Experimental Cannabinoid Receptor 2 Activation in Lipopolysaccharide-Induced Interstitial Cystitis in Mice

by

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DEDICATION PAGE

I would like to dedicate this thesis to my family, friends, and colleagues who have encouraged and supported me throughout the entirety of my degree. Thank you.

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Abstract

Interstitial cystitis (IC) is a chronic pain disorder of the urinary bladder that causes symptoms of persistent pelvic pain, nocturia, and dysuria with no curative treatment. Medical management is therefore directed toward pain and symptom management. However, many patients remain dissatisfied with the management of their condition. The endocannabinoid system has been identified as a key regulator of immune function, with experimental evidence demonstrating the involvement of cannabinoid receptors in bladder inflammation. Eliciting anti-inflammatory and analgesic effects derived from cannabinoid receptor 2 (CB_2R) activation the aim of this study. Evaluating the therapeutic potential of entities that result in local CB_2R activation in experimental IC by direct and indirect mechanisms is the objective of this study.

Direct activation of the CB_2R with β-Caryophyllene (BCP), a dietary sesquiterpenoid and phyto-derived CB_2R agonist, or indirect activation through the inhibition of endocannabinoid breakdown with JZL184 exerted anti-inflammatory analgesic effects in experimental IC. Intravital microscopy (IVM) and Luminex immunoassays showed that intravesical treatment with either compound reduced leukocyte adhesion in the bladder microcirculation and inhibited proinflammatory cytokine production, respectively. Von Frey aesthesiometry and behavioral assessment also revealed analgesic effects consistent with activation of the CB_2R. However, some results showed confounding effects of JZL184 vehicle treatment, and potential CB_2R-independent anti-inflammatory effects of BCP. Our results suggest that pharmacological activation of the CB_2R could underscore a novel treatment strategy for reducing IC-induced pain and inflammation.

List of Abbreviations and Symbols Used

ABHD6/12 – Alpha/beta domain hydrolases 6/12

AC – Adenylyl cyclase

ACEA – Arachidonyl-2'-chloroethylamide

AEA – Anandamide

2-AG – 2-Arachidonoylglycerol

ANOVA – Analysis of variance

AP-1 – Activator protein 1

ATF – Activation transcription factor

AUA – American Urological Association

BCA – Bicinchonic acid (protein assay)

BCP – β-Caryophyllene

BTX-A – Botulinum toxin A

BPS – Bladder pain syndrome

cAMP – Cyclic adenosine monophosphate

CACF – Carleton Animal Care Facility

CAM – Cellular adhesion molecule

CB_1R – Cannabinoid receptor 1

CB_2R – Cannabinoid receptor 2

CGRP – Calcitonin gene-related peptide

CNS – Central nervous system

CREB – cAMP response element binding protein

CRP – C-reactive protein

CUA – Canadian Urological Association

CYP450 – Cytochrome P450

DAG – Diacylglycerol

DAGL – Diacylglycerol lipase

DMSO – Dimethyl sulfoxide

DRG – Dorsal root ganglion

DSS – Dextran sodium sulfate

ECS – Endogenous cannabinoid system

ERK 1/2 – Extracellular signal-regulated kinase 1/2

FAAH – Fatty acid amide hydrolase

FITC – Fluorescein isothiocyanate

FDA – United States Food and Drug Administration

GAGs – Glycosaminoglycans

GFR – Glomerular filtration rate

GIRKs – G-protein coupled inward rectifying K^+ channel

GPCR – G-Protein coupled receptor

GRAS – Generally regarded as safe

H_2RA – Histamine$_2$-receptor antagonist

IBD – Inflammatory bowel disease

IC – Interstitial cystitis

ICAM – Intercellular adhesion molecule

Ig – Immunoglobulin

IL – Interleukin

i.p. – Intraperitoneal

i.v. – Intravenous

IVM – Intravital microscopy

JNK – c-Jun N-terminal kinase

LUT – Lower urinary tract

MAGL – Monoacylglycerol lipase

MAPK – Mitogen-activated protein kinase

MAPP – Multi-disciplinary approach to the study of Chronic Pelvic Pain (MAPP)

MD-2 – Myeloid differentiation factor 2

NAE – N-acylethanolamine

NAPE – N-arachidonoyl phosphatidyl ethanol

NBF – Neutral buffered formalin

NF-$\varkappa$B – Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF – Nerve growth factor

NIDDK – National Institute of Diabetes, Digestive, and Kidney Diseases

NO – Nitric oxide

OAB – Overactive bladder disorder

OEA – Oleoylethanolamide

PBS – Painful bladder syndrome

PE – Phosphatidylethanolamine

PEA – Palmitoylethanolamine

PECAM – Platelet and endothelial cell adhesion molecule 1

PC – Phosphatidylcholine

PFD – Pelvic floor dysfunction

PGE_2 – Prostaglandin E_2

PGD_2 – Prostaglandin D2

$PGF_{2\alpha}$ – Prostaglandin F2-alpha

PLC – Phospholipase C

PMN – Polymorphonuclear

PPAR – Peroxisome proliferator-activated receptors

PPS – Pentosan polysulfate sodium

PST – Potassium sensitivity test

PTX – Pertussis toxin

PI – Protease inhibitor

RICE – Rand Interstitial Cystitis Epidemiology Study

RBC – Red blood cell

RCT – Randomized controlled trial

SCF – Stem cell factor

SD – Standard deviation

sLe^x – Sialyl $Lewis^x$

SNM – Sacral nerve stimulation

SUFU – Society for Urodynamics and Female Urology

STAT – Signal transducer and activator of transcription

TCA – Tricyclic antidepressant

TEM – Transendothelial migration

THC – Δ9-tetrahydrocannbinol

TLR – Toll-like receptor

TNBS – 2,4,6-trinitrobenzene sulfonic acid

T-PER – Tissue protein extraction reagent

TRPV1 – Transient receptor potential vanilloid 1

UC – Ulcerative colitis

VCAM – Vascular cell adhesion molecule

WHO – World Health Organization

* – Indicates statistical significance between specified groups

– Indicates statistical significance between specified groups

ϕ – Indicates statistical significance between specified groups

$ – Indicates statistical significance between specified groups

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Chapter 1: Introduction

1.1 Definition, Diagnosis, and Epidemiology of Interstitial Cystitis

The definition of interstitial cystitis (IC) has been subject to continuous refinement since its initial description by Joseph Parrish in a 1836 textbook, citing a condition that causes chronic pelvic pain, increased urinary frequency, and dysuria (Parsons & Parsons, 2004). Years later, glomerulations or "Hunner's ulcers" were discovered on the bladder mucosa of some IC patients by Dr. Guy Hunner, leading to their incorporation into the diagnostic criteria (Hunner, 1915). However, these findings were later shown to be present only in a subset of patients (Jones & Nyberg, 1997), with more recent evidence suggesting that less than 10% of IC patients present with Hunner's ulcers (Teichman, 2009). Therefore, two subtypes of IC were coined: classical, or Hunner type IC (HIC) in which patients show distinct inflammatory lesions on the bladder mucosal surface, and non-Hunner type IC, the more common form, in which these lesions are absent. Painful bladder syndrome (PBS), or bladder pain syndrome (BPS), are used synonymously with IC. However, these are symptom descriptive, and not clinical pathological terms used to encompass disorders of bladder-related pain (Meijlink, 2014), and do not include the inflammatory nature of IC (Birder, 2019). To date, the most widely used definition, established by the Society for Urodynamics and Female Urology (SUFU) in 2010, defines IC as an "unpleasant sensation related to the urinary bladder that is associated with lower urinary tract symptoms for more than six weeks, in the absence of infection or identifiable causes". This definition has been adopted by both the American (AUA) and Canadian Urological Associations (CUA) (Cox & Golda, 2016).

However, many variations of this definition exist. The difficulty in establishing a universally accepted definition is largely due to the lack of a specific diagnostic criteria, variable histopathological changes across patients, and the high variability among symptom presentation and treatment responses (Hanno, 2002).

To date, there remains no gold-standard for the detection and diagnosis of IC (Patnaik et al., 2017), with the diagnosis remaining one of exclusion. An in-depth review of the diagnostic process was outlined by Cox et al. (2016) in the Canadian Journal of Urology. In brief, a thorough medical history is required to identify classical diagnostic symptoms, which include pain in the suprapubic, pelvic, perineal, and inguinal regions, in addition to increased urinary frequency, urgency, or nocturia. Symptoms must be present for a minimum of 6 months in the absence of infection or other identifiable causes (Hanno & Dmochowski, 2009). A number of diagnostic clinical procedures can also be performed which help screen for and exclude other urological, gastrointestinal, or gynecological conditions (Sant, 2002). Differentials such as endometriosis, prostatitis, overactive bladder disorder (OAB), fibromyalgia, and vulvar disorders may present overlapping clinical symptoms (Cox & Golda, 2016). Urinalysis and urine cultures must be performed to eliminate the possibility of bacterial cystitis. Physical exams and pelvic ultrasound are done to screen for masses and hernias, while urinary cytology can rule out some urinary tract malignancies. Cystoscopy is also commonly performed to screen for bladder cancer's, identify Hunner's lesions on the urothelium, and examine the bladder mucosa for erythematous swelling and inflammation (Grover et al., 2011). Other tests, including the potassium sensitivity test (PST), intravesical anesthetic bladder challenge, and hydrodistension can also aid in the diagnostic process (Cox & Golda, 2016).

Widespread variation exists in the reported incidence and prevalence of IC due to the variation in diagnostic criteria's and definitions (Davis et al., 2015). Despite this, it is well reported that IC is much more prevalent in women, with a female-to-male ratio estimated at 9:1 (Bogart et al., 2007), however IC also affects men and children (Close et al., 1996). The RAND Interstitial Cystitis Epidemiology Study (RICE), the largest IC epidemiology study conducted to date, found that between 2.7 and 6.5% of American females report symptoms that are consistent with an IC diagnosis (Berry et al., 2010). This translates to approximately 3.3-7.9 million American females being affected by the symptoms of IC, however, only 9.7% of these females report receiving a definitive IC diagnosis (Berry et al., 2011). Canadian epidemiological data is limited, however it is estimated that IC accounts for approximately 2.8% of outpatient urology clinic visits in Canada (Nickel et al., 2005). While sex currently remains the only definitive risk factor, studies have suggested a possible hereditary predisposition, with one study reporting that adult female first-degree relatives of IC patients have a prevalence of IC 17 times greater than that observed in the general population (Warren et al., 2004). Clinical evidence has also suggested a potential link between diseases of the lower gastrointestinal tract such as inflammatory bowel disease (IBD), and the development of pathologies in adjacent organs including vulvodynia and endometriosis (Grundy et al., 2019).

1.2 Pathophysiology of IC

IC is a chronic pelvic pain syndrome with an unknown etiology (Birder, 2019). The current pathophysiological concepts center around the role of uroepithelial cell dysfunction, inflammation, and afferent nerve hyperexcitability causing visceral

hyperalgesia and allodynia (Gonzalez et al., 2014; Grover et al., 2011; Patnaik et al., 2017).

The urothelium is a specialized, multifunctional epithelium that serves as a barrier between intravesical contents and the underlying vasculature, connective, nervous, and muscular tissue (Birder & Andersson, 2013). It forms a high resistance-barrier to ions, solutes, water, or urinary pathogens (Birder & Andersson, 2013) and transmits physical and chemical signals to afferent nerve fibers innervating the bladder (Birder et al., 2012). The urothelium is lined by a mucosal layer comprised of glycosaminoglycans (GAGs) and glycoproteins that enhance the impermeability of the urothelium, protecting underlying structures from toxic urinary metabolites. Numerous studies have shown that patients with IC have a damaged or diminished GAG layer, resulting in increased urothelial permeability (Hurst et al., 2015; Keay et al., 2014) that allows urinary solutes, including potassium ions, into traverse the urothelium into the suburothelium and bladder interstitium (Parsons, 2007). The diffusion of urinary solutes in this way can depolarize and sensitize innervating bladder afferent nerves, inflame the surrounding tissue, and cascade to the development of lower urinary tract (LUT) symptoms (Gonzalez et al., 2014a). Bladder epithelial cells from IC patients exhibit significantly decreased proliferation compared to control patients (Liu et al., 2011). Antiproliferative factor (APF), which inhibits urothelial cell proliferation is increased in urine samples of IC patients (Keay et al., 2004). Damage to the urothelium is exacerbated following initial insult, with the subsequent release of proinflammatory mediators and infiltration of immune cells, including macrophages, lymphocytes, eosinophils, and mast cells (Erickson et al., 1997; Grover et al., 2011). The increased sensitivity of IC patients to the

potassium sensitivity test, wherein pain is reported following instillation of a potassium solution into the bladder, is thought to be a result of the increased bladder permeability (Y.-H. Jiang et al., 2016). However, uroepithelial dysfunction specific to IC remains undetermined in so far as whether bladder permeability is causal to IC or consequential to an instigated localized inflammation by other means (Grundy et al., 2018).

The exposure of the bladder interstitium to urinary constituents may depolarize muscle cells and innervating urothelial afferents, and inflame the underlying tissue (Parsons, 2011). Irrespective of etiology, inflammation plays a central role in the pathophysiology of IC (Grover et al., 2011). Inflammation in the bladder is characterized by increased vascularization causing erythematous swelling, mucosal irritation, and infiltration of pro-inflammatory mediators (Gonzalez et al., 2014a; Grover et al., 2011). Damaged or dysfunctional urothelial cells produce a range of molecules that perpetuate the immune response, including stem cell factor (SCF), APF, prostaglandin E_2 (PGE_2) and nitric oxide (NO). SCF stimulates the activation of mast cells, which have been identified as crucial effector cells implicated in the pathogenesis of IC (Sant et al., 2007). PGE_2 and NO regulate vasculature homeostasis, and cause angioedema by augmenting arteriolar dilation and increasing microvascular permeability (Funk, 2001; Tripathi et al., 2007). Various other pro-inflammatory mediators have been shown to be increased in the bladders of IC patients, including histamine, nerve growth factor (NGF), bradykinin, and a range of various chemokines and cytokines, including IL-6, IL-8, and IL-1β (Jiang et al., 2013). IL-6 concentrations in urine samples were also found to positively correlate with pain scores (Erickson et al., 1997). These mediators elicit a host of vascular events, including arteriolar vasodilation, plasma extravasation from post-capillary venules,

leukocyte adhesion to the vascular endothelium, and leukocyte migration into the inflamed or damaged tissue (Birder & Kullmann, 2018), resulting in the disruption of tissue structure and function, heightened afferent sensitivity, and altered smooth muscle contractility. These functional changes underly the LUT symptoms of voiding dysfunction, poor bladder compliance, and hyperalgesia (Birder & Kullmann, 2018; Grover et al., 2011).

Increasing evidence also suggests a strong role of neurogenic inflammation in IC, in which neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP) are released from nociceptors and propagate the local inflammatory response (Chiu et al., 2012; Gonzalez et al., 2014a). The infiltration of immune cells and release of pro-inflammatory mediators are known to sensitize peripheral afferent fibers (Ren & Dubner, 2010). Visceral pain arising from the bladders of IC patients may be mediated by alterations in peripheral afferent pathways, such that afferent fibers respond in an exaggerated manner to typically innocuous stimulation (Gonzalez et al., 2014). Algogenic neuropeptides, including SP, NGF, CGRP and bradykinin have been shown to be increased in bladder tissue and urine samples from IC patients (Birder & Kullmann, 2018). The release of these molecules into the periphery causes the sensitization of peripheral afferent fibers (Schaible et al., 2011). For example, overexpression of NGF in mouse urothelial cells has been shown to cause neuronal hyperinnervation, pelvic hypersensitivity, and alternations in bladder function (Schnegelsberg et al., 2010). Acute intravesical challenge with lipopolysaccharide (LPS), similar to the model used in this study, has been shown to significantly increase the bladder content of NGF (Bjorling et al., 2001). In addition, an increase in SP positive nerve fibers, and in mast cell infiltration,

have both been observed in IC patients (Pang et al., 1995). SP is a tachykinin secreted by activated C-fiber afferents and inflammatory cells, that functions as an inflammatory mediator, and is directly involved in the recruitment and degranulation of mast cells (Li et al., 2012). Mast cells store a number of vasoactive, pro-inflammatory, and nociceptive mediators (Sant et al., 2007). Exposure of the bladder to these compounds further sensitizes bladder afferents, and exacerbates the local inflammatory response in a positive-feedback fashion (Grover et al., 2011). The excess release of SP also impacts the smooth muscle, causing bladder spasticity, urgency and pain (Birder & Kullmann, 2018). In mice that are deficient in mast cells, the severity of neurogenic inflammation induced by exogenous SP administration is notably reduced (Bjorling et al., 1999).

The alterations in urothelial permeability, afferent nerve stimulation, and infiltration of mast cells are highly interrelated, and causes both positive and negative feedback loops centered around persistent inflammation (Sant et al., 2007). The cycle continues as immune cells and mediators derived from uroepithelial dysfunction and neurogenic inflammation sustain peripheral and central sensitization, and establish visceral hyperalgesia (Gonzalez et al., 2014). In sum, this process is an intricate interplay between vascular, immune, and nervous systems, resulting in alterations in bladder structure and function, which manifests in dysfunctional voiding symptoms and chronic pelvic pain (Birder & Kullmann, 2018).

1.3 The Recruitment of Immune Cells in IC

The early response to a noxious stimulus or tissue injury occurs in the form of acute inflammation, and is characterized by local vasodilation, increased vascular permeability and leukocyte migration, and activation of inflammatory cascades causing

the release of cytokines, histamines, kinins, and pro-inflammatory mediators (Grover et al., 2011). In the bladder, these mediators cause erythematous swelling, ulceration of the bladder mucosa, and subsequent irritation. If the inflammatory stimulus is not removed, chronic inflammation ensues, leading to irreversible tissue damage and subsequent bladder dysfunction symptoms, such as those observed in IC patients (Grover et al., 2011). One common feature of an immune response is the migration of immune cells from the blood to the tissue (Zarbock & Ley, 2009). The recruitment of leukocyte to sites of inflammation involves a sequence of interactions with the vascular endothelium, and subsequent transmigration through the vessel wall at the target site (Zarbock & Ley, 2009). Various immune cells, including macrophages, lymphocytes, eosinophils, neutrophils, and mast cells infiltrate the bladder during IC (Erickson et al., 1997; Grover et al., 2011). This infiltration process involves the tethering and rolling of the immune cell along the endothelium, firm adhesion to the vessel wall, and transmigration into the tissue (Granger & Senchenkova, 2010). Intravital microscopy (IVM) is a powerful technique that allows for the visualization of the microcirculation under both physiological and pathophysiological conditions, and can be used to visualize and quantify leukocyte recruitment *in vivo* (Pinho et al., 2011).

Leukocyte tethering and subsequent rolling are the initial interactions of leukocytes with the vascular endothelium (Kowalewska et al., 2011). This interaction is mediated by the selectin class of adhesion molecules, which are type-1 transmembrane glycoproteins (Cummings & McEver, 2009). Selectins function to slow the velocity of leukocyte movement along the vascular endothelium to allow for firm adhesion (Granger & Senchenkova, 2010), and are expressed on both endothelial cells and leukocytes. Three

types of selectins are involved in leukocyte tethering; P-selectin, E-selectin, and L-selectin. P-selectin is expressed by endothelial cells and platelets (Zarbock & Ley, 2009). In endothelial cells, P-selectin is stored in secretory granules called Weibel-Palade bodies. Upon stimulation by inflammatory mediators such as histamine, cytokines, and leukotrienes, Weibel-palade bodies fuse with the plasma membrane to increase the surface expression of P-selectin (Wang et al., 2003; Zarbock & Ley, 2009). E-selectin serves a similar role, but unlike P-selectin, is not stored in granules, and is regulated by transcriptional activation (Kraiss et al., 2003). Leukocytes also express L-selectin, and P-selectin glycoprotein ligand 1 (PSGL-1), which assist in the leukocyte tethering process. L-selectin is localized to leukocyte microvilli, facilitating leukocyte-endothelial interactions by binding sialyl Lewis^x (sLe^x) and proteoglycans on the endothelial surface (Ivetic et al., 2019). L-selectin also mediates secondary tethering, whereby free-flowing leukocytes tether to an already rolling leukocyte (Kelly et al., 2007). PSGL-1 is the major P- and E-selectin ligand under flow conditions, and is constitutively expressed on the surface of leukocytes (Kappelmayer & Nagy, 2017).

Acting in opposition to the function of leukocyte and endothelial cell adhesion molecules are the hydrodynamic and shear forces generated by the movement of blood through the microvasculature (Granger & Senchenkova, 2010). Given the higher haemodynamic pressure in the arterial circulation, these forces are significantly higher in arterioles compared to venules. Therefore, leukocyte rolling, and subsequent adhesion, is rarely observed in the arteriolar microcirculation. Furthermore, it has been shown that leukocyte adherence to the venular endothelium induced by an inflammatory stimulus is inversely proportional to the wall shear rate (Slattery et al., 2005). However, the

expression of selectins and other adhesion molecules has also been shown to be significantly higher in post-capillary venules than in arterioles, and is further upregulated during inflammation (Sumagin & Sarelius, 2013) This combination of reduced shear force and higher expression of adhesion molecules makes post-capillary venules the predominant site of leukocyte rolling, adherence and tissue transmigration (Granger & Senchenkova, 2010).

The processes of leukocyte rolling stimulates the activation of signalling pathways that activate integrins, another family of adhesion molecules, which further reduce rolling velocity and mediate firm arrest (Ley et al., 2007). Integrins are heterodimeric proteins comprised of α and β subunits that form strong adhesive interactions with counter receptors on endothelial cells (Granger & Senchenkova, 2010). Different immune cells express different combinations of α and β subunits, the most common of which are the β_1 and β_2 integrins. β_2 integrins have a common β subunit, CD18, and are exclusively expressed on all leukocytes, while β_1 integrins share a common CD29 subunit, and are expressed on eosinophils, lymphocytes, monocytes, and natural killer cells (Krieglstein & Granger, 2001). The α subunits largely dictate ligand specificity of the integrins to immunoglobulin (Ig) supergene cell surface molecules and a range of extracellular matrix proteins (Krieglstein & Granger, 2001).

Leukocyte integrins bind to Ig-like adhesion molecules expressed on the surface of the vascular endothelium to facilitate firm leukocyte adhesion. This class of adhesion molecules includes intracellular adhesion molecule 1 (ICAM-1) and 2 (ICAM-2), vascular cell adhesion molecule 1 (VCAM-1), and platelet endothelial cell adhesion molecule (PECAM-1) (Granger & Senchenkova, 2010). ICAM-1 and PECAM-1 are

constitutively expressed on endothelial cells and are not up-regulated in response to pro-inflammatory mediators. Conversely, ICAM-1 and VCAM-1 expression is highly inducible by cytokines and endotoxins, and exhibit a time- and dose-dependent increase in surface expression (Zhu et al., 2013) facilitated by the transcription factors NF-ϰB and activator protein-1 (AP-1) (Sawa et al., 2008). As mentioned, the α integrin subunit plays a critical role in determining integrin binding specificity for cell adhesion molecules. The very late antigen 4 (VLA-4) integrin ($\alpha_4\beta_1$) specifically binds VCAM-1 (Elices et al., 1990), while the lymphocyte function-associated antigen 1(LFA-1) integrin ($\alpha_L\beta_2$) interacts with ICAM-1 (Long, 2011). Studies using antibody's to block integrin function have shown that firm adhesion of leukocytes to the endothelium becomes significantly reduced (Muller, 2013).

Once firmly adherent, leukocytes begin to emigrate out of the vasculature through either a transcellular or paracellular route (Muller, 2011). The paracellular route involves leukocytes transmigrating to the extravascular tissue from adjacent endothelial cell junctions, whereas the transcellular route sees leukocyte pass directly through the endothelial cells in a transcytotic fashion (Muller, 2011). While the paracellular route is more common, leukocytes are capable of both (Petri & Bixel, 2006). Aside from the aforementioned cellular adhesion molecules (CAM's), a number of key proteins have been identified as important regulators of transendothelial migration (TEM). Junctional adhesion molecule A (JAM-A) is one such protein and is concentrated at endothelial cell borders. During inflammation, JAM-A redistributes from cell-cell junctions to the apical membrane domain (Stamatovic et al., 2012), where it binds to CD18 (LFA-1) integrin on leukocytes (Ostermann et al., 2002). Anti-JAM-A antibodies have been shown to

decrease TEM *in vivo* (Woodfin et al., 2009). PECAM-1 also plays a crucial role in mediating TEM, and is concentrated at endothelial cell borders and diffusely on leukocytes (Muller, 2011). A homophilic interaction between endothelial and leukocyte PECAM is required for TEM (Mamdouh et al., 2003). When this interaction is inhibited, leukocytes remain tightly adhered to the endothelium, but are unable to actively transmigrate (Liao et al., 1995). Furthermore, CD99 is thought to act through a similar homophilic interaction, like PECAM, however it has been shown to regulate a later step in the TEM process (Muller, 2013). CD99 (single-chain type-1glycoprotein) may also function in opening endothelial cell junctions to allow for leukocyte insertion (Petri & Bixel, 2006)

1.4 Microvascular Dysregulation During Inflammation

The leukocyte rolling and adhesion process occurs in the post-capillary venules of the microcirculation, which is the terminal vascular network of the systemic circulatory system comprised of microvessels with diameters of less than 20μm (Guven et al., 2020). These microvessels consist of arterioles, capillaries, and venules. The main cell types in the microvasculature include endothelial cells which line the vessel walls, smooth muscle cells, red blood cells (RBC), leukocytes, and blood plasma components (Ince, 2005). The highly branching network of microvessels is the site of oxygen, nutrient, hormone, and metabolic waste transfer between the blood and tissues. The main function of the microcirculation is to maintain optimal perfusion and oxygen delivery to organs, in accordance with their metabolic demands (Garon, 2015). The regulation of capillary perfusion is a dynamic process, involving myogenic (i.e., vascular tone), metabolic (e.g.,

pCO_2, lactate, H^+) and neurohumoral (e.g., aldosterone, vasopressin) mechanisms (Ince, 2005).

The microcirculation also plays a critical role in inflammation. During an inflammatory event, the microvasculature undergoes a range of functional and structural changes (Granger & Senchenkova, 2010). These changes include an impairment of vasomotor function, a reduction in capillary perfusion, adhesion of leukocytes and platelets in post-capillary venules, enhanced intravascular coagulation, and an increase in vascular permeability (Granger & Senchenkova, 2010). Inflammation is associated with alterations in microvascular blood flow, with hyperemia often being noted during the early phase. The hyperemic response reflects the reaction of arterioles to the rapid release of vasoactive mediators, such as histamine, prostaglandins, and NO (Granger & Senchenkova, 2010). These mediators also greatly increase vascular permeability, allowing for an increased extravasation of not only leukocytes, but also plasma proteins and water, into the interstitial space (Granger & Rodrigues, 2016). Excessive extravascular fluid accumulation leads to the formation of interstitial edema, which can occlude the capillary lumen and impair local tissue perfusion (Granger & Rodrigues, 2016). Interstitial edema and vascular congestion are frequently observed in IC patients (H.-J. Kim, 2016).

The accumulation and migration of leukocytes through the microvasculature can also affect capillary perfusion. In comparison to RBC's, leukocytes are much larger and have diameters greater than capillaries in most tissue (Warnke & Skalak, 1990). Thus, there is a natural tendency for passive leukocytes to slow blood flow as they enter and transit through the capillary network (Mazzoni & Schmid-Schonbein, 1996). This is

exaggerated when there is an increased number of infiltrating immune cells during inflammation, and a decrease in perfusion pressure due to local vasodilation (Harris & Skalak, 1993). A reduction in leukocyte margination has been shown to increase functional capillary density (FCD), an indicator of tissue perfusion, and increase RBC velocity (Kuebler et al., 1997). Therefore, drugs that are able to maintain normal microvascular perfusion by limiting leukocyte aggregation and minimizing microvascular hyperpermeability have received attention as potential therapeutics for acute and chronic inflammatory diseases (Granger & Rodrigues, 2016).

1.5 Current Treatments for IC

IC is a difficult disorder to treat and manage due to unclear understanding of the disorder's etiology, the symptom variability among patients, and the inconsistencies across diagnostic criteria's (Hanno et al., 2011). Given the current lack of a curative treatment for IC, the main goals of management are to provide relief of symptoms and optimize patient quality of life (Cox & Golda, 2016). However, while many therapeutic approaches exist, none have been shown to be consistently effective in providing relief to all patients (Pazin et al., 2016; Rovner et al., 2000). A stepwise approach is taken to IC management, starting with non-invasive "first-line" therapies, and moving towards more invasive treatments that have a higher risk of adverse effects.

The first-line, conservative therapies for IC include patient education, dietary and lifestyle modifications, bladder training, altered fluid intake, and the incorporation of stress management techniques (Cox & Golda, 2016; Foster Jr et al., 2009). Many patients experience exacerbations of symptoms upon ingesting certain foods and drinks, which often include coffee, alcohol, and citrus fruits (Cox & Golda, 2016). Removing these

foods through dietary modification has been shown to improve IC symptoms (Oh-Oka, 2017). Specific bladder training exercises can also be done, with the goal of reducing voiding frequency and urgency, and increasing bladder capacity (Cox & Golda, 2016; C. Parsons & Koprowski, 1991). Finally, stress-reduction strategies, including bathing, yoga, and mediation, can be beneficial to reduce the psychological impacts of IC (Whitmore, 2002).

Second-line treatments for IC include both physical and pharmacological treatment modalities. Many patients with IC experience pelvic floor dysfunction (PFD), and therefore benefit from physiotherapy directed towards relaxation and stretching of the muscles of the pelvic floor (Fitzgerald et al., 2012). Acupuncture, and injection of a local anesthetic into pelvic floor trigger points are also postulated as optional second line physical therapies (Cox & Golda, 2016). Second line pharmacological treatments involve a range of oral medications. Amitriptyline, a common tricyclic antidepressant (TCA), is the most commonly used agent for initial pharmacologic therapy. Amitriptyline has been shown to be most effective at higher doses (> 50 mg daily), however, less than 50% of patients are able to tolerate this high therapeutic dosage (Foster Jr et al., 2009). Pentosan polysulfate (PPS), the only oral medication approved by the FDA and Health Canada (HC) for the treatment of IC (Cox & Golda, 2016; Hanno et al., 2015), is a heparinoid medication that helps reconstitute the deficient GAG layer and correct urothelial permeability (Sokol & Sokol, 2007). The most recent randomized, double-bind, placebo controlled study on the efficacy of PPS treatment revealed no significant treatment benefit (Nickel et al., 2015). Other oral medications used as second line treatments include the antihistamines cimetidine, a histamine$_2$-receptor antagonist (H_2RA), and

hydroxyzine, an histamine$_1$-receptor inverse agonist (Hanno et al., 2015). Support for the use of cimetidine is limited to small scale observational studies lacking long-term follow up data (Dasgupta et al., 2001), while the one randomized controlled trial (RCT) on hydroxyzine showed no difference in symptom improvement between the hydroxyzine and placebo groups (Sant et al., 2003).

Intravesical drug instillations are also second-line treatment options based on the AUA guidelines (Hanno et al., 2015). Intravesical drug delivery allows for a high drug concentration to be delivered directly to the bladder, while reducing systemic exposure and therefore minimizing potential systemic side effects (Zacchè et al., 2015). Currently, dimethyl sulfoxide (DMSO, Rimso-50®) is the only FDA/HC approved intravesical drug treatment. A recent review of patient reported treatment effectiveness showed that only 36.95% of patients treated with DMSO reported improved symptoms, while 32.7% reported worsening of symptoms (Lusty et al. 2018). DMSO has been reported to facilitate detrusor muscle relaxation, has reported anti-inflammatory and analgesic actions, and can inhibit mast cell degranulation (Kurth & Parsons, 2003). Moreover, relief of symptoms is only reported in half of the patients treated (Sant & LaRock, 1994). More recent evidence also suggests intravesical DMSO treatment is only effective for patients with Hunners-type IC (Tomoe, 2015). DMSO is often used in conjunction with other intravesical agents, including local anesthetics (e.g., lidocaine, bupivacaine) and GAG analogues (e.g. chondroitin sulfate, hyaluronic acid, heparin) (Cox & Golda, 2016).

Third-line treatments, and beyond, are more invasive and are reserved for patients who do not respond to first- and second-line therapies. These treatments are typically provided using sedation or general anesthesia (Jerauld et al., 2016). Cystoscopy with

short duration, low pressure hydrodistension is one of the most common third-line treatments (Cox & Golda, 2016), but only provides short-term symptom improvement (Erickson et al., 2007). Observational studies have shown that hydrodistension efficacy rates are highly variable, ranging from 0-70% (Aihara et al., 2009; Cole et al., 2005). Intradetrusor injection of botulinum toxin A (BTX-A), and the implantation of sacral neuromodulatory devices constitute available fourth-line treatments (Jerauld et al., 2016), but should only be used in patients refractory to other treatments (Cox & Golda, 2016). Intadetrusor BTX-A inhibits the release of acetylcholine at the neuromuscular junction preventing detrusor overactivity (Linsenmeyer, 2013; Shenot & Ryann Mark, 2013). Similarly, sacral nerve modulation (SNM) with an implantable neuroprosthetic device modulates the activity of the bladder and pelvic floor muscles, and has shown high efficacy for treating urodynamic symptoms, including urge incontinence, urinary urgency, frequency, and retention (Brookoff & Bennett, 2006; Colaco & Evans, 2015). However, SNM has a high rate of surgical revision, and has multiple potential side effects (Ghazwani et al., 2011), including painful stimulation, infection, and battery site pain (Cox & Golda, 2016).

Finally, immunosuppressive therapy using cyclosporine-A has shown positive effects in multiple observational studies (Forrest et al., 2012; Sairanen et al., 2005). This fifth-line treatment provided a significant improvement in symptoms in one RCT, yet side effects occurred in 94% of patients (Sairanen et al., 2004), with hypertension, elevated serum creatinine, and reduced glomerular filtration rate (GFR), being the most common (Crescenze et al., 2017). Once all treatment options have been exhausted, surgical procedures, including urinary diversion, augmentation cystoplasty, partial or total

cystectomy can be performed (Garzon et al., 2020). Patients who have undergone these surgical treatments report significant improvements in pain, urinary symptoms, and quality of life (Andersen et al., 2012), however these procedures are considered last resort options (Cox & Golda, 2016).

Despite the numerous and diverse treatment options available, many IC patients remain dissatisfied with their symptom management. In one survey of 750 IC patients, 27-50% of patients reported no improvement in their condition across all treatments, with an addition 26-31% reporting that their condition had worsened (Hill et al., 2008). Furthermore, there appears to be a disconnect between IC treatment guidelines based on efficacy data from clinical trials, and patient reported effectiveness in clinical practice (Lusty et al., 2018). There remains a need for safe and effective treatment options for IC patients. Cannabinoids are an emerging therapeutic option for the management of difficult-to-treat pain arising from chronic illnesses (Russo, 2008), and are currently listed as such for the treatment of IC in the CUA guidelines (Cox & Golda, 2016).

1.6 *Cannabis sativa* and The Endogenous Cannabinoid System

Cannabis sativa is one of the oldest plants cultivated by humans for recreational, industrial, and medical purposes, with archeological and historical evidence suggesting its use dating as far back as 12,000 years (Abel, 1970; Adams & Martin, 1996). Ancient Chinese and Greek civilizations utilized constituents of the cannabis plant to create rope, clothing, and other textiles. Historical reports also reference Romans using the strong hemp fibers to create cannabis rope for use in naval construction (Adams & Martin, 1996). The earliest reference of cannabis being used for medicinal purposes stretches

back to 2700BC where it was used to treat ailments including constipation, malaria, and rheumatic pain (Adams & Martin, 1996). It wasn't until the beginning of the 19th century that cannabis was introduced to Western medicine, where its anticonvulsive, analgesic and antiemetic properties were used to treat patients suffering from conditions including seizures, tetanus, rabies, and rheumatism (Adams & Martin, 1996; Mechoulam & Feigenbaum, 1987). Today, cannabis remains one of the most commonly cultivated, trafficked, and illicitly abused drugs worldwide, according to the World Health Organization (WHO) (Bridgeman & Abazia, 2017). Beyond criminalization, the medicalization of cannabis has been hindered due to its psychoactivity, and strong association with the development of mental health disorders, including schizophrenia and other types of psychoses, especially in developing youth (Hall & Degenhardt, 2007; Løberg & Hugdahl, 2009).

More recent interest into the therapeutic potential of cannabis-based medicines was sparked following the elucidation of the endogenous cannabinoid system (ECS). The ECS is involve in a variety of physiological processes, including but not limited to metabolism, neurotransmission, cognition, pain-sensation, and inflammation (Clarke & Watson, 2007; Pertwee, 2006). In short, the ECS is comprised of three major components; the bioactive lipid-based signalling molecules (endocannabinoids), the cannabinoid receptors, and the biosynthetic and metabolic enzymes responsible for production and catabolism of endocannabinoids (Lu & Mackie, 2016; Pertwee & Ross, 2002).

1.6.1 Cannabinoid Ligands

Aside from the well-known psychotropic effects, cannabinoid receptor ligands have been shown to induce a range of biological effects: analgesic and antiemetic, to anti-inflammatory and anti-tumor (Chakravarti et al., 2014; Elikkottil et al., 2009; Fukuda et al., 2014; Toguri et al., 2015). These ligands can be subdivided into three categories, based on their origin; endogenous cannabinoids (endocannabinoids), synthetic cannabinoids, and phytocannabinoids (Howlett et al., 2002).

Endogenous cannabinoids are polyunsaturated fatty acid (i.e. arachidonic acid (AA)) derived eicosanoids that engage cannabinoid receptors (Giuffrida & McMahon, 2010). The first discovered, and best-characterized endogenous cannabinoids are arachidonoyl-ethanolamide (AEA, anandamide), and 2-arachidonoyl glycerol (2-AG) (Lu & Mackie, 2016). Other, less studied endogenous cannabinoids include virodhamine, noladin ether, and N-arachidonoyldopamine (NADA) (Battista et al., 2012). Palmitoylethanolamine (PEA), and N-oleoylethanolamine (NEA), and Oleoylethanolamide (OEA) are endogenous fatty acid ethanolamides, however they exert biological activity through non-cannabinoid receptors (Tsuboi et al., 2018). In contrast to classical neurotransmitters synthesised in advance and stored in and vesicles, endocannabinoids are synthesized and released into the extracellular space on-demand via enzymatic catabolism of membrane lipids (Lu & Mackie, 2016). Activation of a membrane bound receptor, or cellular depolarization, stimulates the conversion of the phospholipid precursors from the lipid bilayer into endocannabinoids (Lu & Mackie, 2016). While AEA and 2-AG are both AA-derived mediators, they differ in their synthetic and degradative pathways, as well as in their cannabinoid receptor affinities (Pertwee, 2006b). While 2-AG functions as a full agonist at both CB_1R and CB_2R, AEA

acts as a partial agonist at both receptors (Reggio, 2010). Under normal physiological conditions, the tissue concentrations of 2-AG are generally hundreds to thousands of times higher than those of AEA (Fezza et al., 2014; Tsuboi et al., 2018), thus 2-AG is considered the natural primary ligand for CB_2R (Sugiura & Waku, 2002). For example, in the central nervous system, 2-AG concentrations are 1000 times higher than that of AEA (Zou & Kumar, 2018). This has also been demonstrated in the a number of peripheral tissues, including the skin (Correia-Sá et al., 2020), intestine (Basu & Dittel, 2011), and urinary bladder (Wang et al., 2015).

The discovery and elucidation of the endogenous cannabinoid ligands prompted the development of selective and non-selective cannabinoid receptor agonists and antagonists (Pertwee, 2006). These synthetic compounds have been designed to structurally resemble the endogenous and phytocannabinoids with varying degrees of receptor binding affinity and efficacy (Zou & Kumar, 2018). Selective, high affinity CB_1R agonists include R-(+)-ethanandamine, Arachidonoyl-2'-chloroethylamide (ACEA), and noladin ether, while selective synthetic agonists for CB_2R include HU-308, AM1241, and JWH-133 (Pertwee, 2010). Several CB_1R and CB_2R selective antagonists have been developed and have demonstrated inverse-cannabimimetic effects (Pertwee, 2010). Synthetic cannabinoids have been approved for clinical use, including Dronabinol® and Nabilone®. Dronabino®l has an identical structure to Δ^9-tetrahydrocannbinol (Δ^9-THC), and is used to treat severe nausea and vomiting caused by cancer chemotherapy, and HIV/AIDS-induced anorexia and weight loss (Badowski & Yanful, 2018; T. P. Freeman et al., 2019). Nabilone ® is a synthetic THC analog with a slightly different chemical structure also used to treat chemotherapy-induced nausea and

vomiting (Freeman et al., 2019). A synthetic CB_1R-selective antagonist indicated for the treatment of obesity (Rimonabant-®) was approved for use in Europe in 2006, but was suspended in 2008 due to risk of severe psychiatric side effects (McLaughlin, 2017).

