CANNABINOID 2 RECEPTOR MODULATION IN EXPERIMENTAL MODELS OF SEPSIS

by

Joel Sardinha

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This thesis is dedicated to my colleagues, friends, and family who have encouraged and supported me throughout this degree.

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ABSTRACT

Sepsis is a critical disease where a dysregulated immune response causes multi-organ dysfunction, leading to organ failure and eventual mortality. Early in the course of sepsis, the microcirculation of the intestine is impaired, leading to tissue hypoperfusion, ischemia and hypoxic cell death, particularly in the inner layer of the intestinal wall, i.e. the mucosa. Once this anatomical and immunological barrier is compromised, intraluminal pathogens can enter the bloodstream and further exacerbate the immune dysregulation. The aim of our research was to examine the impact of cannabinoid 2 receptor modulation on the intestinal microcirculation in different acute murine models of experimental sepsis.

In an endotoxemia model, activation of the CB_2 receptor either through direct agonist (HU308) or enzyme inhibition (URB597 or JZL184) was able to ablate the excessive leukocyte recruitment caused by LPS administration. In a clinically relevant model of sepsis (colon ascendens stent peritonitis – CASP), JZL184 administration was able to minimize the increase in leukocyte adhesion caused by the peritonitis, as well as to improve capillary perfusion of the intestinal mucosa. The use of JZL184 in CB_2 receptor knockout mice showed modest reduction in leukocyte recruitment caused by LPS administration indicating the activation of alternative pathways in CB_2 receptor knockout mice. Overall the effects of activating the CB_2 receptor during acute septic models shows some beneficial effects by minimizing the exaggerated inflammatory response.

LIST OF ABBREVIATIONS USED

Δ^9-THC	Δ^9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
AA	arachidonic acid
AC	adenylate cyclase
ACCP	american college of chest physicians
ACEA	arachidonyl-2'-chloroethylamide
ACPA	arachidonyl-cyclopropylamide
AEA	N-arachidonoylethanolamine
AMP	adenosine monophosphate
ANOVA	analysis of variance
APC	antigen presenting cell
BSA	bovine serum albumin
CACF	carleton animal care facility
cAMP	cyclic adenosine monophosphate
CASP	colon ascendens stent peritonitis
CB_1	cannabinoid receptor 1
CB_2	cannabinoid receptor 2
CBC	cannabichromene
CBD	cannabidiol
CBG	cannabigerol
CBN	cannabinol
CLP	cecal ligation and puncture

COX-2	cyclooxygenase-2
$cPLA_2$	cytosolic phospholipase A_2
DAGL	diacylglycerol lipase
DMSO	dimethyl sulfoxide
EAE	experimental autoimmune encephalomyelitis
ECS	endocannabinoid system
EGDT	early goal directed therapy
ERK	extracellular signal regulated kinases
FAAH	fatty acid amide hydrolase
FCD	functional capillary density
FDA	food and drug administration
FITC	fluorescein isothiocyanate
GPCRs	G protein coupled receptors
I.P.	intraperitoneal
I.V.	intravenous
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ
iNOS	inducible nitric oxide synthase
IVM	intravital microscopy
JAM	junctional adhesion molecules
JNK	c-Jun NH_2-terminal kinase
LFA-1	lymphocyte function associated antigen-1
LOX	lipooxygenase

LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MIP-2	macrophage inflammatory protein-2
NAPE-PLD	N-acyl phosphatidylethanolamine
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
OPS	orthogonal polarization spectral imaging
PAMP	pathogen associated molecular pattern
PECAM	platelet endothelial cell adhesion molecule
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PKA	protein kinase A
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
pO_2	partial pressure of oxygen
PPARγ	peroxisome proliferator-activated receptor γ
PSGL-1	P-selectin glycoprotein ligand-1
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
rhAPC	recombinant human activated protein C

RIG-1	retinoic acid-inducible gene-1
S.C.	subcutaneous
SCCM	society of critical care medicine
SDF	sidestream darkfield imaging
SIRS	systemic inflammatory response syndrome
SSC	surviving sepsis campaign
TLR	toll like receptor
TNF-α	tumor necrosis factor-α
TRAM	TRIF related adapter protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
TXB_2	thromboxane B_2
VCAM	vascular cellular adhesion molecule
VLA-4	very late antigen-4

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CHAPTER 1: INTRODUCTION

1.1 Definition and epidemiology of sepsis

The origin of the word *sepsis* comes from Greek roots which means "to decay" or "to putrefy." Defined in medical terms, sepsis is classified as the presence of pathogenic organisms or their toxins in the blood and tissues, along with a systemic inflammatory response syndrome (Dellinger et al., 2013). Severe sepsis and septic shock are the leading causes of mortality in surgical intensive care unit patients internationally (Vincent et al., 2009). Despite our increasing understanding of the mechanisms propagating sepsis, the incidence of this disease has been increasing over the years with an estimated annual rate of 18 million cases a decade ago (Slade et al., 2003), and current annual rates estimated at 20 to 30 million patients globally (Reinhart et al., 2013).

A variety of different factors seem to be responsible for the increasing rates of sepsis, ranging from the aging population, to the increasing rate of drug-resistant pathogens (Reinhart et al., 2013). The incidence of sepsis in the United States is around 750,000 cases annually, with a mortality rate of 30% (Angus et al., 2001), and around 30,500 annual cases in Canada, also with a 30% mortality rate (Husak et al., 2010). In general, although over the past three decades the mortality rate of patients with sepsis has declined, the concurrent increasing incidence of sepsis within the population has driven the rising number of deaths caused by sepsis (Martin et al., 2003). The criteria required for a medical diagnosis of sepsis is quite variable and clinical guidelines have been updated over the years (Table 1; (Dellinger et al., 2013)). According to a consensus from the American College of Chest Physicians (ACCP) and the Society of Critical Care

Medicine (SCCM), the term sepsis should be used when a known or suspected infection is coupled with at least 2 or more of the requirements for a systemic inflammatory response syndrome (SIRS). The possible criteria for SIRS includes:

a. Body temperature: ≥38ºC or ≤36ºC
b. Heart rate: ≥90 beats/min
c. Hyperventilation: ≥20 breaths per minute or <32 mmHg $PaCO_2$
d. White blood cell count: ≥12,000 cells/μL; or ≤ 4,000 cells/μL; or >10% immature forms.

Table 1: Criteria for the medical diagnosis of sepsis. Modified from (Dellinger et al., 2013).

Infection, documented or suspected, and some of the following:	
General variables	
	Fever (>38.3° C)
	Hypothermia (core temperature <36° C)
	Heart rate >90/min or more than two SD above the normal value for age
	Tachypnea
	Altered mental status
	Significant edema or positive fluid balance (>20 mL/kg over 24 h)
	Hyperglycemia (plasma glucose >140 mg/dL or 7.7 mmol/L) in the absence of diabetes
Inflammatory variables	
	Leukocytosis (white blood cell count >12,000/μL)
	Leukopenia (white blood cell count <4,000/μL)
	Normal white blood cell count with greater than 10% immature forms
	Plasma C-reactive protein more than two SD above the normal value
	Plasma procalcitonin more than two SD above the normal value
Hemodynamic variables	
	Arterial hypotension (systolic blood pressure <90 mmHg, mean arterial pressure <70 mmHg, or a systolic blood pressure decrease >40 mmHg in adults or less than two SD below normal for age)
Organ dysfunction variables	
	Arterial hypoxemia (PaO_2/FiO_2 <300)

	Acute oliguria (urine output <0.5 mL kg^{-1} h^{-1} for at least 2 h despite adequate fluid resuscitation)
	Creatinine increase >0.5 mg/dL or 44.2 μmol/L
	Coagulation abnormalities (INR >1.5 or aPTT >60 s)
	Ileus (absent bowel sounds)
	Thrombocytopenia (platelet count <100,000 μL^{-1})
	Hyperbilirubinemia (plasma total bilirubin >4 mg/dL or 70 μmol/L)
Tissue perfusion variables	
	Hyperlactatemia (>1 mmol/L)
	Decreased capillary refill or mottling

Severe sepsis was defined as sepsis associated with organ dysfunction, while the term septic shock was defined as severe sepsis with hypotension or hypoperfusion (Levy et al., 2003). As patients with sepsis progress to severe sepsis and septic shock, their mortality rates increase with severe sepsis patients showing a mortality of 20 – 50% and septic shock patients showing a mortality of 40 – 80% (Martin, 2012). As a result, the cost of patients with sepsis in the intensive care unit is quite high due to the number of complications, and emergency procedures associated with sepsis. On average it is estimated that each septic case costs around US$25,000 - $50,000 (Martin, 2012), and costs the US health care system around $16.7 billion annually (Angus et al., 2001).

The infections typical in sepsis are mainly bacterial, however there is also increasing incidences of viral and fungal sepsis (Martin et al., 2003). In the past it was believed that sepsis was mainly caused by gram negative bacteria, however more recent epidemiological studies have found that sepsis can be polymicrobial and result from both gram positive and gram negative bacteria (Martin et al., 2003). *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* are the most common bacterial pathogens associated with sepsis (Linnér, 2014). The source of infection in a patient is also an important factor in determining the proper course of

action. As a result of pathogens initiating the sepsis cascade, the infections mostly originate from organs exposed to the environment. Respiratory tract infections are the most common causes of sepsis with the lungs accounting for 68% of septic cases, followed by abdominal infections accounting for 22%, blood infections accounting for 20%, and urinary tract infections accounting for 14% (Martin, 2012; Vincent et al., 2006). However, positive blood cultures are found in only a third of all septic cases (Linnér, 2014). Regardless of the inductive cause of sepsis, it is the over-exaggerated host's immune response that causes the systemic damage and propagates the pathogenesis of sepsis.

1.2 The immune system

A basic understanding of the functions of the immune system is necessary in order to better comprehend the pathogenesis of the septic cascade. The normal human immune system is broadly divided into two categories, the innate and the adaptive immune systems. The innate immune system is the first line of defense the immune system possesses to defend against foreign pathogens. This system responds very rapidly from within minutes to hours of encountering a foreign pathogen (Linnér, 2014). Cells of the innate immune system include neutrophils, monocytes, macrophages, mast cells, granulocytes, and natural killer cells (NK).

The quick response of the immune system is due to the recognition of foreign proteins expressed on pathogens, broadly called pathogen-associated molecular patterns (PAMPs). The most common bacterial PAMPs are lipopolysaccharide (LPS), which is an endotoxin found on the surface of gram negative bacteria, and peptidoglycan which is a common component of the gram positive cell membrane (Linnér, 2014). Immune cells

possess pattern recognition receptors (PRR) on their cell surface, as well as within the cell, allowing them to detect the presence of foreign antigens and thereby activate the innate immune response. A wide variety of PRR families exist on immune and endothelial cells, such as toll like receptors (TLRs), C-type lectin receptors, nucleotide-binding oligomerization domain (NOD) like receptors and retinoic acid-inducible gene-1 (RIG-1) like receptors (Chong & Sriskandan, 2011; Linnér, 2014).

The toll like receptors are the most well studied PRRs with TLR2 and TLR4 being the most studied in sepsis due to their location on immune cells, and their ability to detect the presence of LPS and lipopeptides on gram negative and gram positive bacteria (Chong & Sriskandan, 2011). Toll like receptors are also able to detect innate proteins like alarmins, which are released during tissue damage, stress or trauma. Detection of alarmins is important in diseases like sepsis where endothelial damage is quite prevalent. Activation of these receptors causes downstream signalling that activate a variety of pro-inflammatory cytokines, chemokines, and signalling molecules.

The two most well characterised downstream signalling pathways for the TLRs are the MyD88 pathway and the TRIF/TRAM signalling pathways (Chong & Sriskandan, 2011). Activation of either of these pathways eventually result in gene expression of key inflammatory products through the activation of transcription factor NF-κB (Chong & Sriskandan, 2011; Linnér, 2014). A few important inflammatory products in sepsis that are released by the activation of NF-κB are TNF-α, IL-1β, and IL-6. These signalling molecules, along with a number of other cytokines and chemokines are released during the pro-inflammatory phase early during sepsis pathogenesis. This exaggerated release of cytokines, colloquially referred to as a ‘cytokine storm’ or ‘cytokine soup’ causes the

activation, recruitment and proliferation of leukocytes. Furthermore, due to the systemic presence of these pro-inflammatory cytokine, endothelial cells are also activated, which then release their own inflammatory mediators, further amplifying the exaggerated inflammatory response.

There are only a limited number of PRRs with the ability to detect a finite number of PPARs, therefore immune cells do not have the capability of detecting every single pathogen that exists through PRRs. As a result, the adaptive immune system exists to compensate for this limitation. Cells of the adaptive immune system include B cells, T cells and antigen presenting cells (APCs). The adaptive immune system takes a lot longer to mount an immune response when it first encounters a new pathogen, from days to weeks. However, subsequent encounters with the same pathogen elicits a much quicker and more robust response, due to the memory function of B cells in the adaptive immune system (Linnér, 2014). In order for the adaptive immune cells, specifically T cells, to recognise previously encountered pathogens, they have to be able to detect antigens specific to the particular pathogen. During the initial encounter with a new pathogen, APCs and B cells internalize and process microbial fragments and then present the pathogen's antigens on their surface using a specialized glycoprotein called major histocompatibility complex (MHC). T cell receptors are able to bind to the MHC and recognise the processed antigens, leading to subsequent T cell proliferation and antibody production. Two classes of MHC molecules exist, MHC class I molecules present antigens to $CD8^+$ T cells, while MHC class II molecules present antigens to $CD4^+$ T cells (Chong & Sriskandan, 2011). Despite the predominance of the innate immune system early during an immune challenge, followed by the upregulation of the adaptive immune

response in more chronic inflammatory conditions, these two systems are not mutually exclusive, working in concert with each other to adequately eliminate pathogens. All the experiments conducted for this thesis have simulated an acute experimental model of sepsis and therefore only explored the early pro-inflammatory phase. As a result, all of the experiments have been limited to focus on changes occurring primarily on components of the innate immune system during sepsis.

1.3 Sepsis pathophysiology

Sepsis is a complex immune disorder characterised by a malfunctioning host immune response to an infection. During an infection, the normal host response to combat the pathogen is elicited, however due to the systemic presence of the infection in sepsis, the inflammatory response is hyper-activated. This deregulated hyper-activation of the immune system leads to excessive collateral damage of the endothelial cells lining the blood vessels, and impairments in the circulatory function of blood vessels (N. Matsuda & Hattori, 2007). The endothelial cells are activated by the damage caused by the dysregulated immune system, as well as by the pro-inflammatory mediators released systemically due to the exaggerated immune response. These activated endothelial cells then initiate the release of a wide variety of inflammatory mediators that further exacerbate the pro-inflammatory cascade. Multiple other pathways are also initiated due to the endothelial activation elicited early during sepsis pathogenesis. The complement cascade, the coagulatory cascade, the fibrinolytic cascade, and the nitric oxide pathway are a notable few. Vascular permeability and vessel dilation caused by endothelial damage and nitric oxide release leads to hypotension in septic patients. Coupled with

activated pro-thrombotic and coagulatory cascades, septic patients have impaired microcirculatory function, eventually leading to tissue ischemia, and organ dysfunction.

In a normally functioning immune response to infections, the pro-inflammatory cascade initiated to eradicate the pathogens, is also coupled with an anti-inflammatory response to minimize excessive damage and resolve the immune response upon clearance of the pathogen. This balance between the pro and anti-inflammatory cascades malfunctions during sepsis pathogenesis leading to exaggerated responses from both systems at different time courses in the progression of sepsis pathogenesis (Hotchkiss et al., 2013). The compensatory anti-inflammatory response causes the release of anti-inflammatory mediators like IL-1R antagonists, IL-10, and IL-4 (Cohen, 2002; Hotchkiss et al., 2013; Linnér, 2014). However, due to the systemic presence of the infecting pathogen, the exaggerated pro-inflammatory response outweighs the compensatory anti-inflammatory response during the early stages of sepsis. In more chronic stages of sepsis, once the pathogen has been successfully cleared, the pro-inflammatory cascade gets resolved. However, due to the persistent and exaggerated nature of the pro-inflammatory cascade, there is a delayed compensatory anti-inflammatory cascade that is also exaggerated, leading to immunosuppressed states of patients in more chronic stages of sepsis (Figure 1). During this immunosuppressive phase, patients are more vulnerable to develop secondary infections that can cause further complications and increase mortality.

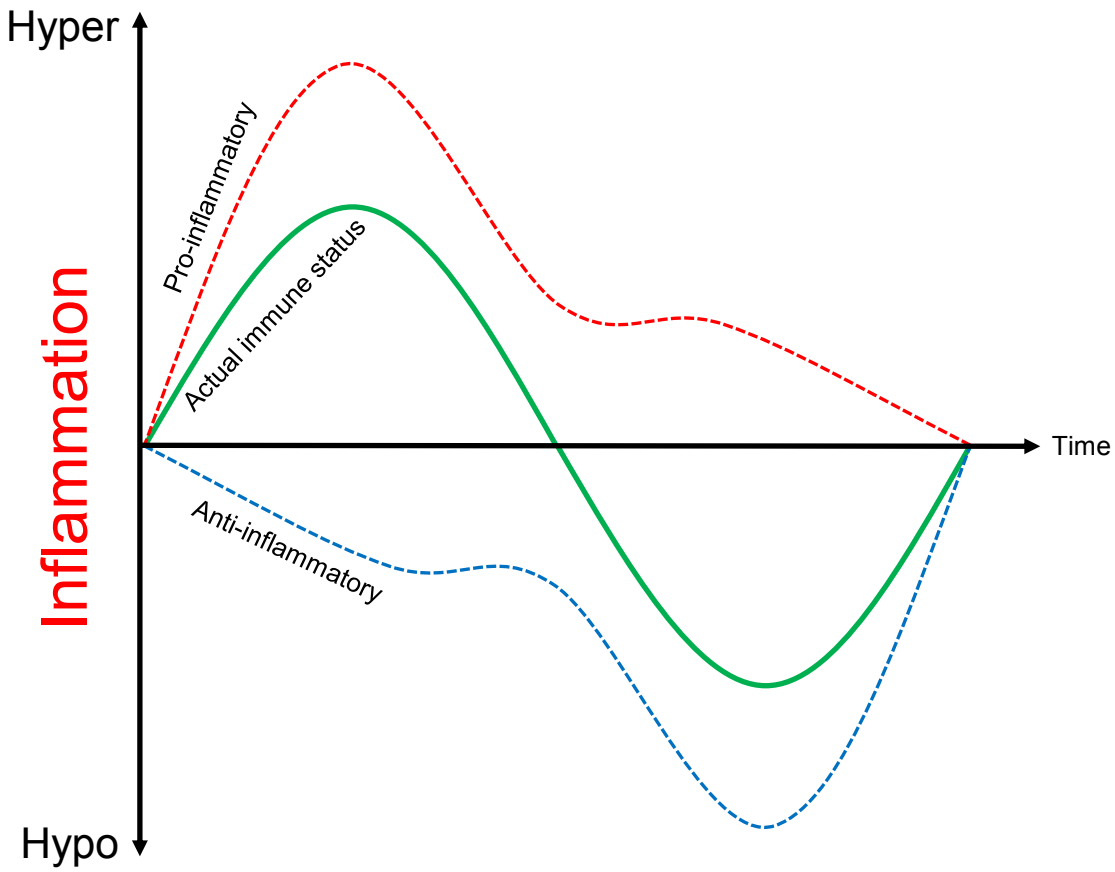

Figure 1: Variable immune statuses of patients with sepsis depending on the progression of their disease. Sepsis is mainly characterised by an early hyper-inflammatory phase, followed by a hypo-inflammatory phase in more chronic stages of sepsis.

1.4 Leukocyte recruitment

Leukocytes encompass all the white blood cells which can be broadly classified as mononuclear cells, polymorphonuclear cells, or granulocytes (Linnér, 2014). A major portion of leukocytes normally circulating in the blood are neutrophils accounting for 40 – 70% of all leukocytes in the blood (Linnér, 2014). Other leukocytes include monocytes, macrophages, lymphocytes, eosinophils, basophils, and dendritic cells. Various signalling mediators in addition to the cytokines and chemokines released during the acute pro-inflammatory phase of sepsis work to mobilize leukocytes to the site of infection. Due to the systemic increase in the levels of these signalling molecules during sepsis, there is an excessive level of leukocyte recruitment and leukocyte adhesion on the endothelial cells. This excessive level of leukocyte adhesion and transmigration causes further activation and damage to the endothelial cells, propagating the inflammatory cascade seen early during sepsis.

Recruitment of leukocytes to the site of infection involves a complex process of tethering, rolling, adhesion, and transmigration (Granger & Senchenkova, 2010; Petri et al., 2008) (Figure 2). A variety of other factors also play a significant role in the transmigration of leukocytes from the blood vessels. The release of adhesion influencing chemical mediators, selective expression of adhesion molecules by endothelial cells and leukocytes, as well as the role of hemodynamic force on leukocyte endothelial interactions will be a few areas covered.

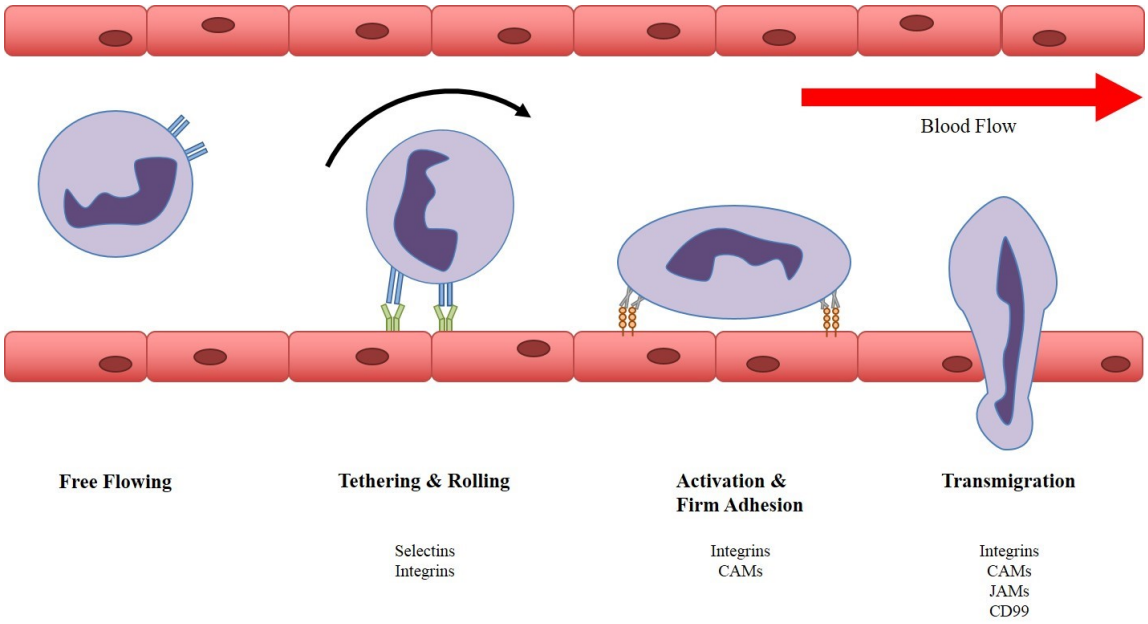

Figure 2: Leukocyte recruitment from the blood to the site of inflammation. The leukocyte transmigration cascade involves a series of sequential steps such as tethering and rolling, activation, firm adhesion, crawling, and transmigration.

1.4.1 Tethering and rolling

In order for free flowing leukocytes in the blood to get to the site of infection, they have to initially interact with the local endothelial wall. This process is accomplished by the expression of molecules known as selectins by both leukocytes and endothelial cells that serve to significantly reduce the velocity of the flowing leukocytes so that they can interact with other molecules for a firm adhesion. Selectins are a family of type I transmembrane, calcium-dependent glycoproteins that initiate the tethering process of leukocytes to the endothelial cells (Petri et al., 2008). Three types of selectins are important in leukocyte tethering, the L-selectins and P-selectin glycoprotein ligand-1 (PSGL-1) which are expressed on leukocytes, E-selectins which are expressed on endothelial cells, and P-selectins which are expressed on platelet cells as well as on endothelial cells (Granger & Senchenkova, 2010).

The constitutive expression of selectins varies depending on the location, the organ, and even vascular cell type. For example, the constitutive expression of P-selectin is much higher in the microvessels of the intestine compared to the vessels of the skin, even though they are both organs exposed to the external environment (Granger & Senchenkova, 2010). Due to the nature of their constitutive expression, P-selectins are stored in secretory granules and rapidly fuse with the cell membrance to express these proteins once the cell is activated. In platelets, these granules are called α-granules, while in the endotheial cells they are called Weibel-Palade bodies (Granger & Senchenkova, 2010; Petri et al., 2008). E-selectin on the other hand is not constitutively expressed or stored, but requires transcriptional activation to be expressed. Transcriptional activation of both P and E-selectins occur in sepsis when elevated levels of cytokines like TNF-α,

IL-1, IL-4, IL-13 are present (Granger & Senchenkova, 2010; Petri et al., 2008). L-selectin is also constitutively expressed on leukocytes, however their role in leukocyte tethering has yet to be fully elucidated. Some studies indicate they interact with P and E-selectins on endothelial cells, while others demonstrate that L-selectin has a secondary tethering function (Petri et al., 2008). Overall, the role of PSGL-1 seems to be more important for leukocyte tethering to the endotheial wall by interacting with E and P-selectins expressed by endothelial cells (Granger & Senchenkova, 2010).

Sheer forces caused by the flow of blood also play a big role in leukocyte rolling and adhesion. The hemodynamic pressure of blood flow is different in various blood vessels thereby altering the ability of leukocytes to adhere to the vessels. In arterioles, the blood pressure is generally much higher than the pressure seen in venules, therefore the sheer force in comparable sized vessels would be much higher in the arteriole (Granger & Senchenkova, 2010). Higher sheer forces overcome the bonds caused by leukocyte endothelial tethering through selectins, and therefore leukocyte rolling is rarely seen in arterioles. However, sheer force is not the only factor responsible for the lack of leukocyte rolling in certain vasculature. The expression of selectins and other adhesion molecules are more prevalant on the the endotheial cells in venules, therefore the combination of a lower sheer force and higher expression of adhesion molecules cause more rolling and transmigration in certain vasculature like post-capillary venules compared to arterioles and capillaries (Granger & Senchenkova, 2010). This phenomenon is quite evident during inflammatory conditions like sepsis when the expression of adhesion molecules is upregulated and blood sheer forces in the microvasculature are reduced due to vascular hypotension.

1.4.2 Leukocyte firm adhesion and transmigration

The transition of leukocyte tethering and rolling along endothelial cells to a firm adhesion and migration involves another set of adhesion molecules expressed on leukocytes called integrins. Integrins are glycoprotein heterodimer complexes consisting of a combination of an α-subunit and a β-subunit. Various leukocytes employ different combinations of these subunits to adhere to the endothelium. Lymphocytes and monocytes employ the β_1 integrins, which is composed of the common β_1 subunit and one of the four different α subunits. The most common β_1 integrin is the very late antigen 4 (VLA-4) which is composed of an α_4 subunit and a β_1 subunit (CD11/CD18) (Granger & Senchenkova, 2010). Neutrophils on the other hand recruit the β_2 family of integrins, the most common of which is lymphocyte function associated antigen (LFA-1) (Petri et al., 2008). LFA-1 is composed of a distinct α_L subunit and a β_2 subunit (CD11a/CD18).

Integrins expressed on leukocytes bind to a family of Immunoglobulin-like adhesion molecules expressed on endothelial cells (Granger & Senchenkova, 2010; Petri et al., 2008). The most common of these adhesion molecules are ICAM-1, ICAM-2, VCAM-1, and PECAM-1. ICAM-1 and ICAM-2 are constitutively expressed on endothelial cells of most vessels, but are found highly expressed in organs exposed to the environment like the lungs and intestine (Granger & Senchenkova, 2010). Furthermore, ICAM-1 and VCAM-1 cellular expression and transcriptional upregulation can be activated in response to cytokines or endotoxin challenge, while ICAM-2 and PECAM-1 expression is constitutive and not affected by signalling mediators (Granger & Senchenkova, 2010). During inflammatory conditions, rather than recycling the membrane bound ICAM-1, the protein is cleaved and released into the blood stream as

soluble ICAM-1 (sICAM-1) before being replaced. This phenomenon is conveniently used as a measure of endothelial cell activation and severity of inflammation in experiential models (Granger & Senchenkova, 2010).

The binding of integrins on the leukocytes to their specific immunoglobulin adhesion molecules on the endothelial cells promotes firm adhesion and eventual emigration of the cell across the vessel into the extraluminal tissue. The integrin VLA-4 on lymphocytes and monocytes binds to VCAM-1, while the LFA-1 integrin on neutrophils binds to ICAM-1 (Petri et al., 2008). Upon binding to the endothelium, the leukocytes try to migrate across the barrier either through a paracellular pathway or a transcellular pathway (Petri & Bixel, 2006). The less often used transcellular pathway involves migrating through the endothelial cells from the apical side, into the cell and out the basal side into the extravascular tissue. The more common approach of most leukocyte transmigrating from the vessels involves a paracellular route that entails squeezing through the intercellular junctions between two endothelial cells (Petri & Bixel, 2006). Once bound to endothelial cells, the leukocytes crawl to the intercellular junction between two endothelial cells and emigrate through the space with the help of a wide variety of junctional molecules.

PECAM-1 is a molecule highly expressed at the intracellular junctions and plays a role in leukocyte paracellular migration. PECAM-1 assists in leukocyte migration either through cell signalling, or through homophillic interactions with the leukocyte to guide it into the paracellular space (Muller, 2010; Petri & Bixel, 2006). The junctional adhesion molecules (JAMs) are another family of immunoglobulin molecules that aid in leukocyte paracellular transmigration. JAM-A is one of the three members of this family of

molecules and functions by directly binding LFA-1 expressed on neutrophils. It has been implicated that LFA-1 may use JAM-A as a binding substrate to activate migration (Petri & Bixel, 2006). Another molecule that plays an important role in leukocyte transmigration is CD99, which is a highly O-glycosylated transmembrane protein expressed on erythrocytes, most leukocytes and endothelial cells (Petri & Bixel, 2006). The mechanism through which CD99 promotes leukocyte transmigration is not completely understood. There are speculations that homophilic interactions between the CD99 on leukocytes and endothelial cells mediate paracellular migration. Alternatively, it has also been proposed that CD99 binding might trigger intracellular signals that modulate the opening of endothelial cell junctions (Muller, 2010; Petri & Bixel, 2006).

1.5 Microcirculation

The circulatory system in our bodies function to deliver oxygen and nutrients, as well as remove waste products from tissues in organs all over our body. The microcirculation is the most important component of the circulatory system as this is where the transfer of oxygen, nutrients, and wastes occur between the blood and tissues. The microcirculation in humans is composed of the smallest blood vessels (<100 μm diameter), comprising of arterioles, capillaries, and venules (Ince, 2005). The most important cell types of the microcirculation are the endothelial cells, smooth muscle cells, red blood cells, leukocytes, and other blood components. The luminal wall of all the micro-vessels in the microcirculation are lined with endothelial cells which are important regulators of nutrient transfer, cell signaling, vascular tone and a variety of other important functions (Kanoore Edul et al., 2011).

The regulation of perfusion in these microcirculatory vessels is a highly dynamic process based on physical factors like the stress and strain of the blood pressure in the vessels, chemical factors like the levels of oxygen, hormones, or lactate levels in the blood, as well as based on direct neuromodulatory control (Ince, 2005). Apart from tissue perfusion, the microcirculation also plays a key role in the function of other systems like the immune system, and thermoregulation (Kanoore Edul et al., 2011). The disruption of this highly important system is clearly evident in critical illnesses like sepsis, where subsequent events resulting from a malfunctioning microcirculation lead to multiple organ failure in patients. Upon understanding the importance of this system, there has been a significant focus in both the clinical and experimental settings to identify, understand, and treat the underlying causes propagating these malfunctions.

