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Inhibiting the Cannabinoid Catabolic Enzyme MAGL to Potentiate the Anti-Arthritic Properties of the Synthetic Glucocorticoid Dexamethasone

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Inhibiting the Cannabinoid Catabolic Enzyme MAGL to Potentiate the Anti-Arthritic Properties
of the Synthetic Glucocorticoid Dexamethasone

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in partial fulfillment of the requirements for the degree of

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Abstract

Inhibiting the Cannabinoid Catabolic Enzyme MAGL to Potentiate the Anti-Arthritic Properties of the Synthetic Glucocorticoid Dexamethasone

Sara R. Nass

Rheumatoid arthritis **(RA, Table 1)** is the most common type of inflammatory arthritis, and is characterized by swelling, inflammation, pain, and destruction of the synovial joints (*i.e.*, knees, elbows, wrists, hips, and fingers). Glucocorticoid receptor agonists are a type of steroid hormone and are among the most common treatments for inflammatory arthritis because of their powerful anti-inflammatory effects. However, harmful side effects are associated with these glucocorticoids (**GCs**), including increasing patients' vulnerability to infections. Cannabinoids (*i.e.*, cannabis-like signaling molecules) exert anti-inflammatory and analgesic effects with limited side effects compared to traditional immunosuppressants making them excellent targets for the development of new arthritic therapeutics. For example, in mice, selective inhibition of the cannabinoid enzyme monoacylglycerol lipase (**MAGL**) reduces acute inflammatory pain and edema. Dual administration of drugs are promising novel treatments because it allows lower doses of drugs to attenuate pain and inflammation, while limiting side effects. Combined administration of an endocannabinoid enzyme inhibitor and nonsteroidal anti-inflammatory drug reduces neuropathic and acute pain. Similarly, inflammatory arthritis is reduced by a GC administered with an anti-inflammatory cytokine (*i.e.*, immune system signaling molecule) in mice. Given the anti-inflammatory properties of MAGL inhibition, it is plausible that MAGL inhibition will increase the analgesic and anti-inflammatory effects of a steroid treatment, perhaps reducing the negative side effects of the steroid. Therefore, the goals of the present studies were to determine the analgesic and anti-inflammatory efficacy of (1) the MAGL inhibitor JZL184; (2) the glucocorticoid steroid dexamethasone (DEX); and (3) the combined administration of both JZL184 and DEX. To these ends, we used the collagen-induced arthritis (**CIA**) mouse model. We found that, although both JZL184 and DEX significantly attenuated proinflammatory cytokine levels in the paws of CIA mice, only DEX decreased pain-related behaviors and paw swelling. Combined administration of a sub-effective dose both drugs was ineffective overall.

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Abbreviation	Full Term
2-AG	2-arachidonoylglycerol
anandamide	N-arachidonoylethanolamine
CB_1	cannabinoid receptor one
CB_2	cannabinoid receptor two
CFA	complete Freund's adjuvant
CIA	collagen induced arthritis
CNS	central nervous system
DEX	dexamethasone
DMARDs	disease-modifying antirheumatic drugs
FAAH	fatty acid amide hydrolase
FLS	fibroblast-like synoviocytes
GCs	glucocorticoids
IFA	incomplete Freund's adjuvant
IL	interleukin
IL-1β	interleukin-1beta
IL-10	interleukin-10
IL-17	interleukin-17
IL-6	interleukin-6
MAGL	monoacylglycerol lipase
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MPO	myeloperoxidase
RA	rheumatoid arthritis
Th1	Type 1 T helper cells
TH17	T helper 17 cells
THC	Δ9-tetrahydrocannabinol
TNFα	tumor necrosis factor alpha
Treg	regulatory T cells

Table 1. Non-standard abbreviations.

Introduction

Rheumatoid Arthritis (**RA**) is a debilitating chronic joint disorder that causes mood disturbances and decreases activity. The most common features are swelling, inflammation, pain, and erosion of the synovial joints (Firestein, 2003). RA pain is primarily induced by chronic inflammation, and has a higher correlation with functional disability compared to joint destruction alone, indicating pain is a greater predictor for RA outcome (Sokka, Kankainen, & Hannonen, 2000). Chronic inflammation and tissue damage in RA is causes by a failure of the host's immune system to discriminate between self and non-self tissue, thus RA is an autoimmune disorder (Lindstrom & Robinson, 2010). The following sections cover a primer on the immune system and the inflammatory response as it relates to RA, followed by background on the endocannabinoid system, and rationale for combined targeting of both immune and cannabinoid systems to combat inflammatory arthritis in the proposed mouse model.

The Immune System

Although the immune system protects the host from pathogens (*e.g.*, viruses and bacteria), an inflammatory response can also be initiated against self-tissue (*i.e.*, autoimmunity) or non-threatening environmental stimuli (*e.g.*, allergy). The two main branches of the immune system are innate and adaptive immunity. Innate immunity is non-specific and mounts a swift immune response in a consistent manner, even after multiple presentations of the same pathogen (Abbas, Litchman, & Pober, 2002). In contrast, a specific response to the presence of pathogens is developed by adaptive (*i.e.*, acquired) immunity. A distinct response and memory for individual pathogens is acquired, so a quicker and more intense response can be mounted after repeat exposure. Adaptive immunity is essential in vaccinations, but also autoimmune disorders such as RA. After immunization with a weakened or killed pathogen, adaptive immunity

develops memory, causing a more powerful immune response when the pathogen is encountered in the future (Abbas et al., 2002). The adaptive immune system is normally nonreactive to tissues originating from the host. However, this tolerance to self-tissue can break down and lead to autoimmunity (Lindstrom & Robinson, 2010).

Although there are many different cells involved in the inflammatory response, only the cells most relevant to the proposed project will be discussed here (see **Figure 1**). The short-lived innate leukocytes (*i.e.*, white blood cells), neutrophils, are the first cells to be activated during the inflammatory response and attract macrophages and dendritic cells to the site of inflammation (Nathan, 2006). Neutrophils express a substantial amount of the enzyme myeloperoxidase (**MPO**), which can be analyzed as an indirect measure of neutrophil activity (Al-Abd et al., 2014). Monocytes are immature innate leukocytes that circulate in the blood and differentiate into macrophages when recruited to inflamed tissue (Abbas et al., 2002). Macrophages release pro-inflammatory cytokines (*i.e.*, the "hormones" of the immune system), and engage in phagocytosis (*i.e.*, engulfing harmful molecules) and antigen presentation (Boissier, Semerano, Challal, Saidenberg-Kermanac'h, & Falgarone, 2012). Dendritic cells bridge the gap between innate and adaptive immunity by engulfing antigens (*i.e.*, substances that induce antibody production) and presenting them via major histocompatibility complex (**MHC**) class II molecules to T cells of the adaptive immune system. Activated helper CD4+T cells then migrate from lymph nodes to the site of inflammation. The Type 1 T helper (**Th1**) and T helper 17 (**Th17**) cell subtypes are considered proinflammatory and activate leukocytes; whereas regulatory T cells (**Tregs**) release anti-inflammatory cytokines (Rabb, 2002). B cells of the adaptive immune system release antibodies that bind to antigens (Rabb, 2002).

Figure 1. Schematic drawing of the immune response in RA. During RA pathogenesis immune cells migrate to the joint synovium. Neutrophils are the first cells recruited to the site of inflammation. Macrophages proliferate in the synovium to ingest foreign bodies and produce proinflammatory cytokines (*e.g.*, IL-1β, TNFα, & IL-6). Cytokines are signaling molecules that mediate inflammation. Dendritic cells present self-antigens to T cells that migrate from the lymph nodes into the synovium. T cells activate leukocytes and also produce proinflammatory cytokines (*e.g.*, IL-17). The anti-inflammatory cytokine IL-10 is also produced by T cells, but there is an imbalance in favor of pro-inflammatory cytokines. B cells produce autoantibodies against self-tissue. The immune cells in the synovium form a pannus (*i.e.*, abnormal layer of tissue) that leads to erosion of cartilage and eventually bone damage.

Cytokines are signaling molecules that modulate the immune response. Cytokines are pleiotropic (*i.e.*, activate a variety of leukocytes) and redundant (*i.e.*, different cytokines have similar effects). Proinflammatory cytokines stimulate leukocyte activation, to eliminate antigens and synthesize more cytokines. The pro-inflammatory cytokines tumor necrosis factor alpha (**TNFα**) and interleukin-1beta (**IL-1β**) are mainly produced by macrophages and stimulate leukocytes; whereas interleukin-17A (**IL-17A**) is produced by Th17 cells and induces proinflammatory cytokine production (Boissier et al., 2012; Shahrara, Huang, Mandelin, & Pope, 2008). The proinflammatory cytokine interleukin-6 (**IL-6**) is released from a variety of

leukocytes and induces antibody production (Boissier et al., 2012). Some cytokines have predominantly anti-inflammatory effects. For example, the anti-inflammatory cytokine interleukin-1 (**IL-10**) is produced by Treg cells and reduces inflammation and regulates the inflammatory response, helping return the host to homeostasis after pathogen elimination (Chen et al., 2012).

Inflammatory Response in Rheumatoid Arthritis

Although the exact etiology of RA is not well understood, the progression of inflammation involves the innate and adaptive immune systems (see **Figure 1**). The synovium (*i.e.*, the soft tissue lining of synovial joints, such as knuckles) is the main site of inflammation and tissue destruction in RA (Smith, 2011). Genetic susceptibility to RA is associated with specific variations of the genes that encode for MHC class II molecules expressed on antigen presenting cells (*e.g.*, dendritic cells and macrophages) (Roudier, 2006). There is an increase in activated macrophages within the RA synovium that release pro-inflammatory cytokines (*e.g.*, TNFα, IL-1β, and IL-6) (McInnes & Schett, 2011). During RA pathogenesis, self-tissues are presented by MHC class II molecules on dendritic cells and macrophages to helper T cells. Within the synovium and blood of RA patients, proinflammatory Th1 and Th17 helper T cell subtypes increase in number, while anti-inflammatory Treg helper T cells decrease (Chen et al., 2012; Shahrara et al., 2008). Immune memory develops when proinflammatory cytokines stimulate cells to produce autoantibodies (*e.g.*, rheumatoid factor) against self-tissue, resulting in further inflammation in the synovium (Takemura, Klimiuk, Braun, Goronzy, & Weyand, 2001). Thus, the initial innate immune response to self-tissue becomes an acquired immune response to self-tissue that generally targets tissues in the synovial joints.

Within the RA synovium there is an abundance of the proinflammatory cytokines TNFα, IL-1β, IL-6, and IL-17 (McInnes & Schett, 2007; Shahrara et al., 2008). These cytokines are also expressed in the serum of RA patients, but not healthy volunteers, indicating an upregulation of proinflammatory cytokines throughout the body (Chen et al., 2012). The presence of IL-10 in RA serum and joints indicates a failure of the immune system to keep RA inflammation in check (Chen et al., 2012). Proinflammatory cytokines induce inflammatory pain by decreasing nociceptor (*i.e.*, free nerve ending that acts as a receptor for pain) activation threshold (Kidd & Urban, 2001).

In addition to leukocytes, fibroblast-like synoviocytes (**FLS**) are mesenchymal cells that proliferate and adhere to the cartilage in RA joints. FLS activated by TNFα and IL-1β aggressively destroy RA joints by releasing more proinflammatory cytokines and matrix metalloproteinase (**MMPs**) that degrade the cartilage extracellular matrix (Karouzakis, Neidhart, Gay, & Gay, 2006). Chronic inflammation and the release of cartilage degrading MMPs causes the synovium to become hyperplastic and develop pannus (*i.e.*, an abnormal tissue layer), eventually leading to cartilage destruction and bone erosion (Karouzakis et al., 2006; McInnes & Schett, 2011).

Current Rheumatoid Arthritis Treatments

Reducing RA symptoms typically involves decreasing joint inflammation and the resulting pain perception. Immunosuppressants, including disease-modifying antirheumatic drugs (**DMARDs**), glucocorticoids (**GCs**), and biologic therapies are the most common pharmacological treatments used to slow RA progression (Singh et al., 2016). However, immunosuppressants can induce aversive side effects, and patients often experience pain despite

improvement in inflammation (Lee, 2013). For example, DMARDs, such as methotrexate reduce pain in addition to inflammation, but need to be administered early to exert analgesic effects. Efficacy of DMARDs as analgesics decreases when introduced even just twelve months after diagnosis (Nell et al., 2004). Biologic therapies suppress specific components of the immune system. However, they are associated with higher annual healthcare expenditure compared to other immunosuppressants, and in some patients are ineffective at slowing the progression of joint damage or reducing symptoms (Walsh & McWilliams, 2012).

Steroids are clinically useful at quickly reducing inflammation and associated pain, but can induce harmful metabolic side effects (Singh et al., 2016). GCs are steroid hormones that are produced from cholesterol in the cortex of the adrenal glands. GCs bind to cytosolic GC receptors within cells to regulate gene expression. Steroids decrease immune cell recruitment through inhibition of the transcription of genes that encode proinflammatory cytokines (Coutinho & Chapman, 2011). In peripheral blood mononuclear cells taken from RA patients, GCs inhibit the production of the proinflammatory cytokines TNFα, IL-1β, IL-6, and IL-17 (Colin et al., 2010; Schlaghecke, Beuscher, Kornely, & Specker, 1994). Due to their potent anti-inflammatory effects, synthetic GCs such as prednisone are effective at treating RA and other inflammatory diseases. However, GCs also activate expression of genes involved in metabolic processes (Coutinho & Chapman, 2011). Therefore, long term use increases the risk of serious aversive effects, including adrenal insufficiency, osteoporosis, diabetes, and cardiovascular risk (Ravindran, Rachapalli, & Choy, 2009). Due to the aforementioned side effects, GCs are mainly administered at low doses for maintenance therapy, or at high doses for short term use to reduce inflammation during "flare-ups" (J. F. Ferreira, Ahmed Mohamed, & Emery, 2016). Although immunosuppressive treatments are effective at slowing disease progression they can lead to

serious side effects because they dampen the immune response. The main obstacle in

immunosuppressant use is the increased susceptibility to infections, such as tuberculosis and

hepatitis (Furst, 2010).

Inflammatory Arthritis Model

Preclinical animal models of inflammatory arthritis are used to study RA mechanisms

and treatments. The collagen-induced arthritis (**CIA**) model of inflammatory arthritis is the most

extensively used animal model to research the pathogenesis of RA progression and potential

novel treatments (Brand, Latham, & Rosloniec, 2007; Williams, Feldmann, & Maini, 1992). CIA

is induced by immunizing mice with a mixture of type II collagen and complete Freund's

adjuvant (**CFA**) (Brand et al., 2007; Kinsey, Naidu, Cravatt, Dudley, & Lichtman, 2011b). Mice

will develop anti-collagen antibodies and three to seven weeks after the initial immunization

swelling and redness develop in one or more paws (Brand et al., 2007). Although the

development of CIA is accelerated compared to RA, the pathogenesis of both is perpetuated by

synovial infiltration of neutrophils as measured by the enzyme myeloperoxidase (**MPO**) (Al-Abd

et al., 2014), autoreactive lymphocytes (*i.e.*, T cells and B cells) (Seki et al., 1988; Svensson,

Jirholt, Holmdahl, & Jansson, 1998), activation of macrophages and FLS (Holmdahl, Tarkowski,

& Jonsson, 1991; Nishioku et al., 2012), and increased proinflammatory cytokine production

(*e.g.*, TNFα, IL-1β, and IL-6) (Rioja, Bush, Buckton, Dickson, & Life, 2004). Similar to RA, the

inflammatory response and synovial pannus in CIA eventually leads to cartilage destruction and

joint damage (Asquith, Miller, McInnes, & Liew, 2009; Brand et al., 2007). Thus, CIA and

human inflammatory arthritis are mechanistically linked.

The Endocannabinoid System

Cannabinoid agonists, such as Δ^9-tetrahydrocannbinol (**THC**), the main psychoactive component of *Cannabis*, bind to and activate the G-protein-coupled receptors, cannabinoid receptor 1 (**CB$_1$**) and cannabinoid receptor 2 (**CB$_2$**) (Mechoulam & Parker, 2013). CB$_1$ mediates the central nervous system (**CNS**) effects of cannabinoids (*e.g.*, pain, hypothermia, and cognitive and behavioral deficits) and is expressed throughout the body, including in the synovium of RA patients and on FLS (Crowe, Nass, Gabella, & Kinsey, 2014; Gui et al., 2014; Richardson et al., 2008). On the other hand, CB$_2$ is expressed mainly on immune cells, but is also expressed at low levels in the CNS, including on activated microglia (*i.e.*, the macrophages of the CNS) (Cabral, Raborn, Griffin, Dennis, & Marciano-Cabral, 2008). In the RA synovium, CB$_2$ is expressed on macrophages, T cells, and B cells, as well as FLS (Fukuda et al., 2014; Gui et al., 2014; Selvi et al., 2008). The presence of cannabinoid receptors in RA joints presents a possible target for novel anti-inflammatory and analgesic treatments.

One of the main mechanisms of cannabinoid modulation of inflammation is the regulation of cytokines. For example, THC attenuates the release of the pro-inflammatory cytokines IL-6 and TNFα *in vitro* from macrophages stimulated by the bacterial endotoxin lipopolysaccharide (Chang, Lee, & Lin, 2001; Fischer-Stenger, Updegrove, & Cabral, 1992). FLS cultured from RA synovial tissue and stimulated with the pro-inflammatory cytokines TNFα or IL-1β, produce and release the pro-inflammatory cytokine IL-6. Pan cannabinoid agonists, such as WIN-55,212-2 and CP55940 (Lowin, Pongratz, & Straub, 2016; Selvi et al., 2008), as well as the selective CB$_2$ agonists, HU-308 and JWH133 (Fukuda et al., 2014; Gui et al., 2014), attenuate the secretion of IL-6 from stimulated FLS. Similarly, the endogenous cannabinoid, anandamide also attenuates TNFα stimulated FLS production of IL-6 and IL-8 (Lowin, Apitz,

Anders, & Straub, 2015). These data suggest that cannabinoids may reduce RA inflammation by attenuating the release of pro-inflammatory cytokines from FLS in the synovium.

