Neisseria gonorrhoeae–Induced Inflammatory Pyroptosis in Human Macrophages is Dependent on Intracellular Gonococci and Lipooligosaccharide

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ABSTRACT: *Neisseria gonorrhoeae*, the human obligate pathogen responsible for the sexually transmitted disease gonorrhea, has evolved several mechanisms to evade the host immune response. One such mechanism is the modulation of host cell death pathways. In this study, we defined cell death pathways induced by *N gonorrhoeae* in human monocyte-derived macrophages (MDMs). In a dose-dependent manner, *N gonorrhoeae* stimulation of MDMs resulted in caspase 1 and 4–dependent cell deaths, indicative of canonical and noncanonical pyroptosis, respectively. Internalization of bacteria or stimulation with lipooligosaccharide (LOS) specifically induced pyroptosis in MDMs and increased secretion of IL-1β. Collectively, our results demonstrate that *N gonorrhoeae* induces inflammatory pyroptosis in human macrophages due in part to intracellular LOS. We propose that this in turn may exacerbate inflammatory outcomes observed during mucosal infection.

KEYWORDS: cell death, *Neisseria gonorrhoeae*, pyroptosis, macrophages, LOS

Introduction

The gram-negative pathogen *Neisseria gonorrhoeae* is the causative agent of the sexually transmitted infection gonorrhea, which affects approximately 100 million people globally.[1] In men, gonorrhea is defined by a marked infiltration of polymorphonuclear neutrophils (PMNs), resulting in symptomatic urethritis and epididymitis. Disease in women is associated with cervicitis which can be asymptomatic leading to continued transmission between sex partners. Moreover, a subset of women with gonococcal infection will develop upper genital tract disease, which can lead to pelvic inflammatory disease, ectopic pregnancy, and tubal infertility.[2,3] Gonococcal colonization of the mucosa and invasion of reproductive tract epithelial cells lead to production of pro-inflammatory cytokines, activation of resident immune cells, and recruitment of circulating immune cells. *Neisseria gonorrhoeae* can also traverse the epithelium, thereby entering the submucosa, where it interacts with both resident and recruited immune cells including macrophages and neutrophils (polymorphonuclear cells; PMN).

Although PMNs appear to play an essential role in clearing *N gonorrhoeae*, several studies have also demonstrated that the gonococcus is capable of survival in these cells.[4-7] The gonococcus evades neutrophil-mediated killing by neutralization of reactive oxygen burst and delaying lysosomal degradation.[5,7,8] Resident immune cells also aid in combating gonococcal infection. Within the submucosa, where there is an abundance of macrophages, which function in pathogen clearance via phagocytosis and production of cytokines that can promote recruitment of other innate and adaptive immune cells and maturation of adaptive immune cells. In the genital mucosa, macrophages are well represented, comprising 30% of all CD45+ cells, a common leukocyte marker.[9,10] The gonococcus has been demonstrated to induce the production of pro-inflammatory cytokines by human macrophages.[11] Furthermore, *N gonorrhoeae* can alter macrophage function by promoting delayed phagosome-lysosome fusion, thereby evading degradation.[11,12] Although the gonococcus can alter macrophage function, the impact on macrophage survival is understudied. In some cell types such as epithelial cells and PMNs, *N gonorrhoeae* has been demonstrated to promote host cell survival.[13-16] Yet, in immune cells such as monocytes and B cells, *N gonorrhoeae* promotes host cell death.[17,18]

One recent report demonstrated that stimulation of macrophages with *N gonorrhoeae* did not induce apoptosis, a well-characterized cell death pathway.[19] Apoptosis is defined by mitochondrial depolarization, activation of caspase-3, nuclear condensation, and membrane blebbing. These authors demonstrated that macrophages fail to undergo apoptosis following *N gonorrhoeae* stimulation; however, this study only examined apoptosis through monitoring of caspase 3 activation.[19] A lack of caspase 3 activation in epithelial cells and neutrophils following *N gonorrhoeae* stimulation has also been reported.[13] In addition to apoptosis, cell death can occur by pyroptosis and necroptosis, which are pro-inflammatory lytic forms of cell death. These pathways are dependent on immune caspases 1 and 4 or kinases such as receptor-interacting protein kinase 1/3 (RPIK1/3) and mixed lineage kinase domain-like protein (MLKL), respectively. Recently, we determined that epithelial cells undergo necroptosis following stimulation with *N gonorrhoeae*.[20]

Several gram-negative pathogens have been demonstrated to induce stimulation of pyroptosis in macrophages.[21-24] Similar to apoptosis, pyroptosis is dependent on caspase activity;

however, unlike apoptosis, it requires immune caspases 1 and 4.[21,25-27] Immune caspases are recruited and activated by the formation of the inflammasome, which is responsible for the processing of pro-inflammatory cytokines such as IL-1β. Previous studies have shown that *N gonorrhoeae* activates the NLRP3 inflammasome and caspase 1 in human monocytes, representing an essential step for the induction of the canonical pyroptotic pathway.[17] With other pathogens, canonical pyroptosis is stimulated by extracellular stimuli such as flagellin and lipopolysaccharide (LPS). The noncanonical pyroptotic pathway can be activated either by extracellular or intracellular stimuli such as intracellular LPS which activates caspase 4, promoting its autoproteolytic cleavage and activation and subsequent induction of pyroptosis. In this study, we demonstrate that *N gonorrhoeae* induces macrophage cell death through pyroptosis, exploiting both the canonical caspase 1–dependent pathway and the noncanonical caspase 4–dependent pathway. We further demonstrate that gonococcal-induced macrophage cell death is dependent on intracellular gonococci and lipooligosaccharide (LOS).

Materials and Methods

Cell culture and bacterial strains

Monocytes were isolated from peripheral blood by Ficoll separation and plated in nontreated Petri dishes at a concentration of 5 × 10^7 cells/mL. Cells were incubated 24 hours in RPMI/10% fetal bovine serum (FBS)/10% human serum. Media was removed from adherent cells after 24 hours, cells were washed with phosphate-buffered saline (PBS), and media was replaced with fresh monocyte-derived macrophage (MDM) media composed of 5 mM HEPES/10%FBS/10% human serum/RPMI. Monocytes were differentiated over the course of 7 days to macrophages with human serum containing macrophage colony-stimulating factor. After 7 days, MDMs were lifted and counted using a hemocytometer to determine cell concentrations for proper plating for each experiment. *Neisseria gonorrhoeae* strain FA1090B (obtained from J. G. Cannon, University of North Carolina School of Medicine Chapel Hill, Chapel Hill, NC, USA) that expresses the OpaB adhesin protein in the absence of all other Opa proteins was used for most of the studies.[28] For confocal microscopy studies, green fluorescent protein (GFP)-expressing *N gonorrhoeae* strain F62 (F62-GFP) was used.[29] The mutant LOS strain 1291Δ*msbB* and wild-type strain 1291 (obtained from Michael Apicella, College of Medicine, University of Iowa, Iowa City, IA, USA) were used to elucidate the role of LOS in the induction of MDM cell death. All strains expressed pili as determined by light microscopy. *Neisseria gonorrhoeae* were grown overnight on chocolate agar plates at 37°C in a 5% CO$_2$ incubator.

