

## Research Article

# AP-1-Targeted Anti-Inflammatory Activities of the Nanostructured, Self-Assembling S5 Peptide

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Received 21 March 2015; Revised 27 April 2015; Accepted 27 April 2015

Academic Editor: Dennis D. Taub

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Peptide-based therapeutics have received increasing attention in medical research. However, the local delivery of such therapeutics poses unique challenges. Self-assembling peptides that use decorated nanofibers are one approach by which these therapeutics may be delivered. We previously found that the self-assembling K5 peptide affects the anti-inflammatory response. The aim of the present study was to investigate another self-assembling peptide, S5. Unlike the K5 peptide which has a positive charge, the S5 peptide has a free hydroxyl (-OH) group. We first examined whether the S5 peptide regulates the inflammatory response in primary cells and found that the S5 peptide reduced the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and tumor necrosis factor (TNF)- $\alpha$  in lipopolysaccharide- (LPS-) treated bone marrow-derived macrophages. Moreover, the S5 peptide significantly downregulated cyclooxygenase- (COX-) 2, TNF- $\alpha$ , and interleukin- (IL-) 1 $\beta$  expression by blocking the nuclear translocation of c-Jun. Consistent with this finding, the S5 peptide diminished the activation of inflammatory signaling enzymes related to p38. The S5 peptide also inhibited the formation of the p38/c-Jun signaling complex in RAW264.7 cells. Similarly, p38 and MKK3/6 were inhibited by the S5 peptide in LPS-activated peritoneal macrophages. Taken together, these results strongly suggest that the S5 peptide could exert anti-inflammatory effects by inhibiting the c-Jun/p38 signaling pathway.

## 1. Introduction

Innate immune responses provide four anatomical, physiological, phagocytic, and inflammatory barriers. Among these barriers, the macrophage-mediated inflammatory barrier is the most important [1]. Macrophages produce proinflammatory cytokines and other signaling molecules such as interleukins (ILs), tumor necrosis factor (TNF)- $\alpha$ , and prostaglandins (PGs), all of which are essential for the initial inflammatory response [2]. Thus, the initial activation of macrophages is a significant event in inflammatory processes. To activate the inflammatory response, macrophage surface receptors such as Toll-like receptors (TLRs) interact with their cognate ligands [3]. Activated macrophages consequently upregulate intracellular signaling pathways to

activate the nuclear factor (NF)- $\kappa$ B pathway and the activator protein- (AP-) 1 pathway, leading to the expression of inflammatory genes [4–6]. To cure inflammatory diseases, effective regulation of the inflammatory response is required. Therefore, targeted inhibition of intracellular inflammatory-related signaling pathways has been considered to be a promising therapeutic strategy for treating various immunological diseases such as cancer, septic shock, diabetes, and atherosclerosis [7–11]. The applications of self-assembling peptides have been widely investigated. Biological hydrogels consisting of self-assembling peptide nanofibers have been shown to have a broad range of potential applications [12–15]. Self-assembling peptides consist of short peptides with 8 to 16 residues that are 2.5 to 5 nm in length, depending on conditions such as pH and ionic strength [16]. These

structural features have made self-assembling systems an attractive option for tissue culture-based and tissue-based research into the mechanisms that control various cellular processes [17]. Although recent studies have indicated that self-assembling peptides have potential applications in drug delivery as a nanomedicine platform [18], the molecular mechanisms underlying these applications are not yet fully understood.

In our previous study, we determined the immune-regulatory functions of a self-assembling peptide, K5, RADARADARADADA-KKKKK. In the present study, we investigated another peptide named S5, RADARADARADADA-SSSSS, with 5 serine residues, which was developed to investigate polarity effects on self-assembling property, plasma protein interactions, and platelet activation [19]. While the K5 peptide exhibits lysine residue-driven net positive charge at a pH of 7.4, disadvantageous in terms of membrane permeability, S5 peptide only maintains free hydroxyl group. Considering chemical property of S5, it is hypothesized that the penetration of S5 into cytoplasmic compartment in a cell might be better than K5. In this study, we aimed to demonstrate the immunopharmacological improvement of the S5 peptide in cellular and molecular inflammatory responses.

