

Full Length Research Paper

Effects of forsythoside on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages

Jiayi Guan[#], Hong Shen^{*}, Yonghong Zhang[#], Defeng Cui

Animal Science and Technology College, Beijing University of Agriculture, Beijing 102206, China.

Accepted 18 June, 2013

Forsythoside (FS) is an active component of *Forsythia suspense* and exhibit a certain functions, such as, anti-inflammation, anti-bacterial, anti-virus, among others. However, immunomodulation effects of FS at cell level have been unclear. This work is to investigate the effects of FS on RAW264.7 cells stimulated with lipopolysaccharide (LPS) by detecting the release of cytokines and chemokines, phagocytosis and surface molecule expression. The results showed that the absorbance value of cells in FS groups at medium and high concentration was significantly lower compared with LPS group in terms of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. There was a marked increase in the release of tumor necrosis factor (TNF)- α and decrease NO production in the experimental groups. FS and LPS-treated RAW264.7 cells strongly exhibited phagocytosis capacity to CFSE-labeled chicken red blood cells, and the number of positive cells expressing MHC-II molecules showed a significant decrease in FS together with LPS groups by flow cytometry analysis. The experiment demonstrated that FS has no significant cytotoxicity at the low concentration, markedly promoted the release of TNF- α and down-expressions of MHC-II surface molecules, and enhanced the phagocytosis capacity of macrophages, whereas inhibited NO production, which suggested that FS has cell immune regulation effect.

Key words: Forsythoside (FS), function, RAW264.7 cells, lipopolysaccharide (LPS).

INTRODUCTION

Forsythia suspense has been known as “Lianqiao” (Chinese) and this genus is widely distributed in Asia countries, such as China, Korea and Japan, and its extracts are used as a Chinese traditional medicine to treat inflammation, pyrexia, ulcer, gonorrhea and erysipelas (State Pharmacopeia Commission of P. R. China, 2005). A number of chemical constituents from this plant with diverse structures, including phenylethanoid glycosides, forsythoside, lignans and flavonoids have been reported, and the other reported that three new caffeoyl phenylethanoid glycosides and six known compounds were isolated (Tokar et al., 2004; Fu et al., 2009). At present many researchers are of the opinion that Forsythoside is the active component of

pharmacological effects of forsythia suspense and exhibits anti-inflammatory, anti-bacterial, and anti-tumor effects, however, its immunomodulatory role and the mechanism at cellular level is not clear.

Macrophages are important immune cells which are the first line of host defense against bacterial infection and cancer growth, and play essential roles in the initiation, maintenance and resolution of inflammation (Yan et al., 2011). When stimulated by lipopolysaccharide (LPS), a bacterial endotoxin, macrophages produce a number of inflammation-related enzymes, cytokines and chemokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, prostaglandin E2 and nitric oxide (NO), cyclooxygenase-2 and inducible nitric oxide synthase for

*Corresponding author. E-mail: shenhong912@sina.com; Tel.: (86) 10-80799469.

[#]These authors contributed equally to this work.

the primary protection of the host (Hu et al., 2008; Tao et al., 2009). These cytokines and chemokines are essential for the inflammatory response to pathogenic germs or toxicants, as well as the elimination of tumor cells (Liew, 2003). However, overproduction of these inflammatory mediators is often associated with numerous diseases, such as rheumatoid arthritis and atherosclerosis (Isomaki, 1997; Libby, 2000). Thus, inhibition of the overproduction of inflammation-related mediators may play a beneficial role in inflammatory diseases. RAW264.7 cells derived from pristane-elicited murine peritoneal macrophages transformed with Abelson leukemia virus and the physiological studies of macrophages have been notably advanced by the availability of cell lines (Raschke, 1978). RAW264.7 cells have been particularly valuable because of their ease of culture, rapid growth rate, and the phenotypic resemblance to primary macrophages (Rouzer et al., 2005).

