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# The *Pseudomonas aeruginosa* HSP70-like protein DnaK induces IL-1β expression *via* TLR4-dependent activation of the NF-κB and JNK signaling pathways

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ABSTRACT

IL-1β expression is increased in response to *P. aeruginosa* infection, but the responsible proteins have not been clearly elucidated. Here, we demonstrate for the first time that IL-1β expression is induced in response to the heat shock protein 70-like protein DnaK. Treatment with recombinant DnaK (rDnaK) increased IL-1β expression in a dose- and time-dependent manner, and the release of mature IL-1β in response to rDnaK was detected to an extent similar to that stimulated by the well-known agonists, lipopolysaccharide and nigericin. rDnaK-mediated IL-1β expression was driven by the NF-κB signaling pathway. In addition, expression was controlled by the JNK signaling pathway, although these two signaling cascades act independently upon rDnaK stimulation. Finally, rDnaK-induced IL-1β expression was initiated *via* the action of TLR4. Taken together, the data reveal that *P. aeruginosa*-derived DnaK induces expression of IL-1β *via* TLR4-dependent activation of the NF-κB and JNK signaling pathways.

## 1. Introduction

Innate immune responses play important roles in defending the host, including animals, against invading pathogens by triggering the action of numerous pro-inflammatory cytokines [1,2]. Cytokine production is driven by the recognition of pathogen-associated molecular patterns (PAMPs) *via* the engagement of Toll-like receptors (TLRs) expressed by diverse immune cells, such as monocytes and macrophages [3]. PAMP recognition leads nuclear translocation of NF-κB, a central regulator of innate immune response activation, resulting in the production of numerous cytokines, including interleukin-1β (IL-1β) [4]. IL-1β is a critical host-encoded pro-inflammatory cytokine that mediates neutrophil recruitment and bacterial clearance during infections [5]. The mature form of IL-1β is produced via a two-step process: an initial priming signal and a subsequent activating signal. In the first step, NF-κB promotes transcription of the IL-1β-encoding gene, which then leads to translation of pro-IL-1β [6]. The second process is mediated by the activation of the multiprotein complex known as the inflammasome, leading to caspase-1 activation; finally, activated caspase-1 cleaves pro-IL-1β to generate mature IL-1β [7–9]. The active form of caspase-1 initiates a form of programmed cell death known as pyroptosis [10]. This process is facilitated by the formation of cleaved gasdermin D, a pore forming protein cleaved by the action of caspase-1 to generate its active form, which in turn results in IL-1β release [11].

This effect has been studied in the context of a number of microbial infections that are recognized as emerging nosocomial infections in veterinary, including those by avian influenza, swine influenza and *Pseudomonas aeruginosa* [12–15]. Higher IL-1β levels were observed in the corneas of mice [16] and the bronchoalveolar lavage fluid of cystic fibrosis patients [17] upon infection with *P. aeruginosa*. The infection stimulates caspase-1 activation and subsequent IL-1β production *via* the action of a functional type III secretion system (T3SS), but not *via* any well-known effector molecules (*i.e.*, ExoS, ExoT, ExoY, and ExoU) [18–20]. These processes also play key roles in activating inflammatory responses against tissue damage *via* the detection of endogenous damage-associated molecular patterns (DAMPs).

Heat shock proteins (HSPs) are a group of highly conserved proteins in both prokaryotic and eukaryotic cells that are conventionally known as intracellular chaperones [21]. However, upon exposure to certain stresses, such as inflammation and infection, eukaryotic HSPs such as

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HSP70 are released into fluids *via* unknown mechanisms where they then act as an endogenous DAMP to transmit a 'danger signal' that modulates the secretion of pro-inflammatory cytokines [22,23]. In support of this concept, elevated levels of HSP70 were found in the bronchoalveolar lavage fluid of individuals with lung inflammation [24]. Similarly, highly conserved and ubiquitously expressed bacterial HSPs have been shown to modulate innate immune responses and contribute to an inflammatory disease pathology. In this context, we have been interested in the effects of *P. aeruginosa*-derived HSPs on IL-1β production. Here, we demonstrate the stimulatory effect of *P. aeruginosa*-derived DnaK, a HSP70-like protein, on IL-1β expression *via* TLR4-dependent activation of the NF-κB and c-Jun *N*-terminal kinases (JNK) signaling pathways.

## 2. Materials and methods

### 2.1. Reagents

BAY 11–7082 and SP600125 were purchased from A.G. Scientific (San Diego, CA, USA). Proteinase K was purchased from Thermo Scientific (Waltham, MA, USA). *P. aeruginosa*-derived lipopolysaccharides (LPS), nigericin and acetyl-tyrosyl-valyl-alanyl-aspartyl-chloromethylketone (ac-YVAD-cmk) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CLI-095 was purchased from Invivogen (San Diego, CA, USA).