Thirdly, the phytocannabinoids are a diverse group of chemical constituents of the cannabis plant. Typical phytocannabinoids are terpenophenolic compounds derived from a C_{21} or C_{19} precursor (Thomas et al., 2016). To date, more than 120 phytocannabinoids have been identified and isolated from the cannabis plant (Morales et al., 2017), the most abundant of which is Δ^9-THC. Discovered in 1964 by Raphael Mechoulam (Gaoni & Mechoulam, 1964), Δ^9-THC is the main constituent of cannabis responsible for the psychoactive effects of cannabis consumption (Morales et al., 2017). Other well-known and abundant phytocannabinoids, which are extensively reviewed by El-Sohly & Gul (2014), include cannabinol (CBN), cannabidiol (CBD), cannabigerol (CBG), Δ^8-tetrahydrocannbinol (Δ^8-THC), cannabivarin (CBV), and cannabichromene (CBC), among others. Of the aforementioned phytocannabinoids, CBD has garnered significant attention due to its well established anti-inflammatory, anti-epileptic, analgesic, and anxiolytic properties, while being devoid of psychoactive effects (Gonçalves et al., 2020). A few phytocannabinoid preparations have been approved for clinical use. For example, Epidiolex ®, an oral CBD solution, was approved in 2018 by the Unites States Food and Drug Administration (FDA) for the treatment of seizures associated with rare forms of childhood epilepsy (Freeman et al., 2019). Moreover, Sativex ®, a buccal spray comprised of a 1:1 ratio of Δ^9-THC and CBD, has been approved in a number of countries, including in Canada, for the adjunctive treatment for symptomatic relief of neuropathic pain in adults with multiple sclerosis (Freeman et al., 2019).

Phytocannabinoids have vastly different affinities and functionalities at the cannabinoid receptors (Morales et al., 2017), and also have non-cannabinoid receptor targets including peroxisome proliferator-activated receptors (PPARs) (O'Sullivan, 2016), transient receptor potential (TRP) channels (Muller et al., 2019) and other GPCR's, such as GPR55 (Sharir & Abood, 2010) and GPR18 (McHugh et al., 2010). Cannabis also contains many terpenes, which are aromatic compounds formed by repeating isoprene units, and give the cannabis plant its distinct aroma (Gonçalves et al., 2020). Various terpenes have demonstrated a range of therapeutic properties (Cox-Georgian et al., 2019), and may be beneficial in the treatment of various cancers, and in the management of chronic pain and inflammatory disorders (Russo & Marcu, 2017). However, few terpenes have been shown to interact directly with the cannabinoid receptors (Gonçalves et al., 2020).

β-Caryophyllene (BCP) is one of the major sesquiterpenes found in cannabis and has strong affinity and full agonist functionality at the CB_2R (K_i= 155 ± 4nM) (Gertsch et al., 2008; Hartsel et al., 2016). BCP is found in large concentrations in the essential oils of common spices and plants, including oregano (*Origanum vulgarae* L.), clove (*Syzygium aromaticum*), and black pepper (*piper nigrum* L.), and has 'generally regarded as safe' (GRAS) designation from FDA as a food additive, and for use in cosmetic products (Gertsch et al., 2008; Hartsel et al., 2016). The acute oral lethal dose (LD_{50}) of BCP in rats is greater than 5000 mg/kg, demonstrating its very low *in vivo* toxicity (da Silva Oliveira et al., 2018; Schmitt et al., 2016). Radioligand binding assays have shown that BCP interacts with the CP55,940 binding site (i.e., THC binding site) of the CB_2R, and exhibits no affinity at the CB_1R (Gertsch et al., 2008). CB_2R activation by BCP

causes inhibition of adenylyl cyclase (AC), triggers stimulation of intracellular calcium transients, and activation of MAP kinases (Gertsch et al., 2008). Consistent with the effects of CB_2R agonists, BCP produces anti-inflammatory and analgesic effects in a range of *in vivo* models, including lipopolysaccharide-induced models of inflammation (Askari & Shafiee-Nick, 2019), and the carrageenan-induced paw edema test (Gertsch et al., 2008). BCP's lack of affinity for the CB_1R, and its well reported anti-inflammatory and analgesic effects have garnered significant interest as a potential therapeutic option for conditions with inflammatory pathologies (Klauke et al., 2014).

1.6.2 Cannabinoid Receptors

Two cannabinoid receptors have been identified to date: cannabinoid receptor 1 (CB_1R) and cannabinoid receptor 2 (CB_2R). Both receptors belong to the superfamily of class A rhodopsin-like G protein-coupled receptors (GPCR), and have seven transmembrane spanning domains (Galiègue et al., 1995; Howlett, 2002). While the two receptors share a 44% amino acid homology (Console-Bram et al., 2012), they differ significantly in their tissue distribution patterns (Howlett, 2002).

The first cloned cannabinoid receptor (Matsuda et al., 1990), CB_1R, is one of the most abundant GPCR's in the CNS, with particularly high expression in the neocortex, hippocampus, basal ganglia, cerebellum, and brainstem (Herkenham et al., 1991; Kendall & Yudowski, 2017). This receptor has been shown to be implicated in a range of higher brain functions, including executive, emotional, reward, and memory processing (Wu, 2019). The CB_1R is also highly enriched at presynaptic and axonal locations (Kendall & Yudowski, 2017; Straiker & Mackie, 2005), where they mediate inhibition of neurotransmitter release from axonal terminals (Howlett et al., 2002). The distribution

pattern of the CB_1R within the CNS accounts for the numerous characteristic effects of CB_1R agonists, including catalepsy, hypokinesia, and psychotropic effects (Howlett, 2002). The CB_1R has also been identified in several extra-neural cells and peripheral tissues, including in the reproductive organs, eyes, vascular endothelium, adrenal glands, bone marrow, and prostate (Galiègue et al., 1995).

The CB_1R and CB_2R are both coupled to pertussis toxin (PTX)-sensitive $G_{i/o}$ protein, which upon activation, suppresses cyclic adenosine monophosphate (cAMP) production through the inhibition of AC (Howlett et al., 2002). The reduction of the intracellular concentration of cAMP decreases the activity of protein kinase A (PKA), which phosphorylates the cAMP-response element binding protein/activation transcription factor (CREB/ATF) family of transcriptional regulators (Berdyshev, 2000). The CB_1R also modulates the activity of several ion channels, including N, P/Q, and R-type Ca^{2+} channels, and G-protein coupled inwardly rectifying K^+ channels (GIRKs) (Zou & Kumar, 2018), altering calcium and potassium currents, neuronal excitability, and neurotransmission (Chevaleyre et al., 2006; A. C. Howlett, 2005). Furthermore, stimulation of CB_1R leads to the phosphorylation and activation of mitogen-activated protein kinase (MAPK) signaling cascades, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, which regulate nuclear transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\varkappa$B), and c-Myc (Howlett, 2005; Panikashvili et al., 2005).

The CB_2 receptor was the second cannabinoid receptor to be cloned, and was isolated from macrophages in the marginal zone of the spleen (Munro et al., 1993). While CB_2 receptors are found in select areas of the CNS including in microglial cells (Atwood

& Mackie, 2010), expression of the CB_2R in the CNS is significantly lower compared to the CB_1R (Lu & Mackie, 2016; Navarro et al., 2016). CB_2 receptors are highly localized to the cells and tissues of the immune system, including the spleen, thymus, tonsils, and lymph nodes (Tsuboi et al., 2018). Among white blood cell populations, expression is highest in B-cells, natural killer cells, monocytes, and polymorphonuclear cells, and lower in $CD4^+$ and $CD8^+$ T-cells (Galiegue et al., 1995). CB_2R expression is also largely dependent on the activation status of the immune cells, suggesting a strong role in immune regulation (Basu & Dittel, 2011; Carlisle et al., 2002). CB_2R expression is highly inducible, with mRNA levels increasing as much as 100-fold following nerve injury or during inflammation (Dhopeshwarkar & Mackie, 2014; Hsieh et al., 2011). Peripheral tissues that express CB_2R include the retina, bone marrow, gastrointestinal tract, and bladder uroepithelium (Atwood & Mackie, 2010; Bjorling & Wang, 2018; Toguri et al., 2014). A defining difference between CB_1R and CB_2R is the lack of psychotropic effects associated with CB_2R activation, which has hindered the development of CB_1R directed therapies (Ashton & Glass, 2007). Therefore, selective CB_2R agonists have the potential to provide the desired anti-inflammatory and immunosuppressive effects, while avoiding undesired psychoactive effects.

Similar to CB_1R, CB_2R signals primarily through heterotrimeric $G_{i/o}$ proteins to modulate a range of signalling pathways, the most studied of which is modulation of AC activity, which is known to play a key role in immune cell proliferation and differentiation (Basu & Dittel, 2011). CB_2R activation also elicits its biological effects through MAPK cascades, including ERK, p38 MAPK, and JNK. These signaling pathways have been shown to modulate cytokine production and immune cell migration

(Basu & Dittel, 2011). Other signalling pathways modulated by CB_2R include GIRK activation, and the recruitment of β-arrestin (Dhopeshwarkar & Mackie, 2014). CB_2R ligands also exhibit significant functional selectivity (biased agonism), whereby different ligands are able to activate or inhibit signalling pathways with different rank order potencies (Atwood et al., 2012; Dhopeshwarkar & Mackie, 2014). However, it remains unknown which signal transduction pathway, or combination of pathways, are most relevant for therapeutic purposes (Soethoudt et al., 2017).

1.6.3 Endogenous Cannabinoid Biosynthesis and Metabolism

The numerous anabolic and catabolic enzymes involved in the biosynthesis and breakdown of endocannabinoids are key components of the endogenous cannabinoid system. The two principal endocannabinoids, AEA and 2-AG, are both AA derivates, however their synthetic and degradative pathways differ significantly (Pál Pacher et al., 2006). In general, endocannabinoids are produced intracellularly in a stimulus-dependent fashion and are released into the extracellular environment where they engage cannabinoid receptors.

2-AG can be biosynthesized through multiple pathways in which all precursors are arachidonic acid-containing glycerophospholipids, abundant in cell membranes (Sugiura et al., 2006). Inositol phospholipids with 2-arachidonoyl groups represent the main precursor for 2-AG synthesis. Inositol phospholipids are hydrolyzed by phospholipase C (PLC) to form 2-arachidonoyl-diacylglycerol, which is deacylated by an *sn*-1 specific diacylglycerol lipase (DAGL) to form 2-AG (Tsuboi et al., 2018). Other glycerophospholipids, including phosphatidic acid and phosphatidylcholine (PC), can also be hydrolyzed to form 2-AG (Bisogno et al., 1999; Tsuboi et al., 2018).

2-AG is metabolized by a number of enzymes, which decrease 2-AG concentrations and terminate 2-AG signalling (Baggelaar et al., 2018). The main catabolic pathway of 2-AG involves hydrolysis into AA and glycerol by the serine hydrolase, monoacylglycerol lipase (MAGL). In the CNS, MAGL accounts for approximately 85% of 2-AG hydrolysis (Blankman et al., 2007), and is also the major contributor to peripheral 2-AG breakdown (Schlosburg et al., 2010a). Mice treated with a MAGL inhibitor showed significantly increased levels of 2-AG (Longn et al., 2009; Muccioli, 2010), and decreased levels of free AA (Long et al., 2009), which is the main substrate for COX-2 mediated production of pro-inflammatory eicosanoids, including prostaglandin E_2 (PGE_2) (Murataeva et al., 2014). 2-AG is also a substrate for COX-2 mediated production of PGE_2, however, MAGL inhibition has been shown to cause significant reductions in numerous pro-inflammatory prostaglandins and eicosanoids, including PGE2, prostaglandin D2 (PGD2), prostaglandin F2-alpha (PGF2α), thromboxane B_2 (Nomura et al., 2011). This finding suggests a duel role of MAGL in attenuating 2-AG signalling and regulating eicosanoid production (Murataeva et al., 2014). Furthermore, JZL184, a selective MAGL inhibitor, increases 2-AG in the CNS and peripheral tissues, resulting in cannabimimetic effects (Long et al., 2009; Muccioli, 2010). Other enzymes involved in 2-AG catabolism include alpha/beta domain hydrolases 6 and 12 (ABHD6/12) (Baggelaar et al., 2018), cytochrome P450 (CYP450) (Zelasko et al., 2015), and lipoxygenases (Kozak et al., 2002), however their relative contributions are minor compared to MAGL (Murataeva et al., 2014).

Anandamide is member of the N-acylethanolamine (NAE) family of bioactive lipids (Lambert & Muccioli, 2007). The majority of NAE's, including AEA, are

generated from an N-arachidonoyl phosphatidyl ethanol (NAPE) precursor. The first pathway elucidated involves the hydrolysis of NAPE by a membrane associated Ca^{2+}-sensitive NAPE-selective phospholipase D (NAPE-PLD) (Muccioli, 2010). NAPE-PDL-mediated AEA synthesis is considered the main synthetic pathway in the CNS (Leung et al., 2006). Peripheral NAE concentrations in NAPE-PDL knockout mice indicate the presence of additional metabolic pathways (Muccioli, 2010). Studied in immune cells, the next best understood pathway involves cleavage of the NAPE phosphodiester bond by NAPE-selective PLC, followed by dephosphorylation of phosphor-anandamide, yielding anandamide (Lu & Mackie, 2016).

A number of NAE's, including N-oleoylethanolamine, N-palmitoylethanolamide, and AEA are hydrolyzed by the membrane-bound amidase, fatty acid amide hydrolase (FAAH), however, FAAH preferentially hydrolyzes AEA over other NAE's (Muccioli, 2010). FAAH terminates AEA-mediated signalling by converting it to AA and ethanolamine, both of which have no affinity towards CB_1R or CB_2R (Ahn et al., 2009). Inhibition of FAAH increases the levels of various ethanolamine's, many of which, unlike AEA, have actions that are not mediated by cannabinoid receptors (Lu & Mackie, 2016; Luchicchi et al., 2010). Peripheral FAAH inhibition has been shown to produce antinociceptive and analgesic effects, but these actions were lost through CB_1R antagonism (Clapper et al., 2010). AEA can also be oxidized by COX-2 to generate prostamides (prostaglandin ethanolamides) (Woodward et al., 2008), and by other enzymes including FAAH-2 and N-acylethanolamine-hydrolyzing acid amidase (Muccioli, 2010)

1.7 Targeting the ECS for Bladder Pain and Inflammation

The widespread expression of CB_2 receptors on immune cells and in numerous peripheral tissues, and the anti-inflammatory effects associated with its activation, make CB_2 receptors an attractive target for treating inflammatory conditions (Pacher & Mechoulam, 2011; Turcotte et al., 2016). Interest in the function of cannabinoid receptors in the bladder and LUT increased following a study that showed beneficial effect of cannabis on urodynamic symptoms in MS patients (Freeman et al., 2006).

Both CB_1R and CB_2R have been identified in the bladders of various species, including rats, monkeys, and humans (Gratzke et al., 2008). Both receptors have been detected in the urothelium, detrusor, sections of lumbosacral spine that innervate the bladder, and in the dorsal root ganglion (DRG) of L5, L6 and S1 (Merriam et al., 2008). CB_2R expression, however, is significantly higher in the urothelium and suburothelium than in the detrusor (Gratzke et al., 2008), and co-localizes with transient receptor potential vanilloid 1 (TRPV1) and calcitonin gene-related protein (GCRP), which function in the transmission of nociceptive signals (Caterina et al., 1997). CB_2R expression, but not CB_1R, has been shown to be upregulated after acute and chronic cystitis, particularly in the mucosa and detrusor (Merriam et al., 2008).

To date, and to the best of our knowledge, few studies have investigated the effects of intravesical CB_2R-targeted treatments with phyto-derived agonists on bladder inflammation. Moreover, it has been demonstrated that 2-AG is capable of inhibiting neuropathic and inflammatory pain (Josée Guindon et al., 2011), however, little investigation has been done on the use of peripheral MAGL inhibitors to manage visceral pain (Bjorling & Wang, 2018). The upregulation of CB_2 receptors in the bladder, anti-

inflammatory and analgesic effects associated with its activation, and the inflammatory pathology of IC, make the CB_2R a viable target for IC pharmacotherapy.

1.8 Central Hypothesis

My research project investigates the effects of ECS modulation, specifically CB_2R activation, on the immune response in experimental cystitis. The overarching hypothesis for this project is that CB_2R activation, either through direct activation with a novel phytocannabinoid, or by enhancing the tone of local endocannabinoid signalling, will provide beneficial anti-inflammatory and analgesic effects during bladder inflammation.

1.9 Study Objectives

The overarching objective of this study was to examine the therapeutic potential of CB_2R activation in an LPS-induced model of cystitis in mice. Given the pre-clinical success of CB_2 targeted treatments in a range of animal models of pain and inflammation (Donvito et al., 2018a), and the need for safe and effective pain management strategies for cystitis patients, I sought to evaluate the therapeutic potential of CB_2 activation through direct agonist activation, and indirectly, by enhancing the tone of local endocannabinoid signalling in the bladder. My first objective was to examine the impact that genetic CB_2R deletion had on the severity of bladder inflammation. Secondly, I sought to then validate the pharmacological target of BCP by treating $CB_2^{-/-}$ animals with the sesquiterpene, which is a phyto-derived and selective CB_2R agonist. My third objective was to then explore the efficacy of intravesical BCP treatment in reducing bladder inflammation and pain and comparing the effects of BCP treatment to that of the

only FDA/HC approved intravesical treatment, 50% a.q. DMSO (Rimso-50®). Finally, as an alternative approach to direct CB_2R activation, my fourth objective was to explore the anti-inflammatory and analgesic potential of local MAGL inhibition, the enzyme responsible for 2-AG catabolism, and terminating 2-AG signaling.

Chapter 2: Materials and Methods

2.1 Animals

All animal experiments and procedures were approved by the Dalhousie University Committee on Laboratory Animals (protocol #19-024) and complied with the guidelines set by the Canadian Council for Animal Care. Experiments with wild type (WT) animals used healthy female BALB/c mice (8-12 weeks old, 18-24g) purchased from Charles River Laboratories (Wilmington, MS, USA). All mice were housed in the Carleton Animal Care Facility (CACF) in the Sir Charles Tupper Medical Building (Halifax, NS, Canada) in climate controlled, ventilated rack cages maintained at 22°C. Animals had *ad libitum* access to standard rodent chow and water, and were acclimatized for at least 1 week in the CACF prior to experimentation.

The *Cnr2*$^{-}$ mice used in this study (female BALB/c, 8-12 weeks old, 18-24g) were obtained in-house from the lab of Dr. Melanie Kelly (Department of Pharmacology, Dalhousie University). The *Cnr2*$^{-}$ colony was bred and maintained by Janette Nason and housed under the same condition as the wild-type animals in the CACF. Confirmation of genetic knockout of CB_2R was also done by personnel in the Kelly lab. In brief, *Cnr2*$^{-}$ mice were obtained by crossing male C57BL/6J *Cnr2*$^{-}$ mice (strain B6.129P2-Cnr2tm1Dgen/J; Jackson Laboratory, Bar Harbor, ME, USA) with inbred female BALB/c mice (Charles River Laboratories) for 10 generations. Loss of *Cnr2*, the gene encoding the CB_2R protein, was confirmed via polymerase chain reaction (PCR) using DNA from ear punches with an Accustart II Mouse Genotyping Kit (Quanta Biosciences, Gaithersburh, MD, USA) (Thapa et al., 2018).

2.2 Drugs and Reagents

The drugs and reagents used in experiments were all purchased from commercial suppliers. Lipopolysaccharide (LPS), BCP, highly refined olive oil (low acidity), and DMSO were all purchased from Sigma-Aldrich (Oakville, ON, Canada). JZL 184 (IC_{50} = 8nM), the selective MAGL inhibitor used in this study, was purchased from Tocris Bio-Techne (Oakville, ON, Canada), and Cremophor from EMD Millipore Corp (Burldington, MA, USA). Saline (0.9% Sodium Chloride Injection USP, B. Braun Medical Inc., Bethlehem, PA, USA) was purchased from the in-house supplier at the Charles Tupper Building.

2.3 Experimental Groups

In total, 12 experimental groups were established for the experiments. Two intravesical instillations were performed on each animal, the first at T_0 (LPS or saline) and the second at T_1 , 30 minutes later, after draining the bladder of the LPS/saline solution. Descriptions of the interventions are shown in Table 1. Section 2.4 describes the experimental timelines.

Table 1: Description of experimental groups.

Group Name	Description of Intervention
Group 1: CON (control) n= 6	50µl saline at T_0 50µl saline at T_1
Group 2: LPS (untreated) n= 5	50µl LPS at T_0 50µl saline at T_1
Group 3: $CB_2^{-/-}$ CON n= 5	50µl saline at T_0 50µl saline at T_1
Group 4: $CB_2^{-/-}$ LPS n= 5	50µl LPS at T_0 50µl saline T_1
Group 5: $CB_2^{-/-}$ LPS + 100mg/kg BCP n=5	50µl LPS at T_0 50µl BCP (40mg/ml) at T_1
Group 6: LPS + 100mg/kg BCP n= 5	50µl in LPS at T_0 50µl BCP (40mg/ml) at T_1
Group 7: LPS + 300mg/kg BCP n= 5	50µl LPS at T_0 50µl BCP (120mg/ml) at T_1
Group 8: LPS + olive oil (vehicle) n= 5	50µl LPS at T_0 50µl olive oil vehicle at T_1
Group 9: LPS + 50% DMSO aq. n= 5	50µl LPS at T_0 50µl 50% DMSO (aq.) at T_1
Group 10: LPS + 100mg/kg BCP in 50% DMSO aq. n=5	50µl LPS at T_0 50µl BCP (40mg/kg) in 50% DMSO aq.
Group 11: LPS + 1:1:18 (vehicle) n= 5	50µl LPS at T_0 50µl of 1:1:18 (cremophor: DMSO: saline) at T_1
Group 12: LPS + 16mg/kg JZL184 n= 5	50µl LPS at T_0 50µl JZL184 (16mg/kg) at T_1

2.4 Experimental Timelines

2.4.1 Intravital Microscopy

The experimental IVM timeline begins with induction of general anesthesia, and surgical preparation of the animal (T: -0:15). The induction of cystitis with intravesical delivery of LPS was considered T: 0:00min. The LPS solution was removed from the bladder after 30 minutes and was replaced by the treatment instillation (sterile saline for controls, T: 0:30min). 15 minutes prior to the start of IVM, the fluorochromes were administered intravenously (i.v.) into the tail vein (T: 1h:45min). IVM was performed exactly 2 hours following LPS challenge (T: 2h:00). Once all videos were recorded, tissue samples were collected (T: 2h30min) and stored appropriately. A graphic of the full IVM timeline is shown in Figure 1.

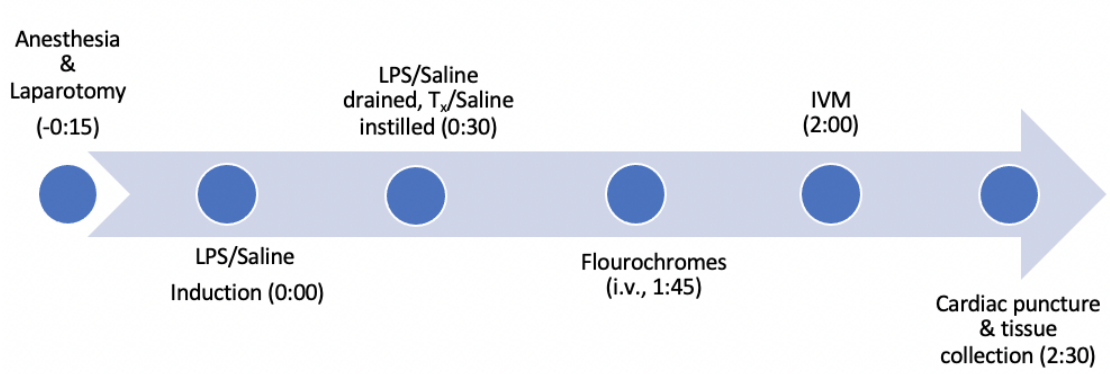


Figure 1: Intravital microscopy experimental timeline.

2.4.2 Von Frey Aesthesiometry and Behavior

The experimental timeline for Von Frey algesiometry and behavioral observation begins with a 15-minute acclimation in the von Frey enclosure (T: -0:30min), after a one-hour acclimation in the quiet procedure room. Baseline measurements were then taken after the 15-minute acclimation in the enclosure (T_0: -0:15min). Five von Frey measurements were recorded for each animal, and a behavioral score assigned. The animals were then anesthetized with isoflurane and LPS/saline instilled into the bladder (T: 0:00min). The LPS solution was manually drained and replaced by the treatment instillation (T: 0:30min). The animals were then kept under anesthesia for an hour and a half to ensure the treatment solution remained in the bladder (T: 2h:00). The animals then recovered for one hour before the second von Frey measurements and behavioral scores were recorded (T_1: 3h:00). A graphic of the full von Frey and behavior timeline is shown in Figure 2.

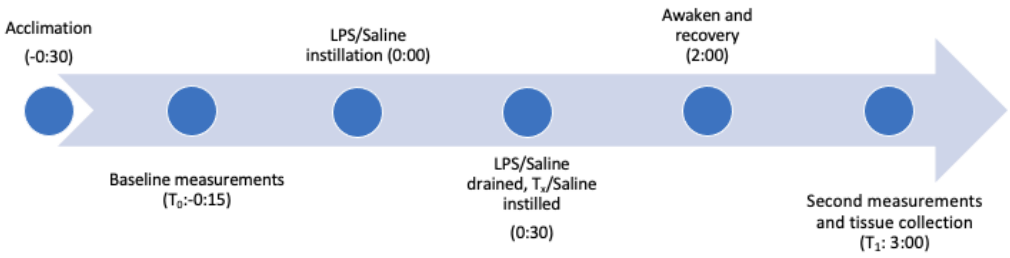


Figure 2: Von Frey aesthesiometry and behavioral assessment timeline.

2.5 Intravital Microscopy

2.5.1 Anesthesia and Surgical Preparation

Every animals' body weight was measured using a portable (PT-600 Sartorius, Göttingen, Germany) laboratory scale. The animal was then anesthetized via intraperitoneal (i.p.) injection of sodium pentobarbital (90 mg/kg, 27 mg/ml, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) and placed on a temperature-controlled heating pad (Coda RightTemp Monitor, Kent Scientific, Torrington, CT, USA). A surgical depth of anesthesia was achieved before any procedure was performed. The depth of anesthesia was monitored by visual assessment of the animals' breathing rate, and the presence/absence of the pedal withdrawal reflex. To maintain anesthetic depth throughout the procedure, sodium pentobarbital (5.4 mg/ml) was administered repeatedly i.p. as needed (0.01-0.06ml). Core temperature was continually monitored using a rectal temperature probe attached to the Coda RightTemp monitor and maintained at 37 ± 1°C. Oxygen was provided to the animals if their breathing became labored or irregular at any point during the procedure.

Once at a surgical depth of anesthesia, the animal was placed in a supine position on the RightTemp heating pad. The lower abdominal and pelvic region were wiped with a 70% isopropyl alcohol swab (HealthCare Plus, Toronto, ON, Canada). The bladder was drained by applying manual pressure to the lower abdomen, similar to Credé's maneuver. A Maylard incision was then made using stainless steel curved surgical scissors (Premium Instruments, Ronkonkoma, NY, USA). The underlying abdominal muscle was gently lifted using dull forceps, and a small incision made along the linea alba. Saline soaked cotton tipped applicators (Puritan Medical Products, Guilford, ME, USA) were

then used to gently exteriorize the bladder. A transurethral catheter, created by inserting the tip of a 30G needle (0.3mm x 13mm, BD PrecisionGlide, Franklin Lakes, NJ, USA) into the lumen of a short segment (4-5cm) of polyethylene tubing (PE10, inner diameter: 0.28mm, BD Intramedic, Sparks, MD, USA) connected to a 1mL glass syringe (Hamilton, Franklin, MD, USA), was then gently inserted into the urethra. Fine tip forceps (Fine Science Tools, North Vancouver, B.C., Canada) were used to very gently hold the urethral meatus, while serrated narrow pattern forceps (Fine Science Tools) were used to hold and insert the catheter into the urethral orifice. The catheter was slowly guided through the urethra, following the natural curvature of the urethra, until approximately 1-1.5cm of the catheter was inserted. If any resistance was encountered, the catheter was slowly withdrawn, and the angle of insertion adjusted accordingly. Once inserted, normal saline or LPS (150µg/ml, 0.375mg/kg) from *Escherichia coli* (Serotype O26:B6, L8274, Sigma-Aldrich, Ontario, Canada) dissolved in normal saline was slowly instilled into the bladder. Once the full volume was instilled, the catheter was gently retracted from the urethra. The dull forceps were then used to apply a small amount of pressure to the external urethral meatus to prevent initial backflow of the inoculum. The catheterization procedure described above was used for all solutions delivered via intravesical instillation. Depending on the treatment group, the LPS solution was replaced by the appropriate treatment 30 minutes later, using Credé's maneuver to empty the bladder. A warm saline soaked 2x2 non-woven sponge (AMD Medicom Inc ®, Montreal, QC, Canada) was constantly kept over the incision until the bladder was exposed for imaging. A small vessel clamp (Fine Science Tools) was applied to the external urethral meatus to avoid leakage of the bladder contents through the urethra during IVM.

2.5.2 Microscopy

Fifteen minutes prior to the start of IVM, a bolus injection of 0.05% Rhodamine-6G (1.5 ml/kg, excitation: 515-560 nm, Sigma-Aldrich) and 5% fluorescein isothiocyanate-bovine serum albumin (FITC-BSA, 1ml/kg, excitation: 450-490 nm, Sigma-Aldrich) fluorochromes was administered via the tail vein (30G X ½ inch, 1ml BD tuberculin syringe). An epifluorescent microscope (Leica, model MST49, Wetzlar, Germany) was used to visualize leukocyte adhesion and capillary perfusion in the bladder microcirculation. With the animal in the supine position, the bladder was gently exteriorized and a circular glass coverslip (18 mm diameter) placed on top of the inflated bladder. The exteriorized bladder was then positioned under the 20X objective (Leica, Germany), and visualized through a 10X eyepiece (HC Plan, Leica, Germany). A mercury-arc light source (LEJ EBQ 100W, Leistungselektronic JENA GmbH, Jena Germany) attached to the microscope was used to illuminate the field of view. The IVM set up is shown in figure 3. Different excitation filters were used to excite the fluorochromes at their respective wavelengths. To visualize leukocyte adhesion, a 530-550nm bandpass excitation filter was applied (which allows only green light to pass through) to excite the Rhodamine-6G (emission 515nm). Six visual fields containing non-branching submucosal venules were selected and put into focus and a 30 second video was recorded for each visual field. For visualization of capillary perfusion, a 460-490nm bandpass filter was applied (which allows only blue light to pass) to excite FITC (emission 520 nm). Six randomly selected visual fields with capillaries in focus were selected and a 30 second video recorded for each visual field

2.5.3 Tissue Collection

After all videos were recorded, the animal was sacrificed via cardiac puncture. Blood was drawn into a 1 ml syringe containing 5-10 µl of heparin. Approximately 0.5 ml of blood was collected from each animal. The blood was transferred to a 1mL Eppendorf tube (Axygen MaxyClear Microtube, Axygen Inc, NY, USA) and immediately centrifuged for 10 minutes at 2500 rpm. The plasma was then removed, placed in a 0.5 mL microtube, snap frozen in liquid nitrogen, and stored at -80°C. The bladder was excised from the animals using fine tip scissors, placed in normal saline on a microscope slide, and cut on a transverse plane to separate the top and bottom sections of the bladder. The top section of the bladder was snap frozen in liquid nitrogen and placed in the -80°C freezer, while the bottom half was placed in a labelled histology cassette and submerged in 10% neutral buffered formalin (NBF).

Figure 3: Intravital microscopy set up.

1) CODA RightTemp® Monitor with attached rectal temperature probe, 2) CODA RightTemp® heating pad with animal placed in supine position, 3) Saline and other reagents on heating pad, 4) Surgical tools (scissors, forceps, tweezers), 5) Gain and brightness control, 6) Mercury-arc light source housing, 7) Epifluorescent Leica Microscope MTS49, 8) C-mounted Horn Imaging video camera, 9) Acer computer monitor, 10) LED light source.

2.5.4 Video Analysis

Videos were recorded using a black and white video camera (30 FPS, Horn Imaging BC-71, Aalen, Germany) C-mounted to the top of the microscope. A bi-directional media converter (Canopus ADVC110, Grass Valley, Montreal, QC, Canada) and image capturing software (WinDV version 1.2.3, Petr Mourek, Czech Republic) were used to capture the videos projected on a computer monitor (Acer LCD AL1716 monitor, Acer, Xizhi, Taiwan). The gain and brightness were adjusted accordingly to optimize image clarity. Adapter (Version 2.1.6, Macroplant LLC, Texas, USA) was used to decompress the AVI video files for offline analysis.

All IVM video recordings were analyzed offline in a blinded fashion using ImageJ software (NIH, USA). Leukocytes that were firmly adherent to the same position of the vessel endothelium for 30 seconds were defined as adherent leukocytes. To quantify leukocyte adhesion, a straight section of the vessel was outlined and measured. The number of adherent leukocytes in within the outlined area were then counted. The area and number of leukocytes were then used to calculate the number of adherent leukocytes on the cylindrical surface of the vessel (#cells/mm^2). Still-frame images from leukocyte trafficking videos obtained from IVM are shown in figure 4.

Capillary perfusion was assessed by quantifying the functional capillary density (FCD) of the bladder microvasculature. FCD was defined as the total length of capillaries with observable erythrocyte movement in relation to a predefined area of the video recording. FCD was quantified by summing the total length of capillaries containing observable erythrocyte movement and dividing by the selected area (cm/cm^2). Images showing capillary perfusion in the bladder microcirculation are shown in figure 5.

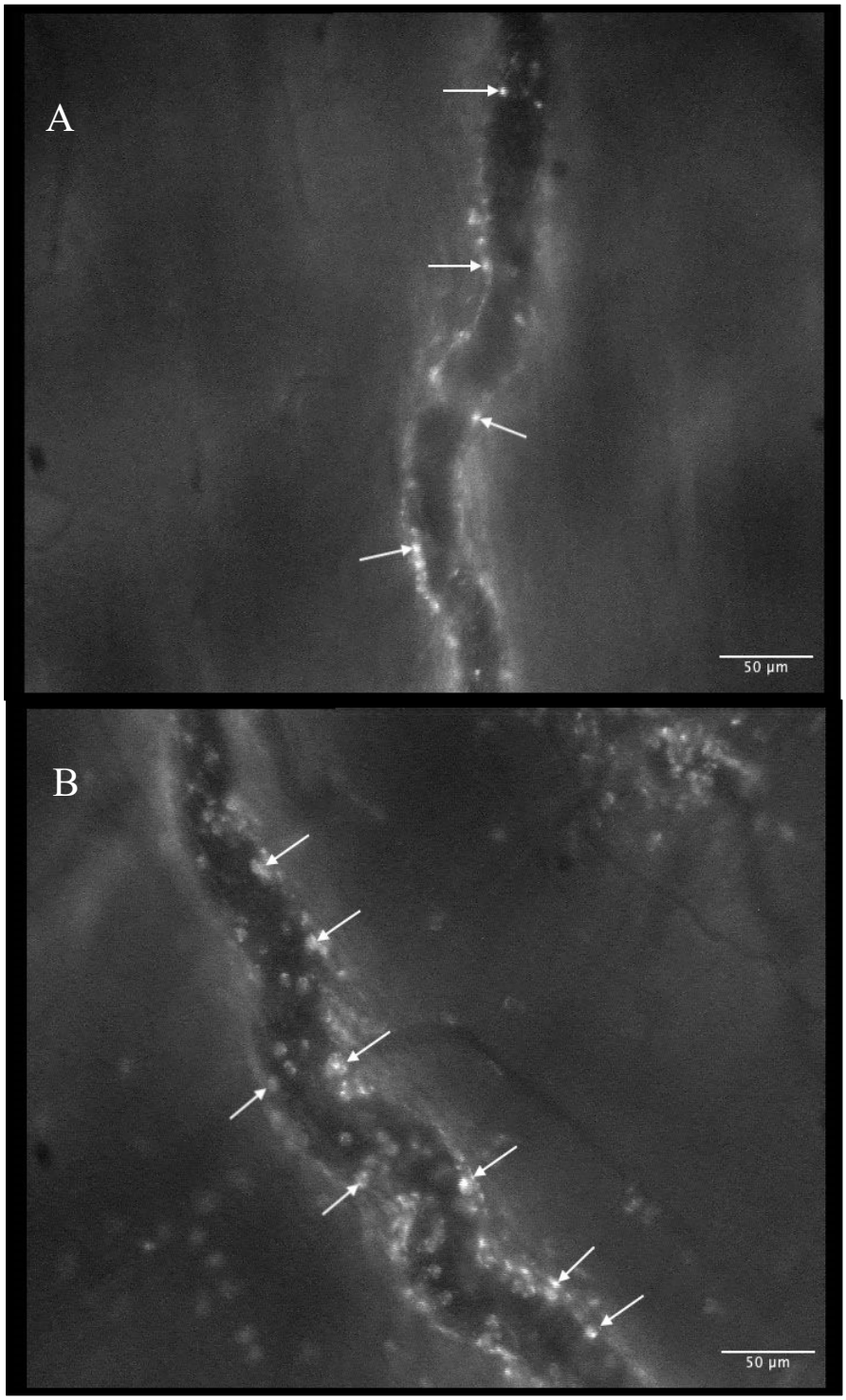


Figure 4: Still-frame images of leukocyte adhesion within the bladder microcirculation of female BALB/c mice. Images obtained by intravital microscopy (magnification = 200x). Images from control (A) and LPS (B) animals are shown. White arrows indicate adherent leukocytes

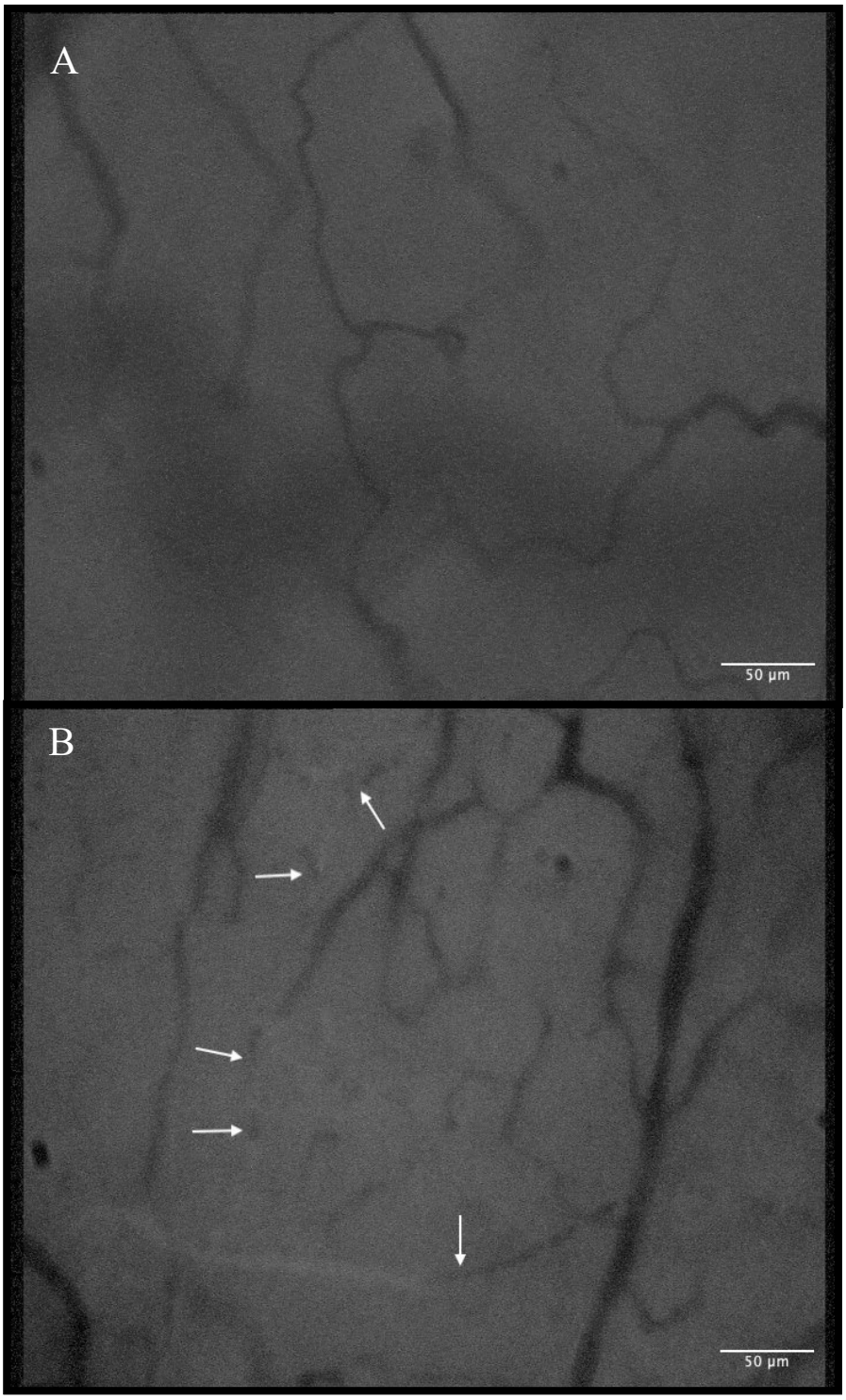


Figure 5: Still-frame images of capillary perfusion within the bladder microcirculation of female BALB/c mice. Images obtained by intravital microscopy (magnification = 200x). Images from control (A) and LPS (B) animals are shown. White arrows indicate areas of irregular capillary perfusion.

