Group B streptococcal membrane vesicles induce proinflammatory cytokine production and are sensed in an NLRP3 inflammasome-dependent mechanism in human macrophages

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ABSTRACT

Group B *Streptococcus* (GBS) is a major cause of fetal and neonatal mortality worldwide. Many of the adverse effects associated with invasive GBS are associated with inflammation that leads to chorioamnionitis, preterm birth, sepsis, and meningitis; therefore, understanding bacterial factors that promote inflammation is of critical importance. Membrane vesicles (MVs), which are produced by many pathogenic and non-pathogenic bacteria, may modulate host inflammatory responses. In mice, GBS MVs injected intra-amniotically can induce preterm birth and fetal death. Although it is known that GBS MVs induce large-scale leukocyte recruitment into infected tissues, the immune effectors driving these responses are unclear. Here, we hypothesized that macrophages respond to GBS-derived MVs by producing proinflammatory cytokines and are recognized through one or more pattern recognition receptors. We show that THP-1 macrophage-like cells produce high levels of neutrophil- and monocyte-specific chemokines in response to MVs derived from different clinical isolates of GBS. Interleukin (IL)-1ß was significantly upregulated in response to MVs, which was independent of NF-kB signaling but dependent on both caspase-1 and NLRP3. These data indicate that MVs contain one or more pathogen-associated molecular patterns that can be sensed by the immune system. Furthermore, this study identifies the NLRP3 inflammasome as a novel sensor of GBS MVs. Our data additionally indicate that MVs may serve as immune effectors that can be targeted for immunotherapeutics, particularly given that similar responses were observed across this subset of GBS isolates.

INTRODUCTION

Group B *Streptococcus* (GBS) is an opportunistic pathogen that colonizes the vaginal or rectal tract of ~30% of women (1). While maternal colonization is often asymptomatic, GBS can cause severe infections in pregnant women and neonates (1). Pregnancy- and neonatal-associated GBS infections are often characterized by pathologies exhibiting a high degree of inflammation. During pregnancy, this can present as placental villitis and preterm birth, whereas in neonates, GBS can cause meningitis and sepsis (2-4). Despite the high colonization frequencies in mothers, only a fraction of women and their neonates develop these threatening infections. The reasons for this discrepancy, however, are incompletely characterized.

We and others have postulated that strain variation contributes to the discrepancy in disease outcome. Indeed, specific phylogenetic lineages of GBS, which are defined by multilocus sequence typing (MLST) are more likely to cause neonatal infections (5-7). Notably, sequence type (ST)-17 strains are more commonly associated with invasive neonatal infections (5, 8, 9), whereas ST-1 strains are associated with invasive disease in adults (10). Conversely, ST-12 strains have been linked to asymptomatic maternal colonization (11). We demonstrated that ST-17 strains elicit stronger proinflammatory immune responses and persist longer inside macrophages than other strains (12, 13). Interestingly, we also found that ST-1 and ST-17 strains induce stronger activation of the proinflammatory transcription factor NF-kB compared to ST-12 strains (13). While ST-17 strains were previously found to have unique virulence gene profiles relative to other lineages, the specific bacterial factor(s) promoting these altered inflammatory responses are not fully understood (14-16).

Recently it was reported that GBS produces membrane vesicles (MVs) that can induce substantial recruitment of neutrophils and lymphocytes into murine extraplacental membranes, which mimicked GBS-associated chorioamnionitis in humans (17, 18). In support of this finding,

GBS MVs were shown to induce production of the neutrophil chemokine CXCL1 in a murine model of *in utero* infection (17, 19), which has been shown in other GBS infection models (20, 21). Further, we recently reported that GBS MV production varies in abundance and protein composition across STs (17, 19, 22). More specifically, several immunomodulatory virulence factors, including hyaluronidase, C5a peptidase, and sialidase were highly and differentially abundant across STs (22). Together these data indicate that MVs promote proinflammatory immune responses; however, no prior studies have comprehensively examined the mechanisms by which human leukocytes respond to GBS MVs.

As sentinel leukocytes at the maternal fetal interface, macrophages play an important role in shaping immune responses. At the maternal-fetal interface macrophages make up 20-30% of leukocytes (23) and play pivotal roles in fertility (24), placental function (25), and host-pathogen interactions at the maternal-fetal interface (26-28). The THP-1 monocytic leukemia cell line can be differentiated with phorbol esters into macrophage-liked cells (29) and serve as a model system to evaluate host responses to GBS (12, 30). Using this model, we previously showed that THP-1 cells produce high levels of proinflammatory cytokines in response to GBS. Interestingly, several cytokines displayed lineage-specific inflammatory responses, with ST-17 strains eliciting a more potent inflammatory response compared to other lineages (13). Here, we examined macrophage responses to GBS MVs isolated from a diverse set of strains and found that these MVs induce the production of proinflammatory cytokines and chemokines. We also identified NLRP3 as a sensor of GBS derived MVs. In all, this study has expanded our current understanding of how host cells respond to GBS MVs. Additionally, by identifying the pathways upregulated by MVs, we have identified the proinflammatory pathways and receptors that could be used as potential immunotherapeutic targets.

METHODS

Bacterial Strains and Culture

GBS strains GB0037 (GB37), GB0411 (GB411), GB0653 (GB653), and GB1455 were isolated as described previously (31, 32). The invasive isolates GB37, GB411, and GB1455, were isolated from the blood or cerebrospinal fluid of infants with early onset GBS disease (31), while the colonizing strain GB653 was isolated from vaginal/rectal swabs collected from an asymptomatically colonized mother before childbirth (32). These isolates were previously characterized by MLST and capsular serotyping (9, 11). The GBS strains analyzed here represent colonizing and invasive isolates belonging to each of three common STs: ST-1 (GB37), ST-12 (GB1455 and GB653), and ST-17 (GB411). Strains were cultured using Todd-Hewitt Broth (THB) or Todd-Hewitt Agar (THA) (BD Diagnostics, Franklin Lakes, New Jersey, USA) overnight at 37°C with 5% CO_2.

Membrane vesicle isolation

MVs were isolated as previously described (22). Briefly, overnight THB cultures were diluted 1:50 into fresh broth and grown to late logarithmic phase (optical density $(OD)_{600} = 0.9$). Cultures were centrifuged at 2000 x g for 20 minutes at 4°C. Supernatants were collected and re-centrifuged at 8500 x g for 15 minutes at 4°C, followed by filtration through a 0.22µm filter and concentration using Amicon Ultra-15 centrifugal filters (10 kDa cutoff) (MilliporeSigma, Burlington, MA, USA). Concentrated supernatants were subjected to ultracentrifugation for 2 hours at 150,000 x g at 4°C. Pellets were resuspended in PBS and purified using qEV Single size exclusion columns (IZON Science, Christchurch, New Zealand) per the manufacturer's

92 instructions. MV fractions were collected and re-concentrated using the Amicon Ultra-4
93 centrifugal filters (10 kDa cutoff) (MilliporeSigma, Burlington, Massachusetts, USA) and
94 brought to a final volume of 100 µL in PBS. MVs were aliquoted and stored at -80°C until
95 further use.
96
97 **Nanoparticle Tracking Analysis**
98 MVs were quantified via nanoparticle tracking analysis using a NanoSight NS300 (Malvern
99 Panalytical Westborough, MA, USA) equipped with an automated syringe sampler as described
100 previously (22, 33, 34). For each sample, MVs were diluted in PBS (1:100 – 1:1000) and
101 injected with a flow rate of 50. Once loaded, five 20-second videos were recorded at a screen
102 gain of 1 and camera level of 13, which were analyzed at a screen gain of 10 and a detection
103 threshold of 4 after capture. Data were subsequently exported to a CSV file for analysis using the
104 R package tidyNano (33).
105
106 **THP-1 Cell Culture**
107 THP-1 cells (TIB-202) were obtained through ATCC (Manassas, VA) and stored according to
108 vendor guidelines (35). Briefly, cells were cultured in RPMI 1640 (Gibco, ThermoFisher,
109 Waltham, MA) supplemented with L-Glutamine, 10% fetal bovine serum (FBS), and 1%
110 antibiotic-antimycotic (100 µg/mL Streptomycin, 0.25 ug/mL Amphotericin B, & 100 U/mL
111 Penicillin; Gibco, ThermoFisher, Waltham, MA) as previously described (12, 13). For
112 experiments, THP-1 cells were only utilized until passage 10. When indicated, THP-1 monocytes
113 were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) as previously

114 described (12, 13). Cells were differentiated in RPMI (without phenol red) supplemented with L-
115 Glutamine, 2% FBS and 100 nM PMA for 24 hours prior to experimentation (12, 13).
116
117 For experiments using GBS treated cells, THP-1 cells were washed twice with PBS prior to
118 infection. The bacteria were resuspended in RPMI and added to the THP-1 cells at a multiplicity
119 of infection (MOI) of 10 bacteria per cell. Cells were incubated for 1 hour and the media was
120 subsequently aspirated. Cells were washed thrice with PBS and fresh RPMI with L-Glutamine
121 (no phenol red) containing 2% FBS, 100 nM PMA, penicillin (5 µg/mL) and gentamicin
122 (100µg/mL) was added (termed RPMI 2/0). Cells were incubated for an additional 24 hours. For
123 MV treatment, cells were washed twice, and fresh RPMI 2/0 containing MVs at an MOI of 100
124 MVs per differentiated macrophage was added and incubated for 25 hours. Cells were treated
125 with LPS (1 µg/ml, clone L2654, Millipore Sigma, Burlington, MA) to serve as positive controls.
126 At the end of each treatment period, supernatants were collected, centrifuged for 10 minutes at
127 4000 rev/min at 4°C and aliquoted. Samples were stored at -80°C until used for downstream
128 analysis.
129
130 **Cytokine and Cytotoxicity Analysis**
131 For semiquantitative analysis of cytokines in supernatants from THP-1 cultures, we employed a
132 human cytokine antibody microarray (ab133998, Abcam, Cambridge, UK) according to
133 manufacturer's instructions as previously described (13). Cells were seeded into 6-well plates at
134 a density of 4 x 10^6 per well and treated as described above. Membranes were imaged using an
135 Amersham Imager 600 (GE Life Sciences), and densitometry was performed using ImageJ
136 software. Cytokines falling above a fold change of 2 relative to mock treated were considered

upregulated and further analyzed. For subsequent analyses of cytokine production, caspase-1 activation, and cell death, cells were seeded into 12-well plates at a density of 2 x 10^6 cells per well and treated as described above. Cytokines with more than a 2-fold change relative to mock-treated cells were verified using a custom ProcartaPlex bead assay (ThermoFisher, Waltham, MA) as described by the manufacturer. These assays were read and analyzed using a Luminex 200 and Luminex xPONENT v3.1 software, respectively (Luminex Corp., Austin, Texas). Cellular cytotoxicity was assessed using a CyQuant lactate dehytrogenase (LDH) assay (Invitrogen, Waltham, MA) per the manufacturer's instructions.

Immunofluorescence Staining and Microscopy Analysis

THP-1 cells were differentiated into 4-well Nunc Lab-Tek II Chamber slides (ThermoFisher, Waltham, MA) at a density of 10^5 cells per well and differentiated as described above. Cells were treated with either MVs (MOI 100) or LPS (1 μg/mL) for 0.5 or 2 hours and stained for the NF-κB subunit p65 by immunofluorescence as described (36). Briefly, the cells were fixed using 4% paraformaldehyde in PBS for 10 minutes, washed three times with ice cold PBS, and permeabilized for 10 minutes in 0.2% Triton-X in PBS. Cells were washed three more times in PBS and blocked in 10% goat serum/1% BSA/0.3% Tween in PBS for 20 minutes. Rabbit anti-NF-kB antibody (1:1600; clone D14E12; Cell Signaling Technology, Danvers, MA) was added to cells and incubated overnight at 4°C. Cells were washed three times and incubated with Alexa Fluor Goat anti-rabbit 546nm secondary antibody (10 μg/mL; Invitrogen, Waltham, MA) for 1 hour, and washed again in PBS. Coverslips were mounted using Vectashield DAPI (Vector Laboratories, Inc., Burlingame, CA), and representative images were obtained using a Nikon

Eclipse Ti outfitted with a 20x plan fluor objective. Immunofluorescent microscopy was performed in biological triplicate for each timepoint and treatment.

Caspase-1 Activity, Responses, and Inhibition

After treatment of THP-1 cells, caspase-1 activity was quantified in supernatants using a commercially available assay (Caspase-GLO 1 Assay; Promega, Madison, WI) according to the manufacturer's instructions. Caspase-1 activity in supernatants was quantified using a GloMax Navigator (Promega, Madison, WI). To assess the impact of caspase-1 on MV-induced IL-1β production, PMA-differentiated THP-1 cells were seeded into 12-well plates and pretreated with 50μM of the caspase-1 inhibitor, Ac-YVAD-CHO (Cayman Chemical Company, Ann Arbor, MI), or 10 μM of the NLRP3 inhibitor, MCC950 (Invitrogen, Waltham, MA), for 30 minutes. Cells were treated with either LPS or GBS as described above, and IL-1β concentrations were measured using a ProcartaPlex simplex assay (ThermoFisher, Waltham, MA).

qPCR Analysis

PMA-differentiated THP-1 cells, which were seeded in 12-well plates at a density of 2×10^6 cells per well, were left untreated or treated with either GBS bacteria, LPS, or MVs as described above. After 2 or 4 hours, supernatants were aspirated and cells were lysed by adding 1mL Trizol reagent (Invitrogen, Waltham, MA) and gentle scraping. Samples were stored at -20ºC until RNA extraction was performed using Phase Lock gel heavy tubes per manufacturer's instructions (Quanta Bio, Beverly, MA). RNA was quantified using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -20 ºC until use. Reverse transcription was performed on 0.5 μg of total RNA using Quantitect Reverse Transcription Kit

(Qiagen, Hilden, Germany), and 2 µL of the resulting cDNA was amplified by PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA) with Taqman probes specific for pro-IL-1β (Assay ID: Hs00174097_m1) and GAPDH (Assay ID: Hs99999905_m1). PCR was performed in a QuantStudio 5 real time thermal cycler for 35 cycles (Applied Biosystems, Waltham, MA).

