Title
Linking Cannabinoid Receptor Type II (CB2) Biology to Function

Author
Castaneda, Julie Theresa

Publication Date
2016

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Linking Cannabinoid Receptor Type II (CB_2) Biology to Function

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Toxicology

by

Julie Theresa Castaneda

2016

ABSTRACT OF THE DISSERTATION

Linking Cannabinoid Receptor Type II (CB_2)

Biology to Function

By

Julie Theresa Castaneda

Doctor of Philosophy in Molecular Toxicology

University of California, Los Angeles, 2016

Professor Michael D. Roth, Chair

Cannabinoids, the primary bioactive components in marijuana, bind and signal through endogenous cannabinoid receptors. mRNA encoding for the cannabinoid receptor type II (CB_2) predominates in human leukocytes. CB_2 is a G protein-coupled receptor (GPCR) and traditionally thought to be expressed on the cell surface. However, as reliable methods for imaging CB_2 were lacking, we hypothesized that a monoclonal antibody raised against the N-terminus of CB_2 could be combined with conventional and imaging flow cytometry to study CB_2 protein expression. Detection was validated using gene-modified cell lines and isotype control antibodies. When applied to peripheral blood cells, no CB_2 was detected on T cells, monocytes, and dendritic cells,

but it was detected on the surface of B cells. However, following membrane permeabilization, a high concentration of intracellular CB_2 was detected. When B cells were exposed to cannabinoids, surface CB_2 internalized but not in the pattern of pre-existing intracellular CB_2. The expression of GPCRs at different cellular locations can promote functional heterogeneity with respect to downstream signaling and function. As such, we hypothesized that this differential expression of CB_2 by leukocytes is likely a highly-regulated event and plays an important role in cannabinoid function. In order to further assess, we studied the expression on human B cells from different tissue sources and identified that surface CB_2 was present in naïve and memory B cells but lacking on the surface of activated B cells. Furthermore, B cell lymphomas with an activated phenotype exhibited the same pattern. Naïve cord blood B cells were therefore activated *in vitro*, allowing us to directly link the acquisition of an activated phenotype to the loss of surface CB_2. Findings were confirmed with confocal microscopy and demonstrated a diffuse but punctate intracellular distribution of CB_2 that did not overlap with either lysosomal or mitochondrial staining. Our findings document a novel and dynamic multi-compartment expression pattern for CB_2 in B cells that is specifically modulated during B cell activation. The intracellular location of CB_2 and the specific role of different receptors on biologic function remains to be determined but will likely be very informative in understanding cannabinoid biology.

The dissertation of Julie Theresa Castaneda is approved.

Oliver Hankinson

Robert H. Schiestl

Catia Sternini

Michael D. Roth, Committee Chair

University of California, Los Angeles

2016

To the fallen warriors and newly acquired guardian angels: Karen Loarca, Baruka, and Bagel.

Forever in my heart.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 3

Differential Expression of Intracellular and Extracellular CB_2 Cannabinoid Receptor Protein by Human Peripheral Blood Leukocytes (previously published in *Journal of NeuroImmune Pharmacology*):

Exposure to Δ9-Tetrahydrocannabinol Impairs the Differentiation of Human Monocyte-derived Dendritic Cells and their Capacity for T Cell Activation (previously published in *Journal of NeuroImmune Pharmacology*):

Chapter 4

Dynamic Regulation of Cell Surface CB_2 Receptor during Human B Cell Activation and Differentiation (manuscript in preparation for publication in *Journal of NeuroImmune Pharmacology*):

LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AEA	anandamide
CB_1	cannabinoid receptor type I
CB_2	cannabinoid receptor type II
CBD	cannabidiol
CBG	cannabigerol
CBN	cannabinol
DEA	Drug enforcement agency
GPCR	G protein-coupled receptor
Ig	immunoglobulin
LSD	lysergic acid diethylamide
mAB	monoclonal antibody
MAP	mitogen-activated kinase
MFI	mean fluorescent intensity
NIDA	National Institute on Drug Abuse
PBL	peripheral blood leukocytes
THC	delta-9-tetrahydrocannabinol

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Michael Roth, for allowing me to join his lab and pursue a career in a field that I have always felt passionately about. I thank him for all of his guidance over the past years. He has been such a great mentor working very hard along myside to shape me into the scientist who I am today. I have looked up to him many times as a father figure. I thank him for his patience and great advice throughout the years, since I was a very green scientist when I joined his lab. He has been nothing but supportive and has allowed me to work independently and run freely with my project. I am forever grateful for his close supervision and his hands on teaching. Not many students can say that they have had such an involved mentor throughout the course of their dissertation work, and I think his close involvement reflects deeply on the quality of my work.

I would also like to thank all of the past and present members of the Roth lab for teaching me on a constant basis and never closing the door when I had many, many questions. I would specifically like to thank Dr. Airi Harui, who has been another mentor to me since the first day I joined the lab. She trained me during my rotation and has continued to give me advice until this day. She has truly played a great role into shaping me into the person I am today. She has also served as a tremendous friend along these years. I look at her as a friend, sister, mother, and foremost, a magnificent role model. I would also like to thank all of my committee members, Dr. Hankinson, Dr. Schiestl, and Dr. Sternini for all of their advice throughout the years. They were always willing to meet with me anytime I needed help or guidance with my academic career. I am truly grateful for picking such a great committee.

I would also like to thank my family and friends. Everyone has been my rock and has supported me unconditionally. I would not be obtaining this degree if it were not for them. They

have been there for me when I doubted my abilities as a scientist. They have always been my #1 fans, and they have given me the strength to push forward. My support system is truly a tremendous asset. I could never repay them for the amount of support I have received.

I would also like to thank the UCLA Flow Cytometry, Virology, Translational Pathology, and Advanced light Microscopy/Spectroscopy core for lending their services that have heavily contributed to my research and specifically, to Jeff Calimlim and Dr. Matthew Schibler for training me in flow cytometry and confocal microscopy. I would also like to thank all of the anonymous people who have donated peripheral blood, cord blood, and tonsils to my research. Without them, my research would not be possible. I would like to thank my multiple funding sources including the NIH, American College of Toxicology, and UCLA. These funds made my doctoral work possible. I would also like to thank the multiple organizations, including the Society of NeuroImmune Pharmacology and American College of Toxicology, who have awarded me multiple travel awards and poster awards throughout the years. These awards helped solidify my ability to communicate science to a broad audience.

Chapter 3 is a version of two previous publications: Castaneda JT, Harui A, Kiertscher SM, Roth JD, Roth MD. Differential expression of intracellular and extracellular CB_2 cannabinoid receptor protein by human peripheral blood leukocytes. J Neuroimmune Pharm. 8(1):323-332, 2013 and Roth MD, Castaneda JT, Kiertscher SM. Exposure to Δ9-Tetrahydrocanninol Impairs the Differentiation of Human Monocyte-derived Dendritic Cells and their Capacity for T cell Activation. J Neuroimmuno Pharmacol. 10:333-343, 2015. I would like to acknowledge the contributions of the co-authors in the paper: Jeffrey Roth for contributing to the experimental methods and experimental work, Dr. Airi Harui and Dr. Sylvia Kiertscher for the help with designing the experiments and interpretation of data, and Dr. Michael Roth for designing

experiments, interpretation of data, providing insight regarding the writing, and editing the publication. I would also like to acknowledge Benjamin Alderete and Raymond Kong from Amnis Corporation for their aid in obtaining the ImageStream images.

Chapter 4 is a version of a manuscript in preparation for the *Journal of NeuroImmune Pharamcology*. I would like to acknowledge the contributions of the co-authors: Dr. Airi Harui for the help with designing the experiments and interpretation of data and Dr. Michael Roth who helped with designing the experiments, interpreting the data, providing insight regarding the writing, and editing the manuscript. I would also like to acknowledge Dr. Sylvia Kiertscher for consulting with the interpretation of data.

Dr. Michael Roth is the principal investigator who directed and supervised the research in this dissertation. He contributed by providing insight regarding the writing and editing of each chapter.

Julie Theresa Castaneda

EDUCATION

PhD, Molecular Toxicology, UCLA, Expected December 2016 **GPA: 3.7/4.0**
B.S. Forensic Science, Eastern Kentucky University, August 2010 **GPA: 3.8/4.0**

PUBLICATIONS

1. **Castaneda JT**, Harui A, Kiertscher SM, Roth JD, Roth MD. Differential Expression of Intracellular and Extracellular CB_2 Cannabinoid Receptor Protein by Human Peripheral Blood Leukocytes. J Neuroimmune Pharmacol. 8(1):323-332, **2013**. PMC3587044
2. Roth MD, **Castaneda JT**, Kiertscher SM. Exposure to Δ9-Tetrahydrocannabinol Impairs the Differentiation of Human Monocyte-derived Dendritic Cells and their Capacity for T Cell Activation. J Neuroimmune Pharmacol. 10(2):333-43, **2015**. PMC4470806
3. **Castaneda JT**, Harui A, Roth MD. (**2016**). Dynamic Regulation of Cell Surface CB_2 Receptor during Human B Cell Activation and Differentiation. Manuscript in preparation. J Neuroimmune Pharmacol.
4. **Castaneda JT**, Harui A, Roth MD. (**2016**). *In vitro* Activation of Naïve Mature B cells Induces Isotype Switching and Down-Regulation of CB2 Expression. Manuscript in preparation.

PRESENTATIONS AND POSTERS

Oral Presentations

1. Linking Cannabinoid Receptor Type 2 (CB2) Biology to Function. Gordon Research Conference (GRC): Cellular and Molecular Mechanisms of Toxicity, Aug 9-14, 2015, Andover, NH.

Posters

1. **Castaneda JT**, Kiertscher SM, Roth JD, Roth MD, Harui A. Protein expression and gene regulation of CB2 in human immune cells. The Young Investigators Poster Session, 17th Scientific Conference of the **Society on NeuroImmune Pharmacology (SNIP)**, Apr 5-10, **2011**, Clearwater Beach, FL. J Neuroimmune Pharmacol, 6 (Suppl 1):S26–S27, **2011**, and **The Department of Medicine Research Day**, Sept 24, **2011**, UCLA.
2. **Castaneda JT**, Kiertscher SM, Harui A, Roth MD. Intracellular CB2 receptor and receptor trafficking in human immune cells. The Young Investigators Poster Session, 18th Scientific Conference of **SNIP**, Apr 24-28, **2012**, Honolulu, HI. J Neuroimmune Pharmacol (**2012**) 7 (Suppl 1):S32–S33.
3. **Castaneda JT**, Kiertscher SM, Harui A, Roth MD. Differential Expression of Cannabinoid Type 2 (CB2) Receptors by Human B cells, T cells, and Monocytes. Poster sessions: 33rd Annual Meeting of the **American College of Toxicology (ACT)**, Nov 04-07, **2012**, Champions Gate, FL. Int J Toxicol (**2013**) 32 (1): 74-75., and **The Department of Medicine Research Day**, Oct 06, **2012**, UCLA.
4. **Castaneda JT**, Harui A, Kiertscher SM, Roth MD. Distribution Patterns for the CB2 Receptor Vary Between Human B Cells, T Cells, and Malignant Cell Lines. Poster sessions: 34th Meeting of **ACT**, Nov 03-06, **2013**, San Antonio, TX. Int J Toxicol (**2014**) 33 (1): 45., the

Southern California Chapter of the Society of Toxicology (SCCSOT) Meeting, Oct 17, 2013, Irvine, CA, and **Department of Medicine Research Day**, Oct 05, **2013**, UCLA.

5. **Castaneda JT**, Harui A, Kiertscher SM, Roth MD. Expression of CB_2 – A dynamic feature of B cells. The Young Investigators Poster Session, 20th Scientific Conference of **SNIP**, Mar 26-29, **2014**, New Orleans, LA. J Neuroimmune Pharmacol (**2014**) 9: 9.
6. **Castaneda JT**, Harui A, Kiertscher SM, Roth MD. The impact of B cell activation and isotype switching on CB2 expression. Poster sessions: 35th Meeting of **ACT**, Nov 09-12, **2014**, Orlando, FL. Int J Toxicol (2015) 34 (1): 81., and **Department of Medicine Research Day**, Oct 11, **2014**, UCLA.
7. **Castaneda JT**, Harui A, Kiertscher SM, Roth MD. Role of Differential CB2 Expression Patterns in the Biologic Function of B Cells. Poster sessions: **GRS and GRC on Cellular and Molecular Mechanisms of Toxicity**, Aug 08-14, **2015**, Andover, NH., the **SCCSOT** Annual Meeting, Oct 12, **2015**, Isis Pharmaceuticals, Carlsbad, CA, and **The Department of Medicine Research Day**, Oct 03, **2015**, UCLA, and the 36th Meeting of **ACT**, Nov 08-11, **2015**, Summerlin, NV. Int J Toxicol (2016) 35 (1): 58.

FELLOWSHIPS

1. American College of Toxicology (ACT) North American Graduate Fellowship, 2014-2016
2. Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (Parent F31), National Institute of Health (NIH), **2014 – 2016**
3. Graduate Student Mentorship Fellowship, UCLA Graduate Division **2012 – 2013**
4. Eugene-Cota Robles Fellowship, UCLA Graduate Division, **2010 – 2014**
5. National Science Foundation Graduate Research Fellowship - Honorable Mention – **2012**
6. UCLA Competitive Edge Program, Funded by the National Science Foundation (NSF) Alliance for Graduate Education and the Professoriate (AGEP) grant, **07/2010 – 09/2010**

HONORS/AWARDS

1. ACT travel grant for the 37th annual meeting of American College of Toxicology (ACT) Baltimore, MD–Nov, 2016.
2. ACT travel grant for the 36th annual meeting of ACT Summerlin, NV–Nov, **2015**.
3. 1st place poster award at the Southern California Chapter of the Society of Toxicology (SCCSOT) Annual Meeting at Isis Pharmaceuticals in Carlsbad, CA–Oct, **2015**.
4. Above and Beyond Award at the Gordon Research Conference (GRC) on Cellular and Molecular Mechanisms of Toxicity in Andover, NH–Aug, **2015**.
5. Funding award for the Gordon Research Seminar (GRS) on Cellular and Molecular Mechanisms of Toxicity in Andover, NH–Aug, **2015**.
6. ACT travel grant for the 35th annual meeting of ACT in Orlando, FL–Nov, **2014**.
7. 1st Place Arthur Falek Early Career Investigator Award at the 20th annual meeting of the Society of NeuroImmune Pharmacology (SNIP) in New Orleans, LA – Mar, **2014**
8. SNIP Travel Award for the 20th annual meeting of SNIP in New Orleans, LA – Mar, **2014**
9. ACT Furst Award: Best student paper at the 34th meeting of ACT in San Antonio, TX - Nov, **2013**
10. ACT travel grant for the 34th annual meeting of ACT in San Antonio, TX– Nov, **2013**
11. Training scholarship for "Flow Cytometry Bootcamp," at SoCal Flow Summit, UC Irvine – Mar, **2013**.
12. ACT travel grant for the 33rd annual meeting of ACT in ChampionsGate, FL – Oct, **2012**
13. SNIP Travel Award for the 18th annual meeting of SNIP in Honolulu, HI – Apr, **2012**

CHAPTER 1

BACKGROUND

According to the Monitoring the Future Survey [Johnston 2016], sponsored by the National Institute on Drug Abuse (NIDA), marijuana is the most commonly abused illicit drug in the United States. As of 2015, ~23% of young adults (age 19-28) responding to this national survey reported consumption of marijuana in the past 30 days. By comparison, during this same time interval, the national prevalence for tobacco smoking by young adults was reported at 16%, followed by amphetamine consumption at 3% and cocaine consumption at 2%. These striking differences in use are fueled in part by the growing perception that marijuana use is not harmful [Okaneku 2015] and by the growing number of states that have legalized marijuana for medicinal (4 U.S. states plus the District of Colombia) and/or recreational use (19 U.S. states) [Wilkinson 2016]. This setting and the growing interest in medicinal applications of cannabis products makes it essential that we understand the human biology and toxicology of marijuana.

Marijuana is a term that describes the dry leaves, stems, flowers, and seeds of the *Cannabis sativa* plant. It has been prohibited in the United States by federal law since the 1937 Marijuana Tax Act and the US Drug Enforcement Agency (DEA) has classified it as an illegal schedule I drug (i.e. classified as having no medical use and a high risk for abuse). Schedule I drugs are considered the most dangerous class of drugs with potential for severe psychological and physiological dependence. Other schedule I drugs include heroin, ecstasy, and lysergic acid diethylamide (LSD). Marijuana can be smoked in hand-rolled cigarettes (joints), in cigars that have been emptied out of its contents and refilled with marijuana (blunts), through vaporizers to avoid inhaling smoke, consumed in edibles, or brewed as tea. While the use of marijuana for medicinal, religious, and recreational purposes dates back 5000 years, the discovery of cannabinoid molecules and our understanding of how they interact with our endogenous human cannabinoid signaling

system represents a relatively recent area of investigation [Aizpurua-Olaizola 2016, Herring 1998, Pertwee 2006].

Marijuana is composed of over 400 different compounds, including more than 100 different cannabinoids [Aizpurua-Olaizola 2016, Greydanus 2013]. Cannabinoids are the primary bioactive constituents of marijuana and the main psychoactive cannabinoid is delta-9-tetrahydrocannabinol (THC). The THC content in the average illicit marijuana cigarette is reported by the Potency Monitoring Project to comprise approximately 12% by weight [ElSohly 2014]. Other cannabinoids also found in the *Cannabis sativa* plant include cannabidiol (CBD), cannabigerol (CBG), and cannabinol (CBN), but these are not considered to play a role in the psychoactive effects associated with marijuana consumption. Upon combustion and smoking, marijuana also liberates an array of polycyclic aromatic hydrocarbons including the known carcinogens benzo(α)pyrene and benz(α)anthracene, which are components of the particulate phase of smoke [Roth 2001]. Toxic substances such as carbon monoxide, hydrogen cyanide, and nitrosamines are also released as part of the gas phase of marijuana smoke. While all of these released constituents may have biologic and/or toxic consequences, the focus of this thesis work is on the cannabinoid constituents and specifically on the biology of the human type 2 cannabinoid receptor.

Endogenous Cannabinoid System

The development of synthetic cannabinoids eventually led to the discovery of a human endogenous cannabinoid system that is comprised of at least two arachidonic acid-derived endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide (AEA), their biosynthetic and degradative enzymes, and two cannabinoid receptors, CB_1 and CB_2. [Bisogno 2005, Cabral 2015]. The endocannabinoid system has been found to play a role in immunomodulation, metabolic

regulation, bone growth, pain, cancer, and psychiatric disorders [Aizpurua-Olaizola 2016, Kleyer 2012]. Endocannabinoids are thought to be enzymatically produced and released "on demand" [Cabral 2015]. They bind and activate seven-transmembrane G protein-coupled receptors (GPCRs) type I (CB_1) and type II (CB_2) and are linked to intracellular signaling cascades including adenylyl cyclase, cAMP, mitogen-activated protein (MAP) kinase, and intracellular calcium [Howlett 2002, Maccarrone 2015].

Cannabinoid receptors, CB_1 and CB_2, share 44% amino acid homology and bind THC with relatively equal affinity [Cabral 2015, Shire 1996]. They are expressed in most organ systems, and their activation by marijuana smoke can have wide-ranging health effects [Grotenhermen 2003, Volkow 2014, Turcotte 2016]. The CB_1 receptor is mostly found in the central nervous system and mediates the psychoactive components associated with cannabinoids. CB_1 has been described to play a role in memory, pain regulation, stress response, and the regulation of metabolism [Busquets Garcia 2016, Cabral 2015]. The CB_2 receptor is mostly found in peripheral tissue and mediates the immune regulating components of cannabinoids. More specifically, the highest concentration of CB_2 is found in immune cells in addition to lower concentrations found in bone cells, keratinocytes, adipocytes, and renal tissue [Basu 2011, Mackie 2006]. The CB_2 receptor is suggested to play a role in immunomodulatory mechanisms that regulate inflammation and also play a role in host defense [Basu 2011, Herring 1998, Turcotte 2016].

Effects of THC on the Human Immune System

Despite the widespread use of marijuana and its increasing legalization across multiple states in the U.S., there is relatively little information known about the effects of cannabinoids on

human immunity. Cannabinoids have been described to have anti-inflammatory effects on leukocytes. [Cabral 2015, Roth 2015, Volkow 2014]. In mouse studies, the CB_2 receptor has been found to play a role in the responsiveness to infectious pathogens and play a role in immune homeostasis [Newton 1994, Newton 2009]. In human studies, alveolar macrophages from the lungs of marijuana smokers have been found to be deficient in the production of cytokines, nitric oxide, and mediation of bacteria killing [Baldwin 1997, Roth 2002, Shay 2003]. Human T cells activated in the presence of THC have also been found to result in a T helper type 2 (Th2)-skewed pattern of cytokine production with limited proliferation [Yuan 2002].

With the highest levels of expression on immune cells, the CB_2 receptor is suggested to mediate the immune regulating effects of cannabinoids [Cabral 2015, Roth 2015, Volkow 2014, Turcotte 2016]. In support of this statement, there are several studies done with animal models, including CB_2 knock-out mice [Liu 2009, Turcotte 2016, Ziring 2006]. Although murine CB_2 and human CB_2 share 82% amino acid homology of the coding regions, there are significant differences in non-coding regions of their respective genes, suggesting that some inter-species differences likely exist with respect to regulation and expression [Liu 2009]. This potential difference argues that a combination of both animal models and human studies are required to understand the regulation and function of the CB_2 receptor with respect to the immune system. Nonetheless, CB_2 knock-out mice have been reported to exhibit higher levels of leukocyte recruitment and an over-production of pro-inflammatory cytokines [Buckley 2008]. While these mice do not exhibit obvious morphological differences they have also been noted to have abnormalities in the formation of several T cell and B cell subsets within lymphoid organs, making the CB_2 receptor vital for the formation of T cell and B cells subsets involved in immune homeostasis [Turcotti 2016, Ziring 2006]. An increase in IgE production and allergic diseases

would be expected in a model that is driven towards Th2 skewing [Agudelo 2008]. Surprisingly, THC treated CB_2 knockout mice showed increased levels of IgE serum production, suggesting a role for CB_2 receptor in the regulation of IgE [Newton 2012]. Immune suppression was also observed when THC was administered to tumor-bearing mice, which promoted tumor growth in a CB_2-dependent manner [Zhu 2000]. Translating *in vivo* and *in vitro* experiments performed in animal or cell line models into an understanding of the biology in humans is also challenging because of the route of consumption, amount of exposure, and the pattern of use in marijuana users is entirely different, and there are often concurrent exposures of humans to tobacco, alcohol, and other substances that might affect the immune system in an additional or different manner.

Potential Use of Marijuana as a Therapeutic Agent

The previously described work suggests that cannabinoid receptors may be centrally involved in immune function, and therefore, the CB_2 pathway may represent an attractive target for cannabinoid-based drugs. Cannabinoids have been promoted as a new class of drugs with the potential for beneficial anti-inflammatory, immunoregulatory, and anti-fibrotic effects [Atwood 2012, Pacher 2011, Turcotti 2016]. CB_2 agonists have already been shown to reduce inflammation through the p38-MK2 pathway [Turcotti 2016]. There are currently multiple FDA-approved cannabinoid based medications. Marinol (Schedule III drug) and Cesamet (Schedule II drug) have been prescribed for the treatment of chemotherapy induced nausea and vomiting. Marinol has also been prescribed as an appetite stimulant and as a treatment for glaucoma by lowering intraocular pressure. Recently in July of 2016, Syndros^{TM} (scheduling pending), an orally administered liquid formulation of dronabinol, has also received FDA approval. It has been prescribed to treat anorexia associated weight loss in AIDS patients and chemotherapy induced nausea and vomiting.

Also, Sativex, a sublingual spray that is composed of equal concentrations of THC and CBD, has received FDA approval to proceed with phase III clinical trials for the treatment of pain in patients with advanced cancer. It is also prescribed for the treatment of spasticity due to multiple sclerosis. CBD is of great therapeutic interest since it has been shown to have anti-emetic, anti-inflammatory, and anti-psychotic effects [Bergamaschi 2011, Cabral 2015, Turcotti 2016]. There have also been no effects observed on blood pressure, pulse, body temperature, or gastrointestinal and psychological function [Bergamaschi 2011]. Another cannabinoid formulation that contains only CBD, Epidiolex, is also undergoing phase III testing for the treatment of a rare genetic seizure disorder (Dravet Syndrome). Despite the Schedule I DEA classification assigned to marijuana (having no medical use and a high risk for abuse), there is obvious evidence that strategies focused on regulating CB_2 signaling might represent promising treatments for autoimmune or chronic inflammatory diseases. Understanding the expression and function of the human CB_2 receptor may provide an important key to unlocking further cannabinoid-based drug development.

Understanding CB_2 Receptor as a GPCR

The CB_2 receptor has traditionally been described as a cell surface GPCR. GPCRs respond to a wide variety of stimuli and play crucial roles in neurotransmission, cellular metabolism, secretion, differentiation, growth, inflammation, and immune responses. GPCR activation is initiated by ligand binding, an event that usually occurs at the cell surface. Ligand binding induces a conformational change that activates heterotrimeric G-protein signaling and a subsequent cascade of events leading to internalization of the receptor and linkage with other signaling pathways [Jean-Alphonse 2011, Syrovatkina 2016].

The CB_2 receptor has been reported to exhibit a complex pharmacology (drug interaction profile), signaling (second messenger pathways) and trafficking pattern [Aizpurua-Olaizola 2016, Basu 2011, Howlett 2005]. The characterization of THC has led to the synthesis of cannabinoid analogs classified as synthetic cannabinoids, which are used to study structure-activity relationships, characterize cannabinoid-mediated bioactivity, and contribute to the understanding of mechanism of action by which endocannabinoids and phytocannabinoids exert their effects on the immune system [Cabral 2015]. The development of new ligands that can mimic the protective effects of cannabinoids has proven particularly difficult due to the constant discovery of multiple endogenous ligands, targets, and sites of interaction. Further research is needed to understand the mechanism of action of cannabinoids since the patterns of activation and induction of intracellular signaling differs with each compound.

As demonstrated in CB_2 transfected CHO cells, human HL-60, human bronchial epithelial cells, murine microglial cells, and a murine macrophage cell line, CB_2 signaling is initiated through its interaction with heterotrimeric G_i-proteins and the inhibition of adenylyl cyclase [Turcotte 2016]. CB_2 signaling has been linked to phosphorylation of MAP kinase, phosphorylation of AKT, modulation of intracellular calcium, and generation of intracellular ceramide [Basu 2011, Brown 2012, Chen 2012, Cudaback 2010, Howlett 2005, Turcotte 2016]. The mechanisms responsible for this signaling diversity have not been adequately explained.

In studies with other GPCRs, it is often the process of receptor internalization that allows the receptor to become associated with an array of adaptor and signaling molecules [Calebiro 2010, Jean-Alphonse 2011]. The finding that CB_1 receptor is expressed at intracellular sites and can mediate signaling adds further support for CB_2 to play a role in mediating intracellular signaling [Rozenfeld 2011]. Rab proteins direct receptor trafficking to specific intracellular organelles, and

CB_2 receptors have been suggested to internalize via Rab-mediated endocytosis and initiate downstream intracellular signaling [Calebiro 2010, Grimsey 2011]. In artificial cell constructs, CB_2 has been observed to undergo both constitutive and ligand-based internalization and traffic through endosomal and lysosomal compartments [Atwood 2010, Grimsey 2011, Kleyer 2012]. Blocking internalization or shifting the use of adaptor proteins has been observed to shift intracellular versus extracellular GPCR distribution [Grimsey 2011]. The dynamic balance between CB_2 receptors at the cell surface and at possible intracellular sites might play a vital role in understanding cannabinoid receptor biology. The availability of cell surface receptors for ligand interaction can determine the responsiveness of a cell and further induction of intracellular signaling. Receptor availability for ligand binding is a very important feature in order to understand drug action and how the CB_2 receptor can be exploited for therapeutic purposes. There is great diversity in the trafficking of GPCRs, and it is vital to understand the specific pathways involved with CB_2.

