Original Paper

Pam3CSK4 Induces MMP-9 Expression in Human Monocytic THP-1 Cells

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Key Words

Pam3CSK4 • MMP-9 • TLR2 • THP-1 cells

Abstract

Background: Matrix metalloproteinase (MMP)-9 is known to degrade the extracellular matrix and increased MMP-9 levels are related with the pathogenesis of many inflammatory conditions including obesity. Pam3CSK4 is a synthetic triacylated lipopeptide (LP) which is a potent activator of immune cells and induces cytokine production. However, it is unclear whether Pam3CSK4 is able to induce MMP-9 expression in monocytic cells. We, therefore, determined MMP-9 production by Pam3CSK4-treated THP-1 cells and also investigated the signal transduction pathway(s) involved. **Methods:** MMP-9 expression was determined by real-time qPCR and ELISA. MMP-9 activity was assessed by zymography. THP-1 cells, THP1-XBlue™ cells, THP1-XBlue™-defMyD cells, anti-TLR2 mAb and selective pharmacological inhibitors were used to study signaling pathways involved. Phosphorylated and total proteins were detected by western blotting. **Results:** Pam3CSK4 induced MMP-9 expression ($P < 0.05$) at both mRNA and protein levels in human monocytic THP-1 cells. Increased NF- κ B/AP-1 activity was detected in Pam3CSK4-treated THP-1 cells and MMP-9 production in these cells was significantly suppressed by pre-treatment with anti-TLR2 neutralizing antibody or by inhibition of clathrin-dependent endocytosis. Also, MyD88-/- THP-1 cells did not express MMP-9 following treatment with Pam3CSK4. Inhibition of JNK, MEK/ERK, p38 MAPK and NF- κ B significantly suppressed MMP-9 gene expression ($P < 0.05$). **Conclusion:** Pam3CSK4 induces MMP-9 production in THP-1 cells through the TLR-2/MyD88-dependent mechanism involving MEK/ERK, JNK, p38 MAPK and NF- κ B/AP-1 activation.

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Introduction

Matrix metalloproteinases (MMPs) are metal-dependent proteolytic enzymes that degrade components of the extracellular matrix (ECM) [1]. MMP family proteins are involved in important processes such as cell migration, proliferation, and apoptosis [2–4]. So far

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known, five main groups of MMPs include: gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), stromelysins (MMP-3, MMP-10, and MMP-11), membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and matrilysins (MMP-7 and MMP-26) [5]. The expression and activity of several MMPs have been addressed in many inflammatory disorders including obesity, arthritis, multiple sclerosis, CNS inflammation and cancer [6-10]. MMP-9 is produced by monocytes/macrophages at the sites of chronic inflammation such as arthritic areas, atherosclerosis, and periodontal disease. The expression of MMP-9 by inflammatory or M1-type macrophages exacerbates the inflammatory response by ECM degradation and recruitment of T-helper (Th)-17 cells and neutrophils leading to substantial tissue damage [11, 12]. The activity of MMPs is tightly regulated at levels of gene expression, spatial localization, and proenzyme activation. MMP-9 expression is dependent on the activation of innate immune Toll-like receptors (TLRs) which are pattern recognition receptors highly expressed on immune cells [13]. TLR binding to pathogen-associated molecular patterns (PAMPs) triggers signaling via myeloid differentiation (MyD)-88 downstream adaptor protein to activate a signaling cascade leading to the activation of mitogen-activated protein kinases (MAPKs) and transcription factors (NF- κ B and AP-1). The activation of transcriptional factors leads to the expression of proinflammatory cytokines as well as MMP production [14-16].

