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dependent tumor clearance“

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ABSTRACT

Inflammasomes are supermolecular complexes that form in response to a variety of different pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). These molecules are sensed by a variety of inflammasome sensors, for example NOD-, LRR- and pyrin domain-containing 3 (NLRP3) senses mitochondrial dysfunction and *Bacillus anthracis* lethal toxin is sensed by NOD-, LRR- and pyrin domain-containing 1 (NLRP1)¹. Following nucleation and oligomerization of the sensor an adaptor protein Apoptosis-associated speck-like protein containing a CARD (ASC) is recruited. A zymogen Caspase-1 is activated and cleaves Gasdermin-D (GSDMD), pro-interleukin-18 (pro-IL-18) and pro-interleukin-1 β (pro-IL-1 β). Plasma membrane is permeabilized by GSDMD pores and secretion of the active form of proinflammatory cytokines interleukin-18 (IL-18) and interleukin-1 β (IL-1 β). This leads to an inflammatory form of cell death called pyroptosis. However, there are still many details concerning inflammasome activation that are unknown. For example, we don't know exactly which molecules inflammasome sensors react to and there likely exist other sensors that haven't been discovered yet. The potential to form inflammasomes still has to be demonstrated for several inflammasome sensors, namely for interferon-inducible protein 16 (IFI-16) and retinoic acid-inducible gene I (RIG-I). Additionally, it still hasn't been elucidated which factor leads to oligomerisation of NLRP3 inflammasome. Therefore, research in this field is necessary for the better understanding of inflammasome complex formation. Discovering novel compounds that would modulate the inflammasome would also be beneficial to many patients, because inflammasomes are implicated in a variety of different diseases such as autoinflammatory diseases¹ for instance Cryopyrin-associated periodic syndromes (CAPS) and even cancer²⁻⁴.

The aim of our study was to design a system to discover novel inflammasome modulators or even novel inflammasome sensors. We developed a flow cytometry based technique for screening non-adherent cell populations and a microscopy based technique for screening adherent cell populations. The screens are based on sensing ASC speck formation with the help of fluorescent protein gene tags or using a fluorescently labeled ASC antibody. We used ASC-Citrine reporter mice⁵ and created a human monocytic leukemia THP-1 ASC-GFP reporter cell line. Our tools can be used as either basic biology discovery tools to identify novel inflammasome sensors and modulators or as drug discovery tools.

The human gut microbiota produces thousands of unique small molecules that can potentially affect nearly all aspects of human physiology, from immune modulation to dysbiosis^{6,7}. Small molecules produced by gut microbiota are very diverse, the diversity of chemical classes they produce rivals that of microorganisms from any other ecological niche⁶. It has been previously described that microbiota-associated metabolites help shape the host-microbiome interface by modulating NLRP6 inflammasome signaling, epithelial IL-18 secretion, and the generation of downstream antimicrobial peptides⁸. Additionally, bile acid analogues, modified by gut microbiota,

are activators of pyrin inflammasome⁹. Thus, we suspected that the microbiota metabolome could be a rich source of potential inflammasome modulators and that the gut microbiome plays a role in inflammasome regulation. For screening, we used a collection of 144 supernatants of human gut microbes monocultured in appropriate media⁷. These supernatants contain a large variety of complex and structurally diverse molecules, that have previously been shown to activate G-protein coupled receptors (GPCRs). Uncovering the function of any of these microbial products would elucidate host-microbiota connections and provide us with an understanding of their biological relevance.

We discovered a few promising bacterial candidates that are able to activate the NLRP3 inflammasome. Our future direction is to elucidate which exact molecule from the supernatant activates the inflammasome and if this molecule also induces IL-18 and IL-1 β secretion.

INTRODUCTION

Inflammasome

The inflammasome is a component of the innate immune system that allows the host to respond to microbial infection and tissue damage¹⁰. Inflammasomes are key signaling platforms that detect a variety of different PAMPs and DAMPs. They activate the pro-inflammatory cytokines IL-1 β and IL-18¹¹. Inflammasomes are multi-protein complexes that consist of a sensor, an adaptor protein ASC (encoded by PYCARD) and an effector protein Caspase-1 (*Figure 1*). Inflammasome sensors contain death domain motifs, either a pyrin domain or CARD domain, that are protein-protein interaction domains^{12,13}. Upon inflammasome stimulation, they bind to CARD or pyrin domains of other inflammasome components. This is followed by oligomerization and ASC recruitment which causes ASC filament formation. Multiple ASC filaments fuse into a single macromolecular focus, known as an ASC speck¹⁴. Nucleated ASC recruits a zymogen pro-Caspase-1 thus enabling proximity induced Caspase-1 self-cleavage and activation¹⁵. Active Caspase-1 then proteolytically activates the pro-inflammatory cytokines pro-IL-1 β ¹⁶, pro-IL-18 and a pore-forming protein called Gasdermin-D (GSDMD) that normally exists in an autoinhibited state¹⁷. GSDMD has a N-terminal and a C-terminal domain that are linked by a long loop that caspase-1 cleaves at an aspartate site¹⁸. This causes the formation of GSDMD pores in cell membrane that disrupt the osmotic potential, causing cell swelling and consequent lysis¹⁸. Active forms of IL-1 β and IL-18 are then released through GSDMD pores and the cell goes through an inflammatory form of cell death called pyroptosis that leads to the recruitment of other immune cells, such as neutrophils.

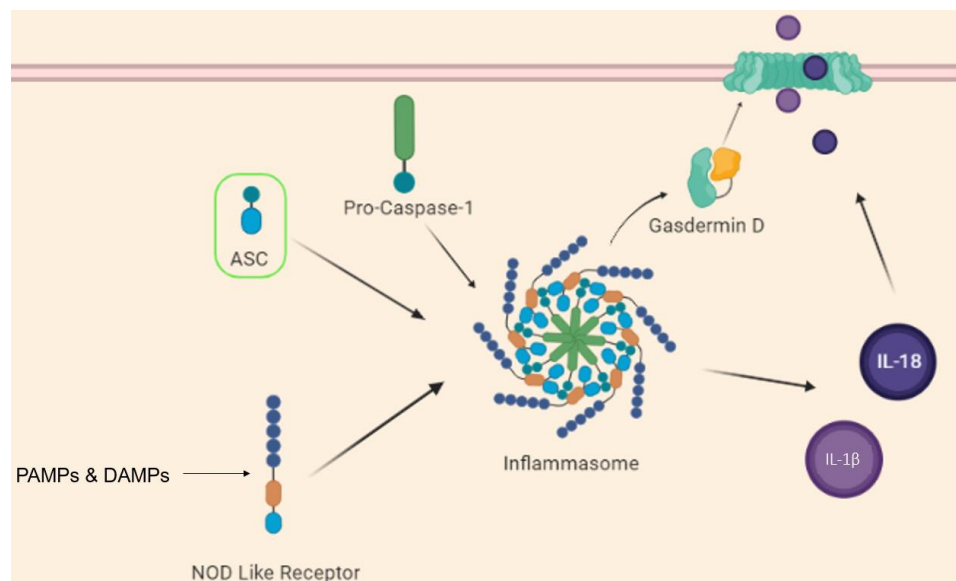


Figure 1 Inflammasome activation pathway. NOD like receptors respond to different PAMPs and DAMPs and form a supermolecular complex called the inflammasome, together with an adaptor protein called ASC and pro-caspase-1. The active form of Caspase-1 cleaves pro-IL 1 β and pro-IL 18 that are released in their active form through GSDMD pores.

Several inflammasome sensors that can trigger the formation of inflammasomes have been described (*Figure 2*). Most are a part of the NOD-like receptor (NLR) family, namely NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4 (NOD-, LRR- and CARD-containing 4). These sensors, with the exception of NLRP1, have three domains; they contain an amino-terminal death-fold domain (NLRPs contain a pyrin domain, whereas NLRC4 contains a CARD domain), a central NACHT nucleotide-binding domain and carboxy-terminal leucine-rich repeats (LRRs)¹⁹. The death-fold domains interact with ASC and Caspase-1, the NACHT domain has ATPase activity and has a role in the oligomerization of the proteins and the LRRs have regulatory functions that might be involved in ligand interaction¹¹.

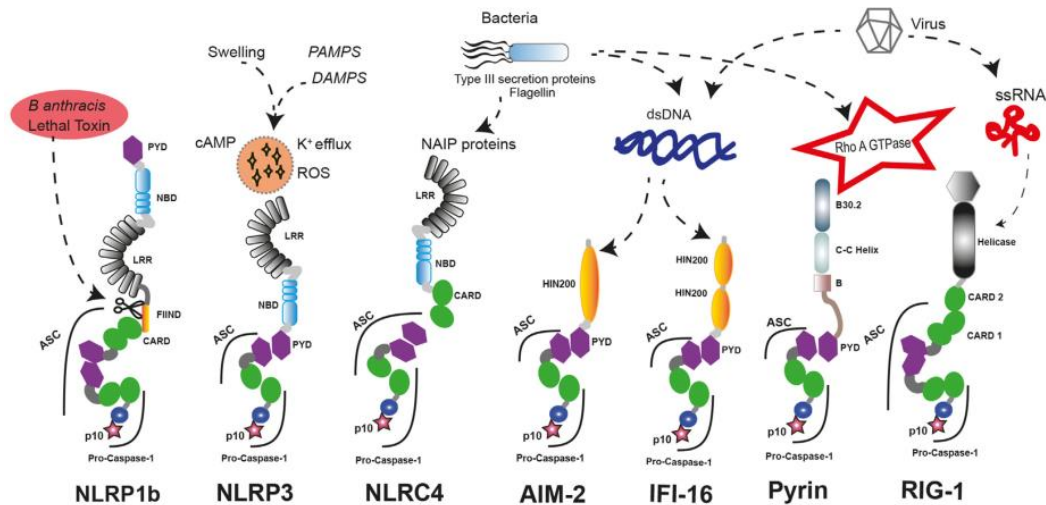


Figure 2 Commonly studied inflammasome sensors and activators. NLRP1b responds to lethal toxin of *Bacillus anthracis*. NLRP3 is a general sensor of cellular damage that responds to disturbance in ion signalling and mitochondrial integrity. NLRC4 recognizes bacterial proteins. AIM2 and IFI-16 sense dsDNA; RIG-1 detects ssRNA. Pyrin inflammasome is induced by bacterial toxins that modify RhoA GTPase¹.

Other proteins have also been described to be able to form functional inflammasomes such as Pyrin, absent in melanoma-2 (AIM2), interferon-inducible protein 16 (IFI-16) and retinoic acid-inducible gene I (RIG-I)¹. Pyrin contains a pyrin domain that is responsible for interaction with ASC and later activation of Caspase-1. The pyrin-inflammasome assembly is likely triggered by bacterial toxins that modify Rho family proteins²⁰. AIM2 and IFI-16 both recognize cytosolic nucleic acids by the HIN domains and have a pyrin domain to recruit ASC¹¹. RIG-I protein is a sensor for RNA and is thought to assemble an inflammasome and recruit ASC with its CARD domains²¹. However, it should be noted that for some of these inflammasome sensors (including RIG-I and IFI16), additional studies are needed to demonstrate their potential to form inflammasomes.

One of the most commonly studied inflammasome sensors is NLRP3. It is likely a global sensor for pathogens and cellular damage, as it responds to a wide variety of triggers that cause disruption of ion signaling or loss of mitochondrial integrity²². Mitochondrial dynamics are involved in the regulation of the NLRP3 inflammasome, for example it

has been shown that hexokinase dissociation from mitochondrial outer membrane is sufficient to initiate an NLRP3 inflammasome activating cascade²³. NLRP3 activation is a two-step process — first step is priming, and the second is activation. Priming is induced through the recognition of various PRR ligands and cytokines, an example of the first signal is LPS²⁴, that activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). NF- κ B induces the transcriptional expression of NLRP3, pro-IL-1 β and pro-IL-18 as well as post-translational modifications of NLRP3²⁵. Full activation and inflammasome assembly follows the recognition of a second signal that causes cellular stress. An example of a second signal molecule is Nigericin, a carboxylic ionophore that disrupts ion signaling²⁶. Nigericin causes cytosolic depletion of K⁺ that mediates NLRP3 inflammasome activation, similarly to ATP - another classical NLRP3 activator²⁷.

Mutations in different inflammasomes can lead to autoinflammatory diseases. Some examples are NLRP3 associated cryopyrin-associated periodic syndrome (CAPS) and familial Mediterranean fever (FMF) or Pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND). CAPS is a disease resulting from a gain-of-function mutation of the NLRP3 gene leading to an overproduction of IL-1 β and subsequent severe cutaneous, neurological, ophthalmologic and rheumatologic symptoms of the disease²⁸. FMF is the most frequent hereditary inflammatory disease, symptoms of which include periodic attacks of fever and serositis, inflammation of the serous tissues of the body²⁹. It is caused by mutations in the *MEFV* gene, which encodes pyrin²⁹. PAAND is caused by gain of function mutations in the *MEFV* gene that causes a disease distinct from FMF including the formation of subcorneal neutrophilic aggregates³⁰.

Alongside this, inflammasomes are also involved in the pathophysiology of other illnesses, including degenerative processes, fibrosis, or metabolic diseases. NLRP3 has also been associated with cancer^{2,3}, it has been shown that aberrant activation of NLRs occurs in various cancers³¹, orchestrating the tissue microenvironment and potentiating neoplastic risk³². However, our group has shown that mice with a gain of function mutation of NLRP3 develop smaller tumors after injection (Brewer et al, unpublished). Additionally, it has been shown that that transmembrane protein 176B (TMEM176B) inhibits NLRP3 inflammasome activation and that the inhibition of TMEM176B enhances tumor infiltration by CD8+ T cells and improves the efficacy of immune checkpoint blockers³³. This data together suggests that different patients could possibly benefit from both activators and inhibitors of the inflammasome and that drug development targeting inflammasomes is a promising field expansion. Several NLRP3 inflammasome activation inhibitors were recently patented from naturally derived and synthetic agents mainly by academic researchers³¹. One such modulator is MCC950, a potent and specific small-molecule inhibitor of NLRP3, that directly interacts with the Walker B motif within the NLRP3 NACHT domain, thereby blocking ATP hydrolysis and inhibiting NLRP3 activation and inflammasome formation³⁴. It has already shown therapeutic potential in mouse models for treatment of dermal and pulmonary inflammation³⁵ and

Parkinson's disease³⁶. Hence, an important focus of future research is to find effective inflammasome modulators and determine their therapeutic potential.

