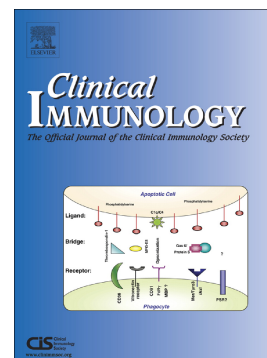# Accepted Manuscript

An optimized whole blood assay measuring expression and activity of NLRP3, NLRC4 and AIM2 inflammasomes

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An optimized whole blood assay measuring expression and activity of NLRP3, NLRC4 and AIM2 inflammasomes

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Abstract

The proinflammatory protease caspase-1 plays pivotal roles in central pathways of innate immunity, thereby contributing to pathogen clearance. Beside its physiological role, dysregulated activity of caspase-1 is known to contribute to an increasing number of diseases. In this study we optimized and validated a low-volume human whole blood assay facilitating the measurement of caspase-1 activation and inflammasome-related gene expression upon stimulation of the NLRP3, NLRC4 or AIM2 inflammasome. Using the NLRP3 inflammasome specific inhibitor MCC950 we were able to measure the activity of canonical or alternative NLRP3 pathways, AIM2 and NLRC4 inflammasomes in whole blood. Based on our data we assume a superposition of NLRP3 and NLRC4 inflammasome activities in human whole blood following stimulation with *S. typhimurium*. The optimized whole blood assay may be suitable for diagnostic and research purposes for pediatric patients who can only donate small amounts of blood.

Keywords

procaspase-1, inflammasome, whole blood assay, NLRP3, AIM2, NLRC4

Conflicts of interest

The authors declare no conflict of interest.

1. Introduction

The proinflammatory protease caspase-1 plays pivotal roles in central pathways of innate immunity, thereby contributing to pathogen clearance. Beside its physiological role, caspase-1 mediated inflammation is known to contribute to an increasing number of diseases like gout, type 2 diabetes, bronchopulmonal dysplasia or periodic fever syndromes [1]. Activation of caspase-1 is initiated through proximity-induced autoproteolysis of caspase-1 precursor molecules, orchestrated by a large multi-protein complex called the inflammasome. Most inflammasomes consist of at least three components: an intracellular sensor (pattern recognition receptor, PRR) for the recognition of specific pathogen- or danger-associated molecular patterns (PAMPs, DAMPs), the adaptor protein apoptosis speck-like protein containing a CARD (ASC), and procaspase-1. The current model of inflammasome activation consists of two steps. Firstly, transmembrane bound Toll-like receptors (TLR) located at the cell surface recognize extracellular DAMPs or PAMPs leading to enhanced transcription of proinflammatory target genes and priming of the inflammasome. Secondly, intracellular DAMPs or PAMPs trigger the intracellular sensor, leading to inflammasome assembly and caspase-1 activation [2]. To date, nine different intracellular sensor proteins are known to form inflammasomes in response to stimulation with distinct triggers. The signal specificity for the different inflammasomes is realized through the second signal only, which is mediated through diverse agents like ATP, bacterial toxins or crystalline materials for the NLRP3-, bacterial flagellin or components of bacterial type 3 secretion systems for the NLRC4-, and cytosolic dsDNA for the AIM2-inflammasome [3]. Active caspase-1 induces the maturation and secretion of the proinflammatory cytokines IL-1β and IL-18 and mediates pyroptosis, a programmed proinflammatory cell death [1]. Typically, studies analyzing disease-specific inflammasome activity in patients are performed *ex vivo* using peripheral blood mononuclear cell (PBMC) based assays [4-7]. Soluble plasma-factors as well as cellular interactions, both known to modulate the innate immune response [8,9], are lost with the PBMC isolation. Caspase-1 is expressed not only in monocytes, macrophages and dendritic cells, but also in PMNs representing the major leukocyte subset in peripheral blood, typically absent in PBMC preparations [10]. Thus, analyzing inflammasome/caspase-1 activity in purified PBMCs seems to be highly artificial. Furthermore, PBMC purification requires large amounts of blood, thereby rendering the assay impractical for the use in small children or neonates. In this study we optimized and validated a low-volume human whole blood assay (WBA) facilitating the measurement of caspase-1 activation and inflammasome-related gene expression upon specific stimulation of the NLRP3, NLRC4 or AIM2 inflammasome.

2. Material and methods

2.1. Whole Blood Assay

Hirudin or heparin coated tubes (Sarstedt 04.1944.001 or 01.1604.001) were used to collect blood from volunteers. Unless stated otherwise, hirudin was used. Blood samples were distributed on 96 well plates using 140 μl per well. Priming for NLRP3- and AIM2-assays was performed using 1 μg/ml ultra-pure LPS (Invivogen, tlrl-3pelps) for 5.5 h. Plates were incubated on a shaker (450 rpm) in a humidified incubator with 37 °C, 5 % CO_2. For NLRP3 activation, additional incubation with ATP for 30 min was performed. The activation of the AIM2 inflammasome was performed by transfection of double stranded DNA (poly(dA:dT), Invivogen, tlrl-patn). Therefore, 0.8 μg Lipofectamine 2000 (ThermoFisher Scientific) and 0.4 μg poly(dA:dT) (Invivogen, tlrl-patn) were diluted each in a total volume of 10 μl in OptiMem medium.

Both volumes were mixed in a ratio of 1:1 and incubated for 5 min at room temperature. The total volume of 20 µl was added per well (final concentration 4 µg/ml Lipofectamine 2000, 2 µg/ml polydA:dT) for either 6 h or 24 h. For some experiments poly(dA:dT)/LyoVec complexes (Invivogen, tlrl-patc) were used. The NLRC4 inflammasome was stimulated with *Salmonella typhimurium* (strain SL1344) from an overnight culture in LB broth containing 300 mM NaCl and 200 µg/ml Streptomycin. Salmonella were washed with PBS twice. An estimated MOI of 10/leukocyte was used. Therefore, a leukocyte count of 10.0×10^9/L was assumed for all patients. After $0.5 - 4$ h of stimulation, 20 µg/ml Gentamicin was added to the cells followed by a second incubation step of 2 h. For NLRC4 activation, cells were not agitated during stimulations. The total volume for all inflammasome assays was 200 µl/well. At the end of all stimulations 100 µl of PBS was added to each well, the plates were centrifuged (1200 rpm, 5 min, room temperature) and the supernatant from each well was frozen at -80 °C. IL-1β or IFNα levels in the supernatant were analyzed using a cytometric bead assay (CBA, Becton Dickinson) according to the manufacturers' instructions. Unless stated otherwise, the results are expressed as mean ±SD, n=3. The study was approved by the responsible ethics committee (Ethics committee of the TU Dresden, EK97032014) before start. Human whole blood was drawn from healthy volunteers after obtaining written informed consent.

2.2. Inhibitors

Stock solutions of potassium chloride (KCL, 3 M), MCC950 (10 mM) and Ac-YVAD-CHO (100 mM) were dissolved in PBS and used at the specified final concentrations. The inhibitors were added to whole blood samples together with the priming stimuli. The total volume for all assays including inhibitors was 220 µl per well.

