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Extract from *Coriolus versicolor* fungus partially prevents endotoxin tolerance development by maintaining febrile response and increasing IL-6 generation

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ABSTRACT

Endotoxin tolerance is defined as a reduced endotoxin-induced fever following repeated injections of lipopolysaccharide (LPS). Clinical examples of endotoxin tolerance include sepsis or cystic fibrosis. This state is characterized by inhibition of pro-inflammatory cytokines production and decrease in nuclear factor-kappa B (NF-κB) activation. Extract from *Coriolus versicolor* (CV) fungus is classified as a biological response modifier, which exhibits various biological activities, including immunopotentiating properties. The aim of study was to examine the effect of CV extract injection on body core temperature of Wistar rats during LPS-induced endotoxin tolerance. Body temperature was measured using biotelemetry. CV extract was injected intraperitoneally (100 mg kg^{-1}) 2 h prior to the first LPS peritoneal administration (50 μg/kg). Endotoxin tolerance was induced by three consecutive daily injections of LPS at the same dose. We also investigated the influence of CV extract pre-injection on the properties of peripheral blood mononuclear cells (PBMCs) isolated from LPS-treated rats in response to LPS stimulation *ex vivo*. PBMCs were isolated 2 h after the first LPS injection. After 24 h pre-incubation, the cells were stimulated with LPS (1 μg ml^{-1}) for 4 h. Our results revealed that CV extract partially prevents endotoxin tolerance through maintaining febrile response in rats following consecutive exposure to LPS. This state was accompanied by the ability of PBMCs isolated from rats injected with CV extract and LPS to release larger amounts of interleukin 6 and greater NF-κB activation in response to LPS stimulation *ex vivo* compared with the cells derived from rats injected only with LPS. Data also showed that CV extract augmented mitogenic effect of LPS on PBMCs and caused increase in reactive oxygen species generation. We concluded that CV extract, by a modifying effect on body temperature during endotoxin tolerance, can be consider as the immunostimulating agent, which prevents the non-specific refractoriness described in patients with sepsis or ischemia.

1. Introduction

Endotoxin tolerance (ET) was initially described when it was observed that animals survived a lethal dose of bacterial endotoxin if they had been previously treated with a sublethal injection. In animal models, two phases of endotoxin tolerance are described, an early phase associated with altered cellular activation and a late phase related to the development of specific antibodies against the polysaccharide side chain of Gram-negative organisms (West and Heagy, 2002). Interestingly, successive injections of lipopolysaccharide (LPS) either intravenously (i.v.) or intracerebroventricularly induced pyrogenic tolerance to LPS associated with the inhibition of fever. However, the transmission of the pyrogenic tolerance between brain and peripheral tissues has not been ascertained (Kozak et al., 1990). Moreover, the occurrence of lack of cross tolerance between different pyrogens (Soszynski et al., 1991; Roth et al., 1997) and the fact that tolerance state can be broken resulting in the development of fever, when a higher dose of the same pyrogen are used indicates, that the process should be interpreted as a process of refractoriness and adaptation of the immune receptors and effectors to a given dose of endotoxin, rather than a state of simple hyporesponsiveness to LPS (Soszynski, 2002; Cross, 2002). In support, it has been observed that innate immune cells, such as macrophages and monocytes, when pre-treated *ex vivo* with a low level of LPS, they revealed a transient state of cell unresponsiveness to a subsequent LPS exposure (West and Heagy, 2002). It was characterized by the inhibition of LPS-stimulated pro-inflammatory

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cytokines production, including tumor necrosis factor (TNF) α, interleukin (IL) 6 and IL-1β, inhibition of mitogen-activated protein kinase activation, and decrease in activation of nuclear factor-kappa B (NF-κB) (Biswas and López-Collazo, 2009).

A clinically relevant example of endotoxin tolerance was observed in patients with sepsis. It is also believed that *ex vivo* model of endotoxin tolerance partially reproduce sepsis-induced innate immune alterations and this phenomenon might be associated with the refractory state that was primarily observed during late-stage sepsis (Pena et al., 2014). Sepsis similarly like ET results from a deregulated innate cell immune response subsequent to infection by bacteria (Cavaillon and Adib-Conquy, 2006). Blood leukocytes from septic patients exhibit similar phenotype to endotoxin tolerant cells. For instance, neutrophils from septic patients are refractory to production of inflammatory mediators while they upregulate anti-inflammatory molecules (transforming growth factor β, IL-10) when exposed to secondary TLR stimuli (Vergadi et al., 2018). Similarly, blood monocytes derived from sepsis patients prove to be like endotoxin tolerant cells, because they have a serious reduction of pro-inflammatory cytokine production (e.g. IL-1α, IL-1β, TNF-α and IL-6) when re-stimulated *in vitro*, in comparison to healthy volunteer cells (Draisma et al., 2009; Muñoz et al., 1991). Moreover, peripheral blood mononuclear cells (PBMCs) of patients with sepsis show patterns of nuclear factor-kappa B (NF-κB) expression that resemble those reported during LPS tolerance, including low expression of both active (p65p50) and inactive (p50p50) forms of NF-kB after LPS stimulation. Importantly, the PBMCs of these patients were not able to perform NF-kB translocation upon LPS activation, similar to endotoxin-tolerized cells (Adib-Conquy et al., 2000). Downregulation of major histocompatibility (MHC) class II, CD86, and class II transactivator (CIITA) has also been observed in circulating cells from patients with sepsis and in endotoxin tolerance, which in both cases, is associated with impaired antigen presentation capacity (López-Collazo and del Fresno, 2013). Microarray studies of human PBMCs showed also that the expression profile of several dozen pro-inflammatory genes of sepsis patients is strongly associated with the signature for endotoxin tolerance. Furthermore, in early stage of sepsis, the risk for the development of a confirmed sepsis or organ dysfunction was predicted by this endotoxin tolerance signature. Finally, endotoxin tolerance-induced refractoriness observed in sepsis patients leads to pathologic alteration and mortality. In consequence, sepsis continues to be the major infection-related cause of death globally (Pena et al., 2014).

