

**Innate Recognition and Inflammasome
Activation in Human Myeloid Immune Cells by
Methanogenic Archaea**

Dissertation

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Tim Vierbuchen

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Erster Gutachter: Prof. Dr. Holger Heine

Zweiter Gutachter: Prof. Dr. Thomas Röder

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Abbreviations

AIM2	Absent in melanoma 2
AMP	Anti-microbial peptide
ANOVA	Analysis of variation
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
BF	Brightfield
BFP	Blue fluorescent protein
BLaER1	B cell leukemia C/EBP α ER clone 1
bp	Base pair
C/EBP α	CCAAT/enhancer-binding protein alpha
CARD	Caspase recruitment domain
Cas9	CRISPR-associated protein 9
CatB	Cathepsin B
CatX	Cathepsin X
CCR7	C-C chemokine receptor 7
CD	Cluster of differentiation
cGAMP	2',3'-cyclic GMP-AMP
cGAS	cGAMP synthetase
crRNA	CRISPR RNA
CSLM	Confocal laser scanning microscopy
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSB	Double strand break
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
FACS	Fluorescence-activated cell sorting
GALT	gut-associated lymphoid tissue
GFP	Green fluorescent protein
gRNA	guide RNA
GSDMD	Gasdermin D

HDR	Homology-directed repair
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	Intestinal epithelial cell
IgA	Immunoglobulin A
IL	Interleukin
ILC	Innate lymphoid cell
IFN	Interferon
InDel	Insertion and deletion mutations
IRAK	IL-1R-associated kinases
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
KO	Knockout
LDH	Lactate dehydrogenase
LGP2	laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
M cell	Microfold cell
MAMP	Microbe-associated molecular pattern
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation antigen 5
MIC	Minimal inhibitory concentration
M Φ	Macrophage
moDC	Monocyte-derived dendritic cell
MyD88	Myeloid differentiation primary response 88
NEK7	NIMA related kinase 7
NHEJ	Non-homologous end joining
NF- κ B	Nuclear factor kappa B
NK cell	Natural killer cell
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NOD1/2	Nucleotide-binding oligomerization domain-containing protein 1/2
OMV	Outer membrane vesicle
PAM	Palindrome adjacent motif
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PMN	Polymorphonuclear leukocyte
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
S-layer	Surface layer
sgRNA	Single guide RNA
STING	Stimulator of interferon genes
TIDE	Tracking of InDels by decomposition
TIR	Toll/IL-1 resistance domain
TLR	Toll-like receptor
TMA	trimethylamine
TNF- α	Tumor necrosis factor alpha
tracrRNA	Trans-activating crRNA
TRAF	TBF receptor-associated factor
TRIF	TIR domain-containing adapter-inducing interferon β
UNC93B1	UNC93 homolog B 1
WT	Wildtype

II Abstract

1 English

The importance of the microbiota on health and immune homeostasis is widely accepted and many studies are currently dissecting the interaction between the microbiota and our body. However, most of these studies are focusing on bacteria alone, although viruses, fungi and archaea were also found in this microbial community. Recent studies showed that archaea are present at nearly every part of the body but their contribution to health and disease is not understood. The gut-associated methanogenic archaeon *Methanosphaera stadtmanae* induces inflammatory responses, however, the mechanism how this archaeon is sensed by the immune system has not been evaluated until now. In contrast, another methanogenic gut inhabitant, *Methanomassiliicoccus luminyensis*, is discussed for supplementation in humans as it has the unique metabolic capability to degrade trimethylamines, which can be harmful for the human host. Yet, its immunostimulatory activities have not been investigated. This study aims (1) to elucidate the receptors, archaeal structures and signaling pathways that are engaged upon activation of human immune cells by *M. stadtmanae* and (2) to analyze immunological features of *M. luminyensis* in order to exclude excessive immune stimulation.

Human monocytes, monocyte-derived dendritic cells (moDCs) as well as the monocytic cell line BLaER1 were used to characterize *M. stadtmanae*-induced cellular activation on the molecular level showing that this archaeon is a strong activator of the innate immune system capable of inflammasome activation and induction of type I interferons. Stable knockout cell lines were generated using CRISPR/Cas9 to specifically identify TLR7 and TLR8 as key receptors in recognition of *M. stadtmanae*. Furthermore, our data demonstrate a TLR8-dependent activation of the NLRP3 inflammasome sharing major characteristics with the LPS-induced alternative pathway. *M. luminyensis* on the other hand induces only a weak response in moDCs compared to *M. stadtmanae*, probably due to its significantly lower phagocytosis rate. Moreover, *M. luminyensis* is sensitive towards anti-microbial peptides and exhibits typical characteristics of a commensal gut inhabitant which supports its application as probiotic supplement in humans for TMA degradation.

For the first time, this work describes specific recognition of an archaeon by human immune cells on the molecular level. As some studies indicate a potential connection of *M. stadtmanae* to inflammatory bowel disease and lung hypersensitivity, our findings might help to understand how archaea are involved in inflammatory disease progression.

2 Deutsch

Die Mikrobiota des Menschen ist wichtig für dessen Gesundheit und hat großen Einfluss auf die Immunhomöostase. Die detaillierte Erforschung der Wechselwirkung zwischen Mikrobiota und menschlichem Körper ist aktuell Gegenstand vieler Studien. Häufig wird allerdings nur die bakterielle Vielfalt betrachtet und dabei andere Mitglieder dieser mikrobiellen Gemeinschaft wie Viren, Pilze und Archaeen vernachlässigt. Erst kürzlich konnte gezeigt werden, dass Archaeen auf allen Körperstellen zu finden sind, ihr Einfluss auf Gesundheit und Krankheit ist jedoch weitgehend unbekannt. *Methanosphaera stadtmanae* ist ein methanogenes Archaeon, welches im Darm beheimatet ist und inflammatorische Immunantworten auslösen kann. Der molekulare Mechanismus, wie *M. stadtmanae* vom Immunsystem erkannt wird, ist nicht bekannt. Im Gegensatz dazu wird der Einsatz eines anderen Darmarchaeons, *Methanomassiliicoccus luminyensis*, als Probiotikum diskutiert, da es die einzigartige metabolische Fähigkeit besitzt, Trimethylamin zu verstoffwechseln, welches gesundheitsschädliche Eigenschaften besitzt. Seine immunstimulatorische Aktivität wurde bisher nicht beschrieben. Ziel dieser Arbeit ist es (1) die Rezeptoren, archaeellen Strukturen und Signalwege zu identifizieren, die wichtig für die Erkennung von *M. stadtmanae* durch Immunzellen sind und (2) die immunologischen Eigenschaften von *M. luminyensis* zu analysieren, um übermäßige Immunantworten durch diese Spezies auszuschließen.

Humane Monozyten, Monozyten-generierte dendritische Zellen (moDCs) sowie die Monozytenzelllinie BLaER1 wurden verwendet, um die durch *M. stadtmanae*-vermittelte Immunantwort zu untersuchen. Dabei wurde gezeigt, dass diese Immunantwort vor allem durch Aktivierung des Inflammasoms und Expression von Typ I Interferonen gekennzeichnet ist. Mit Hilfe von CRISPR/Cas9-generierten *knockout* Zelllinien konnten TLR7 und TLR8 als Schlüsselrezeptoren identifiziert werden. Zudem wird eine TLR8-abhängige Inflammasomaktivierung induziert, die Eigenschaften der LPS-abhängigen alternativen Aktivierung aufweist. *M. luminyensis* löst dagegen nur schwache Immunantworten aus, wahrscheinlich aufgrund seiner geringen phagozytotischen Eigenschaften. Da dieses Archaeon sensitiv gegenüber antimikrobiellen Peptide ist, besitzt es typische Eigenschaften eines kommensalen Darmbewohners, was für einen Einsatz als Probiotikum spricht.

Zum ersten Mal konnte auf molekularer Ebene die Erkennung eines Archaeons durch menschliche Immunzellen beschrieben werden. Da ein Zusammenhang von *M. stadtmanae* mit chronisch entzündlichen Darmerkrankungen und Lungenhypersensitivität vermutet wird, kann diese Arbeit dazu beitragen, den Zusammenhang zwischen Archaeen und entzündlichen Erkrankungen besser zu verstehen.

III General Introduction

1 The Innate Immune System

In almost every situation, each individual must cope with a high quantity of microbes on and in our body. Prokaryotic as well as eukaryotic microorganisms live on our skin and mucosal surfaces, yet, most of these organisms are commensals, which means that they benefit from their host (e.g., by nutrient supply) without harming it. Nevertheless, our body must also handle manifold pathogens, in form of bacteria and viruses but also eukaryotic microorganisms like protozoa, worms, and algae or even pathogenic proteins called prions. The immune system has evolved to defend the body from those pathogens but also from toxins and cancer cells. In vertebrates, the immune system has two major strands: on one hand the innate immune system, building the first line of defense and immunological memory, and on the other hand the adaptive immune system with its variable receptors. Key cells of the adaptive immune system are T and B lymphocytes, both named after their organ of maturation, which is the thymus (T) and the *Bursa fabricii* (B, an organ only found in birds), respectively. In mammals, B lymphocytes mature in the bone marrow, their main function is the humoral response through secretion of antibodies. T cells mediate cellular immunity as their main function, which includes activation of B cells and killing of virus-infected cells (1).

Main features of the innate immune system are highly conserved as they are not restricted to vertebrates but can be found in plants and invertebrates (2). The innate immune system is based on natural barriers like epithelial cells, humoral factors like anti-microbial peptides (AMPs) or the complement system as well as cellular immunity (3). Various hematopoietic cell types belong to the innate immune system with each cell type having specific tasks in the immune defense. The most abundant cells of the innate immune system are the neutrophils (also called polymorphonuclear leukocytes: PMNs) whose main function is to enter sites of infection and to kill invading microorganisms through phagocytosis or granules containing microbicidal agents (4). Together with basophils and eosinophils they form the family of granulocytes. Other important innate immune cell types are mast cells, natural killer (NK) cells, innate lymphoid cells (ILCs) or $\gamma\delta$ T cells. Monocytes make about 10% of the circulating white blood cells. They engulf microbes through phagocytosis and can recognize and respond to most microbes *via* a large receptor repertoire. Monocytes migrate from the blood into various tissues, where they differentiate into tissue-resident macrophages (M Φ) or dendritic cells (DCs) (5). M Φ and DCs are both antigen presenting cells (APCs) which means that they can take up foreign antigens and present them after processing on their cell surface to

T cells. Especially DCs can initiate an adaptive immune response as they travel to the lymph nodes after antigen uptake and activate naïve T cells, thereby linking the innate and the adaptive immune system (6, 7). Due to their capability of phagocytosis and their large receptor repertoire, this study mainly deals with monocytes/M Φ and DCs.

1.1 Toll-like Receptors

The innate immune system has a broad repertoire of receptors that specifically recognize microbial structures. These structures are conserved across many different microbes and include cell wall components, lipids and nucleic acids. Initially, these structures were termed pathogen-associated molecular patterns (PAMPs) (8), however, as they are not only restricted to pathogens but rather linked to most microorganisms, they are now termed microbe-associated molecular patterns (MAMPs) (9). The receptors that recognize MAMPs are the pattern recognition receptors (PRRs). Each cell of the immune system has a specific subset of PRRs which is defined by the cell type and its role in the immune system. PRRs are divided into different subclasses of which the Toll-like receptors (TLRs) are the first-described and most prominent. All TLRs share a common structure with a cytosolic domain called Toll-IL-1-resistance (TIR) domain which is important for signal transduction, a transmembrane domain, and an ectodomain containing leucine-rich repeats (LRRs) which forms a horseshoe-shaped structure. The ectodomain accounts for the ligand specificity and binding (10). In humans, there exist ten different TLRs, either located at the cell surface or the endosome, whereas twelve TLRs are known in mice (Table III-1). TLRs recognize bacterial cell wall components like lipopolysaccharide (LPS) or lipopeptides, proteins like flagellin or profilin or different nucleic acids.

After ligand binding, TLRs form homo- or heterodimers and, subsequently, signaling adapter molecules such as myeloid differentiation primary-response protein 88 (MyD88) or TIR domain-containing adaptor protein inducing IFN- β (TRIF) are engaged. Signal transduction pathways of TLRs involve IL-1R-associated kinases (IRAKs) and TIR domain-containing adaptor protein inducing IFN- β receptor-associated factors (TRAFs), finally leading to activation and nuclear translocation of transcription factors. These transcription factors include, amongst others, the nuclear factor kappa B (NF- κ B) and interferon-regulatory factors (IRFs) (11). NF- κ B is an essential transcription factor for many pro-inflammatory cytokines, such as interleukin 6 (IL-6), interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α). Cytokines are important players in the immune response as they induce an acute-phase response, increase body temperature and cause swelling. They recruit other immune cells like PMNs and initiate the

adaptive immune response (12, 13). Depending on the TLR, certain IRFs are activated and translocated into the nucleus. IRF3, -5 and -7 are the main factors for the induction of type I interferons (IFNs) including IFN- α and IFN- β . While IFN- α is predominantly expressed by hematopoietic cells, IFN- β can be produced by most cells of the body. IFNs protect viral-infected cells but also non-infected neighboring cells from viral replication. They enhance antigen presentation and can activate the adaptive immune system (14, 15).

Table III-1. Overview of TLRs in mice and humans.

Name	Species	Ligand	Location	Adapter molecules	Reference
TLR2/TLR1 (heterodimer)	H, M	diacyl lipopeptides	cell surface	MyD88	(16)
TLR2/TLR6 (heterodimer)	H, M	triacyl lipopeptides	cell surface	MyD88	(17)
TLR3	H, M	dsRNA	endosomal compartment	TRIF	(18)
TLR4	H, M	LPS	cell surface	MyD88/TRIF	(19)
TLR5	H, M	flagellin	cell surface	MyD88	(20)
TLR7	H, M	ssRNA	endosomal compartment	MyD88	(21)
TLR8	H, (M*)	ssRNA	endosomal compartment	MyD88	(21)
TLR9	H, M	CpG DNA	endosomal compartment	MyD88	(22)
TLR10	H, (M**)	unknown	cell surface	unknown	(23)
TLR11	M	profilin	endosomal compartment	MyD88	(24)
TLR12	M	profilin	endosomal compartment	MyD88	(25)
TLR13	M	bacterial 23S rRNA	endosomal compartment	MyD88	(26)

H, Human; M, Mouse

* mTLR8 was originally thought to be non-functional. It is controversially discussed if natural ligands for mTLR8 exist or if it has just regulatory functions (27).

** mTLR10 only present as pseudogene.

1.2 Recognition of Nucleic Acids by the Innate Immune System

As nucleic acids are conserved and basic structural components for all living organisms, these molecules serve as important MAMPs for the innate immune system. Therefore, recognition of nucleic acids greatly expands the possibility to detect foreign organisms. In contrast to many other MAMPs, nucleic acids are not exclusively produced by microbes but also by the host itself. For instance, damaged host-derived cells and tissue release nucleic acids that might induce an immune response. Thus, nucleic acids can additionally be classified as danger-associated molecular patterns (DAMPs). Although nucleic acids largely extend the repertoire of molecular patterns, it implicates an increased risk of autoimmunity and sterile inflammation if endogenous nucleic acids trigger immune responses without acute danger or damage. Autoimmune diseases, in which the recognition of endogenous nucleic acids is involved, include systemic lupus erythematosus and type I interferonopathies (28). Nevertheless, nucleic acid-specific receptors have been shown to play a crucial role in immune responses against a variety of different pathogens including bacteria, viruses and protozoa (29, 30).

Nucleic acid-sensing receptors are effective inducers of type I IFN responses, but they can also trigger secretion of pro-inflammatory cytokines like TNF- α and IL-1 β dependent on the receptor type. In principal, PRRs that detect nucleic acids can be divided into two subclasses. One group includes TLRs located on endosomal and lysosomal membranes, the other group includes cytoplasmic receptors originated from different receptor families (Figure III-1). In humans, endosomal TLRs comprise TLR3 as well as TLR7-9, whereas mice additionally express TLR13. TLR3 recognizes dsRNA and utilizes TRIF as adaptor molecule, while signal transduction of TLR7-9 is dependent on MyD88. TLR7 and TLR8 both recognize ssRNA, TLR9 is specific for unmethylated CpG DNA (31). The most recent identified nucleic acid-sensing TLR is TLR13 which recognizes a specific sequence in bacterial 23S rRNA (32). The function of all endosomal TLRs depends on a protein called UNC93 homolog B1 (UNC93B1). UNC93B1 interacts with the transmembrane domain of endosomal TLRs and directs them from the endoplasmatic reticulum (ER) to the endo(lyso-)somes (33). It was shown by Tabeta, *et al.* (34) that mice with a missense mutation in UNC93B1 (referred to as '3d' mutation) no longer respond to ligands specific for endosomal TLRs, whereas TLRs on the surface remain functional.

As TLRs exclusively recognize nucleic acids in the endosomal or lysosomal compartment, pathogens that successfully entered the cytosol cannot be detected efficiently by

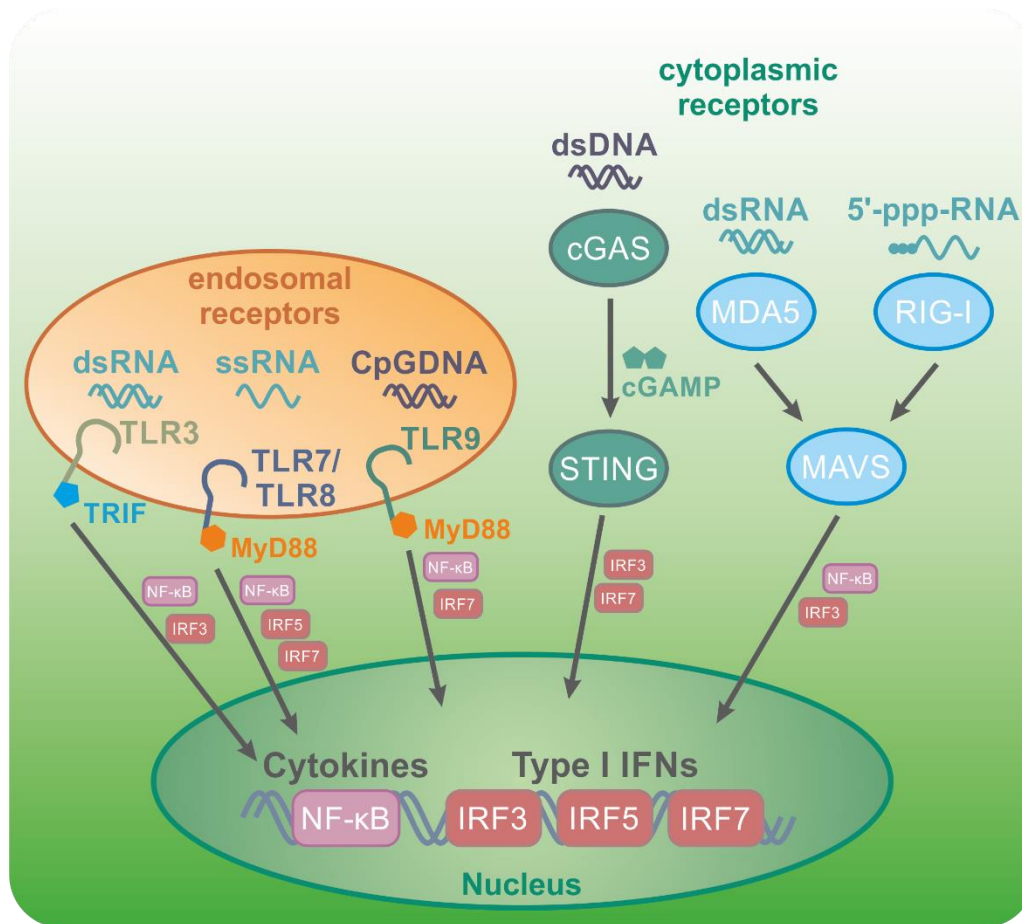


Figure III-1. Overview of endosomal and cytoplasmic nucleic acid-specific receptors in humans.

Nucleic acid-specific receptors differ in their cellular location (endosome or cytosol) and the type of nucleic acid that they can recognize. TLR3 and TLRs7-9 are localized in the endosome and bind to dsRNA (TLR3), ssRNA (TLR7 and TLR8) or unmethylated CpG DNA (TLR9). TLR3 signals *via* the adaptor protein TRIF, whereas TLR7-9 are dependent on MyD88. Activation of TLRs leads to nuclear translocation of NF-κB and a certain subset of IRFs to induce the production of a specific subset of cytokines and IFNs. Important cytoplasmic nucleic acid-specific receptors are cGAS, which binds dsDNA, MDA5, which binds dsRNA, and RIG-I binding 5'-triphosphate RNA or short dsRNA. After engagement of cGAS by dsDNA, the intermediate cGAMP is produced from GTP and ATP by cGAS. cGAMP hereupon activates STING leading to production of IFNs through nuclear localization of IRF3 and IRF7. MDA5 and RIG-I both signal through the adaptor protein MAVS after ligand binding. MAVS implements the production of a certain subset of cytokines and IFNs through nuclear localization of IRF3 and NF-κB. (cGAS, cyclic GMP-AMP synthase; cGAMP, 2'3'-cyclic GMP-AMP; IFN, interferon; IRF, interferon regulatory factor; MAVS, mitochondrial antiviral signaling; MDA5, melanoma differentiation-associated gene 5; MyD88, myeloid differentiation primary-response protein 88; NF-κB, nuclear factor kappa B; RIG-I, retinoic acid-inducible gene I; STING, stimulator of IFN gene; TLR, Toll-like receptor; TRIF, TIR domain-containing adaptor protein inducing IFN-β)

these TLRs. To detect bacteria or viruses located in the cytosol, cells express a variety of different cytosolic receptors. Important cytosolic RNA receptors are Retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2, also known as DHX58), which all belong to the family of RIG-I-like receptors (RLRs). The ligands for RIG-I and MDA5 are 5'triphosphate RNA and (long) dsRNA, respectively, whereas little is known about natural ligands for LGP2. Although it was shown that LGP2 has the ability to bind RNA, it is thought to have rather regulatory

functions on RIG-I and MDA5 (35). Mitochondrial antiviral signaling protein (MAVS) is essential for RLR function as it aggregates on the mitochondrial membrane after RLR engagement and activates IRF3 to induce an antiviral response (36). Foreign and host-derived DNA in the cytosol is detected by cyclic GMP-AMP synthetase (cGAS) (37). Once activated by dsDNA, cGAS catalyzes the production of 2',3'-cyclic GMP-AMP (cGAMP) from GTP and ATP. cGAMP itself acts as a second messenger and binds to stimulator of interferon genes (STING) which in turn utilizes IRF3 to induce expression of type I IFNs. An additional function of STING in human cells was recently described by Gaidt, *et al.* (38), who showed that STING can induce lysosomal cell death leading to inflammasome activation and secretion of IL-1 β . Taken together, recognition of nucleic acids by the innate immune system plays a substantial role in host defense as it enables not only a potent mechanism to detect and respond to viruses but also to bacterial and eukaryotic pathogens.

1.3 Inflammasomes

Inflammasomes are large cytosolic multiprotein complexes that recognize various pathogenic or environmental stimuli. Once activated, the inflammasome induces autocatalytic cleavage of the zymogen pro-caspase-1 into its active form. An important function of caspase-1 is the catalytical processing of the inflammatory cytokines IL-1 β and IL-18 into mature, functional peptides (39). On the one hand, inflammasome activation supports clearance of microbial infection, on the other hand, if malfunctioning, inflammasome activation can lead to sterile inflammation and can contribute to diseases such as cancer, diabetes and Alzheimer's disease (40-42). There exist several different inflammasomes in humans and mice, which differ in their receptor (sensor) domain. This receptor domain is either a NOD-like receptor (NLR) such as NLRP1, NLRP3, NLRC4, or the non-NLR AIM2. The sensor defines the stimuli that can activate the inflammasome e.g., NLRC4 recognizes bacterial flagellin, AIM2 binds to dsDNA and NLRP1 is activated by muramyl dipeptide or *Bacillus anthracis* lethal toxin (43-45). In contrast, for the intensively-studied inflammasome NLRP3 there is no specific stimulus defined. It is reported that NLRP3 is activated by various, structurally unrelated stimuli including monosodium urate crystals, alum, asbestos, bacterial toxins like nigericin, extracellular ATP or cytoplasmic bacterial RNA. It is unlikely that NLRP3 itself can bind to all these different molecules and stimuli. Therefore, NLRP3 is nowadays often referred to as a general cellular stress sensor (46). All inflammasome receptors are linked through a protein called ASC (Apoptosis-associated speck-like protein containing a CARD) to pro-caspase-1

with exception of NLRP1 and NLRC4, which can directly interact with pro-caspase-1, independently of ASC (42).

In general, three different activation pathways for NLRP3 are known, namely canonical, non-canonical and alternative activation. The canonical activation comprises a two-step process in which the first signal is needed to induce the expression of pro-IL-1 β and therefore termed priming step (Figure III-2A). Then, a second signal is mandatory to initiate the actual inflammasome assembly. In case of the NLRP3 inflammasome, various endogenous and microbial danger signals are known that can serve as a second signal and induce inflammasome activation. For many of these stimuli, cellular efflux of potassium ions is of importance (47) as this process activates NIMA-related kinase 7 (NEK7) which was shown to be essential for IL-1 β secretion from cells *in vitro* and *in vivo* (48-50). Still, the exact mechanism of how NEK7 activates NLRP3 on the molecular level is unresolved. Subsequently, canonical activation of the NLRP3 inflammasome leads to formation of ASC specks (also called pyroptosomes), large macromolecular, filamentous complexes that can be visualized by electron microscopy (51, 52). These ASC specks are initiated by activation of NLRP3 which forms a ring-like structure and recruits ASC proteins that linearly polymerize. Pro-caspase-1 is then recruited to the ASC specks, activated and mature caspase-1 is formed by autocatalytically processing (53). Besides ASC speck formation, the activation of gasdermin D (GSDMD) is another characteristic feature of canonical inflammasome activation. Caspase-1 cleaves GSDMD which forms pores in the cell membrane leading to rapid efflux of intracellular components and finally to pyroptosis, a highly inflammatory form of programmed cell death. GSDMD only kills the cell where it was activated but not neighboring cells (54). As the question how processed IL-1 β is secreted from the cells was unresolved for a long time, it was recently shown by Evavold, *et al.* (55) that the pores formed by GSDMD can serve as a channel for IL-1 β to exit the cell.

The second NLRP3 activation pathway is the non-canonical activation. In this setting, caspase-11 in mice (56) or caspase-4/-5 in humans (57, 58) are activated through specific binding of intracellular LPS. Similar to caspase-1 activation, the activation of caspase-4/-5/-11 induces pyroptosis which causes efflux of cellular potassium, yet caspase-4/-5/-11 cannot trigger conversion of IL-1 β itself. In fact, the potassium efflux activates the NLRP3 inflammasome downstream of caspase-4/-5/-11, which results in activation of caspase-1 and secretion of IL-1 β (59).

The alternative NLRP3 inflammasome activation is the most recently described activation pathway. It is a species- and cell-specific pathway, so far only reported for human and porcine monocytes but it could not be demonstrated in murine cells. The alternative

activation diverges from canonical activation as it only requires a single stimulus and not a two-step activation (Figure III-2B). The alternative pathway is induced by activation of TLR4 through binding of LPS. Then, a TRIF-dependent signaling pathway is initiated which includes the molecules RIPK1, FADD and caspase-8, finally leading to NLRP3 activation. Alternative activation is different from canonical activation as no pyroptosis is induced, no ASC specks are formed and no potassium efflux is required (60). However, the role of alternative inflammation in infection or autoimmunity is unknown and needs to be determined.

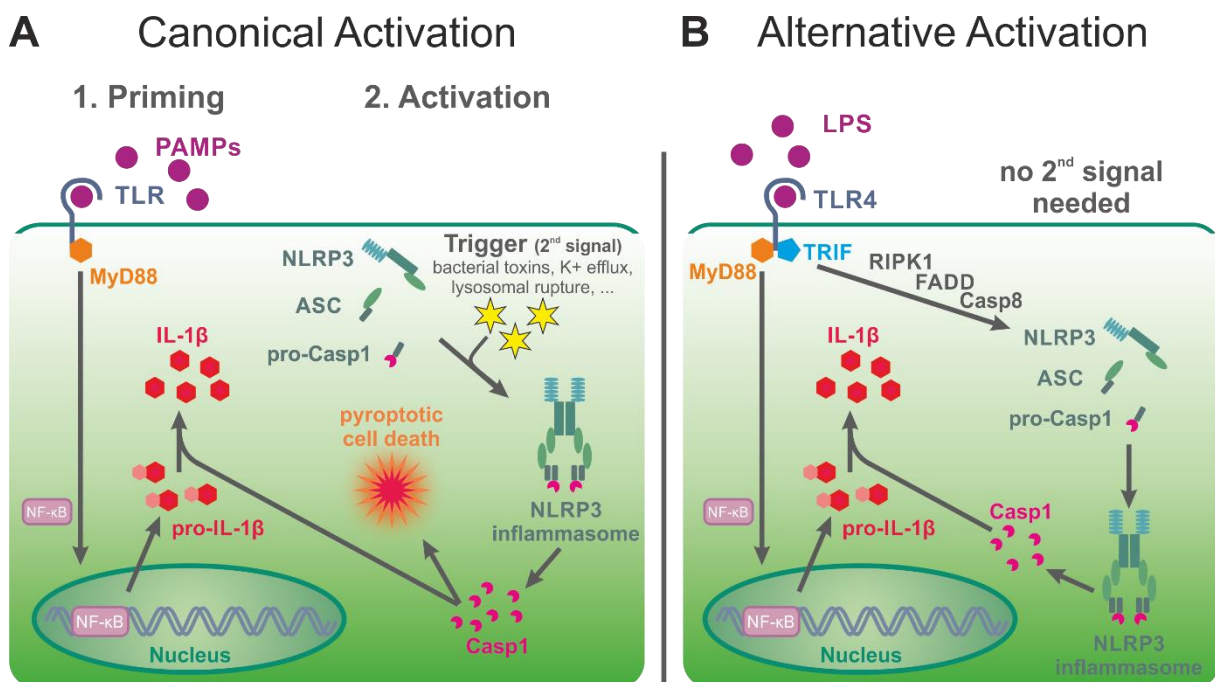


Figure III-2. Canonical and alternative activation of the NLRP3 inflammasome.

(A) In the canonical NLRP3 inflammasome activation process, two signals are required. The first one is the priming step where the expression of pro-IL-1 β is induced e.g., through engagement of a TLR. The second signal triggers the formation of the NLRP3 inflammasome complex consisting of NLRP3, ASC and pro-caspase-1 (often referred to as pyroptosome or ASC speck). This trigger is not a specific ligand but a rather a general stress signal in the cell that induces inflammasome activation. Once activated, the inflammasome turns pro-caspase-1 into its active form which then cleaves pro-IL-1 β . This process results in activation of gasdermin D, a pore forming protein, inducing pyroptosis. (B) Alternative NLRP3 inflammasome activation is a process that takes place in human monocytes but not in murine cells. In this setting, LPS binds to TLR4 which induces expression of pro-IL-1 β . No additional trigger is needed as TLR4 signals *via* TRIF-RIPK1-FADD-Caspase 8 to induce NLRP3 inflammasome activation without pyroptosome formation. Caspase-1 is converted into its active form and cleaves pro-IL-1 β . However, in alternative activation, no pyroptosis is induced. (Casp1, Caspase-1; Casp8, Caspase-8; IL-1 β , Interleukin 1 β ; PAMP, Pathogen associated molecular pattern; TLR, Toll-like receptor)

2 Archaea and the Human Microbiota

2.1 Importance of the Microbiota on Health and Disease

Given the fact that the human body is home to numerous microorganisms, at least equal to its own cell number (61), humans are often referred to as supra- or meta-organisms (62, 63).

As most human-associated microbes are considered to be commensals, others are mutualists, which means that the host benefits from their presence. These benefits include the synthesis of vitamin K, vitamin B12 and folic acid, deconjugation of bile acids or the prevention of colonization with pathogens (64). In the gut, microbes are separated by intestinal epithelial cells (IECs) from the underlying tissue, thereby forming a physical barrier (Figure III-3). The epithelial layer is in close connection to the immune system through the gut-associated lymphoid tissue (GALT). Microfold (M) cells within the epithelial cell layer take up MAMPs and antigens and deliver them to DCs. In addition, DCs are able to sample antigens themselves by stretching their dendrites between the IECs. In the Peyer's patches and isolated lymphoid follicles, which are located closely to the IECs in the lamina propria, DCs can present the antigens to T cells and induce differentiation of B cells into immunoglobulin A (IgA)-secreting plasma cells. IgA is steadily translocated into the intestinal lumen. Together with AMPs, which are secreted by specialized IECs - the so-called Paneth cells -, both types of molecules control the microbial balance in the gut (65-67).

