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ABSTRACT. Inflammatory caspases are involved in the innate immune response, in which cell damage and microorganism invasion lead to cell death via pyroptosis and the release of chemical signals that produce inflammation. Currently, little is known pertaining to the mechanism of activation for one of the major caspase isoforms, caspase-4, other than its ability to bind lipopolysaccharides (LPS) and become more active upon binding. It has been theorized that LPS promotes caspase-4 aggregation through a non-canonical pathway leading to its subsequent activation. Alternatively, caspase-4 may be activated after interacting with LPS micelles rather than monomeric units. Herein, we sought to incorporate LPS into previously optimized enzymatic assay conditions to potentially induce aggregation via direct binding or micelle formation as a scaffold. It was proposed that pro-caspase-4 zymogens would either bind LPS micelles to form a complex that would activate caspase-4 upon cleavage, or that activation could occur through the binding of monomeric LPS directly and oligomerization of caspase-4. However, enzymatic assays performed with caspase-4 and various amounts of LPS showed no significant change in activity. The lack of change does not validate one mechanism of activation over the other, as both mechanisms are LPS dependent. Further studies of the activation mechanism and the presence of aggregates will be needed to determine the role of LPS in caspase-4 activation.

INTRODUCTION

The inflammatory caspase family of enzymes are invoked during the innate immune response, often as a consequence of pathogen associated molecular patterns (PAMPS) or damage associated molecular patterns (DAMPS).[1] Caspases respond to these stimuli by cleaving interluekin 1-β (IL-1β) and gasdermin D (GSDMD), amoung others, into their active form, generating the inflammatory cascade. When this inflammatory response is dysregulated, disease states such as sepsis, gout, and other autoinflammatory disorders can occur.[2,3]

Caspase-1 activation has long been understood to incorporate the aggregation of nucleotide oligomerization domain (NOD-), leucine rich repeat (LRR) and pyrin domain-containing protein 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and pro-caspase 1 to form the canonical NLRP3 inflammasome (Figure 1, left side). However, little is known regarding the caspase-4 activation pathway. Current studies suggest that caspase-4 activation

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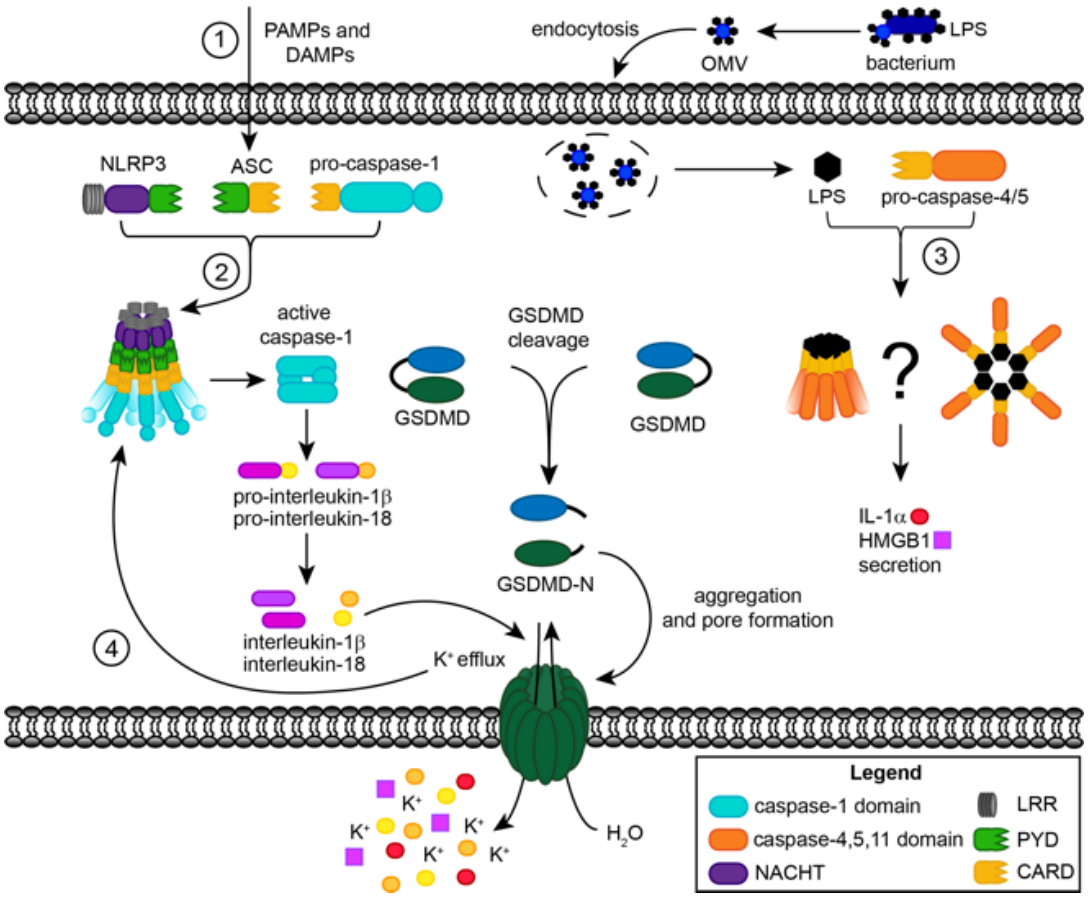

