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THE EFFECT OF A CANNABINOID RECEPTOR 2 (CB₂) AGONIST ON ALLERGIC
AIRWAY INFLAMMATION IN A MOUSE MODEL OF ASTHMA

By

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B.S., University of Washington, Seattle, Washington, 2010

Thesis

presented in partial fulfillment of the requirements
for the degree of

Master of Science
In Toxicology

The University of Montana
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Autumn 2012

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ABSTRACT

Hong, Soram, M.S., Autumn 2012

Toxicology

The effect of a cannabinoid receptor 2 (CB₂) agonist on allergic airway inflammation in a mouse model of asthma

Chairperson: Dr. Kevan Roberts

Allergic asthma is a chronic inflammatory disease that affects approximately 300 million people worldwide. The health problem is compounded by the fact that the prevalence of the disease is increasing in the Western world. Thus, there is a continued need for new and improved therapies for the disease. Recently, it has become evident that cannabinoids have immunosuppressive properties and can be used as therapeutics for various inflammatory diseases. The main aim of this study was to explore the anti-inflammatory effects of CB₂ agonists on allergic airway inflammation using a mouse model of asthma and resolve any mechanisms that underpin the immunosuppressive properties observed using *in vitro* assays. The murine model of allergic lung inflammation entailed transferring ovalbumin (OVA) specific CD4⁺ T cells into normal BALB/c mice which then inhaled OVA for 7 days. Mice were treated daily with either CB₂ compound or vehicle, and the anti-inflammatory effect of the CB₂ agonist was examined by monitoring the effect of the treatment on the level of inflammation and histological changes. Intra-nasal administration of CB₂ analog suppressed the development of a pulmonary eosinophilia and the recruitment of allergen-specific CD4⁺ T cells into the airways. To assist in resolving the mode of action of these compounds the expression of CB₂ receptors on Th2 cells was examined using a novel NBD-labeled CB₂-selective compound. In additional experiments, cells were cultured with CB₂-selective agonist and the effect on Th2 cytokine production was determined. Our data suggest that the attenuation of inflammation mediated by CB₂ agonists is associated with the reduction of pro-inflammatory cytokines and the induction of anti-inflammatory cytokine.

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LIST OF ABBREVIATIONS AND SYMBOLS

AHR: airway hyperresponsiveness

APC (FACS): allophycocyanin

APC: antigen presenting cell

BALF: bronchoalveolar lavage fluid

BSA: bovine serum albumin

C: celcius

CB: cannabinoid

CD: cluster of differentiation

d: day

DC: dendritic cell

ELISA: enzyme-linked immunosorbent assay

EPO: eosinophil peroxidase

FACS: fluorescence-activated cell sorting

F_C: fragment crystallizable region

FITC: fluorescein-5-isothiocyanate

Foxp3: forkhead box P3

g: gram

h: hour

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP: horseradish peroxidase

Ig: immunoglobulin

IL: interleukin

LPS: lipopolysaccharides

M: molar

MDSC: myeloid-derived suppressor cells

mg: milligram

min: minute

mM: millimolar

NBD: nitro-benzoxadiazole

nm: nanometer

NO: nitric oxide

NOS: nitric oxide synthases

OVA: ovabumin

PBS: phosphate buffered saline buffer

PE: phycoerythrin

RPMI: Roswell Park Memorial Institute medium

SEM: standard error of the mean

TGF: transforming growth factor

Th: T helper

THC: delta-9-tetrahydrocannabinol

TMB: 3,3',5,5'-Tetramethylbenzidine

T_{reg}: T regulatory

WB: washing buffer

μl: microliter

μM: micromolar

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CHAPTER 1

INTRODUCTION

Allergic asthma is a major public health burden, affecting some 300 million individuals worldwide. The prevalence of the disease has dramatically increased in the recent decades, and thus there is a continued need for new and improved therapies. Allergic asthma is a chronic inflammatory airway disease characterized by pulmonary eosinophilia, increased mucus production by goblet cells, elevated IgE levels and structural remodeling of the airway wall, leading to airway obstruction and airway hyperreactivity (AHR) to nonspecific stimuli. Allergen-specific CD4⁺ Th2 cells that produce IL-4, IL-5, IL-9 and IL-13 take primacy in driving the mucosal inflammation in asthma. In addition, there is abundant evidence pointing to an important role for structural cells in the pathogenesis of bronchial asthma, that include epithelial cells, endothelial cells, airway smooth muscle cells and fibroblasts. A prominent role in the inflammatory process is played by lung vascular endothelial cells that provide a gateway allowing the recruitment of leukocytes to the site of inflammation.

The disease is characterized by bronchial inflammation and airway hyperreactivity (AHR). The inflammatory process is believed to result from allergens inducing the infiltration of CD4⁺ Th2 cells, eosinophils and other inflammatory cells. Th2 cells play an important role in the asthmatic response. These cells produce cytokines, such as IL-4, IL-5 and IL-13. These Th2 cytokines are responsible for Th2 phenotype commitment, eosinophil development and recruitment. Although asthma is most commonly associated with an aberrant Th2 cell response, severe disease is characterized by additional increased production of the cytokine IL-17. However,

studies dissecting the role of IL-17 in mouse models of asthma have generated variable results and the exact role of this cytokine and the mechanisms that underlie its production remain poorly understood.

It is reported that approximately 10% of the population of the United States is affected by the disease. Thus, there is a continued need for new and improved therapies for the disease. Cannabinoid 2 (CB₂) receptor is a receptor expressed by immune cells. Recently, there is increasing interest in cannabinoid-based compounds that selectively bind the CB₂ receptor as targets for treating chronic inflammatory disease. Given the anti-inflammatory properties of the CB₂ receptor, the aim is to examine the effect of the CB₂ agonist in allergic asthma using a mouse model of the disease.

1.1 Allergic Asthma

Allergic asthma is a complex airway inflammatory condition characterized by increased levels of inflammatory cell infiltration into the airways, airway hyperreactivity (AHR), IgE secretion, mucus production and airway remodeling (Robinson et al. 1992). These inflammatory effects lead to episodic shortness of breath and airway obstruction caused by the secretion of mucus and shedding of airway epithelial cells. Towards the severe end of the spectrum the smaller airways can become occluded by plugs of epithelial cells contained in mucous (Creola bodies).

1.1.1 Characteristics of Allergic Airway Inflammation

Allergic asthma is a multi-cellular process involving mainly eosinophils, neutrophils, CD4⁺ Th2 cells and mast cells (Kay et al. 2005). CD4⁺ Th2 cells are responsible for driving the inflammatory response. Thus, in order to understand the pathogenesis in allergic asthma, it is important to understand how CD4⁺ Th2 cells mediate the inflammatory response. Figure 1 illustrates a schematic representation of the inflammatory cascade in allergic asthma (Bradding et al. 2006). Allergen sensitization is a prerequisite to development of the inflammatory cascade. Antigen-presenting cells (APC) uptake and process inhaled allergens (Bloemen et al. 2007; Verstraelen et al. 2008). The processed allergens are presented to naïve T cells. Then, mucosal T cells in the airways are polarized into Th2 cells and pro-inflammatory cytokines are expressed (Bloemen et al. 2007; Verstraelen et al. 2008). Through pro-inflammatory cytokine production, Th2 cells have the capacity recruit secondary effector cells such as macrophages, basophils and eosinophils into the inflammatory site where these cells become primed and activated for mediator secretion, (Bloemen et al. 2007; Verstraelen et al. 2008). IL-4, IL-5 and IL-13 are cytokines expressed by Th2 cells. IL-4 promotes IgE production and involves in growth and activation of mast cells (Bloemen et al. 2007; Verstraelen et al. 2008). IL-5 promotes eosinophil development, activation, and tissue recruitment. IL-13 stimulates mucus production and secretion.

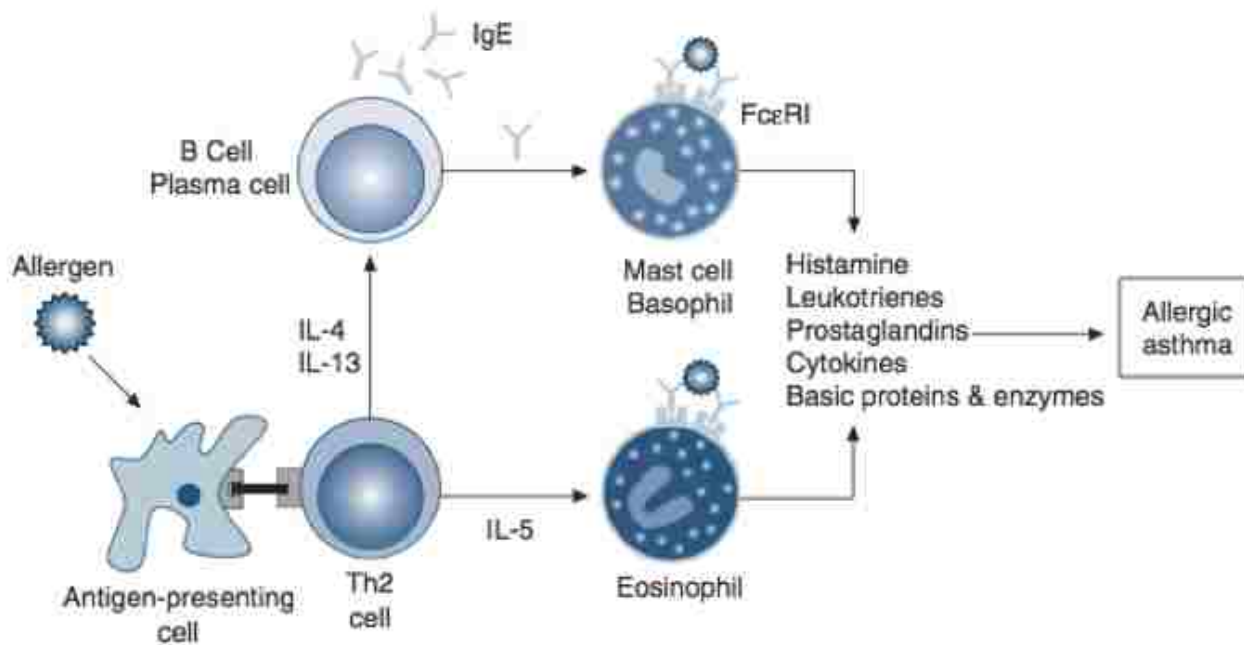


Figure 1. Inflammatory cascade in allergic asthma (Bradding et al. 2006)

1.1.2 Airway Hyperresponsiveness (AHR) during Allergic Lung Inflammation

AHR is an exaggerated airway narrowing in response to a nonspecific stimulus. There is evidence suggests that the degree of airway inflammation is causally related to AHR (Jeffery et al. 1989). The severity of AHR is associated with asthmatic conditions, which include reduced airway diameter, increased smooth muscle contractility and degree of epithelial injury, dysfunctional neuronal regulation, increased micro-vascular permeability and many inflammatory mediators eg IL-4 & IL-13 (Holgate 2008). These asthmatic conditions lead to episodic shortness of breath and airway obstruction.

1.1.3 Eosinophil Recruitment to the Airways during Allergic Lung Inflammation

Eosinophil infiltration in airways is a hallmark of allergic airway inflammation. IL-5 promotes maturation of eosinophils from the bone marrow contributes to their activation and recruitment to the airway. Eosinophils are a rich source of cytotoxic proteins, lipid mediators, oxygen free radicals and cytokines (Ricci et al. 1997). The contents of eosinophils are toxic for epithelial cells. De-granulation and release of toxic substances lead to damage on epithelial cells. Figure 2 illustrates role of eosinophils in asthmatic reaction (Filipovic et al. 2001).

