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Recombinant human KAI1/CD82 attenuates M1 macrophage polarization on LPS-stimulated RAW264.7 cells via blocking TLR4/JNK/NF-κB signal pathway

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KAI1/CD82, a membrane tetraspanin protein, can prevent various cancers and retinal disorders through its anti-angiogenic and anti-metastatic capacity. However, little is known about its anti-inflammatory effect and molecular mechanism. Therefore, the present study aimed to inLPSvestigate effect of a recombinant protein of the large extracellular domain of human KAI1 (Gly 111-Leu 228, *rh*KAI1) on lipopolysaccharides (LPS)-stimulated RAW264.7 macrophage-like cells and mouse bone marrow-derived macrophages (BMDM) and to identify its underlying mechanism. Our data showed that *rh*KAI1 suppressed expression levels of classically macrophages (M1) phenotype-related surface markers F4/80$^+$CD86$^+$ in LPS-stimulated BMDM and RAW264.7 cells. In addition, LPS markedly increased mRNA expression and release levels of pro-inflammatory cytokines and mediators such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α, cyclooxygenase-2, nitric oxide and prostaglandin E$_2$, whereas these increases were substantially down-regulated by *rh*KAI1. Furthermore, LPS strongly increased expression of NF-κB p65 in the nuclei and phosphorylation of ERK, JNK, and p38 MAPK. However, nuclear translocation of NF-κB p65 and phos-

phorylation of JNK were greatly reversed in the presence of *rh*KAI1. Especially, *rh*KAI1 markedly suppressed expression of toll-like receptor (TLR4) and prevented binding of LPS with TLR4 through molecular docking predict analysis. Importantly, Glu 214 of *rh*KAI1 residue strongly interacted with Lys 360 of TLR4 residue, with a binding distance of 2.9 Å. Taken together, these findings suggest that *rh*KAI1 has an anti-inflammatory effect on LPS-polarized macrophages by interacting with TLR4 and down-regulating the JNK/NF-κB signaling pathway. [BMB Reports 2023; 56(6): 359-364]

INTRODUCTION

Macrophages can act as effector defensive cells against pathogens and play a key regulator role in innate and adaptive immune responses (1). Under the influence of various environmental signals, macrophages present two functionally distinct phenotypes, classically activated macrophages (M1) and alternatively activated macrophages (M2) (2). M1-like macrophages are polarized by lipopolysaccharide (LPS) either alone or together with T helper type 1 (Th1) cytokines such as interferon gamma, granulocyte-macrophage colony-stimulating factor (GM-CSF) to promote T helper type 1 (Th1) response (3, 4). M1 macrophages are mainly involved in pro-inflammatory responses characterized by production of pro-inflammatory and Th1 cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and chemokines (3). In contrast, M2-like polarized macrophages contribute to tissue remodeling, repair, angiogenesis, and homeostasis through Th2 immune response (5). M2 macrophages are involved in anti-inflammatory responses by producing Th2 cytokines (such as IL-4 and IL-10) and transforming

growth factor beta (TGF-β) (6). In this regard, numerous studies have reported that imbalance of macrophage polarization contributes to inflammation and dysregulation of the immune system, affecting various diseases such as obesity, cancer, and rheumatoid arthritis (4, 7, 8). Therefore, modulating the activation state of macrophages might be one of the effective therapeutic strategies.