Detection of inflammasome activation

As previously described ASC is an adaptor protein involved in inflammasome formation. Upon inflammasome activation it oligomerizes and forms a large protein complex called an “ASC speck”. These specks reach a size of around 1 μm and there is usually only one speck per cell. Therefore, ASC speck formation can be used as a read out of inflammasome activation. Therefore, fluorescently tagged ASC has been used as a reporter. For example, Tzeng et al⁵, have developed an ASC-citrine transgenic mouse reporting inflammasome activation, where ASC-citrine retains the function. With such tags we are able to visualize ASC in the cell; ASC is dispersed throughout the cell during homeostatic conditions and condensed in to a speck during stimulated conditions (Figure 3). The Citrine tag, as a detection method, reports inflammasome formation in individual cells using fluorescence microscopy. Inflammasome activation can also be detected with an imaging flow cytometer such as AMNIS, where it is possible to calculate whether ASC has oligomerized or not with the help of a brightfield over citrine ratio (Figure 4)⁵.

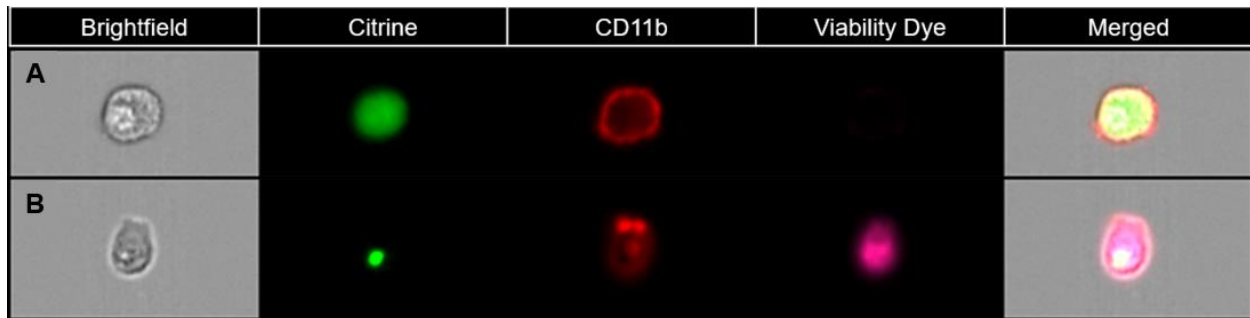


Figure 3 Analysis of ASC Speck Formation in mouse BMDCs by Image-Based Flow Cytometry. Primary BMDC culture was made from ASC Citrine mice. Cells were primed with 100 ng/ml LPS for 2 hr, followed by stimulation with nigericin for 1 hr for positive control. BMDCs were harvested after stimulation for image-based flow cytometry analysis and stained for the expression of CD11b and viability dye. **A** Unstimulated cell in homeostatic condition, primed with LPS only **B** Cell stimulated with LPS and Nigericin, ASC is oligomerized

ASC oligomerization can also be observed with a regular flow cytometer by detecting the change in ASC distribution within the cell³⁷. The transit of ASC into the speck can be identified by a decreased width of the pulse of emitted fluorescence.

Microbial metabolites

Gut microbiota is the collection of microbes in the host gastrointestinal tract (GIT) from all three life domains – bacteria, archaea and eukaryotes^{38,39}. These microbial communities consist of hundreds of abundant bacterial species which reflects co-evolution and selection for specific microbes whose emergent collective behavior is beneficial to the host³⁹. Gut commensal bacteria have a role in nutrient and drug metabolism, they compete with pathogenic microorganisms and thus help prevent their

colonization of the host and help maintain intestinal barrier function. Additionally, gut microbes produce thousands of unique small molecules that can potentially affect nearly all aspects of host physiology, from immune system modulation to dysbiosis^{6,7}. An important source of small molecules is host dietary input that is converted by gut microbiota into a variety of metabolic end products with different biological activities. For example short chain fatty acids (SCFAs) are produced by host microbiota from dietary input and they can directly affect host health through a range of mechanisms such as maintaining glucose homeostasis, immunomodulation and even appetite regulation⁴⁰. Small molecules produced by the microbiota are very diverse as they cover the entire spectrum of chemical classes discovered so far produced by any bacterial species on earth, including mediators of microbe-host and microbe-microbe interactions⁶. They are very abundant as well, as they are present at high micromolar, some even at tens of millimolar in concentration⁴¹. For example SCFAs can be found in the concentration range between 70 and 140 mM, depending on the diet, in the proximal colon⁴². Exploring their function can thus provide understanding of the effects of the microbiota on human health and disease. *Morganella morgani* is able to shape colonic motility by converting dietary histidine into histamine and is able to convert L-Phenylalanine (L-Phe) into phenethylamine, a potent psychoactive molecule⁷. *Bacteroides thetaiotamicron* is a prolific producer of the essential amino acid L-Phe, that is an agonist for G protein-coupled receptors (GPCRs) named GPR56 and GPR97⁷. Therefore showing that metabolites produced by the human microbiota can also function as agonists for a wide range of GPCRs, making metabolome screening a useful tool for identifying metabolic exchanges between gut microbiota with effects on host physiology⁷. Uncovering the function of any of microbiome produced molecules would elucidate host-microbiota connections and provide us with an understanding of their biological relevance. It has been previously described that Microbiota-associated metabolites shape the host-microbiome interface by modulating NLRP6 inflammasome signaling, epithelial IL-18 secretion, and the generation of downstream antimicrobial peptides⁸. Additionally, bile acid analogues, modified by gut microbiota, are activators of pyrin inflammasome pathway. It has been shown that bile acid analogues named BAA485 and BAA473 can activate pyrin in monocytes and intestinal epithelial cells, that are both relevant to maintaining intestinal immune homeostasis⁹. Thus, microbiota metabolome could be a rich source of potential inflammasome modulators as the gut microbiome seems to play a role in inflammasome regulation.

MATERIALS AND METHODS

Cell Culture

Cryopreserved human monocytic leukemia cells THP1 (ATCC, Virginia, USA) were thawed in a water bath at 37°C and freezing media was removed by centrifugation. After the cells were thawed they were resuspended in RPMI-1640 media (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, New York, USA), 100 u/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific, Massachusetts, USA) and 2 mM glutamine (Thermo Fisher Scientific, Massachusetts, USA). They were plated in T-75 flasks (Eppendorf, Hamburg, Germany) and kept in an incubator at 37°C and 5% CO₂ and split as necessary, approximately once a week. They were used for the generation of an ASC GFP reporter cell line.

Cryopreserved FreeStyle™ 293-F Cells (Thermo Fisher Scientific, Massachusetts, USA) were thawed as previously described and resuspended in Dulbecco's Modified Eagle's (DMEM) media (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% FBS (Corning, NY, USA), 100 u/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific, Massachusetts, USA) and 2 mM glutamine (Thermo Fisher Scientific, Massachusetts, USA). They were plated and kept in an incubator at 37°C and 5% CO₂. Cells were passaged every 3 days and used for producing a lentivirus.

Generating primary mouse bone marrow derived macrophages and stimulation

In order to examine inflammasome activity in mouse bone marrow derived macrophages (mBMDMs), mice were sacrificed and their limbs removed. The cells were prepared by flushing femur and tibia with a media filled 25 gauge syringe. Bone marrow derived cells were plated on 15 cm plates (Corning, New York, USA) and cultured in DMEM (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% FBS (Corning, New York, USA), 100 u/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific, Massachusetts, USA), 2 mM glutamine (Thermo Fisher Scientific, Massachusetts, USA) and 25 ng/mL macrophage colony-stimulating factor (M-CSF) (Biolegend, California, USA). M-CSF is a growth factor that influences progenitor cells to commit to the macrophage lineage. Fresh M-CSF in new supplemented media was added again after 7 days of culture, because it gets inactivated with time. Cells were kept in an incubator at 37°C and 5% CO₂ for two weeks. On day 14 mouse BMDMs were harvested by scraping the plates and re-plated at 2x10⁴ cells/well (Z = 0.65) in 384-well glass bottom plates (Corning, New York, USA) for image-based analysis.

To examine inflammasome activity in blood cells and neutrophils, blood was collected by cardiac puncture from ASC Citrine Casp1^{-/-}, Casp1^{-/-} and WT mice. Blood samples were treated with heparin to prevent blood clotting and with an Ammonium-Chloride-Potassium (ACK) lysis buffer to lyse red blood cells prior to stimulating. Neutrophils were isolated using the MojoSort™ Mouse Neutrophil Isolation Kit (BioLegend, California, USA). After isolation, neutrophils were cultured in RPMI-1640 media (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% Fetal Bovine Serum

(FBS) (Corning, New York, USA), 100 u/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific, Massachusetts, USA) and 2 mM glutamine (Thermo Fisher Scientific, Massachusetts, USA) placed in an incubator at 37°C and 5% CO₂ and immediately stimulated.

For stimulation, BMDMs, neutrophils and mouse whole blood were primed with ultrapure LPS (Enzo Life Sciences, New York, USA) 100 ng/mL for 2 hours, followed by stimulation with 10 mM nigericin (Sigma Aldrich, Missouri, USA) for 1 hour as a positive control, 3 hours LPS as a negative control and only BMDMs 2 hours LPS followed by 2 or 24 hours with 129 microbial supernatant collection⁷. They were additionally also stimulated with a NLRP3 inhibitor 0,5 µM MCC950 (Invivogen, California, USA) from the start simultaneously with other treatments. To assess the necessary concentration of a Caspase-1 inhibitor, we also treated them with VX765 (Invivogen, California, USA) at different concentrations. Speck formation was assessed by confocal microscopy or flow cytometry after stimulation.

Generating THP1 reporter cell line and stimulation

Plasmids pMD2.G VSVG (Addgene, Massachusetts, USA), psPAX2 Gag Pol (Addgene, Massachusetts, USA) and ASC GFP were separately introduced to Stable bacteria 2064964 (Invitrogen, California, USA) using heat shock transformation and cultured overnight on Ampicillin agar plates. The next day the plasmids were isolated using the EndoFree Plasmid Maxi Prep Kit (Qiagen, Hilden, Germany). Afterwards, plasmids were digested with ClaI enzyme and CutSmart buffer (New England Biolabs, Massachusetts, USA) for 3 hours at 37°C and 20 minutes at 65°C to create linear vectors. Ethanol precipitation was used to remove the restriction enzyme and all three linear plasmids were separately dissolved in purified water. Subsequently, lentivirus containing these vectors was produced by transfecting FreeStyle™ 293-F Cells (Thermo Fisher Scientific, Massachusetts, USA) using the LipoD293 transfection reagent (SigmaGen, Maryland, USA). On day 3 supernatants were collected and ran through a 0.22 µm filter, virus producing cells were treated with 10% bleach for 15 minutes and discarded in special waste. To generate the ASC GFP reporter cell line THP1 CASP1^{-/-} cells (Artner et al. unpublished) were spinfected at 2500 rpm for 1,5 hours at 37°C with viral supernatant at a 1:100 dilution and 10 µL/mL Polybrene (Sigma Aldrich, Missouri, USA). Cells were then cultured in RPMI-1640 media (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, New York, USA), 100 u/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific, Massachusetts, USA) and 2 mM glutamine (Thermo Fisher Scientific, Massachusetts, USA) and kept in the incubator at 37°C and 5% CO₂ for 48 hours. Afterwards, we obtained single clones by doing serial dilutions of the sorted cells expressing ASC GFP. For stimulation THP1 Casp1^{-/-} ASC GFP cells were primed with 100 ng/mL ultrapure LPS (Enzo Life Sciences, New York, USA) for 2 hours, followed by stimulation with 10 mM nigericin (Sigma Aldrich, Missouri, USA) for 1 hour as a positive control, 3 hours LPS as a negative control.

Mice

Fluorescent reporter ASC Citrine mice were acquired from Golenbock laboratory at University of Massachusetts Medical School⁵. ASC Citrine Casp1^{-/-} mice were previously generated in our laboratory by breeding Casp1^{-/-} mice with ASC Citrine mice. All mice were bred and maintained in specific pathogen-free conditions; mouse protocols were approved by the Institutional Animal Care and Use Committee at the Yale University.

Quantitative polymerase chain reaction (qPCR)

THP1 cells cultured in RPMI-1640 media (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, New York, USA), 100 u/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific, Massachusetts, USA) and 2 mM glutamine (Thermo Fisher Scientific, Massachusetts, USA) in an incubator at 37°C and 5% CO₂. Cells were split into two groups consisting of triplicates, one group was left untreated and the other was treated with 100 ng/mL ultrapure LPS (Enzo Life Sciences, New York, USA) for 2 hours. Subsequently, mRNA was extracted using a protocol combining Trizol extraction and RNeasy Mini Kit (Qiagen, Hilden, Germany) protocol. Cells were harvested and homogenized using 1 ml per 0.1g of mRNA of Trizol (Sigma Aldrich, Missouri, USA). The homogenates were incubated at room temperature for 5 minutes and then centrifuged for 10 minutes at 12000 xg at 4°C to eliminate debris and polysaccharides. Afterwards, 0.2 ml Chloroform (Sigma Aldrich, Missouri, USA) per ml Trizol was added to the homogenates and shaken vigorously for 20 seconds, then the samples were incubated at room temperature for 3 minutes. The samples were then centrifuged at 10000 xg for 18 minutes at 4°C. The aqueous phase was carefully removed by aspiration and transferred to new sterile RNase-free tubes. Slowly an equal volume of 100% RNA-free Ethanol was added. The samples were then loaded into RNeasy columns provided in the RNeasy kit and the instructions from the kit were followed until elution. The mRNA was stored at – 80°C until use. The concentration of the eluted mRNA was measured by a spectrophotometer, 1 µg of mRNA was used for cDNA synthesis following the instructions of the SuperScript™ III First-Strand Synthesis System Kit (Invitrogen, California, USA). Afterwards, qPCR master mixes were created with the cDNA, SYBR Green (Invitrogen, California, USA) and 10 pmol/µL of the respective primers. Primers were ordered from Merck (Merck, Darmstadt, Germany) for genes involved in the inflammasome complex, specifically for *AIM2*, *CARD8*, *CASP4*, *CASP5*, *GSDMD*, *MEFV*, *NLRC4*, *NLRP1*, *NLRP3*, *NLRP6*, *NLRP9*, *NLRP12*, *PYCARD* and the housekeeping gene *UBC*. The experiment was repeated twice for higher statistical significance and gene expression was calculated with the formula 2^{-(dCt)}.