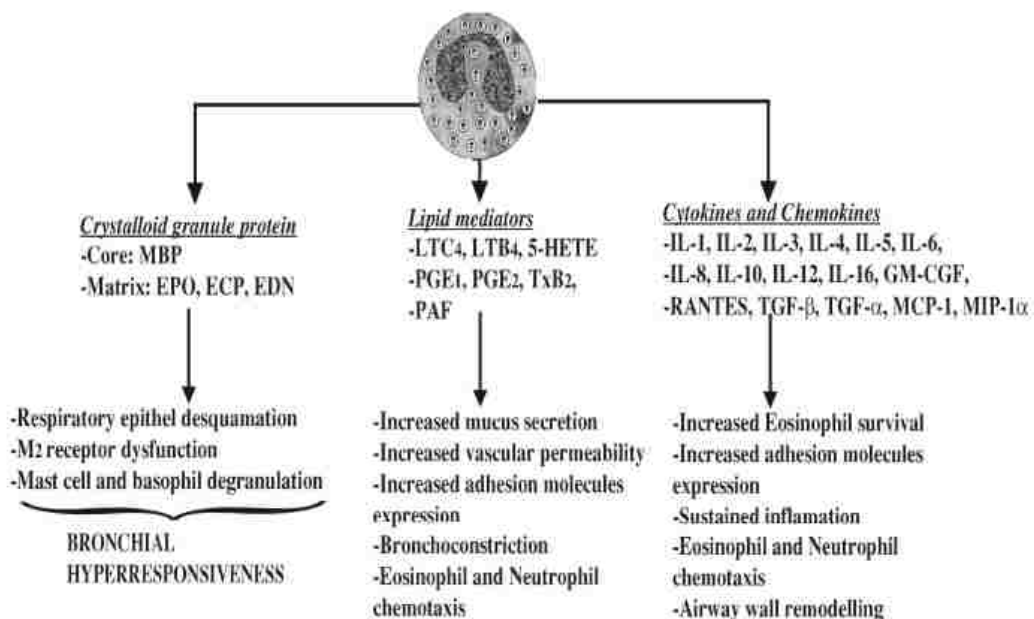


Figure 2. Role of eosinophils in the asthmatic reaction (Filipovic 2001)

1.1.4 Involvement of CD4+ Th17 cells in allergic lung inflammation

Th2 cells are the primary cell type responsible for airway inflammation in allergic asthma. Th17, another CD4⁺ pro-inflammatory effector cells, are also known to involved in the chronic inflammatory response (Jaffar et al. 2011). Th17 cells produce IL-17 during infection

with specific pathogens and this cytokine has a role for pathogen clearance (Korn et al. 2009). IL-17 is a pro-inflammatory cytokine that associated with the release of “airway remodeling” cytokines, IL-6 and IL-8 (Molet et. al. 2001). IL-6 and IL-8 are responsible for neutrophils accumulation in the airways (Laan et al. 1999). The role of IL-17 in asthma is an area of much current investigation. Asthmatic patients have elevated levels of IL-17A and IL-17F that correlate to disease severity, suggesting an important role for these cytokines in severe asthma. Indeed, IL-17A enhances human airway smooth muscle contraction, and elevated IL-17A levels correlate to increased neutrophilic inflammation, a characteristic of severe and steroid-resistant asthma. Increased IL-17A has also been correlated to increased AHR in asthmatics. The cellular source of IL-17 in asthma is unclear, although a recent study has shown the presence of a significant number of CD4⁺ Th17 cells in asthmatic tissues. Interestingly, IL-17 expression by T cells was evident in allergic but not nonatopic asthma. These studies implicate IL-17 in asthma pathogenesis, particularly severe, neutrophilic form of the disease. In mice, there is increased IL-17A levels in lung and IL-17R^{-/-} mice displayed reduced OVA-induced airway eosinophilia, IgE, and Th2 cytokines. In addition, adenoviral expression of IL-17A was sufficient to induce AHR in mice, and overexpression of IL-23 in the lung was sufficient to enhance antigen-dependent eosinophilia, Th2 cytokines, IL-17 production, and AHR. In HDM model, AHR-susceptible mice have enhanced myeloid dendritic cell production of IL-23, which drives Th17 polarization and subsequent AHR. Importantly, studies showed that IL-17A and IL-22 production by Th17 cells was steroid insensitive in vitro. Glucocorticoid treatment in vivo did not abrogate Th17-driven responses but was effective in inhibiting Th2-mediated inflammation. Moreover, our lab and others have shown that transfer of OVA-specific Th17 cells into host mice resulted in neutrophilic airway inflammation, AHR, and mucus metaplasia following OVA challenge. In

summary, these data indicate a role for Th17 and IL-17 in promoting allergic airway inflammation and AHR.

1.1.5 The anti-inflammatory effects of Foxp3⁺ Treg cells on allergic lung inflammation

Tregs control reactivity of self-reactive T cells and are responsible for maintaining immunologic homeostasis. Several types of Tregs have been described on their basis of their origin, generation, and mechanism of action, with two principal subsets identified: naturally occurring Foxp3⁺ Tregs (referred to as nTregs) and inducible Tregs (iTregs). iTregs develop in the periphery from conventional CD4⁺ T cells after exposure to signals such as regulatory cytokines, immunosuppressive agents, or antigen-presenting cells, whereas nTregs are thymus-derived natural CD4⁺ cells that express CD25, the alpha chain of IL-2 receptor and thus present in the host before pathogen exposure. Foxp3 is a key regulatory transcription factor required for the development of the T regulatory phenotype, Treg cells. T regulatory cells play an important role in maintaining immune homeostasis and produce anti-inflammatory cytokines eg TGF- β . These anti-inflammatory cytokines help to regulate immune responses and inflammatory pathologies (Hawrylowicz 2005). The expression of forkhead box P3 (Foxp3), a transcription factor that is critical for the development of Foxp3⁺ Tregs, can also be induced de novo in conventional CD4⁺ T cells, rendering the distinction between the two types of Tregs less obvious. The main iTregs populations include (i) TGF- β -producing Tregs; (ii) T regulatory 1 (Tr1) cells, which secrete IL-10; and (iii) inducible Foxp3⁺ Tregs. IL-4 inhibits TGF- β -induced iTregs and retinoic acid favors maturation of iTregs. Both nTregs and iTregs share similar phenotype and have a contact-dependent mechanism of action that is not fully understood. nTregs express CTLA-4 (cytotoxic T lymphocyte antigen), GITR (glucocorticoid-induced tumor necrosis antigen

4), CCR4 (chemokine receptor) and are generally previously activated (CD45RB^{low} in mice and CD45RO in human). IL-2 and TGF- β play important role in the maintenance and survival of both Treg subsets. However, nTregs and iTregs differ in their principal antigen-specificities and in T cell receptor signal strength and costimulatory requirements needed for their generation. Importantly, IL-6 can convert nTregs to Th17 cells, whereas iTregs, induced by IL-2 and TGF- β , are resistant to this cytokine and thereby may retain their suppressive function at inflammatory sites. This raises the possibility that nTregs and Tregs have separate functions in the adaptive immune response.

1.1.6 The anti-inflammatory effects of IL-10 on allergic lung inflammation

IL-10 is known as an important immunosuppressive cytokine. Several studies suggest that IL-10 inhibits the production of pro-inflammatory cytokines (Moore et al. 2001). During airway inflammation, Th2 cells produce inflammatory cytokines that involve in the development and recruitment of inflammatory cells. IL-10 is able to suppress the development of eosinophils and inhibit the recruitment of eosinophils to the inflammatory site in the airways (Kosaka et al. 2011). T regulatory (T_{reg}) cells are known to produce the large amounts of IL-10. The chronic activation of CD4⁺ cells in the presence of IL-10 in vitro significantly induces the development of T_{reg} cells, which mainly produce IL-10 (Groux et al. 1997). Moreover, there is evidence that IL-10 production is low in asthmatic individuals (Takanashi et al. 1999).

1.1.7 Arginase Activity and Allergic Lung Inflammation

L-arginine is a precursor in protein synthesis and a substrate for enzymes. Arginase is an enzyme involved in L-arginine metabolism since it plays an important role in the urea cycle by generating urea from ornithine. As such it competes with NOS for L-arginine as a common substrate (Maarsingh et al. 2008; Morris et al. 2007). The arginase-mediated degradation of arginine reduces the level of this substrate available to NOS. This "substrate competition" may cause the inhibition of NOS activity. Many studies suggest that alterations in L-arginine and NO metabolic pathways in the lung contribute to asthma pathophysiology (Morris et al. 2004; Yang et al. 2006; Zimmermann et al. 2006). One study shows that the inhibition of NOS activity reduces allergen-induced airway hyperresponsiveness in mice model (Muijsers et al. 2001). In addition, the administration of exogenous L-arginine prevents the allergen-induced hyperresponsiveness after the early asthmatic reaction in *ex vivo* guinea pig model (Boer et al. 1999).

1.1.8 Current Treatments in use for Asthma

Asthma remains a major public health problem that has increased in developed countries. Clearly asthma represents a major and worsening global health problem that despite new therapeutic approaches, many patient symptoms are not adequately controlled. Inhaled corticosteroids and long acting β_2 -agonists are currently the most effective treatment for asthma. However, some patients are corticosteroid resistant, and there is concern about side effects associated with long-term use of corticosteroids, particularly in children (Barnes 2004). This concern leads to a search for new approaches or improved therapies for asthma.

1.2 Cannabinoid Receptors and cannabinoids

Cannabis sativa is an annual herbaceous plant, which is historically known for its medicinal properties against various diseases. In 1964, delta-9-tetrahydrocannabinol (THC) was identified as the major psychoactive of cannabis (Gaoni et al. 1971). A number of studies on synthetic cannabinoid analogs followed the discovery of THC. These cannabinoid analog studies eventually led to the search for specific receptors, effects and mode of action.

1.2.1 Cannabinoid receptors

Cannabinoid 1 (CB₁) receptor was identified in 1988 and cloned in 1990 (Devane et al. 1988; Matsuda et al. 1990). The CB₁ receptor is highly expressed in the central nervous system and mediates many of the neurobehavioral and psychotropic effects (Brown 2007). In 1993, cannabinoid 2 (CB₂) receptor was discovered and cloned (Munro et al. 1993). Unlike the CB₁ receptor, the CB₂ receptor is predominantly expressed by lymphoid tissues (Brown 2007). The CB₁ and CB₂ receptors are G protein-coupled receptors, the lipid receptors (Figure 3).

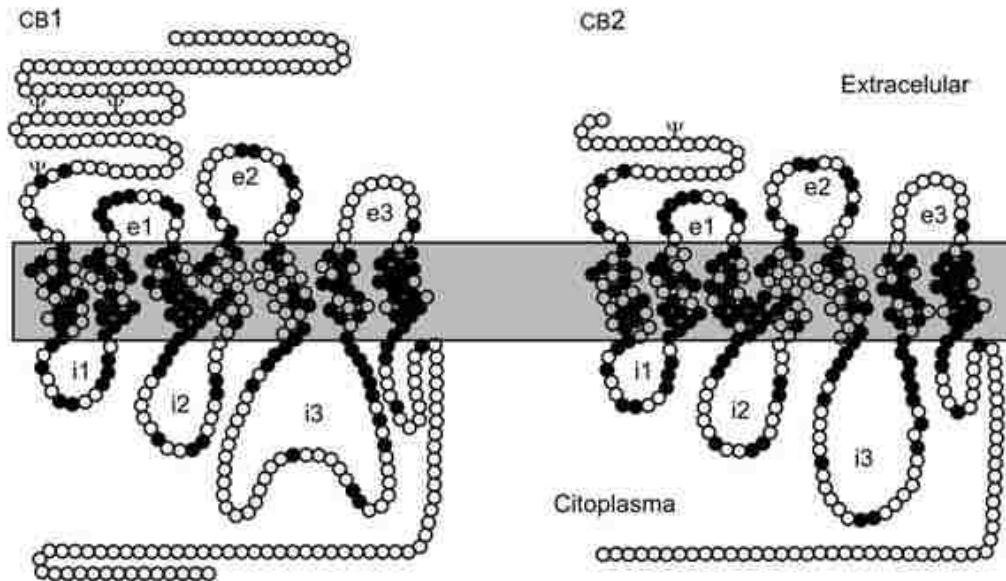


Figure 3. Cannabinoid receptors: CB₁ and CB₂

1.2.2 Endocannabinoids

Endocannabinoids are endogenous ligands for the cannabinoid receptors. N-arachidonylethanolamide, anandamide (AEA), is the first endocannabinoid identified in 1992. Subsequently, a second endocannabinoid, 2-arachidonoylglycerol (2-AG), was discovered in 1995 (Devane et al. 1992; Mechoulam et al. 1995). AEA, acts as a neurotransmitter, is selective for the CB₁ receptor with a much lower affinity for the CB₂ receptor (Cabral et al. 2009). Conversely, 2-AG has a great affinity for the CB₂ receptor compared to the CB₁ receptor (Cabral et al. 2009). It is suggested that 2-AG is the natural ligand for the CB₂ receptors (Cabral et al. 2009).

