MODULATION OF THE IMMUNE RESPONSE TO INFLUENZA VIRUS BY THE CANNABINOID RECEPTORS 1 AND 2

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ABSTRACT

MODULATION OF THE IMMUNE RESPONSE TO INFLUENZA VIRUS BY THE CANNABINOID RECEPTORS 1 AND 2

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Cannabinoids are bioactive signaling molecules exerting several effects, including modulation of immune function. One of the agents studied for its immunosuppressive activity, Δ9-tetrahydrocannabinol (Δ9-THC), is thought to exert part of its activity on immune cells via interaction with cannabinoid receptors 1 (CB1) and 2 (CB2). In previous studies Δ9-THC caused an increase in steady state viral hemagglutinin RNA levels, suggesting decreased viral clearance due to compromised immune function. In addition, in CB1 and CB2 double knock out (CB1-/-CB2-/-) mice, a reduced viral burden and greater magnitude in the immune response were observed, suggesting a role for CB1 and/or CB2 in regulating immune function. The identity of the functionally dysregulated immune population(s) as a result of CB1 and CB2 deletion were unknown. In addition, the effect of Δ9-THC treatment on the cellular immune response to influenza remained to be elucidated. Overall, this dissertation project was designed to test the hypothesis that Δ9-THC suppresses immune responses generated by influenza virus by perturbing an endogenous signaling pathway relying in part on the presence of CB1 and/or CB2. In the present studies, Δ9-THC suppressed cytotoxic T lymphocyte (CTL) function independent of CB1 and CB2, suggesting an alternate mechanism for immune modulation by Δ9-THC in these cells. Second, a kinetic study of influenza infection demonstrated greater immune response magnitude in CB1-/-CB2-/- compared to WT mice. This was evidenced by

elevated steady-state mRNA levels for genes involved in the inflammatory response, earlier T cell activation, greater percentages of cytokine-secreting effector cells, and increased immunopathology in lung tissue. These events likely lead to lower viral burden at the expense of greater tissue damage in CB1-/-CB2-/- mice compared to WT. These differences observed *in vivo* were not reproduced by stimulation of T cells *in vitro*, suggesting differences in antigen presenting cells (APC), which elicit effector cells. Dendritic cells (DC) generated from bone marrow (bmDC) and alveolar macrophages (AM) isolated from CB1-/-CB2-/- mice had greater maturation with and without Toll-like receptor (TLR) stimulation compared to WT mice and bmDC elicited antigen-specific T cells without the need for a maturation stimulus. Third, focusing on the peak day of the inflammatory response induced by influenza virus at day 3, the immune response of APC was characterized in WT and CB1-/-CB2-/- mice in the presence of Δ9-THC. Δ9-THC suppressed influx of APC, including conventional DC (cDC), plasmacytoid DC (pDC), and AM into the lung after influenza infection in WT only. While there was no change in maturation of lung-isolated APC due to Δ9-THC treatment, only mature APC reach the lung, thus Δ9-THC might have affected maturation of APC prior to migration to the lung. Indeed, Δ9-THC suppressed DC maturation and ability to elicit antigen-specific T cells *in vitro*. Taken together, Δ9-THC impairs the immune response to influenza, in part, by suppressing APC function in a CB1 and/or CB2 dependent manner, while suppressing CTL elicitation in a CB1 and CB2 independent manner. Also, the presence of CB1 and/or CB2 on APC prevents exacerbated immune responses after influenza infection. These studies suggest that CB1 and/or CB2 maintain immune homeostasis and prevent excessive reactivity after immune stimulation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

-ssRNA	Negative single stranded RNA
Δ9-THC	Δ9-Tetrahydrocannabinol
11-OH	11-hydroxy
2-AG	2-arachidonoylglycerol
A/PR/8/34	Influenza virus strain A, Puerto Rico, Isolate 8, 1934
AEA	*N*-arachidonoylethanolamide or anandamide
AIDS	Acquired immunodeficiency syndrome
AM	Alveolar macrophage
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ASK1	Apoptosis signal-regulating kinase 1
AT-II	Alveolar type II cells
BALF	Broncho-alveolar lavage fluid
BALT	Bronchus associated lymphoid tissue
BCS	Bovine calf serum
bmDC	Bone marrow-derived dendritic cell
C	Celsius
CASP-1	Caspase-1
CB1	Cannabinoid receptor 1