### 2.2. Bacterial strains and culture conditions

*P. aeruginosa* wild-type strain PAK and a *P. aeruginosa* PAK isogenic *exoSTY* deletion mutant [25] were cultivated on Luria (L) agar or in L broth rich medium (yeast extract, 0.5 %; tryptone, 1 %; and NaCl, 1 %; all w/v) at 37 °C. To prepare live bacteria, bacterial cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4 °C after overnight growth in L broth, and the bacteria pellet was then suspended in phosphate-buffered saline (PBS). To obtain culture supernatant (Sup), bacterial cells were harvested after overnight growth in minimal medium A broth (MinA; $K_2HPO_4$ 1.05 %; $KH_2PO_4$ 0.45 %; $(NH_4)_2SO_4$ 0.1 %; sodium citrate•$2H_2O$ 0.05 %; glutamate 0.845 %; and glycerol 1 %; all w/v except glycerol, v/v) at 37 °C [26]. The Sup was filtered through a low protein-binding membrane with a 0.22 μm pore size (Sartorius, Goettingen, Germany) to completely remove bacterial cells. To prepare concentrated filtrates, the Sup was filtered through Amicon Ultra centrifugal filter units (EMD Millipore, Darmstadt, Germany) with 10, 30, or 50 kDa pore sizes.

### 2.3. Cell culture

All media described below were supplemented with 10 % heat-inactivated fetal bovine serum (Access, Vista, CA, USA), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). A549 (human alveolar epithelial cells) and THP-1 (human monocytes) cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; HyClone). THP-1 cells were differentiated *via* incubation with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 16 h, and the resulting cells were designated as dTHP-1 cells in this study. Cells were maintained at 37 °C in a humidified 5 % $CO_2$ air-jacketed incubator. Unless otherwise indicated, dTHP-1 cells were exposed to recombinant DnaK (rDnaK) protein at 1 μg/ml for 4 h.

### 2.4. Construction of the His-tagged DnaK plasmid and recombinant protein purification

The coding region of the *dnaK* locus (PA4761) was amplified from *P. aeruginosa* strain PAK genomic DNA *via* polymerase chain reaction (PCR) using the following primer pair, which contain restriction enzyme recognition sites for *Bam*HI and *Sac*I (underlined) respectively: 5′−CCGGATCCTATGGGCAAAATCATTGGCATC-3′ and 5′−CCGAGCT CGTTTCGCCATAACCTTTC-3′. The 1.9 kb PCR product was cloned into the pETDuet-1 vector (Novagen, Germany) *via* the two restriction sites. The construction was validated *via* sequence analysis with the pET Upstream (#69214-3) and DuetDOWN1 (#71179-3) primers. DnaK was expressed as a His-tagged fusion protein in *Escherichia coli* BL21 (DE3) (Invitrogen, San Diego, CA, USA). After purifying the fusion protein using an Ni-NTA Purification system (Thermo Scientific), endotoxin removal was performed using phase separation treatment with Triton X-114, as described previously [[27], Liu, 1997 #6161]; the Triton X-114 was removed by Bio-beads™ SM-2 Adsorbent Media (Bio-Rad, Hercules, CA, USA). Concentration of the residual endotoxin was measured at less than 0.5 ng/ml as evaluated using a Pierce limulus amebocyte lysate (LAL) Chromogenic Endotoxin Quantitation Kit. The DnaK concentration was quantified *via* the BCA Protein Assay Kit (Pierce Thermo), adjusted to 500 μg/ml, and stored at −80 °C. For a control extract, an *E. coli* BL21 (DE3) strain harboring a pETDuet-1 vector was subjected to the same procedures.

### 2.5. Quantitative real-time PCR (Q-PCR)

Total RNA was isolated using TRIzol® Reagent following Invitrogen's instructions. SYBR Green PCR Master Mix (KAPA Biosystems, Woburn, MA, USA) was used for the Q-PCR. Synthesis of cDNA from total RNA was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems). Primer sequences were as follows: human *il-1β*, 5′-AAACAGATGAAGTGCTCCTTCCAGG-3′ and 5′-TGGAGAACACCACTTGTTGCTCCA-3′; *P. aeruginosa dnaK*, 5′−CCA CACCGTCATCGTCTATG-3′ and 5′-CGCTTTCCTTCTTGAACTCG-3′. The reactions were performed with a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using the following thermal conditions: stage 1, 50 °C for 2 min and 95 °C for 10 min; stage 2, 95 °C for 15 s and 60 °C for 1 min. The relative levels of *il-1β* mRNA were calculated using the comparative CT method with normalization to human GAPDH (5′−CCCTCCAAAATCAAGTGG-3′ and 5′−CCATCCACAGTCTTCTGG-3′) to control for the amount of RNA used in each reaction. Relative quantities of *dnaK* mRNA were normalized against *P. aeruginosa* 16 s RNA (5′-TGTGAAGAAGGTCTTCGGATTG-3′ and 5′-CGAAGTTAGCCGGTGCT TAT-3′).

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

The amount of IL-1β released into culture supernatants was assayed using the human IL-1β ELISA Kit (Thermo Scientific) according to the manufacturer's instructions.

