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Anti-inflammatory effects of a methanol extract from *Pulsatilla koreana* in lipopolysaccharide-exposed rats

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To investigate the therapeutic effect of a Korean herbal medicine *Pulsatilla koreana* as an anti-septic agent, anti-inflammatory effects of the herbal medicine were determined in lipopolysaccharide (LPS)-exposed rats. Treatment with a methanol extract from *Pulsatilla koreana* significantly inhibited LPS-induced inflammatory responses. Results from ELISA analysis showed that *Pulsatilla koreana* decreased the plasma and hepatic levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α while increased the level of anti-inflammatory cytokine IL-10 in LPS-exposed rats. *Pulsatilla koreana* also decreased the plasma levels of other inflammatory mediators such as NO_3^-/NO_2^-, ICAM-1, PGE_2, and CINC-1 in LPS-exposed rats. Although no significant effects were observed in the phagocytic activities, the distribution of lymphocyte population was significantly shifted by the treatment with *Pulsatilla koreana*. All together, *Pulsatilla koreana* exerts anti-inflammatory activities in the immune-challenged animals implicating that this Korean herbal medicine is therapeutically useful for the treatment of inflammatory diseases like sepsis. [BMB Reports 2012; 45(6): 371-376]

INTRODUCTION

Sepsis is a common clinical problem and cause of death, with extremely high mortality rates in patients undergoing major surgery (1). In its inflammatory state, sepsis stimulates the production of various endogenous inflammatory mediators and profoundly alters immune responses and organ functions. Septic shock causes the appearance of series of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF-α), IL-8 or IL-6. Septic shock is also associated with the appearance of anti-inflammatory cytokines such as the IL-1 receptor antagonists (RA) and IL-10 (2, 3). Successful treatment of sepsis in humans continues to be a great clinical challenge (2), and glucocorticosteroids remain the most effective therapy for inflammatory disorders. Glucocorticosteroids inhibit many functions of activated macrophages, including secretion of cytokines such as IL-1β and TNF-α from cells. Despite the rapid and proven efficacy of topical glucocorticosteroids, the side effects limit their clinical usefulness (4). Thus, development of anti-inflammatory agents with less adverse effects and better curative effects is needed. Various agents are being investigated for the treatment of inflammatory diseases like sepsis. Among them, traditional oriental medicines have been gaining great interest. Several traditional oriental medicines, including *san-huang-xie-xin-tang* (5), *Clematis mandshurica* Rupr (6), *Quercus infectoria* (7), have been investigated for their anti-inflammatory effects. No satisfactory results, however, have been reported so far.

Pulsatilla koreana is a perennial herb of the group *Ranunculaceae* and has been used in oriental herbal medicines for its anti-inflammatory effects. The major components of the methanol extract of *Pulsatilla koreana* are triterpenoid compounds, such as hederagenin, saponins, ursolic acid, monodesmoside (pulsatilla saponin A, B, D), and bisdesmoside (pulsatilla saponin F, H) (8). Those compounds are proposed to be responsible for the major biological functions of *Pulsatilla koreana* such as lowering blood glucose, cholesterol level and lowering blood pressure (9, 10). However, no study has been done to elucidate the plausible mechanism of anti-inflammatory actions of *Pulsatilla koreana*. In this study, we evaluated anti-inflammatory effects of *Pulsatilla koreana* extract by measuring the level of various inflammatory mediators after oral administration of *Pulsatilla koreana* extract at varying doses in LPS-exposed rats.

RESULTS

Cytokines

The levels of plasma cytokines were measured before and after LPS injection and data are shown in Fig. 1. The levels of plasma IL-1β increased exponentially by two orders over 5 hrs upon LPS injection. The treatment with *Pulsatilla koreana* extract, however, attenuated the magnitude of the increase in plasma IL-1β levels in a dose-dependent manner, allowing the dose of 200 and

