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An Analog of the Antimicrobial Peptide CopA5 Inhibits Lipopolysaccharide-Induced Macrophage Activation

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We previously reported that the CopA3 peptide (LLCIALRKK, D-form) originally isolated from the Korean dung beetle has antimicrobial and immunosuppressive effects. However, the high cost of producing the synthetic peptide, especially the D-form, has limited the development of CopA3 for therapeutic purposes. Here, we investigated whether the CopA3 deletion derivative, CopA5, which is composed of only five amino acids (LLCIA) and has the L-form structure, could inhibit the lipopolysaccharide (LPS)-induced activation of macrophages. Peritoneal exudate macrophages (PEM) were isolated from mice and exposed to LPS in the presence or absence of CopA5, and biomarkers of macrophage activation were measured. Our results revealed that LPS-induced nitric oxide (NO) production, tumor necrosis factor (TNF)-α secretion, and phagocytic activity of PEM were significantly inhibited by CopA5 treatment. Similar to CopA3, the structurally modified CopA5 peptide had no cell toxicity (as assessed by measurement of cell viability loss and apoptosis) in PEM. Moreover, the LPS-induced upregulation of the activating phosphorylation of signal transducer and activator of transcription 1 (STAT1) was markedly inhibited by CopA5 treatment. These results suggest that, similar to CopA3, CopA5 inhibits macrophage activation by inhibiting STAT1 phosphorylation and blocking the release of NO and TNF-α. CopA5 may therefore prove therapeutically useful in the realm of immune suppression.

Keywords: Antimicrobial peptide, lipopolysaccharide, macrophages, immunosuppressive agent, signal transduction, STAT1

Introduction

Inflammation is associated with the activation of various immune cells. Macrophages, which are known to be involved in inflammatory and immune responses [1, 2], play key roles in both adaptive and innate immune responses [3–7]. Activated macrophages secrete the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6), which are essential for host survival and tissue injury repair [8]. Macrophages also produce nitric oxide (NO), which is toxic to bacteria and intracellular parasites [9]. However, the malfunction of macrophages has been associated with atherosclerosis [10–14] and rheumatoid arthritis [10–12]. Therefore, macrophage activation must be carefully regulated to ensure the appropriate modulation of inflammation [15]. Various agents can alter macrophage activation, such as ginger extract [15], lipoproteins, and IL-10. All of them have been shown to block the lipopolysaccharide (LPS)-induced activation of macrophages, which is a widely used research model of macrophage activation [4].

We previously demonstrated that the CopA3 peptide isolated from the Korean dung beetle possesses antibacterial activity against various pathogenic bacteria [16] and inhibits the production of proinflammatory cytokines in LPS-treated RAW264.7 macrophages [17]. CopA3 was also reported to exert anti-inflammatory effects on acute colitis in the *Clostridium difficile* toxin A-injected mouse model [18]. These results suggest that CopA3 could be a potent and specific agent for inhibiting immune responses.

The CopA3 peptide is composed of nine amino acids

(LLCIALRKK) and is dimerized via the cysteine located in the middle domain [17]. The D-type form of CopA3, which is preferred for oral treatment because it resists degradation in the gastrointestinal tract [18], has been used in studies verifying its biological activity. However, this form is structurally complicated and it is costly to produce the synthetic peptide, limiting the relevance of CopA3 for drug development. The current form of CopA3 therefore needs to be changed if we hope to further develop this peptide as a drug candidate. Since well-known strategies for developing synthetic peptide drugs involve the use of short sequences and the natural form of amino acid with L-type, we synthesized a 5-mer peptide derived from CopA5: LLCIA. Designated CopA5, this peptide lacked the three positively charged amino acids at the C-terminus (RKK), theoretically increasing its binding affinity for the plasma membrane via the hydrophobicity of four of its component amino acids (L, L, I, A). All of the amino acids of CopA5 were in the L-form, but the cysteine (and thus the ability to dimerize) was preserved in an effort to maintain the well-known biological activity of CopA3 [19]. Here, we examined whether CopA5, as a peptide derivative of CopA3, shows similar inhibitory effects on the LPS-induced activation of macrophages.