The proinflammatory cytokines TNF- α , IL-1 β , and IL-18 activate MMP-9 gene expression in monocytic cells [17, 18]. We earlier reported that palmitate, a saturated free fatty acid found in obesity, induced TLR4-dependent activation of MMP-9 gene expression through the mechanism requiring MyD88 recruitment and activation of NF- κ B/AP-1 transcription factors [19]. It is well known that bacterial components induce and regulate MMP-9 production in immunocytes. Peptidoglycan, a major cell wall component of gram positive bacteria, increases MMP-9 expression during injury and sepsis [20]. Lipopolysaccharide (LPS), cell wall component of gram negative bacteria, induces several MMPs including MMP-2 and MMP-9 in monocytes [21, 22]. We recently found that FSL-1 (Fibroblast-stimulating lipopeptide-1; S-(2, 3 bispalmitoyloxypropyl)-CGDPKHSPKSF) which is a synthetic diacylated lipopeptide mimicking a 44kDa lipoprotein of *Mycoplasma salivarium*, induced MMP-9 gene expression in monocytic cells through activation of MAPK pathway and the regulatory mechanism involving NF- κ B/AP-1 transcription factors [23]. We earlier reported that heat-killed *Listeria monocytogenes* (HKLM) activated MMP-9 gene expression in human monocytic THP-1 cells via the activation of TLR2/MAPK/NF- κ B pathways [24].

The synthetic bacterial triacylated lipopeptide Pam3-Cys-Ser-Lys4 (Pam3CSK4) is a potent activator of the proinflammatory transcription factor NF- κ B. Pam3CSK4 was shown to augment interferon- γ -induced nitric oxide production in mouse vascular endothelial cells via a physical association between IFN- γ R α and MyD88 [25]. However, the role of Pam3CSK4 in regulating MMP-9 induction in monocytic cells remains to be elucidated. Herein, we show that Pam3CSK4 activates MMP-9 gene expression in THP-1 monocytic cells through the TLR-2/MyD88-dependent mechanism involving activation of MEK1/2/ERK, JNK, p38 MAPK and NF- κ B/AP-1 transcription factors.

Materials and Methods

Cell Culture

Human monocytic leukemia THP-1 cell line was purchased from American Type Culture Collection (ATCC) and grown in RPMI-1640 culture medium (Gibco, Life Technologies, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY, USA), 2mM glutamine (Gibco, Invitrogen, Grand Island, NY, USA), 1mM sodium pyruvate, 10mM HEPES, 100 μ g/ml Normocin 50 U/ml penicillin and 50 μ g/ml streptomycin (P/S; (Gibco, Invitrogen, Grand Island, NY, USA), and incubation at 37°C (with humidity) in 5% CO₂. THP-1-XBlue cells stably expressing a secreted embryonic alkaline phosphatase (SEAP) reporter inducible by NF- κ B and AP-1 were purchased from InvivoGen (InvivoGen, San Diego, CA, USA). THP-1-XBlue™-defMyD cells (MyD88-/- THP-1 cells) were also purchased from InvivoGen

(InvivoGen, San Diego, CA, USA). THP-1-XBlue cells were cultured in complete RPMI medium containing Zeocin (200µg/ml) (InvivoGen, San Diego, CA, USA) to select for cells expressing SEAP-NF-κB/AP-1 reporter. THP-1-XBlue™-defMyD cells were cultured in complete RPMI medium containing Zeocin (200µg/ml) and HygroGold (100µg/ml) (InvivoGen, San Diego, CA, USA). Prior to stimulation, THP-1 cells were transferred into normal medium and plated in 12-well plates (Costar, Corning Incorporated, Corning, NY, USA) at 1×10^6 cells/well cell density unless indicated otherwise.

Cell stimulation

THP-1 cells cultured in 12-well plates (Costar, Corning Incorporated, Corning, NY, USA) at a cell density of 1×10^6 cells/well were stimulated with Pam3CSK4 (InvivoGen, San Diego, CA, USA) (200ng/ml) or TNF-α (10ng/ml) for 24hr at 37°C. Cells were harvested for RNA isolation and conditioned media were collected and stored at -80°C until use for measuring secretory MMP-9 protein and SEAP activity.

Measurement of NF-κB/AP-1 activity

THP-1 XBlue cells (InvivoGen, San Diego, CA) are THP-1 cells stably transfected with a reporter construct expressing SEAP under the control of a promoter inducible by NF-κB and AP-1 transcription factors. Following stimulation, NF-κB and AP-1 are activated and promote the secretion of SEAP in supernatant. THP-1 XBlue cells were stimulated with Pam3CSK4 (200ng/ml) or TNF-α (10ng/ml) used as a positive control for 24hr at 37°C. SEAP levels were detected in conditioned media after 4 hr incubation of supernatants with Quanti-Blue medium (InvivoGen, San Diego, CA, USA) analyzed by ELISA reader at 650nm wave length.