In the present study, we aimed to investigate the mechanisms underlying the immunoregulatory effects of FS on LPS-stimulated RAW264.7 cells by evaluating the level of NO and TNF- α , the phagocytosis and surface molecule expression.

MATERIALS AND METHODS

Reagents

Forsythoside (FS) was purchased from China institute of Veterinary Drug Control (Beijing, China). RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone Laboratories, Inc. (Logan, Utah, USA). Thiazolyl blue tetrazolium bromide (MTT) and LPS (purified lyophilized powder) from *Escherichia coli* O111:B4 were purchased from Sigma-Aldrich (St. Louis, USA). Rat monoclonal [NIMR-4] to MHC Class II (FITC) and TNF- α mouse ELISA Kit were purchased from Abcam (Cambridge, UK). CFSE was obtained from eBioscience (San Diego, C.A.).

Macrophage culture

RAW264.7 cells were cultured in 75-cm² plastic flasks and DMEM medium containing heat-inactivated 10% FBS and antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin) at 5% CO₂ and a 37° humidified atmosphere. Adherent cells were detached by scraping and then cultured in either 96-well plates (2 \times 10⁵ cells/well) or six-well plates (2 \times 10⁶ cells/well) for experiments at 85% confluence.

Assessment of cell viability

RAW264.7 cells were seeded into 96-well plates at a density of 1 \times 10⁴ cells/well and incubated in the presence of LPS (1 μ g/ml final concentration) and different concentrations of FS. After incubation for 24 h, 100 μ l of MTT (0.5 mg/ml final concentration) was added and incubation was continued for another 4 h. Mitochondrial succinate dehydrogenase in live cells converted MTT into visible formazan crystal during incubation. The formazan crystals were then solubilized in dimethylsulphoxide and the absorbance was measured at 570 nm by using an enzyme linked immuno sorbent

assay (ELISA) microplate reader. Relative cell viability was calculated and compared with the absorbance of the untreated control group.

Assay of cellular NO production

RAW264.7 cells were cultured in 96-well plates using DMEM medium containing an 1 μ g/ml final concentration of LPS for 24 h and then incubated for another 24 h by adding different concentrations of FS in the media. After incubation, the supernatant medium was collected and 100 μ l of cellular supernatant media containing NO²⁻ (stable oxidation product of NO) was mixed with the same volume of Griess reagent for the incubation of 15 min. The absorbance of the mixture at 550 nm was measured with an ELISA microplate reader. The values obtained were compared with those of standard concentrations of sodium nitrite dissolved in DMEM medium, and the concentrations of nitrite in the supernatant media of treated-cell groups were calculated.

Detection of tumor necrosis factor (TNF)- α release

The production of TNF- α in RAW264.7 cells was detected using ELISA kits following the manufacturer's instructions. RAW264.7 cells were stimulated by a 1 μ g/ml final concentration of LPS for 24 h incubation and then treated with different concentrations of FS for another 24 h. After incubation, the supernatant medium was collected and used for the detection of TNF- α . 50 μ l of TNF- α standards (prepared for calibration), or the same volume of each group medium, was added to the wells of TNF- α antibody-coated 96-well plates in triplicate. Biotinylated antibody reagent (50 μ l) was added and incubated for 3 h at room temperature. The reaction mixture was aspirated and washed using PBS. Streptavidin-horseradish peroxidase conjugate (100 μ l) was added and incubated for 30 min at room temperature. After a thorough washing, 100 μ l of TMB substrate solution was added and incubated for 30 min at room temperature, and the reaction was stopped by the addition of 50 μ l of stop solution and the absorbance was determined at 450 nm using the microplate reader. A TNF- α standard curve was used to quantify the amount of TNF- α released by RAW264.7 cells.

