

## Supplementary Data

# Recombinant human KAI1/CD82 attenuates M1 macrophage polarization on LPS-stimulated RAW264.7 cells *via* blocking TLR4/JNK/NF- $\kappa$ B signal pathway

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## MATERIALS AND METHODS

### *Recombinant human KAI1*

*rhKAI1* protein (catalog No. 12275-H08H) was purchased from Sino Biological Inc. (Beijing, China), as previously described (1). A DNA sequence encoding the second extracellular domain of human KAI1 (P27701-1) (Gly 111–Leu 228) was fused with a poly-histidine tag at the C-terminus and a signal peptide at the N-terminus. Glu-214 mutant of *rhKAI1* (Thr 171–Leu 228) and human recombinant TLR4 was obtained from ANYGENE (Gwangju, Korea).

### *Cell culture analysis*

Isolation and cultivation of bone marrow-derived macrophages (BMDM) was performed as described earlier with some modifications (2, 3). All animal experiments were approved by the Institutional Animal Care and Use Committee of Pusan National University (approval No. 2022-031-A1C0) and conducted in accordance with established guidelines for the care and use of laboratory animals. Eight-week old male C57BL/6 mice were purchased from Samtako Bio Korea Co. (Osan, Korea) and acclimated for 1 week. Mice were sacrificed and their legs were dissected to extract their bone marrow from the tibia and the femur bones. In brief, surrounding muscles were removed following cutting of the joints and the exposed bone marrow was flushed out with PBS. Subsequently, cells were passed through a 70  $\mu$ m cell strainer and centrifuged at  $200 \times g$  for 5 min at 4 °C. Then pellet cells were resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen-Gibco, Carlsbad, CA, USA), 1% penicillin-streptomycin solution (Invitrogen-Gibco) and 50 ng/mL GM-CSF (R&D systems, Inc., Minneapolis, MN, USA). On day 6, BMDM cultures at nearly 90% confluency were used for all experiments. Mouse macrophage-like RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC; Manassas, MD, USA) and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin solution.

### *Cell viability*

The effect of *rhKAI1* on the cell viability of BMDM and RAW 264.7 cells in the presence or absence of LPS was determined using a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay as previously described (1). Briefly, the cells were untreated or pre-treated with various concentration of *rhKAI1* for 2 h, followed by 100 ng/mL LPS (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 24 h, and the addition of WST-1 reagent

(Roche Diagnostics, Indianapolis, IN, USA) for 2 h. The optical density was measured with a microplate spectrophotometer (Genius, Männedorf, Switzerland) set at 450 nm. The results are expressed as the percentage of the treated cells compared to untreated cells. The cell morphology was observed using an inverted-phase contrast microscope (Olympus, Tokyo, Japan).

#### *FACS analysis of M1 macrophage phenotype-related surface markers*

BMDM and RAW 264.7 cells were pre-treated with the indicated concentrations of *rhKAI1* for 2 h, following by 100 ng/mL LPS treatment for 24 h. The cells were then washed twice with PBS and centrifuged at 1,700 rpm for 5 min at 4 °C. The supernatant was removed, and the RAW 264.7 cells were re-suspended and incubated in 100 µL of FACS buffer (1% FBS and 0.1% bovine serum albumin in PBS) containing PerCP anti-mouse CD86 (catalog No. 105026, BioLegend, San Diego, CA, USA) and APC/Cyanine 7 anti-mouse F4/80 (catalog No. 123118, BioLegend) antibodies for 30 min at 4 °C. In a parallel experiment, BMDM cells were incubated with FACS buffer containing CD11b-PE monoclonal antibody (catalog No. #12-0112-82, Invitrogen, Waltham, MA, USA), PerCP anti-mouse CD86 antibody (catalog No. 105026, BioLegend), and APC/Cyanine 7 anti-mouse F4/80 antibody (catalog No. 123118, BioLegend) for 30 min at 4 °C. Data were acquired with a FACSCanto™ II flow cytometer (BD, Franklin Lakes, NJ, USA) and analyzed with the FlowJo Software v10.8.1 (BD, Ashland, OR, USA)