Localization of receptors at the cell membrane has been described to determine signaling via G protein pathways. Kleyer and associates also describe that the amount of cannabinoid receptor on the surface can directly determine receptor function. Interestingly, they also describe that cannabinoid receptors in primary human cells do not only internalize upon agonist interaction. They describe movement of the receptors between cytoplasm and cell membranes by ligand-independent trafficking mechanisms, such as triggering by hydrogen peroxide that is present during inflammation and triggering by nonspecific protein tyrosine phosphatase inhibitors [Kleyer 2012]. They even describe these methods to be responsible for externalization of the cannabinoid receptor in T cells. A phenomenon that we have never seen before. Previous studies have demonstrated that intracellular CB_1 receptors located in lysosomes and mitochondria are able to

induce intracellular signaling, suggesting that intracellular stores of CB_1 receptor are functional [Kleyer 2012, Rozenfeld 2011]. The impact of intracellular CB_2 receptor signaling on cell physiology remains to be determined. Understanding CB_2 receptor trafficking could help determine cell-type specific effects of cannabinoids and the regulation of the CB_2 receptor in different immune cells.

Role of the CB_2 receptor in B cells

While GPCRs are integral membrane proteins, there has been increasing interest in their expression and function at sites other than the extracellular membrane [Jean-Alphonse 2011]. Relatively little is known about the expression and distribution of CB_2 protein in human leukocytes. CB_2 has been traditionally described as a cell membrane GPCR expressed primarily by B cells while CB_2 mRNA has been identified in most leukocytes but with expression levels to also be the highest in B cells [Carayon 1998, Mackie 2006, Sanchez Lopez 2015]. However, experimental data supporting the expression of the CB_2 receptor in human leukocytes has been limited and sometimes contradictory, mostly due to the lack of reliable tools for detecting CB_2 protein in cells of interest. CB_2 protein detection had been particularly difficult due to non-specific staining of primary antibodies and use of CB_2 polyclonal antibodies that can be cross-reactive to other proteins [Graham 2010]. In contrast to our preliminary findings, Graham and associates have suggested that CB_2 was highly expressed on all PBMC, but they used commercial polyclonal rabbit or goat antibodies from different companies without any controls. Staining was different for every manufacturer, from batch to batch, and from subject to subject.

Cannabinoids have also been variably described to promote or suppress B cell proliferation and to bias immunoglobulin (Ig) class switching, suggesting cannabinoids might play a role in B cell activation, differentiation, and maturation [Agudelo 2008, Basu 2013, Newton 1994, Newton 2009, Ngaotepprutaram 2013]. Previous evidence from mouse studies has also suggested that exposure to cannabinoids can bias the response toward a Th2 response (immunosuppressive response) through a mechanism involving CB_2 receptors [Agudelo 2008, Carayon 1998]. Detailed studies in CB_2-knockout mice revealed deficient B cell subsets in several lymphoid organs [Buckley 2012, Ziring 2006]. The CB_2 receptor has also been identified to have chemotactic effects, and cannabinoids have been described to have a role in B cell trafficking within mouse lymph nodes [Basu 2011]. The specific role of CB_2 in the biologic function of B cells, especially in humans, remains to be studied in detail.

The primary function of B cells is to secrete antibodies and thereby provide the humoral component of immunity. Additionally, B cells play important roles in presenting antigens and by secreting cytokines, therefore exerting an influence over the other arms of the immune system [Hoffman 2016]. However, in their naïve state, B cells exhibit limited function and must be activated by exposure to antigens and co-stimulatory signals. Activation in this manner promotes the naïve B cell to enlarge, clonally expand, differentiate, and eventually undergo isotype switching, which results in the production of mature antibodies and differentiation into memory or plasma B cells [Hoffman 2016]. Different antibody isotypes are elicited in response to different pathogens and directly influenced by the nature of costimulatory signals and the local cytokine environment [Hoffman 2016]. Isotype switching in human B cells can be induced by CD40L when combined with IL-21 and modulated by several other cytokines including IL-2, IL-4, IL-10 [Avery 2008, Moens 2014]. The natural process of activation, expansion and differentiation is antigen-

specific and directly enhances the capacity for a host to respond rapidly and effectively to future immune challenges by that same antigen [Hoffman 2016]. Dysregulated activation and isotype switching is also important as it can lead to autoimmune disorders or immunodeficiency [Hoffman 2016]. Some B cell activating signals can also promote allergic or suppressive immune reactions [Hoffman 2016, Taylor 2006]. Employing a mouse model where-in animals were infected with the legionella pneumophila organism, a number of studies were carried out to study the impact of cannabinoid exposure (particularly THC) on the host antibody response to this infection [Cabral 2009, Newton 2004]. This approach demonstrated that exposure to THC and to other CB_2-specific ligands during B cell activation is associated with Ig class switching and the generation of allergic or immunosuppressive responses [Agudelo 2008, Moens 2014, Newton 1994, Newton 2009]. IL-4 is a potent B cell activating cytokine. In the presence of IL-4, CB_2 message has been demonstrated to increase in B cells and to skew isotype switching. Carlisle and associates described that CB_2 might be particularly responsive to agonists when in a responsive or activated state. The immunomodulatory activity of CB_2 might be dependent on the activation of the target as well [Carlisle 2002]. Persidsky and associates found that activation of CB_2 blocks monocyte migration and reduces secretion of pro-inflammatory cytokine, TNF-α [Persidsky 2015]. Further investigation regarding the effects of cannabinoids and the role of the CB_2 receptor on B cell activation and isotype switching is vital in order to characterize potential mediators in controlling isotype switching and assuring the appropriate immune response is induced following antigen exposure.

In conclusion, there are conflicting data regarding the effects of cannabinoids on B cell function. These conflicting results can be attributed to inconsistencies in studies done with mixed immune cell populations versus isolated B cells, comparison of naïve versus activated cells, and

peripheral blood B cells versus tonsillar or splenic B cells. Through the work of this dissertation, we hope to better understand how CB_2 receptor location, the dynamic balance between extracellular and intracellular receptors, activation, and isotype switching link to the biologic effects of human leukocytes. We hypothesize that CB_2 is an immunoregulatory molecule and its expression may be directly tied to the level and/or type of cell activation. Further research on the immunotoxic effects of marijuana and its effects on cannabinoid receptor biology are needed in order to develop a clear understanding of the balance between extracellular and intracellular CB_2 receptors and the impact of intracellular location on cannabinoid-mediated signaling. These results will be of vital interest to the field of cannabinoid receptor biology and directly relevant to understanding the potential toxic effects of cannabinoids on immune function and how the cannabinoid/CB_2 pathway can be exploited for immunotherapeutic purposes.

REFERENCES

1. Agudelo M, cabraNewton C, Widen R, Sherwood T, Nong L, Friedman H, Klen TW. Cannabinoid receptor 2 (CB_2) mediates immunoglobulin class switching from IgM to IgE in cultures of murine-purified B lymphocytes. J Neuroimmune Pharamcol. 3: 35-42, 2008.

2. Aizpurua-Olaizola O, Elezgarai I, Rico-Barrio I, Zarandona I, Etxebarria N, Usobiaga A. (2016). Targeting the endocannabinoid system: future therapeutic strategies. Drug Discov Today. Advance Online Publication. doi: 10.1016/j.drudis.2016.08.005.

3. Atwood B, Wager-Miller J, Haskins C, Straiker A, Mackie K. Functional selectivity in CB2 receptor signaling and regulation: implications for the therapeutic potential of CB2 ligands. Mol Phamacol. 81(2)250-63, 2012.

4. Avery DT, Bryant VL, Ma CS, de Waal Malefyt MR, Tangye S. IL-21-Induced Isotype Switching to IgG and IgA by Human Naïve B Cells is Differentially Regulated by IL-4. J immunol. 181:1767-1779, 2008.

5. Baldwin GC, Tashkin DP, Buckley DM, Park AN, Dubinett SM, Roth MD. Marijuana and Cocaine Impair Alveolar Macrophage Function and Cytokine Production. 156:1606-1613, 1997.

6. Basu S, Dittel BN. Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. Immunol Res. 51:26-38, 2011.

7. Basu S, Ray A, Dittel BN. Cannabinoid Receptor 2 (CB2) Plays a Role in the Generation of Germinal Center and Memory B Cells, but Not in the Production of Antigen-Specific IgG and IgM, in response to T-dependent Antigens. PLoS One. 27;8(6):e67587, 2013.

8. Bergamaschi MM, Queiroz RH, Zuardi AW, Crippa JA. Safety and side effects of cannabidiol: a *Cannabis sativa* constituent. Curr Drug Saf. 6(4):237-249, 2011.

9. Bisogno T, Ligresti A. Di Marzo V. The endocannabinoid signaling system: biochemical aspects. Pharmacol Biochem Behav. 81(2):224-238, 2005.

10. Brown I, Cascio MG, Rotondo D, Pertwee RG, Heys SD, Wahle KW. Cannabinoids and omega-3/6 endocannabinoids as cell death and anticancer modulators. Prog Lipid Res. 52(1):80-109, 2012.

11. Buckley NE. The peripheral cannabinoid receptor knockout mice: an update. Br J Pharmacol. 153(2):309-318, 2008.

12. Busquets Garcia A, Soria-Gomez E, Bellocchio L, Marsicano G. (2016). Cannabinoid receptor type-1: breaking the dogmas. F1000Res. Advance Online Publication. doi: 10.12688/f1000research.8245.1.

13. Cabral GA, Griffin-Thomas L. Emerging Role of the CB_2 Cannabinoid Receptor in Immune Regulation and Therapeutic Prospects. Expert Rev Mol Med. 11:e3, 2009.

14. Cabral GA, Rogers TJ, Lichtman AH. Turning over a new leaf: cannabinoid and endocannabinoid modulation of immune function. J Neuroimmune Pharmacol. 10:193-203, 2015.

15. Calebiro D, Nikolaev VO, Persani L, Lohse MJ. Signaling by internalized G-protein-coupled receptors. Trends Pharmacol Sci.31(5):221-8, 2010.

16. Carayon P, Marchand J, Dussossoy D, Derocq JM, Jbilo O, Bord A, Bouaboula M, Galiegue S, Mondiere P, Penarier G, Le fur G, Defrance T, Casellas P. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood. 92:3605-3615, 1998.

17. Chen W, Kaplan BL, Pike ST, Topper LA, Lichorobiec NR, Simmons SO, Ramabhadran R, Kaminski NE. Magnitude of stimulation dictates the cannabinoid-mediated differential T cell response to HIVgp120. J Leukoc Biol. 92(5):1093-102, 2012.

18. Cudaback E, Marrs W, Moeller T, Stella N. The expression level of CB1 and CB2 receptors determines their efficacy at inducing apoptosis in astrocytomas. PLoS ONE 5(1): e8702. 2010.

19. ElSohly MA. Potency Monitoring Program quarterly report no.123 — reporting period: 09/16/2013-12/15/2013. Oxford: University of Mississippi, National Center for Natural Products Research, 2014.

20. Graham ES, Angel CE, Schwarcz LE, Dunbar PR, Glass M. Detailed characterization of CB2 receptor protein expression in peripheral blood immune cells from healthy human volunteers using flow cytometry. Int J Immunopathol Pharmacol. 23(1):25-34, 2010.

21. Greydanus DE, Hawver EK, Greydanus MM, Merrick J. Marijuana: current concepts. Front Public Health. 1:42, 2013.

22. Grimsey NL, Goodfellow CE, Dragunow M, Glass M. Cannabinoid receptor 2 undergoes Rab5-mediated internalization and recycles via a Rab11-dependent pathway. Biochim Biophys Acta. 1813:1554-15560, 2011.

23. Grotenhermen F. Pharmacokinetics and pharmacodynamics of cannabinoids. Clin Pharmacokinet. 42(4):327-60, 2003.

24. Herring AC, Koh WS, Kaminski NE. Inhibition of the cyclic AMP signaling cascade and nuclear factor binding to CRE and κB elements by cannabinol, a minimally CNS-active cannabinoid. Biochem Pharmacol. 55:1013-1023, 1998.

25. Hoffman W, Lakkis FG, Chalasani G. B Cells, Antibodies, and More. Clin J Am Soc Nephrol. 11(1):137-154, 2016.

26. Howlett AC. The cannabinoid receptors. Prostaglandins Other Lipid Mediat. 68-69:619-631, 2002.

27. Howlett AC. Cannabinoid receptor signaling. Handb Exp Pharmacol. (168):53-79. 2005.

28. Jean-Alphonse F and Hanyaloglu AC. Regulation of GPCR signal networks via membrane trafficking. Mol Cell Endocrin 331:205–214, 2011.

29. Johnston, LD, O'Malley PM, Bachman JG, Schulenberg, JE, Miech RA. (2016). Monitoring the Future national survey results on drug use, 1975–2015: Volume 2, College students and adults ages 19–55. Ann Arbor: Institute for Social Research, The University of Michigan. Available at http://monitoringthefuture.org/pubs.html#monographs

30. Kleyer J, Nicolussi S, Taylor P, Simonelli D, Furger E, Anderle P, Gertsch J. Cannabinoid receptor trafficking in peripheral cells is dynamically regulated by a binary biochemical switch. Biochem Pharm 83:1393-1412, 2012.

31. Liu QR, Pan CH, Hishimoto A, Li CY, Xi ZX, Llorente-Berzal A, Viveros MP, Ishiguro H, Arinami T, Onaivi ES, Uhl GR. Species differences in cannabinoid receptor 2 (CNR2 gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. Genes Brain Behav. 8(5):519-530, 2009.

32. Maccarrone M, Bab I, Biro T, Cabral GA, Dey SK, Di Marzo V, Konje JC, Kunos G, Mechoulam R, Pacher P, Sharkey KA, Zimmer A. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 36(5):277-96, 2015.

33. Mackie B. Cannabinoid receptors as therapeutic targets. Annu. Rev. Pharmacol. Toxicol. 46:101-22, 2006.

34. Newton CA, Chou PJ, Perkins I, Klein TW. CB(1) and CB(2) cannabinoid receptors mediate different aspects of delta-9-tetrahydrocannabinol (THC)-induced T helper cell shift following immune activation by Legionella pneumophila infection. J Neuroimmune Pharmacol. Mar;4(1):92-102, 2009.

35. Newton CA, Klein TW. Cannabinoid 2 (CB2) receptor involvement in the down-regulation but not up-regulation of serum IgE levels in immunized mice. J Neuroimmune Pharmacol. 7(3):591-598, 2012.

36. Newton CA, Klein TW, Friedman H. Secondary immunity to Legionella pneumophila and Th1 activity are suppressed by delta-9-tetrahydrocannabinol injection. Infect Immun. Sep;62(9):4015-20, 1994.

37. Newton CA, Lu T, Nazian SJ, Perkins I, Friedman H, Klein TW. The THC-induced suppression of Th1 polarization in response to Legionella pneumophila infection is not mediated by increases in corticosterone and PGE2. J Leukoc Biol. 76(4):854-861, 2004.

38. Ngaotepprutaram T, Kaplan BLF, Carney S, Crawford R. Suppression by Δ^9-tetrahydrocannabinol of the primary immunoglobulin M response by human peripheral blood B cells is associated with impaired STAT3 activation. Toxicol. 310(2013)84-91, 2013.

39. Okaneku J, Vearrier D, McKeever RG, LaSala GS, Greenberg MI. Change in perceived risk associated with marijuana use in the United States from 2002 to 2012. Clin Toxicol (Phila). 53(3):151-5, 2015.

40. Persidsky Y, Fan S, Dykstra H, Reichenbach NL, Rom S, Ramirez SH. Activation of cannabinoid type two receptors (CB2) diminish inflammatory responses in macrophages and brain endothelium. J Neuroimmune Pharamcol. 10(2):302-308, 2015.

41. Pertwee RG. Cannabinoid pharmacology: the first 66 years. British Journal of Pharmacology. 147: S163–S171, 2006.

42. Roth MD, Marques-Magallanes JA, Yuan M, Sun W, Tashkin DP, Hankinson O. Induction and regulation of the carcinogen-metabolizing enzyme CYP1A1 by marijuana smoke and delta (9)-tetrahydrocannabinol. Am J Respir Cell Mol Biol. 24(3):339-44, 2001.

43. Roth MD, Cheng Q, Harui A, Basak SK, Mitani K, Low TA, SM Kiertscher. Helper-dependent adenoviral vectors efficiently express transgenes in human dendritic cells but still stimulate antiviral immune responses. J Immunol. 169:4651-4656, 2002(a).

44. Roth MD, Castaneda JT, Kiertscher SM. Exposure to Δ9-Tetrahydrocanninol Impairs the Differentiation of Human Monocyte-derived Dendritic Cells and their Capacity for T cell Activation. J Neuroimmuno Pharmacol. 10:333-343, 2015.

45. Rozenfeld R. Type I cannabinoid receptor trafficking: all roads leads to lysosome. Traffic. 12:12-18, 2011.

46. Sanchez Lopez AJ, Roman-Vega L, Ramil Tojeiro E, Giuffrida A, Garcia-Merino A. Regulation of cannabinoid receptor gene expression and endocannabinoid levels in lymphocyte subsets by interferon-beta: a longitudinal study in multiple sclerosis patients. Clin Exp Immunol 179(1):119–127, 2015.

47. Shay A, Choi R, Whittaker K, Salehi K, Tashkin DP, Roth MD, Baldwin GC. Impairment of Antimicrobial Activity and Nitric Oxide Production in Alveolar Macrophages from Smokers of Marijuana and Cocaine. J Infect Dis. 187(4):700-704, 2003.

48. Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessegue B, Bonnin-Cabanne O, Le Fur G, Caput D, Ferrara P. Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. Biochim Biophys Acta. 1307(2):132-136, 1996.

49. Syrovatkina V, Alegre KO, Dey R, Huang XY. (2016). Regulation, Signaling, and Physiological Functions of G-Proteins. J Mol Biol. Advance Online Publication. doi: 10.1016/j.jmb.2016.08.002.

50. Moens L, Tangye SG. Cyotkine-Mediated Regulation of Plasma Cell Generation: IL-21 Takes Center Stage. Front Immunol. 18;5:65, 2014.

51. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis C. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-β: the role of T regulatory cells. Immunology. 117:433-42, 2006.

52. Turcotte C, Blanchet MR, Laviolette M, Flamand N (2016) The CB_2 receptor and its role as a regulator of inflammation. Cell. Mol. Life. Sci. Advance online publication. DOI 10.1007/s00018-016-2300-4

53. Volkow ND, Baler RD, Compton WM, Weiss SR. Adverse health effects of marijuana use. N Engl J Med 370(23):2219-2227, 2014.

54. Wilkinson ST, Yarnell S, Radhakrishnan R, Ball SA, D'Souza DC. Marijuana Legalization: Impact on Physicians and Public Health. Annu Rev Med. 67:453-66, 2016.

55. Yuan M, Kiertscher SM, Cheng Q, Zoumalan R, Tashkin DP, Roth MD. Δ^9-Tetrahydrocannabinol Regulates Th1/Th2 Cytokine Balance in Activated Human T-Cells. J. Neuroimmunol. 133(1-2):124-131, 2002.

56. Ziring D, Wei B, Velazquez P, Schrage M, Buckley NE, Braun J. Formation of B and T cell subsets require the cannabinoid receptor CB2. Immunogenetics. 58(9):714-25, 2006.

57. Zhu LX, Sharma S, Stolina M, Gardner B, Roth MD, Tashkin DP, Dubinett SM. Delta-9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. J Immunol. 165:373-80, 2000.

CHAPTER 2

INTRODUCTION

Cannabinoids, the primary bioactive constituents of marijuana, activate CB_1 and CB_2 receptors and signal through an endogenous cannabinoid system to produce their biologic effects. Expression of the CB_2 receptor predominates in cells from the immune system. However, there is little information known as to how the CB_2 receptor influences human immunity and host defense, the specific location of CB_2 receptors in human leukocytes, and the impact of cannabinoids on its distribution. While it is important to understand the immunotoxic effects that might result from marijuana smoking and exposure to cannabinoids, it is equally important to understand how the CB_2 receptor might be exploited to control inflammation and regulate adaptive immunity from a therapeutic perspective.

The CB_2 receptor has traditionally been described as a cell membrane GPCR expressed primarily by B cells. However, as reliable methods for imaging the CB_2 receptor did not exist, we hypothesized that a monoclonal antibody (mAb) raised against the N-terminus of CB_2 could be combined with conventional and imaging flow cytometry to study CB_2 protein expression. Previous evidence suggested that T cells, which do not express surface CB_2, can mediate the effects of cannabinoids through intracellular CB_2 receptor expression. Therefore, this led us to also hypothesize that intracellular receptors must be able to mediate ligand-induced signaling and biological consequences. After employing a new approach with conventional and imaging flow cytometry, we determined that B cells express CB_2 on the cell surface and at intracellular locations, while T cells, monocytes, and dendritic cells only express CB_2 at intracellular sites. Cell surface CB_2 was responsive to THC by rapidly internalizing when exposed to the ligand. The distribution of this internalized CB_2 did not appear to account for the pre-existing distribution of intracellular CB_2. The reasons as to why CB_2 is expressed on the cell surface of certain cells and not expressed on the cell surface in other cells remains unknown. After concluding with these findings, we

hypothesize the cellular CB_2 receptor location is a key feature that links location to specific biologic outcomes, and the expression of CB_2 at extracellular versus intracellular sites may play an important function in mediating the biologic and toxic effects of cannabinoids.

The expression of GPCRs at different cellular locations can promote functional heterogeneity with respect to downstream signaling and function. As such, we hypothesized that this differential expression of CB_2 by leukocytes is likely a highly-regulated event and plays an important role in cannabinoid function. Activation of CB_2 has been linked to many different signaling pathways and cellular events [Agudelo 2008, Carayon 1996, Ngaotepprutaram 2013]. By focusing on B cells, the only leukocyte discovered to express both CB_2 at the cell surface and at intracellular locations, we hypothesize that B cell activation plays a key role in CB_2 expression and in mediating the biologic function of B cells, such as isotype switching and antibody production**.** In order to investigate the impact of B cell activation on CB_2 expression, we have designed an *in vitro* activation model where we can induce activation and differentiation in naïve mature B cells and track CB_2 expression across the different stages of differentiation and maturation. We determined that cells found to be in an activated state and cells activated *in vitro* lacked cell surface CB_2 and expressed high intracellular CB_2. This finding allowed us to directly link the acquisition of an activated phenotype to the loss of surface CB_2. The intracellular location of CB_2 and the specific role of different receptors on biologic function remains to be determined but will likely be very informative in understanding cannabinoid biology.

The experiments detailed in this dissertation are the first steps in determining what major factor controls the distribution of CB_2 in human leukocytes and how this relates to biologic function. Ultimately, we hypothesize that CB_2 receptor expression, location, and trafficking are all critical features that link cannabinoids to specific signaling and functional consequences on human

leukocytes. Understanding how these features are linked to immune regulation could lead to the development of novel therapeutics by targeting specific biologic outcomes, such as apoptosis, cytokine production, and isotype switching. By the conclusion of these studies, we will have established a clear understanding of the differential expression patterns of CB_2 by human B cells and how it relates biologic function.

REFERENCES

1. Agudelo M, Newton C, Widen R, Sherwood T, Nong L, Friedman H, Klen TW. Cannabinoid receptor 2 (CB_2) mediates immunoglobulin class switching from IgM to IgE in cultures of murine-purified B lymphocytes. J Neuroimmune Pharamcol. 3: 35-42, 2008.

2. Carayon P, Marchand J, Dussossoy D, Derocq JM, Jbilo O, Bord A, Bouaboula M, Galiegue S, Mondiere P, Penarier G, Le fur G, Defrance T, Casellas P. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood. 92:3605-3615, 1998.

3. Graham ES, Angel CE, Schwarcz LE, Dunbar PR, Glass M. Detailed characterization of CB2 receptor protein expression in peripheral blood immune cells from healthy human volunteers using flow cytometry. Int J Immunopathol Pharmacol. 23(1):25-34, 2010.

4. Ngaotepprutaram T, Kaplan BLF, Carney S, Crawford R. Suppression by Δ^9-tetrahydrocannabinol of the primary immunoglobulin M response by human peripheral blood B cells is associated with impaired STAT3 activation. Toxicol. 310(2013)84-91, 2013.

CHAPTER 3

ESTABLISHING A MODEL TO DETECT EXTRACELLULAR AND INTRACELLULAR CB_2 DETERMINES DIFFERENTIAL EXPRESSION PATTERNS AMONG HUMAN LEUKOCYTES

Introduction

CB_2 mRNA has been described to be expressed by most leukocytes, with expression levels to be greatest in B cells, less in monocytes, and low in T cells [Carayon 1998, Mackie 2006]. However, experimental data supporting this in humans has been limited. At the protein level, CB_2 has traditionally been described as a cell membrane GPCR expressed primarily by B cells, but our recent findings challenge this description. Preliminary data from our laboratory suggests there is no CB_2 receptor cell surface expression in T cells, but there is previous evidence that states that T cells have reduced T cell proliferation, activation, and cytokine production when exposed to THC [Cabral 2015, Roth 2002, Volkow 2014]. These findings led us to hypothesize that CB_2 receptors must be present at intracellular locations, and these receptors must be capable of mediating ligand-induced signaling and biologic function.

The distribution of the CB_2 receptor in human leukocytes and the reason as to why extracellular CB_2 is not expressed in T cells is not known. It is not clear whether a difference in distribution of CB_2 represents variable rates of internalization and recycling or whether cell-specific differences related to activation and maturation result in these differential expression patterns. It is also not clear what role these CB_2 expression patterns have in mediating the biologic and toxic effects of cannabinoids on immune function. We hypothesize that the presence of CB_2 at different cellular locations is an important feature that promotes functional heterogeneity with respect to downstream signaling and biologic responses.

At this point in cannabinoid receptor biology, relatively little is known about the expression and distribution of CB_2 protein. No one has been able to characterize CB_2 at a different locations of the cell other than the cell surface. If CB_2 does exist at different locations, there is currently no evidence as to whether these receptors are functional or not. This gap in the field was mostly due

to a lack of a set of reliable tools to measure cannabinoid receptor expression at the cell surface and at intracellular locations. There was also conflicting published results regarding cell surface receptor expression, which lacked reliable controls. In addition, these conflicting data result from the comparison of naïve versus activated cells, use of polyclonal versus monoclonal antibodies, and animal versus human CB_2. In chapter 3, we describe in Castaneda et al. and Roth et al. how we have designed a novel cellular and molecular approach to investigate the expression, cellular distribution, and trafficking of the CB_2 receptor in primary human cells in order to develop a better understanding of how these features impact cannabinoid-mediated signaling and biologic function.

Interestingly, Kaplan and associates describe that there is also evidence that the CB_1 receptor, despite its predominant presence in the central nervous system, can mediate many immune system effects, including direct modulation of immune function by endogenous and exogenous cannabinoids in T cells and innate cells [Kaplan 2013]. With this novel flow cytometry approach, we have also proven that this innovative detection method has the same capability of detecting CB_1 in human leukocytes in a reliable and specific manner. With the new assay created in Castaneda et al. and further described in Roth et al., we strive to measure the distribution of cell surface and intracellular CB_2 receptor expression in human immune leukocytes in order to further understand the role of the CB_2 receptor in human immunity for the possible development of future therapeutics.

References

1. Cabral GA, Rogers TJ, Lichtman AH. Turning over a new leaf: cannabinoid and endocannabinoid modulation of immune function. J Neuroimmune Pharmacol 10:193-203, 2015.

2. Carayon P, Marchand J, Dussossoy D, Derocq JM, Jbilo O, Bord A, Bouaboula M, Galiegue S, Mondiere P, Penarier G, Le fur G, Defrance T, Casellas P. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood. 92:3605-3615, 1998.

3. Castaneda JT, Harui A, Kiertscher SM, Roth JD, Roth MD. Differential expression of intracellular and extracellular CB_2 cannabinoid receptor protein by human peripheral blood leukocytes. J Neuroimmune Pharm. 8(1):323-332, 2013. PMC3587044

4. Kaplan BLF. The Role of CB_1 in immune modulation by cannabinoids. Pharmacology & Therapeutics. 137:365-374, 2013.

5. Mackie B. Cannabinoid receptors as therapeutic targets. Annu. Rev. Pharmacol. Toxicol. 46:101-22, 2006.

6. Roth MD, Castaneda JT, Kiertscher SM. Exposure to Δ9-Tetrahydrocanninol Impairs the Differentiation of Human Monocyte-derived Dendritic Cells and their Capacity for T cell Activation. J Neuroimmuno Pharmacol. 10:333-343, 2015.

7. Volkow ND, Baler RD, Compton WM, Weiss SR. Adverse health effects of marijuana use. N Engl J Med 370(23):2219-2227, 2014.