The disruption of microcirculatory function is a key event early in the pathogenesis of sepsis characterized by a variety of factors but mainly attributed to endothelial dysfunction as a result of leukocyte adherence, rolling and migration (Kanoore Edul et al., 2011). These changes manifest into heterogeneous blood flow to the capillaries, where some capillaries are inadequately perfused, some are normally perfused, and some even have a higher than normal rate of perfusion (Ince, 2005). In diseases like sepsis, the hypoxic nature of tissues caused by a heterogeneous perfusion of capillaries, leads to redistribution of blood flow to maintain adequate perfusion in more vital organs. This process is termed functional shunting, where blood destined for the microvascular tissues is shunted from arterioles to venules resulting in further hypoxic conditions in the microcirculation (Ince, 2005; Kanoore Edul et al., 2011). Due to the hypoxic and ischemic conditions in the microvasculature, other pathways are also up-

regulated and malfunction. The nitric oxide pathway is one key pathway in maintaining the proper homeostasis of the microcirculation, and is severely altered during sepsis. The expression of inducible nitric oxide synthase (iNOS) as well as altered nitric oxide (NO) release from endothelial cells and red blood cells, further exacerbates the heterogeneous shunting of blood in the microcirculation (Ince, 2005).

Another mechanism severely altered in sepsis is the deformability of the red blood cells which are important in proper oxygen delivery. In septic conditions, the red blood cells lose their ability to properly deform in order to travel through the capillaries and therefore aggregate and further block the microvasculature. The lack of proper microvascular perfusion and reduced red blood cell transport in capillaries causes tissue hypoxia and ischemia, propagating the shunting of blood in the circulation. The dramatic reduction in partial pressure of oxygen (pO_2) in the microcirculation compared to the venous partial pressure of oxygen, as a result of functional blood shunting, has been termed the pO_2 gap (Ince, 2005; Kanoore Edul et al., 2011). The pO_2 gap has been used as a quantitative measure of the severity in functional shunting, and in sepsis this phenomenon is more damaging than a hemorrhage (Ince, 2005).

Overall the distributive shunting, coupled with increased microvascular permeability, vessel dilation due to nitric oxide, impaired red blood cell deformability, immune malfunction, and coagulatory disruption, all lead to an exaggerated microvasculatory dysfunction in sepsis. If left untreated, these malfunctioning pathways will continue to worsen the microvascular dysfunction and eventually lead to organ failure (Ince, 2005). Due to the functional shunting of blood, measurement of systemic vital parameters might appear to be normal and not be indicative of the microcirculatory

dysfunction occurring in septic patients. Therefore monitoring microcirculatory function is critical in the early stages of sepsis pathogenesis, where rapid detection and therapeutic intervention can lead to the best patient outcomes.

Monitoring the microcirculatory parameters in patients is quite challenging due to the limited supply of easily accessible microcirculatory beds, as well as a lack of effective methods to observe and quantify microcirculatory function. Significant advances in technology over the years have provided us with better tools to assess and observe microcirculatory parameters in patients. Earlier techniques involved measuring CO_2 levels from different microvascular beds like sublingual and subcutaneous (Ince, 2005). Additionally, hemoglobin saturation was also measured in these vessels through the use of near infrared spectroscopy. Following these techniques came direct observation of the microcirculation through different microcopy techniques.

Orthogonal polarization spectral (OPS) imaging developed by Groner et al., allowed for noninvasive observation of microcirculation through accessible mucous membranes and even on the surface of solid organs (Groner et al., 1999). OPS imaging involves illuminating the tissue of interest with linearly polarized light and recording remitted light that has been orthogonally polarized through a polarizing filter. Only depolarized photons scattered in deep tissue contribute to the image (Groner et al., 1999). Further enhancement to this observational technique came through the creation of side stream dark-field (SDF) imaging (Ince, 2005). This technology allowed for the visualization of blood cells flowing through capillaries through the use of green light (530nm), which is absorbed by hemoglobin. This technique avoids the scattered light inference that was the limiting factor in OPS imaging. In the clinical setting,

measurement of sublingual microcirculation in septic patients showed a correlation with outcome. Septic patients with altered microcirculation had a worse prognosis in terms of outcome compared to other. Furthermore, septic patients that survived had a higher proportion of perfused capillaries than non-surviving septic patients (De Backer et al., 2013).

1.5.1 Intestinal microcirculation

The intestine is an important organ in the pathogenesis of sepsis because it has an extensive and intricate microcirculatory network that is frequently disrupted during sepsis. The luminal gut contents are composed of a plethora of different micro biota including pathogens that can infect the host. As a result, maintenance of the integrity of the intestinal barrier is critical in septic patients as bacteria and other pathogens can translocate into the abdominal cavity and cause secondary infections in patients. As mentioned earlier, the immune status of septic patients is quite heterogeneous with phases of hyper-inflammation, as well as immunosuppression in more chronic stages. Therefore the translocation of pathogens from the disrupted intestinal barrier could further exacerbate the pro-inflammatory state of certain septic patients, as well as endanger immunosuppressed patients due to their vulnerability of acquiring secondary infections.

The concept of a gut derived state of sepsis was first proposed in the 1980s based on the notion that the disruption of the intestinal barrier can cause translocation of pathogens into the abdomen and spread to the rest of the body through mesenteric lymph nodes (Meakins & Marshall, 1986). This concept has been studied over the years and been altered due to a variety of findings. It has been shown that systemic bacterial presence (bacteremia) thorough gut translocation and distal organ injury can be

independent factors (Deitch, 2012). The concept has now evolved to encompass the possible lack of bacterial presence in systemic organ failure. The amended concept states that bacterial and inflammatory invoking mediators translocate through gut barrier disruption caused by intestinal microcirculatory ischemia and invoke an inflammatory response in the host that eventually causes multiple organ failure (Deitch, 2012). Regardless of the presence of the pathogen in systemic circulation, preserving the functionality of the gut microcirculation can prevent translocation of additional inflammatory triggers that contribute to the pathogenesis of sepsis. Therefore assessing the microcirculatory function of the intestine in experimental models of sepsis can be beneficial in improving understanding, as well as developing effective therapies to prevent the progression of sepsis in patients.

1.6 Current therapies for sepsis

Septic shock and severe sepsis are critical conditions that require immediate medical interventions to help stabilize the patient. It is generally accepted that immediate early interventions during these critical stages can significantly influence the outcome of the patient. Unfortunately, due to the diverse and unspecific symptoms of sepsis, proper and timely diagnosis of septic patients is usually delayed. To date there is still no cure for sepsis, however decades of research and multiple trials have tremendously improved our understanding of the disease and helped advance the care administered to septic patients. In 2004 an international consortium of medical experts, researchers, societies and groups involved in sepsis, came together to form the Surviving Sepsis Campaign (SSC) and establish medical guidelines for the proper management of severe sepsis and septic shock in the clinical setting. Since then, the guidelines have been updated twice with the first

occurring in 2008 and the most recent update in 2013 (Dellinger et al., 2013). The fundamental recommendations of the SSC guidelines include immediate interventions to treat the hypotension due to septic shock through the administration of fluid resuscitation and vasopressors, blood culturing and broad spectrum antibiotics after infection confirmation, and other recommendations for measuring parameters like mean arterial pressure, blood glucose levels, and lactate levels (Dellinger et al., 2013). Studies implementing the SSC care guidelines have shown that there are improved outcomes for patients (Levy et al., 2010; Linnér, 2014).

One of the main recommendations of the SSC treatment bundle guidelines involves timely interventions to detected problems. Early goal directed therapy (EGDT) is a regimen used in critical care to rapidly respond to changes in systemic parameters. Due to alterations in microcirculation and hypoxic conditions in tissues, the balance between oxygen demand and oxygen delivery is affected in sepsis, eventually leading to septic shock. EGDT aims to restore the balance between adequate oxygen demand and delivery. This method involves careful monitoring and aggressive response to alterations in various hemodynamic parameters like central venous pressure, mean arterial pressure and central venous oxygen saturation. Changes to central venous pressure were treated with fluid resuscitation, alterations in mean arterial pressure were controlled with administration of vasopressors and vasodilators, and inadequate central venous oxygen saturation was remedied with red blood cell transfusions and inotropic agents (Rivers et al., 2001). Overall, EGDT aims to maintain cardiac preload (volume), afterload (blood pressure), and contractility (stroke volume) with the intent of preserving adequate oxygen delivery. This aggressive regiment administered early to patients with severe sepsis and

septic shock, showed significant benefits in terms of survival and outcomes (Rivers et al., 2001).

As mentioned earlier, septic pathogenesis involves a complex dysregulation of multiple systems like the immune system, coagulation system, thrombotic cascade and complement system to name a few. As a result, aggressive fluid resuscitation and antibiotics to combat the hypotension and infections in patients still are not enough to prevent progression of the septic cascade. Therapies that target one or more of these malfunctioning systems early are also needed to reduce the progression of sepsis to stages of severe sepsis and septic shock.

Recombinant activated protein C (rhAPC) was one such molecule that showed significant promise, due to its combination of anti-inflammatory, anti-thrombotic, profibrinolytic and anti-coagulant properties. Protein C is an intrinsic inhibitor of coagulation in our bodies, however in septic patients protein C levels are depleted, leading to systemic unregulated intravascular coagulation (Sarangi et al., 2010). In 2001, a formulation of recombinant human activated protein C (Xigris®) from Eli Lilly was approved by the FDA after showing benefit in a phase III clinical trial. Activated protein C was then adopted into the 2008 SSC treatment guidelines for patients at high risk for mortality. However, due to APC's potent anti-thrombotic and pro-fibrinolytic effects, patients are also at a heightened risk for severe bleeding. On 25th October 2011, the European Medicines Agency issued a worldwide withdrawal of Xigris® for failing to replicate the initially indicated beneficial results. A Cochrane review of various clinical trials also showed that there was no significant benefit of APC to outweigh the severe risk of bleeding in patients (Martí-Carvajal et al., 2012). The latest update to the SSC

guidelines have also been amended to reflect the withdrawal of this compound (Dellinger et al., 2013).

Another therapeutic approach in the treatment of sepsis is to target the excessive dysregulation of the inflammatory mediators released throughout the disease. Studies looking at blocking pro-inflammatory cytokines like TNFα and IL-1 early in the stages of sepsis have been successful in animal studies. However a multitude of clinical trials conducted over the years focusing on various chemokines and cytokines have not shown much promise (Arndt & Abraham, 2001). Due to the variability in the immune statuses of septic patients in the hospital, simply blocking important pro-inflammatory mediators like TNFα, IL-1β, IL-6, and IL-8 can also be detrimental. During states of immunosuppression, further blockade of these leukocyte chemo-attractant cytokines and chemokines, can leave patients vulnerable to develop secondary infections. In animal models, the induced septic state is highly controlled and reproducible, therefore specific targeting of these immune molecules can show survival benefit compared to septic patients in clinical settings.

Other experimental methods of modulating the immune system during sepsis include immunoglobulin therapy. The hypothesis behind this therapy is that administration of immunoglobulins into septic patients can neutralize the endotoxins found on bacteria and other pathogens. This therapeutic approach involves pooling immunoglobulins from many donors to ensure high polyspecificity for multiple pathogens and administering it to septic patients. Apart from endotoxin neutralization, immunoglobulin therapy was also postulated to stimulate leukocyte recruitment, as well as increase innate bactericidal activity (Alejandria et al., 2013). However, multiple

clinical trials in septic patients have shown conflicting evidence over the years, and this therapy has not shown any substantial evidence in improving survival rates of septic patients (Alejandria et al., 2013).

1.7 The endocannabinoid system

The endocannabinoid system (ECS) is a complex system in our body that derives its name from the *Cannabis sativa* plant. The cannabis plant has been used throughout history for a variety of purposes from raw materials to medicine (Adams & Martin, 1996). Early Roman, Greek, Chinese, and American societies cultivated Cannabis plants for their useful hemp fibers. These tough fibers were used in making ropes, clothes, textiles and other goods (Adams & Martin, 1996). The medical properties of the cannabis plant were also known throughout history, and some believe it to be one of the oldest drugs in human history (Adams & Martin, 1996; Mechoulam & Feigenbaum, 1987). Ancient Chinese, Egyptian, Indian, and Middle Eastern cultures used the Cannabis plant in herbal medications to treat a wide variety of ailments like constipation, malaria, fever, pain, and sleeping disorders (Adams & Martin, 1996; Mechoulam & Feigenbaum, 1987). Prior to the development of synthetic medications, western medicine also adopted the use of cannabis for its anticonvulsant, antiemetic, antianxiety, and analgesic properties (Adams & Martin, 1996; Mechoulam & Feigenbaum, 1987).

The endocannabinoid system can be simplified into 3 elements that interact with each other and function to maintain this signalling system. The first elements are the signalling molecules, or the bioactive ligands (Luchicchi & Pistis, 2012). The second elements are the endocannabinoid receptors that recognise the signalling molecules and

transduce the downstream effects (Howlett, 2005). Finally, the third elements are the enzymes and the transporters that facilitate the production and rapid removal of the signalling molecules to avoid excessive activity of the endocannabinoid receptors (Ahn et al., 2008). The signalling molecules that can affect the endocannabinoid system can be further categorized into 3 different types: phytocannabinoids, endocannabinoids, and synthetic cannabinoids. The phytocannabinoids are chemicals that are naturally produced by the cannabis plant, the endocannabinoids are normally produced by enzymes within our bodies, and the synthetic cannabinoids are artificially manufactured compounds. All of these compounds have the ability to either activate or block the function of cannabinoid receptors within our bodies. The two most well characterized receptors of the endocannabinoid system are the cannabinoid receptor 1 (CB_1) and the cannabinoid receptor 2 (CB_2) (Howlett et al., 2002). The most well-known enzymes responsible for the degradation of the endocannabinoids are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Ahn et al., 2008).

1.7.1 Cannabinoid ligands

The therapeutic properties of the cannabis plant stem from the over 421 chemical compounds it contains, of which over 80 are active phytocannabinoids (Angelo Izzo et al., 2009; Pertwee, 2006). These chemical compounds are able to bind and modulate the activity of different receptors expressed in our body, eliciting their beneficial effects. The most abundant of these phytocannabinoids is Δ^9-tetrahydrocannabinol (THC)(Gaoni & Mechoulam, 1964), which is responsible for eliciting the psychotropic effects associate with smoking cannabis. Due to its psychoactive properties, the use of Δ^9-THC has been limited in the medical field. Other active phytocannabinoids with minimal psychoactive

properties include cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), cannabichromene (CBC), etc. (Angelo Izzo et al., 2009). Cannabidiol has recently received increasing attention due to its therapeutic properties without the unwanted psychoactive properties associates with cannabis. Cannabidiol elicits a range of effects by binding to a wide variety of receptors in addition to the cannabinoid receptors. Some of the beneficial effects of CBD include anti-inflammation, anti-oxidative, anti-tumorous, anti-anxiety, anti-emetic, and anti-nausea to name a few (Mechoulam et al., 2007).

Apart from the plant-derived phytocannabinoids, our bodies synthesize their own natural ligands to the cannabinoid receptors from arachidonic acid (AA) in the cell membranes. These bioactive lipid compounds called endocannabinoids are tightly regulated in their synthesis and degradation by various enzymes in the endocannabinoid system (Luchicchi & Pistis, 2012). The two most well studied endocannabinoids are arachidonylethanolamide (AEA; also known previously as anandamide) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995). Both these endocannabinoids have similar structure and function, however they are quite different in their synthesis, receptor affinities, and degradation enzymes (Luchicchi & Pistis, 2012). Anandamide is synthesized by a specific enzyme called N-acyl phosphatidylethanolamine phospholipase-D (NAPE-PLD), while 2-AG is synthesized through the cooperation of phospholipase C (PLC) and diacylglycerol-lipase (DAGL) (Luchicchi & Pistis, 2012). Anandamide and 2-AG exhibited higher affinities for the cannabinoid CB_1 receptor than for the CB_2 receptor (Reggio, 2002), however 2-AG was found to be much more abundant, as well as 3 times more potent than anandamide at the CB_2 receptor (Gonsiorek et al., 2000; Reggio, 2002).

Administration of chemically manufactured synthetic ligands are yet another method of directly activating the endocannabinoid system through their receptors. Since the discovery of the cannabinoid receptors, over the last 25 years, different compounds have been manufactured that possess varying degrees of affinity and efficacy toward the cannabinoid receptors. Some compounds are able to bind both cannabinoid receptors and have agonistic properties. Examples of these compounds are (–) 11-hydroxy- Δ^8-THC-dimethylheptyl (HU-210), CP55940, and R-(+)-WIN55212 (Pertwee, 2006). Specific agonists for either the CB_1 or CB_2 receptors have also been developed over the years, with newer compounds showing increasing specificity for their respective receptor. Some common CB_1 receptor specific agonists are R-(+)-methanandamide (AM-356), arachidonyl-2'-chloroethylamide (ACEA), arachidonyl-cyclopropylamide (ACPA), and O-1812 (Pertwee, 2006). Common CB_2 receptor selective agonists are HU-308, AM1241, GP1a, JWH-133, L-759633, and L-759656 (Pertwee, 2006). Development of antagonists were also important for the selective blockade of the effects of the cannabinoid receptors. CB_1 receptor selective antagonists or inverse agonists include AM251, AM281, LY320135, and SR141716A (Pertwee, 2006). Antagonists selective for the CB_2 receptor include SR144528 and AM630 (Pertwee, 2006).

1.7.2 Cannabinoid receptors

The cannabinoid receptors belong to the large Class A Rhodopsin-like family of G-protein coupled receptors (GPCRs), which have seven transmembrane domains (Howlett et al., 2002). Currently, two cannabinoid receptors have been classified: the CB_1 and CB_2 receptors. The differences between the two receptors are based on their predicted amino acid sequence, their downstream signaling mechanisms, and their tissue

distribution within the body (Howlett et al., 2002). The CB_1 receptor was the first cannabinoid receptor to be cloned from rat brain cDNA (L. Matsuda et al., 1990). The CB_1 receptor is found most abundantly in the central nervous system but also can be found at lower levels in peripheral tissues. CB_1 receptor expression has been found in the heart, testis, bone marrow, adrenal glands, lungs, tonsils, prostate, uterus, thymus, and ovaries (Galiègue et al., 1995). Due to the higher presence of CB_1 receptors in the CNS, the psychotropic effects associated with cannabis use have been linked to the activity of this receptor (Chevaleyre et al., 2006).

The activation of CB_1 receptors can cause a variety of downstream signalling pathways which occur mainly through the $G_{i/o}$ subunit of G proteins (Howlett et al., 2002). CB_1 receptors are mainly found on presynaptic axon terminals, therefore activating them causes an inhibition of neurotransmitter release from the presynaptic neuron (Chevaleyre et al., 2006). This effect is mainly achieved through the hyperpolarization of the presynaptic membrane (Ladak et al., 2011). Modulation of intracellular cyclic AMP concentrations through the activation of CB_1 receptors results in net phosphorylation of ion channels causing changes in ion currents and general neuronal excitability. Overall, CB_1 receptor activation causes an activation of inwardly rectifying K^+ channels and an inhibition of voltage gated Ca^{2+} channels (Howlett et al., 2002; Pertwee et al., 2010), leading to decreased GABAergic and glutamatergic signalling (Ladak et al., 2011).

Alterations in the activity of transcription factors has also been shown through the activation of CB_1 receptors (Howlett, 2005; Howlett et al., 2002; Ladak et al., 2011). The activation of CB_1 receptors has been shown to be coupled to MAP kinases through the

$G_{i/o}\beta\gamma$ dimer subunit (Bouaboula et al., 1995; Howlett, 2005). Activation of MAP kinases results in sequential phosphorylation of various kinases such as ERK1/2, and p38 MAPK (Howlett, 2005; Ladak et al., 2011). The downstream effects of these kinases causes changes in the activity of various transcription factors like c-Jun, c-Myc, and c-fos that eventually regulate the expression of various genes like Krox-24 (Bouaboula et al., 1995; Howlett, 2005; Ladak et al., 2011). Alterations in gene expression patterns caused by CB_1 receptor activation causes changes in cellular functions like cell differentiation, cell growth, synaptic plasticity, and cell survival (Howlett, 2005).

The CB_2 receptor was the second cannabinoid receptor cloned from macrophages in the marginal zone of the spleen (Munro et al., 1993). Unlike the CB_1 receptor, the CB_2 receptor was found mostly outside the central nervous system and expressed primarily on immune cells. The differential expression levels of CB_2 receptors on immune cells is highest in B cells, followed by NK cells, monocytes, neutrophils, $CD8^+$ T cells, and $CD4^+$ T cells (Galiègue et al., 1995). Lower levels of CB2 receptors are also expressed in the tonsils, pancreas, bone marrow, thymus, adult rat retina, and mouse vas deferens (Felder et al., 2006). The higher expression of CB_2 receptors on immune cells suggests their involvement in modulating functions of the immune system. Overall, activation of the CB_2 receptors serves to downregulate inflammation and leukocyte infiltration. This effect is achieved through the inhibition of cytokine and chemokine release from inflammatory cells, inhibition of leukocyte adhesion and migration, and apoptosis of activated immune cells (Klein, 2005; Lunn et al., 2006).

The exact signalling pathway of the CB_2 receptor has still not yet been fully elucidated, and there are multiple different downstream cascades associated with this

receptor (Basu & Dittel, 2011; Demuth & Molleman, 2006; Ellert-miklaszewska et al., 2013). In general, the CB_2 receptor signals primarily through the $G_{i/o}$ heterotrimeric proteins, similar to the CB_1 receptor (Figure 3). The most studied pathway modulated by these G proteins is the adenylate cyclase (AC) pathway, which has been shown to be both activated and inhibited by the CB_2 receptor in different circumstances (Basu & Dittel, 2011). Activation of adenylate cyclase converts AMP into cyclic AMP (cAMP) which then accumulates within the cell and activates protein kinase A (PKA). Activated PKA then phosphorylates various transcription factors that play a role in cell survival, cytokine and chemokine expression, cell proliferation, differentiation, and apoptosis (Basu & Dittel, 2011).

The MAPK pathways are another important signalling cascade activated by the CB_2 receptors. Similar to adenylate cyclase modulation, the CB_2 receptor has been shown to both activate and inhibit various MAP kinases (Figure 3). The CB_2 receptor was shown to regulate extracellular signal regulated protein kinases (ERK), p38 MAPK, , and c-Jun NH_2-terminal kinases (JNKs) (Basu & Dittel, 2011; Bouaboula et al., 1995; Howlett, 2005; Ladak et al., 2011). Interestingly, various studies have shown that even if the cell type and environment is kept constant, different CB_2 ligands can elicit different signalling cascades (Basu & Dittel, 2011). This phenomenon might be explained by the different allosteric binding sites used by various CB_2 ligands, as well as the differences in their inherent efficacies towards stimulating or blocking the CB_2 receptor (Basu & Dittel, 2011).

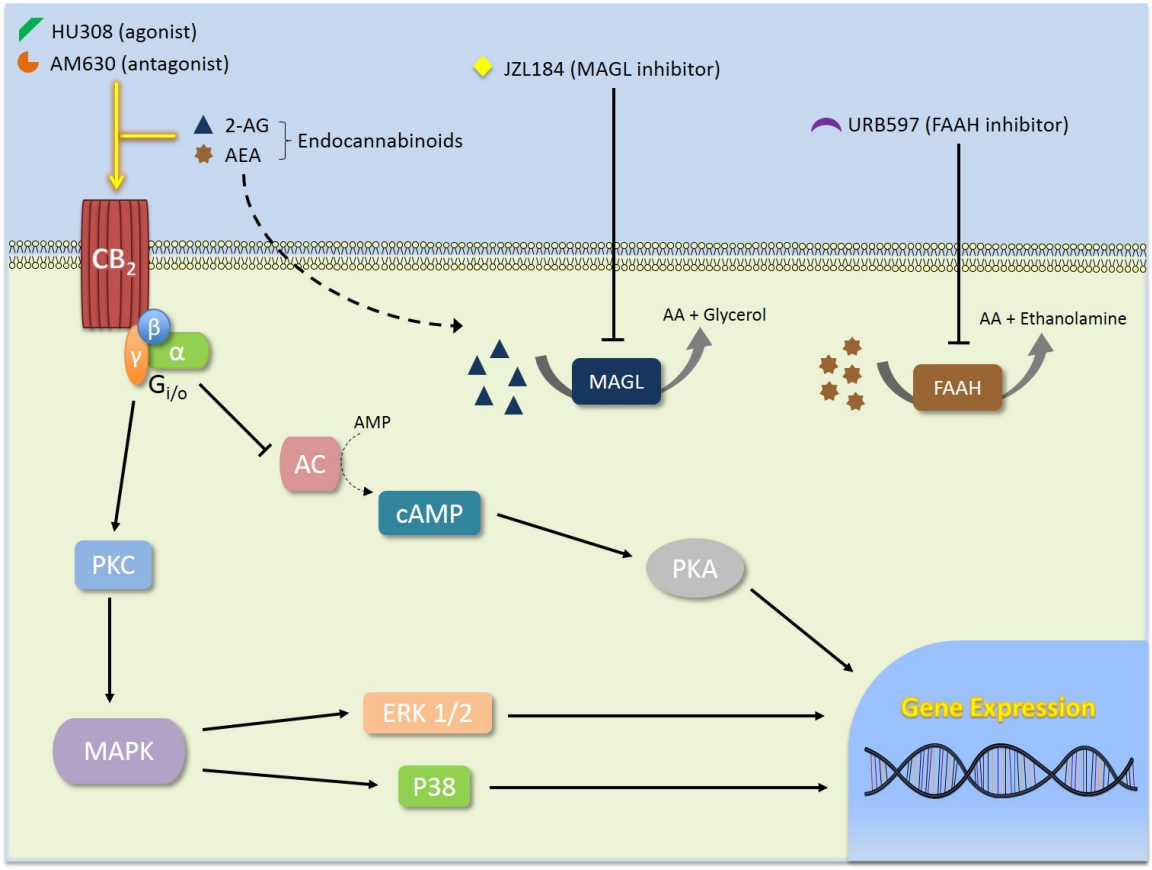

Figure 3: Schematic representation of the cannabinoid 2 receptor pathway.

1.7.3 Endocannabinoid metabolic enzymes

In order to avoid excessive cannabinoid receptor signalling, the cannabinoid system has an intrinsic mechanism designed to eliminate endocannabinoids after they have been released. The eliminiation of endocannabinoids involves a two step process that first involves translocating the endocannabinoids from the extracelluar environment, into the cell, follwed by enzymatic hydrolysis of the endocannabinoids by specific enzymes. The transport of cannabinoids into the cell can occur through multiple different mechanisms, due to their chemical properties (Felder et al., 2006). The main method of translocation occurs via putative membrane carrier mediated transporters (Beltramo et al., 1997; Bisogno et al., 2001). The rate of endocannabinoids was shown to be saturatable, indicating the use of membrane transporters as an important mechanism. Furthermore, specific inhibitors of these membrane transporters also reduces the rate of endocannabinoid translocation. The other method endocannabinoids can enter the cell is through passive diffusion. Due to their chemical properties of being synthesized from membrane lipids, the endocannabinoids are lipophillic and can easily diffuse across the cell membrane.

The two most well characterized endocannabinoid hydolyzing enzymes are fatty acid amide hydrolase (FAAH) which primarily metabolises anadamide, and monoacyl glycerol lipase (MAGL) which primarily hydrolyses 2-AG (Ahn et al., 2008; Kinsey et al., 2009; Long et al., 2009; Petrosino & Di Marzo, 2010) (Figure 4). Alternate pathways that are less prominent but also involved in endocannabinoid metabolism are cyclooxygenases (COX-2), lipooxygenases (LOX), and cytochrome P450, and serine hydrolases like ABHD6 and ABHD12 (Basavarajappa, 2007; Savinainen et al., 2012).

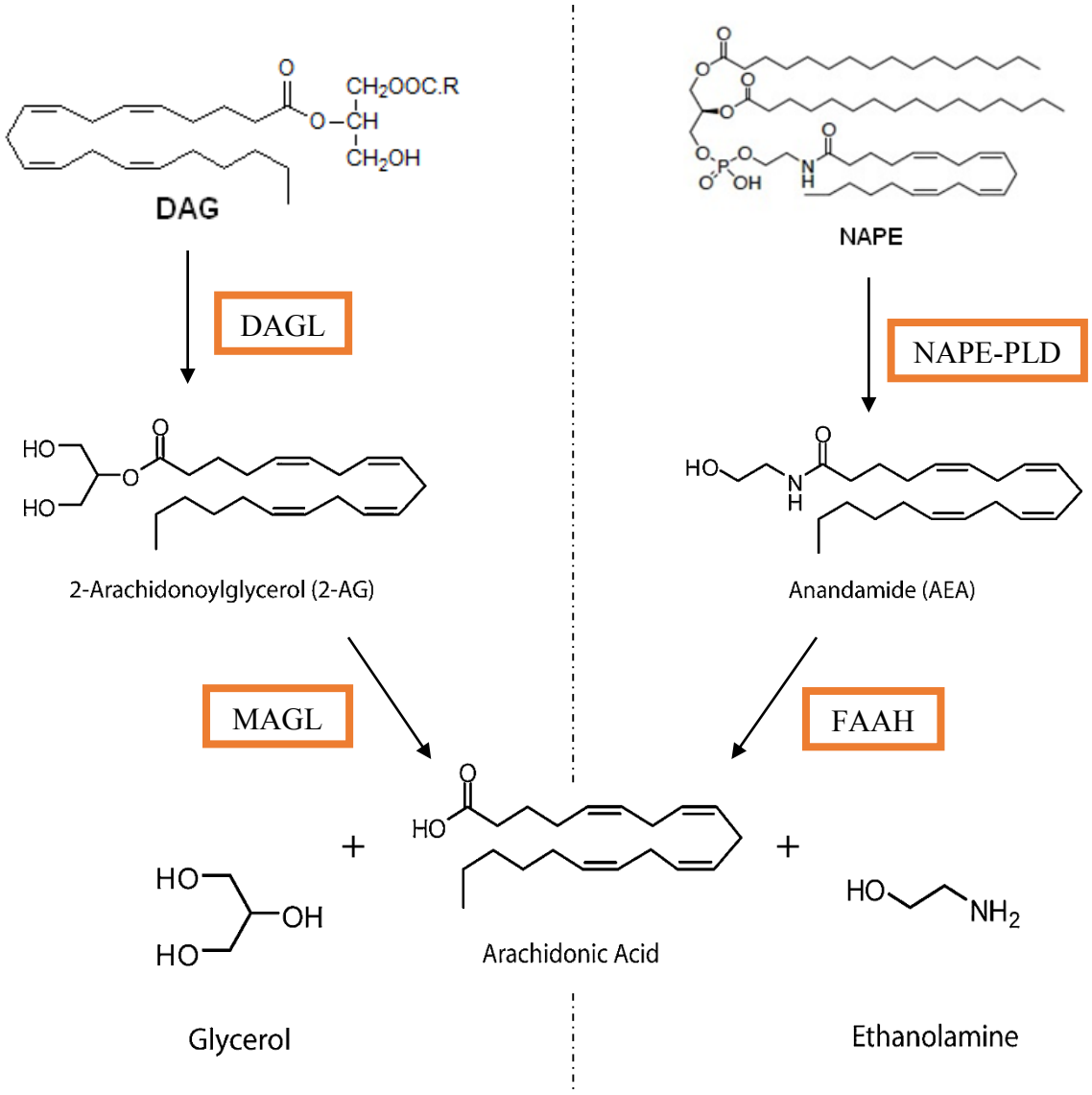

Figure 4: Primary enzymes responsible for the synthesis and degradation of the endocannabinoids anandamide and 2-AG.

