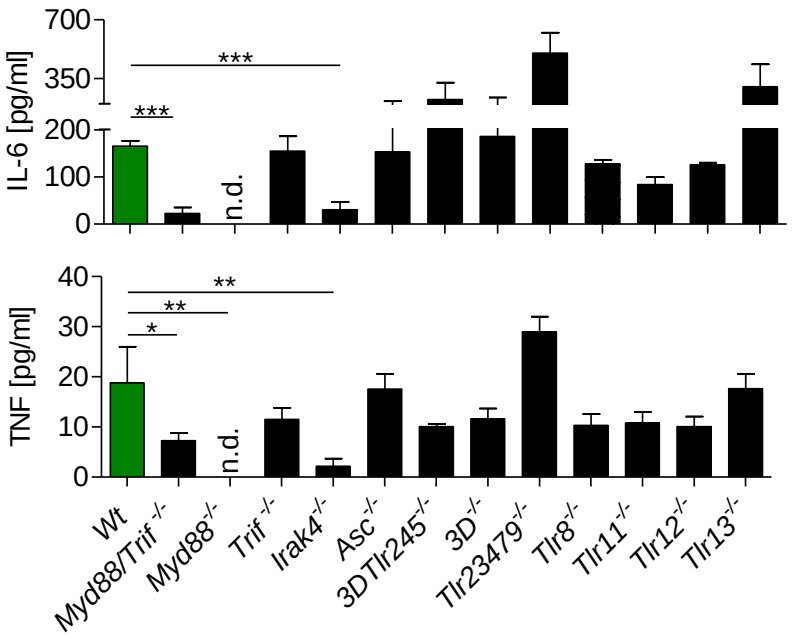


Figure 29: Immune activity 6 h post IIR is MyD88 and IRAK4 dependent. Wild type and the indicated knock out mice were injected intravenously with murine IFN-γ (50 μg/kg body weight). 45 min later, mice were exposed to 30 Gy of total body IIR. Blood was withdrawn 6 h post irradiation. Cytokines (IL-6 and TNF) concentrations were determined from plasma by luminex assay. Each figure represents the pooled data from two to three independent experiments. (wt, wildtype n=18; *Myd88/Trif*$^{-/-}$ n=6; *Trif*$^{-/-}$, *Irak4*$^{-/-}$, *Asc*$^{-/-}$-, *3DTlr245*$^{-/-}$, *3D*, *Tlr23479*$^{-/-}$, *Tlr13-/-*, n=6; *Tlr8*$^{-/-}$, *Tlr11*$^{-/-}$, *Tlr12*$^{-/-}$, n=2). n.d., not detected; TLR, toll like receptor; MYD88, myeloid differentiation factor protein 88; TRIF, toll interleukin-1 protein containing interferon beta; IRAK, IL-1 receptor associated kinase; ASC, apoptosis-associated speck like protein; 3D, defective Unc39B1, a 12x transmembrane ER, endosome TLR transporter affecting TLR3, 7, 8, 9, 11, 12, and 13 function. The graphs show mean ± SD represent pooled data from two to three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Unpaired t-test

Sterile tissue damage induces cell death and particulate stimuli such as uric acid crystals drive interleukin family member cytokine production (Rock et al., 2010). IL-1 like cytokine family members are implicated as “alarmins” and thus DAMP-like host molecules. According to the current literature, specifically IL-33 is a prominent alarmin (Liew et al., 2010; Martin and Martin, 2016; Rider et al., 2017). Therefore, mice lacking expression of the receptor for IL-33, namely ST2 were employed and analyzed by us. *St2*$^{-/-}$ mice mounted a similar immune response compared to that of the wt counterparts (Fig. 30), indicating absence of a role of IL-33 in IIR driven early cytokine production.

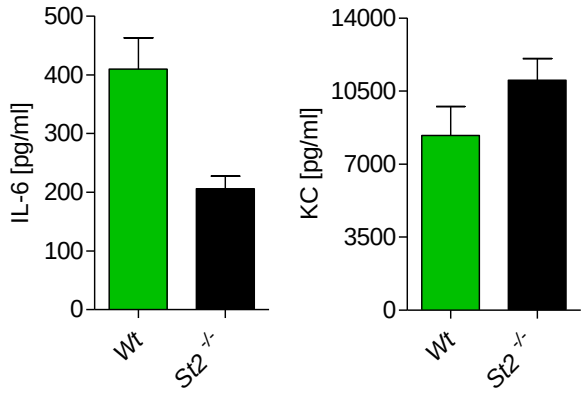


Figure 30: Immune activity 6 h post IIR is IL-33 receptor (ST2) independent. Balb/c mice of the genotypes indicated were injected intravenously with murine IFN-γ (50 μg/kg body weight). 45 min later, mice were exposed to 30 Gy of total body IIR. Blood was withdrawn 6 h post IIR. Cytokine (IL-6) and chemokine (KC) concentrations were determined from plasma by luminex assay. ST2, IL-33 receptor. The graphs show mean ± SD of 3 mice per genotype. Experiment was performed once.

Next, we analyzed *Il1r1*$^{-/-}$ mice lacking responsiveness to IL-1 cytokine family members, namely IL-1α and IL-1β. *Il1r1*$^{-/-}$ mice similar to mice lacking MyD88 and IRAK4 expression failed to mount immune response towards IIR (Fig. 31). IL-1α is a DNA damage sensor triggering sterile inflammation (Cohen et al., 2016). IL-1β plays a critical role in radiation-induced fibrosis (Liu et al., 2006a). However, both *Il1α*$^{-/-}$ and *Il1β*$^{-/-}$ mice were refractory in respect to IIR driven KC release while the former but not the later displayed an IL-6 specific phenotype (Fig. 31). In contrast, *Il1α/β*$^{-/-}$ (double knock out) mice were resistant to IIR challenge. Moreover, *Il1r1*$^{-/-}$ mice displayed a robust phenotype, which indicates both, IL-1α and IL-1β in a crosswise largely compensatory manner as important mediators of IIR driven cytokine and

chemokine production. Caspase-11 has been implicated as so called non-canonical inflammasome driver of IL-1 type cytokine maturation and pyroptosis driver (Broz and Dixit, 2016; Hagar et al., 2013; Kayagaki et al., 2013). In order to evaluate caspase-11, we applied also Sv129 mice known to carry a non-active caspase-11 mutant (Kayagaki et al., 2011). They responded normally towards IIR challenge, indicating caspase-11 is not involved in IIR driven immune activation.

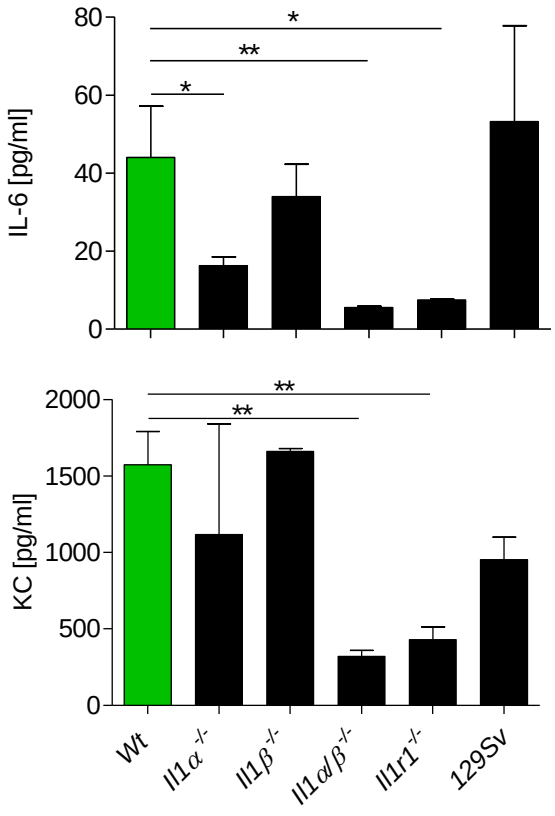


Figure 31: Immune activity 6 h post irradiation is IL-1RI and IL-1α/β dependent, yet caspase-11 independent. Wild type and the indicated knock out mice were injected intravenously with murine IFN-γ (50 μg/kg body weight). 45 min later mice were, exposed to 30 Gy total body IIR. Subsequently, blood was withdrawn 6 h post IIR. Cytokine (IL-6) and chemokine (KC) concentrations were determined from plasma by luminex assay. The graphs show mean ± SD of 3 mice per genotype. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; unpaired t-test. A representative result of two independent experiments performed at different times - at which results were similar.

3.3.2 Long term immune stimulatory response by IIR.

Advances in radiation delivery using megavoltage and intensity- modulated radiation therapy has permitted delivery of higher doses of irradiation to well defined targets. Injury to normal tissues and organs, however, poses a substantial threat in IIR treatment of cancers. Emerging understanding of the radiation-induced normal tissue toxicity suggest that the recovery and repopulation of stromal stem cells are chronically impaired by pro-inflammatory cytokines and chemokines released chronically upon damage after radiation exposure (Chung et al., 2016). Better understanding of the mechanisms underlying generation of pro-inflammatory cytokines and molecular signaling pathways towards chemokine production might reveal novel targets for mitigating radiation injury of tissues and organs or at least the immunological effects of it. In order to analyze long term effects of IIR induced damage, we aimed at establishment of a 2 to 4 day IIR model. Mice were first exposed to 30 Gy of total body irradiation. Two days post IIR, mice were sensitized with murine IFN-γ. Blood was withdrawn at 2, 3 and 4 days post irradiation (Fig. 32). Plasma chemokines (CXCL1/KC, CXCL2/MIP-2) and cytokines (IL-6, TNF) content in the blood were highest at 4 d (96 h) post IIR. IFN-γ priming, in contrary to the short term model (Fig. 25), rather inhibited chemokine plasma release for this reason we abandoned IFN-γ priming for subsequent experiments and withdraw samples at 4 d post IIR (Fig. 33).

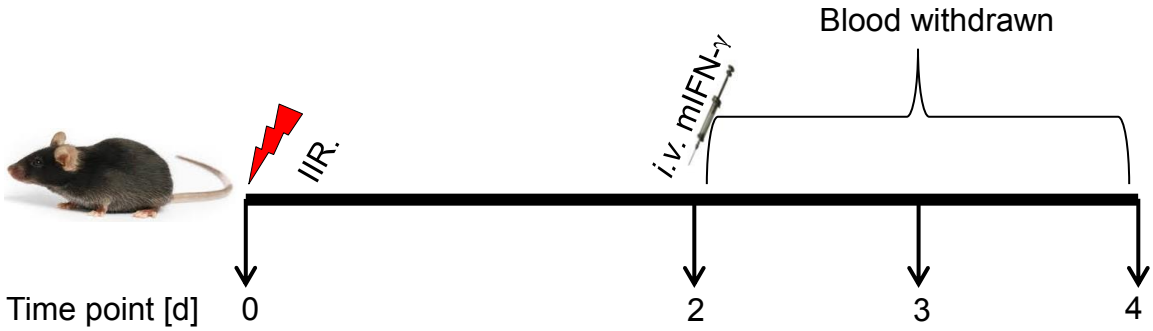


Figure 32: Long term response-to-IIR experimental model. Mice were exposed to 30 Gy of total body IIR. Two days post irradiation mice were sensitized with IFN-γ (50 μg/kg body weight). Blood was harvested at 2 d, 3 d and 4 d post irradiation. Plasma cytokine (IL-6) and chemokine (KC) concentrations were determined from plasma by luminex assay. IIR: Ionizing irradiation; d: Day.

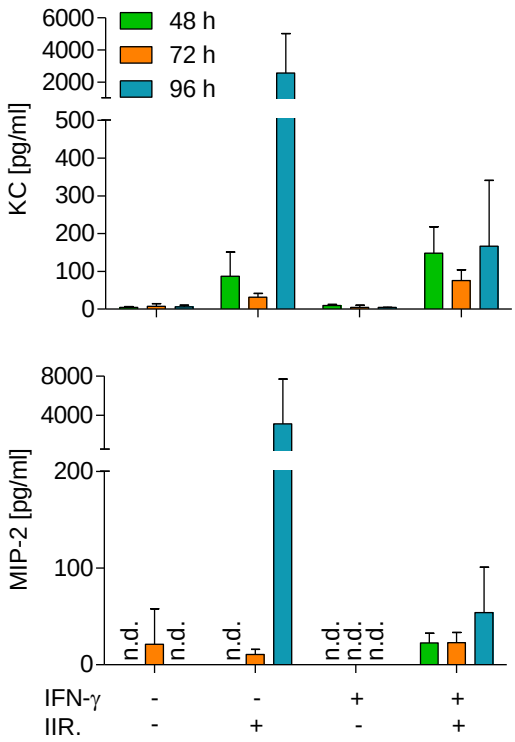


Figure 33: Serum chemokine release peaks at 96 h post IIR and is inhibited by IFN-γ priming. Mice were irradiated with 30 Gy. 2 days later mice were intravenously injected either with IFN-γ (50 μg/kg body weight) or PBS. Blood was withdrawn at the indicated time points. Plasma chemokine (CXCL1/KC, CXCL2/MIP-2) concentrations were analyzed by luminex assay. The graphs show mean ± SD of at least 3 mice per group. Experiment was performed once. n.d., not detected.

Gastrointestinal injury post irradiation is one of the major side effects disturbing the integrity of the epithelial mucosal barrier and thereby inducing bacterial translocation and damage of the intestine. At the 6 h time point post IIR, contribution of the gut microbiota was not detectable (Fig. 26). To evaluate the effect of gut microbiota on “long term” IIR driven immune activation, axenic (germ free) mice were exposed to 30 Gy of total body irradiation. Our results implicate the microbiota as a major contributor to irradiation-induced inflammation. Specifically, the systemic release of chemokines KC and MIP-2 post irradiation was significantly lower in axenic mice as compared to controls, yet not abrogated (Fig. 34) indicating a rather minor yet substantial involvement of DAMP and/or alarmin triggering inflammation.

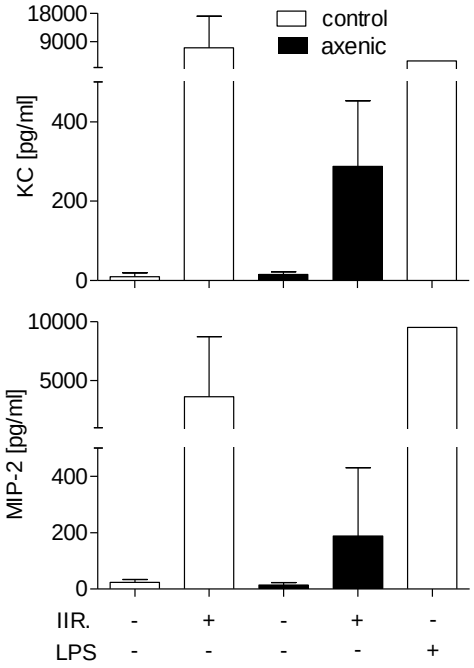


Figure 34: Plasma chemokine content of axenic mice 96 h post ionizing irradiation is diminished, yet substantial. SPF (control) and germ free (axenic) Balb/c mice were exposed to 30 Gy total body irradiation. Blood was withdrawn 96 h post IIR. Chemokine (CXCL1/KC, CXCL2/MIP-2) concentrations were determined by luminex assay. The graphs show mean ± SD of at least 3 mice per group. A representative result of two independent experiments performed at different times - at which results were similar.

Pretreatment with TLR4 or TLR5 agonists protects gut mucosal tissue against radiation induced tissue damage (Burdelya et al., 2008; Riehl et al., 2000). Similarly, TLR2 activation decreases intestinal damage after irradiation (Ciorba et al., 2012) indicating a potential role of endogenous TLR ligands in induction of IIR driven immune activation towards protection from IIR pathology. Although these findings do not imply an involvement in IIR sensing or consequent immune activation beyond microbiota gut barrier transgression immune activation and since a substantial portion of the late inflammatory IIR response was microbiota independent, we considered MyD88, TRIF, and IRAK4 (Kawagoe et al., 2007; Kim et al., 2007; Koziczak-Holbro et al., 2007) as good candidates for signaling molecules mediating IIR driven late immune activation that we had observed at the 96 h time point. We analyzed *Myd88/Trif*$^{-/-}$ and *Irak4*$^{-/-}$ mice first. Both genotypes displayed substantial phenotypes again, as if the TLR/IL-1 receptor pathway mediates both early and late inflammatory immune responses upon IIR (Fig. 35). While the *Myd88*$^{-/-}$ phenotype

was as strong as those mentioned before. Therefore, also in the long term post IIR immune activity observed was MyD88 and IRAK4 dependent. Systemic analysis of MyD88/IRAK4 employing TLRs for their involvement was our consequent incentive. Numerous TLRs such as TLR4 have been implicated as drivers of sterile inflammation in IIR or chemotherapy induced tissue damage (Apetoh et al., 2007). Therefore, we employed different mouse strains- the ones lacking more than one activating gene had been bred by us- that are devoid of TLR activities. Specifically, *Tlr23479*$^{-/-}$ mice were applied first because these five TLRs were considered for their implication in numerous autoimmune activities. Since they lacked a phenotype, we next employed TLR8, -11, -12, and -13 in IIR driven immune activity. As we did not observe remarkable variation as compared to wt controls and since a single TLR activity might be compensated by other TLRs if a set of TLRs rather than a singular TLR would mediate the phenomenon observed by us, we next applied 3d mice principally lacking all endosomal TLR activity (Brinkmann et al., 2007; Tabeta et al., 2006). The negative result of their analysis prompted us to analyze mice comprehensively devoid of all, cell superficial and endosomal TLR activity, namely *3d/Tlr245*$^{-/-}$ mice (Fig. 35). Their unremarkable IIR responsiveness excludes TLR involvement in IIR sensing upstream of MyD88-IRAK4 activity. Although ASC-inflammasomes are implicated in cell death and DAMP driven immune activation (Stoecklein et al., 2015), *Asc*$^{-/-}$ mice exposed to 30 Gy of total body irradiation elicited a normal immune response (Fig. 35), excluding also ASC- inflammasome involvement in IIR driven serum pro-inflammatory mediator release.

