Biochemical and Biophysical Research Communications 397 (2010) 100–105

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

# Toll-like receptor 4-mediated c-Jun N-terminal kinase activation induces gp96 cell surface expression via AIMP1 phosphorylation

Gyuyoup Kim [a], Jung Min Han [a,b], Sunghoon Kim [a,c,*]

[a] Center for Medicinal Protein Network and Systems Biology, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea
[b] Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea
[c] Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Suwon 443-270, Republic of Korea

ARTICLE INFO

*Article history:*
Received 7 May 2010
Available online 16 May 2010

*Keywords:*
gp96
AIMP1
TLR4
LPS
MyD88
JNK

ABSTRACT

The presentation of the endoplasmic reticulum resident chaperone protein, gp96 on the cell surface have been considered as a phenomenon of the immunogenic process activation. Previously, we showed aminoacyl-tRNA synthetase-interacting multifunctional protein 1 (AIMP1) can form a molecular complex with gp96, regulate the ER retention of gp96 through KDEL receptor, and suppress its cell surface expression. However, the physiological conditions that modulate AIMP1–gp96 interaction and cell surface expression of gp96 are not known. In this study, we investigated the process that which can modulate dissociation of AIMP1 and gp96 by using Toll-like receptor (TLR) activation. MyD88 pathway by LPS-mediated TLR4 activation induced the cell surface presentation of gp96 through c-Jun N-terminal kinase (JNK). AIMP1 was phosphorylated by JNK upon LPS stimulation and gp96 was dissociated from phosphorylated AIMP1. We further demonstrated that serine-140 residue of AIMP1 was phosphorylated by JNK and alanine mutation of serine-140 suppressed LPS-induced cell surface expression of gp96. Altogether, these results suggest that AIMP1 is phosphorylated by JNK through TLR-MyD88 pathway and lose the regulatory activity for ER retention of gp96, resulting in the increase of cell surface expression of gp96, and provide a new molecular mechanism underlying TLR-mediated gp96 regulation.



## 1. Introduction

Toll-like receptors (TLRs) have been studied as pattern-recognition receptors to defend against pathogen-associated molecular patterns (PAMPS), the first barrier from microbes in innate immunity [1]. There are at least 10 TLR families have been described in murine [2], that recognize a broad spectrum range of PAMPS such as lipids, lipopeptides, proteins, and nucleic acids [3]. TLRs are type-1 transmembrane receptors, containing a ligand binding domain of leucine rich repeats and a cytoplasmic Toll/IL-1 receptor (TIR) domain that interacts with TIR domain containing adaptor molecules, of which there are five: MyD88, Mal, TRIF, TRAM, and SARM [4]. MyD88 is utilized by all of the TLRs with the exception of TLR3 [5]. Once recruited to the receptor, MyD88 lead a common signaling pathway culminating in the activation of the nuclear factor κB (NFκB) and the mitogen-activated protein kinase p38 and Jun N-terminal kinase (JNK) [6,7].

Heat shock protein (HSP) gp96, or grp94 is an endoplasmic reticulum (ER)-resident member of HSP90 family [8]. Like other ER-resident proteins, gp96 contains a C-terminal KDEL sequence, which is involved in retrograde transport from Golgi to ER. However, despite this KDEL sequence, cell surface gp96 presentation has been demonstrated on mouse Meth-A sarcomas but not on normal embryonic fibroblasts [9]. In addition, it has been reported that gp96 is expressed on murine thymocytes [10], which indicates that cell surface gp96 expression is not restricted to tumor cells. Also, gp96 has been implicated in the activation or maturation of dendritic cells (DCs) [11]. Proinflammatory cytokine secretion [12] and MHC class I/II up-regulation [13] can be induced by direct interaction between gp96 and DCs via CD91 [14] and TLR2/TLR4 [12] resulted in DC maturation. These reports support that extracellular gp96 is involved in innate and adaptive immunity [15]. In a transgenic mouse model 96tm-Tg, enforced cell surface expression of gp96 induced significant DC activations and showed spontaneous systemic lupus erythematosus (SLE)-like autoimmune phenotypes [16]. In addition, gp96 is increased in synovial fluid from the joints of rheumatoid arthritis (RA) patients and activates macrophages that promote the chronic inflammation of RA [17]. Recently, we have shown that *in vivo* administration of the chemical compound reduces SLE-like phenotypes in 96tm-TG mice

* Corresponding author at: Center for Medicinal Protein Network and Systems Biology, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea. Fax: +82 2 875 2621.
*E-mail address:* sungkim@snu.ac.kr (S. Kim).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.05.075

via ER retention of gp96 [18]. Thus, uncontrolled gp96 cell surface expression leads to the breakdown of immunologic tolerance with autoimmune disease-like pathological states.

AIMP1 (also known as p43) was identified as one of three auxiliary factors of mammalian multi-ARS complex [19]. AIMP1 binds and helps the catalytic reaction of arginyl-tRNA synthetase [20]. AIMP1 is also involved in diverse physiological processes [21], like a cytokine activity on monocytes [22–24], endothelial cells [25], fibroblasts [26], and even a glucagons-like hormonal activity [27]. Recently, we found that AIMP1 physically interacts with gp96 and controls ER retention of gp96, preventing its extracellular presentation [28]. In addition, AIMP1-deficient mice showed the increased levels of gp96 on the cell surface, thereby displaying the phenotypes similar to those of gp96 transgenic mice [28]. Although all of these previous studies demonstrated the importance of the ER retention of gp96 for immune tolerance, it is not yet determined which pathway controls the dissociation of gp96 from AIMP1 and localization of gp96 on the cell surface. To address this question, we first confirmed the innate immune activation via TLR4 can induce the surface expression of gp96, and then investigated the dissociation mechanism between gp96 and AIMP1 in the activation of TLR4 signal pathway.

