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RESEARCH ARTICLE

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Cyclophilin A is an endogenous ligand for the triggering receptor expressed on myeloid cells-2 (TREM2)

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Abstract

Triggering receptor expressed on myeloid cells 2 (TREM2) is a cell surface receptor expressed on macrophages, microglial cells, and pre-osteoclasts, and that participates in diverse cellular function, including inflammation, bone homeostasis, neurological development, and coagulation. In spite of the indispensable role of the TREM2 protein in the maintenance of immune homeostasis and osteoclast differentiation, the exact ligand for TREM2 has not yet been identified. Here, we report a putative TREM2 ligand which is secreted from MC38 cells and identified as a cyclophilin A (CypA). A specific interaction between CypA and TREM2 was shown at both protein and cellular levels. Exogenous CypA specifically interacted and co-localized with TREM2 in RAW264.7 cells, and the physical interactions were shown to regulate TREM2 signaling transduction. The Pro^{144} residue in the extracellular domain of TREM2 was found to be the specific binding site of CypA. When considered together, this provides evidence that CypA interacts specifically with TREM2 as a potent ligand.

Abbreviations: Aβ, amyloid beta; ApoE, apolipoprotein E; BMDMs, bone marrow-derived macrophages; CsA, cyclosporin A; CypA, cyclophilin A; DAP12, DNAX-activating protein 12; DMI, dexamethasone–methylisobutylxanthine–insulin; EMMPRIN, extracellular matrix metalloproteinase inducer; FACS, fluorescence activated cell sorter; FBS, fetal bovine serum; IL-1β, interleukin-1 beta; IL-6, interleukin-6; ITAM, immunoreceptor tyrosine-based activation motif; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; NFAT, nuclear factor activating T-cells; PLCγ1, phospholipase C gamma 1; PPIase, peptidyl-prolyl cis-trans isomerase; SHIP1, SH2 domain-containing 356 inositol phosphatase-1; sTREM2, soluble TREM2; TLR, Toll-like receptor; TNF, tumor necrosis factor; TREM2, Triggering receptor expressed on myeloid cells 2; TREM2-CTF, C-terminal fragment of TREM2; TREM2-ICD, intracellular domain of TREM2.

Kon-Young Ji, Su-Man Kim and Su-Min Yee contributed equally to this work.

KEYWORDS
anti-inflammation, CD147, cyclophilin A, TREM2, TREM2 ligand

1 | INTRODUCTION

The triggering receptor expressed on myeloid cells 2 (TREM2) is an innate immune receptor expressed on the cell surface of pre-osteoclasts, microglial cells, dendritic cells, and macrophages.[1-4] A role of TREM2 in the immune system has been shown to block macrophage activation by signaling through Toll-like receptor (TLR) 4.[5,6] During development of immune cells, TREM2 induces the differentiation of osteoclast, microglial cells, and monocytes to generate dendritic cells.[7,8] TREM2 consists of an extracellular domain, a transmembrane domain, and a short cytoplasmic tail lacking the signaling motifs. TREM2 delivers intracellular signaling through the adaptor protein DAP12 that contains an immunoreceptor tyrosine-based activation motif (ITAM), which provides a docking site for Syk.[6,9] Numerous TREM2 ligands, such as anionic, zwitterionic lipids, and amyloid-beta have been identified,[10] and recent studies have also reported that ApoE can bind to TREM2 and enhance phagocytosis of amyloid beta in-vitro.[11,12] However, ApoE-mediated activation of TREM2 signaling is still unclear. Moreover, a recent study has suggested that ApoE binding to TREM2 is questionable, given that TREM2 does not have the classical ApoE-binding sequence, and that a large amount of ApoE is secreted to serve as an autocrine signal in microglia.[13]

Previously, Cyclophilin A (CypA) has been shown to have both intracellular and extracellular activities.[14] CypA is best known as a specific binding protein for cyclosporin A (CsA) within cells, and these CypA–CsA complex block calcineurin-dependent activation of nuclear factor activating T-cells (NFAT).[15,16] Another function of CypA is to regulate protein folding as a molecular chaperone via peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, catalyzing *cis-trans* isomerization at proline residues.[17,18] CypA is secreted by stimulation of an inflammatory response[19,20] and oxidative stress via a vesicular pathway.[21,22] Extracellular CypA is bound to CD147, which is also known as an extracellular matrix metalloproteinase inducer (EMMPRIN), and has been identified as a receptor of CypA.[16] Extracellular CypA activity induces the signaling transduction of CD147 through peptidyl-prolyl *cis-trans* isomerization of proline residues in CD147.[17,23,24]

The purpose of this study is to identify and characterize a novel ligand for TREM2. Here, we demonstrate for the first time that CypA acts as a novel ligand for TREM2 by specific interaction and co-localization of CypA with TREM2, which suppressed the expression of LPS-stimulated pro-inflammatory cytokines. The present study will provide information that helps to further elucidate a potential regulatory mechanism by which TREM2 operates in myeloid cells and during inflammatory disease.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

MC38 colon carcinoma cells, HEK293T cells, and RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics at 37°C in a humidified 5% CO_2 incubator.

2.2 | Mice

Wild-type (WT) and TREM2$^{-/-}$ mice were maintained under specific pathogen-free conditions, and any offspring were genotyped using RT-PCR prior to use in the experiments. Mice were individually housed and maintained on a 12 hours light–dark cycle at 22 ± 1°C.

2.3 | Generation of TREM2-Ig fusion protein

The mouse TREM2 extracellular domain was subcloned into a pcDNA3.1(+) vector-containing the human-Ig (Supplementary Figure S1A). The pcDNA3.1(+) vector-containing TREM2-Ig cDNA was shown that the nucleotide sequences were 1,209 base pairs, and its predicted molecular weight was approximately 44.6 kDa (Supplementary Figure S1B). HEK293T cells were plated 1×10^6 cells into 10 cm^2 cell culture dish for 24 hours before transfection at 60% confluence and transfected with pcDNA3.1(+) vector-containing TREM2-Ig cDNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as described in the manufacturer's protocol. At 4-h post-transfection, the DNA-containing medium was replaced with fresh medium supplemented with 10% FBS and incubated for 2 days. The transfected HEK293T cells were changed with serum-free media and incubated for another 2 days. The serum-free culture supernatant was harvested and concentrated by ultrafiltration. The concentrated supernatant was purified by

protein A column after ammonium sulfate precipitation. The purified mouse TREM2-Ig was observed at approximately ~30–50 kDa using silver staining and western blot assay (Supplementary Figure S1C, D).

