



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



The antiviral activity of poly- γ -glutamic acid, a polypeptide secreted by *Bacillus* sp., through induction of CD14-dependent type I interferon responses



Wooseong Lee^a, Seung-Hoon Lee^a, Dae-Gyun Ahn^a, Hee Cho^a, Moon-Hee Sung^b,
Seung Hyun Han^c, Jong-Won Oh^{a,*}

^a Department of Biotechnology and Center for Protein Function Control, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, Republic of Korea

^b Department of Advanced Fermentation Fusion Science and Technology, Kookmin University, Seoul 136-702, Republic of Korea

^c Department of Oral Microbiology and Immunology, Dental Research Institute, School of Dentistry, Seoul National University, Seoul 110-749, Republic of Korea

ARTICLE INFO

Article history:

Received 1 August 2013

Accepted 21 August 2013

Available online 6 September 2013

Keywords:

Poly- γ -glutamic acid

Interferon

TLR4

SARS coronavirus

Hepatitis C virus

Antiviral agent

ABSTRACT

Poly- γ -glutamic acid (γ -PGA) is an anionic polypeptide secreted by *Bacillus* sp. that has been shown to activate immune cells through interactions with toll-like receptor 4 (TLR4). However, its ability to induce the type I interferon (IFN) response has not yet been characterized. Here, we demonstrate that γ -PGA induces type I IFN signaling pathway via the TLR4 signaling pathway. The induction required both myeloid differentiation factor 2 (MD2) and the pattern-recognition receptor CD14, which are two TLR4-associated accessory proteins. The γ -PGA with high molecular weights (2000 and 5000 kDa) was able to activate the subsequent signals through TLR4/MD2 to result in dimerization of IRF-3, a transcription factor required for IFN gene expression, leading to increases in mRNA levels of the type I IFN-response genes, 2'-5' OAS and ISG56. Moreover, γ -PGA (2000 kDa) displayed an antiviral activity against SARS coronavirus and hepatitis C virus. Our results identify high-molecular weight γ -PGA as a TLR4 ligand and demonstrate that γ -PGA requires both CD14 and MD2 for the activation of type I IFN responses. Our results suggest that the microbial biopolymer γ -PGA may have therapeutic potential against a broad range of viruses sensitive to type I IFNs.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Toll-like receptor 4 (TLR4) is a receptor for lipopolysaccharide (LPS), a potent, pathogen-associated microbial pattern (PAMP) released by Gram-negative bacteria. LPS is recognized by the TLR4-myeloid-differentiation-factor-2 (-MD2) complex through hydrophobic interactions [1]; its recognition activates two downstream pathways: the myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway and the TIR domain-containing adaptor protein-inducing IFN- β (TRIF)-dependent pathway [2–5]. Recent evidence suggests that TLR4 signaling can also be activated by several non-LPS TLR4 ligands; a cell-wall skeletal component derived from *Mycobacterium* sp. and OK-432 derived from *Streptococcus* sp. [6] induce TLR4-mediated innate and acquired immune responses through NK and T-cell activation. In addition, increasing numbers of proteins that are TLR4 ligands have been identified [7].

For example, a 55-kDa protein isolated from the parasitic plant *Aeginetia indica* [8], the *Escherichia coli* fimbriae H protein [9], and the heat shock proteins Hsp60 and Hsp70 [10,11] have all been recently proposed as TLR4 ligands [12].

Poly- γ -glutamic acid (γ -PGA) is an anionic polypeptide in which D- and/or L-glutamate is polymerized via γ -amide linkages between the α -amino and γ -carboxylic acid functional groups. Anionic γ -PGA molecules with various molecular weights are produced and secreted by *Bacillus* species such as *Bacillus subtilis* (*natto*), a strain producing the traditional Japanese fermented food “natto”, and *B. subtilis* sp. Chungkookjang, a strain used for the production of a transitional Korean fermented seasoning made from soybeans [13]. This biodegradable, non-toxic microbial polymer has been used as a delivery agent for therapeutic proteins, protein vaccines, and anti-tumor drugs [14,15]. γ -PGA was also shown to activate immune cells, including macrophages, dendritic cells, and T lymphocytes, and induce the production of proinflammatory cytokines such as interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and interleukin-1 [16,17]. It was proposed that this *in vivo* activation of immune cells by γ -PGA is mediated by the

* Corresponding author. Tel.: +82 2 2123 2881; fax: +82 2 362 7265.
E-mail address: jwoh@yonsei.ac.kr (J.-W. Oh).

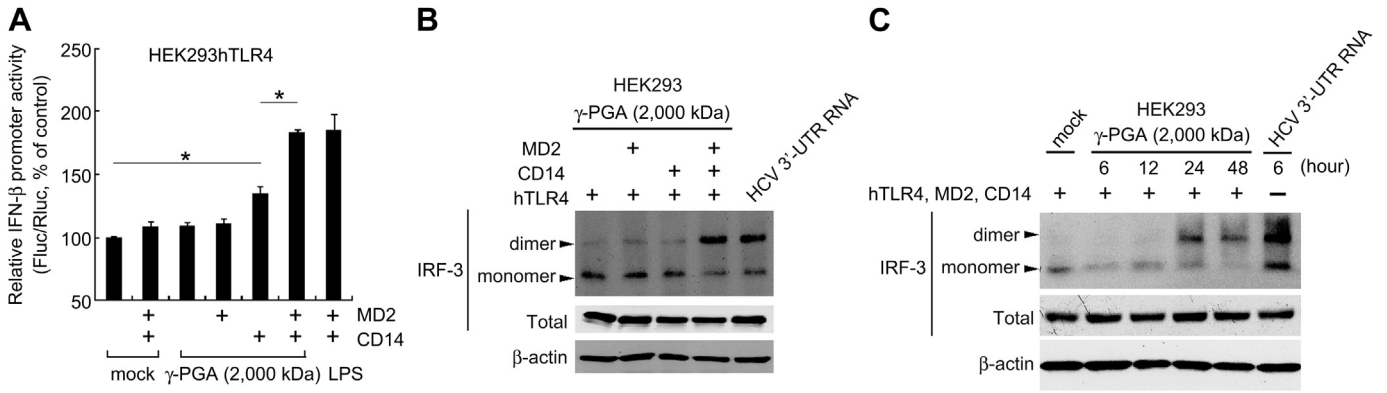


Fig. 1. Stimulation of TLR4 by γ -PGA requires the accessory factors MD2 and CD14 for induction of IFN- β production. (A) HEK293hTLR4 cells were transfected with plasmids to express the indicated proteins (MD2 and CD14) and with the luciferase expressing plasmids (IFN- β -pGL3 and pRL-TK) for the IFN- β promoter activity assay. After 12 h, the cells were treated with 100 μ M γ -PGA (MW = 2000 kDa) or 200 ng/ml LPS for 30 h and then harvested for dual luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity from the pRL-TK plasmid. Normalized luciferase activity (Fluc/Rluc) of mock-treated cells was defined as 100. Data are presented as means \pm SD of three measurements from three independent experiments. * $P < 0.05$ (Student *t*-test). (B and C) Dimerization of IRF-3 by γ -PGA treatment. HEK293 cells were transfected with plasmids individually expressing the indicated proteins. After 6 h, cells were treated with γ -PGA (MW = 2000 kDa) for 48 h (B) or for the indicated time periods (C). HEK293 cells transfected with HCV 3'-UTR transcripts (1 μ g/ml) using a liposome was analyzed at 6 h post-transfection. Cleared cell lysates were resolved on a native polyacrylamide gel (top panel) or a denaturing gel (middle and bottom panels) and analyzed by immunoblotting to detect IRF-3 and β -actin.

