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Dopamine selectively modulates lipopolysaccharide-induced TNF-alpha, IFN-gamma and IL-10 within mice tissues

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Key words: dopamine; cytokines; cytokine modulation; tissues; dopamine antagonists; IL-10; IFN-γ; TNF-α

Abstract

Dopamine (DA) administration in sepsis is used to modulate the hypotensive condition and to normalize the blood vessels perfusion. However, whether this administration of DA has an effect on the release of cytokines in vivo deserves investigation.

Pre-exposure of DA (1 μg/ml) to whole blood enhanced IL-10 (30%) production level following LPS stimulation. This IL-10 enhancement became statistically significant ($p<0.001$) upon the addition of D2-DA receptor (DAR) antagonists, Clozapine or Haloperidol. Furthermore, systemic administration of DA (0.5–50 mg/kg) in mice suppressed significantly LPS-induced TNF-α levels in blood, liver, spleen, brain, and lungs; IL-10 levels in blood, brain and liver; and IFN-γ levels in blood, liver, brain, and lungs. On the other hand, DA enhanced significantly LPS-induced IL-10 production in the lungs and spleen, and IFN-γ levels in the spleen. Administration of Clozapine (54 mg/kg) or Haloperidol (62 mg/kg) with LPS (1 μg) and

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DA (5 mg/kg) reversed DA suppressive effects on LPS-induced cytokines in blood, IFN-γ in brain and lungs, and enhanced significantly LPS-induced IL-10 production in blood, spleen, liver, and lungs.
These results indicate that DA modulatory effect on LPS-induced blood cytokines-producing cells is mediated mainly by D2-DAR (D2/ D3/D4) through enhancing immune cells migration and extravasation into tissues. Furthermore, DA selectivity on cytokines modulation is tissue specific, mediated by the type of DAR expressed and on the immune cells lodged in each tissue.

INTRODUCTION

Sepsis is the most serious infectious consequence causing high mortality (Moreno *et al.* 2006). In severe cases, sepsis is usually combined with hypotension and hypo-perfusion resulting in organ dysfunction syndrome (Moreno *et al.* 2006). The pathogenesis of sepsis involves an excessive production of pro-inflammatory cytokines in response to microbial secretion of chemicals such as lipopolysaccharide, lipoteichoic acid, or protein exotoxins, leading to a cytokine storm (Sriskandan & Altmann 2008). Dopamine (DA) administration in sepsis is used to modulate the hypotensive condition and to normalize the blood vessels perfusion (De Backer *et al.* 2010). However, whether this administration of DA has an effect on the release of cytokines *in vivo* deserves investigation.

DA is a neurotransmitter that exerts its function through stimulating DA receptors (DAR) expressed on the cell surface of several types of brain cells as well as kidneys, lungs, adrenal glands, spleen, and blood cells (Missale *et al.* 1998; Basu & Dasgupta 2000; Pivonello *et al.* 2001; Watanabe *et al.* 2006). There are two main types of DAR; D1-DAR (D1 and D5), and D2-DAR (D2, D3 and D4). D1-DAR are coupled with Gαs (stimulatory) class of G proteins whereas D2-DAR are coupled with Gαi (inhibitory) class of G proteins (Sibley *et al.* 1993; Missale *et al.* 1998; Pivonello *et al.* 2001; Neve at al. 2004). For instance stimulation of D1/5 DAR on human T cells and cerebrovascular endothelium cells resulted in activation of adenylate cyclase leading to an increase in c-AMP which in turn activates protein kinase A (PKA) (Bacic *et al.* 1991; Saha *et al.* 2001). This process inhibits translocation of nuclear factor-κB (NF-κB) through retarding the degradation of the inhibitor (IκB-α) of NF-κB and thus suppresses different pro-inflammatory cytokines genes (Abraham *et al.* 2001). On the other hand, stimulation of D2-DAR, which are expressed on

lymphocytes, endothelial cells, neutrophils and platelets, showed contradictory results on the NF-κB dependent pathway (Bacic *et al.* 1991; Emerson *et al.* 1999; Basu & Dasgupta 2000). It has been shown that stimulating D2-DAR promotes inhibition of c-AMP synthesis in HeLa cells (Yang *et al.* 2003). Other studies, however, have shown that D1 and D2-DAR can be coupled to other Gα proteins, thus triggering different signaling pathways to increase or decrease c-AMP production respectively (Missale *et al.* 1998; Sidhu 1998; Neve *et al.* 2004). This differential coupling of D1 and D2-like receptors may indicate that DA signal distinct cellular effects in two different kinds of cells expressing the same DAR. Furthermore, differential expression of DAR on different tissues also contributes to DA exerts distinct effects on those cells (Missale *et al.* 1998; Pacheco *et al.* 2009).

In vitro, DA concentrations at 10^{-16} to 10^{-4}M were found to activate resting human T cells via D2 and D3 receptors (Levite *et al.* 2001), and secrete TNF-α through activation of D3 or D5 and IL-10 through activation of D2 or D5 receptors (Besser *et al.* 2005). On the other hand, DA or DA agonists were found to inhibit cytokines production like TNF-α, IL-1, IL-12, IL-6, and IL-8 from monocytes/macrophages during the early phase of inflammation (Beck *et al.* 2004). Furthermore, at high concentrations, DA activates α and β adrenoceptors as has been demonstrated using monocytic cell lines (Platzer *et al.* 2000; Bergquist *et al.* 2000; Farmer *et al.* 2000; Brenner *et al.* 2003). It has been shown that DA activates α2 adrenoceptors and induces pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, and anti-inflammatory cytokines (e.g. IL-10) through activation of NF-κB, whereas DA stimulates β adrenoceptors and inhibits the transcription of NF-κB similar to D1-like receptors pathway (Bergquist *et al.* 2000, Farmer *et al.* 2000). *In vivo,* administration of DA (0.1 nmol) in mice selectively attracted naïve CD8 T cells into peritoneal cavity and treatment with D3 antagonists reduced homing of those naïve T cells into lymph nodes (Watanabe *et al.* 2006). These DA effects on cytokine production show the importance of DA in delivering signals from the brain to T cells and other immune cells which are important for effective, proper, regulated and orchestrated immune responses (Levite 2008).