## 2. Materials and methods

### 2.1. Cells and vectors

HL-60 cells was grown in RPMI 1640 medium with supplementing 10% FBS and antibiotics. Mouse splenocytes were isolated from pathogen-free 12-week-old mouse (C57BL/B6) [28]. Dominant negative mutant of JNK was the kind gift of Dr. D. Levens (National Institutes of Health, USA). AIMP1 point mutants were made by using site-directed mutagenesis Kit (Invitrogen) with specific primers: S140A-(forward), 5′-AGAAGGAGAAAAAACAGCAAGCAATAGCTGGAAG-3′; S140D-(forward), 5′-AGAAGGAGAAAAAACAG CAAGACATAGCTGGAAGTGCC-3′; S140-(reverse), 5′-TTGCTGTTTT TTCTCCTTCTTCTCTCCTTTC-3′; S153A-(forward), 5′-ACTCTAAGC CAATAGATGTTGCCCGTCTGGATCT-3′; S153D-(forward), 5′-ACTCTAAGCCAATAGATGTTGACCGTCTGGATCTTCGA-3′; S153-(reverse), 5′-AACATCTATTGGCTTAGAGTCGGCACTTCCA-3′; T 287A-(forward), 5′-CTAATGATGAGTGTGTGGCTGCATACAAAGGAGT-3′; T 287-(reverse), 5′-AGCCACACACTCATCATTAGTGTGAAGATC-3′. Transfection was performed by using electroporator (INCYTO).

### 2.2. Antibodies and reagents

TLR2, TLR4, TLR9, MyD88, c-Myc (9E10), HA, and phosphor-serine antibodies were purchased from Santa Cruz Biotechnology. gp96 antibody was from Stressgen. Heat-killed *Escherichia coli* (XL1-blue strain) were prepared by boiling for 30 min. LPS (from *E. coli*, O127: B8) was purchased from Sigma–Aldrich. All other TLR agonists were obtained from InvivoGen. SB203580, PD98059, and SP600125 were from Calbiochem. Recombinant JNK and c-Jun were from Millipore and Cell Signaling Technology, respectively.

### 2.3. Flow cytometry

Surface gp96 staining of cells and flow cytometry were performed as described in [28]. Collected cells were washed with 1× PBS, and resuspended in FACS buffer (1× PBS containing 2% FBS, and 1% BSA) for 30 min. After then, cells were stained with monoclonal gp96-phycoerythrin conjugated antibody (Assay Designs) for 30 min and washed with 1× PBS for three times. Cells were analysed on a FACScan flow cytometry using CellQuest software (Becton Dickinson Biosciences).

### 2.4. Transfection of small interfering RNAs (siRNA)

Endogenous TLR family or AIMP1 were depleted using siRNA transfection method. For each transfection, HL-60 suspension containing $8 \times 10^5$ cells in 500 μL of RPMI 1640 with 10% FBS but without antibiotics, were added to a well of a 24 well plate, and cells were transfected with siRNA duplexes (final concentration, 50 nmol/L) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Pre-designed siRNAs (Invitrogen) were 25-nucleotide duplexes and had the following sequences: TLR2, 5′-UUCAGAGUGAGCAAAGUCUCUCCGG-3′; TLR4, 5′-UUCAACUUCCACCAAGAGCUGCCUC-3′; TLR9, 5′-AGUAUUUGCAGGGCACUCGCCAGGG-3′; AIMP1, 5′-GGAGCUGAAUCCUAAGAAGAAGA UU-3′. MyD88–siRNA containing a pool of four target-specific 20–25 nucleotides was purchased from Santa Cruz Biotechnology.

### 2.5. Recombinant AIMP1 mutant purification

AIMP1 wild-type and all other mutants were sub-cloned into pET 28a vector (Novagen). His-tagged proteins were expressed in Rosetta (DE3) *E. coli* strain (Novagen), purified by nickel affinity chromatography as described previously [20].

### 2.6. Two-dimensional electrophoresis

2D samples were prepared by solubilizing in 2D-lysis buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v ASB-14, and 2 mM tributylphosphine), loaded onto the immobilized pH gradient (IPG) strip gels (linear pH gradient 7–10, 7 cm), and focused with PROTEAN isoelectric focusing cell (Bio-Rad). After equilibration with 375 mM Tris–HCl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol, 2% DTT, and 2.5% iodoacetamide, the IPG strips were embedded on top of 10% SDS–PAGE gels, sealed with 1% agarose, and electrophoresed.

### 2.7. Binding assay

For immunoprecipitation, the cell was solubilized with lysis buffer (25 mM Tris–HCl, pH 7.4, 10 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% Triton X-100, 2 mM DTT, and 1 mM PMSF with protease inhibitor cocktail). Extracted proteins were mixed with gp96 antibody (Santa Cruz Biotechnology) pre-bound with protein A/G agarose for 2 h, and non-specific proteins were washed out with lysis buffer. The proteins were denatured by boiling after adding 5× sample buffer, and then subjected to SDS–PAGE for Western-blot analysis with AIMP1 antibody. To address that the interaction of AIMP1 and gp96 could be affected by AIMP1 phosphorylation, the cell lysate was treated with phosphatase, then incubated with recombinant GST–gp96 protein [28], and immunoprecipitated with AIMP1 antibody.

