Spirobenzofuran (SBF): An isolate from *Acremonium sp. HKI 0230* and *Coprinus echinosporus* suppresses inflammation in RAW 264.7 cells

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Abstract: Inflammation is an aggregate of different pathologic responses in body that leads to life threatening conditions if not combated at early stages. A variety of chemical medications from low quality to high quality are available in market for treatment of inflammation. However the side effects posed by these medications cannot be ignored. Here in our study we have shown for the first time, the anti-inflammatory effects of SBF compound that is obtained from wild mushroom species that are *Acremonium sp. HKI 0230* and *Coprinus echinosporus*. We employed Nitric oxide determination, cell viability assay, RT-PCR and western blot analysis to check the anti-inflammatory effects of SBF. The antioxidant activity of this compound has been studied in detail in past, but our results have shown that SBF potently suppressed the Nitric oxide production (NO) without any cytotoxicity to the model cell line; RAW 264.7 cells. It also inhibited the production of major proinflammatory mediators and cytokines i.e. iNOS, COX-2, IL-1β, IL-6 and TNF-α. SBF elicited its anti-inflammatory effects via the canonical NF-κB and MAPK pathway. Taken together, our results have shown that SBF exhibits excellent anti-inflammatory activity in vitro and further experimentations may warrant its application as a commercial herbal remedy for inflammation related anomalies.

Keywords: Inflammation, RAW 264.7 cells, SBF, proinflammatory cytokines, NF-κB, MAPK

INTRODUCTION

Inflammation is well coordinated response of physiological system of cells towards any kind of endogenous or exogenous invasion. This includes the signaling of inflammation specific cells towards the site of insult via blood stream(Barton, 2008). In case of acute inflammation, it is the sentinel cells, specifically the macrophages that act as first line of defense by engulfing and killing the foreign invasive material. When these macrophages are stimulated, they also release certain kinds of proinflammatory mediators and anti-inflammatory cytokines that serve to control or aggravate inflammation (Medzhitov, 2008; Gordon and Taylor, 2005). Basically the type of cytokines that are released depends on what kind of pathogen associated molecular pattern (PAMP), is activated by receptors. Due to the binding of Toll like receptors (TLR's) with their specific PAMP, majorly TNF-α and IL-6 is released on the very first hand that secrete nitric oxide synthase 2(NOS2), due to which there is a simultaneous production of nitric oxide (Serhan, 2007). After this a series of reactions and activations are regulated that trigger the release of other proinflammatory cytokines and mediators like IL-1β, COX-2 and iNOS. These all entities together produce strong inflammatory response that if on the lower scale (which depends on the quantity and intensity of foreign substance), is inhibited by endogenous anti-inflammatory cytokines like interferons (IFN-γ) and IL-10 (Dinarello, 2000). But if the production of proinflammatory cytokines is greater than the counteracting anti-inflammatory cytokines, there needs to be an anti-inflammatory agent given exogenously.

Genus *Acremonium* that is also called *Cephalosporium* is a kind of wild cosmopolitan fungus which is obtained from soil and other dead plant materials. It consists of around 150 species. In 1953, the antimicrobial investigation of this fungus led to its name being called as *Cephalosporium acremonium* which terminated into the development of Cephalosporin class of antibiotics. Basically this fungus is an opportunistic pathogen and 9 out of 150 of its species are mycologic disease causing agents (Das *et al.*, 2010; Lee *et al.*, 2009; Omolo *et al.*, 2011). But here in our study we used spirobenzofuran (SBF), that is isolated from the *Acremonium sp. HKI 0320*, with a molecular weight of 262.1g/mol having waxy, white solid appearance (Kleinwachter *et al.*, 2001). Another source for SBF isolation is from the *Coprinus echinosporus* species of genus *Coprinus*. This genus has been reported to possess anti-inflammatory, anti-bacterial and antioxidant properties but the anti-inflammatory activity of SBF obtained from this species has remained unexplored (Ki *et al.*, 2015; El-Seedi *et al.*, 2010; Kettering *et al.*, 2005; Liu *et al.*, 2012; Pettit *et al.*, 2010).