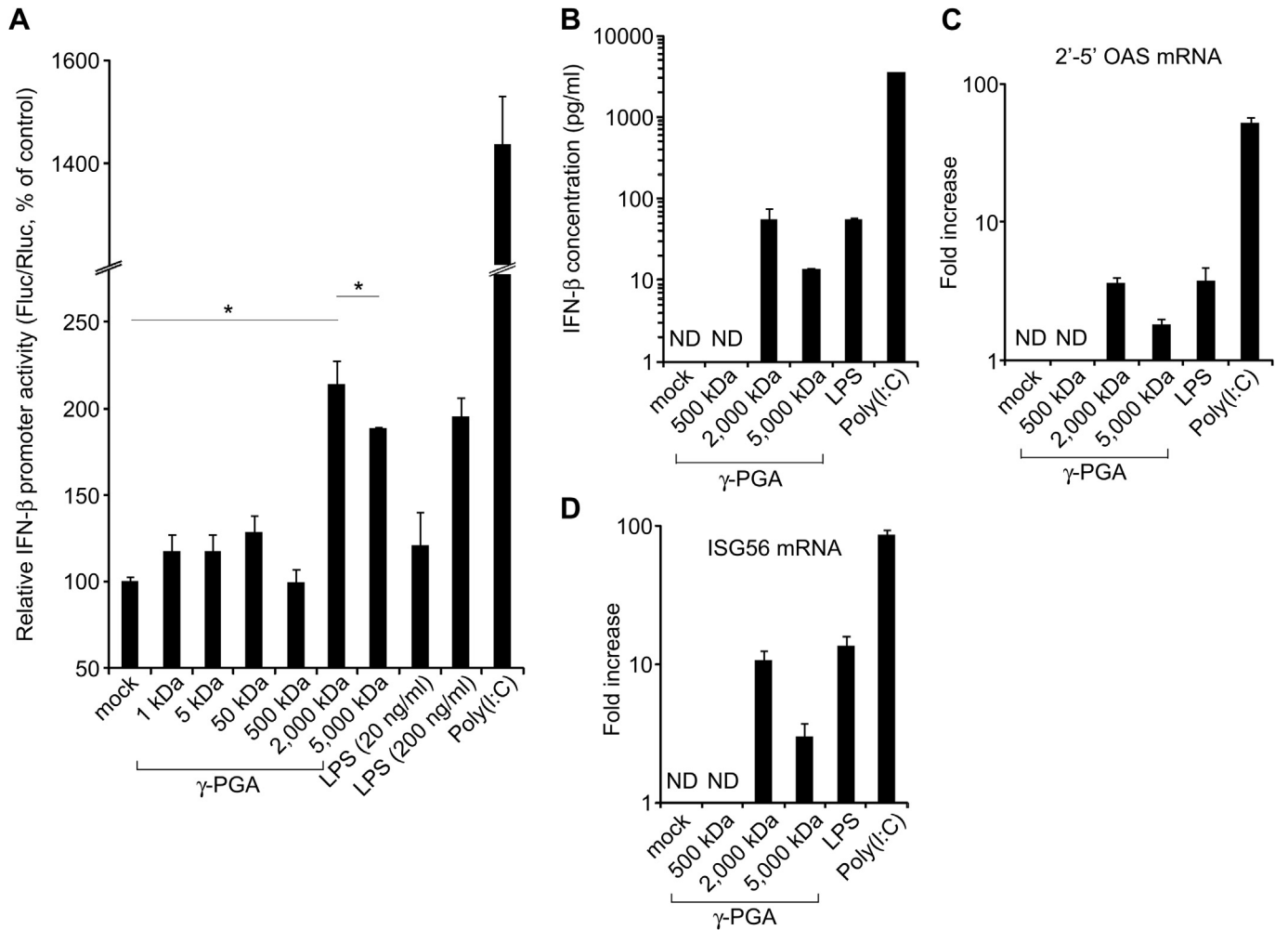


Fig. 2. Induction of type I IFN responses by high-molecular weight γ -PGAs. (A) HEK293hTLR4 cells grown in 6-well plates were transfected with plasmids expressing MD2 and CD14 (500 ng each/well) and with the luciferase expressing plasmids for the IFN- β promoter activity assay as in Fig. 1A. The transfected cells were treated with 100 μ M γ -PGAs with different molecular weights. As positive controls for the assay, the indicated concentrations of LPS and 0.2 μ g/ml poly(I:C) were used to stimulate IFN- β promoter activity. Data are presented as means \pm SD of three measurements from three independent experiments. * $P < 0.05$. (B–D) HEK293hTLR4 cells were transfected with plasmids expressing MD2 and CD14 and exposed to 100 μ M γ -PGAs with different molecular weights, 200 ng/ml LPS, or 0.1 μ g/ml poly(I:C) for 30 h and then harvested for analysis of the secreted IFN- β levels by ELISA (B), and the mRNA levels of the type I IFN-inducible genes 2'-5' OAS (C) and ISG56 (D) by real-time qRT-PCR. The GAPDH-normalized mRNA level in the mock-treated cells is considered one unit and the increase in mRNA abundance is shown as fold induction. ND = not detected. The data shown are from one representative of duplicate experiments with triplicate assays. The two experiments gave highly similar results.

TLR4-MyD88 signaling pathway. Furthermore, a recent report demonstrated the prophylactic effectiveness of intra-nasally administered γ -PGA against the influenza virus in mice [18]. The details, however, of how γ -PGA activates TLR4 and possibly induces the type I interferon (α and β) response have not yet been investigated.

This study was performed to uncover the molecular mechanism underlying γ -PGA-mediated activation of TLR4 signaling pathway and its impact on RNA virus replication. We investigated whether MD2 and the plasma membrane-localized pattern recognition receptor (PRR) CD14 are required as accessory molecules for γ -PGA to induce the TLR4-mediated IFN responses. We also assessed the antiviral activity of γ -PGA against severe acute respiratory syndrome coronavirus (SARS-CoV) and hepatitis C virus (HCV). Our

data validate the potential of γ -PGA as a broad-spectrum antiviral agent that triggers type I IFN signaling.

2. Materials and methods

2.1. Cells and culture

Human embryonic kidney 293 (HEK293) and HEK293hTLR4 (InvivoGen, San Diego, CA, USA) cell lines stably expressing human TLR4 were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 μ g/ml blasticidin S hydrochloride (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere of 5% CO₂. The Huh7 human hepatocellular carcinoma cell line was grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin and 1% nonessential amino acids. The plasmids expressing human CD14, MD2, and TLR4 were described previously [19,20]. The