### 2.8. JNK in vitro kinase assay

Recombinant JNK1 protein was mixed in kinase buffer (25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 2 mM DTT, 0.2 mM ATP, and 0.1 mM $Na_3VO_4$ with protease inhibitor cocktail) and combined with 10 μCi of [$\gamma$-$^{32}$P] ATP, 5 μg recombinant AIMP1 mutant. The reaction was incubated for 30 min at 30 °C, terminated by adding 5× SDS sample buffer and boiled to denature samples for SDS–PAGE. The phosphorylated AIMP1 and c-Jun (as positive control) were determined by autoradiography of the dried blot.

### 2.9. Statistical analysis

The Student's *t*-test was used for statistical analysis. *P* values of <0.05 were considered to represent statistically significant differences.

## 3. Results

### 3.1. TLR activation induces the cell surface gp96 expression

To address the immunogenic function of cell surface expressing gp96, we treated an innate immunogenic trigger; Gram negative bacteria (heat-killed *E. coli*) to the monocyte cell line (HL-60) and mouse primary splenocyte for 24 h and analysed cell surface expressed gp96 by using flow cytometry. As a result, when the HL-60 was treated with *E. coli*, cell surface expression of gp96 significantly increased. In addition, isolated splenocytes from mouse also express the gp96 on the cell surface in response to *E. coli* treatment (Fig. 1A). To investigate whether the immune response by *E. coli* that increased cell surface gp96 is by TLR activation, we treated purified-LPS instead of *E. coli* on HL-60. The cell surface gp96 was increased by LPS in dose-dependent manner (Fig. 1B and C).

Since LPS is known as an activator for TLR4 in innate immunity, we examined other TLR agonists can induces cell surface gp96 expression. Most TLRs activation showed no increase of gp96 on cell surface, except TLR4 and TLR7 (Fig. 1D). Interestingly, activation of endosomal TLR9 showed clearance of gp96 on the cell surface. The reason why TLR9 activation suppresses cell surface