Many exogenous cannabinoid agonists have undesirable behavioral and cognitive effects, including deficits in attention, memory, and motor function. Furthermore, chronic administration can lead to tolerance and abuse (Mechoulam & Parker, 2013). One way to bypass the abuse potential and psychomimetic side effects of cannabinoid agonists is to target the endogenous cannabinoid (*i.e.*, endocannabinoid) system. The two major endocannabinoids, N-arachidonoylethanolamine (**anandamide**) and 2-arachidonoylglycerol (**2-AG**), are synthesized *de novo* from phospholipid precursors, and bind to and activate both cannabinoid receptors (Mechoulam & Parker, 2013). Within the CNS, 2-AG is more abundant than anandamide, and acts as a full agonist at the CB_1 and CB_2 receptors (Ahn, McKinney, & Cravatt, 2008). 2-AG is also present in the synovium of RA patients and throughout other areas of the body, including the spleen (*i.e.*, lymphoid organ important in the immune system) (Hillard, 2000; Richardson et al., 2008). Monoacylglycerol lipase (**MAGL**) is the predominant catabolic enzyme for 2-AG, and metabolizes 2-AG into glycerol and arachidonic acid (Blankman, Simon, & Cravatt, 2007). Pharmacological inhibition of MAGL, for example by the highly selective MAGL inhibitor JZL184, increases 2-AG systemically (Long et al., 2009). JZL184 attenuates acute inflammatory pain and edema induced by injecting the seaweed extract carrageenan (Ghosh et al., 2012). However, there are no published reports on the effects of MAGL inhibition in chronic inflammatory pain models, such as arthritis.

Cannabinoids in Inflammatory Arthritis

Although cannabinoids are anti-inflammatory and analgesic with limited side effects compared to traditional immunosuppressants, research focused on the effects of cannabinoids in chronic inflammatory arthritis *in vivo* is limited. The oromucosal mouth spray Sativex, which contains a mixture of the *Cannabis* extracts THC and cannabidiol, decreases self-reports of RA-induced pain in humans (Blake, Robson, Ho, Jubb, & McCabe, 2006). In the preclinical CIA model, repeated administration of cannabidiol, or its synthetic cannabinoid derivatives, decreases paw swelling and cartilage destruction (Haj et al., 2015; Malfait et al., 2000; Sumariwalla et al., 2004). Cannabidiol also attenuates the production of TNF-α from CIA synovial cells (Malfait et al., 2000). Similarly, CIA-induced inflammation, cartilage degradation, and bone erosion is also attenuated by the repeated administration of the selective CB$_2$ agonists JWH133 and HU-308 (Fukuda et al., 2014; Gui, Liu, Liu, Su, & Dai, 2015).

In addition to synthetic cannabinoid agonists, there is evidence that the endocannabinoid system modulates CIA. Just as MAGL catabolizes 2-AG, fatty acid amide hydrolase (**FAAH**) catabolizes the other endocannabinoid, anandamide. FAAH inhibition or genetic deletion increases tissue levels of anandamide. Genetic deletion of FAAH or repeated administration of the FAAH inhibitors URB597 or JNJ1661010 attenuates clinical signs of arthritis, and genetic deletion of FAAH attenuates joint damage in mice subjected to CIA (Kinsey et al., 2011b; Lowin et al., 2015). Interestingly, the FAAH inhibitor URB597 or genetic deletion of FAAH also reduces CIA-induced thermal hyperalgesia (*i.e.*, hypersensitivity to noxious heat) (Kinsey et al., 2011b). These data from human and animal studies suggest that endocannabinoids attenuate inflammatory joint pain and overt signs of joint inflammation.

Combination Treatments

In contrast to the classic approach of targeting a disease with a single drug, combined administration of multiple drugs offers several advantages. Combination therapies may maintain analgesic and anti-inflammatory efficacy by using lower doses of each drug, thereby decreasing the negative side effects (van Laar et al., 2012). In addition, chronic pain is often induced by multiple mechanisms. Although inflammation is the main perpetrator of RA pain, many patients also develop central sensitization, an increase in CNS pain pathway activity that causes hypersensitivity (Lee, 2013). Therefore, addressing multiple targets may increase the efficacy of the drug regimen.

The synthetic glucocorticoid receptor agonist dexamethasone (**DEX**) is used in preclinical research and reduces inflammation in the CIA model (Inglis et al., 2007a). A combination of DEX and the anti-inflammatory cytokine IL-4 attenuates CIA-induced paw inflammation (Kang et al., 2000; Kawalkowska et al., 2016). Interestingly, the combination of DEX and IL-4 also prevents the resurgence of CIA-induced paw swelling after treatment is terminated, indicating combination therapies may limit the need for long term use of glucocorticoids (**GCs**), thereby reducing undesirable side effects (Kawalkowska et al., 2016). Thus, there is evidence to support the idea that combining therapeutics with GCs may also reduce pain and inflammation in models of inflammatory arthritis with increased efficacy and decreased side effects. Endocannabinoid catabolic enzyme inhibition attenuates hyperalgesia and clinical signs of arthritis in the CIA model (Kinsey et al., 2011b), and the synthetic glucocorticoid receptor agonist DEX attenuates CIA-induced inflammation (Inglis et al., 2007a). The present study was designed to determine the analgesic and anti-inflammatory efficacy of (1) the MAGL inhibitor JZL184; (2) the glucocorticoid steroid dexamethasone (DEX); and (3) the

combined administration of both JZL184 and DEX. Our overall goal was to ascertain whether pain and inflammation caused by inflammatory arthritis are further reduced by targeting both glucocorticoid and cannabinoid receptors than by targeting either receptor system alone.
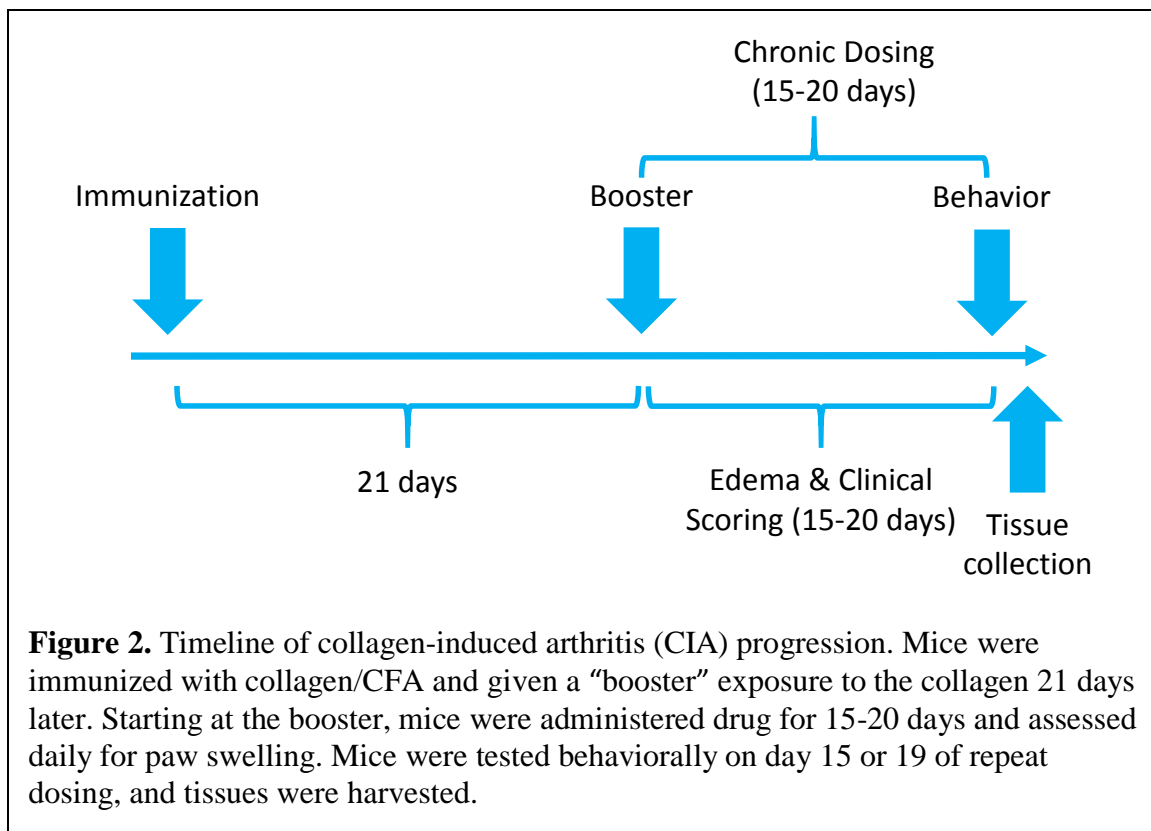
Methods

Animals

Subjects were 226 male DBA/1J (Jackson Laboratory, Bar Harbor, ME) mice 9-12 weeks old at the start of the experiment. Males were chosen because multiple reports (Holmdahl, Jansson, & Andersson, 1986; Jansson & Holmdahl, 2001; Nilsson, Andren, Diaz de Stahl, & Kleinau, 2009) indicate that female mice are protected from collagen induced arthritis and rarely develop arthritis. Mice were housed 3-5 per cage in a temperature (20-22°C) and humidity controlled, AAALAC accredited facility, with *ad libitum* access to food and water, on a 12:12 light:dark cycle. The Institutional Animal Care and Use Committee at West Virginia University approved all the experimental protocols (14-1007).

Collagen Induced Arthritis (CIA) Inflammatory Arthritis

A timeline of the treatments is illustrated in **Figure 2**. Mice were anesthetized with isoflurane (Phoenix Pharmaceuticals, Burlingame, CA), and approximately 100 μl of an emulsion of bovine type II collagen (2 mg/ml), dissolved in 0.05 M acetic acid, in an equal volume of CFA was injected intradermally into the tail. Twenty-one days later (Brand et al., 2007; Impellizzeri et al., 2013), mice were given a secondary "booster" exposure to the collagen preparation, in an equal volume of incomplete Freund's adjuvant (**IFA**), slightly proximal from the first injection (Brand et al., 2007). Control mice were administered an emulsion of IFA and acetic acid, without collagen, at both exposures (Brand et al., 2007). The difference between

CFA and IFA is that CFA contains heat-inactivated bacteria, to cause an innate immune response at the injection site. The CFA in the proposed experiment consisted of *M. tuberculosis* (4 mg/ml) (Becton, Dickinson and Company, Franklin Lakes, NJ) suspended into 85% paraffin oil (Fisher Scientific, Pittsburgh, PA) and 15% mannide monooleate (Fisher Scientific, Pittsburgh, PA) (Brand et al., 2007).

At the conclusion of the experiments, mice were humanely euthanized and hind limb tissue were collected, snap frozen in liquid nitrogen, and stored at -80°C until assay.

Figure 2. Timeline of collagen-induced arthritis (CIA) progression. Mice were immunized with collagen/CFA and given a "booster" exposure to the collagen 21 days later. Starting at the booster, mice were administered drug for 15-20 days and assessed daily for paw swelling. Mice were tested behaviorally on day 15 or 19 of repeat dosing, and tissues were harvested.

Paw edema and clinical scoring

After the "booster" injection, all four paws were examined daily for arthritic signs using a semi-quantitative clinical scoring system as follows: 0, normal; 1, erythema and mild swelling

confined to the ankle joint or toes; 2, erythema and mild swelling extending from the ankle to the

midfoot or ankle joint; 3, erythema and moderate swelling extending from the ankle to the

metacarpal/metatarsal joints; 4, erythema and severe swelling encompassing the ankle, foot, and

digits (Kinsey et al., 2011b). The scores for each limb were summed for each mouse, resulting in

a composite arthritis score with a maximum of 16 total points. Hind paw thickness was also

measured with a digital micrometer, daily until the end of the experiment (Wixey WR100). Data

from our lab as well as others indicate that CIA does not develop in our model until the "booster"

collagen administration and daily handling before the booster reduces arthritis incidence (Brand

et al., 2007).

Behavioral Assessments

Behavioral assessments of mechanical allodynia (*i.e.*, pain perception to non-noxious

touch) and thermal hyperalgesia were conducted on mice subjected to the CIA model of

inflammatory arthritis. Mice were randomly assigned to each drug treatment at the start of each

experiment and repeatedly dosed for 15-20 days (see **Table 2**). Repeated dosing and behavioral

testing schedules were based on the established timeline for mice to develop signs of arthritis and

preliminary data (see **Figure 2** and results**)** (Brand et al., 2007; Gao et al., 2015). Given that the

effects of MAGL inhibition on chronic inflammation are unknown, we used a prophylactic

design, with the goal of increasing the likelihood of detecting an effect of the novel drug

treatments. On behavioral testing days, mice were tested for allodynia followed by hyperalgesia.

The experimenter was blinded to treatments throughout testing. Independent experimenter

reliabilities for behavioral tests and caliper measurements were performed at multiple time points

throughout the experiment and were correlated to > 0.98.

Mechanical allodynia. On test days, mice were brought into the testing room, weighed and injected. Immediately afterwards, mice were placed in ventilated polycarbonate chambers (7.5 x 9 cm) on an aluminum mesh table where they were allowed to acclimate for at least 60 min before testing mechanical allodynia (Kinsey et al., 2009). Mechanical allodynia was tested by stimulating the plantar surface of each mouse hind paw with von Frey filaments (North Coast Medical, Morgan Hill, CA) using the "up-down" method (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994; Crowe et al., 2015). Von Frey filaments are commonly used to test touch sensitivity in humans and other animals and are calibrated to bend at established weights. Each hind paw is stimulated approximately twice per second with each filament (0.16-6.0 g), starting with the 0.6 g filament. Hind paws were stimulated in ascending filament order until the mouse clutched or lifted its paw at least three out of five times. This was considered a positive response, and the sensitivity of each paw was tested by presenting the filaments in descending order to establish a sensory threshold.

Thermal Hyperalgesia. Mice were placed in ventilated polycarbonate chambers (7.5 x 9 cm) on a glass table and acclimated for at least 60 min before testing thermal hyperalgesia (Lichtman, Shelton, Advani, & Cravatt, 2004). Thermal hyperalgesia was tested using the plantar stimulator apparatus (Hargreaves, Dubner, Brown, Flores, & Joris, 1988; Lichtman et al., 2004). A plantar stimulator with a noxious heat source was positioned directly beneath the hind paws of each mouse, under a glass table. The latency for the mouse to withdraw its hind paw from the initiation of the radiant heat was scored as the dependent variable. A 20 s cutoff time was used to avoid the possibility of tissue damage (Kinsey et al., 2011a; Lichtman et al., 2004).

Cytokine and Myeloperoxidase (MPO) Enzyme-Linked Immunosorbent Assay (ELISA)

Paw cytokine and MPO protein levels were quantified by sandwich ELISA, per manufacturer's protocol (R&D Systems, Minneapolis, MN). Paw samples were separated at the ankle joint, cut into small pieces, and homogenized in 1.5 ml phosphate-buffered saline using a Tissue Tearor (Bartlesville, OK). Proinflammatory (TNFα, IL-1β, IL-6, IL-17) and anti-inflammatory (IL-10) cytokines, and MPO were quantified in duplicate.

In brief, 96 well plates were coated with an anti-mouse capture antibody (derived from goat or rat immune cells that were exposed to the target mouse protein) and incubated at room temperature overnight. The following day, the samples and standards were added to each plate and incubated at room temperature for 2 h. Samples were decanted and each plate was washed 3 times with wash buffer (0.05% Tween 20 in phosphate buffered saline). A detection antibody was then added to each well and incubated at room temperature for 2 h. The protein of interest (*i.e.*, cytokine or MPO) became "sandwiched" between the capture antibody and detection antibody. Horseradish peroxidase (Streptavidin-HRP) was then added to each well and incubated for 20 min. HPR linked the detection antibody to a chemical tag (*i.e.*, a 1:1 mixture of H_2O_2 and Tetramethylbenzidine) that produced color. The color reaction was halted after 20 min incubation in the dark by 2 N H_2SO_4. Optical densities were determined with a V max kinetic microplate reader (Molecular Devices, Sunnyvale, CA) and read at 450 nm with a 560 nm correction. Values for known standards on each plate were plotted. The optical density of each unknown sample was fitted to this standard curve to determine the concentration of cytokine or MPO in each sample. Data are reported as the mean of the duplicates. No values were excluded for being above detection limit. The detection limit (protein (min/max)) in pg/ml for each assay

were as follows: TNFα (2000/31.3), IL-1β (1000/15.6), IL-6 (1000/15.6), IL-17 (1000/15.6), IL-10 (2000/31.3), and MPO (16000/250).

Drugs

The MAGL inhibitor JZL184 was purchased from Cayman Chemical (Ann Arbor, MI). The synthetic glucocorticoid DEX was purchased from Sigma-Aldrich (St Louis, MO). Both drugs were dissolved in a vehicle consisting of ethanol, Cremophor (Sigma-Aldrich, St Louis, MO), and saline in a ratio of 1:1:18 parts (Crowe et al., 2015; Kinsey & Cole, 2013). All solutions were administered at room temperature at a volume of 10 µl/g body weight.