J Neuroimmune Pharmacol (2013) 8:323–332
DOI 10.1007/s11481-012-9430-8

ORIGINAL ARTICLE

Differential Expression of Intracellular and Extracellular CB_2 Cannabinoid Receptor Protein by Human Peripheral Blood Leukocytes

Julie T. Castaneda · Airi Harui · Sylvia M. Kiertscher · Jeffrey D. Roth · Michael D. Roth

Received: 22 October 2012 / Accepted: 16 December 2012 / Published online: 10 January 2013

Abstract mRNA encoding for the CB_2 cannabinoid receptor is expressed by many subsets of human peripheral blood leukocytes (PBL), but little is known about the resulting protein expression and function. Employing clones from the A549 and 293T cell lines that were constructed to express both full-length human CB_2 and GFP, we developed a flow cytometry assay for characterizing CB_2 protein expression. A monoclonal antibody directed against human CB_2 selectively stained the surface of transduced but not parental cell lines. When cells were fixed and permeabilized, imaging flow cytometry identified large stores of intracellular protein. Total cellular staining for CB_2 corresponded closely with the level of GFP expression. When exposed to Δ^9-tetrahydrocannabinol, CB_2-expressing cells internalized cell surface CB_2 receptors in a time- and dose-dependent manner. Applying these approaches to human PBL, CB_2 protein was identified on the surface of human B cells but not on T cells or monocytes. In contrast, when PBL were fixed and permeabilized, intracellular CB_2 expression was readily detected in all three subsets by both conventional and imaging flow cytometry. Similar to the protein expression pattern observed in fixed and permeabilized PBL, purified B cells, T cells, and monocytes expressed relatively equal levels of CB_2 mRNA by quantitative real-time RT-PCR. Our findings confirm that human PBL express CB_2 protein but that its distribution is predominantly intracellular with only B cells expressing CB_2 protein at the extracellular membrane. The differential role of intracellular and extracellular CB_2 receptors in mediating ligand signaling and immune function remains to be determined.

Keywords Cannabinoids · Cannabinoid receptor CB_2 · G-protein coupled receptors · Intracellular membrane receptors · Leukocytes · Imaging flow cytometry · Tetrahydrocannabinol

J. T. Castaneda · A. Harui · S. M. Kiertscher · J. D. Roth · M. D. Roth (✉)
Division of Pulmonary and Critical Care Medicine, Department of Medicine, CHS 37–131, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095-1690, USA
e-mail: mroth@mednet.ucla.edu

J. T. Castaneda · M. D. Roth
Inter-Departmental Program in Molecular Toxicology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Introduction

While the use of Cannabis (marijuana) for medicinal, religious, and recreational purposes dates back 5,000 years, the identification of cannabinoids and the discovery of an endogenous cannabinoid ligand and receptor signaling pathway in human cells represents a relatively recent discovery (Mechoulam 1986; Pertwee 2006). Cannabinoid receptor subtype 1 (CB_1) is highly expressed in the brain and well known for mediating the psychoactive effects of marijuana, while the highest expression of mRNA encoding for cannabinoid receptor subtype 2 (CB_2) exists in peripheral tissues and particularly within cells of the immune system (Basu and Dittel 2011; Bouaboula et al. 1993; Galiègue et al. 1995; Munro et al. 1993). Both receptors are membrane-associated G-protein coupled receptors (GPCR) and bind Δ^9-tetrahydrocannabinol (THC) with relatively equal affinity (Munro et al. 1993; Shire et al. 1996). However, a number of other ligands have been identified, which express high selectivity for CB_2 (Pertwee 2006). Using these reagents, it has been shown that activation of CB_2 receptor can regulate both innate and adaptive immunity including the ability to suppress anti-cancer responses (McKallip et al. 2005; Zhu et al. 2000) and host defenses against pneumonia (Klein et al. 2000; Newton et al. 2009; Shay et al. 2003), promote

apoptosis of antigen presenting cells and T cells (Do et al. 2004; McKallip et al. 2002), alter cytokine production and antibody isotype switching (Agudelo et al. 2008; Cencioni et al. 2010; Srivastava et al. 1998; Yuan et al. 2002), modulate the infectivity and replication of HIV virus (Gorantla et al. 2010; Roth et al. 2005), regulate the inflammatory aspects of atherosclerosis (Mach and Steffens 2008), and play a role in several autoimmune diseases (Malfait et al. 2000; Sipe et al. 2005). This body of work has lead to considerable interest in understanding the role that endogenous cannabinoids have in the immune system and in developing CB_2-selective therapies (Klein 2005; Mackie 2006). However, the direct examination of CB_2 protein on human cells has been limited by an inability to reliably detect and quantitate receptor protein. While mRNA profiles have suggested that there is differential expression of CB_2 by B cells, T cells, and other leukocyte subsets, there have been very few studies evaluating differences in protein expression or cellular distribution. The current research focuses on the development and validation of a flow cytometry approach for measuring and tracking CB_2 receptor protein in human cells. The findings provide a flexible method for receptor study in primary cells and new insights regarding the differential expression of CB_2 receptors at intracellular versus extracellular locations in human B cells, T cells, and monocytes.

Methods

Primary cells and cell lines

Following informed consent, peripheral blood leukocytes (PBL) were isolated by Ficoll-gradient centrifugation from the blood of healthy human donors. Cell subsets were identified by flow cytometry using fluorescent-labeled monoclonal antibodies (mAb) directed against B cells (anti-CD20, BD Biosciences, San Jose, CA), T cell subsets (anti-CD3, Invitrogen, Camarillo, CA or in combination with anti-CD4 or anti-CD8, BD Biosciences), and monocytes (anti-CD13, Invitrogen). Purified subsets were prepared from primary PBL using lineage-specific mAb and magnetic nanoparticles (StemCell Technologies, B.C., Canada). Purities for each subset were confirmed by flow cytometry.

The human embryonic kidney cell line 293T and lung cancer epithelial cell line A549 (ATCC, Manassas, VA) were maintained in culture as adherent monolayers in complete medium composed of DMEM or RPMI-1640, respectively (Cellgro, Manassas, VA), supplemented with 10 % fetal bovine serum (Omega Scientific, Tarzana, CA) and antibiotics. The 293T/CB_2-GFP and A549/CB_2-GFP cell lines were constructed by transducing the corresponding parental lines with a self-inactivating lentivirus expressing full-length human CB_2 receptor cDNA and green fluorescent protein (GFP) as previously described (Sarafian et al. 2008). Expression of CB_2 was regulated by a hCMV promoter with the expression of GFP linked through an internal ribosomal entry site. Transduced cells were sorted by flow cytometry (FACSVantage SE cell sorter, Becton Dickinson, San Jose, CA) for GFP-expressing clones, and aliquots of the expanded cell lines were cryopreserved for subsequent use.

Δ^9-tetrahydrocannabinol (THC)

A 50 mg/ml stock of THC in ethanol was obtained from the National Institute on Drug Abuse (Bethesda, MD) and immediately prior to use was diluted serially in DMSO and culture medium to produce a final ethanol concentration in assays of 0.01 % (v/v) and DMSO concentration at ≤0.25 % (v/v). Diluent controls were prepared using the same dilution strategies with ethanol and DMSO that did not contain THC.

Detection of extracellular CB_2 receptor

Adherent cell lines were treated with 0.1 % trypsin (Cellgro) for 1 min, quenched with 10 % fetal bovine serum, and then washed with phosphate-buffered saline (PBS) containing 0.2 mg/ml EDTA (Invitrogen, Carlsbad, CA) to produce a single cell suspension. Extracellular CB_2 was detected by pre-treating with 50 μl human AB Serum (Omega Scientific) for 1 min followed by a 30 min incubation on ice with unlabeled primary mouse IgG_2 mAb directed against either human CB_2 (clone #352114, 0.5 μg/tube, R&D System, Minneapolis, MN) or isotype-matched mAbs against an irrelevant antigen, mouse NK1.1 (clone #PK136, 0.5 μg/tube, BD Biosciences) or mouse Thy1.2 (clone #30-H12, 0.5 μg/tube, BD Biosciences). After washing twice with PBS/2 % AB serum, cells were incubated with an APC-labeled goat anti-mouse $F(ab')_2$ mAb (APC-labeled GAM, 0.5 μg/tube, Invitrogen) for 30 min on ice. To identify different PBL subsets, cells were incubated with lineage-specific fluorescent-labeled mAb for 20 min and washed. All cells were then fixed and incubated with 1 % paraformaldehyde for 20 min at 4 °C and washed. The cells were covered to protect from light and stored at 4 °C until analyzed.

Detection of total cellular CB_2 expression

In order to detect intracellular CB_2 receptor, single cell suspensions of either PBL or indicated cell lines were fixed with 1 % paraformaldehyde (Sigma Aldrich, St. Louis, MO) and treated with permeabilizing solution (BD Biosciences). Cells were washed once with PBS/2 % AB serum, resuspended in permeabilizing solution, and blocked with 50 μl human AB serum. Staining with primary unlabeled mAb (against CB_2, NK1.1, or Thy1.2) and secondary APC-labeled GAM were carried out as detailed in the extracellular staining protocol except that incubation times were prolonged to 60 min on ice.

After washing, PBL were further stained with fluorescent-labeled antibodies directed against lineage-specific markers and then fixed again with 1 % paraformaldehyde prior to storage and analysis.

Conventional and imaging flow cytometry

Conventional multiparameter flow cytometry was carried out using a FACScan II-plus cytometer (BD Biosciences) with the acquisition of 5,000–30,000 events depending upon the assay conditions. Analysis of acquired data was performed using FCS Express V3 software (De Novo Software, Los Angeles, CA). Flow cytometry results are presented as histograms or two-parameter dot plots with indicated values representing the mean linear fluorescence intensity (MFI) of the gated population. Fluorescent images were acquired using an ImageStreamX® cytometer (Amnis Corporation, EMD Millipore, Seattle, WA) capable of simultaneously acquiring high resolution digital images at up to 60X magnification, in each of 12 channels, as cells pass through the cytometer's detection chamber. Analysis was performed using IDEAS Software (Amnis Corporation) in which image display characteristics were first optimized for background and range of fluorescent intensity and then applied equally to all image within an analysis set. Gates were set to display representative cell images expressing the mean fluorescent intensity for each analysis channel. Internalization scores were derived using an eroded-pixel mask generated from the bright field image to determine the ratio of intracellular to extracellular signal for each marker of each cell.

CB_2 receptor internalization assay

Single cell suspensions of 293T/CB_2-GFP cells were suspended in X-VIVO media (Lonza, Walkersville, MD) and after incubating with THC or diluents alone under different conditions (of concentration and time), the reactions were quenched with ice-cold PBS. Cells were then stained to determine the residual expression of extracellular CB_2 as already described. Alternatively, the trafficking of fluorescent-labeled extracellular CB_2 was assessed using the imaging cytometer. 293T/CB_2-GFP cells were first stained with anti-CB_2 mAb and APC-labeled GAM, washed to remove excess antibodies, and then antibody-stained cells were incubated with either diluent alone or 8 μM THC at 37 °C for 40 min. At the completion of the incubation period, cells were fixed with 1 % paraformaldehyde and 3,000–5,000 cell events of interest acquired using the ImageStreamX® cytometer.

Quantitative real-time RT-PCR

cDNA was prepared directly from 2×10^5 whole PBL or purified B cell, T cell, and monocyte subsets using a FastLane cDNA kit (QIAGEN, Valencia, CA). qRT-PCR analysis was performed in batch with three replicates/sample using a StepOne real-time PCR system (Applied Biosystems, Foster City, CA) and labeled probes for CB_2 and a housekeeping gene, GAPDH (both from Applied Biosystems). Relative expression of CB_2 mRNA was corrected for GAPDH and represented as the average 1/Δ-CT values obtained from triplicate wells.

Statistics

Individual conditions were assayed in triplicate within an experiment and a minimum of three replicate experiments carried out for each assay. Flow cytometry histograms and dot-plots display results from a single representative tube of a single experiment. All other data are presented as means of replicate tubes or experiments as detailed. The difference between means was determined using a Student's *t*-test with a $p<0.05$ accepted as statistically significant.

Results

Anti-CB_2 mAb detects cell membrane expression of CB_2 receptor

The A549 and 293T cell lines and their transduced clones expressing human CB_2 and GFP were used to optimize a flow cytometry assay for measuring cell surface CB_2. Isotype-matched mAbs directed against irrelevant antigens (mouse NK1.1 and Thy1.2) were also employed to assess background staining and specificity. As demonstrated by results from a representative flow cytometry experiment in Fig. 1, no CB_2-specific staining was observed when A549 cells (or 293T cells, data not shown) were stained with anti-CB_2 mAb. However, there was a clear fluorescent signal when anti-CB_2 mAb was used to stain the surface of A549/CB_2-GFP cells (MFI=29.6±2.1 for CB_2 vs 5.6±2.5 for NK1.1, $p<0.05$, averaged results from 3 experiments) and a much brighter signal when used to stain 293T/CB_2-GFP cells (MFI=871.2±19.2 for CB_2 vs 7.1±0.9 for NK1.1, $p<0.05$, averaged results from 3 experiments). Assessing GFP expression as an independent measure of transgene expression by these two cell lines confirmed the relatively low expression by A549/CB_2-GFP cells and the much higher expression by 293T/CB_2-GFP cells.

Cell permeabilization exposes intracellular CB_2 protein

While GPCR are integral membrane proteins, there has been increasing interest in their expression and function at sites other than the extracellular membrane (Jean-Alphonse and Hanyaloglu 2011). Cells were therefore probed for the

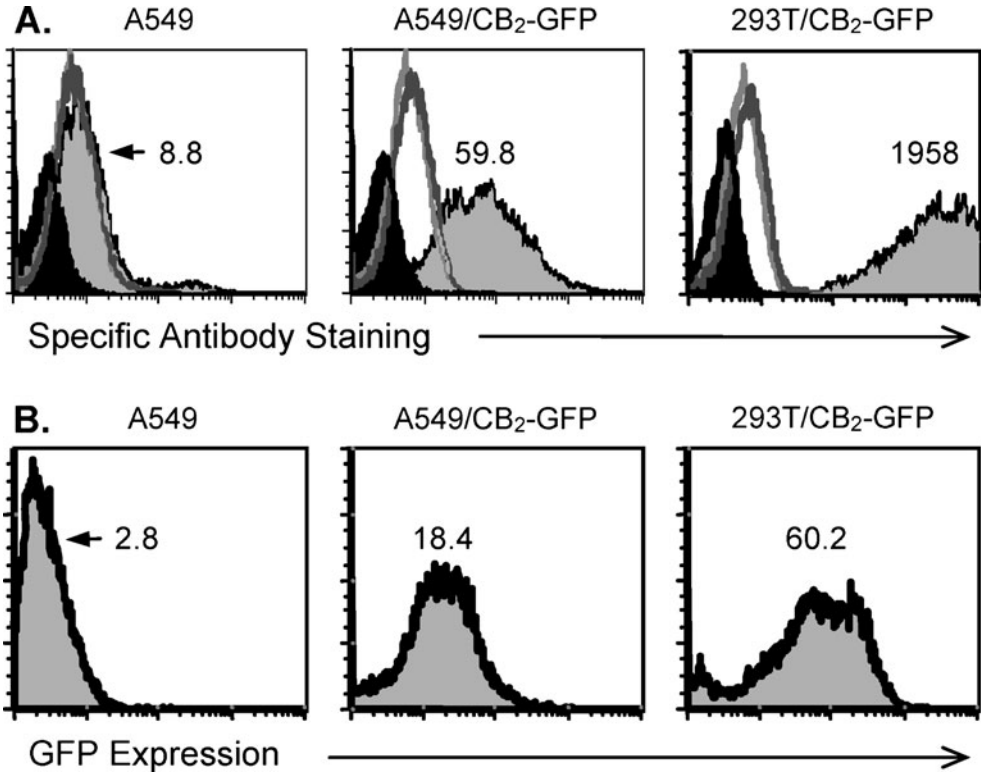


Fig. 1 Anti-CB_2 mAb detects cell membrane expression of CB_2 receptor. Parental cell lines (A549 and 293T; data shown only for A549) and stable clones expressing both CB_2 and GFP (A549/CB_2-GFP and 293T/CB_2-GFP) were incubated with a primary unlabeled mAb against CB_2 protein or an isotype-matched irrelevant target (anti-mouse NK1.1 or Thy1.2) and then stained with APC-labeled GAM. Representative experiment shown, n=3. (**a**) Cells were analyzed by flow cytometry for APC fluorescence and mean fluorescent intensities (MFI) for CB_2-stained cells indicated for each condition. Black fill=unstained cells; white fill=isotype controls; shaded fill=CB_2 stained. (**b**) Corresponding GFP expression with net MFI values shown

expression of intracellular CB_2 protein by adding fixation and permeabilization steps to our standard flow cytometry protocol (Fig. 2). While surface staining of viable 293T/CB_2-GFP cells revealed high levels of CB_2 expression, there was a 50 to 60 % drop in fluorescent intensity when cells were stained after fixation and permeabilization suggesting an impact of the fixation process on antigen-antibody binding affinity. As a result, fluorescent intensity values could not be used to directly compare the levels of extracellular to intracellular protein. Imaging flow cytometry was therefore

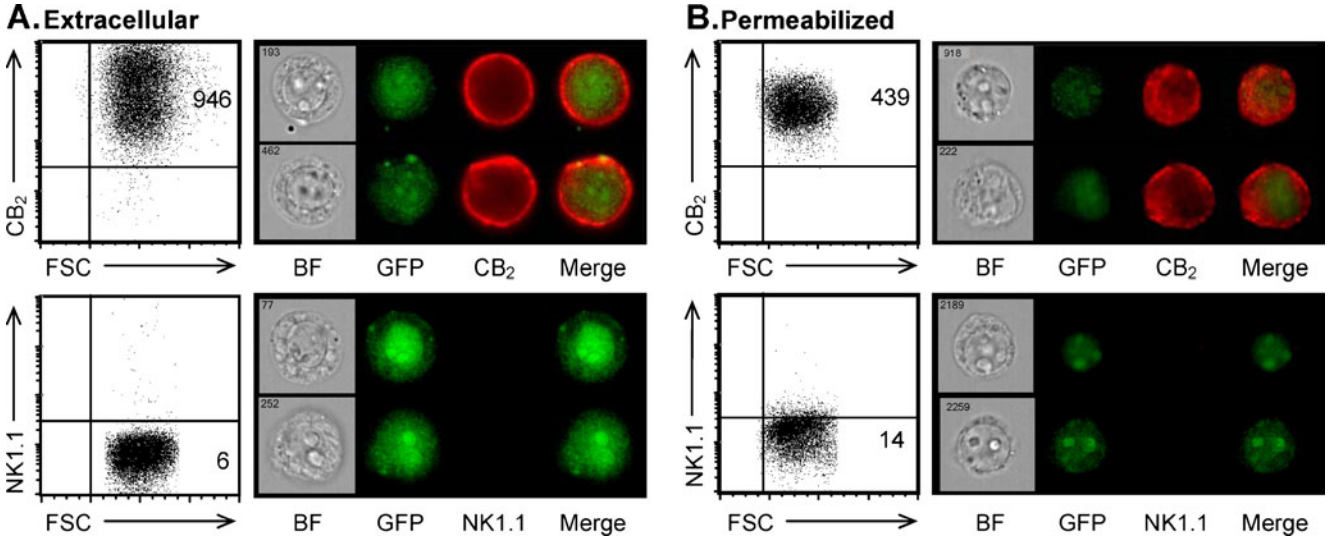


Fig. 2 Cell permeabilization exposes intracellular CB_2 protein. For each condition, a representative dot-plot from conventional flow cytometry is shown (MFI indicated) followed by image sets obtained from two representative cells captured by imaging flow cytometry. Images were selected from cells expressing the mean GFP expression and mean CB_2 or NK1.1 expression, respectively. Bright field (BF), GFP fluorescence, APC fluorescence, and merged fluorescence images are shown. (**a**) For assessing extracellular CB_2 protein, viable 293T/CB_2-GFP cells were incubated at 4 °C for 30 min with 0.25 μg of CB_2 mAb (top panel) or 0.25 μg of an isotype control mAb, NK1.1 (bottom panel), washed, and then incubated with APC-labeled GAM for detection. (**b**) For assessing total cellular CB_2 expression, 293T/CB_2-GFP cells were fixed and permeabilized prior to staining at 4 °C for 60 min with 0.5 μg of CB_2 mAb (top panel) or 0.5 μg of an isotype control, NK1.1 (bottom panel) followed by detection with APC-labeled GAM

employed to localize antibody binding sites. As demonstrated in Fig. 2a, imaging of 293T/CB_2-GFP cells that were stained with anti-CB_2 mAb using the extracellular protocol revealed an intense rim of fluorescence associated with the extracellular membrane. However, when the same cells were stained following fixation and permeabilization, an entirely different CB_2 expression pattern emerged (Fig. 2b). Rather than an intense rim of membrane fluorescence, the majority of the CB_2 signal was associated with the cytoplasmic compartment.

CB_2 receptor internalization and trafficking following exposure to THC

In order to assess trafficking between extracellular and intracellular CB_2 receptors, we employed two complementary approaches to assess for ligand-induced receptor internalization. Using the 293T/CB_2-GFP cell line as a model, we assessed changes in expression of extracellular CB_2 in response to treatment with THC. Incubating cells with a 4 μM concentration of THC for up to 80 min at 37 °C was associated with a time-dependent decrease in cell surface CB_2 expression (Fig. 3a). Similarly, exposing cells for 40 min (at 37 °C) to increasing concentrations of THC from 0 to 8 μM resulted in a concentration-dependent decrease in surface staining by anti-CB_2 mAb (Fig. 3b). THC-dependent changes did not occur when cells were maintained at 4 °C (data not shown) confirming an energy-dependent process.

In a second approach, imaging flow cytometry was used to assess the impact of THC exposure on receptor location (Fig. 3c). Viable 293T/CB_2-GFP cells were stained with anti-CB_2 mAb and secondary APC-labeled GAM and then incubated at 37 °C with either 8 μM THC or diluent alone for 40 min. While conventional flow cytometry demonstrated no change in overall fluorescent signal (data not shown), fluorescent imaging demonstrated trafficking and coalescence of the fluorescent signal within the cytoplasm in response to THC. Cells labeled with anti-CB_2 mAb and incubated at 37 °C, in the absence of THC, did show evidence of antibody-induced capping and early vacuolization, but extensive trafficking and coalescence of the CB_2 label within the cytoplasm occurred only in the presence of THC. This visual assessment was confirmed by using quantitative measurement of the intracellular to extracellular fluorescent ratios for the two conditions, which demonstrated a significant intracellular shift in response to THC.

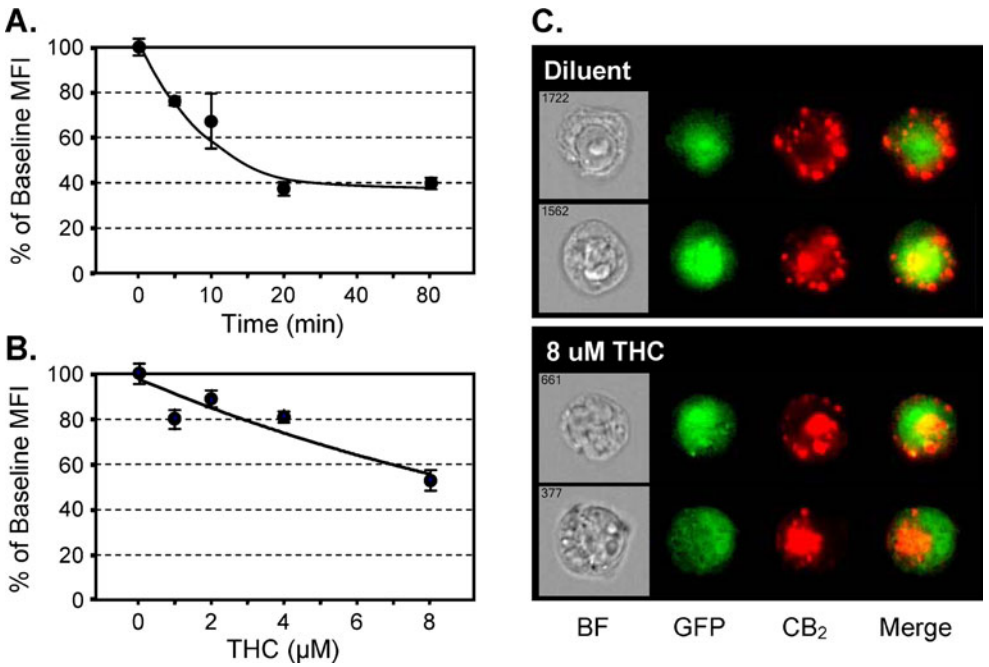


Fig. 3 CB_2 receptor internalization and trafficking following exposure to THC. Viable 293T/CB_2-GFP cells were incubated in X-VIVO media at 37 °C with (**a**) 4 μM THC for different time intervals or (**b**) with increasing concentrations of THC (0–8 μM) for 40 min. After the defined incubations, reactions were quenched with excess ice-cold PBS, cells incubated with 0.25 μg of CB_2 or NK1.1 mAb for 30 min on ice, and then stained with APC-labeled GAM for detection. Data for both assays are expressed as the percentage of baseline expression (MFI), which was obtained from cells maintained at 4 °C with exposure to diluent alone throughout the entire assay. Graphs display the mean (± SE) for three independent experiments, each performed in triplicate. **c** Imaging flow cytometry was also employed to assess CB_2 receptor internalization. Cells were first incubated at 4 °C with CB_2 or NK1.1 mAb, followed by APC-labeled GAM for detection, and then exposed to either diluent control (top panel) or 8 μM THC (bottom panel) for 40 min at 37 °C. Reactions were then quenched with excess cold PBS, cells fixed, and analyzed within 48 h by imaging flow cytometry. Image sets were obtained from two representative cells expressing the mean levels of GFP and CB_2 expression. Bright field (BF), GFP fluorescence, APC fluorescence, and merged fluorescence images are shown

Distribution of CB_2 expression in peripheral blood leukocytes (PBL)

Having validated the capacity for flow cytometry to assess cell surface and total cellular CB_2 expression in cell lines, we assessed whether this approach could detect CB_2 expression in primary human PBL. Blood samples were obtained from healthy non-smoking subjects in order to avoid any impact of exogenous THC exposure on receptor expression. As demonstrated in Fig. 4a, cell surface staining with the anti-CB_2 mAb was only observed in B cells. There was no difference in fluorescence staining between the anti-NK1.1 and anti-CB_2 mAb when T cells (including both the CD4 and CD8 subsets; data not shown) and monocytes were examined.

However, the CB_2 expression pattern was entirely different after fixation and permeabilization (Fig. 4b). In addition to B cells, a fluorescent signal for CB_2 was detected in all T cells and monocytes. In contrast to studies with 293T/CB_2-GFP cells where fixation and permeabilization was associated with a decrease in the MFI for CB_2 staining, there was a marked increase in CB_2 fluorescent intensity when B cells were stained after fixation and permeabilization. This finding would suggest that a much higher percentage of CB_2 protein is expressed in the cytoplasm of B cells as compared to expression on the cell surface. Furthermore, while T cells failed to exhibit any CB_2 expression on their cell surface, they exhibited high levels of intracellular fluorescence. Intracellular staining of monocytes was also consistently positive for cytoplasmic CB_2 protein, and there were no consistent differences in the level of expression between B cells, T cells, and monocytes. The fluorescent staining intensity exhibited by these different subsets, broken down into cell surface staining and total cellular staining, are summarized in Table 1. Visual confirmation of antibody binding location was obtained using the ImageStreamX® imaging cytometer (Fig. 5). As in our cell lines, intracellular staining revealed diffuse cytoplasmic staining in B cells, T cells, and monocytes. There was no fluorescent signal when cells were stained with anti-NK1.1 mAb.

As had been observed when cells from the 293T/CB_2-GFP line were exposed to THC, the extracellular expression of CB_2 by CD20+ B cells was also down-regulated when PBMC were exposed to THC in the range of 0.5 to 2.0 μM (results not shown). No change was observed for monocytes or T cells, which did not demonstrate extracellular CB2 staining under any conditions.