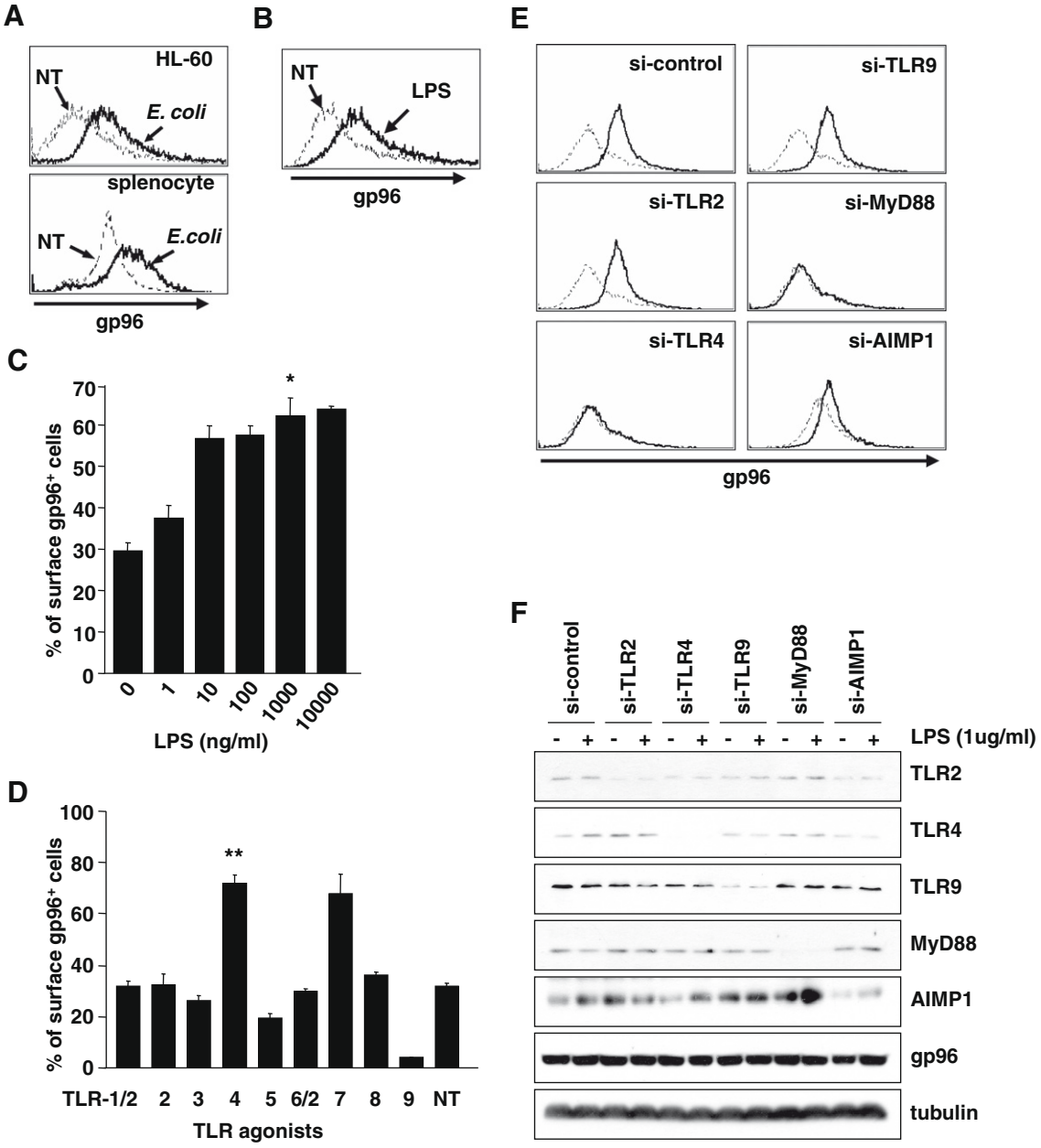


**Fig. 1.** Cell surface gp96 induction by TLR activation. (A) Heat-killed *E. coli* treated for 24 h (solid line) or not treated (NT; dotted line) HL-60 cells and splenocytes were stained for cell surface gp96, followed by flow cytometry analysis. HL-60 cells were treated with 1 μg/ml LPS (B) or in dose-dependent manner (C) for 24 h and stained for cell surface gp96, followed by flow cytometry analysis (*$P = 0.0002$). (D) To investigate the cell surface gp96 expression upon the variable TLR agonists, HL-60 cells were treated with Pam3CSK (1 μg/ml for TLR1/2), heat-killed *Listeria monocytogenes* (HKLM; $10^8$ cells/ml for TLR2), Poly(I:C) LMW (10 μg/ml for TLR3), LPS (1 μg/ml for TLR4), Flagellin (10 μg/ml for TLR5), Pam2CGDPKHPKSF (1 μg/ml for TLR6/2), Imiquimod (10 μg/ml for TLR7), ssRNA40 (10 μg/ml for TLR8), and ODN2006 (5 μM for TLR9) for 24 h and stained for cell surface gp96, followed by flow cytometry analysis (**$P < 0.0001$). (E) HL-60 cells were transfected with each siRNA for 48 h, treated with LPS for 24 h (solid line) or not treated (dotted line), and stained for cell surface gp96, followed by flow cytometry analysis. (F) Knockdown of each gene was validated by Western-blot analysis. Data represent the means ± SD.

expression of gp96 needs further investigation. Despite all kinds of TLRs commonly used the MyD88 as an adaptor molecule with an exception of TLR3, LPS and Imiquimod significantly increase the cell surface gp96 expression through TLR4 and TLR7, respectively. To investigate the gp96 localization dependent on the MyD88 related pathway obviously, we used TLR4 model in the following experiments.

To confirm that LPS-induced cell surface expression of gp96 is dependent on the TLR activation and not by other effect, TLR2, TLR4, and TLR9 genes were depleted by using each siRNAs before cells were treated with LPS, and monitored the surface levels of gp96 by using flow cytometry. When TLR4 was depleted, the cell surface gp96 was not increased by LPS, but in the case of TLR2 and TLR9 knockdown showed the increase of gp96 on the cell surface by LPS in the same manner with si-control (Fig. 1E and F). In addition to this result, most TLR's common adaptor molecule, MyD88 knockdown also showed similar phenomenon of TLR4. Therefore, we supposed that MyD88 pathway through TLR activation is important to determine the cell surface gp96 localization.

### 3.2. JNK activation is related to the expression of gp96 on the cell surface

MyD88 stimulation by LPS leads TRAF6 downstream pathway activation including p38MAPK, ERK, and JNK [7]. To address the involvement of these kinases on the gp96 localization, cells were pre-treated with each kinase inhibitor (10 μM) and then treated with LPS for 24 h. It was observed that SP600125 inhibited the cell surface gp96 expression, but other inhibitors did not (Fig. 2A and B). To determine the relationship with JNK activation and gp96 presentation on the cell surface more detail, we obtained dominant-negative JNK expression, and then we found that the LPS-induced cell surface expression of gp96 was inhibited (Fig. 2C). Therefore, these results suggest that innate immunogenic environment such as LPS induce the expression of gp96 on the cell surface through JNK activation.

### 3.3. AIMP1 phosphorylation by JNK induces AIMP1–gp96 dissociation

Previously, we found that AIMP1 associated with gp96 in the ER, to prevent cell surface export of gp96 [28]. The gp96-binding region of AIMP1 (amino acids 54–192) contains lysine-rich sequences and the C-terminal dimerization domain of gp96 contains glutamate-rich sequences. In this reason, we supposed this interaction is due to the charge–charge interaction. The post-translational modification such as phosphorylation can change the charge of protein, thus we investigated that TLR trigger could phosphorylate AIMP1. AIMP1 extracted from the control and LPS-treated cells were separated by 2D gel electrophoresis. Although AIMP1 of the control cells was detected mainly as a single spot, a few additional spots were generated in the more acidic region upon LPS treatment, but disappeared upon LPS with JNK inhibitor treatment (Fig. 3A), indicating that AIMP1 is phosphorylated by TLR4 activation. Consistently, *in vitro* binding assay showed that serine residue of AIMP1 was phosphorylated (Fig. 3B) by LPS but the phosphorylation of threonine residue was not observed (data not shown). Furthermore, the GST-fused gp96 was detected upon alkaline phosphatase treatment, but reduced on the control (Fig. 3B), indicating that TLR4 activation release gp96 from AIMP1 by serine residue specific phosphorylation on AIMP1.

