International Immunopharmacology 50 (2017) 61–68

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

# Roles of serum in innate immune responses of human leukocytes to synthetic lipopeptide

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ARTICLE INFO

*Keywords:*
LPS binding protein
Neutrophil
$Pam_3CSK_4$
Priming
Respiratory burst
Serum

ABSTRACT

Tripalmitoyl-*S*-glyceryl-L-Cys-Ser-$(Lys)_4$ ($Pam_3CSK_4$) is a highly conserved molecular motif found in various classes of lipoproteins. The requirement for leukocyte to respond to synthetic $Pam_3CSK_4$ were studied. $Pam_3CSK_4$ primed neutrophils for a respiratory burst in a serum-dependent manner. $Pam_3CSK_4$ upregulated CD11b, CD14, and cytochrome $b_{558}$, and downregulated Leu-8. Treatment of neutrophils with anti-CD14 antibodies and treatment of serum with anti-LPS binding protein (LBP) antibodies resulted in the inhibition of priming for respiratory burst by $Pam_3CSK_4$. It should be noted that LBP could not replicate the effects of serum in priming of neutrophils for respiratory burst by $Pam_3CSK_4$. Serum LBP bound to immobilized $Pam_3CSK_4$. $Pam_3CSK_4$ induced the interleukin-8 (IL-8) production by leukocytes in a serum-dependent manner. Further, $Pam_3CSK_4$-induced priming of neutrophils for respiratory burst was not inhibited by the LPS antagonists LA-14-PP, *Rhodobacter sphaeroides* LPS, or E5531, and $Pam_3CSK_4$-induced IL-8 production by leukocytes was not affected by LPS antagonist, E5531, indicating that $Pam_3CSK_4$ was recognized by a different receptor than LPS. Thus, $Pam_3CSK_4$ and LPS had similar biological activities and similar requirement to act on leukocytes, but were recognized by different receptors. Serum in the action of $Pam_3CSK_4$ on leukocytes was not replicated by LBP, suggesting that $Pam_3CSK_4$ might be disaggregated by serum to result in the activation of leukocytes.

## 1. Introduction

Outer membrane of gram negative bacteria contains many lipoproteins that have been shown to be the causative agent of diseases such as outer surface proteins of *Borrelia* in Lyme disease. Understanding the role of lipoproteins in pathogenesis of diseases provides novel therapeutic targets of the diseases [5]. Lipoproteins have been shown to elicit a strong host innate inflammatory response [5]. Tripalmitoyl-*S*-glyceryl-L-Cys-Ser-$(Lys)_4$ ($Pam_3CSK_4$) is a highly conserved molecular motif found in lipoproteins of various classes of bacteria [5]. The activation of immune cells by lipopolysaccharide (LPS) involves LPS binding protein (LBP) and CD14 molecules; LBP binds LPS and catalyzes the transfer of LPS to CD14 [37]. Recently, we reported that disaggregation is essential for LBP to support the action of LPS on neutrophils and that disaggregation is mediated by serum, serum albumin, or high-density lipoprotein [15]. Albumin-mediated disaggregation of LPS has been reported to result in LBP-mediated formation of a CD14-LPS complex [10]. Toll-like receptor (TLR) 4 is the central transmembrane component of the LPS receptor, and TLR2 has been shown to mediate the recognition of bacterial components, including lipoprotein or lipoteichoic acid [24]. It has been reported that an allergen, German cockroach frass induces TLR2-mediated activation of neutrophils [23]. Lipoprotein and lipopeptide act on neutrophils, resulting in mobilization of granules and priming for a subsequent respiratory burst [11,17,22,32,33,36]. Lipoprotein-induced activation of neutrophils or monocytes has been shown to be CD14-dependent [16,29,30,32,36].