2.6 Von Frey Aestheiometry

2.6.1 Aesthesiometry

Electric von Frey aesthesiometry (IITC Life Sciences Inc. 2390 Series, Woodland Hills, California, USA) was performed to assess mechanical allodynia in experimental animals. The animals were allowed to acclimate in a quiet procedure room for one hour, with 15 minutes in a plexiglass enclosure with a mesh floor (IITC Inc. Life Sciences) before baseline measurements were recorded. Von Frey force measurements were taken following behavioral assessment. The von Frey device was calibrated with a 5g calibration weight before animal measurements were taken. Similar to behavioral assessment, healthy animals were scored prior to IC induction to set a baseline for each individual animal. A rigid tip probe attached to the aesthesiometer was directed at the suprapubic region of the lower abdomen through the mesh floor, until a withdrawal response was observed. The highest exerted force applied before withdrawal was recorded (in grams). Five measurements were recorded for each animal. The same procedure was then performed following LPS and treatment instillations. The von Frey set-up is shown in figure 6

2.6.2 Anesthesia

Following baseline measurements, animals were anesthetized with isoflurane vapor (2-5%, Isoflurane USP 99.9%, Fresenius Kabi, Toronto, ON, Canada). LPS (or saline) was then instilled into the bladder (see section 2.6.1 for catheterization procedure). Animals were kept on a heating pad (Physitemp TACT-2LV with RET-3 rectal probe, Clifton, NJ, USA) for the duration of the procedure. After 30 minutes, the LPS solution

was manually drained, and the treatment solution instilled. The treatment remained in the bladder for 1.5 hours, similar to the IVM timeline. The animal was then awoken from anesthesia and allowed to recover for one hour in the quiet procedure room with half of the cage on a water heating pad (HTP-1500, Adriot Medical Systems, Loudon, TN, USA). The second set of measurements were then recorded following the procedure outlined in section 2.7.1. After the second measurements were taken, the animal was then sacrificed, and the tissue collected as outlined in section 2.5.3.

2.7 Behavioral Assessment and Scoring

As done for the von Frey aesthesiometry, all mice were scored before any intravesical instillations to set an individual baseline score for each animal, and again following saline/LPS and subsequent treatment. Three behavioral parameters were evaluated: posture, motor activity, and position of the eyelid. The scoring system used in this study was adapted from Boucher et al., (2000). The maximum score for each behavioral category was 10. The three parameters were also summed to provide a cumulative behavioral score, with the maximum cumulative score being 30. Scoring details are outlined in table 2.

Table 2: Description of parameters used in behavioral scoring system.

Behavioral Parameter	**Description**
Eye opening/closing	Completely opened eye lids receive a score of 0, half closed lids received a score of 5, and fully closed lids received a score of 10. Scores of 2 and 7 were assigned based on intermediate positions of the eyelids and observer discretion
Posture	Normal posture (i.e. no back curvature, full extension of the body) received a score of 0, 5 for moderately hunched (some attempt at extension, and 10 for fully rounded (no attempts at extending body). Scores of 2 and 7 were assigned based on intermediate postural position and observer discretion
Motor Activity	Activity (e.g. exploring, grooming) within a 20 second time window (e.g. No movement for 10 seconds (=50%) = score of 5

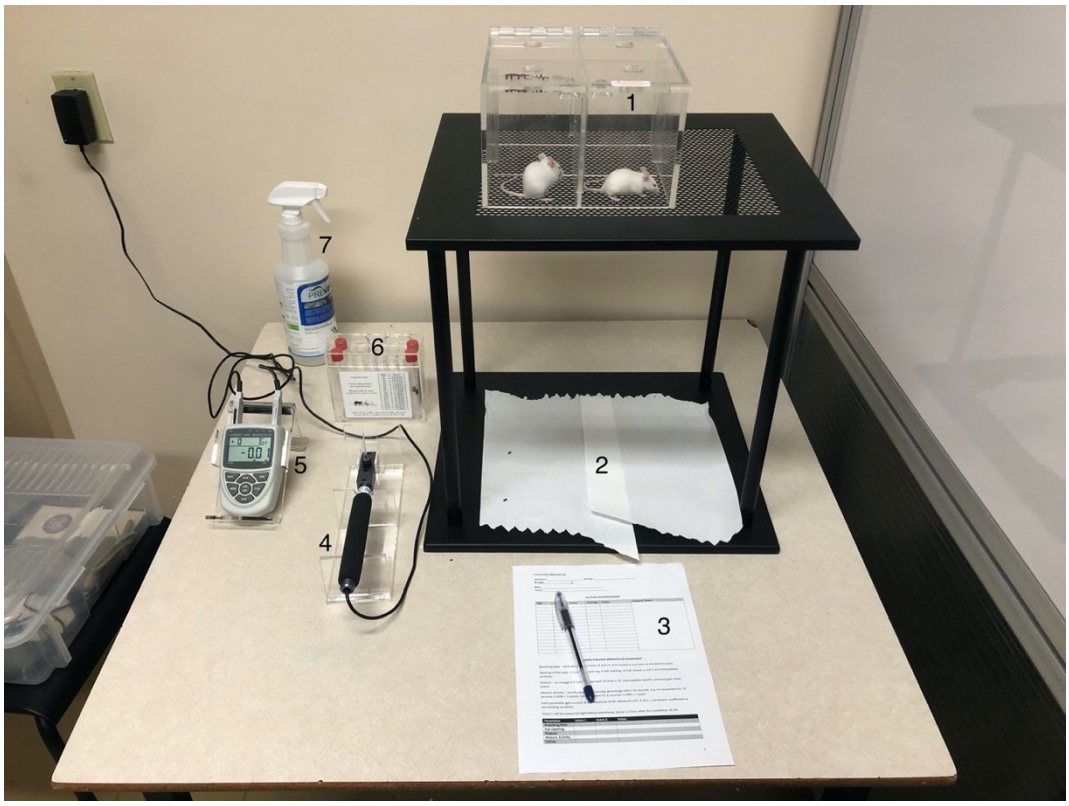


Figure 6: von Frey Aesthesiometry set up. 1) Plexiglass animal enclosure housing experimental animals 2) Mesh stand with paper towel 3) von Frey and behavior scoring sheet 4) Electronic von Frey probe 5) Force readout device 6) von Frey tips 7) Plexiglass and mesh cleaner. All von Frey equipment is from IITC Life Science Inc.

2.8 Bladder Histopathology

2.8.1 Tissue Processing and Staining

Following sacrifice, the bladder was excised and fixed in 10% neutral buffered formalin for 48 hours in a labelled histology cassette. After 48 hours, the cassettes were transferred and stored in 70% ethanol. The tissue samples were then processed using an ASP6025 Automated Vacuum Tissue Processor (Leica Biosystems, Richmond Hill, Canada) and embedded in paraffin. The paraffin-embedded tissue blocks were then cut in 5µm sections using a HistoCore Multicut Semi-automated Rotary Microtome (Leica Biosystems). Three sections were taken from each tissue block. The sections were then transferred onto a float water bath (Lipshaw MFG Co. Detroit, MI, USA) set at 42℃, and carefully transferred onto the glass slide (Fisherbrand Microscope Slides, Thermo Fisher Scientific, Ottawa, Ontario, Canada). The slides were then placed in an oven set at 60℃ overnight to melt excess paraffin.

Tissues were then stained using the hematoxylin and eosin (H&E) staining protocol shown in table 3. For steps requiring multiple immersions or dips (ex. Xylene, 5mins immersion x3), each subsequent immersion or dip was done using clean reagents. Briefly, the slides were deparaffinized using a series of washes in Xylene and varying concentrations of ethanol, then placed in tap water. The slides were then stained with H&E and rinsed under running tap water. The quality of the staining was assessed on a light microscope before proceeding to dehydration steps. Slides that were well stained were then washed in 70%, 95%, and 100% ethanol, and xylene to dehydrate the tissue. Cover slips were then placed on the slides using Cytoseal-60 mounting media (Electron Microscopy Sciences, Fort Washington, PA, USA) and allowed to dry in a fume hood.

Once dry, the slides were examined and scored using an adapted score from Hopkins et al., (1998)

Table 3: Detailed hematoxylin and eosin staining procedure used to stain bladder tissue sections. Procedures requiring multiple immersions or dips (ex. 5min Xylene immersion, x3) used new reagent for each subsequent immersion or dip.

Reagent	Procedure
Xylene	5min immersion (x3)
100% Ethanol	2min immersion (x3)
95% Ethanol	2min immersion (x2)
70% Ethanol	2min immersion (x2)
Running Tap Water	5min immersion
Harris Hematoxylin	3min immersion
Running Tap Water	Until solution clear
1% Acid (HCl) Alcohol	3 dips
Running Tap Water	Until solution clear
Scott's Tap Water	1min immersion
Running Tap Water	Until solution clear
Eosin	10 Dips
70% Ethanol	10 Dips (x3)
95% Ethanol	10 Dips (x2)
100% Ethanol	10 Dips (x3)
Xylene	5min immersion (x3)

2.8.2 Bladder Histopathology Scoring

Following staining, bladder sections were examined and scored according to a bladder inflammation grading scale established by (Hopkins et al., 1998). The score was adapted slightly due to the absence of epithelial denudation and necrosis in the cystitis model used in this project. For each animal, one bladder section was scored. The bladder section was divided into four quadrants, with each quadrant receiving an individual score. Sections were examined and scored using an Optika B-290TB brightfield microscope (Optika Microscopes, Ponterica, Italy) with N-PLAN 40x objective lens, connected to a Windows 10 tablet (Microsoft, Redmond, WA, USA). The average of the four quadrant scores was then taken. A description of the scoring system used is provided in table 4

Table 4: Histopathological grading scale used to quantify bladder inflammation. Adapted from Hopkins et al. (1998)

Grade	Histopathological description
0	No evidence of inflammatory cell infiltration or edema
1	Submucosal inflammatory cell infiltration (focal)
2	Submucosal edema and inflammatory cell infiltration (multifocal or diffuse)
3	Submucosal edema and inflammatory cell infiltration that extends into the mucosal epithelium
4	Inflammatory cell infiltrate extends into detrusor muscle, in addition to criteria for grade 3

2.9 Tissue Cytokine and Adhesion Molecule Analysis

2.9.1 Tissue Preparation and Bicinchonic acid (BCA) Protein Assay

A BCA protein assay was performed to determine the total protein concentration in the bladder samples prior to the Luminex assay. Bladder tissue samples were removed from the -80ºC freezer and thawed on ice the day of the protein assay in 400µl of Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific) containing protease inhibitor (PI, 10ml T-PER:1 PI tablet, Cat. #A32953, Thermo Fisher Scientific) Bladder samples were homogenized on ice in the T-PER buffer using a TissueRuptor (Qiagen, Velno, Netherlands) for 30 seconds. The samples were then spun in a refrigerated centrifuge (4ºC) at 10,000g for 10 minutes. The supernatant was isolated, transferred to a clean 1.5ml Eppendorf tube, and diluted 10-fold in T-PER (+PI) buffer (10µl sample, 90µl buffer). The BCA assay was then performed according to the manufacturer's protocol for a 96 well plate (Rapid Gold BCA Protein Assay, Thermo Fisher Scientific). The protein concentration in the samples was calculated using the equation provided from the standard curve on Microsoft Excel, accounting for the 1:10 dilution. All samples were aliquoted and frozen in a -80ºC freezer.

2.9.2 Mouse Magnetic Luminex Assay

A custom designed mouse cytokine 10-plex kit (Kit Lot # L131954, R&D Systems, Minneapolis, MN, USA) was used to determine the concentration of the following cytokines, chemokines, and adhesion molecules in bladder tissue homogenates: keratinocyte-derived chemokine (KC, mouse homolog of human interleukin 8), macrophage inflammatory protein-2 (MIP-2), interferon gamma (IFN-ɣ), lipopolysaccharide-induced CXC chemokine (LIX), tumor necrosis factor alpha (TNF-α),

interleukin 6 (IL-6), intercellular adhesion molecule 1 (ICAM-1), interleukin 1β (IL-1β), interleukin 10 (IL-10), and P-selectin (CD26P). Bladder tissue homogenates were diluted using T-PER (+PI) reagent to a final protein concentration of 500µg/ml. All samples were run in technical duplicates. A 7-point standard curve was generated for each analyte, and the assay performed according to manufacturer's protocol.

In brief, 50µl of standard or sample was added to each well of a flat-bottom 96-well plate. 50µl of the microparticle cocktail containing the magnetic detection antibody beads for each analyte was added to each well and incubated at room temperature in the dark for two hours on a horizontal orbital microplate shaker (0.12" orbit, 800 rpm). The plate was then placed in a Bio-Plex Pro magnetic wash station (Bio-Rad) and washed 3 times to remove unbound beads. Next, 50µl of diluted Biotin detection antibody cocktail was added to each well and incubated in the dark for 1 hour on the same microplate shaker settings. Unbound beads were again washed off using the magnetic wash station for 3 cycles. Finally, 50µl Streptavidin-PE was added to each well and incubated in the dark for 30 minutes on the microplate shaker. Another cycle of 3 washes was performed. The beads were then resuspended by adding 100µl of supplied R&D wash buffer to each well. The plate was then incubated for 2 minutes at room temperature on the microplate shaker, and then read using a Bio-Plex 200 Analyzer and Bio-Plex Manager software (Bio-Rad, Mississauga, ON, Canada), which was calibrated prior to use.

2.10 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8.6 (GraphPad Software Inc, La Jolla, CA, USA). All data is presented as the mean ± standard deviation

(SD). Homogeneity of variance and normal distribution of the residuals were confirmed using Bartlett's test and Kolmogorov-Smirnov test, respectively. Differences between groups was analyzed using a one-way analysis of variance (ANOVA) for the IVM, cytokine, and histology data. Von Frey aesthesiometry and behavior data was analyzed using a two-way repeated measures ANOVA. Tukey's HSD post-hoc was performed to examine group wise comparisons. Analysis involving comparison of two-groups was done using a two-tailed t-test. Statistical significance was considered at $p < 0.05$. All data in the results are expressed as mean ± standard deviation (SD), with the individual data points also being shown for von Frey and behavioral data.

Chapter 3: Results

3.1 Examining the Impact of Genetic *Cnr2* Deletion on the Response to LPS-induced Bladder Inflammation

3.1.1 Intravital Microscopy

Leukocyte adherence to the endothelial wall of submucosal venules in the bladder microcirculation was examined by IVM two hours following intravesical instillation of sterile saline or 150µg/ml LPS solution in wildtype (WT) and cannabinoid 2 receptor knockout ($CB_2^{-/-}$) mice. The number of adherent leukocytes was minimal in wildtype control animals. LPS instillation in WT mice significantly ($p < 0.05$) increased the number of adherent leukocytes in submucosal venules compared to the control group. Leukocyte adherence was increased approximately 3-fold following LPS instillation (Figure 7). The same effect was observed in $CB_2^{-/-}$ control and LPS animals, whereby leukocyte adhesion was significantly ($p < 0.05$) increased in the $CB_2^{-/-}$ LPS group relative to the $CB_2^{-/-}$ controls. $CB_2^{-/-}$ animals in the control and LPS groups showed a significantly higher number of adherent leukocytes compared to WT animals that received the same intravesical solution ($p < 0.05$, Figure 7).

Intravesical administration of LPS in WT animals did not significantly affect the functional capillary density in the bladder microcirculation compared to WT controls ($p > 0.05$). There was also no significant effect ($p > 0.05$) of LPS administration on FCD in $CB_2^{-/-}$ animals, however, FCD was found to be significantly higher in the $CB_2^{-/-}$ LPS group compared to the WT counterparts ($p < 0.05$, Figure 8).

3.1.2 Luminex Assay

The levels of KC, MIP-2, ICAM, IFN-ɣ, IL-1β, IL-6, IL-10, LIX, P-Selectin, and TNF-α were measured in bladder tissue samples using a custom designed Luminex assay. Incomplete data was obtained for IFN-ɣ, IL-10, LIX and TNF- α. Measurements were found to be below the range of their respective standard curves and were therefore not included in analysis. Intravesical LPS instillation significantly elevated the bladder tissue concentrations of KC, IL-1β, IL-6, P-Selectin, and MIP-2 in both WT and $CB_2^{-/-}$ mice, compared to the respective control groups ($p < 0.05$, Figure 9).

KC concentrations in both WT and $CB_2^{-/-}$ LPS mice were both elevated approximately 3-fold, with no significant difference observed between genotypes ($p < 0.05$, Figure 9A). IL-1β concentrations were significantly elevated after LPS instillation in both WT and $CB_2^{-/-}$ animals ($p <0.05$). The concentrations of IL-1β in $CB_2^{-/-}$ LPS treated mice were elevated 1.8-fold and were significantly higher than the concentrations observed in the WT LPS group ($p < 0.05$, Figure 9B). The concentrations of IL-6 increased in WT and $CB_2^{-/-}$ LPS treated animals by approximately 3-fold ($p < 0.05$, Figure 9C), with no difference being detected between genotypes of the same treatment group. P-selectin was also significantly increased by 1.5-fold in both groups ($p <0.05$, Figure 9D), with no difference being detected between the genotypes of the respective treatment groups. Finally, MIP-2 concentrations were increased by approximately 3-fold in WT mice, and 8-fold in $CB_2^{-/-}$ mice following LPS instillation ($p < 0.05$, Figure 9E). MIP-2 concentrations were also found to be significantly higher in $CB_2^{-/-}$ LPS mice compared to the LPS treated WT mice.

While trends also show an increase in ICAM following LPS instillation in both genotypes, this effect was not statistically significant ($p > 0.05$, Figure 9F).

3.1.3 Bladder Histopathology

Bladder cross-sections were stained with H&E and scored using a histopathology score that was adapted from Hopkins et al. (1998). Intravesical LPS administration caused a significant increase in the bladder inflammation scale in both WT and $CB_2^{-/-}$ animals, characterized by multifocal and/or diffuse inflammatory cell infiltration, and mild subepithelial edema ($p < 0.05$). No significant difference was observed between the two genotypes of the control or LPS groups ($p > 0.05$, Figure 10).

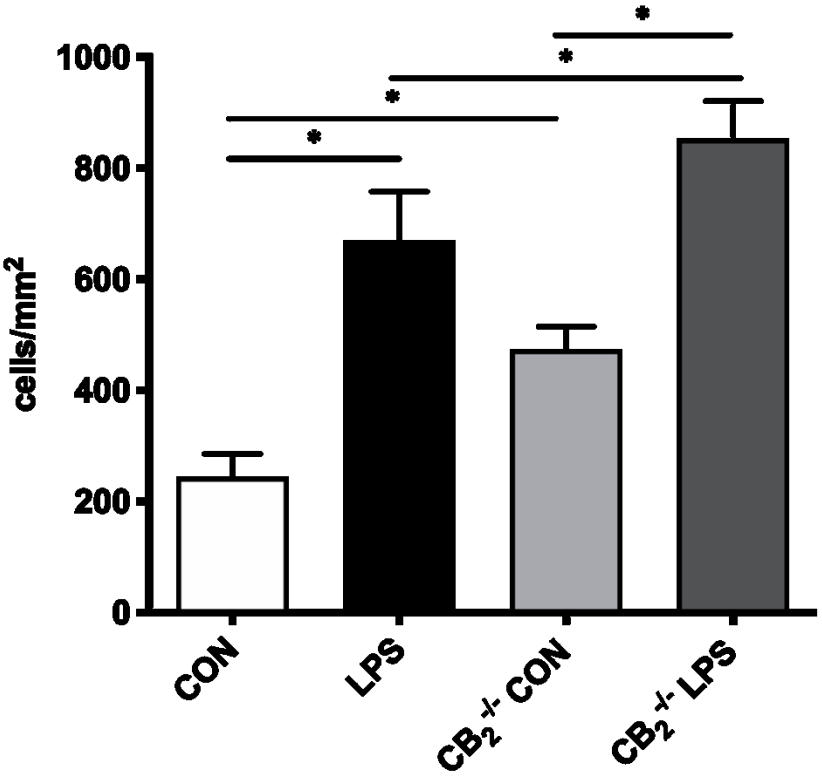


Figure 7: Leukocyte adhesion in the submucosal bladder venules of WT and $CB_2^{-/-}$ female BALB/c mice belonging to the following experimental groups: control (CON, n=6), LPS-induced IC (LPS, 0.375mg/kg, n=5), $CB_2^{-/-}$ control ($CB_2^{-/-}$ CON, n=5), and LPS-induced IC in $CB_2^{-/-}$ mice ($CB_2^{-/-}$ LPS, n=5). Leukocyte adhesion was assessed via intravital microscopy 2 hours following intravesical administration of LPS or saline (control). Data presented as mean ± SD. * $p < 0.05$

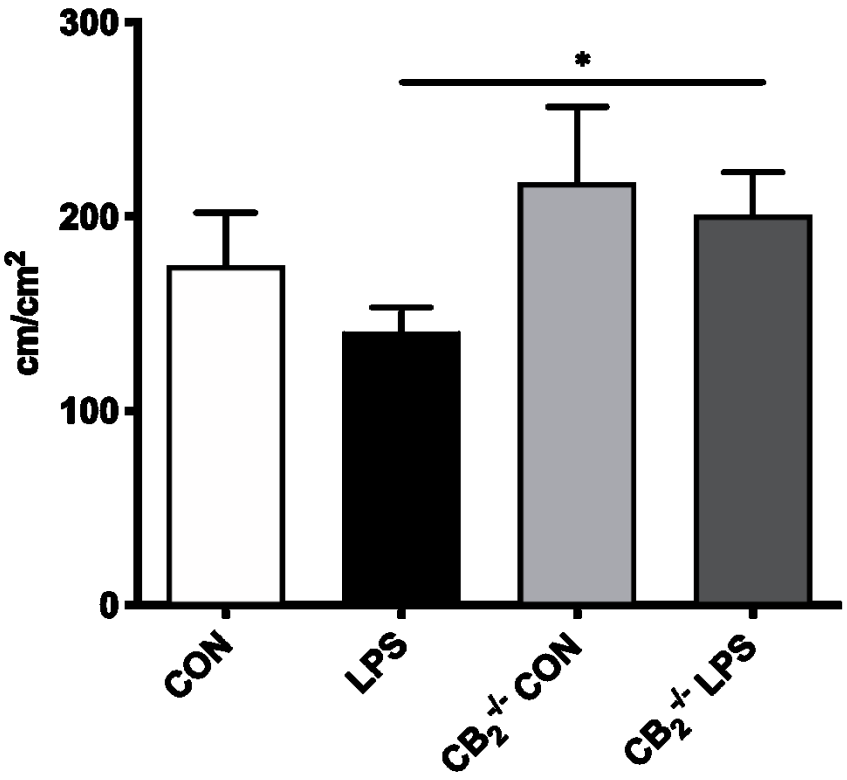


Figure 8: Capillary perfusion quantified through the functional capillary density (FCD) of the bladder microcirculation of WT and $CB_2^{-/-}$ female BALB/c mice belonging to the following experimental groups: control (CON, n=6), LPS-induced IC (LPS, 0.375mg/kg, n=5), $CB_2^{-/-}$ control ($CB_2^{-/-}$ CON, n=5), and LPS-induced IC in $CB_2^{-/-}$ mice ($CB_2^{-/-}$ LPS, n=5). Capillary perfusion was assessed via intravital microscopy 2 hours following intravesical administration of LPS or saline (control). Data presented as mean ± SD. * $p < 0.05$.

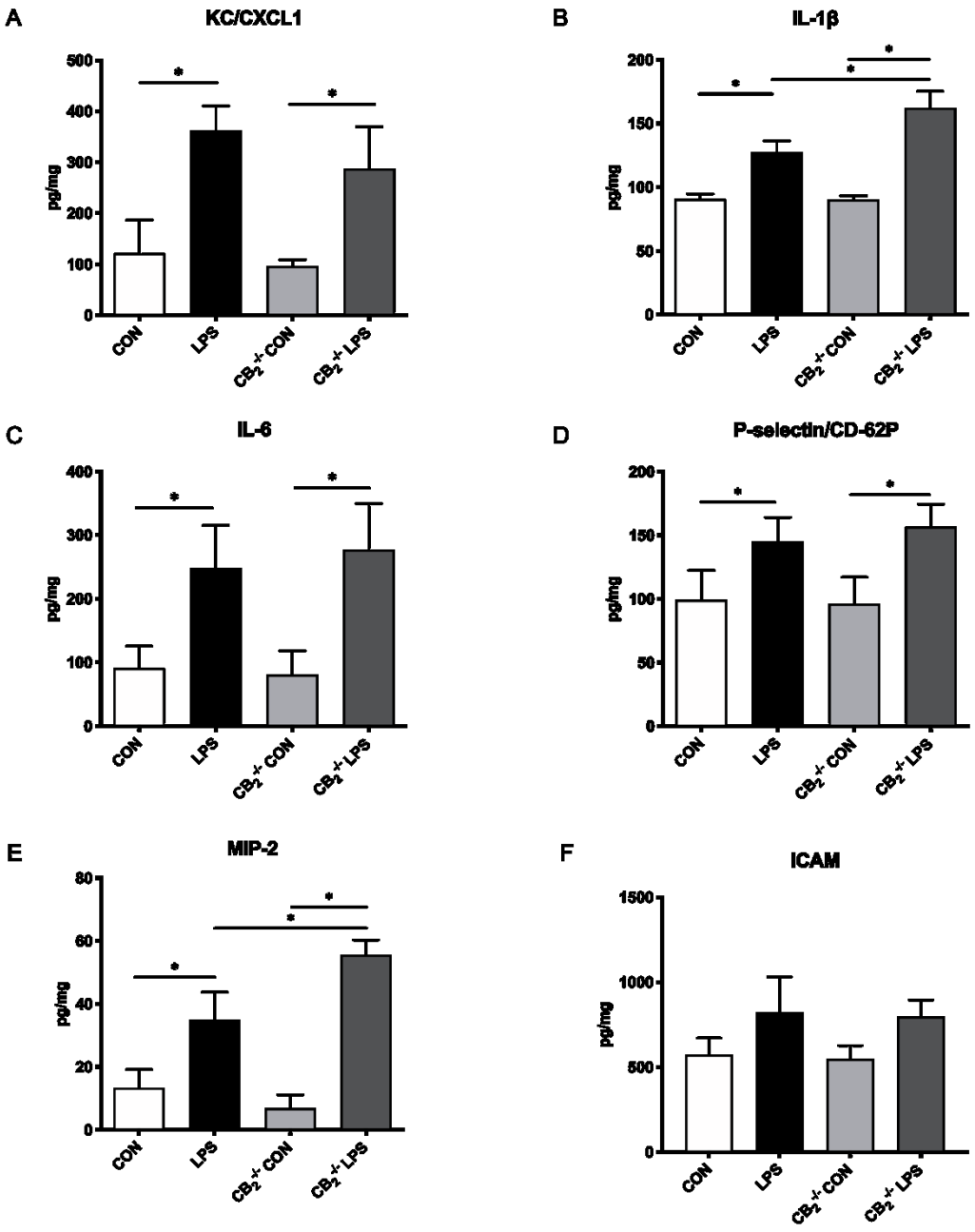
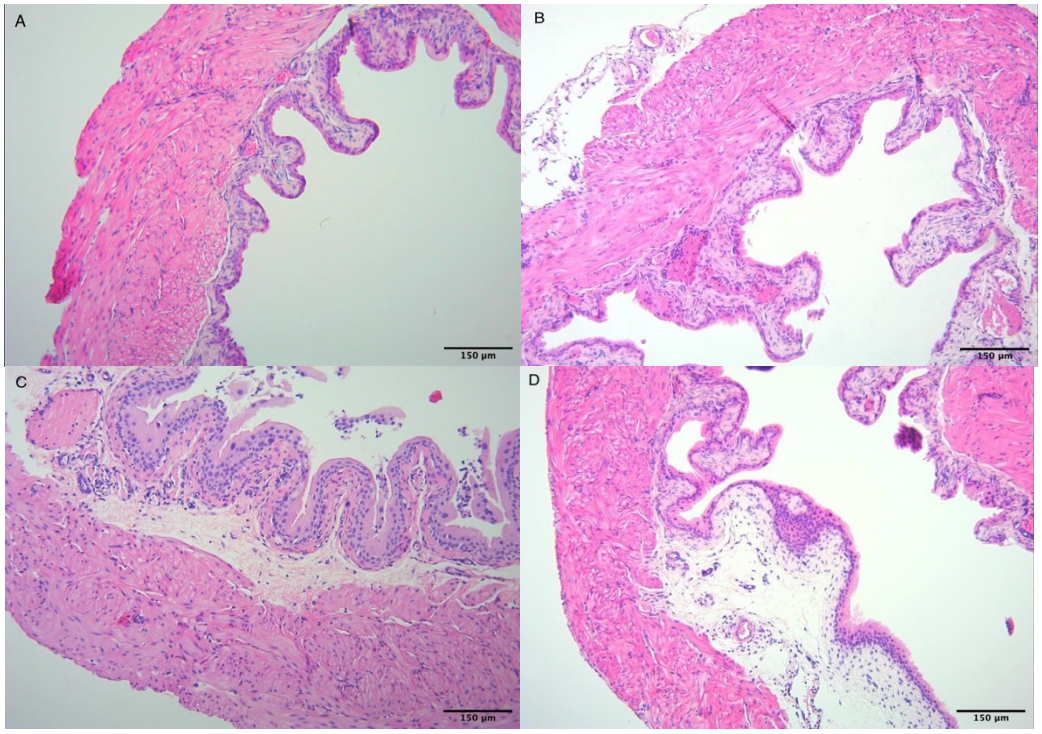


Figure 9 (A-F): Bladder tissue concentrations of KC/CXCL1 (A), IL-1β (B), IL-6 (C), P-selectin/CD-62P (D), MIP-2 (E), and ICAM (F) in WT and $CB_2^{-/-}$ female BALB/c mice belonging to the following experimental groups: control (CON, n=5), LPS-induced IC (LPS, 0.375mg/kg, n=5), $CB_2^{-/-}$ control ($CB_2^{-/-}$ CON, n=5), and LPS-induced IC in $CB_2^{-/-}$ mice ($CB_2^{-/-}$ LPS, n=5). Sterile saline (control) and LPS were administered via intravesical instillation. Two technical replicates were performed for each sample. Data presented as mean ± SD. * $p < 0.05$.



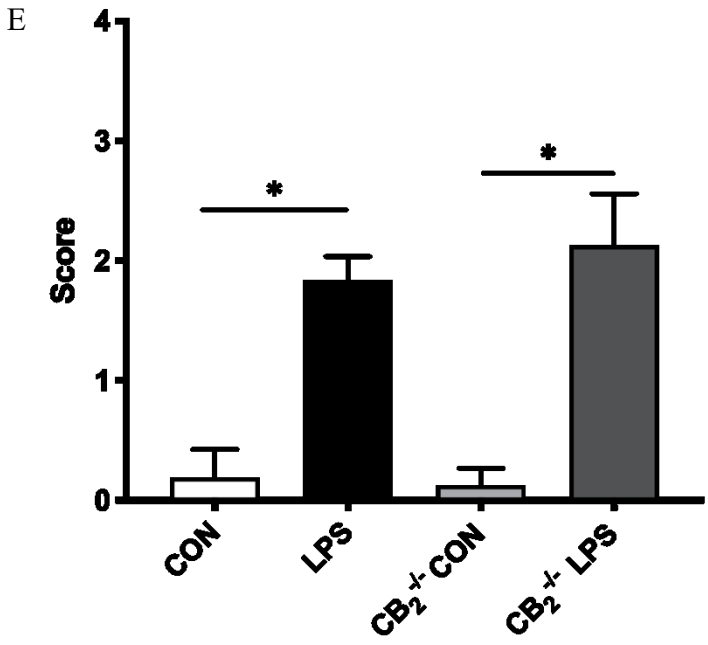


Figure 10 (A-E): Representative histology images and bladder histopathology scores. Slides were stained with H&E and scored based on an adapted scoring system from Hopkins et al. 1998. Panel A shows a representative score of 0, B shows a score of 1, C shows a score of 2, and D shows a score of 3. Scores from the following experimental groups are shown: control (CON, n=5), LPS-induced IC (LPS, 0.375mg/kg, n=5), $CB_2^{-/-}$ control ($CB_2^{-/-}$ CON, n=5), and LPS-induced IC in $CB_2^{-/-}$ mice ($CB_2^{-/-}$ LPS, n=5). Data presented as mean ± standard deviation. * $p < 0.05$.

3.2 Validating the Pharmacological Target of β-Caryophyllene in Cannabinoid 2 Receptor Knockout Mice.

3.2.1 Intravital Microscopy

$CB_2^{-/-}$ mice were treated with intravesical BCP following experimental LPS-induced cystitis to assess the pharmacological target of BCP, which has been reported to be a selective agonist for the CB_2R (Gertsch et al., 2008). Intravesical administration of BCP (100mg/kg, 40mg/ml) in $CB_2^{-/-}$ mice, following LPS-induced bladder inflammation, did not significantly reduce the number of adherent leukocytes in the submucosal venules of the bladder microcirculation ($p > 0.05$, Figure 11).

Functional capillary density, a measurement of microvascular perfusion, can be altered during inflammatory conditions. Local administration of LPS into the bladder of $CB_2^{-/-}$ mice did not significantly alter the FCD (Figure 8). Subsequent intravesical treatment with 100mg/kg BCP in $CB_2^{-/-}$ mice did not significantly alter the FCD ($p > 0.05$, Figure 12).

3.2.2 Luminex Assay

LPS instillation into the bladders of $CB_2^{-/-}$ mice caused a significant increase in the bladder tissue concentrations of KC, IL-1β, IL-6, P-Selectin and MIP-2 (Figure 9). Subsequent intravesical BCP treatment (100mg/kg, 40mg/ml) in $CB_2^{-/-}$ mice was able to significantly reduce ($p < 0.05$) the elevated levels of KC by over 2-fold (Figure 13A), P-Selectin by roughly 1.7x (Figure 13D) and MIP-2 by 2-fold (Figure 13E). IL-1β, IL-6 and ICAM concentrations were unaffected by 100mg/kg BCP instillation

3.2.3 Bladder Histopathology

Intravesical LPS administration into the urinary bladder caused a significant increase in inflammatory cell infiltration and subepithelial edema in both WT and $CB_2^{-/-}$ mice (Figure 10). Subsequent intravesical treatment of $CB_2^{-/-}$ mice with 100mg/kg (40mg/ml) BCP had no effect on the bladder histopathology score ($p > 0.05$, Figure 14).

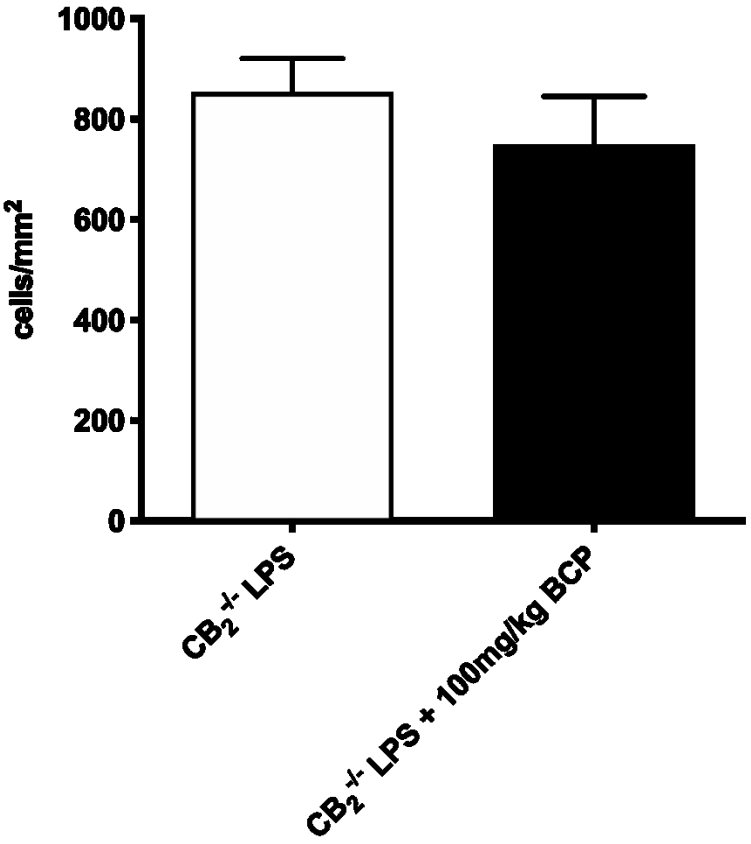


Figure 11: Leukocyte adhesion in the submucosal bladder venules of $CB_2^{-/-}$ female BALB/c mice belonging to the following experimental groups: LPS-induced IC ($CB_2^{-/-}$ LPS, 0.375mg/kg, n=5), and LPS-induced IC treated with BCP ($CB_2^{-/-}$ LPS + 100mg/kg BCP, n =5). Leukocyte adhesion was assessed via intravital microscopy 2 hours following intravesical administration of LPS. Data presented as mean ± SD. A two-tailed t-test was performed to compare group means.

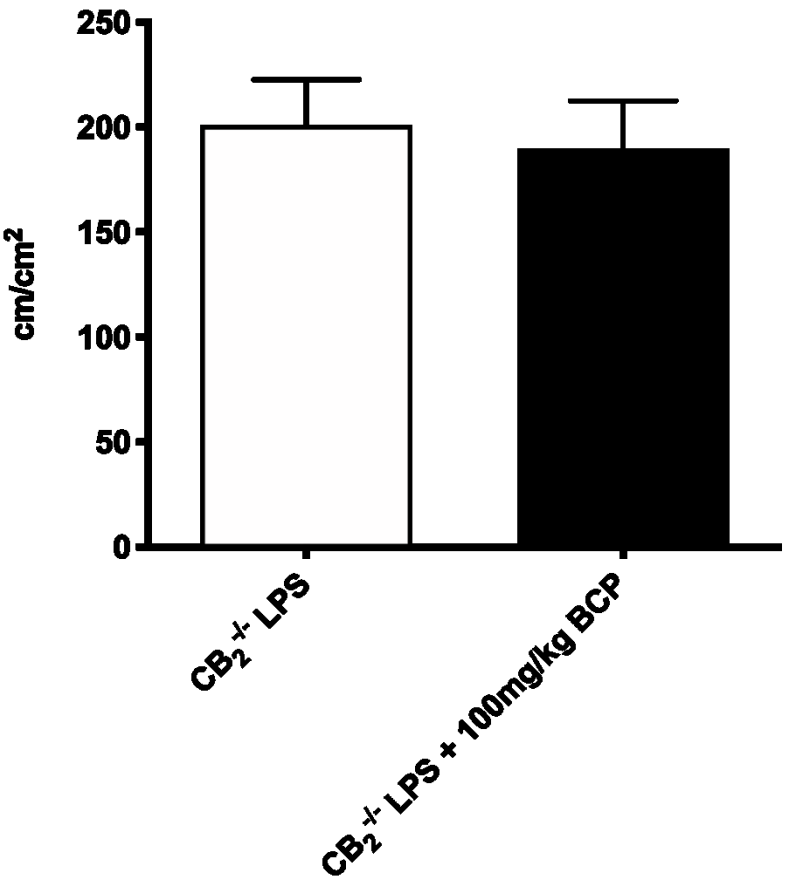


Figure 12: Capillary perfusion quantified through the functional capillary density (FCD) of the bladder microcirculation of $CB_2^{-/-}$ female BALB/c mice in the following experimental groups: LPS-induced IC ($CB_2^{-/-}$ LPS, 0.375mg/kg, n=5), and LPS-induced IC treated with BCP ($CB_2^{-/-}$ LPS+100mg/kg BCP, n=5). Capillary perfusion was assessed via intravital microscopy 2 hours following intravesical administration of LPS. Data presented as mean ±SD. A two-tailed t-test was performed to compare group means.

