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DISSERTATION

Receptors and Signaling Pathways for the Detection of Viable Bacteria by Human Phagocytes

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Abstract

The innate immune system serves as the host's first line of defense against microbial invasion. *Per definitionem*, all microbes contain highly conserved molecular structures termed pathogen-associated molecular patterns (PAMPs). PAMPs are sensed by innate immune cells through a vast array of germ-line encoded receptors, called pattern recognition receptors (PRRs). Beyond the discrimination between 'self' and 'non-self' structures by PRR ligation, the innate immune system makes finer distinctions. It was previously shown that mouse phagocytes differentiate between viable and dead bacteria independently of replication or virulence factors. Bacterial viability is indicated by the presence of bacterial mRNA, which was previously identified as the first so-called 'viability-associated-PAMP' (*vita*-PAMP).

The present study systematically analyzes the capacity of human primary phagocytes to distinguish viable from dead bacteria and the immunological consequences. In contrast to earlier observations in murine phagocytes, human cells produce low levels of interferon-β (IFN-β) in response to live but also dead bacteria. Instead, human cells selectively induced tumor necrosis factor α (TNFα) in response to live bacteria, which is independent of bacterial viability in mice. As in murine cells, the release of mature Interleukin (IL)-1β was induced only upon detection of viable bacteria. Using pharmacological inhibitors as well as lentivirally transduced shRNA-mediated gene silencing, we observed a dichotomous signaling pattern of cytokine production in response to live *Escherichia coli*. In $CD14^{+}CD16^{-}$ monocytes or monocyte-derived dendritic cells, respectively, the recognition of bacterial viability differentially drives IL-1β and TNFα responses via two seemingly separate signaling pathways. Production of mature IL-1β required NLR family pyrin domain containing 3 (NLRP3), and Apoptosis-associated speck-like protein containing a CARD (ASC), and was at least partially dependent on the signaling adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF), Toll like receptor (TLR)-4, and Bruton's tyrosine kinase (BTK). In contrast, TNFα production was largely dependent on TLR8 and Phosphoinositide 3-kinase (PI3K) signaling. Similar to murine cells, bacteria-induced IFN-β production required TLR4 and TRIF. These data demonstrate the capacity of human phagocytes to sense and differentially respond to live and dead bacteria. The study sheds lights on the signaling requirements for *vita*-PAMP-sensing in human phagocytes, and it highlights differences from and similarities to the murine system. The findings make an important contribution

to our understanding of host-pathogen interaction in humans and provide potential molecular targets for vaccine adjuvants or host-directed adjuvant immunotherapies.

Zusammenfassung

Das angeborene Immunsystem bildet die erste Verteidigungslinie gegen mikrobielle Invasion. *Per definitionem* enthalten alle Mikroben hoch konservierte, molekulare Strukturen, die als Pathogen-assoziierte molekulare Muster (PAMPs) bezeichnet werden. PAMPs werden durch angeborene Immunzellen über ein breites Repertoire an in der Keimbahn kodierten Rezeptoren, die so genannten pattern recognition receptors (PRRs), erkannt. Neben der Unterscheidung von körpereigenen und körperfremden Strukturen nimmt das angeborenen Immunsystem eine weitere, feinere Unterscheidung der detektierten Strukturen vor. So konnte gezeigt werden, dass murine Phagozyten lebende von toten Bakterien unterscheiden können, unabhängig von Replikation und dem Vorhandensein von Viruzlenzfaktoren.

Die vorliegende Arbeit analysiert systematisch die Fähigkeit humaner, primärer Phagozyten zwischen lebenden und toten Bakterien zu unterscheiden sowie die sich daraus ergebenden immunologischen Konsequenzen. Im Gegensatz zu den Beobachtungen mit murinen Phagozyten produzierten humane Zellen geringe Mengen an Interferon-β (IFN-β) in Antwort auf lebende und tote Bakterien. Humane Zellen induzierten hingegen Tumornekrosefaktor (TNF) α selektiv nach Detektion lebender Bakterien, welches murine Zellen unabhängig von bakterieller Vitalität produzieren. Wie murine Zellen, sezernierten humane Zellen biologisch aktives Interleukin (IL)-1β nur nach Detektion lebender Bakterien. Mit Hilfe von pharmakologischen Inhibitoren und shRNA-vermittelten Gen-silencing mittels lentiviraler Transduktion, konnte eine Dichotomie in den Signalwegen der Zytokinantwort humaner Phagozyten gezeigt werden. Die Detektion lebender Bakterien induzierte die Produktion von IL-1β und TNFα durch $CD14^{+}CD16^{-}$ Monozyten bzw. von Monozyten abgeleitete dendritische Zellen über zwei scheinbar separate Signalwege. Die Produktion des prozessierten, biologisch wirksamen IL-1 β setzte das Vorhandensein von NLR Family Pyrin Domain Containing 3 (NLRP3) und Apoptosis-associated speck-like protein containing a CARD (ASC) voraus, und hing zumindest teilweise vom Signaladaptormolekül adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF), Toll like receptor (TLR)-4 und Bruton's tyrosine kinase (BTK) ab. Im Gegensatz dazu zeigte sich die Produktion von TNFα abhängig von TLR8 and Phosphoinositide 3-kinase (PI3K). Ähnlich zu murinen Zellen erfordert die Bakterien-induzierte Produktion von IFN-β durch humane Zellen TLR4 und TRIF.

Die vorliegenden Daten zeigen die Fähigkeit humaner Phagozyten lebende und tote Bakterien zu detektieren und mit einer differenzierten Immunantwort zu reagieren. Ferner beleuchtet die Studie die notwendigen Signalwege zum Erkennen von *vita*-PAMPs durch humane Phagozyten. Diese Erkenntnisse liefern einen wichtigen Beitrag zu unserem Verständnis von Wirt-Pathogen-Interaktionen und zeigen mögliche Zielmoleküle für Impfstoffadujuvantien oder Wirt-zentrierte Immunotherapeutika auf.

1 Introduction

1.1 Innate Immune Systems Components and their Function

From the moment of birth, we engage in a continuous interaction with microorganisms, which may include potentially dangerous encounters with pathogens. To protect from infection, all known organisms (including microorganisms) have evolved early defense systems. Conceptually, such systems would ideally fulfill the following criteria; *i*) immediate availability and responsiveness, *ii*) a capacity for precise risk evaluation and, importantly, *iii*) ability for rapid and effective host-protective responses. In general, these criteria are thought to be met by the innate immune system. In most of the cases, it detects microbial threats and efficaciously clears microbial invaders prior to full-blown infection and the appearance of clinical symptoms[1]. Hence, the innate immune system has a remarkable capacity for risk assessment[2].

The innate immune system consists of a large variety of cells and mediator and effector molecules[3]. Epithelia cells as well as associated structures like mucus form a first border at body surfaces such as the skin or the lung. Epithelia also possess direct antimicrobial functions e.g. through the secretion of antimicrobial peptides. A broad range of invading or colonizing microbes can be taken up by specialized cells, called professional phagocytes. This group of cells encompasses macrophages, monocytes, dendritic cells (DCs), and granulocytes, most prominently neutrophils. Macrophages, neutrophils, and monocytes elicit multiple, direct effector responses to defend the host[3, 4]. In contrast, DCs contribute indirectly to host defense by activating the adaptive arm of the immune system[4]. DCs process the microbial cargo and generate antigenic peptides, which are presented to T cells in combination with co-stimulatory molecules. Through the production of distinct cytokines, DC can shape and control the ensuing adaptive immune response. Further cellular components include natural killer cells, which play a major role in controlling viral infections and eosinophils, basophils and mast cells, which in turn are essential to defend parasites.[3] More recently, innate lymphoid cells (ILC) were discovered and described as an innate immune cell type that contributes to host defense and inflammation at mucosal surfaces[5]. Besides these cellular components, the innate immune system also comprises a large array of humoral sensors and effectors, including acute phase proteins like pentraxins, the complement system, antimicrobial peptides, and type I interferons (IFNs). Produced early during infection, acute phase

proteins act as opsonins, which tag microbes for phagocytosis, as regulators of coagulation, and activators of the complement system[3]. The complement system is activated by different triggers, which results in membrane destabilization and ultimately the killing of microbes and infected cells. Also, the complement system contributes to chemotaxis of phagocytes to the site of infection, their activation and phagocytosis of microbes through opsonization[6]. Type I IFNs are crucial for the defense against viruses, but they can exert both beneficial and detrimental effects during bacterial infection[3, 7]. Proinflammatory cytokines, released during infection upon activation of immune cells, are critical orchestrators of innate and adaptive immune responses to infection. Particularly, tumor necrosis factor (TNF)α, Interleukin (IL)-6 and IL-1β are known to be essential drivers of host protective immunity. Upon activation, several nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) assemble into large cytosolic protein complexes termed inflammasomes, which are required for the processing of prol-IL1β and pro-IL-18 into the active cytokines[3].

The efficacious and adequate function of the innate immune system quintessentially depends on its capacity for accurate detection and measurement of the microbial threat imposed by a given microbial encounter[8].

1.2 Recognition of Microbial Patterns by the Innate Immune System

In his now famous 1989 conference concept paper, the late Charles Janeway Jr. outlined what later became known as the “pattern recognition theory”. He predicted correctly that the innate immune system is activated through the detection of unique, highly conserved molecular structures, which he termed pathogen-associated molecular patterns (PAMPs)[9]. *Per definitionem*, PAMPs are essential structural components of microorganisms such as bacterial or fungal cell wall components or viral nucleic acids, which uniquely mark them as “non-self”. Hence, the detection of PAMPs through pattern recognition receptors (PRRs) expressed by cells of the innate immune system makes it possible to discriminate between self and non-self structures. In most cases, PRRs can be activated by several ligands[10]. The PRR family comprises several groups of evolutionarily conserved receptors: Toll-like receptors (TLRs), NLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs). The more recently discovered cytosolic deoxyribonucleic acid (DNA) receptor cyclic-GMP-AMP synthase (cGAS, cGAMP synthase) does so far not belong to a larger family of PRRs. Recognition of the

respective ligands by the PRRs results in the transcription of chemokines, cytokines and type I IFNs, and also influences cellular processes such as cytokine processing or cell death[11].

1.2.1 TLRs

Since the discovery of the first mammalian member, TLR4[12], the family of TLRs has grown to ten human and twelve murine identified type I transmembrane glycoproteins.
Depending on their cellular localization, TLRs can be divided into two groups: cell surface TLRs, such as TLR1, -2 and -4 to -6, sense extracellular PAMPs. For instance, TLR4 detects the major cell wall component of gram-negative bacteria lipopolysaccharide (LPS), and TLR5 detects flagellin present in flagellated bacteria. Endosomal TLRs 3, 7, 8 and 9 recognize nucleic acids. TLR3 detects double-stranded ribonucleic acid (dsRNA) found in viruses, TLR7 and TLR8 sense single-stranded RNA (ssRNA), and TLR9 detects CpG-rich DNA[13]. Recently, TLR8 has been suggested as a sensor of bacterial RNA[14, 15]. Activation of TLRs through their respective ligands requires leucine-rich repeats (LRRs) and induces receptor homo- or heterodimerization. Subsequently, the cytosolic Toll/Interleukin-1 receptor (TIR) domain undergoes changes in conformation and recruits adaptor molecules such as Myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF) to induce intracellular signaling cascades. All TLRs signal via MyD88, whereas TLR3 exclusively requires TRIF for downstream signaling. TLR4 forms an interesting exception. During the early phase of activation, it signals via MyD88. Upon recruitment to the endo(lyso-)somal compartment, TLR4 signals primarily through TRIF[13]. It has been suggested that TLR5 can also signal via TRIF[16]. However, the adaptor molecules MyD88 and TRIF do not exclusively associate with TLRs. MyD88 is a critical signaling component of cytokine receptors IL-1R1 and transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI)[17]. TRIF has been shown to interact with RNA-helicases DEAD-box helicase (DDX) 1, DDX21, and DEAH-box helicase (DHX) 36[18].
TLR-signaling via MyD88 induces the assembly of a multimeric protein complex, sometimes referred to as the ‘Myddosome’, consisting of multimers of MyD88 and members of the interleukin-1 receptor-associated kinase (IRAK) family. The downstream signaling cascade involves transforming growth factor beta-activated kinase 1 (TAK1), which activates nuclear factor 'kappa-light-chain-enhancer' of

activated B-cells (NF-κB) and mitogen-activated protein kinases *(*MAPK). NF-κB relocates into the nucleus and initiates the transcription of inflammatory cytokine-encoding genes such as TNFα, IL-6 or pro-IL-1β. MAPK like p38 or c-Jun N-terminal kinase (JNK) also activate transcription factors for various cytokines. In the case of plasmacytoid DCs (pDCs), MyD88 serves as the adaptor for TLR7- and TLR9-derived signals to induce interferon regulatory factor (IRF) 7-dependent transcription of type I IFN secretion, in addition to NF-κB activation. TRIF signals mainly via TAK1 to trigger NF-κB and MAPK activation, and via TANK-binding kinase 1 (TBK1) to activate IRF3-mediated production of type I IFNs.[19]

1.2.2 NLRs

NLRs form a large family of 22 proteins identified in humans and 34 in mice. The receptors are characterized by a tripartite organization: the LRRs at the C-terminus, a central NOD domain and a N-terminal domain required for downstream protein-protein interaction[20]. The first identified NLRs, NOD1 and NOD2, are activated upon cytosolic detection of specific peptidoglycan fragments. The oligomerized receptors activate NF-κB and MAPK, resulting in the production of cytokines and antimicrobial effectors and also induce type I IFNs[21].

A large group of NLRs share the ability to assemble into inflammasomes. First described in 2002[22], inflammasomes form a cytosolic surveillance system that is formed upon detection of multiple microbial stimuli and host derived molecular signatures indicating danger[23]. For example, NLRC4 activation by cytosolic flagellin requires NAIP5[24]. Interestingly, so far no inflammasome-forming NLR has been shown to directly bind its respective stimulus[23]. Activation of most inflammasomes recruits the adaptor molecule apoptosis-associated speck-like protein containing CARD (ASC). This complex in turn engages procaspase-1. The subsequent autocatalytic cleavage and activation of caspase-1 promotes the processing of pro-IL-1β and pro-IL-18 into the secreted proinflammatory effectors and also induces a form of inflammatory cell death, termed pyroptosis[23] through the cleavage of gasdermin-D[25].

Intensive research has focused on the NLR family pyrin domain containing 3 (NLRP3) inflammasome. Given the highly inflammatory potential of inflammasome-derived effectors such as IL-1β[26], activation of NLRP3 is tightly regulated on multiple levels[27].

First, priming signals are required. The activation of NF-κB enhances transcription of *NLRP3* as well as *pro-IL-1β* and *pro-caspase-1.* NF-κB activation can, among others,

result from TLR-signaling, which illustrates a functional link and coordination between different groups of PRRs. Also, several posttranslational NLRP3 modifications have been described as NLRP3 priming steps[27, 28]. Second, specific stimuli trigger inflammasome assembly and activation[27]. Depending on the mode of activation, canonical and non-canonical pathways have been described. Canonical stimuli (e.g. Nigericin) activate the NLRP3 inflammasome most likely through triggering cellular alterations such as production of (mitochondrial) reactive oxygen species (ROS) or release of mitochondrial DNA and potassium efflux. Non-canonical NLRP3 inflammasome activation, in contrast, additionally requires caspase-11 in mice, and caspase-4 in humans. Caspase-4/11 activation is triggered by cytosolic recognition of LPS, independently of the well-known LPS sensor TLR4, lymphocyte antigen 96 (MD2) and cluster of differentiation (CD)14[23].

Interestingly, NLRP3 inflammasome functions not only in the induction of protective immune responses against bacterial, viral and fungal infections, but it has also been associated with detrimental inflammatory responses in various infection models[29]. NLRP3 also plays a critical role in the pathogenesis of several inflammatory or degenerative disorders, including Alzheimer's disease, insulin resistance, type 2 diabetes, atherosclerosis, and gout[30].

1.2.3 Other PRRs

The family of RLR comprises RIG-I, melanoma differentiation antigen 5 (MDA-5) and Laboratory of Genetics and Physiology 2 (LGP2). They are found in a broad range of cell types and play a crucial role in the control of various viral infections. Located in the cytosol, RIG-I and MDA-5 sense viral RNA, or RNA transcripts associated with viral replication. Both receptors recruit the adapter molecule ‘mitochondrial antiviral-signaling protein’ (MAVS). Activation of downstream molecules, including NF-κB, IFR3 and -7, mediates the production of type I IFNs and proinflammatory cytokines. For LGP2, a regulatory function has been suggested, however, its role remains only incompletely understood[31].

Characterized by a C-type-like domain, the CLR-family of PRRs often recognize carbohydrate structures (e.g. Dectin-1 detects β-glucan), but also other molecular classes like lipids. CLRs are well known for their protective role against fungal infections. Recent evidence also suggests CLRs as key players in the immunity against

mycobacterial infections. Upon activation, CLRs activate various Syk kinase-dependent and -independent signaling cascades to control inflammatory immune responses[32].

There are two known ALRs: AIM2 and interferon-γ-inducible protein 16 (IFI16). AIM2 binds cytosolic dsDNA, like viral DNA, which triggers inflammasome assembly, IL-1β release and cell death. IFI16 is thought to induce the production of type I IFNs. Interestingly, IFI16 also recognizes viral DNA in the nucleus and needs to be relocated to the cytosol for downstream signaling[11].

cGAS was recently discovered as a cytosolic enzyme, which upon binding of dsDNA synthesizes the second messenger molecule cyclic di-nucleotide cAMP-cGMP (cGAMP). cGAMP in turn is sensed by the cytosolic receptor protein stimulator of interferon genes (STING), leading to the transcription of type-I IFNs and also NF-kB-dependent signaling[33]. cGAS plays a critical role in the control of viral infections[34, 35].

1.3 The Innate Immune System Detects Bacterial Viability

1.3.1 Adding a New Layer to Innate Immune Recognition: Discrimination Between Viable and Dead Bacteria

The detection of PAMPs by the innate immune system indicates the presence of non-self, microbial structures. However, since PAMPs are present in pathogenic as well as non-pathogenic organisms and pathogens are able to bypass the detection of their PAMPs, Vance *et al.* have previously argued that the immune systems needs to be provided with additional information to scale the infectious risk. They hypothesized "patterns of pathogenesis", characteristic microbial processes such as replication, cytosolic invasion or alteration of the host cell's cytoskeleton that might be sensed by the immune system. Remarkably, microbial growth and death were suggested to be represented by distinct molecules and termed PAMP-*per vita* and PAMP-*post mortem*[36].

Indeed, more recently, it was demonstrated that the innate immune system of mice possesses the ability to detect bacterial viability independently of replication or virulence factors. Bone marrow-derived macrophages (BMDMs) show a more robust inflammatory response after infection with viable, apathogenic thymidine auxotrophic (*ThyA*$^-$) and therefore replication-deficient *Escherichia coli* (*E. coli*) in comparison to stimulation with their dead counterparts[37]. Only viable *E. coli* induce the secretion of IL-1β, and caspase-1-dependent cell death (pyroptosis), and also lead to increased production of type-I IFN. The levels of many other proinflammatory cytokines such as

TNFα, IL-6, and also pro-IL-1β are similar upon stimulation with either live or dead bacteria. Both types of bacteria activate NF-κB and MAPK. The transcription of pro-IL-1β requires TLR signaling through MyD88, but not TRIF. The secretion of IL-1β requires NLRP3, ASC and caspase-1. Surprisingly, TRIF-deficient macrophages fail to secrete IL-1β after infection with viable bacteria, suggesting that TRIF plays an important role in NLRP3 inflammasome activation, which was independent of transcriptional regulation. Also, TRIF plays a key role for IFN-β production, while MyD88 is dispensable. Experiments *in vivo* confirmed that viable bacteria induce high levels of IL-1β in a TRIF-, ASC-, and NLRP3-dependent manner[37].

Heat killing of bacteria quickly leads to a loss of bacterial RNA, whereas other PAMPs such as LPS or DNA are preserved. Addition of bacterial RNA to heat-killed *E. coli* restores IL-1β and IFN-β responses in BMDMs. Of all tested RNA types, messenger RNA (mRNA) is the most effective in triggering inflammasome activation, when administered together with heat-killed *E. coli*. However, this requires the phagocytosis and intracellular release of bacterial mRNA. Thus, mRNA was identified as the first member of a new class of PAMPs, which signal the presence of viable microbes. It was therefore termed 'viability-associated PAMP' (*vita*-PAMP). RIG-I, known to sense viral nucleic acids[31], was suggested to be dispensable for the detection of bacterial mRNA[37].

Interestingly, immunization experiments in mice using either viable or dead *E. coli* alone or in combination with total bacterial RNA, respectively, revealed that vaccination with heat-killed *E. coli* plus total bacterial RNA is equieffective as immunization with viable *E. coli.* Thus, innate immune detection of bacterial viability efficaciously induces innate and adaptive immunity[37].

1.3.2 Molecular Mechanisms and Signaling Pathways Involved in the Detection of Bacterial Viability

Further work is needed to gain deeper insights into the mechanism underlying the detection of microbial viability and cellular signaling pathways that transduce the signal viability into innate immune responses. A previous study reported DHX33 as a sensor for bacteria-derived RNA and dsRNA upstream of NLRP3. Experiments targeting DHX33 expression by short hairpin RNA (shRNA) revealed a reduced ability of THP-1 macrophages to secrete IL-1β and IL-18 in response to bacterial and retroviral RNA. Co-precipitation experiments suggested that DHX33 directly binds total RNA of *E. coli* and viral dsRNA and activate the NLRP3 inflammasome[38]. Another study specified

differences in the detection of cytosolic bacterial RNA in murine and human cells. In line with data from our group[37], it was found that only mRNA induces NLRP3 inflammasome activation in murine BMDMs. In contrast, *E. coli* mRNA and also transfer RNA (tRNA) as well as several types of ribosomal RNA (rRNA) promote inflammasome-dependent IL-1β release of human monocyte-derived macrophages (MoMs) and the human monocytic cell line THP-1 cells, suggesting the engagement of more promiscuous receptors in human cells [39]. Further experiments performed with THP-1 macrophages demonstrated the dispensability of 5'end trisphosphate moieties[39], in line with previous observations in BMDMs[37]. Additionally, inflammasome activation does not depend on double strandedness of bacterial RNA, the full length or sequence of individual RNA transcripts. Also, the authors demonstrated that the cytosolic presence of bacterial RNA of gram-positive and gram-negative bacteria mediate inflammasome activation[39].

Rathinam *et al.* further characterized the observed TRIF-dependence of NLRP3 activation. BMDMs infected with enterohemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* secreted type I IFNs in a TRIF- and TLR4-dependent manner, which in turn enhances caspase-11 expression and its autoactivation. Subsequently, caspase-11 promotes cleavage of pro-IL-1β through caspase-1 by cooperating with the assembled NLRP3 inflammasome. This assembly, however, was found to be caspase-11-independent and was suggested to be induced by the presence of prokaryotic mRNA in the cytosol. The caspase-11 mediated link between TRIF and the NLRP3 inflammasome was confirmed for other gram-negative bacteria *in vitro* and *in vivo*, but not for gram-positive bacteria. In summary, the authors suggested the activation of the NLRP3 inflammasome requires first the TLR4-MyD88 mediated transcription of NLRP3 and pro-IL-1β, second the cytosolic presentation of bacterial mRNA for inflammasome assembly and third the TRIF-induced type I IFN-mediated caspase-11 expression[40].

Recently, caspase-11 was demonstrated also to be activated by intracellular LPS delivered by outer membrane vesicles (OMV) derived from gram negative bacteria[41]. Viable, but not dead gram-negative bacteria produce OMV containing LPS, which gain cytosolic access trough endocytosis and release from early endosomes. OMV trigger IL-1β secretion and cell death of several murine cell types and human cell lines through caspase-11 activation. *In vivo* experiments confirmed cytosolic LPS sensing requires caspase-11 in mice[41].

Interestingly, a recent study, which was carried out in collaboration with our laboratory, revealed changes in the mitochondrial respiratory chain upon detection of viable

bacteria, which are required for a protective immune response[42]. It was found that that viable, but not heat-killed *E. coli* increase the activity of complex II (CII), a member of the electron-transport chain, in BMDMs and human $CD14^{+}CD16^{-}$ monocytes. Blocking CII decreases levels of IL-1β and enhances anti-inflammatory IL-10 and susceptibility to *Salmonella enterica* (*S. enterica*) Typhimurium *in vivo*. Consistently, BMDMs show inefficient bacterial killing, reduced *Il1b* and *Ifnb* mRNA levels as well as secretion of IL-1β after challenge with *E. coli* and inhibition of CII. Strikingly, treating mice with a C II inhibitor reduces IL-1β and IL-10 production after infection with viable *E. coli* to the levels secreted after infection with heat-killed *E. coli*. The detection of microbial RNA or RNA mimics, in particular *E. coli* RNA, TLR3 ligand poly(inosinic:cytidylic) acid (poly I:C) and R848 by BMDMs and human $CD14^{+}CD16^{-}$ monocytes, but not LPS or TLR9 ligand CpG, activates CII. The activation of CII requires TRIF and MyD88, and it was proposed that phagosomal TLR signaling induces viability-associated CII activation. Additionally, CII activation was demonstrated to depend on NLRP3, caspase-1 and caspase-11. Collectively, the data suggest alterations of the respiratory chain complexes as an important regulatory response to the detection of live bacteria, and the importance of TLR signaling for viability-associated immune responses[42].

1.3.3 Other Possible Indicators of Bacterial Viability

As indicated above, it has been proposed that bacterial RNA, and specifically mRNA is part of a larger group of viability-associated molecules, *vita*-PAMPs, which the immune system senses as indicators of elevated infectious threat[8].

A recent study confirmed that viable but not dead EHEC activate the NLRP3 inflammasome independently of major virulence in BMDMs and BMDCs[43]. Further experiments revealed that bacterial RNA:DNA hybrids, which are physiologically formed during DNA replication and transcription, access the cytosol from phagosomes co-localized with NLRP3 and activate the inflammasome[43]. Therefore, RNA:DNA hybrids might represent a novel *vita*-PAMP that could utilizes different or similar signaling pathways upstream of NLRP3.

Also, viable bacteria produce LPS-rich OMV. Therefore, the detection of OMV through the hosts cytosolic surveillance mechanisms serves as an indicator of live, replicating and potentially dangerous bacteria, which require an adjusted immune reponse[41]. Moreover, OMVs could also contain other *vita*-PAMPs such as bacterial RNA, which are delivered simultaneously into the cytosol.

Another study suggested that bacterial metabolites could indicate the presence of viable bacteria. The accumulation of bacterial citrate was identified as an activator of the NLRP3 inflammasome in BMDMs infected with *S. enterica* Typhimurium mutants. The authors proposed that citrate could be detected either directly or indirectly by modulation of other bacterial metabolites by the innate immune system as an additional group of *vita*-PAMPs[44].

Furthermore, quorum-sensing molecules and bacterial second messengers like 3'-5' diguanylate (c-di-GMP) have been suggested as molecules associated with bacterial growth and viability[36]. Interestingly, bacterial second messengers have been identified as activators of the NLRP3 inflammasome independently of mitochondrial ROS and STING, an earlier identified receptor[45, 46].

1.3.4 Sensing Bacterial Viability as Part of an Immunological Risk Assessment Process and Future Implications

The physiological relevance of the recognition of bacterial viability has been integrated in the innate immune system's ability to assess the infectious risk. Sander and Blander postulated five innate immune checkpoints, which help to determine the microbial threat and regulate subsequent protective immune responses. This tight control ensures efficicaious pathogen clearance, while avoiding unnecessary inflammatory damage to host tissues. The presence of (multiple) PAMPs as integral components of microbes poses a higher infectious risk than the detection of individual, soluble PAMPs. Likewise, viable bacteria and also the detection of pathogenicity represent a serious threat to the host. However, the detection of microbial danger has to be assessed in context with indicators of invasiveness in order to discriminate between infection and colonization. Finally, the immune response has to be adapted to the specificities of the affected tissue.[8]

Manipulation of the receptors and signaling pathways involved in the detection of microbial viability, that so far have not been fully investigated, might serve as a strategy to therapeutically direct immune responses. Given the robust immune response and induction of adaptive immunity in mice upon detection of bacterial viability, associated receptors and signaling pathways pose potential targets of vaccine adjuvants or antimicrobial drugs[2, 37, 47].

1.4 Aim of the Study

The sensing of bacterial viability has been described and further characterized primarily for mice, *in vitro* and *in vivo*, and limited data is available on human cell lines. However, the immune response of primary human immune cells, namely antigen presenting cell (APC) to bacterial viability, has yet to be investigated in detail.

The first aim of my study was to examine whether the differential cytokine response pattern of murine cells to viable and dead bacteria[37] is conserved in various human primary immune cells. This also comprised dose-response effects of increasing multiplicity of infection (MOI).

The second aim was to investigate the involvement of distinct PRR and downstream signaling molecules in the detection of viability in human primary immune cells.

As an initial screen, a variety of small molecule inhibitors targeting candidate innate immune pathways were used. This ready-to-use system served as a pre-screening approach to identify targets for lentiviral-vector-mediated shRNA-mediated gene silencing. In addition, given the central role of TRIF for the induction of immune responses to viable bacteria in mice and the requirement of MyD88 for transcription of pro-IL-1β, ASC and the NLRP3 inflammasome for secretion of IL-1β[37], these molecules were of great interest and specifically examined using the lentiviral transduction approach.

2 Material and Methods

2.1 Monocyte Purification and Culture Conditions

Human peripheral blood mononuclear cells (PBMCs) were isolated from Buffy Coats provided by the Red Cross Blood Transfusion Service, Berlin, Germany.

Buffy Coats were diluted 1:1 in RPMI-ethylenediaminetetraacetate (EDTA) wash buffer (RPMI (from Life Technologies; Darmstadt, Germany) containing 5% fetal calf serum (FCS) and 0.2 mM EDTA (both from Sigma-Aldrich; Steinheim, Germany)). First, PBMC were isolated by density centrifugation over Histopaque-1077 (Sigma-Aldrich; Steinheim, Germany) for 25 minutes at 800 g at 20°C. Isolated mononuclear cells were then washed in RPMI-EDTA wash buffer for 10 minutes at 200 g and 160 g, respectively, for the removal of platelets until the supernatants appeared clear. $CD14^+CD16^-$ monocytes were purified by immunomagnetic separation using human EasySep monocyte isolation kits with CD16 depletion (STEMCELL Technologies; Grenoble, France) following the manufacturer's instructions. $CD14^+CD16^-$ monocytes are hereafter referred to as "classical monocytes". Purified monocytes were seeded at a density of $1x10^6$/ml. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 1% glutamine, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1% non-essential amino acids (all from Sigma-Aldrich; Steinheim, Germany*)* at 37°C in humidified incubator maintaining a carbon dioxide (CO_2) level of 5%. Hereafter, this medium is referred to as “complete medium”.

2.2 Differentiation of Monocytes into Monocyte-Derived Dendritic Cells

Classical monocytes were differentiated into monocyte-derived dendritic cells (MoDCs) by culture for four days in complete medium containing 10 ng/ml recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml recombinant IL-4 (both purchased from BioLegend; San Diego, USA). Cells were maintained in optimal culture conditions by replacing 50% MoDC-complete medium on day one and three of differentiation. MoDCs were stimulated on day four of differentiation and analyzed as described below.