Statistical Analyses

Induction of CIA effect (as compared to non-CIA controls), was determined using unpaired t-tests with mechanical allodynia, thermal hyperalgesia, cytokine level, or MPO level as the dependent variable. All other data were analyzed using analysis of variance (ANOVA), followed by Tukey *post hoc* comparisons, with the exception of the DEX dose response data, for which Dunnett's *post hoc* comparison was used instead (all doses compared with CIA/VEH). For the first experiment, JZL184 data and DEX dose response data were analyzed using one way between subjects ANOVA with treatment (CIA/VEH, CIA/JZL184, CIA/DEX) as the independent variable and mechanical allodynia, thermal hyperalgesia, cytokine level, or MPO level as the dependent variable. In the second experiment, a one-way between subjects ANOVA of treatment condition (CIA/VEH, CIA/JZL184, CIA/DEX, CIA/JZL184+DEX) as the independent variable and mechanical allodynia, thermal hyperalgesia, cytokine level, or MPO level as the dependent variable was conducted, followed by Tukey *post hoc* comparisons. Data for clinical scores and edema were compared using mixed design ANOVA with days post immunization as the within subject variables and treatment as the between subjects variables. All

data are presented as mean ± S.E.M. Differences were considered statistically significant if $p <$

0.05.
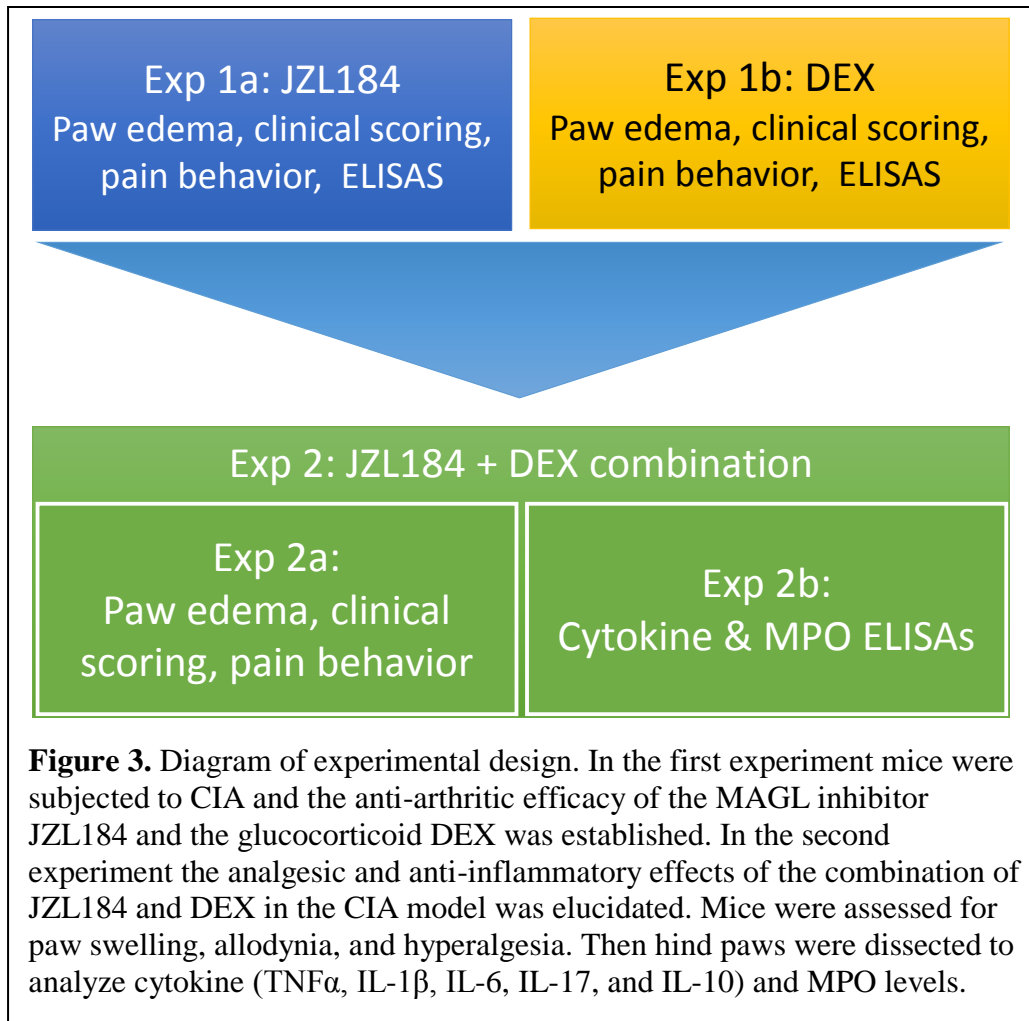
Figure 3. Diagram of experimental design. In the first experiment mice were subjected to CIA and the anti-arthritic efficacy of the MAGL inhibitor JZL184 and the glucocorticoid DEX was established. In the second experiment the analgesic and anti-inflammatory effects of the combination of JZL184 and DEX in the CIA model was elucidated. Mice were assessed for paw swelling, allodynia, and hyperalgesia. Then hind paws were dissected to analyze cytokine (TNFα, IL-1β, IL-6, IL-17, and IL-10) and MPO levels.

Experimental Design

An outline of the experimental design is summarized in **Figure 3**. Preliminary

experiments were performed to determine the optimal behavioral testing days for mice subjected

to CIA. Mice were administered a collagen/CFA emulsion to induce CIA, followed 21 days later

by a booster exposure to collagen. Separate non-CIA control mice were also tested. Paw edema

was assessed daily using digital calipers and arthritis severity was scored using a semi-

quantitative clinical scale. Mice were tested for mechanical allodynia and thermal hyperalgesia

(Impellizzeri et al., 2013; Kinsey et al., 2009; Kinsey et al., 2011b) on days 22, 25, 28, 31, and

34 post immunization to determine the optimal behavioral testing timeline. Planned comparisons

were used to establish appropriate testing days for the proceeding experiments, as choosing the

optimal test day was the rationale for running these experiments.

The goal of the first experiment was to determine the appropriate doses of the MAGL

inhibitor JZL184 (Exp1a) or the steroid DEX (Exp1b) to combine in the CIA model of

inflammatory arthritis.

Exp1a. Determine anti-arthritic efficacy of the selective MAGL inhibitor JZL184. Mice

were subjected to CIA or control procedures. Starting at the booster individual mice were

administered a dose of JZL184 (8 mg/kg) (Kinsey et al., 2013; Schlosburg et al., 2010) or

vehicle once daily, for 15 consecutive days (*i.e.*, days 21-35; see **Table 2**). Separate non-

CIA control mice were also tested. Paw edema was assessed daily using digital calipers

and arthritis severity was scored using a semi-quantitative clinical scale. Mice were tested

for mechanical allodynia and thermal hyperalgesia (Impellizzeri et al., 2013; Kinsey et

al., 2009; Kinsey et al., 2011a) on day 14 of repeated dosing. On the 15th day of repeated

dosing, mice were humanly euthanized and hind limb tissues were collected. It was

hypothesized that JZL184 (8 mg/kg) would attenuate CIA-induced paw swelling and

behavioral pain responses.

A separate group of mice were subjected to CIA and administered a dose of

JZL184 (4 mg/kg) (Kinsey et al., 2013; Schlosburg et al., 2010) or vehicle once daily, for

15 consecutive days. Disease progression was assessed via paw swelling, clinical scoring,

allodynia, and hyperalgesia (see **Table 2**). On the 15th day of repeated dosing, mice were

humanly euthanized and hind limb tissues were collected. Due to a lack of allodynic or hyperalgesic effect of CIA, mice were dosed consecutively for an additional three days and tested for grip strength (see **Appendix A**) on day 16 and humanly euthanized and hind limb tissue were collected on day 18. It was hypothesized that JZL184 (4 mg/kg) would be subeffective at attenuating CIA-induced paw swelling and behavioral pain responses.

Exp1b. Determine anti-arthritic efficacy of the synthetic glucocorticoid steroid dexamethasone. As in Exp1a, CIA was induced and a dose response curve was constructed to establish the anti-inflammatory and analgesic potency of the synthetic steroid dexamethasone (DEX; 0.0625, 0.125, 0.25, or 0.5 mg/kg) (Inglis et al., 2007a). For 15 consecutive days, mice were administered a dose of DEX or vehicle and then assessed for paw swelling, disease progression (*i.e.*, clinical scoring), allodynia, and hyperalgesia (see **Table 2**). Due to the powerful anti-inflammatory effects of DEX, a second cohort of mice was run to test even lower doses of dexamethasone (*i.e.*, 0.015625, 0.03125, or 0.5 mg/kg) than originally proposed. For 15 consecutive days, mice were administered a dose of DEX or vehicle and then assessed for paw swelling, disease progression, allodynia, and hyperalgesia (see **Table 2**). Due to a lack of a behavioral effect of CIA, mice were dosed consecutively for an additional five days and retested on day 19, and humanly euthanized, and hind limb tissue were collected on day 21. It was hypothesized that DEX would dose dependently attenuate CIA-induced paw swelling and pain responses.

The goal of the <u>second experiment</u> was to determine the analgesic and anti-inflammatory effects of repeated combined administration of both the MAGL inhibitor JZL184 and the synthetic glucocorticoid DEX in the CIA model of inflammatory arthritis.

<u>Exp2a. Determine the behavioral and arthritic effects of dual administration of subeffective doses of both JZL184 and dexamethasone.</u> Mice were administered JZL184 (4 mg/kg), DEX (0.015625 mg/kg), JZL184 (4 mg/kg) +DEX (0.015625 mg/kg), or vehicle once daily, for 15 consecutive days following the booster exposure to collagen (*i.e.*, day 21) (see **Table 2**). During repeated drug administration, mice were assessed daily for gross signs of paw inflammation and disease progression was assessed by clinical scoring. After 15 days of treatment (*i.e.*, day 35), each mouse was tested for allodynia, and hyperalgesia, as detailed above. Again, due to a null CIA effect in the behavioral tests, mice were consecutively dosed for another four days and retested on day 19, humanly euthanized, and hind limb tissues were collected. It was hypothesized that the combination of subeffective doses of JZL184 and DEX would attenuate CIA-induced paw swelling and pain responses to a greater degree than either drug alone.

Experiment 1a: JZL184 Dosing

Condition	Control	CIA	CIA	Condition	Control	CIA	CIA
JZL184 (mg/kg)	0	0	8	JZL184 (mg/kg)	0	0	4
Mice	12	12	12	Mice	10	10	10

Experiment 1b: DEX DRC

Condition	Control	CIA	CIA	CIA	CIA	CIA
DEX (mg/kg)	0	0	0.0625	0.125	0.25	0.5
Mice	10	10	9	10	9	10

Condition	Control	CIA	CIA	CIA	CIA
DEX (mg/kg)	0	0	0.015625	0.03125	0.5
Mice	10	10	9	9	9

Experiment 2: Combination JZL + DEX

	Control	CIA	
	VEH	VEH	JZL184
Vehicle	9	11	8
DEX	-	9	9

Table 2. Treatment groups and total sample size for each experiment.

Exp2b. Determine the inflammatory effects of dual JZL184/DEX administration. Hind paws were dissected and homogenized to analyze proinflammatory (TNFα, IL-1β, IL-6, and IL-17) and anti-inflammatory (IL-10) cytokine and myeloperoxidase (MPO) levels using sandwich ELISA. It was hypothesized that the combination of JZL184 and DEX would decrease proinflammatory cytokine levels and increase anti-inflammatory cytokine levels.

Results

Preliminary data: CIA induces paw swelling and thermal hyperalgesia

Due to the inconsistencies with the CIA model in our lab in the past, we first ran a pilot group of mice to demonstrate that we could induce CIA and to establish a behavioral timeline. Mice were immunized with collagen to induce CIA, and given a booster exposure to collagen 21 days later. A group of non-CIA control mice were given control emulsions. CIA induced an increase in arthritic clinical scores [$F(1, 15) = 21.547$; p <.01; **Figure 4A**] and paw thickness [$F(1, 15) = 7.693$; p <.01; **Figure 4B**] compared to non-CIA controls.

Mice were tested for mechanical allodynia and thermal hyperalgesia on days 22, 25, 28, 31, and 34 post immunization. In the von Frey test, there was no interaction between treatment and days post immunization [$F(4, 22) = .6786$, p = .1347; **Figure 4C**], nor was there a main effect of treatment [$F(1, 22) =.8031$, p = .8031] or days post immunization [$F(4, 22) =.579$, p = .6786], indicating CIA did not induce mechanical allodynia. Although in the plantar stimulator test there was also no interaction between treatment and days post immunization [$F(4, 22) = 1.828$, p = .1305; **Figure 4D**] or main effect of days post immunization [$F(4, 22) =2.173$, p = .0786], there was a main effect of treatment [$F(1, 22) = 15.612$, p <.01], indicating CIA induced thermal hyperalgesia. Although there was only a main effect of collagen treatment and not an interaction effect in the plantar stimulator test, data for each individual testing day were analyzed based on a planned comparison to establish a behavioral timeline. *Post hoc* analyses revealed that CIA induced hyperalgesia on days 22, 31, and 34, but not days 25 or 28 post immunization. Based on these data behavioral testing was performed at least 31 days post immunization in all proceeding experiments. Although CIA did not induce mechanical allodynia in the preliminary

experiment, mechanical allodynia was still tested in experiments one and two, because it previously has been the most consistent behavioral test for the CIA model in our lab.

Experiment 1a: Repeated MAGL inhibitor JZL184 partially attenuates CIA-induced paw cytokine level increase

We next performed repeated administration of the MAGL inhibitor JZL184 (8 or 4 mg/kg) to establish a subthreshold dose to be combined with the steroid DEX in experiment two. Tolerance develops after repeated dosing (6 days) of JZL184 (16 or 40 mg/kg) (Kinsey et al., 2013; Schlosburg et al., 2010). Therefore, we hypothesized that a lower dose of JZL184 (4 mg/kg) would be ineffective at attenuating CIA-induced pain and inflammation without causing tolerance after repeated administration.

We first ran mice with only a moderate dose of JZL184 (8 mg/kg) to establish an effective dose of JZL184. Acute administration of JZL184 (8 mg/kg) attenuates CIA-induced thermal hyperalgesia (Nass, 2015), but these data were obtained before the model was optimized. Mice were administered the MAGL inhibitor JZL184 (8 mg/kg, *s.c.*) or vehicle once daily for 15 consecutive days starting at the booster. There was an interaction in paw swelling between treatment and days post immunization as measured by arthritic clinical scores [$F(30, 33) =$ 10.544; p <.01; **Figure 5A**] and paw thickness [$F(30, 33) = 7.693$; p <.01; **Figure 5B**]. *Post hoc* comparisons revealed that CIA increased arthritic scores and paw thickness. Furthermore, *post hoc* analyses also indicated a statistical increase in paw swelling in JZL184/CIA mice compared to CIA. However, based on data from other cohorts of mice the effect is probably due to differences in paw swelling across collagen exposed mice and unlikely to replicate. Thus, the JZL184 effect is probably not biologically relevant.

Mice were tested for mechanical allodynia and thermal hyperalgesia on day 14 post booster. There was a significant effect of drug treatment on mechanical allodynia [$F(2, 33) = 4.653$, p <.05; **Figure 5C**], although *post hoc* analyses indicated CIA did not induce mechanical allodynia. CIA induced thermal hyperalgesia [$F(2, 33) = 11.623$, p <.01; **Figure 5D**], but JZL184 (8 mg/kg) did not reverse this CIA-induced thermal hyperalgesia.

Mice were humanely euthanized day 15 post booster and hind limb tissue were harvested. Right hind paws were separated and homogenized. A sandwich ELISA was used to analyze proinflammatory (TNFα, IL-1β, IL-6, and IL-17) and anti-inflammatory (IL-10) cytokine and MPO levels. CIA significantly increased paw levels of the proinflammatory cytokines TNFα [$F(2, 33) = 7.298$, p <.01; **Figure 6A**], IL-1β [$F(2, 33) = 7.149$, p <.01; **Figure 6B**], IL-6 [$F(2, 33) = 9.188$, p <.01; **Figure 6C**], and IL-17 [$F(2, 33) = 7.069$, p <.01; **Figure 6D**], the anti-inflammatory cytokine IL-10 [$F(2, 33) = 8.978$, p <.01; **Figure 6E**], and the neutrophil marker MPO [$F(2, 33) = 16.859$, p <.01; **Figure 6F**]. *Post hoc* analyses revealed that the JZL184/CIA mice did not significantly differ in proinflammatory (TNFα, IL-1β, IL-6, and IL-17) and anti-inflammatory (IL-10) cytokine from either the CIA or control mice. These results suggest that JZL184 (8 mg/kg) partially attenuates cytokine levels in CIA paws. MPO levels were not affected by JZL184 (8 mg/kg).

Due to the partial attenuation of CIA paw inflammation by JZL184 (8 mg/kg), we next tested a separate group of mice with a low dose of JZL184 (4 mg/kg) to establish a subeffective dose. For 18 consecutive days, mice were administered the MAGL inhibitor JZL184 (4 mg/kg, *s.c.*) or vehicle once daily. There was an interaction between treatment and days post immunization in clinical scores [$F(34, 27) = 5.854$; p <.01; **Figure 7A**] and caliper

measurements [$F(34, 27) = 6.310$; p <.01; **Figure 7B**]. *Post hoc* comparisons revealed that CIA increased paw swelling, but JZL184 (4 mg/kg) was ineffective.

On day 15 post booster, mice were tested for pain-related behaviors. CIA did not induce mechanical allodynia [$F(2, 27) = 1.701$; p =.2015; **Figure 7C**] or thermal hyperalgesia [$F(2, 27) = 1.722$; p =.4340; **Figure 7D**]. Due to the lack of CIA effect, there was no JZL184 (4 mg/kg) attenuation of pain-related behaviors.

On day 18 post booster mice were humanely euthanized and hind limbs were harvested. Similar to the previous experiment, CIA significantly increased paw levels of cytokines TNFα [$F(2, 27) = 7.298$, p <.01; **Figure 8A**], IL-1β [$F(2, 27) = 5.662$, p <.01; **Figure 8B**], IL-6 [$F(2, 27) = 7.378$, p <.01; **Figure 8C**], IL-17 [$F(2, 27) = 12.035$, p <.01; **Figure 8D**], IL-10 [$F(2, 27) = 7.547$, p <.01; **Figure 8E**]. However, CIA did not significantly increase MPO [$F(2, 27) = 3.294$, p =0525; **Figure 8F**] levels. *Post hoc* analyses revealed that JZL184 (4 mg/kg) had no effect on pro and anti-inflammatory (IL-10) cytokine levels or MPO.

These data reveal that JZL184 (4 mg/kg) is subeffective in CIA-induced paw swelling and inflammatory mediators.

