

ORIGINAL RESEARCH

NOD-Like Receptor Protein 3 Inflammasome Priming and Activation in Barrett's Epithelial Cells



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SUMMARY

Microbial products activate Toll-like receptors and inflammasomes. Gram-negative bacteria (which dominate the microbiome of reflux esophagitis) produce lipopolysaccharide, a Toll-like receptor-4 ligand. To explore this system's contribution to esophageal inflammation, we studied lipopolysaccharide effects on inflammasome priming and activation in esophageal cells.

BACKGROUND & AIMS: Microbial molecular products incite intestinal inflammation by activating Toll-like receptors (TLRs) and inflammasomes of the innate immune system. This system's contribution to esophageal inflammation is not known. Gram-negative bacteria, which dominate the esophageal microbiome in reflux esophagitis, produce lipopolysaccharide (LPS), a TLR4 ligand. TLR4 signaling produces pro-interleukin (IL)1 β , pro-IL18, and NOD-like receptor protein 3 (NLRP3), which prime the NLRP3 inflammasome. Subsequent NLRP3 inflammasome activation cleaves caspase-1, inducing secretion of proinflammatory cytokines and pyroptosis (inflammatory cell death). We explored LPS effects on NLRP3 inflammasome priming and activation in esophageal cells.

METHODS: We exposed esophageal squamous and Barrett's epithelial cells to LPS and measured the following: (1) TLR4, pro-IL1 β , pro-IL18, and NLRP3 expression; (2) caspase-1 activity; (3) tumor necrosis factor- α , IL8, IL1 β , and IL18 secretion; (4) lactate dehydrogenase (LDH) release (a pyroptosis marker); and (5) mitochondrial reactive oxygen species (ROS). As inhibitors, we used acetyl-Tyr-Val-Ala-Asp-CHO for caspase-1, small interfering RNA for NLRP3, and (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride for mitochondrial ROS.

RESULTS: Squamous and Barrett's cells expressed similar levels of TLR4, but LPS induced TLR4 signaling that increased tumor necrosis factor- α and IL8 secretion only in Barrett's cells. Barrett's cells treated with LPS showed increased expression of pro-IL18, pro-IL1 β , and NLRP3, and increased mitochondrial ROS levels, caspase-1 activity, IL1 β and IL18 secretion, and LDH release. Acetyl-Tyr-Val-Ala-Asp-CHO, NLRP3 small interfering

RNA, and Mito-TEMPO all blocked LPS-induced IL1 β and IL18 secretion and LDH release.

CONCLUSIONS: In Barrett's cells, LPS both primes and activates the NLRP3 inflammasome, causing secretion of proinflammatory cytokines and pyroptosis. By triggering molecular events promoting inflammation, the esophageal microbiome might contribute to inflammation-mediated carcinogenesis in Barrett's esophagus. (*Cell Mol Gastroenterol Hepatol* 2016;2:439-453; <http://dx.doi.org/10.1016/j.jcmgh.2016.03.006>)

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Gastroesophageal reflux disease (GERD) causes esophageal inflammation (reflux esophagitis) that damages the esophageal squamous epithelium and enables its replacement by a metaplastic, intestinal-type columnar epithelium (Barrett's esophagus).¹ GERD-induced chronic inflammation in Barrett's esophagus is thought to contribute to the development of esophageal adenocarcinoma, a tumor whose frequency has increased at an alarming rate in

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Abbreviations used in this paper: Ac-YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-CHO; AIM, absent in melanoma; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; ATP, adenosine triphosphate; DAMP, damage-associated molecular pattern; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GERD, gastroesophageal reflux disease; IL, interleukin; LPS, lipopolysaccharide; LDH, lactate dehydrogenase; mRNA, messenger RNA; NF- κ B, nuclear factor- κ B; NLRP3, NOD-like receptor protein 3; NOD, nucleotide-binding domain, leucine-rich repeat containing proteins; PAMP, pathogen-associated molecular pattern; PBS, phosphate-buffered saline; pro, protein; PRRs, pattern-recognition receptors; qPCR, quantitative reverse-transcription polymerase chain reaction; ROS, reactive oxygen species; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; TLR, Toll-like receptor; TNF, tumor necrosis factor.

Most current article

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Western countries.^{2,3} A number of proinflammatory cytokines have been found in biopsy specimens of inflamed Barrett's metaplasia, with especially high levels of interleukin (IL)1 β .⁴ Mice genetically engineered to express high levels of IL1 β in the esophagus develop esophagitis with a Barrett's-like metaplasia that progresses to adenocarcinoma, suggesting that IL1 β might play a key role in Barrett's-associated tumorigenesis.⁵ GERD can cause the esophagus to secrete proinflammatory cytokines,⁶ but there also is reason to believe that the esophageal microbiome might contribute to esophageal inflammation with production of IL1 β .

IL1 β and IL18 (another proinflammatory cytokine) can be generated through proteolytic cleavage of their precursor proteins (pro-IL1 β and pro-IL18) by the cysteine protease caspase 1.⁷⁻⁹ In addition to activating these proinflammatory cytokines, caspase-1 can induce pyroptosis, a unique form of programmed cell death in which the dying cells release their proinflammatory cytoplasmic contents into the extracellular space, causing further inflammation.^{7,10} Thus, caspase-1 can play a major role in promoting inflammation, and caspase-1 can be activated by a cytoplasmic protein complex called an *inflammasome*.¹¹

Inflammasomes contain pattern-recognition receptors (PRRs) that recognize certain pathogen-associated molecular patterns (PAMPs) produced by microbes, and certain damage-associated molecular patterns (DAMPs) produced by damaged cells.¹² There are a number of families of PRRs, including the nucleotide-binding domain, leucine-rich repeat containing proteins (NOD; NOD-like receptors [NLRs]), and the absent in melanoma 2 (AIM)-like receptors,¹² and different inflammasomes usually are named for the PRRs they harbor.¹³ After the sensing of PAMPs or DAMPs, the PRRs assemble a large macromolecular complex containing the PRR itself and an adapter protein called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). This complex activates caspase-1 in canonical inflammasomes, and/or murine caspase-11 (caspase-4/-5 in human beings) in noncanonical inflammasomes.^{13,14}

Canonical and noncanonical inflammasome activation is a 2-step process. The first step primes the inflammasome by increasing the expression of pro-IL1 β and pro-IL18. This priming step in canonical and noncanonical inflammasomes is virtually identical, and is accomplished by activation of Toll-like receptors (TLRs), NOD1 or NOD2, or by signaling through tumor necrosis factor (TNF)- α or IL1.^{13,14} In contrast, canonical and noncanonical inflammasomes differ in the second activating step. In canonical inflammasomes, activation involves the sensing of PAMPs or DAMPs by the PRRs, which then activate caspase-1. The activated caspase-1 cleaves the pro-IL1 β and pro-IL18 produced during the priming step into mature active forms that are secreted into the extracellular space. Activated caspase-1 also induces pyroptosis, which promotes further inflammation.¹³ In noncanonical inflammasomes, the PRRs recognize intracellular lipopolysaccharide (LPS) (a product of gram-negative bacteria) to activate caspase-11.¹⁴ Activated caspase-11 can mediate pyroptosis directly, but the secretion of IL1 β and IL18 induced by caspase-11 is an indirect process that requires caspase-1, ASC, and the NOD-like receptor protein

3 (NLRP3) PRR to convert pro-IL1 β and pro-IL18 into their active forms.^{14,15}

TLRs also are PRRs, and some of the same molecular patterns that activate the PRRs in inflammasomes also activate the TLRs located on the cell surface or in endosomes. In collaboration with inflammasomes, TLRs protect against microbial infection by triggering an innate immune response.¹⁶ However, inappropriate TLR activation can lead to uncontrolled chronic inflammation and promote carcinogenesis.^{17,18} Chronic inflammation caused by inappropriate TLR activation can, in some cases, be attributed to disturbances in the local microbiome, which is the collection of microbes and their genes that reside in a biological niche.¹⁹ The human microbiome comprises bacteria, fungi, protozoa, and viruses, although bacteria have been the most well characterized and studied.¹⁹

In some cell types, LPS produced by gram-negative bacteria has been shown to function as a PAMP that activates TLR4 and primes the inflammasome (step 1). In nonesophageal cells, LPS also has been shown to cause inflammasome activation (step 2).^{13,20,21} Gram-negative bacteria dominate the esophageal microbiome of patients with GERD and Barrett's esophagus,²²⁻²⁴ and one earlier study found that Barrett's epithelial cells express TLR4 that can be activated by LPS.²⁵ In this study, we have explored the effects of LPS on TLR4 signaling and on priming and activation of the inflammasome in esophageal squamous and Barrett's epithelial cells.

Materials and Methods

Esophageal Squamous and Barrett's Epithelial Cell Primary Cultures, and Barrett's Epithelial Cell Lines

We established primary cultures of esophageal squamous epithelial cells (NES-B3, NES-B10, NES-G2, and NES-G4) using biopsy specimens of esophageal squamous epithelium from 4 patients with GERD, and primary cultures of Barrett's epithelial cells (BAR-12, BAR-15, and BAR-18) using biopsy specimens of Barrett's metaplasia from 3 patients with nondysplastic Barrett's esophagus as previously described.²⁶ We used 2 non-neoplastic, telomerase-immortalized Barrett's epithelial cell lines (BAR-T, BAR-10T) that were developed in our laboratory^{27,28} for the mechanistic studies described later, and we compared the response of the Barrett's cell lines to that of the primary cultures of Barrett's epithelial cells. These studies were approved by the Institutional Review Board of the Dallas VA Medical Center. Primary cultures and BAR-T cell lines were co-cultured with a fibroblast feeder layer and maintained in growth medium as previously described.^{26,27} Primary cultures and cell lines were maintained at 37°C in a 5% CO₂ incubator. For individual experiments, primary esophageal cells and BAR-T cell lines were seeded equally into collagen IV-coated wells (BD Biosciences, San Jose, CA), in the absence of fibroblast feeder layers, and maintained in growth medium.

