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Evaluation of a Serine Hydrolase Inhibitor JZL184 as an Immunomodulator against Avian Pathogenic Escherichia Coli O78 in Chickens

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Evaluation of a serine hydrolase inhibitor JZL184 as an immunomodulator against avian pathogenic *Escherichia coli* O78 in chickens

By

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Chickens in the poultry industry are reared under intensive conditions where they are often exposed to opportunistic pathogens. *Escherichia coli* strain O78 is responsible for about half of the cases of avian colisepticemia. Potential therapeutic treatments have been proposed to inhibit the hydrolases that controls the endogenous levels of the endocannabinoid, 2-arachidonoylglycerol (2-AG). 2-AG is the full agonist at the CB2 receptors of the endocannabinoid system expressed among leukocytes and it plays a role in mediating the activation of phagocytic macrophages. It is speculated that elevating 2-AG levels could increase macrophage cytokines and promote the recruitment of immune cells at the infected tissues. The purpose of this study was to investigate the immunomodulating effect of the 2-AG hydrolase inhibitor, JZL184 in chickens. The treatments could potentially up-regulate the innate immune responses in chickens during an *E. coli* infection by conveying a message from the endocannabinoid system to the immune system.

Keywords: Avian, immunity, immunomodulation, endocannabinoid system, 2-arachidonoylglycerol, JZL184, carboxylesterase, proinflammatory cytokine, interleukin-1β, *Escherichia coli*, colibacillosis, airsacculitis.

DEDICATION

First and foremost, I want to give all thanks to God, who has directed and blessed my life more than I deserve or could ever ask for. I would like to dedicate this research to my parents, Mingtsu Ho and Wenhsin Chiang, for all of the love and support they have given me throughout my lifetime. I would also like to dedicate this research to my fiancé, Jason Chan, for all of the love and support he has given me during my time throughout this Master's program.

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CHAPTER I

LITERATURE REVIEW

1.1 The endocannabinoid system

The endogenous cannabinoid system is a neurotransmission pathway with a lipid-signaling network named after the plant Cannabis. The psychoactive components of Cannabis, Δ^9-tetrahydrocannabinol (THC), and the endocannabinoids, 2-arachidonylglycerol (2-AG) and anandamide (AEA), bind to the membrane-bound G-protein coupled receptors, cannabinoid receptors type 1 and 2 (CB1 and CB2) (1, 2). The endocannabinoid system responds to endogenous signaling lipids to modulate an extensive range of physiological processes and regulate important immune cell functions.

1.1.1 Synthesis of endocannabinoids

In the central nervous system, endocannabinoid signaling is found to occur by a non-vesicular calcium-dependent retrograde mechanism (3). Endocannabinoids are synthesized and released after stimulations that occur at the post-synaptic neuron during neurotransmissions (4). 2-AG is found to form rapidly on demand upon cellular stimulation from membrane phospholipids, making the endocannabinoid system capable of rapidly adapting to changing conditions (5). In physiological activities, 2-AG is produced by cells at the postsynaptic cleft upon depolarization where it gets released into

the synapse (6). When pre-synaptic cannabinoid receptors are activated by newly synthesized 2-AG, it results in a reduction in neurotransmitter release.

In the immune system, 2-AG and AEA were observed to be synthesized by inflammatory cells after cell stress or being stimulated with G-protein-coupled receptor agonists or Ca^{2+} ionophores (7). The major biosynthetic pathway consists of sequential hydrolysis of inositol phospholipids through diacylglycerol (DAG) by β-type phospholipase C and diacylglycerol lipase (DAGL) (8).

1.1.2 Endocannabinoid receptors

Most studies have found evidence that endocannabinoids regulate CB1 receptors in the brain and affect the central nervous system while endocannabinoids primarily regulate CB2 receptors in the peripheral immune system and mircoglia (9). CB1 and CB2 receptors were first successfully cloned in 1990 and 1993, respectively. The discoveries of cannabinoid receptors have led to the identification of their endogenous ligands, 2-AG and AEA, and the agonists to the cannabinoid receptors have helped us understand the roles of the endocannabinoid system. Endocannabinoid 2-AG is a full agonist to CB1 and CB2 receptors, whereas AEA is the second major cannabinoid and a partial agonist to CB1 receptors (10). Differences between these receptors include their predicted amino acid sequence, signaling mechanisms, tissue distribution, and sensitivity to certain potent agonists and antagonists that show marked selectivity for one or the other receptor type (11).

1.1.2.1 Cannabinoid type I (CB1) receptors

CB1 receptors are primarily found in the brain and located in the central and peripheral nervous systems. Upon 2-AG synthesis, the released 2-AG functions as a retrograde messenger by activating the CB1 receptors at the presynaptic cleft and interrupting the calcium influx, inhibiting the release of neurotransmitters (12). Responses of CB1 to agonists have been found to stimulate the central nervous system and to provide beneficial effects, including analgesia, attenuation of nausea and vomiting in cancer chemotherapy, reduction of intraocular pressure, appetite stimulation in wasting syndromes, relief from muscle spasms/spasticity in multiple sclerosis, and decreased intestinal motility (11). Despite the benefits, over-stimulation of the CB1 receptors in the central nervous system could cause alterations in cognition, memory, euphoria, and sedation (11).

1.1.2.2 Cannabinoid type II (CB2) receptors

CB2 receptors were cloned from the human promyelocytic cell line HL-60 and are mainly expressed in the peripheral tissues, lymphoid tissues, and myeloid cells (13). According to the mRNA data from studies that measured the level of expression of CB2 receptors in leukocytes, dendritic cells, mast cells, macrophages, monocytes, neutrophils, NK cells, and T lymphocytes have all been detected to express CB2 receptor mRNA, providing evidence of the presence of CB2 receptors in immune cells (7). It was then speculated that endocannabinoids and cannabinoids would modulate immune cell functions. Some studies have shown immunosuppressive effects induced by CB2 receptor

activation where immune responses were down regulated and promoted the proliferation of pathogens and even cancer cells (7).

1.1.2.3 Cannabinoid receptor agonists and antagonists

Ligands that show no marked selectivity toward CB1/CB2 include 2-AG, HU-210, CP55940, THC, and WIN55212 (11). Ligands with the highest to lowest selectivity towards CB1 are ACEA, O-1812, SR141716A, AM281, ACPA, 2-AG metabolite (2-arachidonylglyceryl ether), LY320135, and methanandamide. As for CB2, the ligands that are selective towards this receptor are JWH-015, JWH-051, L-768242, JWH-139, AM630, JWH-133, L-759633, L-759656, HU-308, and SR144528 (in the order with decreasing CB2/CB1 selectivity) (11).

1.2 Metabolism of endocannabinoids

Since endocannabinoids are synthesized on demand and broken down almost immediately, the degradative routes and enzymes of 2-AG and AEA have received much attention as promising targets as therapeutic treatments (14). While the hydrolysis of AEA by fatty acid amide hydrolase (FAAH) is well characterized, less is known about the degradation pathways of 2-AG because it has been found that multiple enzymes facilitate the breakdown of 2-AG. Monoacylglycerol lipase (MAGL) has been found to be the principal 2-AG hydrolase in the rodent brain where 2-AG gets broken down to arachidonic acid and glycerol. In mouse brain, MAGL was revealed to hydrolyze approximately 85% of 2-AG, where FAAH, ABHD6 and ABHD12 were found to mediate the hydrolysis of the remaining 15% (15). 2-AG hydrolyzing enzymes have been found to be different in various animals. Carboxylesterases 1 and 2 were found to be

involved in the breakdown of 2-AG in cultured human THP-1 cells (16, 17). It was suggested by Blankman et al. that for there to be multiple enzymes displaying 2-AG hydrolyzing characteristics could indicate these enzymes exhibit distinct cellular or subcellular distributions and possibly undergo different forms of regulated expression (15). The enzymes with different biological activities also imply the enzymes that degrade 2-AG in the brain are distinct from those in the peripheral tissues.

1.2.1 Endocannabinoid catabolic enzyme inhibitors

JZL184 is a carbamate compound developed to covalently modify the active site serine residue of serine hydrolases such as MAGL (18). Carboxylesterase in rodent liver has been identified as a major off-target in JZL184 inhibition of FAAH and MAGL (19, 20). Crow et al. compared the chemical reactivity of JZL184 with MAGL and human carboxylesterases CES1 and CES2. JZL184 was found to potently inhibit MAGL and CES1 (20). In addition, under circumstances of oral administration, CES1 and CES2 may significantly metabolize JZL184 and other pharmaceutical agents via first pass metabolism post intestinal absorption (20).

Due to observed low-level cross-reactivity of JZL184 (and other MAGL inhibitors) with FAAH and peripheral carboxylesterases, Chang et al. compared the selectivity and potency between inhibitors from the *O*-aryl carbamate class and the *O*-hexafluoroisopropyl (HFIP) carbamate class (21). It was discovered that the HFIP analog of JZL184, KML29 have higher selectivity and greater potency towards MAGL in brain tissues of mice (21).

1.3 Endocannabinoid effects

1.3.1 Endocannabinoid effects on the central nervous system

Endocannabinoids have been found to be involved in the regulation of a variety of physiological and pathophysiological processes that reflect functions in both the central nervous system and peripheral tissues (22). Nass et al. inhibited MAGL and FAAH in mice with JZL184 and PF-3845, respectively, to study the effects of endocannabinoids 2-AG and AEA on thermoregulation and induced hypothermia (23). The body temperature of mice was found to decrease under stressors of cold ambient temperature and systemic inflammation of LPS. It was also discovered that endocannabinoids attenuate fever in response to endotoxin challenges in that study. In another study, Benamar et al. used CB1 receptor antagonist rimonabant and CB2 receptor selective antagonist SR144528 after inducing fevers in rats with LPS (24). They found that CB1 receptors play a crucial role in the anti-pyrogenic effect of WIN 55,212-2.

1.3.2 Endocannabinoid effects on the tissues

Endocannabinoids have been shown to have both anti-inflammatory and proinflammatory effects on immune responses *in vivo*. In studies of experimental multiple sclerosis and LPS-induced pulmonary inflammation models, evidence has shown that there is a decreased inflammatory cell recruitment and enhanced anti-inflammatory cytokine secretion when the endocannabinoid system is activated (7). In studies that showed a proinflammatory role of 2-AG *in vivo,* the effects include enhanced leukocyte recruitment, release of proinflammatory cytokines and autacoids, and phagocytosis, where endocannabinoids played roles in augmenting host defense (7).

For a study where the model was LPS-induced expression of cytokines, the up-regulated 2-AG levels caused by the inhibition of MAGL caused a decrease in the expressions of IL-1β, IL-6, IL-10, and TNF-α (10). In that study, Alhouayek et al. experimentally induced colitis with trinitrobenzene sulfonic acid (TNBS) in mice and inhibited MAGL with the selective antagonist JZL184 to increase 2-AG levels. The increased 2-AG levels counteracted colitis and related systemic inflammation with immunosuppressing effect and decreased brain and liver cytokine IL-1β, IL-1, TNF-α and chemokine MCP-1 levels. This experiment demonstrated the protective role of both cannabinoid receptors type 1 and 2 in mice against colitis and suggested both CB1 and CB2 receptors to be targeted instead of just either of the receptors specifically. Hsu et al. confirmed DAGLβ inhibition by KT109 and KT172 in living systems (25). They discovered KT109 and KT172 potently and selectively inactivated DAGLβ *in vitro* and *in vivo*, hindering functions of DAGLβ in regulating arachidonic acid, prostaglandins and TNF-α release in peritoneal macrophages by inhibiting the biosynthesis of 2-AG in mice. Reductions of PGD_2 and PGE_2 after DAGLβ inactivation indicate a regulating role of 2-AG in the production of prostaglandins in macrophages. Reduced secretion of IL-1β from LPS-treated macrophages may suggest the inflammatory cytokine is regulated by DAGLβ and ABHD6 (25). Time-course studies show both inhibitors producing complete inhibition of macrophage DAGLβ by 1h after *in vivo* treatments (25).