### 2.7. Immunoblot analysis

Cells were collected and lysed on ice for 10 min in 20 mM Tris−HCl (pH 7.4), 50 mM NaCl, 50 mM Na pyrophosphate, 30 mM NaF, 5 μM zinc chloride, 2 mM iodoacetic acid and 1 % Triton X-100 in distilled water supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM sodium orthovanadate (Sigma-Aldrich). The lysates were centrifuged at 10,000 × *g* for 15 min at 4 °C, and the protein concentrations were measured using the BCA Protein Assay Kit (Pierce Thermo). About 25 μg/well of each protein extract was separated on 12 % SDS-PAGE gels, and the proteins were then transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 5 % non-fat dry milk solution at room temperature for 1 h and incubated at 4 °C overnight with the appropriate antibodies: p-IKKα/β (16A6), p-IκBα (14D4), IκBα, IKKα, IKKβ, p-JNK (98F2), JNK and β-actin (D6A8) (Cell Signaling Technology, Danvers, MA, USA). After washing, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using an ImageQuant LAS-4000 system (GE Healthcare Life Sciences) following addition of WEST-ZOL®plus

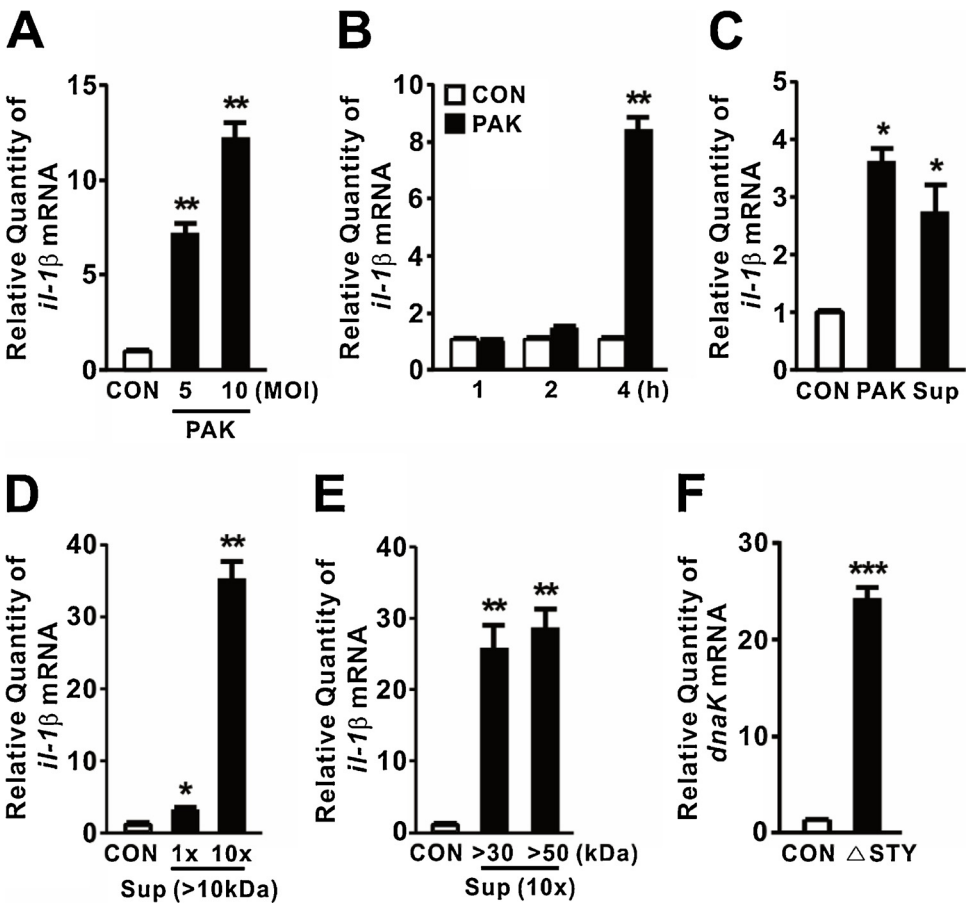


**Fig. 1. *P. aeruginosa*-derived culture supernatant induces *il-1β* expression.** (**A**, **B**) A549 cells were treated with PAK at MOIs of 5 or 10 for 4 h (**A**) or at an MOI of 10 for the indicated times (**B**). (**C**) A549 cells were treated for 4 h with either PAK at an MOI of 10 or with 100 μl of culture supernatant (Sup) obtained from PAK cultivated in MinA medium. (**D**) A549 cells were treated for 4 h with 100 μl of either 1×- or 10×-concentrated filtrates of PAK cultures size-fractionated on a membrane filter with a 10 kDa cutoff. (**E**) A549 cells were treated for 4 h with 100 μl of 10× concentrated filtrates of PAK cultures size-fractionated on membrane filters with either a 30 kDa or a 50 kDa cutoff. (**F**) A549 cells were treated for 4 h with a *P. aeruginosa* strain PAK *exoSTY* deletion mutant at an MOI of 200. After treatment, the increase in *il-1β* or *dnaK* mRNA levels was measured by Q-PCR. Data are expressed as the mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ *vs.* CON. PBS (**A** and **B**) and MinA (**C**-**F**) were used as a control (CON). MOI, multiplicity of infection.

