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ORIGINAL ARTICLE

Measurement of protein cytokines in tissue extracts by enzyme-linked immunosorbent assays: Application to lipopolysaccharide-induced differential milieu of cytokines

Khalid Z. Matalka[1], Maha F. Tutunji[2], Mona Abu-Baker[3] & Yousef Abu Baker[3]

[1] Faculty of Pharmacy and Medical Technology, University of Petra, Amman, Jordan.
[2] Pharmaceutical Research Unit, Royal Scientific Society & Chemistry Department, Faculty of Science, University of Jordan, Amman, Jordan.
[3] Genome for Scientific Research, Beirut, Lebanon.

Correspondence to: Prof. Khalid Z. Matalka, PhD
Faculty of Pharmacy and Medical Technology
University of Petra,
P.O.Box 961343, Amman, JORDAN
EMAIL: kzm@uop.edu.jo or kzm@go.com.jo
FAX: +962 6 571-5570, TEL: +962 6 571-5546

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Abstract

OBJECTIVES: Determination of protein cytokines in local tissues would help to evaluate their local role in health, sickness behavior and immune-mediated diseases. Therefore, developing a simple quantitative method of protein cytokines in tissues/organs is highly important.

METHODS: Mouse tissues were collected following intraperitoneal administration of endotoxin-free PBS or lipopolysaccharide. A mild detergent, 0.1% Igepal, was added in a buffer to enhance cytokines extraction. The tissues were then disrupted, homogenized, centrifuged and the supernatants were collected and assayed using solid-phase immunoassays.

RESULTS: The presence of 0.1% Igepal extracted significantly more TNF-α from liver (322%: $p<0.01$), brain (358%: $p<0.05$), lungs (1600%: $p<0.01$), and more IL-10 from liver (220%: $p<0.001$), brain (4650%: $p<0.001$) than PBS alone. On the other hand, using 0.1% Igepal did not increase IFN-γ extraction from liver, spleen, brain, lungs, skin and kidneys more than PBS alone. Furthermore, i.p. administration of LPS induced a differential milieu of cytokines. LPS increased significantly the production of TNF-α, IFN-γ, and IL-10 from liver (521%, 123%, 72%: $p<0.01, 0.04, 0.04$), brain (470%, 122%, 280%: $p< 0.01, 0.03, 0.01$), peritoneal lavage ($p<0.001$) and blood ($p<0.001$). However, the pattern of increase was different for the above cytokines in spleen, skin, lungs and kidneys.

CONCLUSIONS: The extraction of protein cytokines from tissues was superior with addition of mild detergent. Furthermore, our results showed a differential cytokines response to LPS with respect to tissue and cytokine type. This method should provide an important tool for studying local protein cytokines in behavioral pattern, sickness behavior, and immune-mediated diseases as well as to determine local therapeutic efficacy of immunomodulatory drugs.

Introduction

Cytokines, as pro-inflammatory or anti-inflammatory, are the main regulators of local tissue immune response. Thus, quantitating cytokines in tissues would help to evaluate their local role in health, sickness behavior and immune-mediated diseases. The available methodologies for determining cytokines in tissues are based on the assessments of immunohistochemical method [3,5], cytokines mRNA expression [20], or culturing mononuclear cells from tissues [8,21]. The detection by immunohistochemical is still semi-quantitative in spite of the availability of newer image analysis techniques. In addition, tissue mRNA expression methods either using *in situ* hybridization or polymerase chain reaction are semi-quantitative. The real-time PCR is now the quantitative method of choice for cytokine gene expression [2] but still is difficult to reproduce, expensive and is less relevant to the function of cytokines. On the other hand, mononuclear cells culture methods are difficult to perform on every organ, and do not represent the actual in vivo cellular and behavioral interactions. Therefore, it would be very important to have an absolute quantitative method of protein cytokines in tissues such as spleen, liver, intestine, lungs or brain without the need of culturing the mononuclear isolated cells.

