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ORIGINAL ARTICLE

Pyroptosis induced by TLR9 ligand, ODN1826, requires ROS production and caspase-11 activation in Raw264.7 cells

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Abstract

Background: Toll-like receptor 9 (TLR9), located in the endosomal compartment, is known to play a role in inflammation by recognizing oligonucleotides that contain CpG motive (CpG-ODN). Signaling by TLR9 leads to the production of proinflammatory cytokines and can trigger cell death.

Objective: This study aims to investigate the molecular mechanism of pyroptosis induced by ODN1826 in the mouse macrophage cell line (Raw264.7).

Methods: The protein expression and the amount of lactate dehydrogenase (LDH) of ODN1826-treated cells were determined by immunoblotting and LDH assay, respectively. In addition, the level of cytokine production was observed by ELISA assay and the ROS production was determined by flow cytometry.

Results: Our results showed that ODN1826 induced pyroptosis as judged by LDH releases. Furthermore, caspase-11 and gasdermin D activation, which are the key molecules in pyroptosis, were also observed in ODN1826-activated cells. Moreover, we also demonstrated that Reactive Oxygen Species (ROS) production by ODN1826 is essential for caspase-11 activation and gasdermin D release, which leads to pyroptosis.

Conclusion: ODN1826 induces pyroptosis in Raw264.7 cells via caspase-11 and GSDMD activation. Moreover, the production of ROS by this ligand plays an essential role in the regulation of caspase-11 and GSDMD activation, which then controls pyroptosis in TLR9 activation.

Key words: caspase-11, ODN1826, pyroptosis, ROS production, TLR9 ligand

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Introduction

Toll-Like Receptors (TLRs) have been known to play an essential role in infectious and inflammatory diseases. Among them, TLR9, an endosomal TLR, expresses in various cell types, including macrophages, dendritic cells and B cells.[1] This receptor recognizes oligodeoxynucleotides that contain unmethylated CpG motifs (CpG-ODN), a common motif in bacterial and viral DNA, but uncommon in the vertebrate genome. Besides bacterial DNA, TLR9 can also recognize endogenous molecules ejected from mitochondria (mtDNA) when the cells are under stress or damage.[2] In addition, recent evidence demonstrates that mtDNA can mediate inflammation via TLR9, inflammasome and STING pathways.[2] Targeting the immune reactions mediated by TLR9 is a potential therapeutic strategy to control inflammation and autoimmune diseases.[3]

Since TLR9 is located in the endosomal compartment. CpG DNA can enter the cells by binding to the cell surface before entering early endosomes through a clathrin-dependent, caveolin-independent pathway.[4] The early endosome containing CpG DNA TLR9 complex needs to fuse with a lysosome, thereby maturing into a late endosome. TLR9, like other endosomal TLRs (TLR3 and TLR7), requires receptor proteolysis in the endolysosome. In the endolysosomes of macrophages, lysosomal cathepsins and endopeptidases, which function only at acidic pH, cleave the TLR9 ectodomain.[5,6] This process is necessary to recruit MyD88, an adaptor protein, which initiates signal transduction pathways leading to activation of NF-κB, IRFs, or MAP kinases resulting in regulation of the expression of cytokines, chemokines, and type I IFNs that ultimately protect the host from microbial infection.[5] Phosphoinositides (PIs), a key component of cell membranes, control of TLR7 and TLR9 localization and signaling.[7,8] Recent evidence shows that a phosphoinositide kinase (PI kinase), particularly PI(3) P 5-kinase, known as PIKfyve, regulates colocalization of TLR9 and its ligand which contributes to the activation of signaling of this receptor.[9]

Pyroptosis, an inflammatory programmed cell death, is characterized by gasdermin-mediated pore formation in the plasma membrane.[10] Emerging evidence has recently indicated that pyroptosis contributes to infection and several non-infectious diseases, such as cancer.[11] Pyroptosis can be caused by both the canonical and non-canonical inflammasome pathways. The canonical inflammasome requires activation of the inflammasome sensor, NLRP3, to form a caspase-1 inflammasome complex. In contrast, non-canonical inflammasome, caspase-1-independent pathway, is induced by human caspase-4/5 or mouse caspase-11, which is activated by lipopolysaccharide (LPS) of Gram-negative bacteria, suggesting that signaling from TLRs also play role in pyroptosis by regulating through caspase enzymes.[12] Activation of caspase both canonical and noncanonical pathway results in the cleavage of gasdermin D (GSDMD) into a 31 kDa pore-forming N-terminal effector domain of GSDMD and triggers pyroptotic cell death.