#### *Real-time quantitative polymerase chain reaction (q-PCR) analysis*

RAW 264.7 cells were pre-treated with the indicated concentrations of *rhKAI1* for 2 h, following by 100 ng/mL LPS treatment for 24 h. Thereafter, the total RNA was extracted using Tri-RNA Reagent (FAVORGEN, Ping-Tung, Taiwan) and the cDNA was synthesized using a cDNA synthesis kit (AccuPower®, Bioneer, Daejeon, Korea) in accordance with the manufacturer's instructions. qPCR was carried out utilizing the SYBR Premix Ex Taq Kit (Takara Biotechnology, Otsu, Japan) and amplified by the ABI QuantStudio3 (Applied Biosystems, Carlsbad, CA, USA). All primers used in this study are listed in the supplementary Table S1.

#### *Measurement of nitric oxide (NO), prostaglandin E2 (PGE<sub>2</sub>) and cytokines*

RAW 264.7 cells were pre-treated with the indicated concentrations of *rhKAI1* for 2 h, following by 100 ng/mL LPS treatment for 24 h. Supernatants were collected, and the levels of NO, PGE<sub>2</sub>, and cytokines were measured. NO production was estimated by the quantity of nitrite released using Griess reagent colorimetric assays, as previously described (4). Briefly, supernatants were mixed with an equal volume of

1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride, and then incubated in the dark for 10 min. Thereafter the absorbance was measured at 540 nm using a microplate spectrophotometer and was calculated by comparison to a sodium nitrite standard curve. An enzyme-linked immunosorbent assay (ELISA) was carried out to quantify the secreted levels of PGE<sub>2</sub> (catalog No. KEG004B, R&D systems, Inc.), IL-1 $\beta$  (catalog No. MLB00C, R&D systems, Inc.), and IL-6 (catalog No. M6000B, R&D systems, Inc.) according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### *Immunofluorescence analysis*

RAW 264.7 cells were pre-treated with the indicated concentration of *rhKAI1* for 2 h, following by 100 ng/mL LPS treatment for 1 h, cells fixation and permeabilization with 0.2% Triton X-100. After blocking with 3% bovine serum albumin in PBS, cells were immersed in anti-toll-like receptor 4 (TLR4; catalog No. #PA5-23124, Invitrogen), anti-LPS (catalog No. # MA5-41631, Invitrogen), and anti-nuclear factor kappa-light-chain-enhancer of activated B (NF- $\kappa$ B) p65 (catalog No. sc-8008, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for 3h. This was followed by the proving of Alexa Fluor™ 488-labeled goat anti-mouse (catalog No. A11001, Invitrogen), Alexa Fluor™ 568-labeled goat anti-mouse (catalog No. A11004, Invitrogen), and Alexa Fluor™ 488-labeled goat anti-rabbit (catalog No. A11008, Invitrogen) IgG secondary antibodies for 30 min. The cells were incubated in 4',6'-diamidino-2-phenylindole (DAPI; Sigma - Aldrich Chemical Co.) for 10 min to visualize the nucleus. Immunofluorescence images were obtained with a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

### *Western blots*

RAW 264.7 cells were pre-treated with the indicated concentration of *rhKAI1* for 2 h, following by 100 ng/mL LPS treatment for 24 h, harvesting and total protein lysates extraction (5). Equal amounts of protein were loaded, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20, subsequently probed with primary antibodies overnight at 4 °C, and then immunoblotted with the corresponding secondary antibodies for 1 h at room temperature. All primers used in this study are listed in the supplementary Table S2. The membranes were then exposed to an enhanced chemiluminescence solution (Thermo Fisher Scientific) and visualized using a LAS-3000 Imaging System (Fujifilm Image Reader, Valhalla,

NY, USA). Densitometric analysis of the data was performed using the ImageJ® software (v1.48, NIH, Bethesda, MD).