2.4 | Protein analysis by Nano-LC/ESI-MS/MS

Lyophilized peptide samples were dissolved in 0.1% formic acid for Nano-LC/ESI-MS/MS. We performed using a nano-LC/MS system consisting of an equipped with a Nano-source. An autosampler was used to load 5 μL aliquots of the peptide solutions into a C_{18} trap-column of I.d.75 μm, length 10 mm, and particle size 5 μm (Waters, in USA). The peptides were desalted and concentrated on the column for 5 minutes at a flow rate of 5 μL/min. Then, the trapped peptides were separated on a microcapillary column composed of C18 analytical column of I.d. 75 μm, length 150 mm, and particle size 3 μm (Waters, in USA). The mobile phase A was composed of 100% water with 0.1% formic acid. The mobile phase B was contained 100% acetonitrile with 0.1% formic acid. The gradient began with 5% B for 0.33 minutes, ramped to 35% B over 39.67 minutes, to 50% over 10 minutes, to 95% over 0,5 minutes, remained at 95% B over 9.5 mutesin, and to 5% B for another 0.5 minutes. The column was equilibrated with 5% B for 14.5 minutes before the next run. The voltage applied to produce an electrospray was 2.2 kV, and the cone voltage was 35 eV. Argon was introduced as collision gas at a pressure of 20 pounds per square inch (psi). MS scan range was 200–2000 m/z (profile mode). Data-dependent peak selection of the three most abundant MS ions from MS was used, where the collision energy was used Charge State Recognition. The distinct protein was excised from the SDS–PAGE gel. After trypsin digestion, the enzymatic peptide fragments were subjected to nano-LC/ESI-MS/MS analysis to identify the distinct protein. Based on MS and MS/MS data, the protein was successfully identified based on 95% or a higher confidence interval of their scores in the MASCOT search engine (Matrix Science Ltd).

2.5 | Western blot and far-western blot analysis

Cell lysates were prepared using radio-immunoprecipitation assay (RIPA) (Enzo Life Sciences, Farmingdale, NY, USA) lysis buffer that contains protease and phosphatase inhibitor cocktails. The protein concentrations were measured by Bradford reagent (Bio-Rad Inc., Hercules, CA). Equal amounts of protein were subjected to 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to an immunoblot polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was incubated with blocking buffer (1% BSA and 5% skim milk in TBS) at 4°C overnight and washed with TBS-T (50 mM Tris (pH 7.4), 150 mM NaCl, 0.05% Tween 20). The membrane was incubated with the individual antibodies against cyclophilin A, TREM2, apolipoprotein E (Abcam, Inc., Cambridge, MA), HA-Tag, FLAG-Tag, Syk, phospho-Syk, PI3K, phospho-PI3K, Akt, phospho-Akt, PLCγ1, phospho-PLCγ1, NFATc1, phospho-NFATc1, and alpha-tubulin (Cell Signaling Technology, Danvers, MA). In far-western blot analysis, the membrane was incubated with the recombinant fusion proteins such as TREM2-Ig, Axl-Ig, LTβR-Ig, and CD147-Ig (Sino Biological Inc., Beijing, China). After incubation, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) and developed using enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA). Protein activity was determined using a LAS-3000 image analyzer (Fujifilm, Tokyo, Japan) and Image Gauge software (Fujifilm).

2.6 | ELISA-based receptor–ligand binding assay

Recombinant CypA was serially diluted to indicate concentration in coating buffer and incubated for 2h on 96-well plates (Nunc Maxisorp, Rochester, NY). After three times washing with washing buffer (PBS containing 0.05% Tween20), the plates were blocked with blocking buffer (PBS containing 5% BSA) for 2 hours. The recombinant mouse TREM2-Ig, recombinant CD147, and site-directed mutagens of TREM2-Ig were diluted in blocking buffer and incubated in each well for 2 hours at RT. After three times washing, the binding between r.CypA and TREM2-Ig or mutagens of TREM2-Ig was detected with HRP-conjugated anti-human IgG antibody and tetramethylbenzidine (TMB) as substrate. Absorbance was measured at 450 nm after stopping the reaction with 10% sulfuric acid.

2.7 | Co-immunoprecipitation and immunoblot assay

The HEK293T cells were transfected with CypA-Flag and/or TREM2-HA plasmid. At 48 hours post-transfection, the cells were lysed in 1 mL of RIPA lysis buffer for 30 minutes at 4°C and centrifuged at 13,000 rpm for 20 minutes. The lysates were subjected to immunoprecipitation with indicated antibodies and coupled to protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were washed three times with phosphate-buffer saline

(PBS, pH 7.4) and treated with 5× protein sample buffer. The samples were boiled, and immunoblotting was performed. Immunoblot assay was examined with the indicated antibodies.

2.8 | Flow cytometry analysis

Cells were stained with the indicated antibodies for 30 minutes on ice and analyzed by two-color flow cytometry on FACS Calibur using Cell Quest software (BD Bioscience, Mountain View, CA).

2.9 | Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde for 15 minutes on ice and then incubated in blocking buffer (PBS containing 5% FBS) for 1 hour on ice. The cells were incubated with the indicated primary antibodies overnight at 4°C. After three times washing with blocking buffer, the indicated secondary antibodies were incubated for 1 hour on ice. The stained cells were washed three times and mounted with ProLongGold antifade reagent with DAPI (Invitrogen, Carlsbad, CA).

2.10 | Site-directed mutagenesis

Site-directed mutagenesis of cDNA encoding TREM2-Ig was performed using a QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's instruction. The mutations of TREM2-Ig were verified via automatic DNA sequencing.

2.11 | Real-time (RT)-PCR analysis

Total RNA from RAW 264.7 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified total RNA was synthesized to cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Gene expression was quantified using the ABI QuantStudio 6 Flex RT-PCR system using the TaqMan universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Primers for *β-actin*, *interleukin (IL)-1β*, *IL-6,* and *tumor necrosis factor* (*TNF*) were purchased from the TaqMan Gene Expression Assay (Applied Biosystems). The relative mRNA expression levels of *IL-1β*, *IL-6,* and *TNF* were normalized to that of *β-actin* and calculated using the ΔΔCt method.

2.12 | Statistical analysis

For the statistical analyses of the data, P values were calculated using one-way or two-way analysis of variance (ANOVA) and Student's *t*-test. The results were considered statistically significant when P values were $< .05$.

