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Research paper

Pneumococcal DNA-binding proteins released through autolysis induce the production of proinflammatory cytokines via toll-like receptor 4

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ABSTRACT

Streptococcus pneumoniae is a leading cause of bacterial pneumonia. Our previous study suggested that *S. pneumoniae* autolysis-dependently releases intracellular pneumolysin, which subsequently leads to lung injury. In this study, we hypothesized that pneumococcal autolysis induces the leakage of additional intracellular molecules that could increase the pathogenicity of *S. pneumoniae.* Liquid chromatography tandem-mass spectrometry analysis identified that chaperone protein DnaK, elongation factor Tu (EF-Tu), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were released with pneumococcal DNA by autolysis. We demonstrated that recombinant (r) DnaK, rEF-Tu, and rGAPDH induced significantly higher levels of interleukin-6 and tumor necrosis factor production in peritoneal macrophages and THP-1-derived macrophage-like cells via toll-like receptor 4. Furthermore, the DNA-binding activity of these proteins was confirmed by surface plasmon resonance assay. We demonstrated that pneumococcal DnaK, EF-Tu, and GAPDH induced the production of proinflammatory cytokines in macrophages, and might cause host tissue damage and affect the development of pneumococcal diseases.

1. Introduction

Streptococcus pneumoniae, also known as pneumococcus, is a Gram-positive diplococcus and major human pathogen. This bacterium asymptomatically colonizes the upper respiratory airway and causes common clinical syndromes, such as otitis media, sinusitis, bronchitis, and empyema, or even severe life-threatening diseases, including pneumonia, meningitis, and septicemia [1,2]. Pneumococcal infections have led to significant morbidity and mortality worldwide especially in children under 5 years old and adults over 65 years old in developing countries [3].

A variety of pneumococcal virulence factors, including the autolytic enzyme LytA, contribute to the development of pneumococcal diseases [4]. LytA is responsible for the characteristic autolytic behavior associated with pneumococcus. It has been reported that LytA potentially contributes to pneumococcal pathogenesis by catalyzing the release of intracellular toxins and generating proinflammatory cell wall fragments [5]. Our previous study suggested that *S. pneumoniae* autolysis-dependently releases pneumolysin (PLY), which is a cholesterol-dependent cytolytic pore-forming toxin, that induces the disruption of pulmonary immune defenses [6]. Therefore, autolysis plays a central role in the pathogenesis of pneumococcal diseases.

The innate immune response is the first line of defense against any bacterial infection. In this regard, macrophages respond immediately to diverse microbial pathogens and control the replication of invading pathogens [7,8]. Macrophages express various pattern recognition receptors, such as toll-like receptors (TLRs), which activate downstream signaling and induce the production of proinflammatory cytokines.

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TLRs play a crucial role against microbial infections, initiating and activating both host innate and adaptive immunity [9]. They recognize the presence of microbial pathogens via detection of conserved structures. It has been shown that the cell wall components of pneumococci, such as lipoteichoic acid and peptidoglycan are recognized by TLR2 [10]. TLR4 is a key component of the innate response to Gram-negative infections through the recognition of lipopolysaccharide (LPS). In addition, PLY is also considered to be a TLR4 ligand. TLR4-mutant mice are more susceptible to lethal infection after intranasal infection with pneumococcus [11]. It has also been reported that TLR9 plays an important role in the recognition of pneumococcus [12]. However, the possibility that other pneumococcal virulent factors interact with the host innate immune system and participate in pneumococcal pathogenesis remains to be explored.

In this study, we hypothesized that pneumococcal autolysis induces the leakage of additional intracellular virulent factors, which increase the pathogenicity of *S. pneumoniae*. Here, we demonstrated that chaperone protein DnaK, elongation factor Tu (EF-Tu), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were released into the pneumococcal culture supernatant by autolysis. In addition, we examined their activity of inducing the production of proinflammatory cytokines in mouse peritoneal macrophages and THP-1-derived macrophage-like cells.

2. Materials and methods

2.1. Bacterial strains and reagents

S. pneumoniae D39 (NCTC 7466) strain was purchased from the National Collection of Type Cultures (Salisbury, UK). Inactivation of *lytA* gene in *S. pneumoniae* D39 strain was performed as described previously [13]. *S. pneumoniae* was grown in tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ, USA), with 100 μg/mL spectinomycin (Sigma-Aldrich, St. Louis, MO, USA) added to the medium to allow for *lytA*-negative mutant-strain (Δ*lytA*) selection. Recombinant (r) LytA protein was kindly provided by Dr. Yuuki Sakaue (Niigata University, Niigata, Japan). Polyinosinic-polycytidylic acid [TLR3 ligand; Poly (I:C)], LPS (TLR4 ligand) from *Escherichia coli*, resiquimod (TLR7 ligand; R848), and cytosine-phosphate-guanosine oligodeoxynucleotides 1668 (TLR9 ligand; CpG ODN 1668) were purchased from InvivoGen (Toulouse, France). Antibodies against pneumococcal DnaK, EF-Tu, and GAPDH were generated by Eurofins Genomics K.K. (Tokyo, Japan). Briefly, rabbits were immunized intracutaneously with 200 μg of rDnaK, rEF-Tu, or rGAPDH emulsified with an equal volume of Freund's complete adjuvant. Two weeks later, booster immunizations were done using the same amount of each recombinant protein emulsified with incomplete Freund's adjuvant. Second booster immunizations were done 2 weeks later. Ten days after the second booster immunizations, rabbits were sacrificed and sera were extracted from blood collected by cardiac puncture. Immunoglobulin G (IgG) antibodies were purified using protein A column.

2.2. Animals

In this study, male, 8–10 weeks old 3d mice (*Unc93b1* mutant; TLR3, TLR7, and TLR9 ligand-unresponsive), its wild-type littermate control mice, C3H/HeN, C3H/HeJ mice, and BALB/c mice were used. 3d mice were obtained as described previously [14], and all other mice were purchased from Nihon CLEA (Tokyo, Japan). Mice were maintained under standard conditions in accordance with our institutional guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee of Niigata University.