Previous studies have shed light on the anti-bacterial properties of spirobenzofuran. But here in our research, we report the anti-inflammatory activity of SBF for the first time. Our results demonstrated that SBF potently suppressed the NO production in RAW 264.7 cells

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without any cytotoxicity over the selected dosage used. Furthermore it also inhibited the production of proinflammatory mediators and cytokines and has elicited its anti-inflammatory effects via canonical NF-κB and MAPK pathway.

MATERIALS AND METHODS

Materials

Spirobenzofuran (SBF) was a kind gift from Professor Bong-Sik Yun from Chonbuk National University, Gobong-ro 79, Iksan 570-752, Korea. It was identified as spirobenzofuran by the above mentioned professor according to previous report (Kleinwachter *et al.*, 2001) and the chemical structure is shown in (fig. 1). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were bought from WelGene Co. (Deagu, Korea). The total RNA extraction kit was from Invitrogen (Carlsbad, CA, USA). Oligo dT, iNOS, COX-2, TNF-α, IL-6 and IL-1β primers were obtained from Bioneer Co. (Daejeon, Korea). LPS (Escherichia coli 055:B5) and 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbro mide (MTT) were purchased from Sigma (St Louis, MO, USA). Specific antibodies used against phospho- and/or total form of ERK, JNK, p38, IKK α/β, IκB, NFκB p65, iNOS, COX-2, β-actin and secondary antibody rabbit HRP linked antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents and chemicals were obtained from Sigma Aldrich Co.

Cell culture

RAW 264.7, Murine macrophage cell line, originating from American Type culture collection (ATCC) was being cultured in complete DMEM supplemented with 5% FBS and 1% penicillin (100 IU/ml) and streptomycin sulfate (100µg/ml) in humidified 5% CO_2 incubator at 37ºC.

Nitric oxide (NO) assay

Griess reaction was the basis of nitric oxide (NO) measurement. Briefly, RAW 264.7 cells were seeded in 24 well plate and incubated with or without LPS (0.1µg/ml) in absence or presence of SBF at concentration of 12.5-50µM for 18hrs. Next day, the culture supernatants (100µl) were mixed with Griess reagent (0.2% naphthyl ethylene diamine dihydrochloride, and 2% sulphanilamide in 5% phosphoric acid) in DDW at equal volumes and incubated for 5min at room temperature. The absorbance in each well was then read at 540nm in ELISA reader (Versamax microplate reader, Molecular devices, LLC. CA, USA).

Cell viability (MTT) assay

To analyze the cytotoxic effects of SBF, cell viability assay was performed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent which was added to culture medium at a final concentration of 0.1mg/ml in 24 well plate. After 4 hr of incubation at 37ºC in 5% CO_2, the violet colored crystals were dissolved in Dimethyl sulfoxide (DMSO) 100µl/ well and absorbance values were read at 560 nm (Versamax microplate reader, Molecular devices, LLC. CA, USA).

RNA extraction and qRT-PCR

For RNA extraction**,** RAW 264.7 cells were pretreated with or without SBF at indicated concentrations for 30 min and then stimulated with LPS (0.1µg/ml) for 18h in 6 well plates. TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions was used for extraction of total RNA. Later experimental steps were according to our previous reports (Saba *et al.*, 2015; Saba *et al.*, 2015). Quantitative PCR primers sequence is given in table 1.

Western blot analysis

RAW264.7 cells were treated or untreated with SBF (12.5 - 50µM/ml) in the presence or absence of LPS (0.1µg/ml) in 6 well plates. Cytosolic and nuclear proteins were extracted according to the instructions of NE-PER® Nuclear and cytosolic extraction reagents (Thermo scientific, #78833 & #78835). Protein quantification was then done using PROMEASURE assay kit (PRO-PREP, iNtRON Biotechonology, Gyeonggi-do, Korea). The remaining method employed was according to our previous report (Lee *et al.*, 2014).

STATISTICAL ANALYSIS

Data is presented as mean ± SEM. One-way analysis of variance followed by Dunnett's *t*-test was used for statistical analysis. SAS 9.3 software (SAS institute Inc, Gary NC, USA) was applied for analysis. *P* values less than 0.01 were considered as statistically significant.

