Experimental endotoxemia induces leukocyte adherence and plasma extravasation within the rat pial microcirculation

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Short running title: Pial microcirculation in endotoxemic rats

Key words: sepsis, endotoxin, intravital microscopy, blood brain barrier.

Abstract

Objectives: Disturbance of capillary perfusions due to leukocyte adhesion, disseminated intravascular coagulation, tissue edema is critical components in the pathophysiology of sepsis. Alterations in brain microcirculation during sepsis are not clearly understood. The aim of this study is to gain an improved understanding of alterations through direct visualization of brain microcirculations in an experimental endotoxemia using intravital microscopy (IVM).

Methods: Endotoxemia was induced in Lewis rats with Lipopolysaccharide (LPS, 15 mg/kg i.v.). The dura mater was removed via a cranial window to expose the pial vessels on the brain surface. Using fluorescence dyes, plasma extravasation of pial venous vessels and leukocyte-endothelial interaction were visualized by intravital microscopy 4h after LPS administration. Plasma cytokine levels of IL1-β, IL-6, IFN-γ, TNF-α and KC/GRO were evaluated after IVM.

Results: A significant plasma extravasation of the pial venous vessels was found in endotoxemia rats compared to control animals. In addition, a significantly increased number of leukocytes adherent to the pial venous endothelium was observed in septic animals. Endotoxemia also induced a significant elevation of plasma cytokine levels of IL1-β, IL-6, IFN-γ, TNF-α and KC/GRO.

Conclusion: Endotoxemia increased permeability in the brain pial vessels accompanied by an increase of leukocyte-endothelium interactions and an increase of inflammatory cytokines in the plasma.

Introduction

Sepsis is the leading cause of death in intensive care units resulting from a systemic inflammatory response to infection. In response to pathogen associated molecular patterns (PAMPs), large quantities of proinflammatory cytokines are released into the circulation and initiate an unregulated immune cascade that can cause multiple organ failure (Streck *et al.* 2008; Wang *et al.* 2008). Alterations in the microcirculation have been suggested to play a critical role in pathophysiology of sepsis (Lundy *et al.* 2009).

One of the first organs affected in sepsis is the brain. The brain has an immunologic advantage due to its anatomical separation from the immune system by the blood brain barrier (BBB), a lack of lymphatic drainage system, and low expression of histocompatibility complex antigens. The BBB plays an important role in controlling the entry of inflammatory cells and macromolecules into the brain via the selective permeability of microvascular endothelial cell tight junctions and associated astrocytes (Pytel *et al.* 2009). Although the mechanism is not clear, disruption of the BBB and the resulting brain edema has been proposed as a major factor for the pathophysiology of septic encephalopathy and subsequent sepsis induced brain dysfunction. Conflicting evidence exists showing both increased permeability of the BBB in septic encephalopathy (Mayhan 1998; Siami *et al.* 2008) and unchanged BBB permeability with intact tight junctions of endothelial cell in endotoxemic animals (Bickel *et al.* 1998; Rosengarten *et al.* 2008).

To investigate the effect of endotoxin on permeability of the BBB, we used intravital fluorescence microscopy and fluorescence dyes to directly visualize the interaction of leucocytes and endothelium and variation in permeability of the BBB in an *in vivo* endotoxemia rat model.

Material and Methods

Animals

Male Lewis rats (weight 250-300g) were purchased from Charles River (Wilmington, MA, USA) and maintained in the animal care facility of the Faculty of Medicine at Dalhousie University. Animals were provided with water and rodent chow *ad libitum* under standard 12-hour light/dark rhythmic conditions. All animal experimentations were undertaken in compliance with the guidelines of the Canadian Council on Animal Care.

Anaesthesia and preparation

Rats were anaesthetized with an initial intraperitoneal (i.p.) injection of sodium pentobarbital (65 mg/kg, Ceva Sante Animale, Montrol, QC, Canada) and maintained by repeated intravenous (i.v.) injections of pentobarbital (15 mg/kg). The head and right groin regions were shaved. To maintain stable body temperature, rats were placed in the dorsal position on a heating pad. Core body temperature was monitored through a rectal probe. An oxygen tube was connected to the mouth for additional oxygen supply. An incision was made in the skin of the right groin exposing both the femoral artery and vein. After insertion of a 22G 1″ JELCO® I.V. Catheter into the femoral artery arterial blood pressure, heart rate and pulse oximetry were continuously monitored via a Datex Engstrom monitor (Salo, Finland). A second 22G 1″ JELCO® I.V. Catheter then inserted into the femoral artery for a continuous infusion of 0.9% sodium chloride (Montreal, QC, Canada) at a rate of 1 ml/h.