3 | RESULTS

3.1 | Identification of novel TREM2 ligand secreted from MC38 cells

In our unpublished data, we observed that TREM2-specific cellular responses in various cells were elicited by treatment with culture supernatant of MC38 colorectal carcinoma cells in a dose-dependent manner, suggesting that the endogenous TREM2 ligand may exist in the supernatant of MC38 cells. To identify a putative TREM2 ligand, serum-free culture supernatant of MC38 cells was therefore harvested and concentrated as a source of the potential TREM2 ligand. A distinct band of approximately 18 kDa was found in lanes loaded with reactant between the supernatants and TREM2-Ig compared with a media-only control (Figure 1A). The distinct band was excised from the silver-stained SDS–PAGE gel and proteins from the peptide sequence databases by LC/ESI-MS/MS and MASCOT protein identification program. The highest protein score was 223, suggesting that a distinct protein is identical or has extensive homology to a specific protein (Individual ions scores >43, $P < .05$) (Figure 1B, left panel). The sequence coverage of the distinct protein was 69% against peptidyl-prolyl cis-trans isomerase A (IPI00554989), also known as cyclophilin A (CypA), based on the mascot search results in the mouse IPI database (Figure 1B, right panel). A set of proteins that were highly correlated with the protein scores are listed in Supplementary Table S1, and the theoretical molecular weight of CypA was identical to the estimated value. To verify the correspondence of the distinct protein with CypA and its ability to interact with TREM2, western and far-western blot analyses were performed using anti-CypA and TREM2-Ig, respectively. CypA was consistently observed at the position corresponding to CypA in the supernatant of MC38 cells (Figure 1C), which interacted with TREM2-Ig (Figure 1D). These data suggest a potential interaction between CypA and TREM2.

3.2 | Comparison of binding ability of TREM2 and CD147 to CypA

Given that the culture supernatant of MC38 cells contains numerous proteins, there are a number of non-specific

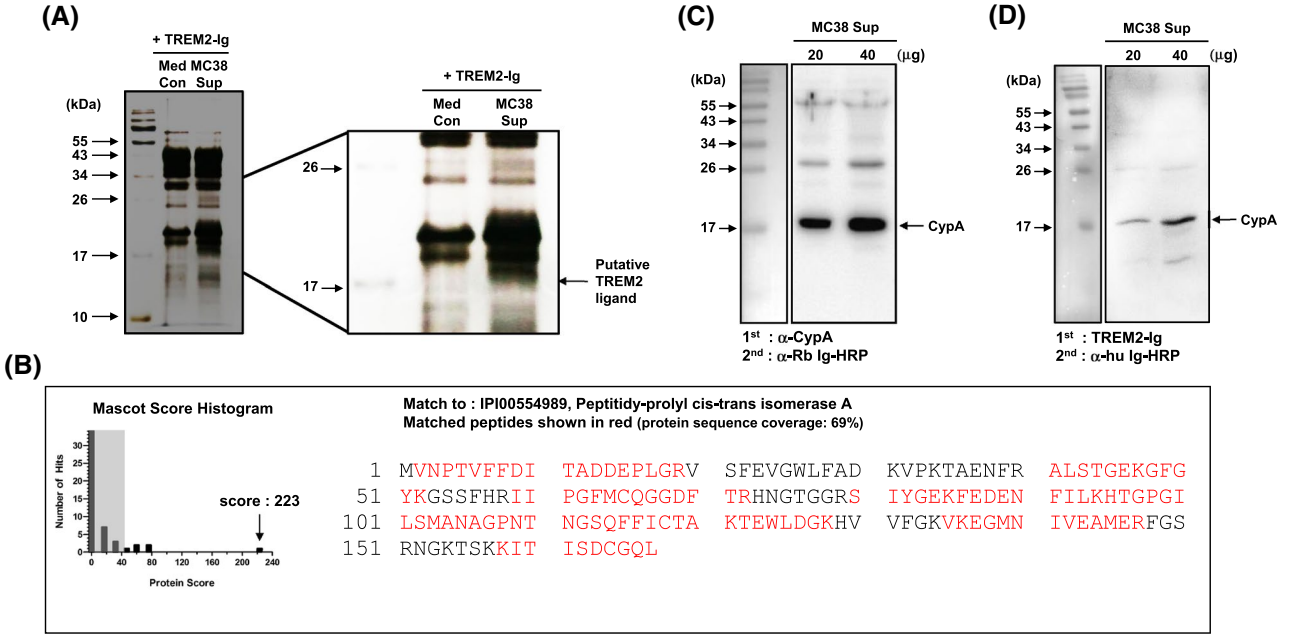

FIGURE 1 Identification of putative TREM2 ligand from the concentrated serum-free culture supernatant of MC38 cells. Serum-free culture supernatant of MC38 cells was harvested and concentrated by ultrafiltration. A, The unreacted TREM2-Ig and the reactant between TREM2-Ig and the concentrated MC38 culture supernatant were immune-precipitated with protein A/G agarose beads and separated by silver staining after being fractionated by SDS–PAGE. B, Sequence information obtained from ESI-MS of excised protein bands was taken and submitted to MASCOT (Matrix Science Ltd) for protein identification using the National Center for Biotechnology Information (NCBI) protein database. The concentrated MC38 culture supernatant was analyzed by western blot (C) and far-western blot assay (D) using anti-CypA antibody and mouse TREM2-Ig fusion protein, respectively

protein bands other than CypA, as shown in Figure 1C,D. In addition, CypA was originally known as a ligand for CD147.[25] Therefore, we examined whether recombinant mouse CypA (rCypA) is capable of binding with CD147, as well as TREM2. As shown in Figure 2A, rCypA was bound to both TREM2-Ig (left) and CD147-Ig (middle panel), but a much stronger binding signal is apparent between rCypA and TREM2 than with CD147. However, rCypA did not bind to HRP-conjugated anti-human Ig (α-hu Ig-HRP) which was used as a control for the secondary antibody (Figure 2A, right panel). Next, to rule out the possibility of non-specific binding of CypA to human Ig fused in TREM2-Ig or CD147-Ig, the binding capacity was evaluated using other cell surface receptors fused with human Ig, such as Axl-Ig and LTβR-Ig. In contrast to the specific interaction of rCypA with TREM2-Ig, rCypA did not bind to Axl-Ig, LTβR-Ig, or human Ig, providing evidence that rCypA specifically interacts with TREM2 or CD147, and that this did not result from nonspecific binding to human Ig. A dose-dependent interaction of rCypA with TREM2-Ig or CD147-Ig was also observed in ELISA-based receptor–ligand binding assay, with a much higher binding affinity of rCypA to TREM2-Ig (Kd = 0.97 μg/mL) than CD147-Ig (Kd = 1.40 μg/mL) (Figure 2C). These data are consistent with TREM2 interacting with CypA as a ligand and having a stronger binding capacity and smaller Kd than its interaction with CD147.