2.3. Cell Viability

For viability analysis, cells were stimulated as described before and 0.1% Triton X-100 treated whole blood samples were used as cell death positive control. Cells from triplicate samples were pooled and erythrocyte lysis was performed (BD PharmLyse, 555899) followed by a washing step with PBS. The cell pellet was resuspended in 600 µl PBS and distributed into 3 wells of a 96-well plate. Then, 20 µl of AlamarBlue Reagent (ThermoFisher Scientific, DAL1100) were added per well. Plates were incubated on a shaker (450 rpm) in a humidified incubator with 37 °C, 5 % CO_2 for 1 h. Fluorescence signals were measured at 560/585 nm (excitation/emission).

2.4. Immunoprecipitation

Serum containing supernatants from three samples were pooled and pre-cleared using 200µl Protein A Agarose (Santa Cruz Biotechnology, sc-2001) per 500 µl sample (2 h, 4 °C, rotation). For immunoprecipitation, supernatants were incubated with 3 µg anti-IL-1β antibody (Santa Cruz Biotechnology, sc-7884) (1 h, 4 °C, rotation). Subsequently, the immune complexes were incubated with 20 µl Protein A agarose overnight (4 °C, rotation). Precipitates were analysed by SDS-PAGE and western blotting using an anti-IL-1β antibody (Santa Cruz Biotechnology, sc-1250, 1:100). Densitometric analysis was performed using ImageJ (http://imagej.nih.gov/ij/).

2.5. Gene Expression Analysis

Following erythrocyte lysis, total RNA from the leukocyte pellets was purified using the RNeasy micro kit (Qiagen). Subsequently, first strand cDNA was generated using the Sensiscript RT kit (Qiagen). Both kits were used according to the manufacturer's instructions. Gene expression analysis was performed as reported earlier [11]. The following primers were used: *CASP1* 5'-TTT CCG CAA GGT TCG ATT TTC A-3' and 5'- GGC ATC TGC GCT CTA CCA TC -3'; *NLRP3* 5'- TAG CCA CGC TAA TGA TCG ACT-3' and 5'- TTG ATC GCA GCG AAG ATC CAC-3'; AIM2 5'- TGG CAA AAC GTC TTC AGG AGG-3' and 5'- AGCTTGACTTAGTGGCTTTGG-3'; *NLRC4* 5'-GAA CTG ATC GAC AGG ATG AAC G-3' and 5'-ACC CAA GCT TGA CGA GTT GT-3'; *ASC* 5'- TGG ATG CTC TGT ACG GGA AG-3' and 5'- CCA GGC TGG TGT GAA ACT GAA-3'; *IL1B* 5'-CAC GAT GCA CCC TGT ACG ATC A-3' and 5'-GTT GCT CCA TAT CCT GTC CCT-3'; *HPRT1* 5'- CCT GGC GTC GTG ATT AGT GAT-3' and 5'-AGA CGT TCA GTC CTG TCC ATA A-3'. Unless stated otherwise, the results are expressed as mean ±SD, n=3.

3. Results

3.1 Activation of the NLRP3 inflammasome can be quantified in whole blood

Priming of whole blood samples with 1 µg/ml ultra-pure LPS resulted in immediate IL-1β secretion. This effect is probably mediated through human monocytes featuring a constitutively active caspase-1 now known to be regulated *via* a monocyte specific alternative NLRP3 inflammasome pathway [12,13]. Additional stimulation with ATP led to further increase of IL-1β secretion due to canonical NLRP3 activation. In order to minimize unspecific cell death in the samples we decided to use 1mM ATP for all subsequent experiments (Fig. 1A). High extracellular potassium concentrations are known to inhibit the activation of the NLRP3 inflammasome. Using our WBA, extracellular potassium inhibited the NLRP3-inflammasome-mediated IL-1β secretion in a dose-dependent manner, affecting not only the canonical but also the alternative NLRP3 pathway (Fig. 1B). Next, we tested the NLRP3-specific inhibitor MCC950 [14]. MCC950 also inhibited the canonical and alternative NLRP3 dependent IL-1β secretion in a dose-dependent manner, but high concentrations of MCC950 up to 10 µM were needed for full inhibition of Il-1β secretion (Fig. 1C). Pre-incubation for 1 h with MCC950 before onset of the stimulation did not reduce the required concentration of MCC950 (Fig. 1D). However, MCC950 is known to be highly bound to plasma proteins; thereby potentially explaining the need for high MCC950 concentrations in the WBA. Given the fact that MCC950 is NLRP3-specific in concentrations up to 10 µM *in vitro* [14], we decided to use 1 and 10 µM MCC950 for further analyses. Neither 80 mM extracellular potassium nor 10 µM MCC950 reduced the cell viability; thereby excluding increased cell death rates as a cause for the reduced IL-1β secretion (Fig. 1E).

3.2 DNA transfection is feasible in whole blood and can activate the AIM2 inflammasome

DNA of cytosolic bacteria or DNA viruses activates the AIM2 inflammasome. For *in vitro* studies, activation of the AIM2 inflammasome is typically induced *via* transfection of synthetic double-stranded DNA (dsDNA) into cells. First we followed standard transfection protocols using Lipofectamine 2000 and poly(dA:dT) for 24 h after 5.5 h of LPS priming. For non-primed (PBS-primed) whole blood, unloaded Lipofectamine induced IL-1β secretion, which was not further amplified using Lipofectamine together with poly(dA:dT). Poly(dA:dT) alone did not induce IL-1β secretion. LPS priming of whole blood induced

