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CD14 directly binds to triacylated lipopeptides and facilitates recognition of the lipopeptides by the receptor complex of Toll-like receptors 2 and 1 without binding to the complex

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Running title: CD14-induced enhancement of recognition of lipopeptides by TLRs

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Summary

It has demonstrated that the recognition of triacylated lipopeptides by Toll-like receptor (TLR) 2 requires TLR1 as a coreceptor. In the NF- κ B reporter assay system in which human embryonic kidney 293 cells were transfected with TLR2 and TLR1 together with an NF- κ B luciferase reporter gene, S-(2,3-bisphosphatidyl)-N-palmitoyl-Cys-Lys-Lys-Lys-Lys (Pam₃CSK₄) and Pam₃CSSNA were recognized by TLR 2/TLR1, but the recognition level was unexpectedly very low. However, cotransfection of CD14 drastically enhanced the recognition of triacylated lipopeptides by TLR2/TLR1. The CD14-induced enhancement did not occur without cotransfection of TLR1. Both CD14^{dS39-A48}, a mutant with deletion of the part of possible N-terminal ligand-binding pocket, and anti-CD14 monoclonal antibody reduced the CD14-induced enhancement. Transfection of a TIR domain-deficient mutant of TLR2 (TLR2^{dE772-S784}) or TLR1 (TLR1^{dQ636-K779}) completely abrogated the CD14-induced enhancement. Soluble recombinant CD14 added extracellularly enhanced the recognition of Pam₃CSSNA by TLR2/TLR1. Immunoprecipitation analysis demonstrated that CD14 was not associated with TLR2 but that TLR1 was associated with TLR2. In addition, surface plasmon resonance-based assay demonstrated that CD14 binds to Pam₃CSK₄ at a dissociation constant of 5.7 μ M.

This study suggests that CD14 directly binds to triacylated lipopeptides and facilitates recognition of the lipopeptides by the TLR2/TLR1 complex without binding to the receptor complex.

Introduction

Lipoprotein (LP) is a bacterial cell wall component involved in the systemic inflammatory response caused by gram-negative and gram-positive bacteria as well as in chronic inflammatory disorders caused by spirochetes *Borrelia burgdorferi* and *Treponema pallidum* (1-4). LP was first characterized and sequenced from *Escherichia coli* by Braun (5), and it has been demonstrated to be biologically active (6-10). The part of LP responsible for biological activities has been demonstrated to be the N-terminal lipopeptide (LPT) moiety, the structure of which is S-(2,3-bispalmitoyloxypropyl)-N-palmitoyl-Cys-Ser-Ser-Asn-Ala- (Pam₃CSSNA-) (6).

Mycoplasmas, wall-less microorganisms, also possess LP/LPT capable of activating macrophages or fibroblasts (11-15). Mühlrad *et al.* (12, 13) identified a 2-kD LPT called MALP-2 from *Mycoplasma fermentans* that is capable of activating monocytes/macrophages, and they determined the structure to be Pam₂CGNNDENISFKEK. We have also found that a 44-kD LP (LP44) from *M. salivarium* activates normal human gingival fibroblasts and macrophages (14, 15). In addition, the structure of the N-terminal LPT moiety of LP44 has been determined to be Pam₂CGDPKHPKSFTGWVA- (15). On the basis of the N-terminal structure of LP44, the diacylated LPT Pam₂CGDPKHPKSF (FSL-1) possessing the same activity as that of LP44 was synthesized (15).

Microbes and their components are capable of activating innate immune responses through being recognized by Toll-like receptors (TLRs) (16). To date, more than ten human TLRs have been identified and have been shown to be critical for signaling by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan (PGN), and LP/LPT (16). The activation of innate immunity by TLRs also leads to the development of antigen-specific adaptive immunity. Thus, TLRs play a key role in bridging between innate immunity and adaptive immunity (16).

TLR2, of all TLRs identified, has the broadest specificity. That is, TLR2 recognizes a wide variety of structurally different PAMPs, such as LP/LPT, PGN, lipoarabinomannan, glycosylphosphatidylinositol- anchored protein of the protozoa *Trypanosoma cruzi*, neisserial porin and yeast zymosan (16). Therefore, we are very much interested in knowing how TLR2 recognizes these PAMPs. It has been demonstrated that TLR2 requires TLR6 and TLR1 as a coreceptor for the recognition of diacylated and triacylated LPTs, respectively (16). However, we have found that TLR6 is dispensable for the recognition of FSL-1 by TLR2 in the NF- κ B luciferase reporter assay, although the cotransfection of TLR6 enhances the recognition of FSL-1 by TLR2 (17, 18). Very recently, Buwitt-Beckmann *et al.* (19) have found that TLR2 does not always require TLR6 for the recognition of diacylated LPTs by TLR2 and that the requirement is dependent upon the amino acid sequence and length of the peptide portion and the fatty acid of LPTs. Thus, details of the molecular mechanism by which TLR2 recognizes microbial LP/LPT still remain unknown. Therefore, we are now trying to determine the molecular mechanism (17, 18). We have so far demonstrated that leucine residues located at the conserved region of a leucine-rich repeat motif play a key role in the recognition of FSL-1 and PGN by TLR2 and that both lipid and peptide portions of FSL-1 were recognized by TLR2 (17, 18).

Recent studies have revealed that another LPS receptor, CD14, facilitates the binding of LPS to the TLR4–MD-2 complex and consequent intracellular signaling (20). CD14 also interacts with soluble TLR2 (sTLR2) and facilitates the binding of sTLR2 to PGN (21). Using fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) imaging techniques to study molecular associations, Manukyan *et al.* (22) found that binding of triacylated LPT to CD14 is the first step in the LPT recognition, inducing physical proximity of CD14 and LPT with the TLR2/TLR1 complex and formation of the TLR2 signaling complex. Thus, they suggest that CD14, triacylated LPT, TLR2 and TLR1 form a tetramolecular complex, which is required for the activation of macrophages by LPT. However, the molecular mechanism by which CD14 plays a key role in the recognition of LPT by the TLR2/TLR1 complex is not fully understood yet.