Animals were randomly assigned to experimental (septic) or control groups. In the experimental group, lipopolysaccharide (LPS, from *Escherichia coli*, serotype O26:B6, Sigma-Aldrich, Oakville, ON, Canada) was given i.v at 15 mg/kg. Control animals were given an equal volume of normal saline.

Cranial window surgery

After stabilization in a stereotactic frame, a midline incision of the scalp was made. The underlying soft tissue was removed and bleeding was stopped. Using a low speed drill, a cranial window (approximately 3x3 mm) was made right of the middle sagittal line between the bregma and lambdoid sutures. Constant irrigation with an artificial cerebrospinal fluid was used during drilling to avoid brain overheating. Then, dura mater was removed using micro-iris scissors (Codman & Shurtleff Inc., Raynham, MA, USA) and the cerebral surface was covered with artificial cerebrospinal fluid.

Fluorescence intravital microscopy (IVM)

Four hours after injection of LPS or normal saline, rats were injected 5% fluorescein isothiocyanate (FITC)-albumin (Sigma-Aldrich, ON, Canada) solution (i.v.; 50 mg/kg) and 0.05% Rhodamine 6G (Sigma-Aldrich) solution (i.v.; 0.75 mg/kg). Intravital microscopy was performed with an upright fluorescence microscope (DM5000B, Leica, Richmond Hill, ON, Canada) equipped with a mercury bulb (Short ARC, HBO, 100W, Osram, Germany), an external light source (Leica CTR 5000,), eGFP blue and N21 green filters, objectives (N-PLAN L 20x/0.4, HC PLAN 10x/22 n) and a digital high-speed camera (EM-CCD, C9100, Hamamatsu, Japan). Images were captured using Volocity 5.0 software (PerkinElmer, Woodbridge, ON, Canada).

Five small pial venous vessels (diameter < 100 μm) with clear focus were randomly selected in each animal for 30 s video recording of leukocyte-endothelium interaction and 3 s recording for plasma

extravasation. The sensitivity of brightness was adjusted for appropriate exposure to capture the complete range of brightness. Images were recorded with a final magnification of 200x.

Plasma cytokine multiplex assay

Arterial blood samples were collected at the end of experiments using heparinized syringes. Animals were sacrificed by overdose of pentobarbital (100 mg/kg i.v.). Plasma was separated by centrifugation and stored at -80^{o}C for later cytokine analysis. Plasma cytokine levels were examined using a Meso Scale Discovery multiplex spot assay for rat interleukin 1 beta (IL1- β), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), interferon γ (IFN-γ), and growth-related oncogene (KC/GRO, MSD, Gaithersburg, MD). Samples were measured using a MSD SECTOR Imager 2400 and analyzed with MSD Discovery Workbench software.

Data Analysis

A well focused frame of the video clip was selected and exported as uncompressed TIFF-image with 1000x1000 pixels resolution (8 bit gray scale) from Volocity to Image J (National Institutes of Health, Bethesda, MD). To evaluate plasma extravasation, we measured the intensity of FITC fluorescence outside and inside venous vessels from each image and calculated the ratio of pixel intensity. Only the focused regions of the image were selected for this measurement.

To evaluate leukocyte-endothelium interactions, a 100 x 100 pixel (64μm x 64μm) grid was applied to the exported pictures. Using the video clip from the still images captured and noted above, the number of leukocytes firmly adherent to the vessels for 30 s in the focused squares was counted as

adhesion leukocytes or stickers and the mean density of leukocytes per square was determined. The evaluation of plasma extravasation and leukocyte-endothelium interaction was performed in a blinded fashion.

Statistical analysis

All data were analyzed with Sigma Stat/Plot (Systat Software Inc., San Jose, CA). Normal distribution of data was tested using the Kolmogorov-Smirnov test. T-tests and Mann-Whitney-Rank-Sum-Tests (MWRS) were performed for parametric and non-parametric data analysis, respectively. All normally distributed data are presented as mean ± standard deviation. Non-normally distributed data are presented as median in Box-Plots, in which the boxes described the 25%-75% percentile and whiskers the 10%-90% percentile. Mean arterial pressure and heart rate were analyzed by a two-way analysis of variance (repeated measures in the factor of time), followed by the Bonferroni test. Probability values less than 0.05 were considered significant.