There is inconsistency in the requirement for serum or LBP in the action of lipoproteins or lipopeptide on leukocytes [11,17,22,29,30,32,33,36]. Previous reports have indicated that cell activation by lipoproteins is serum-independent [17,22,32,36], whereas synthetic lipopeptides require serum or LBP [25,27]. It has been reported that $Pam_3CSK_4$ primes mononuclear cells for an IL-1β and TNF-α response of a lower magnitude compared to LPS in the presence of serum [26]. Although human filarial *Wolbachia* synthetic lipopeptide has been shown to activate human neutrophils in a serum-

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http://dx.doi.org/10.1016/j.intimp.2017.06.006
Received 17 March 2017; Received in revised form 8 June 2017; Accepted 9 June 2017
Available online 19 June 2017

independent manner [33], to our knowledge, requirement for serum in neutrophil responses to $Pam_3CSK_4$ have not been studied. LBP has been shown to mediate the binding of lipopeptide to CD14 to induce the production of cytokines by monocytes [27]. In TLR1/TLR2-transfected cells, LBP and CD14 independently mediated formation of a lipoprotein- or $Pam_3CSK_4$-TLR1/TLR2 complex that induced cell activation [25].

In addition, $Pam_3CSK_4$-induced activation via transfected TLR2 was facilitated by exogenous soluble CD14 [12,21,34], through mediation of the formation of a $Pam_3CSK_4$-TLR2/TLR1 complex [21,34]. As shown with LPS [10,15], lipopeptide is reported to be highly aggregated, and biological activity of lipopetptide critically depends on solubilization by treatment with a detergent and dilution in a solution containing bovine serum albumin (BSA) or serum [35].

In the present study, we showed that $Pam_3CSK_4$ primed neutrophils for enhanced respiratory burst, upregulated CD11b, CD14, and cytochrome $b_{558}$, and downregulated Leu-8 in a serum-dependent manner. Priming for enhanced respiratory burst by $Pam_3CSK_4$ was inhibited by anti-CD14 antibodies or anti-LBP antibodies; however, the effects of serum could not be replicated by LBP, suggesting that $Pam_3CSK_4$ required factors in addition to LBP to act on neutrophils. $Pam_3CSK_4$-induced production of cytokines by leukocytes was serum-dependent. Leukocyte responses to $Pam_3CSK_4$ were not modulated by LPS antagonists. Thus, $Pam_3CSK_4$ and LPS had similar biological activities, with similar requirements to mediate activation of leukocytes, but were recognized by different receptors. Possible requirement for disaggregation of $Pam_3CSK_4$ by serum in the action of the lipopeptide on leukocytes was discussed.

## 2. Materials and methods

### 2.1. Reagents

$Pam_3CSK_4$ was purchased from EMD Biosciences, Inc. (La Jolla, CA). LPS from *Escherichia coli* K235 [18] was a gift from Floyd McIntire (University of Colorado, Denver). A synthetic lipid A precursor (LA-14-PP) was purchased from Daiichi Pure Chemicals (Tokyo, Japan). LPS from *Rhodobacter sphaeroides* was purchased from List Biological Laboratories Inc. (Campbell, CA). E5531 was a gift from Eisai Co. Ltd. (Tokyo, Japan). Cytochrome c, BSA and Histopaque 1077 were purchased from Sigma Chemical Co. (St. Louis, MO). N-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Vega Biochemical (Tucson, AZ). Dextran (molecular weight 200,000–300,000, LPS-free) was purchased from ICN Biomedicals Inc. (Aurora, OH). Recombinant human LBP and mouse anti-human LBP monoclonal antibody (mAb 2B5) were gifts from P. *S. Tobias* (Scripps Research Institute, La Jolla, CA). Rabbit anti-human LBP antibody was purchased from Novus Biologicals USA (Littleton, CO). Type IV collagen was obtained from Koken (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-conjugated anti-Leu-8 mAb was purchased from Becton Dickinson (San Jose, CA). FITC-conjugated anti-CD11b mAb (Bear-1) was purchased from SanBio (Mountain View, CA). Anti-CD14 mAb (MY4) and FITC-conjugated MY4 were purchased from Coulter Immunology (Hialeah, FL). Anti-cytochrome $b_{558}$ (7D5) mAb was a gift from M. Nakamura (Nagasaki University) [19]. FITC-labeled goat anti-mouse immunoglobulin G (IgG) was purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). Anti-IL-8 mAb, rabbit anti-IL-8 antibody, IL-8 standard, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from R & D systems (Minneapolis, MN).