We found that CopA5 treatment inhibited LPS-induced NO production and TNF-α secretion in murine peritoneal exudate macrophages (PEM). The LPS-induced increase in the phagocytic activity of PEM was markedly blocked by CopA5, and CopA5 pretreatment significantly decreased the LPS-induced activating phosphorylation of signal transducer and activator of transcription 1 (STAT1). These findings suggest that, similar to CopA3, CopA5 could be a potent agent for inhibiting immune responses mediated by the activation of macrophages.

Materials and Methods

Synthesis of the 5-mer CopA5 (L-Type) and Determination of Dimer Peptide Structure

CopA5 peptide was synthesized by AnyGen (Korea) [18]. The peptide was purified by reverse-phase high-performance liquid chromatography (HPLC) using a Capcell Pak C18 column (Shiseido, Japan); a linear gradient of water-acetonitrile (0–80%) containing 0.1% trifluoroacetic acid was used to elute the peptide (45% recovery). The identity of the peptide was confirmed by electrospray ionization (ESI) mass spectrometry (Platform II; Micromass, UK). The interchain disulfide bond was formed by dissolving the synthetic peptide in an acetonitrile:H_2O (50:50) solution and then oxidized by incubating in an aqueous 0.1 M NK_4HCO_3 solution (pH 6.0–6.5) for 24 h. The disulfide pattern of the dimeric form of CopA5 was determined by analyzing the peptide solution by HPLC and ESI mass spectrometry.

Cell Culture and Reagents

Murine PEM were harvested by peritoneal lavage from male ICR mice by intraperitoneal injection of 10 ml of sterile ice-cold PBS (10 mM). The cells were washed with PBS and cultured in DMEM containing 10% FCS for 2 h, and non-adherent cells were washed away before use [15]. This study was approved by the Animal Care and Use Committee of Daejin University (Korea). Polyclonal antibodies against phospho-STAT1, phospho-p38MAPK, phospho-ERK1/2, and phospho-NF-κB were obtained from Cell Signaling Technology (USA). Anti-β-actin antibody, propidium iodide (PI), LPS, dichlorodihydrofluorescein diacetate (DCF-DA) and staurosporine were purchased from Sigma Aldrich (USA).

Cell Viability

PEM (10^5 cells/well) were treated with CopA5 (10 or 50 μg/ml) and/or LPS, as indicated in the text, for 48 h, and then incubated with 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye for 2 h. The solubilization reagent was added, and absorbance was determined at 570 nm on a microplate spectrophotometer (Model 3550; Bio-Rad, Canada) [20].

Phagocytosis Assay

PEM (10^5 cells/well) were pretreated with CopA5 (10 μg/ml) for 1 h and then incubated with medium alone (con), LPS (1 μg/ml) alone, or LPS plus CopA5. After 48 h, cells were further incubated with 50 μg of fluorescein-labeled bioparticles (Molecular Probes, USA) in 1 ml of medium for 5 h. The bioparticle-containing medium was removed and immediately replaced with 300 μl of a trypan blue solution (0.25 mg/ml) to quench extracellular bioparticles. The cells were washed with PBS, trypsinized, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA) [21].

Immunoblot Analysis

PEM were pretreated with CopA5 (10 μg/ml) for 1 h and then further incubated with medium alone (con), LPS alone, or LPS plus CopA5 for 2 h. Cells were washed with cold PBS, lysed in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Nonidet P-40), and then equal amounts of protein were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The appropriate antibodies were applied, and antigen-antibody complexes were detected with the LumiGlo reagent (New England Biolabs, USA). The immunoblots were visualized with a chemiluminescence image analyzer (Fusion FX; Vilber Lourmat, Germany) [22].

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay

PEM were treated with 10 μg/ml CopA5 or 1 μM staurosporine for 24 h and then fixed with 4% paraformaldehyde for 20 min. Cell

staining and detection of cells with fragmented nuclear DNA were conducted according to the manufacturer's instructions, as previously described [23]. Nuclei were visualized by PI counterstaining at room temperature. Cells were analyzed using a Nikon Eclipse E600 epifluorescence microscope (Nikon Instruments, Inc., USA).

Measurement of TNF-α in Conditioned Culture Medium

For measurement of TNF-α, PEM (10^5 cells/well) were treated with medium (con), LPS alone, CopA5 alone, or CopA5 plus LPS for 24 h. Supernatants were collected, and the concentration of TNF-α was measured using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, USA) [24].