KAI1, also known as CD82, is a protein belonging to the tetraspanin protein family. It has four transmembrane domains, two extracellular loops, a very small intracellular loop, and short N- and C-terminal cytoplasmic tails (9, 10). KAI1 can interact with integrins, epidermal growth factor receptor, protein kinase C, Src kinase, and small GTPase. It is involved in cancer progression through regulation of cellular events including cell adhesion, migration, and survival (9, 10). Several tetraspains including KAI1 can interact with pattern recognition receptors such as C-type lectin receptors, TLRs, and Fc receptors (11, 12). In particular, both macrophages and dendritic cells can specifically recruit KAI1 to phagosomes upon phagocytosis of various fungi (13). In 2019, Tam *et al*. suggested that a lack of KAI1 reduced macrophage fungal killing and its single-nucleotide polymorphisms modulated cytokine production (11). Recently, it has been reported that KAI1 can restrain phagocyte migration in inflammation murine models and that it is required for normal morphology and activation of M2 macrophages (14). Furthermore, several studies have demonstrated that the large extracellular loop (LEL) of KAI1 is necessary for biological activities of tetraspanins with several pharmaceutical benefits (15-17). In 2021, Lee *et al*. demonstrated that KAI1 could directly bind to vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) and block their signaling mechanism, ultimately having an anti-angiogenic effect (15). They also reported that recombinant human LEL protein of KAI1 (*rh*KAI1) could suppress tumor metastasis in B16 melanoma cells-incorporated mice and prevent neovascularization in oxygen-induced retinopathy mice. More recently, we have uncovered that *rh*KAI1 could partially interact with TGF-β binding sites of TGF receptor I/II, integrin β1, and integrin αv (16). Additionally, we have found that *rh*KAI1 could suppress TGF-β-induced epithelial-mesenchymal transition (EMT) of retinal pigment epithelial cells by blocking Smad signaling pathway (16). Although several studies have suggested that KAI1 could prevent various cancers and retinal disorders through its anti-angiogenic and anti-metastatic capacity, few studies have reported effects of KAI1 on macrophage polarization and its potential anti-inflammatory efficacy. Therefore, the present study aimed to determine effects of *rh*KAI1 on M1 polarized inflammatory response and identify the underlying molecular mechanism using LPS-stimulated mouse RAW 264.7 macrophage-like cells and primary bone marrow-derived macrophages (BMDM).

RESULTS AND DISCUSSION

*rh*KAI1 suppresses expression of M1 macrophage phenotype-related surface markers in LPS-stimulated BMDM and RAW264.7 cells

Cytotoxic effects of *rh*KAI1 on primary BMDM and RAW 264.7 cells were examined with a WST-1 assay. At concentrations up to 800 ng/ml, *rh*KAI1 had no cytotoxic effect on BMDM or RAW 264.7 cells with or without LPS stimulation (Data not shown). Furthermore, a cell morphology comparison showed that both unstimulated-BMDM and -RAW 264.7 cells had a round shape, whereas LPS-stimulated cells had an irregular shape with polygonal spindle-shaped pseudopodia (Fig. 1A). However, such morphological change induced by LPS was diminished by *rh*KAI1 treatment in RAW264.7 cells, while no apparent change was observed in BMDM cells. To investigate effects of *rh*KAI1 on expression levels of M1 macrophage phenotype-related surface markers in LPS-stimulated condition, populations of $F4/80^+CD86^+$ cells and $F4/80^+CD80^+$ cells were examined by flow cytometry. Results of flow cytometry indicated that 100 ng/ml LPS significantly increased $F4/80^+CD86^+$ cells to 4.60-fold of those in the control BMDM. However, such increase in the population of LPS-induced $F4/80^+CD86^+$ cells was markedly decreased by *rh*KAI1 pre-treatment in a concentration-dependent manner (Fig. 1B-D). In addition, the population of $F4/80^+CD86^+$ and $F4/80^+CD80^+$ cells was substantially enhanced by LPS treatment to 90.47-fold of that in control RAW 264.7 cells, while such increase was significantly suppressed by pretreatment with *rh*KAI1 (Fig. 1E, F). Quantitative mean fluorescence intensity results were similar to results of population analysis using M1 surface markers (Fig. 1G). Results of morphological observation and flow cytometry for $F4/80^+CD86^+$ cells and $F4/80^+CD80^+$ cells in BMDM and RAW264.7 cells revealed that RAW 264.7 cells were more sensitive to *rh*KAI1 than BMDM. Therefore, RAW264.7 cells were selected and used in subsequent experiments to investigate the protective effect of *rh*KAI1 on LPS-induced M1 polarization. To re-verify the inhibitory effect of *rh*KAI1 on the increase of $F4/80^+CD86^+$ cells induced by LPS treatment, we carried out an immunofluorescence analysis for CD86 in RAW 264.7 cells. As shown in Fig. 1H, the expression of CD86, a surface marker of M1 macrophage polarization, was markedly increased by LPS stimulation. However, such increase of CD86 induced by LPS was noticeably suppressed in the presence of *rh*KAI1, consistent with results obtained by flow cytometry.