1.3.3 Serine hydrolase inhibitors on macrophage inflammatory responses

The effects of serine hydrolase inhibitors on macrophage inflammatory responses in mice were studied by Hsu et al. via blockade of DAGL-β in peritoneal macrophage

where it was found to affect macrophage metabolism (25). They regulated 2-AG by preventing its production via DAGL inhibition. LC/MS analysis detected a reduced 2-AG content in macrophages of mice treated with DAGL-β inhibitors KT109 and KT172 (5mg/kg). DAGL-β was also found to play a role in regulating PGE_2, where the inhibition of DAGL-β strongly decreased this prostaglandin in macrophages from KT109-treated PLA2G4A (arachidonic acid producing enzyme) knockout mice (25). Regulation of the 2-AG biosynthesizing enzyme, DAGL-β, was discovered to regulate endocannabinoids, diacylglycerols, and prostaglandins in mice macrophages. They found that serine hydrolase inhibitors also inhibit ABHD6 and off-targets like carboxylesterases (CES3, CES2G). DAGL blockade significantly reduced 2-AG levels along with macrophage-secreted cytokines, IL-1β and TNF-alpha, in lipopolysaccharide-stimulated macrophages (25). It was therefore speculated that elevating 2-AG levels could increase macrophage cytokines and promote the recruitment of immune cells at the infected tissues.

1.3.4 Therapeutic applications of ECS-related drugs

In cardiovascular diseases, CB2 signaling seems to play a protective role where its activation in the cells of the cardiovascular system (endothelial and vascular smooth muscle cells, cardiomyocytes, fibroblasts, and resident immune cells) prevents the proinflammatory response and decreases pathological proliferation (26). There is evidence supporting the idea that dysregulated endocannabinoid systems often occur in cardiovascular diseases, and that the stimulation of CB1 receptors promote injuries, whereas the CB2 receptors have been shown to attenuate inflammation (26). Therapeutic effects of endocannabinoid and cannabinoids in the gastrointestinal tract have been

discovered due to THC inhibiting acetylcholine release from enteric nerves in the gut. All gut functions are regulated by the endocannabinoid system where it helps the central nervous system in maintaining metabolic and homeostatic functions in the body (27). Endocannabinoids also signal through cannabinoid receptors to regulate immune cell functions and activities, especially toward adaptive immune responses (28). The activation of the cannabinoid receptors causes transcriptional modulations in immune cell gene expression to regulate cell migration, production of cytokines, chemokines, and other mediators. For example, AEA was found to inhibit the production of proinflammatory cytokines, IL-6 and IL-8, in human monocytes (29). Sugiura et al. found 2-AG to induce rapid increase in levels of intracellular free calcium in HL-60 cells that express the CB2 receptors and this effect could be blocked by CB2 antagonists (30). This observation led to their conclusion that 2-AG is the physiological ligand for the CB2 receptor and not AEA. The regulation of immune functions by endocannabinoids and cannabinoid receptors suggests various therapeutic approaches to modulate immunity.

1.4 Immunology: Innate immunity

1.4.1 Macrophages

Macrophages are mainly developed from monocytes derived from bone marrow precursor cells. These stem cells of the granulocytic-monocytic lineage derive into a variety of macrophages upon stimulation by granulocyte macrophage stimulating factor or IL-3 (31). Stem cell differentiation is guided by various cytokines. Monocytes enter blood circulation after leaving from the bone marrow within 24 h and differentiate into

macrophages during immune responses where they exit capillary walls and enter connective tissues to engulf pathogens and foreign agents.

1.4.2 Cytokine therapy: Macrophage activation

Nau et al. studied host-pathogen interactions using a comparative analysis of gene expression in human macrophages infected with Gram-positive bacteria, Gram-negative bacteria, and mycobacteria (32). Macrophages were cultured with specific bacterial components including LPS (Gram-negative), lipoteichoic acid and protein A (Gram-positive), heat shock proteins (hsps) and mannosylated proteins. LPS, lipoteichoic acid, muramyl dipeptide, and hsps were found to be the bacterial components capable of inducing macrophage activation due to their function as agonists for toll-like receptors. However, other bacterial components do not stimulate TLRs, suggesting macrophage activation is mainly facilitated by macrophage TLRs. It was also found that, without bacterial stimulation, the gene expressions associated with antigen-presenting functions of macrophages were absent. The pathogen-specific responses of *M.tuberculosis* inhibiting IL-12 production in human macrophages suggest that there are signaling pathways responsible for carrying out the distinctive changes in gene expression other than those mediated by TLRs. The cytokine-repressing effect on macrophages also entails the evolution of bacteria selected for their ability to survive within macrophages and counter the host's innate immune responses, such as host resistance in tuberculosis-infected mice and humans. The observation of IL-12 repression in tuberculosis-infected animals gave rise to effective cytokine treatments where supplemental and exogenous IL-12 successfully helped clear bacterial infections. The study provides useful data for

applications in designing vaccines and cytokine therapies that aim to strengthen host innate immune responses (32).

1.5 Chicken innate immune system: chicken macrophages

Chicken macrophage lineage cells differentiate from embryonic hemopoietic progenitors before chickens are hatched. Broiler type chickens have lower numbers of macrophage-type colonies compared to Leghorn chickens, thus broilers are more prone to perinatal infections (33). Chickens in general have little to no resident macrophages present in the abdominal cavity until inflammation occurs and monocytes are recruited into the tissue site (33).

There have been successful cases in modulating immune cells in chickens. Djeraba and Quere introduced Acemannan (ACM1) to vaccines against avian viral diseases and found an increased *in vivo* capacity of NO and cytokine IL-1, IL-6 production. ACM 1 had an immunomodulating effect on macrophage activation that targeted mannose receptors and had a priming effect on chicken monocytes (34).

Sevimli et al. investigated the roles of IL-1β, IL-6 and TNF-α in amyloid arthropathic layer chickens with mild to severe amounts of amyloid accumulation in the surface epithelium of the synovial membranes where macrophages and heterophils infiltrate after inflammation was induced (35). IL-1β and TNF-α were found to be the two important proinflammatory cytokines in the occurrence of avian amyloidosis, whereas the results for IL-6 suggest otherwise (35).

1.6 Cannabinoid effects on chickens

Alizadeh et al. evaluated the effects of cannabinoid on food intake behavior in neonatal layer-type chicken with intracerebroventricular (ICV) injections of cannabinoid ligands 2-AG and JWH015 in separate experiments (36). Hyperphagia was induced dose-dependently by ICV injections of cannabinoid receptor agonist 2-AG and JWH015 while co-injections of selective cannabinoid receptor antagonists SR141716A and AM630 minimized and decreased the effect of amplified food intake, suggesting that CB1 and CB2 receptors play important roles in modulating feeding behavior (36, 37). Emadi et al. also studied endocannabinoid effects on food intake and metabolism in neonatal chickens using CB65, a selective CB2 agonist, and AM630, a selective CB2 antagonist, to support that CB2 agonists have an effect on increasing chicken food intake (9).

1.7 *Escherichia coli* infections in chickens

The economic impact of avian pathogenic *Escherichia coli* (APEC) is mainly due to mortality, slower growth rates, and carcass downgrading (38). In commercial broiler operations, APEC infections are controlled indirectly by vaccination against other respiratory diseases and minimizing stress conditions, and directly by administration of antimicrobial agents to suppress symptoms in infected flocks. The development of efficacious APEC vaccines has potential in combating this important poultry disease, however, cost, vaccine delivery method and timing of vaccination has been the major challenges for vaccine developers (38). With APEC being a global pathogen that can cause diseases that affect all age groups, a successful vaccine needs to not only be safe but also provide a certain degree of protection. To facilitate a better understanding of

chicken immune responses to APEC, it will require new technologies and reagents to better improve the designs of the vaccines (39).

In poultry, colisepticemia is known to be the most severe manifestation of *E. coli* infections where the sickness often begins as an upper respiratory infection after an initial primary mycoplasmal or viral infection, leading to infiltration into the blood and internal organs and the development of pericarditis, perihepatitis, airsacculitis, and salpingitis (40). *E. coli* strain O78 is responsible for about half of the cases of avian colisepticemia where the virulent strain adheres to avian epithelial tissues and starts by invading the trachea (41). Although colisepticemia is usually observed in chicks older than 4 weeks, from a management prospective, early vaccination with vaccines delivered in drinking water is a suitable practice for the poultry industry (41). In the study on live attenuated bacterial vaccines by Nolan et al., they found that the *E. coli* O78:K80 vaccine candidate is not only inexpensive but can also be easily administered, providing the poultry industry a feasible method of vaccinating chickens (40).

1.7.1 Host Immune Responses to APEC

Microbial-induced activation of immune cells leads to the instigation of intracellular signaling pathways related to microbial killing mechanisms and the production of pro- and/or anti-inflammatory cytokines (42). Bar-Shira and Friedman demonstrated chicken innate preparedness and gradual development of pro-inflammatory functions under environmental stimuli of feed and bacteria where a significant increase in IL-1β and IL-8 was observed (43). The results indicated possible recruitment of bone marrow-derived heterophils with an elevation in *β*-defensin gene expression and the

recruitment of lymphocytes explained by the pro-inflammatory activity in the developing gut. They discovered exposure of the gut to feed and bacteria triggered a low yet significant increase in proinflammatory cytokines, IL-1β and IL-8 (43).

1.8 Avian therapeutics

Lowenthal et al. summarized the therapeutic pros and cons of cytokine therapy for avian species (44). They suggested cytokine levels have an influence on the growth of animals due to both illness and environmental stress could hinder the ability for animals to grow at an optimal rate. Studies have shown that growth is heavily influenced by the interactions between the immune and the central nervous system (45). The activation of the immune system releases cytokines and involves systemic effects by altering body metabolism and neuroendocrine functions. Cytokines play an important role in orchestrating those systemic responses in animals by conveying information about the immunologic activities to the brain and the peripheral tissues (46). In immunologically challenged animals, cytokines IL-1β, IL-6, and TNF-α were found to decrease overall food intake (46). Sick animals become anorexic and lose weight, leading to a significant reduction in both animal muscle mass and monetary value to the livestock industry. The cytokines mentioned above are also known as the pro-inflammatory cytokines that are secreted by monocytes, macrophages, and microglia upon exposure to antigens. Studies on IL-1β showed the cytokine depresses appetite where effects of negative nitrogen balance and increased plasma corticosterone were observed when recombinant IL-1β was chronically injected into central and peripheral tissues to mimic diseases (47, 48). Despite anorexia and weight loss of animals, these studies in the 1980s and 1990s suggested the

loss of appetite as an evolved strategy to facilitate recovery (45, 46, 49). Evidence showed weight loss having positive effects on the survival rate of *Listeria monocytogenes*-infected mice where free-feeding mice only consumed 58% of what the non-infected control consumed but were more likely to survive (50).

