MODULATION OF ENDOCANNABINOID SYSTEM IN EXPERIMENTAL ENDOTOXEMIA

by

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This Masters of Science thesis is dedicated to my husband, Kian Behizadeh for his patience, love support and encouragement.

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ABSTRACT

Impairment of the intestinal microcirculation (IMC) plays a critical role in the pathogenesis of sepsis. Consequently, the protection of the IMC represents a pivotal therapeutic target in severe sepsis. The aim of this study was to examine the effects of endocannabinoid system modulation on the IMC. Experimental animals groups were: control, endotoxemic animals (lipopolysaccharide; LPS), LPS + CB1R agonist, LPS + CB1R antagonist, LPS + CB1R agonist + CB1R antagonist, LPS + CB2R agonist, LPS + CB2R antagonist, LPS + CB2R agonist + CB2R antagonist, LPS + cannabinoid degradation enzyme inhibitor and LPS + enzyme inhibitor + CB2R antagonist. Endotoxemia significantly increased leukocyte adhesion in intestinal submucosal venules, and significantly reduced capillary perfusion of the muscular and mucosal layers of the intestinal wall. In acute experimental endotoxemia, IMC was significantly improved (by reducing leukocyte adhesion and increasing capillary perfusion) with CB1R inhibition or CB2R activation or inhibition of endocannabinoid degradation.

List of Abbreviations Used

ACCP	American College of Chest Physicians
AC	adenylyl cyclase
AEA	anandamide
2-AG	2-arachidonoylglycerol
APC	activated protein C
BP	blood pressure
BCP	B-caryophyllene
Ca^{2+}	calcium
CASP	colon ascendents stent peritonitis
CARS	compensatory anti-inflammatory response syndrome
cAMP	cyclic adenosine 3,5-monophosphate
CBR	cannabinoid receptors
CB	cannabinoid
CBN	cannabinol
CBD	cannnabidiol
CB1R	cannabinoid subtype 1 receptor
CB2R	cannabinoid subtype 2 receptor
CNS	central nervous system
CS	corticosteroids
CIHI	Canadian Institute for Health Information
CLP	cecal ligation and puncture
CVP	central venous pressure
DAG	diacylglycerol
DMSO	dimethyl sulfoxide

DIC	disseminated intravascular coagulation
EAE	encephalomyelitis
ECS	endocannabinoid system
EGDT	early goal directed therapy
ELAM-1	endothelial leukocyte adhesion molecule 1
eCB	endocannabinoid
FAAH	fatty acid amide hydrolase
FCD	functional capillary density
FDA	food and drug administration
FITC	fluorescein isothiocyanate
GPCR	G protein coupled receptor
GI	gastrointestinal
$G_{i/o}$	inhibitory G protein
HR	heart rate
H&E	hematoxylin and eosin
ICAM	intracellular adhesion molecule
IFN-γ	interferon- gamma
IL-1β	interleukin-1 beta
IL-1ra	IL-1 receptor antagonist
I.P.	intraperitoneally
I.V.	intravenously
IVM	intravital microscopy
ICU	intensive care unit
IMC	intestinal microcirculation
JAM	junctional adhesion molecule
K^+	potassium
KCL	potassium chloride

LPS	lipopolysaccharide
LMs	lipid mediators
MAGL	monoacylglycerol lipase
MAP	mean arterial pressure
MAPK	mitogen activated protein kinases
MOF	multiple organ failure
MODS	multiple organ dysfunction syndrome
MIF	macrophage inhibiting factor
MIP-2	macrophage inflammatory protein 2
NAPE	N-arachidonyol phosphatidyl ethanolamine
NAPE-PLD	N-arachidonyol phosphatidyl ethanolamine phospholipase D-like enzyme
NF-κB	nuclear factor κB
OPS	orthogonal polarization spectral imaging
PAF	platelet activating factor
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered solution
PECAM-1	platelet/endothelial cell adhesion molecule-1
PBS-T	phosphate buffered solution with tween 20
PMN	polymorphonuclear neutrophils
PRRs	pattern recognition receptors
rhAPC	recombinant human activated protein C
RhoA	Ras homolog gene family member A
SCCM	Society of Critical Care Medicine
SDF	sidestream dark-field imaging
SIRS	systemic inflammatory response syndrome
SSC	Surviving Sepsis Campaign

SvO2	mixed venous oxygen saturation
$S_{cv}O_2$	central venous (superior vena cava) saturation
Temp	temperature
TLRs	toll-like receptors
TNF-α	tumor necrosis factor-alpha
TM	transmembrane
THC	delta-9-tetrahydrocannabinol
VCAM	vascular cell adhesion molecule

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CHAPTER 1: INTRODUCTION

1.1 Sepsis

Infection that causes excessive inflammation throughout the body (sepsis) is the most important complication in the surgical patient. Sepsis, severe sepsis and septic shock are the most frequent causes of death in surgical intensive care unit (ICU) patients (Bone *et al*., 1997; Vincent *et al*., 2009). Sepsis affects 18 million people worldwide every year (Slade *et al*., 2003). The Canadian Institute for Health Information (CIHI) reported that the number of hospitalized patients with sepsis were about the same as stroke hospitalized patients, however, the number of deaths from sepsis was three times more than deaths from strokes or heart attacks (Canadian Institute for Health Information. 2010).

During infection, the host immune system functions to destroy the causative pathogen such as bacteria (gram negative, gram positive), fungi and viruses (Martin *et al*., 2003). The innate immunity (first line of defense against pathogens) consists of receptors called pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) expressed by these pathogens. In normal immune response, PRRs are expressed on innate immune cells, responsible for up-take and killing of pathogens (Chong & Sriskandan, 2011; Cinel & Opal, 2009). In sepsis the immune system becomes highly (hyper-) activated, and systemic inflammation can affect all organs of the body. The inflammatory response to an initial infection becomes amplified and leads to an excessive release of several pro-inflammatory cytokines (TNF-α and IL-1β) and chemokines (IL-8), which then increase the recruitment of neutrophils, and leads to tissue damage and multiple organ failure (Ness *et al*., 2012; Cinel & Opal, 2009). This

excessive production of anti-inflammatory cytokines and chemokines, results in subsequent immunosuppression, thus increasing the body's susceptibility to secondary infection and increase mortality in sepsis (Ashare *et al*., 2005; Rudiger *et al*., 2008). While there have been significant advances in the understanding pathophysiology and treatment of sepsis, current therapies are not effective at reducing mortality rate of sepsis. Therefore, the search for novel therapies for sepsis remains paramount to treating this disease (Ness *et al*., 2012; Zanotti *et al*., 2002).

1.1.1 Definitions

In 1992, the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) defined sepsis as a systemic inflammatory response to an infection (see Table 1 for definition of commonly used terms) (Bone *et al*., 1997; Bone *et al*., 1992; Levy *et al*., 2003). A systemic inflammatory response syndrome (SIRS) can also arise in the absence of infection, e.g. during pancreatitis, ischemia, trauma and burns (Figure 1).

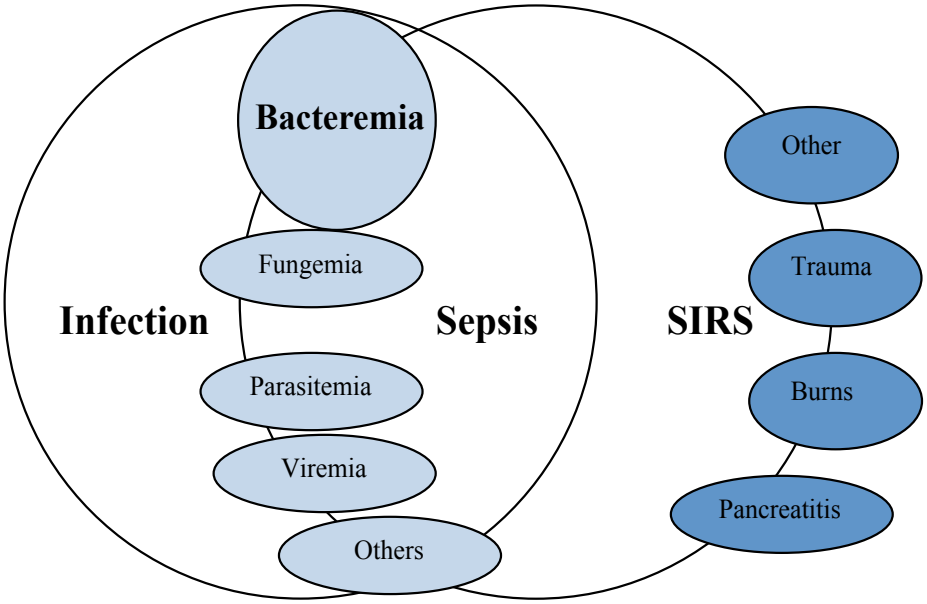


Figure 1. Relationship between SIRS, sepsis, and infection (modified from Bone *et al*., 1992).

Table 1. Definitions

Terms	Definition ACCP/SCCM
Infection	Infection is microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by pathogenic or potentially pathogenic organisms. The site, type and extent of the infection have a significant impact on the severity of prognosis of sepsis.
Bacteremia	The presence of viable bacteria in the blood.
SIRS	A systemic inflammatory response to a variety of infectious and non-infectious causes, such as ischemia, multiple trauma, burn injury, non-infectious pancreatitis, hemorrhagic shock, transplant rejection. SIRS manifested by two or more of following symptoms: 1) Body temperature $\geq 38°C$ or $\leq 36°C$ 2) Heart rate ≥ 90 beats min^{-1} 3) Respiratory rate ≥ 20 beats min^{-1} or a PaCO2 of ≤ 32mm Hg 4) White blood cell count $\geq 12{,}000$ cells μL^{-1} or $\leq 4{,}000$ μL^{-1}
Sepsis	Sepsis is the systemic inflammatory response to infection.
Severe sepsis	Sepsis associated with one of the following symptoms: organ dysfunction, hypoperfusion, or sepsis-induced hypotension (manifested <90mm Hg in systolic blood pressure or reduction of >40mm Hg from baseline with exclusion of other causes for hypotension)
Septic shock	Severe sepsis plus a persistent arterial hypotension, despite adequate fluid resuscitation. In addition, septic shock in adults is defined as a state of acute circulatory failure shown by hypotension.

1.1.2 Epidemiology and etiology

Sepsis is a common disease with a high mortality rate and significant costs for health care resources. In Canada, the CIHI reported that in 2008-2009 more than 30,500 cases of sepsis with mortality rate of over 30% (Husak *et al*., 2010). In the United States, the national incidence of sepsis accounts for over 751,000 cases annually with a mortality rate of 28.6% (Angus *et al*., 2001). During the past two decades, despite the decrease in overall mortality rate among hospitalized sepsis patients, the incidence of sepsis is increasing (Martin *et al*., 2003). The incidence of sepsis is projected to rise significantly as the population ages and the presence of comorbidities increases (Angus *et al*., 2001; Monneret *et al*., 2007). Sepsis is more common in men than women, but the mortality rates are not differing according to sex (Martin *et al*., 2003). The sepsis syndromes represent significant cost to the health care system; the average cost for treatment per patient estimated $22,000. The annual cost for sepsis treatment in the USA is estimated to be $16.7 billion (increasing by 1.5% per year) (Martin *et al*., 2003; Angus *et al*., 2001; Slade *et al*., 2003). Currently, there are no specific medications approved for the treatment of sepsis.

The most common origins of infection in sepsis are the lungs (68%), followed by abdomen (22%), blood (20%) and urinary tract (14%). Gram-positive pathogens are found in 40% of patients' cultures and gram-negative bacteria in 38% of cultures. The most common pathogens that caused infection were *Staphylococcus aureus* (14%), *Pseudomonas species* (14%) and *Escherichia coli* (13%) (Vincent *et al*., 2006).

1.1.3 Organ dysfunctions and failure in clinical sepsis

Multiple Organ Dysfunction Syndrome (MODS) is defined as a progressive and reversible dysfunction in two or more organs as a result of physical insults or impaired homeostasis. MODS is the consequence of sustained SIRS (Fry, 2012). The most common causes of MODS are sepsis, trauma, severe tissue injury, severe infection, hypoperfusion/ischemia, burns and inflammation (Barie *et al*., 2009; Wang & Ma, 2008). In 1975, Baue *et al*., described Multiple Organ Failure (MOF), as a failure of many or all systems after an overwhelming injury or operation. In 1980, Fry *et al*., described the linear relation between number of failed organs and mortality rate in ICU patients (Fry *et al*., 1980; Barie *et al*., 2009). The pathophysiology of MODS consists of two pathological phases (refer to Figure 2). The early phase starts with overproduction of inflammatory mediators in response to an infection. Cytokine overproduction leads to activation of leukocytes and endothelial cells, increase of endothelial permeability and polymorphonuclear neutrophils (PMN) adhesion and migration. This is followed by production of the first cytokine storm by inflammatory cells in the spleen, liver and bloodstream (Wang & Ma, 2008). Presence of infection or physical insult leads to release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-alpha) and Interleukin-1beta (IL-1beta). Circulating cytokines then activate endothelial cells, which up-regulate expression of adhesion molecules: endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1). These adhesion molecules cause adhesion of monocytes, T-cells and PMNs to endothelial cells. Systemic leukocyte activation and extravasation causes tissue damage, which contribute to the later phase of MODS (Wang & Ma, 2008). In the later phase, adhesion of leukocytes to the

endothelium is followed by migration to interstitial space. Extravasated inflammatory cells and cytokines amplify the inflammation. High concentrations of cytokines increase the infiltration of neutrophils, and excessive production of cytokines, which then leak into the bloodstream. This phenomenon may cause damage to remote organs, by activating inflammatory cells in those organs (Figure 2) (Wang & Ma, 2008). One of the main scientific approaches used so far in the treatment of MODS is the inhibition of inflammation, for example by removal of endotoxin and cytokines from sepsis patients by specific antibodies; however, this therapeutic approach seems to not improve the outcome (Li *et al*., 2007; Fry, 2012).

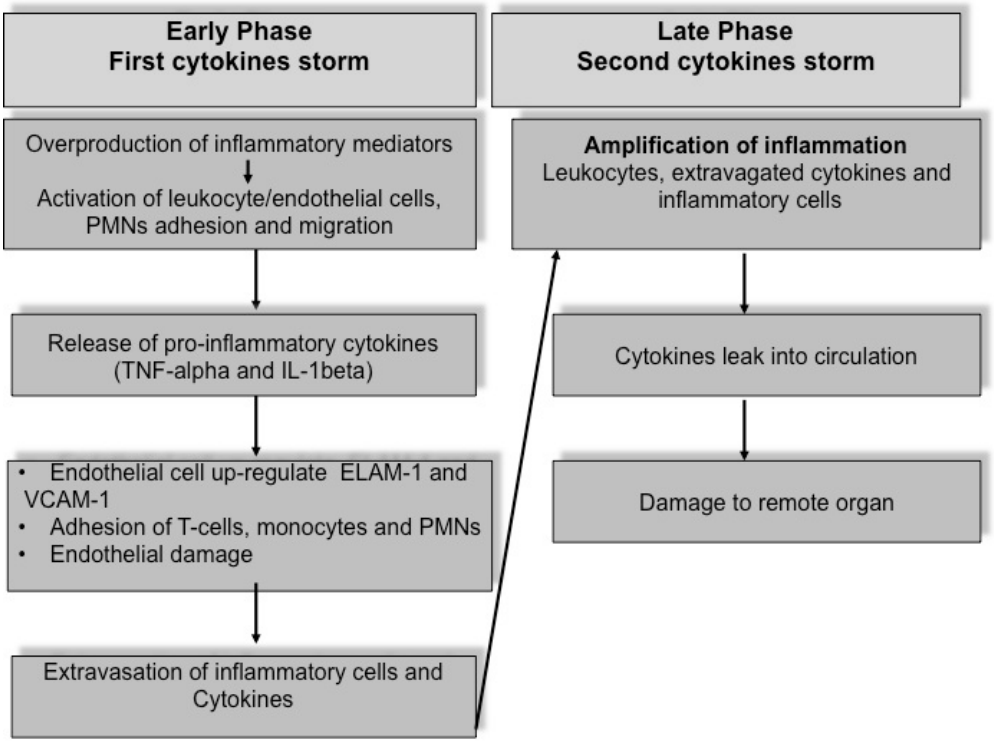


Figure 2. Early and late phases of MODS. ELAM-1 endothelial-leukocyte adhesion molecule 1; PAF, platelet-activating factor; TNF-α, tumor necrosis factor-α; PMNs polymorphonuclear neutrophils; VCAM-1, vascular cell adhesion molecule 1 (modified from Wang & Ma, 2008).

1.2 Microcirculatory changes in sepsis

1.2.1 Capillary perfusion

The microcirculation represents the smallest blood vessels with diameter <100 μm and consists of the capillaries, arterioles and venules (Figure 3). The microcirculation is the site where exchange of oxygen, nutrients, and waste takes place, and its main role is to provide oxygen and nutrients to the tissue cells and removing waste and carbon dioxide (Johnson, 2008). The wall of arterioles consists of endothelial layer surrounded by multiple layers of vascular smooth muscle (Figure 3). The primary role of the arterioles is the regulation of blood flow. The capillaries, are highly permeable, and are the site of exchange for nutrients, gases and water between blood and tissues. The venules, drain the blood from the capillaries for return to the heart, and contain intercellular endothelial junctions that allow plasma proteins and circulating cells (leukocytes) escape from the blood stream. Venules are also the major site of transvascular protein exchange and leukocyte-endothelial cell interaction (Johnson, 2008; Granger & Senchenkova, 2010).

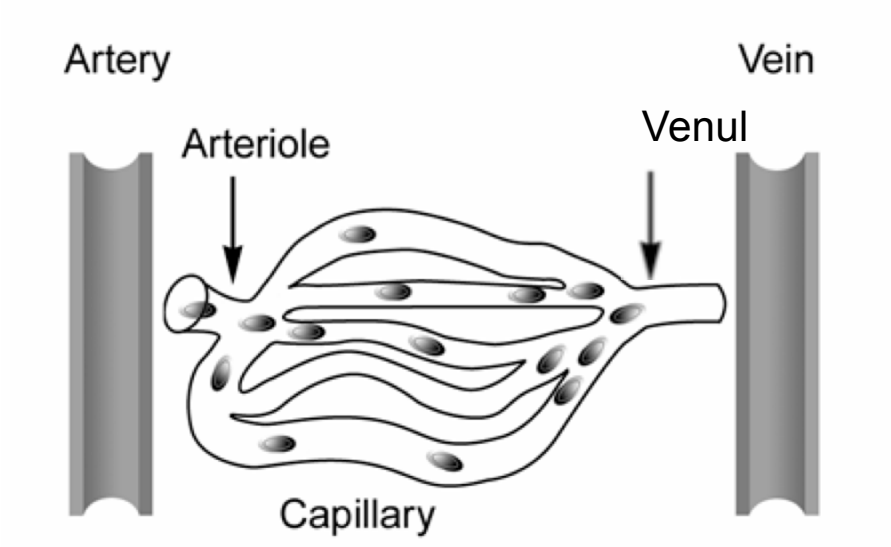


Figure 3. The diagram of microcirculation units. Arteriole, capillary bed and venule (modified from Duling & Desjardins, 1987)x

In sepsis microcirculation is impaired this leads to decreased oxygen supply for tissues (Lundy & Trzeciak, 2009). Impairment of capillary perfusion represents a key event in the pathophysiology of sepsis. These abnormalities are characterized by some capillaries being under-perfused, and others having normal or high perfusion rate (Ince, 2005). In sepsis, capillary perfusion is compromised due to: decreased deformability of red blood cells, increased blood viscosity, adhering neutrophils, activation of clotting cascade and other factors (Lehr *et al., 2000;* Spronk *et al*., 2004).

Studies of human microcirculation are limited to epi-illumination (involves reflecting an energy source) of organs and tissues in which the microcirculation is very near to the surface such as skin, tongue, retina (Johnson, 2008). Human capillary perfusion in these microvascular beds can be evaluated by microvideoscopic techniques such as orthogonal polarization spectral (OPS) imaging and sidestream dark-field (SDF) imaging (Ince, 2005; Nencioni *et al*., 2009; Lundy & Trzeciak, 2009). Both OPS and SDF are video-microscopic imaging techniques that allow evaluation of microcirculation in critically ill patients. These techniques are based on application of different wavelength of lights to the tissue, with the reflection of the light providing images from microvascular vessles (De Backer *et al*., 2010). Another technique that has been recently used for assessment of microcirculatory functions is laser doppler imaging, which provides a measurement of both flux and velocity of red blood cells (Johnson, 2008; De Backer *et al*., 2010). Changes in microcirculation are commonly seen in clinically ill patients, especially sepsis patients. Therefore, monitoring the changes in microcirculation could contribute to the diagnosis of sepsis and to identify novel therapeutic strategies to improve the outcome of microcirculatory dysfunction in septic patients.

1.2.2 Leukocyte recruitment

The most important characteristic of sepsis is leukocyte (over-)recruitment. The endothelium plays an important role in leukocyte activation. Under normal conditions endothelial cells are highly active. When pathogens invade tissues, endothelial cells release inflammatory mediators to recruit leukocytes, which eradicate the pathogens (Granger & Senchenkova, 2010). Leukocytes consist of different types of blood cells including: neutrophils, monocytes, eosinophils, basophils, lymphocytes and dendritic cells. In healthy human blood, the majority of leukocytes are in an inactive state; however, upon interaction with pro-inflammatory mediators they become activated and phagocytose and destroy the pathogen. Migration of leukocytes to the site of injury or infection is important to produce an inflammatory response (Petri & Bixel, 2006). Therefore, the leukocyte–endothelial cell interaction is an essential defence against bacterial invasion. However, in sepsis there is an extensive, sustained and generalized activation of endothelium (Aird, 2003). The process is hyper-activated, attacks healthy tissue that are not part of infected site and it happen in all organs in the body (Phillipson & Kubes, 2011).

A variety of chemical mediators that are released from inflamed tissue act on leukocytes and or endothelial cells to promote or inhibit leukocyte-endothelial interaction. These include histamine, leukotrienes, platelet activating factor (PAF), cytokines and chemokines. Histamine, leukotrienes and PAF released from mast cells and/or macrophages, signal for rapid induction of leukocyte rolling. The leukotrienes and PAF can also activate the rolling leukocytes and initiate their transition to firm adhesion. Cytokines and chemokines released from vascular cells stimulate expression of E-selectin

and P-selectin as well as increase the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Granger & Senchenkova, 2010).

The process of leukocyte recruitment into the site of injury is a multistep process of interaction between adhesion molecules on the leukocytes and endothelium, which are: tethering and rolling, activation, firm adhesion, crawling and transmigration from the blood vessel to the extravascular tissue (Figure 4).

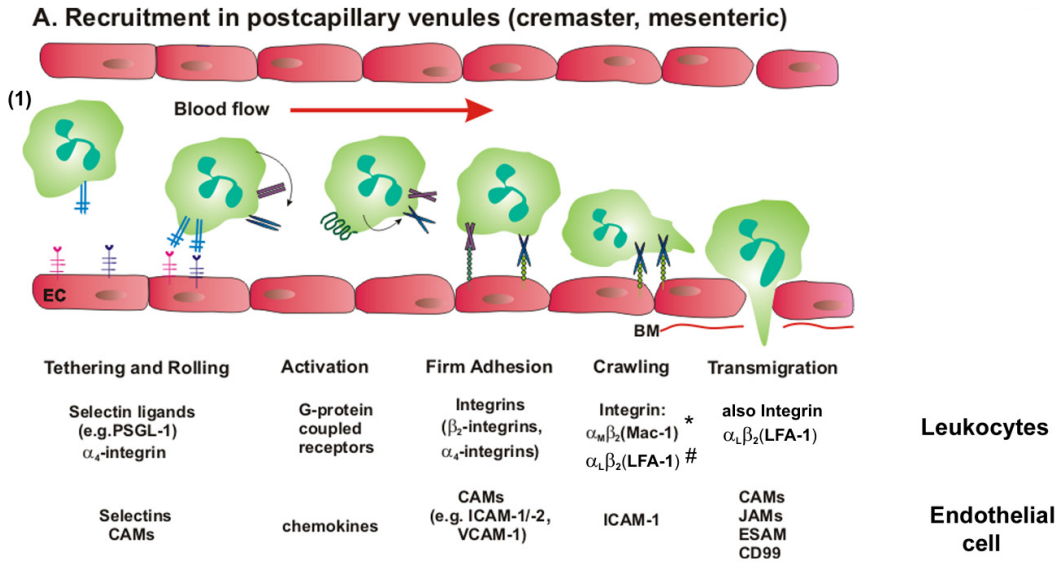


Figure 4. **Leukocyte recruitment to the site of injury.** Leukocyte recruitment to the site of injury involves multiple steps such as tethering and rolling, activation, activation, firm adhesion, crawling and transmigration of leukocytes from blood endothelium to the site of injury (Petri *et al*., 2008).

1) Tethering and rolling

Tethering is the initial interaction between leukocytes and the endothelium of a vessel wall, followed by movement of leukocytes along the endothelial wall, known as rolling (refer to Figure 4). These steps are dependent on up-regulation of selectins. The selectins are lectin-like adhesion glycoproteins, which have a distal lectin-like domain that enable them to bind to specific carbohydrate groups (Kelly *et al*., 2007). Three selectins have been identified: L-selectin, P-selectin and E-selectin. L-selectins are expressed on the surface of neutrophils, monocytes, lymphocytes and other leukocytes. L-selectin mediate leukocyte rolling by interacting with P- and E-selectins expressed on the endothelial cells. P-selectins are constitutively expressed and stored in Weibel-Palade bodies of endothelial cells and in α-granules of platelets. When endothelium or platelets gets activated (by inflammatory stimuli) the granules fuse with the plasma membrane resulting in expression of P-selectins on the cell surface (Sanz & Kubes, 2012). Endothelial P-selectins can support both leukocyte- and platelet-endothelial interactions (Ley *et al*., 1995). E-selectins are synthesized and expressed on the vascular endothelium in the presence of mediators such as LPS, IL-1 and TNF-α (Kelly *et al*., 2007). Similar to other selectins, E-selectins support leukocyte rolling (Petri & Bixel, 2006; Petri *et al*., 2008; Kelly *et al*., 2007). In general, E-selectins and P-selectins on the endothelium bind to the leukocytes, slow down the velocity of leukocytes thereby causing the rolling of the leukocytes on the vascular wall (Petri & Bixel, 2006).

2) Leukocyte Activation and Firm Adhesion

The weak interactions (rolling) between leukocytes and endothelium are strengthened as a result of leukocyte activation (Figure 4). Leukocyte activation is mediated by chemokine-dependent and chemokine-independent mechanisms (Granger & Senchenkova, 2010). Chemokines (a family of small polypeptide cytokines) are heterodimeric proteins expressed by leukocytes, macrophages, mast cells and tissue cells that facilitate adhesion of leukocytes to vascular endothelium. After induction of chemokines by inflammatory cytokines, such as IL-1 or TNF-α, chemokines binds to chemokine receptors at the surface of leukocytes and induce leukocyte activation (Kelly *et al*., 2007). The firm adherence of leukocytes to the endothelium requires adhesion molecules called integrins. Integrins (such as VLA-4 and CD11/CD18), are a large family of adhesion molecules consisting of (α) and (β) subunits. The α4β1-integrins (VLA-4), enable lymphocytes and monocytes to bind to VCAM on endothelial cells. The β2-integrins (CD11/CD18) mediate the firm adhesion of neutrophils to the endothelium (Granger & Senchenkova, 2010). Different integrins are expressed by different leukocytes; this allows them to interact with different cellular adhesion molecules (CAMs). The CAMs, including ICAM-1, ICAM-2, VCAM-1 and PECAM-1, belong to an immunoglobulin superfamily expressed on vascular endothelium and function as ligands for leukocyte integrins. For example, ICAM-1 and VCAM-1 are involved in firm adhesion of leukocytes to endothelium (Ley *et al*., 2007; Petri & Bixel, 2006). Endothelial ICAM-1 expression is increased by inflammatory cytokines (Ley, 2010). ICAM-1 is the major ligand supporting the binding of α1β2-integrins (Kelly *et al*., 2007). Most endothelial cells express VCAM-1 upon cytokine stimulation. VCAM-1 is the most

important ligand for α4-integrins and is involved in monocyte recruitment to the site of inflammation (Ley, 2010).

Leukocyte Crawling

Another step in the recruitment cascade is the crawling of leukocytes (Figure 4). Adhering leukocytes crawl along the luminal surface of vessels in search of transendothelial migration. Crawling is mediated by the αMβ2-integrins (Mac-1) (Phillipson *et al*., 2006; Sanz & Kubes, 2012), Mac-1-dependent crawling is mediated by ICAM-1, but not ICAM-2 (Phillipson *et al*., 2006; Kelly *et al*., 2007; Petri *et al*., 2008)

3) Transmigration

Transmigration of the leukocyte from the circulation through the vascular endothelium into the underlying tissue is the final step of leukocyte recruitment into the site of injury (Figure 4). Transmigration requires endothelial junctional molecules, including an immunoglobulin superfamily platelet/endothelial cell adhesion molecule-1 (PECAM-1), and junctional adhesion molecule (JAM), as well as non-immunoglobulin molecules such as CD99 (Petri & Bixel, 2006). Petri (2006) reviewed the two proposed molecular mechanism that leukocytes can use to transmigrate across the vascular endothelium: paracellular or transcellular pathway (Petri & Bixel, 2006). In the paracellular pathway, leukocytes migrate through interendothelial junctions, passing between the adjacent endothelial cells; in the transcellular pathway, leukocytes migrate through an individual endothelial cell (Petri & Bixel, 2006; Kelly *et al*., 2007).

1.2.3 Role of the intestinal microcirculation

Intestinal hypoperfusion occurs frequently during the disease development and reperfusion may result in an additional release of pro-inflammatory mediators into the systemic circulation. This enhances the SIRS and contributes to early MOF and death (Moore, 1999). Intestinal mucosal hypoperfusion can also causes a breakdown of gut epithelial barrier function thus releasing bacteria and their toxins into the systemic circulation and creating a gut-derived septic state (Rowlands *et al*., 1999). Therefore, the IMC has been suggested to act as the "motor" of MOF in systemic inflammation (Meakins & Marshall, 1986). The study of IMC is a therapeutic target in severe systemic inflammation, sepsis.

1.3 Therapeutic Approaches

1.3.1 General principles

Identification of therapies that can target the different cellular and inflammatory components of the compromised microcirculation in sepsis is essential in order to preserve barrier function, ensure adequate tissue oxygenation, and maintain immune function (Ince, 2005). Several different strategies are used at different stages of sepsis progression (Figure 5): pathogen removal (strategy A), modulation of receptors and mediator release (strategy B) and adjuvant therapy (strategy C). While pathogen removal is necessary in every case, it does not guarantee a positive outcome. Adjuvant therapy is necessary in many cases, but does not directly influence the pathogenesis of the disease. Therefore, modulation of receptors and mediator release represent a preferred option for the development of novel approaches to improve the IMC in severe inflammation and sepsis.