1.2.3 Exogenous cannabinoids

Exogenous cannabinoids are cannabinoids extracted from the cannabis sativa plant or synthetically formulated. Delta-9-tetrahydrocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD) have been the most studied exogenous cannabinoids.

1.3 Cannabinoids interaction with CB2 receptor

Cannabis plants, known as marijuana, have been used both for recreational and medicinal purposes for several centuries. Cannabinoids are the major active components found in the cannabis plant (Nocerino et al. 2000). Classical and recent studies suggest that cannabinoids are effective in the treatment of nausea and vomiting associated with cancer chemotherapy, anorexia and cachexia seen in HIV/AIDS patients, as well as neuropathic pain, and spasticity in multiple sclerosis (Nocerino et al. 2000). Recently, interest in the anti-inflammatory properties of cannabinoids has increased. Interestingly, several studies have reported that various cannabinoids have been shown to affect the functional activities of immune cells from rodents and humans, including B cells, T cells, macrophages and natural killer cells (Klein et al. 2003).

The mechanism of immunosuppression by cannabinoids has been investigated both in vitro and in vivo studies. There is little clear scientific evidence to fully understand the underlying mechanism how cannabinoid receptors are mediated by cannabinoids for exhibiting their immunosuppressive properties. However, recent cannabinoid studies indicate that cannabinoids clearly modulate immune responses during inflammatory processes (Croxford et al. 2005). Many in vitro experiments suggest that cannabinoid may exert their immunosuppressive properties in

four main ways: induction of apoptosis, inhibition of cell proliferation, inhibition of cytokine and chemokine production, and induction of regulatory T cells (Croxford et al. 2005).

Hegde et al. have done extensive experiments in order to demonstrate activation of cannabinoid receptors through administration of the cannabinoid THC (Figure 4) in mice (Hegde et al. 2010). In this study, THC activated both the CB₁ and the CB₂ receptors. Activation of both CB receptors led to induction of myeloid-derived suppressor cells (MDSC). MDSC are a heterogeneous cell population which include immature macrophages, granulocytes, DC and other myeloid cells, and mediate potent immunosuppressive properties (Gabrilovich et al. 2009). The THC - mediated induction of MDSCs reduced the proliferation of T cells and T cell- mediated inflammation, which may lead to immunosuppression on inflammatory response.

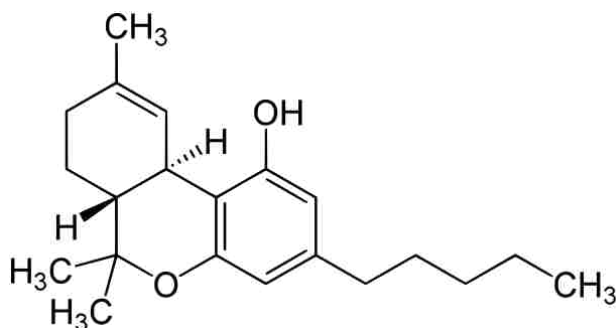


Figure 4. Structure of Delta-9-tetrahydrocannabinol (THC)

1.4 Allergic Asthma and Cannabinoids as a Potent Therapeutic

The main drawback for using a cannabinoid agonist as a potential therapeutic agent for asthma treatment is the psychotropic effect mediated by activation of CB₁ receptor. In order to

resolve the undesired CB₁ effect, a cannabinoid agonist, specifically designed to target the CB₂ receptor, was developed and formulated by Dr. Philippe Diaz (CLNP, Department of Biomedical and Pharmaceutical Sciences at the University of Montana). Figure 5 presents the progressive discovery paradigm. As the result of his investigation, the CB₂ agonist, NMP7, was formulated and provided to investigate its potential therapeutic for asthma.

In asthma, the lung inflammation is elicited by CD4⁺ Th2 cells. Based on the immunosuppressive nature of the CB₂ receptors and our CB₂ agonist, we carried out in vivo experiments to evaluate anti-inflammatory effect of the agonist on a mouse model of asthma. In addition, in vitro experiments were carefully designed to investigate possible cellular mechanisms how the agonist exert its anti-inflammatory property.

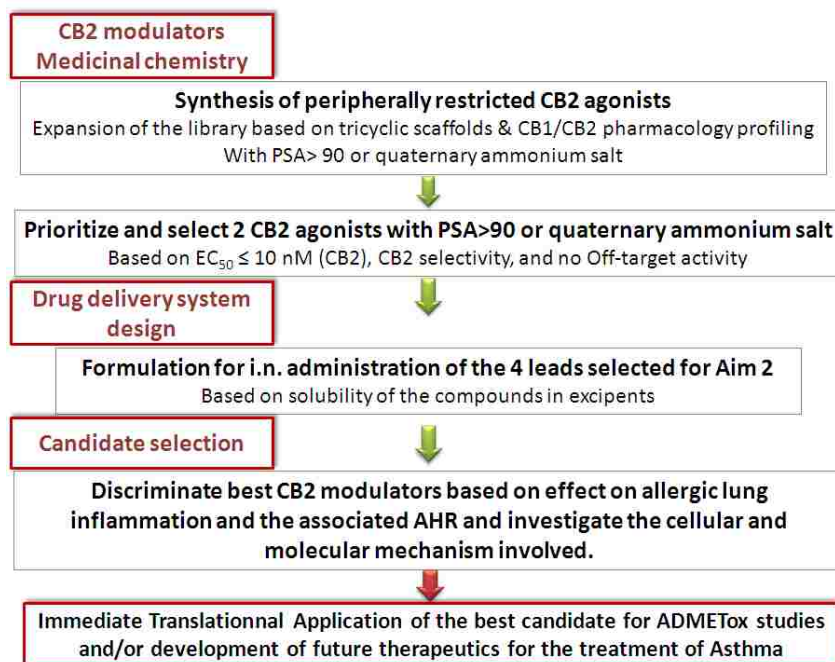


Figure 5. The progressive discovery paradigm with the descriptions of the key experiments with the advancing measures

CHAPTER 2

MATERIAL AND METHODS

2.1 Animals

BALB/c, C57BL6, DO11.10 and Foxp3-GFP transgenic mice were purchased from Jackson Laboratory, Bar Harbor ME. All mice were maintained in pathogen-free conditions in the animal facility at the University of Montana (Missoula, MT). All experiments were performed to the guidelines of the National Institutes of Health and approved by the University of Montana Institutional Animal Care and Use Committee (IACUC).

2.2 Media

Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, L-glutamine (Life Technologies, Carlsbad CA), penicillin and streptomycin (Life Technologies), HEPES (Life Technologies), sodium pyruvate (Life Technologies), and 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO).

2.3 Cannabinoid Agonist (NMP7)

Peripherally acting cannabinoid agonist, NMP7, was designed and provided by Dr. Philippe Diaz (Core Laboratory for Neuromolecular Production, Department of Biomedical and Pharmaceutical Sciences, University of Montana).

2.4 Mouse Model of Asthma

2.4.1 Preparation of DO11.10 CD4⁺ Th2 cells

Peripheral lymph node cells (PLN) were obtained from DO11.10 mice. CD8⁺ cells were first depleted using MACS beads (Miltenyi Biotech, Auburn, CA). Cells (5×10^5 /ml) were incubated in complete RPMI media for 4 d in the presence of OVA₃₂₃₋₃₃₉ peptide (1 µg/ml, Mimotopes, San Diego, CA) and murine IL-4 (2 µg/ml; R&D Systems), plus mAb anti-IFN-gamma (5 µg/mL R4-6A2, American Type Tissue Collection (ATCC), Manassas, VA). After 4 d of incubation, cells were re-stimulated using culture conditions identical to those previously used, but this time also in the presence of exogenous IL-2 (10 ng/mL R&D Systems) for a further 4 d. On d 8, the cells were depleted of class II⁺ cells by panning by incubating with anti-class II mAb (5 µg/ml M5/114; ATTC) for 30 min, then plated-bound mouse anti-rat IgG (10 µg/mL; Jackson ImmunoResearch, West Grove, PA) for 1 h. Non-adherent (Class II- cells) CD4⁺ Th2 cells were collected for subsequent culture.

2.4.2 Adoptive Transfer of CD4⁺ Th2 cells into Mice and Ovalbumin Aerosol challenge

Eight-day polarized DO11.10 CD4⁺ Th2 cells (10^7 cells/mouse) were adoptively transferred into BALB/c animals by intravenous injection. Mice (four per group) were then intranasally challenged by exposure in a chamber to aerosolized solutions of OVA (0.5%, Grade V; Sigma-Aldrich) for 20 min/day, over 7 consecutive days using a Wright's nebulizer (Buxco). Control mice were exposed to OVA aerosols but did not received DO11.10 Th2 cells.

2.5 In Vivo Experiments

2.5.1 Administration of NMP7

Prior to OVA aerosol challenge, Mice were treated daily with the CB₂ agonist (5 mg/kg of body weight) or vehicle by intra-nasal and intra-peritoneal injection.

2.5.2 Cell Differential Count

Cytospin preparations were performed on 5×10^4 cells followed by staining the cells using a Wright-Giemsa-protocol (Hema 3 Staining kit, Fisher Scientific, Houston, TX). Cell differential percentages were determined by light microscopic evaluation of stained and expressed as absolute cell numbers.

2.5.3 Eosinophil Peroxidase (EPO) Assay

The EPO activity in BAL cells was determined by colorimetric assay. 100 μ l of PBS was added to each well in a 96 well flat-bottomed plate (Falcon). The cells from the BAL fluid were resuspended in PBS pH 7.0 in a final volume of 300 μ l. In triplicate for each sample, 100 μ l of the cell suspension was added to the top well and serial diluted through the 8th well. The substrate solution was prepared by crushing one tablet of orthophenylene diaminedihydrochloride, (OPD, Sigma) (final concentration of 0.1%) in 50 μ M Tris-HCl (Sigma) containing 0.1% Triton X-100 (Sigma) and 1 mM hydrogen peroxide (Sigma). 100 μ l of the substrate solution was added to each well and the plates were then incubated at room temperature for 30 min or until sufficient color development has occurred at which time 50 μ l of 0.3 M sulfuric acid (Sigma) was added to stop the reaction. The absorbance was measured using an automatic plate reader (Molecular Devices

VersaMax microplate reader) at 495 nm. The measurements taken were total and not released EPO, and were indicative of the number of eosinophils present in the BAL.

2.5.4 Flow Cytometry Analysis on In Vivo Experiments

Cells were stained and analyzed on a FACSAria cell sorter (BD Biosciences, San Joes, CA) using FACVSDiVa software to enumerate CD4⁺ T cells (APC-Cy7, BD Biosciences), OVA-specific T cells (KJ1-26, APC, BD Biosciences), CD11c cells (APC, BioLegend) and Class II⁺ cells (APC, BioLegend). Flow cytometric analysis of CD4⁺ vs. KJ1-26⁺ and CD11c⁺ vs. ClassII⁺ were performed.

2.6 In Vitro Experiments

2.6.1 In Vitro Analysis of CB2 Agonist

12-well culture plates were pre-coated with anti-CD3 (2µg/ml, 2C11; ATTC) and incubated 24 h. Cells were isolated from DO11.10 mouse spleens. Cells were cultured either in pre-coated anti-CD3 culture plate or in un-coated culture plate with soluble OVA-peptide (1µg/ml, Mimotopes, San Diego, CA) in the presence of different concentrations of the CB₂ agonist, NMP7 (0, 0.1, 0.5, 2.5 µM) for 2 days. Supernatants were harvested and stored at -4°C for cytokine measurement.