In this study we found that CD14 was able to enhance the recognition of triacylated LPTs, Pam₃CSSNA and Pam₃CSK₄, by the TLR2/TLR1 complex, that the N-terminal part of CD14 was involved in the enhancement, and that the signal of the CD14-induced enhancement was mediated by both TLR2 and TLR1 of the receptor complex, whereas CD14 itself was not associated with the TLR2/TLR1 complex. In addition, we found that CD14 directly bound to Pam₃CSK₄ at a dissociation constant (K_D) of 5.7 μ M but that CD14 did not bind to the diacylated LPT FSL-1.

Results

CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1

Gene targeting studies have clearly demonstrated that the recognition of diacylated and triacylated LPTs by TLR2 requires TLR6 and TLR1 as a coreceptor, respectively (16). To confirm this in our NF- κ B reporter assay system in which human embryonic kidney 293 (HEK293) cells were transfected with TLR2, TLR1 or TLR6 together with an NF- κ B luciferase reporter gene, we examined whether FSL-1 as well as Pam₃CSK₄ and Pam₃CSSNA were recognized by TLR2/TLR6 or TLR2/TLR1. It was found that FSL-1 was recognized by TLR2/TLR1 as well as TLR2/TLR6 in this system, although the recognition level by TLR2/TLR6 was higher than that by TLR2/TLR1 (Fig. 1). However, it is thought that cotransfection of TLR1 has no effect on the recognition of FSL-1 by TLR2, because we have already found that FSL-1 is recognized by only TLR2 in this system (17). In addition, it was found that Pam₃CSK₄ and Pam₃CSSNA were recognized by TLR2/TLR1, but, unexpectedly, the recognition level was very low in this system (Fig. 1). Therefore, we thought that other molecules such as CD14 are required for the full recognition, since CD14 is known to enhance the recognition of PGN, LP and LPS by TLRs (20, 21, 23-26). It was found that the cotransfection of CD14 drastically enhanced the recognition of Pam₃CSSNA by TLR2/TLR1 (Fig. 2A), whereas CD14 did not enhance the recognition of the diacylated LPT FSL-1

by TLR2/TLR1 (Fig. 2B). Interestingly, the CD14-induced enhancement did not occur without cotransfection of TLR1 (Fig. 2A). In addition, the CD14-induced enhancement of the recognition was dependent on the amount of CD14 gene transfected (data not shown), confirming that CD14 has activity to enhance the recognition of triacylated LPTs by TLR2/TLR1.

Knockdown of TLR1 by RNA interference

In order to further confirm the necessity of TLR1 in the CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1, TLR1 was silenced by transiently transfecting HEK293 cells with a silencing vector expressing a siRNA targeting the human TLR1 gene (psiRNA-hTLR1) construct together with genes of TLR2 and CD14. First, we investigated whether the transcription of TLR1 was downregulated by the transfection with psiRNA-hTLR1, and found that the transcription of TLR1 was clearly suppressed by the transfection (Fig. 3A). Next, experiments were carried out to determine whether the CD14-induced enhancement of the recognition of triacylated LPT by TLR2/TLR1 was downregulated by silencing of TLR1. It was found that the CD14-induced enhancement of the recognition of Pam₃CSK₄ was reduced by knockdown of TLR1 (Fig. 3B), whereas knockdown of TLR1 had no effect on the recognition of FSL-1 by TLR2 and/or TLR1 (Fig. 3C). These results suggest that TLR1 is indispensable for the CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1.

Effect of CD14^{dS38-A48} on the recognition of triacylated LPTs by TLR2/TLR1

Muroi *et al.* (27) demonstrated that the amino acid regions of 35-44 and 235-243 of mouse CD14 are required for TLR2-mediated activation of NF- κ B. More recently, Kim *et al.* (28) have presented the crystal structure of mouse CD14 at a 2.5 Å resolution and speculated that a large hydrophobic pocket found on the N-terminal side of the horseshoe-like structure of mouse CD14 is the main

component of the LPS-binding site. On the basis of these findings, we speculated that the N-terminal portion of CD14 is involved in the CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1. Therefore, we first made a deletion mutant of S39-A48 of CD14 (CD14^{dS39-A48}), the region of which almost corresponds to S35-A44 of mouse CD14 (27), and examined whether CD14^{dS39-A48} was able to enhance the recognition of Pam₃CSSNA and Pam₃CSK₄ by TLR2/TLR1. The effect of an anti-CD14 monoclonal antibody (Ab), which is known to suppress the biological functions of CD14, on the CD14-induced enhancement was also examined. It was found that the recognition of Pam₃CSK₄ and Pam₃CSSNA by TLR2/TLR1 was enhanced by transfection of CD14^{dS39-A48}, but the enhancement level was significantly lower than that obtained by transfection of CD14 (Fig. 4A and 4B). The anti-CD14 monoclonal Ab also significantly, but not completely, downregulated the CD14-induced enhancement of the recognition (Fig. 4A and 4B). These results suggest that the amino acid region of S39-A48 of CD14 plays a key role in the CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1.

Signaling of the CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1

It is of great interest to know which coreceptor, TLR2 or TLR1, forming the receptor complex triggers signal of the CD14-induced enhancement of the recognition of triacylated LPTs by the receptor complex. Therefore, we made mutants of TLR2 (TLR2^{dE772-S784}) and TLR1 (TLR1^{dQ636-K779}) in which the TIR domain was deleted from TLR2 and TLR1, respectively. These mutant genes were transfected into HEK293 cells instead of TLR2 or TLR1, and then the NF- κ B reporter activity was measured after stimulation with Pam₃CSSNA. The transfection of TLR2^{dE772-S784} or TLR1^{dQ636-K779} completely abrogated the CD14-induced enhancement of the recognition of Pam₃CSSNA by TLR2/TLR1 (Fig. 5A and 5B).

Taken together, these results suggest that the signal of CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1 is mediated by signaling pathways triggered by

both TLR2 and TLR1 of the receptor complex.

