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Dear Editor:

On behalf of my co-authors, I am pleased to submit the enclosed manuscript, titled "Mixtures of various recombinant growth factors inhibits pro-inflammatory mediators and cytokines in LPS-stimulated RAW 264.7 cells via inactivating ERK and NF-κB pathways", to *International Journal of Molecular Medicine*.

In the present paper, we investigated the anti-inflammatory effects of mixtures of recombinant growth factors (MRGFs) on the generation of several chemokines, cytokines, and enzymes involved in the inflammatory process in LPS-stimulated RAW 264.7 cells.

Mixtures of recombinant growth factors (MRGFs) has the potential to prevent inflammatory diseases through the down-regulation of pro-inflammatory mediators which is partly mediated by inhibition of ERK and NF-κB pathways in LPS-stimulated RAW 264.7 cells.

I hope you will find our paper interesting and will consider it for publication in *International Journal of Molecular Medicine.*

Sincerely

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# Mixtures of various recombinant growth factors inhibits pro-inflammatory mediators and cytokines in LPS-stimulated RAW 264.7 cells via inactivating ERK and NF-κB pathways

**Running Title:**

The Anti-inflammatory Activity of Mixtures of Various Recombinant Growth Factors on LPS-stimulated RAW 264.7 cells

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**Abbreviations:** NO, nitric oxide; MRGFs, mixtures of recombinant growth factors; MAPK, mitogen-activated protein kinase; NF- κB, nuclear factor-κB; LPS, lipopolysaccharide; IκB, inhibitory factor-κB

**Key words:** Growth factor, RAW 264.7 cells, inflammation, mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB)

**Abstract**

Growth factors are important for regulating a variety of cellular processes and typically act as signaling molecules between cells. In this study, we examined the mechanism underlying the inhibition of nitric oxide (NO) and pro-inflammatory cytokine production by mixtures of recombinant growth factors (MRGFs) via the mitogen-activated protein kinase (MAPK) and the nuclear factor-κB (NF-κB) signal transduction pathways in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The results showed that MRGFs significantly attenuated LPS induction of pro-inflammatory cytokines and NO production in a dose-dependent manner. To elucidate the mechanism underlying the inhibitory effect of MRGFs, we examined the effect of LPS-stimulated phosphorylation of MAPKs and the NF-κB signaling pathway on the stabilization of NF-κB nuclear translocation and inhibitory factor-κB (IκB) degradation. In conclusion, we clearly demonstrated that treatment with MRGFs resulted in a reduction in the phosphorylation of the ERK and NF-κB signaling pathways, whereas the phosphorylation of JNK and p38 was not affected. Taken together, the results suggest that MRGFs inhibit the production of pro-inflammatory cytokines and NO by down-regulating iNOS gene expression and blocking the phosphorylation of the ERK and NF-κB signaling pathways. Thus, these findings may provide direct evidence for the potential application of

MRGFs in the prevention and treatment of inflammatory diseases.

## Introduction

Various growth factors, also known as cytokines or steroid hormones, regulate a variety of cellular processes, as well as the development of tumors, inflammation, and wound healing. These growth factors differ from well-known polypeptide hormones, such as insulin and adrenocorticotropic hormone, not only in the response elicited but also in the mode of delivery from the secreting to the responding cell. Growth factors also have different cell-type specificities and different functions (1-4). For example, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF), are thought to play a role in wound healing and in the regulation of class II Major histocompatibility complex, macrophages, and lymphocytes. During this process, inflammation initiates healing. The regulation of inflammation is so important that homeostatic mechanisms evolved to control this process (1, 5-9). Insulin-like growth factor (IGF)-I displays pleiotropic properties, including the ability to promote cellular proliferation, differentiation, and processes involved in metabolism/hypertrophy, such as nutrient transport, energy storage, gene transcription, and protein synthesis (10). Superoxide dismutase (SOD), one of the most important antioxidants, is an enzyme that catalyzes the dismutation of superoxide ($O_2^-$) into oxygen and hydrogen peroxide, and it serves as a key antioxidant in cells (11). Above

mentioned several growth factors including EGF, bFGF, KGF, IGF-I, and SOD, which act by binding to their respective receptor tyrosine kinases, followed by downstream signaling and activation of the protein kinase C, AKT, and ERK signaling pathways. In particular, mitogen-activated protein kinase (MAPK) signaling, including ERK, plays a critical role in innate immune responses (8, 11, 12).