Real Time Quantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The cDNA was synthesized using 1µg of total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, CA, USA). For real-time PCR, cDNA template (50ng) was amplified using Inventoried TaqMan Gene Expression Assay products (MMP-9: Hs00234579_m1; GAPDH: Hs03929097_g1) containing two gene-specific primers and one TaqMan MGB probe (6-FAM dye-labeled) with a TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster city) and a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The mRNA levels were normalized against GAPDH mRNA and MMP-9 mRNA expression relative to control was calculated using $2^{-\Delta\Delta}$ Ct-method [20]. Relative mRNA expression was shown as fold expression over average of control gene expression. MMP-9 gene expression level in control treatment was taken as 1 and the data were presented as mean±SEM values. The results were analyzed statistically and $P < 0.05$ was considered significant.

Quantification of secretory MMP-9

MMP-9 secreted protein in the supernatants of THP-1 cells stimulated with Pam3CSK4 or TNF-α was quantified using sandwich ELISA following the manufacturer's instructions (R&D systems, Minneapolis, USA).

Gelatin zymography

THP-1 cells were incubated with Pam3CSK4 and TNF-α. After incubation for 24 hrs, conditioned media were collected and mixed with Zymogram sample buffer, BioRad (62.5mM Tris-HCl, pH 6.8, 25% glycerol, 4%SDS and 0.01% bromophenol blue) and loaded on to a 10% polyacrylamide gel with gelatin (10% Ready Gel® Zymogram Gel, Biorad) for electrophoresis. The gel was incubated with renaturing buffer, BioRad (2.5% Triton X-100) for 1 hour at room temperature and incubated with zymogram developing buffer, BioRad (50mM Tris-HCl, pH 7.5, 200mM NaCl and 5mM CaCl₂) for 24 hrs at 37°C. Gels were stained with staining solution (0.5% Coomassie Brilliant Blue R-250, 40% Methanol, 10% Acetic Acid) for 2 hrs and then destained with destaining solution (40% Methanol, 10% Acetic Acid) until the bands appeared. Proteolytic activity was indicated as clear bands against the black background of stained gel.

Western blotting

THP-1 cells were harvested and incubated for 30 min with lysis buffer (Tris 62.5 mM (pH 7.5), 1% Triton X-100, 10% glycerol). The lysates were then centrifuged at 14000×g for 10 min and the supernatants were

collected. Protein concentration in lysates was measured by Quickstart Bradford Dye Reagent, 1x Protein Assay kit (Bio-Rad Laboratories, Inc, CA). Protein (20 µg) samples were mixed with sample loading buffer, heated for 5 min at 95°C and resolved by 12% SDS-PAGE. Cellular proteins were transferred to Immuno-Blot PVDF membrane (Bio-Rad Laboratories, USA) by electroblotting. The membranes were then blocked with 5% non-fat milk in PBS for 1h, followed by incubation with primary antibodies against p-ERK1/2, p-SAPK/JNK, p-p38, p-c-Jun, p-IKKα/β, p-IKB, p-NF-κB and β-actin in 1:1000 dilution at 4°C overnight. All primary antibodies were purchased from Cell Signaling (Cell Signaling Technology, Inc). The blots were then washed four times with TBS and incubated for 2h with HRP-conjugated secondary antibody (Promega, Madison, WI, USA). Immunoreactive bands were developed using an Amersham ECL plus Western Blotting Detection System (GE Health Care, Buckinghamshire, UK) and visualized by Molecular Imager® VersaDoc™ MP Imaging Systems (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA, USA). All data are expressed as mean±SEM values. Multiple groups were compared by one-way ANOVA followed by post-hoc Tukey's comparison test. Two groups were compared by unpaired Student's t-test. For all analyses, P-values <0.05 were considered significant.