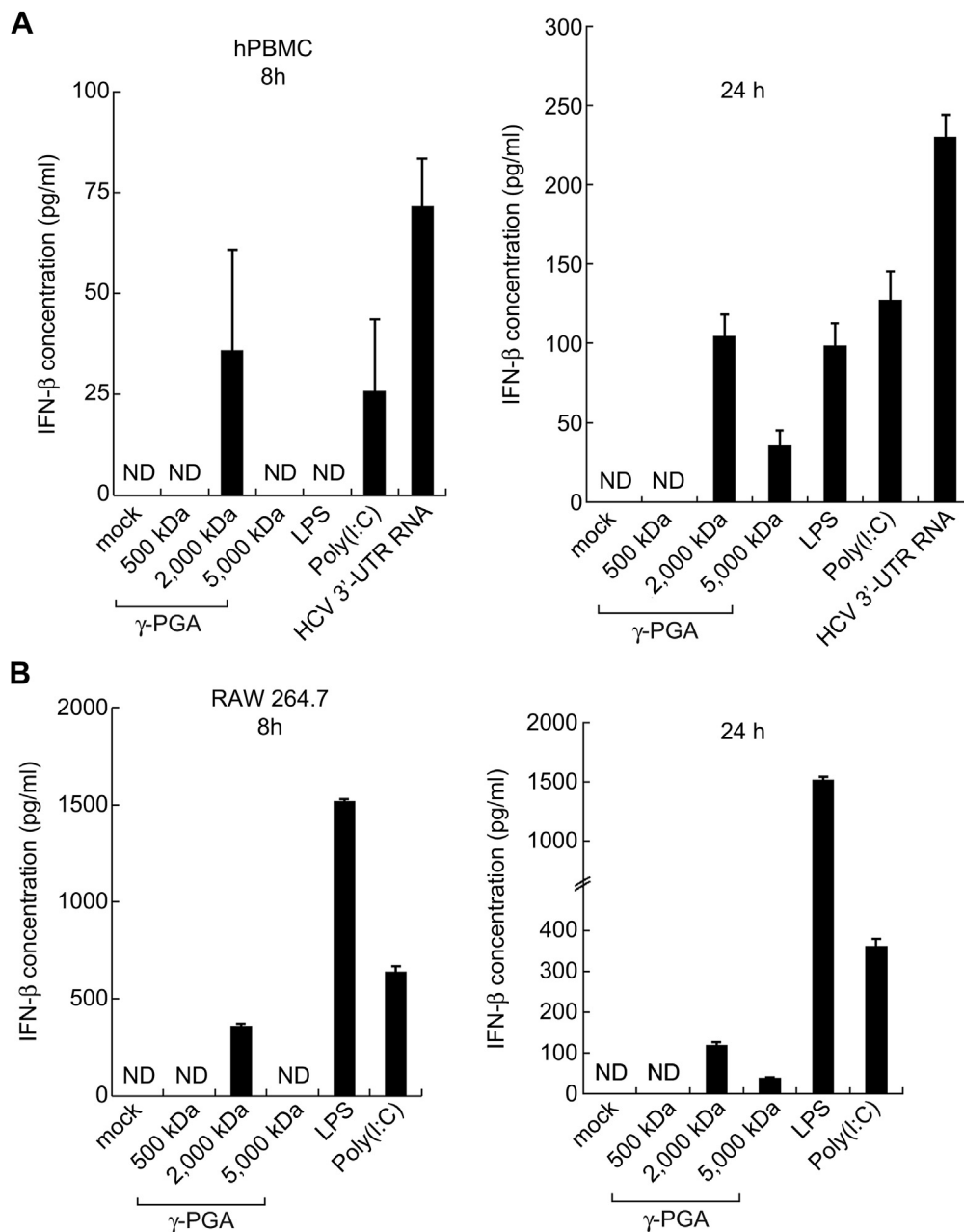


Fig. 3. Induction of IFN- β production by γ -PGA in hPBMC and mouse macrophage cells. hPBMCs (A) or RAW 264.7 cells (B) were treated with γ -PGA, LPS, or poly(I:C) as in Fig. 2A or transfected with HCV 3'-UTR *in vitro* transcripts as in Fig. 1C, for 8 h or 24 h. Levels of secreted IFN- β were measured by ELISA. Data from one of two independent experiments with similar results are shown.

experiment with human peripheral blood mononuclear cells (hPBMCs) was approved by the Institutional Review Board of Yonsei University. Human blood was obtained from the Blood Services, Western Blood Center of Korean Red Cross. PBMCs were isolated from heparinized peripheral blood from healthy volunteers by ficoll density gradient centrifugation. hPBMCs and the murine macrophage cell line RAW 264.7 were grown in DMED supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.2. Reagents and transfection

Endotoxin-free γ -PGAs with different average molecular weights (1, 5, 50, 500, 2000, and 5000 kDa) were prepared as described previously [16] and provided by BioLeaders (Daejeon, Korea). *E. coli* (O26:B6) LPS and polyinosinic:polycytidylic acid [poly(I:C)], a double-stranded RNA analog, were obtained from Sigma–Aldrich. Anti-IRF-3 (FL-425) and anti- β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. Interferon (IFN)- α A (I-4276) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Plasmids were transfected into cells using Fugene HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA). In the plasmid transfections, we added empty vector to normalize the total amounts of transfected DNA. Poly(I:C) and HCV 3'-untranslated region (UTR) *in vitro* transcript, prepared by T7 RNA polymerase-mediated *in vitro* transcription as previously described [21], were delivered into HEK293 using Lipofectamin RNAiMAX (Invitrogen, Carlsbad, CA, USA). hPBMCs and RAW 264.7 cells were transfected with those PAMP RNAs using the lipidoid ND98 (98N₁₂₋₅) (kindly provided by Prof. Seung-Woo Cho, Yonsei

University, Korea). The lipidoid-RNA complexes were prepared, as previously described [22]. HCV RNA was transfected into cells using TransIT-mRNA Transfection kit (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions or by electroporation as previously described [23].

2.3. Interferon β reporter assay

HEK293hTLR4 cells grown in 24-well plates were transfected with the plasmid expressing either human CD14 or MD2 (150 ng each) plus 150 ng of IFN β -pGL3, which expresses firefly luciferase under the control of the IFN β promoter and 150 ng of pRL-TK reporter (Promega, Madison, WI, USA), which expresses *Renilla* luciferase as an internal control. Twelve hours after transfection, cells were washed and fresh medium was added to the cells before stimulation with 100 µM γ -PGA, LPS (20 or 200 ng/ml), or poly(I:C) (200 ng/ml). Cells were harvested 30 h later and luciferase assays were performed using the Dual-Glo luciferase assay system (Promega), as described previously [24]. Firefly luciferase activity was normalized against *Renilla* luciferase activity to yield relative luciferase activity. All experiments were repeated at least three times and performed in triplicate. Data are presented as mean \pm SD.

2.4. Analysis of IRF-3 dimerization

Cells were lysed in a lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM tetrasodium pyrophosphate, 17.5 mM β -glycerophosphate, 1 mM 1 mM Na₃VO₄) supplemented with an EDTA-free protease