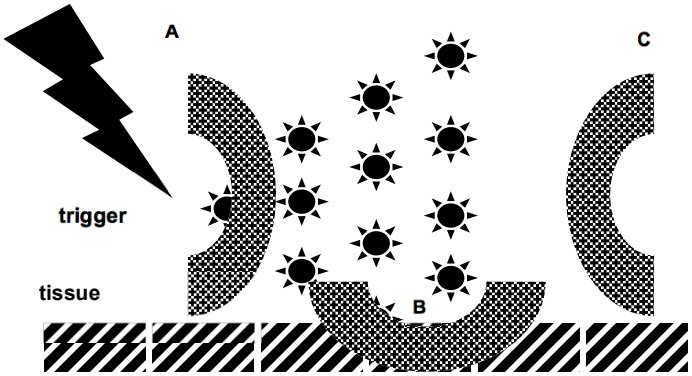


Figure 5. Anti-inflammatory strategies. A – inhibition of inflammatory triggers; B – modulation of receptors and mediators; C – substitution / adjuvant therapy.

Therapeutic approaches to treat clinical sepsis are needed to improve the outcomes in severe sepsis patients. Since microcirculation is affected in many septic patients a number of different approaches have been studied to resuscitate the microcirculaiton. According to the latest international guideline from Surviving Sepsis Campaign (SSC) for management of severe sepsis and septic shock the recommended therapeutic approaches for treatment of sepsis are: initial resuscitation, antibiotic therapy, fluid therapy, vasopressors, (Dellinger *et al*., 2008). Therapeutic approaches are based on the current International Guidelines for Management of Severe Sepsis and Septic shock: 2008 (Dellinger *et al*., 2008; 2004).

1.3.1.1 Early goal-directed therapy

Early recognition of sepsis can improve the patient's outcome and reduce mortality (Funk *et al*., 2009). Sepsis is identified by an alternation of clinical parameters such as temperature, blood pressure, heart rate and respiratory rate (Refer to table 1)(Bone *et al*., 1992). Early goal directed therapy (EGDT) is a method used in critical care medicine, involving monitoring and management of hemodynamics in patients with high risk of mortality (Rivers *et al*., 2001). The goal of EGDT is to restore systemic oxygen delivery by manipulating cardiac preload (volume), afterload (blood pressure), and contractility (stroke volume), and it is conducted by monitoring central venous pressure (CVP), mean arterial pressure (MAP) and central venous (superior vena cava) saturation ($S_{cv}O_2$) to preserve the effective tissue perfusion within the first 6 hours of disease recognition (Rivers *et al*., 2001). According to the SSC guideline (2008) the general purpose of initial resuscitation is to achieve all of these:

- Central venous pressure (CVP) ≥8-12 mm Hg

- Mean arterial pressure (MAP) ≥65 mm Hg
- Urine output ≥0.5 ml/kg/hr
- Central venous (superior vena cava) saturation ($S_{cv}O_2$) ≥70% or mixed venous oxygen saturation (SvO2) ≥65%

1.3.1.2 Antibiotic Therapy

Intravenous antibiotic therapy within the first hours of diagnosis of severe sepsis or septic shock is highly recommended; a delay in effective antibiotic therapy increases the risk of mortality (Dellinger *et al*., 2008; Kumar *et al*., 2006; Textoris *et al*., 2011). Choosing the appropriate antibiotic should be based on the host characteristic including drug intolerances, underlying disease; the site of infection, local ecology, pharmacokinetic and pharmacodynamics of the antibiotic. In addition, clinicians should be aware of the patient's history such as drug resistance and underlying diseases (Textoris *et al*., 2011; Dellinger *et al*., 2008). Antibiotic therapy is recommended for duration of 7-10 days, but for patients with slow clinical response the treatment could continue for longer courses (Dellinger *et al*., 2008; Vincent *et al*., 2011).

1.3.1.3 Fluid Therapies

Intravenous fluid administration is a common approach to provide nutrients and maintain blood volume in critically ill patients (Villela *et al*., 2009). Fluid resuscitation targets CVP ≥8-12 mm Hg. This approach requires a fixed amount of fluid (colloids or crystalloids) to be administered over a short period of time; 1000 ml of crystalloids or 300-500 ml of colloids during 30 minutes, while hemodynamic responses (BP and HR) and safety limits (e.g. CVP) are monitored. In cases of sepsis-induced tissue hypoperfusion, more rapid and larger volume of fluids may be required (Dellinger *et al*.,

2008; Vincent *et al*., 2011).The most common crystalloid solutions that used are 0.9% sodium chloride (normal saline) and lactated Ringer's solution. The most commonly used colloid solution is hydroxyethyl starch (Hollenberg *et al*., 2004). Resuscitation with crystalloid or colloid suffers from specific limitations. For example, colloids administration could increase the risk of acute renal failure. The volume of distribution is much larger for crystalloid than colloids, thus a larger volume of crystalloid is needed to achieve the same level of volume expansion and results in more edema. However, crystalloids are less expensive (Dellinger *et al*., 2008; Daniels, 2011).

1.3.1.4 Vasopressors

If an adequate arterial pressure is not restored with fluid therapy, vasopressor therapy is required to achieve MAP ≥65 mm Hg (Marik, 2011; Dellinger *et al*., 2004). Vasopressor therapy is used to maintain perfusion and restore hypotension in septic shock. It is recommended if the clinical signs of shock continue despite an adequate fluid replacement. Based on SSC guidelines vasopressor drugs such as norepinephrine, dopamine, epinephrine, and phenylephrine are used to maintain perfusion and treat low blood pressure by causing vasoconstriction (Dellinger *et al*., 2008).

1.3.1.5 Corticosteroids

The SSC guidelines (2008) recommended intravenous use of hydrocortisone only in adult patients with septic shock who were unresponsive to fluids and vasopressor therapy (Chipp *et al*., 2010; Cohen, 2011; Dellinger *et al*., 2008). Corticosteroids (CS) affect metabolism, the cardiovascular and immune system, and has an anti-inflammatory action that interferes with components of the inflammatory response; this can be beneficial in diseases such as sepsis where the inflammatory process does not recognize

normal host cells from invasive cells (Ile & Ouest, 2005; Cohen, 2011). The early use of corticosteroids started in 1980s, but high dose and short duration treatment with steroids was not found to be effective for resolution of sepsis. Current treatment is carried out with low dose steroid and longer duration of treatment and only in septic shock patients unresponsive to other therapies (Vincent *et al*., 2011; Batzofin, *et al*., 2011). Clinically effective corticosteroid treatments of sepsis include hydrocortisone and fludrocortisone (Dellinger *et al*., 2008). The adverse effects of corticosteroid treatment are increased risk of gasterointestinal bleeding, superinfection, or hyperglycaemia (Ile & Ouest, 2005) .

1.3.2 Adjuvant therapies

1.3.2.1 Recombinant Human Activated Protein C

In 2001, the USA Food and Drug Administration (FDA) approved the use of activated protein C (APC; Xigris®) for the treatment of severe sepsis. In 2008, SSC guidelines recommended APC for treatment of adult patients with sepsis-induced organ dysfunction associated with a high risk of death (Dellinger *et al*., 2008). APC is an endogenous protein that has pro-fibrinolytic, anti-thrombosis, anti-coagulant and anti-inflammatory properties, and therefore is an important modulator of coagulation and inflammation associated with severe sepsis (Bernard *et al*., 2001). Severe sepsis leads to disseminated intravascular coagulation (DIC). This reduces the amount of blood that reaches different organs, suggesting that treatment with APC can improve microcirculatory perfusion and thus prevent MODS (Bernard, 2003). In the intestinal microcirculation, APC administration has been shown to increase the functional capillary density (FCD) and reduce leukocyte adhesion to the venules (Lehmann *et al*., 2006). Furthermore, in patients with severe sepsis, administration of APC improved capillary

perfusion (De Backer *et al*., 2006; Nencioni *et al*., 2009). However, it should be pointed out that the main disadvantage associated with the use of APC is the risk of serious bleeding (Woodward & Cartwright, 2009; Bernard *et al*., 2001; Bernard, 2003). APC was the first and only anti-inflammatory agent that has proved effective in the treatment of sepsis to reduce mortality. However, on October 25, 2011, the European Medicines Agency issued a worldwide withdrawal of APC from the market by Eli Lilly since it did not meet the primary endpoint of the PROWESS-Shock study, which was a statistically significant reduction in 28-day mortality (Martí-Carvajal *et al*., 2012).

1.3.2.2 Lipid signaling in inflammation and sepsis

Acute inflammation is the host response to an infection or injury. However when inflammatory reactions become uncontrollable, they progress from local to systemic inflammation. Uncontrolled inflammation is a characteristic of many diseases such as atherosclerosis, asthma, cancer and sepsis (Serhan *et al*., 2008; Norling & Serhan, 2010). Increasing evidence suggests that inflammation and inflammatory disorders, including sepsis, can be modulated by endogenous chemical mediators, including lipid mediators (LMs). Lipid mediators regulate resolution of inflammation by activating anti-inflammatory and pro-resolving signaling pathways to terminate acute inflammation. The arachidonic acid-derived eicosanoid, lipoxins, are the first recognized LMs that have anti-inflammatory and pro-resolving (resolution of inflammation is the reduction or removal of neutrophils infiltration to inflamed sites) properties (Norling & Serhan, 2010; Serhan *et al*., 2008). Lipoxins appear to terminate inflammation by reducing neutrophil infiltration to the site of inflammation, and increasing the uptake and clearance of apoptotic neutrophils by macrophages (Norling & Serhan, 2010; Serhan *et al*., 2008;

Walker *et al*., 2011).

1.3.2.3 Immunomodulatory Therapy

Production of pro-inflammatory cytokines is the part of the body's immune response to an infection that allows the immune cells to be activated. In sepsis bacterial endotoxin binds to immune cells via TLRs, such as dendritic cells and macrophages, and causes excessive production of pro-inflammatory cytokines, including TNF-α and IL-1β (Chong & Sriskandan, 2011). The extensive release of these cytokines leads to organ dysfunction and mortality in sepsis. Thus blocking the level of TNF-α and IL-1β could be used as effective strategy to improve outcome of sepsis. Despite that, treatment with anti-cytokine agents can be harmful as a consequence of interfering with the beneficial effects of cytokines in stimulating inflammatory mechanisms (Dinarello & Abraham, 2002). The function of anti-inflammatory agents depends on the time course of sepsis. The early phase of sepsis is characterized by increases in inflammatory mediators, this phase is known as hyper-inflammatory state manifested as SIRS; but as sepsis persists, there is a shift toward a hypo-inflammatory immunosuppressive state manifested by compensatory anti-inflammatory response syndrome (CARS) (Figure 6). Anti-inflammatory strategies that are used in the early course of sepsis when the patient shows a hyper-inflammatory response may improve the treatment; alternatively, anti-inflammatory strategies that been used in the hypo-inflammatory phase of sepsis may worsen the outcome of sepsis (Figure 6)(Hotchkiss & Karl, 2003; Christaki *et al*., 2011).

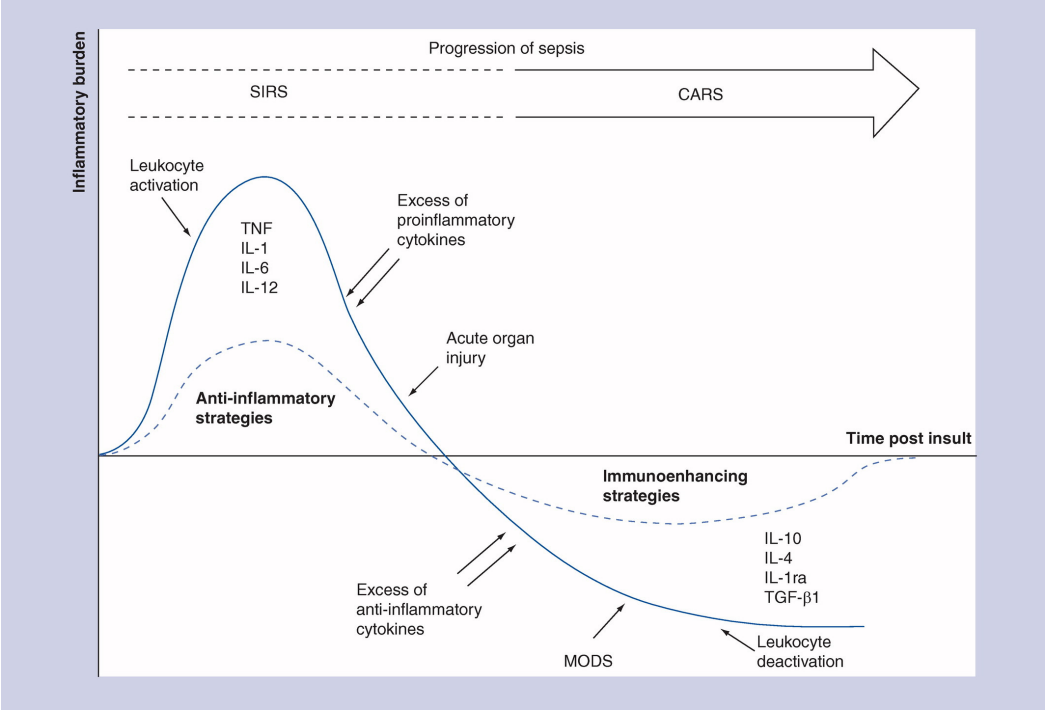


Figure 6. Anti-inflammatory strategies: Anti-inflammatory strategies are used in the early phase of sepsis, hyper-inflammatory state, while immunoenhancing strategies are used in hypo-inflammatory immunosuppressive state. CARS: Compensatory anti-inflammatory response syndrome; IL-1ra: IL-1 receptor antagonist; MODS: Multiple organ dysfunction syndrome; SIRS: Systemic inflammatory response syndrome (Christaki *et al*., 2011).

1.4 Experimental Sepsis Models

In order to more effectively study the pathology of sepsis, a number of experimental approaches and models have been developed. There are two main categories of experimental sepsis models: A) Toxin models and B) Intra-abdominal sepsis models.

A) The endotoxin model, which is produced through direct administration of endotoxin (lipopolysaccharide, LPS), is widely used to examine the pathophysiology of sepsis. The endotoxin model has the advantages of being easy to use, and endotoxins are causing similar pathophysiological changes as sepsis (Remick &Ward, 2005; Deitch, 2005). LPS is the main component of the gram-negative bacterial cell wall, and is known to stimulate the release of inflammatory mediators from various cells and induce acute infectious symptoms upon administration. The main aim of endotoxin injection is to mimic the septic response. There are pathophysiological similarities between injection of endotoxin and sepsis. However, there are also differences (Remick &Ward, 2005). Several studies using the endotoxin model of sepsis have reported that the main potential mediators of sepsis are the pro-inflammatory cytokines; TNF-α and IL-1. High levels of TNF-α were reported in sepsis patients (Waage & Halstensen, 1987), and in humans that received LPS (Michie *et al*., 1988; Remick & Ward, 2005). It is also known that there is a direct relation between the amount of TNF-α produced in serum and the mortality rate in the sepsis patients (Remick & Ward, 2005). Endotoxin challenge results in rapid increases in the plasma level of TNF-α; it also increases the IL-1 production, slightly later than TNF-α. Sepsis patients also express TNF-α, but in a much lower level than endotoxin-treated volunteers (Remick & Ward, 2005). In other sepsis models such as the

cecal ligation and puncture (CLP) model, TNF-α appears in plasma later and in lower concentrations than in the acute model of sepsis (endotoxemia). Although acute endotoxin models are useful models for their reproducibility and similarities to pathophysiology of sepsis, the disadvantage of these models are the massive and rapid production of several pro-inflammatory cytokines after endotoxin injection, in contrast to the lower and more prolonged cytokine production in clinical sepsis patients (Remick & Ward, 2005; Dejager *et al*., 2011). These differences may explain why therapeutic strategies that might be effective in endotoxin models are not effective in clinical sepsis patients (Remick & Ward, 2005; Riedemann *et al*., 2003).

B) The intra-abdominal sepsis models are generated through the introduction of bacteria into the body. As with other models, the intra-abdominal models have their own advantages and disadvantages. These models are clinically more relevant compared to endotoxin models. The main classes of intra-abdominal sepsis model are: 1) the fecal pellet model 2) the defined bacterial inoculum model and 3) the endogenous fecal contamination model (Deitch, 2005).

The fecal pellet model is produced by administration of feces plus an adjuvant (such as barium) within a pellet, in a gelatin capsule or as a part of fibrin clot. The role of adjuvant is to prevent rapid clearance of bacteria by the host and to modulate the mortality rate. The mortality rate in this model depends on dose and composition of the bacteria of the feces. The disadvantage of this model is the differences in the amount of bacteria and species between different feces samples, results in variable sepsis severity. Overall, the feces pellet model is not as useful as models that have a defined bacterial amount (Deitch, 2005).

The defined bacterial inoculum model is very similar to the fecal pellet model. The only difference is that the bacterial population is defined. Generally *Escherichia coli* (*E. coli*) is the strain of bacteria that is mixed with sterilized cecal contents and introduced into the peritoneum. The main advantage of this model is that the mortality rate is controllable by modifying the dose of *E. coli* (Deitch, 2005).

The endogenous fecal contamination model further consists of at least two models: CLP and Colon Ascendens Stent Peritonitis (CASP). Over the past 30 years the CLP has been extensively used to study the underlying mechanism of sepsis (Rittirsch *et al*., 2009; Dejager *et al*., 2011). The CLP involves a combination of 3 factors: tissue trauma due to laparotomy, necrosis due to ligation of the cecum and infection due to leakage of feces into the peritoneum (Dejager *et al*., 2011). In CLP model, the cecum is ligated and punctured with a needle. The advantage of this model is that mortality rate and time of death can be modulated by the size of the needle and the number of punctures made in the cecum (Rittirsch *et al*., 2009); CLP shows a high degree of similarity to human sepsis progression (Wichterman *et al*., 1980; Dejager *et al*., 2011). However, the disadvantages of this model are that animals are not fluid resuscitated and do not receive antibiotics, which are both standard therapeutic strategies for sepsis treatment (Deitch, 2005). The second type of fecal contamination model is the CASP model, which is performed by surgical insertion of a stent with a defined diameter into the ascending colon. Thus allows feces and bacteria to reach the peritoneal cavity continuously. The main advantage of the presence of the stent is to ensure continuous fecal and bacteria contamination. Similarly to the CLP model, by modulating the size of the stent, the severity of the inflammatory response and mortality rate is controllable (Deitch, 2005).

Compared to CLP, CASP is a newer model with fewer variables; however, CASP is more difficult surgical procedure that can be a weakness in terms of reproducibility (Dejager *et al*., 2011). A study of the CASP model showed that it is a clinically relevant model as it closely mimics symptoms of clinical sepsis (Lustig *et al*., 2007).

1.5 The Endocannabinoid System

1.5.1 Physiology and Pharmacology

Cannabis sativa contains more than 60 active phytocannabinoids, and it is one of the oldest plants to be used not only for its psychoactive effects but also for medical purposes. Cannabinoids have been shown to have analgesic, anti-emetic, anti-inflammatory and immunomodulatory effects, and have been shown to be effective in prevention of nausea/vomiting and the treatment of obesity and cancers (Vemuri *et al*., 2008; Pertwee, 2006b; Izzo *et al*., 2009). The endocannabinoid system (ECS) is an endogenous lipid signaling system. Endocannabinoids are arachidonic acid derivatives and part of the bioactive lipid signaling system. This suggests that the possible role of endocannabinoids in treatment of uncontrolled inflammatory diseases such as sepsis. The ECS consists of cannabinoid receptors cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R), their endogenous ligands (endocannabinoids), and the enzymes involved in their synthesis and inactivation (Pertwee, 2006b).

Cannabinoid receptors are members of the G protein coupled receptor (GPCR) family. CBRs activate multiple signaling pathways including coupling to inhibitory G proteins ($G_{i/o}$) to activate downstream signaling pathways consequently reduction in Cyclic adenosine 3,5-monophosphate (cAMP) production in most tissues. Additionally,

CBRs regulate phosphorylation and activation of different members of the family of mitogen activated protein kinases (MAPKs) (Figure 7)(Bosier *et al*., 2010). In 1990, Matsuda *et al*. cloned the CB1R (Matsuda *et al*., 1990). CB1R is a seven transmembrane (TM) domain protein of GPCR family A. CB1R is highly expressed in the CNS, especially in the mammalian brain, and has a role in a modulation of synaptic transmission (Herkenham *et al*., 1991; Matsuda *et al*., 1990; Bouaboula *et al*., 1993; Ishac *et al*., 1996). CB1R expression in the CNS is responsible for the psychoactive effects of cannabinoids (Chevaleyre *et al*., 2006). Activation of presynaptic CB1Rs causes inhibition of neurotransmitter release in the brain and peripheral nerves and therefore modulates inhibitory and excitatory neurotransmission in the CNS (Chevaleyre *et al*., 2006; Guggenhuber *et al*., 2010). Stimulation of presynaptic CB1R inhibits neurotransmitter release through stimulation of potassium (K^+) channels and inhibition of calcium (Ca^{2+}) channels (Turu & Hunyady, 2010; Chevaleyre *et al*., 2006). Postsynaptic CB1R are also expressed in a variety of peripheral tissues and cells, including the vasculature and gut; activation of these receptors produces hypotension and regulates emesis and feeding, respectively (Godlewski *et al*., 2004; Hornby & Prouty, 2004). CB1Rs are also expressed in peripheral tissues such as adrenal glands, heart, lung, spleen, tonsils and on immune cells (Bouaboula *et al*., 1993; Felder *et al*., 2006; Buckley, 2008; Galiègue *et al*., 1995). CB1R expression on leukocytes is B cells > NK cells > neutrophils > CD8+ cells > monocytes > CB4+ cells (Pertwee, 1997; Bouaboula *et al*., 1993; Galiègue *et al*., 1995). The therapeutic potential for CB1R ligands has been extensively studied. For example, Rimonabant (CB1R antagonist) was shown to have a beneficial effect in weight loss in obesity (Howlett *et al*., 2004; Turu & Hunyady, 2010).

However, it was withdrawn from the market (in 2008) because of severe psychiatric side effects. CB1R is a promising therapeutic target for treatment of many disorders (such as metabolic disorders, including obesity, type 2 diabetes), however the effort for developing CB1R ligands (antagonist) should avoid the psychological adverse effects (Wu *et al*., 2011).

In 1993 Munro *et al*. identified the peripheral CB2R (Munro *et al*., 1993). The CB2R is mainly expressed on immune cells and is known to have a role in immune responses and regulation of inflammatory reactions (Felder *et al*. 2006; Berdyshev, 2000). The rank order of CB2R expression in immune cells is: B cells > NK cells > monocytes > neutrophils > CD8+ cells > CD4+ cells (Galiègue *et al*., 1995). CB2R is also expressed in the brain, bone marrow, spleen, thymus and through out the gastrointestinal system (Schatz *et al*., 1997; Van Sickle *et al*., 2005; Ofek *et al*., 2006; Felder *et al*., 2006; Howlett *et al*., 2004; reviewed by Buckley, 2008). CB2R expressed on immune cells show various immunomodulatory effects. Furthermore, CB2R are also present in endothelial cells, which can limit the inflammatory response and inflammatory cell activation and adhesion. The mechanism that CB2Rs use to mediate biological activity is the cAMP signaling pathway. CB2R are coupled to $G_{i/o}$. As a result of G_i coupling, activation of CB2R causes inhibition of adenylyl cyclase (AC), consequently reducing the intracellular level of cAMP production (Figure 7) (Howlett *et al*., 2004). cAMP is an important regulator of neutrophil function, since high levels of cAMP inhibit neutrophil function and activation. This clarifies the importance of CB2R modulation in regard to cAMP production and subsequent neutrophil activation (Tschöp *et al*., 2009). The therapeutic potential for CB2R modulation has been extensively studied. For example,

activation of CB2R had an anti-inflammatory effect that includes inhibition/reduction of pro-inflammatory cytokines production and adhesion molecule expression (Gertsch, 2008; Bento *et al*., 2011; Lehmann *et al.*, 2012; Bátkai *et al*., 2007; Horváth *et al*., 2012).

Ligands for cannabinoid receptors are divided into three main classes: (1) phytocannabinoids, (2) endogenous cannabinoids (endocannabinoids) and (3) synthetic cannabinoids. Cannabinol (CBN), cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) are the best known phytocannabinoids (Gaoni & Mechoulam, 1964; Mechoulam & Gaoni, 1967; Hanus, 2008). CBN is a psychoactive component from the cannabis plant, and acts as a weak agonist of CB1R and CB2R. It was the first phytocannabinoid that was isolated by Wood *et al*. in 1899 (Hanus, 2008). CBD is a non-psychoactive constituent of cannabis (Mechoulam *et al*., 2007) that was isolated by Mechoulam's lab in 1963 (Mechoulam and Shvo, 1963); it has a very low affinity for both CB1R and CB2R (Pertwee, 2008). CBD produces a range of effects including anti-inflammatory, anti-oxidant, anti-convulsive, anti-anxiety, anti-nausea, anti-tumor and neuroprotective effects (Mechoulam *et al*., 2007; Ruiz-Valdepenas *et al*., 2011; Mechoulam & Hanus, 2002; Capasso *et al*., 2008). In 1964, Mechoulam's group isolated the psychoactive constituent of cannabinoid plant, Δ9-THC (Gaoni & Mechoulam, 1964; Mechoulam & Gaoni, 1967). In addition to psychotropic effects, Δ9-THC possess analgesic (Walker & Huang, 2002) and anti-emetic (Kwiatkowska *et al*., 2004) properties. It also stimulates appetite and has neuroprotective properties (Mechoulam & Hanus, 2001). However, the use of Δ9-THC is limited by its psychoactive side effects (Pertwee, 2006a). Δ9-THC has the ability to activate both CB1R and CB2R (Pertwee, 2008).

Endocannabinoids are endogenous arachidonic acid derivatives, which are the

body's natural agonists for cannabinoid receptors. The two best known endocannabinoids, are arachidonylethanolamide (anandamide) (Devane *et al*., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al*., 1995; Sugiura *et al*., 1995); both function as neurotransmitters. Anandamide (AEA) has a stronger affinity for CB1R, than for CB2R. 2-AG acts as a full agonist at both CB1R and CB2R (Hanus, 2007, 2008). 2-AG and AEA are both synthesized on demand through multiple biosynthetic pathways (Hanus, 2007). AEA is formed from N-arachidonoyl phosphatidylethanolamine (NAPE) by an enzyme named phospholipase D-like (NAPE-PLD). 2-AG is formed from diacylglycerol (DAG) by a selective enzyme, DAG Lipase (André & Gonthier, 2010).

Synthetic cannabinoids bind to either CB1R or CB2R, or to both receptors and are structurally similar to phytocannabinoids (Howlett *et al*., 2002). The most frequently used synthetic cannabinoid ligands are agonists such as HU308, ACEA and inverse agonists (shows opposite pharmacological effects to an agonist) such as SR141716A and AM630.

Lastly, termination of the signaling function of the endocannabinoids is a two-step process, which requires specific reuptake proteins or transporters and subsequent hydrolysis of the molecules with hydrolysis enzymes (Basavarajappa, 2007). To date, two enzymes have been identified that are required for inactivation of endocannabinoids, namely, fatty-acid amide hydrolyse (FAAH) and monoacylglycerol lipase (MAGL) (Jayamanne *et al*., 2006; Jhaveri *et al*., 2007). AEA is metabolized by FAAH; 2-AG is metabolized by MAGL and FAAH (Basavarajappa, 2007). FAAH is a membrane bound enzyme, which belongs to the family of serine hydrolase and MAGL is a also a serine-hydrolase. FAAH are highly expressed in the brain and liver; MAGL are highly expressed in brain (Rodríguez de Fonseca *et al*., 2005). Inhibition of FAAH elevates the

endogenous levels of AEA and consequently prolongs its biological effects, such as anti-inflammatory and analgesic effects (Seierstad & Breitenbucher, 2008). Recently, it was reported that genetic deletion or inactivation of FAAH in rats, results in analgesia, anti-inflammatory, and anti-depressant effects without psychotropic side effects (Bachovchin & Cravatt, 2012). Inhibition of MAGL leads to increase the level of 2-AG in the brain that may result in CB1R-mediated analgesic effects (Long *et al*., 2009; Jhaveri *et al*., 2007).

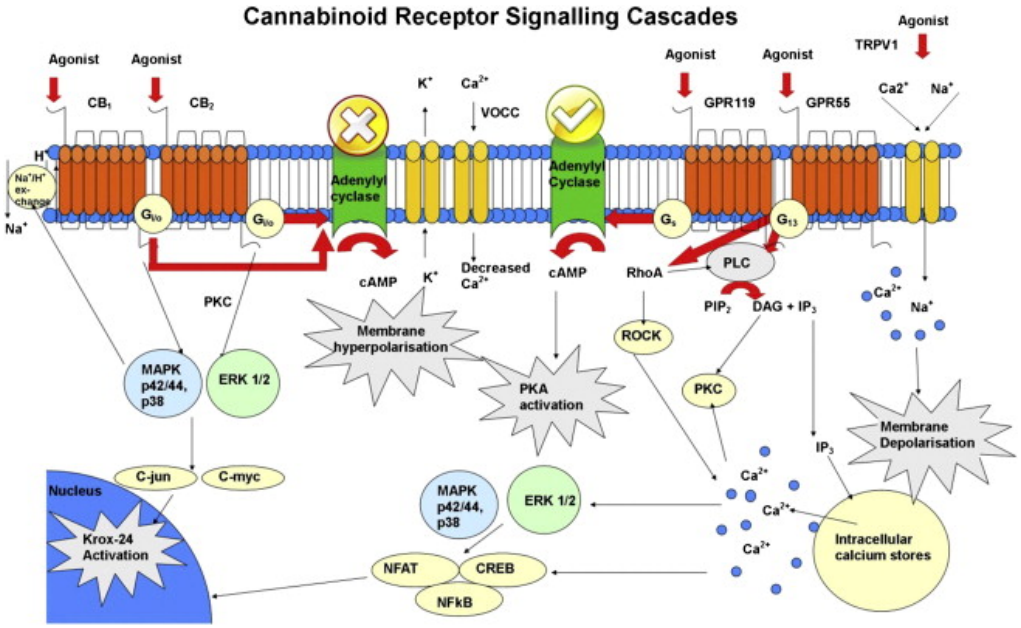


Figure 7. Cannabinod Receptor Signaling Cascade. Activation of the CB1 receptor results in Gi/o coupling, with inhibition of adenylyl cyclase, modulation of membrane ion channels and activation of MAPK/ERK signaling and transcription factors, causing membrane hyperpolarization. Activation of the CB2 receptor results in Gi/o coupling, with inhibition of adenylyl cyclase, and activation of MAPK/ERK signaling and transcription factors (Ladak *et al.*, 2011).

