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Serum Amyloid A3 Binds MD-2 To Activate p38 and NF-κB Pathways in a MyD88-Dependent Manner

Atsuko Deguchi,* Takeshi Tomita,* Tsutomu Omori,* Akiko Komatsu,* Umeharu Ohto,† Satoshi Takahashi,‡ Natsuko Tanimura,§ Sachiko Akashi-Takamura,§ Kensuke Miyake,§ and Yoshiro Maru*†

Serum amyloid A (SAA) 3 is a major component of the acute phase of inflammation. We previously reported that SAA3 served as an endogenous peptide ligand for TLR4 to facilitate lung metastasis. Because these experiments were performed with SAA3 recombinant proteins purified from Escherichia coli or mammalian cells, we could not rule out the possibility of LPS contamination. In this study, we used SAA3 synthetic peptides to eliminate the presence of LPS in SAA3. We found that the SAA3 synthetic peptide (aa 20–86) (20–86) stimulated cell migration and activated p38 in a manner dependent on TLR4, MD-2, and MyD88. SAA3 (20–86) also activated NF-κB and Rho small GTPase. Using surface plasmon resonance analysis, the binding constant \( K_d \) values between SAA3 (20–86) or SAA3 (43–57) and TLR4/MD-2 protein highly purified by the baculovirus system were 2.2 and 30 \( \mu \)M, respectively. Flag-tagged SAA3 tightly bound to protein A–tagged MD-2, but not to TLR4 in baculovirus coinfection experiments. Although SAA3 (20–86) caused a low, but appreciable level of endocytosis in TLR4, it induced the upregulation of both IL-6 and TNF-α, but not IFN-β. An i.v. injection of SAA3 (43–57) induced the lung recruitment of CD11b^Gr-1^ cells at an estimated serum concentration around its \( K_d \) value toward TLR4/MD-2. Taken together, these results suggest that SAA3 directly binds MD-2 and activates the MyD88-dependent TLR4/MD-2 pathway. The Journal of Immunology, 2013, 191: 000–000.

Well-known host receptors that recognize extrinsic molecules include morphine receptor and TLR. In contrast to morphine of botanical origin, the endogenous ligands of morphine receptor were identified and include neuronal peptides such as endorphin. In addition to LPS of bacterial origin (1) and paclitaxel of botanical origin (2), TLR4 has been shown to recognize the endogenous polypeptide ligands of mammalian cell origin, including HMGB1 (3), heat shock protein (Hsp)70 (4), S100A8 (5, 6), serum amyloid A (SAA3) (7, and HMGN1 (8). However, the potential coexistence of Escherichia coli–derived LPS during the preparation of these peptide ligands for experimental usage has been of major concern. For example, disagreements on Hsp70 as an endogenous ligand have been reported, and, by referring to the literature on Hsp70 and TLR4 interactions, binding was concluded to be an artifact due to the contamination of LPS in Hsp70 proteins (9).

SAA is an acute-phase protein involved in the homeostasis of inflammatory responses. Four mouse SAAs (SAA1, SAA2, SAA3, SAA4), with >40% amino acid identity, and three human SAAs (SAA1, SAA2, SAA4) have been identified (10). Human SAA3 has been characterized as a pseudogene. SAA1, which is produced by both hepatocytes and melanoma cells, was shown to bind neutrophil FPR2 to induce IL-10 expression. This resulted in the suppression of cytotoxic T cells and NK cell stimulation, suggesting that SAA1 is a double-edged sword (11). We previously reported that tumor-derived growth factors such as TNF-α and vascular endothelial growth factor induced the expression of two endogenous TLR4 ligands, S100A8 and SAA3, in the lungs of tumor-bearing mice before lung metastasis (so-called premetastatic lungs), and that SAA3 functions as a TLR4-mediated chemotactic agonist for both myeloid cells and tumor cells (7). An anti-SAA3 neutralizing Ab inhibited lung metastasis. The autoamplification of SAA3 by lung-specific Clara cells may contribute to organotropism in lung metastasis (12).

TLRs recognize specific structural motifs of various pathogens, known as pathogen-associated microbial patterns, and activate innate immune responses. The ligand-induced dimerization of TLRs is known to trigger the recruitment of adaptor proteins to intracellular Toll/IL-1R domains to initiate signaling. Signaling cascades via the Toll/IL-1R domains were shown to be mediated by specific adaptor molecules, including MyD88, MAL (also known as TIRAP), Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) (13). TLR4 associates with coreceptor MD-2; LPS binding to MD-2 induces the dimerization of TLR4 (14); and a single MD-2 mutation that substitutes cysteine 95 with tyrosine (C95Y) completely abolishes LPS responsiveness (15). A previous study showed that TLR4 activates the NF-κB pathway through both MyD88-dependent signaling as an early-phase regulation and TRIF-dependent signaling as a late-phase regulation (16). TRIF signaling initially increases transcriptional levels of IFNs in an IFN regulatory factor 3–dependent manner (17).