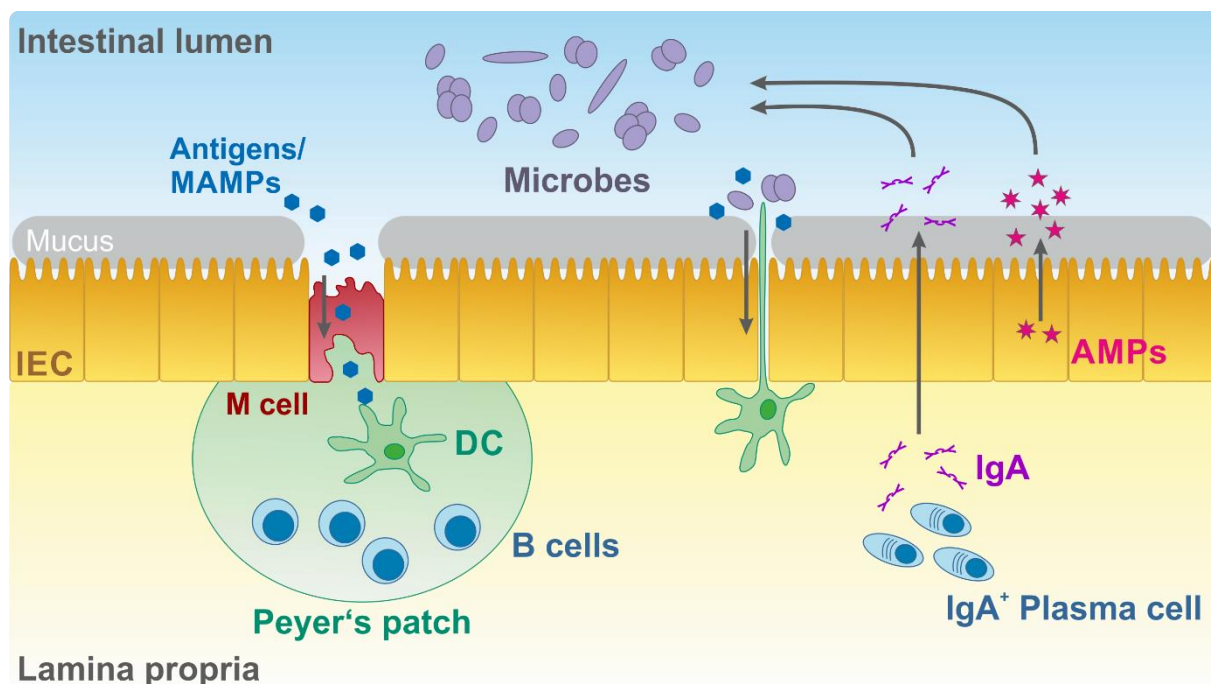


Figure III-3. Schematic overview of the mucosal immune system in the gut.

IECs form a physical barrier against microbes in the gut and protect the body from microbial infiltration. The immune system plays an important part in shaping the composition of the microbiota. Microbial antigens and MAMPs are directly sensed by IECs or sampled by M cells, thereby transferring the antigens to DCs in the Peyer's patches or isolated lymphoid follicles. DCs themselves can also directly take up microbes and antigens and present them to T and B cells. Activated B cells may differentiate into IgA-secreting plasma cells. IgA and AMPs are constitutively secreted into the intestinal lumen to contain homeostasis of the microbiota. (AMP, anti-microbial peptide; DC, dendritic cell; IEC, intestinal epithelial cell; IgA, immunoglobulin A; M cell, microfold cell; MAMP, microbe-associated molecular pattern)

The microbiota also has direct implications on the immune system as it trains the immune system from early life on and the immune system itself shapes the composition of the microbiota (68, 69). The importance of the microbiota is explicitly shown by the usage of germ-free animals. These animals have an increased susceptibility towards pathogens caused by underdeveloped lymphatic tissues as well as a reduced production of mucus and AMPs. Both, adaptive and innate immune responses are highly compromised, demonstrating that the natural microbiota is essential for the development of a functional immune system (70, 71). The immune system senses members of the microbiota through PRRs which are mandatory to balance the composition of the microbiota. PRRs are not exclusively expressed by hematopoietic cells but also by the IECs. Their activation through MAMPs is necessary for steady production and secretion of AMPs and IgA, and for the epithelial barrier function in general (72-75). Stimulation of IECs by the natural microbiota is essential for specific cytokines that lead to the development of tolerogenic immune responses. If the barrier function of IECs is disturbed through certain genetic and environmental factors, pro-inflammatory responses might be induced that can break the microbial homeostasis (65). This so-called dysbiosis of the microbiota can have adverse effect on the host as imbalances can contribute to several diseases including inflammatory bowel disease (IBD) (76, 77), irritable bowel syndrome (IBS), obesity (78) or asthma and allergy (79).

2.2 Archaea

In 1977, Carl Woese claimed that archaea form an own prokaryotic domain distinct from bacteria, based on 16S rRNA analysis (80). Although archaea share prokaryotic features like the absence of intracellular compartments, the lack of a nucleus, or the circular genomic DNA with bacteria, other elements are closely related to eukaryotes. These include the ribosomal structure, DNA replication, and the translation and transcription machinery (81-83). A *new view on the tree of life* by Hug, *et al.* (84), which is based on ribosomal protein sequences, places archaea closely to eukaryotes. In addition, the recently discovered phylum of Lokiarchaeota strengthens the hypothesis that eukaryotes have been evolved from a common archaeal ancestor (85, 86). Archaea have a unique cellular structure as their cell membrane is built from ether-linked lipids instead of ester-linked lipids that are utilized by bacteria and eukaryotes (87). Furthermore, the archaeal cell wall is not composed of peptidoglycan (murein) but rather diverse across different archaeal phylogenetic groups. For instance, surface layer (S-layer) proteins, pseudomurein or methanochondroitin can form the archaeal cell envelope, which are all unique for this domain (88).

Originally, archaea were regarded as extremophilic organisms that colonize habitats with high temperature, low pH or high salt concentrations. Over the time, archaea have also been discovered in rather moderate environments such as oceans, freshwater or soil (89, 90). The first group of archaea that were described as members of the human microbiota are the methanogens, originally isolated from feces of animals and later also from humans (91, 92). Methanogenic archaea are strictly anaerobic and utilize C1 or C2 carbon sources such as CO₂ or acetate to produce methane. As many methanogens depend on hydrogen supply, they frequently live in syntrophy with fermentative bacteria. The bacteria produce hydrogen which is used by archaea for methanogenesis and the bacteria in turn are dependent on a low hydrogen partial pressure to render the fermentation process thermodynamically favorable (93). Therefore, methanogens are thought to be an important factor in the metabolic function of the microbiota. This assumption is supported by the findings of Dridi, *et al.* (94), who showed that the most abundant methanogen, *Methanobrevibacter smithii*, is found in 96% of human guts and well adapted to its host, where it can make up to 10% of the anaerobic biomass (95). Other common methanogenic gut inhabitants are *Methanosphaera stadtmanae* and *Methanomassiliicoccus luminyensis*. *Methanobrevibacter oralis* is commonly found in the oral cavity (96). Yet, not all human-associated archaea are methanogens. In the gut, archaea from the orders Halobacteriales, Sulfolobales, Nitrosphaerales, Desulfococcales, and others have been discovered (97, 98). Furthermore, it was recently shown that Thaumarchaeota are present on the skin (99) and even in the lung, Woese archaeota have been detected (100). However, the actual diversity of the human archaeome is probably vastly underestimated. Studies have shown that ‘universal’ 16S rRNA primers, which were thought to amplify both bacterial and archaeal rRNA, are highly biased towards bacteria and are not suitable for detection of most archaea in the microbiota (100-102). This lack of information contributes to the fact that little is known about the function and interaction of archaea with their host. Besides the metabolic functions of archaea like methane production and depletion of trimethylamine (TMA) by *M. luminyensis* (103), the actual impact of different archaeal species on health and disease remains largely unknown.

2.3 Potential Disease Association of Archaea

Although no archaeal pathogen has been identified so far, it cannot be excluded that they do exist. Archaea can be detected in notable abundancies on every body site and they live in close relationship to their host and other members of the microbiota. This fact makes a potential pathogenicity of certain archaeal species conceivable, yet, our current methods to

detect archaea in our microbiota are apparently a hindrance. Nonetheless, several studies suggest an involvement of archaea in different diseases. It was shown that *M. smithii* promotes obesity in gnotobiotic mouse model (95) and its number is increased in humans with obesity (104, 105). Furthermore, a strong correlation between the occurrence of methanogenic archaea and periodontal disease (106-109) or brain abscesses (110) was observed. However, it is unclear if archaea are directly involved in disease development or if their presence promotes the occurrence of pathogenic bacteria which are actually causing the disease. In IBD, *M. stadtmanae* is detectable in patients more frequently, and higher antibody titers against this archaeon compared to healthy individuals have been found (111). *M. smithii* and *M. stadtmanae* are both gut-associated archaea, yet they can also be detected in high amounts in bioaerosols of large, industrial animal farms (112). When these archaea are aerosolized and injected into the respiratory system of mice, they induce an immune response, at which *M. smithii* causes a mild immune activation and *M. stadtmanae* induces a considerably stronger response in form of lung hypersensitivity (113). These studies indicate that archaea might be harmful to farm workers and could cause chronic respiratory diseases.

The different immune responses mounted by *M. smithii* and *M. stadtmanae* were also investigated *in vitro* using human PBMCs and monocyte-derived dendritic cells (moDCs). In both studies, *M. stadtmanae* induced a significantly stronger release of pro-inflammatory cytokines such as IL-1 β and TNF- α compared to *M. smithii* (111, 114). As IL-1 β is detectable after stimulation of moDCs with *M. stadtmanae*, the archaeon presumably activates the inflammasome. Moreover, Bang, *et al.* (114) observed that the expression of the co-stimulatory molecule CD86 and the C-C chemokine receptor type 7 (CCR7), which are both markers of activation and maturation of DCs, is increased on the cell surface of moDCs after stimulation with *M. stadtmanae*. The response to *M. stadtmanae* requires phagocytosis and endosomal acidification (114), which indicates that the actual recognition process takes place in an endosomal or intracellular compartment and not on the cell surface. In addition to pro-inflammatory cytokines, *M. stadtmanae* also induces expression of different AMPs (114) and both, *M. smithii* and *M. stadtmanae* are susceptible to natural and synthetic AMPs in a low μ M-range (115). These studies demonstrate that the human immune system can specifically recognize and cope with different archaeal species. Yet, it is largely unknown if the host benefits from the presence of archaea or if certain archaeal species even harm the body and cause diseases.

3 Cellular Systems and CRISPR/Cas9 Technology

3.1 BLaER1 Cell Line

Besides PBMCs, primary monocytes and moDCs, the BLaER1 cell line is used in this study as a monocytic cell model system to investigate the response of myeloid immune cells against *M. stadtmanae*. Genetic manipulations like gene knockouts or construction of fusion proteins through the CRISPR/Cas9 system are applicable in these cells. BLaER1 cells are based on RCH-ACV, a B cell precursor leukemia cell line, which was genetically modified with a C/EBP α -ER-IRES-GFP construct by Rapino, *et al.* (116). These cells can be transdifferentiated into a monocytic cell line by cultivation with β -estradiol, interleukin 3 (IL-3) and macrophage colony-stimulating factor (M-CSF or CSF-1) for 7 days (Figure III-4). C/EBP α is an important transcription factor for monocytic and granulocytic cells during hematopoiesis (117). In BLaER1 cells, C/EBP α is translocated into the nucleus upon addition of β -estradiol which drives the transdifferentiation from a B lymphocytic cell line into a monocytic cell line. This process is supported by IL-3, a proliferative factor for myeloid cells (118) as well as M-CSF, a growth and differentiation factor for monocytes, macrophages and their respective progenitor cells (119). Rapino, *et al.* (116) showed that after transdifferentiation into monocyte/M Φ -like cells, BLaER1 cells become quiescent and functional phagocytes. Their expression profile is similar to that of primary macrophages. Furthermore, BLaER1 monocytes have a fully functional NLRP3 inflammasome complex and, in contrast to conventional monocytic cell lines like THP-1, alternative inflammasome activation can be triggered in BLaER1 monocytes (60).

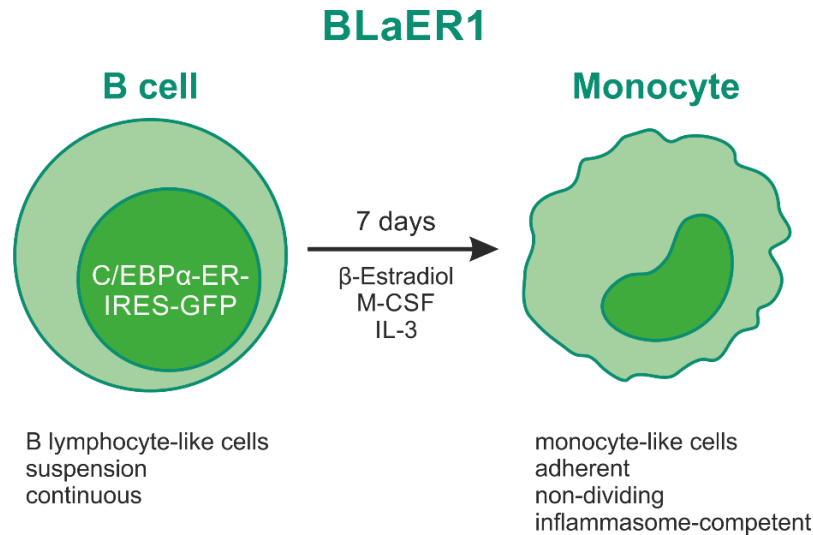


Figure III-4. Transdifferentiation of BLaER1 B cells into monocytic cells.

The BLaER1 cell line is based on the RCH-ACV cell line and was stably transduced with a C/EBP α -ER-IRES-GFP construct. Transdifferentiation of BLaER1 B cells into BLaER1 monocytes is achieved by cultivation of the cells with β -estradiol for 7 days, which leads to the translocation of the myeloid transcription factor C/EBP α . M-CSF and IL-3 are added to support the transdifferentiation process. (C/EBP α , CCAAT/enhancer-binding protein alpha; ER, Estradiol receptor; GFP, Green fluorescent protein; IL-3, Interleukin 3; IRES, internal ribosomal entry site; M-CSF, Macrophage colony-stimulating factor).

3.2 CRISPR/Cas9 as a Tool for Genetic Engineering

CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated protein 9) is a fast-developing technology which can be used for genetic manipulation of various cells and organisms. The technology is based on a mechanism that was originally described as an ancient defense mechanism of bacteria and archaea against phages and viruses (120, 121) and therefore often referred to as a prokaryotic adaptive immune system (122). CRISPR is used by microbes to excise foreign DNA from their genome. It comprises various enzymes including the RNA-guided nuclease Cas9 which is used for genetic engineering. Cas9 is associated with a CRISPR RNA (crRNA) array consisting of an auxiliary trans-activating crRNA (tracrRNA) and a guide RNA (gRNA). In order to simplify the system for genetic engineering, both RNAs can be fused to a single guide RNA (sgRNA) (123). The gRNA or sgRNA guides the Cas9 to a target DNA sequence *via* a 20 bp pairing (Figure III-5). This 20 bp sequence has only one restriction as a palindrome adjacent motif (PAM) must follow at the 3'-end of the 20 bp pairing sequence. The PAM sequence is species-dependent and can vary in its length. For the widely used Cas9 from *Streptococcus pyogenes* the PAM is 'NGG' (121). After binding of Cas9 to the target sequence, a double strand break (DSB) is catalyzed three bp downstream of the PAM. Once the target DNA is cleaved, mutations can be generated either randomly by non-homologous end joining (NHEJ) or specifically by homology-directed repair (HDR). In case of NHEJ, the cell repairs the DSB through a fast but error-prone

mechanism. This often leads to formation of insertion or deletion mutations (InDels). If these InDels are located within an exon, a frameshift can be introduced, and this can be used to generate a gene knockout. A donor (repair) template can be delivered with the Cas9 enzyme in order to use HDR to change specific nucleic acids or even add larger sequences like fluorescent proteins. The donor template can be given as ssDNA or dsDNA sequences or as plasmid DNA (124).

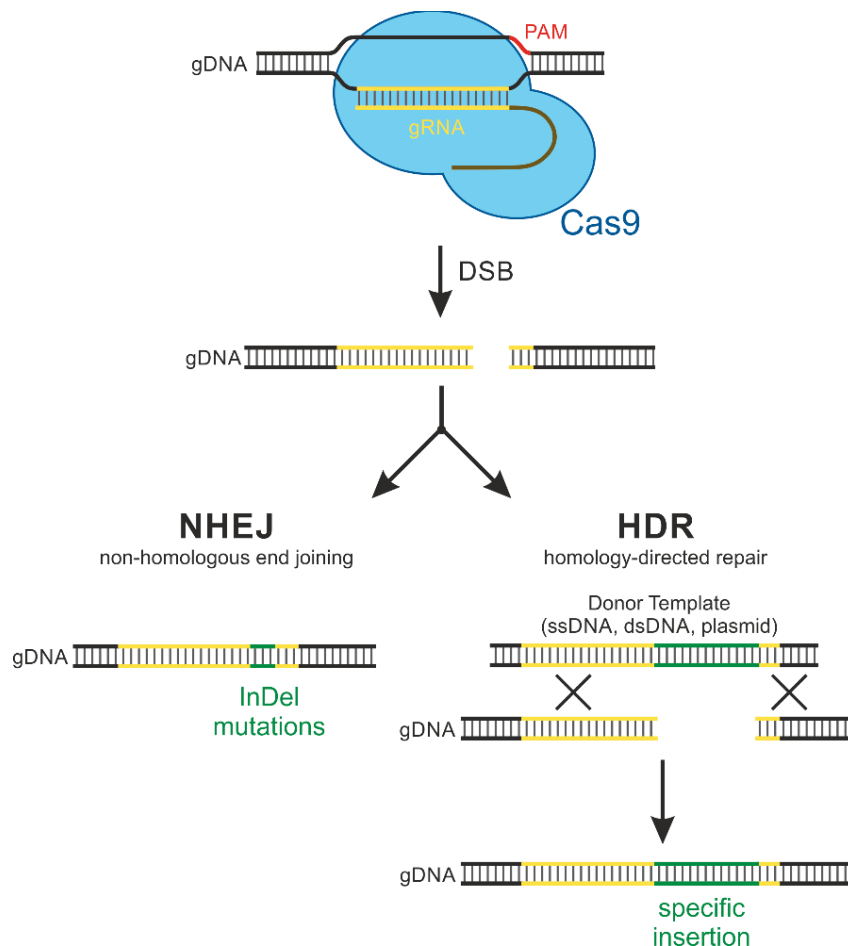


Figure III-5. CRISPR/Cas9 as tool for genetic engineering in mammalian cells.

The gRNA forms a complex with the Cas9 enzyme and directs it to complementary sequences in the gDNA of the cells. Binding between gRNA and the target sequence in the gDNA is only achieved if the PAM is present in the gDNA. After binding of Cas9 to the DNA, the enzyme catalytically cleaves the DNA *via* a DSB. The DSB is located three bases downstream of the PAM. The DSB is repaired by NHEJ which leads to the formation of InDel mutations. If a donor template is present, specific insertions can be introduced *via* HDR. (DSB, double strand break; gDNA, genomic DNA; gRNA, guide RNA; HDR, homology-directed repair; InDel, insertion or deletion of bases; NHEJ, non-homologous end joining; PAM, protospacer adjacent motif).

To generate clonal BLaER1 knockout cell lines, a method adapted from Ran, *et al.* (123) is used (Figure III-6). At first, BLaER1 cells are transfected with plasmid which encodes a target gene-specific gRNA and the Cas9 enzyme which is linked *via* a T2A self-cleaving peptide to blue fluorescent protein (BFP) (125). BFP positive cells are single cell-sorted *via* fluorescence activated cell sorting (FACS) and expanded. The clones are finally analyzed for

InDel mutations in the target gene by sequencing and tracking of InDels by decomposition (TIDE) analysis.

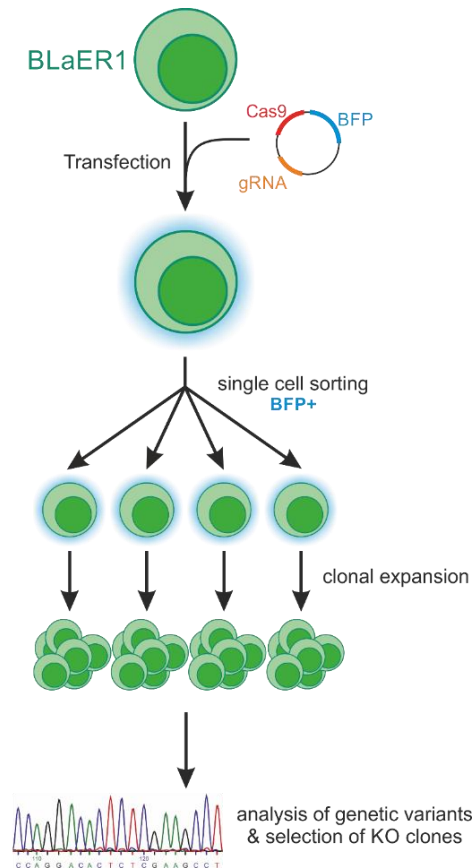


Figure III-6. Generation of BLaER1 KO cell lines using CRISPR/Cas9

For generation of clonal BLaER1 KO cell lines, the cells are transfected with a CRISPR/Cas9 plasmid by electroporation. The plasmid encodes the SpCas9 enzyme, the gRNA and BFP as selection marker. After transfection, BFP+ cells are single cell sorted by FACS and expanded for 2-3 weeks. The occurrence of InDel mutations in the gene of interest in the different clones is analyzed by sequencing. Clones with frameshift mutations on all alleles are selected for further analysis and characterization. (BFP, blue fluorescent protein; FACS, fluorescence-activated cell sorting; gRNA, guide RNA; InDel, insertion or deletion mutations; KO, Knockout). Adapted and modified from Ran, *et al.* (123).

4 Aim of this Study

This study aims to investigate the interaction of human-associated methanogenic archaea with the immune system. The first part is focused on *M. luminyensis*, a methanogenic archaeon that was just recently described as an inhabitant of the human gut. Its ability to degrade TMA suggests that it provides benefits to the host. However, it is still unknown which effects *M. luminyensis* has on the immune system. Therefore, the strength and quality of the response of myeloid immune cells towards *M. luminyensis* will be analyzed. In addition, the effect of AMPs on the growth of *M. luminyensis* will be investigated.

The second part deals with the immune response of human myeloid cells against *M. stadtmanae* as it was shown that this archaeon is a strong inducer of pro-inflammatory cytokines. The exact recognition mechanism is unknown, as no archaeal MAMP could be identified yet. Thus, the study aims to identify the MAMP(s) from *M. stadtmanae* that is/are recognized by immune cells and to determine the receptor(s) involved in this process. The inflammasome components and their activation process, which are induced by *M. stadtmanae*, will also be investigated. Accordingly, the results can contribute to an improved understanding of the principles of archaeal recognition by the human immune system and how archaea might impact on inflammatory diseases.

IV Publication I: Immunogenic properties of the human gut-associated archaeon *Methanomassiliicoccus luminyensis* and its susceptibility to antimicrobial peptides

Bang C, Vierbuchen T, Gutschmann T, Heine H, Schmitz RA

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1 Abstract

The methanogenic archaeon *Methanomassiliicoccus luminyensis* strain B10^T was isolated from human feces just a few years ago. Due to its remarkable metabolic properties, particularly the degradation of trimethylamines, this strain was supposed to be used as “Archaeobiotic” during metabolic disorders of the human intestine. However, there is still no data published regarding adaptations to the natural habitat of *M. luminyensis* as it has been shown for the other two reported mucosa-associated methanoarchaea. This study aimed at unraveling susceptibility of *M. luminyensis* to antimicrobial peptides as well as its immunogenicity.

By using the established microtiter plate assay adapted to the anaerobic growth requirements of methanogenic archaea, we demonstrated that *M. luminyensis* is highly sensitive against LL32, a derivative of human cathelicidin (MIC=2 µM). However, the strain was highly resistant against the porcine lysin NK-2 (MIC=10 µM) and the synthetic antilipoplysaccharide peptide (Lpep) (MIC>10 µM) and overall differed from the two other methanoarchaea, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* in respect to AMP sensitivity. Moreover, only weak immunogenic potential of *M. luminyensis* was demonstrated using peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (moDCs) by determining release of pro-inflammatory cytokines.

Overall, our findings clearly demonstrate that the archaeal gut inhabitant *M. luminyensis* is susceptible to the release of human-derived antimicrobial peptides and exhibits low immunogenicity towards human immune cells *in vitro* – revealing characteristics of a typical commensal gut microbe.

2 Introduction

The human gut microbiota is by far described to be dominated by bacteria (126), although a large number of microeukaryotes, fungi, viruses as well as archaea also form part of it (127). In contrast to bacteria, fungi and viruses, the immunological impact of archaea on the human immune homeostasis has rarely been described. This underrepresentation is mainly because still no archaeal pathogen is known and the challenges of growing these microorganisms in the laboratory.

The two methanoarchaeal strains *Methanosphaera stadtmanae* and *Methanobrevibacter smithii* were isolated and cultivated from human feces over 30 years ago (128, 129). Recently, human immune cell activation in response to these two strains was investigated (111, 114) and it was shown that they are prone to the lytic effects of antimicrobial peptides, similarly to those described for bacteria, though they differed in sensitivities (115). Only recently, several strains from the newly identified methanogenic order Methanomassiliicoccales were also found to inhabit the human intestine with low abundances (130-132) but only the strain *Methanomassiliicoccus luminyensis* strain B10^T was cultured and biochemically characterized in 2014 (133). Importantly, this strain was shown to degrade trimethylamine (TMA) *via* H₂-dependent reduction of methyl-compounds in the process of methanogenesis (133). Thus, the authors hypothesized that human-associated Methanomassiliicoccales strains might be used as probiotics against metabolic disorders associated with TMA produced by gut bacteria (103). These disorders include trimethylaminuria (134) as well as the development of cardiovascular (135) and chronic kidney disease (136). However, besides its natural metabolic capacity to diminish TMA very little is known about the functional role of Methanomassiliicoccales within the human intestine and its microbiota. When discussing a potential use of Methanomassiliicoccales as “Archaeobiotics” (103), particularly their impact on human immune homeostasis has to be considered.

As mentioned above, the predominant mucosa-associated archaeal strains *M. stadtmanae* and *M. smithii* appear to have highly different effects on human immune cells, particularly with respect to their overall immunogenicity and sensitivity to mammalian-derived antimicrobial peptides (AMPs) (114, 115). The corresponding information is still lacking for the new order of Methanomassiliicoccales but it is crucial for a holistic view on its functional role within the human intestine. Therefore, the current study aimed at evaluating those parameters for the cultivable strain *M. luminyensis*.

3 Materials and Methods

Ethics statement

Approval for these studies was obtained from the Institutional Ethics Committee at the University of Lübeck (Lübeck, Germany; Az. 12-202A) according to the Declaration of Helsinki. All donors gave written informed consent.

Growth of *M. luminyensis*

M. luminyensis (DSM 25720) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and was grown at 37 °C in 5 or 50 ml minimal medium (Medium 120, DSMZ) under strict anaerobic conditions as described earlier (137). The medium was reduced with the reductant cysteine (2 mM final concentration) prior to inoculation, 150 mM methanol was added as carbon and energy source and 152 kPa H₂/CO₂ (80/20 vol/vol) was used as gas phase. To prevent bacterial contamination the medium was supplemented with 100 µg/ml ampicillin. *M. luminyensis* cell numbers were counted in precultures by using a *Thoma* counting chamber and concurrent determination of optical turbidity at 600 nm (OD₆₀₀) during the growth period. For immune cell stimulation, exponentially growing *M. luminyensis* cells were harvested at 3,200 x g for 30 min, washed and resuspended in 50 mM Tris-HCl (pH 7.0).

Antimicrobial peptides (AMPs)

The AMPs tested in this study were a derivative of the human cathelicidin LL37 (LL32) (138), one derivative of porcine NK-lysin (NK2) (139), and a synthetic antilipoplysaccharide peptide Lpep 19-2.5 (140) (all purified from chemical peptide synthesis, and kindly provided by O. Holst, Division of Structural Biochemistry, Research Center Borstel, Borstel, Germany). Peptides were stored in stock solutions at -20°C and diluted in anaerobic Aqua_{dest} prior to use.

Microtiter plate assay for AMP susceptibility test

The antimicrobial activity of the peptides against *M. luminyensis* was determined by growth inhibition in microtiter plates as described earlier (115). In brief, 2x10⁷ cells from mid-exponential growth phase of precultures were inoculated into 250 µl minimal medium in U-bottom polystyrene microtiter plates (MICROLON® - Greiner Bio-One GmbH, Frickenhausen, Germany) and supplemented with different AMP-concentrations. Incubation of these cultures was performed in an anaerobic jar (Schuett-Biotec GmbH, Göttingen, Germany) and 152 kPa H₂/CO₂ was continuously supplied during incubation except during monitoring of optical

densities in a plate reader that was performed in an anaerobic chamber. After stationary phase was reached, cultures were randomly picked for phase contrast microscopy.

Cell culture

For peripheral blood mononuclear cell (PBMC) isolation, heparinized blood of donors was prepared by Ficoll (Merck KGaA, Darmstadt, Germany) separation (141). PBMCs were resuspended at a concentration of 2×10^6 cells/ml in RPMI medium (Merck KGaA) supplemented with 10% FCS (Merck KGaA) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin (both Merck KGaA). Preparation of monocyte-derived dendritic cells was performed by harvesting PBMCs as described above and subsequent isolation of monocytes by counter flow elutriation centrifugation (142). MoDCs were generated by addition of interleukin 4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) to PBMCs in 6-well plates for 7 days as described previously (143), harvested and re-cultured in complemented RPMI medium for stimulation experiments. PBMCs as well as moDCs were grown and incubated in a humidified atmosphere of 5% carbon dioxide at 37 °C.

Cytokine measurements

Cytokine releases of stimulated PBMCs and moDCs were quantified after 20 h by using commercial ELISA Kits (Life Technologies GmbH, Darmstadt, Germany) specific for IL-1 β and TNF- α in supernatants.

Confocal laser scanning microscopy

For confocal laser scanning microscopy 10^5 moDCs were incubated at 37 °C for 2 h on VI channel μ -slides (Ibidi, Martinsried, Germany) before stimulation with 10^7 Fluorescein isothiocyanate (FITC)-labeled (1 mg/ml, Sigma-Aldrich Chemie GmbH, Hamburg, Germany) methanoarchaeal cells for 16 h. Subsequently, cells were fixed in 3% paraformaldehyde (Biolegend) and labeled with Hoechst 33342 (3 μ M, (Life Technologies GmbH)). Images were captured using Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with Leica confocal software.

Statistical analysis

The data were analyzed for statistical significance using Graph Pad Prism 7.02 software. P values ≤ 0.05 were considered to be statistically significant.

4 Results

The present study mainly aimed at evaluating the sensitivity of *M. luminyensis* to epithelial-derived antimicrobial substances as well as its recognition by humane immune cells with respect to the activation of pro-inflammatory pathways.

4.1 Growth inhibition of *M. luminyensis* by AMPs

M. luminyensis is a coccoid methanoarchaeon that is very small in size (approximately 0.85 μm in diameter) (130). Earlier studies have shown that this methanoarchaeon can grow on methanol and trimethylamines as carbon sources, but for energy metabolism urgently needs hydrogen as electron donor (130). To meet the specific growth conditions of *M. luminyensis*, the microtiter plate assay that was recently established for anaerobic microorganisms (115) was adjusted. Under optimized growth conditions at 37 °C (see Material and Methods) stationary growth phase of *M. luminyensis* was reached after 200 h in 250 μl at an optical density of approximately 0.6 (Fig. 1A, Control). Cell morphology after growth in microtiter wells was assessed by phase contrast microscopy (Fig. 1C, Control). Microscopy also served for exclusion of contaminations within the cultures.