Mushrooms have an established history of human use for thousands of years in traditional oriental therapies. Moreover, modern clinical practice in Japan, China or Korea continues to depend on mushroom-derived preparation. *Coriolus versicolor* fungus (CV; also known as *Trametes versicolor)*, among others, exhibits the anti-tumor, anti-inflammatory, anti-oxidant, anti-viral, anti-bacterial, and immunomodulatory properties (Barros et al., 2016). CV extract stimulates both humoral and cell-mediated immune responses. It directly affects the release of many pleiotropic cytokines with predominantly a pro-inflammatory profile, such as IL-1β, IL-6, IL-12, TNF-α, interferon (IFN) γ and transforming growth factor (TGF) β (Saleh et al., 2017; Ho et al., 2004). Moreover, the CV extract-stimulated immune cells produce colony stimulating factors, including granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) (Li et al., 2010; Liu et al., 1996). Polysaccharopeptides isolated from CV fungus have also a wide range of stimulatory effects on many immune cell types, enhancing their proliferation. The *in vitro* studies show that CV extract augments proliferative response of splenocytes, human and rodent PBMCs, lymphocytes T and B or monocytes (Sekhon et al., 2013; Jędrzejewski et al., 2016; Lee et al., 2010). Similar proliferation and activation responses were observed in various animal studies. After administration of CV extract to animals, isolated peritoneal macrophages as well as lymphocytes demonstrate proliferative mitogenic response *ex vivo* (Wang et al., 1996; Liu et al., 1993). Furthermore, the treatment of mice with CV extract resulted in an increased white blood and neutrophil count (Sze and Chan, 2009). Finally, CV extracts stimulation increases the phagocytic activity of macrophages (Sze and Chan, 2009), enhances NK cells cytotoxic properties and activates lymphocytes B to promote antibody formation and complement system activation (Saleh et al., 2017). Experimental and clinical studies demonstrate also that CV extracts show non-toxic effects and they are clinically well-tolerated (Cheng and Leung, 2008). Taking into consideration all these findings, CV extracts appears to be a factor, which modulates a number of pathophysiological responses.

Our previous studies revealed that CV extract provokes an anapyrexia-like and TNF-α-dependent response in rats (Jedrzejewski et al., 2014), as well as it is able to induce the prolongation of endotoxin fever in IL-6 related manner (Jedrzejewski et al., 2015). Based on these findings, in the present experiments we have decided to examine the *in vivo* and *ex vivo* effect of CV extracts on the endotoxin tolerance induced by LPS in rats, especially since a number of recent reports indicate that clinical examples of endotoxin tolerance include not only sepsis but also diseases, such as cystic fibrosis and acute coronary syndrome (López-Collazo and del Fresno, 2013). Furthermore, in these pathologies mortality rates often remain high, despite modern medical advances, including new antibiotics and vaccines, best practice treatments, and well-equipped intensive care units (Angus et al., 2001).

Our results have revealed that CV extract injection attenuates established endotoxin tolerance through maintaining febrile response in rats, and this phenomenon is associated with immunostimulating effect of CV extract on the PBMCs in *ex vivo* studies. These data are encouraging for the management of refractoriness, which is observed in sepsis and/or non-infectious shock.

2. Materials and methods

2.1. Animals

Male Wistar rats [Strain:WistarCrl:WI(Han)] aged 8–12 weeks and weighting from 250 g to 300 g were purchased from the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw (Poland) and allowed to acclimatize for 10 days before starting the experiments. Rats were housed in individual plastic cages in a room at constant relative humidity (60 ± 5%), temperature (24 ± 1 °C), with a 12:12 h light-dark photoperiod, with lights on at 7:00 a.m. Rodent laboratory food and drinking water were provided *ad libitum*. All procedures were approved by the local Bioethical Committee for Animal Care in Bydgoszcz (Poland, permission no. 17/2013).

2.2. Drugs preparations

Extract from *Coriolus versicolor* (CV extract) fungus (Cov 1 strain, MycoMedica, Czech Republic) was dissolved in a sterile 0.9% sodium chloride (saline) and injected intraperitoneally (i.p.) at a dose of 100 mg kg^{-1}. This dose of CV extract was selected for experiments since we have noticed that it has modulated normal T_b in Wistar rats (Jedrzejewski et al., 2014) and has induced interleukin 6-related extension of endotoxin fever (Jedrzejewski et al., 2015). Lipopolysaccharide (LPS) derived from *Escherichia coli* (0111:B4, Sigma Chemicals, St. Louis, MO, USA) was dissolved in a sterile 0.9% sodium chloride. Before injection, the stock solution of LPS (2.5 mg ml^{-1}) was diluted in a warm sterile saline to the desired concentration.

2.3. Experiments in vivo: Body core temperature

To monitor body core temperature (T_b) the rats, under sterile condition, were implanted intra-abdominally with temperature-sensitive, miniature and battery-operated biotelemeters (PhysioTel®, model TA10TA-F40; Data Sciences International, St. Paul, MN, USA) as we described previously (Wrotek et al., 2011). Described experiments were

started 10 days after surgery.

Pyrogenic tolerance to LPS was induced by three consecutive daily injections of LPS at the same dose (50 μg/kg) as described previously (Piotrowski et al., 2014). All injection solutions were warmed to 37 °C before administration. CV extract was injected at 7:00 a.m., 2 h prior to the first LPS administration (9:00 a.m.). The next two LPS injections were made every 24 h at 9:00 a.m. The control rats were administered i.p. with an equivalent volume of pyrogen-free saline or CV extract solution. The rats were briefly restrained and not anesthetized during the procedures. The animals were weighed before injections to determine the precise doses of LPS and CV extract.

2.4. Experiments in vitro

2.4.1. Rat PBMCs fraction isolation

Blood was collected 2 h after the first LPS injection from anesthetized rats (intramuscularly injection of mixture: 87 mg kg^{-1} ketamine and 13 mg kg^{-1} xylazine) by cardiac puncture into the solution of ethylenediamine tetraacetic acid (MP Biomedicals, San Diego, CA, USA). Peripheral blood mononuclear cells (PBMCs) isolation was performed according to the density gradient centrifugation method, as we described previously (Jędrzejewski et al., 2014) from the four groups of animals (four individuals in each group): the rats treated with CV extract or saline (control vehicle for CV extract) followed by the first LPS injections (CV/LPS and saline/LPS group, respectively), the animals injected with CV extract before the first saline administrations (control vehicle for LPS; CV/saline group) and the control rats injected only with saline (saline/saline group). The whole collected blood was diluted 1:1 (*v/v*) with phosphate buffered saline (PBS, pH 7.4), carefully layered onto the separation medium (Ficoll-Paque Plus, Amersham Biosciences, Piscataway, NJ, USA) and centrifuged (35 min, 400 × *g*, 23 °C). After centrifugation, the PBMCs fraction was collected and suspended in the complete RPMI medium (RPMI-1640 with L-glutamine; Sigma Aldrich, Germany) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAA Laboratories GmbH, Cölbe, Germany), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (PAA Laboratories GmbH).

2.4.2. PBMCs culture and treatment

Before culturing, PBMCs fractions isolated from the four rats belonging to each experimental group were mixed. Then, the count and viability of PBMCs were determined by trypan blue exclusion test, using the LUNA™ automated cell counter (Logos Biosystems, Annandale, VA, USA). For MTT test and reactive oxygen species (ROS) accumulation measurement, the cells were seeded in a 96-well culture plate (Corning, NY, USA) at a density of 1×10^5 cells/well in a total volume of 100 μL RPMI medium supplemented with 10% FBS and antibiotics. For NF-κB p65 or IL-6 assessment, 1×10^6 cells/well were seeded in a 24-well culture plate in a total volume of 1 mL supplemented RPMI medium. Before stimulation, PBMCs were pre-incubated at 37 °C for 24 h in an incubator providing a humidified (95%) atmosphere containing 5% of CO_2. After pre-incubation, PBMCs were treated with LPS (extracted from *Escherichia coli* 0111:B4, Sigma Aldrich, Germany) at a concentration of 1 μg mL^{-1} or incubated with pyrogen-free saline (control incubation for LPS treatment) for the next 4 h. For the stimulation, LPS was diluted in a pyrogen-free saline and added to the culture in a volume of 100 μL for IL-6 and NF-κB p65 assessment or in a volume of 10 μL for the MTT assay, respectively.