To determine the binding of AIMP1 and gp96 is altered by TLR4 activation, LPS-treated cell lysates were immunoprecipitated with anti-gp96 antibody, and blotted with anti-AIMP1 antibody. Although AIMP1 of the control cell was immunoprecipitated with gp96, it was disappeared upon LPS treatment (Fig. 3C and D). However, the dissociation of AIMP1 and gp96 by TLR4 activation was blocked on the dominant-negative JNK overexpression (Fig. 3D), correlated with the result that cell surface expression of gp96 (Fig. 2C). These results suggest that AIMP1 could be phosphorylated by activation of JNK pathway on TLR4 signaling.

### 3.4. Phosphorylation of AIMP1 serine-140 regulates cell surface expression of gp96

To address which residue of AIMP1 could be phosphorylated by JNK activation, we searched the possible site by structural modeling, and collected a few candidates among those lists by stand on the gp96-binding region. Candidates (from Ser to Ala or Asp) were expressed on the cell, and treated with LPS. Myc-tagged AIMP1

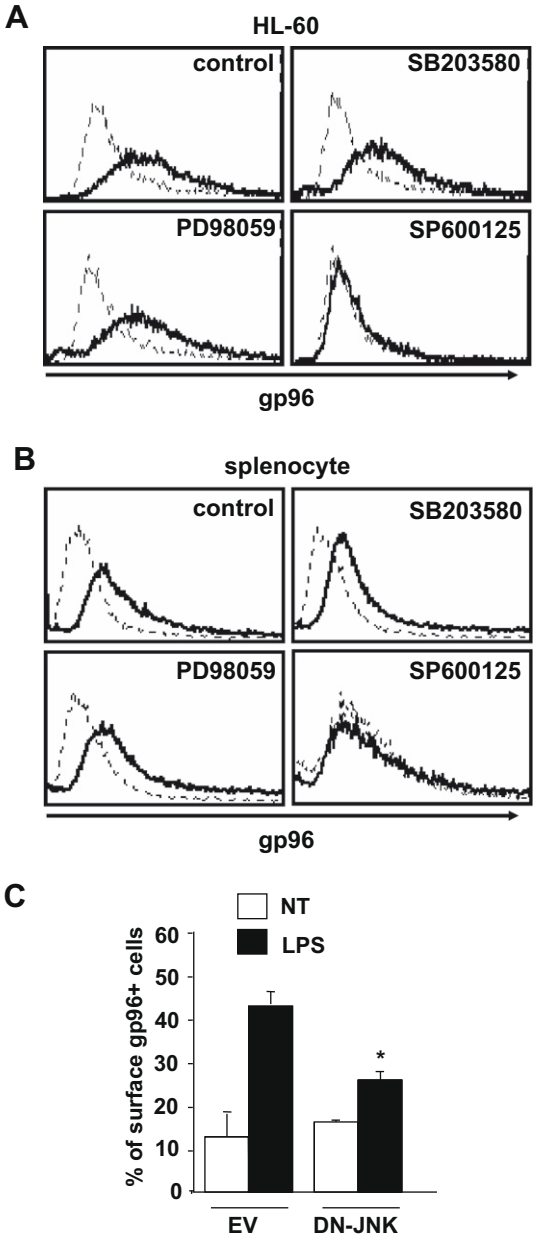


**Fig. 2.** Cell surface gp96 presenting related with JNK activation. (A) HL-60 cells and (B) splenocytes were pre-treated with each kinase inhibitor SB203580 (p38MAPK), PD98059 (ERK1), and SP600125 (JNK), and treated with LPS (solid line) or not (dotted line) for 24 h. Surface gp96 was determined by flow cytometry. (C) HL-60 cells were transfected with empty vector (EV) or dominant-negative JNK (DN-JNK) for 24 h and treated with LPS for 24 h, followed flow cytometry analysis for surface gp96 presentation (*$P = 0.002$). Data represent the means ± SD.