Analysis of phagocytic activity

A single-cell suspension of chicken red blood cells (cRBCs) was obtained freshly and washed three times with PBS. 1 \times 10⁷ cells/ml cRBCs in PBS were labeled with 5.0 μ M CFSE for 15 min at 37°C. These cells were then washed thoroughly with in-complete medium and re-suspended in the complete medium. RAW264.7 cells were adjusted to 2 \times 10⁵ cells /well and cultured overnight in 24-well culture plate. Then 100 μ l of 2 \times 10⁹/ml CFSE-labeled cRBCs were added in the 24-well culture plate containing RAW264.7 cells and incubated for 4 h at 37°C. Non-phagocytic cells were removed by rinsing with PBS, and phagocytic cells were collected by repeated pipetting, and then flow cytometric analysis was performed on a FACS Caliber.

Evaluation of MHC-II molecule expression

RAW264.7 cells were stimulated by a 1 μ g/ml final concentration of LPS for 24 h incubation and then treated with different concentrations of FS for another 24 h. Cells were collected and 5 \times 10⁵ harvested macrophages were washed once with FACS buffer. For one-color staining, cells were stained with fluorescein isothiocyanate (FITC)-labeled mouse MHC-II antibody. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least ten thousand cells were assayed using a FASCalibur flow cytometry, and data

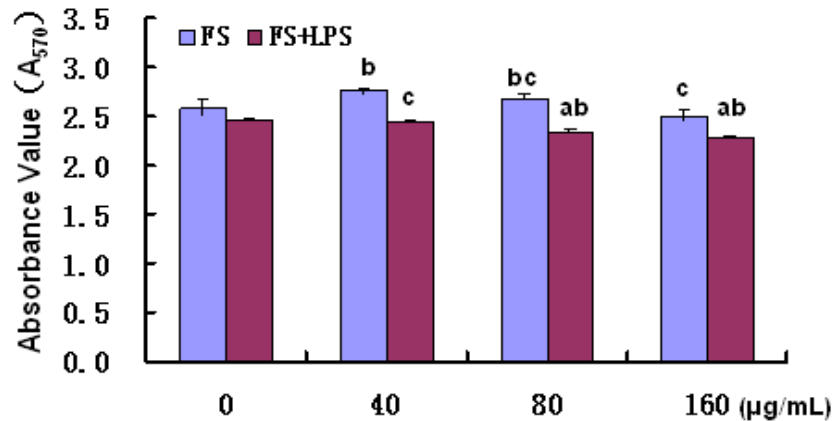


Figure 1. Effects of FS on the viability of RAW264.7 cells at different concentration. a ($p < 0.01$) vs FS (0 µg/ml), b ($p < 0.01$) vs LPS (0 µg/ml), c ($p < 0.01$) means compared between FS and FS+LPS at same concentration.

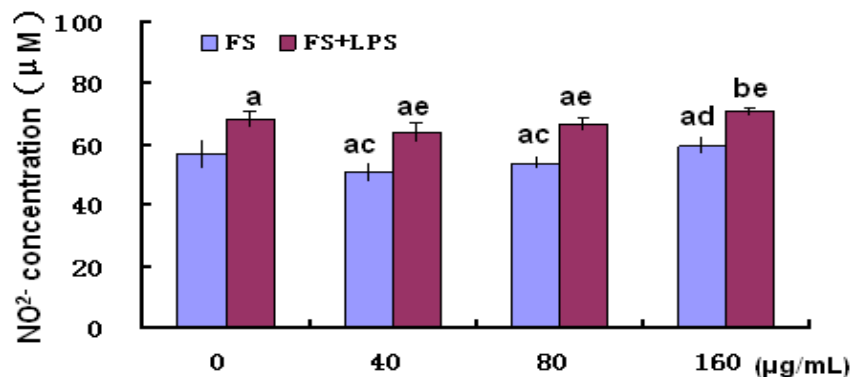


Figure 2. Effects of FS on NO production of RAW264.7 cells treated with FS and LPS. a vs 0 ($p < 0.05$), b vs 0 ($p < 0.01$), c vs LPS ($p < 0.01$), d vs LPS ($p < 0.05$), e vs 40, 80, 160 ($p < 0.01$) at same concentration respectively.

were analyzed with CellQuest software. Dead cells were excluded using the vital nucleic acid stain propidium iodide (PI). The percentage of cells stained with a particular reagent was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAb.