2.3 Isolation of Human Alveolar Macrophages

Bronchioalveolar lavages (BAL) from anonymous patients were provided by BAL diagnostics laboratory at our Department (Department of Internal Medicine/Infectious Diseases and Pulmonary Medicine, Charité – Universitätsmedizin Berlin, Germany). BAL was centrifuged at 1300 rpm for 5 minutes. Cells were cultured overnight at a density of $1x10^6$/ml in complete medium containing penicillin (100 units/ml), streptomycin (100 µg/ml; both purchased from Sigma-Aldrich; Steinheim, Germany) and amphotericin B (0.25 µg/ml, from PAA Laboratories GmbH; Cölbe, Germany) to clear possible mycotic or bacterial contamination. Human alveolar macrophages (hAM) were purified by overnight adhesion to the culture dish. The following day, cells were washed twice in antibiotic/antimycotic-free complete medium to remove remaining non-adherent cells and achieve a high purity hAM culture (adapted from reference[48]). Alveolar macrophages were further cultured in complete medium and infected as described below. Supernatants were analyzed for IL-1β, TNFα, IL-6 and IL-10 release by enzyme-linked immunosorbent assay (ELISA) as described below.

2.4 Generation of Viable and Heat-Killed *ThyA⁻ E. coli*

Naturally occurring *ThyA⁻ E. coli* K12, strain DH5α (from Invitrogen; Karlsruhe, Germany) were selected streaking wild type *E. coli* DH5α on Luria-Bertani (LB) agar plates supplemented with 50 µg/ml trimethoprim and 500 µg/ml thymidine (both purchased from Sigma-Aldrich; Steinheim, Germany) at room temperature for 3 days, as described previously[37, 49, 50]. LB agar recipe is provided in Supplementary Tab. 1 in the Appendix.

Subsequent inoculation and overnight culture of single colonies in LB medium verified bacterial growth only in thymidine-containing medium and resistance to trimethoprim. Viable *ThyA⁻ E. coli* were grown to mid lo phase, aliquoted and stored in LB medium supplemented with 500 µg/ml thymidine, 50 µg/ml trimethoprim and 30% glycerol at -80°C. Heat killing was performed by growing *ThyA⁻ E. coli* to mid log phase (optical density of 600 nm (OD_{600}) of 0.6) washed twice in phosphate buffered saline (PBS, from Life Technologies; Darmstadt, Germany) and diluted to OD_{600} of 0.6, and subsequently killed by incubation in a water bath at 60°C for 90 minutes. Efficacious killing was confirmed by streaking onto LB agar supplemented with thymidine and trimethoprim and incubation in an overnight culture containing the supplements at 37 °C. Heat-killed *E.*

coli (HKEC) were used either directly after cooling to room temperature or stored in PBS containing 30% glycerol at -80°C.

2.5 Infection and Stimulation Experiments in Various Human Phagocytes

For infection experiments, a *ThyA*$^-$ *E*. *coli* preculture was prepared from frozen stock in LB medium supplemented with 50 µg/ml trimethoprim and 500 µg/ml thymidine and incubated in a shaking incubator over night at 37 °C the day prior to infection. The overnight culture was diluted in supplemented LB medium to an OD_{600} of 0.1, and subsequently re-grown into mid-log phase (OD_{600} of 0.65 - 0.85). Both viable mid-log phase *ThyA*$^-$ *E*. *coli* as well as freshly prepared or thawed heat-killed *ThyA*$^-$ *E*. *coli* were washed twice in PBS and resuspended in PBS. The concentration of bacteria was adjusted to $1x10^9$ bacteria/ml (conversion of OD_{600} values to colony forming units was calculated on previously established growth curve measurements in the group). Unless indicated otherwise, classical monocytes were infected with a MOI of 1 or stimulated with HKEC at a 1:1 cell to bacteria ratio, and MoDCs and hAM were stimulated with a MOI of 10 / 1:10 ratio. All infection experiments were performed in antibiotic-free complete medium. 90 minutes post infection (p.i.) or stimulation, penicillin (100 units/ml) and streptomycin (100 µg/ml) were added to the culture medium to ensure killing of remaining non-phagocytosed, extracellular bacteria.

2.6 Inhibitor Screening Experiments

Purified classical monocytes were treated with small molecule inhibitors as specified in Supplementary Tab. 2.

After addition of inhibitors and respective preincubation times as given in Supplementary Tab. 2, cells were infected with viable and dead bacteria as described above. Overnight stimulation with TLR ligands was performed to assess efficicacy and specificity. A list of all TLR ligands used in this study is provided in Supplementary Tab. 3 in the Appendix. The following day, cell culture supernatants were collected for cytokine quantification by ELISA.

2.7 Production of Lentiviral Vectors and Transduction of MoDCs

The protocol for gene transduction in human MoDCs was based on a protocol for human immunodeficiency virus 1 (HIV-1)-derived lentiviral vectors published by Satoh and Manel [51] and was adapted to meet the conditions for infection experiments.
Figure (Fig. 1 provides an overview on the principle of the performed experiments.

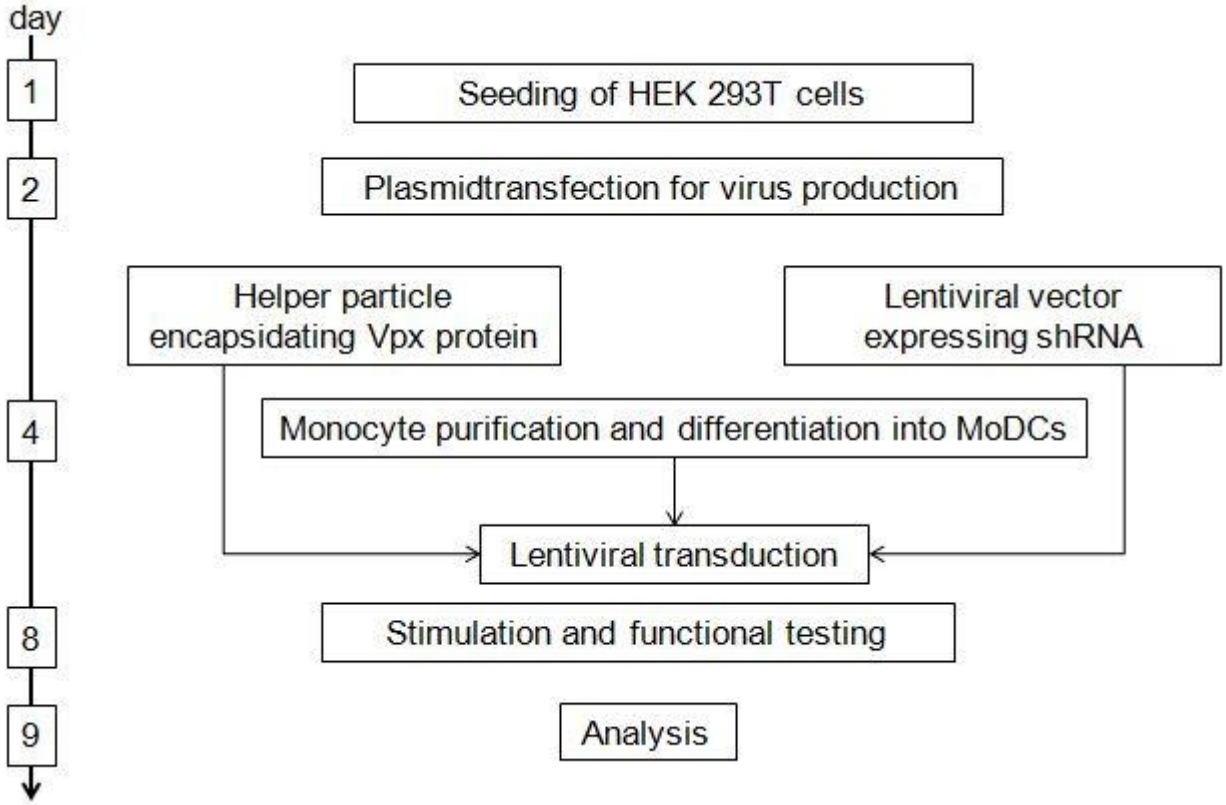


Fig. 1: Principle of lentiviral shRNA-mediated gene silencing in human MoDCs. On day 1, HEK293T cells were seeded and cultured overnight. The next day, cells were separately transfected with plasmids encoding for either helper-particles, encapsidating the Vpx protein, or lentiviral vectors expressing target-specific shRNA. 2 days after transfection, classical monocytes were purified and cultured in differentiation medium containing GM-CSF and IL-4. The transduction was performed directly after plating the monocytes by adding equal volumes of helper-particle and lentiviral vector containing HEK293T culture supernatants. 4 days after transduction, differentiated MoDCs were subjected to infection and functional testing, e.g. by stimulation with LPS. ELISA was performed to assay cytokines in cell culture supernatants. Cell lysates were either subjected to RNA isolation or Western Blot analysis to assess target gene or protein expression.

Lenti-X Human embryonic kidney cells 293T (HEK 293T, from Clontech; Mountain View, USA) were cultured in Dulbecco's Modified Eagle's medium *(*DMEM, from Life Technologies; Darmstadt, Germany*)* supplemented with 10% FCS, hereafter referred to as lentivirus (LV)-medium. One day prior to transfection, HEK 293T cells were detached using 1xTrypsin-EDTA solution (from Sigma-Aldrich; Steinheim, Germany) and subsequently plated at a density of $6x10^5$/ml in a six well plate, at 2 ml/well. After

overnight incubation, the medium was aspirated and replaced with 2 ml of prewarmed, fresh LV medium avoiding HEK 293T cell-detachment. The plasmid transfection was performed at least 6 hours after addition of fresh medium.

The following plasmids were used for helper particle and lentiviral vector production: pLKO.puro (from Addgene, plasmid #8453; Cambridge, USA), psPAX2 (from Addgene, plasmid #12260; Cambridge, USA), pCMV-VSVG (from Addgene, plasmid #8454; Cambridge, USA), pSIV3+ (gift from Philippe-Emmanuel Mangeot). All shRNA constructs based on p.LKO.1 vector were purchased from GE Healthcare Dharmacon; Lafayette, USA, including the targets and sequences listed in Supplementary Tab. 4 in the Appendix. 100 µg/ml ampicillin (from Sigma-Aldrich; Steinheim, Germany) was added to LB agar plates and LB medium culture to select pLKO.puro, psPAX2, pCMV-VSVG and pSIV3+ containing *E. coli*. 100 µg/ml carbenicillin (from Thermo Scientific; Braunschweig, Germany) was added to LB agar plates and LB medium culture to select shRNA constructs based on p.LKO.1 vector containing a carbenicillin resistance-gene.

Plasmid DNA was purified using the PureLink® HiPure Plasmid Midiprep Kit (from Invitrogen; Karlsruhe, Germany) following the manufacturer's recommendations. *Trans*IT®-293 transfection reagent (Mirus Bio LLC; Madison, USA) was warmed up to room temperature and vortexed before use, and 8 µl of *Trans*IT were used to transfect a total of 3 µg plasmid DNA/well. For one transfection 8 µl of *Trans*IT®-293 were added to plain DMEM, vortexed for 5 seconds, and incubated at room temperature for 5 minutes. Plasmid DNA, to produce either helper particles or lentiviral vectors, was added as indicated in Tab. 1 and mixed by tapping.

Tab. 1: Quantity of transfected plasmid DNA per well in a six well plate for production of helper particles and lentiviral vectors. Table adapted from reference [51].

Helper particle	Lentiviral vector
2.6 µg pSIV3+	1.6 µg pLKO.1 + target-shRNA
	1.0 µg psPAX2
0.4 µg pCMV-VSVG	0.4 µg pCMV-VSVG

After 15 minutes incubation time at room temperature, the mixture was added dropwise and cells were incubated at 37°C in humidified incubator. 18 hours after transfection, the medium was aspirated and 3 ml of prewarmed, fresh LV medium was added for an additional 24 to 26 hours.

Two kinds of supernatant were produced containing either HIV-1-derived lentiviral vector particles, or simian immune deficiency virus (SIV)-mac-derived helper particles. The lentiviral vectors are replication-deficient. They contain the genetic information encoding for target-specific shRNA. After reverse transcription, the genetic information, including the DNA encoding for the target-specific shRNA is integrated into the genomic DNA of the monocytes and the desired shRNA is constantly expressed during the differentiation into MoDCs, targeting the sequence-specific mRNA.

However, myeloid cells express SAM domain and HD domain-containing protein 1 (SAMHD1) which inhibits the lentiviral reverse transcriptase by decreasing the concentration of available intracellular deoxynucleoside triphosphates and therefore infection with HIV-1[52]. The SIV-derived helper particle carries Vpx protein, which abolishes SAMHD1-mediated inhibition of reverse transcription and therefore allows infection of myeloid cells by HIV-1[53]

After purification, $7.5x10^5$ monocytes per well were seeded in a 24 well plate in differentiation medium as described above. Virus containing supernatants were collected and filtered separately through 0.45 µm cellulose acetate syringe filters (Kisker Biotech; Steinfurt, Germany). Lentiviral shRNA clones were tested individually for their silencing efficiency. Usually we tested four to five clones per target gene. Clones with sufficient silencing efficacy were pooled together before addition to the cells. 375 µl of each supernatant were added to transduce cells. 1.5 µl of 8 mg/ml filtered protamine solution (from Sigma-Aldrich; Steinheim, Germany) were added and cell-virus-protamine suspension was mixed gently. Fresh differentiation medium was added as described above. 4 days after transduction, MoDCs were stimulated with various stimuli, including live and dead bacteria. An overview of targeted genes and their respective functional controls is given in Tab. 2. After overnight incubation cell supernatants were collected for ELISA and cell-lysates were generated for either RNA isolation or Western Blot.

Tab. 2: Target-specific functional control to assess efficiency of lentivirus-mediated gene silencing.

Silenced gene	Control	Concentration	Incubation	Manufacturer
ASC, NLRP3	LPS, ultrapure + Nigericin	100 ng/ml 10 µM	4 hours 30 minutes	InvivoGen; San Diego, USA Merck Millipore; Darmstadt, Germany
MyD88, TLR8	CL075 R848	1 µg/ml 1 µg/ml	overnight overnight	Both from InvivoGen; San Diego, USA
TLR4	LPS ultrapure	100 ng/ml	overnight	InvivoGen; San Diego, USA
TRIF	MPLA	1 µg/ml	overnight	InvivoGen; San Diego, USA

2.8 RNA Isolation and Complementary DNA Synthesis

MoDCs were washed once with PBS and lysed in 300 µl lysis buffer included in the GeneJET RNA Purification Kit (from Life Technologies; Darmstadt, Germany). RNA isolation was performed as recommended by the manufacturer's protocol. Contaminating genomic DNA was removed by DNAse (from Life Technologies; Darmstadt, Germany) treatment according to the manufacturer's instructions. RNA quantity was measured using Nanodrop 2000 Spectrophotometer (Thermo Scientific; Braunschweig, Germany). Complementary (c) DNA was generated using RevertAid First Strand cDNA Synthesis Kit (from Life Technologies; Darmstadt, Germany) following the manufacturer's recommendations and used for real-time polymerase chain reaction (PCR) reactions.

2.9 Quantitative Real-time PCR

Quantitative real-time RT–PCR (qRT-PCR) was performed using SYBR Select Master Mix (from Life Technologies; Darmstadt, Germany) with the primer pairs specified in Tab. 3.

qRT-PCR was run on the Applied Biosystems 7300 Real-Time PCR System. Relative expression was calculated using the $\Delta\Delta C^t$ method relative to unstimulated control-lentivirus-transduced MoDCs.

Tab. 3: Primer pairs used for qRT-PCR.

Target	Forward	Reverse
β-actin	5'-GGATGCAGAAGGAGATCACT-3'	5'-CGATCCACACGGAGTACTTG-3'
DHX33	5'-AgTCCCTACCCTgAAgCTgT-3’	5’-ATAgCCAACCgATgAggACC-3’
TLR4	5′- AAGCCGAAAGGTGATTGTTG-3’	5'-GATACCAGCACGACTGCTCA-3'
TLR8	5′-AgTTTCTCTTCTCggCCACC-3′	5′-ACATgTTTTCCATgTTTCTgTTgT-3′
TRIF	5′-CCCggATCCCTgATCTgCTTg-3′	5′-ggTgAAggCATgTTCCACACT-3’

2.10 Western Blot

Cells were washed twice with phosphoprotein wash buffer and lysed in lysis buffer. Total cell lysates were cleared by centrifugation at 20800g for 10 minutes. Supernatants were denatured in Laemmli buffered at 95°C for 5 minutes, or for TLR4 detection at 70°C for 5 minutes. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 8% polyacrylamide for detection of TRIF, NLRP3, and TLR4; 10% polyacrylamide for detection of ASC, IL-1β and MyD88). Separated proteins were transferred onto a nitrocellulose blotting membrane (GE Healthcare Life Sciences; Freiburg, Germany). Membranes were blocked using Odyssey® Blocking Buffer (LI-COR Biotechnology GmbH; Bad Homburg, Germany) and exposed to primary antibodies at 4°C overnight followed by incubation with secondary antibodies at room temperature for 1 hour. Proteins were detected using an Odyssey® infrared imaging system (LI-COR Biotechnology GmbH; Bad Homburg, Germany). Primary and secondary antibodies used are indicated in Tab. 4 and Tab. 5, respectively. A complete list of buffers and other required reagents used is provided in Supplementary Tab. 5.

Tab. 4: Primary antibodies used for Western Blot analysis

Primary antibody	Manufacturer	kDA	species	dilution
ASC	Santa Cruz Biotechnology; Dallas, Texas, USA	24	rabbit	1:500
β-Actin		42	goat	1:1000
IL-1β	Cell signaling; Frankfurt am Main, Germany	31, 17	rabbit	1:1000
IL-1β	Santa Cruz Biotechnology; Dallas, Texas, USA	31	mouse	1:200
MyD88		33	rabbit	1:200
NLRP3	Cell signaling; Frankfurt am Main, Germany	110, 85	rabbit	1:1000
TLR4	abcam; Cambridge, United Kingdom	80	rabbit	1:400
TRIF	Cell signaling; Frankfurt am Main, Germany	98	rabbit	1:400

Tab. 5: Secondary antibodies used for Western Blot analysis

Secondary Antibody	Manufacturer	Species	dilution
anti-goat	Rockland Immunochemicals Inc.; Limerick, USA	donkey, labelled with IRDye 800	1:2000
anti-mouse		goat, labelled with Cy 5.5	1:5000
anti-rabbit		goat, labelled with Cy 5.5	1:2000
anti-rabbit		goat, labelled with IRDye 800	1:2000

2.11 ELISA

IL-1β, TNFα, IL-6 and IL-10 concentrations in cell culture supernatants were measured by ELISA using *Ready*-SET-*Go* human *ELISA* kits (eBioscience*;* San Diego, USA) following the manufacturer's recommendations. Intracellular IL-1β was measured in cell

lysates using the *Ready*-SET-*Go* human *ELISA* kit for IL-1β. Cell lysis was performed by at least five freeze/thaw cycles using liquid nitrogen.

IFN-β was measured by ELISA using the Human IFN Beta ELISA construction kit purchased from Antigenix America (Huntington Station, USA). ELISA was performed following the manufacturer's recommendations.

All samples were analyzed for absorbance at 450 nm using FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices; Biberach an der Riss, Germany).

2.12 Cell Death Measurement by Lactate Dehydrogenase Release Assay

Cytosolic lactate dehydrogenase (LDH) release was quantified with the use of CytoTox 96 ® Non-Radioactive Cytotoxicity Assay Kit (Promega Corporation; Madison, USA) following the manufacturer's protocol. An uninfected control was lysed 1 hour prior to collection of cell-free supernatans using the lysis buffer provided with the kit . It served as a maximum value. The basal LDH release was quantified in an uninfected, unlysed, control sample. Specific LDH release was determined by subtracting basal LDH release from all samples, including the maximum value and calculating the percentage of the highest possible LDH release represented by the maximum value to assess cell death.

2.13 Statistical Analysis

Data was analyzed using the GraphPad Prism software, Version 5.01. Statistical tests were performed as indicated in the figure legends.

3 Results

3.1 Human Phagocytes Respond Differentially to Viable and Heat-Killed *E. coli*

3.1.1 Classical Monocyte Responses to Bacterial Viability

Classical human monocytes were isolated and stimulated with either viable or killed *E. coli* at different MOIs, as described in detail in the 'Material and Methods' section (2.1 and 2.5). Viable bacteria induce robust levels of IL-1β, which is not observed with heat-killed bacteria (Fig. 2A).

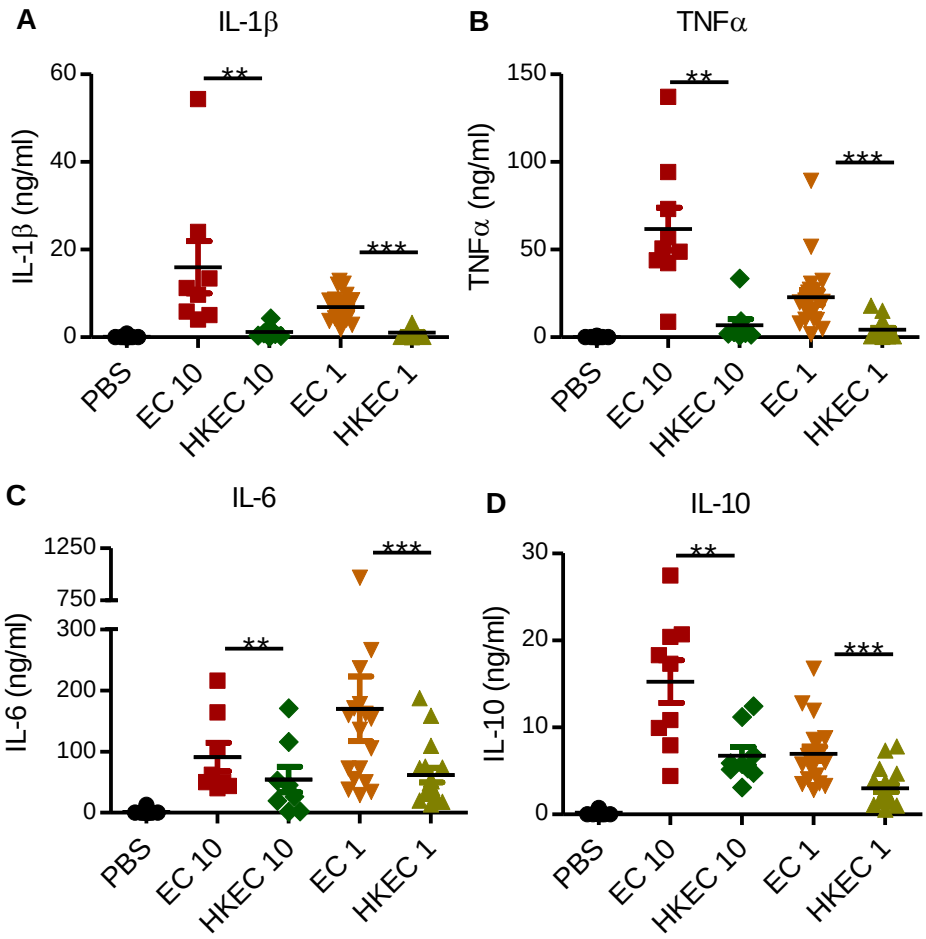


Fig. 2: Cytokine production by classical monocytes in response to viable and dead *E. coli*. Classical monocytes were stimulated with viable or dead bacteria at MOI 10 or 1, respectively. Release of IL-1β (A), TNFα (B), IL-6 (C) and IL-10 (D) was measured by ELISA 18 hours p.i. Data are shown as mean + SEM. Each symbol represents an independent experiment (=donor). Wilcoxon matched pairs test was applied. For MOI 10 n=8-9 , for MOI 1 n=17-20, depending on the measured cytokine. ** = $p < 0.01$, *** = $p < 0.001$.

Unlike murine phagocytes[37] and hAM (see below, section 3.1.3), classical monocytes produce TNFα nearly exclusively in response to viable bacteria, which is only weakly

induced by killed bacteria, even at a bacteria to cell ratio of 10:1 (Fig. 2B). Slightly higher levels of IL-6 (Fig. 2C) and anti-inflammatory IL-10 (Fig. 2D) are produced in response to live bacteria. Considerable levels of IL-6 and IL-10 were also produced in response to HKEC (Fig. 2C,D).

3.1.2 MoDCs Responses to Bacterial Viability

DCs are critical for the initiation of adaptive immune responses[3]. To assess the potential of DCs to detect bacterial viability, classical monocytes were differentiated into MoDCs as described in section 2.2 and subsequently stimulated with live or dead *E. coli*.

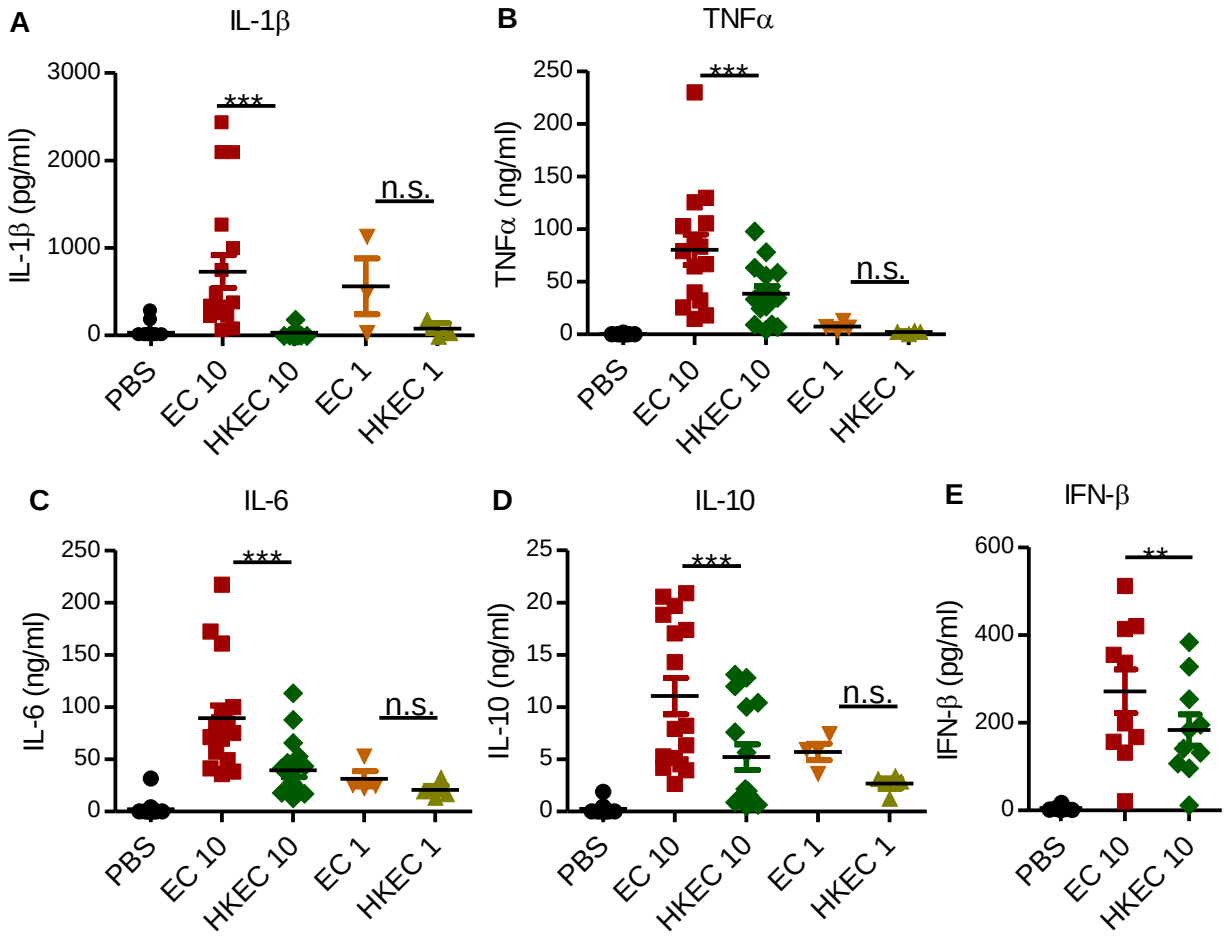


Fig. 3: Cytokine production by human MoDCs after infection with viable and dead *E. coli*. MoDCs were infected with viable or dead bacteria at MOI 10 or 1, respectively. Release of IL-1β (A), TNFα (B), IL-6 (C), IL-10 (D) and IFN-β (E) was measured by ELISA 18 hours p.i. Data are shown as mean + SEM. Each symbol represents an independent experiment (=donor). Wilcoxon matched pairs test was applied. For MOI 10 n=15-17, depending on the measured cytokine, for MOI 1 n=3-4. IFN-β n= 10. n.s. = not significant, ** = $p < 0.01$, *** = $p < 0.001$.

MoDCs produce IL-1β only after infection with viable *E. coli* (Fig. 3A). In contrast to monocytes, MoDCs secrete TNFα in response to bacteria, almost regardless of viability (Fig. 3B). Similar responses can be observed for IL-6 (Fig. 3C) and IL-10 (Fig. 3D).

However, MoDCs infected with an MOI of 10 of viable bacteria, produce significantly more cytokines compared to the stimulation with dead bacteria.

Since it has been shown for BMDMs that type I IFN signaling contributes to caspase-11 activation and subsequent IL-1β release after infection with gram-negative bacteria[40], I quantified IFN-β release in response to viable and dead *E. coli*. Indeed, viable *E. coli* induce a slight, but significantly elevated IFN-β response, although the overall IFN release was rather low (Fig. 3E).

3.1.3 hAM Responses to Bacterial Viability

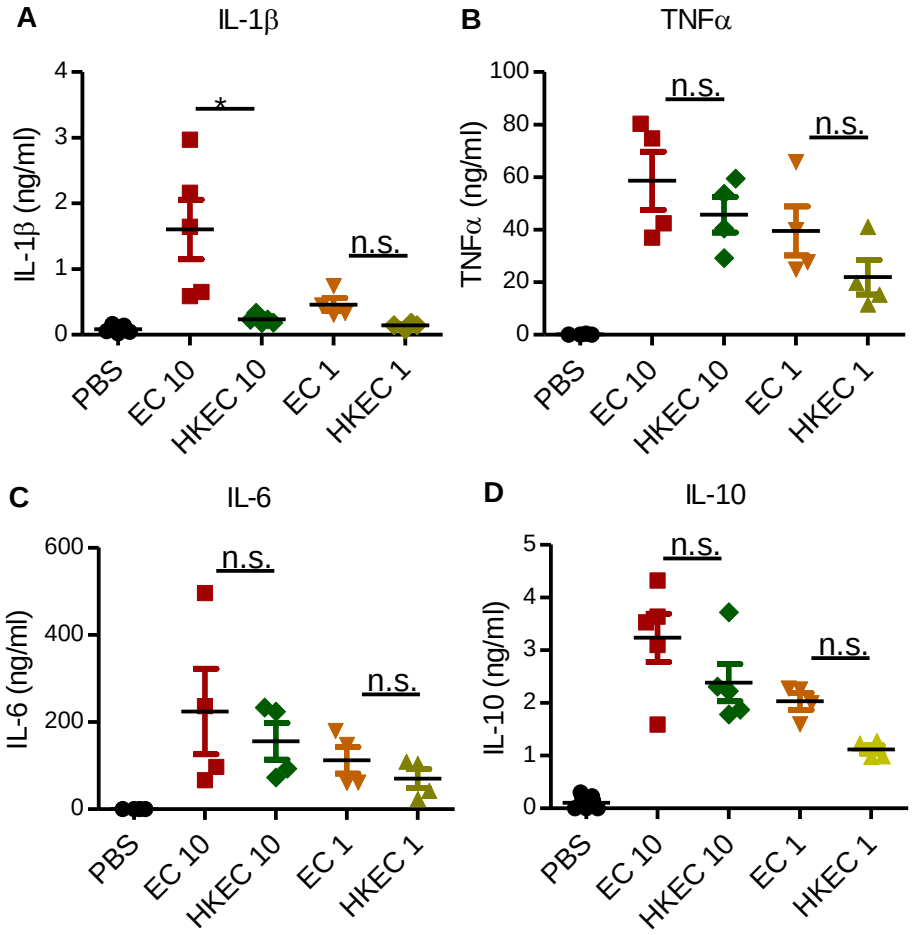


Fig. 4: Cytokine production by hAM in response to viable and dead *E. coli*. hAM were infected with viable or dead bacteria at MOI 10 or 1, respectively. Release of IL-1β (A), TNFα (B), IL-6 (C) and IL-10 (D) was measured by ELISA 18 hours p.i. Data are shown as mean + SEM. Each symbol represents an independent experiment (=donor). Wilcoxon matched pairs test was applied. For MOI 10 n=4-5, depending on the measured cytokine, for MOI 1 n=4. n.s.= not significant, * = $p < 0.05$.

hAM were treated and infected with viable and dead *E. coli* as described in sections 2.3 and 2.5.