Very few reports [4,15–17] described methods to measure tissue cytokines following i.p. administration of lipopolysaccharide (LPS). One of these reports [4] showed very low levels of cytokines in tissues even though 100 μg of LPS was administered per mouse. The latter report used a homogenizer and PBS or Tris buffer to extract the tissue cytokines. Other reports [15–17] used a tissue homogenizer with buffer containing 0.5% Trition as a detergent but without showing the possible interference of such detergent concentration on the cytokines measurement using enzyme linked-immunosorbent assays [19]. Recently, Rosengren et al [19] showed the effects of different non-ionic detergents to detect different cytokines using enzyme linked-immunosorbent assay. Furthermore, they demonstrated the superiority of using 0.1% Igepal to extract cytokines from human synovial tissues obtained from rheumatoid arthritis and osteoarthritis patients [19]. In the latter work, it was found that the use of 0.1% Igepal resulted in ~67% increase in IL-6 detection from synovial tissue from rheumatoid arthritis patients. The present study extends the latter observations and describes a method for determining cytokines in different tissues/organs from normal mice such as liver, spleen, kidneys, lungs, brain and skin as well as organs/tissues obtained from mice following intraperitoneal administration of LPS. The tissues/organs are placed in a buffer containing 0.1% Igepal, a non-ionic detergent, to facilitate the extraction of cytokines. The tissues, then, were disrupted, homogenized, centrifuged and the supernatants were assayed using solid-phase immunoassays. In addition, in vitro testing was performed to show the effect of using such detergent in whole blood culture for detecting cytokines using solid phase immunoassays. Measuring tissue cytokines, as described here, should provide a highly important tool for studying local protein cytokines in normal behavioral pattern, sickness behavior, immune-mediated diseases as well as local therapeutic efficacy of immunomodulatory drugs.

Materials and methods

Reagents. The following reagents: RPMI 1640, penicillin-streptomycin, L-glutamine, lipopolysaccharide (LPS, L-6143), phytohemeagglutinin (PHA-L, L-4144), endotoxin-free Dulbecco's phosphate buffer (without calcium and magnesium) and bovine serum albumin (BSA) were purchased from Sigma. Culture 6-well plates and maxisorp 96-well flat bottom plates were purchased from Nunc International (Denmark).

Mice. Twenty-eight Balb/c mice weighing 20–30 g were used in the study and divided into three groups. Groups 1 (n=6) and 2 (n=11) of mice were injected i.p. with 1 ml of endotoxin-free PBS and group 3 (n=11) was injected i.p. with 1 ml of 5 μg LPS. Two hours later, mice were sacrificed by cervical dislocation and tissues/organs were collected within 6–10 minutes. The tissues were blood, peritoneal lavage, lungs, spleen, liver, kidneys, skin, and brain.

Blood. After sacrificing the animal, blood was collected from cardiac chamber and left to clot under ice. The blood was then centrifuged for 4 min and serum collected, stored at –30 °C for cytokine analysis.

Peritoneal lavage. After sacrificing the animal and taking blood sample, 2 ml of ice-cold endotoxin-free PBS were injected into the peritoneum. Peritoneal lavage was harvested after gentle massage of the peritoneum, centrifuged and the supernatant was stored at –30 °C for cytokine analysis.

Extraction of cytokines from tissues/organs. Lungs, liver, spleen, kidneys, skin and brain, respectively, were weighed, and 2 ml of ice-cold endotoxin-free PBS containing 0.1% Igepal CA-630 nonionic detergent were added to the tissues left for 10 minutes under ice before homogenizing the tissues with a tissue disrupter (Janke and Kundel). The concentration 0.1% of Igepal was based on a previous study [19]. The tissues then were centrifuged for 5 min, and the supernatants were collected, stored at –30 °C for cytokine analysis.

Human whole blood culture. Blood samples were drawn from three healthy volunteers into sterilized sodium heparin tubes (Vacutainer, Becton-Dickinson) and processed within 45 minutes. The production of cytokines from whole blood was performed as described elsewhere [13,14]. The blood was diluted with 1:10 with RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, without exogenous serum. To each well of the 6-well culture plates, 2.0 ml of the diluted blood was added. A mixture of PHA+LPS in 40 μl volume were added to give a final concentrations of 5 μg/ml and 1 μg/ml for PHA and LPS, respectively, and incubated in 5% CO2 at 37 °C for 48 h. At the end of incu-

