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The role of phagocytosis in IL-8 production by human monocytes in response to lipoproteins on *Staphylococcus aureus*

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ABSTRACT

Cytokine responses to microbes are triggered by pattern recognition receptors, such as Toll-like receptors (TLRs), which sense pathogen-associated molecular patterns. Cell wall-associated triacylated lipoproteins in *Staphylococcus aureus* are known to be native TLR2 ligands that mediate host inflammatory responses against *S. aureus*. However, the mechanism by which these lipidated lipoproteins, which are buried under the thick *S. aureus* cell wall, work to stimulate TLR2 remains unclear. Heat-killed wild type *S. aureus* cells activated human monocytic THP-1 cells to produce proinflammatory cytokines, including interleukin (IL)-8, whereas the lipoprotein lipidation-deficient *lgt* mutant induced less than an eighth of the amount of IL-8 induced by the wild type. IL-8 induction in response to heat-killed *S. aureus* cells in THP-1 cells was not inhibited by a blocking antibody against cell surface TLR2, suggesting that intracellular TLR2 might be involved in the induction of IL-8 by *S. aureus* lipoprotein. The relationship between phagocytosis and IL-8 production in THP-1 cells was analyzed on a single-cell level by flow cytometry using fluorescein-labeled *S. aureus* cells and phycoerythrin-labeled anti-IL-8 antibody. Production of intracellular IL-8 was correlated with phagocytosis of *S. aureus* cells in THP-1 cells and in human peripheral blood mononuclear cells. Opsonization of *S. aureus* cells enhanced both the phagocytosis of *S. aureus* cells and the production of intracellular IL-8 in THP-1 cells. These results suggest that lipidated lipoproteins on *S. aureus* cells stimulate human monocytes after phagocytosis.

1. Introduction

The innate immune system, which is crucial for host defense against microbial infection, relies on humoral effectors, such as cytokines and complement proteins and cellular immune responses, such as phagocytosis [1]. Phagocytosis is the process by which phagocytic cells recognize non-self and self particles, such as infectious agents and apoptotic cells, and induce receptor-mediated internalization of these particles [2]. Particles engulfed by phagocytic cells are delivered into an intracellular organelle called the phagosome, which is remodeled by fusion and fission with endosomes and lysosomes to generate a bactericidal phagolysosome [3]. The phagolysosome has the capacity to enzymatically digest the internalized contents into individual molecules and plays key roles in both innate and adaptive immunity, leading to pathogen killing and antigen presentation, respectively [3–5].

Phagocytic cells modulate innate immune responses through the production of proinflammatory cytokines in response to invading microbes; this response is triggered by germline-encoded pattern-recognition receptors (PRRs) [6]. The Toll-like receptors (TLRs), a family of mammalian PRRs, are type I integral transmembrane glycoproteins with extracellular domains containing leucine-rich repeats, which recognize microbial products, and a cytoplasmic Toll/interleukin (IL)-1 receptor homology domain, which transfers signals leading to the production of proinflammatory cytokines [7]. Some TLRs (e.g. TLR1, TLR2, TLR4, TLR5 and TLR6) are expressed on the cell surface and recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and flagellin from Gram-negative bacteria and lipoprotein from Gram-positive bacteria, whereas TLR7, TLR8 and TLR9 are exclusively found within intracellular endosomes and mainly sense microbial nucleic acids. Even though the TLR2/TLR1 and TLR2/TLR6 complexes that sense

Abbreviations: PRR, pattern-recognition receptor; TLR, Toll-like receptor; IL, interleukin; PAMP, pathogen-associated molecular pattern; LPS, lipopolysaccharide; Lgt, lipoprotein diacylglycerol transferase; Lnt, lipoprotein *N*-acyl transferase; Δ*lgt*, *lgt* gene deletion; PBS, phosphate buffered saline; PBMC, peripheral blood mononuclear cell; FBS, fetal bovine serum; TNF, tumor necrotizing factor; MOI, multiplicity of infection; PE, phycoerythrin; MFI, mean fluorescence intensities.

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diacylated lipoproteins or triacylated lipoproteins, respectively, are known to be expressed on the phagocytic cell surface [8], these TLR2 complexes are also reported to be recruited to macrophage phagosomes, where they discriminate pathogens and induce pro-inflammatory signals for host defense [9–11].

Bacterial lipoproteins are synthesized as precursors with an N-terminal signal peptide and translocated into cell membranes by the Sec machinery [12]. The first enzyme for bacterial lipoprotein biosynthesis, lipoprotein diacylglycerol transferase (Lgt), catalyzes the transfer of a diacylglycerol group to a cysteine residue in the signal peptide of the lipoprotein precursor. The second enzyme, lipoprotein signal peptidase (Lsp), cleaves the N-terminal signal peptide at the cysteine residue, and the third enzyme, lipoprotein *N*-acyl transferase (Lnt), transfers an acyl group to the α-amino group of the cysteine residue, resulting in a triacylated lipoprotein. Whereas lipoproteins in Gram-positive *Staphylococcus aureus* were thought to be diacylated, due to the absence of an *Escherichia coli*-type Lnt, we recently found that *S. aureus* lipoproteins are *N*-acylated, and, thus, triacylated [13,14]. In the *lgt* gene deletion (Δ*lgt*) mutant bacteria, lipoprotein processing is blocked, resulting in the accumulation of precursor lipoproteins [15,16]. Whereas wild type *S. aureus* induces cytokine production in macrophages and is also capable of inducing inflammatory responses *in vivo*, Δ*lgt* mutant *S. aureus* fails to induce cytokine responses [15,16]. Recently, we demonstrated that staphylococcal cell wall-associated lipoprotein, SitC, functions as a native ligand of cell surface-expressed TLR2 [13]. However, it is not known how lipoproteins on the *S. aureus* cell surface are efficiently recognized by TLR2. In this study, we found that cytokine responses in human monocytes induced by mature lipoproteins embedded under the *S. aureus* cell wall occurred after phagocytosis. The cytokine response to *S. aureus* is enhanced by an increase in intracellular bacterial uptake at a single-cell level and this phagocytosis-coupled cytokine response is mainly attributable to staphylococcal lipoprotein.