Rho and Rho kinases are involved in NF-κB activation, which suggests their potential role in LPS-induced inflammation (18,
19). Rho kinases are known to act as molecular switches controlling several critical cellular functions, such as actin cytoskeleton organization, cell migration and adhesion, reactive oxygen species formation, and apoptosis.

In this study, we investigated whether SAA3 could serve as a physical and functional ligand of endogenous origin for TLR4 and attempted to identify differences between SAA3 and LPS using synthetic peptides that completely eliminated the presence of LPS.

Materials and Methods

Chemicals and Abs

LPS (E. coli, 055:B5) and polyoxymycin B were purchased from Sigma-Aldrich (St. Louis, MO); synthetic lipid A (compound 506) was purchased from Peptide Institute (Osaka, Japan); and synthetic monophosphoryl lipid A (MPLA) was purchased from InvivoGen. Rabbit polyclonal anti-phospho-p38, anti-phospho-JNK1, anti-phospho-ERK2, anti-phospho-p38, anti-phospho-Akt, anti-p65, anti-JNK1, anti-ERK2, anti-Akt1, and anti-p65 Abs were from Cell Signaling Technology (Danvers, MA). Anti-CD11c, anti-Gr-1 Abs were from Bio-Rad Laboratories (Hercules, CA). Both mouse Abs and rabbit polyclonal TLR4 Abs were purchased from Abcam. Anti-β-actin mAb and Rho activation assay kit were purchased from Millipore. The SAA3 (20–86) peptide was synthesized by Thermo Fisher (97.7% purity by HPLC analysis) or MBL International (100% purity by HPLC analysis). Mouse SAA3 (43–57) and SAA3 (43–57)-K44E were synthesized by Operon, and their peptide purities were 99.7 and 99.7%, respectively. As a control peptide, the SAA3 (43–57) sequence was computationally scrambled (amino acid sequences; KYKHWNKDRSKFA). Alexa Fluor 594-labeled LPS was purchased from Invitrogen. Alexa Fluor 594 Microscale Protein Labeling Kit (Invitrogen).

Animal study

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan), and Thra−/− and Myd88−/− mice were purchased from Oriental BioService (Kyoto, Japan). MD-2−/− mice were provided by RIKEN (20). Mice were used for experiments at 2–3 mo of age. For in vivo stimulation with the SAA3 (43–57) peptide, C57BL/6 mice were i.p. injected with 100 µg SAA3 (43–57). Eight hours after the i.p. peptide administration, mice were sacrificed and minced mouse lungs were digested with collagenase/dispase/DNase solution. Recruits were incubated with anti-CD11b-FITC and anti-Gr-1-PE Abs. The recruitment of CD11b+Gr-1+ cells in the lungs was determined by flow cytometry, as described previously (12). All procedures performed with mice were approved by the Animal Research Committee of Tokyo Women’s Medical University.

In vitro migration assay

Murine peritoneal macrophages were harvested 4 d after an i.p. injection of 100 µl 10% thiglycolate broth. Thiglycolate broth–elicited peritoneal macrophages were harvested by using PBS. The migration of RAW264.7 cells or peritoneal macrophages was evaluated using a culture insert (BD Falcon) in DMEM supplemented with 0.1% BSA. The level of high-density lipoprotein in 1% BSA was below the detection limit (the high-density lipoprotein test was analyzed by Nagahama Life Science Laboratory). Cells were added into the upper well of the culture insert (8 µm pore size; Falcon), and indicated peptides were added to the lower wells. An aliquot (200 µl) of the cell suspension (5 × 10^5 cells/well) was seeded in each of the upper wells and incubated for 6 h at 37°C with 5% CO₂. Cells that migrated through the filter were counted with crystal violet staining.