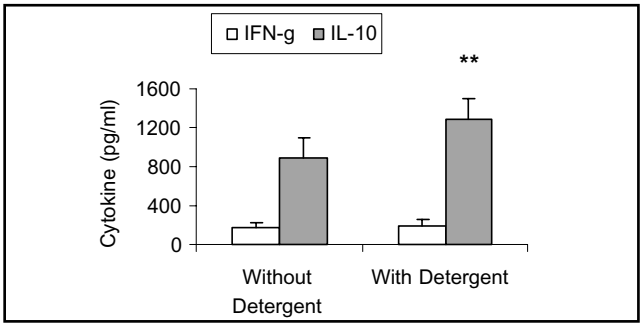

Figure 1. Human IFN-γ and IL-10 detection levels from PHA+LPS stimulated whole blood. The harvested cells either were centrifuged (without detergent) or 0.1% Igepal was added (with detergent). Using 0.1% Igepal following harvesting the cells enhanced significantly (**: p<0.03) IL-10 levels detection.

Table 1. Recovery of cytokines in a buffer containing 0.1% Igepal

Cytokine*	Concentration (pg/ml) Added	% Percent Recovered
hIL-10	300	108±1
hIFN-γ	300	95±5
mIL-10	400	118±6
mIFN-γ	400	105±3
mTNF-α	400	113±2

* h: human cytokines
m: mouse cytokines

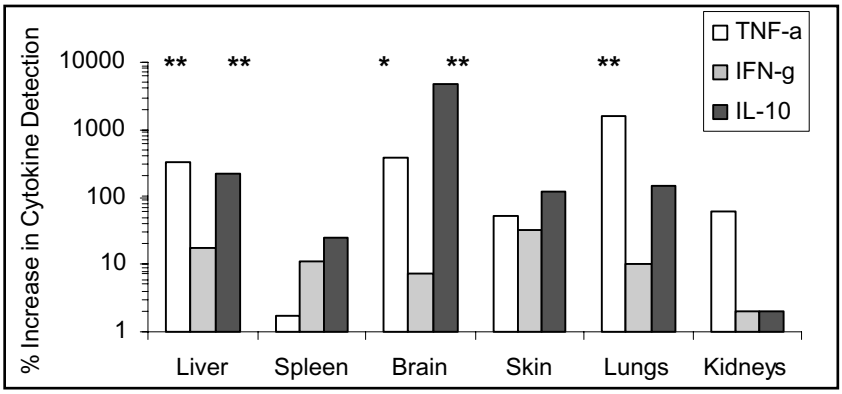

Figure 2. The percent increase in mouse tissue TNF-α, IFN-γ, and IL-10 detection when 0.1% Igepal in the extracting buffer was used. **: p< 0.01, *: p< 0.05

bation, blood was collected from wells into sterilized tubes and each well was washed with 0.5 ml of RPMI to ensure removal of all well content. The amount was divided into two tubes; one centrifuged and supernatant was separated, and to the other portion 0.1% of Igepal was added. The supernatants and the detergent-containing blood were aliquoted and stored in sterilized tubes at –30 °C until assayed.

Cytokine analysis. Mouse tissue-extracted cytokines (TNF-α, IL-10 and IFN-γ) and human cytokines (IL-10 and IFN-γ) were assayed using ELISAs adapting the procedures recommended by the manufacturer (mouse and human DuoSet, respectively, R&D Systems, UK). Briefly, captured antibodies for all cytokines were coated as recommended by the manufacturer in PBS pH 7.2–7.4. All samples were diluted 1:5 with samples assay buffer. All standards and samples were run in duplicates. Anti-cytokine-biotinylated detector antibodies were used at 300 μg/ml for TNF-α, and 400 ng/ml for IFN-γ and IL-10. Strepavidin-Horseradish peroxidase conjugate with H2O2-Tetramethylbenzidene (R&D, UK) substrate was used. Plates were read by SCO GmbH (Dingelstadt, Germany) ELISA plate reader and absorbance was transformed to cytokine concentrations (pg/ml) and then to fg/mg of tissues using a standard curve computed on Excel. The sensitivities for all cytokines were between 4 to 6 pg/ml.

Data analysis. The data in figures 1 and 3 present the cytokine concentrations (± SE). The data are expressed in fg/mg of tissue except for blood and peritoneal lavage which are expressed in pg/ml (± SE). Figure 2 presents the percent increase in cytokine detection when 0.1% Igepal was used in the extraction buffer. Comparisons between groups were performed by using Wilcoxon-rank sum test when n < 10 and unpaired two tailed student t-test when n >10.