MDM stimulation and cell death analysis

Neisseria gonorrhoeae were grown in chemically defined media to an optical density (OD) ~0.6 to 0.7, centrifuged and resuspended in MDM media at an OD of 1.0, and added to MDMs at multiplicity of infection (MOI) equivalent to 10 and 100. Cocultures were maintained at 37°C in a 5% CO$_2$ incubator and harvested at several time points over the course of 24 hours. Supernatants were collected and centrifuged for 10 minutes at 700 relative centrifugal force (RFCs) to pellet nonadherent cells and cellular components. Adherent cells were incubated with trypsin (Corning, Corning, NY, USA) for 10 minutes followed by addition of 10% FBS/RPMI. Trypsinized cells were combined with nonadherent cells for analysis of cell death by trypan blue exclusion, flow cytometry, or caspase activity. Staurosporine (STS, 3 μM; Sigma, St. Louis, MO, USA) was used as a positive control for the induction of apoptosis. Monocyte-derived macrophages were transfected with either purified *Escherichia coli* LPS (100 ng/mL) or purified *N gonorrhoeae* LOS (100 ng/mL) for 2 hours and analyzed for cell death using confocal microscopy. Lipopolysaccharide was purchased from Invitrogen (*E coli* O111:B4; Invitrogen, San Diego, CA, USA) and LOS purified from *N gonorrhoeae* strain F62 was a kind gift from Sunita Gulati (University of Massachusetts Medical School, Worcester, MA, USA). Lipooligosaccharide purity was confirmed with stimulation of HEK cells expressing either TLR2 or TLR4. To evaluate the ability of LOS to stimulated HEKs, cells were stimulated with 100 ng/mL of purified LOS or control LPS (*E coli* O111:B4, Invitrogen) for 6 hours in the absence of a transfection agent. Lipooligosaccharide was deemed pure (lacking TLR2-stimulating ligands) if it only stimulated TLR4-expressing HEK cells but not TLR2-expressing HEK cells. Stimulation was confirmed by detection of IL-8 by enzyme-linked immunosorbent assay (ELISA; BD Biosciences, East Rutherford, NJ, USA).

Flow cytometry

Monocyte-derived macrophages were stimulated for 6 hours with 100 ng/mL LPS plus 5 mM adenosine triphosphate (ATP), 3 μM STS, or *N gonorrhoeae* strain FA1090B at an MOI of 10 or 100. Following stimulation, cells were lifted and washed 3 times in 0.5% FBS/PBS. Cells were stained for CD11b and CD68 expression (CD11b-APC; BioLegend, San Diego, CA, USA and CD68-FITC; BioLegend) for 30 minutes at 4°C and then washed. Prior to analysis, cells were stained with the live-dead stain, propidium iodide (PI; BioLegend) and analyzed on a BD LSR II (San Jose, CA, USA). To evaluate perturbation to the mitochondrial membrane potential, MDMs were stained with Rhodamine 123, a mitochondrial membrane potential dye. Cells were incubated with 1 μM of Rhodamine 123 (Molecular Probes, Eugene, OR, USA), for 30 minutes at 37°C. Following staining, cells were lifted and stained for macrophage markers as described above and were immediately analyzed by flow cytometry. For Rhodamine 123 staining, CD68-PerCP/Cy5.5 and CD11b-APC (BioLegend) were used to delineate macrophage populations.

Caspase 3 activity assay

Caspase 3 activity was determined with an EnzChek Caspase-3 Assay (Molecular Probes), which measures generation of fluorescence following the cleavage of the fluorogenic substrate, Z-DEVD-AMC. Combined trypsin/supernatant pellets were lysed overnight at –80°C, thawed, and centrifuged at 13 000 rpm for 10 minutes to pellet cell debris. Supernatants were transferred to a 96-well plate and incubated with Z-DEVD-AMC for 1 hour. Absorbance was evaluated in a fluorescence microplate reader using an excitation wavelength of 365 nm and emission detection at 465 nm. Data were collected using a Molecular Devices SpectraMax M5 with corresponding SoftMax Pro 4.8 software (Molecular Devices, Sunnyvale, CA, USA).

Immune caspase activity assays

Caspase 1 and caspase 4 activities were determined with either Caspase-1/ICE Colorimetric Assay (BioVision, Milpitas, CA, USA) or Caspase-4 Colorimetric Assay (MBL International Corporation, Woburn, MA, USA), whereby the generation of colorimetric products following cleavage of caspase-specific substrates (YVAD-pNA or LEVD-pNA) was monitored. Combined trypsin/supernatant pellets were lysed on ice for 10 minutes and centrifuged at 10 000g for 1 minute to pellet cell debris. Supernatants were transferred to a 96-well plate and incubated with YVAD-pNA or LEVD-pNA for 2 hours at room temperature (RT) and absorbance was evaluated at 400 nm on a Molecular Devices SpectraMax M5 with corresponding SoftMax Pro 4.8 software (Molecular Devices).

Caspase inhibition

Prior to bacterial stimulation, MDMs were incubated with the following inhibitors: Caspase-1 Inhibitor (Z-WEHD-FMK; R&D Systems, Inc., Minneapolis, MN, USA) and Caspase-4 Inhibitor (Z-YVAD-FMK; R&D Systems, Inc.). Inhibitors were added at a concentration of 100 nM 1 hour prior to bacterial stimulation with FA1090B, 1291, or 1291Δ*msbB* for 6 hours, harvested as above, and analyzed for cell death by trypan blue staining and IL-1β (ELISA; R&D Systems, Inc.).

LOS transfection

Monocyte-derived macrophages were plated at a density of 5 × 10^5/mL 24 hours prior to transfection. To prepare for transfection either *E coli* LPS (*E coli* O111:B4; Invitrogen) or *N gonorrhoeae* LOS (100 ng/mL) was diluted in HEPES-buffered saline (HBS) to a concentration of 100 ng/mL in a volume of 50 μL. Separately, 300 ng of DOTAP (Sigma) was combined with HBS to a volume of 100 μL. The 2 reactions were then combined for a total volume of 150 μL and incubated at RT for 20 minutes. DOTAP LPS mixtures were then added to MDMs for 2 hours prior to analysis by confocal microscopy.

Confocal microscopy

Sterile coverslips were placed in nontreated 12-well plates prior to the addition of MDMs. Cells were plated at a concentration of $0.3 × 10^6$ to $0.5 × 10^6$ cells/mL for 24 hours. Monocyte-derived macrophages were either left untreated or treated for 1 hour with 2 μg cytochalasin D. Cells were then either cocultured with strain F62-GFP or transfected with *E coli* LPS (*E coli* O111:B4; Invitrogen) or *N gonorrhoeae* LOS.[29] After stimulation, cells were then treated with Zombie Red (BioLegend) as directed by the manufacturer. Cells were fixed with 4% paraformaldehyde solution in PBS for 10 minutes at RT and permeabilized with 0.1% Triton X-100. Cells were washed, blocked with 2% bovine serum albumin/PBS solution, and stained with phalloidin Alexa Fluor647 (Molecular Probes). Cells were washed a final time, air-dried, and mounted with Vectashield DAPI Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and stored in the dark. Readings for GFP, DAPI, Zombie Red, and Alexa Fluor647 were performed at 470, 405, 624, and 675 nm, respectively, using a Nikon AR1 confocal microscope.

ELISA for pro-inflammatory cytokines

Supernatants from stimulated cultures were collected at 6 hours and stored at –80°C until analysis. The ELISA analysis was performed using commercially available kits for human IL-1β, interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) (BD Biosciences).