Results

Pam3CSK4 induces MMP9 expression in the human monocytic THP-1 cells

To determine whether Pam3CSK4 induced MMP-9 expression in monocytic cells, we treated THP-1 cells with 200ng/mL of Pam3CSK4 for 24 hrs. The data show that Pam3CSK4 induced a significant upregulation of MMP-9 expression at both mRNA (Fig. 1A; $P=0.0021$) and protein (Fig. 1B; $P=0.0021$) levels. Gelatin zymography of the conditioned media obtained from the cells treated with Pam3CSK4 also showed high level of MMP-9 activity (Fig. 1C).

TLR2 neutralization antibody blocks Pam3CSK4 induced MMP-9 upregulation

Pam3CSK4 can activate cells through TLR2 and produce inflammatory mediators [25]. Therefore we hypothesized that TLR-2-mediated signaling was involved in MMP-9 production in monocytic THP-1 cells treated with Pam3CSK4. This was accomplished by

Fig. 1. Pam3CSK4 induces MMP-9 expression in human monocytic THP-1 cells. THP-1 cells were treated with the 200ng/mL Pam3CSK4 for 24h. Total cellular RNA was isolated and MMP-9 gene expression was determined by qRT-PCR. Relative mRNA expression was expressed as fold expression over average of gene expression in vehicle-treated cells. The average gene expression level in vehicle-treated cells was assumed to be 1 (A). Cell culture supernatants were used to determine MMP-9 secretory protein by ELISA (B). Gelatin zymography was performed on the conditioned media to analyze MMP-9 activity (C). In all cases, TNF-α (10ng/ml) treated THP-1 cells

were used as a positive control. Data are represented as mean ±SEM. Statistical analysis was done using One Way-ANOVA (Tukey's multiple comparisons test). $P<0.05$ was considered as statistically significant (*), $P<0.01$ as highly significant (**), and $P<0.001/P<0.0001$ were considered as extremely significant (***/****).

Fig. 2. Pam3CSK4 induced expression of MMP-9 is TLR2 dependent. THP-1 cells were treated with 1 μ g/ml of anti-huTLR2-IgA (TLR2 Nab) or isotype-matched control antibody (IgA) for 30 min. Cells were then exposed to Pam3CSK4 (200ng/mL). Cells and conditioned media were collected after 24h. MMP-9 mRNA expression was determined by RT-PCR (A). Secreted MMP-9 protein was assessed by ELISA (B). Data are represented as mean $\pm$ SEM. Statistical analysis was done using One Way-ANOVA (Tukey's multiple comparisons test). $P < 0.05$ was considered as statistically significant (*), $P < 0.01$ as highly significant (**), and $P < 0.001/P < 0.0001$ were considered as extremely significant (***/****).

Fig. 3. Pam3CSK4-induced, TLR2-mediated MMP-9 expression in THP-1 cells involves clathrin-dependent endocytosis. THP-1 cell cultures were treated with pharmacological endocytosis inhibitors; chlorpromazine (an inhibitor of clathrin-dependent endocytosis; 10 μ M) and Methyl- β -cyclodextrin (MBCD; an inhibitor clathrin-independent endocytosis; 2mM) for 1hr prior to treatment with Pam3CSK4 for 24hr [23]. Cells and conditioned media were collected and used for MMP-9 expression. MMP-9 gene expression was determined by real-time RT-PCR (A). Cell culture supernatants were used to determine secreted MMP-9 protein by ELISA (B). Data are represented as mean $\pm$ SEM. Statistical analysis was done using One Way-ANOVA (Tukey's multiple comparisons test). $P < 0.05$ was considered as statistically significant (*), $P < 0.01$ as highly significant (**), and $P < 0.001/P < 0.0001$ were considered as extremely significant (***/****).

treating cells with TLR-2 blocking antibody and isotype control antibody before stimulation with Pam3CSK4. TLR2 blocking significantly decreased Pam3CSK4-induced MMP-9 gene expression in THP-1 cells (Fig. 2A; $P = 0.0005$) as well as reduced secreted MMP-9 levels in culture media (Fig. 2B; $P < 0.0001$).