LPS and Adenosine Triphosphate Treatment

In preliminary experiments, we treated telomerase-immortalized, non-neoplastic esophageal squamous^{29,30}

and BAR-T cell lines with LPS (Sigma, St. Louis, MO) for 24 and 48 hours in concentrations of 0.1, 1, and 10 $\mu\text{g}/\text{mL}$, and found that the 1 and 10 $\mu\text{g}/\text{mL}$ concentrations decreased cell viability in some of the cell lines at 48 hours. At 24 hours, LPS at a dose of 10 $\mu\text{g}/\text{mL}$ reduced cell viability in BAR-10T cells only. Therefore, we used LPS at a concentration of 1 $\mu\text{g}/\text{mL}$ for up to 24 hours for all further experiments in BAR-T cell lines and in primary esophageal cells. For some studies, Barrett's cell lines were stimulated with 100 $\mu\text{g}/\text{mL}$ adenosine triphosphate (ATP) (Sigma) for 30 minutes just before the collection of cells for analysis.

Inhibition of TLR4 Signaling

BAR-10T cells were pretreated with 1 $\mu\text{mol}/\text{L}$ TAK-242 (CalBiochem, Billerica, MA), a selective TLR4 inhibitor, after which LPS was added to the TAK-242-containing media for either 30 minutes or 4 hours.

NLRP3 RNA Interference

BAR-10T cells were plated equally in 24-well tissue culture plates and transfected using Lipofectamine RNAi-MAX Regent (Invitrogen, Carlsbad, CA) and OptiMEM (Life Technologies, Grand Island, NY) with 25 pmol/mL of the SMARTpool ON-TARGETplus NLRP3 small interfering RNA (siRNA) (Thermo Scientific, Waltham, MA) for 72 hours per the manufacturer's instructions. As a control, cells were transfected with ON-TARGETplus nontargeting siRNA #1 (Thermo Scientific). After transfection, the medium was removed, and replaced with growth medium. The efficiency of the siRNA for inhibiting NLRP3 expression was determined by Western blot at 72 hours. siRNA knockdown was performed in 2 independent experiments.

Measurement of Caspase-1 Activity

Caspase-1 activity was measured using the caspase-1 assay kit (Fluorometric; Abcam, Cambridge, MA), which recognizes the sequence YVAD, per the manufacturer's instructions. This assay is based on the detection of cleavage of the substrate YVAD-7-amino-4-trifluoromethyl coumarin, which emits a blue light at 400 nm. Upon cleavage of the substrate by caspase-1, the free YVAD-7-amino-4-trifluoromethyl coumarin emits a yellow-green fluorescence at 505 nm. The fold increase in caspase-1 activity was determined by comparing the fluorescence intensity between treated and untreated BAR-T cells. Fluorescence intensity was detected using the Infinite 200 PRO series multimode microplate reader (Tecan US, Research Triangle Park, NC). All assays were performed in triplicate in at least 2 independent experiments.

Inhibition of Caspase-1

BAR-10T cells were incubated with 50 or 100 $\mu\text{mol}/\text{L}$ acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO; Merck Millipore, Guyancourt, France), a potent and specific inhibitor of caspase-1,³¹ with or without 1 $\mu\text{g}/\text{mL}$ LPS for 24 hours. All assays were performed in triplicate in at least 2 independent experiments.

Mitochondrial Reactive Oxygen Species Detection

To measure mitochondrial reactive oxygen species (ROS), the fluorescent probe MitoSOX Red (Life Technologies) was used as previously described.³² In brief, BAR-10T cells were placed in 2-well, Lab-Tek II chamber slides (Nalge Nunc, Rochester, NY) with a chamber volume of 1 mL at 1×10^5 cells per well. Cells were treated with or without 1 $\mu\text{g}/\text{mL}$ LPS for 24 hours, after which the cells were washed 2 times with phosphate-buffered saline (PBS). Cells were loaded with 5 $\mu\text{mol}/\text{L}$ MitoSOX Red for 10 minutes at 37°C, and then washed 2 times with PBS. Cells were fixed in 2% paraformaldehyde for 3–5 minutes, and washed 2 times with PBS. Then the cells were stained with 4',6-diamidino-2-phenylindole for 1 minute and washed with PBS 3 times. For some studies, cells were treated with 100 $\mu\text{mol}/\text{L}$ Mito-TEMPO (Enzo Life Sciences, Farmingdale, NY) and LPS for 24 hours followed by loading with MitoSOX as described earlier. Cells were imaged with a Leica DM6000 B fluorescence microscope (Leica Microsystem, Buffalo Grove, IL) and fluorescence was quantitated using National Institutes of Health ImageJ software (version 1.48; Bethesda, MD) from 5 separate high-power fields (40 \times) per well, and then averaged. All assays were performed in at least 2 independent experiments.

Quantitative and Qualitative Reverse-Transcription Polymerase Chain Reaction

Total RNAs were isolated by using the RNeasy Mini kit (Qiagen, Valencia, CA) per the manufacturer's instructions, and quantitated using the Nanophotometer (Implen, Westlake Village, CA). Reverse-transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen) per the manufacturer's instructions. The primer sequences (Table 1) were designed using Primer Express (Applied Biosystems, Foster City, CA) and manufactured by Integrated DNA Technologies (Coralville, IA). Each 50- μL polymerase chain reaction (PCR) reaction contained 1–5 μL of complementary DNA (corresponding to 25 or 125 ng total RNA), 2.5 $\mu\text{mol}/\text{L}$ of each primer (12.5 pmol total), and 25 μL of 2 \times GoTaq Green Master Mix (Promega, Madison, WI). For reverse-transcription (RT)-PCR analysis of pro-*IL18*, PCR conditions consisted of 95°C for 5 minutes followed by 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; for pro-*IL1 β* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PCR conditions consisted of 95°C for 5 minutes followed by 25 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and for NLRP3, PCR conditions consisted of 95°C for 5 minutes followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. After amplification, PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. GAPDH transcripts served as internal controls. All RT-PCR analyses were performed in 2 independent experiments. In addition to conventional PCR, real-time quantitative RT-PCR (qPCR) was performed using rapid cycling with the StepOnePlus Real-Time PCR System and SYBR Green Master Mix (Applied Biosystems). The

Table 1. Oligonucleotide Primers

Primer	Sequence, 5' to 3'	Location	Use
ASC-5'	TCCTGACGGATGAGCAGTA	Sense	qPCR
ASC-3'	GGCTGGTGTGAAACTGAAGA	Antisense	
AIM2-5'	CTCCTGAGTCTCTGCTAGTTA	Sense	qPCR
AIM2-3'	ACTCTCCATCTGACAACTTTGG	Antisense	
NLRP1-5'	GGTTCAGGGATGCTGGAATA	Sense	qPCR
NLRP1-3'	CCAAGTGGCCAACGTAGAA	Antisense	
NLRP3-5'	GAAGAGGAGTGATGGGTTTAC	Sense	qPCR
NLRP3-3'	TCTGCTTCTCACGACTTTCTG	Antisense	
NLRC4-5'	CATCCACGAAACTACCTTCAACTCC	Sense	qPCR
NLRC4-3'	GACAAGCAGCAGGAGACTAAT	Antisense	
GAPDH-5'	TCCCACCTTTCTCATCCAAG	Sense	qPCR and qualitative PCR
GAPDH-3'	GTCTGCAAAAGGAGTGAGGC	Antisense	
Pro-IL1 β -5'	AGTAGCAACCAACGGGAAGG	Sense	Qualitative PCR
Pro-IL1 β -3'	TTGAGGGCGTCATTACAGGAT	Antisense	
Pro-IL18-5'	AGATGATGAAAACCTGGAATCAGA	Sense	qualitative PCR
Pro-IL18-3'	TGTTCTCACAGGAGAGAGTTGA	Antisense	
NLRP3-5'	GCTGCGATCAACAGGAGAGA	Sense	qualitative PCR
NLRP3-3'	GCTCACACTCTCACCCAGAC	Antisense	

primer sequences for *ASC*, *AIM2*, *NLRP1*, *NLRP3*, and *NLRC4* messenger RNAs (mRNAs) are listed in Table 1. The reference gene *GAPDH* served as an internal control. The relative quantity of mRNA was normalized to *GAPDH*, which was expressed at similar levels in all samples, using the delta delta CT method of relative quantification, where CT is the threshold cycle. All qPCR assays were performed in triplicate in at least 2 independent experiments.

Protein Extraction and Immunoblotting

Total protein was extracted using 200 μ L of 1 \times cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with 0.5 mmol/L phenylmethylsulfonyl fluoride according to the manufacturer's instructions (Cell Signaling Technology). Protein concentrations were determined using the BCA-200 Protein Assay kit (Pierce, Rockford, IL). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with primary antibodies overnight at 4°C (Table 2). Secondary antibody was either goat anti-rabbit, horse anti-mouse IgG (Cell Signaling Technology), or donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated with horseradish peroxidase (Cell Signaling Technology), and chemiluminescence was determined using the enhanced chemiluminescence detection system (Pierce). The membranes were stripped and re-probed with mouse anti- β -tubulin (Sigma) as a loading control. Proteins were quantified using ImageJ software version 1.48, and the relative quantity of protein with respect to the loading control was calculated. All immunoblots were performed in 2 independent experiments.