Figure 1. Schematic of canonical and non-canonical pathways of inflammasome activation and subsequent steps leading to pyroptosis.

occurs through binding of gram negative lipopolysaccharide (LPS) to the caspase activation and recruitment (CARD)-domain of pro-caspase-4, stimulating oligomerization of the LPS-bound caspase-4.[4] More recently, it has been theorized that pro-caspase-4 binds to large LPS micelles at its CARD domain followed by sequential proteolysis of the complex leading to active caspase-4.[5] The exact oligomerization state and the overall structure of the combined complex is yet to be uncovered. This is due in part to the autoproteolytic activity of caspase-4, which makes the complex difficult to isolate. Previous experiments have been performed using mutagenically inactivated caspase-4 to ameliorate this issue, however this methodology does not provide an accurate representation of what occurs *invivo*. Herein, *in vitro* assay conditions were optimized in order to monitor caspase-4 activity with the incorporation of LPS. Variables examined included the concentration of caspase-4, incubation times, temperature, buffer components, and the addition of activating agents such as dithiothreitol (DTT). If LPS indeed activates caspase-4, increased activity is expected for upon addition of LPS within *in vitro* assays. For this reason, LPS titration studies were performed in order to understand its role in the activation of non-canonical caspase inflammasomes.

METHODS

Standard Fulorogenic Assay with Caspase-4

All enzymatic assays were performed using the Biotek H1 Synergy 3000 Hybrid plate reader for fluorescence measurement. Wells contained caspase-4 (100 nM), the peptide-based substrate WEHD-AMC (20 μM), where AMC is 7-amino-4-methylcoumarin, and DTT (3 mM). Wells were supplemented with caspase buffer (50 mM HEPES, 50 mM NaCl, 0.1 % CHAPS, 10 mM

EDTA, 5 % glycerol, pH 7.2) to achieve a total volume of 100 μL. Kinetic data were obtained using previously determined fluorescence assay conditions for the fluorophore AMC linked to the substrate (excitation 380 nm, emission 460 nm).[6] Each experiment was run in triplicate for a duration of 90 minutes. Depending on the variables tested, a prior 30-minute incubation of caspase 4 with DTT was allotted in certain cases. Temperature was also manipulated in certain cases, in which samples were set to 37 °C during florescent monitoring.

LPS Titration Assay with Caspase-4

LPS titration assays were performed using similar methodology to that of the standard flourgenic assays with varrying LPS concentrations (0- 10 μM). All wells were supplemented with caspase buffer to achieve a total volume of 100 μL each. Manipulated variables for this assay included varying incubation time (0, 10, 40 min) of LPS with caspase-4, the addition of heat (37 °C) in certain cases, and the addition of DTT (3 mM). CHAPS, from the caspase buffer used, was tested usingdynamic light scattering (DLS) for observation of micellular formation as a preventative measure to ensure it was not disrupting potential LPS micelle formation in solution. DLS measurements were conducted using an Anton Paar Litesizer™ 100 instrument.

RESULTS & DISCUSSION

The mechanism of activation for caspase-1 is characterized by the aggregation of inactive pro-caspase-1 with NLRP3 and ASC following a PAMP or DAMP signal (Figure 1). The complex formed undergoes proteolysis to generate active caspase-1. The active enzyme can then cleave the inactive substrates, pro-interleukin-1β and GSDMD, into their respective active forms causing a potassium efflux and cell rupture. For caspase-4/5 the mechanism of activation is far less understood. It has been proposed that LPS-rich outer-membrane vesicles of bacteria are endocytosed, after which they bind to the CARD domain of pro-caspase 4/5 in the cytosol.
However, the complex formed and its mechanism of dissociation into active caspase-4 remains undetermined (Figure 1). What is known about this cellular process is that, similar to caspase-1, caspase-4 is able to cleave GSDMD into its active form (GSDM-N) causing the same down stream effect of potassium efflux leading to pyroptosis.