2.4.3. MTT assay

In order to determine the effect of LPS treatment on the proliferation and viability of PBMCs isolated from the CV extract or/and LPS-injected rats the MTT assay was made, according to the previously described procedure (Jędrzejewski et al., 2016). Briefly, following the stimulation of cells with LPS or incubation with pyrogen-free saline, 100 μl of fresh RPMI culture medium without phenol red was added to each well to re-suspend the cells. Then, 10 μl of MTT solution (at a concentration of 5 mg mL^{-1}) dissolved in a plain RPMI medium was added to each well and the plate was incubated at 37 °C, in a humidified atmosphere containing 5% CO_2 for 4 h. Subsequently, the plate was centrifuged, culture medium was removed and 100 μl of dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to each well. Finally, the absorbance in the LPS- or pyrogenic-free saline-treated cells was measured at 570 nm (with reference wavelength of 630 nm) using Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). All absorbance values were corrected against blank wells, which contained growth media alone. The results were also expressed as the mean percentage MTT absorbance of PBMCs isolated from saline/saline-treated rats, which was served as 100.

2.4.4. Nuclear NF-κb p65 activation assay

The level of NF-κB p65 activation was estimated in the PBMCs isolated from the rats belonging to each experimental group, which were stimulated with LPS or incubated with sterile saline *ex vivo*. After stimulation, nuclear proteins from the cells were extracting using Nuclear Extraction kit from Abcam (London, UK; cat. No. ab113474) according to the manufacturer's instructions. The obtained supernatants were used to analysis the quantify NF-κB p65 activation using NF-κB p65 Transcription Factor Assay Kit (Colorimetric) from Abcam (cat. No. ab210613) according to the manufacturer's instructions. In the final supernatants, total nuclear protein concentration was also measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The level of NF-κB p65 activation were expressed as the ratio of the measured absorbance (OD 450 nm) expressing the quantify NF-κB p65 activation per 1 mg of total nuclear protein. Colorimetric changes in the assays were detected using Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.4.5. IL-6 assay

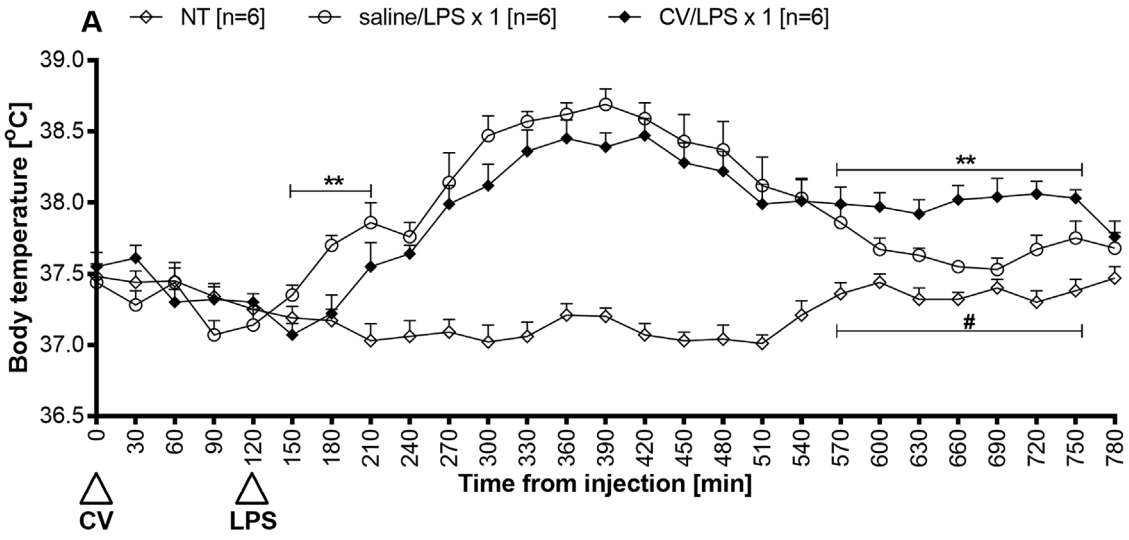
The final culture supernatants were analyzed for the release of IL-6 from the PBMCs. The level of this cytokine was determined by a standard sandwich ELISA kit from R&D Systems (Minneapolis, MN, USA; cat. No. R6000B) according to the manufacturer's instructions. Colorimetric changes in the assays were detected using Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The sensitivity of the IL-6 kit was less than 21 pg mL^{-1}.

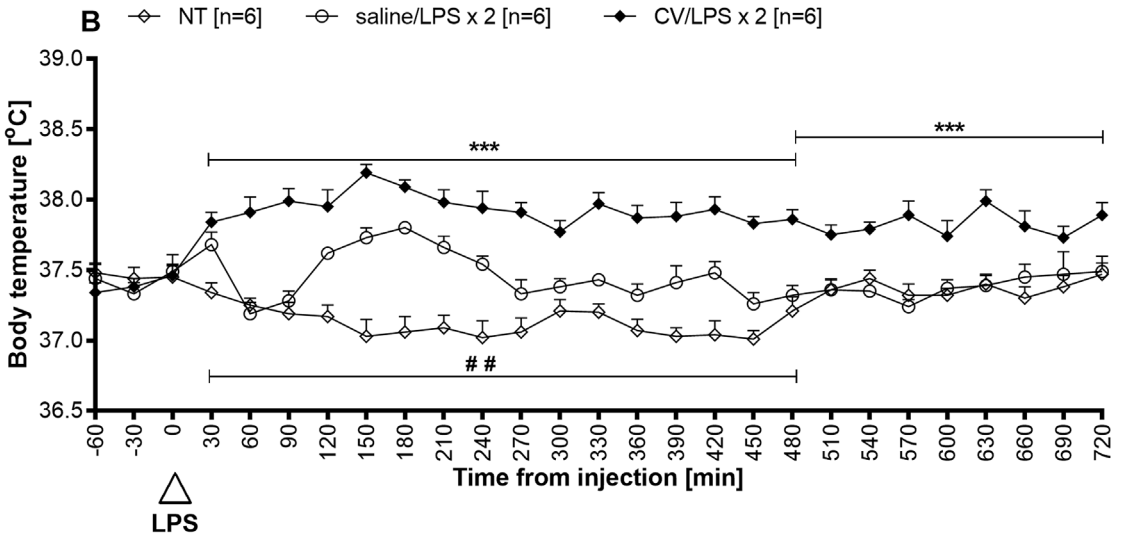
2.4.6. ROS assay

To evaluate the generation of reactive oxygen species in LPS-stimulated PBMCs isolated from the CV extract or/and LPS-injected rats, ROS accumulation was detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich). DCFH-DA is a non-fluorescent compound which upon taken up by passive diffusion into cells is hydrolyzed by esterases to yeld non-permeable DCFH. In the presence of ROS, DCFH is oxidized to the fluorescent DCF. The cells were seeded into 96-well microtiter plates at 1×10^5 cells per well and pre-incubated at 37 °C for 24 h Then, the cells were treated with LPS or incubated with sterile saline. After 4 h, PBMCs were washed twice with PBS and then, incubated with 20 μM DCFH-DA (200 μL/well) at 37 °C for 30 min in the dark. Following incubation, DCFH-DA solution was removed and 100 μL of PBS was added to each well. The fluorescence was measured in a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) using excitation at 485 nm and emission at 528 nm. The results were expressed as the fold change relative to equivalent control untreated cells.