1.5.2 Endocannabinoid system as therapeutic target in sepsis

The peripheral ECS is up regulated during septic shock (Wagner *et al*., 1998; Maccarrone *et al*., 2002). Endocannabinoids are released from endothelial cells, macrophages, dendritic cells, platelets and parenchymal cells in response to inflammatory stimuli and oxidative stress. Furthermore, elevated levels of endocannabinoids have been reported in the sera of patients and animals in septic shock (Varga *et al*., 1998; Wagner *et al*., 1998; Pacher, *et al*., 2005; Orliac *et al*., 2003; Csoka *et al*., 2009). This suggests the importance of ECS modulations in sepsis.

The purpose of this research project was to examine the effects of manipulating the ECS during acute systemic inflammation (ie, sepsis). A crucial endpoint of this study was the preservation of the intestinal microcirculation, a key therapeutic target in the treatment of sepsis. This could involve attenuation of immune cell recruitment and improvements in microcirculatory perfusion. In our experimental approaches we used cannabinoid receptor-selective ligands, as well as enzyme inhibitors that prevent endocannabinoid degradation. Our aim was to determine their effects on the intestinal microcirculation (leukocyte-endothelial interactions, functional capillary density) in an *in vivo* model of experimental sepsis.

1.6 Hypotheses and objectives

The overall hypothesis of this study was that modulation of ECS during acute phase of sepsis would improve microcirculation and reduce the severity of systemic inflammation. The overall hypothesis is further divided into two testing hypotheses: 1.

Modulation (activation or inhibition) of cannabinoid receptors by cannabinoid ligands preserves the intestinal microcirculation in experimental sepsis and 2. Modulation of ECS by inhibition of endocannabinoid degradation preserves the IMC in endotoxemia sepsis model.

The main objectives of this study were:

Objective 1. to examine the effects of CB1R modulation on systemic hemodynamics, intestinal leukocyte endothelial interactions and functional capillary density and histology in experimental sepsis.

Objective 2. to examine the effects of CB2R modulation on systemic hemodynamics, intestinal leukocyte endothelial interactions and functional capillary density, plasma levels of cytokines and adhesion molecules and histology in experimental sepsis.

Objective 3. to examine the effects of inhibition of endocannabinoid degradation on systemic hemodynamics, intestinal leukocyte endothelial interactions and functional capillary density and histology in experimental sepsis.

CHAPTER 2: MATERIAL AND METHODS

2.1 Animals

130 Male Lewis rats (weight, 250 ± 50 g) were purchased from Charles River Laboratories International Inc. (Wilmington, MS, USA) and housed in chip-bedded cages. Prior to experiments, animals were acclimatized for one week in the air-filtered institutional Carleton Animal Care Centre at Dalhousie University, Halifax, Canada. Animals were kept on a 12 hr light/dark cycle, with the room temperature maintained at 22°C and humidity at 55-60%. A standard diet of rodent chow and sterile drinking water was available *ad libitum*. All experimental procedures and protocols were performed in accordance with the standards and procedures set forth by the Canadian Council on Animal Care (protocol No. 11072).

2.2 Anesthesia

Rats were weighed with a commercially available scale (Sartorius, Goettingen, Germany) prior to anesthesia followed by an intraperitoneal (i.p.) administration of sodium pentobarbital (54.7mg/ml, 54.6 mg/kg body weight) (Ceva Sante Animale, Montreal, QC, Canada). After 15 to 20 min of initial anesthetic induction, the animals were prepared for surgical procedures. Throughout the experiment, the depth of anesthesia was assessed by checking the animal's reaction to tail pinch and, when needed, an additional 0.05 ml pentobarbital (54.7mg/ml, 9.1mg/kg body weight) was administered.

2.3 Surgical preparation

Animals were placed in a supine position on an operation table. The neck region was shaved to facilitate access and the surgical area cleaned and disinfected with sterile alcohol swabs (Health care, Toronto, Canada). A vertical incision was made in the neck using a scalpel (Fine Science Tools, British Columbia, Canada) and the surrounding tissue was dissected with the use of forceps (Fine Science Tools, British Columbia, Canada). Visualization of the jugular vein and the carotid artery was facilitated by dissection using blunt-tip scissors and then the vagus nerve, that runs along with the carotid artery, was gently separated away from the artery using fine-forceps (Figures 8a & b). The vein and artery were then separated and approximately 5-7 mm of the length of these vessels was isolated for cannulation. Catheters were fabricated by enveloping a 23 gauge needle with Intramedic Non-radiopaque polyethelene tubing (PE 50, Clay Adams, Sparks, MD, USA). For catheter placement, a piece of sterile silk black braided string (Ethicon, New Jersey, USA) was placed under the blood vessel. Then, a surgical knot was tied at the distal end of the blood vessel and a loose knot was made at the proximal side of the blood vessel. Using micro-dissecting scissors (Fine Science Tools, British Columbia, Canada), a small incision was made close to the distal end of the vessels. A fine-tipped micro-forceps (Fine Science Tools, British Columbia, Canada) was placed into the incision site and, using another pair of forceps, the polyethelene tubing was gently placed into the vessels (Figure 9). A PE 50 catheter was inserted into the left jugular vein was for intravenous (i.v.) administration of drugs, florochomes, LPS and anesthetics. Aproximately 1 cm of catheter tubing was inserted into the vessels and secured using silk string tightened with a triple knot. The patency of the catheter was

checked by flushing a small amount of saline through it. The right carotid artery was cannulated for monitoring of arterial blood pressure and heart rate and the carotid artery cannulation followed the same procedure as the vein cannulation, except that a surgical vessel clamp was used to hold the proximal end of the vessel because the arterial blood pressure is high. During the two hours of observation period, mean arterial pressure (MAP), heart rate (HR) and temperature (Temp) was monitored and recorded every 15 minutes. Animals were placed in a supine position on a heating pad (thermostatic platform) and kept at a body temperature of 37± 0.5°C (98.6°F) measured via a rectal thermometer.

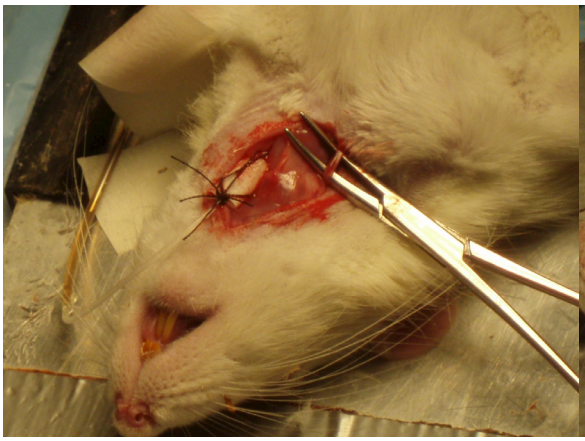


Figure 8 (a). Insertion of polyethylene catheter in the jugular vein.

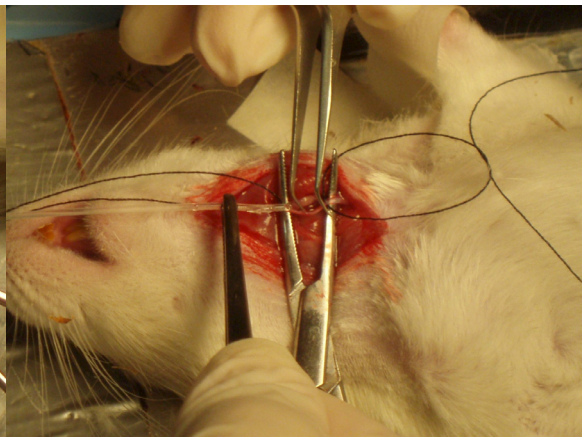


Figure 8 (b). Dissection of carotid artery.

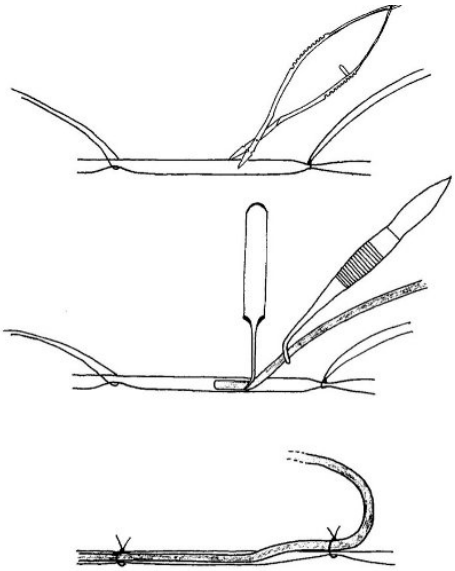


Figure 9. Catheter placement. Using micro-dissecting scissors place a small incision approximately ¼ of the way through the vessel and place fine-tipped forceps into the incision and using another pair of forceps move the catheter into the vessel (middle). Upon completion of the placement of the catheter suture the catheter in place (bottom) (Jespersen *et al*., 2012).

A tracheostomy was performed to access the airway and maintain patency. During this procedure, the muscle tissue was dissected away from the trachea with blunt forceps (Fine Science Tools, British Columbia, Canada). The trachea was lifted slightly and an incision was made between the annular hyaline cartilage in the trachea with the scalpel (Fine Science Tools, British Columbia, Canada). Using cotton tipped applicators (Puritan medical products company, Maine, USA), any blood in the surrounding area was removed. A 16 gauge catheter (Cathlon, Smiths Medical International Ltd, Lancashire, UK) was placed into the trachea. Insertion of the catheter was followed by securing (tied 2-3 surgical knots at the proximal and distal end of the trachea) the catheter to the trachea. A 3 ml syringe (Becton Dickinson and Company, NJ, USA) connected to a short cannula was used as suction for removal of fluids that could be obstructing the trachea.

2.4 Endotoxemia protocol

After surgical preparation was completed, endotoxemia was produced by the administration of lipopolysaccharide from *Escherichia coli*, (serotype O26:B6, Sigma-Aldrich, Oakville, ON, Canada). Lipopolysaccharide (LPS) was dissolved in sterile saline (0.9% Sodium Chloride, Hospira, Montreal, QC, Canada) (50 mg/10 ml). LPS was administered intravenously (i.v.) at 0 min or 60 min in the experimental time-course (Table 2)(Figures 10a & b). LPS administration was carried out gradually over 15 min, while monitoring the blood pressure (BP) in order to ensured that the mean arterial pressure (MAP) maintained above 60 mmHg. If blood pressure dipped < 60 mmHg, oxygen was administered via the tracheotomy until BP recovered to ≥ 60 mmHg. For the

post-treatment groups (Figure 10a), drugs were administered 15 min following LPS challenge. For the pre-treatment groups (Figure 10b), drugs were administered 1 hr prior to LPS challenge. All cannabinoid drugs were administered over the duration of a 10-15 min period.

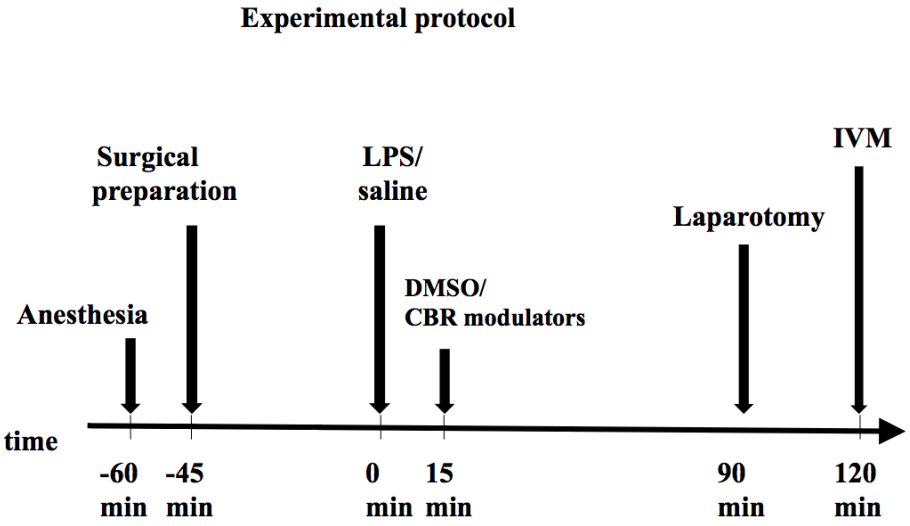


Figure 10(a). Experimental timeline (post-treatment) for IVM in the intestinal microcirculation. Times for anesthesia, surgical preparation, endotoxin, cannabinoid receptor modulator injections, laparotomy and IVM are indicated.

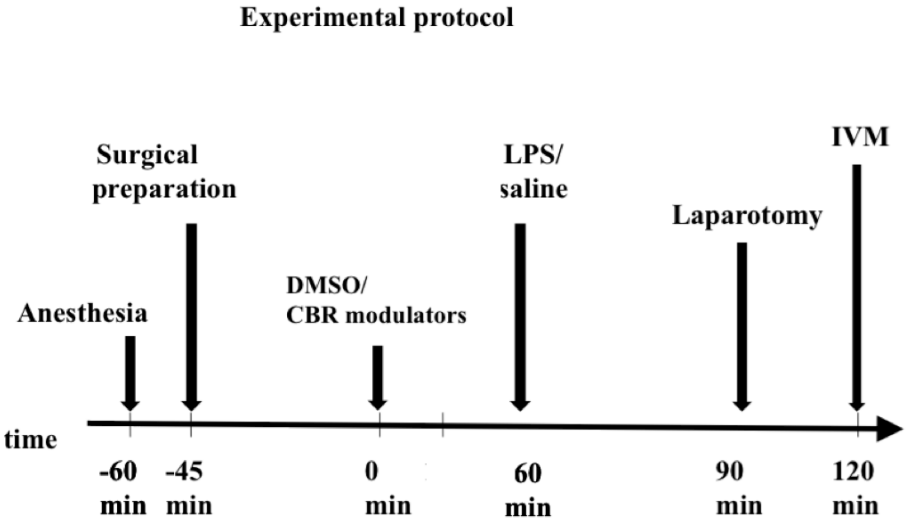


Figure 10(b). Experimental timeline (pre-treatment) for IVM in the intestinal microcirculation. Times for anesthesia, surgical preparation, endotoxin, cannabinoid receptor modulator injections, laparotomy and IVM are indicated.

2.5 Pharmacological Agents

ACEA is a potent and highly selective CB1 receptor agonist (K_i=1.4 nM) that displays >1400-fold selectivity for CB1 versus CB2 receptors (Tocris Bioscience, Ellisville, Missouri, USA)(Hillard *et al*., 1999). ACEA (2.5 mg/kg) was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Canada) (10 mg/4 ml) and further diluted in saline (1:2).

AM281 is a potent, selective CB1 receptor antagonist/inverse agonist, with K_i values of 12 nM and 4200 nM for CB1 and CB2 receptors, respectively (Tocris Bioscience, Ellisville, Missouri, USA)(Lan *et al*., 1999). AM281 (2 mg/kg) was dissolved in DMSO (10 mg/500 µl) and further diluted in saline (1:5). All the above treatments were administered i.v. to animals 15 min after endotoxin administration (Protocol#1; Figure 10a).

HU308 is a potent and selective CB2 receptor agonist with K_i values of 22.7 nM and 10 µM for CB2 and CB1 receptors, respectively (Tocris Bioscience, Ellisville, Missouri, USA) (Hanus *et al*., 1999). HU308 (2.5 mg/kg) was dissolved in DMSO (10mg/4ml) and stock solution further diluted in saline (1:2).

GP1a is a highly selective CB2 receptor agonist; K_i values are 0.037 nM and 363 nM for CB2 and CB1 receptors, respectively (Tocris Bioscience, Ellisville, Missouri, USA)(Tschöp *et al*., 2009). GP1a (2.5 mg/kg) was dissolved in DMSO (10mg/200µl) and further diluted in saline (1:5).

AM630 is a CB2 receptor antagonist/inverse agonist, with K_i value of 31.2 nM that displays 165-fold selectivity for CB2 receptors over CB1 receptors and behaves as a

weak partial/inverse agonist at CB1 receptor (Tocris Bioscience, Ellisville, Missouri, USA)(Ross *et al*., 1999). AM630 (2.5 mg/kg) was dissolved in DMSO (10mg/200µl) and further diluted in saline (1:5).

URB597 is a potent selective and irreversible inhibitor of FAAH (Cayman Chemical, Ann Arbor, MI, USA). URB597 inhibits FAAH activity with an IC_{50} value of 4 .6 ±1.6 nM in brain (Holt *et al*., 2005). URB597 (0.3mg/kg) and (0.6 mg/kg) was given i.v. to animals 1 hr prior to endotoxin administration URB597 was dissolved in DMSO (6mg/10ml) and further diluted with saline (1:3) (Protocol#2; Figure 10b).

2.6 Experimental groups

A total of 130 rats were randomly assigned to 13 experimental groups (each group was 10 animals). Group 1 served as the healthy control group (Control). Group 2 and 3 were untreated LPS animals (LPS 5 or 20 mg/kg, respectively). Details for treatment groups are provided in sections 2.6.1, 2.6.2 and 2.6.3 (see Table 2).

2.6.1 CB1R experiments

In group 4, the CB1R agonist, ACEA (2.5 mg/kg), was administered and group 5 received the CB1R antagonist, AM281 (2 mg/kg), i.v. after 15 min of endotoxemia. In group 6, we administered the CB1R antagonist, AM281 (2 mg/kg), after 15 min of endotoxemia and CB1R agonist, ACEA (2.5 mg/kg), 10 min after the AM281.

2.6.2 CB2R experiments

Group 7 received LPS (20 mg/kg) and the CB2R agonist, HU308 (2.5 mg/kg). In group 8, we administered another CB2R agonist GP1a (2.5 mg/kg). Group 9 received the CB2R antagonist, AM630 (2.5 mg/kg), i.v. after 15 min of endotoxemia. Group 10 was administered the CB2R antagonist, AM630 (2.5 mg/kg), after 15 min of endotoxemia, and the CB2R agonist, HU308 (2.5 mg/kg), 10 min after the AM630.

2.6.3 Enzyme inhibitor experiments

Three groups were examined. In group 11, URB597 (0.3 mg/kg) was administered 1 hr prior to endotoxin administration and in group 12, URB597 (0.6 mg/kg) was administered 1 hr prior to endotoxin administration; group 13 received the URB597 (0.6 mg/kg) 1 hr prior to endotoxin administration and AM630 (2.5 mg/kg) 5 min after URB597 administration.

Table 2- Experimental groups

Groups	Targets	Substrate	Dosages
Group 1.	Control	Saline	
Group 2.	Endotoxemia	LPS	5 mg/kg LPS
Group 3.	Endotoxemia	LPS	20 mg/kg LPS
Group 4.	CB1R (agonist)	LPS (5 mg/kg) + ACEA	2.5 mg/kg ACEA
Group 5.	CB1R (antagonist)	LPS (5 mg/kg) + AM281	2 mg/kg AM281
Group 6.	CB1R (agonist) + CB1R (antagonist)	LPS (5 mg/kg) + ACEA + AM281	2.5 mg/kg ACEA 2 mg/kg AM281
Group 7.	CB2R (agonist)	LPS (20 mg/kg) + HU308	2.5 mg/kg HU308
Group 8.	CB2R (agonist)	LPS (20 mg/kg) + GP1a	2.5 mg/kg GP1a
Group 9.	CB2R (antagonist)	LPS (20 mg/kg) + AM630	2.5 mg/kg AM630
Group 10.	CB2R (agonist) + CB2R (antagonist)	LPS (5 mg/kg) + HU308 + AM630	2.5 mg/kg HU308 2.5 mg/kg AM630
Group 11.	FAAH (inhibition)	URB597 (0.3 mg/kg) + LPS (5 mg/kg)	0.3 mg/kgURB597
Group 12.	FAAH (inhibition)	URB597 (0.6 mg/kg) + LPS (5 mg/kg)	0.6 mg/kgURB597
Group 13.	FAAH (inhibition) + CB2R (antagonist)	URB597 (0.6mg/kg) + AM630 + LPS (5 mg/kg)	0.6 mg/kgURB597 2.5 mg/kg AM630

2.7 Intravital microscopy

IVM was carried out it all groups at 2 hr in the protocol (mortality in this model of endotoxemia was starting at 4 hr and 100% at 8 hr in pilot experiments). Procedures for animal preparation and IVM are outlined below (sections 2.7.1-6).

2.7.1 Laparatomy

Laparotomy was performed at time point 1.5 hr to provide a stabilization period of 30 minutes before IVM at time point 2 hrs (Figures 10a & b). The surgical area (abdomen) was cleaned and disinfected using alcohol swabs. By using a scalpel, a superficial skin cut was made on the abdomen. The muscular layer was lifted and a scalpel was used to cut right along the linea alba to open up the muscular layer. The purpose of lifting the muscle layer was to avoid cutting and damaging the intestine and other organs in the abdominal cavity. After opening of the abdomen a 3-4 cm long segment of the small intestine (terminal ileum) was exteriorized and placed carefully on the specially designed stage and held by a supporting device. The exteriorized portion of the intestine was surrounded with gauze sponges soaked in the saline to keep the intestine moist throughout the experiment. During the microscopic procedure, the intestine was perfused with thermostat-controlled (37°C/ 98° F) 0.9% sodium chloride solution (Hospira, Montreal, QC, Canada) to avoid drying. Briefly, by adjusting the tube that was attached to the specially designed stage, next to the intestine the saline was allowed to pull itself between intestine and cover slip (Propper, Bev-L-Edge Microscope slide, 0.9-1.0mm, NY, USA). Out of this tube heated saline dripped constantly. A flow of 5 ml/hr was used to make sure that the gut, which was swapped out of the abdominal cavity

remained warm and moist. A cover slip was placed without any compression or traction on the section of the terminal ileum that was chosen for the intestinal microcirculation (approximately one square cm). The animal along with the heating pad was then transferred onto the microscope stage.

2.7.2 Fluorescent staining

Fifteen minutes before the start of the IVM, leukocytes were stained by the i.v. injection of Rhodamine-6G (1.5ml/kg) (Sigma-Aldrich, ON, Canada) and the plasma was stained with fluorescein isothiocyanate (FITC)-albumin (1ml/kg) (Sigma-Aldrich, ON, Canada). FITC facilitated evaluation of the capillary flow by amplified contrast of the plasma. Rhodamine-6G and FITC both were administered into the jugular vein and flushed with small amount of saline to ensure the complete administration and circulation in the body. This process occurred in minimal light to prevent photo bleaching of the fluorochromes.

2.7.3 IVM equipment

Intravital fluorescent video microscopy was performed using the following devices:

- Epifluorescent microscope (Leica DMLM, Wetzlar, Germany)
- Light source (LEG EBQ 100, Jena, Germany)
- Lens (Leica N PLAN L 20X/0.40)
- Filter for examination with Rhodamine 6G (Leica)
- Filter for examinations with FITC-albumin (Leica)
- Black and white DAGE CCD video camera (DAGE MTI Inc., Michigan City, IN)
- Video tape recorder (DSR-25 DVCAM SONY, Halifax, Canada)
- Black and white monitor (Speco technologies, Texas, USA)

2.7.4 Leukocyte adherence

The microscope was set to focus upon the submucosa level of the prepared intestinal section, for investigation of leukocyte recruitment. Six visual fields containing non-branching, submucosal collecting venules (V1) and postcapillary venules (V3) over a length of at least 300 μm were observed and recorded for 30 sec each.

2.7.5 Functional capillary density

For investigation of functional capillary density (FCD), the filter was changed for examinations with FITC, and the setting was focused. Video sequences (30 sec) of six randomly selected fields of the capillaries within the longitudinal musculature and six fields of the capillaries within the circular muscle were recorded.

Lastly, the examination of the mucosa was performed through the opening of the intestinal lumen over a length of 2 cm (according Bohlen *et al*., 1976). A microcautery knife (Medtronic, FL, USA) was used to make a cut on the intestine, opposite to the direction of vessels. The sections that were filled with feces were used to avoid any alterations in heat temperatures among the mesenteric wall. The intestinal content was flushed with warm (37^{o}C) saline and the intestine was lifted again and held by the supporting device. Next, cotton swabs soaked in saline were used to remove the feces. A cover slip was placed on the prepared segment and the rest of IVM microscopy for the mucosa was performed. The cover slip was placed but not too tight, and the hanging drop tube was placed again close to intestine. Then six, 30 sec video sequences of randomly chosen mucosa sections were recorded. The duration of each experiment, including induction of anesthesia, did not exceed 240 min.

2.7.6 Video analysis

Evaluation of all the video sequences was carried out off-line on a video monitor. The following parameters were analyzed: adhering leukocytes [the number of leukocytes that during an observation period stayed immobile for at least 30 sec to an oblique, cylindrical endothelial surface; (sticker) = cells per square millimeter], flow of rolling leukocytes [the number of leukocytes that during an observation period of 30 sec passed in a rolling motion through a selected vascular diameter; (roller-flow) = cells per minute]. Vessel lengths and diameters were also recorded and measured. Functional capillary density (FCD) [the length of capillaries with observable erythrocyte perfusion in relation to a predetermined rectangular field; cm/cm2] according to Schmid-Schoenbein *et al*. (1977) was calculated. According to their method, the number of intersections between the capillaries and the grid were counted which was used to calculate the length of capillaries per area.

2.8 Blood/Tissue Sampling

At the end of the experiment, 1 ml of arterial blood was drawn into a syringe containing 0.1 ml heparin (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON, Canada) to prevent blood coagulation. The blood sample was then centrifuged (Brinkmann microcentrifuge model 5415, obtained from Eppendorf, Hauppauge, NY, USA) at 5000 rpm for 10 min at room temperature to obtain plasma. The plasma was collected in 0.5 ml microtubes and stored at -80°C until further measurement.

Next, tissue samples were collected from the distal small intestine. The samples were perfused with saline thoroughly, then fixed in 10% formalin for histology. After all the recordings, and blood and tissue samples were taken, the animals were euthanized by potassium chloride (KCl, 149 mg/ml saline, 142 mg/kg) (EDM Chemicals Inc., Gibbstown, N.J., USA), which was delivered i.v.

2.9 Histology

The intestinal samples were stored in 10% formalin. Then tissues were placed into plastic cassettes, washed with 70% ethanol twice. Samples were embedded in paraffin overnight by using an automated tissue processor (Leica Micosystems Inc., Richmond Hill, ON, Canada). The paraffin-embedded tissues were then cut into 5 μm sections using a microtome (Jung AG, Heidelberg, Germany) and tissue float water bath (Lipshaw MFG Co, Detroit, MI, USA). The tissue samples were then transferred onto glass microscope slides and dried in an oven (56-57°C) overnight.

The slides were then stained with hematoxylin and eosin to examine the morphological changes in the rat small intestine tissues. The paraffinized slides were de-paraffinized by sequential washing with xylene, 100%, 95%, 70% EtOH and running tap water. After rinsing off the EtOH using water, the slides were stained with filtered hematoxylin for 2 minutes. The excessive stain was quickly rinsed off with water, Scott's H_2O Solutions (2 minutes), 0.2% nitric acid and rinsed again with water. Then the slides were rinsed and they were stained with 1% eosin. To ensure proper staining, the slides were dipped in eosin solution 13 times. The excessive eosin was washed away with 70%,

95%, 100% EtOH and xylene washes (each 3x). Then glass coverslips were mounted to the slides and sealed with Cytoseal solution (Electron Microscopy Sciences, Fort Washington, PA, USA). The slides were left to dry under the fume hood overnight.

The degree of histological injury in the small intestine was assessed blinded based on a grading scale. The presence and severity of damage was rated from 0 to 4. A score of 0 represented normal histology; 1, represented slight disruption of the surface epithelium; 2, epithelial cell loss and injury at villus tip; 3, mucosal vasocongestion, hemorrhage and focal necrosis with loss of less than one-half of villi; and 4, damage extending to more than one-half of villi (Chiu *et al*., 1970).

2.10 Plasma Cytokine Analysis

Mediator release was evaluated using a 10-plex Procarta Multiplex Cytokine Assay kit (Affymetrix Inc. Santa Clara, CA, USA). The cytokines and adhesion molecules measured were TNF-α, IL-1β, IL-6, IFN-γ, ICAM and VCAM. The plasma samples collected from all animals at the end of each experiment were analyzed using the cytokine assay kit and Luminex Technology Analyzer and BioPlex Manager software (Bio-Rad, Mississauga, ON, Canada). According to the Procarta cytokine assay manual, the luminex instruments were calibrated before each experiment and validated once every 30 days. The experiments were performed only when the calibration and validation were successful. Briefly, the plates were pre-wet with 150 µl reading buffer. 50 µl of the antibody beads were added to each well of the pre-wet 96-well filter bottom plates and washed with washing buffer by vacuum filtration. Rat-specific plasma buffer (25 µl/well)

was added to all the wells. The standards were prepared by reconstituting the lypophilized premixed standard powder in 250 µl of rat-specific plasma standard buffer and incubated for 25 min. Eight standards were prepared starting at 20,000 pg/ml and decreasing 4-fold with each standard. All standards and samples (25 µl/well) were loaded to the 96-well filter plate in duplicate (25 µl/well for final volume of 50 µl/well) and incubated for 60 min at room temperature on a shaker (500 rpm). Then, after washing with 1X wash buffer, the detection antibody (25 µl/well) was added to the plate and incubated for 30 min at room temperature on a shaker (500 rpm). After removing the detection antibody by vacuum filtration, the plates were washed with 1X washing buffer (150 µl/well). Streptavidin-PE (50 µl/well) was added to the each well and incubated for 30 min at room temperature on a shaker (500 rpm). Streptavidin was removed with vacuum filtration and washed with the 1X washing buffer (150 µl/well), subsequently 120 µl/well of the reading buffer was added to all wells and placed on a shaker (500 rpm) for 5 min and analyzed on Luminex instrument. Standard curves were plotted for each cytokine that was measured. The kit sensitivity (Limit of Detection, LOD) was ≤ 1 pg/ml for each cytokine. The final dilution of samples was 1:8, and the final concentrations of each cytokine were calculated based on the dilution. Each sample and standard were run in duplicates, so the mean of measured concentration was used. The cytokine concentrations were expressed as pg/ml.

2.11 Statistical Analysis

All data are expressed as means ± standard deviation (SD). Statistical analyses of the results were performed using the software GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA). Differences between groups were analyzed using one-way ANOVA (analysis of variance, ANOVA), followed by the Bonferroni corrected Student`s t-test for group wise comparisons. Mean arterial pressure, heart rate and temperature were analyzed by a two-way analysis of variance for repeated measures followed by Scheffé's test. The significance level was considered at $p<0.05$.

CHAPTER 3: RESULTS

3.1 CB1R experiments

The objective of the first part of the experiments was to study CB1R modulation in experimental endotoxemia. We used a specific CB1R agonist and antagonist and the combination of both.

3.1.1 Vital parameters

Time course of mean arterial pressure (MAP) is shown in Figure 11A. MAP remained within the physiological range, in control, LPS and treatment groups during the observation period. Immediately after LPS administration, a slight drop in MAP was observed, however, at the time of IVM (120 min) all animal showed comparable MAP values (Figure 11A). The heart rate (HR) remained unchanged in control and LPS groups during the experiments. The slight decrease in the ACEA+AM281 treated endotoxemic animals was still in the physiological range (Figure 11B). Temperature remained stable in the control, LPS and all treatment groups (Figure 11C).