Statistical analysis

All data were presented as the mean \pm SD. Individual values were compared by Dunnett's test and a p value less than 0.05 was considered to be statistically significant.

RESULTS

Cell viability of RAW264.7 cells treated with FS

Cell viability was detected by MTT assay and the results are shown in Figure 1. Compared with LPS group, there was significant decrease in cell absorbance value in

FS+LPS groups at the 80 and 160 µg/ml of FS, respectively, however there was not marked effect on cell viability in LPS+40 µg/ml FS. The results indicated FS was helpful to cells survival and relieve effects of LPS on RAW264.7 cells at the low concentration.

NO production and TNF- α release from RAW264.7 cells

There was a significant enhancement of the nitrite concentration in RAW264.7 cells stimulated with LPS in conditioned medium compared with that in LPS non-stimulated cells (Figure 2), which indicated that a stable NO₂⁻ product was released into the culture medium and there was an increased NO production. RAW264.7 cells treated with FS showed a reduction in the production of NO following those stimulated with LPS at the low and medium concentration of FS, however, the level of NO

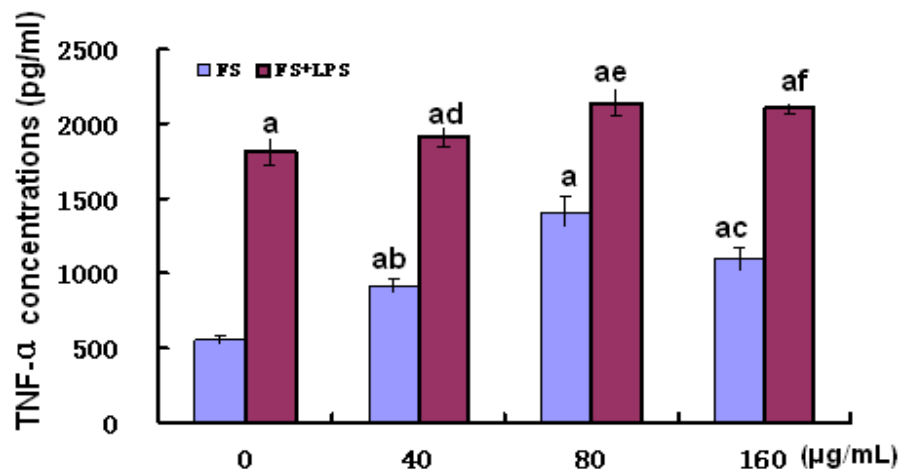


Figure 3. Effects of FS on TNF- α secretion from RAW264.7 cells. a vs 0 ($p < 0.01$), b vs LPS ($p < 0.01$), c vs LPS ($p < 0.05$), d vs 40 ($p < 0.01$), e vs 80 ($p < 0.01$), f vs 160 ($p < 0.01$).