Recombinant proteins

SAA3 (20–122), SAA3 (20–98), SAA3 (20–86), SAA3 (20–68), or SAA3 (20–62) recombinant proteins were prepared from E. coli (BL21 strain). Each deletion mutant was generated by PCR using specific primers. After the purification of GST-tagged SAA3 proteins, GST was cleaved by thrombin or PreScission protease (GE Healthcare), and SAA3 (20–122), SAA3 (20–98), SAA3 (20–68), or SAA3 (20–62) was purified by ion exchange chromatography. His-tagged SAA1, SAA2, SAA3, and SAA4 recombinant proteins were prepared from E. coli (BL21-CodonPlus-RIL). To prepare mammalian SAA3 recombinant proteins, Flp-In 293 cells were cotransfected with recombinase-expressing pOG44 and C-terminal FLAG-tagged SAA3-inserted pcDNA/RIT/TOPO expression vectors. SAA3-integrated cells were selected in DMEM containing 30 µg/ml blasticidin and 200 µg/ml hygromycin B. Cells were treated with 1 µg/ml doxycycline for 72 h and lysed in FLAG lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium cholate, 1 mM EDTA) containing the Complete Mini protease inhibitor mixture (Roche Applied Science). Anti-FLAG Ab-conjugated agarose beads were added to the supernatant and incubated for 2 h at 4°C by end-over-end rotation. Beads were washed twice with FLAG lysis buffer and three times by PBS. To elute FLAG-tagged SAA3 proteins, beads were mixed for 30 min with 100 µg/ml (total 500 µl) FLAG peptides in PBS containing 0.5% sodium cholate. The eluate was desalted and subjected to purification by ion exchange chromatography. The purity of all recombinant proteins was >98%, as judged by SDS-PAGE analysis. Endotoxin levels determined by Limulus amebocyte lysate test (WAKO) were very low (<1 EU/ml).

Pull-down assay

The mouse truncated TLR4 and protein A-tagged MD-2 were cloned into the pACGSP7 vector (BD Biosciences), as previously reported (22). TLR4 and MD–2–protein A baculoviruses were generated using the Baculovirus Expression Vector System (BD Biosciences) in Sf9 cells (Invitrogen). C-terminal FLAG-tagged SAA3 was cloned into the pFastBac vector (Invitrogen), and the SAA3 baculovirus was generated using the Baculovirus system in Sf9 cells (Bac to Bac Baculovirus Dual Expression Systems; Invitrogen). High Five cells (Invitrogen) were cotransfected with TLR4, MD-2, and/or SAA3 baculoviruses(es). One milliliter of culture medium was immunoprecipitated using 1 µg anti-mouse TLR4 mAb. To analyze MD-2 immunoprecipitates, 10 µM Human IgG-Sepharose 6 FastFlow (GE Healthcare) was used for 1 ml baculovirus-coinfected High Five culture medium. The immunoprecipitates were separated by SDS-PAGE and transferred to an Immobilon P membrane (Millipore, Billerica, MA). After blocking with Blocking One (Nicalai Tesque, Kyoto, Japan), the membranes were probed with the indicated Ab, followed by HRP-labeled anti-rabbit IgG and anti-mouse IgG (GE Healthcare). Signals were visualized by SuperSignal West Pico Chemiluminescnet Substrate (Pierce).

Detection of the Rho-GTP form

The Rho-GTP form was determined using the Rho activation kit (Millipore). Rho activity was analyzed using a manufacturer’s protocol. Briefly, the GTP-bound form of Rho was collected by immunoprecipitation with Rho-Trap beads. We used GTPγS- or GDP-loaded cell lysates as a positive or negative control, respectively. A total of 20 µg cell lysates was used for Western blotting.

Quantitative PCR analysis

Cells were treated with SAA3, LPS, or MPLAs in DMEM supplemented with 0.1% BSA for cytokine induction. Total RNA was extracted using RNeasy mini (Qiagen) and reverse transcribed to cDNA using SuperScript III reverse transcriptase with an oligo dt primer (Invitrogen). Quantitative PCR analysis was carried out using the SYBR Green master mixture (Applied Biosystems, Carlsbad, CA) and StepOnePlus Real Time PCR System (Applied Biosystems). Gene expression levels were calculated from cycle threshold values, and the relationship between the cycle threshold value and a logarithm of the copy number of a target gene was confirmed to be on a linear line using the corresponding isolated DNA and its serial dilutions as a standard. Thus, gene expression levels for IL-6, TNF-α, IL-1β, IL-10, and IL-12 were normalized against those of β-actin and GAPDH. The following primers were used: β-actin, 5′-CTTTCTGACGCTTCTGTTG-3′ and 5′-ATGGAAGGTAAACACGCC- C-3′; IL-6, 5′-AACGTGATGACCTTGGCAAGA-3′ and 5′-TGGTACTCT CAAGAGAAGCAGG-3′; Ifnβ1, 5′-TCCTCAAGAGAGGATGGC-3′ and 5′-ACCCAGTGCTGGAAAGATTG-3′; TNF-α, 5′-ATGAGAGGAG GCACATTGG-3′ and 5′-CACTGCTCTCTCATCCTG-3′; IL-10, 5′-A CGTGAAAGACCTTCTAGAGT-3′ and 5′-TGCTGCTCTGTAAGCACCTTG- G-3′; CSF2, 5′-CGAATCTTTCTGCCGCGTG-3′ and 5′-CATCAAGAA GCCCTGTAACC-3′; Cxcl1, 5′-TCTCCTGATTCTGGAGCACAC-3′ and 5′-A CCACCAAACCGAATCATGAC-3′; Ccl2, 5′-GGGATCATCTTCGTTGGA- AA-3′ and 5′-AGGCTCCCTGATCCTGCTG-3′; Ccl3, 5′-GGAATATGGC CGTGGAAATCT-3′ and 5′-CTGACCTCCTCCTTCCTC-3′; Saa3, 5′-CC CGAGATGGGAAGTATTTG-3′ and 5′-GGTACAAGCCAAAGATGGGT-3′.
Surface plasmon resonance analysis