Results

Igepal at 0.1% does not interfere with cytokines measurement. The effect of 0.1% Igepal on cytokines recovery is presented in Table 1. The results showed that 0.1% of Igepal did not influence the recovery of any cytokine tested whether it was a human or mouse origin and therefore no interference with the detection of cytokines in enzyme immunoassay was observed.

Concentration of cytokines in supernatants versus whole blood containing 0.1% Igepal from stimulated whole blood cultures. The difference in cytokines measured in the supernatants and blood containing 0.1% Igepal from stimulated whole blood is presented in figure 1. The concentration of IL-10 from stimulated (PHA+LPS) samples in blood containing 0.1% Igepal was significantly more than cell culture supernatant (48%: p< 0.03) (Figure 1). However, this was not observed with IFN-γ. It has to be mentioned that adding 0.1% Igepal to blood caused hemolysis, which indicate that using 0.1% Igepal destroys the cell membrane of red blood cells and probably as well mononuclear cells and thus facilitate the detection of extra-

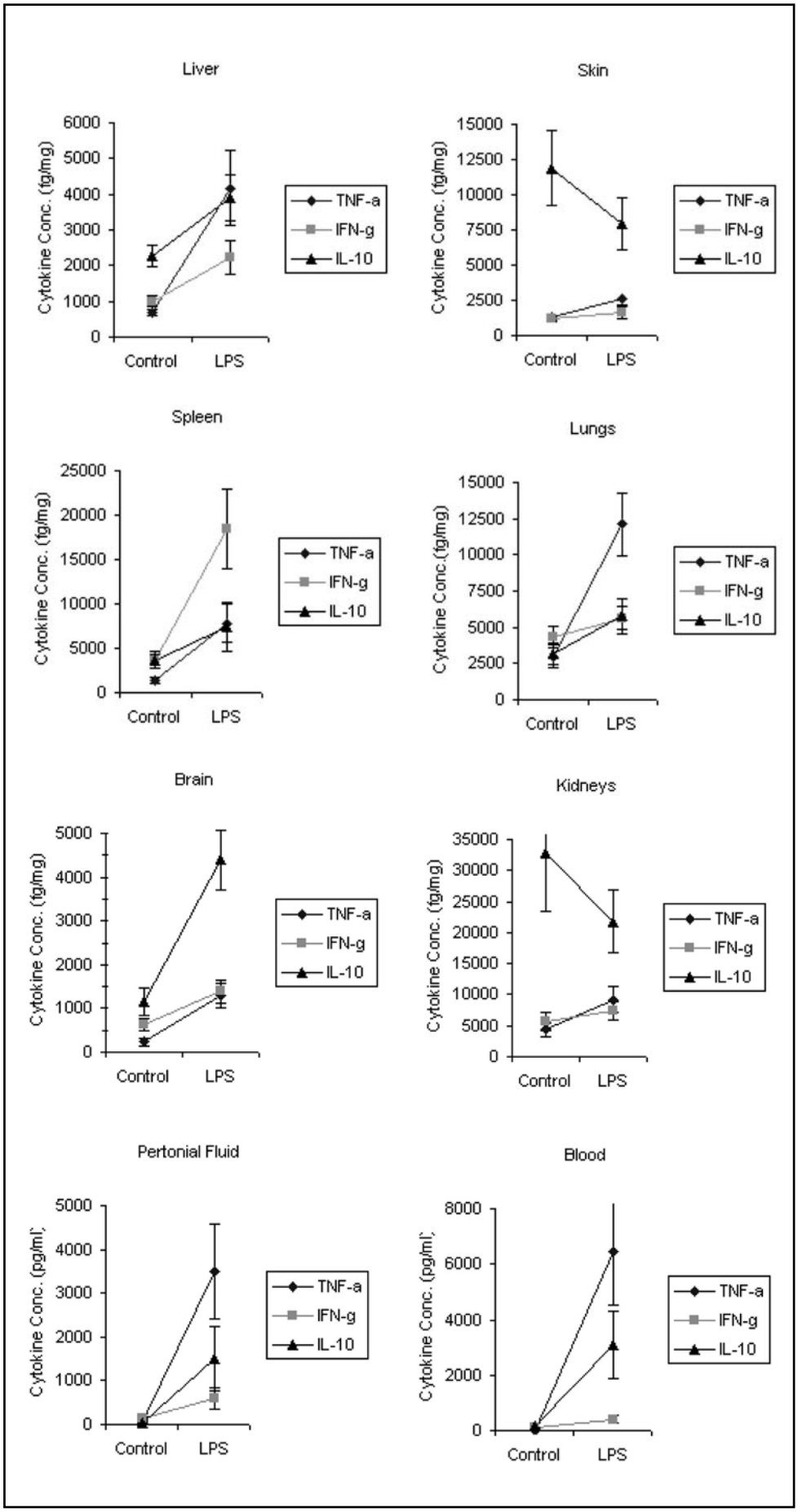

Figure 3. TNF-α, IFN-γ, and IL-10 levels in tissues following 2 hours of injecting endotoxin-free PBS (control) or 5 μg LPS. LPS increased significantly the production of TNF-α in liver ($p<0.01$), brain ($p< 0.01$), peritoneal lavage ($p< 0.001$), blood ($p<0.001$), spleen ($p<0.05$), skin ($p<0.05$) and lungs ($p<0.01$). LPS increased significantly the production of IFN-γ in liver ($p<0.05$), brain ($p<0.05$), peritoneal lavage ($p<0.001$), blood ($p< 0.001$), and spleen ($p<0.01$). Also, LPS increased significantly IL-10 levels in liver ($p<0.05$), brain ($p<0.01$), peritoneal lavage ($p<0.001$), and blood ($p<0.001$).

cellular, intracellular, as well as membrane-bound cytokines.

Detection of extracted cytokines from tissues. The presence of 0.1% Igepal (in comparison to PBS alone) significantly extracted more TNF-α from liver (322%: p<0.01), brain (358%: p<0.05), lungs (1600%: p<0.01), showed a trend towards an increase from skin (54%) and kidneys (59%) but no difference was observed from spleen (Figure 2). In addition, 0.1% Igepal significantly extracted more IL-10 from liver (220%: p<0.001), brain (4650%: p<0.001) than PBS alone, showed a trend towards an increase from lungs (144%) and skin (121%) but no difference was observed from spleen and kidneys. On the other hand, using 0.1% Igepal did not increase IFN-γ extraction from liver, spleen, brain, lungs, skin and kidneys more than PBS alone.