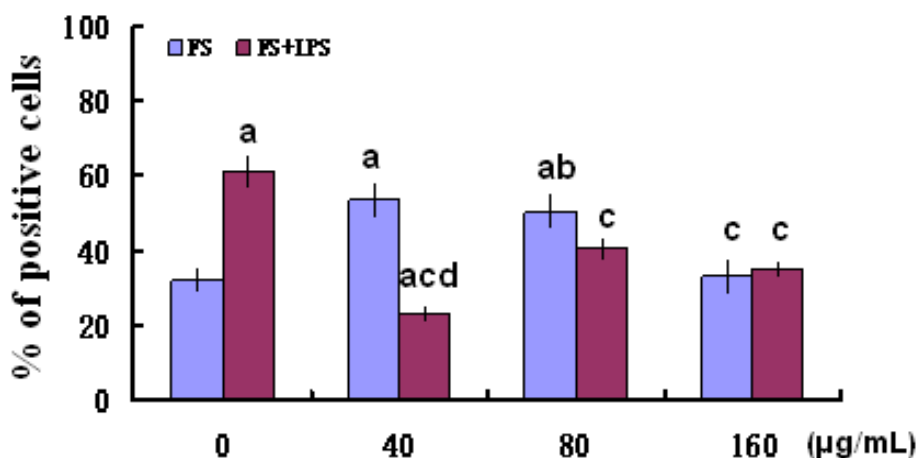


Figure 4. Cell surface expression of MHC-II molecules in RAW264.7 cells treated with FS. a vs 0 ($p < 0.05$), b vs LPS ($p < 0.05$), c vs LPS ($p < 0.01$), d vs 40 ($p < 0.01$).

production was a little more at high dose of FS. The results suggested that FS had a degree inhibitory effect on NO production from the macrophages. The amount of TNF- α released from LPS-treated RAW264.7 cells together with FS was assessed using anti-TNF- α coated ELISA plates to determine the effects of FS. There was a significant increase in TNF- α release of FS and LPS-stimulated cells compared with that of LPS non-treated RAW264.7 cells (Figure 3), which showed FS had the potential to promote the release of TNF- α from LPS-induced RAW264.7 cells.

Phagocytic ability and MHC-II surface molecule expression in RAW264.7 cells

To determine the expression level of MHC-II surface

molecule in cells treated with FS and LPS, the positive cells were detected using FCM (Figure 4). There was a marked decrease in positive cells treated with LPS together with FS at different concentration compared with the LPS-stimulated cell group, which showed down-expression of MHC-II surface molecules. The results indicated that FS had the potential to inhibit the expression of MHC-II surface molecules. Phagocytic capacity is a very important function for macrophages. In order to analyze phagocytic activity of macrophages treated with FS, RAW264.7 cells stimulated with LPS were cultured in the presence of different concentration of FS. cRBCs were collected and labeled with CFSE. Treated RAW264.7 cells were co-cultured for 2-4 h with CFSE-labeled cRBCs, and phagocytosis was evaluated in term of the uptake of macrophages to CFSE-labeled cRBC using FCM. There was a significant increase in