*rh*KAI1 inhibits LPS-induced inflammatory response in RAW 264.7 cells

LPS is considered an initiator of M1 macrophages polarization. It can induce the production of key inflammatory mediators such as inflammatory cytokines and chemokines (3, 4). Extensive studies have shown that LPS can enhance pro-inflammatory cytokines (such as IL-1β, IL-6, IL-12 and TNF-α) and representative inflammatory mediators (e.g., NO and PGE_2)
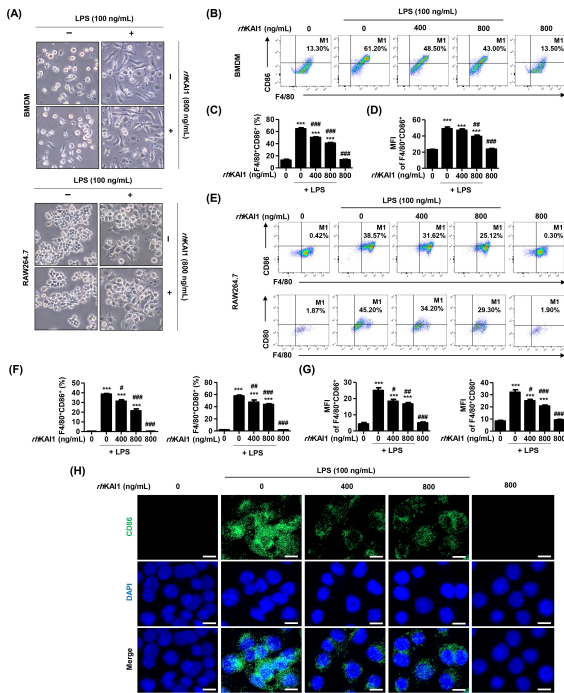
Fig. 1. *rh*KAI1 reduces the expression of LPS-stimulated M1 macrophage phenotype-related surface markers in primary mouse bone marrow-derived macrophages (BMDM) and RAW 264.7 mouse macrophage-like cells. Cells were stimulated with various concentrations of *rh*KAI1 for 24 h or pre-treated with or without *rh*KAI1 for 2 h, following by 100 ng/ml LPS treatment for 24 h. (A) Representative microscopy images of the cell. Cell morphology was observed using an inverted-phage contrast microscope, and acquired at 100× magnification. (B) Representative flow cytometry plots of F4/80$^+$CD86$^+$ in BMDM cells. (C) Quantification for M1 phenotype are displayed as the percentages of F4/80$^+$CD86$^+$ cells from CD11b-PE-gated cells in BMDM. (D) Quantitative mean fluorescence intensity (MFI) of F4/80$^+$CD86$^+$ cells in BMDM. (E) Representative flow cytometry plots of F4/80$^+$CD86$^+$ and F4/80$^+$CD80$^+$ in RAW 264.7 cells. (F) Quantification for M1 phenotype are displayed as the percentages of F4/80$^+$CD86$^+$ cells and F4/80$^+$CD80$^+$ cells from total live-gated cells in RAW 264.7 cells. (G) Quantitative MFI of F4/80$^+$CD86$^+$ cells and F4/80$^+$CD80$^+$ cells in RAW264.7 cells. Data are expressed as the mean ± SD (n = 5). ***$P <$ 0.001 compared to control. $^#P <$ 0.05, $^{##}P <$ 0.01 and $^{###}P <$ 0.001 compared to LPS-stimulated cells. (H) Representative images were acquired using a confocal laser scanning microscopy. Confocal images show the expression of CD86 (green) and DAPI (blue). Scale bar = 50 μm.