As observable with classical monocytes and MoDCs, IL-1β production was dependent on bacterial viability (Fig. 4A), whereas no major differences are observed for TNFa, IL-6 and IL-10 production regardless of bacterial viability (Fig. 4B-D).

The enhanced IL-1β production is clearly dose dependent (Fig. 5A), whereas stimulation with HKEC does not increase IL-1β production even at higher doses (Fig. 5A). Furthermore, very high bacterial loads (MOI 100, live *E. coli*) lead to reduced IL-1β release, presumably due to enhanced cell death (Fig. 5A). Accordingly, IL-6 responses after infection with high doses of viable bacteria are decreased (Fig. 5C), while TNFα production shows an increase independently of viability correlating with the MOI (Fig. 5B). Interestingly, high doses of viable *E. coli* reduce the production of IL-10, while comparable doses of dead bacteria maintain levels similar to those of lower MOIs (Fig. 5D).

Taken together, hAM respond differentially to viable and dead bacteria, suggesting resident innate immune cells of the lung can differentiate bacterial threat by detection of viability, yet their response pattern is different from circulating monocytes.

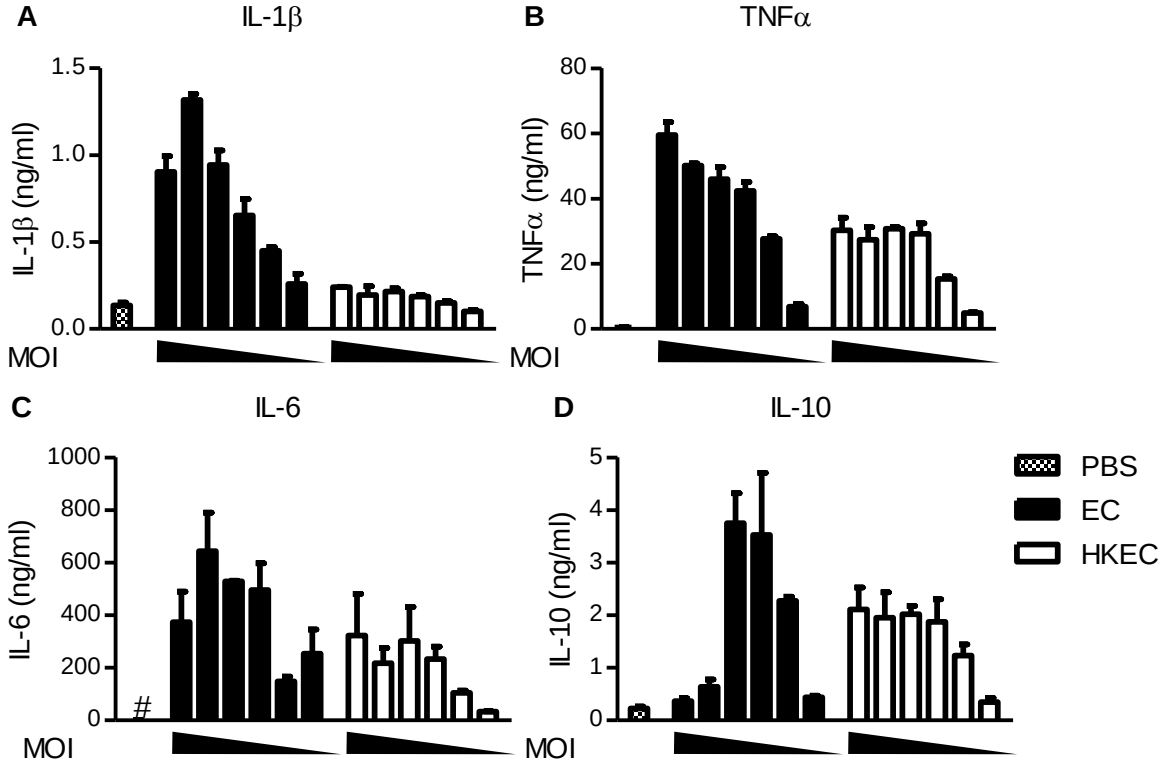


Fig. 5: Dose-responses of human alveolar macrophages infected with live and dead *E. coli*. hAM were infected at MOIs ranging from 100 to 0.1. Cytokine response was determined 18 hours p.i. by ELISA for IL-1β (A), TNFα (B), IL-6 (C) and IL-10 (D). No statistical analysis was performed due to the sample size of n=1.

Collectively, all three investigated cell types adapt their cytokine responses to the detection of bacterial viability and produce IL-1β nearly exclusively in response to viable bacteria. The magnitude of cytokine levels varied depending on the cell type and on the MOI.

3.2 Small Molecule Inhibitor Screen for Pathways Involved in the Detection of Bacterial Viability in Classical Monocytes

3.2.1 Inhibition of NF-κB Transcription Factor and MAPK Abolishes Cytokine Responses to Viable and Dead *E. coli*

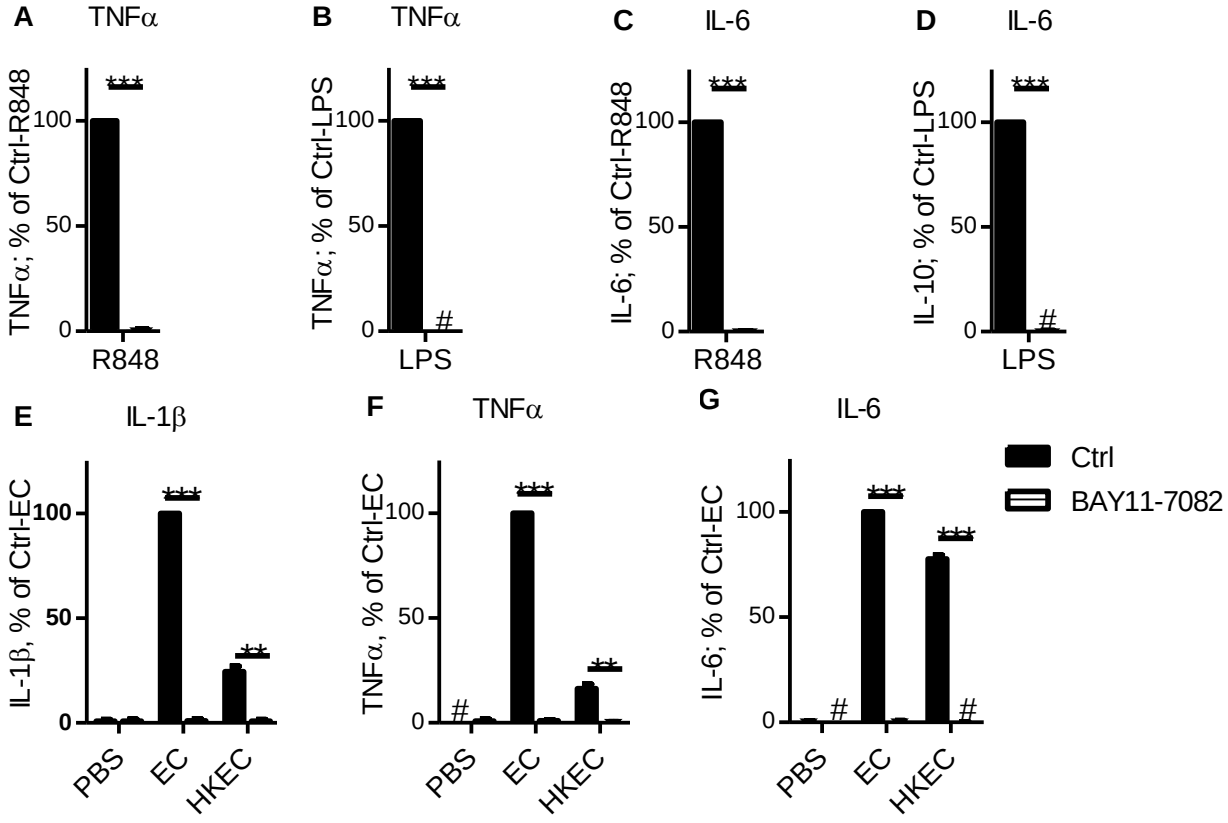


Fig. 6: Cytokine production by IκBα-inhibitor BAY11-7082-pretreated classical monocytes after stimulation with TLR-agonists or viable and dead *E. coli*. Cells were pretreated with BAY11-7082 for 1 hour and subsequently stimulated with R848, LPS (A-D) or infected with viable and dead *E. coli* (E-G) at MOI 1. Cytokine release was measured 18 hours post stimulation by ELISA (all n=2). All data are expressed as percentage of untreated cells stimulated with R848, LPS or infected with viable *E. coli.* Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, ** = $p < 0.01$, *** = $p < 0.001$, # = not detected.

First, we tested the role of major proinflammatory signaling pathways known to mediate many innate immune responses, for their possible involvement in the discrimination process between viable and dead bacteria.

We first tested the transcription factor NF-κB, due to its known function in TLR signaling and inflammatory immune responses[54]. The frequently used inhibitor of kappa B α (IκB-α) BAY11-7082 causes a nearly complete abolishment of TNFα and IL-6 responses after TLR4 (LPS) and TLR7/8 (R848) stimulation (Fig. 6A-D). In line with the TLR agonist data, IκB-α inhibition robustly reduces all tested cytokines following infection with both viable and dead bacteria (Fig. 6E-G). We concluded that NF-κB is a mandatory component of downstream signaling upon detection of both viable and dead bacteria, but it does not directly participate in the immunological decision making process dependent on the detection of bacterial viability.

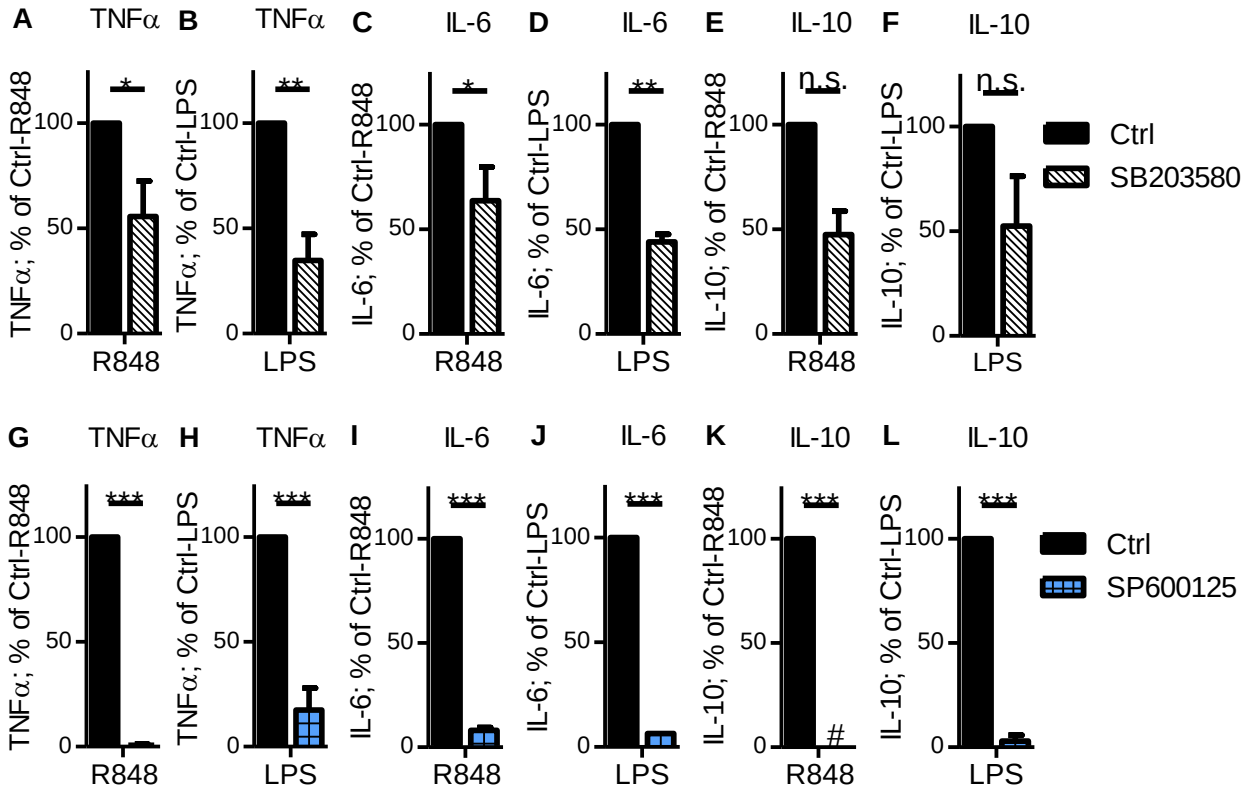


Fig. 7: Pretreatment of classical monocytes with p38/RK MAPK-inhibitor SB203580 (A-F) and JNK-inhibitor SP600125 (G-L) reduces cytokine responses after TLR stimulation. Cells were pretreated with SB203580 or SP600125 for 1 hour and subsequently stimulated with R848 or LPS. Cytokine release was measured 18 hours post stimulation by ELISA (for SB203580 n=4, for SP600125 n=3). All data are expressed as percentage of untreated cells stimulated with R848 or LPS. Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests. n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, # = not detected.

Next, the involvement of MAPK was investigated by using the inhibitory molecules SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor). To quantify the inhibitory capacity, cytokine responses of control and pretreated classical monocytes were monitored after stimulation with LPS or R848. TNFα, IL-6, and IL-10 responses are strongly diminished by pretreatment with both MAPK inhibitors (Fig. 7).

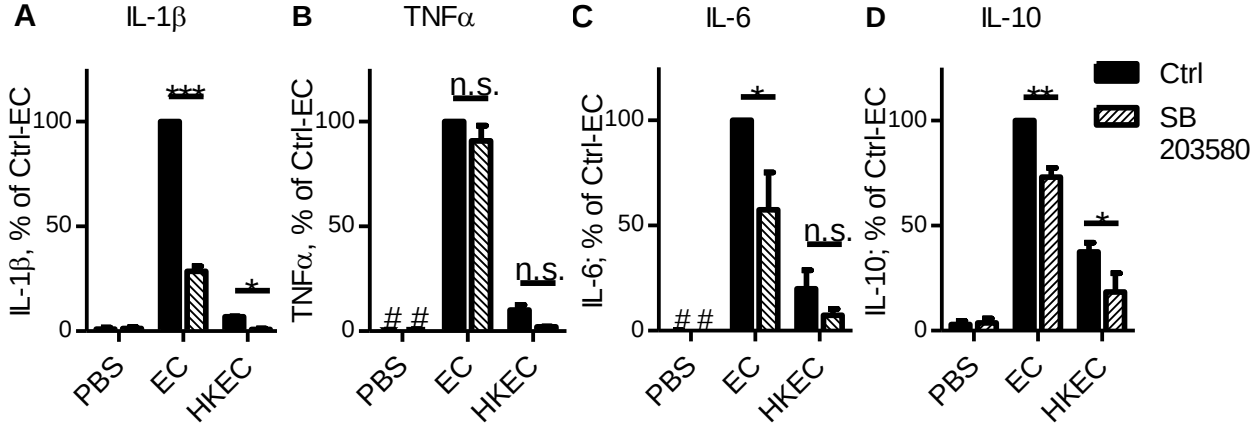


Fig. 8: Cytokine production by p38/RK MAPK-inhibitor SB203580-pretreated classical monocytes after infection with viable and dead *E. coli*. Cells were pretreated with SB203580 for 1 hour and subsequently stimulated with viable and dead *E. coli* at MOI 1. Cytokine release was measured 18 hours p.i. by ELISA (all n=3). All data are expressed as percentage of untreated cells stimulated with viable *E. coli.* Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments were separated from functional controls for the sake of clatrity. n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, # = not detected.

Independently of viability, p38/RK MAPK inhibitor SB203580 pretreated monocytes produce significantly less IL-1β and IL-10 after infection. Similar inhibitory effects are observable for IL-6, however, the reduction after infection with heat killed bacteria did not reach statistical significance (Fig. 8A, C-D). In contrast, TNFα secretion is not modified by the application of p38/RK MAPK-inhibitor SB203580 (Fig. 8B). Furthermore, p38/RK MAPK-inhibition by SB203580 induces no detectable cell death (Data not shown).

Inhibition of JNK, a member of MAPK signaling[55], by SP600125 reduces the secretion of IL-1β not only after infection with viable but also heat-killed *E. coli* (Fig. 9A). Preincubation with SP600125 completely abolishes secretion of TNFα, IL-6 and IL-10 (Fig. 9B-D).

Cells preincubated with JNK-inhibitor tend to release more LDH in any of the tested conditions (data not shown). The observed cell death might partially account for the reduced cytokine response.

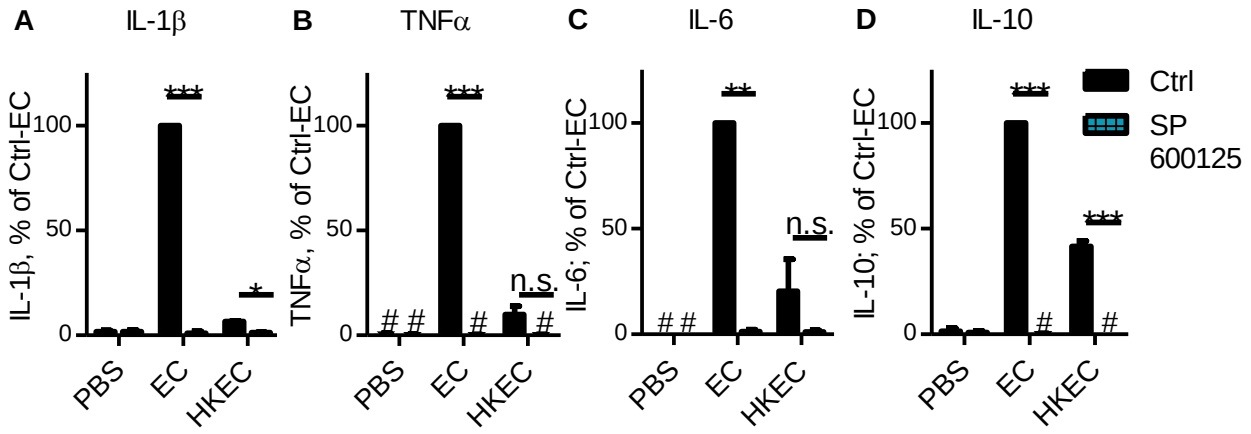


Fig. 9: Cytokine production by JNK-inhibitor SP600125-pretreated classical monocytes after infection with viable and dead *E. coli*. Cells were pretreated with SP600125 for 1 hour and subsequently infected with viable and dead *E. coli* at MOI 1. Cytokine release was measured 18 hours p.i. by ELISA (all n=2). All data are expressed as percentage of untreated cells stimulated with viable *E. coli.* Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments were separated from functional controls for the sake of clatrity. n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, # = not detected.

Taken together, these data suggest that MAPK play a role in the detection of bacteria, not necessarily dependent on the detection of viability. A possible contribution of p38 MAPK to hypothesized dichotomy of IL-1β and TNFα cytokine responses (see below) following recognition of viable bacteria remains to be investigated.

3.2.2 Inhibition of Phosphoinositide 3-kinase Decreases TNFα Response to Viable *E. coli* and TLR7/8 Stimulation

Phosphatidylinositol 3-kinase (PI3K) is critically involved in host defense against bacterial infections, by regulating important cellular processes, including phagocytosis[56], autophagy[57] and TLR signaling[58]. The role for PI3K in sensing bacterial viability is unknown.

We used PI3K inhibitor LY294002 to assess the function of PI3K and PI3K-dependent cellular processes in responses to viable and killed bacteria. Preincubation of human monocytes with LY294002 does not alter IL-6, but reduces TNFα and IL-10 responses by 50% to TLR7/8 agonist R848 (Fig. 10A, C, E), however, the effect did not reach statistical significance. In contrast, LPS-induced cytokine production is slightly or significantly *increased* by LY294002 preincubation (Fig. 10B, D, F). Although statistically not fully conclusive, the data suggest an inhibitory capacity of LY294002 for TLR8-mediated immune responses.

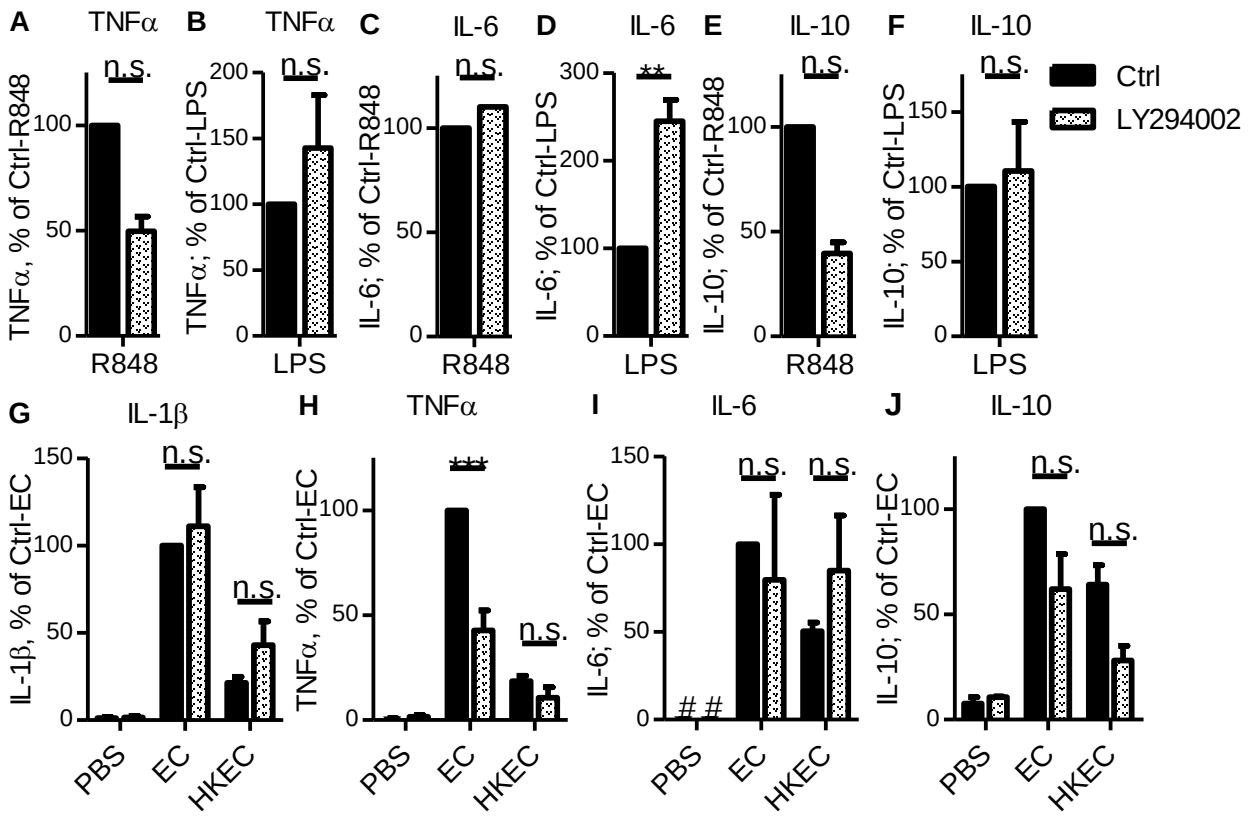


Fig. 10: Cytokine production by PI3-kinase inhibitor LY294002-pretreated classical monocytes after TLR stimulation or infection with viable and dead *E. coli*. Cells were pretreated with LY294002 for 1 hour and subsequently stimulated with R848, LPS (A-F) or infected with viable and dead *E. coli* (G-J) at MOI 1. Cytokine release was measured 18 hours post stimulation by ELISA (all n=3 except E, F, I, J n=2). All data are expressed as percentage of untreated cells stimulated with R848, LPS or infected with viable *E. coli.* Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, ** = $p < 0.01$, *** = $p < 0.001$.

Interestingly, PI3K inhibition by LY294002 causes a highly significant reduction of TNFα levels after the detection of viable bacteria (Fig. 10H). The residual TNFα response after infection with heat-killed *E. coli* is not significantly affected, but also reduced. leaving the question open if a different, PI3K-independent signaling pathway controls the residual TNFα response. Furthermore, inhibition of PI3K does not significantly alter IL-1β, IL-6 or IL-10 cytokine response after stimulation with either viable or dead *E. coli* (Fig. 10G, I, J). Results from experiments performed by our group with the autophagy inhibitor wortmannin and Cytochalasin D revealed a strongly reduced TNFα production in response to viable but not dead *E. coli*. (personal communication Matteo Ugolini, Sander laboratory, data not shown, unpublished). Therefore, LY294002-mediated inhibition of autophagy might contribute to the observed results.

Taken together, the data might suggest an important role for PI3K and potentially autophagy for the induction of TNFα responses upon detection of bacterial viability. Furthermore, these results in conjunction with data from section 3.2.4 and 3.2.5 suggest a dichotomic regulation of IL-1β- and TNFα-responses after infection with viable *E. coli*.

3.2.3 Inhibition of Bruton's Tyrosine Kinase Diminishes Cytokine Production in Response to Viable *E. coli*

Bruton's tyrosine kinase (BTK) is an enzyme involved in B cell biology and Fc receptor signaling[59], and it was shown to positively regulate TLR8 signaling[60]. Inhibition of BTK by LFM-A13 was reported to impair TLR2 and TLR4 downstream signaling[61]; it was therefore examined for its role in bacteria-induced cytokine induction.

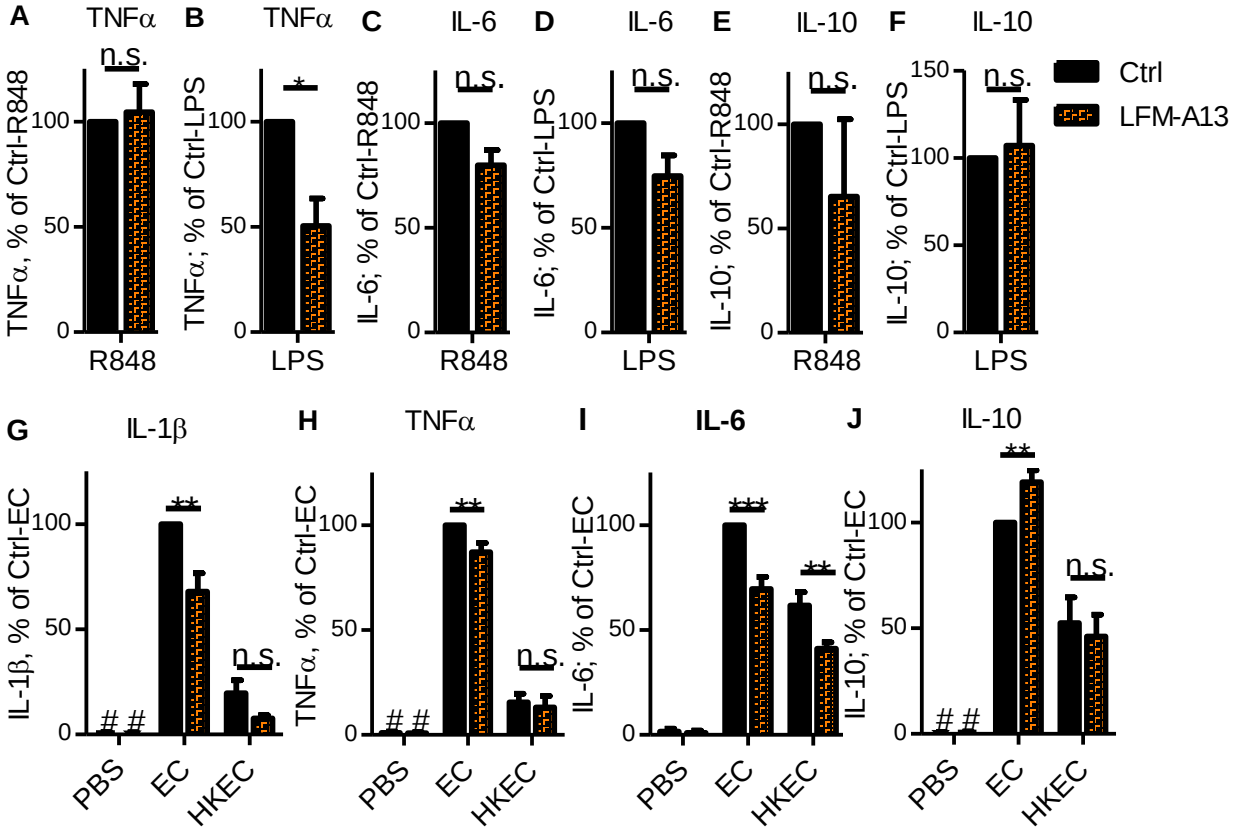


Fig. 11: Cytokine production by BTK-inhibitor LFM-A13-pretreated classical monocytes after stimulation with TLR-agonists or live and dead *E. coli*. Cells were pretreated with LFM-A13 for 1 hour and subsequently stimulated with R848, LPS (A-F) or viable and dead *E. coli* (G-J) at MOI 1. Cytokine release was measured by ELISA 18 hours post stimulation (G, all n=4, except A, B, E, F n=3). All data expressed as percentage of solvent (0.5% DMSO) treated cells stimulated with R848 or infected with viable *E. coli*. Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, # = not detected.

Experiments to characterize the specificity of LFM-A13 reveal a significant reduction of TNFα and IL-6 responses after LPS, but not R848 stimulation (Fig. 11A-D), indicating an involvement of BTK in TLR4, but not TLR7/8 signal transduction in our system. IL-10 levels are not significantly altered (Fig. 11E, F). However, the IL-10 responses after inhibitor application and R848 stimulation show a strong variation, allowing no final exclusion of TLR8 inhibition capacity. The inhibitor application does not display relevant cytotoxic effects (data not shown).