Enzyme-Linked Immunosorbent Assays for IL8, TNF- α , IL1 β , and IL18

Supernatants from esophageal cell cultures were collected and centrifuged to remove cellular debris. The

amounts of IL8, TNF- α , IL1 β , and IL18 in the culture supernatants were determined by using commercially available, cytokine-specific enzyme-linked immunosorbent assays (IL18: MBL, Nagoya, Japan; IL8 and TNF- α : Life Technologies; or IL1 β : R&D Systems, Minneapolis, MN) per the manufacturer's instructions. All assays in cell lines were performed in triplicate in at least 2 independent experiments.

Measurement of Lactate Dehydrogenase

To evaluate pyroptotic cell death, lactate dehydrogenase (LDH) release in the supernatants was measured using the Cytotoxicity Detection kit (Roche Applied Science, Indianapolis, IN) per the manufacturer's instructions.³³

Data Analyses

Quantitative data are expressed as means \pm SEM. Statistical analyses were performed using an unpaired Student

Table 2. Antibodies Used

Antibody	Source information	Dosage	Use
NLRP3	Rabbit monoclonal (Cell Signaling Technology)	1:1000 dilution	WB
TLR4	Rabbit monoclonal (Santa Cruz Biotechnology)	1:500 dilution	WB
Phospho-NF- κ B p65(Ser536)	Rabbit monoclonal (Cell Signaling Technology)	1:1000 dilution	WB
Total NF- κ B	Rabbit monoclonal (Cell Signaling Technology)	1:2000 dilution	WB
Pro-IL1 β	Goat polyclonal (R&D Systems)	1:1000 dilution	WB
β -tubulin	Mouse monoclonal (Sigma)	1:2000 dilution	WB

WB, western blot.

t test or, for multiple comparisons, an analysis of variance, and the Student–Newman–Keuls multiple-comparisons test using the Instat for Windows statistical software package (GraphPad Software, San Diego, CA).

P values of .05 or less were considered significant for all analyses.

Results

Primary Cultures of Esophageal Squamous and Barrett's Epithelial Cells Express Similar Levels of TLR4, but LPS Induces Secretion of TNF- α and IL8 Only in the Primary Cultures of Barrett's Epithelial Cells

By using Western blot, we found that primary esophageal squamous cells and primary Barrett's epithelial cells expressed similar levels of TLR4 (Figure 1A). In some cell

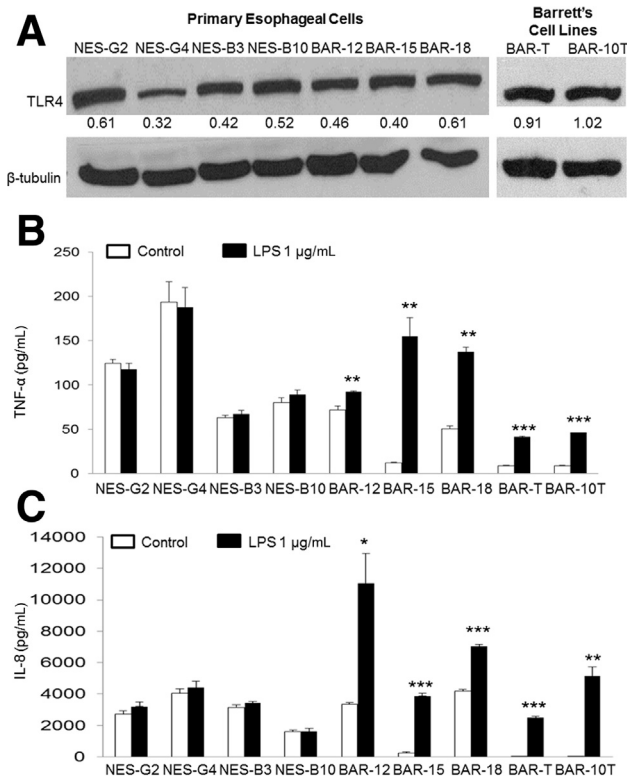


Figure 1. Primary cultures of esophageal squamous epithelial cells (NES-B3, NES-B10, NES-G2, and NES-G4), and primary cultures of Barrett's epithelial cells (BAR-12, BAR-15, and BAR-18), express similar levels of TLR4, but LPS significantly increases secretion of TNF- α and IL8 only in the primary Barrett's cells. Similar to primary Barrett's cells, Barrett's epithelial cell lines (BAR-T and BAR-10T) also express TLR4 and significantly increase secretion of TNF- α and IL8 in response to LPS. (A) Western blot for basal expression of TLR4 protein. Numbers represent the relative quantity of protein with respect to the loading control. (B and C) Enzyme-linked immunosorbent assays for (B) TNF- α secretion and (C) IL8 secretion after 24 hours of LPS stimulation. Bar graphs represent the means \pm SEM. **P* \leq .05 compared with untreated control. ***P* \leq .01 compared with untreated control. ****P* \leq .0001 compared with untreated control.

types, LPS produced by gram-negative bacteria (which dominate the esophageal microbiome of GERD patients^{22–24}) is known to activate TLR4 to increase levels of inflammatory cytokines such as TNF- α and IL8.^{25,34} We found that treatment with LPS (1 μ g/mL for 24 hours) caused a significant increase in the secretion of TNF- α (Figure 1B) and IL8 (Figure 1C) in all 3 primary Barrett's cell cultures, but not in any of the primary cultures of squamous cells.

Barrett's Epithelial Cell Lines Express TLR4, and LPS Induces Them to Secrete TNF- α and IL8

Similar to our primary Barrett's epithelial cells, our telomerase-immortalized Barrett's epithelial cell lines (BAR-T and BAR-10T) also expressed TLR4 (Figure 1A). Treatment with LPS (1 μ g/mL for 24 hours) significantly increased secretion of TNF- α (Figure 1B) and IL8 (Figure 1C) in both cell lines, as it did in our primary cell cultures.

Barrett's Cell Lines Express High Levels of NLRP3 mRNA, and Barrett's Cell Lines and Primary Barrett's Cells Express Similar Levels of NLRP3 Proteins

Inflammasomes are named for their PRR (eg, NLRP1, NLRP3, NLRC4, AIM2), and the caspase-1 in the inflammasome complex can interact with the PRR directly, or indirectly via the adaptor protein ASC.¹³ By using qPCR, we assessed the mRNA expression of inflammasome PRRs and ASC in our Barrett's cell lines. Both BAR-T and BAR-10T expressed NLRP1, NLRP3, and ASC mRNAs; neither cell line expressed appreciable levels of NLRC4, and only BAR-T expressed some AIM2 mRNA (Figure 2A). NLRP3 was the PRR expressed at the highest mRNA level in the BAR-T and BAR-10T cell lines. By using Western blot, we found that our Barrett's cell lines and primary cultures of Barrett's epithelial cells expressed similar levels of NLRP3 protein (Figure 2B).

LPS Induces Pro-IL1 β , Pro-IL18, and NLRP3 mRNA and Protein Expression That Is Dependent on TLR4 Signaling in Barrett's Cell Lines

In most cell types, inflammasome functioning usually requires 2 signals. The first signal induces the expression of pro-IL1 β and pro-IL18, which primes the inflammasome for activation by the second signal (Figure 2C).¹³ A prototypical example of priming is the binding of LPS to TLR4, leading to activation of nuclear factor- κ B (NF- κ B) and the transcriptional up-regulation of pro-IL1 β , pro-IL18, and NLRP3 mRNAs.¹³ In BAR-T and BAR-10T cells treated with LPS for up to 8 hours, RT-PCR showed increased expression of pro-IL1 β , pro-IL18, and NLRP3 mRNA (Figure 3A). Western blot showed increases in pro-IL1 β and NLRP3 protein levels during 24 hours of treatment with LPS (Figure 3B); antibodies against IL18 failed to detect a signal in our positive control, which precluded evaluation of IL18 protein levels in our samples. To confirm the role of TLR4 in this LPS-