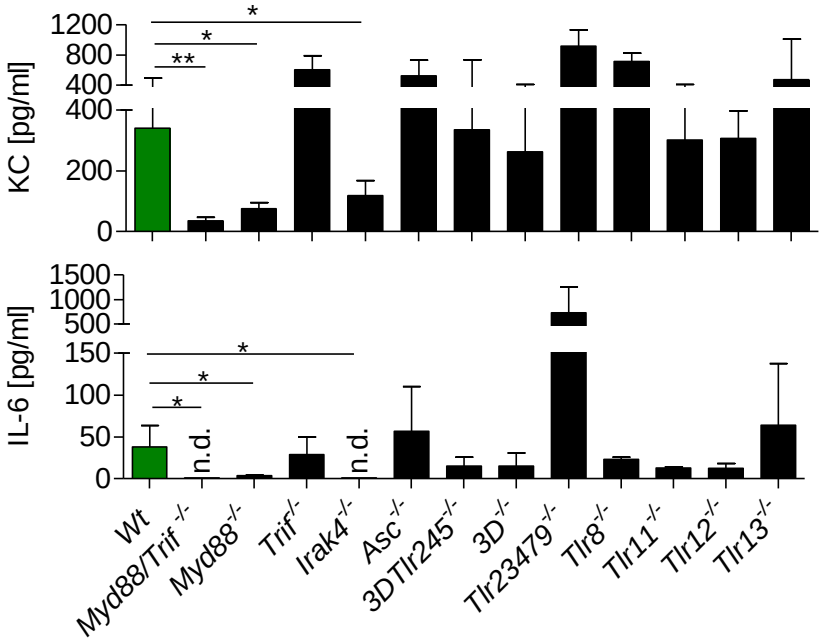


Figure 35: Plasma cytokine and chemokine content 96 h post IIR is MyD88 and IRAK4 dependent. Mice were exposed to 30 Gy total body IIR. Blood was withdrawn 96 h post IIR. Plasma cytokine and chemokine contents were determined by luminex assay. wt, wildtype n=18; *Myd88/Trif*$^{-/-}$ n=6; *Trif*$^{-/-}$, *Irak4*$^{-/-}$, *Asc*$^{-/-}$-, *3DTlr245*$^{-/-}$, *3D*, *Tlr23479*$^{-/-}$, *Tlr13*$^{-/-}$, n=6; *Tlr8*$^{-/-}$, *Tlr11*$^{-/-}$, *Tlr12*$^{-/-}$, n=2; TLR, toll like receptor; MYD88, myeloid differentiation factor protein 88; TRIF, toll interleukin-1 protein containing interferon beta; IRAK, IL-1 receptor associated kinase; ASC, apoptosis-associated speck like protein; 3D, defective Unc39B1, a 12x transmembrane ER, endosome TLR transporter affecting TLR3, 7, 8, 9, 11, 12, and 13 function. The graphs show mean ± SD. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; Unpaired t-test. Each graph represents data pooled from two to three independent experiments.

Inhibition of IL-1R1/MyD88 signaling promotes stem cell-driven tissue regeneration (Martino et al., 2016). Mice lacking IL-13 expression were resistant to radiation induced lung injury (Chung et al., 2016). In order to examine members of the IL-1 type cytokine family for a potential impact on the immune response to long term immune activity upon IIR (Liew et al., 2010; Martin and Martin, 2016; Rider et al., 2017), mice lacking receptor for IL-33, namely ST2 expression, were analyzed by us. *St2*$^{-/-}$ mice mounted a similar or even stronger immune response compared to that of the wt controls (Fig. 36).

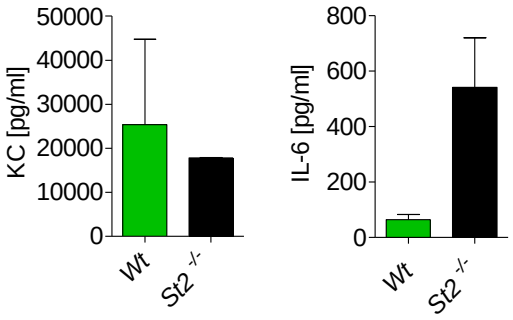


Figure 36: Plasma cytokine and chemokine 96 h post irradiation in IL-33 receptor k.o. mice. Mice were exposed to 30 Gy total body irradiation. Blood was withdrawn 96 h post irradiation. Plasma cytokine and chemokine content were determined by luminex assay. The graphs show mean ± SD of 3 mice per genotype. Experiment was performed once.

Implication of IL-1α as DNA damage mediator (Cohen et al., 2016) and IL-1β mediation of radiation-induced fibrosis (Liu et al., 2006a) motivated us towards a consequent analysis of both, *Il1α*$^{-/}$ and *Il1β*$^{/-}$ mice. While in the former mice immune activity was unremarkable, the latter displayed a fulminant phenotype (Fig. 37). This result is very surprising given that neither *Il1α/β*$^{/-}$, nor *Il1r1*$^{-/-}$ mice resembled the *Il1β*$^{/-}$ phenotype (Fig. 37). We conclude that IL-1β activity cannot be compensated by IL-1α activity, yet IL-1α might have suppressed the expression of another molecule which compensates for IL-1β absence. Only thereby could be explained as to how a single knock out (k.o.) phenotype is masked in a double k.o. genotype. Possibly, IL-1α to some extent act as anti-inflammatory function absence of which allows for a pro inflammatory activity that is absent from *Il1β*$^{/-}$ mice and acts in a IL-1RI independent manner (Fig. 37)

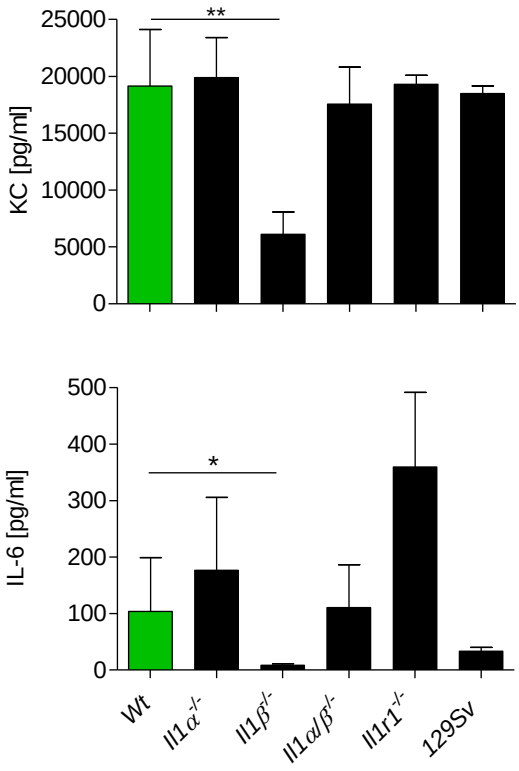


Figure 37: Plasma cytokine and chemokine 96 h post IIR is IL-1β dependent given that both IL-1α and IL-1R1 functions are operative. Mice were exposed to 30 Gy total body irradiation. Blood was withdrawn 96 h post irradiation. Plasma cytokine and chemokine were analyzed by luminex assay. The graphs show mean ± SD of at least 3 mice per group. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; n.s. not significant. Unpaired t-test. A representative result of two independent experiments performed at different times - at which results were similar.

Endogenous DAMP or alarmin release elicited such as by preconditioning through radiotherapy or chemotherapy might establish a pro-inflammatory milieu possibly contributing to successful cancer therapy. Moreover, beneficial graft-versus-leukemia reactions (GvLR), potentially also triggering adversative graft-versus-host disease (GvHD) are exploited for the persistent eradication of hematologic malignances upon hematopoietic stem cell transplantation. Since *Myd88/Trif* $^{-/-}$ mice failed to mount an immune response towards IIR and recent studies implicate IL-1R1/MyD88 as bone regeneration inhibitory (Martino et al., 2016), we assessed a role of MyD88 activity in BMT. First, mice were either treated for 10 days with antibiotics or left untreated prior to exposure to 9 Gy of total body irradiation. Regardless of the antibiotic treatment, *Myd88/Trif*$^{-/-}$ mice died early upon allogeneic bone marrow transplantation (Fig. 38).

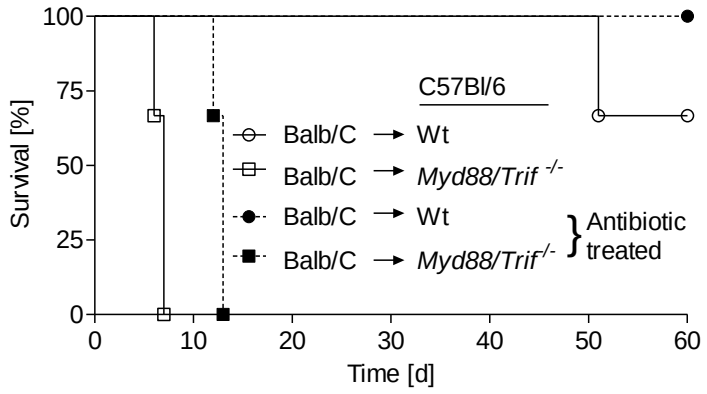


Figure 38: MyD88 and/or TRIF signaling is critical for allogeneic bone marrow transplantation. Mice were treated with drinking water supplemented with 0.33mg/ml of metronidazole and ciprofloxacin or left untreated and then exposed to 9 Gy of total body irradiation. One day later, $10x10^6$ bone marrow cells were injected intravenously. Survival was monitored for 60 days. n= 3 mice per genotype.

To reveal whether the allogeneic bone marrow transplantation is responsible for the lethality, we next transplanted syngeneic bone marrow cells into *Myd88/Trif*$^{-/-}$ mice. Surprisingly, engraftment with syngeneic bone marrow cells failed to rescue *Myd88/Trif*$^{-/-}$ mice (Fig. 39).

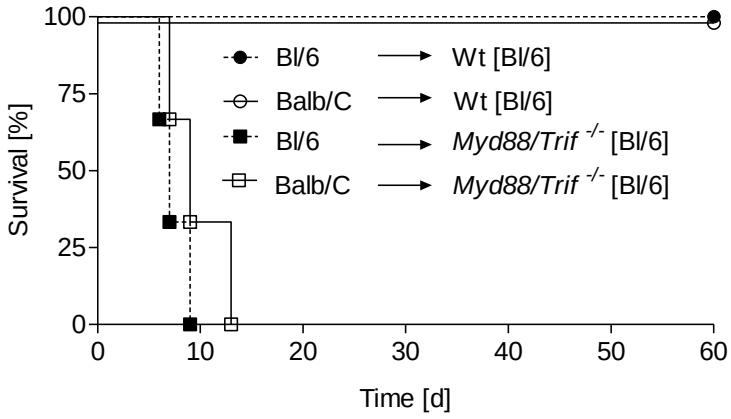


Figure 39: Syngeneic bone marrow transplant failed to rescue *Myd88/Trif*$^{-/-}$ mice. Mice exposed to 9 Gy of total body irradiation received one day later $10x10^6$ bone marrow cells from the indicated strain by intravenous injection. n= 3 mice per genotype.

To exclude an influence of the BMT such as by infection through intravenous injection on early lethality, we only irradiated mice. *Myd88/Trif*$^{-/-}$ mice as well as *Irak4*$^{-/-}$ mice exposed to 9 Gy of total body IIR “still” succumbed significantly earlier as compared to wt controls, indicating that these mice are sensitive to irradiation

(Fig. 40). As if general lack of TLR/IL-1R activity renders mice irradiation insensitive that is not influenced by BMT.

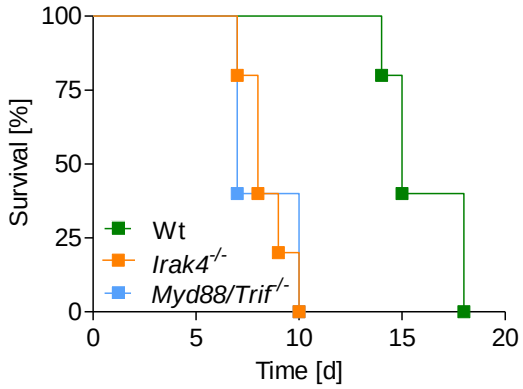


Figure 40: *Myd88/Trif*$^{-/-}$ and *Irak4*$^{-/-}$ mice are radio-hypersensitive. Survival analysis of Wild type (Wt), *Myd88/Trif*$^{-/-}$ and *Irak4*$^{-/-}$ mice exposed to 9 Gy of total body irradiation. Figure represents pooled data from two independent experiments. n = 5 mice per genotype.

Radiation elicits wanted and unwanted immune activity such as towards GvL and GvHD, respectively as well as cell death and damage in a dose dependent manner. Radiation targets primarily dividing cells such as hematopoietic cells. According to our genetic implications, both IL-1α and IL-1β contribute to immune responses towards IIR. We comparatively analyzed susceptibility of both bone marrow and splenocytes to IIR. Cell death correlated with the irradiation dosage, yet dose dependently inhibited cytokine induction (Fig. 41)

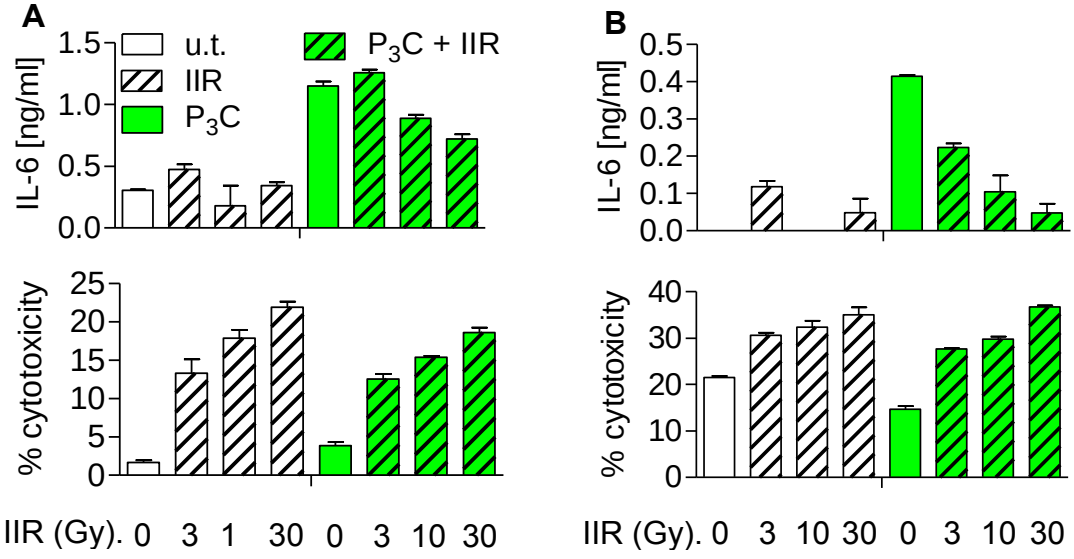


Figure 41: Irradiation induced cytotoxicity and the corresponding cytokine production from fresh bone marrow cells (A) and splenocytes (B). Five million

murine fresh bone marrow cells and splenocytes were challenged with P_3C (1 µg/ml) or left unchallenged followed by exposure to IIR at the indicated doses. 16 hours post irradiation supernatants were harvested and analyzed for the cytokine and LDH content. (u.t., untreated; IIR, exposed to IIR; P_3C, Pam_3CSK_4 priming). The graphs show mean ± SD of duplicates in each case. A representative result of two independent experiments performed at different times - at which results were similar.

Cells signal chromatin damage by release of IL-1α. IL-1α senses DNA damage directly and acts as a signal for genotoxic stress without the loss of cell membrane integrity (Cohen et al., 2016). IL-1β plays a critical role in the radiation induced fibrosis (Liu et al., 2006a). Il-1α released from dying cells initiates sterile inflammation by recruiting neutrophils, whereas IL-1β promotes the recruitment and retention of macrophages (Rider et al., 2011). Conceptually integrating these findings experimentally, we irradiated primed BMMs to analyze pro or cleaved form of IL-1β post irradiation (Fig. 42) because primed or unprimed bone marrow cells and splenocytes failed to release detectable amounts of both IL-1 isoforms upon irradiation (not shown).

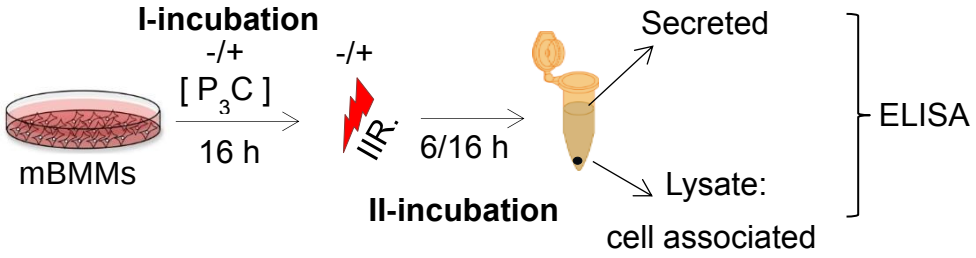


Figure 42: Schematic regimen for in vitro IIR of primed murine BMMs towards IL-β production and toxicity analysis.

Five million murine BMMs were pretreated with Pam_3CSK_4 (P_3C) or left untreated for the indicated time points. Subsequently, cells were exposed to 30 Gy of IIR. 6 or 16 hours post irradiation supernatants and lysates were harvested and analyzed for their cytokine and LDH content. I-Incubation, time of incubation post P_3C priming; II-Incubation, time of incubation post radiation exposure; h, hours.

TLR2 ligand primed murine BMMs exposed to IIR not only succumbed to pyroptosis, but also released substantial amounts of IL-1β highlighted with blue rectangles (Fig. 43). Our results indicate IIR driven release of IL-1β from primed but not from unprimed BMMs, as well as priming independent yet IIR dependent cytotoxicity.

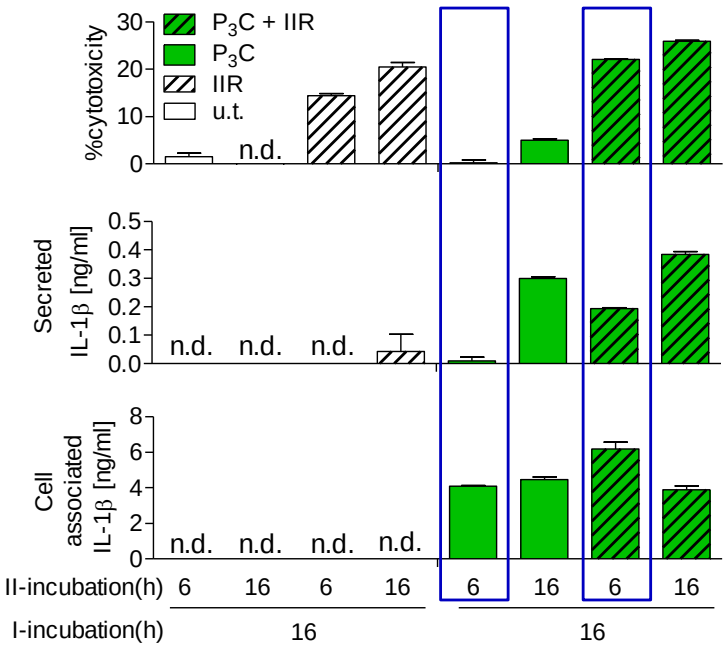


Figure 43: Ionizing irradiation induced toxicity and IL-1β release from primed BMMs. Five million murine BMMs were pretreated with P_3C or left untreated for the indicated time periods. Subsequently, cells were exposed to 30 Gy of irradiation. 6 and 16 hours post IIR, supernatants and lysates were harvested and analyzed for the cytokine and LDH content by ELISA. (n.d., not detected; u.t., un treated; IIR, exposed to ionizing radiation; P_3C, Pam_3CSK_4 primed; I-Incubation (h), time of incubation post P_3C priming; II-Incubation (h), time of incubation post radiation exposure; h, hours). The graphs show mean ± SD of duplicates in each case. Experiment was performed once.