Determination of minimal inhibitory concentrations (MIC) of selected AMPs was performed by adding various concentrations from 5 nM up to 20 μM at the beginning of growth. LL32 is the shortest active unit of human-derived cathelicidin (138), whereas NK2 is a lysin originally isolated from pig (139) and Lpep19.2-5 is a synthetically modified peptide optimized for therapeutic use (144). Not expected, complete growth inhibition of *M. luminyensis* by LL32 was obtained already at low concentrations (2 μM , Tab. 1), whereas inhibition by NK2 as well as Lpep19.2-5 was observed only at relatively high concentrations, 10 μM and 20 μM respectively. In addition, microscopic evaluation during the stationary phase of *M. luminyensis* in the presence of AMPs revealed different morphological properties. LL32 resulted in swelling of cells (up to 50% of cell size), which in case of strongly effected cells lead to release of cytoplasmic material from the cells (Fig. 1C). In contrast, micrographs of *M. luminyensis* after incubation with high concentrations of NK2 and Lpep19.2-5 revealed increased cell coagulation, however only minor effects on overall cell morphology (Fig. 1C).

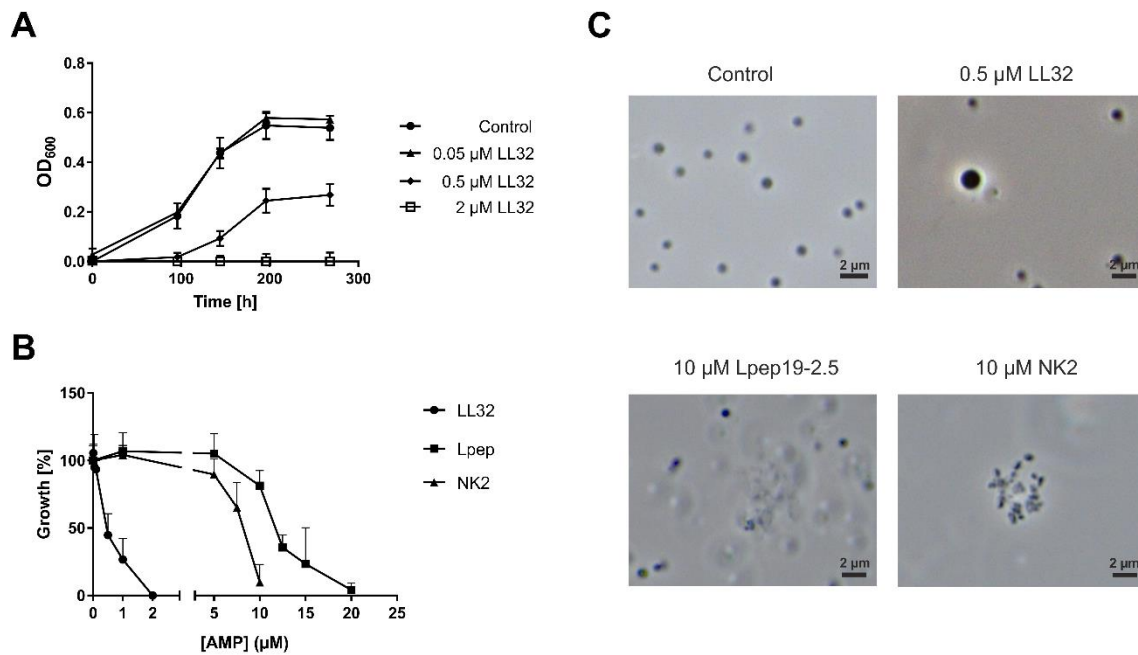


Figure 1: Growth inhibition of *M. luminyensis* by various AMPs

(A) 2×10^7 cells of *M. luminyensis* incubated with depicted concentrations of LL32 at 37 °C in 250 μl minimal medium under anaerobic conditions (see Materials and Methods). Turbidity of cultures at 600 nm (OD_{600}) was monitored over time. Error bars represent standard deviation of three biological replicates in one experimental setup. (B) 2×10^7 cells incubated with various concentrations of the peptides LL32, Lpep 19-2.5 or NK2 at 37 °C in 250 μl minimal medium. Turbidity of control cultures at 600 nm (OD_{600}) after 200 h of growth was set to 100%. Error bars represent standard deviation of three biological replicates. (C) Phase-contrast micrographs taken after 200 h of growth with the indicated concentrations of the respective added AMPs.

4.2 Immune cell activation by *M. luminyensis*

In the current study human PBMCs as well as moDCs were used to evaluate the immunogenic potential of *M. luminyensis*, as previously done for the other investigated mucosa-associated methanoarchaea. 1×10^6 or 1×10^7 cells of *M. luminyensis* were added to preparations of 1×10^5 PBMCs as well as moDCs. After 20 h of incubation, supernatants were taken and cytokine release of TNF- α and IL-1 β was measured by ELISA. *M. stadtmanae* served as positive control in these experiments due to its capability to induce very high cytokine releases in primary human immune cells (112, 114). Compared to *M. stadtmanae*, stimulation with *M. luminyensis* only led to low amounts of released pro-inflammatory cytokines TNF- α and IL-1 β in both, PBMCs (Fig. 2A) and moDCs (Fig. 2B). This effect was observed in a concentration-dependent manner as was also shown for the control stimulus *M. stadtmanae* (Fig. 2A+B, 1×10^6 and 1×10^7 cells). In addition to cytokine release, phagosomal uptake by moDCs was evaluated by using CLSM. 1×10^7 FITC-labeled methanoarchaeal cells were added to 1×10^5 moDCs seeded in VI channel μ -slides. After 16 h of incubation, DNA was stained with Hoechst 33342 and visualized using confocal microscopy. Whereas the uptake of *M. stadtmanae* occurred in

high numbers, *M. luminyensis* was only rarely found to be phagocytosed by moDCs as depicted in Fig. 2C. In addition, the typical change of morphology after activation of immune responses in moDCs such as formation of dendrites was only observed for the positive control *M. stadtmanae*.

5 Discussion

Although bacteria represent the major microbial part of the human gut ecosystem, recently available molecular tools revealed the constant presence of archaeal species in the gastrointestinal tract (145). Besides the most prevalent methanoarchaeal strains *M. stadtmanae* and *M. smithii*, three novel methanoarchaeal species belonging to the Methanomassiliicoccales were isolated and their respective genomes sequenced during the last years (130-132). Due to their metabolic capability to degrade trimethylamine (TMA) (133) a potential application of naturally occurring Methanomassiliicoccales members as “Archaeobiotics” was proposed recently (103). Since information on the general immunogenic impact of these strains is still missing, the current study mainly aimed to elucidate the molecular cross-talk between components of the human immune system and the cultivable strain *M. luminyensis*.

5.1 Susceptibility of *M. luminyensis* to AMPs

As a part of the human microflora, methanogenic archaea such as *M. stadtmanae*, *M. smithii* and *M. luminyensis* are exposed to various epithelial as well as immune defense mechanisms that prevent invasion or colonization of organs and maintain homeostasis (146). One crucial line of epithelial defense is the secretion of AMPs that represent an essential part of immunity within the human intestine (147). Since AMPs in general exhibit various structural motifs, structurally different AMPs, like a cathelicidin and a NK-lysin derivative as well as a synthetic antilipopopolysaccharide peptide (Lpep) were chosen in order to examine the general susceptibility of *M. luminyensis*. Moreover, lytic effects of these AMPs on other mucosa-associated methanoarchaeal strains were demonstrated earlier (115). We demonstrated that *M. luminyensis* is indeed susceptible to the human AMP-derivative LL32 in low μM -ranges (see Table 1). In addition, we show that this strain is more resistant to AMPs from other origins such as NK2 and Lpep19-2.5. The latter results resemble those of the other two mucosa-associated methanoarchaeal strains, *M. stadtmanae* and *M. smithii* summarized in Table 1. When compared to the sensitivity of the other two strains, *M. luminyensis* appears to be similarly resistant against the lytic effects of NK2 and Lpep19-2-5 as shown for *M. stadtmanae* (115).

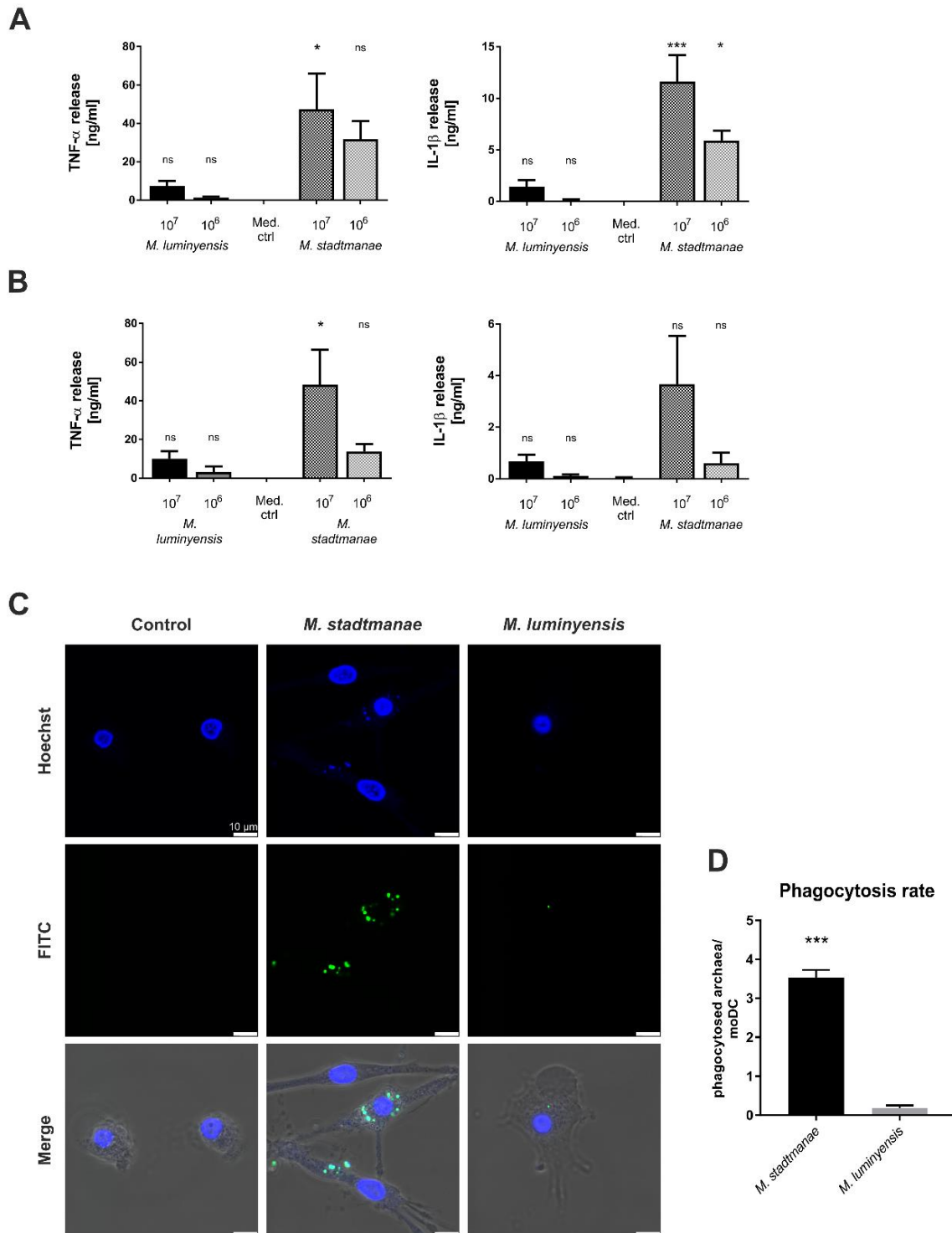


Figure 2: Immune cell activation after stimulation with *M. luminyensis*

Cytokine release after stimulation of 1×10^5 PBMCs (A) as well as 1×10^5 moDCs (B) with 1×10^6 and 1×10^7 *M. luminyensis* or *M. stadtmannae* cells for 20 h was quantified using commercial ELISA-Kits. Unstimulated cells (Med. ctrl.) were used as negative controls. Depicted data are means of at least 3 independent biological replicates with their respective standard errors of the mean (SEM). Values are compared to medium control. ns: not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (one-way ANOVA with Bonferroni post hoc test). (C) 1×10^5 moDCs were stimulated with 1×10^7 FITC-labeled methanoarchaeal cells in VI channel μ -slides for a period of 16 h. After incubation, moDCs were washed, fixed with 3% paraformaldehyde and DNA was labeled with Hoechst 33342. Images were captured using Leica SP5 confocal microscope with Leica confocal software and are representative of the respective samples (three independent biological replicates). (D) Phagocytosis rate of *M. stadtmannae* and *M. luminyensis* by moDCs was determined by counting phagocytosed archaeal cells in image sections of three biological replicates. Values are compared to each-other. *** $P \leq 0.001$ (two-tailed unpaired t-test).

However, with respect to this, LL32 showed higher activity against *M. luminyensis*. On the other hand, *M. luminyensis* is more resistant against all peptides tested than *M. smithii*. Concerning the latter it has to be mentioned that based on the small cell size of *M. luminyensis*, twice as much cells were used for inoculation (for the reason of growth monitored by turbidity). During earlier studies it was already shown that the cell membrane charge of the tested methanoarchaeal strains, as well as the cell wall structure, strongly influence the interaction and lytic activity of the used cationic charged AMPs (115, 148, 149). Although the cell wall composition of *M. luminyensis* has not yet been fully characterized, transmission electron micrographs indicated a double-layer cell wall with one thin electron-dense layer and one thick transparent layer for this strain (130). As has been shown for *M. stadtmanae* and *M. smithii*, *M. luminyensis* was found to behave like a Gram-positive in Gram staining assays suggesting that its cell wall might be also composed of pseudomurein (150), however this speculation has to be proven in future studies. With respect to the cell membrane composition, a recent publication demonstrated an unusual membrane lipid composition for *M. luminyensis* (151). Whereas the cell membranes of *M. smithii* and *M. stadtmanae* are composed of already high amounts of caldarchaeols (13 - 40%), the cell membrane of *M. luminyensis* contains approximately 58% (151). These caldarchaeols form mono-layer tetraether lipids and thus exhibit a high ordered structure of the membrane. Besides, the cell membrane of *M. luminyensis* has been shown to contain only low amounts (~ 33%) of hydrophilic head groups, such as phosphatidylglycerol, and thus probably is less negatively charged, but stabilized by high amounts of lipids with glycosidic head groups (151, 152). Thus, the differences obtained for *M. luminyensis* might be mainly due to its unique cell wall and cell membrane architecture (130, 151). With respect to the effects of AMPs on structural changes of *M. luminyensis*, phase-contrast micrographs revealed swelling of cells after incubation with LL32, but not after treatment with the other peptides. This swelling was most likely due to the osmotic stress after lysis of the cell membrane. During the treatment with NK2 and Lpep19.2-5 *M. luminyensis* cells appeared rather to coagulate – an effect that was also observed for *M. stadtmanae* during earlier studies and thus coagulation of the cells might resemble a general preventive function against the lytic effects of naturally-occurring AMPs (115). In conclusion, the results of AMP-treatment regarding growth inhibition and morphological changes of *M. luminyensis* revealed that the cell membrane as well as the cell wall appears to be well adapted to mammalian-derived antimicrobial peptides that obtain homeostasis within the intestine.

Table 1: Antimicrobial activity of various AMPs against methanogenic archaea

(MIC minimal inhibitory concentration)

Strain	MIC (μM)			Ref.
	LL32	NK2	Lpep 19-2.5	
<i>M. luminyensis</i>	2	10	> 10	This study
<i>M. stadtmanae</i>	5	10	> 10	(115)
<i>M. smithii</i>	1	3	3	(115)

5.2 Immunogenic potential of *M. luminyensis*

Previous studies on immune cell activation and responses focusing on cytokine release and CLSM demonstrated that the mucosa-associated methanoarchaeons *M. smithii* and *M. stadtmanae* led to differential immune cell activation and responses (112, 114). Based on these results, the question arose, if and how human immune cells respond to *M. luminyensis*. Interestingly, we found that stimulation of human immune cells with *M. luminyensis* only led to low amounts of the released cytokines TNF- α and IL-1 β as it has been demonstrated for the commensal methanoarchaeal strain *M. smithii*. In addition, phagocytosis by moDCs as well as the formation of typical dendrites revealing activation was only rarely seen after stimulation with *M. luminyensis*, whereas strong activation was obtained after stimulation with *M. stadtmanae* as has been observed earlier (114). These findings suggest that – as it is known for bacteria – diverse (methano)archaeal strains appear to possess structurally different molecular patterns, which serve to a greater or lesser extent as immune activators.

To date, the respective involved human receptor that recognizes archaeal molecular patterns has not been described. However, the pseudomurein-containing cell wall of both, *M. stadtmanae* and *M. smithii*, is surrounded by a second layer composed of heteropolysaccharides whose structure remains to be elucidated (153). As mentioned above, the cell wall architecture of *M. luminyensis* also appears to be composed of two layers with different structural properties (130). Since numerous membrane-bound and extracellular receptors are known to be involved in the recognition of bacteria-associated heteropolysaccharides (154), it is conceivable that the outer layer of *M. luminyensis* as well as *M. smithii* cell walls prevents these strains from phagocytosis by human immune cells. However, in order to support this hypothesis, data on the respective involved human pattern-recognition receptor as well as on the chemical properties of mucosa-associated methanoarchaeal cell wall composition are urgently needed.

Recent studies with particular focus on genomic adaptations demonstrated that *M. luminyensis* appears to be the less common gut inhabitant when compared to other members of the Methanomassiliicoccales in the human intestine (155). In detail, phylogenetic analysis of the so far in human stool detected Methanomassiliicoccales strains revealed that *M. luminyensis* is more related to soil and sediment methanogens, whereas the later described strain "Candidatus Methanomethylophilus alvus" was found to be genetically more related to gastrointestinal methanogens [11]. The additional identified strain "Candidatus Methanomassiliicoccus intestinalis" is phylogenetically also more related to soil and sediment methanogens, however it has a reduced genome and an up to 20-fold higher prevalence when compared to its closest relative *M. luminyensis*. Thus, Borrel and colleagues concluded that *M. luminyensis* might not be the best model to study the interactions of the representatives of this archaeal order with their human host (155). Indeed, it could be speculated that other members of Methanomassiliicoccales might resemble different immunogenetic properties when compared to *M. luminyensis*. However, with respect to its metabolic capabilities of TMA depletion, its general appearance in the human intestine and the herein observed overall mild human immune response particularly of this Methanomassiliicoccales strain might be proposed for a potential application as an "Archaeobiotic" (103).

6 Conclusion

Taken together, this study substantiated adaptation of the intestinal archaeal strain *M. luminyensis* to its natural habitat and underlines previous findings on diverse physiological and immunomodulatory roles of other mucosa-associated methanoarchaeal strains in the human intestine.

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8 Author Contributions

Conceptualization: Corinna Bang, Thomas Gutschmann, Holger Heine, Ruth A. Schmitz.

Data curation: Corinna Bang, Tim Vierbuchen.

Formal analysis: Corinna Bang.

Funding acquisition: Holger Heine, Ruth A. Schmitz.

Investigation: Tim Vierbuchen.

Methodology: Corinna Bang, Tim Vierbuchen.

Project administration: Holger Heine, Ruth A. Schmitz.

Resources: Thomas Gutschmann, Holger Heine, Ruth A. Schmitz.

Supervision: Holger Heine, Ruth A. Schmitz.

Visualization: Corinna Bang, Tim Vierbuchen.

Writing ± original draft: Corinna Bang, Tim Vierbuchen.

Writing ± review & editing: Thomas Gutschmann, Holger Heine, Ruth A. Schmitz.

V Publication II: The Human-Associated Archaeon *Methanosphaera Stadtmanae* is Recognized by Its RNA and Induces TLR8-Dependent NLRP3 Inflammasome Activation

Vierbuchen T, Bang C, Rosigkeit H, Schmitz RA, Heine H

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1 Abstract

The archaeon *Methanosphaera stadtmanae* is a member of the gut microbiota; yet, the molecular cross-talk between archaea and the human immune system and its potential contribution to inflammatory diseases has not been evaluated. Although archaea are as bacteria prokaryotes, they form a distinct domain having unique features such as different cell wall structures and membrane lipids. So far, no microbe-associated molecular patterns of archaea which activate innate immune receptors have been identified. By stimulating human myeloid cells with *M. stadtmanae* and purified archaeal nucleic acids, we identified both the microorganism and its RNA as potent stimuli for the innate immune system. To dissect the recognition and activation pathways induced by *M. stadtmanae*, human monocytic BLaER1 knockout cells were generated using the CRISPR/Cas9 system targeting components of TLR and inflammasome signaling. While the recognition of *M. stadtmanae* is mediated by TLR7 and TLR8, activation of the NLRP3 inflammasome depends solely on TLR8 engagement. Notably, this process resembles hallmarks of both the canonical and the recently described alternative inflammasome activation. Thus, we have demonstrated for the first time the specific recognition of and response to an archaeon by human cells at the molecular level.

2 Introduction

During the last decade, the extensive use of modern molecular approaches has revealed the existence of trillions of microorganisms in the human intestine that form a complex ecological community (126, 156). Though these microorganisms interact closely with their human hosts to provide many physiological benefits, this community has also been implicated in the development of an increasing number of diseases – in particular, those associated with chronic inflammation (157). Although much research has focused upon bacterial species (which dominate the human gut microbiota), species of the archaeal domain are also stable components

of the gut microbiota (127, 158, 159). Relatively little is known as to how these less-studied organisms influence human health, although several studies have suggested that methanogenic species of archaea, such as *Methanosphaera stadtmanae*, might be involved in the development of systemic diseases such as obesity (104, 105, 160, 161), cancer (162-164), lung hyperresponsiveness (113) and inflammatory bowel disease (IBD) (111). Understanding the molecular mechanisms through which these microorganisms induce inflammation is thus an important step in uncovering how such diseases might develop.

M. stadtmanae is currently known to be the second most abundant archaeon in the human intestine (94, 129). During the last few years, we and others have demonstrated the high immunogenic potential of *M. stadtmanae* in human peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (moDCs) (111, 112, 114) – strong innate and adaptive immune responses, including the secretion of pro-inflammatory cytokines TNF- α and IL-1 β , were detected. Although neither the pattern recognition receptors (PRRs) nor the respective microbe-associated molecular patterns (MAMPs) involved in the response to *M. stadtmanae* have yet been identified, we showed previously that phagocytosis and endosomal acidification are required for recognition of *M. stadtmanae* and resulting cytokine release by both human PBMCs and moDCs (114). IL-1 β secretion, as seen following exposure to *M. stadtmanae*, results from activation of the inflammasome, a cytosolic multiprotein complex required for processing pro-IL-1 β (165). In general, inflammasomes consist of a cytosolic sensor protein, the adaptor protein ASC and the effector caspase caspase-1. This sensor protein can be a member of the NLR family or AIM2, a cytosolic DNA sensor (44). NLRP3 is the best-studied member of the NLR family and senses cellular stress signals like potassium efflux and lysosomal leakage (46).

Endosomal recognition of microorganisms often shows an anti-viral type response that is induced through the detection of nucleic acids and includes type I interferons (IFN- α/β) (31). As a result, we hypothesized that intracellular PRRs might recognize archaeal cellular structures, in particular, their nucleic acids and potentially leading to inflammasome activation. Various cytosolic or endosomal receptors have been identified that are capable of sensing nucleic acids of bacterial or viral origin (29) and might also be involved in the detection of *M. stadtmanae* – for example, Toll-like receptors (TLRs), nucleotide-binding oligomerization domain receptors (NLRs), and retinoic acid-inducible gene 1-like receptors (RLRs). However, receptor silencing and inhibition using RNAi constructs or antagonists, are often challenging and inefficient in primary human immune cells (166), and this makes it difficult to identify which of these receptors might recognize such archaea. We therefore adopted a loss-of-function

approach using CRISPR/Cas9-mediated mutagenesis to obtain stable human knockout cells with a particular focus on intracellular receptors and signaling molecules.

Through this approach, we identified archaeal RNA as the pivotal MAMP of *M. stadtmanae* that activates TLR8 and, to a lesser extent, TLR7. Moreover, *M. stadtmanae* triggers a so far undescribed TLR8-dependent NLRP3 inflammasome activation pathway in human monocytes that shares elements of canonical and alternative inflammasome activation. Thus, our findings describe in detail the molecular mechanisms by which *M. stadtmanae* induces inflammatory responses in human monocytes, which will provide the first steps towards understanding how archaea interact with their host in the gut microbiota and elucidating the potential role of these microorganisms in inflammatory diseases such as IBD or lung hyperresponsiveness.

3 Results

3.1 *M. stadtmanae* RNA is a MAMP inducing an antiviral type I/III interferon response in human monocytes

The aim of this study was to elucidate the cellular receptors and MAMPs that are involved in sensing the methanogenic archaeon, *M. stadtmanae* (98, 111). Based on our hypothesis that intracellular PRRs and nucleic acids might be involved, recognition of *M. stadtmanae* should lead to an anti-viral type cellular immune response. Thus, we first analyzed the time-dependent mRNA expression of type I and type III IFNs in moDCs (Figure 1A) and PBMCs (Figure 1B). Expression of the genes encoding IFN- α 14, IFN- β and IFN- λ 1 (IL-29) was upregulated in PBMCs and moDCs upon exposure to *M. stadtmanae*. As NF- κ B, IRF1 and IRF5 are reported to be involved in the response against pathogens and particularly IRF5 in the induction of type I and type III IFN responses (167), we next examined the subcellular localization and expression of these transcription factors following cellular activation by *M. stadtmanae*. Using confocal microscopy, we observed that 4 h after stimulation, all three transcription factors were translocated from the cytoplasm into the nucleus (Figure 1C). These findings indicated that these transcription factors were active, and provided further evidence that exposure to *M. stadtmanae* induces an anti-viral type I/III IFN response.

As such immune responses are often induced in response to viral or bacterial nucleic acids, either detected in the cytosol or endocytic compartments, and given that the response to *M. stadtmanae* is also dependent on phagocytosis (114), we hypothesized that the

nucleic acids from *M. stadtmanae* might similarly act as MAMPs and activate immune cells. To determine the immunogenicity of archaeal nucleic acids, we transfected purified DNA or RNA into PBMCs using the liposomal transfection reagent DOTAP, and analyzed the secretion of two pro-inflammatory cytokines, TNF- α and IL-1 β . Transfection of archaeal DNA did not induce secretion of TNF- α or IL-1 β in human PBMCs (Figure S1). In contrast, RNA from *M. stadtmanae* induced TNF- α and IL-1 β release from moDCs (Figure 1D), as well as PBMCs (Figure 1E), which was absent upon addition of RNase A to the transfection mix. To analyze if the RNA-dependent activity is restricted to certain RNA species, we purified single ribosomal 5S, 16S, and 23S RNA and examined their capability to activate moDCs (Figure 1F) and PBMCs (Figure 1G). In this experimental setup, no significant changes in cytokine secretion by the different rRNA fractions were observed.

3.2 *M. stadtmanae* is recognized by innate immune cells through TLR7 and TLR8

Having demonstrated that RNA from *M. stadtmanae* is a potent activator of human immune cells, our next step was to identify the cognate PRR involved in its recognition by human monocytes. Since knockdown approaches in primary human monocytes and dendritic cells were unable to achieve a complete inhibition of gene expression (data not shown), we decided to instead use the BLaER1 cell line (116), in which genetic modifications and stable knock-out *via* CRISPR/Cas9 are possible (60). This line is a B-ALL-derived cell line that can be transdifferentiated into a monocyte-like cell type, so we first confirmed whether these BLaER1 cells, upon differentiation, respond to *M. stadtmanae* in a similar manner to PBMCs and moDCs, and thus whether they would provide a suitable model in which to unpick the molecular mechanisms involved in recognition of this archaeon. Exposure of BLaER1 monocytes to *M. stadtmanae* led to an increase in TNF- α , IL-6 and IL-1 β secretion 18 h after stimulation, in a similar manner to that observed with moDCs. Using Cytochalasin D, an inhibitor of phagocytosis, and Bafilomycin A1, an inhibitor of endosomal acidification, we confirmed that the recognition of *M. stadtmanae* in BLaER1 monocytes is also dependent on both mechanisms (Figure S2A), similarly to our findings in human moDCs (114). The phagocytosis of *M. stadtmanae* by BLaER1 monocytes was verified by confocal microscopy (Figure S2B). These data confirmed the use of BLaER1 monocytes as a suitable model system for this study.

Our data suggest that *M. stadtmanae* RNA is detected by intracellular PRRs in human monocytes. All human nucleic acid-specific TLRs (TLR3, TLR7-9) are located in the

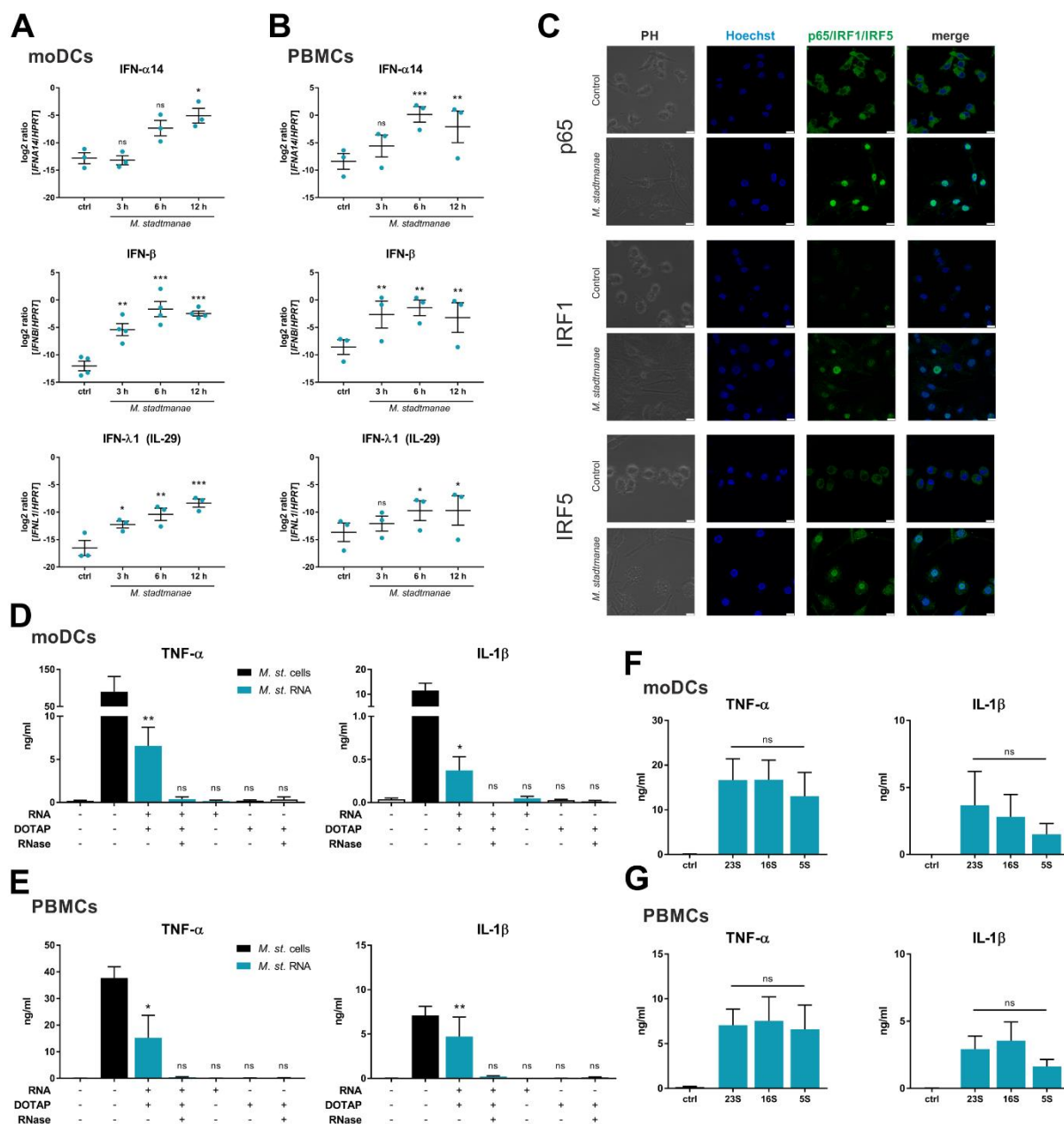


Figure 1. *M. stadtmanae* and its RNA induce an immune response with antiviral characteristics. (A, B) The expression of genes encoding for IFN- α 14, IFN- β and IFN- λ 1 in moDCs (A) and PBMCs (B) after stimulation with *M. stadtmanae* for 3, 6 and 12 h was measured by qRT-PCR. The log₂ ratios of all three gene to the reference gene HPRT are presented. The data from at least three different donors are shown as the mean \pm SEM (n = 3-4). ns: not significant, * P \leq 0.05, ** P \leq 0.01, *** P \leq 0.001 (all compared to unstimulated control group; repeated measures one-way ANOVA with Dunnett post hoc test). (C) Confocal microscopy of cellular location of NF- κ B p65, IRF1, and IRF5 (green) in moDCs after stimulation with *M. stadtmanae* for 4 h by immunolabelling. Nuclei were counterstained with Hoechst 33342 (blue). Scale bars: 10 μ M. The images shown are representative examples from one of three independent experiments (n = 3). (D, E) ELISA of TNF- α and IL-1 β in the supernatants of stimulated moDCs (D) or PBMCs (E) after 18 h. Cells were either untreated, treated with 10⁷ cells of *M. stadtmanae*, or with 5 μ g/ml of total RNA from *M. stadtmanae*. RNA was complexed to DOTAP and pre-treated for 30 min at 37 $^{\circ}$ C with RNase A where indicated. (F, G) ELISA of TNF- α and IL-1 β in the supernatants of moDCs (F) or PBMCs (G) stimulated for 18 h with 10⁷ cells of *M. stadtmanae* or 2.5 μ g/ml of purified rRNAs (complexed to DOTAP). In (D-G), the data shown are the mean \pm SEM of at least four different donors (n = 4-7). ns: not significant, * P \leq 0.05, ** P \leq 0.01 (one-way ANOVA with Tukey post hoc test; in (D, E) all compared to unstimulated control group and in (F, G) the rRNA fractions are compared to each other).

endosomal compartment, and these receptors rely on the protein UNC93B1, which is mandatory for their trafficking from the endoplasmic reticulum (ER) to the endosomes (168). To confirm our assumption that *M. stadtmanae* is recognized by endosomal TLRs, we generated UNC93B1^{-/-} cells (see Figures S3A and S3B for further information regarding all CRISP/Cas9-generated BLaER1-KO cells in this study) and determined whether these cells retained an ability to respond upon exposure to archaeal cells. UNC93B1^{-/-} BLaER1 monocytes failed to secrete any TNF- α and IL-1 β after stimulation with *M. stadtmanae* (Figure 2A), confirming the involvement of either TLR3, or TLR7-9 in the induction of cytokine responses.