in macrophages, ultimately promoting an inflammatory response (18). Inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) are enzymes responsible for the production of NO and PGE$_2$ that are also positively correlated with pro-inflammatory cytokines (19). Therefore, levels of pro-inflammatory cytokines and inflammatory mediators have been applied as indicators of anti-inflammatory efficacy (18, 19). In the present study, we assessed effects of *rh*KAI1 on expression levels of inflammatory cytokines in LPS-stimulated RAW 264.7 cells.
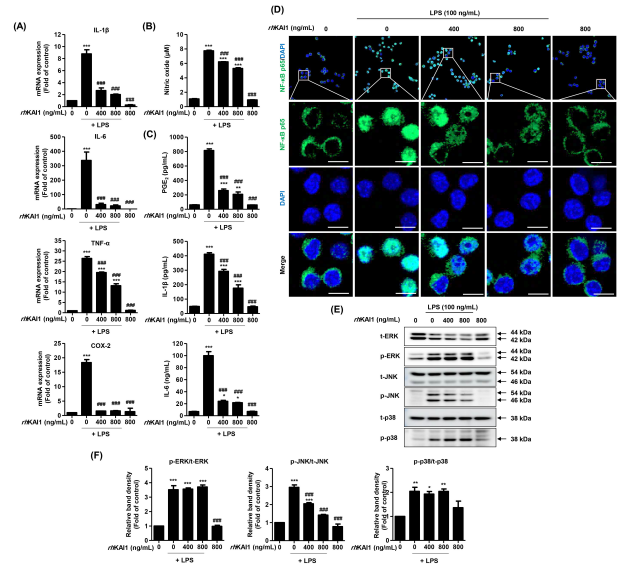

Fig. 2. *rh*KAI1 suppress LPS-induced inflammatory response in RAW 264.7 macrophages via blocking of JNK/NF-κB signaling pathway. (A) Relative levels of mRNA expression for IL-1β, IL-6, TNF-α, and COX-2 were expressed as fold of control. (B) In the supernatants, NO concentration was measured by the Griess reaction. (C) The secretion levels of PGE$_2$, IL-1β and IL-6 levels were determined using commercial ELISA kits. Data are expressed as the mean ± SD (n = 4). ***$P <$ 0.001 compared to control. $^{###}P <$ 0.001 compared to LPS-stimulated cells. (D) The cells subjected to immunofluorescence staining with NF-κB p65 antibody and representative images were acquired using a confocal laser scanning microscopy. Green fluorescence indicates the localization of NF-κB p65, and blue fluorescence by DAPI allows the nuclei. Scale bar = 50 μm. (E) The protein expression of MAPK signaling molecules including ERK, p-ERK, JNK, p-JNK, p38 MAPK and p-p38 MAPK. (F) Bar diagram showing the relative protein density after normalization with actin based on Western blot analysis. *$P <$ 0.05, **$P <$ 0.01, and ***$P <$ 0.001 compared to control. $^{###}P <$ 0.001 compared to LPS-stimulated cells.

Real-time qPCR results (Fig. 2A) revealed that LPS remarkably increased mRNA expression levels of pro-inflammatory cytokines and mediators including IL-1β, IL-6, TNF-α, and COX-2. However, such increases were substantially down-regulated by *rh*KAI1 pre-treatment. Especially, expression levels of IL-1β, IL-6, and COX-2 following *rh*KAI1 treatment were similar to those in untreated control cells. Next, to determine whether *rh*KAI1 could also regulate the release of inflammatory cytokines and mediators, secretion levels of NO, IL-1β, IL-6, and PGE$_2$ in cell supernatants were analyzed. As shown in Fig. 2B, secretion levels of NO were markedly increased in LPS-stimulated cells (10.90 ± 1.13 μM, P < 0.001) compared to those in untreated cells (1.69 ± 0.47 μM). However, the secretion of NO was significantly reduced in 800 ng/ml *rh*KAI1 pre-treated cells (8.39 ± 0.76 μM, P < 0.05) compared with that in LPS-stimulated cells. Release levels of other inflammatory mediators such as PGE$_2$, IL-1β, and IL-6 were also greatly increased by