RESULTS

SBF suppresses LPS induced inflammation in RAW 264.7 cells

Bacterial lipopolysaccharides (LPS) are the toxic components of outer membrane of almost all gram negative bacteria. Once LPS enters into the living cell, it activates the production of inflammatory mediators, Nitric oxide (NO) being the first one (Sharma *et al.*, 2007). Therefore, we investigated whether SBF affects LPS-induced NO production in RAW 264.7 cells. As shown in fig. 2a, SBF potently suppressed LPS induced NO production when injected to culture media 30 min after the sample injection. Furthermore, no cytotoxic effects were observed for the dosage of SBF used, (fig. 2b).

Expression of proinflammatory mediators and cytokines under SBF treatment

As a result of LPS bondage with TLR4 receptors, a number of proinflammatory mediators and cytokines are released that signals the cells to secrete anti-inflammatory

cytokines that would suppress the inflammation (Beutler, 2000). If the threshold of endotoxin or exotoxin is below the levels that can be counteracted by system's own endogenous inflammatory suppressors, there is no need of an external agent. However in most cases, when the toxin threshold is above the acquired immunity, there is a need of some exogenous or external treatment. We, therefore, checked whether SBF affected the proinflammatory mediators and cytokine expression in RAW 264.7 cells. SBF significantly inhibited the expression of iNOS, COX-2, IL-1β, IL-6 and TNF-α mRNA expression in a dose dependent manner (figs. 3a, b).

Reduction in protein expression levels of iNOS and COX-2 by SBF

In the former result we have shown the inhibition of iNOS and COX-2 at transcriptional levels. We then geared to check if SBF affected these factors at translational level, so immunoblotting was performed to check their expression. As shown in fig. 4, SBF inhibited the protein expression of both iNOS and COX-2 in a concentration dependent manner.

Involvement of NF-κB pathway in SBF signal transduction

The most common pathway followed by any agent to reveal its anti-inflammatory effects that is stimulated via TLR-4 receptors is NF-κB pathway. This pathway regulates the expression of numerous genes and particularly those genes involved in inflammation and immunity. As shown in (fig. 5a), SBF following the canonical NF-κB pathway, firstly inhibited the phosphorylation of IRAK-1 and then moving downstream it suppressed the phosphorylation levels of P-TAK1, P-IKK α/β, P-IκB/α and NF-κB. Basically NF-κB is sequestered in cytoplasm by IκB kinase family of factors. It only gets translocated to nucleus when IKKα/β type of factors disrupts this complex on the stimulation of TLR-4 ligand. Then it exhibits its inflammatory activates by turning on the various inflammatory stimulating genes. Fig. 5b shows the relative ratio of these proteins when compared to β-actin as our house keeping gene.

C-Jun n-terminal kinase (JNK)/MAPK pathway suppression by SBF

Mitogen activated protein kinase pathway (MAPK) is another universal pathway for induction of inflammatory cascade in cells that are under stress. In our study we used LPS as an exogenous inflammatory stimulator which posed stress on cells, so we geared to check the signal transduction of SBF via MAPK pathway. SBF showed dose dependent inhibitory effect on MAPK pathway starting from the level of diminution of phosphorylation of MEK and MKK as shown in (figs. 6a, b). Furthermore it also dose dependently inhibited the phosphorylation of JNK, ERK and P38 (figs. 6c, e).

DISCUSSION

The extra ordinary qualities that exist in mushrooms either of edible nature or non-edible type cannot be denied. These qualities include on the very first hand, the antioxidant properties which means they have nutritional and metabolic enzymes that scavenge free radicals from the body and decrease the amount of Reactive oxygen species (ROS) (Kozarski *et al.*, 2015). Other beneficial properties include anti-inflammatory effects, anti-cancerous effects and so on. Mushroom is basically a fungus that is grown by nature itself and since almost 19th century, it is grown very effectively by human hands when the beneficial qualities of this plant were and now continuously being unraveled (Mattila *et al.*, 2000; Sullivan *et al.*, 2006). Here in our study we have shown the anti-inflammatory aspects of a single compound SBF that is being isolated from two kinds of mushroom species from the Genus *Acremonium* and Genus *Coprinus.*

The major players in inflammation are the macrophage cells that engulf the invading pathogen and moreover release chemicals called proinflammatory mediators and cytokines that recruit more inflammatory responsive cells (Serhan and Savill, 2005). Nitric oxide is a gaseous moiety that is produced due to oxidation of L-arginine under the action of enzyme nitric oxide synthase (NOS). There are basically three isoforms of NOS but it is inducible NOS (iNOS) among them that is responsible for the overproduction of nitric oxide that aggravates the inflammation (Korhonen *et al.*, 2005). In our study, SBF has dose dependently decreased the nitric oxide production in a dose dependent manner indicating that on the very first hand it can combat the excessive production of NO that can be deleterious for the cells if not controlled. SBF also did not show any cytotoxicity as measured by the MTT assay (fig. 2).