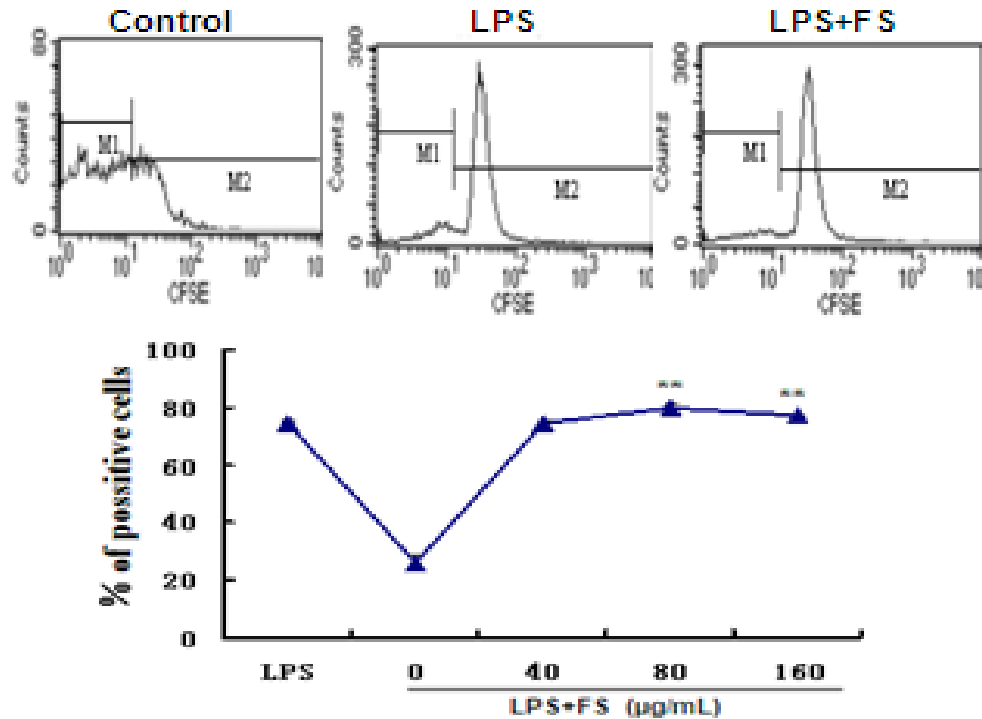


Figure 5. Phagocytic activity of RAW264.7 cells treated with FS to CFSE-labeled cRBCs.

phagocytes of macrophages cultured in the media containing FS at comparative high concentration, which showed FS improved the phagocytotic capacity of macrophages (Figure 5).

DISCUSSION

Macrophages play a crucial role in the inflammatory and immune responses and are major sources of inflammatory mediators, including prostaglandins (PGs), NO and TNF- α . RAW264.7 mouse macrophage cell line has long been used as an *in vitro* model to study inflammatory molecules synthetic response to a wide variety of stimuli (Shin, 2008; Jeong, 2009). It is known that macrophages produce multiple inflammatory molecules such as NO, TNF- α , IL-1, IL-12, interferon- γ and chemokines in response to the stimulation of some lectins (Kesharwani et al., 2007), such as phytohemagglutinin to stimulate RAW264.7 cells could induce the production of NO (Daekyung et al., 2011). Nitric oxide is a multifunctional biomolecule involved in a variety of physiological and pathological processes, such as vascular regulation, host immune defense, neurotransmission and other systems (Paige, 2007). Vascular dysfunctions are associated with the impaired production of NO, whereas bacterial septic shock, certain inflammatory and autoimmune are associated with NO overproduction (Moncada et al., 1992). NO plays a bene-

ficial role in anti-tumor and anti-virus replication, and in anti-inflammatory processes and the suppression of NO overproduction has become a new therapeutic strategy for the treatment of inflammatory-related diseases (Pacher, 2007). In our study, we found that the exposure of RAW264.7 cells to LPS caused a significant increase in NO production, and FS reduced to a degree NO production in LPS-stimulated RAW264.7 cells, which suggested that FS had the potential of anti-inflammatory action. This observation is consistent with the findings previously reported by other researchers (Hu et al., 2008; Lin et al., 2008).

Cytokines play important roles in the regulation of inflammation. TNF- α , IL-1 β and IL-6 are multifunctional pro-inflammatory cytokines and exhibit various pro-inflammatory effects in certain inflammatory diseases, such as rheumatoid arthritis and atherosclerosis (Dayer, 2004). It has been reported that TNF- α elicits downstream pro-inflammatory events, such as the release of IL-6, certain inflammatory cytokines (Straub et al., 2000). TNF- α has long been considered as a key molecule for the induction of apoptosis and the development of the humoral immune response. In this experiment, RAW264.7 cells treated with LPS and FS showed a significant increase in TNF- α release from RAW264.7 cells, which indicated that FS is capable to promote LPS-induced production of TNF- α .

Major histocompatibility (MHC) is a large cluster of genes, which encode antigen presenting molecules and

therefore play a key role in molecular self/non-self discrimination and in the activation of adaptive immune response (Janeway et al., 2005). Its identification is considered as a major breakthrough in the identification as a key component of the adaptive immune system to respond to pathogen infection (Christophe et al., 2011). MHC-II molecules are cell surface glycoproteins that bind self and antigenic peptides and present them to T cells to initiate an immune response (Germain et al., 1993). One of the most important functions for macrophages is to present peptides derived from exogenous antigens to surrounding T cells through their cell-surface interactions and the expression of co-stimulatory molecules in antigen presenting cells (APCs), which is crucial in determining the nature and extent of the immune responses (Ma et al., 2008). Phagocytosis of macrophages is a part of innate immunity for protection against foreign pathogens, microorganism or dead cells (Gordon et al., 2005). Previous reports showed that alternatively activated macrophages have an enhanced phagocytosis ability and a lower antigen presenting ability (Bouhleb et al., 2007). In our experiment, we found that positive cells expressing MHC-II molecules in RAW264.7 cells treated with FS and LPS was significantly lower, and cells stimulated with LPS and FS showed significantly higher phagocytosis capacity against cRBCs compared with the control, which might be related to the activation stages of macrophages (Liu et al., 2007). These results were consistent with the concept in alternative activated macrophages the antigen presenting ability is reduced (Weigert et al., 2007).