## 2. Materials and Methods

**2.1. Materials.** The S5 peptide was purchased from SynBioSci (Livermore, CA, USA). Tetrazole (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), prednisolone and indomethacin, and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme immunoassay (EIA) kits and enzyme-linked immunosorbent assay (ELISA) kits for determining the levels of PGE<sub>2</sub> and TNF- $\alpha$  were purchased from Thermo Scientific (NYSE: TMO) and Amersham (Little Chalfont, Buckinghamshire, UK). Fetal bovine serum (FBS), RPMI1640 and DMEM media were purchased from Thermo Scientific (NYSE: TMO). RAW264.7 and HEK293 cells were obtained from ATCC (Rockville, MD, USA). Phospho- and total protein antibodies against c-Jun, extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), p38, MAP kinase kinase 3/6 (MKK3/6), TGF- $\beta$  activated kinase 1 (TAK1), lamin A/C, and  $\beta$ -actin were purchased from Cell Signaling (Beverly, MA, USA) and Santa Cruz (Santa Cruz, CA, USA).

**2.2. Animals.** Male ICR mice (6 weeks old, 17 to 21 g) were purchased from DAEHAN BIOLINK (Chungbuk, Korea) and housed in plastic cages under conventional conditions. Water and food pellets (Samyang, Daejeon, Korea) were supplied *ad libitum*. All studies were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Sungkyunkwan University (approval ID: SKKUBBI 12-6-2).

**2.3. Preparation of Bone Marrow-Derived Macrophages.** Bone marrow- (BM-) derived macrophages were obtained from

ICR male mice (6 weeks old, 17 to 21 g) by severing the bone of the femoral region. Bone marrow-derived macrophages were flushed out into Petri dishes with PBS. After harvesting bone marrow cells by centrifugation at 1,200 rpm for 5 minutes at 4°C, the cells were resuspended in RPMI1640 medium containing 5% FBS. Finally, bone marrow-derived macrophages ( $1 \times 10^6$  cells/mL) were seeded in 100 mm tissue culture dishes and grown for 4 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**2.4. Cell Culture.** Primary cells (bone marrow-derived macrophages) and cell lines (RAW264.7 and HEK293 cells) were cultured with RPMI1640 medium supplemented with 10% heat-inactivated FBS, glutamine, penicillin, and streptomycin at 37°C under 5% CO<sub>2</sub>.

**2.5. Determination of PGE<sub>2</sub> and TNF- $\alpha$  Production.** RAW264.7 cells and bone marrow-derived macrophages ( $1 \times 10^6$  cells/mL) were preincubated for 18 h, after which cells were treated with the S5 peptide (0 to 100  $\mu$ g/mL) or standard compounds (prednisolone and indomethacin) for 30 min and then additionally treated with LPS (1  $\mu$ g/mL) for 24 h. Production and the release of PGE<sub>2</sub> and TNF- $\alpha$  were determined by EIA and ELISA kits, as described previously [20].

**2.6. Cell Viability Assay.** Bone marrow-derived macrophages or RAW264.7 cells ( $1 \times 10^6$  cells/mL) were treated with S5 peptide (0 to 100  $\mu$ g/mL) for 24 h. Cytotoxic effect of S5 peptide was evaluated by conventional MTT assay as described previously [21, 22].

**2.7. mRNA Expression Analysis by Quantitative Reverse Transcriptase-Polymerase Chain Reaction.** To evaluate inflammatory gene mRNA expression levels, RAW264.7 cells pretreated with S5 peptide (0 to 100  $\mu$ g/mL) for 30 min were incubated with LPS (1  $\mu$ g/mL) for 6 h. Total RNA from the cells was then isolated with TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions and stored at -70°C until use. The levels of *IL-1 $\beta$* , *TNF- $\alpha$* , and *COX-2* mRNA expression levels were quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) with SYBR Premix Ex Taq according to the manufacturer's instructions (Takara, Japan). Reactions were run in a real-time thermal cycler (Bio-Rad, USA), as reported previously [23, 24]. Results are expressed as relative ratios of gene expression normalized to the internal control GAPDH. Primers were ordered from Bioneer (Seoul, Korea) and their sequences are listed in Table 1.

**2.8. Preparation of Total Lysates and Nuclear Extracts, Immunoblotting, and Immunoprecipitation.** Preparation of total lysates and nuclear extracts from LPS-treated RAW264.7 cells pretreated with S5 peptide was used, a method previously published [25]. Immunoblotting analysis of phosphorylated and total protein [c-Jun, lamin A/C, MAPK (ERK, p38, and JNK), MKK3/6, MEK1/2, MKK4/7, MKK4, TAK1, lamin A/C,