We next generated KO cells lacking MyD88 or TRIF, which are the central adapter molecules used by different TLRs for signal transduction (169). Upon exposure to *M. stadtmanae*, TRIF^{-/-} cells showed a slightly decreased but still high secretion of TNF- α and no reduction in the levels of IL-1 β and RANTES (CCL5), compared to that induced in stimulated WT cells (Figure 2B). In contrast, this response was completely absent in MyD88^{-/-} cells exposed to this archaeal species (Figure 2B). Our results showed that *M. stadtmanae* is recognized through one or more MyD88-dependent TLRs by human monocytes.

As TLR9 recognizes DNA, TLR3 signals *via* TRIF, and human TLR7 and TLR8 are the only RNA-specific receptors that signal *via* MyD88 and are dependent on UNC93B1, we assumed that either or both of TLR7 and TLR8 were involved in the detection of *M. stadtmanae*. To identify which of these receptors recognizes *M. stadtmanae*, we generated single-gene knockout cell lines for each these receptors, as well as a double knockout (TLR7^{-/-} \times TLR8^{-/-}) cell lines. Using these cells, we demonstrated that the secretion of TNF- α in response to *M. stadtmanae* can be induced *via* TLR7 as well as TLR8, with reduced levels of cytokine secretion detected compared to that produced by stimulated WT cells in both knockout cell lines. TLR8^{-/-} cells did, however, show a greater decrease in TNF- α secretion compared to that of the TLR7^{-/-} cells (Figure 2C). Unexpectedly, the secretion of IL-1 β was entirely dependent on TLR8, as no secretion of this cytokine was detected in TLR8^{-/-} cells after stimulation with *M. stadtmanae*. The TLR7/8 agonist R848, the TLR7 agonist CL264, and the TLR8 agonist TL8-506 were used as controls to confirm the specificity of the clones used in this study (Figure 2C). Additionally, we transfected purified RNA from *M. stadtmanae* into TLR7^{-/-}, TLR8^{-/-}, and TLR7^{-/-} \times TLR8^{-/-} BLaER1 monocytes using DOTAP and measured the secretion of TNF- α and IL-1 β after 18 h. Similar to whole archaea, RNA from *M. stadtmanae* is recognized by TLR7 and TLR8, but the knockout of TLR8 revealed that the latter receptor has a stronger effect (Figure 2D).

Recently, TREML4 was shown to be involved in the recruitment of MyD88 to TLR7, thereby amplifying its signaling(170). However, TREML4^{-/-} BLaER1 monocytes showed no alterations in secretion of IL-6 or TNF- α after stimulation with *M. stadtmanae* or different TLR7/8 agonists (Figure S4) indicating that this protein, at least in human monocytes, is not essential for TLR7/8 activation. To further characterize the individual roles of TLR7 and TLR8 in the response to *M. stadtmanae*, we used qRT-PCR to analyze the expression of type I and type III IFN mRNAs in the different TLR-KO cell lines after 8 h stimulation with *M. stadtmanae*. In TLR7^{-/-} cells, only IFN- β showed a slight reduction in expression compared to the levels in WT stimulated monocytes (Figure 2E), whereas expression of genes encoding all three IFNs, IFN- α 14, IFN- β and IFN- λ 1, were significantly reduced from WT levels in TLR8^{-/-} cells, and an even stronger effect was detected in double-KO cells (Figure 2E). These data demonstrated that not only pro-inflammatory cytokines such as TNF- α and IL-1 β but also type I/III IFNs are induced by activation of TLR8 (and to a minor degree TLR7) with *M. stadtmanae*.

As mice and humans differ in their repertoire of RNA-specific TLRs (171), we also analyzed the cytokine response of bone marrow-derived dendritic cells (BMDCs) from different knockout mice after stimulation with *M. stadtmanae* (Figure S5). Using these cells, we demonstrated that in mice, the recognition of this archaeon is completely dependent on mTLR7, whereas the absence of mTLR8 or mTLR13 had no effect on the secretion of IL-6.

3.3 *M. stadtmanae* induces the secretion of IL-1 β via activation of the NLRP3 inflammasome

Maturation and release of IL-1 β along with that of the pro-inflammatory cytokine IL-18, occurs upon activation of caspase-1, following activation of the inflammasome and recruitment of apoptosis associated speck-like protein containing a CARD (ASC) in response to PRR signaling. Inflammasomes are cytosolic multiprotein complexes that differ in their utilization of receptor proteins such as NLRP3 or AIM2 (165). To clarify the process of IL-1 β release and inflammasome activation induced by *M. stadtmanae*, we first analyzed the release of active caspase-1 p20 and mature IL-1 β p17 in the supernatant of human monocytes using immunoblotting. After stimulation for 18 h with *M. stadtmanae*, caspase-1 p20 and IL-1 β p17 were detected in the supernatant of both primary human monocytes (Figure 3A) and BLaER1 monocytes (Figure 3B), indicative of inflammasome activation in both these cell types. To

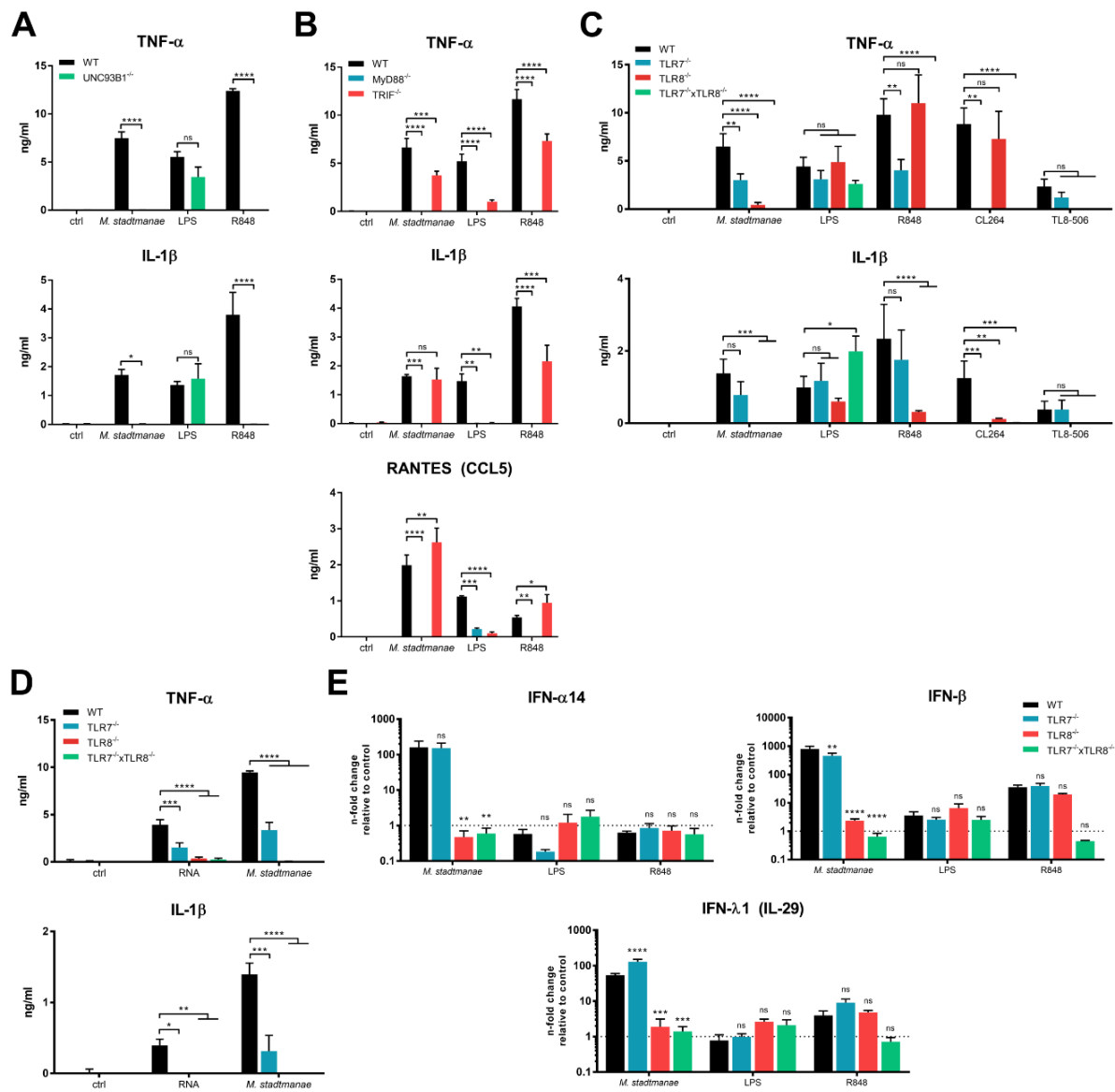


Figure 2. *M. stadtmanae* induces cytokine responses in BLaER1 monocytes through TLR7 and TLR8.

(A) Secretion of TNF- α , IL-1 β , and RANTES (CCL5) in the supernatants of BLaER1 WT and UNC93B1^{-/-} monocytes as measured by ELISA. Cells were stimulated with 10^7 cells of *M. stadtmanae*, 50 ng/ml LPS or 5 μ g/ml R848 for 18 h. (B) Secretion of TNF- α and IL-1 β in the supernatants of BLaER1 WT and MyD88^{-/-} or TRIF^{-/-} monocytes stimulated as in (A). (C) TNF- α and IL-1 β secretion in the supernatants of BLaER1 WT, TLR7^{-/-}, TLR8^{-/-} or TLR7^{-/-}xTLR8^{-/-} monocytes stimulated for 18 h with either 10^7 cells of *M. stadtmanae*, 50 ng/ml LPS, or 5 μ g/ml of either R848, CL264, and TL8-506. (D) Secretion of TNF- α and IL-1 β secretion in the supernatants of BLaER1 WT, TLR7^{-/-}, TLR8^{-/-} or TLR7^{-/-}xTLR8^{-/-} monocytes stimulated for 18 h with 10^7 cells of *M. stadtmanae* or 5 μ g/ml RNA purified from *M. stadtmanae* and complexed to DOTAP. In (A-D), ns: not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ (repeated measures two-way ANOVA with Bonferroni post hoc test). The data from one representative clone of two are shown as the mean \pm SEM of at least three independent experiments ($n = 3-4$). (E) The expression of genes encoding IFN- α 14, IFN- β , and IFN- λ 1 in BLaER1 WT and TLR7^{-/-}, TLR8^{-/-} or TLR7^{-/-}xTLR8^{-/-} monocytes after stimulation with *M. stadtmanae*, 50 ng/ml LPS, or 5 μ g/ml R848 for 8 h was measured by qRT-PCR. The expression ratios of all three IFN genes to the reference gene *HPRT* are shown as n-fold induction relative to the unstimulated control. ns: not significant, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ (repeated measures two-way ANOVA with Bonferroni post hoc test in (A) and Dunnett post hoc test in (B-E)). The data are shown as the mean \pm SEM of three independent experiments ($n = 3$).

identify the type of inflammasome that is activated by *M. stadtmanae*, we first determined the effects of specific inhibitors for caspase-1 (Ac-YVAD-cmk) and NLRP3 (MCC950) (172) upon the induction of pro-inflammatory cytokine responses. Both inhibitors almost completely abolished the release of IL-1 β observed in untreated primary human monocytes (Figure 3C) and BLaER1 monocytes (Figure 3D) after stimulation with *M. stadtmanae*, though release of IL-6 remained unaffected by either inhibitor confirming their specificity. These findings demonstrated the dependency of *M. stadtmanae*-induced inflammasome activation on caspase-1 and NLRP3. An equivalent observation was made for primary moDCs and PBMCs (Figures S6A and S6B).

To confirm activation of the NLRP3 inflammasome by *M. stadtmanae*, we generated additional BLaER1 cell lines in which NLRP3, ASC, or caspase-1 were knocked out, and stimulated these cells with *M. stadtmanae* (Figure 3E). The results were consistent with those obtained using inhibitors, showing that all three molecules were required for the secretion of IL-1 β . However, NLRP3^{-/-} and caspase-1^{-/-}, but not ASC^{-/-}, cells also showed a decrease in IL-6 secretion after stimulation with *M. stadtmanae*. In addition, we tested the involvement of NEK7, a molecule that was recently shown to be required for NLRP3 inflammasome activation (50). BLaER1 NEK7^{-/-} cells also displayed a significant decrease in IL-1 β secretion (Figure 3E), but the effect was not as strong as that in the NLRP3, ASC, and caspase-1 knockouts.

Pyroptosis, an inflammatory form of cell death, has been reported to follow NLRP3 inflammasome activation, and to be involved in the release of pro-inflammatory cytokines (165). To test whether pyroptosis was a consequence of *M. stadtmanae*-induced NLRP3 activation, cell death was measured by detecting the release of lactate dehydrogenase (LDH) from stimulated and unstimulated BLaER1 WT and KO monocytes. BLaER1 WT cells showed a release of approximately 20% of the amount of LDH released by the cell lysis control after stimulation with *M. stadtmanae*. By contrast, LDH release, and thus cell death, was undetectable in cells lacking the inflammasome components NLRP3, ASC, caspase-1 or NEK7 (Figure 3F), and was also absent from TLR8^{-/-} and TLR7^{-/-}×TLR8^{-/-} cells (Figure 3G) indicating that activation of TLR8 by *M. stadtmanae* hence leads to induction of pyroptosis.

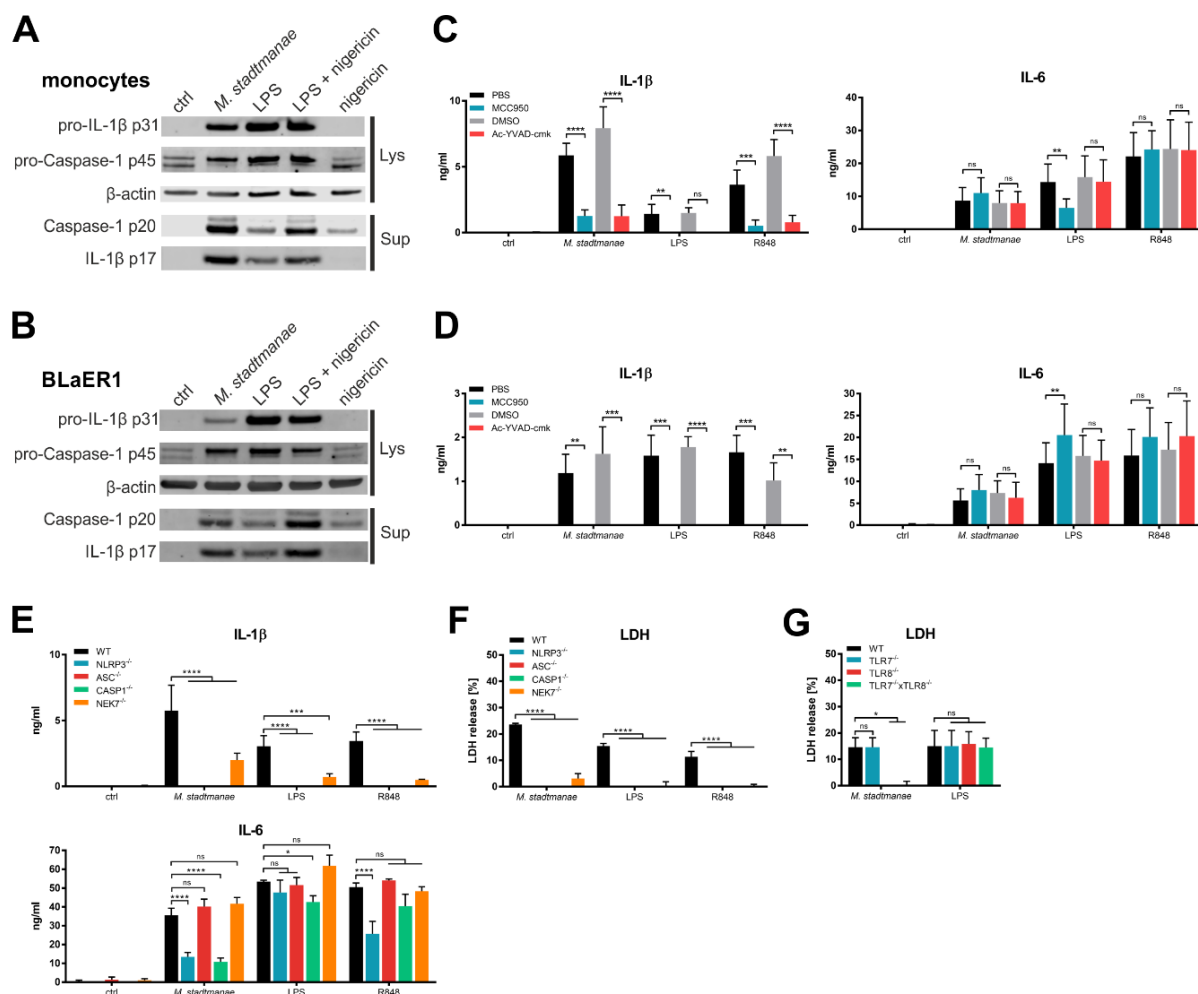


Figure 3. *M. stadtmanae* activates the NLRP3 inflammasome in primary and BLaER1 monocytes.

(A, B) Immunoblotting of cell lysates and supernatants of primary monocytes (A) and BLaER1 WT monocytes (B) showing the activation status of caspase-1 (p20) and IL-1β (p17). Additionally, the pro-forms of both proteins, namely caspase-1 p45 and IL-1β p31 were analyzed in the cell lysate. Analysis of β-actin was used as loading control. Cells were stimulated with *M. stadtmanae* (100:1) or 50 ng/ml LPS for 18 h. 13.4 μM nigericin was added where indicated for the last 2 h. Blots are shown either from one representative donor of three in (A) or as one representative of three independent experiments in (B) (n = 3). (C, D) The secretion of IL-6 and IL-1β in the supernatants of stimulated primary monocytes (C) or BLaER1 WT monocytes (D) as measured using ELISA. Inhibitors of NLRP3 and caspase-1 (MCC950 and Ac-YVAD-cmk, respectively) and control substances (medium and DMSO, respectively) were added 1 h before stimuli were added. Cells were stimulated with 10⁷ cells of *M. stadtmanae*, 50 ng/ml LPS, or 5 μg/ml R848 for 18 h. ns: not significant, * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001 (repeated measures two-way ANOVA with Tukey post hoc test). The data from at least three different donors in (C) (n = 3-4) or three independent experiments in (D) (n = 3) are shown as the mean ± SEM. (E) The secretion of IL-6 and IL-1β in the supernatants of stimulated primary monocytes or BLaER1 WT and KO monocytes (as indicated in the figure) was measured using ELISA. Cells were stimulated with 10⁷ cells of *M. stadtmanae*, 50 ng/ml LPS, or 5 μg/ml R848 for 18 h. ns: not significant, * P≤0.05, *** P≤0.001, **** P≤0.0001 (repeated measures two-way ANOVA with Dunnett post hoc test). The data from one representative clone of two (except for NLRP3) of three independent experiments (n = 3) are shown as the mean ± SEM. (F, G) Detection of LDH in the supernatant of stimulated BLaER1 WT and KO monocytes (as indicated in the figure). Cells were stimulated with 10⁷ cells of *M. stadtmanae*, 50 ng/ml LPS, or 5 μg/ml R848. ns: not significant, * P≤0.05, **** P≤0.0001 (repeated measures two-way ANOVA with Dunnett post hoc test). The data from one representative clone of two (except for NLRP3) of three independent experiments (n = 3) are shown as the mean ± SEM.

3.4 *M. stadtmanae* induces the secretion of IL-1 β without the formation of ASC specks in BLaER1 monocytes

Several pathways for inflammasome activation have previously been described, including canonical, non-canonical, and alternative activation. They differ in terms of the stimuli responsible, and the formation of ASC specks: in particular, alternative activation in response to LPS stimulation and TLR4 signaling was recently shown to be independent of ASC speck formation (60) otherwise characteristic of both canonical and non-canonical inflammasome activation. To distinguish which of these activation pathways was induced upon stimulation of human monocytes by *M. stadtmanae*, we examined whether ASC specks could be observed in these cells. As expected, ASC speck formation was detectable in control BLaER1 cells treated with LPS and nigericin for 4 h to induce canonical inflammasome activation (Figure 4A). Conversely, in cells treated with LPS alone, which induces the alternative activation pathway, ASC specks were absent both 4 and 18 h after treatment (Figure 4A). Although we were able to show TLR8-dependent induction of pyroptosis in BLaER1 monocytes, usually a hallmark of canonical activation, cells stimulated with *M. stadtmanae* showed no ASC speck formation, a phenotype more comparable to that of cells stimulated with LPS alone and alternative activation. Secretion of IL-1 β in BLaER1 monocytes after 4 h with *M. stadtmanae* or LPS alone was relatively weak (Figure 4B), however, by 18 h, the level of IL-1 β secreted by these cells was much higher and comparable to that induced by canonical inflammasome activation, despite the lack of formation of ASC specks. Taken together, these results demonstrated that *M. stadtmanae* triggers a TLR8-dependent NLRP3 inflammasome activation pathway in human monocytes that shares characteristics of canonical as well as alternative inflammasome activation.

4 Discussion

Only recently have researchers begun to appreciate the full diversity of our microbiome and to include genetic data from other domains (94). Due to methodological challenges (101), the function and influence of archaea on the human immune system have yet to be determined and, the exact mechanism by which *M. stadtmanae* induces inflammation remains unknown. Though much is known of the molecular interactions involved in bacterial-induced inflammation, these findings are not immediately applicable to the detection of archaea by immune cells: bacteria and archaea are genetically and structurally different. In particular, the

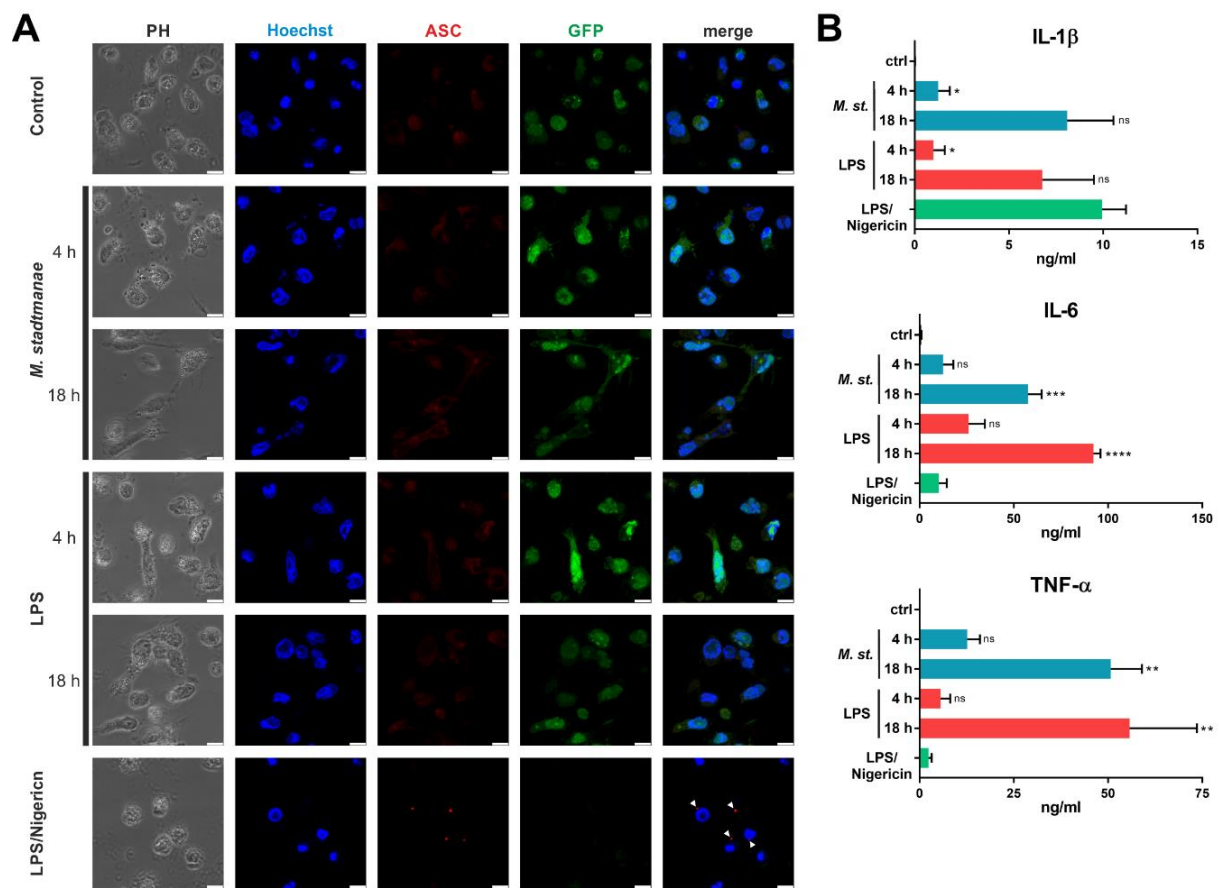


Figure 4. *M. stadtmanae* does not induce formation of ASC specks in BLaER1 monocytes.

(A) Confocal microscopy was used to visualize fixed BLaER1 monocytes stained with ASC-specific antibodies (red). Cells were stimulated for 4 or 18 h with 10^7 cells of *M. stadtmanae* or 100 ng/ml LPS. LPS/nigericin stimulation was performed for 4 h, with 13.4 μ M nigericin being added 3 h after initial LPS stimulation. After fixation, cells were stained with anti-ASC antibodies. DNA was stained with Hoechst 33342 (blue), and cytoplasmic GFP expression is shown in green. White arrowheads depict ASC specks. Scale bars: 10 μ M. The data are shown from one representative of three independent experiments ($n = 3$). (B) The secretion of IL-1 β , IL-6 and TNF- α from cells shown in (A) was measured using ELISA. Values are compared to those obtained using LPS/nigericin stimulation. ns: not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ (one-way ANOVA with Dunnett post hoc test). The data are from three independent experiments ($n = 3$) and shown as the mean \pm SEM.

various archaeal cell wall structures and membrane lipids are very different from those of bacteria (*e.g.*, the methanoarchaea *M. stadtmanae* and *Methanobrevibacter smithii* do not engage NOD2, TLR2, or TLR4 (114)), and their translation and transcription machineries resemble eukaryotic processes (98). Here, we describe for the first time the key archaeal MAMP, in the form of *M. stadtmanae* RNA which is recognized by human immune cells through TLR8 and, to a lesser extent, through TLR7.

We consider activation of human myeloid cells by *M. stadtmanae* to be reminiscent of antiviral characteristics, as it induces the gene expression of type I and type III IFNs and the translocation of IRF1 and IRF5 into the nucleus. The cytokine response is completely dependent on MyD88 and UNC93B1 but not on TRIF - these findings indicate the involvement of nucleic

acids, specifically ssRNA, in the recognition process. We clearly demonstrate that archaeal RNA, but not DNA, can induce a pro-inflammatory response. Recently, there has been great interest in bacterial RNA as a potent MAMP sensed by TLR7 (173, 174) and TLR8 (175, 176) in human immune cells. The activation of these receptors through bacterial RNA leads to secretion of pro-inflammatory cytokines and type I IFNs, such as IFN- α and IFN- β (31). Archaeal RNA activates immune cells in a similar manner and irrespective of the rRNA species. This observation is consistent with the recently unraveled crystal structures and binding mechanisms of TLR7 (177) and TLR8 (178), as both receptors bind to the degradation products of RNA. In contrast to murine TLR13, which binds to a specific sequence in bacterial 23S rRNA (179), the activation of TLR7 and TLR8 is rather sequence-independent. Both receptors concurrently recognize single nucleotides and short oligonucleotides (2-3mers), which should be present in all fractions of RNA from *M. stadtmanae*. This mechanism appears to be promiscuous and capable of recognizing a wide range of organisms, thus, it is not restricted to bacteria but likely involved in the recognition of viruses, and presumably other archaeal species besides *M. stadtmanae*. In BLaER1 monocytes, the recognition of *M. stadtmanae* is mediated primarily by TLR8 and to a lesser extent by TLR7. Notably, the induction of IFN- α 14 and IFN- λ 1 as well as the secretion of IL-1 β is fully dependent on TLR8. This difference in receptor involvement might be due to the unequal expression of the two receptors, as TLR8 rather than TLR7 seems to be the predominant receptor in human monocytes (180). Additionally, TLR8 and TLR9 seem to outperform TLR7 in terms of UNC93B1-mediated trafficking to the endosomes (181), which may explain the stronger influence of TLR8 on *M. stadtmanae*-induced secretion of TNF- α and IL-6.

We demonstrated that in addition to inducing pro-inflammatory cytokines and IFNs, *M. stadtmanae* also potently activates the NLRP3 inflammasome. As secretion of IL-1 β is completely lost in NLRP3^{-/-} cells, it clearly indicates that the NLRP3 inflammasome is the essential executor activated by *M. stadtmanae*. The contribution of other inflammasome complexes (*e.g.*, in priming steps), however, cannot be completely ruled out. With the use of inhibitors and BLaER1 KO monocytes, we revealed that inflammasome activation is TLR8-dependent and RNA-mediated. This process is most likely related to the activation process induced by bacterial RNA, which was also shown to trigger the NLRP3 inflammasome, leading to caspase-1 activation and the conversion of pro-IL-1 β into its active form (182, 183). However, the process of *M. stadtmanae*-induced inflammasome activation differs from classical activation pathways, namely, canonical and non-canonical activation, which both lead to the formation of ASC specks and cell death after short stimulation times (184). In the case

of *M. stadtmanae*, inflammasome activation is delayed compared to classical NLRP3 activation (e.g., by LPS/nigericin stimulation). Furthermore, *M. stadtmanae* did not induce ASC speck formation in BLaER1 monocytes, although IL-1 β was clearly present in the supernatant after 18 h. This activation resembles the recently described process of alternative inflammasome activation (60). However, in contrast to this TLR4- and TRIF-dependent alternative activation, *M. stadtmanae* induces TLR8-dependent inflammasome activation and TLR8- and NLRP3-dependent cell death, known as pyroptosis, independently of TRIF. Furthermore, we detected LDH release after stimulation with LPS alone. One possible explanation is that this mechanism is caspase-4-mediated, as Gaidt et al. used caspase-4^{-/-} BLaER1 monocytes as control cells.