Table 1 Expression of CB_2 by human PBL subsets

	Extracellular		Permeabilized	
	CB_2[a]	NK1.1	CB_2	NK1.1
B cells[b]	46.5±6.1[*]	3.9±0.4	91.0±15.2 [*, **]	2.4±1.1
T cells	4.2±0.6	4.1±0.5	80.4±18.7 [*, **]	2.3±1.3
Monocytes	5.9±0.5	5.5±0.5	68.2±14.3 [*, **]	4.8±2.4

[a] Human PBL were stained for cell membrane expression (extracellular protocol) or total cellular expression (permeabilized protocol) with either unlabeled anti-CB_2 or anti-NK1.1 mAbs, followed by detection with fluorescent-labeled goat anti-mouse $F(ab')_2$, and analyzed by flow cytometry. Results reported as MFI±SE, n=4 paired experiments

[b] Lineage-specific markers were used to identify B cells (CD20+), T cells (CD3+), and monocytes (CD13+ and/or CD14+)

[*] $p<0.05$ comparing CB_2 expression to NK1.1 expression

[**] $p<0.05$ comparing CB_2 expression detected by the extracellular versus permeabilized staining protocols

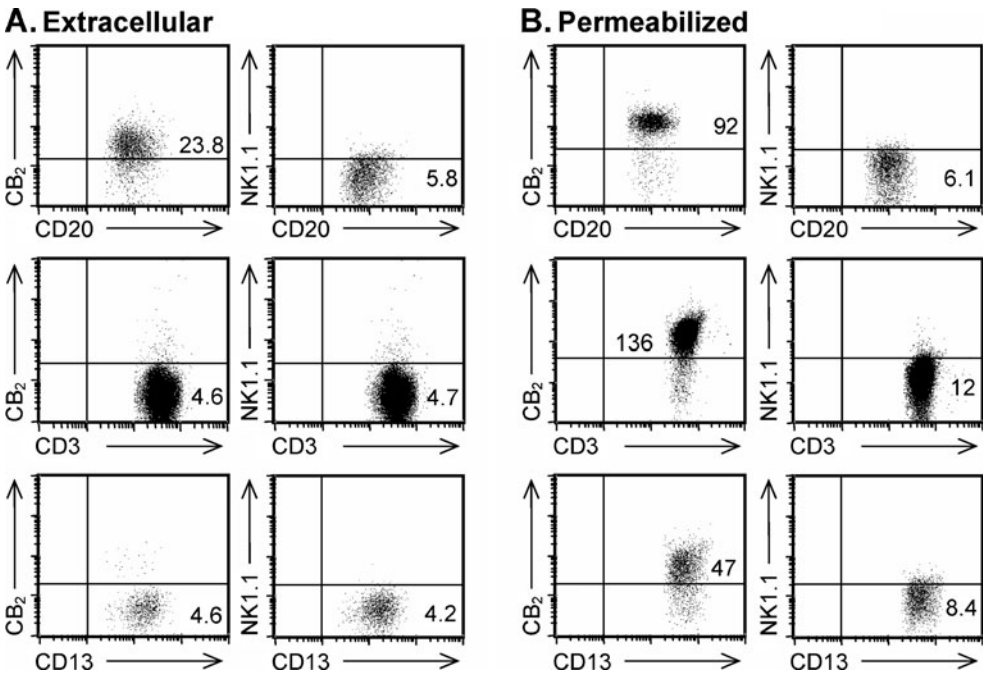


Fig. 4 Distribution of CB_2 expression in peripheral blood leukocytes (PBL). PBL were purified by density gradient centrifugation, washed, and then stained while still viable for (**a**) detection of extracellular CB_2 +or (**b**) following fixation and permeabilization for detection of total cellular CB_2 expression as previously described in Fig. 2, followed by counterstaining with lineage-specific fluorescent-conjugated antibodies for detection of CD20+ B cells (top), CD3+ T cells (middle), and CD13+ monocytes (bottom). MFI values for each population are listed. Representative experiment is shown, n=4

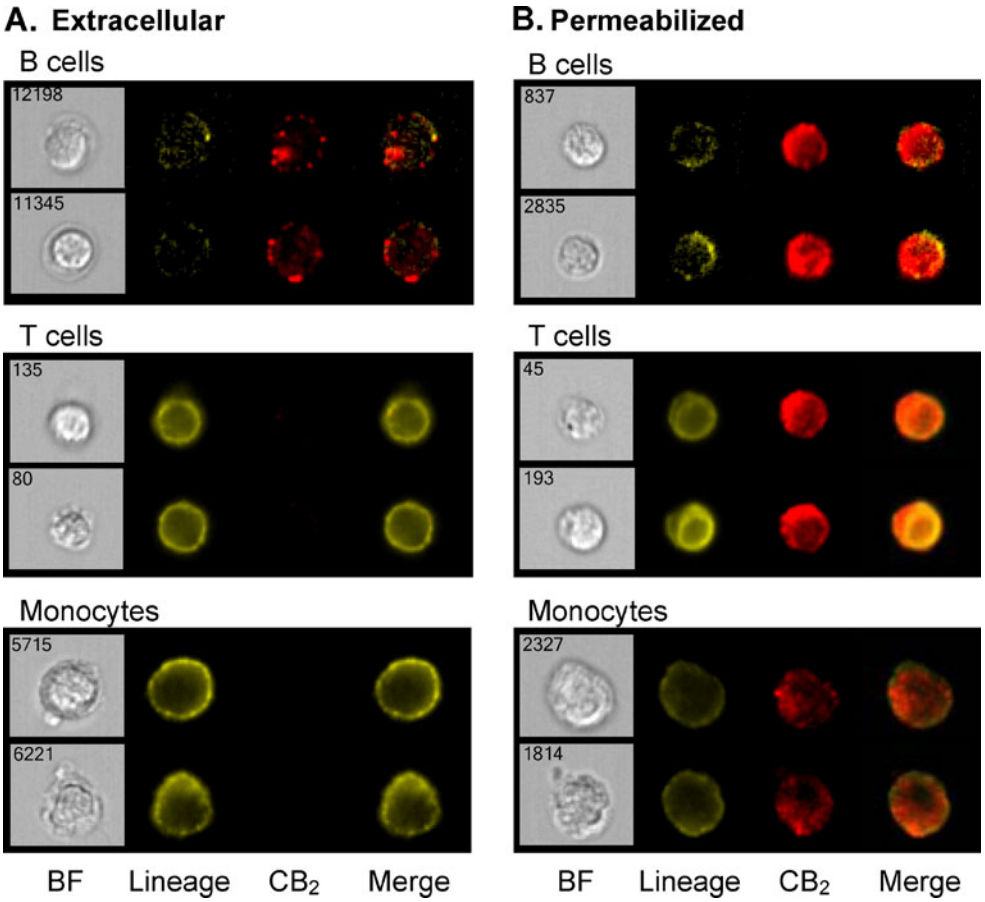


Fig. 5 Fluorescent imaging confirms differential staining pattern in human PBL subsets. **a** Viable or (**b**) fixed and permeabilized human PBL that had been stained as detailed in Fig. 4 were fixed with 1 % paraformaldehyde and analyzed by imaging flow cytometry within 48 h. Gating was used to select for CD20+ B cells (top panels), CD3+ T cells (middle panels), and CD13+ monocytes (bottom panels) with image sets shown from two representative cells captured by imaging flow cytometry. Images were selected from cells expressing the mean GFP and mean CB_2 expression for each gated population. Bright field (BF), GFP fluorescence, APC fluorescence, and merged fluorescence images are shown

Quantitative real-time RT-PCR confirms the expression of CB_2 mRNA which correlates with total cellular CB_2 protein levels

Expression of CB_2 protein by flow cytometry was correlated with mRNA expression using a quantitative real-time RT-PCR assay. Labeled probes for CB_2 and a housekeeping gene, GAPDH, were quantitated simultaneously in the same well, allowing the relative level of CB_2 expression to be described by the relative differences in cycle times ($1/\Delta$-CT). Immunomagnetic selection was used to isolate B cell, T cell, and monocyte subsets with purity confirmed by flow cytometry at an average of 83.2±5.3 % for B cells, 96.5± 3.5 % for T cells, and 94.5±2.4 % for monocytes (Fig. 6a–b, n=4 separations). Similar to the results for total cellular CB_2 expression, all of these subsets expressed similar levels of CB_2 mRNA with no statistically-significant difference noted between groups (Fig. 6c).

Discussion

The CB_2 gene was cloned from a human leukemia cell line in 1993 and found to encode for a GPCR that bound cannabinoids with high affinity, but unlike CB_1, it was expressed primarily in lymphoid organs by lymphocytes, monocytes, and polymorphonuclear cells (Bouaboula et al. 1993; Galiègue et al. 1995; Munro et al. 1993; Schatz et al. 1997). The functional consequences of cannabinoids on immunity have turned out to be extensive with the capacity to regulate chemotaxis, phagocytosis, bacterial killing, antigen processing and presentation, T cell activation and cytokine production, and B cell differentiation and isotype switching (Basu and Dittel 2011; Klein 2005; Klein and Cabral 2006). This has led to considerable interest in developing therapeutic drugs based on their interaction with CB_2 receptor (Guindon and Hohmann 2008; Klein 2005; Mackie 2006). However, there is relatively little information regarding the expression and distribution of CB_2 protein on target cells. In this study, we constructed cell lines expressing different levels of human CB_2 and used a commercial anti-CB_2 mAb to develop a sensitive and specific flow cytometry assay for detecting CB_2 protein. This mAb was developed using gene-modified cells expressing full length human CB_2 as the immunogen. It readily detects CB_2 expressed on the cell membrane, and in our hands, cell staining was not blocked by pre-incubation with a 50-mer N-terminal peptide (results not shown), suggesting that it may be directed against one of the extracellular loops of the GPCR structure. With its high throughput and the capacity for multiplexing, this assay should provide an important tool for

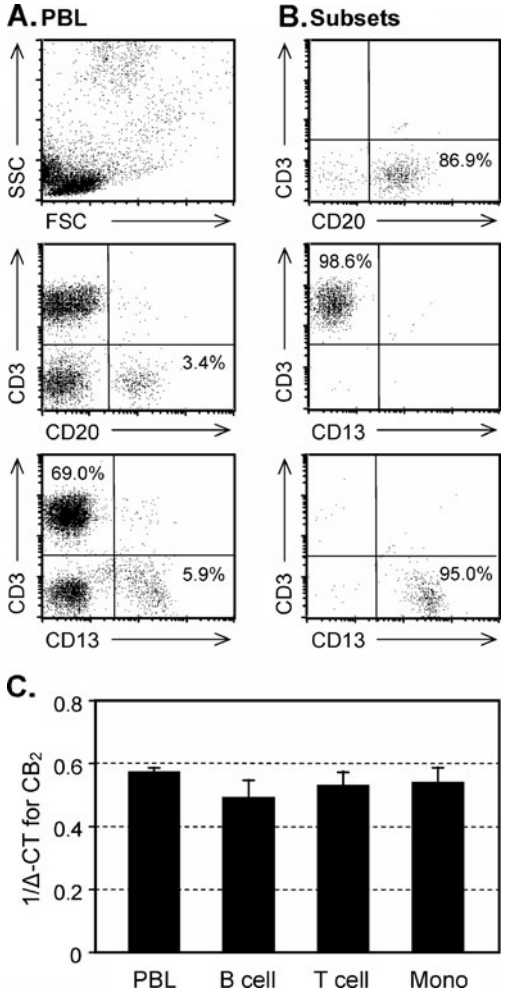


Fig. 6 CB_2 mRNA levels from purified human B cells, T cells, and monocytes. **a** Human PBL isolated by density gradient centrifugation and analyzed with lineage-specific markers to identify the B cell, T cell, and monocyte subsets, and their respective percentages within the entire PBL sample. **b** Each subset was then individually purified by immunomagnetic depletion with the final purity determined by flow cytometry using lineage-specific makers. Dot plots are from a representative experiment with the average purity from 5 different isolations being 83.2±5.3 % for B cells, 96.5±3.5 % for T cells, and 94.5±2.4 % for monocytes. **c** cDNA was prepared from 2×10^5 PBL or the purified subsets using a FastLane cDNA kit and quantitative real-time RT-PCR for all cells isolated from a single donor carried out in triplicate using labeled probes for CB_2 and GAPDH. Relative expression of CB_2 between groups is displayed as 1/Δ-CT representing average data from triplicate determinations for 3 different normal donors. No significant differences were identified between groups

probing CB_2 receptor status in cells of interest. More importantly, with the addition of cell permeabilization and imaging flow cytometry, our findings challenge the longstanding notion that CB_2 functions primarily as a cell surface receptor (Basu and Dittel 2011; Dainese et al. 2010). GPCR have the capacity to traffic between different cell compartments where they can interact with different adaptor proteins and signaling pathways (Jean-Alphonse and Hanyaloglu 2011). When examining primary human B cells, our studies identified CB_2 protein at both extracellular and intracellular locations. However, while B cells, T cells, and monocytes expressed similar levels of CB_2 mRNA, CB_2 protein expression was restricted entirely to intracellular sites in T cells and monocytes. In an analogous manner, a number of research groups have recently described a primary intracellular distribution of CB_1 protein within different sets of neurons (Rozenfeld 2011). Cannabinoids are highly lipophilic molecules, and it has been shown that both extracellular and intracellular CB_1 receptors can mediate signaling and biologic responses when exposed to ligands (Brailoiu et al. 2011; Rozenfeld 2011; Thomas et al. 1990). Others have also recently begun to evaluate CB_2 receptor internalization and trafficking (Atwood et al. 2012; Grimsey et al. 2011). Using gene-modified cell lines and epitope-tagged CB_2 molecules, a complex relationship between CB_2 ligand exposure, receptor internalization, and cell signaling has been reported (Atwood et al. 2012). In this setting, our results suggest a similar paradigm for the expression of native human CB_2 by PBL and make it likely that the differential expression of CB_2 at extracellular and intracellular sites plays an important role in the immune responses to cannabinoids. This differential expression of CB_2 may also be linked to the variety of signaling pathways that have been associated with CB_2 activation (Howlett 2005).

While others have used flow cytometry to evaluate CB_2 receptor expression on cells (Carayon et al. 1998; Cencioni et al. 2010; Graham et al. 2010), there are several features which distinguish our assay from past studies with human PBL. Carayon and associates (Carayon et al. 1998) generated and purified polyclonal rabbit anti-CB_2 antibody directed against the C-terminal (intracellular tail portion) of human CB_2. As fixation and permeabilization were required for antigen detection, their approach precluded a comparison between extracellular and intracellular staining. A fluorescent signal was detected from stained B cells and was inhibited by excess peptide, but the findings were much less convincing with respect to the staining of other cell types. More recently, Graham and coworkers (Graham et al. 2010) evaluated polyclonal antibodies from several commercial manufacturers and reported that human B cells, T cells, monocytes, NK cells, and polymorphonuclear cells all express high levels of extracellular CB_2. However, the staining patterns in their report were highly-variable from manufacturer to manufacturer and from batch to batch. Furthermore, in the absence of appropriate control antibodies or the inclusion of known positive and negative controls, one cannot really draw conclusions about sensitivity and specificity. Based on these concerns we focused on a defined mAb with the ability to detect extracellular CB_2 expression. In order to optimize and validate staining patterns, we constructed cell lines expressing defined levels of human CB_2 (A549/CB_2-GFP and 293T/CB_2-GFP) and compared staining patterns to those observed with parental cells (A549 and 293T). During the optimization process, it was obvious that non-specific background staining could easily be mistaken for receptor

expression if antibodies were not carefully titrated and appropriate isotype controls employed. By including the expression of a linked GFP reporter gene in our vector construct, we also possessed a mechanism for independently assessing expected CB_2 staining patterns. Perhaps the most important technical advancement was the inclusion of both intracellular and extracellular staining protocols. In this respect, our studies were also aided by the use of an ImageStreamX® cytometer. Due to the impact of fixation and permeabilization on antibody staining, we could not use MFI to directly compare extracellular and intracellular protein levels by conventional flow cytometry. However, visual inspection of captured images readily identified the cytoplasmic compartment as the primary source of our CB_2 signal. Imaging also allowed us to independently confirm the process of receptor internalization in response to ligand exposure. Given the controls and approaches employed, there should be little doubt regarding the performance characteristics of this flow cytometry approach.

In summary, we describe a rapid and flexible approach for detecting and localizing human CB_2 protein expression in cell lines and primary human cells. This approach uses commercially available reagents and should have wide applicability. In addition, for the first time, we report that CB_2 receptor is primarily located at intracellular sites in PBL and that expression is not limited to the cell membrane as previously thought. Even in B cells, which express both extracellular and intracellular CB_2, the majority of receptor protein is located within the cell. Our findings and related investigations carried out with CB_2 suggest that there is trafficking between receptor locations and that intracellular receptors are likely to be biologically active. Future studies focused on understanding the role of differential CB_2 receptor location on cannabinoid function are warranted.

Acknowledgments We thank Benjamin Alderete and Raymond Kong for their assistance with ImageStreamX® fluorescent imaging and analysis services that were graciously provided by Amnis Corporation, EMD Millipore, Seattle, WA. This work was supported by National Institutes of Health, National Institute on Drug Abuse [Grant R21-DA021813, R01-DA03018]. (M.D.R.). Flow cytometry was performed utilizing the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility supported by National Institutes of Health [Grant CA-16042, AI-28697]; the Jonsson Comprehensive Cancer Center, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA. Julie T. Castaneda was supported by institutional awards through the UCLA Competitive Edge, E. Eugene Cota Robles, and Graduate Mentored Research programs. The authors declare that they have no conflict of interest.

References

Agudelo M, Newton C, Widen R, Sherwood T, Nong L, Friedman H, Klein TW (2008) Cannabinoid receptor 2 (CB_2) mediates immunoglobulin class switching from IgM to IgE in cultures of murine-purified B lymphocytes. J Neuroimmune Pharmacol 3:35–42

Atwood B, Wager-Miller J, Haskins C, Straiker A, Mackie K (2012) Functional selectivity in CB_2 receptor signaling and regulation: implications for the therapeutic potential of CB_2 ligands. Mol Pharmacol 81:250–263

Basu S, Dittel BN (2011) Unraveling the complexities of cannabinoid receptor 2 (CB_2) immune regulation in health and disease. Immunol Res 51:26–38

Bouaboula M, Rinaldi M, Carayon P, Carillon C, Delpech B, Shire D, Le Fur G, Casellas P (1993) Cannabinoid-receptor expression in human leukocytes. Eur J Biochem 214:173–180

Brailoiu GC, Oprea TI, Zhao P, Abood ME, Brailoiu E (2011) Intracellular CB_1 cannabinoid receptors are activated by anandamide. J Biol Chem 286:29166–29174

Carayon P, Marchand J, Dussossoy D, Derocq JM, Jbilo O, Bord A, Bouaboula M, Galiegue S, Mondiere P, Penarier G, Le Fur G, Defrance T, Casellas P (1998) Modulation and functional involvement of CB_2 peripheral cannabinoid receptors during B-cell differentiation. Blood 92:3605–3615

Cencioni MT, Chiurchiù V, Catanzaro G, Borsellino G, Bernardi G, Battistini L, Maccarrone M (2010) Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB_2 receptors. PLoS One 5:e8688

Dainese E, Oddi S, Maccarrone M (2010) Interaction of endocannabinoid receptors with biological membranes. Curr Med Chem 17:1487–1499

Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS (2004) Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. J Immunol 173:2373–2382

Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur J Biochem 232:54–61

Gorantla S, Makarov E, Roy D, Finke-Dwyer J, Murrin LC, Gendelman HE, Poluektova L (2010) Immunoregulation of a CB_2 receptor agonist in a murine model of neuroAIDS. J Neuroimmune Pharm 5:456–468

Graham ES, Angel CE, Schwarcz LE, Dunbar PR, Glass M (2010) Detailed characterisation of CB_2 receptor protein expression in peripheral blood immune cells from healthy human volunteers using flow cytometry. Int J Immunopathol Pharmacol 23:25–34

Grimsey NL, Goodfellow CE, Dragunow M, Glass M (2011) Cannabinoid receptor 2 undergoes Rab5-mediated internalization and recycles via a Rab11-dependent pathway. Biochim Biophys Acta 1813:1554–1560

Guindon J, Hohmann AG (2008) Cannabinoid CB_2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. Br J Pharmacol 153:319–334

Howlett AC (2005) Cannabinoid receptor signaling. Handb Exp Pharmacol 168:53–79

Jean-Alphonse F, Hanyaloglu AC (2011) Regulation of GPCR signal networks via membrane trafficking. Mol Cell Endocrinol 331:205–214

Klein TW, Newton CA, Nakachi N, Friedman H (2000) Delta 9-tetrahydrocannabinol treatment suppresses immunity and early IFN-gamma, IL-12, and IL-12 receptor beta 2 responses to Legionella pneumophila infection. J Immunol 164:6461–6466

Klein TW (2005) Cannabinoid-based drugs as anti-inflammatory therapeutics. Nat Rev Immunol 5:400–411

Klein TW, Cabral GA (2006) Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. J Neuroimmune Pharm 1:50–64

Mach F, Steffens S (2008) The role of the endocannabinoid system in atherosclerosis. J Neuroendocrinol 20:S53–S57

Mackie B (2006) Cannabinoid receptors as therapeutic targets. Annu Rev Pharmacol Toxicol 46:101–122

Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreakos E, Mechoulam R, Feldmann M (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. PNAS 97:9561–9566

McKallip RJ, Lombard C, Martin BR, Nagarkatti M, Nagarkatti PS (2002) Delta(9)-tetrahydrocannabinol-induced apoptosis in the thymus and spleen as a mechanism of immunosuppression in vitro and in vivo. J Pharmacol Exp Ther 302:451–465

McKallip RJ, Nagarkatti M, Nagarkatti PS (2005) Delta-9-tetrahydrocannabinol enhances breast cancer growth and metastasis by suppression of the antitumor immune response. J Immunol 174:3281–3289

Mechoulam R (1986) The pharmacohistory of Cannabis sativa. In: Mechoulam R (ed) Cannabinoids as therapeutic agents. CRC Press, Boca Raton, pp 1–19

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61–65

Newton CA, Chou PJ, Perkins I, Klein TW (2009) CB(1) and CB(2) cannabinoid receptors mediate different aspects of delta-9-tetrahydrocannabinol (THC)-induced T helper cell shift following immune activation by Legionella pneumophila infection. J Neuroimmune Pharm 4:92–102

Pertwee RG (2006) Cannabinoid pharmacology: the first 66 years. Br J Pharmacol 147:S163–S171

Roth MD, Tashkin DP, Whittaker KM, Choi R, Baldwin GC (2005) Tetrahydrocannabinol suppresses immune function and enhances HIV replication in the huPBL-SCID mouse. Life Sci 77:1711–1722

Rozenfeld R (2011) Type I cannabinoid receptor trafficking: all roads lead to lysosome. Traffic 12:12–18

Sarafian T, Montes C, Harui A, Beedanagari SR, Kiertscher S, Stripecke R, Hossepian D, Kitchen C, Kern R, Belperio J, Roth MD (2008) Clarifying CB_2 receptor-dependent and independent effects of THC on human lung epithelial cells. Toxicol Appl Pharmacol 231:282–290

Schatz AR, Lee M, Condie RB, Pulaski JT, Kaminski NE (1997) Cannabinoid receptors CB_1 and CB_2: a characterization of expression and adenylate cyclase modulation within the immune system. Toxicol Appl Pharmacol 142:278–287

Shay A, Choi R, Whittaker K, Salehi K, Tashkin DP, Roth MD, Baldwin GC (2003) Impairment of antimicrobial activity and nitric oxide production in alveolar macrophages from smokers of marijuana and cocaine. J Infect Dis 187:700–704

Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessegue B, Bonnin-Cabanne O, Le Fur G, Caput D, Ferrara P (1996) Molecular cloning, expression and function of the murine CB_2 peripheral cannabinoid receptor. Biochim Biophys Acta 1307:132–136

Sipe JC, Arbour N, Gerber A, Beutler E (2005) Reduced endocannabinoid immune modulation by a common cannabinoid 2 (CB_2) receptor gene polymorphism: possible risk for autoimmune disorders. J Leukoc Biol 78:231–238

Srivastava MD, Srivastava BIS, Brouhard B (1998) Δ^9 Tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. Immunopharmacology 40:179–185

Thomas BF, Compton DR, Martin BR (1990) Characterization of the lipophilicity of natural and synthetic analogs of delta 9-tetrahydrocannabinol and its relationship to pharmacological potency. J Pharmacol Exp Ther 255:624–630

Yuan M, Kiertscher SM, Cheng Q, Zoumalan R, Tashkin DP, Roth MD (2002) Δ^9-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T-cells. J Neuroimmunol 133:124–131

Zhu LX, Sharma S, Stolina M, Gardner B, Roth MD, Tashkin DP, Dubinett SM (2000) Delta-9-tetrahydrocannabinol inhibits antitumor immunity by a CB_2 receptor-mediated, cytokine-dependent pathway. J Immunol 165:373–380

This work was presented in abstract form at the 18th Scientific Conference of the Society on NeuroImmune Pharmacology, April 26, 2012, Honolulu, HI.

J Neuroimmune Pharmacol (2015) 10:333–343
DOI 10.1007/s11481-015-9587-z

ORIGINAL ARTICLE

Exposure to Δ9-Tetrahydrocannabinol Impairs the Differentiation of Human Monocyte-derived Dendritic Cells and their Capacity for T cell Activation

Michael D. Roth · Julie T. Castaneda · Sylvia M. Kiertscher

Received: 5 December 2014 / Accepted: 14 January 2015 / Published online: 23 January 2015

Abstract The capacity for human monocytes to differentiate into antigen-presenting dendritic cells (DC) can be influenced by a number of immune modulating signals. Monocytes express intracellular cannabinoid type 1 (CB1) and 2 (CB2) receptors and we demonstrate that exposure to Δ9-tetrahydrocannabinol (THC) inhibits the forskolin-induced generation of cyclic adenosine monophosphate in a CB2-specific manner. In order to examine the potential impact of cannabinoids on the generation of monocyte-derived DC, monocytes were cultured in vitro with differentiation medium alone [containing granulocyte/macrophage-colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4)] or in combination with THC. The presence of THC (0.25–1.0 μg/ml) altered key features of DC differentiation, producing a concentration-dependent decrease in surface expression of CD11c, HLA-DR and costimulatory molecules (CD40 and CD86), less effective antigen uptake, and signs of functional skewing with decreased production of IL-12 but normal levels of IL-10. When examined in a mixed leukocyte reaction, DC that had been generated in the presence of THC were poor T cell activators as evidenced by their inability to generate effector/memory T cells or to stimulate robust IFN-γ responses. Some of these effects were partially restored by exposure to exogenous IL-7 and bacterial superantigen (*S. aureus* Cowans strain). These studies demonstrate that human monocytes express functional cannabinoid receptors and suggest that exposure to THC can alter their differentiation into functional antigen presenting cells; an effect that may be counter-balanced by the presence of other immunoregulatory factors. The impact of cannabinoids on adaptive immune responses in individuals with frequent drug exposure remains to be determined.

Keywords Δ9-tetrahydrocannabinol · Cannabinoid receptor · Monocyte · Dendritic cell · T cell activation · Cytokines

M. D. Roth (✉) · S. M. Kiertscher
Department of Medicine, Division of Pulmonary and Critical Care, David Geffen School of Medicine at UCLA, CHS 37-131, Los Angeles, CA 90095-1690, USA
e-mail: mroth@mednet.ucla.edu

J. T. Castaneda
Department of Medicine, Interdepartmental Program in Molecular Toxicology and the Division of Pulmonary and Critical Care, David Geffen School of Medicine at UCLA, CHS 37-131, Los Angeles, CA 90095-1690, USA

Introduction

The expression of cannabinoid receptors by human leukocytes suggests that both endogenous ligands and inhaled marijuana smoke might exert immunoregulatory properties that are distinct from their effects on the brain (Klein and Cabral 2006; Klein et al. 2005). Furthermore, while brain cells exclusively express cannabinoid receptor type 1 (CB1), leukocytes express both CB1 and CB2, with CB2 reported as the predominant subtype (Bouaboula et al. 1993, Munro et al. 1993; Nong et al. 2002). Both CB1 and CB2 are transmembrane G-protein coupled receptors that inhibit the generation of cyclic adenosine monophosphate (cAMP) and can signal through a variety of pathways including PI3-kinase, MAP kinase, NF-κB, AP-1, and NF-AT (Basu and Dittel 2011; Bosier et al. 2010). The resulting effects on host immunity have primarily been studied in animal models and suggest a coordinated down-regulation of cellular responses that can occur through altered trafficking, selective apoptosis, or functional skewing of antigen presenting cells and T cells away from T helper type 1 (Th1) or Th17 response patterns (Klein

et al. 2000; Zhu et al. 2000; Nagarkatti et al. 2009; Rieder et al. 2010; Karmaus et al. 2013; Kong et al. 2014). Similar results have been observed when purified human T cells are stimulated in vitro in the presence of Δ9-tetrahydrocannabinol (THC) (Yuan et al. 2002). However, the extent to which the effects are observed in humans in vivo is unclear. Daily administration of marijuana or oral THC to research subjects in a prospective and randomized study had no obvious effect on T cell proliferation or cytokine production when blood cells were subsequently isolated and stimulated in vitro (Bredt et al. 2002). Sipe et al. (2005) examined the distribution and function of a common polymorphism in the human CB2 gene associated with the replacement of a glutamine by an arginine at amino acid position 63. Functionally, lymphocytes from subjects with either of these genotypes proliferated normally when stimulated with anti-CD3 antibody. However, when stimulated in the presence of an endocannabinoid, lymphocytes expressing the glutamine residue at position 63 were markedly inhibited while those expressing the arginine were only modestly suppressed. The arginine substitution also correlated with the prevalence of autoimmune disease in the subjects tested. Collectively, this body of work suggests that cannabinoids are biologically active immune regulators in humans.