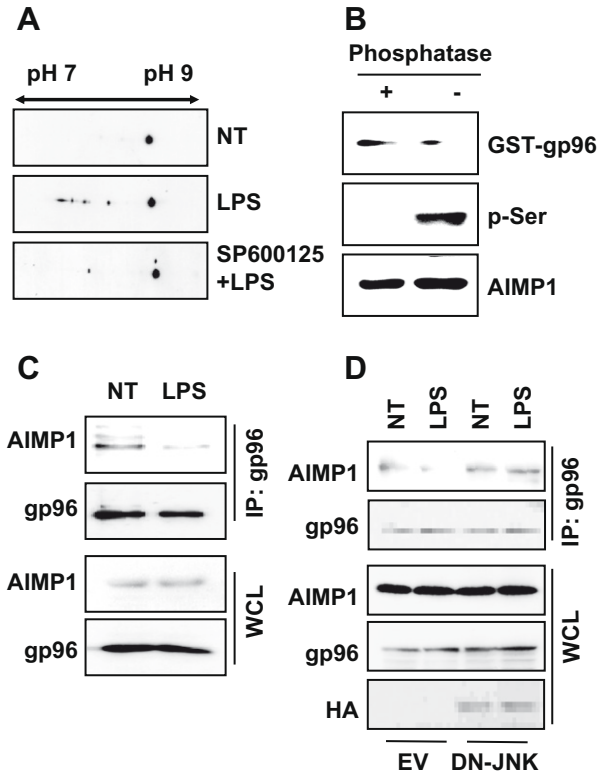


**Fig. 3.** AIMP1 is phosphorylated by LPS via JNK activation. (A) Cell extracts from non-treated (top), or LPS-treated (middle) were separated by 2D gel electrophoresis and subjected to Western blotting with anti-AIMP1 antibody. To determine the JNK-dependent phosphorylation of AIMP1, SP600125 pre-treated cell was treated with LPS (bottom). (B) LPS-treated cell lysate was combined with GST-fused gp96 protein *in vitro*, immunoprecipitated with anti-AIMP1 antibody and immunoblotted with anti-phospho-Ser or anti-GST antibody. To determine whether the binding of AIMP1 and gp96 is affected by phosphorylation of AIMP1, the lysate was reacted with alkaline phosphatase. (C) LPS-induced dissociation of AIMP1 and gp96 was determined by immunoprecipitation. Proteins extracted from non-treated (NT) or LPS-treated HL-60 cells were immunoprecipitated with anti-gp96 antibody and precipitated proteins were immunoblotted using anti-AIMP1 antibody. WCL indicate whole cell lysate. (D) To determine the relation of JNK to phosphorylation of AIMP1, HL-60 cells were transfected with empty vector (EV) or dominant-negative JNK (DN-JNK), and immunoprecipitated as described former. HA-tagged DN-JNK overexpression was determined anti-HA probe antibody. Data are representative of three independent experiments.

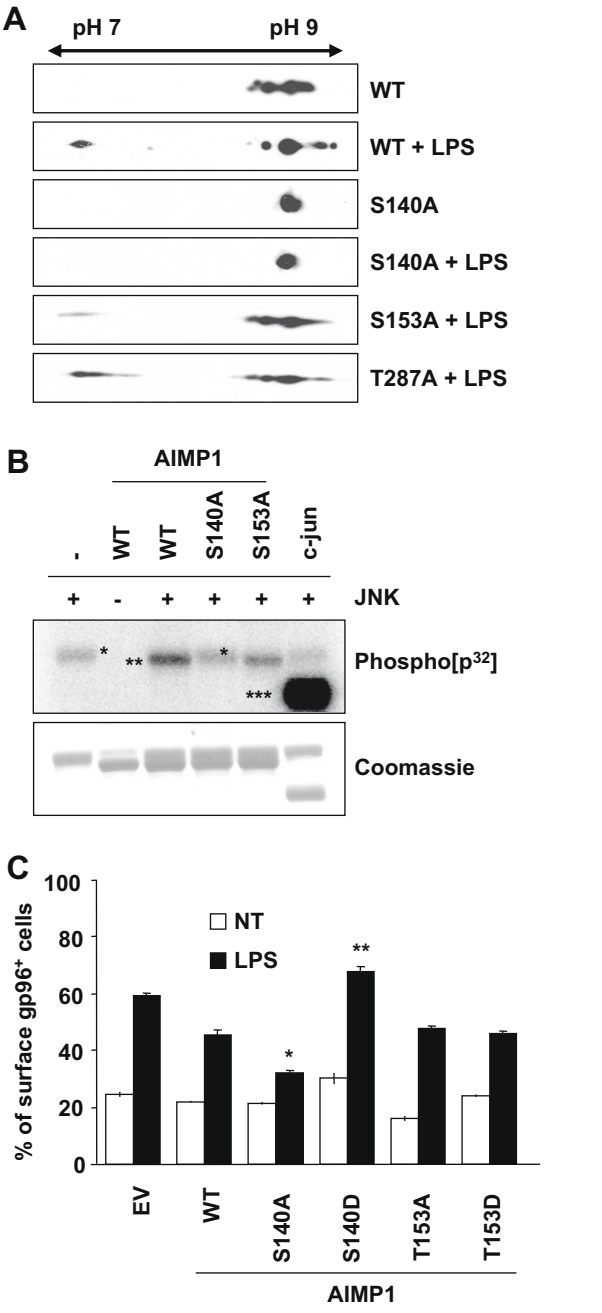


**Fig. 4.** Ser-140 residue of AIMP1 phosphorylation by JNK. (A) HL-60 cells were transfected with Myc-tagged AIMP1 wild-type or mutants (as indicated) and treated with LPS or not. Proteins were extracted from cells, separated by 2D gel electrophoresis, and subjected to Western blotting with anti-c-Myc antibody. (B) Recombinant AIMP1 proteins were incubated with JNK in [$\gamma$-$^{32}$P] ATP contained reaction buffer as described in Section 2. After 30 min, samples were separated by SDS–PAGE, stained with coomassie staining solution and analysed the band by phosphoimager (* indicates JNK, ** indicates AIMP1, and *** indicates c-Jun as positive control). (C) After transfection of 24 h with AIMP1 wild-type (WT) or mutants (as indicated), HL-60 cells were treated with LPS for 24 h and analysed by flow cytometry for surface gp96 presentation (*$P < 0.0001$, **$P = 0.0012$). Data represent the means ± SD.

were separated by 2D gel electrophoresis. Overexpressed AIMP1 was responded to LPS treatment and additional spots were detected in the acidic region, with the exception of S140A mutant (Fig. 4A). To evaluate that JNK could phosphorylate AIMP1 directly, *in vitro* kinase assay was performed by using bacterial expressed recombinant proteins. In our system, AIMP1 could be phosphorylated by JNK, indicating that AIMP1 is one of the substrate for JNK. JNK could not phosphorylated the S140A mutant, but other candidate S153A could be phosphorylated (Fig. 4B). Furthermore, the surface gp96 was not responsible on S140A mutant to LPS, while S140D mutant showed highly expressed surface gp96 either treated with LPS or not (Fig. 4C). These results show that the induction of surface gp96 by LPS or innate immune trigger is due to the gp96 dissociation from the phosphorylated AIMP1 at serine-140 residue by JNK activation that was stimulated by TLR/MyD88 signal pathway.