### *Molecular docking*

To predict the interaction of *rhKAI1* and TLR4, the three-dimensional (3-D) structures of TLR4 and *rhKAI1* were obtained from the Protein Data Base (PDB; <https://www.rcsb.org>) and AlphaFold (<https://alphafold.ebi.ac.uk>), respectively. The PDB ID code of TLR4 is 3VQ2 and the AF ID code of *rhKAI1* is P277701. The binding affinity of TLR4 and *rhKAI1* was calculated with the PyRx virtual screening program (The Scripps Research Institute, San Diego, CA, USA, <https://pyrx.sourceforge.io>) and the binding complex was visualized by the PyMOL molecular graphics system (Schrodinger, Inc., New York, NY, USA, <https://pymol.org>). After the binding of TLR4 and *rhKAI1*, the most stable complex was selected from the top 10 complexes obtained through the ZDOCK server (<https://zdock.umassmed.edu>)

### *Determination of binding affinity ( $K_D$ ) using BLItz label-free biosensor system*

BLItz label-free biosensor system for biolayer interferometry assay (Sartorius AG, Göttingen, Germany) was used for the binding test and the determination of binding affinity ( $K_D$ ). *rhKAI1* (500 nM) was loaded onto HIS1K biosensor for immobilization. Various concentration of *rhTLR4* (5 ~ 40  $\mu\text{g}/\text{mL}$ ) were incubated with the aptamer-immobilized HIS1K sensor in binding buffer. Both association and dissociation events were recorded for 120 s for the kinetic analysis after a waiting time for baseline to stabilize. Binding kinetics data were analyzed using BLItz Pro 1.3 software.

### *Statistical Analysis*

GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA) was utilized for the statistical analysis. Data are presented as the mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA) and Tukey's post-hoc analysis were performed for comparisons between groups at  $p < 0.05$ .

**Supplementary Table S1.** Information of primers used for qPCR analysis.

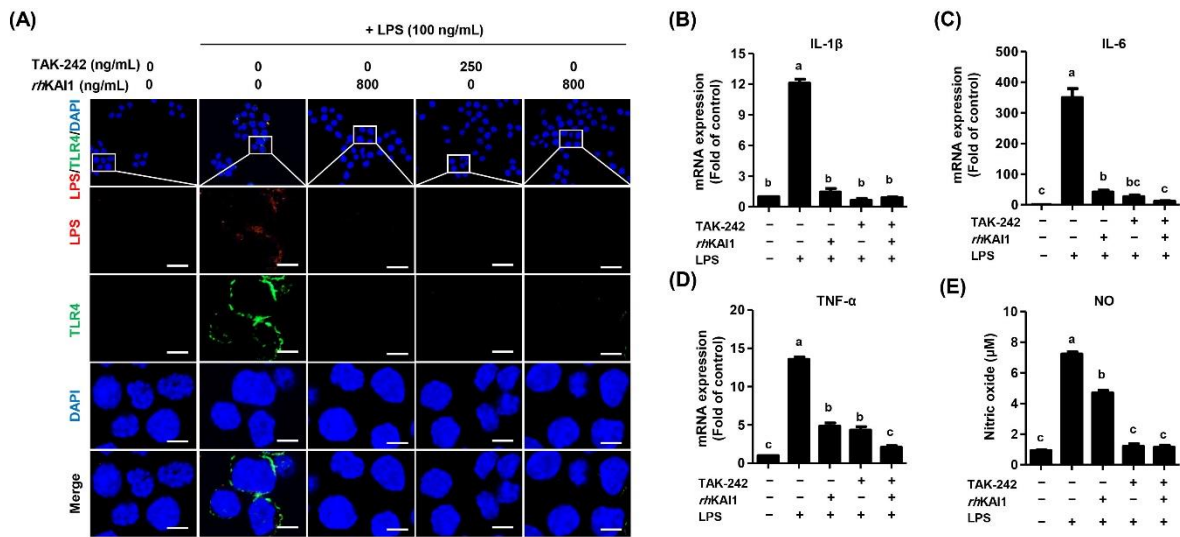
Gene	Primer Sequence (5'-3')	
	Forward	Reverse
COX-2	GCGACATACTCAAGCAGGAGCA	AGTGGTAACCGCTCAGGTGTTG
GAPDH	CATCATGCCACCCAGAAGACTG	CTCCACGACGTAICTCAGCG
IL-1 $\beta$	TGGGAAACAACAGTGGTCAGG	CCATCAGAGGCAAGGAGGAA
IL-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
TNF- $\alpha$	GAGTGACAAGCCTGTAGCC	CTCCTGGTATGAGATAGCAA