Expanding upon this hypothesis, we examined the expression of cannabinoid receptors by human monocytes and the impact of THC on their differentiation into monocyte-derived dendritic cells (DC). Exposing monocytes to THC blocked many of the features normally associated with their differentiation into functional DC and impaired their capacity for T cell activation. Furthermore, the T cell activation that did occur was associated with a change in T cell phenotype and cytokine secretion. However, the impact of THC was partially overcome when DC and T cells were exposed to a combination of activation signals and exogenous cytokines. Our findings suggest that cannabinoids are capable of altering the differentiation and activation of cells involved in human cell-mediated immunity.

Materials and Methods

Primary Cells and Cell Lines

Human peripheral blood was obtained from healthy volunteers according to a protocol approved by the UCLA Institutional Review Board. Mononuclear cells (PBMC) were isolated by ficoll density gradient centrifugation. Chinese hamster ovary (CHO) cells were transfected with a plasmid encoding for human CB2 and transfectants (CHO-CB2) selected by growth in Kaighn's F-12 medium containing 0.2 mg/ml G418 (Calbiochem, San Diego, CA).

Reagents and Antibodies

THC and SR144528 (selective CB2 antagonist) were provided by the National Institute on Drug Abuse (NIDA, Bethesda, MD). JWH-015 (selective CB2 agonist) was obtained from Enzo Life Sciences (Farmingdale, NY). All cannabinoids were solubilized in ethanol and diluted serially in DMSO and then culture medium prior to use (final ethanol concentration≤0.01% and DMSO≤0.125%). Interleukin (IL)-10, and IL-12 ELISA kits, fluorescent-labeled monoclonal antibodies (mAbs) directed against CD11c, CD13, CD14, CD40, CD45RA, CD86, and HLA-DR, and mAbs used for cell depletion and purification were all from BD-Biosciences (San Jose, CA). Monoclonal anti-CB2 and fluorescent-labeled anti-CB1 antibodies and recombinant human IL-4, IL-7, IL-12 and IL-15 were from R&D Systems (Minneapolis, MN). APC-labeled goat anti-mouse $F(ab')_2$ mAb, fluorescent-labeled anti-CD25 antibody and fluorescein-labeled dextran (FITC-dextran, MW 40,000) were purchased from Invitrogen (Carlsbad, CA). Granulocyte/macrophage-colony stimulating factor (GM-CSF) was obtained from Berlex Laboratories, Inc. (Richmond, CA).

Preparation of Monocytes, DC and T cells

Human monocytes were prepared from PBMC by immunomagnetic depletion (Miltenyi Biotec, Auburn, CA) and T cells were purified using a combination of mAb (anti-CD14, anti-CD16, anti-CD19) and anti-mouse Ig-conjugated immunomagnetic beads (Dynal, Lake Success, NY). DC were differentiated from monocyte precursors by culturing adherent PBMC in X-VIVO 15 medium (Lonza; Walkersville, MD) supplemented with GM-CSF (800 U/ml) and IL-4 (100 to 500 U/ml) according to a standard protocol (Kiertscher and Roth 1996). The effects of THC on this differentiation process were assessed by adding THC (0.25 to 1.0 μg/ml) or diluent alone (containing ethanol/DMSO) 10 min before the addition of GM-CSF and IL-4. Dendritic cells were recovered from the flasks on day 7 and the expression of cell surface markers characterized by fluorescence-activated cell sorting (FACS) using a FACS Calibur® cytometer with CellQuest® analysis software (Becton Dickinson, San Jose, CA). For mixed leukocyte reactions (MLR) and cytokine assays, DC were further purified by depleting T cells, natural killer cells and B cells using lineage-specific mAb (anti-CD3, anti-CD19, anti-CD56) and immunomagnetic beads (Dynal).

Analysis of CB1 and CB2 Expression

For CB1 and CB2 mRNA expression, total cellular RNA was isolated using Rneasy mini-kits (Qiagen, Valencia, CA) and RT-PCR was carried out as detailed below or through a commercial vendor employing a quantitative RT^2 Profiler™ PCR

Array (Qiagen). Total RNA was reverse-transcribed using the cDNA Cycle® kit from Invitrogen (Carlsbad, CA) and 2 μl of the reverse transcription (RT) product used in a 20 μl RT-PCR reaction containing 0.4 mM dNTM mix, 2 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, PCR buffers and 0.5 μM each of the forward and reverse primers for either CB1 (5'-caccttccgcaccatcaccac-3'; 5'-gtctcccgcagtcatcttctcttg-3'), CB2 (5'-catggaggaatgctgggtgac-3'; 5'-gaggaaggcgatgaacaggag-3') or β-actin (5'-tgatggtgggcatgggtcag-3'; 5'-gtgttggcgtacaggtcttt-3'), all from Invitrogen. RT-PCR cycling conditions for CB1 and CB2 included an initial 5 min denaturation @ 94 °C followed by 35 cycles consisting of 45 s @ 94 °C, 45 s @ 64 °C, and 1 min @ 72 °C, with a final extension for 7 min @72 °C. Cycling conditions for β-actin were similar except for the use of only 30 cycles and an annealing temperature of 62 °C. RT-PCR products were resolved on 2% agarose gels and imaged with a UV transilluminator and a Polaroid photodocumentation camera. Expression of β-actin was used to control for loading and signal intensities measured by densitometry using NIH Imager software (NIH, Bethesda, MD). Using this approach, serial two-fold dilutions of total RT product from CHO-CB2 cells demonstrated a linear relationship between dilution factor and signal intensity over an 8-fold range.

Cell surface and intracellular expression of CB1 and CB2 receptors were determined by FACS analysis as previously described (Castaneda et al. 2013). Briefly, cell surface CB2 was detected with unlabeled mouse mAb directed against human CB2, followed by APC-labeled goat anti-mouse $F(ab')_2$. Isotype-matched mAb against an irrelevant antigen (mouse NK1.1) was used as a control. Cell surface CB1 was measured by anti-CB1-PE, with anti-mouse NK1.1-PE serving as a negative isotype control. For the detection of intracellular CB1 and CB2 receptor, cells were fixed with 1 % paraformaldehyde/PBS (Sigma Aldrich, St. Louis, MO) and treated with permeabilizing solution (BD Biosciences) prior to staining with mAb.

Forskolin-Induced cAMP Assay

Functional coupling of cannabinoid receptors to G-protein activity was assessed by measuring forskolin-induced cAMP levels in CHO-CB2 cells and fresh human monocytes. CHO-CB2 cells were cultured overnight at 5×10^5 cells/well in a 6-well plate. The next day, DMSO was added (50 μM) and cells cultured for an additional 18 h. THC (0.5 μg/ml), JWH-015 (0.025 μM), the combination of SR144528 (1 μM) and THC (0.5 μg/ml), or diluent alone, were then added to the wells and incubated for 15 min prior to an 18 h stimulation with 50 μM forskolin (Sigma-Aldrich). For studies with monocytes, 5×10^6 PBMC were placed into each well of a 24-well plate and monocytes allowed to adhere for 2 h in X-VIVO-15 medium. Non-adherent cells were then removed, wells rinsed, and the remaining monolayer treated in a manner identical to that described for CHO-CB2 cells. For both cell types, supernatants were harvested at the end of the forskolin stimulation and stored at −80 °C until assayed in duplicate for cAMP using a standard competitive enzyme immunoassay kit (Cayman Chemicals; Ann Arbor, MI). The final reaction product was read in a plate reader at 405 nm (Spectra/SLT Lab Instruments; Salzburg, Austria) and the amount of cAMP determined by regression analysis.

DC Endocytosis and Cytokine-Secretion Assays

DC that had been generated in the presence or absence of THC (0.5 μg/ml) for 7 days were cultured with FITC-dextran (1 mg/ml) for 60 min at either 4 °C or 37 °C and the assay terminated by adding 3 ml of ice-cold PBS containing 0.1% azide. Cell pellets were extensively washed and the DC immediately analyzed for accumulation of intracellular fluorescence by FACS. The degree of endocytosis was determined by comparing the intracellular uptake at 37 °C to that at 4 °C (i.e., difference in mean fluorescence intensity). In order to evaluate their capacity for cytokine production, control and THC-treated DC were also cultured for an additional 48 h at 0.5×10^6 cells/ml in X-VIVO 15 medium supplemented with GM-CSF (800 U/ml), IL-4 (500 U/ml) and 20 μg/ml of heat-killed, formalin-fixed *Staphylococcus aureus* Cowan (SAC, Calbiochem) as a cytokine-inducing agent. Supernatants were harvested and replicate samples assayed for the concentration of IL-10 and IL-12 by cytokine-specific ELISA. Results from duplicate wells were analyzed using a microplate reader and automated regression software (Spectra/SLT).

MLR and Cytokine Assays

DC and THC-DC were evaluated for their capacity to activate T cells in a standard MLR assay (Kiertscher and Roth 1996). Allogeneic CD45RA+ T cells were isolated by negative selection with specific antibody (anti-CD14, anti-CD16, anti-CD19, anti-CD45RO) and immunomagnetic beads, then labeled using the Vybrant CDSE/CFSE Cell Tracer Kit (Invitrogen-Molecular Probes, Eugene, OR) according to the manufacturer's protocol. DC were cultured with 2×10^5 T cells at 1:50 DC:T cell ratios in X-VIVO 15 medium in 96 well round-bottom plates at 37 °C in a humidified CO_2 incubator. For some experiments, DC and THC-DC were matured by culture with 20 μg/ml SAC for 18–24 h prior to co-culture with the T cells. In other experiments, the co-cultures were supplemented with 2 ng/ml of either IL-7, IL-12 or IL-15. On day 5 of co-culture,

the T cells were collected and analyzed by FACS for proliferation (by CFSE dilution) and cell surface marker expression (by addition of marker-specific fluorescent antibodies). Cell-free supernatants were collected from the wells and assessed for cytokines by custom multiplex analysis (Aushon BioSystems, Billerica, MA). Each cytokine was measured in duplicate and represented as the average value±SD.

Statistical Analysis

Data from individual experiments are represented as the mean±SD for the indicated number of replicates. Pooled data from multiple experiments are represented as mean values or as a percentage of control, ± SE. Comparisons involving multiple groups were assessed by one-way ANOVA for the presence of an overall treatment effect at a level of $p<0.05$. If an overall difference was identified, then a post-hoc analysis was performed to compare individual groups of interest using either a paired or unpaired *t*-test as appropriate for the relationship between experimental groups. Statistically significant differences were determined by the presence of $p\leq0.05$ for single comparisons or with a Bonferroni correction for multiple comparisons.

Results

Human Monocytes Express Functional Cannabinoid Receptors

As an initial step in understanding the potential interaction between cannabinoids and human monocyte-derived DC, monocytes were evaluated for the expression of the CB1 and CB2 receptor subtypes by RT-PCR (Fig. 1a) and flow cytometry (Fig. 1b). RT-PCR studies were carried out on monocytes that had been purified to >90% purity by either negative depletion or fluorescent cell sorting. mRNA encoding for both CB1 and CB2 were detected, although expression of CB2 predominated whether analyzed by standard RT-PCR (Fig. 1b, representative experiment) or by an automated quantitative RT-PCR using cells from 4 different donors (average CB2:CB1 ratio=4.0; range =0.15 to 10.34).

Despite the presence of mRNA, standard flow cytometry failed to detect CB1 or CB2 receptor protein on the cell surface of monocytes even though antibodies were directed against their N-terminal epitopes. However, when cells were fixed and permeabilized, specific staining for both CB1 and CB2 was detected, consistent with the presence of intracellular protein (Fig. 1b). Intracellular background staining with isotype control mAb was minimal for CB1 but dimly-

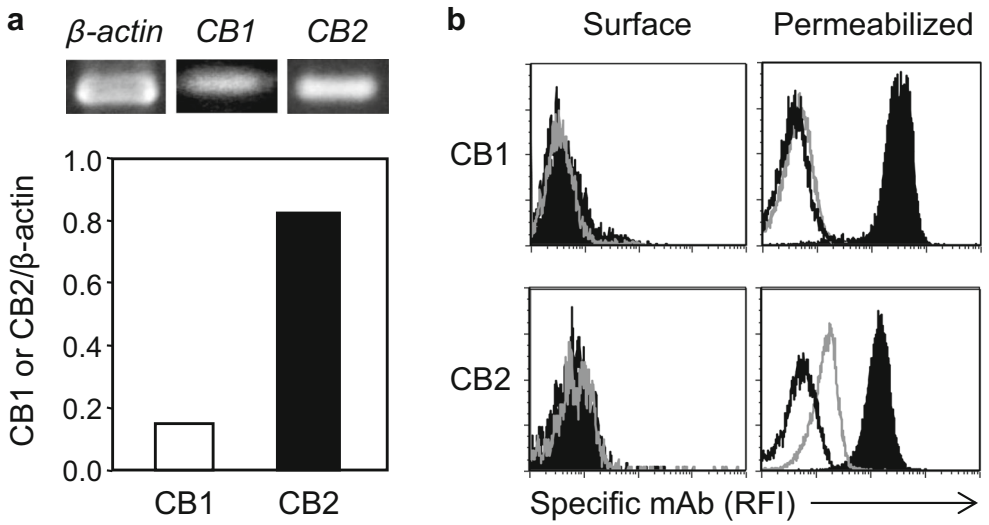


Fig. 1 Monocytes express high levels of CB2 mRNA and intracellular CB1 and CB2 protein. **a**: Peripheral blood monocytes were purified from PBMC by negative depletion using immunomagentic beads and the relative expression of CB1 and CB2 mRNA determined by semi-quantitative RT-PCR using densitometry and normalized for the expression of β-actin. Representative results from one of two determinations. **b**: mAbs specific for the N-terminal domains of CB1 and CB2 were used to identify expression of the corresponding proteins on the cell surface or at intracellular sites following permeabilization. Anti-CB1 was a fluorochrome-conjugated mAb while anti-CB2 was an unlabeled mAb detected by secondary stain with fluorochrome-conjugated goat-anti-mouse IgG antibody. For the intracellular analysis, cells were fixed with paraformaldehyde and permeabilized prior to mAb staining. Results are gated for CD13+ or CD14+ peripheral blood monocytes by flow cytometry and expressed as histograms with relative fluorescence intensity (RFI) on the X-axis. Black line (no fill)=unstained cells; Gray line (no fill)=isotype control mAb for CB1 (top panels) or isotype control mAb with secondary detection by GAM for CB2 (bottom panels); Black solid fill=staining by anti-CB1 (top panels) or anti-CB2 mAb with secondary detection by GAM (bottom panels). Representative results from one of 4 determinations

positive for CB2, likely reflecting the need for APC-labeled goat anti-mouse F(ab')$_2$ as a secondary detection reagent. Due to these differences in fluorescent labels and staining protocols, the relative fluorescent intensity for CB1 and CB2 cannot be directly compared as measures of receptor concentration.

The presence of functional CB2 receptor complexes was then assessed by measuring the impact of different cannabinoids on forskolin-induced generation of cAMP (Fig. 2a). Using CHO-CB2 cells as a model, we confirmed that treatment with THC (0.5 μg/ml=1.59 μM) significantly inhibited the generation of cAMP, as did JWH-015 (0.025 μM; selective CB2 agonist) at $p<0.01$. Furthermore, the inhibition of cAMP by THC was blocked by pretreatment with SR144528, a selective CB2 receptor antagonist ($p<0.01$). The same assays were repeated using purified human monocytes (Fig. 2b). Again, an overall CB2 agonist treatment effect was present. Pretreatment with either THC or JWH-015 inhibited the forskolin-induced generation of cAMP (68.0+4.2% and 58.3+5.7% of control levels, respectively) and the effects of THC were blocked by SR144528 ($p<0.01$ for all comparisons). While monocytes express both CB1 and CB2, the predominance of CB2 mRNA and the response of these cells to CB2-selective agents suggest that CB2 acts as the dominant cannabinoid signaling pathway.

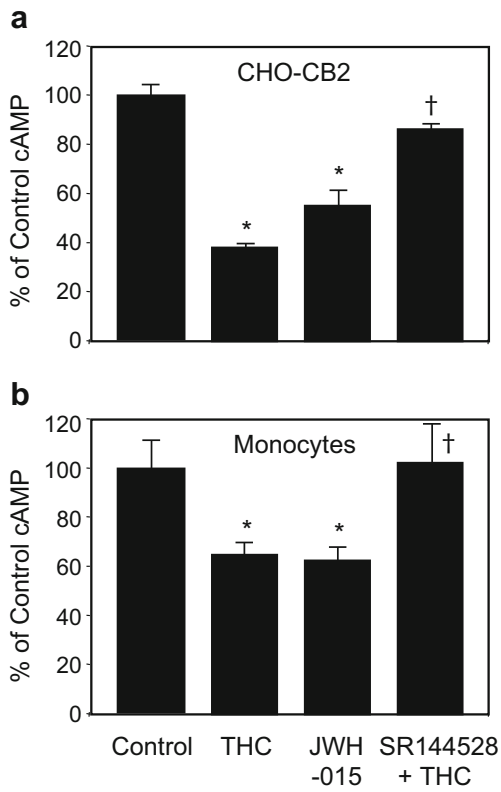


Fig. 2 CB2 receptors on monocytes are coupled to G_i proteins and activated by THC. CHO cells expressing human CB2 (CHO-CB2) (**a**) and adherent human monocytes (**b**) were pre-treated for 15 min with either diluent alone (control), THC (0.5 μg/ml), JWH-015 (0.025 μM), or the combination of SR144528 (1 μM) and THC (0.5 μg/ml), followed by an 18 h stimulation with forskolin (50 μM). Accumulation of cAMP in the culture supernatant was determined by a competitive enzyme immunoassay and presented as mean values±SD of replicate measurements. JWH-015 is a selective CB2 agonist and SR144528 a selective CB2 antagonist. *$p<0.01$ compared to control. †$p<0.01$ compared to THC treatment alone. Representative results from one of 4 experiments

Exposure to THC Alters the Phenotype of Monocyte-Derived DC

The differentiation of human monocytes into DC is associated with characteristic changes in cell surface proteins involved in antigen presentation (Kiertscher and Roth 1996). To evaluate the effects of THC on this aspect of differentiation, adherent PBMC were cultured for 7 days with GM-CSF and IL-4 and examined for the expression of typical monocyte and DC markers by flow cytometry (Fig. 3). Exposure to THC (0.25 to 1.0 μg/ml) did not prevent the normal down-regulation of CD14, but did inhibit the upregulation of other cell surface markers characteristic of antigen presenting cells including CD11c, HLA-DR, CD40 and CD86. The effects were concentration-dependent, with 0.5 μg/ml THC inhibiting expression of all of these markers by 40–60%. Interestingly, the response profiles were not uniform for every protein. THC produced a uniform decrease in the expression of CD11c and CD40 on all of the cells but resulted in two distinct subsets with respect to the expression of HLA-DR and CD86 – one population that did not express these markers and one that expressed relatively normal levels (Fig. 3). In the latter case, the relative proportions of these two subsets depended upon the concentration of THC, with higher levels of THC resulting in fewer marker-positive cells.

Cannabinoids have been reported to promote the apoptosis of mouse bone marrow-derived DC under certain conditions (Do et al. 2004). In order to assure that the phenotypic changes observed in our studies were not the result of poor viability, DC that had been differentiated in the presence of either THC (0.25 to 1.0 μg/ml) or JWH-015 (0.25 to 0.75 μM) were stained with propidium iodide and Annexin-V-FITC. There was no significant impact of either cannabinoid on the number of recovered cells or on the frequency of apoptotic or dead cells (data not shown).

Dendritic Cells Generated in the Presence of THC are Functionally Altered

In addition to their high level expression of major histocompatibility complex and costimulatory molecules, monocyte-derived DC are usually characterized by their capacity for antigen uptake, as well as their secretion of

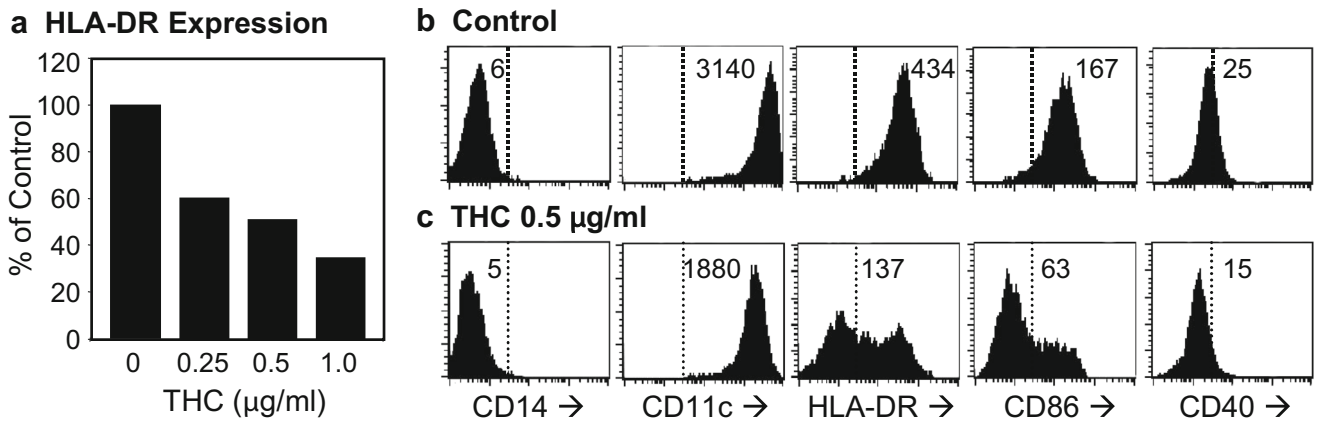


Fig. 3 THC inhibited the expression of HLA-DR and other DC surface markers in a concentration-dependent manner. **a**: Adherent PBMC were cultured for 7 days with GM-CSF and IL-4 to induce their differentiation into DC in the presence of diluent alone or increasing concentrations of THC as indicated. Cell surface HLA-DR expression was measured by flow cytometry (percent of control mean fluorescence intensity). Representative experiment, $n=5$. **b**-**c**: Adherent PBMC were cultured for 7 days with GM-CSF and IL-4 to induce their differentiation into DC in the presence of diluent alone (**b**) or THC at 0.5 μg/ml (**c**) added at the beginning of culture. Cell surface expression of CD14, CD11c, HLA-DR, CD86 and CD40 were measured by flow cytometry. Numbers represent mean fluorescent intensity for the entire population and the dashed line indicates the highest fluorescence intensity value for unstained cells. Data are representative from one of 9 experiments

cytokines that promote cell mediated immunity. Receptor-mediated endocytosis was measured by the uptake of FITC-dextran and was dramatically suppressed in cells that had been exposed to THC (Fig. 4a). The production of IL-10 and IL-12 was also assessed by stimulating cells with SAC and measuring cytokines released into the culture media at 48 h following stimulation. Interestingly, while the production of IL-12 was significantly suppressed ($p<0.01$), the secretion of IL-10, which can bias T cell activation toward T helper type 2 (Th2) and/or T regulatory (Treg) phenotypes, was not altered (Fig. 4b). This differential effect on cytokine production is consistent with an immunoregulatory effect rather than a global suppression of DC function.

THC-DC are Poor T cell Stimulators and Fail to Induce Effector T cells

The capacity for DC to stimulate the activation and proliferation of antigen-specific T cells represents a final integrated measure of their function. DC that had been generated in the presence or absence of THC (THC-DC) were recovered from the 7 day culture of adherent PBMC, purified by negative depletion, and cultured with allogeneic T cells in a MLR assay (Fig. 5). In order to avoid direct effects on responder T cells, no further THC was added during the 5 days of DC:T cell co-culture. Proliferation was monitored by labeling cells with CFSE, which also allowed the phenotype of responder cells to be tracked with each cell division over time. While control

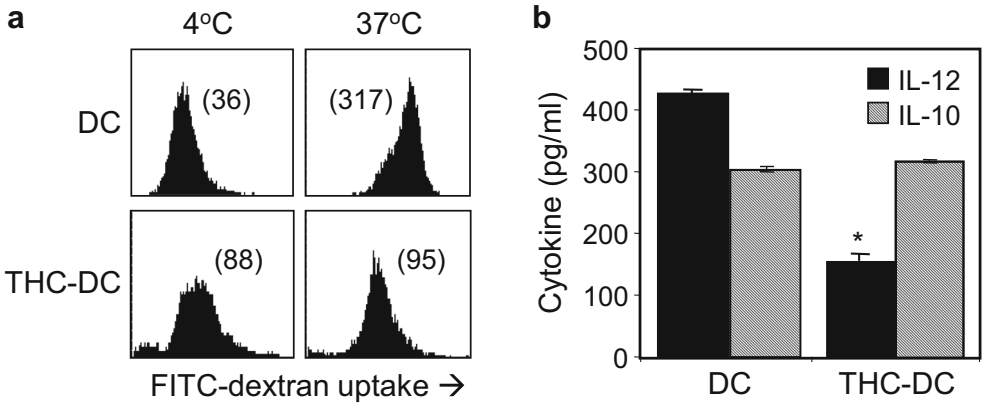
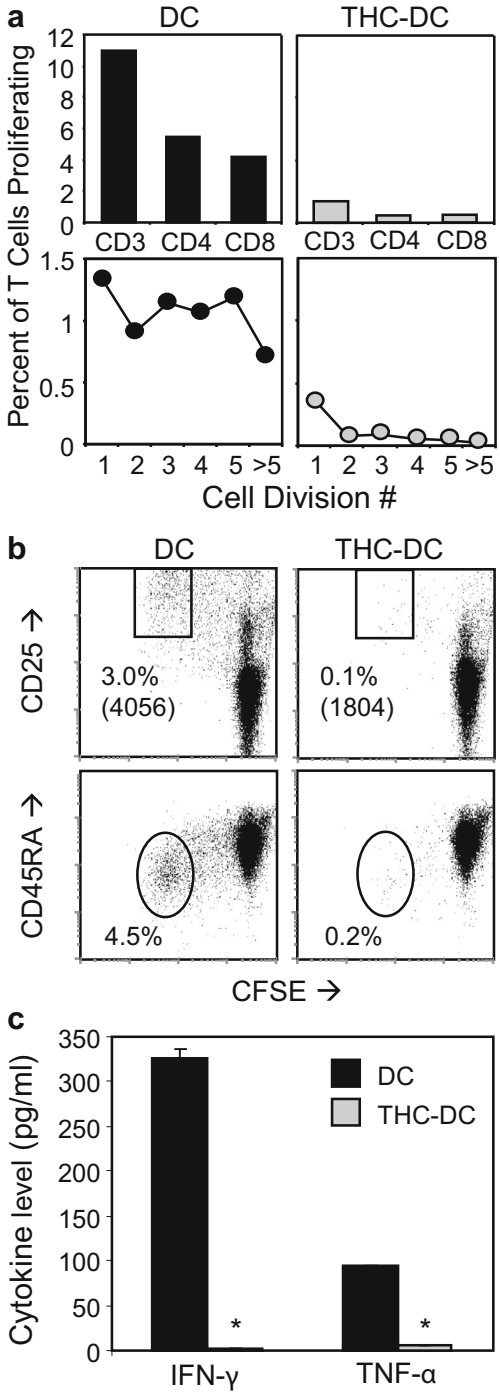


Fig. 4 Exposure to THC impairs the capacity for receptor-mediated endocytosis and alters the relative production of IL-10 and IL-12 by activated DC. **a**: Purified DC that had been generated in the presence or absence of THC (0.5 μg/ml) for 7 days were cultured for 1 h with FITC-dextran (1 mg/ml) at either 4 °C or 37 °C and then analyzed by FACS for uptake. Numbers represent mean fluorescence intensity. **b**: Purified DC were cultured for an additional 48 h (0.5×10^6 cells/ml) with 20 μg/ml SAC as a cytokine-inducing agent. Release of IL-10 and IL-12 was measured by cytokine-specific ELISA and represented as mean values± SD for replicate measurements. *$p<0.01$ compared to control. $N=3$ experiments



◀ **Fig. 5** Exposure to THC impairs the ability for DC to activate allogeneic T cells in a MLR assay. DC or THC-exposed DC were cultured with 2×10^5 CFSE-labeled allogeneic CD45RA+ T cells at a 1:50 DC:T cell ratio in a standard MLR assay. Following 5 days of culture, T cells were collected and stained with fluorescently-labeled monoclonal antibodies specific for CD8, CD45RA, and CD25. **a**: The percentage of T cells and T cell subsets that proliferated was determined by measuring CFSE dilution and cell surface marker expression (top panels). The percentage of T cells corresponding to each cell division number is displayed in the bottom panels. **b**: The proliferating cells were further analyzed for the development of an effector memory phenotype (upregulation of CD25, downregulation of CD45RA). The percentages indicate the percent of total T cells within the specified regions, with the numbers in parentheses indicating the MFI of those cells. **c**. The levels of IFN-γ and TNF-α present in the co-cultures were determined by multiplex analysis of the day 5 supernatants. *$p<0.01$ comparing control DC and THC-DC. Representative results from one of 6 experiments (**a-b**), and one of two experiments (**c**)

DC were potent stimulators of both CD4+ and CD8+ responder T cells, the proliferative response to THC-DC was severely blunted (Fig. 5a). THC-treated DC induced T cell proliferation that ranged from only 17 to 32% of control values ($p<0.01$, $n=6$ experiments). Furthermore, while control DC promoted the conversion from naïve CD45RA+ T cells to activated CD45RO+ clones expressing the high affinity IL-2 receptor (CD25), this functional transformation into effector/memory T cells was almost completely absent when T cells were stimulated with THC-DC (Fig. 5b). There was a similar impact on the generation of effector cell cytokines as measured by the release of IFN-γ and TNF-α into the culture supernatant (Fig. 5c, $p<0.01$).