3.3 | Direct interaction of CypA with TREM2 in vitro

To validate the specific binding of CypA to TREM2 in vitro, the binding capability was analyzed by co-immunoprecipitation in HEK293T cells transfected with FLAG-tagged CypA and/or HA-tagged TREM2 plasmids. In HEK293T cells co-transfected with both plasmids, the protein band at the position corresponding to HA-TREM2 was detected in immunoblots using anti-HA, following co-immunoprecipitation with anti-FLAG (Figure 3A). In our previous study, we found that endogenous expression of TREM2 is gradually upregulated during adipocyte development.[26] We therefore further investigated the intrinsic interaction between CypA and TREM2 in adipocytes that were not required for their transfection. We differentiated 3T3-L1 preadipocytes into adipocytes for 10 days (upper panel), and endogenous expression of CypA and TREM2 was confirmed by western blot analysis (Figure 3B, lower panel). The intrinsic CypA-TREM2 interaction was then verified by the presence of increased CypA signal in immunoprecipitated TREM2 on day 10 (Figure 3C).

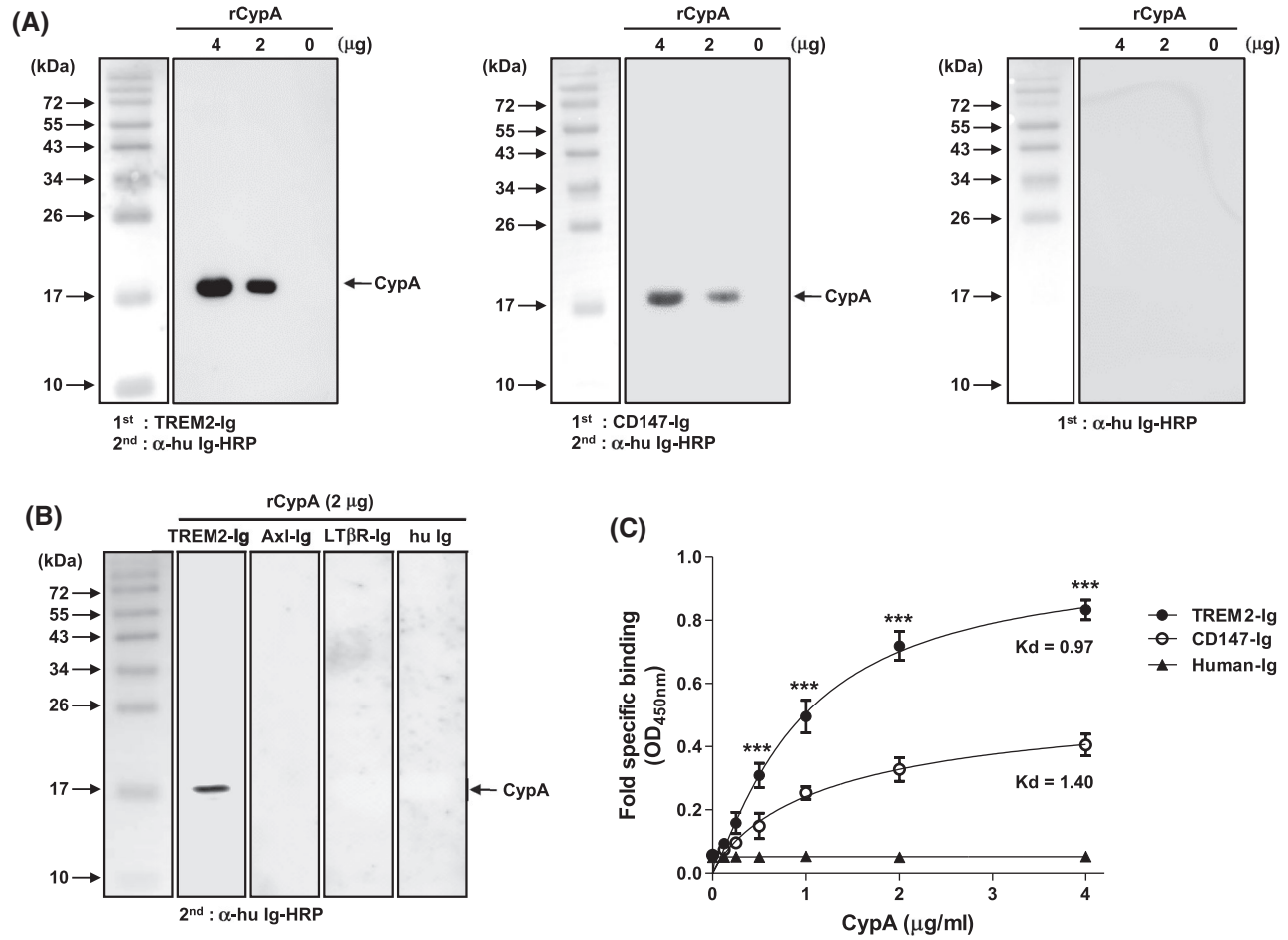

FIGURE 2 Receptor–ligand binding assay of CypA to TREM2 and CD147 by far-western blot and ELISA-based receptor–ligand binding assay. CypA was separated by 15% SDS–PAGE and transferred to PVDF membranes. A, For far-western blotting of CypA, membranes were incubated with the TREM2-Ig, CD147-Ig, or HRP-conjugated secondary antibody as negative controls. B, Far-western blotting was analyzed by the indicated fusion proteins, and HRP-conjugated secondary antibody was used as negative controls. C, The binding affinity of rCypA for TREM2-Ig and CD147-Ig was determined in a dose-dependent manner by ELISA-based receptor–ligand binding assay. Data are shown as the mean ± SEM and significant (***$P < .001$) difference between the value for CD147-Ig and TREM2-Ig

These data suggested that CypA might act as a specific ligand for TREM2.