Bacterial viability

Bacterial viability was enumerated in MDMs stimulated with either FA1090B or F62-GFP for 6 hours. Prior to bacterial stimulation, MDMs were either left untreated or treated for 1 hour with 2 μg of cytochalasin D or vehicle control, dimethyl sulfoxide (DMSO). Bacterial viability was analyzed in 3 different fractions: supernatants (extracellular), MDM associated, and MDM intracellular. For culture supernatants, 100 μL of supernatant was diluted in culture media and plated on chocolate agar plates. Monocyte-derived macrophage–associated bacteria were cultured following washing of MDMs 3 times with PBS followed by incubation for 10 minutes at 37°C with a 1% saponin solution. The saponin solution was then diluted and plated on chocolate agar. To determine the number of intracellular bacteria, MDMs were incubated with 50 μg/mL gentamicin for 1.5 hours at 37°C. After incubation with gentamicin, cells were washed and then lysed with 1% saponin for 10 minutes at 37°C; the saponin solution was then diluted and plated on chocolate agar. All plates were incubated at 37°C overnight.

Statistical analysis

Data were analyzed using analysis of variance with either Bonferroni posttest or Dunnett posttest as indicated within
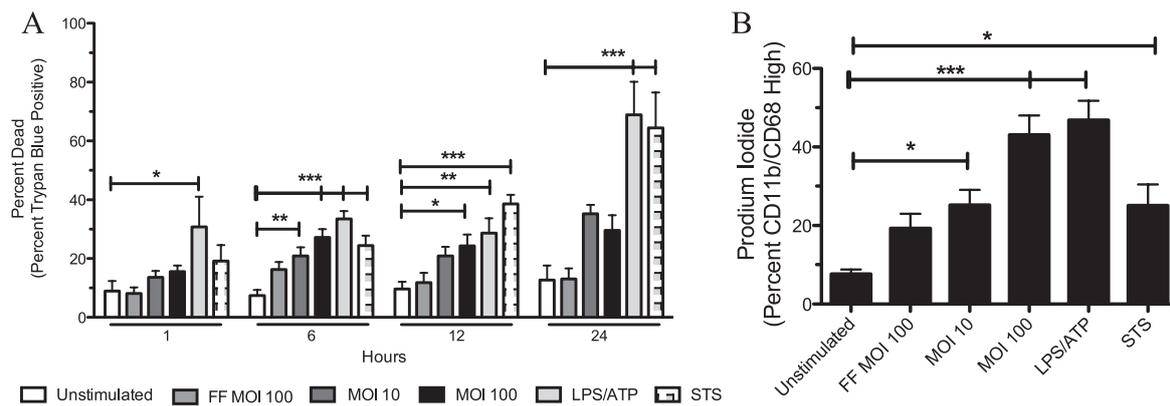
Figure 1. *Neisseria gonorrhoeae* induces cell death in monocyte-derived macrophages. MDMs were stimulated with medium alone (unstimulated), LPS (100 ng/mL, *Escherichia coli* O111:B4) and 5 mM ATP (LPS/ATP), 3 μM staurosporine (STS), formalin-fixed bacteria MOI 100 (FF MOI 100), or live bacteria MOI 10 or 100 of strain FA1090B. (A) Cell death was determined by trypan exclusion at 1, 6, 12, and 24 hours. (B) Cell death at 6 hours was confirmed by flow cytometry as the percent of total MDMs staining positive for propidium iodide. The graphs depict the mean ± SEM from 8 donors; 1-way ANOVA with a Dunnett multiple comparisons posttest was performed at each time point (*$P < .05$; **$P < .01$; ***$P < .001$). ANOVA indicates analysis of variance; ATP, adenosine triphosphate; LPS, lipopolysaccharide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection.

each figure legend. A *P* value of <.05 was considered statistically significant.

Results

N gonorrhoeae induces cell death in human MDMs in a nonapoptotic manner

To establish whether gonococcal stimulation of human macrophages induced cell death, we analyzed bacteria-stimulated MDMs over the course of 24 hours. We observed elevated levels of cell death in *N gonorrhoeae*–treated cultures over the course of 24 hours as measured by trypan blue exclusion (Figure 1A). Compared with unstimulated MDMs, cultures incubated with *N gonorrhoeae* strain FA1090B (MOI of 10 and 100) displayed characteristics of cell death as early as 6 hours. Macrophage cell death in live bacteria–treated cocultures (MOI 10 and 100) exhibited 2-fold to 3-fold increase in cell death as compared with unstimulated cultures at 6 hours. Bacteria-induced cell death was also observed at 12 hours in MOI 10 and MOI 100 cocultures. There was a small but not statistically significant increase in MDM cell death in MOI 10–treated cultures at 24 hours as compared with earlier time points (Figure 1A). To rule out the contribution of confounding factors of cell death by-products at later time points and to examine the direct effects of gonococcal stimulation on cell death, we focused subsequent studies on early time points (6 hours). As observed with trypan blue exclusion, MDMs treated with live bacteria exhibited a 2-fold to 3-fold increase in cell death compared with unstimulated MDMs as determined by PI incorporation as measured by flow cytometry (Figure 1B). We observed a minimal increase in MDM cell death in cultures stimulated with formalin-fixed bacteria compared with unstimulated controls as measured by trypan exclusion or PI incorporation studies (Figure 1A and B). When compared with cell death

levels induced by live bacteria at a similar MOI, formalin-fixed bacteria induced approximately 50% less cell death. These results demonstrate that *N gonorrhoeae* stimulation induces macrophage cell death in a dose-dependent and time-dependent manner and that the induction of cell death is dependent on bacterial viability.

To determine whether *N. gonorrhoeae*–induced macrophage cell death resulted from the induction of apoptosis, we next monitored 2 apoptotic markers, host cell mitochondrial membrane potential and caspase 3 activity, in bacterial cocultures.[30] Loss of mitochondrial membrane potential was monitored by Rhodamine 123 staining, a dye that is only maintained in cells in which mitochondrial membrane integrity is unperturbed. Coculture of MDM with *N gonorrhoeae* failed to alter Rhodamine 123 staining compared with unstimulated controls, indicating maintenance of the mitochondrial membrane potential (Figure 2A). We did not observe increased caspase 3 activity, an executioner caspase responsible for the degradation of intracellular components during apoptosis following gonococcal stimulation with either MOI of 10 or 100 (Figure 2B).[31] Stimulation with STS resulted in diminished Rhodamine 123 staining and increased caspase 3 activity as compared with unstimulated controls consistent with induction of apoptosis, at 6 hours (Figure 2A and B). Collectively, these data indicate that *N gonorrhoeae* does not specifically induce apoptosis in MDMs.