Inhibition of BTK leads to a significantly reduced secretion of IL-1β and IL-6 after infection with viable *E. coli*. (Fig. 11G, I). The cytokine response to dead bacteria is also significantly reduced for IL-6, and by trend for IL-1β without statistical significance (Fig. 11G, I). Inhibition of BTK causes a small but significant decrease in TNFα production after infection with viable but not dead *E. coli* (Fig. 11E). BTK blockade does not show any effect on the IL-10 response after infection with either viable or dead bacteria (Fig. 11G).

In conclusion, BTK seems to be involved mainly in the induction of IL-1β and IL-6 after infection with both type of *E. coli*. Additionally, the collected data could indicate a minor involvement in the signaling pathways leading to secretion of TNFα upon detection of viable bacteria.

3.2.4 Inhibition of TLR4 Reduces the IL-1β Response through the Reduction of Intracellular pro-IL-1β

Unpublished data from our group revealed that macrophages derived from TLR4-deficient mice showed impaired IL-1β responses, suggesting an involvement of TLR4 in the immune response to bacterial viability (personal correspondence, L. E. Sander). Here, CLI-095 was used to inhibit TLR4 signaling.

The inhibition specificity of TLR4-signaling by CLI-095 in human classical monocytes was investigated by stimulation with selective TLR ligands. Indeed, CLI-095-treated monocytes produce significantly less TNFα and IL-6 (Fig. 12B, D) after LPS-stimulation, whereas responses to R848 (Fig. 12A, C) are unaltered. The IL-10 production is reduced for both ligands, which might indicate an off-target or downstream inhibitory effect of CLI-095 (Fig. 12E, F). Since neither LPS nor R848 alone promote a sufficiently detectable IL-1β response, the inhibition quality could not be evaluated for this inflammasome-dependent cytokine after ligand stimulation (Data not shown). Despite the slight unspecific effects, we deemed CLI-095 sufficient for further screening.

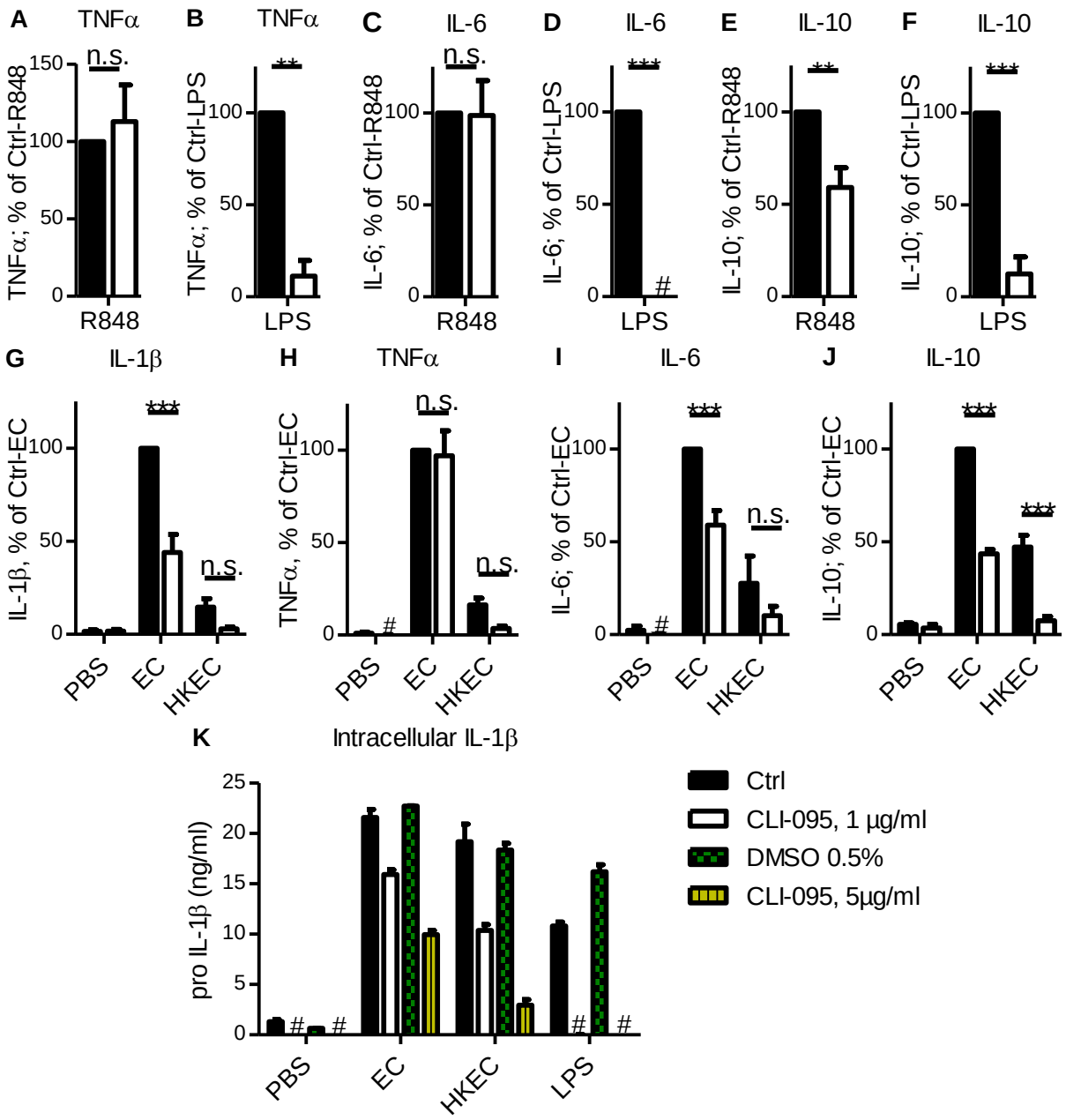


Fig. 12: Cytokine secretion and pro-IL-1β production by TLR4-signaling inhibitor CLI-095-pretreated classical monocytes in response to TLR stimulation or live and dead *E. coli*. Cells were pretreated with CLI-095 for 1 hour and subsequently stimulated with R848 or LPS (A-F, K) or infected with viable and dead *E. coli* (H-J) at MOI 1. Cytokine values were measured by ELISA 18 hours post stimulation (n=5 for all except C, D, I; n=4; K n=1). All data are expressed as percentage of untreated cells stimulated with R848, LPS or infected with viable *E. coli.* Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, ** = $p < 0.01$, *** = $p < 0.001$, # = not detected.

Infection experiments with viable bacteria reveal that CLI-095 pretreated cells secrete significantly less IL-1β, IL-6 and IL-10, but release similar amounts of TNFα (Fig. 12G-J) compared to control cells. Although pretreatment also reduces the cytokine responses after infection with heat killed *E. coli*, this difference does not reach statistical

significance, except for IL-10 (Fig. 12G-J). The residual TNFα release observed upon HKEC stimulation is largely blocked by CLI-095 (albeit not statistically significant), indicating that this very minor portion of the TNF response is LPS-driven (Fig. 12H). Additionally, CLI-095 treatment does not significantly induce or reduce LDH release as a measure of monocyte cell death, after infection or TLR-ligand stimulation (data not shown). Adressing the question of whether the effect of TLR4-signaling inhibition on IL-1β release affects intracellular (mainly pro-IL-1β) levels, cytosolic pro-IL-1β was determined as described in the methods section. Interestingly, pro-IL-1β levels induced by viable and dead *E. coli* are comparable (Fig. 12K). And indeed, CLI-095 treatment decreases intracellular pro-IL-1β levels after stimulation with both viable and dead *E. coli* in a dose dependent manner and completely abolishes intracellular pro-IL1β induced by LPS-stimulation (Fig. 12K).

These data suggested two important conclusions. First, live and dead *E. coli* equally induce intracellular pro-IL-1β, whereas only live bacteria induced IL-1β release, indicating that recognition of live bacteria regulates inflammasome activation, as observed in mice[37]. Second, TLR4 plays a crucial role in the in the induction of intracellular pro-IL-1β, irrespective of bacterial viability.

3.2.5 TLR8 Inhibition by Imiquimod Selectively Impairs TNFα Responses after Infection with Viable *E. coli*

Imiquimod (R837) is a known agonist of TLR7, but it has also been reported to act as an inhibitor on TLR8[62]. Imiquimod was first investigated for inhibition specificity by using specific TLR4 and TLR7/8 ligands, LPS and R848, respectively. IL-1β could not be used as a reliable readout, since TLR stimulation alone is not reliably sufficient to induce IL-1β production by classical monocytes.

Imiquimod preincubation tend to reduce IL-6, IL-10 responses to TLR8 but not to TLR4 ligands (Fig. 13C-F) and TNFα production after administration of both stimuli (Fig. 13A, B). None of these observations reached statistical significance. Hence, these data indicate only a limited specific, weak inhibitory effect of Imiquimod on TLR8 signaling in human monocytes.

In contrast, Imiquimod in fact modulates cytokine responses to infection with *E. coli*. While IL-1β release is not significantly changed but rather enhanced (Fig. 13G), Imiquimod pretreatment significantly reduces TNFα production only after infection with viable *E. coli* (Fig. 13H). IL-6 and IL-10 responses are not affected upon stimulation with

either viable or dead bacteria (Fig. 13I, J). Additionally, cell death assays show no significant cytotoxic effects of the inhibitor application only (Data not shown).

Taken together, these data again strongly indicate that IL-1β and TNFα responses are differentially regulated, and Imiquimod specifically diminishes TNFα secretion, which could suggest an involvement of TLR8 signaling in this process.

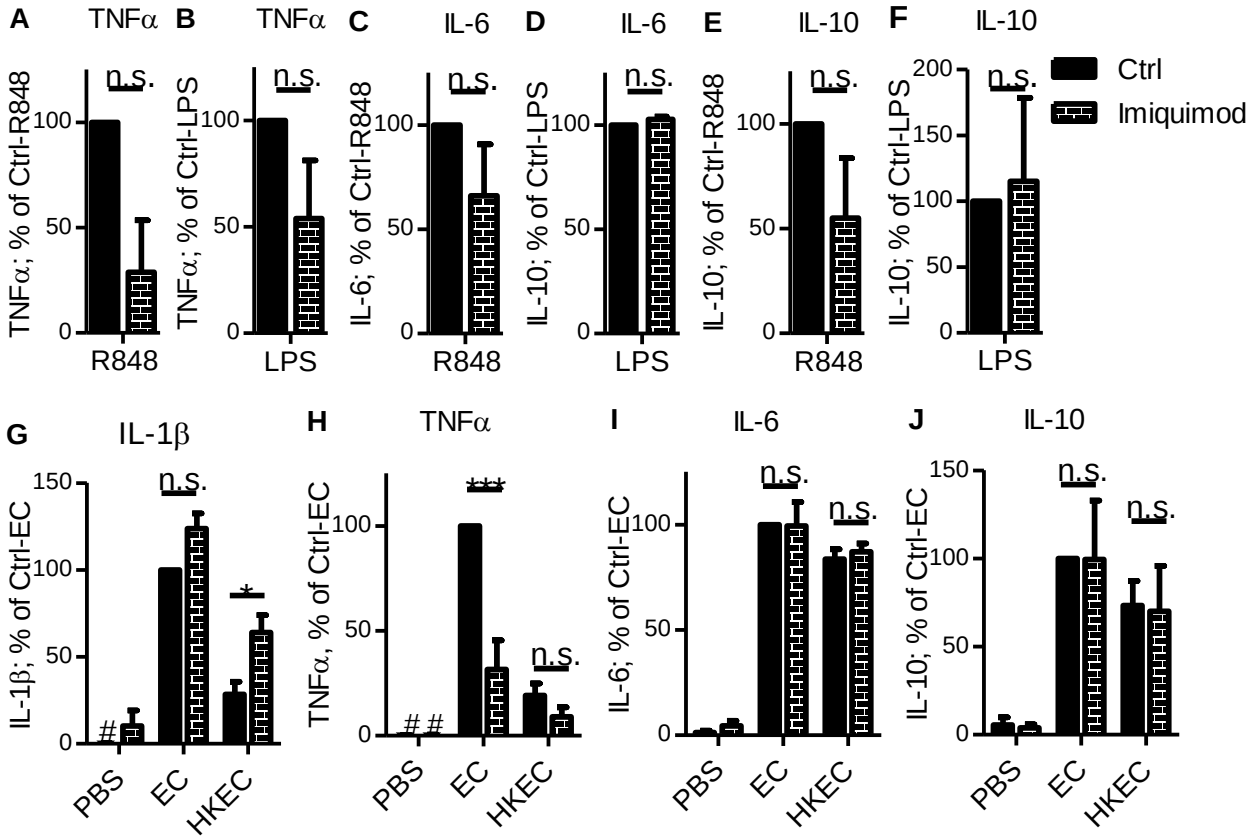


Fig. 13: Effect of TLR8-inhibitor Imiquimod pretreatment on cytokine production of classical monocytes in response to stimulation with TLR ligands or viable and dead *E. coli*. Cells were pretreated with Imiquimod for 45 minutes and subsequently stimulated with R848 or LPS (A-F) or viable and dead *E. coli* (G-J) at MOI 1. Cytokine release was measured 18 hours post stimulation by ELISA. All data are expressed as percentage of solvent (0.5% DMSO) treated cells stimulated with R848 or infected with viable *E. coli* (all n=3). Data are shown as mean + SEM. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, * = $p < 0.05$, *** = $p < 0.001$, # = not detected.

3.2.6 Summary of Signaling Inhibitor Screen

In summary, the inhibitor screening experiments support the hypothesis that proinflammatory innate immune responses, namely IL-1β and TNFα, are differentially induced via distinct signaling pathways upon detection of *E. coli.* TLR4 and BTK and potentially p38 MAPK play a dominant role in IL-1β secretion, while the TLR8-PI3K (autophagy) axis might be an important component in TNFα responses to live and

partially also dead bacteria. Cytokine responses of monocytes to bacterial stimulation require JNK and NF-κB independently of the detection of bacterial viability. The results of the small-molecule inhibitor screen are summarized in .

Tab. 6.

Tab. 6: Alteration of cytokine response by monocytes after inhibitor application and infection with viable *E. coli* (A) or heat killed *E. coli* (B) in % compared to respective control samples.

A

Inhibitor	Target	IL-1β	TNFα	IL-6	IL-10
BAY11-7082	IκBα	- 99.1	- 96.8	- 98.6	- 95.7
SB203580	p38	- 71.4	- 9.2	- 42.6	- 27.0
SP600125	JNK	- 99.0	- 99.7	- 98.7	- 99.8
CLI-095	TLR4	- 53.2	- 15.2	- 32.8	- 55.0
LFM-A13	BTK	- 38.3	- 14.2	- 31.2	+ 18.6
LY294002	PI3K	+ 10.9	- 57.3	- 3.1	- 38.1
Imiquimod	TLR7/8	+ 23.8	- 68.4	- 0.7	- 0.5

B

Inhibitor	Target	IL-1β	TNFα	IL-6	IL-10
BAY11-7082	IκBα	- 96.6	- 97.2	- 99.9	- 93.7
SB203580	p38	- 87.9	- 80.0	- 63.6	- 51.0
SP600125	JNK	- 79.4	- 97.1	- 94.5	- 100.0
CLI-095	TLR4	- 79.8	- 78.5	- 62.2	- 84.1
LFM-A13	BTK	- 60.9	- 15.5	- 33.4	- 12.3
LY294002	PI3K	+ 101.3	- 42.6	+ 68.2	- 56.2
Imiquimod	TLR7/8	+ 144.8	- 51.2	+ 2.5	- 16.1

3.3 Evaluation of Candidate Molecules Involved in the Detection of Bacterial Viability Using Lentiviral shRNA Vectors

Initially, the effect of MoDCs differentiation in the presence of LV-medium instead of standard growth medium was controlled. Separate analysis of both approaches revealed no relevant effect of the chosen culture media on cytokine production. Since MoDCs were cultured in a higher volume for lentiviral transduction and cytokines were

therefore diluted, cytokine responses were calculated as percentages of the *E. coli* infected samples set as 100% for both approaches. No appreciable differences between the two groups were detectable (data not shown).

3.3.1 TRIF Deficiency Impairs IL-1β Precursor Cleavage in Response to Viable *E. coli* and Secretion of other Cytokines by MoDCs

As mentioned above, the intracellular adaptor molecule TRIF is known to be essential for the cytokine responses to bacterial viability in murine cells[37]. Also, it is a known adaptor molecule for TLR4 and TLR3[13], and might also be involved in the signaling of intracellular RNA helicases, which sense dsRNA[18]. It has important functions in cytokine induction and host defense[63], thus, TRIF was a very promising target to investigate.

First, individual lentiviral vectors encoding for single TRIF-specific shRNAs were tested for their knockdown efficacy by stimulation of transduced MoDCs with TLR3-TRIF ligand poly I:C. Hereafter, lentiviral vectors encoding for TRIF-specific shRNA are referred to as TRIF-LV, whereas lentiviral vectors expressing no specific shRNA are referred to as Ctrl-LV. Due to the lack of cytokine responses of MoDCS to poly I:C, qRT-PCR for TRIF expression was performed and three of the four vectors tested efficaciously down-regulate TRIF mRNA expression (Fig. 14A). Further experiments to functionally assess TRIF knockdown efficiency were performed using the licensed vaccine adjuvant and TRIF-biased TLR4 agonist monophosphoryl lipid A (MPLA)[64]. Transduction of MoDCs with three of the clones in combination significantly reduces MPLA-induced TNFα and IL-6 responses (Fig. 14B-C). IL-1β is not significantly reduced due to weak secretion after TLR4 stimulation (Data not shown). As with monocytes, IL-1β is not secreted to considerable levels after TLR4 stimulation (Data not shown). An efficient knockdown efficiency could be further confirmed in infection experiments, both on the mRNA and the protein level (Fig. 14D, I).

Interestingly, TRIF gene silencing in MoDCs impairs IL-1β responses to viable bacteria, while the response to dead *E. coli* is not significantly reduced. TNFα secretion is diminished and IFN-β response is almost completely blocked independently of bacterial viability. TRIF silencing does not affect IL-6 responses to either bacterial stimulus (Fig. 14E-H). The observed effect on IL-1β secretion is not a result of insufficient inflammasome priming, since NLRP3 and pro-IL-1β expression are not affected by TRIF knockdown. Importantly, MPLA-TLR4/TRIF induced pro-IL-1β expression (which

requires TRIF) is completely abolished upon TRIF silencing, further confirming the specificity of the knockdown (Fig. 14I).

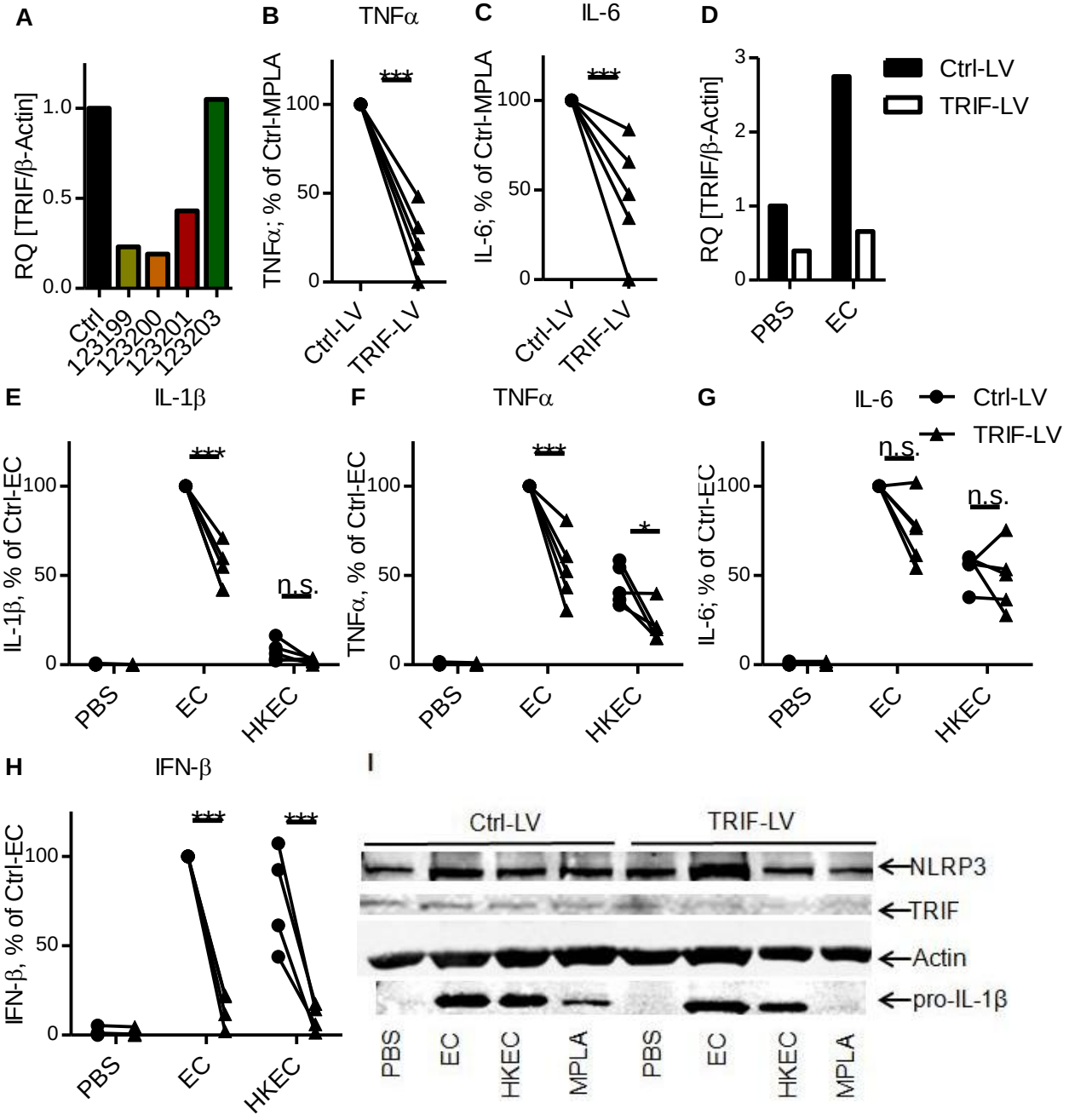


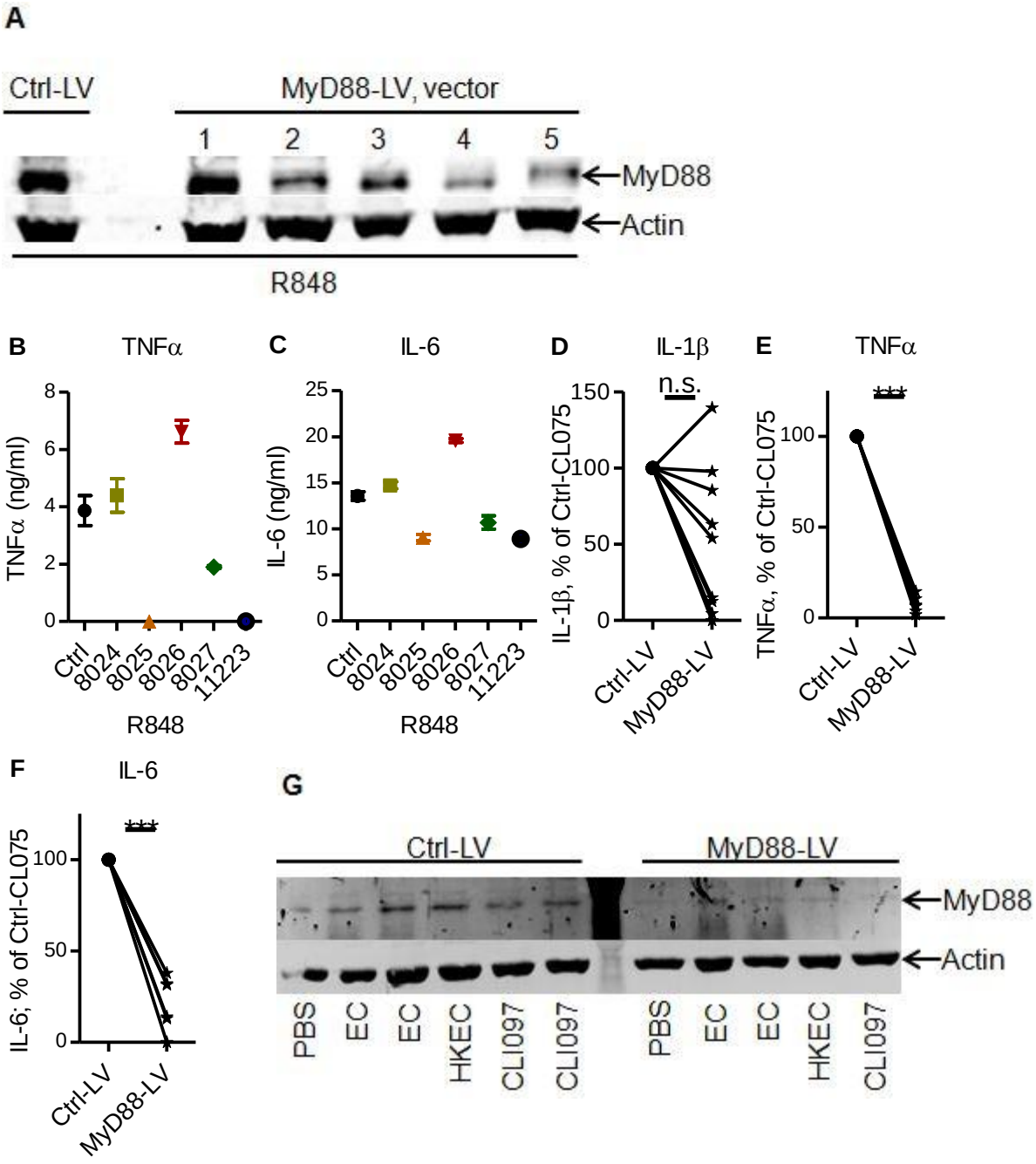
Fig. 14: TRIF silencing differentially alters cytokine responses of MoDCs after infection with viable and dead *E. coli* without effecting NLRP3 or pro-IL-1β expression. Knockdown efficiency of single lentiviral shRNA vectors was examined by qRT-PCR (A, n=1), and tested by MPLA stimulation of Ctrl-LV- and TRIF-LV-transduced MoDCs for 18 hours and subsequent cytokine quantification by ELISA (B,C, n=5). Knockdown efficiency after infection with viable *E. coli* was confirmed by qRT-PCR (D) and Western Blot (I). Transduced MoDCs were infected with viable and dead *E. coli* at MOI 10. Cytokine release was measured 18 hours post stimulation (all n=5 except H n=4). All data are expressed as percentage of Ctrl-LV-treated cells stimulated with MPLA or viable *E. coli*. Each symbol represents an individual experiment. NLRP3 and pro-IL-1β expression in TRIF-deficient MoDCs was investigated by Western Blot (I). Two-way Anova was performed, followed by

Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, * = p < 0.05, *** = p < 0.001.

Taken together, these data indicate a major role for TRIF in the viability-induced secretion of mature IL-1β, without affecting inflammasome priming.

3.3.2 MyD88 is a Key Signaling Component for Cytokine Production by MoDCs in Response to Bacterial Stimulation

Previous findings in murine phagocytes suggested a critical role of MyD88 in the detection of bacterial viability. Therefore, it was of great interest to specify the function of MyD88 in human MoDCs.



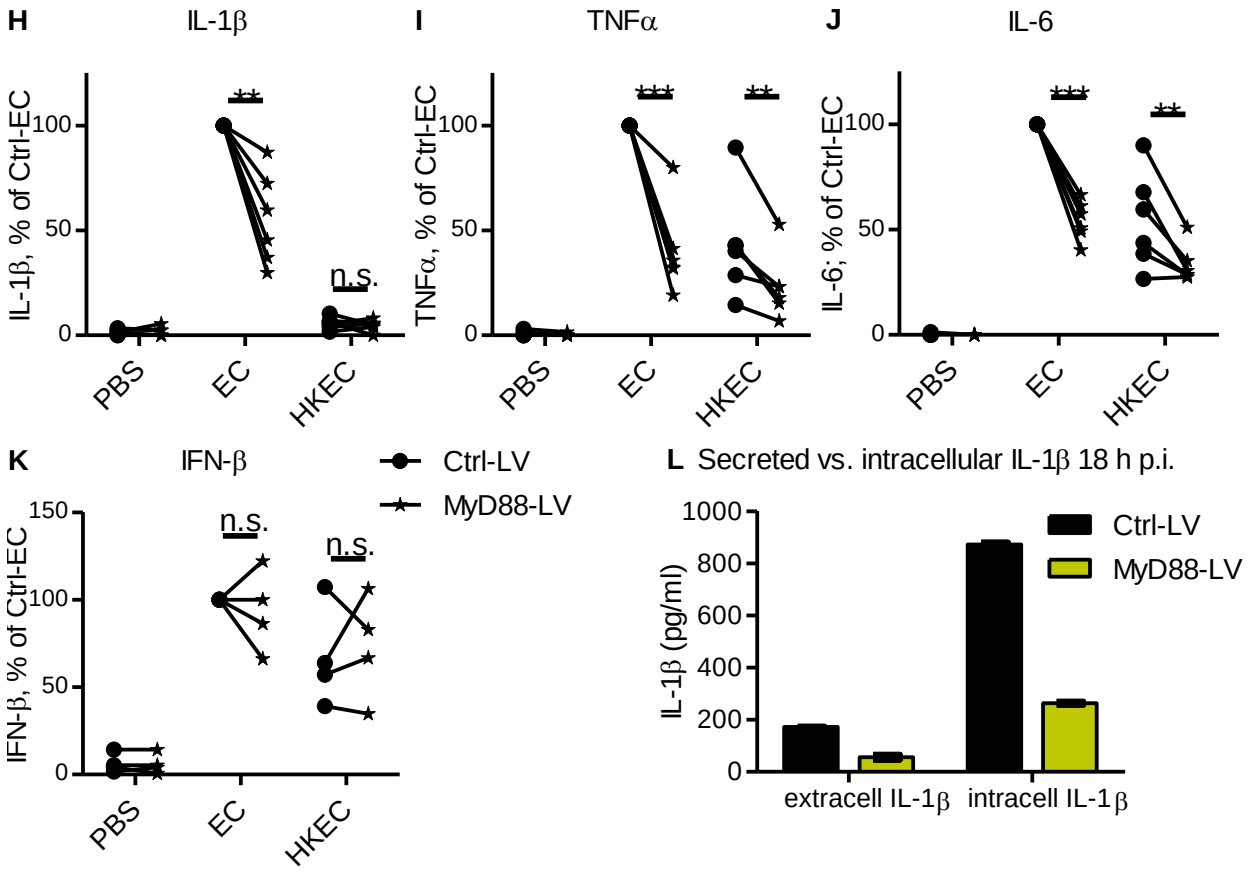


Fig. 15: MyD88 plays a major role in the induction of cytokine responses by MoDCs after infection with viable and dead *E. coli*. Knockdown efficiency for MyD88-LV was measured by Western Blot (A) and examined by stimulating transduced MoDCs with R848 for 18 hours and subsequent cytokine quantification by ELISA (B, C, n=1). Silencing efficiency of three pooled MyD88-LV clones was monitored by stimulating transduced MoDCs and subsequent cytokine quantification (D, n=5; E-F, n=6). Knockdown efficiency was also monitored by Western Blot (G, representative of three independent experiments).Ctrl-LV- and MyD88-LV-transduced MoDCs were infected with viable and dead *E. coli* at MOI 10. Cytokine secretion was measured 18 hours post stimulation (all n=6 except K, n=3). Results are expressed as percentage of Ctrl-LV-transduced cells stimulated with CLI075 or viable *E. coli*. Each symbol represents an individual experiment. pro-IL-1β was measured as described above (L, n=1). Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, ** = $p < 0.01$, *** = $p < 0.001$.