2.5. Statistical analysis

In *in vivo* experiments, temperature values are reported as means ± standard error mean (SEM) calculated for six rats for each experimental group. Five-minute temperature recordings were pooled into 30-min averages for presentation. In *ex vivo* studies mean





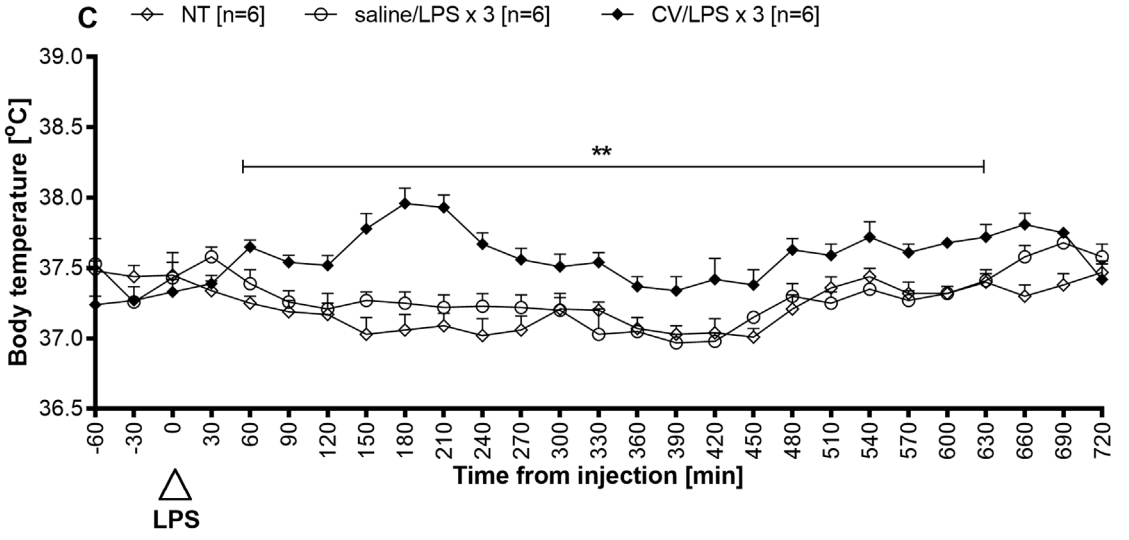


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Fig. 1. Changes of body temperature (°C) over time (minutes post-injection) of rats pre-treated with Coriolus versicolor extracts (CV; 100 mg kg^{-1} i.p.; black diamonds) or saline (open circle) followed by LPS administration (50 μg kg^{-1} i.p.) in comparison with non-treated animals (NT; open diamonds). The graphs present Tb of rats treated with LPS once (A), twice (B) or three times (C). LPS injections were made at 24 h intervals. Values are means ± S.E.M. at 30-min averages. Letter n indicates sample size in a respective group. White triangles indicate the time of CV extracts or LPS administration. Asterisks denote significant differences between experimental groups (CV/LPS vs. saline/LPS; **P < 0.01; ***P < 0.001, respectively). Hash mark indicate significant differences between saline/LPS-injected rats and non-treated animals (#P < 0.05; # #P < 0.01, respectively).

values ± SEM of IL-6 (in the culture supernatants) and NF-κB (in the nuclear extracts) concentrations were calculated for four samples from three independent experiments, whereas, in the case of MTT assay and ROS measurement mean values ± SEM presented the results for six samples from three independent experiments. The results were analyzed using the nonparametric Kruskal-Wallis one-way ANOVA test with the level of significance set at $p < 0.05$.

3. Results

3.1. Pre-treatment with CV extract abolished the attenuation of febrile response during development LPS-induced tolerance in rats

The effect of CV extract on the febrile response during development on tolerance to LPS is illustrated in Fig. 1. The rats were injected i.p. with CV extract at a dose of 100 mg kg^{-1} at 7:00 a.m. 2 h prior to the first LPS administration. The next two LPS injections were made every 24 h at 9:00 a.m. In order to increase the transparency of the presented results, T_b of rats pre-treated with CV extract or saline (control vehicle for CV extract) 2 h prior to triple saline administration (control vehicle for LPS) was not included in the graphs. Body temperature of these animals measured in the second and third days was comparable to control (non-treated; NT) rats (data not shown).

As can be seen in Fig. 1A, pre-treatment of the animals with CV extract resulted in significant alterations of the post-LPS T_b that can be regarded as a protraction of the time course of fever response to the first administration of endotoxin. The T_b of rats pre-treated with CV extract followed by the first LPS administration (38.19 ± 0.13 °C) was comparable to the T_b of saline/LPS-treated rats (38.38 ± 0.14 °C) measured from 240 to 540 min post-injection ($p = 0.25$). The differences in T_b between CV/LPS-treated rats (38.01 ± 0.11 °C) and saline/LPS-injected rats (37.57 ± 0.08 °C) were observed from 570 to 750 min post-injection ($P < 0.01$).

The presented results related to the effect of CV extracts on the endotoxin fever are consistent with those described in Jedrzejewski et al. (2015), however, they were not the purpose of the present studies. The main aim of this publication was to examine the influence of CV extracts on the febrile response during development of endotoxin tolerance in rats. Therefore, the most important findings are shown in Fig. 1B–C, after the second and third injection of LPS with 24 h intervals, when significance differences in endotoxin tolerance were observed. After the second LPS administration (Fig. 1B), the mean body temperature calculated for the period from 30 to 480 min post-injection was significantly higher in rats pre-treated with CV extract (CV/LPS group; 37.93 ± 0.08 °C) in comparison with saline/LPS-treated animals (37.47 ± 0.07 °C) ($P < 0.001$). However, saline/LPS-injected individuals also showed higher T_b than control non-treated rats (37.12 ± 0.09 °C) ($P < 0.01$). Moreover, from 480 to 720 min post-injection only the rats pre-treated with CV extracts demonstrated higher T_b (37.83 ± 0.08 °C) compared to control animals (37.36 ± 0.07 °C) ($P < 0.001$), whereas T_b of saline/LPS-treated rats was similar reaching the average value of 37,43 ± 0.08 °C ($P = 0.21$). The third administration of LPS (Fig. 1C) provoked increase in T_b measured from 60 to 630 min post-injection only in CV-pre-treated rats (37.61 ± 0.08 °C) in comparison with saline/LPS-injected (37.22 ± 0.08 °C) and control animals (37.17 ± 0.08 °C) ($P < 0.01$).