strong IL-1β secretion, which was further amplified by unloaded Lipofectamine. Again, using Lipofectamine together with poly(dA:dT) did not further amplify IL-1β secretion (Fig. 2A). To clarify if DNA transfection into whole blood cells was successful, we examined IFNα secretion after 24 h. Poly(dA:dT) or Lipofectamine only treated cells did not secrete any IFNα. However, cells treated with Lipofectamine together with poly(dA:dT) produced a strong IFNα signal, indicating the recognition of dsDNA via cytosolic DNA sensors (Fig. 2B). Next, we tested high extracellular potassium in order to block the NLRP3-mediated IL-1β signal induced by the LPS priming. Interestingly, 40 mM potassium fully inhibited the IL-1β secretion for all PBS-primed samples. For the LPS-primed samples, 40mM potassium reduced the IL-1β secretion upon stimulation with LPS, LPS/Lipofectamine, and LPS/poly(dA:dT) to approximately 50%. Interestingly, the LPS primed sample stimulated with Lipofectamine and poly(dA:dT) was the only one in which 40 mM potassium inhibited the IL-1β secretion to a lower extent. Furthermore, 80 mM extracellular potassium fully blocked IL-1β secretion for all samples in this assay (Fig. 2C). In order to clarify the roles of NLRP3 and AIM2 in this DNA transfection assay we used the NLRP3 specific inhibitor MCC950. Again, MCC950 fully blocked IL-1β secretion for all PBS-primed samples. In the LPS primed samples 10 μM MCC950 blocked IL-1β secretion upon stimulation with LPS, LPS/Lipofectamine, and LPS/poly(dA:dT). Therefore, these stimuli directly increase NLRP3 activation. IL-1β secretion of LPS primed samples stimulated with Lipofectamine and poly(dA:dT) was only partially inhibited by MCC950, indicating an AIM2-dependent IL-1β secretion (Fig. 2D). Hence we used LPS-primed cells together with MCC950-mediated NLRP3 inhibition to uncover the AIM2 signal for all following AIM2 activation assays. In order to reduce the assay time we compared 6 h to 24 h of transfection following LPS priming (Fig. 2E). The absolute IL-1β secretion for LPS/Lipofectamine/poly(dA:dT)/MCC950 activated samples was higher for 24 h of transfection when compared to 6 h (2398 pg/ml *vs.* 1271 pg/ml). However, ratios between the LPS/Lipofectamine/poly(dA:dT)/MCC950 activated and the LPS/Lipofectamine/MCC950 control samples were nearly the same for both time points (3.4 (6 h) vs. 3.6 (24 h)). Therefore, we decided to use the 6h protocol for further analyses (Fig. 2F). Next, we compared the Lipofectamine 2000 poly(dA:dT) transfection protocol with a commercially available product in which poly(dA:dT) is complexed to the cationic transfection reagent LyoVec. Samples stimulated with LPS/poly(dA:dT)/Lyovec/MCC950 did not secrete more IL-1β than LPS/MCC950 stimulated samples 6 h or 24 h after transfection (Fig. 2F). In contrast to the NLRP3 activation assay, using a concentration of 80 mM extracellular potassium reduced cell viability in the AIM2 activation assay. This effect could probably be explained by the prolonged incubation time. However, NLRP3 inhibition using MCC950 did not impair cell viability (Fig. 2G).

3.3 Time-dependent superposition of NLRP3 and NLRC4 inflammasome activation following infection with *S. typhimurium*

Stimulation of the NLRC4 inflammasome was performed using *S. typhimurium* with an estimated MOI of 10. Gentamicin was added to the medium 30 min to 4 h after infection to restrict extracellular growth of the bacteria and incubation was continued for additional 2 h. Stimulation of whole blood using *S. typhimurium* led to fast and strong secretion of IL-1β, which could be detected already after 0.5+2 h of stimulation, reaching its maximum after 2+2 h (Fig. 3A). 40 mM extracellular potassium showed a strong inhibition of the *S. typhimurium* induced IL-1β secretion at early time points. Later on, an increasing IL-1β

signal could be detected despite extracellular potassium. Just as for the NLRP3 and AIM2 activation assays, 80 mM potassium fully blocked IL-1β secretion (Fig. 3 B). Specific inhibition of the NLRP3 inflammasome using MCC950 revealed a strong inhibition of IL-1β secretion at early time points, indicating a strong NLRP3 dependency. For longer stimulations, the inhibitory effect of MCC950 on IL-1β secretion faded, thereby uncovering an increased NLRC4 dependency. Hence, we assume a superposition of NLRP3 and NLRC4 inflammasome activation after stimulation with *S. typhimurium* (Fig. 3C). Neither 80 mM extracellular potassium nor 10 μM MCC950 reduced the cell viability, thereby excluding that increased cell death caused the reduced IL-1β secretion (Fig. 3D).

3.4 Secreted IL-1β is mature and specifically cleaved by caspase-1

The secretion of mature IL-1β into the cell culture supernatant was measured as surrogate marker of inflammasome/caspase-1 activity in this study. Most commercially available IL-1β-antibodies detect mature as well as uncleaved pro-forms of IL-1β. Hence, we analyzed the specificity of the used cytometric bead array for mature IL-1β. IL-1β derived from the serum containing cell culture supernatant was concentrated using immunoprecipitation and subsequently quantified by western blotting. When compared, the IL-1β signal from the cytometric bead array and the western blot band intensity of mature IL-1β strongly correlated with each other (Fig. 4A). Beside caspase-1, several other proteases are able to convert pro-IL-1β into its biologically active form in certain conditions [15]. Using the caspase-1 inhibitor Ac-YVAD-CHO, we analyzed the caspase-1 dependency of the measured IL-1β signal. 100 μM YVAD-CHO, with or without 10 μM MCC950, led to strong inhibition of the IL-1β secretion following stimulation with LPS, LPS/ATP, LPS/Lipofectamin/poly(dA:dT) as well as *S. typhimurium* (Fig. 4B). Therefore, our WBA specifically measures mature IL-1β, processed by active caspase-1.

3.5 The WBA is influenced by the anticoagulant used, sample agitation and storage time

Whole blood assays can be influenced by the anticoagulants used and hirudin is known to be the best anticoagulant for WBA using transfected DNA as stimuli [16]. Therefore, we used hirudin-anticoagulated whole blood initially. After establishing the assays we compared the influence of the anticoagulants hirudin and heparin. No difference in the secretion of IL-1β was observed following NLRP3 or NLRC4 stimulations (Fig. 5A). However, following Lipofectamine 2000 mediated DNA transfection for the AIM2 assay, we observed a difference between hirudin- or heparin-anticoagulated samples. The IL-1β signal observed was much stronger for hirudin-anticoagulated whole blood, indicating a more effective transfection of DNA in these samples (Fig. 5A). When used in clinical settings, blood volumes obtained from patients, especially from children, can strongly vary in test tubes. Therefore, we analyzed the influence of different hirudin concentrations to the WBA. Neither NLRP3 nor AIM2 or NLRC4 stimulations were affected by different starting volumes and hence were independent of the hirudin concentration in the samples (Fig. 5B). Using non-agitated whole blood for *in vitro* assays leads to sedimentation of cells during the incubation. We analyzed the influence of agitation on the activation of NLRP3 and AIM2 inflammasomes. Interestingly, LPS priming and stimulation of the AIM2-inflammasome were independent of sample agitation. However, sample-agitation was mandatory for the activation of NLRP3 following ATP stimulation (Fig. 5C). Therefore, stimulation of NLRP3 and AIM2 were performed using continuous

agitation during the assays. Due to technical limitations, we were not able to analyze the influence of agitation on the activation of the NLRC4 inflammasome. Hence, stimulation of the NLRC4 inflammasome using *S. typhimurium* was performed without agitation. Next we analyzed the effect of storage time on the WBA. Blood was drawn simultaneously and was stored at room temperature in the dark. The WBA was started after 0, 4, 8, 12, 16, and 24 h of storage. Interestingly, LPS priming as well as activation of NLRP3 and AIM2 inflammasomes led to a robust IL-1β signal, even after 24 h of storage time (Fig. 5D). The ATP mediated canonical NLRP3 activation resulted in elevated IL-1β secretion levels for later time points. In contrast, activation of the NLRC4 inflammasome was dependent on the duration of storage with a decrease of the IL-1β secretion (Fig. 5D).