2.3. Preparation of bacterial supernatants and purification of extracellular DNA

S. pneumoniae D39 and Δ*lytA* strains were grown statically in TSB at 37 °C under aerobic conditions. Overnight cultures were inoculated into fresh TSB to allow continued bacterial growth. Optical densities of 600 nm at each time points were analyzed using miniphoto518R (TAITEC, Saitama, Japan). After 6-, 12-, and 24-h incubation, bacterial supernatants were collected by centrifugation at 3000 × *g* for 10 min and cells were removed by filtration using a 0.22-μm pore size membrane filter (Merck Millipore, Billerica, MA, USA). Real-time polymerase chain reaction (PCR) was performed to quantify extracellular DNA (eDNA) in the bacterial supernatant, as described below. After 12-h incubation, eDNA was also purified from pneumococcal culture supernatants by phenol-chloroform purification and ethanol precipitation, and then stored in Tris-EDTA buffer (10 mM Tris; 0.1 mM EDTA; pH 8.0) at −20 °C until it was required for further assays.

2.4. Quantification of DNA by real-time PCR

To determine the concentration of eDNA in bacterial culture supernatants, absolute quantification in real-time PCR was performed with the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) using the SYBR Green detection protocol. Optimization of the real-time PCR reaction was performed according to the manufacturer's instructions. To prepare a standard curve, *S. pneumoniae* DNA was extracted and purified using GenElute™ bacterial genomic DNA kit (Sigma-Aldrich) according to the kit manual, and then the concentration was determined using a spectrophotometer (e-spect; Malcom, Tokyo, Japan). The primers used for real-time PCR were designed to target a fragment of PLY-encoding gene of *S. pneumoniae*, and were based on a published sequence [15]. The forward primer oligonucleotide sequence was 5′-AGCGATAGCTTTCTCCAAGTGG-3′, and the reverse primer sequence was 5′-CTTAGCCAACAAATCGTTTACCG-3′.

2.5. Cell preparation and culture

Mice were injected intraperitoneally with 4 mL of 4% thioglycolate medium (Becton Dickinson, Franklin Lakes, NJ, USA). Four days later, peritoneal macrophages were isolated by peritoneal lavage using 10 mL sterile phosphate buffered saline. The cells (1×10^5 cells/200 μL) were seeded onto a 96-well plate (Becton Dickinson) and cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Wako Pure Chemical Industries, Osaka, Japan) at 37 °C in 95% air and 5% CO_2. After 60 min, cells were washed with RPMI 1640 to remove non-adherent cells. The recovered adherent cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Japan Bio Serum, Hiroshima, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako Pure Chemical Industries).

Human monocytic cell line THP-1 (ATCC TIB-202) were obtained from RIKEN Cell Bank (Ibaraki, Japan). The cells (1×10^5 cells/200 μL) were incubated in RPMI 1640 supplemented with 200 nM phorbol 12-myristate 13-acetate (Cayman Chemical, Ann Arbor, MI, USA) to induce differentiation into macrophage-like cells at 37 °C in 95% air and 5% CO_2. After 48 h of incubation, the cells were washed with RPMI 1640 and cultured further in the medium without FBS for 24 h, and then the medium was changed to remove the cytokines induced by cell adherence. The recovered adherent cells were grown in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.6. Protein identification

Pneumococcal eDNA from the bacterial culture supernatant, purified DNA, or TSB as control was mixed with 2% sodium dodecyl sulfate (SDS) sample buffer, heated at 99 °C for 3 min, separated by SDS-

Table 1
PCR primer sequences.

Gene	Sequence (5′–3′)	Length (bp)
dnaK	Forward: GATGACGATGACAAAATGTCTAAAATTATCGGTAT Reverse: CATCCTGTTAAGCTTTTACTTTTCCGTAAACTCTC	607
tuf	Forward: GATGACGATGACAAAATGGCAAAAGAAAAATACGA Reverse: CATCCTGTTAAGCTT TTAAGCTTCGATTTCTGTAA	398
gapdh	Forward: GATGACGATGACAAAATGGTAGTTAAAGTTGGTAT Reverse: CATCCTGTTAAGCTTTTATTTAGCAATCTTTGCGA	335

polyacrylamide gel electrophoresis (PAGE) using 12.5% gels (Gellex International, Tokyo, Japan), and analyzed using Pierce™ silver stain kit (Thermo Fisher Scientific). In comparison with purified DNA and TSB, clearly stained three bands from eDNA were identified by Liquid chromatography tandem-mass spectrometry (LC-MS/MS). This analysis was performed by Japan Bio Services (Saitama, Japan).

2.7. Expression and purification of recombinant proteins

To avoid contamination of LPS, recombinant proteins were expressed in *Brevibacillus choshinensis*, which is a Gram-positive bacterium, by using *Brevibacillus* secretory expression system (TaKaRa, Shiga, Japan) [16,17] according to the manufacturer's instructions. Briefly, the pneumococcal *dnaK* gene (Ref. Seq. WP_000034662. 1), *tuf* gene (Ref. Seq. WP_001040724.1), and *gapdh* gene (Ref. Seq. WP_000260666.1) were amplified from the genomic DNA of the *S. pneumoniae* D39 strain by PCR. Sequences of all primers used in this study are listed in Table 1. The primers were designed with 15-base 5′ overhangs that were homologous to pBIC expression vector, and with sequences (20 bases) for amplifying the target gene at the 3′ ends. The resulting PCR fragments were mixed with the vector and transformed into *Brevibacillus*-competent cells. Cells were grown in medium supplemented with 50 μg/mL neomycin (Thermo Fisher scientific) for selection, after which rDnaK, rEF-Tu, and rGAPDH with an N-terminal His_6-tag were expressed in the culture supernatant and purified using an Ni-nitrilotriacetic acid column (Qiagen, Hilden, Germany). The concentration of proteins was measured by bicinchoninic acid protein assay kit (Thermo Fisher Scientific). The amount of LPS in 1 μg of purified proteins was determined to be < 2 pg according to an LPS-detection kit (GenScript, Piscataway Township, NJ, USA).

2.8. Detection of DnaK, EF-Tu, and GAPDH in pneumococcal culture supernatant

For detection of DnaK, EF-Tu, and GAPDH, the supernatants were collected from *S. pneumoniae* D39 and Δ*lytA* strains after 12-h incubation. The supernatants were then mixed with 2% SDS sample buffer, heated at 99 °C for 3 min, separated by SDS-PAGE using 12.5% gels, and transferred to polyvinylidene difluoride membranes (Merck Millipore). The membranes were incubated with blocking reagent (Nacalai Tesque, Kyoto, Japan) to inhibit non-specific binding, and probed with anti-DnaK, anti-EF-Tu, or anti-GAPDH antibody diluted in Tris-buffered saline containing 0.05% Tween 20 (TaKaRa). The membrane was then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signalling Technologies, Danvers, MA, USA) in Tris-buffered saline containing 0.05% Tween 20. The membrane was treated with HRP substrates (GE Healthcare, Buckinghamshire, UK) and analyzed by a chemiluminescence detector (Fujifilm, Tokyo, Japan). Each band was analyzed using Image Studio Lite (LI-COR Bioscience, Lincoln, NE, USA).