The present study demonstrates the *in vivo* effect of pharmacological doses of DA (Basu & Dasgupta 2000; Basu *et al.* 2001) on LPS-induced IFN-γ, TNF-α and IL-10 in several organs. The use of LPS represents a mild-moderate sepsis condition. The cytokines selected herein represent proinflammatory and anti-inflammatory cytokines with respect to the type of cells producing them. IFN-γ is produced primarily by T helper-1 (Th1) lymphocytes, natural killer (NK) lymphocytes and also B lymphocytes, whereas TNF-α is produced by

variety of cells including monocytes/macrophages (resident macrophages), T, B and NK lymphocytes, astrocytes, endothelial cells, and smooth muscle cells (Abbas *et al.* 1996; Laengle *et al.* 2003; Cosentino *et al.* 2005; Lund 2008). IL-10, on the other hand, is produced by Th2 cells, monocytes/ macrophages (resident macrophages), B lymphocytes, keratinocytes, and glial cells (Abbas *et al.* 1996; Cosentino *et al.* 2005; Lund *et al.* 2008). The objective is to show DA effect on the production of IFN-γ, TNF-α and IL-10 following LPS activation from several organs/lodging cells expressing different DA receptors and to reveal whether this effect is mediated through DAR. The long term objective is to utilize such knowledge in formulating further studies on DA immunomodulatory effect in sepsis and specific autoimmune mediated diseases.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS, L-6143), endotoxin-free phosphate buffer saline (PBS), RPMI-1640, L-glutamine, penicillin-streptomycin and Igepal CA-630 were purchased from Sigma (St. Louis, MO, USA). Cytokine Duoset kits were purchased from R&D systems, UK. Bovine serum albumin (BSA) was purchased from Research Organics (Cleveland, OH, USA). DA (Dopamine HCL 50 mg/5 ml), as a product of DEMO S.A. Pharmaceutical industry (Kifissia, Greece) and Haloperidol tablets as a product of Janssen-Cilag Pharmaceutical Company (Beerse, Belgium) were utilized in the experiments. Clozapine was provided from the Novartis Pharmaceutical scientific office in Amman, Jordan. The 6-well culture plates and Maxisorp 96-well flat-bottomed microtiter plates were purchased from Nunc International (Roskilde, Denmark).

Subjects

Ten healthy human volunteers (6 males and 4 females) with a mean average age of 25±0.4 years old were enrolled in the study after signing an informed consent. The females were in the early to mid follicular phase (3–9 days) of their menstrual cycle (Matalka 2003a). None of the volunteers has taken any medication for at least a week, did any exercise, or ate before the blood sample was drawn. All blood samples were drawn in the morning between 8 and 9:30 a.m.

Whole human blood culture for dopamine study

Blood samples were withdrawn from healthy subjects (males and females) into sterile vacutainer heparinized tubes (Becton Dickinson). Whole blood

was diluted (1:10) with RPMI 1640 media, containing 2 mM L-glutamine, 100 u/ml penicillin and 100 µg/ml streptomycin, without exogenous serum and containing different concentrations of (0, 0.0001, 0.001, 0.01, 0.1, 1 µg/ml) of DA (Matalka 2003b). The plates were incubated for 24 h at 37 °C in 5% CO_2 incubator. On the second day and after the first incubation, 40 µL volume of PBS, or LPS (1 µg/ml) was added, and plates were further incubated for another 24 hours. After the second incubation, blood was collected from wells into sterilized tubes and each well was washed with 0.5 ml of sterile PBS solution to be sure that all content of the well has been removed. Tubes were centrifuged and the supernatants were collected and stored in sterilized tubes at –30 °C until assay. Previous studies from our lab and others demonstrated that LPS does not induce IFN-γ from whole blood cultures (Elenkov *et al.* 1998; Matalka 2003a), therefore IFN-γ concentrations were not studied following *in vitro* LPS stimulation. Finally, mixing studies of DA (0, 0.1 and 1 µg/ml) with excess of DA antagonists (12.3 µg/ml for Haloperidol; 10.7 µg/ml for Clozapine), without or with LPS stimulation were performed as above.

Animals

Male healthy Balb/c mice of 12–14 weeks old weighing 35 ± 3 g were used in the study. Animals were housed on a 12:12 hr light cycle at 22 ± 2 °C with food and water available *ad libitum*. All animal experiments were performed in compliance with Petra University guidelines. Mice were divided into groups (5 mice/group) and each group of mice was injected intraperitoneally (i.p.) with 1 ml of the following solutions: endotoxin-free PBS, 1 µg of LPS, 0.5 mg/kg of DA plus 1 µg of LPS, 5 mg/kg of DA plus 1 µg of LPS, and 50 mg/kg of DA plus 1 µg of LPS ((Basu & Dasgupta 2000; Basu *et al.* 2001). For the DA antagonists study, Clozapine and Haloperidol were used due to their selective and non-selective D2-DAR antagonistic effects, respectively, as well as their possible anti-inflammatory effects (Moots *et al.* 1999; Song *et al.* 2000). Mice were divided into groups (5 mice/group) and the antagonist solutions were filtered through 0.2 µm sterile filters. Each group of mice was injected i.p. with 1 ml of the following solutions: endotoxin-free PBS, 1 µg of LPS, 5 mg/kg of DA plus 1 µg of LPS, 5 mg/kg of DA plus 1 µg of LPS mixed with 62 mg/kg of Haloperidol, and 5 mg/kg of DA plus 1 µg of LPS mixed with 54 mg/kg of Clozapine.

Mice tissue extracts for cytokine assay

Following administration of solutions, animals were sacrificed after two hours. It has been shown earlier that 2–6 h time points are considered the best

times for LPS-induced cytokine production in mice (Gao *et al.* 2000; Matalka *et al.* 2005). Cytokines were extracted from mice tissues in accordance with a method described elsewhere (Matalka *et al.* 2005). Briefly, two hours after administration mice were scarified by cervical dislocation and organs were collected within 6–10 minutes. After cervical dislocation, blood was collected from cardiac chamber, put into a tube and weighed, followed by adding 2 ml ice-cold endotoxin-free PBS containing 0.1% Igepal CA-630 and left incubating for 10 minutes under ice. Lungs, liver, spleen, and brain were removed from mice, put into tubes, weighed and incubated with 2 ml of ice-cold endotoxin-free PBS containing 0.1% Igepal CA-630 for 10 minutes under ice. This was followed by homogenizing the tissues/organs by a tissue disrupter (Janke and Kundle). Tubes were centrifuged for 5 min, and the supernatants were collected into labeled sterile curvets, stored at –30 °C for cytokine analysis.