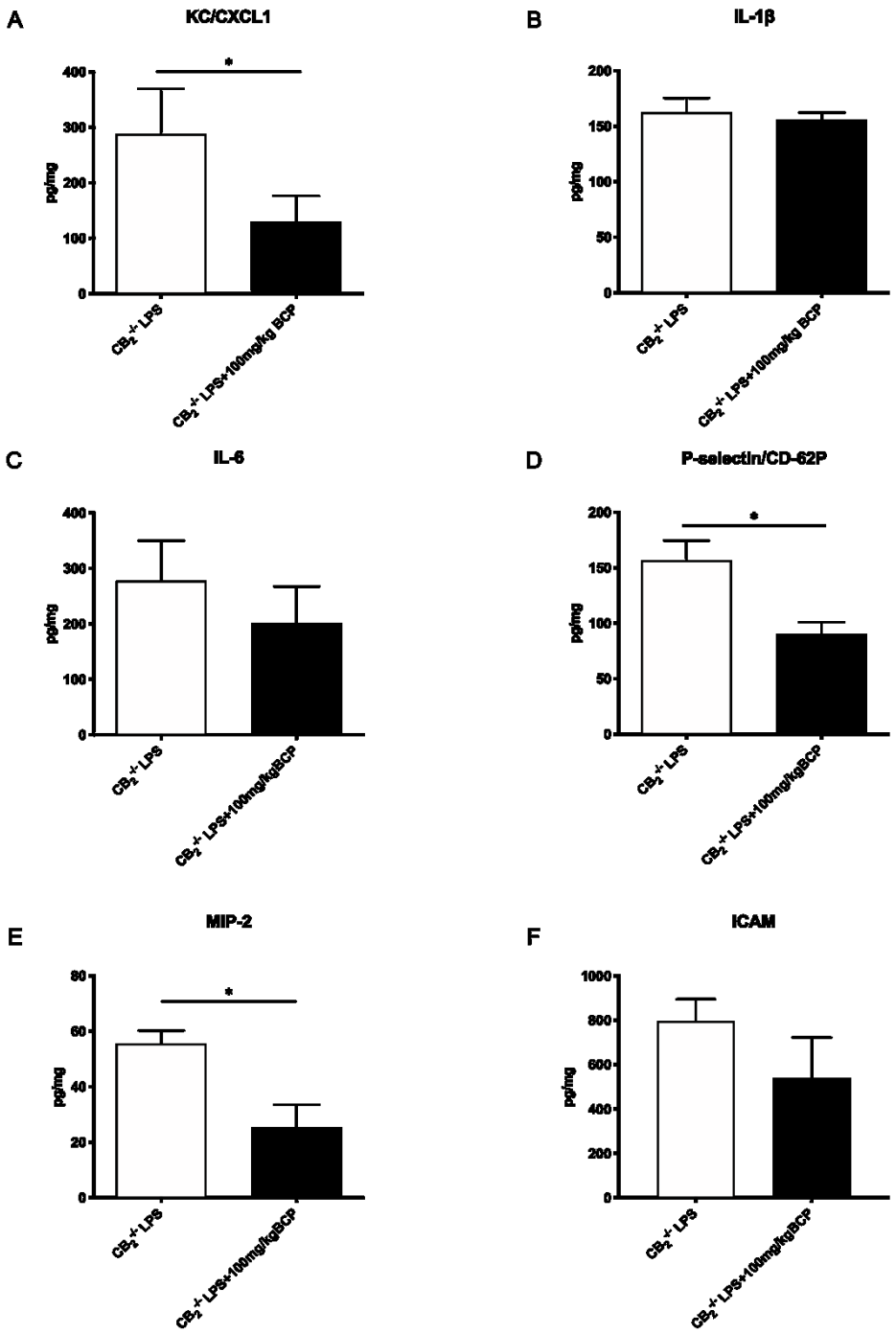


Figure 13 (A-F): Bladder tissue concentrations of KC/CXCL1 (A), IL-1β (B), IL-6 (C), P-selectin/CD-62P (D), MIP-2 (E), and ICAM (F) in $CB_2^{-/-}$ female BALB/c mice from the following experimental groups: LPS-induced IC ($CB_2^{-/-}$ LPS, 0.375mg/kg, n=5), and LPS-induced IC treated with BCP ($CB_2^{-/-}$ LPS+ 100mg/kg BCP, n=5). Sterile saline (control) and LPS were administered via intravesical instillation. Two technical replicated were performed for each sample. Data presented as mean ± standard deviation. * $p <$ 0.05. A two-tailed t-test was performed to compare group means.

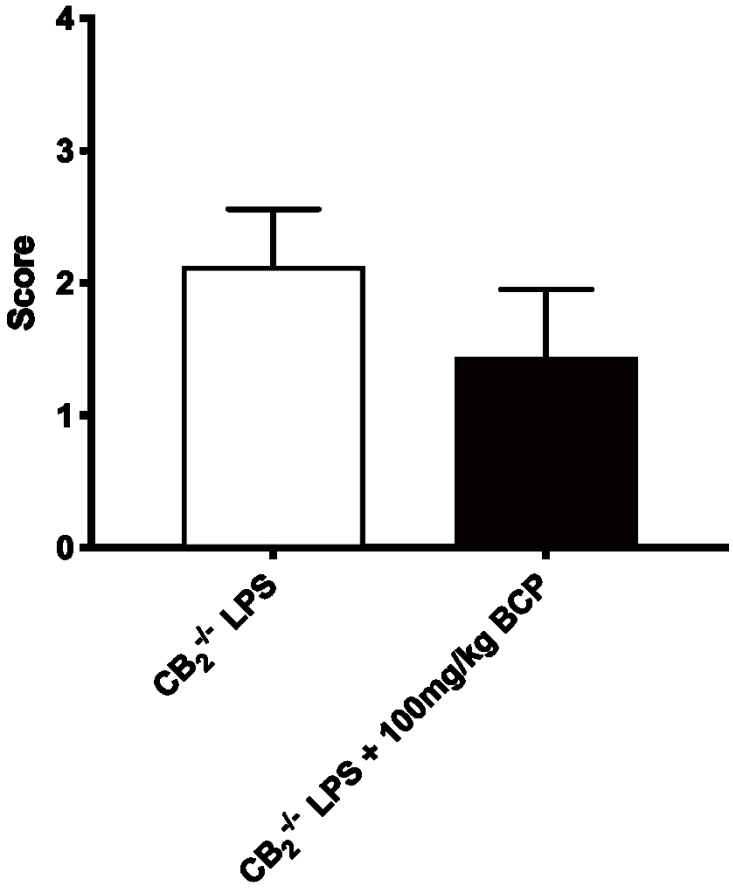


Figure 14: Bladder histopathology scores of $CB_2^{-/-}$ female BALB/c mice in the following experimental groups: LPS-induced IC ($CB_2^{-/-}$ LPS, 0.375mg/kg, n=5), and LPS-induced IC treated with BCP ($CB_2^{-/-}$ LPS+ 100mg/kg BCP, n=5). Slides were stained with H&E and scored based on an adapted scoring system from Hopkins et al. 1998. LPS and BCP were administered via intravesical instillation. Data presented as mean ± SD. A two-tailed t-test was performed to compare group means.

3.3 Exploring the Efficacy of Intravesical β-Caryophyllene Treatment in LPS-induced Experimental Cystitis and Comparing β-Caryophyllene Treatment to the Standard Clinical Intravesical Treatment for IC, 50% DMSO (Rimso-50 ®).

3.3.1 Intravital Microscopy

Intravesical LPS administration (150µg/ml) significantly increased the number of adherent leukocytes in post-capillary venules of the bladder microcirculation, compared to control wild type animals. Subsequent intravesical treatment with highly refined olive oil, the vehicle used for all BCP instillations, did not significantly reduce the number of adherent leukocytes ($p > 0.05$). BCP treatment at dosages of 100mg/kg (40mg/ml) and 300mg/kg (120mg/ml) significantly reduced the number of adherent leukocytes relative to the vehicle treated group ($p < 0.05$, Figure 15), however, there was no significant difference in the number of adherent leukocytes between the two dosages ($p > 0.05$). In contrast, mice treated with 50% DMSO (a.q.) following LPS challenge did not show a significant reduction in leukocyte adherence ($p < 0.05$), compared to the untreated LPS group. The number of adherent leukocytes in the combination treatment group, consisting of 100mg/kg BCP in 50% DMSO was significantly lower than that observed in the untreated-LPS and vehicle treated-groups ($p < 0.05$) but was not significantly lower than the 50% DMSO alone treatment ($p > 0.05$). In contrast, the number of adherent leukocytes in the 100mg/kg BCP group was significantly lower than that of the 50% DMSO treatment group ($p < 0.05$). The effects of the aforementioned intravesical treatments on leukocyte adhesion in the bladder microcirculation is shown in Figure 15.

The functional capillary density of the bladder microcirculation was not significantly affected by intravesical LPS instillation. Treatment with 50% DMSO significantly increased the FCD relative to both the control and untreated-LPS groups (p

< 0.05). Moreover, this increase was significantly higher than the100mg/kg BCP and vehicle treatment groups, which were not significantly different from the untreated-LPS and control groups ($p > 0.05$, Figure 16)

3.3.2 Luminex Assay

Vehicle treatment did not significantly reduce the tissue concentration of KC relative to the LPS group ($p > 0.05$). Both dosages of BCP significantly reduced the concentrations of KC by approximately 2-fold relative to the vehicle treated group ($p < 0.05$). Likewise, intravesical 50% DMSO instillation reduced KC concentrations to a similar extent relative to the untreated LPS group ($p<0.05$, Figure 17A).

IL-1β concentrations were unaffected by treatment with olive oil vehicle, 100mg/kg BCP, and 50% DMSO. Only the 300mg/kg BCP treatment significantly reduced KC concentrations relative to the untreated LPS group ($p<0.05$). However, this effect was not discernable from the vehicle treatment ($p>0.05$).

Vehicle treatment had no significant effect on IL-6 concentrations ($p>0.05$). BCP treatment at the 300mg/kg dose, but not the 100mg/kg dose, significantly reduced the concentrations of IL-6 in the bladder tissue by 2-fold relative to the vehicle treatment ($p<0.05$). 50% DMSO treatment did not significantly reduce the concentration of IL-6 ($p>0.05$).

Similarly, olive oil vehicle had no effect on the tissue concentrations of P-selectin relative to the -LPS group ($p>0.05$). While both BCP dosages significantly reduced P-selectin levels relative to the LPS group, only the 100mg/kg dosage lowered the concentration to a level discernable from the vehicle treatment group, producing a 1.5-

fold decrease in the concentration ($p<0.05$). 50% DMSO treatment was also effective in significantly lowering P-selectin levels to a level comparable to the 100mg/kg BCP treatment.

The concentrations of MIP-2 were only significantly reduced by treatment with 300mg/kg BCP ($p<0.05$). This decrease was significant relative to the vehicle treatment group, which had no effect relative to the LPS group ($p>0.05$).

None of the instillation treatment produced a significant decrease in ICAM concentrations, however trends show a non-significant decrease in ICAM levels with an in BCP dosage. Concentrations observed in the 50% DMSO group were comparable to the BCP treatments, albeit not significant from the LPS group ($p >0.05$)

3.3.3 Bladder Histopathology

LPS instillation caused a significant increase in the bladder histopathology score. Intravesical treatment with highly refined olive oil (vehicle), 100mg/kg BCP, or 50% DMSO had no effect on the histopathology score ($p > 0.05$). However, the 300mg/kg BCP treatment caused a significant decrease in the grading score relative to the untreated-LPS group ($p < 0.05$, Figure 18), however, it was not discernable from the vehicle treatment ($p>0.05$)

3.3.4 von Frey Aesthesiometry

von Frey aesthesiometry was performed to assess mechanic sensitivity of the pelvic region animals in order to evaluate the potential local analgesic effects of the various intravesical treatments used in this study. Mechanical allodynia was measured in

all animals before the first instillation (T_0) to set an individual baseline measurement. There was no significant difference in baseline (T_0) values between any of the experimental groups (Figure 19, $p < 0.05$). The second measurement was recorded 3 hours following LPS instillation (T_1), and 2.5 hours following treatment. The mean change in the withdrawal force between the first and second measurements was then calculated for each animal. Figure 2 in section 2.4.2 details the full von Frey aesthesiometry experimental timeline.

Control animals showed no change in the withdrawal threshold between T_0 and T_1 ($p > 0.05$). Conversely, animals in the LPS group showed a significant decrease in the withdrawal threshold, with the mean T_1 withdrawal threshold being significantly lower than that of control group ($p < 0.05$). Animals in the LPS group showed a mean decrease of 6.5g in the withdrawal threshold between T_0 and T_1. Vehicle (olive oil) treatment following LPS challenge did not significantly affect the withdrawal threshold relative to the untreated LPS group ($p > 0.05$). BCP treatment at a dosage of 100mg/kg significantly increased the withdrawal threshold following LPS instillation ($p > 0.05$), however the increase was not significant in the 300mg/kg dose treatment group ($p > 0.05$). Similarly, intravesical treatment with 50% DMSO resulted in a non-significant increase in the withdrawal threshold compared to the untreated LPS group ($p < 0.05$, Figure 19).

3.3.5 Behavior

Observational analysis of animal behavior was performed to further evaluate the analgesic effects of the experimental treatments. Three parameters were recorded;

position of the eye lids, animal posture, and animal motor activity. The three scores were also summed to provide a cumulative behavior score.

Control animals that received intravesical instillations of sterile saline showed no change in the position of the eye lids between T_0 and T_1. LPS instillation caused a significant increase in the eye lid position score compared to the control animals ($p < 0.05$). Treatment with olive oil vehicle did not significantly affect the LPS-induced increase in eye lid position score ($p > 0.05$). The 300mg/kg BCP treatment group was the only group that showed a significant reduction in T_1 score relative to the LPS and vehicle groups. The 100mg/kg BCP and 50% DMSO treatment groups did not significantly improve the LPS-induced increase in eye-lid position score (Figure 20A).

Control animals also showed no change in the posture score between T_0 and T_1. LPS administration into the bladder caused a significant increase in the postural score at T_1 compared to the control group ($p < 0.05$). Vehicle treatment had no significant effect on T_1 posture score compared to the LPS group ($p > 0.05$). However, all three treatments were able to significantly reduce the postural score at T_1 relative to the untreated-LPS group ($p < 0.05$). Moreover, the T_1 postural score for both BCP treatment groups were significantly lower the vehicle treatment group ($p< 0.05$, Figure 20B)

Control animals also showed no significant difference in motor activity score between T_0 and T_1 ($p >0.05$), whereas the motor activity score of animals in the LPS group was significantly elevated at T_1, indicating a significant reduction in motor activity. BCP treatment dosed at 100mg/kg and 300mg/kg was able to significantly reduce the motor activity score relative to T_1 of the LPS group, however the effects were not

discernable from vehicle treatment ($p>0.05$). Vehicle and 50% DMSO treatment did not significantly reduce the LPS-induced increase in motor activity score ($p > 0.05$).

The scores for the three behavior parameters assessed were summed to generate a cumulative behavioral score. Control animals showed a minimal, non-significant increase in the total cumulative score. Both the LPS and Vehicle treated groups showed significant increases from T_0 to T_1 ($p <0.05$). The cumulative score for the vehicle treatment group at T_1 was not significantly different from the LPS group at T_1. However, both BCP treatment groups, in addition to the 50% DMSO treatment group, were able to significantly decrease the LPS-induced increase in behavioral score ($p < 0.05$). The BCP treatments groups also showed a significant decrease in cumulative score at T_1 relative to the vehicle treatment group at T_1.

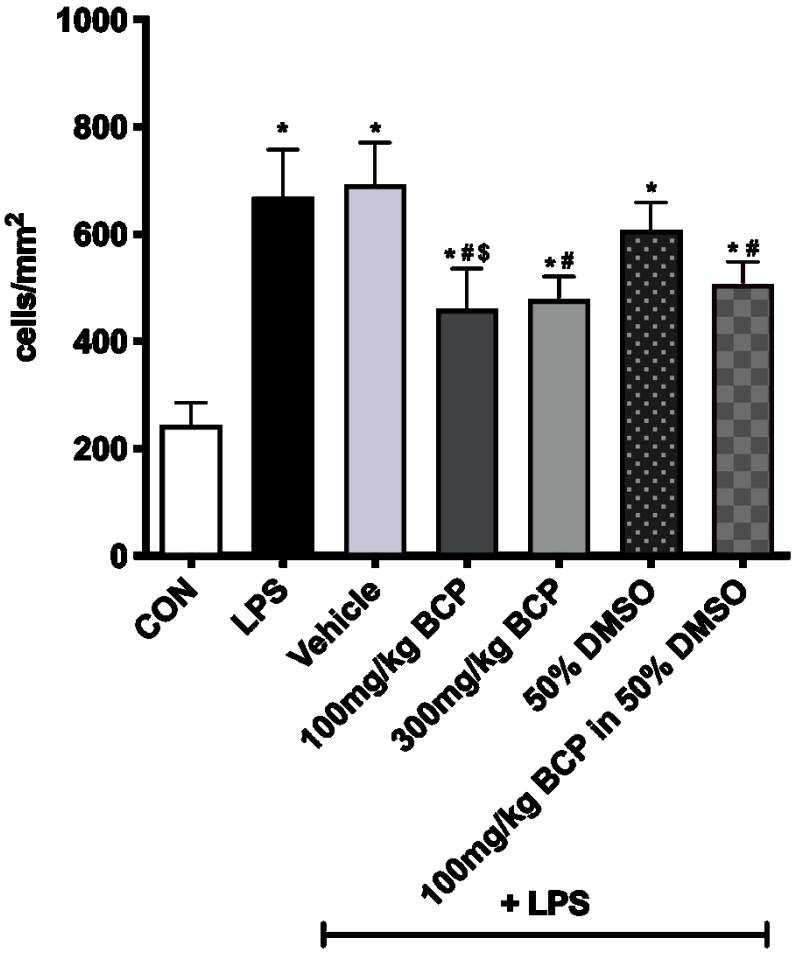


Figure 15: Leukocyte adhesion in the submucosal venules of WT female BALB/c mice belonging to the following experimental groups: control (CON, n=6), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle (LPS +Vehicle, n =5), 100mg/kg BCP (LPS+100mg/kg BCP, n=5), 300mg/kg BCP (LPS+300mg/kg BCP, n=5), 50% DMSO (LPS+50%DMSO, n =5), and 100mg/kg BCP in 50% DMSO (LPS+100mg/kg BCP in 50% DMSO, n=5). Leukocyte adhesion was assessed via intravital microscopy 2 hours following intravesical administration of LPS. Data presented as mean ± SD. * $p < 0.05$ versus CON, # $p < 0.05$ versus LPS+Vehicle, $ $p < 0.05$ versus LPS+50%DMSO

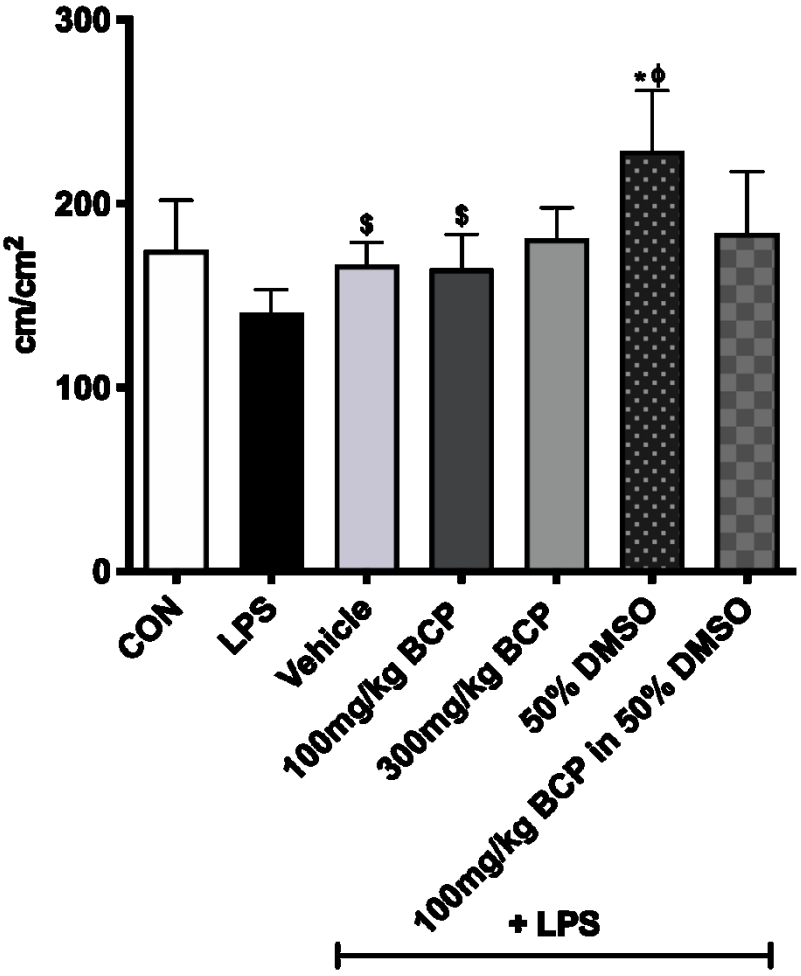


Figure 16: Capillary perfusion, quantified through the functional capillary density of the bladder microcirculation, in WT female BALB/c mice belonging to the following experimental groups: control (CON, n=6), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle (LPS +Vehicle, n =5), 100mg/kg BCP (LPS+100mg/kg BCP, n=5), 300mg/kg BCP (LPS+300mg/kg BCP, n=5), 50% DMSO (LPS+50%DMSO, n =5), and 100mg/kg BCP in 50% DMSO (LPS+100mg/kg BCP in 50% DMSO, n=5). FCD was assessed by intravital microscopy 2 hours following intravesical administration of LPS. Data presented as mean ± SD. * $p < 0.05$ vs CON, ϕ $p < 0.05$ versus LPS, $ $p < 0.05$ vs LPS+50%DMSO.

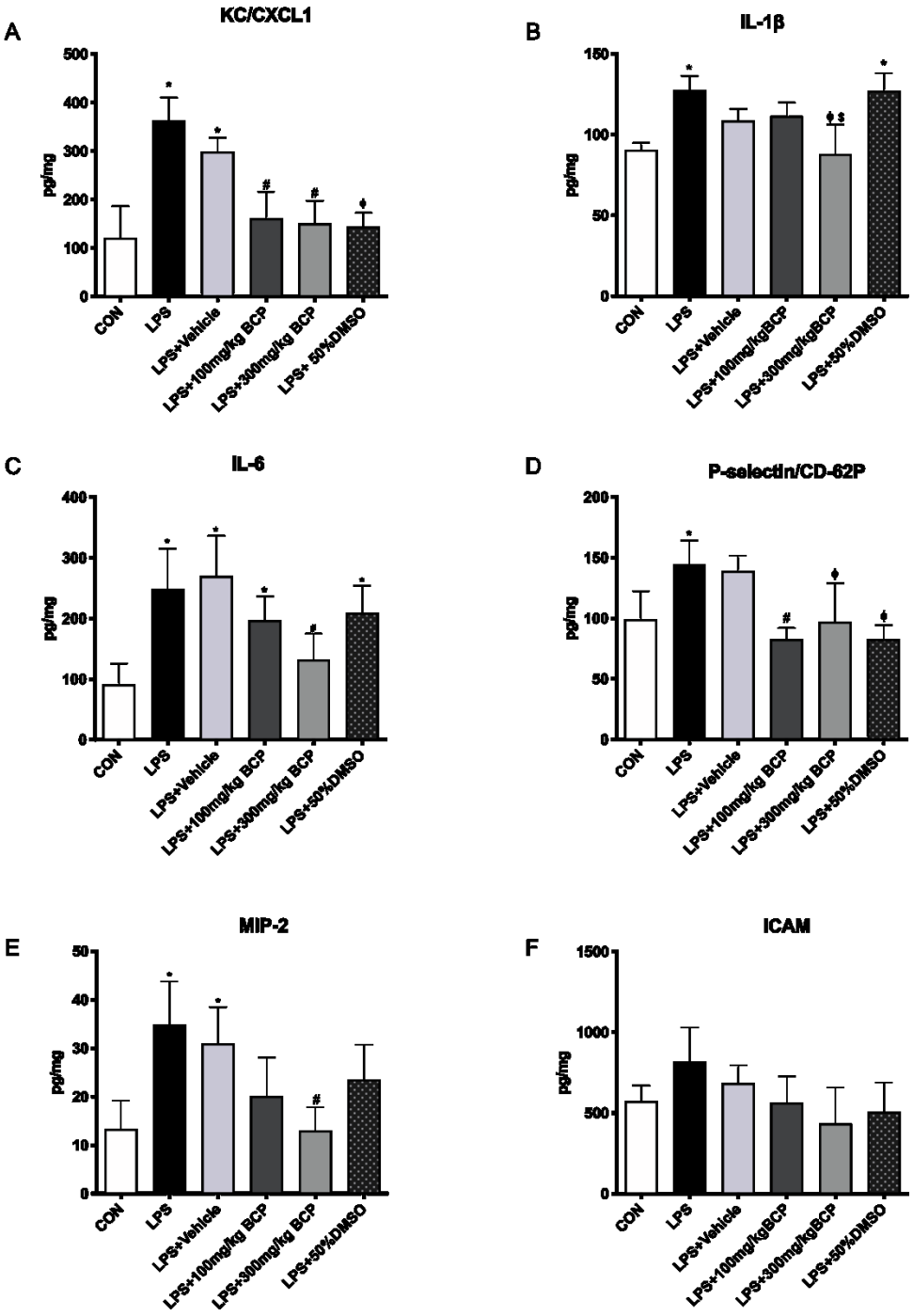


Figure 17 (A-F): Concentrations of KC/CXCL1 (A), IL-1β (B), IL-6 (C), P-selectin/CD-62P (D), MIP-2 (E), and ICAM (F) the in bladder tissue of WT female BALB/c mice belonging to the following experimental groups: control (CON, n=5), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle (LPS +Vehicle, n =5), 100mg/kg BCP (LPS+100mg/kg BCP, n=5), 300mg/kg BCP (LPS+300mg/kg BCP, n=5), and 50% DMSO (LPS+50%DMSO, n =5).Two technical replicates were performed for each sample. Data presented as mean ± SD. * $p < 0.05$ vs CON, ϕ $p < 0.05$ versus LPS, # $p > 0.05$ vs LPS+Vehicle, $ $p < 0.05$ vs LPS+50%DMSO.

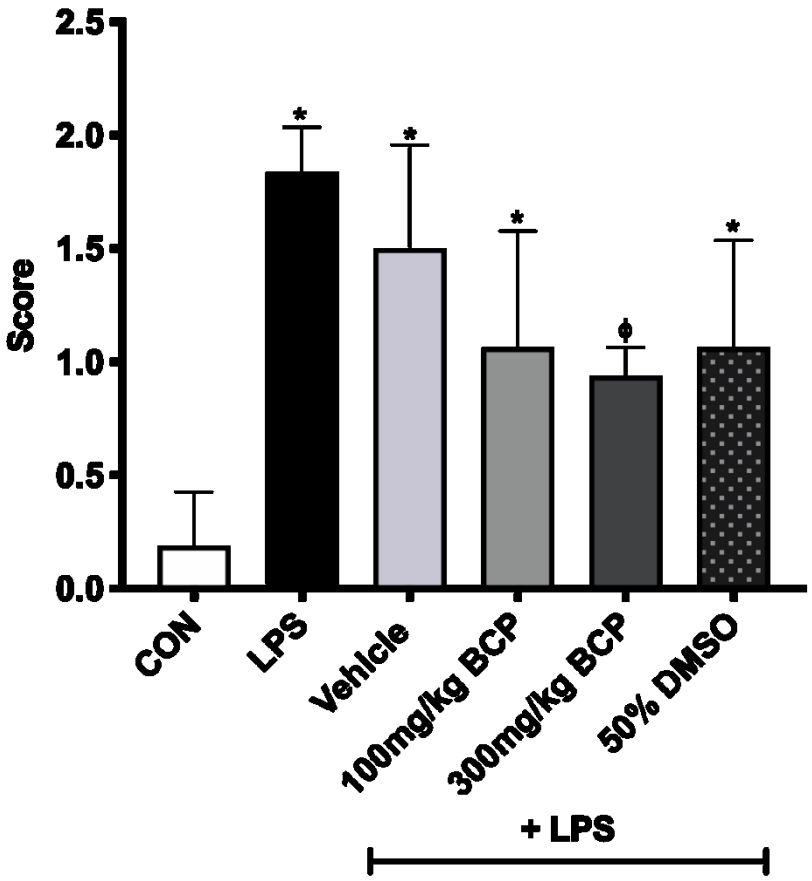


Figure 18: Bladder histopathology scores of WT female BALB/c mice belonging to the following experimental groups: control (CON, n=5), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle (LPS +Vehicle, n =5), 100mg/kg BCP (LPS+100mg/kg BCP, n=5), 300mg/kg BCP (LPS+300mg/kg BCP, n=5), 50% DMSO (LPS+50%DMSO, n =5). Slides were stained with H&E and scored based on an adapted scoring system from Hopkins et al. 1998. LPS and BCP were administered via intravesical instillation. Data presented as mean ± SD. * $p < 0.05$ vs CON, ϕ $p < 0.05$ versus LPS

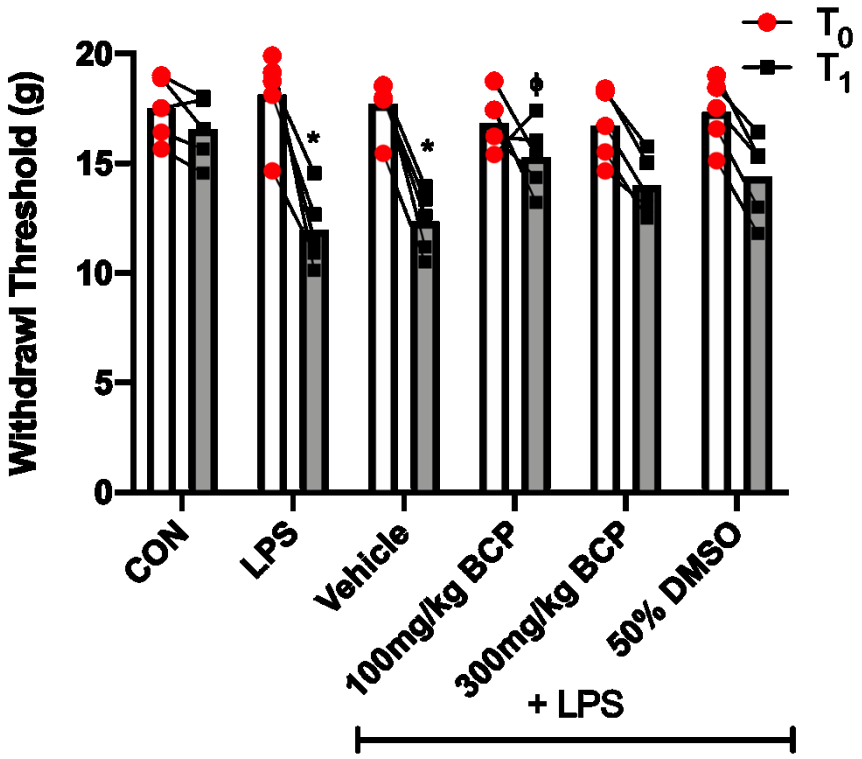


Figure 19: Withdrawal threshold (g) of female BALB/c mice before cystitis induction (T_0) and after treatment (T_1), measured by an electronic von Frey aesthesiometer. The following experimental groups are shown: control, (CON, n =5), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle (LPS+Vehicle, n=5), 100mg/kg BCP (LPS+100mg/kg BCP, n=5), 300mg/kg BCP (LPS+300mg/kg BCP, n=5), and 50% DMSO. Individual data points and group means are shown. There was no difference in the mean T_0 withdrawal threshold between any treatment groups. * $p < 0.05$ vs CON T_1, ϕ $p < 0.05$ vs LPS T_1.

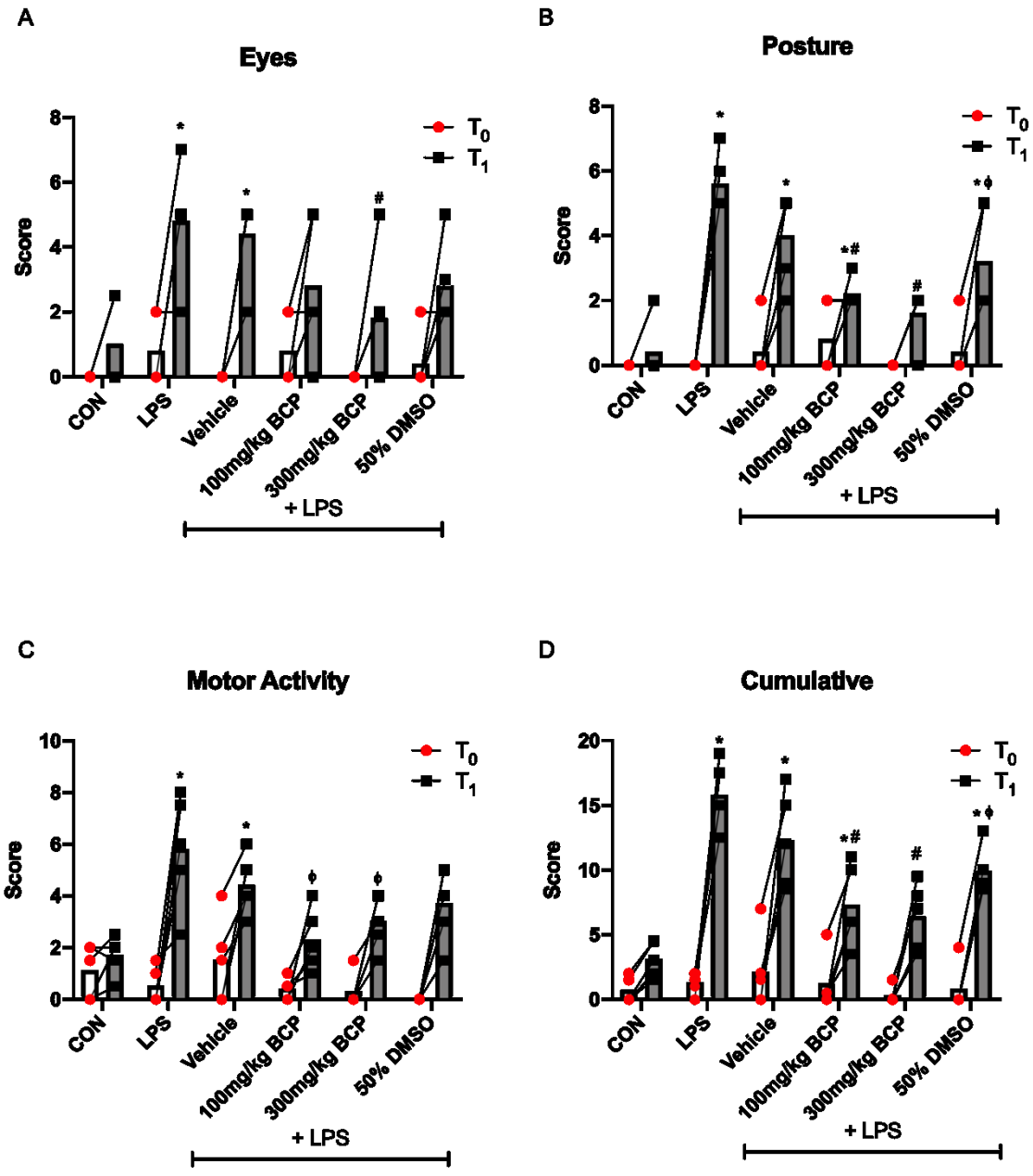


Figure 20 (A-D): Score for each behavioral parameter (A-C), and the cumulative behavior score (D), before cystitis induction (T_0) and after treatment (T_1). The following experimental groups are shown: control, (CON, n =5), untreated LPS-induced IC (LPS, 0.375mg/kg n=5), vehicle (LPS+Vehicle, n=5), 100mg/kg BCP (LPS+100mg/kg BCP, n=5), 300mgkg BCP (LPS+300mg/kg BCP, n=5), and 50% DMSO (LPS+50%DMSO, n=5). There was no difference in the mean T_0 scores between any treatment groups. Individual data points and group mean are shown. * $p < 0.05$ vs CON T_1, ϕ $p < 0.05$ vs LPS T_1, # $p < 0.05$ vs LPS+Vehicle T_1.

3.4 Exploring the Therapeutic Potential of Indirect CB_2R Activation by Increasing Local Endocannabinoid Concentrations Through the Inhibition of Monoacylglycerol Lipase

3.4.1 Intravital Microscopy

JZL184, at a dosage of 16mg/kg, was administered intravesically following LPS challenge. JZL184 was administered in a 1:1:18 ratio vehicle consisting of DMSO, cremophor, and saline. Vehicle treated mice showed a non-significantly reduction in the number of adherent leukocytes compared to the untreated LPS animals ($p>0.05$). JZL 184 treatment significantly reduced the number of adherent leukocytes in the bladder microcirculation relative to the LPS group ($p<0.05$), however this reduction was not significantly lower than vehicle treatment ($p>0.05$) An unpaired t test between the vehicle treated group and the JZL184 treated group did however reveal a significant reduction in leukocyte adherence (Figure 21).

The FCD of the bladder microcirculation was non-significantly reduced by LPS instillation, however, local administration of JZL184 restored FCD levels back to those observed in control animals and was significantly higher than that observed in the LPS group ($p<0.05$). Vehicle treatment had no significant effect on FCD (Figure 22).

3.4.2 Luminex Assay

1:1:18 (Cremophor: DMSO: Saline) vehicle treatment caused a non-significant decrease in KC concentrations relative to the LPS group. Treatment with JZL184 significantly reduced the KC concentrations by more than 50% relative to the LPS group ($p<0.05$). No significant difference was detected between the vehicle and JZL184 treatment groups ($p >0.05$, Figure 23A).

Similar results were observed for IL-1β, whereby vehicle treatment produced a non-significant decrease in IL-1β concentrations ($p>0.05$). JZL184 treatment significantly reduced the concentration to control levels, however this reduction was not significantly lower relative to the vehicle treatment group ($p<0.05$, Figure 23B).

Il-6 concentrations were also unaffected by vehicle treatment ($p>0.05$). However, treatment with JZL184 did significantly reduce the concentrations of IL-6 relative to the vehicle treatment group ($p<0.05$). JZL184 treatment reduced by IL-6 levels by approximately 50% (Figure 23C).

The concentrations of P-selectin were not significantly reduced with vehicle treatment ($p>0.05$), however JZL184 did cause a significant reduction relative to the LPS group ($p<0.05$). No difference was detected between the vehicle and JZL184 groups ($p>0.05$, Figure 23D).

MIP-2, while significantly elevated by LPS instillation, was unaffected by both vehicle and JZL184 treatment. Both the vehicle and JZL184 treatments produced slight, non-significant reductions in MIP-2 concentrations ($p>0.05$, Figure 23E).

ICAM concentrations were unaffected by both the vehicle and JZL184 treatment ($p>0.05$).

3.4.3 Bladder Histopathology

LPS instillation caused a significant increase in the bladder histopathology score (Figure 10) which was characterized by multifocal and diffuse inflammatory cell infiltration and mild subepithelial edema. Intravesical treatment with 1:1:18 vehicle did not significantly reduce the histopathology score ($p>0.05$). However, treatment with

16mg/kg JZL184 was able to significantly reduce the histopathology score ($p<0.05$), although this reduction was not significantly different from the vehicle treatment group ($p>0.05$, Figure 24).