It is well documented that TLRs and NOD-like Receptor (NLRs) signaling are essential for pyroptosis induction.[13] Recent evidence shows that acyclic diterpenoid acid of geranylgeranoic acid (GGA), recognized as an acyclic retinoid, is able to induce caspase-4 activation via TLR4 leading to the production of N-terminal fragment of GSDM (GSDM-NT) in human hepatoma-derived HuH-7 cells. Furthermore, the induction of pyroptosis by GGA is also accompanied by the increase of reactive oxygen species (ROS), leading to non-canonical activation of caspase-4 to activate the NLRP3 during cell death.[14] It has also been demonstrated that ROS production by *Citrobacter rodentium*-infected bone marrow-derived macrophages (BMDMs) regulates caspase-11 expression and activation leading to pyroptosis.[15] These results suggest that ROS production also plays an important role in pyroptosis. Although the role of ROS production in pyroptosis has been extensively investigated in several surface TLRs such as TLR4 and TLR2, very limited information of interrelationships between endosomal TLRs and ROS production. In the present study, we investigate the significance of ROS production in pyroptosis induction of the mouse macrophage cell line activated with ODN1826, a ligand of TLR9.

Materials and Methods

Cell line and culture condition

Raw264.7 macrophages (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco Labs, Grand Island, NY, USA) at 37°C under a 5% CO_2 atmosphere.

TLR ligands and chemical reagents

Lipopolysaccharide (LPS) (from an *Escherichia coli*), CpG Class B ODN 1826 (5′-TCCATGACGTTCCTGACGTT-3′) and non-CpG ODN2138 (GC control of CpG ODN1826) (5′-TCCATGAGCTTCCTGAGCTT-3′) were purchased from Invivogen. Diphenyleneiodonium (DPI) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich. YM201636 was purchased from MedChem Express.

Activation of Raw264.7 macrophages with TLR ligands

Raw264.7 macrophages (0.5×10^6 cells/well) were seeded overnight in a 6-well plate. The overnight cultures of Raw264.7 cells were treated with ODN1826 or non-CpG ODN2138 at a concentration of 1 μg/ml. For treatment with inhibitors, the cells were pretreated with 10 μM of diphenyleneiodonium (DPI) or 1 μM of YM201636 for 1 h prior to the activation with ODN1826 (1 μg/ml) or *E. coli* LPS (100 ng/ml).

LDH assay

The release of lactate dehydrogenase (LDH) in the culture supernatants was measured by cytotoxicity assay to determine the level of pyroptosis. At the indicated time intervals, the supernatants of treated cells were collected and LDH activity was detected by using the CytoTox 96® non-radioactive cytotoxicity assay (Promega) according to the manufacturer's instructions. The supernatants were applied to each well of flat-bottom 96-well enzymatic assay plate (NUNC™ Brand Product). The 50 μl of reconstituted substrate mix was added and incubated at room temperature in the dark for 20 min. To stop the reaction, 50 μl of stop solution was applied to each well. The LDH measurements recorded absorbance at 490 nm. For maximum LDH release, untreated cells were added with a 10× lysis solution and performed the LDH detection in the same manner. The percentage of LDH release was calculated followed by the formula: % LDH release = (Experimental LDH release − Spontaneous LDH release/Maximum LDH release) × 100.

Immunoblotting

The treated cells were lyzed in a lysis buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates were separated on 7%, 10% and 15% SDS-PAGE gels. Proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). The non-specific binding sites on the membrane were blocked with 5% blocking solution (Roche Diagnostics) for 1 h before proteins were allowed to react with specific primary antibodies against caspase-11 (Abcam), GSDMD (Abcam) and Actin (Merck Millipore) at 4°C overnight. The membranes were washed three times with 0.1% PBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (R&D Systems) for 1 h at room temperature. Thereafter, the membranes were washed four times with 0.1% PBST before a chemiluminescence substrate (Roche Diagnostics) was added and protein brands were detected by enhanced chemiluminescence.