LPS stimulates cytokine synthesis in organs. Intraperitoneal administration of LPS increased significantly the production of TNF-α, IFN-γ, and IL-10 in liver (521%, 123%, 72%: p<0.01, 0.05, 0.05), brain (470%, 122%, 280%: p< 0.01, 0.05, 0.01), peritoneal lavage (p<0.001) and blood (p<0.001) (Figure 3). However, the pattern was different in spleen, skin, lungs and kidneys. In spleen, i.p.administration of LPS increased significantly the production of TNF-α and IFN-γ (476%, 400%: p<0.05, 0.01) but showed a trend towards an increase in IL-10 levels (101%). In skin, i.p. administration of LPS increased significantly TNF-α (95%: p<0.05), but no change in IFN-γ and IL-10 levels were observed. In lungs, i.p. administration of LPS increased significantly TNF-α levels (305%: *p*<0.01), showed a trend towards an increase in IL-10 (84%) but no change in IFN-γ levels were observed. In kidneys, i.p. administration of LPS showed only a trend towards an increase in TNF-α (113%) but no change in IFN-γ and IL-10 levels were observed (Figure 3).

Discussion

This study describes a simple method for measuring cytokines in tissues. The method is based on using a mild detergent, 0.1% Igepal, that facilitates the extraction of cytokines following tissue/organ homogenization. Such detergent concentration did not interfere with detection of human TNF-α, IL-6, IL-1β, IL-8 [19], as also seen in the present study with human IFN-γ, IL-10 and mouse IFN-γ, IL-10, TNF-α. Furthermore, 0.1% Igepal increased IL-6 extraction from synovial tissue by ~67% when compared with PBS alone. The extracted amount was ~80% of what is present in the synovial tissues when determined by western blot [19]. Higher concentrations of Igepal, however, did not increase the recovered cytokines. In the present study different tissues were used and different cytokines were extracted. Using 0.1% Igepal, the detection of IL-10 and TNF-α was significantly more from liver (> 200% and >300%, respectively), brain (>4000% and >300%, respectively), and lungs (>100% and 1000%, respectively) but this was not seen with IFN-γ.