3.4.4 von Frey Aesthesiometry

Control animals showed no significant change in the measured withdrawal threshold between T_0 and T_1 ($p>0.05$), while untreated-LPS animals showed a significant decrease in the withdrawal threshold, with the mean T_1 threshold being significantly lower than that of the control group ($p<0.05$). Subsequent intravesical treatment with 1:1:18 vehicle did not significantly improve the withdrawal threshold at T_1 compared to T_1 of the LPS group ($p>0.05$). The withdrawal threshold of animals in the 16mg/kg JZL184 treatment group was significantly higher at T_1 compared to animals in the untreated LPS at T_1. However, no significant difference in the T_1 withdrawal thresholds between the vehicle and JZL184 treatment groups was detected ($p>0.05$, Figure 25).

3.4.5 Behavior

Control animals showed no change in the position of the eyelids between T_0 and T_1 ($p>0.05$). Conversely, LPS instillation caused a significant increase in the eye lid position score, indicating a more closed position of the eyelid at T_1 ($p<0.05$). Both vehicle and JZL184 treatment caused a significant reduction in eyelid position score ($p<0.05$), however no significant difference was detected between the vehicle and JZL184 treatment groups ($p>0.05$, Figure 26A).

No change in the posture score was detected between T_0 and T_1 in the control group, whereas animals in the LPS group showed a significant increase in posture score

($p<0.05$). JZL184 treatment significantly reduced the LPS-induced increase in postural score ($p <0.05$). The T_1 postural score of animals in the JZL184 treatment group was also significantly lower than the T_1 score of the vehicle treated group ($p<0.05$). Vehicle treatment had no effect on the LPS-induced increase in posture score ($p>0.05$, Figure 26B).

Motor activity score was also significantly increased following intravesical LPS administration compared to control animals ($p<0.05$). Vehicle treatment, nor JZL184 treatment significantly affected the LPS-induced increase in motor activity score ($p >0.05$, Figure 26C).

Summing the scores of the three behavior parameters provided the cumulative behavior score. There was no significant difference between the T_0 and T_1 scores in the control group ($p> 0.05$). Animals in the LPS group showed a significant increase in the cumulative score ($p <0.05$). The T_1 cumulative score in the JZL184 treatment group was significantly lower than the cumulative T_1 score for the LPS group ($p< 0.05$), however, the T_1 score was not significantly lower than the vehicle treatment group ($p>0.05$). The cumulative T_1 score for the vehicle group was not significantly lower than the LPS group ($p>0.05$, Figure 26D).

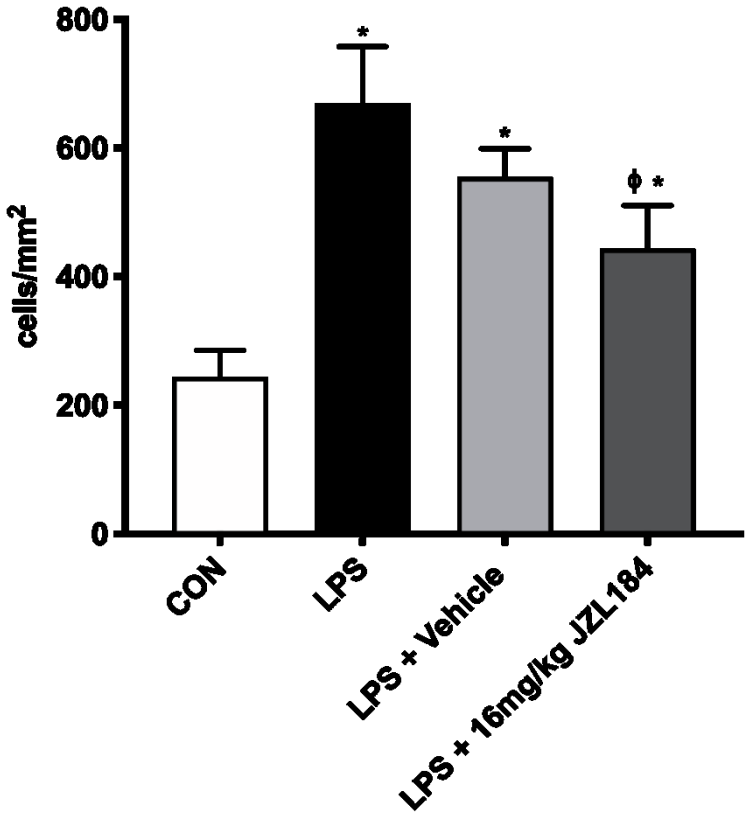


Figure 21: Leukocyte adhesion in the submucosal venules of WT female BALB/c mice in the following experimental groups: control (CON, n=6), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle treatment (LPS+Vehicle, n=5), and JZL184 treatment (LPS+JZL184, 16mg/kg, n=5). Leukocyte adhesion was assessed by intravital microscopy 2 hours following intravesical administration of LPS or sterile saline (control). Data presented as mean ± SD. ϕ $p < 0.05$ vs LPS

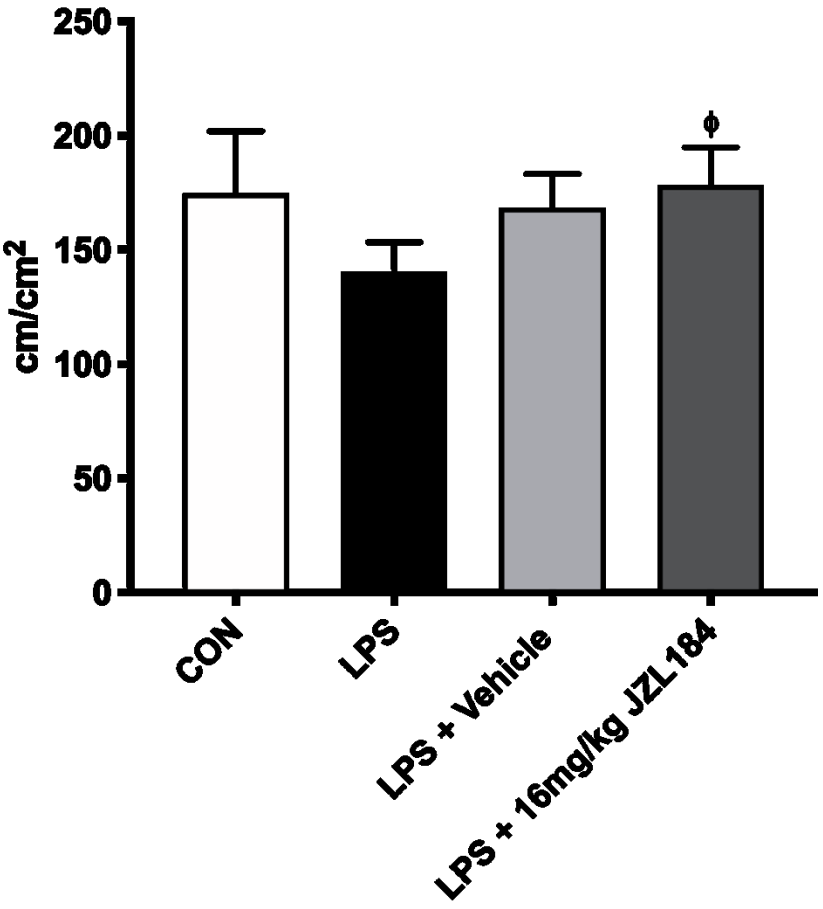


Figure 22: Capillary perfusion, quantified through the functional capillary density of the bladder microcirculation, in WT female BALB/c mice belonging to the following experimental groups: control (CON, n=6), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle treatment (LPS+Vehicle, n=5), and JZL184 treatment (LPS+JZL184, 16mg/kg, n=5). FCD was assessed by intravital microscopy 2 hours following intravesical administration of LPS or saline (control). Data presented as mean ± SD. * $p <$ 0.05 vs CON, ϕ $p <$0.05 vs LPS.

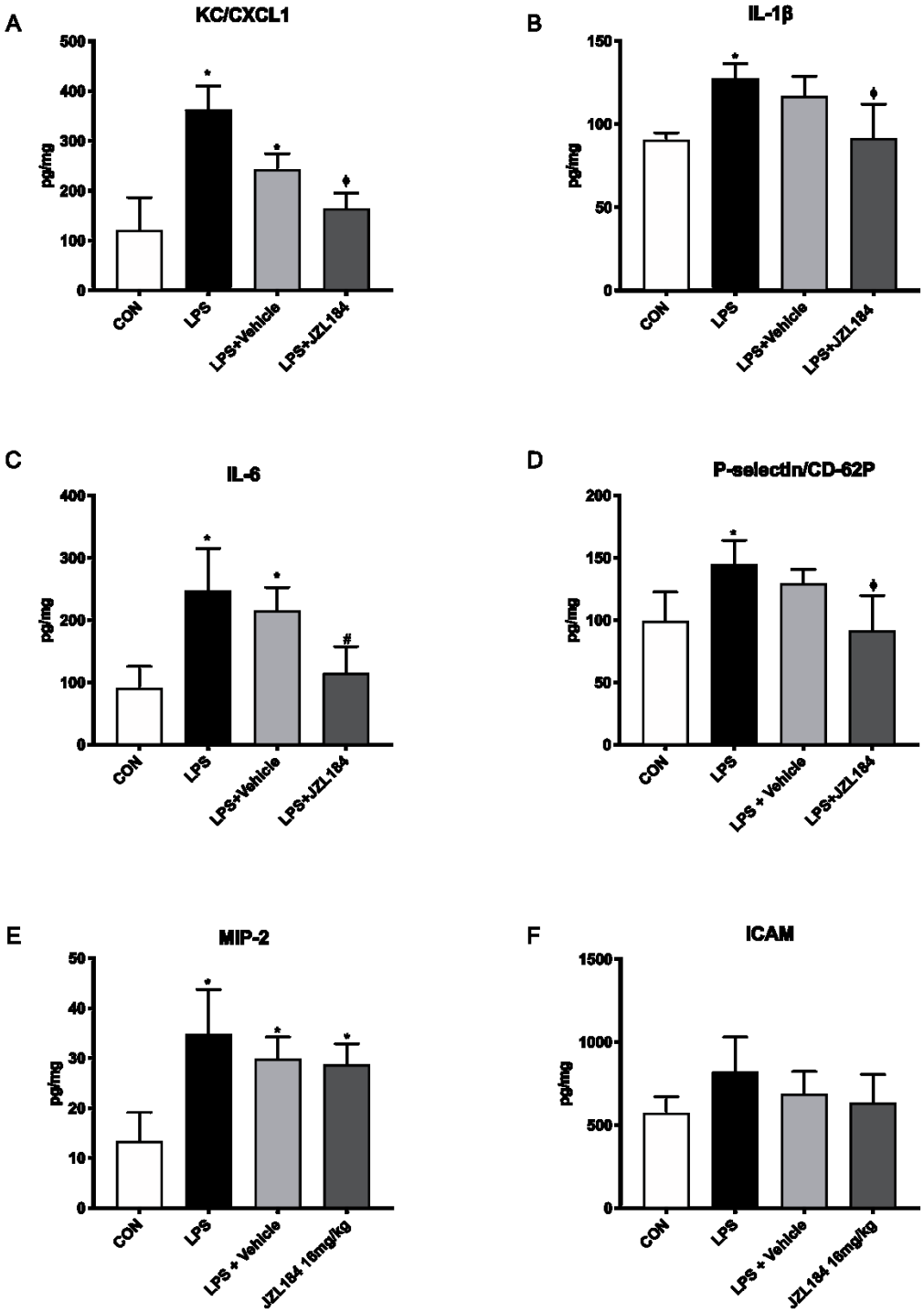


Figure 23(A-F): Concentrations of KC/CXCL1 (A), IL-1β (B), IL-6 (C) P-selectin/CD-62P (D), MIP-2 (E), and ICAM (F) in bladder tissue of female wild-type BALB/c mice belonging to the following experimental groups: control (CON, n=5), untreated LPS-induced IC (LPS, 0.375mg/kg n=5), vehicle treatment (LPS+Vehicle, n=5) JZL184 treatment (LPS+JZL184, 16mg/kg, n=5). Two technical replicated were performed for each sample. Data presented as mean ± SD. * $p < 0.05$ vs CON, # $p < 0.05$ vs LPS+Vehicle, ϕ $p < 0.05$ vs LPS.

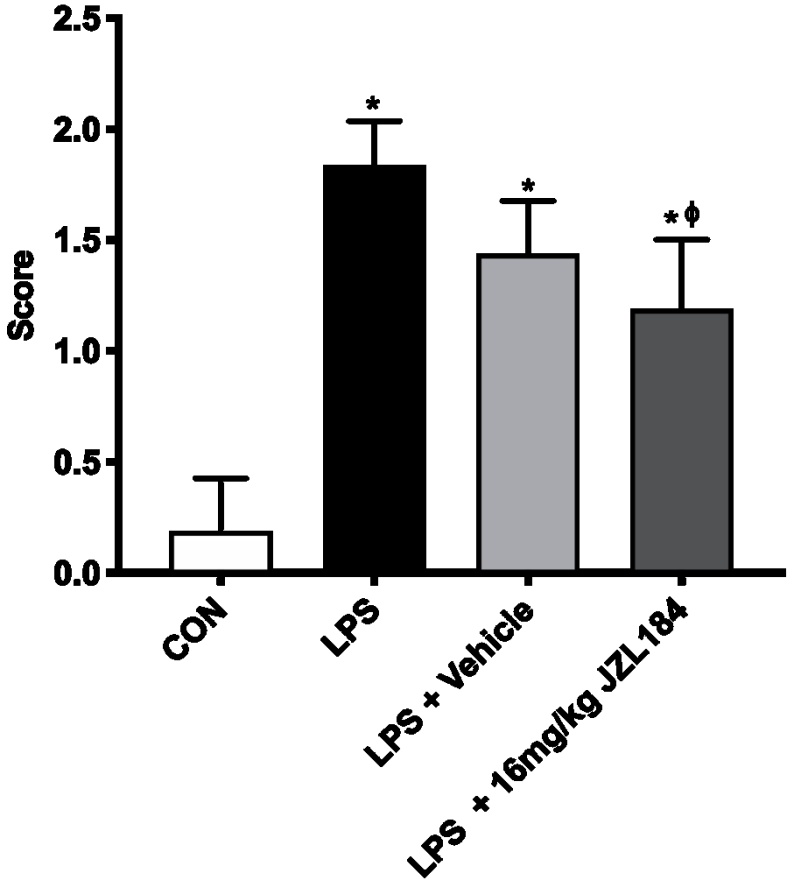


Figure 24: Bladder histopathology scores of WT female BALB/c mice in the following experimental groups: control (CON, n=5), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle treatment (LPS+Vehicle, n=5), and JZL184 treatment (LPS+JZL184, 16mg/kg, n=5). Slides were stained with H&E and scored based on an adapted scoring system from Hopkins et al. 1998. Data presented as mean ± SD. * $p < 0.05$ vs CON, ϕ $p < 0.05$ vs LPS

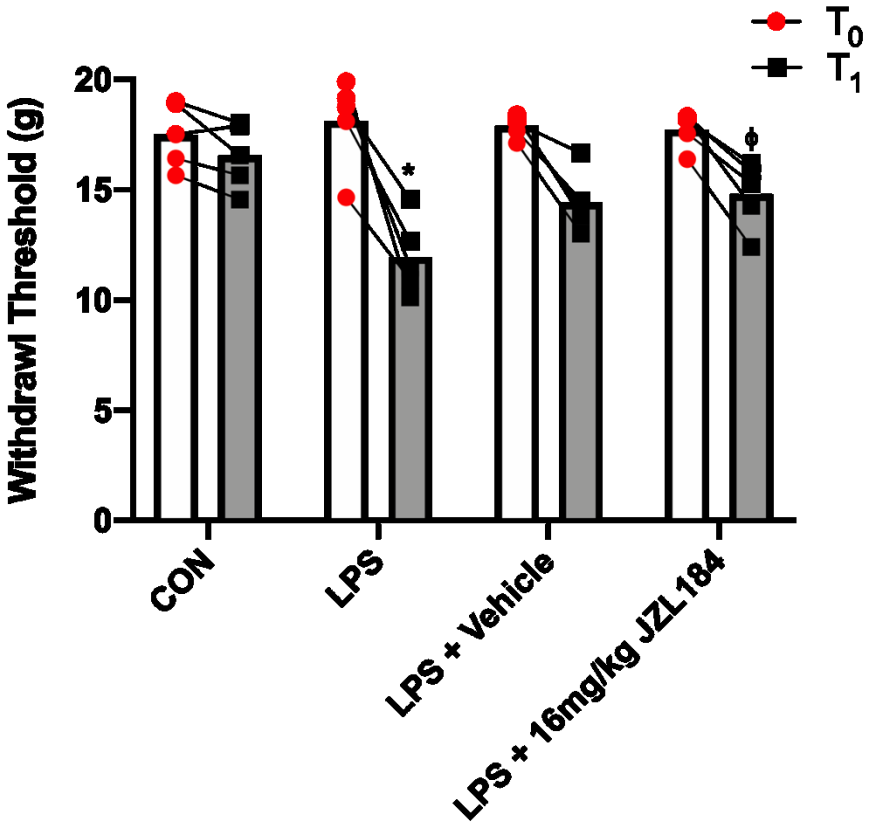


Figure 25: Withdrawal threshold (g) of female BALB/c mice before cystitis induction (T_1) and after treatment (T_0), measured by an electronic von Frey aesthesiometer. The following experimental groups are shown control (CON, n=5), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle treatment (LPS+Vehicle, n=5), and JZL184 treatment (LPS+JZL184, 16mg/kg, n=5). Individual data points and group mean is shown. There was no difference in the mean T_0 withdrawal threshold between any treatment groups. * $p < 0.05$ vs CON T_1, ϕ $p < 0.05$ vs LPS T_1.

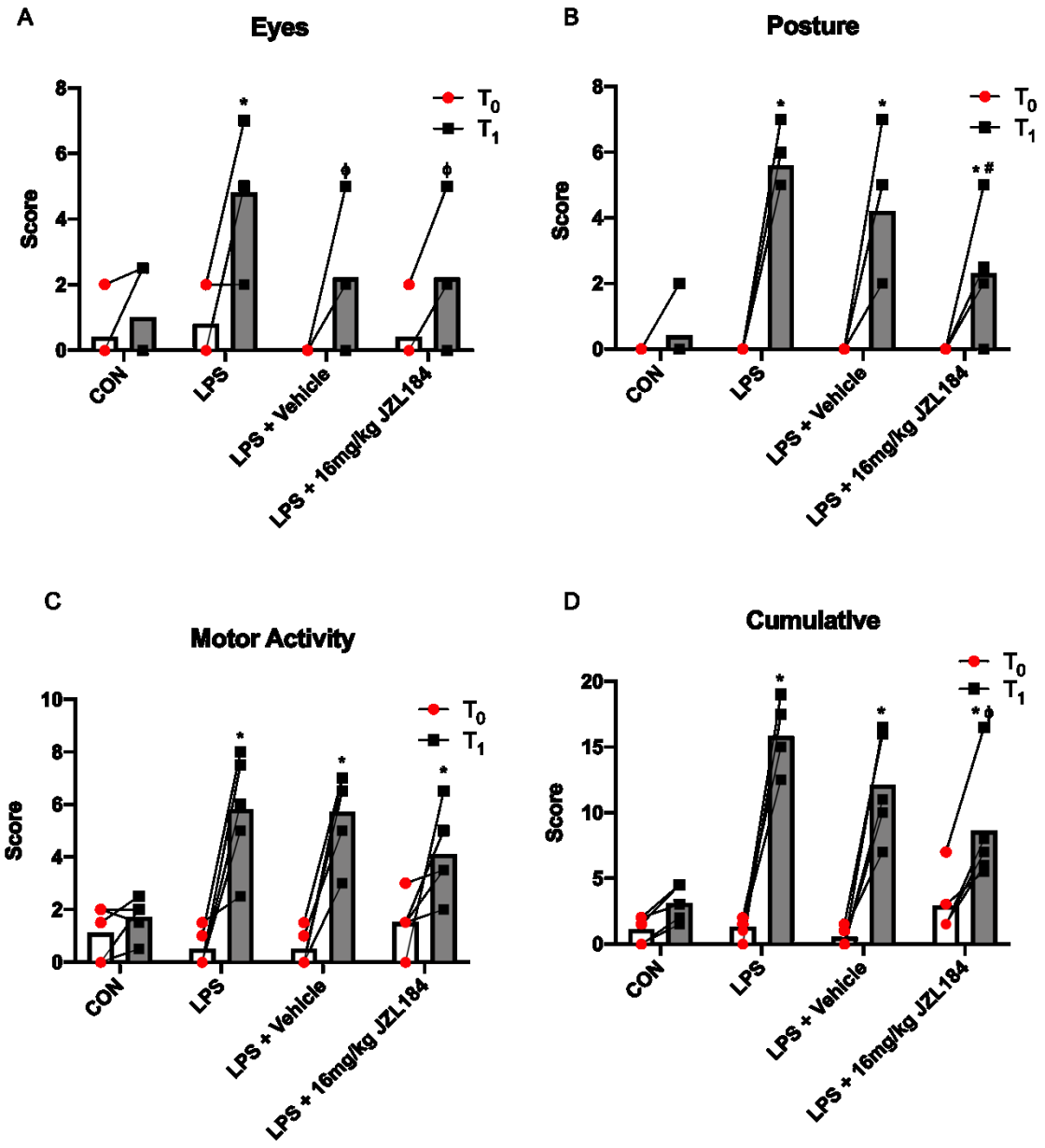


Figure 26 (A-D): Score for each behavioral category, and the cumulative score, before cystitis induction (T_0) and after treatment (T_1). The following experimental groups are shown: control (CON, n=5), untreated LPS-induced IC (LPS, 0.375mg/kg, n=5), vehicle treatment (LPS+Vehicle, n=5), and JZL184 treatment (LPS+JZL184, 16mg/kg, n=5). There was no difference in the mean T_0 scores between any treatment groups. Individual data points and group mean are shown. * $p < 0.05$ vs CON T_1, ϕ $p < 0.05$ vs LPS T_1, # $p < 0.05$ vs LPS+Vehicle T_1.

Chapter 4: Discussion

IC is a debilitating chronic pain condition of the urinary bladder, and is well known to have a significant negative impact on a patients' quality of life (Vasudevan & Moldwin, 2017). Despite a wide range of potential therapeutic options being available, many IC patients remain dissatisfied with their symptom management (Hill et al., 2008), and no treatment has been shown to be effective for all patients (Pazin et al., 2016). The ECS has emerged as a promising target to alleviate acute and chronic pain episodes, and CB_2R activation has been shown to inhibit inflammation (Manzanares et al., 2006).The CB_2R, typically referred to as the peripheral cannabinoid receptor, has been identified in the bladder of various species, including humans (Gratzke et al., 2009). Given the well reported immunosuppressive and anti-inflammatory effects of CB_2R agonists, and lack of associated euphoric effects, the CB_2R may represent a beneficial target for IC pharmacotherapy.

To our knowledge, this is the first study to show that intravesical administration of the phyto-derived CB_2R agonist, BCP, exerts analgesic and anti-inflammatory effects in a model of acute cystitis. Furthermore, this work adds to the growing body of literature that demonstrates anti-inflammatory and analgesic effects of enhancing local 2-AG levels through MAGL inhibition (reviewed by Mulvihill & Nomura, 2013).

4.1 The Impact of Genetic *Cnr2*$^{-}$ Knockout on the Severity of Bladder Inflammation.

The use of transgenic *Cnr2*$^{-}$ ($CB_2^{-/-}$) mice has contributed greatly to the understanding of the roles of the CB_2R in human diseases, including in a range of

inflammatory conditions. Studies using *Cnr2*$^{-}$ mice to study the role of this receptor under inflammatory conditions have shown that the loss of endogenous CB_2R activity results in an exacerbated inflammatory response compared to WT animals (summarized by Turcotte et al., 2016). Here, we utilized $CB_2^{-/-}$ mice to assess the role of this receptor, and endogenous CB_2R activity, in response to LPS-induced inflammatory cystitis.

The results of this study are in partial agreeance with the findings of the above-mentioned studies. Here, mice lacking global expression of CB_2R showed a significantly higher number of adherent leukocytes in the venules of the bladder microcirculation compared to WT counterparts, in both saline treated controls and LPS challenged animals. This finding parallels those from a recent study by Kapellos et al. (2019), who showed that global CB_2R deficiency resulted in significantly enhanced neutrophil and monocyte recruitment in the dorsal air pouch model of acute inflammation, compared to WT littermates (Kapellos et al., 2019). Importantly, the enhanced neutrophil recruitment was shown to be independent of blood leukocyte numbers. The CB_2R deficient neutrophils isolated from the dorsal air pouch exhibited an enhanced migratory transcriptional profile, and overexpressed a number of genes associated with chemotaxis and inflammatory cell recruitment (Kapellos et al., 2019). They also showed that immune cells lacking the CB_2R had a significantly higher adherence to immobilized ICAM-1, which would enhance their ability to egress from the intravascular space into the tissue. This finding could explain the increased numbers of adherent leukocytes observed in control $CB_2^{-/-}$ animals.

To our knowledge, no studies to date have examined the impact of CB_2R deletion specific to bladder inflammation, however, a number of studies using a range of animal

models of inflammation have shown an exacerbated response in the absence of endogenous CB_2R input. For example, in a mouse model of proliferative vitreoretinopathy, genetic CB_2R knockout resulted in worsening of ocular histopathology, characterized by an increase in inflammatory cell infiltration in the retina, and an increase in the expression of pro-inflammatory mediators (Szczesniak et al., 2016). Similarly, mice lacking CB_2R showed an exaggerated response to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, in which expression of IL-1β and tumour necrosis factor alpha (TNF-α) levels were significantly elevated, and histopathological scores showed exacerbated colon necrosis and cellular infiltration compared to the WT littermates (Engel et al., 2010). The number of neutrophils isolated from the bronchoalveolar lavage fluid of septic $CB_2^{-/-}$ mice was also shown to be significantly higher than that found in septic WT animals (Tschop et al., 2009). While histopathological evaluation of the urinary bladder in this study showed no significant difference between the two genotypes, potentially due to the acute nature of the model used, IL-1β and MIP-2 concentrations were both significantly elevated in $CB_2^{-/-}$ mice following LPS administration into the bladder compared to the WT mice. Taken together, these findings suggest that endocannabinoid signalling, specifically through the CB_2R, is capable of altering signalling pathways that regulate pro-inflammatory cytokine and chemokine production, which directly impacts intravascular leukocyte recruitment (Massena & Phillipso, 2012).

The elevated levels of IL-1β and MIP-2 in $CB_2^{-/-}$ mice observed in this study, as well as the upregulation of pro-inflammatory mediators in the aforementioned studies, supports the notion that the presence and endogenous activation of CB_2R may function to limit the inflammatory response and minimize the degree of tissue damage following

insult (Donvito et al., 2018b; Lehmann et al., 2012). This is also supported by the finding that endocannabinoid levels appear to be transiently elevated under a variety of inflammatory conditions (Vincenzo Di Marzo & Petrosino, 2007; Turcotte et al., 2015), For example, AEA levels in the colon of rats treated with TNBS are significantly elevated, with the same trend being present in biopsies of patients with ulcerative colitis (UC) (D'Argenio et al., 2006). Moreover, 2-AG and AEA are also both elevated in the synovial fluid of rheumatoid and osteo-arthritis patients (Barrie & Manolios, 2017). The upregulation of endocannabinoid levels following inflammation may help to re-establish homeostasis, and limit tissue inflammation (D'Argenio et al., 2006). Whether or not inflammation of the bladder increases the local synthesis of endocannabinoids remains unclear, as studies have reported conflicting evidence (Merriam et al., 2011; Wang et al., 2015). Using an acrolein-induced model of IC, Merriam et al. (2011) reported a significant increase in tissue concentrations of the endocannabinoid AEA, as well as in the N-acylethanolamines OEA and PEA, which have well reported anti-inflammatory effects (Orio et al., 2019; Passavanti et al., 2019). Interestingly, PEA has also been shown to downregulate FAAH expression and activity (Di Marzo et al., 2001), which may function to indirectly activate CB_1R and CB_2R by increasing the local concentration of AEA. Conversely, Wang et al. (2015) showed no change in tissue concentrations of AEA or 2-AG following cyclophosphamide induced cystitis. The discrepancy in the literature on the change in endocannabinoid levels in the bladder following inflammation should be clarified in future studies.

In this study, capillary perfusion was examined by quantifying the functional capillary density in the bladder microcirculation. The rate and extent of capillary

perfusion is a tightly regulated processes, influenced by numerous factors including upstream vascular and precapillary sphincter tone, endothelial wall integrity, leukocyte recruitment, intravascular coagulation, and local perfusion pressure (Granger & Senchenkova, 2010). This finely tuned process becomes dysregulated during acute and chronic inflammatory conditions (Granger & Rodrigues, 2016). The initial response to inflammation is characterized by increased microvascular permeability to plasma proteins and leukocyte recruitment into post capillary venules. The increased leukocyte influx contributes to microvascular barrier dysfunction, and increases the probability of capillary plugging, both of which reduce capillary blood flow (He et al., 2020). Moreover, the accumulation of slow-rolling leukocytes in the microvasculature causes steric hindrance-related malperfusion of capillaries (Granger & Senchenkova, 2010). The vasoactive molecules released from the endothelium and infiltrating immune cells, including eicosanoids (ex. PGE_2), nitric oxide (NO), and histamine (Abdulkhaleq et al., 2018), enhance local vasodilation and amplify the hyperemic response. While intravesical LPS instillation had no significant effect on FCD in WT or $CB_2^{-/-}$ animals, it produced a slight negative effect in both genotypes. The lack of significant change may be due to the short observation time, as capillary perfusion was assessed only 2 hours following LPS administration. However, the FCD measured in $CB_2^{-/-}$ mice was significantly higher than that of WT counterparts following LPS challenge. To our knowledge, few studies have investigated the impact of the CB_2R on perfusion at the capillary level. While this process is highly dynamic and influenced by multiple factors, this observation may be potentially due to an amplified hyperemic response in $CB_2^{-/-}$ animals due to an increased response to leukocyte and endothelial-derived vasoactive mediators. For example, CB_2R agonists

have been shown to downregulate eNOS expression (Huang et al., 2012), therefore, abolishing endogenous CB_2 input may result in increased local blood flow during acute phase inflammation. However, overtime, as fluid and protein escapes the intravascular space, the interstitial fluid pressure rises to a level sufficient to occlude the capillary lumen (Granger & Rodrigues, 2016). Decreased microvascular density (Rosamilia et al., 2001) and impaired bladder perfusion (Pontari et al., 1999) have been reported in bladders of IC patients, therefore, maintaining microvascular homeostasis is an important therapeutic consideration.

Von Frey aesthesiometry and behavioral observations were not performed using $CB_2^{-/-}$ mice in this study due to the observation that $CB_2^{-/-}$ animals displayed significantly heightened anxiogenic behaviors. While these behaviors may be variable by strain, behavioral alterations, such as those observed in preclinical animal models of schizophrenia, including altered locomotor behavior activity and anxiety-like behaviors, have been observed in animals in which the *Cnr2* gene has been deleted (Ortega-Alvaro et al., 2011). These behavioral abnormalities did not allow for the effective collection of behavioral data or von Frey recordings to be performed in this study. However, a number of studies have used these transgenic mice to study the role of the CB_2R in pain modulation. For example, in a partial sciatic nerve ligation model of neuropathic pain, $CB_2^{-/-}$ mice showed an increase in mechanical allodynia and neuropathic pain phenotype (Nent et al., 2019). Similarly, in a murine model of join pain induced by intra-articular injection of monosodium iodoacetate, mechanical allodynia was shown to be was significantly enhanced in $CB_2^{-/-}$ animals (La Porta et al., 2013). A number of studies have also shown that CB_2R antagonists diminish the analgesic effects of locally administered

CB_2R agonists (Donvito et al., 2018), providing further support for CB_2R-mediated peripheral antinociception.

4.2 Assessing the Pharmacological Target of Intravesical Beta-Caryophyllene.

β-Caryophyllene is one of the major sesquiterpenes found in the cannabis plant, and is also found in a wide range of the essential oils of common spices and food plants (Klauke et al., 2014). The sesquiterpene has been reported as natural selective agonist at the CB_2R, which no significant affinity for the CB_1R (Gertsch et al., 2008). BCP has been shown to possess a number of therapeutic properties, including anti-inflammatory (Gertsch et al., 2008) and local anesthetic (Ghelardini et al., 2001). In this study, $CB_2^{-/-}$ mice were treated with BCP to determine if its anti-inflammatory effects in the bladder were CB_2R-dependent.

IVM of the urinary bladder of $CB_2^{-/-}$ mice treated with 100mg/kg BCP following LPS-induced inflammation revealed no significant change in the number of adherent leukocytes compared to untreated $CB_2^{-/-}$ LPS mice. Moreover, no difference in the histopathology score was observed between the BCP treated and untreated animals. These results suggest that BCP does not inhibit inflammation in mice lacking CB_2R expression. Our IVM and bladder histology findings are consistent with those of Gertsch et al. (2008). Using the carrageenan-induced paw edema test, a well-known model of acute inflammation in which carrageenan is injected into the sub-plantar surface of the rodent paw, Gertsch et al. (2008) showed that BCP treatment was ineffective in reducing edema in $CB_2^{-/-}$ animals. Conversely, in WT animals, BCP inhibited paw edema by 70%, and was shown to be more effective than treatment with JWH133, a highly selective

CB_2R agonist (Ki = 3.4nm) (Gertsch et al., 2008). Similar findings were shown by Bento et al. (2011), who showed that pharmacological blockade of CB_2R with AM630, a selective CB_2R antagonist, abolished the protective effects of BCP against colonic tissue damage in a model of dextran sodium sulfate (DSS)- induced colitis. In their study, pre-incubation with AM630 reversed the inhibitory effect of BCP on CXCL1 (KC) and TNF-α protein levels (Bento et al., 2011).

Although BCP was suggested to act predominantly through the CB_2R, the highest dosage of BCP administered by Gertsch and colleagues (2008) were shown to induce anti-edematogenic effects in CB_2R knockout mice, suggesting that BCP may not act exclusively through this receptor. The dosages eliciting these anti-edematogenic effects (50mg/kg) were lower than both the dosages used in this study. Here, intravesical BCP treatment at a dose of 100mg/kg in $CB_2^{-/-}$ animals significantly reduced the tissue concentrations of KC, P-Selectin, and MIP-2 following LPS challenge, suggesting its anti-inflammatory effects may not be mediated solely through the CB_2R. These results are in discord with our IVM results. One possible explanation to this finding is that the magnitude of the non-CB_2R mediated anti-inflammatory effect elicited by BCP was not sufficient to cause an inhibitory effect on leukocyte adherence. BCP is a highly lipophilic molecule that can easily cross the cellular membrane, and therefore, potentially exert its pharmacological effects by acting directly on intracellular pathways (Sharma et al., 2016). BCP, in addition to a variety of cannabinoids, has been shown to interact with peroxisome proliferator-activated receptors (PPAR), specifically the PPAR-α and PPAR-ɣ isoforms (O'Sullivan, 2016; Sharma et al., 2016). These receptors are both expressed in the urothelium (Varley & Southgate, 2008), have a structurally diverse array of ligands,

and have demonstrated anti-inflammatory effects upon activation (Martin, 2010). PPAR's, similar to cannabinoids, regulate the transcription of inflammatory response genes by antagonizing the AP-1, NF-ϰB, and signal transducer and activator of transcription (STAT) pathways (Vanden Berghe et al., 2003). PPAR activation by cannabinoids may occur by either direct engagement of the nuclear receptor, or by initiating intracellular signalling cascades via cell surface cannabinoid receptors which lead to PPAR stimulation (Irrera et al., 2019).

Few studies have provided *in vivo* evidence that the anti-inflammatory effects of BCP involves crosstalk between CB_2 R and PPAR-ɣ pathways. Bento et al. (2011) showed that the beneficial anti-inflammatory effects of BCP treatment on the disease activity index of DSS-induced colitis was markedly reduced by the selective PPAR antagonist, GW9662. This finding demonstrates an important role of this nuclear transcription factor in the mechanism of the anti-inflammatory actions of BCP (Bento et al., 2011). More recent evidence has also shown that while CB_2R dependent, PPARɣ activation is also involved in BCP-evoked suppression of vascular inflammation (Youssef et al., 2019). To our knowledge, few studies have investigated the therapeutic potential of PPARɣ agonists in bladder inflammation. However, Mahal et al. (2018) showed that treatment with the PPARɣ agonist, pioglitazone, preserved uroepithelial integrity in cyclophosphamide-induced IC. BCP's ability to preserve the urothelial barrier has not been directly evaluated. This may represent a potential key therapeutic application, given the increased urothelial permeability and barrier dysfunction is reported in IC patients (Birder, 2019). While we cannot conclude from this study that PPARɣ is directly implicated in BCP's anti-inflammatory activity, future studies could incorporate use

selective PPAR antagonists to evaluate its contribution to BCP's anti-inflammatory effects.

4.3 Exploring the Efficacy of Intravesical BCP Treatment, and Comparing the Effects to the Standard Clinical Treatment, Intravesical 50% a.q. DMSO (Rimso-50 ®).

The third objective of this study aimed to evaluate the anti-inflammatory and analgesic properties of BCP treatment in the bladder, and to compare these effects to the only HC and FDA approved intravesical therapy for IC, 50% DMSO (a.q.). Numerous studies using various animal models of inflammation have shown a beneficial role of CB_2R activation, however, few have investigated the therapeutic potential of local activation of the CB_2R in the context of bladder inflammation.

Using IVM to examine the murine urinary bladder microcirculation *in vivo* during inflammation represents a novel methodological approach. Kowalewska et al., (2011) utilized IVM to visualize leukocyte-endothelial interactions and endothelial expression of inflammatory mediators in the bladder microcirculation during inflammation. Similar to our results, local LPS administration caused a significant increase in leukocyte recruitment. The authors demonstrated that this process was P-selectin and ICAM dependent. Their study revealed a low number of adherent leukocytes in saline controls, similar to the results observed from this study, however, histological evaluation showed no difference in leukocyte infiltration between saline controls having undergone IVM, and saline controls having not undergone IVM (Kowalewska et al., 2011). While IVM of the bladder has been performed, we are unaware of any studies using IVM of the bladder to evaluate the anti-inflammatory effects of CB_2R target treatment. It has been widely demonstrated that activation of the CB_2R induces an anti-inflammatory and response both

in vivo and *in vitro* (Turcotte et al., 2016). CB_2R ligands exert these effects through the suppression of pro-inflammatory cytokine production, and subsequent inhibition of immune cell activation and recruitment (Turcotte et al., 2016). Intravital microscopy allows for the leukocyte recruitment process to be visualized and quantified *in vivo,* making it a valuable tool to study the effects of ECS modulation. In this study, IVM was used to quantify leukocyte adherence in the bladder microcirculation to assess the local anti-inflammatory effects of BCP. Intravesical delivery of BCP at both tested dosages caused a significant reduction in the number of adherent leukocytes relative to vehicle-treated and untreated animals. Previous studies have used IVM to evaluate the effect of CB_2R activation on leukocyte recruitment in other organs and have shown similar results. Sardinha et al., (2014) showed that i.v treatment with HU308 (CB_2R agonist) significantly reduced the number of adherent leukocytes in V1 and V3 venules of the intestinal microcirculation following induction of endotoxemia. Likewise, Porter et al., (2019) performed IVM on the iridial microvasculature, and showed that topical treatment with three structurally diverse CB_2R-selective ligands reduced leukocyte adhesion in a model of endotoxin-induced uveitis. Together, these results support the use of IVM as a method to assess the effect of ECS-targeted treatments on leukocyte recruitment and show that CB_2R agonists decrease leukocyte adhesion in a range of tissues under inflammatory conditions.

A number of inflammatory mediators have been shown to be increased in serum, urine, and biopsy samples from patients with IC (Jiang et al., 2013; Kuo, 2014). In this study, intravesical instillation of 150µg/ml *E.coli* LPS caused a significant increase in the tissue concentrations of KC and MIP-2 (IL-8 homologues), IL-1β, IL-6, and P-selectin.

These inflammatory mediators are implicated in the direct sensitization of afferent fibers causing exaggerated nociceptive transmission, and function as leukocyte chemoattractants (Gonzalez et al., 2014b).