Cytokine treatments became popular as they present a natural and feasible approach with the successful cloning of avian cytokine genes. Successful cytokine therapeutics was proposed to increase growth rate and productivity utilizing the strategies of either administration of recombinant cytokines or inhibition of harmful endogenous cytokines (51). Interferons and anti-TNF agents were mentioned in a review paper as two of the most successful cytokine therapies that were developed (51). To reduce inflammation, IL-1 receptor antagonists were postulated to have therapeutic potential. The effectiveness of cytokine therapy for an animal also requires successful cloning of the full repertoire of genes. For poultry, cytokine therapy finally became more feasible with the cloning of a number of avian cytokine genes (44). One major challenge to cytokine therapy is ensuring safe delivery of the cytokines as they typically degrade within a short time frame and act locally. Delivery of cytokines to chickens became commercially feasible using live viral vectors that provided long-term *in vivo* cytokine production and were found to be cost-effective without the need of frequent injections to induce long-term protection.

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CHAPTER II

EVALUATION OF A SERINE HYDROLASE INHIBITOR JZL184 AS AN IMMUNOMODULATOR AGAINST AVIAN PATHOGENIC *ESCHERICHIA COLI* O78 IN CHICKENS

2.1 Introduction

Endocannabinoid research has spawned the introduction of new therapies that have revolutionized the treatment of many important inflammatory diseases (1, 2, 3, 4). The endocannabinoid system has important functions in regulating immune responses in animals (5). The cannabinoid receptors types 1 and 2 (CB1 and CB2) of the endocannabinoid system respond to endogenous signaling lipids to modulate an extensive range of physiological processes and regulate important immune cell functions (6). 2-arachidonoylglycerol (2-AG), the full agonist of the CB2 receptors that are commonly expressed among leukocytes, has been widely used to study the endocannabinoid effects on immune cells (7). Potential therapeutic treatments have been proposed to inhibit the hydrolases that controls the endogenous levels of endocannabinoids. For example, inhibitors targeting 2-AG degradative enzymes, including monoacylglycerol lipase (MAGL), a/b-hydrolase domain 6 (ABHD6) and 12 (ABHD12), and carboxylesterases (CES) were designed and developed to block the enzyme functions and elevate the endogenous levels of the endocannabinoid (8-11). Among all the inhibitors known to inhibit degradative enzymes of 2-AG, JZL184 is one of the most potent and selective

antagonists to the serine hydrolase, MAGL, in mouse brain (8). A wide array of studies on murine and rodent models has helped unveil the anti- and pro-inflammatory effects on immune responses when the level of the endocannabinoid, 2-AG, is elevated indirectly via selective blockade of 2-AG hydrolysis in the physiological systems (12).

However, little research has been done to study the therapeutic potential of JZL184 in chickens. Due to the potential risks of antimicrobial agents (AMAs) there is a need for alternative therapeutics and non-AMA alternatives to antimicrobial growth promoters (AGPs) to maintain livestock health and increase livestock production in the poultry industry (13). Chickens in the poultry broiler industry are reared under intensive conditions where they are often exposed to opportunistic pathogens such as *E. coli*. *E. coli* O78 is responsible for about half of the cases of avian colisepticemia where the virulent strain adheres to avian epithelial tissues and invades the trachea (14). In general, livestock with clinical and subclinical infections eat less, grow poorly, and have higher feed conversion ratios (15). For chickens, vaccines have been developed to provide long-term immunity against infectious diseases. However, with live-attenuated vaccines as the most effective form of immunization, the rise of virulent strains poses a serious concern. An additional concern is that some livestock industries still rely on the use of AMAs to enhance growth performance and prevent infections. Given the broad-spectrum protection, this makes AMAs an effective method for treating, preventing, and controlling avian diseases (16). However, the requirement for routine use contributes to antimicrobial-resistant pathogens that are harmful to human health, forcing the government to limit AMAs as growth promoters. The reduction in antibiotic-use in poultry could tackle resistance but in turn affect poultry production and its profitability

(17). The restrictions on AMAs in the U.S. urge food animal industries to advance and produce innovative yet safe therapeutic measures to guard livestock health and increase production.

Levels of the endocannabinoid, 2-AG, have been found to play a role in mediating the activation of phagocytic macrophages, conveying a message from the endocannabinoid system to the immune system (18). In studies where 2-AG levels were significantly reduced by DAGL inhibitors, macrophage-secreted cytokines, IL-1β and TNF-α, in lipopolysaccharide-stimulated macrophages were reduced as well (19). We hypothesize that elevating 2-AG levels could increase macrophage cytokine production and promote the recruitment of immune cells at the infected sites. The goal of this study was to investigate the effects of JZL184 on the systemic inflammation and the inflammatory mediator, Interleukin 1β. IL-1β is an important proinflammatory cytokine that activates macrophages and T lymphocytes, causes local tissue damage, fever, and loss of function (12). This cytokine is secreted by macrophages in response to TLR signaling at the sites of local pathogen invasion to induce inflammation. To study the effectiveness of JZL184 treatments in the control of avian pathogenic *E. coli* (APEC) infection in intensive livestock, we used an *in vivo* systemic model in which 63 5-week-old birds were infected intratracheally with 10^8 colony forming unit (CFU) of *E. coli* strain O78. The purpose of this study is to investigate the immunomodulating effect of JZL184 and whether the treatment could up-regulate the innate immune response in chickens during an *E. coli* infection.

2.2 Materials and methods

2.2.1 Animals

Chickens used in the study were hatched from fertile White Leghorn specific-pathogen-free eggs, and they were obtained from Charles River Labs (Storrs, CT). A total of 63 chickens were reared in negative pressure isolators at the facility in the Biomedical building located at Mississippi State University College of Veterinary Medicine until the completion of the study. Animals were under veterinary care with feed and water available *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Mississippi State University.

Table 2.1 Time points and number of birds to be sacrificed for each group.

Groups	Vehicle (*E. coli* challenge)			10 mg/kg JZL184 (*E. coli* challenge)			40 mg/kg JZL184 (*E. coli* challenge)			Negative control		
Time points	8h	28h	56h	8h	28h	56h	8h	28h	56h	8h	28h	56h
# of birds	6	6	6	6	6	6	6	6	6	3	3	3

2.2.2 Study design

Sixty-three chickens were divided into 4 groups listed in Table 2.1. At 5 weeks of age, the vehicle control (4:1 ratio of polyethylene glycol 300: Tween 80), low dose (10 mg kg^{-1}), and high dose (40 mg kg^{-1}) groups were injected intraperitoneally with JZL184/vehicle treatment 4 h prior to the *E. coli* challenge. Two different groups of chickens were separately pretreated with a low dose (10 mg kg^{-1}) and a high dose (40 mg kg^{-1}) of the selective 2-AG degradative enzyme inhibitor, JZL184, 4 h prior to being

challenged with APEC strain O78. JZL184 was dissolved in a 4:1 ratio of polyethylene glycol 300: Tween 80, as described by Long et al (8).

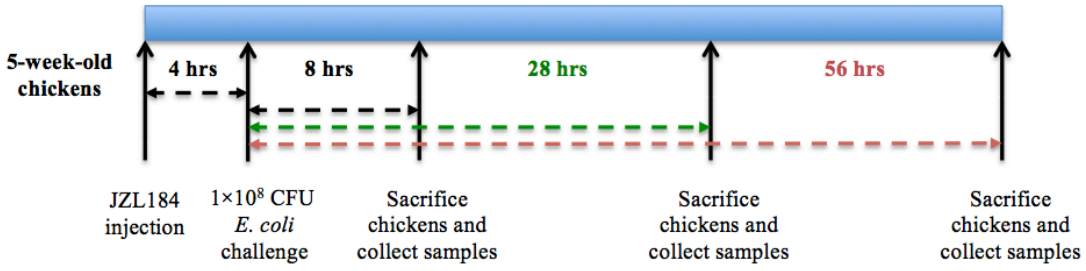

Figure 2.1 Study design timeline.

The experiment started with injection of JZL184 at 0, 10, or 40 mg kg^{-1} to 5-week-old chickens intraperitoneally. *E. coli* challenge of 1×10^8 CFU APEC O78 was administered to groups at 4 h post JZL184 injection. Necropsies and sample collections were performed at 3 different time points (8, 28, and 56 h) post infection.

2.2.3 Infection of chickens with *E. coli* O78

The *E. coli* O78 strain was a field virulent strain isolated from chickens with airsacculitis. *E. coli* O78 was cultured in brain-heart infusion (BHI) broth overnight and then transferred to fresh BHI broth to culture for 4 hours. The bacterial culture was enumerated using a serial dilution and plated on Tryptic soy agar plates. The virulence of APEC strain O78 was tested in a systemic chicken infection model, as described by Ragione et al (20). At 5 weeks of age, each chicken except the negative control group was challenged intratracheally with 1 ml of PBS suspension containing 10^8 CFU of APEC O78. As shown in Figure 2.1, chickens were given JZL184 treatments by intraperitoneal injection and after 4 h they were then challenged with 10^8 CFU of *E. coli*

O78. Images of sick and healthy chickens were taken during necropsy showing healthy organs compared to infected organs with signs of airsacculitis, perihepatitis, and pericarditis.

2.2.4 *E. coli* re-isolation and culture

At 8, 28, and 56 h p.i., 21 chickens were euthanized at each time point and bacteria were isolated from the blood, heart, liver, and air sacs of each bird with sterile cotton swabs and plated on MacConkey agar (see example in Figure 2.2). The plates were incubated at 37 °C for 24 h and examined for bacterial colonies.

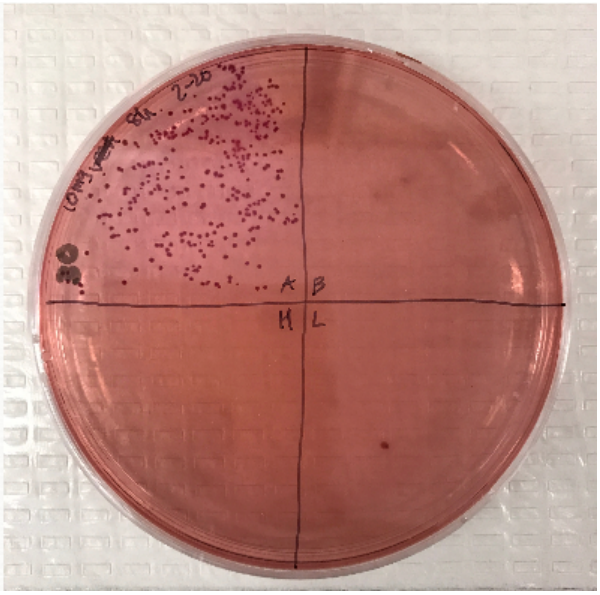

Figure 2.2 An example of *E. coli* re-isolation on a MacConkey agar plate.

MacConkey agar plates are labeled with the corresponding tag number of each individual chicken and divided into four sections prior to the *E. coli* re-isolation. The blood (B), air sac (A), heart (H), and liver (L) of each chicken was swabbed with sterile cotton swabs and inoculated onto the designated quadrants. The inoculated MacConkey agar plates were incubated at 37 °C for 24 h.