Image Acquisition

Mouse BMDMs generated from ASC Citrine Casp1^{-/-} mice, were stimulated with ultrapure LPS (Enzo Life Sciences, New York, USA) 100 ng/mL for 2 hours, followed by stimulation with 10 mM nigericin (Sigma Aldrich, Missouri, USA) for the positive control

and without it for the negative control. Other samples were treated with the Noah Palm laboratory essential collection of microbial supernatants⁷ in duplicates for either 2 hours or 24 hours. Subsequently media was removed from all samples and they were stained with 5 µg/mL wheat germ agglutinin (WGA) Alexa Fluor 647 conjugate (Invitrogen, California, USA) that stains cell membrane and 10 µg/mL Hoechst 33342 (Invitrogen, California, USA) that stains cell nuclei. After the dye was washed away the cells were imaged in the Live Cell Imaging Solution (Invitrogen, California, USA) with a high throughput microscope The IN Cell Analyzer 2200 (GE Healthcare, Illinois, USA). Three fields of view per well and three images per field of view were acquired. One image with a GFP channel (ASC-Citrine), another with a Cy5 channel (WGA) and another with a DAPI channel (Hoechst). The plates were discarded after imaging.

Image analysis and statistics

Image analysis was done with Cell Profiler 3.1.8 software. A pipeline was created to identify cells based on overlaying detected nuclei based on Hoechst staining with detected cell membranes based on WGA staining. Subsequently, ASC specks were identified based on Citrine signal and overlaid with previously defined cells to get a percentage of cells were ASC was oligomerized in each field of view. JMP software was used to average the percentages from per field of view to per well. To merge images ImageJ software was used. Statistics was calculated with GraphPad Prism software. Multiple group comparisons were analyzed by one-way ANOVA test with Dunett's test and adjusted p-value was used to identify statistically significant differences between groups.

Flow Cytometry

Blood cells, mouse BMDMs and mouse neutrophils were treated with inflammasome stimulus, namely ultrapure LPS (Enzo Life Sciences, New York, USA) 100 ng/mL for 2 hours, followed by stimulation with 10 mM nigericin (Sigma Aldrich, Missouri, USA) for 1 hour for the positive control and without it for the negative control. Blood cells were stained with CD8α-Pac Blue, CD4-PerCP, Ly6C-BV605, Ly6G-APC, TCRβ-BV711, CD11b-AF700, NK1.1-PE and Fixable Viability Dye (FVD) eFluor 506 (Invitrogen, California, USA). Neutrophils were stained with CD45-Pac Blue, CD11b-PerCP CY5.5, Ly6G-APC and NK1.1, CD8α, TCRβ, NKp46 all in PE and FVD eFluor 506. BMDMs were stained with CD11b-APC and FVD eFluor 506. WT THP1 cells were stained with anti-ASC (ATTO 647N) in APC and FVD eFluor 506, THP1 CASP1-/- ASC GFP were stained with FVD eFluor 506. All cells were fixed with 4% PFA for 10 minutes in the dark. Cells were then resuspended in FACS buffer and ran either on LSRII (BD Biosciences, California, USA) or CytoFLEX (Beckman Coulter, California, USA) flow cytometers. The results were analyzed with the FlowJo software.

Imaging Flow Cytometry

Mouse BMDMs were treated with inflammasome stimulus, ultrapure LPS (Enzo Life Sciences, New York, USA) 100 ng/mL for 2 hours, followed by stimulation with 10 mM nigericin (Sigma Aldrich, Missouri, USA) for 1 hour. Then they were stained with CD11b

in APC and FVD eFluor 780 and fixed with 4% PFA for 10 minutes in the dark. Cells were resuspended in FACS buffer for running on Amnis Imagestream-X MarkII Imaging Flow Cytometer (Luminex Corporation, Texas, USA). Five out of twelve channels were used, Channels 1 and 9 were used for brightfield detection, Channel 2 for the Citrine signal, Channel 11 for CD11b-APC signal and Channel 12 for the FVD eFluor 780 signal; 488 nm and 642 nm excitation lasers were used. Single color compensation controls and fully stained samples were acquired and analyzed. Integrated software INSPIRE (MilliporeSigma, Massachusetts, USA) was used for data collection and the results were analyzed with the IDEAS software.

RESULTS AND DISCUSSION

Method to detect NLRP3 inflammasome modulation in non-adherent cell populations

To detect inflammasome modulation in immune cells we developed a flow cytometry based assay. We expected a reduction in the width of emitted fluorescence (*Figure 4D, 4E*) based on Sester et al publication describing a flow cytometry based method detecting the change in ASC distribution within the cell³⁷. Therefore, the assay to detect inflammasome activation is dependent upon the rapid oligomerization of ASC protein in the cell following inflammasome activation. We first used image-based flow cytometry AMNIS to quantitatively characterize speck positive cells by calculating brightfield over citrine signal ratio (*Figure 4A*) in ASC Citrine Casp1^{-/-} mouse BMDMs. With the help of this sensitive method we optimized our treatment protocol. We found that the optimal time of stimulation is 2 hours priming with LPS followed with 1 hour of nigericin for the positive control and 3 hours LPS for the negative control (*Figure 4B, 4C*).

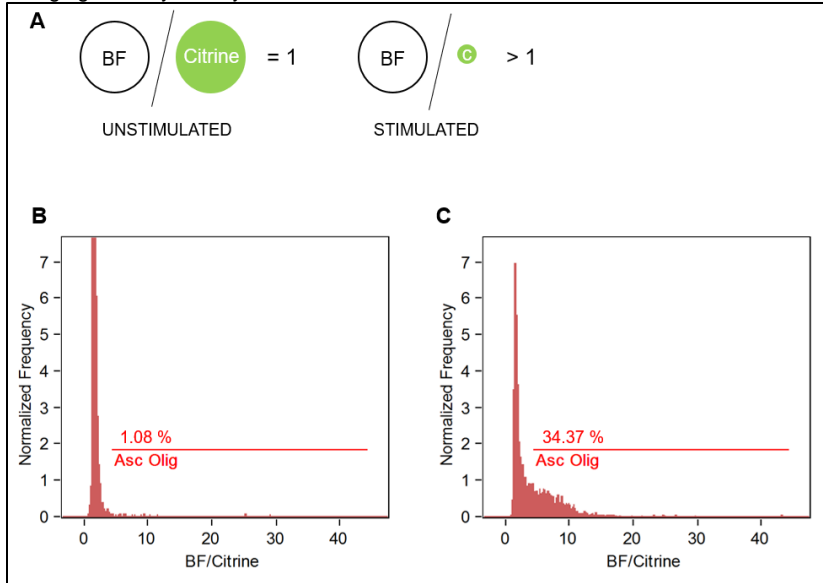
We then generated a reporter THP1 CASP1^{-/-} ASC GFP cell line. THP1 cells are a human monocytic leukemia cell line which we chose, because we previously observed that we can detect a high percentage of ASC Oligomerization in monocytes (*Figure 6C, 6H*) and because finding novel inflammasome modulators in human cells is potentially clinically relevant. LPS-primed THP1 CASP1^{-/-} ASC GFP cells were treated with or without nigericin for 1 hour, stained with a Viability dye (FVD) and fixed with 4% PFA. Analyzed on cytometers capable of evaluating pulse width in fluorescence channels namely LSRII BD Biosciences and CytoFLEX Beckman Coulter, we observed a distinct population with a low width profile in nigericin stimulated samples (*Figure 4G*). Concluding, that we have successfully generated a reporter cell line and that our detection method works for detecting ASC oligomerization in human monocytes.

Additionally, we wanted to test which inflammasome sensors were expressed by THP1 cells to get a better idea on what type of hits we could expect. We did a quantitative polymerase chain reaction (qPCR) analysis and found that only NLRP3, CARD8 and caspase-4 were expressed (*Supplementary figure 1*), among the sensors we tested for.

To make it easier to use our tool directly on primary human material, we repeated the experiment on cells that haven't been genetically modified to express a fluorescent ASC protein. We stimulated THP1 CASP1^{-/-} cells and stained them with an ASC antibody. We could still observe and quantify ASC oligomerization (*Figure 4F, 8B*) based on low ASC width/area ratio.

Thus, we propose a high throughput flow cytometry based method to detect NLRP3 inflammasome modulation in non-adherent cell populations (*Figure 4H*).

Imaging Flow Cytometry



Flow Cytometry

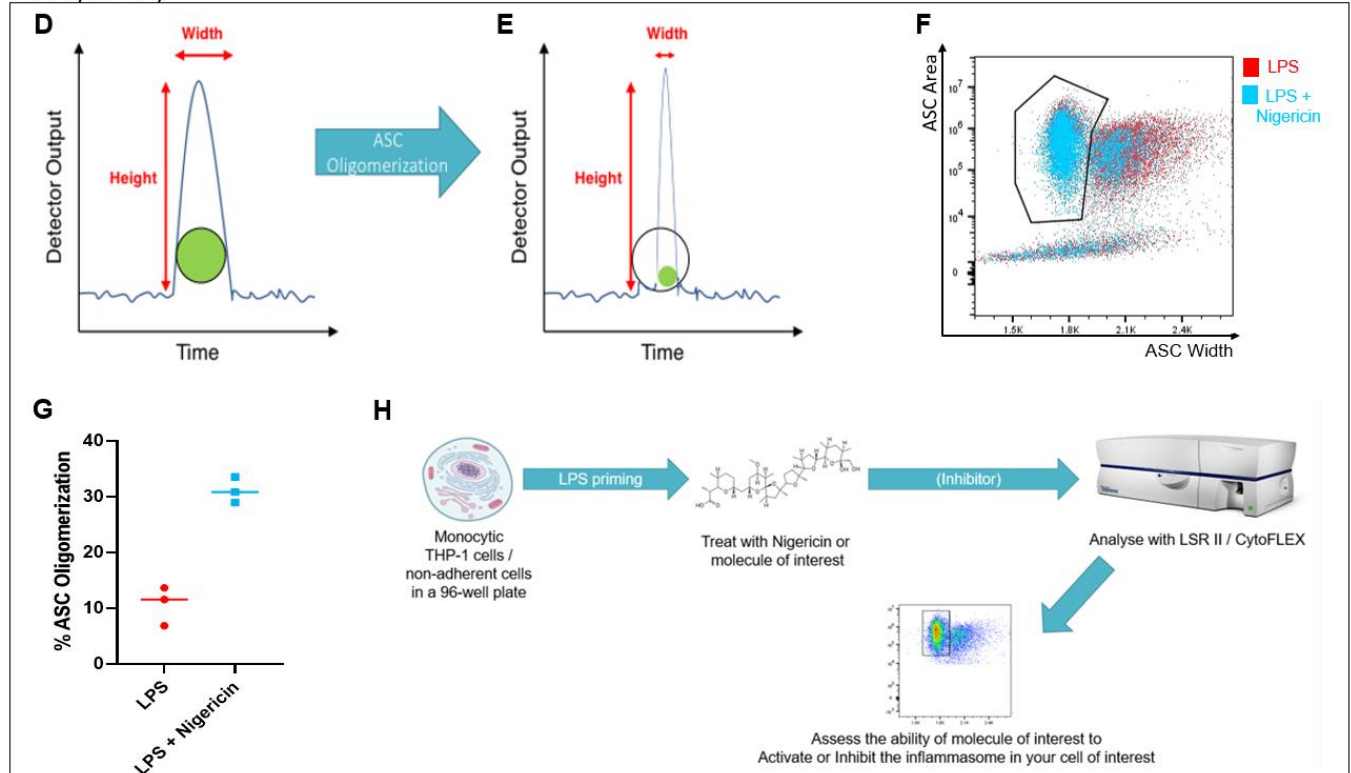


Figure 4 Assessment of ASC oligomerization by Flow Cytometry **A** Schematic explaining the brightfield over citrine ratio; in unstimulated cells ASC is dispersed throughout the cell therefore the ratio of the brightfield signal over the citrine signal is equal to one, in stimulated cells ASC oligomerizes in a single speck and the BF/citrine > 1 **B** A histogram showing the percentage of ASC oligomerization in LPS treated BMDMs, assessed by AMNIS **C** A histogram showing the percentage of ASC oligomerization in LPS and nigericin treated BMDMs, assessed by AMNIS **D** Cell in homeostasis; ASC (in green) is dispersed throughout the cell, the width of the signal is as large as the cell. **E** After ASC (in green) oligomerizes it becomes a single speck, the height of the signal remains the same, however the width of the signal is smaller than in homeostasis (cell is represented by the black circle). **F** Flow cytometry graph showing the ASC width low population in THP1 CASP1^{-/-} ASC GFP cells **G** Quantification of ASC oligomerization in THP CASP1^{-/-} cells stained with an ASC Ab **H** Proposed method to detect NLRP3 inflammasome modulation in non-adherent cell populations

Caspase-1 limits ASC speck detection

Caspase-1 a protein that is synthesized as an inactive zymogen, after it is recruited into the inflammasome complex, containing the adaptor protein ASC and the sensor, it is activated by autoproteolytic cleavage²². Autoproteolysis of caspase-1 results in release of IL-1 β and IL-18 and an inflammatory form of cell death, pyroptosis.

We hypothesized that a knock-out of Caspase-1 would prevent pyroptosis and improve our ability to detect ASC specks, as pyroptosis results in cell lysis⁴³, which might make it harder to detect inflammasome formation. To test our hypothesis, we treated wildtype (WT) and Caspase-1 knock out (CASP1^{-/-}) BMDMs generated from ASC Citrine reporter mice with LPS as a negative control and with LPS and Nigericin as a positive control (*Figure 5A, Supplementary figure 2*). We then analyzed the cells with flow cytometry (*Figure 4H*) and found that we can observe more ASC oligomerization in CASP1^{-/-} BMDMs compared to the WT BMDMs (*Figure 5B*). We still observed 31,9% ASC oligomerization in the dead cell population of CASP1^{-/-} cells (*Figure 5A*), therefore Caspase 1 deficient cells still die albeit at lower levels or altered kinetics. Additionally, we observed 66,8% of ASC oligomerization in live cells in CASP1^{-/-}, which is much higher compared to 12,9% of ASC oligomerization in live WT cells (*Figure 5C*).

Additionally, we tested a Caspase-1 inhibitor VX765 to determine if we could use wildtype cells, instead of genetically modified caspase-1 knock-out cells and treat them with the inhibitor. We stimulated ASC Citrine mouse derived BMDMs with different concentrations of VX765 and observed successful Caspase-1 inhibition at 20 μ M concentration with approximately 4,83 % of ASC oligomerization (*Figure 6 A-G, Supplementary figure 3*).