1.7.4 Targeting the endocannabinoid system in sepsis

The peripheral presence of CB_2 receptor on all immune cells and the overall anti-inflammatory properties elicited by activating this receptor, make it a unique therapeutic target in inflammatory conditions like sepsis (Di Marzo et al., 2004; Klein, 2005; Lunn et al., 2006; Pacher & Mechoulam, 2011). The endocannabinoid system may play an integral role in modulating the immune system during inflammatory diseases like sepsis, evidenced by the fact that elevated levels of 2-AG and anandamide have been found in the serum of septic patients and inflammatory animal models (Klein, 2005; Varga et al., 1998). Furthermore, the expression levels of the CB_2 receptor on leukocytes were increased after being primed with inflammatory mediators like thioglycolate and interferon gamma (IFNγ), or in inflammatory condition like experimental autoimmune encephalomyelitis (EAE) (Carlisle et al., 2002; Maresz et al., 2005). Therefore, modulating the activity of the CB_2 receptor during systemic inflammatory conditions like sepsis may help minimize inflammatory damage caused by excessive immune cell recruitment and release of pro-inflammatory mediators (Sardinha et al., 2013).

1.8 Hypothesis

The overall hypothesis of this project was that CB_2 receptor modulation during the acute hyperinflammatory phase of sepsis will preserve the microcirculation of the intestine. Specifically, we hypothesized that specific activation of the CB_2 receptor via various mechanisms like direct agonist stimulation or indirect stimulation through increased endocannabinoid levels by blocking their degrading enzymes may be therapeutically beneficial during the early pro-inflammatory states of sepsis pathogenesis. Furthermore, blocking the activity of the CB_2 receptor through specific CB_2 receptor

antagonists will exaggerate the pro-inflammatory response and further disrupt proper microcirculatory function. Finally, we hypothesized that eliminating the CB_2 receptor pathway through the use of CB_2 receptor knockout mice will abolish the beneficial effects elicited by activating the CB_2 receptor.

1.9 Study objectives

The goal of this research project was to examine the systemic effects of modulating the activity of the CB_2 receptor on immune cell recruitment and microcirculatory integrity in acute models of murine sepsis. As mentioned earlier, intestinal mucosa barrier disruption after tissue ischemia is an important step in the pathogenesis of sepsis, leading to the translocation of additional pathogens into the blood stream and the development of secondary infections further exacerbating the septic condition. Therefore maintenance of the intestinal microcirculation is a crucial therapeutic target early in the septic cascade, and impairments to this extensive microcirculatory network may be indicative of subsequent disease progression.

Our primary goal was to assess the physiological changes in intestinal microcirculatory parameters after acute experimental sepsis and the impact of CB_2 pathway modulation on these parameters. Within this goal, our primary endpoint was to assess the changes in systemic leukocyte activation by measuring leukocyte endothelial interactions in the submucosal venules of the terminal ileum. The secondary endpoint was to assess the robustness of organ perfusion by quantifying the density of functional capillaries in various layers of the intestinal wall. Our secondary goal was to assess molecular changes occurring systemically during acute experimental sepsis and the

impact of the CB_2 pathway in altering these molecular changes. One endpoint within this goal was to measure plasma levels of systemically circulating cytokines as an indication of immune activity. The final endpoint was to measure mRNA expression levels of the CB_2 receptor and an important inflammatory transcription factor NF-κB in the tissue of organs like the intestine and spleen. For this research project we chose to assess the intestinal microcirculatory integrity in two different experimental models of acute sepsis in mice.

Experimental models

We chose to first assess the effect of CB_2 receptor modulation in an endotoxemia model, which involved administration of lipopolysaccharide (LPS) intravenously into wild-type mice. This model simulates a bacteremia state based on the excessive presence of bacterial antigens systemically. The excessive presence of bacterial antigens causes an exaggerated pro-inflammatory immune response by the innate immune system, leading to dysregulated activation and damage to endothelial cells, propagating sepsis pathogenesis. However, in an endotoxemia model of LPS administration, the presence of viable bacteria is missing, therefore the specific damage and pathogenesis associated with live bacteria is also missing in this model. Despite the lack of pathogenic bacteria, LPS administration is a valid model of acute sepsis as it simulates the overall pro-inflammatory immune state seen in during the initial stages of sepsis pathogenesis. In this model we chose to use three unique methods to activate the CB_2 receptor. We used HU308 which is a specific CB_2 receptor agonist to directly activate the CB_2 receptor. We also used URB597 which is a fatty acid amide hydrolase (FAAH) inhibitor to increase endogenous levels of the endocannabinoid anandamide to increase CB_2 receptor

activation. Similarly, we used JZL184 which is a monoacylglycerol lipase (MAGL) inhibitor to increase endogenous levels of the endocannabinoid 2-Arachidonoylglycerol (2-AG) to increase CB_2 receptor activation. Alternatively, we used AM630 which is a CB_2 receptor specific antagonist/inverse agonist to inhibit CB_2 receptor activation.

For our second model of experimental sepsis we chose to assess a clinically relevant model of polymicrobial sepsis. Colon Ascendens Stent Peritonitis (CASP model) simulates abdominal sepsis by disrupting the intestinal barrier through an implanted stent. The presence of the stent provides an avenue for the luminal contents of the intestine to translocate into the abdominal cavity, thereby introducing pathogens and inflammatory mediators that can cause infections and peritonitis. The CASP model overcomes the limitations of the LPS model by introducing live pathogens and inflammatory mediators into animals, simulating a more clinically relevant model of sepsis. Similar to clinical sepsis, the CASP model presents a greater degree of variability in sepsis pathogenesis compared to the endotoxemia model. In this model we chose to assess CB_2 receptor activation through JZL184 administration.

Finally, for our last model we chose to assess the impact of CB_2 receptor modulation on our various systemic parameters in an endotoxemic model with CB_2 receptor knockout mice. In order to validate the role of the CB_2 receptor in our experimental models of sepsis we chose to repeat our endotoxemia experimental approach with CB_2 receptor knockout mice. We chose to use the endotoxemia model of experimental sepsis and focused on the effects of the enzyme inhibitor JZL184 to increase endocannabinoid levels and activate the CB_2 receptor.

Intravital Microscopy

In all three studies we first assessed the therapeutic benefit of modulating the activity of the CB_2 receptor by using intravital microscopy to observe changes in various microcirculatory parameters. The main advantage of this technique is that it allows real time observation of microcirculatory parameters *in vivo*. We assessed physiological changes in immune response through two different parameters of leukocyte endothelial interactions. We measured levels of leukocyte adherence as well as leukocyte rolling as an indication of leukocyte activation and recruitment. Furthermore, we observed capillary perfusion in the intestinal muscle layers and in the mucosa, using functional capillary density to quantify the robustness of tissue perfusion. Using intravital microscopy we were able to assess physiological changes occurring at the microcirculatory level after insult and any benefit conferred after treatment.

Multiplex assay

After assessing the physiological changes occurring in the microcirculation due to CB_2 receptor manipulation we also wanted to assess the underlying molecular changes caused by modulating the CB_2 receptor. We chose to measure the circulating levels of specific cytokines and chemokines in the blood as they are the signalling molecules released by different cells to modulate the function of the immune system. We assessed changes in the levels of systemically circulating pro-inflammatory and anti-inflammatory cytokines and the influence of the CB_2 receptor in altering their levels.

Quantitative RT-PCR

In order to further understand the molecular changes occurring in sepsis and the potential impact of the CB_2 receptor, we decided to measure the changes in mRNA

expression of the CB_2 receptor and NF-κB p65 subunit in the tissue of the intestine and the spleen. Since we were observing the physiological microcirculatory parameters in the intestine, we also wanted to assess if these two important proteins might be the underlying mechanisms behind the physiological changes in this tissue. The spleen is an important organ in the immune system therefore we decided to assess the possible influence of the CB_2 receptor pathway in changing the expression levels of NF-κB and CB_2 receptor expression in splenocytes.

CHAPTER 2: MATERIAL AND METHODS

2.1 Animals

In all experiments involving wild type animals, healthy adult male C57BL/6 mice (6 - 8 weeks old; 20-30g) were purchased from Charles River Laboratories International Inc. (Wilmington, MS, USA). Animals were housed in ventilated rack cages and allowed to acclimatize for a week in the Carleton Animal Care Facility (CACF) of the Faculty of Medicine at Dalhousie University, Halifax, Nova Scotia, Canada. For our cannabinoid 2 receptor knockout experiments, DeltaGen CB_2R-/- mice were purchased from Jackson Laboratories and a colony was established in house at CACF. All animals used for the CB_2R-/- experiments were bred by mating two homozygous CB_2R knockout mutant parents and were used after 8 weeks of age. All animals were kept on a standard 12 hrs light/dark cycle, with standard room temperature 22°C and humidity 55% – 60%. Animals had access to a standard diet of rodent chow and water *ad libitum*. This study was conducted in accordance with the guidelines and standards set forth by the Canadian Council on Animal Care and approved by the University Committee on Laboratory Animals at Dalhousie University (protocol No. 13-061).

2.2 Experimental groups

Endotoxemia: A total of 11 experimental groups were tested for the endotoxemia model with 3 – 5 animals per group. Group 1 was a healthy control group (control), group 2 was the endotoxemia challenge (Lipopolysaccharide (LPS); 5 mg/kg), and group 3 was the vehicle control (30% DMSO solution).

Group 4 received LPS (5 mg/kg) as well as HU308 (2.5 mg/kg). HU308 is a potent and selective synthetic agonist of the CB_2 receptor with K_i values of 22.7 nM and

an EC_{50} = 5.57 nM. In contrast the K_i value of HU308 for the CB_1 receptor is >10 μM (Hanus et al., 1999). HU308 (2.5 mg/kg) was dissolved in 10% DMSO vehicle solution and administered I.V.

Group 5 received LPS (5 mg/kg) as well as the synthetic CB_2 receptor antagonist/inverse agonist AM630 (2.5 mg/kg; Tocris Bioscience, Ellisville, Missouri, USA). AM630 has a K_i value of 31.2 nM for the CB_2 receptor and displays 165-fold selectivity for CB_2 receptors over CB_1 receptors (Ross et al., 1999). AM630 was dissolved in 30% DMSO vehicle solution and administered I.V.

Group 6 received LPS (5 mg/kg) as well as the fatty acid amide hydrolase (FAAH) inhibitor URB597 (0.6 mg/kg; Cayman Chemical, Ann Arbor, MI, USA). URB597 has an IC_{50} value of 4.6 ± 1.6 nM for FAAH in the brain (Kathuria et al., 2003). URB597 was dissolved in 10% DMSO vehicle solution and administered I.V.

Group 7 received LPS (5 mg/kg) as well as the monoacylglycerol lipase (MAGL) inhibitor JZL184 (16 mg/kg; Tocris Bioscience, Ellisville, Missouri, USA). JZL184 has an IC_{50} value of 8 nM in the mouse brain (Long et al., 2009), and exhibits >300-fold selectivity for MAGL over FAAH in vitro. JZL184 was dissolved in 30% DMSO vehicle solution and administered I.V.

Groups 8 - 11 were the treatment control groups and received saline instead of LPS. Group 8 received HU308 (2.5 mg/kg) dissolved in 10% DMSO, group 9 received AM630 (2.5 mg/kg) dissolved in 30% DMSO, group 10 received URB597 (0.6 mg/kg) dissolved in 10% DMSO, and group 11 received MAGL (16mg/kg) dissolved in 30% DMSO.

CASP: A total of 4 groups were tested for the colon ascendens stent peritonitis model with 6 – 8 animals per group. Group 12 was a sham control that had the surgical procedure performed, but no stent was implanted into the ascending colon. Group 13 was the CASP group that had a stent placed and fecal matter was allowed to pour out into the abdominal cavity for 6 hours. Group 14 was the vehicle control group that had the CASP surgery and received 30% DMSO vehicle solution. Group 15 had the CASP surgery and received JZL184 (16 mg/kg) dissolved in 30% DMSO.

CB_2R -/-: A total of 3 groups were tested in the *CB_2R -/-* model with 3 animals per group. Similar to the endotoxemia model, animals in Group 16 were the controls, Group 17 received LPS (5 mg/kg), and Group 18 received LPS (5 mg/kg) and JZL184 (16 mg/kg) in 30% DMSO.

Table 2: Experimental Groups

Groups	**Model**	**Intervention**
Group 1 - Control	Endotoxemia	Saline
Group 2 - Challenge	Endotoxemia	LPS (5 mg/kg)
Group 3 - Vehicle Control	Endotoxemia	LPS (5 mg/kg) + 30% DMSO
Group 4 – LPS + CB_2R agonist	Endotoxemia	LPS (5 mg/kg) + HU308 (2.5 mg/kg)
Group 5 – LPS + CB_2R antagonist	Endotoxemia	LPS (5 mg/kg) + AM630 (2.5 mg/kg)
Group 6 – LPS + FAAH inhibitor	Endotoxemia	LPS (5 mg/kg) + URB597 (0.6 mg/kg)
Group 7 – LPS + MAGL inhibitor	Endotoxemia	LPS (5 mg/kg) + JZL184 (16 mg/kg)
Group 8 - CB_2R agonist control	Endotoxemia	Saline + HU308 (2.5 mg/kg)
Group 9 - CB_2R antagonist control	Endotoxemia	Saline + AM630 (2.5 mg/kg)
Group 10 – FAAH inhibitor control	Endotoxemia	Saline + URB597 (0.6 mg/kg)
Group 11 - MAGL inhibitor control	Endotoxemia	Saline + JZL184 (16 mg/kg)
Group 12 - SHAM	CASP	Saline
Group 13 - CASP	CASP	CASP (20 gauge)
Group 14 - Vehicle Control	CASP	CASP (20 gauge) + 30% DMSO
Group 15 - MAGL inhibitor	CASP	CASP (20 gauge) + JZL184 (16 mg/kg)
Group 16 - Control	CB_2R -/-	Saline
Group 17 - Challenge	CB_2R -/-	LPS (5 mg/kg)
Group 18 – MAGL inhibitor	CB_2R -/-	LPS (5 mg/kg) + JZL184 (16 mg/kg)

2.3 Anesthesia

Prior to the start of intravital microscopy every animal's body weight was measured using a commercially available weighing scale and sodium pentobarbital (90 mg/kg, 54 mg/mL; Ceva Sante Animale, Montreal, QC, Canada) was administered by intraperitoneal (I.P.) injection at a 50% dilution with 0.9% sodium chloride (Hospira, Montreal, QC, Canada). Mice were properly anesthetized prior to any procedure and the depth of anesthesia was regularly checked with a toe pinch reflex. An additional 0.1mL of sodium pentobarbital (20 mg/kg, 54 mg/mL) was administered intravenously (I.V.) at a 90% dilution with 0.9% sodium chloride whenever required to maintain a sufficient depth of anesthesia.

For the colon ascendens stent peritonitis surgery induction of anesthesia was achieved by placing the mice in an enclosed environment with 5% isoflurane and an O_2 flow rate of 2 L/hr using an anesthetic vaporizer (VetEquip, Pleasanton, CA, USA) until sufficient anesthesia was achieved. To maintain proper anesthesia, mice continuously breathed 1 - 2% isoflurane at an O_2 flow rate of 0.6 – 0.8 L/hr. Ketoprofen (1 mg/mL) was injected subcutaneously (S.C.) at the start of surgery for post-operative analgesia.

2.4 Surgical procedure

Endotoxemia: Once animals were sufficiently anesthetized, they were placed on a heating pad in a supine position and their body temperature was maintained at 37 ± 0.5°C (98.6°F) measured via a rectal thermometer. Rectal body temperature was monitored and recorded every 15 minutes for the duration of the experiment. The neck region distal to the trachea was shaved on the left side and cleaned with isopropyl alcohol swabs (Health Care, Toronto, Canada). A small superficial incision was made to the skin using fine

scissors (Fine Science Tools, British Columbia, Canada) and blunt forceps were used to separate the underlying connective tissue. A longitudinal incision was made to the skin using tissue scissors to expose the underlying tissue and the jugular vein was isolated from the surrounding tissue using blunt forceps. Using a dissecting microscope, a 5 – 7 mm section of the jugular vein was further isolated from the surrounding tissue using fine forceps. A 10 cm length of black braided silk string (Ethicon, New Jersey, USA) was used to tie a surgical knot around the superior end of the vein and restrict blood flow. A second silk string was used to tie a loose knot at the inferior end of the vessel and haemostats were used to hold the strings and place adequate tension on the vessel to have a small pool on blood trapped in the vessel length between the two knots. Using micro-dissecting scissors (Fine Science Tools, British Columbia, Canada), a small incision was made close to the superior end of the vessel, and using fine tipped micro forceps a catheter was placed into the lumen of the vessel through the incision site. The catheter was made by placing an intramedic non-radiopaque polyethelene tubing (PE 10, Clay Adams, Sparks, MD, USA) over a 30 gauge needle and securing it with waterproof glue. Approximately 1 cm of the catheter tubing was inserted into the vessels and the patency of the catheter was tested by flushing a small amount of saline. The catheter was then firmly secured by tightening the inferior knot with a triple knot over the vessel with the inserted catheter tube. The loose ends of the superior knot was used to secure the catheter tubing above the vessel by making a secure double knot. LPS, treatment drugs, saline, fluorochromes and anesthetics were administered intravenously through the catheter. Animals breathed room air spontaneously, but oxygen was provided if breathing got laboured. Mucous and saliva buildup was also extracted from the throat using a short

cannula connected to a 10 ml syringe (Becton Dickinson and Company, NJ, USA) as a suction device to prevent obstruction of the trachea.

Colon Ascendens Stent Peritonitis: Once the mice were adequately anesthetized, they were placed on a heating pad in a supine position. The abdominal area was shaved using hair clippers and the skin was sterilized by applying chlorhexidine gluconate, 70% isopropyl alcohol, and iodine soaked on gauze sponges in a sequential manner respectively. The surgery was carried out aseptically so all instruments and materials used during the surgery were autoclaved or came sterilely packaged. The surgeon wore sterile gloves, a surgical cap, and a mask in accordance with Dalhousie University CACF guidelines.

At the start of the surgery a sterile surgical drape was used to cover the animal with an opening for the area of surgery. A midline incision was made longitudinally along the abdomen with a scalpel. Blunt forceps was used to separate the skin from the abdominal muscle layer. Using fine tissue scissors, a cut was made longitudinally along the abdominal muscle layer on the *linea alba*. Saline soaked cotton tipped applicators were used to isolate the ascending colon and place it on saline soaked gauze sponge pads. The intestinal tissue was kept moist during the surgical procedure using normal saline. An 8-0 polypropylene monofilament suture was used to place a superficial surgical knot 5 mm past the ileocaecal valve on the surface of the ascending colon. A 20 gauge catheter was inserted distally from the knot into the ascending colon lumen approximately 5 mm intraluminally and the needle was removed. The loose suture ends of the knot were used to secure the catheter tube. A second surgical knot was made superficially on the ascending colon wall using the remaining 8-0 polypropylene monofilament suture 3 – 4

mm distally from the inserted catheter tube, and the loose ends were used to secure the knot to the catheter tube. The catheter tube was cut to leave 2 – 3 mm of the tube projecting out of the ascending colon leaving a stent. The fecal matter should automatically fill the stent, but the colon can be gently massaged to fill the stent with stool. The intestinal loop was repositioned back into the abdominal cavity and 0.5 mL of saline was administered into the abdominal cavity for fluid resuscitation. 15 minutes later the treatment compound was administered into the abdominal cavity. The abdominal muscle layer was sutured using a 6-0 polypropylene monofilament absorbable suture with a running uninterrupted stitch. The skin incision was sutured using a 4-0 braided silk suture with a multiple interrupted knot stitch. The mouse was placed in a clean cage in the recovery room with half the cage over a heating pad and the mouse was assessed until fully conscious and mobile. The mice had *ad libitum* access to rodent chow mash and drinking water within their cages. The animals were allowed to recover for 5 hours in the animal recovery room.

2.5 Experimental timeline

Endotoxemia: The experimental timeline for the endotoxemia protocol begins with general anesthesia of the animal and cannulating the left jugular vein as described in the previous section. Once the jugular vein was cannulated, endotoxemia was induced by intravenous administration of lipopolysaccharide from Escherichia coli, (serotype O26:B6, Sigma-Aldrich, Oakville, ON, Canada). Lipopolysaccharide (LPS; 5 mg/kg, 1 mg/mL) was dissolved in sterile saline (0.9% Sodium Chloride) and administered intravenously (I.V.) at 0 minutes in the experimental time-course (Figure 5). LPS administration was carried out as a short-time infusion (5 min). All treatment compounds

were administered 15 minutes after LPS challenge as post treatments to the insult. Animals were subsequently monitored for 2 hours until intravital microscopy (IVM). Within the 2 hours period, body temperature was monitored and recorded every 15 minutes. Additionally, breathing rate and anesthetic depth was also monitored, with oxygen being provided if breathing got labored, and additional sodium pentobarbital administered if anesthetic depth was insufficient. 30 minutes prior to intravital microscopy, the fluorochromes (details in section 2.6) were administered I.V. via the catheter. Subsequently, a laparotomy was performed (details in section 2.6) and the animal was prepared for IVM. Upon completion of IVM recordings (details in section 2.6), whole blood and tissue samples of small intestine and spleen were collected and stored (details in section 2.7).

Colon Ascendens Stent Peritonitis: The experimental timeline for CASP begins with anesthesia and surgical implantation of the stent into the ascending colon (described in section 2.4). Time 0 of the experimental timeline was upon the completion of the CASP surgery, and 15 minutes later the treatment drug was administered (Figure 6). The animal was allowed to recover after surgery and 5 hours later the animal was once again anesthetized with sodium pentobarbital (90 mg/kg) in preparation for IVM. The same procedure was carried out for IVM as the endotoxemia timeline with the exception of the implantation of a catheter into the jugular vein. For the CASP protocol, fluorochromes and saline were administered I.V. through tail vein injections.

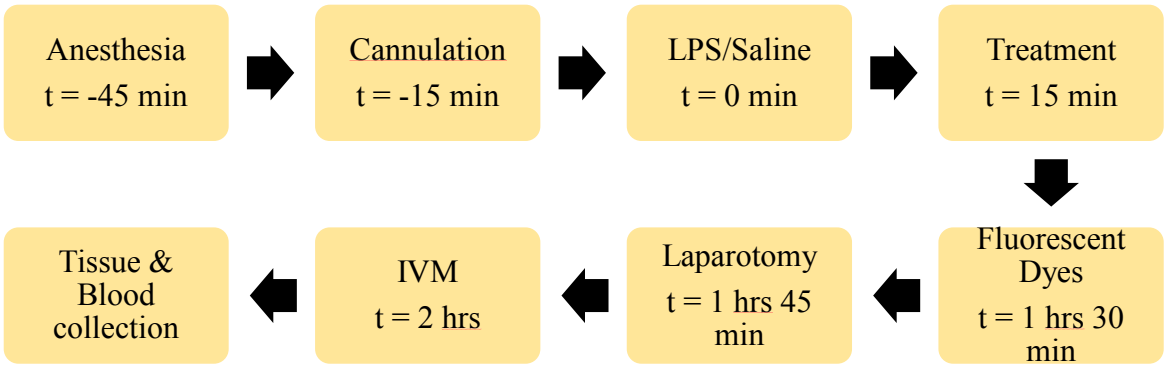

Figure 5: Experimental timeline for endotoxemia studies.

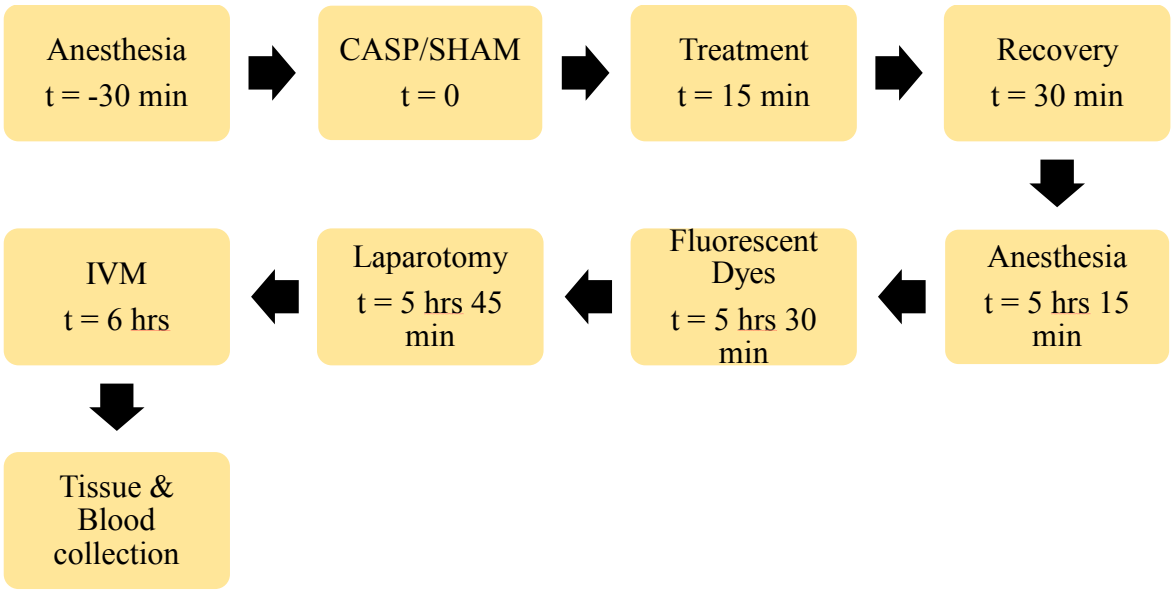

Figure 6: Experimental timeline for CASP study.

2.6 Intravital microscopy

Fluorochromes: Thirty minutes prior to the start of the IVM, fluorochromes were administered I.V. through the catheter for the endotoxemia model or through tail vein injection for the CASP model. Rhodamine-6G (1.5 mL/kg) (Sigma-Aldrich, ON, Canada) was used to stain leukocytes and FITC-labeled BSA (fluorescein isothiocyanate-labeled bovine serum albumin; 1 mL/kg) was used to help enhance blood contrast and observe perfusion in capillaries.

Laparotomy and IVM setup: Fifteen minutes before IVM, the laparotomy was performed after ensuring the animal's anesthetic depth was properly sufficient. A midline incision was made on the animal's abdomen using a scalpel and scissors were used to cut away enough skin to expose the abdominal muscle layer. Using fine tissue scissors, the muscle layer was cut along the *linea alba* to expose the abdominal cavity and prevent bleeding. Using saline soaked cotton tipped applicators the caecum was located and a portion of the terminal ileum was exteriorized onto a hook of a specially designed apparatus (Figure 7) that was fixed to the heating pad. The animal was placed on its side to minimize the tension on the intestine and saline was perfused over the intestine to keep it moist. The apparatus enabled continuous thermostat-controlled (37°C/ 98° F) saline to be perfused over the exposed intestinal section to reflect physiological conditions of moisture and temperature. The saline was pumped at a rate of 5 mL/hr and a glass slide was used to make a liquid contact with the section of intestine placed on the hook. The heating pad containing the animal and intestine on the apparatus was placed under the microscope for observation.

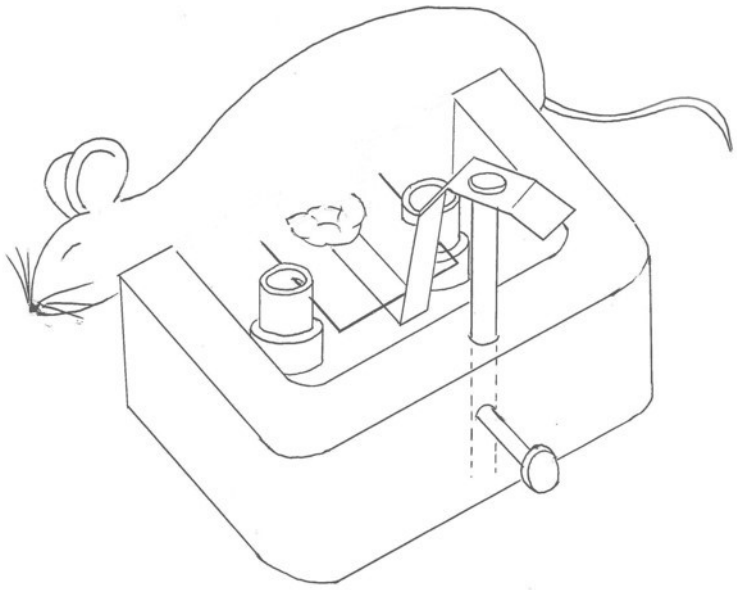

Figure 7: Specially designed aparatus used to maintain intestinal section at physiological conditions during intravital microscopy.

Microscopy: To observe the animal's intestinal micro-hemodynamics, specifically distinguishing leukocyte activity and capillary perfusion, we used an epifluorescent microscope (Leica DMLM, Wetzlar, Germany). An attached mercury-arc light source (LEG EBQ 100, Jena, Germany) was used to illuminate the observed area and different excitation filters were used to allow specific wavelengths of light to specifically excite the different fluorochromes. A 460-490 nm band pass excitation filter, which allows blue light to pass through but blocks all other wavelengths, was used to excite FITC and allow observation of the capillary perfusion. A 530-550 nm band pass excitation filter, which allows only green light to pass, was used to excite Rhodamine and allow observation of leukocytes. A water immersion lens (Leica N PLAN L 20X/0.40) was used to make a liquid contact between the lens and glass slide over the section of intestine for observation. A black and white DAGE CCD video camera (DAGE MTI Inc., Michigan City, IN), mounted on the microscope was used to observe and record video directly onto

a computer through a video converter (DFG/USB2PRO, The Imaging Source, Germany) and imaging software (IC capture, The Imaging Source, Germany).

Leukocyte activity: Using the green excitation filter, the microscope was used to focus on the submucosal venules. Six visual fields containing non-branching, submucosal collecting venules (V1) and postcapillary venules (V3) containing a length of at least 300 μm were observed and recorded for 30 seconds each in every animal. Collecting venules were classified as vessels with a diameter of more than 50μm and accompanied by an adjacent arteriole. Post capillary venules were classified as vessels typically under 50μm in diameter.

Functional Capillary Density: Using the blue excitation filter, the microscope was used to focus on the muscular layers of the small intestine. The longitudinal and circular muscle layers in the mouse intestine are quite thin that it was only possible to focus on both these layers at the same time. Six randomly selected areas of intestinal muscle layers were recorded for 30 seconds each. To observe the capillary perfusion of the mucosal villi the observed section of small intestine was superficially cauterized longitudinally using a microcautery knife (Medtronic, FL, USA). Then using fine tissue scissors the intestinal wall was cut open to expose the lumen and 0.9% saline was use to flush out the luminal contents. Using saline soaked cotton tipped applicators, the intestine was gently manipulated to maximize exposure to the lumen and the glass slide was once again placed over the exposed intestinal lumen to create a liquid contact between the tissue and slide. Once again the microscope was used to focus on the mucosal villi and six randomly selected areas were recorded, with each field containing at least five villi for analysis.

2.7 Blood & tissue collection

Following completion of intravital microscopy, the animal was euthanized by cardiac puncture where blood was drawn into a heparinized syringe. Three microliters of heparin (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON, Canada) was place into a 30 gauge needle to prevent blood coagulation. On average 0.5mL of blood was obtained, which was then placed in a 1mL Eppendorf tube and centrifuged at room temperature for 10 minutes at 10,000 x g. The plasma was then isolated and aliquoted into 0.5 mL microtubes and stored at -80°C until further use. Immediately after cardiac puncture, tissue samples of the small intestine and spleen were also taken. A section of the terminal ileum was isolated, longitudinally cut open, and the luminal contents flushed with saline. The tissue section was then placed into a cryogenic tube and frozen in liquid nitrogen. Similarly, whole spleen was also isolate from the mice and frozen in liquid nitrogen. Both tissue samples were placed at -80°C for long term storage until further use.

2.8 Video analysis

All video recordings were carried out offline using ImageJ software (NIH, US). According to our established criteria (Lehmann et al., 2012; Menger et al., 1992), rolling leukocytes were classified as those visibly moving along the endothelial wall and crossing a predetermined cross-sectional line of vessel. The number of rolling leukocytes were counted for 30 seconds and used to estimate the number of rolling leukocytes per minute. Adherent leukocytes were those that were immobile on the endothelial wall for the entire duration of the 30 second recording. A predetermined area of vessel was measured and the numbers of adherent leukocytes within that area was counted. These two values were then used to estimate the numbers of adherent leukocytes on the

cylindrical surface of the luminal side of the vessel. To quantify capillary perfusion we used functional capillary density (FCD), which is a measure of the length of capillaries with observable erythrocyte perfusion in any given area in $\mu m/\mu m^2$. For the muscular layers, a rectangular area was measured and the length of all perfused vessels within that area were added to calculate FCD. For the mucosal villi, five unique villi in each recording were analysed. The area of each villus and the length of the perfused capillaries in each villus was measured and added to obtain a total area and total perfused capillary length, which was then used to calculate FCD.