Highly pure mouse TLR4/MD-2 proteins (purity >99.5%), which were used for crystallization analysis (21), were used for the surface plasmon resonance assay. Briefly, the extracellular domain of TLR4 (22–627) and MD-2 (19–160) was inserted into the expression vector pMT/BiP/V5-His of the Drosophila Expression System. The TLR4/MD-2 complex was purified by IgG-Sepharose affinity chromatography, protein A tag cleavage by thrombin, and Superdex 200 gel filtration chromatography. Mouse MD-2 (purity >99.5%) recombinant proteins were also purified by a similar procedure with a baculovirus expressing MD-2 alone. The TLR4/MD-2 protein or MD-2 was immobilized to the GLM sensor chip of the ProteOn XPR36 protein interaction array system (Bio-Rad, Hercules, CA). Immobilization was performed by first injecting 250 μl of a 1:1 mixture of 0.2 M 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide and 0.05 M sulpho-N-hydroxysuccinimide, followed by the TLR4/MD-2 complex or MD-2 to obtain ~1000 resonance units. The heat-denatured TLR4/MD-2 complex or MD-2 was used as a control. A quantity amounting to 1 M ethanolamine was then applied to block the remaining active amino groups at the surfaces. PBS-Tween 20 buffer was used as a running buffer with 100 mM Na-2-hydroxysuccinamate. To completely eliminate the possibility of contamination by sub-molar amounts of LPS in the ligand preparation, we attempted to chemically synthesize SAA3 peptides. Successful peptide synthesis is known to technically depend on its length or amino acid sequence (23). Because of the technical difficulty associated with synthesizing the full-length SAA3 peptide without the signal sequence (103 mer), we attempted to shorten the SAA3 peptide sequence while retaining its biological activity. We first examined the effect of a series of SAA3 deletion mutants produced in E. coli on cell migration to evaluate regions responsible for its activity (Supplemental Fig. 1A). Although very low LPS levels, which have been referred to as “endotoxin-free” in the literature, were found in the ligand preparation, we still used polymyxin B to chelate unavoidably contaminated LPS in the recombinant proteins (see Materials and Methods). Whereas SAA3 (20–122), SAA3 (20–98), or SAA3 (20–86) recombinant proteins significantly stimulated cell migration in RAW264.7 cells, shorter mutants such as SAA3 (20–68) or SAA3 (20–62) still retained the ability to stimulate cell migration, although it was diminished (Supplemental Fig. 1B). Therefore, we decided to use the synthetic SAA3 (20–86) peptide (67 mer) for all subsequent assays. SAA3 (20–86) peptides were directly labeled with Alexa Fluor 594. After fixation with 4% paraformaldehyde, cells were stained with the anti-TLR4 mAb (mouse TLR4 mAb; Abcam), followed by incubation with anti-mouse IgG Alexa Fluor 488. Endocytic vesicles were recognized by double staining with TLR4/MD-2 (MTS510; eBioscience) Ab, followed by streptavidin-PE for flow cytometric analysis. Staining was assessed with Cytomics FC500 (Beckman Coulter).

Statistical analysis

Data, expressed as the mean ± SD, were analyzed using the Student t test, and p values <0.05 were considered significant.