Given that TLR8, but not TLR7, is involved in ROS production by the NADPH oxidase NOX2 complex in human neutrophils (185) and a relationship between the NLRP3 inflammasome and NOX2 was previously demonstrated in relation to phagosomal acidification (186), we hypothesize that *M. stadtmanae*-induced TLR8-dependent NLRP3 activation is associated with the production of reactive oxygen species (ROS) (187). ROS production might provide a necessary signal for inflammasome activation, especially as these reactive intermediates can serve as a NEK7-mediated activation signal of the NLRP3 inflammasome (188). Thus, ROS may provide a feasible mechanism promoting *M. stadtmanae*-induced NLRP3 activation. Using BLaER1 NEK7^{-/-} monocytes, we were able to confirm the involvement of NEK7 in *M. stadtmanae*-induced cytokine production, though this phenotypic loss was not as strong as that observed in cells lacking ASC, caspase-1, NLRP3 or TLR8. As a result, the detailed mechanism of how NEK and ROS influence NLRP3 activation will require further clarification.

The findings regarding the interaction between the methanogenic archaeon *M. stadtmanae* and human immune cells indicate that archaea represent an important part of our microbiota. There is a great need for further investigation of human-associated archaea, if one considers that many inflammatory diseases are associated with both the inflammasome (189, 190) and the composition of the microbiota (157). As we identified *M. stadtmanae* as a strong activator of pro-inflammatory immune responses *in vitro*, it is conceivable that it could also contribute to an over-activation of the inflammasome *in vivo*. Therefore, future studies should adapt their experimental design to consider these important components of our microbiota and better understand the involvement of archaea in the development of inflammatory diseases such as IBD and lung hypersensitivity, and thus shape possible treatment approaches.

5 Materials and Methods

Ethics approval statement

Approval for these studies was obtained from the Institutional Ethics Committee at the University of Lübeck (Lübeck, Germany; Az. 12-202A) according to the Declaration of Helsinki. All donors gave written informed consent.

M. stadtmanae growth and media

M. stadtmanae (DSM 3091) was grown as previously described (115). Immune cell stimulation experiments were carried out with exponentially growing *M. stadtmanae* cells that were centrifuged at $3200 \times g$ for 30 min, washed, and suspended in aerobic 50 mM Tris-HCl (pH 7.0).

Isolation of archaeal RNA and DNA

For the isolation of nucleic acids, *M. stadtmanae* cells were grown as previously described (115), harvested at 4 °C ($3200 \times g$ for 30 min) and lysed in liquid nitrogen using a Mikro-Dismembrator S laboratory ball mill (Sartorius) for 3 min at 1600 bpm. RNA was isolated by TRIzol extraction followed by DNase I treatment. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. Concentration and purity were determined using the DS-11 spectrophotometer (DeNovix). RNA with an A_{260}/A_{280} ratio ≥ 2.0 was considered as pure RNA; DNA with an A_{260}/A_{280} ratio of approximately 1.8 was considered as pure DNA.

The separation of ribosomal RNA fractions (5S, 16S and 23S rRNA) was carried out by excision of the respective fractions after agarose gel electrophoresis and subsequent purification using NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's protocol (Macherey-Nagel).

Cell culture

Peripheral blood mononuclear cells (PBMCs) were prepared from the heparinized blood of donors by gradient centrifugation (141) using Biocoll (Merck). Subsequently, monocytes were isolated by counter-flow elutriation centrifugation (142). Monocyte-derived dendritic cells (moDCs) were then generated from monocytes as previously described (143). All cells were cultured in RPMI 1640 medium with stable glutamine supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin (P/S; all from Merck)) from now

on referred to as complete medium. Cells were grown and incubated in a humidified atmosphere of 5% carbon dioxide at 37 °C.

BLaER1 cells were a gift from Thomas Graf (Center for Genomic Regulation, Barcelona, Spain). The cells were cultured under the same conditions as the moDCs and maintained at a cell density between $1 \times 10^5 - 2 \times 10^6$ cells/ml. Transdifferentiation was induced by cultivating 3×10^5 cells/ml for 6-7 days in complete medium with 10 ng/ml IL-3, 10 ng/ml M-CSF (both Peprotech), and 100 nM β -estradiol (Sigma), as previously described (116). For stimulation, cells were re-plated in complete medium without IL-3, M-CSF, or β -estradiol. The process of transdifferentiation was regularly checked using standard flow cytometry analysis with CD19-BV421, CD11b-APC and CD14-PE antibodies (all from BioLegend) on a MACSQuant Analyzer 10 (Miltenyi Biotec).

Cell stimulation

PBMC and moDC were stimulated for 18 h at a cell density of 1×10^5 cells/well in a 96-well flat-bottom plate with a total volume of 200 μ l. Monocytes and BLaER1 cells were stimulated at a cell density of 0.25×10^5 and 0.5×10^5 cells/well, respectively. Whole cells of *M. stadtmanae* were applied at 10^7 cells per well, unless otherwise indicated. LPS (from *E. coli* O111, a gift from Otto Holst, Research Center Borstel) was added at a final concentration of 50 ng/ml. R848, CL264, and TL8-506 (all from InvivoGen) were used at 5 μ g/ml. Archaeal RNA was complexed with the liposomal transfection reagent DOTAP (Carl Roth) prior to stimulation at a ratio of 6.5 μ l DOTAP per 1 μ g RNA in 50 μ l of pure RPMI 1640. Total RNA was added at a concentration of 5 μ g/ml, and purified rRNA was added at 2.5 μ g/ml. Three hours after the addition of RNA, 50 μ l of complete RPMI 1640 was added to the cells. When indicated, RNA was digested using RNase A, DNase I or Proteinase K (Thermo Fisher) prior to complexing with DOTAP as recommended by the manufacturer. For the inhibition experiments, 7.5 μ M MCC950, 50 μ M Ac-YVAD-cmk (both Sigma-Aldrich), 2 μ M Cytochalasin D (Sigma), or 10 nM Bafilomycin A1 (Merck) was added to cells 1 h prior to stimulation.

Generation of stable knockout cell lines using CRISPR/Cas9

Gene-specific gRNA sequences were designed using Benchling online software (<https://benchling.com>), in which off-target scores were kept as low as possible. The gRNA sequences that were used in this study are listed in Supplementary Table 1. The BbsI restriction site was used to clone ODNs (Thermo Fisher) encoding gRNA sequences into pU6-(BbsI)-

CBh-Cas9-T2A-BFP (a kind gift from Ralf Kuehn (125); Addgene plasmid #64323) as previously described (123). Using the Human B Cell Nucleofector Kit and Nucleofector I device (program U-15; both Lonza), 1×10^6 cells were transfected with 2 μg plasmid DNA. Forty-eight hours after transfection, GFP⁺/BFP⁺ cells were sorted into 96-well plates (1 cell/well) by the Fluorescence Cytometry core unit at the Research Center Borstel using a FACSAria IIu (BD Biosciences). Two to three weeks after sorting, DNA from the clones was isolated using QuickExtract DNA Extraction Solution (Epicenter). Specific gene fragments were amplified using standard PCR methods and sequenced at Eurofins Genomics. The occurrence of InDel mutations was analyzed using the Tracking of Indels by Decomposition (TIDE) online software (191) (<https://tide.nki.nl/>). Only clones with frameshift mutations on all target alleles were used for further experiments. Clones were characterized using Western blot and/or ELISA depending on the availability of specific antibodies. The results of the immunoblot and TIDE analysis are shown in Figure S3A and S3B, respectively.

Cytokine Measurements and LDH release assay

The concentrations of released cytokines in the supernatants were determined after 16 h using commercial ELISA kits specific for hIL-1 β , hIL-6, mIL-6, hTNF- α (Thermo Fisher), and hRANTES (CCL5) (R&D Systems). LDH release was measured using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher) according to the manufacturer's instructions. LDH release (%) was calculated as follows: (stimulated sample – unstimulated sample) / (lysis control – unstimulated sample) \times 100.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

For expression analysis *via* qRT-PCR, 0.5×10^6 or 1×10^6 PBMCs, moDCs, or BLaER1 monocytes were stimulated for the indicated time periods (3, 6 and 12 h for PBMCs and moDCs; 8 h for BLaER1 monocytes) with *M. stadtmanae* cells (100:1) in a total volume of 0.5 or 1 ml at 37 °C. After stimulation, cells were harvested, and RNA was isolated using the NucleoSpin RNA Isolation Kit (Macherey-Nagel) according to the manufacturer's instructions. The concentration and purity of RNA were analyzed using a DS-11 spectrophotometer (DeNovix). Reverse Transcription of RNA into cDNA was achieved using SuperScript III, RNase Out, dNTPs, and oligo dT primer (all from Thermo Fisher) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green Master Mix on a LightCycler 480 II (both Roche). All primers were purchased from Thermo Fisher, and their sequences are listed in Supplementary Table 2. The following cycling conditions were

applied: 95 °C for 10 min, (95 °C for 10 s; 63 -58 °C for 10 s with -0.5 °C/cycle and 72 °C for 6 s) × 45 cycles. The data were analyzed using LightCycler 480 software (v. 1.5.1) by calculating the ratio of the target gene expression to expression of the reference gene *HPRT*. Data were presented either as log₂ ratio or as n-fold induction compared to unstimulated cells.

Immunoblotting

Human monocytes or BLaER1 cells were stimulated with *M. stadtmanae* at a ratio of 1:100 in RPMI 1640 with 1% P/S (both Merck) for 18 h. LPS at 50 ng/ml was used as a control, and 13.4 μM nigericin (Invivogen) was added for the last 2 h. Supernatants were precipitated with trichloroacetic acid (1:10, Sigma), and the pellet was washed three times with acetone and suspended in LDS buffer (Thermo Fisher) with 5% β-mercaptoethanol (Carl Roth). Cell lysates were generated by direct lysis of the cells in LDS buffer with 5% beta-mercaptoethanol. To analyze KO clones, cell lysates from BLaER1 KO monocytes were generated in the same manner. Gel electrophoresis was performed using NuPage Novex 12% Bis-Tris protein gels (Thermo Fisher) for 45 min at 200 V, and proteins were subsequently blotted onto PVDF membranes (Carl Roth) for 1 h at 30 V. Membranes were blocked using Roti-Block (Carl Roth), and primary antibodies were added overnight at 4 °C. Caspase-1 p20 (Bally-1, 1:1000, Adipogen, used for detection of p45 and p20), IL-1β (D3U3E, 1:1000, used for detection of p31 and p17), α-actin (8H10D10, 1:5000, both Cell Signaling), ASC (B-3, 1:1000, Santa Cruz Biotechnology), NLRP3 (Cryo-2, 1:1000, Adipogen), and NEK7 (EPR4900, 1:5000, Abcam) were used as primary antibodies. Goat-anti-mouse-Alexa 680 (Thermo Fisher) and goat-anti-rabbit-IRDye 800CW (LI-COR Biosciences) were used as secondary antibodies at 1:10000 dilution. The blots were visualized with the LI-COR Odyssey system (LI-COR Biosciences).

Confocal laser scanning microscopy

For confocal laser scanning microscopy, 10⁵ moDC were incubated at 37 °C for 2 h on 6 channel μ-Slide (Ibidi) and subsequently stimulated for 4 h with 10⁷ *M. stadtmanae* cells. After fixation in 3% paraformaldehyde, primary antibodies were added at 1:100 in PBS with 3% BSA and 0.1% saponin and incubated overnight at 4 °C. Cells were incubated with anti-NF-κB p65 (1:100, F-6), anti-IRF1 (1:100, B-1), or anti-IRF5 (1:100, H-56, all from Santa Cruz Biotechnology) overnight at 4 °C, followed by staining with an Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (1:300, Invitrogen). The nuclei were counterstained using Hoechst 33342 dye (1:3000, Life Technologies). For detection of ASC specks, BLaER1 cells were stimulated for 4 and 18 h, and staining was performed as

previously described. ASC (1:100, B-3, Santa Cruz Biotechnology) was used as the primary antibody, and Alexa Fluor 546-conjugated goat anti-mouse IgG (H+L) was used as the secondary antibody (1:300, Invitrogen). Images were captured using the TCS SP5 confocal microscope and LAS AF software (both from Leica).

Statistical analysis

The data were analyzed for statistical significance using a one-way or two-way ANOVA with a Bonferroni, Dunnett or Tukey post hoc test using Graph Pad Prism 7.02 software. *P* values ≤ 0.05 were considered to be statistically significant.

6 Author Contributions

TV, CB, RAS, HH designed the research; TV, CB, HR performed the research; TV, CB, HR, HH analyzed the data; and TV, CB, RAS, HH wrote the paper.

7 Acknowledgements

We gratefully acknowledge the fluorescence cytometry core unit at the Research Center Borstel and Marten Holtermann, Ina Goroncy, Katrin Böhnstedt and Cornelia Goldberg for excellent technical assistance. We greatly thank Thomas Graf (Barcelona, Spain) for providing the BLaER1 WT cells, Douglas Golenbock (Worcester MA, USA) for providing the murine TLR7/TLR8-KO bone marrow cells, and Carsten Kirschning (Essen, Germany) for providing the murine TLR13-KO bone marrow cells.

8 Funding

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9 Supplementary Material

9.1 Supplementary Figures

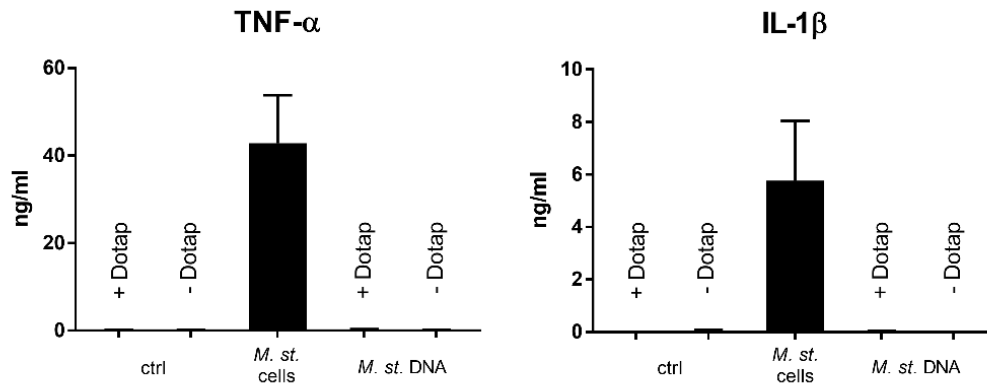


Figure S1. *M. stadtmanae* DNA does not induce an immune response in human PBMCs.

Quantification of TNF- α (left) and IL-1 β (right) by ELISA of the supernatant of PBMCs either left unstimulated, stimulated by *M. stadtmanae* cells, or by purified DNA. DNA was complexed to the liposomal transfection reagent DOTAP and added to human PBMCs for 18 h. The data are from three different donors ($n = 3$) and shown as the mean \pm SEM.

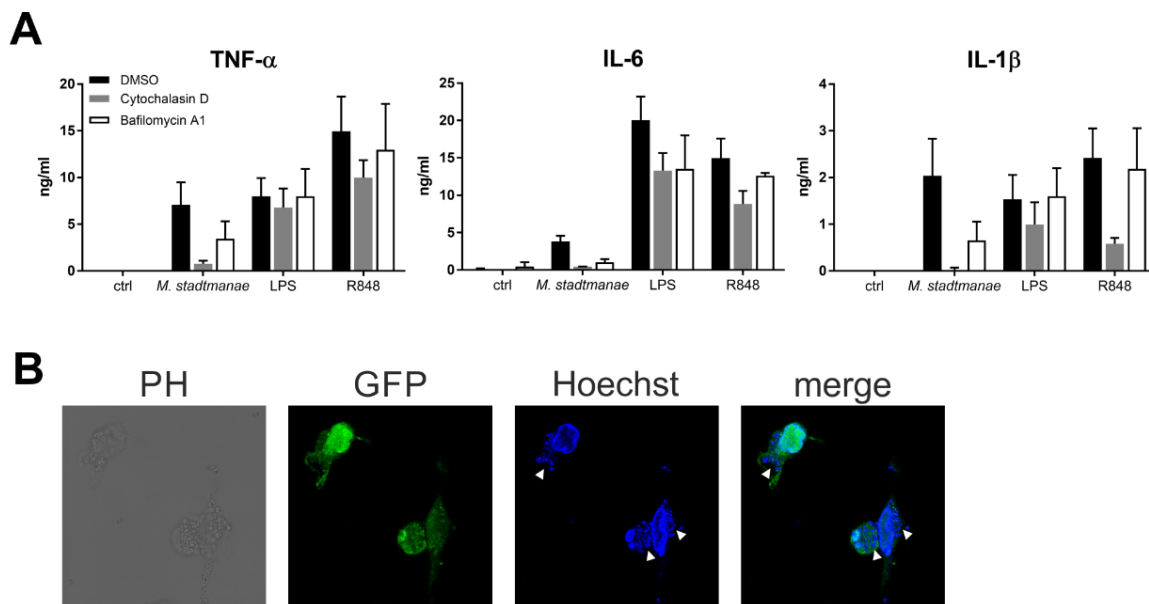


Figure S2. Response of BLaER1 monocytes to *M. stadtmanae*.

(A) Quantification of TNF- α , IL-6, and IL-1 β in the supernatant of BLaER1 monocytes either left unstimulated or stimulated with 10^7 *M. stadtmanae* cells, 50 ng/ml LPS or 5 μ g/ml R848 using ELISA. Cytochalasin D and Bafilomycin A (inhibitors of phagocytosis and endosomal acidification, respectively) were added 1 h prior to stimulation. The data from three independent experiments ($n = 3$) are shown as the mean \pm SEM. (B) Confocal microscopy of BLaER1 monocytes 4 h after the addition of *M. stadtmanae*. GFP expression is shown in green and Hoechst 33342 staining in blue. White arrows indicate the points where *M. stadtmanae* is phagocytosed by BLaER1 cells. Scale bars: 10 μ m. The images shown are representative examples from one of two independent experiments ($n = 2$).

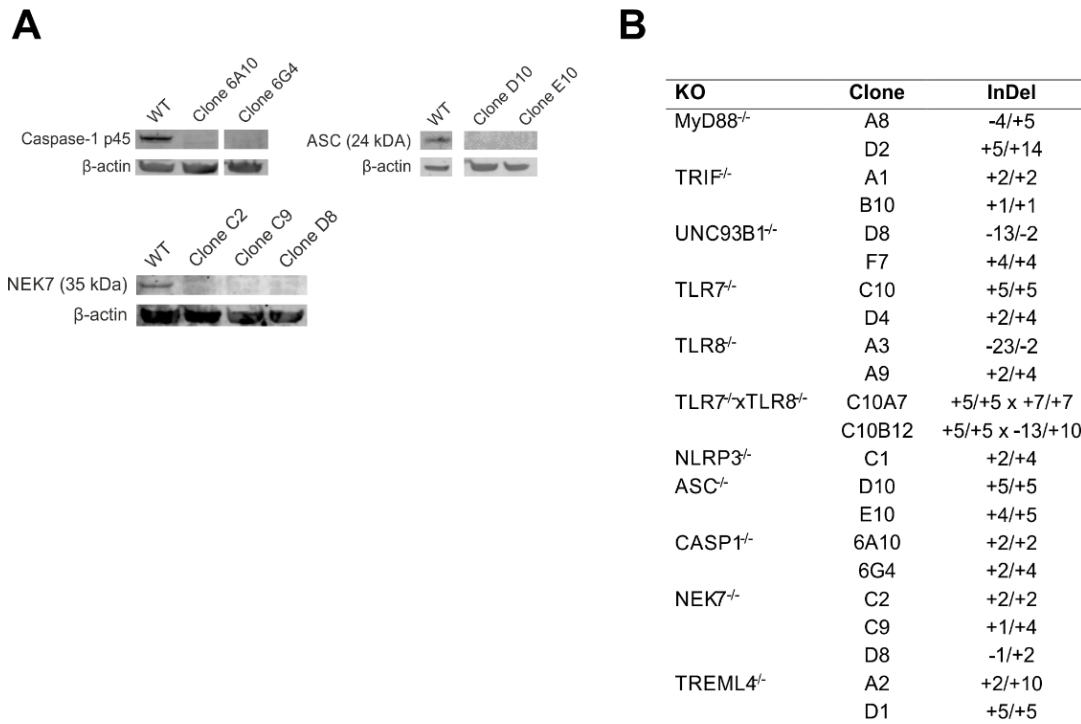


Figure S3. Generation of BLaER1 KO cell lines.

(A) Immunoblotting of BLaER1 KO cell lines was performed if specific antibodies were available. β -actin was used as the loading control. (B) Types of InDel mutations that were identified by TIDE analysis for all KO clones used in this study. All clones show frameshift mutations on both alleles.

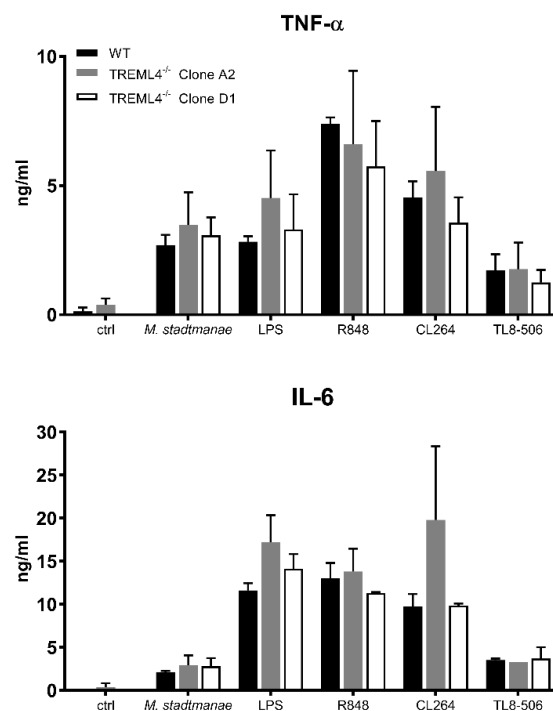


Figure S4. Response of BLaER1 TREML4^{-/-} to *M. stadtmanae* and TLR7/8 agonists.

Quantification of TNF- α and IL-6 in the supernatant of BLaER1 WT and TREML4^{-/-} monocytes stimulated with 10^7 *M. stadtmanae* cells, 50 ng/ml LPS or 5 μ g/ml R848, CL264 or TL8-506 for 18 h using ELISA. The data from two independent experiments (n = 2) and two different knockout clones are shown as the mean \pm SEM.

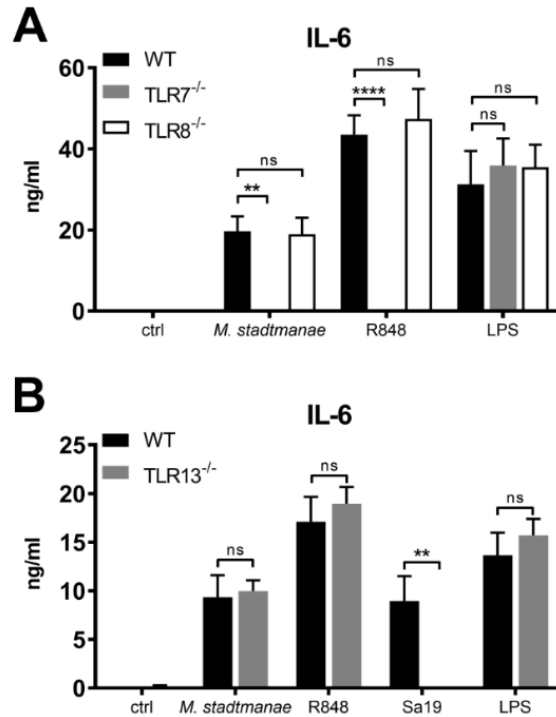


Figure S5. Stimulation of IL-6 secretion in response to *M. stadtmanae* is dependent on mTLR7 but not on mTLR8/mTLR13 in murine bone marrow-derived dendritic cells (BMDCs).

(A) Quantification of IL-6 in the supernatant of wild-type (WT), TLR7^{-/-} or TLR8^{-/-} murine BMDCs stimulated for 18 h with 10⁷ cells of *M. stadtmanae*, 50 ng/ml LPS, 5 µg/ml R848 as measured by ELISA. (B) Quantification of IL-6 in the supernatant of WT or TLR13^{-/-} murine BMDCs stimulated for 18 h with 10⁷ cells of *M. stadtmanae*, 50 ng/ml LPS, 5 µg/ml R848 or 5 µM Sa19 (TLR13 agonist) was measured by ELISA. (A, B) ns: not significant, ** $P \leq 0.01$, **** $P \leq 0.00001$ (two-way ANOVA with Bonferroni *post hoc* test). The data from two to four independent experiments ($n = 2-4$) are shown as the mean \pm SEM.

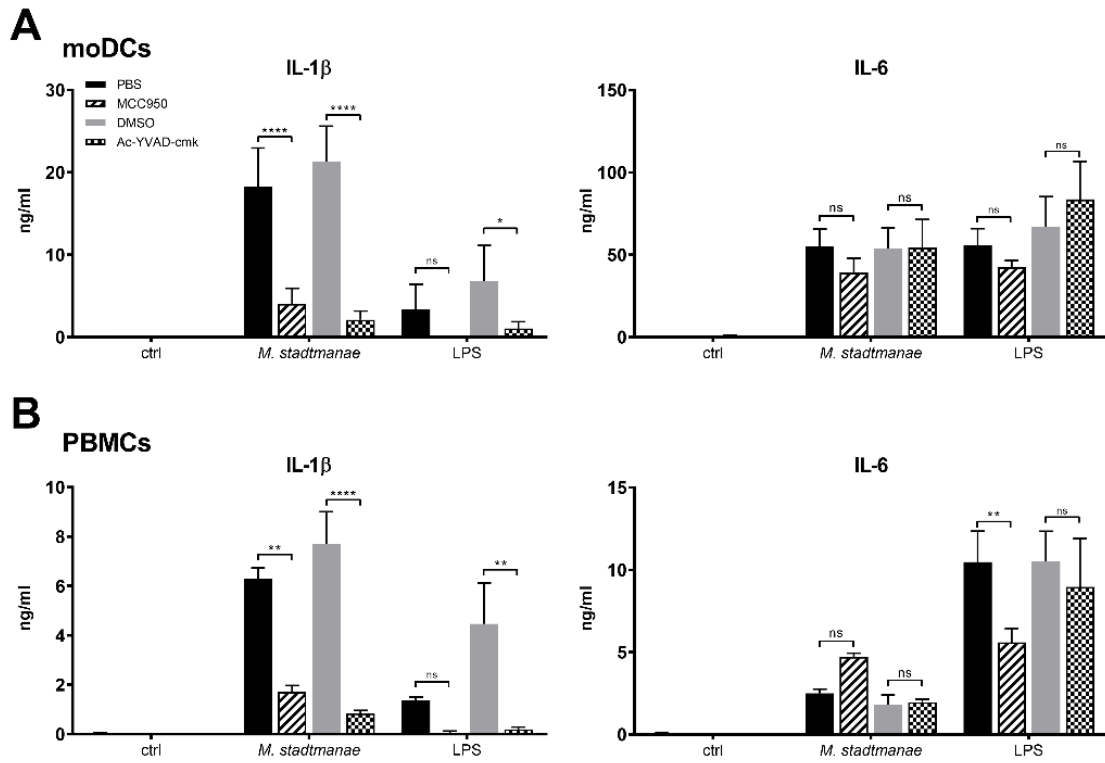


Figure S6. Secretion of IL-1 β in human moDCs and PBMCs is dependent on NLRP3 and caspase-1.

(A, B) Secretion of IL-6 (left) and IL-1 β (right) in the supernatants of stimulated human moDCs (A) and PBMCs (B) was measured using ELISA. Cells were treated with either MCC950 or Ac-YVAD-cmk (inhibitors of NLRP3 and caspase-1, respectively), or control substances (medium and DMSO, respectively) 1 h prior to stimulation with 10^7 cells of *M. stadtmanae* or 50 ng/ml LPS for 18 h. ns: not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ (repeated measures two-way ANOVA with Tukey *post hoc* test). The data from at least three different donors ($n = 3-5$) are shown as the mean \pm SEM.

9.2 Supplementary Tables

Table S1. Guide RNA sequences

Gene	gRNA sequence (5'-3') + PAM
ASC	ATGGACAGCAGCGCGCCCCG CGG
CASP1	ACAGACAAGGGTGCTGAACA AGG
MyD88	ACCACACTTGATGACCCCT GGG
Nek7	TGTGGTTGGAAGTGAAGAAC AGG
NLRP3	GTCTTCCTGGCATATCACAG TGG
TLR7	ACTGTGTACCTATTCCACTG GGG
TLR8 (double KO)	CGTTCTGGTGCTGTACATTG GGG
TLR8 (single KO)	ACAGGAAGTTCCCCAAACGG TGG
TREML4 A2	AAGAGTCCACATAGGAGACG TGG
TREML4 D1	AGCCAGGGAAGAGTCCACAT AGG
TRIF	GATGAGGCCCGAAACCGGTGT GGG
UNC93B1	CTACGACGAGACCTACCGCG AGG

Table S2. Primer for qRT-PCR

Gene	Name	Sequence (5'-3')	Reference
IFNL1 (IFN- λ 1)	huIFN-L1-fw	CGCCTTGGAAGAGTCACTCA	(192)
	huIFN-L1-rv	GAAGCCTCAGGTCCCAATTC	
IFNA14 (IFN- α 14)	huIFN-a14-fw	AGGAGGAATTTGATGGCAAC	(193)
	huIFN-a14-rv	AGCAGCAGATGAGTTCTTTG	
IFNB1 (IFN- β)	huIFN-b-fw	AAGCAGCAATTTTCAGTGTCAG	
	huIFN-b-rv	CCTCAGGGATGTCAAAGTTCA	
HPRT	huHPRT_fw	GTCAGGCAGTATAATCCAAAGA	
	huHPRT_rv	GGGCATATCCTACAACAAACT	

9.3 Supplementary Methods

Stimulation of murine BMDCs

Bone marrow cells from C57BL/6 WT, TLR7^{-/-}, TLR8^{-/-} (both are a kind gift from Douglas Golenbock, UMASS, Worcester, USA), or TLR13^{-/-} (a gift from Carsten Kirschning, University of Duisburg-Essen, Essen, Germany) were differentiated into BMDCs as described previously (176). Cells were seeded at 1×10^5 cells/ml in a 96-well plate and stimulated for 18 h with 10^7 cells of *M. stadtmanae*, 50 ng/ml LPS, 5 μ g/ml R848, or 5 μ M Sa19 (both from Invivogen).

VI Additional Results

1 Materials and Methods

This section offers additional materials and methods that were not mentioned in chapters IV or V before. If not indicated otherwise, experiments were performed as described in these sections.

Cell stimulation

Besides the different stimuli described in chapters IV and V, cells have been additionally stimulated with *Lactococcus lactis* or the lipopeptide Pam3CSK4 (EMC microcollections) where indicated. *L. lactis* has been prepared as described by Stein, *et al.* (176) and added in a final load of 10^7 bacteria/ml. Pam3CSK4 has been used in a final concentration of 10 μ M.