LPS, whereas such increases were suppressed by *rh*KAI1 pre-treatment before LPS treatment (Fig. 2C). These results suggest that *rh*KAI1 can suppress LPS-induced inflammatory response by modulating the production of pro-inflammatory cytokines and mediators. Findings of the present study are consistent with multiple previous studies suggesting that some members of the tetraspanin family, CD9, CD53, CD63, and CD81 can suppress inflammatory responses (20).

*rh*KAI1 attenuates JNK/NF-κB p65 signaling pathway in LPS-stimulated RAW 264.7 cells

Inhibiting nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) and mitogen-activated protein kinases (MAPK) pathways has been suggested as the main mechanism underlying the attenuation of LPS-induced inflammatory cytokine production (19, 21). Among various intracellular signaling pathways, NF-κB has been identified as the key transcription factor in regulating the expression of inflammatory factors under LPS stimulation (19). Inactive NF-κB heterodimer p65/50 can interact with IκB-α in the cytosol to form a complex. Upon stimulation with LPS, IκB-α is phosphorylated and degraded subsequently. Released NF-κB dimers then translocate to the nucleus to initiate inflammatory gene transcription (21). Therefore, we further assessed whether the anti-inflammatory effect of *rh*KAI1 involved the NF-κB signaling pathway through immunofluorescence analysis. As shown in Fig. 2D, NF-κB p65 expression was significantly increased in the nuclei of LPS-stimulated RAW 264.7 cells than in control cells. However, such increase of NF-κB p65 expression induced by LPS was greatly reversed in the presence of *rh*KAI1. Next, to investigate the molecular mechanism by which *rhKAI1* regulated LPS-induced inflammation, MAPKs signaling pathways including extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK were analyzed. MAPKs can phosphorylate different intracellular proteins and transcription factors, subsequently regulating gene expression. ERK proteins are activated by endogenous or exogenous mitogens, cytokines, and growth factors to regulate cell proliferation (22). JNK and p38 MAPK are predominantly activated by inflammatory cytokines and bacterial LPS (23). In the present study, we found that phosphorylation levels of ERK, JNK, and p38 MAPK were substantially increased by LPS treatment (Fig. 2E, F). However, phosphorylation level of JNK was significantly reduced by *rh*KAI1 pre-treatment in a dose-dependent manner, while phosphorylation levels of ERK and p38 were not affected by *rh*KAI1. This result indicates that *rh*KAI1 can attenuate LPS-induced inflammatory response by blocking the JNK/NF-κB p65 signaling pathway.