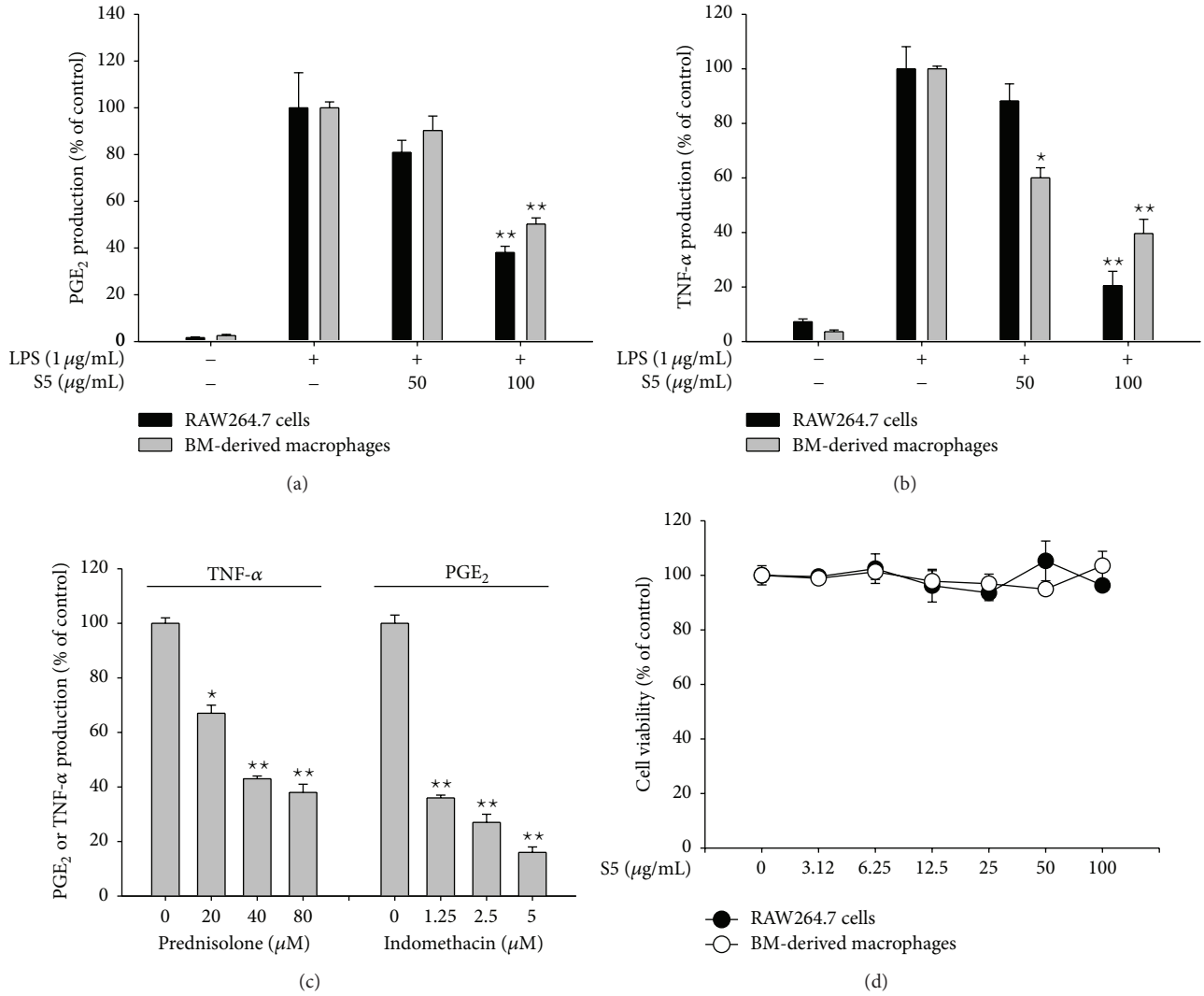


FIGURE 1: Effects of the S5 peptide on TNF-α and PGE<sub>2</sub> production and viability of LPS-treated RAW264.7 cells and BM-derived macrophages. (a, b, and c) The levels of NO, PGE<sub>2</sub>, and TNF-α in the supernatants of RAW264.7 cells or bone marrow-derived macrophages that had been treated with the S5 peptide (0 to 100 μg/mL) or standard compounds (prednisolone and indomethacin) in the presence or absence of LPS (1 μg/mL) for 6 (TNF-α) or 24 (PGE<sub>2</sub>) h were analyzed by EIA and ELISA, respectively. (d) The viability of RAW264.7 cells and BW-derived macrophages was determined by the MTT assay. \*  $P < 0.05$  and \*\*  $P < 0.01$  compared with control cells; \*  $P < 0.05$  compared with normal cells.

TABLE 1: Sequences of primers used in real-time PCR analysis.