3.4 TLR4 mediates necrotic hepatocyte homogenate driven immune cell activation.

Ischemia/reperfusion injury (I/RI) precipitates a large array of pathological conditions such as myocardial infarction, cerebral stroke, and hepatic cirrhosis (Akhtar et al., 2013; Land et al., 2016). A hallmark of these pathologies is excessive inflammation. TLRs are pathogen recognition receptors which orchestrate the innate immune response and the subsequent adaptive immune response. TLRs are involved in organ rejection and tolerance, I/RI, and infection pathologies post transplantation. TLRs bind exogenous ligands expressed on invading pathogens and endogenous ligands such as low-molecular hyaluronic acid, fibronectin, heat shock protein 70, and heparin sulfate cleaved upon exposure through cell injury (Barrat et al., 2005; Nicholas et al., 2011; O'Neill et al., 2014). Such “DAMPs” activate TLR2 and TLR4 to mediate an inflammatory response in the absence of pathogens.

In order to analyze necrotic cells for their potential to activate immune cells, we adopted an in vitro homogenate preparation method (Kono et al., 2010b). Specifically, hepatocytes isolated from rat liver solid organs were either stored at 4°C overnight or cells cultured normally mimicking the storage phase during organ transportation upon which cells were heat shocked at 45°C for 10 minutes to mimic reperfusion associated “heat shock”. Cells were then cultured in medium containing histidine, tryptophan, and ketoglutarate (HTK) either for five hours or overnight under normal incubation conditions to mimic the engraftment phase of a transplanted organ. Cells were then harvested, frozen and thawed three times and subsequently subjected to ultra-sonication. Murine BMMs were confronted with the resultant homogenates which were referred to as Ischemia/reperfusion-like (I/R-like) incubated hepatocytes (Fig. 44).

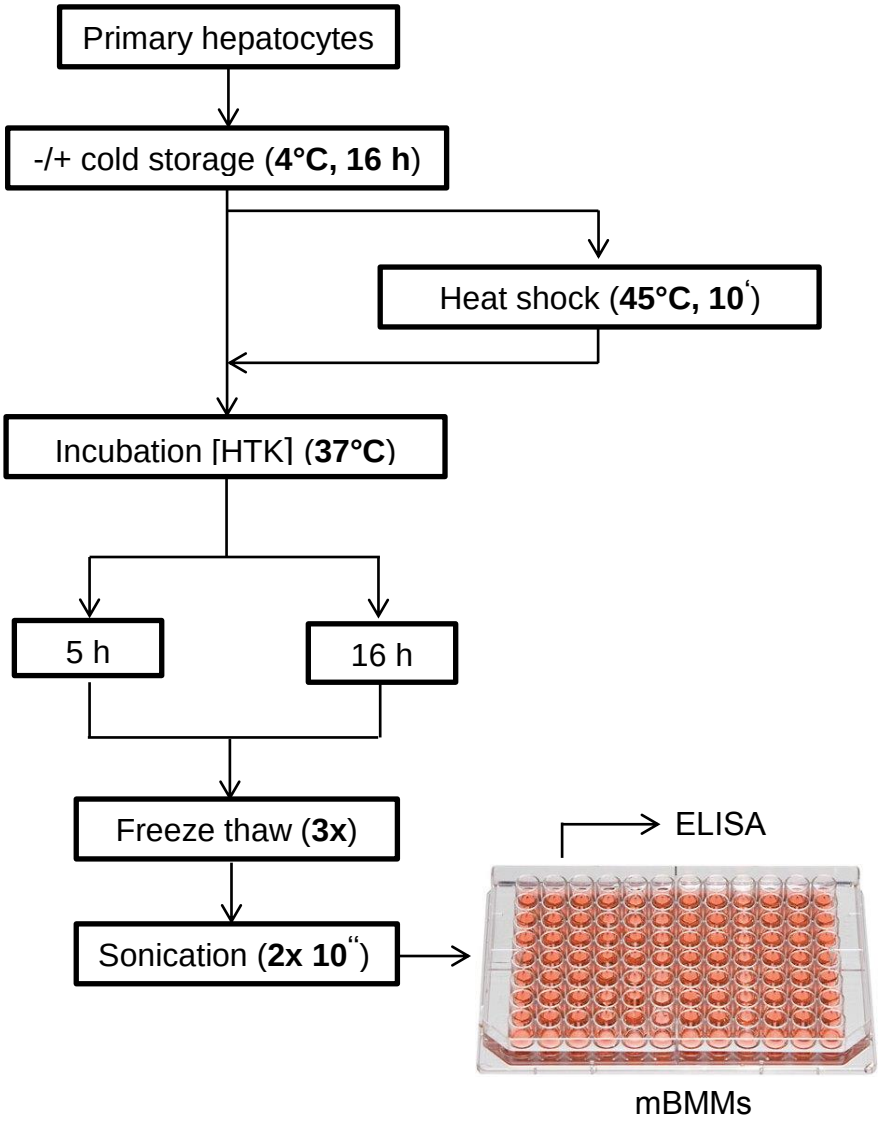


Figure 44: Schematic representation and analysis of I/R-like treatment of primary hepatocytes and their immune stimulatory potential.

-/+, w/o; HTK, histidine, tryptophan, and ketoglutarate containing medium; mBMMs, murine bone marrow derived macrophages; h, hour.

TLRs are widely expressed within the liver. They were proposed to contribute to immune responses towards I/R-like incubated hepatocyte homogenates indeed mounted an immune response, the latter produced IL-6 in an incubation time dependent, yet prior cold storage independent manner (Fig. 45).

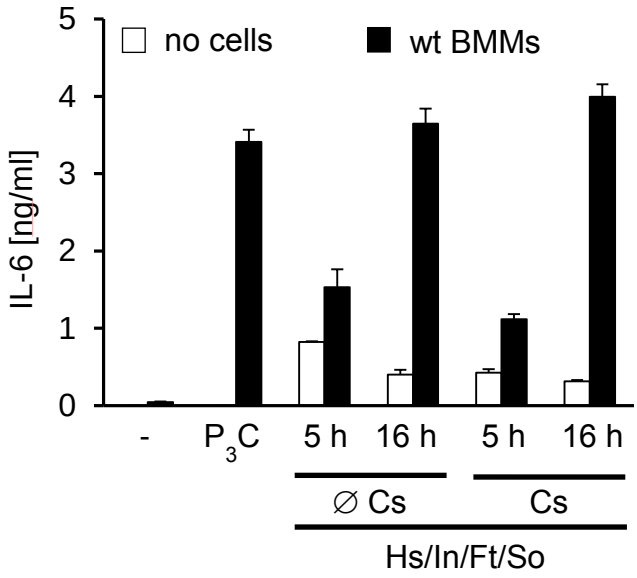


Figure 45: Cold storage does not influence the stimulatory capacity of I/R-like incubated hepatocyte lysates. BMMs were primed with 20ng/ml of murine IFN-γ and challenged with TLR specific stimuli (P_3C 1 μg/ml) or I/R-like incubated hepatocyte homogenates for 16 hours. (BMMs, Bone marrow derived macrophages; P_3C, Pam_3CSK_4; ∅ Cs, no cold storage; Cs, cold storage; Hs, heat shock; In, incubation; Ft, freeze-thaw; So, sonication; h, hour). Supernatants were analyzed for cytokine content. The graphs show mean ± SD of duplicates in each case. A representative result of three independent experiments performed at different times - at which results were similar.

Neutrophils are major effector cells of acute inflammation (Phillipson and Kubes, 2011). They are the first leukocytes recruited to an inflammatory site. Antimicrobial function of phagocytes partially depends on the generation of superoxide anions. Resultant oxidative stress derives from an imbalance between the generation of ROS and effective antioxidant activity, and contributes to ischemia pathology (Akhtar et al., 2013). ROS is a major inducer of tissue damage in the post ischemic phase as well.

ROS release as an additional parameter of I/RI driven immune activation. Conditional Hoxb8 neutrophils (see 2.2.6.2) were confronted with hepatocyte homogenates. IFN-γ priming substantially increased the amounts of ROS produced by neutrophils upon treatment of hepatocyte lysate challenge (Fig. 46). Our results identify I/R-like incubated hepatocytes are equipped with sterile immune stimulatory DAMP.

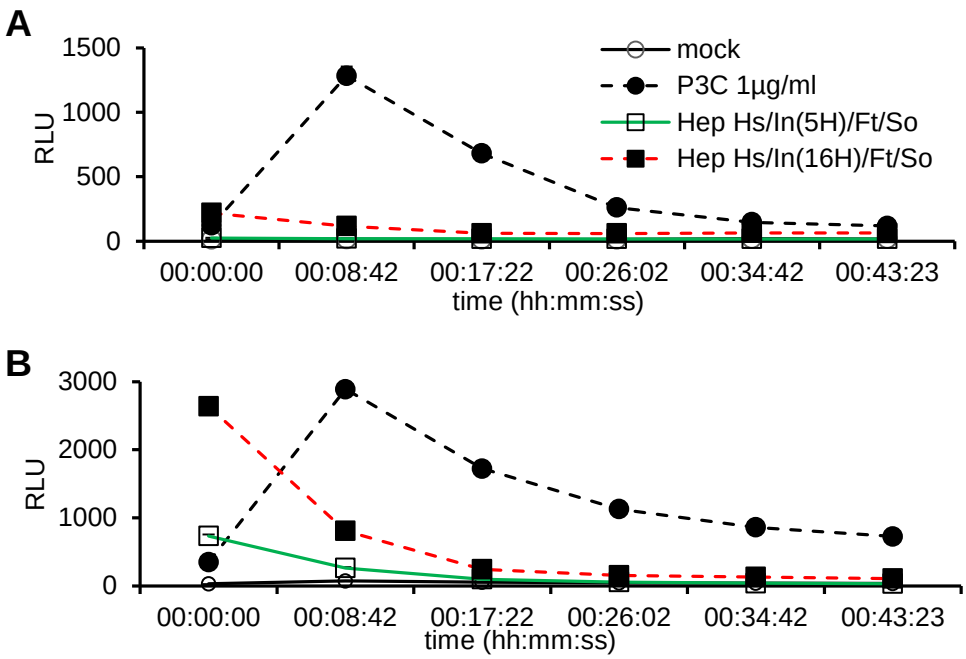


Figure 46: ROS inducing activity of I/R-like incubated hepatocyte homogenates is incubation time dependent. Hoxb8 neutrophils which were untreated (A) or IFN-γ primed (B) were challenged with differentially I/R-like incubated primary rat hepatocyte homogenates. (P_3C, Pam_3CSK_4; hep, hepatocytes; ∅ Cs, no cold storage; Cs, cold storage; Hs, heat shock; In, incubation; Ft, freeze-thaw; So, sonication; h, hour. 5H, culture for 5 h; 16H, culture for 16 h). Resultant oxidative burst was measured by lucigenin based chemiluminescence assay 30 mins post incubation. A representative result of two independent experiments performed at different times at which results were similar is shown.

We next challenged *Myd88/Trif*$^{-/-}$ BMMs with the I/R-like incubated hepatocyte homogenates to narrow down PRRs such as TLRs for their possible involvement in the recognition of DAMPs. *Myd88/Trif*$^{-/-}$ BMMs failed to release IL-6 upon challenge with I/R-like incubated hepatocyte homogenates (Fig. 47). Consequently, we challenged *3d/Tlr24*$^{-/-}$ mBMMs which lack both, functional Unc93B1 mediating ER-endosome shuttling endosomal TLRs, as well as TLR2 and TLR4 expression with the necrotized hepatocyte homogenates. *3d/Tlr24*$^{-/-}$ BMMs failed to release

detectable amounts of IL-6 in to the supernatants upon I/R-like treated hepatocyte homogenate challenge. Our results indicate the TLR/IL-1 receptor pathway system as a mediator of I/RI driven immune activation. Specifically, the global signaling molecules MyD88 and/or IL-1 receptors and endosomal TLRs and/or superficial TLR2 or -4 mediate the cell activation observed by us (Fig. 47).

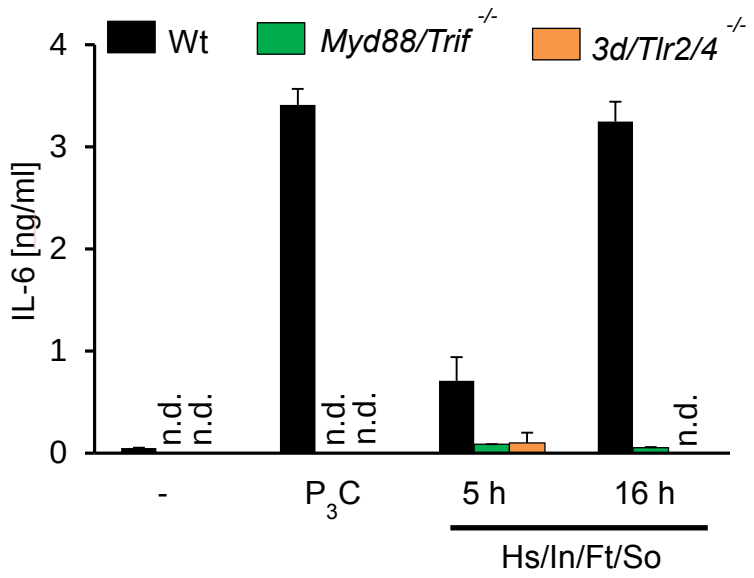


Figure 47: I/R-like incubated hepatocyte homogenate driven BMM activation is MyD88, TRIF, UNC93B1, TLR2, and/or TLR4, as well as incubation time dependent. Indicated BMMs were primed with 20 ng/ml IFN-γ and subsequently challenged with P_3C (1 μg/ml) along with indicated I/R-like incubated hepatocyte homogenates for 16 h. Supernatants were analyzed for IL-6 concentrations (Hs, heat shock; In, incubation; Ft, freeze-thaw; So, sonication; h, hour; P_3C, Pam_3CSK_4; n.d., not detected). The graphs show mean ± SD of duplicates. A representative result of two independent experiments performed at different times - at which results were similar.

The liver consists of parenchymal cells (hepatocytes) and nonparenchymal cells (NPCs), including Kupffer cells, sinusoidal endothelial cells, stellate cells, and hepatic dendritic cells. TLR4 is operative on NPCs but not on hepatocytes (Tsung et al., 2007). However, we prepared homogenates from isolated hepatocytes which had been treated in an I/RI-like manner. In order to investigate whether TLR2 and/or TLR4 mediate the response to the hepatocyte homogenates, we challenged BMMs derived from mice lacking expression of TLR2, TLR4, or both TLRs with I/RI- like hepatocyte homogenates. In respect to IL-6 production, neither *Tlr2/4*$^{-/-}$, nor *Tlr4*$^{-/-}$ BMMs responded to homogenates of primary hepatocytes while *Tlr2*$^{-/-}$ BMMs were

unremarkable. Thus, our results indicate TLR4 as mediator of necrotic primary hepatocyte driven IL-6 production by BMMs (Fig. 48). Taken together our results indicate I/R-like incubated hepatocyte homogenates not only as TLR4 dependent cytokine, but also ROS production inducing agent.

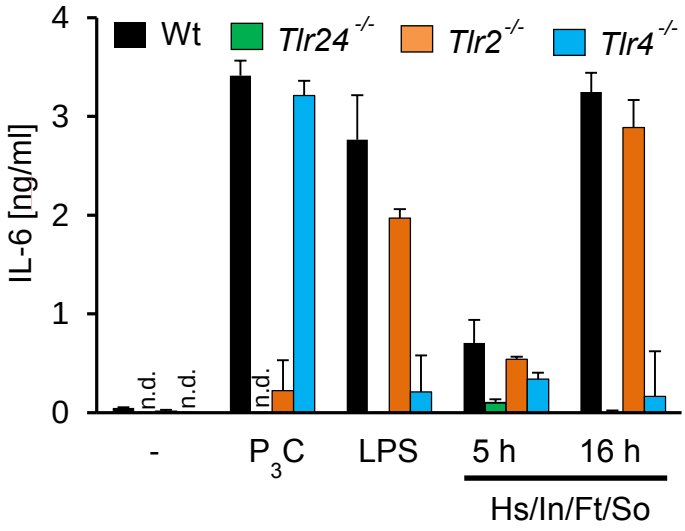


Figure 48: TLR4 mediates I/RI-like incubated hepatocytes driven BMM IL-6 release. Indicated BMMs were primed with 20 ng/ml of murine IFN-γ and subsequently challenged with P_3C (1 μg/ml), LPS (0.1 μg/ml) or differentially incubated (5 h Vs 16 h incubated) necrotic hepatocyte homogenates for 16 hours. Supernatants were analyzed for cytokine content. (P_3C, Pam_3CSK_4; Hs, heat shock; In, incubation; Ft, freeze-thaw; So, sonication; n.d., not detected; h, hour). The graphs show mean ± SD of duplicates. A representative result of two independent experiments performed at different times - at which results were similar.

In respect to chemokine KC production, however, a yet unspecified endosomal TLR is operative in respect to I/R- like treated hepatocyte homogenate sensing (Fig. 49). Specifically, wt, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ BMMs released murine IL-8 homolog “KC” into their supernatants upon confrontation with I/R-like treated hepatocyte homogenates. In contrast, *3d/Tlr2/4*$^{-/-}$ BMMs were refractory. As if different aspects of I/R driven cell activity might be mediated by different sensors from within the molecular family of TLRs.

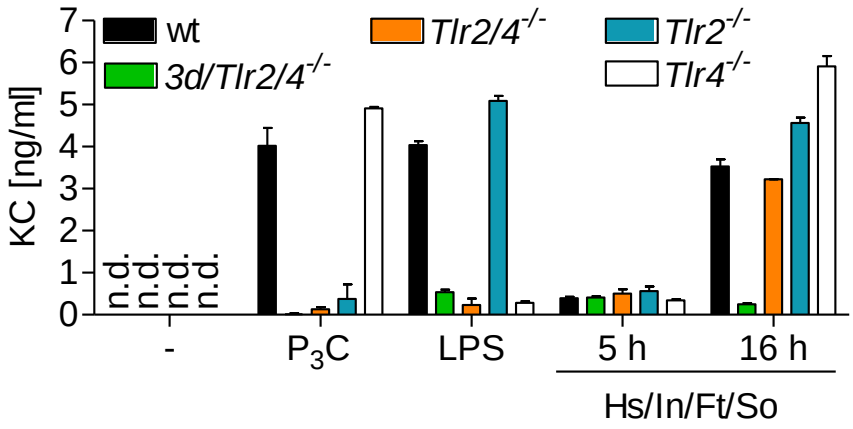


Figure 49: I/R-like incubated hepatocyte driven KC production is endosomal TLR dependent but independent of TLR4. Indicated mBMMs were primed with 20 ng/ml of murine IFN-γ and subsequently challenged with P_3C (1 μg/ml), LPS (0.1 μg/ml) or differentially incubated (5 h Vs 16 h incubated) necrotic hepatocyte homogenates for 16 hours. Supernatants were analyzed for KC content. (P_3C, Pam_3CSK_4; Hs, heat shock; In, incubation; Ft, freeze-thaw; So, sonication; n.d., not detected; h, hour). The graphs show mean ± SD of duplicates. A representative result of two independent experiments performed at different times - at which results were similar.