2.6.2 Measurement of cytokines

IL-4 and IL-5 Measurement

The protocols used to assay cytokines IL-4 and IL-5 are as follows: 50 µl of anti-IL4

(5µg/ml generated from the 11B11 hybridoma cultivated in our laboratory) and IL-5 (Pharmingen) capture antibodies [5.0 µg/mL final], diluted in PBS, were pipetted into a 96 well plate and stored at 4°C overnight followed by 2 washes on an automated plate washer (Thermo Electron Well wash 5 MK2 Plate washer, Fisher Scientific) with ELISA wash buffer (WB) (PBS & 0.5% Tween). Plates were blocked by adding 200 µl of blocking buffer (1% bovine serum albumin (BSA) (Sigma) in 1 mM carbonate/bicarbonate buffer, pH 9.6) was added and incubated at room temperature for a minimum of 2 h; the excess was then washed off with WB x 3. Standards and samples were added (50 µl/well) and incubated over night at 4°C. The plates were washed 3X with WB. Biotin-conjugated detection antibodies (anti-IL-4 (Pharmingen), IL-5 (Pharmingen) were diluted to 2.5 µg/mL in blocking buffer and 50 µl was added to each well and allowed to incubate at room temperature for 45 min followed by washing 3X with WB. Streptavidin-conjugated horseradish-peroxidase (SA-HRP) (Jackson ImmunoResearch Laboratories Westgrove , PA) was diluted 1:4000 in blocking buffer. 100 µl of SA-HRP was added to each well and incubated for 30 min at room temperature. The plates were washed 5X with WB and blotted dry. 100µl of 3,3',5,5' Tetramethylbenzidine (TMB) (Sigma) substrate was added to each well, incubated up to 30 min at room temperature at which point the reaction is stopped with 100 µl of 0.3 M H₂SO₄ (Sigma). The absorbance was read at 495 nm.

IL-10 Measurement

The R&D systems (Minneapolis, MN) IL-10 ELISA kit was used to quantify this cytokine. The protocol was similar to IL-4 and IL-5 protocol with the exception of the following: The capture antibody was diluted 1:200 in 0.2 M sodium phosphate buffer (pH 6.5) and 100 µl added per well in a 96 well plate. The plates were sealed and incubated at 4°C overnight. The plates

were washed 3X with WB . 200 µl of blocking buffer (1% BSA in PBS) was added to each well and the plates were incubated for 1 h at room temperature. The plates were washed 3X with WB . 100 µl of samples or standards (diluted in blocking buffer) were added to the appropriate well and incubated 2 h at room temperature. The plates were washed 3X with WB. 100 µl of detection antibody (1:200) was added per each well and the plates were incubated for 2 h at room temperature. The plates were washed 3X with WB . 100 µl of Avidin-HRP solution (1:1000) was added to each well and incubated for 30 min at room temperature in the dark. The plates were washed 3X with WB . 100 µl of TMB was added to each well and the plate was incubated in the dark for up to 30 min. The reaction was stopped with 0.3M H₂SO₄. The absorbance was measured at 450nm on the plate reader.

IL-17 Measurement

The R&D systems (Minneapolis, MN) IL-17 ELISA kit was used to quantify this cytokine. The protocol was similar to IL-10 protocol with the exception of the following: The capture antibody was diluted to 2 µg/ml in PBS and 100µl was added per each well in a 96 well plate. The detection antibody was diluted to 400 ng/ml. SA-HRP solution was diluted to 1 ng/ml. After addition of SA-HRP, the plates were incubated for 20 min at room temperature in the dark. After addition of TMB, the plates were incubated in the dark for up to 20 min.

2.6.3 Determining Expression of Foxp3⁺ in CB₂ Culture

12-well culture plates were pre-coated with anti-CD3 (2µg/ml, 2C11; ATTC) and incubated 24 h. Cells were isolated from Foxp3-GFP mouse spleens and cultured in pre-coated

plates with various doses of NMP7 (0, 0.1, 0.5, 2.5 μ M) for 2 days. Cells were harvested and analyzed by flow cytometry.

2.6.4 Determining the effect of CB₂ agonists on Treg numbers using Foxp3-GFP mice and intra-cellular Foxp3 staining.

Foxp3 - GFP mice (B6.Cg-Foxp3^{tm2Tch/J}, Jackson Laboratories) were used to enumerate Treg cell numbers in cultures treated with anti-CD3 and CB₂ agonists. GFP expression was determined by flow cytometry in conjunction with labeled secondary antibodies to CD4 & CD8.

2.6.5 Arginase Activity Assay

This assay was used for measuring arginase activity of the cells. The conversion of L-arginine to L-ornithine was measured. 2×10^6 peritoneal cells from CB₂ treated or control mice were cultured with or without lipopolysaccharide (LPS, 1 μ l/ml, brand) and incubated 24 h. After 24 h, cells were harvested and lysated with cell lysis buffer containing protease inhibitor cocktail. The cell lysates were activated for the activation of arginase in the lysate. 25 μ l of 10 mM/L MnCl₂ was added to 25 μ l of cell lysate in the tube and incubated at 55°C for 20 min. 150 μ l of carbonate buffer (100 mM/L, pH10, brand) was added to the tube containing cell lysate for the initiation of arginase reaction. 50 μ l of 100mM/L L-arginine was added to the tube and incubated at 37 °C for 1.5 h. 750 μ l of glacial acetic acid was added to stop the reaction. Standards were prepared with known amount of L-ornithine (8 mM to 250 mM, brand) and treated the same as the samples. 250 μ l of ninhydrin reagent (2.5 g of ninhydrin in 40 ml of 6 M phosphoric acid and 60 ml of glacial acetic acid) was added to samples and standards. Samples and standards were

boiled at 90-100 °C for 1h. The absorbance was measured at 515 nm on the plate reader (Molecular Devices VersaMax microplate reader).

2.7 CB₂ receptor expression

2.7.1 Intracellular cannabinoid receptor staining

CB₂ receptor binding by CD4⁺ Th2 cells using NBD-labeled CB₂ ligand by confocal microscopy. Th2 cells, with or without blocking with cold CB₂ agonist, were stained with PE-conjugated anti-CD4 antibody (red) and the NBD-labeled CB₂-selective compound at 5 μM, on ice for 30 min (green) and then visualized by confocal microscopy (40x). The cells with cold CB₂ agonist were pre-incubated with the agonist prior to staining.

2.7.2 Expression of CB₂ receptor activated B and dendritic cells

Lung mononuclear and spleen cells from C57BL6 mice (1X10⁶ cells/ml) were initially stimulated with LPS (10 μg/ml) for 48h. The stimulated cells were Fc blocked and stained with NBD-labeled CB₂ ligand (5 μM) and allophycocyanin-labeled anti-CD11c or anti CD45 B220 mononuclear antibody in the absence or presence of unlabeled (cold) CB₂ agonist (100 μM, as competitive ligand) for 30 min. Expression of CB₂ receptors (FITC channel) on the activated cells were analyzed by FACS.

2.8 Statistical Analysis

Data are expressed as means SEM. Data obtained from in vitro experiments comparing two variables, were analyzed using the students t test. In vivo experiments comparing variables were

analyzed using the Mann-Whitney U test. Differences with p values <0.05 were considered statistically significant. To determine statistical significance of groups, two-way ANOVA tests were used for analysis. The Prism software package was used in all cases.

CHAPTER 3

RESULTS

3.0 Specific Aims

The overall goal of this study was to examine whether treatment with CB₂ agonist reduced the airway inflammation in a mouse model of asthma. The treatment of mice with CB₂ agonists was expected to have minimal psychotropic effects while suppressing allergic airway inflammation.

Specific Aim 1: Effect of treatment of mice with CB₂ agonists on allergic lung inflammation.

To evaluate whether the CB₂ agonist attenuated airway inflammation mice were treated intranasally with the agent. The effect on the following responses was evaluated:

- (i) Pulmonary inflammation
- (ii) Cytokines present in bronchoalveolar lavage fluid (BALF)

Specific Aim 2: What is the effect of CB₂ agonists on T cells in vitro - effect on cytokine production, Treg numbers and arginase activity.

To test whether our CB₂ agonist will modulate Th2 cytokine production, immunosuppressive cytokine production and arginase activity, in vivo experiments were carried out. This aim was to determine CB₂ mode of action using cells isolated from mouse spleens cultured with the agonist.

- (i) Effect on Th2 cytokines: IL4- and IL-5
- (ii) Effect on IL-10 and IL-17
- (iii) Effect on Foxp3⁺ cell expansion

- (iv) Effect on arginase activity

Specific Aim 3: Monitor CB₂ receptor expression by immune cells using NBD labeled agonists.

To examine the expression of CB₂ receptors on the immune cells, cells were isolated and stained with NBD-labeled CB₂ ligand. This approach was developed to examine the cellular expression of the receptor during allergic airway inflammation and cell activation and possible action site of our CB₂ agonist. Th2 cells and activated immune cells were stained with NBD-labeled CB₂ ligand. The unlabeled agonist, NMP7, was added prior to the cell staining to observe binding activity.

- (i) Monitor CB₂ expression on Th2 cells
- (ii) Monitor CB₂ expression on activated B and dendritic cells (DC)

3.1 In Vivo Experiments

Recent research suggests that the CB₂ agonist indicated anti-inflammatory properties. Given the anti-inflammatory properties of the CB₂ receptor, in vivo experiments were designed to test if our CB₂ agonist can attenuate airway inflammation in a mouse model of asthma.

3.1.1 OVA-specific CD4⁺ cells and CD11c⁺ClassII⁺ antigen presenting cells

OVA-specific CD4⁺ cells and CD11c⁺ClassII⁺ APCs are responsible for initiating the inflammatory cascade in a mouse model of asthma. Inhibition of these cells is evidence for the attenuation of airway inflammation. To evaluate whether CB₂ agonist inhibits inflammatory phenotype, BALF cells and lung mononuclear cells were stained for flow analysis. Figure 6 illustrates that the agonist treated mice demonstrated reduction in numbers of OVA-specific CD4⁺ cells and CD11c⁺ClassII⁺ APCs (approximately 50% for both) compared to vehicle mice.

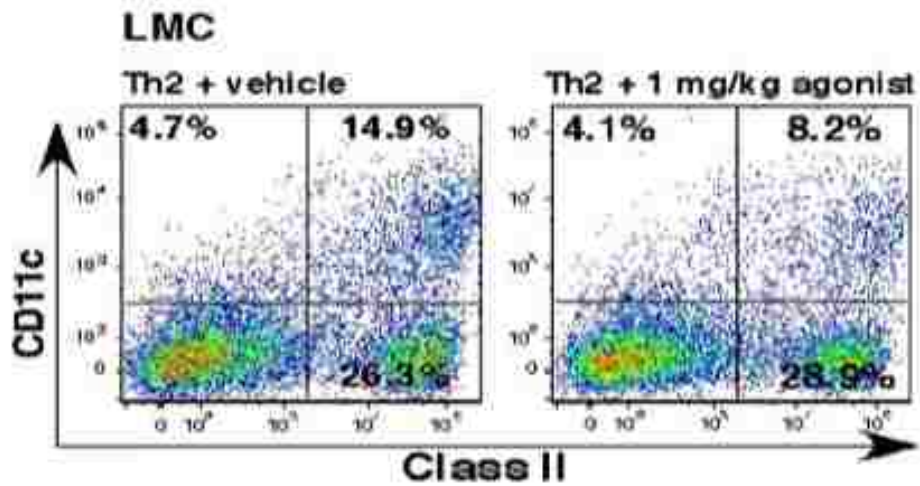
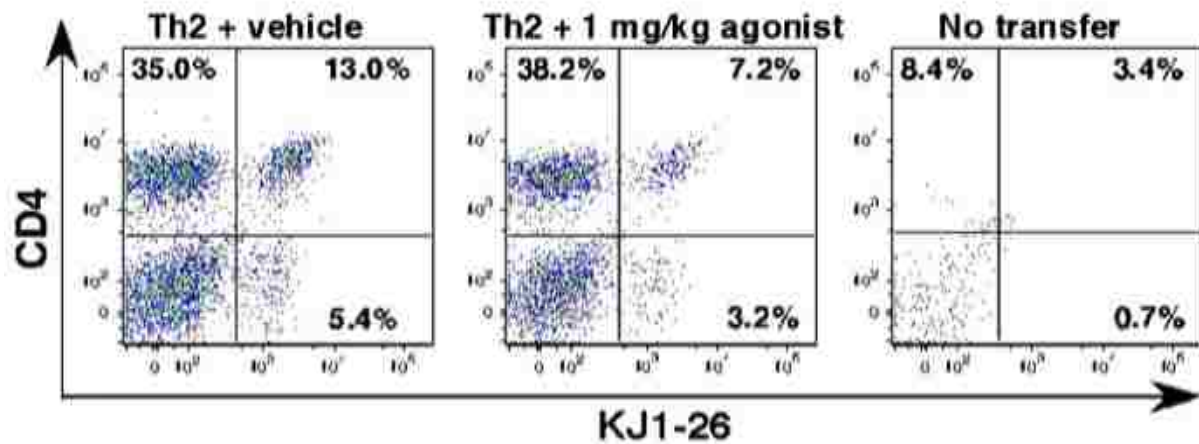
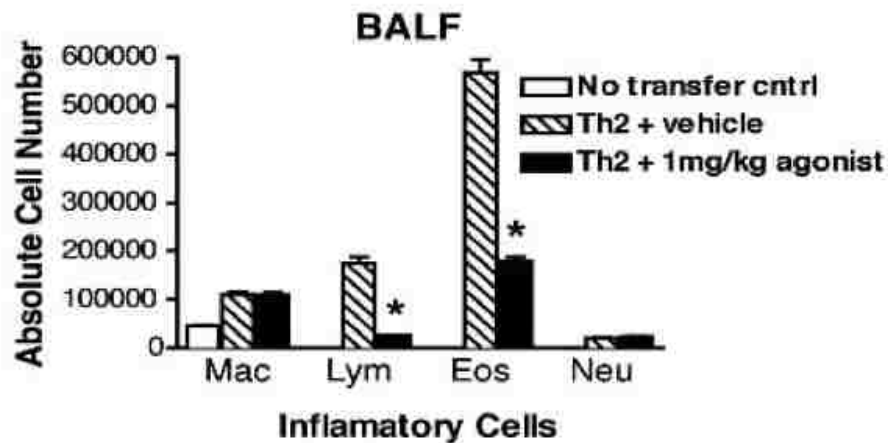