Out of five MyD88-LV tested in MoDCs, three are sufficient to suppress MyD88 expression (Fig. 15A). Accordingly, TNFα, and IL-6 responses after stimulation with R848, a TLR7/8 agonist known to require MyD88 are efficiently reduced in MoDCs transduced with the three selected MyD88-LV (Fig. 15B,C). Functional knockdown controls using CL075 as another MyD88-dependent stimulus confirm the potency of MyD88-LV-mediated silencing (Fig. 15D-F). Western Blot analysis verifies a sufficient knockdown of MyD88 after stimulation and infection (Fig. 15G). MyD88-LV-transduced MoDCs secrete significantly less IL-1β after infection with viable *E. coli* (Fig. 15H).

Interestingly, MyD88 silencing reduced TNFα and IL-6 in response to bacteria, independently of viability (Fig. 15H-J). MyD88 knockdown does not significantly affect IFN-β production after infection with viable or dead bacteria (Fig. 15K). Silencing of MyD88 reduces the level of intracellular pro-IL-1β by 68% compared to Ctrl-LV-transduced cells (Fig. 15L). Notably, this reduction correlates with the decrease of secreted IL-1β (Fig. 15H), but is only demonstratetd by a single experiment.

In summary MyD88 is a key molecule in the induction of cytokine responses to viable and dead bacteria. Most likely, IL-1β secretion is regulated mainly by MyD88 prior to cleavage and secretion, presumably on the level of pro-IL-1β induction.

3.3.3 ASC Silencing Strongly Impairs Secretion of IL-1β and other Cytokines in Response to *E. coli*

After analyzing the involvement of major TLR adaptor proteins TRIF and MyD88, the inflammasome adaptor protein ASC was investigated for its role in IL-1β secretion upon detection of bacterial viability. A role for ASC was already demonstrated for murine innate immune cells[37], but not examined in human phagocytes.

First, individual ASC-LV plasmids were used separately to produce lentiviral vectors and checked for their silencing efficiency by functional and Western Blot analysis. Interestingly, plasmids sufficient to reduce the LPS plus Nigericin-induced IL-1β responses also diminish TNFα and IL-6 production (Fig. 16A-C). Although functional analysis suggests three suitable plasmids, Western Blot analysis shows no obvious decrease in ASC protein expression (Blot not shown). Nonetheless, a combination of the three selected plasmids after functional analysis leads to a considerable reduction of ASC expression in all tested conditions (Fig. 16G).

Additionally, ASC-LV-transduced MoDCs infected with viable *E. coli* show strongly reduced IL-1β, and unexpectedly also diminished TNFα as well as IL-6 responses. However, the cytokine levels induced by dead *E. coli* remain unaltered (Fig. 16H-J). Similarly, silencing of ASC reduces the secretion of IFN-β only after the detection of bacterial viability (Fig. 16K).

In line with the data obtained for the murine innate immune system, ASC seems to play a critical role in IL-1β secretion by primary human immune cells after infection with viable *E. coli*. However, the data obtained could suggest a relevant degree of non-specific effects of ASC-silencing or a broader role of ASC in immune response induction, given the unexpected reduction of TNFα and IL-6 upon detection of *E. coli*.

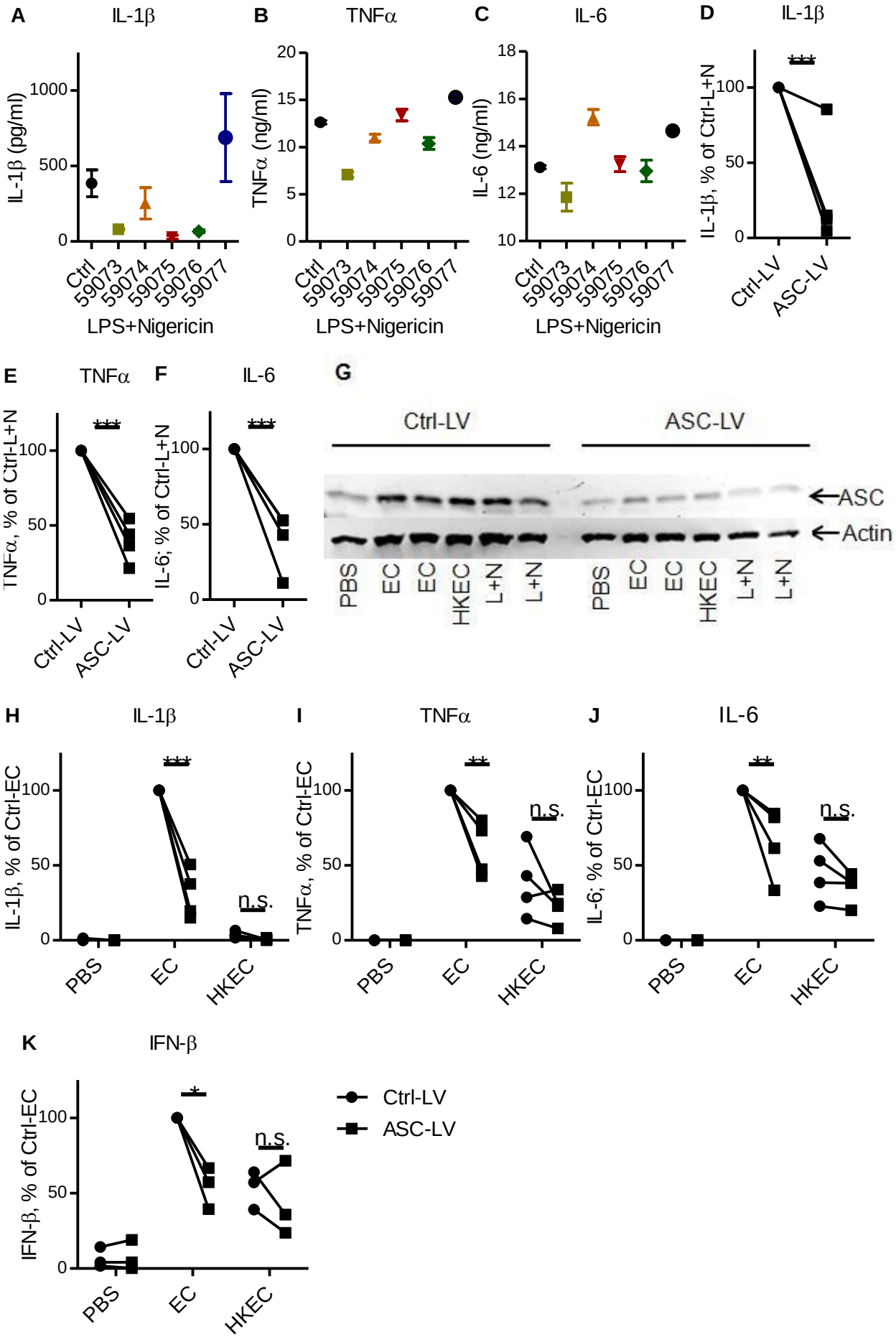

A
IL-1β
IL-1β (pg/ml)
1000
500
0
Ctrl
59073
59074
59075
59076
59077
LPS+Nigericin
B
TNFα
TNFα (ng/ml)
20
15
10
5
0
C
IL-6
IL-6 (ng/ml)
18
16
14
12
10
D
IL-1β
IL-1β, % of Ctrl-L+N
***
Ctrl-LV
ASC-LV
E
TNFα
TNFα, % of Ctrl-L+N
F
IL-6
IL-6; % of Ctrl-L+N
G
Ctrl-LV
ASC-LV
←ASC
←Actin
PBS
EC
HKEC
L+N
H
IL-1β
IL-1β, % of Ctrl-EC
**
n.s.
PBS
EC
HKEC
I
TNFα
TNFα, % of Ctrl-EC
J
IL-6
IL-6; % of Ctrl-EC
K
IFN-β
IFN-β, % of Ctrl-EC
*
Ctrl-LV
ASC-LV

Fig. 16: ASC silencing reduces IL-1β, TNFα and IL-6 responses upon detection of viable but not dead *E. coli*, and impairs IFN-β production independently of viability. Knockdown efficiency of single lentiviral shRNA vectors was functionally examined by stimulating ASC-LV-transduced MoDCs for 4 hours with LPS, followed by 30 minutes with Nigericin and subsequent cytokine quantification by ELISA (A-C, n=1). Silencing efficiency of three selected ASC-LV was assessed by stimulating ASC-LV-transduced MoDCs with LPS+nigericin and subsequent cytokine quantification by ELISA (D-F, n=4). Knockdown efficiency was confirmed by Western Blot (G). G shows a representative blot of three independent experiments. Ctrl-LV- and ASC-LV-transduced MoDCs were infected with viable and dead *E. coli* at MOI 10. Cytokine release was measured by ELISA 18 hours post stimulation (all n=4 except K n=3). Data are expressed as percentage of Ctrl-LV-treated cells stimulated with either LPS and Nigericin or viable *E. coli*. Each symbol represents an individual experiment. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.3.4 NLRP3 Inflammasome is Required to Promote IL-1β Secretion by MoDCs after Infection with Viable *E. coli*

It has previously been shown in murine phagocytes that detection of bacterial viability or bacterial *vita*-PAMPs induces the activation of the NLRP3 inflammasome[37]. Thus, we tested the role for NLRP3 in the innate immune response to bacterial viability. A total of five lentiviral plasmids encoding for NLRP3 shRNA were used simultaneously to generate a pool of NLRP3-LV. As expected, NLRP3-LV-transduced MoDCs produce significantly less IL-1β after stimulation with the known NLRP3 activator LPS plus Nigericin, while other cytokines are not significantly affected (Fig. 17A-C).

Silencing efficiency was confirmed by Western Blot analysis (Fig. 17D).

IL-1β secretion of MoDCs transduced with NLRP3-LV is highly significantly reduced specifically after infection with viable *E. coli* (Fig. 17E). All other cytokines measured are not significantly affected (Fig. 17F-H). However, IFN-β production is reduced in two out of three experiments but induced in one experiment after infection with viable *E. coli* (Fig. 17H), leaving the role of NLRP3 for IFN-β response unclear.

Taken together, the NLRP3 inflammasome is critically required for IL-1β secretion upon detection of bacterial viability.

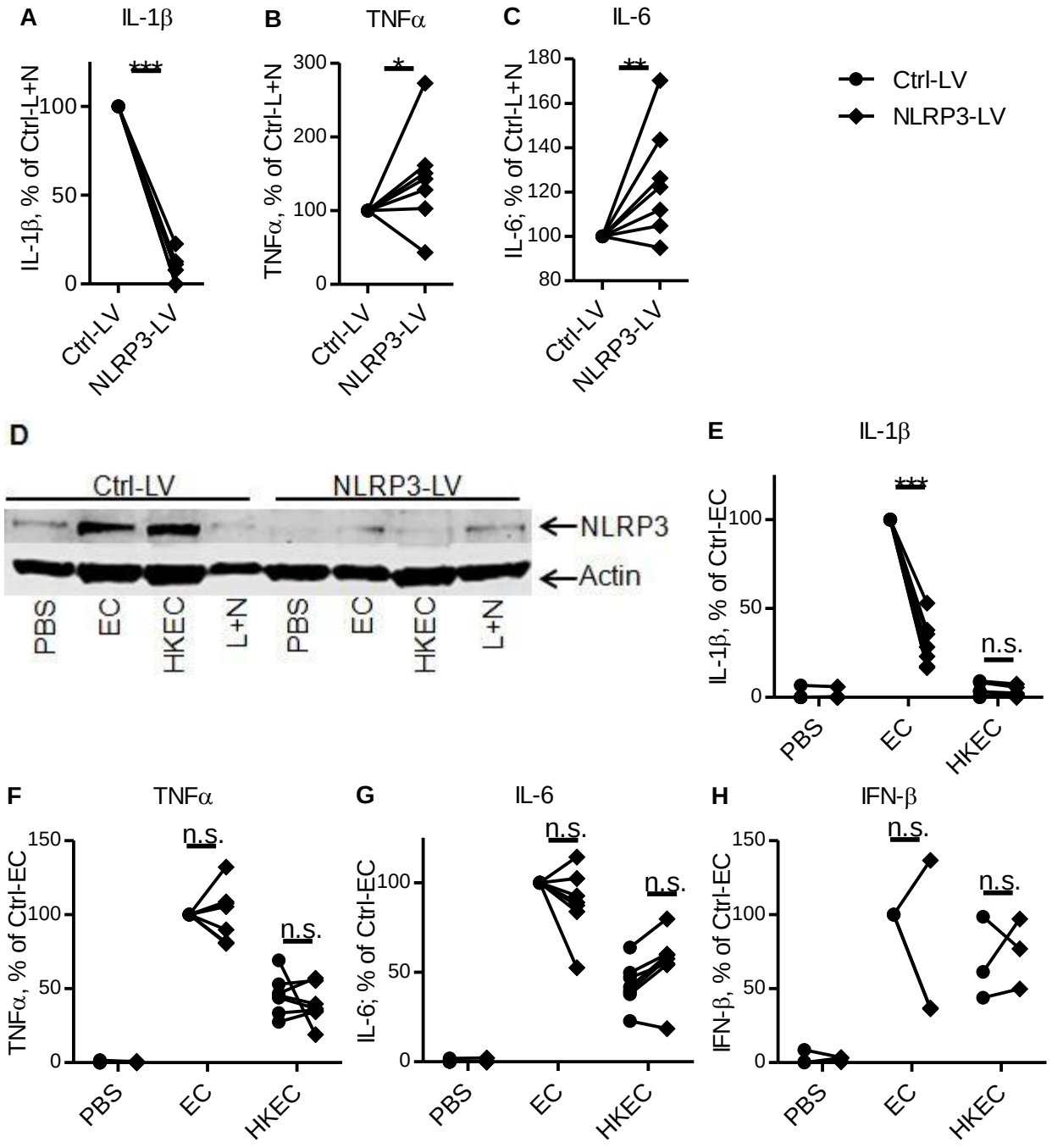


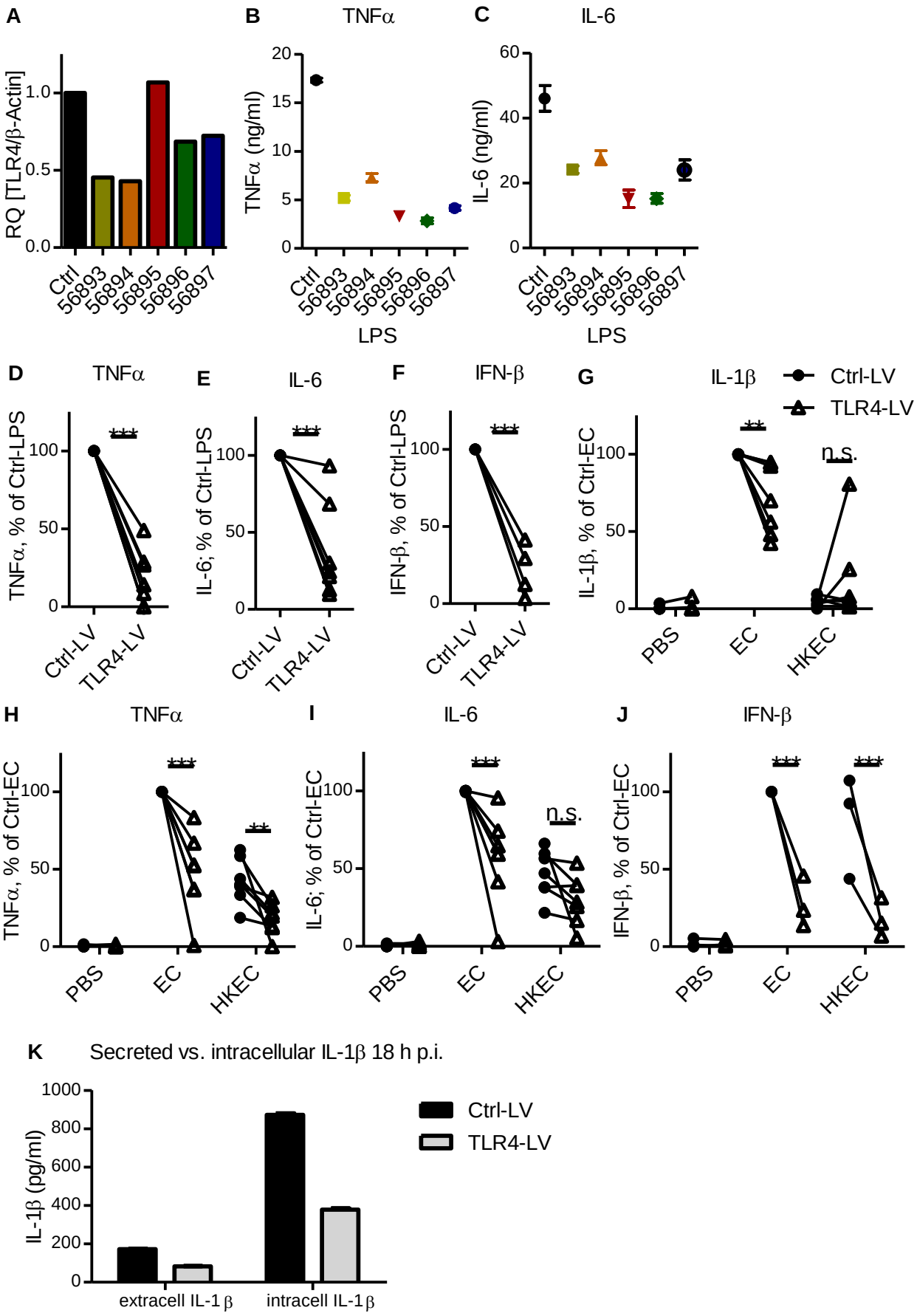
Fig. 17: NLRP3 silencing impairs MoDCs to secrete IL-1β after infection with viable *E. coli* but does not alter other cytokine responses. Knockdown efficiency was functionally examined by stimulating transduced MoDCs for 4 hours with LPS followed by 30 minutes with Nigericin and subsequent cytokine quantification by ELISA (A-C, n=7). Western Blot analysis confirmed efficient silencing (D). The blot shown is representative of three comparable experiments. Ctrl-LV- and NLRP3-LV-transduced MoDCs were infected with viable and dead *E. coli* at MOI 10. Cytokine release was measured by ELISA 18 hours post stimulation (all n=7 except H n=3). All data are expressed as percentage of Ctrl-LV treated cells stimulated with either LPS and Nigericin or viable *E. coli*. Each symbol represents an individual experiment. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the purpose of clatrity. n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, # = not detected.

3.3.5 TLR4 Silencing Reduces IL-1β and IL-6 Secretion after Detection of Viable *E. coli*

Since the inhibitor screening approach suggests an involvement of TLR4 in the induction of pro-IL-1β and additionally an important role of IL-6, IL-10 but not TNFα responses, lentiviral experiments were carried out to specifically examine the function of TLR4.

Testing of individual lentiviral shRNA vectors showed that four out of five TLR4-LV reasonably reduce TLR4 mRNA levels, although none of the TLR4-LV alone reduces expression by more than 60% (Fig. 18A). All of the tested TLR4-LV suppress TNFα and IL-6 release after stimulation with TLR4 ligand LPS (Fig. 18B, C). Subsequently, a combination of plasmids was used for TLR4-LV production. The experiments show a significantly decreased TNFα, IL-6 and IFN-β response of TLR4-LV pool-transduced MoDCs after stimulation with LPS (Fig. 18D-F). Multiple attempts to assess TLR4 silencing efficiency by Western Blot failed, since reliable TLR4 signals could not be detected (data not shown). In accordance with the inhibitor experiments, TLR4 silencing diminishes IL-1β and IL-6 secretion after infection with viable bacteria (Fig. 18G, I). Surprisingly, TLR4-LV-transduced MoDCs respond less effectively in terms of TNFα after infection with viable and dead *E. coli*, indicating an additional signal transduction role of TLR4 in the induction of TNFα secretion by MoDCs (Fig. 18H). TLR4 silencing strongly reduces the capability of MoDCs to produce IFN-β in response to viable and dead *E. coli* (Fig. 18J). Consistently with results obtained with CLI095, TLR4 deficiency decreases the levels of intracellular pro-IL-1β. The reduction of intracellular pro-IL-1β correlates with the quantified decrease of secreted IL-1β (Fig. 18K).

Taken together, the important role of TLR4 for IL-1β production, presumably by induction of intracellular pro-IL-1β, is confirmed using lentiviral gene silencing. Additionally, MoDCs require TLR4 for the induction of IL-6 production after infection with viable *E. coli* and for TNFα as well as IFN-β responses after infection with both viable and dead bacteria, indicating a major role of TLR4 in the cytokine response of MoDCs. The results also indicate an important difference between primary classical monocytes and MoDCs (Fig. 2B, Fig. 3B, Fig. 12), both in their capacity to produce TNFα in response to dead *E. coli*, as well as the role of TLR4 in this process. Overall, it appears that MoDCs are more responsive to TLR4 stimulation.


A
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0.5
0.0
Ctrl
56893
56894
56895
56896
56897
LPS
B
TNFα
TNFα (ng/ml)
20
15
10
5
0
C
IL-6
IL-6 (ng/ml)
60
40
20
0
D
TNFα
TNFα, % of Ctrl-LPS
100
50
***
Ctrl-LV
TLR4-LV
E
IL-6
IL-6; % of Ctrl-LPS
F
IFN-β
IFN-β, % of Ctrl-LPS
G
IL-1β
Ctrl-LV
TLR4-LV
IL-1β, % of Ctrl-EC
**
n.s.
PBS
EC
HKEC
H
TNFα
TNFα, % of Ctrl-EC
I
IL-6
IL-6; % of Ctrl-EC
J
IFN-β
IFN-β, % of Ctrl-EC
K
Secreted vs. intracellular IL-1β 18 h p.i.
IL-1β (pg/ml)
1000
800
600
400
200
0
extracell IL-1β
intracell IL-1β
Ctrl-LV
TLR4-LV

Fig. 18: Cytokine release by TLR4-LV-transduced MoDCs in response to LPS and viable and dead *E. coli*. Knockdown efficiency of single lentiviral shRNA vectors was monitored by qRT-PCR (A, n=1) and functionally examined by stimulating transduced MoDCs with LPS for 18 hours and subsequent cytokine quantification by ELISA (B, C, n=1). Silencing efficiency of three selected vectors was monitored by stimulating transduced MoDC with LPS and subsequent cytokine quantification by ELISA. Ctrl-LV- and TLR4-LV-transduced MoDCs were infected with viable and dead *E. coli* at MOI 10. Cytokine secretion was measured 18 hours post stimulation by ELISA (all n=7, except F , J n=3) Results are expressed as percentage of Ctrl-LV-transduced cells stimulated with LPS or viable *E. coli*. Each symbol represents an individual experiment. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. Intracellular IL-1β levels were measured as described above (K, n=1). n.s. = not significant, ** = $p < 0.01$, *** = $p < 0.001$.

3.3.6 TLR8 Silencing Slightly Alters Cytokine Production by MoDCs

Due to the possible involvement of TLR8 in the induction of TNFα suggested by data obtained with Imiquimod treatment of classical monocytes (Fig. 13), TLR8 expression was targeted by lentiviral transduction.

TLR8-LV produced with each of the available plasmids was used separately for transduction. MoDCs were stimulated with TLR8 agonist CL075, and cytokine measurements show that two plasmids decrease TNFα, and to a lesser extent IL-6 (Fig. 19A, B). These two TLR8-LV clones were used separately or in combination. Silencing efficiency was functionally monitored by CL075 stimulation and qRT-PCR. Western Blot could not be performed due to the unavailability of suitable TLR8 antibodies. Unfortunately, cytokine responses after CL075 are generally variable and not constantly significantly reduced (data not shown), even though qRT-PCR measurements shows a reasonable silencing efficiency on the mRNA level for unstimulated cells in some of the experiments (Fig. 19C), possibly due to the TLR7-costimulatory capacity of CL075 or R848 respectively. Furthermore, qRT-PCR reveals a modest silencing efficiency for TLR8-LV-transduced and *E. coli*-infected cells (Fig. 19C), indicating that infection with viable *E. coli* might stimulate TLR8 expression, overcoming the modest silencing effects achieved by TLR8-LV.

Despite rather weak knockdown efficiencies, similar trends as seen with the Imiquimod-treated monocytes were observable. TLR8-LV-transduced MoDCs secrete lower levels of TNFα, while IL-1β levels are slightly increased upon infection with viable *E. coli* (Fig. 19D, E). Surprisingly, in contrast to inhibitor screening, IL-6 production is also selectively reduced after infection with viable but not heat killed *E. coli* (Fig. 19F).

Furthermore, TLR8-silencing reduces the ability of MoDCs to produce IFN-β in response to *E. coli* independently of viability (Fig. 19G).

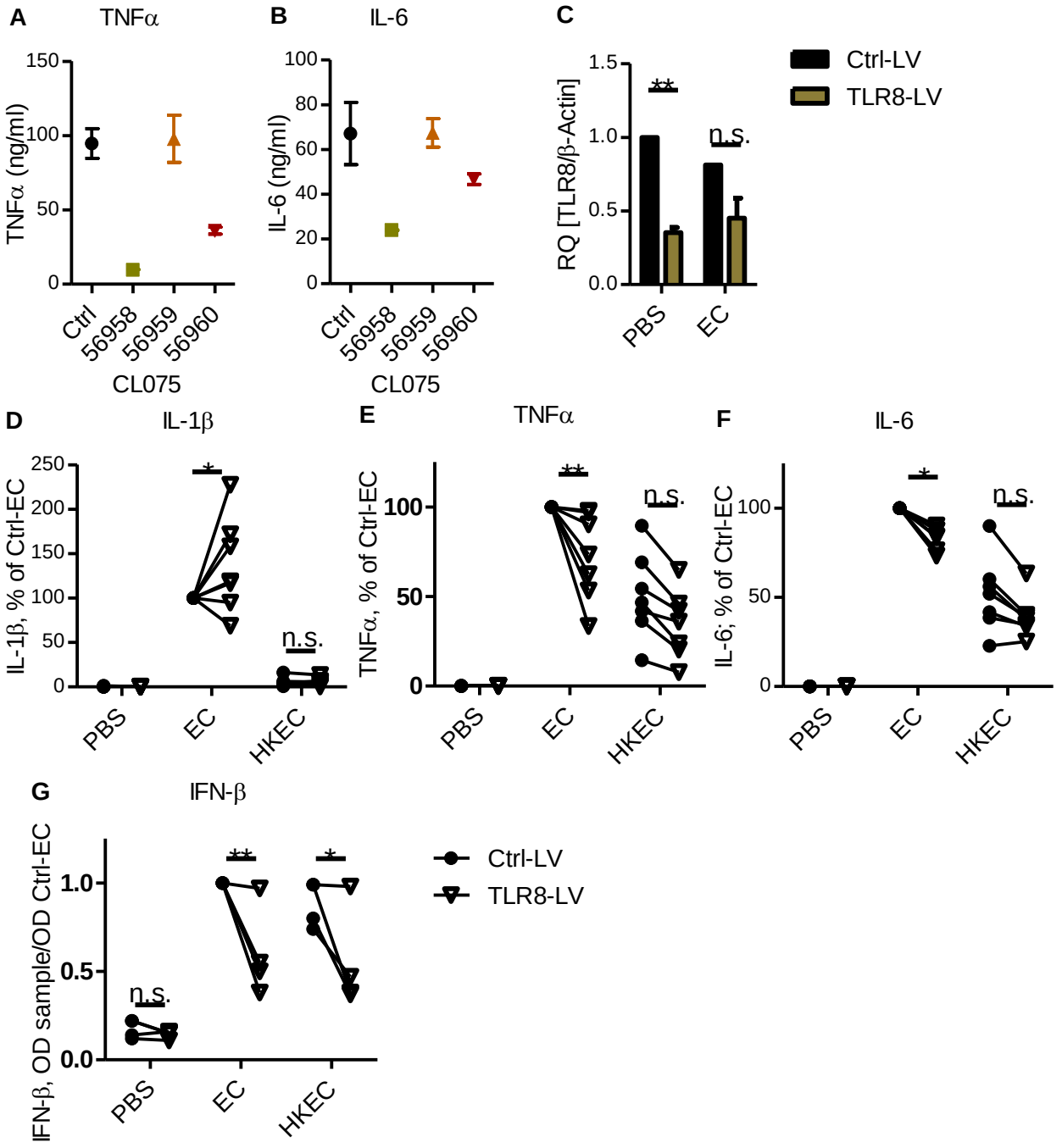

Fig. 19: TLR8-silencing yields variable results. Knockdown efficiency of single lentiviral shRNA vectors was functionally examined by stimulating transduced MoDCs with CL075 for 18 hours and subsequent cytokine quantification by ELISA (A,B, n=1). Knockdown efficiency of vectors was used monitored by qRT-PCR 18 hours p.i. Data are shown as mean + SEM (C, n=4). Ctrl-LV- and TLR8-LV-transduced MoDCs were infected with viable and dead *E. coli* at MOI 10. Cytokine secretion was measured 18 hours post stimulation by ELISA (all n=7). Results are expressed as percentage of Ctrl-LV-transduced cells infected with viable *E. coli*. For IFN-β (K, n=4) OD ratios were calculated. Each symbol represents an individual experiment. Two-way Anova test was performed, followed by Bonferroni posttests for individual cytokines. Graphs for infection experiments and functional controls were separated for the sake of clatrity. n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$.

Taking into consideration the uncertain knockdown efficiency, the data have to be confirmed in another system. The CRISPR/Cas9 gene editing technologies now available have allowed the Sander group to confirm the data in a monocytic cell line using TLR8-deficient cells (data not shown, Sander laboratory, unpublished). However, the data presented here do again support the observation of a dichotomous induction of IL-1β and TNFα following detection of viable bacteria partially depending on TLR8.

3.3.7 DHX33

Since a recent study suggested DHX33 as a sensor for cytosolic RNA and activator of the NLRP3 inflammsome[38], the role of this helicase in the sensing of live bacteria was additionally investigated.

First, DHX33-LV were separately tested for their silencing efficiency by qRT-PCR. Two plasmids show a reliable reduction of *Dhx33* mRNA expression after infection with viable *E. coli* but not after stimulation with poly I:C (Fig. 20A,B). Poly I:C was suggested to activate the NLRP3 inflammasome through DHX33 signaling[38]. However, poly I:C alone does not induce IL-1β secretion in MoDCs (Data not shown). We chose the two DHX33-LV clones with the “best” knockdown efficiency to test their effect on *E. coli*-induced cytokine responses. A synopsis of all experiments suggests that possibly DHX33-deficient MoDCs produce slightly more IL-1β, and slightly less IL-6 after infection with viable *E. coli* only, while TNFα responses seem to be reduced in either condition (Fig. 20C-E). qRT-PCR shows no sufficient DHX33 knockdown of unstimulated MoDCs. In contrast, after infection with viable bacteria, DHX33 expression is diminished in both conditions with a stronger but not significant reduction of DHX33 in DHX33-LV-transduced MoDCs (Fig. 20F).