Results

Systemic hemodynamic changes

All animals survived surgery and IVM examinations without protocol violations and missing data points. MAP remained stable in control animals (Figure 1a). Administration of LPS reduced MAP to the levels below than control animals and reached statistically significance ($p<0.05$) at 60 min. However, none of the animals reached a MAP lower than 65mmHg. Consistent with reduced MAP, the heart rate in LPS challenged rats was higher than that in controls (Figure 1b). Body temperature measured by rectal probe remained at normal levels ($37.2 \pm 0.6^{o}C$) in both control and LPS challenged rats throughout the experiment.

Permeability of blood brain barrier

Using intravital fluorescence microscopy, permeability of the blood brain barrier (BBB) was investigated by measuring FITC labeled albumin leakage from small pial vessels (diameters < 100 μm). Control animals shown in Figure 2, demonstrated that FITC labeled albumin remained mainly inside vessel walls (Figure 2a). Conversely, endotoxemic animals showed a significant amount of FITC-albumin present outside the vessels (Figure 2b), indicating an extravasation of FITC-albumin from the vessels. To quantify the extravasation, the ratio of the fluorescent intensity outside versus inside vessels quantified. As shown in Figure 3, a significant ($p<0.001$) increase in fluorescence ratio was observed in LPS challenged groups comparing to the control group.

Leukocyte adherence

To investigate leukocyte – endothelium interaction, the number of leukocytes firmly adherent to the small vessels (diameter < 100 μm) was evaluated. As shown in Figure 4, LPS challenge significantly increased ($p=0.025$) the number of leukocytes adherent to the vessel wall.

Plasma cytokines

Five hours after administration of normal saline or LPS challenge, cytokine levels of IFN-γ, IL-1, IL-6, TNF-α and KC/GRO in the plasma were significantly (IL-1 $p<0.05$, TNF-α and KC/GRO $p<0.01$, IFN-γ and IL-6 $p<0.0001$,) increased in the LPS challenged animals compared to control animals (Figure 5).

Discussion

Sepsis induced brain dysfunction, septic encephalopathy, is an early clinical symptom in septic patients. Although its pathophysiology is not well understood and controversies exist, it has been suggested that microcirculation is a key organ in septic pathophysiology and plays a critical role in sepsis development (Siami *et al.* 2008). In this study, using a well-established endotoxemia rat model and intravital microscopy, we demonstrated that endotoxemia induced an increase in permeability of the brain pial vessels and that this change was accompanied by an increase of leukocyte–endothelial cell interaction.

Lipopolysaccharide (LPS) is an endotoxin from the outer membrane of gram negative bacteria. It is also one of the most potent microbial mediators implicated in the pathogenesis of sepsis. It has been demonstrated that LPS triggers proinflammatory cytokine production, including TNF-α and IL-1β, and also upregulates expression of their receptor (Alexander *et al.* 2008; Tsao *et al.* 2001). TNF- α acts through its receptor, TNFR1, to induce expression of cell adhesion molecules on endothelial cells (Alexander *et al.* 2008; Tsao *et al.* 2001). The cross-linking of adhesion molecules triggers cytoskeletal remodeling in endothelial cells, which allow leukocyte transendothelial migration to proceed (Gaber *et al* 2004; Hang *et al.* 2004). TNF- α also causes increased permeability of the BBB and anti-TNF- α reduces the BBB permeability induced by sepsis (Hang *et al.* 2004; Tsao *et al.* 2001). In our experiments, systemic LPS administration induced high levels of the pro-inflammatory cytokines IL-6, TNF-α and IL-1β in blood circulation. Such a large amount of proinflammatory cytokines may contribute to the increased leukocyte-endothelium interaction and promote permeability of the BBB in endotoxemic rat brains.

LPS may also directly disrupt the BBB thereby increasing permeability in experimental models (Gaillard *et al.* 2003; Mayhan 1998; Osuchowski *et al.* 2005; Veszelka *et al.* 2007; Xaio *et al.* 2001). It has been demonstrated that injection of LPS transiently disrupted the BBB and allowed ^{14}C-sucrose (340Da) but not ^{14}C-dextran (50-100kDa) to cross-tight junctions (Osuchowski *et al.* 2005; Singh *et al.* 2004; Singh *et al.* 2007). In addition, extravasation of insulin and albumin was demonstrated in other septic brains (Hofer *et al.* 2008; Xaio *et al.* 2001). However, other studies reported that LPS did not acutely disrupt the BBB in rats (Bickel *et al.* 1998; Rosengarten *et al.* 2008). In addition, LPS reduced the permeability of the BBB was reported in other animal models (Ahishali *et al.* 2005; Kaya *et al.* 2004). These conflicting results may be due to different serotypes of LPS, different application procedures and dosage, and different tracers used in various animal models.