### 2.2. Preparation of neutrophils and mononuclear cells

The use of human leukocytes from adult healthy volunteers was approved by the Research Ethics Committee of the Kyushu University. Neutrophils and mononuclear cells were obtained from the freshly drawn venous blood of volunteers, as described [1]. Citrate was used as an anticoagulant. Neutrophils were isolated by dextran sedimentation, followed by Histopaque centrifugation. Erythrocytes were removed by hypotonic lysis. Plasma proteins and hemoglobin were removed by centrifugation of the neutrophils through 20% Histopaque in phosphate buffered saline (PBS). Neutrophils were suspended in PBS, pH 7.2 at $5 \times 10^6$ cells/ml. Neutrophil preparations contained > 96% neutrophils and 0.1% monocytes. Mononuclear cells were recovered from the interface between PBS and Histopaque, washed twice with PBS, and suspended in PBS at $5 \times 10^6$ cells/ml. Mononuclear cells contained 28% monocytes [2].

### 2.3. Incubation of neutrophils with $Pam_3CSK_4$ or LPS

Neutrophils ($1.25 \times 10^5$ cells in 50 μl) were incubated with $Pam_3CSK_4$ or LPS in type IV collagen-coated polystyrene tubes at 37 °C for 30 min [20]. Polystyrene tubes were coated with 30 μg/ml of type IV collagen for 2 h at room temperature and were washed twice with PBS. Collagen was used to prevent activation of neutrophils by foreign surfaces.

### 2.4. Measurement of neutrophil priming for fMLP-stimulated release of superoxide anion ($O_2^-$)

Generation of $O_2^-$ was measured by the superoxide dismutase-inhibitable reduction of cytochrome *c*, as described [1]. After incubation with agents at 37 °C for 30 min, neutrophils were stimulated with 1 μM fMLP in the presence of 20 μM cytochrome *c* at 37 °C for 7 min. After incubation with fMLP, the cell suspensions were centrifuged at 3000 × *g* for 5 min, and the absorbance of each supernatant was determined. The height of the peak at 550 nm, which was due to reduced cytochrome *c*, was determined with reference to the isosbestic points at 542 nm and 556 nm. The extinction coefficient of 0.021 $\mu M^{-1}$ was used to calculate the total amount of $O_2^-$ released.

### 2.5. Expression of CD11b, CD14, Leu-8, or cytochrome $b_{558}$

Neutrophils ($1.25 \times 10^5$ cells in 50 μl) were incubated with FITC-conjugated mAb, anti-CD11b, anti-CD14, or anti-Leu8, at 2 μg/ml for each antibody, in an ice bath for 30 min. Expression of cytochrome $b_{558}$ was determined using mAb 7D5 [14,19]. Neutrophils were incubated on ice for 30 min with 7D5 (at 1:1000 of concentrated ascites). The cells were then washed with ice-cold PBS and incubated for 30 min with FITC-conjugated goat anti-mouse IgG (5 μg/ml). Antibody binding was analyzed for fluorescence intensity using an Ortho Cytoron flow cytometer (Ortho Diagnostic Systems, Westwood, MA). A total of 5000–10,000 gated cells were analyzed, and the results are expressed as mean channel of fluorescence intensity (MFI) [4,14,20].

### 2.6. Binding of LBP to $Pam_3CSK_4$ or LPS

Polystyrene tubes were coated with $Pam_3CSK_4$ (1 μg/ml) or LPS (1 μg/ml) for 2 h and washed twice with PBS. PBS or serum (5% in PBS) was placed in $Pam_3CSK_4$- or LPS-coated tubes for 2 h at room temperature. After washing three times, bound LBP was detected by incubating tubes with anti-LBP antibody (100 ng/ml of 1% BSA) for 1 h, and then horseradish peroxidase-conjugated goat anti-mouse IgG [4].

### 2.7. Measurement of cytokine production

Neutrophils ($2 \times 10^6$ cells/ml) or mononuclear cells ($1 \times 10^6$ cells/ml) were cultured with test samples in RPMI1640 medium supplemented with or without 5% serum in a 5% $CO_2$ humidified atmosphere at 37 °C for 16 h in polystyrene tubes or microplates. After culture, cells were removed by centrifugation at 3000 × *g* for 5 min and IL-1β, TNF-α, or IL-8 in the supernatant was determined by sandwich enzyme linked immunosorbent assay.