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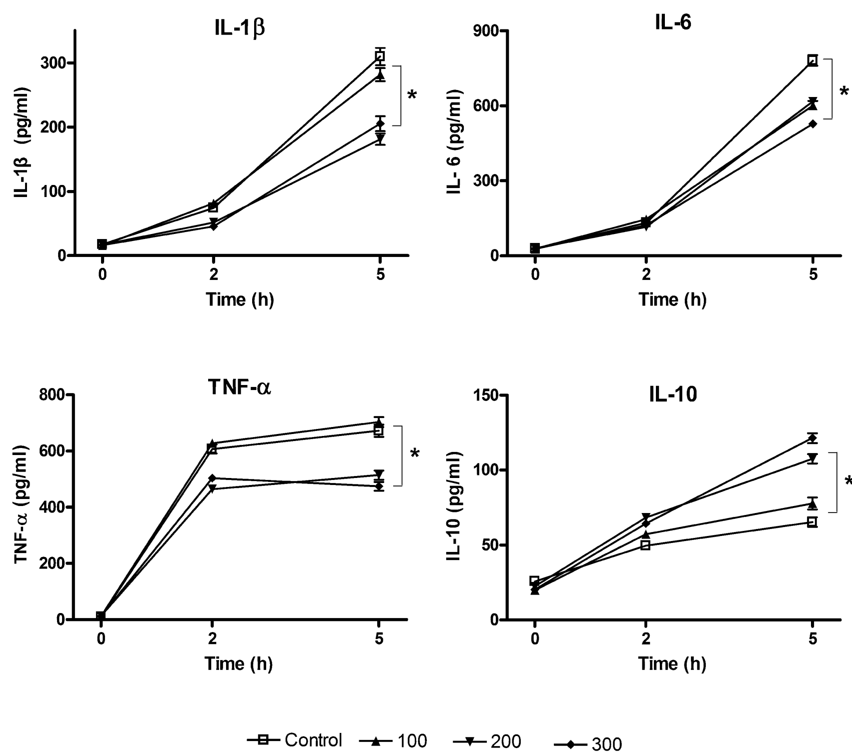

Fig. 1. Effects of a methanol extract from *Pulsatilla koreana* on plasma cytokine levels-time profiles in lipopolysaccharide-exposed rats. The levels of plasma cytokines were measured before and after LPS injection in rats pre-treated with varying doses of *Pulsatilla koreana* extract (*$P < 0.05$).

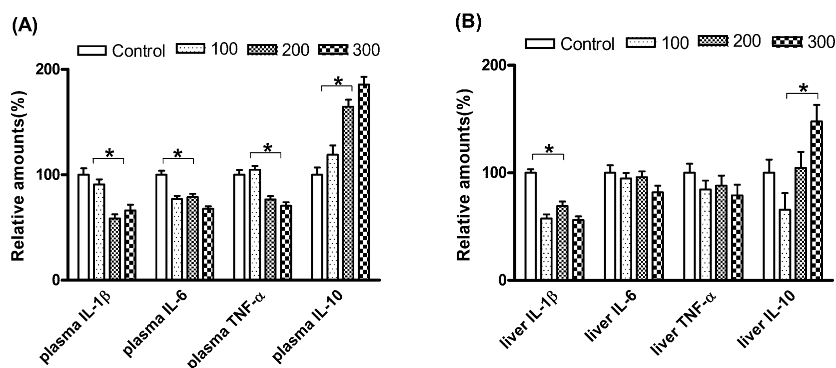

Fig. 2. Effect of a methanol extract from *Pulsatilla koreana* extract on (A) plasma cytokine and (B) liver cytokine levels in lipopolysaccharide-exposed rats. The levels of plasma cytokines and liver cytokines were measured 5 hours after LPS injection in rats pre-treated with varying doses of *Pulsatilla koreana* extract (*$P < 0.05$).

300 mg/kg to decrease the plasma IL-1β level down to almost 50% of that in the control group. The levels of plasma IL-6 also showed an exponential increase by almost three orders over 5 hrs upon LPS injection. The treatment with *Pulsatilla koreana* extract, however, attenuated the magnitude of the increase in plasma IL-6 levels in a dose-dependent manner, allowing the dose of 300 mg/kg to decrease the plasma IL-6 level down to 65% of that in the control group. The level of plasma TNF-α showed a sharp increase by two orders over 2 hrs upon LPS challenge, and remained constant. The treatment with *Pulsatilla koreana* extract, however, attenuated the magnitude of the increase in plasma TNF-α level, allowing the dose of 200 and 300 mg/kg to decrease the plasma TNF-α level down to almost 70% of that in the control group. The levels of plasma IL-10 showed a linear increase by two times over 5 hrs upon LPS injection. The treatment with *Pulsatilla koreana* extract, however, augmented the magnitude of the increase in plasma IL-10 levels in a dose-dependent manner, allowing the dose of 200 and 300 mg/kg to increase the plasma IL-10 level up to almost 200% of that in the control group.