Cell Stimulation with extracellular potassium

To investigate the influence of potassium efflux on IL-1 β secretion, potassium chloride (KCl) was added to the medium before stimulation. Briefly, primary monocytes or BLaER1 monocytes were seeded into a 96-well plate (0.25×10^5 cells/well or 0.5×10^5 cells/well, respectively) and incubated for 2 h. Different concentrations of KCl (Sigma) from a 2 M stock ranging from 1 mM to 50 mM were added to cells and incubation was continued for about 1 h. 10^7 cells of *M. stadtmanae*, 50 ng/ml LPS, 5 μ g/ml R848 or 10 μ M Pam3CSK4 were added and cells were stimulated for 18 h in a total volume of 200 μ l. For the last 2 h of stimulation, 13.4 μ M nigericin (Invivogen) was added where indicated. Concentration of IL-1 β and IL-6 in the supernatant was analyzed by ELISA (see chapter V.5)

Generation of BLaER1 KO cell lines

BLaER1 KO cells were generated as described in chapter V.5. In addition, genes for Cathepsin B, GSDMD, MAVS, RIG-I and STING were knocked out using the CRISPR/Cas9 system. Specific gRNAs for each gene are listed in Table VI-1. Confirmation of successful KOs was done using TIDE analysis (IX.1) and for MAVS^{-/-}, RIG-I^{-/-} and STING^{-/-} additionally by immunoblotting. α RIG-I (D12, 1:200, Santa Cruz), α MAVS (E-3, 1:200, Santa Cruz) and α STING (clone # 723505, 1:2500, R&D systems) have been used.

Table VI-1. gRNA sequences for generation of BLaER1 KO cell lines.

Gene	gRNA sequence (5'-3') + PAM
CTSB (Cathepsin B)	TCAACAAACGGAATACCACG TGG
GSDMD	TTGAGGGCTTCCTAACCACC AGG
MAVS	GTAGATACAACCTGACCCTGT GGG
DDX58 (RIG-I)	TACCTACATCCTGAGCTACA TGG
TMEM173 (STING)	GCTGGGACTGCTGTAAACG GGG

Generation of a BLaER1 ASC-mScarlet reporter cell line *via* homology directed repair (HDR)

The red fluorescent protein mScarlet (194) was c-terminally linked to endogenous ASC *via* a (G₄S)₃ linker peptide using the CRISPR/Cas9 system. Briefly, the gRNA sequence (5'-AGGACCTGGAGCGGAGCTG) targeting the C terminus of the ASC gene was cloned into the plasmid pU6-(BbsI)-CBh-Cas9-T2A-BFP (a kind gift from Ralf Kuehn (125); Addgene plasmid #64323) *via* the BbsI restriction site as described earlier. The donor DNA was generated by amplification of an ASC left and right homology arm (each about 800 bp) closely to gRNA target site and amplification of the mScarlet gene from the pmScarlet_C1 plasmid (pmScarlet_C1 was a gift from Dorus Gadella (194); Addgene plasmid # 85042) with specific overhangs using Herculase II Fusion polymerase (Agilent). The PCR fragments were purified with PureLink PCR Purification kit (Thermo Fisher) and assembled together with a PCR-amplified pUC19 plasmid to generate the HDR donor plasmid pUC19_HDR-ASC_mScarlet *via* the NEBuilder HiFi DNA Assembly Cloning kit (New England Biolabs). Plasmid integrity was verified by restriction digest using BamHI, EcoRI, SmaI, XbaI and NotI (all Thermo Fisher) according to the manufacturer's instructions and by sanger sequencing at eurofins genomics. To generate the endogenous ASC-mScarlet fusion protein, BLaER1 cells were transfected with 1 µg of the CRISPR/Cas9 plasmid pU6-ASC-Cterm-CBh-Cas9-T2A-BFP and 2 µg of the donor template pUC19_HDR-ASC_mScarlet by nucleofection. Two days after nucleofection, single cell sorting of GFP+BFP+ cells was performed by the Fluorescence Cytometry core facility at the Research Center Borstel. Nucleofection and cell sorting are already described in chapter V.5. Positive clones were identified by PCR targeting the mScarlet insert and verified by sequencing. The clone A7 with a heterologous insertion was selected and analyzed by confocal microscopy, Western Blot and ELISA (methods are described in detail in chapter V.5).

2 Results

2.1 Involvement of cytoplasmic nucleic acid-specific receptors in recognition of *M. stadtmanae*

While it was shown in chapter V that RNA from *M. stadtmanae* is mainly recognized by the endosomal receptors TLR7 and TLR8, the role of cytosolic nucleic acid sensors was not evaluated. Most immune cells and many non-immune cells possess a repertoire of different cytosolic nucleic acid-specific receptors. These recognition pathways include the cGAS-STING and the RIG-I/MDA5-MAVS pathway, which detect cytosolic dsDNA or dsRNA/5'-triphosphate RNA, respectively. It was recently shown that the cGAS-STING pathway is important for the response against various pathogens, including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Plasmodium falciparum* or *Neisseria gonorrhoeae* (195-198). DNA from these pathogens becomes accessible either through lysis of the bacteria in the cytosol or through translocation of DNA from the phagolysosome to the cytosol.

As it is unknown if archaeal nucleic acids are recognized in the cytosol or if potential archaeal cyclic dinucleotides are present that can also directly activate STING, we investigated the role of the cGAS-STING and the RIG-I-MAVS pathways in *M. stadtmanae*-induced immune cell activation. To address this question, we generated clonal BLaER1 cell lines deficient for STING, RIG-I or MAVS using the CRISPR/Cas9 system. Three different clones for each gene knockout were verified by TIDE analysis (IX.1) and by Western Blot (Figure VI-1 A-C). As it was recently shown that the cGAS-STING pathway has the potential to activate the NLRP3 inflammasome in human monocytes (38), we first stimulated BLaER1 monocytes with *M. stadtmanae* and analyzed the secretion of IL-1 β and IL-6 in the supernatant (Figure VI-1 D; results for all clones are shown in Figure IX-1). Neither disruption of the cGAS-STING pathway nor the RIG-I- and MAVS-dependent pathways did result in a significant decrease in IL-1 β or IL-6 release. This leads to the suggestion that *M. stadtmanae* -induced inflammasome activation is independent of the STING pathway.

One of the major function of cytosolic nucleic acid receptors is the induction of type I IFNs. To clarify the role of cGAS-STING and RIG-I-MAVS pathways in induction of IFNs by *M. stadtmanae*, BLaER1 STING^{-/-}, RIG-I^{-/-} and MAVS^{-/-} monocytes have been stimulated with *M. stadtmanae* and induction of IFN- β expression has been measured *via* qRT-PCR (Figure VI-1 E; results for all clones are shown in Figure IX-1). The specificity of the different clones was determined by stimulation with pathway-specific activators, namely DNA for the cGAS-STING pathway and 5'-triphosphate RNA for the RIG-I-MAVS pathway. Expression of IFN- β

after stimulation with *M. stadtmanae* was not reduced, neither in the STING^{-/-} nor in the RIG-I^{-/-} and MAVS^{-/-} BLaER1 monocytes. Altogether, these results demonstrate that induction of cytokine secretion and IFNs by *M. stadtmanae* is independent of major cytosolic nucleic acid recognition pathways.

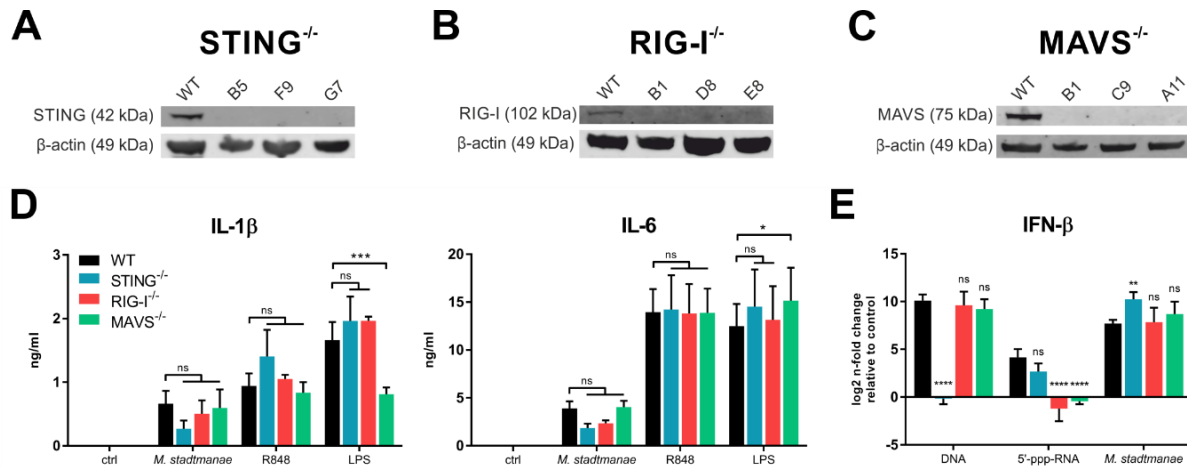


Figure VI-1. Response to *M. stadtmanae* is independent of major cytosolic nucleic acid recognition pathways. (A-C) Immunoblotting of BLaER1 KO cell lines (STING, RIG-I and MAVS) was performed using specific antibodies for each target gene. Three different clones per gene KO were analyzed. β-actin was used as the loading control. (D) IL-1β and IL-6 secretion in the supernatants of BLaER1 WT, STING^{-/-}, RIG-I^{-/-} or MAVS^{-/-} monocytes stimulated for 18 h with either 10⁷ cells of *M. stadtmanae*, 50 ng/ml LPS, or 5 μg/ml R848 was measured by ELISA. (E) The expression of IFN-β in BLaER1 STING^{-/-}, RIG-I^{-/-} or MAVS^{-/-} monocytes after stimulation with *M. stadtmanae* (100:1), 0.5 μg/ml 5'-ppp-RNA, or 1 μg/ml plasmid DNA for 6 h was measured by qRT-PCR. The expression ratios of IFN-β gene to the reference gene HPRT are shown as log₂ n-fold induction relative to the unstimulated control cells. (D-E) ns: not significant, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 (repeated measures two-way ANOVA with Dunnett post hoc test). The data are shown as the mean ± SEM of four independent experiments (n=4) in (D) or three independent experiments (n = 3) in (E) from one representative clone per gene. Three different STING^{-/-} clones and two different clones of each RIG-I^{-/-} and MAVS^{-/-} were used in qRT-PCR and ELISA (Figure IX-1).

2.2 Role of cathepsin B and gasdermin D on cytokine response to *M. stadtmanae*

We clearly demonstrated that recognition of *M. stadtmanae* takes place in the phagolysosome and is mediated through the RNA-specific receptors TLR7 and TLR8. Yet, it is unclear how *M. stadtmanae* activates the NLRP3 inflammasome. A role in lysosomal-dependent NLRP3 inflammasome activation was assigned to cathepsin B (CatB), a cysteine protease important for proteolytic functions in the cytosol (199, 200). Furthermore, it was shown that CatB is important for TLR8-dependent inflammasome activation induced by HIV using CA-074-Me as inhibitor of CatB (187). Based on these publications, a role for CatB in *M. stadtmanae*-induced NLRP3 activation was suggested. Accordingly, a CatB-deficient BLaER1 cell line has been generated using the CRISPR/Cas9 system. The cell line was analyzed for frameshift mutations using TIDE analysis (IX.1). To examine the role CatB, we

stimulated BLaER1 WT and CatB^{-/-} monocytes with *M. stadtmanae* and measured secretion of IL-1 β and IL-6 (Figure VI-2). Stimulation of CatB^{-/-} cells with *M. stadtmanae* results in cytokine secretion levels comparable to WT cells. This leads to the conclusion that CatB is dispensable for recognition of *M. stadtmanae* and inflammasome activation induced by this archaeon. However, a reduction of IL-1 β is detectable after stimulation with LPS. The role of CatB in LPS-induced alternative inflammasome activation was not yet evaluated but it was shown that CatB an important factor for canonical inflammasome activation in THP-1 monocytes through LPS/nigericin (201). Nevertheless, all studies on CatB using the inhibitor CA-074-Me should be looked at critically because CA-074-Me not only inhibits CatB but rather a wide range of different cathepsins and cystatins (202). However, a role of CatB in *M. stadtmanae*- or TLR8-induced inflammasome activation in BLaER1 monocytes could not be observed.

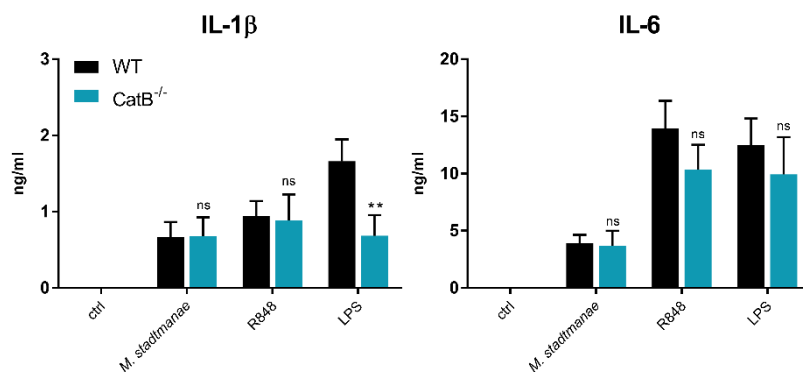


Figure VI-2. Response to *M. stadtmanae* is independent of Cathepsin B.

IL-1 β and IL-6 secretion in the supernatants of BLaER1 WT or CatB^{-/-} monocytes stimulated for 18 h with either 10^7 cells of *M. stadtmanae*, 50 ng/ml LPS, or 5 μ g/ml of R848 is shown. ns: not significant, ** $P \leq 0.01$ (repeated measures two-way ANOVA with Bonferroni post hoc test). The data are shown as the mean \pm SEM of four independent experiments ($n=4$).

In addition to the pro-inflammatory cytokines pro-IL-1 β and pro-IL-18, GSDMD is an additional target that is cleaved and activated by caspase-1. After cleavage of GSDMD, the N-terminal fragment oligomerizes into the cell membrane, where it forms a pore-like structure. These pores impair the cell integrity which can lead to pyroptosis, an inflammatory form of programmed cell death (54). However, it has been recently discovered that GSDMD pores can also function as channels for IL-1 β to passively leave the cell without induction of pyroptosis (55). So far, the role of GSDMD has only been described for canonical and non-canonical inflammasome activation. It is unclear if GSDMD is necessary for the secretion of IL-1 β after alternative inflammasome activation. Since *M. stadtmanae*- and TLR8-dependent inflammasome activation resemble key characteristics of alternative activation, the function of

GSDMD in these pathways is also unknown. To investigate the role of GSDMD in *M. stadtmanae*-induced inflammasome activation, BLaER1 GSDMD^{-/-} monocytes were generated using CRISPR/Cas9. Positive KO clones have been selected based on TIDE analysis (IX.1). Stimulation of GSDMD^{-/-} cells with CL097 or LPS/nigericin leads to a decreased release of IL-1 β and LDH with only minor effects on IL-6 secretion (Figure VI-3; results for all clones are shown in Figure IX-2). As both stimuli are activators of the canonical inflammasome activation pathway, GSDMD is important for IL-1 β release and induction of pyroptosis, which we could reproduce in our experiments. In contrast, stimulation with LPS or *M. stadtmanae* did not lead to a reduction in IL-1 β or LDH release from these cells and we suggest that GSDMD is negligible in alternative inflammasome activation. Yet, IL-1 β secretion in GSDMD^{-/-} cells was largely impaired after stimulation of R848. As R848 should not trigger canonical activation (188), the role of GSDMD in this setting is ambiguous. Although our results demonstrate that GSDMD is dispensable for alternative and *M. stadtmanae*-induced inflammasome activation, the mechanism of how IL-1 β is able to exit the cells without GSDMD pores is still unresolved.

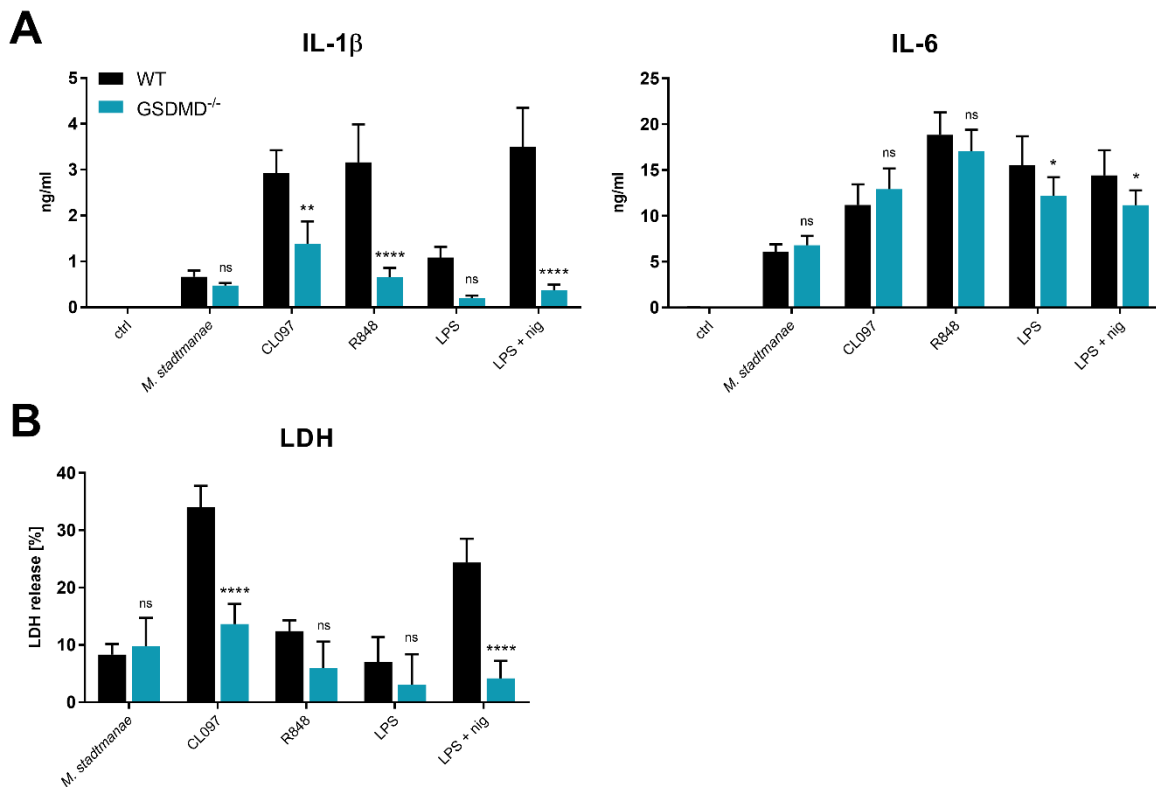


Figure VI-3. Inflammasome activation and LDH release are independent of GSDMD.

(A) IL-1 β and IL-6 secretion and (B) release of LDH in the supernatants of BLaER1 WT or GSDMD^{-/-} monocytes stimulated for 18 h with either 10⁷ cells of *M. stadtmanae*, 5 μ g/ml of CL097 5 μ g/ml R848 or 50 ng/ml LPS is shown. ns: not significant, * P \leq 0.05, ** P \leq 0.01, **** P \leq 0.0001 (repeated measures two-way ANOVA with Bonferroni post hoc test). The data are shown as the mean \pm SEM of four independent experiments (n=4). Data from one of three different clones are shown (Figure IX-2). (LDH, lactate dehydrogenase; nig, nigericin)

2.3 Influence of Potassium Efflux on Inflammasome Activation Induced by *M. stadtmanae*

In case of canonical NLRP3 inflammasome activation, efflux of potassium ions from the cell is a trigger that is shared between most canonical activators (47) with a few exceptions including imiquimod and CL097 (188). If a NLRP3 activator is dependent on potassium efflux, the secretion of IL-1 β can be inhibited by addition of high concentrations of KCl into the surrounding medium (59). To investigate the influence of potassium efflux on *M. stadtmanae*-induced NLRP3 inflammasome activation, primary human monocytes were stimulated with *M. stadtmanae* in medium containing increasing concentrations of KCl. Compared to canonical activation (LPS + nigericin or Pam3CSK4 + nigericin) where a solid inhibition of IL-1 β secretion with increasing concentrations of KCl is detectable, the effect on IL-1 β secretion after stimulation with *M. stadtmanae* is weaker (Fig. VI-4). Similar effects were observed after stimulation with LPS or R848. Like the alternative activation pathway, IL-1 β secretion from monocytes cannot be inhibited by extracellular KCl after stimulation with *M. stadtmanae*. Thus, potassium efflux is not required for *M. stadtmanae*-induced inflammasome activation.

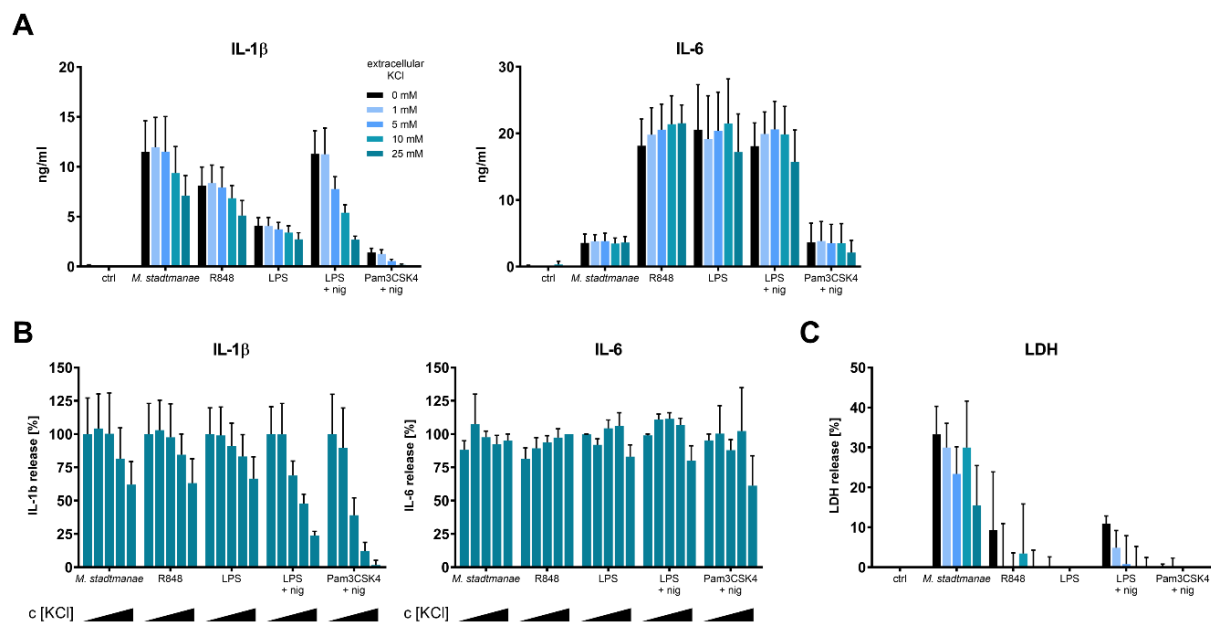


Figure VI-4. Secretion of IL-1 β in primary monocytes after stimulation with *M. stadtmanae* is independent of potassium efflux.

(A-C) Primary human monocytes were stimulated in medium with increasing concentrations of KCl up to 25 mM. 10^7 cells of *M. stadtmanae*, 5 μ g/ml R848, 50 ng/ml LPS or 10 μ M Pam3CSK4 were added for 18 h and 13.4 μ M nigericin were added for the last 2 h where indicated. Concentration of IL-1 β and IL-6 were measured by ELISA and are depicted as absolute concentration in (A) or relative concentration in (B). Release of LDH into the supernatant is shown in (C). In (A-C), the data shown are the mean \pm SEM of at least three different donors (n = 3-4). (LDH, lactate dehydrogenase; nig, nigericin)

2.4 Characterization of ASC-mScarlet BLaER1 reporter cell line

Inflammasome activation induced by *M. stadtmanae* in BLaER1 monocytes does not induce the formation of ASC specks as shown in chapter V. In order to simplify visualization of ASC speck formation and to allow live cell imaging, we generated an ASC reporter cell by fusion of a red fluorescent protein mScarlet to the C-terminus of endogenous ASC in BLaER1 cells *via* a (G₄S)₃ linker peptide. The gene encoding mScarlet was cloned into a pUC19 plasmid with approx. 800 bp homology arms on each site and the linker peptide at the N-terminus of mScarlet. This plasmid served as donor DNA for HDR in BLaER1 cells using the CRISPR/Cas9 system. Single cell clones were analyzed by PCR and the clone A7 was obtained which is heterozygous for the ASC-mScarlet fusion protein. The heterogeneity should ensure a proper formation and activation of the inflammasome as mScarlet could possibly interfere in speck formation. Initially, the clone A7 was analyzed by Western Blot (Figure VI-5 A) showing that this clone expresses WT ASC as well as the ASC-mScarlet fusion protein in comparable amounts. Confocal microscopy confirmed that the ASC-mScarlet protein in clone A7 is functional as red fluorescence of mScarlet is detectable in all cells. The clone also shows GFP expression as well as a similar morphology compared to WT BLaER1 monocytes (Figure VI-5B). The reporter cell line was stimulated with *M. stadtmanae*, LPS and different TLR7/8 stimuli to ensure that the cells secrete similar cytokine levels of TNF- α , IL-6 and IL-1 β compared to WT cells and to show that inflammasome activation is not disturbed (Figure VI-5C). The results clearly demonstrate that the reporter cell line is still capable of inflammasome activation and has no defects in cytokine secretion.

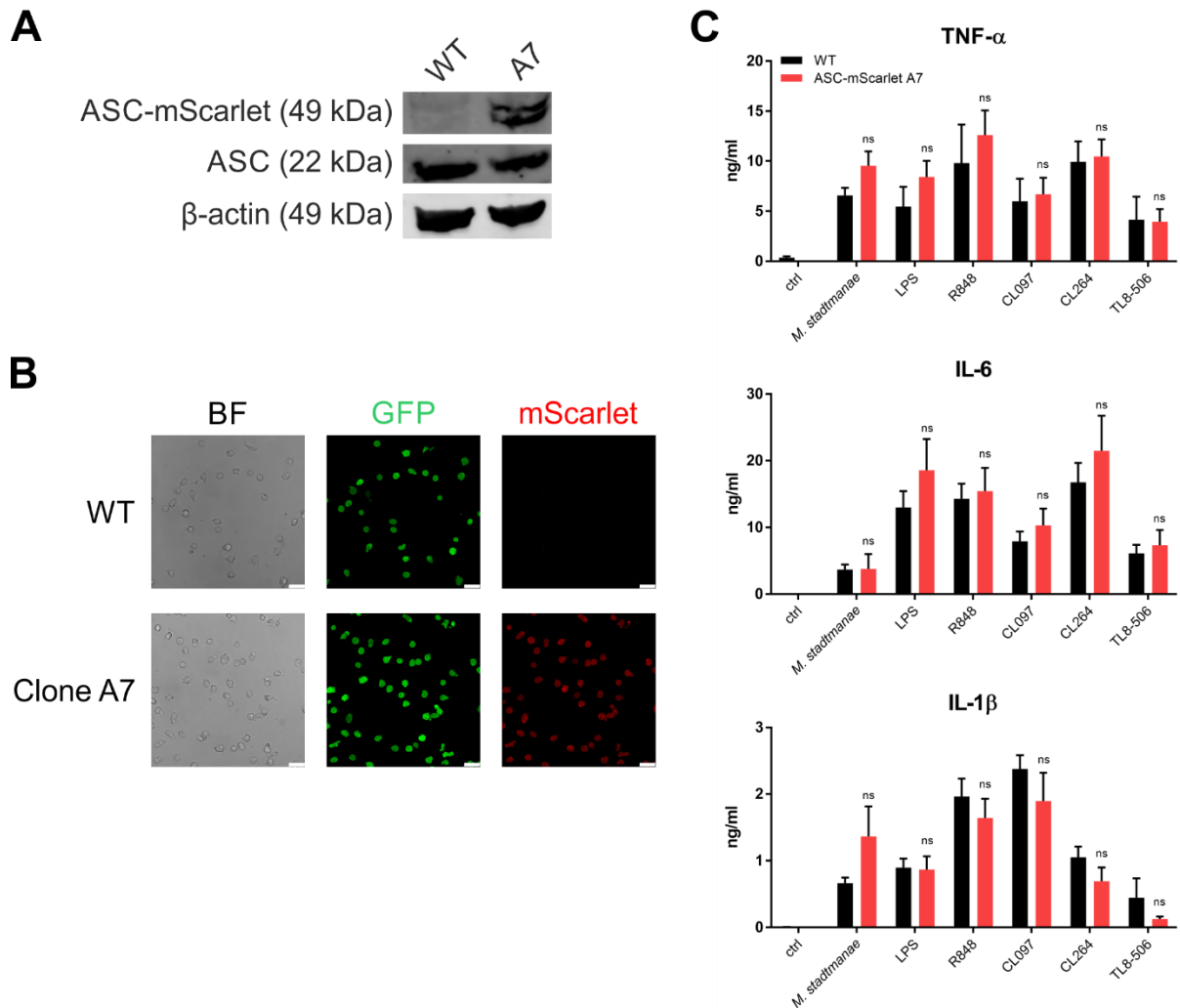


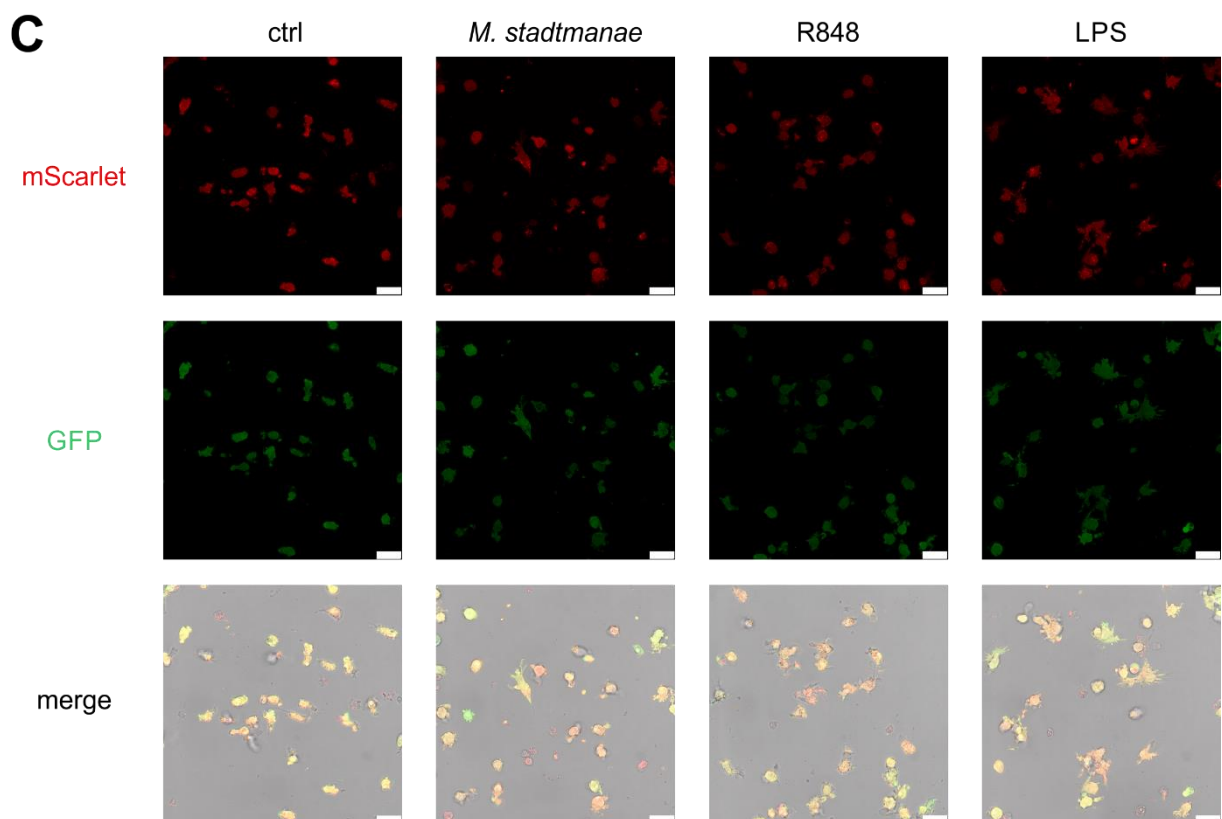
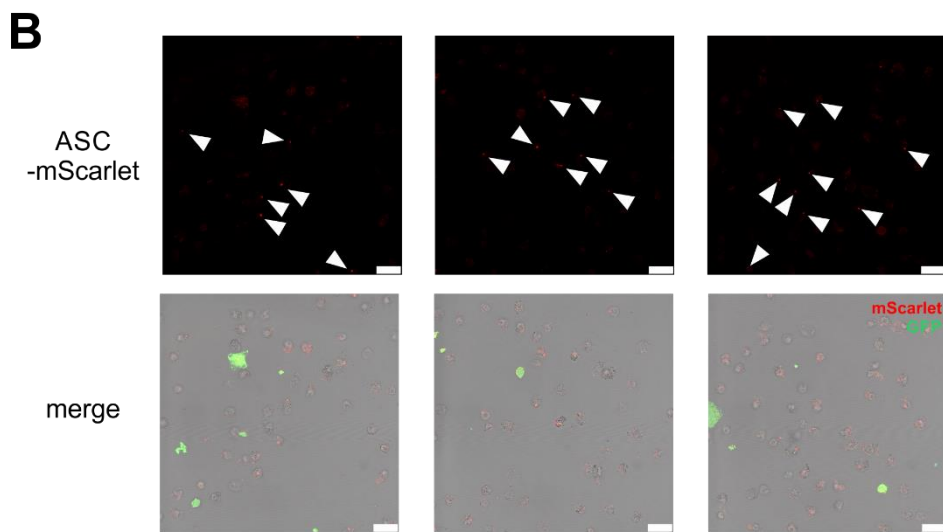
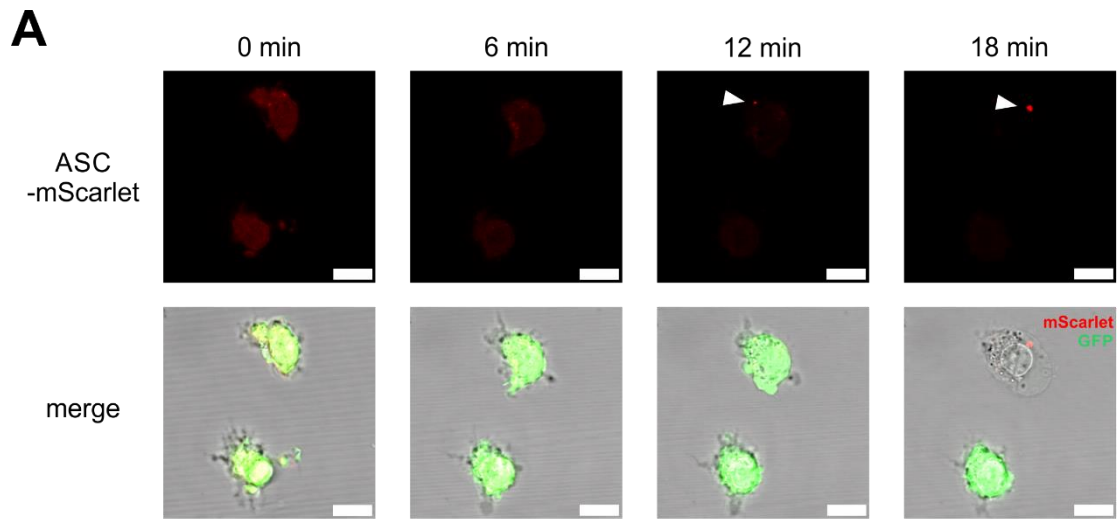
Fig VI-5. BLaER1 ASC reporter cell line A7 shows heterozygous expression of an ASC-mScarlet fusion protein with cytokine secretion levels comparable to WT cells.