IL-6 has been shown to be significantly elevated in urine samples of IC patients, and positively correlates with cystitis pain scores and disease severity (Lamale et al., 2006; Lotz et al., 1994). IL-6 is a pleiotropic inflammatory cytokine that upregulates acute phase proteins, such as c-reactive protein (CRP) (Tanaka et al., 2014), and has also been shown to be increased in chronic inflammatory conditions (Gabay, 2006). IL-6 contributes to the sensitization of bladder afferent pathways (Girard et al., 2011). BCP instillation, at the 300mg/kg dosage was able to significantly reduce the tissue concentrations of IL-6 following LPS instillation, however the lower dosage failed to achieve a significant reduction. Similarly, BCP inhibited IL-6 expression in periodontal (Picciolo et al., 2020) and microglial cells (Chang et al., 2013) following LPS stimulation. Other CB_2R agonists have shown similar effects *in vivo*, reducing IL-6 levels in models of atherosclerosis (Zhao et al., 2010) and ischemic stroke (Zarruk et al., 2012). 50% DMSO instillation did not produce a significant reduction in bladder IL-6 expression, contrary to reports demonstrating anti-inflammatory effects in the bladder (Kim et al., 2011). Differences in the experimental timeline (acute vs chronic) and the method of IL-6 quantification (RT-PCR vs Luminex) may account for these observed differences.

Urinary and serum levels of IL-8 have also been shown to be elevated in patients with active IC (Jiang et al., 2013). IL-8 is a polymorphonuclear (PMN) leukocyte chemoattractant released by epithelial cells, endothelial cells, and a variety of immune

cells leukocytes in response to inflammatory stimuli (El Ayadi et al., 2018). Mice lack the gene encoding IL-8, however the murine MIP-2 and KC have been identified as the functional homologues of IL-8 (Hol et al., 2010). MIP-2 concentrations have been shown to positively correlate with neutrophil egress across the uroepithelium during inflammation of the LUT (Hang et al., 1999), while KC is known to trigger CGRP release from primary nociceptive neurons (Qin et al., 2005). In this study, BCP treatment reduced the bladder concentrations of both MIP-2 and KC. Both dosages produced significant reductions in KC levels, while only the 300mg/kg dose significantly reduced MIP-2 concentrations relative to the vehicle treated group. Bento et al., (2011) also demonstrated a significant inhibitory effect of BCP on colonic KC protein levels in a DSS-induced colitis model. Moreover, BCP supressed the production of KC and MIP-2 from macrophages isolated from the mesenteric lymph nodes stimulated with LPS (Bento et al., 2011). To our knowledge, this is the only other study demonstrating the inhibitory effect of BCP on MIP-2 and KC levels in an *in vivo* model of inflammation. Various synthetic CB_2R agonists, such as JWH015, and WIN55,212-2 have shown similar effects (reviewed by Turcotte et al., 2016). Instillation of 50% DMSO was able to significantly reduce the concentration of KC, however MIP-2 levels were not significantly reduced with DMSO treatment. DMSO's anti-inflammatory mechanism is thought to be related to the suppression of IL-8 production (DeForge et al., 1992)

Production of IL-6 and IL-8 have been shown to be increased by IL-1β, a key inflammatory mediator that has been implicated is a wide range of acute and chronic inflammatory disorders (Ren & Torres, 2009). IL-1β has an extensive, multifunctional role in pain and inflammation (reviewed by Ren & Torres, 2009). Similar to the other

mediators, IL-1β has been shown to be increased in the sera and urine of IC patients (Jiang et al., 2013). In our study, the higher dose (300 mg/kg) BCP treatment after LPS administration produced a significant reduction in IL-1β concentrations. This reduction was significantly lower than untreated animals and the 50% DMSO treatment, however not from the vehicle treatment. CB_2R activation with a wide range of ligands have shown an inhibitory effect on IL-1β levels. This has been demonstrated in the bladder by Tambaro et al. (2014), who showed that i.p. administration of JWH015 inhibited IL-1β mRNA expression following LPS-induced IC. Similarly, BCP has been shown to exert suppressive effects on IL-1β production in animal models of colitis (Bento et al., 2011), kainic-acid induced seizure (Liu et al., 2014), cisplatin-induced kidney damage (Horváth et al., 2012) as well an in LPS-stimulated human whole blood (Gertsch et al., 2008). Conversely, the heightened IL-1β levels were retained following treatment with 50% DMSO.

In this study, we also quantified the levels of two adhesion molecules, P-selectin and ICAM, in the urinary bladder. P-Selectin and ICAM play key roles in mediating inflammation through promoting adherence of leukocytes to the vascular endothelium (Granger & Rodrigues, 2016). Bladder biopsies from human IC patients have reported increased staining for ICAM and P-selectin (Green et al., 2004). Moreover, it has been shown that specific inhibition of ICAM reduces the severity of experimental cystitis (Zhang et al., 2016). In this study, LPS instillation did not cause a significant upregulation of ICAM, however, P-Selectin was significantly upregulated. Treatment with 100mg/kg BCP significantly reduced the tissue concentration on P-selectin. This effect was not significant from the 300mg/kg group. However, a considerably higher

standard deviation was observed in the 300mg/kg BCP treatment group which may account for the lack of statistical significance. While not statistically significant, trends did show a dose dependent decrease in ICAM in this study. A similar effect was found by Varga et al. (2018). In their study, the authors showed that the severity of alcohol-induced steatohepatitis was significant reduced by BCP treatment. BCP decreased the expression of VCAM, ICAM, E-Selectin and P-selectin in hepatic tissue, which corresponded with a significant decrease in hepatic neutrophil infiltration (Varga et al., 2018). Similarly, Horváth et al., (2012) showed that BCP dose-dependently reduced ICAM-1, while ameliorating cisplatin-induced kidney dysfunction, morphological damage and the renal inflammatory response. Our results, in combination with these findings, suggest that BCP may be a promising natural pharmacological agent for the management of inflammatory disorders, acting to attenuate leukocyte infiltration to the inflamed tissue by reducing endothelial adherence within the bladder microcirculation.

Our histopathology data are in accordance with the studies performed by Horváth et al., (2012) and Bento et al., (2011) showing inhibitory effects of BCP treatment on cellular infiltration into the tissue. In this study, BCP treatment at 300 mg/kg was able to significantly reduce the histopathology score relative to the untreated LPS group, however we could not detect a significant difference relative to the vehicle, or other treatment groups. This is likely attributable to the significant variation observed within treatment groups, making differences between treatment groups difficult to discern. In this study, we used a qualitative score to evaluate edema and leukocyte infiltration into the bladder due to technical limitations in quantifying the number and density of leukocyte in the tissue. Given that the majority of the inflammatory infiltrate was located

in the lamina propria and in the marginal areas of the vasculature, we were limited in our ability to detect smaller changes in infiltration without quantifying the number or density infiltrating leukocytes.

In this study, LPS instillation into the bladders of WT mice caused a significant reduction in the withdrawal threshold to mechanical stimulation of the lower abdomen with the von Frey filament. This finding is indicative of heightened nociceptive pain in LPS challenged animals. Suprapubic pain is the most characteristic symptom of IC (Driscoll & Teichman, 2001). Previous studies using von Frey aesthesiometry in animal models of IC have failed to show referred hindpaw tactile sensitivity, however animals do exhibit an increase in pelvic nociception (Lai et al., 2015). Therefore, similar to Cui et al., (2019), we applied the von Frey filament to the lower abdomen and observed a significant decrease in the withdrawal threshold following LPS instillation. Intravesical BCP treatment (100 mg/kg) reversed the LPS-induced reduction in the withdrawal threshold, while the 300 mg/kg dosage did not produce a statistically significant recovery, similar to the 50% DMSO treatment. These results are in accordance with various studies showing anti-nociceptive effects of CB_2R agonists (reviewed by Guindon & Hohmann, 2008). For example, Kuwahata et al. (2012) showed that intraplantar administration of BCP reversed mechanical allodynia in mice induced by partial sciatic nerve ligation. The local anti-allodynic effects of BCP were inhibited by systemic pre-treatment with AM630 (CB_2R antagonist), whereas pre-treatment with AM251 (CB_1R antagonist) did not alter the effect of BCP (Kuwahata et al., 2012), supporting the role of CB_2R-mediated peripheral antinociception. Moreover, BCP treatment was also effective in suppressing mechanical allodynia induced by intraplantar formalin injection (Fiorenzani et al., 2014).

While numerous studies have shown beneficial anti-inflammatory and analgesic effects of CB_2R agonists on referred inflammation and pain stemming from the injury of the limbs, fewer studies have investigated the beneficial effects on visceral pain syndromes such as IBD, prostatitis, pancreatitis, and IC. Using a diet-induced model of pancreatitis, Zhang et al., (2014) showed that the CB_2R agonist LY3038404 HCl reduced pain related behaviors, and inhibited secondary abdominal hyperalgesia induced by the Hargreaves test directed at the abdomen. Similarly, in a model of TNBS-induced colitis, Sanson et al., (2006) showed that JWH015 (CB_2R agonist) inhibited the abdominal contractile response to colorectal distension. To our knowledge, few studies have investigated the use of CB_2R agonists for the treatment of cystitis-induced pain. One study by Wang et al. (2014) showed that systemic treatment with GP1a (CB_2R agonist) inhibited referred mechanical sensitivity of the hind paw in a model of IC induced by intravesical acrolein instillation. Our results are in parallel with these findings and contribute to the growing body of evidence that supports the use of CB_2R agonists in alleviating visceral pain. More specifically, we show that a BCP, a common food additive and commercially available health product, can induce analgesia in a model of visceral pain through the activation of the CB_2R Taken together, these results support the potential use of CB_2R agonists as a possible therapeutic avenue for the management of IC bladder pain.

In the present study, BCP was administered locally into the bladder, which greatly reduces the probability that the analgesic effects were mediated through a central process. While CB_2R agonists have been widely shown to reduce the section of inflammatory mediators that directly contribute to the sensitization of primary afferent neurons (Walker

& Hohmann, 2005), is well reported that cannabinoids affect pain perception through supra-spinal, spinal, and peripheral mechanisms (Anand et al., 2009). For example, BCP has been shown to produce local anesthetic effects, as demonstrated by Ghelardini et al. (2001). BCP was able to significantly reduce, in a dose-dependent manner, the electrically evoked contractions of the rat phrenic hemidiaphragm, with the effects being comparable those of procaine (Ghelardini et al., 2001). However, the involvement of the CB_2R in BCP's local anesthetic effect was not evaluated by Ghelardini et al (2001). The exact mechanism of BCP-mediated analgesia is yet to be fully elucidated. BCP may potentially mediate antinociception through either a direct or indirect mechanism, where direct analgesic activity is exerted through the activation of the CB_2R on primary sensory neurons (Anand et al., 2008). In contrast, indirect analgesic is conferred through the inhibition of pro-inflammatory and/or algogenic substance release, or via the engagement of other systems involved in analgesia, such as the endogenous opioid system (Manzanares et al., 2006). BCP polypharmacological properties, as both an anti-inflammatory and local anesthetic agent holds therapeutic value for inflammatory pain conditions, such as IC.

In addition to evaluating pain induced by mechanical stimulation using von Frey aesthesiometry, we also used behavioral approaches to study non-stimulus evoked nocifensive behaviors. We adapted a scoring system initially developed by Boucher et al., (2000) to study behaviors associated with cystitis-induced pain in rats, however the breathing rate parameter was removed due to the challenge of quantifying this parameter in mice. Locomotory behavior was also quantified, based on the observation that voluntary activity was shown to be significantly reduced in acute cystitis (Bon et al.,

2003). While all behavioral parameters were significantly negatively affected by LPS instillation, BCP treatment produced beneficial effects on all parameters. The 300mg/kg dose significantly improved all parameters relative to vehicle treated animals, with only the motor activity score not being significantly lower than vehicle treatment, but significantly lower than the untreated group. The 100mg/kg dosage also improved the postural score and cumulative score relative to vehicle treated animals, and motor activity relative to untreated animals. These results indicate a beneficial effect of BCP on reducing the LPS-induced increase in nocifensive behaviors. Our results are in accordance with the findings of Klauke et al. (2014), who demonstrated that oral BCP treatment reduced the frequency of nocifensive responses to formalin-induced inflammatory pain (paw licking, shaking, lifting). Klauke and colleagues also showed that BCP treatment was unable to reduce the frequency of pain responses in $CB_2^{-/-}$ animals, and administration of SR144528 (CB_2R antagonist) blocked the analgesic effects conferred by BCP to WT animals. These findings were corroborated by Paula-Friere et al. (2014), who showed that oral BCP treatment dose dependently reduced pain behaviors in both acute (hot plate and formalin test) and chronic models (chronic sciatic nerve constriction) of pain. A key finding from Paula-Freire et al. (2014) was that the anti-nociceptive efficacy of BCP was drastically reduced following pre-treatment with naloxone, an opioid receptor antagonist. CB_2R agonists have been reported to stimulate peripheral release of the endogenous opioid β-endorphin, leading to the activation of mu-opioid receptors in primary afferent neurons, hence producing an enhanced analgesic effect (Ibrahim et al., 2005). In support of this finding, Katsuyama et al. (2013) showed that ineffective doses of BCP significantly enhanced morphine-induced anti-nociception.

These finding have particular relevance to IC pharmacotherapy, as opioids remain the most prescribed class of medications for IC pain management (Lusty et al., 2018), and this trend does not appear to be changing (Zillioux et al., 2020). Numerous pre-clinical and clinical studies have shown that cannabinoids work synergistically to enhance opioid analgesia, and enable reduced opioid dosing without a loss of analgesic efficacy (opioid-sparing effect) (Nielsen et al., 2017). In line with the national initiative to try to reduce opioid prescriptions in light of the opioid epidemic, cannabinoid analgesics, specifically those devoid of psychoactive side effect (ie. selective CB_2R agonists) hold therapeutic promise.

In this study, the analgesic effects of BCP treatment were compared to those conferred by intravesical instillation of 50% DMSO. DMSO (a.q.) instillation has served as one of the primary pharmacological approaches to treating IC since its approval by the FDA in 1978 (Rawls et al., 2017). Its mechanism of action is thought to be a combination of anti-inflammatory effects (inhibition of IL-8, PGE_2 production), nerve blockade, smooth muscle relaxation, and collagen inhibition, however there remains a general lack of understanding surrounding its precise mechanism (Hanno, 2012; Rawls et al., 2017; Shiga et al., 2007). A recent review of patient reported treatment effectiveness showed that only 36.95% of patients treated with DMSO reported improved symptoms, while 32.7% reported worsening of symptoms (Lusty et al., 2018). In the von Frey test, 50% DMSO treatment did not significantly improve the LPS-induced decrease in the withdrawal threshold relative to untreated animals, while BCP (100 mg/kg) treatment did significantly increase the withdrawal threshold. However, we were unable to detect a significant difference between the mean T_1 withdrawal thresholds between the two

treatment groups. Behavioral assessment revealed no significant improvement on eye position score or motor activity with DMSO treatment, however posture and the cumulative score were significantly reduced. Given these results, we cannot fully conclude that BCP treatment provided a superior analgesic effect over DMSO. This may in-part be due to the sample size of the treatment groups, which consisted of 5 animals per group. Increasing the sample size may reveal a difference in efficacy between the two treatment approaches. With respect to the mixed clinical results arising from DMSO treatment, there is currently no consensus as to the optimal dose, bladder dwell time, and number of recommended treatments, which may contribute to variability among patient response to treatment (Rawls et al., 2017).

In sum, we found that CB_2R target treatment via intravesical administration of BCP inhibited the production of inflammatory mediators in the bladder that have been shown to be involved in the sensitization of bladder afferent pathways. The results from this study, in combination with those described in the literature, suggest that suppression of visceral sensitivity associated with bladder inflammation by BCP may be a combined effect of reduced inflammation and local inhibition of afferent signalling. The observed anti-inflammatory and analgesic effects conferred by intravesical BCP treatment warrant further research on this agent as a potential future treatment option for pain and inflammation of the bladder

4.4 Monoacylglycerol Lipase Inhibition as an Alternative Approach to Enhance Local CB_2R Activation.

As an alternative approach to direct agonist administration, the fourth objective in this study aimed to enhance the local lone of endocannabinoid signalling by inhibiting

MAGL, the major enzyme responsible for terminating 2-AG signalling. ECS enzyme inhibitors have been shown to exert cannabimimetic actions by preventing the hydrolysis and oxygenation endocannabinoids (Toczek & Malinowska, 2018). Given that 2-AG, in contrast to AEA, functions as a full agonist at the CB_2R and CB_1R and is more abundant than AEA in the bladder (Wang et al., 2015), we administered JZL184 to selectively inhibit MAGL in order to increase the local 2-AG concentration. This enzyme inhibitor has been shown to cause marked increases in 2-AG accumulation in a range of different organs, without altering the levels of AEA (Long et al., 2009b).

Leukocyte recruitment in the microcirculation of the bladder following LPS challenge and subsequent JZL184 treatment administration was evaluated by IVM. JZL184 treatment administered at a dose of 16mg/kg significantly reduced the number of adherent leukocytes in the bladder relative to untreated animals. While treatment with vehicle caused no significant reduction in leukocyte adhesion, addition of JZL184 to the vehicle conferred a significant reduction. However, no significant difference was detected between the vehicle and JZL184 treatment groups. Similarly, JZL184 treatment significantly increase the FCD relative to untreated animals, but this effect was not significantly different from vehicle treatment. Vehicle-related limitations are discussed in section 4.5.1 of the discussion. Numerous studies using JZL184 and other selective MAGL inhibitors have shown inhibitory effects on leukocyte recruitment and pro-inflammatory cytokine production. For example, JZL184 administered at the same dose used in this study, significantly decreased leukocyte migration to the lungs and decreased vascular permeability in the lungs during acute lung injury (Costola-de-Souza et al., 2013). Similarly, neutrophil and macrophage infiltration into the inflamed gastrocnemius

and soleus of the right posterior rat limb was significantly reduced following treatment with JZL184 (Jiang et al., 2015). A key finding by Jiang and colleagues (2015) was that the anti-inflammatory effects were reversed by both a CB_1R and CB_2R antagonist, suggesting that both cannabinoid receptors play a role in the anti-inflammatory effects of JZL184. This finding was corroborated by Alhouayek et al., (2011) who showed that the ability of JZL184 to counteract TNBS-induced colonic inflammation was dependent on simultaneous activation of both cannabinoid receptors. Studies have positively identified a protective role of endogenous CB_1R activation in peripheral inflammatory disorders (Massa et al., 2004), therefore the CB_1R may be involved in some of the anti-inflammatory elicited by JZL184 that were observed in our study. Moreover, it has previously been shown by Rockwell et al., (2006), that 2-AG is also capable of exerting immunomodulatory effects independent of both CB_1R and CB_2R, as demonstrated by its ability to reduce IL-2 and IFNɣ in CB_1R/CB_2R double knockout leukocytes. While we did not evaluate the relative contribution of each receptor, our results are consistent with studies showing beneficial effects of JZL184 treatment under inflammatory conditions. Future studies could employ the use selective CB_1R and/or CB_2R antagonists, or a duel antagonist, to assess the relative role of different receptors in mediating the anti-inflammatory effects of JZL184 treatment.

In accordance with the reduction in leukocyte recruitment shown via IVM, JZL184 treatment reduced the levels of a number of the pro-inflammatory cytokines that were elevated following LPS instillation. While vehicle treatment did not significantly reduce any of the measured cytokines relative to the untreated group, we were only able to detect a significant difference in IL-6 concentrations between the vehicle and JZL184

groups. However, JZL184 treatment did cause a significant reduction in KC, IL-1β, and P-Selectin compared to the untreated group. Our results from our histological analysis showed similar vehicle-related effects. Regardless, from clinical perspective, the JZL184 treatment did provide an anti-inflammatory benefit, reducing the bladder concentration of a number of clinically relevant pro-inflammatory mediators. Similar to our observations, the aforementioned study by Alhouayek et al. (2011) also showed a significant reduction in IL-1β and IL-6 expression with JZL184 treatment. Similarly, Habib et al., (2019) recently demonstrated hepatoprotective and anti-inflammatory effects of MAGL inhibition in a model of chronic liver injury, where significant reductions in the expression of IL-1β, IL-6 and PGE_2 were observed in isolated Kupffer cells and peritoneal macrophages. Furthermore, intraperitoneal treatment with JZL184 attenuated the LPS-induced increase in IL-1β, IL-6, and TNF-α levels in both the CNS and periphery (Kerr et al., 2013). Interestingly, the authors suggested differing mechanisms of JZL184 in the CNS versus in the periphery based on the observation that 2-AG levels in the frontal cortex remained unchanged despite reductions in pro-inflammatory cytokines. Conversely, MAGL inhibition significantly increased the splenic 2-AG content (Kerr et al., 2013). These results demonstrate the widespread activity of MAGL inhibition, and provide further evidence suggesting that ECS modulation through enhanced 2-AG signalling may be an effective therapeutic approach to both central and peripheral inflammatory disorders.

The local administration of JZL184 into the urinary bladder produced a significant recovery of the LPS-induced reduction in the withdrawal threshold measured by electronic von Frey aesthesiometry. Similar to our IVM and Luminex results, we were

not able to detect a significant difference relative to the vehicle treatment group, however, vehicle alone did not produce a significant recovery in the withdrawal threshold. This finding suggests an enhanced analgesic effect conferred by JZL184 treatment. This finding is agreeance with numerous reports showing the analgesic benefit produced by MAGL inhibition in both acute and chronic pain states. For example, Comelli et al., (2007) showed that acute inflammatory pain induced by intraplantar injection of carrageenan was inhibited by systemic treatment with URB602 (MAGL inhibitor). URB608 treatment significantly reduced the withdrawal latency to the Hargreaves procedure, which allows for assessment of thermal nociception, 2- and 24-hours following carrageenan injection. Notably, SR144528 (CB_2R inverse agonist), but not rimonabant (CB_1R inverse agonist), prevented the analgesic action of URB608. However, studies have reported conflicting evidence as to whether or not the analgesic effects of MAGL inhibitors are dependent on CB_2R expression. For example, Ghosh et al., (2013), showed that the anti-allodynic effects of JZL184 was mediated by input from both the CB_1R and CB_2R, where JZL184 produced analgesic effects in both $CB_1^{-/-}$ and $CB_2^{-/-}$ animals. While these two aforementioned studies used different inhibitors, which may have distinct pharmacological activity aside from MAGL inhibition, Khasabova et al., (2011) also showed that JZL184 reduced mechanical hyperalgesia caused by bone cancer in a CB_2R-dependent mechanism. This dichotomy may be influenced by multiple factors, including the dosage, treatment frequency and duration, as well as the type of pain being studied. While we cannot draw mechanistic conclusions from our study, our results are in general agreement with the literature findings showing analgesic effects of MAGL inhibition. Moreover, we observed significant improvements in the postural score

of JZL184 treated mice relative to vehicle treated animals, in addition to improvements in eye opening and the cumulative score relative to untreated animals. Improved postural position and opening of the eyes are consistent with MAGL inhibition producing anti-nociceptive behaviors. Similarly, Griebel et al., (2015) showed that writhing activity, induced by intraperitoneal injection of phenylbenoquinone, was reduced in a dose-dependent manner by SAR127303. Taken together, these findings support a positive role of MAGL inhibition in alleviating visceral pain.

One of the major limitations to managing pain through the manipulation of 2-AG via the inhibition of MAGL has been the observation that JZL184 produces behavioral effects consistent with cannabimimetics (Long et a l., 2009). In line with these observations, JZL184 has been shown to stimulate hypomotility and hypothermia at higher doses (Toczek & Malinowska, 2018) and induce physical dependence (Schlosburg et al., 2010b). It has also been shown that increased systemic 2-AG enhances the response to painful stimuli due to CB_1R desensitization, resulting in a loss of CB_1 analgesic input (Bjorling & Wang, 2018; Schlosburg et al., 2010b). However, the lower concentrations of 2-AG in the periphery, compared to the CNS, opens the possibility of achieving local increases in 2-AG through tissue-specific regulation of MAGL function without causing CB_1 desensitization (Bjorling & Wang, 2018). Importantly it has also been shown that the anti-inflammatory and anti-nociceptive effects of low dose, but not high dose JZL184 treatment, were retained when JZL184 was administered repeatedly (Ghosh et al., 2013). Given the chronicity of IC, the ability to administer this potential therapeutic agent directly into the bladder via the transurethral route, while avoiding potential drug

tolerance, makes this a highly attractive therapeutic option for bladder pain management worthy of further investigation.

In addition to the well described role of MAGL in terminating 2-AG signalling, MAGL is also involved in the regulation of free arachidonic acid levels, a substrate for COX-2-mediated prostaglandin synthesis (Mulvihill & Nomura, 2013). 2-AG can be metabolized by COX-2 leading to the production of pro-inflammatory prostaglandins (Di Marzo, 2008), which could mitigate the ability of 2-AG to reduce inflammation and associated pain. Therefore, the combined use of MAGL and COX-2 inhibitors has garnered attention. Using IVM, Philpott & McDougall, (2020) showed that the combined effect of KLM29 (MAGL inhibitor) and Celecoxib (COX-2 inhibitor) was greater than the individual treatment effects for reducing leukocyte recruitment in the microcirculation of the knee joint in a rat model of osteoarthritis. Crowe et al., (2015) showed similar effects, whereby JZL184, in combination with diclofenac, attenuated machinal allodynia in a model of chronic neuropathic pain. This beneficial effect was also shown to be in part mediated by the CB_1R.

To date, and to the best of our knowledge, no studies have investigated the use of MAGL inhibitors to alleviate bladder inflammation and accompanying visceral pain. This is the first study to demonstrate that intravesical manipulation of the elements of the endocannabinoid system may have the ability to supress inflammation and pain of the LUT. Future experiments could investigate the combined approach of COX-2 and MAGL inhibition, which has proven efficacious in reducing inflammation in other organs.

4.5 Experimental Model of IC

The etiology of IC remains idiopathic and may involve multiple causes, which complicates the use of animal models to investigate the pathogenesis of IC (Bjorling et al., 2011). Despite this, a number of animal models have been developed to better understand the mechanisms underlying the symptoms associated with IC and inflammation of the LUT. The majority of these models seek to recreate bladder-centric features by administering a noxious substance or chemical irritant via an intravesical instillation (Birder & Andersson, 2018). Of these irritants, *E. coli* lipopolysaccharide, used in this study, is one of the most commonly used exogenous irritants instilled into the bladder to initiate cystitis (Bjorling et al., 2011). Uroepithelial cells lining the urinary bladder mucosa recognize the presence of intravesical LPS predominantly through the toll-like receptor (TLR)-4/cluster of differentiation (CD)14/myeloid differentiation factor-2 (MD-2) receptor complex (Backhed et al., 2002; Schilling et al., 2003). LPS recognition by this receptor complex leads to activation of NF-ϰB, followed by the rapid production of numerous inflammatory mediators, including IL-6, IL-8, IL-1β, and NGF (D. E. Bjorling et al., 2001; Schilling et al., 2003; J. Song et al., 2007). These pro-inflammatory mediators enhance peripheral immune signalling, and cause exaggerated nociceptive transmission (Cui et al., 2019; Nicotra et al., 2012; J. Song et al., 2007).

Numerous animal models of pain have shown that inflammatory signalling secondary to TLR-4 activation plays a critical role in the development of hyperalgesia and allodynia (Schrepf et al., 2014). Pain associated with cystitis is thought to be the summation of the nociceptive effects of the noxious stimuli arising from the cellular release of pro-inflammatory cytokines and other algogenic mediators (Bjorling et al.,

2001). In this study, von Frey aesthesiometry experiments showed that mice treated with LPS had significantly reduced withdrawal thresholds 3 hours post instillation, indicative of heightened pelvic sensitivity due to visceral bladder pain. The multi-disciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network recommends that animals models of IC should recapitulate pelvic nociception in correspondence with alterations in biochemical and/or histopathological changes (Lai et al., 2013). The LPS model used in this study adheres to these recommendations, as shown by the increased visceral sensitivity to mechanical stimuli, the elevated concentrations of numerous pro-inflammatory mediators, and the increased number of leukocytes infiltrating the bladder.

4.5.1 Limitations and Future Considerations

While this model recapitulates certain pathological aspects of IC, there remains a number of inherent limitations to our study, which relate to both the use of our model as well as our experimental design. The precise etiology of IC remains unknown, making it challenging to model the associated pathophysiological elements and symptomatology. As mentioned, experimental models of IC has been induced via the intravesical administration of several chemical irritants, including LPS, turpentine, mustard oil, acrolein, and cyclophosphamide (Bjorling et al., 2011). A significant level of variability exists among studies in terms of the concentration of the irritant, the volume instilled into the bladder, and the exposure time (Birder & Andersson, 2018). Each of these parameters can greatly affect the nature of the interaction between the urothelium and the irritant, producing variable results. In addition to irritant-induced cystitis, neurogenic and autoimmune approaches have been attempted to induce IC symptoms, with variable results (Birder & Andersson, 2018). Although more than 20 animal models of IC have

been used, there remains no standard animal model for IC (Song et al., 2017). The consensus on an extensive review of the animal models used for IC research was that none of the currently available models perfectly mimics clinical IC (Bjorling et al., 2011). While these models may be beneficial to our understanding of the mechanisms of bladder pain and inflammation, they are limited to demonstrating how the bladder responds to a noxious, often exogenous stimuli (Birder & Andersson, 2018). Further disadvantages of these models relate to uncertainty of whether or not the mechanisms that underlie the response of the bladder and nervous system to these compounds are relevant to those that result in inflammation and associated pain in patients with IC. Interestingly, feline IC is a naturally occurring idiopathic condition affecting domestic cats which causes pathophysiological changes that are very similar to those observed in human non-hunners IC (Kullmann et al., 2018). While obvious barriers exist in terms of accessibility, findings using this model may be the most translatable to humans.

One of the major limitations to the conclusions drawn from our findings showing anti-inflammatory and analgesic effects of BCP and JZL184 treatment is the inability to separate some of the treatment effects from the effects exerted by the drug vehicles. For all BCP instillations, BCP was dissolved in highly refined olive oil, whereas JZL184 was administered in a solution consisting of one-part DMSO, one-part Cremophor, and 18-parts sterile saline. Olive oil has previously been used as vehicle for intravesical delivery of Bacillus Calmette-Guerin (BCG) on a study investigating bladder carcinoma (Noguera-Ortega et al., 2016) and is widely used for gavage treatments with BCP (Gertsch et al., 2008; Klauke et al., 2014). We are unaware of any studies showing beneficial effects of olive oil on bladder inflammation, however, intravesical olive oil

instillation has been shown not to provide analgesia in response to bladder distension-induced visceral nociception (Ness & Elhefni, 2004). Some of the phenolic compounds in olive oil such as tyrosol, hydroxytyrosol, and oleuropein may possess anti-oxidant and anti-inflammatory properties (Antunes & de Lourdes Pires Bianchi, 2010; Sun et al., 2017). While we utilized highly refined olive oil, we cannot exclude the possibility that some of these compounds contributed mild anti-inflammatory effects to the vehicle. Intravesical delivery of empty liposomes have recently been shown to counteract mucosal inflammation of the urothelium (Tyagi et al., 2017), and may be a drug delivery system for intravesical BCP instillation to investigate in future studies.

The 1:1:18 vehicle used to deliver JZL184 confounded some of the results obtained from the JZL184 treatment group. Studies have reported various ways of solubilizing JZL184, however the constituents of some of the vehicles and organic solvents used are incompatible with delivery into the bladder, such as ethanol (Kinsey et al., 2013) and dimethylformamide. Studies have used varying ratios of saline, DMSO, polyethylene glycol, Tween 80, and emulphor (Josée Guindon et al., 2011; J. Z. Long, Li, et al., 2009; Marino et al., 2020), while Philpott & McDougall, (2020) used the same vehicle composition ratio used in this study. The benefit in using trace amounts of DMSO derives from the fact that DMSO is a permeability enhancer and can improve cellular uptake of the locally instilled drug. DMSO is able to penetrate the bladder urothelium without causing tissue damage, and has been shown to enhance the transport of various lipophilic chemotherapeutic drugs (ex. cisplatin, pirarubicin) into bladder tumors (Giannantoni et al., 2006). However, as mentioned, DMSO alone is a treatment for IC, and can therefore exert beneficial anti-inflammatory effects on its own, as was observed.

In order to minimize the amount of DMSO used to solubilize JZL184, we combined it with cremophor, a formulation vehicle used to enhance the solubility of poorly water soluble drugs (Gelderblom et al., 2001). However, cremophor can form micelles in aqueous solution (Kessel, 1992). The combination of cremophor and DMSO has been shown to be effective in enhancing drug uptake in the bladder. For example, the commercial formulation of paclitaxel (Taxol ®), a drug used to treat bladder carcinoma, contains cremophor which can entrap the drug, reducing its free fraction. Addition of DMSO significantly reversed the entrapment and increased the bladder tissue delivery of paclitaxel (Chen et al., 2003). Despite containing a relatively small portion of DMSO, it is possible that the DMSO in our vehicle exerted sufficient anti-inflammatory activity to confound the observed results. Furthermore, while we are unaware of any studies investigating therapeutic benefit of cremophor in the bladder, one study did show antinociceptive effects when administered orally, but no mechanistic insight was provided by the authors (Tabarelli et al., 2003). Moving forward with this treatment approach, compatibility and pharmacokinetic/dynamic consideration of the drug vehicle should be made with the goal of optimizing JZL184 delivery. Moreover, the use of novel intravesical drug delivery strategies, such as mucoadhesive carriers and polymeric gels could be investigated in future studies (Guhasarkar & Banerjee, 2010).

Another limitation of this study is that all of the experiments were performed using an acute model of bladder pain and inflammation. LPS instillation did cause inflammation of the bladder and bladder pain, effectively recapitulating cardinal IC symptoms, and adhering to MAPP Network recommendations. However, the acute nature of this model is not representative of clinical time course. Cystitis, by definition, requires

symptoms to be present for at least 6 months. The clinical time course, and resolution of any form of acute inflammation differs from that seen in most IC patients, in whom inflammation does not resolve (Birder & Andersson, 2018). Furthermore, acute models of cystitis do not allow for major structural changes in the bladder to occur, such a fibrosis. In the bladder, fibrosis results in decreased bladder compliance and capacity, causing voiding dysfunction (Fry et al., 2018), which is common among cystitis patients (Grover et al., 2011). Future studies should adopt a more chronic model in order to better mirror the clinical course of IC. Moreover, future research should also attempt to incorporate the use of cytometric techniques in order to assess the effect of the studied treatments on bladder function, given that IC often causes bladder dysfunction.

Moreover, a limitation affecting the external validity of this study's findings is that only female mice were used. While the epidemiological data shows that IC is significantly more prevalent among females, males are also impacted by IC (Arora & Shoskes, 2015). In general, male mice are rarely used in research on the LUT (Lamanna et al., 2020). One of the major obstacles to using male mice for bladder research involving intravesical instillations is the difficulty associated with catheterization of the male urethra. In comparison, visualization and catheterization of female urethra is easy, with significantly less risk of inducing urethral trauma. New methods of transurethral catheterization for male rodents have been designed to attempt to increase the use of male mice in urological research (Lamanna et al., 2020). Employing these methods in future research will help increase the use of male subjects in animal research on LUT pathologies.

Another important consideration for the limitations of this study is the fact that fluorescent dyes were administered to enhance the contrast of white blood cells and to visualize the blood flow. Rhodamine 6G accumulates in the mitochondria of cells, and while highly effective in staining granulocytes and monocytes, it is less effective in staining cell of the lymphoid lineage (Baatz et al., 1995). It has been shown that rhodamine 6G staining maintains a high level of fluorescent intensity in granulocytes and monocytes, however, 15-30 minutes after administration the relative fluorescence intensity of lymphocytes decreases considerably (Baatz et al., 1995). While this timeframe is well within our observation window, it is possible that some infiltrating immune cells were stained inadequately to be effectively visualized and were therefore not counted.

Finally, another limitation in this study is the possibility of experiment bias. While a second party observer was blinded for all IVM video analysis, the same individual who performed the von Frey, behavior, and histology experiments was responsible for data collection. Therefore, the experimenter was aware which experimental group was being performed. However, to minimize experimenter bias, every measured parameter was an average of multiple measurements. Furthermore, to minimize inter-observer variability, only one observer was responsible for data collection for each method. Nevertheless, performing a fully blinded study would eliminate the influence of experimenter bias.

4.6 Conclusion

In this study, we investigated the role of the CB_2R in experimental cystitis, along with the potential anti-inflammatory and analgesic effects associated with its

pharmacological activation. First, we showed that global deletion of the CB_2R conferred an exacerbated inflammatory phenotype, whereby CB_2R deficient animals showed a higher degree of leukocyte adhesion in the bladder microcirculation, and increased levels of two pro-inflammatory mediators. BCP, a phyto-derived CB_2R agonist was then administered to $CB_2^{-/-}$ mice to evaluate its pharmacological target in the bladder. Cytokine analysis revealed a potential off-target effect of BCP, which may mediate part of its anti-inflammatory effects. This was based on observation that inflammatory mediators in the bladder were downregulated following BCP treatment in $CB_2^{-/-}$ mice. In WT animals, intravesical BCP treatment elicited a strong anti-inflammatory response following LPS stimulation, producing a marked decrease in leukocyte adhesion and reducing pro-inflammatory cytokine production. In comparison to 50% DMSO, the standard clinical therapy for IC, BCP showed stronger anti-inflammatory effects. BCP treatment (100mg/kg) was also able to restore the LPS-induced pelvic sensitivity to mechanical stimulation, suggesting a local analgesic effect. Finally, we showed that indirect activation of the CB_2R through the inhibition of 2-AG hydrolysis elicits similar anti-inflammatory and analgesic effects. However, partial vehicle-related effects place limitation these finding. In sum, this study shows that activation of the CB_2R pathway in the urinary bladder produces anti-inflammatory and analgesic effects, and may represent a viable target for future IC pharmacotherapy

References

Abdulkhaleq, L. A., Assi, M. A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y. H., & Hezmee, M. N. M. (2018). The crucial roles of inflammatory mediators in inflammation: A review. *Veterinary world*, *11*(5), 627–635.

Abel, E. L. (1970). Marijuana and Memory. *Nature*, *227*(5263), 1151–1152.

Adams, I. B., & Martin, B. R. (1996). Cannabis: pharmacology and toxicology in animals and humans. *Addiction (Abingdon, England)*, *91*(11), 1585–614.

Ahn, K., Johnson, D. S., & Cravatt, B. F. (2009). Fatty acid amide hydrolase as a potential therapeutic target for the treatment of pain and CNS disorders. *Expert Opinion on Drug Discovery*. NIH Public Access.

Aihara, K., Hirayama, A., Tanaka, N., Fujimoto, K., Yoshida, K., & Hirao, Y. (2009). Hydrodistension under local anesthesia for patients with suspected painful bladder syndrome/interstitial cystitis: safety, diagnostic potential and therapeutic efficacy. *International Journal of Urology*, *16*(12), 947–952.

Alhouayek, M., Lambert, D. M., Delzenne, N. M., Cani, P. D., & Muccioli, G. G. (2011). Increasing endogenous 2-arachidonoylglycerol levels counteracts colitis and related systemic inflammation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *25*(8), 2711–21.

Anand, P., Whiteside, G., Fowler, C. J., & Hohmann, A. G. (2009). Targeting CB2 receptors and the endocannabinoid system for the treatment of pain. *Brain research reviews*, *60*(1), 255–66.

Anand, U., Otto, W. R., Sanchez-Herrera, D., Facer, P., Yiangos, Y., Korchev, Y., Rolfe, B., Benham, C., Bountra, C., Chessell, I. P., & Anand, P. (2008). Cannabinoind receptor CB2 localisation and agonist-mediated inhibition of capsaicin responses in human sensory neurons. *Pain*, *138*(3), 667–680.

Andersen, A., Granlund, P., Schultz, A., Talseth, T., Hedlund, H., & Frich, L. (2012). Long-term experience with surgical treatment of selected patients with bladder pain syndrome/interstitial cystitis. *Scandinavian Journal of Urology and Nephrology*, *46*(4), 284–289.

Antunes, L. M. G., & de Lourdes Pires Bianchi, M. (2010). In vivo Cytogenetic Effects of Multiple Doses of Dietary Vegetable Oils: Position of Olive Oils. In *Olives and Olive Oil in Health and Disease Prevention* (pp. 1071–1077). Elsevier Inc.

Arora, H. C., & Shoskes, D. A. (2015). The enigma of men with interstitial cystitis/bladder pain syndrome. *Translational andrology and urology*, *4*(6), 668–76.

Ashton, J. C., & Glass, M. (2007). The cannabinoid CB2 receptor as a target for inflammation-dependent neurodegeneration. *Current neuropharmacology*, *5*(2), 73–80.

Askari, V. R., & Shafiee-Nick, R. (2019). Promising neuroprotective effects of β-caryophyllene against LPS-induced oligodendrocyte toxicity: A mechanistic study. *Biochemical pharmacology*, *159*, 154–171.

Atwood, B. K., & Mackie, K. (2010). CB2: a cannabinoid receptor with an identity crisis. *British Journal of Pharmacology*, *160*(3), 467.