Data analysis

Data analysis was performed using RStudio. Shapiro-Wilk tests were used to determine whether data followed a normal distribution. Normally distributed data were analyzed for significance using a two-way analysis of variance (ANOVA), followed by a Tukey HSD *post hoc* test. Alternatively, non-parametric data were analyzed using a Kruskal-Wallis test, followed by Dunn's *posthoc* test to test for differences between groups. Multiple hypothesis testing was corrected using Benjamini-Hochberg or Bonferroni correction when necessary. The analyses used for individual experiments are denoted in the figure legends for clarity.

RESULTS

GBS MVs elicit proinflammatory cytokine responses

We first sought to characterize the cytokine response elicited by MVs from a diverse set of GBS strains representing major STs in clinical circulation. Specifically, we characterized the cytokine response to MVs from an ST-1 strain (GB37), two ST-12 strains (GB653 and GB1455), and one ST-17 strain (GB411). Of note, GB37, GB1455, and GB411 were all isolated from infants with invasive infections, whereas GB653 was isolated from an asymptomatically colonized mother. Human cytokine antibody microarrays revealed that MVs from GB411 and GB653 induced cytokine production from THP-1 macrophages. Of the 80 cytokines and chemokines assayed, 7 were upregulated at least 2-fold in comparison to the untreated cells (Supplemental Figure 1). Cytokines upregulated in responses to MVs included the monocyte and neutrophil chemokines, CCL1, CCL2, CXCL1, CCL20, the pyrogen IL-1β, and the proinflammatory cytokine IL-6 (Figure 1, Supplemental Figure 1). Several cytokines were also induced differentially between the two isolates: MVs from GB411 induced CXCL1, CCL1, and IL-1ß more strongly than MVs from GB653 (Figure 1). However, the same trend was not observed when comparing cytokines between bacteria-treated THP-1 cells since GB411 and GB653 elicited similar cytokine responses for each of these targets (Figure 1).

To validate these differences in cytokine production, we used quantitative Luminex-based assays. Consistent with previous results (13), GBS induced a potent proinflammatory response relative to untreated controls (Supplemental Figure 2-3), though IL-6 production remained unchanged by MV exposures (Supplemental Figure 3). Moreover, the MVs induced CCL1, CCL20, CXCL10, CXCL1, and IL-1ß, with no differences between the strains from which the MVs were derived (Figure 2). While CCL2 displayed an elevated response relative to

mock treatment, this induction was only significant for MVs produced by GB37, GB411, and GB1455.

Next, we assessed cytotoxicity for all strains examined above using a lactate dehydrogenase activity assay to ensure that these responses were not biased due to differential cell death. In these analyses, we found that bacteria induced moderate cytotoxicity that varied slightly across bacterial strains (Supplemental Figure 4). Notably, GB37 induced significantly more cytotoxicity than GB1455; however, this cytotoxicity was modest. Although low levels of cytotoxicity were observed during MV treatments, with an average of ~6%, the cytotoxicity levels did not vary across MVs produced by the four different GBS strains.

Membrane vesicles induce caspase-1 activation

Since IL-1ß was significantly increased in response to all GBS MVs regardless of the strain, we sought to classify the inflammatory pathways that impact its production. Using the Caspase-GLO 1 assay, we detected caspase-1 activity in our untreated controls as well as our LPS-stimulated control, albeit at a substantially higher magnitude in our LPS control (Supplemental Figure 5). Detectable caspase-1 activity was also observed in response to MVs and the GBS strains, though some differences were noted. Compared to untreated controls, MVs from GB37, GB411, and GB1455 induced the most potent caspase-1 responses, providing confirmation that MVs were capable of inducing caspase-1 activity (Figure 3A, Supplemental Figure 5). Similarly, GB411 bacteria induced a higher degree of caspase-1 activation compared to untreated controls, which is consistent with our previous findings (Supplemental Figure 6).

Next, we sought to determine if alternative pathways may be contributing to the conversion of pro-IL-1β to mature active IL-1β. To assess this, we pretreated THP-1 cells with

the capsase-1 inhibitor Ac-YVAD-CHO prior to treatment with MVs or LPS for 25 hours. We found that LPS and untreated controls both produced lower amounts of IL-1β when pretreated with Ac-YVAD-CHO compared to the vehicle controls (83% and 90% reduction, respectively); Figure 3B). Furthermore, inhibition of caspase-1 by Ac-YVAD-CHO resulted in almost complete abrogation of MV-stimulated IL-1β secretion (91% reduction) compared to the vehicle control. Importantly, alterations in IL-1β production were not associated with cell death (Supplemental Figure 7). This finding therefore demonstrates that caspase-1 activation is necessary for the maturation of pro-IL-1β to mature IL-1β in response to GBS MVs, regardless of the strain type (Figure 3B).

NLRP3 is essential for MV mediated IL-1B secretion

Having established that caspase-1 is required for IL-1β maturation, we next investigated the upstream sensor of MVs. Because GBS triggers inflammasome activation via a NLRP3-dependent mechanism, we assessed whether inhibition of NLRP3 could impact caspase-1 activation in response to GBS MVs (37). Notably, inhibition of NLRP3 with the MCC950 inhibitor prevented both MV- and GBS-induced caspase-1 activity (Figure 4A and Supplemental Figure 8). We observed a similar trend in our control cells, demonstrating some baseline inflammasome activity in THP-1 cells; however, the magnitude of inflammasome activation was lower in control groups (Figure 4A). Inhibition of NLRP3 also reduced cytotoxicity for both the GBS bacteria- and MV-treated cells; however, this result was not observed for our untreated controls (Figure 4B).

Using a similar approach, we also assessed whether NLRP3 impacted secretion of IL-1β from THP-1 cells. In these experiments, we found that inhibition of NLRP3 signaling

274 significantly decreased IL-1β secretion in both the media and LPS controls relative to the vehicle
275 controls (Figure 4C). While the decrease was significant in both groups, the effect was lower for
276 the untreated controls. Moreover, NLRP3 inhibition reduced IL-1β secretion in response to both
277 GBS and the MVs demonstrating that MV-induced IL-1β requires NLRP3 (Figure 4C).
278
279 **Membrane vesicles do not trigger transcription activation of pro-IL-1B**
280 We next assessed whether the high levels of IL-1ß produced in response to GBS MVs
281 were due to the release of existing pools of pro-IL-1ß, or if MVs could directly induce
282 transcription of pro-IL-1ß. Using RT-qPCR analysis, we observed no significant increase in pro-
283 IL-1ß gene expression relative to untreated cells for LPS, MV, or bacteria treated THP-1 cells at
284 2 hours post infection (Figure 5A). At 4 hours post infection, however, LPS induced a significant
285 increase in pro-IL-1ß gene expression relative to untreated cells, but no similar increases were
286 observed in response to MVs or GBS (Figure 5A). Using immunofluorescence, we similarly
287 found that while LPS rapidly induced the translocation of the NF-κB subunit p65, neither
288 untreated nor MV treated THP-1s induced NF-κB translocation. Notably, MVs induced no NF-
289 κB translocation in response to MVs after a 2-hour exposure (Figure 5B). Similar results were
290 observed at 30 minutes post exposure (Supplemental Figure 9). Together, these data indicate that
291 MVs do not induce a largescale alteration in pro-IL-1ß gene expression or NF-κB activation,
292 suggesting that elevated IL-1β secretion is likely due to post-translational regulation.
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294
295
296

DISCUSSION

Previous studies demonstrated that *in utero* exposure to GBS MVs induced recruitment of neutrophils and lymphocytes into the gestational membranes (17), while MVs induced neutrophil recruitment to the lung in a neonatal sepsis model (19); however, the signals that perpetuate the influx of leukocytes remains unclear. Herein, we have demonstrated that MVs induce expression of proinflammatory cytokines and chemokines in human macrophages *in vitro*, which likely contribute to the inflammatory infiltrate observed *in vivo* (17, 19). Additionally, we found that MVs induce the production of IL-1β by activating pro-IL-1β maturation in an NLRP3 and caspase-1 dependent manner, but independently of NF-κB signaling.

By expanding our current understanding of the cytokine responses towards GBS derived MVs, we have identified the modulators that likely impact the adverse pathologies observed *in vivo*. A previous study demonstrated that the murine chemokine KC, known as CXCL1 in humans, was upregulated in response to GBS MVs (17). In support of these findings, we demonstrate that production of CXCL1 and many additional chemokines are upregulated in human macrophages following challenge with GBS MVs. Notably, CCL1, CCL20, CXCL1, and CXCL10 were all significantly upregulated in response to MVs from four clinical strains. Similarly, the chemokine CCL2 was significantly elevated in response to three of the clinical strains we examined. These chemokines are critical for recruitment of leukocytes to sites of infection, with varying target cell specificities. CXCL1 and CCL20, for example, attract neutrophils (38-40), whereas CCL1 and CCL2 attract monocytes and macrophages (41, 42). Additionally, CCL20 and CXCL10 recruit lymphocytes (40, 43). Unsurprisingly, many of the cytokines have been implicated in GBS-associated disease. For example, CCL20 is upregulated during infection at the blood brain barrier (44). Similarly, CCL2 has been shown to be strongly

upregulated during GBS sepsis cases (45). Taken together these data indicate that GBS MVs serve as a critical initiator of disease associated cytokine responses.

Another cytokine that was significantly upregulated in response to MVs was the pyrogen IL-1ß, which plays a critical role in the host defense to GBS infections by promoting production of additional neutrophil specific chemokines (20, 46). Although IL-1ß does not have direct chemoattractant activity, IL-1ß signaling does impact the production of CXCL1 in GBS infections (20). In fact, IL1R knockout mice display reduced neutrophil recruitment and significant increases in mortality when challenged with GBS (46). Given the abundant recruitment of neutrophils and lymphocytes into MV challenged tissues (17), these data provide critical insights into the mechanisms driving this leukocyte infiltration. Although we and others have shown strain variation in IL-1ß production in response to whole bacteria (13, 47), here we found that MVs consistently elicited a consistent level of IL-1ß from human macrophages, suggesting that it may serve as an important biomarker or possibly a therapeutic target.

Previous studies have highlighted the signaling pathways involved in producing mature IL-1ß. Notably, high levels of this cytokine were only produced when both TLR (toll-like receptor) signaling and inflammasome activation occurred (48). TLR signaling occurs when pathogen-associated molecular patterns (PAMPs) engage their cognate receptor (49, 50), which results in the induction of proinflammatory gene expression, including the inactive form of this cytokine, pro-IL-1ß (50). Canonically, the induction of pro-IL-1ß gene expression depends on translocation of the transcription factor NF-κB, into the nucleus (51).

For pro-IL-1ß to be secreted in its mature, active form, a second signal is required. This signal is typically in the form of a danger associated molecular pattern (DAMPs), such as a change in membrane potential due to membrane damage (52, 53). DAMPs are sensed by NLRPs

(Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain-containing receptors) (52, 54). Once sensed, NLRPs oligomerize with other subunits, forming the inflammasome, (52, 55, 56) which cleaves pro-caspase-1 into its mature, active form (56, 57). Active caspase-1 then cleaves pro-IL-1ß, triggering its release (57). This concerted process results in release of stored pools of pro-IL-1ß, allowing for rapid immune activation.

Notably, we demonstrate that GBS MVs trigger caspase-1 activation in human macrophages and that the secretion of IL-1ß is dependent on caspase-1 activation. Our findings further indicate that MVs do not trigger expression of pro-IL-1β or activation of NF-κB, suggesting that IL-1ß production in response to MVs is largely due to post-transcriptional regulatory mechanisms. Interestingly, we also found that caspase-1 activation is ablated in the absence of NLRP3, suggesting that NLRP3 is a sensor of GBS MVs. While previous reports have demonstrated that GBS induces IL-1β production in a NLRP3-dependent manner, this is the first study to demonstrate that GBS MVs contribute to this response (28, 37, 58). Furthermore, we are not aware of any other studies that have identified a pattern recognition receptor capable of sensing GBS MVs. This newfound information may allow for the development of receptor antagonist therapies targeting the NLRP3 dependent recognition of GBS MVs, which could prevent host inflammation and subsequent adverse pregnancy outcomes.

Our data also indicate that inhibition of the NLRP3 inflammasome reduces MV-induced cytotoxicity of macrophages *in vitro*. Several studies have shown that GBS virulence factors, such as hemolysin can induce NLRP3-dependent pyroptosis (37, 58-60). Other studies indicate that GBS mediated pyroptosis is mediated by the activation of the pore forming mediator of pyroptosis, gasdermin D (61, 62). In our examination of THP-1 macrophages, both the MVs and bacteria mediated a modest amount of cell death, which was dependent on the NLRP3

366 inflammasome, suggesting that MVs can induce pyroptosis, which could suggest gasdermin D
367 activation. While further studies are needed to confirm this hypothesis, the high levels of IL-1β
368 production together with NLRP3 mediated cell death indicate that MVs may be partly
369 responsible for GBS-mediated pyroptosis.