MAPKs are serine/threonine-specific protein kinases (13-16). They are important upstream factors, which lead to the activation of nuclear factor-κB (NF-κB) (6, 17). They are mainly composed of three subfamily members: ERK, JNK, and p38. The MAPK signaling pathway regulates a wide variety of cellular events, including complex cellular programs, such as differentiation, proliferation, apoptosis, and processes involved in immune response (18). Phosphorylation of MAPKs modulates the expression of a variety of genes involved in immune and inflammatory responses, including inducible nitric oxide (iNOS), cyclooxygenase-2 (COX-2).

In addition, NF-κB nuclear translocation, IκB phosphorylation, and degradation are important inflammatory factors. The expression of pro-inflammatory cytokines is mainly regulated by the NF-κB pathway (19). In unstimulated cells, NF-κB resides in the cytoplasm as an inactive NF-κB-IκB complex (20).

Despite previous studies of growth factors and pathways associated in inflammation

(8, 12, 21), the mechanism underlying the inflammatory response, particularly the response to mixture of mentioned five growth factors has not been studied.

In this study, the anti-inflammatory effects of mixtures of recombinant growth factors (MRGFs) on the generation of several chemokines, cytokines, and enzymes involved in the inflammatory process, such as inducible iNOS, COX-2, IL-1β, IL-6, IL-10, IL-12p40, GM-CSF, MCP-I, TNF-α, and NO in LPS-stimulated RAW 264.7 cells were investigated. We also investigated whether MRGFs influence the LPS-stimulated ERK and NF-κB signaling pathway.

## Materials and Methods

### Materials

Mixed in same ratios, recombinant human EGF, recombinant human bFGF, recombinant human KGF, recombinant human IGF-I, and recombinant human SOD were provided from a Nutrex Technology Co. Ltd. (Seoul, Korea).

3- (4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide (MTT), Lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A) and DMEM media, fetal calf serum (FBS), trypsin EDTA, Phosphate buffered saline (PBS), Penicillin/streptomycin were purchased from WelGENE Co. (Korea). Antibodies specific to COX-2, GAPDH was purchase from Santa Cruz (CA, USA) and iNOS was purchased from PharMingen (BD Biosciences, CA, USA). Antibodies against p-ERK, ERK, p-JNK, JNK, p-p38, p38, NF-κB, p-IκB, IκB and Lamin B1 were purchased from Cell Signaling Technology (MA, USA). PD98059 and IKK inhibitor Ⅶ were purchased from Sigma Chemical Co. and Calbiochem (San Diego, USA). Secondary antibodies specific for anti-goat IgG, anti-mouse IgG, and anti-rabbit IgG were purchased from Vector Laboratories (Burlingame, CA). iNOS, COX-2 and GAPDH oligonucleotide primers were obtained from Bioneer (Seoul, Korea).

**Cell cultures of Raw 264.7 cells**

Raw 264.7 murine macrophage cells were maintained at 37 $^{o}$C in a humidified atmosphere of 95% air and 5% $CO_2$ in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 10 μg/ml streptomycin.

**Cell viability**

Cell viability was assessed using a MTT assay that was performed by a slight modification of the method described by Wasserman et al (22). Seeded RAW 264.7 cells in a 12-well plate were treated with LPS and MRGFs after 24 h incubation. Supernatant removed and a MTT solution was added to each well and further incubated for 4h in incubator. Subsequently, 1.5ml of dimethyl sulfoxide (DMSO) was treated to each well to solubilize any deposited formazon. After an incubation of 10 min at room temperature, optical density (OD) was determined at 540nm on an ELISA plate reader (Thermomax, Molecular Devices, Sunnyvale, CA, USA).

**NO assay**

NO production was detected by assessment of nitrite accumulation. After RAW

264.7 cells were seeded in a 12-well plate, LPS (100 ng/ml) and indicated concentration of MRGFs (0.01, 0.1, 1 and 10 μg/ml) were added to the culture medium, and incubated for 24h in incubator. The concentration of nitrite in spent media was determined via Griess reaction. A 100μl Supernatant of each well transferred 96-well plate and mixed with 100μl of Griess reagent (Thermo Fisher scientific, Wilmington, Delaware, U.S.A) in a separate 96-well plate. After an incubation of 10 min at room temperature, OD was determined at 540nm on an ELISA plate reader.