Enhancement of the TLR2/TLR1-mediated recognition of triacylated LPTs by soluble recombinant CD14

As a first step to clarify the mechanism by which CD14 enhanced the recognition of Pam₃CSSNA by TLR2/TLR1, we examined whether soluble recombinant CD14 enhanced the recognition. It was found that CD14 added extracellularly also enhanced the recognition of Pam₃CSSNA by TLR2/TLR1 in a dose-dependent manner and that 5 µg/ml of soluble recombinant CD14 enhanced the recognition to the same level as did 150 ng of CD14 gene used for transfection (Fig. 6A). This result suggests that CD14 affects the interaction of triacylated LPTs with the receptor complex of TLR2 and TLR1 on the cell surface of the transfectants.

Effects of CD14 on the surface expression of TLR2 in HEK293 cells

To define the molecular mechanisms underlying the CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1, we speculated that the CD14-induced enhancement is due to upregulation of TLR2 surface expression in HEK293 transfectants by cotransfection of CD14 and/or TLR1. Therefore, we examined whether surface expression of TLR2 in HEK293 cells was upregulated by cotransfection of CD14 and/or TLR1 and found that the surface expression of TLR2 was slightly enhanced by cotransfection of TLR1 and/or CD14 (Fig. 6B).

Thus, the CD14-induced enhancement of the recognition of triacylated LPTs by TLR2/TLR1 might be partially, but not completely, explained by upregulation of the TLR2 surface expression caused by cotransfection with TLR1 and/or CD14.

Interaction between CD14 and TLR2/TLR1 complex

Judging from the results described above, we first speculated that CD14 formed a complex with TLR2 or TLR1 or both. It has already been shown that CD14 interacts with TLR2 (21), but it remains unknown whether CD14 interacts with TLR1 or the complex of TLR2 and TLR1. Therefore, we examined whether CD14 transfected was associated with TLR2 or TLR2/TLR1 by using an immunoprecipitation assay. First, we established HEK293 cells stably transfected with both FLAG-tagged TLR2 and HA-tagged TLR1 (HEK293.2.1). After transient transfection with Myc-tagged CD14, the cells were lysed in a lysis buffer and immunoprecipitated with anti-FLAG-coupled beads. Proteins immunoprecipitated were separated by SDS-PAGE and detected with anti-FLAG, anti-HA or anti-Myc Ab. CD14 transiently transfected was detected in the cell lysate but was not associated with TLR2 and TLR1, which were associated with each other (Fig. 7A). In addition, it was also found that stimulation with Pam₃CSSNA, Pam₃CSK₄ or FSL-1 did not induce the association of CD14 with TLR2/TLR1 (Fig. 7A and 7B).

These results suggest that TLR1 forms a complex with TLR2 in the membrane of HEK293 transfectants regardless of stimulation but that CD14 is not associated with the receptor complex.

Binding of a triacylated LPT to soluble CD14 determined by surface plasmon resonance studies

We also considered the possibility that CD14 is bound to LPTs as ligands and then passes them to the receptor complex of TLR2/TLR1. In order to verify this possibility, the surface plasmon resonance (SPR) technique was used to determine whether CD14 directly bound to Pam₃CSK₄. The interaction between Pam₃CSK₄ and immobilized CD14 was monitored by flowing various concentrations of Pam₃CSK₄ over a CD14-coated chip surface. It was found that Pam₃CSK₄ directly bound to CD14 and the K_D value of the binding was calculated to be 5.7 μ M from the overlaid dose-response binding curve, whereas the diacylated LPT FSL-1 did not (Fig. 8). Weak interaction of Pam₃CSK₄ with lysozyme as a protein unrelated to CD14 was also observed, but the affinity (K_D

= 2.7 mM) was approximately 500-fold lower than that (5.7 μ M) to CD14 (Fig. 8). This weak affinity of Pam₃CSK₄ with CD14 may be explained as a nonspecific binding due to hydrophobicity of the LPT.

Discussion

Microbial LPs responsible for the activation of macrophages, B lymphocytes and fibroblasts are divided into two groups, diacylated and triacylated LPs, that are mainly produced from mycoplasmas and bacteria, respectively. It has been demonstrated that TLR2 requires TLR6 and TLR1 as a coreceptor for the recognition of diacylated and triacylated LPTs, respectively (16). However, several lines of evidence have indicated that TLR6 is not always required for the recognition of diacylated LPTs by TLR2 (17-19). We have also found that the diacylated LPT FSL-1 is recognized by TLR2 transfected into HEK293 cells without cotransfection of TLR6 (17, 18). However, there are no reports that TLR1 is not required for the recognition of triacylated LPTs by TLR2. In order to confirm the requirement of TLR1 in the recognition of triacylated LPTs by TLR2 in our NF- κ B reporter assay system, we examined whether Pam₃CSK₄ and Pam₃CSSNA were recognized by the TLR2/TLR1 complex. It was found that Pam₃CSK₄ and Pam₃CSSNA were recognized by the TLR2/TLR1 complex, but, unexpectedly, the recognition level was very low in this system (Fig. 1). Therefore, we thought that other molecules such as CD14 are required for the full recognition, since CD14 is known to enhance the recognition of PGN by TLR2 (21, 24) and LPS by TLR4 (20, 29) and CD14 mediates activation of monocytes by LP from *Treponema pallidum* and *Borrelia burgdoferi* (25, 26). It was found that the cotransfection of CD14 drastically enhanced the recognition of Pam₃CSSNA by the TLR2/TLR1 complex in a dose-dependent manner (Fig. 2). Interestingly, the CD14-induced enhancement did not occur without cotransfection of TLR1 (Fig. 2A). Furthermore, CD14-induced enhancement was significantly reduced by knockdown of TLR1 (Fig. 3), suggesting that TLR1 is absolutely required for the recognition of

triacylated LPTs, but not the diacylated LPT FSL-1, by TLR2. More recently, it has been demonstrated that CD14 was absolutely required for LPS-induced activation of the TRIF-TRAM pathway and, to a lesser extent, CD14 also participated in signaling mediated by the TLR2/TLR6 receptor complex (29). However, the present study demonstrated that CD14 was involved in the recognition of triacylated LPTs, but not diacylated LPTs, by the TLR2/TLR1 receptor complex. In addition, the results obtained by an SPR assay clearly demonstrated that triacylated LPTs bound to CD14, whereas the diacylated LPT FSL-1 did not bind to CD14 (Fig. 8). This may explain the results (Fig. 2B) showing that CD14 did not enhance the diacylated LPT FSL-1 by the TLR2/TLR1 complex. If the binding of LPT to CD14 is the first step for the recognition of LPTs by TLR2 as suggested by Manukyan *et al.* (22), it is speculated that CD14 is not involved in TLR2/TLR6-mediated signaling.