In conclusion, the observed immunoregulatory effects of FS may be mediated partly by the promotion of TNF- α secretion and phagocytosis ability, the inhibition of NO production, down-regulation of MHC-II expression, which suggest that FS, an essential active constituent of forsythia suspense, may be a potential chemical agent for the disease treatment.

ACKNOWLEDGEMENTS

This work was supported by a grant from Beijing Municipal Education Commission (PXM2013_014207_000067 and PXM2012_014207_000013). And the Funding Project for Academic Human Resources Development in Institutions of Higher Learning under the Jurisdiction of Beijing Municipality (PHR201107134).

ABBREVIATIONS

LPS, Lipopolysaccharide; **FS**, forsythoside; **DMEM**, Dulbecco's modified Eagle's medium; **FBS**, fetal bovine serum; **ELISA**, enzyme linked immuno sorbent assay; **TNF**, tumor necrosis factor; **cRBCs**, chicken red blood cells; **FITC**, fluorescein isothiocyanate; **PI**, propidium iodide; **PGs**, prostaglandins; **MHC**, major histocompatibility; **APCs**, antigen presenting cells.

REFERENCES

- Bouhleb MA, Derudas B, Rigamonti E, et al (2007). PPAR γ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab.* 6(2):137-143.
- Christophe Eizaguirre, Tobias L. Lenz, Ralf D, Sommerfeld, Chris H, Martin K, Manfred M (2011). Parasite diversity, patterns of MHC II variation and olfactory based mate choice in diverging three-spined stickleback ecotypes, *Evo.l Ecol.* 25:605-622.
- Daekyung Kim, Yasuhiro Yamasaki, Zedong Jiang, et al (2011). Comparative study on modeccin- and phytohemagglutinin (PHA)-induced secretion of cytokines and nitric oxide (NO) in RAW264.7 cells. *Acta Biochim. Biophys. Sin.* 43(1): 52-60.
- Dayer JM (2004). The process of identifying and understanding cytokines: from basic studies to treating rheumatic diseases. *Best Pract Res. Clin. Rheumatol.* 18: 31-45.
- Fu Nan Wang, Zhi Qiang Ma, Ying Liu, et al (2009). New Phenylethanoid Glycosides from the Fruits of *Forsythia Suspense* (Thunb.) Vahl. *Molecules.* 14:1324-1331.
- Germain R N, Margulies D H (1993). The biochemistry and cell biology of antigen processing and presentation. *Ann.Rev.Immunol.* 11(1): 403-450.
- Gordon S, Taylor PR (2005). Monocyte and macrophage heterogeneity. *Nature Rev.* 5:953-964.
- Hu XD, Yang Y, Zhong XG, et al (2008). Anti-inflammatory effects of Z23 on LPS-induced inflammatory responses in RAW264.7 macrophages. *J. Ethnopharmacol.* 120: 447-451.
- Isomaki P, Punnonen J (1997). Pro and anti-inflammatory cytokines in rheumatoid arthritis. *Ann. Med.* 29: 499-507.
- Janeway CA, Travers P, Walport M, Sclomchik MJ (2005). *Immunobiology: the immune system in health and disease.* Garland Sci. Publishing, New York.
- Jeong GS, Lee DS, Kim YC (2009). Cudraticus xanthone A from *Cudrania tricuspidata* suppresses pro-inflammatory mediators through expression of anti-inflammatory hemeoxygenase-1 in RAW264.7 macrophages. *Int. Immunopharmacol.* 9: 241-246.