2.9 Plasma cytokine and chemokine analysis

Systemically circulating cytokine and chemokine levels were analysed in plasma samples using a multiplex assay. A custom 8-plex ProcartaPlex™ Immunoassay Kit - Magnetic Beads from eBioscience to perform quantitative, multiplexed immunoassays based on the Luminex® technology. Specific analytes of TNF-α, IL-1β, IL-6, IL-10, IL-17a, and MIP-2 were measured using a 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. The multiplex assay was carried out using the standard Affymetrix multiplex immunoassay protocol for mice. Briefly, 50µl of the detection antibody magnetic beads for each analyte was added to each well of the 96-well flat bottom plate. The 96-well plate was secured to a hand held magnetic plate washer and the liquid was discarded by inverting the plate. The magnets located around the bottom of the well attract the beads to the side, thus retaining them in the well while the

liquid contents are discarded. 150µL of 1X Wash Buffer was added into each well and decanted by placing the plate on the magnetic washer and inverting. 25µL of Universal Assay Buffer was added into each well, followed by 25µL of standards or samples into dedicated wells in duplicates. A 4-fold standard dilution was used to create a 7-point standard curve. An additional 25µL of Universal Assay Buffer was added to the blank wells instead of sample. The plate was sealed and incubated at room temperature for 60 minutes at 500rpm on a plate shaker. Following incubation, the wells were washed 3-4 times using 150µL of 1X Wash Buffer and decanted by inversion. Then 25µL of detection antibodies were added to each well and incubated at room temperature for 30 minutes at 500rpm. Once more, the wells were washed 3-4 times with 150µL of 1X Wash Buffer and decanted by inversion. 50µL of Streptavidin-PE was then added to each well and incubated at room temperature for 30 minutes at 500rpm. Another 3-4 washes of 150µL of 1X Wash Buffer per well was carried out and the liquid contents were drained by inversion. Finally, 120µL of Reading Buffer was added to each well and incubated at room temperature for 5 minutes at 500rpm.

2.10 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA isolation: RNA was harvested from whole spleen and small intestine by homogenizing in 1mL TRIzol® (Invitrogen, Burlington, ON) and following manufacturer's protocol. Briefly, 0.2 mL chloroform was added to each homogenized sample in TRIzol®, briefly vortexed, incubated at room temperature for 3 minutes, and centrifuged at 4°C for 15 minutes at 12,000 x g. The aqueous phase was then separated and added to 0.5 mL 100% isopropanol, incubated at room temperature for 10 minutes, and centrifuged at 4°C for 10 minutes at 12,000 x g. The supernatant was removed and

the RNA pellet was washed in 1 mL 75% ethanol, briefly vortexed, and then centrifuged 4°C for 5 minutes at 7,500 x g. The supernatant was allowed to air dry for 10 minutes, then the pellet was resuspended in RNase-free water, followed by an incubation at 60°C for 15 minutes, and finally stored at -80°C.

Reverse Transcription: Reverse transcription reactions were carried out with Super-Script III® reverse transcriptase (Invitrogen, Burlington, ON). Briefly, 2 μg of RNA sample was added to a solution of RNase free water, 1μL of dNTP mix (Invitrogen, Burlington, ON), and 1 μL of Oligo$(dT)_{12\text{-}18}$ primer (Invitrogen, Burlington, ON). The solutions were incubated at 65°C for 5 minutes and then placed immediately on ice. Then for each sample a +RT (containing Super-Script III® reverse transcriptase) reaction and a –RT (dH_2O instead of Super-Script III® reverse transcriptase) reaction as a negative control was made for use in the subsequent qPCR experiment. Each RT reaction contained 4 μL of 5X first-strand buffer, 1 μL of 0.1M DTT, 1 μL RNaseOUT™ Recombinant RNase Inhibitor, and 1 μL of either SSIII® (+RT) or dH_2O (-RT). The tubes were incubated at 50°C for 1 hour, followed by a second incubation at 70°C for 15 minutes. The samples were then diluted by adding 20 μL of dH_2O per tube, aliquated and stored at -80°C.

qPCR: qPCR was conducted using the LightCycler® system and software (version 3.0; Roche, Laval, QC). Each reaction composed of 2 μL cDNA sample, 0.5 μM each of forward and reverse primers (β-Actin, CB_2R, NF-κB), 2mM primer-specific concentration of $MgCl_2$, 2 μL of LightCycler® FastStart Reaction Mix SYBR Green I, and dH_2O to get a total volume of 20 μL. CB_2 receptor primer sequence (5′–3′) Forward GGATGCCGGGAGACAGAAGTGA’ and Reverse

'CCCATGAGCGGCAGGTAAGAAAT' designed and optimized by (Laprairie et al., 2014). β-Actin forward primer 'AAGGCCAACCGTGAAAAGAT' and reverse primer 'GTGGTACGACCAGAGGCATAC' designed by (Blázquez et al., 2011). NF-κB forward primer 'GCGTACACATTCTGGGGAGT' and reverse primer 'CCGAAGCAGGAGCTATCAAC' designed by (C. Cao et al., 2006). In addition to the negative controls (-RT), a dH_2O control, as well as a primer standard control containing product-specific cDNA of a known concentration was included in each experimental run. The PCR program was: 95°C for 10 minutes, 50 cycles of 95°C for 10 seconds, a primer-specific annealing temperature of 59°C for β-Actin/NF-κB or 57°C for CB_2R for 5 seconds, and 72 °C for 10 seconds. cDNA abundance was calculated by comparing the cycle number at which a sample entered the logarithmic phase of amplification (crossing point) to a standard curve generated by amplification of cDNA samples of known concentration.

2.11 Statistical analysis

All data presented in the results 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). The Kolmogorov-Smirnov test was used to assess normality of the data. In the intravital microscopy data, differences between groups were analyzed using one-way ANOVA (analysis of variance, ANOVA), followed by the Newman-Keuls test for group wise comparisons. The significance level was considered at $p < 0.05$. In the cytokine and qRT-PCR data, differences between groups were compared using one tailed unpaired t-test with significance considered at $p < 0.05$.

CHAPTER 3: RESULTS

3.1 Endotoxemia

3.1.1 Intravital microscopy

Leukocyte Adherence (V1): Leukocyte adherence on the endothelial wall of submucosal collecting venules (V1) was examined after treatment with various CB_2 receptor activity modulators (Figure 8). The levels of adherent leukocytes in the control group were minimal and LPS administration significantly ($p < 0.05$) increased the number of adherent leukocytes in V1 venules compared to controls (Figure 8). After LPS administration, the leukocyte adherence increased almost a 100-fold compared to controls. CB_2 receptor activation through the administration of HU308 (2.5 mg/kg), 15 minutes after LPS administration, was able to minimize the extent of leukocyte adherence caused by LPS (Figure 8). Leukocyte adherence in V1 venules after LPS and HU308 administration was significantly ($p < 0.05$) lower compared to LPS treatment alone, and not significantly ($p > 0.05$) higher than controls. CB_2 receptor inhibition through the administration of AM630 (2.5 mg/kg), 15 minutes after LPS administration, did not significantly ($p > 0.05$) increase the levels of leukocyte adherence in V1 venules compared to LPS (Figure 8). Similar to the LPS group, the LPS + AM630 group was significantly ($p < 0.05$) higher compared to controls for V1 leukocyte adherence. CB_2 receptor activation by increasing levels of anandamide through FAAH inhibition by administration of URB597 (0.6 mg/kg), 15 minutes after LPS administration, was able to significantly ($p < 0.05$) reduce levels of leukocyte adherence in V1 venules caused by LPS (Figure 8). Similarly, CB_2 receptor activation by increasing levels of 2-AG through MAGL inhibition by administration of JZL184 (0.6 mg/kg), 15 minutes after LPS

administration, was able to significantly ($p < 0.05$) reduced levels of leukocyte adherence in V1 venules caused by LPS (Figure 8). The levels of leukocyte adherence for both the URB597 and JZL184 groups were not significantly ($p > 0.05$) higher compared to controls. The levels of leukocyte adherence in V1 venules for all the treatment control groups showed no difference to saline controls (data not shown).

Leukocyte Adherence (V3): Similar trends as seen in V1 for leukocyte adherence was also seen in post capillary venules (V3) for all groups. LPS administration caused a significant ($p < 0.05$) increase in leukocyte adherence compared to controls (Figure 9). The LPS group had a roughly 10-fold increase in leukocyte adhesion compared to controls. HU308 administration after LPS was able to significantly ($p < 0.05$) reduce levels of leukocyte adhesion in V3 venules caused by LPS. However, the level of leukocyte adherence in V3 venules after HU308 treatment was still significantly ($p < 0.05$) higher than controls (Figure 9). AM630 administration after LPS did not significantly ($p > 0.05$) exacerbate levels of leukocyte adhesion in V3 venules compared to LPS administration alone. URB597 administration after LPS was able to significantly ($p < 0.05$) reduce levels of leukocyte adhesion caused by LPS, but levels were still significantly ($p < 0.05$) higher than controls. JZL184 administration also showed similar results after LPS administration, as levels of adherent leukocytes in V3 venules were significantly ($p < 0.05$) reduced compared to LPS, but still significantly ($p < 0.05$) higher than controls (Figure 9). Once again the levels of leukocyte adherence in V3 venules of all treatment control groups showed no difference to saline controls (data not shown).

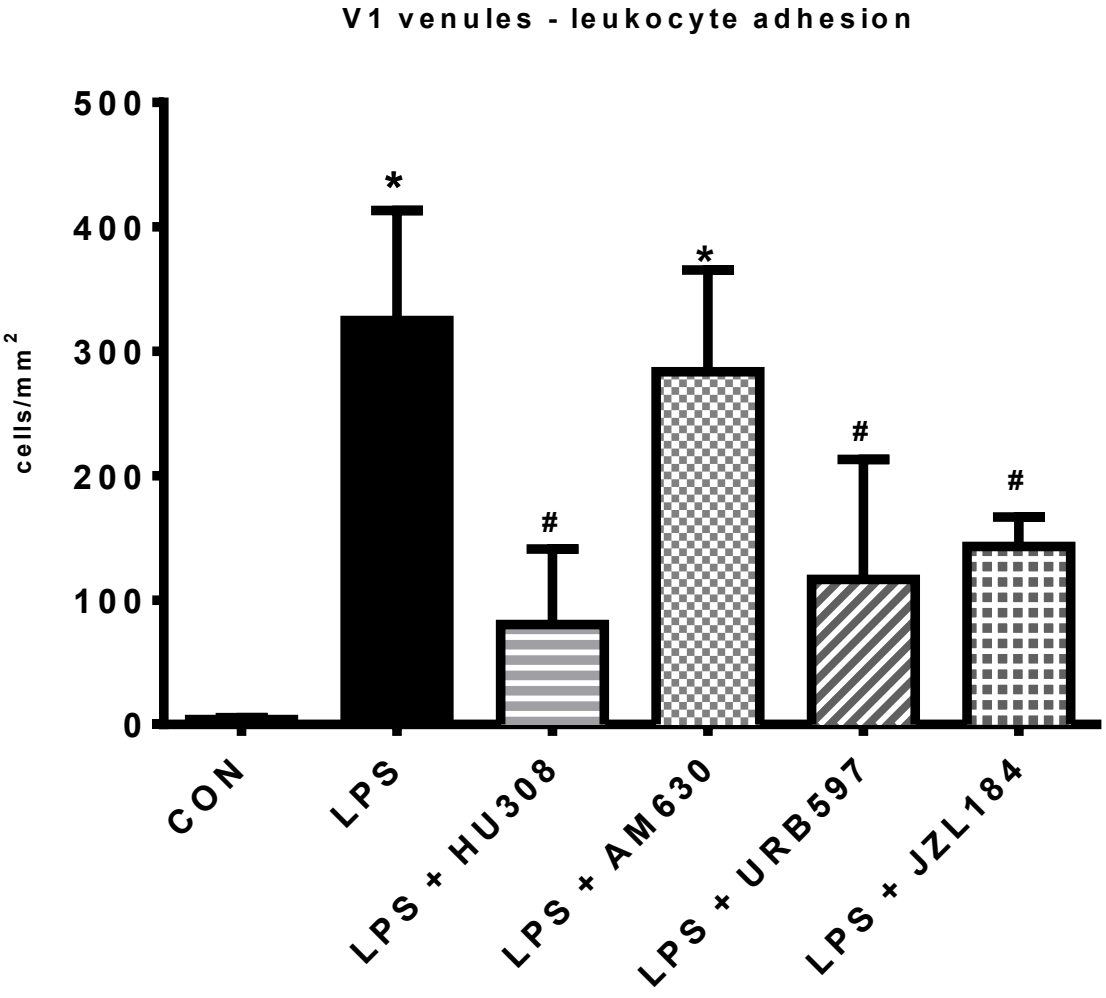

Figure 8: Leukocyte adhesion in collecting venules (V1; >50µm vessel diameter). Control group (CON); endotoxemia group LPS (5 mg/kg); LPS + HU308 (2.5 mg/kg) a CB2 receptor agonist; LPS + AM630 (2.5mg/kg) a CB2 receptor antagonist/inverse agonist; LPS + URB597 (0.6mg/kg) a FAAH inhibitor; and LPS + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control. # $p < 0.05$ versus LPS.

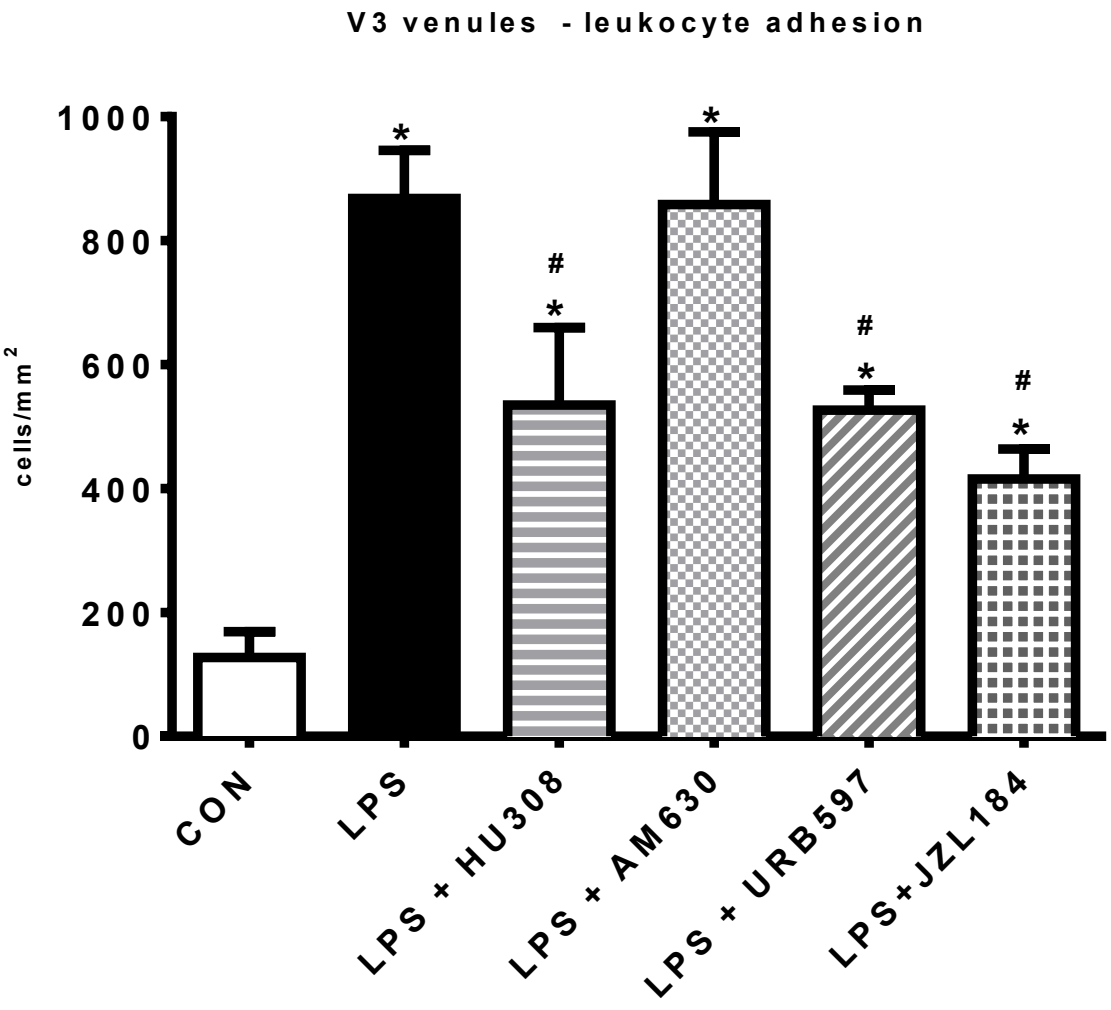

Figure 9: Leukocyte adhesion in post capillary venules (V3; <50μm vessel diameter). Control group (CON); endotoxemia group LPS (5 mg/kg); LPS + HU308 (2.5 mg/kg) a CB2 receptor agonist; LPS + AM630 (2.5mg/kg) a CB2 receptor antagonist/inverse agonist; LPS + URB597 (0.6mg/kg) a FAAH inhibitor; and LPS + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control. # $p < 0.05$ versus LPS.

Leukocyte Rolling (V1): Endotoxin challenge through LPS administration caused a significant ($p < 0.05$) reduction in the numbers of rolling leukocytes in V1 venules compared to controls (Figure 10). Administration of HU308, URB597, or JZL184 after LPS, did not significantly ($p > 0.05$) increase the number of rolling leukocytes compared to LPS. AM630 administration after LPS did not significantly ($p > 0.05$) reduce the number of rolling leukocytes compared to LPS (Figure 10). Leukocyte rolling in V1 venules for all treatment control groups showed no difference to saline controls (data not shown).

Leukocyte Rolling (V3): Similar to V1 venules, LPS administration caused significant ($p < 0.05$) reduction in the number of rolling leukocytes compared to controls in V3 venules (Figure 11). Administration of HU308, URB597, or JZL184 after LPS, did not significantly ($p > 0.05$) increase the number of rolling leukocytes compared to LPS. AM630 administration after LPS did not significantly ($p > 0.05$) further reduce the number of rolling leukocytes compared to LPS (Figure 11). Leukocyte rolling in V3 venules for all treatment control groups showed no difference to saline controls (data not shown).

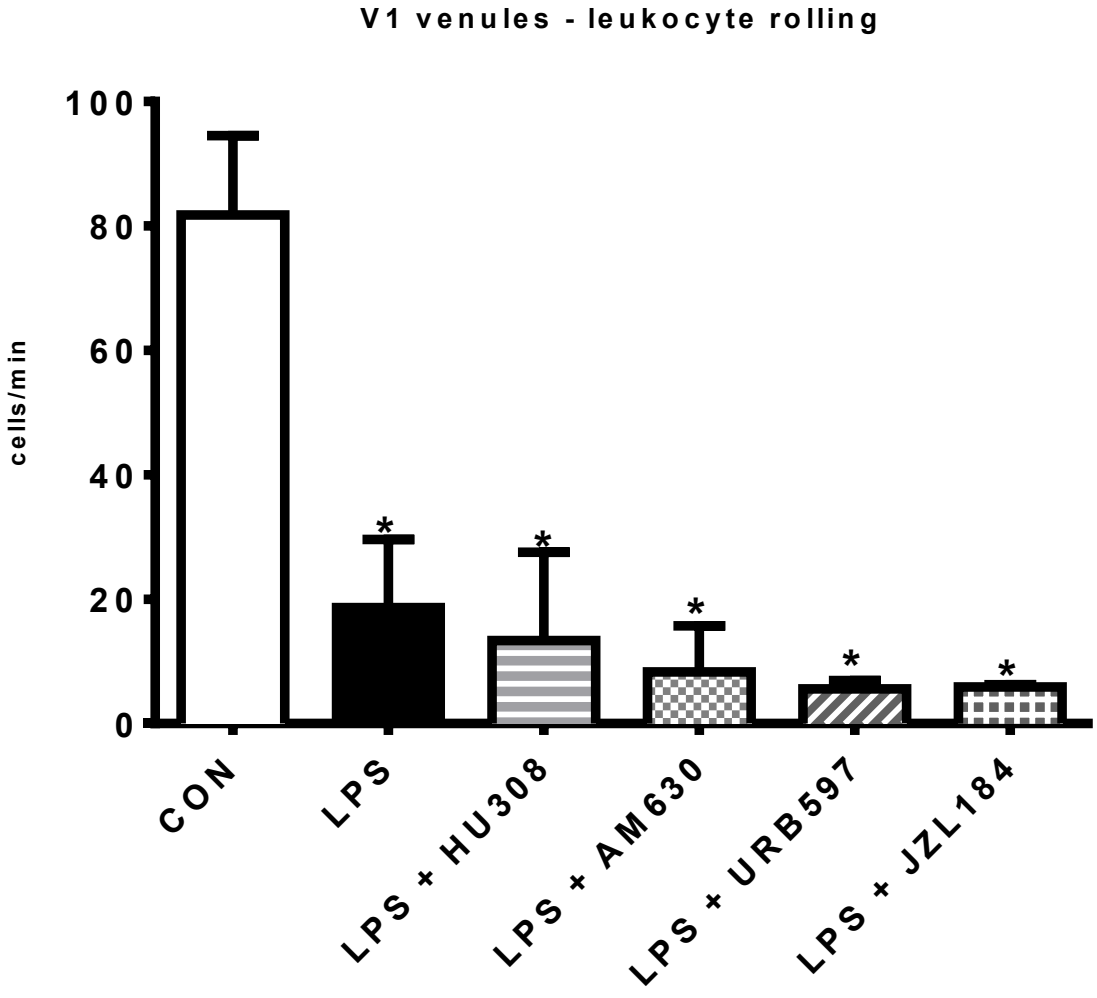

Figure 10: Leukocyte rolling in collecting venules (V1; >50μm vessel diameter). Control group (CON); endotoxemia group LPS (5 mg/kg); LPS + HU308 (2.5 mg/kg) a CB2 receptor agonist; LPS + AM630 (2.5mg/kg) a CB2 receptor antagonist/inverse agonist; LPS + URB597 (0.6mg/kg) a FAAH inhibitor; and LPS + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. $* p < 0.05$ versus control.

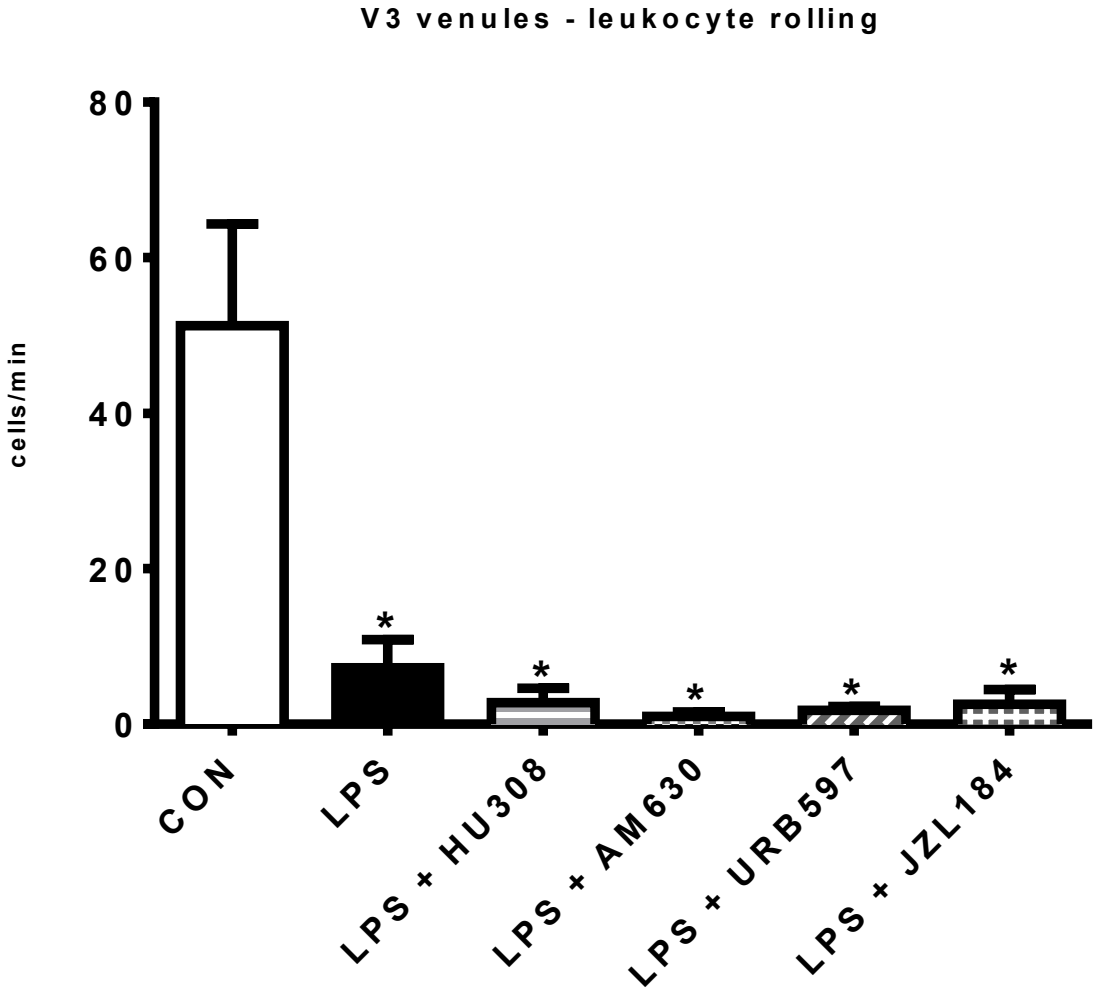

Figure 11: Leukocyte rolling in post capillary venules (V3; <50μm vessel diameter). Control group (CON); endotoxemia group LPS (5 mg/kg); LPS + HU308 (2.5 mg/kg) a CB2 receptor agonist; LPS + AM630 (2.5mg/kg) a CB2 receptor antagonist/inverse agonist; LPS + URB597 (0.6mg/kg) a FAAH inhibitor; and LPS + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. $* p < 0.05$ versus control.

Functional Capillary Density (Intestinal Muscle Layers): Administration of LPS did not significantly ($p > 0.05$) reduce functional capillary density compared to controls (Figure 12). Only AM630 administration after LPS was able to significantly ($p < 0.05$) reduce FCD compared to controls. HU308, URB597, or JZL184 administration after LPS did not significantly ($p > 0.05$) change FCD compared to LPS. JZL184 administration did show similar levels of FCD to controls, however they were not significantly ($p < 0.05$) different than LPS.

Functional Capillary Density (Mucosa): Administration of LPS significantly ($p > 0.05$) reduced functional capillary density in mucosal villi compared to controls (Figure 13). HU308, URB597, or JZL184 administration after LPS did not significantly ($p > 0.05$) improve FCD compared to LPS. AM630 administration after LPS did not significantly ($p < 0.05$) further reduce FCD compared to LPS (Figure 13). All treatment groups had significantly ($p < 0.05$) lower FCD compared to controls.

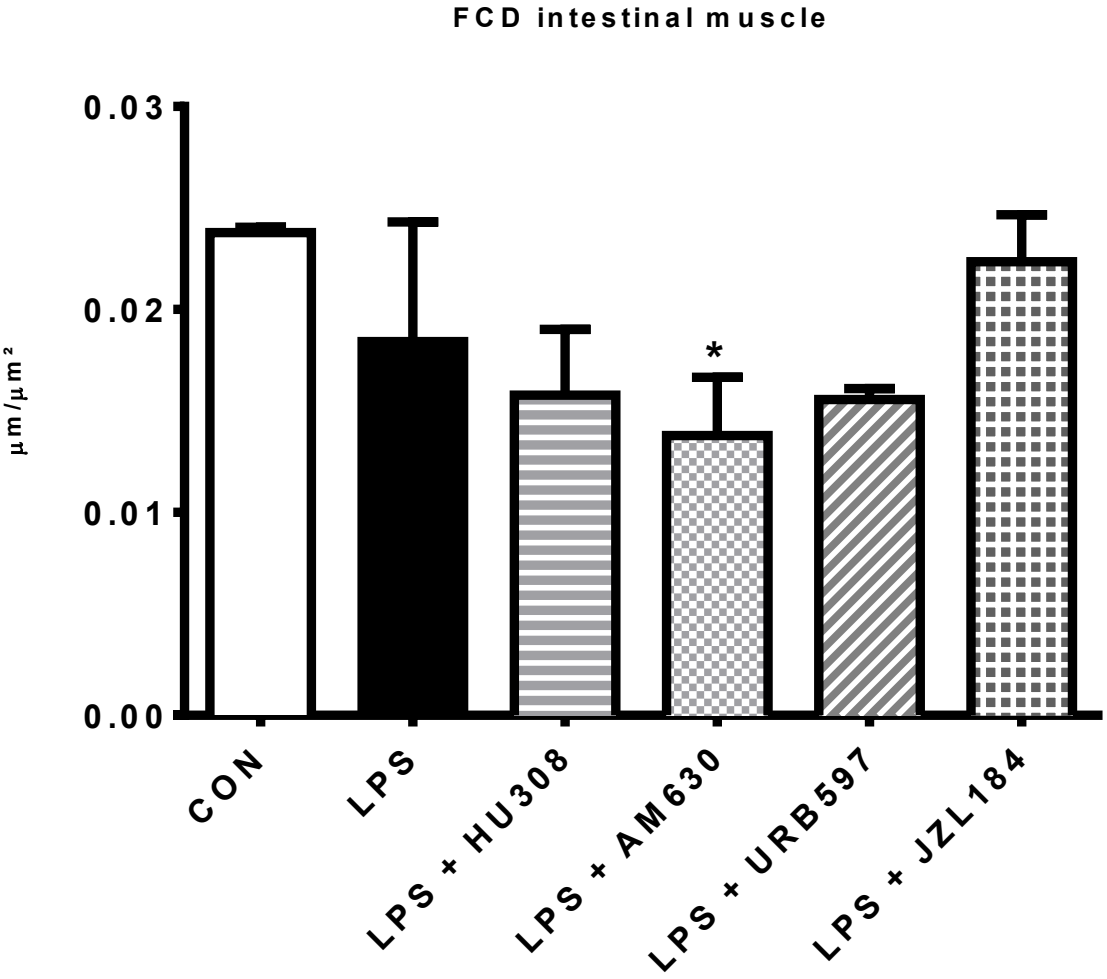

Figure 12: Capillary perfusion quantified through functional capillary density (FCD) within the submucosal muscle layers of the intestine. FCD quantified as total length of well perfused capillaries within a predetermined rectangular area of the observed field. Control group (CON); endotoxemia group LPS (5mg/kg); LPS + HU308 (2.5mg/kg), a CB2 receptor agonist; LPS + AM630 (2.5mg/kg), a CB2 receptor antagonist/inverse agonist; LPS + URB597 (0.6mg/kg), a FAAH inhibitor; and LPS + JZL184 (16mg/kg), a MAGL inhibitor. Data presented as mean ± standard deviation. $* p < 0.05$ versus control.