N gonorrhoeae induces pyroptosis in MDMs

Activation of immune caspases and subsequent production of pro-inflammatory cytokines occur normally as the result of pathogen stimulation for the processing of cytokines. However, under some circumstances, immune caspase activation leads to the induction of cell death. Caspase 1 and caspase 4 activities have been shown to be essential to the initiation of canonical
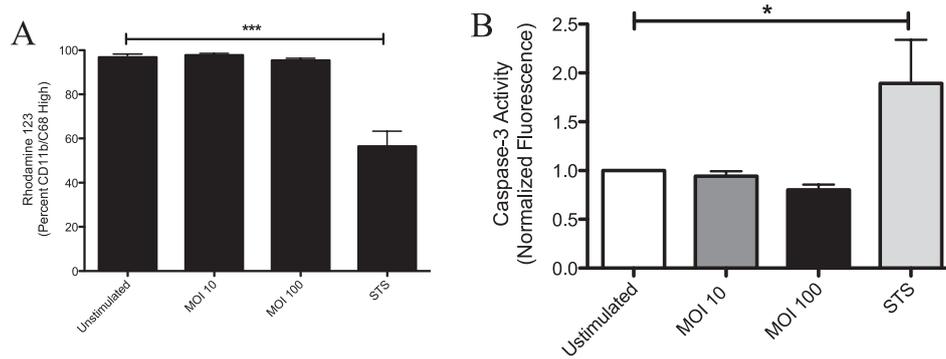
Figure 2. *Neisseria gonorrhoeae* does not induce apoptotic markers in MDMs. MDMs were treated with medium alone (unstimulated), 3 μM staurosporine (STS), or live bacteria strain FA1090B MOI 10 or 100 and either stained with (A) Rhodamine 123 or (B) analyzed for caspase 3 activity after 6 hours. Incorporation and retention of Rhodamine 123 by mitochondria are dictated by the mitochondrial membrane potential state, where depolarized organelles release the dye. Values are expressed as the percent of total MDMs staining positive for Rhodamine 123. Caspase 3 values were normalized to the unstimulated control at each time point. Rhodamine 123 graph depicts the mean ± SEM (n = 3 donors) 1-way ANOVA with a Dunnett multiple comparisons posttest. Caspase 3 graphs depict the mean ± SEM (n = 6 donors) 1-way ANOVA with a Dunnett multiple comparisons posttest (*$P < .05$; ***$P < .001$). ANOVA indicates analysis of variance; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection.
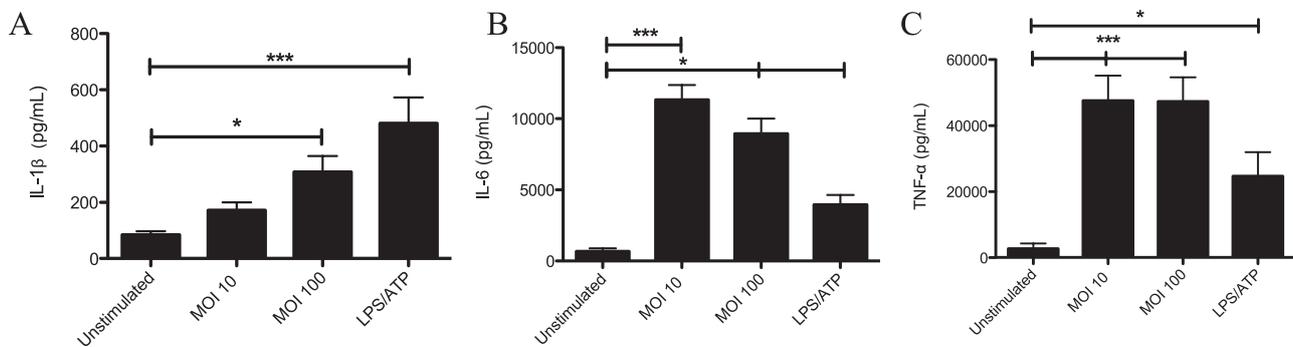
Figure 3. *Neisseria gonorrhoeae* induces pro-inflammatory responses in monocyte-derived macrophages. MDMs were incubated with 100 ng/mL LPS (*Escherichia coli* O111:B4) and 5 mM ATP (LPS/ATP), live bacteria strain FA1090B at MOI 10 or MOI 100 for 6 hours, and medium alone as a negative control (unstimulated). (A) IL-1β, (B) IL-6, and (C) TNF-α were measured in cell culture supernatants by ELISA. Values depict the mean ± SEM from 3 donors; 1-way ANOVA with a Dunnett multiple comparisons posttest (*$P < .05$; **$P < .01$; ***$P < .001$). ANOVA indicates analysis of variance; ATP, adenosine triphosphate; IL-6, interleukin 6; LPS, lipopolysaccharide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection; TNF-α, tumor necrosis factor α.

and noncanonical pyroptosis, respectively.[25-27,32-35] Previous studies reported that *N gonorrhoeae* activates the NLRP3 inflammasome, resulting in caspase 1 activation and pro-inflammatory cytokine production, specifically IL-1β.[11,17] Thus, we next analyzed the inflammatory response of MDM–stimulated *N gonorrhoeae* (MOI of 10 and 100) by ELISA. We observed increased IL-1β in MDM supernatants stimulated with *N gonorrhoeae* as compared with unstimulated controls (Figure 3). In addition, we also observed increased IL-6 and TNF-α in MDM supernatants stimulated with *N gonorrhoeae*. Increased secretion of these cytokines is in agreement with previous published studies in human macrophages following gonococcal stimulation.[11,36]

We observed increased caspase 1 activity in MDMs treated with *N gonorrhoeae* for 6 hours at an MOI 100 (Figure 4A). Similarly, stimulation of MDMs with *N gonorrhoeae* resulted in a significant increase in caspase 4 activity compared with unstimulated controls (Figure 4B). Stimulation of MDMs with LPS plus ATP also resulted in increased caspase 1 and caspase 4 activities as compared with unstimulated controls at 6 hours. To determine whether caspase 1 and caspase 4 activities are required for *N gonorrhoeae*–induced MDM death, we used caspase-specific inhibitors. Inhibition of caspase 1, with the selective inhibitor Z-WEHD-FMK, led to diminished cell death with a 18% and 36% increase in cell viability in MOI 10 and MOI 100–stimulated cultures as compared with the DMSO control, respectively (Table 1). We also observed an increase in MDM viability following *N gonorrhoeae* stimulation in MDMs pretreated with the caspase 4 inhibitor, Z-YVAD-FMK. In caspase 4 inhibitor–treated MDMs, a 21% and 37% increase in viability of MOI 10 and MOI 100–stimulated cocultures were observed, respectively, as compared with DMSO controls (Table 1). As expected, inhibition of the apoptotic caspase, caspase 3, did not alter MDM viability following *N gonorrhoeae* stimulation when compared with DMSO controls (Table 1). These results indicate that *N gonorrhoeae* induces cell death via pyroptosis, exploiting
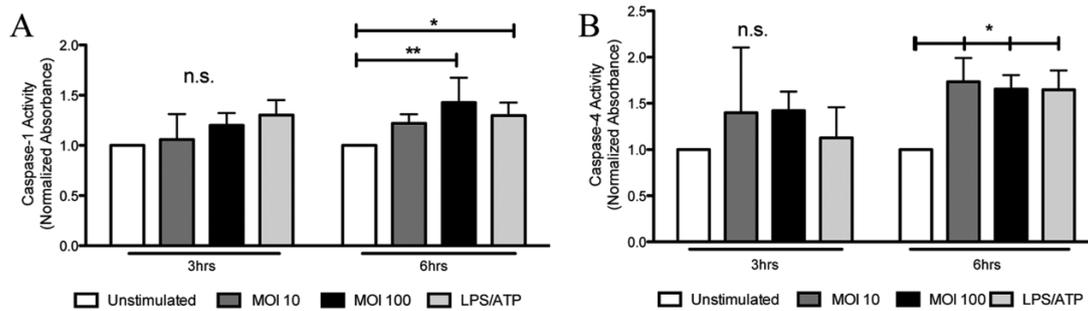
Figure 4. *Neisseria gonorrhoeae* activates immune caspases in MDMs. MDMs were incubated for 3 or 6 hours with medium alone (unstimulated), LPS (100 ng/mL, *Escherichia coli* O111:B4), and 5 mM ATP (LPS/ATP), or live bacteria MOI 10 or 100. MDMs were collected, lysed, and examined for (A) caspase 1 activity or (B) caspase 4 activity. Values were determined by normalizing absorbance values to their respective unstimulated controls for each experiment and are depicted by mean ± SEM (n = 7 donors); 1-way ANOVA with a Dunnett multiple comparisons posttest to unstimulated controls at each time point (*$P < .05$). ANOVA indicates analysis of variance; ATP, adenosine triphosphate; LPS, lipopolysaccharide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection.