We concluded that Caspase-1 limits ASC speck detection and that a Caspase-1 knock-out improves cell viability and sensitivity of the assay. Therefore, we performed all our further experiments with CASP1^{-/-} cells.

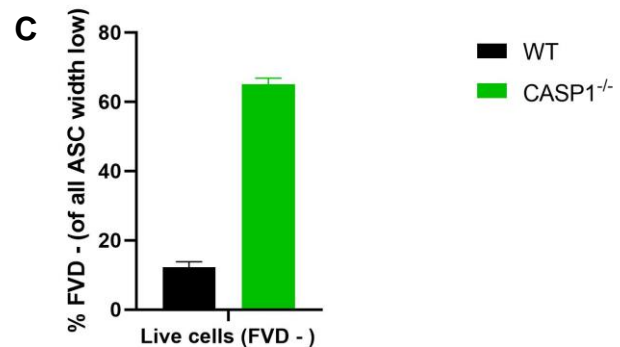
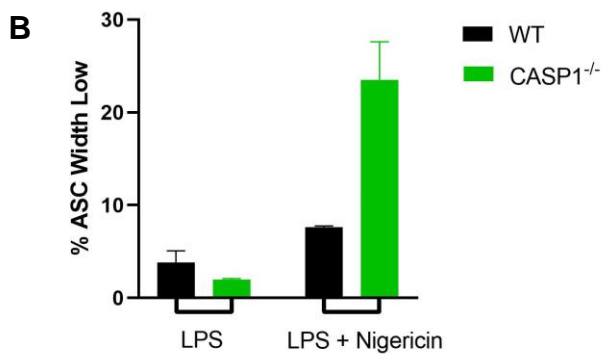
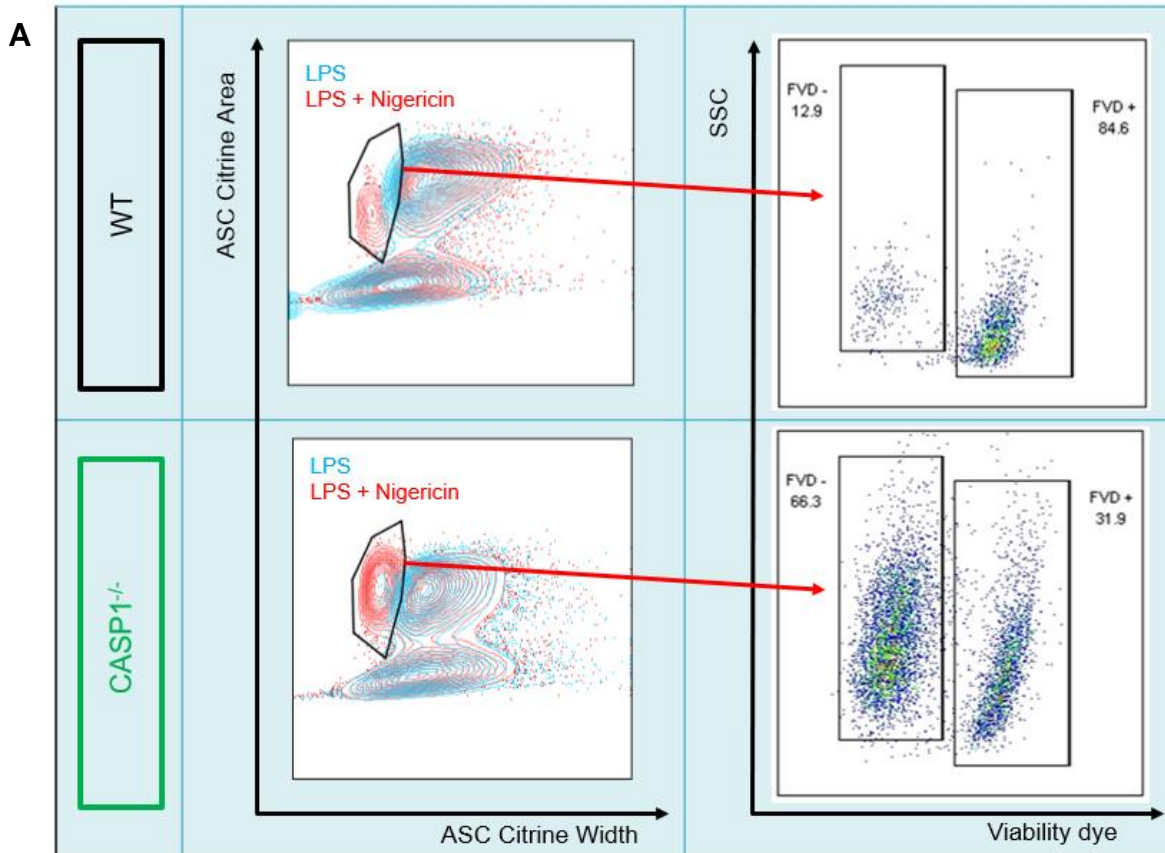


Figure 5 Investigating the role of Caspase-1 in ASC speck detection in mouse ASC Citrine BMDMs, cells were stained with a fixable viability dye to assess whether they are alive or they have undergone pyroptosis and analyzed by flow cytometry **A** Comparison of ASC oligomerization in WT BMDMs vs CASP1^{-/-} by flow cytometry; in the overlay the population in red is the positive control – cells treated with LPS and Nigericin, the population in blue is the negative control – cells treated only with LPS **B** Quantification of % of ASC Oligomerization in WT cells compared to CASP1^{-/-} cells **C** Quantification of % of ASC oligomerization in the live cell population in WT compared to CASP1^{-/-} cells.

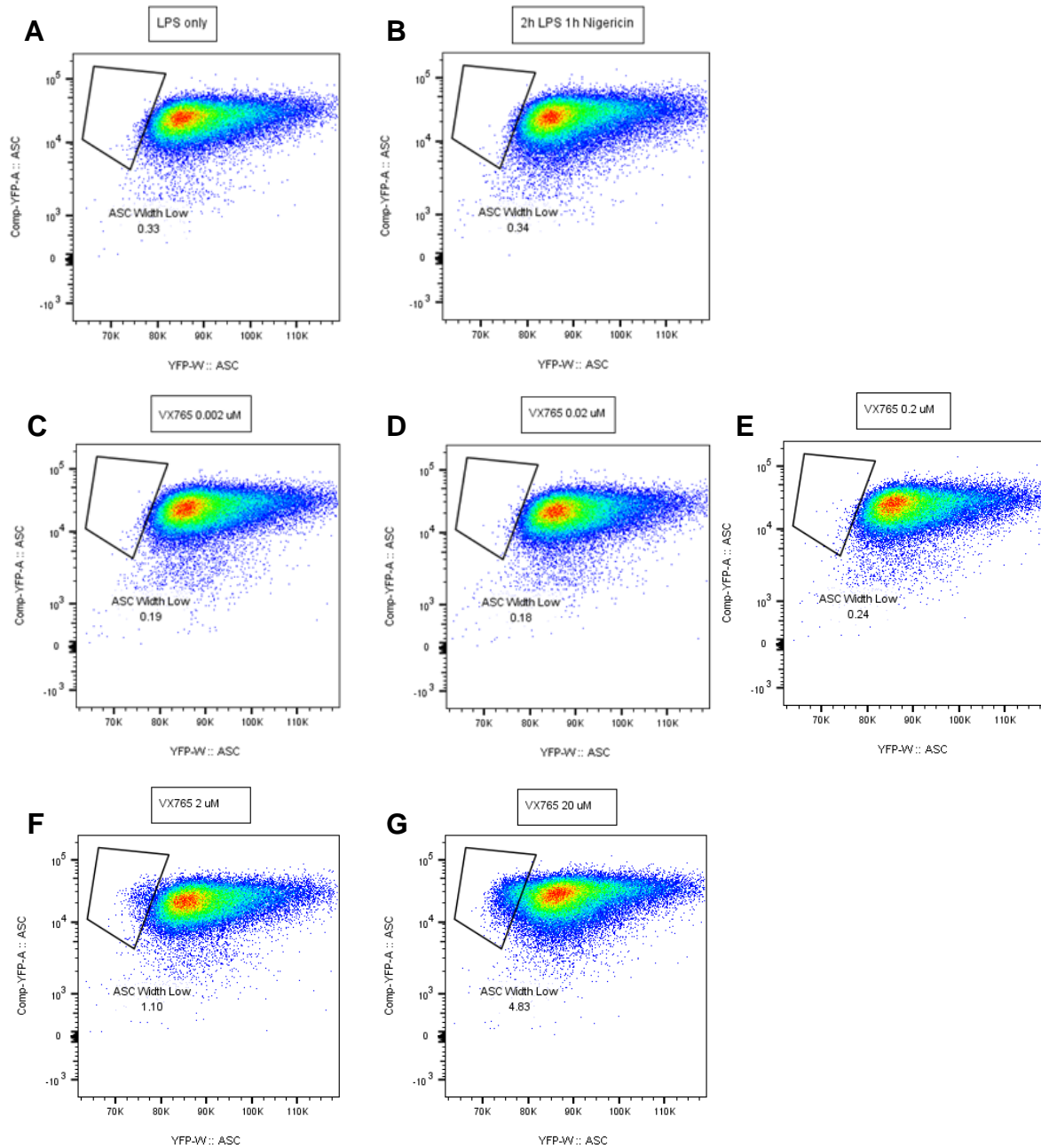


Figure 6 ASC Citrine mouse BMDMs, fixable viability dye negative, stimulated with 2h LPS and 1h Nigericin, treated with different concentrations of PanCaspase Inhibitor VX765 **A** Cells treated with LPS only **B** Cells treated with 2h of LPS and 1 hour Nigericin without VX765 **C** Cells stimulated with 2h LPS and 1h Nigericin treated with 0,002 μ M VX765 **D** Cells stimulated with 2h LPS and 1h Nigericin treated with 0,02 μ M VX765 **E** Cells stimulated with 2h LPS and 1h Nigericin treated with 0,2 μ M VX765 **F** Cells stimulated with 2h LPS and 1h Nigericin treated with 2 μ M VX765 **G** Cells stimulated with 2h LPS and 1h Nigericin treated with 20 μ M VX765

NLRP3 dependent ASC oligomerization can be detected in neutrophils and monocytes but not in T cells, NK cells and granulocytes

To investigate in what type of immune cells NLRP3 dependent ASC oligomerization can be detected with our method (*Figure 4H*), we extracted blood from Casp1^{-/-} mice by cardiac puncture. After obtaining the maximum volume of blood possible, we lysed red blood cells and stimulated the remaining cells with 2 hours of LPS priming and 1 hour of Nigericin stimulation. We then stained them with an ASC antibody, relevant immune cell markers and a viability dye and ran the samples on a flow cytometer. Data was analyzed with FlowJo software.

We observed ASC oligomerization in neutrophils and monocytes but not in T cells, NK cells and granulocytes (*Figure 7A-F, 8*). To confirm our results, we repeated the experiment by isolating neutrophils from whole blood of ASC Citrine Casp1^{-/-} mice and thus further supported that NLRP3 dependent inflammasome formation can be observed in neutrophils (*Figure 7G,9A*).

Furthermore, the experiment has been repeated in ASC Citrine Casp1^{-/-} BMDMs and in monocytic THP1 cells (*Figure 7H, 9B*), which also confirmed that we can also observe NLRP3 dependent inflammasome formation in monocytes and macrophages.

Therefore, our method to find novel NLRP3 inflammasome modulators in non-adherent cell populations can be successfully utilized in multiple cells at once, for example directly in whole blood. It thus has a potential to be used on human blood, which would add to the relevance of the potential discovery of novel modulators. Additionally, by using different stimuli our method could be used to screen multiple cells for novel inflammasome sensors.

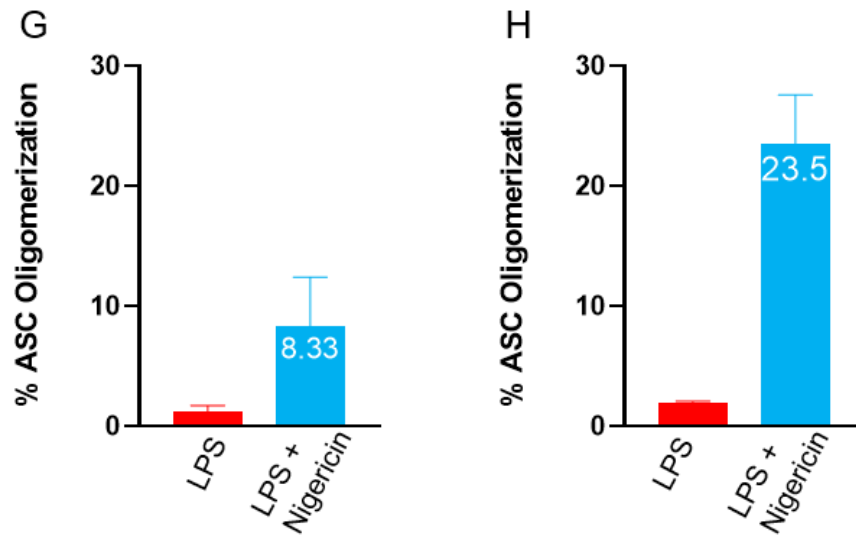
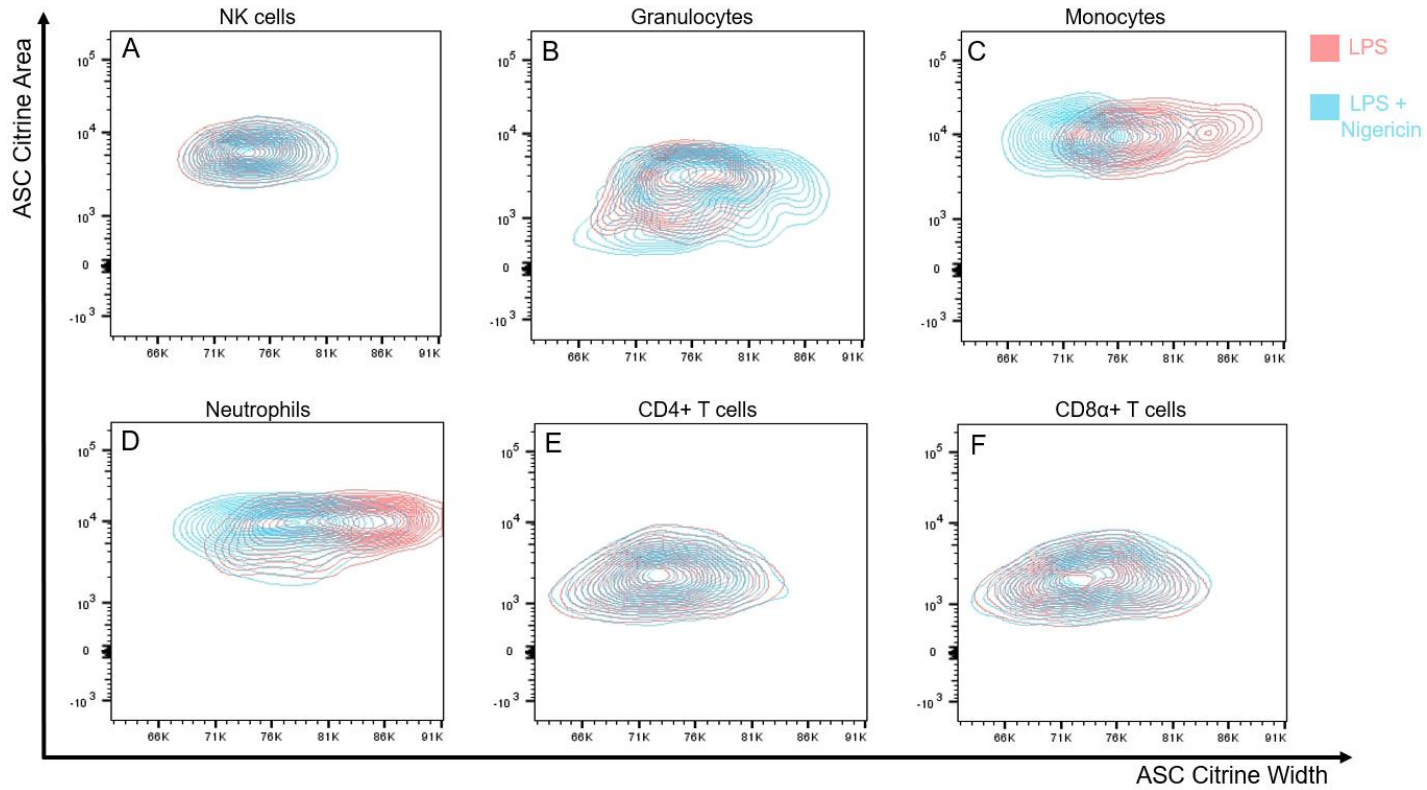