3.2. Mitogenic effect of Coriolus versicolor extract administration in vivo on the PBMCs stimulated with LPS ex vivo

To determine the influence of CV extract injection on the LPS-induced proliferation and viability of PBMCs isolated from LPS-treated rats, the cells were pre-incubated for 24 h after isolation and then stimulated with LPS or incubated with pyrogen-free saline for the next 4 h. The amount of metabolically active cells were measured by MTT test. As shown in Fig. 2A, LPS-stimulated cells isolated from the all experimental groups of rats, except for saline/LPS-injected animals, exhibited significant increase in cell proliferation and viability compared to the respective PBMCs incubated with pyrogen-free saline. All results of MTT assay were also expressed as percentage of control cells isolated from saline/saline-injected rats and stimulated with LPS (Fig. 2B) or incubated with pyrogen-free saline (Fig. 2C), which were served as 100%. As can be seen in Fig. 2B, the cells isolated from LPS-treated rats showed significant decrease in cell proliferation and viability in response to LPS-stimulation *ex vivo*. Interestingly, this effect was more noticeable for cells isolated from saline/LPS-injected rats (29.1 ± 7.6%) than for PBMCs derived from CV/LPS-treated animals (61.3 ± 3.2%; $P < 0.001$). On the other hand, mitogenic activity of LPS stimulation was observed for cells isolated from CV/saline-injected rats, when cell proliferation reached 122.4 ± 3.2% compared to control PBMCs isolated from saline/saline-treated rats ($P < 0.01$). Finally, cell incubation with pyrogen-free saline did not provoke any significant changes in the rate of cell proliferation and viability for the cells derived from all experimental groups of rats (116.3 ± 8.3%, 98.1 ± 4.8%, 110.6 ± 11.1% for CV/LPS-, saline/LPS, CV/saline-injected rats, respectively) compared to control PBMCs.

3.3. In vivo Coriolus versicolor extract administration increases the ability of PBMCs isolated from LPS-injected rats to up-regulate activation of NF-κB, synthesis of IL-6 and increase ROS production in response to LPS challenge ex vivo.

Since it is known that during endotoxin tolerance immune cells such as macrophages, neutrophils, dendritic cells or lymphocytes T show decreased secretion of inflammatory cytokines, i.a. IL-6, TNF-α or IL-1 (Cavaillon and Adib-Conquy, 2006; Mengozzi and Ghezzi, 1993) and diminished expression of p50:p65 NF-κB heterodimers (Abraham, 2003), in the present study we examine the effect of CV extract injection on the NF-κB p65 activation and synthesis of IL-6 by PBMCs isolated from LPS-treated rats in response to LPS stimulation *ex vivo*.

The level of NF-κB p65 activation in the PBMCs isolated from LPS-treated rats in response to LPS stimulation *ex vivo* was measured in the nuclear extracts obtained after cell stimulation per 1 mg of total nuclear protein. As can be seen in Fig. 3, the lowest NF-κB p65 activation was observed in the PBMCs isolated from saline/LPS-injected animals. Importantly, pre-injection of CV extract in LPS-treated rats provoked a higher activation of NF-κB p65 in the cells stimulated with LPS *ex vivo* ($P < 0.01$). These values were significantly lower in comparison with CV/saline- and saline/saline-treated rats, however the greatest NF-κB p65 activation was observed in CV/saline-injected rats ($P < 0.05$ compared to saline/saline-treated animals). Finally, we did not observe any differences in NF-κB p65 activation between PBMCs isolated from the all experimental groups of rats in response to sterile saline incubation *ex vivo*. The level of NF-κB activation in these nuclear extracts was comparable to that observed in non-stimulated cells and these