DC Activators and Supplemental Cytokines Partially Restore the Function of THC-DC

A number of factors can help restore function to impaired antigen presenting cells or enhance their capacity to stimulate T cell responses. Given our findings with THC-DC, we hypothesized that a combination of DC activation and cytokine replacement might be effective for this purpose. In initial experiments, DC and THC-DC were exposed to heat-killed and fixed SAC for 18–24 h prior to co-culture with T cells. The goal was to replicate bacterial activation signals that might occur during an immune challenge in vivo. In other experiments, the co-cultures were supplemented with IL-7, IL-12 or IL-15 to replace key cytokines known to be involved in the proliferation and differentiation of effector/memory T cells. As demonstrated in Fig. 6, pre-treating control DC with SAC enhanced their capacity to stimulate T cell proliferation and maturation. In addition, exposing THC-DC to SAC restored some of their capacity to generate mature (CD45RO+/CD25+) responder T cells. This effect correlated with the upregulation of HLA-DR, CD80 and CD86 on THC-DC (data not shown). In addition, supplementing the co-cultures with IL-7 helped SAC-stimulated DC to further promote the expansion and phenotypic maturation of effector T cells, a synergistic effect that was not observed with either IL-12 or IL-15. When assessed in a limited number of experiments, IL-7 also increased the production of IFN-γ and TNF-α, consistent with a

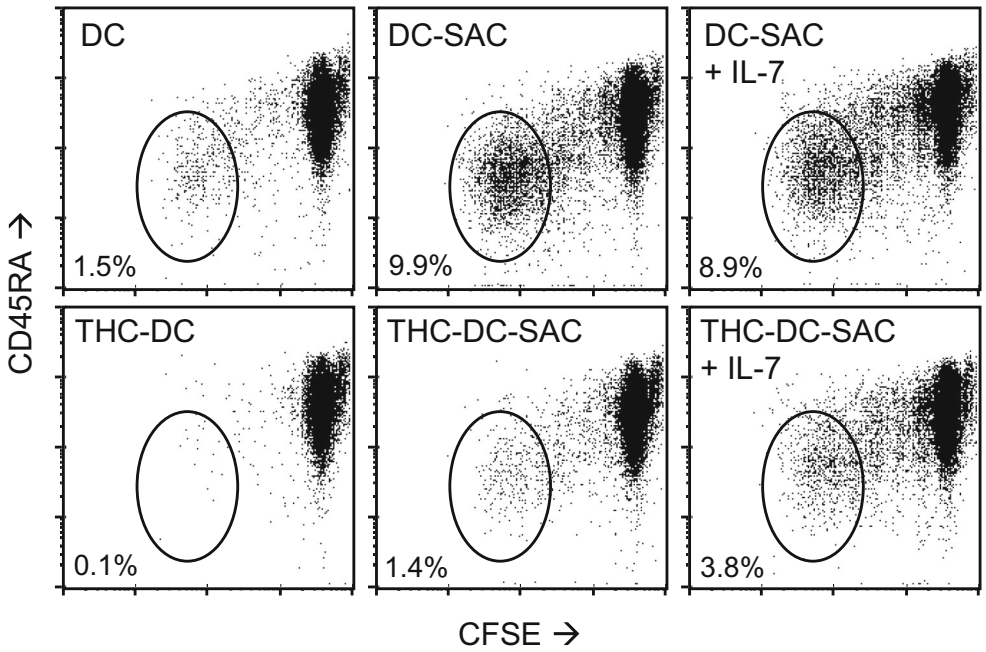


Fig. 6 Addition of SAC or IL-7 can partially compensate for the diminished T cell stimulatory capacity of THC-exposed DC. DC or THC-exposed DC were cultured with 2×10^5 CFSE-labeled allogeneic CD45RA+ T cells at a 1:50 DC:T cell ratio as in Fig. 5. As indicated, some of the DC and THC-exposed DC were further matured by an 18–24 h exposure to 20 μg/ml SAC prior to co-culture with the T cells. Additionally, some of the DC-T cell co-cultures were supplemented with 2 ng/ml rhIL-7. The CFSE dilution and CD45RA staining profiles are shown for the various experimental groups, with the percentages indicating the percent of total T cells within the CFSE-diluted/CD45RA-dim regions. Representative results one of 6 experiments

restoration of their effector/memory function (data not shown).

Discussion

The human CB2 receptor was first cloned from a human myeloid cell line (Munro et al. 1993) and has been reported as the predominant cannabinoid receptor subtype expressed by immune cells (Galiègue et al. 1995). Consistent with this, we found that expression of CB2 mRNA predominated over CB1 when fresh human monocytes were purified and assayed by semi-quantitative RT-PCR techniques. However, neither cannabinoid receptor could be detected on the extracellular surface of monocytes when stained with mAbs known to be specific for their N-terminal sequences. We recently reported that CB2 may exist as an intracellular protein in immune cells (Castaneda et al. 2013) and others have suggested that CB1 may also function as an intracellular receptor (Brailoiu et al. 2011). Consistent with these observations, the addition of an initial fixation and permeabilization step resulted in positive staining by both anti-CB1 and anti-CB2 mAbs, but not by their respective isotype controls. Functional receptor protein was confirmed by assaying the capacity for cannabinoids to inhibit forskolin-induced changes in cAMP. Addition of THC, a pan-agonist with equal affinity for CB1 and CB2, blocked forskolin-induced cAMP in both transduced CHO-CB2 cells and in fresh human monocytes. In addition, this effect was recapitulated by exposure to JWH-015, a selective CB2 agonist, and the effects of THC were completely blocked by SR144528, a selective CB2 antagonist. These findings confirm reports that CB2 predominates as the functional cannabinoid receptor pathway in human monocytes and add the caveat that receptor expression occurs at an intracellular location rather than on the cell surface.

Monocytes act as myeloid precursors that can differentiate along a number of functionally-distinct pathways depending upon their interaction with cytokines, growth factors, infectious signals and other regulatory mediators (Sica and Mantovani 2012). When driven to differentiate into monocyte-derived DC under the influence of GM-CSF and IL-4 (Kiertscher and Roth 1996; Roth et al. 2000), their function can also be modulated by a variety of factors (Alonso et al. 2011). Concurrent exposure to IL-6 and macrophage-colony stimulating factor can divert differentiation toward macrophages instead of DC (Chomarat et al. 2000). Transforming growth factor (TGF)-β and IL-23 promote the development of DC that promote Th17 biased responses (Rajkovic et al. 2011). IL-10 promotes tolerogenic and Th2-

promoting features (Steinbrink et al. 1997), while a variety of toll-like receptor ligands and immunostimulatory cytokines will promote DC that stimulate effector/memory T cells (Banchereau et al. 2000; Lanzavecchia and Sallusto 2000). In this setting, we hypothesized that exposure to THC during the process of DC differentiation would provide valuable insight regarding its immunoregulatory properties. Further, given the immunosuppressive effects that cannabinoids have on antigen-specific T cell responses in animals in vivo (Klein et al. 2000; Zhu et al. 2000) and on human T cell activation in vitro (Yuan et al. 2002; Sipe et al. 2005), we hypothesized that cannabinoids might render DC tolerogenic or otherwise skew their stimulatory activity.

Only a few studies have examined the interaction of cannabinoids with DC and in most cases the focus has been on murine models or on the effects of cannabinoids on differentiated DC (Do et al. 2004; Lu et al. 2006; Karmaus et al. 2013). Do et al. (2004) suggested that THC can impair immune responses by inducing DC apoptosis. However, they studied mouse bone marrow-derived DC and apoptosis occurred primarily when THC concentrations exceeded 5 μM. In our studies, immunoregulatory effects on human monocyte-derived DC were observed at lower THC concentrations (0.8–3.2 μM), more akin to peak levels that occur in the blood of marijuana smokers (Kosel et al. 2002), and had no effect on cell recovery or surface staining by Annexin-V. Instead of apoptosis, we observed broad-ranging effects of THC on the expression of MHC class II and costimulatory molecules, and the capacity for antigen uptake and IL-12 production. Furthermore, DC that had been exposed to THC during their in vitro differentiation (THC-DC) were impaired in their capacity to activate T cells – including both CD4+ and CD8+ responders. T cell proliferation and the acquisition of a memory/effector phenotype were both impaired, as was the release of Th1 cytokines. These effects of THC on the capacity for monocyte-derived DC to stimulate T cells are almost identical to the direct effects of THC on T cell activation (Yuan et al. 2002; Robinson et al. 2013), suggesting a coordinated immunoregulatory effect. It is interesting that other immunosuppressive factors, including IL-10 and TGF-β, share this capacity to act in a coordinated manner on both DC and T cells (Steinbrink et al. 1997; Rajkovic et al. 2011). As is the case with IL-$10^{-/-}$ knockout mice (Davidson et al. 1996), $CB1^{-/-}$/$CB2^{-/-}$ double-knockout mice exhibit elevated levels of activated T cells and respond to antigen challenges by producing a higher number of activated effector cells and stronger IFN-γ responses (Karmaus et al. 2011). Collectively, these findings suggest an intrinsic role for endocannabinoid signaling as a homeostatic regulator of T cell activation.

There are a number of critical features that develop during the transition from monocytes into DC that enable them to activate antigen specific T cells (Banchereau et al. 2000; Lanzavecchia and Sallusto 2000). Among these are high levels of antigen expression in the context of cell-surface MHC, the upregulation of adhesion and costimulatory molecules, and the elaboration of immunostimulatory cytokines. Our studies suggest that cannabinoid receptor activation impacts on all of these. Exposure to THC during the differentiation of monocyte-derived DC impaired antigen uptake and prevented the normal upregulation of MHC class II. These findings are consistent with earlier reports by McCoy et al. (1999), where THC was found to impair the presentation of whole hen egg lysozyme, which required uptake and processing, but not the presentation of its immunodominant peptides, which bound directly to existing cell surface MHC. Dendritic cells that present antigen in the absence of adequate costimulatory molecules cannot fully activate T cells and may contribute to the development of T cell anergy (Banchereau et al. 2000; Lanzavecchia and Sallusto 2000). The inhibitory effects of THC on the expression of CD40, CD86 and other costimulatory molecules likely contributed to the failure of THC-DC to stimulate T cell proliferation. Finally, the relative production of IL-10 and IL-12 by DC plays a central role in their capacity to activate either Th1 (requiring IL-12) or Th2 (dependent upon IL-10) responses. In our studies, THC-DC produced only limited amounts of IL-12 but normal levels of IL-10. Lu et al. (2006b) reported a similar suppressive effect of THC on the expression of MHC and costimulatory molecules and on production of IL-12 by mouse bone marrow-derived DC that had been infected with *Legionella pneumophila*.

While these findings add to other compelling evidence that cannabinoids can exert important immunosuppressive effects, clinical evidence that marijuana smoking significantly impairs immune function in humans is limited. One explanation may be that inhaled THC never produces sufficient systemic levels, or that exposures may not be sustained for a sufficient period of time. to mediate immunosuppressive effects (Kosel et al. 2002; Desrosiers et al. 2014). Another explanation may be that the effects are short-lived or counterbalanced by the presence of other immune regulatory factors. The study of purified cells in vitro culture does not adequately replicate the complex environment that occurs during an immune challenge in vivo. In this study we hypothesized that the processes of DC activation and cytokine exposure that occur in response to an infectious challenge might modulate the impact of THC. Exposing DC and THC-DC to heat-killed and fixed SAC for 18–24 h enhanced their capacity for T cell activation; an effect that was more pronounced with THC-DC than with control DC. Adding IL-12 and IL-15 to the DC:T cell co-culture also enhanced T cell activation and proliferation, but these effects occurred equally with control and THC-DC. Furthermore, these cytokines promoted T cell proliferation and cytokine production even in the absence of stimulation by DC (data not shown). However, the addition of IL-7 to DC:T cell co-cultures had a dramatic effect on T cell proliferation,

maturation and cytokine production that was restricted in part to co-cultures containing THC-DC. These studies suggest that the immunoregulatory effects of THC might be counterbalanced by the presence of a combination of DC activating signals and the production of cytokines by other cell types present in the local immune environment.

In summary, our experiments demonstrate that human monocytes express functional cannabinoid receptors, even if they are not detectable on the cell surface, and that exposure to THC alters their capacity to differentiate into immunostimulatory DC with prominent effects on antigen uptake and presentation, expression of costimulatory molecules, and production of IL-12. The end result is the generation of DC that fail to stimulate T cell proliferation or promote maturation into functional effector/memory T cells. While the effects are relatively potent when studied in isolation in vitro, there may be a number of immunoregulatory factors that could counteract or moderate the impact of cannabinoid exposure in vivo. The functional role that marijuana smoking has on host immunity and the response to immune challenges in vivo remains to be clarified.

Acknowledgments Research reported in this publication was supported by the National Institute on Drug Abuse, National Institutes of Health, under award numbers 5-R01-DA003018 and 1-R01-DA037102. JT Castaneda was supported by a Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship to Promote Diversity in Health-Related Research from the National Institute on Drug Abuse, National Institutes of Health, under award numbers 1 F31 DA036293. Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research (CFAR) Flow Cytometry Core Facility that is supported by National Institutes of Health awards CA-16042 and AI-28697, and by the JCCC, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA.

Conflicts of Interest The authors declare that they have no conflict of interest.

Statement of Human Rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

Alonso MN, Wong MT, Zhang AL, Winer D, Suhoski MM, Tolentino LL, Gaitan J, Davidson MG, Kung TH, Galel DM, Nadeau KC, Kim J, Utz PJ, Söderström K, Engleman EG (2011) TH1, TH2, and TH17 cells instruct monocytes to differentiate into specialized dendritic cell subsets. Blood 118(12):3311–3320. doi:10.1182/blood-2011-03-341065

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000) Immunobiology of dendritic cells. Annu Rev Immunol 18:767–811. doi:10.1146/annurev.immunol.18.1.767

Basu S, Dittel BN (2011) Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. Immunol Res 51(1):26–38. doi:10.1007/s12026-011-8210-5

Bosier B, Muccioli GG, Hermans E, Lambert DM (2010) Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities. Biochem Pharmacol 80(1):1–12. doi:10.1016/j.bcp.2010.02.013

Bouaboula M, Rinaldi M, Carayon P, Carillon C, Delpech B, Shire D, LeFur G, Casellas P (1993) Cannabinoid-receptor expression in human leukocytes. Eur J Biochem 214(1):173–180. doi:10.1111/j.1432-1033.1993.tb17910.x

Brailoiu GC, Oprea TI, Zhao P, Abood ME, Brailoiu E (2011) Intracellular cannabinoid type 1 (CB1) receptors are activated by anandamide. J Biol Chem 286(33):29166–29174. doi:10.1074/jbc.M110.217463

Bredt BM, Higuera-Alhino D, Shade SB, Hebert SJ, McCune JM, Abrams DI (2002) Short-term effects of cannabinoids on immune phenotype and function in HIV-l-infected patients. J Clin Pharmacol 42(11 Suppl):82S–89S. doi:10.1177/0091270002238798

Castaneda JT, Harui A, Kiertscher SM, Roth JD, Roth MD (2013) Differential expression of intracellular and extracellular CB(2) cannabinoid receptor protein by human peripheral blood leukocytes. J Neuroimmune Pharmacol 8(1):323–332. doi:10.1007/s11481-012-9430-8

Chomarat P, Banchereau J, Davoust J, Palucka AK (2000) IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat Immunol 1(6):510–514. doi:10.1038/82763

Davidson NJ, Leach MW, Fort MM, Thompson-Snipes L, Kühn R, Müller W, Berg DJ, Rennick DM (1996) T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. J Exp Med 184(1):241–251

Desrosiers NA, Himes SK, Scheidweiler KB, Concheiro-Guisan M, Gorelick DA, Huestis MA (2014) Phase I and II cannabinoid disposition in blood and plasma of occasional and frequent smokers following controlled smoked cannabis. Clin Chem 60(4):631–643. doi:10.1373/clinchem.2013.216507

Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS (2004) Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. J Immunol 173(4):2373–2382. doi:10.4049/jimmunol.173.4.2373

Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur J Biochem 232(1):54–61. doi:10.1111/j.1432-1033.1995.tb20780.x

Karmaus PW, Chen W, Crawford RB, Harkema JR, Kaplan BL, Kaminski NE (2011) Deletion of cannabinoid receptors 1 and 2 exacerbates APC function to increase inflammation and cellular immunity during influenza infection. J Leukoc Biol 90(5):983–995. doi:10.1189/jlb.0511219

Karmaus PWF, Chen W, Crawford R, Kaplan BLF, Kaminski NE (2013) Δ9-Tetrahydrocannabinol impairs the inflammatory response to influenza infection: role of antigen-presenting cells and the cannabinoid receptors 1 and 2. Toxicol Sci 131(2):419–433. doi:10.1093/toxsci/kfs315

Kiertscher SM, Roth MD (1996) Human CD14+ leukocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GM-CSF and IL-4. J Leukoc Biol 59(2):208–218

Klein TW (2005) Cannabinoid-based drugs as anti-inflammatory therapeutics. Nat Rev Immunol 5(5):400–411. doi:10.1038/nri1602

Klein TW, Cabral GA (2006) Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. J Neuroimmune Pharmacol 1(1):50–64. doi:10.1007/s11481-005-9007-x

Klein TW, Newton CA, Nakachi N, Friedman H (2000) Delta9-tetrahydrocannabinol treatment suppresses immunity and early

IFN-gamma, IL-12, and IL-12 receptor beta2 responses to Legionella pneumophila infection. J Immunol 164(12):6461–6466. doi:10.4049/jimmunol.164.12.6461

Kong W, Li H, Tuma RF, Ganea D (2014) Selective CB2 receptor activation ameliorates EAE by reducing Th17 differentiation and immune cell accumulation in the CNS. Cellular Immunol 287(1):1–17. doi:10.1016/j.cellimm.2013.11.002

Kosel BW, Aweeka FT, Benowitz NL, Shade SB, Hilton JF, Lizak PS, Abrams DI (2002) The effects of cannabinoids on the pharmacokinetics of indinavir and nelfinavir. AIDS 16(4):543–550

Lanzavecchia A, Sallusto F (2000) Dynamics of T lymphocyte responses: iIntermediates, effectors, and memory cells. Science 290(5489):92–97. doi:10.1126/science.290.5489.9

Lu T, Newton C, Perkins I, Friedman H, Klein TW (2006a) Cannabinoid treatment suppresses the T-helper cell-polarizing function of mouse dendritic cells stimulated with Legionella pneumophila infection. J Pharmacol Exp Ther 319(1):269–76. doi:10.1124/jpet.106.108381

Lu T, Newton C, Perkins I, Friedman H, Klein TW (2006b) Role of cannabinoid receptors in Delta-9-tetrahydrocannabinol suppression of IL-12p40 in mouse bone marrow-derived dendritic cells infected with Legionella pneumophila. Eur J Pharmacol 532(1–2):170–177. doi:10.1016/j.ejphar.2005.12.040

McCoy KL, Matveyeva M, Carlisle SJ, Cabral GA (1999) Cannabinoid inhibition of the processing of intact lysozyme by macrophages: evidence for CB2 receptor participation. J Pharmacol Exp Ther 289(3):1620–1625

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365(6441):61–5. doi:10.1038/365061a0

Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M (2009) Cannabinoids as novel anti-inflammatory drugs. Future Med Chem 1(7):1333–1349. doi:10.4155/fmc.09.93

Nong L, Newton C, Cheng Q, Friedman H, Roth MD, Klein TW (2002) Altered cannabiniod receptor mRNA expression in peripheral blood mononuclear cells from marijuana smokers. J Neuroimmunol 127(1–2):169–176. doi:10.1016/S0165-5728(02)00113-3

Rajkovic I, Dragicevic A, Vasilijic S, Bozic B, Dzopalic T, Tomic S, Majstorovic I, Vucevic D, Djokic J, Balint B, Colic M (2011) Differences in T-helper polarizing capability between human monocyte-derived dendritic cells and monocyte-derived Langerhans'-like cells. Immunology 132(2):217–25. doi:10.1111/j.1365-2567.2010.03356.x

Rieder SA, Chauhan A, Singh U, Nagarkatti M, Nagarkatti P (2010) Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression. Immunobiology 215(8):598–605. doi:10.1016/j.imbio.2009.04.001

Robinson RH, Meissler JJ, Breslow-Deckman JM, Gaughan J, Adler MW, Eisenstein TK (2013) Cannabinoids inhibit T-cells via cannabinoid receptor 2 in an in vitro assay for graft rejection, the mixed lymphocyte reaction. J Neuroimmune Pharmacol 8(5):1239–1250. doi:10.1007/s11481-013-9485-1

Roth MD, Gitlitz BJ, Kiertscher SM, Park AN, Mendenhall M, Moldawer N, Figlin RA (2000) Granulocyte/macrophage-colony stimulating factor and interleukin-4 enhance the number and antigen-presenting activity of circulating CD14+ and CD83+ cells in cancer patients. Cancer Res 60(7):1934–1941

Sica A, Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122(3):787–795. doi:10.1172/JCI59643

Sipe JC, Arbour N, Gerber A, Beutler E (2005) Reduced endocannabinoid immune modulation by a common cannabinoid 2 (CB2) receptor gene polymorphism: possible risk for autoimmune disorders. J Leukoc Biol 78(1):231–238. doi:10.1189/jlb.0205111

Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH (1997) Induction of tolerance by IL-10-treated dendritic cells. J Immunol 159(10):4772–4780

Yuan M, Kiertscher SM, Cheng Q, Zoumalan R, Tashkin DP, Roth MD (2002) Δ9-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T-cells. J Neuroimmunol 133(1–2):124–131. doi:10.1016/S0165-5728(02)00370-3

Zhu LX, Sharma A, Stolina S, Gardner B, Roth MD, Tashkin DP, Dubinett SM (2000) Δ-9 tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. J Immunol 165(1):373–80. doi:10.4049/jimmunol.165.1.373

CHAPTER 4

DYNAMIC REGULATION OF CELL SURFACE CB_2 RECEPTOR DURING HUMAN B CELL ACTIVATION AND DIFFERENTIATION

Dynamic Regulation of Cell Surface CB_2 Receptor during Human B Cell Activation and Differentiation.

Julie T. Castaneda, Airi Harui, and Michael D. Roth

Division of Pulmonary and Critical Care Medicine (J.T.C., A.H., M.D.R.) and the Inter-Departmental Program in Molecular Toxicology (J.T.C., M.D.R.), Department of Medicine, CHS 37-131, David Geffen School of Medicine at UCLA, Los Angeles, California

Corresponding Author:

Julie T. Castaneda,

Department of Medicine, CHS 37-131,

Geffen School of Medicine at UCLA

Los Angeles, CA 90095-1690

Telephone: (310) 825-9393, FAX: (310) 267-2020, jtcastaneda@mednet.ucla.edu.

(pending submission to the Journal of NeuroImmune Pharmacology)

ABSTRACT

Cannabinoid receptor type 2 (CB_2) is the primary receptor pathway mediating the immunologic consequences of cannabinoids. We recently reported that human peripheral blood B cells express CB_2 on both the extracellular membrane and at intracellular sites, where-as monocytes and T cells only express intracellular CB_2. To better understand the pattern of CB_2 expression by human B cells, we examined $CD20^+$ B cells from three tissue sources. Both surface expression and intracellular staining were present and uniform in cord blood B cells, where all cells exhibited a naïve mature phenotype ($IgD^+/CD38^{Dim}$). While naïve mature and quiescent memory B cells ($IgD^-/CD38^-$) from tonsils and peripheral blood exhibited a similar pattern, tonsillar activated B cells ($IgD^-/CD38^{bright}$) expressed little to no surface CB_2. We hypothesized that regulation of the surface CB_2 receptor may occur during B cell activation. Consistent with this, a B cell lymphoma cell line known to exhibit an activated phenotype (SUDHL-4) was found to lack cell surface CB_2 but express intracellular CB_2. Furthermore, *in vitro* activation of human cord blood resulted in a down-regulation of surface CB_2 on those B cells acquiring the activated phenotype but not on those retaining IgD expression. Using a CB_2 expressing cell line (293T/CB2-GFP), confocal microscopy confirmed the presence of both cell surface expression and multifocal intracellular staining, the latter of which did not co-localize with either mitochondria, lysosomes, or nucleus. Our findings suggest a dynamic multi-compartment expression pattern for CB_2 in B cells that is specifically modulated during the course of B cell activation.

INTRODUCTION

Cannabinoids, the primary bioactive constituents of marijuana, activate cannabinoid receptor type 1 (CB_1) and type 2 (CB_2) and signal through an endogenous human cannabinoid system to produce their biologic effects [Aizpurua-Olaizola 2016, Cabral 2015, Maccarrone 2015, Pacher 2006]. Expression of CB_2 predominates in cells from the immune system [Castaneda 2013, Schmöle 2015], and cannabinoids have been described to exert potent immunosuppressive effects on antigen presenting cells [Klein 2006, Roth 2015], B cells and antibody production [Agudelo 2008, Carayon 1998], T cell responsiveness and cytokine production [Eisenstein 2015, Yuan 2002], and monocyte/macrophage function [Hegde 2010, Roth 2004]. However, the majority of these findings stem from studies employing agonists and antagonists with defined CB_2 binding specificities, and only limited insight has been available regarding the actual expression patterns and dynamic regulation of CB_2 protein. CB_2 has traditionally been described as a seven-transmembrane G protein-coupled receptor (GPCR) expressed on the cell surface and responsive to extracellular ligand binding. Ligand binding has been shown to initiate both receptor internalization [Atwood 2012] and a diverse number of intracellular signaling cascades, including adenylyl cyclase, cAMP, mitogen-activated protein (MAP) kinase, and intracellular calcium [Howlett 2005, Jean-Alphonse 2011, Maccarrone 2015]. However, after using a highly sensitive and specific monoclonal anti-CB_2 antibody and fluorescent imaging, we were surprised to find that CB_2 was expressed exclusively in the intracellular compartment of human monocytes, dendritic cells, and T cells without detectable cell surface staining [Castaneda 2013, Roth 2015]. Only human B cells expressed CB_2 on the cell surface, which internalized in response to ligand exposure, as well as within the intracellular compartment [Castaneda 2013]. These findings challenge our understanding of the CB_2 receptor and identify the need for additional insight.