370 Our analyses also suggest that MVs do not induce pro-IL-1ß gene expression. Indeed,
371 while LPS induced a potent upregulation of pro-IL-1ß by 4 hours post-exposure, we observed no
372 upregulation of pro-IL-1ß in response to MVs or bacteria at either timepoint. Additionally, this
373 lack of induction correlated with the activation of NF-κB signaling, which suggests the
374 following: 1) MVs do not overwhelmingly induce the expression of pro-IL-1ß; and 2) the
375 upregulation of IL-1ß signaling is likely due to the activation of inflammasome signaling in
376 primed macrophages. Taken together, these data indicate that GBS MVs induce the production
377 of IL-1ß in primed macrophages, which is likely a conserved feature of GBS MVs.

378 A prior study demonstrated that GBS MVs contain active hemolysin and that MV-
379 associated hemolysin exacerbates neonatal sepsis *in vivo* (19). Although it was suggested that
380 GBS-mediated caspase-1 induction requires GBS hemolysin (37), our data indicate that MVs
381 from a non-hemolytic strain of GBS (GB0037) still induce a robust IL-1ß response and activate
382 caspase-1 (63). This finding indicates that other factors associated with MVs also induce
383 caspase-1 activation. Indeed, use of proteomics in our prior study found that MVs of different
384 genetic backgrounds contained multiple virulence factors that have been linked to inflammatory
385 responses previously (22). Several factors known to promote immune evasion, such as
386 hyaluronidase, sialidase, and C5a peptidase, were present in GBS MVs at variable levels across
387 diverse phylogenetic backgrounds (22). While these factors can diminish host sensing of GBS,
388 other MV-derived factors likely promote these inflammatory responses. Nonetheless, future

studies are required to classify the role that these other factors play in activating these signaling cascades.

Despite advancing our current understanding of the host response elicited towards GBS MVs, it is important to recognize the limitations of our study. Although no strain-specific immune responses towards GBS MVs were observed, we only examined 4 distinct clinical isolates that could have limited our ability to detect differences. Furthermore, our cytokine analysis was limited to those included in the antibody microarrays; hence, it is likely that other responses may also be important. Although our results are consistent with previous reports regarding the host response to GBS MVs (17, 19), it is possible that our system lacks the appropriate complexity to fully model the host response to GBS MVs. Indeed, although THP-1 cells have been shown to largely recapitulate the responses elicited from peripheral blood mononuclear cells, the magnitude of their responses can vary between these two systems (64). Furthermore, the use of cells in monoculture does not capture the complexity of the host responses observed *in vivo*. Therefore, future studies using alternative model systems are warranted.

Overall, data from this study enhance our understanding of how GBS MVs promote both adverse pregnancy and neonatal infection outcomes (Figure 6). It has been established that GBS MVs promote adverse outcomes partly by enhancing neutrophil recruitment (17, 19). In conditions such as chorioamnionitis, we suggest that the sensing of MVs by macrophages may promote proinflammatory immune signaling. Consistent with these findings, we have demonstrated that MVs promote the release of many neutrophil recruiting chemokines as well as the pyrogen IL-1β, which are important for neutrophil recruitment that promote tissue damage via net-osis (20, 40, 65, 66). We also demonstrate that the MV-mediated induction of IL-1β is

412 dependent on caspase-1 activation, which further promotes a proinflammatory environment.
413 Through both direct and indirect tissue damage, MVs likely play a role in weakening gestational
414 membranes, inducing chorioamnionitis, and promoting preterm labor due to enhanced induction
415 of these inflammatory responses (Figure 6). Collectively, these findings expand our
416 understanding of how the immune system respond to these bacterial components that contain
417 important virulence factors capable of initiating an inflammatory response. While the specific
418 PAMPs and DAMPs contained in MVs are not known, this study provides a foundation for
419 future studies aiming to classify the specific factors within MVs that trigger these responses.
420 These data illustrate that GBS MVs can induce potent proinflammatory cytokine
421 responses, which is due in part to the activation of the NLRP3 inflammasome. This study
422 advances our understanding of how GBS MVs interact with the host, by identifying the cytokine
423 response towards GBS MVs as well as by identifying NLRP3 as a sensor of MVs. Furthermore,
424 because these cytokine responses are largely conserved across genetically distinct clinical GBS
425 isolates, these responses may represent important targets for immunotherapy or as biomarkers for
426 disease status. Taken together, this study has provided mechanistic insight into the immune
427 response elicited towards GBS MVs.
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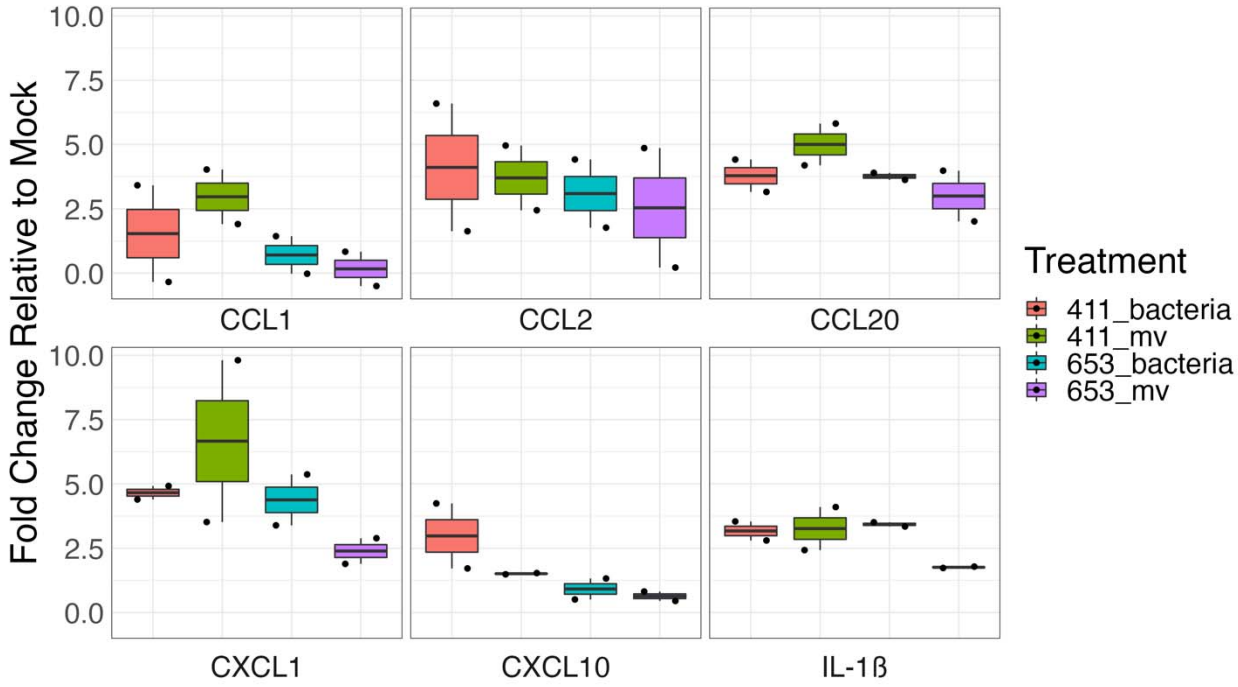
Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions Statement

CRM, MGP, and SDM designed the study; CRM performed the laboratory work and conducted the analysis; MGP, SDM, DMA, and JGA provided institutional support, guidance and resources, and CRM drafted the manuscript. All authors contributed to and approved of the manuscript content.

458 **Figures**:



459 **Figure 1: Human antibody cytokine microarray reveals highly upregulated cytokines in**

460 **response to GBS and GBS MVs.**

461 Human cytokine antibody microarrays (Abcam) were probed with supernatants from untreated,

462 bacteria-treated, or MV-treated THP-1-derived macrophages. Membrane densitometry was

463 assessed using ImageJ software. The bacterial strains used are an invasive ST-17 strain (GB411)

464 and a colonizing ST-12 strain (GB653). Shown here are hits of interest that displayed greater

465 than 2-fold change (FC) induction relative to untreated controls in at least one group. Black dots

466 indicate a single biological replicate. n = 2/treatment.

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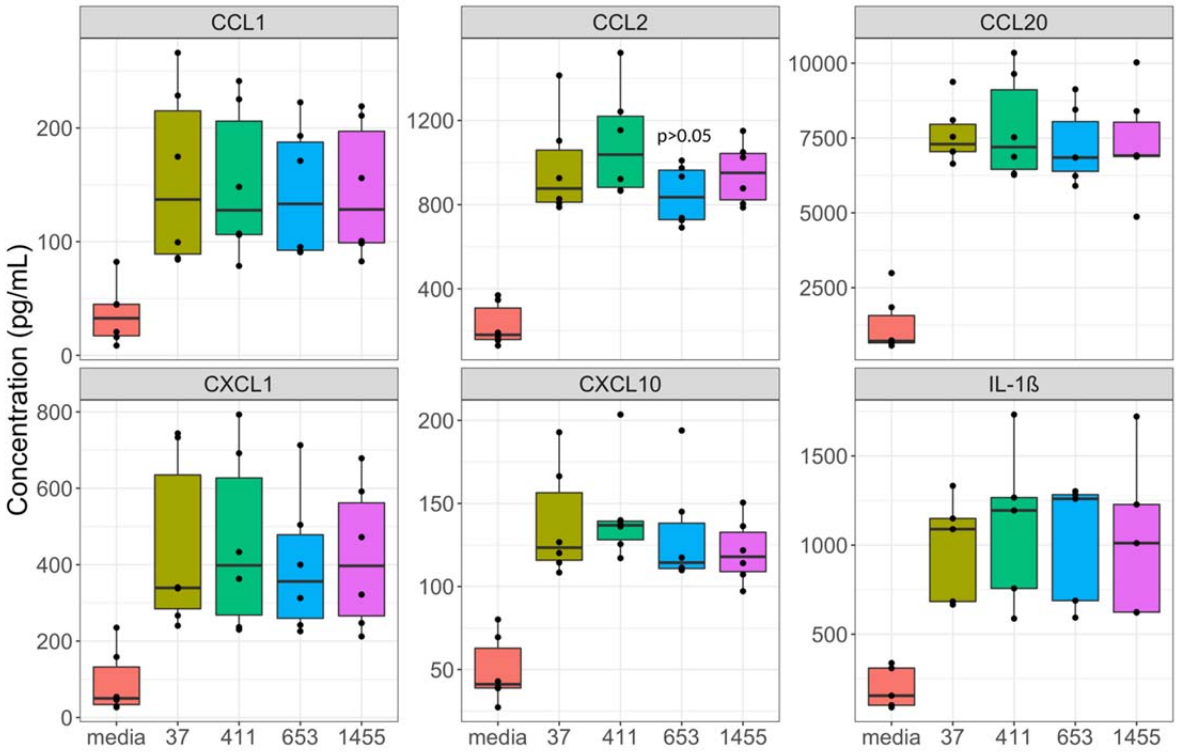

Figure 2: MVs induce proinflammatory cytokine and chemokine responses

Supernatants from THP-1 derived macrophages which were untreated or treated with MVs (MOI 100) for 25 hours were assessed for cytokine production using ProcartaPlex multiplex or singleplex (IL-1B) bead-based assays. Individual black dots indicate a single biological replicate (n = 5-6 for each group). Statistics were determined using either an ANOVA with a Tukey HSD post hoc or a Kruskal Wallis test with a Dunn Test post hoc when appropriate. All comparisons to mock treatment were significant ($p<0.05$) unless noted with a specific p-value.