Table 1. *Neisseria gonorrhoeae* induction of cell death in MDMs is dependent on caspase 4 activity.

HUMAN MONOCYTE-DERIVED MACROPHAGE CELL VIABILITY			
	DMSO	C1 INHIBITOR	C4 INHIBITOR
Unstimulated	84.4 ± 9	79.3 ± 9	88.9 ± 8
MOI 10	65.8 ± 7	77.6 ± 11	79.6 ± 7.1*
MOI 100	56.6 ± 10	77.1 ± 16*	77.7 ± 5***
LPS/ATP	57.5 ± 10	66.8 ± 4	70.1 ± 12*
	DMSO	C3 INHIBITOR	
Unstimulated	84.4 ± 9	88.1 ± 6	
MOI 10	65.8 ± 7	70.8 ± 8	
MOI 100	56.6 ± 10	65.2 ± 8	
STS	62.0 ± 1	75.4 ± 11*	

Abbreviations: ATP, adenosine triphosphate; LPS, lipopolysaccharide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection; STS, staurosporine. MDMs were incubated with caspase 1, caspase 4, or caspase 3 inhibitors at a concentration of 100 nM for 1 hour prior to addition of LPS (100 ng/mL, *E coli* O111:B4) and 5 mM ATP (LPS/ATP) or live bacteria strain FA0190B MOI 10 or MOI 100 for 6 hours. Cells were collected and viability assessed by trypan blue staining. Inhibitors: caspase 1 inhibitor (Z-WEHD-FMK), caspase 4 inhibitor (Z-YVAD-FMK), and caspase 3 inhibitor (Z-DEVD-FMK). Table contains data from 7 donors and are shown as mean ± SEM; 2-way analysis of variance with a Bonferroni multiple comparisons posttest on each inhibitor treatment as compared with DMSO control (*$P < .05$; **$P < .01$).

both the canonical (caspase 1 dependent) and noncanonical (caspase 4 dependent) pathways.

Macrophage cell death is associated with intracellular gonococci

To further examine the interaction of *N gonorrhoeae* with MDMs that resulted in bacteria-induced pyroptosis, MDMs were treated for 6 hours with GFP-expressing *N gonorrhoeae* strain F62, followed by treatment with the viability dye, Zombie Red. Confocal imaging of MDMs demonstrated that the fraction of cells staining positive for bacteria alone (GFP+, Zombie Red-) was about the same as the fraction staining for both bacteria and loss of membrane integrity (GFP+, Zombie Red+) in cultures treated with an MOI of either 10 or 100 (Figure 5). These results indicate that a portion of

macrophages associated with bacteria remain viable. However, most of the Zombie Red+ cells were also GFP+, indicating that MDM cell death was enhanced following stimulation with *N gonorrhoeae*.

Although MDM cell death was enhanced in the presence of *N gonorrhoeae* as determined by confocal imaging, we found an equal portion of MDMs that were positive for GFP+ single positive or for GFP+/Zombie Red+. Variation in MDM survival despite bacterial association could result from variation in the localization of cell-associated *N gonorrhoeae*. Using sequential z-stack confocal imaging, we next examined the presence of extracellular and intracellular bacteria in MDM-stimulated cultures (Figure 6A). This analysis demonstrated that MDMs with intracellular bacteria were 3 times more likely to be GFP+/Zombie Red+ as compared GFP+ single positive in both MOI 10 and MOI 100–treated
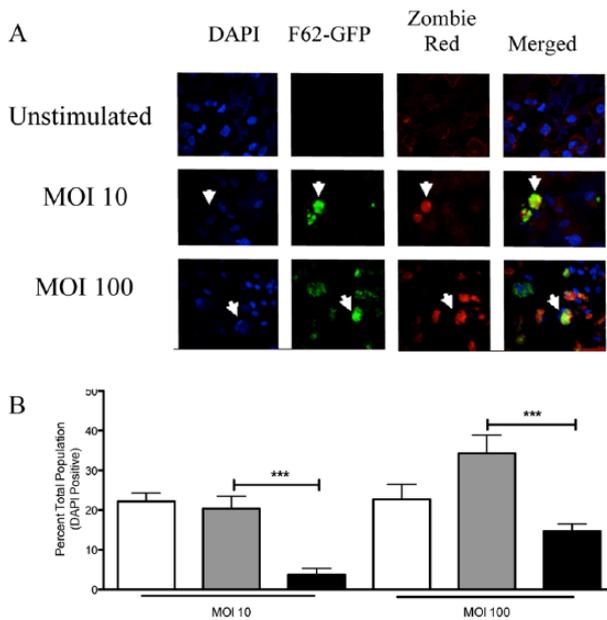
Figure 5. *Neisseria gonorrhoeae*–induced MDM cell death is associated with intracellular bacteria. MDMs were either left unstimulated or treated with *N gonorrhoeae* F62-GFP corresponding to MOI of 10 and 100 for 6 hours. Following stimulation, cells were stained with Zombie Red and mounted with a mounting media containing DAPI. (A) Representative images for unstimulated MOI 10 and MOI 100. White arrows indicate F62-GFP bacteria and Zombie Red+ cells. (B) Cells were enumerated as being positive for GFP+ (white), GFP+/Zombie Red+ (gray), and Zombie Red+ (black). Quantification of confocal images of 4 fields per donor, mean ± SEM (nv6 donors). Each MOI was analyzed by 1-way ANOVA with a Dunnett posttest (***$P < .01$). ANOVA indicates analysis of variance; MOI, multiplicity of infection.

cocultures (Figure 6A and B). In contrast, MDMs with extracellular bacteria were primarily GFP+ single positive (Figure 6A and B).

To further confirm the requirement for intracellular *N gonorrhoeae* for the induction of MDM cell death, cultures were pretreated with cytochalasin D. As expected, pretreatment of MDMs with cytochalasin D lowered levels of bacteria-induced cell death as compared with DMSO controls (Figure 6C). Cytochalasin D treatment reduced *N gonorrhoeae*–induced MDM cell death ~80% in MOI 10 cocultures and ~75% in MOI 100 cocultures as compared with DMSO controls. The extent of bacterial internalization, in both cytochalasin D–treated and control DMSO–treated samples, was assessed by an antibiotic protection assay and revealed, as expected, lower levels of intracellular bacteria in cytochalasin D–treated samples as compared with the DMSO control consistent with inhibition of *N gonorrhoeae* entry by cytochalasin D (Table 2). Despite lower levels of intracellular bacteria, cytochalasin D–treated cultures had similar levels of total bacteria within cocultures, showing no overall effect of cytochalasin D treatment on bacterial viability. Collectively, these results indicate that *N gonorrhoeae* induction of MDM pyroptosis is associated with intracellular bacteria.

Gonococcal LOS induces cell death in MDMs

The human caspase, caspase-4 like murine caspase-11, has been shown to interact with *E coli* LPS within the cytosol via the LPS lipid A moiety.[37] The lipid A component of *E coli* LPS and *N gonorrhoeae* LOS are similar in overall immune-stimulatory capacity despite differing acyl chain length and arrangement, leading us to postulate that MDM pyroptosis was induced by gonococcal LOS.[38] We observed that stimulation of MDM with transfected intracellular *N gonorrhoeae* LOS resulted in cell death as observed by Zombie Red staining when compared with unstimulated cultures (Figure 7A; Supplemental Figure 2). Incubation of MDMs with intracellular LOS also resulted in caspase 4 activation (Figure 7B). As a positive control of intracellular LPS-induced macrophage cell death, we incubated MDMs with *E coli* LPS. Consistent with previous reports, *E coli* LPS resulted in increased cell death and elevated levels of caspase 4.[37] Neither intracellular *E coli* LPS nor *N gonorrhoeae* LOS induced activation of caspase 1 or caspase 3 (Supplemental Figure 3). Combined, these findings indicate that gonococcal LOS induces MDM cell death via noncanonical pyroptosis.