Figure 7 Analysis of ASC Oligomerization in cell types from mouse whole blood by flow cytometry, red in overlay are cells treated only with LPS (negative control) and in blue are cells treated with LPS and Nigericin (positive control) **A** Natural killer (NK) cells **B** Granulocytes **C** Monocytes **D** Neutrophils **E** CD4+ T cells **F** CD8 α + T cells **G** Quantification of ASC oligomerization in Neutrophils detected by flow cytometry, results pooled from two separate experiments **H** Quantification of ASC Oligomerization in Monocytes detected by flow cytometry

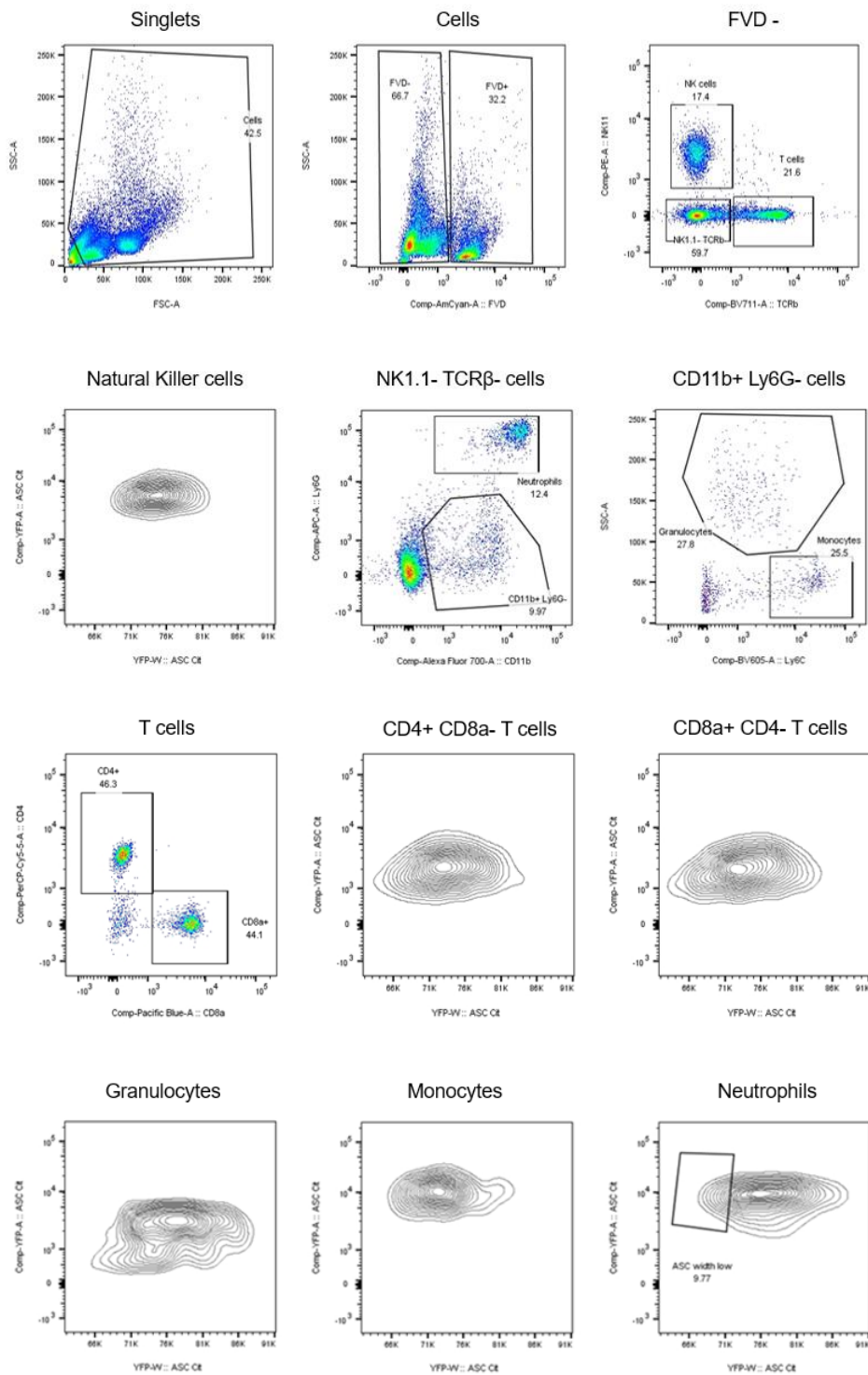


Figure 8 Gating strategy for analysis of ASC Oligomerization in cell types from mouse whole blood by flow cytometry

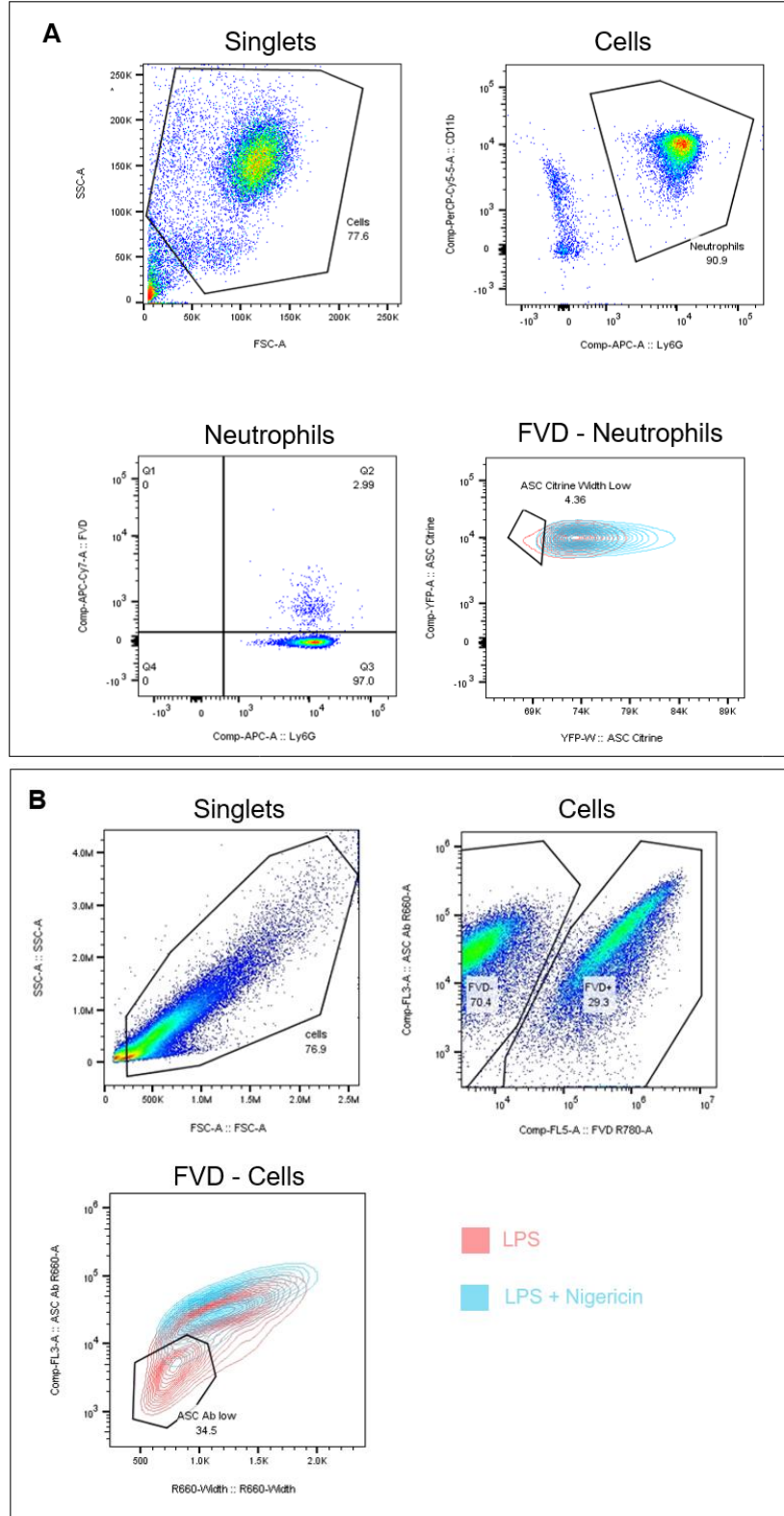


Figure 9 Gating strategy for flow cytometry analysis **A** ASC Citrine Casp1^{-/-} mouse Neutrophil isolation from whole blood and ASC oligomerization analysis after stimulation **B** THP1 CASP1^{-/-} cells stained with ASC Ab, ASC oligomerization analysis and quantification

Method to detect inflammasome activation in adherent cell populations

The cytometry based tool we developed (*Figure 4H*), didn't work as well for adherent cells as it did for non-adherent cells. Additionally, cytometers are limited in the number of wells per plate that can be screened and by time. To develop a more compelling tool with direct visualization by microscopy that is even more high-throughput than our previous method, with which we would be able to screen thousands of molecules at once, we developed a microscopy based screening tool.

Microscopes such as the IN Cell Analyzer 2200 have been developed to screen multiple 384-well plates at once in a matter of a couple of hours. However, since microscopy based systems are usually limited to adherent cells, we developed a this tool to detect inflammasome activation in adherent cell populations.

We first generated mouse BMDMs from Casp1^{-/-} ASC Citrine mice and seeded them in glass bottom 384-well plates. Subsequently, we stimulated the cells with LPS and Nigericin and stained them with WGA and a nuclear stain. We then used the IN Cell Analyzer microscope to screen the plates which resulted in many microscopy images that had to be analyzed. For this purpose, we wrote a pipeline in Cell Profiler (*Figure 10A*) and analyzed the data with statistical analysis tools JMP and Prism.

With this method we could detect ASC specks in LPS and nigericin stimulated mouse BMDMs (*Figure 10B, 10C*) based on the principle that ASC citrine fluorescent signal oligomerizes into a single speck, becoming much stronger compared to homeostasis when ASC citrine is dispersed throughout the cell. We optimized the cell density to 20.000 cells per well (*Supplementary figure 4*).

Thus, we propose a high throughput microscopy based method to detect NLRP3 inflammasome modulation in adherent cell populations (*Figure 10D*).