2.9. In vitro cytokine assay

Mouse peritoneal macrophages (1×10^5 cells/200 μL) were stimulated with pneumococcal eDNA (5 μg/mL), rDnaK (5 μg/mL), rEF-Tu (5 μg/mL), rGAPDH (5 μg/mL), Poly (I:C) (5 μg/mL), LPS (5 ng/mL), R848 (5 μg/mL), or CpG ODN 1668 (5 μg/mL). To determine whether the DNA induced immune-stimulatory activity, eDNA was pre-incubated in the presence or absence of DNase I (400 U/mL; TaKaRa) for 1 h at 37 °C followed by DNase I inactivation by incubation at 80 °C for 2 min with 0.5 M EDTA. To determine whether the immune-stimulatory activity is due to endotoxin contamination, eDNA (5 μg/mL), rDnaK (5 μg/mL), rEF-Tu (5 μg/mL), rGAPDH (5 μg/mL), and LPS (5 ng/mL) were pre-incubated in the presence or absence of proteinase K (20 μg/mL; Wako Pure Chemical Industries) for 2 h at 37 °C followed by proteinase K inactivation by boiling for 5 min. The mouse peritoneal macrophages were then incubated in the presence of these stimulants for 6 h. The concentrations of interleukin (IL)-6 and tumor necrosis factor (TNF) in the culture supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kit (Biolegend, San Diego, CA, USA) according to the manufacturer's protocol.

THP-1 macrophages (1×10^5 cells/200 μL) were also stimulated with pneumococcal eDNA (5 μg/mL), rDnaK (5 μg/mL), rEF-Tu (5 μg/mL), rGAPDH (5 μg/mL), or LPS (5 ng/mL) for 6 h. Then, the concentrations of IL-6 and TNF in the culture supernatants were determined using ELISA kit (Biolegend) according to the manufacturer's protocol.

2.10. Immunofluorescence analysis

THP-1 macrophages treated with rDnaK, rEF-Tu, or rGAPDH for 6 h were fixed using 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries), followed by incubation of the cells in a blocking solution (Thermo Fisher Scientific) for 30 min. For rDnaK, rEF-Tu, and rGAPDH detection, samples were stained with rabbit anti-DnaK, anti-EF-Tu, or anti-GAPDH antibody in the blocking solution. After overnight incubation at 4 °C, the secondary AlexaFluor 594-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific) in blocking buffer was added, followed by a 2-h incubation in the dark. For TLR4 detection, samples were stained with mouse anti-TLR4 antibodies (HTA125; Abcam, Cambridge, UK). After overnight incubation at 4 °C, the secondary AlexaFluor 488-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific) was added, followed by incubation for 2 h. Samples were then observed with a confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

2.11. Estimation of DNA-binding activity of the proteins by surface plasmon resonance assay

The molecular basis of the binding of rDnaK, rEF-Tu, and rGAPDH to pneumococcal DNA was investigated using surface plasmon resonance (SPR)-based binding techniques. SPR measurements were performed using Biacore X100 instrument (GE Healthcare) at 25 °C. As the running buffer, 100 mM HEPES containing 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P 20 was used at a flow rate of 10 μL/min. Purified pneumococcal DNA was digested with micrococcal nuclease (TaKaRa) at 37 °C for 30 min. The digested DNA was labeled with biotin using biotin 3′ end DNA labeling kit (Thermo Fisher Scientific). Biotin-labeled DNA (10 μg) was dissolved in 500 mM NaCl running buffer and immobilized on SA sensor chip (GE Healthcare) by streptavidin-biotin interaction. Bovine serum albumin (BSA; 100 μg/mL; Sigma-Aldrich), rDnaK (100 μg/mL), rEF-Tu (100 μg/mL), and rGAPDH (100 μg/mL) were loaded on the chip using running buffer. Regeneration of the sensor chip surface was achieved by 30-s pulse of 50 mM NaOH. The data was analyzed by Biacore X100 evaluation software (GE Healthcare).