Experiment 1b: Repeated steroid dexamethasone attenuates CIA-induced pain and inflammation

We next performed a dose response curve of repeated administration of the steroid DEX to establish a subthreshold dose to be combined with JZL184 in experiment two. Repeated (15 days) DEX (0.5 mg/kg) administration attenuates CIA-induced paw swelling, allodynia, and hyperalgesia (Unpublished data from our lab). Therefore, we hypothesized that repeated administration of a lower dose of DEX (0.25 mg/kg or below) would yield a subthreshold dose

that does not attenuate arthritic signs. CIA induced paw swelling as measured by clinical scores [$F(15, 18) = 5.259$; p <.01; **Figure 9A**] and paw thickness [$F(15, 18) = 4.527$; p <.01; **Figure 9B**] compared to non-CIA controls. Mice were administered the steroid DEX (0.0625, 0.125, 0.25, or 0.5 mg/kg; *s.c.*) or vehicle once daily for 15 consecutive days. In CIA mice there was an interaction between drug treatment and days post immunization in clinical scores [$F(4, 60) = 4.902$; p <.01; **Figure 9A**] and paw thickness [$F(4, 60) = 4.388$; p <.01; **Figure 9B**]. *Post hoc comparisons* revealed that all doses of DEX attenuated CIA-induced increase in arthritic scores and paw diameter.

Mice were tested for allodynia and hyperalgesia on day 15 post booster. CIA had no effect on mechanical allodynia, compared to control mice [$t(18) = 1.828$, p = .0842; **Figure 9C**]. However, DEX (0.0625, 0.125, or 0.5 mg/kg) decreased paw withdrawal thresholds in the von Frey test [$F(4, 43) = 4.537$; p < .01; **Figure 9C**], indicating an anti-allodynic effect. Similarly, CIA *per se* did not induce thermal hyperalgesia in the plantar stimulator test [$t(18) = 1.144$, p = .2674; **Figure 9D**]. DEX had no effect on thermal hyperalgesia as compared with CIA mice [$F(4, 43) = .184$; p = .9456; **Figure 9D**]. Due to the robust reduction in paw swelling by DEX (all doses), ELISAs were not run to determine cytokine and MPO levels in these mice.

Because DEX ($\geq$0.0625 mg/kg) attenuated CIA-induced paw swelling, we ran a second dose response curve with lower doses of DEX (*i.e.*, 0.015625 or 0.03125 mg/kg) to find a subthreshold dose. The highest dose (*i.e.*, 0.5 mg/kg) and negative controls were also included. CIA increased arthritic clinical scores [$F(14, 18) = 11.310$; p <.01; **Figure 10A**] and paw thickness [$F(14, 18) = 6.493$; p <.01; **Figure 10B**] compared to non-CIA controls. For 20 consecutive days mice were administered DEX or vehicle once daily. There was a significant interaction between DEX treatment and days post immunization in mice administered collagen in

clinical scores [$F_{(42, 32)}$ = 6.493; p <.01; **Figure 10A**] and paw thickness [$F_{(42, 32)}$ = 5.470; p <.01; **Figure 10B**]. *Post hoc* analyses revealed all doses of DEX (≥0.015625 mg/kg) attenuated CIA-induced hind paw thickness, but clinical scores were reduced at (≥0.03125 mg/kg DEX).

On day 19 post booster, CIA increased mechanical allodynia [$t_{(18)}$ = 5.916, p <.01; **Figure 10C**] and thermal hyperalgesia [$t_{(18)}$ = 4.829, p <.01; **Figure 10D**], as compared to non-CIA control mice. Interestingly, in CIA mice, only the high dose DEX (0.5 mg/kg) attenuated mechanical allodynia [$F_{(3, 33)}$ = 4.919; p < .01; **Figure 10C**] and thermal hyperalgesia [$F_{(3, 32)}$ = 3.559, p <.05; **Figure 8D**].

Mice were humanely euthanized on day 20 post booster and proinflammatory (TNFα, IL-1β, IL-6, and IL-17) and anti-inflammatory (IL-10) cytokine and MPO paw levels were analyzed by sandwich ELISA. CIA significantly increased paw levels of IL-1β [$t_{(18)}$ = -2.791, p <.05; **Figure 11B**], IL-6 [$t_{(18)}$ = -3.043, p <.01; **Figure 11C**], and IL-10 [$t_{(18)}$ = -2.605, p <.05; **Figure 11E**] as compared to non-CIA controls. The CIA increase in IL-1β [$F_{(3, 32)}$ = 5.317, p <.01; **Figure 11B**], IL-6 [$F_{(3, 32)}$ = 6.900, p <.01; **Figure 11C**], and IL-10 [$F_{(3, 32)}$ = 4.573, p <.01; **Figure 11E**] tissue levels was attenuated by all doses of DEX (≥0.015625 mg/kg). Similarly, the paw levels of TNFα [$t_{(18)}$ = -2.703, p <.05; **Figure 11A**], IL-17 [$t_{(18)}$ = -2.112, p <.05; **Figure 11D**], and MPO [$t_{(18)}$ = -4.363, p <.01; **Figure 11F**] were increased in CIA mice compared to non-CIA controls. However, only DEX (≥0.03125 mg/kg) attenuated the CIA increase in paw TNFα [$F_{(3, 32)}$ = 4.552, p <.01; **Figure 11A**], IL-17 [$F_{(3, 32)}$ = 3.724, p <.05; **Figure 11D**], and MPO [$F_{(3, 32)}$ = 6.941, p <.01; **Figure 11F**].

These data indicate that DEX (0.015625 mg/kg) was ineffective at attenuating CIA-induced arthritic clinical scores, pain-related behavior, and proinflammatory cytokines TNFα and IL-17 and MPO paw levels. Therefore, DEX (0.015625 mg/kg) was used in Experiment Two.

Experiment 2a: Repeated dual administration of the MAGL inhibitor JZL184 and the

steroid dexamethasone attenuates CIA-induced paw swelling

In Experiment Two, we determined the effects of dual administration of subeffective doses of the MAGL inhibitor JZL184 (4 mg/kg) and the steroid DEX (0.015625 mg/kg), as established in Experiment One. We hypothesized that combined JZL184 and DEX would attenuate CIA-induced arthritic signs, but would be ineffective alone. CIA increased arthritic clinical scores [$F(19, 18) = 11.098$; $p < .01$; **Figure 12A**] and paw thickness [$F(19, 18) = 6.348$; $p < .01$; **Figure 12B**] compared to non-CIA controls. Mice were administered the MAGL inhibitor JZL184 (4 mg/kg), the steroid DEX (0.015625 mg/kg), a combination of JZL184 (4 mg/kg) and DEX (0.015625 mg/kg), or vehicle subcutaneously once daily for 19 consecutive days. In CIA mice, there was a significant interaction between treatment and days post immunization in clinical scores [$F(60, 43) = 4.902$; $p < .01$; **Figure 12A**] and paw thickness [$F(60, 43) = 4.388$; $p < .01$; **Figure 12B**]. *Post hoc* comparisons revealed that DEX and coadministration of JZL184/DEX attenuated CIA-induced arthritic clinical scores and paw thickness.

Mice were tested for mechanical allodynia and thermal hyperalgesia on day 19 post booster. CIA-induced mechanical allodynia [$t(18) = 2.210$, $p < .05$; **Figure 12C**] compared to non-CIA control mice. However, in mice administered collagen JZL184, DEX, or JZL/DEX did not attenuate the CIA-induced increase in paw withdrawal thresholds in the von Frey test [$F(3, 33) = 1.729$; $p = .1801$; **Figure 12C**]. CIA did not induce thermal hyperalgesia [$t(18) = 1.660$, $p = .1142$; **Figure 12D**]. Due to the lack of a CIA effect, *post hoc* comparisons revealed no significant attenuation of hyperalgesia by JZL184, DEX, or JZL/DEX [$F(3, 33) = 1.611$, $p = .2056$; **Figure 12D**]. Taken together, these results suggest that JZL/DEX attenuates CIA-induced paw swelling, but the effect is probably driven by DEX.

Experiment 2b: Repeated dual administration of the MAGL inhibitor JZL184 and the steroid dexamethasone did not attenuate CIA-induced increase in paw cytokines and MPO

Mice were humanely euthanized on day 19 post booster and hind limbs were taken. Proinflammatory (TNFα, IL-1β, IL-6, and IL-17) and anti-inflammatory (IL-10) cytokine and MPO levels were analyzed by sandwich ELISA. CIA significantly increased paw levels of the proinflammatory cytokines TNFα [$t(18) = -2.521$, p <.05; **Figure 13A**], IL-1β [$t(18) = -2.635$, p <.05; **Figure 13B**], IL-6 [$t(18) = -32.535$, p <.05; **Figure 13C**], IL-17 [$t(18) = -2.416$, p <.05; **Figure 13D**], and the neutrophil marker MPO [$t(18) = -4.036$, p <.01; **Figure 13F**] as compared to non-CIA controls. CIA paw tissue increase in TNFα [$F(3, 33) = 1.058$, p =.3801; **Figure 13A**], IL-1β [$F(3, 33) = .569$, p =.6392; **Figure 13B**], IL-6 [$F(3, 33) = .856$, p =.4733; **Figure 13C**], IL-17 [$F(3, 33) = .985$, p =.4118; **Figure 13D**], and MPO [$F(3, 33) = 1.100$, p =.3632; **Figure 13F**] was unaffected by JZL184 (4 mg/kg), DEX (0.015625 mg/kg), or JZL184 (4 mg/kg) + DEX (0.015625 mg/kg). Similarly, CIA did not increase paw tissue levels of IL-10 [$t(18) = -1.475$, p =.1575; **Figure 13E**] and JZL184, DEX, or JZL/DEX had no effect on IL-10 [$F(3, 33) = 1.1274$, p =.2995; **Figure 13E**].

Taken together, these data indicate that dual administration of JZL184/DEX did not affect the CIA-induced increase in proinflammatory cytokines or MPO paw tissue levels.

Discussion

The overall goal of the present study was to elucidate the effects of targeting cannabinoid and glucocorticoid receptors alone and in combination, to attenuate pain and inflammation caused by the collagen-induced arthritis (**CIA**) mouse model of inflammatory arthritis. This is the first study to investigate monoacylglycerol lipase (**MAGL**) inhibition in a chronic pain model, either alone or in combination with the synthetic steroid dexamethasone (**DEX**).

The first aim (Exp 1a) of the present study was to determine the efficacy of the selective MAGL inhibitor JZL184 in attenuating inflammatory arthritis. Repeated administration of JZL184 (4 or 8 mg/kg, *s.c.*) did not attenuate CIA-induced paw swelling. However, JZL184 (8 mg/kg) partially attenuated the CIA-increased proinflammatory (IL-1β, TNFα, IL-6, & IL-17) and anti-inflammatory (IL-10) cytokines. These data align with previous studies demonstrating that MAGL inhibition by JZL184 attenuates proinflammatory (IL-1β, TNFα, & IL-6) and anti-inflammatory (IL-10) cytokines in other preclinical models of inflammation (Kerr et al., 2013; Kinsey et al., 2011c).

The second Aim (Exp 1b) of the present study was to determine the efficacy of the synthetic glucocorticoid receptor agonist dexamethasone on CIA-induced inflammation and pain. Repeated administration of DEX (≥0.03125 mg/kg, *s.c.*) attenuated CIA-induced arthritic clinical scores. DEX (≥0.015625 mg/kg, *s.c.*) attenuated the CIA-increased proinflammatory (IL-1β & IL-6) and anti-inflammatory (IL-10) cytokines, whereas DEX (≥0.03125 mg/kg, *s.c.*) attenuated the CIA-increased proinflammatory (TNFα and IL-17) cytokines and MPO. These results confirm previous reports that DEX attenuates CIA-induced paw swelling and cytokine levels (Inglis et al., 2007a; Kawalkowska et al., 2016; Pu et al., 2016). This is the first report to show that DEX (0.5 mg/kg, *s.c.*) also attenuates CIA-induced allodynia and hyperalgesia.

The third Aim (Exp 2) of the present study was to determine the behavioral and immune effects of dual administration of subeffective doses of both JZL184 and dexamethasone. Due to its absence of anti-inflammatory effects in overt paw swelling and paw cytokine levels, the 4 mg/kg dose of JZL184, was chosen. Similarly, the 0.015625 mg/kg dose of DEX was chosen, due to its lack of attenuation of CIA-induced arthritic clinical scores and paw cytokine (IL-1β, IL-6, & IL-10) levels. Repeated dual administration of JZL184 (4 mg/kg) and DEX (0.015625

mg/kg) attenuated CIA-induced paw swelling. However, solo DEX (0.015625 mg/kg) also

reduced paw swelling to a similar extent as JZL184/DEX, indicating that this effect is probably

driven by DEX. JZL184/DEX did not attenuate the CIA-induced increase in pro-inflammatory

cytokine and MPO paw levels.

There is a paucity of research on behavior using the CIA model of inflammatory arthritis.

Hence, we first performed a behavioral time course and determined that in our lab, CIA-induced

thermal hyperalgesia starting 31 days post immunization, which corresponds to the beginning of

a significant increase in paw swelling in CIA mice. These results align with previous reports that

the onset of CIA-induced thermal hyperalgesia coincides with a significant increase in paw

swelling in rats (Nieto, Clark, Grist, Chapman, & Malcangio, 2015). It was surprising that

repeated administration of JZL184 (8 mg/kg) did not attenuate CIA-induced thermal

hyperalgesia, because MAGL inhibition attenuates acute inflammatory pain induced by

intraplantar injection of carrageenan (Ghosh et al., 2012; Ignatowska-Jankowska et al., 2013), a

seaweed extract known to cause paw swelling. One possible explanation for the weak MAGL

effect is that the inflammatory response in our model is more robust than other models. In

support of this idea, JZL184 (8 mg/kg) did not reduce overt paw swelling despite partially

attenuating paw cytokine levels. Alternatively, the mice may have developed partial tolerance to

repeated JZL184 administration. Repeated administration of high doses (*e.g.*, 40 mg/kg or 16

mg/kg) of JZL184 reduces CB_1 expression and sensitization (Schlosburg et al., 2010).

Furthermore, tolerance develops to the analgesic and gastroprotective effects of JZL184 after

repeated administration (6 days) of high doses (40 mg/kg or 16 mg/kg), but not low dose (4

mg/kg) in mice (Kinsey et al., 2013; Schlosburg et al., 2010). Although the lower dose of

JZL184 (8 mg/kg) used in the present study did not show tolerance at the cytokine level after 15

days of repeated dosing, the effects of a 15 day treatment of JZL184 on cannabinoid receptor

expression and sensitization are unknown and may reflect differences in CB_1 vs. CB_2

susceptibility to tolerance.

Tolerance to JZL184 may be prevented at low doses (Kinsey et al., 2013). Therefore, we

ran a separate cohort of mice to investigate the effects of JZL184 (4 mg/kg) on CIA-induced pain

and inflammation. JZL184 (4 mg/kg) did not attenuate CIA-induced paw swelling or paw

cytokine levels, indicating that 4 mg/kg is a subeffective dose in the CIA model. That said, there

was no CIA effect in either the von Frey or plantar stimulator tests to use as a comparison group

to properly determine the potential anti-analgesic effects of JZL184 (4 mg/kg). Although the von

Frey and plantar stimulator tests are the most commonly reported preclinical pain tests in the

CIA model (Gao et al., 2015; Impellizzeri et al., 2013; Inglis et al., 2007b), in our hands,

behavior in either test was highly variable across cohorts. Therefore, we also piloted grip

strength, which may discern CIA-induced loss of muscle function. In the grip strength test, a

mouse is inverted on a wire cage bottom and the latency to fall from the wire is measured

(Hutter-Saunders, Gendelman, & Mosley, 2012). We found the paw swelling interferes with the

ability to grip the wire, as CIA mice showed a significant reduction in latency to fall from the

wire that was unaffected by JZL184 treatment (please see **Appendix A**).

The overall goal of the present study was to find a subeffective dose of the steroid DEX

to combine with the MAGL inhibitor JZL184 to reduced CIA-induced pain and inflammation.

The steroid DEX is a well-known anti-inflammatory drug that attenuates CIA-induces paw

swelling (Inglis et al., 2007a; Kawalkowska et al., 2016) and proinflammatory cytokines (*e.g.,*

IL-1β, TNFα, IL-6, & IL-17) in the serum of RA patients and rats subjected to CIA (Colin et al.,

2010; Pu et al., 2016; Schlaghecke et al., 1994). Furthermore, DEX attenuates LPS-induced paw

edema and tissue increase of the proinflammatory cytokines IL-1β and TNFα (Naidu, Kinsey,

Guo, Cravatt, & Lichtman, 2010). Due to its potent anti-inflammatory effects, it was difficult to

select a subeffective dose of DEX in the present study. After running two dose response curves it

was determined that DEX (≥0.03125 mg/kg) attenuates CIA-induced paw swelling and increase

in paw cytokine levels. DEX (0.015625 mg/kg) did not reduce CIA-induced arthritic clinical

scores, mechanical allodynia, or thermal hyperalgesia and therefore, was chosen as the dose

coadministered with JZL184.

We confirmed data from published reports that high dose DEX attenuates CIA

inflammation, including paw swelling, clinical scores, and proinflammatory cytokine levels

(Inglis et al., 2007a; Kawalkowska et al., 2016; Pu et al., 2016). We extended these findings by

demonstrating that repeated DEX (0.5 mg/kg) administration also attenuates CIA-induced

mechanical allodynia and thermal hyperalgesia. The observed analgesic and anti-inflammatory

effects of DEX on inflammatory pain and paw swelling in the chronic CIA model is consistent

with previous literature that DEX attenuates pain and edema in acute models of inflammatory

pain (S. H. Ferreira et al., 1997; Naidu et al., 2010; Sasso et al., 2012). The present data, together

with previous studies, suggest that DEX attenuates both chronic and acute inflammatory pain and

inflammation. However, from a practical point of view, steroid hormones are not a viable long-

term treatment for RA, especially at high doses, due to their harmful side effects such as

metabolic problems, adrenal insufficiency, osteoporosis, and mood changes (Nelson, 2011).