CB1-/-	Mice null for CB1
CB2	Cannabinoid receptor 2
CB2-/-	Mice null for CB2
CB1-/-CB2-/-	Mice null for both CB1 and CB2
CCL20	Chemokine (C-C motif) ligand 20 also known as Macrophage Inflammatory Protein-3 alpha (MIP-3α)
CCSP	Clara cell secretory protein
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CO	Corn oil
CO2	Carbon dioxide
COOH	Carboxylic acid
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T lymphocyte
CYP	Cytochrome P450 enzyme
DAG	Diacylglycerol
DC	Dendritic cell
DHBP	Dihydrobenzopyran
dsRNA	Double stranded RNA
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine Serum

GFP	Green fluorescent protein
GMCSF	Granulocyte macrophage colony-stimulating factor
GMCSFR	Granulocyte macrophage colony-stimulating factor receptor
GPCR	G-protein coupled receptor
GRK	G protein-coupled receptor kinases
H	Hemagglutinin
h	Hours
HAART	Highly active antiretroviral therapy
HSV	Herpes simplex virus
IFN	Interferon
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IL	Interleukin
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-12p40	Interleukin 12 beta subunit 40kD
IL-17	Interleukin 17
Ig	Immunoglobulin
IκB	Inhibitor of kappa B
Io	Ionomycin
IP3	Inositol trisphosphate

IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
JAK-STAT	Janus tyrosine kinase-signal transducer and activator of transcription
JNK3	C-Jun N-terminal kinase 3
Kv channel	Voltage-gated potassium channel
LD50	Lethal dose at which half of the tested animals die
LPI	Lysophosphatidylinositol
LPS	Lipopolysaccharide
M1	Matrix protein (influenza)
M2	Ion channel protein (influenza)
Mac/Mθ	Macrophage/monocyte
MAP kinase	Mitogen-activated protein kinase
MDA5	Melanoma differentiation associated gene-5
MDSC	Myeloid derived suppressor cells
MGL	Monoglyceride lipase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MKK4	Mitogen-activated protein kinase kinase 4
MyD88	Myeloid differentiation primary response gene (88)
N	Neuraminidase
NA	Naïve
NAD	Normal antibody diluent

NALT	Nose associated lymphoid tissue
NFκB	Nuclear factor kappa B
NFAT	Nuclear factor of activated T cells
NK cell	Natural killer cell
NLR	NOD-like receptors
NLRP3	NLR pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain-containing (NOD)
NP	nucleoprotein
OT-1	TCR receptor transgenic mice for specific for OVA257-264 (SIINFEKL)
PA	Polymerase subunit A (influenza)
PAMP	Pathogen-associated molecular pattern
PB1	Polymerase subunit B1 (influenza)
PB2	Polymerase subunit B2 (influenza)
pDC	Plasmacytoid dendritic cell
pfu	Plaque-forming units
PI	Phosphatidylinositol
PKR	Double-stranded RNA-dependent protein kinase
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PPARγ	Peroxisome proliferator-activated receptor gamma
PR8	A/PR/8/34 (short form)
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid-induced gene-I

ROS	Reactive oxygen species
RXRα	Retinoid X receptor alpha
SAL	saline
SAS	Statistical Analysis System
Sp	Surfactant protein
SPLC	Splenocytes
ssRNA	Single stranded RNA
TAK1	Transforming growth factor β–activated kinase 1
TAB	TAK1 binding protein
TBS	Tris buffered saline
TGFβ	Transforming growth factor beta
Th cell	T helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta
TRPC1	Transient receptor potential channel 1
UC	Ulcerative colitis
VH	vehicle
vRNA	Viral RNA
WT	Wild type

INTRODUCTION

I. Respiratory Tract and Lung Associated Immune Defenses against Pathogens

A) Respiratory Tract Anatomy and Function

The respiratory tract's main function in healthy individuals is to facilitate gas exchange of oxygen acquired from the atmosphere and carbon dioxide produced inside the body at the alveolus. The main purpose of oxygen inside the cell is to serve as an electron acceptor in the generation of energy in the form of adenosine triphosphate from carbon-containing energy sources inside the mitochondria. The respiratory tract starts at the nose, continues through the trachea, bronchi, lung lobes, bronchioles, and alveoli [1]. Furthermore, due to its large surface area and contact with the external environment it is a site for antigen exposure and mucosal immune reactions [2-4].