Measurement of intracellular reactive oxygen species (ROS) production

The intracellular ROS levels were measured using cell-permeable non-fluorescence probe 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich). Briefly, the stimulated cells were washed twice with pre-warmed PBS buffer and loaded with 10 μM DCFH-DA detection reagent. After incubation in darkness for another 30 min at 37°C, cells were washed twice with an ice-cold PBS buffer and measured immediately. The fluorescence representing intracellular ROS levels was measured with CytoFLEX flow cytometer (Beckman Coulter) using CytExpert.

Determination of TNF-α and IFN-β production

The supernatant from activated cells was collected and the cytokine production levels were measured by mouse TNF-α kit (BD Bioscience, San Diego, USA) and mouse IFN-β kit (R&D Systems) following the manufacturer's instructions.

Statistical analysis

All experiments were performed at least three independent times. The results were expressed as mean ± SEM. All data were analyzed by the Prism software (GraphPad) by using Student's *t* test or one-way ANOVA test followed by a post-hoc multiple comparison test based on specific experiments. Asterisks indicate statistically significant differences based on *p*-values as indicated here: ** for $p < 0.01$ and **** for $p < 0.0001$.

Results

Induction of pyroptosis in ODN1826-treated Raw264.7 macrophages

Raw264.7 macrophages were used to assess the role of ODN1826 in the stimulation of pyroptosis. The cells were treated with ODN1826 for 18 h. Raw264.7 cells that undergo pyroptosis were determined by measuring the release of the enzyme lactate dehydrogenase (LDH). As shown in **Figure 1a**, ODN1826 was able to induce LDH release. It is well documented that gasdermin D (GSDMD), the key substrate for caspase-11, is essential in pyroptosis induction.[16] This protein can be cleaved by caspase-11 to create an N-terminal fragment that is a crucial determinant for proinflammatory cell death, pyroptosis. As expected, the active form of caspase-11 and N-terminal fragment of GSDMD (GSDMD-NT) were also observed in ODN1826-treated Raw264.7 macrophages (**Figure 1b**). In addition, the results indicated that pyroptosis induced via TLR9 was also associated with caspase-11 and GSDMD.

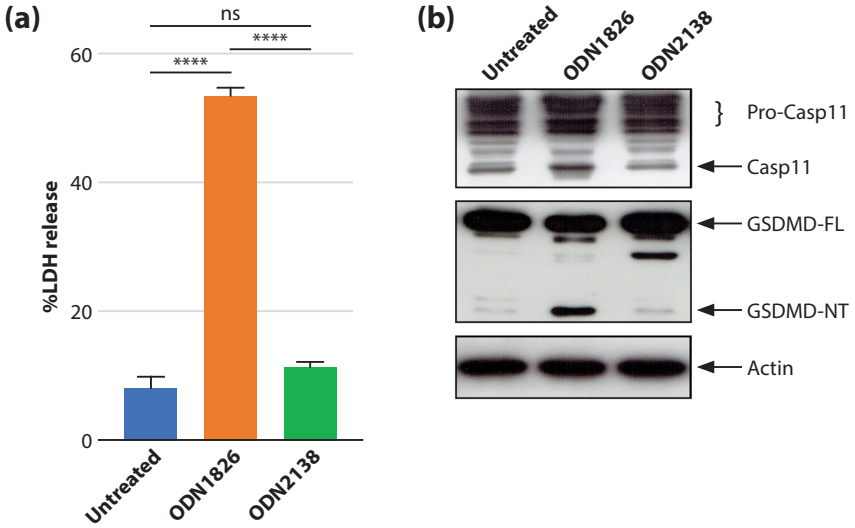

Figure 1. Induction of pyroptosis in ODN1826-treated Raw264.7 cells. Mouse macrophages were treated with ODN1826 (1 μg/ml) or non-CpG ODN2138, as a negative control (1 μg/ml) for 18 h. The treated cells were collected for analysis. (a) The amount of LDH release in supernatants was measured by using the LDH assay. Data are means ± SEM from at least three independent experiments; ****$p < 0.0001$ were analyzed by two-tailed unpaired Student's *t*-test. (b) Cell lysates were prepared and analyzed by immunoblotting. Representative bands are shown from three independent experiments.