2.2.5 IL-1β serum cytokine ELISA

Blood samples were collected from the wing vein. The serum was obtained after centrifugation and stored at -20 °C until measurement of the pro-inflammatory cytokine, IL-1β. For *in vitro* quantitative detection of IL-1β in chicken serum, concentrations of the cytokine in each sample were determined using an ELISA kit purchased from MyBiosource, Inc (San Diego, CA). The double-antibody sandwich ELISA employs an anti-chicken IL-1β monoclonal antibody as the pre-coat and biotin-labeled polyclonal antibody as the detecting antibody. 100 µL of serum samples and chicken IL-1β standard samples were added to the antibody-precoated plate and incubated at 37 °C for 90 min. The plate was washed three times before 100 µL of biotinylated anti-chicken IL-1β antibody was added to each well and incubated at 37 °C for 60 min. After three washes, 100 µL enzyme-conjugate liquid was added to each well and incubated at 37 °C for 30 min. The plate was washed five times before the addition of the color reagent liquid (100 µL) to each well and incubated in the dark at 37 °C until the chicken IL-1β standards have darkened and displayed a color gradient. The OD values were measured at 450 nm within 10 min.

2.2.6 Preparation of tissue for proteome and protein quantification

The liver and spleen were harvested and stored in -80 °C freezer until utilized. At time of processing, organs were thawed and ~200 mg of tissue was homogenized with dounce tissue grinders on ice with 1 ml of 50 mM Tris-HCl (pH 7.4). Homogenates were centrifuged at 4 °C for 30 min at 10,000 ×g and the supernatant (post-mitochondrial) of each sample were collected and stored at -80 °C until utilized. Protein concentrations for

liver were determined with supernatants diluted 1:20 v/v in deionized distilled water and incubated with Quick Start Bradford Dye Reagent (Bio-Rad, Hercules, California) for 5 min at room temperature. Absorbance values of liver homogenates were measured at 595 nm with a plate reader and compared against a bovine serum albumin standard to determine protein concentrations.

2.2.7 Gel-based activity-based protein profiling (ABPP)

Activity ABPP is a widely adapted chemical proteomic strategy to characterize enzyme functions directly in native biological systems on a global scale. It enables the activities of enzymes with conserved catalytic mechanisms to be evaluated in their native environments within tissues (21). The activity of 2-AG degrading enzymes was investigated via gel-based ABPP. Liver homogenates containing native tissue proteomes were diluted to a final protein concentration of 2 mg/mL in a 50 µL reaction volume with 50 mM Tris–HCl (pH 7.4). 1 µL of 8 µM FP-biotin was added to each sample and incubated for 60 min in RT. Negative controls included at least one proteome sample that was denatured at 90 °C for 5 min prior to the addition of FP-biotin. The samples were mixed with 10 µL of 6X SDS-PAGE loading buffer to terminate the reaction and then heated on a thermo-mixer at 90 °C for 5 min. After the samples cooled to RT, 25 µL of each sample was loaded onto the 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel where electrophoresis proceeded at 100 V for 15 min and 120 V for 70 min on 8-well gels (90 min on 16-well gels). The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane at 20V for 30 min. Following the transfer, the membranes were blocked in 5% (w/v) non-fat milk at 4 °C overnight.

After washing with tween buffer the membranes were incubated with an avidin-horseradish peroxidase conjugate (1:2000 v/v), in 1% (w/v) non-fat milk for 2 h. The membranes were washed with tween buffer for 10 min for a total of three times before they were incubated with chemiluminescent substrate for 5 min. Images were obtained using X-ray film for various exposure times.

2.2.8 Enzyme assay

The potency of the drug, JZL184, was measured by its inhibition of the 2-AG degrading enzyme, carboxylesterases, in chicken blood serum. JZL184 was dissolved in dimethylsulfoxide (DMSO) and added to the reaction mixture to give final concentrations of 0, 0.1, 1, and 10 μM. JZL184 was incubated with 5 μl of undiluted chicken serum in a 96-well plate in a total volume of 155 μL in 50 mM Tris–HCl (pH 7.4) for 30 min at 37 °C. p-NPV was added to a final concentration of 500 μM and the hydrolysis reactions were measured at 405 nm for 5 min. The slopes were determined to calculate the activity of the carboxylesterase enzyme family. The data were best fit to an equation that describes noncompetitive enzyme inhibition.

2.2.9 Statistical analysis

The mean ± SE was determined for each treatment group. Comparison of chicken serum IL-1β between different groups and enzymatic activities of CES in chicken liver, spleen, and serum were assessed by a Student's t-test. The measure of JZL184 efficacy was assessed by the comparative protection from clinical colibacillosis, defined as the presence of lesions typical of colibacillosis in liver, heart, or air sacs.

2.3 Results

2.3.1 Pathological lesions

The pathogenic strain O78 of avian *E. coli* produces systemic infections causing airsacculitis, pericarditis, and perihepatitis. Air sacs, hearts, and livers were examined for gross lesions at 8, 28, and 56 h post infection. The gross lesions of *E. coli* infection are typically marked with thickening and development of suppurative exudate in the air sacs, liver focal necrosis, and inflammation of the pericardium and excess of fluid (Figure 2.3). As shown in Table 2.2, airsacculitis lesions in APEC challenged chickens formed as early as 8 h p.i., while the other two lesions, perihepatitis and pericarditis, developed slower and presented at later time points.

E. coli re-isolation provided information on a possible trend for the bacteria to be eliminated faster in JZL184-treated birds compared to vehicle-treated birds (Table 2.3). Figure 2.4 demonstrated the trend of *E. coli* elimination in chickens. At the 8 h time point, JZL184 treated groups were found to have a higher number of chickens positive for *E. coli* in their air sacs. Despite beginning with more infected birds, the JZL184 treated groups had equal or less amount of birds positive for *E. coli* by the last time point, 56 h p.i.

Throughout the duration of the study, only one chicken, which was the vehicle-treated bird, was found dead between 28 to 56 h p.i. from the systemic infection induced by APEC. Gross postmortem examination revealed the dead bird had developed airsacculitis lesions. As shown in Figure 2.5, the number of chickens with airsacculitis in each group stayed constant after 28 h p.i. The higher dose (40 mg kg^{-1}) of JZL184 treatment led to 100% occurrence of airsacculitis lesions compared to all other treatments

observed (see Figure 2.5). As shown in Figure 2.6, the percentage of birds found healthy and without any lesions varied in each group. The negative control birds were used as reference with 100% rate of healthy chickens. Of the birds infected with *E. coli*, the vehicle control birds had 33.3% of birds healthy by the end of the study, whereas the JZL184 treatments were found to be associated with increased lesion development following APEC inoculation. The lower dose of JZL184 treatment displayed similar results as the higher dose; however, 16.7% of the chickens in the low-dose group remained lesion-free until the end of the study, whereas no birds were lesion-free in the high-dose group.

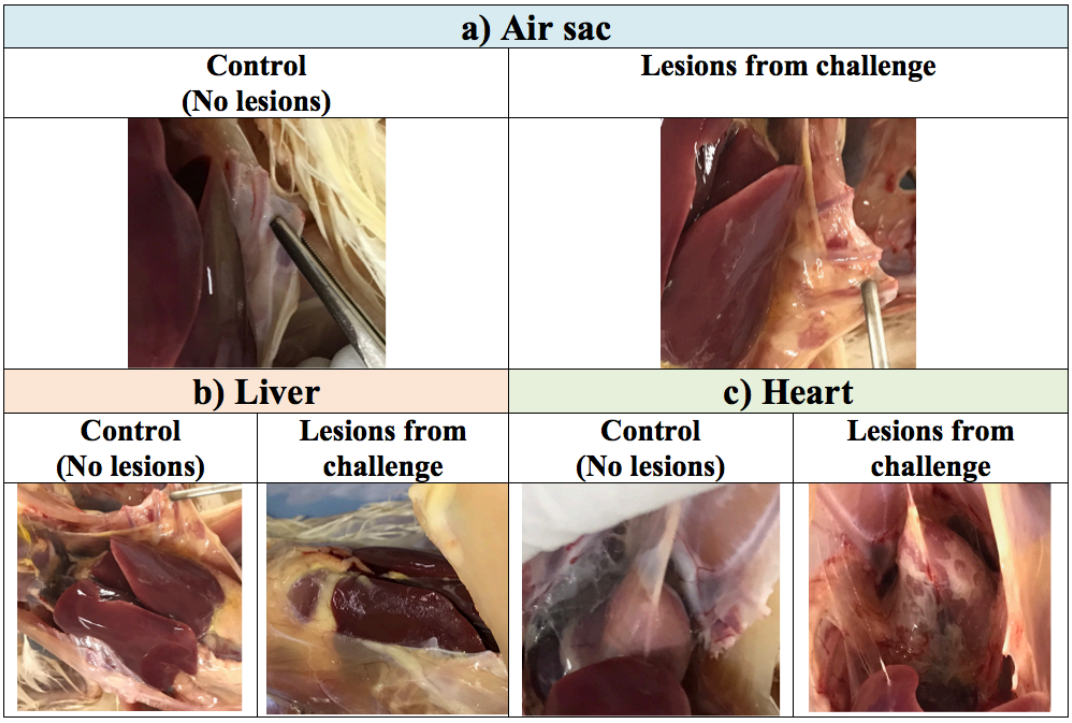

Figure 2.3 Gross lesions of chickens challenged with 1×10^8 CFU of APEC O78.

The gross lesions of *E. coli* infection are typically marked with a) endothelial thickening and development of suppurative exudate in the air sacs; b) Inflammation of the coating of the liver develops and c) inflammation of the pericardium develops on the heart.

Table 2.2 Pathological findings on chicken treated with or without JZL184 and then challenged with or without APEC O78.

Gross lesions[#] (No. positive/total)	Airsacculitis			Perihepatitis			Pericarditis			No lesions		
Hours (p.i.)*	8	28	56	8	28	56	8	28	56	8	28	56
Negative control	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3
Vehicle control + *E. coli*	1/6	4/6	4/6	0/6	0/6	3/6	0/6	1/6	1/6	5/6	2/6	2/6
10 mg/kg JZL184 + *E. coli*	3/6	5/6	5/6	0/6	0/6	1/6	0/6	0/6	1/6	3/6	1/6	1/6
40 mg/kg JZL184 + *E. coli*	3/6	6/6	6/6	0/6	2/6	0/6	1/6	1/6	0/6	3/6	0/6	0/6

Number of chickens positive for airsacculitis, perihepatitis, and pericarditis were accounted for and the rates of *E. coli* occurrence were presented as the number of positive over the total number of birds tested in each group.
*Euthanasia and necropsy were performed at 8, 28, and 56 h p.i.

Table 2.3 *E. coli* re-isolation findings on chickens treated with or without JZL184 and then challenged with or without APEC O78.

Microbiology* (No. positive[#]/total)	Air sac			Liver			Heart			Blood		
Hours (p.i.)	8	28	56	8	28	56	8	28	56	8	28	56
Negative control	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Vehicle control + *E. coli*	4/6	3/6	2/6	2/6	1/6	1/6	2/6	0/6	1/6	0/6	0/6	0/6
10 mg/kg JZL184 + *E. coli*	6/6	4/6	1/6	3/6	1/6	0/6	3/6	1/6	0/6	1/6	0/6	0/6
40 mg/kg JZL184 + *E. coli*	5/6	4/6	2/6	2/6	1/6	0/6	1/6	0/6	0/6	0/6	0/6	0/6

* At each time point, chickens were sacrificed and their air sacs were swabbed and plated on MacConkey agar and incubated at 37 °C for 24 h.
# Number of chickens positive for *E. coli* were accounted for and the rates of *E. coli* occurrence are presented as the number of positive over the total number of birds tested in each group.