Figure 11A. Mean arterial pressure [mmHg]; Control, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist (2.5 mg/kg); LPS+AM281, endotoxin plus CB1R antagonist (2 mg/kg); and LPS+ACEA+AM281, endotoxin plus CB1R agonist (2.5 mg/kg) plus CB1R antagonist (2 mg/kg), means ± standard deviation.

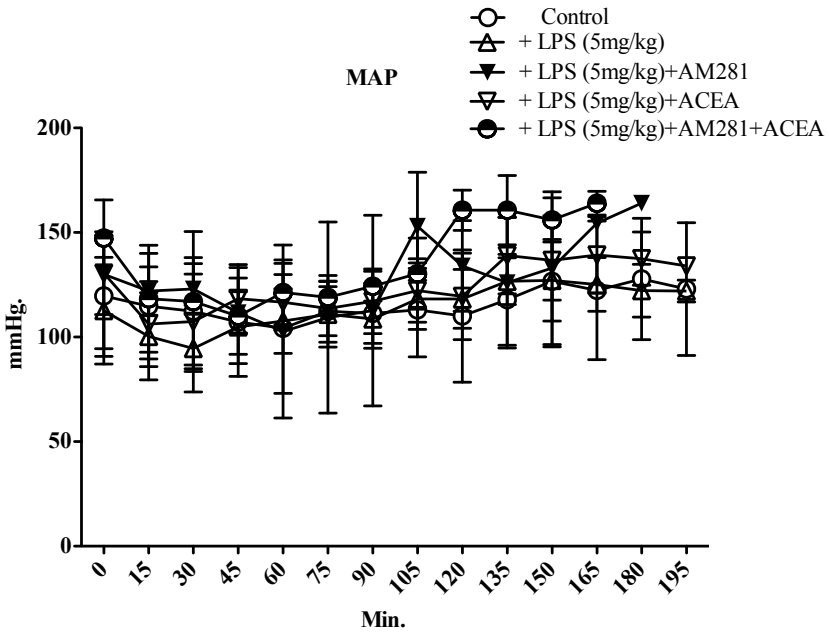


Figure 11B. Heart rate [bpm]; Control, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist (2.5 mg/kg); LPS+AM281, endotoxin plus CB1R antagonist (2 mg/kg); and LPS+ACEA+AM281, endotoxin plus CB1R agonist (2.5 mg/kg) plus CB1R antagonist (2 mg/kg), means ± standard deviation.

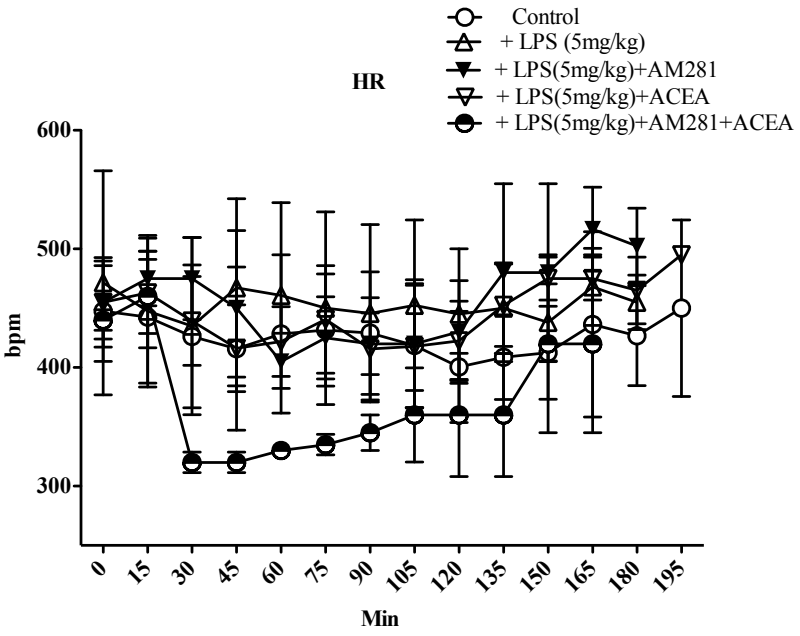
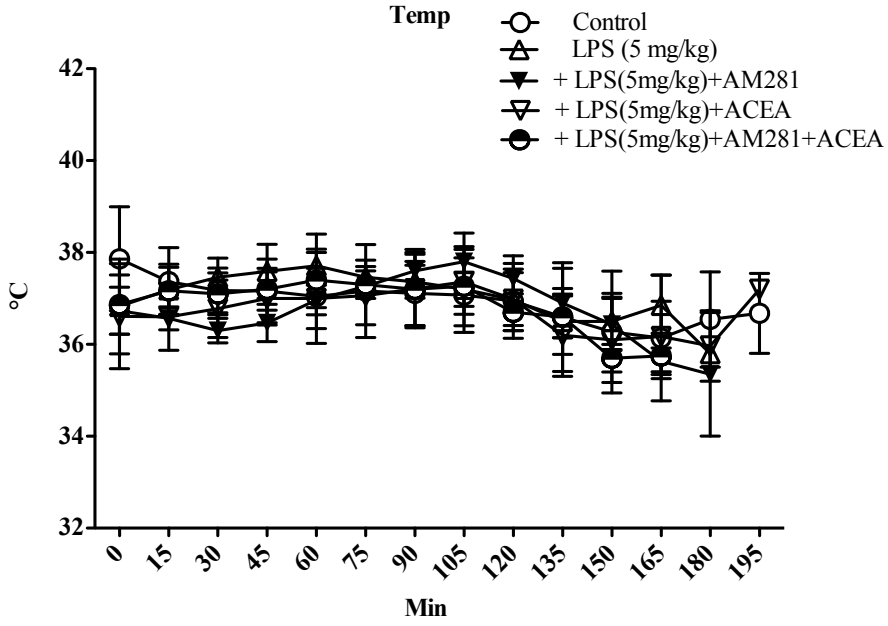


Figure 11C. Temperature [°C]; Control, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist (2.5 mg/kg); LPS+AM281, endotoxin plus CB1R antagonist (2 mg/kg); and LPS+ACEA+AM281, endotoxin plus CB1R agonist (2.5 mg/kg) plus CB1R antagonist (2 mg/kg), means ± standard deviation.



3.1.2 Leukocyte adherence

IVM measurements examining adherent leucocytes in intestinal submucosal venules (V1) are shown in Figure 12A. LPS administration significantly ($p<0.05$) increased the number of adherent leukocytes in V1 venules comparing to control (Figure 12A). Treatment with the CB1R agonist (ACEA) also increased the leukocyte recruitment ($p<0.05$). Treatment with the CB1R antagonist, AM281, and the combination treatment with CB1R agonist (ACEA) plus CB1R antagonist (AM281) showed no significant increase in leukocyte adhesion.

IVM measurements examining adherent leucocytes in intestinal postcapillary venules (V3) are shown in Figure 12B. LPS administration significantly ($p<0.05$) increased the number of adherent leukocytes in V3 venules compared to controls (Figure 12B). Treatment with the CB1R agonist (ACEA) further increased the leukocyte recruitment ($p<0.05$). Treatment with the CB1R antagonist, AM281, and the combination treatment with CB1R agonist (ACEA) plus CB1R antagonist (AM281) showed no significant increase in leukocyte adhesion.

Figure 12A. Leukocyte adhesion in collecting venules (V1) [n/mm2]; Control, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist; LPS+AM281, endotoxin plus CB1R antagonist, and LPS+ACEA+AM281, endotoxin plus CB1R agonist (2.5 mg/kg) plus CB1R antagonist (2 mg/kg), means ± standard deviation; N/A: numbers per area. *p<0.05 versus Control.

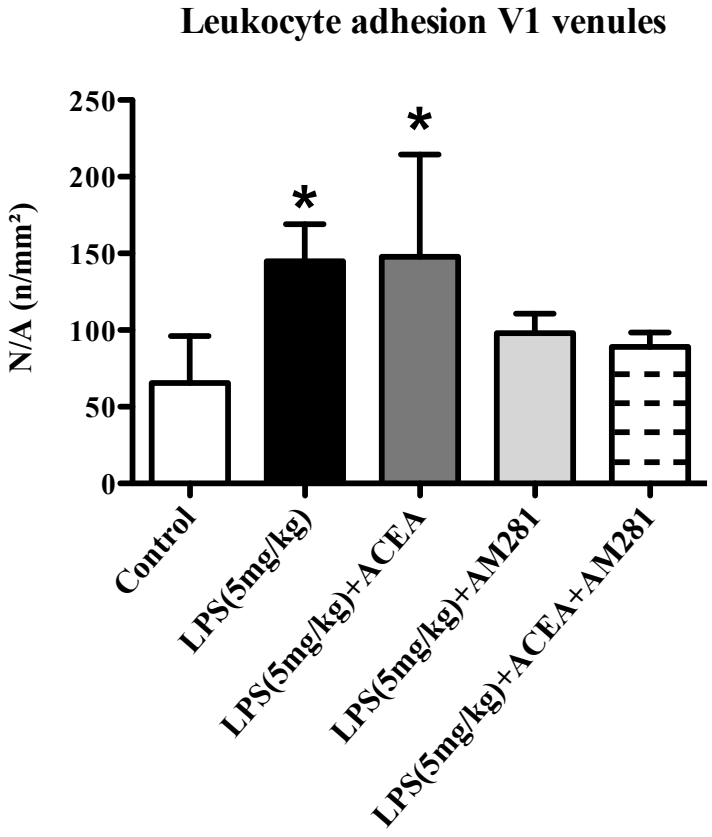
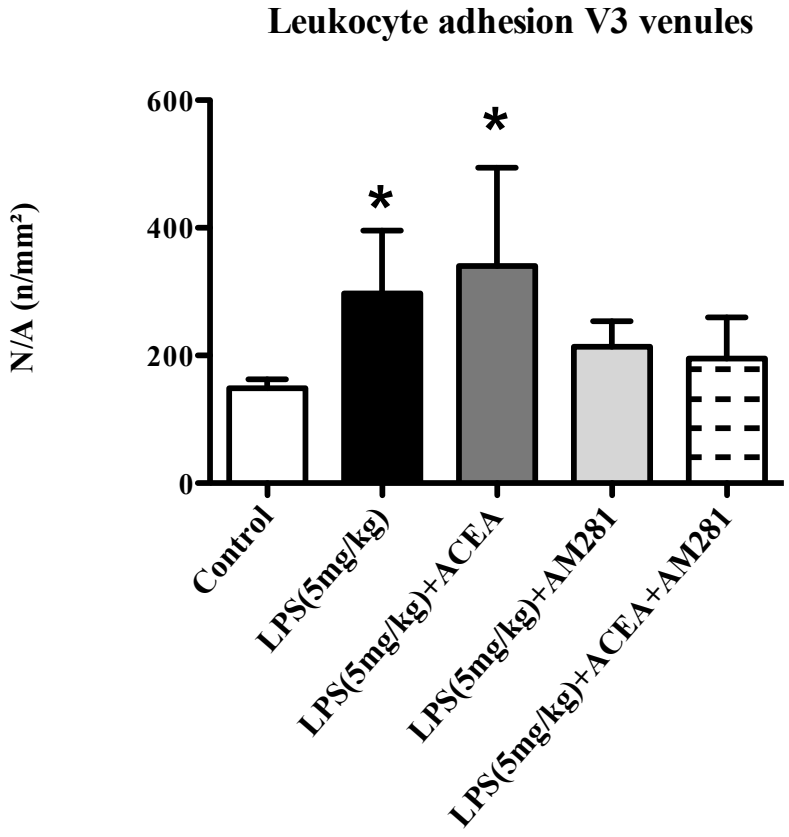


Figure 12B. Leukocyte adhesion in postcapillary venules (V3) [n/mm2]; Control, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist; LPS+AM281, endotoxin plus CB1R antagonist, and LPS+ACEA+AM281, endotoxin plus CB1R agonist (2.5 mg/kg) plus CB1R antagonist (2 mg/kg), means ± standard deviation; N/A: numbers per area. *p<0.05 versus Control.



3.1.3 Rolling leukocytes

IVM measurements examining rolling leucocytes in intestinal V1 and V3 venules are shown in Figure 13A & B, respectively. The endotoxin challenge resulted in a significant decrease in temporarily adherent leukocytes (roller) in V1 and V3 venules compared to control group, 2 hr after start of endotoxemia. Treatment groups showed no difference in the number of rolling leukocytes in the V1 and V3 venules in comparison to untreated LPS animals (Figure 13A & B).

Figure 13A. Leukocyte rolling in collecting venules (V1) [n/min]; Control, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist; LPS+AM281, endotoxin plus CB1R antagonist, and LPS+ACEA+AM281, endotoxin plus CB1R agonist (2.5 mg/kg) plus CB1R antagonist (2 mg/kg), means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Control.

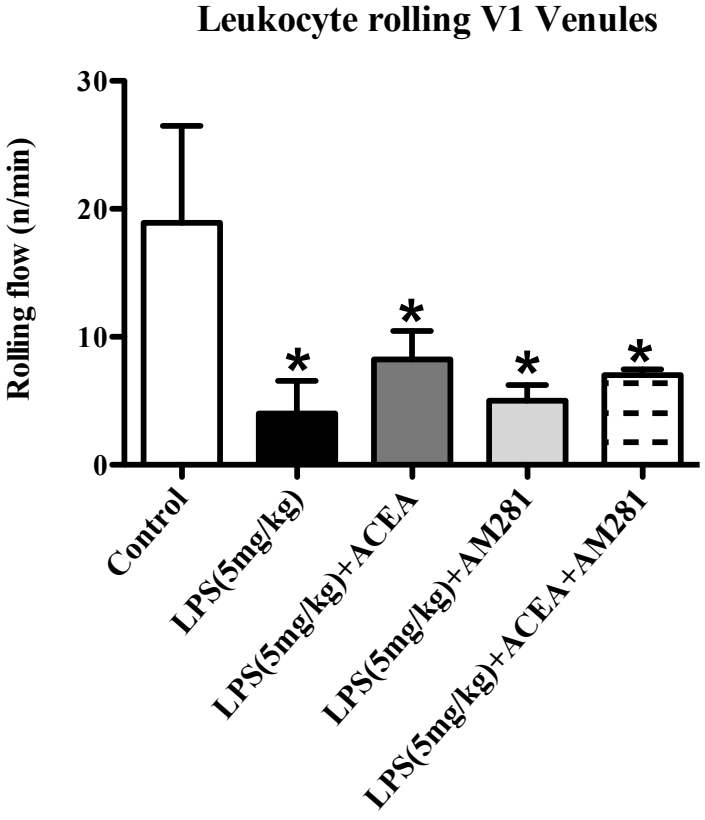
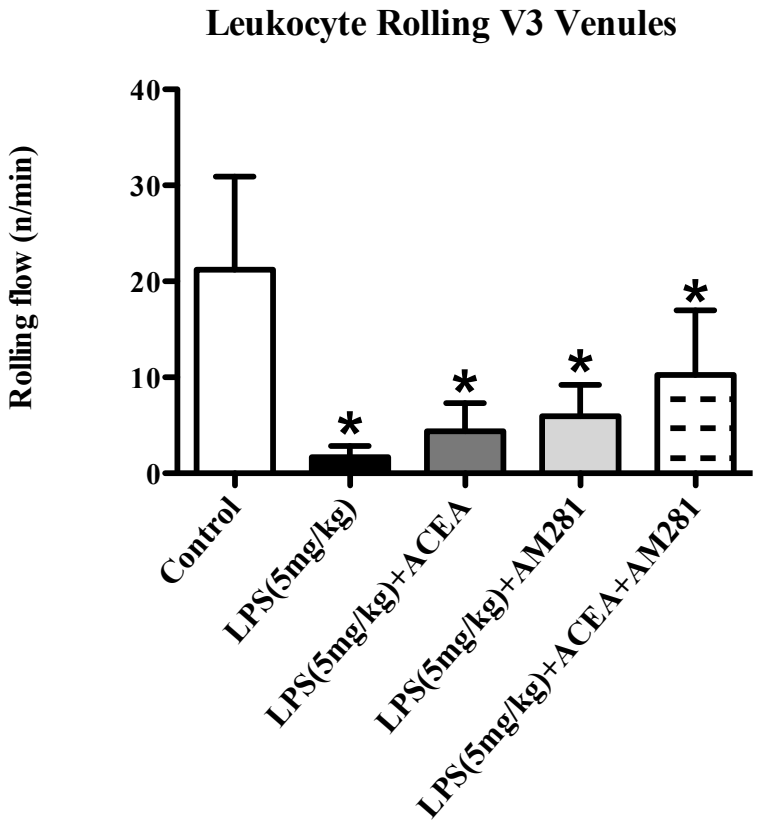


Figure 13B. Leukocyte rolling in postcapillary venules (V3) [n/min]; Control, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist; LPS+AM281, endotoxin plus CB1R antagonist, and LPS+ACEA+AM281, endotoxin plus CB1R agonist (2.5 mg/kg) plus CB1R antagonist (2 mg/kg), means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Control.



3.1.4 Functional capillary density

Administration of LPS significantly reduced functional capillary density ($P<0.05$ vs. Control) in longitudinal (Figure 14A), circular muscle layers (Figure 14B) as well as mucosal layer (Figure 14C). Treatment with AM281 after LPS administration significantly increased FCD in longitudinal muscular ($P<0.05$ vs. LPS), the circular muscular ($P<0.05$ vs. LPS) and mucosal layer of intestinal wall ($P<0.05$ vs. LPS). ACEA and ACEA+AM281 treatment did not increase FCD in the several layers of the intestinal wall as compared to the LPS group (Figure 14A, B & C).

Figure 14A. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in longitudinal muscle layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist; LPS+AM281, endotoxin plus CB1R antagonist, and LPS+ACEA+AM281, endotoxin plus CB1R agonist plus CB1R antagonist, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls; # p<0.05 versus LPS.

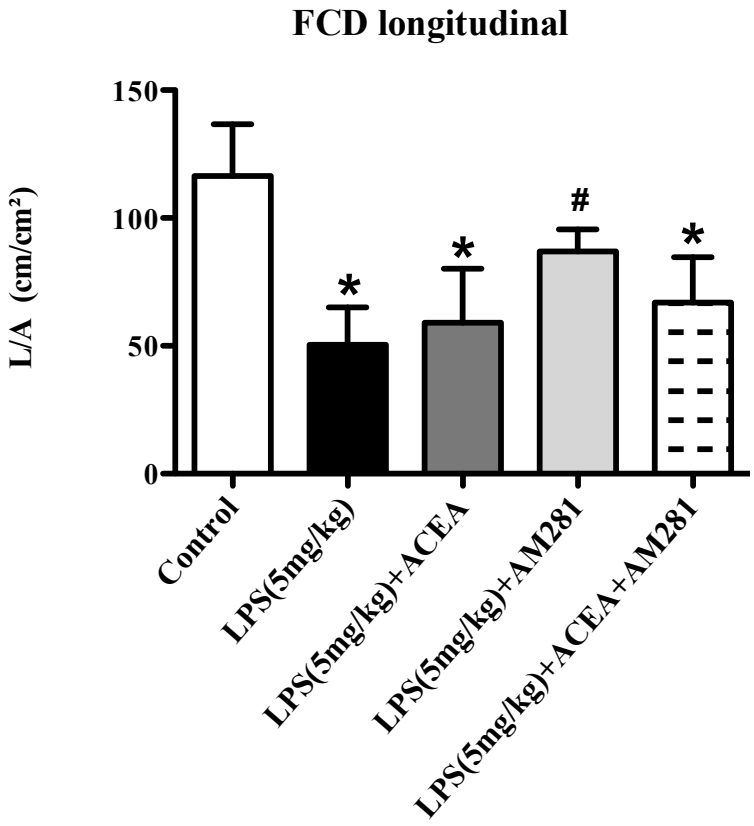


Figure 14B. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in circular muscle layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist; LPS+AM281, endotoxin plus CB1R antagonist, and LPS+ACEA+AM281, endotoxin plus CB1R agonist plus CB1R antagonist, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls; # p<0.05 versus LPS.

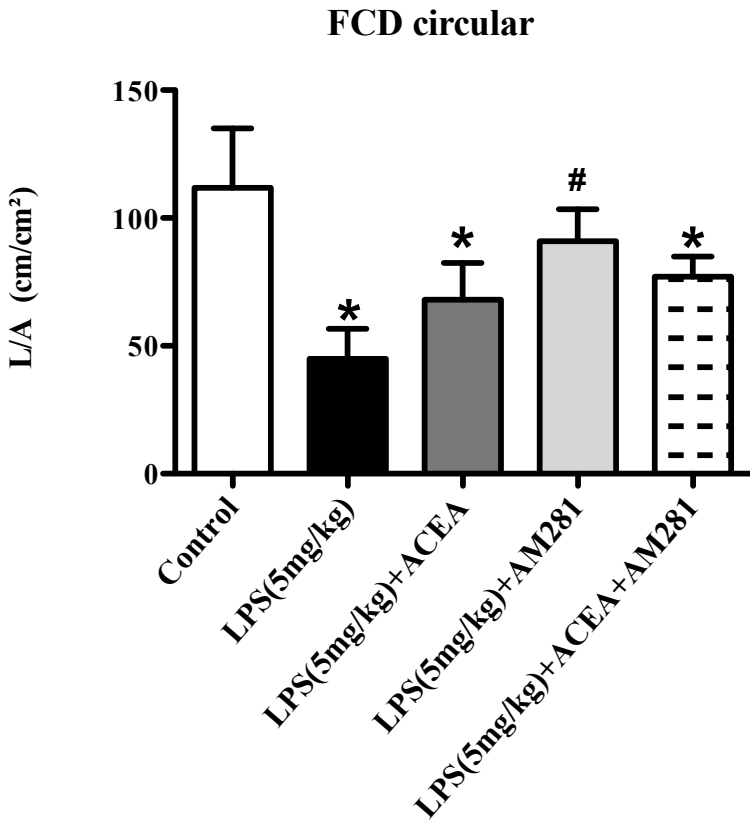
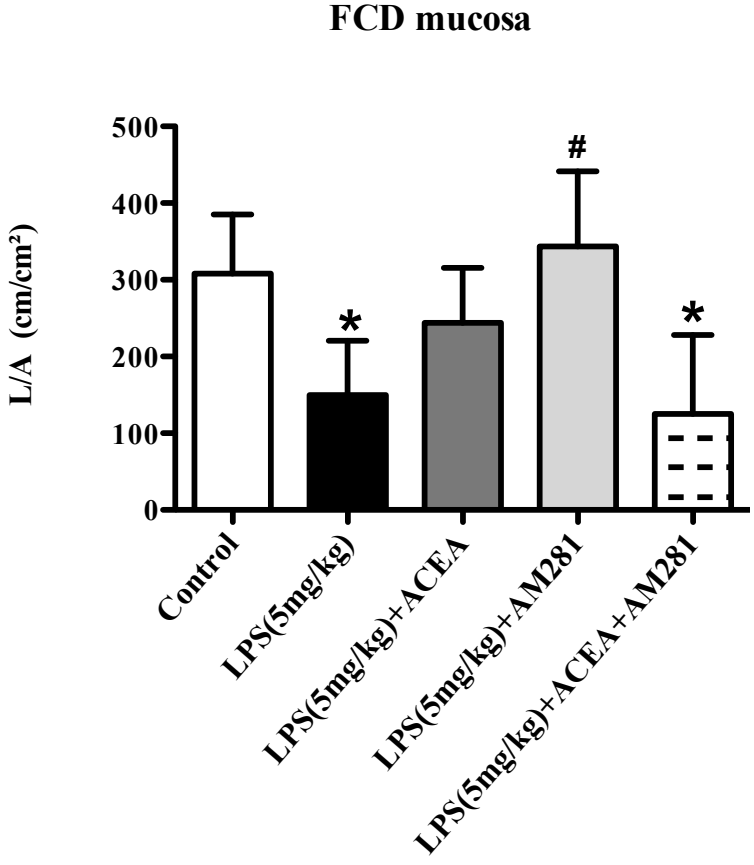


Figure 14C. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in mucosal layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+ACEA, endotoxin plus CB1R agonist; LPS+AM281, endotoxin plus CB1R antagonist, and LPS+ACEA+AM281, endotoxin plus CB1R agonist plus CB1R antagonist, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls; # p<0.05 versus LPS.

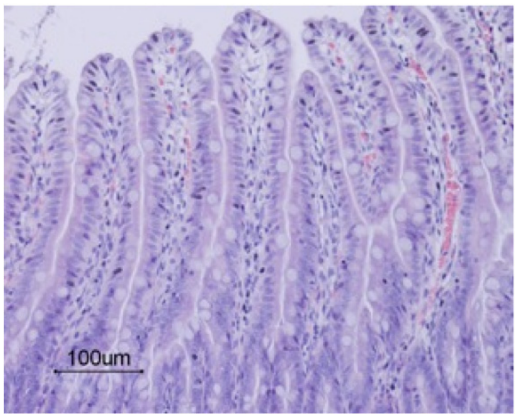


3.1.5 Histology

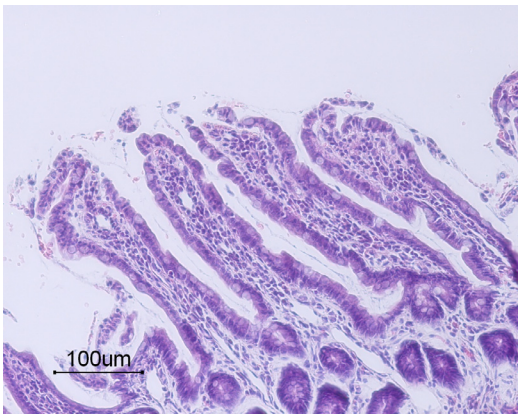
Control animals showed a normal mucosa (Grade 0, according to Chiu *et al*., 1970). Short time endotoxin challenge resulted in sub-epithelial space formation at villus tips and slight disruption of the surface epithelium. The histological score changed to Grade 1-2. The histological score was 0 (normal histology) for the tissue from the CB1R agonist or antagonist and 0-1 for combination treatment with CB1R agonist and antagonist treated endotoxemic animals. (Figure 15A, B, C, D & E)

Figure 15. Histology: Morphological changes in the rat small intestine after 3 hours of endotoxemia (H&E staining). **A**-Control, **B**-LPS, **C**-LPS+ACEA, **D**-LPS+AM281, **E**-LPS+ACEA+AM281.

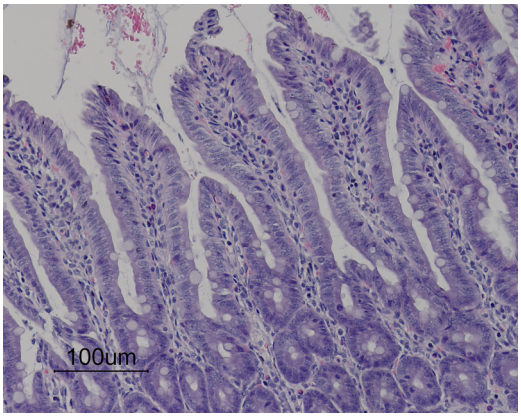
A) Control



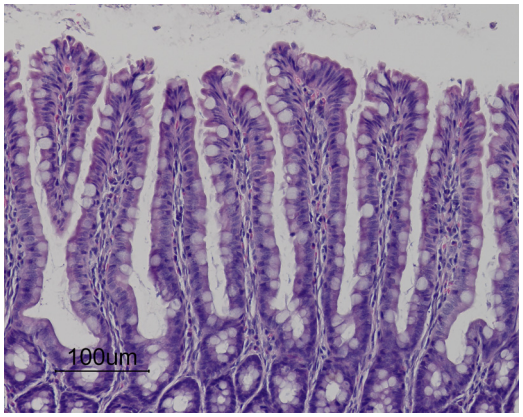
B) LPS(5mg/kg)



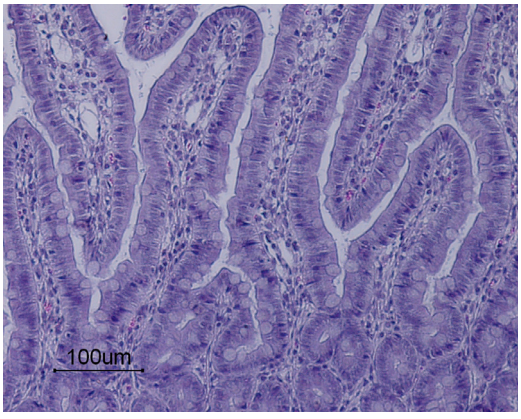
C) LPS(5mg/kg)+ACEA



D) LPS(5mg/kg)+AM281



E) LPS(5mg/kg)+ACEA+AM281



3.2 CB2R experiments

The objective of the second part of the experiments was to study CB2R modulation in experimental endotoxemia. We used specific CB2R agonists and a CB2R antagonist and the combination of both.

3.2.1 GP1a

3.2.1.1 Vital parameters

Time course of MAP is shown in Figure 16A. MAP remained within the physiological range, in control, LPS and GP1a treatment group during the observation period (Figure 16A). The HR remained unchanged in control, LPS and GP1a group during the experiments (Figure 16B). Temperature remained stable in the control, LPS and GP1a treatment group (Figure 16C).

Figure 16A. Mean arterial pressure [mmHg]; Control, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist (2.5 mg/kg), means ± standard deviation.

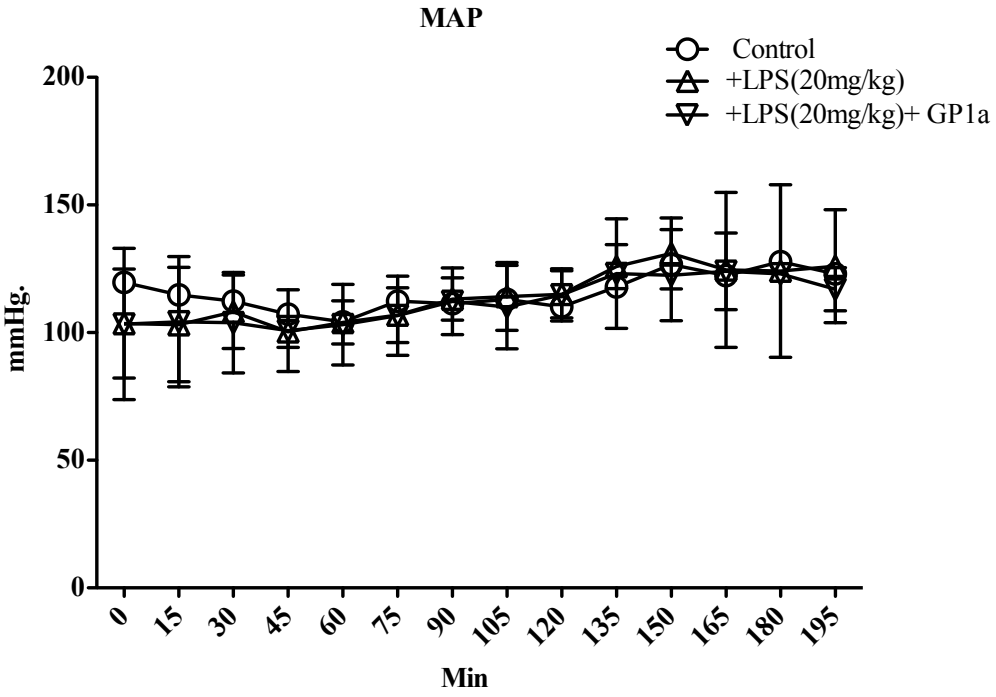


Figure 16B. Heart rate [bpm]; Control, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist (2.5 mg/kg), means ± standard deviation.