Measurement of Cellular Reactive Oxygen Species (ROS)

PEM were incubated in DCF-DA (50 μM) for 40 min, and then incubated with medium, LPS alone, or LPS plus CopA5 for 30 min. Changes in fluorescence intensity were monitored using a GloMax 20/20 (Promega, USA) [25].

Statistical Analysis

The results are presented as mean values ± SEM. Data were analyzed using the SIGMA-STAT professional statistics software program (Jandel Scientific Software, USA). Analyses of variance with protected *t* tests were used for intergroup comparisons.

Results and Discussion

CopA5, a CopA3 Analog, Reduces LPS-Induced NO Production in PEM

CopA3 is known to have antimicrobial activity [18] and can inhibit the LPS-stimulated activation of Raw 264.7 macrophages [17]. Here, we examined whether the structurally modified analog of CopA3, CopA5 (Fig. 1A), has similar effects on LPS-triggered macrophage activation. We isolated PEM from mice, exposed them to 1 μg/ml LPS [24, 26] for 24 h, and then measured the NO concentration in the conditioned culture medium using the Griess reagent and spectrometric analysis [27]. We found that LPS stimulation increased NO production (Fig. 1B), but that this change was significantly decreased by CopA5 cotreatment (Fig. 1B). CopA5 alone had no effect on NO production in PEM.

This indicates that, similar to its parent peptide CopA3, the 5-mer CopA5 peptide (LLCIA) appears to inhibit LPS-induced macrophage activation. CopA5 may be more hydrophobic because it lacks three positively charged amino acids of CopA3. Importantly, our present findings suggest that these positively charged amino acids are not necessary for the ability of this peptide to inhibit the LPS-mediated stimulation of macrophages. As the CopA5

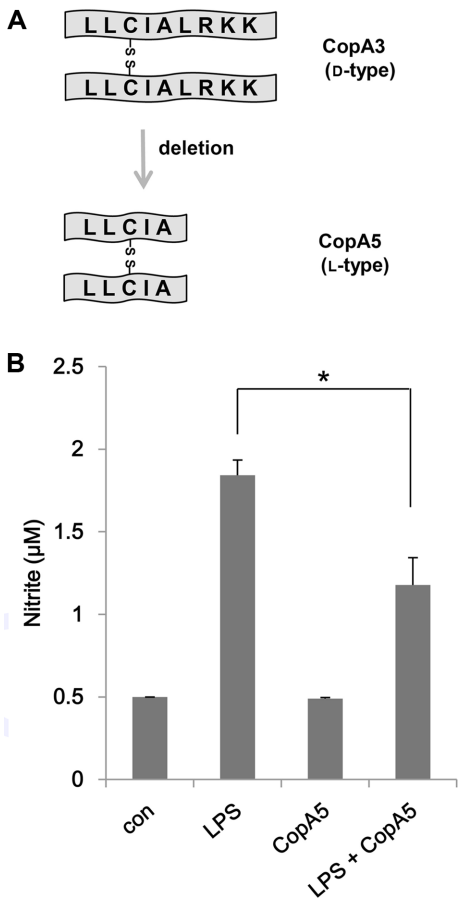

Fig. 1. CopA5 inhibits LPS-induced NO production in peritoneal exudate macrophages (PEM).
(**A**) Synthesis of the L-type, 5-mer dimerized peptide, CopA5. (**B**) PEM isolated from male ICR mice were pretreated with CopA5 (10 μg/ml) for 1 h, and further incubated with medium only (con), LPS (1 μg/ml) alone, CopA5 (10 μg/ml) alone, or LPS plus CopA5 for 24 h. The concentrations of NO in the conditioned media were measured using the Griess reagent and spectrometric analysis. The bars represent the mean ± SEM of three experiments performed in triplicates (*, $p < 0.005$).

peptide was in the L-form, not the D-from, there was some concern that it could be proteolytically degraded, either by reacting with itself in the culture medium or by proteolytic processes inside cells. However, our finding that CopA5 markedly inhibited the LPS-induced production of NO by PEM suggests that this peptide may be relatively resistant to degradation despite its L-form structure.