Results

The SAA3 synthetic peptide stimulates cell migration in a manner dependent on TLR4, MD-2, and MyD88

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Statistical analysis

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stimulated cell migration in RAW264.7 cells, and the maximal effect was displayed at 0.1 μM SAA3 (20–86) (Fig. 1A). By testing three different concentrations (0, 0.05, 0.1 μM) of SAA3 (20–86) in both upper and lower chambers, we found that SAA3 (20–86) induced cell migration in a concentration gradient-dependent manner, suggesting that the effect of SAA3 (20–86) preferentially induced chemotactic cell migration (Supplemental Fig. 1D). The effective dosages of SAA3 (20–86) peptides on cell migration were similar to those of mammalian cell– or E. coli–derived SAA3 proteins (Supplemental Fig. 1E). We used circular dichroism (CD) spectroscopy to investigate structural differences between SAA3 (20–86) peptides and naturally produced SAA3 proteins. CD spectroscopy is a useful technique to analyze the secondary structures of polypeptides. The results showed the far-UV CD spectra of SAA3 (20–86) peptides, purified SAA3 proteins from E. coli, and mammalian cells. These spectra exhibited slightly negative values in the region of 200–230 nm, indicating that there is almost no solid secondary structure. Based on spectra analysis, we assume that the structures of both SAA3 (20–86) peptide and naturally produced SAA3 protein are very similar to each other in the PBS solution (Supplemental Fig. 2).

To examine whether the synthetic SAA3 (20–86) peptide increased cell migration through the TLR4/MD-2 pathway, we performed a cell migration assay using peritoneal macrophages obtained from wild-type (wt), Tlr4−/−, or MD-2−/− mice. The Fpr2 agonist MMK-1 (24), a general chemoattractant, was used as a positive control in these assays. The SAA3 (20–86) peptide stimulated cell migration in peritoneal macrophages obtained from wt mice, but not from Tlr4−/− or MD-2−/− mice (Fig. 1B, 1C). Because TLR4/MD-2 can activate both the MyD88-dependent and TRIF-dependent (MyD88-independent) pathway, we further investigated the effect of the peptides on cell migration in MyD88−/−peritoneal macrophages. The SAA3 (20–86) peptide failed to induce cell migration in these macrophages (Fig. 1D). In contrast, MMK-1 increased cell migration independently of TLR4, MyD88, or MD-2 (Fig. 1B–D), and the SAA3 (20–86) failed to induce cell migration in TLR4−/−, MyD88−/−, or MD-2−/−deficient macrophages. Because SAA1/2 is known to increase cell migration through FPR1 (Fpr2), we then examined whether SAA3 (20–86) could enhance cell migration through FPR1 (Fpr2). We tested the effect of SAA3 (20–86) on cell migration in FPR1 (Fpr2)-knockdown RAW264.7 cells. SAA3 (20–86), but not MMK-1, still increased cell migration in two different FPR1 (Fpr2)-knockdown RAW264.7 cells (Supplemental Fig. 3B). In addition, we generated HEK293 FPR1 (Fpr2)-overexpressing cells to investigate the involvement of FPR1 (Fpr2) in the SAA3 effect. SAA3 did not enhance cell migration, whereas both SAA1 and SAA2 did (Supplemental Fig. 3C). These results indicate that both TLR4/MD-2 and MyD88, but not Fpr2, are necessary for cell migration by SAA3.

**SAA3 activates p38, JNK, and Akt, but not ERK2**

The authentic TLR4 ligand, LPS, was shown to activate the p38, JNK, ERK pathway (25). These MAPKs are known to be upstream of NF-kB signaling (26). SAA3 (20–86) dose dependently activated p38, JNK1, and Akt, but not ERK2 (Fig. 2A). We then examined the time dependency of p38 activation by SAA3 (20–86), and found that both p38 and p65 phosphorylation significantly increased after 30 min (Fig. 2B) with the SAA3 (20–86) treatment. In addition to these kinases, Rho kinases are known to act as molecular switches that control several cellular functions, such as actin cytoskeleton organization, cell migration and adhesion, reactive oxygen species formation, and apoptosis (27). Therefore, we tested the effects of the SAA3 (20–86) peptide on Rho activity. As shown in Fig. 2C, the Rho-GTP form increased in SAA3 (20–86)-treated RAW264.7 cells.