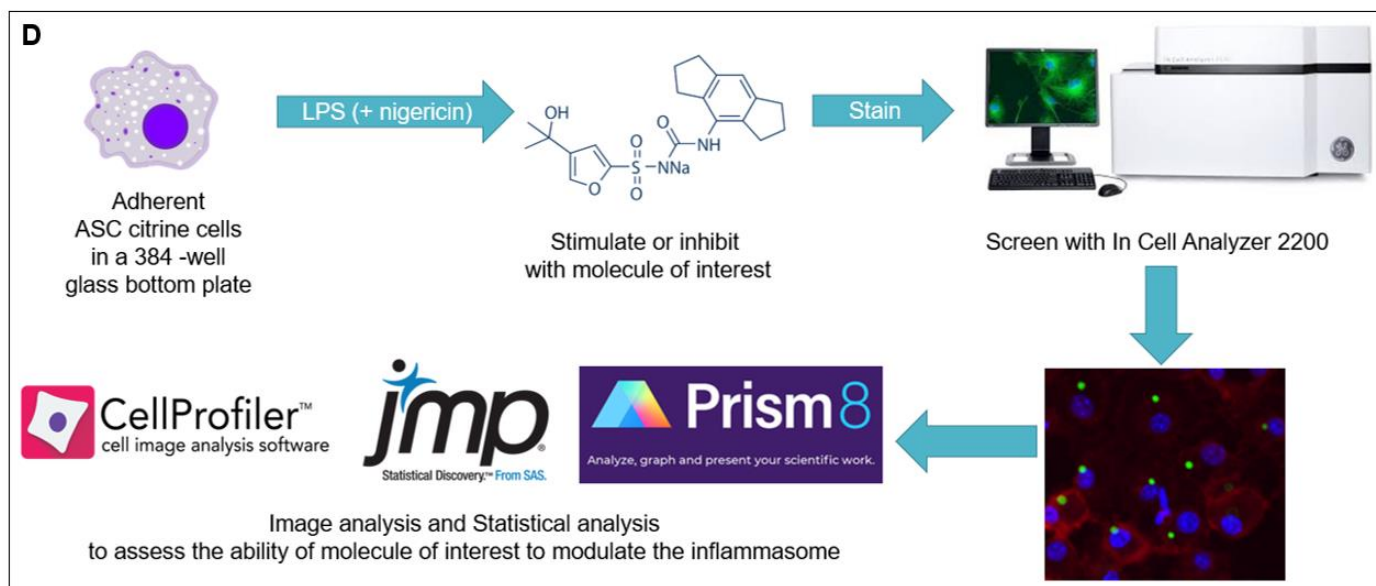
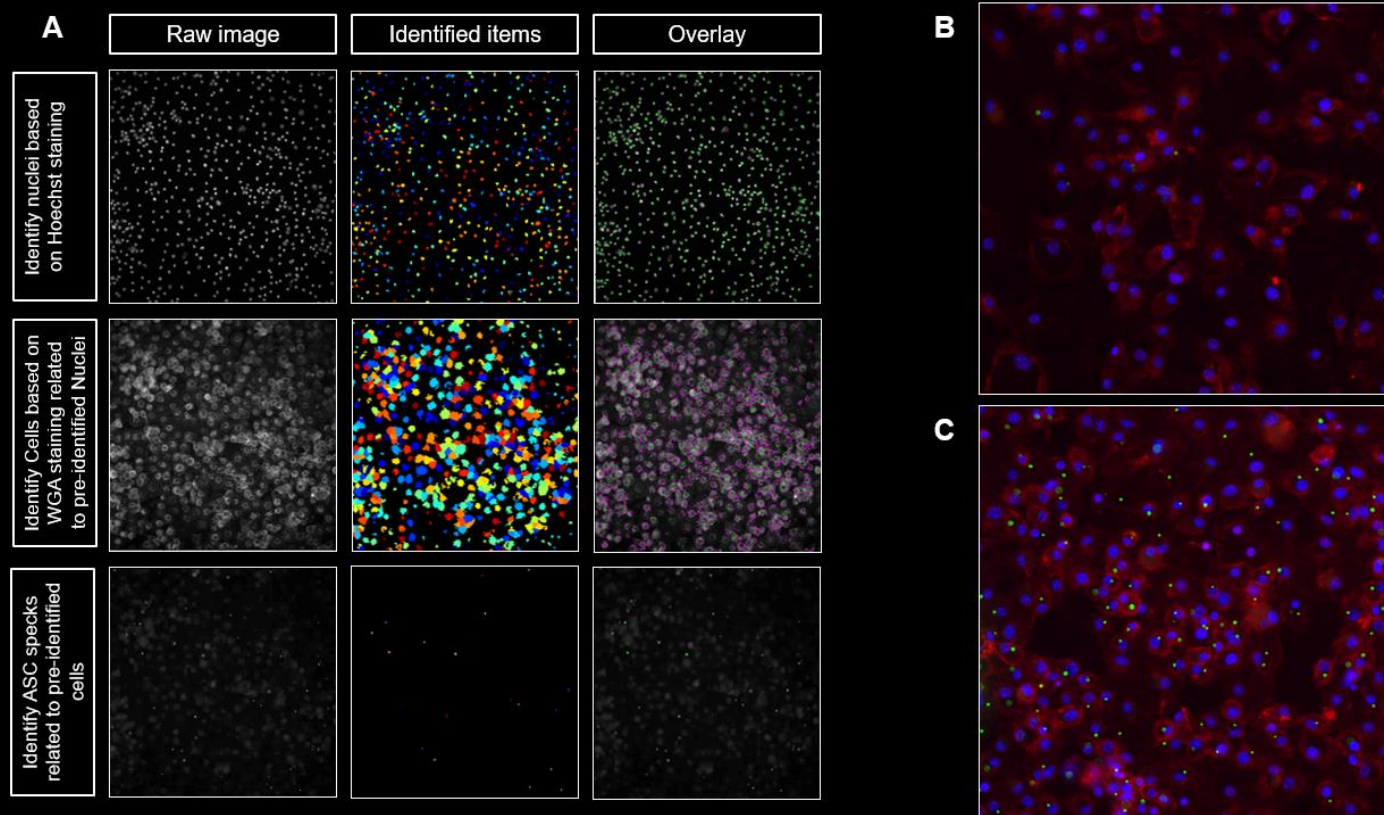


Figure 10 Developing a microscopy based approach to screen for novel inflammasome modulators **A** The approach we have taken for designing the pipeline for image analysis for the screen **B-C** An overlay of the images from all three channels where ASC specks are clearly visible, Hoechst in blue, WGA in red and ASC citrine in green, **B** LPS only treated sample **C** LPS and nigericin stimulated sample **D** Proposed method to detect NLRP3 inflammasome modulation in adherent cell populations

Several microbes from the gut secrete molecules that activate the inflammasome

To investigate which microbially produced molecules modulate the inflammasome and which inflammasome sensors are involved we screened microbial supernatants. We procured the microbial supernatant library from Noah Palm laboratory⁷. A selection of human gut bacteria was monocultured in appropriate media for two days, when the cultured bacteria reached stationary phase, their supernatants were collected by centrifugation. Samples were filtered and heat inactivated.

We screened with the help of our, previously described, microscopy based approach (Figure 10D). We seeded Casp1^{-/-} ASC citrine mouse BMDMs in 384-well glass bottom plates and primed them for two hours with LPS. Then we treated the cells with the microbial supernatants in duplicates for two hours and separately for twenty-four hours. After treatment, we stained the cells with wheat germ agglutinin (WGA), a cell membrane dye and Hoechst, a nuclear dye. The plates were imaged with the IN Cell Analyzer 2200 microscope. The acquired images were analyzed with a pipeline we created with the Cell Profiler software (Figure 10A). Statistical analysis was done with JMP and Prism programs.

We have identified seven microbial supernatants that can activate the inflammasome after 2 hours of stimulation (Figure 11A) and fourteen microbial supernatants that can activate it after 24 hours of stimulation (Figure 11B) in a statistically significant manner ($p < 0,01$).

Supernatants of *Clostridium perfringens* and *Bacteroides fragilis* were capable of stimulating the inflammasome at both time points. Both are anaerobic bacteria with previously known ability to activate the NLRP3 inflammasome. Gram-positive pathogen *C. perfringens* that causes gas gangrene, activates it with a pore forming protein Perfringolysin O⁴⁴. Gram-negative commensal *B. fragilis* activates the NLRP3 inflammasome by a mechanism that involves potassium efflux and that does not require bacterial viability or phagocytosis, however the exact molecule involved is thus far unknown⁴⁵.

Additionally, after two hours of stimulation supernatants of bacteria from the Enterobacteriaceae family, *Streptococcus spp.* and *Morganella spp.* were able to activate the inflammasome, all with previously described potential to stimulate the NLRP3 inflammasome⁴⁶⁻⁴⁸. *Streptococcus spp.* likely activates it with the help of M protein⁴⁷, whereas *Morganella spp.* activates it with a pore forming protein Hemolysin A^{48,49}. For Enterobacteriaceae the molecule that induces inflammasome activation is unknown, however a member of the family *Proteus mirabilis* can induce a robust IL-1 β release with the help of an unknown molecule⁴⁶. Additionally, a member of the *Bifidobacterium* genus also activated the inflammasome after two hours of stimulation, which hasn't been described in the literature to the best of our knowledge.

After 24 hours of stimulation we observed inflammasome activation in a sample treated with the supernatant of a different strain from the genus *Bifidobacterium* than in the two hour treatment. We also observed ASC oligomerization after stimulation with the supernatant of *Fusobacterium spp.* bacteria. Knowing that *Fusobacterium nucleatum* infection of gingival epithelial cells leads to NLRP3 inflammasome-dependent secretion of IL-1 β ⁵⁰ makes it likely that the strain we screened also secretes a similar protein and thus activates the NLRP3 inflammasome.

Additionally, we also observed ASC oligomerization after 24 hours of stimulation with supernatants of *Lactobacillus reuteri*, *Bacteroides ovatus*, *Bacteroides uniformis* and *Megasphaera spp.*, none of the four bacteria has a previously described ability to activate the inflammasome to the best of our knowledge.

In conclusion, we found that several bacteria from the human gut are capable of activating the inflammasome. Some have already been described and some are novel, however for most the exact molecule and mechanism are unknown.

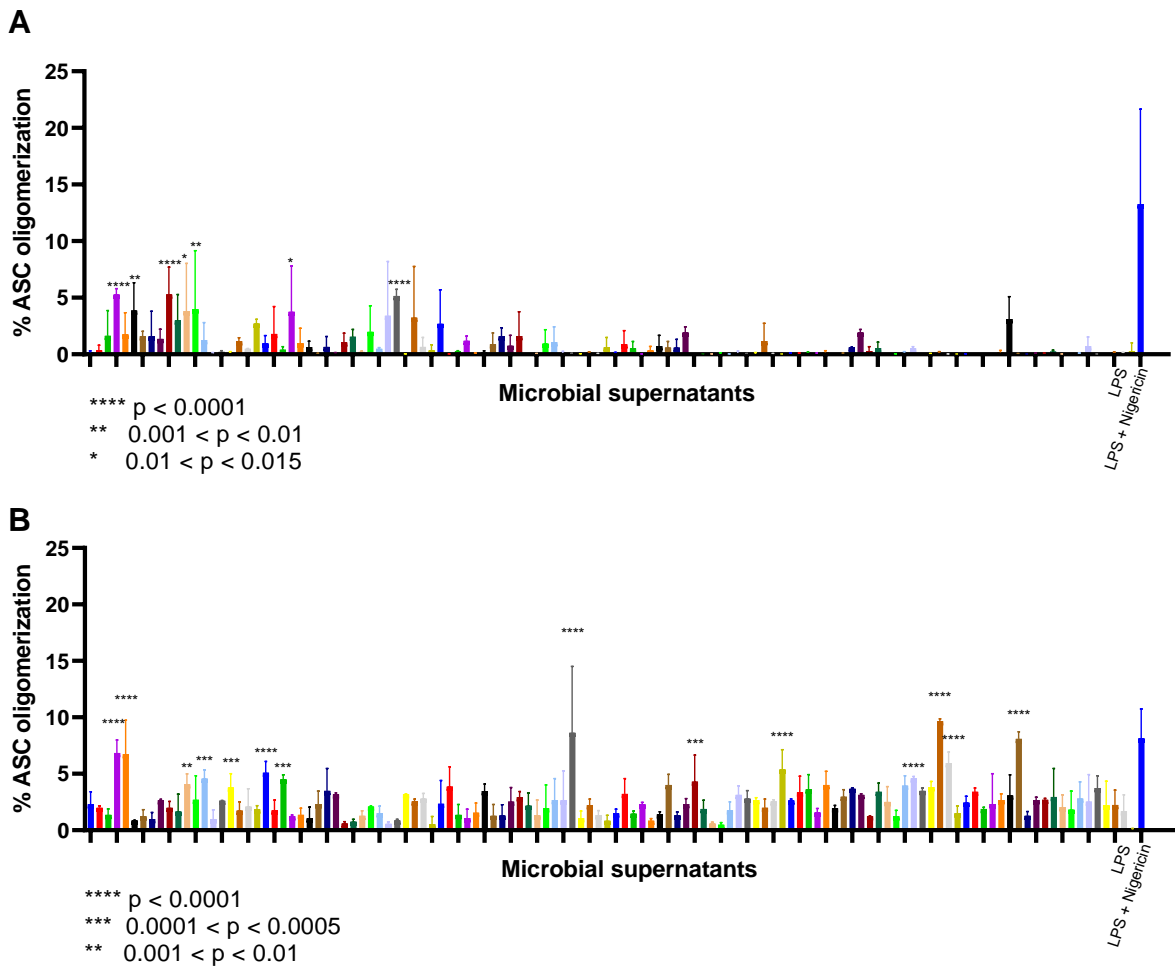


Figure 11 Microbial supernatants screen on *Casp1*^{-/-} ASC citrine mouse BMDMs **A** Samples primed for 2 hours with LPS followed by 2 hour microbial supernatant stimulation **B** Samples primed for 2 hours with LPS followed by 24 hour microbial supernatant stimulation

Molecules produced by gut microbiota activate the NLRP3 inflammasome

To identify the inflammasome sensor involved in the activation following stimulation with each of the supernatants, we repeated the experiment by adding MCC950, a known specific inhibitor of NLRP3³⁵.

First, we treated ASC Citrine Casp1^{-/-} mouse derived BMDMs with LPS and Nigericin and added MCC950 in different concentrations to establish a dose response and find out which concentration will be suitable to repeat our screen with. We observed an inhibition of NLRP3 activation, with only 0,88% ASC oligomerization, when treated with 1 μ M concentration of MCC950 (*Figure 12 A-G, Supplementary figure 3*).

All samples were treated with 1 μ M concentration of MCC950 simultaneously with LPS stimulation. After 2 hours microbial supernatants were added in duplicates for either 2 hours or 24 hours. Nigericin was added to the positive control. We observed that MCC950 treatment prevented ASC oligomerization in all samples, indicating that all of the molecules that activated the inflammasome in the previous screen seemed to have stimulated the NLRP3 sensor (*Figure 13B-C*). This result is expected, because NLRP3 sensor has the broadest spectrum of activation and because we tested the samples on mouse bone marrow derived macrophages that highly express NLRP3 (*Supplementary figure 5*). Additionally, we screened the supernatants by pre-treating them with LPS which is required for NLRP3 inflammasome formation.

Therefore, we conclude that all of the hits observed in the previous screen (*Figure 11A - B*) were NLRP3 dependent and that several human gut bacteria are capable to activate the NLRP3 inflammasome in BMDMs.