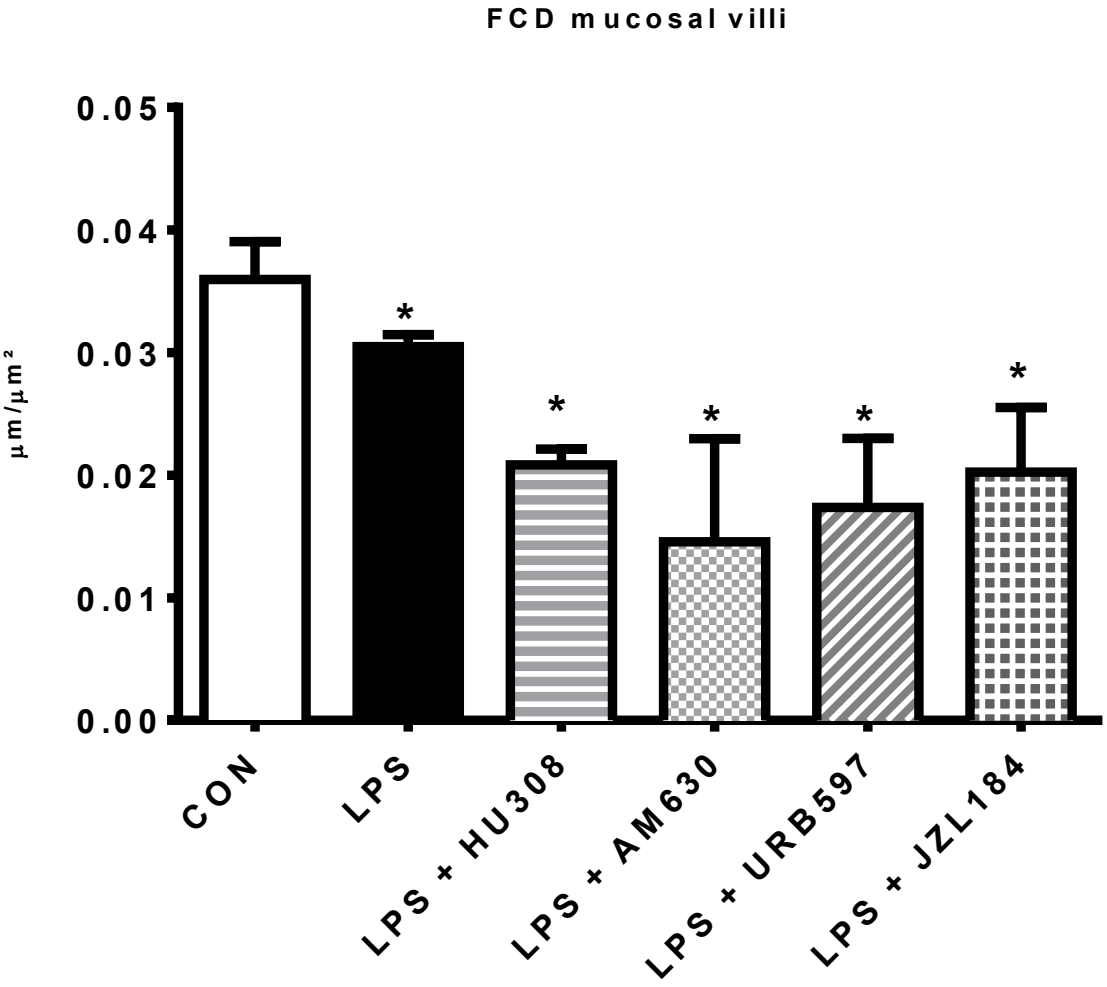

Figure 13: Capillary perfusion quantified through functional capillary density (FCD) within the mucosal villi of the intestinal lumen. FCD quantified as total length of well perfused capillaries within individual villi. Control group (CON); endotoxemia group LPS (5mg/kg); LPS + HU308 (2.5mg/kg), a CB2 receptor agonist; LPS + AM630 (2.5mg/kg), a CB2 receptor antagonist/inverse agonist; LPS + URB597 (0.6mg/kg), a FAAH inhibitor; and LPS + JZL184 (16mg/kg), a MAGL inhibitor. Data presented as mean ± standard deviation. $* p < 0.05$ versus control.

3.1.2 Multiplex assay

Circulating TNF-α levels were significantly ($p < 0.05$) elevated in the LPS group compared to controls (Figure 14). The treatment group JZL184 after LPS administration was able to significantly reduce the levels of TNF-α compared to the LPS group, but the levels were still significantly higher compared to controls (Figure 14).

IL-1β levels in mice were significantly elevated after LPS administration compared to controls (Figure 15). JZL184 treatment after LPS administration was unable to reduce levels of IL-1β compared to LPS. IL-1β levels were also significantly elevated compared to controls (Figure 15).

IL-6 levels were undetectable in the control group, and LPS administration significantly ($p < 0.05$) increased levels of circulating IL-6 compared to controls (Figure 16). JZL184 administration was unable to reduce the elevated levels of IL-6 caused by LPS. JZL184 treatment group also had significantly ($p < 0.05$) elevated levels of IL-6 compared to controls (Figure 16).

Baseline IL-10 levels in controls were evident, and LPS administration significantly ($p < 0.05$) elevated levels of IL-10 compared to controls (Figure 17). JZL184 administration after LPS did not significantly reduce levels of IL-10 compared to the LPS group, but there was a sufficient reduction which additionally made it also not significantly ($p > 0.05$) different than controls (Figure 17).

MIP-2 levels in the LPS group were significantly ($p < 0.05$) elevated compared to the control group (Figure 18). Treatment with JZL1844 significantly ($p < 0.05$) reduced MIP-2 levels compared to LPS, but they were still significantly ($p < 0.05$) higher compared to control (Figure 18).

IL-17A levels were elevated in the LPS group, but they were not significantly higher compared to controls (Figure 19). JZL184 treatment caused elevated levels of IL-17A, which was significant ($p < 0.05$) compared to both LPS and control groups (Figure 19).

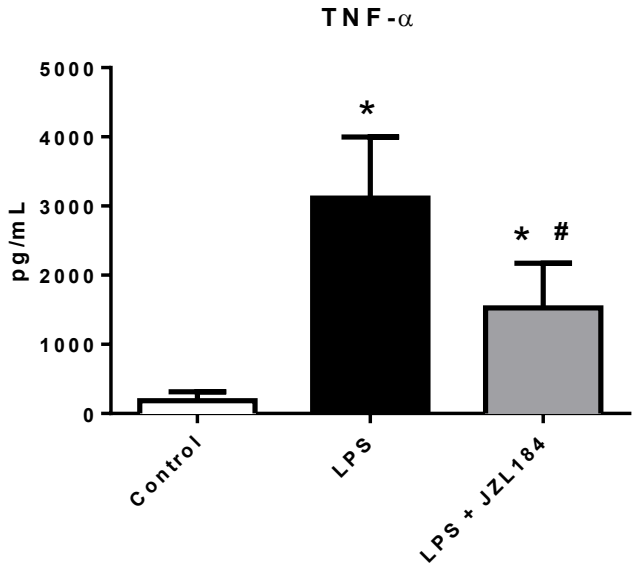

Figure 14: Circulating plasma concentrations (pg/mL) of TNF-α in mice. Groups include controls, LPS (5mg/kg), and LPS (5mg/kg) + JZL184 (16mg/kg). Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control. # $p < 0.05$ versus LPS.

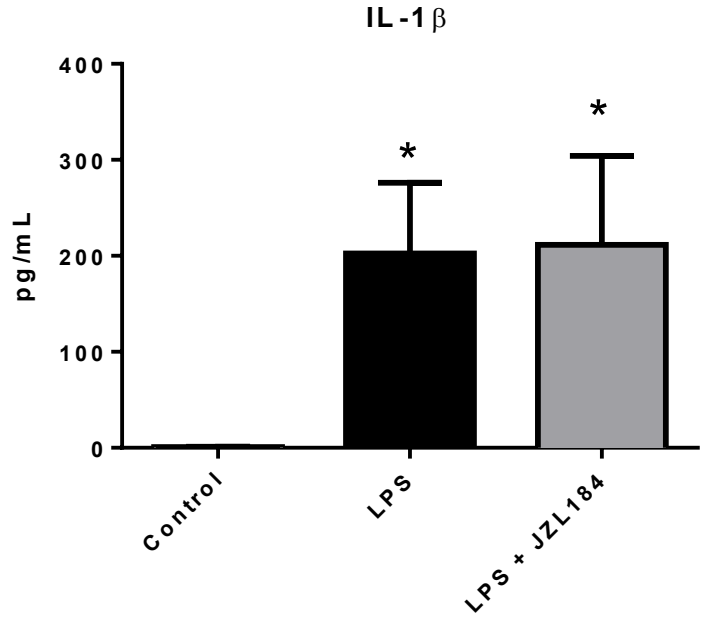

Figure 15: Circulating plasma concentrations (pg/mL) of IL-1β in mice. Groups include controls, LPS (5mg/kg), and LPS (5mg/kg) + JZL184 (16mg/kg). Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control.

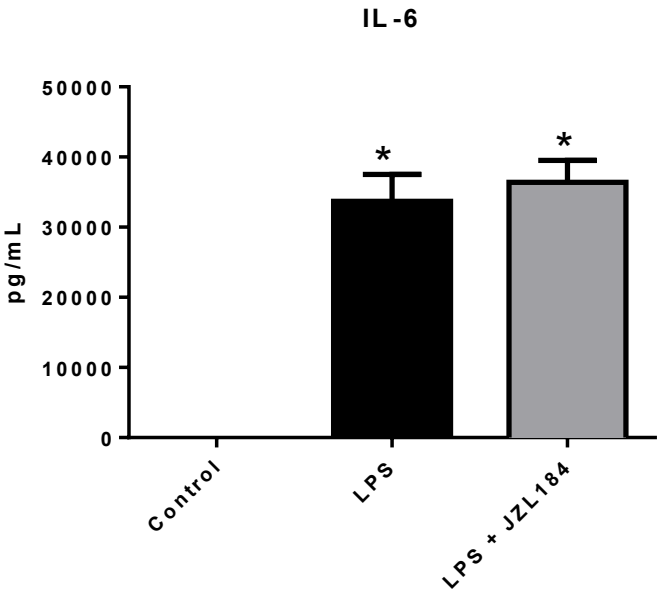

Figure 16: Circulating plasma concentrations (pg/mL) of IL-6 in mice. Groups include controls, LPS (5mg/kg), and LPS (5mg/kg) + JZL184 (16mg/kg). Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control.

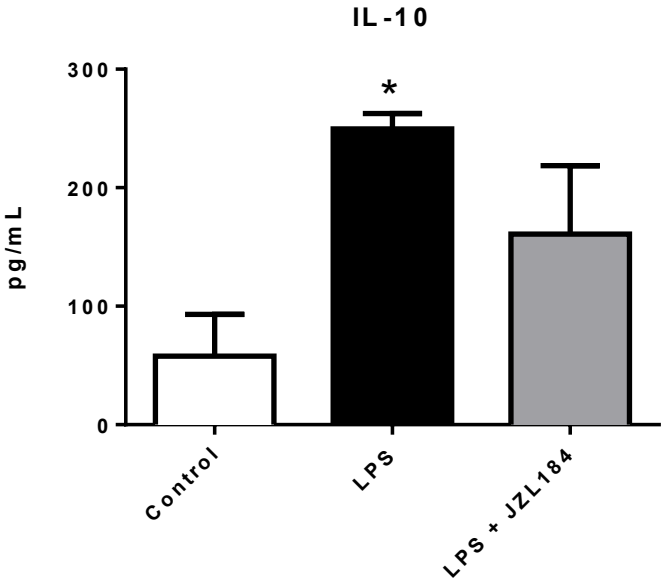

Figure 17: Circulating plasma concentrations (pg/mL) of IL-10 in mice. Groups include controls, LPS (5mg/kg), and LPS (5mg/kg) + JZL184 (16mg/kg). Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control.

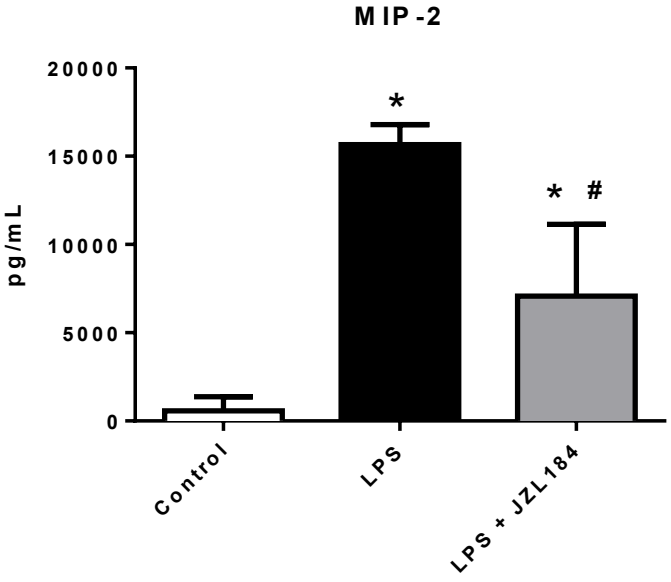

Figure 18: Circulating plasma concentrations (pg/mL) of MIP-2 in mice. Groups include controls, LPS (5mg/kg), and LPS (5mg/kg) + JZL184 (16mg/kg). Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control. # $p < 0.05$ versus LPS.

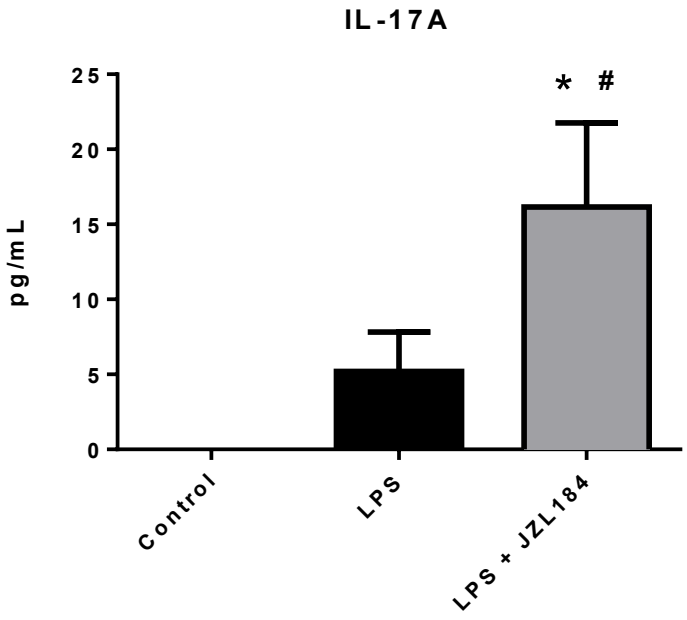

Figure 19: Circulating plasma concentrations (pg/mL) of IL-17a in mice. Groups include controls, LPS (5mg/kg), and LPS (5mg/kg) + JZL184 (16mg/kg). Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus control. # $p < 0.05$ versus LPS.

3.2 Colon ascendens stent peritonitis (CASP)

3.2.1 Intravital microscopy

Leukocyte Adherence (V1): Inducing abdominal sepsis through CASP surgery caused a significant ($p < 0.05$) increase in the number of adherent leukocytes on V1 venules compared to controls within 6 hours of observation time (Figure 20). Administration of the vehicle control DMSO after CASP surgery did not significantly ($p > 0.05$) alter the levels of adherent leukocytes in V1 venules compared to CASP surgery alone. Administration of JZL184 after CASP surgery significantly ($p < 0.05$) reduced the number of adherent leukocytes in V1 venules compared to CASP surgery alone (Figure 20). Furthermore, the number of adherent leukocytes in V1 venules after CASP surgery and JZL184 administration was not significantly ($p > 0.05$) different than controls.

Leukocyte Adherence (V3): CASP surgery also caused a significant ($p < 0.05$) increase in the number of adherent leukocytes in V3 venules compared to controls (Figure 21). Administration of the vehicle control DMSO after CASP surgery did not significantly ($p > 0.05$) alter the levels of adherent leukocytes in V3 venules compared to CASP surgery alone. Administration of JZL184 after CASP surgery did not significantly ($p > 0.05$) reduce the number of adherent leukocytes in V3 venules compared to CASP surgery alone. However, JZL184 administration did show a trend towards improvement as the number of adherent leukocytes in V3 venules was also low enough to be not significantly different than controls (Figure 21).

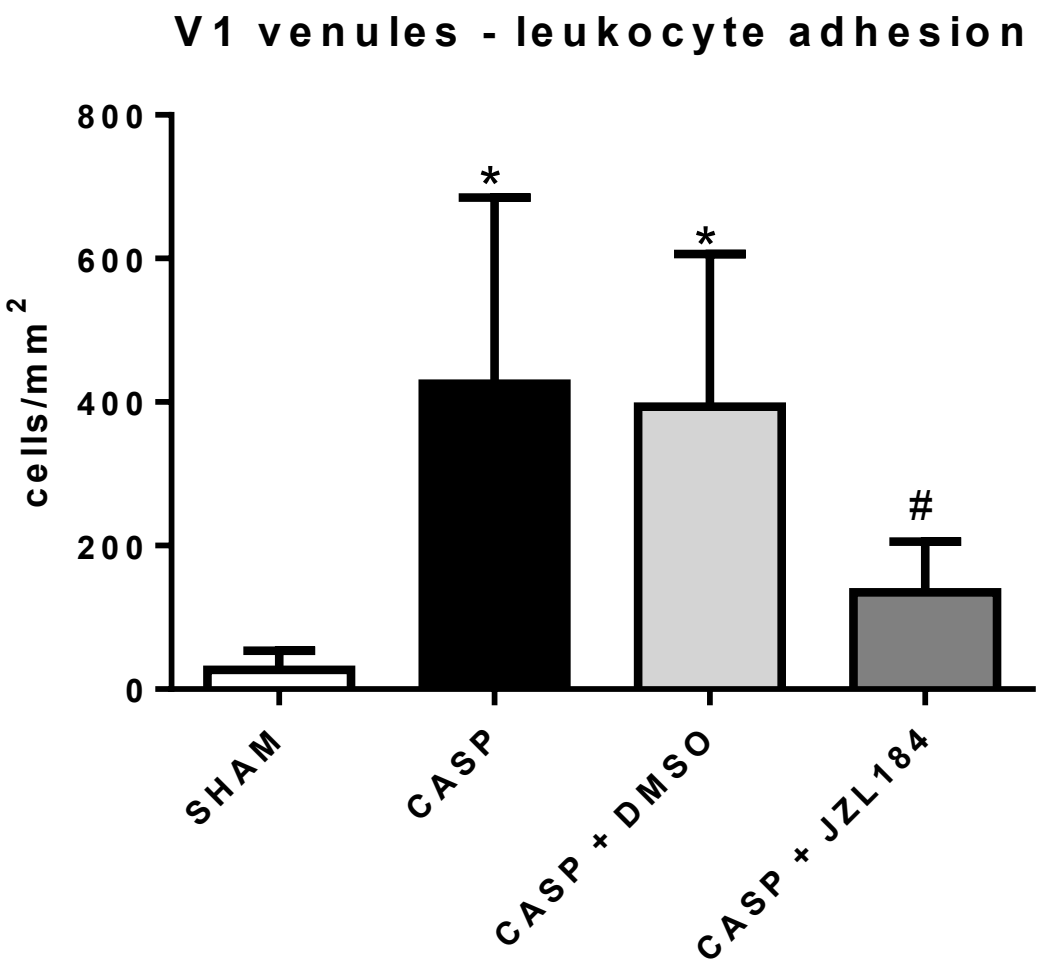

Figure 20: Leukocyte adhesion in collecting venules (V1; >50μm vessel diameter). Groups include: control surgery (SHAM); Colon ascendens stent peritonitis surgery (CASP); CASP + DMSO (vehicle control); CASP + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus SHAM. # $p < 0.05$ versus CASP.

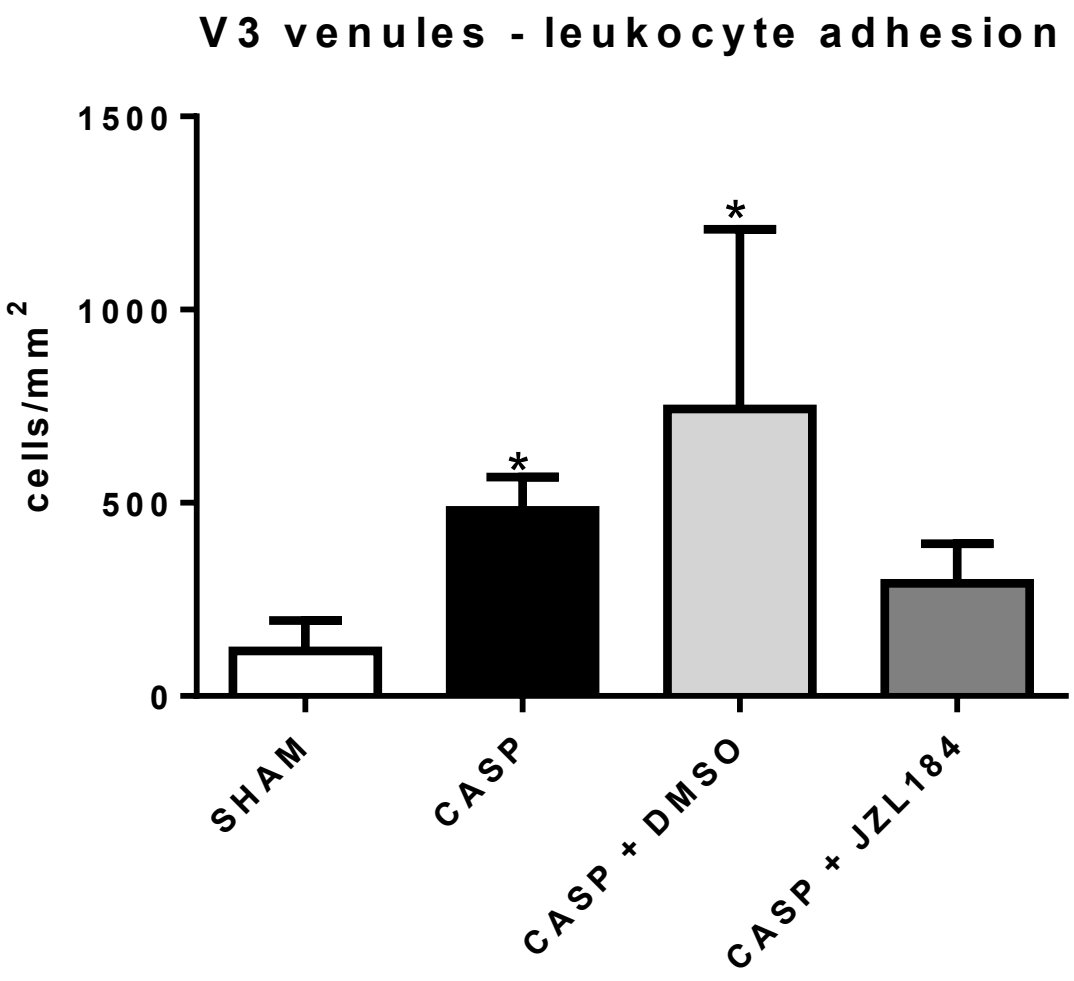

Figure 21: Leukocyte adhesion in post capillary venules (V3; <50μm vessel diameter). Groups include: control surgery (SHAM); Colon ascendens stent peritonitis surgery (CASP); CASP + DMSO (vehicle control); CASP + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus SHAM.

Leukocyte Rolling (V1): CASP surgery significantly reduced the number of rolling leukocytes in V1 venules compared to SHAM controls (Figure 22). Administration of vehicle control DMSO after CASP surgery did not significantly ($p > 0.05$) alter the number of rolling leukocytes in V1 venules compared to CASP surgery alone. Administration of JZL184 after CASP surgery did not significantly ($p > 0.05$) increase the number of rolling leukocytes in V1 venules compared to CASP surgery alone (Figure 22).

Leukocyte Rolling (V3): Similar results to V1 venules were seen in V3 venules for rolling leukocyte counts. CASP surgery significantly ($p < 0.05$) reduced the number of rolling leukocytes in V3 venules compared to SHAM controls (Figure 23). Administering the vehicle control DMSO after CASP surgery did not significantly ($p > 0.05$) alter the number of rolling leukocytes in V3 venules compared to CASP surgery alone. Administration of JZL184 after CASP surgery did not significantly ($p > 0.05$) increase the number of rolling leukocytes in V3 venules compared to CASP surgery alone (Figure 23).

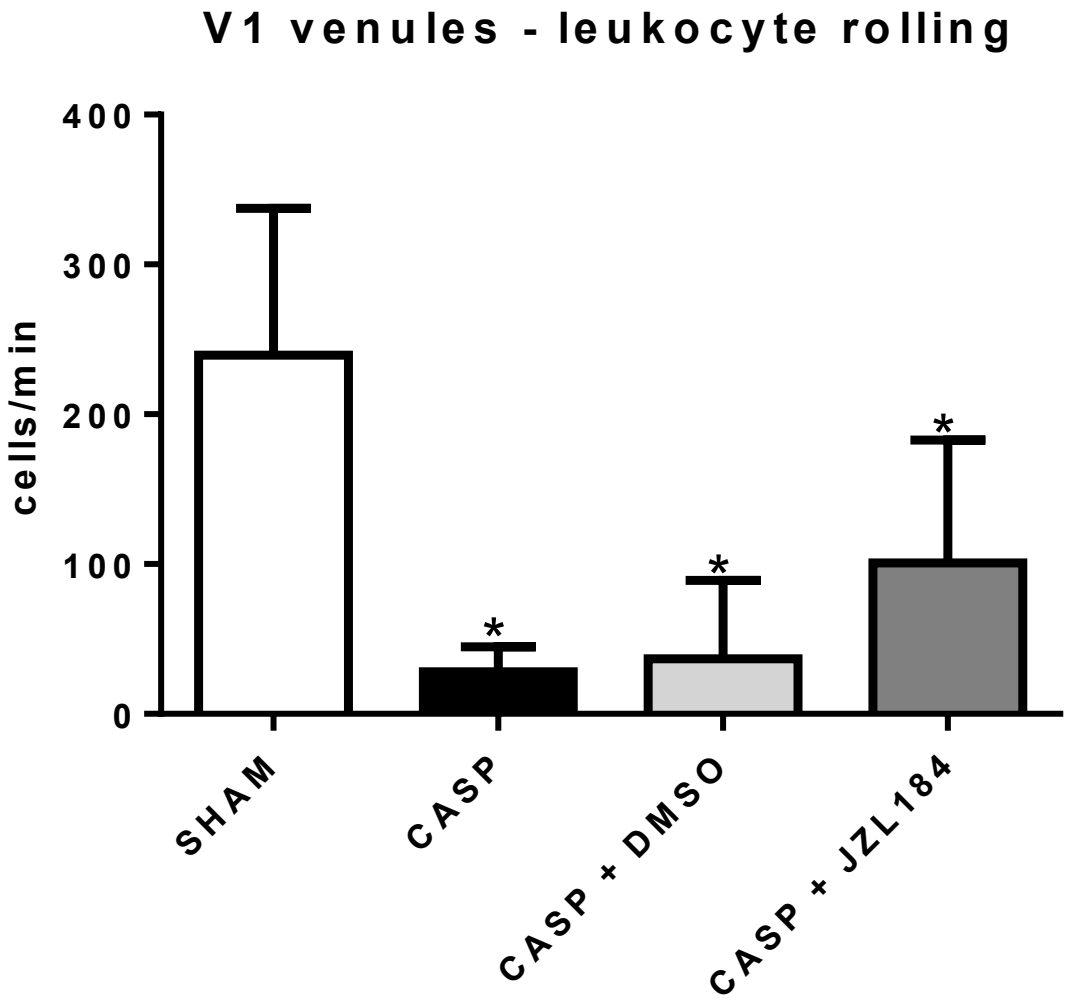

Figure 22: Leukocyte rolling in collecting venules (V1; >50μm vessel diameter). Groups include: control surgery (SHAM); Colon ascendens stent peritonitis surgery (CASP); CASP + DMSO (vehicle control); CASP + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. $*$ $p < 0.05$ versus SHAM.

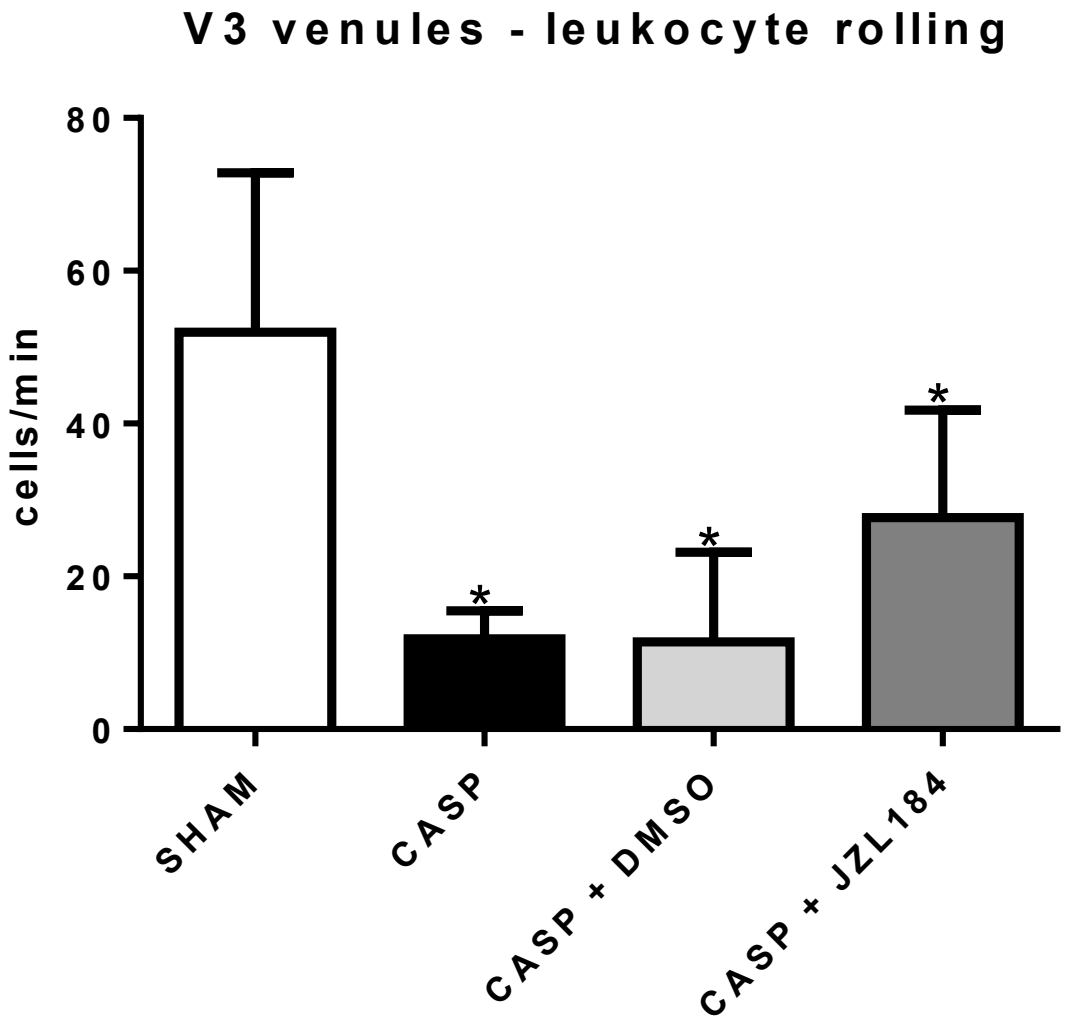

Figure 23: Leukocyte rolling in post capillary venules (V3; <50μm vessel diameter). Groups include: control surgery (SHAM); Colon ascendens stent peritonitis surgery (CASP); CASP + DMSO (vehicle control); CASP + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus SHAM.

Functional Capillary Density (Muscle Layers): In the muscle layers, abdominal CASP surgery did not significantly ($p > 0.05$) reduce functional capillary density compared to SHAM controls (Figure 24). Administration of vehicle control (DMSO) after CASP surgery did not significantly ($p > 0.05$) alter the functional capillary density of the intestinal muscle layers compared to CASP surgery alone. Administration of JZL184 after CASP surgery showed similar levels of muscular FCD to controls, but was not significantly ($p > 0.05$) different than CASP surgery alone (Figure 24).

Functional Capillary Density (Mucosa): In the intestinal mucosal villi, CASP surgery significantly ($p < 0.05$) reduced the FCD compared to SHAM surgery (Figure 25). Administration of vehicle control (DMSO) after CASP surgery did not significantly ($p > 0.05$) alter the functional capillary density of the intestinal muscle layers compared to CASP surgery alone. Administration of JZL184 after CASP surgery showed significant ($p < 0.05$) improvement in mucosal villi FCD compared to CASP alone, and was not significantly ($p > 0.05$) different than the SHAM controls (Figure 25).