Gene		Primer sequence
TNF-α	F	5'-TGCCTATGTCTCAGCCTCTT-3'
	R	5'-GAGGCCATTTGGAACCTTCT-3'
COX-2	F	5'-CACTACATCCTGACCACTT-3'
	R	5'-ATGCTCCTGCTTGAGTATGT-3'
IL-1β	F	5'-TAGAGCTGCTGGCCTTGTTA-3'
	R	5'-ACCTGTAAAGGCTTCTCGGA-3'
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

and β-actin] levels was performed, according to published methods [26]. For immunoprecipitations, cell lysates were

generated from RAW264.7 cells ( $1 \times 10^7$  cells/mL) that had been treated either with or without LPS (1 μg/mL) for 90 min. Lysates containing equal amounts of protein (500 μg) were precleared with 10 μL protein A-coupled Sepharose beads (50% v/v) (Amersham, UK) for 1 h at 4°C. Precleared samples were incubated overnight at 4°C with 5 μL antibody to p38. Immune complexes were captured with 10 μL protein A-coupled Sepharose beads (50% v/v) by rotation for 3 h at 4°C.

**2.9. Statistical Analysis.** Data (Figures 1 and 2) are expressed as means ± standard deviations (SDs) and were calculated from one of two independent experiments. Each experiment was performed with six technical replicates. The data shown in this paper are representative of three independent experiments, each of which yielded similar results. For statistical