The PIKfyve inhibitor prevents ODN1826-induced pyroptosis

Previous study demonstrated that TLR9 signaling requires PI(3) P 5-kinase (PIKfyve) to phosphorylate phosphatidylinositol to control membrane trafficking of ODN and TLR9.[9] To illustrate the significance of this kinase in pyroptosis induced by ODN1826, YM201636 was used as a specific inhibitor of PIKfyve. In the presence of YM201636, the level of IFN-β significantly decreases (**Figure 2a**). This result is consistent with a previous report which demonstrated that YM201636 can interfere with co-localization and signaling of CpG-ODN and TLR9, leading to the inhibition of IFN-β.[9] To examine if PIKfyve is specific only for endosomal TLR, *E. coli* LPS (a TLR4 ligand) was used as comparison. As shown in **Figure 2b**, ODN1826-induced production of TNF-α was significantly decreased in the presence of YM201636, while *E. coli* LPS-treated was not affected. We further investigated if membrane trafficking of ODN1826 and TLR9 is essential for pyroptosis. The LDH release from ODN1826 activated Raw264.7 cells in the presence of YM201636 was assayed. As shown in **Figure 2c**, YM201636 suppresses LDH release in ODN1826-activated cells. This result is also consistent with decreased caspase-11 activation and cleavage of gasdermin D (**Figure 2d**).