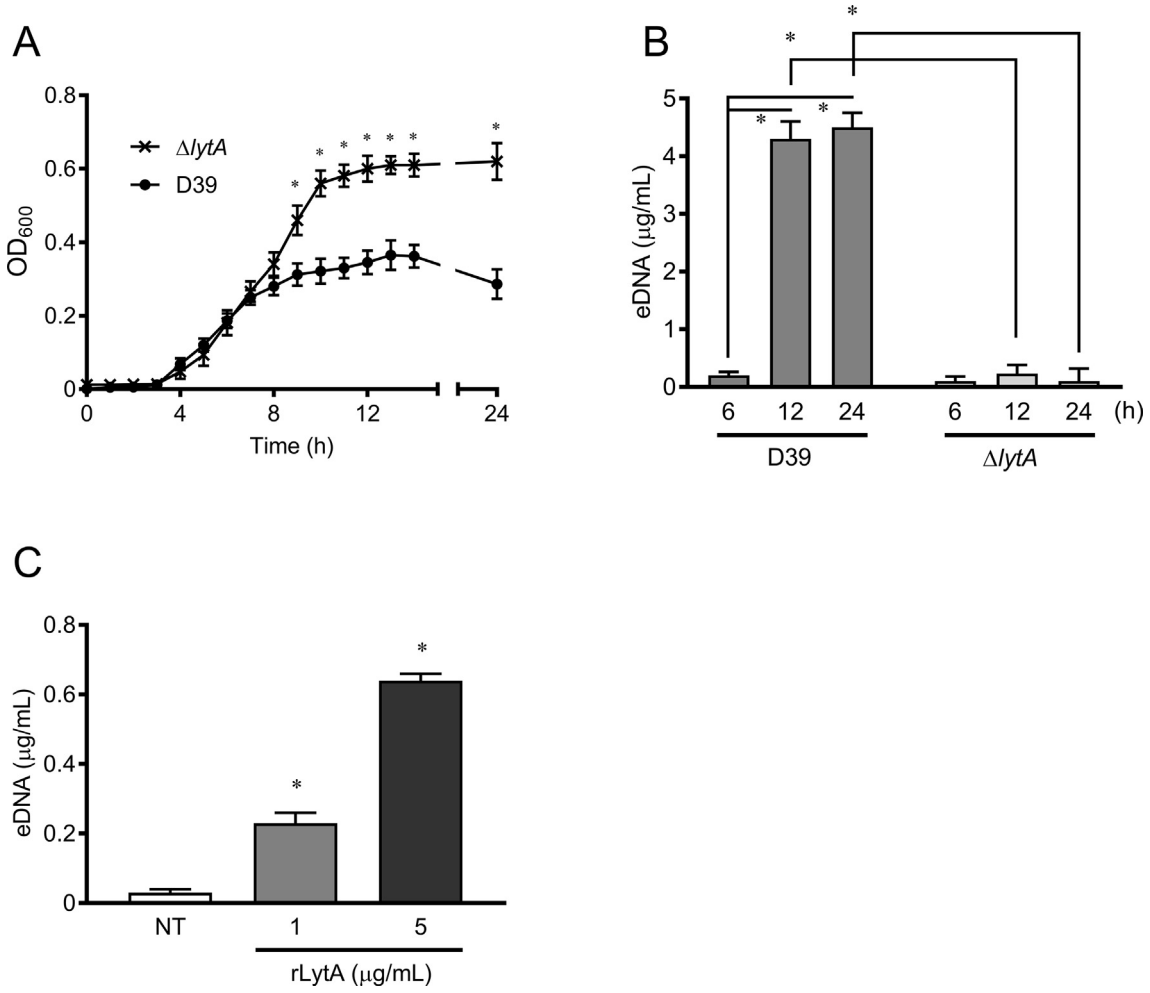

Fig. 1. Pneumococcal autolysis causes DNA release. (A) *S. pneumoniae* D39 and Δ*lytA* strains were grown statically in TSB at 37 °C under aerobic conditions. Overnight cultures were inoculated into fresh TSB to allow continued bacterial growth. Optical densities of 600 nm at each time points were analyzed. (B) *S. pneumoniae* D39 and Δ*lytA* strains were grown statically in TSB for 6, 12, and 24 h. Pneumococcal eDNA in the culture supernatant was then quantified by real-time PCR. (C) One or five micrograms per mL of rLytA was added to *S. pneumoniae* Δ*lytA* culture during the stationary phase and incubated for an additional 12 h. Real-time PCR was performed to quantify pneumococcal eDNA in the culture supernatant. Data are shown as the mean ± SD of quadruplicate determinants, and compared using Student's *t*-test (A), one-way analysis of variance with Tukey's multiple-comparisons test (B), and Dunnett's multiple-comparisons test (C). $^{*}p < .05$ was considered statistically significant.

2.12. Statistical analysis

All data represent the means ± standard deviation (SD) of quadruplicate determinants and were evaluated using GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA, USA). Values of $p < .05$ were considered statistically significant.

3. Results

3.1. S. pneumoniae autolysis causes DNA leakage into the bacterial supernatant

We first investigated whether pneumococcal autolysis can cause DNA leakage into the bacterial supernatant. Fig. 1A shows that both *S. pneumoniae* D39 and Δ*lytA* (autolytic enzyme gene *lytA*-negative mutant) strains reached a stationary growth phase after a 12-h incubation. The optical density of *S. pneumoniae* D39 strain was significantly lower than that of Δ*lytA*, which was due to *S. pneumoniae* D39 autolysis. Fig. 1B shows that significantly higher level of eDNA (extracellular DNA) was released into the culture supernatant of *S. pneumoniae* D39 as compared with that of *S. pneumoniae* Δ*lytA* after 12- and 24-h incubation. However, no significant difference in the level of eDNA released into the culture supernatant was observed between *S. pneumoniae* D39 and Δ*lytA* after a 6-h incubation. Additionally, treatment with rLytA induced eDNA release from *S. pneumoniae* Δ*lytA* in a dose-dependent manner (Fig. 1C). These findings suggest that autolysis of *S. pneumoniae* D39 strain occurs after reaching a stationary growth phase followed by DNA release into the bacterial supernatant.

3.2. eDNA induces IL-6 production in a TLR3-, TLR7-, and TLR9-independent manner

We next investigated whether eDNA shows proinflammatory activity. Fig. 2 shows that eDNA induced IL-6 production in mouse peritoneal macrophages derived from wild-type littermate control mice, whereas DNase I-digested eDNA also induced equal levels of IL-6 production compared with untreated eDNA. To determine whether eDNA causes IL-6 production via TLR3, TLR7, or TLR9, peritoneal macrophages derived from 3d mice (TLR3, TLR7, and TLR9 ligand-unresponsive) were stimulated with eDNA. However, eDNA induced IL-6 production in peritoneal macrophages derived from 3d mice. Therefore, pneumococcal eDNA induced IL-6 production in a TLR3-, TLR7-, and TLR9-independent manner.

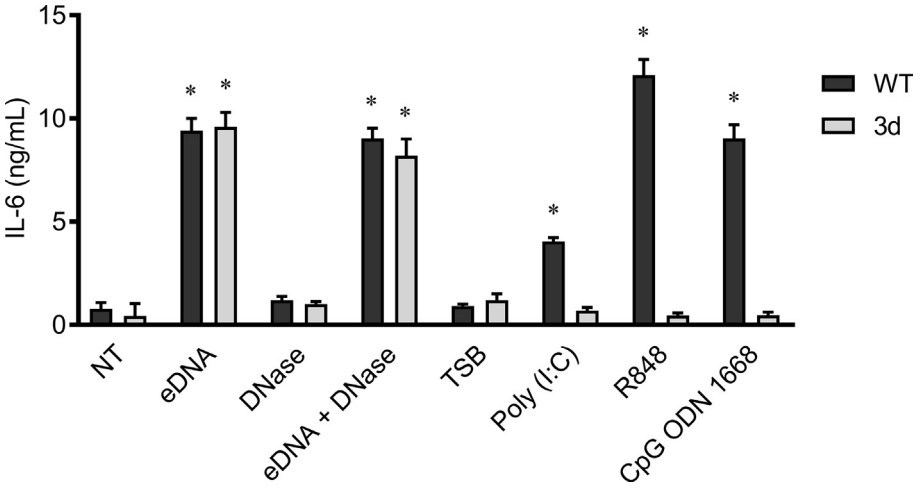

Fig. 2. Pneumococcal eDNA induces IL-6 production in mouse peritoneal macrophages Peritoneal macrophages (1×10^5 cells/200 μL) derived from 3d mice and its wild-type littermate control mice were stimulated with 5 μg/mL of eDNA, DNase I-digested eDNA, TSB, Poly (I:C) (TLR3 ligand), R848 (TLR7 ligand), or CpG ODN 1668 (TLR9 ligand) for 6 h at 37 °C. The concentration of IL-6 in cell culture supernatants was determined by ELISA. Data are shown as the mean ± SD of quadruplicate determinants. The group means were compared using one-way analysis of variance with Dunnett's multiple-comparisons test compared with non-treated (NT) group. $^{*}p < .05$ was considered statistically significant.