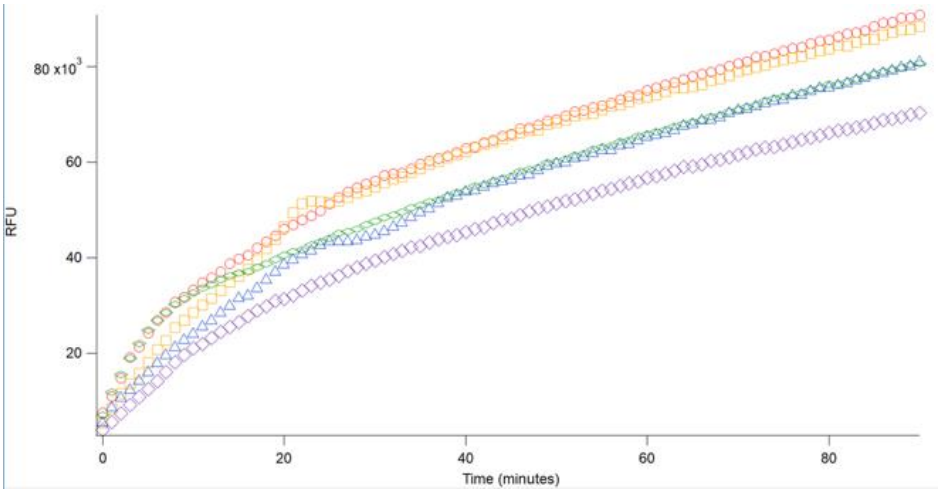

Figure 2. LPS Titration Progress Curve. Progress curves of caspase-4 with WEHD-AMC and LPS (Red: 0 μM LPS, Orange: 1 μM LPS, Green: 2 μM LPS, Blue: 5 μM LPS, and Purple: 10 μM LPS). Reactions were performed at 37 °C using the LPS titration assay protocol.

Table 1. Manipulated Assay Conditions for Caspase-4 and Their Effect on Kinetic Activity[a]

	Conditions			
Trial	DTT (Inubation)	LPS (incubation)	Heat	Change in Activity
1	-	-	-	Control
2	-	-	+	Heat Control
3	+ (30 min)	-	+	Increase
4	+ (none)	-	+	Increase
5	+ (30 min)	+ (10 min)	+	No Change
6	-	+ (10 min)	+	No Change
7	+ (30 min)	+ (40 min)	+	No Change
8	+ (none)	+ (none)	+	No Change
9	+ (none)	+ (none)	+	No Change
10	+ (none)	+ (none)	+	No Change

[a]DTT and LPS concentrations were kept constant at 3 mM and 2 μM, respectively, while incubation time with caspase-4 was varied for each. The substrate WEHD-AMC (20 μM) was also incorporated for each reaction. When implemented, heat was maintained at 37 ^{o}C for the duration of fluorescence monitoring.

Despite the apparent need for LPS to function as a PAMP to induce high levels of caspase-4 activity, this relationship was not observed through conducted *in vitro* enzymatic assays. To test the effect of LPS on the enzymatic activity of caspase-4, an LPS titration assay was performed (Figure 2). Through the obtained results, it is evident that despite the increasing concentration of LPS, the activity of caspase-4 did not change. Interestingly, samples that contained zero or low (1 μM) LPS concentrations had slightly larger initial reaction velocities than that observed for the higher LPS concentrations (10 μM). An initial justification for the observed trend was that caspase-4 requires LPS micelles to bind, and that their formation was being hinderd by pontential aggregation of the detergent CHAPS buffer component. To rule this out as a possibility, CHAPS was tested at varying percentages using DLS in an attempt to observe aggregates, however no aggregation was detected. LPS was also tested through DLS at its reported critical micelle concentration of 5 μM.[7] Interestingly, no micelle formation was detected through DLS for LPS. It is not clear if this is an instrument sensitivity issue, or if micelles were truly absent. Additional work is needed to determine the degree to which LPS aggregates are present at these low concentrations.

Based on the DLS results, the next justification for the trend observed in Figure 2 was the assay conditions being used for enzymatic testing, or that LPS requires a time dependent incubation with caspase-4 before the addition of substrate. Variables including addition of DTT, LPS incubation time, and incorporation of heat were manipulated to determine if they functioned as a potential limiting factor for LPS activation (Table 1). LPS incubation with caspase-4 consisted of 0, 10, or 40 minutes, while also manipulating the addition of DTT and its respective incubation time with caspase-4. As previously determined, DTT induced an increase in caspase-4 activity (trials 3 and 4), however, no conditions that incorporated LPS resulted inincreased enzymatic activity, as seen with trials 5-10 (Table 1).

As none of the manipulated assay conditions allowed for LPS to induce higher levels of activity for caspase-4, other possible explanations to justify this rationale could simply be that the variant of LPS used is not compatible with the caspase CARD domain. Ostensibly, LPS is required to remain

integrated within outer-membrane vesicles for pro-caspase-4 recognition. The future direction of our research is to incorporate new strains of LPS while also performing additional dynamic light scattering experiments at varying concentrations to observe possible LPS micelle formation. Further oligomerization studies will be performed on caspase-4 to determine the quantity of aggregates needed to form the possible non-canonical structure required for its activation.

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