## 4. Discussion

This study demonstrates the molecular mechanism of gp96 surface localization responding to innate immune trigger. TLR4 signaling by LPS leads JNK activation through MyD88-dependent pathway, resulting in AIMP1 phosphorylation. This leads collapse of AIMP1–gp96 complex and surface presentation of gp96 through ER–Golgi pathway. The gp96-binding region of AIMP1 has positive charge by lysine-rich sequences and the AIMP1-binding region of gp96 has negative charge by glutamate-rich sequences. Therefore, the association between AIMP1 and gp96 may be due to the

charge–charge interaction. Through JNK-mediated phosphorylation, AIMP1 gets negative charge within gp96-binding region, resulting in reduced binding between AIMP1 and gp96.

LPS was the most potent agent for cell surface expression of gp96 among the TLR agonists on the HL-60 cell (Fig. 1D). All TLRs utilize the adaptor molecule MyD88 commonly, with the exception of TLR3. Despite MyD88 lead a common signal pathway, individual TLR family members induce different signaling pathway by assembling of other adaptor molecules of kinases [29]. In this reason, we supposed that the surface gp96 was decreased or not changed on the other agonist-treated cells than control. Unexpectedly, TLR9 agonist ODN2006 eliminated the surface gp96 completely (Fig. 1D). Related with this phenomenon, it was reported that the proportion of B cells and monocytes expressing TLR9 is increased in SLE patients [30]. It may be supposed that both TLR9 activation and surface gp96 expression cause chronic inflammatory response, culminating SLE-like diseases, there are maybe a negative feedback each other to avoid unnecessary activation.

Although HSPs have been implicated as endogenous activators for DCs, chronic or uncontrolled expression of gp96 on the cell surface induces significant DC activation and spontaneous SLE-like autoimmune phenotypes in mice [16,28]. gp96 is increased in synovial fluid from the joints of human rheumatoid arthritis patients and the expression of gp96 shows a correlation with inflammation and synovial lining thickness, further supporting the pathological association of gp96 with autoimmune diseases [17]. These results indicate that gp96 is a potential new therapeutic target, and previously we screened and identified a gp96-binding chemical that suppresses surface translocation of gp96. In mice, maturation of DCs, populations of APCs, and activated B and T cells were significantly reduced by administration of the candidate compound [18]. The compound also alleviated the SLE-like symptoms. Thus, the control of gp96 localization is very important event for maintaining the immune tolerance.

In summary, we demonstrate that LPS induces the dissociation of gp96 from AIMP1 by phosphorylation through TLR4/MyD88-mediated JNK activation, resulting in translocation of gp96 on the cell surface.

## Acknowledgments

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2008-359-C00024), by FPR08-B1-250 of 21C Frontier Functional Proteomics Project from Korean Ministry of Science & Technology, by 2009-0063498 of the Acceleration Research of KOSEF.