It is not yet clear whether cannabinoids routinely bind and activate intracellular CB_2, but there is at least one report providing direct experimental evidence for this [Brailoiu 2014]. It is also not clear why B cells exhibit a receptor expression pattern that is distinct from other leukocytes or whether this is a unique feature in cells obtained from peripheral blood or related to the specific stage of cell activation or differentiation. B cell activation has been suggested to play a role in the pattern of CB_2 expression in a prior report [Carayon 1998]. In order to better understand CB_2 expression patterns exhibited by human B cells, this report examines cells obtained from three different tissue sources (adult peripheral blood, cord blood, and tonsils), evaluates the relationship between defined B cell subsets and CB_2 expression patterns, and uses an *in vitro* model for activating B cells in order to examine changes in CB_2 expression as they correlate to the life cycle of functional B cell responses.

METHODS

Primary cells and cell lines: Following informed consent, peripheral blood leukocytes (PBL) were isolated by Ficoll-gradient centrifugation (GE HealthCare, Chicago, IL) from the blood of healthy human donors. Human umbilical vein cord blood leukocytes were obtained from anonymous donors through the UCLA Virology Core and isolated in the same manner. Fresh human tonsillar tissue was also obtained in an anonymous manner through the UCLA Translational Pathology Core from patients undergoing routine elective tonsillectomies. Tonsillar tissue was handled in a sterile manner, minced, and then extruded through a sterile 100 uM filter to produce single cells. Filtered cells were then rinsed with PBS (Cellgro, Manassas, VA) and processed in the same manner as PBL. Cell subsets were identified by flow cytometry using fluorescent-labeled monoclonal antibodies (mAb) directed against T cells (anti-CD3, Invitrogen, Camarillo, CA), B cells (anti-CD20, BD Biosciences, San Jose, CA), and B cell subsets (anti-IgD and anti-IgM, Biolegend, San Diego, CA and anti-CD27 and anti-CD38, BD Biosciences).

The human B cell non-Hodgkin's lymphoma cell line, SUDHL-4 (gifted by Dr. John Timmerman) was cryopreserved, and when needed, it was cultivated in suspension in complete medium composed of RPMI-1640 (Cellgro) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA), 50 uM 2-mercaptoethanol (MP Biomedicals, Santa Ana, CA), and 1% antibiotic-antimycotic solution (Cellgro).

Detection of CB_2 receptor by flow cytometry: CB_2 on the extracellular membrane was detected as previously described [Castaneda 2013]. In summary, cells were pre-treated with human AB

Serum (Omega Scientific) followed by a 30 min incubation with unlabeled primary mouse IgG_2 mAb directed against either human CB_2 (clone #352114, 0.5 μg/tube, R&D Systems, Minneapolis, MN) or isotype-matched mAb against an irrelevant antigen, mouse NK1.1 (clone #PK136, 0.5 μg/tube, BD Biosciences). After washing, cells were incubated with an APC-labeled goat anti-mouse F(ab')2 mAb (APC-labeled GAM, 0.5 μg/tube, Invitrogen) for 30 min. To identify different leukocyte subsets, cells were incubated with lineage-specific fluorescent-labeled mAb for 20 min and washed. All cells were then fixed with 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and washed. Samples were protected from light and stored at 4°C until analyzed.

In order to detect total cellular CB_2 expression (extracellular + intracellular), cell suspensions were fixed (1% paraformaldehyde), permeabilized (permeabilizing solution, BD Biosciences), and blocked with human AB serum. Staining with primary unlabeled mAb (against CB_2 or NK1.1) and secondary APC-labeled GAM were carried out as already detailed except for the use of a 60 minute incubation time and the presence of permeabilizing solution. After washing, leukocytes were further stained with fluorescent-labeled antibodies as indicated for individual experiments, fixed, and stored for analysis.

In order to identify total cellular CB_2 expression in specific B cells subsets, cells were pre-stained with B cell subset markers prior to fixation, permeabilization, and staining for CB_2. This step prevented the detection of intracellular subset markers (IgD, IgM, CD27, and CD38), which can otherwise result in misclassification. After staining, cells were fixed with 1% paraformaldehyde, washed, and cryopreserved in PBS with 2% human AB serum and 10% dimethyl sulfoxide (Sigma-Aldrich). On the day of CB_2 analysis, cells were rapidly thawed at 37°C, treated with permeabilizing solution and stained for 30 min with Alexa Fluor ® 647-labeled mouse IgG2a mAb directed against either human CB_2 (clone #352114, 2 μg/tube, Novus

Biologicals, Littleton, CO) or isotype-matched mAb against an irrelevant antigen, mouse NK1.1 (clone #PK136, 2 μg/tube, Biolegend) and with fluorescent-labeled antibodies directed against CD20 and CD3. Cells were fixed once again with 1% paraformaldehyde prior to storage and analysis.

***In vitro* activation and differentiation of naïve mature B cells:** B cells obtained from umbilical vein cord blood were cultured for 5 days at 1 x 10^6 cells/mL in RPMI-1640 supplemented with 10% human AB serum and 1% antibiotic-antimycotic solution in combination with the following activating reagents: a) 5 μg/mL anti-IgM (Jackson ImmunoResearch, West Grove, PA); b) 100-250 ng/mL mega-CD40L (Enzo Life Sciences, Farmingdale, NY); c) 100 ng/mL IL-21 (Peprotech, Rocky Hill, NJ); and d) 100 ng/mL IL-4 (R&D Systems). Leukocytes were phenotyped at day 0 (before culture) and day 5 to identify B cell subsets and determine CB_2 expression as already described.

Multiparameter flow cytometry: Multiparameter flow cytometry was carried out using a FACScan II-plus cytometer and SORP BD HTLSRII (BD Biosciences) with the acquisition of 5,000–40,000 events depending upon the assay conditions. Analysis utilized FCS Express V3 or V5 software with gating on $CD20^+/CD3^-$ events followed by subset analyses (De Novo Software, Ontario, Canada). Flow cytometry results are presented as two-parameter dot plots with reported values representing the mean linear fluorescence intensity (MFI) of the gated population.

Confocal microscopy: Pre-cleaned coverslips were coated overnight with 0.1 mg/mL poly-l-lysine hydrobromide (Sigma-Aldrich), washed with sterile culture water (Cellgro), and plated in

12 well plates with 1.5 x10^5 293T or 293T/CB_2-GFP cells/mL for 48 hrs at 37°C in complete medium composed of DMEM 1X (Cellgro), 10% fetal bovine serum, and 1% antibiotic-antimycotic solution. Cells were then washed with PBS and incubated with 150 nM MitoTracker® Orange CMTMRos (Molecular Probes, Eugene, OR) in serum free media or 100 nM LysoTracker® Red DND-99 (Molecular Probes) in complete medium for 2 hr at 37°C. Cells were then washed with pre-warmed DMEM or complete medium. MitoTracker-stained cells were fixed and incubated with 1% paraformaldehyde for 20 min at 4°C. LysoTracker-stained cells were mounted and imaged immediately without fixation. Coverslips were mounted onto slides with one drop of SlowFade® Diamond Antifade Mountant with DAPI (Molecular Probes). For co-localization studies, MitoTracker stained cells were fixed and incubated with 1% paraformaldehyde and treated with permeabilizing solution. Cells were washed and followed by a 30 min incubation with an Alexa Fluor® 647-labeled mAbs (against CB_2 or NK1.1). After washing, cells were fixed with 1% paraformaldehyde and mounted for image analysis. Slides were imaged on SP2 1P-FCS or SP5 Blue confocal microscopes in 10-20 sections.

Statistics:

A minimum of three replicate experiments were carried out for each assay. Flow cytometry dot-plots display results from a single representative tube of a single experiment. All other data are presented as means of replicate tubes or experiments as detailed. The difference between means was determined using a Student's T-test with a $p < 0.05$ accepted as statistically significant.

RESULTS

Heterogeneity of B cell populations and CB_2 expression in leukocytes obtained from cord blood, adult peripheral blood, and tonsils. In order to determine if surface expression of CB_2 is a uniform feature of all B cell populations or whether it varies with the local environment or state of differentiation, flow cytometry was used to examine the $CD20^+/CD3^-$ population recovered from three different sources including umbilical vein cord blood, adult peripheral blood, and tonsils (**Figure 1**). $CD20^+$ B cells recovered from cord blood exhibited a homogeneous phenotype consistent with naive mature B cells ($IgD^+/CD38^{dim}$), while cells recovered from peripheral blood exhibited markers suggestive of both naive mature and quiescent memory ($IgD^-/CD38^-$) subsets. Cells recovered from tonsils demonstrated features of all three subsets: naive mature, activated ($IgD^-/CD38^+$), and quiescent memory B cells (**Figure 1A)**. Similarly, the staining by anti-CB_2 mAb ranged from homogeneous and positive on cord blood and peripheral blood B cells to heterogeneous on tonsillar B cells (**Figure 1B**). Consistent with our prior findings with peripheral blood, no surface CB_2 staining was observed on $CD3^+$ T cells regardless of the source of cells (data not shown).

Surface expression of CB_2, but not total cellular CB_2, is limited in activated B cells recovered from tonsils. In order to directly examine the relationship between B cell subsets and CB_2 expression, gated $CD20^+/CD3^-$ B cells from human tonsils were classified into three defined subsets, naïve mature, activated, and quiescent memory (**Figure 2A**), and evaluated for both surface expression (**Figure 2B**) and total cellular CB_2 expression (**Figure 2C**) by flow cytometry. Non-specific background staining on these gated subsets was assessed using an isotype control mAb directed against an irrelevant target (mouse NK1.1). This approach clarified that the

expression of CB_2 on the surface of both naïve mature ($IgD^+/CD38^{dim}$) and quiescent memory ($IgD^-/CD38^-$) subsets was relatively homogeneous and strongly-positive, while surface CB_2 expression on the activated subset ($IgD^-/CD38^+$) ranged from negative to dim-positive with the majority of cells exhibiting no detectible extracellular membrane expression of CB_2. Using a permeabilization step, total cellular expression of CB_2 (intracellular plus cell membrane) was also assessed. Interestingly, in contrast to the findings on the cell membrane, all three B cell subsets exhibited high expression of CB_2 with the highest expression by the activated subset.

Malignant B cell lines expressing an activated phenotype exhibit the same pattern of CB_2 expression as that observed with primary activated B cells from tonsils. CB_2 has been described as an oncogene with enhanced expression of CB_2 by leukemia and lymphoma cell lines [Jorda 2003, Perez-Gomez 2015]. Given the expression pattern observed with tonsillar B cells, we hypothesized that altered CB_2 expression might be associated with an activated phenotype and that expression by these cells might reside primarily at an intracellular location. A B cell lymphoma cell line described as exhibiting the characteristics of activated B cells, SUDHL-4, was therefore assessed for both B cell subset markers, IgD, CD38, and CD27 (**Figure 3A**), and for cell surface (**Figure 3B**) and total CB_2 expression (**Figure 3C**) by flow cytometry. As expected for the activated phenotype, these cells were $IgD^-/CD38^+/CD27^+$ (tonsillar activated B cells were also $CD27^+$, data not shown). Following the same pattern as activated B cells from tonsils, SUDHL-4 cells did not express cell surface CB_2, but exhibited high total cellular CB_2 after being fixed, permeabilized, and stained with anti-CB_2 mAb. Similar findings were observed with two other human malignant B cell lymphomas tested (Ramos and Granta-519 cells, data not shown).

Changes in CB_2 expression when human naïve B cells are activated *in vitro* and acquire the phenotype of activated B cells. The difference in CB_2 expression between activated B cells and other subsets lead us to hypothesize that CB_2 expression is modulated as part of the activation process. In order to directly test this hypothesis, cord blood B cells were activated by cross-linking the B cell receptor in combination with mega-CD40L, IL-21, and IL-4 as physiologic costimulatory signals. Activation was assessed by changes in expression of cell surface IgD, IgM, CD27, and CD38 (**Figure 4A**). At day 0, B cells start out in a naïve mature state ($IgD^+/CD38^{Dim}$). By day 5, two distinct sub-populations emerge, one still phenotypically naïve ($IgD^+/CD38^{Dim}$) and the other with an activated phenotype ($IgD^-/CD38^+$). Each population was examined for the expression of both cell surface CB_2 and total cellular CB_2. On day 0, cell surface CB_2 expression was obviously positive as was intracellular expression. Similarly, the B cells that remained phenotypically naïve on day 5 exhibited both cell surface and intracellular CB_2. However, surface expression of CB_2 was absent on those day 5 cells that had acquired an activated phenotype. As was the case with activated B cells recovered from tonsils, the activated cells generated *in vitro* still exhibited intracellular CB_2 even though surface expression had been lost (**Figure 4B**).

Intracellular CB_2 is expressed in a diffuse but punctate pattern that is distinct from the distribution of the lysosomal and mitochondrial compartments. As an integral transmembrane GPCR, CB_2 has classically been viewed as a cell surface receptor. However, the current findings suggest that it is the intracellular form of CB_2 that represents the most consistent and predominant form. Confocal microscopy was therefore employed to investigate the distribution and location of intracellular CB_2 in peripheral blood B cells, the activated B cell lymphoma cell line, SUDHL-4, and in a 293T cell line transduced to stably express CB_2 (293T/CB_2-GFP) (**Figure 5A**). An

identical appearing diffuse, but punctate, cytoplasmic distribution of CB_2 was observed in all three cases. Cells stained in an identical manner using the isotype control NK1.1 mAb exhibited no detectible fluorescence (data not shown). In order to compare expression patterns to other organelle markers, cells from the 293T/CB_2-GFP line were also stained with MitoTracker and LysoTracker reagents to determine mitochondrial and lysosomal staining patterns, respectively (**Figure 5B**). Lysosomal staining shared no obvious features with the staining pattern for CB_2, but the mitochondrial staining also exhibited a diffuse but punctate pattern. Given prior evidence that Δ-9-tetrahydrocanabinol (THC), a prototypic cannabinoid that binds to the CB_2 receptor, has potent effects on cell energetics and mitochondrial function when incubated with a CB_2-expressing cell line [Sarafian 2003], co-localization studies were carried out to assess whether CB_2 is expressed in the mitochondrial membrane (**Figure 5C**). Despite some similarity in staining pattern, there was no co-localization of fluorescent images for mitochondrial and CB_2 staining. Cell surface CB_2 did co-localize with cell membrane markers (data not shown).

DISCUSSION

The concept of CB_2 as a simple GPCR expressed on the surface of human leukocytes [Graham 2010, Klein 2003] is being challenged by a number of recent findings, including our imaging studies that employ a mAb against the N-terminal domain of CB_2 to detect protein expression [Castaneda 2013, Roth 2015]. Using a combination of multi-parameter flow cytometry and flow-based imaging, we observed that CB_2 can be expressed on the cell surface, as expected, but is also present within the cytoplasm. Furthermore, the expression pattern for CB_2 was not uniform across cell types. The intracellular expression, rather than the extracellular expression, was the predominant form [Castaneda 2013]. While peripheral blood B cells expressed both cell surface and intracellular CB_2, T cells, monocytes, and dendritic cells exhibited only the intracellular form of CB_2. Even though cell surface CB_2 can rapidly internalize when exposed to a ligand, the distribution of this internalized CB_2 did not appear to account for the pre-existing distribution of intracellular CB_2. The biologic basis underlying these different CB_2 expression patterns has not yet been fully delineated, but there is growing evidence that the presence of GPCRs at different cellular locations is an important feature of these receptors that promotes functional heterogeneity with respect to downstream signaling and biologic responses [Flordellis 2012, Gaudet 2015]. Along these lines, there is growing evidence that intracellular forms for both CB_1 and CB_2 are common and exert distinct biologic effects [Brailoiu 2011, Bernard 2012, Gómez-Cañas 2016]. In this setting, understanding the distribution, regulation, and dynamic balance between cell surface and intracellular CB_2 receptors is likely to provide important insight regarding cannabinoid receptor biology.

The unique expression of CB_2 on the surface of peripheral blood B cells led us to question whether this represented an intrinsic and stable feature of B cells in general or was more characteristic of those in peripheral blood. B cells were therefore obtained from three sources for comparison including umbilical vein cord blood, adult peripheral blood, and tonsils. B cell subsets from these different sources were characterized as either naïve mature, activated, or memory B cells based on their expression of IgD, IgM, CD27 and CD38 [Ettinger 2005]. When analyzed in this manner, it became clear that all naïve and memory B cells, regardless of source, expressed both cell surface and intracellular CB_2. On the other hand, B cells with an activated phenotype ($IgD^-/IgM^-/CD38^+/CD27^+$) expressed only the intracellular form of CB_2, and in most cases the level of intracellular CB_2 was higher than that observed in naïve or memory B cells obtained from the same sample. Prior studies had noted that $IgD^-/CD38^+$ germinal center B cells, consistent with the activated tonsillar B cells studied here, express a different pattern of CB_2 protein staining than other B cells. However, they were using a polyclonal rabbit antibody that targeted a C-terminal CB_2 peptide sequence and concluded that their findings represented the transition of CB_2 from an inactive to an "activated/phosphorylated" state [Carayon 1998, Rayman 2004]. It is plausible that their findings actually mirrored ours, but features related to receptor localization were not appreciated due to technical limitations.

Given the unique CB_2 signature of the activated B cell population, we entertained two possible hypotheses based on the existing literature. The simplest hypothesis being that B cell activation is associated with a down-regulation of the surface CB_2 receptor. Alternatively, it has been reported that CB_2 can form heterodimers with the CXCR4 chemokine receptor and has chemotactic properties that result in the selective homing of CB_2^+ and CB_2^- B cells to different regions of lymphoid follicles [Basu 2013, Coke 2016]. We addressed the potential linkage between

B cell activation and CB_2 expression using two different approaches. CB_2 is known to be expressed by B cell lymphomas and has been described as an oncogene [Jorda 2003, Perez-Gomez 2015]. We therefore examined a human B cell lymphoma cell line, SUDHL-4, that had been described to express an activated B cell phenotype. Consistent with a linkage between activation state and CB_2 expression pattern, this cell line and two other lymphoma lines that exhibited an "activated" phenotype were found to exhibit high intracellular CB_2 but no surface staining. In order to more directly test the linkage between B cell activation and CB_2 expression pattern, we employed an *in vitro* model in which naïve mature human B cells obtained from umbilical vein cord blood were activated with a combination of receptor signaling and supporting cytokines [Ettinger 2005]. After 5 days in culture, the initial homogeneous population of naïve B cells had evolved into two obvious subsets: one that retained the naïve B cell phenotype (IgD^+) and the other that exhibited an activated B cell phenotype (IgD^-). When examined for the expression of CB_2, there was a clear distinction between these two subsets with a loss of extracellular CB_2 only on the activated subset. Collectively, the evidence presented in this report points to a clear linkage between the acquisition of an "activated" B cell phenotype and specific regulation of CB_2 protein expression.

With limited information regarding the nature of intracellular CB_2, we employed a combination of confocal microscopy and marker co-localization studies to evaluate the distribution and location of intracellular CB_2. It exhibited a diffuse but punctate pattern within the cytoplasm. This appearance was the same regardless of the type of cells studied – primary peripheral blood B cells, the SUDHL-4 cell line, or the 293T/CB_2-GFP line that we had previously described [Castaneda 2013]. Using the 293T/CB_2-GFP line, we compared the distribution of CB_2 staining to the staining of mitochondrial and lysosomal markers. The sparse and well defined features of lysosomal staining did not match and were not pursued further. On the other hand, the punctate

but diffuse pattern of mitochondrial staining shared some similarities to the pattern observed with CB_2. This represented an interesting observation given our prior findings that THC can disrupt cell energetics and mitochondrial transmembrane potential in airway epithelial cells in a CB_2-dependent manner [Sarafian 2008]. Along the same lines, Bernard and associates identified a similar effect on neuronal cells but ascribed this effect to intracellular CB_1, which localized to mitochondria in their studies. However, there was no obvious co-localization between the CB_2 receptor and mitochondrial markers when directly examined by dual staining and confocal microscopy.

In summary, we can conclude that the expression of CB_2 in human leukocytes appears to be specifically regulated with respect to the cellular location (cell membrane versus intracellular distribution), the cell lineage being studied (B cells as compared to T cells, monocytes, and dendritic cells), and the state of B cell activation and differentiation (activated versus the naïve and memory subsets). The presence of an activated phenotype on B cells is specifically associated with down-regulation of the surface CB_2 receptor, a feature identified in B cells recovered from human tonsils and also observed *in vitro* when naïve B cells were stimulated to acquire an activated phenotype. Given the capacity for cell surface CB_2 to form heterodimers with chemokine receptors and promote migration and homing and given the location of CB_2^+ and CB_2^- B cells in different compartments within lymphoid follicles [Basu 2013, Coke 2016], it is possible that modulating surface CB_2 during B cell activation plays an important role in trafficking. The capacity for T cells, dendritic cells, and malignant B cells to respond to cannabinoids in a CB_2-dependent manner has been well characterized [McKallip 2002, Roth 2015, Yuan 2002], yet these cells do not express CB_2 on the cell surface. The logical conclusion is that intracellular CB_2 must also be capable of mediating ligand-induced signaling and biological consequences. With the recent report by

Brailoiu et al (2014), there is now direct evidence for this. Given the high membrane solubility of cannabinoids, we hypothesize that the presence of CB_2 at different locations within a cell provides a mechanism for cells to link receptor activation to different signaling and biologic consequences, resulting in an expanded functional heterogeneity of cannabinoids. The intracellular location of CB_2 and the specific role of different receptors on biologic function remains to be determined but will likely be very informative in understanding cannabinoid biology.

REFERENCES

1. Agudelo M, Newton C, Widen R, Sherwood T, Nong L, Friedman H, Klein TW. Cannabinoid receptor 2 (CB2) mediates immunoglobulin class switching from IgM to IgE in cultures of murine-purified B lymphocytes. J Neuroimmune Pharmacol. 3(1):35-42, 2008.

2. Aizpurua-Olaizola O, Elezgarai I, Rico-Barrio I, Zarandona I, Etxebarria N, Usobiaga A. (2016). Targeting the endocannabinoid system: future therapeutic strategies. Drug Discov Today. Advance Online Publication. doi: 10.1016/j.drudis.2016.08.005.

3. Basu S, Dittel BN. Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. Immunol Res. 51:26-38, 2011.

4. Basu S, Ray A, Dittel BN. Cannabinoid Receptor 2 (CB2) Plays a Role in the Generation of Germinal Center and Memory B Cells, but Not in the Production of Antigen-Specific IgG and IgM, in Response to T-dependent Antigens. PLoS One. 27;8(6):e67587, 2013.

5. Bernard G, Massa F, Puente N, Lourenco J, Bellocchio L, Soria-Gomez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, Hebert-Chatelain E, Mulle C, Ortega-Gutierrez S, Martin-Fontecha M, Klugmann M, Guggenhuber S, Lutz B, Gertsch J, Chaouloff F, Lopez-Rodriguez ML, Grandes P, Rossignol R, Marsicano G. Mitochondrial CB1 receptors regulate neuronal energy metabolism. Nat. Neurosci. 15, 558–564, 2012.

6. Brailoiu GC, Deliu E, Marcu J, Hoffman NE, Console-Bram L, Zhao P, Madesh M, Abood ME, Brailoiu E. Differential activation of intracellular versus plasmalemmal CB2 cannabinoid receptors. Biochemistry. 5;53(30):4990-9, 2014.

7. Brailoiu GC, Oprea TI, Zhao P, Abood ME, Brailoiu E. Intracellular cannabinoid type 1 (CB1) receptors are activated by anandamide. J. Biol. Chem. 286, 29166–29174, 2011.

8. Cabral GA, Rogers TJ, Lichtman AH. Turning over a new leaf: cannabinoid and endocannabinoid modulation of immune function. J Neuroimmune Pharmacol. 10:193-203, 2015.

9. Carayon P, Marchand J, Dussossoy D, Derocq JM, Jbilo O, Bord A, Bouaboula M, Galiègue S, Mondière P, Pénarier G, Fur GL, Defrance T, Casellas P. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood. 15;92(10):3605-15, 1998.

10. Castaneda JT, Harui A, Kiertscher SM, Roth JD, Roth MD. Differential expression of intracellular and extracellular CB_2 cannabinoid receptor protein by human peripheral blood leukocytes. J Neuroimmune Pharm. 8(1):323-332, 2013. PMC3587044

11. Coke CJ, Scarlett KA, Chetram MA, Jones KJ, Sandifer BJ, Davis AS, Marcus AI, Hinton CV. Simultaneous Activation of Induced Heterodimerization between CXCR4 Chemokine Receptor andCannabinoid Receptor 2 (CB2) Reveals a Mechanism for Regulation of Tumor Progression. J Biol Chem. 6;291(19):9991-10005, 2016.

12. Eisenstein TK, Meissler JJ. Effects of Cannabinoids on T-cell Function and Resistance to Infection. J Neuroimmune Pharmacol. 10(2):204-16, 2015.

13. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE. IL-21 induces differentiation of human naïve and memory B cells into antibody-secreting plasma cells. J Immunol. 175(12):7867-7879, 2005.

14. Flordellis CS. The plasticity of the 7TMR signaling machinery and the search for pharmacological selectivity. Curr Pharm Des. 18(2):145-60, 2012.

15. Gaudet HM, Cheng SB, Christensen EM, Filardo EJ. The G-protein coupled estrogen receptor, GPER: The inside and inside-out story. Mol Cell Endocrinol. 15;418 Pt 3:207-19, 2015.

16. Graham ES, Angel CE, Schwarcz LE, Dunbar PR, Glass M. Detailed characterization of CB2 receptor protein expression in peripheral blood immune cells from healthy human volunteers using flow cytometry. Int J Immunopathol Pharmacol. 23(1):25-34, 2010.

17. Gómez-Cañas M, Morales P, García-Toscano L, Navarrete C, Muñoz E, Jagerovic N, Fernández-Ruiz J, García-Arencibia M, Pazos MR. Biological characterization of PM226, a chromenoisoxazole, as a selective CB2 receptor agonist with neuroprotective profile. Pharmacol Res. 110:205-15, 2016.

18. Hegde VL, Nagarkatti M, Nagarkatti PS. Cannabinoid receptor activation leads to massive mobilization of myeloid-derived suppressor cells with potent immunosuppressive properties. Eur J Immunol. 40(12):3358-71, 2010.

19. Jorda MA, Rayman N, Valk P, De Wee E, Delwel R. Identification, characterization, and function of a novel oncogene: the peripheral cannabinoid receptor CB2. Ann N Y Acad Sci. 996:10-6, 2003.

20. Klein TW, Cabral GA. Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. J Neuroimmune Pharmacol. 1(1):50-64, 2006.

21. Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L, Friedman H. The cannabinoid system and immune modulation. J Leukoc Biol. 74(4):486-96, 2003.

22. Maccarrone M, Bab I, Biro T, Cabral GA, Dey SK, Di Marzo V, Konje JC, Kunos G, Mechoulam R, Pacher P, Sharkey KA, Zimmer A. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 36(5):277-96, 2015.

23. McKallip RJ, Lombard C, Martin BR, Nagarkatti M, Nagarkatti PS. Delta(9)-tetrahydrocannabinol-induced apoptosis in the thymus and spleen as a mechanism of immunosuppression in vitro and in vivo. J Pharmacol Exp Ther. 302(2):451-65, 2002.

24. Pacher P, Bátkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev. 58(3):389-462, 2006.

25. Pérez-Gómez E, Andradas C, Blasco-Benito S, Caffarel MM, García-Taboada E, Villa-Morales M, Moreno E, Hamann S, Martín-Villar E, Flores JM, Wenners A, Alkatout I, Klapper W, Röcken C, Bronsert P, Stickeler E, Staebler A, Bauer M, Arnold N, Soriano J, Pérez-Martínez M, Megías D, Moreno-Bueno G, Ortega-Gutiérrez S, Artola M, Vázquez-Villa H, Quintanilla M, Fernández-Piqueras J, Canela EI, McCormick PJ, Guzmán M, Sánchez C. Role of Cannabinoid Receptor CB_2 in HER2 Pro-oncogenic Signaling in Breast Cancer. J Natl Cancer Inst. 107(6):djv077, 2015.

26. Roth MD, Whittaker K, Salehi K, Tashkin DP, Baldwin GC. Mechanisms for impaired effector function in alveolar macrophages from marijuana and cocaine smokers. J Neuroimmunol. 147(1-2):82-6, 2004.

27. Rayman N, Lam KH, Laman JD, Simons PJ, Löwenberg B, Sonneveld P, Delwel R. Distinct expression profiles of the peripheral cannabinoid receptor in lymphoid tissues depending on receptor activation status. J Immunol. 15;172(4):2111-7, 2004.