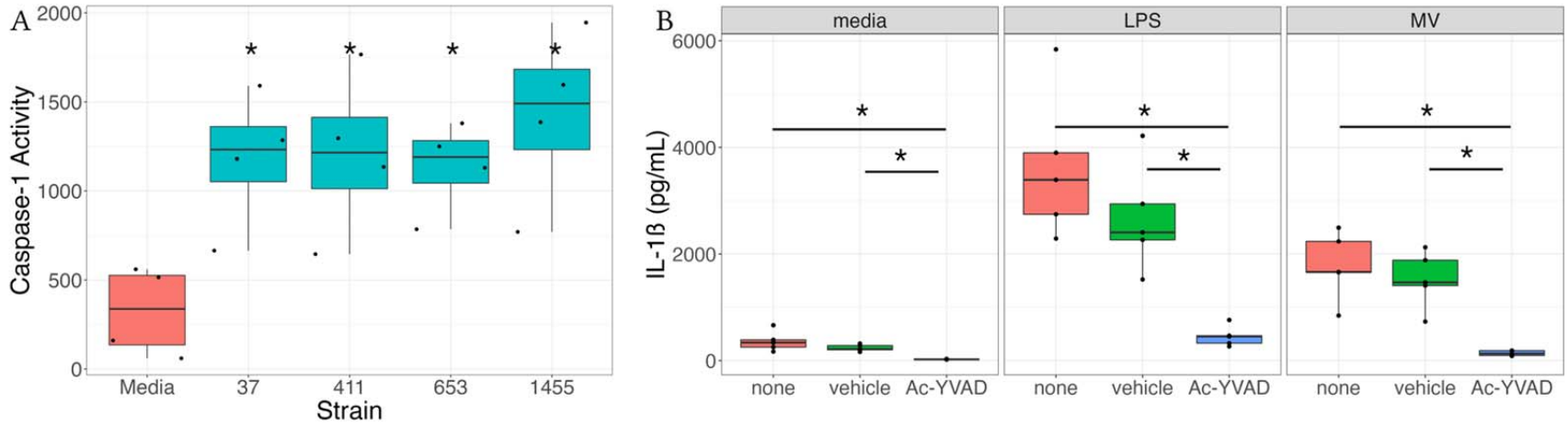

Figure 3: Caspase-1 is critical for the host response to GBS MVs

A.) THP-1 derived macrophages were unstimulated or treated with MVs for 25 hours. Supernatants were then assessed for caspase-1 activity using a caspase-1 GLO assay. Relative light units (RLU) were obtained from a GLO Max Navigator. Data represent the amount of caspase-1 activity (caspase-1 activity = (RLU GLO) - (RLU AC)) from paired samples. B.) THP-1 derived macrophages were pre-treated media, ethanol (vehicle), or Ac-YVAD-CHO for 30 minutes prior to stimulation with LPS, media, or MVs for 25 hours. Supernatants were then assessed for IL-1β concentration using ProcartaPlex bead-based assays. Individual black dots indicate a single biological replicate (n = 4 for each group). Statistical significance is defined as $p<0.05$ as calculated by ANOVA with a Tukey post-hoc and indicated by (*).

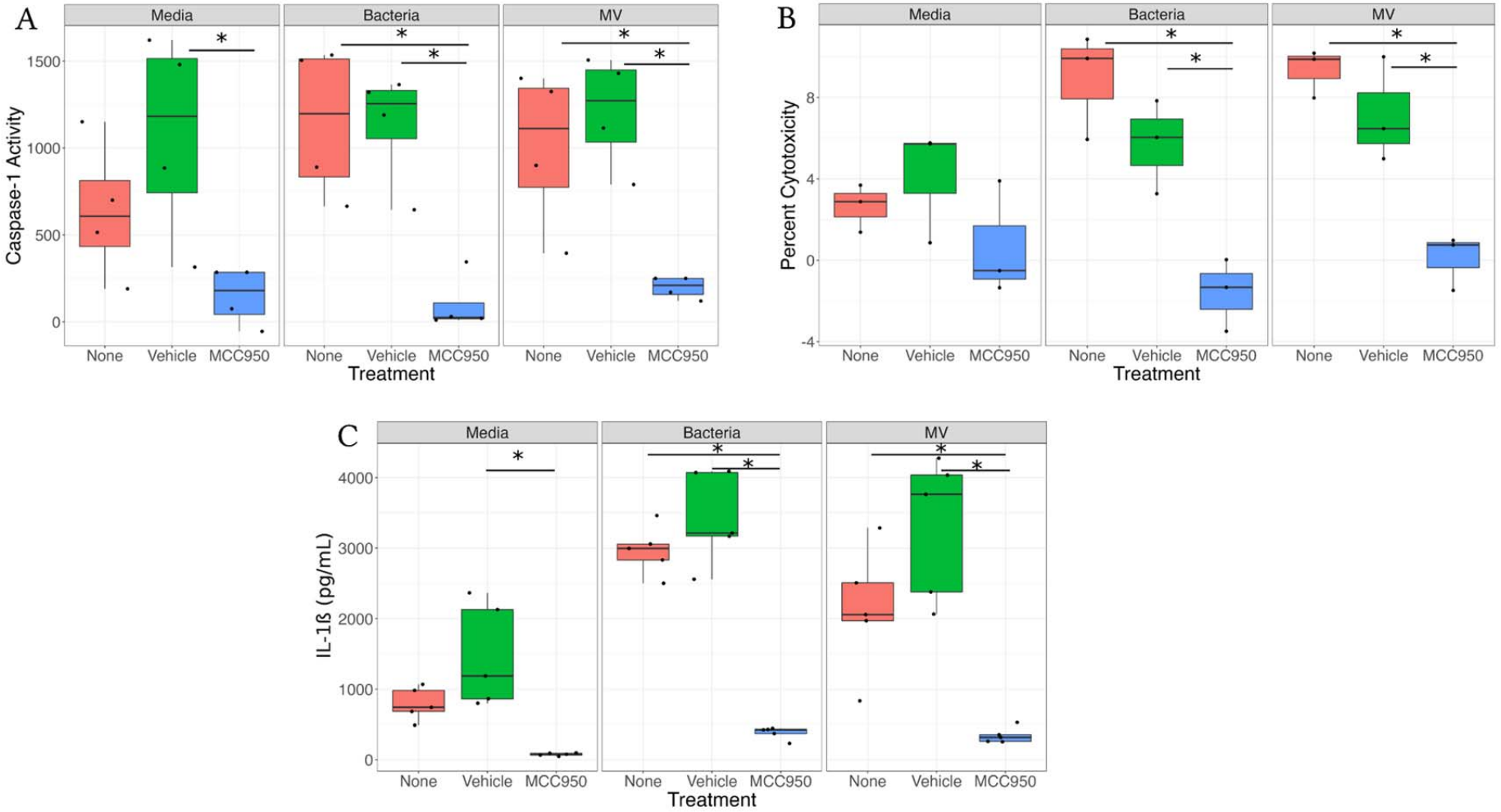

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Figure 4: Inhibition of NLRP3 Ablates Caspase-1 Activity in Response to MVs

THP-1s were treated with the NLRP3 inhibitor MCC950 or DMSO for 30 minutes prior to treatment with bacteria, MVs, or media for 25 hours. A.) Caspase-1 activity was determined using the Caspase-1 GLO assay. Caspase 1 activity = ((GLO reagent) – (Ac-YVAD-CHO + GLO Reagent)). Individual points represent individual biological replicates (n = 4 each group). B.) Supernatants were assessed for cytotoxicity using the CyQuant LDH Assay. Individual black dots indicate a single biological replicate (n = 3 for each group). C.) IL-1ß contained in supernatants was quantified using ProcartaPlex IL-1ß single plex assays. Individual points represent individual biological replicates (n = 5 each group). Statistics were determined using an ANOVA with a Tukey's HSD post-hoc test. Significance was defined as $p<0.05$ and denoted with an (*).

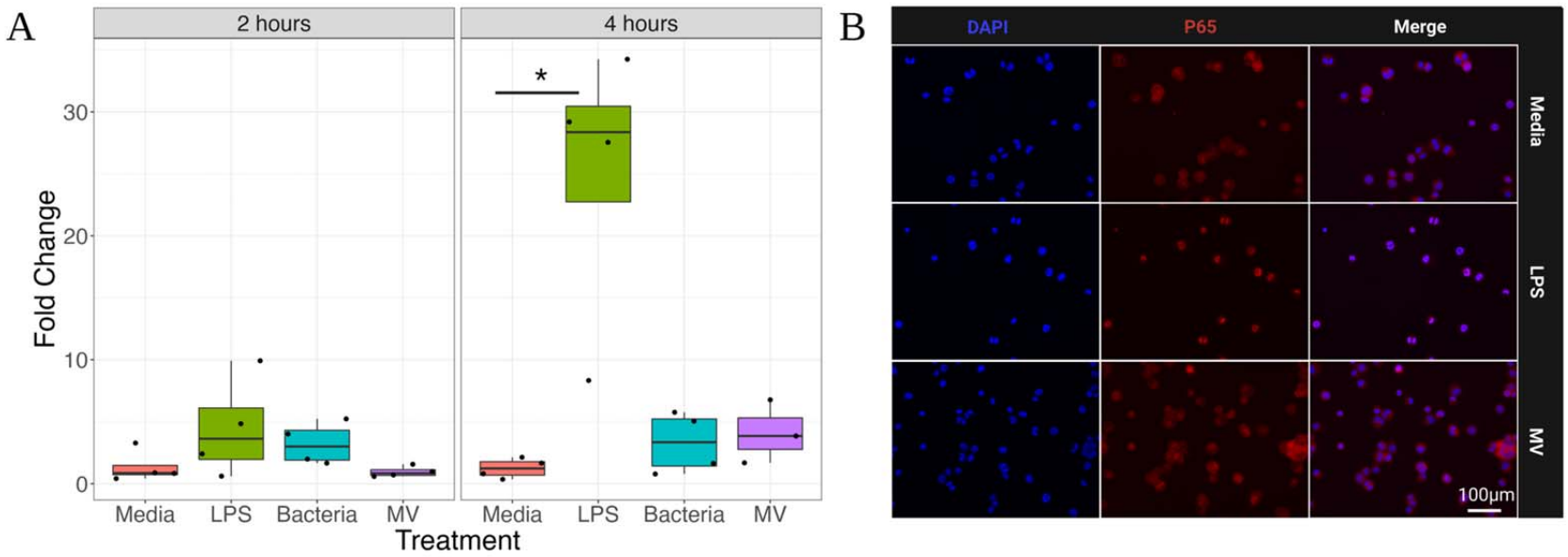


Figure 5: MVs do not prime human macrophages.

A.) Expression of pro-IL-1ß mRNA from THP-1s treated with bacteria, MVs, LPS, or media was quantified using Taqman probes at 2 and 4 hours after treatment. Fold change values were calculated relative to respective media controls. Each individual dot represents a single biological replicate (n = 4/group). Statistics are calculated using a t-test or Wilcoxson test when appropriate. Significance was defined as $p<0.05$ and denoted by (*). B.) Differentiated THP-1 derived macrophages were untreated or treated with LPS or MVs for 2 hours prior to fixation and immunofluorescence staining for NF-kB subunit p65 (stained red). Nuclei are stained using DAPI (blue). Shown here are representative images (n = 5) taken at 40x magnification.