Therefore, the third goal of the present study was to coadminister subeffective doses of

the MAGL inhibitor JZL184 and the steroid DEX to attenuated CIA-induced arthritic signs while

mitigating aversive side effects. The combination of low doses of JZL184 and DEX attenuated

arthritic clinical scores in the present study, but the combination group did not differ from the

DEX treated group, which indicates that the observed anti-inflammatory effect was likely driven

by DEX and not JZL184. Although two dose response curves of DEX were run in present study,

it is plausible that an even lower dose of DEX than we selected would yield a clearer

coadministration effect. Indeed, despite the lack of a reduction in arthritic clinical scores by DEX

(0.015625 mg/kg) in the dose response curve in Exp 1b, this same DEX dose did attenuate paw

swelling in Exp 2. In support of the idea that DEX drove the anti-inflammatory effects, other labs

have reported that glucocorticoids dominate the attenuation of CIA-induced paw swelling in

combined treatments (Joosten et al., 1999; Kang, Lee, & Lee, 2000; Kawalkowska et al., 2016).

For example, dual administration of DEX (0.25 or 0.025 mg/kg) and the anti-inflammatory

cytokine IL-4 reduced paw inflammation, whereas IL-4 alone was ineffective, suggesting that the

anti-inflammation was predominantly driven by DEX (Kang et al., 2000; Kawalkowska et al.,

2016). When DEX is repeatedly administered and then administration in halted, mice subjected

to CIA demonstrate a "rebound" effect with exaggerated paw swelling once DEX is withheld

(Kang et al., 2000; Kawalkowska et al., 2016). Interestingly, mice administered the dual

DEX/IL-4 treatment had less of a rebound of paw swelling than mice in the DEX-only treatment

(Kawalkowska et al., 2016). These data indicate that, although DEX may dominate the anti-

inflammatory effects of combined treatment, it is plausible that dual administration with another

anti-inflammatory mediator may continue to reduce inflammation after DEX treatment is

terminated. Steroid discontinuation-induced inflammation is problematic for patients of chronic

inflammatory disorders because steroids are recommended only for short-term use due to their

aversive side effects (Singh et al., 2016). Unfortunately, in the present study the protection of

JZL184 against DEX discontinuation-induced inflammation was not tested because paw tissue

was harvested to determine for cytokines, but the idea of MAGL inhibition protecting from

"steroid rebound" warrants further investigation. For example, mice may be subjected to CIA and administered DEX (as in Exp 1b) with or without JZL184. When DEX administration is suspended, a rebound effect (*e.g.*, paw swelling) would be expected in DEX-treated mice, but JZL184 may attenuate such paw swelling.

Only one dose of JZL184/DEX coadministration was tested in the present study due to time and expense constraints, but it would be worth exploring multiple doses of JZL184/DEX for dual administration. The goal of the present study was to combine subeffective doses of both drugs to attenuate CIA-induced pain and inflammation to the same degree as an effective dose of either drug alone. Nevertheless, coadministration of subeffective doses of JZL184/DEX only reduced arthritic clinical scores. Although solo DEX attenuated CIA-induced inflammation and pain at high doses, aversive side effects limit the use of steroids in a clinical setting (Ravindran et al., 2009). However, solo JZL184 partially attenuated CIA-induced increase in paw cytokine levels at the 8, but not 4 mg/kg dose. Therefore, subeffective DEX combined with a partially effective dose of JZL184 (*i.e.,* 8 mg/kg) may yield a greater anti-arthritic effect compared to only a subeffective dose of JZL184, while limiting the risks associated with steroid use.

Although dual administration of JZL184 and DEX did not attenuate the CIA-induced increase in paw pro- and anti-inflammatory cytokine levels, the combination treatment partially attenuated MPO, a marker of neutrophil activation. This blunted MPO response may indicate that JZL184/DEX attenuates paw swelling by reducing immune cell activation, rather than by decreasing immune cell signaling. To better address this hypothesis, it would be interesting to quantify paw levels of different types of immune cell using flow cytometry. Dual administration of DEX/IL-4 decreases the amount of pro-inflammatory Th-17 cells, while increasing the amount of anti-inflammatory Treg cells (Kawalkowska et al., 2016). Thus, in addition to

decreasing neutrophil activity, the combination of JZL184/DEX may also attenuate the activity of other immune cells involved in RA, including macrophages, T cells, and B cells.

Due to the decrease in CIA-induced paw swelling by coadministration of JZL184/DEX future studies may also investigate joint tissue damage, using histology. Previous reports indicate that FAAH inhibition or CB_2 receptor agonism attenuates paw swelling and joint degradation (Fukuda et al., 2014; Gui et al., 2015; Kinsey et al., 2011b). These data, along with the present study, suggest that dual administration of JZL184/DEX may also reduce CIA-induced joint damage.

A potential limitation of this study is that the dosing schedule was prophylactic and mice received drug treatment prior to the development of paw swelling. Prophylactic treatments are important for a variety of medical conditions that have an increased likelihood of occurring, including thromboembolic disease or infection after surgeries (Alhassan et al., 2017; Enzler, Berbari, & Osmon, 2011). Further, vaccines are a major source of preventative treatments (Rappuoli, Pizza, Del Giudice, & De Gregorio, 2014). However, most disorders are not detectable until the symptoms are present, and therefore must be treated therapeutically. In preclinical research both types of designs offer distinct advantages. Therapeutic dosing regiments are crucial in preclinical models to increase translatability to many human medical disorders. However, investigating preventative treatment is also useful in preclinical research. Although translatability is essential, it also is important to know if an effect is worth pursing further. In the present study there was a lack of an anti-inflammatory or analgesic effect after preventative treatment with the combination of JZL184 and DEX. Given that JZL/DEX could not prevent paw inflammation from developing, it is unlikely that dual administration would be anti-inflammatory after paw inflammation was already present.

A significant limitation of the CIA model chosen for the present study is that there was insufficient CIA induction of allodynia or hyperalgesia to determine the potential analgesic effects of dual JZL184/DEX treatment. In addition to the behavioral tests used, another potential reason for the inconsistent pain behavior is the CIA model itself. Although CIA is a great model of inflammatory arthritis, due to the strong similarities in pathogenesis with RA, there are many complicating factors in this model that make it difficult to use. For example, the presence of paw swelling is an obvious indication of collagen exposure. In the present study this was mitigated by blinding the experimenter to drug treatment, but due to the nature of the behavioral assays and paw swelling measurements it is impossible to blind an experimenter to the presence of paw swelling. Another limitation is that some of the mice immunized to collagen never develop paw swelling (Brand et al., 2007). The incidence of paw swelling in non-drug treated mice in our lab is typically above 80%, but there is considerable variance between experiments, a reflection of the variance of CIA responses between mouse cohorts. Furthermore, swelling across paws is not uniform in each individual mouse and paw swelling across mice is inconsistent. Multiple methodological refinements were made to increase consistency across cohorts. We also tried to mitigate inconsistencies across cohorts of mice by having only one rater across all experiments to increases internal reliability. Although interrater reliabilities were conducted periodically to minimize drift, having only one rater may decrease reproducibility. A possible remedy to the problem of variance in swelling across paws and incidence across cohorts is to trade the strong mechanistic link between RA and CIA for more control within each experiment by using a different model of inflammatory arthritis.

In contrast to the CIA model, which is mainly used to research RA immunosuppressant treatments, injection of an inflammatory mediator into the footpad is extensively used to

characterize chronic inflammatory pain (Ghasemlou, Chiu, Julien, & Woolf, 2015; Inglis et al., 2007b). For example, intraplantar injection of CFA (without collagen, as is used in the CIA model) into the hind paw footpad of rodents induces chronic inflammatory pain in the injected paw, but not contralateral paw (Bagdas, AlSharari, Freitas, Tracy, & Damaj, 2015). Although intraplantar injection of an inflammatory agent induces fairly consistent edema and swelling across animals, the immune response does not activate lymphocytes of the adaptive immune system, and thus does not model the autoimmune response germane to rheumatoid arthritis RA (Ghasemlou et al., 2015). This disconnect between inflammatory research and pain research limits external validity and translational potential. For example, many immunosuppressant treatments for RA are ineffective at attenuating pain (Lee, 2013). Data from our lab indicate that the CB_2 receptor agonist HU-308 attenuates CIA-induces paw swelling, but has no effect on allodynia; whereas HU-308 attenuates CFA-induced hyperalgesia, but not paw edema (see **Appendix B**). These data indicate the importance of incorporating behavioral tests into traditional models of inflammatory arthritis to develop novel treatments for RA that reduce both pain and inflammation.

Another alternative to the CIA model that is gaining popularity among inflammatory arthritis researchers is the collagen-antibody-induced arthritis (CAIA) model. In the classic CIA model, antibodies to self-collagen are produced by the mice. In the CAIA model, arthritis is induced by administration of these anti-collagen antibodies, along with immune stimulation by the gram-negative bacterial marker lipopolysaccharide (Kagari, Doi, & Shimozato, 2002). Swelling develops in one or more paws within 10 days and lasts approximately three weeks. Inflammation eventually leads to cartilage and bone destruction, similar to CIA and RA. The major benefit of CAIA is that the time course is more rapid than in CIA, but unlike RA and CIA

the joint inflammation in the CAIA model is not perpetuated by the T-cells of the adaptive immune system that are important in the induction of autoimmune disorders (Asquith et al., 2009; Kagari et al., 2002). We conducted a pilot study using the CAIA model (see **Appendix C**), but unfortunately the CAIA mice did not develop significant paw swelling compared to non-CAIA control mice, and thus MAGL inhibition was not anti-inflammatory. We consulted with the vendor, who was unable to explain the lack of arthritic development. Regardless, CAIA is reported to induce arthritis in multiple mouse strains that are resistant to CIA, as well as females (Khachigian, 2006). Thus, CAIA may offer broader opportunities to investigate cannabinoid effects on inflammatory arthritis, for example using genetic knockout mice that do not express MAGL.

In summary, we found that MAGL inhibition partially reduces paw inflammation caused by collagen-induced arthritis. This is the first demonstration of an anti-inflammatory effect of MAGL inhibition and an analgesic effect of DEX in a chronic inflammatory joint pain model. As previously demonstrated, the synthetic glucocorticoid dexamethasone was a potent anti-inflammatory agent. Overall, the effects of either drug varied by test, by model, and by cohort. This variance may explain why behavioral data are rarely reported in the preclinical inflammatory arthritis literature. Given the strong negative side effects of current arthritis treatments, including but not limited to repeated steroid administration, the evidence that MAGL inhibition had anti-inflammatory effects is encouraging and merits additional investigation.