We then examined the effects of the peptides on p38 activation in peritoneal macrophages obtained from Tlr4−/−, MD-2−/−, or MyD88−/− mice. p38 phosphorylation by SAA3 (20–86) was observed after 30 min in wt peritoneal macrophages, but was completely abolished in Tlr4−/−, MD-2−/−, or MyD88−/−mac-

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**FIGURE 2.** The SAA3 (20–86) peptide activates p38, JNK1/2, and Akt, but not ERK2 in RAW264.7 cells. (A) The SAA3 (20–86) peptide increased p38, JNK1/2 phosphorylation in a dose-dependent manner. RAW264.7 cells were treated with the indicated concentrations (0.03, 0.1, 0.3, or 1.0 μg/ml) of the SAA3 (20–86) peptide for 30 min. Similar results were obtained in three independent experiments. (B) SAA3 activated the phosphorylation of p65 and p38 in a time-dependent manner. RAW264.7 cells were treated with 1 μg/ml SAA3 (20–86) peptide for the indicated times. Cell lysates were subjected to Western blotting using the indicated Ab. Similar results were obtained in three independent experiments. (C) The GTP-bound active form of Rho was detected after the SAA3 (20–86) stimulation. RAW264.7 cells were treated with 1 μg/ml SAA3 (20–86) peptide for 30 min. GTPyS- or GDP-loaded cell lysates were used as a positive or negative control, respectively. In the third panel, the vertical axis indicates the fold change in Rho-GTP. *p < 0.05 from three independent experiments.
The SAA3 (20–86) peptide induces the phosphorylation of p38 in a TLR4-, MD-2-, or MyD88-dependent manner. (A) Peritoneal macrophages obtained from wt or TLR4−/− mice were treated with the SAA3 (20–86) peptide at 1.0 μg/ml for the indicated times. Phosphorylated p38, JNK, Akt, and p65 were examined by the anti–phospho-p38, JNK, Akt, or p65 Ab. Similar results were obtained in three independent experiments. (B) Peritoneal macrophages obtained from wt, MD-2, or MyD88−/− mice were treated with the SAA3 (20–86) peptide at 1.0 μg/ml for 30 min, and phosphorylated p38 levels were quantified. The densitometric intensities of phosphorylated p38 were normalized against those of p38. Normalized values at 30 min relative to those at 0 min were presented. Data are the mean ± SD (n = 3). *p < 0.01 compared with 0 min.

FIGURE 3

FIGURE 4. The SAA3 (20–86) peptide causes low, but appreciable levels of endocytosis in RAW264.7 cells. (A) RAW264.7 cells were stimulated for 60 min with 1 μg/ml LPS labeled with Alexa Fluor 594 (red) or 1 μg/ml SAA labeled with Alexa Fluor 594 and costained by the anti-TLR4 Ab and Alexa Fluor 488–conjugated secondary Ab to detect endocytic vesicles. Scale bar, 20 μm. Allows indicated double-positive cells. (B) The number of vesicles double positive for TLR4 and LPS or SAA3 was counted 60 min after the stimulation by LPS or SAA3 (20–86). *p < 0.001 compared with control. (C) Flow cytometric analysis of TLR4 on the cell surface after stimulation with 100 ng/ml lipid A or 1 μg/ml SAA3 (20–86). Treated cells were stained with the biotinylated TLR4/MD-2 (MTS510) Ab, followed by streptavidin-PE. The histograms at the indicated time points showed TLR4/MD-2 (dark histogram) or the isotype control (clear histogram). The data were representative of three independent experiments.
We next examined whether SAA3 would bind to TLR4 or MD-2 directly by a pull-down assay using culture medium obtained from HighFive insect cells coinfected with protein A–tagged MD-2, TLR4, and FLAG-tagged SAA3 encoding baculoviruses. The MD-2 immunoprecipitates contained the SAA3 protein (Fig. 6A, left panel, lane 8). However, TLR4 failed to com Immunoprecipitate with SAA3 (Fig. 6A, right panel, lane 8). These results suggest that SAA3 bound MD-2, but not TLR4.

To further confirm whether the SAA3 (20–86) peptide physically binds the TLR4/MD-2 complex, we performed a surface plasmon resonance assay using mouse TLR4/MD-2 immobilized onto a ProteOn GLM chip. Various concentrations of SAA3 peptides were used as analytes. The sensorgram indicated that SAA3 (20–86) bound to the TLR4/MD-2 complex (Fig. 6C). The \( K_D \) value was calculated as 2.2 \( \mu \)M. Moreover, we evaluated the site in SAA3 responsible for TLR4/MD-2 binding, using overlapping 15-mer peptides by 7 aa (Fig. 6B). Only SAA3 (43–57) showed a marked affinity to TLR4/MD-2 (Fig. 6D). The \( K_D \) value of SAA3 (43–57) toward TLR4/MD-2 was calculated as 30 \( \mu \)M. These results suggest that the amino acid sequences of SAA3 (43–57) are responsible for binding to TLR4/MD-2. We also found that the SAA3 (35–49) peptide showed very weak binding affinity to TLR4/MD-2 in surface plasmon resonance analysis (data not shown); therefore, we suggest that K44 could be essential, but that some tertiary structure involving K44 and aa 49–57 may be required. We further investigated the SAA3/MD-2 interaction using highly purified MD-2 proteins. We generated baculovirus-derived MD-2 recombinant proteins to perform surface plasmon resonance analysis. The \( K_D \) values were calculated as 3.5 and 20 \( \mu \)M using a heterogeneous ligand model (Fig. 6F). These results indicate that SAA3 (20–86) can directly bind MD-2 and are consistent with the results obtained from the immunoprecipitation assay (Fig. 6A).

