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Staphylococcus aureus lipoproteins augment inflammatory responses in poly I:C-primed macrophages

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ABSTRACT

Secondary bacterial infection contributes to severe inflammation following viral infection. Among foodborne pathogenic bacteria, *Staphylococcus aureus* is known to exacerbate severe inflammatory responses after infection with single-stranded RNA viruses such as influenza viruses. However, it has not been determined if *S. aureus* infection enhances inflammatory responses after infection with RNA enteric viruses, including rotavirus, which is a double-stranded RNA virus. We therefore investigated the molecular mechanisms by which a cell wall component of *S. aureus* enhanced inflammatory responses during enteric viral infection using poly I:C-primed macrophages, which is a well-established model for double-stranded RNA virus infection. *S. aureus* lipoproteins enhanced IL-6 as well as TNF-α production in poly I:C-primed macrophages. Pam2CSK4, a mimic of Gram-positive bacterial lipoproteins and *S. aureus* lipoproteins, also significantly enhanced IL-6 production in poly I:C-primed macrophages. While IFN-β expression was increased in poly I:C-primed macrophages treated with Pam2CSK4 or *S. aureus* lipoproteins, the level of IL-6 enhancement in poly I:C-primed macrophages was decreased in the presence of anti-IFN-α/β receptor antibody, suggesting that IFN-β plays an important role in enhanced IL-6 production. Phosphatidylinositol-3-kinase, Akt, ERK and NF-κB were also involved in the enhanced IL-6 production. Collectively, these results suggest that *S. aureus* lipoproteins induce excessive inflammatory responses in the presence of poly I:C.

1. Introduction

Foodborne disease, caused by ingestion of contaminated food or drink, is a prevalent clinical problem. Over 250 different foodborne diseases are associated with various pathogens including fungi, bacteria, viruses, and parasites [1]. A recent study reported that 19,542 infections, 4,445 hospitalizations, and 71 deaths due to foodborne disease occurred in the United States [2]. In 2005, approximately 2 million deaths worldwide occurred due to gastrointestinal illness caused by microbial foodborne infections [3]. Different foodborne pathogens evoke acute and chronic inflammatory responses of different intensities. For example, *Staphylococcus aureus*, which can contaminate food products during food preparation and processing, is a causative agent of serious acute and chronic infections [4,5].

Staphylococcal enteritis is considered one of the most common causes of foodborne diseases and is caused by *S. aureus* enterotoxins [5]. *S. aureus* also contains several virulence factors that contribute to inflammatory responses in the gut [6]. Cell wall components of *S. aureus* such as lipoproteins, lipoteichoic acid, and peptidoglycan stimulate immune responses through the activation of their cognate receptors [7]. Although the activation of Toll-like receptors (TLRs) plays a protective role in microbial infections, it is often associated with the development of inflammation [8,9]. Among the cell wall components of *S. aureus*, lipoproteins are essential for interleukin (IL)-8 production in human intestinal epithelial cells [6]. In addition to bacteria, norovirus, hepatitis A and E viruses, astroviruses, and rotavirus, which replicate in host cells, are an important cause of foodborne diseases [10]. The nucleic acid of viruses can be either DNA or RNA, positive or negative in polarity, and single-stranded or double-stranded [11]. TLRs play an important role in inflammatory responses to viral nucleic acids. For example, TLR3-dependent inflammatory responses are mediated by double-stranded RNA from viruses including rotavirus, while TLR7 and TLR8 usually recognize single-stranded viral RNA [12,13].

Co-infection or secondary bacterial infection often causes severe

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morbidity and mortality [14]. Influenza associated with secondary bacterial infections has been extensively studied, because influenza virus infection is one of the largest disease burdens in humans [15]. In terms of concurrent infections with foodborne bacteria and viruses, a recent study reported that intestinal bacteria promote enteric virus infections [16]. Moreover, it was demonstrated that *Campylobacter* infection is often associated with enteric viral infections that result in severe inflammatory diarrhea [17]. However, the molecular basis of the association between foodborne pathogenic bacteria and viral infections is not yet understood. Therefore, in this study, we investigated the pathogenic mechanism for excessive inflammatory responses caused by a foodborne disease-related bacterium and a virus in macrophages.

2. Materials and methods

2.1. Bacteria and reagents

S. aureus RN4220, a lipoprotein-deficient mutant (Δ*lgt*), a complemented strain of Δ*lgt* harboring the p*Slgt* plasmid (p*Slgt*), and a lipoteichoic acid-deficient mutant (Δ*ltaS*) were used in this study [18]. Lipoproteins of *S. aureus* RN4220 and mutant strains were isolated as described previously [6]. Poly I:C, a synthetic analog of viral double-stranded RNA, was obtained from InvivoGen (San Diego, CA, USA). A mimic of Gram-positive bacterial lipoproteins, Pam2CSK4, was purchased from EMC Microcollections (Tuebingen, Germany). Anti-mouse IFN-α/β receptor antibody was purchased from BD Pharmingen (Franklin Lakes, NJ, USA). LY294002 was obtained from Sigma-Aldrich (St. Louis, MO, USA). U0126 and JAK inhibitor were purchased from Calbiochem (La Jolla, CA, USA). Antibodies specific for Akt, phospho-Akt, ERK, phospho-ERK, IκBα, phospho-IκBα, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2. Preparation of killed bacteria