3.4 | Specific interaction of exogenous CypA with TREM2 on cell surface

To test the specific interaction of CypA with TREM2, flow cytometry-based receptor–ligand binding assay was performed in RAW264.7 cells that endogenously express both TREM2 and CypA. The proportion of the $CypA^{+}TREM2^{+}$ population was increased by treatment of additional exogenous rCypA in a dose-dependent manner (upper panel), which was reduced by the addition of recombinant TREM2 (rTREM2) as a binding competitor for TREM2 on the cell surface, as determined (Figure 4A, lower panel). We next determined whether the treated rCypA was colocalized with TREM2 or CD147 on RAW 264.7 cells. As shown in Figure 4B, treatment of rCypA induced formation of foci in cells fluorescently stained with anti-CypA (red, white arrows). The localization of foci in CypA was consistent with those in TREM2 (green, white arrows). The merged images showed yellow fluorescence at these foci (white arrows), indicating colocalization of rCypA and TREM2. By contrast, the localization of foci formed by treatment of rCypA was not consistent between CypA (red, white arrows) and CD147 (green, yellow arrows) and consequent red fluorescence in the merged images (white arrows) (Figure 4C), suggesting that CypA and CD147 may not be fully colocalized due to less binding affinity than CypA and TREM2.

3.5 | Impaired interaction of CypA with TREM2 mutant

Previous studies have demonstrated that proline residues in the transmembrane domain of CD147 are critical for its interaction with CypA.[17,23,24] To further verify the specific interaction of

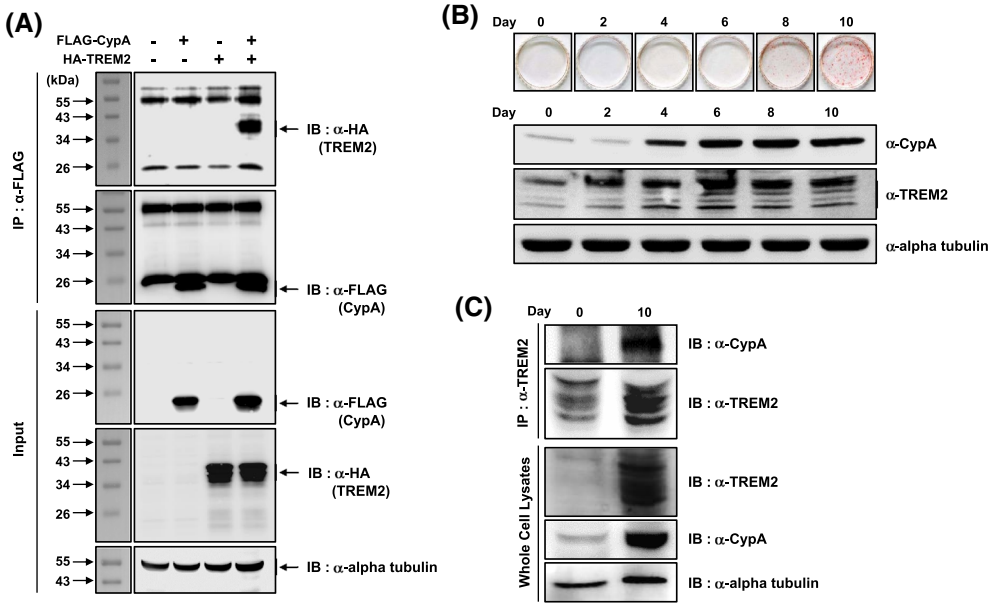

FIGURE 3 Direct interaction of endogenous CypA with TREM2 in vitro. HEK293T cells were transfected with CypA-FLAG and TREM2-HA plasmid in 10 cm^2 cell culture dish and incubated for 2 days. A, Co-immunoprecipitation was performed using anti-FLAG antibody, and the immunoprecipitated immune complexes were identified by immunoblot assay with anti-HA or anti-FLAG antibody. 3T3-L1 pre-adipocyte cells were differentiated to adipocytes using DMI-induction. B, DMI-induced 3T3-L1 cells were stained with Oil-Red O, and protein lysates from pre-adipocytes (Day-0) to adipocytes (Day-10) were analyzed by immunoblotting using the indicated antibodies. C, Immunoblots were analyzed using anti-CypA and anti-TREM2 antibodies after immunoprecipitation by the anti-TREM2 antibody

CypA with TREM2, we generated TREM2 mutants and analyzed their interaction by ELISA. Six proline residues were contained in the extracellular domain of TREM2-Ig, and each proline was converted to alanine using site-directed mutagenesis (M1~M6) (Figure 5A). Among the resulting mutants, the binding of M4 mutant (P144A) to rCypA was markedly inhibited compared with control or other mutants (Figure 5B). A dose dependency of the inhibited binding affinity was also observed consistently in the M4 mutant (Kd = 2.64 μg/mL for control vs Kd = 3.91 μg/mL for M4) (Figure 5C). These data demonstrate the specificity of CypA–TREM2 interaction.

3.6 | The regulation of cellular functions by CypA–TREM2 interaction

TREM2 signaling has been known to occur by phosphorylation of Syk, PI3K/Akt, phospholipase C gamma 1 (PLCγ1), and dephosphorylation of nuclear factor-activated T-cell (NFAT).[27] To determine whether the interaction of CypA with TREM2 is capable of regulating various cellular functions, we investigated the activation of downstream molecules of TREM2 signaling and the consequent cellular functions in rCypA-treated RAW264.7 cells. As expected, the phosphorylation of Syk, PI3K, Akt, and PLCγ1 was increased by rCypA treatment, and NFATc1 was dephosphorylated (Figure 6A). The phosphorylation patterns in rCypA-treated bone marrow-derived macrophages (BMDMs) of WT mice were similar with those in rCypA-treated RAW264.7 cells, but there were no differences in those of $TREM2^{-/-}$ mice (Figure 6B). The activation of TREM2 signaling elicits upregulation of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF.[28] As shown in Figure 6C, treatment of RAW264.7 cells with rCypA resulted in increased mRNA expression of IL-1β, IL-6, and TNF, which was attenuated to control levels by the addition of rTREM2 as a competitor for the interaction of rCypA with cell surface TREM2. TREM2 has also been reported to inhibit inflammatory responses by attenuating toll-like receptor (TLR) signaling.[29] Treatment of lipopolysaccharide (LPS) led to excessive mRNA expression of IL-1β, IL-6, and TNF, which was significantly inhibited by the addition of rCypA (Figure 6D). Inhibition of expression was restored by an additional treatment of rTREM2 with no significant restoration of TNF. The expression patterns were also similar with those of rCypA-treated BMDMs from WT mice, but no significant differences were observed in those of $TREM2^{-/-}$ mice (Figure 6E). These data provide direct evidence that CypA triggers signaling through TREM2 and thereby leads to TREM2-specific cellular functions as a ligand for TREM2.