Effect of the i.v. SAA3 peptide injection on the recruitment of CD11b*Gr-1* cells in the mouse lung
Among the 15-mer peptides of SAA3, only SAA3 (43–57) significantly increased cell migration in RAW264.7 cells (Fig. 7A).
We also examined the dose-response curves of the SAA3 (35–49), SAA3 (43–57), SAA3 (51–65), and SAA3 (59–73) peptides, and suggested that the SAA3 (43–57) peptide efficiently induced cell migration (Supplemental Fig. 4C). In addition, the SAA3 (43–57)-K44E peptide failed to increase cell migration (Fig. 7B). This is consistent with the results of surface plasmon resonance analysis shown in Fig. 6D and 6E. The pulmonary induction of SAA3 in tumor-bearing mice resulted in an increase in the serum levels of SAA3 (29), which in turn stimulated the mobilization of myeloid cells from the bone marrow. Therefore, we examined myeloid cell recruitment in the lungs by injecting the SAA3 (43–57) peptide of 99.7% purity i.v. into nontumor-bearing mice. The results indicated that the SAA3 (43–57) peptide increased the recruitment of CD11bGr-1 cells in the lungs, whereas a scrambled peptide did not affect recruitment (Fig. 7C). Because the SAA3 (20–86) peptide directly bound MD-2 (Fig. 6A, 6F), we then tested the effect of SAA3 (43–57) on the recruitment assay in MD-2–/– mice. The SAA3 (43–57) peptide failed to increase recruitment in MD-2–/– mice (Fig. 7D). The scrambled peptide study and application of MD-2–/– mice confirm the functional capacity of SAA3 (43–57) peptide in vivo. In addition, similar results were obtained from mice injected with mammalian cell–derived full-length SAA3 proteins (Fig. 7E). These results suggest that SAA3 in the serum can serve as an endogenous ligand of TLR4 to induce myeloid cell mobilization from the bone marrow in a MD-2–dependent manner.

**Discussion**

One of the major arguments against endogenous peptide ligands for TLR4 is that their potential contamination with LPS is unavoidable during purification from prokaryotic or eukaryotic cells. To theoretically eliminate this possibility, we chemically synthesized SAA3, which we previously proposed as such an endogenous ligand and evaluated its physical, biochemical, and biological abilities. Compared with naturally produced peptides in cells, chemically synthesized peptides may lack structural maturation. According to structural analysis (CD spectrum), the spectrum of the chemically synthesized SAA3 (20–86) peptide revealed an intrinsically disordered feature very similar to those obtained from E. coli and mammalian cells (Supplemental Fig. 2). These results suggested that the SAA3 (20–86) peptide could almost provide the native activity of SAA3. However, the KD value between the SAA3 (20–86) peptide and TLR4/MD-2 was about three magnitudes larger than that of SAA3 expressed and purified from mammalian cells (7). Motif Scan suggests that protein kinase Cζ may phosphorylate SAA3 at serine 50 in SAA3 (43–57) (30). Posttranslational modifications in SAA3 or conformational changes may be needed for full activity to be achieved. Interestingly, most immune cell interactions are of low affinity; for example, TCR binds to the MHC peptide with a KD in the μM order, CD8 to MHC class I protein with KD > 100 μM, CD2 binds to CD58 with a Kd of 9–22 μM, and binding of CD4 to TCR is even weaker (31–34).