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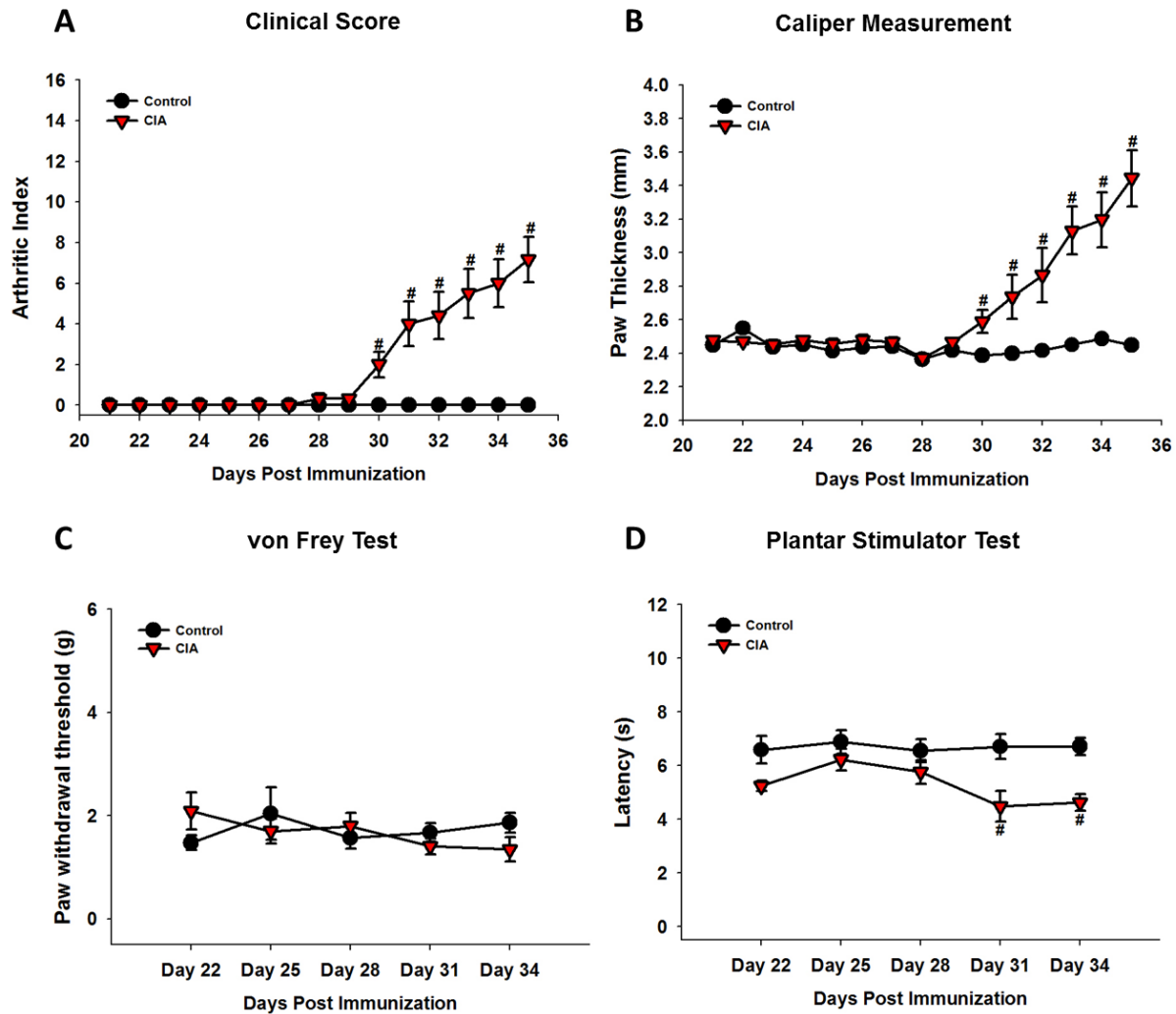
Figure 4. Collagen-induced arthritis (CIA) induces paw swelling and thermal hyperalgesia. Mice were administered an emulsion of collagen/CFA and then given a booster exposure to collagen 21 days later. Control mice were administered an emulsion of acetic acid/IFA. CIA mice developed an increase in arthritic clinical scores (A) and hind paw thickness (B). Mice were tested for pain-related behaviors on days 22, 25, 28, 31, and 34 post immunization. Mice did not develop mechanical allodynia tested via the von Frey assay (C), but did develop thermal hyperalgesia in the plantar stimulator test (D). Data expressed as mean $\pm$ SEM (n = 12). # $p <$ 0.05 vs. control mice.
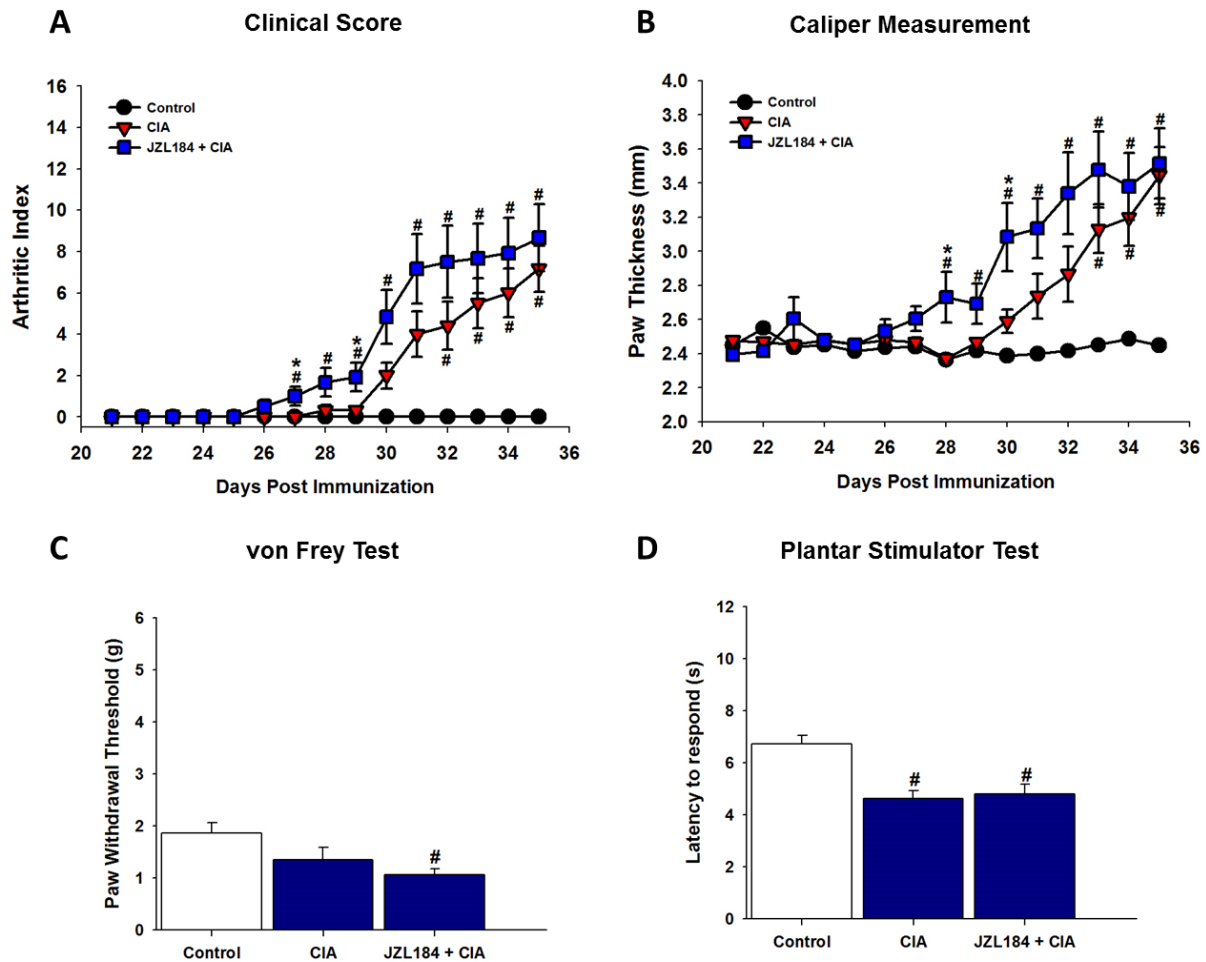
Figure 5. Repeated dosing of the MAGL inhibitor JZL184 (8 mg/kg) did not affect paw swelling, allodynia, or hyperalgesia in mice subjected to CIA. Mice were administered JZL184 (8 mg/kg, *s.c.*) or vehicle for 15 days. JZL184 did not attenuate CIA-induce paw swelling as measured by clinical scores (A) or paw thickness (B). CIA mice did not develop mechanical allodynia (C), but did develop thermal hyperalgesia (D). Data expressed as mean ± SEM (n = 12). * $p < 0.05$ vs. CIA mice; # $p < 0.05$ vs. control mice.
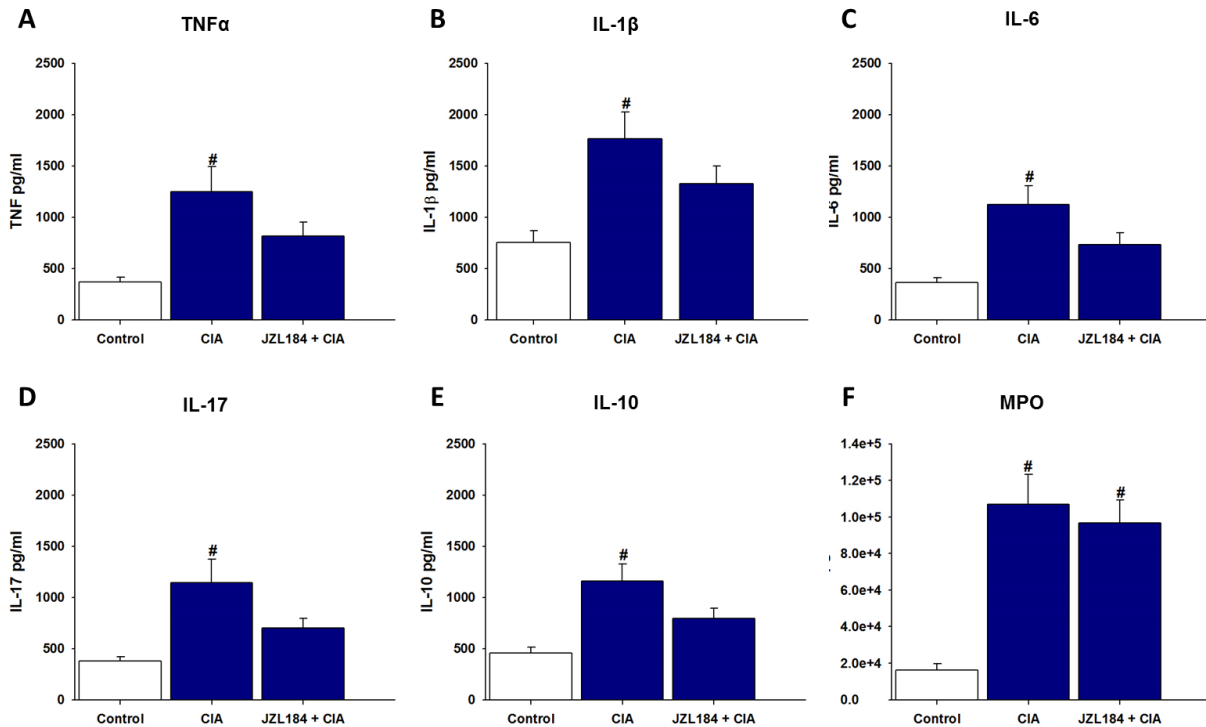
Figure 6. Repeated dosing of the MAGL inhibitor JZL184 (8 mg/kg) partially attenuated CIA-induced increase in paw levels of proinflammatory (TNFα, IL-1β, IL-6, and IL-17) (A-D) and anti-inflammatory (IL-10) (E) cytokines. JZL184 did not affect the CIA-induced increase in MPO (F), a marker of neutrophil activity. Mice were administered JZL184 or vehicle for 15 days. Data expressed as mean ± SEM (n = 12). # $p < 0.05$ vs. control mice.
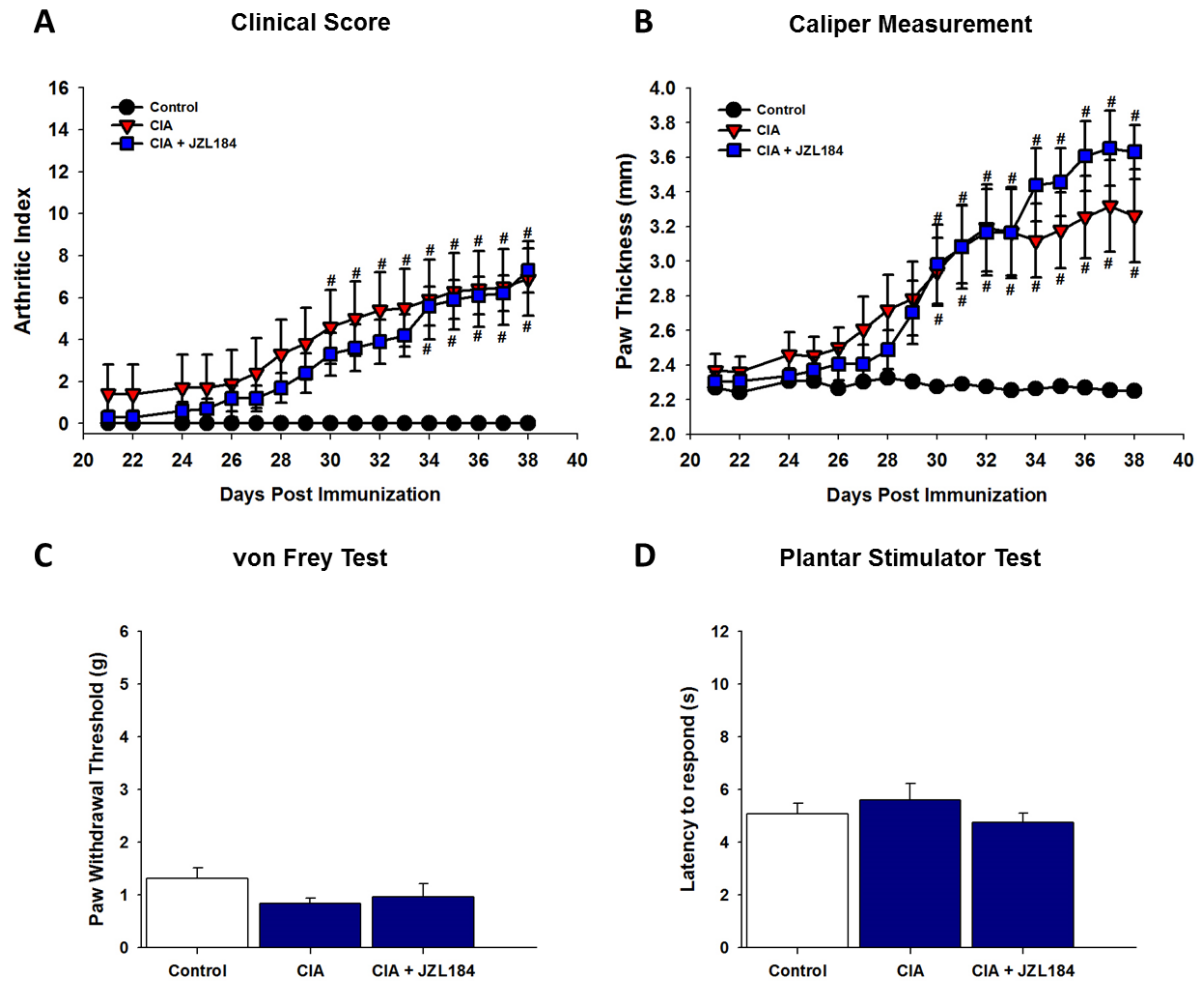
Figure 7. Repeated dosing of the MAGL inhibitor JZL184 (4 mg/kg) was subeffective at attenuating CIA-induced paw swelling. Mice were administered JZL184 or vehicle for 18 days. JZL184 did not attenuate the CIA-induce increase in arthritic clinical scores (A) or hind paw thickness (B). CIA mice did not develop mechanical allodynia (C) or thermal hyperalgesia (D). Data expressed as mean ± SEM (n = 10). # $p < 0.05$ vs. control mice.
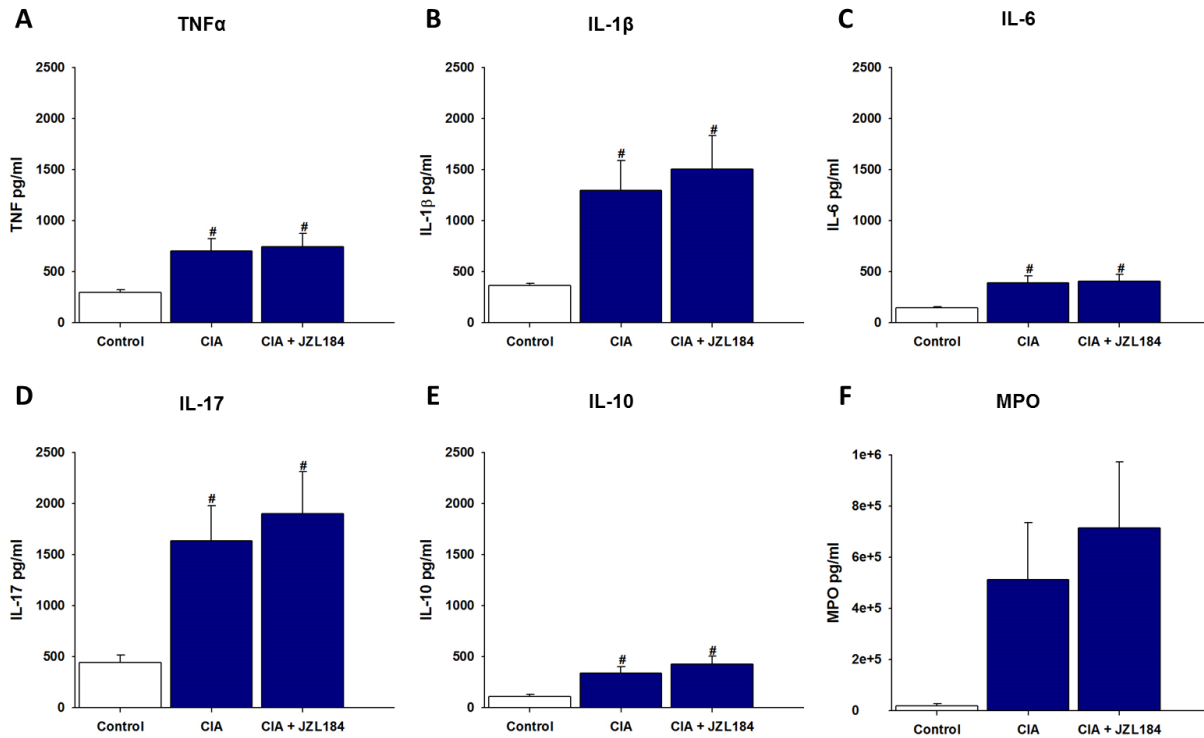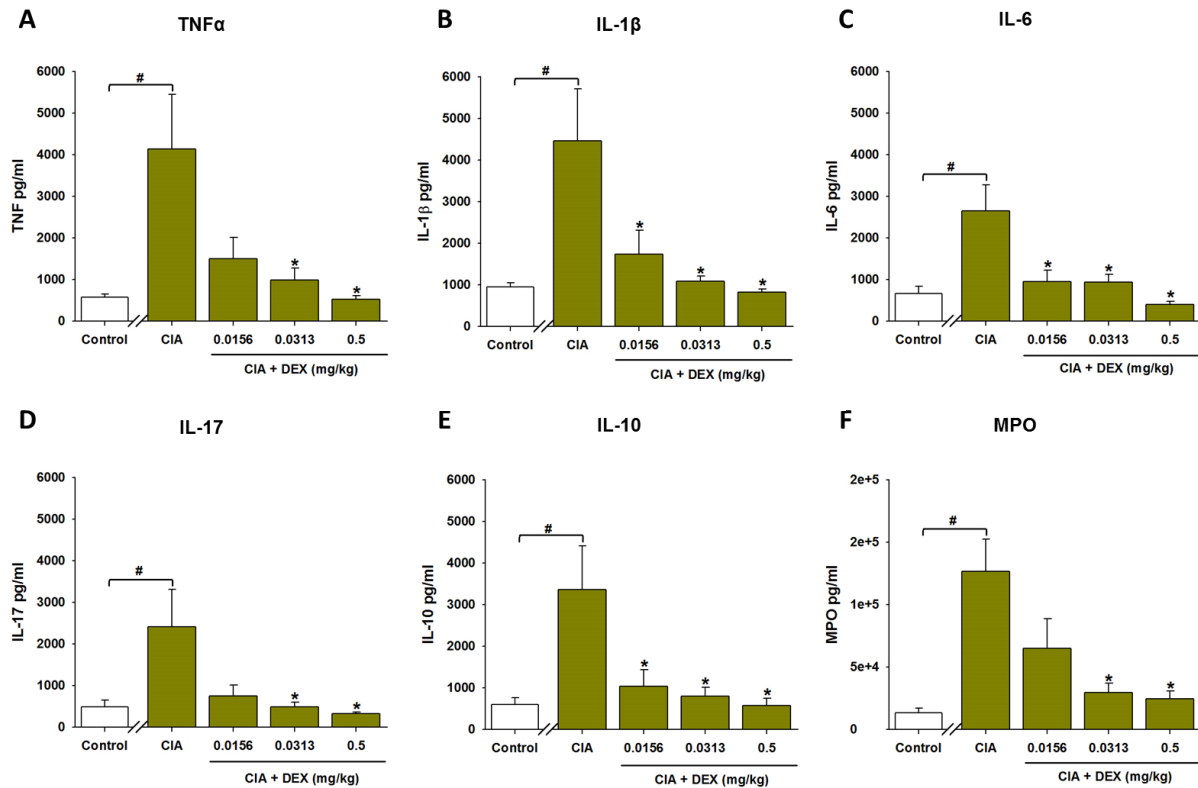
Figure 8. Repeated dosing of the MAGL inhibitor JZL184 (4 mg/kg) was subeffective at attenuating the CIA-induced increase in paw levels of proinflammatory (A-D) and anti-inflammatory (E) cytokines. CIA did not increase paw MPO (F) levels. Mice were administered JZL184 or vehicle for 18 days. Data expressed as mean ± SEM (n = 10). # $p < 0.05$ vs. control mice.
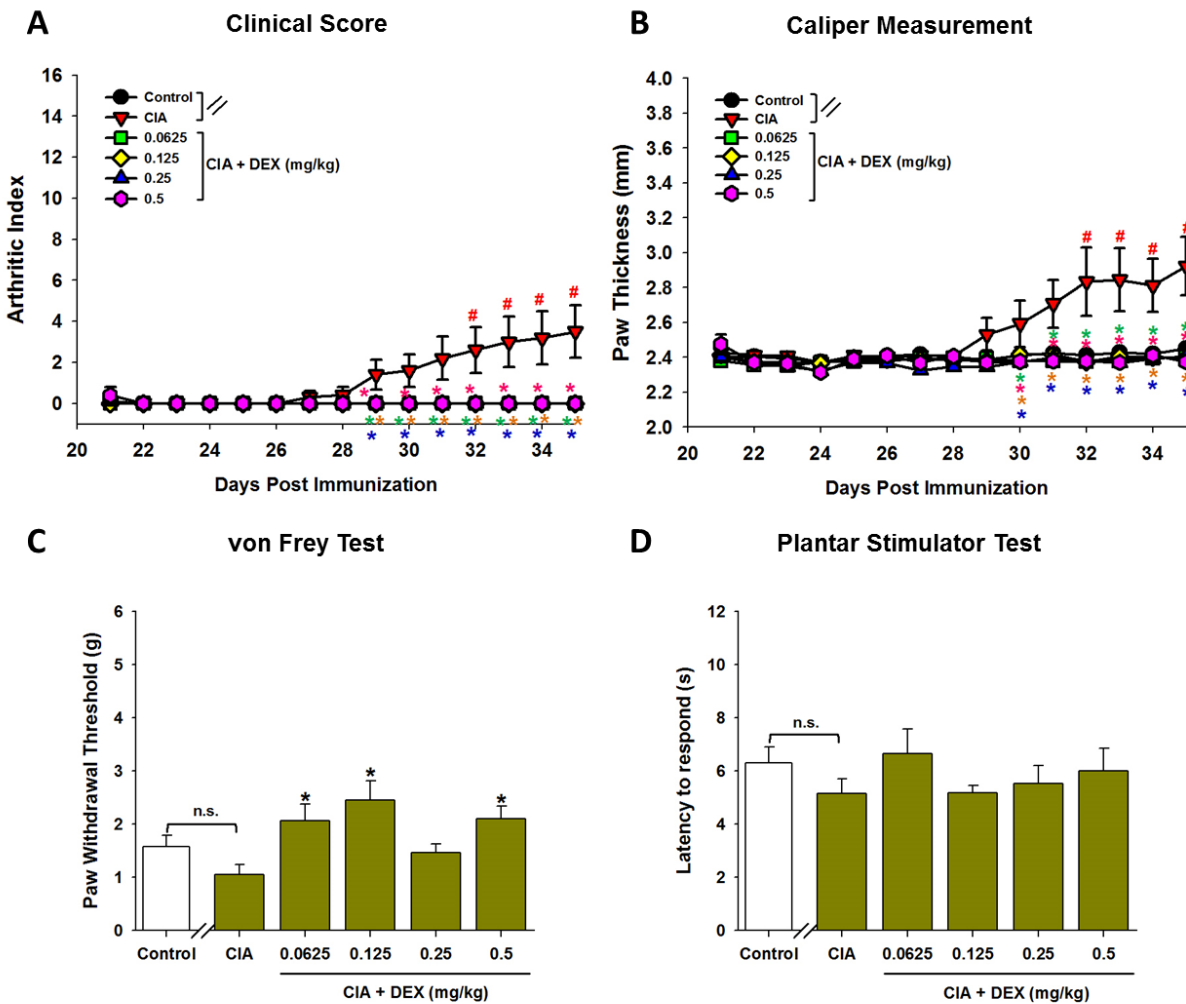
Figure 9. Repeated dosing of the steroid dexamethasone (DEX) attenuated paw swelling and allodynia, in mice subjected to CIA. Mice were administered DEX or vehicle once daily for 15 consecutive days. DEX attenuated CIA-induced clinical scores (A) or paw thickness (B). Although CIA did not induce mechanical allodynia, DEX attenuated mechanical allodynia (C). CIA did not induce thermal hyperalgesia in the plantar test (D). Data expressed as mean $\pm$ SEM (n = 9-10). * $p < 0.05$ vs. CIA mice; # $p < 0.05$ vs. control mice.
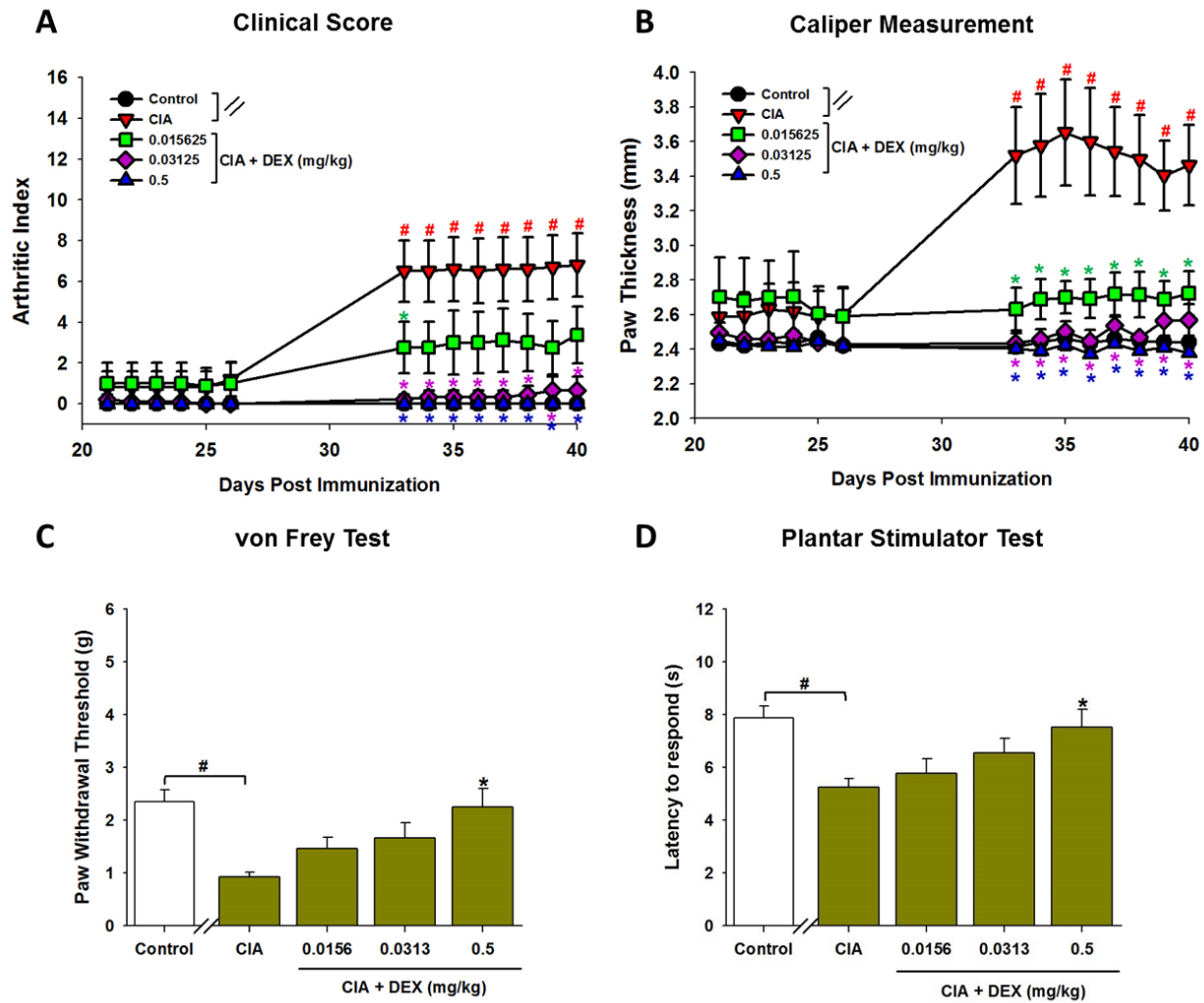
Figure 10. Repeated dosing of the steroid dexamethasone (DEX; 0.015625 mg/kg) was subeffective at attenuating arthritic clinical scores and pain-related behavior in CIA mice. Mice were administered DEX or vehicle once daily for 20 days. DEX attenuated CIA-induced arthritic clinical scores (A) paw thickness (B), mechanical allodynia (C), and thermal hyperalgesia (D). Data expressed as mean ± SEM (n = 9-10). * $p < 0.05$ vs. CIA mice; # $p < 0.05$ vs. control mice.