The levels of plasma cytokines were measured 5 hours after LPS injection and data are shown in Fig. 2A. The levels of plasma IL-1β in the treatment groups were significantly lower than that of the control group, reaching about 50% levels of that in the control group at a dose of 200 mg/kg *Pulsatilla koreana* extract. The levels of plasma IL-6 in the treatment groups were significantly lower than that of the control group, reaching about

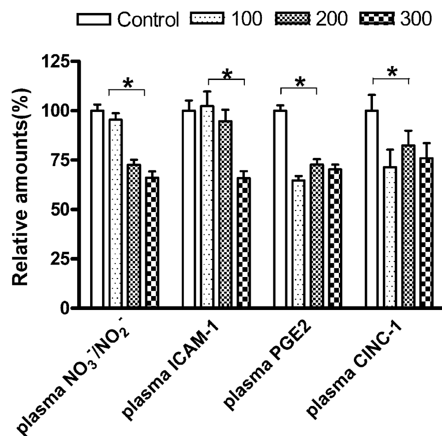

Fig. 3. Effects of a methanol extract from *Pulsatilla koreana* extract on the plasma levels of inflammatory mediators in lipopolysaccharide-exposed rats. The plasma levels of inflammatory mediators were measured 5 hours after LPS injection in rats pre-treated with varying doses of *Pulsatilla koreana* extract (*P $<$ 0.05).

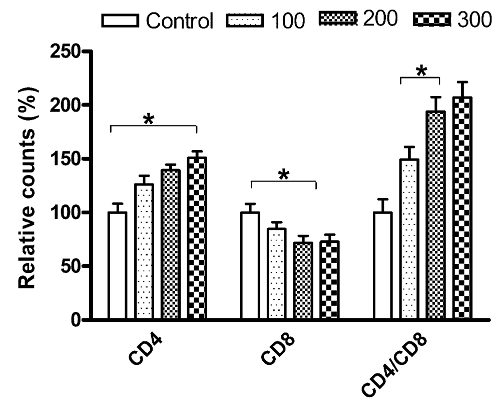

Fig. 4. Effect of a methanol extract from *Pulsatilla koreana* extract on the distribution of blood lymphocytes in lipopolysaccharide- exposed rats. The proportions of CD4-positive and CD8-positive lymphocytes in whole blood samples were measured 5 hours after LPS injection in rats pre-treated with varying doses of *Pulsatilla koreana* extract (*P $<$ 0.05).

70% levels of that in the control group at a dose of 100 mg/kg *Pulsatilla koreana* extract. The levels of plasma TNF-α in the treatment groups were significantly lower than that of the control group, reaching about 70% of that in the control group at a dose of 200 mg/kg *Pulsatilla koreana* extract. No decreases were observed with further increase of doses. The levels of plasma IL-10 in the treatment groups were significantly higher than that of the control group, reaching about 200% of that in the control group at a dose of 200 mg/kg *Pulsatilla koreana* extract.

The levels of liver cytokines were measured 5 hours after LPS injection and data are shown in Fig. 2B. The levels of liver IL-1β in the treatment groups were significantly lower than that of the control group, reaching about 50% of that in the control group at a dose of 100 mg/kg *Pulsatilla koreana* extract. No further lowering was observed with further increase of doses. However, no significant differences in the levels of liver IL-6, TNF-α and IL-10 were observed between the control and treated groups.

NO_3^-/NO_2^-, ICAM-1, CINC-1, and PGE_2

The treatment with *Pulsatilla koreana* extract lowered the plasma levels of NO_3^-/NO_2^- and ICAM-1 in a dose-dependent manner. The levels of NO_3^-/NO_2^- and ICAM-1 were significantly decreased to 65% of that in the control group at the doses of 200 mg/kg and 300 mg/kg, respectively.

The treatment with *Pulsatilla koreana* extract also lowered the plasma levels of CINC-1, and PGE_2. The levels of CINC-1, and PGE_2 were significantly decreased to about 60-70% of that in the control group at the doses of 100 mg/kg and no further decreases were observed with increasing doses (Fig. 3).