3.7 | Higher binding ability of CypA with TREM2 than ApoE

Recent studies have reported that ApoE is a ligand with high-affinity binding for TREM2.[11,12] To compare the binding

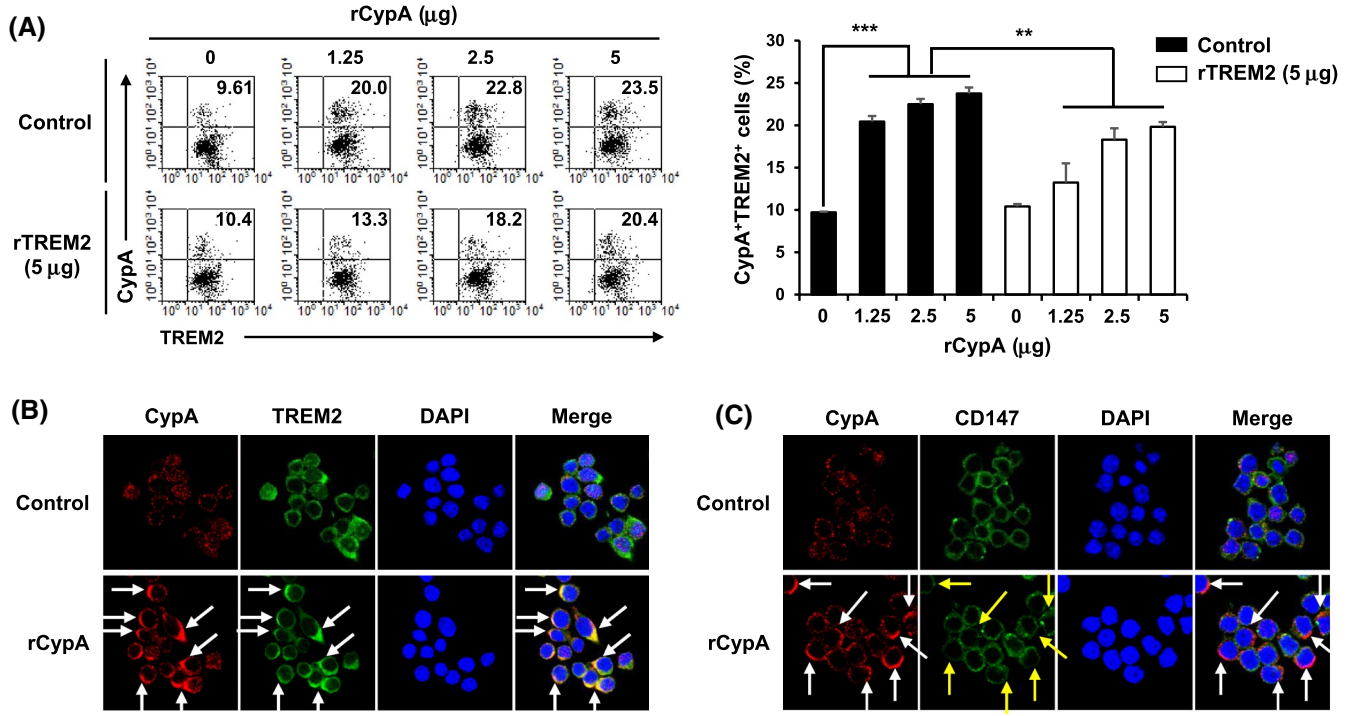

FIGURE 4 Receptor–ligand binding of exogenous CypA with TREM2 in RAW 264.7 cells. A, The RAW 264.7 cells were treated with the indicated concentration of rCypA and rTREM2 for 30 minutes, followed by flow cytometry analysis. The number of each quadrant represents the cell percentage (left panel). The average percentages of $CypA^{+}TREM2^{+}$ cell population obtained from flow cytometry analysis are represented as a bar graph (right). Data shown are representative of three independent experiments and error bars represent mean ± SEM. $^{**}P < .01$; $^{***}P < .001$. B-C, RAW 264.7 cells treated with rCypA were stained with immunofluorescence antibodies such as CypA (Red), TREM2 (Green), and CD147 (Green). And the nucleus was stained with DAPI (Blue). Images for each protein and the merged image are shown

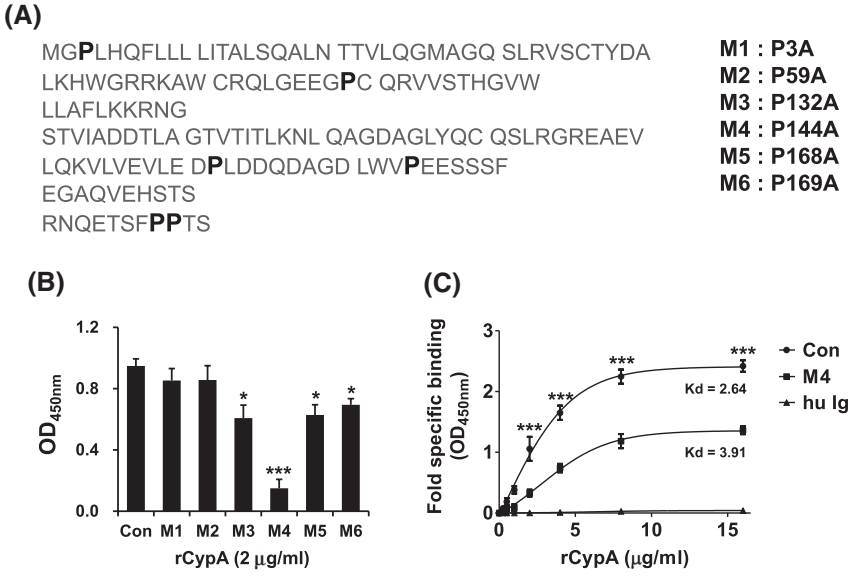

FIGURE 5 Impairment of receptor–ligand binding between CypA and P144A TREM2-Ig mutant. A, Proline residues in the predicted extracellular domain of TREM2 were mutated by site-directed mutagenesis to alanine, respectively. The specific interaction of rCypA with TREM2-Ig mutants (B) and the binding affinity of rCypA with TREM2-Ig and P144A TREM2-Ig mutant was measured in a dose-dependent manner by ELISA-based receptor–ligand binding assay. These data are shown as the mean ± SEM and significant ($^{*}P < .05$; $^{***}P < .001$) difference between the value for control and TREM2-Ig mutants