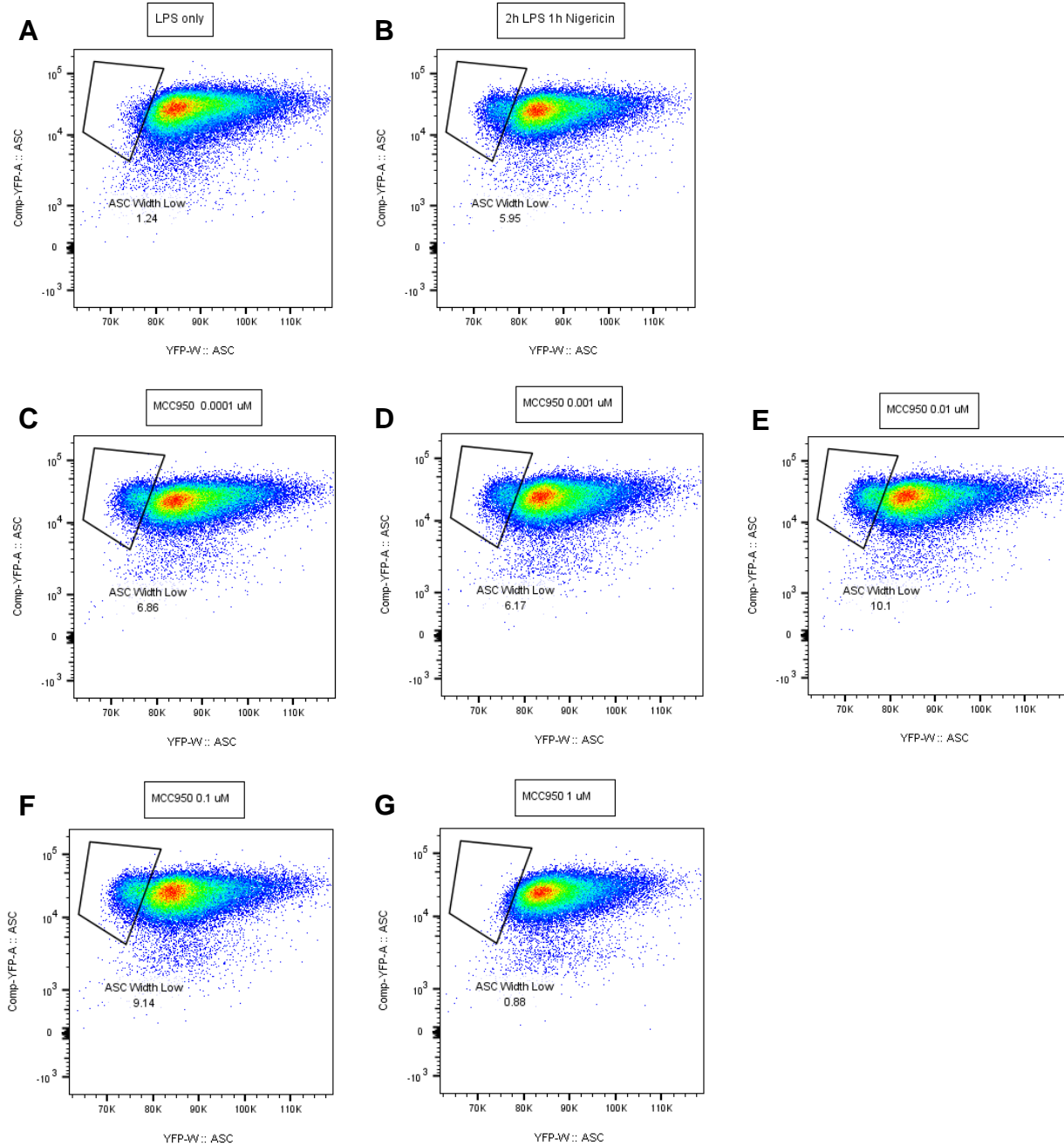


Figure 12 *Casp1*^{-/-} ASC Citrine mouse BMDMs, fixable viability dye negative, stimulated with 2h LPS and 1h Nigericin, treated with different concentrations of NLRP3 inhibitor MCC950 **A** Cells treated with LPS only **B** Cells treated with 2h of LPS and 1 hour Nigericin without MCC950 **C** Cells stimulated with 2h LPS and 1h Nigericin treated with 0,0001 μ M MCC950 **D** Cells stimulated with 2h LPS and 1h Nigericin treated with 0,001 μ M MCC950 **E** Cells stimulated with 2h LPS and 1h Nigericin treated with 0,01 μ M MCC950 **F** Cells stimulated with 2h LPS and 1h Nigericin treated with 0.1 μ M MCC950 **G** Cells stimulated with 2h LPS and 1h Nigericin treated with 1 μ M MCC950

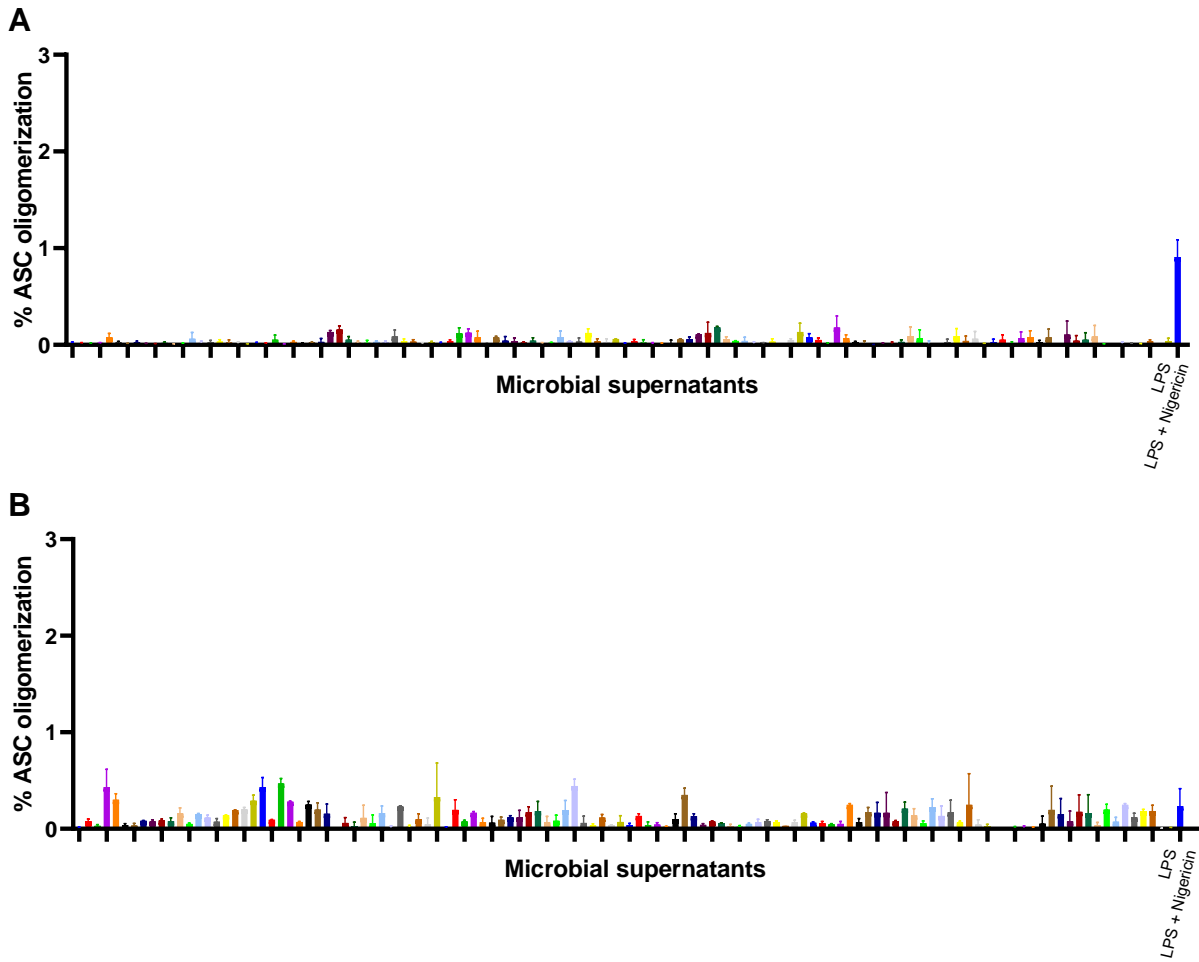


Figure 13 Microbial supernatants screen on *Casp1*^{-/-} ASC citrine mouse BMDMs treated with MCC950 **A** Dose response curve to MCC950 **B** Samples treated with NLRP3 inhibitor MCC950 and 2 hour LPS priming followed by 2 hour microbial supernatant stimulation **C** Samples treated with NLRP3 inhibitor MCC950 and 2 hour LPS priming followed by 24 hour microbial supernatant stimulation

CONCLUSIONS

We developed two different tools for discovery of novel inflammasome modulators and sensors. These tools potentially provide valuable insights in the mechanism of inflammasome formation and a deeper understanding of innate immune mechanisms. With the help of the microscopy based tool we have identified several bacterial supernatants that are able to activate the NLRP3 inflammasome.

We propose two different methods of detecting inflammasome activation, both based on ASC oligomerization. The first method utilizes flow cytometry for detection of ASC oligomerization in non-adherent cell populations in cells where ASC protein is either genetically tagged with a fluorescent protein or the cells are stained with a fluorescently labelled anti ASC antibody (*Figure 4H*). The second method we propose is based on the detection of genetically tagged ASC protein in adherent cell populations with microscopy (*Figure 10D*).

Our research has shown that our tool can be reliably used to study the NLRP3 inflammasome in monocytes macrophages and neutrophils (*Figure 7 A-H*). Additionally, other inflammasome sensors could potentially be studied in any other blood cells. We observed that Caspase-1 knock out greatly improves cell viability and sensitivity (*Figure 5 A-C*), therefore Caspase-1 should either be knocked out or inhibited by VX765 (*Figure 6 A-G*) in the cell of interest when utilizing these methods.

In our effort to make this method applicable on primary human samples, we have shown that it works on blood samples of wildtype mice when stained with an ASC antibody and that a pan-caspase inhibitor can be used with similar results to a caspase 1 knock out. However, we only validated this with the flow cytometry based method, in the future this should be repeated with the microscopy based method as well.

We performed a high throughput microscopy based screen of bacterial supernatants, collected from monocultured human gut bacteria that we procured from the Noah Palm laboratory⁷. We have observed some hits from bacterial supernatants with previously described ability to activate the NLRP3 inflammasome, such as pathogens *Clostridium perfringens*⁴⁴ and *Fusobacterium spp.*⁵⁰ and *Bacteroides fragilis*⁴⁵ an anaerobic comensal bacteria that is part of the normal microbiota. However, not all of the molecules activating the inflammasome produced by these bacteria have been identified.

With the help of the high throughput screen we performed, we also observed NLRP3 inflammasome activation in a total of six samples stimulated with supernatants from bacteria with previously undescribed potential to modify the inflammasome.

Two supernatants collected from two different bacterial strains of the genus *Bifidobacterium* have shown the potential to activate the NLRP3 inflammasome. Bacteria from this genus are gram positive, non motile and among the first microbes to colonize the human gastrointestinal tract⁵¹. As they are believed to be probiotic with a role in preventing colorectal cancer and reduction of inflammatory bowel disease (IBD)

symptoms⁵¹, it would be beneficial to research if their ability to activate the inflammasome plays a role in preventing these diseases.

Similarly, *L. reuteri*, another hit we observed, is also a human commensal probiotic microbe found in four different body sites, namely the gastrointestinal tract, urinary tract, skin and breast milk⁵². It has been linked to production of antimicrobial molecules and subsequent remodeling of the host microbiota and inhibition of growth of pathogenic bacteria⁵². It would be valuable to understand whether the beneficial role of *L. reuteri* is also linked to its ability to activate the NLRP3 inflammasome and how. Interestingly, some *L. reuteri* strains are capable of converting a dietary component L-histidine into the biogenic amine histamine^{53,54} which has been shown to have the potential to inhibit the NLRP3 inflammasome⁸. This could mean that the molecule activating the NLRP3 inflammasome is even more potent when not antagonized by histamine that is likely also present in the collected supernatant.

Additionally, we observed hits from supernatants of two different *Bacteroides* species, namely *B. ovatus* and *B. uniformis*. Obligately anaerobic commensal species from the *Bacteroides* genus are found exclusively in the gastrointestinal tracts of mammals⁵⁵. Thus, they are highly adapted to life in the gut and establish stable, long-term associations with their hosts⁵⁵. One of those adaptations is also *Bacteroides*' ability to influence the host immune system to help with the control of competing pathogens⁵⁶. Therefore, *Bacteroides* bacteria can confer numerous health benefits but are also important clinical pathogens, when they escape the gastrointestinal tract, with an associated mortality rate of more than 19%⁵⁶. Specifically, *B. uniformis* is known to contributing to the shaping of the gut microbiota by secreting the membrane attack complex/perforin (MACPF) that lyses surrounding bacterial pathogens⁵⁵. Like *L. reuteri*, *B. ovatus* is also used as a probiotic⁵⁷. Knowing which molecules secreted by these bacteria activate the NLRP3 inflammasome would help shed the light into the complex world of microbiota-host interactions. Additionally, it might help us understand how these comensals can become pathogenic outside the gut.

Bacteria of the *Megasphaera* genus, of which we found a species that secretes a molecule capable of activating the NLRP3 inflammasome, are relatively unstudied. They are gut commensal gram negative cocci that can produce important metabolites such as short chain fatty acids (butyrate, acetate, formate, caproate), vitamins and essential amino acids, which makes them beneficial to the host⁵⁸. However, they have also been implicated in bacterial vaginosis⁵⁹ and endocarditis⁶⁰. Therefore, understanding how and why this particular strain activates the NLRP3 inflammasome would elucidate its role in the human gut microbiome.

The method we are proposing has some caveats, one that we have observed during our high throughput screen of microbial supernatants is re-discovering known compounds, such as Perfringolysin O produced by *C. perfringens*. To avoid this in the future, we could further filter the supernatants with a 50 kDa filter to exclude large proteins that are

likely known bacterial toxins. We could also screen with a synthetic compound library where the function of the molecules is largely unknown.

Additionally, all our hits were NLRP3 dependent. To get around this, we could be using more uncommon cell types in the future such as CNS microglia, keratinocytes or even intestinal organoids. These cell types likely express different and so far undiscovered inflammasome sensors, as they are less studied in the context of inflammasome modulation. We could also be using NLRP3^{-/-} cells, treat cells with MCC950 or high extracellular potassium in order to specifically avoid NLRP3 dependent hits.

Another possible caveat is that while treating our cells with the supernatants we add some bacterial media along with the bacterial secretions. Various molecules present in bacterial media could potentially be responsible for some of the background noise in our screen. However, as media is present in all of the samples it likely doesn't affect the statistically significant hits, as they rise above the background.

In the future we would like to identify the specific molecules activating the NLRP3 inflammasome we discovered in our screens. Firstly, we would have to test whether stimulation with the supernatants leads to IL-1 β and IL-18 secretion to re-confirm our results. We would also like to test whether the effect we see is limited to mouse BMDMs or if the supernatants we tested also stimulate human cells. Furthermore, with the help of mass spectrometry and fractionation, we would try to find out exactly which molecule causes NLRP3 activation.