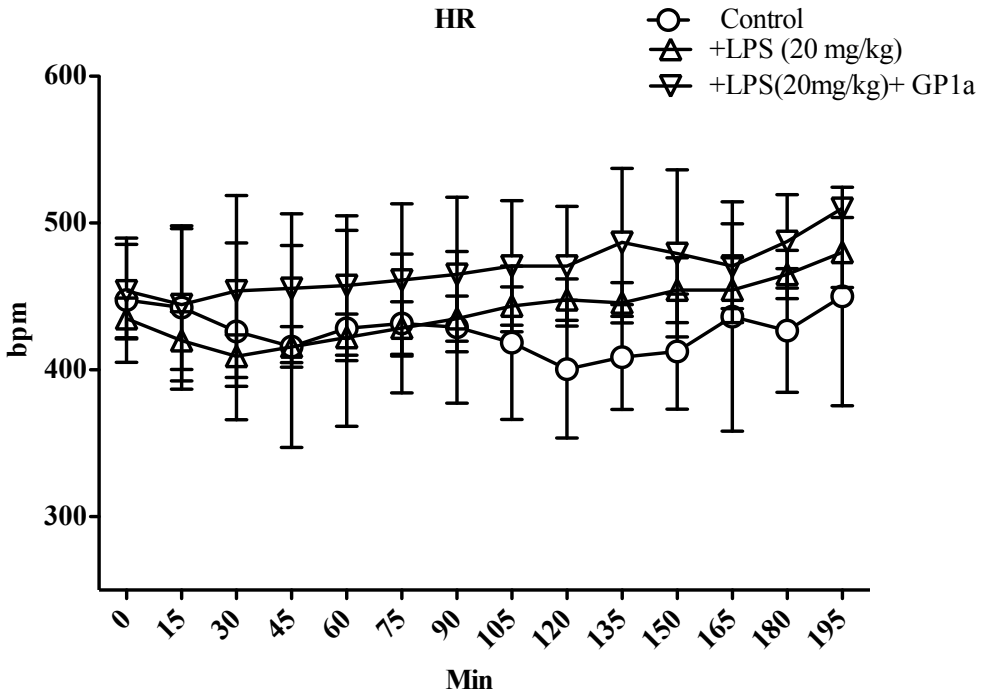
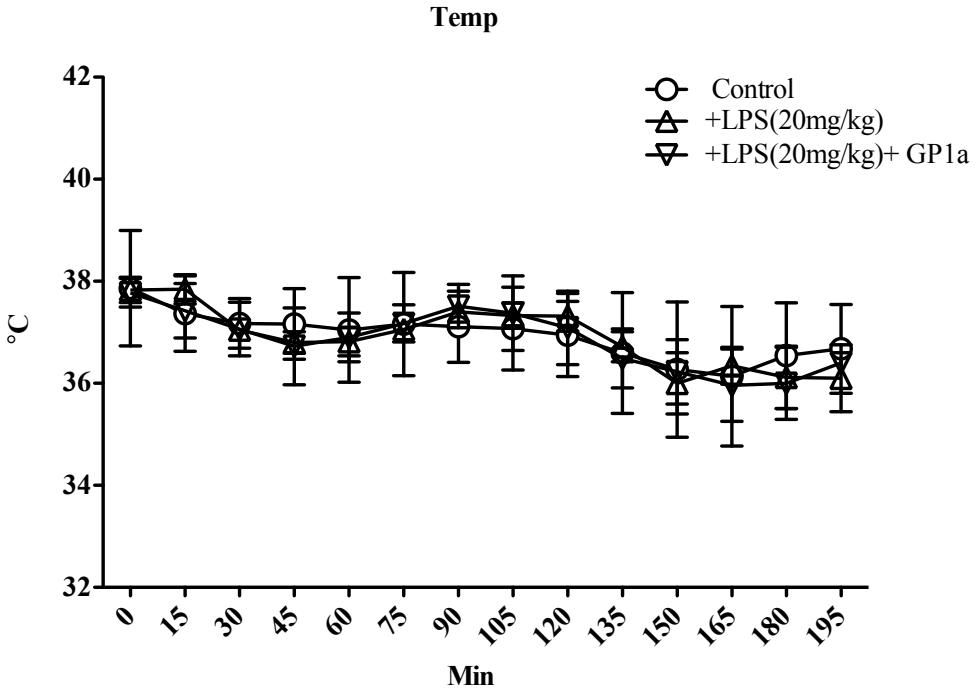


Figure 16C. Temperature [°C]; Control, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist (2.5 mg/kg), means ± standard deviation.



3.2.1.2 Leukocyte adherence

IVM measurements examining adherent leucocytes in (V1) and (V3) are shown respectively in Figure 17A and B. Endotoxin challenge resulted in a 2.3 fold increase ($p<0.0001$) in the number of adherent leukocytes in V1 venules (Figure 17A). Treatment with the CB2R agonist, GP1a, showed no further increase of leukocyte recruitment. LPS administration increased the number of adherent leukocytes in V3 venules comparing to control ($p<0.05$) (Figure 17B). Treatment with the CB2R agonist, GP1a, showed no further increase in the leukocyte recruitment.

Figure 17A. Leukocyte adhesion in collecting venules (V1) [n/mm^2]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a (2.5mg/kg), endotoxin plus CB2R agonist; means ± standard deviation; N/A: numbers per area. *p<0.05 versus Controls.

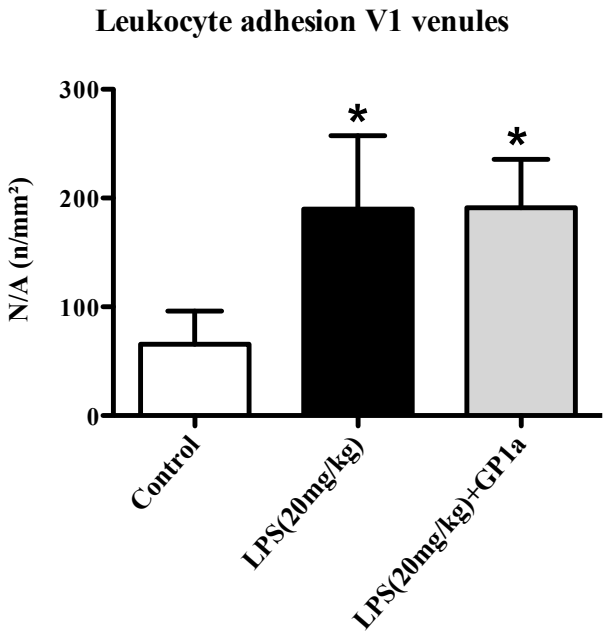
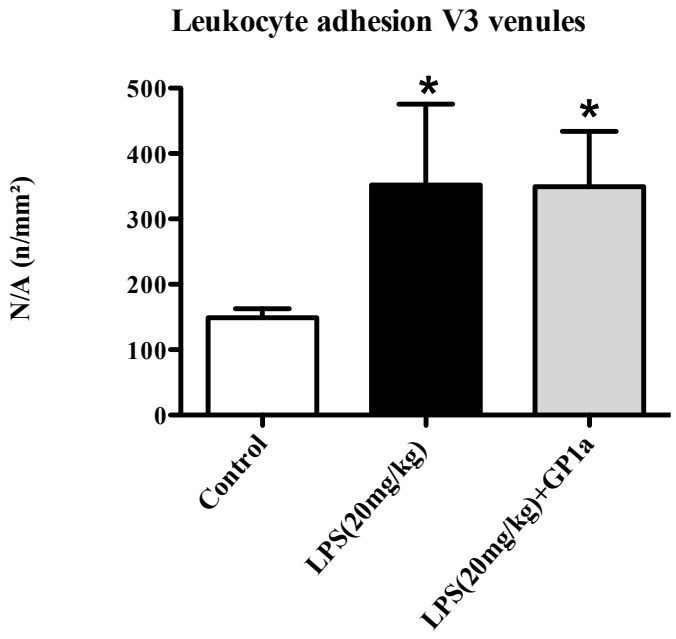


Figure 17B. Leukocyte adhesion in postcapillary venules (V3) [n/mm^2]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a (2.5mg/kg), endotoxin plus CB2R agonist; means ± standard deviation; N/A: numbers per area. *p<0.05 versus Controls.



3.2.1.3 Rolling leukocytes

The endotoxin challenge resulted in a significant ($p<0.0001$) decrease in rollers in V1 and V3 venules 2 hr after start of endotoxemia (Figure 18A & B). Treatment with the CB2R agonist, GP1a also resulted in a significant ($p<0.0001$) decrease of rolling leukocytes in V1 and V3 venules.

Figure 18A. Leukocyte rolling in collecting venules (V1) [n/min]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist; means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Controls.

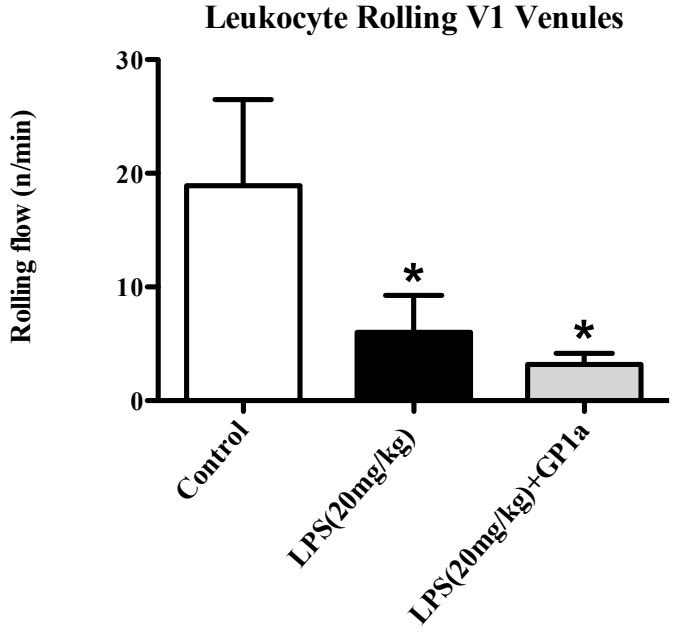
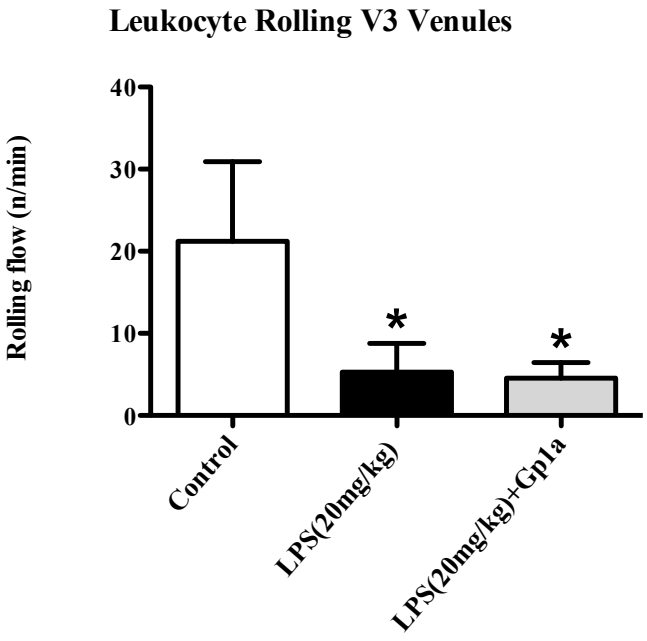


Figure 18B. Leukocyte rolling in postcapillary venules (V3) [n/min]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist; means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Controls.



3.2.1.4 Functional capillary density

The functional capillary density is given (19A, B & C). In the circular muscle layer and longitudinal muscle layers endotoxin challenge resulted in a significant ($P<0.05$) reduction of FCD in endotoxemic groups. Administration of LPS did not influence FCD in the mucosal muscle layers. Treatment with CB2R agonist, GP1a significantly ($P<0.05$) increased FCD in the circular muscle layer and longitudinal muscle layers in comparison to LPS.

Figure 19A. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in longitudinal muscle layer; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist; means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls; # *p*<0.05 versus LPS.

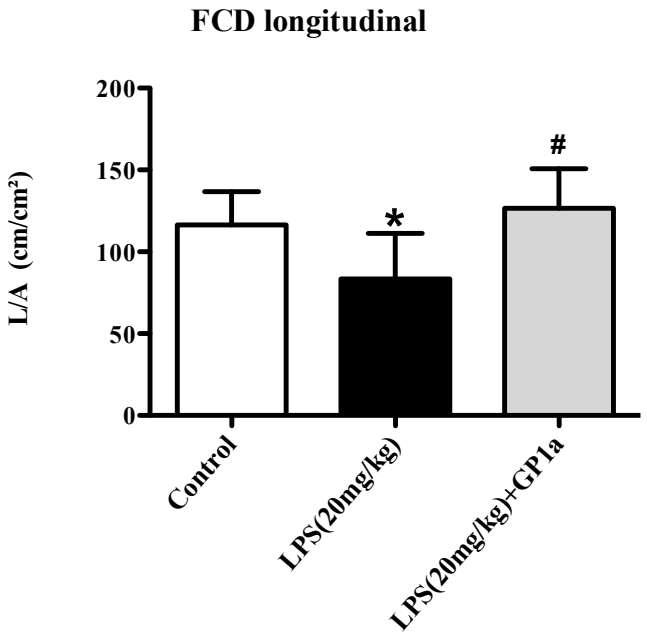


Figure 19B. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in circular muscle layer muscle layer; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist; means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls; # *p*<0.05 versus LPS.

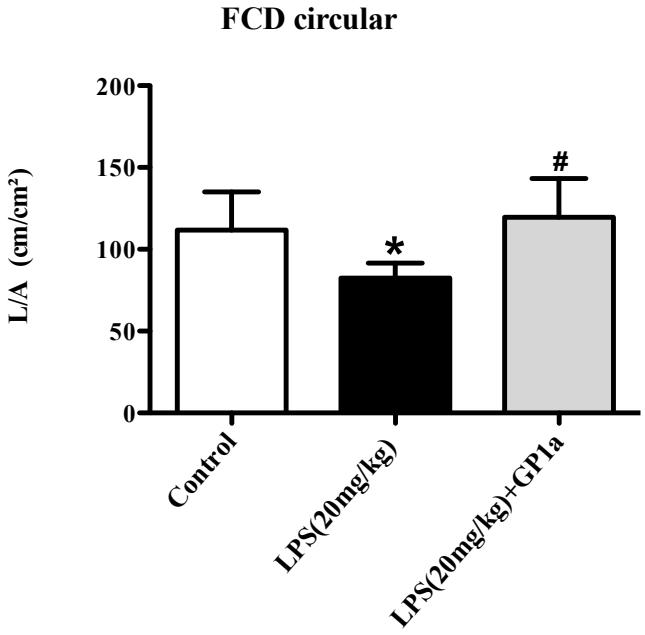
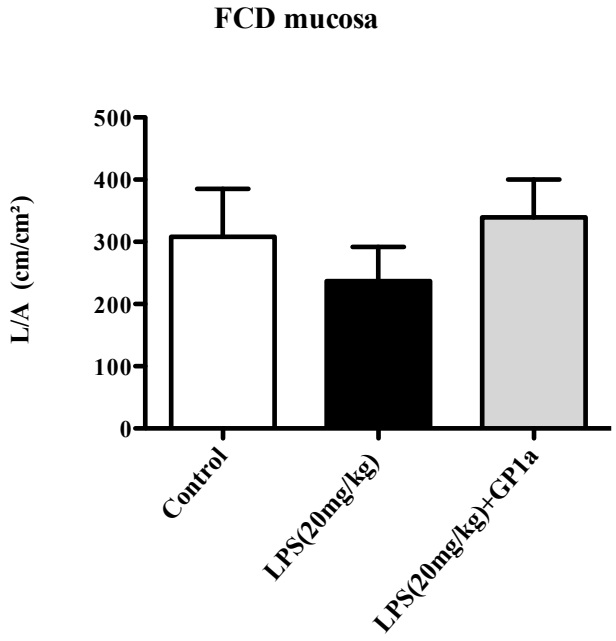


Figure 19C. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in mucosal layer; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+GP1a, endotoxin plus CB2R agonist; means ± standard deviation; L/A: length of capillaries per area.



3.2.2 HU308 and AM630

3.2.2.1 Vital parameters

The time course of MAP is shown in Figure 20A. For the endotoxin groups, MAP remained within the physiological range in control, LPS and treatment groups during the observation period. Immediately after LPS administration, a slight drop in MAP was observed, however, at the time of IVM (120 min) all animals showed comparable MAP values. The HR remained unchanged in control and LPS group; HU308 and AM630 treated endotoxemic animals during the experiments (Figure 20B). Temperature remained stable in the control, LPS and all treatment groups (Figure 20C).

Figure 20A. Mean arterial pressure [mmHg]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist (2.5 mg/kg); LPS+AM630, endotoxin plus CB2R antagonist (2.5 mg/kg); means ± standard deviation.

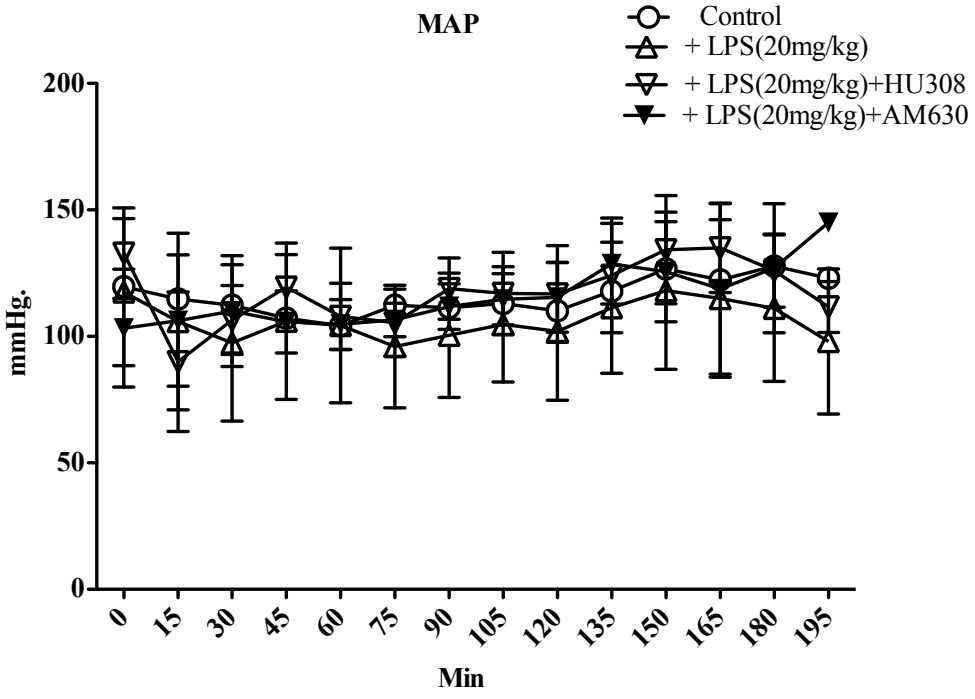


Figure 20B. Heart rate [bpm]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist (2.5 mg/kg); LPS+AM630, endotoxin plus CB2R antagonist (2.5 mg/kg); means ± standard deviation.

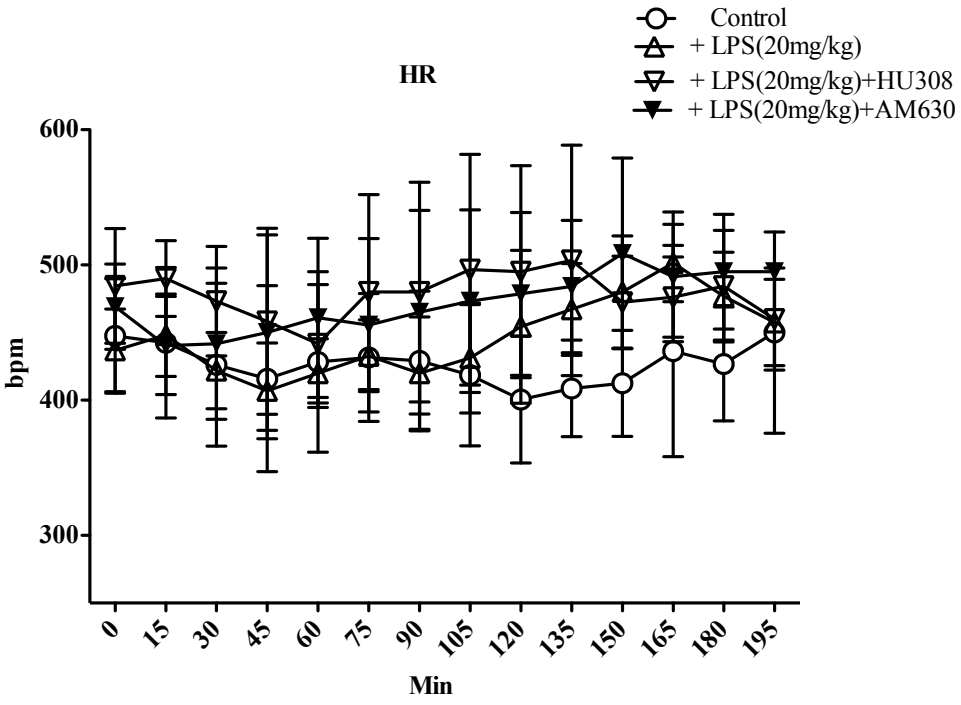
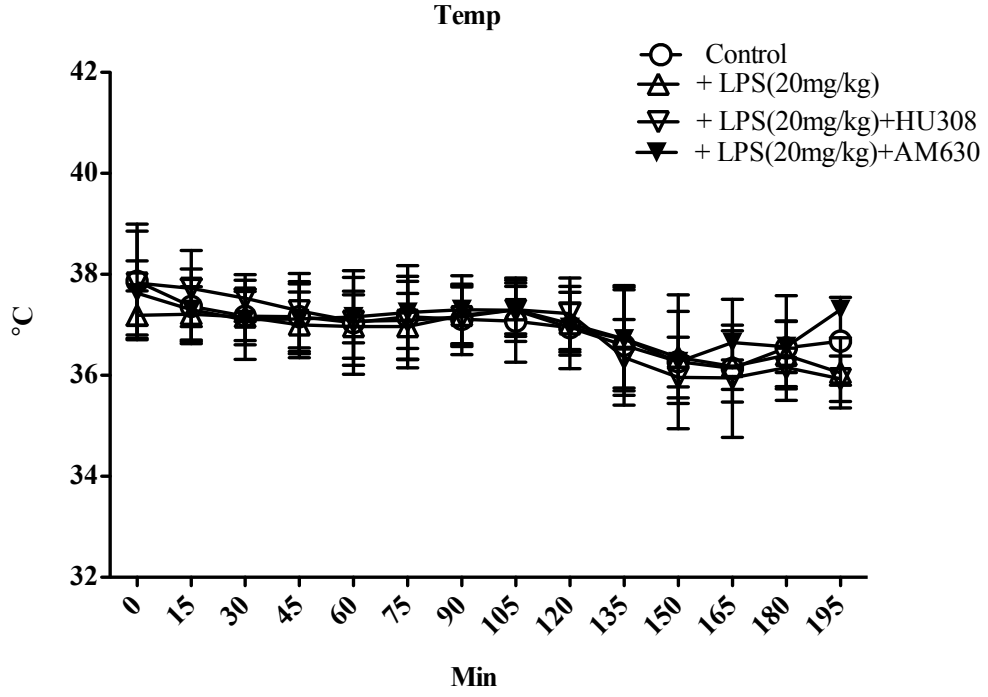


Figure 20C. Temperature [°C]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist (2.5 mg/kg); LPS+AM630, endotoxin plus CB2R antagonist (2.5 mg/kg); means ± standard deviation.



3.2.2.2 Leukocyte adherence

IVM measurements examining adherent leucocytes in intestinal V1 and V3 venules are shown respectively in Figure 21A& B. Endotoxin challenge resulted in a 2.3 fold increase ($p<0.0001$) in the number of adherent leukocytes in V1 venules (Figure 21A). Treatment with the CB2R agonist, HU308, decreased leukocyte adhesion by 57% ($p<0.0001$), while treatment with the CB2R antagonist (AM630) showed no further increase of leukocyte recruitment. LPS administration increased the number of adherent leukocytes in V3 venules comparing to control ($p<0.05$) (Figure 21B). Treatment with the CB2R agonist, HU308, significantly decreased leukocyte adhesion ($p<0.05$). Treatment with the CB2R antagonist (AM630) showed no further increase in the leukocyte recruitment.

Figure 21A. Leukocyte adhesion in collecting venules (V1) [n/mm^2]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+AM630, endotoxin plus CB2R antagonist, means ± standard deviation; n/min: numbers per minute N/A: numbers per area. *p<0.05 versus Controls; # *p*<0.05 versus LPS.

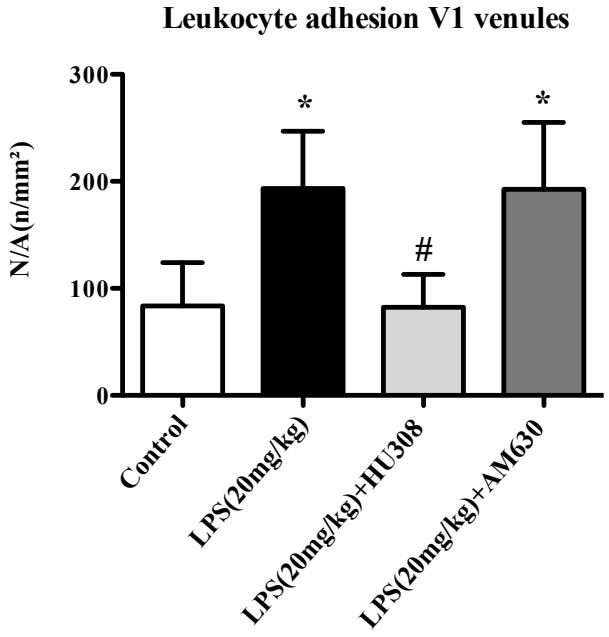
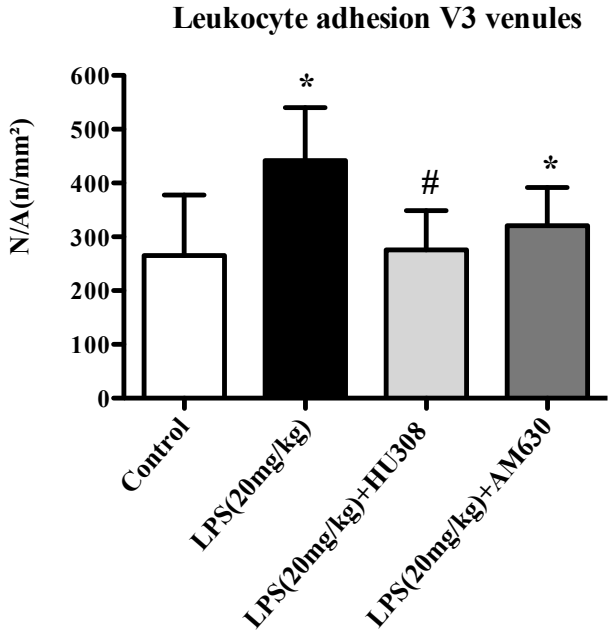


Figure 21B. Leukocyte adhesion in postcapillary venules (V3) [n/mm^2]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+AM630, endotoxin plus CB2R antagonist, means ± standard deviation; N/A: numbers per area. *p<0.05 versus Controls; # *p*<0.05 versus LPS.



3.2.2.3 Leukocyte rolling

The endotoxin challenge resulted in a significant ($p<0.0001$) decrease in temporarily adherent leukocytes (roller) in V1 and V3 venules 2 hours after start of endotoxemia comparing to control (Figure 22A& B). Treatments with CB2R agonist and antagonist also significantly reduced the number of rolling leukocytes in the V1 and V3 venules comparing to control, but not in comparison to LPS.

Figure 22A. Leukocyte rolling in collecting venules (V1) [n/min]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+AM630, endotoxin plus CB2R antagonist, means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Control.

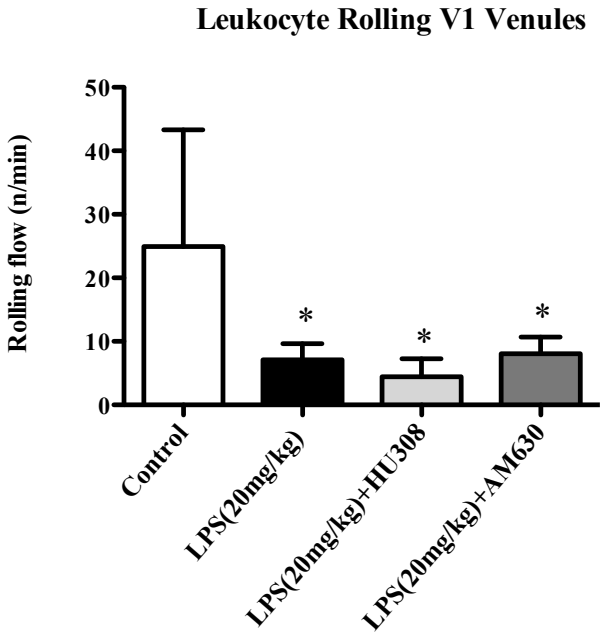
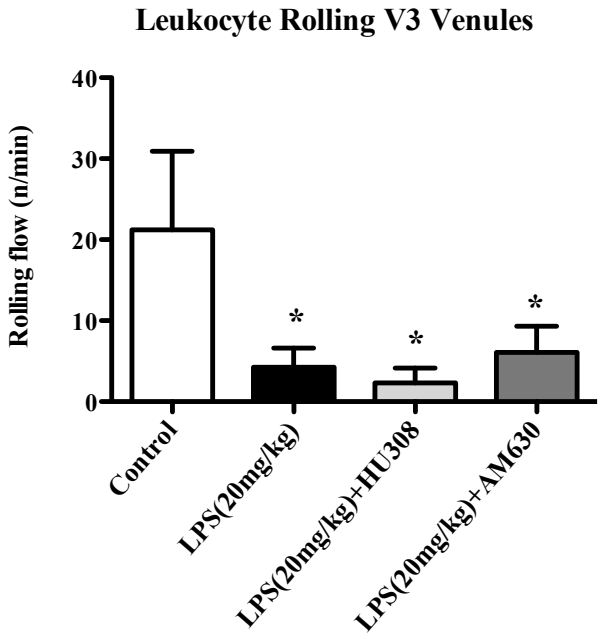


Figure 22B. Leukocyte rolling in postcapillary venules (V3) [n/min]; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+AM630, endotoxin plus CB2R antagonist, means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Controls.