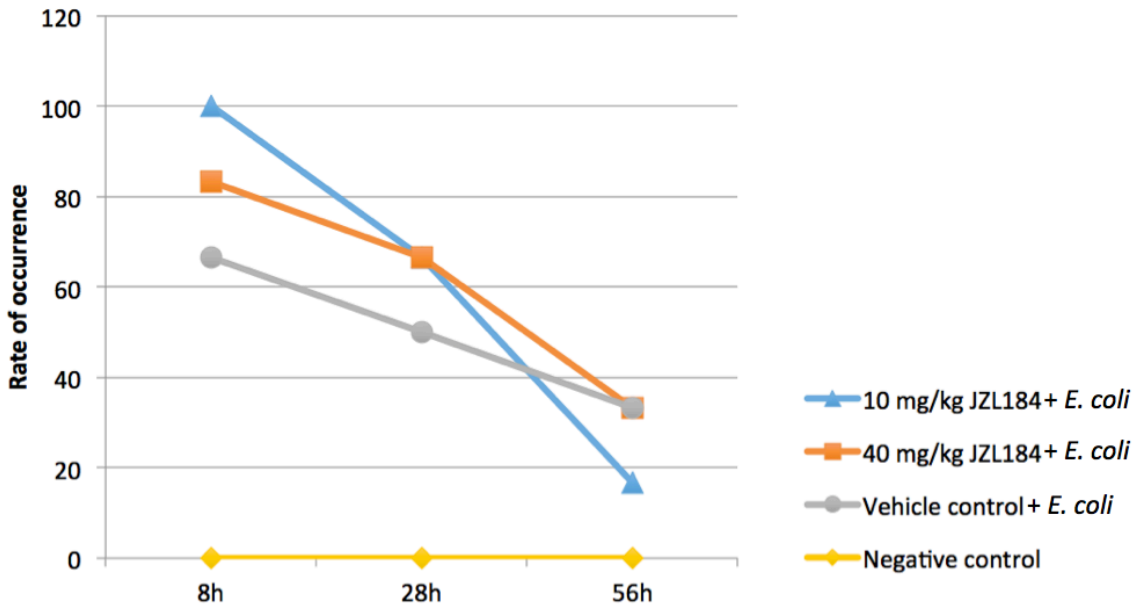

Figure 2.4 Percentage of chickens' air sacs tested positive for *E. coli* after re-isolation for each treatment group at 8, 28, 56 h time points p.i.

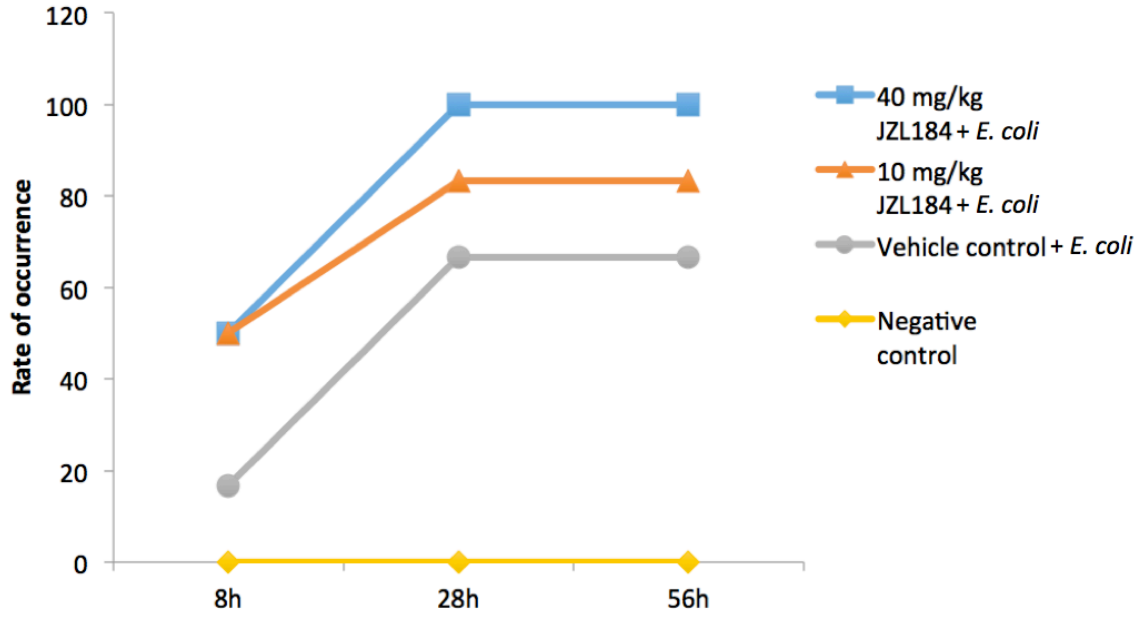

Figure 2.5 Percentage of birds in each group with airsacculitis gross lesions post infection.

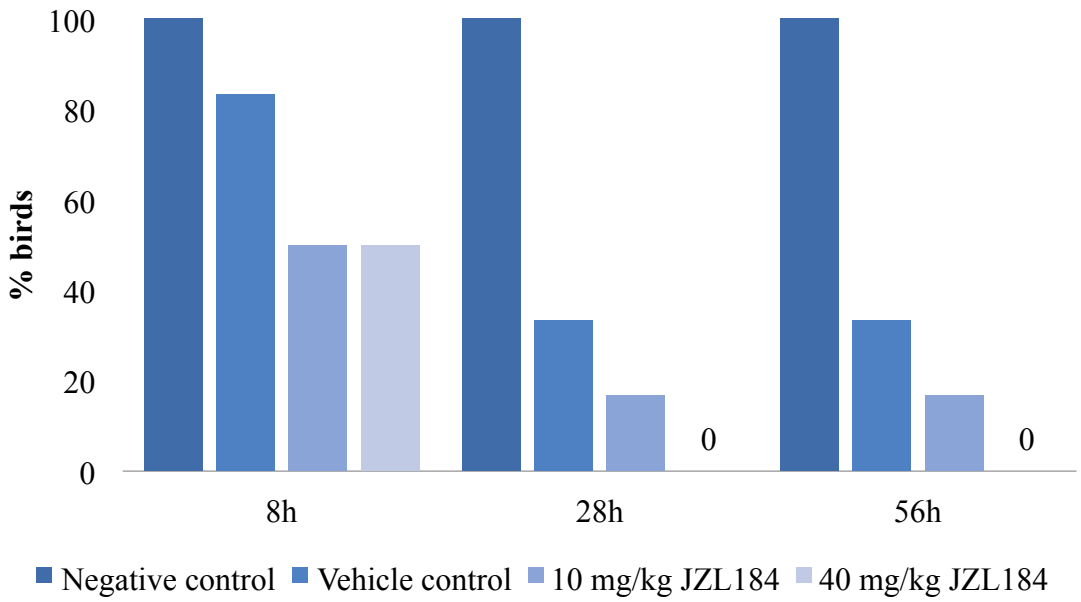

Figure 2.6 Percentage of lesion-free birds within each group over time (p.i.).

At 28 and 56 h p.i., the percent occurrence of lesion-free birds from the 40 mg/kg JZL184 treatment group was 0%.

2.3.2 Cytokine analysis IL-1β ELISA

Many cytokines, such as TNF-α and IL-1β, are known to be important mediators responsible for coordinating the inflammatory responses in the host as a defense mechanism against the endotoxins released by pathogenic bacteria. As shown in Figure 2.7, blood serum IL-1β levels decreased over time for the vehicle-treated and higher dose JZL184-treated groups. For the lower dose, 10 mg kg^{-1} JZL184 treatment group, the serum IL-1β level significantly decreased by the 28 h p.i. time point but later significantly increased within the 56 h p.i time point.

As shown in Figure 2.8, the concentration of IL-1β after JZL184 treatment and *E. coli* challenge presented no significant differences. The data only revealed a significant up-regulation of IL-1β levels between the 10 mg kg^{-1} JZL184-treated group and the vehicle control group at the 56 h p.i. time point. The expressions of IL-1β in all of the groups challenged with *E. coli* were not found significantly different from the negative control groups at all of the time points (8, 28, and 56 h p.i.).

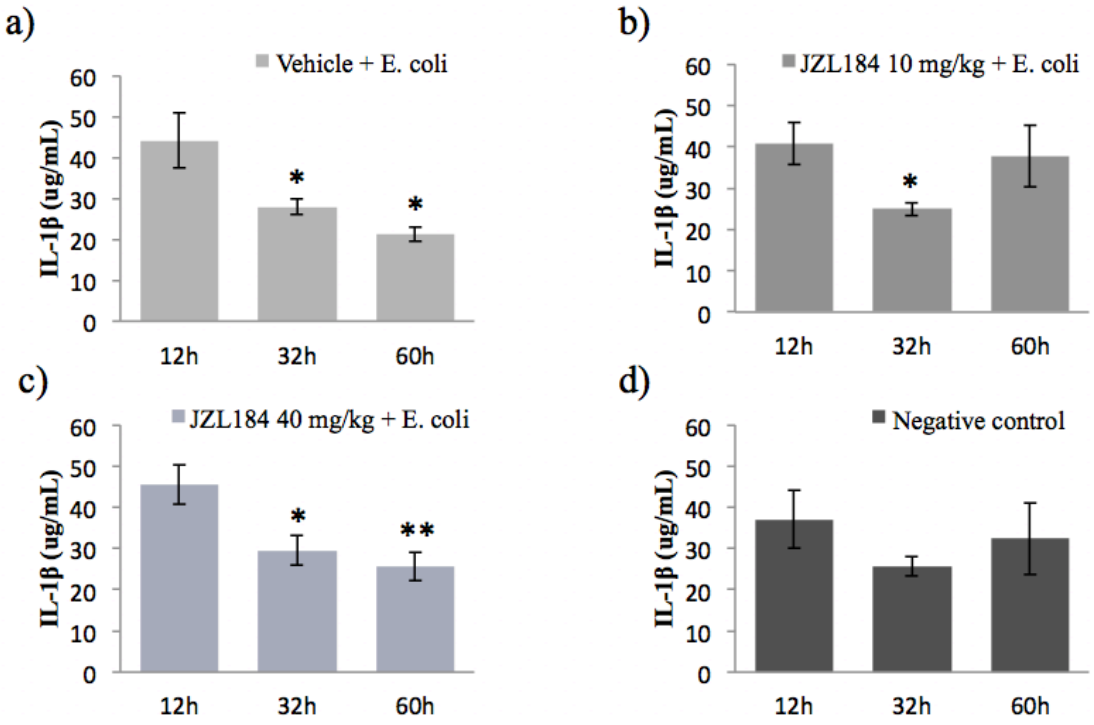

Figure 2.7 IL-1β concentration in chicken blood serum post JZL184 injection

Data are analyzed by comparing the IL-1β levels within the a) vehicle control group, b) JZL184 treatment group (10 mg kg^{-1}), c) JZL184 treatment group (40 mg kg^{-1}), and d) negative control group. *$p < 0.05$ as compared to IL-1β levels at 12 h post JZL184 treatment. **$p < 0.01$ as compared to the IL-1β level at 12 h post 40 mg kg^{-1} JZL184 treatment .