3.6 Inflammasome-related genes are regulated differentially following priming and inflammasome stimulation

Expression of the inflammasome-related genes *NLRP3*, *AIM2*, *NLRC4*, *ASC*, *CASP1* and *IL1B* was analyzed following LPS priming and different inflammasome stimulations. After priming with LPS, *CASP1* and *IL1B* were upregulated, whereas expression of *ASC* and *NLRP3* was not altered (Fig. 6A). The effects mediated by the short ATP stimulation do not depend on variation of gene expression and were therefore not analyzed. For the LPS priming during the AIM2 stimulation, the gene expression pattern of *CASP1*, *IL1B* and *ASC* mimicked the patterns seen after NLRP3 stimulation. The expression of *AIM2* did not increase during LPS priming just as the expression of *NLRP3*. Subsequent activation of the AIM2-inflammasome by DNA transfection reduced the gene expression of *CASP1*, *IL1B*, and *ASC*, while *AIM2* stayed unregulated (Fig. 6B). Stimulation of the NLRC4 inflammasome using *S. typhimurium*, led to downregulation of *ASC* and *NLRC4*. Interestingly, expression of *CASP1* and *IL1B* did not increase and remained at basal levels (Fig. 6C).

4. Discussion

In the present study, we validated a hirudin-based low-volume WBA, capable of analyzing caspase-1 activity as well as inflammasome-related gene expression following specific stimulation of the NLRP3, AIM2 or NLRC4 inflammasome.

Generally, WBAs are quick and easy to perform and allow physiological interactions between soluble plasma factors as well as cellular interactions, both known to modulate the innate immune response [8,9]. The majority of caspase-1 mediated IL-1β secretion in peripheral blood is processed in neutrophils, monocytes, macrophages and dendritic cells. Therefore, WBAs should depict the patient's immune status better than PBMC-based assays. Analysis of inflammasome activation in peripheral blood samples the total signals of all cells. In our WBA, priming with LPS resulted in immediate IL-1β secretion into the supernatant. This effect is probably mediated through human monocytes featuring a constitutively active caspase-1 known to be regulated via a monocyte specific alternative NLRP3 inflammasome pathway [12,13]. Additional stimulation with ATP led to further increase of IL-1β secretion due to canonical NLRP3 activation. Therefore, the difference between the IL-1β signal of LPS/ATP- and LPS-stimulated samples depicts the canonical NLRP3 activation.

The pathway of alternative NLRP3 inflammasome activation complicated the specific analysis of AIM2 and NLRC4 inflammasomes, likely due to LPS or *S. typhimurium* mediated, TLR4 dependent signaling. Hence,

we tried to inhibit the NLRP3 inflammasome using high extracellular potassium in order to uncover the non-NLRP3 specific signals. High extracellular potassium is known to strongly inhibit the activation of the NLRP3 inflammasome [17,18]. Using our WBA we could demonstrate this is also true for human whole blood: A concentration of extracellular potassium as low as 40 mM strongly inhibited the activation of the NLRP3 inflammasome *via* alternative as well as canonical pathways. Although high extracellular potassium concentrations were initially thought to specifically inhibit the NLRP3 inflammasome, it is now known that inhibition of potassium-efflux *via* high extracellular concentrations of potassium also inhibit NLRP1, AIM2, NLRC4 and NLRP7 inflammasomes as well [2]. Interestingly, the concentrations required for inhibition differs between the inflammasomes. As NLRP3 activation can be inhibited with 30 mM extracellular potassium, partial inhibition of the AIM2 and NLRC4 inflammasome can be obtained using concentrations of 80 mM but concentrations of 120 mM or higher are needed for full inhibition [17,19,20]. These findings are in line with the data from our WBA: 40 mM extracellular potassium seems to preferably inhibit NLRP3 activation while 80 mM extracellular potassium fully blocked IL-1β secretion. However, the AIM2 and NLRC4 mediated IL-1β signal was lower when using 40 mM extracellular potassium compared to MCC950 mediated NLRP3 inhibition. Using the highly NLRP3-specific inhibitor MCC950 we were able to subtract the NLRP3-mediated IL-1β signal following AIM2 and NLRC4 stimulations.

Using Lipofectamine 2000 and poly(dA:dT), we could demonstrate that transfection of DNA into cells of whole blood is feasible and effective. Of note, priming of the AIM2 inflammasome using LPS is necessary for effective IL-1β secretion following DNA transfection. Whereas extracellular poly(dA:dT) does not induce a priming signal, Lipofectamine 2000 increased the IL-1β secretion of PBS- as well as LPS-primed cells and therefore seems to activate alternative as well as canonical NLRP3 inflammasome pathways. Lipofectamine 2000 belongs to the class of cationic lipid nanocarriers typically considered neutral tools for gene delivery. However, they are known to activate Toll-like receptor signaling mediated NFκB activation as well as NLRP3 inflammasome assembly *via* induction of reactive oxygen species [21-23]. Activation of both pathways clearly explains the Lipofectamin 2000-mediated effects seen in this assay.

Although *in vitro* studies using murine BMDM led to the assumption that *S. typhimurium* is detected solely by NLRC4 inflammasomes, *in vivo* studies demonstrated that infection with *Salmonella* activates NLRP3 and NLRC4 inflammasomes [24-26]. Following infection *in vivo*, activation of NLRC4 is mediated *via* SPI-2 T3SS dependent flagellin secretion, whereas activation of NLRP3 seems to be mediated by non-canonical NLRP3 activation *via* caspase-4/5 (caspase-11 in mice) [25,27,28]. Infection of BMDM *in vitro* revealed that NLRP3-mediated caspase-1 activation by flagellin involves an efficient priming step with upregulation of NLRP3 [29]. Using *S. typhimurium* as stimulus for our WBA led to mainly NLRP3-mediated IL-1β secretion for the early time points and strong NLRC4 dependent IL-1β secretion later on. This effect could be explained by *S. typhimurium*-mediated signaling through the TLR4 receptor at early time points, leading to enhanced IL-1β expression and activation of the alternative NLRP3 pathway. Later on, NLRC4 is activated *via* T3SS dependent flagellin secretion. The effect of non-canonical NLRP3 activation mediated by sensing of intracellular Salmonella-LPS was not addressed in our study, but should occur at later time points. Stimulation of whole blood with *S. typhimurium* therefore leads to superposition of NLRP3 and NLRC4 activity. This indicates that proinflammatory signaling through inflammasomes, activated by

pathogens *in vivo,* seems to differ dependent on the tissue/niche infected: Local infections are detected by macrophages and dendritic cells and IL-1β is secreted *via* canonical or non-canonical NLRP3, AIM2 or NLRC4 signaling. When the infection enters the blood stream, human monocytes can respond quickly through proinflammatory signaling *via* the alternative NLRP3-inflammasome pathway until the canonical or non-canonical NLRP3, AIM2 or NLRC4 pathways are activated later on.

Different strategies can be used to normalize leukocyte counts in whole blood assays. Blood consists of different leukocyte subsets expressing functional inflammasomes and secreting IL-1β at variable levels. Therefore, it is unclear as to which leukocyte population the IL-1β signal should be normalized to. Furthermore, differences in leukocyte counts and subsets are part of specific patient groups or diseases and absolute cytokine concentrations in plasma are directly affecting the host's organs. Therefore, we did not normalize the measured IL-1β values to cellular counts.