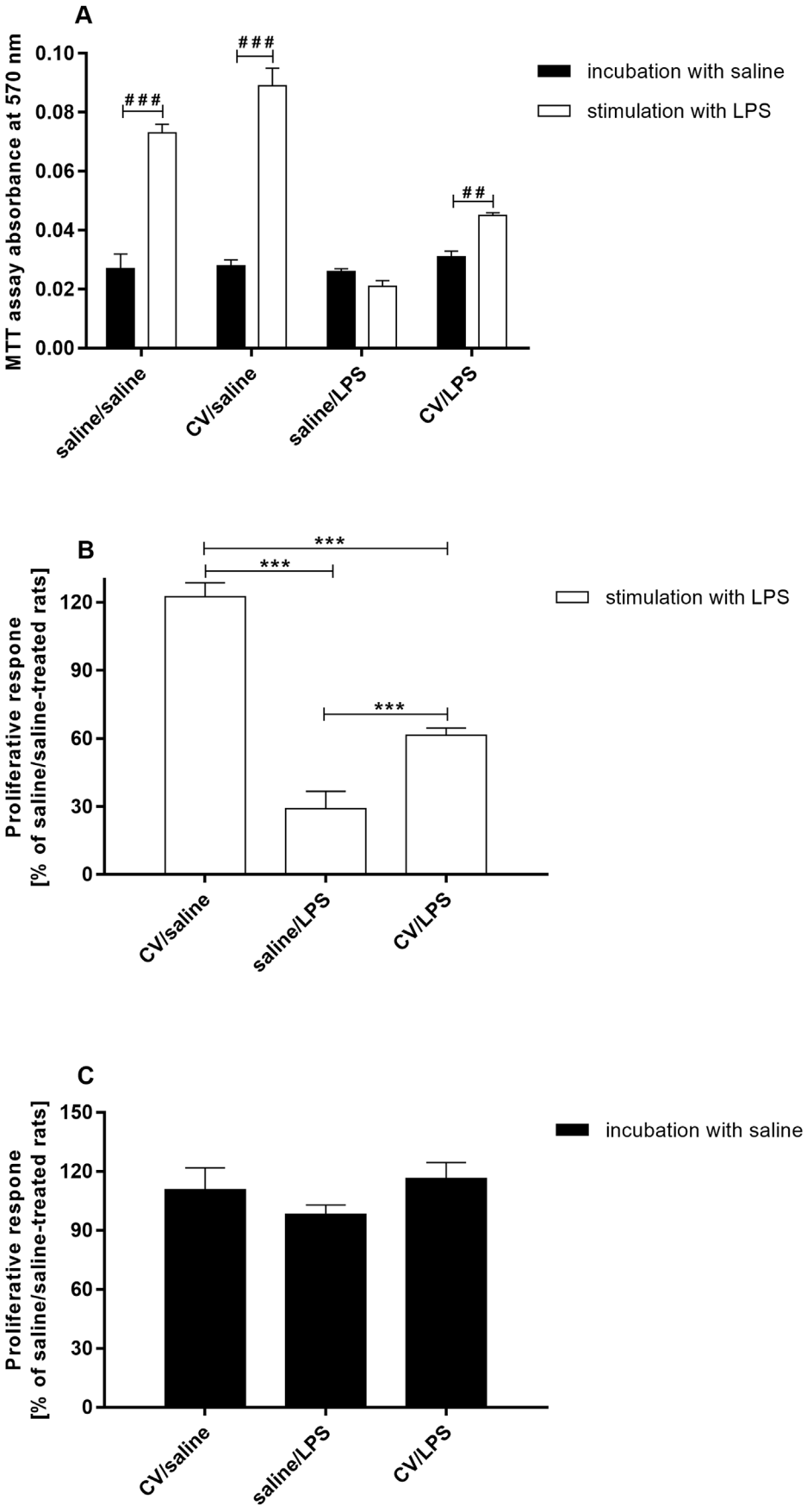

Fig. 2. Proliferation and viability of peripheral blood mononuclear cells (PBMCs) isolated from the rats pre-treated with *Coriolus versicolor* extract (100 mg kg^{-1} i.p.) or saline 2 h followed by LPS administration (50 μg kg^{-1} i.p.) in comparison with control saline/saline-treated animals and CV extract/saline-injected rats. After isolation, PBMCs were pre-incubated for 24 h and, then were stimulated with LPS (1 μg ml^{-1}) or incubated with pyrogen-free saline for the next 4 h. Cell proliferation and viability was evaluated by MTT assay. Data in Fig. (B) and (C) were expressed as the mean % MTT absorbance (ration of absorbance in wells with PBMCs isolated from experimental groups of rats to that with cells derived from control saline/saline-treated animals x 100%) ± SEM of three independent experiments with six wells each. Hash marks in Fig. (A) indicate significant differences between LPS stimulated-PBMCs compared to saline-incubated cells, which were isolated from the same experimental group of rats (# #P < 0.01; # # #P < 0.001, respectively). Asterisks in Fig. (B) denote significant differences between the LPS-stimulated cells isolated from the respective experimental groups of animals as indicated (***P < 0.001).

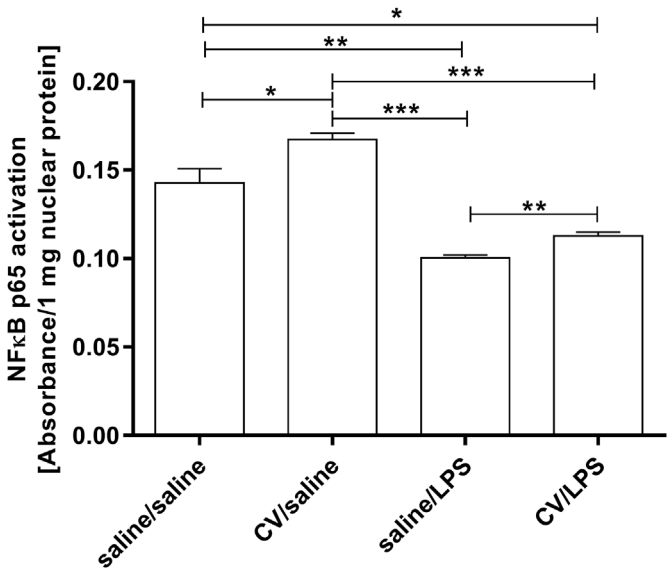

Fig. 3. Level of NF-κB p65 activation measured in the LPS-stimulated PBMCs isolated from the rats belonging to the same experimental groups as described in Fig. 2. After isolation, PBMCs were pre-incubated for 24 h and, then were stimulated with LPS (1 μg ml^{-1}) for the next 4 h. Values are means ± SEM of NF-κB p65 activation in the nuclear extracts obtained after cell stimulation per 1 mg of total nuclear protein. Asterisks denote significant differences between the LPS-stimulated cells isolated from the respective experimental groups of animals as indicated (*P < 0.05; **P < 0.01; ***P < 0.001, respectively).

values were below detection limit of the used assay kit (data not shown).

As can be seen in Fig. 4, PBMCs isolated from CV/LPS-treated rats produced significantly more IL-6 (381.1 ± 5.3 pg mL^{-1}) than cells isolated from saline/LPS-injected animals (297.8 ± 16.1 pg mL^{-1}) in response to LPS stimulation *ex vivo* (P < 0.01). However, these levels of cytokine were lower compared to the concentration of IL-6 measured in the supernatants derived from the cells isolated from CV/saline-injected rats (517.4 ± 7.1 pg mL^{-1}) as well as saline/saline-treated animals (422 ± 7.2 pg mL^{-1}). Finally, we did not observe any differences in IL-6 level between PBMCs isolated from the all experimental groups of rats in response to sterile saline incubation. The concentration of IL-6 in these supernatants was significantly lower compared to LPS-stimulated cells (P < 0.001).

To assess the capacity of the LPS-stimulation *ex vivo* to generate intracellular reactive oxygen species, PBMCs isolated from the all experimental groups of rats were treated with LPS for 4 h and stained with 2′,7′-dichlorofluorescein diacetate (DCFH-DA). As can be seen in Fig. 5, the highest levels of ROS generation (3.32 fold increase in ROS production in comparison to untreated cells) was observed in the cells isolated from the CV/LPS-treated rats. Importantly, these values were greater to that noticed in the PBMCs derived from the saline/LPS-injected rats (2.70 fold increase; P < 0.001). In contrast, ROS production in the cells isolated from non-tolerant rats (CV/saline and saline/saline group) was at lower level, however, the CV extract-injection resulted in higher ROS generation in PBMCs compared to the control saline/saline-treated rats (2.14 *vs.* 1.96 fold increase, respectively; P < 0.01). Finally, we did not observe any differences in ROS production between PBMCs isolated from the all experimental groups of rats in response to sterile saline incubation (data not shown).

4. Discussion

Endotoxin tolerance is defined as a reduced responsiveness to LPS challenge following a first encounter with endotoxin. This phenomenon has been observed both *in vitro* and *in vivo* in animal models as well as in humans (Biswas and López-Collazo, 2009). Importantly, the incidence of endotoxin tolerance has been reported in several disease settings, including sepsis, trauma, surgery, cardiac arrest, resuscitation, and pancreatitis, underlining its clinical significance. Although endotoxin tolerance has been thought as a protective mechanism to avoid systemic inflammation, which should be interpreted as an adapted immune response (Soszynski, 2002), clinically, this state is correlated with a high risk of secondary infection and mortality. Moreover, it can lead to fatal blunting of immunological responses to subsequent infections in the, among others, sepsis survivors (Cavaillon et al., 2003; Monneret et al., 2008; Morrison et al., 1999).

Immunosuppressive diseases have many forms and it is yet to be known if endotoxin tolerance is involved in them, however, certainly the mechanism of pyrogenic tolerance and immunosuppressive disease are both manifested mainly as a reduced ability of the immune cells to release cytokines involved in the activation of the febrile processes in the brain (Soszynski et al., 2013; Cavaillon, 1995). On the other hand, the increase of body temperature (Tb) during fever has several advantages over infections: inhibition of bacterial growth, increased bactericidal activities of neutrophils and macrophages, T cell proliferation and differentiation, B cell proliferation and the production of antibodies or stimulation of acute-phase protein synthesis (Kluger et al., 1996). Thus, it seems to be a significant to search for agents, which will be able to attenuate the established refractory state accompanying the certain diseases, whose mechanism, at least partially, resembles endotoxin tolerance.