CopA5 Treatment of PEM Inhibits LPS-Induced TNF-α Release and Phagocytosis

Next, we measured whether CopA5 could block LPS-induced proinflammatory cytokine production and phagocytic activity in PEM. We exposed PEM to LPS [24, 26] in the

presence or absence of CopA5 for 24 h, and then measured the concentrations of TNF-α in the conditioned media. As expected, exposure of macrophages to LPS increased the secretion of TNF-α (Fig. 2A). However, CopA5 cotreatment significantly inhibited this increase (Fig. 2A). CopA5 also suppressed the LPS-induced phagocytic activity of PEM (Fig. 2B).

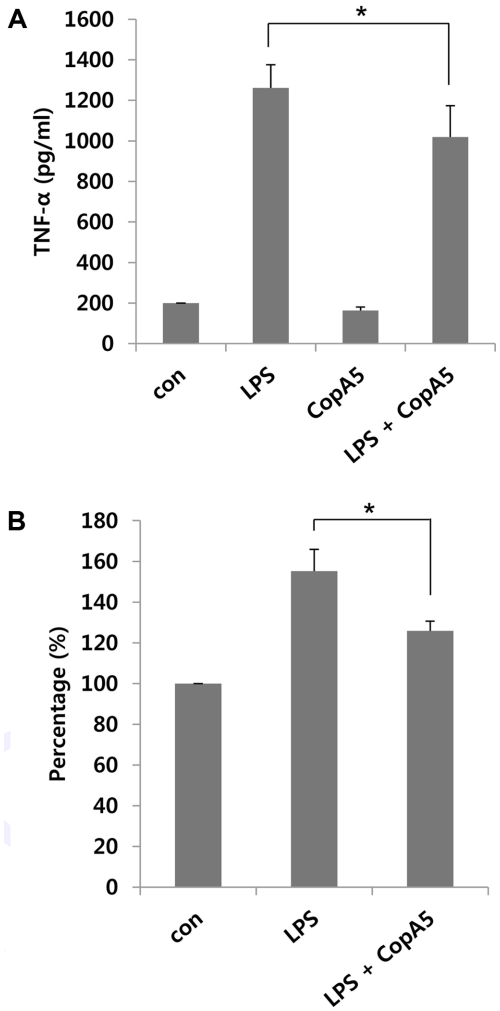

Fig. 2. CopA5 reduces LPS-induced TNF-α release and phagocytosis in macrophages.
(**A**) Peritoneal exudate macrophages (PEM) were pretreated with CopA5 (10 μg/ml) for 1 h, and further incubated with medium only (con), LPS (1 μg/ml) alone, CopA5 (10 μg/ml) alone, or LPS plus CopA5. After 24 h, the concentrations of TNF-α in the conditioned media were measured. The bars represent the mean ± SEM of three experiments performed in triplicates (*, $p < 0.005$). (**B**) PEM were treated with medium (con), LPS alone, CopA5 alone, or LPS plus CopA5 for 48 h, and then incubated with 50 μg of fluorescein-labeled bioparticles for 5 h. Bioparticle-containing cells were analyzed by flow cytometry. The bars represent the mean ± SEM of three experiments performed in triplicates (*, $p < 0.005$).

CopA5 Does Not Show Cytotoxicity against PEM

Since a high concentration of CopA3 (~ 150 μg/ml) reportedly triggers apoptosis in human leukemia cells [28], we assessed whether the apparent ability of CopA3 to inhibit the LPS-induced changes in PEM reflected cytotoxicity. We exposed PEM to different concentrations of CopA5 for 48 h, and used the MTT assay to measure cell viability. Staurosporine, which is known to potently induce apoptosis in macrophages [17], was used a positive control. We found that whereas PEM treated with 1 μM staurosporine displayed an 80% viability loss, treatment with CopA5 did not alter the viability of these macrophages (Fig. 3A). To confirm these findings, we incubated PEM with CopA5 (10–50 μg/ml) or staurosporine (1 μM) for 24 h, and used TUNEL staining to detect DNA fragmentation. As shown in Fig. 3B, staurosporine caused DNA fragmentation in PEM (white spots, TUNEL-positive cells), whereas CopA5 did not. These results suggest that the ability of CopA5 to inhibit the LPS-mediated stimulation of macrophages is not due to cytotoxicity.