As another future direction, we want to screen for molecules that are capable of inhibiting the NLRP3 inflammasome. To do that we would repeat the screen with microbial supernatants on cells that were already pretreated with LPS and Nigericin and observe where the signal is inhibited. We would also like to repeat both screens on different cell types, specifically on primary human whole blood cells.

The methods we are proposing are very versatile tools that can be used for high throughput screens with any kind of molecular library on any type of cells. They offer a unique possibility to further study the inflammasome complex and potentially discover novel drugs to treat inflammasome related diseases.

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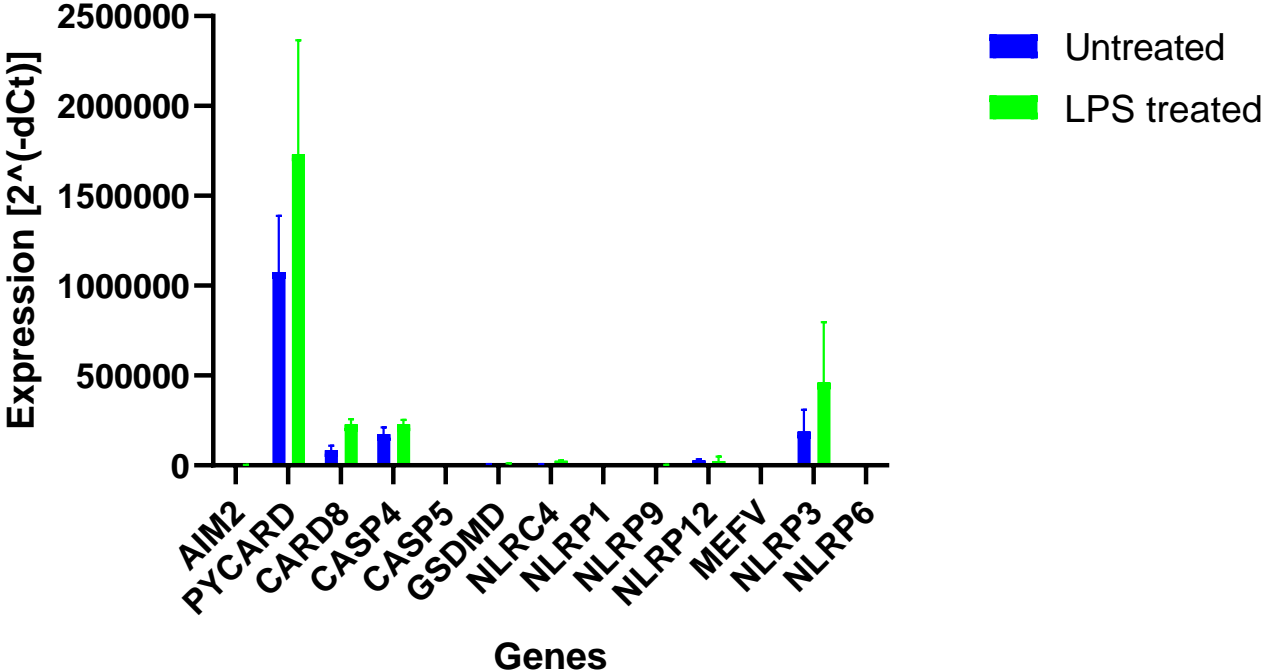
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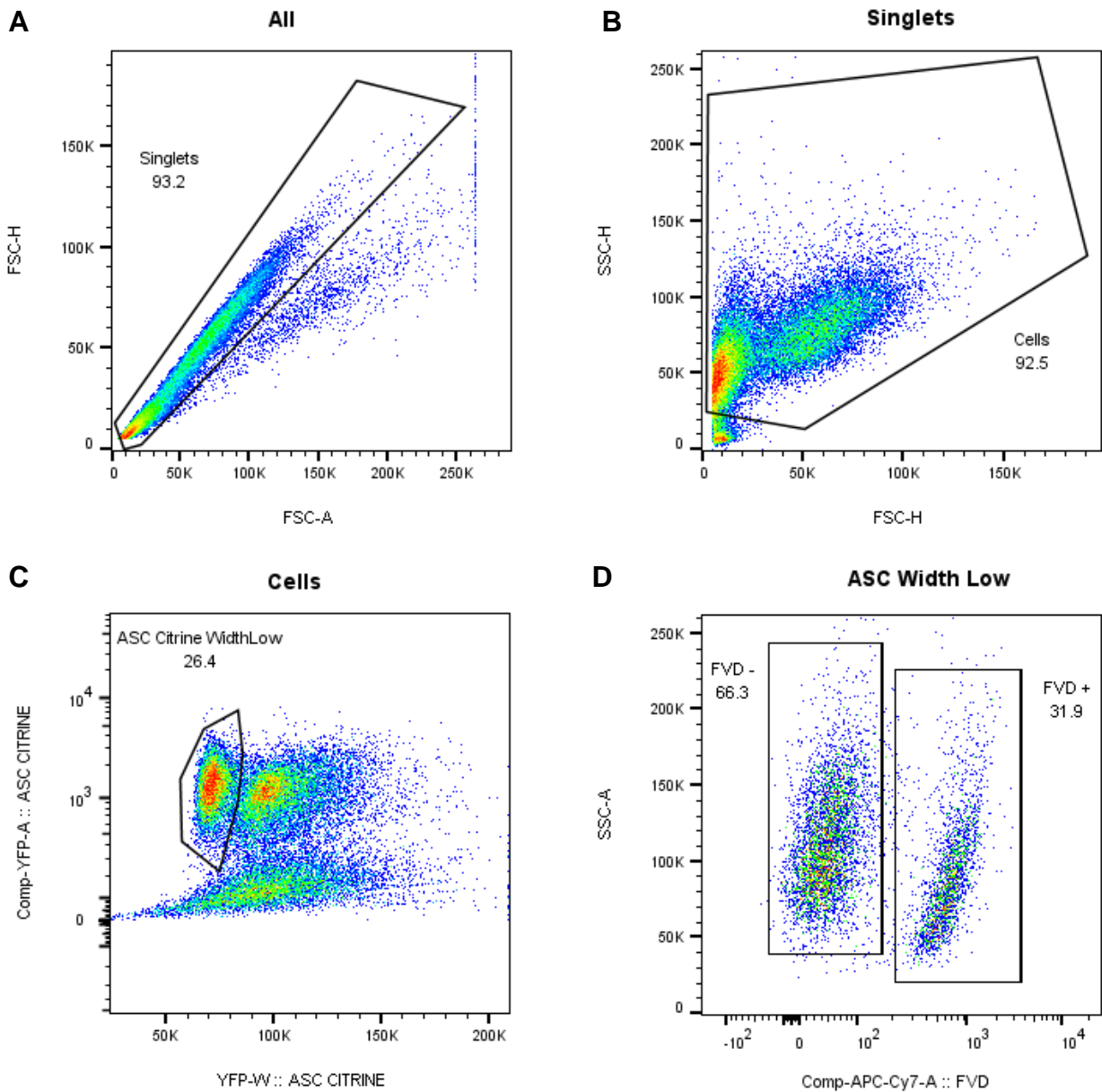
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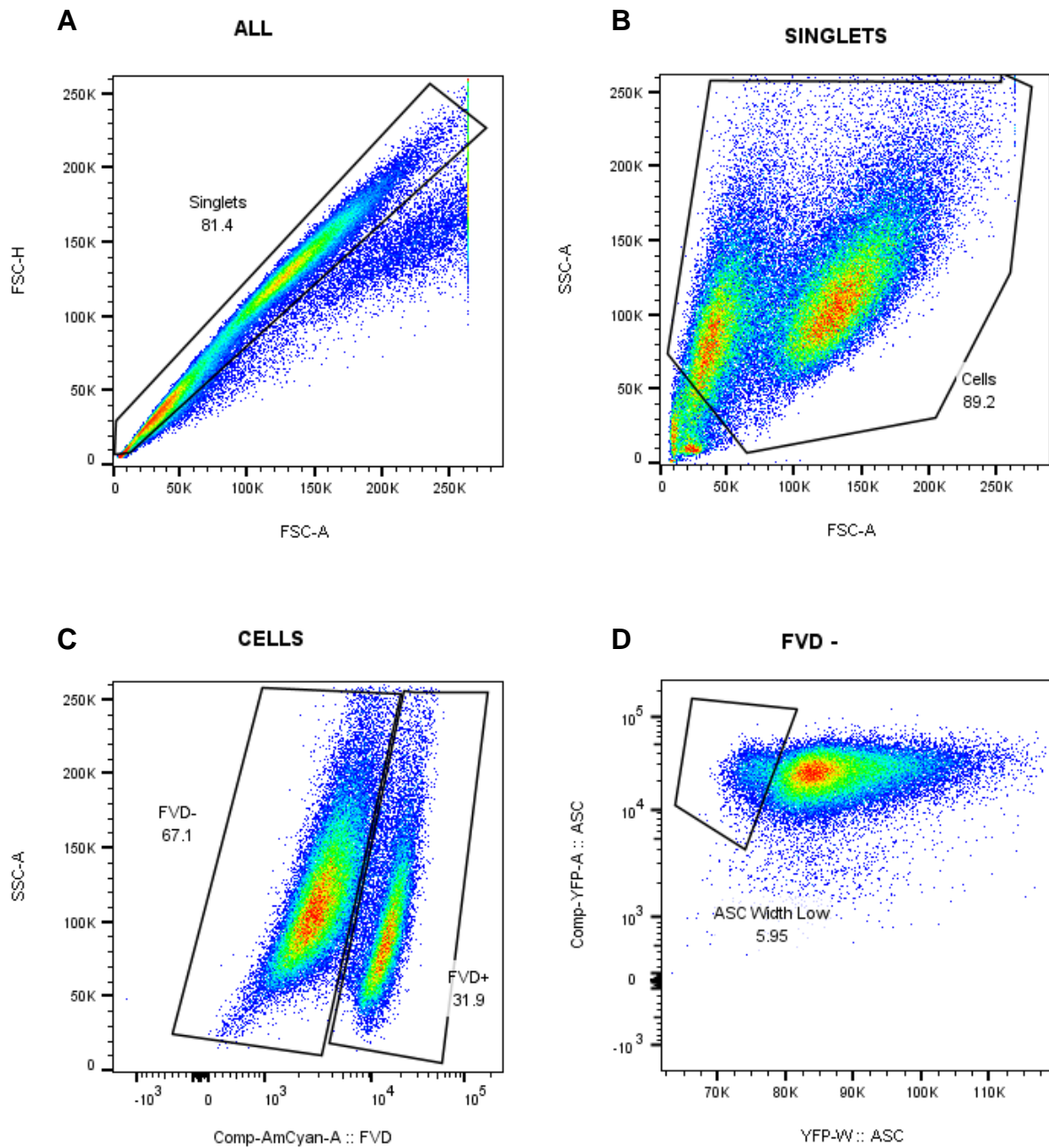
SUPPLEMENTARY INFORMATION



Supplementary figure 1 Inflammasome sensor gene expression in THP1 cells treated with LPS or untreated, quantified by qPCR



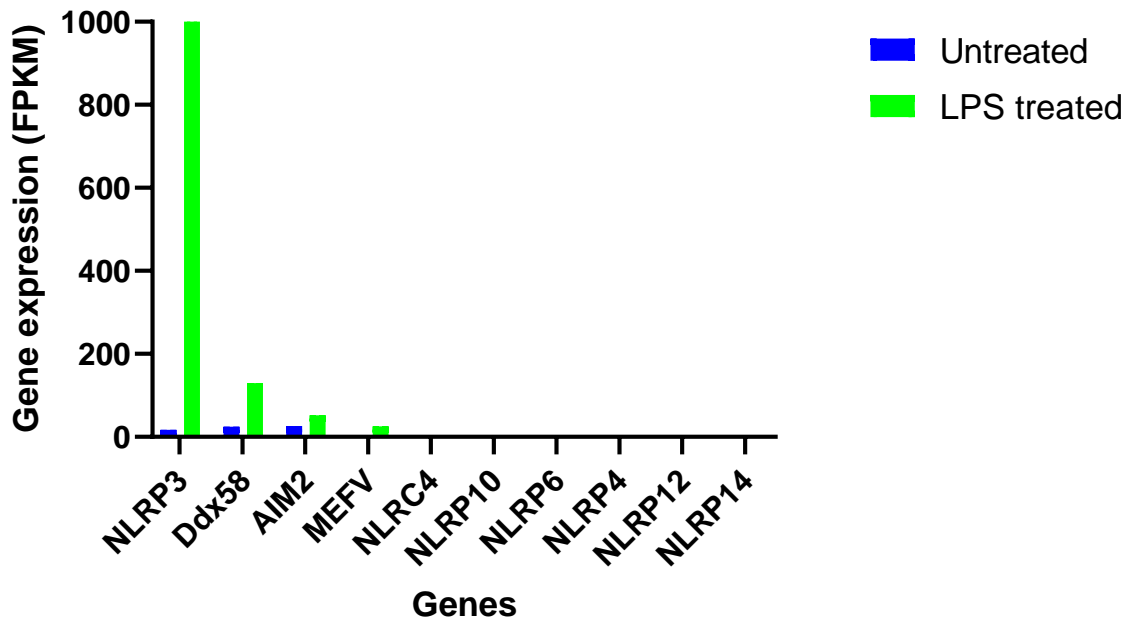
Supplementary figure 2 Gating strategy for comparison of ASC Citrine WT and Casp1^{-/-} mouse BMDMs stained with FVD **A** Gating for singlets on all cells **B** Gating for cells on singlets population with FSC-A and SSC-A **C** Gating for ASC width low on cells population ASC Citrine width and area **D** Gating for FVD negative and positive on ASC width low population with FVD Area and SSC-A



Supplementary figure 3 Gating strategy for dose response to MCC950 and VX765, ASC Citrine mouse BMDMs stained with FVD **A** Gating for cells on singlets population with FSC-A and SSC-A **B** Gating for fixable viability dye on cells population with FVD and SSC-A **C** Gating for ASC width low on FVD – population with ASC width and ASC area

# of cells per well	20x10 ³	10x10 ³	5x10 ³	2,5x10 ³
Z' value	0,65	0,24	-0,30	-7,34

Supplementary figure 4 Optimization of number of cells per well for the microscopy based approach based on Z value



Supplementary figure 5 Inflammasome sensor gene expression in mouse bone marrow derived macrophages treated with LPS or untreated, quantified by RNA sequencing (Ruaidhrí Jackson)