Although NLRP3 and NLRC4 activation assays were independent from the anticoagulant used, the AIM2 assay was not. While hirudin allows effective stimulation of the AIM2 inflammasome, heparin does not. We did not address the reason for this effect in our WBA. However, according to the study from Coch *et al.* we assume that heparin displaces the nucleic acids from the transfection agent, thereby facilitating DNAse-mediated degradation of poly(dA:dT) and inhibiting effective transfection of nucleic acids [16]. The fact that different concentrations of hirudin do not affect the assay, turns hirudin additionally into the favored anticoagulant for this assay.

Following erythrocyte lysis we were able to generate cDNA from remaining leukocyte pellets of low-volume single whole blood samples. LPS stimulated samples showed enhanced expression of *CASP1* and *IL1B,* whereas *ASC*, *NLRP3* and *AIM2* remained at basal levels. This expression pattern is in line with published data [30,31]. Expression of *CASP1*, *IL1B*, *NLRP3* and *AIM2* is known to be strongly time-dependent following LPS stimulation, inducing gene-expression for early time points and returning back to basal levels later on [30,31]. Infection of whole blood samples with *S. typhimurium* led to reduced expression of *ASC* and *NLRC4*. *S. typhimurium* mediated downregulation of *NLRC4* was reported earlier for murine B-cells and to a lesser extend for murine bone marrow-derived macrophages [32]. Therefore, by analyzing inflammasome-related gene expression in control and stimulated whole blood samples, we were able to prove already published expression patterns to be also true for human whole blood.

5. Conclusions

We have optimized a hirudin-based WBA capable of reporting caspase-1 activity and inflammasome-related gene expression. Using the NLRP3 inflammasome specific inhibitor MCC950 we were able to specifically analyze alternative or canonical NLRP3, AIM2, or NLRC4 inflammasome signaling. The assay needs only low-volume whole blood samples, is independent regarding to the hirudin concentration used and shows relatively robust IL-1β signals even after prolonged storage of the whole blood samples. Hence the assay may be suitable for clinical research applications and even in pediatric patients. Given the fact that inflammasome-related signaling pathways are linked to a multitude of disease-causing pathologies, WBAs capable of deciphering specific inflammasome pathways could help to understand their molecular pathophysiology.

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Legends

Fig. 1. Activation and specific inhibition of the NLRP3 inflammasome is feasible in human whole blood.
(A-D) Hirudin-anticoagulated whole blood samples were primed with LPS for 5.5 h and subsequently stimulated with ATP for additional 30 min. At the end of the stimulation IL-1β concentrations in the supernatant were analyzed as surrogate marker of inflammasome/caspase-1 activation. (A) Different ATP concentrations were used for NLRP3 activation after priming with LPS. (B, C) High extracellular potassium and MCC950 were applied showing their NLRP3 inhibitory effect. If not stated otherwise, inhibitors were were added to whole blood samples together with the priming stimuli. (C) n=5. (D) Samples were pre-treated with MCC950 1 h prior to the start of the stimulation. (E) Following stimulation with LPS/ATP and co-treatment with PBS, potassium chloride or MCC950, cell viability was measured using alamarBlue. 0.1% Triton X-100 treated whole blood samples were used as positive control for cell death. nd: not detectable.

Fig. 2. Specific activation of the AIM2 inflammasome in human whole blood.
(A, C-F) IL-1β concentrations in the supernatant were analyzed as surrogate marker of inflammasome/caspase-1 activation after stimulation of hirudin-anticoagulated whole blood by transfecting poly(dA:dT). If not stated otherwise, transfection was performed for 24 h. (A) Transfection of poly(dA:dT) using Lipofectamine 2000 was preceded by a priming step with either PBS or LPS (n=5). (B) 24 h after transfection of poly(dA:dT) using Lipofectamine 2000, the IFNα concentration in the supernatant was analyzed. (C, D) The influence of high extracellular potassium and MCC950 to IL-1β secretion were characterized during transfection of poly(dA:dT). Inhibitors were added to whole blood samples together with the priming stimuli. (E) Transfection of poly(dA:dT) using Lipofectamine 2000 was performed for either 6 h or 24 h. (F) Whole blood was transfected with poly(dA:dT) using Lipofectamine 2000 or LyoVec for 6 h or 24 h. (G) Following LPS priming, poly(dA:dT) transfection and co-treatment with PBS, potassium chloride or MCC950 the cell viability was measured using alamarBlue. 0.1% Triton X-100 treated whole blood samples were used as positive control for cell death. nd: not detectable.

Fig. 3. Salmonella typhimurium activates the NLRP3 and NLRC4 inflammasome in human whole blood.
(A-C) After stimulation of hirudin-anticoagulated whole blood using *Salmonella typhimurium* (MOI 10), IL-1β concentration in the supernatant was analyzed as surrogate marker of inflammasome/caspase-1 activation. After 0.5 – 4 h of stimulation, 20 μg/ml Gentamicin was added to the cells followed by a second incubation step of 2 h. (A) Characterization of the time dependency following stimulation with *S. tyhpimurium*. (B, C) High extracellular potassium and MCC950 were applied during incubation with *S. typhimurium*. Inhibitors were added to whole blood samples together with the *Salmonella*. (D) Cell viability was measured using alamarBlue following incubation with *S. typhimurium* for 4h and further 2h in the presence of gentamicin under co-treatment with PBS, potassium chloride or MCC950. 0.1% Triton X-100 treated whole blood samples were used as positive control for cell death. nd: not detectable.