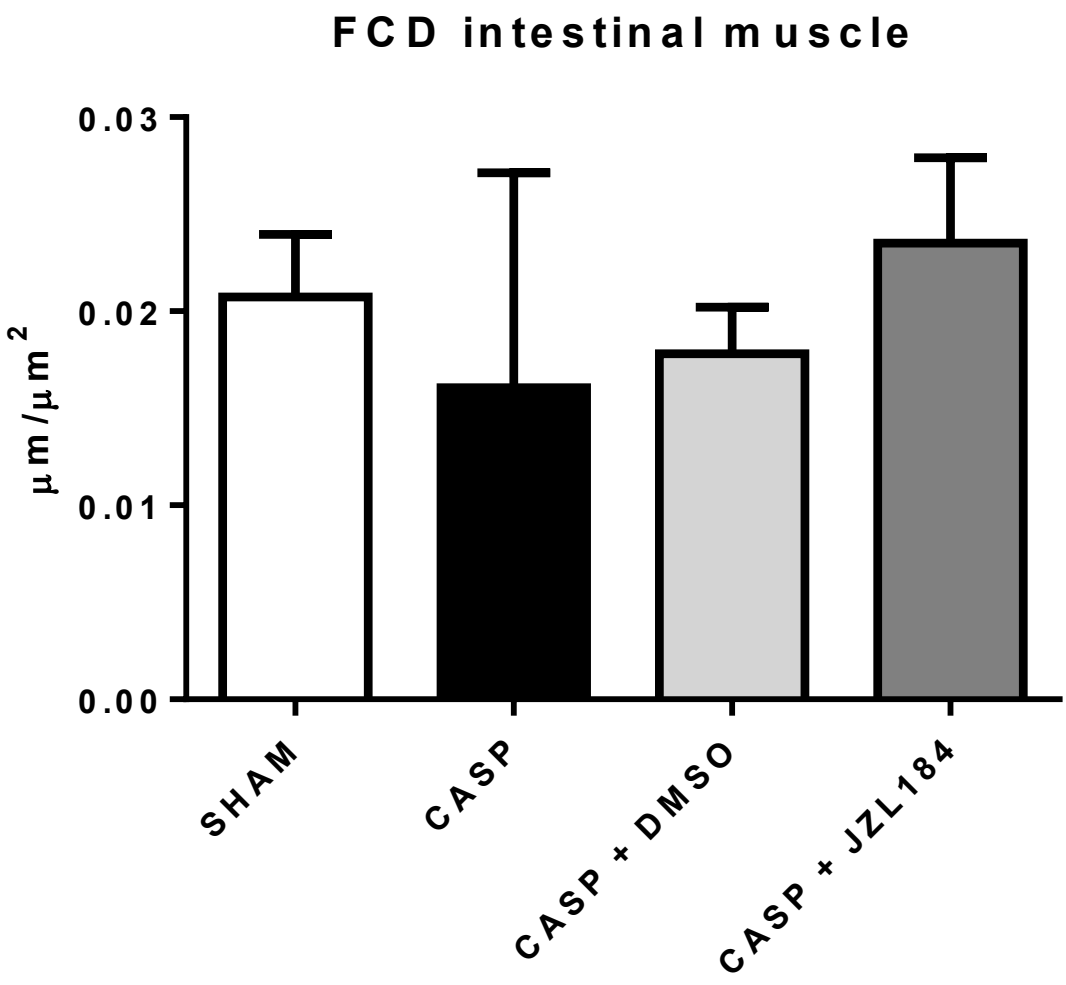

Figure 24: Capillary perfusion quantified through functional capillary density (FCD) within the intestinal submucosal muscle layers. FCD quantified as total length of well perfused capillaries within a predetermined rectagular area of the visible field. Groups include: control surgery (SHAM); Colon ascendens stent peritonitis surgery (CASP); CASP + DMSO (vehicle control); CASP + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation.

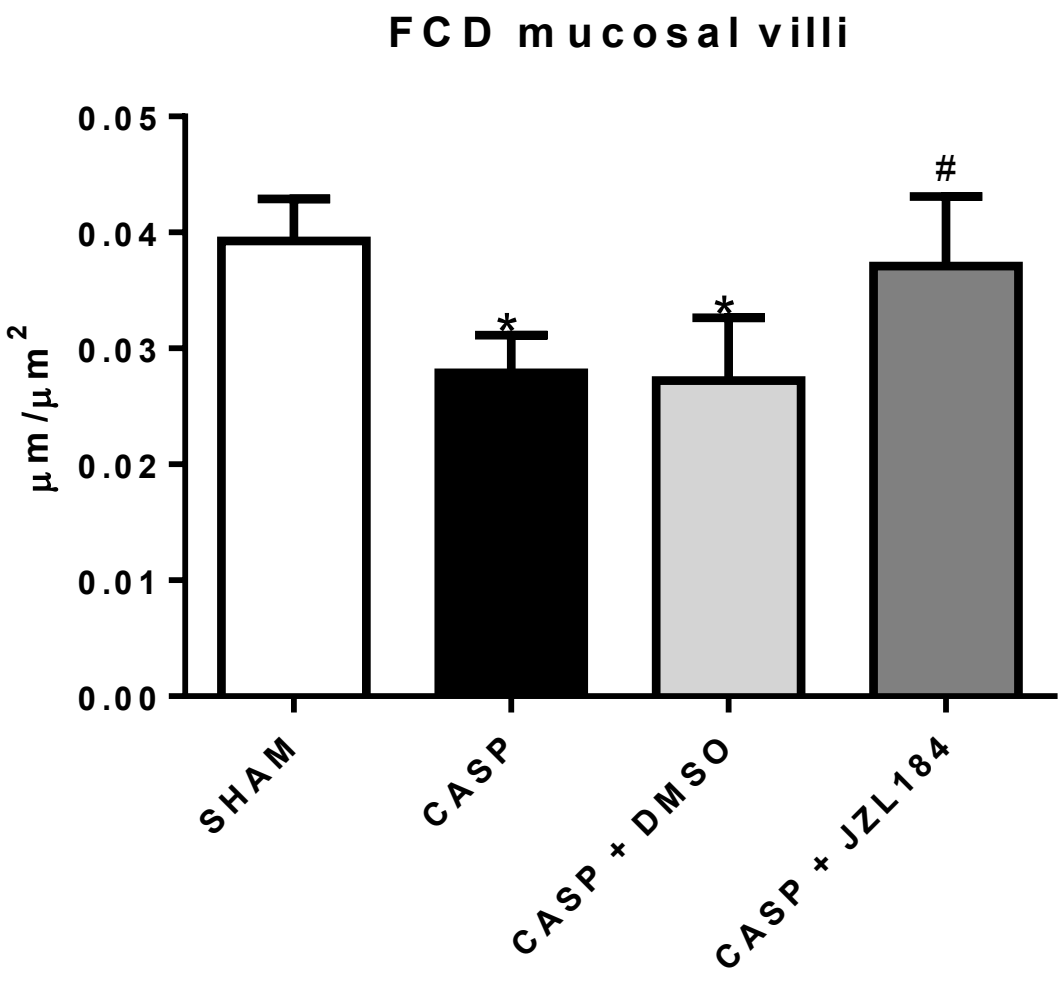

Figure 25: Capillary perfusion quantified through functional capillary density (FCD) within the mucosal villi of the intestinal lumen. FCD quantified as total length of well perfused capillaries within individual villi. Groups include: control surgery (SHAM); Colon ascendens stent peritonitis surgery (CASP); CASP + DMSO (vehicle control); CASP + JZL184 (16mg/kg) a MAGL inhibitor. Data presented as mean ± standard deviation. ∗ $p < 0.05$ versus SHAM. # $p < 0.05$ versus CASP.

3.2.2 Quantitative RT-PCR

Cannabinoid receptor 2 mRNA expression: The expression levels of CB_2 mRNA in the terminal ileum of mice subject to CASP was minimal. The treatment of mice with JZL184 after CASP was unable to significantly ($p > 0.05$) alter the expression levels of CB_2 mRNA in the terminal ileum (Figure 26). The expression levels of CB_2 mRNA in the spleen of mice subject to CASP was relatively higher compared to the intestine. Mice treated with JZL184 after CASP were unable to significantly ($p > 0.05$) alter the expression level of CB_2 mRNA in the spleen (Figure 26).

NF-κB p65 mRNA expression: The expression levels of NF-κB p65 mRNA in the terminal ileum of mice subject to CASP was evident, and the treatment of mice with JZL184 after CASP was unable to significantly ($p > 0.05$) alter the expression levels of NF-κB p65 mRNA in the terminal ileum (Figure 27). Similar levels of NF-κB p65 mRNA expression in the spleen of mice subject to CASP was evident compared to the intestine. Mice treated with JZL184 after CASP were also unable to significantly ($p > 0.05$) alter the expression level of NF-κB p65 mRNA in the spleen (Figure 27).

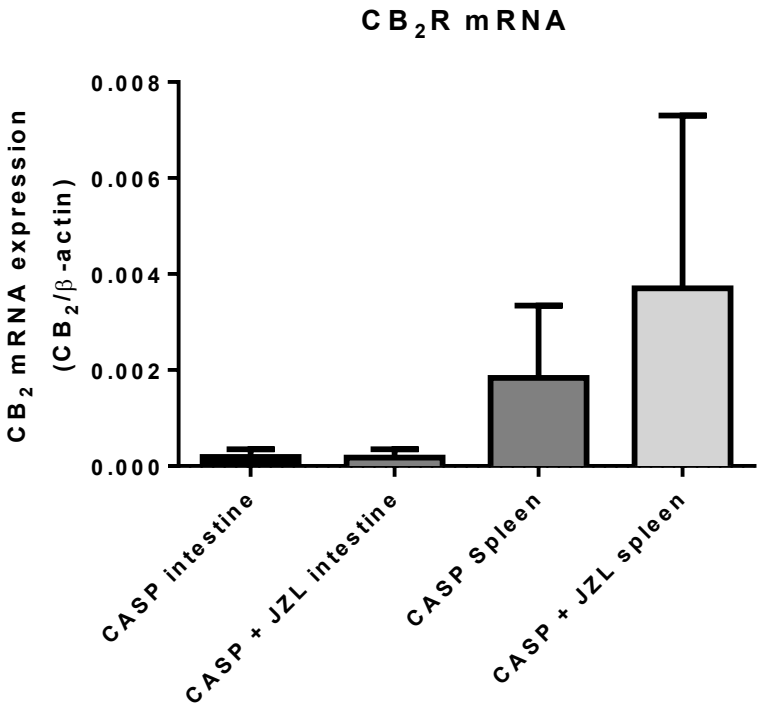

Figure 26: CB_2 receptor mRNA levels from whole spleen and intestinal terminal ileum tissue quantified via qRT-PCR and normalized to sample matched β-actin mRNA levels. Data presented as mean ± standard deviation.

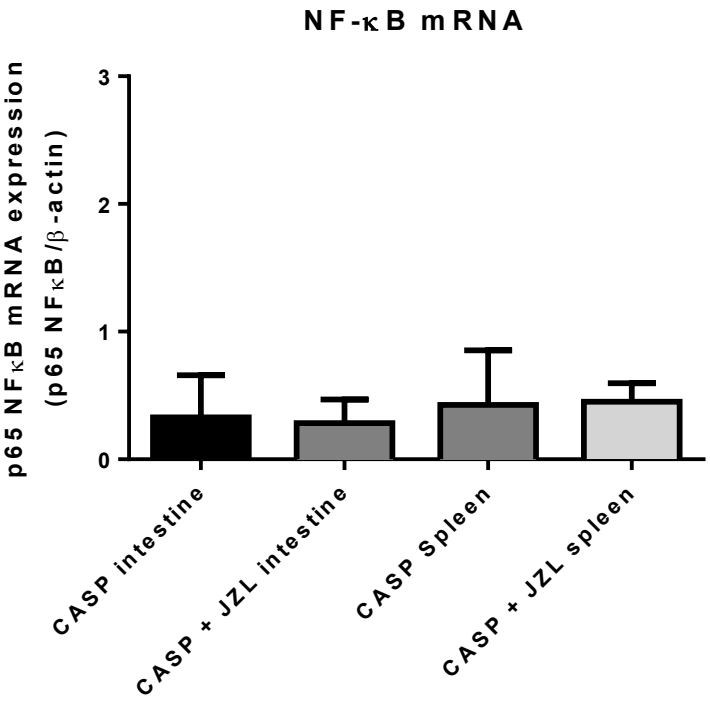

Figure 27: NF-κB p65 subunit mRNA levels from whole spleen and intestinal terminal ileum tissue quantified via qRT-PCR and normalized to sample matched β-actin mRNA levels. Data presented as mean ± standard deviation.

3.3 Endotoxemia in cannabinoid receptor 2 knockout mice (CB_2-/-)

3.3.1 Intravital microscopy

Leukocyte Adherence (V1): Induction of endotoxemia in CB_2R -/- mice through the administration of LPS caused a significant ($p < 0.05$) increase in the number of adherent leukocytes in V1 venules compared to controls (Figure 28). Administration of JZL184 after LPS showed a trend towards a reduction, however the number of adherent leukocytes in V1 venules were not significantly ($p > 0.05$) different than the LPS group. Furthermore, the number of adherent leukocytes in V1 venules of the JZL184 group were not significantly ($p > 0.05$) different than the control group (Figure 28).

Leukocyte Adherence (V3): LPS administration caused a significant ($p < 0.05$) increase in the number of adherent leukocytes in V3 venules compared to controls (Figure 29). Administration of JZL184 after LPS showed a significant ($p < 0.05$) reduction in the number of adherent leukocytes in the V3 venules compared to LPS alone. Furthermore, the number of adherent leukocytes in V3 venules were also significantly ($p < 0.05$) higher compared to controls (Figure 29)

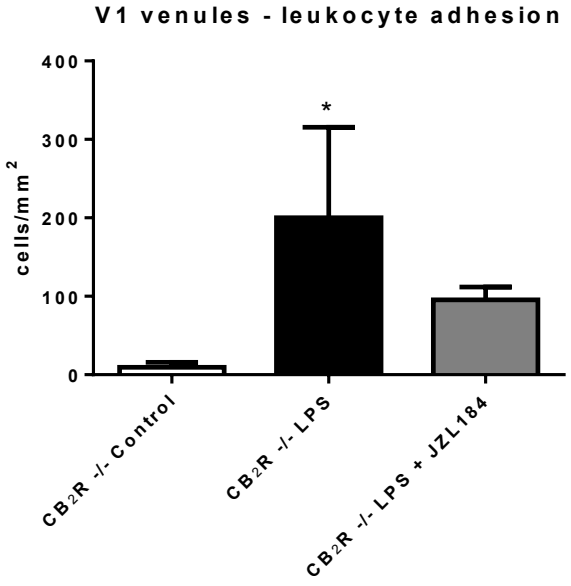

Figure 28: Leukocyte adhesion in collecting venules (V1; >50µm vessel diameter). Groups include: control CB_2 receptor -/- mice ; Endotoxemia group LPS (5 mg/kg) in CB_2 receptor -/- mice; and LPS (5 mg/kg) + JZL184 (16 mg/kg) in CB_2 receptor -/- mice. Data presented as mean ± standard deviation. * $p < 0.05$ versus control.

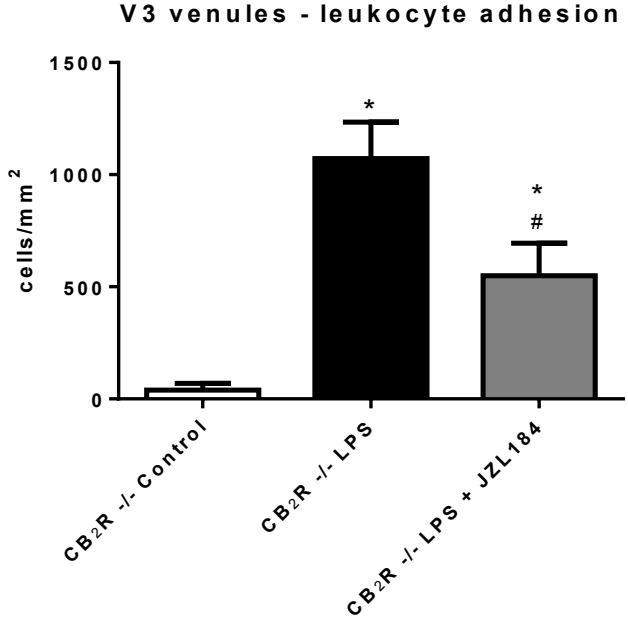

Figure 29: Leukocyte adhesion in post capillary venules (V3; <50µm vessel diameter). Groups include: control CB_2 receptor -/- mice ; Endotoxemia group LPS (5 mg/kg) in CB_2 receptor -/- mice; and LPS (5 mg/kg) + JZL184 (16 mg/kg) in CB_2 receptor -/- mice. Data presented as mean ± standard deviation. * $p < 0.05$ versus control. # $p < 0.05$ versus LPS.

Leukocyte Rolling (V1): Similar to W.T. mice, LPS administration in CB_2R -/- mice caused a significant ($p < 0.05$) reduction in the number of rolling leukocytes in V1 venules compared to controls (Figure 30). JZL184 administration after LPS did not significantly ($p > 0.05$) improve the levels of leukocyte rolling compared to LPS alone in V1 venules.

Leukocyte Rolling (V3): Similar to the V1 venules, LPS administration caused a significant ($p < 0.05$) reduction in the number of rolling leukocytes in V3 venules compared to controls (Figure 31). JZL184 administration after LPS did not significantly ($p > 0.05$) improve the levels of leukocyte rolling in V3 venules compared to LPS alone.

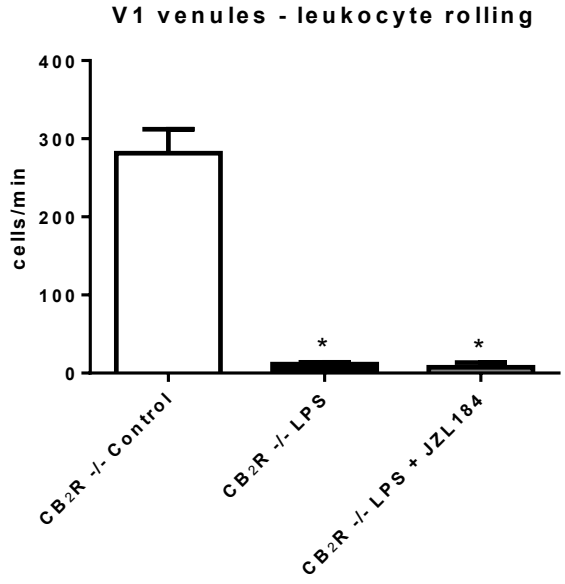

Figure 30: Leukocyte rolling in collecting venules (V1; >50μm vessel diameter). Groups include: control CB_2 receptor -/- mice ; Endotoxemia group LPS (5 mg/kg) in CB_2 receptor -/- mice; and LPS (5 mg/kg) + JZL184 (16 mg/kg) in CB_2 receptor -/- mice. Data presented as mean ± standard deviation. * $p < 0.05$ versus control.

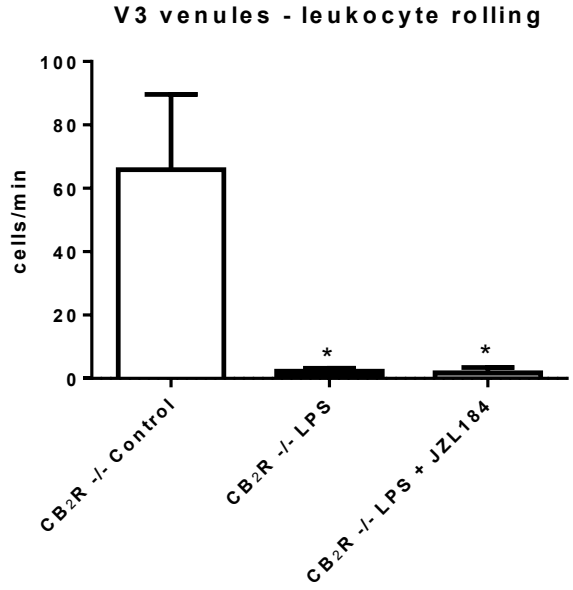

Figure 31: Leukocyte rolling in post capillary venules (V3; <50μm vessel diameter). Groups include: control CB_2 receptor -/- mice ; Endotoxemia group LPS (5 mg/kg) in CB_2 receptor -/- mice; and LPS (5 mg/kg) + JZL184 (16 mg/kg) in CB_2 receptor -/- mice. Data presented as mean ± standard deviation. * $p < 0.05$ versus control.

Functional Capillary Density (Muscle Layers): In CB_2R -/- mice, LPS administration did not significantly ($p > 0.05$) reduce the FCD in the muscle layer compared to the control group (Figure 32). JZL184 administration after LPS did not significantly ($p > 0.05$) alter the intestinal muscular FCD compared to the LPS group (Figure 32).

Functional Capillary Density (Mucosa): LPS administration significantly ($p < 0.05$) reduced the FCD in the mucosal villi compared to the control group (Figure 33). JZL184 administration after LPS did not significantly ($p > 0.05$) improve the FCD in the mucosal villi compared to LPS. Furthermore, the mucosal villi FCD after LPS and JZL184 administration was significantly ($p < 0.05$) lower than the control group (Figure 33).

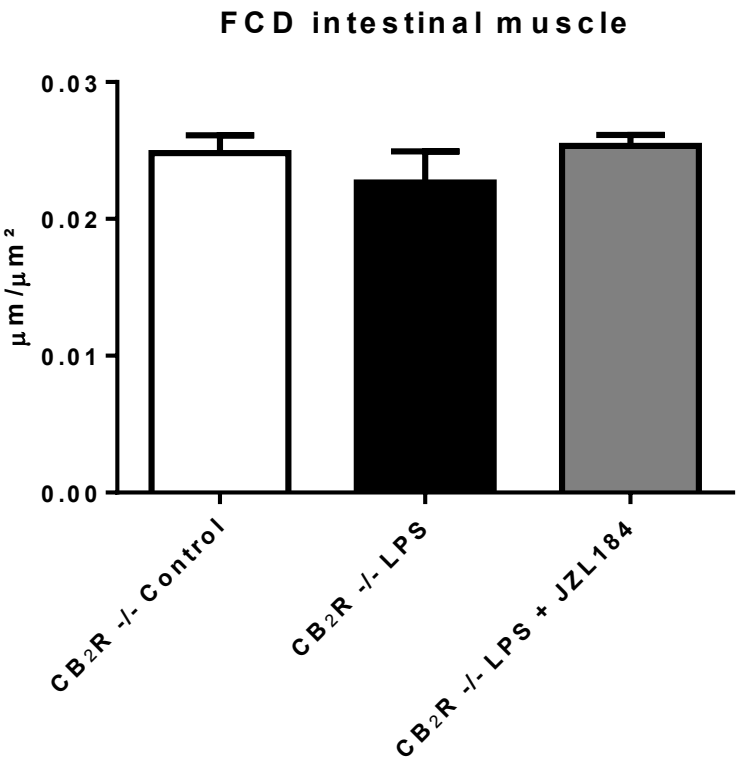

Figure 32: Capillary perfusion quantified through functional capillary density (FCD) within the intestinal submucosal muscle layers. FCD quantified as total length of well perfused capillaries within a predetermined rectagular area of the visible field. Groups include: control CB_2 receptor -/- mice ; Endotoxemia group LPS (5 mg/kg) in CB_2 receptor -/- mice; and LPS (5 mg/kg) + JZL184 (16 mg/kg) in CB_2 receptor -/- mice. Data presented as mean ± standard deviation.

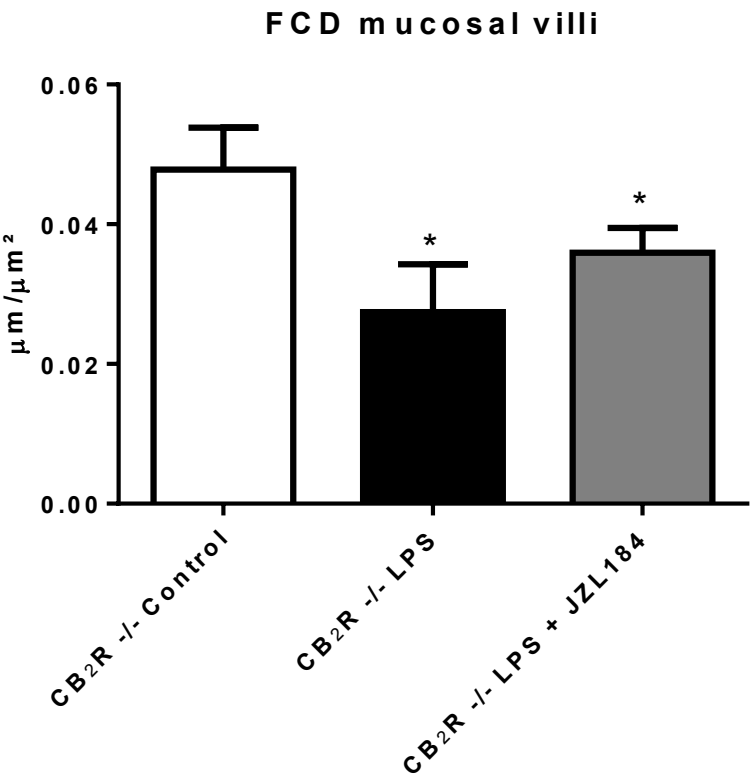

Figure 33: Capillary perfusion quantified through functional capillary density (FCD) within the mucosal villi of the intestinal lumen. FCD quantified as total length of well perfused capillaries within individual villi. Groups include: control CB_2 receptor -/- mice ; Endotoxemia group LPS (5 mg/kg) in CB_2 receptor -/- mice; and LPS (5 mg/kg) + JZL184 (16 mg/kg) in CB_2 receptor -/- mice. Data presented as mean ± standard deviation. * $p < 0.05$ versus control.

3.3.2 Quantitative RT-PCR

Cannabinoid receptor 2 mRNA expression: The expression levels of CB_2 mRNA in the terminal ileum and spleen of CB_2 knockout mice subject to endotoxemia was non-existent (Figure 34). The treatment of CB_2 knockout mice with JZL184 after LPS administration was unable to significantly ($p > 0.05$) alter the expression levels of CB_2 mRNA in the terminal ileum and spleen (Figure 34).

NF-κB p65 mRNA expression: The expression levels of NF-κB p65 mRNA in the terminal ileum of CB_2 knockout mice subject to endotoxemia was minimal, and the treatment of CB_2 knockout mice with JZL184 after LPS administration was unable to significantly ($p > 0.05$) alter the expression levels of NF-κB p65 mRNA in the terminal ileum (Figure 35). The levels of NF-κB p65 mRNA expression in the spleen of CB_2 knockout mice subject to endotoxemia was noticeably elevated compared to the intestine. CB_2 knockout mice treated with JZL184 after LPS showed a significant ($p < 0.05$) reduction in the expression level of NF-κB p65 mRNA within the spleen (Figure 35).

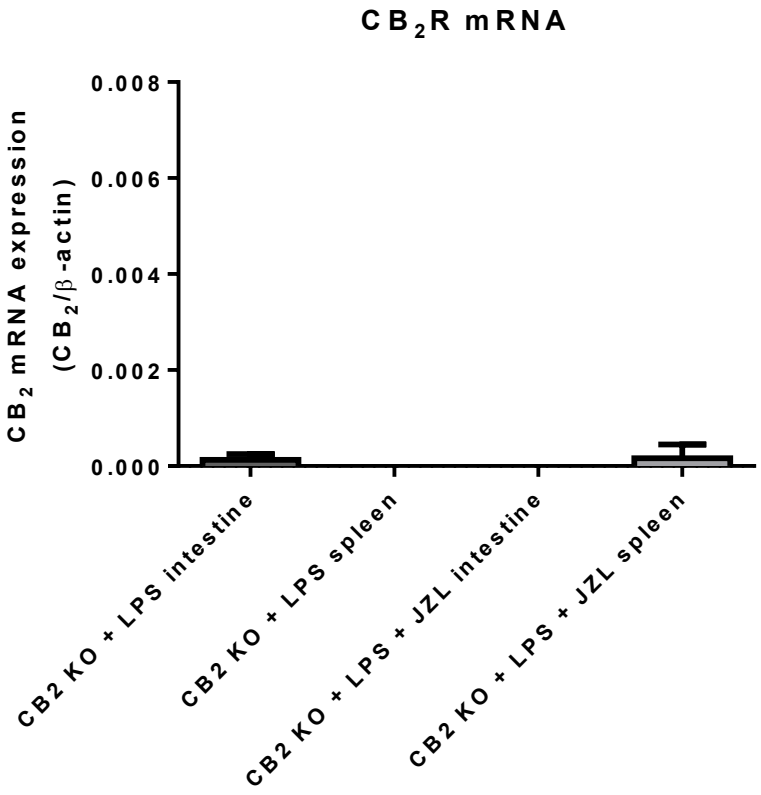

Figure 34: CB_2 receptor mRNA levels from whole spleen and intestinal terminal ileum tissue of CB_2 receptor knockout mice quantified via qRT-PCR and normalized to sample matched β-actin mRNA levels. Data presented as mean ± standard deviation.

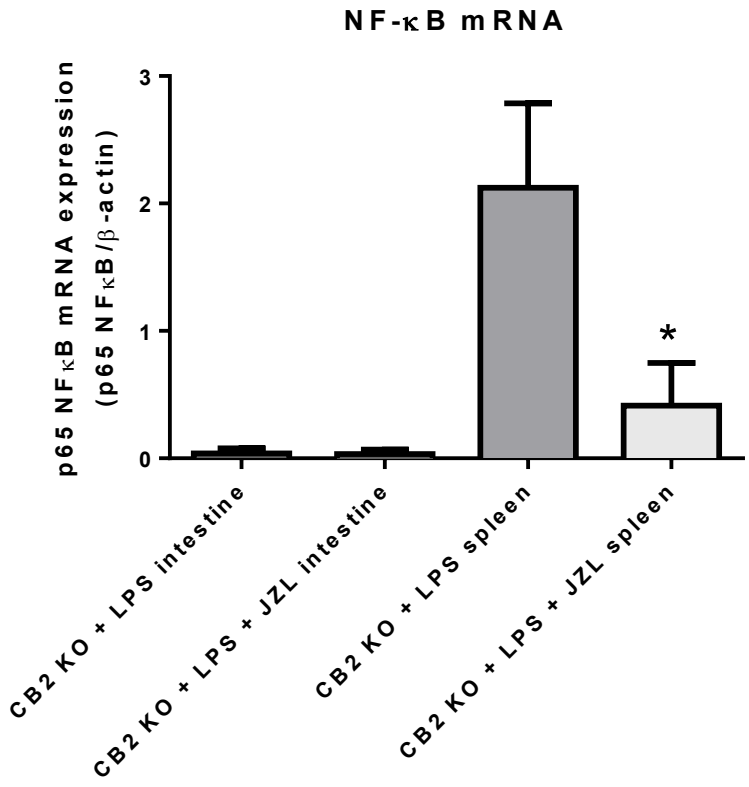

Figure 35: NF-κB p65 subunit mRNA levels from whole spleen and intestinal terminal ileum tissue of CB_2 receptor knockout mice quantified via qRT-PCR and normalized to sample matched β-actin mRNA levels. Data presented as mean ± standard deviation. $*$ $p < 0.05$ versus tissue matched LPS.

CHAPTER 4: DISCUSSION

4.1 General summary of results

The aim of our study was to investigate the potential therapeutic role of CB_2 receptor modulation in experimental sepsis, specifically with regard to changes that occur in the intestinal microcirculation. In general, it was hypothesized that activation of the CB_2 receptor would elicit their anti-inflammatory effects that would be beneficial in an acute setting of experimental sepsis where an excessive inflammatory response propagates the disease.