Effect of endocytosis inhibitor on Pam3CSK4-mediated MMP-9 induction

TLR2-ligand internalization in addition to TLR2-surface binding and receptor-mediated signaling also plays a role in the activation of inflammatory genes and this process

Fig. 4. Pam3CSK4-induced MMP-9 expression in THP-1 cells is MyD88-dependent. THP-1-XBlue™-defMyD cells were treated with 200ng/ml of Pam3CSK4 to induce MMP-9 production. Total cellular RNA was isolated and MMP-9 gene expression was determined by RT-PCR (A). Cell culture supernatants were harvested for ELISA (B). TNF- α (10ng/ml) treated cultures were used as a positive control. Data are represented as mean $\pm$ SEM. Statistical analysis was done using One Way-ANOVA (Tukey's multiple comparisons test) $P < 0.0001$. $P < 0.05$ was considered as statistically significant (*), $P < 0.01$ as highly significant (**), and $P < 0.001/P < 0.0001$ were considered as extremely significant (***/****).

Fig. 5. Pam3CSK4 treatment of THP-1 cells activates MAPK/NF- κ B signaling pathways. THP-1 cells were treated with Pam3CSK4 for different time points. Cell lysates were prepared as described in methods and samples were run on denaturing gels. Phosphorylated ERK1/2, JNK, p-38 and c-Jun are depicted in the upper panels and respective total proteins are shown in the lower panels (A). Phosphorylated IKK α / β , I κ B, and NF- κ B are depicted in the upper panel and β -actin expression is shown in the bottom panel (B). Total protein expression was used as endogenous control for MAPK and β -actin was used as endogenous control for NF- κ B expression in western blots.

is controlled by clathrin-dependent endocytosis mechanism [26]. Therefore, to assess the role of TLR2-ligand internalization in MMP-9 induction, THP-1 cells were pretreated with 10 μ M of Chlorpromazine, an inhibitor of clathrin-dependent endocytosis and 1mM methyl- β -cyclodextrin (MBCD), an inhibitor of clathrin-independent endocytosis followed by treatment with Pam3CSK4 and incubation for 24 hrs. The inhibition of clathrin-dependent endocytosis significantly reduced MMP-9 expression both gene (Fig. 3A; $p < 0.0001$) and protein (Fig. 3B, $p < 0.0001$) levels. No significant change was seen in MMP-9 expression in cultures pretreated with 1mM of MBCD compared to non-MBCD treated controls. These results suggest that TLR2-ligand complex internalization was involved in MMP-9 expression by THP-1 cells treated with Pam3CSK4.

MyD88 deficiency abolishes Pam3CSK4-mediated MMP-9 production

MyD88 is a central adaptor molecule involved in TLR-mediated signaling regulating the inflammatory mediators' gene expression [27]. To test whether MyD88 was involved in Pam3CSK4-induced MMP-9 expression, THP-1-XBlue™-defMyD cells, also known as MyD88-/- THP-1 cells, which are deficient in MyD88 activity were treated with Pam3CSK4 or TNF- α

Fig. 6. Pam3CSK4-induced MMP-9 expression in THP-1 cells is abrogated by inhibition of MAPK/NF- κ B pathways. THP-1 cells were pretreated with MEK-ERK inhibitors (U0126: 10 μ M; PD98059: 10 μ M) or JNK inhibitor (SP600125: 20 μ M) or p38 inhibitor (SB203580: 10 μ M) NF- κ B inhibitor (cyclosporine A; 1 μ M) for 1 hr and then treated with Pam3CSK4 for 24 hr. Cells and supernatants were collected. Cells were used for the isolation of total RNA to assess the MMP-9 gene expression by real-time RT-PCR (A). Secreted levels of MMP-9 protein were determined in supernatants by ELISA (B). Culture supernatants of X-Blue cells and MyDdef cells were harvested after 24h treatments as indicated for assessing SEAP activity indicating the degree of NF- κ B/AP-1 activation) (C and D). Data are represented as mean $\pm$ SEM. Statistical analysis was done using One Way-ANOVA (Tukey's multiple comparisons test). $P < 0.0001$. $P < 0.05$ was considered as statistically significant (*), $P < 0.01$ as highly significant (**), and $P < 0.001/P < 0.0001$ were considered as extremely significant (***/****).