Fig. 4. Secreted IL-1β is mature and specifically cleaved by caspase-1
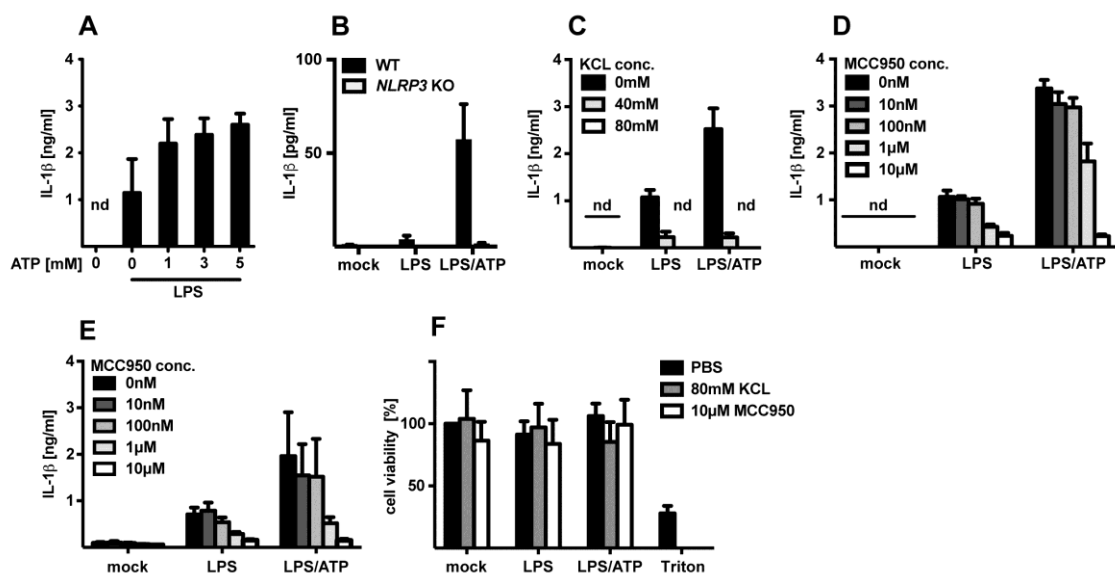
(A) Following stimulation of the NLRP3, AIM2 or NLRC4 inflammasome secreted IL-1β in the supernatant was analyzed using a cytometric bead array or immunoprecipitated and subsequently analyzed by SDS-PAGE and western blotting using an anti-IL-1β antibody. Densitometric analysis of the relative band intensity of mature IL-1β was performed using ImageJ software. Stimulation was performed as follows: NLRP3: 5.5 h 1 µg/ml LPS, 30 min 1 mM ATP. AIM2: 5.5 h 1 µg/ml LPS + 6 h DNA transfection using 2 µg/ml poly(dA:dT) and 4 µg/ml Lipofectamine. NLRC4: *S. typhimurium* (MOI 10) for 4 h followed by 2 h in the presence of gentamicin. When noted, 10 µM MCC950 was added at the beginning of the stimulation. (B) Caspase-1 dependency of the mature IL-1β secretion was analyzed using the caspase-1 inhibitor Ac-YVAD-CHO (100 µM) in the presence or absence of the NLRP3 inhibitor MCC950 (10 µM). Inhibitors were added to whole blood samples together with the priming stimuli. nd: not detectable.

Fig. 5. Influence of assay modifying factors on inflammasome stimulation.
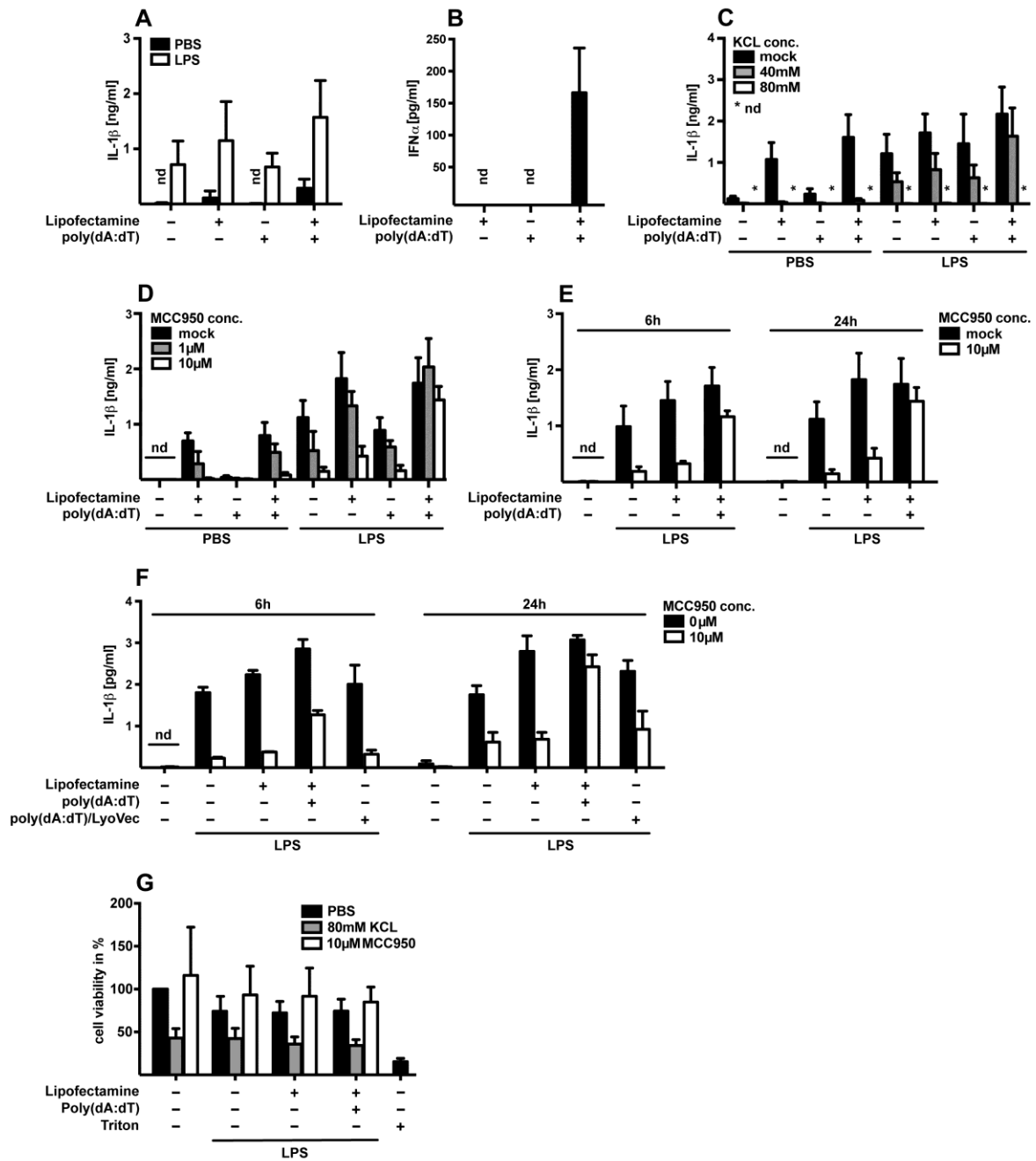
(A-D) NLRP3, AIM2 or NLRC4 specific inflammasome stimulation was applied to anticoagulated whole blood samples as described in Fig. 4A. If not stated otherwise, hirudin was used for anticoagulation. Subsequently, the IL-1β concentration in the supernatant was analyzed as surrogate marker of inflammasome/caspase-1 activation. (A) Hirudin- or heparin-anticoagulated whole blood was used for the inflammasome-specific stimulation. (B) The influence of different hirudin concentrations on inflammasome/caspase-1 activation was analyzed. (C) Whole blood samples were either agitated with 450 rpm or not during specific inflammasome stimulation. (D) Different storage times (room temperature, in the dark) were applied for a given period of time before initiating the inflammasome stimulation. ATU: antithrombin units, nd: not detectable.
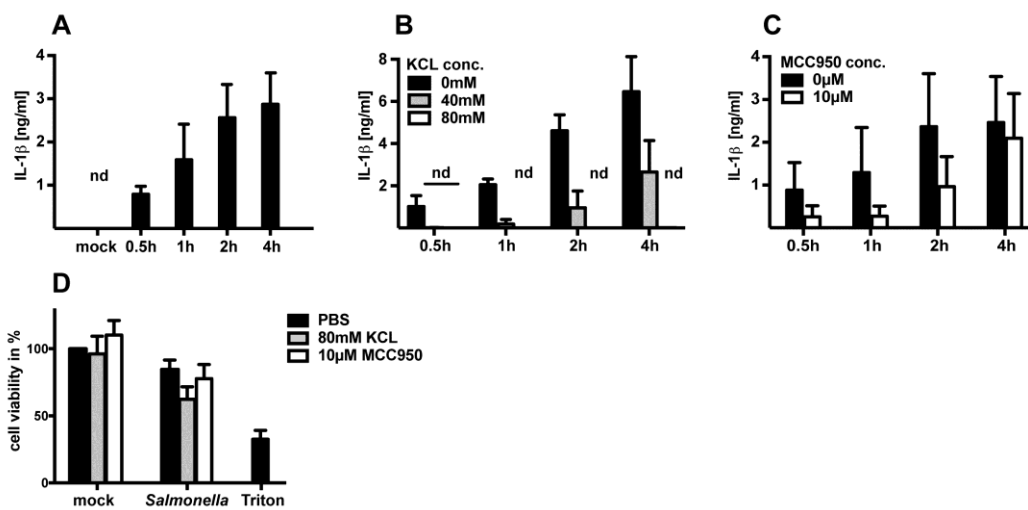
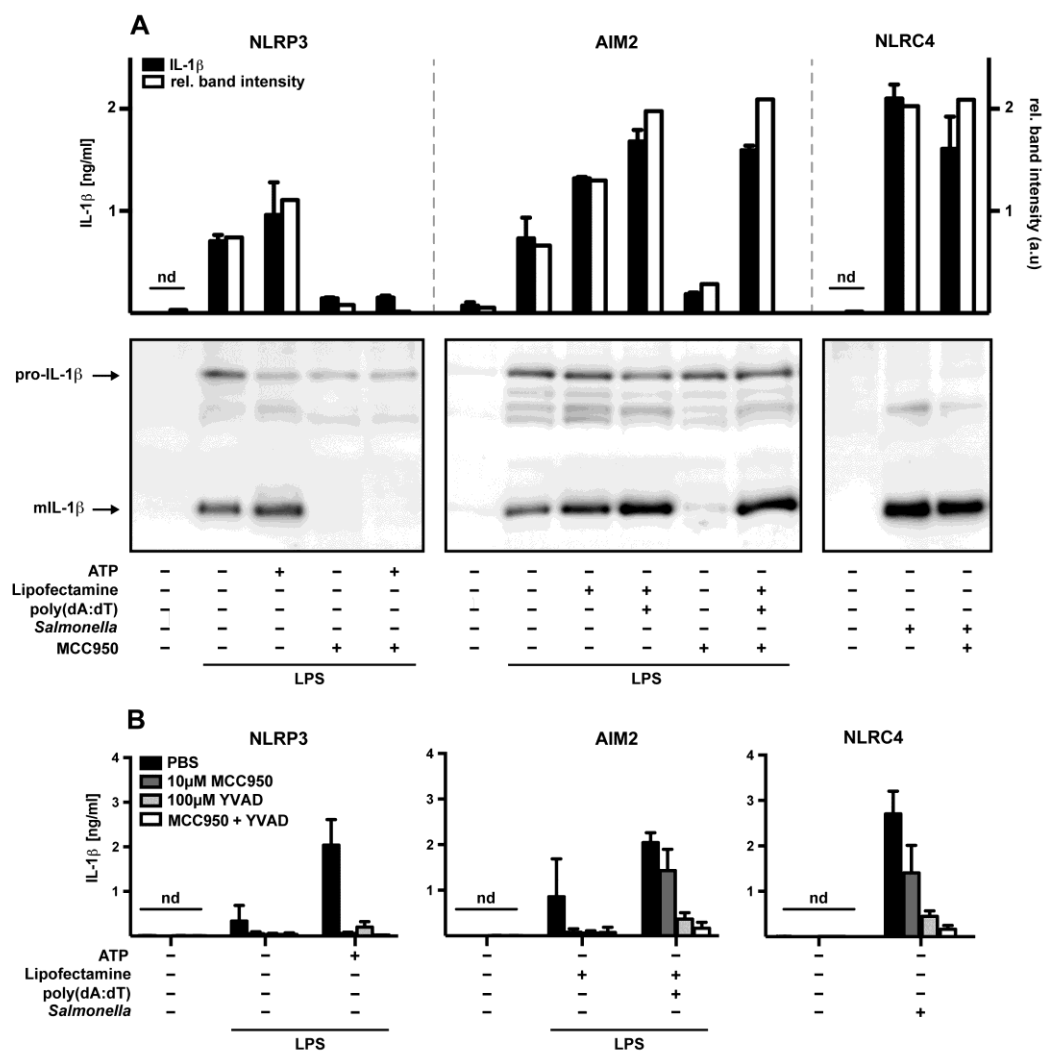*Fig. 6. Quantitative real-time PCR analysis of inflammasome-related genes.*