4. Discussion

4.1 Unraveling the prophylactic and/or therapeutic roles of TLR agonists and antagonists.

The innate immune system is equipped with sensors such as TLRs which detect harmful “non-self” pathogens invading hosts, as well as danger/damage signals released by injured cells upon trauma. To potentially interfere with related pathologies we analyzed TLR agonists and antagonists. Since TLR4 is expressed on the cell surface, interference by soluble compounds might be feasible. TLR4 senses lipopolysaccharide (LPS) from Gram-negative bacteria. In a collaborative effort we analyzed six synthetic tetra-acylated antagonists, namely DA 193, 253, 254, 255, 256, and 257 (Fig. 3) and seven agonists namely FA 99, 125, 146, 147, 148, 151, and 161 for their specificity and TLR4 modulation capacity.

Three synthetic lipid A mimetics carrying specific (*R*)-3-hydroxyacyl chains, namely DA193 (C_{14} at C-2/C-2′, C_{12} at C-3/C-3′), DA253 (C_{14} at C-2/C-2′, C_{10} at C-3/C-3′;) and DA256 (C_{12} at C-2/C-2′ and C-3/C-3′) have substantially antagonized activities in hPBMCs whereas aside from DA256 (acting bi-specifically) the shorter chain DA254 (C_{12} at C-2/C-2′, C_{10} at C-3/C-3′) and DA255 (C_{10} at C-2/C-2′, C_{12} at C-3/C-3′) exhibited high anti-inflammatory activity merely on mBMMs. The species-specific differences in the inhibitory capacity of the DA-compounds might be related to the dissimilarities in the shape and the size of the LPS-binding pocket of h- and mMD-2 (Ohto et al., 2007; Park et al., 2009).

The affinity and strength of the binding of the lipid A mimetics by the hydrophobic binging pocket of MD-2 is strongly dependent on both hydrophobic and ionic interactions, the latter being vital for the binding to hMD-2 which possess strong positively charged Lys and Arg residues at the rim of the binding groove as compared to mMD-2. Along these lines, the appropriate length of the acyl chains (e.g. C_{12}-C_{14} in DA193/DA256) might contribute to sufficient hydrophobic attraction and a correct positioning of the lipid A mimetics within the binding pocket of MD-2, such that the phosphate groups of the ligand establish strong ionic contacts with the positively charged residues at the entrance of the pocket thus ensuring an efficient binding. Since there are significantly less positively charged residues at the rim of the binding groove of mMD-2, the ionic interactions are probably not as important for the

affinity, which, in this instance, might rely mostly on the hydrophobic interactions. The shorter-chain DA-compounds might submerge deep into the binding pocket of mMD-2, which increases the number of hydrophobic interactions and, consequently, enhances the affinity.

We have previously revealed that, in contrast to their natural counterpart lipid IVa, synthetic tetraacylated lipid A mimetics which are based on the conformationally restricted βGlcN(1↔1)αGlcN scaffold (DA-compounds) exhibited species-independent antagonistic properties when applied to cells ectopically overexpressing human and murine TLR4/MD-2 complexes (Artner et al., 2013). Herein we inhibited TLR4/MD-2 complex-mediated pro-inflammatory activity by applying six differently acylated DA-compounds (Fig. 3) which compete with LPS to bind MD-2 on murine BMMs and human PBMCs.

Only three compounds, namely DA254, DA255 and DA256 out of six impaired production of MIP-2 and KC by murine BMMs while IL-6 and NO production was abrogated by all compounds except for DA257 (Fig. 7). However, in hPBMCs TLR4/MD-2 driven TNF and IL-6 release were most significantly inhibited by DA193, -253 and 256 upon LPS challenge (Fig. 8). These results indicate that the latter three DA-compounds - which are being validated through their additional murine cell inhibitory property - as prime anti-inflammatory therapeutic candidates. Whether DA256 or other compounds such as DA193 or -253 prove as best anti-inflammatory drug candidates or not depends upon further analysis. For instance, *in vivo* application and consequent pharmacokinetic analyses will reveal the therapeutic potential of the antagonists.

TLR agonists are used as vaccine adjuvants, in the treatment of Type I allergy, cancer, and infectious diseases. Current clinical research on TLRs mainly depends on the endpoint of stimulated transduction pathways leading to the production of cytokines, activation of innate immune effector cells which further leads to the activation of adaptive immune responses. There are currently molecular mimics of the natural ligands which have improved pharmacodynamics properties such as monophosphoryl lipid A (MPLA). TLR4 recognizes LPS, a glycolipid constituent of the outer membrane of gram negative bacteria. LPS consists of three covalently linked portions, namely a hydrophobic lipid A component, a hydrophilic core oligosaccharide and O-antigen. Lipid A consists of bis phosphorylated-2-*N*-acetyglucosamine

residues, to which multiple fatty acyl side chains are linked (Makkouk and Abdelnoor, 2009). Since LPS is toxic, it is not suited for therapeutic use. Mimics of the Lipid A structure our agonists have the capacity to activate murine BMMs induce cytokine (IL-6) and chemokine (KC) in a TLR4 dependent, yet milder manner (Fig. 9). Whether agonists like 3-*O*-deacylated monophoshoryl lipid A (MPLA) proven to be less toxic yet retaining the ability to activate APCs and induce cytokine cascades necessary for the induction of both Th1- and Th2- type immune responses in the systemic and mucosal compartments of the immune system (Baldridge and Crane, 1999; Kanzler et al., 2007; Martin et al., 2003; Mata-Haro et al., 2007; Rezaei, 2006) has to be analyzed in vivo.

4.2 Sterile inflammation: DAMPs are a PRR specific subset of alarmins.

4.2.1 Experimental models of sterile host injury.

Inflammation is a vital defense mechanism against invasive pathogens. Innate immune cells such as neutrophils and macrophages recruited to the sites of infection phagocytose harmful agents and release cytokines and chemokines that activate lymphocytes and thus adaptive immunity. Besides keeping invading pathogens at bay, the inflammatory response is also crucial for tissue and wound repair. Inflammation as a result of mechanical trauma, ischemia-reperfusion injury (I/RI), and exposure to ionizing irradiation (IIR) leads to release of endogenous components termed damage associated molecular patterns (DAMPs) such as mitochondrial DNA (mtDNA) which can occur in the absence of microorganisms. Strikingly, sterile inflammation similar to microbial agent induced inflammation recruits neutrophils and macrophages (Chen and Nunez, 2010). Although inflammation is important in tissue repair and in the clearance of the invading pathogens, chronic inflammation is harmful to the host organisms.

Alarmins including DAMPs are endogenous factors that normally reside intracellularly and are therefore not exposed to the innate immune system under normal physiological conditions. Alarmins encompass DAMPs and cytokines which are all endogenous molecules released by apoptotic cells. Besides the ER-Golgi secretion pathway, alarmins recruit DCs and thus directly or indirectly promote adaptive immune responses (Bianchi, 2007).

Mouse models of sterile tissue damage driven immune activation include acid-induced lung injury model which identified oxidative stress and TLR4 activity as key mechanism of acute lung injury (Imai et al., 2008). TLR4 binding of HMGB1 was correlated with early relapse after anthracycline based chemotherapy (Apetoh et al., 2007). The Acetaminophen induced liver necrosis model has revealed that CD24 deficient mice displayed increased susceptibility to DAMPs but not to PAMPs clearly discriminating damage induced inflammation from pathogen induced inflammation (Chen et al., 2009).

Using a murine model by administering recombinant bactericidal/permeability-increasing protein (BPI) fragment $rBPI_{21}$ one day post sub lethal dose of IIR has improved the mouse survival which qualified it as an effective radiation mitigating candidate molecule (Guinan et al., 2011). At the start of my study in the year 2011 on involvement of PRRs in trauma pathology we chose IIR induced inflammation as best model of sterile insult. Few studies implicated IIR as the inducer of serum cytokines in mice (Kang et al., 2009; Singh et al., 2009) and radiation has induced temporal changes in multiple cytokines in pulmonary fibrosis sensitive mice (Ao et al., 2009). Considering this literature, plasma cytokines and chemokines were measured for immune activation analysis. In contrast to some literature, it relied largely on IFN-γ priming. Later, inflammatory cytokines release in non-small cell lung cancer patients during and after radiotherapy has been demonstrated and correlated with lung toxicity (Siva et al., 2014). Moreover, ultraviolet- radiation induced TLR4 activation towards neutrophilic inflammation was shown (Bald et al., 2014). Mice deficient of formyl peptide receptor 1 failed to respond to chemotherapy-induced antitumor immunity (Vacchelli et al., 2015). Moreover, MtDNA released during trauma and shock activates neutrophils via the p38 map kinase (Zhang et al., 2010a). Since, mtRNA shares the ligand binding motif core sequence with the 23S r RNA segment Sa19 in bacteria, we analyzed its activation potential in human. Another mode of sterile insult in our focus was I/RI, a major threat to organ rejection (Akhtar et al., 2013). TLRs have been reported to be involved in organ rejection (Shen et al., 2007; Tsung et al., 2007; Tsung et al., 2005). In order to analyze necrotic cells for their potential to activate immune cells, we adopted an in vitro homogenate preparation method (Kono et al., 2010b). To further elucidate the roles of sterile inflammation in respect to an involvement of TLRs, we conclusively employed sterile insults mtRNA

challenge, exposure to IIR, and I/R-like treated hepatocyte homogenate challenge as our experimental models.

4.2.2 16S mt rRNA is a DAMP that activates human TLR8.

We and other groups have identified a 19mer (oligoribonucleiotide) ORN sequence named Sa19 which is located within the bacterial 23S rRNA as the ligand of murine TLR13 (Oldenburg et al., 2012). In search for its human analog, we observed human PBMCs to be responsive to hiSa and even Sa19 itself (Kruger et al., 2015)(Fig. 18) indicating humans as being equipped with sensors that detect bacterial TLR13 ligands while lacking TLR13 expression.

According to the endosymbiotic theory, mitochondria are derived from chemotropic prokaryotic Rickettsiales phagocytosed by prokaryotic Archaea to contribute to eukaryote formation. Mitochondria as endosymbionts lost most of their redundant genetic material over time (Thrash et al., 2011; Williams et al., 2013) which is causative for the reduction in size of large ribosomal (r) 16S and small 12S rRNAs as compared to native bacterial 23S and 16S rRNA, respectively. Considering this prokaryotic origin, we analyzed 16S mt rRNA for the stimulatory properties of its Sa19 like sequence segments.

The sequence and localization of segments in the peptidyl transferase loop V of bacterial 23S rRNA was our primary focus. We compared the mitochondrial 16S rRNA nucleotide sequence of human, mouse, cattle, and rat with bacterial 23S rRNA. We choose two segments the first of which fulfilled both conditions, namely similarity in localization and sequence thus resembling the MLS antibiotic binding site identified as TLR13 ligand in bacterial 23S rRNA. The resultant oligoribunucleotide (ORN) was named “mitochondrial peptidyl transferase loop (*mtPTL)” and is located in loop V of 16S mt rRNA. The second segment lies between loop III and IV of 16S mt rRNA (named BtmtD3_4) and carries a core sequence which is identical with that of the TLR13 ligand (Sa19 “GGAAAGA”). While *mtPTL activated murine BMMs in a TLR13 independent but TLR7 dependent manner (Fig. 14B), BtmtD3_4 despite carrying the Sa19 core sequence “GGAAAGA” did not activate murine BMMs (Fig. 14A) to a detectable degree. However, both ORNs activated human PBMCs (Fig. 16) (Kruger et al., 2015), yet did not trigger release of IFNα indicating lack of its TLR7 specificity (Fig. 18). These results supported our hypothesis of broader ligand specificity of the

human ssRNA receptor as compared to its murine counterpart and acts in a TLR7 independent manner.

It was earlier reported that mtDNA possess immune stimulatory potential. For instance mtDNA elicited secretion of TNF when added to mouse splenocytes and also has the potential to induce arthritis when injected into the mouse joints (Collins et al., 2004). MtDNA released during shock directly bound to TLR9 towards activation of neutrophils (Zhang et al., 2010b). Additional reports implicate mtDNA as immune stimulatory DAMP (Ganguly et al., 2009; Zhang et al., 2010b). Despite the evidence towards mtDNA as a potential immune stimulating DAMP, mtRNA had gone unnoticed. One plausible explanation could be that abundant host ssRNAs are usually hidden within the cell membranes or degraded by RNases available abundantly within the cell, thereby limiting their accessibility to endosomal TLRs. But in the case of a sudden trauma and tissue damage, spatial safety mechanism might be breached (Crozat and Beutler, 2004). Since mitochondria are former bacterial organelles (Bonawitz et al., 2006) and mitochondrial RNA bears Sa19 like core binding motifs, mt RNA isolated from rat liver and human carcinogenic cell line Hep G2 was analyzed for its potential to induce cytokine release from human PBMCs by us.

Previous data suggest presence of GU- or U-rich sequences in synthetic single stranded RNAs as TLR7 and 8 prone (Diebold et al., 2004; Heil et al., 2004). Human TLR7 recognizes bacterial tRNA to induce type I such as IFN-α production. PBMCs, confronted with mitochondrial RNA failed to induce IFNα, which rather excludes an involvement of tRNA in PBMCs activation (Fig. 16). Moreover, PBMCs from a human individual expressing a non-functional Glu53Δ MyD88 mutant (Alsina et al., 2014) failed to respond to Sa19 “like” ORN challenge (Kruger et al., 2015) which indicated involvement of TLRs in bacterial and possibly mt-RNA detection.

Comparative transcriptome profiling of undifferentiated and differentially differentiated THP-1 cells indicated an increased mTLR13 like TLR8 mRNA level in Sa19 responsive THP1 cells upon differentiation for 3 days (3ddi) with phorbol 12-myristate 13-acetate (PMA). Moreover, *Unc93B1*$^{-/-}$ 3ddiTHP-1 cells failed to respond to Sa19 like ORN as well as mt RNA challenge (Fig. 19, Fig. 21). Thus, an endosomal TLR mediates cellular recognition of mt rRNA. Endosomal receptors like TLR3, -7, -8, and -9 have been attributed to specific nucleic acid ligands. Moreover, TLR7 and TLR8

genes are located on the X chromosome (Gantier et al., 2008). Based on our transcriptome analysis and the supporting literature, we considered TLR8 as the most promising candidate among seven candidate membrane receptors the mRNA expression of which was also upregulated in 3ddiTHP-1 cells as compared to normal and 8ddiTHP-1 cells.

Besides *Unc93b1*$^{-/-}$, also *Tlr8*$^{-/-}$-3ddiTHP-1 cells failed to respond to mtRNA challenge (Fig. 21). Our gain of TLR8 function analysis indicated HEK293 cells overexpressing hTLR8 (hTLR8^{+}) as Sa19 like ORN sensor (Fig. 22). Patients carrying *Tlr8* polymorphisms are susceptible to pulmonary tuberculosis (Davila et al., 2008) which might be due their failure to sense mycobacterial and mt RNA. Our finding of mtRNA as TLR8 ligand suggests a therapeutic intervention in sepsis pathogenesis and trauma induced sterile inflammation by blockade of TLR8 activity (Kruger et al., 2015). Activation of DCs through TLR8, however, could elicit Th1 and thus CD8+ T-cell activity which could be exploited towards chronic viral infection and to enhance the efficacy of immunization (Wille-Reece et al., 2005).

4.2.3 IIR driven immune response is detectable 6 hours (short term) and 4 days (long term) later.

Exposure to ionizing radiation (IIR) causes cell damage in a dose dependent manner without affecting outer barriers such as skin was operative in other experimental trauma models. It is a common practice to use radiotherapy along with or alternatively to chemotherapy to treat malignant tumors. Radiotherapy affects actively dividing cells such as cancer cells by damaging nascent DNA. Cells whose DNA is damaged beyond repair stop dividing and succumb to apoptosis. An unwanted side effect of IIR is damage of healthy cells eliciting hyper inflammation. Furthermore, individuals exposed to radiation loose peripheral immune cells to a significant degree which predisposes them to opportunistic infections. On the other hand, endogenous DAMPs released from the disintegrating cells mount an immune response by activating PRRs such as TLRs towards beneficial low-level inflammation.

Radiation is being used for both, diagnostic and therapeutic purposes, such as in cancer therapy. Radiation selectively kills rapidly dividing tumor cells (Baskar et al., 2012). However, radiation is generally considered as a double edged sword, since it counteracts malignant tumor proliferation and on the other hand causes general genome instability. Irradiation is of electromagnetic (X-rays and gamma- rays) and

particulate (electrons, protons, α- particles, neutrons and heavy charged particles) nature. Non-ionizing radiations include ultraviolet (UV), microwave and infrared radiation, as well as IIR which has the highest frequency and shortest wavelength. We chose IIR as a surrogate trigger of sterile tissue damage, which is operative in preconditioning for bone marrow transplantation (BMT). Specifically, resident bone marrow is cleared by irradiation to provide an ecologic niche which allows for the settlement of engrafted bone marrow upon BMT. We observed IFN-γ priming sensitizes *Tlr4*$^{-/-}$ mice which succumb to LPS challenge (Spiller et al., 2008) due to maximal APC activity (Marchi et al., 2014). Accordingly, IFN-γ priming sensitization prior IIR exposure had significantly increased the immune response which peaked 6 h post IIR exposure in our short term model, whereas upon long term IFN-γ priming rather inhibited chemokine and cytokine release possibly in accordance with a report on IFN-γ administered 2 days post exposure to IIR which prevented acute radiation-induced mucositis in the oral cavity, bladder and intestines in an animal model (Yang et al., 2016).

4.2.3.1 Short and long term systemic response to IIR is TLR independent, yet IL-1 dependent.

Wild type mice mounted a strong immune response upon exposure to IIR in both short term and long term radiation regimens (Fig. 25, Fig. 33). Control mice were not exposed to IIR but otherwise treated like irradiated mice (sham mice) and placed for the same time and same irradiation chambers with a volume of 1404 cubic centimeters were used. Plasma cytokines analyzed in sham mice were below detection limit excluding stress as the inducing stimulus. Strikingly, the immune response in axenic mice (germ free) was indistinguishable from that of controls at the short term time point (Fig. 26), and still substantially, yet weaker at the long term time point at which injury to the mucosal barrier might lead to transgression of microbiota. Moreover, PRRs expressed by epithelial cells and intestinal stem cells do play an important role in combating the bacterial translocation from the gut post IIR exposure (Santaolalla and Abreu, 2012). In an IIR-damaged gut, several DAMPs might act along with PAMPs to recruit inflammatory infiltrates and activate the immune defenses. Sterile neutrophilic inflammation was shown to be a key player in the pathogenesis of acute lung injury (Abraham, 2003) and liver injury (Liu et al., 2006b). Blocking macrophage infiltration has mitigated radiation induced skin injury and

radiation lung injury (Thanasegaran et al., 2015). Accordingly, macrophage and neutrophil depletion significantly reduced cytokine production (Fig. 27), indicating macrophages and neutrophils as major sources of the plasma cytokines detected in primed and irradiated mice.