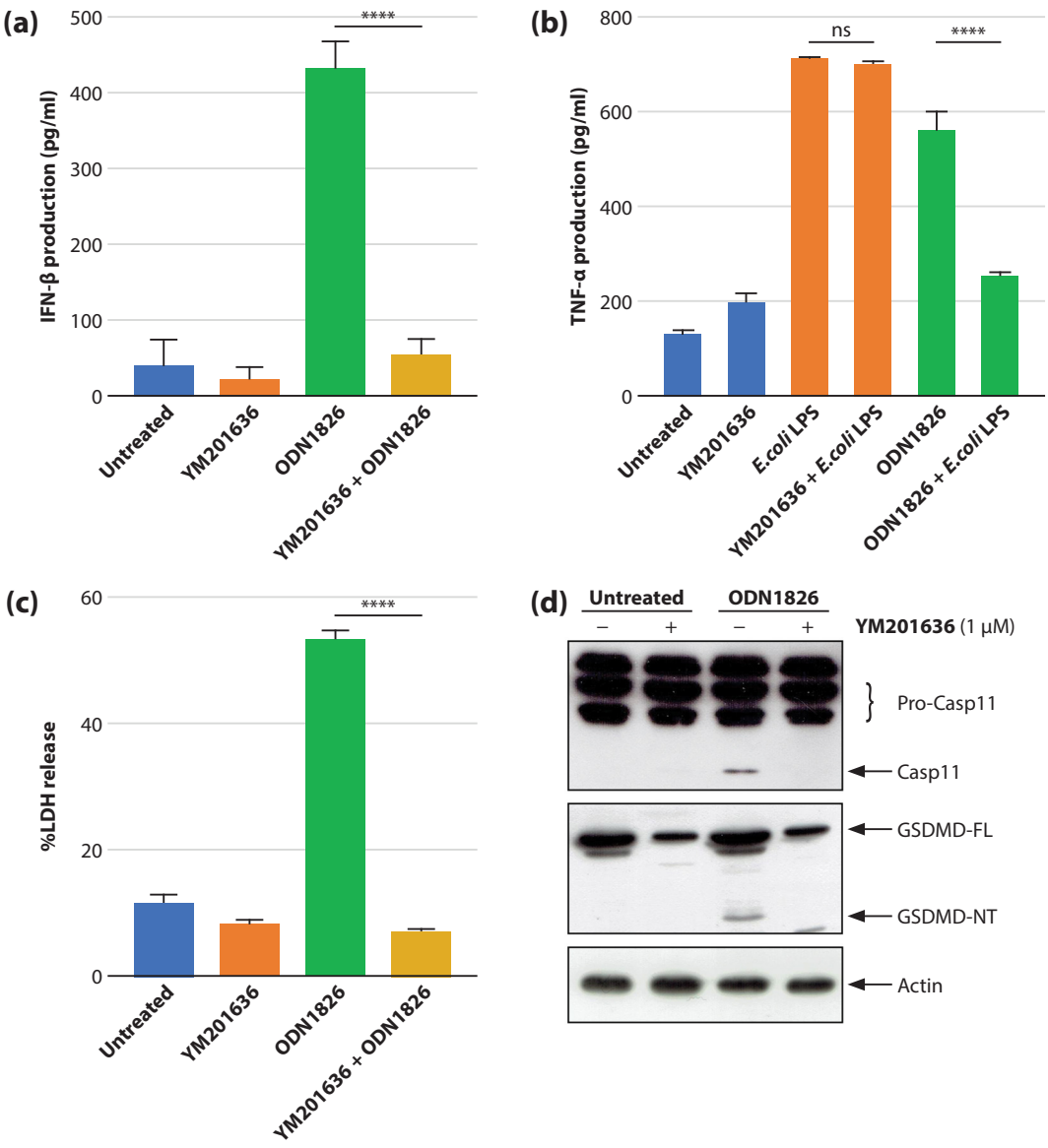

Figure 2. PIKfyve inhibitor, YM201636, interferes with pyroptosis induced by ODN1826 in Raw264.7 cells. Raw264.7 cells were pretreated with PIKfyve inhibitor, YM201636 (1 μM), for 1 h before being stimulated with ODN1826 (1 μg/ml) or *E. coli* LPS (100 ng/ml). After 18 h of stimulation, the supernatant was collected and determined the level of IFN-β (a) and TNF-α (b) production by ELISA assay. Data are means ± SEM from at least three independent experiments. All data were determined by one-way ANOVA followed by Tukey's multiple comparison test. ****$p < 0.0001$. (c) The amount of LDH release in the supernatants was measured using the LDH assay and presented as means ± SEM from at least three independent experiments. All data were determined by one-way ANOVA followed by Tukey's multiple comparison test. ****$p < 0.0001$. (d) The cell lysates were prepared and analyzed by immunoblotting. Representative bands are shown from three independent experiments.