Iwaki *et al.* (21) have recently indicated that the CD14 region spanning amino acids 57–64 is critical for interaction with TLR2 and enhancement of TLR2-mediated PGN signaling. Kim *et al.* (28) presented the crystal structure of mouse CD14 at a 2.5 Å resolution. A large hydrophobic pocket was found on the N-terminal side of the horseshoe-like structure. Previously identified regions involved in LPS binding map to the rim and bottom of the pocket, indicating that the pocket is the main component of the LPS-binding site (28). The present study also demonstrated that the N-terminal amino acid region of S39-A48 of CD14 played a key role in the CD14-induced enhancement of the recognition of triacylated LPTs by the TLR2/TLR1 receptor complex (Fig. 4).

Taken together, the results suggest that the N-terminal part of CD14 is responsible for binding to LP/LPT as well as LPS.

The present study demonstrated that soluble recombinant CD14 was also capable of enhancing the recognition of triacylated LPTs by the TLR2/TLR1 receptor complex (Fig. 6A), suggesting that membrane-bound CD14 is not absolutely required for the CD14-induced enhancement. In addition, it was found that the CD14-induced enhancement could not be completely explained by upregulation of the expression level of TLR2 caused by cotransfection of CD14 (Fig. 6B).

Therefore, we thought that CD14 was involved in binding to LPTs or to the TLR2/TLR1 complex. Analyses by flow cytometry and confocal microscopy by Manukyan *et al.* (22) have recently demonstrated that Pam₃CSK₄ binds to CD14. By FRET analysis, they found that both TLR2 and TLR1 bind to Pam₃CSK₄ and that TLR2 does not bind to CD14 without preincubation with Pam₃CSK₄. They also found by FRAP that LPT binding decreases the mobility of TLR2 on HEK293 cells transfected with TLR2 and/or CD14, suggesting that after LPT stimulation, TLR2 was targeted to a low-mobility complex, which could be formed by TLR2 associations with intracellular adaptor molecules. Thus, they suggest that TLR2, TLR1, CD14 and Pam₃CSK₄ form a tetramolecular complex during activation of cells by Pam₃CSK₄. Our finding obtained by an SPR assay that CD14 bound to Pam₃CSK₄ is in accordance with the finding of Manukyan *et al.* (22). However, our finding obtained by an immunoprecipitation assay that CD14 was not associated with the TLR2/TLR1 complex even after stimulation with Pam₃CSK₄ (Fig. 7) is in contrast to the finding of Manukyan *et al.* (22) that the CD14/LPT complex is associated with the TLR2/TLR1 complex only after stimulation with Pam₃CSK₄.

We speculate that CD14 is not involved in the stability of the TLR2/TLR1/LPT complex by forming a tetramolecular complex but that CD14 just facilitates the binding of LPT to the TLR2/TLR1 receptor complex without binding to the complex as described in the binding of LPS to the TLR4–MD-2 complex (20).

Experimental procedures

Abs and reagents

FITC-conjugated anti- human CD14 monoclonal Ab (MY-4) was purchased from Beckman Coulter Inc. (Miami, FL), anti-CD14 monoclonal Ab (clone 134620) as a blocking Ab was from R&D Systems (Minneapolis, MN), anti-TLR2 monoclonal Ab (IMG-319) was from Biocarta (San Diego,

CA), anti-FLAG M2 monoclonal Ab was from Sigma-Aldrich Co. (St. Louis, MO), anti-V5 Ab was from Invitrogen (Carlsbad, CA), anti-HA Ab was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-Myc was from Cell Signaling Technology, Inc. (Beverly, MA), and Protein G-sepharose was from Amersham Bioscience (Uppsala, Sweden). S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-Cys-Ser-Ser-Asn-Ala (Pam₃CSSNA) was purchased from Bachem AG (Hauptstrasse, Switzerland). FSL-1 [S-(2,3-bispalmitoyloxypropyl) Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, Pam₂CGDPKHPKSF] was synthesized as described previously (15). Pam₃CSK₄ was purchased from InvivoGen (San Diego, CA).

All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

Cloning of human TLR2, its mutants and human TLR6

The cDNAs of TLR2 (2.35 kb) and TLR6 (2.4 kb) were obtained by RT-PCR of RNA isolated from a human monocytic cell line, THP-1 cells. The cDNAs of TLR2, TLR6 and CD14 were cloned into a pEF6/V5-His TOPO vector (hereafter referred to as pEF-TLR2, pEF-TLR6 and pEF-CD14) (Invitrogen). The cDNA clone (KIAA0012) containing human TLR1 obtained from Kazusa DNA Research Institute (Chiba, Japan) was digested by KpnI and NotI. The resulting KpnI-NotI fragment was cloned into a pEF6/V5-His TOPO vector (pEF-TLR1). The deletion mutants of TLR2 (TLR2^{dS39-A48} and TLR2^{dE772-S784}) and TLR1 (TLR1^{dQ636-K779}) were produced from pEF-TLR2 and pEF-TLR1, respectively, by a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. HA-tagged TLR1 (punoha-htlr1) was purchased from InvivoGen. Human TLR2 cDNA(19-784th amino acid; containing preprotrypsin leader sequence and FLAG sequence at the N-terminus) was cloned into pcDNA3 vector (Invitrogen). The full length of hCD14 cDNA cloned from THP-1 cells was subcloned into pcDNA3.1(-) Myc vector (Invitrogen) (pcDNA-CD14).