B) Differences in the Respiratory Tract between Humans and Rodents

There are differences in anatomy, respiration, and cellular composition of the lung in rodents such as the mouse compared to human. Anatomically, aside from obvious size differences, mice have four right (apical, cardiac, azygous, and diaphramatic) lung lobes and one left lung lobe, while humans have three right (superior, middle, and inferior) and two (superior and inferior) left lung lobes. Unlike humans, rodents do not have respiratory bronchioles, their respiratory tract is linear, their noses differ in anatomy and cell distribution, and their airways have a proportionally greater diameter [5-8]. Functionally, rodents are obligate nose breathers, while humans are only obligate nose breathers as infants and later develop into naso-oral breathers [8-10]. Furthermore, the lungs of mice contain more Clara cells than humans [11-13].

Aside from these few differences, the mouse is a suitable model to study immune responses including those in the lung, due to the similarities to immune responses elicited to influenza for example, as discussed below.

C) Lung Associated Lymphoid Organs

The lung consists of a large mucosal surface and due to inhalation presents a site of chronic non-pathogenic and occasional pathogenic antigen exposure [3, 14]. While the nose, mouth, and trachea have a natural flora of bacteria, the airways and alveoli are sterile in healthy individuals [15-17]. The lung harbors resident immune cells, including alveolar macrophages (AM) and dendritic cells (DC). In addition, the lung is highly perfused resulting in frequent traffic of immune cells through lung tissue [14]. Lymphoid structures associated with the respiratory tract include the cervical lymph nodes, hilar lymph nodes and mediastinal lymph nodes, bronchus associated lymphoid tissue (BALT), and nose associated lymphoid tissue (NALT). However, the cervical lymph nodes and NALT seem to be less important in the generation of some immune responses such as those to influenza [18]. Other cells, including epithelial cells, perform immune functions, too, as discussed in more detail below. BALT and NALT consists of immune cell aggregates in the mucosa, including T cells, B cells, DC, macrophages, and high endothelial venules, which allow exit of immune cells from the blood and entry into the lymphatics. In addition, the formation of BALT can be induced by infection, especially during chronic infections [14, 19-22]. The size of the BALT varies dramatically among mouse strains and a particularly small BALT has been observed in the C57Bl/6 strain compared to other strains [23].

D) Mechanical Defenses

The first lines of defense in the respiratory tract are the nose, which acts to filter inhaled air and the larynx, which acts as a sphincter during expectoration and cough, reducing backflow of inhaled substances [24]. The cough reflex acts as a mechanical defense to expel mucosubstances potentially containing pathogens [25]. Despite these first defenses, inhaled particles reach the respiratory epithelium. Due to constant exposure to environmental antigens, the respiratory epithelium has several protective mechanisms against infection and various means to regulate immune responses in the lung [26]. The physical barrier of the respiratory epithelium presents another line of defense to insult. Also, the mucociliary apparatus constantly moves inhaled particles that have deposited on the mucous layer of the respiratory tract via sedimentation, impaction, Brownian diffusion, interception, and electrostatic precipitation out of the airways [27, 28]. Mucus contains several antimicrobial factors including surfactant proteins (Sp), defensins, cathelicidins, lactoferrin and lysozyme as well as additional antimicrobials secreted from immune cells, such as immunoglobulin A (IgA) as discussed later [24, 29].

E) Respiratory Epithelial Cells

There are several types of cells with different functions in the respiratory epithelium in humans and mice including: type I (AT-I) and type II alveolar cells (AT-II), ciliated epithelial cells, mucous (goblet) cells, and Clara cells. While AT-I and AT-II cells are found in the alveoli, the respiratory epithelium in the bronchi and bronchioles is lined with ciliated cells and goblet cells. Clara cells are found in the bronchioles. The glylcoproteins that make up the mucus are produced by goblet cells and the produced mucus is transported by the mucociliary apparatus away from the airways towards the proximal end of the airways by the unidirectional beating of

cilia from ciliated cells. Clara cells are the main secretory cells in the airways and also possess proliferative capacity [11, 30]. Furthermore they are involved in metabolism of xenobiotics in the lung [31]. Type I epithelial cells are involved in the gas exchange and are terminally differentiated. Most of the lung is lined by the type I cells due to their large size and their main function is to oxygenate the blood, but are fewer in number than AT-II cells [32]. In addition, they are able to transfer ions in and out of the respiratory lumen and thus contribute to lung liquid homeostasis [33]. AT-II cells have secretory functions, metabolic capacity, and also are able to differentiate into type I cells to allow for regeneration of the airway epithelium. Pulmonary surfactant is among the secretions of AT-II cells and reduces surface tension of the alveoli and thereby increases pulmonary compliance [31, 34, 35].