Cytokine assays

Extracted mouse cytokines (IL-10, IFN-γ and TNF-α) and human cytokines (IL-10, TNF-α) were assayed using enzyme linked immunosorbent assay adapting the procedures recommended by the manufacturer (mouse or human DuoSet, R&D Systems, UK). The cytokines were measured twice and each time samples were run in duplicate. Absorbance was read at 450 nm and 600 nm as a differential wavelength by ELISA plate reader (SCO GmbH, Dingelsadt, Germany) and then transformed to cytokines concentrations (pg/g) using a standard curve computed on excel sheet after transforming values to log to construct a straight line on log-log graph.

Data Analysis

All mice data are presented as a mean ± SE in pg/g of tissue. For whole blood culture studies, relative concentrations are used instead of absolute concentrations values. This is because each volunteer cytokine levels were normalized to his/her control values (Matalka *et al.* 2003a,b). All data were assessed by using one way ANOVA analysis followed by a Tukey's test (95% confidence) for multiple comparisons using SPSS 11.5. *p*-value less than 0.05 was considered significant.

RESULTS

Pre-exposure of DA modulates IL-10 in LPS-stimulated human whole blood cultures

DA at 0.1 and 1 μg/ml did not increase IL-10 levels more than it is counterpart controls from non-stimulated whole blood cultures. Similarly, DA mixed with 5 molar excess of DA antagonists did not induce any change in IL-10 production from non-stimulated whole blood cultures (Figure 1). Based on these experiments, DA or DA antagonists' administration *in vivo* was not performed.

In LPS-stimulated whole blood cultures, DA enhanced the production of IL-10 level by 30%. These IL-10 levels further increased and became statistically significant upon the addition of DA antagonists; Haloperidol and Clozapine ($p<0.001$) (Figure 1). On the other hand, DA did not modulate TNF-α from LPS-stimulated whole blood cultures (data not shown).

Concurrent DA administration with LPS modulates cytokine production in mice tissue extracts

After two hours of administration, LPS induced IL-10 production in blood, liver, brain and lungs, but not in spleen (Figure 2). DA administration, however, suppressed significantly LPS-induced IL-10 levels in blood (37–52%),

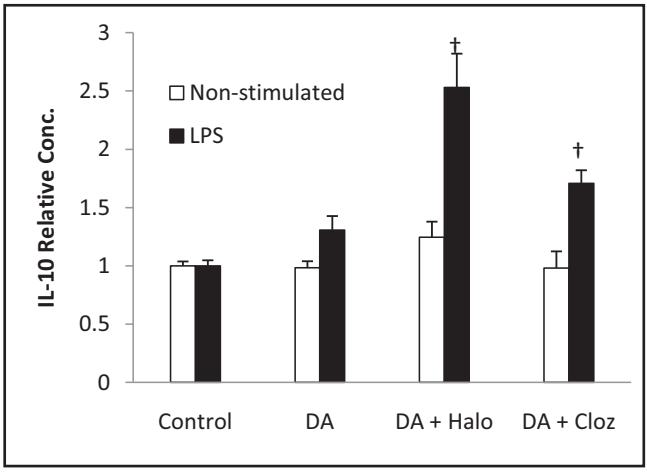

Fig. 1. DA (1 μg/ml) alone or with Haloperidol and Clozapine (5 molar in excess of DA) did not modulate IL-10 production levels in non-stimulated whole blood. In LPS-stimulated whole blood, however, DA antagonists; Haloperidol and Clozapine increased significantly the production of IL-10 levels when they were added to DA (†: $p<0.001$).

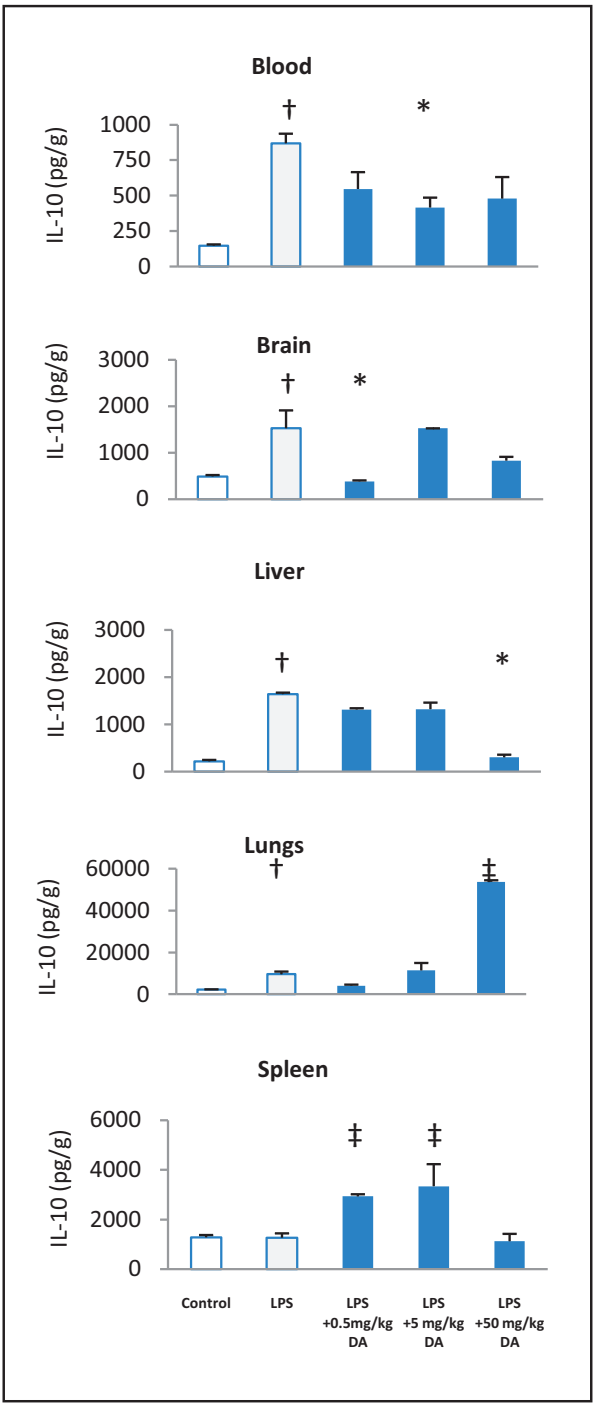

Fig. 2. Systemic DA administration modulated significantly LPS-induced IL-10 in blood ($p<0.006$), brain ($p<0.01$), liver ($p<0.007$), lungs ($p<0.008$) and spleen ($p<0.01$). LPS administration increased significantly IL-10 production levels (†) in blood, brain, liver, and lungs. DA administration, however, suppressed significantly (*) LPS-induced IL-10 production in blood (at 5 mg/kg of DA), brain (at 0.5 mg/kg of DA), and liver (at 50 mg/kg), whereas DA administration enhanced significantly (‡) LPS-induced IL-10 production level in lungs (at 50 mg/kg of DA), and in spleen (at 0.5 and 5 mg/kg of DA). (†: LPS against control; *: DA against LPS if decreased; ‡ DA against LPS if increased) (n=5, ±SE).

brain (52% at 0.5 mg/kg of DA), and liver (80% at 50 mg/kg of DA) (Figure 2). On the other hand, DA administration at 50 mg/kg enhanced LPS-induced IL-10 production in the lungs by 460% (Figure 2). In spleen, no change in IL-10 level was observed following LPS administration, but when DA at 0.5 and 5 mg/kg was co-administered with LPS, IL-10 production levels in spleen increased significantly by 131 and 163%, respectively (Figure 2).