Regarding these inconclusive qRT-PCR results, the role of DHX33 in the signaling process upon detection of bacterial viability remains elusive. Western Blot analysis was not performable due to unavailability of suitable antibodies at the time the experiments were performed.

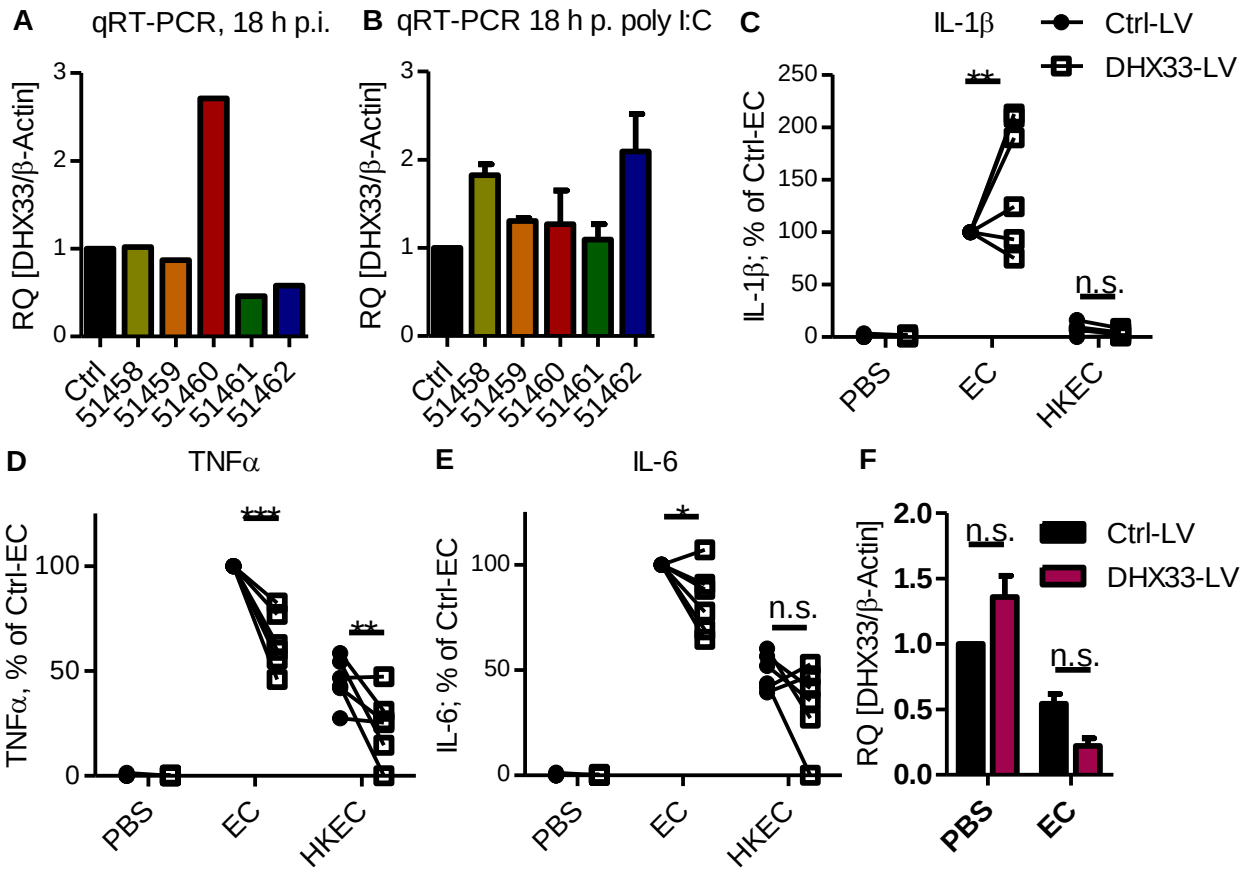

Fig. 20: DHX33-shRNA-transduced MoDCs secrete more IL-1β and less IL-6 in response to viable bacteria and show an impaired TNFα production independently of viability. Knockdown efficiency for DHX33-LV produced with single plasmids was examined by qRT-PCR of MoDCs stimulated with poly I:C (A, n=1) or viable *E. coli* at MOI 10 (B, n=1) for 18 hours. Ctrl-LV- and DHX33-LV-transduced MoDCs were infected with viable and dead *E. coli* at MOI 10. Cytokine secretion was measured 18 hours post stimulation by ELISA (all n=6). Results are expressed as percentage of Ctrl-LV-transduced cells infected with viable *E. coli*. Each symbol represents an individual experiment. Knockdown efficiency of the plasmid combinations used was monitored by qRT-PCR 18 hours p.i. and data are shown as mean + SEM (F, n=3). Two-way Anova test was performed, followed by Bonferroni posttests. n.s. = not significant, * = $p < 0.05$, ** =$p < 0.01$, *** = $p < 0.001$, # = not detected.

4 Discussion

The present study formally demonstrates the ability of human phagocytes to discriminate between viable and dead bacteria, independently of virulence. Surprisingly, in contrast to data obtained from murine BMDMs, especially classical monocytes responded almost exclusively to viable bacteria with a robust production of IL-1β and additionally TNFα. Monocytes (as well as circulating DC, Matteo Ugolini, Sander laboratory, unpublished) seem to "fine tune" their cytokine response to viable bacteria even more than MoDCs, hAM and also murine BMDMs.

The inhibitor screening experiments suggested a dichotomous regulation of IL-1β and TNFα responses to live bacteria in classical monocytes. The tested molecules seem to play a predominant role in priming the cytokine responses, but not exclusively in the signaling process that is initiated upon detection of viable bacteria.

Finally, this study demonstrates in a side by side approach that IL-1β secretion upon infection with viable but not dead *E. coli* required the activation of the NLRP3, likely the adaptor ASC and TRIF signaling.

An overview of the signaling pathways involved in the detection of viable bacteria is given in Fig. 21.

4.1 The Detection of Bacterial Viability is Conserved in Human Phagocytes

The ability of the innate immune system to detect microbial viability was initially demonstrated in murine BMDMs[37]. Whether human innate immune cells share this capacity was unknown. Here, I confirmed that also human primary innate immune cells are capable of discriminating between viable and dead bacteria. However their response to live bacteria differs depending on the cell type investigated. In line with murine BMDMs[37], human MoDCs and hAM show a significantly elevated IL-1β secretion in response to viable bacteria. Classical monocytes, which constitute the largest group of the three known monocyte subsets in the blood[65], additionally adapt TNFα-responses to viability. Since monocytes are key players in the innate immune defense[66] and data obtained with murine monocytes suggest that they can migrate to the site of infection and differentiate into macrophages and monocyte-derived DCs[67], their selective activation might be important for viability-induced migration and activation of resident tissue cells like DCs or macrophages. The surprising observation that

classical monocytes not only regulate IL-1β secretion, but also TNFα production in response to bacterial viability could imply that monocytes engage additional pathways to fine tune the immune response. Given that monocytes can be viewed as sentinels in the circulation, this property may be critical to avoid harmful systemic inflammation. The observed dichotomic signaling pathways of IL-1β and TNFα, (illustrated in Tab. 6) could also serve as a backup strategy to detect microbes that developed mechanisms to evade immune recognition. Given their central role in the immune system[66], this might be crucial for the induction of an efficient antimicrobial response.

The inhibition of TLR4 in classical monocytes predominantly reduced IL-1β and IL-10, but not TNFα (Fig. 12G-I) production after infection with live and dead *E. coli*. In comparison, MoDCs showed also impaired TNFα and IL-6 responses upon TLR4 inhibition (Fig. 18G-I), indicative of the predominant role of LPS sensing for the latter proinflammatory cytokines in MoDCs. In contrast, classical monocytes are less responsive to LPS. Robust TNFα production requires the detection of viable bacteria, possibly as an adaptation to limit the immune response in the blood stream to soluble LPS. As discussed below, TNFα secretion might depend on TLR8-signaling in human monocytes. Murine TLR8 is dispensable in the response induction of TLR7/8 agonist[13], which might help explain the difference between human and murine TNFα responses to bacterial viability.

IL-10 is one of the most important anti-inflammatory cytokines, and therefore tightly regulated[68]. For instance, LPS-mediated IL-10 expression has been recently shown to mediate down-regulation of TLR4 signaling and subsequent proinflammatory cytokine production[69]. Also, continious LPS stimulation triggers IL-10 production, which reduces NLRP3 inflammasome activation and expression in BMDMs in an autocrine manner[70]. Human phagocytes produce slightly more IL-10 to viable bacteria, most likely as a counter-measure to limit inflammatory respones and tissue damage. However, given the rather moderate differences, we decided to focus on the proinflammatory cytokines.

Studying the response of DCs to viability was of great interest for two reasons: firstly, DCs represent a cellular link between innate and adaptive immunity, and secondly[3], they are suitable for gene silencing using lentiviral transduction[51]. However, although human MoDCs are widely used for immunological studies, a corresponding cell type that functions *in vivo* has not been described, and therefore, MoDC remain a model cell[71].

Preliminary data generated by our group suggested no relevant cell death at the tested MOIs, in contrast to data from the murine system[37]. Also, recent studies did not detect significant cell death of monocytes after LPS-mediated inflammasome activation[72, 73]. However, a more detailed investigation of pyroptosis could uncover further similarities or differences in the signaling upon detection of bacterial viability by the murine and human innate immune system.

4.2 Viability-Induced Immune Responses Require Priming Signals and the Engagement of Distinct Signaling Pathways

NF-κB and MAPK play a central role in the downstream signaling of various PRRs[21, 31, 54, 74]. Also, they are essential for the induction of immune responses of BMDMs infected with viable and dead bacteria[37] and these signaling pathways were therefore relevant targets to investigate.

The inhibition of NF-κB activation by BAY11-7082 abolishes cytokine response to viable and dead bacteria alike. This was not unexpected, given the known central role of NF-κB signaling for PRR-induced cytokine induction. However, contribution of off-target inhibitor effects has been reported in the literature and cannot be excluded. It has been shown that BAY11-7082 reduces phosphorylation of IκBα, probably through upstream effects[75], but it may also activate p38 and JNK-1 MAPK upon TNFα stimulation[76], and suppress JNK activation in LPS-stimulated RAW 264.7 macrophages[75]. Furthermore, a direct inhibition of NLRP3 inflammasome activation has been reported *in vitro* and *in vivo*[77, 78].

MAPK form a network of so far 14 identified mammalian members, of which some are clustered into subgroups: first, four isoforms of p38 MAPKs (α-δ), second, JNKs and third extracellular signal-regulated kinase 1 (ERK1) and ERK2. They can be activated by a complex signaling network with shared and individual activators downstream of PRRs, yet not fully elucidated[55].

In addition to the induction of proinflammatory cytokines[55], p38 has been implicated in the TLR and MyD88-dependent induction of phagocytosis and phagosome maturation in *E. coli* infected BMDMs[79]. Accordingly, p38α deficient murine macrophages showed impaired phagocytosis[80]. Phagocytosis is required for cytosolic delivery of mRNA and pro-IL-1β cleavage[37]. However, preliminary experiments performed by our group suggested that phagocytosis was dispensable for IL-1β secretion (see section 3.2.2). Therefore, the observed reduction of IL-1β secretion might be explained by

transcriptional regulation of pro-IL-1β by p38 inhibitor SB203580 after stimulation with bacteria (Fig. 8A). Also, TLR4-TRIF-dependent involvement of p38 in the induction of autophagy has been reported[81]. Furthermore, increased vacuole formation and deficient autophagy are further described as unspecific effects of p38 inhibitor SB302580[82].

SB203580 blocks p38α/β[83], but many studies described unspecific effects. This includes the inhibition of JNKs, Receptor Interacting Protein-2 (RIP2) and ERK signaling[82, 84]. Strikingly, RIP2, which is crucial for NOD2 downstream signaling[85], is inhibited even more efficiently than the original target p38[84]. Additionally, stimulatory effects on ERK, but also NF-κB have been previously discussed, at least in the THP-1 monocytic cell line[86], possibly indicating cell-type dependent off-target effects. More recently, a study using p38γ/δ$^{-/-}$ BMDMs revealed that both isoforms are required for LPS-stimulated ERK activation and production of distinct cytokines[87]. p38γ/δ could potentially have compensated SB203580-mediated inhibition of TNFα, IL-6, and IL-10 responses. SB-resistant cells have been used to determine p38-independent effects[82, 83], which however, is not suitable for experiments in human primary cells and beyond the screening approach planned here. Overall, it can be assumed that p38 plays a role in the induction of cytokine responses to viable and dead *E. coli* in classical monocytes, however, the inhibitor experiments performed by us allow no conclusion on the quantitative contributions or the exact mechanisms of action due to the various assumable unspecific effects.

A potential role of JNK in cytokine induction of viable and dead bacteria has not been investigated. It was shown that JNK-inhibition in CD14^{+} monocytes leads to strong TNFα and weak IL-1β suppression after LPS stimulation. The authors proposed that JNK could stabilize TNFα mRNA and reported concentration-dependent unspecific inhibition of p38 activation, while ERK1, 2 and IκB-α remained uneffected[88]. Another study observed inhibition of the δ-isoform of PI3K in mast cells[89]. Inhibitor experiments in RAW264.7 macrophages suggested a requirement of JNK signaling for phagocytosis of *S.aureus*[90, 91], but not group B streptococcus (GBS)[92]. Also, JNK has been implicated in the regulation of NLRP3 activity through phosphorylation of ASC[93]. JNK-mediated signaling might induce context-dependent additional cellular processes, which could contribute to cytokine induction in our model. Given the limited specificity of available inhibitors such as SP600125, the data obtained here can only suggest a broad function for JNK in cytokine induction in our model, possibly through regulation of multiple cellular processes.

ERK is known to control numerous cytokines[55] and was recently demonstrated to be required for inflammasome priming in human moncytes[94]. The interesting possible contribution of ERK-signaling remains to be evaluated in our model, since initial experiments with ERK-signaling inhibitor PD98059 yielded highly variable inhibition results and were discontinued.

More specific approaches such as RNA interference, or CRISPR/Cas9-mediated gene editing, could help to further dissect and confirm the roles of the investigated molecules in viability-induced signaling in classical monocytes.

TLR4 is well-known to sense LPS derived from gram-negative bacteria. TLR4 signaling induces the production of proinflammatory cytokines[13]. The cleavage of IL-1β into the secreted active form additionally requires activation of the inflammasome[27], which in turn can be regulated by TLR signaling[74].

TLR4 signaling was inhibited using CLI-095, which blocks TLR4 association with its downstream adaptor molecules[95]. In contrast to the other used inhibitor, so far no off-target effects have been published. TLR4 signaling inhibition reduces levels of intracellular IL-1β in response to *E. coli* and completely abolished them after LPS stimulation. Pretreated cells, however, still produce considerable amounts of pro-IL-1β after infection with viable and dead bacteria, either due to insufficient signaling inhibition or additional signaling pathways inducing IL-1β, or both. Also, impaired expression of the inflammasome is likely to have contributed to the reduced IL-1β secretion[74].

Monocytes have been demonstrated to secrete IL-1β upon TLR-stimulation without the requirement of a second inflammasome activating signal[96], which was also observed in our experiments, although not very reliably. A recent study using monocytes purified by positive selection demonstrated that the cleavage of pro-IL-1β requires the internalization of TLR4 and activity of caspase-4/5, that are considered to correspond to murine caspase-11, but not caspase-1[72]. TLR4-TRIF signaling was required for efficient caspase-11-dependent inflammasome activation of BMDMs in response to gram-negative bacteria[40]. In contrast to Viganò *et al.*[72], another study reported the TLR4-dependent LPS-mediated inflammasome activation is induced via TRIF and caspase-8 upstream of NLRP3 and requires downstream activity of caspase-1[73]. Also, a TLR4-independent inflammasome activation by cytosolic LPS in a caspase-4 and caspase-1-dependent manner in THP-1 cells has been reported.[97] Independently of the exact signal transduction, LPS-induced one-step inflammasome activation could account for

the small viability-independent release of IL-1β observed for HKEC and might also contribute to the IL-1β response to viable *E. coli*.

Collectively, TLR4 is very likely to be important for inflammasome priming, and IL-1β responses of monocytes to live *E. coli* and TLR4 inhibition possibly prevented not only transcription, but also the non-canonical inflammasome activation. However, unpublished data from our group demonstrate that live *E. coli* predominantly activate the canonical NLRP3-inflammasome with only a minor contribution of the non-canononical inflammasome and caspase-4 (Moritz Pfeiffer & Elisa T. Helbig *et al.* , Sander group, unpublished). In line with the inhibitor experiments, TLR4 silencing suppresses production of intracellular IL-1β in MoDCs, indicating a role for TLR4 signaling in the priming process, rather than inflammasome activation. In contrast to classical monocytes, TLR4-deficient MoDCs also show impaired TNFα responses, which might be explained through a stronger dependency of cytokine induction from TLR4 signaling.

Since experiments performed by our group suggest the requirement of phagocytosis for TNFα induction in monocytes, additional TLR4-mediated processes in MoDCs might have contributed to the observed effects. For instance, TLR2x4$^{-/-}$ and MyD88$^{-/-}$ BMDMs show impaired internalization of *E. coli* and phagosome maturation. The phagosome maturation process depends on a phagosomal TLR signal presented by bacterial cargo and could not be induced by activation of TLR not colocalized with bacteria[79]. Additionally, TLR4 stimulation induces autophagy in RAW 264.7 macrophages, which was shown to depend on TRIF[81]. It is unlikely that monocytes and MoDCs differ fundamentally in the processes leading to the induction of phagocytosis or autophagy, and also Yates and coworkers reported no relevance of TLR4 signaling for phagosome maturation[98]. Nevertheless the relevance of TLR4 for TNFα secretion through the effects mentioned cannot be excluded, since monocytes and MoDCs indeed differ in their induction of cytokine responses, which is illustrated by the observation that the one-step activation of the inflammasome of monocytes in response to LPS was absent in MoDCs and MoMs[72, 96].

Initially implicated as a crucial player in adaptive immunity[59], a growing number of studies focused on BTK in innate immunity and revealed inconsistent data on an involvement in downstream signaling of TLRs[99-103] or phagocytosis[104, 105]. BTK was also reported as critically required for NLRP3 inflammasome activation[106]. Lee *et al.* showed that BTK-mediated phosphorylation of DDX41 is crucial for IFN-β induction to various stimuli such as transfected *E. coli*[107]. DDX41 senses intracellular DNA and bacterial

cyclic dinucleotides and induces type I IFN via STING[108]. Surprisingly, studies with innate immune cells from X-linked agammaglobulinemia (XLA)-patients yielded contrary data on the role of BTK[61,109-113], indicating cell and stimulus-dependent signaling pathways. The inhibitor-approach using LFM-A13[114] suggests a role for BTK in TLR4 signaling. Experiments using cells from XLA patients would yield more reliable data to determine BTK's role in the detection of viability and to exclude off-target effects[112,115].

The immunostimulatory component Imiquimod induces the production of proinflammatory cytokines through activation of TLR7[116], which is mainly expressed in human pDCs and B cells[117]. Human monocytes were reported to express appreciable levels of *Tlr8*, in contrast to only low expression of *Tlr7* and *Tlr9* mRNA[117]. Moreover, Zhu and colleagues formally demonstrated an inability of Imiquimod to activate human TLR8[118]. Surprisingly, in human MoMs, Imiquimod show inhibitory effects on TLR8[62], and it was therefore used to screen for a potential role of TLR8-signaling in sensing viability. Yet, Imiquimod has been shown to activate the NLRP3 inflammsome[119]. Recently, Gross *et al*. demonstrated a ROS-dependent activation of the NLRP3 inflammasome, which probably may accelerate the IL-1β response to viable and dead bacteria as observed in the experiments performed here. Furthermore, they showed that Imiquimod can mediate endosomal leakage or hinder lysosomal acidification, or possibly both. However, this was suggested not to be required for inflammasome activation[120]. Experiments performed by our group using Bafilomycin to inhibit lysosomal acidification also found reduced TNFα levels, especially after infection with viable *E. coli.* This is in line with data obtained from Imiquimod-pretreated monocytes, which lead to a selective reduction in TNFα release (Fig. 13H). The requirement of lysosomal acidification for TLR8-signaling[121] has been reported, indicating a role for endosomal TLRs, like TLR8 or 9, for the induction of TNFα. However, monocytes do not respond to TLR-stimulus CpG ODN (data not shown) and express only low levels of *Tlr9* mRNA[117].

Yet, as with other pharmacological inhibitors, Imiquimod has additional, modulatory effects. Independently of TLR7, Imiquimod has been reported to induce proinflammatory cytokines through antagonizing adenosine receptors and downstream adenylyl cyclase[122] and also to augment intracellular calcium concentration[123]. Although the studies mentioned above provide good evidence how Imiquimod modifies the cytokine responses to *E. coli*, the experimental approach cannot exclude interfering effects. Therefore, and due to possible viability-dependent signaling, we attempted to silence TLR8 expression using shRNA-expressing LV vectors in MoDCs. However, the

silencing efficiency in the LV-TLR8-transduced MoDC was uncertain. There is growing evidence for the role of TLR8 in the detection of bacterial RNA. Previous experiments performed with human primary monocytes revealed TLR8 as a sensor for *Borrelia burgdorferi* RNA, which induces MyD88-dependent IFN-β and also TNFα release. Bacterial RNA was reported to be sensed exclusively in the phagosome and does not gain cytosolic access[14]. Furthermore, using RNA from various gram-positive bacteria, Eigenbrod and coworkers demonstrated that TLR8 senses bacterial RNA, and silencing TLR8 significantly reduced levels of TNFα, but also IL-6 and IFNβ in human MoMs[15], in accordance with the results obtained here. They also observed the requirement of phagocytosis for the induction of cytokines in a bacteria-dependent manner, suggesting that TLR8-derived signals might contribute differentially to innate immune responses. Silencing experiments confirmed the necessity of TLR8 for a robust induction of cytokines and IFN-β of MoMs in response to *Streptococcus pyogenes*[15].

A recent study reported that TLR8 can sense degradation products of ssRNA and that it contains two binding sites. One causes dimerization upon ligation, and the second facilitates ligand binding to the first[124]. Lysosomal acidification might induce degradation[124], giving a possible explanation for the requirement of acidification for functional TLR8 signaling[121].

In addition, TLR8 signaling itself might also facilitate ligand detection through autophagy induction of cytosolic, microbial RNA. The activation of murine TLR7, a close relative of human TLR8[13], was shown to efficiently provoke autophagy in a MyD88-dependent manner in mouse RAW 264.7 macrophages. Yet, it is less robust in primary macrophages. Also, distinct stimuli of TLR8 activated autophagy in HeLa cells[125].

Improved shRNA sequences for lentiviral transduction or stable human TLR8 knock-out cell lines might help to validate our preliminary results. qRT-PCR analysis showed reduced expression of TLR8, although not significantly, after infection with viable *E. coli*. However, the functional control using CL075 suggested an insufficient knockdown. CL075 is also known to activate TLR7 signaling at higher concentrations[126] than used in the experiments performed here. However, compensatory TLR7-signaling following TLR8 knockdown cannot be excluded and may have accounted for some of the effects. Unpublished data from the Sander laboratory now reveal a clear role for TLR8 signaling in the detection of live bacteria and bacterial RNA (Matteo Ugolini *et al.*, unpublished). These results were obtained in primary monocytes using siRNA-mediated TLR8 knockdown.

The PI3-K family consists of three groups: class I PI3-K, which are engaged by cell surface receptors and mediate activation of multiple signaling pathways to control e.g. cell survival, but also phagocytosis, besides class II and class III PI3-K, which both signal at intracellular membranes to control phagocytosis, endocytosis and autophagy[127, 128]. PI3K are essential for the completion of engulfment of larger particles, but are dispensable for the initiation of phagocytosis[56], and are furthermore required for phagosome maturation[128]. PI3K-mediated autophagy is also know to enhance the delivery of certain PAMPs to endosomal TLR[129]. The inhibition of these processes using LY204002 might have decreased the availability of endosomally sensed ligands, which seem necessary for TNFα production. In line with this, the cytokine responses to R848 are decreased, while LPS stimulation rather enhances cytokine production after inhibitor pretreatment of monocytes.

Autophagy, and therefore PI3K activity, has been also implicated in the control of NLRP3 inflammasome activation through clearance of dysfunctional or damaged mitochondria[130, 131] and the activated inflammasome itself[132]. Also, pro-IL-1β is directly degraded in lysosomes following autophagic processes[133]. Therefore, impaired clearance of the IL-1β processing machinery could account for the unchanged or even enhanced IL-1β levels observed after PI3K inhibition (Fig. 10G). Another study observed decreased TNFα and enhanced IL-1β levels after inhibition of autophagy and stimulation of human PBMCs with TLR ligands. However, they suggest the inhibition of autophagy rather alters transcription of cytokines and do not affect inflammasome activity[134].

Regarding the broad range of functions of PI3K, it is difficult to dissect the inhibition of which process contributed the most to the observed effects. Also, there is evidence that PI3K inhibitor LY294002 targets other kinases apart from the PI3K-family[135]and might have cell-type-dependent additional inhibitory effects. Given that autophagy largely depends on class III PI3K, specific evaluation of this group of proteins required for autophagosome[57], for instance through lentiviral transduction, could help to clarify the role of PI3K and potentially autophagy for the induction of cytokine responses to viable *E. coli.* A very recent publication suggests a critical role of autophagosome formation and endoplasmic reticulum (ER)-phagy in type-I IFN responses to c-di-AMP released as a *vita*-PAMP from live gram-positive bacteria[136].

MyD88 and TRIF are essential adaptor molecules for TLR signaling[13] and play distinct roles in the induction of murine innate immune response to viable *E. coli*[37]. Therefore, they were investigated in human MoDCs.

MyD88 is a critical signaling adaptor for all TLRs, except TLR3, and mediates the induction of proinflammatory cytokines[19]. Not surprisingly, MyD88-silencing significantly reduces levels of all measured cytokines, mostly independently of viability. The reduction of the IL-1β response can be explained through decreased induction of pro-IL-1β, and potentially also though reduced priming of NLRP3, since TLR stimuli serve as priming signals. MyD88 is also known for the induction of type IFN of pDCs in response to TLR7/TLR9 stimuli[19], yet it seems dispensable for IFN-β induction of MoDCs in response to *E. coli,* which rather depend on the TLR4-TRIF axis.

Moreover, MyD88 is a critical adaptor for other TIR-domain-containing receptors, including the IL-1 receptor[137] and the receptor TACI[17]. So far, the role of TACI in innate immunity is poorly characterized. Therefore, the priming signals mentioned above may not only be derived from TLRs.

In accordance with murine data[37], TRIF signaling at least partially mediates the maturation of IL-1β in response to viable *E. coli*. In murine macrophages, NLRP3 expression upon LPS stimulation depended at least partially on TRIF-signaling[138], which could also contribute to reduced IL-1β secretion to viable *E. coli*. However, previous data from BMDMs show no effect of TRIF on *Nlrp3* transcription[37] and Western Blot analysis does not show obvious effects of TRIF silencing on NLRP3 levels in MoDCs after infection with *E coli*.

Rathinam and colleagues suggested that bacterial mRNA mediates inflammasome assembly and activation, and the TLR4-TRIF axis accelerates IL-1β processing through up-regulation of caspase-11 expression via the induction of type IFN[40]. Indeed, viable bacteria provoke MoDCs to secrete significantly more IFNβ, and IFNβ secretion strongly depends on TLR4 and TRIF. The relevance of this autocrine feedback loop in human MoDCs, however, requires further investigation. Experiments using neutralizing IFN-receptor antibodies could provide further evidence for a potential role of IFN in non-canonical inflammasome activation in *E. coli*-infected MoDCs.

Besides, direct sensors of *vita*-PAMPs upstream of TRIF are a considerable option, but there is only little evidence in the literature. A study suggested DDX1 as an upstream sensor of dsRNA in murine DCs, which interacts via DDX21 and DHX36 with TRIF to induce type I IFN[18]. Another study demonstrated that DDX1 is dispensable, but DHX33

is required for the induction of IL-1β release in response to viral infection[139]. DHX33 silencing in THP-1 cells and MoMs reduces IL-1β and IL-18 secretion after stimulation with reoviral RNA. No data have been published concerning stimulation with bacterial RNA, leaving the role of DHX33 in human primary immune cells uncertain. Pulldown experiments performed with total RNA of *E. coli* indicate DHX33 as a sensor for bacterial RNA and showed that DHX33 directly binds NLRP3 inflammasome[38], which was demonstrated to induce the release of IL-1β in human MoDCs in response to viable *E. coli*. The role of DHX33 as a sensor of viral RNA upstream of MAVS was confirmed in murine DCs. DHX33-deficient cells showed impaired type I IFN production[140]. Chakrabarti *et al.* further characterized DHX33 dependent sensing of viral RNA. RNAse L cleaves viral RNA, which is then more efficiently sensed by DHX33 and which induces the formation of a DHX33-MAVS-NLRP3 complex[139]. The question whether a similar mechanism is involved in the detection of bacterial RNA remains unanswered[141].

Importantly, in contrast to murine cells[37], it has recently been shown that various types of bacterial RNA are able to induce the activation of the NLRP3 inflammasome in human cells[39]. Given that total *E. coli* RNA binds DHX33[38], the helicase remains of high interest as a potential sensor of vita-PAMPs. However, due to technical problems and possibly insufficient silencing in my experiments, I did not succeed in conclusively analyzing its role in the detection of live bacteria.

In addition to DHX33, also IFN-induced proteins with tetratricopeptide repeats (IFIT), which are intracellularly located molecules capable of limiting viral infections[142] were suggested as bacterial mRNA sensors. Human IFIT5 and IFIT1 specifically bind single-stranded 5' triphosphorylated RNA (PPP-RNA), and bacterial mRNA has a detectable free 5'PPP group[143]. However, calf intestinal phosphatase treatment of *E. coli* mRNA, which non-specifically catalyzes the dephosphorylation of the 5´ ends, did not modify the proinflammatory effects observed for murine BMDMs[37]. In accordance, inflammasome activation upon detection of bacterial RNA in THP-1 cells does not require 5' triphosphates[39]. Therefore, the suggested IFIT are unlikely to sense bacterial mRNA.

Importantly, TRIF silencing does not completely abolish the IL-1β response, either due to incomplete knockdown, or because of signaling pathways upstream of NLRP3, initiated through the detection of bacterial viability, or direct activation of NLRP3 might account for the persistent IL-1β response

The importance of the NLRP3 inflammasome and adaptor ASC for the IL-1β secretion upon detection of viable, gram negative bacteria has been well-established in murine cells[37, 40] and could be confirmed in this study for human MoDCs. Using lentiviral transduction, NLRP3 silencing almost abolished IL-1β production. The vita-PAMPs that lead to the activation of the NRLP3 inflammasome in human monocytes and MoDCs after infection with viable bacteria are not definitely identified. Candidate molecules such as various bacterial RNA species and bacterial second messengers have been demonstrated to induce NLRP3 inflammasome activation[39, 46]. Gupta *et. al* suggested a possible direct interaction of gram-positive bacterial RNA with the NLRP3 inflammasome[144]. However, it was not formally demonstrated that addition of these molecules to heat killed bacteria is able to restore the IL-1β secretion. Data obtained from human monocytes suggest the dispensability of phagocytosis for NRLP3 activation and IL-1β release in contrast to murine BMDMs[37]. This raises the question as to how putative *vita*-PAMPs access the cytosol. One possibility might be the endocytosis of so-called OMVs, produced only by viable bacteria, which gain cytosolic access from early endosomes as demonstrated by Vanaja and co-workers[41]. Presumably, they could also contain *vita*-PAMPs such as bacterial mRNA. However, unpublished data from the sander laboratory suggest that this pathway plays a rather insignificant role in the context of live bacterial infection of human phagocytes (Moritz Pfeiffer *et al.*, unpublished). Alternatively, RNA may leak from degraded bacteria and may be shuttled from endosomes (or phagosomes) via active transport. A recent publication suggests SID1 transmembrane family member 2 (SITD-2) as an endosomal transporter of dsRNA[145]. Its role in shuttling bacterial RNA is unknown. Furthermore, another study reports that DDX19 binds viral mRNA and facilitates its transport from the nucleus to the cytosol[146], but a possible involvement in intracellular transport of bacterial RNA has not been investigated yet. DDX19 was also shown to activate the NLRP3 inflammasome in response to viral RNA[147]. However, its role in sensing bacterial RNA / *vita*-PAMPs has not been investigated.