3.2.2.4 Functional capillary density

The FCD is given (23A, B& C). FCD was significant decreased following LPS challenge (Figure 23A& B). HU308 and AM630 treated animals showed no significant differences in FCD compared to control animals.

Figure 23A. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in longitudinal muscle layer; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+AM630, endotoxin plus CB2R antagonist, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls.

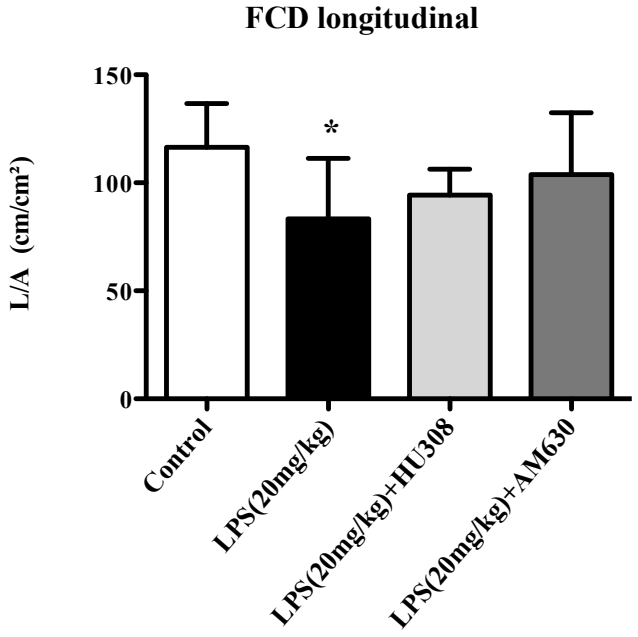


Figure 23B. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in circular muscle layer muscle layer; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+AM630, endotoxin plus CB2R antagonist, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls.

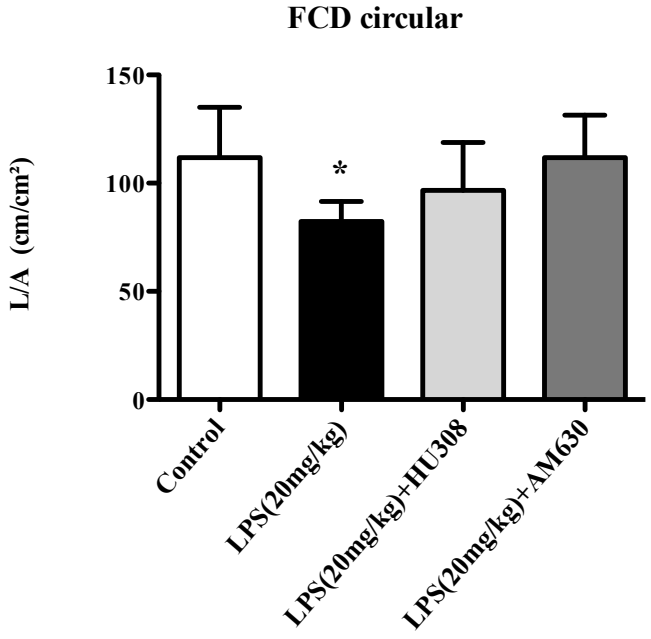
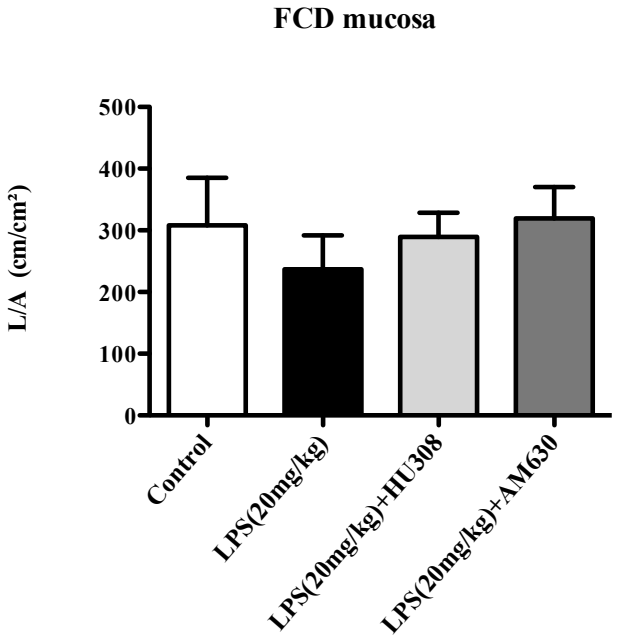


Figure 23C. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in mucosal layer; Controls, control group; LPS, endotoxemia group (20 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+AM630, endotoxin plus CB2R antagonist, means ± standard deviation; L/A: length of capillaries per area.



3.2.3 Combination experiments

3.2.3.1 Vital parameters

The time course of MAP is shown in Figure 24A. For the endotoxin groups, MAP remained within the physiological range in control, LPS and treatment groups during the observation period. At the time of IVM (120 min) all animals showed comparable MAP values. The HR remained unchanged in control and LPS group; HU308 and HU308+AM630 treated endotoxemic animals during the experiments (Figure 24B). Temperature remained stable in the control, LPS and all treatment groups (Figure 24C).

Figure 24A. Mean arterial pressure [mmHg]; Mean arterial pressure [mmHg]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist (2.5 mg/kg); LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5 mg/kg) plus CB2R antagonist (2.5 mg/kg); means ± standard deviation.

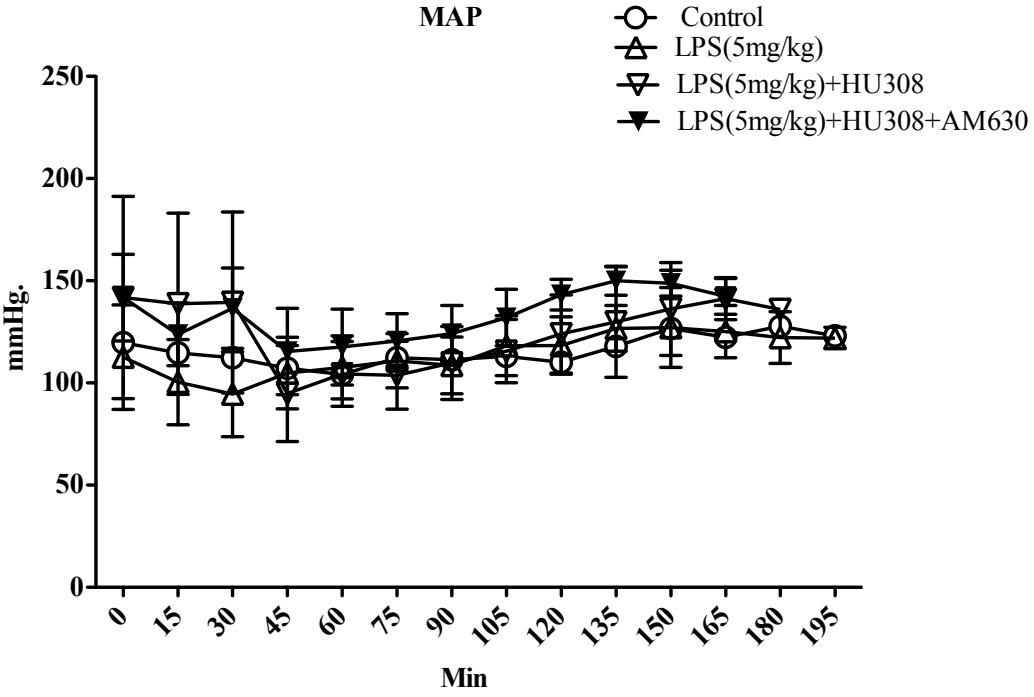


Figure 24B. Heart rate [bpm]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist (2.5 mg/kg); LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5mg/kg) plus CB2R antagonist (2.5 mg/kg); means ± standard deviation.

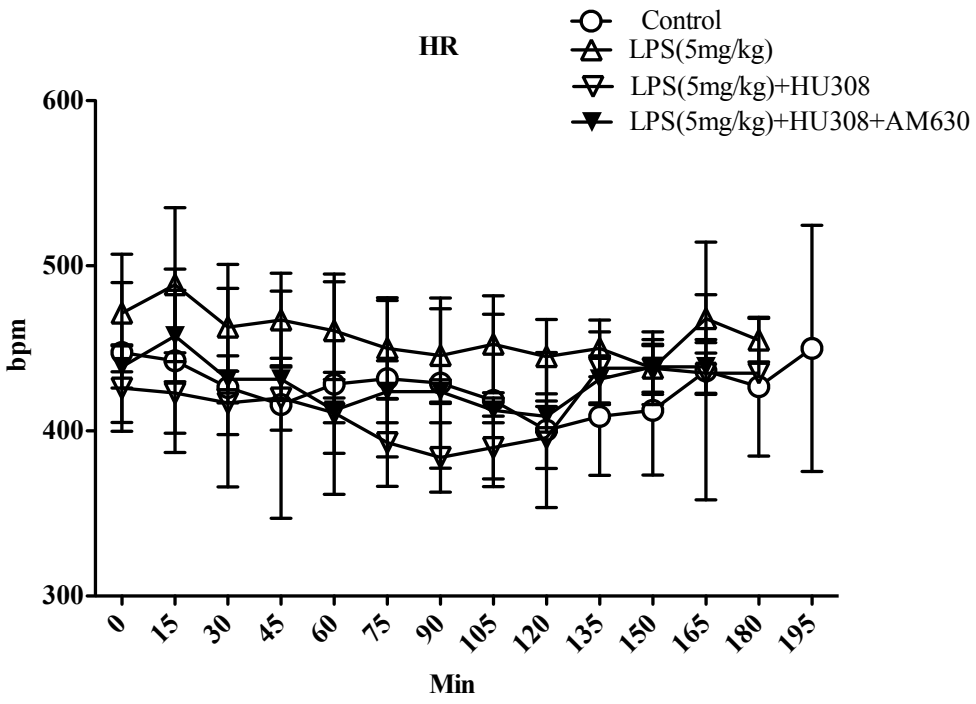
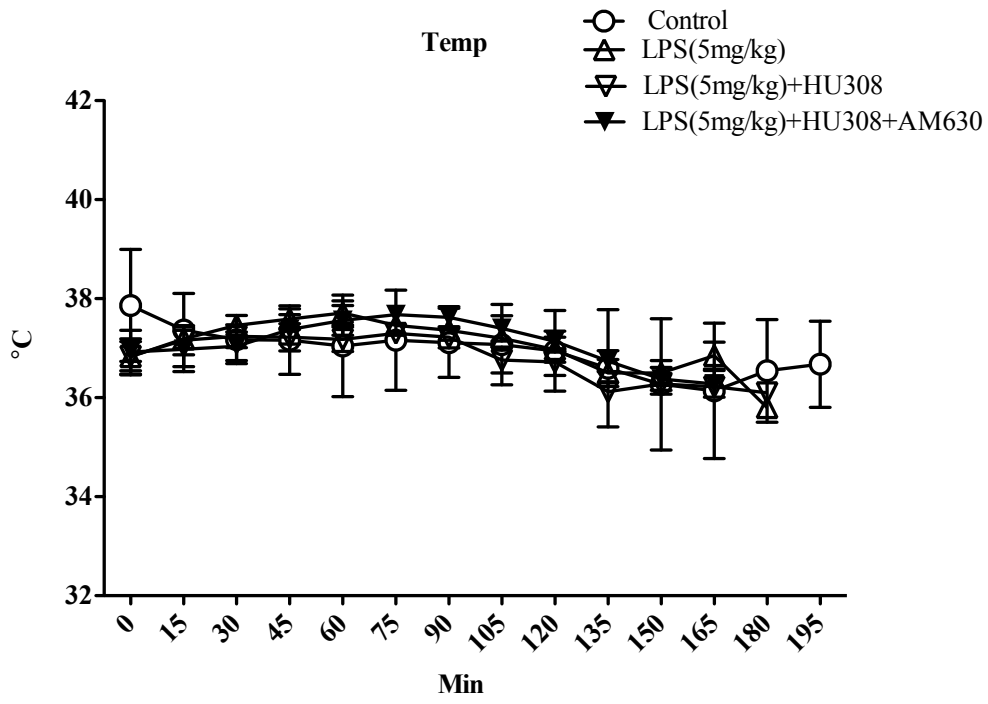


Figure 24C. Temperature [°C]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist (2.5 mg/kg); LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5mg/kg) plus antagonist (2.5 mg/kg); means ± standard deviation.



3.2.3.2 Leukocyte adherence

IVM measurements examining adherent leucocytes in V1 and V3 are shown respectively in Figure 25A& B. Endotoxin challenge resulted in a 2.3 fold increase ($p<0.0001$) in the number of adherent leukocytes in V1 venules (Figure 25A). Treatment with the CB2R agonist, HU308, decreased leukocyte adhesion by 59%, while treatment with the combination treatment with CB2R agonist (HU308) plus CB2R antagonist (AM630) increased leukocyte adhesion. LPS administration increased the number of adherent leukocytes in V3 venules comparing to control ($p<0.05$) (Figure 25B). Treatment with the CB2R agonist, HU308, decreased leukocyte adhesion (ns). Treatment with the combination treatment with CB2R agonist (HU308) plus CB2R antagonist (AM630) increased leukocyte adhesion.

Figure 25A. Leukocyte adhesion in collecting venules (V1) [n/mm2]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5mg/kg) plus CB2R antagonist (2.5mg/kg), means ± standard deviation; N/A: numbers per area. *p<0.05 versus Controls; # *p*<0.05 versus LPS.

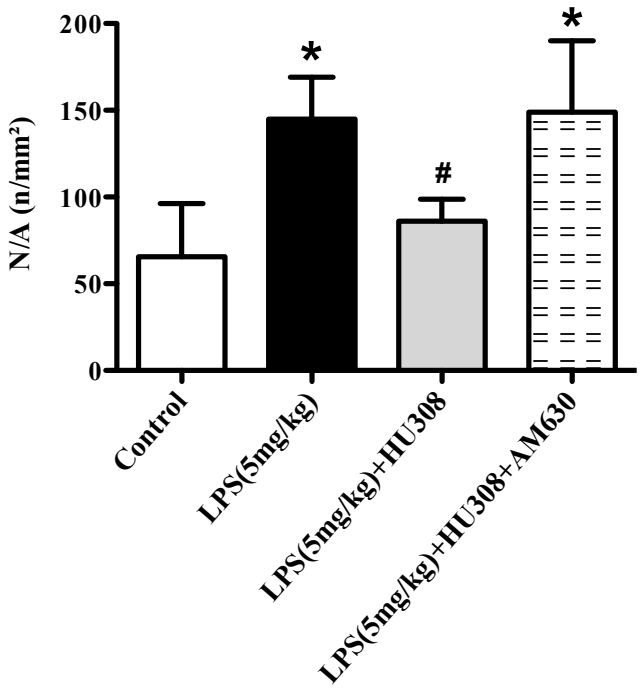
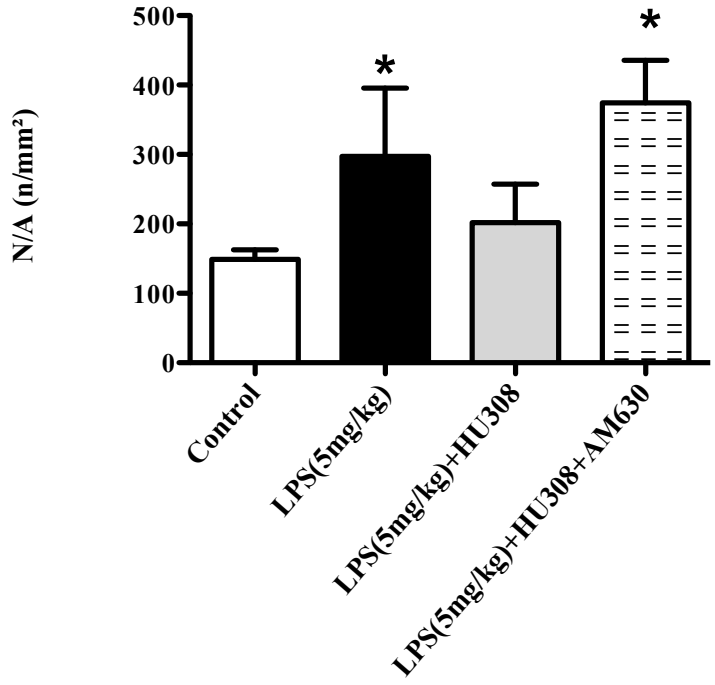


Figure 25B. Leukocyte adhesion in postcapillary venules (V3) [n/mm2]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5mg/kg) plus CB2R antagonist (2.5mg/kg), means ± standard deviation; N/A: numbers per area. *p<0.05 versus Controls.



3.2.3.3 Leukocyte rolling

The endotoxin challenge resulted in a significant ($p<0.05$) decrease in rollers in V1 and V3 venules 2 hours after start of endotoxemia (Figure 26A& B). Treatments with CB2R agonist, HU308 also significantly ($p<0.05$) reduced the number of rolling leukocytes in the V1 and V3 venules comparing to control. The combination treatment with CB2R agonist (HU308) plus CB2R antagonist (AM630) did not resulted in a significant decrease of rolling leukocytes in V1 and V3 venules.

Figure 26A. Leukocyte rolling in collecting venules (V1) [n/min]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5mg/kg) plus CB2R antagonist (2.5mg/kg), means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Controls.

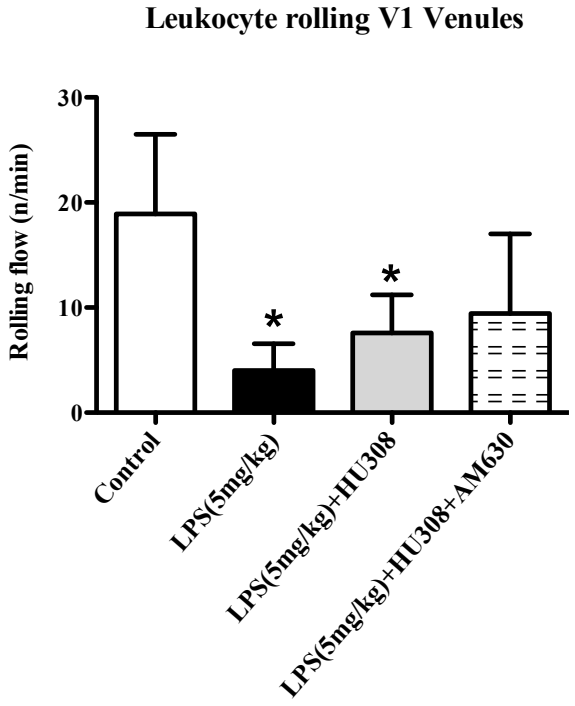
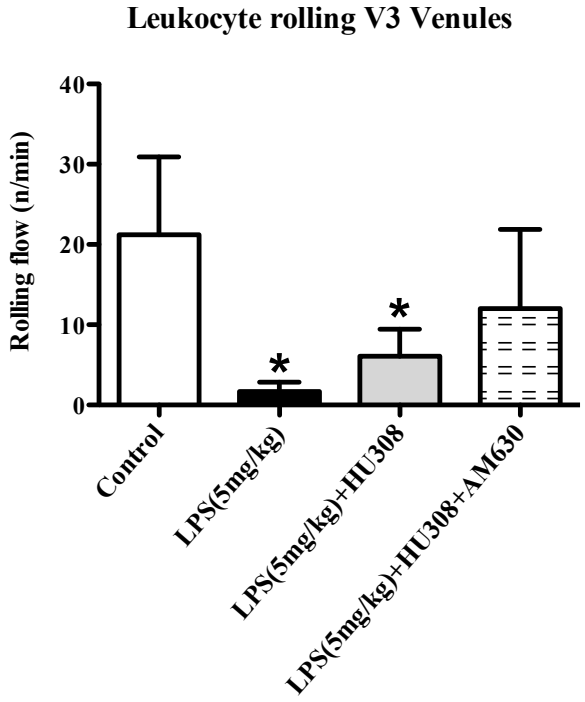


Figure 26B. Leukocyte rolling in postcapillary venules (V3) [n/min]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+HU308+AM630, endotoxin plus CB2R agonist plus CB2R antagonist, means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Controls.



3.2.3.4 Functional capillary density

Administration of LPS significantly reduced FCD ($P<0.0001$ vs. Controls) in longitudinal (Figure 27A), and circular muscle layers (Figure 27B) as well as the mucosal layer ($P<0.05$) (Figure 27C). Treatment with HU308 after LPS administration significantly ($P<0.05$) decreased FCD in longitudinal muscular, the circular muscular but not in mucosal layer of intestinal wall. In circular muscular, treatment with HU308 significantly increased FCD compare to LPS. The HU308+AM630 treatment significantly ($P<0.05$) decreased FCD in the several layers of the intestinal wall as compared to the LPS group.

Figure 27A. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in longitudinal muscle layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5mg/kg) plus CB2R antagonist (2.5mg/kg), means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls.

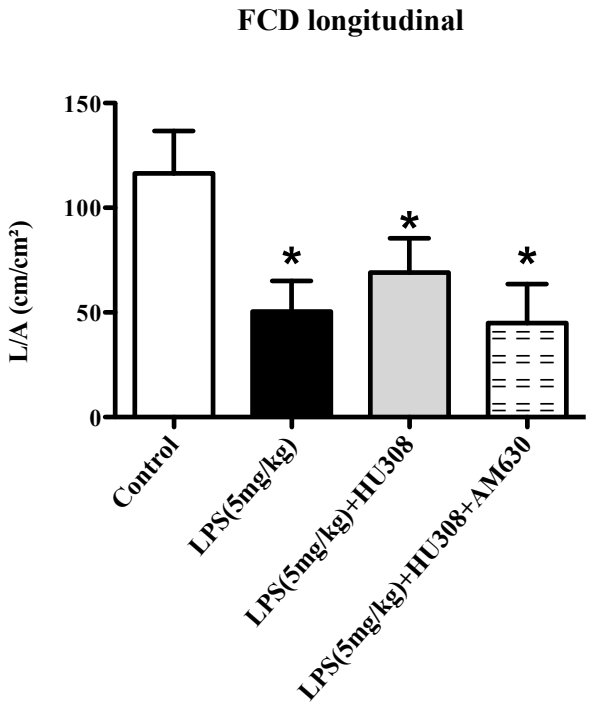


Figure 27B. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in circular muscle layer muscle layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5 mg/kg) plus CB2R antagonist (2.5mg/kg), means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls; # p<0.05 versus LPS.

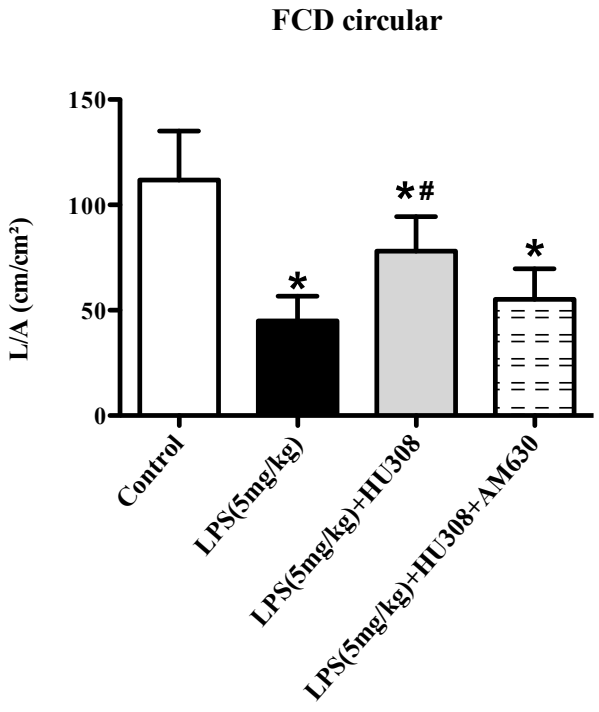
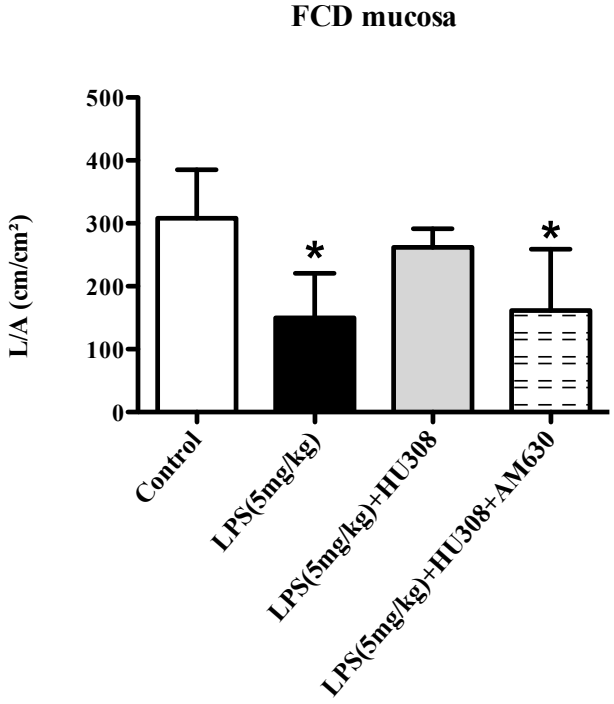


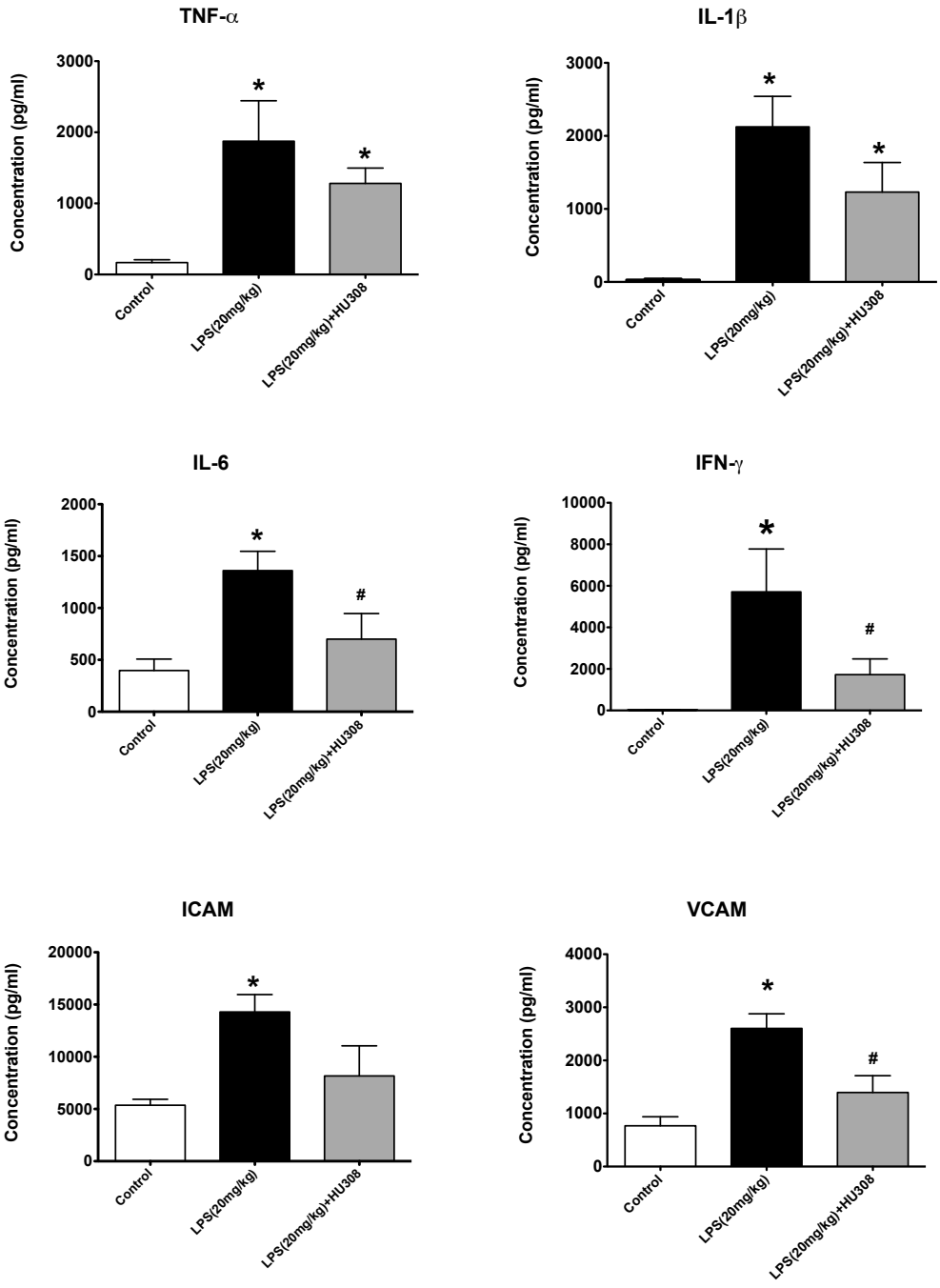
Figure 27C. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in mucosal layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); LPS+HU308, endotoxin plus CB2R agonist; LPS+HU308+AM630, endotoxin plus CB2R agonist (2.5mg/kg) plus CB2R antagonist (2.5mg/kg), means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls.



3.2.4 Cytokines and adhesion molecules

Cytokine analyses of endotoxemia experiments demonstrated that LPS challenge resulted in a significantly elevated level of inflammatory mediators (Figure 28). When LPS-challenged animals received CB2R agonist, HU308 levels of TNF-alpha and IL-1beta were found to be reduced ($p<0.05$). The concentration of IL-6 and IFN-gamma and the adhesion molecules, ICAM and VCAM, was reduced in LPS animals treated with HU308.

Figure 28. Cytokines and adhesion molecules [pg/ml]; A – TNF-alpha, **B** –IL-1beta, **C** –IL-6, **D** –INF-gamma, **E** –ICAM, **F**–VCAM; Controls, control group; LPS, endotoxemia group (20 mg/kg); LPS+HU308; endotoxin plus CB2R agonist, means ± standard error; * $p<0.05$ vs. Control; # $p<0.05$ vs. LPS.