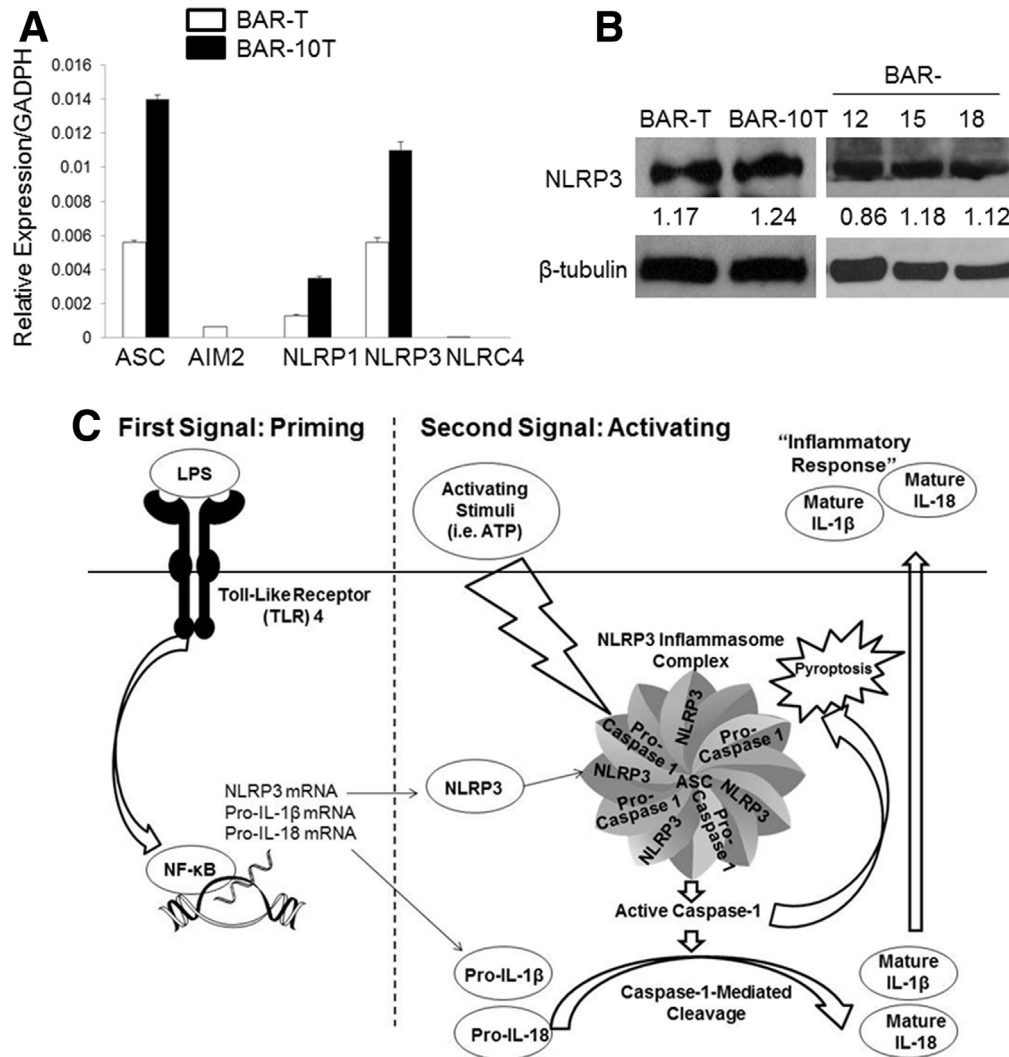


Figure 2. Barrett's cell lines express high levels of NLRP3 mRNA, and the cell lines and primary cultures of Barrett's cells express similar level of NLRP3 protein. (A) Representative experiment showing mRNA expression of ASC and inflammasome PRRs (AIM2, NLRP1, NLRP3, and NLRC4) in BAR-T and BAR-10T cell lines. *Bar graphs* represent the means \pm SEM. (B) Western blot for basal expression of NLRP3 protein in BAR-T and BAR-10T and in primary Barrett's cell cultures (BAR-12, BAR-15, and BAR-18). Numbers represent the relative quantity of protein with respect to the loading control. (C) Schematic showing NLRP3 inflammasome priming and activation. The first signal induces the expression of pro-IL1 β and pro-IL18, which prime the inflammasome for activation by the second signal. In this example, LPS binding to the TLR4 provides the first signal that leads to activation of NF- κ B and the transcriptional up-regulation of pro-IL1 β , pro-IL18, and NLRP3 mRNAs. The second signal activates the inflammasome. In this example, ATP is the stimulus that causes NLRP3 to associate with ASC, which contains a caspase recruitment domain. This NLRP3 inflammasome complex activates caspase-1, which catalyzes the proteolytic cleavage of pro-IL1 β and pro-IL18 to their mature forms, which are secreted from the cell to elicit an inflammatory response. Cleaved caspase-1 also can induce pyroptosis.

induced priming, BAR-10T cells were treated with the specific TLR4 inhibitor TAK-242; efficiency of this inhibitor was assessed by Western blot for phospho-p65, a marker of NF- κ B activation. Treatment with TAK-242 eliminated the LPS-induced increase in phospho-p65 expression (Figure 3C). Moreover, LPS-mediated increases in expression of pro-IL1 β , pro-IL18, and NLRP3 mRNA were reduced markedly by treatment with TAK-242 (Figure 3D), suggesting that inflammasome priming is dependent on TLR4 signaling in Barrett's cells.

LPS Activates Caspase-1, Causes Secretion of IL1 β and IL18, and Induces Release of LDH in Barrett's Cells

After priming, inflammasome complex formation and cleavage of pro-caspase-1 to its active form can be caused by a number of different stimuli including extracellular ATP, a prototypical example of an activating signal (Figure 2C).¹² To explore whether ATP can activate inflammasomes in Barrett's cells, we treated BAR-T and BAR-10T cells with LPS as a priming signal, and then added

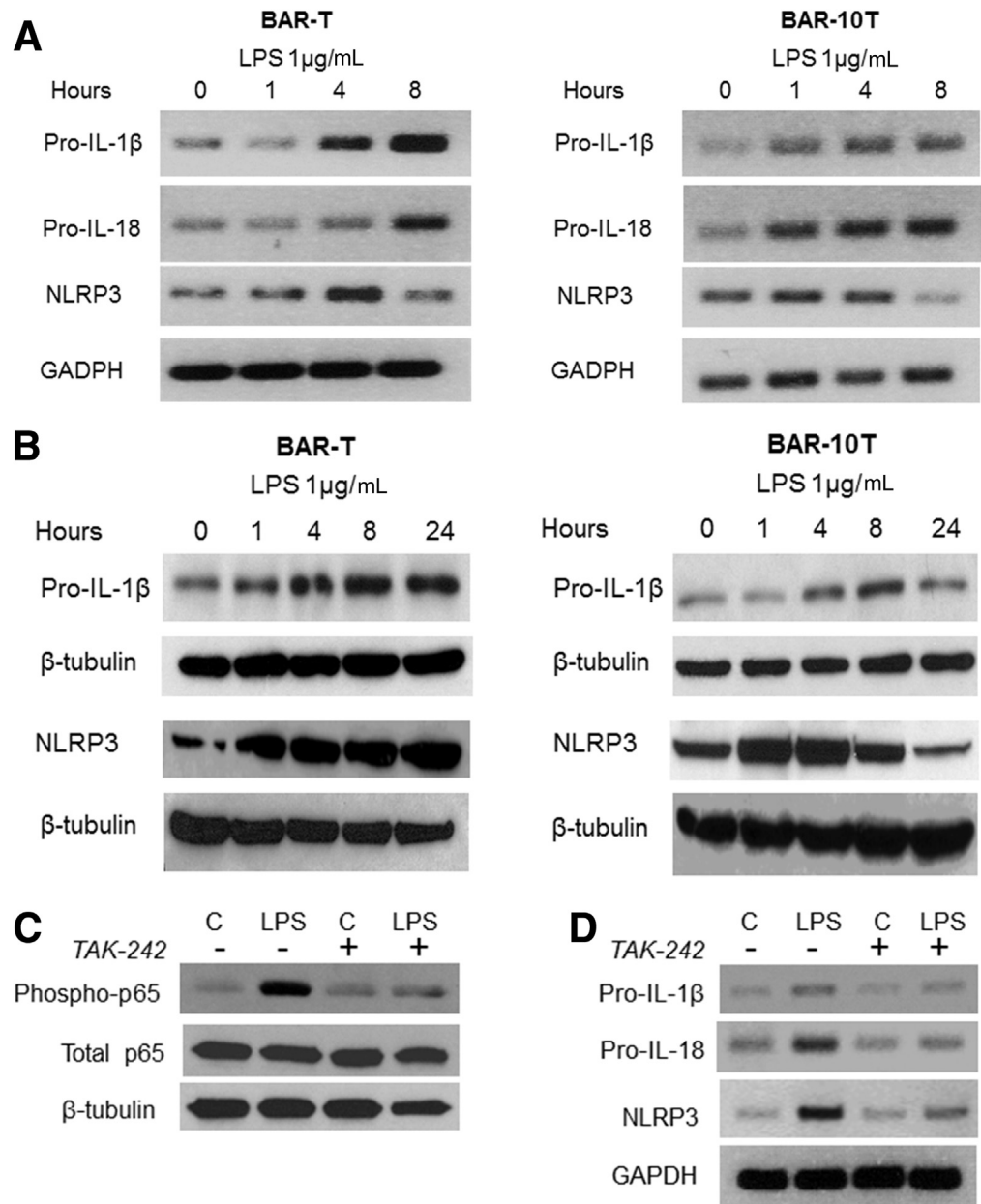


Figure 3. In Barrett's cell lines, LPS treatment induces (A) expression of pro-IL1 β , pro-IL18, and NLRP3 mRNA as determined by RT-PCR, and (B) expression of pro-IL1 β and NLRP3 protein as determined by Western blot. Inhibition of TLR4 signaling by TAK-242 decreases LPS-induced (C) phosphorylation of p65 protein and (D) pro-IL1 β , pro-IL18, and NLRP3 mRNA expression. (C) Vehicle-treated control.

ATP to the media for 30 minutes immediately before collecting the cells for analysis.²¹ In both cell lines, the combination of LPS and ATP caused a significant increase in caspase-1 activity, in the secretion of the active forms of IL1 β and IL18, and in the release of LDH (an indicator of pyroptosis) (Figure 4).

LPS alone (without exogenous ATP) has been shown to both prime and activate the Nlrp3 inflammasome in mouse dendritic cells.²¹ Indeed, when we treated BAR-T and BAR-10T cells with only LPS for up to 24 hours, this treatment also caused a significant increase in caspase-1 activity, secretion of IL1 β and IL18, and release of LDH (Figure 4). Thus, LPS alone or in combination with exogenous ATP can activate the inflammasome in Barrett's

epithelial cell lines. We confirmed these findings using primary cultures of Barrett's cells. As in the cell lines, we found that LPS alone significantly increased the secretion of IL1 β and IL18, and the release of LDH in primary Barrett's epithelial cells (Figure 5). These findings suggest that it is the NLRP3 inflammasome that is activated by LPS in Barrett's cells.