(positive control). Following Pam3CSK4 treatment, MMP-9 expression was abrogated in MyD88 $^{-/-}$ cells at both mRNA and protein levels (Fig. 4A and B). Nevertheless, TNF- α -mediated induction of MMP-9 was not affected in MyD88 $^{-/-}$ cells as it activates MMP-9 gene expression via the MyD88-independent pathway. These data suggest that Pam3CSK4 induces MMP-9 gene upregulation in monocytic cells through the MyD88-dependent signaling cascade.

MAPK and NF- κ B signaling pathways are involved in Pam3CSK4-induced MMP-9 up-regulation

It has been reported that ERK, JNK, p38, c-Jun, and NF- κ B signaling pathways are the downstream activators of TLR2/MyD88. We next asked whether these molecules played a role in Pam3CSK4-induced MMP-9 production. THP-1 cell treatment with Pam3CSK4 increased phosphorylation of ERK, JNK, p38, c-Jun; (Fig 5A) as well as IKK α/β , I κ B and NF- κ B (Fig. 5B). As expected, MMP-9 mRNA expression was reduced (Fig 6A; $P < 0.0001$) by treatment with inhibitors of either MAPK/JNK (SB203580/SP600125) or ERK (PD98059, U0126) or NF- κ B (cyclosporine A). Consistent with qRT-PCR results, MMP-9 levels in culture supernatants of THP-1 cells were significantly suppressed (Fig 6B; $P < 0.0001$) after treatment with inhibitors of either MAPKs or NF- κ B. Since the promoter of MMP-9 gene has NF- κ B/AP-1 binding sites [28], we investigated whether Pam3CSK4 induced enhanced levels of NF- κ B / AP1 activity in THP-1 cells. As expected, NF- κ B/AP-1 activity was strongly induced in culture media from Pam3CSK4-treated cells (Fig. 6C; $P = 0.0031$). This confirmed the dependence of Pam3CSK4-induced MMP-9 production on NF- κ B/AP1 activation by using THP-1 cells

expressing a reporter driven by NF- κ B and AP-1 response elements. Similarly, MyD88 deficiency also significantly inhibited the activation of NF- κ B/AP-1 following Pam3CSK4 treatment (Fig. 6D; $P=0.0197$). These data suggest that Pam3CSK4-induced upregulation of MMP-9 gene expression in monocytic cells involves activation of NF- κ B/AP-1 transcription factors through the MyD88-dependent signaling cascade.

Discussion

MMPs are the major proteases and their dysregulation is involved in the pathogenesis of several pathogenesis disorders [29–31]. In obesity, MMP-9 may facilitate the infiltration of immune cells into the adipose tissue and could promote obesity-associated metabolic inflammation. We earlier showed that microbial components and the free fatty acid palmitate induced robust MMP-9 production in monocytic cells [19, 24]. Bacterial lipoprotein were shown to induce inflammatory mediators including IL-6, TNF- α , IL-8, CSF2, CSF3, and ICAM-1 and IL-1 β [32]. However, the role of bacterial lipoprotein in regulating MMP-9 production in monocytic cells is not defined yet. In this study, we used a synthetic triacylated lipopeptide (Pam3CSK4) that mimics the acylated amino terminus of bacterial lipoprotein. Our data show that Pam3CSK4 induces MMP-9 expression at both mRNA and protein levels in human monocytic THP-1 cells. Moreover, Pam3CSK4-induced MMP-9 production was dependent on the TLR2/MyD88 signaling pathway. TLRs respond to pathogen- or danger-associated molecular patterns by inducing expression of proinflammatory cytokines/chemokines and related inflammatory proteins in monocytes/macrophages and dendritic cells [33]. TLR2/4 were identified as the predominant TLRs that orchestrated the innate immune responses to bacterial infections in a mouse model study through the production of cytokines by macrophages and dendritic cell [34]. Bacterial components such as LPS, lipopeptides, lipoteichoic acids (LTAs), and peptidoglycan stimulate the production of cytokines and other inflammatory mediators [35, 36]. FSL-1 and HKLM were reported to induce MMP-9 production [23, 24, 37]. Herein, we show that Pam3CSK4 induces MMP-9 production in monocytic cells. Of note, the production of proinflammatory cytokines depends on both signaling from the cell surface and endosomal compartments [38]. In agreement with this, our data show that blocking the surface TLR-2-mediated signaling by THP-1 cell treatment with anti-TLR2 neutralizing antibody significantly abrogated MMP-9 production in response to Pam3CSK4 activation.