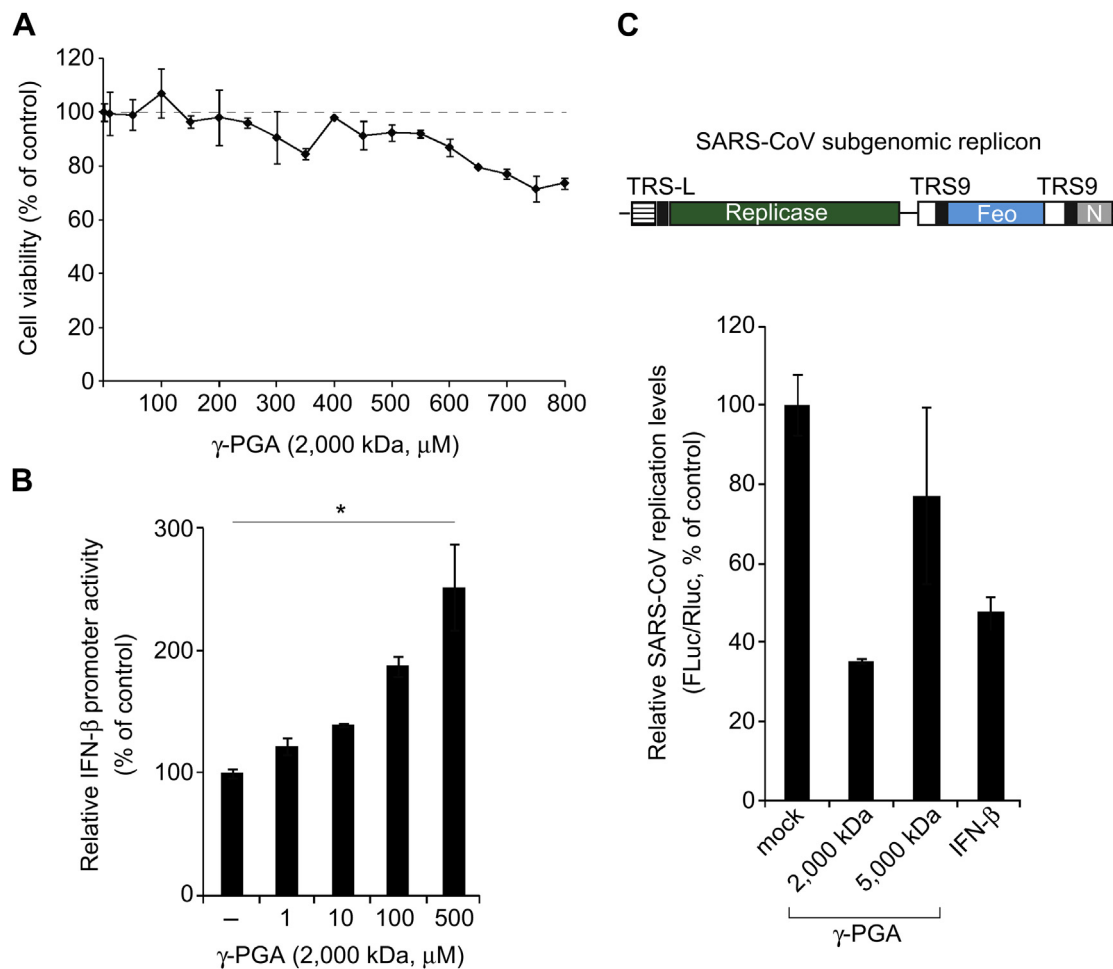
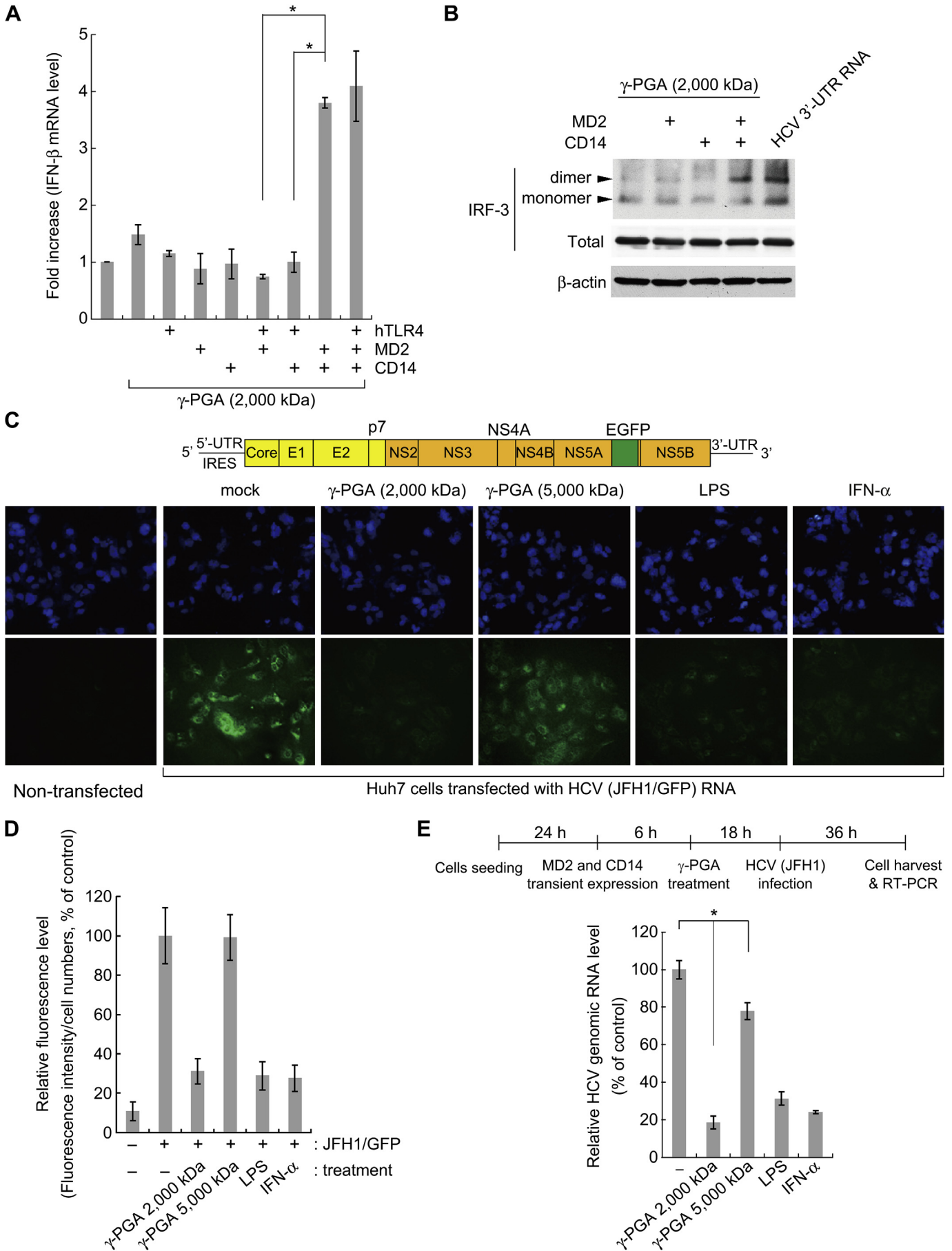


Fig. 4. Inhibition by γ -PGA of SARS-CoV replicon replication. (A) The cytotoxicity of γ -PGA (MW: 2000 kDa) in HEK293hTLR4 cells transiently expressing MD2 and CD14 was evaluated by the MTS assay. Cells were treated with increasing concentrations of γ -PGA for 30 h. Data are expressed as the percentage of the untreated control cells. (B) Relative IFN β -promoter activity was assessed as in Fig. 1 after the cells were treated with increasing concentrations of γ -PGA for 30 h. (C) Schematic diagram of the SARS coronavirus replicon (top panel), which expresses a luciferase reporter gene used for cell-based analysis of the antiviral activity of γ -PGA. The Feo gene (a firefly luciferase gene fused to a neomycin phosphotransferase gene) is expressed from subgenomic mRNA synthesized from transcription-regulating sequence 9 (TRS9), allowing quantitative evaluation of viral genome replication. HEK293hTLR4 cells were transfected with plasmids expressing MD2 and CD14 along with pSARS-REP-Feo producing the SARS-CoV replicon RNA, and pRL-TK. At 12 h post-transfection, cells were treated with 100 µM γ -PGA with the indicated molecular weight for 30 h and harvested for dual-luciferase assays. Firefly luciferase activity from the replicon plasmid was normalized to *Renilla* luciferase activity from the pRL-TK plasmid. The normalized luciferase activity of the mock-treated transfected cells was defined as 100. Data from one of two independent experiments with similar results are shown.



inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) by incubating on ice for 20 min. Protein samples (40 µg) were subjected to electrophoresis on a 7.5% native polyacrylamide gel and transferred to a nitrocellulose membrane. IRF-3 monomer and its dimer form were detected by Western blot analysis using an anti-human IRF-3 antibody and corresponding secondary antibody, as previously described [25]. Protein samples resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were analyzed for total IRF-3 and β-actin to verify equal protein loading. Membrane-bound antibodies were detected with the enhanced chemiluminescence kit (ECL, GE Healthcare Life Sciences, Piscataway, NJ, USA).

2.5. IFN-β measurement

HEK293hTLR4 cells transiently expressing CD14 and MD2 were stimulated with γ-PGA or other type I IFN-inducing molecules as described above for the IFN-β reporter assay. hPBMCs or RAW 264.7 cells were plated at a density of 1×10^6 cells/well in a 96-well round-bottom plate and treated with 100 µM γ-PGA or LPS (200 ng/ml), or transfected with 1 µg/ml of each of poly(I:C) and HCV 3'-UTR. After stimulation for 8 or 24 h, supernatants were collected by centrifugation. The amounts of IFN-β secreted from the stimulated cells were assessed using the VeriKine human or mouse IFN-β enzyme-linked immunosorbent assay (ELISA) kit (PBL Interferon Source, Piscataway, NJ, USA). The detection limits of the human and mouse IFN-β ELISAs were 25 and 15 pg/ml, respectively.

2.6. RT-quantitative PCR

Total RNA was isolated using Trizol reagent (Invitrogen). RT-qPCR analysis was performed using SYBR Premix ExTaq (Takara, Japan) and specific primers for IFN-β, IFN-stimulated gene 56 (ISG56), 2'-5'-oligoadenylate synthetase (OAS), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as described previously [21]. HCV genomic RNA levels were assessed by quantitative reverse transcription real-time PCR (qRT-PCR), as described previously [23].