Figure 6. CB2 agonist inhibits numbers of antigen-specific CD4⁺ cells and antigen presenting cell. Clonotypic CD4⁺KJ1-26⁺ (OVA-specific CD4⁺) cells in the BALF and CD11c⁺ClassII⁺ cells in lung mononuclear cell (LMC) were enumerated by FACS analysis.

3.1.2 Absolute inflammatory cell number

During the inflammatory process, infiltration of inflammatory cells occurs in airways. In order to evaluate whether our CB₂ agonist attenuates airway inflammation, absolute cell numbers in BALF were determined. Figure 7 demonstrates that in the agonist treated mice, a 5-fold decrease in lymphocyte cell number and a 3-fold decrease in eosinophil cell number is seen, compared to vehicle mice. No significant reduction in cell numbers for macrophages and neutrophils were observed.



*Figure 7. CB₂ agonist reduces recruitment of inflammatory cells in airway. Cell differential counts in the BALF were determined by light microscopic evaluation. Results are expressed as total numbers (per mouse) of lymphocytes (Lym), macrophages (Mac), eosinophils (Eos), and neutrophils (Neu). Data are means \pm SEM n=3. * indicates P values < 0.05 compare to vehicle group.*

3.1.3 Eosinophil peroxidase (EPO) level

Eosinophil infiltration in airways is a hallmark of airway inflammation. Eosinophils produce eosinophils peroxidase (EPO), which is a good indicator of airway inflammation. Cell-associated EPO levels were present in BALF collected from control, vehicle, low dose agonist and high dose agonist treated mice. Figure 8 demonstrates that low dose and high dose agonist treated mice, had a 2-fold and 4-fold decrease in EPO levels, compared to vehicle mice. The agonist significantly reduced EPO levels in BALF in a dose-dependent manner.

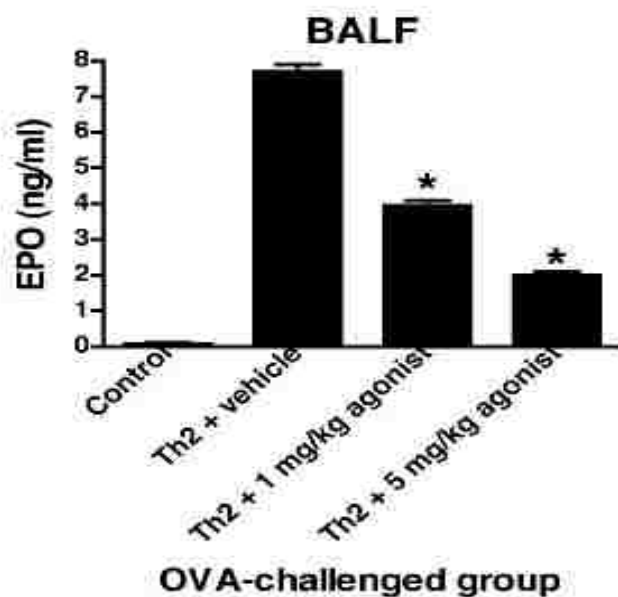
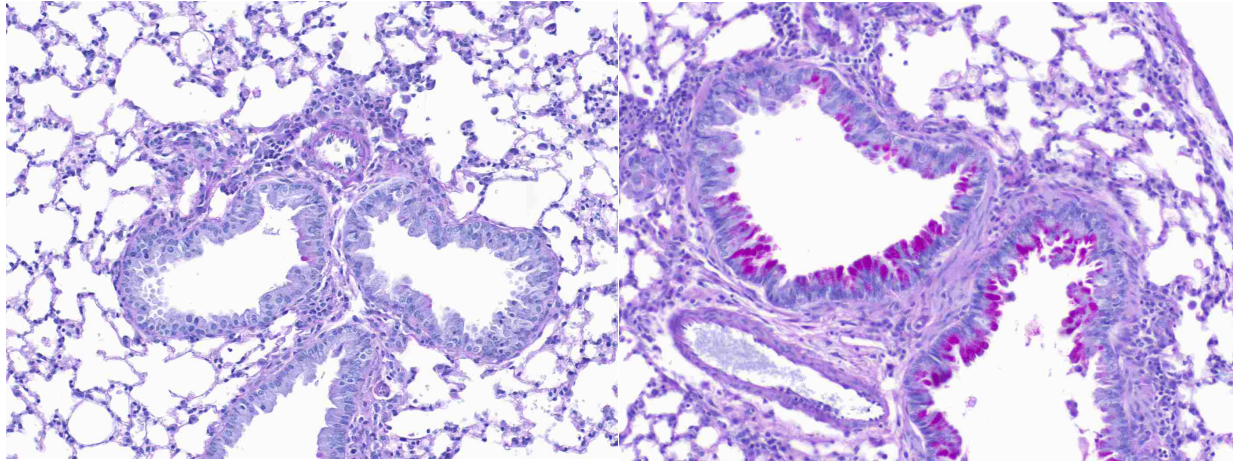


Figure 8. *CB₂ agonist reduces Eosinophil peroxidase (EPO) levels in the BALF. EPO levels in the BALF assessed by colorimetric analysis. Data are means \pm SEM n=3. * indicates P values < 0.05 compare to vehicle group.*

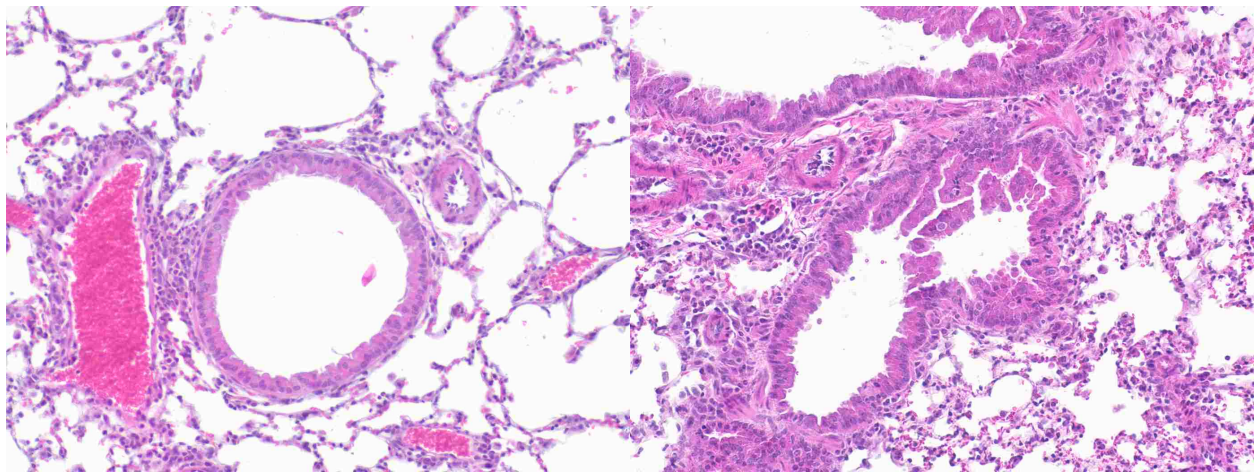
3.1.4. Lung tissue staining

Excessive mucus secretion and the formation of pockets of inflammation around airways occur during the inflammatory process. Lung tissues from asthmatic mice, CB2 treated group and vehicle group, were collected. The collected tissues were stained using PAS and H&E. PAS staining indicates mucus secretion of airways (Figure 9A and 9B). It is showing that non-treated group secretes mucus in airways (9A), which is significantly inhibited by CB2 agonist (9B). H&E staining indicates pockets of inflammation around airways (Figure 9C and 9D). In non-treated group, pockets of inflammation are observed significantly around airways (9C). These inflammatory pockets were significantly inhibited by CB2 agonist (9D).



PANEL A

PANEL B



PANEL C

PANEL D

Figure 9. PAS and H&E staining of lung tissue. Lung tissue was prepared from Aerosol Challenged + CB2 agonist treated mice (A & C) or Aerosol Challenged + no CB2 treatment (B & D). Tissues were stained using PAS (A & B) or H& E (C & D).

3.1.5 The Effect of CB₂ agonist on IL-10

To test whether our CB₂ agonist promotes IL-10 production in airway to suppress the inflammatory process, IL-10 levels in BALF was measured by ELISA. The agonist promoted IL-10 production. Figure 10 demonstrates that the agonist treated mice, a 4-fold increase in IL-10 production, compared to vehicle mice.

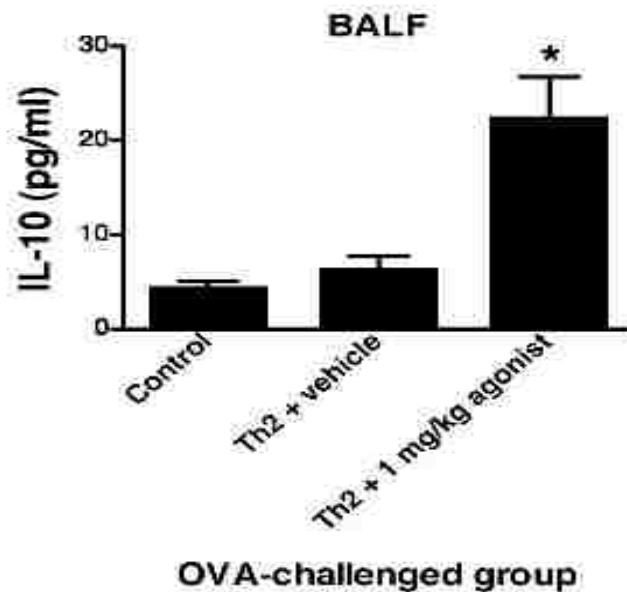


Figure 10. CB₂ agonist promotes IL-10 in vivo model. IL-10 levels in BALF of control and Th2 recipients treated with either agonist or vehicle, measured by ELISA. Data are means \pm SEM $n=3$.

* indicates P values < 0.05 compare to vehicle group.

3.2 In Vitro Experiments

CB₂ receptors are highly expressed on immune cells. Synthetic cannabinoid agonists can mediate their effect by activating cannabinoid receptors. Recent studies suggest that cannabinoids have anti-inflammatory properties and could potentially be used as a new class of anti-inflammatory agents against immune inflammatory disease. However, it is not clearly known how cannabinoids express their immunosuppressive properties. In vitro experiments were designed to investigate possible mechanisms.

3.2.1 The Effect of CB₂ Agonist on IL-10 production

IL-10 is an immunosuppressive cytokine that can attenuate inflammation. Induction of IL-10 could be a possible mechanism for down-regulating the expression and production of inflammatory cells and cytokines (Moore et al. 2001). To determine the effect of the CB₂ agonist on IL-10 production, cells were isolated from DO11.10 mouse spleens and were stimulated with anti-CD3 (Figure 11, white bars) or OVA-peptide (Figure 8, black bars) in the presence of different concentrations of the agonist for 2 days. IL-10 was measured by ELISA as described in the methods and materials. Figure 8 shows that the agonist promoted IL-10 production of the cells. Cultures treated with the agonist were compared with the control cultures (no CB₂ agonist). Both anti-CD3 and OVA-peptide cultures showed statistically significance increment of IL-10 production.