*rh*KAI1 prevents binding of LPS to TLR4s on the cell surface

TLRs expressed on the plasma and endosomal membranes of immune cells including macrophages are of interest to immunologists because of their front-line role in the initiation of innate immunity against invading pathogens (24, 25). Among various TLRs, TLR4 plays a crucial role in inflammatory and immune responses. It can efficiently sense Gram-negative bacterial infections through recognition of LPS, a bacterial membrane component (26). LPS binding to TLR4 leads to the activation and translocation of NF-κB into the nucleus, which then triggers the production of pro-inflammatory cytokines and type-I interferons (26, 27). To determine whether *rh*KAI1 could interact with TLR4 or prevent binding of LPS with TLR4, an immunofluorescence analysis was carried out to calculate molecular docking of each element. As shown in Fig. 3A, increment of TLR4 expression by LPS was markedly suppressed in the presence of *rh*KAI1. Subsequently, to predict the interaction between TLR4 and *rh*KAI1, 3-D structures of
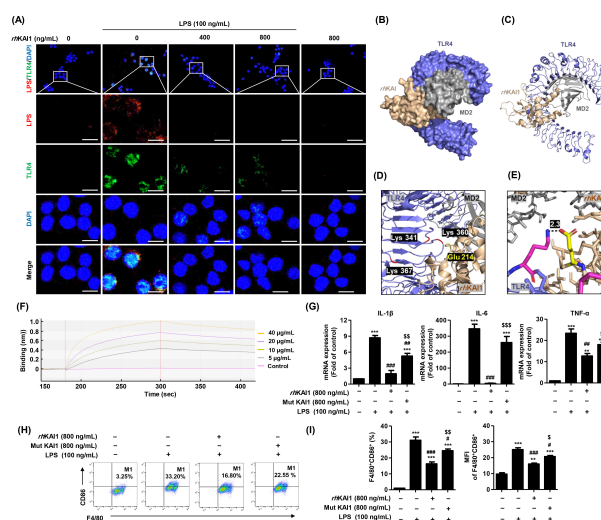

Fig. 3. *rh*KAI1 interrupt interaction of LPS/TLR4 complex in LPS-stimulated RAW 264.7 macrophages. (A) The cells were subjected to immunofluorescence staining with LPS and TLR4 specific antibodies. Representative images were acquired using a confocal laser scanning microscopy. Confocal images show the expression of LPS (red), TLR4 (green), and DAPI (blue). Scale bar = 50 μm. The docking conformations of *rh*KAI1/TLR4 complex are illustrated by the surface (B) and cartoon (C). (D) The interacting residues of *rh*KAI1/TLR4 complex are shown in cartoon representation. (E) Atoms N and O were marked with blue and pink, respectively. The covalent bound of the *rh*KAI1 (yellow, Glu 214)-TLR4 (pink, Lys 360) complex and is illustrated by a black dotted line, and its distance is presented. (F) Binding affinity determination for *rh*KAI1 and *rh*TLR4 using BLItz label-free biolayer interferometry system. Binding (nm) refers to changes in optical interference. (G-I) Cells were pre-treated with *rh*KAI1 or mutant (Glu 214) protein of *rh*KAI1 for 2 h, following by 100 ng/ml LPS treatment for 24 h. (G) Relative levels of mRNA expression for IL-1β, IL-6, and TNF-α was expressed as fold of control. (H) Representative flow cytometry plots of F4/80⁺CD86⁺ in RAW 264.7 cells. (I) Quantification and MFI for F4/80⁺CD86⁺ cells and F4/80⁺CD80⁺ cells from total live-gated cells in RAW 264.7 cells. Data are expressed as the mean ± SD (n = 3). **P < 0.01 and ***P < 0.001 compared to control. #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to LPS-stimulated cells. $P < 0.05, $$P < 0.01 and $$$P < 0.001 compared with LPS+*rh*KAI1 treatment cells.