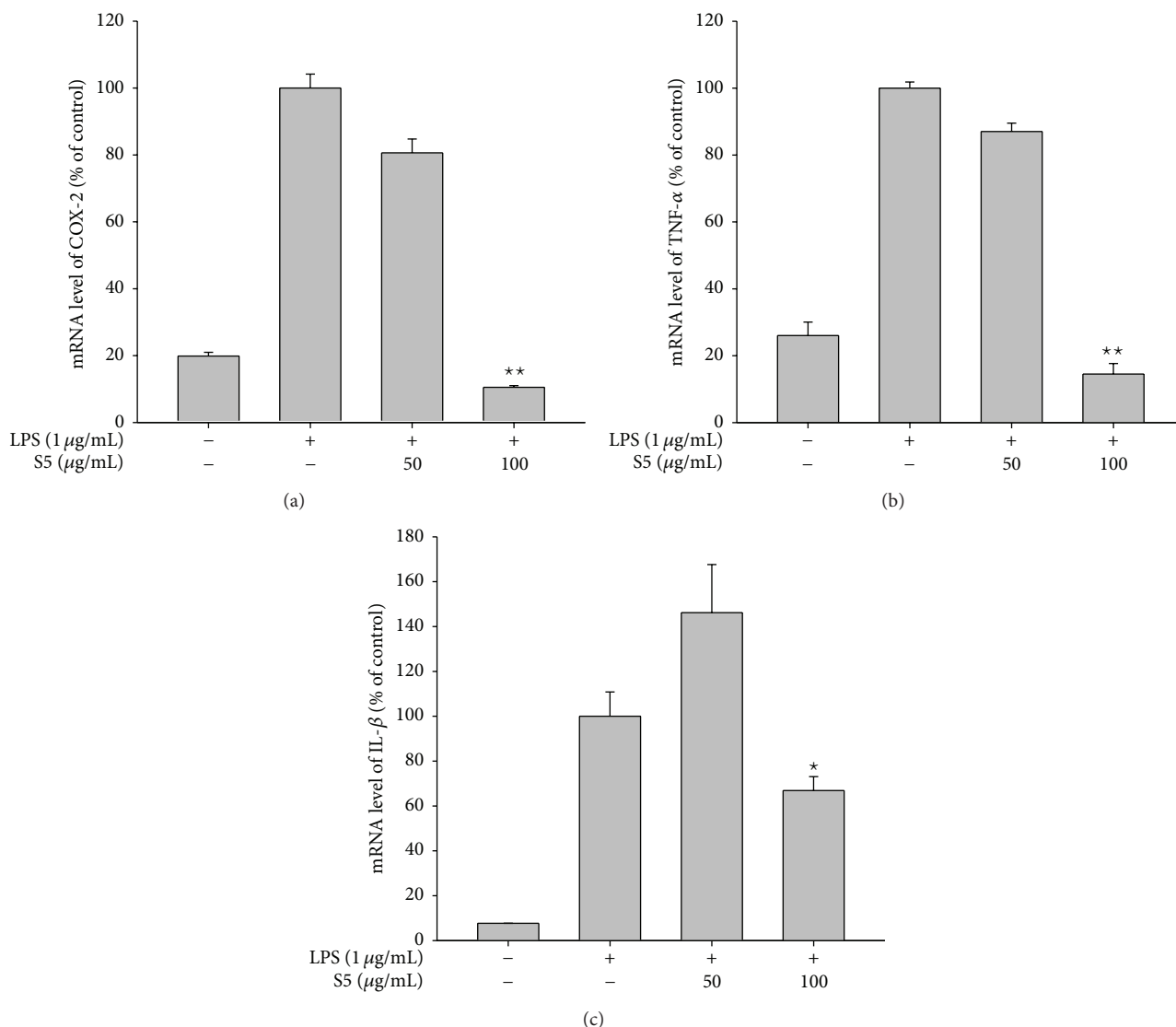


FIGURE 2: Effects of the S5 peptide on the mRNA expression levels of proinflammatory genes. The mRNA levels of TNF- $\alpha$ , COX-2, and IL-1 $\beta$  in RAW264.7 cells that had been pretreated with the S5 peptide and stimulated with LPS (1  $\mu$ g/mL) for 6 h were determined by real-time PCR. \* $P < 0.05$  compared with control cells.

comparisons, results were analyzed using analysis of variance/Scheffé's post hoc test and the Kruskal-Wallis/Mann-Whitney tests. All  $P$  values  $< 0.05$  were considered statistically significant. All statistical tests were carried out using the computer program SPSS (SPSS Inc., Chicago, IL, USA).

### 3. Results and Discussion

We previously reported that the self-assembling K5 peptide, applied as 3D scaffolds for tissue engineering or drug delivery [18, 27], downregulates the macrophage-mediated inflammatory responses [28]. The S5 peptide is part of a family of self-assembling peptides that includes the K5 peptide. However, in contrast to the K5 peptide, the S5 peptide lacks positive charge in this backbone. Since these two peptides exhibit reasonably similar structures with only a minor difference,

we hypothesized that the S5 peptide would also exert anti-inflammatory effects.

Similar to the pharmacological profiles of K5 peptide [28], the S5 peptide appears to be a promising therapeutic molecule for dampening the inflammatory response. For instance, the S5 peptide decreased the production of PGE<sub>2</sub> (Figure 1(a)) and TNF- $\alpha$  (Figure 1(b)) in both malignant RAW264.7 cells and in BM-derived LPS-activated macrophages in a dose-dependent manner as seen in the cases of standard compounds prednisolone and indomethacin (Figure 1(c)); importantly, no cytotoxicity was observed in these cells (Figure 1(d)). These data raised a possibility that S5 peptide can be a peptide drug with anti-inflammatory property as previously suggested with synthetic TIP-like peptide AP318, peptides from bovine  $\beta$ -casein, cathelicidin, and N15P polypeptide [29–31]. Since elucidating the molecular