**FIGURE 6.** SAA3 binds to MD-2. (A) Insect cells were coinfected by baculoviruses expressing FLAG-tagged SAA3 and protein A–tagged MD-2 or TLR4. Culture media were pulled down by IgG (left panel) or the anti-TLR4 Ab (right panel) and subsequently subjected to Western blotting with the anti-FLAG Ab. (B) A series of overlapping SAA3 synthetic peptides. (C) The binding kinetics of TLR4/MD-2 complexes with SAA3 (20–86) measured by surface plasmon resonance analysis. Binding signals were determined by injecting various concentrations (3.87 μM [red line], 1.93 μM [light green line], 0.96 μM [green line], 0.48 μM [light blue line], or 0.24 μM [purple line]) of the SAA3 (20–86) peptide. Sensorsgrams showed the association and dissociation of SAA3 to TLR4. Culture media were pulled down by IgG and subsequently subjected to Western blotting with the anti-FLAG Ab. (D) The binding kinetics of TLR4/MD-2 complexes with SAA3 (43–57) measured by surface plasmon resonance analysis. Binding signals were determined by injecting various concentrations (18 μM [red line], 9 μM [orange line], 4.5 μM [green line], 2.25 μM [blue line]) of SAA3 (43–57). Sensorsgrams showed the association and dissociation of SAA3 to TLR4/MD-2. (E) The amino acid alignment of SAA3 and SAA1 in the SAA3 (43–57) region and surface plasmon resonance analysis with SAA3 (43–57) and SAA3 (43–57)-K44E. Binding signals were determined by injecting the indicated concentrations of SAA3 (43–57) or SAA3 (43–57)-K44E. (F) The binding kinetics of MD-2 with SAA3 (20–86) measured by surface plasmon resonance analysis. Binding signals were determined by injecting various concentrations (12.9 μM [red line], 3.87 μM [blue line], 1.29 μM [green line]) of the SAA3 (20–86) peptide. Sensorsgrams showed the association and dissociation of SAA3 to MD-2.
We found that the SAA3 protein was coimmunoprecipitated with MD-2 in the baculovirus system (Fig. 6A). In addition, \( K_D \) values were calculated as 3.5 and 20 \( \mu M \) by surface plasmon resonance analysis using the SAA3 (20–86) peptide against baculovirus-produced MD-2 (Fig. 6F), suggesting that SAA3 directly binds the MD-2 protein. We emphasize that MD-2 binding is necessary to exert the downstream signaling. This mode of binding is similar to that of LPS, in which contact with MD-2 was shown to induce the dimerization of TLR4. As expected, the SAA3-induced cell migration and mobilization of CD11b+Gr-1+ cells were MD-2 dependent. However, at least two points distinguish SAA3 from LPS. First, as far as cytokine induction in vitro is concerned, LPS was 10-fold more potent than the SAA3 (20–86) peptide. Given that SAA3 can persistently be produced in stimulated cells in vivo, even low levels of SAA3 may be involved in chronic inflammation. Second, SAA3 differs from LPS in that SAA3 fails to activate, at least efficiently, the TRIF-dependent activation of IFN expression even in the presence of CD14 (Fig. 5B). CD14 was shown to promote TLR4 endocytosis at low concentrations of LPS (28). Because RAW264.7 cells endogenously express CD14, LPS can cause CD14-dependent endocytosis. Although SAA3 (20–86) insufficiently induced TLR4 endocytosis (Fig. 4), this contrasts well with the \( \mu \) opioid receptor whose endocytosis is induced by morphine of extrinsic origin, but not the endogenous peptide ligand (35). Interestingly, SAA3 (20–86) still increased the transcription levels of proinflammatory cytokines such as IL-6 and TNF-\( \alpha \). These results suggest that SAA3 mainly activates MyD88-dependent NF-\( \kappa \)B signaling, and not TRIF-dependent transcription. The CD14 independency of SAA3 function was further supported by the results of a cell migration assay with peritoneal macrophages obtained from CD14-deficient mice. No difference was found in SAA3-induced cell migration between wt and CD14-deficient peritoneal macrophages (Supplemental Fig. 3D). Therefore, we suggest that CD14 may be dispensable for SAA3 functions.

SAA1 and SAA2, which contain 68–71% amino acid homology with SAA3, have been shown to be agonists for FPRL1 (Fpr2). The authentic ligand MMK-1 increased cell migration independently of TLR4, MyD88, or MD-2 (Fig. 1B–D), whereas SAA3 (20–86) failed to induce cell migration in TLR4-, MyD88-, or MD-2–deficient macrophages. In contrast, the SAA3 (20–86) peptide still caused cell migration in FPRL1 (Fpr2)-knockdown RAW264.7 cells (Supplemental Fig. 3B). These results clearly showed that the SAA3 peptide induced cell migration in a TLR4-, MD-2–, MyD88-dependent manner, but not through FPRL1 (Fpr2)-mediated signaling. This discrimination between SAA3 and SAA1 led us to narrow down the critical domain of the SAA3 sequence to 43–57, in which a single amino acid change from lysine to glutamic acid was found to affect both physical and biological activities. These results suggest that some tertiary structure involving K44 may be important for binding to MD-2.

Taken together, our results based on physical, cellular, and animal experiments support the idea that SAA3 is a potential endogenous peptide ligand for TLR4/MD-2.
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Disclosures
The authors have no financial conflicts of interest.

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