Previous studies have shown that TLR3 blockade protected mice from radiation induced gastrointestinal syndrome (Takemura et al., 2014). TLR7/8 modulation and radiotherapy synergistically induced systemic antitumor immune activity (Scholch et al., 2015). In my experiments, mice injected intraperitoneally with the TLR7/9 inhibitory oligodeoxyribonucleotide “79i” prior to irradiation substantially reduced cytokine release post IIR exposure (Fig. 28) as if TLR7 and/or TLR9 and other endosomal TLRs might be involved in IIR driven immune activation. We consider MyD88 and TRIF as the major adaptors that mediate signal transduction by TLRs and IL1R1 (Kawai and Akira, 2010; Takeda and Akira, 2004) and as tissue injury triggers release of endogenous danger signals that might act as ligands for TLRs or IL-1R1because *Myd88/Trif* $^{-/-}$, *Myd88* $^{-/-}$ and *Irak4*$^{-/-}$ mice failed to mount an immune response in both our short and long term modes of sterile insult. These results indicate involvement of TLRs and/or IL-1 cytokine receptors in IIR driven immune activity. The identity of the *Myd88*$^{-/-}$ with the *Myd88/Trif*$^{-/-}$ mice phenotypes rather excluded TRIF mediated signaling. Several TLRs are known to be involved in radiation induced tissue damage. For instance TLR4 has been implicated as a key receptor in IIR driven sterile inflammation (Apetoh et al., 2007) mice and mucosal tissue treated with TLR4 or TLR5 agonists were protected against IIR induced tissue damage (Burdelya et al., 2008; Riehl et al., 2000). Our analysis of mice lacking TLR8, -11, -12, and -13 expression was unremarkable in that they responded to a similar degree as compared to the wt counterparts (Fig. 29, Fig. 35). However, the difference between both models might be that a long time course post IIR exposure could elicit indirect effects due to gut-circulation barrier transgression by microbiota.

In contrast, mice carrying an Unc93B1 missense point mutation (*3d* mice) that disables ER-endosome TLR trafficking thereby blunting the endosomal TLR translocation (Brinkmann et al., 2007; Tabeta et al., 2006) mounted an immune response which was similar to that of wild mice at short and long term time points post IIR (Fig. 29, Fig. 35). Finally, *3d* mice lacking TLR2, TLR4 and TLR5 expression (*3D/Tlr245*$^{-/-}$) and thus any TLR activity exhibited no phenotype (Fig. 29, Fig. 35) at

both short and long term time points, as if TLRs are not involved in IIR sensing. Therefore, we focused on the Interleukin-1 (IL-1) family of proteins which encompasses 11 family members, of which only IL-1 receptor antagonist (IL-1Ra) carries a signal peptide (Dinarello, 2009) which allows it to be released as an active molecule without a requirement for its processing. Additionally it was shown IL-1 pathway plays an important role in the development of radio-dermatitis in that mice lacking either IL-1 or the IL-1 receptor expression developed less inflammation (Janko et al., 2012).

Out of all IL-1 type cytokines, proIL-1α was the first cytokine shown to translocate in to the nucleus. Accordingly, it contains a nuclear localization sequence (LKKRRL) and binds to chromatin might act as a transcription factor (Cohen et al., 2010; Stevenson et al., 1997; Werman et al., 2004). IL-1α is synthesized in response to tissue injury (Dinarello, 2011; Toldo et al., 2015; Van Tassell et al., 2013) and its expression is elevated in the lungs and heart after exposure to IIR (Kruse et al., 2001; Rubin et al., 1995). In contrast, even though total body radiation induced IL-1β, IL-18, and IL-33 expression in mouse thymus, spleen, and bone marrow cells, IL-18 alone is stable and increased in mouse serum in a dose dependent manner for up to 13 days (Ha et al., 2014). According to numerous reports, IL-33 is a widely operative alarmin (Liew et al., 2010). Mice lacking the receptor for IL-33, namely *St2*$^{-/-}$ mice, however, responded normally to both short and long term irradiation regimens upon IIR (Fig. 30, Fig. 36) indicating that Il-33 is not an alarmin involved in the IIR driven immune response observed by us.

Next, we therefore analyzed *Il1α*$^{-/-}$ and *Il1β*$^{-/-}$ mice for their possible responsiveness to IIR in respect to an immune activity such as serum cytokine release. However, these mice were unremarkable. In contrast, *Il1r1*$^{-/-}$ mice displayed “the” MyD88 and IRAK4 phenotype. Since both IL-1α and IL-1β are known to employ the IL-1RI, we next recruited also double knock out, namely *Il1α/β*$^{-/-}$ mice for our analysis. Both, IFN-γ primed *Il1r1*$^{-/-}$ and *Il1α/β*$^{-/-}$ mice were largely unresponsive 6 h post IIR challenge (Fig. 31). These findings accord with IL-1β implication in development of fibrosis upon chronic radio-dermatitis (Liu et al., 2006a) and murine lung fibrosis post IIR (Rubin et al., 1995).

At the start of my project an older ^{60}Co radiation source (12.5 cGy/min) was used by us, upon discontinuation of its application we used a replacing electrical X-ray (X strahl 320) of high dose rate of 161.55 cGy/min. Accordingly, exposure times were shortened by 10 fold. A conceptual “problem” of our previous studies had been a lack of IL-1α and IL-1β contents in the sera of the irradiated mice that contained substantial amounts of other cytokines (IL-6, TNF) and chemokines (KC, MIP-2) discussed above in the light of the *Il1r1*$^{-/-}$ and *Il1α/β*$^{-/-}$ phenotypes which implicated IL-1α and IL-1β conceptually. Moreover, it is difficult to explain why *Il1β*$^{-/-}$ mice released significantly less IL-6 and KC as shown in Figure 37 96 h upon irradiation while *Il1α/β*$^{-/-}$ mice were unremarkable. As if long term (but not short term) IL-1α is anti-inflammatory in the absence of IL-1β. While application of 30 Gy IIR took 4 h, the exposure time was reduced to 18 min with the electric X-ray source. On the one hand, the change in the dose rate/time applied to mice possibly influences data although the amounts of all plasma cytokines analyzed by us were robust and regardless of the source applied. On the other hand, “6 h” is no longer the minimal time period from the start of the IIR exposure. We will now analyze serum at earlier time points wherein a time period of 30 min will be the limit according to the practical constraints. Possibly, the maximum of cytokine levels attributed to the 6 h time point (Fig. 25) might be put into relation by the values to be obtained at earlier time points.

Moreover, mice lacking MyD88 or IRAK4 expression were sensitive to radiation insult (Fig 40) supporting a recent report according to which *Il1r1*$^{-/-}$ and *Myd88*$^{-/-}$ mice had improved mesenchymal stem cell driven bone regeneration as compared to the wt counterparts indicating that IL-1R1/MyD88 signaling negatively regulates bone regeneration (Martino et al., 2016). Recent studies have shown that upon a sub lethal body irradiation in which limbs and head were shielded to avoid hematopoietic damage, mice deficient in a dsDNA sensor, namely absent in melanoma 2 (AIM2), were resistant to gastrointestinal syndrome. Accordingly, AIM2 senses radiation-induced DNA damage in the nucleus and drives caspase-1 dependent death of intestinal epithelial cells and bone marrow cells (Hu et al., 2016). In contrast our results show the radiation induced immune response to be independent of ASC in both short and long term IIR exposure. Therefore, in our model and in the time course of an immune response we analyzed the involvement of any ASC such as AIM-2 inflammasome activity is not operative.

However, our implication of both IL-1α and IL-1β and their receptor in IIR driven early and late immune activation does not exclude involvement of non-ASC inflammasomes such as the NLRC4 inflammasome, for instance it has been shown that microbiota induce IL-1β release via NLRC4 inflammasome (Franchi and Nunez, 2012). Regardlessly, we favor a scenario according to which unprocessed IL-1α and IL-1β possibly at undetectable levels drive IIR induced immune activity. We will have to detect both cytokines and chemokines in bone marrow cells and splenocytes of irradiated mice or upon culture and ex-vivo in which we failed yet (not shown). Our results hint at IL-1α and IL-1β as promising candidate molecules mediating radiation induced inflammation. Radiation induced cell death inflammatory molecules such as uric acid and ATP besides IL-1α and IL-1β (Glaccum et al., 1997; McDonald et al., 2010). We therefore support the notion that IIR induced damage release DAMPs (Kono et al., 2010b) to mediate IL-1 dependent inflammation wherein the IL-1 might be “the” alarmins themselves.

4.2.3.2 “In vitro” analysis to unravel IIR driven immune response.

Several known intracellular alarmins such as high mobility group box-1 (HMGB-1), members of the IL-1 cytokine family the receptors of which signal through MyD88/IRAK4, cytosolic calcium-binding proteins of the S100 family, heat shock proteins 70/90, peroxiredoxin-1, and nucleosomes have been implicated as inflammatory stimulants. For instance, HMGB-1 binds to cell surface scavenger receptor for advanced glycation end products (RAGE) (Muller et al., 2001; Wang et al., 1999). Several reports illustrate effects of IIR on gene expression of cytokine mRNAs (Brach et al., 1993; Ishihara et al., 1993; Sherman et al., 1991) and NF-κB activation (Raju et al., 1999). IIR induced macrophage responses are involved in rejection of tumor cells (Chiang et al., 1997). Recent studies implicated several inflammasomes in IIR mediated cell and tissue damage. For instance, AIM2 mediates the caspase-1 dependent death of intestinal epithelial cells and bone marrow cells in response to DNA double strand breaks caused by IIR (Hu et al., 2016), while NLRP3 inflammasome activation also mediates IIR induced immune cell death. Accordingly, *Nlrp3*$^{-/-}$ mice displayed improved survival. Moreover, the quantity of cleaved caspase-1, IL-1β, and IIR induced pyroptosis was significantly decreased in mBMMs (Liu et al., 2017). However, the separate implication of both, AIM2 and NLRP3 inflammasomes, in IIR driven effects in two separate studies in which each

molecule was indicated as exclusively essential is difficult to conceive. *Aim2*$^{-/-}$ mice express NLRP3 and *Nlrp3*$^{-/-}$ express AIM2, putting into relation both implications at least.

Our in vitro IIR experiments showed that IIR induced pyroptosis are directly proportional to the irradiation dose regardless of the cell type (Fig. 41). Secondly, IIR triggered significant release of IL-1β from primed mBMMs indicating probably pro-IL-1β release post IIR exposure could be operative to alarm and trigger sterile inflammation (Fig. 43). The question arising is that for the function of it. As emphasized earlier, tissue damage repair is a canonical goal of inflammation. A prevalent damage IIR drives is that of DNA. The cellular DNA damage detection is rapid and elicits DNA repair (Ding et al., 2016). Therefore, a transient and rapid immune response observed by us could promote DNA repair.

Radiotherapy causes DNA single strand breaks, crosslinks and double strand breaks (DSBs) (Prise et al., 2005). Exposure of a typical cell to 1 Gy of IIR induces 1000 single-strand breaks, 40 DSBs and around 3000 damaged bases (Ward, 1994). Cells respond to IIR driven DNA damage by activating cell cycle checkpoint kinases which promote DNA repair thereby preventing transmission of damage DNA to daughter cells. Deficiencies in DNA repair mechanisms predispose some patients to adverse side effects. Non-homologous end joining (NHEJ), homologous recombination (HR) and single-strand alignment (SSA) are the prominent DSB repair mechanisms operative in healthy cells. Vital chromatin remodeling and DNA repair proteins include H2a histone family member X (H2AX), ataxia telangiectasia mutated (ATM), phosphatase and tensin homolog (PTEN), and cell division protein kinases (CDKs) (Iyama and Wilson, 2013).

Some mechanisms underlying hypersensitivity to IIR are still elusive, but it is estimated that 70% of hypersensitivity cases are due to genetic variants (Turesson et al., 1996). Specifically, mutations in the *ATM* gene are responsible for extreme hypersensitivity to IIR (Masuda and Kamiya, 2012) and polymorphisms in genes like *XRCC3* and *RAD51* increased the risk of radio sensitivity (Vral et al., 2011). Different types of repair are operative in different cell types (Iyama and Wilson, 2013). An important aspect that needs to be analyzed in our IIR driven immune response setting is the involvement of NF-κB in the IIR induced inflammation. NF-κB is a known master regulator of both innate and adaptive immunity. NF-κB is a

transcription factor composed of homo- heterodimers of the NF-κB/Rel family. Radiation activates NF-κB via recognition of DNA-DSBs by ATM (Brach et al., 1991; Piret et al., 1999). Upon recruitment to DSBs in the nucleus, the ATM-DSBs complex translocate to the cytoplasm to activate the p50/p65 NF-κB complex. Recently, the NF-κB inducing IL-1α sensed DNA damage which links genotoxic stress signaling to sterile inflammation wherein IL-1α is recruited to DNA damage sites (Cohen et al., 2016). Therefore, analyzing the involvement of DNA-DSBs in our in vivo and in vitro models towards its relation to IL-1α and IL-1β driven cell activation could reveal a mechanism of IIR induced tissue damage immune activation.

4.2.4 Immune activation upon challenge with I/R-like incubated hepatocyte homogenates might be TLR dependent.

Solid organ transplantation is the only therapeutic option upon end-stage organ failure. Yet, several measures such as genetic expression tests, alloMap molecular expression, analysis of ABO compatibility, tissue typing and lifelong immunosuppressive treatment are being involved in preparation and maintenance of patients preventing organ rejection either hyper acutely or chronically. Several studies indicate ischemia/reperfusion (I/R) injury as an inflammatory trigger of adversative immune activity which poses a potentially lethal threat to the solid organ allograft recipient (Akhtar et al., 2013; Land et al., 2016). The cooling of the organ has beneficial effects such as diminishing biological deterioration and inhibiting hypoxic injury by reducing cellular metabolism. Simple static storage (SCS) is the approved storage for liver preservation. SCS relies on the effects of cooling alone in the absence of perfusion and is supplemented by storage in specific preservation solutions the formulation of which aims at modifying inevitable molecular changes (McAnulty, 2010).

4.2.4.1 TLR4 and endosomal TLR are involved in I/RI during the course of liver transplantation - like primary hepatocyte treatment.

According to a common view, I/RI in partial hepatectomy and liver transplantation is associated with the release of various endogenous ligands for PRRs such as TLRs expressed on hepatic tissue or recipient immune cells to activate signaling pathways that induce neutrophils and T-lymphocytes towards inflammation and injury (Kawai and Akira, 2007; Tsung et al., 2005; Zhai et al., 2004). For instance, previous reports

suggest modulation of TLR2 and TLR4 expression in the donor organs upon I/RI (Chang and Toledo-Pereyra, 2012; Evankovich et al., 2010). TLR9 affinity towards pathogen and host-derived DNA is considered to mediate I/RI driven neutrophil activation, liver necrosis, and cytokine release (Bamboat et al., 2010; Chang and Toledo-Pereyra, 2012; Yasuda et al., 2005). Hypothermic stress induces cell death and possibly release of alarmins or in general DAMPs specifically binding to PRRs or cytokine receptors expressed on phagocytes that subsequently secrete pro-inflammatory cytokines towards inflammation eliciting primary graft non-function upon liver transplantation (Evankovich et al., 2010).

To mimic solid organ storage driven immune activation, I cultivated primary rat hepatocytes and applied their homogenates to immune cells the activation of which was analyzed. This part of my study was initiated to evaluate the formulated/modified organ preservation medium containing histidine, tryptophan, and ketoglutarate (HTK) provided by Prof. Rauen (Institute for Physiological Chemistry, University Hospital Essen) which was shown to improve the preservation of liver grafts (Stegemann et al., 2010). Our initial set of experiments was performed with rat hepatocytes cold stored for 16 h in HTK medium. Subsequently cold stored hepatocytes were injected intraperitoneally into wt mice. 16 h later, peritoneal lavage was analyzed for antigen presenting cell (APC), namely macrophage and neutrophil, infiltration. Cold stored hepatocytes recruited significant numbers of APCs into wt mice wherein the number of APCs recruited into *3d/Tlr2/4*$^{-/-}$ and *Tlr2/4*$^{-/-}$ mice abdominal cavities were similar to that of the wt controls. We therefore searched for ways to increase the stimulatory potential of cold stored primary hepatocytes. In this regard we adapted an *in vitro* homogenate preparation method which employed heat shock (45°C for 10 min). First, we tested different treatments such as cells were either cold stored or left untreated and subjected to heat shock alone or heat shocked followed by incubation, and final contents were ultra-sonicated to generate a homogenous lysates (Kono et al., 2010a) We observed that neither overnight hypothermic (4°C) preservation, nor rewarming of the hepatocytes towards 37°C induced a detectable stimulatory capacity (Fig. 45). In contrast, 45°C heat shock for 10 minutes followed by overnight incubation at regular cell culture conditions (HS-cult) thus even in the absence of a cold storage resulted in profound cell stimulation upon hepatocyte homogenate challenge.

We also observed that the hepatocyte homogenates induce ROS production (Fig. 46) which accords with findings according to which vascular cells are considered to be the first line source of ROS within the first few minutes of reperfusion (Land, 2012). Moreover, lack of activation of *Myd88/Trif*$^{-/-}$ BMMs in response to I/R-like incubated hepatocyte homogenates suggested the involvement of a TLR in cell activation (Fig. 47). I/R-like incubated hepatocyte homogenates failed to induce both IL-6 and KC from *3d/Tlr2/4*$^{-/-}$ BMMs (Fig. 47, 49). Since neither *Tlr2/4*$^{-/-}$, nor *Tlr4*$^{-/-}$ BMMs responded to I/R-like incubated hepatocyte homogenates whereas *Tlr2*$^{-/-}$ BMMs were unremarkable, IL-6 induction by I/R-like incubated hepatocyte homogenates was TLR4 dependent (Fig. 48). This result was particularly remarkable given that the chemokine KC inducing property of I/R-like incubated hepatocyte homogenates was not TLR4 dependent. However, KC production was endosomal TLR dependent (Fig. 49). This contrasting result could be due to sensing of different products of stored and I/R-like treated primary hepatocytes, namely a TLR4 and an endosomal TLR ligand. Obviously, TLR4 activation is prone to IL-6, while “the” endosomal TLR activation is KC prone. LPS as TLR4 ligand and several endosomal TLR ligands should be applied to wt BMMs next to analyse release of both IL-6 and KC in a ligand dose dependent manner. Chemokines induce cell migration and activation by binding to specific cell surface receptors on the target cells. Moreover, it has been shown that different cells bear different chemokine receptors (Premack and Schall, 1996). Whether TLR4 and endosomal TLR blockade would impact I/R-like incubated hepatocyte homogenate driven immune activation is likely according to our results although it needs to be elucidated. These in vitro data shall be followed up by in vivo transplantation model analyses to evaluate the therapeutic potential of our findings.

5. Summary

Toll- like receptor (TLR) 4 mediates septic and sterile inflammation. To inhibit TLR4-mediated pro-inflammatory signaling and the resultant pathologies, we analyzed synthetic tetraacylated antagonists. Effective inhibition of the LPS-induced pro-inflammatory activity of human PBMCs was achieved by the long-chain LPS mimetics DA193, DA253, and DA256. In contrast, in mouse BMMs DA-compounds possessing shorter β-hydroxy- fatty acids, namely DA256, DA255, and DA254 exhibited the most pronounced antagonistic effect. These mimetics were TLR4 specific as they did not impair Cl075 (TLR7/8) driven cell activation. Synthetic agonists induced cytokine (IL-6) and chemokine (CXCL1/KC) production in a TLR4-dependent manner. TLR4 antagonists and agonists are promising candidates for development of therapeutically applicable anti-inflammatory and adjuvant compounds.