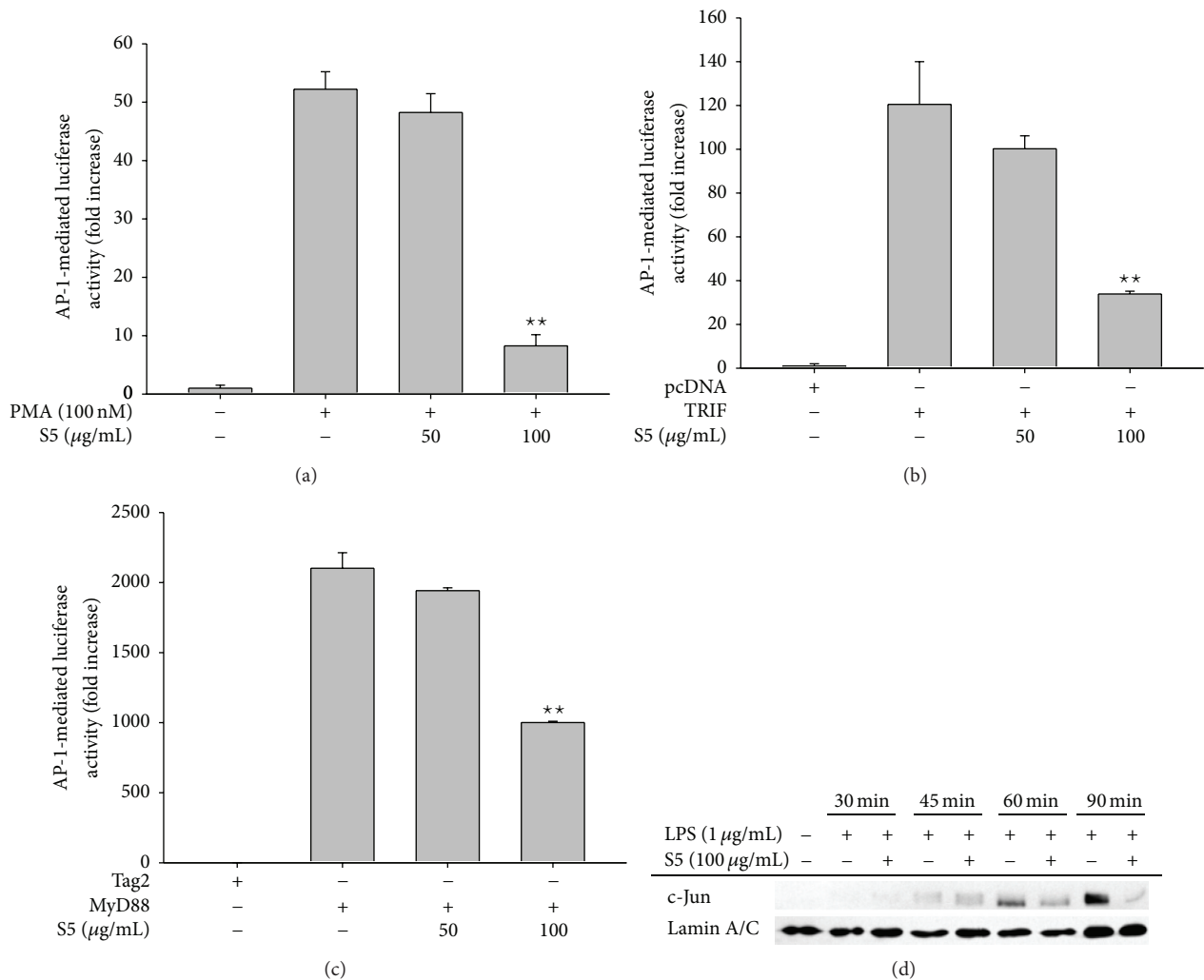


FIGURE 3: Effects of the S5 peptide on the activation of transcription factors. (a, b, and c) HEK293 cells that had been cotransfected with constructs driving the expression of AP-1-Luc, TLR adaptor molecules (MyD88 and TRIF), and  $\beta$ -gal (as a control) were treated with the S5 peptide (0 to 100  $\mu$ g/mL) in the presence or absence of PMA (100 nM). The resultant luciferase activity was measured with a luminometer. (d) The nuclear translocation level of a transcription factor in the AP-1 family (c-Jun) was assessed by immunoblotting analysis with antibodies against total c-Jun. \*  $P < 0.05$  and \*\*  $P < 0.01$  compared with control cells.

mechanisms of these effects is important for initiation of drug discovery process, we next examined the anti-inflammatory mechanism of the S5 peptide.

To examine the effects of the S5 peptide on the inflammatory response on the transcriptional level, the mRNA levels of *COX-2* (Figure 2(a)), *TNF- $\alpha$*  (Figure 2(b)), and *IL-1 $\beta$*  (Figure 2(c)) were measured by real-time PCR in cells that had been stimulated with LPS. These experiments showed that the S5 peptide (100  $\mu$ g/mL) downregulated the expression of *COX-2*, *TNF- $\alpha$* , and *IL-1 $\beta$* . To investigate whether the S5 peptide regulates DNA promoter activity, a luciferase reporter assay was used that resulted in cell luminescence. The S5 peptide remarkably decreased AP-1-mediated transcriptional activation in response to PMA, MyD88, or TRIF; moreover, this effect was dose-dependent (Figures 3(a), 3(b), and 3(c)). Activation of the inflammatory response is known to result in nuclear translocation of the AP-1 transcription

factor, which results in transcription of AP-1-driven target genes.

To examine the nuclear translocation of c-Jun in the inflammatory response, we determined the levels of c-Jun in the nuclear fractions of LPS-treated RAW264.7 cells. This is because peptide K5 displayed predominant inhibitory activity on AP-1 translocation. Interestingly, these experiments showed that the S5 peptide prevented the nuclear translocation of c-Jun at 60 and 90 min (Figure 3(d)). This result implies that the S5 peptide may regulate the AP-1 signaling pathway. To investigate the activation status of upstream signaling molecules in the AP-1 pathway, the phosphorylation of various MAPKs (ERK, p38, and JNK) was analyzed by Western blotting. The phosphorylation of p38 and JNK was decreased at 30 and 15 min, while ERK phosphorylation was not decreased by the S5 peptide (Figure 4(a)). Interestingly, the S5 peptide only prevented phosphorylation of MKK3/6,