- Kesharwani V, Sodhi A (2007). Differential activation of macrophages in vitro by lectin Concanavalin A, Phytohemagglutinin and Wheat germ agglutinin: production and regulation of nitric oxide. *Nitric Oxide* 16: 294-305.
- Libby P, Aikawa M, Schönbeck U (2000). Cholesterol and atherosclerosis. *Biochim. Biophys. Acta.* 1529: 299-309.
- Liew FY (2003). The role of innate cytokines in inflammatory response. *Immunol Lett.* 85: 131-134.
- Lin QY, Jin LJ, Cao ZH, Xu YP (2008). Inhibition of inducible nitric oxide synthase by *Acanthopanax senticosus* extract in RAW264.7 macrophages. *J. Ethnopharmacol.* 118: 231-236.
- Liu G, Ma H, Jiang L, et al (2007). The immunity of splenic and peritoneal F4/80(+) resident macrophages in mouse mixed allogeneic chimeras. *J. Mol. Med.* 85(10):1125-35.
- Ma H, Liu G, Ding W, Wu Y, Cai L, Zhao Y (2008). Diabetes-induced alteration of F4/80(+) macrophages: a study in mice with streptozotocin-induced diabetes for a long term. *J. Mol. Med.* 86(4):391-400.
- Moncada S, Palmer RMJ, Higgs DA (1992). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 43:109-142.
- Pacher P, Joseph S, Beckman JS, Liaudet L (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* 87: 315-424.
- Paige JS, Jaffrey SR (2007). Pharmacologic manipulation of nitric oxide signaling: targeting NOS dimerization and protein-protein interactions. *Curr Top Med Chem.* 1:97-114.
- Raschke WC, Baird S, Ralph P, Nakoinz I (1978). Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell.* 15: 261-267.
- Rouzer Carol A, Jacobs Aaron T, Nirodi Chetan S, et al (2005). RAW264.7 cells lack prostaglandin-dependent autoregulation of tumor necrosis factor- α Secretion. *J. Lipid Res.* 46:1027-1037.
- Shin EM, Zhou HY, Guo LY, Kim JA, et al (2008). Anti-inflammatory effects of glycyrol isolated from *Glycyrrhiza uralensis* in LPS-stimulated RAW264.7 macrophages. *Int. Immunopharmacol.* 1524-1532.
- State Pharmacopeia Commission of P. R. China (2005). *Pharmacopeia*

- of the P. R. China; People's Health Publishing House: Beijing, 1: p117.
- Straub RH, Linde DN, Mannel J, Scholmerich W, Falk A (2000). Abacteria-induced switch of sympathetic effect or mechanisms augments local inhibition of TNF- α and IL-6 secretion in the spleen. *FASEB J.* 14: 1380-1388.
- Tao JY, Zheng GH, Zhao, Wu JG, et al (2009). Anti-inflammatory effects of ethyl acetate fraction from *Melilotus suaveolens* Ledeb on LPS-stimulated RAW264.7 cells. *J Ethnopharmacol.* 123: 97-105.
- Tokar M, Klimek B (2004). Isolation and identification of biologically active compounds from *Forsythia viridissima* flowers. *Acta Pol. Pharm.* 61:191-197.
- Weigert A, Tzieply N, Von Knethen A, et al (2007). Tumor cell apoptosis polarizes macrophages role of sphingosine-1-phosphate. *Mol. Biol Cell.* 18(10): 3810-3819.
- Yan-Fang Xian, Yu-Cui Li, Siu-PO IP, et al (2011). Anti-inflammatory effect of patchouli alcohol isolated from *Pogostemonis Herba* in LPS-stimulated RAW264.7 macrophages. *Exp. Therap. Med.* 2: 545-550.