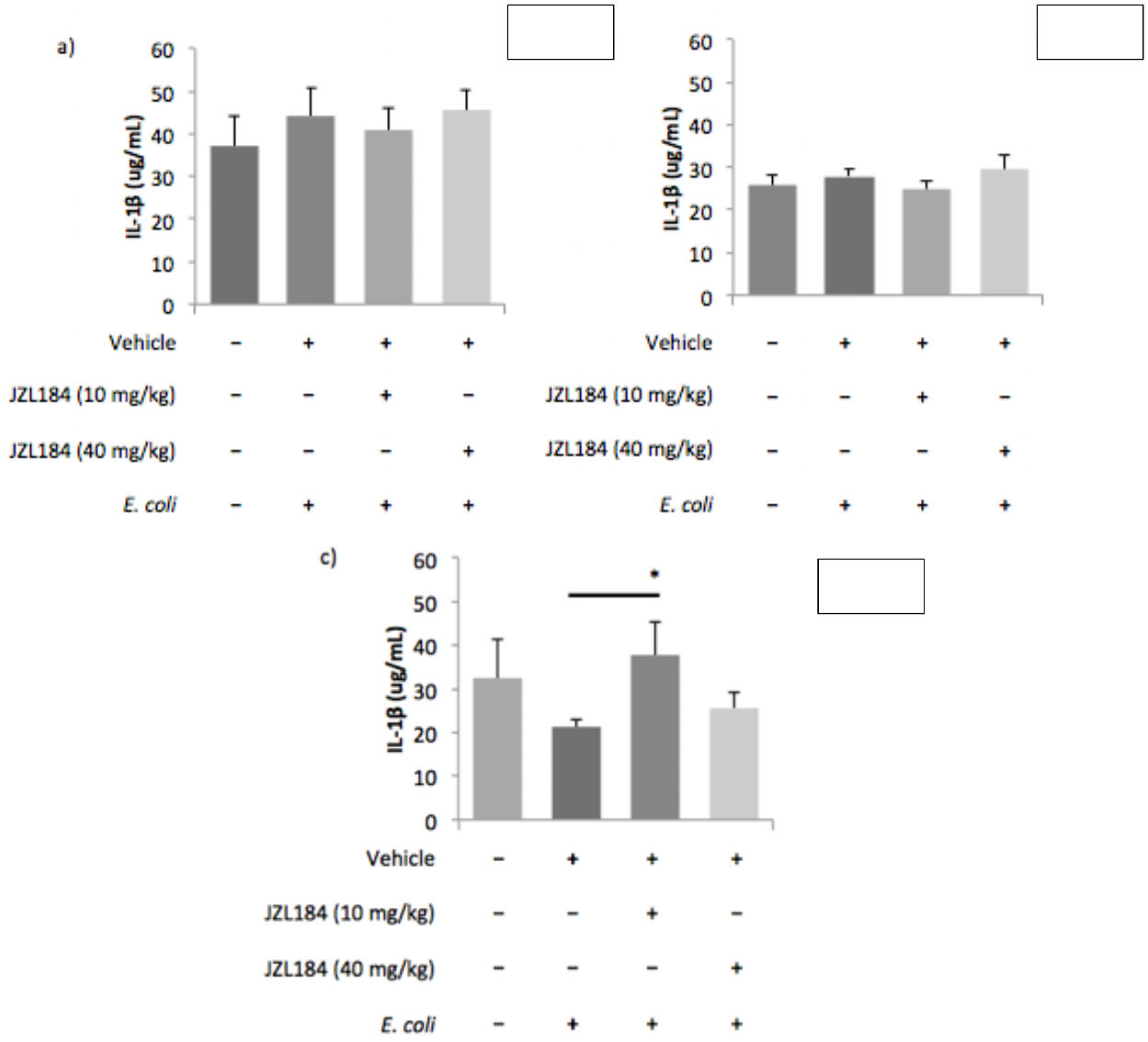

Figure 2.8 Chicken serum proinflammatory cytokine IL-1β levels

Concentrations of IL-1β over three different time points, a) 8 h, b) 28 h, and c) 56 h p.i. were compared between control groups and treatments. No significant differences were observed between the vehicle control (challenged with *E. coli*), low-dose treatment (10 mg/kg JZL184), and high-dose treatment (40 mg/kg JZL184) when compared to the negative control ($p < 0.05$).

2.3.3 Carboxylesterase enzyme inhibition by JZL184

The mode of inhibition of CES enzyme activity by JZL184 in chicken blood serum was investigated. Analysis of the fractional inhibition data was best described by an inhibition model (Figure 2.9; r^2 =0.99). The IC_{50} value was 3.015 µM.

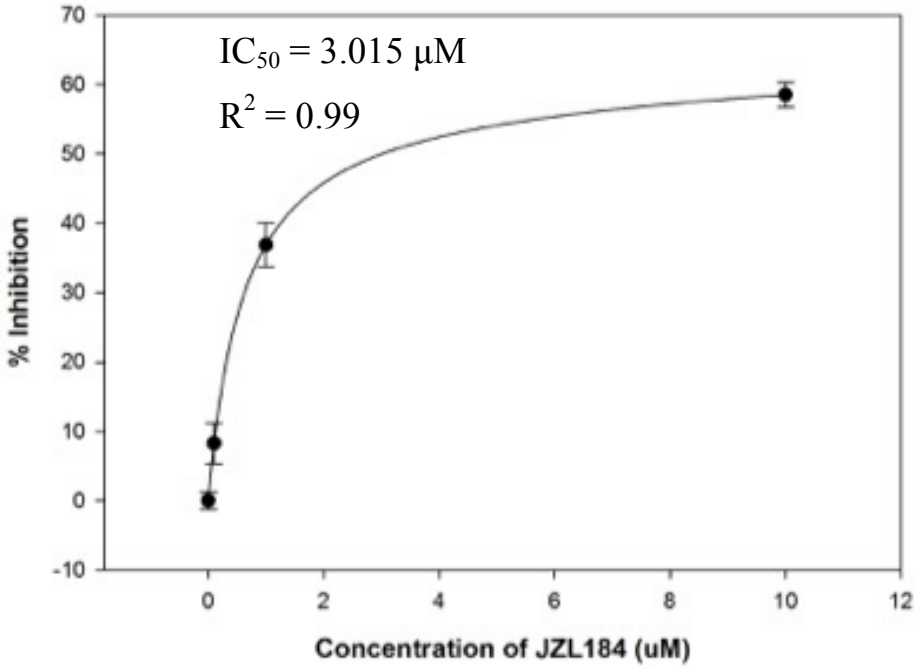

Figure 2.9 Inhibition of the carboxylesterase activity by JZL184 detected in chicken serum

2.3.4 Competitive ABPP

SDS-PAGE was used in this study to assess 2-AG degrading enzymes activities in liver and spleen proteomes in response to JZL184 using FP-biotin as the activity probe. Results in the gel images (Figure. 2.10-2.14) revealed no evidence of noticeable serine hydrolase inhibition or CES enzyme inhibition by JZL184 treatments.

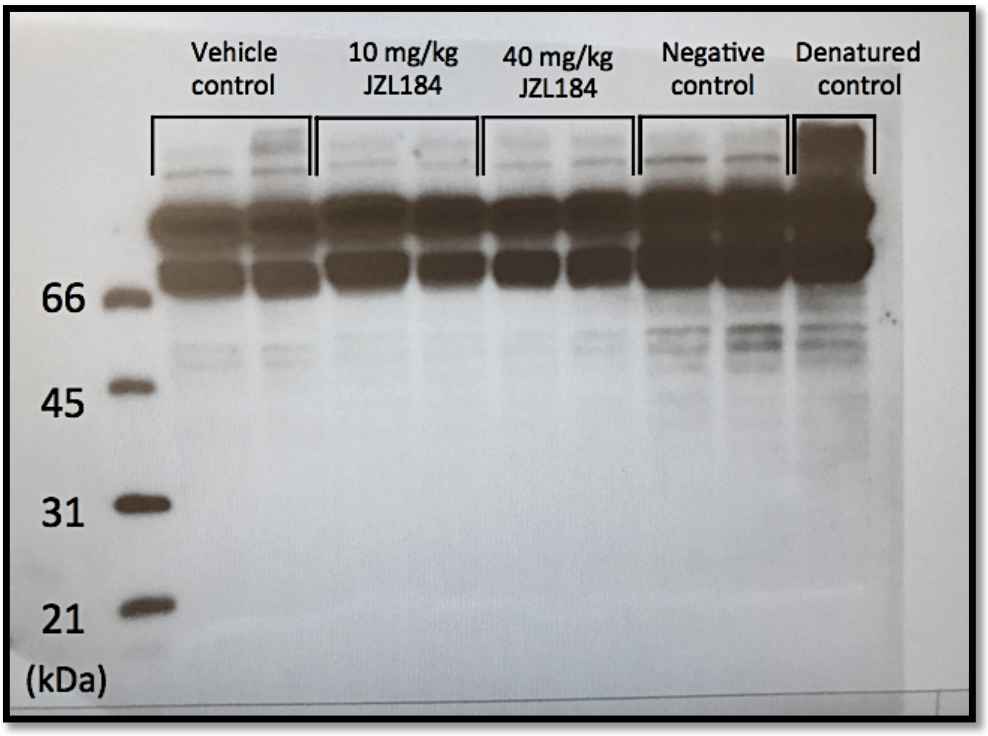

Figure 2.10 SDS-PAGE results of chicken liver homogenates (8-well) (32 h post JZL184 injection)

Two liver homogenate samples from each treatment group sacrificed at 32 h post JZL184 injection were randomly selected for SDS-PAGE. The denatured control was a negative control liver homogenate boiled at 95 °C for 5 min.