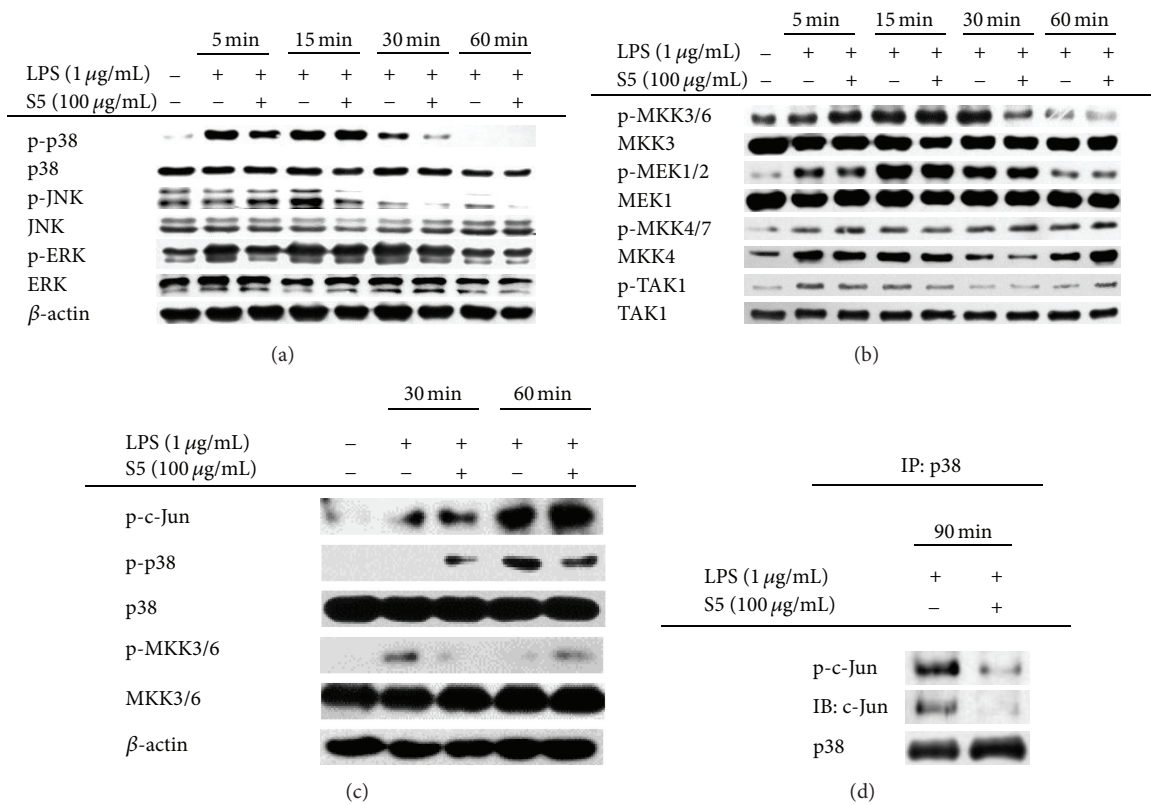


FIGURE 4: Effects of the S5 peptide on the activation of the AP-1 signaling pathway. (a and b) Phosphoprotein and total protein levels of p38, JNK, ERK, MEK1/2, MKK3/6, MKK4/7, TAK1, and  $\beta$ -actin in the lysates of RAW264.7 cells were analyzed by Western blotting. (c) The levels of total and phosphorylated c-Jun, p38, MKK3/6, and  $\beta$ -actin in lysates from peritoneal macrophages were determined by immunoblotting analysis. (d) The effect of the S5 peptide on the c-Jun/p38 interaction was determined by immunoprecipitation analysis with antibody against p38 and by immunoblotting analysis with antibodies against p-c-Jun, c-Jun, and p38.

an upstream kinase of p38 [32], whereas the S5 peptide did not prevent the phosphorylation of the upstream molecule JNK, which is downstream of MKK4/7. Phosphorylation of TAK1, a kinase that is upstream of the MKKs [33], was also decreased by the S5 peptide (Figure 4(b)). To determine whether the effects of the S5 peptide were recapitulated in primary cells, peritoneal macrophages were harvested from ICR mice and the phosphorylation status of various signaling molecules was investigated by Western blotting. This experiment revealed that the S5 peptide decreased the phosphorylation of c-Jun, p38, and MKK3/6 in LPS-driven signaling (Figure 4(c)). Moreover, the S5 peptide also dramatically blocked the interaction of phospho-p38 with c-Jun and its phosphoform, as revealed by immunoprecipitation and immunoblotting analyses (Figure 4(d)). Based on these results, we conclude that the S5 peptide is capable of preventing AP-1 signaling events. Some of strong anti-inflammatory remedies such as medicinal plant-derived extracts including *Dryopteris crassirhizoma* and *Archidendron clypearia* and their individual components including andrographolide, ginsenosides, curcumol, and lutein are known to suppress AP-1 function in their pharmacological actions [34–38], suggesting that AP-1 could play a critical role in inflammation-regulatory functions. Due to their size and charge, some peptides are regarded as one of membrane nonpermeable