Furthermore, human TLR8 not only replaces TLR13 but in addition senses mitochondrial rRNA and 16S rRNA derived oligo deoxyribonucleotide (BtmtD3_4) in a UR/R motif dependent manner. Responsiveness of *Tlr8*$^{-/-}$- and *Unc93b1*$^{-/-}$ THP-1 cells towards mitochondrial RNA was abrogated. TLR8 gain of function experiments further indicated 16S mitochondrial rRNA derived ORN (BtmtD3_4) as TLR8-specific. Blocking TLR8 at the early phase of bacterial sepsis besides antibiotic therapy is thus a promising strategy.

Severe trauma through tissue damage triggers an inflammatory response which correlates with severity of injury and is associated to mortality and morbidity as in sepsis. Exposed to ionizing radiation as a surrogate trigger of sterile tissue damage, *Myd88*$^{-/-}$ and *Irak4*$^{-/-}$ mice quickly succumbed to irradiation sickness pathology. Systemic immune response of comprehensively TLR activity deficient *3d/Tlr2/4/5*$^{-/-}$ and germ-free axenic mice exposed to ionizing radiation was unremarkable, whereas *Myd88*$^{-/-}$, *Irak4*$^{-/-}$, and *Il1*$\alpha/\beta^{-/-}$ mice were insensitive. Accordingly, radiation insult of primed murine BMMs not only induced necroptosis, but also a substantial extracellular accumulation of IL-1β. Our results implicate release of unprocessed IL-1β rather than PRR activation as a major source of ionizing irradiation driven sterile inflammation. Whether DNA damage response proteins and caspases are involved needs to be analyzed.

Ischemia/reperfusion injury elicits inflammatory responses in the absence of a microbial component, yet poses a potentially lethal threat to the host and solid organ allograft, also with profound influence on acute as well as long-term graft function. In an in vitro model mimicking the organ storage phase prior to transplantation, necrotized cell homogenates induced inflammatory mediator production in a TLR4 and endosomal TLR dependent manner.

6. Zusammenfassung

Toll-like receptor (TLR) 4 vermittelt septische und sterile Entzündungen. Zur möglichen Inhibition von TLR4 vermittelten pro-inflammatorischen Signalweiterleitung und daraus resultierenden Pathologien untersuchten wir synthetische tetra-acylierte Antagonisten. Eine effektive Inhibition von LPS induzierter pro-inflammatorischer Aktivität von humanen PBMCs wurde durch langkettige Antagonisten DA193, DA253 und DA256 erreicht. Im Gegensatz dazu zeigten in murinen BMMs die DA-Substanzen mit kurzen ß-hydroxy-Fettsäuren, namentlich DA256, DA255 und DA254, den größten antagonistischen Effekt. Diese Mimetika waren TLR4 spezifisch, da sie Cl075 (TLR7/8) getriebene Zellaktivierung nicht beeinflussten. Die synthetischen Agonisten induzierten eine TLR4 abhängige Zytokin- (IL-6) und Chemokin- (CXCL1/KC) Produktion. Unseren Ergebnissen zufolge sind Antagonisten und Agonisten vielversprechende Kandidaten für die Entwicklung von therapeutischen anti-inflammatorischen oder adjuvantiven Medikamenten.

Des Weiteren fungiert humaner TLR8 nicht lediglich als Analog von murinem TL13, sondern erkennt auch mitochondrielle rRNA und aus ihr 16S rRNA abgeleitete Oligodeoxyribonukleotide in einr UR/R- Motiv abhängigen Weise. *Tlr8*$^{-/-}$- und *Unc93b1*$^{-/-}$ THP-1 Zellen reagierten nicht auf mitochondrielle RNA. Dies wurde durch Wiederherstellung der Funktion (*gain of function*) von TLR8 bestätigt. TLR8 Blockierung in der frühen Phase einer bakteriellen Sepsis bei Antibiotika Gabe ist demnach eine vielversprechende Therapieoption.

Traumen durch Gewebeschäden induzieren eine Entzündungsreaktion, die mit der Schwere der Verletzung korreliert und eine der Sepsis vergleichbare Mortalität und Morbidität aufweist. Mit ionisierender Strahlung als Model einer sterilen Gewebe-Schädigung konfrontierte *Myd88*$^{-/-}$ und *Irak4*$^{-/-}$ Mäuse sind hypersensitiv für ein letales Syndrom. Die systemische Immunantwort von ionisierender-Strahlung-ausgesetzten und TLR-Aktivität freien *3d/Tlr2/4/5*$^{-/-}$ - und keimfreien Mäusen war unauffällig. Im Gegensatz dazu waren *Myd88*$^{-/-}$, *Irak4*$^{-/-}$ und *Il1*$\alpha/\beta^{-/-}$ Mäuse unempfindlich. Konsequenterweise induzierte Bestrahlung von vorstimulierten murinen BMMs nicht lediglich Nekroptosis, sondern auch eine messbare extrazelluläre Akkumulation von biologisch aktivem pro-IL-1β. Unsere Ergebnisse implizieren die Freisetzung von unprozessierten IL-1β und nicht PRR Aktivierung als Hauptauslöser einer sterilen

Entzündungsreaktion. Eine mögliche Involvierung von Proteinen der DNA Schadensantwort und Caspasen gilt es nun zuklären.

Ischämie-/Reperfusionsschaden verursachen Entzündungsreaktionen in Abwesenheit mikrobiologischer Komponenten. Gleichwohl stellen Sie eine potentiell tödliche Bedrohung des Organismus z.B. bei allogener Organtransplantation dar und haben einen großen Einfluss auf akute- und spätere Transplantatfunktionen. Ein *in vitro* Model für Organ Transplantation zeigt, dass Homogenate nekrotisierte Hepatozyten die Produktion inflammatoischer Mediatoren induzieren können und diese Immunantwort TLR4 und womöglich von einem endosomalen TLR abhängig ist.

7. Bibliography

Abraham, E. (2003). Neutrophils and acute lung injury. Critical care medicine *31*, S195-199.

Akhtar, M.Z., Henderson, T., Sutherland, A., Vogel, T., and Friend, P.J. (2013). Novel approaches to preventing ischemia-reperfusion injury during liver transplantation. Transplant Proc *45*, 2083-2092.

Ali, S., Huber, M., Kollewe, C., Bischoff, S.C., Falk, W., and Martin, M.U. (2007). IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. Proc Natl Acad Sci U S A *104*, 18660-18665.

Alsina, L., Israelsson, E., Altman, M.C., Dang, K.K., Ghandil, P., Israel, L., von Bernuth, H., Baldwin, N., Qin, H., Jin, Z.*, et al.* (2014). A narrow repertoire of transcriptional modules responsive to pyogenic bacteria is impaired in patients carrying loss-of-function mutations in MYD88 or IRAK4. Nat Immunol *15*, 1134-1142.

Ao, X., Zhao, L., Davis, M.A., Lubman, D.M., Lawrence, T.S., and Kong, F.M. (2009). Radiation produces differential changes in cytokine profiles in radiation lung fibrosis sensitive and resistant mice. J Hematol Oncol *2*, 6.

Apetoh, L., Ghiringhelli, F., Tesniere, A., Criollo, A., Ortiz, C., Lidereau, R., Mariette, C., Chaput, N., Mira, J.P., Delaloge, S.*, et al.* (2007). The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunol Rev *220*, 47-59.

Artner, D., Oblak, A., Ittig, S., Garate, J.A., Horvat, S., Arrieumerlou, C., Hofinger, A., Oostenbrink, C., Jerala, R., Kosma, P.*, et al.* (2013). Conformationally constrained lipid A mimetics for exploration of structural basis of TLR4/MD-2 activation by lipopolysaccharide. ACS Chem Biol *8*, 2423-2432.

Bald, T., Quast, T., Landsberg, J., Rogava, M., Glodde, N., Lopez-Ramos, D., Kohlmeyer, J., Riesenberg, S., van den Boorn-Konijnenberg, D., Homig-Holzel, C.*, et al.* (2014). Ultraviolet-radiation-induced inflammation promotes angiotropism and metastasis in melanoma. Nature *507*, 109-113.

Baldridge, J.R., and Crane, R.T. (1999). Monophosphoryl lipid A (MPL) formulations for the next generation of vaccines. Methods *19*, 103-107.

Bamboat, Z.M., Balachandran, V.P., Ocuin, L.M., Obaid, H., Plitas, G., and DeMatteo, R.P. (2010). Toll-like receptor 9 inhibition confers protection from liver ischemia-reperfusion injury. Hepatology (Baltimore, Md) *51*, 621-632.

Barrat, F.J., Meeker, T., Gregorio, J., Chan, J.H., Uematsu, S., Akira, S., Chang, B., Duramad, O., and Coffman, R.L. (2005). Nucleic acids of mammalian origin can act

as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. J Exp Med *202*, 1131-1139.

Baskar, R., Lee, K.A., Yeo, R., and Yeoh, K.W. (2012). Cancer and radiation therapy: current advances and future directions. International journal of medical sciences *9*, 193-199.

Beutler, B., and Rietschel, E.T. (2003). Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol *3*, 169-176.

Bianchi, M.E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. Journal of leukocyte biology *81*, 1-5.

Biragyn, A., Ruffini, P.A., Leifer, C.A., Klyushnenkova, E., Shakhov, A., Chertov, O., Shirakawa, A.K., Farber, J.M., Segal, D.M., Oppenheim, J.J.*, et al.* (2002). Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. Science *298*, 1025-1029.

Bonawitz, N.D., Clayton, D.A., and Shadel, G.S. (2006). Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. Mol Cell *24*, 813-825.

Botos, I., Segal, D.M., and Davies, D.R. (2011). The structural biology of Toll-like receptors. Structure (London, England : 1993) *19*, 447-459.

Boyton, R.J., and Openshaw, P.J. (2002). Pulmonary defences to acute respiratory infection. Br Med Bull *61*, 1-12.

Brach, M.A., Gruss, H.J., Kaisho, T., Asano, Y., Hirano, T., and Herrmann, F. (1993). Ionizing radiation induces expression of interleukin 6 by human fibroblasts involving activation of nuclear factor-kappa B. J Biol Chem *268*, 8466-8472.

Brach, M.A., Hass, R., Sherman, M.L., Gunji, H., Weichselbaum, R., and Kufe, D. (1991). Ionizing radiation induces expression and binding activity of the nuclear factor kappa B. J Clin Invest *88*, 691-695.

Brikos, C., and O'Neill, L.A. (2008). Signalling of toll-like receptors. Handb Exp Pharmacol, 21-50.

Brinkmann, M.M., Spooner, E., Hoebe, K., Beutler, B., Ploegh, H.L., and Kim, Y.M. (2007). The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. J Cell Biol *177*, 265-275.

Broz, P., and Dixit, V.M. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol *16*, 407-420.

Burdelya, L.G., Krivokrysenko, V.I., Tallant, T.C., Strom, E., Gleiberman, A.S., Gupta, D., Kurnasov, O.V., Fort, F.L., Osterman, A.L., Didonato, J.A.*, et al.* (2008). An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. Science *320*, 226-230.

Chang, W.J., and Toledo-Pereyra, L.H. (2012). Toll-like receptor signaling in liver ischemia and reperfusion. Journal of investigative surgery : the official journal of the Academy of Surgical Research *25*, 271-277.

Chen, C.J., Shi, Y., Hearn, A., Fitzgerald, K., Golenbock, D., Reed, G., Akira, S., and Rock, K.L. (2006). MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. J Clin Invest *116*, 2262-2271.

Chen, G.Y., and Nunez, G. (2010). Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol *10*, 826-837.

Chen, G.Y., Tang, J., Zheng, P., and Liu, Y. (2009). CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science *323*, 1722-1725.

Chiang, C.S., Syljuasen, R.G., Hong, J.H., Wallis, A., Dougherty, G.J., and McBride, W.H. (1997). Effects of IL-3 gene expression on tumor response to irradiation in vitro and in vivo. Cancer research *57*, 3899-3903.

Chung, S.I., Horton, J.A., Ramalingam, T.R., White, A.O., Chung, E.J., Hudak, K.E., Scroggins, B.T., Arron, J.R., Wynn, T.A., and Citrin, D.E. (2016). IL-13 is a therapeutic target in radiation lung injury. Sci Rep *6*, 39714.

Ciorba, M.A., Riehl, T.E., Rao, M.S., Moon, C., Ee, X., Nava, G.M., Walker, M.R., Marinshaw, J.M., Stappenbeck, T.S., and Stenson, W.F. (2012). Lactobacillus probiotic protects intestinal epithelium from radiation injury in a TLR-2/cyclo-oxygenase-2-dependent manner. Gut *61*, 829-838.

Cohen, I., Rider, P., Carmi, Y., Braiman, A., Dotan, S., White, M.R., Voronov, E., Martin, M.U., Dinarello, C.A., and Apte, R.N. (2010). Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. Proc Natl Acad Sci U S A *107*, 2574-2579.

Cohen, I., Rider, P., Vornov, E., Tomas, M., Tudor, C., Wegner, M., Brondani, L., Freudenberg, M., Mittler, G., Ferrando-May, E.*, et al.* (2016). Corrigendum: IL-1alpha is a DNA damage sensor linking genotoxic stress signaling to sterile inflammation and innate immunity. Sci Rep *6*, 19100.

Collins, L.V., Hajizadeh, S., Holme, E., Jonsson, I.M., and Tarkowski, A. (2004). Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses. Journal of leukocyte biology *75*, 995-1000.

Colonna, M. (2007). TLR pathways and IFN-regulatory factors: to each its own. Eur J Immunol *37*, 306-309.

Crozat, K., and Beutler, B. (2004). TLR7: A new sensor of viral infection. Proc Natl Acad Sci U S A *101*, 6835-6836.

Czabotar, P.E., Lessene, G., Strasser, A., and Adams, J.M. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol *15*, 49-63.

Dambuza, I.M., and Brown, G.D. (2015). C-type lectins in immunity: recent developments. Curr Opin Immunol *32*, 21-27.

Davila, S., Hibberd, M.L., Hari Dass, R., Wong, H.E., Sahiratmadja, E., Bonnard, C., Alisjahbana, B., Szeszko, J.S., Balabanova, Y., Drobniewski, F.*, et al.* (2008). Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. PLoS Genet *4*, e1000218.

DeMarco, M.L., and Woods, R.J. (2011). From agonist to antagonist: structure and dynamics of innate immune glycoprotein MD-2 upon recognition of variably acylated bacterial endotoxins. Mol Immunol *49*, 124-133.

Di Virgilio, F., and Vuerich, M. (2015). Purinergic signaling in the immune system. Auton Neurosci *191*, 117-123.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science *303*, 1529-1531.

Dinarello, C.A. (1991). Interleukin-1 and interleukin-1 antagonism. Blood *77*, 1627-1652.

Dinarello, C.A. (2009). Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol *27*, 519-550.

Dinarello, C.A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood *117*, 3720-3732.

Ding, D., Zhang, Y., Wang, J., Zhang, X., Gao, Y., Yin, L., Li, Q., Li, J., and Chen, H. (2016). Induction and inhibition of the pan-nuclear gamma-H2AX response in resting human peripheral blood lymphocytes after X-ray irradiation. Cell death discovery *2*, 16011.

Evankovich, J., Billiar, T., and Tsung, A. (2010). Toll-like receptors in hepatic ischemia/reperfusion and transplantation. Gastroenterology research and practice *2010*.

Fearon, D.T., and Locksley, R.M. (1996). The instructive role of innate immunity in the acquired immune response. Science *272*, 50-53.

Fitzgerald, K.A., Palsson-McDermott, E.M., Bowie, A.G., Jefferies, C.A., Mansell, A.S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M.T.*, et al.* (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature *413*, 78-83.

Forsbach, A., Nemorin, J.G., Montino, C., Muller, C., Samulowitz, U., Vicari, A.P., Jurk, M., Mutwiri, G.K., Krieg, A.M., Lipford, G.B., *et al.* (2008). Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. J Immunol *180*, 3729-3738.

Franchi, L., and Nunez, G. (2012). Immunology. Orchestrating inflammasomes. Science *337*, 1299-1300.

Freaney, J.E., Kim, R., Mandhana, R., and Horvath, C.M. (2013). Extensive cooperation of immune master regulators IRF3 and NFkappaB in RNA Pol II recruitment and pause release in human innate antiviral transcription. Cell Rep *4*, 959-973.

Gal, P., and Ambrus, G. (2001). Structure and function of complement activating enzyme complexes: C1 and MBL-MASPs. Curr Protein Pept Sci *2*, 43-59.

Galanos, C., Freudenberg, M.A., and Reutter, W. (1979). Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc Natl Acad Sci U S A *76*, 5939-5943.

Galluzzi, L., Buque, A., Kepp, O., Zitvogel, L., and Kroemer, G. (2017). Immunogenic cell death in cancer and infectious disease. Nat Rev Immunol *17*, 97-111.

Ganguly, D., Chamilos, G., Lande, R., Gregorio, J., Meller, S., Facchinetti, V., Homey, B., Barrat, F.J., Zal, T., and Gilliet, M. (2009). Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. J Exp Med *206*, 1983-1994.

Gantier, M.P., Tong, S., Behlke, M.A., Xu, D., Phipps, S., Foster, P.S., and Williams, B.R. (2008). TLR7 is involved in sequence-specific sensing of single-stranded RNAs in human macrophages. J Immunol *180*, 2117-2124.

Gao, B., and Tsan, M.F. (2003). Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. J Biol Chem *278*, 174-179.

Gautier, V., Cayrol, C., Farache, D., Roga, S., Monsarrat, B., Burlet-Schiltz, O., Gonzalez de Peredo, A., and Girard, J.P. (2016). Extracellular IL-33 cytokine, but not endogenous nuclear IL-33, regulates protein expression in endothelial cells. Sci Rep *6*, 34255.

Gay, N.J., and Keith, F.J. (1991). Drosophila Toll and IL-1 receptor. Nature *351*, 355-356.

Gehrig, S., Eberle, M.E., Botschen, F., Rimbach, K., Eberle, F., Eigenbrod, T., Kaiser, S., Holmes, W.M., Erdmann, V.A., Sprinzl, M., *et al.* (2012). Identification of modifications in microbial, native tRNA that suppress immunostimulatory activity. J Exp Med *209*, 225-233.

Glaccum, M.B., Stocking, K.L., Charrier, K., Smith, J.L., Willis, C.R., Maliszewski, C., Livingston, D.J., Peschon, J.J., and Morrissey, P.J. (1997). Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. J Immunol *159*, 3364-3371.

Greaves, D.R., and Gordon, S. (2009). The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. J Lipid Res *50 Suppl*, S282-286.

Greber, B.J., Boehringer, D., Leitner, A., Bieri, P., Voigts-Hoffmann, F., Erzberger, J.P., Leibundgut, M., Aebersold, R., and Ban, N. (2014). Architecture of the large subunit of the mammalian mitochondrial ribosome. Nature *505*, 515-519.