Chemiluminescent Substrate (Intron, South Korea).

### 2.8. Statistical analysis

Statistical analyses were performed with Student's *t* test or one-way ANOVA followed by Tukey's post-hoc multiple range test using the Instat package from GraphPad (GraphPad Software, San Diego, CA, USA). $p < 0.01$ was considered statistically significant.

## 3. Results

### 3.1. P. aeruginosa-derived culture supernatant induces il-1β expression.

We previously showed that IL-1β expression is induced in response to *P. aeruginosa* strain PAO1 [28]. Here, we followed up that investigation by examining the IL-1β expression in response to another well-known *P. aeruginosa* strain, PAK. As shown in Fig. 1**A** and **B**, *il-1β* expression was gradually elevated in a dose- and a time-dependent manner, reaching a maximum at a multiplicity of infection (MOI) of 10 after treatment for 4 h. Due to cytotoxicity caused by the infection, we could not extend the induction over the MOI and incubation time used in this study. These results indicate that *P. aeruginosa* PAK can induce *il-1β* expression. *P. aeruginosa* possesses diverse virulence factors that act as PAMPs; these include flagellin and LPS, which are recognized by TLR5 [29] and TLR4 [30], respectively. In addition, some of virulence factors are released *via* secretion systems and also contribute to *P. aeruginosa* pathogenicity [31,32]. To discern whether secreted proteins are involved in the induction of *il-1β* expression, cells were treated with either *P. aeruginosa* PAK or a supernatant (Sup) obtained from the PAK culture. As shown in Fig. 1C, *il-1β* expression was induced in response to the Sup to a level similar to that caused by PAK, suggesting the involvement of secreted proteins for the induction. To verify this effect, cells were treated with 1-fold and 10-fold concentrated filtrates obtained using filter units with a 10 kDa pore size, and this treatment resulted in a dramatic induction of *il-1β* expression in a concentration-dependent manner (Fig. 1D). To further determine the size of the responsible proteins, we obtained 10-fold concentrated filtrates using filter units with either 30 or 50 kDa pore sizes. Both filtrates clearly induced *il-1β* expression, indicating that the molecular weight of the proteins could exceed 50 kDa (Fig. 1E). Next, we examined whether the genetic locus of *dnaK* is induced in the *P. aeruginosa* strain upon infection of host cells. As shown in Fig. 1F, there was clear induction of *dnaK* gene expression when cells were exposed to the *P. aeruginosa* strain, suggesting that expression is stimulated by infection. Taken together, these results indicate that *P. aeruginosa*-derived proteins larger than 50 kDa induce the IL-1β expression.

### 3.2. P. aeruginosa-derived DnaK induces IL-1β expression

Given that endogenous HSPs such as HSP70 transmit a "danger signal" and that DnaK (an HSP70-like protein) has a molecular weight of approximately 79 kDa, we were interested in knowing whether DnaK contributes to induction of IL-1β expression. To determine the role of *P. aeruginosa*-derived DnaK, Triton X-114-pre-treated rDnaK was obtained. As shown in Fig. 2**A** and **B**, the rDnaK treatment clearly induced *il-1β* expression in a dose- and a time-dependent manner. By contrast, pre-treatment with proteinase K prevented this induction, suggesting a role for DnaK. DnaK-stimulated induction was further analyzed relative to the time-dependent release of mature IL-1β stimulated by two well-known agonists, LPS and nigericin; the results demonstrated that DnaK is a potent stimulator of IL-1β production (Fig. 2C). Next, we determined whether caspase-1 was required for rDnaK-stimulated IL-1β production. As shown in Fig. 2D, IL-1β production was reduced significantly after treatment with ac-YVAD-cmk, a specific chemical