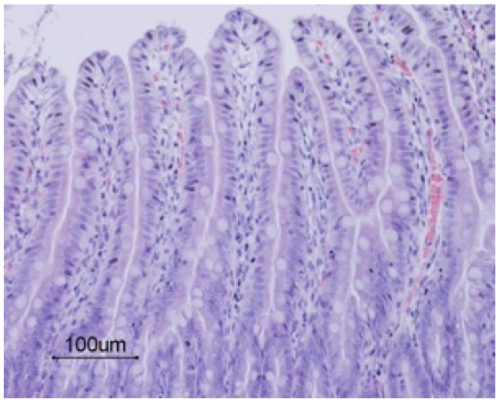


3.2.5 Histology

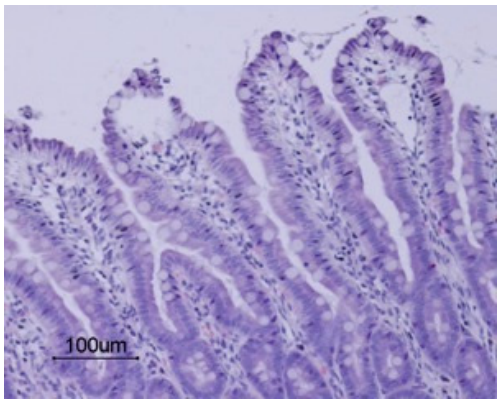
Control animals showed a normal mucosa (Figure 29). Short time endotoxin challenge resulted in subepithelial space formation at villus tips and slight disruption of the surface epithelium. The histological score changed to Grade 1-2. The histological score for the CB2R agonist, HU308 or GP1a treated animals was 0 (normal histology). The CB2R antagonist, AM630-treated endotoxemic animals, and the CB2R combination LPS+HU308+AM630 scored 1 at maximum, showing a slight disruption of the surface epithelium and visibility of spaces at the tips of villi.

Figure 29. Histology; Morphological changes in the rat small intestine after 3 hours of endotoxemia (H&E staining). **A**-Control, **B**-LPS, **C**-LPS+HU308, **D**-LPS+AM630, **E**-LPS+HU308+AM630, **F**-LPS+GP1a.

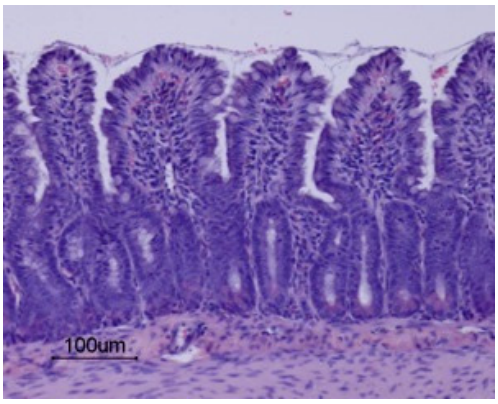
A) Control



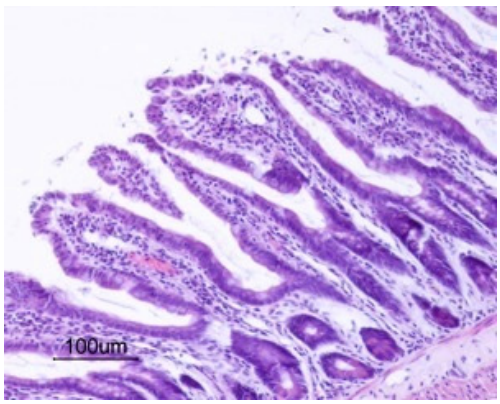
B) LPS(20mg/kg)



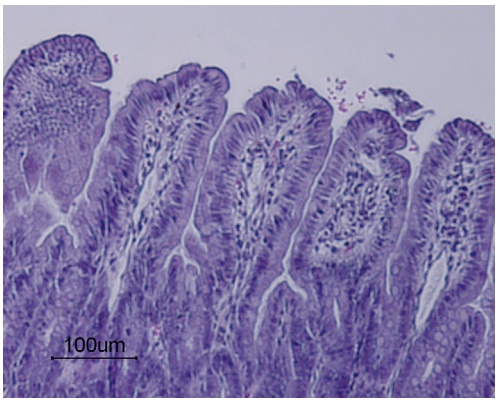
C) LPS(20mg/kg)+HU308



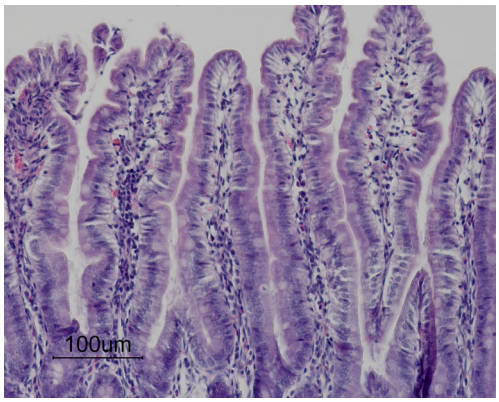
D) LPS(20mg/kg)+AM630



E) LPS(5mg/kg)+HU308+AM630



F) LPS(20mg/kg)+GP1a



3.3 Enzyme inhibitor experiments

The objective of this part of the experiments was to study FAAH inhibition in experimental endotoxemia. We used a specific FAAH inhibitor (URB597) and the combination of FAAH inhibitor with a CB2R antagonist.

3.3.1 Vital parameters

MAP and HR values are shown in Figure 30 A, B& C. Blood pressure and heart rate remained stable in the control group and in the LPS group. In LPS+URB597+AM630 treatment group, a slight increase in first 15 min in mean arterial pressure was apparent, however, at the time of IVM (180 min) all animal showed comparable MAP values (Figure 30A). Heart rate and temperature in the all groups remained stable throughout the experiment (Figure 30 B& C).

Figure 30A. Mean arterial pressure [mmHg]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6 mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor (0.6 mg/kg) plus CB2R antagonist (2.5 mg/kg) plus endotoxin; means ± standard deviation.

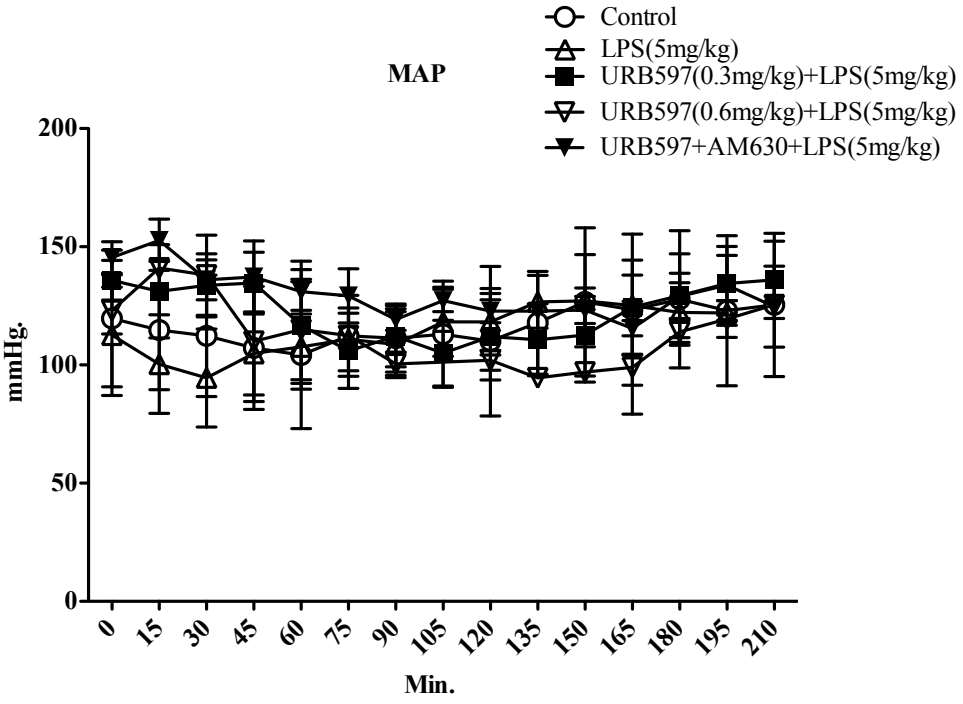


Figure 30B. Heart rate [bpm]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6 mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor (0.6 mg/kg) plus CB2R antagonist (2.5 mg/kg) plus endotoxin; means ± standard deviation.

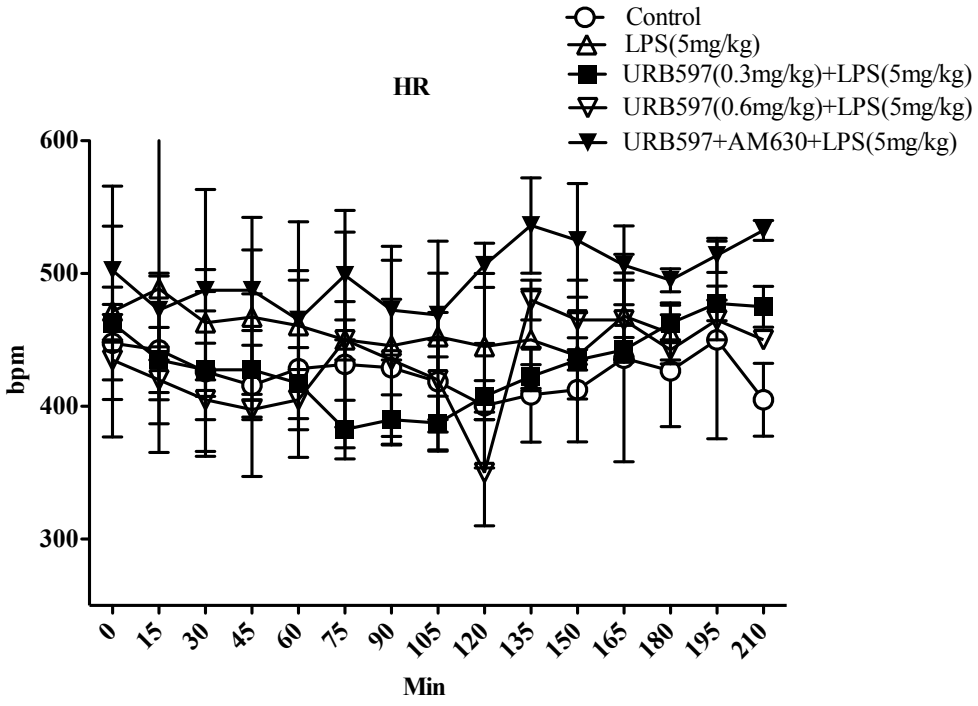
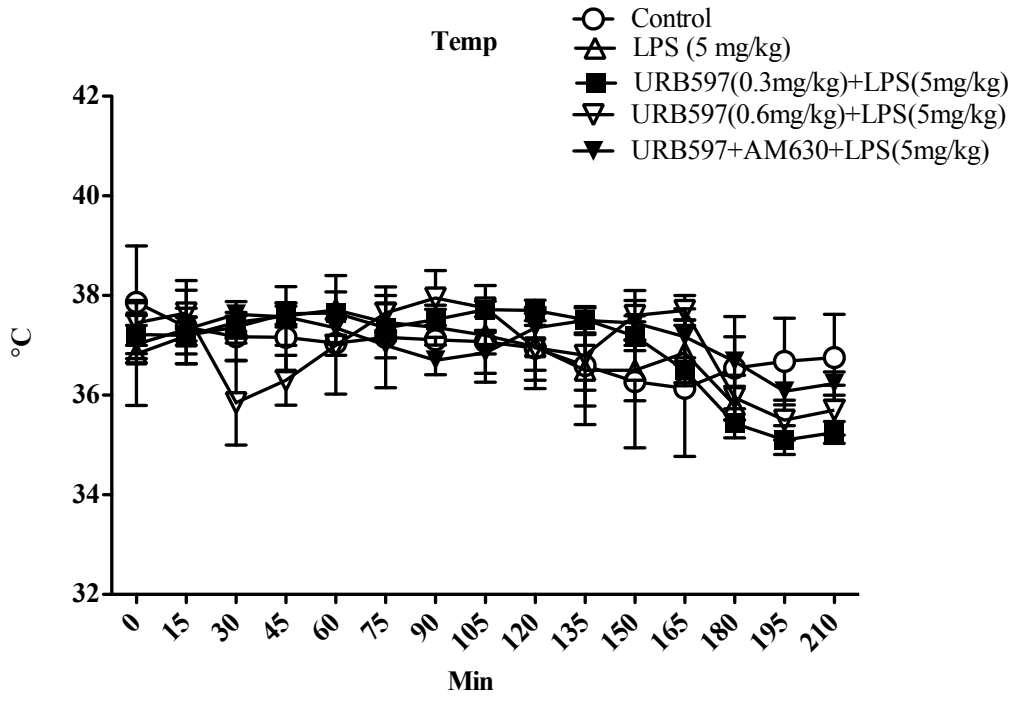


Figure 30C. Temperature [°C]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6 mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor (0.6 mg/kg) plus CB2R antagonist (2.5 mg/kg) plus endotoxin; means ± standard deviation.



3.3.2 Leukocyte adherence

IVM measurements examining adherent leucocytes in intestinal V1 venules are shown in Figure 31A. LPS administration significantly ($p<0.05$) increased the number of adherent leukocytes in V1 venules comparing to control (Figure 31A). Treatment with higher dosages (0.6 mg/kg) of the FAAH enzyme inhibitor, URB597, showed a trend towards decrease leukocyte adhesion. The combination treatment with URB597 (enzyme inhibitor) plus CB2R agonist (AM630) increased leukocyte adhesion compare to control.

The endotoxin challenge resulted in a significant increase in adherent leukocytes in V3 venules 2 hours after start of endotoxemia. Treatment with both dosages of the URB597 resulted in a decrease of adhering leukocytes in V3 venules (not statistically significant) and the combination treatment of URB597+AM630 increased leukocyte adhesion compare to control (Figure 31B).

Figure 31A. Leukocyte adhesion in collecting venules (V1) [n/mm2]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor (0.6mg/kg) plus CB2R antagonist plus endotoxin, means ± standard deviation; N/A: numbers per area. *p<0.05 versus Controls.

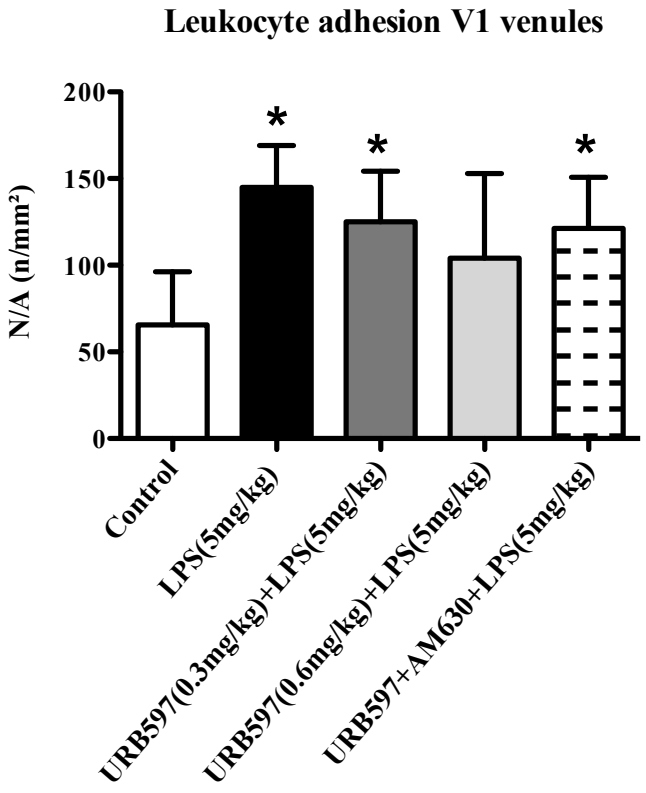
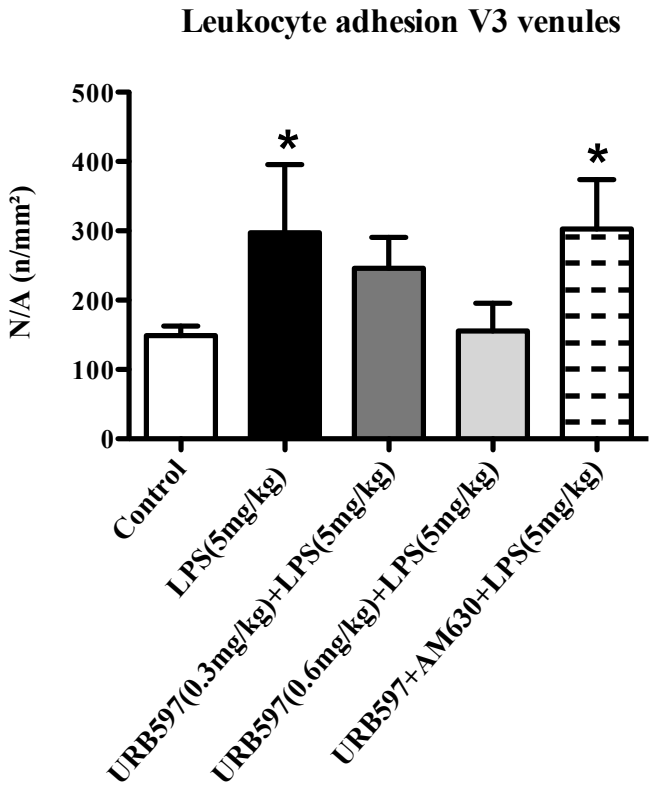


Figure 31B. Leukocyte adhesion in postcapillary venules (V3) [n/mm2]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor (0.6mg/kg) plus CB2R antagonist plus endotoxin, means ± standard deviation; N/A: numbers per area. *p<0.05 versus Controls.



3.3.3 Rolling leukocytes

The endotoxin challenge resulted in a significant ($p<0.0001$) decrease in temporarily adherent leukocytes (roller) in V1 and V3 venules, 2 hr after start of endotoxemia comparing to control (Figure 32A& B). Treatments with both dosages of the URB597 and URB597+AM630 also significantly ($p<0.05$) reduced the number of rolling leukocytes in the V1 and V3 venules comparing to control.

Figure 32A. Leukocyte rolling in collecting venules (V1) [n/min]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor (0.6mg/kg) plus CB2R antagonist plus endotoxin, means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Controls.

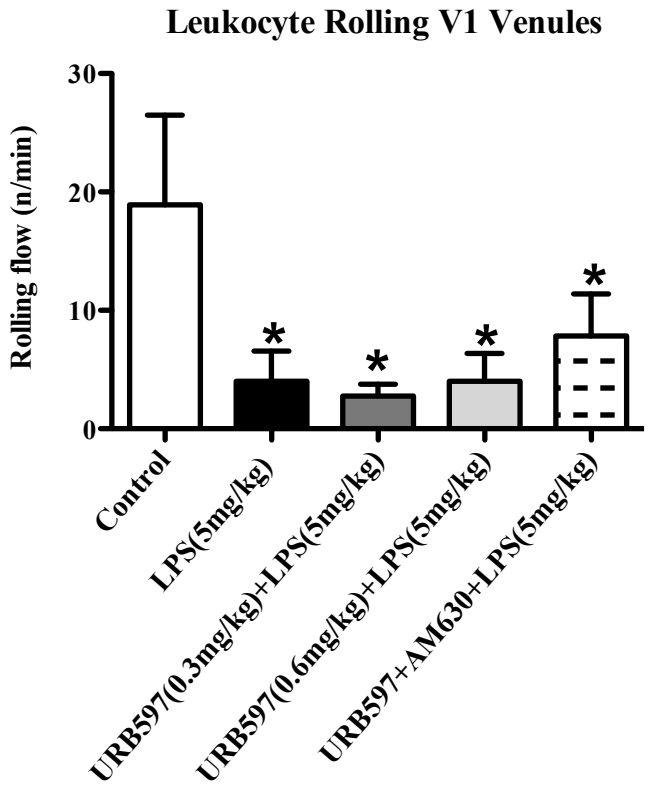
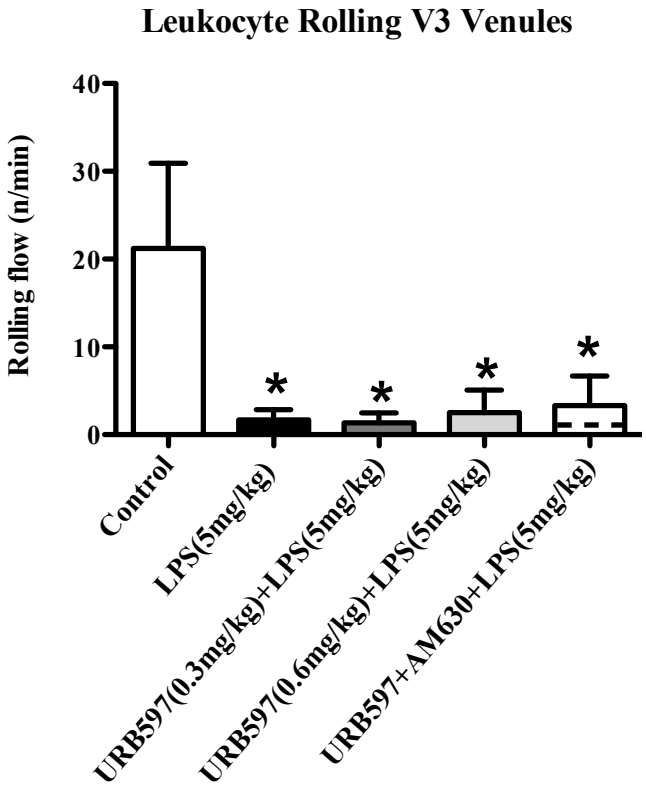


Figure 32B. Leukocyte rolling in postcapillary venule (V3) [n/min]; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor (0.6mg/kg) plus CB2R antagonist plus endotoxin, means ± standard deviation; n/min: numbers per minute. *p<0.05 versus Controls.



3.3.4 Functional capillary density

The functional capillary density is given 33 A, B& C. FCD was significantly ($p<0.05$) decreased after endotoxin challenge in longitudinal (Figure 23 A), circular muscle layer (Figure 33 B), and in mucosal layers (Figure 33 C). Both dosages of the URB597 and combination treatment with URB597+AM630 tend to improve FCD in longitudinal, circular muscle layer and mucosa of intestinal wall (Figure 33 A, B& C) In the circular muscle layer, URB597+AM630 treatment significantly increased FCD in compare to LPS group.

Figure 33A. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in longitudinal muscle layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor plus CB2R antagonist plus endotoxin, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls.

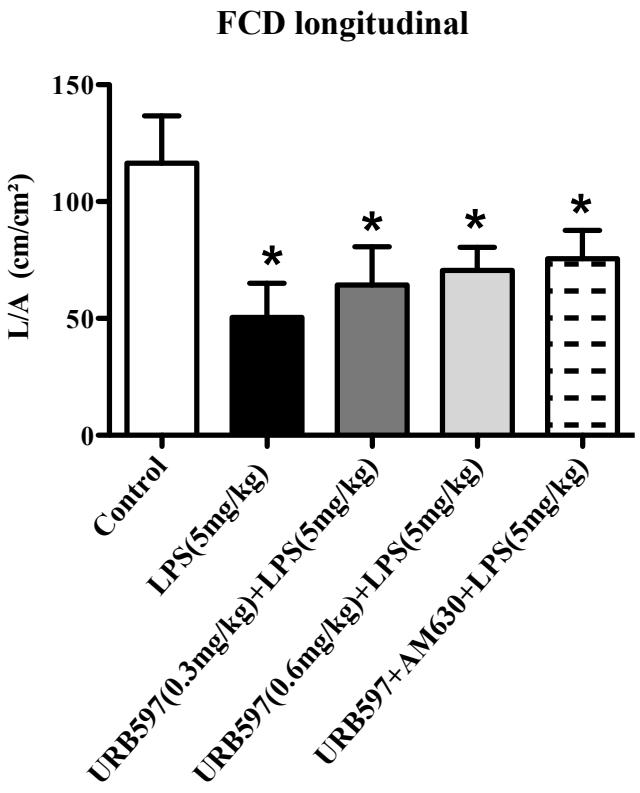


Figure 33B. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in circular muscle layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor plus CB2R antagonist plus endotoxin, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls; # p<0.05 versus LPS.

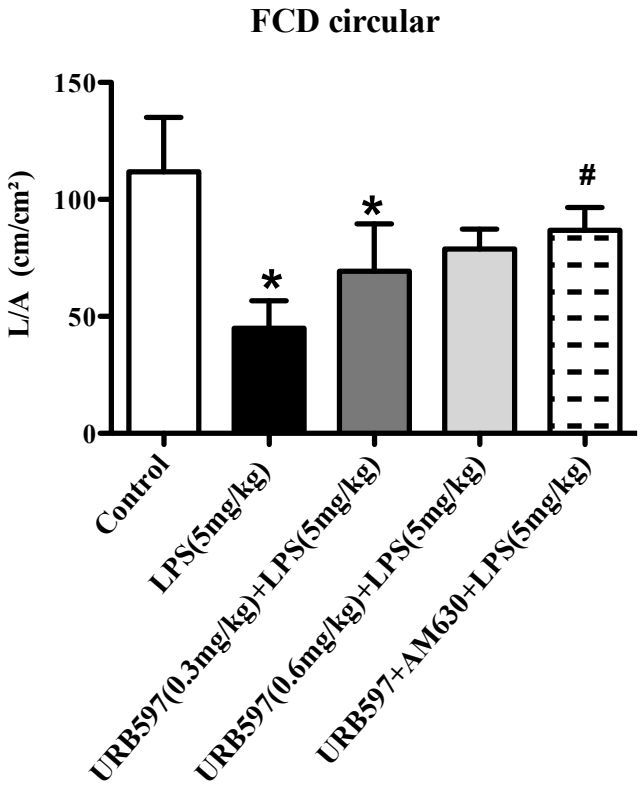
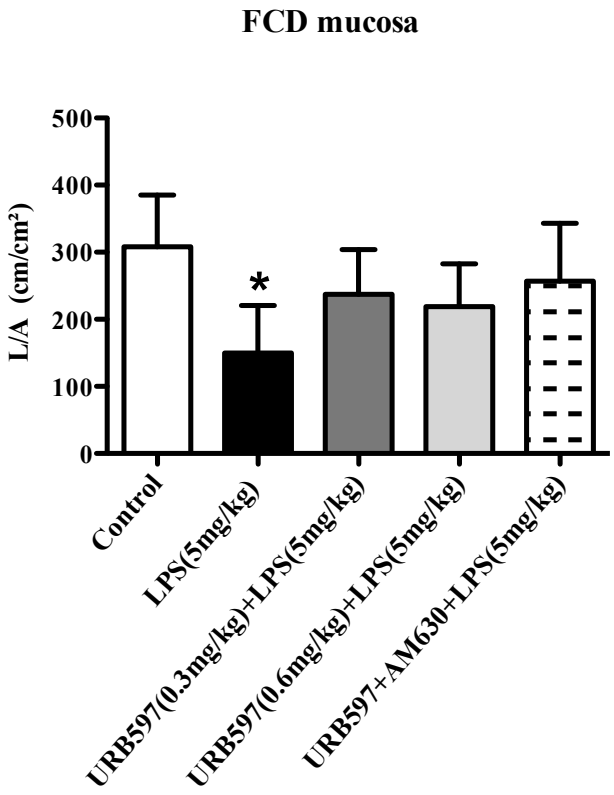


Figure 33C. Functional capillary density (FCD) [cm/cm^2]; functional capillary density in mucosal muscle layer; Controls, control group; LPS, endotoxemia group (5 mg/kg LPS at time 0); URB597 (0.3mg/kg)+LPS, enzyme inhibitor (0.3mg/kg) plus endotoxin; URB597 (0.6mg/kg)+LPS, enzyme inhibitor (0.6mg/kg) plus endotoxin; URB597+AM630+LPS, enzyme inhibitor plus CB2R antagonist plus endotoxin, means ± standard deviation; L/A: length of capillaries per area. *p<0.05 versus Controls.

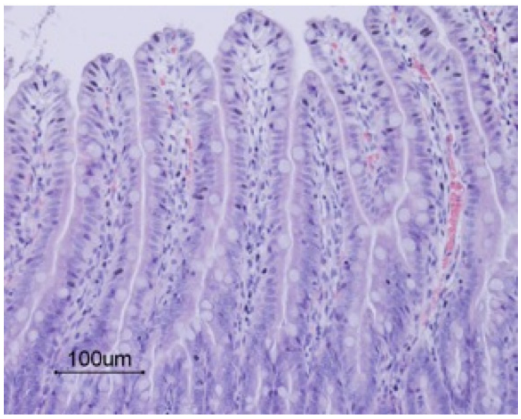


3.3.6 Histology

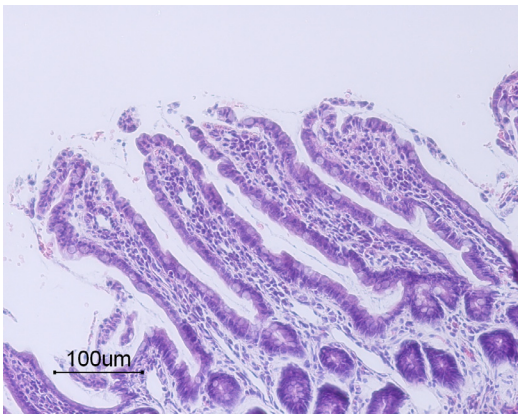
Control animals showed a normal mucosa (Grade 0, according to Chiu *et al*.). Short time endotoxin challenge resulted in sub-epithelial space formation at villus tips and slight disruption of the surface epithelium. The histological score changed to Grade 1-2. The histological score was 0-1 for the tissue from the URB597 treated endotoxemic animals and 0 (normal histology) for LPS+URB597+AM630 animals (Figure 34).

Figure 34. Histology; Morphological changes in the rat small intestine after 3 hours of endotoxemia (H&E staining). **A**-Control, **B**-LPS, **C**-URB597+LPS, **D**-URB597+AM630+LPS

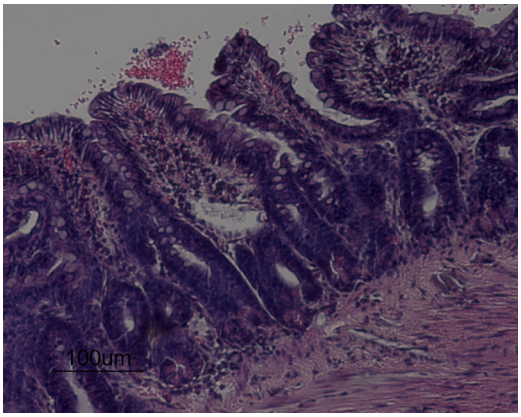
A) Control



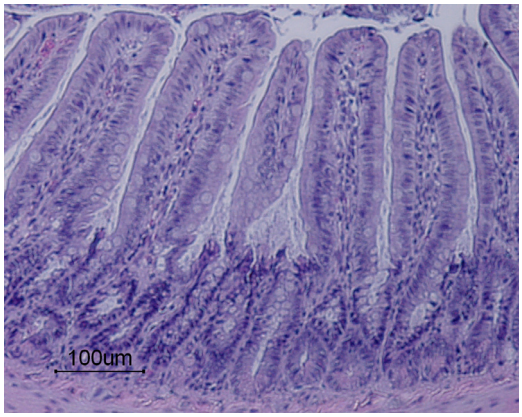
B) LPS(5mg/kg)



C) URB597(0.6mg/kg)+LPS(5mg/kg)



D) URB597+AM630+LPS(5mg/kg)



CHAPTER 4: DISCUSSION

4.1 Overview of Findings

The intestinal microcirculation (IMC) plays a critical role in the pathogenesis of sepsis since disturbance of the IMC may cause a breakdown of the gut mucosal barrier, releasing bacteria and their toxins into the systemic circulation. Therefore, preservation of the IMC is a key therapeutic target in the treatment of sepsis and involves attenuation of immune cell recruitment and improvement in microcirculatory function, both of which result in prevention of pathogen translocation from the intestine to the circulation. This research project focused on manipulation of the endocannabinoid system (ECS) during acute systemic inflammation. The aim of this study was to determine the effect of the modulation (activation or inhibition) of cannabinoid receptors by cannabinoid receptor-selective ligands (agonist and/or antagonists), as well as drugs that prevent endocannabinoid degradation, on intestinal leukocyte recruitment and gut capillary perfusion in experimental sepsis. It was hypothesized that modulation of ECS during the acute phase of sepsis would improve microcirculation and reduce the severity of systemic inflammation. The results of this study demonstrated that CB1R inhibition, but not CB1R activation, reduced leukocyte recruitment within the intestinal microvasculature in experimental endotoxemia. Concomitant with reduced leukocyte recruitment, CB1R antagonist administration also improved capillary perfusion in the sub-layers of the intestinal wall. Moreover, CB2R activation (HU308), but not CB2R inhibition, reduced leukocyte recruitment within the intestinal microvasculature in acute experimental sepsis models. Furthermore, this work showed that URB597, an inhibitor for the FAAH enzyme that increases AEA levels, inhibits leukocyte activation and improves capillary perfusion.