CopA5 Blocks the LPS-Induced Stimulation of PEM by Inhibiting the Activating Phosphorylation of STAT1

Given that LPS is known to stimulate macrophages by activating the NF-κB and Janus kinase (JAK)/STAT pathways [9, 26, 29–36], we examined the effect of CopA5 on these pathways. PEM were exposed to medium, LPS alone, CopA5 alone, or LPS plus CopA5 for 2 h, and cell lysates were subjected to immunoblotting. LPS-treated PEM exhibited a marked increase in STAT1 phosphorylation, but this effect was significantly inhibited by CopA5 treatment (Fig. 4A). In contrast, LPS induced the phosphorylation of p38MAPK, CREB, and NF-κB, but these changes were not affected by CopA5 (Fig. 4A). The latter finding was somewhat surprising, as NF-κB is essential for the production of NO and inflammatory cytokines [36], and we had observed that CopA5 treatment reduced the ability of LPS to upregulate NO (Fig. 1B) and TNF-α (Fig. 2A). These results indicate that the ability of CopA5 to block the LPS-stimulated activation of PEM involves inhibition of STAT1 but not NF-κB-mediated signaling.

Finally, as the LPS-induced activation of macrophages is known to be highly dependent on ROS [37], which are essential for IL-1 gene expression in LPS-treated macrophages [31], we examined whether CopA5 blocked ROS production in LPS-stimulated macrophages. PEM were exposed to medium, LPS alone, or LPS plus CopA5 for 30 min, and ROS levels were measured using DCF-DA fluorescein. However, CopA5 treatment did not reduce the LPS-induced

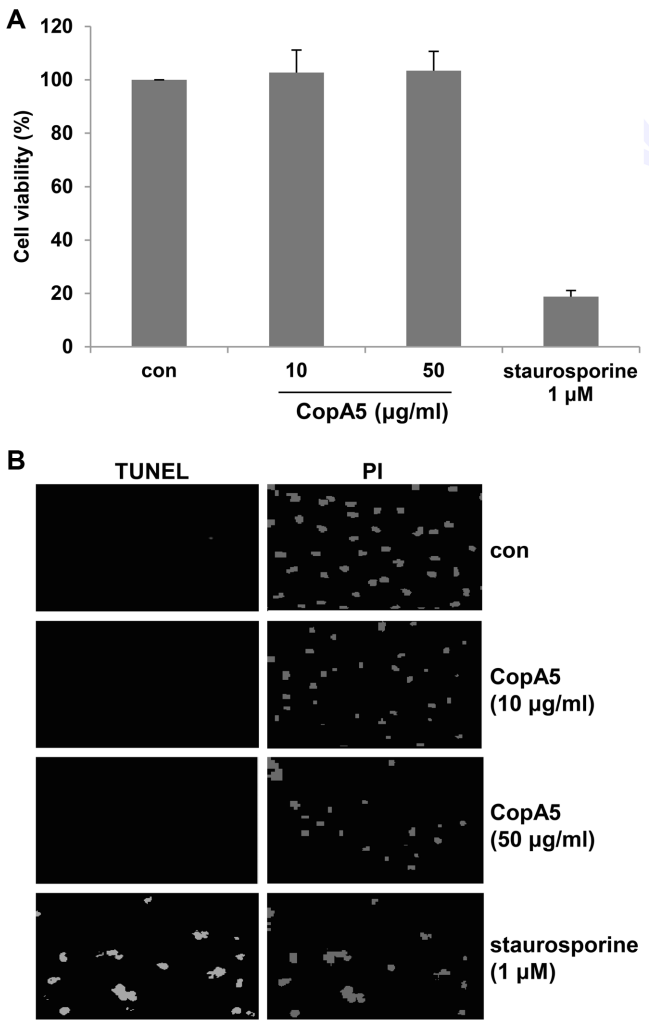

Fig. 3. CopA5 does not show cytotoxicity against peritoneal exudate macrophages (PEM).

(**A**) PEM (10^5 cells/well) were incubated with medium (con), CopA5 (10–50 μg/ml), or staurosporine (1 μM) for 48 h, and cell viability was measured by MTT assay. The bars represent the mean ± SEM of three experiments performed in triplicates. (**B**) PEM (10^4 cells/well) were treated with medium (con), CopA5 (10–50 μg/ml), or staurosporine for 24 h, and DNA fragmentation was measured by TUNEL assay. Nuclei were visualized by propidium iodide (PI) counterstaining. The results shown are representative of three separate experiments.