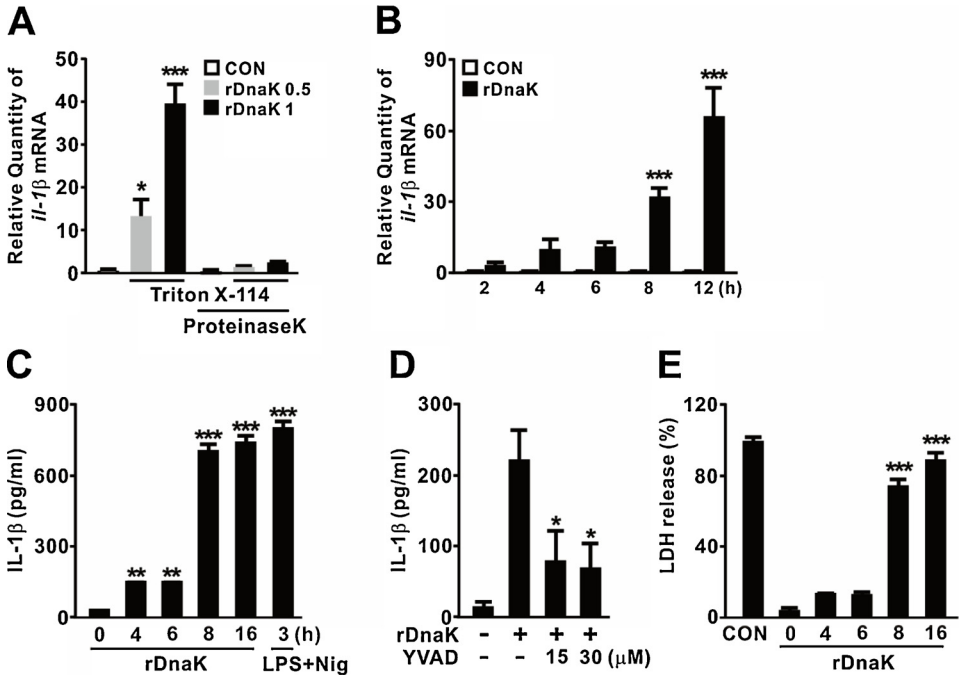


**Fig. 2. *P. aeruginosa*-derived DnaK induces IL-1β expression.** (**A**) dTHP-1 cells were incubated with Triton X-114-pre-treated rDnaK (0.5 or 1 μg/ml) for 4 h. A control extract was used as a negative control (CON), and proteinase K treatment (20 μg/ml) was performed for 1 h. (**B**) dTHP-1 cells were treated with rDnaK at 1 μg/ml for the indicated times. Next, *il-1β* mRNA levels were quantitated by Q-PCR. (**C**) dTHP-1 cells were treated with rDnaK at 1 μg/ml for the indicated time. Cells treated with LPS (100 ng/ml) and nigericin (5 μM) for 3 h were used as positive controls. (**D**) dTHP-1 cells were pre-treated with the indicated amounts of caspase-1 inhibitor (YVAD) for 1 h, followed by rDnaK treatment at 1 μg/ml for 8 h. After treatment, release of IL-1β from dTHP-1 cells were measured in an ELISA. (**E**) dTHP-1 cells were treated with rDnaK (1 μg/ml) for the indicated times. Cytotoxicity was assessed *via* an LDH release assay. Data are expressed as the mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *vs.* CON (**A** and **B**), no treatment (**C** and **E**) and treatment with rDnaK alone (**D**). A control extract (**A**), PBS (**B**) and NaOH (**E**) were used as controls (CON).

inhibitor of caspase-1, prior to addition of rDnaK, suggesting that IL-1β production is controlled by caspase-1. Next, we measured the biological activity of rDnaK-activated caspase-1 by performing a lactate dehydrogenase (LDH) release assay (Fig. 2E), which is based on a reduction in cell viability caused by formation of membrane pores by caspase-1 during a unique form of programmed cell death known as pyroptosis. Therefore, we conclude that *P. aeruginosa*-derived DnaK supports induction of IL-1β expression.

### 3.3. rDnaK-induced IL-1β expression is mediated by the NF-κB and JNK signaling pathways

The NF-κB transcription factor and mitogen-activated protein kinases (MAPKs) are the central regulators of the expression of numerous inflammatory mediators. In this context, we determined whether NF-κB and MAPKs are involved in triggering the rDnaK-mediated IL-1β expression by pre-treating cells with specific chemical inhibitors. As shown in Fig. 3A, pre-treatment with either BAY 11-7082, a specific chemical inhibitor of NF-κB, or SP600125, a specific chemical inhibitor of JNK, significantly reduced *il-1β* expression in a dose-dependent manner. Moreover, combined treatment with both inhibitors reduced the *il-1β* expression to a greater extent than did the individual treatments, suggesting the possibility that there is no crosstalk between the two signaling cascades. Unlike the positive result obtained after JNK inhibition, extracellular signal-regulated kinase and p38 did not appear to be significantly involved (data not shown). The effects of NF-κB and JNK were further verified by measuring the release of mature IL-1β, and the results supported the notion that NF-κB and JNK stimulate IL-1β expression (Fig. 3B). Next, we determined whether rDnaK treatment activates NF-κB and JNK signaling *via* immunoblot analysis. As shown in Fig. 3**C** and **D**, IκBα degradation and JNK phosphorylation were observed after 1 h of incubation, indicating that both NF-κB and JNK are activated after rDnaK treatment and that the activation of each protein might not be connected. To determine whether NF-κB and JNK activation are independent of each other, cells were pre-treated with either SP600125 or BAY 11-7082 and analyzed by immunoblotting. As shown in Fig. 3**C** and **D**, pre-treatments did not alter IκBα degradation or JNK phosphorylation, verifying that there is no crosstalk between the two signaling cascades. Taken together, these results show that rDnaK-mediated IL-1β expression is controlled by both the NF-κB and JNK signaling pathways.