LPS administration in mice induced TNF-α production in all examined tissues (Figure 3). DA administration, however, suppressed significantly LPS-induced TNF-α production levels in blood (52–78%), brain (30–49%), liver (40–84%), lungs (33% at 5 and 50 mg/kg of DA), and spleen (23–77%) (Figure 3).

As for IFN-γ, LPS administration in mice induced its levels in blood, brain, liver, lungs, and spleen (Figure 4). DA administration, however, suppressed significantly LPS-induced IFN-γ production in the blood (40–62%), brain (29–55%), liver (45–83%), and lungs (43–51%) (Figure 4). In the spleen, co-administration of DA at 5 mg/kg increased significantly LPS-induced IFN-γ production by 63%, but the latter level was not observed at a higher dose of DA (Figure 4).

Effects of D2-DAR antagonists on DA modulation of LPS-induced cytokine production in selected tissue/organs

In the following sets of experiments DA at a dose of 5 mg/kg has been chosen based on its modulatory effect on TNF-α, IFN-γ and to a lesser extent on the anti-inflammatory IL-10 levels to investigate the role of D2-DAR on DA-selective cytokine modulation within tissues, as well as their anti-inflammatory effects. Similar to the results of previous experiments, DA at 5 mg/kg suppressed LPS-induced IL-10 level in blood only, and enhanced its level in the spleen (Figures 2 and 5, Table 1). When Haloperidol or Clozapine was co-administered with DA, both of them reversed DA modulatory effect on LPS-induced IL-10 production level in the blood (Figure 5, Table 1). Furthermore, administration of these two antagonists with DA enhanced significantly LPS-induced IL-10 production level in blood, liver, lungs and spleen but with different effects (Figure 5). Haloperidol was more effective in enhancing LPS-induced IL-10 production in lungs, whereas Clozapine was more effective in spleen (Figure 5). In the brain, however, LPS-induced IL-10 production level was decreased significantly following co-administration of Haloperidol with DA.

As for LPS-induced TNF-α production level, DA administration at 5 mg/kg suppressed significantly its levels in blood, liver, spleen and brain (Figures 3

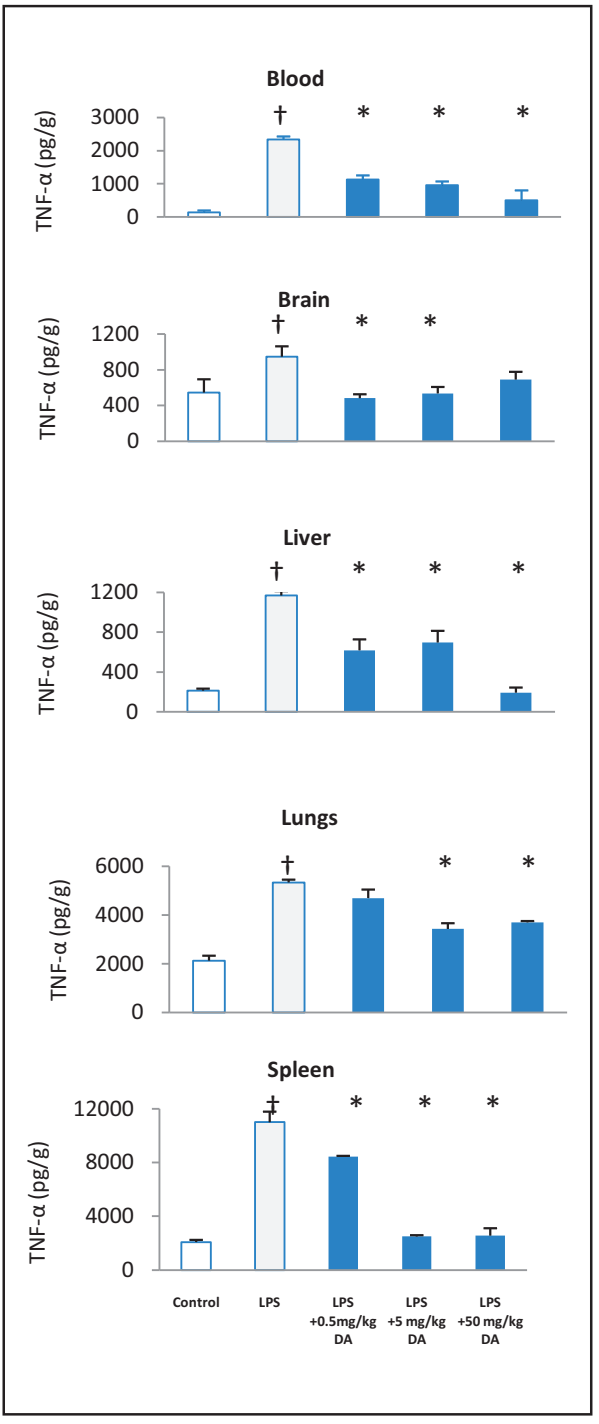

Fig. 3. Systemic DA administration modulated significantly LPS-induced TNF-α in blood ($p<0.0001$), brain ($p<0.02$), liver ($p<0.0001$), lungs ($p<0.001$) and spleen ($p<0.0001$). LPS administration increased significantly TNF-α production levels (†) in blood, brain, liver, lungs and spleen. DA administration, however, suppressed significantly (*) LPS-induced TNF-α production in blood, brain (at 0.5 and 5 mg/kg of DA), liver, lungs (at 5 and 50 mg/kg of DA), and spleen. (†: LPS against control; *: DA against LPS if decreased; ‡ DA against LPS if increased) (n=5, ±SE).