Excessive generation of NO in turn activates the production of Cyclooxygenase (COX) and particularly COX-2 type of moiety that causes the conversion of arachidonic acid to prostaglandins causing the physical manifestations of inflammation that are swelling, reddening and pain. Furthermore there are also some other major proinflammatory cytokines released like IL-1β, IL-6 and TNF-α (Alexander and Rietschel, 2001). These cytokines also aggravate the inflammatory response if not properly controlled at appropriate time. SBF in our studies has potently inhibited the production of these proinflammatory mediators at mRNA levels and also the levels of iNOS and COX-2 are suppressed by SBF at protein levels (figs. 3, 4).

Inflammation can be characterized as pathologically very important pathway for almost every disease. So it is inevitable that there are so many pathways for activation of inflammation. Two of those most common pathways

that initiate inflammation are the NF-κB and MAPK pathways. NF-κB pathway is having further two types that is canonical and non-canonical pathway (Kopp and Medzhitov, 1999). In our study SBF strongly inhibited the phosphorylation of the Canonical NF-κB pathway (fig. 5).

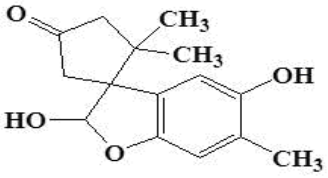

Fig. 1: Chemical structure of SBF

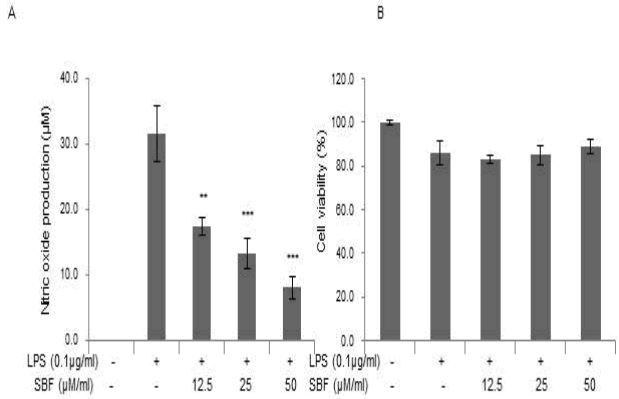

Fig. 2: Suppression of NO production by SBF. a RAW 264.7 cells were preincubated with SBF for 30 mins and then stimulated with LPS (0.1µg/ml) of 18hrs. 100µl of cell supernatant was transferred to 96 well plate and reacted with equal amounts of Griess reagent, and then NO production was measured at 540nm in spectrophotometric micro plate reader. b Cell viability was measured by MTT assay. Values in bar graph are mean ± SEM of at least 3 independent experiments. ***p<0.001 and **p<0.05 compared to LPS.