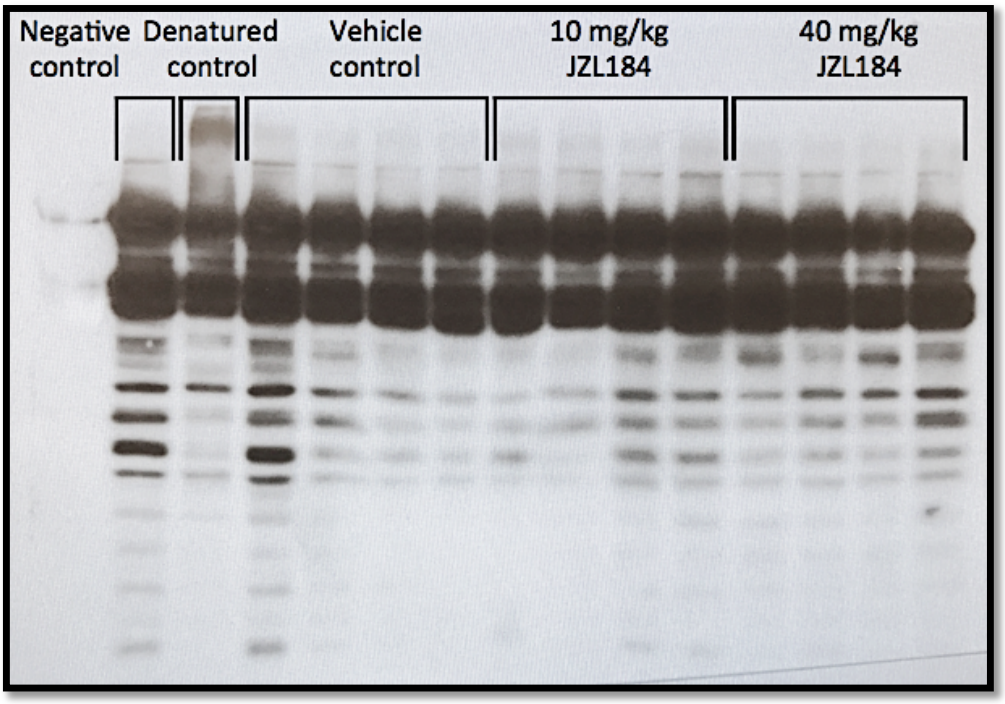

Figure 2.11 SDS-PAGE results of chicken liver homogenates (16-well) (32 h post JZL184 injection)

One liver homogenate of a chicken sacrificed at 32 h post JZL184 injection from the negative control group was randomly selected to be used for both the negative control and denatured control sample. The denatured control sample was a negative control liver homogenate sample boiled at 95 °C for 5 min. Four liver homogenates of chickens sacrificed at 32 h post JZL184 injection were randomly selected from groups that had treatments of vehicle, 10 mg/kg JZL184, and 40 mg/kg JZL184. A total of 14 samples from chickens sacrificed at the 32 h post JZL184 injection time point were analyzed with SDS-PAGE to examine for enzyme activity.

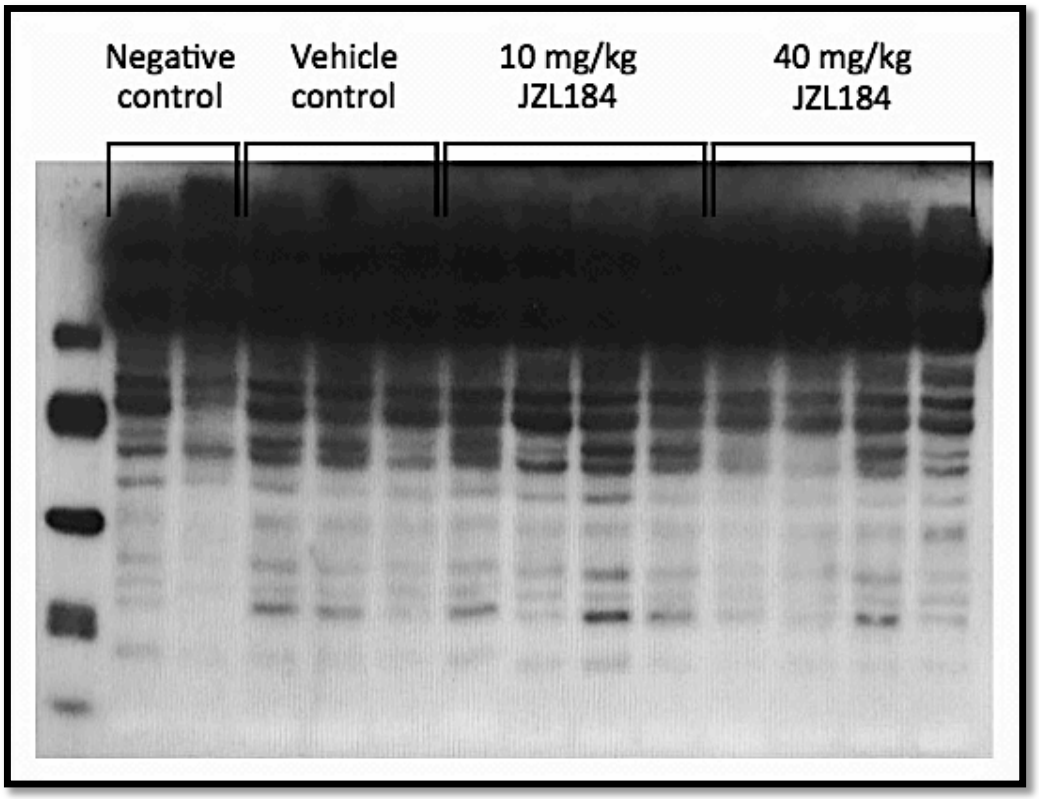

Figure 2.12 SDS-PAGE results of chicken liver homogenates (16-well) (60 h post JZL184 injection)

Two and three liver homogenates of chickens sacrificed at 60 h post JZL184 injection from the negative control group were randomly selected to be used for the negative and vehicle control samples, respectively. Four liver homogenates of chickens sacrificed at 60 h post JZL184 injection were randomly selected from the10 mg/kg JZL184 and 40 mg/kg JZL184 treatment groups. A total of 14 samples from chickens sacrificed at the 60 h post JZL184 injection time point were analyzed with SDS-PAGE to examine for enzyme activity.

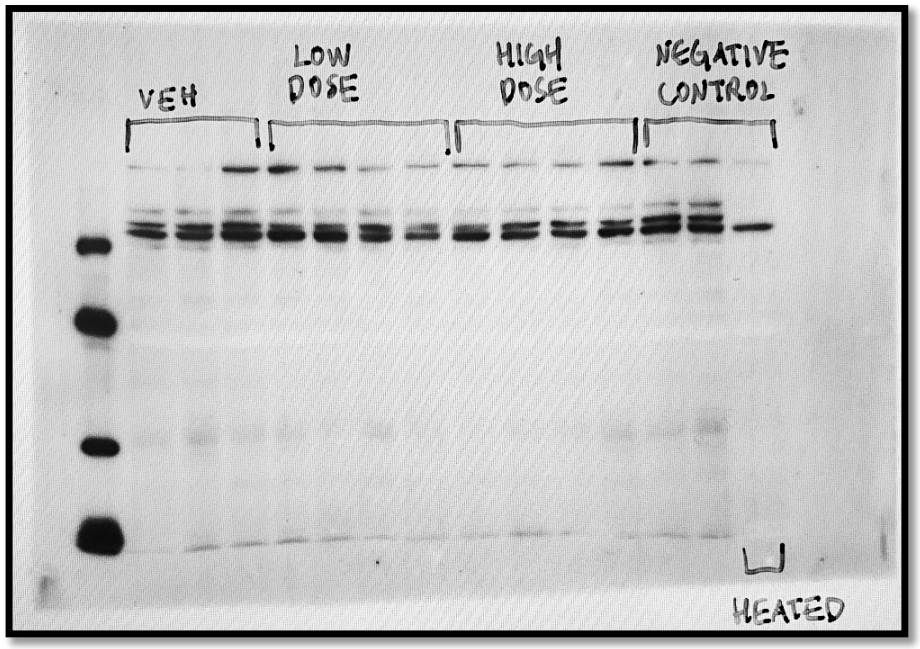

Figure 2.13 SDS-PAGE results of chicken spleen homogenates (16-well) (32 h post JZL184 injection)

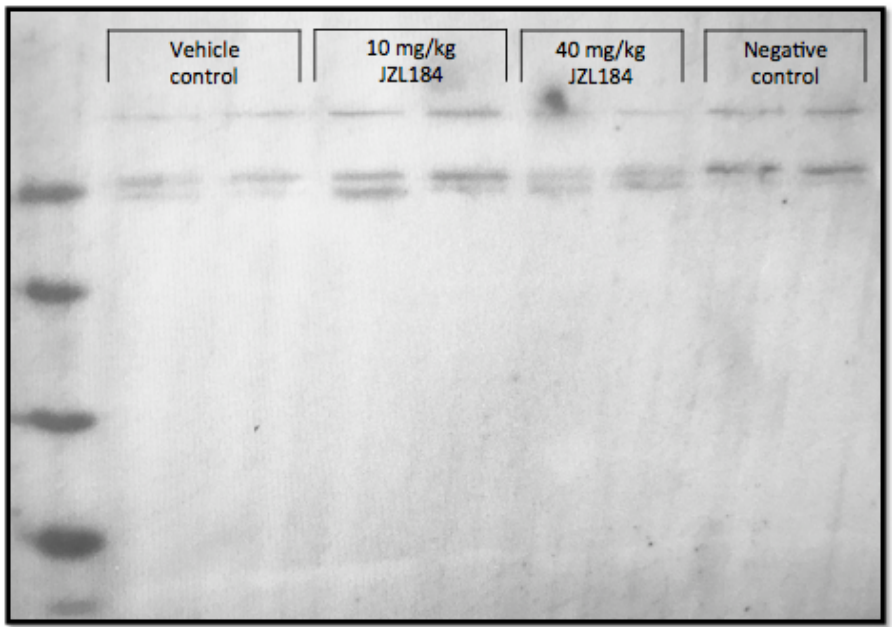

Figure 2.14 SDS-PAGE results of chicken lung homogenates (8-well) (32 h post JZL184 injection)

2.3.5 Carboxylesterase enzyme activity

Activities of CES enzymes were measured to investigate whether there were inhibiting effects of JZL184 after 32 and 60 h post JZL184 injection. No significant differences were found between the vehicle and the JZL184 treatments at the two later time points. JZL184 did not affect CES enzymes in spleen samples collected at 32 and 60 h post JZL184 treatment. It is suggested that the pharmacological effects of the serine hydrolase inhibitor, JZL184, were already depleted within 32 h.

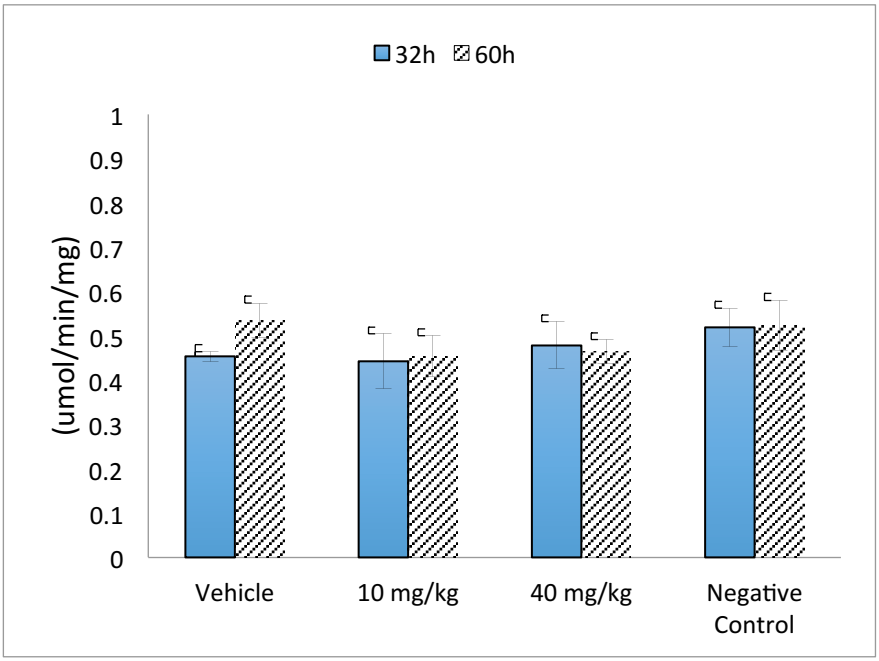

Figure 2.15 Spleen CES enzymes activity

Spleen homogenates from 32 and 60 h post JZL184 treatments were analyzed for CES enzyme activity. Earlier time points were not available. The levels of the CES enzyme activity are presented in the units, μmol per min per mg of homogenate. No significant differences were found between JZL184-treated birds and the vehicle-treated birds at both time points.