**Cell lysate preparation and Western blot analysis**

Treated whole cell extracts were lysed in RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.1% Sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO, U.S.A) and a protease inhibitor cocktail tablet (Roche Diagnostics indianapolis, IN, U.S.A) for preparation of cellular extracts. Cytoplasmic and unclear were extracted using buffer A (HEPES 10mmol/L, pH 7.9, KCl 10mmol/L, 2mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1mmol/L, 1mM EGTA, dithiothreitol (DTT) 1mmol/L, aprotinin 1mg/L, protease inhibitor cocktail tablet 5mg/ml) and buffer B (HEPES 20mmol/L, pH 7.9, NaCl 420mmol/L, edetic acid 0.1mmol/L, egatazic acid 0.1mmol/L, PMSF 1mmol/L, DTT 1mmol/L, aprotinin 1mg/L, protease inhibitor

cocktail tablet 1mg/ml) (23). The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard (24).

For Western blot analysis, each cell lysates containing 20μg of proteins were resolved on 10~12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a Polyvinylidene fluoride (PVDF) membranes. The membrane was washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies. The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the enhanced chemiluminescence (ECL) Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

**Reverse transcription polymerase chain reaction (RT-PCR)**

Total RNA was extracted using the TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions after treatment and Quantified by ND1000 spectrophotometer (Thermo Fisher scientific, Wilmington, Delaware, U.S.A) of the absorbance at 260nm. cDNA was synthesized with 2μg of denatured total RNA in a

final volume of 20μl of buffer containing MgCl2, KCl, dNTPs, and oilgo-dT reverse transcriptase by incubation at 42℃ for 60 min. The cDNA obtained was amplified with the following primers: iNOS forward (F): 5'- CTA CCT ACC TGG GGA ACA CCT GGG -3', iNOS reverse (R): 5'- GGA GGA GCT GAT GGA GTA GTA GCG G -3', COX-2 forward (F): 5'- CTG TAT CCC GCC CTG CTG GTG -3', COX-2 reverse (R): 5'- ACT TGC GTT GAT GGT GGC TGT CTT -3', and GAPDH forward (F): 5'- GCC AAA AGG GTC ATC ATC TC -3', GAPDH reverse (R): 5'- GGT CCT CAG TGT AGC CCA AG -3'. Application was performed using PCR Master Mix (Takara Bio Inc., Tokyo, Japan) in total volume 20μl. PCR cycling conditions were consisted of denaturation at 94℃ for 30 seconds, annealing at 60℃ for 1 min, and extension at 72℃ for 30 seconds. The products were electrophoresed for 30 minutes at 100V on a 1% agarose gel. Gels were visualized by Molecular Imager® Gel Doc™ XR imaging system (Bio-Rad Laboratories, Inc., Hercules, CA).

**Real-time RT-PCR**

Total RNA and the cDNAs were generated as previously described. Real-time PCR was performed with a C1000™ Termal Cycler (Bio-Rad, Hercules, CA, U.S.A) using SYBR Green (Takara, Shiga, Japan). Reactive mixtures were incubated for 40 cycles at

95℃ for 15 seconds; 58℃ for 45 seconds' and 72℃ for 20 seconds. Gene expression was normalized to those of the housekeeping gene encoding GAPDH.

**ELISA assay for detecting cytokine production**

After the RAW 264.7 cells were seeded in a 24-well plate, LPS and MRGFs were treated to the each well and were incubated for 24h. Cytokine concentrations of the culture media were measured using each cytokine's ELISA kit (eBioscience, San Diego, CA, U.S.A).

**Statistical analysis**

Statistical analyses were performed using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Results are expressed as the mean ± standard deviation (25). Data was analyzed One-way ANOVA followed by a Duncan's test for multiple comparison and two-tailed value of $p<0.05$ was considered statistically significant.

## Results

### Effect of MRGFs on cell viability and NO production

After each five growth factors including EGF, bFGF, KGF, IGF-I, and SOD, which were measured NO production, we studied the effects of MRGFs on the production of NO. As none of the five growth factors decreased cell viability or NO production up to a concentration of 100 ng/ml in LPS-stimulated RAW 264.7 cells (data not shown), we prepared MRGFs by mixing each 100 ng/ml of five growth factors in the same ratio. As the MRGFs did not decrease the viability of RAW 264.7 cells up to a concentration of 10 μg/ml (data not shown), this concentration was used in subsequent experiments. The RAW 264.7 cells were incubated with LPS (100 ng/ml) and MRGFs at concentrations of 0.01, 0.1, 1 and 10μg/ml for 24 h, and the NO production was measured. Fig 1. shows the levels of NO production following the treatment of the RAW264.7 cells with the MRGFs. After treatment with 10 μg/ml of MRGFs, NO production decreased significantly in the RAW 264.7 cells compared to an LPS-stimulated control ($p<0.05$). These results demonstrate that MRGFs inhibit the production of NO by suppressing iNOS activity.