LPS Causes the Secretion of IL1 β and IL18, and Induces Pyroptosis in Barrett's Cells via the NLRP3 Inflammasome

To confirm that LPS signals through the NLRP3-containing inflammasome in Barrett's epithelial cells, we

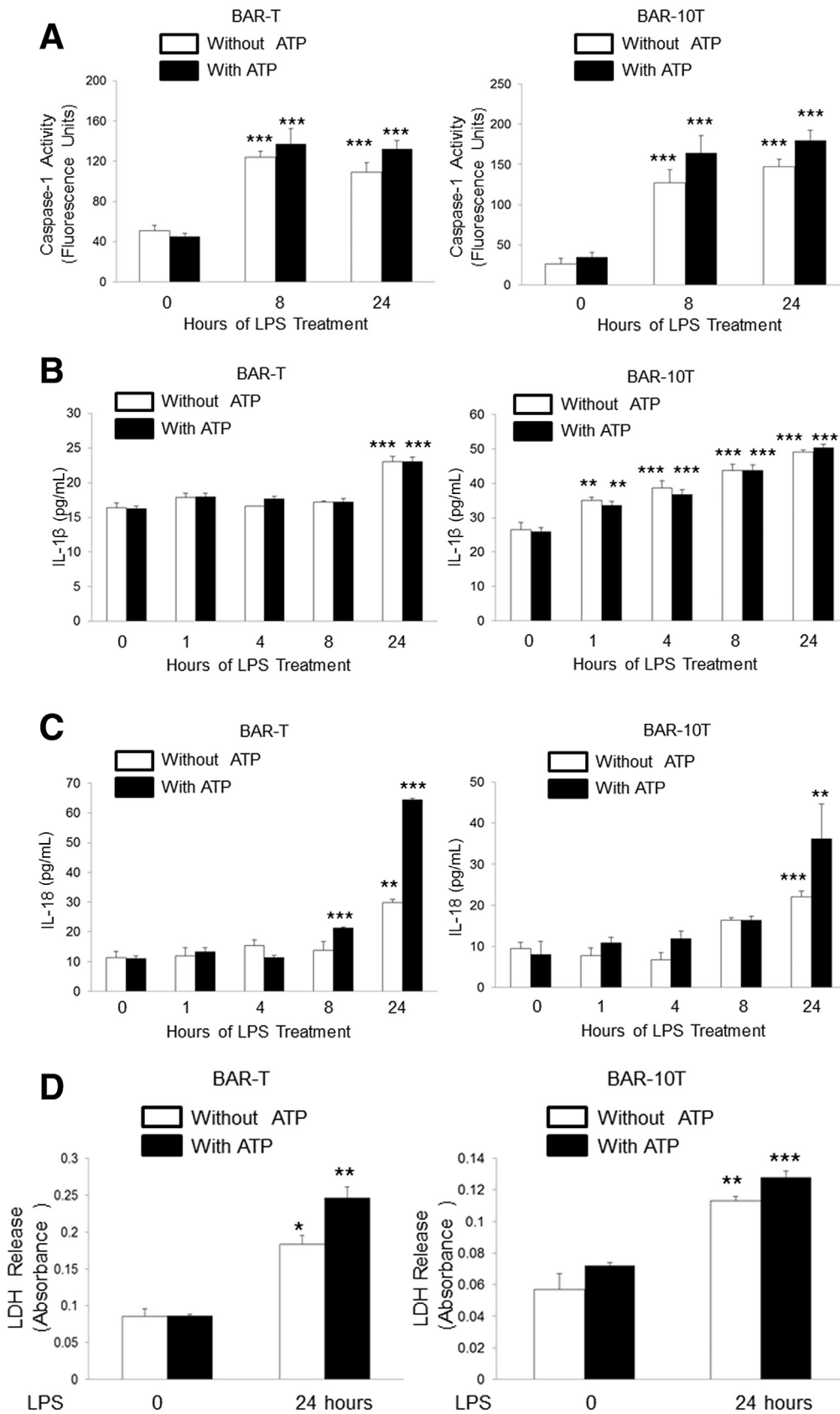


Figure 4. LPS activates caspase-1, causes secretion of IL1 β and IL18, and induces release of LDH (an indicator of pyroptosis) in Barrett's cell lines. BAR-T and BAR-10T cells were treated with LPS for up to 24 hours, with and without the addition of ATP for 30 minutes just before performing (A) caspase-1 activity assay and (B–D) enzyme-linked immunosorbent assays for (B) IL1 β , (C) IL18, and (D) LDH release. Bar graphs represent the means \pm SEM. * $P < .05$ compared with non-treated corresponding control. ** $P \leq .01$ compared with nontreated corresponding control. *** $P \leq .001$ compared with nontreated corresponding control.

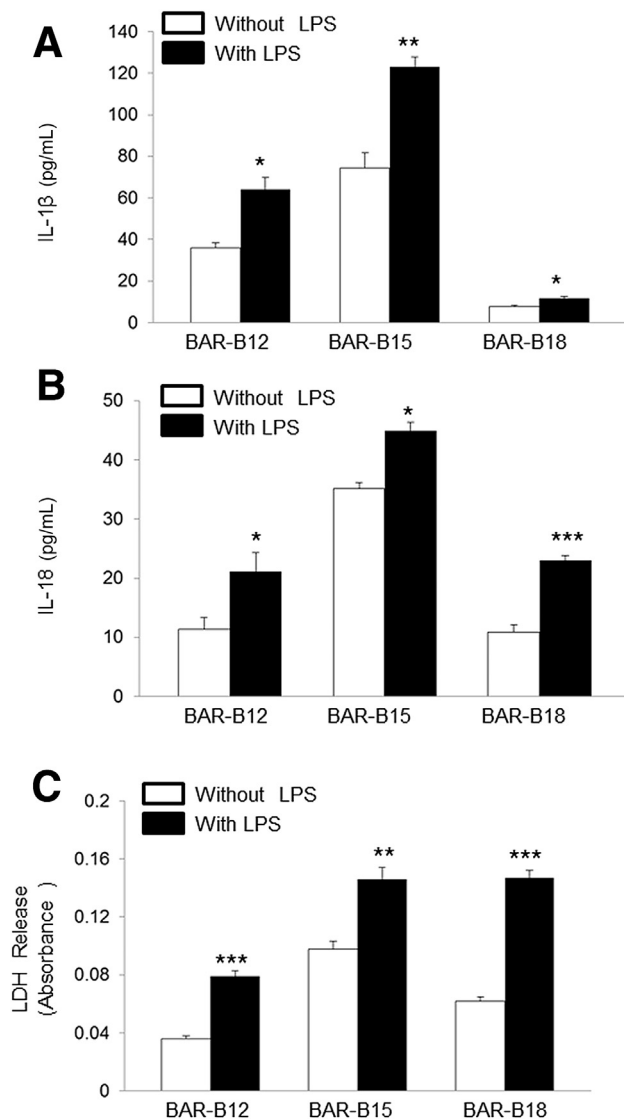


Figure 5. LPS alone causes secretion of IL1 β and IL18, and induces release of LDH in primary cultures of Barrett's epithelial cells (BAR-12, BAR-15, and BAR-18). Enzyme-linked immunosorbent assays for (A) IL1 β secretion, (B) IL18 secretion, and (C) LDH release after 24 hours of LPS stimulation. *Bar graphs* represent the means \pm SEM. * $P < .05$ compared with nontreated control. ** $P < .001$ compared with nontreated control. *** $P < .0001$ compared with nontreated control.

knocked down NLRP3 in BAR-10T cells using a specific siRNA. Figure 6A shows that NLRP3 siRNA blocked NLRP3 protein expression at baseline and after LPS stimulation. NLRP3 siRNA virtually abolished the LPS-induced increases in the secretion of IL1 β and IL18 and in the release of LDH in BAR-10T cells (Figure 6B–D). These findings show that LPS signals through the NLRP3 inflammasome in Barrett's epithelial cells. Furthermore, the near-complete elimination of LPS effects by NLRP3 siRNA suggests that NLRP3 is the primary LPS-responsive inflammasome in Barrett's epithelial cells.

The Secretion of Proinflammatory Cytokines and the Induction of Pyroptosis Triggered by LPS in a Barrett's Cell Line Both Depend on the Activation of Caspase-1

All inflammasomes can activate the protease caspase-1, which promotes inflammation by cleaving pro-IL1 β and pro-IL18 into their active forms and by inducing pyroptosis.^{7,12} To show that the secretion of proinflammatory cytokines and the induction of pyroptosis that are induced by LPS depend on caspase-1 in Barrett's cells, we treated BAR-10T cells with LPS in the presence of Ac-YVAD-CHO, a specific caspase-1 inhibitor.³¹ We found that Ac-YVAD-CHO treatment in concentrations of 50 and 100 μ g/mL abolished the increase in the secretion of IL1 β , IL18, and in the release of LDH by LPS (Figure 7). These findings show that LPS signaling through the NLRP3 inflammasome leads to caspase-1 activation, which triggers the release of proinflammatory cytokines and induces pyroptosis in Barrett's epithelial cells. Furthermore, the almost complete elimination of LPS effects on pyroptosis by Ac-YVAD-CHO suggests that LPS induces canonical (caspase-1-mediated) NLRP3 inflammasome activation in Barrett's epithelial cells.