Distribution of blood lymphocyte subpopulations

The proportions of CD4-positive and CD8-positive lymphocytes in whole blood samples were measured and data are shown in Fig. 4. The treatment with the *Pulsatilla koreana* extract led to dose-dependent continuous increases in the level of CD4-positive lymphocytes, reaching a significantly different level at the dose of 300 mg/kg. Contrarily, the treatment with the *Pulsatilla koreana* extract led to continuous decreases in the level of CD8-positive lymphocytes, but failed to reach a significantly different level at the doses tested. The ratio of the CD4-positive to CD8-positive lymphocytes showed a dose-dependent increase by the treatment with *Pulsatilla koreana* extract, leading to a significant increase at the dose as low as 100 mg/kg and about a two-time increase at the dose of 300 mg/kg. The treatment with the *Pulsatilla koreana* extract led to continuous increases in the phagocytic acivities as the doses increased. However, no significant difference was observed at the doses tested.

DISCUSSION

In this study, we investigated the anti-inflammatory activity of an methanolic extract of *Pulsatilla koreana* in lipopolysaccharide-exposed rats. The extent of inflammatory responses was monitored by measuring the production levels of various inflammatory mediators, i.e., cytokines (IL-1β, IL-6, TNF-α, IL-10) in plasma and liver, and MCP-1, CINC-1, PGE_2 in plasma and peritoneal lavage fluid (PLF), NO_3^-/NO_2^- and ICAM-1. Effects of *Pulsatilla koreana* on the distribution of blood lymphocyte subpopulation (CD4, CD8) and phagocytosis activity of polymorphonuclear neutrophils (PMN) were also measured. The plasma levels of cytokines were measured at 2 and 5 hours after LPS challenges and the extent of changes from the basal levels were compared to the non-treated control group. The other inflammatory responses were measured at 5 hours after LPS challenges and compared to those of the non-treated control group. The measurement time points of 5 hours (11) and the LPS dose of 5 mg/kg (12) were chosen to obtain maximum levels of cytokine production.

Lipopolysaccharides (LPS) are structural components that make up the outer membranes of Gram-negative bacteria and are associated with tissue injury and fatal outcome in septic shock. Pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF-α), IL-8 or IL-6, and anti-inflammatory cytokines, such as IL-1 receptor antagonist (RA) and IL-10, are produced in response to LPS (13-15). Effective immune-modulatory agents usually inhibit the synthesis or activity of the cytokines stimulated by LPS challenge in animals. The administration of a specific antibody against TNF-α (16) or a receptor antagonist for IL-1 (17) improved survival rates of the subject animals after lethal LPS administration.

TNF-α is released by monocytes and macrophages in response to various stimuli including bacterial LPS (18) and a principal mediator of the deleterious effects of endotoxin (19). TNF-α is also secreted by Kuffer cells upon LPS shock and causes hepatic injury and hepatocyte apoptosis (20). Since over-production of TNF-α is associated with a wide range of pathologic conditions, many recent efforts have been made to find ways to down-regulate its production or inhibit its effects *in vivo* (21). IL-1β and IL-6 as TNF-α are also pro-inflammatory cytokines that have been implicated as mediators of LPS toxicity *in vivo* and *in vitro*. The biological properties of those pro-inflammatory cytokines are remarkably similar to TNF-α and the synergistic effects are evident in several models. In this experiment, we showed that the effects of LPS challenge on the pro-inflammatory cytokine production, i.e., the exponentially elevated plasma levels upon LPS exposure, were significantly attenuated by the treatment with *Pulsatilla koreana* extract, supporting its anti-inflammatory effect.

IL-10 is an anti-inflammatory cytokine (22). It controls the production of the other cytokines such as IL-6 and TNF-α and decreases the T-cell activation *in vitro* and *in vivo* (23, 24). In this study, we showed that the treatment with *Pulsatilla koreana* extract significantly increased the plasma levels of the anti-inflammatory cytokines in a dose-dependent manner in LPS-challenged rats. The same effects were also observed in the hepatic levels of the anti-inflammatory cytokines. These augmenting effects of *Pulsatilla koreana* extract on the anti-inflammatory cytokine production strongly supports its anti-inflammatory effect of this extract.