(A) NLRP3, (B) AIM2 or (C) NLRC4 specific inflammasome stimulation was applied to hirudin-anticoagulated whole blood samples. Stimulation was performed as follows: (A) 5.5 h 1 µg/ml LPS. (B) 5.5 h 1 µg/ml LPS followed by DNA transfection (2 µg/ml poly(dA:dT) and 4 µg/ml Lipofectamine) or PBS for 6 h. (C) *S. typhimurium* (MOI 10) for 4 h followed by 2 h in the presence of gentamicin. The NLRP3 inhibitor MCC950 was not applied. Relative gene expression is displayed as ratio of stimulated samples to mock samples.
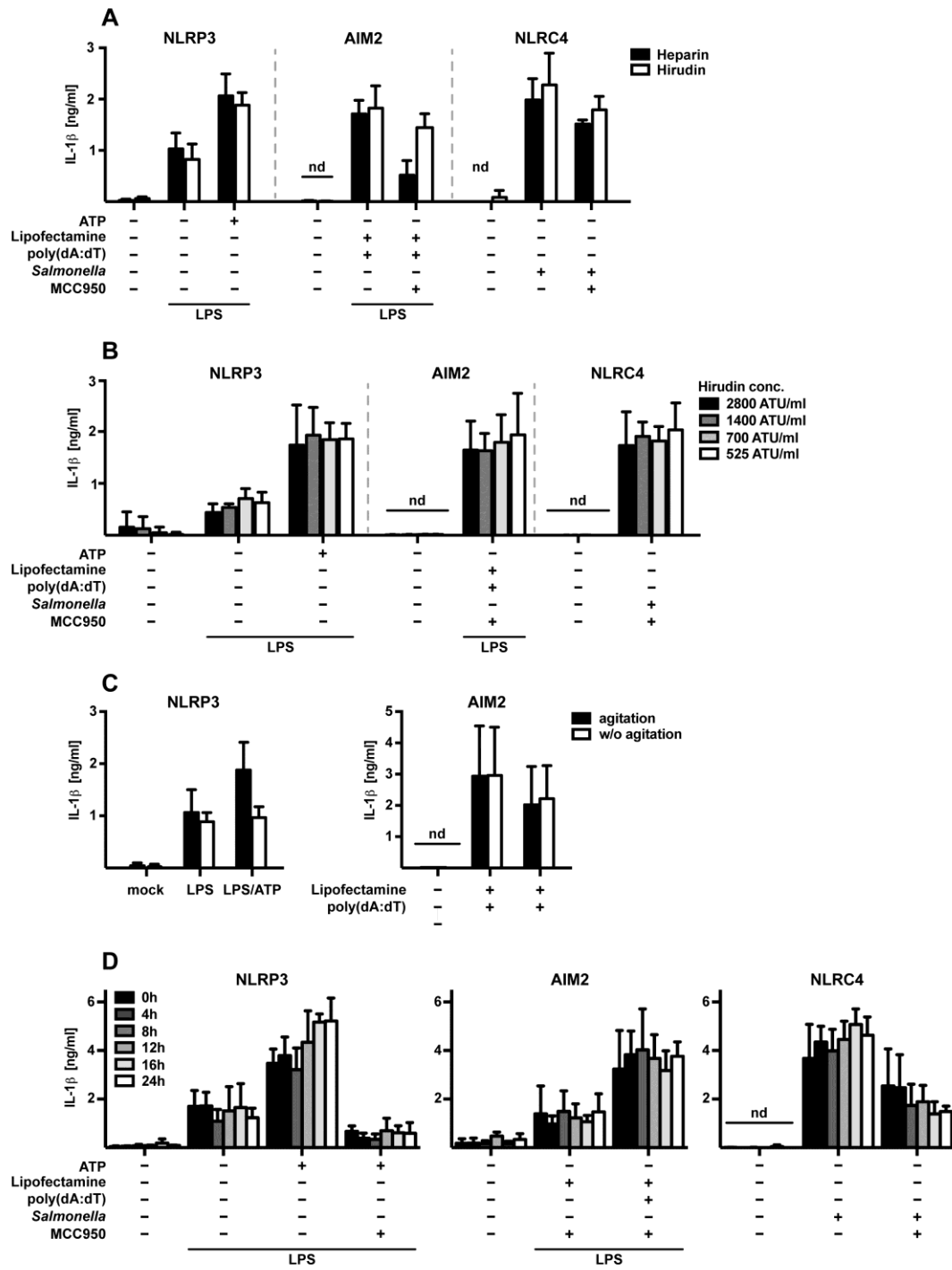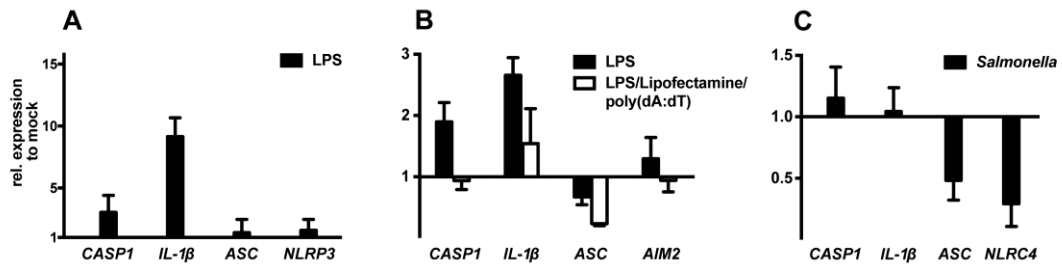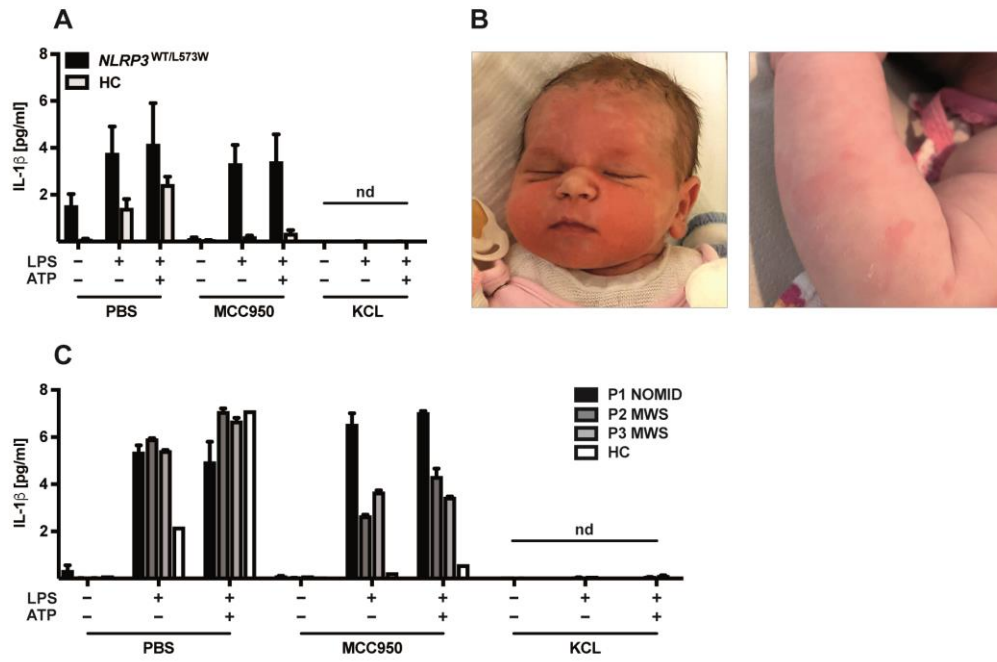
A