Independent of TLR4 Signaling, LPS Induces Mitochondrial Production of ROS, Which Mediate Activation of the NLRP3 Inflammasome in a Barrett's Cell Line

Mitochondrial production of ROS is known to play a major role in NLRP3 inflammasome activation.³⁵ In cultured mouse astrocytes, LPS has been found to stimulate the mitochondrial production of ROS that activate the NLRP3 inflammasome.²⁰ To explore the role of mitochondrial ROS in NLRP3 inflammasome activation in Barrett's cells, we used MitoSox Red to measure ROS superoxide produced in the mitochondria of BAR-10T cells at baseline and after stimulation with LPS. LPS treatment caused a significant increase in the intensity of MitoSox Red immunostaining (ie, more mitochondrial superoxide) (Figure 8A). This LPS-induced increase in mitochondrial superoxide was abolished by treatment with Mito-TEMPO, a mitochondrial-targeted antioxidant (Figure 8A). Mito-TEMPO also blocked LPS-induced increases in the secretion of IL1 β and IL18, and in the release of LDH (Figure 8B–D). Inhibition of TLR4 signaling with TAK-242 had no effect on LPS-induced mitochondrial superoxide (Figure 8E). These findings show that LPS activates the NLRP3 inflammasome in Barrett's epithelial cells by increasing mitochondrial production of ROS in a fashion that is independent of TLR4 signaling. A schematic model summarizing the mechanisms elucidated by our study is provided in Figure 9.

Discussion

Our exploration of the effects of LPS on TLR4 signaling and on priming and activation of the inflammasome in esophageal squamous and Barrett's epithelial cells have uncovered several novel findings. We have shown that, although esophageal squamous cells and Barrett's epithelial

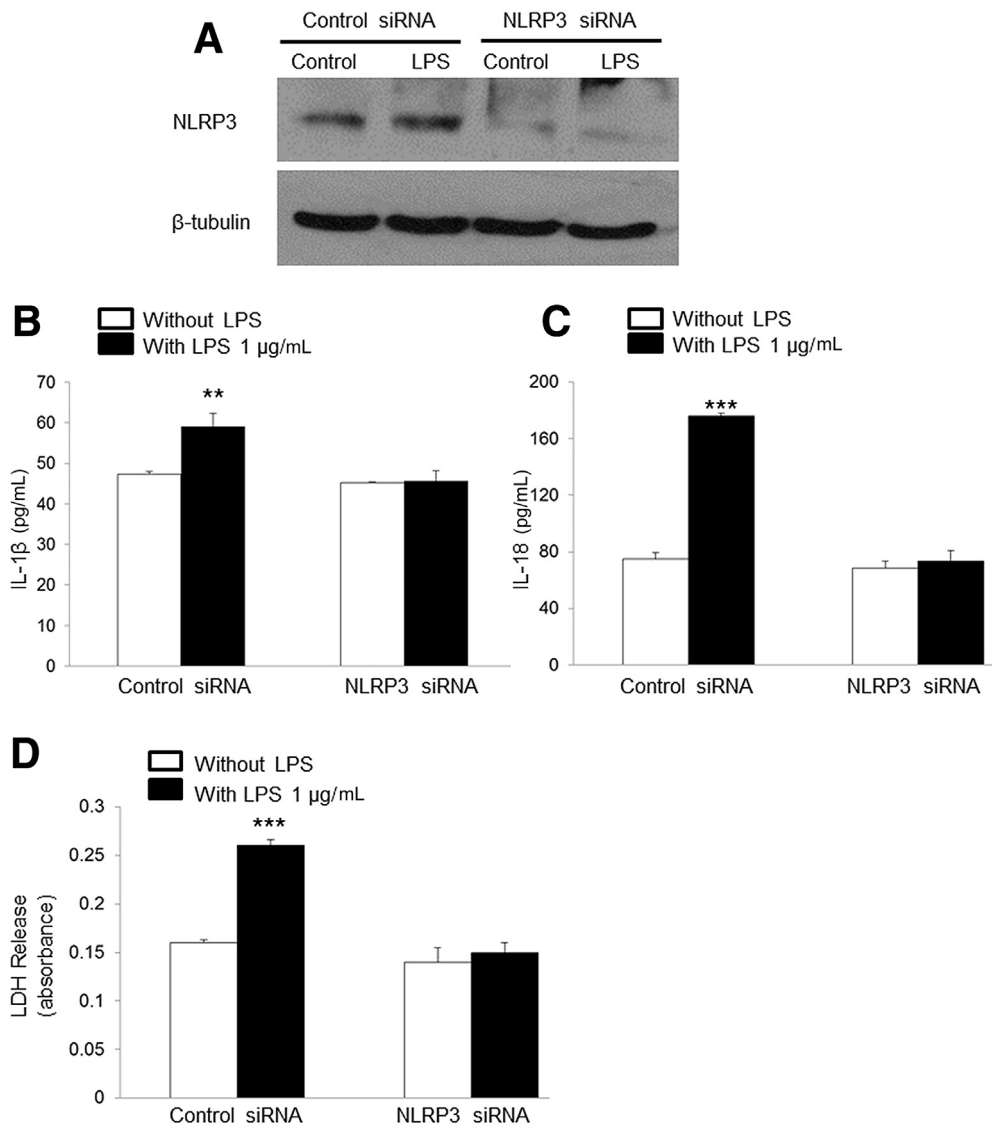


Figure 6. LPS causes the secretion of IL1 β and IL18, and induces pyroptosis in Barrett's cells by signaling through the NLRP3 inflammasome. (A) Representative Western blot showing that NLRP3 siRNA knocks down NLRP3 protein expression both at baseline and after 24 hours of LPS stimulation in BAR-10T cells. BAR-10T cells transfected with control siRNA or NLRP3 siRNA were treated with LPS for 24 hours, and enzyme-linked immunosorbent assays were performed for (B) IL1 β secretion, (C) IL18 secretion, and (D) LDH release. Bar graphs represent the means \pm SEM. ** $P \leq .01$ compared with nontreated corresponding control. *** $P \leq .0001$ compared with nontreated corresponding control.

cells express similar levels of TLR4, LPS induces TLR4 signaling that increases the secretion of TNF- α and IL8 only in the Barrett's cells. We have shown that LPS alone (without exogenous ATP) both primes and activates the NLRP3 inflammasome, events that might enable the gram-negative bacteria-dominated esophageal microbiome to contribute to inflammation-mediated carcinogenesis in Barrett's esophagus. Finally, we have shown that LPS increases mitochondrial production of ROS in a fashion independent of TLR4 signaling, and that it is those ROS that mediate canonical, caspase-1-dependent activation of the NLRP3 inflammasome in Barrett's epithelial cells.

The gut microbiome recently was recognized as a key factor that influences both human health and disease.¹⁹ Microbial interactions with their hosts have been proposed to contribute to benign disease and carcinogenesis through effects on inflammation, cell proliferation, and the generation of genotoxic agents.^{34,36} Microbial interactions also might contribute to cancer development through effects

on immune surveillance, angiogenesis, and cellular metabolism, and, for patients with cancer, the microbiome can affect host susceptibility to chemotherapeutic drugs.³⁶ However, few studies have addressed the role of the esophageal microbiome in esophageal disease. Macfarlane et al²³ reported high levels of pathogenic, nitrate-reducing *Campylobacter* species in the esophagus of patients with Barrett's metaplasia, but not in control patients. Conceivably, these bacteria might contribute to esophageal disease by enhancing the production of damaging, reactive nitrogen species. Other studies have shown that the esophageal microbiome of patients with reflux esophagitis and Barrett's esophagus is dominated by gram-negative bacteria that produce LPS, the natural ligand of TLR4.²²⁻²⁴

TLRs are critical sensors of the PAMPs produced by viral, bacterial, and fungal microorganisms. Such PAMPs can activate TLR signaling, triggering an inflammatory response that might protect the host from microbial invasion.³⁷ TLR4 recognizes PAMPs of the endotoxin LPS, a component of the

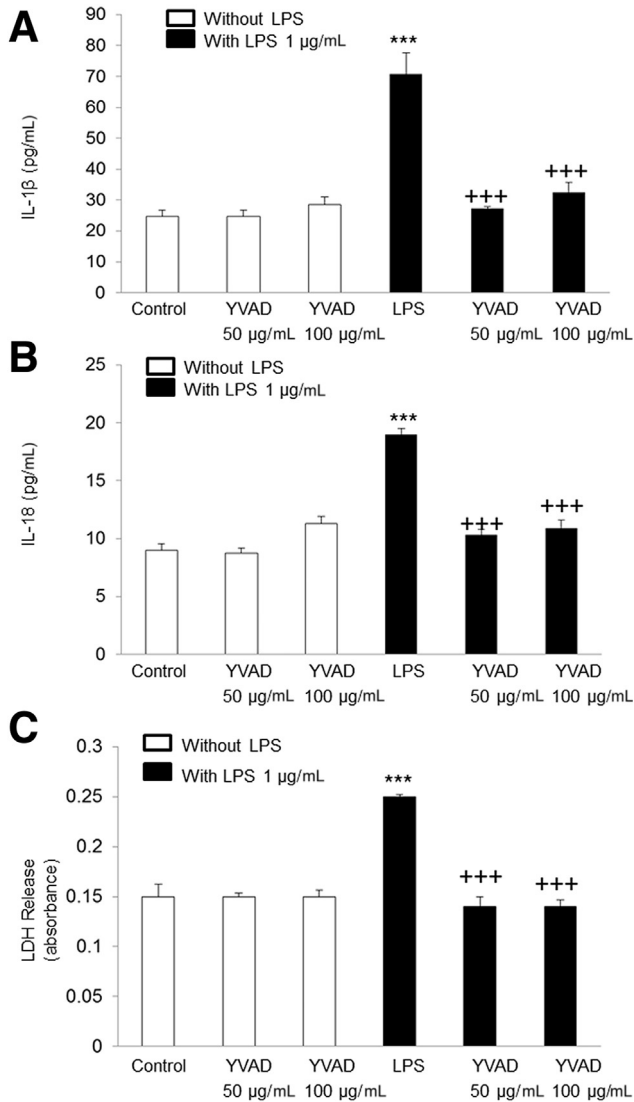


Figure 7. LPS signaling through the NLRP3 inflammasome causes caspase-1-dependent increases in the secretion of IL1β and IL18 and in the release of LDH. Enzyme-linked immunosorbent assays for (A) IL1β secretion, (B) IL18 secretion, and (C) LDH release in BAR-10T cells treated with LPS for 24 hours in the presence of 50 or 100 µg/mL of YVAD, a specific caspase-1 inhibitor. Bar graphs represent the means ± SEM. ****P* ≤ .001 compared with nontreated control. +++*P* ≤ .001 compared with LPS-treated cells.