In the endotoxemia model, the various approaches employed to increase the activation of the CB_2 receptor all showed a benefit as they were able to ablate the LPS-induced increase in leukocyte recruitment within submucosal intestinal venules. Pro-inflammatory cytokine levels were elevated in the plasma of endotoxemic mice, however only JZL184 administration was able to attenuate the levels of TNF-α and MIP-2. These results indicate that CB_2 receptor activation during systemic inflammation can help minimize the excessive leukocyte activation, thereby minimizing subsequent microvascular damage.

According to our results from the endotoxemia experiments we continued our research in the CASP model with a focus on JZL184 administration to activate the CB_2 receptor. JZL184 administration after CASP surgery was able to reduce the peritonitis-induced increase in leukocyte-endothelial adhesion significantly. CB_2 receptor expression in the spleen was higher than in the intestine after CASP, however JZL184 administration did not significantly alter CB_2 mRNA levels. NF-κB p65 mRNA levels were similar in both spleen and intestine, and JZL184 had no impact on altering the expression levels of

this transcription factor in both tissues. Overall these results indicate that JZL184 administration does have some therapeutic benefit in reducing leukocyte endothelial adherence in an infectious inflammatory state.

Finally, we completed our studies using CB_2 receptor knockout mice looking once again specifically at JZL184 administration in experimental sepsis (endotoxemia model). We observed a modest reduction in leukocyte activation with JZL184 administration in the collecting venules and a significant reduction in leukocyte adherence in the post capillary venules. Furthermore, the mRNA levels of NF-κB p65 in the spleen were elevated after LPS administration and significantly reduced after JZL184 administration. Overall these results indicate that in CB_2 receptor knockout mice administered JZL184, there may be alternative CB_2 receptor independent mechanisms or off target effects that are employed to elicit the anti-inflammatory effects seen.

4.2 Endotoxemia model

Endotoxemia models have long been used to study various inflammatory diseases both *in vivo* and *in vitro* (Binion et al., 1997; Haraldsen et al., 1996; Miura & Fukumura, 1996). The endotoxin model is based on the premise that sepsis progression is caused by the exaggerated host's immune response to the systemic presence of pathogenic antigens, rather than the pathogen itself propagating the septic cascade (Deitch, 2005). Therefore, the administration of pathogenic endotoxins like lipopolysaccharide (LPS), which is the main pathogen associated molecular pattern (PAMP) of Gram-negative bacteria that is recognised by our immune system, are widely used to study the inflammatory response in septic conditions. The main advantage of the endotoxemia model is the consistent response elicited by the immune system to the systemic presence of pathogenic antigens.

The exaggerated release of inflammatory mediators like TNF-α and IL-1 are similar to the high levels of these same mediators seen in septic patients (D. G. Remick & Ward, 2005). Many studies showed that high levels of these inflammatory mediators were damaging to the host, and sustained levels of these mediators were responsible for organ injury and death in sepsis (D. G. Remick & Ward, 2005). Other lines of evidence also indicated the importance of these inflammatory mediators in sepsis. Higher levels of these mediators correlate with a higher rate of mortality, blocking these mediators in experimental endotoxin models of sepsis improved outcome, and direct administration of these inflammatory mediators induced similar pathological changes as seen in sepsis (D. G. Remick & Ward, 2005). Another advantage of endotoxin models are their relative ease of administration and their high rate of reproducibility. In experimental models of endotoxemic sepsis, the severity of induced inflammation can be easily controlled based on the dosage of administered toxin. Therefore we chose to first assess the role of modulating the activity of the CB_2 receptor in an endotoxemia inflammatory model.

4.2.1 Intravital microscopy

Intravital microscopy allowed us to observe leukocyte recruitment in the microcirculatory venules of the intestine in an *in vivo* setting after LPS and treatment administration. Assessment of inflammation by studying leukocyte-endothelial interactions has been well established in our lab with studies involving rat and mice models (Kianian, Al-banna, et al., 2013; Lehmann et al., 2011; Sardinha et al., 2014), and also assessed in various microcirculatory beds like the iris, the intestine, the brain and others (Brady, 1994; Jädert et al., 2012; Ramirez et al., 2012; Toguri et al., 2014). In this study we showed that intravenous administration of LPS (5 mg/kg) caused a significant

increase in the number of adherent leukocytes in both V1 and V3 venules compared to saline controls. A concomitant reduction in rolling leukocytes was also evident in V1 and V3 venules after LPS administration, indicating that the inflammatory stimuli caused most of the rolling leukocytes to adhere to the endothelial surface. It has been well established that LPS administration increases leukocyte adhesion to the endothelial cells in the intestinal microvasculature (N. Matsuda & Hattori, 2007; Miura & Fukumura, 1996; Salomao et al., 2012). This increase in leukocyte adhesion is mostly due to the increase in the expression of adhesion molecules, selectins, and integrins by both leukocytes and the endothelial cells once activated by bacterial antigens like LPS (Cinel & Opal, 2009; Haraldsen et al., 1996). Interestingly, studies using various inflammatory models have shown both increased (Ni et al., 2004; Zhang et al., 2007) or decreased (Hayes et al., 2004) leukocyte rolling compared to controls. This discrepancy may be attributable to the difference in local vs systemic inflammatory models used by the different studies. The local induction of inflammation will intuitively cause an increase in the number of rolling leukocytes to the site of infection, while in a systemic inflammatory model, the disseminated inflammation may cause more leukocyte adhesion systemically, thereby reducing leukocyte rolling. Furthermore, in our study, the significant reduction in leukocyte rolling may be indicative of the excessive immune activation caused by the dose of LPS administered, resulting in robust leukocyte adherence and minimal rolling.

Sepsis is characterised by alterations in the microcirculation, caused by altered capillary perfusion, eventually leading tissue ischemia and organ dysfunction (Ince, 2005). Alterations to capillary perfusion are thought to be caused by a variety of different mechanisms involved in systemic inflammation such as endothelial cell dysfunction,

altered red blood cell deformability, increased leukocyte recruitment, and activation of the coagulatory cascade (De Backer et al., 2011). Furthermore, a clinical study with septic patients indicated that among the microcirculatory variable measured, the proportion of perfused small vessels was the best predictor of survival outcome in septic patients (De Backer et al., 2013). Therefore, we also measured intestinal tissue perfusion and found LPS administration significantly reduced functional capillary density in the mucosal villi but was not significant in the muscle layers compared to controls. Excessive leukocyte adhesion during early stages of sepsis can disrupt normal endothelial function resulting in activation of various cascades (e.g. coagulation cascade) impacting capillary perfusion (Chong & Sriskandan, 2011; N. Matsuda & Hattori, 2007; Schouten et al., 2008). The lack of significant reduction in intestinal muscle capillary perfusion may be attributed to the short timeframe in our experiment. Since we observed capillary perfusion 2 hours after LPS administration, this short time frame may not have been sufficient to cause a significant disruption of the capillary perfusion in the intestinal muscle layer.

In our study we activated the CB_2 receptor after inducing an endotoxemic inflammatory state by administering the synthetic CB_2 receptor agonist HU308, which significantly reduced the levels of leukocyte adhesion in both V1 and V3 venules. In the V1 venules, the levels of leukocyte adherence were sufficiently reduced that they were not significantly different than controls. Adherent leukocytes in the V3 venules were reduced after HU308 administration compared to the LPS group, but they were still significantly elevated compared to controls. Studies using HU308 in various inflammatory conditions have shown a reduction in the number of infiltrating leukocytes

at different organs of the body (P. Mukhopadhyay, Rajesh, et al., 2010; Oka et al., 2006; Rajesh, Pan, et al., 2007). In these studies, HU308 administration reduced leukocyte adhesion by reducing chemokine and cytokine expression, expression of adhesion molecules, and activation of immune cells.

In our study, the number of rolling leukocytes after HU308 administration was not returned back to control levels in both V1 and V3 venules. The reduction in adherent leukocytes after HU308 administration was expected to cause a concomitant increase the levels of rolling leukocytes, however the number of rolling leukocytes were not significantly different than the LPS group. The most probable explanation for the reduced leukocyte adherence and rolling with HU308 administration is based on the suppression of adhesion molecules and prevention of leukocyte activation. In the control group basal levels of leukocyte rolling are achieved though the expression of selectin and integrins on leukocytes and endothelial cells. However, after HU308 administration, the activated CB_2 receptor pathway may cause a reduction in the expression of selectins, integrin, and adhesion molecules minimizing both the adherence and rolling of leukocytes. HU308 administration can also prevent leukocyte activation and the release of pro-inflammatory cytokines thereby minimizing the chemotactic ability of leukocytes subsequently lowering their rolling numbers.

Another potential benefit of activating the CB_2 receptor pathway in a septic state is minimizing the disruption of proper capillary perfusion in organs. Therefore we assessed the impact of HU308 administration in an endotoxemic state and found that functional capillary density in the mucosal villi were not significantly improved. The functional capillary density in the intestinal muscle layers was not significantly different

from either the LPS group or controls. This result may be explained by the heterogeneous nature of the septic state in activating a wide variety of inflammatory pathways that affect capillary perfusion, and activating the CB_2 receptor pathway might help minimize the effects of a few of these pathways, but not completely ablate them.

Since the CB_2 receptor has been implicated in minimizing leukocyte adhesion, we assessed the impact of blocking this receptor with an antagonist/inverse agonist during an endotoxemia state. We hypothesized that blocking the basal levels of CB_2 receptor activity would further exacerbate the inflammatory condition leading to higher levels of leukocyte adhesion. In our study, AM630 administration after LPS showed no significant elevation to the number or adherent leukocytes in both V1 and V3 venules compared to the LPS group, as well as no significant alterations to the number of rolling leukocytes in both V1 and V3 venules. This same phenomenon was observed in previous studies in our lab with rats (Kianian, Al-banna, et al., 2013; Lehmann et al., 2012) and it was attributed to the probable maximal leukocyte adherence elicited with the high dose of LPS administration that could not be further augmented by blocking the CB_2 receptor with AM630. Current literature assessing immune cell migration in different inflammatory diseases also support a role of the CB_2 receptor in inflammation which is attenuated with AM630 administration (Miller & Stella, 2008; Rajesh, Mukhopadhyay, et al., 2007; Romero-Sandoval et al., 2009). Overall, these studies show that the increased recruitment of immune cells during inflammatory conditions can be attenuated with the activation of the CB_2 receptor, while subsequent administration of antagonists specific to this receptor minimizes its anti-inflammatory effects. Further support for the role of the CB_2 receptor pathway in mediating inflammation comes from our assessment of capillary perfusion.

Administration of AM630 after LPS presumably blocked constitutive levels of CB_2 receptor activity and therefore further exacerbated the endotoxemia inflammatory response. Supporting this explanation was the observation that AM630 administration after LPS caused a significant decrease in functional capillary density in our intestinal muscle layer compared to controls, while LPS administration alone was unable to reduce FCD under the same parameters.

CB_2 receptor agonists as well as antagonists have been implicated in modulating leukocyte chemotaxis (Lunn et al., 2006). Various studies reviewed by Lunn et al (2006) have shown contradictory results showing enhanced or attenuated cellular migration with CB_2 receptor agonists or antagonists. There seems to be an intricate involvement of the cannabinoid receptors on the immune cells in determining their function. Modulating the activity of the CB_2 receptor at different stages of the inflammatory response may explain the discrepancy. Two unique steps are required by the immune system during an infection or insult. First, leukocytes are needed to localize to the site of injury, and secondly they need to be activated once at the insult. Therefore the role of activating or blocking the CB_2 receptor at these different stages may explain the contradictory roles of CB_2 receptor agonists and antagonists in cell migration assays. Lunn et al. (2006) propose that systemic CB_2 receptor agonist administration causes a dispersed chemotactic environment for the leukocytes therefore minimizing their specified migration to the site of insult. The elevated levels of CB_2 receptor agonist may increase nonspecific chemotactic movement of leukocytes as well as randomize cell polarity thereby ablating the accumulation of excessive leukocyte migration to sites of insult and minimize further exacerbating the inflammation. On the other hand, inverse agonist administration may

simply disrupt the normal chemotactic ability of leukocytes, thereby achieving a similar result as CB_2 receptor agonists, where leukocyte chemotaxis to the site of infection is minimized.

As an alternative approach to activate the CB_2 receptor, we decided to assess the impact of inhibiting the activity of the endogenous hydrolysing enzyme FAAH, which metabolizes anandamide. Inhibiting FAAH activity would elevate endogenous levels of anandamide thereby increasing and prolonging the activation of the CB_2 receptor. In our study, URB597 administration significantly reduced levels of leukocyte adherence after LPS administration in both V1 and V3 venules similar to effect seen with HU308 administration. The number of rolling leukocytes in V1 and V3 venules after LPS and URB597 administration were not returned to levels seen in the control group, which again may be explained by the effects of CB_2 receptor activation in reducing the expression of adhesion molecules. Similar to HU308 administration, URB597 was unable to significantly improve the functional capillary density after LPS administration in both the intestinal muscle layers and in the mucosal villi. Multiple studies have investigated the role of FAAH as a therapeutic target in treating pain and inflammation in a variety of diseases (Holt et al., 2005; Naidu et al., 2010; Nicotra et al., 2013; Schlosburg et al., 2009). Overall the results of these studies align with our findings where disruption of FAAH, either through selective blockers like URB597 or the generation of FAAH knockout mice, can be beneficial in an inflammatory state by reducing cellular infiltration, edema formation, release of pro-inflammatory cytokines, and tissue damage.

Another important endocannabinoid in activating the CB_2 receptor pathway is 2-AG, therefore we assessed the impact of inhibiting MAGL in an endotoxin model. 2-AG

and anandamide were shown to have similar affinities for the CB_2 receptor, however 2-AG has a greater efficacy, making it a full agonist of the CB_2 receptor, while anandamide is only a partial agonist (Gonsiorek et al., 2000; Sugiura et al., 2002). In our study, the MAGL inhibitor JZL184 was able to ablate the LPS induced leukocyte adhesion in both V1 and V3 venules similar to URB597 and HU308. Also similar to URB597 and HU308, JZL184 administration was unable to restore normal leukocyte rolling after LPS administration in V1 and V3 venules. Due to the relatively recent synthesis of JZL184 (Long et al., 2009), few studies have explored the effects of this compound in inflammatory damage. Overall, studies using JZL184 in inflammatory conditions like acute lung injury (Costola-de-Souza et al., 2013), inflammatory bowel disease (Alhouayek et al., 2011), hepatic ischemia/reperfusion injury (Z. Cao et al., 2013) have shown anti-inflammatory effects like reduced leukocyte infiltration, attenuated pro-inflammatory cytokine levels, and minimized tissue injury (Kohnz & Nomura, 2014). The beneficial effects of JZL184 in these different inflammatory conditions, support our findings that JZL184 was able to ablate the LPS induced leukocyte adhesion in intestinal submucosal venules. Furthermore, JZL184 administration was the only group to show a similar level of capillary perfusion in the intestinal muscle layers compared to the control group.

It may be more beneficial to modulate endocannabinoid levels rather than use synthetic agonists because they are synthesized and released at specific sites and act at local environments. As mentioned earlier, the use of various synthetic ligands can have variable affinities and efficacies for the CB_2 receptor (Atwood et al., 2012). Furthermore, they may bind to allosteric sites on the CB_2 receptor, therefore the specificity of the

downstream signalling cascades are much more variable when synthetic agonists are used. On the other hand blocking the endogenous metabolizing enzymes to elevate endocannabinoid levels confers a relatively lower level of variability due to the intrinsic activation of the CB_2 receptor with its natural ligands. Although the metabolism may be hindered by the administered enzyme blockers, other pharmacokinetic properties of the endocannabinoids will be able to function normally. Synthetic agonists on the other hand have altered chemical structures compared to the endocannabinoids which conferring their unique characteristic. Therefore the pharmacokinetics of the different synthetic drugs can be quite variable and as a result intrinsic mechanisms like membrane transporters and metabolizing enzymes may be unable to efficiently eliminate the drugs. Prolonged availability of the synthetic drugs may cause potential side effects like excessive receptor desensitization and tachyphylaxis.

4.2.2 Cytokine levels

Cytokines are signalling molecules released by a wide variety of different cells and are important in a host of various homeostatic immune responses. However, during a septic state, excessive levels of cytokines are released by a variety of different cells leading to disseminated activation of various immune cascades, disruption of normal immune function and widespread tissue injury (Blackwell & Christman, 1996). Septic patients were shown to have elevated levels of various cytokines, especially prominent pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 (Arnalich et al., 2000; Casey et al., 1993). Therefore we wanted to assess the systemic levels of various inflammatory cytokines in an endotoxemia model, and the impact on these cytokine levels after CB_2 receptor activation.

LPS administration to mice in our study showed significantly elevated plasma levels of TNF-α, IL-1β, IL-6, and MIP-2. TNF-α is an important pro-inflammatory cytokine that is released early during an inflammatory cascade. TNF-α plays a significant role in mobilizing an acute immune response, as well as inducing the production of other pro-inflammatory cytokines like IL-1β and IL-6 (Blackwell & Christman, 1996). Several studies on humans have shown that endotoxin administration in patients cause a rapid increased in plasma TNF-α levels which reach their peak levels within 1 – 2 hours after administration (Kemna et al., 2005; Michie et al., 1988). In our study administration of JZL184 after LPS was able to significantly ablate the release of TNF-α into circulation. Many studies in the past have assessed the anti-inflammatory effects of activating the CB_2 receptor by measuring the changes in TNF-α levels in the plasma. Overall, using various CB_2 agonists and antagonists, studies have shown that activating the CB_2 receptor in inflammatory conditions, reduced the plasma levels of TNF-α (Greineisen & Turner, 2010; Gui et al., 2013; Smith et al., 2000). More specifically, studies assessing the impact of JZL184 administration in various inflammatory conditions also showed a similar decrease in plasma TNF-α levels (Alhouayek et al., 2011; Z. Cao et al., 2013; Costola-de-Souza et al., 2013; Kerr et al., 2013).

IL-1β is another important pro-inflammatory cytokine released early during the inflammatory cascade and plays a role in leukocyte recruitment as well as activating the production of other cytokines like IL-6 and TNF-α (Blackwell & Christman, 1996). Similar to TNF-α, IL-1β levels in the plasma rise early during an inflammatory insult, and may be responsible for the autocrine regulation of leukocyte stimulation and recruitment. The importance of IL-1β has been studied in septic patients through the use

of IL-1 receptor antagonist (IL-1ra) to block the activity of IL-1β on its endogenous receptor (Dinarello, 2005). Overall, IL-1ra administration helped reduce septic mortality in mice, as well as minimize the production of related cytokines like IL-6, TNF-α, and IL-8 (Blackwell & Christman, 1996; Lamacchia et al., 2010).

The role of the cannabinoid system has also been investigated *in vitro* and *in vivo* over its influence in modulating the levels of IL-1β during inflammatory conditions. Overall, activation of the CB_2 receptor with various ligands during inflammation has shown a reduction in the levels of IL-1β secretion by most immune cells (Di Filippo et al., 2004; Greineisen & Turner, 2010). In our study, administration of JZL184 after LPS induction had no significant impact on reducing IL-1β levels. Other studies also assessing the role of JZL184 in inflammation show inconsistent results. Some studies show similar results to our study where JZL184 administration does not produce a significant reduction in plasma IL-1β levels (Alhouayek et al., 2011; Kerr et al., 2013), while others do show a significant reduction in IL-1β levels (Z. Cao et al., 2013). The discrepancy in the results is probably due to the different inflammatory models being assessed in each study, thereby causing a differential response by the immune system to JZL184 administration.

IL-6 is an inflammatory cytokine that has a variety of different effects during an immune response. Initially, IL-6 was thought to be a pro-inflammatory cytokine because elevated levels of IL-6 were detected in patients with inflammatory conditions and persistent elevated levels of IL-6 correlated negatively with survival (Pinsky et al., 1993; Riedel & Carroll, 2013; Waage et al., 1989). Furthermore, the administration of IL-1β or TNF-α into mice increased the levels of plasma IL-6, and the combination of both cytokines synergistically increased IL-6 levels (Shalaby et al., 1989). Now it is

understood that IL-6 has both pro- and anti-inflammatory activities, and its role is to regulate the transition of the inflammatory cascade from acute to chronic inflammation (Jones, 2005). The early peak plasma levels of proximal cytokines like TNF-α and IL-1β during an inflammatory response eventually cause the release of temporally delayed cytokines like IL-6 which reaches peak plasma levels around 3 – 4 hours after LPS administration (Kemna et al., 2005). This later rise in IL-6 levels help resolve the acute innate immune response by minimizing neutrophils recruitment and activate the chronic immune response by stimulating lymphocyte and macrophage recruitment (Gabay, 2006). In our study, JZL184 administration did not significantly alter the IL-6 plasma levels induced by LPS administration. One explanation for the lack of effect by JZL184 might be the relatively acute time point of analysing IL-6 levels in our experiment. A study assessing acute lung injury, assessed IL-6 levels 2 hours after LPS administration and found no significant reduction in IL-6 levels with JZL184 treatment (Kerr et al., 2013). A more chronic study assessed IL-6 plasma levels after 3 days in a TNBS induced colitis model and showed a reduction in IL-6 levels after JZL184 administration (Alhouayek et al., 2011). These results may explain the lack of effect seen in our study, where plasma was also isolated 3 hours after LPS and JZL184 administration.

IL-10 is the most prominent anti-inflammatory and immunosuppressive cytokine that is released by lymphocytes, monocytes, and endothelial cells (Oberholzer et al., 2002). IL-10 is thought to be important in helping resolve an inflammatory cascade primarily because it supresses the production of many inflammatory cytokines like IL-1β, TNF-α, IL-6, IL-8, GM-CSF, as well as inhibiting the production of vasodilators like nitric oxide (Oberholzer et al., 2002). Endotoxemia studies in mice have shown that IL-

10 dose dependently improves survival rates by limiting the release of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6, and antibodies against IL-10 inhibit its protective effects and exacerbate mortality (Gerard et al., 1993; Howard & Muchamuel, 1993; Standiford et al., 1995). One of the mechanisms that the CB_2 receptor confers its anti-inflammatory properties is by stimulating the release of anti-inflammatory cytokines like IL-10. Activating the CB_2 receptor during various inflammatory conditions has been shown to increase the release of IL-10 by many different types of immune cells (Correa et al., 2005; Greineisen & Turner, 2010; Smith et al., 2000). In our study, activation of the CB_2 receptor through JZL184 administration did not significantly increase the levels of IL-10 induced by LPS, but rather we saw a slight decrease in IL-10 plasma levels. A study by Kerr et al., also assessed the role of JZL184 in LPS induced plasma cytokines and found that LPS expectedly increase IL-10 levels, but subsequent JZL184 administration surprisingly reduced IL-10 levels (Kerr et al., 2013). These results were attributed to a CB_1 mediated pathway as administration of AM251 (CB_1 antagonist) was able to block these effects, while AM630 (CB_2 antagonist) administration showed minimal effects (Kerr et al., 2013).

Macrophage inflammatory protein 2 (MIP-2) is a pro-inflammatory chemokine secreted by monocytes and macrophages, and plays an important role in neutrophil and polymorphonuclear cell recruitment during sepsis (Osuchowski et al., 2006; D. Remick et al., 2000; Tsujimoto et al., 2005). These studies have shown that early elevated levels of MIP-2 were detected in LPS induced inflammatory models, while a gradual increase in MIP-2 was detected in CLP models of sepsis, and sustained elevated levels of this chemokine was associated with increased mortality. In our study, LPS administration

significantly increased MIP-2 plasma levels, and JZL184 administration was able to significantly ablate this response. Multiple other studies have assessed the role of the CB_2 receptor in various experimental inflammatory diseases and show that CB_2 receptor activation helps reduce the release and mRNA expression of MIP-2, as well as regulate MIP-2 expression by showing that CB_2 receptor knockout mice have significantly elevated MIP-2 levels (Bátkai et al., 2007; P. Mukhopadhyay, Rajesh, et al., 2010; Rajesh, Pan, et al., 2007). To our knowledge the only other study that assessed the role of MAGL inhibition on MIP-2 levels in a hepatic ischemia reperfusion model showed a reduction in plasma MIP-2 levels after JZL184 administration as well as in MAGL knockout mice (Z. Cao et al., 2013).

IL-17a is a pro-inflammatory cytokine that is known to be produced mainly by Th17 cells, but can also be produced by neutrophils, eosinophils, NKT cells and NK cells (Korn et al., 2009). Multiple studies using distinct methods of activating the CB_2 receptor have shown that plasma IL-17a levels induced by inflammatory insults can be reduced by activating the CB_2 receptor. In viral infected mice, IL-17 levels were significantly increased, and activation of the CB_2 receptor either by anandamide administration, FAAH inhibition, or Δ^9-THC administration reduced the levels of IL-17a (Correa et al., 2011; Karmaus et al., 2013). Furthermore, CB_2 receptor knockout mice showed increased IL-17 expression in a hepatic ischemia model, and *in vitro* CB_2 receptor agonist administration reduced Th17 cell differentiation as well as IL-17 mRNA expression (Guillot et al., 2014). In our study, LPS administration alone did not significantly increase IL-17 levels, however subsequent administration of JZL184 caused a significant increase in plasma IL-17 levels. To our knowledge, no other study has investigated the effects of JZL184 on IL-

17 levels in the plasma. The increase in IL-17 levels with JZL184 administration contradicts the known literature which indicates that CB_2 receptor activation would reduce IL-17 levels. One possible explanation for the discrepancy lies with JZL184's unique method of activating the CB_2 receptor by increasing levels of 2-AG. As will be discussed in more detail in a later section, increased levels of 2-AG may concurrently cause an increase in prostaglandin levels which are also pro-inflammatory and may be responsible for the increased IL-17 levels. Studies have shown that prostaglandins like PGE_2 can influence IL-17 levels in various inflammatory conditions (Napolitani et al., 2009; Schiffmann et al., 2014)

Overall the cytokine results from our study correlate well with the study by Kerr et al (2013) which was the first study to assess the impact of JZL184 administration on plasma cytokines in an acute lung injury model, showing that JZL184 ablated the LPS induced increase in TNF-α and IL-10 levels, while having minimal impact on IL-1β and IL-6 levels (Kerr et al., 2013). Our results indicate that JZL184 administration does increase overall CB_2 receptor activation and can cause a modest anti-inflammatory effect by minimizing the release of acute pro-inflammatory cytokines.

4.3 Colon ascendens stent peritonitis

Apart from its many advantages, the endotoxemia model of sepsis does also have some drawbacks. The main drawback of this model is the lack of viable pathogens infecting the host, therefore the complete pathophysiology of sepsis is not well represented. In endotoxemia models, the administration of LPS causes an immediate and rapid response of the immune system, though a rapid release of pro-inflammatory mediators and a relatively quick resolution of the inflammatory response. In contrast,

although heightened levels of pro-inflammatory mediators are present in septic patients and animals, the levels of these mediators are usually lower, and more importantly, they increase much more gradually and are sustained for longer periods of time (Deitch, 2005; D. G. Remick & Ward, 2005). This might be one of the reasons for the failure of many clinical trials focusing on blocking the effects of inflammatory mediators like TNF-α for a therapeutic benefit in septic patients (Arndt & Abraham, 2001).

In order to overcome the drawbacks of the endotoxemia model, experimental studies of sepsis employed models that use endogenous fecal contamination through organ barrier disruption. The two most common fecal contamination models are cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP). CLP has been the most commonly used model of polymicrobial sepsis over the past few decades (Hubbard et al., 2005), and has generally been considered the standard model for pre-clinical trials of various experimental sepsis therapeutics (Dejager et al., 2011). This technique was developed by Wichterman and colleagues in the 1970s and has several advantages over other septic models. The CLP model offers a combination of 3 insults that are similar to insults seen in septic patients. The first insult occurs from the tissue trauma caused by the abdominal laparotomy to access the cecum. The second insult comes from the eventual development of necrotic tissue that occurs from ligating the cecum. The final insult comes from perforating the cecum and allowing the gut contents to cause peritonitis and septicemia (Dejager et al., 2011). The severity of peritonitis can also be controlled based on the gauge of needle used for perforation and the number of perforations made. One drawback to this model is the formation of abscesses over the

perforations, blocking continuous fecal translocation, and preventing adequate peritonitis progression (Schabbauer, 2012).

The CASP model is very similar to the CLP model because it is also a model of inherent polymicrobial fecal translocation. Similar to the CLP model, the severity of the induced peritonitis can be modulated based on the gauge of stent implanted. However, the main advantage that the CASP model confers over the CLP model is that the presence of the stent prevents abscess formation over the site of organ barrier disruption and therefore continuous fecal translocation is much more reliable in the CASP model compared to CLP (Stephen Maier et al., 2004). Similar to the CLP model, the CASP model also offers the initial tissue trauma from the laparotomy, and a secondary insult of fecal translocation into the abdominal cavity.

Both the CASP and CLP models of endogenous fecal translocation generate immune responses that are similar to clinical sepsis compared to the other models of sepsis. There are however molecular differences in the inflammatory pathway between both models that have translated into various outcomes in animal studies (Dejager et al., 2011; Schabbauer, 2012). In general it seems that the CLP method shows limited bacteremia and a delayed systemic hyper-inflammatory response compared to CASP (Stephen Maier et al., 2004; Schabbauer, 2012). The CASP model has tended to generate a much more robust cytokine release and bacterial loads at earlier time points compared to the CLP model (Stephen Maier et al., 2004; Schabbauer, 2012). Many of the expected pro-inflammatory cytokines and chemokines like TNF-α, IL-1, IFN-γ, IL-18 are released within a few hours after implanting the stent. Furthermore, anti-inflammatory cytokines levels like IL-10 are also increased very rapidly after surgery, which might be reflective

of the compensatory anti-inflammatory response also seen in septic patients (Schabbauer, 2012). Inherent with both the CASP and CLP models are the higher levels of variability in the inflammatory response compared to endotoxemic models. However, for experimental therapeutic testing, this higher degree of variability might better reflect the heterogeneous septic conditions normally encountered in clinical settings, thereby validating these models. Based on the benefits conferred by the CASP model we decided to assess the role of modulating the CB_2 receptor through JZL184 administration in the clinically relevant CASP model of sepsis.

4.3.1 Intravital microscopy

Similar to our endotoxemia model, we decided to first observe the detrimental changes in microcirculatory parameters induced by fecal peritonitis and the potential benefits of activating the CB_2 receptor pathway in mitigating these effects. Our sham operated mice showed some basal levels of leukocyte adhesion in both the V1 and V3 submucosal intestinal venules as expected and may be attributed to the trauma and inflammation caused by the laparotomy surgery. The CASP surgery caused a significant elevation in leukocyte adhesion in both V1 and V3 venules compared to the sham control group. Furthermore, the density of leukocyte adhesion in the CASP group were in a similar range to the levels seen in the endotoxemia group for both V1 and V3 venules. As expected, the variability in the levels of leukocyte adhesion was much higher in the CASP model compared to the LPS model. Various other CASP studies have also shown increases in leukocyte adhesion and leukocyte infiltration in various organs throughout the body, validating the systemic inflammatory response induced by this model (Feterowski et al., 2004; Lustig et al., 2007; Stephan Maier et al., 2000).

A prior study in our lab investigated the role of the CB_2 receptor in a rat model of CASP and found that a high dose of HU308 was able to significantly reduce the induction of adherent leukocytes in both V1 and V3 venules (Lehmann et al., 2011). To our knowledge no other study has assessed the role of the CB_2 receptor in a CASP model of sepsis. A few studies have assessed the properties of the CB_2 receptor in CLP models of sepsis but their results are conflicting (Csoka et al., 2009; M. Liu et al., 2014; Tschop et al., 2009). Overall these studies have shown that CB_2 receptor knockout mice induce higher levels of leukocyte recruitment after CLP, and activation of the CB_2 receptor attenuates leukocyte infiltration. However, the survival rate, organ injury, and systemic cytokines show conflicting results in the two CB_2 receptor knockout studies (Csoka et al., 2009; Tschop et al., 2009). In our study, administration of JZL184 was able to significantly ablate the levels of leukocyte adhesion in V1 venules, and moderately reduce leukocyte adherence in V3 venules. These results support the understanding that activating the CB_2 receptor pathway during an inflammatory state will minimize leukocyte recruitment and may prevent subsequent microcirculatory dysfunction and tissue ischemia. Similar to our endotoxemia results, CASP surgery caused a significant reduction in the number of rolling leukocytes in both V1 and V3 venules, presumably due to their concomitant increase in their adherence to the endothelial cells. JZL184 administration was able to moderately increase rolling leukocyte numbers in both sets of venules, however this increase was not significantly different than the CASP group. Once more, the lack of improvement in leukocyte rolling with JZL184 administration may be attributable to the reduction in expression of adhesion molecules on leukocytes and the

endothelial surface caused by the activation of the CB_2 receptor, preventing normal leukocyte rolling.