Atwood, B. K., Wager-Miller, J., Haskins, C., Straiker, A., & Mackie, K. (2012). Functional Selectivity in CB 2 Cannabinoid Receptor Signaling and Regulation: Implications for the Therapeutic Potential of CB 2 Ligands. *Molecular pharmacology*, *81*, 250–263.

Baatz, H., Steinbauer, M., Harris, A. G., & Krombach, F. (1995). Kinetics of white blood cell staining by intravascular administration of rhodamine 6G. *International journal of microcirculation, clinical and experimental*, *15*(2), 85–91.

Backhed, F., Meijer, L., Normark, S., & Richter-Dahlfors, A. (2002). TLR4-dependent recognition of lipopolysaccharide by epithelial cells requires sCD14. *Cellular Microbiology*, *4*(8), 493–501.

Badowski, M., & Yanful, P. (2018). Dronabinol oral solution in the management of anorexia and weight loss in AIDS and cancer. *Therapeutics and clinical risk management*, *14*, 643–651.

Baggelaar, M. P., Maccarrone, M., & van der Stelt, M. (2018). 2-Arachidonoylglycerol: A signaling lipid with manifold actions in the brain. *Progress in Lipid Research*, *71*, 1–17.

Barrie, N., & Manolios, N. (2017). The endocannabinoid system in pain and inflammation: Its relevance to rheumatic disease. *European journal of rheumatology*, *4*(3), 210–218.

Basu, S., & Dittel, B. N. (2011). Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. *Immunologic research*, *2*

Battista, N., Di Tommaso, M., Bari, M., & Maccarrone, M. (2012). The endocannabinoid system: an overview. *Frontiers in behavioral neuroscience*, *6*, 9.

Bento, A. F., Marcon, R., Dutra, R. C., Claudino, R. F., Cola, M., Leite, D. F. P., & Calixto, J. B. (2011). β-caryophyllene inhibits dextran sulfate sodium-induced colitis in mice through CB2 receptor activation and PPARγ pathway. *American Journal of Pathology*, *178*(3), 1153–1166.

Berdyshev, E. V. (2000). Cannabinoid receptors and the regulation of immune response. *Chemistry and physics of lipids*, *108*(1–2), 169–90.

Berry, S. H., Bogart, L. M., Pham, C., Liu, K., Nyberg, L., Stoto, M., Suttorp, M., & Clemens, J. Q. (2010). Development, validation and testing of an epidemiological case definition of interstitial cystitis/painful bladder syndrome. *The Journal of urology*, *183*(5), 1848–52.

Berry, S. H., Elliott, M. N., Suttorp, M., Bogart, L. M., Stoto, M. A., Eggers, P., Nyberg, L., & Clemens, J. Q. (2011). Prevalence of symptoms of bladder pain syndrome/interstitial cystitis among adult females in the United States. *The Journal of urology*, *186*(2), 540–4.

Birder, L., & Andersson, K.-E. (2013). Urothelial Signaling. *Physiological Reviews*, *93*(2), 653.

Birder, L., & Andersson, K.-E. (2018). Animal Modelling of Interstitial Cystitis/Bladder Pain Syndrome. *International neurourology journal*, *22*(Suppl 1), S3-9.

Birder, L A, Ruggieri, M., Takeda, M., van Koeveringe, G., Veltkamp, S., Korstanje, C., Parsons, B., & Fry, C. H. (2012). How does the urothelium affect bladder function in health and disease? ICI-RS 2011. *Neurourology and urodynamics*, *31*(3), 293–9.

Birder, Lori A. (2019). Pathophysiology of interstitial cystitis. *International Journal of Urology*, *26*(S1), 12–15.

Birder, Lori A, & Kullmann, F. A. (2018). Role of neurogenic inflammation in local communication in the visceral mucosa. *Seminars in immunopathology*, *40*(3), 261–279.

Bisogno, T., Melck, D., De Petrocellis, L., & Di Marzo, V. (1999). Phosphatidic acid as the biosynthetic precursor of the endocannabinoid 2-arachidonoylglycerol in intact mouse neuroblastoma cells stimulated with ionomycin. *Journal of neurochemistry*, *72*(5), 2113–9.

Bjorling, D. E., Jacobsen, H. E., Blum, J. R., Shih, A., Beckman, M., Wang, Z. Y., & Uehling, D. T. (2001). Intravesical Escherichia coli lipopolysaccharide stimulates an increase in bladder nerve growth factor. *BJU International*, *87*(7), 697–702.

Bjorling, D., Wang, Z.-Y., & Bushman, W. (2011). Models of Inflammation of the Lower Urinary Tract. *Neurourology and Urodynamics*, *30*(5), 673–682.

Bjorling, Dale E., Jerde, T. J., Zine, M. J., Busser, B. W., Saban, M. R., & Saban, R. (1999). Mast cells mediate the severity of experimental cystitis in mice. *Journal of Urology*, *162*(1), 231–236.

Bjorling, Dale E, & Wang, Z.-Y. (2018). Potential of Endocannabinoids to Control Bladder Pain. *Frontiers in systems neuroscience*, *12*, 17.

Blankman, J. L., Simon, G. M., & Cravatt, B. F. (2007). A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chemistry & biology*, *14*(12), 1347–56.

Bogart, L. M., Berry, S. H., & Clemens, J. Q. (2007). Symptoms of Interstitial Cystitis, Painful Bladder Syndrome and Similar Diseases in Women: A Systematic Review. *Journal of Urology*.

Bon, K., Lichtensteiger, C. A., Wilson, S. G., & Mogil, J. S. (2003). Characterization of cyclophosphamide cystitis, a model of visceral and referred pain, in the mouse: Species and strain differences. *Journal of Urology*, *170*(3), 1008–1012.

Boucher, M., Meen, M., Codrom, J.-P., Coudore, F., Kemeny, J.-L., & Eschalier, A. (2000). Cyclophosphamide-incuced cystitis in freely-moving conscious rats: behavioral approach to a new model of visceral pain. *Journal of Urology*, *164*(1), 203–208.

Bridgeman, M. B., & Abazia, D. T. (2017). Medicinal Cannabis: History, Pharmacology, And Implications for the Acute Care Setting. *P&T: a peer-reviewed journal for formulary management*, *42*(3), 180–188.

Brookoff, D., & Bennett, D. (2006). Neuromodulation in Intractable Interstitial Cystitis and Related Pelvic Pain Syndromes. *Pain Medicine*, *7*(1), S166–S184.

Carlisle, S. J., Marciano-Cabral, F., Staab, A., Ludwick, C., & Cabral, G. A. (2002). Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *International immunopharmacology*, *2*(1), 69–82.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., & Julius, D. (1997). The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature*, *389*(6653), 816–824.

Chakravarti, B., Ravi, J., & Ganju, R. K. (2014). Cannabinoids as therapeutic agents in cancer: current status and future implications. *Oncotarget*, *5*(15), 5852–72.

Chang, H. J., Kim, J. M., Lee, J. C., Kim, W. K., & Chun, H. S. (2013). Protective effect of β-caryophyllene, a natural bicyclic sesquiterpene, against cerebral ischemic injury. *Journal of Medicinal Food*, *16*(6), 471–480.

Chen, D., Song, D., Guillaume Wientjes, M., & Au, J. (2003). Effect of dimethyl sulfoxide on bladder tissue penetration of intravesical paclitaxel. *Clinical cancer research*, *9*(1), 363–369.

Chevaleyre, V., Takahashi, K. A., & Castillo, P. E. (2006). Endocannabinoid-mediated synaptic plasticity in the CBS. *Annual Review of Neuroscience*, *29*(1), 37–76.

Chiu, I. M., von Hehn, C. A., & Woolf, C. J. (2012). Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. *Nature neuroscience*, *15*(8), 1063–7.

Clapper, J. R., Moreno-Sanz, G., Russo, R., Guijarro, A., Vacondio, F., Duranti, A., Tontini, A., Sanchini, S., Sciolino, N. R., Spradley, J. M., Hohmann, A. G., Calignano, A., Mor, M., Tarzia, G., & Piomelli, D. (2010). Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nature Neuroscience*, *13*(10), 1265–1270.

Clarke, R. C., & Watson, D. P. (2007). Cannabis and Natural Cannabis Medicines. In *Marijuana and the Cannabinoids* (pp. 1–15). Totowa, NJ: Humana Press.

Close, C. E., Carr, M. C., Burns, M. W., Miller, J. L., Bavendam, T. G., Mayo, M. E., & Mitchell, M. E. (1996). Interstitial cystitis in children. *The Journal of urology*, *156*(2 Pt 2), 860–2.

Colaco, M., & Evans, R. (2015). Current guidelines in the management of interstitial cystitis. *Translational andrology and urology*, *4*(6), 677–683.

Cole, E., Scarpero, H., & Dmochowski, R. R. (2005). Are patient symptoms predictive of the diagnostic and/or therapeutic value of hydrodistention? *Neurourology and urodynamics*, *24*(7), 638–642.

Comelli, F., Giagnoni, G., Bettoni, I., Colleoni, M., & Costa, B. (2007). The inhibition of monoacylglycerol lipase by URB602 showed an anti-inflammatory and anti-nociceptive effect in a murine model of acute inflammation. *British journal of pharmacology*, *152*(5), 787–94.

Console-Bram, L., Marcu, J., & Abood, M. E. (2012). Cannabinoid receptors: nomenclature and pharmacological principles. *Progress in neuro-psychopharmacology & biological psychiatry*, *38*(1), 4–15.

Correia-Sá, I. B., Carvalho, C. M., Serrão, P. V., Loureiro, A. I., Fernandes-Lopes, C., Marques, M., & Vieira-Coelho, M. A. (2020). A new role for anandamide: defective link between the systemic and skin endocannabinoid systems in hypertrophic human wound healing. *Scientific Reports*, *10*(1), 11134.

Costola-de-Souza, C., Ribeiro, A., Ferraz-de-Paula, V., Calefi, A. S., Aloia, T. P. A., Gimenes-Júnior, J. A., de Almeida, V. I., Pinheiro, M. L., & Palermo-Neto, J. (2013). Monoacylglycerol Lipase (MAGL) Inhibition Attenuates Acute Lung Injury in Mice. (M. Idzko, Ed.) *PLoS ONE*, *8*(10), e77706.

Cox-Georgian, D., Ramadoss, N., Dona, C., & Basu, C. (2019). Therapeutic and Medicinal Uses of Terpenes. In *Medicinal Plants* (pp. 333–359). Cham: Springer International Publishing.

Cox, A., & Golda, N. (2016). CUA Guideline: Diagnosis and treatment of interstitial cystitis. *Canadian Urological Association Journal*, *10*, E36–E55.

Crescenze, I., Tucky, B., Li, J., Moore, C., & Shoskes, D. (2017). Efficacy, Side Effects, and Monitoring of Oral Cyclosporine in Interstitial Cystitis-Bladder Pain Syndrome. *Urology*, *107*, 49–54.

Crowe, M. S., Leishman, E., Banks, M. L., Gujjar, R., Mahadevan, A., Bradshaw, H. B., & Kinsey, S. G. (2015). Combined inhibition of monoacylglycerol lipase and cyclooxygenases synergistically reduces neuropathic pain in mice. *British journal of pharmacology*, *172*(7), 1700–12.

Cui, X., Jing, X., Lutgendorf, S. K., Bradley, C. S., Schrepf, A., Erickson, B. A., Magnotta, V. A., Ness, T. J., Kreder, K. J., O'Donnell, M. A., & Luo, Y. (2019). Cystitis-induced bladder pain is Toll-like receptor 4 dependent in a transgenic autoimmune cystitis murine model: a MAPP Research Network animal study. *American Journal of Physiology-Renal Physiology*, *317*(1), F90–F98.

Cummings, R. D., & McEver, R. P. (2009). *C-type Lectins*. *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press.

D'Argenio, G., Valenti, M., Scaglione, G., Cosenza, V., Sorrentini, I., & Di Marzo, V. (2006). Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. *The FASEB Journal*, *20*(3), 568–570.

da Silva Oliveira, G.-L., Conceicao Machado, K., Conceicao, Machado, K., dos Santos, A.-P., Feitosa, C.-M., & de Castro Almeida, F.-R. (2018). Non-clinical toxicity of β-caryophyllene, a dietary cannabinoid: Absence of adverse effects in female Swiss mice. *Regulatory Toxicology and Pharmacology*, *92*, 338–346.

Dasgupta, P., Sharma, S. D., Womak, C., Blackfod, H., & Dennis, P. (2001). Cimetidine in painful bladder syndrome: a histopathological study. *British journal of urology*, *88*(3), 183–186.

Davis, N. F., Gnanappiragasam, S., & Thornhill, J. A. (2015). Interstitial cystitis/painful bladder syndrome: the influence of modern diagnostic criteria on epidemiology and on Internet search activity by the public. *Translational andrology and urology*, *4*(5), 506–11.

DeForge, L. E., Fantone, J. C., Kenney, J. S., & Remick, D. G. (1992). Oxygen radical scavengers selectively inhibit interleukin 8 production in human whole blood. *The Journal of clinical investigation*, *90*(5), 2123–9.

Dhopeshwarkar, A., & Mackie, K. (2014). CB2 Cannabinoid receptors as a therapeutic target-what does the future hold? *Molecular pharmacology*, *86*(4), 430–7.

Di Marzo, V. (2008). Endocannabinoids: synthesis and degradation. *Reviews of physiology, biochemistry and pharmacology*, *160*, 1–24.

Di Marzo, V, Melck, D., Orlando, P., Bisogno, T., Zagoory, O., Bifulco, M., Vogel, Z., & De Petrocellis, L. (2001). Palmitoylethanolamide inhibits the expression of fatty acid amide hydrolase and enhances the anti-proliferative effect of anandamide in human breast cancer cells. *The Biochemical journal*, *358*(Pt 1), 249–55.

Di Marzo, Vincenzo, & Petrosino, S. (2007). Endocannabinoids and the regulation of their levels in health and disease. *Current Opinion in Lipidology*, *18*(2), 129–140.

Donvito, G., Nass, S. R., Wilkerson, J. L., Curry, Z. A., Schurman, L. D., Kinsey, S. G., & Lichtman, A. H. (2018a). The Endogenous Cannabinoid System: A Budding Source of Targets for Treating Inflammatory and Neuropathic Pain. *Neuropsychopharmacology*, *43*(1), 52–79.

Donvito, G., Nass, S. R., Wilkerson, J. L., Curry, Z. A., Schurman, L. D., Kinsey, S. G., & Lichtman, A. H. (2018b). The Endogenous Cannabinoid System: A Budding Source of Targets for Treating Inflammatory and Neuropathic Pain. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *43*(1), 52–79.

Driscoll, A., & Teichman, J. M. H. (2001). How do patients with interstitial cystitis present? *Urology*, *166*(6), 2118–20.

El Ayadi, A., Herndon, D. N., & Finnerty, C. C. (2018). Biomarkers in burn patient care. In *Total Burn Care: Fifth Edition* (pp. 232-235.e2). Elsevier Inc.

Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M. E., & Lobb, R. R. (1990). VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell*, *60*(4), 577–84.

Elikkottil, J., Elikottil, J., Gupta, P., & Gupta, K. (2009). The analgesic potential of cannabinoids. *Journal of opioid management*, *5*(6), 341–57.

Ellis, C. G., Jagger, J., & Sharpe, M. (2005, August). The microcirculation as a functional system. *Critical Care*. BioMed Central.

ElSohly, M. A., & Gul, W. (2014). Constituents of Cannabis Sativa. In Roger G Pertwee (Ed.), *Handbook of Cannabis* (pp. 3–22). Oxford University Press.

Engel, M., Kellerman, C., Burnat, G., Hahn, E., & Rau, T. (2010). Mice lacking cannabinoid CB1-, CB2-receptors or both receptors show increased susceptibility to trinitrobenzene sulfonic acid (TNBS)-induced colitis. *The journal of physiology and pharmacology*, *61*(1), 89–97.

Erickson, D., Kunselman, A., Bentley, C., Peters, K., Rovner, E. S., Demers, L., Wheeler, M., & Keay, S. (2007). Changes in urine markers and symptoms after bladder distention for interstitial cystitis. *Journal of Urology*, *177*(2), 556–560.

Erickson, D. R., Belchis, D. A., & Dabbs, D. J. (1997). Inflammatory cell types and clinical features of interstitial cystitis. *The Journal of urology*, *158* (1), 790–3.

Fezza, F., Bari, M., Florio, R., Talamonti, E., Feole, M., & Maccarrone, M. (2014). Endocannabinoids, related compounds and their metabolic routes. *Molecules*, *19*(11), 17078–106.

Fiorenzani, P., Lamponi, S., Magnani, A., Ceccarelli, I., & Aloisi, A. M. (2014). In vitro and in vivo characterization of the new analgesic combination beta-caryophyllene and docosahexaenoic acid. *Evidence-based Complementary and Alternative Medicine*, *2014*.

Fitzgerald, M. P., Payne, C. K., Lukacz, E., Yang, C., Peters, K., Chai, T. C., Nickel, J. C., Hanno, P. M., Kreder, K. J., Burks, D. A., Mayer, R., Kotarinos, R., Fortman, C., Allen, T., Fraser, L., Mason-Cover, M., Furey, C., Odabachain, L., Sanfield, A., Chu, J., Huestis, K., Tata, G., Dugan, N., Sheth, H., Bewyer, K., Anaeme, A., Newton, K., Featherstone, W., Halle-Podell, R., Cen, L., Landis, J., Propert, K., Foster Jr, H., Kusek, J. W., & Nyberg, L. M. (2012). Randomized multicenter clinical trial of myofascial physical therapy in women with interstitial cystitis/painful bladder syndrome and pelvic floor tenderness. *Journal of Urology*, *187*(6), 2113–2118.

Forrest, J. B., Payne, C. K., & Erickson, D. R. (2012). Cyclosporine A for refractory interstitial cystitis/bladder pain syndrome: experience of 3 tertiary centers. *Journal of Urology*, *188*(4), 1186–1191.

Foster Jr, H., Hanno, P., Nickel, J. C., Payne, C. K., Mayer, R. D., Burks, D. A., Yang, C. C., Chai, T. C., Kreder, K. J., Peters, K., Lukacz, E., Fitzgerald, M. P., Cen, L., Landis, J. R., Propert, K. J., Yang, W., Kusek, J. W., & Nyberg, L. M. (2009). Effect of amitriptyline on symptoms in treatment naïve patients with interstitial cystitis/painful bladder syndrome. *Journal of Urology*, *185*(5), 1853–1858.

Freeman, R. M., Adekanmi, O., Waterfield, M. R., Waterfield, A. E., Wright, D., & Zajicek, J. (2006). The effect of cannabis on urge incontinence in patients with multiple sclerosis: A multicentre, randomised placebo-controlled trial (CAMS-LUTS). *International Urogynecology Journal*, *17*(6), 636–641.

Freeman, T. P., Hindocha, C., Green, S. F., & Bloomfield, M. A. P. (2019). Medicinal use of cannabis based products and cannabinoids. *BMJ (Clinical research ed.)*, *365*, l1141.

Fry, C. H., Kitney, D. G., Paniker, J., Drake, M. J., Kanai, A., & Andersson, K. E. (2018). Fibrosis and the bladder, implications for function ICI-RS 2017. *Neurourology and Urodynamics*, *37*(S4), S7–S12.

Fukuda, S., Kohsaka, H., Takayasu, A., Yokoyama, W., Miyabe, C., Miyabe, Y., Harigai, M., Miyasaka, N., & Nanki, T. (2014). Cannabinoid receptor 2 as a potential therapeutic target in rheumatoid arthritis. *BMC musculoskeletal disorders*, *15*, 275.

Funk, C. D. (2001, November 30). Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science*. American Association for the Advancement of Science.

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research and Therapy*, *8*(SUPPL. 2), S3.

Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Fur, G., & Casellas, P. (1995). Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *European Journal of Biochemistry*, *232*(1), 54–61.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., Fur, G. LE, & Casellas, P. (1995). Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *European Journal of Biochemistry*, *232*(1), 54–61.

Gaoni, Y., & Mechoulam, R. (1964). Isolation, structure and partial synthesis of an active constituent of hashish. *J Am Chem Soe*, *86*, 1646–1647.

Garon, A. (2015). Vascular Stenosis: An Introduction. In *PanVascular Medicine, Second Edition* (pp. 781–868). Springer Berlin Heidelberg.

Garzon, S., Lagana, A., Casarin, J., Raffaelli, R., Cromi, A., Sturla, D., Franchi, M., & Ghezzi, F. (2020). An update on treatment options for interstitial cystitis. *Menopause Review*, *19*(1), 35–43.

Gelderblom, H., Verweij, J., Nooter, K., & Sparreboom, A. (2001). Cremophor EL: The drawbacks and advantages of vehicle selection for drug formulation. *European Journal of Cancer*, *37*(13), 1590–1598.

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J.-Z., Xie, X.-Q., Altmann, K.-H., Karsak, M., & Zimmer, A. (2008). Beta-caryophyllene is a dietary cannabinoid. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(26), 9099–104.

Ghazwani, Y., Elkelini, M., & Hassouna, M. (2011). Efficacy of sacral neuromodulation in treatment of bladder pain syndrome: long-term follow-up. *Neurourology and urodynamics*, *30*(7), 1271–1275.

Ghelardini, C., Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G., & Bartolini, A. (2001). Local anaesthetic activity of β-caryophyllene. *Il Farmaco*, *56*(5), 387–389.

Ghosh, S., Wise, L. E., Chen, Y., Gujjar, R., Mahadevan, A., Cravatt, B. F., & Lichtman, A. H. (2013). The monoacylglycerol lipase inhibitor JZL184 suppresses inflammatory pain in the mouse carrageenan model. *Life sciences*, *92*(8–9), 498–505.

Giannantoni, A., Di Stasi, S. M., Chancellor, M. B., Costantini, E., & Porena, M. (2006, December 1). New Frontiers in Intravesical Therapies and Drug Delivery. *European Urology*. Elsevier.

Girard, B. M., Cheppudira, B. P., Malley, S. E., Schutz, K. C., May, V., & Vizzard, M. A. (2011). Increased expression of interleukin-6 family members and receptors in urinary bladder with cyclophosphamide-induced bladder inflammation in female rats. *Frontiers in Neuroscience*, *5* (FEB).

Giuffrida, A., & McMahon, L. R. (2010). In vivo pharmacology of endocannabinoids and their metabolic inhibitors: therapeutic implications in Parkinson's disease and abuse liability. *Prostaglandins & other lipid mediators*, *91*(3–4), 90–103.

Gonçalves, E. C. D., Baldasso, G. M., Bicca, M. A., Paes, R. S., Capasso, R., & Dutra, R. C. (2020). Terpenoids, Cannabimimetic Ligands, beyond the Cannabis Plant. *Molecules (Basel, Switzerland)*, *25*(7).

Gonzalez, E. J., Arms, L., & Vizzard, M. A. (2014a). The Role(s) of Cytokines/Chemokines in Urinary Bladder Inflammation and Dysfunction. (M. Winder, Ed.)*BioMed Research International*, *2014*, 120525.

Gonzalez, E. J., Arms, L., & Vizzard, M. A. (2014b). The role(s) of cytokines/chemokines in urinary bladder inflammation and dysfunction. *BioMed research international*, *2014*, 120525.

Granger, D. Neil, & Senchenkova, E. (2010). Inflammation and the Microcirculation. *Colloquium Series on Integrated Systems Physiology: From Molecule to Function*, *2*(1), 1–87.

Granger, Daniel Neil, & Rodrigues, S. F. (2016). Microvascular Responses to Inflammation. In *Compendium of Inflammatory Diseases* (pp. 942–948). Springer Basel.

Gratzke, C., Christ, G. J., Streng, T., Park, A., Stief, C. G., Hedlund, P., & Andersson, K.-E. (2008). Morphological and Functional Characterization of CB1 and CB2 Receptors in Rat, Monkey, and Human Urinary Bladder. *Journal of Urology*, *179*(4S), 351–351.

Gratzke, C., Streng, T., Park, A., Christ, G., Stief, C. G., Hedlund, P., & Andersson, K.-E. (2009). Distribution and function of cannabinoid receptors 1 and 2 in the rat, monkey and human bladder. *The Journal of urology*, *181*(4), 1939–48.

Green, M., Fillippou, A., Sant, G., & Theoharides, C. (2004). Expression of Intracellular Adhesion Molecules in The Bladder of Patients with Interstitial Cystitis. *Urology*, *63*, 688–693.

Griebel, G., Pichat, P., Beeske, S., Leroy, T., Redon, N., Jacquet, A., Françon, D., Bert, L., Even, L., Lopez-Grancha, M., Tolstykh, T., Sun, F., Yu, Q., Brittain, S., Arlt, H., He, T., Zhang, B., Wiederschain, D., Bertrand, T., Houtmann, J., Rak, A., Vallee, F., Michot, N., Auge, F., Menet, V., Bergis, O. E., George, P., Avenet, P., Mikol, V., Didier, M., & Escoubet, J. (2015). Selective blockade of the hydrolysis of the endocannabinoid 2-arachidonoylglycerol impairs learning and memory performance while producing antinociceptive activity in rodents. *Scientific Reports*, *5*(1), 1–16.

Grover, S., Srivastava, A., Lee, R., Tewari, A. K., & Te, A. E. (2011). Role of inflammation in bladder function and interstitial cystitis. *Therapeutic advances in urology*, *3*(1), 19–33.

Grundy, L., Caldwell, A., & Brierley, S. M. (2018). Mechanisms Underlying Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome. *Frontiers in neuroscience*, *12*, 931.

Grundy, L., Erickson, A., & Brierley, S. M. (2019). Visceral Pain. *Annual review of physiology*, *81*, 261–284.

Guhasarkar, S., & Banerjee, A. (2010). Intravesical drug deliver: challenges, current status, opportunities and novel strategies. *Journal of Controlled Release*, *148*(2), 147–159.

Guida, F., Luongo, L., Boccella, S., Giordano, M. E., Romano, R., Bellini, G., Manzo, I., Furiano, A., Rizzo, A., Imperatore, R., Iannotti, F. A., D'Aniello, E., Piscitelli, F., Sca Rossi, F., Cristino, L., Di Marzo, V., de Novellis, V., & Maione, S. (2017). Palmitoylethanolamide induces microglia changes associated with increased migration and phagocytic activity: involvement of the CB2 receptor. *Scientific reports*, *7*(1), 375.

Guindon, J, & Hohmann, A. G. (2008). Cannabinoid CB 2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. *British Journal of Pharmacology*, *153*(2), 319–334.

Guindon, Josée, Guijarro, A., Piomelli, D., & Hohmann, A. G. (2011). Peripheral antinociceptive effects of inhibitors of monoacylglycerol lipase in a rat model of inflammatory pain. *British Journal of Pharmacology*, *163*(7), 1464–1478.

Guven, G., Hilty, M. P., & Ince, C. (2020). Microcirculation: Physiology, Pathophysiology, and Clinical Application. *Blood Purification*, *49*(1–2), 143–150.

Habib, A., Chokr, D., Wan, J., Hegde, P., Mabire, M., Siebert, M., Ribeiro-Parenti, L., Le Gall, M., Lettéron, P., Pilard, N., Mansouri, A., Brouillet, A., Tardelli, M., Weiss, E., Le Faouder, P., Guillou, H., Cravatt, B. F., Moreau, R., Trauner, M., & Lotersztajn, S. (2019). Inhibition of monoacylglycerol lipase, an anti-inflammatory and antifibrogenic strategy in the liver. *Gut*, *68*(3), 522–532.

Hall, W., & Degenhardt, L. (2007). Prevalence and correlates of cannabis use in developed and developing countries. *Current opinion in psychiatry*, *20*(4), 393–7.

Hang, L., Haraoka, M., Agace, W., Leffler, H., Burdick, M., Strieter, R., & Svanborg, C. (1999). Macrophage inflammatory protein-2 is required for neutrophil passage across the epithelial barrier of the infected urinary tract. *Journal of Immunology*, *162*(5), 3037–3044.

Hanno, P., & Dmochowski, R. (2009). Status of international consensus on interstitial cystitis/bladder pain syndrome/painful bladder syndrome: 2008 snapshot. *Neurourology and Urodynamics*, *28*(4), 274–286.

Hanno, P. M. (2002). Interstitial cystitis-epidemiology, diagnostic criteria, clinical markers. *Reviews in urology*, *4 Suppl 1*(Suppl 1), S3-8.

Hanno, P. M. (2012). Bladder Pain Syndrome (Interstitial Cystitis) and Related Disorders. In *Campbell-Walsh Urology* (pp. 357-401.e18). Elsevier.

Hanno, P. M., Burks, D. A., Clemens, J. Q., Dmochowski, R. R., Erickson, D., Fitzgerald, M. P., Forrest, J. B., Gordon, B., Gray, M., Mayer, R. D., Newman, D., Nyberg, L., Payne, C. K., Wesselmann, U., & Faraday, M. M. (2011). AUA Guideline for the Diagnosis and Treatment of Interstitial Cystitis / Bladder Pain Syndrome. *The Journal of urology*, *185*(6), 2162–2170.

Hanno, P. M., Erickson, D., Moldwin, R., Faraday, M. M., & American Urological Association. (2015). Diagnosis and Treatment of Interstitial Cystitis/Bladder Pain Syndrome: AUA Guideline Amendment. *The Journal of Urology*, *193*(5), 1545–1553.

Harris, A. G., & Skalak, T. C. (1993). Effects of leukocyte activation on capillary hemodynamics in skeletal muscle. *American Journal of Physiology - Heart and Circulatory Physiology*, *264*(3 33-3).

Hartsel, J. A., Eades, J., Hickory, B., & Makriyannis, A. (2016). Cannabis sativa and Hemp. *Nutraceuticals*, 735–754.

He, P., Talukder, M. A. H., & Gao, F. (2020). Oxidative Stress and Microvessel Barrier Dysfunction. *Frontiers in Physiology*, *11*, 472.

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R., & Rice, K. C. (1991). Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *11*(2), 563–83.

Hill, J. R., Isom-Batz, G., Panagopoulos, G., Zakariasen, K., & Kavaler, E. (2008). Patient Perceived Outcomes of Treatments Used for Interstitial Cystitis. *Urology*, *71*(1), 62–66.

Hol, J., Wilhelmsen, L., & Haraldsen, G. (2010). The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies. *Journal of Leukocyte Biology*, *87*(3), 501–508.

Hopkins, W. J., Gendron-Fitzpatrick, A., Balish, E., & Uehling, D. T. (1998). Time Course and Host Responses to Escherichia coli Urinary Tract Infection in Genetically Distinct Mouse Strains. *Infection and Immunity*, *66*(6), 2798.

Horváth, B., Mukhopadhyay, P., Kechrid, M., Patel, V., Tanchian, G., Wink, D. A., Gertsch, J., & Pacher, P. (2012). β-Caryophyllene ameliorates cisplatin-induced nephrotoxicity in a cannabinoid 2 receptor-dependent manner. *Free radical biology & medicine*, *52*(8), 1325–33.

Howlett, A. C. (2005). Cannabinoid Receptor Signaling. In *Cannabinoids* (pp. 53–79). Berlin/Heidelberg: Springer-Verlag.

Howlett, A C, Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin, B. R., Mechoulam, R., & Pertwee, R. G. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacological reviews*, *54*(2), 161–202.

Howlett, Allyn C. (2002). The cannabinoid receptors. *Prostaglandins & other lipid mediators*, *68–69*, 619–631.

Hsieh, G. C., Pai, M., Chandran, P., Hooker, B. A., Zhu, C. Z., Salyers, A. K., Wensink, E. J., Zhan, C., Carroll, W. A., Dart, M. J., Yao, B. B., Honore, P., & Meyer, M. D. (2011). Central and peripheral sites of action for CB_2 receptor mediated analgesic activity in chronic inflammatory and neuropathic pain models in rats. *British journal of pharmacology*, *162*(2), 428–40.

Huang, H.-C., Wang, S.-S., Hsin, I.-F., Chang, C.-C., Lee, F.-Y., Lin, H.-C., Chuang, C.-L., Lee, J.-Y., Hsieh, H.-G., & Lee, S.-D. (2012). Cannabinoid receptor 2 agonist ameliorates mesenteric angiogenesis and portosystemic collaterals in cirrhotic rats. *Hepatology*, *56*(1), 248–258.

Hunner, G. L. (1915). A Rare Type of Bladder Ulcer in Women; Report of Cases. *The Boston Medical and Surgical Journal*, *172*(18), 660–664.

Hurst, R. E., Greenwood-Van Meerveld, B., Wisniewski, A. B., VanGordon, S., Lin, H., Kropp, B. P., & Towner, R. A. (2015). Increased bladder permeability in interstitial cystitis/painful bladder syndrome. *Translational andrology and urology*, *4*(5), 563–571.

Ibrahim, M. M., Porreca, F., Lai, J., Albrecht, P. J., Rice, F. L., Khodorova, A., Davar, G., Makriyannis, A., Vanderah, T. W., Mata, H. P., & Malan, T. P. (2005). CB2 cannabinoid receptor activation produces antinociception by stimulating peripheral release of endogenous opioids. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(8), 3093–3098.

Ince, C. (2005). The microcirculation is the motor of sepsis. *Critical care (London, England)*, *9 Suppl 4*(Suppl 4), S13-9.

Irrera, N., D'ascola, A., Pallio, G., Bitto, A., Mazzon, E., Mannino, F., Squadrito, V., Arcoraci, V., Minutoli, L., Campo, G. M., Avenoso, A., Bongiorno, E. B., Vaccaro, M., Squadrito, F., & Altavilla, D. (2019). β-Caryophyllene Mitigates Collagen Antibody Induced Arthritis (CAIA) in Mice Through a Cross-Talk between CB2 and PPAR-γ Receptors. *Biomolecules*, *9*(8).

Ivetic, A., Hoskins Green, H. L., & Hart, S. J. (2019). L-selectin: A Major Regulator of Leukocyte Adhesion, Migration and Signaling. *Frontiers in Immunology*, *10*, 1068.

Jerauld, A., Wormuth, L., & Carlson, B. (2016). New Approaches in Managing Interstitial Cystitis/Bladder Pain Syndrome. *US Pharmacist*, *41*(9), 29–33.

JH, S., & HC, K. (2011). Higher levels of cell apoptosis and abnormal E-cadherin expression in the urothelium are associated with inflammation in patients with interstitial cystitis/painful bladder syndrome. *BJU international*, *108* (Pt 2).

Jiang, S.-K., Zhang, M., Tian, Z.-L., Wang, M., Zhao, R., Wang, L.-L., Li, S.-S., Liu, M., Li, J.-Y., Zhang, M.-Z., & Guan, D.-W. (2015). The monoacylglycerol lipase inhibitor JZL184 decreases inflammatory response in skeletal muscle contusion in rats. *European journal of pharmacology*, *761*, 1–10.

Jiang, Y.-H., Jhang, J.-F., & Kuo, H.-C. (2016). Revisiting the Role of Potassium Sensitivity Testing and Cystoscopic Hydrodistention for the Diagnosis of Interstitial Cystitis. *PloS one*, *11*(3), e0151692.

Jiang, Y.-H., Peng, C.-H., Liu, H.-T., & Kuo, H.-C. (2013). Increased Pro-Inflammatory Cytokines, C-Reactive Protein and Nerve Growth Factor Expressions in Serum of Patients with Interstitial Cystitis/Bladder Pain Syndrome. (J. Spencer, Ed.)*PLoS ONE*, *8*(10), e76779.

Jiang, Y. H., Peng, C. H., Liu, H. T., & Kuo, H. C. (2013). Increased Pro-Inflammatory Cytokines, C-Reactive Protein and Nerve Growth Factor Expressions in Serum of Patients with Interstitial Cystitis/Bladder Pain Syndrome. *PLoS ONE*, *8*(10).

Jones, C. A., & Nyberg, L. (1997). Epidemiology of interstitial cystitis. *Urology*, *49*(5), 2–9.

Kapellos, T. S., Taylor, L., Feuerborn, A., Valaris, S., Hussain, M. T., Rainger, G. E., Greaves, D. R., & Iqbal, A. J. (2019). Cannabinoid receptor 2 deficiency exacerbates inflammation and neutrophil recruitment. *The FASEB Journal*, *33*(5), 6154–6167.

Kappelmayer, J., & Nagy, B. (2017). The interaction of selectins and PSGL-1 as a key component in thrombus formation and cancer progression. *BioMed Research International*. Hindawi Limited.

Katsuyama, S., Mizoguchi, H., Kuwahata, H., Komatsu, T., Nagaoka, K., Nakamura, H., Bagetta, G., Sakurada, T., & Sakurada, S. (2013). Involvement of peripheral cannabinoid and opioid receptors in b-caryophyllene-induced antinociception. *European Journal of Pain (United Kingdom)*, *17*(5), 664–675.

Keay, S. K., Birder, L. A., & Chai, T. C. (2014). Evidence for bladder urothelial pathophysiology in functional bladder disorders. *BioMed research international*, *2014*, 865463.

Keay, S. K., Szekely, Z., Conrads, T. P., Veenstra, T. D., Barchi, J. J., Zhang, C. O., Koch, K. R., & Michejda, C. J. (2004). An antiproliferative factor from interstitial cystitis patients is a frizzled 8 protein-related sialoglycopeptide. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(32), 11803–11808.

Kelly, M., Hwang, J. M., & Kubes, P. (2007). Modulating leukocyte recruitment in inflammation. *The Journal of allergy and clinical immunology*, *120*(1), 3–10.

Kendall, D. A., & Yudowski, G. A. (2017). Cannabinoid Receptors in the Central Nervous System: Their Signaling and Roles in Disease. *Frontiers in Cellular Neuroscience*, *10*, 294.

Kerr, D. M., Harhen, B., Okine, B. N., Egan, L. J., Finn, D. P., & Roche, M. (2013). The monoacylglycerol lipase inhibitor JZL184 attenuates LPS-induced increases in cytokine expression in the rat frontal cortex and plasma: differential mechanisms of action. *British journal of pharmacology*, *169*(4), 808–19.

Kessel, D. (1992). Properties of cremophor EL micelles probed by fluorescence. *Photochemistry and Photobiology*, *56*(4), 447–451.

Khasabova, I. A., Chandiramani, A., Harding-Rose, C., Simone, D. A., & Seybold, V. S. (2011). Increasing 2-arachidonoyl glycerol signaling in the periphery attenuates mechanical hyperalgesia in a model of bone cancer pain. *Pharmacological research*, *64*(1), 60–7.

Kim, H.-J. (2016). Update on the Pathology and Diagnosis of Interstitial Cystitis/Bladder Pain Syndrome: A Review. *International neurourology journal*, *20*(1), 13–7.

Kim, R., Liu, W., Chen, X., Kreder, K. J., & Luo, Y. (2011). Intravesical dimethyl sulfoxide inhibits acute and chronic bladder inflammation in transgenic experimental autoimmune cystitis models. *Journal of biomedicine & biotechnology*, *2011*, 937061.

Kinsey, S. G., Wise, L. E., Ramesh, D., Abdullah, R., Selley, D. E., Cravatt, B. F., & Lichtman, A. H. (2013). Repeated low-dose administration of the monoacylglycerol lipase inhibitor JZL184 retains cannabinoid receptor type 1-mediated antinociceptive and gastroprotective effects. *Journal of Pharmacology and Experimental Therapeutics*, *345*(3), 492–501.

Klauke, A.-L., Racz, I., Pradier, B., Markert, A., Zimmer, A. M., Gertsch, J., & Zimmer, A. (2014). The cannabinoid CB2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. *European Neuropsychopharmacology*, *24*(4), 608–620.

Kowalewska, P. M., Burrows, L. L., & Fox-Robichaud, A. E. (2011). Intravital Microscopy of the Murine Urinary Bladder Microcirculation. *Microcirculation*, *18*(8), 613–622.

Kozak, K. R., Gupta, R. A., Moody, J. S., Ji, C., Boeglin, W. E., DuBois, R. N., Brash, A. R., & Marnett, L. J. (2002). 15-Lipoxygenase metabolism of 2-arachidonylglycerol. Generation of a peroxisome proliferator-activated receptor alpha agonist. *The Journal of biological chemistry*, *277*(26), 23278–86.

Kraiss, L. W., Alto, N. M., Dixon, D. A., McIntyre, T. M., Weyrich, A. S., & Zimmerman, G. A. (2003). Fluid flow regulates e-selectin protein levels in human endothelial cells by inhibiting translation. *Journal of Vascular Surgery*, *37*(1), 161–168.

Krieglstein, C., & Granger, D. N. (2001). Adhesion molecules and their role in vascular disease. *American Journal of Hypertension*, *14*(11), S44–S54.