In our laboratory, we have developed and demonstrated impaired microcirculation of intestine (Lehmann C. *et al.* 2006; Lehmann Ch *et al.* 2007) and mesentery (Birnbaum *et al.* 2006; Lehmann C. *et al.* 2004) in endotoxemic rats. Using the same model, we demonstrated significant extravasation of labeled albumin in the pial vessels of endotoxemic rats, suggesting that endotoxin induces opening of the blood brain barrier. Our results are consistent with previous results that found LPS increases permeability of the BBB (Hofer *et al.* 2008; Xaio *et al.* 2001).

IL-6 is a multifunctional cytokine with diverse actions. Increased IL-6 production is often associated with sepsis and disruption of the BBB in septic brains (Kabir *et al.* 2003; Paul *et al.* 2003). Conversely, IL-6 also acts as an anti-inflammatory cytokine to reduce the migration of leukocytes across the BBB in mouse bacterial meningitis (Paul *et al.* 2003). In our experiments, increased plasma

levels of IL-6 following LPS treatment correlated with increased permeability of the BBB and increased leukocyte-endothelium interactions.

Adhesion of leukocytes in post-capillary venules is an early inflammatory response induced in different tissues by a variety of stimuli. LPS induced recruitment of leukocytes adherent to small venules has been reported in several experimental models (Lehmann C. *et al.* 2001; Lehmann Ch *et al.* 2006). Stimulation by LPS or TNF-α up regulates adhesion molecules on endothelial cells resulting in increased leukocyte interactions with the endothelium (Alexander *et al.* 2008). In addition, low blood pressure with low shear rates also contributes to increased leukocyte adhesion (Russell *et al.* 2003). In our experiments, increased number of adherent leukocytes was observed as the result of LPS stimulation. However, it is not clear whether decreased blood pressures in all the endotoxemic rats are significant enough to reduce the shear rates and contribute to increased leukocyte-endothelium interaction.

Systemic hypotension and hypoxia may contribute to disruption of the BBB, therefore, MAP, HR and O_2 saturation were closely and continuously monitored. Rapid administration of LPS induced a transient drop in MAP. Using a slower rate of LPS administration (about 0.1 ml/min) none of the rats exhibited a MAP lower than 65 mmHg, the minimal level required to maintain the integrity of the BBB (Ahishali *et al.* 2005). In addition, all animals were supplied with oxygen so oxygen saturation did not drop below 97%. Other factors which may damage the BBB, such as local mechanical or chemical stimulation, potential excitatory light and temperature of artificial cerebrospinal fluid were controlled in both control and endotoxemic animals. These data suggest that increased permeability of the BBB in

LPS challenged rats was not due to the drop of MAP or lack of oxygen, but due to direct immune response to endotoxemia.

Many factors may influence these complex animal experiments. Careful consideration must be given and steps taken to avoid situations, described above, that results in transient drops in MAP, fluid balance during endotoxemia experiments and minor injury/bleeding during cranial window preparation.

In conclusion, using florescence intravital microscopy, we were able to directly examine the cerebral microcirculation through a cranial window. We also demonstrated that endotoxin induced permeability changes in the brain pial vessels and that these changes were accompanied by an increase in leukocyte-endothelial cell interactions. This animal model can be used to study mechanisms of sepsis in the brain and effects of therapeutic strategies at the microcirculation levels.

Figure legends

Figure 1: a) Arterial blood pressure (MAP) and b) heart rate (HR) in control and endotoxemic (LPS) animals at the time 0 (from beginning of injection of LPS or normal saline) and every15 min interval during experimentation. * indicates a significant difference between the two groups (t-test, $p<0.05$).

Figure 2: Images of pial vessels and surrounding tissues on the control (a) and endotoxemic (b) rat brain 4 hours after saline or LPS administration.

Figure 3: Vascular permeability in pial microvasculature of control and endotoxemic rats. Ratio of FITC-albumin pixel intensity within vasculature and outside the vasculature was examined in no LPS (Control) and endotoxemic (LPS) rat brains. * indicates a significant difference between the two groups (Mann-Whitney, $P<0.001$). n=14.

Figure 4: Number of adherent leukocytes in the pial microvasculature of control and endotoxemic (LPS) rats. The number of adherent rhodamine 6G labelled leukocytes was determined per square (64 μm x 64μm) in the focused venous pial vessels. * indicates a significant difference between the two groups (Mann-Whitney, $p=0.025$). Boxplot with median and 25%/ 75% percentile. n=14.