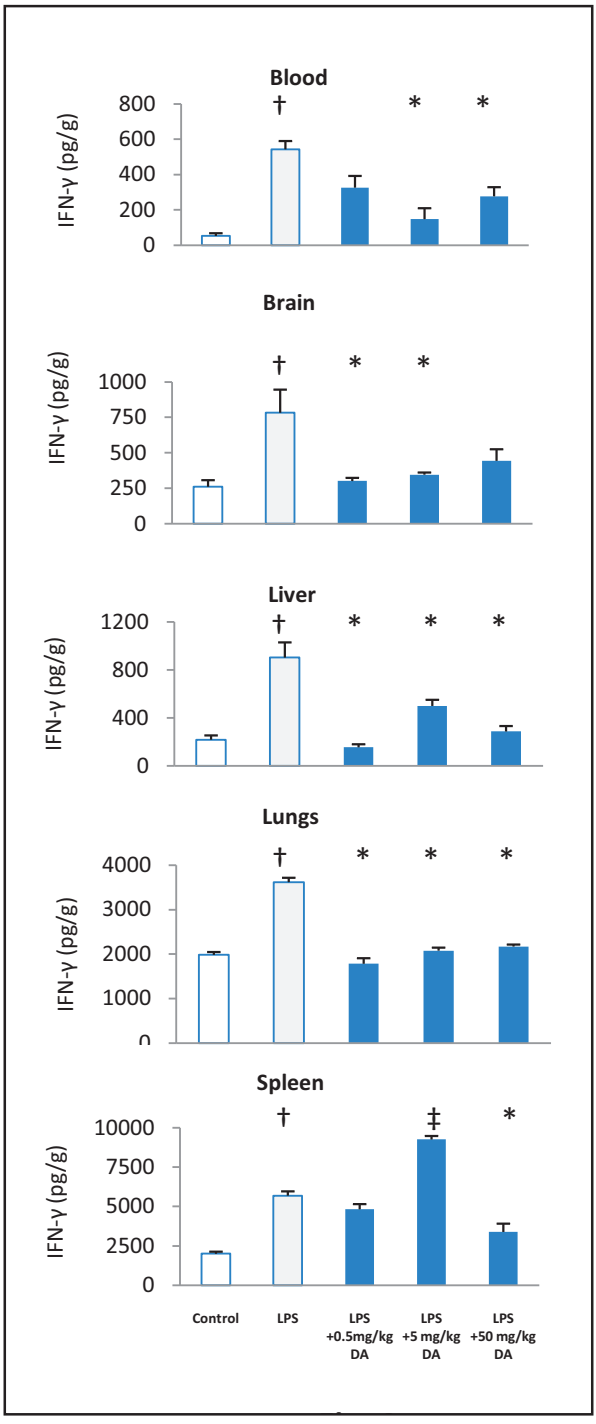

Fig. 4. Systemic DA administration modulated significantly LPS-induced IFN-γ in blood ($p<0.001$), brain ($p<0.01$), liver ($p<0.0001$), lungs ($p<0.0001$) and spleen ($p<0.0001$). LPS administration increased significantly IFN-γ production levels (†) in blood, brain, liver, lungs and spleen. DA administration, however, suppressed significantly (*) LPS-induced IFN-γ production levels in the blood (at 5 and 50 mg/kg of DA), brain (at 0.5 and 5 mg/kg of DA), liver, lungs and spleen at 50 mg/kg of DA), whereas DA at 5 mg/kg enhanced significantly (‡) LPS-induced IFN-γ production level in the spleen. (†: LPS against control; *: DA against LPS if decreased; ‡ DA against LPS if increased) (n=5, ±SE).

and 6). When Haloperidol or Clozapine was co-administered with DA, both of them reversed DA modulatory effect on LPS-induced TNF-α production level in blood (Figure 6, Table 1). In brain, liver and spleen, however, Haloperidol and Clozapine enhanced the suppressive effect of DA on LPS-induced TNF-α production level, whereas Clozapine showed similar effects in lungs (Figure 6, Table 1).

In case of LPS-induced IFN-γ production level, similar DA modulatory effects were obtained (Figures 4 and 7). When Haloperidol or Clozapine was co-administered with DA, Clozapine reversed DA modulatory effect on LPS-induced IFN-γ production level in liver, lungs and blood, whereas Haloperidol reversed such effect in the lungs, brain, spleen and partially in blood (Figure 7, Table 1). In the brain, however, Haloperidol enhanced significantly LPS-induced IFN-γ production level by 146% (Figure 7).

Tab. 1. Dopamine modulatory effect on LPS-induced IL-10, TNF-α and IFN-γ production in several tissues and its mediation by type-2 DAR expression.

Organ	Cytokine	DA (5 mg/kg)	DA+Halo	DA+Cloz	DAR Expression*
Blood	IL-10	↓**	R↑	R↑	D2,D3, D4,D5
Brain	IL-10	↔	↓	NC	D1, D2, D3, D4, D5
Liver	IL-10	↔	↑	↑	D1, D4, and D5
Lungs	IL-10	↔	↑↑	↑	D1, D2, D3, D4, D5
Spleen	IL-10	↑	↑↑	↑↑↑	D3 and D4
Blood	TNF-α	↓	R	R	D2,D3, D4,D5
Brain	TNF-α	↓	↓↓	↓↓	D1, D2, D3, D4, D5
Liver	TNF-α	↓	↓↓	↓↓	D1, D4, and D5
Lungs	TNF-α	↔↓	NC	↓↓	D1, D2, D3, D4, D5
Spleen	TNF-α	↓	↓↓	↓↓	D3 and D4
Blood	IFN-γ	↓	RP	R	D2,D3, D4,D5
Brain	IFN-γ	↓	R↑	R	D1, D2, D3, D4, D5
Liver	IFN-γ	↓	NC	R	D1, D4, and D5
Lungs	IFN-γ	↓	R	R	D1, D2, D3, D4, D5
Spleen	IFN-γ	↑	R	NC	D3 and D4

* as has been identified and detected by references (7, 15, 35, 39, 49, 50)
** ↔: no effect; ↓: Decreased (significant); ↑: Increased (significant); NC: No change;
R: Reversed (significant); RP: Partial Reversion (significant); R↑: not only reversed but also significantly