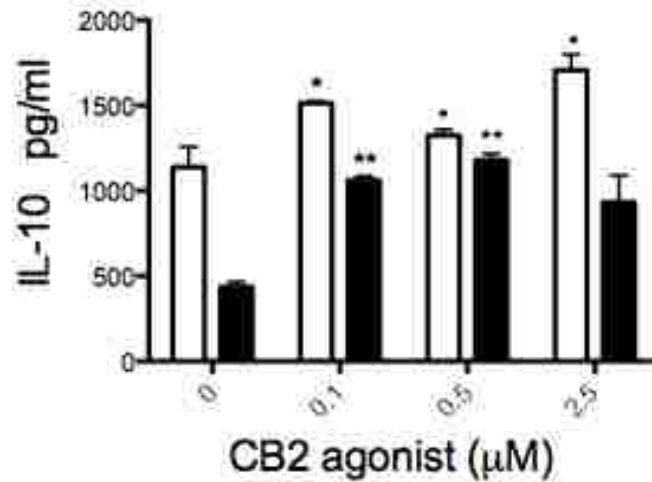


Figure 11. CB_2 agonist promotes anti-inflammatory cytokine IL-10. Cells, isolated from DO11.10 mouse spleen, were stimulated with anti-CD3 (white bars) or OVA-peptide (black bars) in the presence of different concentrations of the CB_2 agonist for 2 days. Supernatants were harvested and anti-inflammatory cytokine, IL-10, was measured by ELISA. Data are means \pm SEM $n=3$. * indicates P values < 0.05 . ** indicates P values < 0.01 compared to no CB_2 .

3.2.2 The Effect of CB₂ Agonist on IL-17 production

Another cytokine, IL-17 is produced during infection with specific pathogens. Th17 cells produce IL-17 in response to specific pathogens for pathogen clearance (Korn et al. 2009). It is suggested that IL-17 expression is associated with allergic response (Jaffar et al. 2011). IL-17 may serve a potentially important role in the mediation of dysregulated Th2 response, which causes allergic airway inflammation (Laan et al. 1999). To determine the effect of our CB₂ agonist on IL-17 production, cells were isolated from DO11.10 mouse spleens and stimulated with anti-CD3 (Figure 12, white bars) or OVA-peptide (Figure 12, black bars) in the presence of different concentrations of the agonist for 2 days. IL-17 was measured by ELISA. Cultures treated with the agonist were compared with control cultures (no CB₂ agonist). Our CB₂ agonist did not inhibit IL-17 production in anti-CD3 cultures. However, in OVA-peptide cultures, the agonist significantly inhibited IL-17 production.

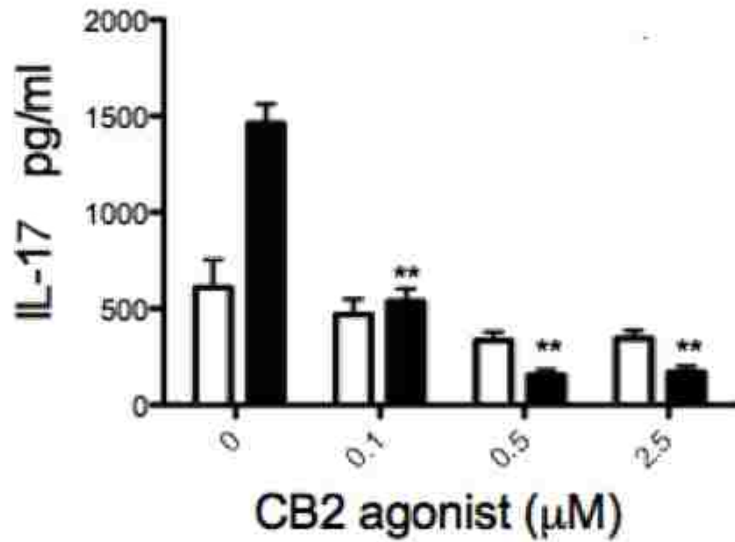


Figure 12. *CB₂ agonist inhibits Th17 cytokine IL-17. Cells, isolated from DO11.10 mouse spleen were stimulated with anti-CD3 (white bars) or OVA-peptide (black bars) in the presence of different concentrations of the CB₂ agonist for 2 days. Supernatants were harvested and anti-inflammatory cytokine, IL-10, measured by ELISA. Data are means ± SEM n=3. ** indicates P values < 0.01 compared to no CB₂.*

3.2.3 The Effect of CB₂ Agonist on pro-inflammatory cytokines, IL-4 and IL-5

IL-4 and IL-5 are key pro-inflammatory cytokines in airway inflammation. To determine the effect of our CB₂ agonist on IL4 and IL-5 production, cells were isolated from DO11.10 mouse spleens and stimulated with anti-CD3 (Figure 13, white bars) or OVA-peptide (Figure 13, black bars) in the presence of different concentrations of the agonist for 2 days. The cytokine levels were measured by ELISA. Cultures treated with the agonist were compared with control cultures (no CB₂ agonist). Our CB₂ agonist effectively inhibited IL-4 production. Also, IL-5 production was significantly inhibited for anti-CD3 cultures.

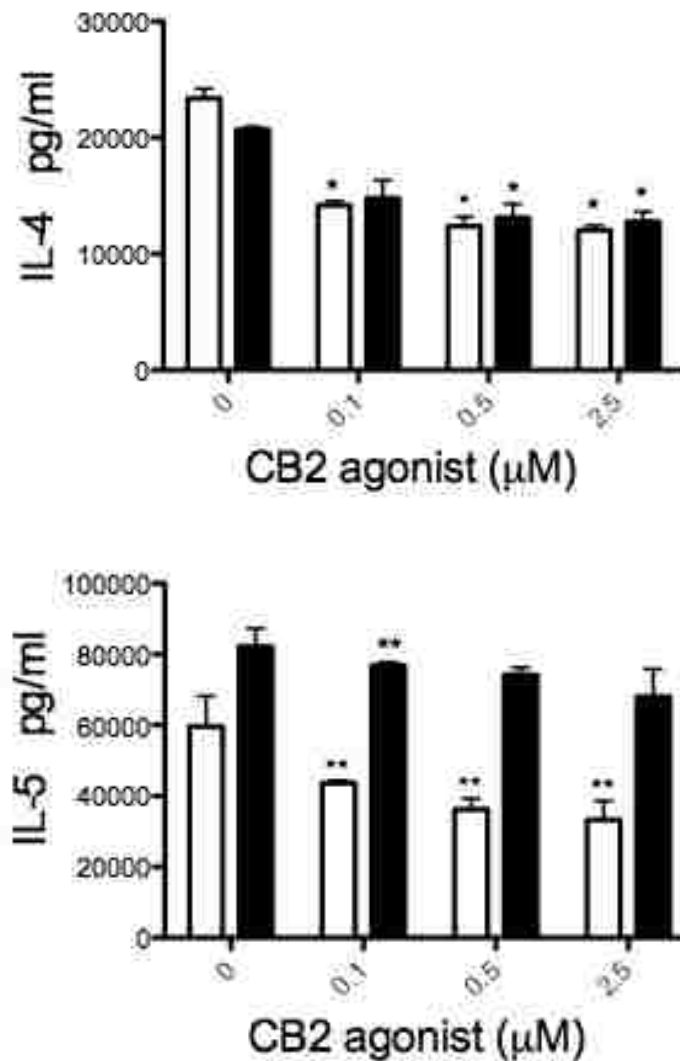


Figure 13. *CB₂ agonist inhibits pro-inflammatory cytokines IL-4 and IL-5. Cells, isolated from DO11.10 mouse spleen, were stimulated with anti-CD3 (white bars) or OVA-peptide (black bars) in the presence of different concentrations of the CB₂ agonist for 2 days. Supernatants were harvested and anti-inflammatory cytokine, IL-10, measured by ELISA. Data are means ± SEM n=3. * indicates P values < 0.05. ** indicates P values < 0.01 compared to no CB₂.*

3.2.4 The Effect of CB₂ Agonist on Expression of Foxp3⁺

Foxp3 is a key regulatory transcription factor required for the development of the T regulatory phenotype. T regulatory cells produce anti-inflammatory cytokines (TGF- β or IL-10), which helps to prevent inflammation. In order to test if our CB₂ agonist induces the expression of Foxp3⁺, cells were isolated from Foxp3-GFP mouse spleens and cultured with the agonist for 2 days. Foxp3 expressions in different types of immune cells were analyzed. Foxp3 expressions on four different types of immune cells (B cells, CD4⁺ T cells, macrophages and CD25⁺ T cells) were analyzed by flow cytometry (Figure 14). Our CB₂ agonists did not induce Foxp3 expression; Foxp3⁺ population remained similarly for CB₂ or no CB₂ groups.

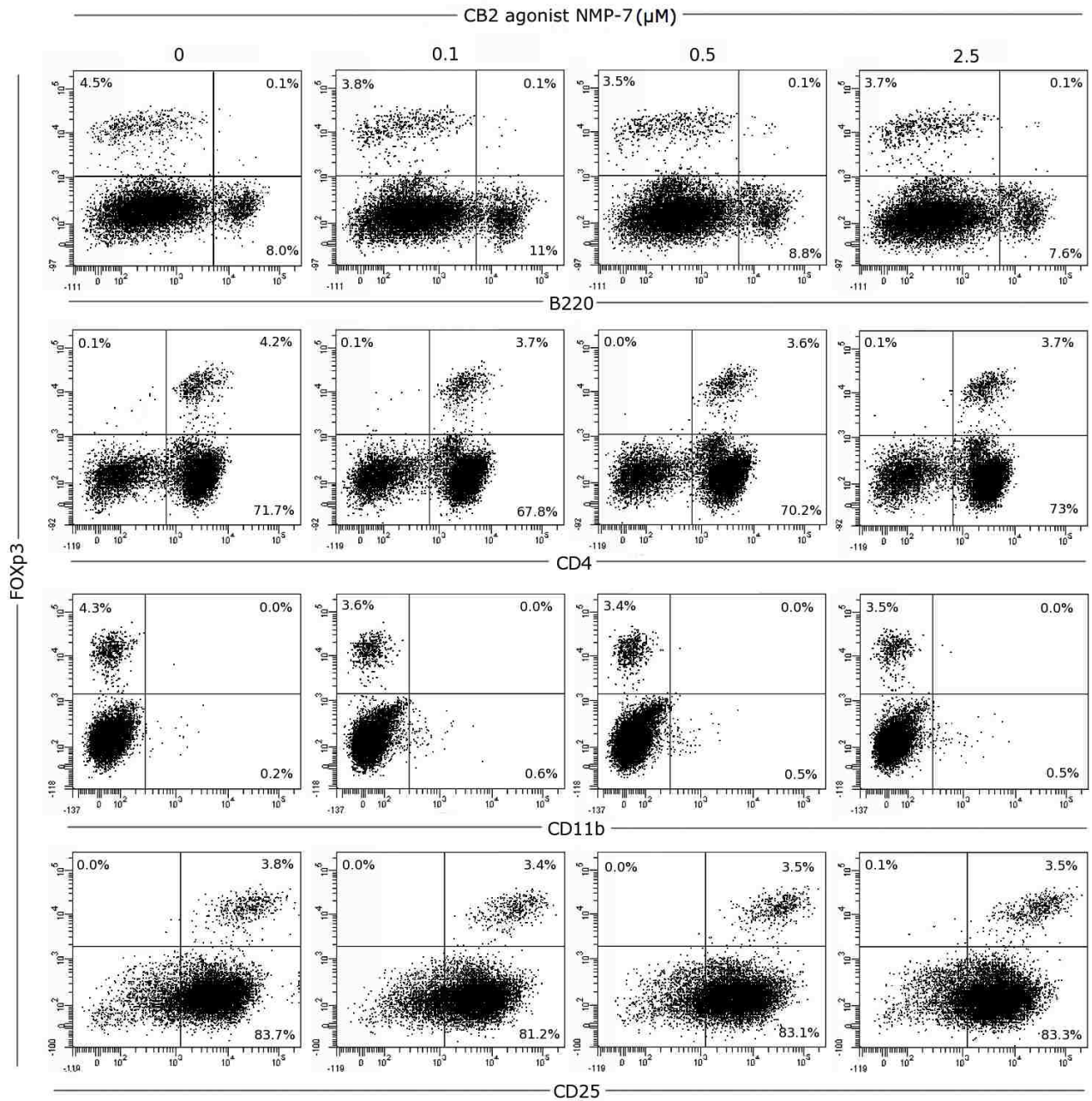


Figure 14. *Foxp3* expression by various cells in the cultures were enumerated by FACS analysis. Each row represents cell types B cells (B220), CD4⁺T cells (CD4), macrophages (CD11b) and CD25⁺ cells (CD25). Each column represents different doses of the CB₂ agonist (0, 0.1, 0.5 and 2.5 μM) added to cultures.