28. Sarafian T, Kouyoumjian S, Khoshaghideh F, Tashkin DP, Roth MD. Delta 9-tetrahydrocannabinol disrupts mitochondrial function and cell energetics. AM J Physiol Lung Cell Mol Physiol. 284:L298-L306, 2003.

29. Sarafian T, Montes C, Harui A, Beedanagari SR, Kiertscher S, Stripecke R, Hossepian D, Kitchen C, Kern R, Belperio J, Roth MD. Clarifying CB2 receptor-dependent and independent effects of THC on human lung epithelial cells. Toxicol Appl Pharmacol. 231(3):282-90, 2008.

30. Schmöle AC, Lundt R, Gennequin B, Schrage H, Beins E, Krämer A, Zimmer T, Limmer A, Zimmer A, Otte DM. Expression Analysis of CB2-GFP BAC Transgenic Mice. PLoS One. 10(9):e0138986, 2015.

31. Yuan M, Kiertscher SM, Cheng Q, Zoumalan R, Tashkin DP, Roth MD. Δ^9-Tetrahydrocannabinol Regulates Th1/Th2 Cytokine Balance in Activated Human T-Cells. J. Neuroimmunol. 133(1-2):124-131, 2002.

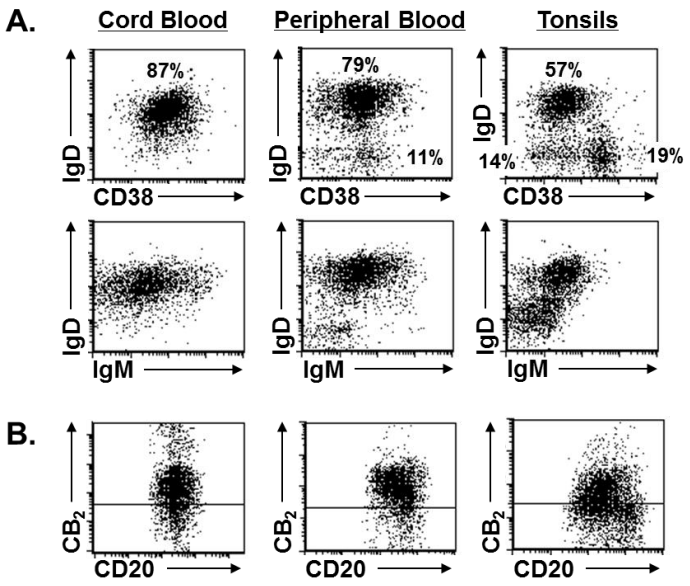


Fig 1. Heterogeneity of B cell populations and CB_2 expression in leukocytes obtained from cord blood, adult peripheral blood, and tonsils. (A) Leukocytes from umbilical vein cord blood, peripheral blood, and tonsils were stained with fluorescent antibody and gated to express only viable events within the $CD20^+/CD3^-$ B cell region. 5-color staining was used to identify the distribution of cells exhibiting IgD and IgM, expressed only on naive mature B cells, and CD38, which is dim on naive mature B cells, positive on activated B cells, and dim/negative on quiescent memory B cells. Percentages for each population are listed. **(B)** Cells within the $CD20^+/CD3^-$ gate were also evaluated for expression of extracellular CB_2 using a primary unlabeled mAb against CB_2 protein followed by secondary staining with APC-labeled GAM. Background staining (horizontal line) was set by staining cells with an unlabeled isotype-matched irrelevant target (anti-mouse NK1.1) followed by secondary staining with APC-labeled GAM. Representative experiment shown, n = 6.

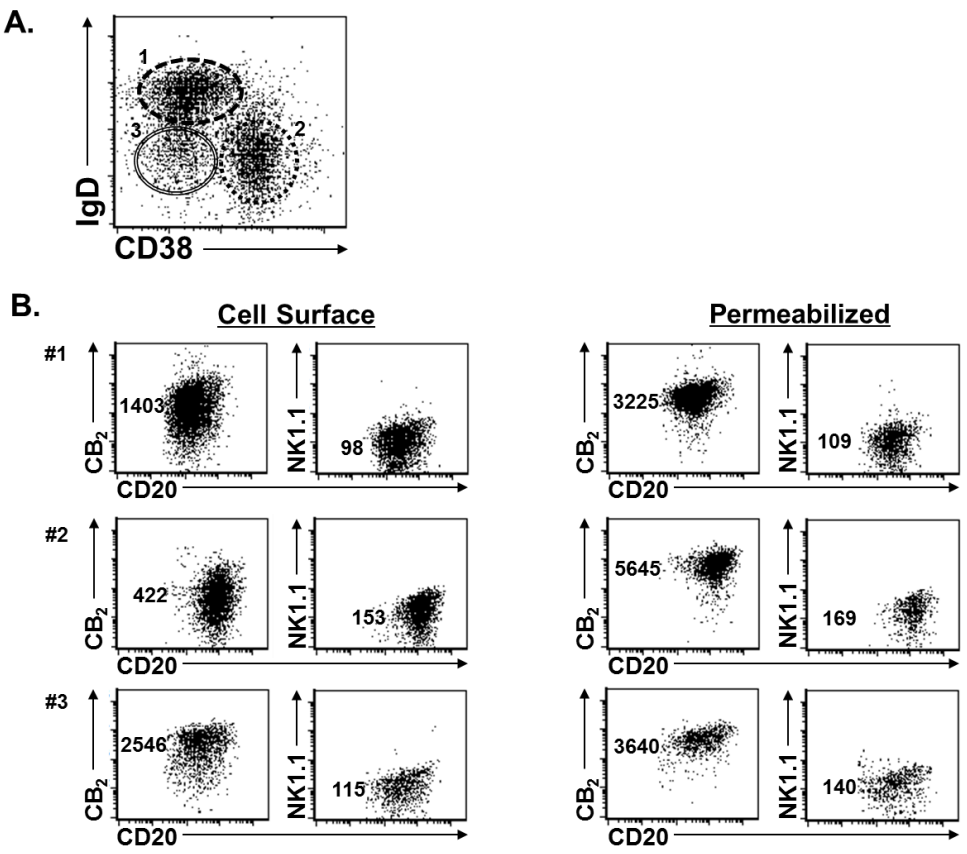


Fig 2. Surface expression of CB_2, but not total cellular CB_2, is limited in activated B cells recovered from tonsils. (A) B cells within the gate for viable $CD20^+/CD3^-$ events obtained from mechanically-digested human tonsils were classified into three subsets based on IgD and CD38 expression patterns: (#1) naïve mature (dashed line), (#2) activated (dotted line), and (#3) quiescent memory (solid line). **(B)** B cells within each of these three subset classifications (#1-#3, correspondingly) were then stained with either anti-CB_2 or anti-NK1.1 (isotype control) while still viable for the detection of cell surface expression (left panel) or after being fixed and permeabilized for the detection of total cellular expression (right panel). Numbers represent relative mean fluorescent intensity for staining on the Y axis. Representative experiment, n= 3.

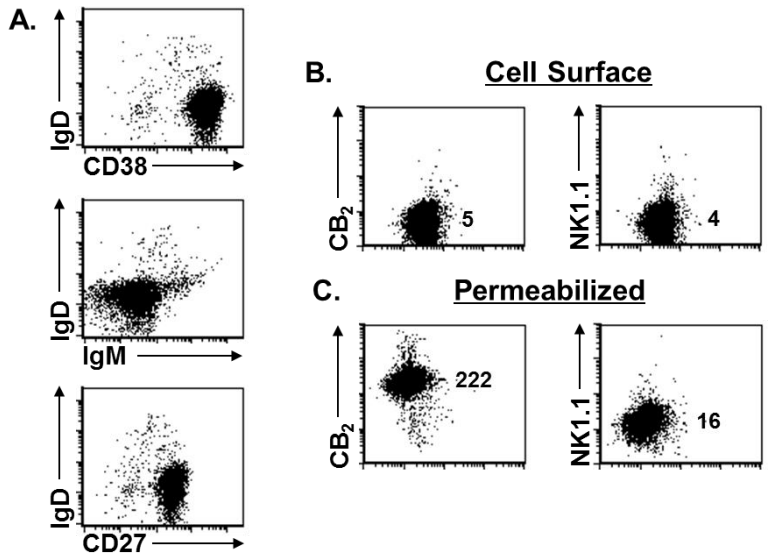


Fig 3. A malignant B cell line expressing an activated phenotype exhibits the same pattern of CB_2 expression as that observed with primary activated B cells from tonsils. (A) Cells from the malignant B cell lymphoma cell line, SUDHL-4, exhibited cell surface markers consistent with an activated B cell subset based on the expression pattern for IgD (negative), anti-CD38 (positive), and anti-CD27 (positive). **(B)** Cells were stained while still viable for detection of cell surface CB_2 with a primary unlabeled mAb against CB_2 protein or isotype control, NK1.1, and then stained with APC-labeled GAM. **(C)** For total cell expression of CB_2, cells were fixed and permeabilized prior to specific staining. Numbers represent relative mean fluorescent intensity for staining on the Y axis. Representative experiment shown, n = 3

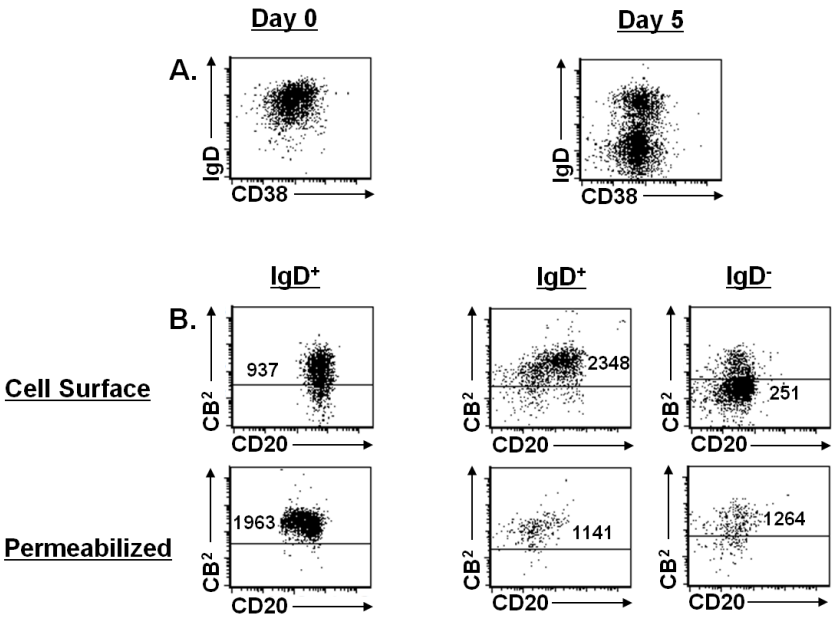


Fig 4. Changes in CB_2 expression when human naïve B cells are activated *in vitro* and acquire the phenotype of activated B cells. (A) 1 x 10^6 umbilical vein cord blood B cells/mL were cultured for 5 days at 37°C and activated *in vitro* by addition of 100 ng/mL IL-21, 100-250 ng/mL mega-CD40L, 5 µg/mL anti-IgM and 100 ng/mL IL-4 in complete medium. Fresh resting cells (day 0) and activated cells (day 5) were gated on the viable $CD20^+/CD3^-$ population and analyzed by flow cytometry for the expression of subset markers to identify naïve mature B cells ($IgD^+/CD38^{Dim}$) and activated B cells ($IgD^-/CD38^+$). **(B)** Gated cells exhibiting the phenotype of either naïve (IgD^+) or activated (IgD^-) B cells were then independently evaluated for fluorescence produced by anti-CB_2 mAb. Live cells were stained to measure cell surface staining while total cell expression of CB_2 (intracellular plus extracellular) was determined in cells that were fixed and permeabilized prior to CB_2 staining. Numbers represent relative mean fluorescent intensity for staining on the Y axis. Representative experiment shown, n = 6

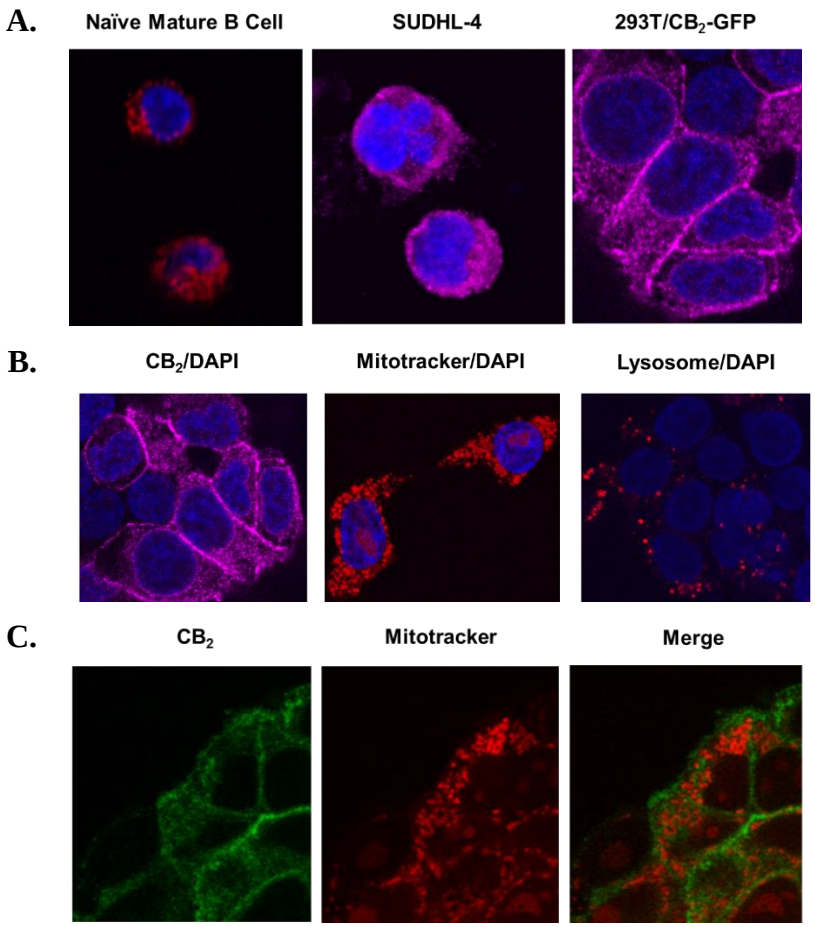


Fig 5. Intracellular CB_2 is expressed in a diffuse but punctate pattern that is distinct from the distribution of the lysosomal and mitochondrial compartments. (A) $CD20^+/CD3^-$ naïve mature B cells purified from peripheral blood, cells prepared from the SUDHL-4 lymphoma cell line, and 293T/CB_2-GFP cells were fixed, permeabilized, and stained with mAb against CB_2 protein (displayed as red/magenta) and mounted with SlowFade® Diamond Antifade Mountant with DAPI (blue) prior to examination by confocal fluorescence microscopy. Magnification 63X; 10-20 sections/cell with an SP5 blue confocal microscope. **(B)** 293T/CB_2-GFP cells were grown on poly-l-lysine coated coverslips, fixed, permeabilized and stained with either CB_2 mAb (left, magenta), MitoTracker (middle, red), or LysoTracker (right, red) prior to mounting with SlowFade® Diamond Antifade Mountant with DAPI (blue). CB_2 and MitoTracker stained

cells were fixed with 1% paraformaldehyde for 20 min at 4°C and later imaged. LysoTracker stained cells were imaged immediately without fixation. Cells were imaged in 10-20 sections with an SP5 blue confocal microscope. Magnification 63X. **(C)** For co-localization imaging, 293T-CB_2-GFP cells grown on poly-l-lysine coated coverslips were first stained with MitoTracker (Red) for 2 hr at 37°C, then fixed, permeabilized, and stained with anti-CB_2 mAb (green) for 30 min at 4°C. Cells were fixed, mounted and imaged in 10-20 sections with an SP5 blue confocal microscope. Magnification 63X.

CHAPTER 5

SUMMARY AND DISCUSSION

As of the year 2016, four states and Washington D.C. have legalized the use of marijuana for recreational purposes, and an additional 19 states have legalized marijuana for medicinal purposes. The rise of legalization has also led to a rise in usage due to the assumption that marijuana has no overall effect on human health. Despite the widespread use of marijuana and its controversial toxic versus medicinal effects, there is relatively little information known about the effects of cannabinoids and CB_2 on human immunity. The research detailed in this dissertation focuses on the CB_2 receptor and how understanding the differential CB_2 expression patterns could link the CB_2 receptor to the biologic function of human leukocytes. While it is important to understand the immunotoxic effects that might result from marijuana smoking, it is equally important to understand how the CB_2 receptor might be exploited to control inflammation and regulate adaptive immunity from a therapeutic perspective. This work will promote a better understanding of how the CB_2 receptor in leukocytes can initiate downstream signaling and induce both the toxic consequences and potential beneficial effects of cannabinoids, which could eventually lead to the development of new targeted ligands.

The concept of CB_2 as a simple GPCR expressed on the surface of human leukocytes [Graham 2010, Klein 2003] has been challenged by a number of recent findings. Addressing this statement had been particularly difficult due to the lack of reliable tools that could measure CB_2 at the cell surface or intracellular locations. CB_2 protein detection had been challenging due to non-specific staining of primary antibodies and use of CB_2 polyclonal antibodies that can be cross-reactive to other proteins [Graham 2010]. The use of CB_2 antibodies with western blots had also been problematic due to the presence of multiple bands of similar molecular weight expected to be CB_2 isoforms [Marchalant 2014]. In addition to the technical difficulties, Graham and associates have suggested that CB_2 was highly expressed on all PBMC, contrasting with our preliminary

findings. This discrepancy can be attributed to the use of commercial polyclonal rabbit or goat antibodies from different companies without any controls. The staining varied for every manufacturer, from batch to batch, and from subject to subject.

Previous evidence from our laboratory describes a lack of cell surface expression in T cells, but T cells have been described to have reduced proliferation, activation, and cytokine production when exposed to THC [Chen 2012, Eisenstein 2015, Yuan 2002]. The logical conclusion is that CB_2 must exist at an intracellular location and that intracellular CB_2 must also be capable of mediating ligand-induced signaling and biological consequences. In chapter 3, Castaneda et al. describes that in order to overcome this hurdle, we constructed cell lines expressing different levels of human CB_2 and used a commercial anti-CB_2 mAb to develop a sensitive and specific flow cytometry assay for detecting CB_2 protein at the cell surface and at intracellular locations. We reported that CB_2 is expressed on the cell surface of B cells and at intracellular locations, while T cells and monocytes lack CB_2 cell surface expression but express CB_2 at intracellular locations. The significance of the differential expression of CB_2 at extracellular and intracellular locations remains unknown but can possibly play an important role in the immune response to cannabinoids.

Also in chapter 3, Roth et al., describes how we successfully applied the model that we designed in Castaneda et al. to the CB_1 receptor. The presence of CB_1 in the human immune system is not commonly described since CB_2 is the more prevalent receptor. With our novel flow cytometry approach, we report that CB_1 is not present on the cell surface of monocytes but indeed present at intracellular sites. This finding is vital for the understanding of how cannabinoids exert their immunotoxic effects since intracellular binding can occur to both CB_1 and CB_2 receptors in human leukocytes versus the common concept of only binding to CB_2. Further understanding of the balance between extracellular and intracellular cannabinoid receptor expression could also

provide insight as to how cannabinoids mediate their toxic effects and induce intracellular signaling.

As previously stated, B cells are the only leukocyte that we have examined to express CB_2 on both the cell surface and at intracellular locations. The biologic basis as to why extracellular CB_2 is not expressed in T cells, monocytes, and dendritic cells is not known, but there is evidence that suggests that the presence of GPCRs at different cellular locations can promote functional heterogeneity with respect to downstream signaling and biologic responses [Flordellis 2012, Gaudet 2015]. Also intracellular forms for both CB_1 and CB_2 have been described to exert distinct biologic effects [Brailoiu 2011, Bernard 2012, Gómez-Cañas 2016]. Understanding the distribution, regulation, and dynamic balance between cell surface and intracellular CB_2 receptors will likely provide important insight regarding cannabinoid receptor biology.

In order to further assess the role of CB_2 at different locations, chapter 4 describes how we investigated the expression of CB_2 in B cells from multiple sources in the human body. Cord blood was composed of mostly naïve mature B cells, while peripheral blood included a mixture of naïve mature and memory B cells. Tonsils were the only source of B cells to express cells at three different stages of differentiation: naïve mature, activated, and memory. Interestingly, out of all of the subsets that were phenotyped, the only subset of B cells to not express cell surface CB_2 was the activated subset of B cells found in tonsils. All B cells from the three sources expressed intracellular CB_2. In accordance with this finding, a B cell lymphoma cell line, SUDHL-4, that was characterized as "activated" due to its IgD vs CD38 profile also did not express CB_2 on the cell surface but had high intracellular stores of CB_2. This finding correlates with the pattern observed in the activated subset of B cells in tonsils. In summary, we observed that B cells in an activated state do not express cell surface CB_2.

In order to further determine the role of activation in differential CB_2 expression patterns, we activated naïve mature cord blood B cells *in vitro* by engaging the B cell receptor with co-stimulatory molecules and cytokines. We were able to induce activation *in vitro* and track the different stages of B cell differentiation and maturation along with CB_2 expression. We determined that the naïve mature B cells that underwent activation showed a decrease in cell surface CB_2 expression. Cells that did not undergo activation after culture and still expressed maturation markers, IgD and IgM, expressed CB_2 on the surface. This finding determined that there is a link between loss of CB_2 expression and activation and that there is a novel and dynamic multi-compartment expression pattern for CB_2 in B cells that is specifically modulated during B cell activation. Further investigation is needed in order to better understand the two-way interaction between activation and CB_2 expression.

The presence of CB_2 at different locations within a cell can possibly provide a mechanism for cells to link receptor activation to different signaling and biologic consequences, resulting in an expanded functional heterogeneity of cannabinoids. In order to further determine the role of intracellular CB_2, we examined intracellular CB_2 expression patterns and potential colocalization with different organelle markers through the use of confocal microscopy. We determined that the CB_2 receptor does not colocalize with mitochondrial or lysosomal markers. This is a very interesting finding due to the fact that after exposure to THC, lung epithelial cells, A549, have reduced mitochondrial function and cell energetics [Sarafian 2003]. The intracellular location of CB_2 and the specific role of receptor location on biologic function remains to be determined.

Based on the findings in chapter 4, we can conclude that differential CB_2 expression patterns are not just a product of cell to cell differences. Distribution is different based on activation, and activation is a key event in isotype switching. With the *in vitro* activation assay that

we developed in chapter 4, we are also able to isotype switch cells *in vitro* and assess the role of CB_2 in isotype switching. In order to assess isotype switching, B cells must lose both cell surface maturation markers, IgD and IgM, and switch to a different immunoglobulin subtype: IgG, IgA, or IgE. We are particularly interested in IgE expression since IgE expression is characteristic of an allergic phenotype. Marijuana usage has been associated with symptoms similar to an allergic reaction [Mimura 2012]. With the model that we employed by activating B cells with a combination of mega-CD40 ligand, IL-21, and IL-4, we were able to skew isotype switching *in vitro* towards an IgE subtype. This isotype switching model will now be crucial to determine how exposure to cannabinoids can affect CB_2 expression patterns and the biologic function of B cells, such as isotype switching and antibody production.

In conclusion, we have developed sensitive and specific models to measure cell surface and intracellular expression of the CB_2 receptor, and we are able induce *in vitro* activation and isotype switching in human leukocytes. We can conclude that the expression of CB_2 in human leukocytes appears to be specifically regulated with respect to the cellular location (cell membrane versus intracellular distribution), the cell lineage being studied (B cells as compared to T cells, monocytes, and dendritic cells), and the state of B cell activation and differentiation (activated versus the naïve and memory subsets). Cannabinoid ligands can access and activate receptors at cell surface and intracellular locations and perhaps with different relative efficiencies. These results suggest that it is possible that modulating surface CB_2 during B cell activation can play an important role in trafficking and contribute to the prevention of autoreactive responses. These results are of primary interest to the field of cannabinoid receptor biology and directly relevant to the understanding of the potential toxic effects of cannabinoids on immune function and how the cannabinoid/CB_2 pathway might be exploited for immunotherapeutic purposes. Our novel studies

provide insight into the biologic role and function of intracellular CB_2 and the importance of receptor location on function. Understanding the dynamic balance between CB_2 receptor location and intracellular signaling could play a role in humoral memory immune responses that could eventually lead to long-term protective therapeutics or vaccinations. Further research is needed to provide new insight into the differential expression of the CB_2 receptor and the complex role of cannabinoids on immune responses and contribute to the characterization of defects that lead to autoimmune diseases.

REFERENCES

1. Bernard G, Massa F, Puente N, Lourenco J, Bellocchio L, Soria-Gomez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, Hebert-Chatelain E, Mulle C, Ortega-Gutierrez S, Martin-Fontecha M, Klugmann M, Guggenhuber S, Lutz B, Gertsch J, Chaouloff F, Lopez-Rodriguez ML, Grandes P, Rossignol R, Marsicano G. Mitochondrial CB1 receptors regulate neuronal energy metabolism. Nat. Neurosci. 15, 558–564, 2012.

2. Brailoiu GC, Oprea TI, Zhao P, Abood ME, Brailoiu E. Intracellular cannabinoid type 1 (CB1) receptors are activated by anandamide. J. Biol. Chem. 286, 29166–29174, 2011.

3. Chen W, Kaplan BL, Pike ST, Topper LA, Lichorobiec NR, Simmons SO, Ramabhadran R, Kaminski NE. Magnitude of stimulation dictates the cannabinoid-mediated differential T cell response to HIVgp120. J Leukoc Biol. 92(5):1093-102, 2012.

4. Castaneda JT, Harui A, Kiertscher SM, Roth JD, Roth MD. Differential expression of intracellular and extracellular CB_2 cannabinoid receptor protein by human peripheral blood leukocytes. J Neuroimmune Pharm. 8(1):323-332, 2013. PMC3587044

5. Eisenstein TK, Meissler JJ. Effects of Cannabinoids on T-Cell Function and Resistance to Infection. J Neuroimmune Pharmacol. 10(2)204-216, 2015.

6. Flordellis CS. The plasticity of the 7TMR signaling machinery and the search for pharmacological selectivity. Curr Pharm Des. 18(2):145-60, 2012.

7. Gaudet HM, Cheng SB, Christensen EM, Filardo EJ. The G-protein coupled estrogen receptor, GPER: The inside and inside-out story. Mol Cell Endocrinol. 15;418 Pt 3:207-19, 2015.

8. Gómez-Cañas M, Morales P, García-Toscano L, Navarrete C, Muñoz E, Jagerovic N, Fernández-Ruiz J, García-Arencibia M, Pazos MR. Biological characterization of PM226, a chromenoisoxazole, as a selective CB2 receptor agonist with neuroprotective profile. Pharmacol Res. 110:205-15, 2016.

9. Graham ES, Angel CE, Schwarcz LE, Dunbar PR, Glass M. Detailed characterization of CB2 receptor protein expression in peripheral blood immune cells from healthy human volunteers using flow cytometry. Int J Immunopathol Pharmacol. 23(1):25-34, 2010.

10. Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L, Friedman H. The cannabinoid system and immune modulation. J Leukoc Biol. 74(4):486-96, 2003.

11. Marchalant Y, Brownjohn PW, Bonnet A, Kleffmann, Ashton JC. Validating Antibodies to the Cannabinoid CB2 Receptor: Antibody Sensitivity Is Not Evidence of Antibody Specificity. J Histochem Cytochem. 62(6):395-404, 2014.

12. Mimura T, Ueda Y, Watanabe Y, Sugiura T. The cannabinoid receptor-2 is envolved in allergic

inflammation. Life Sci. 90:862-66, 2012.

13. Roth MD, Castaneda JT, Kiertscher SM. Exposure to Δ9-Tetrahydrocanninol Impairs the Differentiation of Human Monocyte-derived Dendritic Cells and their Capacity for T cell Activation. J Neuroimmuno Pharmacol. 10:333-343, 2015.

14. Sarafian T, Kouyoumjian S, Khoshaghideh F, Tashkin DP, Roth MD. Delta 9-tetrahydrocannabinol disrupts mitochondrial function and cell energetics. AM J Physiol Lung Cell Mol Physiol. 284:L298-L306, 2003.

15. Yuan M, Kiertscher SM, Cheng Q, Zoumalan R, Tashkin DP, Roth MD. Δ^9-Tetrahydrocannabinol Regulates Th1/Th2 Cytokine Balance in Activated Human T-Cells. J. Neuroimmunol. 133(1-2):124-131, 2002.