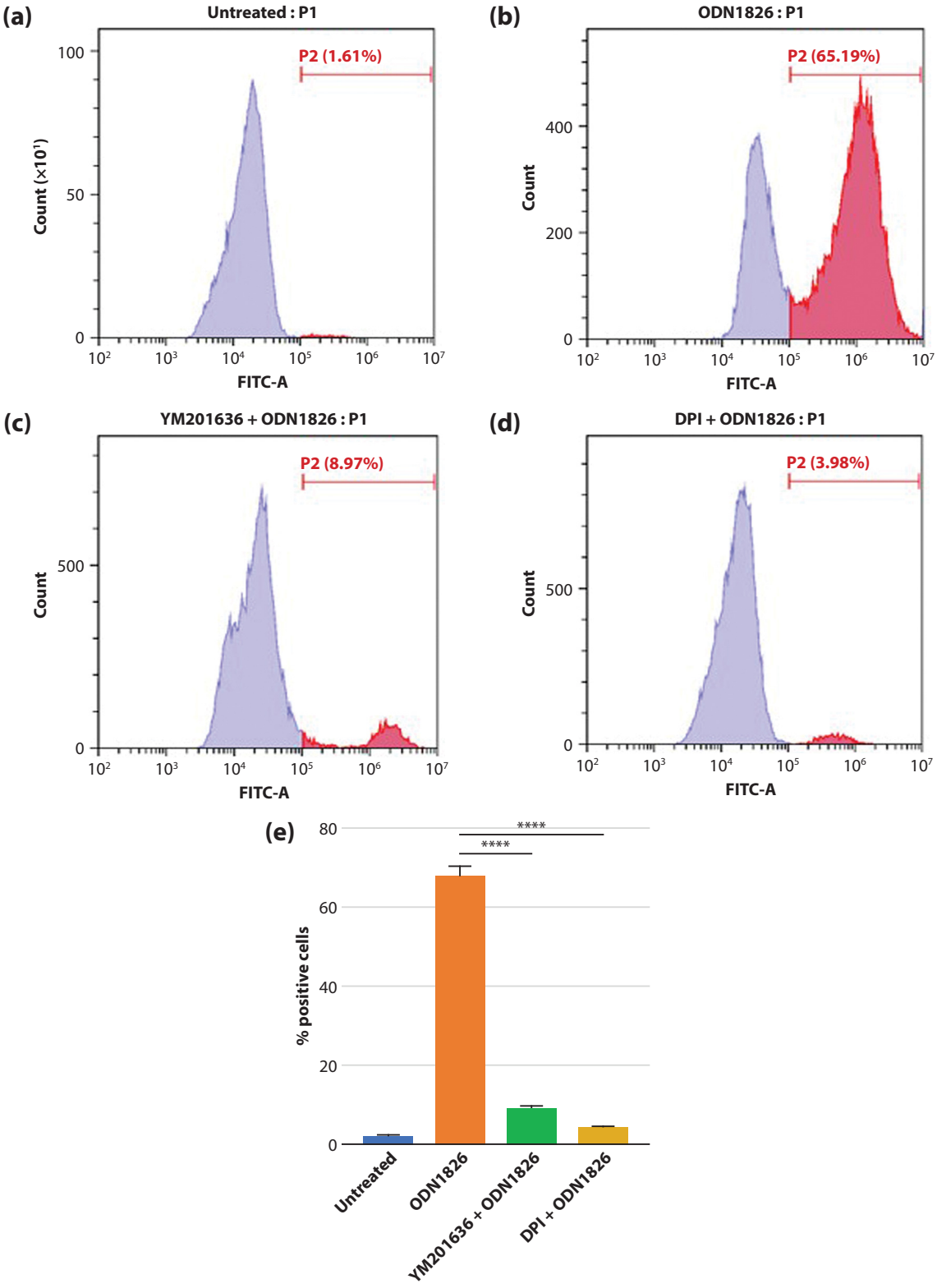

Figure 3. YM201636 inhibits ROS production in OND1826-activated Raw264.7 cells. (a-d) Raw264.7 cells were pretreated with YM201636 (1 μM) or diphenyleneiodonium (DPI) (10 μM) for 1 h before stimulation with ODN1826 (1 μg/ml) for 18 h. After the indicated time point, the treated cells were washed and incubated with 10 μM of DCFH-DA for 30 min at 37°C. Then, the ROS production was analyzed by using flow cytometry. Representative histograms of three independent experiments were performed in duplicate (n = 6). (e) The percentage of positive cells (MFI) from DCFH-DA. The results are presented as means ± SEM of three independent experiments performed in duplicate (n = 6). All data were determined by one-way ANOVA followed by Tukey's multiple comparison test. ****$p < 0.0001$.

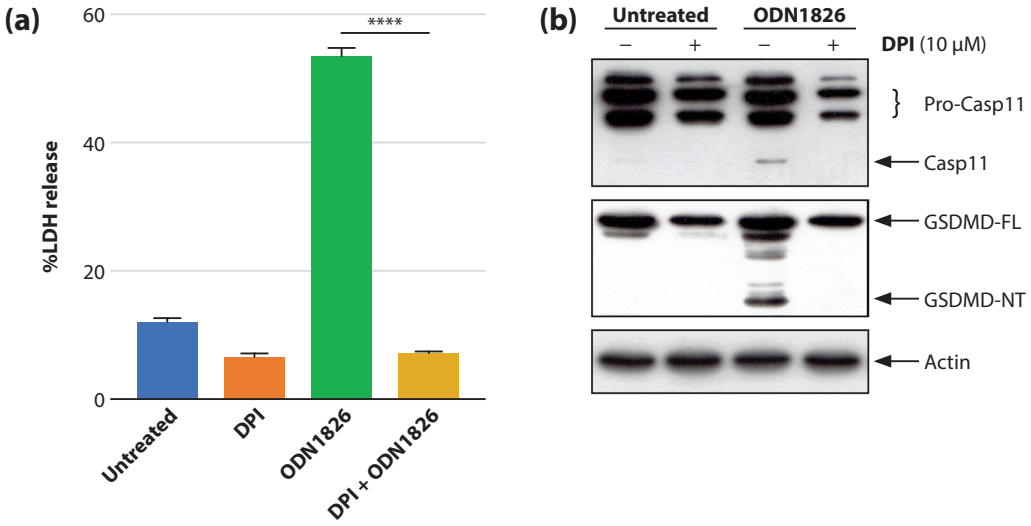

Figure 4. Effects of ROS inhibitor on ODN1826-induced pyroptosis. Raw264.7 cells were pretreated with DPI (10 μM) for 1 h before being stimulated with ODN1826 (1 μg/ml). After 18 h of stimulation, the supernatant was harvested for analysis. (a) The amount of LDH release in supernatants was measured using the LDH assay and presented as means ± SEM from at least three independent experiments. Data were determined using one-way ANOVA followed by Tukey's multiple comparison test. ****$p < 0.0001$. (b) Cell lysates were prepared and analyzed by immunoblotting. Representative bands are shown from three independent experiments.