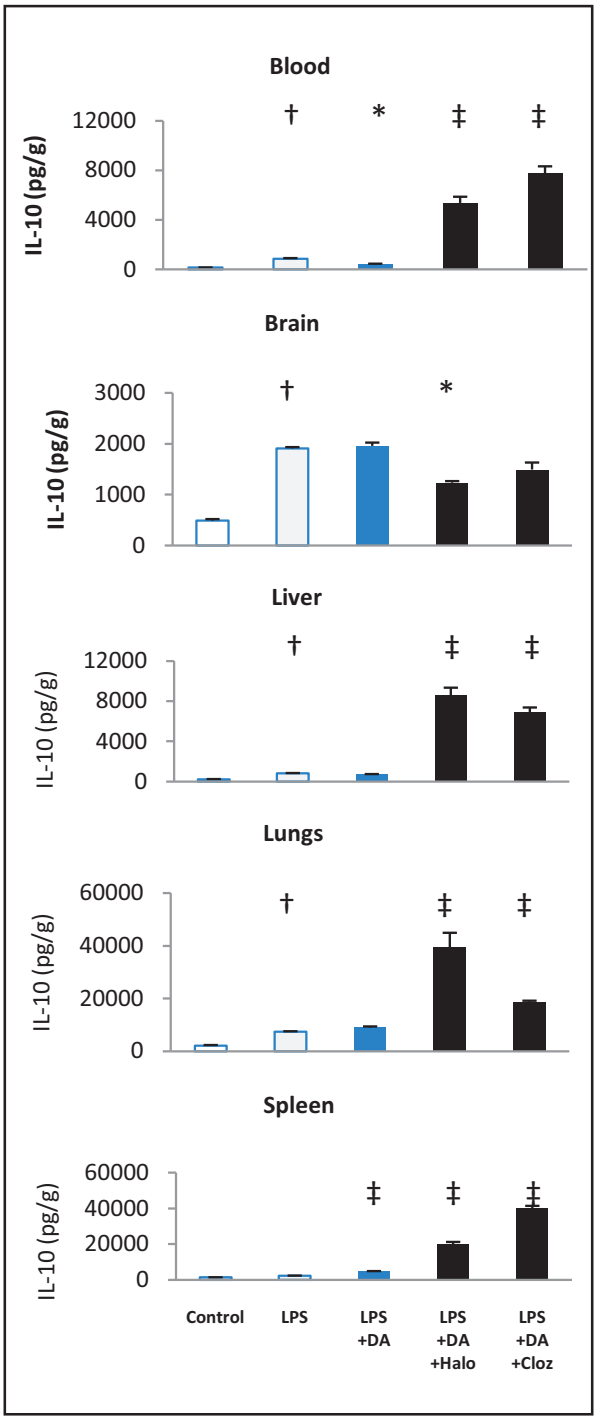

Fig. 5. Systemic administration of DAR antagonists (Haloperidol and Clozapine) not only reversed DA suppressive effects on LPS-induced IL-10 but also enhanced (‡) IL-10 production significantly in blood ($p<0.0003$), liver ($p<0.0001$), lungs ($p<0.0001$) and spleen ($p<0.0001$). On the other hand, Haloperidol with DA suppressed significantly (*) LPS-induced IL-10 levels in the brain ($p<0.001$). (†: LPS against control; *: DA against LPS if decreased; ‡ DA antagonist + DA against LPS if increased) (n=5, ±SE).

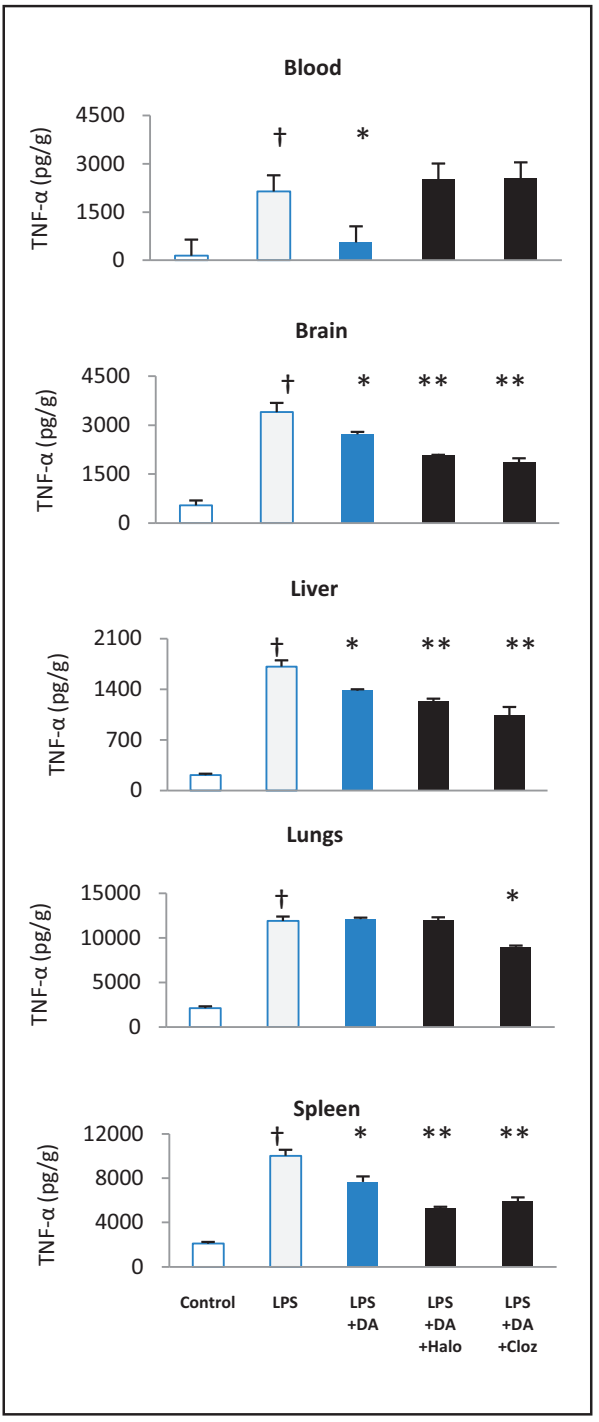

Fig. 6. Systemic administration of DAR antagonists (Haloperidol and Clozapine) reversed the suppressive effect of DA on LPS-induced TNF-α in blood (p<0.0001). On the other hand, Haloperidol and Clozapine enhanced the suppressive effects (**) of DA on LPS-induced TNF-α production in brain (p<0.001), liver (p<0.001) and spleen (p<0.0001), whereas Clozapine only showed similar effects on TNF-α production in lungs. (†: LPS against control; *: DA or DA antagonist + DA against LPS if decreased; ** DA antagonist + DA against DA) (n=5, ±SE).