Guinan, E.C., Barbon, C.M., Kalish, L.A., Parmar, K., Kutok, J., Mancuso, C.J., Stoler-Barak, L., Suter, E.E., Russell, J.D., Palmer, C.D.*, et al.* (2011). Bactericidal/permeability-increasing protein (rBPI21) and fluoroquinolone mitigate radiation-induced bone marrow aplasia and death. Sci Transl Med *3*, 110ra118.

Ha, C.T., Li, X.H., Fu, D., Moroni, M., Fisher, C., Arnott, R., Srinivasan, V., and Xiao, M. (2014). Circulating interleukin-18 as a biomarker of total-body radiation exposure in mice, minipigs, and nonhuman primates (NHP). PLoS One *9*, e109249.

Hacker, H., Tseng, P.H., and Karin, M. (2011). Expanding TRAF function: TRAF3 as a tri-faced immune regulator. Nat Rev Immunol *11*, 457-468.

Hagar, J.A., Powell, D.A., Aachoui, Y., Ernst, R.K., and Miao, E.A. (2013). Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science *341*, 1250-1253.

Han, D., Williams, E., and Cadenas, E. (2001). Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. Biochem J *353*, 411-416.

Hansson, G.K., and Edfeldt, K. (2005). Toll to be paid at the gateway to the vessel wall. Arterioscler Thromb Vasc Biol *25*, 1085-1087.

Hashimoto, C., Hudson, K.L., and Anderson, K.V. (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell *52*, 269-279.

Hauser, C.J., Sursal, T., Rodriguez, E.K., Appleton, P.T., Zhang, Q., and Itagaki, K. (2010). Mitochondrial damage associated molecular patterns from femoral reamings activate neutrophils through formyl peptide receptors and P44/42 MAP kinase. J Orthop Trauma *24*, 534-538.

Hawlisch, H., and Kohl, J. (2006). Complement and Toll-like receptors: key regulators of adaptive immune responses. Mol Immunol *43*, 13-21.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science *303*, 1526-1529.

Horng, T., Barton, G.M., Flavell, R.A., and Medzhitov, R. (2002). The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature *420*, 329-333.

Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A.*, et al.* (2005). Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med *11*, 263-270.

Hoving, J.C., Wilson, G.J., and Brown, G.D. (2014). Signalling C-type lectin receptors, microbial recognition and immunity. Cell Microbiol *16*, 185-194.

Hu, B., Jin, C., Li, H.B., Tong, J., Ouyang, X., Cetinbas, N.M., Zhu, S., Strowig, T., Lam, F.C., Zhao, C.*, et al.* (2016). The DNA-sensing AIM2 inflammasome controls radiation-induced cell death and tissue injury. Science *354*, 765-768.

Hubbard, W.J., Bland, K.I., and Chaudry, I.H. (2004). The role of the mitochondrion in trauma and shock. Shock *22*, 395-402.

Imai, Y., Kuba, K., Neely, G.G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., Ermolaeva, M., Veldhuizen, R., Leung, Y.H., Wang, H.*, et al.* (2008). Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. Cell *133*, 235-249.

Inohara, Chamaillard, McDonald, C., and Nunez, G. (2005). NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu Rev Biochem *74*, 355-383.

Ishihara, H., Tsuneoka, K., Dimchev, A.B., and Shikita, M. (1993). Induction of the expression of the interleukin-1 beta gene in mouse spleen by ionizing radiation. Radiat Res *133*, 321-326.

Itoh, H., Tatematsu, M., Watanabe, A., Iwano, K., Funami, K., Seya, T., and Matsumoto, M. (2011). UNC93B1 physically associates with human TLR8 and regulates TLR8-mediated signaling. PLoS One *6*, e28500.

Iyama, T., and Wilson, D.M., 3rd (2013). DNA repair mechanisms in dividing and non-dividing cells. DNA repair *12*, 620-636.

Janko, M., Ontiveros, F., Fitzgerald, T.J., Deng, A., DeCicco, M., and Rock, K.L. (2012). IL-1 generated subsequent to radiation-induced tissue injury contributes to the pathogenesis of radiodermatitis. Radiat Res *178*, 166-172.

Jiang, R., Ye, J., Zhu, B., Song, Y., Chen, H., and Cao, S. (2014). Roles of TLR3 and RIG-I in mediating the inflammatory response in mouse microglia following Japanese encephalitis virus infection. J Immunol Res *2014*, 787023.

Jockel, S., Nees, G., Sommer, R., Zhao, Y., Cherkasov, D., Hori, H., Ehm, G., Schnare, M., Nain, M., Kaufmann, A.*, et al.* (2012). The 2'-O-methylation status of a single guanosine controls transfer RNA-mediated Toll-like receptor 7 activation or inhibition. J Exp Med *209*, 235-241.

Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol *9*, 361-368.

Kang, S., Lee, E.S., Choi, B., Lim, H.C., Chun, M., Lee, S., and Sohn, S. (2009). Effects of irradiation on cytokine production in a mouse model of Behcet's disease. Clinical and experimental rheumatology *27*, 54-63.

Kanneganti, T.D., Ozoren, N., Body-Malapel, M., Amer, A., Park, J.H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P.*, et al.* (2006). Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. Nature *440*, 233-236.

Kanzler, H., Barrat, F.J., Hessel, E.M., and Coffman, R.L. (2007). Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med *13*, 552-559.

Kaplanski, G., Farnarier, C., Benoliel, A.M., Foa, C., Kaplanski, S., and Bongrand, P. (1994). A novel role for E- and P-selectins: shape control of endothelial cell monolayers. J Cell Sci *107 (Pt 9)*, 2449-2457.

Kasamatsu, J. (2013). Evolution of innate and adaptive immune systems in jawless vertebrates. Microbiol Immunol *57*, 1-12.

Kashuba, V.I., Protopopov, A.I., Kvasha, S.M., Gizatullin, R.Z., Wahlestedt, C., Kisselev, L.L., Klein, G., and Zabarovsky, E.R. (2002). hUNC93B1: a novel human gene representing a new gene family and encoding an unc-93-like protein. Gene *283*, 209-217.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O.*, et al.* (2005). Cell type-specific involvement of RIG-I in antiviral response. Immunity *23*, 19-28.

Kawagoe, T., Sato, S., Jung, A., Yamamoto, M., Matsui, K., Kato, H., Uematsu, S., Takeuchi, O., and Akira, S. (2007). Essential role of IRAK-4 protein and its kinase activity in Toll-like receptor-mediated immune responses but not in TCR signaling. J Exp Med *204*, 1013-1024.

Kawai, T., and Akira, S. (2007). TLR signaling. Semin Immunol *19*, 24-32.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol *11*, 373-384.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., and Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol *6*, 981-988.

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S.*, et al.* (2011). Non-canonical inflammasome activation targets caspase-11. Nature *479*, 117-121.

Kayagaki, N., Wong, M.T., Stowe, I.B., Ramani, S.R., Gonzalez, L.C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W.P., Muszynski, A.*, et al.* (2013). Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science *341*, 1246-1249.

Kenny, E.F., Talbot, S., Gong, M., Golenbock, D.T., Bryant, C.E., and O'Neill, L.A. (2009). MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. J Immunol *183*, 3642-3651.

Kerrigan, A.M., and Brown, G.D. (2009). C-type lectins and phagocytosis. Immunobiology *214*, 562-575.

Kim, H.M., Park, B.S., Kim, J.I., Kim, S.E., Lee, J., Oh, S.C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O.J.*, et al.* (2007). Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell *130*, 906-917.

Kirschning, C.J., Wesche, H., Merrill Ayres, T., and Rothe, M. (1998). Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J Exp Med *188*, 2091-2097.

Kono, H., Chen, C.J., Ontiveros, F., and Rock, K.L. (2010a). Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest *120*, 1939-1949.

Kono, H., Karmarkar, D., Iwakura, Y., and Rock, K.L. (2010b). Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. J Immunol *184*, 4470-4478.

Koziczak-Holbro, M., Joyce, C., Gluck, A., Kinzel, B., Muller, M., Tschopp, C., Mathison, J.C., Davis, C.N., and Gram, H. (2007). IRAK-4 kinase activity is required for interleukin-1 (IL-1) receptor- and toll-like receptor 7-mediated signaling and gene expression. J Biol Chem *282*, 13552-13560.

Kruger, A., Oldenburg, M., Chebrolu, C., Beisser, D., Kolter, J., Sigmund, A.M., Steinmann, J., Schafer, S., Hochrein, H., Rahmann, S.*, et al.* (2015). Human TLR8 senses UR/URR motifs in bacterial and mitochondrial RNA. EMBO Rep *16*, 1656-1663.

Kruse, J.J., Zurcher, C., Strootman, E.G., Bart, C.I., Schlagwein, N., Leer, J.W., and Wondergem, J. (2001). Structural changes in the auricles of the rat heart after local ionizing irradiation. Radiother Oncol *58*, 303-311.

Kunes, P., Holubcova, Z., Kolackova, M., and Krejsek, J. (2010). Interleukin-33, a novel member of the IL-1/IL-18 cytokine family, in cardiology and cardiac surgery. Thorac Cardiovasc Surg *58*, 443-449.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J.*, et al.* (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol *1*, 398-401.

Lamkanfi, M., and Dixit, V.M. (2012). Inflammasomes and their roles in health and disease. Annu Rev Cell Dev Biol *28*, 137-161.

Lamkanfi, M., and Dixit, V.M. (2014). Mechanisms and functions of inflammasomes. Cell *157*, 1013-1022.

Land, W. Allograft injury mediated by reactive oxygen species: from conserved proteins of Drosophila to acute and chronic rejection of human transplants. Part III: interaction of (oxidative) stress-induced heat shock proteins with toll-like receptor-bearing cells of innate immunity and its consequences for the development of acute and chronic allograft rejection. Transplant Rev (Orlando) *17*, 67-86.

Land, W. (2002). Postischemic reperfusion injury to allografts -- a case for 'innate immunity'? European surgical research Europaische chirurgische Forschung Recherches chirurgicales europeennes *34*, 160-169.

Land, W.G. (2004). Ageing and immunosuppression in kidney transplantation. Exp Clin Transplant *2*, 229-237.

Land, W.G. (2005). The role of postischemic reperfusion injury and other nonantigen-dependent inflammatory pathways in transplantation. Transplantation *79*, 505-514.

Land, W.G. (2012). Emerging role of innate immunity in organ transplantation: part I: evolution of innate immunity and oxidative allograft injury. Transplant Rev (Orlando) *26*, 60-72.

Land, W.G., Agostinis, P., Gasser, S., Garg, A.D., and Linkermann, A. (2016). Transplantation and Damage-Associated Molecular Patterns (DAMPs). American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.

Lee, B.L., Moon, J.E., Shu, J.H., Yuan, L., Newman, Z.R., Schekman, R., and Barton, G.M. (2013). UNC93B1 mediates differential trafficking of endosomal TLRs. Elife *2*, e00291.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973-983.

Li, X., Fang, P., Mai, J., Choi, E.T., Wang, H., and Yang, X.F. (2013). Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. J Hematol Oncol *6*, 19.

Liew, F.Y., Pitman, N.I., and McInnes, I.B. (2010). Disease-associated functions of IL-33: the new kid in the IL-1 family. Nat Rev Immunol *10*, 103-110.

Litman, G.W., Rast, J.P., and Fugmann, S.D. (2010). The origins of vertebrate adaptive immunity. Nat Rev Immunol *10*, 543-553.

Liu, S., Chen, J., Cai, X., Wu, J., Chen, X., Wu, Y.T., Sun, L., and Chen, Z.J. (2013). MAVS recruits multiple ubiquitin E3 ligases to activate antiviral signaling cascades. Elife *2*, e00785.

Liu, W., Ding, I., Chen, K., Olschowka, J., Xu, J., Hu, D., Morrow, G.R., and Okunieff, P. (2006a). Interleukin 1beta (IL1B) signaling is a critical component of radiation-induced skin fibrosis. Radiat Res *165*, 181-191.

Liu, Y.G., Chen, J.K., Zhang, Z.T., Ma, X.J., Chen, Y.C., Du, X.M., Liu, H., Zong, Y., and Lu, G.C. (2017). NLRP3 inflammasome activation mediates radiation-induced pyroptosis in bone marrow-derived macrophages. Cell death & disease *8*, e2579.

Liu, Z.X., Han, D., Gunawan, B., and Kaplowitz, N. (2006b). Neutrophil depletion protects against murine acetaminophen hepatotoxicity. Hepatology (Baltimore, Md) *43*, 1220-1230.

Lotze, M.T., and Tracey, K.J. (2005). High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol *5*, 331-342.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A., and Flavell, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A *101*, 5598-5603.

Makkouk, A., and Abdelnoor, A.M. (2009). The potential use of Toll-like receptor (TLR) agonists and antagonists as prophylactic and/or therapeutic agents. Immunopharmacol Immunotoxicol *31*, 331-338.

Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R., and Golenbock, D.T. (2003). Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci U S A *100*, 1966-1971.

Man, S.M., and Kanneganti, T.D. (2015). Regulation of inflammasome activation. Immunol Rev *265*, 6-21.

Marchi, L.F., Sesti-Costa, R., Ignacchiti, M.D., Chedraoui-Silva, S., and Mantovani, B. (2014). In vitro activation of mouse neutrophils by recombinant human interferon-gamma: increased phagocytosis and release of reactive oxygen species and pro-inflammatory cytokines. Int Immunopharmacol *18*, 228-235.

Martin, M., Michalek, S.M., and Katz, J. (2003). Role of innate immune factors in the adjuvant activity of monophosphoryl lipid A. Infect Immun *71*, 2498-2507.

Martin, N.T., and Martin, M.U. (2016). Interleukin 33 is a guardian of barriers and a local alarmin. Nat Immunol *17*, 122-131.

Martino, M.M., Maruyama, K., Kuhn, G.A., Satoh, T., Takeuchi, O., Muller, R., and Akira, S. (2016). Inhibition of IL-1R1/MyD88 signalling promotes mesenchymal stem cell-driven tissue regeneration. Nat Commun *7*, 11051.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell *10*, 417-426.

Masuda, Y., and Kamiya, K. (2012). Molecular nature of radiation injury and DNA repair disorders associated with radiosensitivity. International journal of hematology *95*, 239-245.

Mata-Haro, V., Cekic, C., Martin, M., Chilton, P.M., Casella, C.R., and Mitchell, T.C. (2007). The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science *316*, 1628-1632.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu Rev Immunol *12*, 991-1045.

McAnulty, J.F. (2010). Hypothermic organ preservation by static storage methods: Current status and a view to the future. Cryobiology *60*, S13-19.

McBride, W.H., Chiang, C.S., Olson, J.L., Wang, C.C., Hong, J.H., Pajonk, F., Dougherty, G.J., Iwamoto, K.S., Pervan, M., and Liao, Y.P. (2004). A sense of danger from radiation. Radiat Res *162*, 1-19.

McDonald, B., Pittman, K., Menezes, G.B., Hirota, S.A., Slaba, I., Waterhouse, C.C., Beck, P.L., Muruve, D.A., and Kubes, P. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science *330*, 362-366.

Medzhitov, R., and Janeway, C.A., Jr. (1997). Innate immunity: the virtues of a nonclonal system of recognition. Cell *91*, 295-298.

Merlin, T., Sing, A., Nielsen, P.J., Galanos, C., and Freudenberg, M.A. (2001). Inherited IL-12 unresponsiveness contributes to the high LPS resistance of the Lps(d) C57BL/10ScCr mouse. J Immunol *166*, 566-573.

Mitcham, J.L., Parnet, P., Bonnert, T.P., Garka, K.E., Gerhart, M.J., Slack, J.L., Gayle, M.A., Dower, S.K., and Sims, J.E. (1996). T1/ST2 signaling establishes it as a member of an expanding interleukin-1 receptor family. J Biol Chem *271*, 5777-5783.

Moulin, D., Donze, O., Talabot-Ayer, D., Mezin, F., Palmer, G., and Gabay, C. (2007). Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. Cytokine *40*, 216-225.

Moussion, C., Ortega, N., and Girard, J.P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? PLoS One *3*, e3331.

Muller, F. (2000). The nature and mechanism of superoxide production by the electron transport chain: Its relevance to aging. J Am Aging Assoc *23*, 227-253.

Muller, S., Scaffidi, P., Degryse, B., Bonaldi, T., Ronfani, L., Agresti, A., Beltrame, M., and Bianchi, M.E. (2001). New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. Embo J *20*, 4337-4340.

Netea, M.G., Van der Graaf, C., Van der Meer, J.W., and Kullberg, B.J. (2004). Recognition of fungal pathogens by Toll-like receptors. Eur J Clin Microbiol Infect Dis *23*, 672-676.

Nicholas, S.A., Coughlan, K., Yasinska, I., Lall, G.S., Gibbs, B.F., Calzolai, L., and Sumbayev, V.V. (2011). Dysfunctional mitochondria contain endogenous high-affinity human Toll-like receptor 4 (TLR4) ligands and induce TLR4-mediated inflammatory reactions. Int J Biochem Cell Biol *43*, 674-681.

O'Neill, L.A., and Bowie, A.G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol *7*, 353-364.

O'Neill, L.A., Golenbock, D., and Bowie, A.G. (2013). The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol *13*, 453-460.

O'Neill, S., Harrison, E.M., Ross, J.A., Wigmore, S.J., and Hughes, J. (2014). Heat-shock proteins and acute ischaemic kidney injury. Nephron Exp Nephrol *126*, 167-174.

Ohto, U., Fukase, K., Miyake, K., and Satow, Y. (2007). Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science *316*, 1632-1634.

Ohto, U., Fukase, K., Miyake, K., and Shimizu, T. (2012). Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. Proc Natl Acad Sci U S A *109*, 7421-7426.

Oldenburg, M., Kruger, A., Ferstl, R., Kaufmann, A., Nees, G., Sigmund, A., Bathke, B., Lauterbach, H., Suter, M., Dreher, S.*, et al.* (2012). TLR13 recognizes bacterial

23S rRNA devoid of erythromycin resistance-forming modification. Science *337*, 1111-1115.

Ozinsky, A., Underhill, D.M., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B., Schroeder, L., and Aderem, A. (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*, 13766-13771.

Park, B.S., and Lee, J.O. (2013). Recognition of lipopolysaccharide pattern by TLR4 complexes. Exp Mol Med *45*, e66.

Park, B.S., Song, D.H., Kim, H.M., Choi, B.S., Lee, H., and Lee, J.O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature *458*, 1191-1195.

Peri, F., and Piazza, M. (2012). Therapeutic targeting of innate immunity with Toll-like receptor 4 (TLR4) antagonists. Biotechnol Adv *30*, 251-260.

Phillipson, M., and Kubes, P. (2011). The neutrophil in vascular inflammation. Nat Med *17*, 1381-1390.

Philpott, D.J., Sorbara, M.T., Robertson, S.J., Croitoru, K., and Girardin, S.E. (2014). NOD proteins: regulators of inflammation in health and disease. Nat Rev Immunol *14*, 9-23.

Piret, B., Schoonbroodt, S., and Piette, J. (1999). The ATM protein is required for sustained activation of NF-kappaB following DNA damage. Oncogene *18*, 2261-2271.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C.*, et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085-2088.

Power, C., Fanning, N., and Redmond, H.P. (2002). Cellular apoptosis and organ injury in sepsis: a review. Shock *18*, 197-211.