Activation of caspase 4 and subsequent pyroptosis have been shown to result from direct interaction with lipid A, the component of LPS which contains acyl chains of varied arrangement and length that are pathogen specific.[37] To determine whether the immune-stimulatory activity of *N gonorrhoeae* LOS is dependent on specific lipid A structures, we used a *N gonorrhoeae* strain that produces a penta-acylated LOS lipid A, which elicits a reduced cytokine response and prolonged survival in epithelial cells.[39] Monocyte-derived macrophages were stimulated with *N gonorrhoeae* wild-type strains FA1090B or 1291, and the LOS mutant strain (1291Δ*msbB*) and MDM cell death are monitored by trypan blue exclusion. We observed similar levels of cell death following stimulation of MDMs with all 3 strains as determined by trypan blue exclusion (Table 3). *Neisseria gonorrhoeae* strain 1291Δ*msbB* was also demonstrated to activate immune caspases 1 and 4 to similar levels as the wild-type strain 1291 (Figure 8). Selective inhibition of immune caspases demonstrated that both strains 1291 and 1291Δ*msbB*, exploited caspase 1 and caspase 4 pyroptotic pathways. Only caspase 4 inhibition pretreatment of MDMs stimulated with either 1291 or 1291Δ*msbB* (MOI 100) resulted in an ~29% and ~18% increase in viability, respectively, as compared with DMSO controls (Table 3). However, pretreatment of MDMs with caspase 1 or caspase 4 inhibitors prior to stimulation with 1291 or 1291Δ*msbB* of an MOI of 10 resulted in increased cell viability (Table 3). Together, these results indicate that *N gonorrhoeae* 1291Δ*msbB* induces both canonical caspase 1 and noncanonical caspase 4 pyroptosis.

Discussion

In this study, we demonstrate that *N gonorrhoeae* induces nonapoptotic cell death in human macrophages via
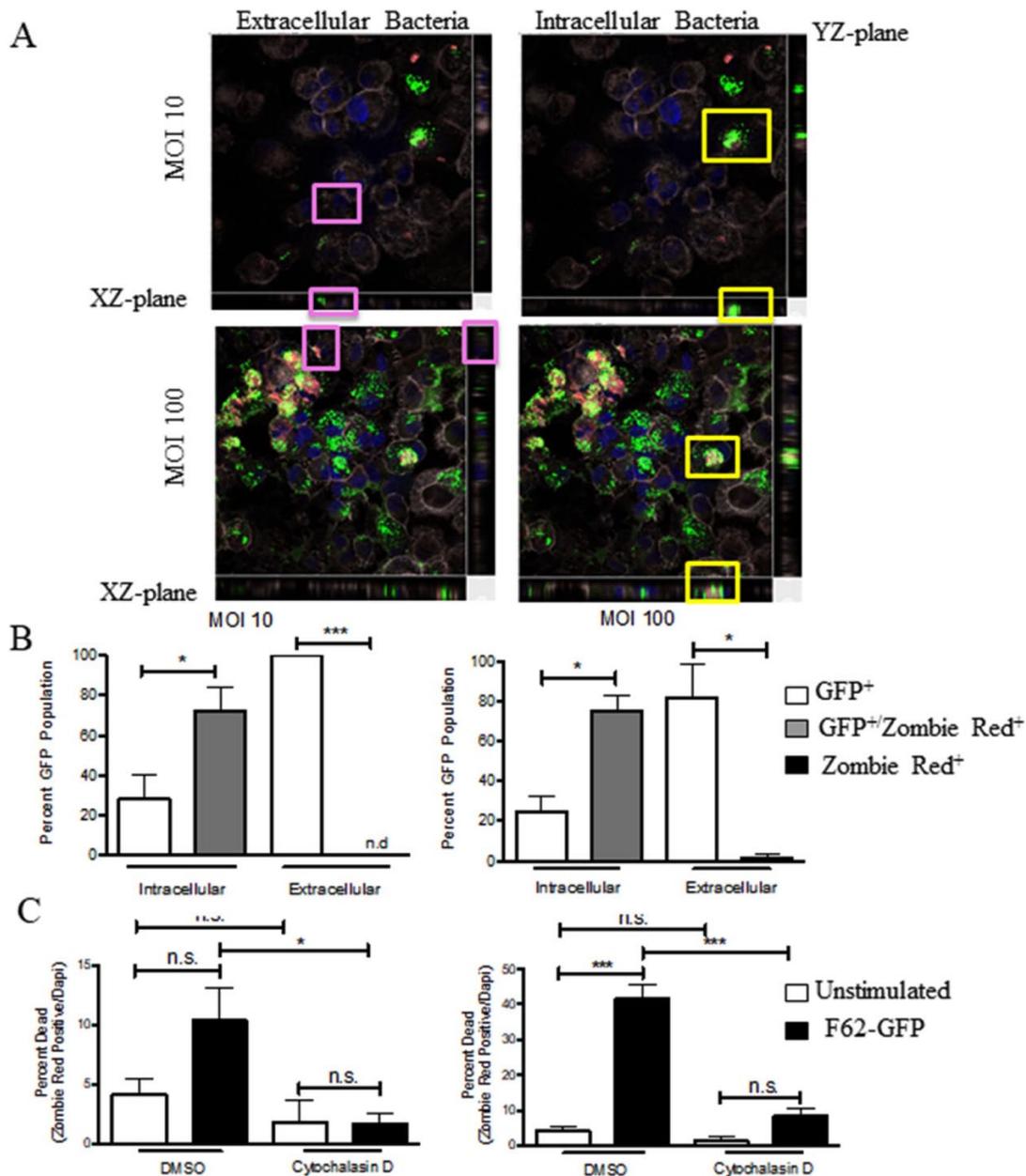
Figure 6. Colocalization of intracellular *Neisseria gonorrhoeae* with dead MDMs. MDMs were either left unstimulated or treated with *N gonorrhoeae* F62-GFP corresponding to MOI of 10 and 100 for 6 hours. Following stimulation, cells were stained with Zombie Red and Phallodin Alexa Fluor647 (actin stain) and mounted with a mounting media containing DAPI. (A) Orthogonal view of z-axis images for MOI 10 and MOI 100. The extracellular bacteria are boxed in pink and the intracellular bacteria are boxed in yellow. (B) Quantification of confocal images of 4 fields per donor. Cells were analyzed for number of cells that were positive for GFP+ (white) or cells that were GFP+/Zombie Red+ (gray). (C) MDMs were treated with cytochalasin D (1 hour) and then with F62-GFP (6 hours), then stained with Zombie Red and mounted with media containing DAPI. Images were analyzed for cell death by Zombie Red+ staining. Cells were either left unstimulated (white) or stimulated with live bacteria (black). Values represent the mean ± SEM (n = 3 donors). All experiments were analyzed by 1-way ANOVA with a Dunnett multiple comparisons posttest for each MOI (n.s., nonsignificant; n.d., not detectable; *$P <$ .05; ***$P <$ .01). ANOVA indicates analysis of variance; DMSO, dimethyl sulfoxide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection.

pyroptosis. This mechanism of cell death can proceed through 2 pathways: caspase 1 dependent (canonical pathway) and caspase 4 dependent (noncanonical pathway). *Neisseria gonorrhoeae*–induced pyroptosis in MDMs occurred through both the canonical and noncanonical pathways. Previous studies have reported that noncanonical pyroptosis is induced by intracellular gram-negative bacteria and intracellular LPS.[37] Indeed, we found that MDM cell death was associated with intracellular gonococci and that cytochalasin D treatment, which inhibited internalization of gonococci and diminished gonococcal-induced cell death. Furthermore, stimulation of MDM with transfected gonococcal LOS also induced pyroptosis and increased secretion of the pro-inflammatory cytokine IL-1β.