In our CASP study we also measured functional capillary density and found similar results to our endotoxemia study where CASP did not significantly reduce FCD in the intestinal muscle layers, but was able to significantly reduce FCD in the mucosal villi. Another CASP study assessing microcirculatory integrity showed no significant reduction in functional capillary density in the intestinal muscle layers and mucosal villi of rats even 18 hours after CASP surgery (Lustig et al., 2007). The short time frame until observation, just 6 hours after implanting the stent, may not have been long enough to cause the severity of peritonitis needed to show a significant FCD decrease in the muscle layer, but was severe enough to reduce mucosal villi FCD. Once again, administration of JZL184 showed no significant change in intestinal muscle layer FCD, however it was able to significantly prevent the CASP induced reduction in FCD in the mucosal villi. To our knowledge this is the first time that the MAGL inhibitor JZL184 has been used to assess changes in microcirculatory parameters in an experimental sepsis model. These results suggest that activating the CB_2 receptor pathway by blocking MAGL activity early during an inflammatory septic state may help minimize the subsequent microcirculatory dysfunction that ensues in disease pathophysiology.

4.3.2 Quantitative RT-PCR

We also wanted to assess CB_2 and NF-κB mRNA expression in different tissues (intestine, spleen) by quantitative RT-PCR. It was important to examine intestinal tissue because our experiments showed physiological changes in intestinal microcirculation, therefore we wanted to explore any possible transcriptional changes occurring in the

intestinal tissue. We also chose to examine transcriptional changes in the spleen due to the importance of splenocytes in the immune response. The spleen and tonsils were shown to contain the highest level of CB_2 receptor expression in the body (Galiègue et al., 1995), and changes in receptor expression occur over longer timeframes, therefore in our short experimental timeframe any changes associated with CB_2 receptor transcription would most likely be prominently evident in the spleen. Furthermore, transcriptional changes to inflammatory proteins in naïve splenocytes may eventually play a role in the immune response at latter stages of disease progression when the mature splenocytes are circulating in the blood. Therefore observing any early transcriptional changes to inflammatory modulators may be indicative of a more regulated immune response and a better prognosis during sepsis.

Studies have shown that cell surface expression and mRNA expression of the CB_2 receptor seems to differ in a time dependent manner when different leukocytes are administered LPS (Kasten et al., 2010). Peritoneal macrophages exposed to IFN-γ and LPS showed decreased mRNA CB_2 receptor expression compared to controls (Carlisle et al., 2002). Alternatively, RAW 264.7 cells showed a time dependent increase in cell surface CB_2 receptor protein expression after LPS administration (S. Mukhopadhyay et al., 2006). Overall these two studies measured two different aspects of CB_2 receptor expression, possibly suggesting that LPS might differentially regulate these two mechanisms by up-regulating CB_2 receptor surface expression, while down-regulating CB_2 receptor mRNA expression (Kasten et al., 2010). In our CASP model we showed minimal expression of CB_2 receptor mRNA in the intestine and a higher expression in the spleen. The increased levels of CB_2 receptor mRNA in the spleen aligns with the known

literature which indicates a higher expression of the CB_2 receptor in immune cells and immune organs like the spleen and tonsils compared to other tissue like the intestine (Galiègue et al., 1995; Gong et al., 2006).

Relative CB_2 receptor mRNA expression in control mice intestine and colon was shown to be higher (Aguilera et al., 2013; A. A. Izzo et al., 2012; Storr et al., 2009) than levels seen in our study, and administration of a phytocannabinoid called cannabichromene was shown to reduce the levels of CB_2 receptor mRNA expression in the intestine (A. A. Izzo et al., 2012). Cannabichromene uses the same principle as JZL184 to activate the cannabinoid receptors by preventing endocannabinoid reuptake by blocking the membrane transporters and weakly inhibiting MAGL to increase the available levels of endocannabinoids (A. A. Izzo et al., 2012). In our CASP study, JZL184 administration after CASP surgery showed a slight increase in CB_2 receptor mRNA expression in the spleen, however the increase was not significantly higher than the CASP group. This result may be explained by the elevated levels of 2-AG caused by JZL184 administration, prompting the up-regulation of CB_2 receptor mRNA in the naïve splenocytes. Another study assessing a lung injury model showed similar results where administration of paraquat poison reduced CB_2 receptor expression and administration of JWH133 a CB_2 receptor agonist caused an increase in the CB_2 receptor expression of lung tissue (Z. Liu et al., 2014). The expression of the CB_2 receptor on immune cells or organ tissue may vary depending on the immune cell type and the phase of the immune response. Early during an inflammatory response, it is likely that CB_2 receptor expression is down-regulated so that a pro-inflammatory response can effectively eliminate the invading pathogen. However, in later stages of inflammation it is possible that CB_2

receptor expression is upregulated to help resolve the earlier pro-inflammatory state. Similarly, immune cell populations involved in the acute inflammatory response have been shown to express lower levels of CB_2 receptor compared to immune cells like B and T lymphocytes that are more prominent in chronic models of inflammation (Galiègue et al., 1995).

NF-κB is a prominent transcription factor that is involved in regulating the expression of multiple genes including pro-inflammatory cytokines and adhesion molecules. NF-κB is shown to play an important role in the pathophysiology of the septic cascade in animal models of sepsis and in septic patients (S. F. Liu & Malik, 2006). Elevated levels of NF-κB have been shown in various organs throughout the body in experimental septic models as well as septic patients, and higher levels of NF-κB have correlated with increased mortality (Bao et al., 2010; S. F. Liu & Malik, 2006; Yu et al., 2007). Furthermore, blocking or disrupting the NF-κB pathway in septic conditions has generally been associated with improved outcomes (S. F. Liu & Malik, 2006; N. Matsuda et al., 2005; Ye et al., 2008). Some of the anti-inflammatory effects of activating the CB_2 receptor pathway have been proposed to be due to inhibition of NF-κB activity. Few studies have assessed the role of the CB_2 receptor on altering NF-κB expression in septic mouse models (Correa et al., 2010; Gui et al., 2013; Nakajima et al., 2006). Overall, these studies indicate that activating the CB_2 receptor attenuates the inflammation induced increase in NF-κB activity thereby minimizing the damage caused by the pro-inflammatory response elicited by the experimental insult. In our study, the administration of JZL184 after CASP surgery showed no significant change in the expression levels of p65 NF-κB in both the spleen and intestinal tissue compared to NF-

κB levels found in CASP animals. One possible explanation for the lack of change in NF-κB expression might be the short duration of the experiment. The organ tissue was harvested just 7 hours after administration of JZL184, and this short duration may not have been sufficient for the activation of the CB_2 receptor pathway or the severity of peritonitis to elicit a significant change in NF-κB mRNA expression. Studies showing the beneficial impact of the CB_2 receptor pathway on NF-κB expression in CLP models harvested organ samples more than 16 hours after treatment (Csoka et al., 2009). Furthermore, various inflammatory studies have shown similar levels of NF-κB mRNA expression in control mice to the levels seen in our study, indicating that a significant increase in NF-κB expression was not established in our study after CASP surgery (Bao et al., 2010; Guleng et al., 2010; Wang et al., 2013; Yu et al., 2007). Another possibility is that CASP surgery did not cause the proper dissociation of the inhibitory IκBα protein from the NF-κB. Therefore simply measuring levels of NF-κB might not properly reflect the inflammatory response. As an additional method, measuring the levels of phosphorylated IκBα protein will help establish the proper activation of the NF-κB transcription factor pathway.

4.4 CB_2 receptor knockout mice

In order to validate our initial results and further assess the anti-inflammatory role of activating the CB_2 receptor during experimental septic models, we decided to repeat our endotoxemia study focusing once again specifically on JZL184 in CB_2 receptor knockout mice. Knockout mice confer the ability to assess the specific role of target proteins by eliminating their genes. In our experiments we used CB_2 receptor knockout mice to assess the specific role of the CB_2 receptor after the administration of JZL184.

There are several advantages of using knockouts over pharmacological agents like antagonists. Primarily, ablation of the target gene in knockout mice ensures that any effect conferred by the CB_2 receptor would be completely eliminated. With synthetic antagonists, pharmacological properties like receptor specificity, affinity, and efficacy, as well as pharmacokinetic and pharmacodynamics properties of the synthetic compounds may vary, thereby altering interpretations of the results. Another advantage of using knockout mice is that CB_2 receptor activation can be prevented without any pharmacological side effects. Administration of synthetic pharmacological agents has the disadvantage of causing unwanted side effects, which may have confounding effects on the experimental results. The one major disadvantage of using knockout mice is the possible alteration in the activity of other similar pathways to overcome the absence of the missing pathway. This issue is especially prominent in proteins like the CB_2 receptor due to their involvement in a variety of different responses, as well as their co-operation with other receptors and pathways in eliciting specific responses.

4.4.1 Intravital microscopy

Similar to our other models we assessed various intestinal microcirculatory parameters in CB_2 receptor knockout mice. Minimal basal levels of leukocyte adhesion in control CB_2 receptor knockout mice were similar to those found in wild-type controls from our endotoxemia study for both V1 and V3 venules. Administration of LPS to CB_2 receptor knockout mice also caused a significant increase in leukocyte adhesion levels that were similar to the levels found in our wild-type mice administered LPS in the endotoxemia study. These results indicate that the inflammatory response to LPS in CB_2 receptor knockout mice is not significantly altered from their normal wild-type littermates

even though they genetically lacked the CB_2 receptor pathway throughout development. Most inflammatory studies using CB_2 receptor knockout mice have shown exaggerated inflammation, tissue damage, and cellular infiltration in challenged CB_2 receptor knockout mice, alluding to the lack of a proper CB_2 receptor mediated anti-inflammatory response (Bátkai et al., 2007; Engel et al., 2010; P. Mukhopadhyay, Rajesh, et al., 2010; Tschop et al., 2009). The absence of further elevated levels of leukocyte adherence in our LPS administered CB_2 receptor knockout mice compared to wild-type mice might once again be explained by a maximal level of leukocyte adhesion as seen in the endotoxemia study. The effect of leukocyte rolling in CB_2 receptor knockout mice once again mirrored the effects seen in our CASP and endotoxemia studies where administration of LPS reduced the levels of rolling leukocytes and administration of JZL184 was unable to ablate this response. In this case the lack of CB_2 receptors may play a role in the absence of any effect, but it can also equally be attributed to the same reasons discussed in the CASP and endotoxemia studies.

The role of the CB_2 receptor in septic models has been controversial based on the conflicting results shown by two studies using CB_2 receptor knockout mice (Csoka et al., 2009; Tschop et al., 2009). Both studies used the CLP model to induce an experimental septic state in CB_2 receptor knockout mice. Csoka et al. (2009) showed better survival, reduced organ injury, diminished NF-κB expression, reduced bacterial load, and attenuated cytokine levels in CB_2 receptor knockout mice. On the other hand, Tschop et al. (2009) showed reduced survival, increased organ injury, elevated bacterial loads, and elevated cytokine levels. Furthermore, they also showed attenuation of these parameters in wild-type mice with the administration of GP1a (a CB_2 receptor agonist). The only

discrepancy between the two studies that may be attributed to the difference is the severity of insult, where the Csoka study employed a more severe model of sepsis by using a 20 gauge needle and perforating the cecum 4 times, while the Tschop study generated a moderate septic state by using a 23 gauge needle to perforate the cecum just once. The lack of the anti-inflammatory effect in the CB_2 receptor knockout mice may have been beneficial in the Csoka study by allowing for a more efficient clearance of the bacteria and therefore increased survival. Alternatively, in the Tschop study, the exaggerated inflammatory response in the CB_2 receptor knockout mice subject to a less severe CLP model may have been detrimental because of the augmented damage caused by an uncontrolled inflammatory response. This dichotomous result makes it challenging to understand the role of the CB_2 receptor during an inflammatory response.

In our study JZL184 administration after LPS in CB_2 receptor knockout mice showed a modest but insignificant reduction in leukocyte adhesion in V1 venules and a significant reduction in V3 venules. The administration of JZL184 in CB_2 receptor knockout mice was expected to cause no significant difference due to the lack of the CB_2 receptor. However, these results indicate that the elevated levels of 2-AG through JZL184 administration have some anti-inflammatory effects that are mediated by alternative mechanisms. Several possible CB_2 receptor independent mechanisms may be responsible for the anti-inflammatory seen in our knockout mice administered JZL184.

The other prominent cannabinoid receptor, the CB_1 receptor, may be involved in some of the anti-inflammatory effects seen in our study due to the elevated levels of 2-AG, which is an endogenous ligand for this receptor. Many studies have assessed the role of the CB_1 receptor in various peripheral inflammation models using agonists,

antagonists, enzyme inhibitors, and knockouts (Li et al., 2010; Massa et al., 2004; Storr et al., 2008). Overall, inflammatory conditions were associated with increases in CB_1 receptor expression in intestinal tissue and mesenteric neurons (A Izzo et al., 2001; Kimball et al., 2006; Massa et al., 2004), but the role of CB_1 receptor activation is conflicting. Some studies have shown that activating the CB_1 receptor during inflammatory conditions can be detrimental and inhibiting the CB_1 receptor can be beneficial (Croci et al., 2003; Kianian, Kelly, et al., 2013; P. Mukhopadhyay, Pan, et al., 2010). On the other hand, studies have also shown that CB_1 receptor knockout mice or CB_1 receptor antagonist administration exaggerates the pro-inflammatory response in various models and administration of a CB_1 receptor agonist attenuates this effect (Kimball et al., 2006; Massa et al., 2004; Smith et al., 2001). Based on these results, and the lack of CB_2 receptors in our knockout mice, CB_1 receptor expression may have been increased and also be responsible for the observed anti-inflammatory effects seen in our study elicited by JZL184 administration.

Other inflammatory studies using cannabinoid receptor knockout mice have also shown that some cannabinoid agonists may mediate their anti-inflammatory effects through cannabinoid independent mechanisms (Braun et al., 2011; Rockwell et al., 2006; Schicho et al., 2011). Rockwell et al. (2006) assessed the cellular mechanisms of the anti-inflammatory properties of 2-AG in supressing cytokine release from immune cells. They showed that 2-AG was able to supress IL-2 secretion in PMA/ionomycin activated leukocytes, and this activation was independent of the cannabinoid pathway (Rockwell et al., 2006). Furthermore, 2-AG was able to exert its suppressive effects even when the cells were pretreated with SR141716A (CB_1 receptor antagonist) and SR144528 (CB_2

receptor antagonist). Additionally, the anti-inflammatory effects of 2-AG were still present in CB_1/CB_2 receptor double knockout leukocytes, and the dose dependent effects were similar to those seen in leukocytes from wild type mice, implicating an alternative mechanism (Rockwell et al., 2006).

The peroxisome proliferator-activated receptor γ (PPARγ) is probably involved in inducing some of the anti-inflammatory effects seen in our CB_2 receptor knockout mice due to its implicated role in inflammation modulation (Chinetti et al., 2000). Rockwell et al. (2006) showed that 2-AG exerts its suppressive effects on IL-2 expression by activating PPARγ in leukocytes, and using a PPARγ specific antagonist T0070907 blocked the effects of 2-AG (Rockwell et al., 2006). Furthermore, they showed that 2-AG supresses the activity of important inflammatory transcription factors like NFκB in a dose dependent manner through PPARγ, leading to reduced expression of inflammatory mediators like IL-2, IL-4, and IFNγ (Rockwell et al., 2006). An alternative study used the same model of PMA/ionomycin activated splenocytes and showed that 2-AG inhibited IFNγ production in a dose dependent manner (Kaplan et al., 2005). The study also used leukocytes from CB_1/CB_2 receptor double knockout mice showing that 2-AG can exert its effects independent of the cannabinoid system. They showed that 2-AG produces its effects by disrupting intracellular calcium levels and subsequently inhibits the proper translocation of nuclear factor of activated T cells (NFAT) into the nucleus (Kaplan et al., 2005). Overall, these studies indicate that 2-AG can impose some of its anti-inflammatory effects using a combination of a variety of different pathways. The plethora of alternative pathways available to 2-AG might explain the modest anti-inflammatory

effects of leukocyte adhesion seen *in vivo* with the administration of JZL184 in our CB_2 receptor knockout mice.

Apart from 2-AG directly activating PPARγ, other lipid derived mediators like the prostaglandins are also intrinsic ligands for the various PPARs (Chinetti et al., 2000; Daynes & Jones, 2002). The prostaglandins are a subclass of eicosanoids that are bioactive lipid molecules which can have both pro-inflammatory and anti-inflammatory effects (Harris et al., 2002). Normally phospholipases and cyclooxygenases convert available arachidonic acid from membrane phospholipids or freely available arachidonic acid into the various eicosanoids. Alternatively, in our JZL184 administered mice, the elevated levels of 2-AG can also be used as a substrate by cyclo-oxygenase 2 (COX-2) to produce prostaglandins. COX-2 is known to be able to use endocannabinoids as substrates for the production of various prostaglandins (Smid, 2008). One anti-inflammatory prostaglandin meditated pathway is through the PGD_2 metabolite 15-d-PGJ_2 which has been shown to bind PPAR-γ (Chinetti et al., 2000; Daynes & Jones, 2002; Jiang et al., 1998). As mentioned earlier, activation of PPARγ can have many immunoregulatory activities like NF-κB inhibition and suppression of various pro-inflammatory cytokines (Chinetti et al., 2000; Daynes & Jones, 2002). Therefore, the anti-inflammatory effects of leukocyte endothelial interactions in CB_2 knockout mice administered JZL184 could also be attributed to the possible effects of elevated prostaglandin production and subsequent PPARγ activation.

As an alternate dichotomous possibility, if eicosanoid levels are not significantly elevated by inhibiting endocannabinoid degradation (Rouzer & Marnett, 2011), the anti-inflammatory effects of JZL184 in our CB_2 knockout mice may also have occurred

indirectly by preventing the pro-inflammatory effects of the eicosanoid pathway (Alhouayek et al., 2014). The metabolism of 2-AG by MAGL results in the production of arachidonic acid, which itself is a bioactive lipid in addition to being a prostaglandin precursor (Alhouayek et al., 2014). Therefore, preventing the degradation of 2-AG with JZL184 causes a systemic buildup of 2-AG and may minimize the availability of arachidonic acid for prostaglandin production (Alhouayek et al., 2014; Z. Cao et al., 2013; Kohnz & Nomura, 2014). Cytosolic phospholipase A_2 ($cPLA_2$), has been thought to be the primary enzymes responsible for arachidonic acid production involved in cyclooxygenase mediated prostaglandin production (Buczynski et al., 2009). However, a study by Nomura et al. (2011) showed that brain arachidonic acid levels were unaltered in $cPLA_2$ deficient mice, and LPS challenge in these mice moderately reduced prostaglandin levels, which was most pronounced after MAGL inhibition with JZL184. This result shows that 2-AG degradation in the brain through MAGL is a significant source of arachidonic acid which then is used as a substrate for prostaglandin synthesis. The same study also measured the peripheral roles of MAGL and $cPLA_2$ on arachidonic acid and prostaglandin levels and found differential contributions by both enzymes in various locations of the body (Nomura et al., 2011). In LPS challenged mice, MAGL was shown to control prostaglandin levels in the liver and lung, while $cPLA_2$ was more prominent in the gut and spleen (Nomura et al., 2011). A recent study by Cao et al. (2013) showed that MAGL inhibition by JZL184 administration or MAGL knockout mice had lower levels of eicosanoids in the liver after hepatic ischemia reperfusion injury (Z. Cao et al., 2013). This study showed a reduction in liver injury which was attributed to a combination of increased CB_2 receptor activation and a reduction in eicosanoid levels after MAGL

inhibition. Both pharmacological and genetic inhibition of MAGL reduced hepatic levels of arachidonic acid, prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), and thromboxane B_2 (TXB_2), suggesting that 2-AG metabolism may be important for eicosanoid production (Z. Cao et al., 2013). Overall these results show that peripheral prostaglandin levels may be affected by MAGL inhibition, thereby playing a role in eliciting some of the anti-inflammatory effects seen in our CB_2 knockout mice administered JZL184.

4.4.2 Quantitative RT-PCR

We once again assessed changes in the mRNA expression levels of the CB_2 receptor and NF-κB in the spleen and intestine of CB_2 receptor knockout mice. We assessed CB_2 receptor mRNA in the spleen and intestine as a positive control. As expected, there was no CB_2 receptor expression in both organs, and JZL184 administration did not change CB_2 receptor expression in these mice. A negligible level of CB_2 receptor mRNA was detected in the LPS administered intestine and JZL184 treated spleen, however these levels were probably artifacts or minimal contamination.

Csoka et al. (2009) demonstrated that western blots of spleens from CB_2 receptor knockout mice had higher levels of Iκ-Bα after CLP compared to wild-type mice. Since Iκ-Bα inhibits NF-κB activation, they concluded that CB_2 receptor knockout mice had decreased NF-κB activation compared to wild-type mice when subject to CLP (Csoka et al., 2009). This result should however be interpreted with some caution as the study by Tschop et al. (2009) demonstrated opposing effects with CB_2 receptor knockout mice, but they did not assess NF-κB expression (Tschop et al., 2009). To our knowledge, no other studies have assessed changes of NF-κB expression in CB_2 receptor knockout mice. Based on studies showing a reduction in NF-κB expression with CB_2 receptor activation

in wild-type mice (Correa et al., 2010; Gui et al., 2013; Nakajima et al., 2006), one would expect that in CB_2 receptor knockout mice the levels of NF-κB expression would be elevated since the suppressive effects of the CB_2 receptor pathway is eliminated.

In our study, the intestinal tissue of CB_2 receptor knockout mice administered LPS showed minimal NF-κB expression and JZL184 had no effect on their levels. This result was similar to the levels of NF-κB expression seen in our CASP study. Interestingly, the levels of NF-κB expression in the spleens of CB_2 receptor knockout mice administered LPS were significantly higher than the levels seen in our CASP study with normal wild-type animals. This result supports our assumption that the genetic ablation of the CB_2 receptor gene may cause an increased expression of NF-κB with LPS administration. Furthermore, administration of JZL184 significantly ablated the LPS induced increase in NF-κB expression within the spleen. This result supports our prediction that the anti-inflammatory effects elicited by JZL184 in our CB_2 receptor knockout mice are working through alternative mechanisms. As discussed previously, the proposed possible mechanisms of either CB_1 receptor or PPARγ mediated anti-inflammatory effects are further supported by these results as other studies have shown the inhibition of NF-κB activity through the activation of these two pathways (Du et al., 2011; Fakhfouri et al., 2012; Panikashvili et al., 2005; Ribeiro et al., 2013). It is possible that there is a complex cooperation between the PPARγ, CB_1, and CB_2 receptors to elicit anti-inflammatory effects in wild-type mice when endocannabinoid levels are increased. Therefore when the CB_2 receptor gene is eliminated, like in our CB_2 receptor knockout mice, there may be a compensatory up-regulation in the activity of the other two pathways to maintain

adequate immune responses. Mechanisms to elucidate the role of the other two pathways will be discussed in the following section.

4.5 Limitations and future studies

One limitation with endocannabinoid manipulation through systemic JZL184 administration is unexpected ***central effects***. Increased levels of the circulating endocannabinoid 2-AG after JZL184 administration may also have central effects, even though JZL184 was administered peripherally. Since 2-AG is lipophilic, it may cross the blood brain barrier and exert some effects on the brain causing centrally activated anti-inflammatory effects. Peripheral administration of JZL184 intraperitoneally (16 mg/kg) in mice was shown to increase 2-AG levels in the brain within 30 minutes of administration (Long et al., 2009). Furthermore, the levels of 2-AG in the brain were elevated for 24 hours before returning to baseline levels. Therefore, peripherally administered JZL184 may still elevate central levels of 2-AG and may cause activation of CB_1 receptors due to their relatively high abundance within the central nervous system. Activation of the CB_1 receptor centrally may cause changes in a wide variety of systemic parameters than can ultimately have an impact on microcirculatory integrity and leukocyte endothelial interaction. A study by Smith et al. (2001) studied the effects of cannabinoid agonists on leukocyte migration and cytokine release in two models of peritonitis using thioglycollate or Staphylococcus enterotoxin A (Smith et al., 2001). They showed that non selective cannabinoids HU-210 and WIN 55212-2 blocked neutrophil migration into the peritoneal cavity and MCP-1 release when administered both intracerebroventricularly and subcutaneously. The anti-inflammatory effect was more pronounced when the agonists were administered centrally and the CB_1 receptor

was implicated to be responsible for the beneficial effects because the use of a selective CB_1 receptor antagonist ablated most of the benefit (Smith et al., 2001). The one major difference between the study by Smith et al. 2001, and our study was the presence of a CB_1 receptor agonist centrally compared to the presence of the endocannabinoid 2-AG centrally in our study. Although 2-AG levels were shown to increase 8-fold centrally with JZL184 administration peripherally (Long et al., 2009), this endocannabinoid has a lower affinity for the CB_1 receptor compared to the synthetic agonists used by Smith et al. 2001, in their study. Therefore, although they did show changes to leukocyte recruitment peripherally by activating central CB_1 receptors, in our study this effect might be minimized. One method of overcoming this limitation would be to administer a specific CB_1 receptor antagonist centrally in conjunction with JZL184 peripherally so that any CB_1 receptor mediated effects are minimized.

Another important limitation of this novel pharmacological approach is the potential impact on ***mean arterial pressure and heart rate***. Unfortunately, we were not able to monitor macrocirculation parameters throughout the experiment. The sepsis cascade is associated with systemic haemodynamic changes to macrovascular and microvascular parameters. The activation of various inflammatory pathways like the coagulation, complement, and nitric oxide pathways, coupled with endothelial dysfunction and increased vascular permeability, causes changes to vital parameters like blood pressure during sepsis. Systemic hypotension of both macro and microvasculature are prominent in sepsis, leading to septic shock. Therefore maintenance (or restoration) of adequate blood pressure and heart rate are important during septic conditions.

To overcome prolonged periods of sepsis induced systemic hypotension throughout the experiment, saline was periodically administered intravenously throughout the experiment. Oxygen was also supplemented whenever the animal's breathing got labored which can also help increase blood pressure. Furthermore, transient drops in blood pressure are evident after intravenous administration of LPS (Kianian, Kelly, et al., 2013; Panayiotou et al., 2010). This temporary drop in blood pressure was shown to automatically recover within a few minutes of LPS administration, indicating that the hypotension was stabilized long before intravital microscopy was performed on the animals. In future studies equipment designed for accurate measurement of mouse MAP and heart rate should be applied to early identify sepsis-induced changes in the microcirculation and to monitor the impact of novel therapeutic substances.

Another limitation in our study is the possibility of ***experimenter bias***. Since the same experimenter conducted the experiment, analysed the videos, and quantified the results, the experimenter could not be completely blinded and elimination of all bias in this study was not possible. However, many steps were taken to minimize the amount of experimenter bias at each stage of the study, and since the experimenter was consistent for all the studies, any unavoidable biases were also consistent between the experiments, allowing us to compare the results. One source of bias during the experiment is when the microcirculatory parameters are being recorded. Since the experimenter had to administer the treatment drugs, they were aware of treatment condition and may have shown some bias during data recording. In order to minimize this effect, every parameter had six unique fields randomly selected and recorded, which were then averaged to produce a single value representing that parameter. This method gave a better representation of the

individual parameter since it was an average of six values, as well as minimized any experimenter bias because it encompassed six unique values.

The ***lack of drug combination groups*** to elucidate CB_2 receptor activity was another limitation to our study. In our study design we decided to pursue CB_2 receptor knockout mice to assess the role of CB_2 receptors in inflammatory conditions. As discussed earlier, knockouts provided a number of benefits over using pharmacological agents like CB_2 receptor antagonists. However, they also possess a few challenges like the possible up regulation of alternative pathways. In future studies, administration of AM630 prior to JZL184 will further help elucidate if the anti-inflammatory effects are abolished or still persist, elucidating the specific role of the CB_2 receptors in wild-type mice. Prior studies in our lab with rats have shown that AM630 pre-treatment was able to ablate the anti-inflammatory effects elicited by the FAAH inhibitor URB597 (Kianian, Al-banna, et al., 2013). In the future, administration of URB597 in CB_2 receptor knockout mice will help elucidate the role of enzyme inhibitors and elevated endocannabinoid levels in knockout mice. Furthermore, pre-treatment of AM630 in combination with CB_2 receptor agonists or endocannabinoid hydrolysing enzyme inhibitors in wild-type mice will help elucidate the specific role of CB_2 receptors in inflammatory conditions.

Based on the possibility of alternative pathways eliciting the anti-inflammatory effects seen in our CB_2 receptor knockout study, administration of specific antagonists to those receptors will be important in future studies. One of the possible receptors discussed was the CB_1 receptor, therefore administration of a specific CB_1 receptor antagonist prior to JZL184 will help determine the role of the CB_1 receptor in our study.

Alternatively, CB_1/CB_2 receptor double knockouts are also viable, therefore administration of JZL184 in these mice will help determine if either two of the cannabinoid receptors are responsible for the effects observed in our study. Another receptor discussed with a possible anti-inflammatory role in our CB_2 receptor knockout study was PPARγ. In order to assess the role of this receptor, future studies should include administration of a specific PPARγ antagonist before JZL184 administration in CB_2 receptor knockout mice. The results of this group will help elucidate if 2-AG levels elicit their anti-inflammatory effects in CB_2 receptor knockout mice through PPARγ. Also discussed earlier was the possibility of a reduction in prostaglandin production caused by the lack of 2-AG metabolism through JZL184 administration. Therefore, another future avenue for investigation would be to measure the circulating levels of various prostaglandins in m receptor knockout mice administered JZL184. If levels of pro-inflammatory prostaglandins were reduced after JZL184 administration compared to control in CB_2 receptor knockout mice, then the subsequent step would be to measure if prostaglandin antagonists are able to mimic the anti-inflammatory effects seen with JZL184.

The results presented in this thesis have shown that various methods of manipulating the CB_2 receptor activation can be beneficial in different models of sepsis. To make the results more clinically applicable, the next step would be to conduct a survival study with the different compounds. A survival study will help determine if early administration of CB_2 receptor activating compounds can resolve inflammation and improve long-term survival in septic models. Furthermore, the use of various methods of

activating the CB_2 receptor will help elucidate the conflicting results shown in CB_2 receptor knockout studies.

4.6 Conclusion

In this study, we investigated the effects of modulating CB_2 receptor activity using various pharmacological mechanisms in different models of acute sepsis in mice. Systemic activation of the CB_2 receptor using either a direct agonist (HU308) or endocannabinoid enzyme inhibitors (URB597 or JZL184) showed therapeutic benefit in an endotoxemia model by improving microcirculatory parameters, e.g. reduction of leukocyte endothelial adhesion and restoration of capillary perfusion. Furthermore, improvements of molecular parameters were also evident based on reductions in inflammatory cytokine levels in the blood. In a CASP model, systemic 2-AG elevation through JZL184 administration also had a beneficial effect on microcirculatory parameters by minimizing inflammatory cell recruitment and minimizing the disruption of intestinal capillary perfusion. The beneficial effects of JZL184 on microcirculatory parameters and molecular parameters were still partially evident in an endotoxemia study with CB_2 receptor knockout mice, indicating that elevated 2-AG levels can also activate CB_2 receptor independent pathways to elicit their anti-inflammatory properties. Overall, this study has shown that the CB_2 receptor pathway can be an important modulator in the inflammatory cascade during sepsis, and in its absence other pathways may be upregulated to elicit similar effects.

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