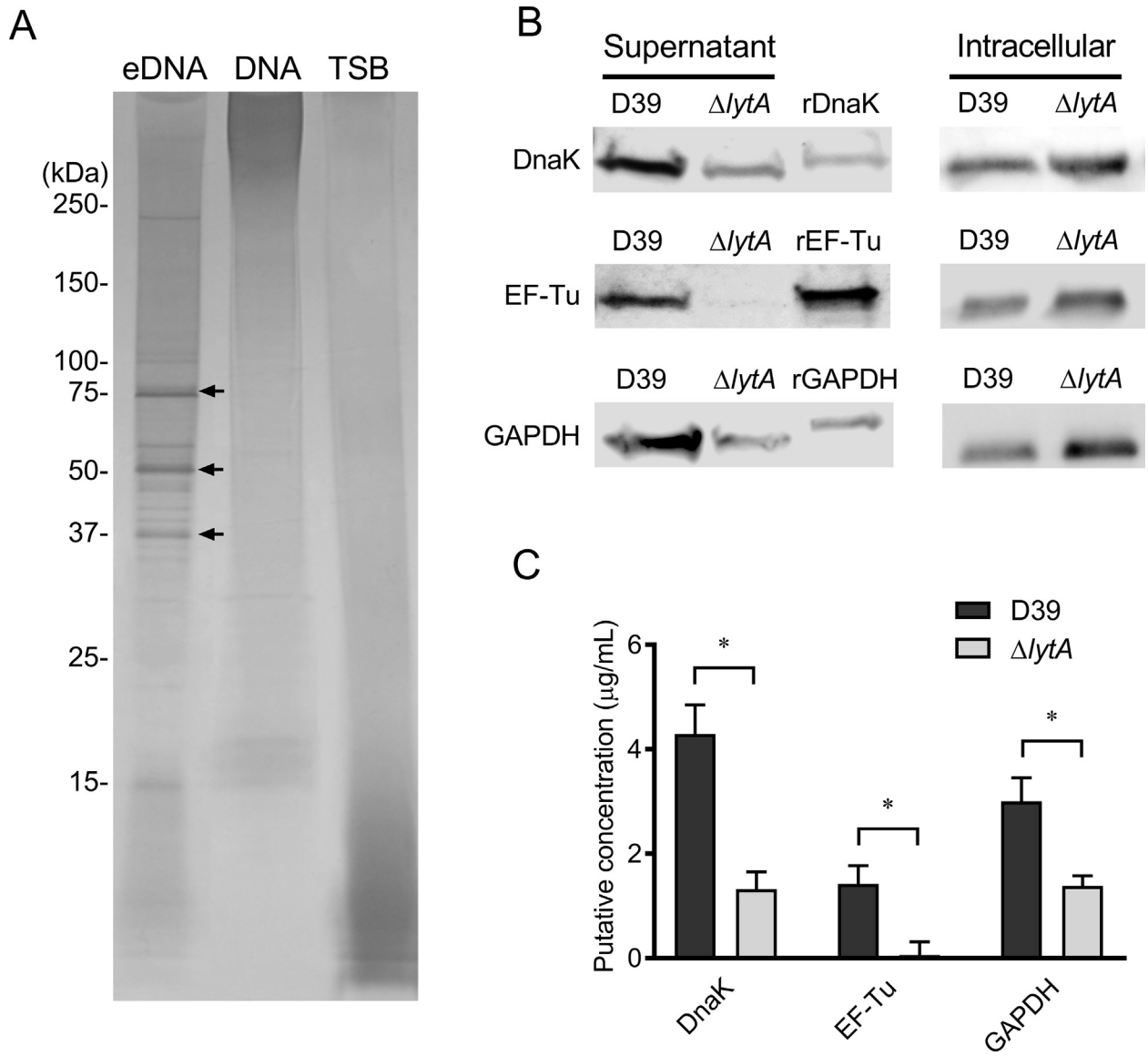

Fig. 3. *S. pneumoniae* autolysis causes DnaK, EF-Tu, and GAPDH release. (A) Silver-stained bands of pneumococcal eDNA from the culture supernatant, purified DNA, and TSB on SDS-PAGE gels. Arrows indicate DnaK, EF-Tu, and GAPDH in order from largest to smallest, which were identified by LC-MS/MS. (B) Intracellular and extracellular (culture supernatant) expression of DnaK, EF-Tu, and GAPDH were analyzed by western blotting. (C) Putative concentration of DnaK, EF-Tu, and GAPDH in the culture supernatant was analyzed from the intensity of western blotting signals by densitometry as compared with that of recombinant proteins. Data are shown as the mean ± SD of quadruplicate determinants. The group means were compared using Student's *t*-test. $^{*}p < .05$ was considered statistically significant.

Table 2
LC-MS/MS data.

Identified protein	Accession No.	Mass	Score	Number of peptides matched
DnaK	COE13114	65269	153	10
EF-Tu	COH15957	65273	48	4
GAPDH	CVY04434	34931	146	5

3.3. Identification of DNA-binding proteins

We hypothesized that eDNA included some proteins in the process of autolysis. Therefore, we performed SDS-PAGE for the separation of eDNA and identified 3 silver-stained bands, which were analyzed by LC-MS/MS. DnaK, EF-Tu, and GAPDH were identified in that order from the top (Fig. 3A, Table 2). *S. pneumoniae* D39 culture supernatant exhibited significantly higher levels of these protein s as compared with that of Δ*lytA* after a 12-h incubation by western blotting (Fig. 3B and C). These findings suggest that both pneumococcal DNA and these proteins were released by autolysis. Additionally, Fig. S1 shows that *S. pneumoniae* strain 12F7 (serotype 12F), which was clinically isolated from patient with invasive pneumococcal disease, also released these proteins in the culture supernatant, whereas its *lytA* mutant derivative (12F7Δ*lytA*) culture supernatant exhibited significantly lower levels of these proteins. These findings suggested that autolysis-dependent release of these proteins is not limited to *S. pneumoniae* D39 strain.