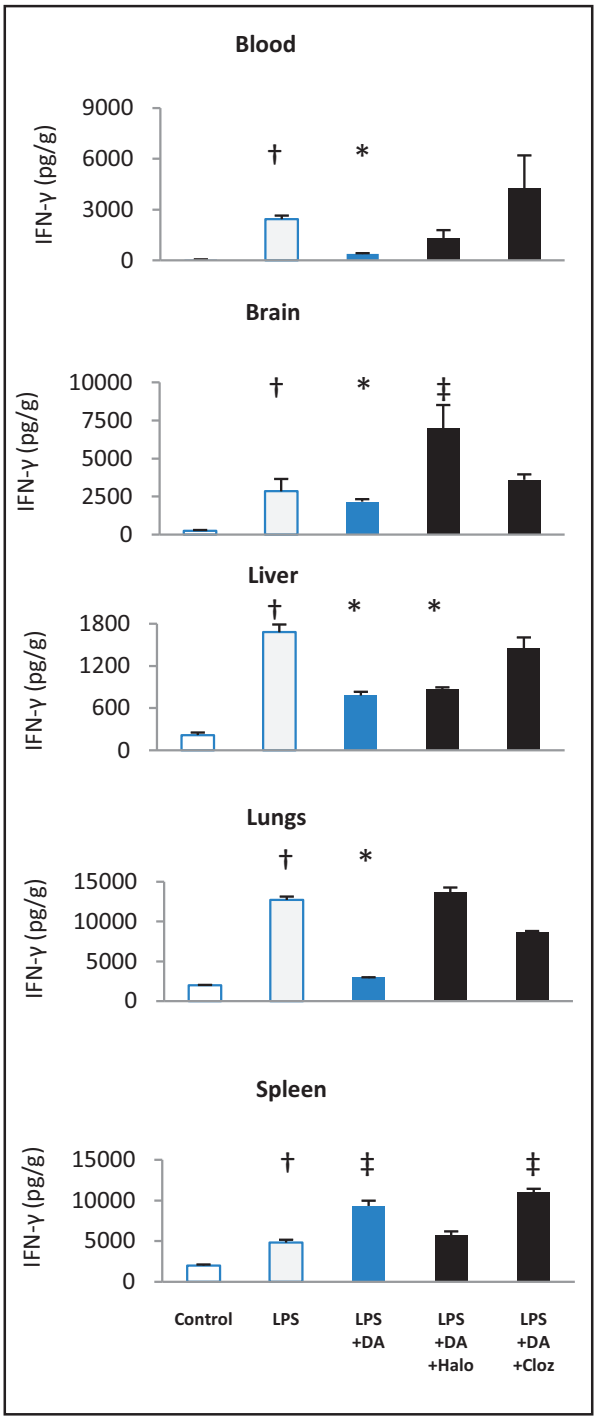

Fig. 7. Systemic administration of DA antagonists, Haloperidol and Clozapine, reversed the suppressive effects of DA on LPS-induced IFN-γ in blood ($p<0.001$), brain ($p<0.001$), and lungs ($p<0.0001$) whereas Clozapine only showed similar effects on IFN-γ production in the liver. In addition, Haloperidol reversed the enhancing effect of DA on LPS-induced IFN-γ in spleen and enhanced (‡) LPS-induced IFN-γ in brain ($p<0.001$). (†: LPS against control; *: DA or DA antagonist + DA against LPS if decreased; ‡ DA or DA antagonist + DA against LPS if increased) (n=5, ±SE).

DISCUSSION

LPS stimulates immune cells including monocytes/macrophages, dendritic cells, T lymphocytes, B lymphocytes and non-immune cells such as, endothelial cells, vascular smooth muscles, fibroblasts, glial cells through binding to plasma membrane-expressing CD14 (Vink *et al.* 2002; Laengle *et al.* 2003; Cosentino *et al.* 2005; Li *et al.* 2007). LPS-bound CD14 forms a complex with Toll like receptors 4 (TLR4) and initiates the TLR signaling pathway and thus cytokine production (Li *et al.* 2010). In addition, an extracellular adaptor protein MD2, which interacts with TLR4, is critical for TLR4 mediated LPS recognition and signaling (Li *et al.* 2010). These TLR4 signaling pathways include NF-κB, phosphatidylinositol 3-kinases (PI-3k), interferon regulatory factors (IRFs), and mitogen-activated-protein-kinases (MAPK; ERK, P53, JNK) stimulation (Pacheco *et al.* 2009; Li *et al.* 2010). The above mentioned immune cells also express DARs as well as brain cells, lungs, liver and spleen cells. Except for brain and lungs, D1-DAR and D2-DAR are differentially expressed on the latter cells and tissues (Table 1). Therefore, when DA at 0.5–50 mg/kg was administered with LPS, the production levels of IL-10, IFN-γ and TNF-α were modulated differently in the above organs. At these concentrations DA also stimulates α and β adrenergic receptors as well as DAR (Bergquist *et al.* 2000; Farmer *et al.* 2000; Brenner *et al.* 2003; Beck, *et al.* 2004).

It is known that DA does not cross the blood brain barrier; however, DA co-administration with LPS modulated cytokines in the brain of mice. This can be explained by the ability of LPS to disrupt the blood brain barrier (Pitossi *et al.* 1997; Jaeger *et al.* 2009) and therefore facilitates DA entry to the brain; both LPS and DA induce a transmission of information from the immune system to the brain via cytokines produced in close proximity of the brain and circulate through the blood stream to the brain cavity (Jaeger *et al.* 2009); and/or influence the migration of DA-modified immune cells such as T regulatory cells inside the brain (Ilani *et al.* 2004). Furthermore, dopamine administration was found to enhance migration and extravasation of immune cells such CD8+ve T cells into secondary lymphoid organs (Watanabe *et al.* 2006) through increasing the expression of integrins, and thus mediated T and probably other immune cells adhesion and extravasation (Levite *et al.* 2001; Ilani *et al.* 2004; Watanabe *et al.* 2006). Similarly, the present study showed that DA administration suppressed all LPS-induced cytokines production in peripheral blood and these effects were reversed by blocking D2-DAR (D2/D3/D4) via administration of Haloperidol and Clozapine. Since D2-DAR were found to be expressed on endothelial cells (Bacic *et al.* 1991; Basu *et al.* 2001), our results indicate that DA enhances migration or extravasation of

cytokines-producing immune cells from blood to tissues mediated mainly by D2-DAR (D2/D3/D4).