4.2 Vital Parameters and Sepsis

Sepsis is associated with changes in hemodynamics. These include changes in mean arterial pressure (MAP), heart rate (HR) and cardiac output (Dyson *et al*., 2012). In the microcirculatory units, leukocyte adherence in venules and blood flow in capillaries are dependent on the MAP, which influences the blood flow velocity. A number of studies have suggested that one potential explanation for increased leukocyte-endothelial cell adhesion is decreased blood flow and low blood pressure with low shear force (Sanz *et al*., 1999; Russell *et al*., 2003). In this study, however, the vital parameters of all experimental groups were stable at the baseline and throughout the experiments. Furthermore, the MAP remained constant and showed no significant differences in either the control group or the treatment groups. In support of this, other studies such as Lehmann *et al*. (2001), (2006), (2007), and Zhou *et al*. (2011) also showed MAP stability in endotoxemia animals. The i.v. administration of 20 mg/kg LPS or 5 mg/kg LPS led to short-term drop in blood pressure within the first 30 min and it lasted only for a few minutes. This is consistent with other studies that showed stable hemodynamics in endotoxemia, and only transient decrease in blood pressure after i.v. injection with LPS (Schmidt *et al*., 1996; Panayiotou *et al*., 2010). However, stabilization of blood pressure in our experiments was only possible by fluid resuscitation and oxygen administration. The initial drop in blood pressure caused by LPS administration can be variable depending on endotoxin serotype, lot, dosage, duration and method of LPS administration. At the time of IVM (120 min), the MAP of all experimental groups were not significantly different.

With respect to the role of cannabinoid ligands in maintaining stable hemodynamics, several studies have suggested that activation of CB1R located on the presynaptic terminals of autonomic nerves or the vascular walls contributes to the hypotension associated with septic shock (Godlewski *et al*., 2004; Varga *et al*., 1998; Wagner *et al*., 1998; Hillard, 2000; Pacher, *et al*., 2005). In keeping with this, blocking CB1Rs by SR141716A has been shown to reduce the hypotension associated with hemorrhagic shock (Cainazzo *et al*., 2002), and to prevent LPS-induced hypotension in septic shock rats (Varga *et al*., 1998; Bátkai *et al*., 2004). Similarly, Kadoi *et al*. (2005), reported the beneficial effect of AM281, a CB1R antagonist, on the systemic hemodynamics parameters in experimental septic shock. An important feature of our endotoxemia model was the i.v. administration of fluid (normal saline) for resuscitation to balance vasodilation that occurs during sepsis. Our experimental model was not associated with septic shock; we did not observe a significant reduction in the MAP (hypotension). Therefore, we were not able to study the beneficial effects of cannabinoid ligands particularly CB1R antagonist on sepsis-induced hemodynamic changes.

The HR in the control groups remained stable throughout the course of our experiments. On the other hand, the HR of animals challenged with endotoxin, increased after 1 hour after exposure to endotoxemia, as compared to baseline (time 0), and remained elevated throughout the experiment. Again, this effect is consistent with other findings, which reported the elevated HR level after induction of endotoxemia (Lehmann *et al*., 2006; Birnbaum *et al*., 2008; Lehmann *et al*., 2004; Zhou *et al*., 2011; Sitina *et al*., 2011).

The body temperature was also stable throughout the experiments in all groups (constant at 37 °C). This was achieved through the use of the adjustable heating pad during the surgical procedure and the local water heating system for microscopy. Other animal studies have shown a drop in a body temperature (hypothermia) in association with sepsis (Steiner *et al*., 2011). LPS administration to rats causes hypothermia. This effect results from vasodilation, which causes heat loss in animals (Cox *et al*., 2003; Steiner *et al*., 2011). In order to study the changes in microcirculation, we had to keep the animal's body temperature as constant as possible, and therefore all the precautions were taken to achieve this goal. With respect to the role of cannabinoid ligands in maintaining constant temperature, Steiner *et al*. (2011) studied the role of CBR in LPS-induced hypothermia. Their study reported that blocking CB1R abolished the LPS-induced hypothermia. Our experimental model was not associated with hypothermia. Therefore, we were not able to study the beneficial effects of cannabinoid ligands particularly CB1R antagonist on sepsis-induced hemodynamic changes.

4.3 Intravital Microscopy

The focus of the intravital microscopy study was to characterize the acute inflammatory response in endotoxemia by examining intestinal leukocyte recruitment and the capillary perfusion of the intestinal wall. We observed a significant increase in the number of adhering leukocytes in V1 and V3 venules of the intestinal microvasculature of endotoxin treated groups compared to the saline control group. LPS-induced recruitment of leukocytes to small venules has been reported by number of studies

(Lehmann *et al*., 2001; 2006; 2012; Wu *et al*., 2012). The initial step for recruitment of leukocyte is the activation of endothelium, which is mediated by up-regulation of adhesion molecules. LPS can stimulate the up-regulation of adhesion molecules on the surface of endothelium (Wu *et al*., 2012; Ramirez *et al*., 2012). It was hypothesized that the LPS model of sepsis produces an acute inflammatory response in the treated animals, manifested with the increased recruitment of leukocytes and decreased perfusion in capillaries.

Leukocyte Adhesion

Our finding showed a significant increase in the number of adhering leukocytes in the intestinal microvasculature caused by LPS challenge in comparison to the control group. We demonstrated for the first time that CB1R inhibition, but not CB1R activation, decreased leukocyte recruitment within the intestinal microvasculature in experimental endotoxemia (Figure 12A & B). Treatment with the CB1R antagonist, (AM281) showed a reversal of the LPS-induced increase in leukocyte adhesion in V1 and V3 venules of the intestinal wall; while, treatment with the CB1R agonist (ACEA) had no impact on leukocyte recruitment. In support of our findings, several studies have suggested that CB1R antagonists exert immunosuppressive and anti-inflammatory actions, for example CB1R inactivation with AM281 reduces mortality in CLP-induced septic shock (Kadoi *et al*., 2005). Rossi *et al*., (2011) showed that the CB1R antagonists/inverse agonists, AM251, SR141716A and AM281, inhibited the adhesion of T cells in inflamed brain venules (Rossi *et al*., 2011). Consistent with this, CB1R inverse agonism reduced neutrophil infiltration in a rat model of endotoxemia (Caraceni *et al*., 2009). In contrast, Mestre *et al*., showed that anandamide was effective in reducing endothelial VCAM-1

expression via CB1R (Mestre *et al*., 2011). Consistent with a beneficial effect of CB1R antagonism, inactivation of CB1R (CB1R knockout mice) significantly reduced leukocyte infiltration into skin lesions in an inflammation-induced model of fibrosis (Marquart *et al*., 2010). In our study, the CB1R antagonist induced a decrease of leukocyte activation that could not be reversed by subsequent CB1R stimulation. This observation may be related to a number of factors, including CB1R ligand concentrations at the receptor, but likely relates to the time course of LPS plasma levels during acute endotoxemia; the majority of leukocytes are activated during the initial LPS concentration peak, which could be prevented by CB1R inhibition. In this respect, later application of CB1R agonists had no significant effect on leukocyte recruitment.

CB2R are expressed on macrophages, neutrophils, lymphocytes, mast cells and dendritic cells and there is strong evidence that activation of these receptors is associated with anti-inflammatory effects (Miller & Stella 2008; Maccarrone *et al*., 2002; Ramirez *et al*., 2012). Ramirez *et al*. (2012) also showed that CB2R activation, using JWH133 or O-1966 cannabinoid ligands, reduces expression of adhesion molecules needed for leukocyte endothelial interactions (Ramirez *et al*., 2012). In our setting, the CB2R agonist, GP1a had no significant effects on the leukocyte adhesion in the V1 and V3 venules compare to LPS group (Figure 18A & B). This is in contrast to previous studies; Gorantla *et al*. (2011) reported GP1a had a strong immunosuppressive effect by reducing leukocyte infiltration into the brain in a murine model of AIDS. Similarly, another study by Xu *et al*. (2007) showed that the CB2R agonist, JWH133, suppressed leukocyte trafficking in inflamed retina. However, this study found that the CB2R agonist, HU-308, given in a dosage of 2.5 mg/kg i.v., significantly reduced the number of adherent

leukocytes in V1 and V3 venules (Figure 22A & B). This finding suggested that CB2R antagonism, in contrast to CB2R activation, would increase leukocyte adhesion. However, following administration of the CB2R antagonist, AM630, we observed no significant effect on LPS induced leukocyte recruitment (Figure 22A & B). This lack of efficacy of AM630, may be explained by the fact that LPS-induced leukocyte adhesion was already at its maximum level, and thus could not be further exacerbated by CB2R antagonist. Consistent with our findings of an anti-inflammatory role for CB2R in endotoxemia, a study by Caldwell *et al*. in 2010 reported that CB2R activation caused a reduction in the number of leukocyte and macrophages at the site of infection, while increasing their phagocytic activity (Caldwell *et al*., 2010). Our laboratory showed that in clinically more relevant sepsis model, CASP-induced sepsis, the agonist dosage used in the LPS endotoxemia experiments was not effective in producing a significant effect on leukocyte adhesion; an increase in the dosage of HU308 to 10 mg/kg was required to obtain the same extent of reduction of intestinal leukocyte adhesion (Lehmann *et al*., 2012). In support of results in the CASP model, Ni *et al*. (2004) also showed in an experimental inflammation model (autoimmune encephalomyelitis), the CB1/CB2 agonist, WIN 55212-2, was only able to attenuate leukocyte-endothelial interactions in the cerebral microcirculation if given in a higher dosage (10 mg/kg). Several other studies had pointed out a role for CB2R in inflammation and sepsis; for example, Tschöp *et al*. (2009) and Csoka *et al*. (2009) examined the impact of CB2R modulation on survival in experimental sepsis induced by CLP using CB2R knockout mice. Despite both groups using similar strains of animals, they reported the opposite effects. In first study, CB2R knock out mice, following CLP-induced sepsis, had a higher mortality and the

administration of a selective CB2R agonist (GP1a) improved survival of wild-type mice (Tschöp *et al*., 2009). In contrast, the Csoka *et al*. study demonstrated that CB2R knockout mice had a better survival from sepsis than wild-type mice (Csoka *et al*., 2009). Although the CLP model was used to induce sepsis in both studies, the severity of sepsis was different in those studies, mild (one puncture) and severe (two punctures). In moderate models of sepsis (Tschöp *et al*., 2009), inefficient clearance of bacterial pathogen leads to prolongation of infection; CB2R activation enhances the immune response that leads to bacterial clearance thus increased survival. Conversely, in severe sepsis (Csoka *et al*., 2009) where neutrophil recruitment and function can be detrimental to the host through increased tissue damage cardiac arrest and death, CB2R inactivation (genetic deletion) reduced mortality. Therefore, the different effects of CB2R modulation can be related to the different severity of the inflammatory response in these experiments; CB2R activation was only beneficial in moderate sepsis, which is comparable to endotoxemia used in our study.

Taken together, these findings indicate that the effect of activation of CB2R in inflammation is dose-dependent and varies according to the experimental conditions. We aimed to investigate a reversal of the CB2R activation by HU308 using a CB2R antagonist in combination in LPS-induced endotoxemia to further confirm that the observed effects are mediated via CB2Rs. After LPS challenge, CB2R were blocked with the antagonist, AM630, and 10 minutes later CB2R were activated with the agonist, HU308. It was seen that CB2R agonist-induced decrease of leukocyte activation could be reversed by subsequent CB2R inhibition (Figure 27A & B). Thus, we can claim that the observed effect of HU308 is indeed CB2R dependent. In support of our findings, a

number of studies have shown that the actions of HU308 can be completely blocked by CB2R antagonists (AM630) or genetic deletion of CB2R. For example, HU308 significantly decreased the level of reactive oxygen species and TNF-α in infarct and ischemia-reperfusion heart injury; these effects were blocked by AM630 (Wang *et al*., 2012); HU308 stimulatory effect on osteoblast migration was blocked by AM630 or in CB2R knock out mice (Sophocleous *et al*., 2011). Batkai *et al*. showed that the protective effect (decreasing neutrophil infiltration) of CB2R agonist (JWH133) in hepatic ischemia-reperfusion was attenuated by AM630 and in CB2R knock out mice (Batkai *et al*., 2007).

Studies showed that the URB597 produces stable and persistent increase of AEA, the effect of URB597 was dose dependent. Studies reported that URB597 maximal effect on FAAH inhibition and subsequent increase AEA level was at the dose of 0.3 mg/kg (Piomelli *et al*., 2006; Jayamanne *et al*., 2006). We have also demonstrated that LPS-induced leukocyte adhesion tends to be decreased in V1 and V3 venules in animals treated with the selective FAAH inhibitor, URB597; FAAH inhibition with URB597 results in increased levels of AEA (Figure 33A & B). Consistent with these findings, several studies have suggested that inhibition/genetic deletion of FAAH exerts anti-inflammatory actions (Naidu *et al*., 2010). A study by Kunos & Pacher reported that using the FAAH inhibitors augmented the levels of endogenous AEA (has anti-inflammatory action) and amplified the protective action of AEA by inhibiting TNF-α production, consequently decreasing the leukocyte infiltration (Kunos & Pacher, 2008). Maccaroone *et al*., reported that AEA is present on rat both endothelial cells and macrophages, suggesting an important role in modulating vascular tone (Maccarrone *et*

al., 2001). Holt *et al*., has shown the anti-inflammatory effect of FAAH inhibitor in carrageenan model of inflammation (Holt *et al*., 2005). In our experiments, the FAAH inhibitor, URB597, had an anti-inflammatory effect. To identify whether or not the FAAH enzyme inhibitor effect is related to the CB2R pathway, the use of CB2R antagonist could provide an insight into the URB597-mediated anti-inflammatory effect. In our study, FAAH inhibition-induced a decrease in leukocyte activation that may was blocked by subsequent CB2R inhibition (31A & B). This observation indicates that the FAAH enzyme inhibitor effect is related to the CB2R pathway. In support of our results, using URB597 in a neuropathic and inflammatory pain model reduced the inflammatory conditions associated with this model (Jayamanne *et al*., 2006). The effects of URB597 in this study were mediated by both CB1R and CB2R. The use of URB597 in an experimental colitis model was also found to significantly reduce inflammation, an action that was abolished in genetic mice knock-out models of CB1R and CB2R. Taken together, these studies indicate a role for both of these receptors in the protective effects seen after block of endocannabinoid degradation (Storr *et al*., 2008). Given the promising beneficial effects of FAAH inhibition in these inflammatory experimental models, future experiments that clarify the involvement of CB1R as well as CB2R in the therapeutic actions of URB597 are warranted.

Leukocyte Rolling

Our findings demonstrated that LPS administration significantly decreased the number of rolling leukocytes in comparison to the saline control group. Under experimental conditions, administration of LPS results in a massive increase in leukocyte adherence, and reduces the number of temporary interacting leukocytes with the

endothelium. Consistent with our findings, others have reported the significant reduction in the rolling leukocytes 1-4 hours after LPS challenge (Hayes *et al*., 2004; Birnbaum *et al*., 2008). Alongside with our findings, Yipp *et al*. (2002) showed that the systemic administration of LPS caused a significant reduction in the circulating leukocytes (Yipp *et al*., 2002).

LPS administration induces the recruitment of leukocytes, which is a crucial feature in sepsis. However, there are different responses to LPS based on the method of administration. Local LPS administration results in an increase in the leukocyte-endothelial interaction (increase in both rolling and adhesion), while systemic administration of LPS decreases circulating leukocytes counts and increases peripheral neutrophil adhesion (Yipp *et al*., 2002). The same study showed that concomitant to the reduction of circulating leukocytes, there was a significant increase in accumulation of neutrophils in the lung tissues, which was also similar to our findings. This thesis showed that the number of rolling leukocytes was significantly reduced after LPS challenge, while the count of firmly adherent cells was increased, which is also a typical finding in sepsis models (Birnbaum *et al*., 2008).

We have seen that treatment with all the CBR agonists, antagonists and combinations of agonist and antagonist significantly reduced the number of rolling leukocytes in the V1 and V3 venules. Treatments with both dosages of the URB597 and URB597 + CB2R antagonist also significantly reduced the number of rolling leukocytes in the V1 and V3 venules compared to controls. Our findings demonstrated that the CBR ligands along with the enzyme inhibitor decreased the leukocyte rolling. Alongside with our findings, Ni *et al*. (2004) showed in an experimental inflammation model the

CB1/CB2 agonist, WIN 55212-2 decreased the leukocyte rolling in the cerebral microcirculation; Xu *et al*. (2007) showed that the CB2R agonist, JWH133, suppressed leukocyte rolling in inflamed retina, suggesting that leukocyte rolling could be attenuated by activation of CBRs.

Functional Capillary Density

Sepsis-induced changes in the microcirculation are also characterized by a decrease in capillary density with increased number of stopped flow capillaries (Backer *et al*., 2011). There are several proposed mechanisms that could be involved in microcirculation alteration in sepsis such as endothelial dysfunction by decreased sensitivity to vasoactive substances, impaired RBC deformability and most importantly, rolling and adhesion of leukocytes (Backer *et al*., 2011; Ince, 2005). In acute experimental endotoxemia, we observed a significant impairment of the FCD in the intestinal mucosa and in the longitudinal and circular muscle layers of the intestinal wall due to LPS challenge. In this study, administration of both dosages of LPS, 5 mg/kg and 20 mg/kg induced a significant decrease in FCD in the longitudinal, circular muscle layers and mucosa of intestinal wall after 2 hours of endotoxemia. This is similar to the results of number of previous studies (Birnbaum *et al*., 2006; Lehmann *et al*., 2004; 2006).

The CB1R antagonist, AM281, significantly improved capillary perfusion in the sub-layers of the intestinal wall in endotoxemic animals (Figure 14A, B & C). Liu *et al*. also showed, in a diabetic rat model, that SR141716, a CB1R antagonist, is able to attenuate skin capillary loss and increase skin blood flow in tissue (Liu *et al*., 2010). The

increased intestinal capillary perfusion in our experiments may be due to peripheral microvascular vasodilation, decreased WBC adhesion and increased blood pressure. The reversal of the beneficial effect of CB1R antagonist administration by CB1R agonist administration supports this explanation. Furthermore, this thesis also showed that treatment with the CB2R agonist, GP1a, significantly improved the FCD in all the muscle layers. While treatment with the CB2R agonist, HU308, also improved FCD, it was only significant in circular muscle layers (Figure 29B). It is not exactly known how the tested CB2R agonists improve capillary perfusion; however it is thought to be mediated by increasing microvascular blood flow, e.g. by reducing leukocyte endothelial interactions. The administration of URB597, to increase endocannabinoid levels, improved capillary perfusion in the sub-layers of the intestinal wall (Figure 29A, B & C). However, treatments such as the CB1R agonists, ACEA, and the CB2R antagonist, AM630, failed to improve the FCD in the LPS treated animals. These findings can be explained by the involvement of other factors causing FCD impairment, for example, disseminated intravascular coagulation and decreased RBC deformability (Chierego *et al*., 2006; Backer *et al*., 2011).

4.4 Cytokines

The excessive production of pro-inflammatory cytokines represents an important mechanism for the early inflammatory damage in sepsis. Our cytokine data provided evidence that the LPS-induced sepsis model generated an acute pro-inflammatory cytokine and adhesion molecule profile. LPS administration is known to increase plasma levels of pro-inflammatory cytokines (Scheiermann *et al*., 2011). Initiation of host

response during sepsis involves activation of pattern-recognition receptors, such as toll-like receptors (TLRs), and associated activation of intracellular signal transduction pathways that lead to activation of transcriptional activators. These include cytosolic nuclear factor-kappa B (NF-κB), which induces activation of genes for a number of acute phase proteins, including pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 (Cinel & Opal, 2009). Excessive elevation of pro-inflammatory cytokines in the plasma of animal and patients with sepsis has been reported (Angele & Chaudry, 2005; Grundling *et al*., 2008; Lehmann *et al*., 2012). TNF-α produced by many cell types, including macrophages, lymphocytes and dendritic cells, is a key pro-inflammatory cytokine involved in mediating endotoxemia and sepsis. Endotoxin challenge results in TNF-α production, which reaches its peak level 1-3 hours after challenge (Lehmann *et al*., 1999). Therefore, inhibition of TNF-α production has a high therapeutic potential in managing sepsis. IL-1β, one of the most important mediators of inflammation, is a potent pro-inflammatory cytokine that can be released by monocytes, dendritic cells and macrophages. IL-1β, in order to be released, requires activation of the cells with TLRs for ligands such as LPS (Eder, 2009).

The CB2R agonist, HU308, reduced the release of the pro-inflammatory cytokines, IL-1β and TNF-α. Furthermore, administration of HU308 decreased the levels of IL-6 and INF-γ (Figure 28). Several studies have now reported that endocannabinoids and synthetic cannabinoids can modulate the release of pro-inflammatory mediators via CB2R-related pathways. For example, an *in vitro* study examined the effect of endocannabinoids on the levels of the pro-inflammatory cytokine, TNF-α. The authors reported that both the endocannabinoids, AEA and 2-AG, as well as synthetic

cannabinoids (WIN 55,212-2 and HU210) inhibited the production of TNF-α released from LPS-treated rat microglial cells (Facchinetti *et al*., 2003). Another study showed that 2-AG inhibits the TNF-α release from LPS-treated murine macrophages both *in vitro* and *in vivo* (Gallily *et al*., 2000). Ouyang *et al*. demonstrated that 2-AG inhibited interleukin 2 (IL-2) secretion in murine splenocytes *in vitro* (Ouyang *et al*., 1998). A study using B-caryophyllene (BCP), a CB2R agonist, reported that BCP inhibition of LPS-stimulated IL-1β and TNF-α expression in human peripheral blood (Gertsch *et al*., 2008). Another in vitro study, using BCP to evaluate it's effects on cytokines released from LPS-stimulated macrophages, showed that treatment with BCP significantly reduced the level of the cytokines (Bento *et al*., 2011).

We also observed a reduction in the levels of soluble adhesion molecules (ICAM and VCAM) by CB2R activation (HU308) in LPS-induced sepsis (Figure 28). It was shown by Brede *et al*. (2001), that the levels of soluble adhesion molecules are correlating with sepsis severity and outcome. The observed decrease in the levels of adhesion molecules in this study is in-line with our IVM findings of attenuated leukocyte recruitment within the intestinal microvasculature by CB2R activation. Studies using other CB2R agonists, e.g. JWH133, HU308 and HU309, during hepatic ischemia/reperfusion injury, demonstrated that CB2R activation also resulted in a reduction of TNF-α and MIP-2 levels, and tissue expression of adhesion molecule ICAM-1 and VCAM-1 (Rajesh *et al*., 2007(b); Batkai *et al*., 2007; Horvath *et al*., 2011). The expression of inflammatory mediators is regulated by various signaling pathways, including NF-κB activation (Cinel & Opal, 2009), which are affected by CB2R modulation. For example, Rajesh *et al*. demonstrated that CBR activation attenuates

TNF-α-triggered NF-κB and RhoA activation (Rajesh *et al*., 2007(a). Furthermore, noladin ether, a stable analog of the endocannabinoid, 2-AG, inhibited the nuclear translocation of NF-κB that leads to the arrest of the cell cycle and inhibition of growth of prostate carcinoma cells (Nithipatikom *et al*., 2011). Interestingly, in this study we have not seen a reduction in TNF-α production in HU308 treated group. This may be due to the activation of TNF-α that peaks 1-3 hours after endotoxin challenge. Since cytokine measurements in our experiments were made later than 3 hours after endotoxin challenge, peak TNF-α activation may have been missed at these later time-points. Taken together, these studies suggest that CB2R modulation is able to alter signaling pathways that regulate production of inflammatory mediators.

4.5 Histology

Histological sections with H&E staining allowed the observation of the intestinal tissue integrity. No damage to intestinal tissues was observed in the control animals as expected (Figure 15A, 29A & 34A). This suggests that our surgical manipulation does not cause intestinal disturbances in the control saline animals. Slight light-microscopic changes were observed in the tissues from animals treated with LPS and LPS + cannabinoid ligands (Figure 15, 29 & 34). Tissues from a LPS treated animals, showed slight disruption of the surface epithelium with the appearance of Gruenhagen's spaces. These spaces are the earliest lesion that can be seen with light microscopy in the intestinal mucosa; are the development of sub-epithelial spaces near the tips of villi (Chiu *et al*., 1970). The minor histological changes that we observed are possibly because of the

early observations (tissue samples were collected 3 hours after endotoxemia) in our acute model. However, by observing histological changes of intestinal tissue, which is an important assessment of tissue damages, we were not able to detect significant morphological changes in our model as early as 3 hours after LPS injection. This is an important finding because our results exhibit promising results for evaluating the early diagnosis of LPS induces changes in monitoring microcirculation, but not for examination of histological changes. And these changes need to be further studied in longer term LPS model or in more chronic model of sepsis.

Several studies reported the beneficial effect of ECS modulation of variety of functions (motility, secretion, immune activity) in GI tract, however, there are no previous studies that have studied the effects of cannabinoid ligands on LPS-induced GI damage. We showed that the CB1R ligands agonist, antagonist and the combination of agonist + antagonist improved the minor LPS-induced damages to the intestinal tissues (Figure 15 C, D& E). The CB2R agonist treatment, HU308 and GP1a, both produced an intestinal integrity score comparable to control saline (Figure 29 C & F). However, the CB2R antagonist, AM630, and the CB2R combination LPS + HU308 + AM630 treated endotoxemic animals, showed slight disruption of epithelium, which indicates that inhibition of CB2R could not improve the minor LPS-induced damages (Figure 29 D & E). The tissues from the URB597 treated endotoxemic animals and URB597 + AM630 treated endotoxemic animals also had intestinal integrity comparable with the control saline (Figure 34 C & D). This indicates that the CB2R are not involved in the protective effect of URB597 on the intestinal integrity. Thus further studies could examine the role of other receptors involve in the function of URB597 such as CB1R.

4.6 Limitations and future Directions

One of the major problems during imaging of the intestinal microcirculation was the respiration-associated movement of the intestine. These movements often resulted in the loss of focus of the camera. By using few drops of topical administration of Isoproterenol we could stabilize the intestinal movement that allowed for proper imaging the intestinal microcirculation.

The fluorescence dye Rhodamine 6G non-specifically labels the mitochondria of all leukocytes and platelets. So the usage of this dye did not permit differentiation between different leukocyte subcategories such as lymphocytes, macrophages, neutrophils, basophils, or monocytes. Further studies could use ex *vivo* labeling technique to identify the specific leukocytes subtypes.

Future studies could examine the effect of substances that we have shown to be anti-inflammatory in combination. For instance, using CB1R antagonist plus CB2R agonist in combination to see whether the anti-inflammatory effect could be amplified. Or use either of above substances in combination with URB597 to see the possible amplified beneficial effect.

In order to have supplementary data for our findings, we could examine CBR and non-CBR signaling pathways that may affect leukocyte migration by *in vitro* Neutrophil Chemotaxis/Migration Assay (Luo, 2001). We could use isolated purified rat neutrophils for migration assays. Neutrophil suspensions plus or minus test agents (CBR and non-CBR agonists/antagonists), will be tracked through the chemotaxis chamber (Neuro

Probe; MD, USA). We could quantify the number of cells that migrated through chamber and count them using a hemocytometer.

4.7 Clinical Implications

The finding of this study may be clinically relevant for sepsis patients. There has been no effective treatment for sepsis so far. The ECS has been evaluated in experimental and clinical studies; however, to date most of the therapeutic cannabinoid ligands been only used in experimental models due to their psychotropic side effects, despite their potential in reducing inflammation. The findings of this study, using LPS endoxemia, and additional work in a more clinically relevant model of sepsis (CASP) carried by our laboratory (Küster *et al*., 2012) indicate that modulation of the ECS using cannabinoid ligands and endocannabinoid enzyme inhibitors has the potential to attenuate systemic inflammation in these two sepsis models. Therefore, results from our experiments could be used for future therapeutic approaches designed to decrease leukocyte activation in blood vessels, increase capillary perfusion and thereby improve the intestinal microcirculation. However, additional research is still needed to further clarify the mechanisms of action and therapeutic effects of cannabinoid substances in endotoxemia (acute), CASP, CLP (chronic) and other experimental sepsis models of sepsis. These studies should examine different dosages and treatment regimens, methods of administration and the associated side effects. These experiments may allow us to better understand the effects of cannabinoid substances *in vivo* so that it may be possible to use them in clinic safely to attenuate inflammation and improve the sepsis patient's survival.

4.8 Conclusions

The present study investigated the effects of ECS modulation in experimental endotoxemia. Systemic inflammatory response was studied by evaluation of intestinal leukocyte recruitment and capillary perfusion. In support of the *in vivo* approach, analysis of cytokines and adhesion molecules allowed us to assess the LPS-induced inflammatory response. The patterns of pro- and anti-inflammatory cytokines and adhesion molecules, together with analysis of neutrophil adhesion and microcirculatory function, provided a means to obtain further insights into the pathophysiology of sepsis and to allow the identification of therapeutic windows for initiating selected therapies at different stages of severe sepsis. This study was one of the first studies that examined the effect of ECS modulation in the intestinal microcirculation in a rat model of endotoxemia. We have demonstrated the therapeutic potential of ECS modulation with CB1R inhibition, CB2R activation and inhibition of endocannabinoids degradation attenuated the endotoxin induces changes in microcirculation and reduced some of inflammatory mediators.

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