3.4. rDnaK, rEF-Tu, and rGAPDH induce the production of proinflammatory cytokines via TLR4

It has been reported that DnaK from *Francisella tularensis*, EF-Tu from *Francisella novicida*, and GAPDH from *S. pneumoniae* induces proinflammatory activation dependent on TLR4 [18–20]. Therefore, we investigated whether rDnaK, rEF-Tu, and rGAPDH activate the production of proinflammatory cytokines in mouse peritoneal

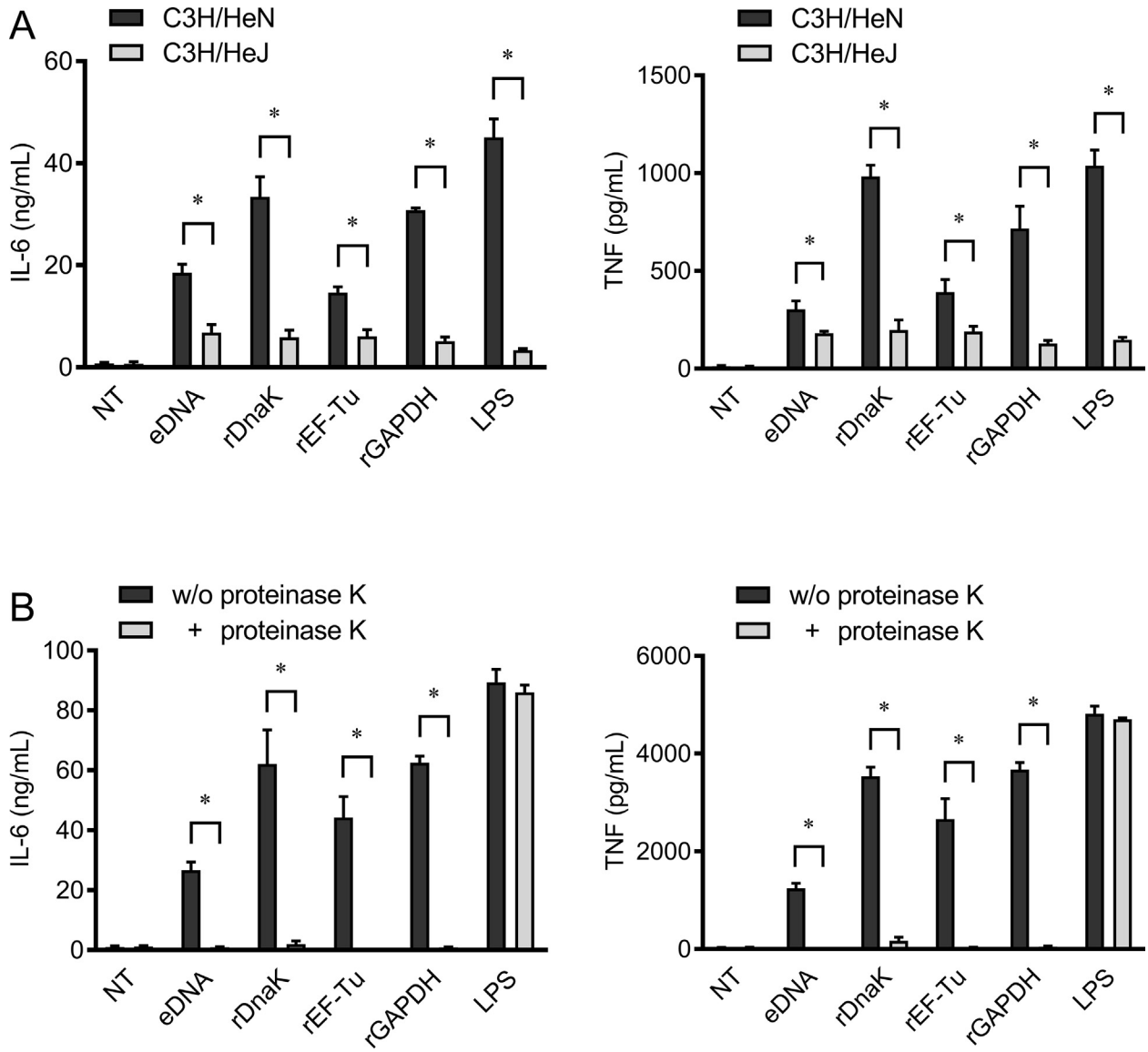

Fig. 4. DNA-binding proteins induce the production of proinflammatory cytokines in mouse peritoneal macrophages. (A) Peritoneal macrophages (1×10^5 cells/200 μL) derived from C3H/HeN or C3H/HeJ mice were stimulated with eDNA (5 μg/mL), rDnak (5 μg/mL), rEF-Tu (5 μg/mL), rGAPDH (5 μg/mL), or LPS (5 ng/mL) for 6 h at 37 °C. The concentrations of IL-6 and TNF in cell culture supernatants were determined by ELISA. **(B)** eDNA (5 μg/mL), rDnaK (5 μg/mL), rEF-Tu (5 μg/mL), rGAPDH (5 μg/mL), and LPS (5 ng/mL) were incubated in the presence or absence of proteinase K (20 μg/mL) for 2 h at 37 °C. Thereafter, the samples were used as stimulants against BALB/c mouse peritoneal macrophages (1×10^5 cells/200 μL) after a 6-h incubation. The concentrations of IL-6 and TNF in cell culture supernatants were determined by ELISA. Data are shown as the mean ± SD of quadruplicate determinants. The group means were compared using Mann-Whitney's *U* test. $^*p < .05$ was considered statistically significant.