Premack, B.A., and Schall, T.J. (1996). Chemokine receptors: gateways to inflammation and infection. Nat Med *2*, 1174-1178.

Prise, K.M., Schettino, G., Folkard, M., and Held, K.D. (2005). New insights on cell death from radiation exposure. The Lancet Oncology *6*, 520-528.

Qiang, X., Yang, W.L., Wu, R., Zhou, M., Jacob, A., Dong, W., Kuncewitch, M., Ji, Y., Yang, H., Wang, H.*, et al.* (2013). Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. Nat Med *19*, 1489-1495.

Qureshi, S.T., Lariviere, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P., and Malo, D. (1999). Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med *189*, 615-625.

Raetz, C.R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. Annu Rev Biochem *71*, 635-700.

Raghavan, R., Sloan, D.B., and Ochman, H. (2012). Antisense transcription is pervasive but rarely conserved in enteric bacteria. MBio *3*.

Raju, U., Gumin, G.J., and Tofilon, P.J. (1999). NF kappa B activity and target gene expression in the rat brain after one and two exposures to ionizing radiation. Radiation oncology investigations *7*, 145-152.

Rezaei, N. (2006). Therapeutic targeting of pattern-recognition receptors. Int Immunopharmacol *6*, 863-869.

Rider, P., Carmi, Y., Guttman, O., Braiman, A., Cohen, I., Voronov, E., White, M.R., Dinarello, C.A., and Apte, R.N. (2011). IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. J Immunol *187*, 4835-4843.

Rider, P., Voronov, E., Dinarello, C.A., Apte, R.N., and Cohen, I. (2017). Alarmins: Feel the Stress. J Immunol *198*, 1395-1402.

Riehl, T., Cohn, S., Tessner, T., Schloemann, S., and Stenson, W.F. (2000). Lipopolysaccharide is radioprotective in the mouse intestine through a prostaglandin-mediated mechanism. Gastroenterology *118*, 1106-1116.

Rittirsch, D., Flierl, M.A., Nadeau, B.A., Day, D.E., Huber-Lang, M., Mackay, C.R., Zetoune, F.S., Gerard, N.P., Cianflone, K., Kohl, J.*, et al.* (2008). Functional roles for C5a receptors in sepsis. Nat Med *14*, 551-557.

Rock, K.L., Latz, E., Ontiveros, F., and Kono, H. (2010). The sterile inflammatory response. Annu Rev Immunol *28*, 321-342.

Rubin, P., Johnston, C.J., Williams, J.P., McDonald, S., and Finkelstein, J.N. (1995). A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. Int J Radiat Oncol Biol Phys *33*, 99-109.

Rus, H., Cudrici, C., and Niculescu, F. (2005). The role of the complement system in innate immunity. Immunol Res *33*, 103-112.

Ryan, A., Lynch, M., Smith, S.M., Amu, S., Nel, H.J., McCoy, C.E., Dowling, J.K., Draper, E., O'Reilly, V., McCarthy, C.*, et al.* (2011). A role for TLR4 in Clostridium difficile infection and the recognition of surface layer proteins. PLoS Pathog *7*, e1002076.

Saha, S., Bhanja, P., Liu, L., Alfieri, A.A., Yu, D., Kandimalla, E.R., Agrawal, S., and Guha, C. (2012). TLR9 agonist protects mice from radiation-induced gastrointestinal syndrome. PLoS One *7*, e29357.

Santaolalla, R., and Abreu, M.T. (2012). Innate immunity in the small intestine. Current opinion in gastroenterology *28*, 124-129.

Schmid-Burgk, J.L., Schmidt, T., Gaidt, M.M., Pelka, K., Latz, E., Ebert, T.S., and Hornung, V. (2014). OutKnocker: a web tool for rapid and simple genotyping of designer nuclease edited cell lines. Genome Res *24*, 1719-1723.

Schmidt, M., Raghavan, B., Muller, V., Vogl, T., Fejer, G., Tchaptchet, S., Keck, S., Kalis, C., Nielsen, P.J., Galanos, C.*, et al.* (2010). Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. Nat Immunol *11*, 814-819.

Scholch, S., Rauber, C., Tietz, A., Rahbari, N.N., Bork, U., Schmidt, T., Kahlert, C., Haberkorn, U., Tomai, M.A., Lipson, K.E.*, et al.* (2015). Radiotherapy combined with TLR7/8 activation induces strong immune responses against gastrointestinal tumors. Oncotarget *6*, 4663-4676.

Schroder, K., and Tschopp, J. (2010). The inflammasomes. Cell *140*, 821-832.

Schumann, R.R. (2011). Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. Biochem Soc Trans *39*, 989-993.

Seong, S.Y., and Matzinger, P. (2004). Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol *4*, 469-478.

Shen, X.D., Ke, B., Zhai, Y., Gao, F., Tsuchihashi, S., Lassman, C.R., Busuttil, R.W., and Kupiec-Weglinski, J.W. (2007). Absence of toll-like receptor 4 (TLR4) signaling in the donor organ reduces ischemia and reperfusion injury in a murine liver transplantation model. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society *13*, 1435-1443.

Sherman, M.L., Datta, R., Hallahan, D.E., Weichselbaum, R.R., and Kufe, D.W. (1991). Regulation of tumor necrosis factor gene expression by ionizing radiation in human myeloid leukemia cells and peripheral blood monocytes. J Clin Invest *87*, 1794-1797.

Shi, Y., Evans, J.E., and Rock, K.L. (2003). Molecular identification of a danger signal that alerts the immune system to dying cells. Nature *425*, 516-521.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med *189*, 1777-1782.

Shirey, K.A., Lai, W., Scott, A.J., Lipsky, M., Mistry, P., Pletneva, L.M., Karp, C.L., McAlees, J., Gioannini, T.L., Weiss, J.*, et al.* (2013). The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature *497*, 498-502.

Singh, V.K., Grace, M.B., Parekh, V.I., Whitnall, M.H., and Landauer, M.R. (2009). Effects of genistein administration on cytokine induction in whole-body gamma irradiated mice. Int Immunopharmacol *9*, 1401-1410.

Sistigu, A., Yamazaki, T., Vacchelli, E., Chaba, K., Enot, D.P., Adam, J., Vitale, I., Goubar, A., Baracco, E.E., Remedios, C.*, et al.* (2014). Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. Nat Med *20*, 1301-1309.

Siva, S., MacManus, M., Kron, T., Best, N., Smith, J., Lobachevsky, P., Ball, D., and Martin, O. (2014). A pattern of early radiation-induced inflammatory cytokine expression is associated with lung toxicity in patients with non-small cell lung cancer. PLoS One *9*, e109560.

Smyth, M.J., Dunn, G.P., and Schreiber, R.D. (2006). Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Adv Immunol *90*, 1-50.

Spiller, S., Dreher, S., Meng, G., Grabiec, A., Thomas, W., Hartung, T., Pfeffer, K., Hochrein, H., Brade, H., Bessler, W.*, et al.* (2007). Cellular recognition of trimyristoylated peptide or enterobacterial lipopolysaccharide via both TLR2 and TLR4. J Biol Chem *282*, 13190-13198.

Spiller, S., Elson, G., Ferstl, R., Dreher, S., Mueller, T., Freudenberg, M., Daubeuf, B., Wagner, H., and Kirschning, C.J. (2008). TLR4-induced IFN-gamma production increases TLR2 sensitivity and drives Gram-negative sepsis in mice. J Exp Med *205*, 1747-1754.

Stegemann, J., Hirner, A., Rauen, U., and Minor, T. (2010). Use of a new modified HTK solution for machine preservation of marginal liver grafts. The Journal of surgical research *160*, 155-162.

Stevenson, F.T., Turck, J., Locksley, R.M., and Lovett, D.H. (1997). The N-terminal propiece of interleukin 1 alpha is a transforming nuclear oncoprotein. Proc Natl Acad Sci U S A *94*, 508-513.

Stoecklein, V.M., Osuka, A., Ishikawa, S., Lederer, M.R., Wanke-Jellinek, L., and Lederer, J.A. (2015). Radiation exposure induces inflammasome pathway activation in immune cells. J Immunol *194*, 1178-1189.

Tabeta, K., Hoebe, K., Janssen, E.M., Du, X., Georgel, P., Crozat, K., Mudd, S., Mann, N., Sovath, S., Goode, J.*, et al.* (2006). The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol *7*, 156-164.

Takeda, K., and Akira, S. (2004). TLR signaling pathways. Semin Immunol *16*, 3-9.

Takemura, N., Kawasaki, T., Kunisawa, J., Sato, S., Lamichhane, A., Kobiyama, K., Aoshi, T., Ito, J., Mizuguchi, K., Karuppuchamy, T.*, et al.* (2014). Blockade of TLR3 protects mice from lethal radiation-induced gastrointestinal syndrome. Nat Commun *5*, 3492.

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

Takeuchi, O., and Akira, S. (2009). Innate immunity to virus infection. Immunol Rev *227*, 75-86.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. Cell *140*, 805-820.

Tan, Y., and Kagan, J.C. (2014). A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. Mol Cell *54*, 212-223.

Tang, D., Kang, R., Zeh, H.J., 3rd, and Lotze, M.T. (2011). High-mobility group box 1, oxidative stress, and disease. Antioxid Redox Signal *14*, 1315-1335.

Tanimura, N., Saitoh, S., Matsumoto, F., Akashi-Takamura, S., and Miyake, K. (2008). Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. Biochem Biophys Res Commun *368*, 94-99.

Tanji, H., Ohto, U., Shibata, T., Taoka, M., Yamauchi, Y., Isobe, T., Miyake, K., and Shimizu, T. (2015). Toll-like receptor 8 senses degradation products of single-stranded RNA. Nat Struct Mol Biol *22*, 109-115.

Termeer, C., Benedix, F., Sleeman, J., Fieber, C., Voith, U., Ahrens, T., Miyake, K., Freudenberg, M., Galanos, C., and Simon, J.C. (2002). Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med *195*, 99-111.

Thanasegaran, S., Ito, S., Nishio, N., Uddin, M.N., Sun, Y., and Isobe, K. (2015). Recruitment of Gr1(+)CD11b (+)F4/80 (+) population in the bone marrow and spleen by irradiation-induced pulmonary damage. Inflammation *38*, 465-475.

Thariat, J., Hannoun-Levi, J.M., Sun Myint, A., Vuong, T., and Gerard, J.P. (2013). Past, present, and future of radiotherapy for the benefit of patients. Nat Rev Clin Oncol *10*, 52-60.

Thieblemont, N., Thieringer, R., and Wright, S.D. (1998). Innate immune recognition of bacterial lipopolysaccharide: dependence on interactions with membrane lipids and endocytic movement. Immunity *8*, 771-777.

Thrash, J.C., Boyd, A., Huggett, M.J., Grote, J., Carini, P., Yoder, R.J., Robbertse, B., Spatafora, J.W., Rappe, M.S., and Giovannoni, S.J. (2011). Phylogenomic evidence for a common ancestor of mitochondria and the SAR11 clade. Sci Rep *1*, 13.

Ting, J.P., Lovering, R.C., Alnemri, E.S., Bertin, J., Boss, J.M., Davis, B.K., Flavell, R.A., Girardin, S.E., Godzik, A., Harton, J.A.*, et al.* (2008). The NLR gene family: a standard nomenclature. Immunity *28*, 285-287.

Toldo, S., Mezzaroma, E., Mauro, A.G., Salloum, F., Van Tassell, B.W., and Abbate, A. (2015). The inflammasome in myocardial injury and cardiac remodeling. Antioxid Redox Signal *22*, 1146-1161.

Tracey, K.J. (2010). Understanding immunity requires more than immunology. Nat Immunol *11*, 561-564.

Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R.S., Madan, R., Thorne, P.S., Wills-Karp, M., Gioannini, T.L., Weiss, J.P.*, et al.* (2009). Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature *457*, 585-588.

Troutman, T.D., Hu, W., Fulenchek, S., Yamazaki, T., Kurosaki, T., Bazan, J.F., and Pasare, C. (2012). Role for B-cell adapter for PI3K (BCAP) as a signaling adapter linking Toll-like receptors (TLRs) to serine/threonine kinases PI3K/Akt. Proc Natl Acad Sci U S A *109*, 273-278.

Tsung, A., Klune, J.R., Zhang, X., Jeyabalan, G., Cao, Z., Peng, X., Stolz, D.B., Geller, D.A., Rosengart, M.R., and Billiar, T.R. (2007). HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. J Exp Med *204*, 2913-2923.

Tsung, A., Sahai, R., Tanaka, H., Nakao, A., Fink, M.P., Lotze, M.T., Yang, H., Li, J., Tracey, K.J., Geller, D.A.*, et al.* (2005). The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med *201*, 1135-1143.

Turesson, I., Nyman, J., Holmberg, E., and Oden, A. (1996). Prognostic factors for acute and late skin reactions in radiotherapy patients. Int J Radiat Oncol Biol Phys *36*, 1065-1075.

Underhill, D.M., and Ozinsky, A. (2002). Phagocytosis of microbes: complexity in action. Annu Rev Immunol *20*, 825-852.

Vacchelli, E., Ma, Y., Baracco, E.E., Sistigu, A., Enot, D.P., Pietrocola, F., Yang, H., Adjemian, S., Chaba, K., Semeraro, M.*, et al.* (2015). Chemotherapy-induced antitumor immunity requires formyl peptide receptor 1. Science *350*, 972-978.

Van Tassell, B.W., Toldo, S., Mezzaroma, E., and Abbate, A. (2013). Targeting interleukin-1 in heart disease. Circulation *128*, 1910-1923.

Vanaja, S.K., Rathinam, V.A., and Fitzgerald, K.A. (2015). Mechanisms of inflammasome activation: recent advances and novel insights. Trends Cell Biol *25*, 308-315.

Vijay-Kumar, M., Aitken, J.D., Sanders, C.J., Frias, A., Sloane, V.M., Xu, J., Neish, A.S., Rojas, M., and Gewirtz, A.T. (2008). Flagellin treatment protects against chemicals, bacteria, viruses, and radiation. J Immunol *180*, 8280-8285.

Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A., Nacken, W., Foell, D., van der Poll, T., Sorg, C.*, et al.* (2007). Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med *13*, 1042-1049.

Vral, A., Willems, P., Claes, K., Poppe, B., Perletti, G., and Thierens, H. (2011). Combined effect of polymorphisms in Rad51 and Xrcc3 on breast cancer risk and chromosomal radiosensitivity. Molecular medicine reports *4*, 901-912.

Walker, N.P., Talanian, R.V., Brady, K.D., Dang, L.C., Bump, N.J., Ferenz, C.R., Franklin, S., Ghayur, T., Hackett, M.C., Hammill, L.D.*, et al.* (1994). Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. Cell *78*, 343-352.

Wallin, I.E. (1922). A Note on the Morphology of Bacteria Symbiotic in the Tissues of Higher Organisms. J Bacteriol *7*, 471-474.

Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J.M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L.*, et al.* (1999). HMG-1 as a late mediator of endotoxin lethality in mice. Science *285*, 248-251.

Wang, H., Yang, H., and Tracey, K.J. (2004). Extracellular role of HMGB1 in inflammation and sepsis. J Intern Med *255*, 320-331.

Ward, J.F. (1994). DNA damage as the cause of ionizing radiation-induced gene activation. Radiat Res *138*, S85-88.

Werman, A., Werman-Venkert, R., White, R., Lee, J.K., Werman, B., Krelin, Y., Voronov, E., Dinarello, C.A., and Apte, R.N. (2004). The precursor form of IL-1alpha is an intracrine proinflammatory activator of transcription. Proc Natl Acad Sci U S A *101*, 2434-2439.

Werts, C., Tapping, R.I., Mathison, J.C., Chuang, T.H., Kravchenko, V., Saint Girons, I., Haake, D.A., Godowski, P.J., Hayashi, F., Ozinsky, A.*, et al.* (2001). Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol *2*, 346-352.

West, A.P., Koblansky, A.A., and Ghosh, S. (2006). Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol *22*, 409-437.

Wille-Reece, U., Flynn, B.J., Lore, K., Koup, R.A., Kedl, R.M., Mattapallil, J.J., Weiss, W.R., Roederer, M., and Seder, R.A. (2005). HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. Proc Natl Acad Sci U S A *102*, 15190-15194.

Williams, T.A., Foster, P.G., Cox, C.J., and Embley, T.M. (2013). An archaeal origin of eukaryotes supports only two primary domains of life. Nature *504*, 231-236.

Yang, R.B., Mark, M.R., Gray, A., Huang, A., Xie, M.H., Zhang, M., Goddard, A., Wood, W.I., Gurney, A.L., and Godowski, P.J. (1998). Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature *395*, 284-288.

Yang, Y., Liu, Z., Wang, J., Chai, Y., Su, J., Shi, F., Wang, J., and Che, S.M. (2016). The effect of interferon gamma on conventional fractionated radiation-induced damage and fibrosis in the pelvic tissue of rabbits. Therapeutics and clinical risk management *9*, 755-762.

Yasuda, K., Yu, P., Kirschning, C.J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S., Hochrein, H., and Wagner, H. (2005). Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J Immunol *174*, 6129-6136.

Yoneyama, M., and Fujita, T. (2008). Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity *29*, 178-181.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., and Kagan, J.C. (2011). CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell *147*, 868-880.

Zhai, Y., Shen, X.D., O'Connell, R., Gao, F., Lassman, C., Busuttil, R.W., Cheng, G., and Kupiec-Weglinski, J.W. (2004). Cutting edge: TLR4 activation mediates liver ischemia/reperfusion inflammatory response via IFN regulatory factor 3-dependent MyD88-independent pathway. J Immunol *173*, 7115-7119.

Zhang, Q., Itagaki, K., and Hauser, C.J. (2010a). Mitochondrial DNA is released by shock and activates neutrophils via p38 map kinase. Shock *34*, 55-59.

Zhang, Q., Raoof, M., Chen, Y., Sumi, Y., Sursal, T., Junger, W., Brohi, K., Itagaki, K., and Hauser, C.J. (2010b). Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature *464*, 104-107.

Zitvogel, L., Kepp, O., and Kroemer, G. (2010). Decoding cell death signals in inflammation and immunity. Cell *140*, 798-804.

8. List of figures

9. List of tables

10. Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

11. Acknowledgements

It’s a pleasure to thank those who made this thesis possible. First of all, I would like to thank my institute director Prof. Dr. Jan Buer for giving me an opportunity to pursue my thesis. A special thanks to my supervisor Prof. Dr. Carsten Kirschning for being patient, supervising my thesis, for his helpful discussions and significant criticisms during the study. I also want to thank the members of the PhD committee.

My gratitude further extends to Sabine Schimanski, Thomas Scholtysik for assisting and discussing several laboratories techniques, to all other colleagues and friends from the University hospital Essen whose help was invaluable. Personally, I would like to thank my family for their unfailing love and support throughout my life and to all my friends for their support and encouragement during my stay in Germany.

12. Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Mechanisms of sterile Inflammation and modulation of TLR4 activity“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Chiranjeevi Chebrolu befürworte.

Essen, den _____ _____ Prof. Dr. C. Kirschning

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den _____ _____ Chiranjeevi Chebrolu

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, e und g der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den _____ _____ Chiranjeevi Chebrolu