outer membrane of the gram-negative bacteria¹⁶ that dominate the esophageal microbiome of patients with reflux esophagitis with and without Barrett's esophagus.²²⁻²⁴

In earlier studies, Verbeek et al²⁵ found TLR4 expression in esophageal squamous cells and in Barrett's epithelial cells. They reported that LPS caused a significant increase in the expression of cyclooxygenase-2 in biopsy specimens of Barrett's metaplasia that were cultured ex vivo, but not in similarly cultured biopsy specimens of squamous esophageal mucosa. In support of our findings, Verbeek et al²⁵ found that LPS increased IL8 secretion in the Barrett's biopsy specimens but, in contrast to our findings, they found that LPS also caused a small, but statistically significant, increase in IL8

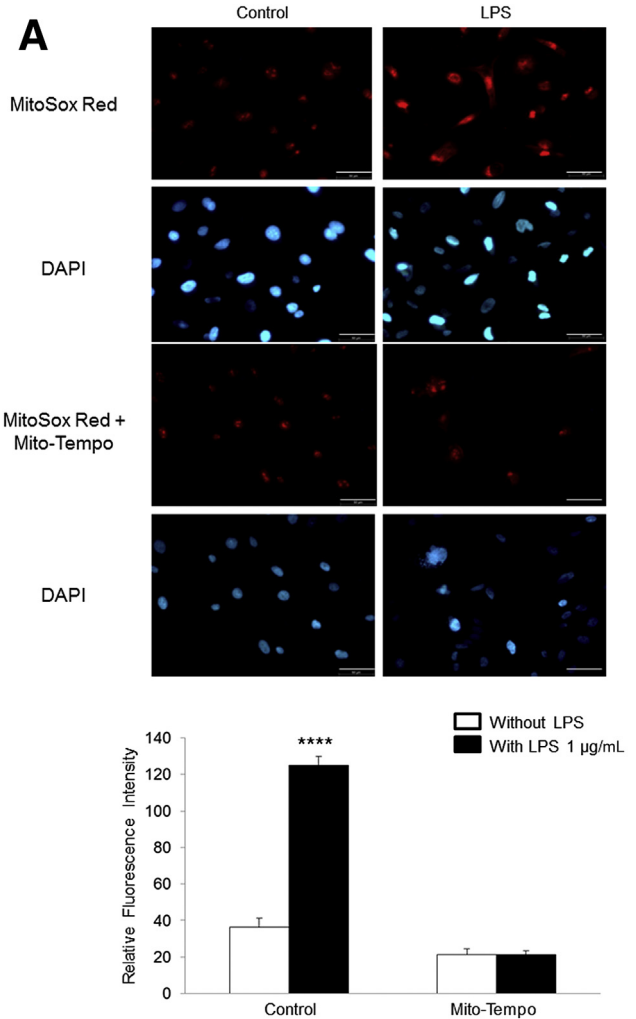


Figure 8. LPS increases mitochondrial ROS production, which activates the NLRP3 inflammasome in BAR-10T cells. (A) MitoSOX Red immunostaining for mitochondrial ROS in BAR-10T cells treated with LPS. Treatment with Mito-TEMPO (a mitochondrial-targeted antioxidant) confirms the mitochondrial origin of the ROS. 4',6-diamidino-2-phenylindole (DAPI) indicates the number of nuclei in the same field. Scale bar: 50 µmol/L. Quantification of MitoSOX Red fluorescence intensity in BAR-10T cells treated with LPS and Mito-TEMPO. (B-D) Enzyme-linked immunosorbent assays for (B) IL1β secretion, (C) IL18 secretion, and (D) LDH release in BAR-10T cells treated with LPS and Mito-TEMPO. (E) MitoSOX Red immunostaining for mitochondrial ROS in BAR-10T cells treated with LPS and TAK-242. DAPI indicates the number of nuclei in the same field. Scale bar: 50 µmol/L. Quantification of MitoSOX Red fluorescence intensity in BAR-10T cells treated with LPS and TAK-242. Bar graphs represent the means ± SEM. **P* ≤ .05 compared with nontreated corresponding control. ***P* ≤ .01 compared with nontreated corresponding control. ****P* ≤ .001 compared with nontreated control. *****P* < .0001 compared with nontreated control.

secretion in the squamous biopsy specimens. It is not clear why Verbeek et al²⁵ found LPS-induced TLR4 signaling in esophageal squamous tissue when we did not, but this disparity might be owing to differences in experimental conditions. We studied squamous epithelial cells in culture,

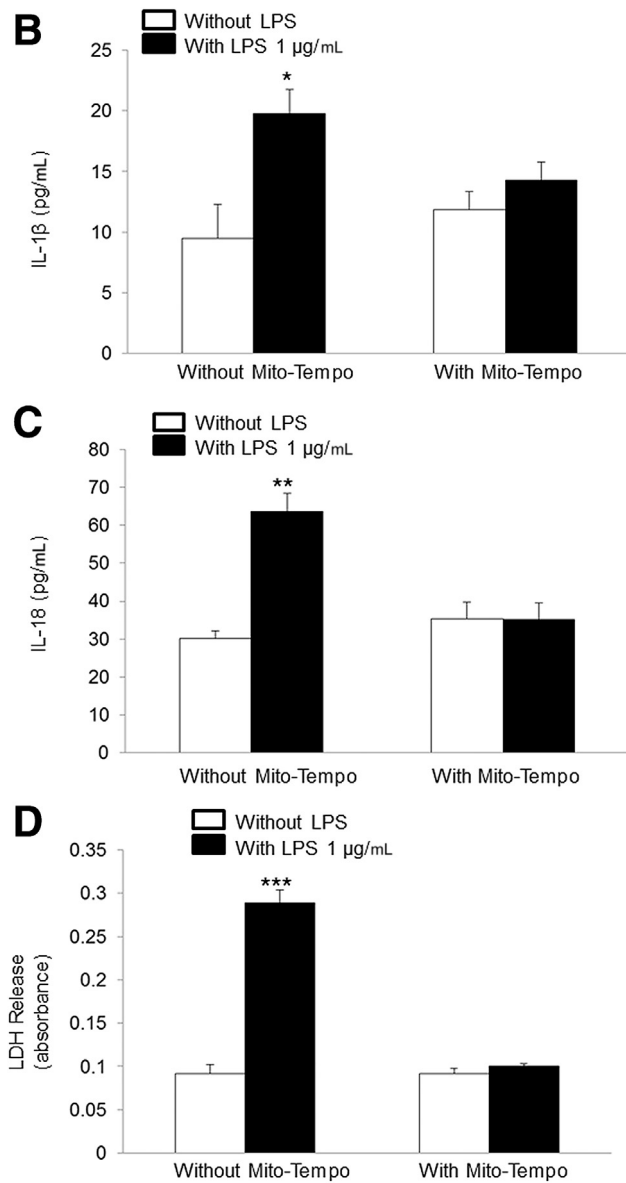


Figure 8. (continued).

whereas Verbeek et al²⁵ used esophageal squamous biopsy specimens that included inflammatory and stromal cells in addition to epithelial cells. Those nonepithelial cell types also express TLR4, and the ex vivo model did not distinguish between TLR4 signaling by epithelial and nonepithelial cells.²⁵ Thus, the small increase in IL8 secretion induced by LPS in the squamous biopsy specimens of Verbeek et al²⁵ might have resulted from TLR4 signaling in inflammatory or stromal cells, rather than in squamous cells.