Ethanol-inactivated *S. aureus* RN4220 and all other mutant strains were prepared as described previously [19]. Bacterial strains were grown in Luria Bertani medium (BD Biosciences, San Jose, CA, USA) at 37 °C to mid-log phase. After washing with phosphate-buffered saline (PBS), bacterial cells were suspended in 70% ethanol for 3 h with shaking. Then, bacterial cells were washed extensively in PBS, adjusted to the appropriate concentration, and stored at −20 °C until use.

2.3. Culture of RAW 264.7 cells

The mouse macrophage cell line, RAW 264.7 (TIB-71), was obtained from the American Type Culture for Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Burlington, ON, Canada), 100 U/ml penicillin, and 100 μg/ml streptomycin (HyClone, Logan, UT, USA) in a 5% CO_2-humidified incubator.

2.4. Preparation and culture of primary macrophages

Animal ethics approval for all experiments was obtained from the Institutional Animal Care and Use Committee of Seoul National University (SNU-140512-6-2). After intraperitoneal injection of 3% Brewer thioglycollate medium (Sigma-Aldrich), primary peritoneal macrophages of C57BL/6 mice were collected by injecting the peritoneal cavity with 10 ml of cold PBS, massaging, and drawing the fluid back into the syringe at day 3 post-thioglycollate infection. After removal of erythrocytes using a Red Blood Lysis Buffer (Sigma-Aldrich), peritoneal macrophages were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol at 37 °C in a 5% CO_2-humidified incubator.

2.5. Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells or peritoneal macrophages (3×10^5 cells/ml) were plated on a culture plate and pre-treated with poly I:C (10 μg/ml) for 18 h. Cells were then treated with the indicated stimuli for an additional 24 h and cell culture supernatants were collected. IL-6 and TNF-α productions were determined using commercial IL-6 and TNF-α ELISA kits (R&D Systems, Minneapolis, MN, USA), respectively.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instruction and was then reverse-transcribed to complementary DNA (cDNA) with random hexamers and reverse transcriptase (Promega, Madison, WI, USA). PCR was performed to amplify cDNA in 20 μl reaction volume with rTaq DNA polymerase (0.5 unit) and 10 pmol of primers specific for murine interferon (IFN)-β (5′-TGAGGACATCTCCCACGTCAA-3′ and 5′-TCCAAG AAAGGACGAACATTCG-3′) or β-actin (5′-GTGGGGCGCCCCAGGCA CCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′). PCR amplification of IFN-β was conducted as follows: an initial denaturation step at 94 °C for 5 min; 30 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s, and 72 °C for 7 min. PCR amplification of β-actin was conducted as follows: an initial denaturation step at 95 °C for 5 min; 25 cycles of 5 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min. Amplified PCR products were visualized on a 1.5% agarose gel by staining with ethidium bromide.

2.7. Western blot analysis

RAW 264.7 cells (3×10^5 cells/ml) were plated on a 6-well culture plate and pre-treated with poly I:C (10 μg/ml) for 18 h. After pre-treatment, cells were treated with the indicated stimuli for 30 min and then washed with cold PBS and lysed in PRO-PREP lysis buffer (iNtRON Biotechnology, Seongnam, Korea). Equal amounts of whole cell lysates were resolved on 10% SDS-PAGE gels and electro-transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were incubated with blocking buffer at room temperature for 1 h and further incubated with blocking buffer containing specific antibodies against Akt, phospho-Akt, ERK, phospho-ERK, IκBα, phospho-IκBα, or β-actin overnight at 4 °C. Subsequently, the membranes were washed and incubated with HRP-conjugated anti-rabbit IgG in blocking buffer for 1 h. Immunoreactive bands were detected with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). Images were captured with a gel documentation system (Bio-Rad, Hercules, CA, USA) as described previously [20].

2.8. Statistical analysis

Treatment groups were compared with appropriate control groups. Statistical significance was measured using unpaired two tailed *t*-test and differences were considered significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. S. aureus lipoproteins are essential for the augmentation of inflammatory responses in poly I:C-primed macrophages

It is known that *S. aureus* lipoproteins are associated with increased expression of TNF-α, IL-6, and IL-8 in inflammatory responses [6,21]. Fig. 1A illustrates that ethanol-inactivated *S. aureus* wild-type strain (*S. aureus* WT), but not a lipoprotein-deficient *S. aureus* strain (*S. aureus* Δ*lgt*), significantly induced IL-6 production in RAW 264.7 cells. Moreover, a complemented *S. aureus* Δ*lgt* strain (p*Slgt*) showed restored IL-6