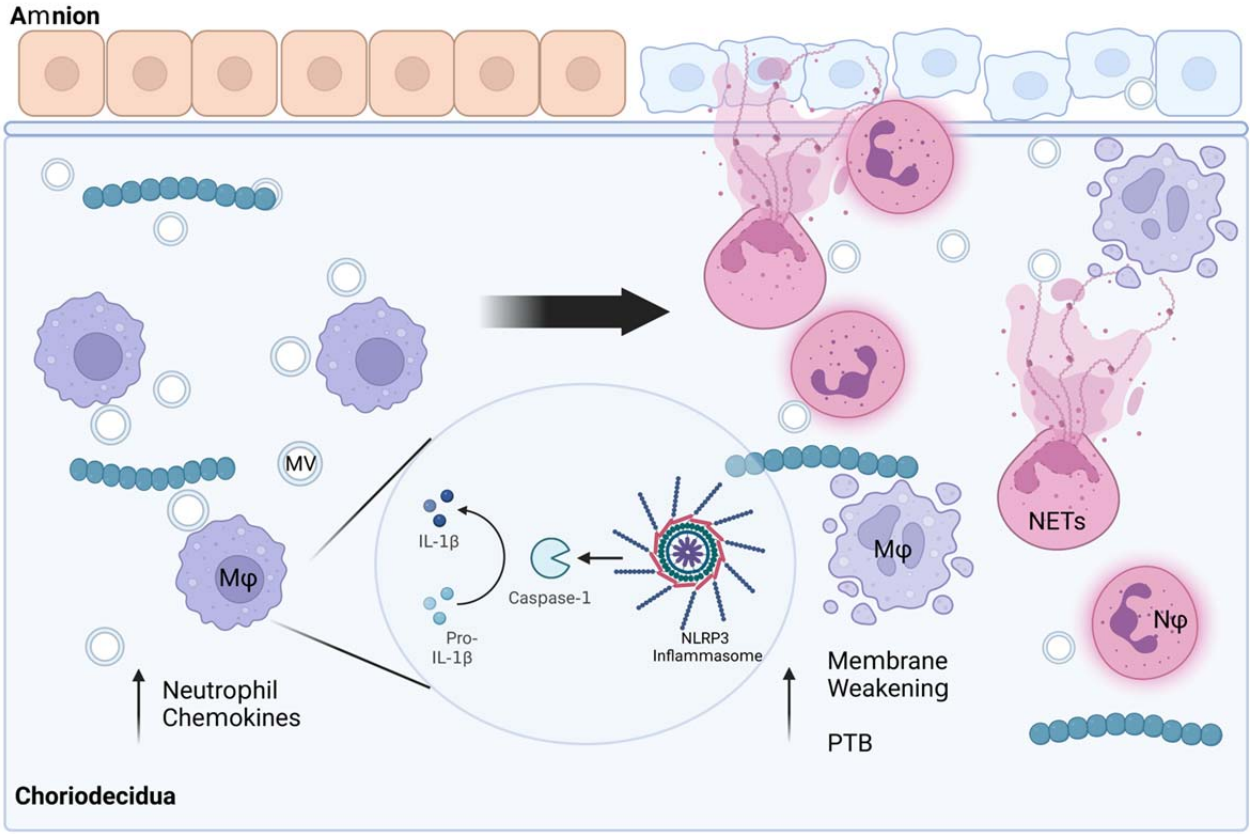
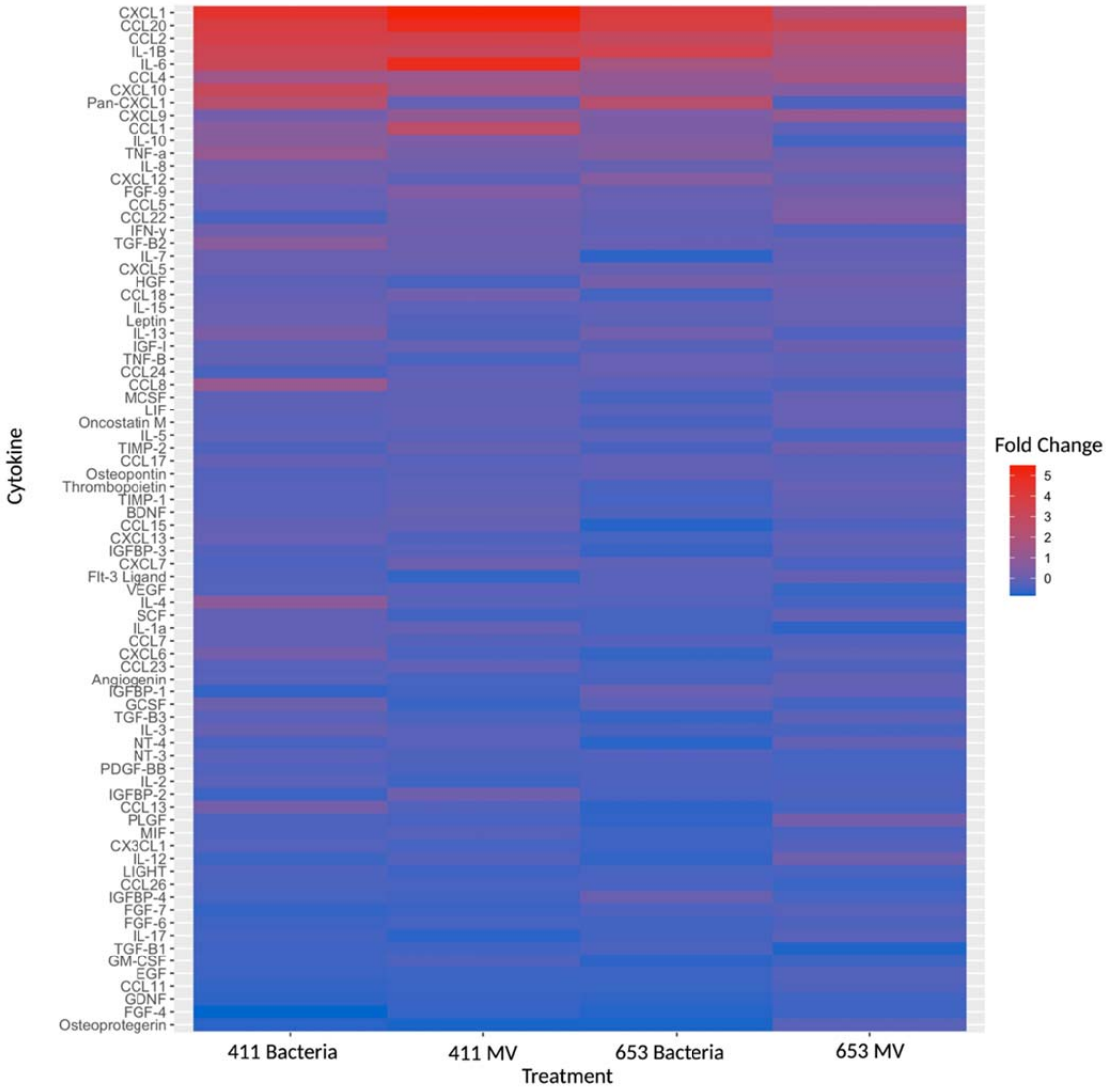


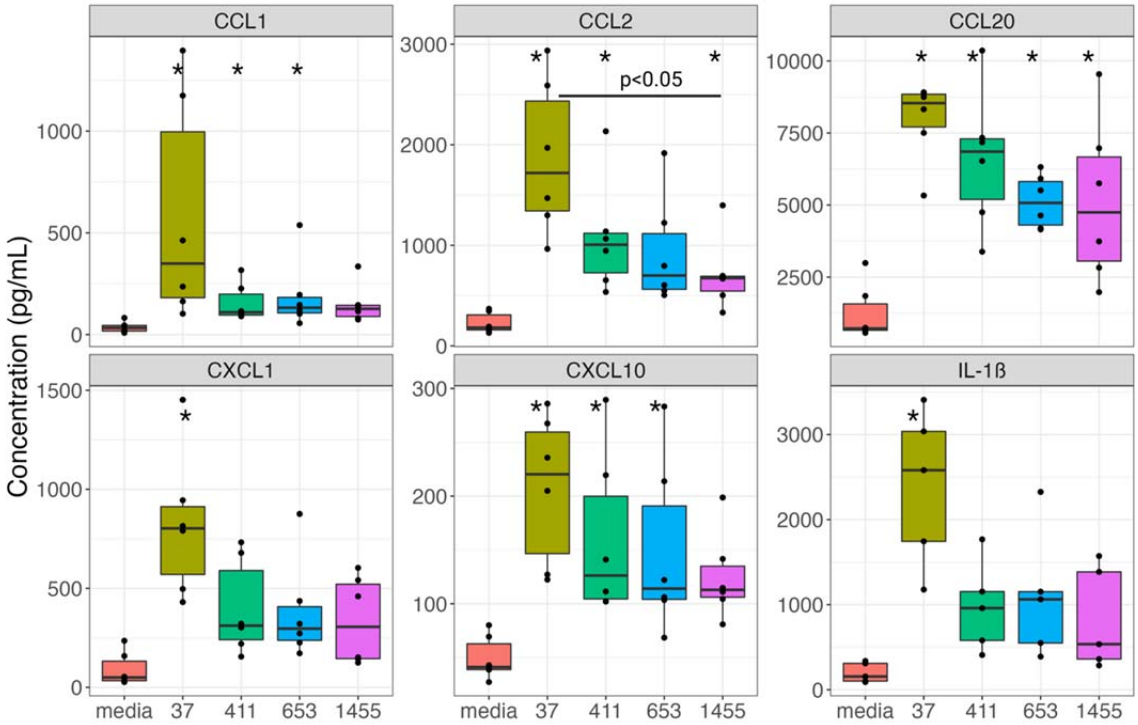
Figure 6: Model of GBS Mediated Chorioamnionitis

GBS is a frequent cause of chorioamnionitis. As sentinel cells at the maternal-fetal interface, macrophages play a critical role in shaping how inflammatory responses are initiated. We show here that macrophages respond to MVs by releasing proinflammatory cytokines and chemokines, many of which recruit neutrophils to the site of infection. Additionally, we show that MVs activate the NLRP3 inflammasome, triggering release of the pyrogen IL-1β. Together these processes promote an influx of neutrophils and leukocytes into the site of infection. In cases such as chorioamnionitis, neutrophils undergo processes including NET-osis, which promote tissue weakening and subsequent preterm birth. Taken together these findings demonstrate mechanistically how MVs may promote preterm birth and chorioamnionitis *in vivo*.



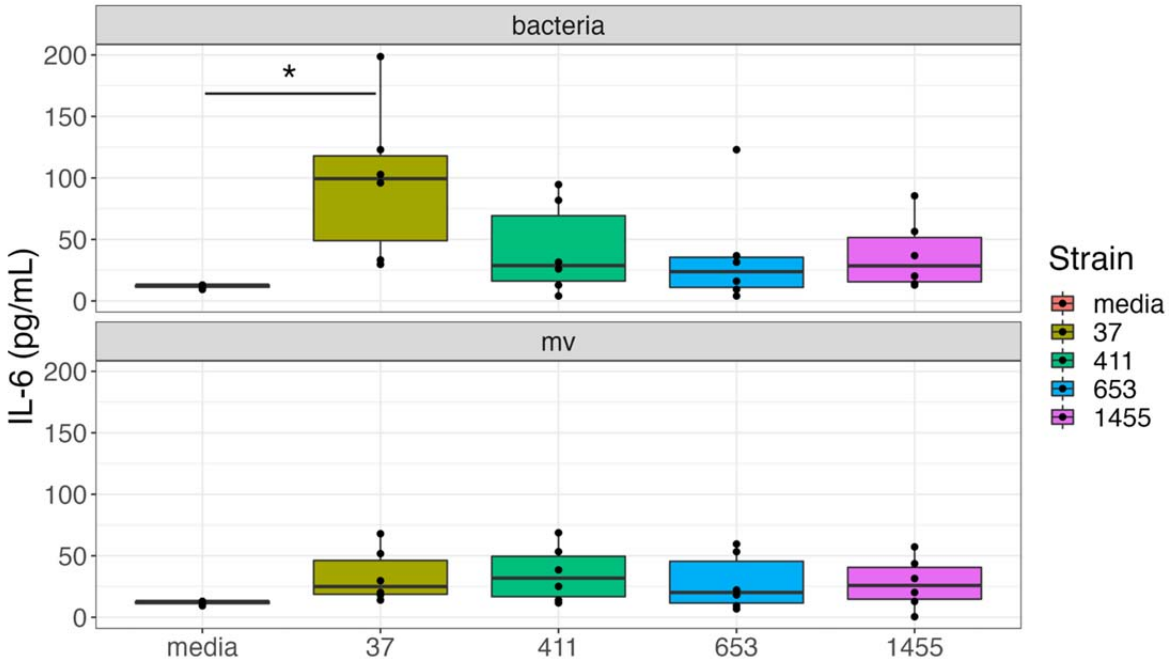
Supplemental Figure 1: Profiling of cytokine responses elicited towards MVs

THP-1s were treated with bacteria (multiplicity of infection (MOI) = 10) or MVs (MOI 100) for 25 hours prior to supernatant collection. Cytokine production was analyzed using a human cytokine antibody microarray (Abcam). Shown here is semi-quantitative densitometry analysis (ImageJ) of cytokine production for all 80 cytokines examined. Color denotes fold change relative to untreated controls. All groups were performed in biological duplicate. Boxes indicate mean fold change for each condition.



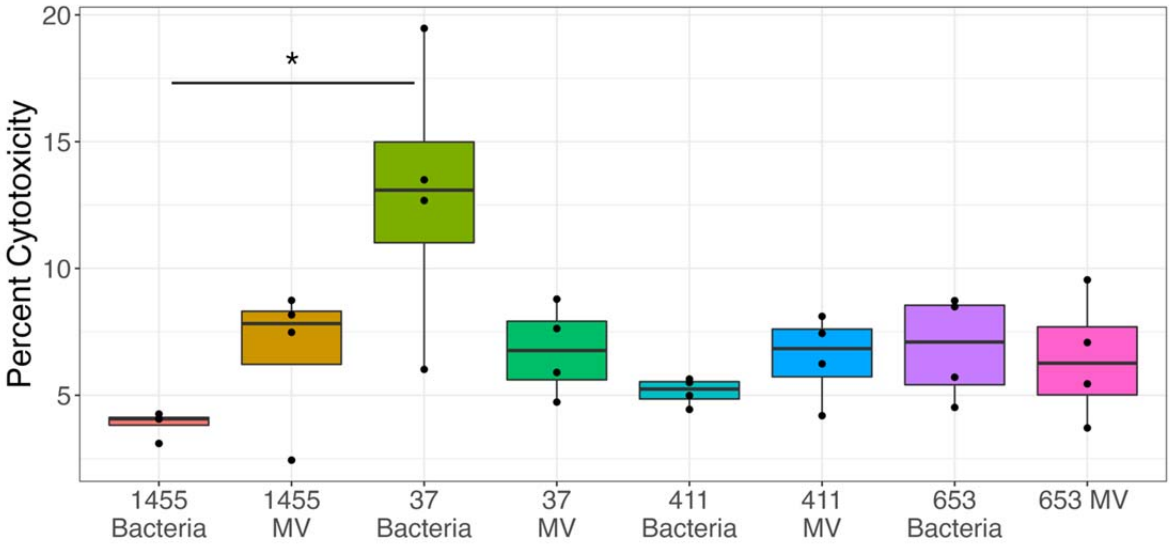
Supplemental Figure 2: Bacteria elicit proinflammatory immune responses from THP-1 macrophages.

Supernatants from THP-1 derived macrophages, which were untreated or treated with bacteria (MOI 10) for 25 hours were assessed for cytokine production using ProcartaPlex multiplex bead-based assays. Each black dot indicates a single biological replicate (n = 5-6 for each group). Data were analyzed by one-way ANOVA with a Tukey HSD post hoc test, or for non-parametric data, a Kruskal Wallis test with a Dunn Test post hoc test. Comparisons with $p < 0.05$ relative to untreated are denoted with (*). Significant differences between strains are denoted with a specific p-value.