The role of ASC in NLRP3 inflammasome activation is undisputable. Therefore, it was not surprising that we observed an inhibition of IL-1β production upon ASC silencing. However, we noted significant reduction of other cytokines as well (Fig. 16I-K). These alterations may be caused either by off-target effects of the shRNA, or by auto/paracrine effects due to reduced IL-1β secretion. Nevertheless, a few reports have also focused on inflammasome-independent functions of ASC. Using a collagen-induced arthritis

model, Ippagunta and co-workers suggested the requirement of ASC in DCs for antigen-specific priming of murine T cells[148]. Further studies confirmed impaired antigen-uptake and presentation of ASC-deficient BMDCs through dysfunctional actin polymerization, independently of the inflammasome and TLR-signaling[149]. Also, using THP-1 cells and murine macrophages, it was found that ASC is required for the induction of various chemokines and cytokines, including TNFα, in response to *Porphyromonas gingivalis* through activation of MAPK in an inflammasome-independent manner. ASC-dependent activation of ERK was also observed for stimulation of TLR2, 4, 5 and infection with *E. coli*[150]. Another study reported ASC-dependent inhibition of caspase-1-mediated NF-κB activation[151], which was later suggested to restrict growth of *Legionella pneumophila* in human monocytes[152]. Therefore, the data could also indicate a broader, inflammasome-independent function of ASC in the cytokine response of MoDCs to *E. coli*, which might be interesting to investigate.

4.3 Concluding Remarks

The study demonstrates that the detection of bacterial viability is conserved in human antigen-presenting cells. The signaling pathways involved, known from murine BMDMs[37], are also widely conserved in human phagocytes, with the notable exception of TNFα production, which is also controlled by viability in human monocytes. Hence, the human innate immune system engages additional pathways and possibly receptors to discriminate between viable and dead bacteria.

Also, it is conceivable that different *vita*-PAMPs elicit robust proinflammatory responses or the same *vita*-PAMP represents different immunostimulatory properties depending on the cellular context in human and murine APC. Further work is required to identify the exact receptors for bacterial viability and *vita*-PAMPs in human APC. The approaches used here, such as gene silencing experiments, can only approximate a requirement of the investigated molecule in the sensing process. Now, techniques such as gene editing by CRISPR/Cas9 and other methods, may shed new light on these processes. However, additional approaches need to be applied to demonstrate a direct interaction of the *vita*-PAMP and a putative receptor.

Emerging target molecules could be investigated by co-immunoprecipitation assays for their RNA binding capacity as performed by Mitoma and colleagues, using labeled total *E. coli* RNA[38].

There is strong evidence in the literature, and unpublished data from our group indicating that TLR8 plays an important role in the detection of bacterial RNA and viable bacteria.

Finally, the detailed study of viability-induced effects on adaptive immunity are of great interest and could help in the development of new adjuvants for safe vaccines[2].

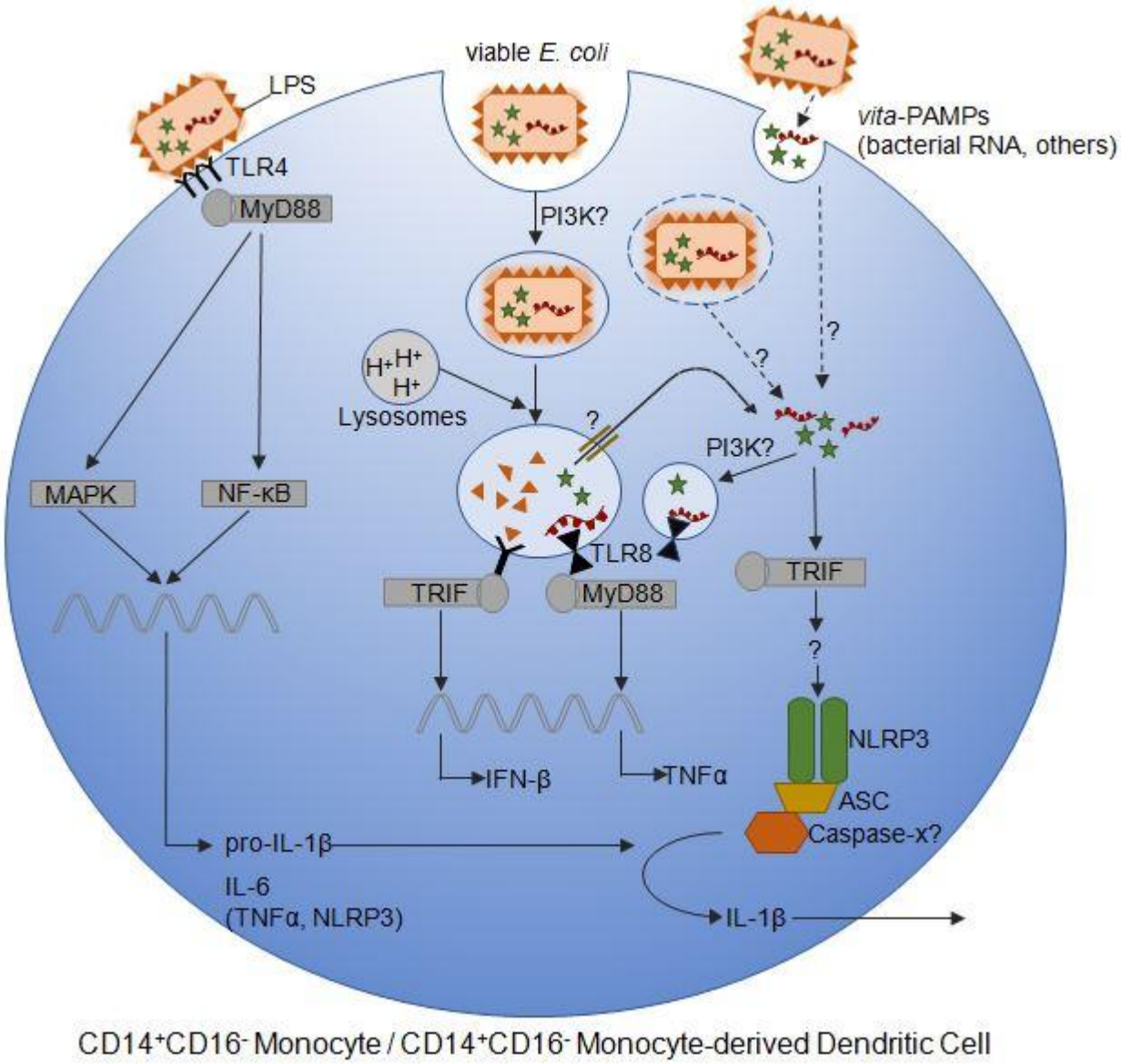

Fig. 21: Human Phagocytes detect viable bacteria and induce a robust proinflammatory cytokine response. Human $CD14^+CD16^-$ monocytes and $CD14^+CD16^-$ monocytes-derived dendritic cells secrete IL-1β in response to viable bacteria. This response requires priming signals via TLR4 and TRIF-dependent activation of the NLRP3 inflammasome and ASC. The effector caspases involved remain to be determined, however, a critical role of caspase-1 is highly likely. Also, the detectable *vita*-PAMP(s), the respective receptor and cytosolic delivery of the *vita*-PAMP need further investigation. IFN-β secretion requires the TLR4-TRIF signaling axis. $CD14^+CD16^-$ monocytes secrete TNFα almost exclusively to viable *E. coli*, which probably depends on TLR8 signaling of bacterial RNA and might require PI3K mediated processes such as phagocytosis and autophagy.

5 References

1. Janeway, C. A.; Medzhitov, R., Innate immune recognition. *Annu Rev Immunol* **2002,** *20*, 197-216.

2. Sander, L. E., Improved vaccines through targeted manipulation of the body's immunological risk-assessment? *Bioessays* **2012,** *34* (10), 876-84.

3. Medzhitov, R., Recognition of microorganisms and activation of the immune response. *Nature* **2007,** *449* (7164), 819-26.

4. Bieber, K.; Autenrieth, S. E., Insights how monocytes and dendritic cells contribute and regulate immune defense against microbial pathogens. *Immunobiology* **2015,** *220* (2), 215-26.

5. Diefenbach, A., Innate lymphoid cells in the defense against infections. *Eur J Microbiol Immunol (Bp)* **2013,** *3* (3), 143-51.

6. Freeley, S.; Kemper, C.; Le Friec, G., The "ins and outs" of complement-driven immune responses. *Immunol Rev* **2016,** *274* (1), 16-32.

7. Boxx, G. M.; Cheng, G., The Roles of Type I Interferon in Bacterial Infection. *Cell Host Microbe* **2016,** *19* (6), 760-9.

8. Blander, J. M.; Sander, L. E., Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nat Rev Immunol* **2012,** *12* (3), 215-25.

9. Janeway, C. A., Jr., Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* **1989,** *54 Pt 1*, 1-13.

10. Kumar, H.; Kawai, T.; Akira, S., Pathogen recognition by the innate immune system. *Int Rev Immunol* **2011,** *30* (1), 16-34.

11. Brubaker, S. W.; Bonham, K. S.; Zanoni, I.; Kagan, J. C., Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol* **2015,** *33*, 257-90.

12. Medzhitov, R.; Preston-Hurlburt, P.; Janeway, C. A., A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **1997,** *388* (6640), 394-7.

13. Kawai, T.; Akira, S., The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **2010,** *11* (5), 373-84.

14. Cervantes, J. L.; La Vake, C. J.; Weinerman, B.; Luu, S.; O'Connell, C.; Verardi, P. H.; Salazar, J. C., Human TLR8 is activated upon recognition of Borrelia burgdorferi RNA in the phagosome of human monocytes. *J Leukoc Biol* **2013,** *94* (6), 1231-41.

15. Eigenbrod, T.; Pelka, K.; Latz, E.; Kreikemeyer, B.; Dalpke, A. H., TLR8 Senses Bacterial RNA in Human Monocytes and Plays a Nonredundant Role for Recognition of Streptococcus pyogenes. *J Immunol* **2015,** *195* (3), 1092-9.

16. Choi, Y. J.; Im, E.; Chung, H. K.; Pothoulakis, C.; Rhee, S. H., TRIF mediates Toll-like receptor 5-induced signaling in intestinal epithelial cells. *J Biol Chem* **2010,** *285* (48), 37570-8.

17. He, B.; Santamaria, R.; Xu, W.; Cols, M.; Chen, K.; Puga, I.; Shan, M.; Xiong, H.; Bussel, J. B.; Chiu, A.; Puel, A.; Reichenbach, J.; Marodi, L.; Döffinger, R.; Vasconcelos, J.; Issekutz, A.; Krause, J.; Davies, G.; Li, X.; Grimbacher, B.; Plebani, A.; Meffre, E.; Picard, C.; Cunningham-Rundles, C.; Casanova, J. L.; Cerutti, A., The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nat Immunol* **2010,** *11* (9), 836-45.

18. Zhang, Z.; Kim, T.; Bao, M.; Facchinetti, V.; Jung, S. Y.; Ghaffari, A. A.; Qin, J.; Cheng, G.; Liu, Y. J., DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. *Immunity* **2011,** *34* (6), 866-78.

19. Kawasaki, T.; Kawai, T., Toll-like receptor signaling pathways. *Front Immunol* **2014,** *5*, 461.

20. Motta, V.; Soares, F.; Sun, T.; Philpott, D. J., NOD-like receptors: versatile cytosolic sentinels. *Physiol Rev* **2015,** *95* (1), 149-78.

21. Philpott, D. J.; Sorbara, M. T.; Robertson, S. J.; Croitoru, K.; Girardin, S. E., NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol* **2014,** *14* (1), 9-23.

22. Martinon, F.; Burns, K.; Tschopp, J., The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* **2002,** *10* (2), 417-26.

23. Vanaja, S. K.; Rathinam, V. A.; Fitzgerald, K. A., Mechanisms of inflammasome activation: recent advances and novel insights. *Trends Cell Biol* **2015,** *25* (5), 308-15.

24. Kofoed, E. M.; Vance, R. E., Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* **2011,** *477* (7366), 592-5.

25. Shi, J.; Zhao, Y.; Wang, K.; Shi, X.; Wang, Y.; Huang, H.; Zhuang, Y.; Cai, T.; Wang, F.; Shao, F., Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **2015,** *526* (7575), 660-5.

26. Dinarello, C. A., A clinical perspective of IL-1β as the gatekeeper of inflammation. *Eur J Immunol* **2011,** *41* (5), 1203-17.
27. Sutterwala, F. S.; Haasken, S.; Cassel, S. L., Mechanism of NLRP3 inflammasome activation. *Ann N Y Acad Sci* **2014,** *1319*, 82-95.
28. Stutz, A.; Kolbe, C. C.; Stahl, R.; Horvath, G. L.; Franklin, B. S.; van Ray, O.; Brinkschulte, R.; Geyer, M.; Meissner, F.; Latz, E., NLRP3 inflammasome assembly is regulated by phosphorylation of the pyrin domain. *J Exp Med* **2017,** *214* (6), 1725-1736.
29. Franchi, L.; Muñoz-Planillo, R.; Núñez, G., Sensing and reacting to microbes through the inflammasomes. *Nat Immunol* **2012,** *13* (4), 325-32.
30. Wen, H.; Ting, J. P.; O'Neill, L. A., A role for the NLRP3 inflammasome in metabolic diseases--did Warburg miss inflammation? *Nat Immunol* **2012,** *13* (4), 352-7.
31. Goubau, D.; Deddouche, S.; Reis e Sousa, C., Cytosolic sensing of viruses. *Immunity* **2013,** *38* (5), 855-69.
32. Dambuza, I. M.; Brown, G. D., C-type lectins in immunity: recent developments. *Curr Opin Immunol* **2015,** *32*, 21-7.
33. Sun, L.; Wu, J.; Du, F.; Chen, X.; Chen, Z. J., Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **2013,** *339* (6121), 786-91.
34. Gao, D.; Wu, J.; Wu, Y. T.; Du, F.; Aroh, C.; Yan, N.; Sun, L.; Chen, Z. J., Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. *Science* **2013,** *341* (6148), 903-6.
35. Lahaye, X.; Satoh, T.; Gentili, M.; Cerboni, S.; Conrad, C.; Hurbain, I.; El Marjou, A.; Lacabaratz, C.; Lelièvre, J. D.; Manel, N., The capsids of HIV-1 and HIV-2 determine immune detection of the viral cDNA by the innate sensor cGAS in dendritic cells. *Immunity* **2013,** *39* (6), 1132-42.
36. Vance, R. E.; Isberg, R. R.; Portnoy, D. A., Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe* **2009,** *6* (1), 10-21.
37. Sander, L. E.; Davis, M. J.; Boekschoten, M. V.; Amsen, D.; Dascher, C. C.; Ryffel, B.; Swanson, J. A.; Müller, M.; Blander, J. M., Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature* **2011,** *474* (7351), 385-9.
38. Mitoma, H.; Hanabuchi, S.; Kim, T.; Bao, M.; Zhang, Z.; Sugimoto, N.; Liu, Y. J., The DEAH box RNA helicase DHX33 senses cytosolic RNA and activates the NLRP3 inflammasome. *Immunity* **2013,** *39* (1), 123-35.

39. Sha, W.; Mitoma, H.; Hanabuchi, S.; Bao, M.; Weng, L.; Sugimoto, N.; Liu, Y.; Zhang, Z.; Zhong, J.; Sun, B.; Liu, Y. J., Human NLRP3 inflammasome senses multiple types of bacterial RNAs. *Proc Natl Acad Sci U S A* **2014,** *111* (45), 16059-64.
40. Rathinam, V. A.; Vanaja, S. K.; Waggoner, L.; Sokolovska, A.; Becker, C.; Stuart, L. M.; Leong, J. M.; Fitzgerald, K. A., TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* **2012,** *150* (3), 606-19.
41. Vanaja, S. K.; Russo, A. J.; Behl, B.; Banerjee, I.; Yankova, M.; Deshmukh, S. D.; Rathinam, V. A., Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. *Cell* **2016,** *165* (5), 1106-19.
42. Garaude, J.; Acín-Pérez, R.; Martínez-Cano, S.; Enamorado, M.; Ugolini, M.; Nistal-Villán, E.; Hervás-Stubbs, S.; Pelegrín, P.; Sander, L. E.; Enríquez, J. A.; Sancho, D., Mitochondrial respiratory-chain adaptations in macrophages contribute to antibacterial host defense. *Nat Immunol* **2016,** *17* (9), 1037-45.
43. Kailasan Vanaja, S.; Rathinam, V. A.; Atianand, M. K.; Kalantari, P.; Skehan, B.; Fitzgerald, K. A.; Leong, J. M., Bacterial RNA:DNA hybrids are activators of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* **2014,** *111* (21), 7765-70.
44. Wynosky-Dolfi, M. A.; Snyder, A. G.; Philip, N. H.; Doonan, P. J.; Poffenberger, M. C.; Avizonis, D.; Zwack, E. E.; Riblett, A. M.; Hu, B.; Strowig, T.; Flavell, R. A.; Jones, R. G.; Freedman, B. D.; Brodsky, I. E., Oxidative metabolism enables Salmonella evasion of the NLRP3 inflammasome. *J Exp Med* **2014,** *211* (4), 653-68.
45. Burdette, D. L.; Monroe, K. M.; Sotelo-Troha, K.; Iwig, J. S.; Eckert, B.; Hyodo, M.; Hayakawa, Y.; Vance, R. E., STING is a direct innate immune sensor of cyclic di-GMP. *Nature* **2011,** *478* (7370), 515-8.
46. Abdul-Sater, A. A.; Tattoli, I.; Jin, L.; Grajkowski, A.; Levi, A.; Koller, B. H.; Allen, I. C.; Beaucage, S. L.; Fitzgerald, K. A.; Ting, J. P.; Cambier, J. C.; Girardin, S. E.; Schindler, C., Cyclic-di-GMP and cyclic-di-AMP activate the NLRP3 inflammasome. *EMBO Rep* **2013,** *14* (10), 900-6.
47. Helbig, E. T.; Opitz, B.; Sander, L. E., Adjuvant immunotherapies as a novel approach to bacterial infections. *Immunotherapy* **2013,** *5* (4), 365-81.
48. Culpitt, S. V.; Rogers, D. F.; Shah, P.; De Matos, C.; Russell, R. E.; Donnelly, L. E.; Barnes, P. J., Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **2003,** *167* (1), 24-31.

49. Maurelli, A. T.; Baudry, B.; d'Hauteville, H.; Hale, T. L.; Sansonetti, P. J., Cloning of plasmid DNA sequences involved in invasion of HeLa cells by Shigella flexneri. *Infect Immun* **1985,** *49* (1), 164-71.

50. Wing, H. J.; Yan, A. W.; Goldman, S. R.; Goldberg, M. B., Regulation of IcsP, the outer membrane protease of the Shigella actin tail assembly protein IcsA, by virulence plasmid regulators VirF and VirB. *J Bacteriol* **2004,** *186* (3), 699-705.

51. Satoh, T.; Manel, N., Gene transduction in human monocyte-derived dendritic cells using lentiviral vectors. *Methods Mol Biol* **2013,** *960*, 401-9.

52. Lahouassa, H.; Daddacha, W.; Hofmann, H.; Ayinde, D.; Logue, E. C.; Dragin, L.; Bloch, N.; Maudet, C.; Bertrand, M.; Gramberg, T.; Pancino, G.; Priet, S.; Canard, B.; Laguette, N.; Benkirane, M.; Transy, C.; Landau, N. R.; Kim, B.; Margottin-Goguet, F., SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nat Immunol* **2012,** *13* (3), 223-8.

53. Laguette, N.; Sobhian, B.; Casartelli, N.; Ringeard, M.; Chable-Bessia, C.; Ségéral, E.; Yatim, A.; Emiliani, S.; Schwartz, O.; Benkirane, M., SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* **2011,** *474* (7353), 654-7.

54. Kawai, T.; Akira, S., Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med* **2007,** *13* (11), 460-9.

55. Arthur, J. S.; Ley, S. C., Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol* **2013,** *13* (9), 679-92.

56. Schlam, D.; Bagshaw, R. D.; Freeman, S. A.; Collins, R. F.; Pawson, T.; Fairn, G. D.; Grinstein, S., Phosphoinositide 3-kinase enables phagocytosis of large particles by terminating actin assembly through Rac/Cdc42 GTPase-activating proteins. *Nat Commun* **2015,** *6*, 8623.

57. Shibutani, S. T.; Saitoh, T.; Nowag, H.; Münz, C.; Yoshimori, T., Autophagy and autophagy-related proteins in the immune system. *Nat Immunol* **2015,** *16* (10), 1014-24.

58. Troutman, T. D.; Bazan, J. F.; Pasare, C., Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K. *Cell Cycle* **2012,** *11* (19), 3559-67.

59. López-Herrera, G.; Vargas-Hernández, A.; González-Serrano, M. E.; Berrón-Ruiz, L.; Rodríguez-Alba, J. C.; Espinosa-Rosales, F.; Santos-Argumedo, L., Bruton's

tyrosine kinase--an integral protein of B cell development that also has an essential role in the innate immune system. *J Leukoc Biol* **2014,** *95* (2), 243-50.

60. Sochorová, K.; Horváth, R.; Rozková, D.; Litzman, J.; Bartunková, J.; Sedivá, A.; Spísek, R., Impaired Toll-like receptor 8-mediated IL-6 and TNF-alpha production in antigen-presenting cells from patients with X-linked agammaglobulinemia. *Blood* **2007,** *109* (6), 2553-6.

61. Gray, P.; Dunne, A.; Brikos, C.; Jefferies, C. A.; Doyle, S. L.; O'Neill, L. A., MyD88 adapter-like (Mal) is phosphorylated by Bruton's tyrosine kinase during TLR2 and TLR4 signal transduction. *J Biol Chem* **2006,** *281* (15), 10489-95.

62. Sacre, S. M.; Lo, A.; Gregory, B.; Simmonds, R. E.; Williams, L.; Feldmann, M.; Brennan, F. M.; Foxwell, B. M., Inhibitors of TLR8 reduce TNF production from human rheumatoid synovial membrane cultures. *J Immunol* **2008,** *181* (11), 8002-9.

63. Ullah, M. O.; Sweet, M. J.; Mansell, A.; Kellie, S.; Kobe, B., TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target. *J Leukoc Biol* **2016,** *100* (1), 27-45.

64. Mata-Haro, V.; Cekic, C.; Martin, M.; Chilton, P. M.; Casella, C. R.; Mitchell, T. C., The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* **2007,** *316* (5831), 1628-32.

65. Ziegler-Heitbrock, L.; Ancuta, P.; Crowe, S.; Dalod, M.; Grau, V.; Hart, D. N.; Leenen, P. J.; Liu, Y. J.; MacPherson, G.; Randolph, G. J.; Scherberich, J.; Schmitz, J.; Shortman, K.; Sozzani, S.; Strobl, H.; Zembala, M.; Austyn, J. M.; Lutz, M. B., Nomenclature of monocytes and dendritic cells in blood. *Blood* **2010,** *116* (16), e74-80.

66. Gren, S. T.; Grip, O., Role of Monocytes and Intestinal Macrophages in Crohn's Disease and Ulcerative Colitis. *Inflamm Bowel Dis* **2016,** *22* (8), 1992-8.

67. Kratofil, R. M.; Kubes, P.; Deniset, J. F., Monocyte Conversion During Inflammation and Injury. *Arterioscler Thromb Vasc Biol* **2016**.

68. Saraiva, M.; O'Garra, A., The regulation of IL-10 production by immune cells. *Nat Rev Immunol* **2010,** *10* (3), 170-81.

69. Curtale, G.; Mirolo, M.; Renzi, T. A.; Rossato, M.; Bazzoni, F.; Locati, M., Negative regulation of Toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. *Proc Natl Acad Sci U S A* **2013,** *110* (28), 11499-504.

70. Gurung, P.; Li, B.; Subbarao Malireddi, R. K.; Lamkanfi, M.; Geiger, T. L.; Kanneganti, T. D., Chronic TLR Stimulation Controls NLRP3 Inflammasome Activation

through IL-10 Mediated Regulation of NLRP3 Expression and Caspase-8 Activation. *Sci Rep* **2015,** *5*, 14488.

71. Haniffa, M.; Bigley, V.; Collin, M., Human mononuclear phagocyte system reunited. *Semin Cell Dev Biol* **2015,** *41*, 59-69.

72. Viganò, E.; Diamond, C. E.; Spreafico, R.; Balachander, A.; Sobota, R. M.; Mortellaro, A., Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nat Commun* **2015,** *6*, 8761.

73. Gaidt, M. M.; Ebert, T. S.; Chauhan, D.; Schmidt, T.; Schmid-Burgk, J. L.; Rapino, F.; Robertson, A. A.; Cooper, M. A.; Graf, T.; Hornung, V., Human Monocytes Engage an Alternative Inflammasome Pathway. *Immunity* **2016,** *44* (4), 833-46.

74. Bauernfeind, F. G.; Horvath, G.; Stutz, A.; Alnemri, E. S.; MacDonald, K.; Speert, D.; Fernandes-Alnemri, T.; Wu, J.; Monks, B. G.; Fitzgerald, K. A.; Hornung, V.; Latz, E., Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* **2009,** *183* (2), 787-91.

75. Strickson, S.; Campbell, D. G.; Emmerich, C. H.; Knebel, A.; Plater, L.; Ritorto, M. S.; Shpiro, N.; Cohen, P., The anti-inflammatory drug BAY 11-7082 suppresses the MyD88-dependent signalling network by targeting the ubiquitin system. *Biochem J* **2013,** *451* (3), 427-37.

76. Pierce, J. W.; Schoenleber, R.; Jesmok, G.; Best, J.; Moore, S. A.; Collins, T.; Gerritsen, M. E., Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* **1997,** *272* (34), 21096-103.

77. Juliana, C.; Fernandes-Alnemri, T.; Wu, J.; Datta, P.; Solorzano, L.; Yu, J. W.; Meng, R.; Quong, A. A.; Latz, E.; Scott, C. P.; Alnemri, E. S., Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. *J Biol Chem* **2010,** *285* (13), 9792-802.

78. Zhao, J.; Zhang, H.; Huang, Y.; Wang, H.; Wang, S.; Zhao, C.; Liang, Y.; Yang, N., Bay11-7082 attenuates murine lupus nephritis via inhibiting NLRP3 inflammasome and NF-κB activation. *Int Immunopharmacol* **2013,** *17* (1), 116-22.

79. Blander, J. M.; Medzhitov, R., Regulation of phagosome maturation by signals from toll-like receptors. *Science* **2004,** *304* (5673), 1014-8.

80. Kang, Y. J.; Chen, J.; Otsuka, M.; Mols, J.; Ren, S.; Wang, Y.; Han, J., Macrophage deletion of p38alpha partially impairs lipopolysaccharide-induced cellular activation. *J Immunol* **2008,** *180* (7), 5075-82.

81. Xu, Y.; Jagannath, C.; Liu, X. D.; Sharafkhaneh, A.; Kolodziejska, K. E.; Eissa, N. T., Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* **2007,** *27* (1), 135-44.

82. Menon, M. B.; Kotlyarov, A.; Gaestel, M., SB202190-induced cell type-specific vacuole formation and defective autophagy do not depend on p38 MAP kinase inhibition. *PLoS One* **2011,** *6* (8), e23054.

83. Bain, J.; Plater, L.; Elliott, M.; Shpiro, N.; Hastie, C. J.; McLauchlan, H.; Klevernic, I.; Arthur, J. S.; Alessi, D. R.; Cohen, P., The selectivity of protein kinase inhibitors: a further update. *Biochem J* **2007,** *408* (3), 297-315.

84. Godl, K.; Wissing, J.; Kurtenbach, A.; Habenberger, P.; Blencke, S.; Gutbrod, H.; Salassidis, K.; Stein-Gerlach, M.; Missio, A.; Cotten, M.; Daub, H., An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc Natl Acad Sci U S A* **2003,** *100* (26), 15434-9.

85. Jun, J. C.; Cominelli, F.; Abbott, D. W., RIP2 activity in inflammatory disease and implications for novel therapeutics. *J Leukoc Biol* **2013,** *94* (5), 927-32.

86. Numazawa, S.; Watabe, M.; Nishimura, S.; Kurosawa, M.; Izuno, M.; Yoshida, T., Regulation of ERK-mediated signal transduction by p38 MAP kinase in human monocytic THP-1 cells. *J Biochem* **2003,** *133* (5), 599-605.

87. Risco, A.; del Fresno, C.; Mambol, A.; Alsina-Beauchamp, D.; MacKenzie, K. F.; Yang, H. T.; Barber, D. F.; Morcelle, C.; Arthur, J. S.; Ley, S. C.; Ardavin, C.; Cuenda, A., p38γ and p38δ kinases regulate the Toll-like receptor 4 (TLR4)-induced cytokine production by controlling ERK1/2 protein kinase pathway activation. *Proc Natl Acad Sci U S A* **2012,** *109* (28), 11200-5.

88. Bennett, B. L.; Sasaki, D. T.; Murray, B. W.; O'Leary, E. C.; Sakata, S. T.; Xu, W.; Leisten, J. C.; Motiwala, A.; Pierce, S.; Satoh, Y.; Bhagwat, S. S.; Manning, A. M.; Anderson, D. W., SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A* **2001,** *98* (24), 13681-6.

89. Tanemura, S.; Momose, H.; Shimizu, N.; Kitagawa, D.; Seo, J.; Yamasaki, T.; Nakagawa, K.; Kajiho, H.; Penninger, J. M.; Katada, T.; Nishina, H., Blockage by SP600125 of Fcepsilon receptor-induced degranulation and cytokine gene expression

in mast cells is mediated through inhibition of phosphatidylinositol 3-kinase signalling pathway. *J Biochem* **2009,** *145* (3), 345-54.

90. Fang, L.; Wu, H. M.; Ding, P. S.; Liu, R. Y., TLR2 mediates phagocytosis and autophagy through JNK signaling pathway in Staphylococcus aureus-stimulated RAW264.7 cells. *Cell Signal* **2014,** *26* (4), 806-14.

91. Wu, H. M.; Wang, J.; Zhang, B.; Fang, L.; Xu, K.; Liu, R. Y., CpG-ODN promotes phagocytosis and autophagy through JNK/P38 signal pathway in Staphylococcus aureus-stimulated macrophage. *Life Sci* **2016,** *161*, 51-9.