### Inhibitory effect of MRGFs on iNOS and COX-2 proteins and mRNA expression

**in LPS-stimulated RAW 264.7 cells**

We investigated the effects of MRGFs on LPS-stimulated iNOS and COX-2 protein by Western blot analysis and examined mRNA expression by RT-PCR and real-time PCR. After the cells were co-treated with LPS (100 ng/ml) at concentration 0.01, 0.1, 1 and 10 μg/ml of MRGFs for 24 h and 6 h, we harvested protein and mRNA samples. MRGFs at a concentration of 10 μg/ml significantly decreased iNOS and COX-2 protein and mRNA expression in the LPS-stimulated cells (Fig. 2). Therefore, the MRGFs showed significant inhibition on the production of pro-inflammatory mediators such as iNOS, COX-2 in LPS-stimulated RAW 264.7 cells.

**Inhibitory effects of MRGFs on MAPK phosphorylation in LPS-stimulated RAW 264.7 cells**

We examined the effects of MRGFs on LPS-stimulated MAPK phosphorylation in RAW 264.7 cell. After the cells were stimulated with LPS, western blot analysis was performed to analyze the total and phosphorylated levels of ERK. Compared with a control, phosphorylated ERK significantly increased after the cells were stimulated with LPS for 2 h (Fig. 3A.). The cells were them co-treated with LPS (100 ng/ml) at concentration 0.01, 0.1, 1 and 10 μg/ml of MRGFs for 2 h, and we investigated the

311 effect of (10 μg/ml) on the inhibition of the phosphorylation of ERK but not that of JNK
312 and p38 in LPS-stimulated RAW 264.7 cells (Fig. 3B.). To confirm the causal link
313 between the inhibition of phosphorylation of MAPKs and MRGFs, Raw 264.7 cells
314 were pre-treated with ERK inhibitor (PD98059, 30 μM) for 1h. After treatment with
315 LPS (100 ng/ml) and MRGFs (10 μg/ml) for 2 h, we confirmed that LPS-stimulated
316 pro-inflammatory mediators, such as iNOS and COX-2, and ERK phosphorylation was
317 both inhibited by 30 μM/ml of PD98059 and 10 μg/ml of MRGFs (Fig. 3C.).
318
319 **Inhibitory effects of MRGFs on NF-κB nuclear translocation, IκB phosphorylation,**
320 **and degradation in LPS-stimulated RAW 264.7 cells**
321 The cells were co-treated with LPS (100ng/ml) at concentration 0.01, 0.1, 1 and 10
322 μg/ml of MRGFs for 4 h. The effect of MRGFs on NF-κB nuclear translocation, IκB
323 phosphorylation and degradation of the cytoplasm and nucleus were then assessed by
324 Western blot. Fig 4A. shows that LPS-stimulated IκB phosphorylation and degradation
325 was significantly inhibited by MRGFs (10 μg/ml) in cytoplasm of RAW 264.7 cells.
326 Also, amount of NF-κB in nucleus was markedly increased upon exposure to LPS alone,
327 but MRGFs (10 μg/ml) inhibited LPS-stimulated nuclear translocation of NF-κB. To
328 confirm whether the MRGFs suppressed the activation of NF-κB, the cells were

pretreated with the upstream inhibitor of NF-κB, IKK inhibitor VII, for 2 h and exposed to LPS (100ng/ml) and MRGFs for 4 h. We confirmed that activation of the LPS-stimulated NF-κB pathway was both inhibited by 1 μM/ml of the IKK inhibitor VII and 10 μg/ml of the MRGFs (Fig. 4B.).

**Inhibitory effects of MRGFs on inflammatory cytokines in LPS-stimulated RAW 264.7 cells**

Inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, were released as a result of the activation of the NF-κB pathway in the LPS-stimulated RAW 264.7 cells. We have already confirmed that MRGFs reduce the activation of NF-κB pathway in the above results. The effects of the MRGFs on the release of cytokines were assessed with an ELISA assay. Table 1. shows the effect of the MRGFs on the inhibition of inflammatory cytokines (IL-1β, IL-6, IL-10, IL-12p40, GM-CSF, MCP-I, TNF-α) in the LPS-stimulated RAW 264.7 cells. Specifically, a concentration of 10 μg/ml of MRGFs decreased the production of IL-10, IL-12p40, GM-CSF, MCP-I, and TNF-α in the LPS-stimulated RAW 264.7 cells (Fig 5.). The results indicate that MRGFs can modulate the synthesis of several cytokines involved in the inflammatory process.