## References

[1] R. Medzhitov, C.A. Janeway Jr., Decoding the patterns of self and nonself by the innate immune system, Science 296 (2002) 298–300.
[2] J.C. Roach, G. Glusman, L. Rowen, A. Kaur, M.K. Purcell, K.D. Smith, L.E. Hood, A. Aderem, The evolution of vertebrate Toll-like receptors, Proc. Natl. Acad. Sci. USA 102 (2005) 9577–9582.
[3] T. Kawai, S. Akira, TLR signaling, Cell Death Differ. 13 (2006) 816–825.
[4] A.F. McGettrick, L.A.J. O'Neill, The expanding family of MyD88-like adaptors in Toll-like receptor signal transduction, Mol. Immunol. 41 (2004) 577–582.
[5] E.F. Kenny, L.A.J. O'Neill, Signalling adaptors used by Toll-like receptors: an update, Cytokine 43 (2008) 342–349.
[6] S. Janssens, R. Beyaert, A universal role for MyD88 in TLR/IL-1R-mediated signaling, Trends Biochem. Sci. 27 (2002) 474–482.
[7] A. Dunne, L.A.J. O'Neill, The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense, Sci. STKE 171 (2003) re3.
[8] Z. Li, J. Dai, H. Zheng, B. Liu, M. Caudill, An integrated view of the roles and mechanisms of heat shock protein gp96-peptide complex in eliciting immune response, Front. Biosci. 7 (2002) d731–d751.
[9] A. Altmeyer, R.G. Maki, A.M. Feldweg, M. Heike, V.P. Protopopov, S.K. Masur, P.K. Srivastava, Tumor-specific cell surface expression of the-KDEL containing, endoplasmic reticular heat shock protein gp96, Int. J. Cancer 69 (1996) 340–349.
[10] D.L. Wiest, A. Bhandoola, J. Punt, G. Kreibich, D. McKean, A. Singer, Incomplete endoplasmic reticulum (ER) retention in immature thymocytes as revealed by surface expression of "ER-resident" molecular chaperones, Proc. Natl. Acad. Sci. USA 94 (1997) 1884–1889.
[11] N. Hilf, H. Singh-Jasuja, P. Schwarzmaier, C. Gouttefangeas, H.-G. Rammensee, H. Schild, Human platelets express heat shock protein receptors and regulate dendritic cell maturation, Blood 99 (2002) 3676–3682.
[12] R.M. Vabulas, S. Braedel, N. Hilf, H. Singh-Jasuja, S. Herter, P. Ahmad-Nejad, C.J. Kirschning, C. da Costa, H.-G. Rammensee, H. Wagner, H. Schild, The endoplasmic reticulum-resident heat shock protein gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway, J. Biol. Chem. 277 (2002) 20847–20853.
[13] H. Singh-Jasuja, H.U. Scherer, N. Hilf, D. Arnold-Schild, H.-G. Rammensee, R.E.M. Toes, H. Schild, The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor, Eur. J. Immunol. 30 (2000) 2211–2215.
[14] R.J. Binder, D.K. Han, P.K. Srivastava, CD91: a receptor for heat shock protein gp96, Nat. Immunol. 1 (2000) 151–155.
[15] P. Srivastava, Roles of heat-shock proteins in innate and adaptive immunity, Nat. Rev. Immunol. 2 (2002) 185–194.
[16] B. Liu, J. Dai, H. Zheng, D. Stoilova, S. Sun, Z. Li, Cell surface expression of an endoplasmic reticulum resident heat shock protein gp96 triggers MyD88-dependent systemic autoimmune diseases, Proc. Natl. Acad. Sci. USA 100 (2003) 15824–15829.
[17] Q.Q. Huang, R. Sobkoviak, A.R. Jockheck-Clark, B. Shi, A.M. Mandelin II, P.P. Tak, G.K. Haines III, C.V. Nicchitta, R.M. Pope, Heat shock protein 96 is elevated in rheumatoid arthritis and activates macrophages primarily via TLR2 signaling, J. Immunol. 182 (2009) 4965–4973.
[18] J.M. Han, N.H. Kwon, J.Y. Lee, S.J. Jeong, H.J. Jung, H.R. Kim, Z. Li, S. Kim, Identification of gp96 as a novel target for treatment of autoimmune disease in mice, PLoS ONE 5 (2010) e9792.
[19] S. Quevillon, F. Agou, J.-C. Robinson, M. Mirande, The p43 component of the mammalian multi-synthetase complex is likely to be the precursor of the endothelial monocyte-activating polypeptide II cytokine, J. Biol. Chem. 272 (1997) 32573–32579.
[20] S.G. Park, K.H. Jung, J.S. Lee, Y.J. Jo, H. Motegi, S. Kim, K. Shiba, Precursor of pro-apoptotic cytokine modulates aminoacylation activity of tRNA synthetase, J. Biol. Chem. 274 (1999) 16673–16676.
[21] S.W. Lee, G. Kim, S. Kim, Aminoacyl-tRNA synthetase-interacting multifunctional protein 1/p43 and emerging therapeutic protein working at systems level, Expert Opin. Drug Discov. 3 (2008) 945–957.
[22] Y.-G. Ko, H. Park, T. Kim, J.-W. Lee, S.G. Park, W. Seol, J.E. Kim, W.-H. Lee, S.-H. Kim, J.-E. Park, S. Kim, A cofactor of tRNA synthetase, p43, is secreted to up-regulate proinflammatory genes, J. Biol. Chem. 276 (2001) 23028–23033.
[23] H. Park, S.G. Park, J. Kim, Y.-G. Ko, S. Kim, Signaling pathways for TNF production induced by human aminoacyl-tRNA synthetase-associating factor, p43, Cytokine 20 (2002) 148–153.
[24] H. Park, S.G. Park, J.-W. Lee, T. Kim, G. Kim, Y.-G. Ko, S. Kim, Monocyte cell adhesion induced by a human aminoacyl-tRNA synthetase-associated factor, p43: identification of the related adhesion molecules and signal pathways, J. Leukoc. Biol. 71 (2002) 223–230.
[25] S.G. Park, Y.-S. Kang, Y.H. Ahn, S.H. Lee, K.-R. Kim, K.-W. Kim, G.Y. Koh, Y.-G. Ko, S. Kim, Dose-dependent biphasic activity of tRNA synthetase-associating factor, p43, in angiogenesis, J. Biol. Chem. 277 (2002) 45243–45248.
[26] S.G. Park, H. Shin, Y.K. Shin, Y. Lee, E.-C. Choi, B.-J. Park, S. Kim, The novel cytokine p43 stimulates dermal fibroblast proliferation and wound repair, Am. J. Pathol. 166 (2005) 387–398.
[27] S.G. Park, Y.S. Kang, J.Y. Kim, C.S. Lee, Y.G. Ko, W.J. Lee, K.-U. Lee, Y.I. Yeom, S. Kim, Hormonal activity of AIMP1/p43 for glucose homeostasis, Proc. Natl. Acad. Sci. USA 103 (2006) 14913–14918.
[28] J.M. Han, S.G. Park, B. Liu, B.-J. Park, J.Y. Kim, C.H. Jin, Y.W. Song, Z. Li, S. Kim, Aminoacyl-tRNA synthetase-interacting multifunctional protein 1/p43 controls endoplasmic reticulum retention of heat shock protein gp96: its pathological implications in lupus-like autoimmune diseases, Am. J. Pathol. 170 (2007) 2042–2054.
[29] G.M. Barton, R. Medzhitov, Toll-like receptor signaling pathways, Science 300 (2003) 1524–1525.
[30] E.D. Papadimitraki, C. Choulaki, E. Koutala, G. Bertsias, C. Tsatsanis, I. Gergianaki, A. Raptopoulou, H.D. Kritikos, C. Mamalaki, P. Sidiromos, D.T. Boumpas, Expansion of Toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: implications for the induction and maintenance of the autoimmune process, Arthritis. Rheum. 54 (2006) 3601–3611.