Chronic inflammation is well known to be associated with a number of gastrointestinal tract cancers, but the role of inflammasomes in the development of those malignancies has not been widely appreciated. There is evidence that inflammasome activation contributes to tumorigenesis in *Helicobacter pylori*-associated gastric cancer and in colon cancer (reviewed by Kolb et al¹³), but little was known about the role of inflammasomes in Barrett's esophagus. We

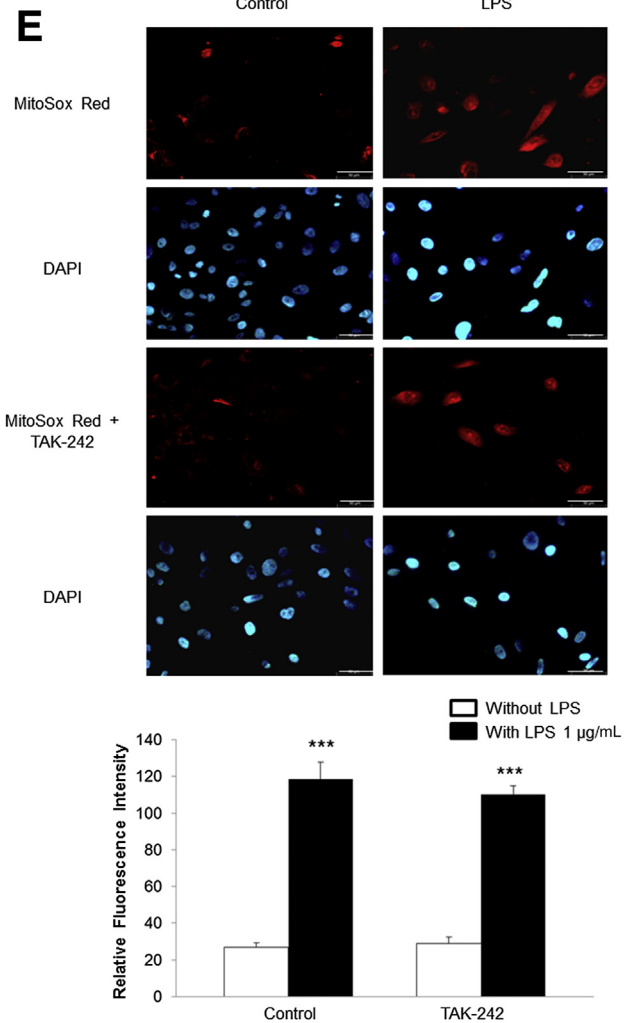


Figure 8. (continued).

have shown that Barrett's cell lines express several inflammasome PRR mRNAs including NLRP1, NLRP3, and AIM2, but NLRP3 predominates and Barrett's cell lines and primary cultures of Barrett's epithelial cells express similar levels of NLRP3 protein. In a number of cell types, NLRP3 inflammasome functioning requires 2 signals: a priming signal (eg, LPS binding to TLR4) that induces the expression of pro-IL1 β and pro-IL18, and an activation signal (eg, extracellular ATP) that results in the secretion of the active forms of IL1 β and IL18 and in the induction of pyroptosis. In mouse macrophages, NLRP3 inflammasome functioning requires LPS for priming and requires extracellular ATP for activation,^{21,38} whereas LPS alone (without exogenous ATP) can both prime and activate the NLRP3 inflammasome in mouse dendritic cells, mouse astrocytes, and human monocytes.^{20,21} Because NLRP3 inflammasome functioning in Barrett's epithelial cells had not been described before, it was not clear whether 1 or 2 separate signals would be required. We found that LPS primed the Barrett's cell NLRP3 inflammasome by up-regulating the expression of pro-IL1 β and pro-IL18. We then added exogenous extracellular ATP to the media as an activation signal positive

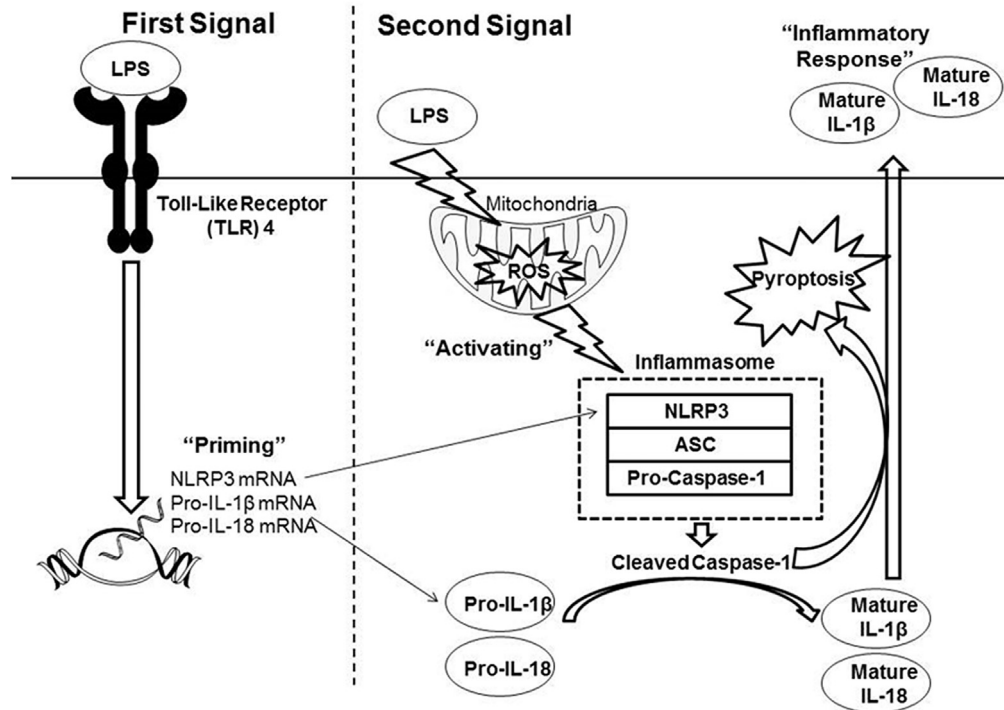


Figure 9. Schematic showing how LPS both primes and activates the NLRP3 inflammasome in Barrett's epithelial cells. LPS provides the first signal by activating TLR4, which induces expression of NLRP3, pro-IL1 β , and pro-IL18. This primes the inflammasome. LPS also provides the second activating signal by increasing mitochondrial production of ROS. These ROS activate the NLRP3 inflammasome, leading to caspase-1 activation, which triggers the release of proinflammatory cytokines and induces pyroptosis. By triggering molecular events that both prime and activate the NLRP3 inflammasome, LPS produced by the esophageal microbiome might contribute to inflammation-mediated carcinogenesis in Barrett's esophagus.

control, and found that this combination of LPS and ATP indeed activated the inflammasome, causing a significant increase in caspase-1 activity, in the secretion of the active forms of IL1 β and IL18, and in the release of LDH. We also found that LPS alone (without exogenous ATP) activated that inflammasome in Barrett's cells. Thus, in Barrett's cells, LPS can both prime and activate the NLRP3 inflammasome. We also showed that a specific NLRP3 siRNA abolished these LPS effects, confirming that LPS signals through the NLRP3 inflammasome.

Recently, it was shown that intracellular LPS can activate caspase-11 directly, which then can activate the NLRP3 inflammasome through the noncanonical inflammasome pathway without signaling through TLR4.¹⁵ Activation of caspase-11 by intracellular LPS can cause pyroptosis directly, but caspase-11-induced secretion of IL1 β and IL18 is an indirect process mediated through the NLRP3/caspase-1 inflammasome.¹⁵ If the pyroptosis we observed in our LPS-treated Barrett's cells was the result of intracellular LPS activating caspase-11, then LPS treatment should have caused pyroptosis even in the presence of a caspase-1 inhibitor.³⁹ However, we found that YVAD, a specific caspase-1 inhibitor, completely abolished pyroptosis in addition to eliminating LPS-mediated secretion of IL1 β and IL18. These findings suggest that the LPS effects we observed in our Barrett's cells all were mediated through canonical inflammasome activation of caspase-1.

ROS also have been shown to play a major role in activating the NLRP3 inflammasome, and mitochondrial ROS production appears to be essential for this process.^{35,40} In our studies in Barrett's cells, MitoSOX Red immunostaining showed that LPS significantly increased mitochondrial ROS production, which was eliminated by treatment with the mitochondrial-targeted antioxidant Mito-TEMPO, but not by treatment with the TLR4 inhibitor TAK-242. These findings suggest that the LPS-induced generation of mitochondrial ROS in Barrett's cells does not rely on TLR4/NF- κ B signaling. In support of our findings, a recent study found that levornidazole (a novel, third-generation, nitromidazole-derivative antibiotic) blocked activation of the NLRP3 inflammasome by suppressing LPS-mediated production of mitochondrial ROS without affecting LPS-mediated NF- κ B activity.⁴¹ In addition, mito-TEMPO abolished LPS-induced increases in IL1 β and IL18 secretion, and in the release of LDH. These findings show that LPS causes a TLR4-independent increase in mitochondrial production of ROS, which in turn activate the NLRP3 inflammasome in Barrett's epithelial cells.

In conclusion, our study supports a role for the esophageal microbiome in activating the NLRP3 inflammasome in Barrett's esophagus. We have shown that the LPS produced by gram-negative bacteria in the esophagus can activate TLR4 signaling in Barrett's cells, but not in esophageal squamous cells. We have found that LPS primes the

Barrett's cell inflammasome for activation by inducing the expression of NLRP3, pro-IL1 β , and pro-IL18. Moreover, we have shown that LPS increases mitochondrial production of ROS in a fashion independent of TLR4 signaling, and that those ROS mediate canonical, caspase-1-dependent inflammasome activation. The activated caspase-1 catalyzes the proteolytic cleavage of pro-IL1 β and pro-IL18 to their mature, active forms, which are secreted from the cell to elicit an inflammatory response that can be exacerbated by caspase-1-induced pyroptosis. By triggering molecular events that both prime and activate the NLRP3 inflammasome, LPS produced by the esophageal microbiome might contribute to inflammation-mediated carcinogenesis in Barrett's esophagus, a biologically significant event. This study suggests the intriguing possibility that manipulation of the esophageal microbiome could be a novel strategy to prevent cancer in Barrett's esophagus.

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Conflicts of interest

The authors disclose no conflicts.

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