## Discussion

The major finding of this study is that MRGFs play an important role in the LPS-stimulated inflammatory response. Previous studies (1-4) revealed that various growth factors regulate a variety of cellular processes, such as cell growth, differentiation, and proliferation, by binding to specific high-affinity cell membrane receptors. Many studies found a correlation between growth factors and wound healing, cancer, and DNA synthesis in various cells (13, 16). Nonetheless, little information was available with respect to the molecular mechanisms underlying the anti-inflammatory effect of a combination of growth factors.

Activation of macrophage plays an important role in the initiation and propagation of inflammatory responses by the inflammatory mediators (26). Therefore, LPS-stimulated macrophage activation increased the production of cytokines such as IL-1β, TNF-α, GM-CSF and NO, which is modulated by the up-regulation of iNOS (27). iNOS and COX-2 are often present together, share a number of similarities, and play fundamental roles in similar pathophysiological conditions, such as inflammation and cancer (28, 29).

The MAPK and NF-κB signaling pathways are important in the regulation of inflammatory mediators. Members of the MAPK family, including ERK, JNK, and p38,

365 are frequently involved in LPS-stimulated inflammation and play a critical role in the
366 regulation of cell growth and differentiation and in the control of cellular responses to
367 cytokines and stresses.

368 The transcription factor NF-κB controls a number of inflammatory mediators, such
369 as iNOS, COX-2, and cytokines, and the factor is important for immunity and
370 inflammation (30). Previous studies reported that LPS-stimulated stimulation of NF-κB
371 signaling activity leading to the activation of MAPK is a major mechanism underlying
372 NO production by iNOS (31). When RAW 264.7 cells are stimulated by LPS, IκB is
373 phosphorylated and separates from NF-κB, resulting in the translocation of NF-κB to
374 the nucleus (32).

375 Cytokines can be used as markers of inflammation (33). The cytokines TNF-α and
376 IL-1β are closely related to each other, and they share many biological activities:
377 pyrogenicity, activation of T lymphocytes, stimulation of fibroblast proliferation, and
378 neutrophil activation (34). IL-10 is a pleiotropic cytokine that modulates the adaptive
379 immune-related cell function. It possesses immune stimulatory properties, including the
380 ability to activate T cells, B cells, NK cells, and mast cells (35,36). In addition, previous
381 studies demonstrated that IL-6, MCP-1, GM-CSF, and IL-12p40 are related
382 inflammatory mediators (35). In particular, IL-12p40 was recently reported to markedly

up-regulate the expression of TNF-α and induce the expression of iNOS in a dose-dependent manner (37).

In this study we treated LPS-stimulated RAW 264.7 cells with various concentrations of MRGFs. The aim was to elucidate the pharmacological and biological effects of MRGFs, which include EGF, bFGF, KGF, IGF-I, and SOD, on the inhibition of inflammatory mediators in macrophages. Our data clearly indicated that MRGFs suppress the production of NO, iNOS, and COX-2 in LPS-stimulated RAW 264.7 cells. In addition, inhibiting the phosphorylation of ERK and NF-κB decreases the production of these inflammatory mediators. Furthermore, the data demonstrated that treatment with MRGFs decreases the production of inflammatory cytokines, such as IL-10, IL-12p40, GM-CSF, MCP-I, and TNF-α, in LPS-stimulated RAW 264.7 cells.

In summary, MRGFs have the potential to decrease inflammatory mediators, such as iNOS, COX-2, IL-10, IL-12p40, GM-CSF, MCP-I, and TNF-α, by inhibition of the phosphorylation of the ERK and NF-κB signaling pathways. These findings suggest that MRGFs may prevent inflammatory diseases by suppressing MAPKs and NF-κB-mediated inflammatory genes.

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## Figure legends

**Fig 1. Inhibition of NO production by MRGFs in LPS-stimulated RAW 264.7 cells.**

After RAW 264.7 cells were incubated for 24 h in 12-wells plate, cells were co-treated with various concentrations of MRGFs (0, 0.01, 0.1, 1 and 10 μg/ml) and 100 ng/ml LPS for 24 h. The nitrite concentration in the culture medium was determined by Griess reagent. Data are expressed as mean ± S.D. (*n=3*). Data are mean ± S.D. (*n=3*). † $p<0.05$ versus control, * $p<0.05$ versus LPS-stimulated.