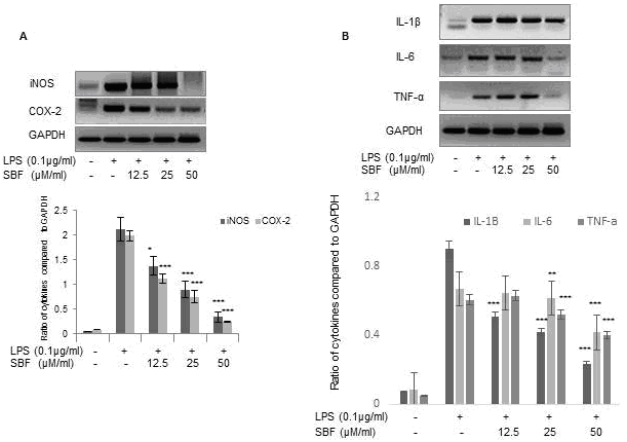

Fig. 3: SBF inhibited the mediators and cytokine expression under LPS stimulation. For mRNA expression, RAW 264.7 cells were pretreated with SBF for 30 mins and then stimulated with LPS for 18 hrs. Total RNA was isolated as described previously and mRNA expression of COX-2, iNOS, IL-1β, IL-6 and TNF-α was determined by RT-PCR. a Suppression of inflammatory mediators iNOS and COX-2 by SBF. b Inhibition in the proinflammatory cytokines expression by SBF. GAPDH was used as housekeeping gene. Images are representative of 3 independent experiments. Values in bar graph are mean ± SEM of 3 independent experiments. ** p<0.005, *** p<0.001 compared to LPS.

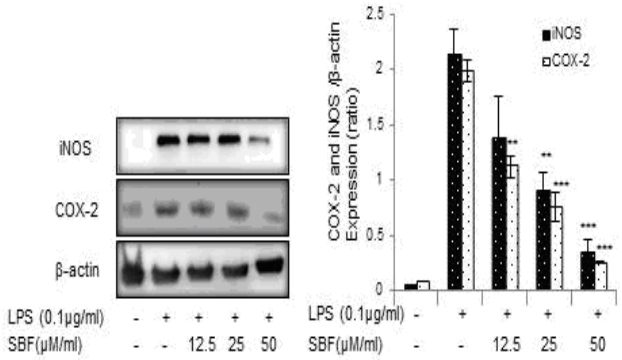

Fig. 4: Diminution of protein expression of iNOS and COX-2 by SBF. For iNOS and COX-2 protein expressions, total protein was extracted from RAW 264.7 cells after pretreatment with SBF and stimulation with LPS for 18 hrs. Proteins were run on SDS-PAGE and analyzed by ECL chemiluminensence. Western blot images are representative of 3 independent experiments. Images were analyzed by image J software and bar values of ***p<0.001 and **p<0.05 were considered statistically significant.

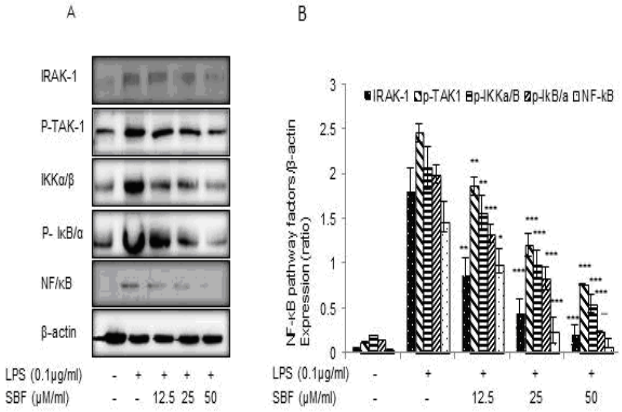

Fig. 5: Signal transduction of SBF via canonical NF-κB pathway. Nuclear and cytosolic proteins from RAW 264.7 cells treated with SBF and stimulated with LPS were extracted using NE-PER extraction kit. ECL chemiluminisence was used to visualize the protein blots. a The images are representative of at least 3 independent experiments. β-actin was taken as housekeeping and reference gene for comparison with other genes. b Bar values shown are average ± SEM of 3 independent experiments. p<0.01 were considered statistically significant compared to LPS only.

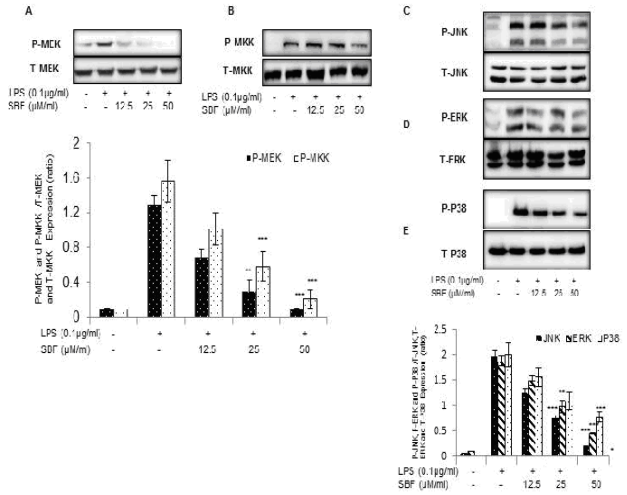

Fig. 6: Attenuation of MAPK pathway by SBF. RAW 264.7 cells were pretreated with SBF and stimulated with or without LPS for 18hrs. Total proteins were extracted and run on SDS-PAGE. Proteins were then analyzed by ECL chemiluminisence system. Images of a, b, c. d and e are representative of at least 3 independent experiments. Values in bar graph are mean± SEM of three independent experiments. $p<0.01$ were considered statistically significant compared to LPS.