ODN1826-induced ROS production leading to pyroptosis

To investigate the ability of ODN1826 in ROS production, the cells were activated with ODN1826 for 18 h before 2′,7′ DCFH-DA dye was added, and the ROS production was analyzed by using flow cytometer. As shown in **Figure 3**, ODN1826 induced ROS production (**Figure 3b**) compared to untreated cells (**Figure 3a**). However, the ROS level was significantly decreased in the presence of YM201636 (**Figure 3c and 3e**), suggesting that PIKfyve is also involved in ROS production in ODN1826-activated Raw264.7 cells. To further analyze the involvement of ROS in pyroptosis induced by ODN1826, the cells were pretreated with the ROS inhibitor DPI before LDH was determined. DPI can prevent ROS production (**Figure 3d and 3e**) and inhibits LDH release from the ODN1826-activated Raw264.7 cells (**Figure 4a**). Furthermore, the attenuation of LDH release is also directly correlated with a decrease in caspase-11 and GSDMD activation (**Figure 4b**). These results imply that ROS production plays an essential role in pyroptosis via caspase-11 activation in ODN1826-activated Raw 264.7 cells.

Discussion

Among TLRs, TLR9 is one of a few that expresses intracellularly within the endosomal compartment. This TLR is specialized for recognizing and responding to pathogen DNA, particularly bacterial DNA containing unmethylated CpG oligonucleotides. Like surface TLR, TLR9 has been known to play a role in combating pathogens. For example, by generating ROS, TLR9 can mediate *Staphylococcus aureus* killing.[17] TLR9 also contributes to many inflammatory and autoimmune diseases, such as systemic lupus erythematosus (SLE).[3] Recent evidence shows that TLR9 can induce programmed cell death via apoptosis in many cell types, such as neurons.[18] In contrast to apoptosis, pyroptosis has been extensively studied in other TLRs, particularly TLR4 but not TLR9.[14] There is limited information, particularly on the molecular level of pyroptosis and TLR9. However, a recent study shows that *Porphyromonas gingivalis* LPS induced pyroptosis of vascular smooth muscle cells involving TLR9.[19] In the present study, we demonstrated that activation of TLR9 can also cause pyroptosis in Raw264.7 macrophages.

Caspase-11 is known to be essential for pyroptosis induction by Gram-negative bacteria. This enzyme can be activated by binding the LPS of the bacteria in the cytosol. Activated caspase-11 cleaves gasdermin D and initiates pyroptosis by forming pores on the cell membrane, resulting in cell lysis and the release of a robust inflammatory response.[10] We observed that ODN1826 also promotes the activation of caspase-11 and GSDMD. Since TLR9 is known to locate in the intracellular compartments, the maturation of CpG-containing endosomes is crucial for initiating TLR9-induced signaling.[9] Recent report demonstrated that PIKfyve, PI(3) P 5-kinase, is important in controlling membrane trafficking and signaling of TLR9.[20] We also demonstrated that PIKfyve is essential for pyroptosis in ODN1826-activated Raw264.7 cells

Several factors have been reported to influence pyroptosis. Recently, our group reported that the increase of IFN-β production could mediate the activation of caspase-11 and GSDMD, leading to pyroptosis in *B. pseudomallei*-infected Raw264.7 cells.[21] Furthermore, besides cytokine, ROS production has been shown to regulate caspase-11 expression and activation in *C. rodentium*-infected BMDMs.[15] ROS generation induced by NLRP3 inflammasome activation has been demonstrated