Figure 5: Plasma cytokine concentration in control and endotoxemic rats (LPS). IL-1β: interleukin 1 beta, IL-6: interleukin 6, TNF-α: tumor necrosis factor-α, IFN-γ: interferon γ and KC/GRO: growth-related oncogene. * indicates a significant difference between the two groups (t-test, *$p<0.05$, **$P<0.01$, ***$p<0.0001$) n=14.

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Figure 1

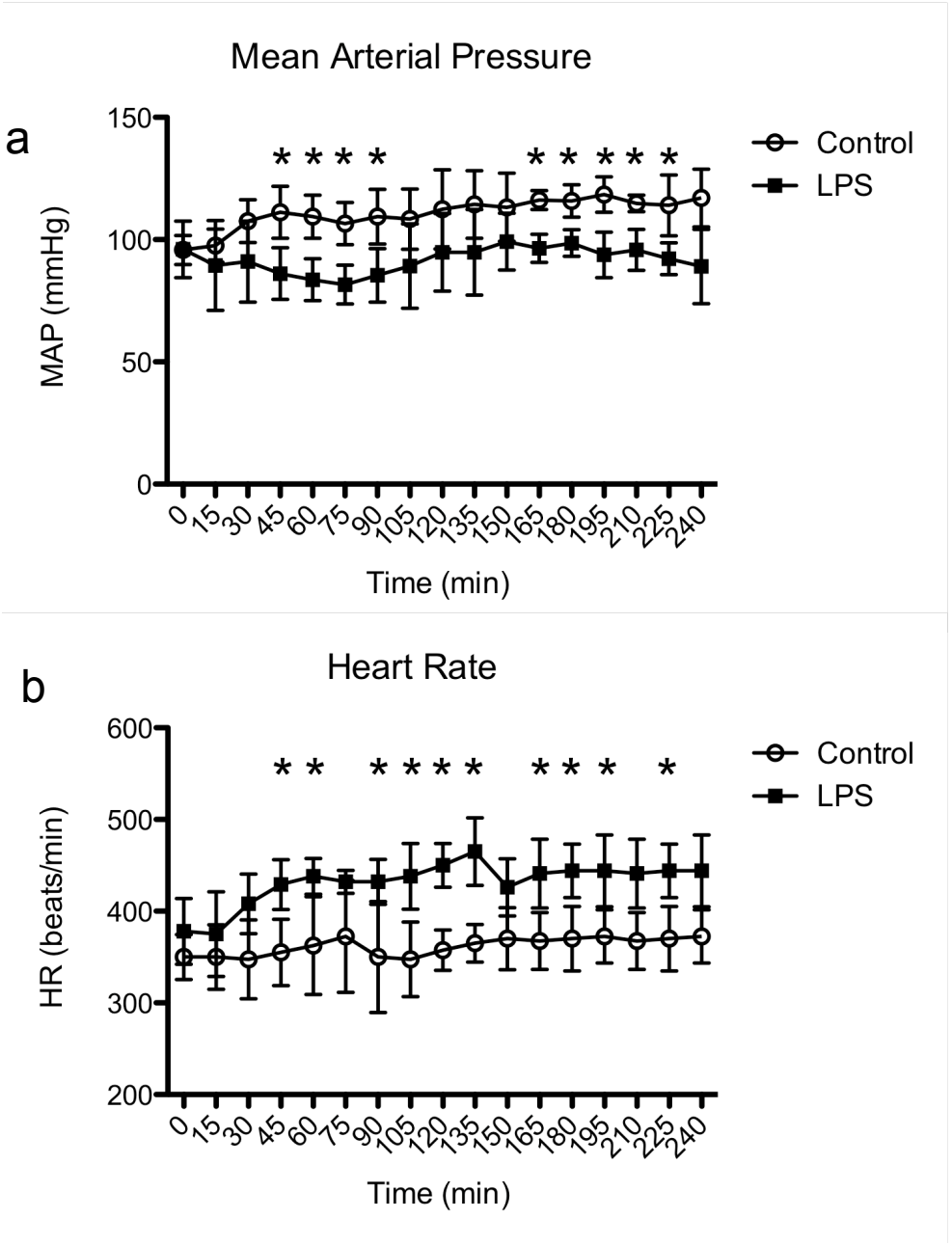
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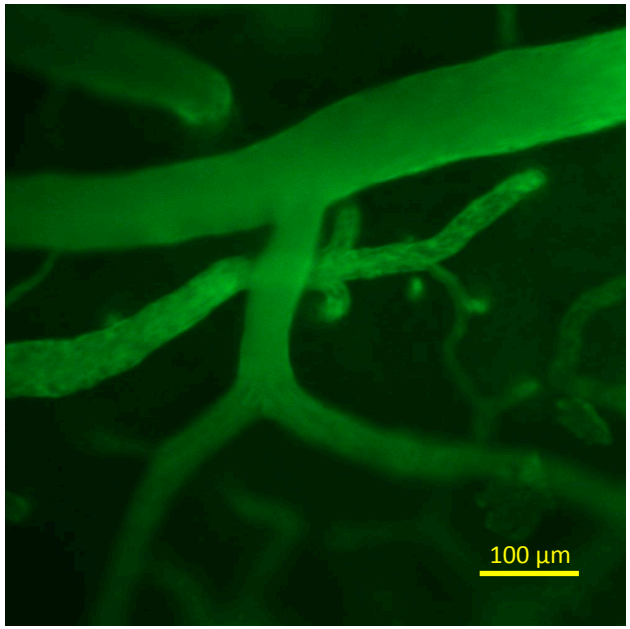
Figure 2