2.7. Cell viability

The cytotoxicity of γ-PGA was measured using MTS [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent as described previously [24].

2.8. SARS-CoV replicon assay

HEK293hTLR4 cells ($\sim 8 \times 10^4$) plated onto each well of 24-well plates were transfected with 150 ng of plasmids expressing CD14 and MD2, respectively, and pRL-TK (150 ng) along with 1 µg pSARS-REP-Feo, a SARS-CoV replicon expressing the firefly luciferase reporter [21]. After 12 h, the transfected cells were treated with 100 µM γ-PGA for 30 h and harvested for dual luciferase assays as described previously [21].

2.9. HCV replication assay

HCV RNA genome with GFP reporter inserted at the NS5A-coding gene [26] was prepared by *in vitro* transcription using the MEGAScript T7 kit (Ambion, Austin, TX, USA) and used for transient HCV replication assays in Huh7 cells. Huh7 cells transfected with plasmids individually expressing MD2 and CD14 were treated with 100 µM PGA (MW 2000 or 5000 kDa) or 1 µg/ml LPS, prior to transfection with the chimeric HCV RNA encoding GFP. IFN-α (500 U/ml) was used as a positive control. At 24 h post-transfection of the HCV genome, cell images were captured with a confocal fluorescence microscope (Zeiss LSM 510) and GFP signal intensities were quantified using the Zeiss Zen 2011 software. Infectious JFH1 HCV (genotype 2a) RNA was prepared by *in vitro* transcription and electroporated into Huh7 cell, as previously described [27]. Virus particles were collected from the culture medium by centrifugation at 2000 ×g for 10 min, followed by passing through a 0.45 µm filter. Filtered culture medium was used for infection of Huh7.

3. Results

3.1. Activation of the IRF-3-dependent IFN-β promoter activity by γ-PGA requires the CD14 for signal transduction through the TLR4-MD2 complex

TLR4 is known to require multiple cofactors, including LPS-binding protein, MD2, and CD14, to respond to LPS [12]. CD14 is a glycosylphosphatidylinositol-anchored protein found on the surface of many TLR4-expressing cells [28]. CD14 interacts with various ligands including a variety of microbial products such as LPS [29–32] and peptidoglycan [33]; synthetic molecules such as the negatively charged, high-molecular weight nucleic acid poly(I:C) [34]; and CpG DNA [12,35]. We hypothesized that the TLR4 cofactors MD2 and/or CD14 may be required for γ-PGA to activate TLR4 and subsequent type I IFN responses. To test this hypothesis, we first assessed the ability of γ-PGA to activate the IFN-β promoter using a luciferase reporter plasmid carrying the binding site of IRF-3, a transcription factor activated by Tank-binding kinase 1 (TBK1) upon TLR4 stimulation [4,5,36]. We used the HEK293hTLR4 cell line stably expressing human TLR4, because the parental cell line HEK293 does not express TLR4 [37]. Moreover, other accessory molecules required for TLR4-mediated LPS sensing are deficient in HEK293 cells, making them unresponsive to LPS [37]. HEK293hTLR4 cells were thus transfected with sets of plasmids expressing either MD2, or CD14, or both, along with the reporter plasmids for the IFN-β promoter assay. After treating the transfected cells with 100 µM of 2000-kDa γ-PGA for 30 h, we performed a dual luciferase-activity assay. As shown in Fig. 1, γ-PGA led to activation of IFN-β promoter in a CD14-dependent manner. Expression of CD14 alone increased the promoter activity by 35%, whereas expression of MD2 alone failed to trigger the promoter activation. This result suggests that CD14 may act as a PRR for γ-PGA. Co-expression of MD2 with CD14 further increased the IFN-β promoter activity by up to 84%, which is comparable to the response induced by 200 ng/ml LPS. This result underscores the critical role of MD2 in the TLR4-mediated signaling pathway.

Activation of IRF-3 by TBK1-mediated phosphorylation upon TLR4 stimulation results in its dimerization and nuclear localization, which in turn activate the transcription of type I IFNs and other IFN-induced genes [4,5,36]. As shown in Fig. 1B, when MD2 and CD14 were co-expressed, IRF-3 dimerization was induced by γ-PGA, further confirming essential roles of these proteins in the activation of TLR4 signaling by γ-PGA. The degree of IRF-3 dimerization was comparable to that induced by HCV 3'-UTR transcripts containing 5' terminal triphosphate and poly-U/UC region, which is known to activate IRF-3 by being recognized by cytosolic retinoic acid-inducible gene I (RIG-I) [25]. The activation of IRF-3 by γ-PGA was delayed compared to 3'-UTR-mediated intracellular activation of type I interferon signaling pathway, probably because transfected genes had to be expressed prior to the TLR4 signaling activation (Fig. 1C). Collectively, these results show that γ-PGA is capable of eliciting the IFN-β response via TLR4 in a MD2- and CD14-dependent manner.

Fig. 5. Inhibition of HCV replication by γ-PGA by induction of type I IFN responses in the hepatocellular carcinoma cell line Huh7. (A) Stimulation of TLR4 by γ-PGA in Huh7 cells requires MD2 and CD14. Huh7 cells grown in 6-well plates were transfected with plasmids (500 ng each/well) individually expressing the indicated proteins for 24 h prior to treatment with 100 µM γ-PGA (MW = 2000 kDa). After 18 h, cells were harvested for analysis of the mRNA levels of IFN-β by real-time qRT-PCR. The GAPDH-normalized mRNA level in the mock-treated cells is considered one unit and the increase in mRNA abundance level is shown as fold induction. **P* < 0.01. (B) Huh7 cells were transfected with plasmids to express TLR4 along with MD2 and/or CD14 for 6 h prior to treatment with 100 µM γ-PGA (MW = 2000 kDa) or transfection with HCV 3'-UTR *in vitro* transcript used as a positive control. Dimerization of IRF-3 was analyzed as in Fig. 1B. (C and D) Huh7 cells transiently transfected with plasmids to express MD2 and CD14 were treated with 100 µM γ-PGA (MW 2000 or 5000 kDa) or 1 µg/ml LPS, prior to transfection with the chimeric HCV genome encoding GFP. GFP signal was detected under a fluorescence microscope at 24 h post-transfection (C) and its intensity was quantified (D). Data from one representative experiment from two independent experiments with similar results are shown. (E) Huh7 cells transiently expressing MD2 and CD14 were treated with γ-PGA or LPS as in (C) for 18 h prior to HCV infection. HCV genome copy number was estimated by qRT-PCR at 36 h post-transfection with HCV RNA and expressed as the percentage of the mock-treated control cells. Data present means ± SD of triplicate measurements from three independent experiments. **P* < 0.01.

3.2. Molecular weight-dependent induction of type I IFN responses by γ -PGA

γ -PGA is produced in various forms with diverse molecular weights by *Bacillus* sp. [13]. We tested whether the TLR4/MD2/CD14-mediated IFN- β induction by γ -PGA was dependent on the molecular weight of γ -PGA. We measured IFN- β promoter activity, as described above, in cells treated with γ -PGAs with average molecular weights of 1, 5, 50, 500, 2000, and 5000 kDa, respectively. As shown in Fig. 2A, we observed significant enhancement of IFN- β promoter activity only in cells treated with γ -PGAs with average molecular weights of 2000 kDa or greater. We also confirmed this molecular weight dependence by observing increased levels of IFN- β mRNAs in HEK293hTLR4 cells transiently expressing both MD2 and CD14 (data not shown). Consistent with the reporter assay, γ -PGA with a molecular weight of 2000 kDa resulted in the greatest IFN- β induction, as assessed by ELISA; whereas 500-kDa γ -PGA did not induce a detectable amount of IFN- β (Fig. 2B). These results suggest that TLR4 stimulation by γ -PGA might not simply be attributable to the amino acid repeat sequences of γ -PGA. Instead, more complex structures formed by HMW γ -PGAs might be critical to IFN- β production. The level of induction by this HMW γ -PGA was similar to that achieved by 200 ng/ml LPS, a well-characterized TLR4 ligand, but was lower than that achieved by transfection with poly(I:C). We used this dsRNA analog as a positive control because it is known as a potent viral double strand RNA mimic triggering type I IFN production through the cytosolic nucleic acid-recognition receptors including melanoma differentiation factor 5 (MDA5) and RIG-I [38].