Figure 11. Repeated dosing of the steroid dexamethasone (DEX) was subeffective at attenuating the CIA-induced increase in proinflammatory cytokine (TNFα and IL-17) and MPO paw levels. Mice were administered DEX or vehicle once daily for 20 days. DEX attenuated the CIA-induced paw increase in proinflammatory cytokines (A-D) and the neutrophil marker MPO (F). Data expressed as mean ± SEM (n = 9-10). * $p < 0.05$ vs. vehicle CIA mice; # $p < 0.05$ vs. control mice.
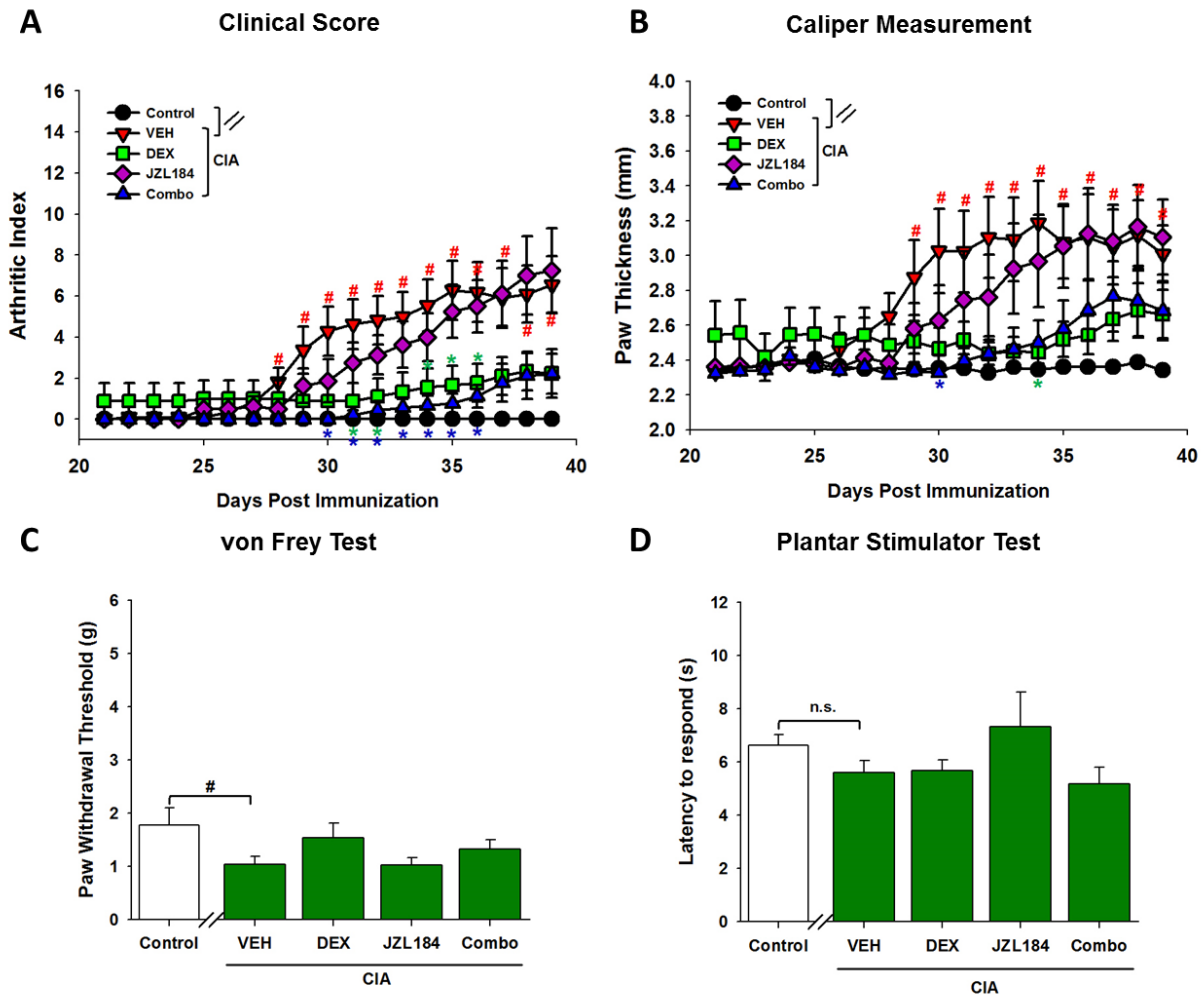
Figure 12. Repeated, dual administration of JZL184/DEX, or DEX alone, attenuated CIA-induced paw swelling. Mice were administered the JZL184 (4 mg/kg*; s.c.*), DEX (0.015625 mg/kg*; s.c.*), both JZL184 (4 mg/kg; *s.c.*) and DEX (0.015625 mg/kg; *s.c.*), or vehicle once daily for 19 days. DEX or JZL184/DEX attenuated CIA-induce arthritic clinical scores (A) and caliper measurements (B). CIA-induced mechanical allodynia (C) was not attenuated by drug treatment. There was no CIA-induced thermal hyperalgesia (D). Data expressed as mean ± SEM (n = 9-11). * $p < 0.05$ vs. CIA mice; # $p < 0.05$ vs. control mice.
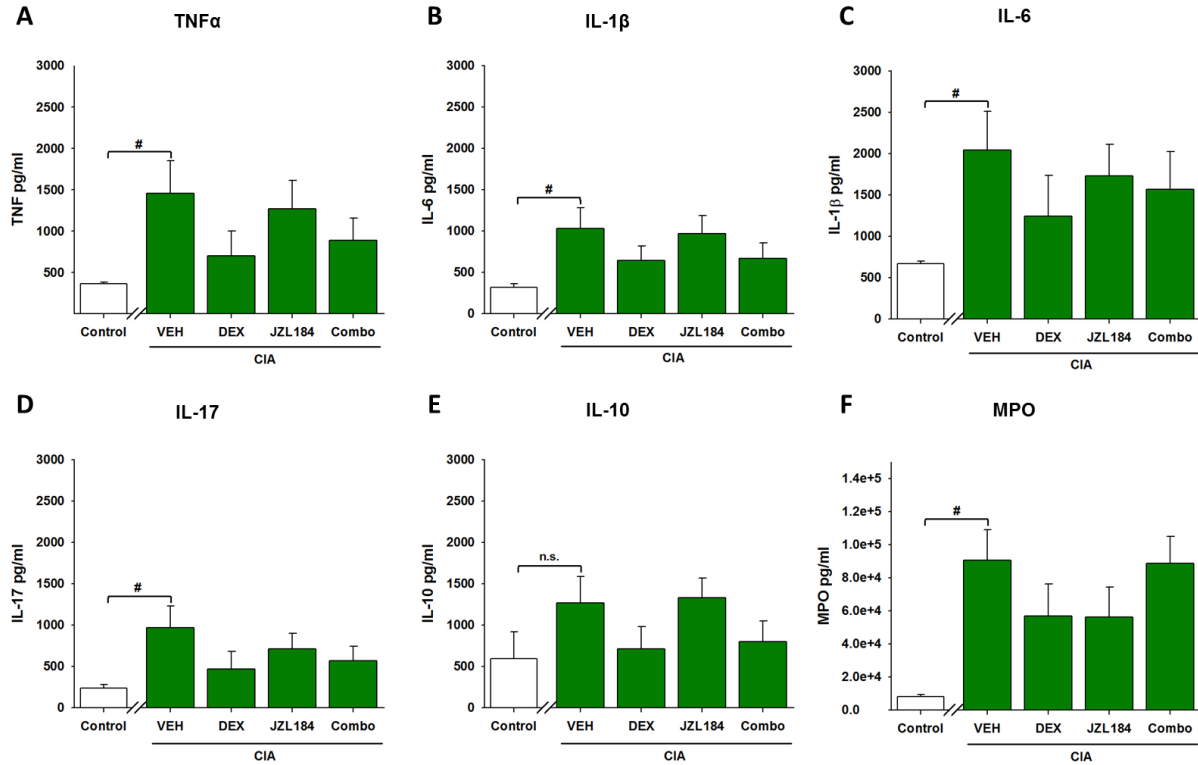
Figure 13. Repeated dual administration of JZL184/DEX did not affect CIA-induced increase in paw levels of proinflammatory cytokine (A-D) and MPO (F). Mice were administered the JZL184 (4 mg/kg; *s.c.*), DEX (0.015625 mg/kg; *s.c.*), a combination of JZL184 (4 mg/kg; *s.c.*) + DEX (0.015625 mg/kg; *s.c.*), or vehicle once daily for 19 days. CIA did not affect IL-10 levels (E). Data expressed as mean ± SEM (n = 9-11). * p < 0.05 vs. CIA mice; # p < 0.05 vs. control mice.

Appendix A.

Grip strength as a measure of CIA-induced loss of paw function

Background

As proposed, the present study focused primarily on pain-induced behaviors such as mechanical allodynia (increased sensitivity to touch) or thermal hyperalgesia (increased sensitivity to noxious heat). These assays were chosen because they are among the most commonly used behavioral tests for pain induced by arthritis in rodents (Gao et al., 2015; Inglis et al., 2007b). That said, the vast majority of arthritis related reports focus exclusively on inflammation and not its resulting pain.

In addition, pain-induced behaviors are potentially problematic in that they are easily confounded by sedation. For example, mechanical allodynia is reversed by high dose opioids (Nagakura et al., 2003), which effectively sedate the test subject. On the other hand, pain-suppressed behaviors are reversed when the animal increases its behavior, and are thus less susceptible to sedative confounds. Thus, the goal of this experiment was to examine a pain-suppressed behavior as well as a test of functional ability, grip strength.

Methods

Thirty male DBA1/J mice were subjected to CIA or control, as described in the main body of the document. Half of the CIA mice were repeatedly administered JZL184 (4 mg/kg, *s.c.*) or vehicle for 16 days. The inverted cage lid test (Brooks, Higgs, Jones, & Dunnett, 2012) was used to measure grip strength. Mice were placed on the top of a wire cage bottom and the wire was briefly shaken to cause the mice to grip the wire. The wire was gently flipped upside-down along

the mouse's y-axis (*i.e.*, pitch, with the mouse's head going forward and down) and suspended approximately 20 cm above a cage filled with soft bedding. The latency for the mouse to drop from the wire was measured by a second experimenter using a stopwatch.

Results

CIA treatment significantly decreased latency to fall [$F_{(2, 27)} = 6.395$; $p < .01$; **Figure A1**]. *Post hoc* comparisons revealed that repeated JZL184 (4 mg/kg) treatment had no effect on grip strength.

Discussion

The inverted cage lid test, a test of grip strength, was surprisingly effective at detecting CIA-induced loss of paw function. This low-tech alternative to allodynia and hyperalgesia tests has not been reported with CIA in mice, and offers some advantages over established pain models. The results align with a previous report indicating that CIA in rats attenuates grip strength as measured by the force used to grip a wire grid to resist the tail being pulled by a spring balance (Sasakawa, Sasakawa, Ohkubo, & Mutoh, 2005). One advantage of the method used in the present study is that the test requires no special equipment and training is minimal.

Watching the mice cling to the wire lid, the differences between CIA and control mice was striking. Mice that had significant paw swelling, especially in the forepaws, had the shortest latencies to fall from the wire. Mice with overt swelling in both forepaws fell from the lid almost immediately. Conversely, mice without paw swelling were able to ambulate along the underside of the lid until they reached the maximum cutoff time of one minute.

The idea to assess grip strength came about at the end of the present project, and so there was inadequate time to incorporate the test more broadly. So, this brief study was limited to only one test point in one cohort of CIA mice. Although MAGL inhibition had no effect on grip strength on day 16, time course data may reveal a delay in functional loss. It may be informative to measure grip strength repeatedly over the course of CIA development, as a measure of functional loss of paw strength.