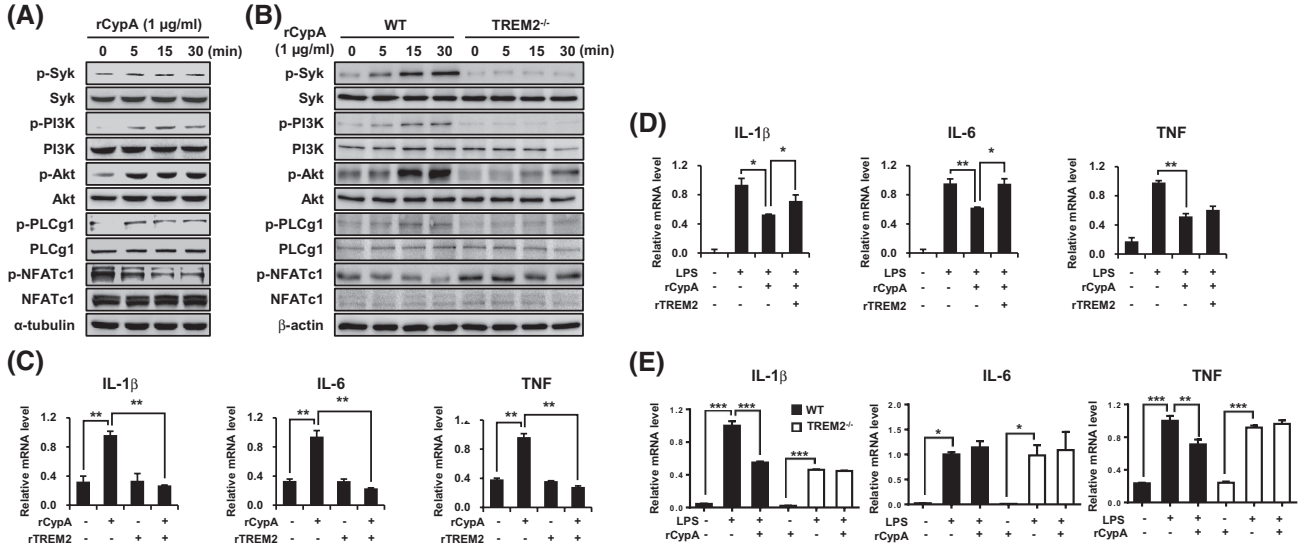

FIGURE 6 The regulation of cellular functions by the interaction of CypA and TREM2. A, B, RAW 264.7 cells and bone marrow-derived macrophages (BMDMs) were stimulated with 1 μg/mL of rCypA, and the phosphorylation of signaling molecules including Syk, PI3K, Akt, PLCγ1, and NFATc1 was investigated by western blot analysis. Expression was normalized to that of α-tubulin or β-actin. C, RAW 264.7 cells were stimulated with 1 μg/mL of rCypA for 24 hours, and the gene expression of pro-inflammatory cytokines was analyzed by RT-PCR. D, E, RAW264.7 cells and BMDMs were stimulated by 100 ng/mL of LPS for 24 hours after interacted with 500 ng/mL of rCypA for 1 hours, and the gene expression of pro-inflammatory cytokines was analyzed by RT-PCR. The data are shown as the mean ± SEM and significant difference indicated (*$P < .05$; **$P < .01$; ***$P < .001$)

ability of CypA and ApoE with TREM2, a receptor–ligand binding assay was performed by far-western blot and ELISA using rCypA or rApoE. In contrast to data reported in the recent studies,[11,12] we found that a dose-dependent binding was observed between rCypA and TREM2-Ig, but not between rApoE and TREM2-Ig (Figure 7A). Given that western blot analysis showed bands that were recognized by anti-ApoE in a dose-dependent manner, no binding between rApoE and TREM2-Ig was observed in far-western blot, and this was not due to differences arising from loading or transfer of proteins (Figure 7B). Furthermore, a much higher binding affinity was also shown between rCypA and TREM2-Ig than between rApoE and TREM2-Ig, as determined by ELISA (Figure 7C). These data suggest that CypA interacts more specifically with TREM2 and acts as a more potent ligand than ApoE.

4 | DISCUSSION

To date, our understanding of TREM2 function has been severely limited due to the fact that no specific ligand for TREM2 has been reported. This study aimed to identify and elucidate a novel ligand for TREM2, and we demonstrated that CypA specifically interacts with TREM2. Although several previous studies have been reported that CypA is a ligand for CD147,[17,23,25] our findings showed that CypA has a much higher binding affinity for TREM2 than CD147. Recently, ApoE has been reported to bind to TREM2 and upregulate phagocytic activity in microglia.[11,12] However, we found that CypA preferentially binds to TREM2 when compared with ApoE. Furthermore, we found that CypA directly induces-TREM2 signaling and cytokine production. Therefore, this study demonstrates that CypA is very likely to be a specific ligand for TREM2.

In our unpublished findings, cell growth, proliferation, and differentiation of TREM2 expressing cells were found to be increased by treatment of culture supernatants of MC38 cells in a dose-dependent manner. We, therefore, used the culture supernatant of MC38 cells as a source of TREM2 ligand and generated recombinant mouse TREM2-Ig fusion proteins for identification of a novel ligand for TREM2 (Supplementary Figure S1). TREM2-Ig produced by HEK293T cells was shown to identify multiple bands in the range of 30–50 kDa using silver staining and western blot analysis (Figure 1A, Supplementary Figure S1c, d). In addition, similar protein bands of TREM2 were observed in co-immunoprecipitation assays of adipocyte and HEK293T cells (Figure 3B,C). These findings are consistent with previous studies that detect multiple protein bands corresponding to secreted fragments of TREM2 (soluble TREM2) in the range of 36–50 kDa and the full-length membrane-bound TREM2 between 36 and 60 kDa as well as its 17 kDa membrane-bound C-terminal fragment (CTF).[30-32] These previous studies have also shown that the full-length membrane-bound TREM2 is shed by ADAM10, resulting in the generation of soluble TREM2 and CTF. The CTF is further cleaved by γ-secretase to produce