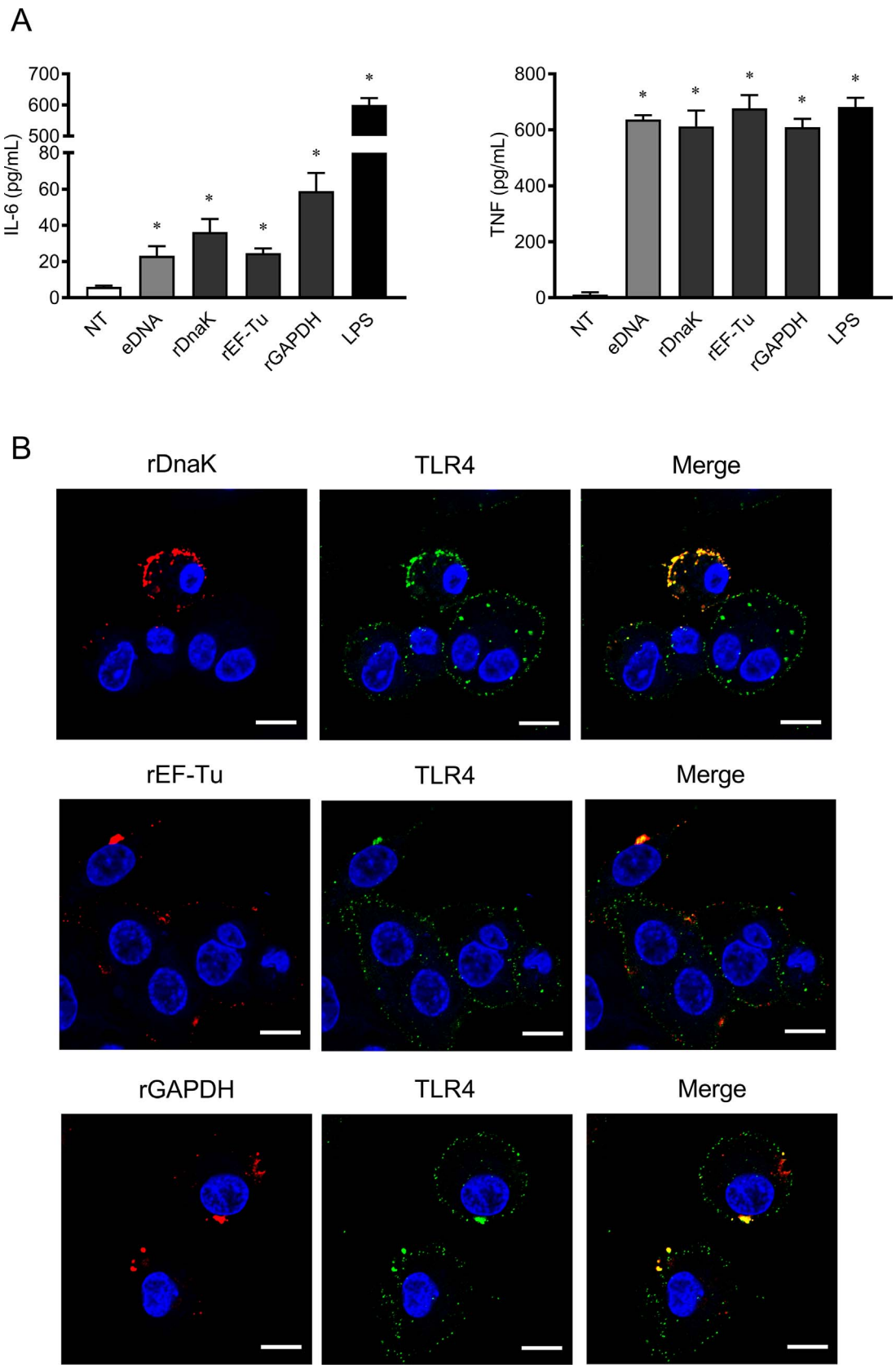

Fig. 5. DNA-binding proteins induce the production of proinflammatory cytokines in THP-1 macrophages. THP-1 macrophages (1×10^5 cells/200 μL) were stimulated with pneumococcal eDNA (5 μg/mL), rDnaK (5 μg/mL), rEF-Tu (5 μg/mL), rGAPDH (5 μg/mL), or LPS (5 ng/mL) for 6 h. (A) The concentrations of IL-6 and TNF in the culture supernatants were determined using ELISA. Data are shown as the mean ± SD of quadruplicate determinants and were evaluated using one-way analysis of variance with Dunnett's multiple-comparisons test compared with non-treated (NT) group. (B) Representative fluorescence-microscopy images of THP-1 macrophages stained for DNA (DAPI; blue), recombinant proteins (red), and TLR4 (green). Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

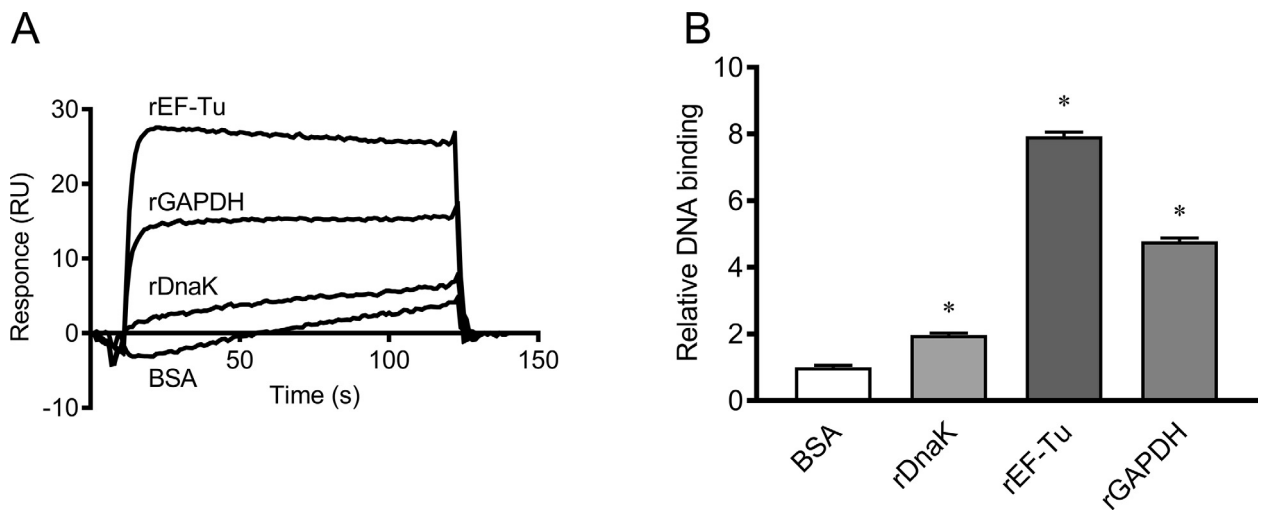

Fig. 6. Binding activity of proteins to pneumococcal DNA. (A) The molecular basis of the binding of rDnaK, rEF-Tu, and rGAPDH to pneumococcal DNA was investigated using SPR-based binding techniques with Biacore X100. (B) Relative binding activity to pneumococcal DNA compared with BSA. Data are shown as the mean ± SD of quadruplicate determinants. The group means were compared using one-way analysis of variance with Dunnett's multiple-comparisons test. $^{*}p < .05$ was considered statistically significant.