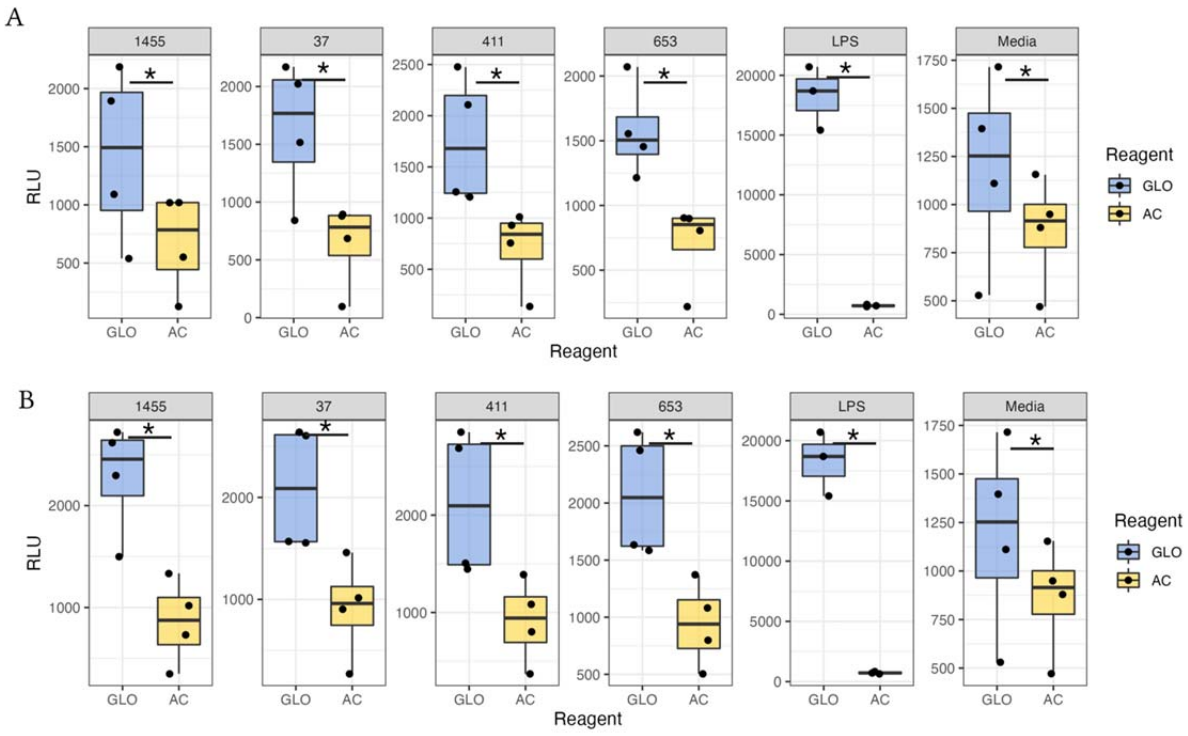
Supplemental Figure 3: IL-6 is not produced in response to GBS MVs

Supernatants from unstimulated or MV-treated THP-1 derived macrophages were assessed for IL-6 using ProcartaPlex multiplex bead-based assays. Individual black dots indicate a single biological replicate (n = 5-6 for each group). Statistics were determined by one-way ANOVA with a Tukey HSD post hoc, or for non-parametric data, a Kruskal Wallis test with a Dunn post hoc test. Significantly different comparison between groups (P-value < 0.05) are denoted with (*).



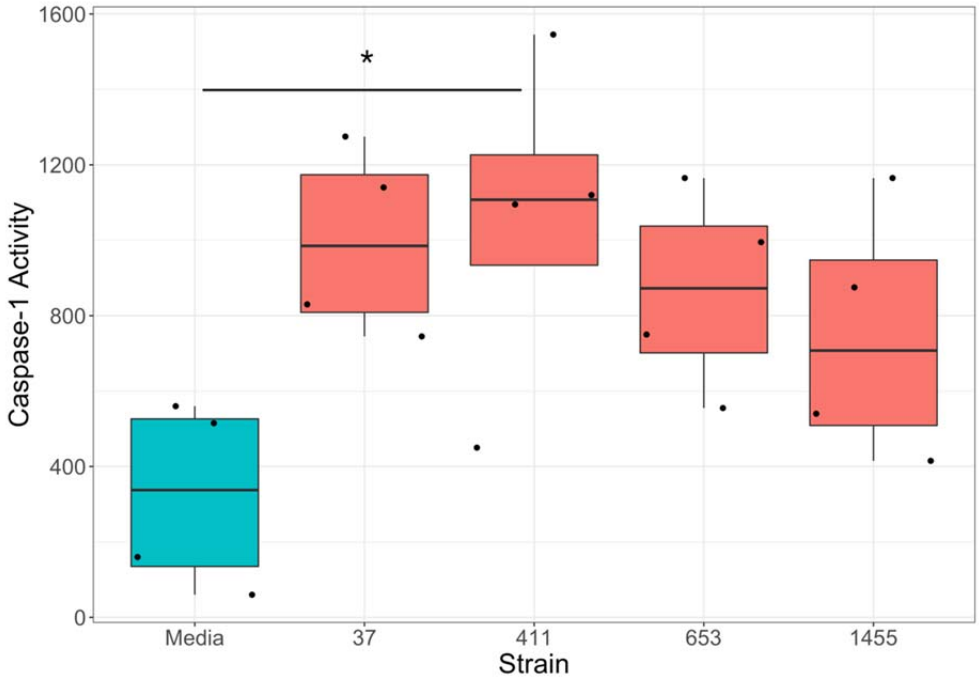
Supplemental Figure 4: MVs induce a low amount of cell death in THP-1s

THP-1 derived macrophages were unstimulated or treated with MVs for 25 hours. Supernatants were assessed for cytotoxicity using the CyQuant LDH Assay. Percent Cytotoxicity is expressed as a percentage relative to untreated cells. Each black dot represents a single biological replicate (n = 4 /group). Data were anlalyzed using either a one-way ANOVA with a Tukey HSD post hoc test (MV treated groups), or a Kruskal Wallis test with a Dunn Test post hoc (Bacteria-treated groups). Significantly different comparison within groups (P-value < 0.05) are denoted with (*). All other comparisons were not significantly different.



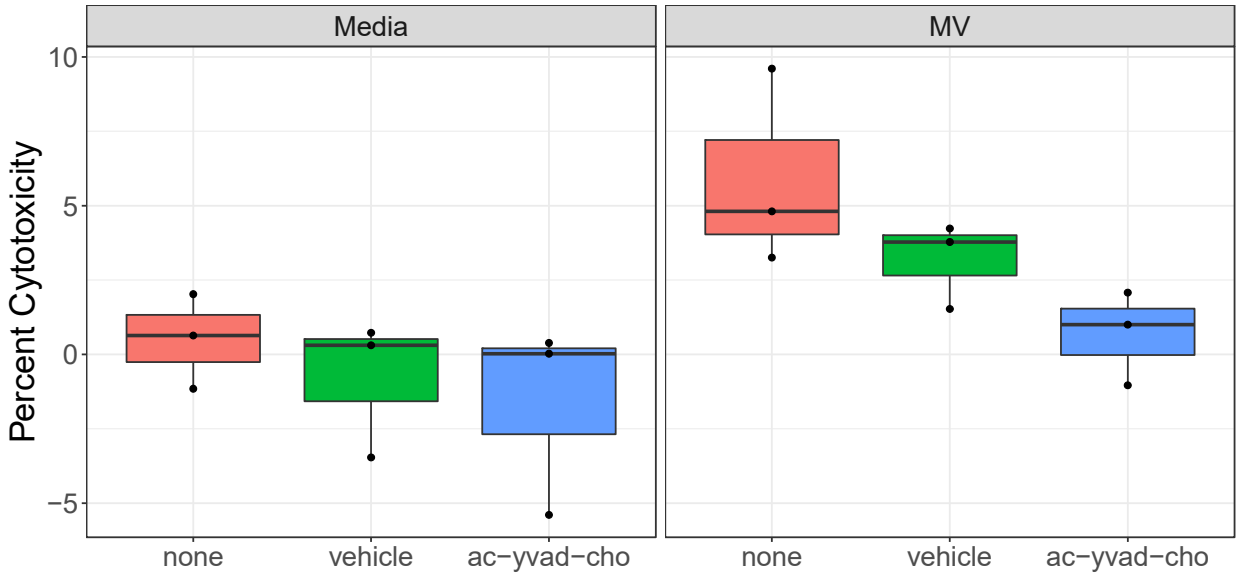
Supplemental Figure 5: MVs induce caspase-1 activity

Supernatants from THP-1 derived macrophages which were unstimulated or treated with bacteria, MVs for 25 hours. Alternatively, cells were stimulated with LPS for 2 hours. Supernatants were then assessed for caspase-1 activity using a caspase-1 GLO assay. A.) Activity from THP-1s treated with bacteria. B.) Activity from THP-1s treated with MVs. Relative light units (RLU) were determined using a GLO Max Navigator. Individual black dots indicate a single biological replicate (n = 3-4 for each group). Statistics were determined using a two-sided, paired t-test. P-value < 0.05 relative to mock treatment is denoted with a (*).



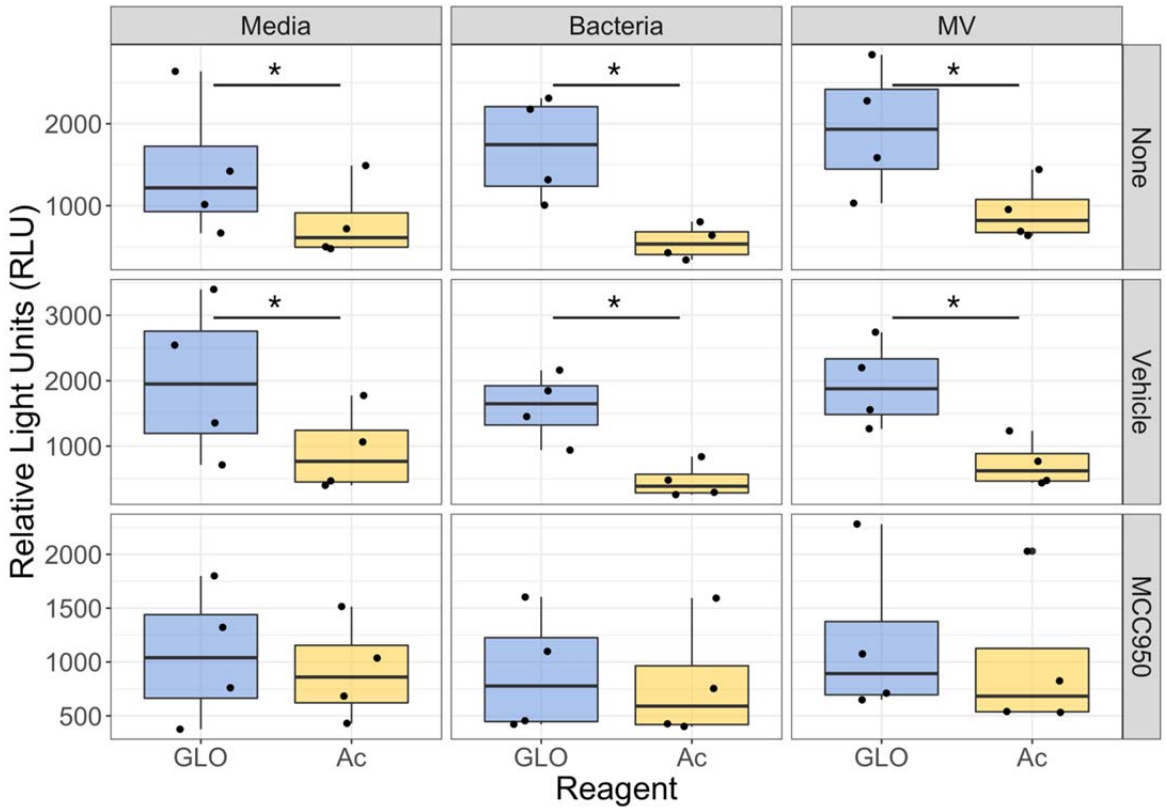
Supplemental Figure 6: Bacteria induce caspase-1 activation in THP-1s

THP-1 derived macrophages were unstimulated or treated with bacteria for 25 hours. Supernatants were then assessed for caspase-1 activity using a caspase-1 GLO assay. Data represent the amount of caspase-1 activity (caspase-1 activity = (RLU GLO) - (RLU AC)) from paired samples. Individual black dots indicate a single biological replicate (n = 4 for each group). Statistical significance is defined as $p<0.05$ as calculated by ANOVA with a Tukey post-hoc and indicated by (*).



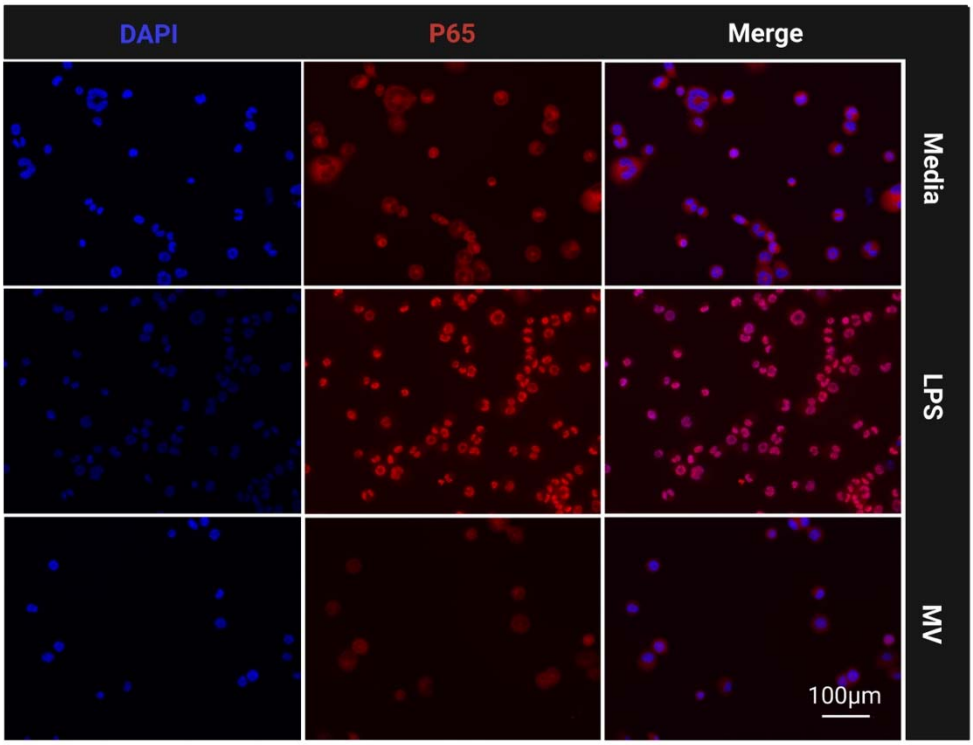
Supplemental Figure 7: Caspase-1 inhibition does not impact cell death responses

THP-1s were untreated, treated with ethanol, or Ac-YVAD-CHO for 30 minutes. Supernatants from THP-1 derived macrophages, which were subsequently unstimulated or treated with MVs for 25 hours were assessed for cytotoxicity using the CyQuant LDH Assay. Individual black dots indicate a single biological replicate (n = 3 for each group). Statistics were determined using either an ANOVA with a Tukey HSD post hoc. No significant difference relative to non-pretreated cells were detected for either group.