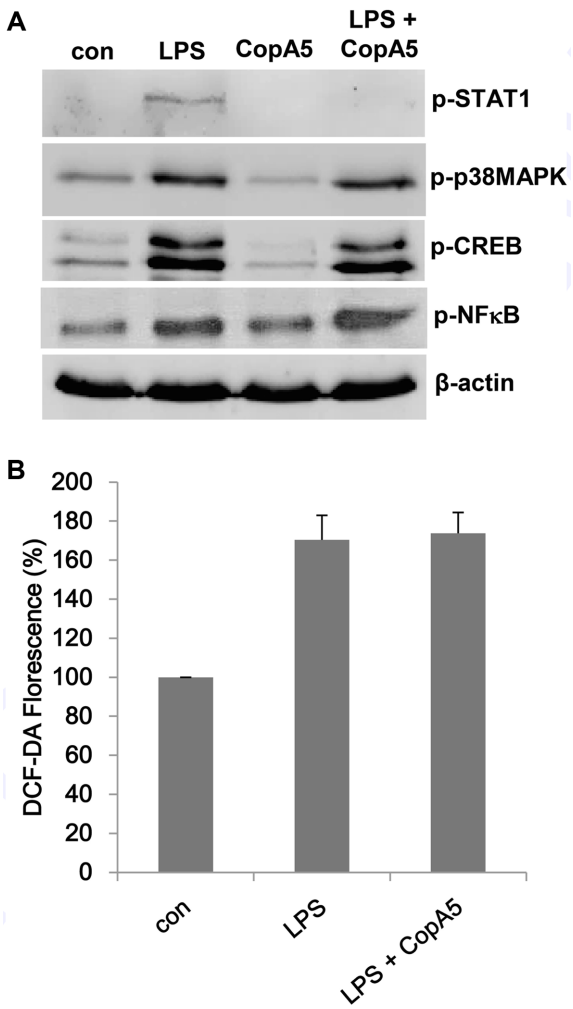

Fig. 4. CopA5 blocks the LPS-induced stimulation of peritoneal exudate macrophages (PEM) by inhibiting the activation of STAT1.

(**A**) PEM (10^5 cells/well) were pretreated with CopA5 (10 μg/ml) for 1 h and then further incubated with medium alone (con), LPS alone, CopA5 alone, or LPS plus CopA5 for 2 h. Cells were lysed, cellular proteins were resolved by 10% SDS-PAGE, and blots were probed with the indicated antibodies. The results presented are representative of three independent experiments. (**B**) PEM (10^5 cells/well) were incubated with DCF-DA (50 μM) for 40 min and then incubated with medium (con), LPS alone, or LPS plus CopA5 for 30 min, and changes in fluorescence intensity were monitored. The bars represent the mean ± SEM of three experiments performed in triplicates.

production of ROS in PEM (Fig. 4B).

Although NF-κB is known to critically mediate signal transduction pathways in LPS-treated macrophages [38, 39], numerous papers have reported that STAT1 signaling is also important to this model of macrophage activation [26, 29–34]. For example, STAT1 is known to combine intracellular signals during macrophage activation [26], and STAT1 knockout mice exhibited severe reductions in the mRNA expression of NO synthase and the level of NO compared with control mice [33]. Toll-like receptor-2 and -4 can also activate STAT1 phosphorylation in macrophages [30]. Moreover, LPS-induced endotoxin signals reportedly require STAT1 activation [40]. Based on our present results, we speculate that CopA5 may inhibit the activity of JAK,

thereby blocking the activation of STAT1 and the downstream upregulation of TNF-α production and NO secretion in LPS-activated macrophages.

In summary, we herein demonstrated that a structurally modified CopA3 analog, CopA5, inhibits the LPS-induced activation of PEM without causing any cytotoxicity. We further showed that this inhibitory effect of CopA5 is associated with its ability to block the activation of STAT1. These novel findings suggest that, similar to CopA3, CopA5 could be a potent agent for inhibiting immune responses mediated by macrophage activation.

Acknowledgments

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