macrophages and THP-1 macrophages. Fig. 4A shows that eDNA, rDnaK, rEF-Tu, and rGAPDH induced IL-6 and TNF production in TLR4-susceptible C3H/HeN peritoneal macrophages. However, peritoneal macrophages derived from TLR4-defective C3H/HeJ mice produced low levels of IL-6 and TNF. Fig. 4B shows that peritoneal macrophages treated with eDNA, rDnaK, rEF-Tu, and rGAPDH digested by proteinase K also produced low levels of IL-6 and TNF. Taken together, eDNA and these pneumococcal proteins induced the production of proinflammatory cytokines via TLR4 without endotoxin contamination. Additionally, eDNA and these proteins also induced IL-6 and TNF production in THP-1 macrophages and immunofluorescence confocal microscopy analyses showed that rDnaK, rEF-Tu, and rGAPDH were colocalized with TLR4 on the surface of THP-1 macrophages (Fig. 5A and B).

3.5. rDnaK, rEF-Tu, and rGAPDH bind pneumococcal DNA

We confirmed whether rDnaK, rEF-Tu, and rGAPDH bind pneumococcal DNA using SPR analysis. Biacore experiments indicated that rDnaK, rEF-Tu, and rGAPDH bound stronger to pneumococcal DNA than to BSA (Fig. 6A and B).

4. Discussion

In this study, we showed that pneumococcal autolysis induces DNA leakage. Although eDNA itself did not induce the production of proinflammatory cytokines in mouse peritoneal macrophages, DnaK, EF-Tu, and GAPDH were released as DNA-binding proteins and induced the production of proinflammatory cytokines in mouse peritoneal macrophages and THP-1 macrophages via TLR4.

LytA is an enzyme that degrades the peptidoglycan backbone of bacterial organisms [21], leading to bacterial autolysis. This enzyme is located in the cytoplasm and is involved in a variety of other physiological cell functions [22]. We observed that pneumococcal autolysis induces DNA leakage in the culture supernatant. Therefore, we first hypothesized that pneumococcal eDNA has a proinflammatory activity which contributes to its pathogenicity.

Bacterial DNA has inflammatory properties resulting from the presence of unmethylated CpG motifs. Unmethylated CpG motifs have been shown to be recognized by TLR9 [23]. It has been reported that TLR9 plays an important role in host protection at an early stage of pulmonary infection of *S. pneumoniae* [24]. However, in this study, pneumococcal eDNA induced the production of proinflammatory cytokines in peritoneal macrophages derived from 3d mice, indicating means that eDNA does not activate TLR3, TLR7, or TLR9. Considering that these TLRs are located and act within the endosomes [25], it can be interpreted that mouse peritoneal macrophages do not uptake and recognize pneumococcal DNA within the endosomes. It has been reported that bacterial DNA uptake in macrophages depends on its length, which means that increasing the length of DNA enhances endocytic uptake activity [26]. In this study, it is suspected that eDNA was digested into small fragments before or after release into the culture supernatant, and could not activate uptake activity against the macrophages.

Reportedly, bacterial heat shock protein, which has an activity of binding DNA, was able to activate TLR2 and TLR4 [27,28]. Therefore, we next hypothesized that some DNA-binding proteins induce the production of proinflammatory cytokines in mouse peritoneal macrophages. Here, we found for the first time and demonstrated that DnaK, EF-Tu, and GAPDH, along with pneumococcal DNA were released into the culture supernatant by autolysis. DnaK is a member of heat shock proteins, which are highly conserved, stress-inducible, and ubiquitous proteins that maintain homeostasis in both eukaryotes and prokaryotes [29,30]. EF-Tu belongs to the group of G-proteins and plays a crucial role in the elongation cycle of translation as a universal carrier of aminoacyl-tRNA from the cytosol of the cell to the A site of the ribosome [31,32]. GAPDH is a key enzyme in the glycolytic pathway, catalyzing the conversion of glyceraldehyde-3-phosphate to d-glycerate 1, 3-bisphospate, in the presence of nicotinamide adenine dinucleotide (NAD^+) and inorganic phosphate, and then mediates formation of NADH and adenosine triphosphate [33,34]. It has been demonstrated that DnaK, EF-Tu, and GAPDH have DNA-binding activity [35–37]. Consistent with this, rDnaK, rEF-Tu, and rGAPDH bound to pneumococcal DNA. Further investigation is needed to determine how DnaK, EF-Tu, and GAPDH interact with pneumococcal DNA in the process of autolysis, and to identify the DNA-binding sites of DnaK, EF-Tu, and GAPDH.

Cytokines play an important role in host defense against *S. pneumoniae*. IL-6 protects the host from pneumococcal infection through reducing the growth of pneumococci and prolonging survival of mice [38]. TNF also contributes to inhibiting the growth and transmission of pneumococci [39]. However, it has been suggested that high serum levels of IL-6 and TNF are associated with early death in patients of sepsis caused from severe pneumonia [40]. Excessive inflammatory reaction, known as cytokine storm, occurs when the host immune response becomes hyperactive against bacterial infections. Several experimental studies and clinical trials have suggested that cytokine

storm correlated directly with tissue injury and an unfavorable prognosis of severe pneumonia [41,42]. Further study is needed to explore whether pneumococcal DnaK, EF-Tu, and GAPDH released by autolysis into the blood stream are associated with extreme production of proinflammatory cytokines in severe pneumococcal pneumonia.

In conclusion, our present study demonstrated that pneumococcal DNA was released by autolysis. Detection of pneumococcal DNA in the sputum or blood by means of PCR may facilitate early diagnosis of pneumonia and sepsis. In addition, we proved that DnaK, EF-Tu, and GAPDH activate host defense responses, and might cause host tissue damage and affect the development of pneumococcal diseases. Furthermore, the immune-stimulatory potential of these proteins makes them attractive candidates for devising protective measures against this deadly infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2018.01.006.

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