In vitro data showed that adding 0.1% Igepal to the harvested cultured cells increased significantly the detection of IL-10, but not for IFN-γ, when compared to the supernatant counterparts. Adding 0.1% Igepal probably lysed white blood cell membrane and thus the captured immunoassay measured the total level: extracellular, intracellular and membrane-bound cytokines. The in vitro data was coherent with the in vivo data since IL-10 detection levels were higher when detergent was used. Previously, it has been shown that when mitogen-stimulated peripheral blood cells were lysed, the measured amount of IL-2 was significantly more than the supernatant counterpart [12], indicating that some cytokines are produced in larger amounts inside the cells more than what is released. In addition, some precursor cytokines such as TNF-α could be present as membrane-bound prior to its release [1]. Therefore, using Igepal as a detergent might as well release such membrane-bound cytokines. The latter suggestion was documented earlier with membrane bound sialidase in pig liver [9] and also the protein refolding yield was enhanced when the same detergent was used [22].

Many studies have used LPS to induce cytokines in tissues such as spleen, liver, lungs, and brain. Almost all of these studies checked the induced cytokines following isolation of mononuclear cells from the tissues/organs. To the authors knowledge, very few reports measured protein cytokines in tissues [4,15–17]. One recent report measured cytokines (IFN-γ, IL-10, and TGF-β) from tissues following disruption and homogenization [4]. In the latter study, the concentrations of cytokines following LPS administration were very low even though they used 100 μg of LPS/mouse. This lower recovery due to; not using a detergent to facilitate the extractions of cytokines from tissues, time of tissue collection after LPS administration (8 h versus 2h in the present study), mice genotype, and the type of LPS used. The present work demonstrated superiority, after using the detergent, to extract cytokines from tissues, including the percent of increase in cytokines concentrations following peripheral LPS administration.

It has been demonstrated that cytokines mRNA (IL-1β, TNF-α, IL-6) are increased in the brain, spleen, liver and adipose tissue following LPS administration [15,16,20]. The consistent increase in the mRNA levels of such cytokines in peripheral organs is also associated with increase of protein cytokines in blood which suggests that LPS induces a series of events in local cytokine component up-regulation in various compartments including brain. In the present study, LPS administration increased TNF-α, IFN-γ and IL-10 levels in most of tissues tested. However, the degree of increase varied between tissues indicating a differential cytokine response to LPS with respect to tissue and cytokine types [17]. This is due to LPS effect on the amount/types of immune cells (lymphocytes and macrophages) in tissues, its ability to induce such cytokines from non-immune cells such as tissue fibroblasts, endothelial cells and enterocytes via their

expression of CD14 or Toll-like receptor 4 [7], and to induce glucocorticoids and catecholamines in certain organs. These neuro-hormonal mediators are known to induce a balance between tissue pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory (IL-10) cytokines [10,11,15–17]. Such cytokine-cytokine interactions with positive or negative feedback, may be pivotal for modulating the cascade of events associated with neurological manifestations of LPS administration. In the present study, IL-10 levels (in molar concentrations) were significantly more than the two pro-inflammatory cytokines; TNF-α and IFN-γ, in the brain, skin and kidneys before and after LPS administration, even though the percent increase in TNF-α level was highest following LPS administration in the investigated tissues. It has been shown that peripheral LPS administration activated hypothalamus-pituitary-axis and sympathetic nervous system to release glucocorticoids, catecholamines and β-endorphins [17,20]. Thus, what kept IL-10 levels predominant in the brain, for instance, following LPS administration would be the high induction of catecholamines in the brain tissue [18]. Regarding kidneys and skin tissues, the slight drop in the IL-10 levels following LPS administration could be explained by the regulatory effect following an increase in pro-inflammatory cytokines. However, the latter observation needs further investigation.

In conclusion, the present study describes a simple method for the determination of protein cytokines in tissues which reflects the actual function of such cytokines. Such method should provide an important tool for studying the local protein cytokines in behavioral pattern, sickness behavior, and immune-mediated diseases as well as to determine local therapeutic efficacy of immunomodulatory drugs.

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