A   **Grip Strength Test**
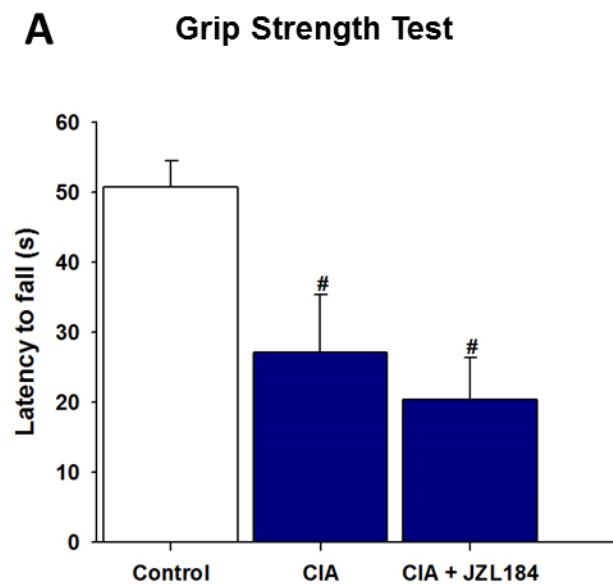
Figure A1. Collagen-induced arthritis (CIA) decreases grip strength. Mice were subjected to CIA. Mice were administered JZL184 (4 mg/kg, *s.c.*) or vehicle for 16 days, then grip strength was tested using the inverted cage lid test. Data expressed as mean ± SEM (n = 10). # $p < 0.05$ vs. non-CIA control mice.

Appendix B.

Anti-arthritic effects of CB_2 agonism in CIA and CFA-induced arthritis models

<u>Background</u>

The goals of the present study were (1) to evaluate the possible analgesic effects of the CB_2 agonist HU-308 in collagen-induced arthritis (CIA) mouse model and (2) to compare the anti-inflammatory and analgesic effects of CB_2 agonism by HU-308 in the CIA model of inflammatory arthritis and the complete Freund's adjuvant (CFA) model of inflammatory pain. There are a variety of selective CB_2 agonists that have been synthesized to have a greater binding affinity for the CB_2 receptor over the CB_1 receptor. CB_2 agonists provide an opportunity to harness the anti-inflammatory and analgesic effects of exogenous cannabinoids without the undesirable cognitive and behavioral side effects exhibited by more traditional cannabinoid agonists, such as THC (Hanus et al., 1999; LaBuda, Koblish, & Little, 2005). Acute administration of CB_2 agonists reduces pain and inflammation in several animal models of acute inflammatory pain, including intraplantar injection of CFA (Clayton, Marshall, Bountra, & O'Shaughnessy, 2002; Kinsey et al., 2011a; Valenzano et al., 2005). However, despite the ability of CFA to induced chronic inflammatory pain that can last for weeks, the analgesic effects of CB_2 agonism have not been evaluated beyond 48 h of inflammatory pain. Further, repeated administration of CB_2 agonists reduces the inflammation and joint destruction caused by collagen induced arthritis (CIA) (Fukuda et al., 2014; Gui et al., 2015), but the analgesic effects are unknown. The present study tested the hypothesis that the CB_2 selective agonist HU-308 decreases inflammation and pain caused by CIA or CFA.

Collagen-induced arthritis

For the CIA model, male DBA1/J mice were immunized with an emulsion of collagen and CFA followed 21 days later by a secondary "booster" exposure to the collagen emulsion, to induce anti-collagen autoimmunity. On day 28 mice were administered 25 µg lipopolysaccharide (LPS) in 0.1 ml saline (ip) to "synchronize" arthritis induction (Caccese, Zimmerman, & Carlson, 1992; Luo et al., 2011). CIA significantly increased paw swelling (*i.e.* arthritic clinical scores [$F_{(32, 27)}$ = 8.794; p <.01; **Figure A2A**] and caliper measurements [$F_{(32, 27)}$ = 4.136; p <.01; **Figure A2B**]) and mechanical allodynia, per the von Frey test [$F_{(2, 27)}$ = 12.728; p <.01; **Figure A2C**]. Repeated administration of the selective CB$_2$ agonist HU-308 (3 mg/kg, *i.p.*) for 10 days significantly attenuated CIA-induced paw swelling. Surprisingly, HU-308 did not affect CIA-induced mechanical allodynia.

CFA-induced arthritis

The CIA model is extensively used to research immunosuppressant treatments that reduce inflammation and disease progression in inflammatory arthritis. In contrast, intraplantar injection of inflammatory mediators is extensively used to characterize novel analgesic treatments for chronic inflammatory pain. To test this hypothesis, either complete Freund's adjuvant (CFA, 20 µl) or saline was injected directly into each hindpaw of male C57BL/6 mice to induce localized paw inflammation and pain. CFA significantly increased inflammatory pain and edema 24h after administration [$F_{(6, 21)}$ = 5.543; p <.01; **Figure A2D**]. Subchronic administration of HU-308 (50 mg/kg, *i.p.*) attenuated CFA-induced hyperalgesia, per the Hargreaves plantar stimulator test [$F_{(2, 21)}$ = 7.464; p <.01; **Figure A2E**]. In contrast to the CIA model, HU-308 did not significantly affect CFA-induced paw edema.

Discussion

Taken together these results suggest a disconnect between the CIA model of inflammatory arthritis traditionally used to develop immunosuppresants and the CFA model of inflammatory pain used to develop novel analgesic treatments. The CFA model is an attractive alternative to the CIA model because of its relatively fast throughput. However, neither model reflects all aspects of inflammatory arthritis in humans, and the effects of the synthetic CB_2 agonist HU-308 varied greatly between models. Although the present study provides new evidence that CB_2 is a potential target for the development of new analgesic anti-inflammatory treatments for inflammatory arthritis these results may have reduced external validity and translatability.
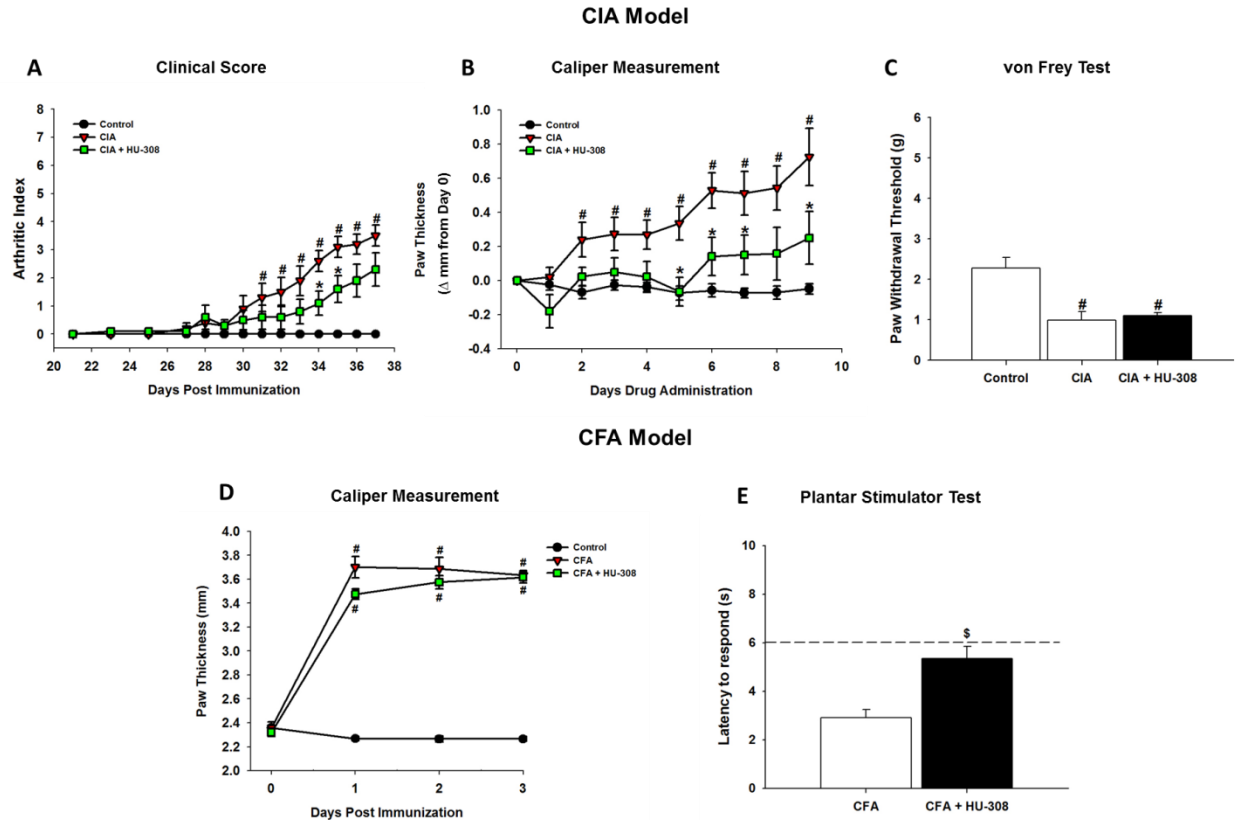
Figure A2. Disconnect between anti-inflammatory and analgesic effects of the CB_2 agonist HU-308 in the CIA model of inflammatory arthritis and the CFA model of inflammatory pain. To test the effects of HU-308 in a model of inflammatory arthritis mice were subjected to CIA and administered HU-308 (3 mg/kg, *i.p.*) for 10 days. HU-308 attenuated CIA-induce paw swelling as measured by clinical scores (A) or paw thickness (B). HU-308 did not affect CIA-induced mechanical allodynia (C). A separate group of mice was injected in the hind paw with CFA and administered HU-308 (50 mg/kg, *i.p.*) for 4 days. HU-308 reduced CFA-induced edema, but did not attenuate CFA-induced hyperalgesia (D). Data expressed as mean ± SEM (n = 8-10). * p < 0.05 vs. CIA; $ p < 0.05 vs. CFA; # p < 0.05 vs. control mice. Dotted line represented control saline paw latencies.

Appendix C.

Collagen Antibody-induced Arthritis model

Background

      The goal of the present study was to pilot the collagen antibody induced arthritis (CAIA) model of inflammatory arthritis in the lab. The production of antibodies against self-peptides (*e.g.*, rheumatoid factor, anti–citrullinated protein, and collagen) by B cells of the adaptive immune system are important in the pathogenesis of both RA and CIA (Asquith et al., 2009; Takemura et al., 2001). In the CAIA model, anti-collagen antibodies generated from mice are passively transferred to test mice to induce paw swelling and pain (Frolov, Yang, Dong, Hammock, & Crofford, 2013; Terato et al., 1992).

      The CAIA model of inflammatory arthritis has several advantages and disadvantages over the more classical CIA model. For example, CAIA develops much faster than CIA (*e.g.*, arthritis develops in one or more paws in two weeks, as opposed to five weeks with CIA). Further, unlike CIA, which can only be induced in susceptible mice with certain haplotypes of the major histocompatibility complex (MHC) class II genes, CAIA can be induced in a wide variety of mice strains (Asquith et al., 2009). This flexibility allows researchers to study inflammatory arthritis using genetic tools, such as gene knockout and transgenic mice that are typically backcrossed on the non-CIA susceptible C57BL/6 mouse strain.

      On the other hand, although the CAIA model has a quicker throughput compared to CIA, arthritis induction is exceedingly more expensive in the CAIA model and the shorter timeline might not translate as well to human chronic inflammatory arthritis (Khachigian, 2006). In addition, CAIA inflammation is mainly perpetuated by the innate immune response and lacks the

critical adaptive T cell immune response needed to perpetuate CIA and RA (Asquith et al., 2009;

Kagari et al., 2002). The present study tested the hypothesis that CAIA would induce paw

swelling and pain-related behaviors that would be attenuated by the MAGL inhibitor JZL184 (4

mg/kg).

Methods

Male BALB/c mice were administered a cocktail of anti-collagen antibodies or saline via tail

vein injection, per manufacturer's protocol (Chondrex, Redmond, WA). Three days later, mice

were administered lipopolysaccharide (LPS, 50 μg, *i.p.*) to stimulate the immune response. Mice

were administered JZL184 (4 mg/kg, *s.c.*) or vehicle (1:1:18 parts ethanol, cremophor, and

normal saline) once daily for 15 days. Mice were assessed daily for arthritis, including clinical

scoring and paw thickness. On the 15th day after immunization, mice were tested for mechanical

allodynia and thermal hyperalgesia, as detailed in the main body of the dissertation.

Results

CAIA did not significantly affect paw swelling (*i.e.*, arthritic clinical scores [$F(2, 24) = 2.161$; p

=.1371; **Figure A3A**] and caliper measurements [$F(2, 24) = 1.411$; p =.2634; **Figure A3B**]) or

thermal hyperalgesia [$F(2, 24) = .482$; p = .6233; **Figure A3D**]. However, CAIA did induce

mechanical allodynia, per the von Frey test [$F(2, 24) = 4.654$; p < .05; **Figure A3C**]. Repeated

JZL184 had no effect on paw swelling or pain-related behaviors.

Discussion

These results suggest that although the CAIA model is broadly reported to be easier to

induce than the CIA model of inflammatory arthritis, the CAIA model, like CIA, still has

optimization problems. Although consultation with the vendor did not yield any obvious

problems with our induction methodology, it is likely we had slight issues not written in published protocols that led to a lower incidence. It is suggested that delivering the antibodies via tail vein injection, versus intraperitoneal injection, produces optimal CAIA incidence (Khachigian, 2006). Thus, it is possible that some mice accidently received a subcutaneous tail injection, which is the least effective method of delivering CAIA. This is unlikely, given that I was thoroughly trained by the Office of Laboratory Animal Resources to inject into the tail vein, a procedure that I practiced extensively prior to injecting collagen antibodies. Moreover, I observed the injection sites for tell-tale blisters that form when injecting subcutaneously or intradermally, and observed neither phenomenon. Nevertheless, my opinion is that, with optimization, CAIA is a promising model to develop novel treatments for inflammatory arthritis.
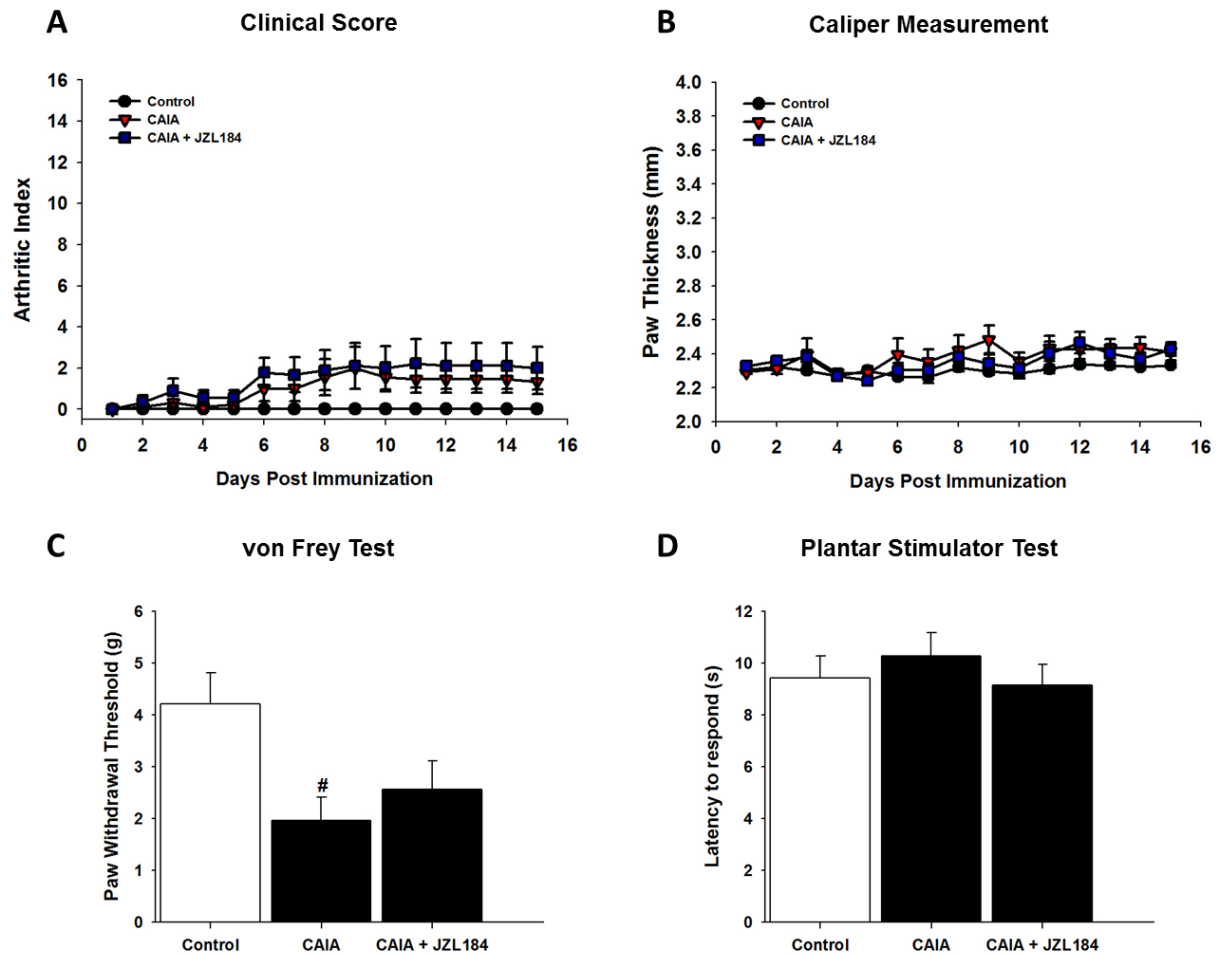
Figure A3. CAIA induced mechanical allodynia, but not paw swelling. Mice were administered an anti-collagen antibody cocktail to induce CAIA or saline via tail vein injection. Three days later mice were administer 50 μg of LPS. Mice were JZL184 (4 mg/kg; *s.c.*) or vehicle once daily for 15 days. CAIA did not induce paw swelling compared to non-CAIA controls as measured by clinical scores (A) and calipers (B). CAIA induced mechanical allodynia in the von Frey assay (C), but not thermal hyperalgesia in the plantar test (D). JZL184 (4 mg/kg) did not affect paw swelling, allodynia, or hyperalgesia. Data expressed as mean ± SEM (n = 9). # p < 0.05 vs. control mice.