IFN- β activates IFN- α expression via autocrine and paracrine pathways, leading to the induction of multiple IFN-stimulated genes (ISGs) including ISG56 and 2'-5' OAS [39]. We also found the expression of these two type I ISGs to be significantly increased by γ -PGA treatment (Fig. 2C and D). IFN- β induction by 2000-kDa γ -PGA, as well as the corresponding stimulation of ISG expression, was stronger than that achieved by 5000-kDa γ -PGA. These results suggest that larger γ -PGAs may form structures that interfere with the TLR4-mediated IFN signaling pathway or inhibit interaction with CD14.

The 2000-kDa γ -PGA also induced the IFN- β production in hPBMCs as effectively as LPS or other RNA PAMPs such as poly(I:C) and HCV 3'-UTR transcripts. The IFN- β induction by γ -PGA appeared to be faster than by LPS, although 24 h after treatment, the levels of IFN- β were comparable (Fig. 3A). The 5000-kDa γ -PGA resulted in IFN- β production at a level slightly higher than the detection limit of this assay (25 pg/ml). In mouse macrophage cells (RAW 264.7), IFN- β production induced by γ -PGA was greater at an early time point (8 h post-treatment) and more effective than in hPBMCs (Fig. 3B). In this cell line, LPS also induced the IFN- β production earlier (at 8 h post-treatment) than in hPBMCs. Altogether, our results clearly demonstrate the ability of HMW γ -PGA to induce type I IFN responses.

3.3. Inhibition of SARS coronavirus replicon replication by γ -PGA

We tested the effectiveness of γ -PGA as a protective antiviral agent. Taking advantage of the fact that among IFN- α , - β , and - γ , IFN- β is the most potent protector against SARS-CoV [40], we assessed the antiviral activity of γ -PGA against SARS-CoV using the pSARS-REP-Feo replicon plasmid (Fig. 4C, top panel). This replicon, which lacks the viral structural genes (except for the N capsid-protein gene), produces the viral RNA transcript from a cytomegalovirus promoter and a subgenomic RNA harboring Feo (a firefly luciferase gene fused to a neomycin phosphotransferase gene) from TRS9 to display luciferase activity. This replicon was

previously shown to replicate in HEK293 cells [21]. As shown in Fig. 4B, 2000-kDa γ -PGA induced IFN- β expression in a dose-dependent manner over the 1–500 μ M range in which γ -PGA showed no significant cytotoxicity on HEK293hTLR4 cells, as assessed by MTS assay (Fig. 4A). We evaluated the antiviral activity of γ -PGA in the HEK293hTLR4 cells transiently transfected with pSARS-REP-Feo and plasmids expressing MD2 and CD14. As shown in Fig. 4C, stimulation of the transfected cells with 100 μ M of 2000-kDa γ -PGA resulted in 65% reduction in luciferase activity, while IFN- β 1a, a potent IFN for suppressing SARS-CoV replication [40], reduced the reporter activity by 52%. The 5000-kDa γ -PGA, which induced IFN- β less efficiently than the 2000-kDa γ -PGA (Figs. 2 and 3), showed reduced antiviral activity, supporting the conclusion that IFN- β induction by γ -PGA is molecular weight dependent.

3.4. Antiviral activity of γ -PGA against hepatitis C virus

The current standard of care for patients with chronic hepatitis C is pegylated IFN plus the nucleoside analogue ribavirin [41]. To test the antiviral activity of γ -PGA against HCV, we first investigated whether the hepatocarcinoma cell line Huh7, which is the only available cell line efficiently supporting HCV replication and propagation [42], requires TLR4, and MD2 and/or CD14 for induction of IFN- β expression by γ -PGA. As shown in Fig. 5A, γ -PGA stimulation increased the IFN- β mRNA levels only when MD2 and CD14 were co-expressed, as demonstrated in HEK293 cells (Fig. 1). In consistent with previous studies demonstrating the expression of TLR4 in hepatocytes [43,44], TLR4 overexpression did not further stimulate the IFN- β expression. This increased IFN- β gene expression level was associated with IRF-3 dimerization (Fig. 5B), further confirming the capacity of γ -PGA to activate the IFN- β signaling pathway. Those MD2 and CD14-expressed Huh7 cells were treated with 2000-kDa γ -PGA and subsequently transfected with HCV genome encoding GFP reporter. Analysis of HCV replication levels, as assessed by GFP signal, showed that the treatment with 2000-kDa γ -PGA resulted in significant inhibition of HCV replication, whereas little inhibition was seen with 5000-kDa γ -PGA. More importantly, in HCV-infected cells, significant reduction in intracellular HCV genome titer (82% inhibition, $p < 0.01$) was observed in cells treated with 2000-kDa γ -PGA, while the 5000-kDa γ -PGA displayed marginal anti-HCV activity (Fig. 5E). The antiviral activity of 100 μ M 2000-kDa γ -PGA was comparable to that achieved with 1 μ g/ml LPS or 500 U/ml IFN- α , verifying the antiviral activity of γ -PGA in virus-infected cells.

4. Discussion

TLR4 activates two distinct signaling pathways that are regulated by the MyD88 and TRIF adaptor proteins to elicit the production of pro-inflammatory cytokines and type I IFNs, respectively [2–5]. The results presented here demonstrate that γ -PGA is an agonist that activates the type I IFN signaling pathway via the TLR4-MD2 complex. The type I IFN responses were only stimulated by γ -PGAs with molecular weights of 2000- and 5000-kDa, with the former one being more effective in stimulation of IFN- β production in both hPBMCs and murine macrophage. The IFN responses required CD14 as a PRR and led to inhibition of SARS-CoV replication, as assessed with a SARS-CoV replicon. In addition, substantial reduction in HCV genome titer was seen in HCV-infected Huh7 cells after treatment with 2000-kDa γ -PGA. In the present work, we used transformed cells such as HEK293 and Huh7 to investigate whether TLR4 and its cofactors are required for IFN- β gene induction by γ -PGA, via transient expression of those proteins, because observations would be blurred if dendritic cells or macrophages expressing multiple TLRs were used. Highlighting a functional role of γ -PGA as a TLR4 ligand, IFN- β secretion was also observed in hPBMCs and murine

macrophage. It is worthy of note that TLR4 expression increases under hepatic inflammation conditions [44] and that HCV infection induces TLR4 expression, leading to enhanced production of IFN- β in human B cells, particularly after LPS stimulation [43]. Furthermore, CD14 expression in hepatocytes is enhanced in inflammatory states [45] and during endotoxemia [46]. Thus, it is likely that IFN- β expression would be highly up-regulated by γ -PGA in virus-infected liver. The chief targets of SARS-CoV infection in the human lung are alveolar epithelium and macrophages, and the latter is predominant infiltrating immune cells in response to the virus infection [47]. Therefore, via paracrine interactions between Kupffer cells and hepatocytes in the liver or pulmonary alveolar epithelium and macrophages in the lungs, γ -PGA would potentially lead to synergistic antiviral activity *in vivo*. Recently, Moon et al. [18] demonstrated *in vivo* antiviral activity of nasally-delivered γ -PGA against influenza A virus, which was proposed to be attributable to the type II IFN (IFN- γ) produced mainly by antigen-presenting cells such as macrophages and dendritic cells. Our results, together with those of previous studies, suggest that γ -PGA can activate host innate immune responses to display antiviral activity by induction of both type I (α and β) and type II (γ) IFNs.