3.2.5 The Effect of CB₂ Agonist on Arginase Activity

L-arginine metabolism is a key characteristic of myeloid-derived suppressor cells (MDSC). MDSC has been shown to express immunosuppressive properties. High levels of THC has been shown to elicit a response by myeloid-derived suppressor cells. In order to test if our CB₂ agonist activates MDSC for expressing anti-inflammatory effect, mice were treated with or without the agonist. Cells were isolated from spleen and stimulated with or without LPS for 24 hours. After 24 hours both cells and supernatants were harvested to evaluate arginase activity. Arginase activity was determined by spectrophotometric assay based on the principle of conversion of substrate L-arginine to L-ornithine (Hegde et al. 2010). No significant difference was observed for arginase activity of CB₂ and no CB₂ groups (Figure 15). The results suggest that our CB₂ agonist is less likely to exert its anti-inflammatory effect through the activation of MDSC.

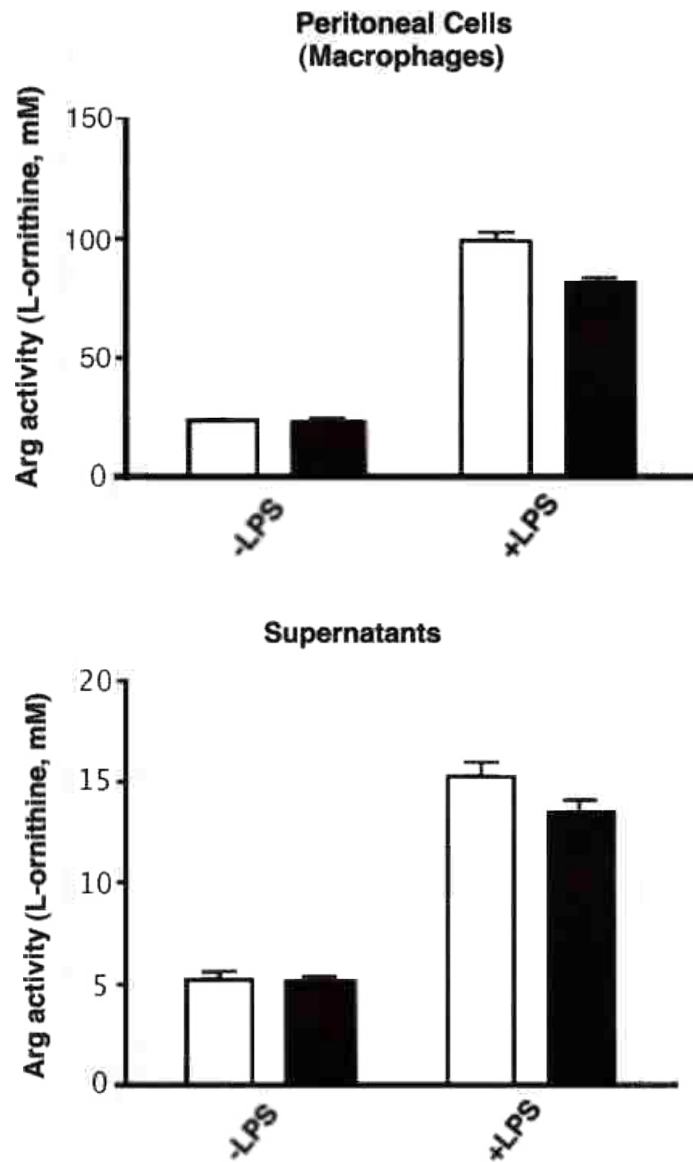


Figure 15. No CB₂ effect on arginase activity. Peritoneal cells (macrophages) were isolated from either CB₂ treated (black bars) or control mice (white bars). Cells were stimulated with or without LPS for 24 h. Cells and supernatants were harvested, arginase activity was measured.

Data are means ± SEM n=4.

3.3 CB2 receptor expression

3.3.1 Intracellular Cannabinoid Receptor Staining

In order to examine whether T cells express the CB2 receptors during allergic inflammation, Th2 cells were stained using the NBD-labeled CB2 ligand. Cells were stained with green (marker for CB₂ receptors) and red (marker for CD4⁺ T cells) stains (Figure 16A). These fluorescent markers demonstrated that CB2 receptors were expressed on Th2 cells. To confirm whether CB₂ receptor expression on Th2 cells is blocked by an unlabeled CB2 agonist, cells were pre-incubated with the unlabeled agonist prior to the receptor staining. Figure 16B illustrates that the cold agonist effectively blocked CB2 receptors. This result suggests that CB₂ receptor is expressed on Th2 cells and the expressed receptors on Th2 cells might be possible action site of CB₂ agonist.

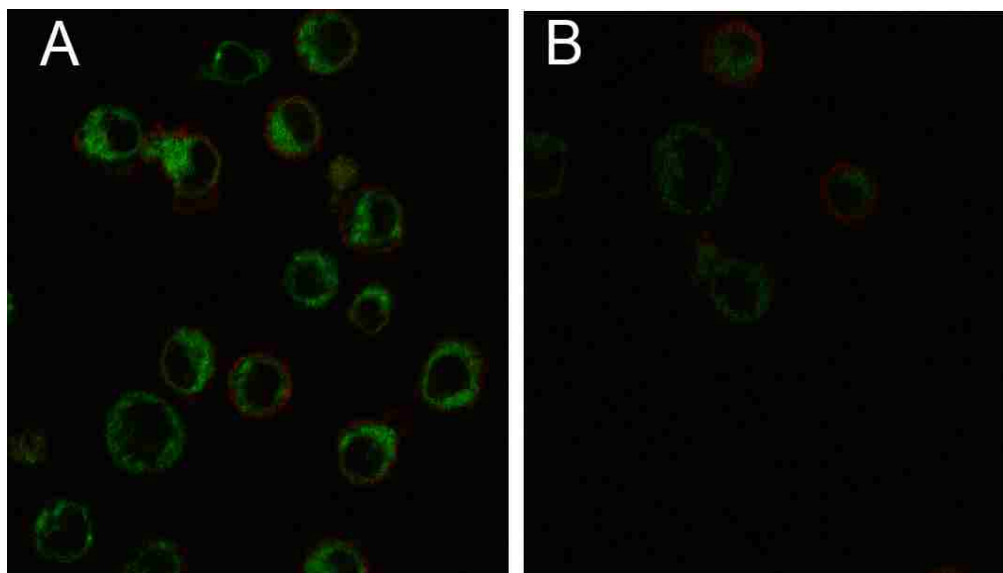


Figure 16. CB_2 receptor binding by $CD4^+$ T cells using NBD-labeled CB_2 ligand

CB_2 receptor binding by $CD4^+$ Th2 cells using NBD-labeled CB_2 ligand by confocal microscopy

(40x). (A) Th2 cells were stained with PE-conjugated anti-CD4 antibody (red) and the NBD-labeled CB_2 -selective compound at 5 μM , on ice for 30 min (green), (B) cells were pre-incubated with un-labeled agonist. The stained cells were visualized by confocal microscopy (Petrov et al.

2011).

3.3.2 Expression of CB₂ receptor activated B and dendritic cells

The expression of CB₂ receptors on immune cells was examined by flow cytometric analysis. Mouse lung and spleen cells were collected. The collected cells were initially stimulated with LPS for B and DC activation. Then, cells were stained with NBD-labeled CB₂ ligand. CB₂ receptors were highly expressed by activated lung B cells and DC (Figure 17, LMC). Additionally, spleen B cells and DC also expressed the receptors (Figure 17, Spleen). In order to determine whether our CB₂ agonist specifically binds to the expressed receptors on the activated cells, the cold agonist was added prior to staining. The addition of the cold agonist effectively blocked CB₂ expression by activated cells (Figure 17, LMC + cold ago). This result suggests that the activated immune cells express CB₂ receptors, and the expressed receptors could be possible action site of CB₂ agonist.

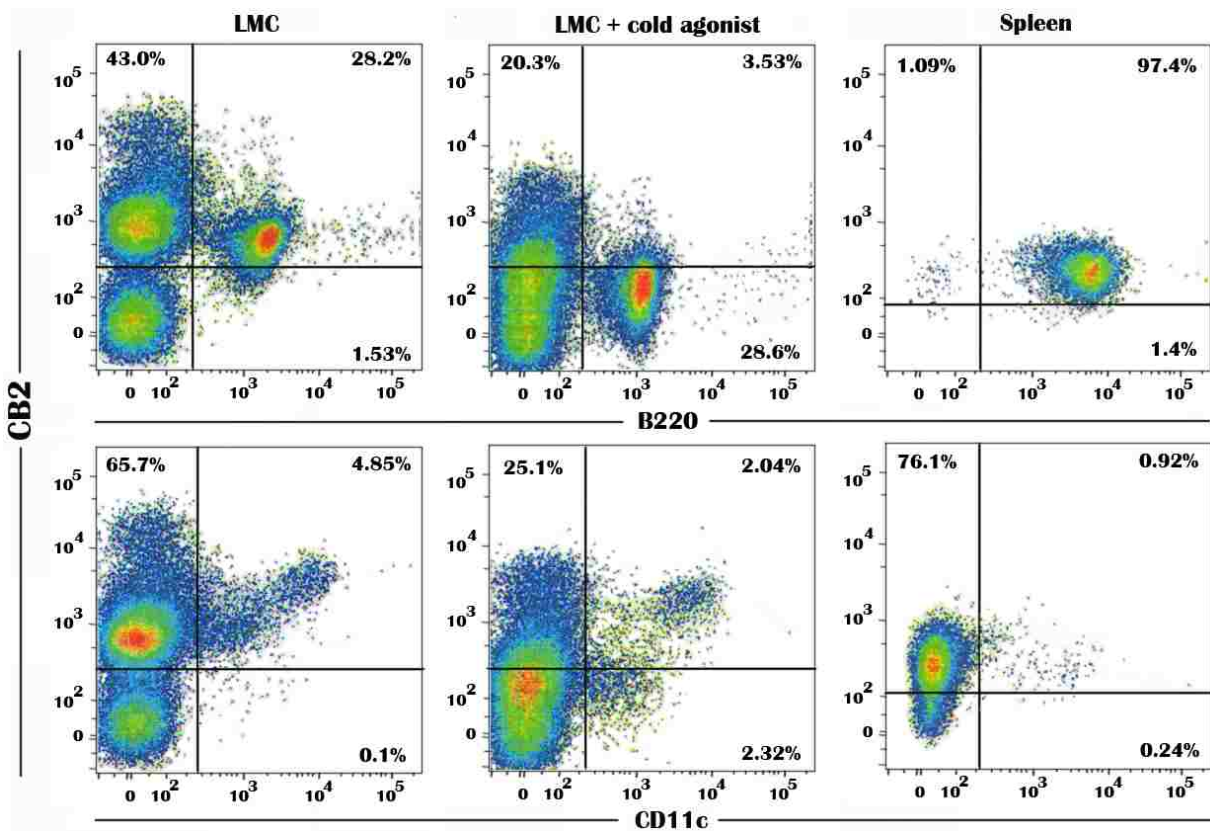


Figure 17. *CB₂ receptor expression by lung mononuclear cell (LMC) and splenic B cells and dendritic cell (DC) using NBD-labeled CB₂ ligand. Each column represents cell types lung mononuclear cells (LMC), lung mononuclear cells treated with the cold agonist (LMC + cold agonist) and spleen cells. FACS was performed to determine the expression of CB₂ receptors on activated B cell (B220⁺) and DC (CD11c⁺) by LMC or Spleen.*

CHAPTER 4

DISCUSSION

Cannabinoids are known for their psychoactive properties and have been used frequently for recreational and sometimes for therapeutic purposes. The effects of both synthetic and endogenous cannabinoids on the immune system have gained great interest in recent years because of their potential use as a new class of anti-inflammatory agents against a number of chronic immune inflammatory diseases. Currently, the therapeutic use of cannabis is gaining mainstream clinical and political acceptance. The main drawback for targeting cannabinoid compounds as potential therapeutic agents is the psychotropic effect primarily mediated by activation of CB₁ receptors. However, there are a number of novel approaches that are being undertaken to circumvent this problem including development of selective CB₂ agonists and peripherally restricted CB₁/CB₂ dual agonists. Currently, cannabinoids are used clinically for the treatment of chemotherapy-induced nausea and vomiting (anti-emetics) and to stimulate appetite.