Supplemental Figure 8: Inhibition of NLRP3 prevents Caspase-1 Activation in Response to MVs

THP-1s were treated with the NLRP3 inhibitor MCC950 prior to treatment with bacteria, MVs, or media. Caspase-1 activity was determined using the Caspase-1 GLO assay. Individual points represent individual biological replicates (n = 4 each group). Statistics were determined using an ANOVA with a Tukey's HSD post-hoc test. Significance was defined as $p>0.05$ and denoted with an (*).



Supplemental Figure 9: MVs do not prime human macrophages

Differentiated THP-1 derived macrophages were untreated or treated with LPS or MVs for 30 minutes prior to fixation and immunofluorescence staining for NF-kB subunit p65 (stained red). Nuclei are stained using DAPI (blue). Shown here are representative images (n = 5) taken at 40x magnification.

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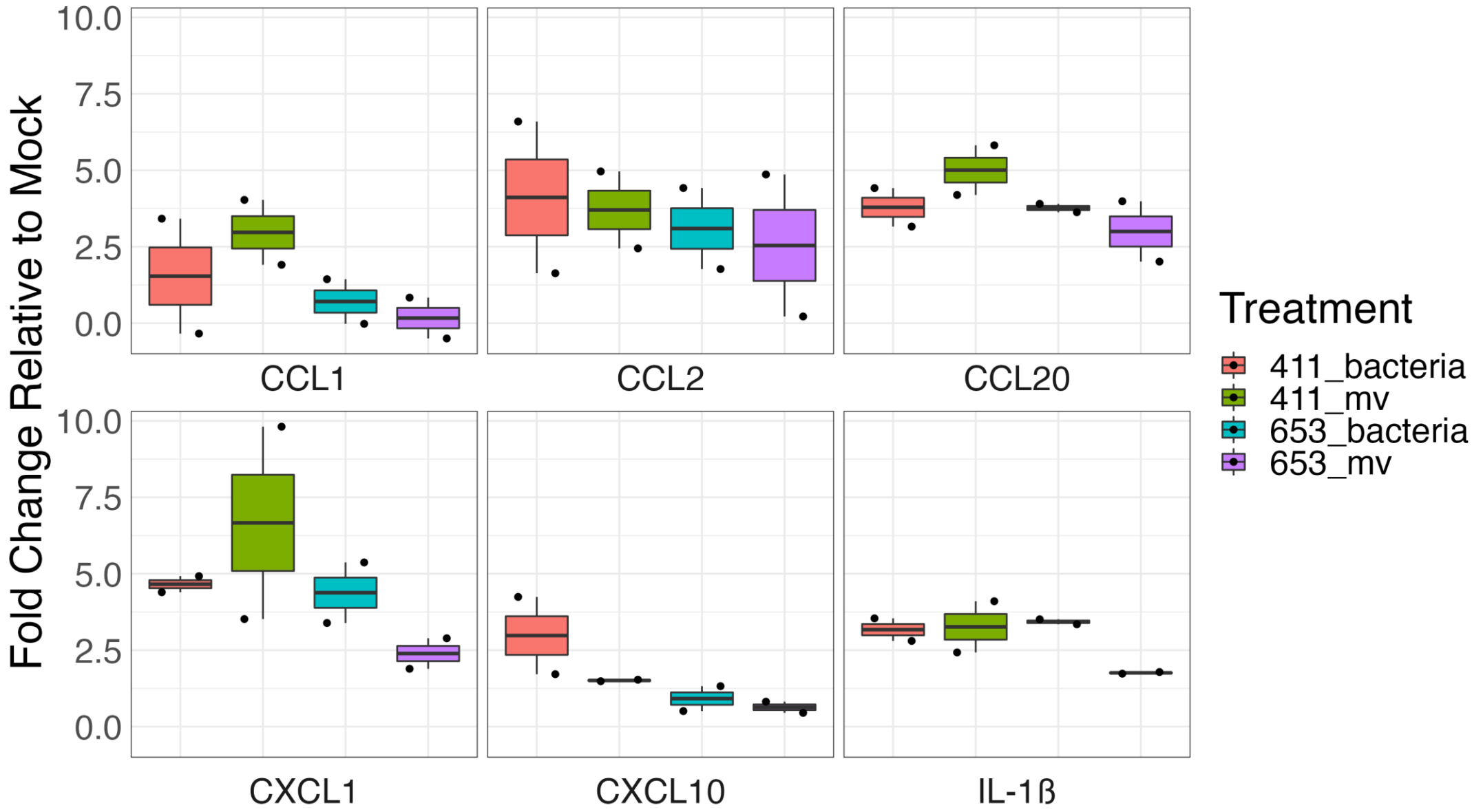
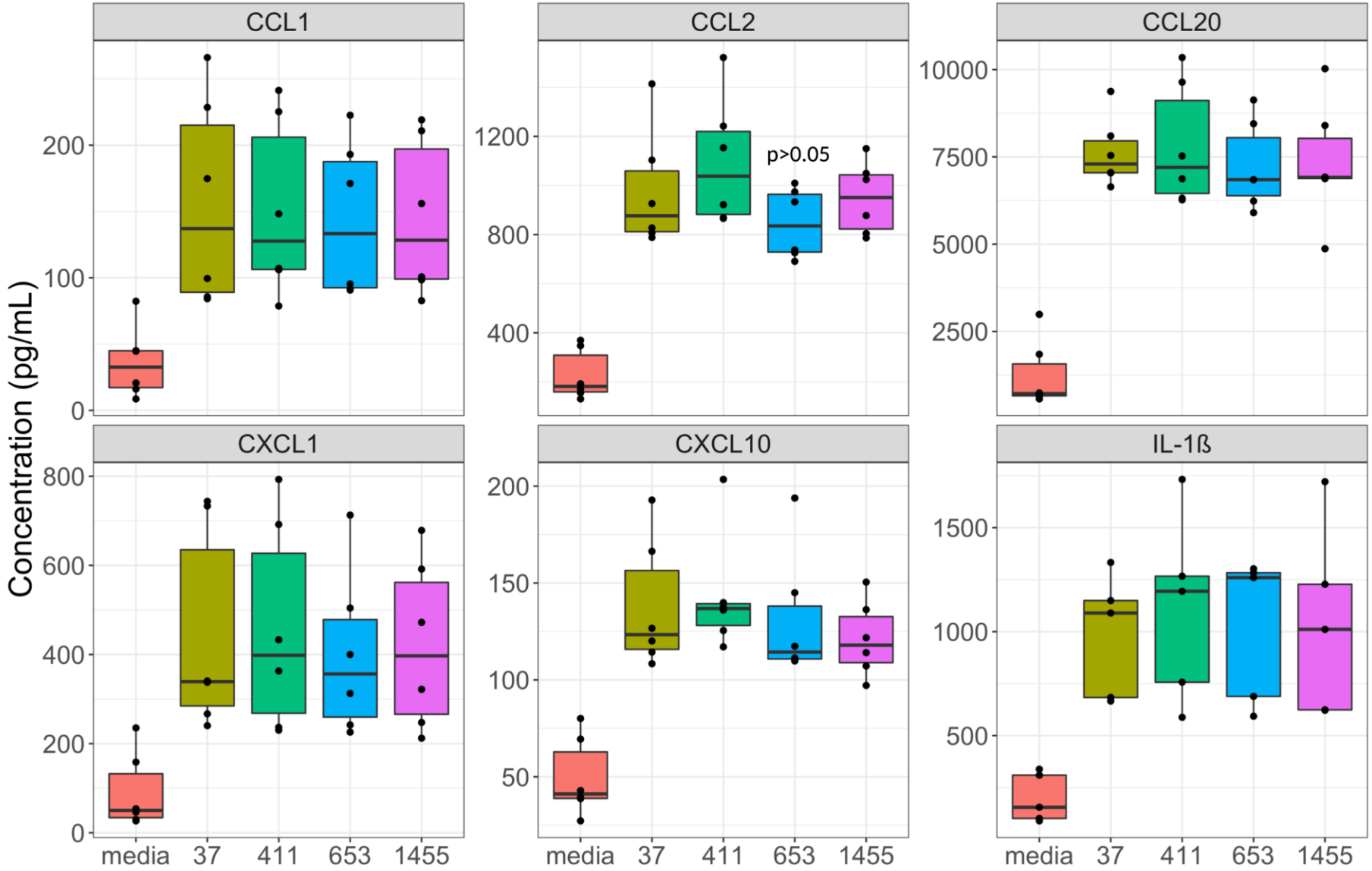
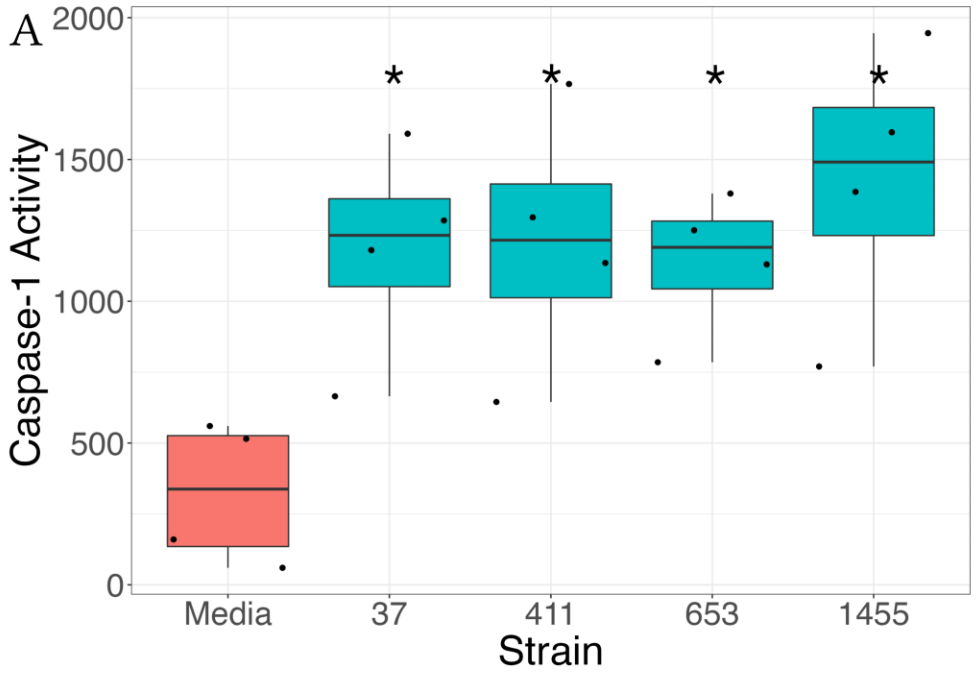
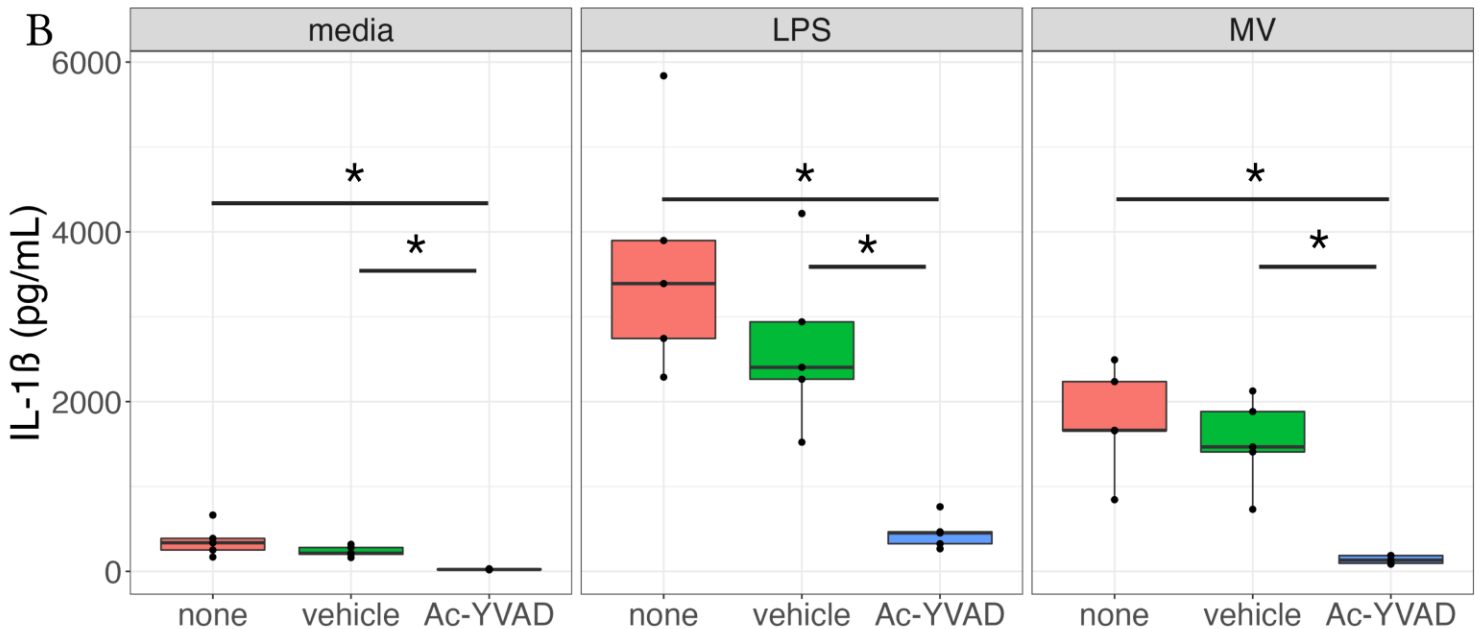