COX-2, cyclooxygenase-2; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IL-1 $\beta$ , interleukin 1 beta; IL-6, interleukin-6; TNF- $\alpha$ , tumor necrosis factor-alpha.

**Supplementary Table S2.** Primary and secondary antibodies used for immunoblotting.

Antibodies	Supplier	Catalog No.	Dilution
ERK	Cell signaling Technology	#4377	1:1,000
JNK	Cell signaling Technology	#9252	1:1,000
p38 MAPK	Cell signaling Technology	#9212	1:1,000
phospho-ERK	Cell signaling Technology	#4370	1:1,000
phospho-JNK	Cell signaling Technology	#9255	1:1,000
phospho-p38 MAPK	Cell signaling Technology	#9215	1:1,000
goat anti-mouse IgG-HRP	Santa Cruz Biotechnology, Inc.	sc-2005	1:2,500
goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology, Inc.	sc-2004	1:2,500

Cell Signaling Technology (Beverly, MA, USA); Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)



**Supplementary Figure S1.** *rhKAI* attenuates LPS-induced M1 macrophage polarization through regulation of TLR4 expression. Cells were stimulated with or without *rhKAI1* (800 ng/mL) and/or 250 ng/mL TAK-242 for 1h and then treated with 100 ng/mL LPS for another 24 h. (A) The cells were subjected to immunofluorescence staining with LPS and TLR4 specific antibodies. Representative images were acquired using a confocal laser scanning microscopy. Confocal images show the expression of LPS (red), TLR4 (green), and DAPI (blue). Scale bar; 50  $\mu$ m. Relative levels of mRNA expression for IL-1 $\beta$  (B), IL-6 (C) and TNF- $\alpha$  (D) were expressed as fold of control. (E) NO concentration was measured by the Griess reaction. Data are expressed as the mean  $\pm$  SD (n = 3). <sup>a-c</sup> Bars with different letters are significantly different at p < 0.05 by Tukey test.



## REFERENCES

1. Lee H, Han JH, Kang YJ et al (2022) CD82 attenuates TGF- $\beta$ 1-mediated epithelial-mesenchymal transition by blocking smad-dependent signaling in ARPE-19 cells. *Front Pharmacol* 13, 991056
2. Zhang X, Goncalves R and Mosser DM (2008) The isolation and characterization of murine macrophages. *Curr Protoc Immunol* Chapter 14, Unit 14.1
3. Bailey JD, Shaw A, McNeill E et al (2020) Isolation and culture of murine bone marrow-derived macrophages for nitric oxide and redox biology. *Nitric Oxide* 100-101,17-29
4. Chae BS (2021) Effect of low-dose corticosterone pretreatment on the production of inflammatory mediators in super-low-dose LPS-primed immune cells, *Toxicol Res* 37, 47-
5. Lee H, Hwang-Bo H, Ji SY et al (2020) Diesel particulate matter<sub>2.5</sub> promotes epithelial-mesenchymal transition of human retinal pigment epithelial cells via generation of reactive oxygen species. *Environ Pollut* 262, 114301