NLRP3   AIM2   NLRC4

IL-1β
rel. band intensity

IL-1β [ng/ml]
rel. band intensity (a.u)

nd

pro-IL-1β →

mIL-1β →

ATP	−	−	+	−	+	−	−	−	−	−	−	−	−	−
Lipofectamine	−	−	−	−	−	−	−	+	+	−	+	−	−	−
poly(dA:dT)	−	−	−	−	−	−	−	−	+	−	+	−	−	−
Salmonella	−	−	−	−	−	−	−	−	−	−	−	−	+	+
MCC950	−	−	−	+	+	−	−	−	−	+	+	−	−	+

LPS   LPS

B

NLRP3   AIM2   NLRC4

PBS
10μM MCC950
100μM YVAD
MCC950 + YVAD

IL-1β [ng/ml]

nd

ATP	−	−	+	−	−	−	−
Lipofectamine	−	−	−	−	+	+	−
poly(dA:dT)	−	−	−	−	−	+	−
Salmonella	−	−	−	−	−	−	+

LPS   LPS

A

rel. expression to mock

LPS

CASP1 IL-1β ASC NLRP3

B

LPS
LPS/Lipofectamine/
poly(dA:dT)

CASP1 IL-1β ASC AIM2

C

Salmonella

CASP1 IL-1β ASC NLRC4

A

B

C

Highlights

- Canonical and alternative NLRP3 pathways can be quantified in whole blood.
- Using MCC950 as specific NLRP3 inhibitor, activation of AIM2 and NLRC4 inflammasomes can be quantified in whole blood.
- *S. typhimurium* infection leads to superposition of NLRP3 and NLRC4 inflammasome activities in human whole blood.