In this study, DA administration was found to suppress LPS-induced production of TNF-α and IFN-γ levels, except for IFN-γ in spleen. It is likely that these effects were in response of DA binding to D1-DAR (D1/D5) and α/β adrenoceptors expressed on the above tissues to induce activation of adenylate cyclase and thus increase intracellular c-AMP level (Beck *et al.* 2004; Besser *et al.* 2005; Watanabe *et al.* 2006). Such elevation of c-AMP inhibits some LPS-TLR4 pathways such as IRFs, PI-3K and stimulates ERKs in dendritic cells and macrophages which lead to a decrease in TNF-α, IL-12 (Beck *et al.* 2004). IL-12 induced IFN-γ production and to an increase in IL-10 production (Hickey *et al.* 2008; Pacheco *et al.* 2009). Since it has been found that activation of D-2 DAR blocks intracellular c-AMP level (Bacic *et al.* 1991; Basu & Dasgupta 2000; Pacheco *et al.* 2009), administering type-2 DAR antagonists should further enhances the suppression of DA on LPS-induced TNF-α in brain, liver, lungs and spleen as seen herein.

DA modulatory effects on LPS-induced IL-10, IFN-γ, and TNF-α in different tissues/organs exhibited somewhat a dose-independent effect (Figures 2–4). This could be due to DA selectivity; migration and extravasation of blood cytokine-producing cells into tissues; and differential DAR expression (Table 1). The differential expression of D1 and D2-DAR (D1/D5 and D2/D3/D4) and α/β adrenoceptors on the cytokine-producing immune cells lodged in such tissues and resident macrophages (e.g. Kupffer cells, microglial cells) determines the effect of dopamine on the LPS-induced cytokines as well as the ability of the non-immune cells (endothelial, fibroblasts, smooth muscles) located in such organs to produce the tested cytokines and TNF-α, respectively upon administrating high DA concentrations (Basu & Dasgupta 2000; Basu *et al.* 2001; Beck *et al.* 2004). Since the expression of DAR on monocytes/macrophages has not been confirmed (Brenner *et al.* 2003; McKenna *et al.* 2002), it is safe to suggest that DA cytokine modulatory effects on the above cells involve α/β adrenoceptors receptors (Platzer *et al.* 2000; Bergquist *et al.* 2000; Farmer *et al.* 2000; Hasko *et al.* 2002; Brenner *et al.* 2003; Beck *et al.* 2004).

The enhancing effects of DA on IL-10 and IFN-γ production levels in spleen and on IL-10 in the lungs (at 50 mg/kg of DA) could partially be explained by the number and type of immune cells lodged in the spleen and lungs. It has been shown previously that B lymphocytes can produce cytokines and can be divided into discrete cytokine-producing effector and regulatory B cell subsets (Lund 2008). The follicular zone B cells and B1 lymphocytes of spleen

can respond rapidly to TLR ligands and produce IFN-γ, while the marginal zone B lymphocytes produce IL-10 upon stimulation of TLR ligands. Since the number of B lymphocytes lodged in the spleen (Yang *et al.* 2003) and lungs (Lindell *et al.* 2009) are more than T cells and also B cells express DAR (D2/D3/D5) (Missale *et al.* 1998), it could be postulated that DA enhancing effect on LPS-production of IFN-γ and IL-10 are from distinct lodged B lymphocytes in the spleen and lungs (Lund 2008; Lindell *et al.* 2008). However, the latter hypothesis needs more evidence-based studies. Furthermore, DA enhancing LPS-induced IL-10 levels in spleen and lungs could also be through stimulation of β2 adrenoceptor mediated IL-10 production from resident macrophages (Platzer *et al.* 2000; Bergquist *et al.* 2000; Farmer *et al.* 2000; Hasko *et al.* 2002; Brenner *et al.* 2003; Beck *et al.* 2004).

It has been shown upon treatment with D2-D2/D3 agonists, blast CD8+ T cells, and to a lesser extent blast CD4+ T cells produced IFN-γ, but not IL-10 (Jaeger *et al.* 2009). This latter observation points out that DA enhancing effect on LPS-induced IFN-γ production in the spleen is mediated by D2-D2/D3 receptors. In the present study, Haloperidol, but not Clozapine (D4 antagonist), reversed DA modulatory effect on LPS-induced IFN-γ production in the spleen. This supports the fact that DA action in the spleen to modulate LPS-induced IFN-γ production is mediated mainly through D2/D3 receptors expressed on lymphocytes (Li *et al.* 2007; Huang *et al.* 2010), by passing the need for IL-12 (Lund 2008).

Since Clozapine is also a 5-hydroxytryptamine (5-HT) receptor antagonist and 5-HT receptors were found to mediate LPS-induced cytokines (Durk *et al.* 2005), it can be postulated that certain cytokine modulatory effects seen herein by Clozapine, and not by Haloperidol, are mediated by 5-HT receptors. For instance, Clozapine reversed DA suppressive effect on LPS-induced IFN-γ level in the liver and decreased LPS-induced TNF-α in the lungs.

A previous observation has shown that short term treatment with Haloperidol improved the activity of rheumatoid arthritis patient that could be due to suppression of anti-inflammatory cytokines such TNF-α (Moots *et al.* 1999). In addition, long-term treatment of Haloperidol is associated with significantly lower hospital mortality in mechanically ventilated patients (Milbrandt *et al.* 2005). These studies led Machado *et al.* (2007) to study the effect of subcutaneous administration of Haloperidol or Clozapine on plasma TNF-α, IL-1β or IL-10 levels in rats that went through a sepsis model, a cecal ligation puncture (CLP) procedure. Even though the plasma cytokine levels were not significantly changed after 12 to 24 hours of Clozapine and Haloperidol administration in

rats with CLP, 40–50% of increase in plasma IL-10 was noted. These studies are in agreement with findings of the present *in vitro* and *in vivo* regarding Haloperidol and Clozapine enhancing LPS-induced IL-10 levels. The use of dopamine in combination of D2-DAR antagonists may have a better effect in increasing the anti-inflammatory cytokines during sepsis. Since D2-DAR receptors are blocked with Haloperidol or Clozapine, all administered dopamine activates D1-DAR which inhibits inflammatory cytokines production, such as TNF-α, leaving LPS-induction of IL-10 pathways unregulated. More studies are warranted to prove such effects.

These patterns of cytokine modulation could draw the attention of utilizing DA or DA agonists alone or in combination with D2-DAR antagonists as an immunomodulatory agent in sepsis. For instance, DA administration showed the suppression effect of DA on LPS-induced cytokines which could reduce the effect of cytokine storm on blood vessels. Furthermore, in specific organs DA alone and with D2-DAR antagonists enhanced LPS-induced IL-10 and IFN-γ production and suppressed TNF-α in the spleen within 2 hours. This might reduce the inflammatory-related effects during sepsis and enhance the anti-inflammatory effects which are needed in the latter condition. However, these cytokine modulation effects by DA or DA agonists and DA antagonists need further studies to show the possibility of using such agents in sepsis and organ-specific autoimmune mediated inflammatory diseases.

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