### 3.4. rDnaK-mediated NF-κB and JNK activation is controlled by TLR4

TLR4 acts as a key receptor involved in HSP70 recognition [33]. To determine whether the HSP70-like protein rDnaK-mediated IL-1β expression is controlled by TLR4, cells were pre-treated with CLI-095, a specific chemical inhibitor of TLR4. As shown in Fig. 4A, this pre-treatment reduced *il-1β* expression, indicating that TLR4 is involved in the induction. The action of TLR4 was further confirmed by measuring the production of mature IL-1β, as shown in Fig. 4**B**. Next, we determined whether TLR4 controls the rDnaK-mediated NF-κB and JNK activation by measuring the phosphorylation of IKKα/β, IκBα, and JNK in the presence of CLI-095. As shown in Fig. 4**C** and **D**, the phosphorylation was markedly reduced in the presence of the inhibitor, suggesting that rDnaK induces the signaling cascades *via* TLR4. Taken together, these results support the conclusion that rDnaK-induced IL-1β expression is controlled by TLR4-dependent activation of the NF-κB and JNK signaling pathways.

## 4. Discussion

As a critical pro-inflammatory cytokine, IL-1β is responsible for recruiting neutrophils and supporting bacterial clearance. In response to *P. aeruginosa* infection, IL-1β expression is induced *via* caspase-1 activation in infected macrophages, and the *P. aeruginosa* T3SS structure (but not the known effectors) was identified as an important inducing factor for this activation and the resulting IL-1β production [18,19,34]. We further investigated *P. aeruginosa*-mediated IL-1β induction and found that nucleoside diphosphate kinase (Ndk), a new T3SS effector, induces IL-1β expression with the support of flagellin during *P. aeruginosa* infection [35]. However, we observed that the IL-1β expression was not reduced during infection with a strain with a mutation in *exsA*, a major transcriptional regulator of T3SS expression, indicating that additional factors other than Ndk and the T3SS structure could be involved in stimulating IL-1β expression. In this study, we demonstrate for the first time that *P. aeruginosa*-derived protein DnaK can induce IL-1β expression and that this DnaK-mediated expression is controlled by TLR4-dependent activation of the NF-κB and JNK signaling pathways.

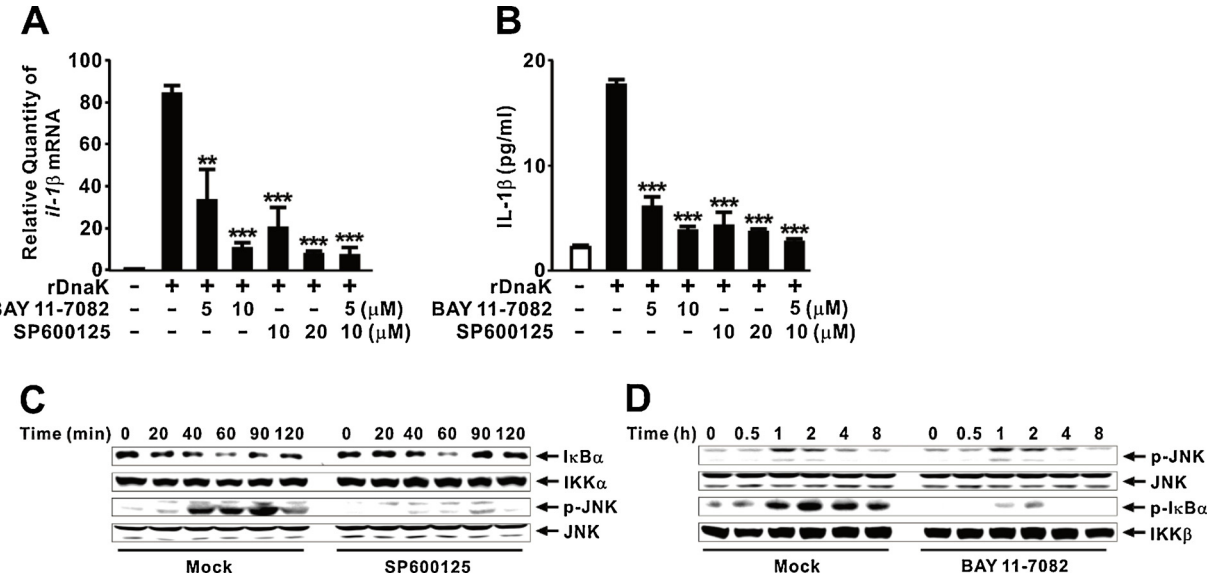


**Fig. 3. rDnaK-induced IL-1β expression is mediated by the NF-κB and JNK signaling pathways.** (**A**, **B**) dTHP-1 cells were pre-treated with either BAY 11-7082 or SP600125 at the indicated amounts for 2 h, followed by rDnaK treatment. Next, *il-1β* mRNA levels were quantitated by Q-PCR (**A**) and the levels of IL-1β released from dTHP-1 cells were measured in an ELISA (**B**). (**C**, **D**) dTHP-1 cells were pre-treated for 2 h with either SP600125 (**C**) or BAY 11-7082 (**D**) (20 μM or 10 μM, respectively), followed by rDnaK (1 μg/ml) for the indicated times. Next, NF-κB and JNK activation levels were assessed *via* immunoblot analysis. The data in **A** and **B** are expressed as mean ± SD ($n = 3$). The data in **C** and **D** are representative of three separate experiments. **, $p < 0.01$; ***, $p < 0.001$ *vs.* treatment with rDnaK alone (**A** and **B**).