a. Control



b. Endotoxemia

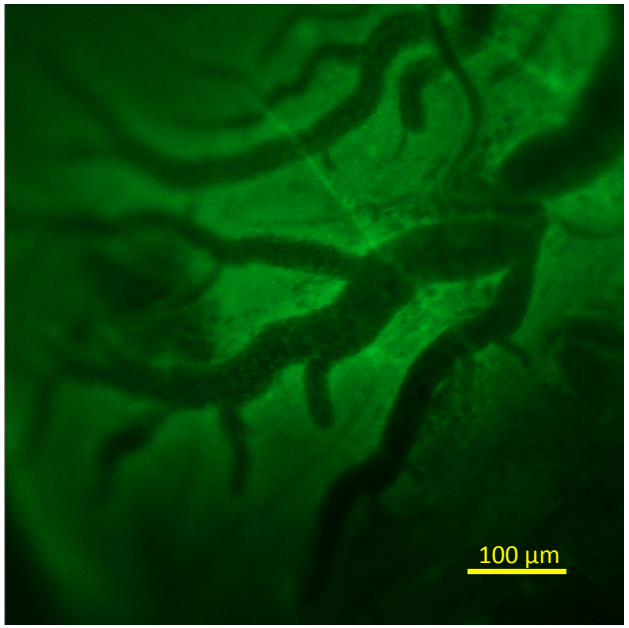


Figure 3

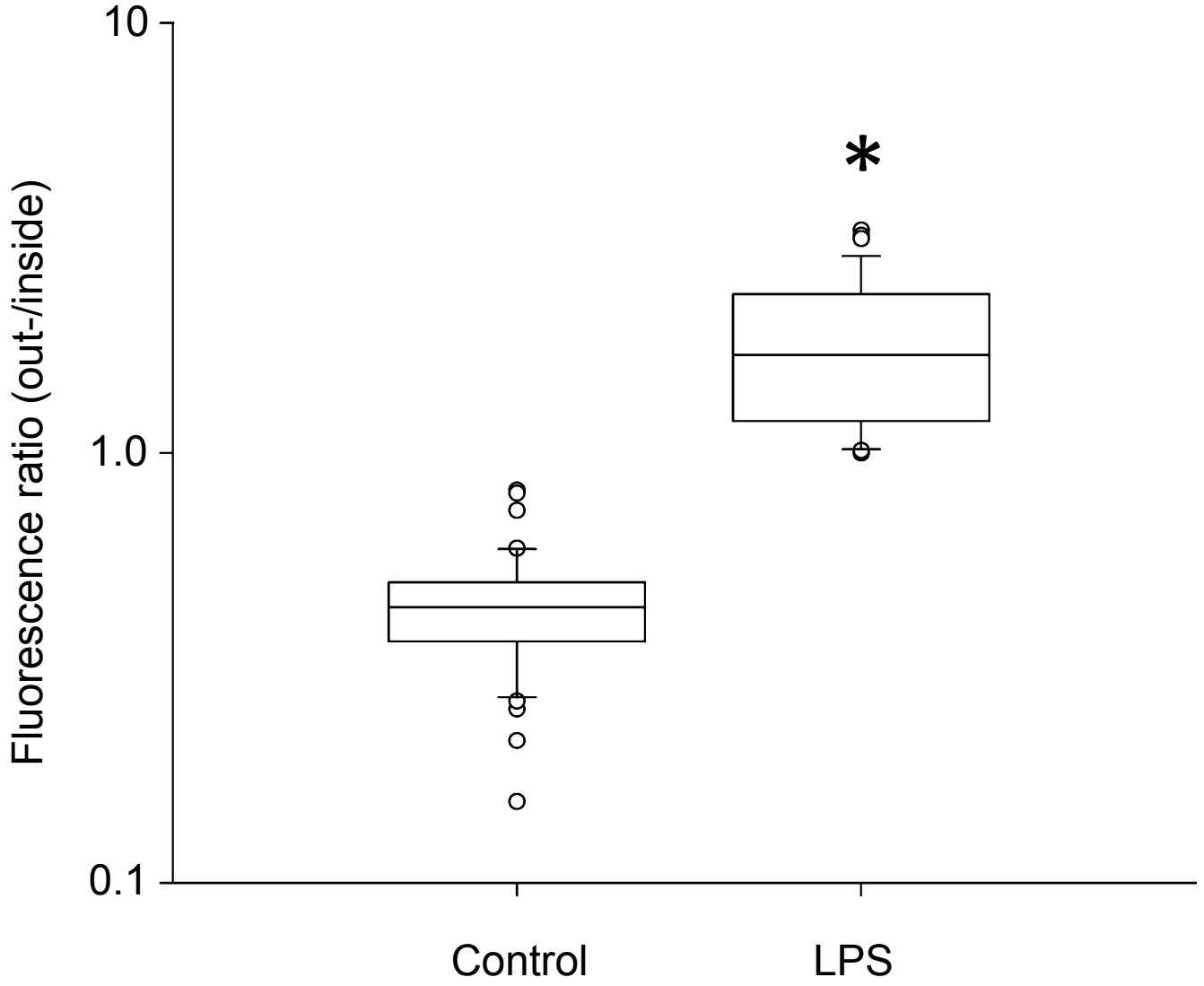