Since we have demonstrated that extract isolated from *Coriolus*

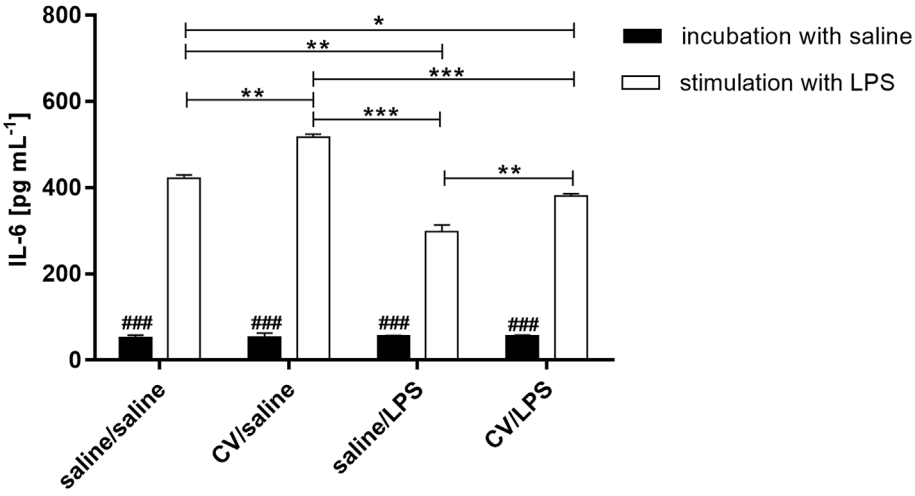

Fig. 4. Synthesis of IL-6 [pg ml^{-1}] in the LPS-stimulated peripheral blood mononuclear cells (PBMCs) isolated from the rats belonging to the same experimental groups as described in Fig. 2. After isolation, PBMCs were pre-incubated for 24 h and, then were stimulated with LPS (1 μg ml^{-1}) or incubated with pyrogen-free saline for the next 4 h. Values are means ± SEM of IL-6 concentration in the final culture medium from three independent experiments. Asterisks denote significant differences between the LPS-stimulated cells isolated from the respective experimental groups of animals as indicated (*P < 0.05; **P < 0.01; ***P < 0.001, respectively). Hash marks indicate significant differences between LPS stimulated-PBMCs in comparison with saline-incubated cells, which were isolated from the same experimental group of rats ($^{\#}$ $^{\#}$ $^{\#}$P < 0.001).

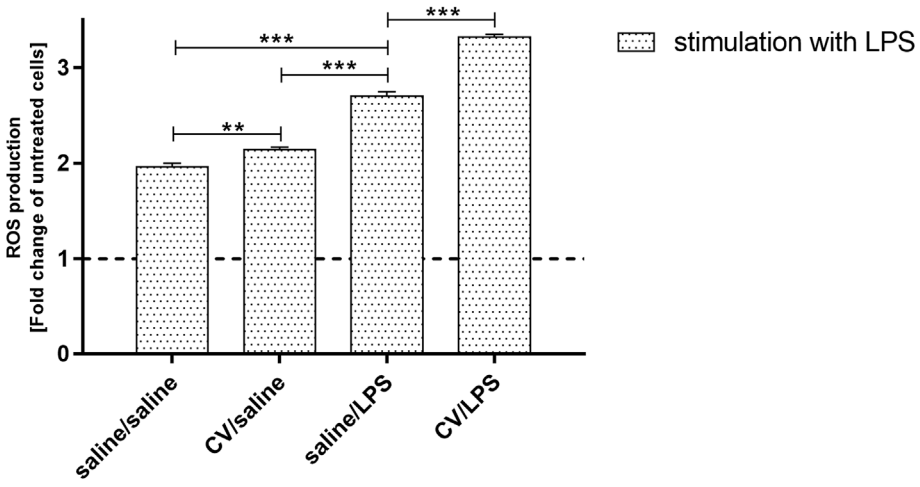

Fig. 5. Reactive oxygen species (ROS) production in peripheral blood mononuclear cells (PBMCs) isolated from the rats belonging to the same experimental groups as described in Fig. 2. After isolation, PBMCs were pre-incubated for 24 h and, then were stimulated with LPS (1 μg ml^{-1}) for the next 4 h. Intracellular production of ROS was determined after stimulation of cells with LPS using DCF-DA. Data show the mean ± SEM of three independent experiments with six wells each. The results were expressed as a fold change compared to the untreated cells Asterisks indicate significant differences between the cells isolated from the respective experimental groups of animals as indicated (**P < 0.01; ***P < 0.001, respectively). The horizontal dashed line represents ROS production in the untreated control cells.

versicolor fungus has been able to induce the prolongation of endotoxin fever in rats (Fig. 1A) in IL-6 related manner (Jedrzejewski et al., 2015), in the present study, we have decided to examine the effect of this extract on the endotoxin tolerance. In animals model this phenomenon development is obtained by several repeated LPS injection result in progressive attenuation of its pyrogenic effects (Roth et al., 1994; Soszynski et al., 1998). Indeed, in the present experiments, triple LPS administration (50 μg ml^{-1}) with 24 h intervals inhibited febrile response to LPS especially after third injection. In contrast, pre-treatment of the LPS-tolerant rats with CV extract resulted in significant increase in T_b on the second and third day post-LPS administration (Fig. 1B and C, respectively). As mentioned above clinical examples of endotoxin tolerance include sepsis. Moreover, changes in body temperature are a characteristic feature of sepsis. Kushimoto et al. (2013) demonstrated that hypothermia is a very important manifestation of infection associated with very high mortality. The results of meta-analyses clearly also showed a negative correlation between body temperature and mortality in sepsis: fever is associated with decrease, whereas hypothermia with increased rate of death (Rumbus et al., 2017). Therefore, the maintaining of CV extract-induced febrile response during endotoxin tolerance it seems to be a favourable phenomenon, which may predict better survival of sepsis patients.