biomaterials. In contrast, most hydrophobic small chemicals are much assessable to be penetrated into the membrane and display higher possibility to directly interact with cytosolic target enzymes. However, chemical drugs showing effective inhibition property toward important signaling enzymes are faced to have another side effect causing nonspecific toxicity. In this respect, peptide drugs are considered to be favorable in terms of drug development, due to their selectivity. Previously, we reported that peptide K5 is able to suppress TNF- $\alpha$  production in LPS-treated RAW264.7 cells up to 48% at 100  $\mu$ g/mL [28]. On the other hand, chemically modified S5 peptide having free OH groups exhibited stronger activity (80% inhibition at 100  $\mu$ g/mL) under the same conditions (Figure 1(b)), implying that membrane permeability of peptide S5 could be more improved than K5. Nonetheless, the fact that K5 was revealed to block the release of TNF- $\alpha$  and PGE<sub>2</sub> between 50 to 100  $\mu$ g/mL [28] is still raising a possibility that some of peptide receptor such as PEPT1, OATP2BA, or OARP1B3 [39, 40] could contribute to the uptake of peptides K5 and S5 into cytoplasm. The point that S5 peptide exhibits higher anti-inflammatory property under improved membrane permeability seems to impose S5 peptide to be developed as anti-inflammatory peptide drug. Indeed, some peptides such as GLP and its analogs, extracellular matrix protein-derived peptides [41],

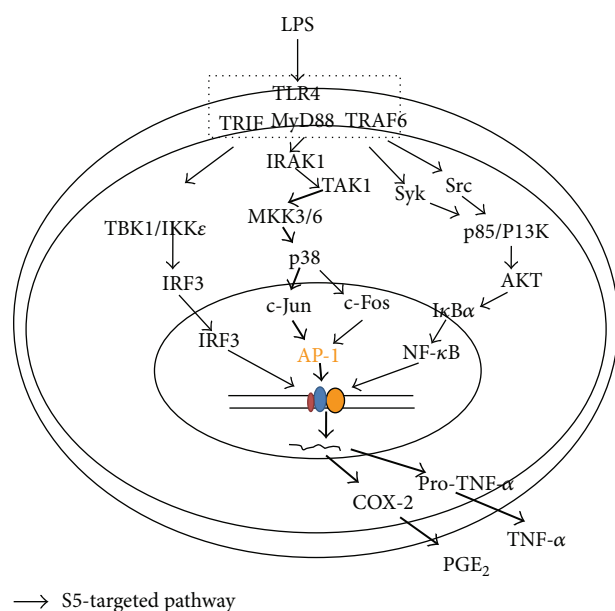


FIGURE 5: Anti-inflammatory effects of the S5 peptide on signaling pathways.

and integrin-binding N-terminal peptides [42] are closed to be developed as antitumorogenic remedy, although the therapeutic use of these peptides is still limited due to high cost, poor stability, and mediocre pharmacokinetics [18]. In cases of the S5 and K5 peptides, these are known to be self-assembling nanopeptides forming a hydrogel applied for drug delivery system and have been shown to be nonimmunogenic and noninvasive in the filling of cavities [15, 18, 43]. According to our results, these peptides are found to have anti-inflammatory properties which can give additional benefits when they are used as carrier to deliver immunomodulatory drugs. Since the S5 peptide itself suppresses events in the TLR4 signaling pathway, including activation of MAPK and AP-1, it may exert a synergetic effect if carrier molecules are NF- $\kappa$ B-targeted anti-inflammatory drugs.

To sum up these results, here we showed that the S5 peptide dampens various components of the TLR4-mediated inflammatory response, such as the production of PGE<sub>2</sub> and TNF- $\alpha$ . In addition, the S5 peptide reduces AP-1 signaling by preventing the phosphorylation of MKK3/6 and TAK1, as summarized in Figure 5. Since the S5 peptide is a self-assembling peptide that could be used for drug delivery, it is possible that the S5 peptide could be loaded with therapeutics that target AP-1 or other compounds that synergize with inflammatory molecules. Future studies will test the efficacy of the S5 peptide as a drug carrier and as an anti-inflammatory agent in an animal model. These experiments have the potential to contribute to the development of an efficacious and novel drug delivery system.

## Disclaimer

The authors alone are responsible for the content and writing of the paper.

## Conflict of Interests

The authors report no conflict of interests.

## Authors' Contribution

Woo Seok Yang, Young-Jin Son, and Mi-Yeon Kim contributed equally to this work.

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