Table 2. Inhibition of actin polymerization reduces intracellular bacteria in MDMs.

NEISSERIA GONORRHOEAE F62-GFP CFU		EXTRACELLULAR BACTERIA (10^7)	CELL-ASSOCIATED BACTERIA (10^7)	INTRACELLULAR BACTERIA (10^5)	TOTAL BACTERIA LEVEL (10^7)
DMSO treatment	MOI 10	2.1 ± 3	0.12 ± 0.008	0.018 ± 0.009	2.2 ± 3.5
	MOI 100	6.6 ± 8	4.7 ± 4	3.3 ± 2	11.3 ± 11.3
Cytochalasin D treatment	MOI 10	5.2 ± 3*	3.2 ± 2**	0.0086 ± 0.01*	3.7 ± 2
	MOI 100	3.2 ± 2	11.5 ± 20	2.4 ± 1*	11.8 ± 19

Abbreviations: CFU, colony-forming unit; DMSO, dimethyl sulfoxide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection.
MDMs were treated with either cytochalasin D or vehicle control DMSO (1 hour) prior to bacterial stimulation (6 hours). Bacterial viability was enumerated as being extracellular, cell associated, or intracellular, as described within the "Materials and Methods" section. Values represent mean ± SEM (n = 3 donors). Two-way analysis of variance comparing cytochalasin D and vehicle DMSO treated with a Bonferroni multiple comparisons posttest (*P < .05; ***P < .01).
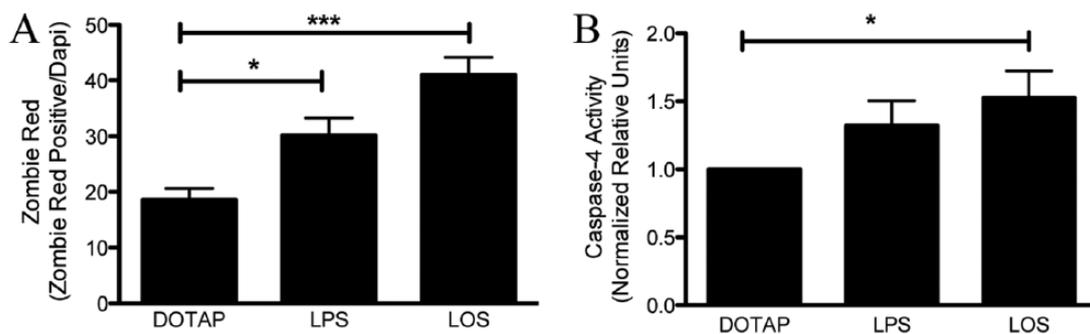
Figure 7. *Neisseria gonorrhoeae* LOS induces MDM cell death. MDMs were grown on sterile coverslips overnight followed by treatment with vehicle control, DOTAP, or purified *Escherichia coli* LPS (LPS, *E coli* O111:B4) and purified *N gonorrhoeae* LOS (LOS) 2 hours. (A) Level of Zombie Red staining was enumerated per condition by confocal microscopy and presented as a percent of all MDMs (4 views per condition per donor). (B) Cell lysates were monitored for caspase 4 activity; values were normalized to vehicle control, DOTAP. Values represent mean ± SEM (n = 3 donors); 1-way ANOVA with a Dunnett multiple comparisons posttest on each analysis (*P < .05; ***P < .001). ANOVA indicates analysis of variance; LPS, lipopolysaccharide; LOS, lipooligosaccharide; MDMs, monocyte-derived macrophages.

The individual roles for caspase 1 and caspase 4 in *N gonorrhoeae*–induced cell death were addressed using inhibitors and revealed that inhibition of each immune caspase resulted in partial rescue in viability of MDM cultures. Because caspase 4 is an upstream activator of caspase 1, its inhibition may have a stronger effect on pyroptosis and IL-1β maturation as compared with inhibition of caspase 1.[40] This is consistent with our findings of increased MDM viability in cultures pretreated with a caspase 4 inhibitor as compared with the caspase 1 inhibitor. We recognize the potential ambiguity in peptide specificity of caspase inhibitors and future studies are required to definitively define the independent or synergistic roles of caspase 1 and caspase 4 in *N gonorrhoeae*–induced pyroptosis.

In addition to examining cell death induced by intact *N gonorrhoeae*, our studies also examine the role of gonococcal LOS in the induction of pyroptosis. Intracellular delivery of LOS in macrophages failed to induce significant activation of caspase 1; in contrast, stimulation with nontransfected LOS plus ATP led to high levels of caspase 1 activity suggesting that this may depend on extracellular TLR4 activation consistent with previous reports using *E coli* LPS.[41,42] Future studies to definitively rule out TLR4-dependent caspase 1 activation in the induction

of pyroptosis in LOS-treated MDMs and will use gene silencing to address the potential role of extracellular TLR4 activation.

To further address the potential role of LOS in intact bacteria-induced MDM cell death, the LOS mutant strain, 1291Δ*msbB*, was used. Previous reports by Apicella and colleagues demonstrated that removal of an acyl chain from gonococcal LOS decreased *N gonorrhoeae*–induced inflammatory cytokine production and prolonged bacterial survival in epithelial cells.[39] Monocyte-derived macrophage stimulation with 1291Δ*msbB* induced comparable cell death with that induced by either the parent strain or FA1090B. Shi and colleagues determined that purified *E coli* lipid A lacking 2 acyl chains retained the ability to induce caspase 4–dependent pyroptosis.[37] Thus, a similar scenario is possible for the 1291Δ*msbB* strain, although other bacterial components may play an additional role. For example, outer membrane components including pili, Opa proteins, and porins may play a role in gonococcal-induced cell death; however, their role in pyroptosis remains unclear.[43-46] Nonetheless, our results indicate that in macrophages, the presence of intracellular gonococci and LOS favors immune caspase activation and pyroptosis (Figure 9).

Table 3. Expression of penta-acylated LOS does not alter *Neisseria gonorrhoeae*–induced cell death in MDMs.