It is well-established that *in vivo* and *in vitro* immunostimulatory activities of CV extract, among others, are associated with activate the cells through NF-kB signaling pathway (Yang et al., 2015; Li et al., 2010) and subsequent de-repression of pro-inflammatory cytokine genes such as IL-1β IL-6 and TNF-α (Chan and Yeung, 2006; Lee et al., 2008). In contrast, many *in vitro*, *in vivo* and *ex vivo* studies have demonstrated that "LPS-tolerant" phenotype is characterized by inhibition of LPS-stimulated TNF-α or IL-6 production as a consequence of the decrease in NF-κB activity (Biswas and López-Collazo, 2009; West and Heagy, 2002). For instance, monocytes from patients with sepsis have a serious reduction of pro-inflammatory cytokine production when re-stimulated *in vitro*, in comparison to healthy volunteer cells (Draisma et al., 2009). The *ex vivo* approach is an intermediate experimental solution that improves researcher control over the secondary endotoxin stimulus (López-Collazo and del Fresno, 2013). Moreover, data obtained from this model are important for highlighting the differences between the more common *in vitro* data and the complex full *in vivo* findings (Kox et al., 2011). Finally, this approach is also useful for determining whether patients' blood cells exhibit endotoxin tolerance (Escoll et al., 2003; del Fresno et al., 2008; del Fresno et al., 2009). Based on these findings, in our study, we have investigated *ex vivo* reflection of *in vivo*-induced tolerance to LPS. PMBCs were isolated 2 h after the first LPS injection and they were re-exposed to LPS *in vitro* (1 μg ml^{-1}) for 4 h. Our *ex vivo* analysis confirms the observation described above. PBMCs isolated from saline/LPS-treated rats produced significantly less IL-6 *in vitro*, than the cells isolated from saline/saline-injected control animals in response to LPS. Importantly, pre-treatment of rats with CV extract before LPS injection stimulated PBMCs to release a higher amounts of IL-6. Moreover, the injection of the CV extract itself (CV/saline group) caused the largest production of this cytokine by the cells (Fig. 4). Similar relationships were observed in the level of NF-κB p65 activation measured in the nuclear extracts derived from the LPS-stimulated PBMCs *ex vivo*. Pre-injection of CV extract in LPS-treated rats provoked a higher activation of NF-κB p65 in the cells stimulated with LPS in comparison with PBMCs isolated from saline/LPS-injected animals. The greatest NF-κB p65 activation was observed in CV/saline-injected rats (Fig. 3). Based on the results, it clearly seen that synthesis of IL-6 by LPS-stimulated cells occurred in a NF-κB p65-dependent manner.

As mentioned above, endotoxin tolerance, clinically, is correlated with a high risk of secondary infection and mortality, among others, due to the occurrence of a refractory state. Therefore, in the present study we have examined whether or not the CV extract may counteract this phenomenon, not only by studying the levels of IL-6 and activation of NF-κB p65, but also by measuring ROS production in LPS-stimulated PBMCs and cell proliferation rate. We have shown that the cells isolated from the CV/LPS-treated rats and stimulated with LPS *in vitro* released larger amounts of ROS than the PBMCs derived from saline/LPS-injected rats. Moreover, the injection of CV extract itself provoked a higher ROS generation in LPS-stimulated PBMCs in comparison with the control saline/saline-treated rats (Fig. 5). It is well established that LPS constitutes a pathogen-associated molecular pattern (PAMP) recognized by the cell surface Toll-like receptor (TLR) type 4 (Wright et al., 1990; Qureshi et al., 1999). Furthermore, LPS is able to trigger mitochondrial ROS generation in the cells through activation of TLR4, thus directly exerting bactericidal activities, and facilitating the anti-infective immune response (West et al., 2011; Lv et al., 2017). Recent findings indicate that the immunoregulatory effects of CV extract, including production of cytokine, is also related to the TLR-4 signaling pathway (Li et al., 2010; Wang et al., 2013), and CV extract activates immune cells to release reactive nitrogen and oxygen intermediates (Chang et al., 2017). However, to the best of our knowledge, it is still unknown if CV extract induces ROS generation via TLR4 receptor. Nevertheless, we suppose that CV extract itself increases ROS production in PBMCs after LPS-stimulation through its synergic effect along with LPS on TLR4 receptors.

Our results corresponding with findings from the others authors, who showed that the macrophages, monocytes or neutrophils from tolerant 'phenotype' exhibited enhanced ROS production (de Lima et al., 2014; Fernandes et al., 2010; Zhu et al., 2016). The increase in ROS generation induced by CV extract administration seems to be a desirable phenomenon since respiratory burst is believed to be an

important host defence strategy utilized to kill invading pathogen (Zhu et al., 2016) and ROS represent a defence mechanism against invading organisms by activated phagocytes (Vega et al., 2002). Destroying microorganisms more efficiently is a favourable state during endotoxin tolerance-induced refractoriness, which is clinically observed in sepsis patients. These patients show increased susceptibility to reinfection, which in turn is associated with worse outcomes, including mortality (Cheng et al., 2016). ROS also act as regulators of transcription factor activities (Thannickal and Fanburg, 2000). For instance, well-known example of redox-sensitive transcription factor is NF-κB. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB (Gloire et al., 2006). Indeed, in our study the level of NF-κB p65 activation in LPS-stimulated PBMCs partly corresponded with the ROS production in these cells. This phenomenon was observed by comparing the cells isolated from CV-extract pre-treated rats and the saline pre-injected animals (compare CV/LPS group and saline/LPS group or CV/saline group *vs*. saline/saline group in Figs. 3 and 5). Based on this results, we presume that the immunostimulating effect of CV extract on the 'tolerant phenotype' of cells is a phenomenon partly dependent on ROS generation.

Finally, interesting differences were also observed in cell proliferation and viability range. LPS-stimulated cells isolated from the all experimental groups of rats, except for saline/LPS-injected animals, exhibited significant increase in cell proliferation and viability in comparison to the cells incubated with saline (Fig. 2A). These observations corresponding with the our previous findings (Jędrzejewski et al., 2016) and the results from the others authors, which indicate that LPS is a potent inducer of the proliferation of B and T cells and even monocytes (Erdogan et al., 2006; Ulmer et al., 2000; Xu et al., 2008). However, as we have shown, the endotoxin tolerance *ex vivo* inhibited mitogenic activity of LPS. On the other hand, pre-treatment of rats with CV extract before LPS injection significantly reduced this phenomenon, and CV extract administration itself increases the mitogenic effect of LPS (Fig. 2B). These results seem to be consistent with the findings of other authors, who have shown that CV extract promotes the proliferation of PBMCs, and T or B lymphocytes (Sekhon et al., 2013; Ho et al., 2004). Moreover, we have also shown previously that LPS significantly enhanced proliferative response to CV extract in a time dependent manner and U-shaped dose response fashion (Jędrzejewski et al., 2016). All these results taken together clearly indicate that CV extract has an immunostimulatory effect on the PBMCs during endotoxin tolerance by its mitogenic properties.

5. Conclusion

Present findings demonstrate that CV extract injection partially prevents endotoxin tolerance development by maintaining febrile response and increasing IL-6 and ROS generation. Moreover, we showed that CV extract partially reverses endotoxin tolerance by causing a shift toward a more pro-inflammatory phenotype and not increasing the pro-inflammatory response at the same time. In consequence, the immunostimulating properties of CV extract may possibly play an important role to prevent the non-specific refractoriness described, among others, in patients with sepsis. We believe that it is a beneficial phenomenon since it has been established that the increase of body temperature during fever has many advantages over infections and fever is associated with reduced mortality in septic patients.

Conflicts of interest

The authors declared no financial or commercial conflict of interest.

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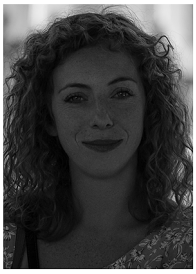

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