We demonstrated that both MD2 and CD14 are required for TLR4-dependent responses to γ -PGA. The crystal structure of TLR4-MD2 in complex with *E. coli* LPS shows that the hydrophobic lipid chains of LPS, a large amphipathic molecule with a molecular weight up to several ten thousand [48], are buried in the hydrophobic pocket of MD2 [1]. In addition, MD2 has been shown to play a role in the dimerization of TLR4; two MD2-LPS complexes are involved in bridging two TLR4 molecules because the two TLR4 molecules in the TLR4-MD2 heterodimer have limited direct interaction, as revealed by the crystal structure of TLR4 in complex with its ligand and MD2 [1]. We found that HEK293/hTLR4 cells transiently expressing MD2 failed to respond to γ -PGA in the absence of CD14. Thus, it is unlikely that MD2 directly interacts with γ -PGA, particularly when considering the hydrophilic nature of γ -PGA. MD2 coexpressed with CD14, however, allowed the activation of the IFN- β promoter and consequently resulted in IRF-3 activation, suggesting a functional role of MD2 in γ -PGA-mediated TLR4-pathway activation. One possible explanation for the requirement of MD2 would be its role in TLR4 expression on the cell surface [49,50].

Our data showed that γ -PGA-induced IFN- β production was increased by 35%, relative to controls, in HEK293/hTLR4 cells transiently expressing CD14 alone, suggesting an indispensable role for CD14 as a PRR of γ -PGA. The crystal structure of CD14 shows that the N-terminal hydrophobic pocket is the principle LPS-binding site [51]. Previous biochemical investigation and structural analysis of CD14, however, strongly suggest that the long, hydrophilic, negatively charged carbohydrate chain of LPS has some affinity for CD14 [51,52]. Consistent with those studies, CD14 does indeed interact with other negatively charged TLR ligands such as CpG DNA [12,35] and poly(I:C) [34]. Thus, the interaction of γ -PGA with CD14 is reminiscent of other hydrophilic molecules interacting with CD14 by as-yet-unknown mechanisms. Our data and the previous results discussed above suggest the role of CD14 as a PRR of multiple damage-associated molecular patterns, including PAMPs and the alarmins released in response to stress and tissue damage [12]. Nevertheless, it remains to be elucidated how CD14 can recognize different molecular compositions, particularly those of negatively charged molecules.

We found that γ -PGA with a molecular weight smaller than 2000 kDa did not induce IFN responses. Furthermore, IFN- β induction by 5000-kDa γ -PGA was attenuated compared with induction by 2000-kDa γ -PGA. It is not clear why γ -PGA activates TLR4-MD2 signaling transduction in such a molecular weight-dependent manner. γ -PGA may form diverse conformations (α -helix, β -sheet, random coil, enveloped aggregation) at different

pHs; it was proposed that at pH 6.0 a lower molecular weight (30 kDa) γ -PGA forms a linear random coil with polyanionic characteristics [14]. Although we still do not know what conformations or structures can be formed by HMW γ -PGAs, hydrophobically modified γ -PGA (molecular weight: 300 kDa), prepared by the covalent attachment of ι -phenylalanine ethyl ester, was shown to form nanoparticles due to its amphiphilic properties [53]. A γ -PGA nanoparticle with a mean diameter of 210 nm was recently shown to induce pro-inflammatory cytokine production via the TLR4 and MyD88 signaling pathways in mouse macrophages [54]. Those results, along with ours, are in some respects similar to the data presented in other recent studies demonstrating that LPS aggregates, but not monomers, are biologically active in the activation of TLR4-mediated inflammatory-cytokine production in human mononuclear cells [55,56]. It remains to be determined how the γ -PGAs, hydrophilic amino-acid-glutamate polymers with different molecular weights and/or structures, act as ligands of CD14 in the activation of the TLR4 and TRIF-dependent signaling pathway. This is a certainly challenging question that remains to be answered.

A recent report may give at least some clue as to the possible mechanisms explaining the attenuated IFN- β induction by the 5000-kDa γ -PGA. Zaroni et al. [28] showed that CD14 is required for LPS-induced TLR4 endocytosis in dendritic cells, which leads to the induction of TRIF signaling to promote IFN expression in a TLR4-containing endosomal vesicle. It is tempting to speculate that the γ -PGA-TLR4 complexes are subjected to CD14-mediated endocytosis, and that γ -PGAs with a molecular weight greater than 2000 kDa interferes with this endocytosis. We favor the hypothesis that, as shown with LPS [28], the γ -PGA-CD14/TLR4-MD2 complex is transported into endosomal vesicles to stimulate TRIF-mediated IFN expression. This hypothesis is consistent with previous findings that CD14 is marginally important for MyD88-dependent TNF- α induction by LPS, but essential for TRIF-mediated IFN expression in macrophages [57]. Although further studies are needed to clarify the precise entry mechanisms of the HMW γ -PGAs, CD14-dependent endocytosis of TLR4- γ -PGA complexes into endosomes and subsequent induction of TRIF-dependent signaling may contribute to the type I IFN responses providing antiviral protection.

5. Conclusions

Our results demonstrate that the anionic polypeptide γ -PGA secreted by *Bacillus* sp. induces innate immune responses through the TLR4-MD2 complex, resulting in an antiviral state. The stimulation of type I IFN production by γ -PGA required CD14 as a cofactor mediating the interaction of γ -PGA with the TLR4-MD2 complex in a molecular weight-dependent manner. Altogether, our results provide evidence that γ -PGA with a molecular weight of 2000 kDa may be used as a broad-spectrum antiviral agent against viruses sensitive to type I IFNs.

Acknowledgments

This work was supported by a grant from the National Research Foundation of Korea (NRF-2012-0008693) and in part by the Technology Innovation Program (MKE 10035159) funded by the Ministry of Knowledge Economy (MKE, Korea) and by grants from the National Research Foundation of Korea funded by the Korean Government (MSIP) (NRF 2009-0092959, 2012-047807, and 2012-0008693).

References

- [1] Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 2009;458:1191–5.