NF- κ B reporter assay

HEK293 cells obtained from ATCC (CRL-1573) were maintained in DMEM (Gibco BRL, Rockville, MD) containing 10% FBS, 100 units/ml penicillin G and 100 μ g/ml streptomycin. HEK293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Eugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) with 30 ng of an NF- κ B reporter plasmid (pNF- κ B-Luc, Stratagene) and 3 ng of a construct directing expression of *Renilla* luciferase under the control of a constitutively active thymidine kinase promoter (pRL-TK, Promega Co., Madison, WI) together with 150 ng of each transfectant gene of pEF-TLR2, pEF-TLR1, pEF-TLR6 or pEF-CD14. Twenty-four h after transfection, the cells were stimulated for 6 h with FSL-1, Pam₃CSNA and Pam₃CSK₄ in the absence of FBS, and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Surface plasmon resonance-based assay

Analysis of direct binding of CD14 to Pam₃CSK₄ was performed using SPR detection on a Biacore X (Biacore AB, Uppsala, Sweden). Soluble recombinant CD14 (R & D Systems) or lysozyme (Wako Pure Chemicals, Tokyo, Japan) was immobilized on a CM5 sensor tip according to the amine coupling method. After washing extensively with a running buffer, HBS-EP (pH7.4) containing 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20, at 25 °C for 15 min, various concentrations of Pam₃CSK₄ (0.66, 6.6, 66 μ M) or FSL-1 (0.6, 6.0, 60 μ M) were injected at a flow rate of 10 μ l/min. The interaction between Pam₃CSK₄ and immobilized CD14 was monitored as a function of time and expressed in response units. Regeneration of the chip was achieved by washes with 5% (w/v) SDS and extensive re-equilibration with the running

buffer.

Knockdown of TLR1 by RNA interference

psiRNA-hTLR1 (InvivoGen) is specifically designed for the cloning of small synthetic oligonucleotides (49-mer) that encode two complementary sequences of 21 nt (5'-gtgactacccggaaagttata-3'/5'-tataactttccgggtagtcac-3'), homologous to a segment of the TLR1 gene, separated by a short spacer region of 7 nt (5'-tcaagaa-3'). The insert is cloned downstream of an RNA polymerase III promoter, the human 7SK promoter. It is transcribed into a short dsRNA with a hairpin structure consisting of a 21 double-stranded region corresponding to the target sequence and a small loop formed by the spacer region.

HEK293.2.1 cells that stably express both FLAG-tagged TLR2 and HA-tagged TLR1 were established in our laboratory. HEK293.2.1 cells were plated at 1×10^6 cells per well in 3.5-cm dishes on the day before transfection. The cells were transfected with 2 μ g of psiRNA-hTLR1 and incubated for another 48 h. Total RNA was prepared from the cells by using an RNeasy kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. The expression of TLR1 mRNA was monitored by RT-PCR using a primer set (5'-cgtaaaactggaagctttgcaaga-3'/5'-ccttgggccattccaaat aagtcc-3').

HEK293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 Transfection Reagent with 30 ng of pNF- κ B-Luc and 3 ng of pRL-TK together with 150 ng of each transfectant gene of pEF-TLR2, pEF-TLR1, pEF-CD14 or psiRNA-hTLR1. Twenty-four h after transfection, the cells were stimulated for 6 h with FSL-1 and Pam₃CSK₄ in the absence of FBS, and luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions.

Immunoprecipitation

HEK293.2.1 cells grown in 10-cm dishes were transfected with 7 µg of Myc-tagged CD14. After 48-h incubation, the cells were lysed in a lysis buffer (pH 7.5) containing 0.4% Triton X-100, 0.3% NP-40, 20 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM NaF, 1 mM Na₃VO₄, 12.5 mM β-glycerophosphate and protease inhibitor cocktail (Roche). The lysates were incubated at 4 °C overnight with an anti-FLAG monoclonal Ab and then precipitated with Protein G Sepharose beads. Proteins included in the precipitates were separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C overnight with Abs against FLAG, HA or Myc and then with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG Ab. Immunoreactive proteins were detected by using ECL detection reagents (Amersham Pharmacia).

Surface expression of TLR2 and CD14 in HEK293 transfectants determined by flow cytometry

In order to assess the surface expression of TLR2 and CD14 in HEK293 cells by flow cytometry, HEK293 cells grown in 6-cm dishes were removed from the plastic dishes 72 h after transient transfection with 1 µg each of TLR2, TLR1 and CD14. The cells were incubated at 4 °C for 1 h with or without isotype-matched mouse IgG or anti-TLR2 monoclonal Ab (IMG-319) and then with FITC-conjugated anti-mouse IgG Ab. The cells were stained with FITC-conjugated MY4 for the surface expression of CD14. The cells were fixed in 0.1 M phosphate buffer containing 0.5% formaldehyde and then analyzed by a FACScan flow cytometer (BD Bioscience, Mountain View, CA). Data for 10000 cells falling within appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using CellQuest software (BD Bioscience).

Statistical analysis

The results of relative luciferase activity were expressed means $\pm$ SD. Means were analyzed by one-way ANOVA and subsequent Tukey's HSD test at a 1% level of significance. All statistical analysis were done using an Kyplot 3.0 for Windows software (Kyence Inc., Japan)

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Figure legends

Fig. 1. NF- κ B activation in HEK293 cells transfected with TLR2 and/or TLR1 or TLR6 by LPTs.

HEK293 cells grown in DMEM were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 with 30 ng of pNF- κ B-Luc and 3 ng of pRL-TK together with 150 ng of each transfectant gene of pEF-TLR2 and/or pEF-TLR1. Twenty-four h after transfection, the cells were stimulated for 6 h with various concentrations (0.1, 1.0, 10 nM) of FSL-1, Pam₃CSSNA or Pam₃CSK₄, and luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions. Results, expressed as the mean $\pm$ SD of triplicate cells, are representative of three separate experiments. * indicates the significant difference compared with Non-stimulated cells transfected with TLR2/TLR6 or TLR2/TLR1, respectively ($p < 0.01$).

Fig. 2. CD14-induced enhancement of the recognition of LPTs by the TLR2/TLR1 complex.

HEK293 cells grown in DMEM were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 with 30 ng of pNF- κ B-Luc and 3 ng of pRL-TK together with 150 ng of each transfectant gene of pEF-TLR2, pEF-TLR1 and/or pEF-CD14. Twenty-four h after transfection, the cells were stimulated for 6 h with various concentrations of Pam₃CSSNA (10, 100 nM) (A) and FSL-1 (1, 10 nM) (B), and luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions. Results, expressed as the mean $\pm$ SD of triplicate cells, are representative of three separate experiments. * indicates the significant difference compared with Non-stimulated cells transfected with TLR2 ($p < 0.01$).