It has been shown that septic conditions cause a decrease in the number of CD4-positive T lymphocytes and an increase in the number of CD8-positive lymphocytes (25, 26), which together results in a decrease in the ratio of CD4-positive/ CD8-positive lymphocytes (27). This shift in the distribution of the lymphocyte population in septic inflammatory conditions was significantly inhibited by the treatment with *Pulsatilla koreana* extract, via dose-dependent increase and decrease in the population of CD4-positive and CD8-positive lymphocytes, respectively. As a result, the ratio of CD4/CD8-positive lymphocytes significantly increased as the dose of *Pulsatilla koreana* extract increased in LPS-exposed rats, strongly supporting the idea that *Pulsatilla koreana* extract exerts anti-inflammatory functions in the inflammatory conditions. However, no significant effects were observed in the phagocytic activities of blood PMNs upon treatment with *Pulsatilla koreana* extract, which is consistent with another study of septic conditions (27).

In this study, we also showed that the treatment with an methanolic extract from *Pulsatilla koreana* significantly decreased the plasma levels of inflammatory mediators such as NO_3^-/NO_2^-, ICAM-1, PGE_2 and CINC-1 in LPS-challenged rats. NO is responsible for vasodilatation, increases in vascular permeability and edema formation at the site of inflammation (28). NO, along with superoxide (O_2^-) and the products of their interaction, also initiate a wide range of toxic oxidative reactions causing tissue injury (29). PGE_2 is implicated in inducing the production of various chemoattractants and pro-inflammatory cytokines (30). Intracellular adhesion molecule-1 (ICAM-1) is a cell surface protein expressed on the vascular endothelium, which is important in the adhesion of leukocytes to activated endothelium (31). The cytokine-induced neutrophil chemoattractant-1 (CINC-1) is a rat homolog for the human interleukin (IL)-8 (32), which is one of the most potent mediators that recruit neutrophils to the site of injury. Together with Il-1β, IL-6, and TNF-α, the decreases in the plasma levels of those inflammatory mediators indicate that the *Pulsatilla koreana* extract is a functional material for its use as an anti-inflammatory agent.

MATERIALS AND METHODS

Materials

Five hundred grams of dry *Pulsatilla koreana* was powdered and then extracted three times with 100% methanol for 5 hours each time in a cooling water-reflux cistern. The methanol extracts were combined and concentrated in a rotary evaporator to yield 110 g of methanol extracts from *Pulsatilla koreana*. All other reagents and chemical were purchased from commercial suppliers.

Animals and treatment

This study was approved by the Animal Care and Use Committee at the Sangji University. Forty Sprague-Dawley male rats of 227.19 ± 6.31 g body weight were housed in temperature and humidity-controlled rooms and allowed free access to basal diet and water *ad lib*. for 1 week before the experiment. Rats were randomly assigned to one of the four groups, i.e., control (normal saline 100 mg/kg), *Pulsatilla koreana* extract 100 mg/kg, *Pulsatilla koreana* extract 200 mg/kg, and *Pulsatilla koreana* extract 300 mg/kg. *Pulsatilla koreana* extract was administered orally using a Jones tube at 5 p.m. every day for four weeks. The control group was given normal saline in the same manner. Dietary and water were *ad lib* provided. After four weeks, a single dose of lipopolysaccharide (LPS, 5 mg/kg per animal) was given to all animals via intraperitoneal injection. The blood samples were taken just before, 2 hr and 5 hr after LPS injection under ether anesthesia by the cardiac puncture method. After the final blood sampling, all animals were subjected to mid-abdominal incision and their livers were harvested.

Measurement of cytokines in plasma and liver
The blood samples were immediately centrifuged (3,000 rpm, 10 min, 4^oC) and plasma were collected, frozen, and kept at -80^oC until use for cytokine analysis. Liver cytokine samples were prepared as follows: 1 g of liver tissues were homogenized on ice using 5 ml of ice-cold phosphate-buffered saline (PBS, pH 7.4) containing a protease inhibitor cocktail (Tablete Complete Roche, Germany). The homogenates were then centrifuged (15,000 rpm, 15 min, 4^oC) and supernatants were filtered through a 0.45 μm filter (Millex-HA, Millipore, France). The filtrates were again centrifuged (15,000 rpm, 15 min, 4^oC) and the supernatants were collected and kept at -80^oC until use for cytokine analysis. The levels of plasma and hepatic cytokines (IL-1β, TNF-α, IL-6 and IL-10) were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Biosource International, USA). The minimum detectable level was 0.7 pg/ml for TNF-α, and 3 to 8 pg/ml for the other cytokines. The levels of plasma cytokines were expressed as picograms per milliliter (pg/ml). The levels of hepatic cytokines in the filtrates were measured and expressed as picograms per milligram (pg/mg).