92. Kenzel, S.; Mancuso, G.; Malley, R.; Teti, G.; Golenbock, D. T.; Henneke, P., c-Jun kinase is a critical signaling molecule in a neonatal model of group B streptococcal sepsis. *J Immunol* **2006,** *176* (5), 3181-8.

93. Hara, H.; Tsuchiya, K.; Kawamura, I.; Fang, R.; Hernandez-Cuellar, E.; Shen, Y.; Mizuguchi, J.; Schweighoffer, E.; Tybulewicz, V.; Mitsuyama, M., Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat Immunol* **2013,** *14* (12), 1247-55.

94. Ghonime, M. G.; Shamaa, O. R.; Das, S.; Eldomany, R. A.; Fernandes-Alnemri, T.; Alnemri, E. S.; Gavrilin, M. A.; Wewers, M. D., Inflammasome priming by lipopolysaccharide is dependent upon ERK signaling and proteasome function. *J Immunol* **2014,** *192* (8), 3881-8.

95. Matsunaga, N.; Tsuchimori, N.; Matsumoto, T.; Ii, M., TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. *Mol Pharmacol* **2011,** *79* (1), 34-41.

96. Netea, M. G.; Nold-Petry, C. A.; Nold, M. F.; Joosten, L. A.; Opitz, B.; van der Meer, J. H.; van de Veerdonk, F. L.; Ferwerda, G.; Heinhuis, B.; Devesa, I.; Funk, C. J.; Mason, R. J.; Kullberg, B. J.; Rubartelli, A.; van der Meer, J. W.; Dinarello, C. A., Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* **2009,** *113* (10), 2324-35.

97. Schmid-Burgk, J. L.; Gaidt, M. M.; Schmidt, T.; Ebert, T. S.; Bartok, E.; Hornung, V., Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *Eur J Immunol* **2015,** *45* (10), 2911-7.

98. Yates, R. M.; Russell, D. G., Phagosome maturation proceeds independently of stimulation of toll-like receptors 2 and 4. *Immunity* **2005,** *23* (4), 409-17.

99. Li, Y. F.; Lee, K. G.; Ou, X.; Lam, K. P., Bruton's tyrosine kinase and protein kinase C μ are required for TLR7/9-induced IKKα and IRF-1 activation and interferon-β production in conventional dendritic cells. *PLoS One* **2014,** *9* (8), e105420.
100. Schmidt, N. W.; Thieu, V. T.; Mann, B. A.; Ahyi, A. N.; Kaplan, M. H., Bruton's tyrosine kinase is required for TLR-induced IL-10 production. *J Immunol* **2006,** *177* (10), 7203-10.
101. Horwood, N. J.; Mahon, T.; McDaid, J. P.; Campbell, J.; Mano, H.; Brennan, F. M.; Webster, D.; Foxwell, B. M., Bruton's tyrosine kinase is required for lipopolysaccharide-induced tumor necrosis factor alpha production. *J Exp Med* **2003,** *197* (12), 1603-11.
102. Liljeroos, M.; Vuolteenaho, R.; Morath, S.; Hartung, T.; Hallman, M.; Ojaniemi, M., Bruton's tyrosine kinase together with PI 3-kinase are part of Toll-like receptor 2 multiprotein complex and mediate LTA induced Toll-like receptor 2 responses in macrophages. *Cell Signal* **2007,** *19* (3), 625-33.
103. Lee, K. G.; Xu, S.; Kang, Z. H.; Huo, J.; Huang, M.; Liu, D.; Takeuchi, O.; Akira, S.; Lam, K. P., Bruton's tyrosine kinase phosphorylates Toll-like receptor 3 to initiate antiviral response. *Proc Natl Acad Sci U S A* **2012,** *109* (15), 5791-6.
104. Mangla, A.; Khare, A.; Vineeth, V.; Panday, N. N.; Mukhopadhyay, A.; Ravindran, B.; Bal, V.; George, A.; Rath, S., Pleiotropic consequences of Bruton tyrosine kinase deficiency in myeloid lineages lead to poor inflammatory responses. *Blood* **2004,** *104* (4), 1191-7.
105. Köprülü, A. D.; Kastner, R.; Wienerroither, S.; Lassnig, C.; Putz, E. M.; Majer, O.; Reutterer, B.; Sexl, V.; Kuchler, K.; Müller, M.; Decker, T.; Ellmeier, W., The tyrosine kinase Btk regulates the macrophage response to Listeria monocytogenes infection. *PLoS One* **2013,** *8* (3), e60476.
106. Ito, M.; Shichita, T.; Okada, M.; Komine, R.; Noguchi, Y.; Yoshimura, A.; Morita, R., Bruton's tyrosine kinase is essential for NLRP3 inflammasome activation and contributes to ischaemic brain injury. *Nat Commun* **2015,** *6*, 7360.
107. Lee, K. G.; Kim, S. S.; Kui, L.; Voon, D. C.; Mauduit, M.; Bist, P.; Bi, X.; Pereira, N. A.; Liu, C.; Sukumaran, B.; Rénia, L.; Ito, Y.; Lam, K. P., Bruton's tyrosine kinase phosphorylates DDX41 and activates its binding of dsDNA and STING to initiate type 1 interferon response. *Cell Rep* **2015,** *10* (7), 1055-65.
108. Jiang, Y.; Zhu, Y.; Liu, Z. J.; Ouyang, S., The emerging roles of the DDX41 protein in immunity and diseases. *Protein Cell* **2016**.

109. Horwood, N. J.; Page, T. H.; McDaid, J. P.; Palmer, C. D.; Campbell, J.; Mahon, T.; Brennan, F. M.; Webster, D.; Foxwell, B. M., Bruton's tyrosine kinase is required for TLR2 and TLR4-induced TNF, but not IL-6, production. *J Immunol* **2006,** *176* (6), 3635-41.

110. Pérez de Diego, R.; López-Granados, E.; Pozo, M.; Rodríguez, C.; Sabina, P.; Ferreira, A.; Fontan, G.; García-Rodríguez, M. C.; Alemany, S., Bruton's tyrosine kinase is not essential for LPS-induced activation of human monocytes. *J Allergy Clin Immunol* **2006,** *117* (6), 1462-9.

111. Lougaris, V.; Baronio, M.; Vitali, M.; Tampella, G.; Cattalini, M.; Tassone, L.; Soresina, A.; Badolato, R.; Plebani, A., Bruton tyrosine kinase mediates TLR9-dependent human dendritic cell activation. *J Allergy Clin Immunol* **2014,** *133* (6), 1644-50.e4.

112. Marron, T. U.; Martinez-Gallo, M.; Yu, J. E.; Cunningham-Rundles, C., Toll-like receptor 4-, 7-, and 8-activated myeloid cells from patients with X-linked agammaglobulinemia produce enhanced inflammatory cytokines. *J Allergy Clin Immunol* **2012,** *129* (1), 184-90.e1-4.

113. Mansell, A.; Smith, R.; Doyle, S. L.; Gray, P.; Fenner, J. E.; Crack, P. J.; Nicholson, S. E.; Hilton, D. J.; O'Neill, L. A.; Hertzog, P. J., Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat Immunol* **2006,** *7* (2), 148-55.

114. Mahajan, S.; Ghosh, S.; Sudbeck, E. A.; Zheng, Y.; Downs, S.; Hupke, M.; Uckun, F. M., Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2, 5-dibromophenyl)propenamide]. *J Biol Chem* **1999,** *274* (14), 9587-99.

115. van den Akker, E.; van Dijk, T. B.; Schmidt, U.; Felida, L.; Beug, H.; Löwenberg, B.; von Lindern, M., The Btk inhibitor LFM-A13 is a potent inhibitor of Jak2 kinase activity. *Biol Chem* **2004,** *385* (5), 409-13.

116. Hemmi, H.; Kaisho, T.; Takeuchi, O.; Sato, S.; Sanjo, H.; Hoshino, K.; Horiuchi, T.; Tomizawa, H.; Takeda, K.; Akira, S., Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* **2002,** *3* (2), 196-200.

117. Hornung, V.; Rothenfusser, S.; Britsch, S.; Krug, A.; Jahrsdörfer, B.; Giese, T.; Endres, S.; Hartmann, G., Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* **2002,** *168* (9), 4531-7.

118. Zhu, J.; Lai, K.; Brownile, R.; Babiuk, L. A.; Mutwiri, G. K., Porcine TLR8 and TLR7 are both activated by a selective TLR7 ligand, imiquimod. *Mol Immunol* **2008,** *45* (11), 3238-43.

119. Kanneganti, T. D.; Ozören, N.; Body-Malapel, M.; Amer, A.; Park, J. H.; Franchi, L.; Whitfield, J.; Barchet, W.; Colonna, M.; Vandenabeele, P.; Bertin, J.; Coyle, A.; Grant, E. P.; Akira, S.; Núñez, G., Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* **2006,** *440* (7081), 233-6.

120. Groß, C. J.; Mishra, R.; Schneider, K. S.; Médard, G.; Wettmarshausen, J.; Dittlein, D. C.; Shi, H.; Gorka, O.; Koenig, P. A.; Fromm, S.; Magnani, G.; Ćiković, T.; Hartjes, L.; Smollich, J.; Robertson, A. A.; Cooper, M. A.; Schmidt-Supprian, M.; Schuster, M.; Schroder, K.; Broz, P.; Traidl-Hoffmann, C.; Beutler, B.; Kuster, B.; Ruland, J.; Schneider, S.; Perocchi, F.; Groß, O., K(+) Efflux-Independent NLRP3 Inflammasome Activation by Small Molecules Targeting Mitochondria. *Immunity* **2016,** *45* (4), 761-773.

121. Gibbard, R. J.; Morley, P. J.; Gay, N. J., Conserved features in the extracellular domain of human toll-like receptor 8 are essential for pH-dependent signaling. *J Biol Chem* **2006,** *281* (37), 27503-11.

122. Schön, M. P.; Schön, M.; Klotz, K. N., The small antitumoral immune response modifier imiquimod interacts with adenosine receptor signaling in a TLR7- and TLR8-independent fashion. *J Invest Dermatol* **2006,** *126* (6), 1338-47.

123. Hwang, H.; Min, H.; Kim, D.; Yu, S. W.; Jung, S. J.; Choi, S. Y.; Lee, S. J., Imiquimod induces a Toll-like receptor 7-independent increase in intracellular calcium via IP(3) receptor activation. *Biochem Biophys Res Commun* **2014,** *450* (1), 875-9.

124. Tanji, H.; Ohto, U.; Shibata, T.; Taoka, M.; Yamauchi, Y.; Isobe, T.; Miyake, K.; Shimizu, T., Toll-like receptor 8 senses degradation products of single-stranded RNA. *Nat Struct Mol Biol* **2015,** *22* (2), 109-15.

125. Delgado, M. A.; Elmaoued, R. A.; Davis, A. S.; Kyei, G.; Deretic, V., Toll-like receptors control autophagy. *EMBO J* **2008,** *27* (7), 1110-21.

126. Gorden, K. B.; Gorski, K. S.; Gibson, S. J.; Kedl, R. M.; Kieper, W. C.; Qiu, X.; Tomai, M. A.; Alkan, S. S.; Vasilakos, J. P., Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J Immunol* **2005,** *174* (3), 1259-68.

127. Vanhaesebroeck, B.; Whitehead, M. A.; Piñeiro, R., Molecules in medicine mini-review: isoforms of PI3K in biology and disease. *J Mol Med (Berl)* **2016,** *94* (1), 5-11.

128. Thi, E. P.; Reiner, N. E., Phosphatidylinositol 3-kinases and their roles in phagosome maturation. *J Leukoc Biol* **2012,** *92* (3), 553-66.
129. Lee, H. K.; Lund, J. M.; Ramanathan, B.; Mizushima, N.; Iwasaki, A., Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* **2007,** *315* (5817), 1398-401.
130. Zhou, R.; Yazdi, A. S.; Menu, P.; Tschopp, J., A role for mitochondria in NLRP3 inflammasome activation. *Nature* **2011,** *469* (7329), 221-5.
131. Nakahira, K.; Haspel, J. A.; Rathinam, V. A.; Lee, S. J.; Dolinay, T.; Lam, H. C.; Englert, J. A.; Rabinovitch, M.; Cernadas, M.; Kim, H. P.; Fitzgerald, K. A.; Ryter, S. W.; Choi, A. M., Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* **2011,** *12* (3), 222-30.
132. Shi, C. S.; Shenderov, K.; Huang, N. N.; Kabat, J.; Abu-Asab, M.; Fitzgerald, K. A.; Sher, A.; Kehrl, J. H., Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol* **2012,** *13* (3), 255-63.
133. Harris, J.; Hartman, M.; Roche, C.; Zeng, S. G.; O'Shea, A.; Sharp, F. A.; Lambe, E. M.; Creagh, E. M.; Golenbock, D. T.; Tschopp, J.; Kornfeld, H.; Fitzgerald, K. A.; Lavelle, E. C., Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. *J Biol Chem* **2011,** *286* (11), 9587-97.
134. Crişan, T. O.; Plantinga, T. S.; van de Veerdonk, F. L.; Farcaş, M. F.; Stoffels, M.; Kullberg, B. J.; van der Meer, J. W.; Joosten, L. A.; Netea, M. G., Inflammasome-independent modulation of cytokine response by autophagy in human cells. *PLoS One* **2011,** *6* (4), e18666.
135. Gharbi, S. I.; Zvelebil, M. J.; Shuttleworth, S. J.; Hancox, T.; Saghir, N.; Timms, J. F.; Waterfield, M. D., Exploring the specificity of the PI3K family inhibitor LY294002. *Biochem J* **2007,** *404* (1), 15-21.
136. Moretti, J.; Roy, S.; Bozec, D.; Martinez, J.; Chapman, J. R.; Ueberheide, B.; Lamming, D. W.; Chen, Z. J.; Horng, T.; Yeretssian, G.; Green, D. R.; Blander, J. M., STING Senses Microbial Viability to Orchestrate Stress-Mediated Autophagy of the Endoplasmic Reticulum. *Cell* **2017**.
137. Cohen, P., The TLR and IL-1 signalling network at a glance. *J Cell Sci* **2014,** *127* (Pt 11), 2383-90.

138. Guarda, G.; Zenger, M.; Yazdi, A. S.; Schroder, K.; Ferrero, I.; Menu, P.; Tardivel, A.; Mattmann, C.; Tschopp, J., Differential expression of NLRP3 among hematopoietic cells. *J Immunol* **2011,** *186* (4), 2529-34.
139. Chakrabarti, A.; Banerjee, S.; Franchi, L.; Loo, Y. M.; Gale, M.; Núñez, G.; Silverman, R. H., RNase L activates the NLRP3 inflammasome during viral infections. *Cell Host Microbe* **2015,** *17* (4), 466-77.
140. Liu, Y.; Lu, N.; Yuan, B.; Weng, L.; Wang, F.; Liu, Y. J.; Zhang, Z., The interaction between the helicase DHX33 and IPS-1 as a novel pathway to sense double-stranded RNA and RNA viruses in myeloid dendritic cells. *Cell Mol Immunol* **2014,** *11* (1), 49-57.
141. Banerjee, S., RNase L and the NLRP3-inflammasome: An old merchant in a new trade. *Cytokine Growth Factor Rev* **2016,** *29*, 63-70.
142. Diamond, M. S.; Farzan, M., The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nat Rev Immunol* **2013,** *13* (1), 46-57.
143. Abbas, Y. M.; Pichlmair, A.; Górna, M. W.; Superti-Furga, G.; Nagar, B., Structural basis for viral 5'-PPP-RNA recognition by human IFIT proteins. *Nature* **2013,** *494* (7435), 60-4.
144. Gupta, R.; Ghosh, S.; Monks, B.; DeOliveira, R. B.; Tzeng, T. C.; Kalantari, P.; Nandy, A.; Bhattacharjee, B.; Chan, J.; Ferreira, F.; Rathinam, V.; Sharma, S.; Lien, E.; Silverman, N.; Fitzgerald, K.; Firon, A.; Trieu-Cuot, P.; Henneke, P.; Golenbock, D. T., RNA and β-hemolysin of group B Streptococcus induce interleukin-1β (IL-1β) by activating NLRP3 inflammasomes in mouse macrophages. *J Biol Chem* **2014,** *289* (20), 13701-5.
145. Nguyen, T. A.; Smith, B. R. C.; Tate, M. D.; Belz, G. T.; Barrios, M. H.; Elgass, K. D.; Weisman, A. S.; Baker, P. J.; Preston, S. P.; Whitehead, L.; Garnham, A.; Lundie, R. J.; Smyth, G. K.; Pellegrini, M.; O'Keeffe, M.; Wicks, I. P.; Masters, S. L.; Hunter, C. P.; Pang, K. C., SIDT2 Transports Extracellular dsRNA into the Cytoplasm for Innate Immune Recognition. *Immunity* **2017,** *47* (3), 498-509.e6.
146. Diot, C.; Fournier, G.; Dos Santos, M.; Magnus, J.; Komarova, A.; van der Werf, S.; Munier, S.; Naffakh, N., Influenza A Virus Polymerase Recruits the RNA Helicase DDX19 to Promote the Nuclear Export of Viral mRNAs. *Sci Rep* **2016,** *6*, 33763.
147. Li, J.; Hu, L.; Liu, Y.; Huang, L.; Mu, Y.; Cai, X.; Weng, C., DDX19A Senses Viral RNA and Mediates NLRP3-Dependent Inflammasome Activation. *J Immunol* **2015,** *195* (12), 5732-49.

148. Ippagunta, S. K.; Brand, D. D.; Luo, J.; Boyd, K. L.; Calabrese, C.; Stienstra, R.; Van de Veerdonk, F. L.; Netea, M. G.; Joosten, L. A.; Lamkanfi, M.; Kanneganti, T. D., Inflammasome-independent role of apoptosis-associated speck-like protein containing a CARD (ASC) in T cell priming is critical for collagen-induced arthritis. *J Biol Chem* **2010,** *285* (16), 12454-62.
149. Ippagunta, S. K.; Malireddi, R. K.; Shaw, P. J.; Neale, G. A.; Vande Walle, L.; Green, D. R.; Fukui, Y.; Lamkanfi, M.; Kanneganti, T. D., The inflammasome adaptor ASC regulates the function of adaptive immune cells by controlling Dock2-mediated Rac activation and actin polymerization. *Nat Immunol* **2011,** *12* (10), 1010-6.
150. Taxman, D. J.; Holley-Guthrie, E. A.; Huang, M. T.; Moore, C. B.; Bergstralh, D. T.; Allen, I. C.; Lei, Y.; Gris, D.; Ting, J. P., The NLR adaptor ASC/PYCARD regulates DUSP10, mitogen-activated protein kinase (MAPK), and chemokine induction independent of the inflammasome. *J Biol Chem* **2011,** *286* (22), 19605-16.
151. Sarkar, A.; Duncan, M.; Hart, J.; Hertlein, E.; Guttridge, D. C.; Wewers, M. D., ASC directs NF-kappaB activation by regulating receptor interacting protein-2 (RIP2) caspase-1 interactions. *J Immunol* **2006,** *176* (8), 4979-86.
152. Abdelaziz, D. H.; Gavrilin, M. A.; Akhter, A.; Caution, K.; Kotrange, S.; Khweek, A. A.; Abdulrahman, B. A.; Grandhi, J.; Hassan, Z. A.; Marsh, C.; Wewers, M. D.; Amer, A. O., Apoptosis-associated speck-like protein (ASC) controls Legionella pneumophila infection in human monocytes. *J Biol Chem* **2011,** *286* (5), 3203-8.

6 Appendix

Abbreviations

AIM2	absent in melanoma 2
ALR	AIM2-like receptor
ASC	apoptosis-associated speck-like protein containing CARD
BAL	bronchoalveolar lavage
BMDCs	bone marrow-derived dendritic cells
BMDMs	bone marrow-derived macrophages
BTK	Bruton's tyrosine kinase
CII	complex II
CD	cluster of differentiation
c-di-GMP	3'-5' diguanylate
cDNA	complementary DNA
CLR	C-type lectin receptor
CO_2	Carbon dioxide
cGAS	cyclic-GMP-AMP synthase
cGAMP synthase	cyclic-GMP-AMP synthase
CpG-ODN	CpG oligodeoxynucleotides
Ctrl	control
DCs	dendritic cells
DDX	DEAD-box helicase
DHX	DEAH-box helicase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
EC	*E. coli*
EDTA	ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
Fig.	figure
GM-CSF	granulocyte macrophage colony-stimulating factor
hAM	human alveolar macrophages
HCl	hydrochloric acid

HEK cells	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid hematopoietic
HIV	human immunodeficiency virus
HKEC	heat killed *E. coli*
IFI16	interferon-γ-inducible protein 16
IFN	interferon
IL	interleukin
ILC	Innate lymphoid cells
IκB	inhibitor of kappa B
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
L+N	LPS plus nigericin
LB	Luria Bertani
LDH	lactate dehydrogenase
LGP2	Laboratory of Genetics and Physiology 2
LMW	low molecular weight
LPS	lipopolysaccharide
LRRs	Leucine-rich repeats
LV	lentivirus
MAVS	mitochondrial antiviral-signaling protein
MAPK	mitogen-activated protein kinases
MD2	Lymphocyte antigen 96
MDA-5	Melanoma differentiation associated gene 5
MoDCs	monocyte-derived dendritic cells
MOI	multiplicity of infection
MoMs	monocyte-derived macrophages
MPLA	monophosphoryl lipid A
mRNA	messenger RNA
MyD88	Myeloid differentiation primary response 88
n.s.	not significant
NF-κB	nuclear factor ‚kappa-light-chain-enhancer' of activated B-cells
NLR	NOD-like receptor
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
OD	optical density
OMV	outer membrane vesicles

PAMPs	pathogen-associated molecular patterns
p.i.	post infection
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDCs	plasmacytoid DCs
PI3K	Phosphatidylinositol 3-kinase
poly I:C	poly(inosinic:cytidylic) acid
qRT-PCR	quantitative real-time PCR
PRR	pattern recognition receptor
RIG-I	retinoic acid inducible gene I
RIP2	Receptor Interacting Protein-2
RLR	RIG-I-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
qRT-PCR	quantitative real-time polymerase chain reaction
S. enterica	*Salmonella enterica*
S. aureus	*Staphylococcus aureus*
SAMHD1	SAM domain and HD domain-containing protein 1
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
SIDT-2	SID1 transmembrane family member 2
SIV	simian immunodeficiency virus
ssRNA	single stranded RNA
STING	stimulator of interferon genes
Tab.	Table
TACI	transmembrane activator and CAML interactor
TAK1	transforming growth factor beta-activated kinase 1
TBK1	TANK binding kinase 1
TEMED	tetramethylethylenediamine
ThyA$^-$	thymidine auxotroph
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
tRNA	transfer RNA

***Vita*-PAMP**	viability-associated PAMP	**XLA**	X-linked agammaglobulinemia

Supplementary Tables

Supplementary Tab. 1: LB medium and agar plate recipe

LB medium		
BBL™ Trypticase™ Soy Broth	Becton, Dickinson and Company; Sparks, USA	10 g
Bacto™ Yeast Extract		5 g
Sodium chloride	Roth; Karlsruhe, Germany	5 g
Deionized water		1000 ml
LB-Agar plates		
Bacto™ Agar	Becton, Dickinson and Company; Sparks, USA	15 g/l LB medium

Supplementary Tab. 2: Inhibitory molecules used for screening experiments

Inhibitor	Manufacturer	Target	Concentration	Preincubation
BAY11-7082	InvivoGen; San Diego, USA	IκBα	10 µM	1 hour
CLI-095	InvivoGen; San Diego, USA	TLR4 signaling	3 µM, 15 µM	1 hour
Imiquimod	Santa Cruz Biotechnology; Heidelberg, Germany	TLR7/8	5 µg/ml	45 minutes
LFM-A13	Sigma-Aldrich; Steinheim, Germany	BTK	5 µM	1 hour
LY294002	InvivoGen; San Diego, USA	PI3-K	5 µM	1hour
SB203580	InvivoGen; San Diego, USA	p38/RK MAPK	1 µM	1 hour

SP600125	InvivoGen; San Diego, USA	JNK	30 µM	1 hour

Supplementary Tab. 3: TLR ligands used in for inhibitor screening experiments

Ligand	Manufacturer	Target	concentration
poly I:C LMW	InvivoGen; San Diego, USA	TLR3	20 µg/ml
LPS ultrapure	InvivoGen; San Diego, USA	TLR4	1 µg/ml, 10 ng/ml
MPLA	InvivoGen; San Diego, USA	TLR4	1 µg/ml
R848	InvivoGen; San Diego, USA	TLR7/8	1 µg/ml
ODN 2395	InvivoGen; San Diego, USA	TLR9	5 µM

Supplementary Tab. 4: Target-specific mature antisense sequences for gene transduction experiments.

Target	Clone ID, Mature Antisense Sequence	Tested?	Used?
ASC	TRCN0000059073 ATCTTGCTTGGGTTGGTGGGC	Yes	Yes
	TRCN0000059074 TGCTGGTCTATAAAGTGCAGG	Yes	No
	TRCN0000059075 TGTGAAACTGAAGAGCTTCCG	Yes	Yes
	TRCN0000059076 TTCAGCTTGAACTTCTTGAGC	Yes	Yes
	TRCN0000059077 TAGGACTGGGACTCCCTTAGG	Yes	No
DHX33	TRCN0000051458 AAGGTCCGATAGATATTGAGC	Yes	Yes
	TRCN0000051459 TTTCAGAGGAAGTTTCCCGAG	Yes	Yes
	TRCN0000051460 AAGCAAAGAGTCTGAAATTGC	Yes	Yes
	TRCN0000051461 AAATGATCACTTTGCGATAGC	Yes	Yes
	TRCN0000051462 ATTTGGGTTCTAAAGGAAATG	Yes	Yes

MyD88	TRCN0000008024 ATCAAGGTACAAAGTTGGTGG	Yes	No
	TRCN0000008025 AAGTCACATTCCTTGCTCTGC	Yes	Yes
	TRCN0000008026 TTCAGTCGATAGTTTGTCTGT	Yes	No
	TRCN0000008027 TTCATTGCCTTGTACTTGATG	Yes	Yes
	TRCN0000011223 ACGTTCAAGAACAGAGACAGG	Yes	Yes
NLRP3	TRCN0000062723 TACTTTCTGTACTTCTTACGG	No	Yes
	TRCN0000062724 ATTCAGTTAGACTCTGGCTGG	No	Yes
	TRCN0000062725 TTTCTGCAGGTTACACTGTGG	No	Yes
	TRCN0000062726 TTCTTGAAGTGTTTCTAACGC	No	Yes
	TRCN0000062727 AAACAGTAGAACAATTCCAGC	No	Yes
TLR4	TRCN0000056893 TATTCAAAGATACACCAGCGG	Yes	Yes
	TRCN0000056894 ATATTAAGGTAGAGAGGTGGC	Yes	Yes
	TRCN0000056895 AAATTCTCCCAGAACCAAACG	Yes	Yes
	TRCN0000056896 ATGATTTACCATCCAGCAGGG	Yes	Yes
	TRCN0000056897 TAAGAAAGCTAGACTACTTGG	Yes	Yes
TLR8	TRCN0000056958 AAGTCAAGTATTTCTAAGCGG	Yes	Yes
	TRCN0000056959 ATTAAGGGATAAATCCAGACG	Yes	No
	TRCN0000056960 TAGTATTGCTTAATGGAATCG	Yes	Yes
TRIF	TRCN0000123199 TATCTTCTACAGAAAGTTGGA	Yes	Yes
	TRCN0000123200 ATCTTCTACAGAAAGTTGGAG	Yes	Yes
	TRCN0000123201 AAGTTGGAGGTGAGAAGTAGG	Yes	Yes
	TRCN0000123203 TTCCGATGATGATTCCAGGGA	Yes	No

Supplementary Tab. 5: Buffer and reagents required for Western Blot.

Phosphoprotein wash buffer		
Sodium orthovanadate 98% 200 mM	Sigma-Aldrich; Steinheim, Germany	5 ml
Sodium pyrophosphate 150 mM		50 ml
Sodium fluoride 99% 1M		50 ml
PBS	Life Technologies; Darmstadt, Germany	500 ml
Lysis buffer		
Tris-HCl, pH 7.4	Sigma-Aldrich; Steinheim, Germany	50 mM
Complete™ Protease Inhibitor Cocktail Tablet	Santa Cruz Biotechnology; Heidelberg, Germany	10 µg/ml
Nonidet® P-40 99%	Fluka Chemie GmbH; Buchs, Switzerland	1%
Phosphoprotein wash buffer		1 ml
Loading buffer (5x)		
Tris-HCl; 0.5M, pH 6.8	Sigma-Aldrich; Steinheim, Germany	1 ml
Glycerol		0.8 ml
SDS (10% w/v)	Serva; Heidelberg, Germany	1.6 ml
Bromophenol blue (1% w/v)	Amersham Pharmacia Biotech; Freiburg, Germany	0.4 ml
β-Mercaptoethanol (1% v/v)	Sigma-Aldrich; Steinheim, Germany	0.4 ml
Deionized water		3.9 ml
Electrode buffer (5x)		
Tris base	Sigma-Aldrich; Steinheim,	3 g

	Germany	
Glycine	Merck Millipore; Darmstadt, Germany	14.4 g
SDS (10% w/v)	Serva; Heidelberg, Germany	1 g
Deionized water		1000 ml
Blot buffer (10x)		
Tris base	Sigma-Aldrich; Steinheim, Germany	3 g
Glycine	Merck Millipore; Darmstadt, Germany	14.4 g
Methanol		20 %
Deionized water		1000 ml
Resolving gel (10%, 2 gels)		
Tris-HCl 1.5 M; pH 8.8	Sigma-Aldrich; Steinheim, Germany	2.5 ml
SDS (10% w/v)	Serva; Heidelberg, Germany	100 µl
Acrylamide solution (40% w/v)		2.5 ml
Ammonium persulfate (10% w/v)		50 µl
Tetramethylethylenediamine (TEMED)		8 µl
Deionized water		4.85 ml
Resolving gel (8%, 2 gels)		
Tris-HCl 1.5 M; pH 8.8	Sigma-Aldrich; Steinheim, Germany	2.5 ml
SDS (10% w/v)	Serva; Heidelberg, Germany	100 µl
Acrylamide solution (40% w/v)		1.89 ml
Ammonium persulfate (10% w/v)		50 µl
TEMED		8µl

Deonized water		5.46 ml
Stacking gel (2 gels)		
Tris-HCl 0.5 M; pH 6.8	Sigma-Aldrich; Steinheim, Germany	1 ml
SDS (10% w/v)	Serva; Heidelberg, Germany	40 µl
Acrylamide solution (40% w/v)		0.53 ml
Ammonium persulfate (10% w/v)		40 µl
TEMED		8 µl
Deonized water		2.43 ml
Protein ladder		
Precision Plus Protein™ Kaleidoscope™	BioRad; Hercules, USA	6 µl

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publications

Helbig ET, Opitz B, Sander LE: "Adjuvant Immunotherapies as a novel approach to bacterial infections", Immunotherapy, Vol. 5, No. 4, pages 365-381.

Pahlitzsch TMJ, **Helbig ET**, Sarioglu N, Hinkson L, von Weizsäcker K, Henrich W. Novel Insights in Fetal Cardiomyopathy due to in utero Herpes Simplex Virus Infection. Fetal Diagn Ther 2017;42:236-9.

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