TLR4 and *rh*KAI1 were obtained from the PDB (PDB ID code of TLR4: 3VQ2) and Alphafold (AF ID code of hKAI1: P277701). Binding affinity was then analyzed and visualized with PyMOL molecular graphics system. As a result, we found that LPS could bind with four TLR4 residues: Glu 214, Lys341, Lys360, Lys367, and Arg434 (28). Among them, Glu 214 of *rh*KAI1 residue could strongly interact with Lys 360 of TLR4 residue. The distance between them was 2.9 Å (Fig. 3B-E). To validate this finding, we assessed the effect of *rh*KAI1 mutant (Glu 214) protein on LPS-stimulated macrophage polarization in RAW 264.7 cells. As shown in Fig. 1G, pretreatment with *rh*KAI1 mutant (Glu 214) protein markedly reversed down-regulation of mRNA expression of pro-inflammatory cytokines by wild type *rh*KAI1. The population and MFI of F4/80$^+$CD86$^+$ M1 surface marker were also reversed in *rh*KAI1 mutant (Glu 214) protein-treated cells (Fig. 3H, I). These results demonstrate that mutation of Glu 214 in *rh*KAI1 can decrease the anti-inflammatory effect of *rh*KAI1. Furthermore, we carried out a surface plasmon resonance binding analysis using BLItz, an optical technique for measuring interactions between proteins, peptides, nucleic acids, small molecules, and/or lipids in real time (29). Results of BLItz analysis showed that *rh*KAI1 strongly bound to recombinant human TLR4 in a highly specific fashion (Fig. 3F). These results indicate that *rh*KAI1 can directly interact with TLR4, which is involved in the suppression of LPS/TLR4-mediated M1 macrophage polarization. In a previous study, Khan *et al.* have demonstrated that KAI1 could interact with TLR9, an endosomal innate immune receptor, and modulate TLR9-dependent NF-κB nuclear translocation as a response to cytosine-phosphate-guanine stimulation in immune cells (30). Khan *et al.*'s results and our findings suggest that KAI1 could directly interact with TLR9 and TLR4 to block TLR-dependent downstream signaling pathway. We further determine whether down-regulation of TLR4 was mediated by the anti-inflammatory effect of *rh*KAI1 in LPS-stimulated cells. We found that the protective ability of *rh*KAI1 on increased cytokine secretion and TLR4 expression was enhanced in the presence of TAK-242, an inhibitor of TLR4 (Supplementary Fig. 1). These results demonstrate that KAI1 is associated with the TLR4 signaling pathway in the inhibition of inflammatory responses.

In conclusion, the current study showed that *rh*KAI1 could attenuate LPS-induced inflammatory responses in RAW 264.7 macrophages-like cells and primary mouse BMDMs. The anti-inflammatory effect of *rh*KAI1 was supported by reduced M1 macrophage phenotype-related surface markers and inhibited production of pro-inflammatory cytokines and mediators. However, many questions remain unsolved regarding macrophage polarization. More efforts are needed to establish specific markers and steps of the differentiation process leading to different subpopulation of macrophages (31). We noted that *rh*KAI1 also suppressed nuclear translocation of NF-κB p65 and phosphorylation of JNK in LPS-stimulated RAW 264.7 cells. In addition, *rh*KAI1 inhibited the expression of TLR4 on the cellular surface and bound strongly to TLR4. Taken together,
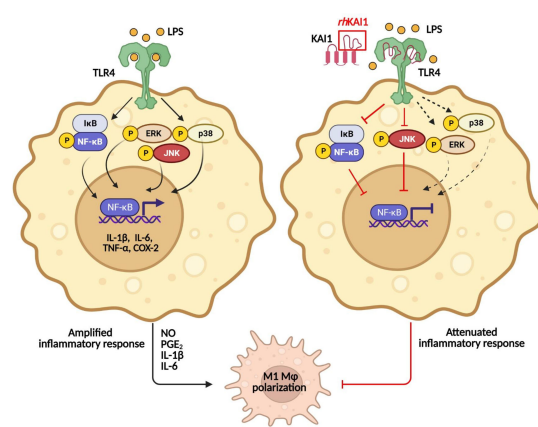

Fig. 4. Schematic summarizing the effect of *rh*KAI1/CD82 against LPS-stimulated inflammatory response in macrophages. *rh*KAI1/CD82 attenuates M1 macrophage polarization on LPS-stimulated RAW264.7 cells via blocking TLR4/JNK/NF-κB signal pathway.

these findings suggest that *rh*KAI1 has an anti-inflammatory effect in LPS-polarized macrophages through its interaction with TLR4 and down-regulation of the JNK/NF-κB signaling pathway (Fig. 4). Although further studies are required to determine the effect of *rh*KAI1 *in vivo*, our findings indicate a pharmacological potential of *rh*KAI1 in the prevention of various inflammatory diseases.

MATERIALS AND METHODS

Details on the used methods are provided in the expanded Materials and Methods section in the online data supplement.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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