**Fig 2. Effect of MRGFs on the protein and mRNA expression of iNOS and COX-2 in RAW 264.7 cells stimulated by LPS.**

(A) RAW 264.7 cells were co-treated with various concentrations of MRGFs (0, 0.01, 0.1, 1 and 10 μg/ml) and LPS (100ng/ml) for 24 h. The 10μg/ml of MRGFs inhibited LPS-stimulated protein expression of iNOS and COX-2 in western blot analysis. (B, C) After cells were co-treated with various concentrations of MRGFs (0, 0.01, 0.1, 1 and 10 μg/ml) and LPS (100ng/ml) for 6 h, total RNA was extracted, and cDNA was synthesized for RT-PCR and real-time PCR. The results presented that 10μg/ml of RGFMs inhibited LPS-stimulated mRNA expression of iNOS and COX-2. (C) Data are mean ± S.D. (*n=3*). † $p<0.05$ versus control, * $p<0.05$ versus LPS-stimulated mRNA expression.

**Fig 3. Effect of MRGFs on the protein expression of phosphorylation ERK of MAPKs.**

(A) RAW 264.7 cells were incubated with LPS for indicated times. The LPS-stimulated cells increased phosphorylation of ERK level at 2 h. (B) RAW 264.7 cells were co-treated with various concentrations of MRGFs (0, 0.01, 0.1, 1 and 10 μg/ml) and LPS (100ng/ml) for 2h. The 10μg/ml of MRGFs inhibited LPS-stimulated phosphorylation ERK of MAPKs in western blot analysis.

(c) After RAW 264.7 cells were pretreated with PD98059 (10, 20 or 30μM/ml) for 1 h, we co-treated with LPS (100ng/ml) and MRGFs (10μg/ml) for 2h. Cell lysates were analyzed by Western blot analysis using various antibodies iNOS and COX-2. PD98059 of ERK inhibitor inhibited LPS-stimulated iNOS and COX-2 in a dose-dependent manner. LPS-stimulated pro-inflammatory mediators, such as iNOS and COX-2, and ERK phosphorylation was both inhibited by 30 μM/ml of PD98059 and 10 μg/ml of the MRGFs

**Fig 4. Effect of MRGFs on LPS-stimulated NF-κB activation, IκB degradation and phosphorylation in RAW 264.7 cells.**

553 (A) After RAW 264.7 cells were co-treated with MRGFs (0, 0.01, 0.1, 1 and 10 μg/ml)
554 and LPS (100 ng/ml), and the cells were incubated for 4 h. Cytoplasm and nuclear
555 extracts of the cells were measured by western blot. The results presented that MRGFs
556 (10μg/ml) inhibited LPS-stimulated IκB phosphorylation and degradation of cytosol and
557 NF-κB phosphorylation of nucleus. (B) After RAW 264.7 cells were pretreated with
558 IKK inhibitor Ⅶ (0.1, 0.5 or 1μM/ml) for 2 h, the cells were co-treated with MRGFs
559 (10μg/ml) and LPS (100ng/ml) for 4 h. The cell lysates were analyzed by Western blot
560 analysis using various antibodies p-IκBα, IκBα and NF-κB. IKK inhibitor Ⅶ (1 μM/ml)
561 and MRGFs(10μg/ml) inhibited LPS-stimulated IκB phosphorylation and degradation
562 of cytosol and NF-κB phosphorylation of nucleus.
563
564 **Fig 5. Effect of MRGFs on cytokine production in LPS-stimulated RAW 264.7 cells.**
565 RAW 264.7 cells were co-treated with different concentrations of MRGFs (0,
566 0.01, 0.1, 1 and 10 μg/ml) and LPS (100 ng/ml) for 24 h. The cytokines
567 concentration in the culture medium was determined by ELISA assay kit. Data are
568 mean ± S.D. (n=3). * $p<0.05$ versus LPS-stimulated cytokine level.
569
570 **Table 1. Effect of RGFMs on pro-inflammatory cytokines production in LPS-**

**stimulated RAW 264.7 cells.** The concentration of cytokines in the culture medium was measured after 24 h incubation. Data are mean ± S.D. (n=3). * $p<0.05$ versus LPS-stimulated cytokine level.