The canonical NF-κB pathway consists on a family of 5 transcriptional factors that are sequestered in an inactive form in the cytoplasm of cell by the inhibitors of NF-κB family (IKB) (Lawrence, 2009; Makarov, 2000). NF-κB is only released and translocated to the nucleus when the IKK which is the IKB complex consisting of IKK-α and IKK-β phosphorylate the IKB and this sets the NF-κB free which moves towards nucleus to produce the inflammatory effects that switch on the genes for inflammation(Lawrence, 2009). IRAK family consists of 4 IRAK members i.e. IRAK-1, IRAK-2, IRAK-4 and IRAK-M. It is the IRAK-1 among these types that is strongly phosphorylated in response to TLR stimulation and is very important for NF-κB signaling (Conze *et al.*, 2008). IRAK-4 is also potently activated by TLR's but it does not activate the NF-κB strongly (Serhan, 2007; Chen *et al.*, 2002). TAK-1 is also another kind of factor that is involved in the activation of both the NF-κB and AP-1 in the nucleus. It is associated with TRAF-6 in the cytoplasm and gets disassociated when TLR stimulation occurs in response to foreign antigen invasion (Vink *et al.*, 2013; Broglie *et al.*, 2010).

The interactive signaling pathways for the extracellular to intracellular or vice versa consist of a coordinated assembly of second messengers, protein kinases and other cellular factors. Mitogen Activated Protein Kinase (MAPK) are one of the major protein phoshorylating cascade that activates multiple stress associated factors and produce an inflammatory response (Pearson *et al.*, 2001; Lewis *et al.*, 1998; Errede *et al.*, 1995). This pathway downstream consists of three different levels of MAPK's. The first level is MAPKinase Kinase Kinase (MAP3K) that consists of MKKK and MEKK, then MAPkinase Kinase (MAP2K) which includes MKK and MEK and finally MAPKinase (MAPK) that consists of ERK, JNK and P-38. Most of the studies are done on the levels of MAP2K and MAPK level. All of these factors in coordinated and sequential manner phosphorylate the proteins downstream and activate the activator protein -1 (AP-1) in the nucleus (Cobb and Goldsmith, 1995; Kyriakis *et al.*, 1994). As can be seen from (fig. 6), SBF has dose dependently phosphorylated the factors of MAPK pathway suggesting that it signal transduces via the MAPK pathway in a potent manner.

In this way we conclude that SBF which is a single compound isolated from the wild mushroom samples exhibit outstanding anti-inflammatory properties and further studies may warrant its use as a functional edible medicine provided the fact that it is obtained from the mushrooms that are reportedly known to be fungal pathogens.

Table 1: Primer sequence for RT-PCR for SBF gene expression

Gene	Primer	Oligonucleotide sequence (5'-3')
GAPDH	F	5'CAATGAATACGGCTACAGCAAC3'
	R	5'AGGGAGATGCTCAGTGTTGG3'
iNOS	F	5'CCCTTCCGAAGTTTCTGGCAGCAGC3'
	R	5'GGCTGTCAGAGCCTCGTGGCTTTGG3'
COX-2	F	5'-TCTCAGCACCCACCCGCTCA-3'
	R	5'-GCCCCGTAGACCCTGCTCGA-3'
IL-1β	F	5'CAGGGTGGGTGTGCCGTCTTTC3'
	R	5'TGCTTCCAAACCTTTGACCTGGGC3'
TNF-α	F	5'TTGACCTCAGCGCTGAGTTG3'
	R	5'CCTGTAGCCCACGTCGTAGC3'
IL-6	F	5'-GTACTCCAGAAGACCAGAGG-3'
	R	5'-TGCTGGTGACAACCACGGCC-3'

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