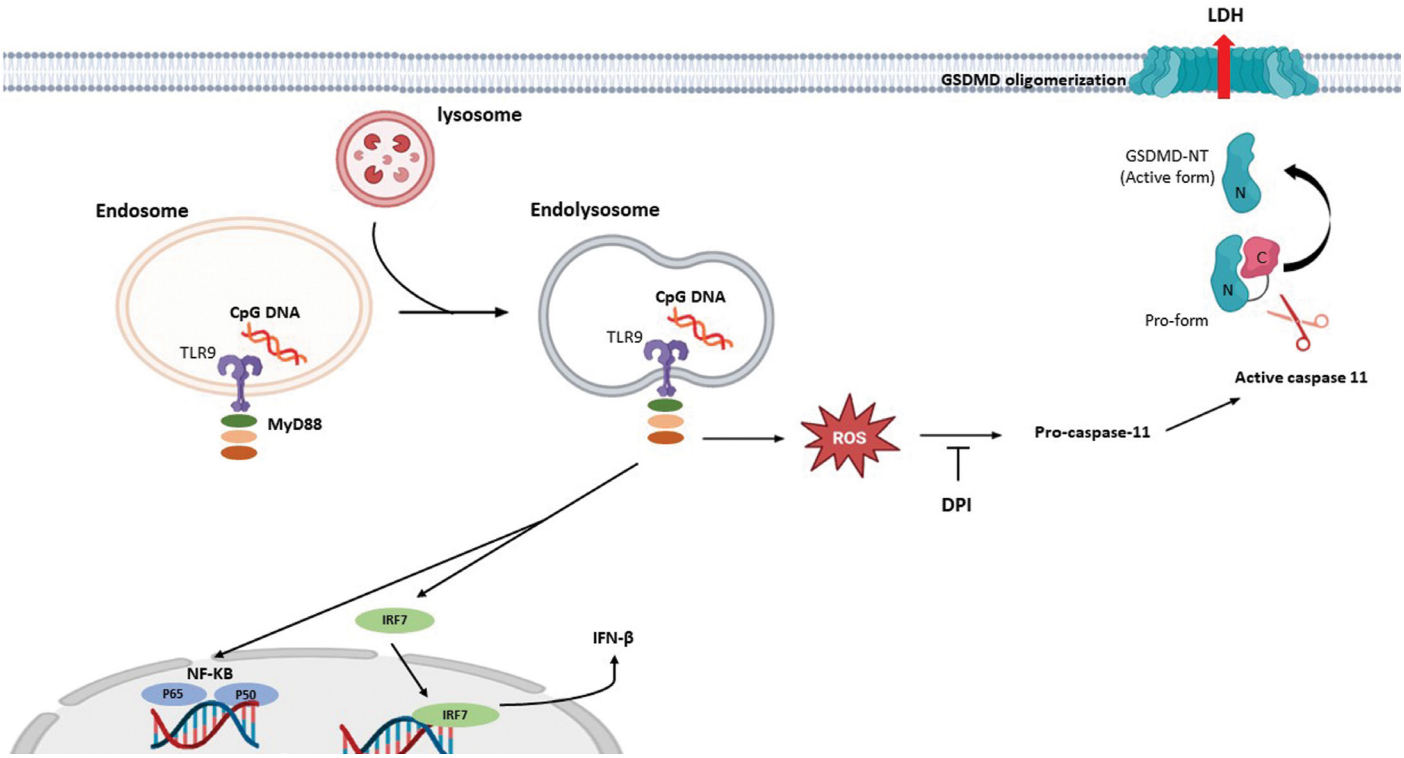

Figure 5. The mechanism model of pyroptosis induced by TLR9 ligand requires ROS production and caspase-11 activation in Raw264.7 cells. ODN1826 is internalized and engages with TLR9 in the endosome. Signaling from TLR9 in endosome mediates ROS production leading to caspase-11 activation. This enzyme cleaves gasdermin D (GSDMD) into GSDMD-NT, leading to LDH release.

to play a role in pyroptosis.[22] Moreover, inhibition of ROS also reduces the cleavage of gasdermin D, suggesting that gasdermin D cleavage occurs downstream of ROS release.[22] In this study, we further demonstrated that the ROS production induced by ODN1826 is an essential factor for regulating caspase-11 and GSDMD activation, leading to pyroptosis.

The molecular mechanism of pyroptosis by TLR has been extensively demonstrated by using LPS (TLR4 ligand).[14] Several lines of evidence also show that cytosolic LPS can activate caspase-11 by binding of LPS to caspase-11 leading to cleavage of gasdermin D.[10] In our study, we clearly demonstrated that by activation of TLR9 by ODN1826 can also activate caspase-11 resulting in the cleavage of gasdermin D followed by LDH release (**Figure 5**). Furthermore, ROS production also plays an essential role in caspase-11 activation.

Conclusion

ODN1826, a TLR9 ligand, induces pyroptosis in Raw264.7 cells via caspase-11 and GSDMD activation. Moreover, the production of ROS by this ligand plays an essential role in the regulation of caspase-11 and GSDMD activation, which then controls pyroptosis in TLR9 activation.

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Author contributions

- Conceived and designed the experiments: MP, RS, PU.
- Performed the experiments: MP, RS, CL.
- Analyzed the data: MP, RS, PU.
- Wrote the manuscript: MP, RS, PU.
- Edited the manuscript: PU.
- Funding acquisition: PU.
- All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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