In the course of this study, we found that supernatant obtained from *P. aeruginosa* cultures can induce IL-1β expression, indicating that stimulating factors are released during the cell growth. LPS has been well characterized as an important PAMP involved in the stimulation of inflammatory responses. Therefore, it is plausible that the culture supernatant may contain *P. aeruginosa*-derived LPS and that this LPS may stimulate the IL-1β expression. However, purified LPS derived from a number of Gram-negative microbes, such as *E. coli*, *Salmonella typhosa*

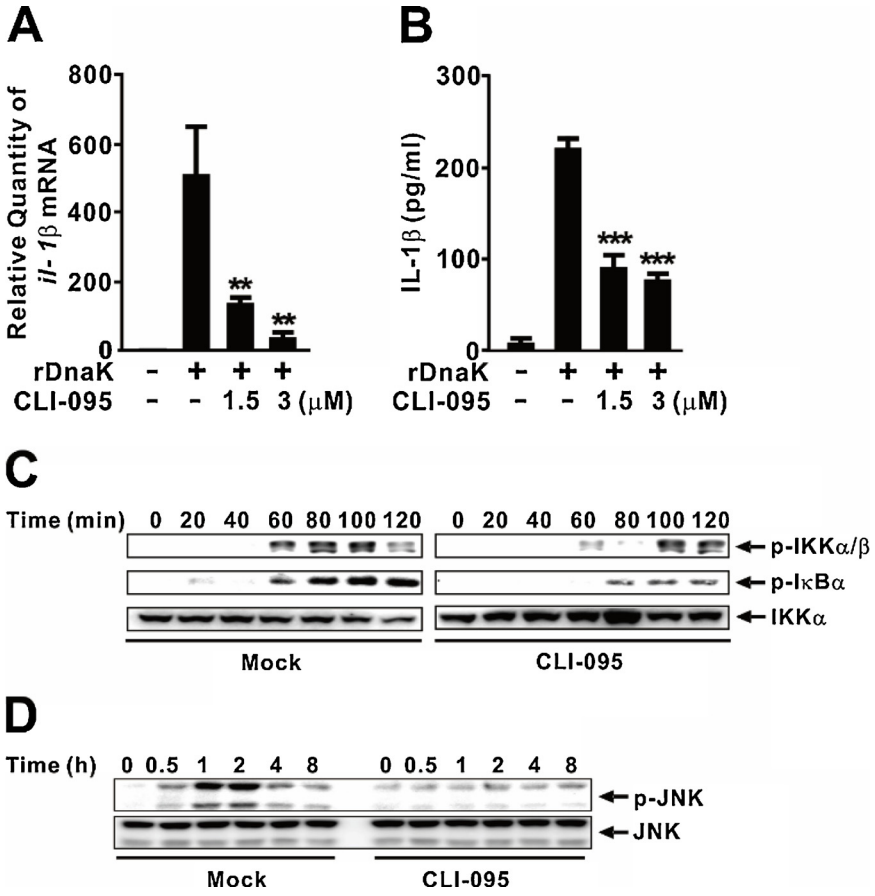


**Fig. 4. rDnaK-mediated NF-κB and JNK activation is controlled by TLR4.** (**A**, **B**) dTHP-1 cells were pre-treated with CLI-095 at the indicated concentrations for 1 h, followed by rDnaK treatment. After the treatment, *il-1β* mRNA levels were measured *via* Q-PCR (**A**), and the levels of IL-1β released from the dTHP-1 cells were measured *via* ELISA (**B**). (**C**, **D**) dTHP-1 cells were pre-treated with CLI-095 at 3 μM for 1 h, followed by rDnaK treatment at 1 μg/ml for the indicated time. After treatment, activation of NF-κB (**C**) and JNK (**D**) was assessed by immunoblot analysis. The data in **A** and **B** are expressed as mean ± SD ($n = 3$). The data in **C** and **D** are representative of three separate experiments. **, $p < 0.01$; ***, $p < 0.001$ *vs.* treatment with rDnaK alone (**A** and **B**).

and *Klebsiella pneumoniae*, but not that from *P. aeruginosa*, showed potent dose-dependent effects on IL-1β expression [36]. In other words, LPS prepared from different bacteria can exhibit disparate effects on IL-1β expression, suggesting that the inducing effect caused by treatment with culture supernatant may not be mediated by *P. aeruginosa*-derived LPS.