Figure 4

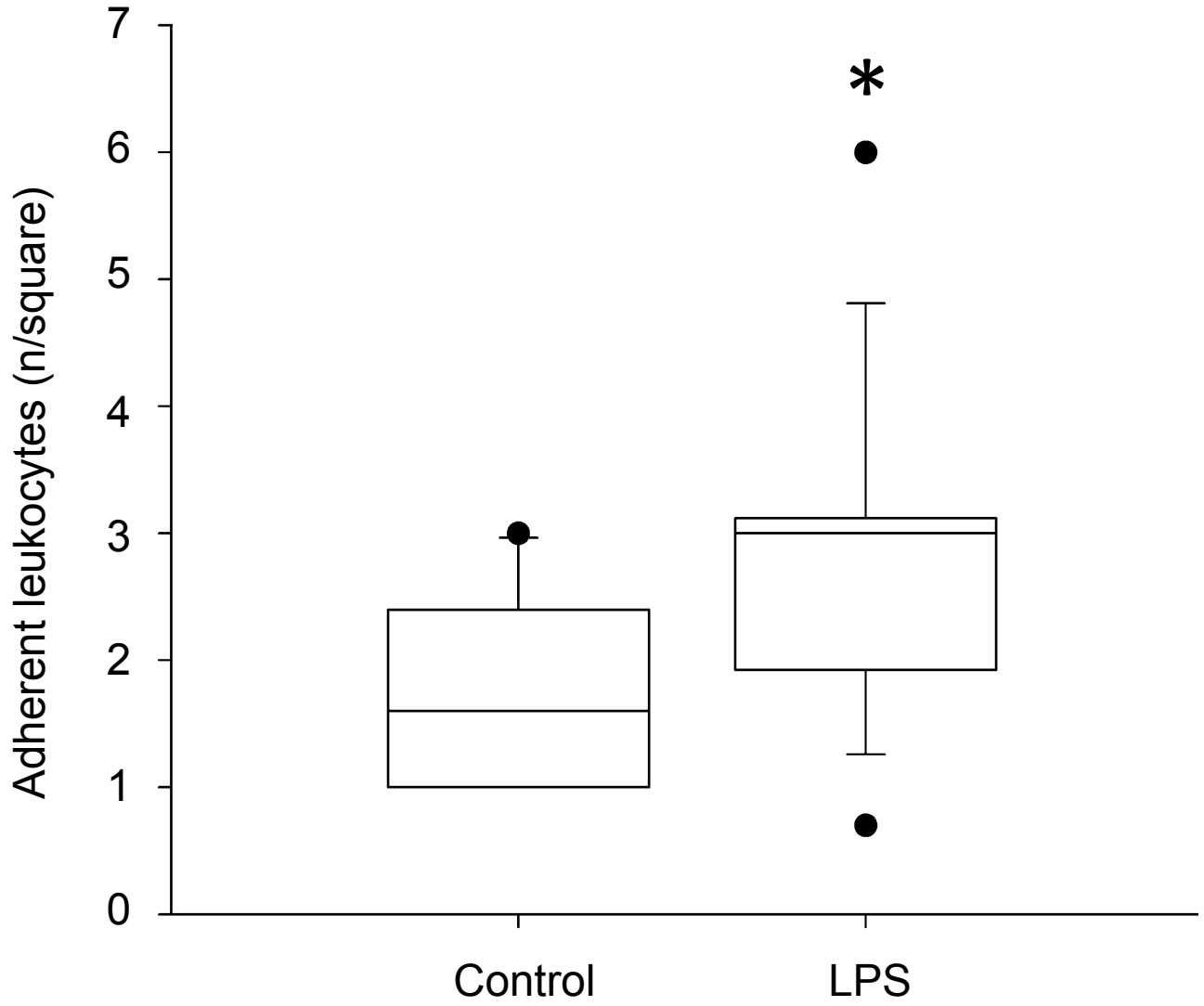
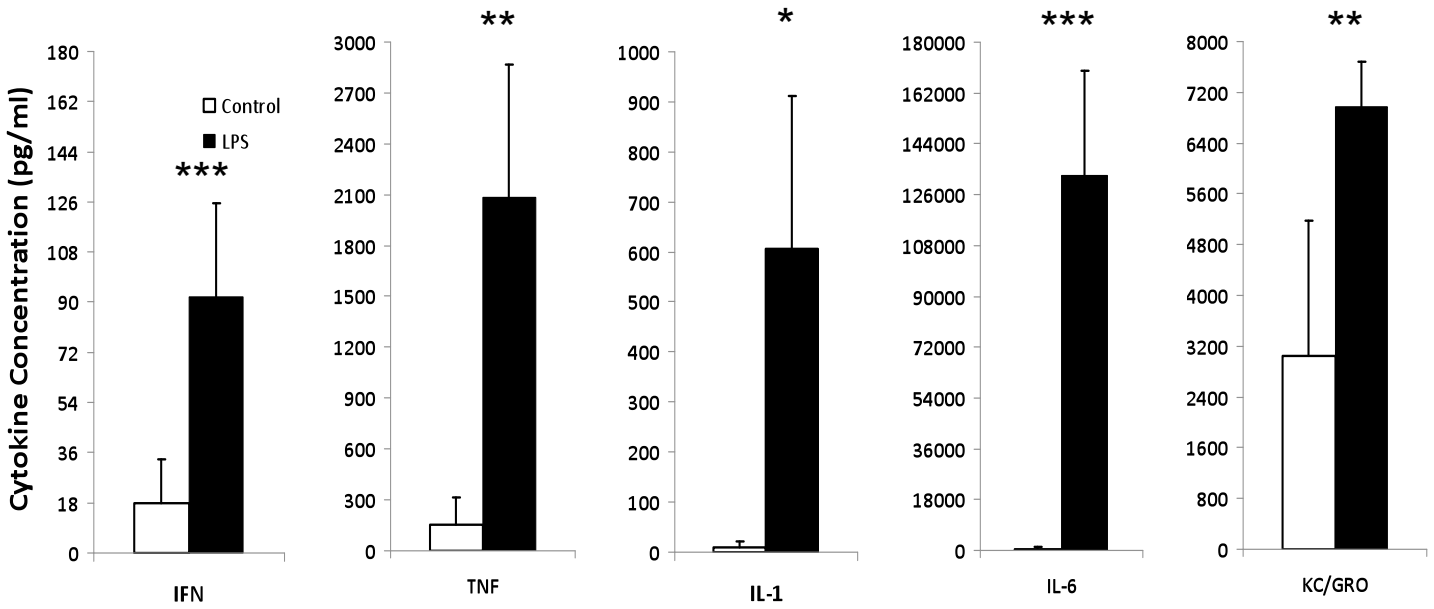


Figure 5


Cytokine Concentration (pg/ml)
Control
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IFN
TNF
IL-1
IL-6
KC/GRO