2. Materials and methods

2.1. Bacteria and human monocytic cells

S. aureus parental RN4220 and Δ*lgt*::pM*lgt* mutant organisms [13] were grown in LB broth (USB Co., Cleveland, OH) at 37 °C overnight with constant shaking. The cells were washed with sterile phosphate buffered saline (PBS), counted in a bacterial counting chamber (depth 0.02 mm; Erma, Tokyo, Japan) and adjusted to the appropriate concentration in the appropriate medium for each assay. THP-1 cells, a human monocytic cell line obtained from the American Type Culture Collection, and human peripheral blood mononuclear cells (PBMCs) isolated from heparinized venous blood of healthy volunteers by density centrifugation on Histopaque (Sigma–Aldrich, St. Louis, MO) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and used as responder cells.

2.2. Preparation of heat-killed S. aureus cells and supernatants for cell stimulation assays

Each *S. aureus* culture was grown in LB broth overnight, inoculated into fresh LB broth and incubated for 3 h to reach the mid-exponential phase of growth. The cells were washed with sterile PBS at 10,000 *g* for 10 min at 4 °C, counted, adjusted to the appropriate concentration and heat-killed at 80 °C for 30 min before being used to stimulate monocytes. To prepare opsonized and non-opsonized bacteria, 35 μl of each serially-diluted suspension of heat-killed bacteria was added to 315 μl of either 50% fresh human serum in Ca^{++} and Mg^{++} enriched gelatin veronal buffer or buffer alone, and the mixtures were incubated at 37 °C for 2 h. The supernatants incubated with bacteria were collected by centrifugation at 10,000 *g* for 10 min at 4 °C, filtered through pyrogen-free round filters (pore size, 0.2 μm; Advantec, Tokyo, Japan), and used for the stimulation of monocytes as a source of free lipoproteins. The sedimented bacteria were resuspended in the original volume of RPMI 1640 (Invitrogen, Carlsbad, CA) and also used for the stimulation of monocytes as opsonized (incubated in 50% fresh human serum) and non-opsonized bacteria (incubated in buffer alone). Newly prepared heat-killed *S. aureus* cells were used for the monocyte stimulation assays.

2.3. Monocyte stimulation and cytokine measurement

THP-1 cells were seeded into each well of 96-well plate at a density of 2×10^4 cells/well in 50 μl of RPMI 1640 and stimulated with 150 μl of prepared heat-killed bacterial cells for 18 h. The supernatants were then harvested, and the concentration of cytokines IL-8, IL-1β, IL-6, IL-10, tumor necrotizing factor (TNF) and IL-12p70 were screened using a human inflammatory cytokine kit with the BD™ cytometric bead array system (BD Biosciences, San Joes, CA). In some experiments, IL-8 concentrations were measured by ELISA (R&D Systems, Inc.) according to the manufacturer's instructions. To clarify the effect of blocking TLR2 on the cell surface, 1 μg/ml murine anti-TLR2 monoclonal antibody or isotype control antibody (eBioscience, Inc., San Diego, CA) was added to each well containing THP-1 cells before stimulation with heat-killed wild type *S. aureus* cells or wild type bacterial supernatant.

2.4. Phagocytosis and intracellular IL-8 detection

To investigate the correlation between internalized bacterial load and IL-8 production, we measured the extent of phagocytosis and intracellular IL-8 at a single-cell level using flow cytometry. Each suspension of wild type or Δ*lgt* mutant *S. aureus* with or without opsonization was fluorescently labeled by incubating the suspension with 1 μM *Bac*Light Green Bacterial Stain™ (Invitrogen) for 15 min; excess dye was removed by washing three times with RPMI 1640. Labeling efficiencies of heat-killed wild type or Δ*lgt* mutant *S. aureus* cells were compared by measuring the total fluorescence intensity of labeled bacterial suspensions at the same concentration using a spectrofluorometer (RF-5301PC, Shimadzu Corp., Japan). THP-1 cells (5×10^5 cells) or PBMCs (1×10^6 cells) were incubated with fluorescein-labeled bacteria at a multiplicity of infection (MOI) of 100 in RPMI 1640 containing 0.1% BD GolgiStop™ (BD Biosciences, San Jose, CA) at 37 °C for 6 h. After incubation, the cells were washed with sterile PBS containing 1% bovine albumin, treated with BD Cytofix/Cytoperm™ (BD Biosciences) according to the manufacturer's instructions and stained with phycoerythrin (PE)-labeled murine anti-human IL-8 antibody (BD Biosciences) for 1 h. After washing, the cells were fixed in paraformaldehyde and analyzed with a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using CellQuest Pro software (BD Biosciences).

2.5. Statistics

Data are expressed as the mean and standard error of the mean of at least three repeated experiments. Differences between groups were compared using a one-way analysis of variance with post hoc testing (Student–Newman–Keuls test). *P*-values less than 0.05 were considered statistically significant.