Fig. 3. Knockdown of TLR1 mRNA in HEK293 cells stably expressing TLR2 and TLR1 by transfection of psiRNA-hTLR1 and its effects on NF- κ B activation.

HEK293.2.1 cells that stably express both FLAG-tagged TLR2 and HA-tagged TLR1 were established in our laboratory. HEK293.2.1 cells were plated at 1×10^6 cells per well in 3.5-cm dishes on the day before transfection. The cells were transfected with (KD) or not transfected with (N) 2 μ g of psiRNA-hTLR1 and incubated for another 48 h. Total RNA was prepared from the cells by using an RNeasy kit according to the manufacturer's instructions. The expression of TLR1 mRNA (A) was monitored by RT-PCR using a primer set (5'-cgtaaaactggaagctttgcaaga-3'/5'-ccttgggccattccaaataagtcc-3') at 20, 22, 24 and 26 amplification cycles (30 s of denaturation at 94 °C, 30 s of annealing at 56 °C and 60 s of extension at 72 °C). For effects of TLR1 knockdown on the NF- κ B activation, HEK293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 with 30 ng of pNF- κ B-Luc and 3 ng of pRL-TK with 150 ng of each transfectant gene of pEF-TLR2, pEF-TLR1 or pEF-CD14 together with (KD) or without (N) 150 ng of psiRNA-hTLR1. Twenty-four h after transfection, the cells were stimulated for 6 h with various concentrations of Pam₃CSK₄ (B) or FSL-1 (C) and luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions. * indicates the significant difference compared with Non-stimulated cells transfected with TLR2 ($p < 0.01$).

Fig. 4. Involvement of the N-terminal region of S39-A48 of CD14 and anti-CD14 monoclonal Ab in CD14-induced enhancement of the recognition of triacylated LPTs by the TLR2/TLR1 complex.

HEK293 cells grown in DMEM were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 with 30 ng of pNF- κ B-Luc and 3 ng of pRL-TK together with 150 ng of each transfectant gene of pEF-TLR2, pEF-TLR1, pEF-CD14 or pEF-CD14^{dS39-A48} instead of pEF-CD14. Twenty-four h after transfection, the cells were stimulated for 6 h with 10 or 100 nM of Pam₃CSSNA (A) or Pam₃CSK₄ (B), and luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions. In order to see the effects of the anti-CD14 monoclonal Ab, which is known to suppress the biological functions of CD14, on the CD14-induced enhancement, the

anti-CD14 monoclonal Ab was added to the cells 1 h before the addition of LPTs at a concentration of 10 µg/ml. Results, expressed as the mean $\pm$ SD of triplicate cells, are representative of three separate experiments. * indicates the significant difference compared with Non-stimulated cells transfected with TLR2 and TLR1 ($p < 0.01$).

Fig. 5. NF- κ B activation in HEK293 cells transfected with TIR domain-deletion mutants of TLR2 or TLR1 by Pam₃CSSNA.

HEK293 cells grown in DMEM were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 with 30 ng of pNF- κ B-Luc and 3 ng of pRL-TK together with 150 ng of each transfectant gene of pEF-TLR2 or pEF-TLR2^{dE772-S784} (A), pEF-TLR1 or pEF-TLR1^{dQ636-K779} (B), or pEF-CD14. Twenty-four h after transfection, the cells were stimulated for 6 h with 10 or 100 nM of Pam₃CSSNA, and luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions. Results, expressed as the mean $\pm$ SD of triplicate cells, are representative of three separate experiments. * indicates the significant difference compared with Non-stimulated cells transfected with TLR2, TLR1 and CD14, respectively ($p < 0.01$).

Fig. 6. (A) Enhancement of the recognition of Pam₃CSSNA by the TLR2/TLR1 complex by soluble recombinant CD14. HEK293 cells grown in DMEM were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 with 30 ng of pNF- κ B-Luc and 3 ng of pRL-TK together with 150 ng of each transfectant gene of pEF-TLR2 or pEF-TLR1. Twenty-three h after transfection, various concentrations (0.15, 1.0, 5 µg/ml) of soluble recombinant CD14 were added to the cultures. After 1-h incubation, the cells were stimulated for 6 h with 10 or 100 nM of Pam₃CSSNA, and luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions. Results, expressed as the mean $\pm$ SD of triplicate cells, are representative of three separate experiments. *

indicates the significant difference compared with Non-stimulated cells transfected with TLR2 and TLR1 ($p < 0.01$).

(B) Upregulation of TLR2 in HEK293 cells by cotransfection of CD14 and/or TLR1. HEK293 cells grown in 6-cm dishes were removed from the plastic dishes 72 h after transient transfection with 1 μ g each of TLR2, TLR1 and CD14. The cells were incubated at 4 °C for 1 h with or without isotype-matched mouse IgG or anti-TLR2 monoclonal Ab and then with FITC-conjugated anti-mouse IgG Ab. The cells were stained with FITC-conjugated MY4 for the surface expression of CD14. The cells were fixed in 0.1 M phosphate buffer containing 0.5% formaldehyde and then analyzed by a FACScan flow cytometer. Data for 10000 cells falling within appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using CellQuest software. See text for details.

Fig. 7. Association of CD14 with the TLR2/TLR1 complex in the presence of LPTs.

HEK293.2.1 cells grown in 10-cm dishes were transfected with 7 μ g of Myc-tagged CD14. After 48-h incubation, the cells were incubated with or without various concentrations (1, 10 100 nM) of Pam₃CSK₄ (A), 100 nM Pam₃CSSNA (B) or 10 nM FSL-1 (B) and then lysed in a lysis buffer. The lysates were incubated at 4 °C overnight with anti-FLAG monoclonal Ab and then precipitated with Protein G Sepharose beads. Proteins included in the precipitates were separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C overnight with Abs against FLAG, HA or Myc and then with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG Ab. Immunoreactive proteins were detected by using ECL detection reagents. See text for details.

Fig. 8. Direct binding of CD14 to Pam₃CSK₄.