- [2] Baccala R, Hoebe K, Kono DH, Beutler B, Theofilopoulos AN. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nat Med* 2007;13:543–51.
- [3] Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls toll-like receptor signaling. *Cell* 2006;125:943–55.
- [4] Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* 2008;9:361–8.
- [5] Tanimura N, Saitoh S, Matsumoto F, Akashi-Takamura S, Miyake K. Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. *Biochem Biophys Res Commun* 2008;368:94–9.
- [6] Okamoto M, Furuichi S, Nishioka Y, Oshikawa T, Tano T, Ahmed SU, et al. Expression of toll-like receptor 4 on dendritic cells is significant for anticancer effect of dendritic cell-based immunotherapy in combination with an active component of OK-432, a streptococcal preparation. *Cancer Res* 2004;64:5461–70.
- [7] Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. *Nat Rev Cancer* 2009;9:57–63.
- [8] Okamoto M, Oh EG, Oshikawa T, Furuichi S, Tano T, Ahmed SU, et al. Toll-like receptor 4 mediates the antitumor host response induced by a 55-kilodalton protein isolated from *Aeginetia indica* L., a parasitic plant. *Clin Diagn Lab Immunol* 2004;11:483–95.
- [9] Abdul-Careem MF, Firoz Mian M, Gillgrass AE, Chenoweth MJ, Barra NG, Chan T, et al. FimH, a TLR4 ligand, induces innate antiviral responses in the lung leading to protection against lethal influenza infection in mice. *Antiviral Res* 2011;92:346–55.
- [10] Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* 2000;164:558–61.
- [11] Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H. HSP70 as endogenous stimulus of the toll/interleukin-1 receptor signal pathway. *J Biol Chem* 2002;277:15107–12.
- [12] Lee CC, Avalos AM, Ploegh HL. Accessory molecules for toll-like receptors and their function. *Nat Rev Immunol* 2012;12:168–79.
- [13] Sung MH, Park C, Kim CJ, Poo H, Soda K, Ashiuchi M. Natural and edible biopolymer poly-gamma-glutamic acid: synthesis, production, and applications. *Chem Rec* 2005;5:352–66.
- [14] Liao ZX, Peng SF, Ho YC, Mi FL, Maiti B, Sung HW. Mechanistic study of transfection of chitosan/DNA complexes coated by anionic poly(gamma-glutamic acid). *Biomaterials* 2012;33:3306–15.
- [15] Xiong Y, Jiang W, Shen Y, Li H, Sun C, Ouahab A, et al. A poly(gamma, l-glutamic acid)-citric acid based nanoconjugate for cisplatin delivery. *Biomaterials* 2012;33:7182–93.
- [16] Kim TW, Lee TY, Bae HC, Hahm JH, Kim YH, Park C, et al. Oral administration of high molecular mass poly-gamma-glutamate induces NK cell-mediated antitumor immunity. *J Immunol* 2007;179:775–80.
- [17] Lee TY, Kim YH, Yoon SW, Choi JC, Yang JM, Kim CJ, et al. Oral administration of poly-gamma-glutamate induces TLR4- and dendritic cell-dependent antitumor effect. *Cancer Immunol Immunother* 2009;58:1781–94.
- [18] Moon HJ, Lee JS, Choi YK, Park JY, Talactac MR, Chowdhury MY, et al. Induction of type I interferon by high-molecular poly-gamma-glutamate protects B6.A2G-Mx1 mice against influenza A virus. *Antiviral Res* 2012;94:98–102.
- [19] Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI. Human toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol* 2002;3:354–9.
- [20] Hajjar AM, O'Mahony DS, Ozinsky A, Underhill DM, Aderem A, Klebanoff SJ, et al. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulins. *J Immunol* 2001;166:15–9.
- [21] Ahn DG, Lee W, Choi JK, Kim SJ, Plant EP, Almazan F, et al. Interference of ribosomal frameshifting by antisense peptide nucleic acids suppresses SARS coronavirus replication. *Antiviral Res* 2011;91:1–10.
- [22] Akinc A, Zumbuehl A, Goldberg M, Leshchiner ES, Busini V, Hossain N, et al. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat Biotechnol* 2008;26:561–9.
- [23] Sun JM, Kim SJ, Kim GW, Rhee JK, Kim ND, Jung H, et al. Inhibition of hepatitis C virus replication by monascus pigment derivatives that interfere with viral RNA polymerase activity and the mevalonate biosynthesis pathway. *J Antimicrob Chemother* 2012;67:49–58.
- [24] Ahn DG, Shim SB, Moon JE, Kim JH, Kim SJ, Oh JW. Interference of hepatitis C virus replication in cell culture by antisense peptide nucleic acids targeting the X-RNA. *J Viral Hepat* 2011;18:e298–306.
- [25] Iwamura T, Yoneyama M, Yamaguchi K, Suhara W, Mori W, Shiota K, et al. Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways. *Genes Cells* 2001;6:375–88.
- [26] Wu Y, Liao Q, Yang R, Chen X. A novel luciferase and GFP dual reporter virus for rapid and convenient evaluation of hepatitis C virus replication. *Virus Res* 2011;155:406–14.
- [27] Kim SJ, Kim JH, Sun JM, Kim MG, Oh JW. Suppression of hepatitis C virus replication by protein kinase C-related kinase 2 inhibitors that block phosphorylation of viral RNA polymerase. *J Viral Hepat* 2009;16:697–704.
- [28] Zanonli I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, et al. CD14 controls the LPS-induced endocytosis of toll-like receptor 4. *Cell* 2011;147:868–80.
- [29] Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249:1431–3.
- [30] da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex: transfer from CD14 to TLR4 and MD-2. *J Biol Chem* 2001;276:21129–35.
- [31] Gioannini TL, Teghanemt A, Zhang D, Coussens NP, Dockstader W, Ramaswamy S, et al. Isolation of an endotoxin-MD-2 complex that produces toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc Natl Acad Sci U S A* 2004;101:4186–91.
- [32] Moore KJ, Andersson LP, Ingalls RR, Monks BG, Li R, Arnaout MA, et al. Divergent response to LPS and bacteria in CD14-deficient murine macrophages. *J Immunol* 2000;165:4272–80.
- [33] Dziarski R, Tapping RI, Tobias PS. Binding of bacterial peptidoglycan to CD14. *J Biol Chem* 1998;273:8680–90.
- [34] Weber C, Muller C, Podszuweit A, Montino C, Vollmer J, Forsbach A. Toll-like receptor (TLR) 3 immune modulation by unformulated small interfering RNA or DNA and the role of CD14 (in TLR-mediated effects). *Immunology* 2012;136:64–77.
- [35] Baumann CL, Aspalter IM, Sharif O, Pichlmair A, Bluml S, Grebien F, et al. CD14 is a coreceptor of Toll-like receptors 7 and 9. *J Exp Med* 2010;207:2689–701.
- [36] Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, et al. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 2002;17:251–63.
- [37] Latz E, Visintin A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA, et al. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J Biol Chem* 2002;277:47834–43.
- [38] Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, et al. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-1 and melanoma differentiation-associated gene 5. *J Exp Med* 2008;205:1601–10.
- [39] Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. *Nat Rev Immunol* 2008;8:559–68.
- [40] Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr HW. Treatment of SARS with human interferons. *Lancet* 2003;362:293–4.
- [41] Foster GR. Pegylated interferons for the treatment of chronic hepatitis C: pharmacological and clinical differences between peginterferon-alpha-2a and peginterferon-alpha-2b. *Drugs* 2010;70:147–65.
- [42] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11:791–6.
- [43] Machida K, Cheng KT, Sung VM, Levine AM, Fong S, Lai MM. Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6. *J Virol* 2006;80:866–74.
- [44] Soares JB, Pimentel-Nunes P, Afonso L, Rolanda C, Lopes P, Roncon-Albuquerque Jr R, et al. Increased hepatic expression of TLR2 and TLR4 in the hepatic inflammation-fibrosis-carcinoma sequence. *Innate Immun* 2012;18:700–8.
- [45] Chou MH, Chuang JH, Eng HL, Chen CM, Wang CH, Chen CL, et al. Endotoxin and CD14 in the progression of biliary atresia. *J Transl Med* 2010;8:138.
- [46] Liu S, Khemlani LS, Shapiro RA, Johnson ML, Liu K, Geller DA, et al. Expression of CD14 by hepatocytes: upregulation by cytokines during endotoxemia. *Infect Immun* 1998;66:5089–98.
- [47] Nicholls JM, Butany J, Poon LL, Chan KH, Beh SL, Poutanen S, et al. Time course and cellular localization of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. *PLoS Med* 2006;3:e27.
- [48] Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002;71:635–700.
- [49] Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4. *J Exp Med* 1999;189:1777–82.
- [50] Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* 2002;3:667–72.
- [51] Kim JI, Lee CJ, Jin MS, Lee CH, Paik SG, Lee H, et al. Crystal structure of CD14 and its implications for lipopolysaccharide signaling. *J Biol Chem* 2005;280:11347–51.
- [52] Kitchens RL, Munford RS. Enzymatically deacylated lipopolysaccharide (LPS) can antagonize LPS at multiple sites in the LPS recognition pathway. *J Biol Chem* 1995;270:9904–10.
- [53] Akagi T, Kaneko T, Kida T, Akashi M. Preparation and characterization of biodegradable nanoparticles based on poly(gamma-glutamic acid) with l-phenylalanine as a protein carrier. *J Control Release* 2005;108:226–36.
- [54] Uto T, Akagi T, Yoshinaga K, Toyama M, Akashi M, Baba M. The induction of innate and adaptive immunity by biodegradable poly(gamma-glutamic acid) nanoparticles via a TLR4 and MyD88 signaling pathway. *Biomaterials* 2011;32:5206–12.
- [55] Mueller M, Lindner B, Kusumoto S, Fukase K, Schromm AB, Seydel U. Aggregates are the biologically active units of endotoxin. *J Biol Chem* 2004;279:26307–13.
- [56] Richter W, Vogel V, Howe J, Steiniger F, Brauser A, Koch MH, et al. Morphology, size distribution, and aggregate structure of lipopolysaccharide and lipid A dispersions from enterobacterial origin. *Innate Immun* 2011;17:427–38.
- [57] Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, et al. CD14 is required for MyD88-independent LPS signaling. *Nat Immunol* 2005;6:565–70.