Bacterial cells have two major chaperone systems known as GroEL (HSP60)-GroES (HSP10) and DnaK (HSP70)-DnaJ (HSP40)-GrpE (HSP20) [37]. We previously showed that *P. aeruginosa*-derived GroEL stimulates an inflammatory response *via* PTX3 production resulting from activation of the NF-κB pathway [38]. Based on the results shown in Fig. 1E, secreted proteins of over 50 kDa are involved in induction of IL-1β expression. In addition to GroEL, DnaK has a molecular weight of over 50 kDa and acts as a periplasmic and extracellular protein [39,40], supporting further investigation of its role in IL-1β induction. A previous study shows that *P. aeruginosa* DnaK plays a role in flagellum formation and motility by forming a complex with the flagella structural protein FliC and nitrite reductase NirS in the periplasm [39]. In addition, DnaK plays a role in bacterial motility and adherence, as well as secretion of toxins, including type III effectors, elastase, and exotoxin A [41]. Here, for the first time, we provide evidence that the *P. aeruginosa* HSP70-like protein DnaK stimulates IL-1β expression in macrophages. One explanation for the IL-1β induction following DnaK treatment is a stimulatory effect of contaminating residual endotoxin following the preparation of recombinant DnaK. To eliminate this possibility, our method for preparing rDnaK included pre-treatment with Triton X-114, which eliminates most of the residual LPS present in purified proteins, and we further verified the LPS content in a LAL endotoxin assay. The results of LAL endotoxin assay confirmed that the pre-treatment reduced the contamination to less than 0.5 ng/ml, which is insufficient to induce IL-1β expression under our experimental conditions. The LPS-free rDnaK still induced expression of IL-1β in a dose-dependent manner; most importantly, proteinase K treatment eliminated the inducing effects of rDnaK, suggesting that DnaK plays a role in induction (Fig. 2A).

In the innate immune response, HSPs promote NF-κB activation and cytokine release in macrophages by engaging signaling receptors such as TLR2 and TLR4 [42,43]. TLR4 and TLR5 but not TLR2 are required for the immune defense responses against *P. aeruginosa* infection [44]. Unlike TLR5, which senses a molecular pattern in flagellin, TLR4 can sense diverse types of intracellular molecules, including the bacterial outer membrane molecule LPS [45]. This versatility suggests that the interaction mechanisms underlying TLR4 signaling are quite diverse and may be more complicated than we understand. Monocytes and macrophages possess multiple HSP70 receptors, including TLR2, TLR4, and Siglecs [46–48]. Siglecs receptor-mediated stimulation of pro-inflammatory responses appears to be specific to human HSP70 and does not recognize *E. coli* DnaK [48], likely reflecting differences in how ligand binding is sensed. Consistent with this idea, we identified TLR4 as the rDnaK recognition receptor that controls induction of IL-1β production. However, we cannot rule out the effects of other receptors on *P. aeruginosa* DnaK-mediated induction of IL-1β.

Macrophages and neutrophils are important sources of IL-1β in response to acute *P. aeruginosa* infections. In this study, we used the human leukemia-derived monocyte THP-1 cell line, which is the most widely used cell line for *in vitro* studies investigating primary human macrophage functions. Once we found the DnaK stimulated expression of *il-1β*, we switched the target cell from A549 epithelial cells to THP-1 cells because A549 cells do not produce caspase-1 [49]. Following PMA treatment, THP-1 cells differentiate to acquire phenotypic and functional characteristics that closely resemble those of primary human macrophages [50]. IL-1β processing in macrophages requires the inflammasome and caspase-1 activation following *P. aeruginosa* exposure [18,19,51]. Consistent with this concept, rDnaK-mediated IL-1β production required active caspase-1 since it was markedly inhibited by pre-treatment with a caspase-1-specific inhibitor (Fig. 2D). Of note, we observed that IL-1β expression was also reduced after pre-treatment with a caspase-8-specific inhibitor (data not shown), indicating some influence from non-canonical inflammasome pathways that involve caspase-8 in transducing the TLR4-mediated signal to stimulate IL-1β production [52]. Unlike in macrophages, IL-1β production in neutrophils is mediated by caspase-1-independent non-canonical inflammasome pathways [53,54], indicating the involvement of diverse inflammasome pathways in the response to DnaK.

Extracellular HSP70 plays a role in inflammation by binding to TLR4, thus leading to NF-κB activation and inflammatory cytokine production [55,56]. However, it was recently shown that intracellular HSP70 regulates inflammation specifically *via* the NLRP3 inflammasome independently of the NF-κB pathway [57]. These observations reflect the diverse roles of HSP70 both in- and outside of mammalian cells. In this study, we reveal that *P. aeruginosa*-derived DnaK protein is sufficient to stimulate IL-1β expression in addition to previously identified T3SS structural proteins and effectors. As a non-self antigen released by *P. aeruginosa* cells, DnaK functions as a PAMP; however, it is a highly conserved protein similar to mammalian HSP70 protein, which can function as a DAMP, a host molecule released from damaged cells under necrotic conditions. In human macrophages, the effects of DnaK are primarily associated with recognition by TLR4 and subsequent signal transduction *via* the NF-κB and JNK signaling pathways. Further studies are necessary to address the roles of the inflammasome pathways, which appear to intensify the inflammatory response to *P. aeruginosa* DnaK during infection. Such studies should enhance our understanding of infectious diseases and provide new opportunities for their treatment.

## Declaration of Competing Interest

None.

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