Kuebler, W. M., Kuhnle, G. E. H., Groh, J., & Goetz, A. E. (1997). Contribution of selectins to leucocyte sequestration in pulmonary microvessels by intravital microscopy in rabbits. *Journal of Physiology*, *501*(2), 375–386.

Kullmann, F. A., McDonnell, B. M., Wolf-Johnston, A. S., Lynn, A. M., Getchell, S. E., Ruiz, W. G., Zabbarova, I. V., Ikeda, Y., Kanai, A. J., Roppolo, J. R., Bastacky, S. I., Apodaca, G., Buffington, C. A. T., & Birder, L. A. (2018). Inflammation and tissue remodeling in the bladder and urethra in feline interstitial cystitis. *Frontiers in Systems Neuroscience*, *12*, 13.

Kuo, H.-C. (2014). Potential urine and serum biomarkers for patients with bladder pain syndrome/interstitial cystitis. *International Journal of Urology*, *21*(S1), 34–41.

Kurth, K., & Parsons, C. L. (2003). The Interstitial Cystitis Syndrome: Intravesical and Oral Treatment. *European Urology Supplements*, *2*(3), 2–9.

Kuwahata, H., Katsuyama, S., Komatsu, T., Nakamura, H., Corasaniti, M. T., Bagetta, G., Sakurada, S., Sakurada, T., & Takahama, K. (2012). Local Peripheral Effects of β-Caryophyllene through CB2 Receptors in Neuropathic Pain in Mice. *Pharmacology & Pharmacy*, *03*(04), 397–403.

La Porta, C., Bura, S. A., Aracil-Fernández, A., Manzanares, J., & Maldonado, R. (2013). Role of CB1 and CB2 cannabinoid receptors in the development of joint pain induced by monosodium iodoacetate. *Pain*, *154*(1).

Lai, H., Gereau, R. W., Luo, Y., O'Donnell, M., Rudick, C. N., Pontari, M., Mullins, C., & Klumpp, D. J. (2015). Animal models of urologic chronic pelvic pain syndromes: Findings from the multidisciplinary approach to the study of chronic pelvic pain research network. *Urology*, *85*(6), 1454–1465.

Lai, M.-C., Kuo, Y.-C., & Kuo, H.-C. (2013). Intravesical hyaluronic acid for interstitial cystitis/painful bladder syndrome: A comparative randomized assessment of different regimens. *International Journal of Urology*, *20*(2), 203–207.

Lamale, L. M., Lutgendorf, S. K., Zimmerman, M. B., & Kreder, K. J. (2006). Interleukin-6, histamine, and methylhistamine as diagnostic markers for interstitial cystitis. *Urology*, *68*(4), 702–706.

Lamanna, O. K., Hsieh, M. H., & Forster, C. S. (2020). Novel catheter design enables transurethral catheterization of male mice. *American Journal of Physiology-Renal Physiology*, *319*(1), F29–F32.

Lambert, D. M., & Muccioli, G. G. (2007). Endocannabinoids and related N-acylethanolamines in the control of appetite and energy metabolism: emergence of new molecular players. *Current opinion in clinical nutrition and metabolic care*, *10*(6), 735–44.

Lehmann, C., Kianian, M., Zhou, J., Küster, I., Kuschnereit, R., Whynot, S., Hung, O., Shukla, R., Johnston, B., Cerny, V., Pavlovic, D., Spassov, A., & Kelly, M. E. (2012). Cannabinoid receptor 2 activation reduces intestinal leukocyte recruitment and systemic inflammatory mediator release in acute experimental sepsis. *Critical care (London, England)*, *16*(2), R47.

Leung, D., Saghatelian, A., Simon, G. M., & Cravatt, B. F. (2006). Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry*, *45*(15), 4720–6.

Ley, K., Laudanna, C., Cybulsky, M. I., & Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews Immunology*, *7*(9), 678–689.

Li, W. W., Guo, T. Z., Liang, D. Y., Sun, Y., Kingery, W. S., & Clark, J. D. (2012). Substance P signaling controls mast cell activation, degranulation, and nociceptive sensitization in a rat fracture model of complex regional pain syndrome. *Anesthesiology*, *116*(4), 882–895.

Liao, F., Huynh, H. K., Eiroa, A., Greene, T., Polizzi, E., & Muller, W. A. (1995). Migration of monocytes across endothelium and passage through extracellular matrix involve separate molecular domains of PECAM-1. *The Journal of experimental medicine*, *182*(5), 1337–43.

Linsenmeyer, T. (2013). Use of botulinum toxin in individuals with neurogenic detrusor overactivity: State of the art review. *The journal of spinal cord medicine*, *36*(5), 402–419.

Liu, H., Song, Z., Liao, D., Zhang, T., Liu, F., Zhuang, K., Luo, K., & Yang, L. (2014). Neuroprotective Effects of Trans-Caryophyllene Against Kainic Acid Induced Seizure Activity and Oxidative Stress in Mice. *Neurochemical Research*, *40*(1), 118–123.

Løberg, E.-M., & Hugdahl, K. (2009). Cannabis use and cognition in schizophrenia. *Frontiers in Human Neuroscience*, *3*, 53.

Long, E. O. (2011). ICAM-1: getting a grip on leukocyte adhesion. *Journal of immunology (Baltimore, Md. : 1950)*, *186*(9), 5021–3.

Long, J. Z., Li, W., Booker, L., Burston, J. J., Kinsey, S. G., Schlosburg, J. E., Pavón, F. J., Serrano, A. M., Selley, D. E., Parsons, L. H., Lichtman, A. H., & Cravatt, B. F. (2009). Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nature chemical biology*, *5*(1), 37–44.

Long, J. Z., Nomura, D. K., & Cravatt, B. F. (2009a). Characterization of Monoacylglycerol Lipase Inhibition Reveals Differences in Central and Peripheral Endocannabinoid Metabolism. *Chemistry & Biology*, *16*(7), 744–753.

Long, J. Z., Nomura, D. K., & Cravatt, B. F. (2009b). Characterization of monoacylglycerol lipase inhibition reveals differences in central and peripheral endocannabinoid metabolism. *Chemistry & biology*, *16*(7), 744–53.

Lotz, M., Villiger, P., Hugli, T., Koziol, J., & Zuraw, B. L. (1994). Interleukin-6 and interstitial cystitis. *The Journal of urology*, *152*(3), 869–73.

Lu, H.-C., & Mackie, K. (2016). An Introduction to the Endogenous Cannabinoid System. *Biological psychiatry*, *79*(7), 516–25.

Luchicchi, A., Lecca, S., Carta, S., Pillolla, G., Muntoni, A. L., Yasar, S., Goldberg, S. R., & Pistis, M. (2010). Effects of fatty acid amide hydrolase inhibition on neuronal responses to nicotine, cocaine and morphine in the nucleus accumbens shell and ventral tegmental area: involvement of PPAR-alpha nuclear receptors. *Addiction biology*, *15*(3), 277–88.

Lusty, A., Kavaler, E., Zakariasen, K., Tolls, V., & Nickel, J. C. (2018). Treatment effectiveness in interstitial cystitis/bladder pain syndrome: Do patient perceptions align with efficacy-based guidelines? *Canadian Urological Association journal = Journal de l'Association des urologues du Canada*, *12*(1), E1–E5.

Mamdouh, Z., Chen, X., Pierini, L. M., Maxfield, F. R., & Muller, W. A. (2003). Targeted recycling of PECAM from endothelial surface-connected compartments during diapedesis. *Nature*, *421*(6924), 748–753.

Manzanares, J., Julian, M., & Carrascosa, A. (2006). Role of the cannabinoid system in pain control and therapeutic implications for the management of acute and chronic pain episodes. *Current neuropharmacology*, *4*(3), 239–57.

Marino, S., Carrasco, G., Li, B., Shah, K. M., Lath, D. L., Sophocleous, A., Lawson, M. A., & Idris, A. I. (2020). JZL184, A Monoacylglycerol Lipase Inhibitor, Induces Bone Loss in a Multiple Myeloma Model of Immunocompetent Mice. *Calcified Tissue International*, *107*(1), 72–85.

Martin, H. (2010). Role of PPAR-gamma in inflammation. Prospects for therapeutic intervention by food components. *Mutation research*, *690*(1–2), 57–63.

Massa, F., Marsicano, G., Hermann, H., Cannich, A., Monory, K., Cravatt, B. F., Ferri, G. L., Sibaev, A., Storr, M., & Lutz, B. (2004). The endogenous cannabinoid system protects against colonic inflammation. *J Clin Invest*, *113*(8), 1202–1209.

Massena, S., & Phillipso, M. (2012). Intravascular Leukocyte Chemotaxis: The Rules of Attraction. In *Hematology - Science and Practice*. InTech.

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., & Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, *346*(6284), 561–564.

Mazzoni, M. C., & Schmid-Schonbein, G. W. (1996). Mechanisms and consequences of cell activation in the microcirculation. *Cardiovascular Research*, *32*(4), 709–719.

McHugh, D., Hu, S. S. J., Rimmerman, N., Juknat, A., Vogel, Z., Walker, J. M., & Bradshaw, H. B. (2010). N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. *BMC neuroscience*, *11*, 44.

McLaughlin, P. J. (2017). Rimonabant. In J. Davies & S. Alexander (Eds.), *Refence Module in Biomedical Sciences*. Edinboro, PA, USA: Elsevier.

Mechoulam, R., & Feigenbaum, J. J. (1987). Towards cannabinoid drugs. *Progress in medicinal chemistry*, *24*, 159–207.

Meijlink, J. M. (2014). Interstitial cystitis and the painful bladder: A brief history of nomenclature, definitions and criteria. *International Journal of Urology*, *21*, 4–12.

Merriam, F. V., Wang, Z., Guerios, S. D., & Bjorling, D. E. (2008). Cannabinoid receptor 2 is increased in acutely and chronically inflamed bladder of rats. *Neuroscience letters*, *445*(1), 130–4.

Merriam, F. V., Wang, Z. Y., Hillard, C. J., Stuhr, K. L., & Bjorling, D. E. (2011). Inhibition of fatty acid amide hydrolase suppresses referred hyperalgesia induced by bladder inflammation. *BJU International*, *108*(7), 1145–1149.

Morales, P., Hurst, D. P., & Reggio, P. H. (2017). Molecular Targets of the Phytocannabinoids: A Complex Picture. *Progress in the chemistry of organic natural products*, *103*, 103–131.

Muccioli, G. G. (2010). Endocannabinoid biosynthesis and inactivation, from simple to complex. *Drug Discovery Today*, *15*(11–12), 474–483.

Muller, C., Morales, P., & Reggio, P. H. (2019). Cannabinoid Ligands Targeting TRP Channels. *Frontiers in Molecular Neuroscience*, *11*, 487.

Muller, W A. (2013). Getting leukocytes to the site of inflammation. *Veterinary pathology*, *50*(1), 7–22.

Muller, William A. (2011). Mechanisms of leukocyte transendothelial migration. *Annual review of pathology*, *6*, 323–44.

Mulvihill, M. M., & Nomura, D. K. (2013). Therapeutic potential of monoacylglycerol lipase inhibitors. In *Life Sciences* (Vol. 92, pp. 492–497). NIH Public Access.

Munro, S., Thomas, K. L., & Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, *365*(6441), 61–65.

Murataeva, N., Straiker, A., & Mackie, K. (2014). Parsing the players: 2-arachidonoylglycerol synthesis and degradation in the CNS. *British journal of pharmacology*, *171*(6), 1379–91.

Navarro, G., Morales, P., Rodríguez-Cueto, C., Fernández-Ruiz, J., Jagerovic, N., & Franco, R. (2016). Targeting Cannabinoid CB2 Receptors in the Central Nervous System. Medicinal Chemistry Approaches with Focus on Neurodegenerative Disorders. *Frontiers in neuroscience*, *10*, 406.

Nent, E., Nozaki, C., Schmöle, A.-C., Otte, D., & Zimmer, A. (2019). CB2 receptor deletion on myeloid cells enhanced mechanical allodynia in a mouse model of neuropathic pain. *Scientific Reports*, *9*(1), 7468.

Ness, T. J., & Elhefni, H. (2004). Reliable visceromotor responses are evoked by noxious bladder distention in mice. *Journal of Urology*, *171*(4), 1704–1708.

Nickel, J. C., Herschorn, S., Whitmore, K., Forrest, J. B., Hu, P., Friedman, A. J., & Baseman, A. S. (2015). Pentosan polysulfate sodium for treatment of interstitial cystitis/bladder pain syndrome: insights from a randomized, double-blind, placebo controlled study. *Journal of Urology*, *193*(3), 857–862.

Nickel, J. C., Teichman, J. M. H., Gregoire, M., Clark, J., & Downey, J. (2005). Prevalence, diagnosis, characterization, and treatment of prostatitis, interstitial cystitis, and epididymitis in outpatient urological practice: The Canadian PIE Study. *Urology*, *66*(5), 935–940.

Nicotra, L., Loram, L. C., Watkins, L. R., & Hutchinson, M. R. (2012). Toll-like receptors in chronic pain. *Experimental Neurology*, *234*(2), 316–329.

Nielsen, S., Sabioni, P., Trigo, J. M., Ware, M. A., Betz-Stablein, B. D., Murnion, B., Lintzeris, N., Khor, K. E., Farrell, M., Smith, A., & Le Foll, B. (2017, August 1). Opioid-sparing effect of cannabinoids: A systematic review and meta-analysis. *Neuropsychopharmacology*. Nature Publishing Group.

Noguera-Ortega, E., Blanco-Cabra, N., Rabanal, R. M., Sánchez-Chardi, A., Roldán, M., Guallar-Garrido, S., Torrents, E., Luquin, M., & Julián, E. (2016). Mycobacteria emulsified in olive oil-in-water trigger a robust immune response in bladder cancer treatment. *Scientific Reports*, *6*(1), 1–12.

Nomura, D. K., Morrison, B. E., Blankman, J. L., Long, J. Z., Kinsey, S. G., Marcondes, M. C. G., Ward, A. M., Hahn, Y. K., Lichtman, A. H., Conti, B., & Cravatt, B. F. (2011). Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. *Science (New York, N.Y.)*, *334*(6057), 809–13.

O'Sullivan, S. E. (2016). An update on PPAR activation by cannabinoids. *British journal of pharmacology*, *173*(12), 1899–910.

Oh-Oka, H. (2017). Clinical Efficacy of 1-Year Intensive Systematic Dietary Manipulation as Complementary and Alternative Medicine Therapies on Female Patients With Interstitial Cystitis/Bladder Pain Syndrome. *Urology*, *106*, 50–54.

Orio, L., Alen, F., Pavón, F. J., Serrano, A., & García-Bueno, B. (2019). Oleoylethanolamide, Neuroinflammation, and Alcohol Abuse. *Frontiers in Molecular Neuroscience*, *11*, 490.

Ortega-Alvaro, A., Aracil-Fernández, A., García-Gutiérrez, M. S., Navarrete, F., & Manzanares, J. (2011). Deletion of CB2 cannabinoid receptor induces schizophrenia-related behaviors in mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *36*(7), 1489–504.

Ostermann, G., Weber, K. S. C., Zernecke, A., Schröder, A., & Weber, C. (2002). JAM-1 is a ligand of the β2 integrin LFA-1 involved in transendothelial migration of leukocytes. *Nature Immunology*, *3*(2), 151–158.

Pacher, P., & Mechoulam, R. (2011, April). Is lipid signaling through cannabinoid 2 receptors part of a protective system? *Progress in Lipid Research*. NIH Public Access.

Pacher, Pál, Bátkai, S., & Kunos, G. (2006). The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacological reviews*, *58*(3), 389–462.

Pang, X., Marchand, J., Sant, G. R., Kream, R. M., Theoharides, T. C., & J. (1995). Increased number of substance P positive nerve fibres in interstitial cystitis. *British Journal of Urology*, *75*(6), 744–750.

Panikashvili, D., Mechoulam, R., Beni, S. M., Alexandrovich, A., & Shohami, E. (2005). CB1 cannabinoid receptors are involved in neuroprotection via NF-kappa B inhibition. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *25*(4), 477–84.

Parsons, C., & Koprowski, P. (1991). Interstitial cystitis: successful management by increasing urinary voiding intervals. *Urology*, *37*(3), 207–212.

Parsons, C. L. (2007). The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis. *Urology*, *69*(4 Suppl), 9–16.

Parsons, C. L. (2011). The role of a leaky epithelium and potassium in the generation of bladder symptoms in interstitial cystitis/overactive bladder, urethral syndrome, prostatitis and gynaecological chronic pelvic pain. *BJU International*, *107*(3), 370–375.

Parsons, J. K., & Parsons, C. L. (2004). The historical origins of interstitial cystitis. *The Journal of urology*, *171*(1), 20–2.

Passavanti, M. B., Alfieri, A., Pace, M. C., Pota, V., Sansone, P., Piccinno, G., Barbarisi, M., Aurilio, C., & Fiore, M. (2019). Clinical applications of palmitoylethanolamide in pain management: protocol for a scoping review. *Systematic Reviews*, *8*(1), 9.

Patnaik, S. S., Laganà, A. S., Vitale, S. G., Butticè, S., Noventa, M., Gizzo, S., Valenti, G., Rapisarda, A. M. C., La Rosa, V. L., Magno, C., Triolo, O., & Dandolu, V. (2017). Etiology, pathophysiology and biomarkers of interstitial cystitis/painful bladder syndrome. *Archives of Gynecology and Obstetrics*, *295*(6), 1341–1359.

Paula-Freire, L. I. G., Andersen, M. L., Gama, V. S., Molska, G. R., & Carlini, E. L. A. (2014). The oral administration of trans-caryophyllene attenuates acute and chronic pain in mice. *Phytomedicine*, *21*(3), 356–362.

Pazin, C., de Souza Mitidieri, A., Moreira Silva, A.-P., Beatriz Ferreira, M., Poli-Neto, O.-B., & Rosa-e-Silva, J.-C. (2016). Treatment of bladder pain syndrome and interstitial cystitis: a systematic review. *International urogynecology journal*, *27*, 697–708.

Pertwee, R G. (2006a). The pharmacology of cannabinoid receptors and their ligands: an overview. *International Journal of Obesity*, *30*, S13–S18.

Pertwee, R G. (2010). Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. *Current medicinal chemistry*, *17*(14), 1360–81.

Pertwee, R G, & Ross, R. A. (2002). Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids*, *66*(2–3), 101–121.

Petri, B., & Bixel, M. G. (2006, October). Molecular events during leukocyte diapedesis. *FEBS Journal*. FEBS J.

Philpott, H. T., & McDougall, J. J. (2020). Combatting joint pain and inflammation by dual inhibition of monoacylglycerol lipase and cyclooxygenase-2 in a rat model of osteoarthritis. *Arthritis Research & Therapy*, *22*(1), 9.

Picciolo, G., Pallio, G., Altavilla, D., Vaccaro, M., Oteri, G., Irrera, N., & Squadrito, F. (2020). β-Caryophyllene reduces the inflammatory phenotype of periodontal cells by targeting CB2 receptors. *Biomedicines*, *8*(6).

Pinho, V., Coelho, F. M., Menezes, G. B., & Cara, D. C. (2011). Intravital microscopy to study leukocyte recruitment in vivo. *Methods in molecular biology (Clifton, N.J.)*, *689*, 81–90.

Pontari, M., Hanno, P. M., & Ruggieri, M. R. (1999). Comparison of bladder blood flow in patients with and without Interstitial Cystitis. *Journal of Urology*, *162*(2), 330–334.

Porter, R. F., Szczesniak, A. M., Toguri, J. T., Gebremeskel, S., Johnston, B., Lehmann, C., Fingerle, J., Rothenhäusler, B., Perret, C., Rogers-Evans, M., Kimbara, A., Nettekoven, M., Guba, W., Grether, U., Ullmer, C., & Kelly, M. E. M. (2019). Selective cannabinoid 2 receptor agonists as potential therapeutic drugs for the treatment of endotoxin-induced uveitis. *Molecules*, *24*(18).

Qin, X., Wan, Y., & Wang, X. (2005). CCL2 and CXCL1 trigger calcitonin gene-related peptide release by exciting primary nociceptive neurons. *Journal of neuroscience research*, *82*(1), 51–62.

Rawls, W. F., Cox, L., & Rovner, E. S. (2017). Dimethyl sulfoxide (DMSO) as intravesical therapy for interstitial cystitis/bladder pain syndrome: A review. *Neurourology and Urodynamics*, *36*, 1–8.

Reggio, P. H. (2010). Endocannabinoid binding to the cannabinoid receptors: what is known and what remains unknown. *Current medicinal chemistry*, *17*(14), 1468–86.

Ren, K., & Dubner, R. (2010, November 14). Interactions between the immune and nervous systems in pain. *Nature Medicine*. Nature Publishing Group.

Ren, K., & Torres, R. (2009). Role of interleukin-1beta during pain and inflammation. *Brain research reviews*, *60*(1), 57–64.

Rockwell, C. E., Snider, N. T., Thompson, J. T., Vanden Heuvel, J. P., & Kaminski, N. E. (2006). Interleukin-2 suppression by 2-arachidonyl glycerol is mediated through peroxisome proliferator-activated receptor gamma independently of cannabinoid receptors 1 and 2. *Molecular pharmacology*, *70*(1), 101–11.

Rosamilia, A., Cann, L., Scurry, J., Rogers, P., & Dwyer, P. (2001). Bladder microvasculature and the effects of hydrodistention in interstitial cystitis. *Urology*, *57*(6), 132.

Rovner, E., Propert, K. J., Brensinger, C., Wein, A. J., Kirkemo, A., Landis, J. R., Kusek, J. W., & Nyberg, L. M. (2000). Treatments used in women with interstitial cystitis: the interstitial cystitis data base (ICDB) study experience. The Interstitial Cystitis Data Base Study Group. *Urology*, *56*(6), 940–945.

Russo, E. B. (2008). Cannabinoids in the management of difficult to treat pain. *Therapeutics and clinical risk management*, *4*(1), 245–59.

Russo, E. B., & Marcu, J. (2017). Cannabis Pharmacology: The Usual Suspects and a Few Promising Leads. *Advances in pharmacology (San Diego, Calif.)*, *80*, 67–134.

Sairanen, J., Forsell, T., & Ruutu, M. (2004). Long-term outcome of patients with interstitial cystitis treated with low dose cyclosporine A. *Journal of Urology*, *6*(1), 2138–2141.

Sairanen, J., Tammela, T., Leppilahti, M., Multanen, M., Paananen, I., Lehtoranta, K., & Ruutu, M. (2005). Cyclosporine A and Pentosan Polysulfase Sodium for the Treatment of Interstitial Cysitis: a Randomized Comparative Study. *Journal of Urology*, *174*(6), 2235–2238.

Sanson, M., Bueno, L., & Fioramonti, J. (2006). Involvement of cannabinoid receptors in inflammatory hypersensitivity to colonic distension in rats. *Neurogastroenterology and Motility*, *18*(10), 949–956.

Sant, G. R. (2002). Etiology, pathogenesis, and diagnosis of interstitial cystitis. *Reviews in urology*, *4 Suppl 1*(Suppl 1), S9–S15.

Sant, G. R., Kempuraj, D., Marchand, J. E., & Theoharides, C. (2007). The Mast Cell in Interstitial Cystitis: Role in Pathophysiology and Pathogenesis.

Sant, G. R., & LaRock, D. R. (1994). Standard intravesical therapies for interstitial cystitis. *Urologic Clinics of North America*, *21*(1), 73–83.

Sant, G. R., Propert, K. J., Hanno, P. M., Burks, D. A., Culkin, D., Diokno, A., Hardy, C., Landis, J., Mayer, R., Madigan, R., Messing, E., Peters, K., Theoharides, C., Warren, J., Wein, A. J., Steers, W., Kusek, J., & Nyberg, L. (2003). A pilot clinical trial of oral pentosan polysulfate and oral hydroxyzine in patients with interstitial cystitis. *Journal of Urology*, *170*(3), 810–815.

Sardinha, J., Kelly, M. E. M., Zhou, J., & Lehmann, C. (2014). Experimental Cannabinoid 2 Receptor-Mediated Immune Modulation in Sepsis. *Mediators of Inflammation*, *2014*, 1–7.

Sawa, Y., Ueki, T., Hata, M., Iwasawa, K., Tsuruga, E., Kojima, H., Ishikawa, H., & Yoshida, S. (2008). LPS-induced IL-6, IL-8, VCAM-1, and ICAM-1 Expression in Human Lymphatic Endothelium. *J Histochem Cytochem*, *56*, 97–109.

Schaible, H. G., Ebersberger, A., & Natura, G. (2011, April 28). Update on peripheral mechanisms of pain: Beyond prostaglandins and cytokines. *Arthritis Research and Therapy*. BioMed Central.

Schilling, J. D., Martin, S. M., Hunstad, D. A., Patel, K. P., Mulvey, M. A., Justice, S. S., Lorenz, R. G., & Hultgren, S. J. (2003). CD14- and Toll-like receptor-dependent activation of bladder epithelial cells by lipopolysaccharide and type 1 piliated Escherichia coli. *Infection and immunity*, *71*(3), 1470–80.

Schlosburg, J. E., Blankman, J. L., Long, J. Z., Nomura, D. K., Pan, B., Kinsey, S. G., Nguyen, P. T., Ramesh, D., Booker, L., Burston, J. J., Thomas, E. A., Selley, D. E., Sim-Selley, L. J., Liu, Q., Lichtman, A. H., & Cravatt, B. F. (2010a). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nature neuroscience*, *13*(9), 1113–9.

Schmitt, D., Levy, R., & Carroll, B. (2016). Toxicological Evaluation of-Caryophyllene Oil: Subchronic Toxicity in Rats. *International journal of toxicology*, *25*(5), 558–567.

Schnegelsberg, B., Sun, T. T., Cain, G., Bhattacharya, A., Nunn, P. A., Ford, A. P. D. W., Vizzard, M. A., & Cockayne, D. A. (2010). Overexpression of NGF in mouse urothelium leads to neuronal hyperinnervation, pelvic sensitivity, and changes in urinary bladder function. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *298*(3).

Schrepf, A., O'Donnell, M., Luo, Y., Bradley, C. S., Kreder, K., & Lutgendorf, S. (2014). Inflammation and inflammatory control in interstitial cystitis/bladder pain syndrome: Associations with painful symptoms. *Pain*, *155*(9), 1755–1761.

Sharir, H., & Abood, M. E. (2010). Pharmacological characterization of GPR55, a putative cannabinoid receptor. *Pharmacology & therapeutics*, *126*(3), 301–13.

Sharma, C., M. Al Kaabi, J., M. Nurulain, S., N. Goyal, S., Amjad Kamal, M., & Ojha, S. (2016). Polypharmacological Properties and Therapeutic Potential of β-Caryophyllene: A Dietary Phytocannabinoid of Pharmaceutical Promise. *Current Pharmaceutical Design*, *22*(21), 3237–3264.

Shenot, P., & Ryann Mark, J. (2013). Intradetrusor onabotulinumtoxinA injection: how I do it. *The Canadian Journal of Urology*, *20*(1).

Shiga, K. I., Hirano, K., Nishimura, J., Niiro, N., Naito, S., & Kanaide, H. (2007). Dimethyl sulphoxide relaxes rabbit detrusor muscle by decreasing the Ca 2+ sensitivity of the contractile apparatus. *British Journal of Pharmacology*, *151*(7), 1014–1024.

Slattery, M. J., Liang, S., & Dong, C. (2005). Distinct role of hydrodynamic shear in leukocyte-facilitated tumor cell extravasation. *American journal of physiology. Cell physiology*, *288*(4), C831-9.

Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., de Petrocellis, L., Ullmer, C., Rothenhäusler, B., Perret, C., van Gils, N., Finlay, D., MacDonald, C., Chicca, A., Gens, M. D., Stuart, J., de Vries, H., Mastrangelo, N., Xia, L., Alachouzos, G., Baggelaar, M. P., Martella, A., Mock, E. D., Deng, H., Heitman, L. H., Connor, M., Di Marzo, V., Gertsch, J., Lichtman, A. H., Maccarrone, M., Pacher, P., Glass, M., & van der Stelt, M. (2017). Cannabinoid CB2 receptor ligand profiling reveals biased signalling and off-target activity. *Nature Communications*, *8*, 13958.

Sokol, A., & Sokol, E. (2007). Interstitial Cystitis and Chronic Pelvic Pain. In M. Deborah (Ed.), *General Gynecology* (pp. 663–685). Mosby.

Song, J., Duncan, M. J., Li, G., Chan, C., Grady, R., Stapleton, A., & Abraham, S. N. (2007). A Novel TLR4-Mediated Signaling Pathway Leading to IL-6 Responses in Human Bladder Epithelial Cells. (S. J. Hultgren, Ed.)*PLoS Pathogens*, *3*(4), e60.

Song, P. H., Chun, S. Y., Chung, J.-W., Kim, Y. Y., Lee, H. J., Lee, J. N., Ha, Y.-S., Yoo, E. S., Kwon, T. G., Kim, J., Kim, D. H., & Kim, B. S. (2017). Comparison of 5 Different Rat Models to Establish a Standard Animal Model for Research Into Interstitial Cystitis. *International neurourology journal*, *21*(3), 163–170.

Stamatovic, S. M., Sladojevic, N., Keep, R. F., & Andjelkovic, A. V. (2012). Relocalization of junctional adhesion molecule A during inflammatory stimulation of brain endothelial cells. *Molecular and cellular biology*, *32*(17), 3414–27.

Straiker, A., & Mackie, K. (2005). Depolarization-induced suppression of excitation in murine autaptic hippocampal neurones. *The Journal of physiology*, *569*(Pt 2), 501–17.

Sugiura, T., Kishimoto, S., Oka, S., & Gokoh, M. (2006). Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. *Progress in lipid research*, *45*(5), 405–46.

Sugiura, T., & Waku, K. (2002). Cannabinoid receptors and their endogenous ligands. *Journal of biochemistry*, *132*(1), 7–12.

Sumagin, R., & Sarelius, I. H. (2013). Emerging understanding of roles for arterioles in inflammation. *Microcirculation (New York, N.Y. : 1994)*, *20*(8), 679–92.

Sun, W., Frost, B., & Liu, J. (2017). Oleuropein, unexpected benefits! *Oncotarget*. Impact Journals LLC.

Szczesniak, A., Porter, R., Toguri, J., Borowska-Fielding, J., Siwakoti, A., Lehmann, C., & Kelly, M. (2016). Cannabinoid 2 receptor is a novel anti-inflammatory target in experimental proliferative vitreoretinopathy. *J Neuropharmcol*.

Tabarelli, Z., Berlese, D. B., Sauzem, P. D., Mello, C. F., & Rubin, M. A. (2003). Antinociceptive effects of Cremophor EL orally administered to mice. *Brazilian Journal of Medical and Biological Research*, *36*(1), 119–123.

Tambaro, S., Casu, M. A., Mastinu, A., & Lazzari, P. (2014). Evaluation of selective cannabinoid CB1 and CB2 receptor agonists in a mouse model of lipopolysaccharide-induced interstitial cystitis. *European Journal of Pharmacology*, *729*(1), 67–74.

Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). Il-6 in inflammation, Immunity, And disease. *Cold Spring Harbor Perspectives in Biology*, *6*(10), 16295–16296.

Teichman, J. M. H. (2009). Hunner's lesions. *Canadian Urological Association journal = Journal de l'Association des urologues du Canada*, *3*(6), 478.

Thapa, D., Cairns, E. A., Szczesniak, A.-M., Toguri, J. T., Caldwell, M. D., & Kelly, M. E. M. (2018). The Cannabinoids Δ8THC, CBD, and HU-308 Act via Distinct Receptors to Reduce Corneal Pain and Inflammation. *Cannabis and cannabinoid research*, *3*(1), 11–20.

Thomas, B. F., ElSohly, M. A., Thomas, B. F., & ElSohly, M. A. (2016). Biosynthesis and Pharmacology of Phytocannabinoids and Related Chemical Constituents. *The Analytical Chemistry of Cannabis*, 27–41.

Toczek, M., & Malinowska, B. (2018). Enhanced endocannabinoid tone as a potential target of pharmacotherapy.

Toguri, J. T., Lehmann, C., Laprairie, R. B., Szczesniak, A. M., Zhou, J., Denovan-Wright, E. M., & Kelly, M. E. M. (2014). Anti-inflammatory effects of cannabinoid CB(2) receptor activation in endotoxin-induced uveitis. *British journal of pharmacology*, *171*(6), 1448–61.

Toguri, J. T., Moxsom, R., Szczesniak, A. M., Zhou, J., Kelly, M. E. M., & Lehmann, C. (2015). Cannabinoid 2 receptor activation reduces leukocyte adhesion and improves capillary perfusion in the iridial microvasculature during systemic inflammation. (L. Prantl, E. M. Jung, & F. Jung, Eds.)*Clinical Hemorheology and Microcirculation*, *61*(2), 237–249.

Tomoe, H. (2015). In what type of interstitial cystitis/bladder pain syndrome is DMSO intravesical instillation therapy effective? *Translational andrology and urology*, *4*(6), 600–4.

Tripathi, P., Tripathi, P., Kashyap, L., & Singh, V. (2007). The role of nitric oxide in inflammatory reactions. *FEMS Immunology & Medical Microbiology*, *51*(3), 443–452.

Tschop, J., Kasten, K. R., Nogueiras, R., Goetzman, H. S., Cave, C. M., England, L. G., Dattilo, J., Lentsch, A. B., Tschop, M. H., & Caldwell, C. C. (2009). The cannabinoid receptor 2 is critical for the host response to sepsis. *J Immunol*, *183*(1), 499–505.

Tsuboi, K., Uyama, T., Okamoto, Y., & Ueda, N. (2018). Endocannabinoids and related N-acylethanolamines: biological activities and metabolism. *Inflammation and regeneration*, *38*, 28.

Turcotte, C., Blanchet, M.-R., Laviolette, M., & Flamand, N. (2016). The CB2 receptor and its role as a regulator of inflammation. *Cellular and Molecular Life Sciences*, *73*(23), 4449–4470.

Turcotte, C., Chouinard, F., Lefebvre, J. S., & Flamand, N. (2015). Regulation of inflammation by cannabinoids, the endocannabinoids 2-arachidonoyl-glycerol and arachidonoyl-ethanolamide, and their metabolites. *Journal of Leukocyte Biology*, *97*(6), 1049–1070.

Tyagi, P., Kashyap, M., Majima, T., Kawamorita, N., Yoshizawa, T., & Yoshimura, N. (2017). Intravesical liposome therapy for interstitial cystitis.

Vanden Berghe, W., Vermeulen, L., Delerive, P., De Bosscher, K., Staels, B., & Haegeman, G. (2003). A paradigm for gene regulation: Inflammation, NF-κB and PPAR. In *Advances in Experimental Medicine and Biology* (Vol. 544, pp. 181–196). Kluwer Academic/Plenum Publishers.

Varga, Z. V, Matyas, C., Erdelyi, K., Cinar, R., Nieri, D., Chicca, A., Nemeth, B. T., Paloczi, J., Lajtos, T., Corey, L., Hasko, G., Gao, B., Kunos, G., Gertsch, J., & Pacher, P. (2018). β-Caryophyllene protects against alcoholic steatohepatitis by attenuating inflammation and metabolic dysregulation in mice. *British journal of pharmacology*, *175*(2), 320–334.

Varley, C. L., & Southgate, J. (2008). Effects of PPAR agonists on proliferation and differentiation in human urothelium. *Experimental and Toxicologic Pathology*, *60*(6), 435–441.

Vasudevan, V., & Moldwin, R. (2017). Addressing quality of life in the patient with interstitial cystitis/bladder pain syndrome. *Asian journal of urology*, *4*(1), 50–54.

Walker, J. M., & Hohmann, A. G. (2005). Cannabinoid Mechanisms of Pain Suppression. In *Cannabinoids* (pp. 509–554). Springer-Verlag.

Wang, Y., Liu, Q., & Thorlacius, H. (2003). Docosahexaenoic acid inhibits cytokine-induced expression of P-selectin and neutrophil adhesion to endothelial cells. *European journal of pharmacology*, *459*(2–3), 269–73.

Wang, Z.-Y., Wang, P., & Bjorling, D. E. (2014). Treatment with a cannabinoid receptor 2 agonist decreases severity of established cystitis. *Journal of Urology*, *191*(4), 1153–1158.

Wang, Z. Y., Wang, P., Hillard, C. J., & Bjorling, D. E. (2015). Attenuation of Cystitis and Pain Sensation in Mice Lacking Fatty Acid Amide Hydrolase. *Journal of Molecular Neuroscience*, *55*(4), 968–976.

Warnke, K. C., & Skalak, T. C. (1990). The effects of leukocytes on blood flow in a model skeletal muscle capillary network. *Microvascular Research*, *40*(1), 118–136.

Warren, J. W., Jackson, T. L., Langenberg, P., Meyers, D. J., & Xu, J. (2004). Prevalence of interstitial cystitis in first-degree relatives of patients with interstitial cystitis. *Urology*, *63*(1), 17–21.

Whitmore, K. (2002). Complementary and Alternative Therapies as Treatment Approaches for Interstitial Cystitis. *Reviews in urology*, *4*(1), S28–S35.

Woodfin, A., Voisin, M.-B., Imhof, B. A., Dejana, E., Engelhardt, B., & Nourshargh, S. (2009). Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. *Blood*, *113*(24), 6246–57.

Woodward, D. F., Liang, Y., & Krauss, A. H.-P. (2008). Prostamides (prostaglandin-ethanolamides) and their pharmacology. *British journal of pharmacology*, *153*(3), 410–9.

Wu, J. (2019). Cannabis, cannabinoid receptors, and endocannabinoid system: yesterday, today, and tomorrow. *Acta Pharmacologica Sinica*, *40*(3), 297–299.

Youssef, D. A., El-Fayoumi, H. M., & Mahmoud, M. F. (2019). Beta-caryophyllene protects against diet-induced dyslipidemia and vascular inflammation in rats: Involvement of CB2 and PPAR-γ receptors. *Chemico-Biological Interactions*, *297*, 16–24.

Zacchè, M. M., Srikrishna, S., & Cardozo, L. (2015). Novel targeted bladder drug-delivery systems: a review. *Research and reports in urology*, *7*, 169–78.

Zarbock, A., & Ley, K. (2009). New Insights Into Leukocyte Recruitment by Intravital Microscopy. *Current topics in microbiology and immunology*, *334*, 129–152.

Zarruk, J. G., Fernández-López, D., García-Yébenes, I., García-Gutiérrez, M. S., Vivancos, J., Nombela, F., Torres, M., Burguete, M. C., Manzanares, J., Lizasoain, I., & Moro, M. A. (2012). Cannabinoid type 2 receptor activation downregulates stroke-induced classic and alternative brain macrophage/microglial activation concomitant to neuroprotection. *Stroke*, *43*(1), 211–219.

Zelasko, S., Arnold, W. R., & Das, A. (2015). Endocannabinoid metabolism by cytochrome P450 monooxygenases. *Prostaglandins & Other Lipid Mediators*, *116–117*, 112–123.

Zhang, L., Kline, R. H., McNearney, T. A., Johnson, M. P., & Westlund, K. N. (2014). Cannabinoid receptor 2 agonist attenuates pain related behavior in rats with chronic alcohol/high fat diet induced pancreatitis. *Molecular pain*, *10*, 66.

Zhang, X., He, H., Lu, G., Xu, T., Qin, L., Wang, X., Jin, X., Liu, B., Zhao, Z., Shen, Z., & Shao, Y. (2016). Specific inhibition of ICAM-1 effectively reduces bladder inflammation in a rat model of severe non-bacterial cystitis. *Scientific Reports*, *6*(1), 35672.

Zhao, Y., Liu, Y., Zhang, W., Xue, J., Wu, Y. Z., Xu, W., Liang, X., Chen, T., Kishimoto, C., & Yuan, Z. (2010). WIN55212-2 ameliorates atherosclerosis associated with suppression of pro-inflammatory responses in ApoE-knockout mice. *European Journal of Pharmacology*, *649*(1–3), 285–292.

Zhu, Y., Shen, T., Lin, Y., Chen, B., Ruan, Y., Cao, Y., Qiao, Y., Man, Y., Wang, S., & Li, J. (2013). Astragalus polysaccharides suppress ICAM-1 and VCAM-1 expression in TNF-α-treated human vascular endothelial cells by blocking NF-κB activation. *Acta Pharmacologica Sinica*, *34*(8), 1036–1042.

Zillioux, J., Clements, M., Pike, C. W., & Rapp, D. (2020). Opioid prescription use in patients with interstitial cystitis. *International Urogynecology Journal*, *31*(6), 1215–1220.

Zou, S., & Kumar, U. (2018). Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *International journal of molecular sciences*, *19*(3).