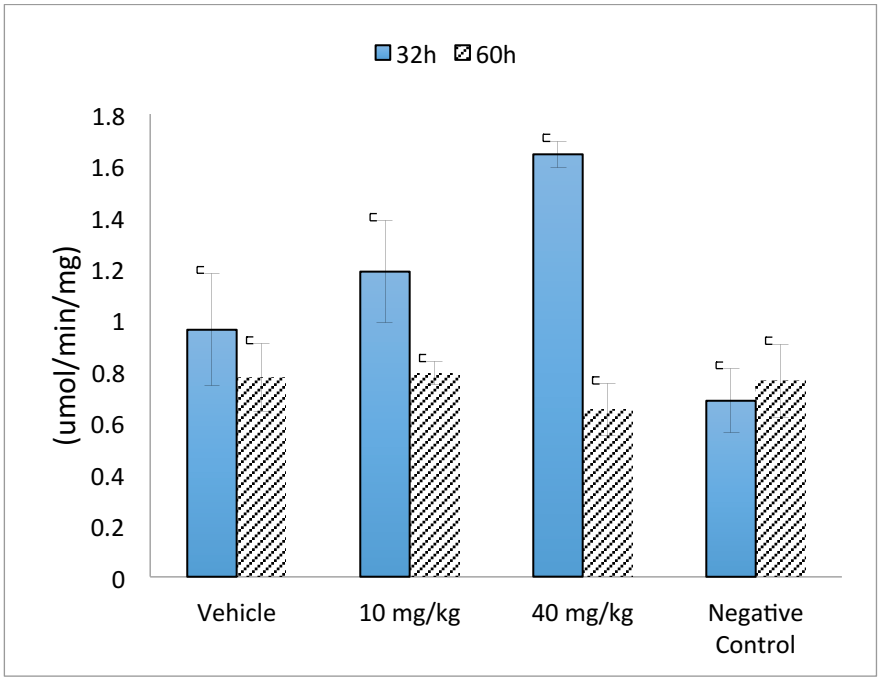

Figure 2.16 Liver CES enzymes activity

Liver homogenates from 32 and 60 h post JZL184 treatments were analyzed for CES enzyme activity. Earlier time points were not available. The levels of the CES enzyme activity are presented in the units, µmol per min per mg of homogenate. No significant differences were found between JZL184-treated birds and the vehicle-treated birds at both time points.

2.4 Discussion

The purpose of this study was to evaluate the effectiveness of JZL184 as an immunomodulatory agent in chickens against APEC strain O78 challenge and to compare their pathological changes as well as their immunological responses. The effects of JZL184 and pathogenicity of APEC strain O78 were assessed by gross lesions and re-isolation of the bacteria from the blood, liver, heart, and air sacs at 8, 28, and 56 h p.i. The *E. coli* challenge provided us with new insight as to how rapid the disease progression in 5-week-old chickens can be. We documented airsacculitis lesions presenting in the challenged chickens' air sacs as early as 8 hr post infection. The

colibacillosis lesions were found to be more severe in chickens given JZL184 treatments. The severity of the lesions ranked from the most severe to the least severe was 40 mg kg^{-1} JZL184-treated group, 10 mg kg^{-1} JZL184-treated group, and vehicle control group. In this study, airsacculitis was the first and major lesion observed from APEC infected chickens. We noticed JZL184 induced the most severe airsacculitis lesions when compared to the vehicle and negative control groups. However, in the elimination of *E. coli*, JZL184-treated birds showed an enhanced ability to clear the bacteria by 56 h p.i. than the control groups. The data on airsacculitis lesions were more indicative than the other lesions (perihepatitis and pericarditis), which allowed us to conclude the drug, JZL184, accelerates the progression of airsacculitis but did not reduce the bird's ability to clear out APEC.

Some of the data from this study suggested effects contrary to a strengthened immune system in chickens (Table 2.2), whereas some of the other data suggested a possibility for JZL184 to have potential in benefiting chickens challenged with APEC (Table 2.3). It is likely that the early onset of tissue damage may be caused by suppression of host immunity by the chemical compound, JZL184, and its vehicle solution (4:1 ratio of polyethylene glycol 300: Tween 80). Therefore, JZL184 may have an immunosuppressive effect instead of an immunomodulating effect on chickens. It could be implied that the inhibition of serine hydrolases may reduce the release of eicosanoids, resulting in the suppression of pro-inflammatory signaling where JZL184 may have potential as an anti-inflammatory drug. JZL184 did not induce of protective immunity against APEC O78, suggesting no prophylactic effect of JZL184 on the immune system 4 h prior to the *E. coli* challenge. Future work may involve experiments

that test the effects of JZL184 on chicken inflammatory responses post *E. coli* infection to investigate whether the drug affects colibacillosis disease progression or enhances the phagocytosis by immune cells during the clearance of pathogens.

Many cytokines, such as TNF-α, IL-1β and IL-6, are known to be important soluble mediators responsible for coordinating rapid inflammatory responses, induction of immune effector responses, and participate in the pathogenesis of hemodynamic involvement and lesions in chickens (22-26). The proinflammatory cytokine, IL-1β is produced by mononuclear phagocytes and other cell types in response to viral, bacterial or protozoal infections in chicken (27). Therefore, we examined the possible roles of IL-1β on the immunomodulating effects of JZL184 in APEC O78-infected chickens. Our study found little to no effects on serum cytokine levels and chicken immune system post injection of JZL184 *in vivo*. The ELISA test revealed the changes of the levels of proinflammatory cytokine IL-1β over time was not affected by JZL184 (Figure 2.8). IL-1β concentrations in the APEC challenged groups were significantly lower at 28 and 56 h p.i. than at 8 h p.i. All three APEC challenged groups showed serum IL-1β levels decreasing over time after 8 h p.i. The expression of IL-1β in the groups challenged by APEC O78 were not different from the control group, indicating the peak of pro-inflammatory cytokine up-regulation during the immune response following *E. coli* infection could have occurred prior to 8 h p.i. It should also be considered that since proinflammatory cytokines are produced on demand and that the *in vivo* concentrations fall in the range of a few Pico-grams to Nano-grams per ml, blood serum levels of the cytokine might not accurately reflect the cytokine levels at the site of inflammation (28). Further investigation on the rate of JZL184 absorption in chickens would be useful in

adjusting sampling time points for future experiments. With little to no stimulation on the secretion of proinflammatory cytokine IL-1β, we postulated that the effects of JZL184 on immune cells are either within the first 4 h post injection or delayed due to slow absorption. The IL-1β levels for the 10 mg kg^{-1} JZL184 treated groups indicate a slow rate of absorption due to the pharmacokinetics of the drug in chickens where significant drug effects were not observed until 60 h post injection. We observed no positive correlation between JZL184 and IL-1β levels in the serum and detected no appreciable differences between IL-1β levels from the *E. coli* infection. IL-1β may be an important cytokine in the occurrence of avian colibacillosis but the findings concerning the serum levels of IL-1β will require further investigation to reach a decision regarding the role of proinflammatory cytokines in APEC infection and the immunomodulating effects of JZL184 in chickens.

Information about JZL184 treatment in chickens is limited. Studies have found the serine hydrolase inhibitor JZL184 to display cross-reactivity with other enzymes, such as FAAH and carboxylesterases, where it may complicate the outcomes in biological studies (29). It is also reasonable to assume MAGL is not likely the major 2-AG metabolizing enzyme in chickens because not all cell lines express MAGL mRNA. In one study, Marrs et al. found ABHD6 controlling 2-AG's efficacy at the CB2 receptors in the cells from the BV-2 microglia cell line and discovered FAAH also played a role in 2-AG hydrolysis (11). How JZL184 affects the endocannabinoid system on a species- and tissue-specific manner is not clear. The discrepancy of JZL184's efficacy in enzyme inhibition between different species and tissues should be further studied using proper

negative and positive controls to confirm the cross-reactivity between mouse, rats, and chickens.

Understanding the chickens *in vivo* responses to different JZL184 solubilizing vehicles would also help ensure the effectiveness of the treatments for future studies. The solubilizing and delivering agents used to dissolve JZL184 and similar inhibitors (KML29) were found to affect both its solubility and efficacy in multiple studies (32-34). Other than the vehicle (4:1 ratio of polyethylene glycol 300:Tween 80) used in both this study and one of the earliest studies by Long et al. (8), there were also saline-emulphor emulsion (saline: cremophor: ethanol; 18: 1: 1) and another saline-based vehicle (15% dimethyl sulfoxide (DMSO), 4.25% Tween 80, 4.25% polyethylene glycol (PEG) 400 and 76.5% saline) (30, 31). It could be in our interest to test and compare the solubility and effects of the different vehicles to find the best suitable formula for safe delivery and administration of JZL184 in future chicken studies.

In conclusion, we demonstrated that JZL184 did not reduce the severity and progression of lesions produced in chickens challenged with 10^8 CFU of APEC O78. It also did not increase the serum levels of IL-1β. Future studies are needed to understand the interactions between the endocannabinoid systems and the immune systems in chickens regarding the reaction mechanisms of JZL184 or the pathogenic challenge models of *E. coli*, as well as immunomodulating treatments for avian infections.

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CHAPTER III

CONCLUSION

Endocannabinoid research has given hope to the treatments of many important inflammatory diseases since it's therapeutic properties has been discovered and introduced. Recognizing and understanding the effects of serine hydrolase inhibitors, such as JZL184, in chickens allows for potential therapeutic or preventative treatments to be established as a combat mechanism against opportunistic pathogens that lead to infectious diseases, such as *E. coli*. It was hypothesized that intraperitoneal injections of JZL184 treatments in 5-week-old chickens could elevate endogenous 2-AG levels to increase macrophage cytokines and promote the recruitment of immune cells at the infected tissues in APEC O78-challenged chickens. The work presented in this thesis showed that 10 mg kg^{-1} and 40 mg kg^{-1} of JZL184 did not reduce the severity and progression of lesions produced in chickens challenged with 10^8 CFU of *E. coli* O78. The JZL184 treatments made colibacillosis lesions in the *E. coli* O78-challenged chickens worse and we did not find evidence of the injections increasing the serum cytokine levels of IL-1β at our sampling times.

Future studies are needed to understand the interactions between the endocannabinoid systems and the immune systems in chickens regarding the reaction mechanisms of JZL184 or the pathogenic challenge models of *E. coli*, as well as immunomodulating treatments for avian infections. Though the focus of this thesis work

had centered on serine hydrolase inhibition and proinflammatory cytokine secretion at 12, 32, and 60 h post JZL184 treatment, the work could be further enhanced with adjustments to both the injection and blood sampling times. This could provide useful data on the efficacy of JZL184 with regards to chicken blood serum IL-1β levels and enzyme activities observed in liver samples. Due to the little amount of research that has been done to study the therapeutic potential of JZL184 in chickens, there is clearly the need to expand the experimental design of *in vivo* studies and fine-tune the techniques for sample analysis that could better characterize and distinguish the inflammatory effects that JZL184 could pose on chickens.