(A) Immunoblotting of cell lysates from BLaER1 WT and ASC-mScarlet A7 monocytes showing the expression of ASC (22 kDa) and ASC-mScarlet fusion protein (49 kDa). Analysis of β -actin (49 kDa) was used as loading control. (B) BLaER1 WT and ASC-mScarlet A7 monocytes were analyzed by confocal microscopy. Brightfield as well as emission of GFP (green) and mScarlet (red) was measured. Scale bars represent 25 μ m. (C) The secretion of TNF- α , IL-6 and IL-1 β in the supernatants of stimulated BLaER1 WT and ASC-mScarlet A7 monocytes was measured using ELISA. Cells were stimulated with 10^7 cells of *M. stadtmanae*, 50 ng/ml LPS, 5 μ g/ml R848, CL097, CL264 or TL8-506 for 18 h. ns: not significant (repeated measures two-way ANOVA with Bonferroni post hoc test). The data of three independent experiments ($n = 3$) are shown as the mean \pm SEM. (BF, brightfield; GFP, green fluorescent protein)

To demonstrate functional ASC speck formation in the ASC-mScarlet cell line, the cells were pre-incubated with LPS and the canonical inflammasome activation was then induced by addition of nigericin. As the reporter cell line can be used for live cell imaging, images were taken every thirty seconds after addition of nigericin. Images for the first 18 minutes in 6-minute steps are shown and demonstrate that nigericin is a fast inducer of inflammasome activation as ASC specks are detectable already after 12 minutes (Figure VI-6A). Two hours after addition of nigericin, ASC specks are clearly formed in many cells (Figure VI-6B). In contrast, to LPS/nigericin stimulation, unstimulated cells show a largely uniform distribution of ASC in cytosol (Figure VI-6C). Stimulation of ASC-mScarlet cells with *M. stadtmanae* did not lead to

formation of ASC specks which is consistent with the results in chapter V. Furthermore, stimulation with R848 or LPS did also not induce formation of ASC specks confirming that alternative inflammasome activation is independent of ASC specks. Our results confirm that the ASC-mScarlet reporter cell line (clone A7) can be used as a functional reporter cell line to detect ASC speck formation in live cell imaging.

Fig VI-6. *M. stadtmanae* does not induce ASC speck formation in a BLaER1 ASC reporter cell line. (A, B) BLaER1 ASC-mScarlet A7 monocytes were pre-incubated for 3 h with 50 ng/ml LPS before 13.4 μ M nigericin has been added. Cells were analyzed directly after addition of nigericin *via* live cell confocal microscopy in (A) or after 2 h in (B). (C) BLaER1 ASC-mScarlet A7 monocytes were stimulated with *M. stadtmanae* (100:1), 5 μ g/ml R848 or 50 ng/ml LPS for 16 h and analyzed by confocal microscopy. (A-C) Brightfield as well as emission of GFP (green) and mScarlet (red) was measured. Scale bars represent 10 μ m in (A) or 25 μ m in (B, C). White arrowheads indicate ASC specks. Representative micrographs from one of two independent experiments are shown.



VII General Discussion

In the last decade, several studies have shown that the microbiota has a great impact on our health and homeostasis. Yet, most of these studies are limited to the bacterial diversity and largely ignore other organisms such as fungi, viruses, and archaea. Particularly archaea have been neglected by medical microbiology. Important reasons for this are methodological limitations as it has been recently shown that so-called ‘universal’ primer for 16S rRNA sequencing, which should amplify both bacterial and archaeal rRNA, are largely biased towards bacteria. In addition, DNA isolation methods and data analyses are often not optimized for the detection of archaea. It is to be assumed that the true diversity of the archaeome is extensively underestimated (100-102).

Already in 2003, the potential existence of archaeal pathogens was discussed by Cavicchioli, *et al.* (203). Although a high diversity of archaea lives in close contact to humans, no pathogenic archaea have been identified by now. The apparent lack of pathogenic archaea contributes to the fact that archaea are widely overlooked as members of our microbiota. Nevertheless, several studies suggest a role of archaea in different diseases, yet exact mechanisms of how archaea might influence disease manifestation and progression are unknown (104, 106, 107, 111, 113). This thesis is focused on the interaction of two distinct archaeal gut inhabitants with the human innate immune system. On the one hand *M. luminyensis*, an archaeon that might provide the host with benefits as it is able to degrade TMA (133), on the other hand *M. stadtmanae* which is able to induce strong immune response in human myeloid cells (114) and which is potentially associated with IBD (111). In the following sections (I) the immune response of *M. luminyensis* and its benefits for the host, (II) the detailed molecular mechanisms of recognition of *M. stadtmanae* and (III) its ability to activate the inflammasome are discussed. Furthermore, (IV) possible other recognition mechanisms of archaea and (V) the potential disease association of *M. stadtmanae* and major implications of this study will be discussed.

1 Immune response to *M. luminyensis* – Is its application as archaebiotic reasonable?

Methanomassiliicoccales describes a new order of methanogenic archaea of which three species have been associated with the human microbiota, namely *Candidatus Methanomethylophilus alva* Mx1201, *Candidatus Methanomassiliicoccus intestinalis* Mx1, and *M. luminyensis*, which has been part of this study (155). These archaea have unique

metabolic functions as they are able to use methylamines as substrates for methanogenesis. In contrast, *M. smithii* or *M. stadtmanae*, the two other important methanogenic gut inhabitants, cannot utilize methylamines. Due to their ability to degrade the methylamine TMA, Methanomassiliicoccales have been discussed as beneficial organisms for the host (103). TMA itself is produced by gut bacteria and can be converted into TMAO by the liver. It is assumed that TMAO is associated with the development of atherosclerosis, cardiovascular disease, and chronic kidney disease (155, 204). Therefore, *Methanomassiliicoccales* could be supplemented as living organisms, a so-called archaebiotic (103), to convert TMA into methane, thereby lowering the amount of TMAO in the body. Other, maybe harmful effects of these archaea on the host and particularly on the immune system, have not been evaluated before. This study is focused on the AMP susceptibility of *M. luminyensis* and the innate immune response against this archaeon.

We demonstrated that the sensitivities of *M. luminyensis* against three AMPs, namely LL32, NK2, and Lpep19.2-5, are comparable to those of *M. stadtmanae*. Although, both archaea are less sensitive to these AMPs compared to *M. smithii*, the MIC is still in a low micromolar range. Thus, the population of *M. luminyensis* can probably be controlled by the immune system and IECs through secretion of AMPs to prevent overgrowth. Moreover, we analyzed the pro-inflammatory response of human PBMCs and moDCs after stimulation with *M. luminyensis*. In comparison to *M. stadtmanae*, *M. luminyensis* induces only a weak secretion of TNF- α and IL-1 β . The levels of secreted cytokines rather resemble the amounts induced by *M. smithii* (111, 114). Interestingly, the mild immune response against *M. luminyensis* correlates with the low phagocytotic activity of moDCs on this archaeon. If we assume that *M. luminyensis* is also recognized intracellularly, the low phagocytosis probably accounts for the low levels of secreted cytokines. The phagocytosis rate of *M. smithii* has not been quantified by Bang, *et al.* (114), yet CSLM and transmission electron microscopy (TEM) micrographs from this study clearly show that it is apparently less phagocytosed compared to *M. stadtmanae*. Overall, *M. luminyensis* is susceptible to AMPs and induces at the same time only a weak immune response. These features are favorable for application as an archaebiotic because the growth of *M. luminyensis* can be controlled through AMPs while excessive release of pro-inflammatory cytokines, which could lead to inflammation and cell damage, has not been observed. Our *in vitro* study does not reveal any concerns for the usage of *M. luminyensis* as TMA degraders in humans, however, more studies including *in vivo* experiments are urgently needed to confirm its safety and to exclude harmful effects of *M. luminyensis* on humans.

2 RNA as key MAMP of *M. stadtmanae*

It has been shown by various studies that *M. stadtmanae* activates the immune system *in vitro* and *in vivo* with a pro-inflammatory phenotype (111-114, 205). Thereby, the observed immune activation is considerably stronger compared to other known methanogenic gut inhabitants *M. smithii* (111, 114) and *M. luminyensis* (this study, chapter IV). Although plenty is known about the function of PRRs and their respective microbial ligands, the molecular mechanism of how *M. stadtmanae* is recognized remained elusive. Since archaea utilize structural components which are different from those of bacteria and eukaryotes, archaeal cell wall structures like pseudomurein or heteropolysaccharides have been discussed as potential MAMPs (98). This study shows that the response of human PBMCs and moDCs does not only include release of pro-inflammatory cytokines such as IL-6 and IL-1 β but is also characterized by nuclear translocation of IRF1 and IRF5 and increased expression of type I and III IFNs.

By using a CRISPR/Cas9-mediated loss-of-function approach of specific receptor and signaling proteins in BLaER1 monocytes, we identified TLR7 and exceedingly TLR8 as key receptors in recognition of *M. stadtmanae* (Figure VII-1). Transfection of purified archaeal RNA into primary PBMCs and moDCs as well as BLaER1 monocytes identified RNA as the major MAMP. In contrast to mTLR13, hTLR7 and hTLR8 do not recognize a specific sequence in the 23S rRNA but are activated by small RNA fragments and degradation products (177, 178, 206-208). This recognition model is consistent with our results as we could not observe significant differences in cytokine secretion levels among different rRNA species (5S, 16S and 23S) of *M. stadtmanae*. Prior to this study, Bang, *et al.* (114) have already utilized a HEK293 transfection system with common human TLRs (TLR2-5 and TLR7-9) as well as the intracellular NLRs, nucleotide-binding oligomerization domain-containing protein (NOD)1 and NOD2, to identify PRRs that specifically recognize *M. stadtmanae*. However, none of these receptors led to IL-8 secretion from HEK293 cells after stimulation with *M. stadtmanae*. Since HEK293 are epithelial kidney cells, phagocytosis does not belong to their principal function unless certain proteins are artificially expressed (209). This might explain why TLR7- and TLR8-transfected HEK293 cells do not respond to *M. stadtmanae* as phagocytosis was shown to be essential to mount a cytokine response in moDCs (114). Therefore, the BLaER1 monocyte model system seems to be a more suitable model to study the function of endosomal TLRs, as this cell line holds monocytic key functions such as phagocytosis and lysosomal degradation of engulfed microbes.

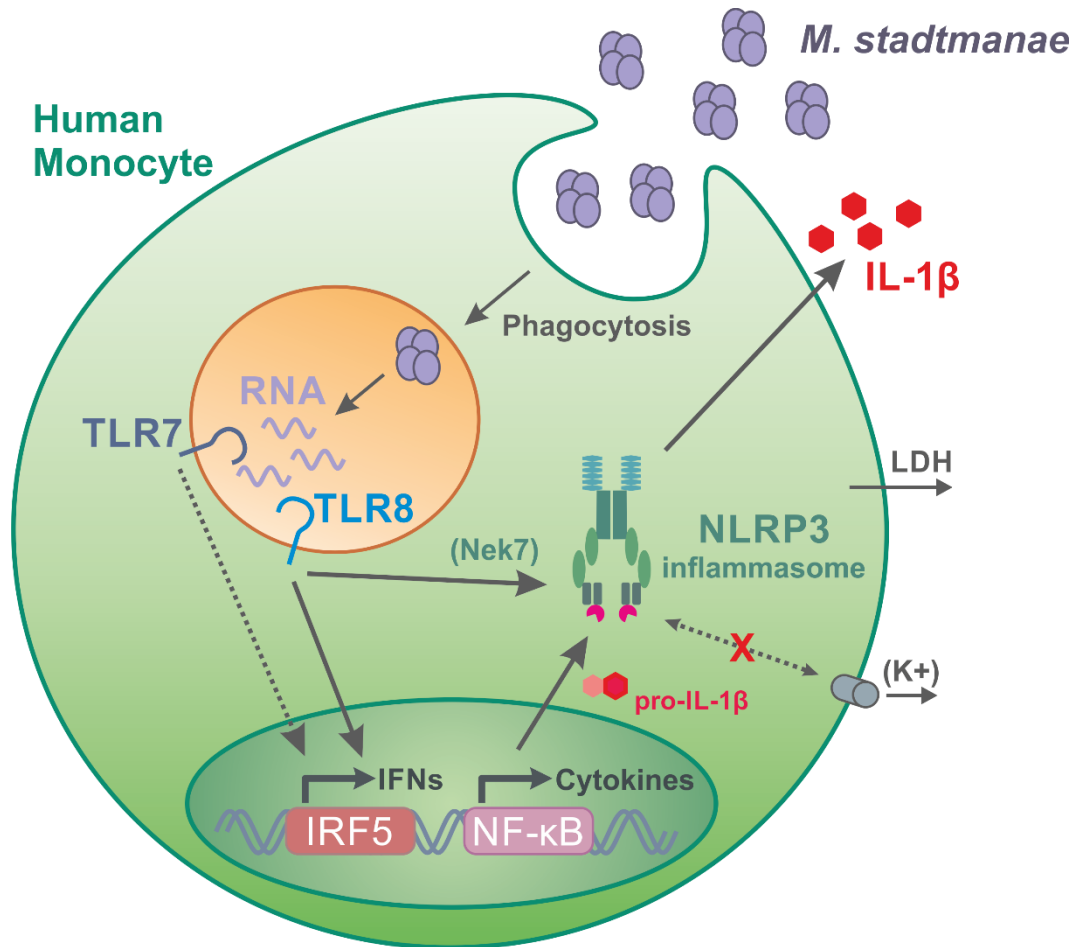


Figure VII-1. Activation model of human monocytes by *M. stadtmanae*.

M. stadtmanae is taken up by myeloid immune cells through phagocytosis. Inside the phagolysosome, the archaea are degraded, and archaeal RNA is released. The RNA can then be recognized by TLR7 and TLR8 and a signaling cascade is induced leading to the nuclear translocation of the transcription factors IRF5 and NF-κB. These transcription factors induce the expression of type I and III interferons as well as proinflammatory cytokines. Through a yet unknown mechanism, TLR8 additionally activates the NLRP3 inflammasome. This process yields to activation of caspase-1 which in turn processes pro-IL-1β into its active form. In contrast to canonical inflammasome activation, potassium efflux is not a feature of *M. stadtmanae*-induced inflammasome activation. (IL-1β, Interleukin 1β; IRF, Interferon regulatory factor; K+, Potassium ions; LDH, Lactate dehydrogenase; NF-κB, Nuclear factor ‘kappa-light-chain-enhancer’ of activated B cells; TLR, Toll-like receptor)

In addition to TLRs, monocytes and DCs possess several intracellular nucleic acid-specific receptors including RIG-I, MDA5 and cGAS (210). Although TLR8 was shown to be a key receptor in recognition of *M. stadtmanae*, an involvement of cytosolic receptors could not be excluded since translocation of other microbes and their nucleic acid from endo(lyso-)somes into the cytosol has been demonstrated (211-214). In VI.2.1 we showed that the cytoplasmic recognition pathways of RIG-I/MDA5-MAVS and cGAS-STING do not affect the response to *M. stadtmanae*. In absence of these pathways, neither secretion of pro-inflammatory cytokines nor the induction of IFN-β was significantly reduced. Moreover, the recently described process of STING-dependent NLRP3 inflammasome activation in human monocytes (38) was not involved as shown by the steady secretion of IL-1β from STING^{-/-} cells after stimulation with

M. stadtmanae. We suggest that the recognition of *M. stadtmanae* is restricted to the endo(lyso-)somal compartment, at least for human monocytes. Involvement of cytosolic recognition pathways in other cell types or in *in vivo* models is still possible and needs to be evaluated.

Even though TLR7 and TLR8 are both specific for ssRNA, our study shows a strong bias towards TLR8 in terms of cytokine secretion and IFN expression after stimulation with *M. stadtmanae* (chapter V). One possible explanation is that *M. stadtmanae*-derived RNA has a stronger affinity to TLR8 than to TLR7. Yet, it is more likely that a higher expression of TLR8 compared to TLR7 in human monocytes accounts for the stronger response through TLR8 (180). A recent study by Eng, *et al.* (215) supports our assumption that the TLR8 pathway is preferred by human monocytes and M Φ compared to the TLR7 pathway. To investigate the capability of TLR7 in recognition of *M. stadtmanae* RNA, another cell type with a higher TLR7 expression such as plasmacytoid DCs (pDCs) (216, 217) could be used. Also, overexpression of TLR7 in BLaER1 monocytes is conceivable. Altogether, this study confirms that TLR7 and TLR8 are versatile receptors which are not restricted to recognize a single group of organisms but rather enable recognition of various microbes. For that reason, RNA can be considered as universal and conserved MAMP across all domains.

3 *M. stadtmanae* activates a new inflammasome activation pathway

In chapter V, we have demonstrated that *M. stadtmanae* induces secretion of IL-1 β from primary and BLaER1 monocytes through activation of the NLRP3 inflammasome. The activation was shown to be dependent on all three inflammasome components, namely NLRP3, ASC, and caspase-1 and moreover dependent on TLR8 and MyD88 (Figure VII-1). This observation is consistent with the findings from Guo, *et al.* (187) who showed that TLR8-dependent NLRP3 inflammasome activation through RNA from HIV in human monocytes is possible. Furthermore, the authors claimed that TLR8-mediated NLRP3 activation is dependent on CatB as they observed a decrease in IL-1 β secretion using the CatB inhibitor CA-074 Me. We could not confirm these findings as BLaER1 CatB^{-/-} monocytes still secreted solid amounts of IL-1 β after stimulation with *M. stadtmanae* or R848 comparably to WT cells. Similar to our findings, Munoz-Planillo, *et al.* (47) showed that, although CA-074 Me inhibited IL-1 β secretion from murine bone marrow-derived macrophages (BMDMs), cells from CatB-deficient mice had no defects in NLRP3 activation. Off-target effects of CA-074 Me are most likely, which is supported by a study showing that CA-074 Me does not only inhibit CatB but also a wide range of different cathepsins (202). The authors mention that instead of CatB

cathepsin X (CatX) could be involved in NLRP3 activation (202). Therefore, the role of CatX in TLR8-mediated NLRP3 activation needs to be further determined.

The alternative activation pathway has been described for human monocytes but is not present in murine cells. Its key characteristics are the secretion of IL-1 β from living cells (no pyroptosis), independency of potassium efflux, and the absence of ASC specks (60). So far, the only known activator of alternative inflammasome activation is LPS. After binding of LPS to TLR4, the NLRP3 inflammasome is activated through a signal cascade initiated by TRIF and involving RIPK1, FADD, and caspase-8. In the case of *M. stadtmanae*- or R848-induced inflammasome activation, TRIF is dispensable as BLaER1 TRIF^{-/-} were still able to secrete normal amounts of IL-1 β , whereas LPS-stimulated cells did not. The exact mechanism of how TLR8 induces activation of the NLRP3 inflammasome remains elusive. While TRIF is negligible in TLR8-dependent inflammasome activation, an involvement of RIPK1, FADD, and caspase-8 cannot be ruled out. Both, TLR4- and TLR8-dependent inflammasome activation, show a delayed IL-1 β secretion. In detail, canonical inflammasome activation through LPS/nigericin leads to high secretion of IL-1 β already after four hours, whereas comparable amounts of IL-1 β in alternative activation are detectable after about 18 hours. A similar delay was demonstrated after stimulation with *M. stadtmanae* in this study.

The NLRP3 inflammasome activation induced by *M. stadtmanae* is distinct from canonical and non-canonical inflammasome activation. In detail, excessive cell death by pyroptosis and formation of ASC specks are key features of classical inflammasome activation pathways. Like LPS-mediated alternative inflammasome activation, *M. stadtmanae* does not induce ASC speck formation and only leads to cell death after comparatively long incubation times. Moreover, most canonical activation pathways can be inhibited by addition of high amounts of extracellular potassium as this blocks the mandatory efflux of cellular potassium (47, 59). Addition of potassium to the culture medium was shown to just have minor effects on *M. stadtmanae*-induced IL-1 β secretion (VI.2.3). Although Gross, *et al.* (188) have shown that canonical inflammasome activation does not necessarily require potassium efflux, the TLR8-mediated inflammasome activation by *M. stadtmanae* does rather reflect key features of the alternative inflammasome activation pathway (Table VII-1).

Canonical inflammasome activation results in pyroptosis through pore formation of GSDMD on the inner cell membrane. GSDMD does not only cause lysis of eukaryotic cells but is also able to kill bacteria, which is thought as an additional mechanism of bacterial clearance in the cytosol of the host cell (54). In contrast to most other cytokines, IL-1 β does not contain a signal leader peptide that results in secretion of the protein through the Golgi apparatus.

Instead, pro-IL-1 β is formed in the cytosol where caspase-1 catalyzes its maturation. However, until GSDMD has been discovered, the molecular mechanism of how active IL-1 β leaves the cell was unknown (218, 219). After GSDMD pores are formed, IL-1 β is passively secreted through the pores. Yet, GSDMD pores do not necessarily induce pyroptosis as also living cells can form GSDMD pores through which IL-1 β can leave the cell (55). It is unknown if GSDMD is also essential for IL-1 β secretion after alternative inflammasome activation. Using GSDMD^{-/-} BLaER1 monocytes, we demonstrated that neither alternative inflammasome activation through LPS nor *M. stadtmanae*-induced inflammasome activation are dependent on GSDMD as these KO cells are still able to secrete IL-1 β . To our knowledge, no other IL-1 β secretion mechanism besides GSDMD pore formation has been described. Identification of this alternative secretion mechanism is of great interest as it could serve as a new target to inhibit excessive and sterile inflammatory responses.

Table VII-1. Key characteristics of different NLRP3 inflammasome activation pathways.

	Canonical	Alternative	<i>M. stadtmanae</i> (TLR8)
NLRP3	Yes	Yes	Yes
ASC specks	Yes	No	No
Pyroptosis	Yes	No	Yes*
K⁺ efflux	Yes	No	No**
NEK7-dependency	Yes	unknown	largely
GSDMD-dependency	Yes	unknown	No
Species	Human & Mouse	Human only	Human only
Activators	cellular stress, bacterial Toxins, crystals, ...	LPS → TLR4 → TRIF → RIPK1/FADD /CASP8	RNA → TLR8 → MyD88

* not for synthetic TLR8 ligands such as R848

** only minor effects

Overall, the TLR8-dependent inflammasome activation induced by *M. stadtmanae* exhibits important key features of alternative activation, which suggests that, in addition to

TLR4, TLR8 can also trigger a variation of the alternative NLRP3 inflammasome activation. Yet, the detailed molecular mechanism needs to be determined. Since TLR8 is located in endo(lyso-)somal compartments, it is possible that engagement of TLR8 induces leakage of lysosomal components into the cytosol. In various settings, lysosomal leakage was shown to trigger NLRP3 inflammasome activation (38, 200, 202). Other possible mechanisms include the formation of reactive oxygen species (ROS) (188, 220) or calcium ion (Ca^{2+}) mobilization from the ER (221, 222). Further clarification is necessary, e.g. through usage of specific inhibitors or generation of KO cells targeting proteins involved in ROS generation or specific Ca^{2+} channels.

4 Is RNA the only key to *M. stadtmanae*-mediated immune responses?

Our study demonstrates that the methanogenic archaeon *M. stadtmanae* is recognized by its RNA through the endosomal receptors TLR7 and TLR8. However, in our setting myeloid immune cells are challenged with *M. stadtmanae* in an oxygenic environment, which does not reflect the situation *in vivo* since *M. stadtmanae* is strictly anaerobic (223). Though the archaeon has two genes encoding for oxygen detoxification proteins (224), it does not survive exposure to oxygen for a longer time. The actual effect of living archaea on immune cells or IECs has so far not been evaluated. Therefore, it cannot be completely ruled out that other mechanisms besides RNA recognition might play a role in the response induction against *M. stadtmanae*. It is possible that living cells of *M. stadtmanae* actively produce and secrete certain molecules like dinucleotides or peptides that could stimulate the immune system. Archaea also have the capability to secrete outer membrane vesicles (OMVs), which could deliver molecules or antigens that stimulate the innate or adaptive immune system (225). Yet, the role of OMVs in *M. stadtmanae*-induced immune cell activation is unclear. Moreover, some bacterial species can actively translocate from the lysosome into the cytosol (211). In this scenario, endosomal receptors are ineffective, while cytosolic receptors become important as they sense cytosolic pathogens. Whether archaea could actively overcome lysosomal degradation through translocation into the cytosol is also unknown. Furthermore, *M. stadtmanae* is able to form biofilms (226) and methanogenic archaea were shown to express surface glycans and adhesion-like proteins (95, 224). Bacteria in biofilms were shown to restrict their clearance through inhibition of phagocytosis and limitation of the immune response (227, 228). Therefore, biofilms can serve as an immune evasion strategy and significantly increase the virulence of pathogens. How biofilms influence the *M. stadtmanae*-induced immune responses is one of the key questions.

Besides the issue of how biofilms and living archaea alter the immune response, the role of archaeal lipids as stimulators of the innate and adaptive immune system also needs to be determined. Several studies have shown that liposomes derived from archaeal lipids, so-called archaeosomes, have a strong adjuvant activity (229, 230). Bacteria and eukaryotes utilize unbranched lipids species which are ester-linked to glycerol. In contrast, archaeal glycerol lipids are ether-linked and based on branched isoprenoid lipids (87). Several mechanisms of how archaeosomes provide an adjuvant function have been discussed (231) including the observation that archaeosomes show a much higher phagocytosis rate (3- to 53-fold) on murine peritoneal J774A.1 MΦs compared to conventional liposomes (232). This could be an explanation for the efficient phagocytic uptake of *M. stadtmanae* which has been described by Bang, *et al.* (114) and was also confirmed in this study (chapter IV). We suggest that the archaeal lipids are recognized by receptors on the surface of phagocytes like monocytes and DCs. These receptors might account for enhanced phagocytosis of *M. stadtmanae*, thus leading transportation of the archaea into the endo(lyso-)somes where the immune response is induced through engagement of TLR7 and TLR8. The just recently identified gut-associated archaeon *M. luminyensis* on the other hand exhibits only a low phagocytosis rate (chapter IV). Becker, *et al.* (233) have shown that *M. luminyensis* carries a unique lipid profile which is distinct from other methanogens. It has been proposed that the uptake of several archaeosomes is mediated through the phosphatidylserine receptor (234). Therefore, it is conceivable that *M. luminyensis* is inefficiently recognized by this (or a similar) receptor which leads to a decreased phagocytosis of *M. luminyensis* compared to *M. stadtmanae*. Archaeal lipids themselves might not necessarily serve as MAMPs but they could enhance the uptake of archaea which is directly linked to a TLR7/TLR8-mediated response as only phagocytosed archaea can be recognized.

5 Is a disease-association of archaea possible? Consequences and future perspectives

The question if archaea may function as pathogens, contribute to disease progression, or promote the growth of pathogenic bacteria through syntrophy has been discussed extensively (98, 203, 204, 235-240). Yet, a pathogenic archaeon has not been identified so far. This study is mainly focused on *M. stadtmanae*, a human gut inhabitant that appears to be associated with IBD (111) or lung hypersensitivity (113). *M. stadtmanae* is recognized through its RNA and induces strong secretion of inflammatory cytokines like TNF- α or IL-6. Moreover, we demonstrated that *M. stadtmanae* activates the NLRP3 inflammasome in TLR8-dependent pathway, while resembling key features of alternative inflammasome activation. This

observation is different from the other intestinal methanogenic archaea *M. smithii* (111, 114) and *M. luminyensis* (this study, chapter IV) which both only induce mild inflammatory responses. Blais-Lecours, *et al.* (111) recognized an increased prevalence of *M. stadtmanae* in IBD patients and elevated levels of antibodies against *M. stadtmanae*. On the other hand, IBD patients were found to have a decreased methane production (241) and lower loads of *M. smithii* (242). Based on these studies, *M. stadtmanae* may replace *M. smithii* as the major intestinal methanogen in IBD patients (Figure VII-2). Thereby, *M. stadtmanae* could induce excessive immune response through its strong immunogenic activity and its potential to activate the NLRP3 inflammasome as demonstrated in this study. Additionally, replacement of *M. smithii* could alter the composition of the whole microbiota as many fermentative bacteria depend on syntrophy with this archaeon. In contrast, *M. stadtmanae* has different metabolic functions which could lead to dysbiosis of the microbiota. The keystone-pathogen hypothesis proposed by Hajishengallis, *et al.* (243) implies that certain low-abundance microorganisms, the so-called keystone-pathogens, could turn a healthy microbiota into dysbiosis, thereby promoting inflammatory diseases. Based on this hypothesis, *M. stadtmanae* could serve as such a keystone-pathogen and alter the composition of the microbiota through replacement of *M. smithii*.

High exposure to bioaerosols in industrial animal farms can lead to pulmonary diseases such as farmer's lung. It was shown that aerosols from dairy barns contain high amounts of *M. smithii* and *M. stadtmanae* (112) and furthermore, Bernatchez, *et al.* (113) demonstrated that aerosolized *M. stadtmanae* induces lung hypersensitivity in mice with symptoms resembling those of farmer's lung. In contrast, *M. smithii* does not induce a strong inflammatory phenotype. In this setting, it is conceivable that *M. stadtmanae* may not act as a 'classical' pathogen but can cause inflammatory diseases through its high immunostimulatory activity. Our propagated model of *M. stadtmanae*-induced immune cell activation needs to be confirmed for this disease model. We have clearly demonstrated that *M. stadtmanae* induces activation of murine dendritic cells through mTLR7 as key receptor. Based on this observation, we assume that *M. stadtmanae*-induced lung hypersensitivity is TLR7-mediated. Therefore, it is necessary to use the same mouse model that was used in the study of Bernatchez, *et al.* (113) and compare the symptoms of WT and mTLR7^{-/-} mice. If our assumption is correct, lung inflammation in TLR7^{-/-} mice should become less severe as the innate immune system should not be activated.