Measurements of ICAM-1, CINC-1, PGE_2, and NO_2^-/NO_3^- in plasma
The levels of plasma ICAM-1, CINC and prostaglandin E_2 (PGE_2) were measured by enzyme-linked immunosorbent assay (ELISA). In brief, plasma samples were pipetted into the wells pre-coated with a monoclonal antibody specific for rat ICAM-1 (R&D Systems, Minneapolis, MN, USA), allowing ICAM-1 in the plasma samples to bind to the immobilized antibody. After washing out, an enzyme-linked polyclonal antibody specific for ICAM-1 was added to the wells. After washing to remove any of the unbound enzyme-linked antibodies, a substrate solution was added to wells and the intensity of color development was measured. The detection limits for ICAM-1 was <17 pg/ml.

The levels of CINC-1 in plasma were measured in the same manner as described above with a monoclonal antibody specific for rat CINC-1 (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The detection limit was 1.3 pg/ml.

The levels of PGE_2 were measured in the same manner with rat monoclonal antibody specific for PGE_2 and acetylcholinesterase covalently coupled to PGE_2 as the enzymatic tracer (R&D Systems). The sensitivity of the PGE_2 assay was <8.3 pg/ml.

The level of stable nitrite (NO_2^-) and nitrate (NO_3^-) in plasma were measured with a commercial kit (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Determination of lymphocyte subpopulation distribution
The blood samples at 5 hr after LPS injection were analyzed for lymphocyte subpopulations. In brief, the blood samples were incubated with fluorescein-conjugated mouse anti-rat CD8 and phycoerythrin-conjugated mouse anti-rat CD4 antibodies (Serotec, Oxford, United Kingdom) for 15 min at 4^oC. After red blood cells were lysed with lysing buffer (Serotec, Oxford, United Kingdom), fluorescence-activated cell sorting (FACS) analysis was carried out on 5×10^4 viable cells using a flow cytometer (Coulter, Miami, FL, USA). The proportions of CD4-positive cytotoxic T cells and CD8-positive Th cells were determined.

Phagocytosis assay of blood polymorphonuclear neutrophils
The phagocytic activity of blood polymorphonuclear neutrophils (PMNs) was determined by a flow cytometric phagocytosis test (33). In brief, each whole blood sample was heparinized and pipetted onto a Falcon polystyrene tube (Becton Dickinson, Oxford, CA, USA) in an ice- water bath. Pre-cooled opsonized Escherichia coli labeled with fluorescein iso-thiocyanate (FITC) (Molecular probes, Eugene, OR, USA) were added to each tube and incubated for precisely 10 min at 37^oC in a shaking water bath. The tubes were then immediately placed in ice water and 100 μl of pre- cooled Trypan Blue (Sigma, St. Louis, MO, USA) solution (0.25 mg/ml in citrate buffer, pH 4.4) was added to quench the fluorescence of the bacteria adhering to the surface of the phagocytized cells. Cells were washed twice with Hank's buffered saline solution, and erythrocytes were lysed using FACS lysing solution (Becton Dickinson). After an additional wash with Hank's buffered saline solution, 100 μl of a propidium iodide (PI) solution (1 μg/ml) was added to stain the nuclear DNA 10 min before FACS analysis (FACSCalibur, Becton Dickinson). A live gate was set on the red (propidium iodide) fluorescence histogram during acquisition to include only those cells with a DNA content equal to human diploid cells. The number of cells with phagocytic activity did not exceed 6% at 0^oC.

Statistical analysis
Results are expressed as mean $\pm$ S.D. Data were statistically analyzed using one-way ANOVA and Duncan's multiple range tests. $P < 0.05$ was considered to be significant.

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