Fold Change Relative to Mock
10.0
7.5
5.0
2.5
0.0
CCL1
CCL2
CCL20
CXCL1
CXCL10
IL-1ß
Treatment
411_bacteria
411_mv
653_bacteria
653_mv


CCL1
CCL2
CCL20
CXCL1
CXCL10
IL-1ß
Concentration (pg/mL)
p>0.05
media
37
411
653
1455


A
2000
1500
1000
500
0
Caspase-1 Activity
Media
37
411
653
1455
Strain


B
media
LPS
MV
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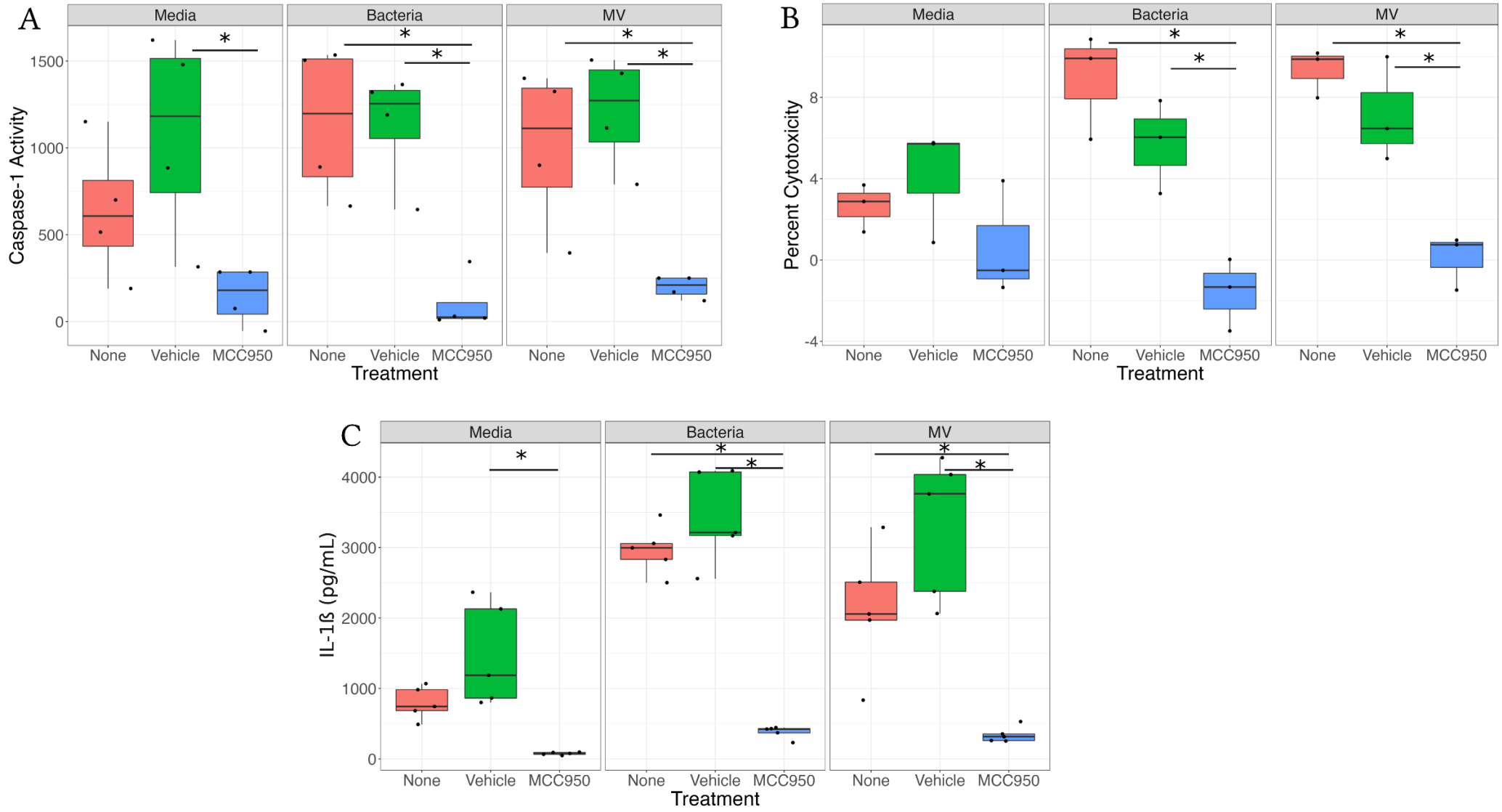
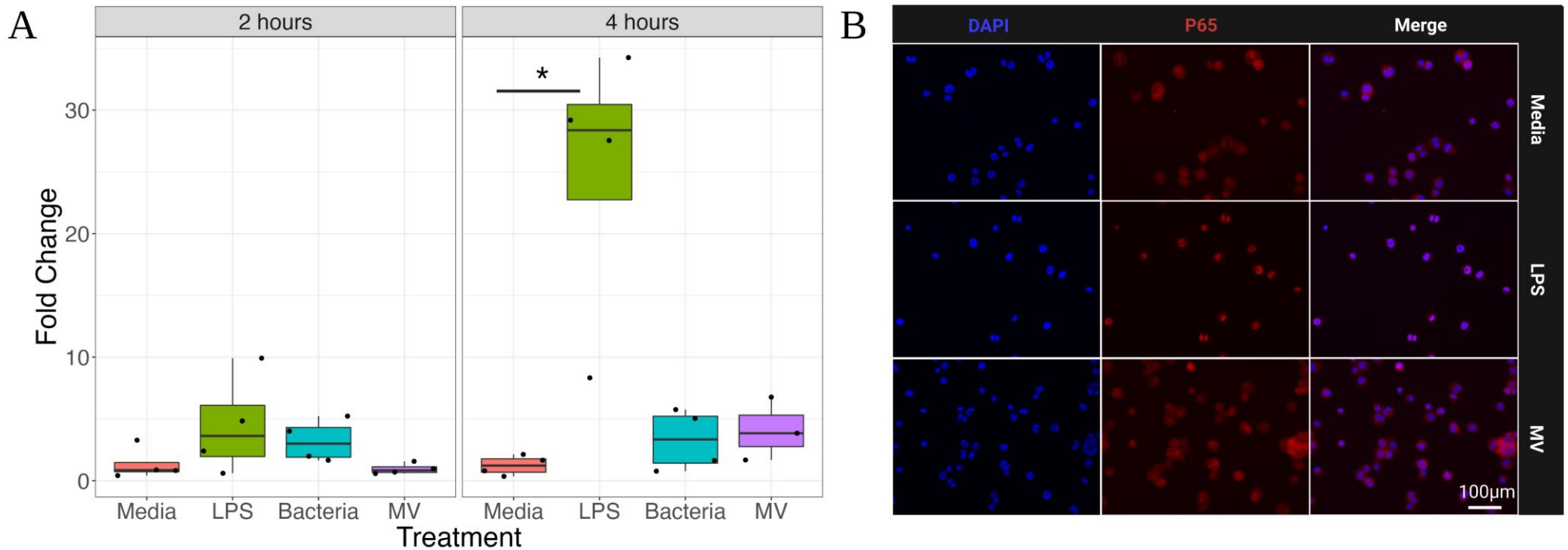
4000
2000
0
IL-1ß (pg/mL)
none
vehicle
Ac-YVAD


A
Media
Bacteria
MV
Caspase-1 Activity
1500
1000
500
0
None
Vehicle
MCC950
Treatment
B
Percent Cytotoxicity
8
4
0
-4
C
IL-1ß (pg/mL)
4000
3000
2000
1000
0
*


A
2 hours
4 hours
*
30
20
10
0
Fold Change
Media
LPS
Bacteria
MV
Media
LPS
Bacteria
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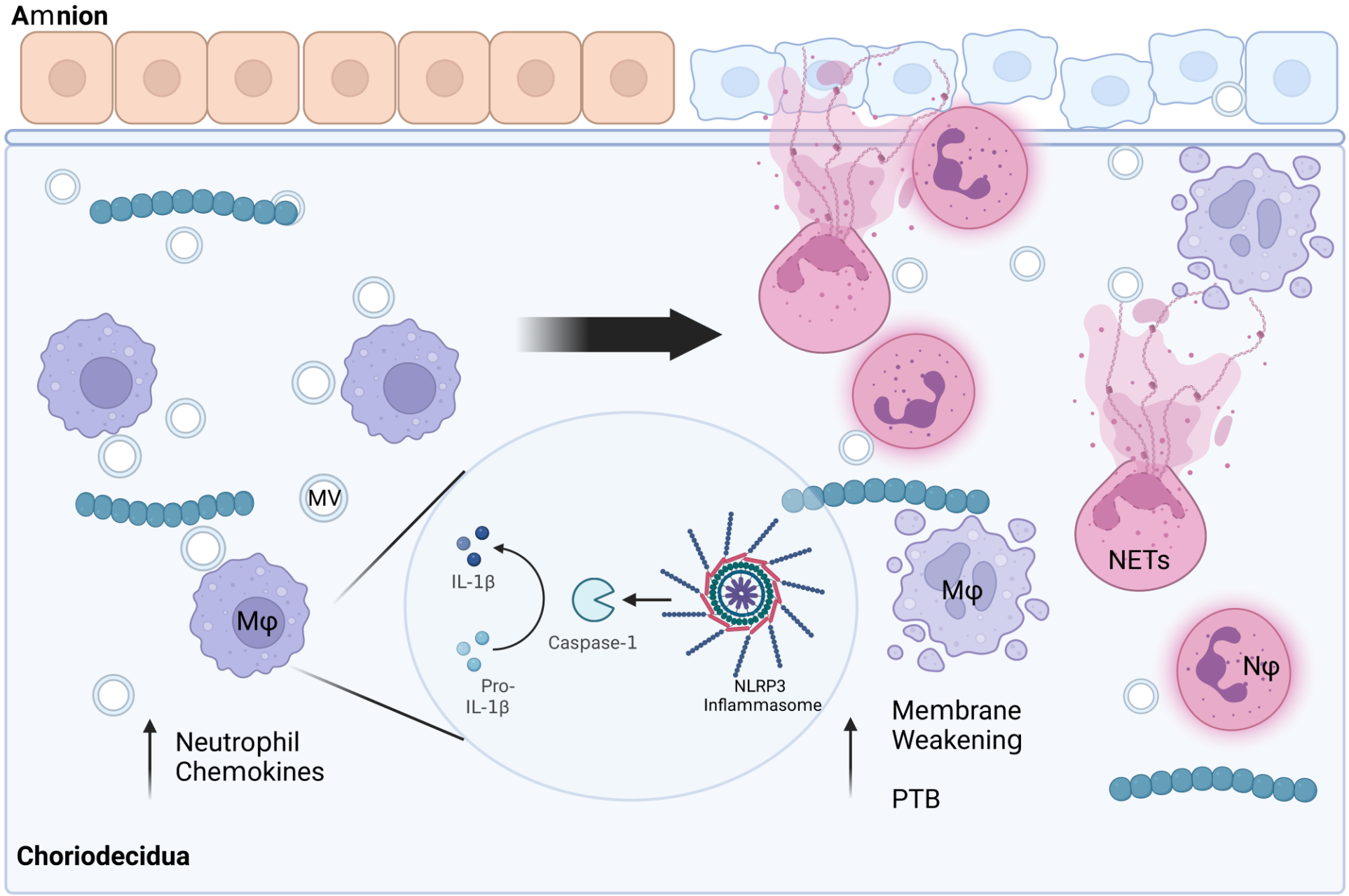
Treatment
B
DAPI
P65
Merge
Media
LPS
MV
100μm


Amnion
MV
Mφ
IL-1β
Pro-
IL-1β
Caspase-1
NLRP3
Inflammasome
Neutrophil
Chemokines
Mφ
NETs
Nφ
Membrane
Weakening
PTB
Choriodecidua