Analysis of direct binding of CD14 to Pam₃CSK₄ was performed using Surface plasmon resonance detection on a Biacore X. Soluble recombinant CD14 or lysozyme was immobilized on a

CM5 sensor tip according to the amine coupling method. After washing extensively with a running buffer, HBS-EP, at 25 °C for 15 min, various concentrations of Pam₃CSK₄ (0.66, 6.6, 66 µM) or FSL-1 (0.6, 6.0, 60 µM) were injected at a flow rate of 10 µl/min. The interaction between Pam₃CSK₄ and immobilized CD14 was monitored as a function of time and expressed in response units. Regeneration of the chip was achieved by washes with 5% (w/v) SDS and extensive re-equilibration with the running buffer. See text for details.

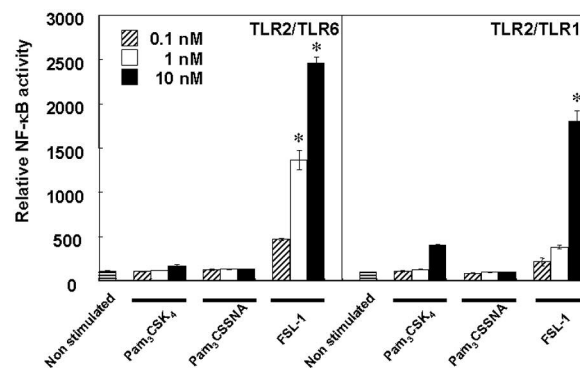


Fig. 1

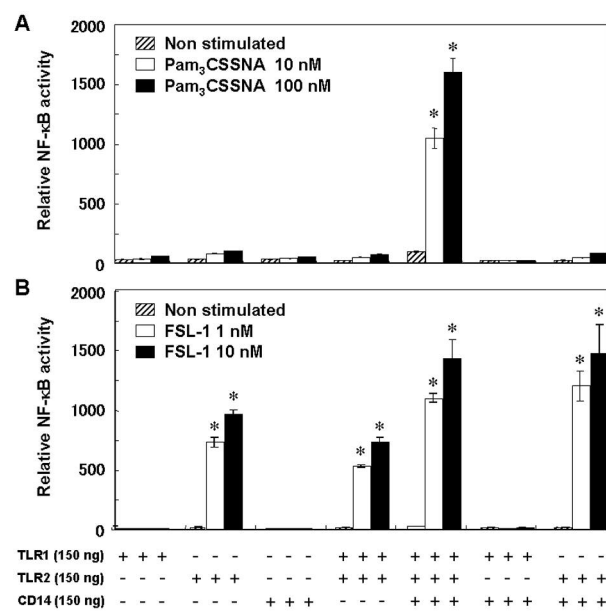


Fig. 2

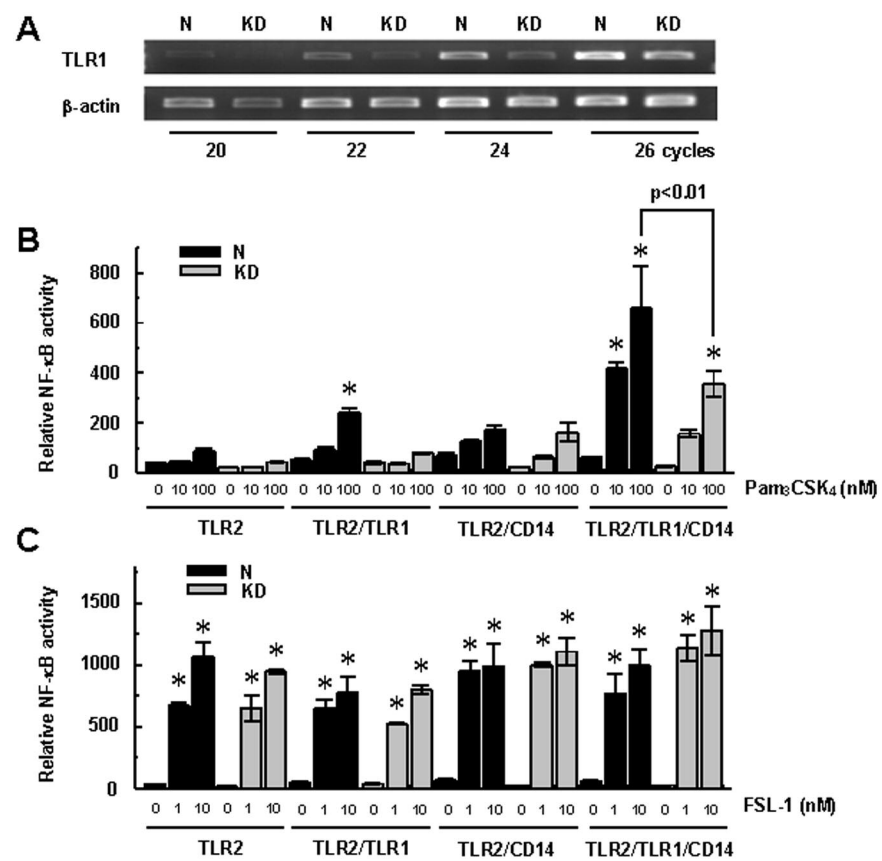


Fig. 3

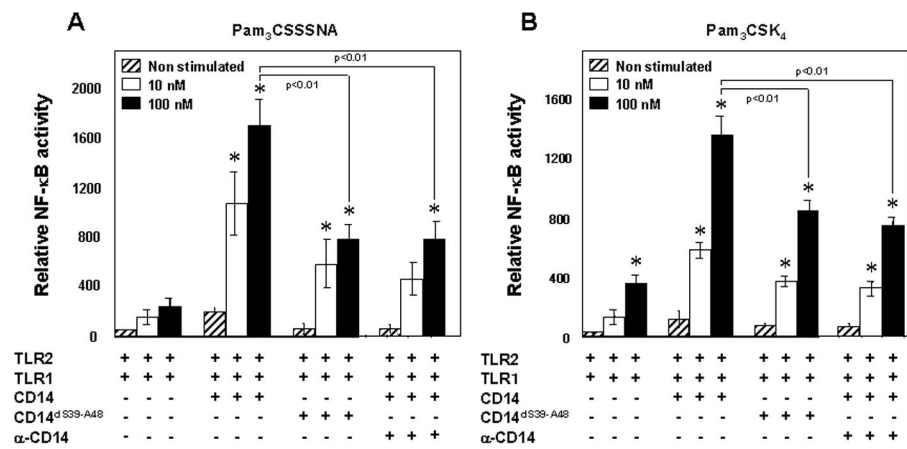


Fig. 4

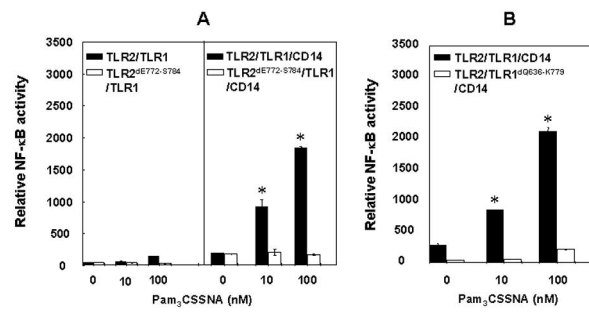


Fig. 5

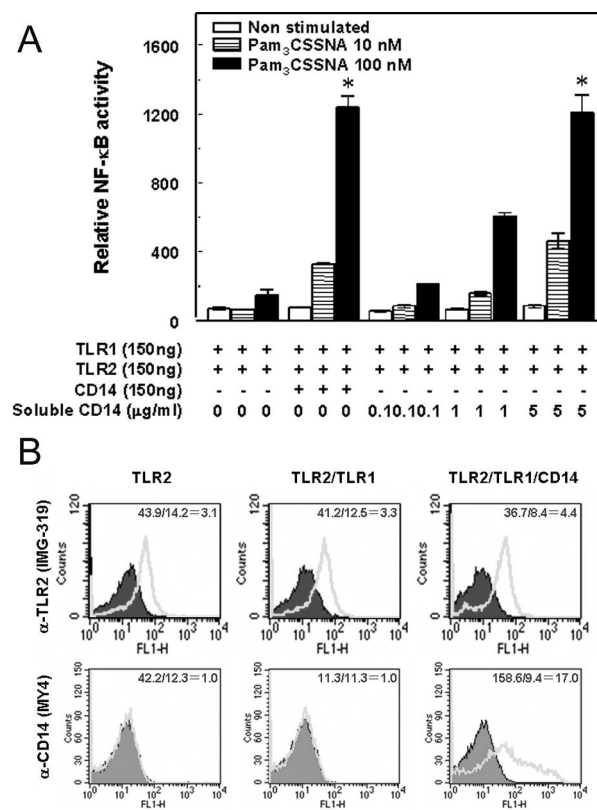


Fig. 6

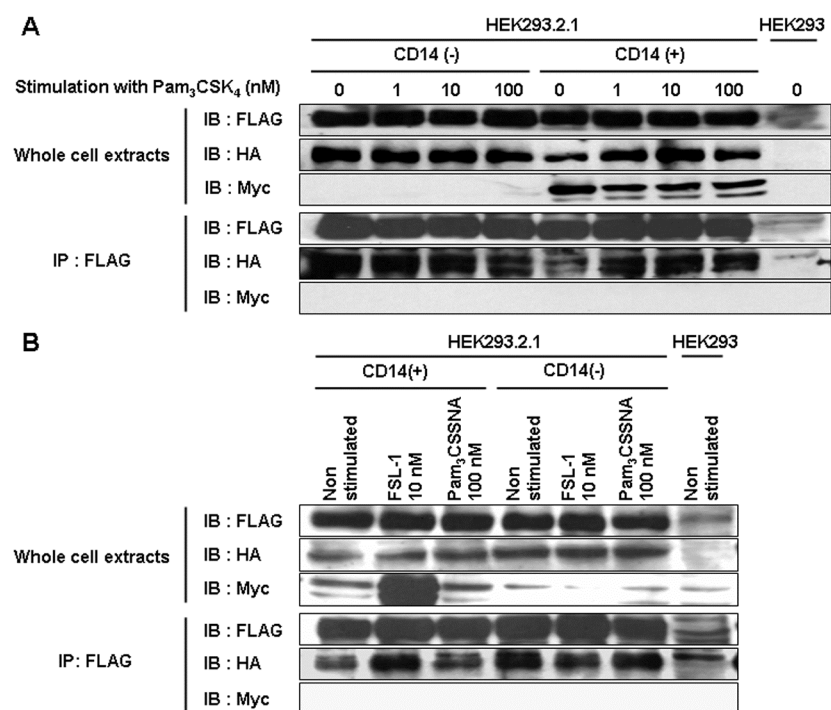


Fig. 7

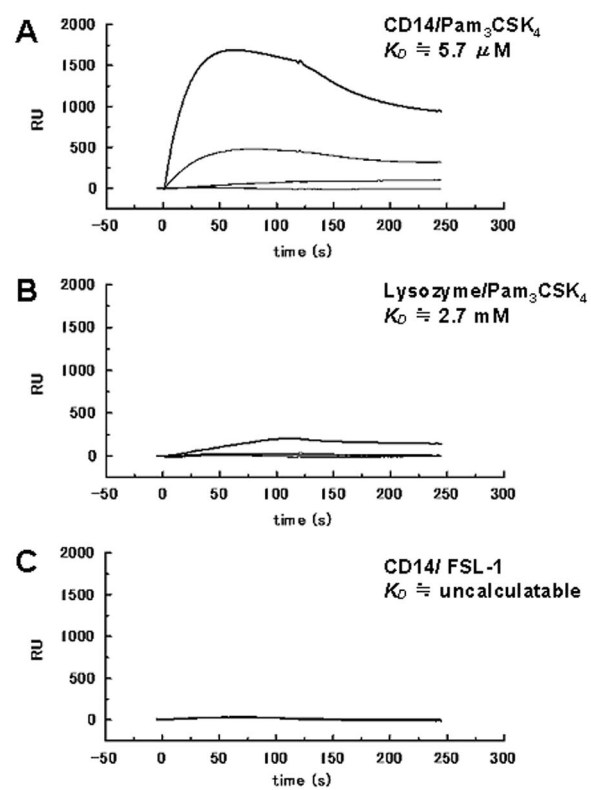


Fig. 8