Our data further show that Pam3CSK4-mediated MMP-9 production was regulated by a mechanism that involved clathrin-dependent endocytosis. Together, these results suggest that Pam3CSK4 activation of THP-1 monocytic cells depends on TLR2 signaling both from cell surface and endosomal compartments. TLR signaling pathways are regulated by TIR domain-containing adaptors such as MyD88, TIRAP/Mal, TRIF and TRAM. MyD88 is a key adaptor protein which is common to all TLRs except TLR3 [39]. The interaction between TLR and MyD88 is required for signaling via the MAPK/NF- κ B pathways [16, 40]. Consistent with the requirement of this adaptor protein for TLR response, we show that MyD88 $-/-$ THP-1 cells did not produce MMP-9 following stimulation with Pam3CSK4.

Our data also show that MyD88 is a key regulatory adaptor molecule in Pam3CSK4-induced AP-1/NF- κ B activation. MMP-9 production was reduced after inhibition of MEK1/2/ERK, JNK, P38 and NF- κ B, all of which are known to be involved in MMP-9 production in other cell systems [41–43]. Our data show that the inhibition of MEK/ERK significantly suppressed the MMP-9 production, suggesting that NF- κ B and MAPK signaling pathways may have a key role in MMP-9 regulation. The interaction of different ligands with the TLRs has been shown to be involved in MMP-9 production. The free fatty acid palmitate is considered as an endogenous ligand for TLR4 and it induces TLR4-dependent activation of MMP-9 gene expression which requires MyD88 recruitment and activation of NF- κ B/AP-1 transcription factor [19]. Likewise, a microbial component FSL-1 was also shown to trigger MMP-9 production in a TLR-2-dependent manner though the recruitment of MyD88 and

activation of MEK1/2, ERK 1/2, MEK5/ERK5, JNK, p38 MAPK and NF- κ B/AP-1 transcription factors [23].

Conclusions

Taken together, our data show for the first time to our knowledge, that synthetic triacylated lipopeptide Pam3CSK4 induces MMP-9 production in human monocytic THP-1 cells through a TLR-2/MyD88-dependent signaling mechanism that involves activation of MEK1/2/ERK, JNK, p38 MAPK and NF- κ B/AP-1 transcription factors.

Abbreviations

AP-1 (Activator protein 1); ATCC (American Type Culture Collection); ECM (Extracellular matrix); ELISA (Enzyme-linked immunosorbent assay); FAA (Free fatty acid); FSL-1 (Fibroblast-stimulating lipopeptide-1); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase); HKLM (Heat killed *Listeria monocytogenes*); Pam3CSK4 (Pam3-Cys-Ser-Lys4); LP (Lipopeptide); LPS (Lipopolysaccharide); LTAs (Lipoteichoic acids); MAPK (Mitogen-activated protein kinase); MBCD (Methyl- β -cyclodextrin); MMP-9 (Matrix metalloproteinase-9); MyD88 (Myeloid differentiation factor 88); NF- κ B (Nuclear factor-kappaB); PA (Palmitate (palmitic acid)); PAMPs (Pathogen-associated molecular patterns); PBMC (Peripheral blood mononuclear cells); PVDF (Polyvinylidene difluoride membrane); SFA (Saturated fatty acid); SEAP (Secreted embryonic alkaline phosphatase); Th17 (T helper-17); THP-1 (A human monocytic cell line); TIR (Interleukin (IL)-1 receptor called toll-IL1-R); TLRs (Toll-like receptors); TNF- α (Tumor necrosis factor-alpha); TRAM (TRIF-related adaptor molecule); TRIF (TIR-domain-containing adapter-inducing IFN- β).

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Disclosure Statement

The authors declare that they have no competing interests.

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