It has become increasingly evident that cannabinoids exert a major influence on the immune system. They modulate immune responses during inflammatory processes and their anti-inflammatory effects have been studied in a number of diseases including rheumatoid arthritis, diabetes, and multiple sclerosis. Moreover, the endogenous cannabinoid AEA, by acting on CB₂ receptors, inhibited IL-12 and IL-23 production but enhanced production the anti-inflammatory cytokine IL-10 by microglial cells. In addition, the immunosuppressive activities of AEA have been attributed to inhibiting proliferative responses of human T cells and IL-17 production, which

is mediated via CB₂ receptors. Generation of the peripheral cannabinoid receptor knockout mice (CB₂^{-/-} mice) has led to important studies implicating the involvement of CB₂ receptors in diverse events including immune cell function and development, autoimmune inflammation, allergic dermatitis, atherosclerosis, apoptosis and chemotaxis.

The anti-inflammatory properties of cannabinoids, and CB₂ ligands in particular, could provide the basis of a new therapeutic approach for allergic asthma. In this study, a CB₂ agonist was designed specifically to bind to the CB₂ but not the CB₁ receptor. The main goal of this approach was to evaluate effect of CB₂ agonist in a mouse model of asthma. In vivo experiments were performed to determine if the CB₂ agonist attenuates airway inflammation. To complement this approach in vitro experiments were designed to elucidate possible mechanisms operative that may underlie the attenuation of airway inflammation. The therapeutic potential of CB₂ agonist on airway inflammation was determined by comparing levels of inflammatory cells and both pro- and anti-inflammatory cytokines in treated and non-treated groups.

The activation of antigen presenting cells (APCs) and antigen-specific CD4⁺ Th2 cells are an important initiating event in driving inflammatory responses in allergic asthma. Comparison was made between non-treated and CB₂ treated groups. The numbers of APCs and CD4⁺ Th2 cells in vehicle group is significantly induced. This induction is significantly inhibited by CB₂ agonist. CB₂ agonist group showed a significant reduction in the number of OVA-specific CD4⁺ cells recruited to the lung, as well as a marked reduction in the number of CD11c⁺ClassII⁺ APCs (Figure 6). The reduction in APCs and antigen-specific Th2 cells suggests that CB₂ agonist has capacity to inhibit early stage of allergic inflammation.

Infiltration of inflammatory cells into the airway is another key feature in airway inflammation. The total cell numbers could be a good indicator of ongoing inflammation. During airway inflammation, pro-inflammatory cytokines are secreted from Th2 cells (IL-2, IL-5 and IL-13). The pro-inflammatory cytokines elicit the development and recruitment of inflammatory effector cells (basophils, eosinophils, macrophages, mast cells and neutrophils). Our primary interest was to assess whether the CB₂ agonist suppressed the mucosal inflammation. To this end the inflammatory cells numbers present in the BAL were counted (eosinophils, lymphocytes, macrophages and neutrophils). In figure 7, the total numbers of eosinophils and lymphocytes in vehicle group are significantly induced. These induced inflammatory cells are effectively inhibited by CB₂ agonist. This significant inhibition of inflammatory cells suggests that CB₂ agonist reduces inflammatory cell infiltration in airways. Consistent with figure 7, eosinophil peroxidase (EPO) level, a good indicator of eosinophil, CB₂ treatment group exhibited a significantly lower level of EPO activity compared to non-treated group (Figure 8). Interestingly, this reduction is observed in dose-dependent manner. The higher dose group (5mg/Kg) showed a more pronounced eosinophil suppression than lower dose group (1mg/Kg) (Figure 8).

IL-10 is an anti-inflammatory cytokine with an important role in preventing inflammatory and autoimmune diseases. Findings from Borish study show that Th2 cells and the production of Th2 cytokines are downregulated by IL-10 (Borish 1999). Kosaka et al. also reported that the production of IL-4 and IL-13 and the resulting eosinophil count, in the BALF in OVA-specific eosinophilic airway inflammation model, are inhibited effectively by the administration of exogenous IL-10 (Kosaka 2011). To consider the anti-inflammatory function of IL-10 in allergic inflammation from previous studies, we determined whether the induction of IL-10 production is

involved in the attenuation by the CB₂ agonist. Consistent with this possibility the level of IL-10 was greatly increased in CB₂ group compared to non-treated group (Figure 10).

According to our *in vivo* experiments, CB₂ treatment shows noticeable inhibition in infiltration of Th2 cells and secondary inflammatory cells (especially eosinophils) in airway. In addition, it was indicated that the agonist has capability of inducing production of anti-inflammatory cytokine, IL-10.

To elucidate how the CB₂ agonist exerts an anti-inflammatory effect, *in vitro* experiments were performed. We addressed the possibility that the CB₂ agonist attenuates airway inflammation by the following mechanisms:

1. ***Inhibiting Th2 and/or Th17 cytokines release***
2. ***Promoting the production of IL-10 and/or expansion of IL-10 producing cells.***
3. ***Elicit an increase in the number of Foxp3⁺ regulatory cells.***
4. ***Promoting expression of arginase.***

Our experimental approaches enabled us to make the following conclusions.

1. *Inhibiting Th2 and/or Th17 cytokines release:* CD4⁺ Th2 cells play a crucial role in orchestrating inflammatory process in allergic airway inflammation. In addition, Th17 cells have also been shown to contribute to the inflammatory process in asthma. In the first instance we examined whether the CB₂ agonist down-regulates Th2 and/or Th17 cytokine production. Cells were isolated from mouse spleens, and the collected cells were activated and cultured in presence of different concentrations of the agonist. We measured the levels of IL-4 and IL-5 (Th2

cytokines) and IL-17 (Th17 cytokine) in the supernatants of cultured T cells. Our results show that IL-4, IL-5 and IL-17 were inhibited in the presence of the CB₂ agonist (Figure 12, Figure 13). In the case of IL-4 and IL-5 a dose-dependent inhibition was not observed since different concentrations of CB₂ agonist exhibited similar degrees of inhibition. However, in IL-17, dose-dependent inhibition was observed.

2. Promoting the production of IL-10 and/or expansion of IL-10 producing cells: *In vivo* experiments, our CB₂ agonist promoted the production of IL-10. Consistently, *in vitro* experiments, higher level of IL-10 was observed in cultures where CB₂ the agonist had been added (Figure. 11).

3. Elicit an increase in the number of Foxp3+ regulatory cells: Foxp3 expressing T cells (T_{regs}) are known to suppress T cell responses and maintain immune homeostasis. Foxp3 is a key transcription factor for T_{reg} development. We investigated the possibility that the culture of T cells in the presence CB₂ agonist may favor the selective expansion of Treg cells with the resultant depression in allergic inflammation. To address this we used the Foxp3-GFP mouse, which has been used to monitor nT_{reg} responses since Foxp3 expression is a marker for T_{reg} cell numbers. Our result showed no significant difference in Foxp3 expression between CB₂ and non-treated groups (Figure 14). This result implied that the CB₂ agonist does not involve promote the development or induction of T_{reg} cells.

4. Promoting expression of arginase: Hegde et al. demonstrated that activation of CB₂ receptor by THC induces MDSC (Hegde et al. 2010). MDSC are a suppressor cell population, which have

been shown to suppress T cell proliferation and possess immunosuppressive properties (Kusmartsev et al. 2000). Induction of arginase activity is known as a major mechanism associated with immunosuppression by MDSC (Gabilovich et al. 2009). In contrast, it is reported that insufficient L-arginine in airway is associated with asthma pathophysiology (Maarsingh et al. 2008). Louis et al. suggests that Th2 cytokines induces arginase activity (Louis et al. 1999). Boer et al. shows that the administration of exogenous L-arginine alleviates allergen-induced hyperresponsiveness in guinea pig model (Boer et al. 1999). In asthmatic patients, arginase activity in serum was increased, whereas the bioavailability of L-arginine declined (Morris et al., 2004). Based on these studies, we evaluated whether the agonist exerts its immune modulatory effects by promoting arginase activity. We found no significant difference in arginase activity between CB₂ and non-treated group was observed (Figure 15) strongly suggesting the attenuation of the immune response is not associated with change in arginase activity and/or MDSC-mediated.

Our *in vitro* findings reveal that CB₂ agonist might exerts its anti-inflammatory effect via inhibiting Th2 (pro-inflammatory) and Th17 cytokines. Our data are supportive of the possibility that the CB₂ agonist mediates this effect by inducing anti-inflammatory cytokine, IL-10, though we were not able to identify the cellular source of the IL-10. No change in arginase activity or number of Foxp3⁺ was observed making it unlikely MDSC or T_{reg} cells play a role in CB₂ - mediated effects.

To understand the mechanisms that underpin CB₂ agonist mediated attenuation, it is crucial to determine cellular expression of CB₂ receptors. CB₂ receptors are known to be expressed by immune cells. In order examine the cellular expression of CB₂ receptors we stained

Th2 cells, B cells and DC with a NBD-labeled CB₂ ligand. We observed CB₂ expression by Th2, B cells and DC (Figure 16A, 17). To confirm the specificity of any binding we monitored staining in the presence of an excess of un-labeled CB₂ agonist. The un-labeled agonist effectively blocked the receptor binding (Figure 16B, 17). Unfortunately several cell types, which included Th2, B cells and DC specifically bound the NBD-labeled material. This makes it difficult to further delineate the point of action of CB₂ agonist and raises the possibility it acts on several cell types.

CHAPTER 5

CONCLUSION AND FUTURE WORK

The main goal of this thesis was to examine and understand the effect of the CB₂ agonist in allergic asthma using a mouse model of the disease. To summarize, our CB₂ agonist, NMP7, was shown to attenuate allergen-induced airway inflammation. The hallmark of airway inflammation (infiltration of inflammatory cells Th2 cells, allergen-specific APC and eosinophils) was noticeably declined in the CB₂ treatment group. We observed that the agonist inhibited the expression of the pro-inflammatory cytokines IL-4, IL-5 and IL-10. Conversely, we also noticed the CB₂ agonist induced expression of the anti-inflammatory cytokine (IL-10). Foxp3⁺ Treg cell appeared not to play a role in the immune suppression mediated by CB₂ agonists. In addition, the agonist did not augment arginase activity suggesting the anti-inflammatory of this agent is not dependent on MDSC. We observed that CB₂ receptors are expressed during the inflammatory response, however, the expression of the receptors was not limited to a specific immune cell type. This implies that the agonist may exert immunosuppression by acting on any of these cells. To conclude, the CB₂ agonist was effective at inhibiting airway inflammation in allergic asthma model most likely by promoting IL-10 expression. Further studies are needed to expand our knowledge in important therapeutic value, particularly in allergic inflammation, of cannabinoid agonist. Our future study will elucidate more precise CB₂ agonist mechanism, as well as addressing any possible side effects of CB₂ agonist. We will need to examine whether IL-10 is involved in anti-inflammatory effect of CB₂ agonist by using co-administration of a neutralizing antibody to IL-10 with CB₂ agonist. Furthermore, we need to do: 1) comparison between CB₁ and CB₂ receptor knockouts to examine if CB₂ receptors are crucial for anti-inflammatory effect of

CB2 agonist, 2) evaluation on which cell type is crucial for CB₂ mediated immune suppression, and 3) investigation on any undesirable side-effects. The elucidation of precise mechanism and side effects will suggest cannabinoids as effective asthma treatment, a cure for asthma.

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