Overall, the role of archaea in human disease is still a mystery that must be solved. This is of clinical importance as treatment strategies of archaea-associated diseases must be

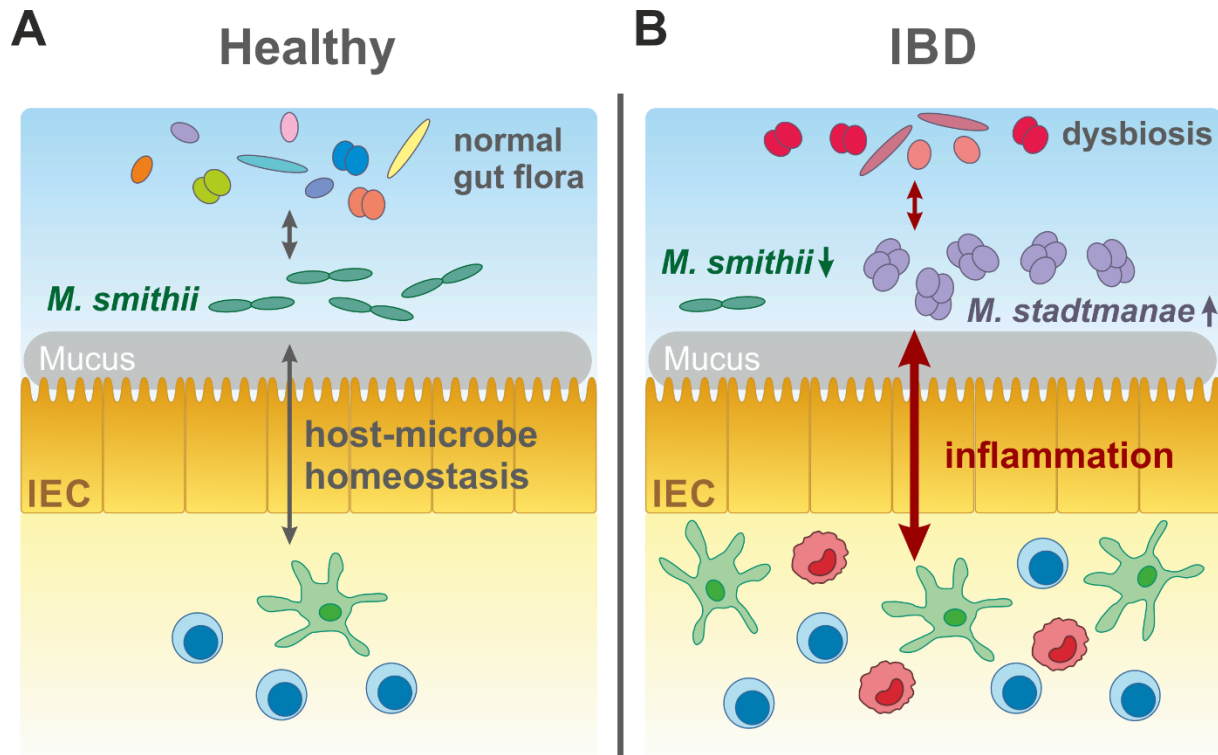


Figure VII-2. Hypothetic model of how *M. stadtmanae* contributes to IBD.

(A) In healthy individuals, *M. smithii* is present in a relatively high abundance and promotes high diversity of the gut microbiota. The immune system and the microbiota homeostatic condition without induction excessive immune responses. (B) In IBD, the abundance of *M. smithii* is decreased, and (partially) replaced by *M. stadtmanae* which promotes alterations in the microbiota with reduced diversity and outgrowth of other, potentially harmful microbes (dysbiosis). The presence of certain microbes including *M. stadtmanae* leads to an increased influx of immune cells and excessive immune responses. (IBD, inflammatory bowel disease; IEC, intestinal epithelial cells)

modified. Since archaeal cell walls and archaeal ribosomes are fundamentally different from those of bacteria, most antibiotics have no effect on archaeal growth and vitality (244). However, methanogenic archaea are susceptible to imidazole derivatives (245) and lovastatin (246). This could be important for the treatment of periodontitis and brain abscesses where methanogenic archaea are most likely involved. For example, the use of metronidazole in brain abscesses has exceptionally improved its clinical outcome. There is a conceivable link between this drug and association of methanogens in this disease because metronidazole is one of the few antibiotics that is also effective against methanogenic archaea (110). To evaluate the disease association of archaea, archaea-specific detection methods should be routinely included in medical microbiology and in future microbiome studies.

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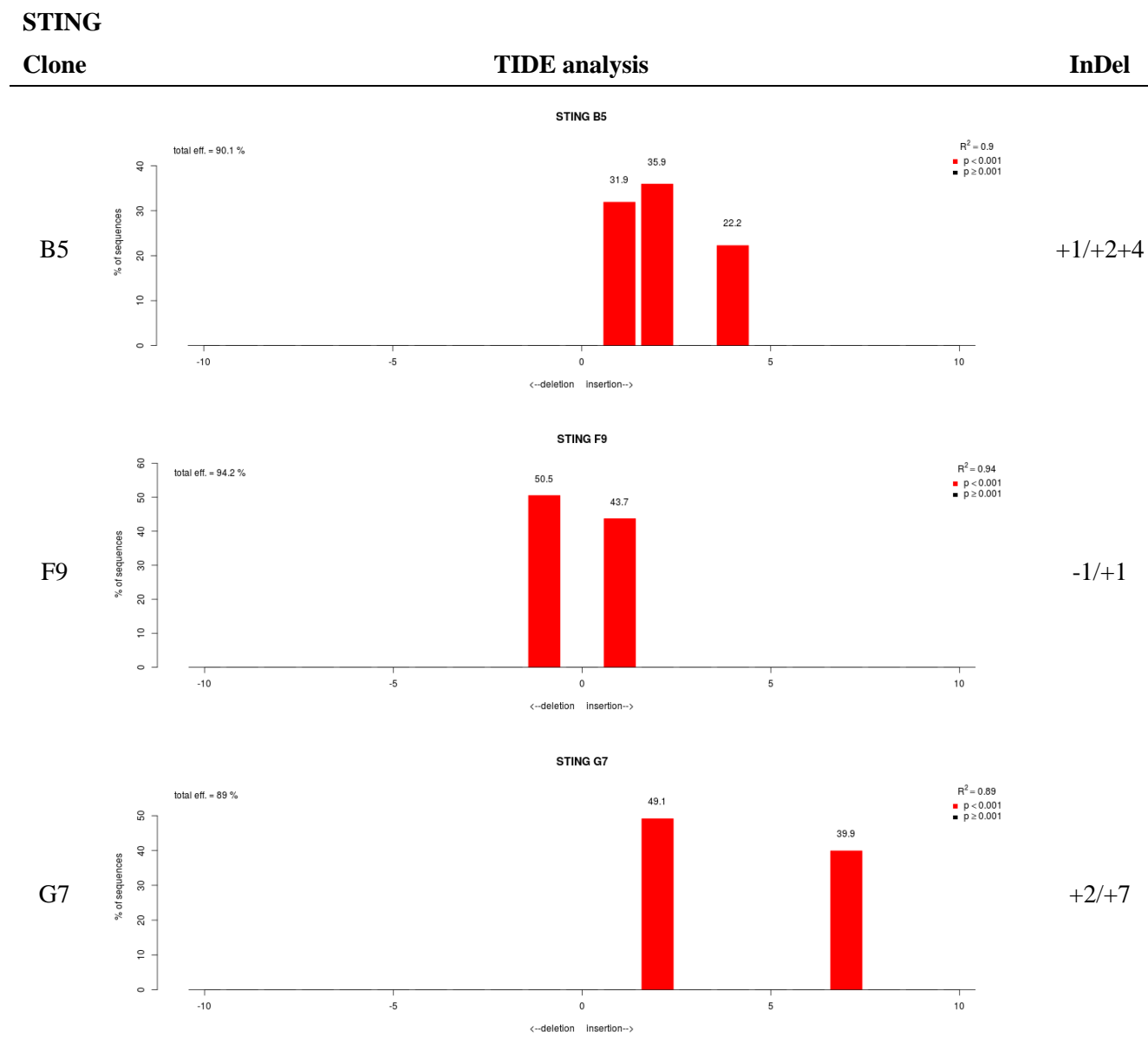
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IX Appendix

1 TIDE analysis



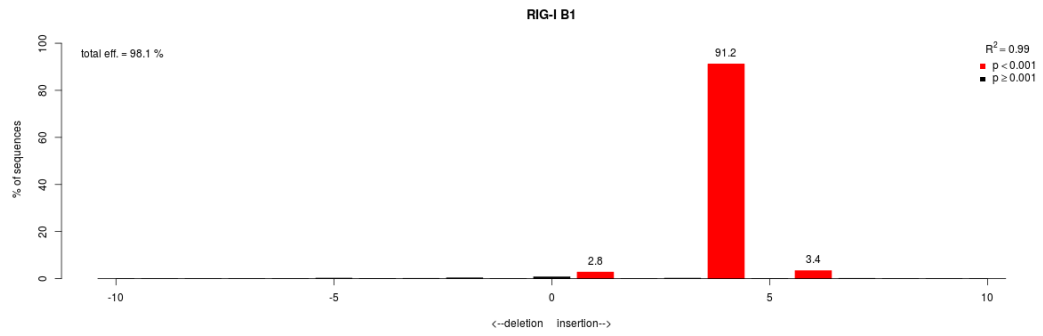
RIG-I

Clone

TIDE analysis

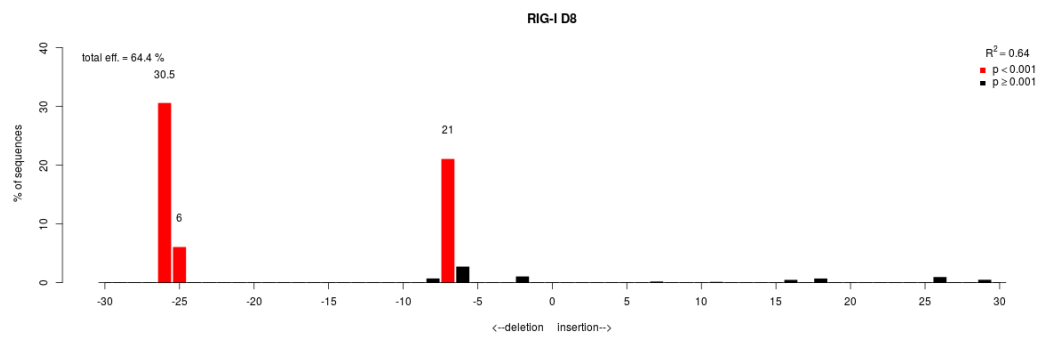
InDel

B1



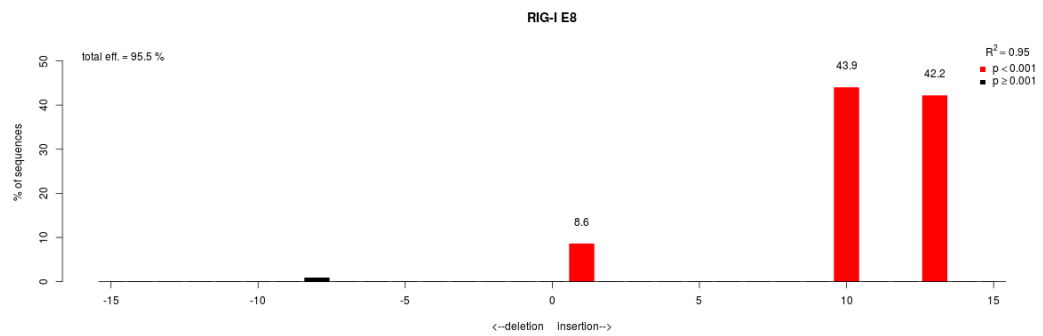
+4/+4

D8



-26/-7

E8



+10/+13

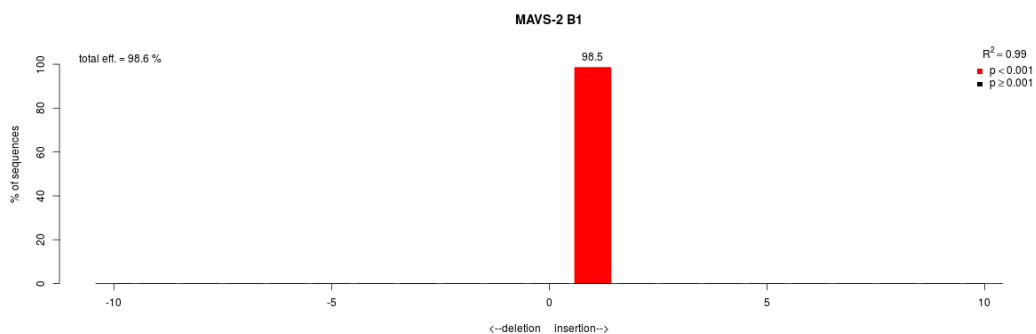
MAVS

Clone

TIDE analysis

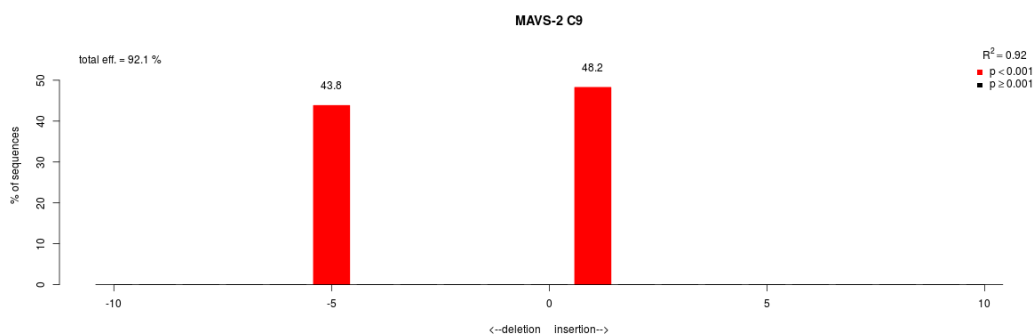
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B1



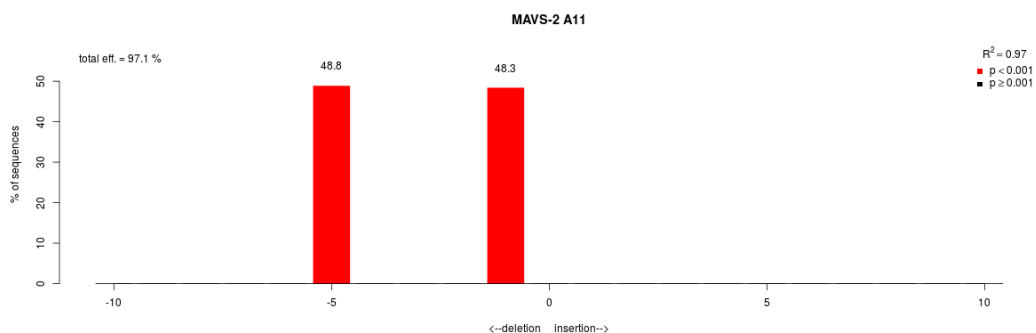
+1/+1

C9



-5/+1

A11



-5/-1

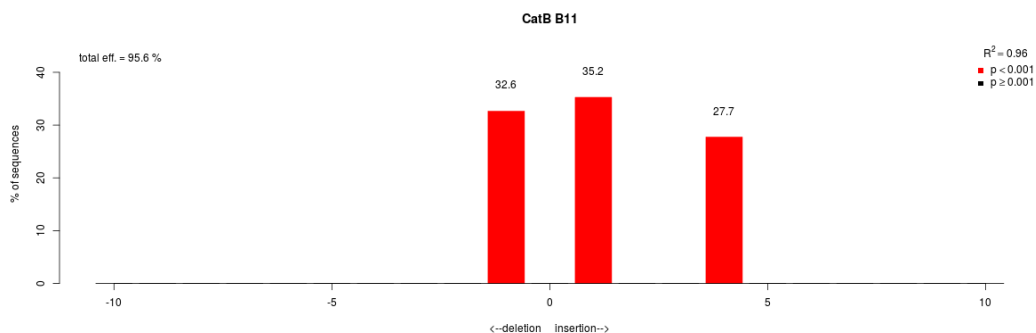
CatB

Clone

TIDE analysis

InDel

B11



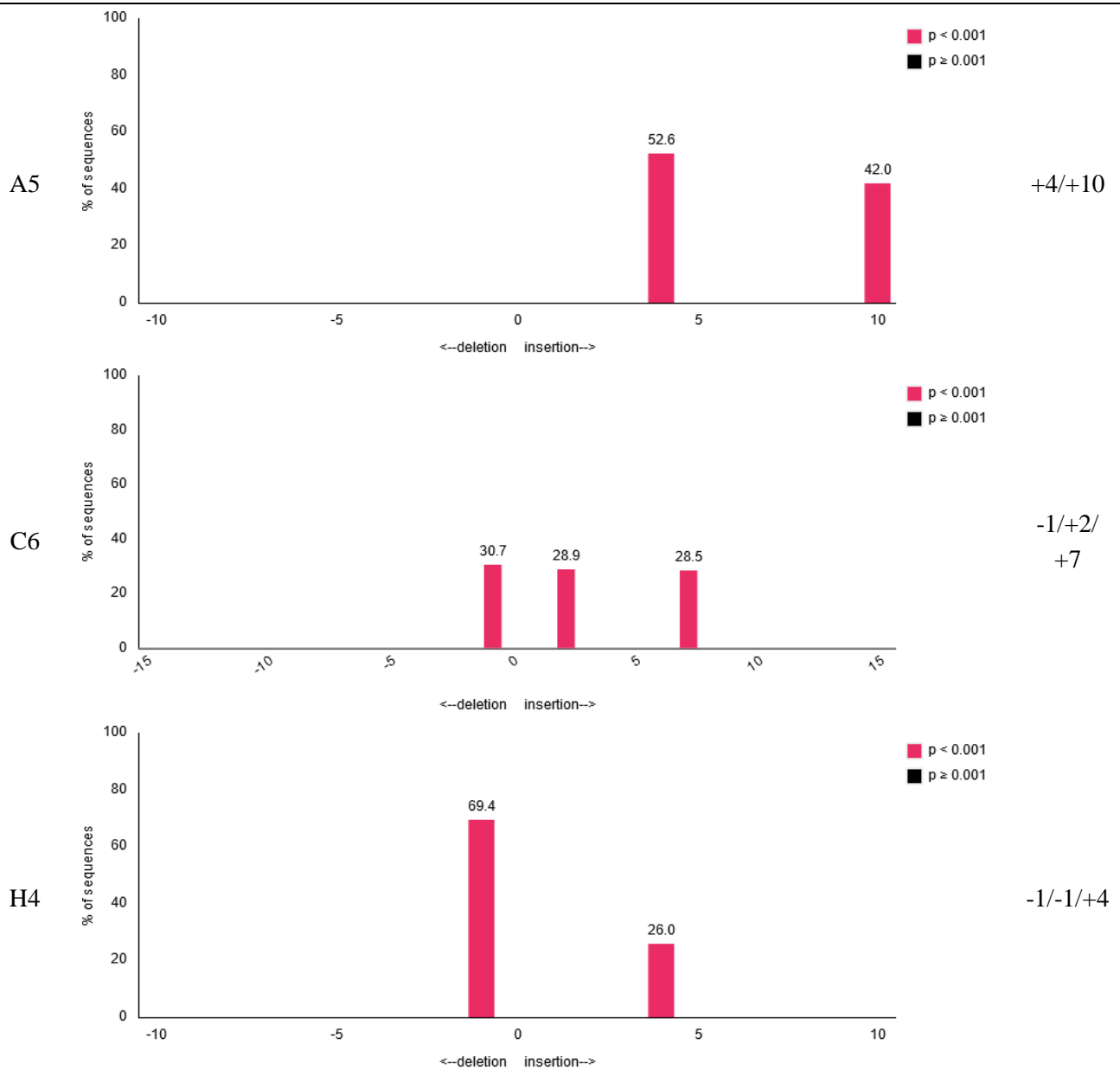
-1/+1/
+4

GSDMD

Clone

TIDE analysis

InDel



2 Figures

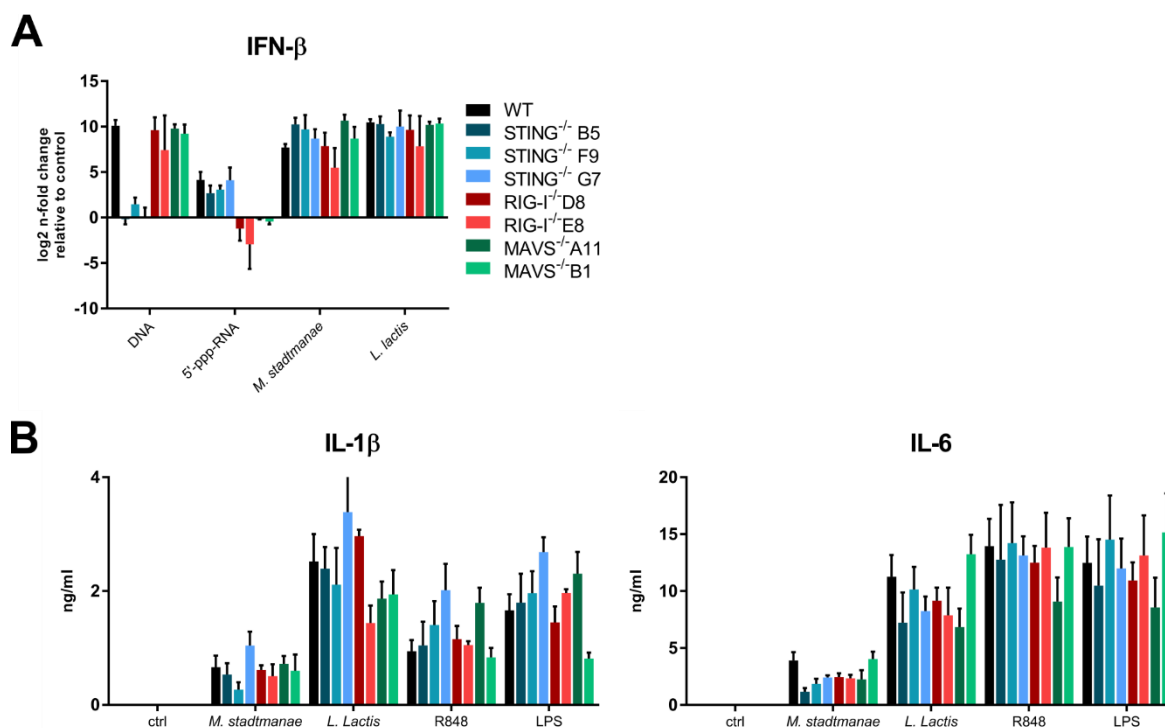


Figure IX-1. Response to *M. stadtmanae* is independent of major cytosolic nucleic acid recognition pathways (all clones).

(A) The expression of IFN- β in BLaER1 STING^{-/-}, RIG-I^{-/-} or MAVS^{-/-} monocytes after stimulation with *M. stadtmanae* (100:1), 0.5 μ g/ml 5'-ppp-RNA, or 1 μ g/ml plasmid DNA for 6 h was measured by qRT PCR. The expression ratios of IFN- β gene to the reference gene HPRT are shown as log₂ n-fold induction relative to the unstimulated control cells. (B) IL-1 β and IL-6 secretion in the supernatants of BLaER1 WT, STING^{-/-}, RIG-I^{-/-} or MAVS^{-/-} monocytes stimulated for 18 h with either 10⁷ cells of *M. stadtmanae*, 10⁷ cells/ml of *L. lactis*, 50 ng/ml LPS, or 5 μ g/ml of R848. The data are shown as the mean \pm SEM of three independent experiments (n=3) in (A) or four independent experiments (n=4) in (B).

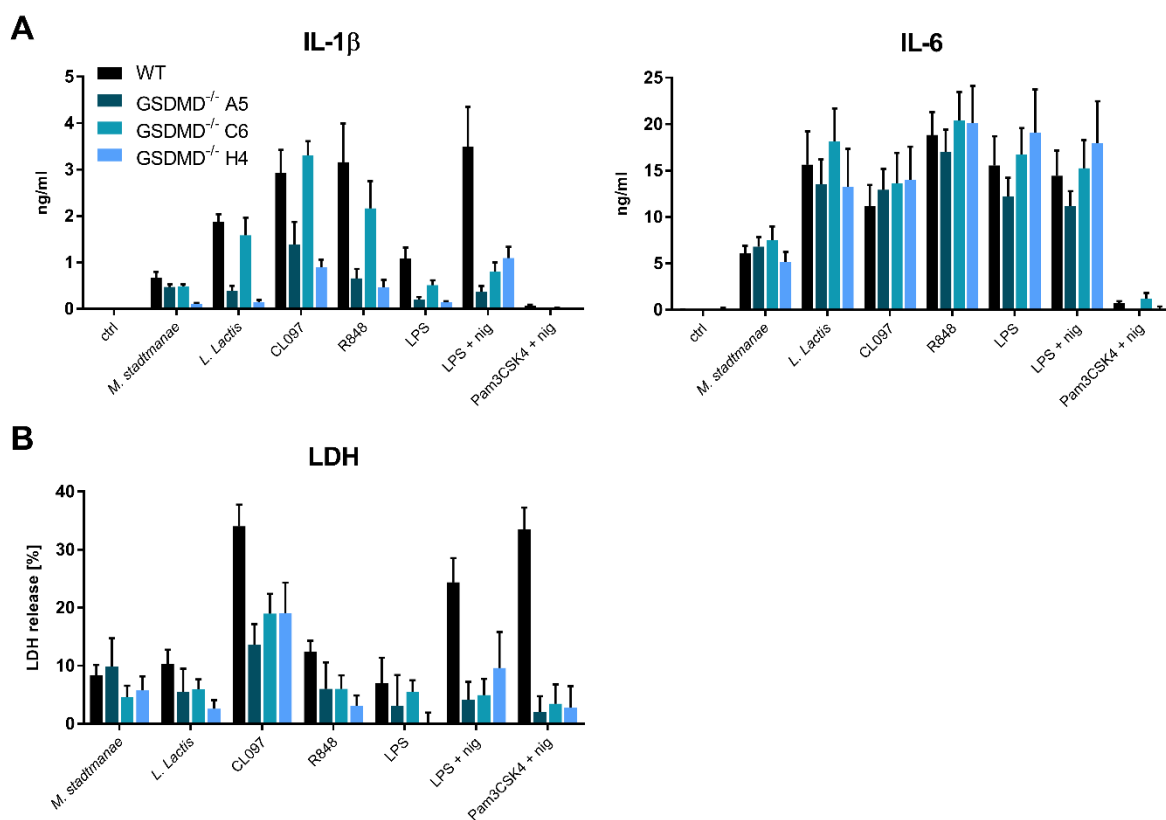


Figure IX-2. Inflammation activation and LDH release are independent of GSDMD (all clones).

(A) IL-1 β and IL-6 secretion and (B) release of LDH in the supernatants of BLaER1 WT or GSDMD^{-/-} monocytes stimulated for 18 h with either 10⁷ cells of *M. stadtmanae*, 5 μ g/ml of CL097 5 μ g/ml R848 or 50 ng/ml LPS. The data are shown as the mean \pm SEM of four independent experiments (n=4). (GSDMD, gasdermin D; IL-1 β , interleukin 1 beta; IL-6, interleukin 6; LDH, lactate dehydrogenase; nig, nigericin)

X Acknowledgements

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XI Curriculum Vitae

Tim Vierbuchen

Nationality German
 Date of birth February 18th, 1989
 Address Alsterdorfer Straße 194
 22297 Hamburg

Education

- 03/2015 – present **PhD thesis** at Research Center Borstel, Division of Innate Immunity, Borstel, Germany
 Supervisor: Prof. Dr. Holger Heine
 Topic: '*Interaction of Methanogenic Archaea with the Human Immune System*'
- Analysis of the immunogenic properties of different human-associated archaea.
 - Identification of RNA as the main MAMP of *Methanosphaera stadtmanae* for human and murine immune cells; mTLR7 and hTLR8 as relevant receptors.
 - Description of a TLR8-dependent NLRP3 inflammasome activation pathway with hallmarks of canonical and alternative inflammasome activation.
 - Supervision of a Master student (Hanna Rosigkeit).
 - PhD representative of Borstel Biomedical Research School (BBS) (02/2016 – 02/2017)
- 07/2013 – 04/2014 **Master thesis** at Helmholtz Institute of Biomedical Engineering, Department of Experimental Medicine and Immunotherapy, Aachen, Germany
 Topic: '*In vitro Characterization of Novel Cytolytic Fusion Proteins and Evaluation of a Synergistic Treatment Strategy*'
- 04/2012 – 04/2014 **MSc, Molecular and Applied Biotechnology**
 RWTH Aachen University, Aachen, Germany
 Major in Red Biotechnology
- 07/2011 – 02/2012 **Bachelor thesis** at University Hospital Aachen, Institute of Medical Microbiology, National Reference Center for Streptococci, Aachen, Germany
 Topic: '*Influence of Subinhibitory Concentrations of Clarithromycin on Resistance Development of Clinical Isolates of Streptococcus pneumoniae*'
- 10/2008 – 02/2012 **BSc, Biotechnology**
 FH Aachen (University of Applied Sciences), Juelich, Germany
 with practical training semester

Employment

- 08/2014 – 02/2015 **Research associate** at University Hospital Hamburg-Eppendorf, Medical Clinic I, Infectious Diseases, Hamburg, Germany
- 02/2012 – 04/2013 **Student research assistant** at University Hospital Aachen, Institute for Medical Microbiology, National Reference Center for Streptococci, Aachen, Germany

Scholarship and Grants

- 09/2017 **EFIS Conference Support Grant**, European Federation of Immunological Societies
- 10/2012 **Education Fund** of RWTH Aachen University supported by Evonik Industries AG
– 04/2014

Membership

- 05/2016 German Society of Immunology (DGfI)
- present

Publications

Vierbuchen T, Bang C, Rosigkeit H, Schmitz RA, Heine H (2017) The Human-Associated Archaeon *Methanosphaera Stadtmanae* is Recognized by Its RNA and Induces TLR8-Dependent NLRP3 Inflammasome Activation. *Front. Immunol.* 8:1535

Bang C, **Vierbuchen T**; Gutschmann T, Heine H, Schmitz RA (2017) Immunogenic properties of the human gut-associated archaeon *Methanomassiliicoccus luminyensis* and its susceptibility to antimicrobial peptides. *PLoS One.* 12(10):e0185919

Cremer C, **Vierbuchen T**, Hein L, Fischer R, Barth S, Nachreiner T (2015) Angiogenin mutants as novel effector molecules for the generation of fusion proteins with increased cytotoxic potential. *J Immunother.* 38(3):85-95

Conference Contributions

- Oral presentation 46th Annual Meeting of the German Society for Immunology (DGfI) 2016, Hamburg, Germany
- Poster presentation Development of Tissue- and Pathogen-specific Cellular Innate Immunity 2017, Freiburg, Germany
47th Annual Meeting of the German Society for Immunology (DGfI) 2017, Erlangen, Germany
Keystone Symposium on Nucleic Acid Sensing Pathways 2016, Dresden, Germany
14th Biennial Meeting International Endotoxin and Innate Immunity Society (IEIIS) 2016, Hamburg, Germany

Workshops/Summer Schools

- 03/2017 13th Spring School on Immunology, DGfI, Ettal, Germany (poster presentation)
- 10/2016 8th Autumn School – Current Concepts of Immunology, DGfI, Merseburg, Germany (oral presentation)

XII Eidesstattliche Erklärung

Hiermit erkläre ich, dass die vorliegende Abhandlung, abgesehen von der Beratung durch die Betreuer, in Inhalt und Form selbständig von mir verfasst wurde, wobei Teile der Arbeit bereits an anderen Stellen veröffentlicht wurden. Hierbei wurden ausschließlich die benannten Hilfsmittel und Quellen verwendet. Wörtlich oder sinngemäß übernommenes Gedankengut wurde als solches kenntlich gemacht. Diese Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden.

Hamburg, den _____

Ort, Datum

Unterschrift