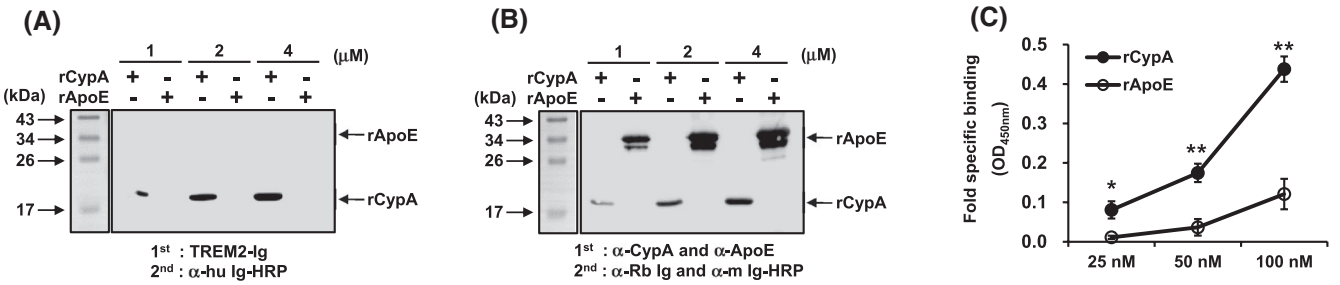

FIGURE 7 Comparison of binding ability of CypA and ApoE to TREM2. A, rCypA and rAopE were subjected to 15% SDS–PAGE and transferred to PVDF membranes. For far-western blotting, the membranes were incubated with the TREM2-Ig. Western blotting was performed using anti-CypA and anti-ApoE antibodies. B, The binding affinity was performed by an ELISA-based receptor–ligand binding assay in a dose-dependent manner. C, The phagocytosis ability of BV2 cells was analyzed by flow cytometry after stimulation with 100 nM of rCypA or rAopE for 2 hours. These data are shown as the mean ± SEM and significant difference indicated (*$P < .05$; **$P < .01$)

the intracellular domain (ICD) of TREM2. Therefore, the multiple bands in our study do not indicate nonspecific binding bands but may be due to cleavage products of full-length TREM2 protein generated by sequential proteolytic processing.

Previous reports have shown that CypA is secreted by various cell types upon stimulation, such as LPS-induced macrophages and LPS-stimulated smooth muscle cells, and then released upon cell death.[14,19-22] Secreted CypA can induce signaling transduction via extracellular binding and peptidyl-prolyl *cis-trans* isomerase activity in proline residues of CD147.[17,23,24] Based on these studies, we showed that treatment of CypA increased TREM2-specific signaling transduction and upregulated the production of proinflammatory cytokines. Moreover, we found that proline residue 144 of TREM2 might be considered as a binding site of CypA, and it was located close to the transmembrane domain of TREM2, similar to proline residues 180 and 211 of CD147. Furthermore, our data showed that the M4 mutant of TREM2-Ig did not completely impair the interaction with CypA, and the M3, M5, and M6 mutants of TREM2-Ig only decreased the interaction slightly. These results suggest that several peptides around proline 144 of TREM2 are involved in the interaction between CypA and TREM2.

The high-affinity stimulation of TREM2 promotes myeloid cell activation, including cell proliferation, cell survival, phagocytosis, production of proinflammatory cytokines, and cytoskeletal rearrangement, by inducing phosphorylation of Syk, PI3K/Akt, PLCγ1, and dephosphorylation of NFAT.[27,28] However, low-affinity stimulation of TREM2 suppresses production of TLR-induced inflammatory cytokines by recruitment of SH2 domain-containing inositol phosphatase-1 (SHIP1) to DNAX-activating protein 12 (DAP12), and it competes with and prevents further recruitment of Syk and PI3K.[27,29] Although we did not compare the signal transduction of TREM2 between high-affinity and low-affinity stimulation of TREM2, the production of proinflammatory cytokines was markedly increased by treatment of rCypA (1ug/ml) and significantly decreased by treatment of rCypA (100 ng/mL) after LPS stimulation. These data indicate that CypA regulates TREM2-specific signal transduction and its cellular functions in myeloid cells as a ligand for TREM2, which may be dependent on the concentration of CypA.

We found that the activated TREM2 signaling triggered by rCypA treatment dephosphorylated NFATc1 and thereby resulted in increased expression of proinflammatory cytokines, such as IL-1β, IL-6, and TNF, which was attenuated to control levels by the addition of rTREM2 as a competitor for the interaction of rCypA with cell surface TREM2 (Figure 6). In addition, the expression of proinflammatory cytokines increased by the interaction between TREM2, and CypA was suppressed by treatment with CsA (Supplementary Figure S2). It has been well-known that CsA as a calcineurin inhibitor leads to immunosuppressive effects by downregulating NFATc1, which inhibits the expression of proinflammatory cytokines.[33,34] TREM2 has been known to be also expressed in tumor-associated macrophages, which create an anti-inflammatory and immunosuppressive microenvironment that promotes tumor escape from immunity, suggesting suppression of anti-tumor responses.[35] CsA promotes the expression of TGFβ, which leads to the creation of an immunosuppressive tumor microenvironment and thereby cancer progression.[36] However, it has not yet been defined the immunosuppressive mechanisms by which TREM2 regulates the CsA-mediated inhibition of calcineurin activation or TREM2 involves in the blockade of calcineurin-mediated NFATc1 inhibition by regulating binding between intracellular CyPA and CsA. The elucidation of the precise mechanism for links between immunosuppressive effect of TREM2 and CsA may be helpful to develop novel therapeutic strategies for different types of immune diseases caused by the malfunction of TREM2.

Recent studies have demonstrated that ApoE may act as a ligand for TREM2 and increase phagocytosis of apoptotic neurons by microglia.[11,12] In this study, specific interaction of rApoE with TREM2-Ig was not observed by far-western blot

analysis, but the interaction of rCypA with TREM2-Ig was shown. These data suggest that CypA has a stronger binding affinity for TREM2 compared with ApoE, and this is consistent with the ELISA-based receptor–ligand binding assay. In a previous study, free ApoE has been reported not to enhance TREM2 signaling or phagocyte activation, due to lack of the ability to binding with TREM2, but plaque-associated ApoE has multiple opportunities to bind with TREM2 and therefore increase phagocytic activity.[12] Thus, CypA might directly bind to TREM2 and upregulate phagocytosis through TREM2 signal transduction, but ApoE might preferentially need to constitute a complex with lipoprotein particles, apoptotic neuronal debris, or Aβ plaques followed by TREM2 recognizing the ApoE complex.

In summary, we suggest that CypA is a novel ligand for TREM2 and may act as a stronger ligand than ApoE. We also provide evidence that CypA has a much higher binding affinity for TREM2 when compared with CD147. These findings provide information that will help to further elucidate the regulatory mechanisms of various cellular functions and diseases that occur due to TREM2 signaling in myeloid cells.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

K.Y. Ji and S.M. Kim designed the experiments, acquired the data, and wrote the manuscript. E.M. Kim, E.H. Lee, H.R. Choi, and C.H. Yun contributed to critical reading of the manuscript and useful discussions. H.S. Kang, and C.W. Lee conducted the experiments. H.B. Lee and H.S. Kang designed the experiments, analyzed the data, reviewed, and edited the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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