HUMAN MONOCYTE-DERIVED MACROPHAGE VIABILITY				
(A)				
	FA1090B	1291	1291Δ*MSBB*	
Unstimulated	86 ± 5	89 ± 6	93 ± 5	
MOI 10	72 ± 11*	73 ± 11**	75 ± 6*	
MOI 100	70 ± 6***	68 ± 6***	60 ± 6***	
LOS/ATP	70 ± 4**	73 ± 13**	71 ± 14*	
STS	65 ± 9***	67 ± 12***	69 ± 14**	
(B)				
STRAIN	1291		1291Δ*MSBB*	
TREATMENT	DMSO	C4	DMSO	C4
Unstimulated	90.2 ± 7	86.5 ± 8	87.9 ± 4	89.5 ± 4
MOI 10	70.4 ± 11	81.4 ± 8	75.7 ± 7	80.8 ± 7
MOI 100	58.5 ± 12	75.4 ± 10*	67.2 ± 8	79 ± 3**
LOS/ATP	68.5 ± 4	69.8 ± 13	64.9 ± 6	76.2 ± 7**
(C)				
STRAIN	1291		1291Δ*MSBB*	
TREATMENT	DMSO	C1	DMSO	C1
Unstimulated	84.9 ± 4	88.4 ± 10	84.9 ± 15	78.0 ± 2
MOI 10	68.8 ± 5	79.2 ± 11	65.5 ± 7	77.6 ± 5
MOI 100	63.7 ± 9	64.4 ± 11	59.7 ± 8	58.3 ± 14
LOS/ATP	1.5 ± 15	66.8 ± 9*	51.0 ± 2	66.7 ± 0*

Abbreviations: ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide; LOS, lipooligosaccharide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection; STS, staurosporine.
Cells were stimulated with FA1090B, 1291, or 1291Δ*msbB* for 6 hours. Cells were collected and viability was assessed by trypan blue staining. MDMs were either left untreated or treated with inhibitors 1 hour prior to stimulation. MDMs were stimulated with LOS (100 ng/mL, strain F62) and 5 mM ATP (LOS/ATP) or live bacteria MOI 10 or MOI 100 for 6 hours. MDMs treated with caspase 1 inhibitor or treated with caspase 4 inhibitor. Cells were collected and viability was assessed by trypan blue staining. Table shows mean ± SEM from 6 donors. Statistical values for (A) were analyzed by 1-way analysis of variance (ANOVA) with a Dunnett multiple comparisons posttest with all values compared with the unstimulated control per condition. Statistical values for (B) and (C) were analyzed by 2-way ANOVA with a Bonferroni multiple comparison posttest where each inhibitor was compared with the DMSO control (*$P < .05$; **$P < .01$; ***$P < .001$).

Neisseria gonorrhoeae is a nonobligate intracellular bacteria, but similar to several other human pathogens has a strict growth requirement for iron, which is not available extracellularly.[47] A recent report observed that stimulation of macrophages with *N gonorrhoeae* correlated with lack of induction of caspase 3 activity, a marker of apoptosis, as a mechanism to promote intracellular survival.[19] A separate report found that stimulation of macrophages with *N gonorrhoeae* resulted in downregulation of host genes involved in iron metabolism and postulated that this leads to increased intracellular iron and gonococcal survival.[48] Collectively, these studies support an important bacteria-induced mechanism to ensure intracellular survival. Our studies, however, show that macrophages undergo a lytic form of cell death, pyroptosis, from gonococcal stimulation. It is possible that induction of lytic cell death is a mechanism implemented by the bacteria to circumvent such lack of iron and escape the cytosol.

As resident immune cells, macrophages are abundant within urogenital mucosa and contribute to both innate and adaptive immune responses following pathogen exposure.[9,10] Induction of host cell death can either promote pathogen clearance or enable pathogens to thrive via the elimination of a phagocytic cell. Inhibition of pyroptosis did not alter the number of viable gonococci recovered from macrophage cultures (data not shown). Furthermore, induction of pyroptosis in *N gonorrhoeae*–stimulated macrophages was associated with the production of pro-inflammatory cytokines. Based on these results, collectively, we propose that *in vivo*, the induction of macrophage cell death contributes to ineffective clearance of both the gonococcus and dying recruited immune cells, such as
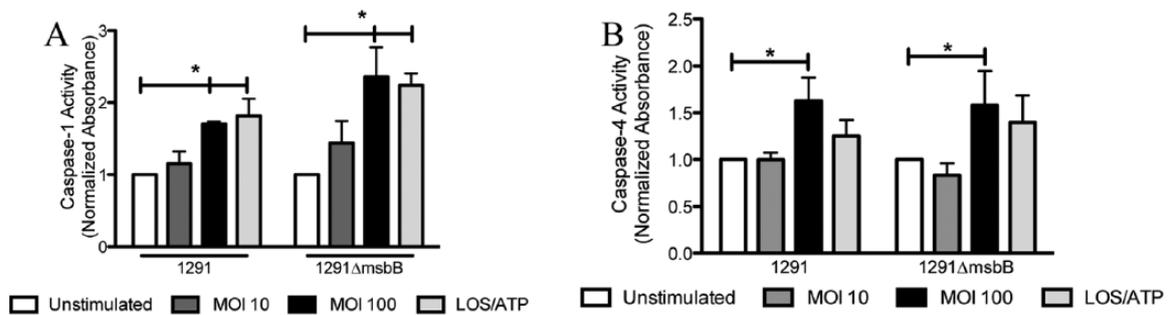
Figure 8. Expression of penta-acylated LOS does not alter *Neisseria gonorrhoeae* induction of caspase 1 and caspase 4 in MDMs. MDMs were incubated for 6 hours with medium alone (unstimulated), LOS (100 ng/mL) and 200 mM ATP (LOS/ATP), or live bacteria MOI 10 or 100. MDMs were collected, lysed and (A) caspase 1 activity assessed by a caspase 1 activity assay (BioVision) and (B) caspase 4 activity assessed by a caspase 4 activity assay (MBL International). Values were determined by normalizing absorbance values to their respective unstimulated controls for each experiment and are depicted by mean ± SEM of 7 donors. Statistics were determined by 1-way ANOVA with a Dunnett multiple comparisons posttest to unstimulated controls at each time point (*$P < .05$). ANOVA indicates analysis of variance; ATP, adenosine triphosphate; LOS, lipooligosaccharide; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection.
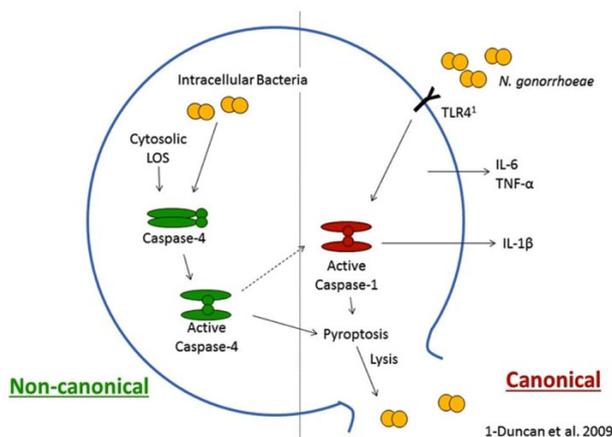
Figure 9. Model for *Neisseria gonorrhoeae*–induced macrophage cell death. Our results indicate that both live gonococci and LOS can induce human macrophage cell death. Activation of pyroptosis by *N gonorrhoeae* was observed to occur via both canonical (caspase 1) and noncanonical (caspase 4 dependent) cell deaths. *Neisseria gonorrhoeae* may induce canonical pyroptosis by extracellular TLR signaling as reported by Duncan and colleagues.[17] We propose that noncanonical pyroptosis is induced by intracellular bacteria. Intracellular *N gonorrhoeae* and/or gonococcal LOS are proposed to escape the phagosome thus preventing gonococcal degradation by the lysosome and activating cytosolic caspase 4. IL-6 indicates interleukin 6; LOS, lipooligosaccharide; TNF-α, tumor necrosis factor α.

neutrophils which have a short half-life during infection.[49] We propose that the ability of *N gonorrhoeae* to induce pro-inflammatory cytokine production combined with lytic pyroptotic cell death in macrophages creates a feed-forward loop for the continual recruitment of neutrophils to the mucosa as observed during human gonococcal infection.

Acknowledgements

Author Contributions

JLR conceived and designed the experiments, wrote the first draft of the manuscript, and analyzed the data. CAG contributed to the writing of the manuscript, made critical revisions, and approved final version. JLR and CAG agree with manuscript results and conclusions and jointly developed the structure and arguments for the paper. All authors reviewed and approved the final manuscript.

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