F) Immune Functions of Type II and Clara Cells

The release of surfactant protein A (SpA) and SpD is mediated by AT-II and Clara cells. Both Sps have the ability to neutralize pathogens and facilitate phagocytosis and to reduce cytokine production by inflammatory cells, thereby protecting lung integrity [36]. In addition, β-defensins and cathelicidins produced by the epithelial cells act as broad spectrum antimicrobials which are thought to occur through electrostatic interactions with membranes of pathogenic bacteria and subsequently inducing permeabilization. In part the release of these soluble mediators is regulated by NaCl concentrations (high [NaCl] is inhibitive) in the lumen, but also induced in response to presence of cytokines [37-39]. Lactoferrin is a protein involved in iron binding, thereby limiting iron to bacteria, but it also acts directly by binding to the membranes of microbes thereby preventing microbe colonization [40, 41]. Lysozyme is directly involved in the

lysis of pathogens, specifically demonstrated for bacteria [42, 43]. In addition the epithelial cells contribute to cytokine production which is discussed in the next paragraphs.

G) Expression of Pathogen Associated Molecular Pattern (PAMP) Receptors in the Airways

The ability of the lung epithelial cells and resident innate immune cells to secrete cytokines in response to presence of pathogens is essential to forming immune responses in the lung. Several PAMP receptors exist and are expressed in the respiratory epithelium or associated cells including AM, DC, lung fibroblasts, airway epithelial cells, lung endothelial cells and lung smooth muscle cells: toll-like receptors (TLR), nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLR), retinoic acid inuduced gene I (RIG-I) like receptors and other cytosolic DNA sensors [44]. Ten TLRs (TRL 1-10) have been identified in the human genome, they respond to PAMPs, and natural or synthetic ligands for these receptors have been identified [45, 46]. Type I epithelial cells in the alveoli express TLRs 1-6 and 9 [47-49]. AM express TLR 1, 2, 4, and 6-8, plasmacytoid DC (pDC) in the lung express TLR 7 and 8 and conventional DC (cDC) TLRs 1-4. In addition, lung fibroblasts, endothelial cells, and smooth muscle cells have been reported to express TLRs 2-4, 8 and 9 [44]. In addition to recognition of foreign material, TLRs are able to recognize intracellular material, usually found in the extracellular fluid after release from necrotic cell death. The intracellular material includes, but is not limited to, heat-shock proteins (bind to TLR 2 and 4) and mRNA (binds to TLR 3) [50-53]. Cytosolic DNA sensors include NLR, RIG-I, melanoma differentiation associated gene-5 (MDA5), as well as double-stranded RNA-dependent protein kinase (PKR) and others [54-56]. Among the NLRs, the most studied and best understood examples is NRL

pyrin domain containing 3 (NLRP3). Expression of NLRP3 was found in neutrophils and macrophages and NLRP3 was highly expressed in monocytes and cDC but not expressed in airway epithelial cells and only low levels in lymphocytes, eosinophils, and pDC [57, 58]. RIG-I expression has been found in airway epithelial cells, cDC and fibroblasts but RIG-I is absent in pDC [59-61]. MDA5 is involved in the cytosolic DNA sensing in macrophages and cDC but its expression can be induced in other cell types by type I interferons (IFNs) [62-64]. PKR is expressed in airway epithelial cells, immune cells and many other tissues and in addition to sensing double stranded RNA (dsRNA) it incorporates other cellular stress signals [65, 66]. Collectively, cells of the respiratory epithelium and immune cells are contributing to the sensing of pathogens by molecular patterns in the lungs.

H) Responses Induced by Activation of PAMP Receptors

After recognition of PAMPs, innate immune responses are initiated. For example, the influenza virus pathogen directly activates TLR 7, NLRs, RIG-I, and MDA5, but other receptors might be indirectly involved as a result of cell damage or as a result of viral replication [67-71]. While TLR 1, 2, 4-6, and 8-10 are expressed on the cellular surface, TLR 3, 7, and 9 are found in the endosome [45, 46, 72]. TLR signaling shares conserved pathways within the receptor family. Specifically, the adapter protein myeloid differentiation primary response gene (88) (MyD88) activates interleukin-1 receptor-associated kinases (IRAK) 4, the subsequently activated kinase IRAK1 then activates TNF receptor associated factor 6 (TRAF6), which results in the activation of two transcription factors: nuclear factor kappa B (NFκB) by phosphorylation and thus degradation of inhibitor of nuclear factor kappa-B (IκB) by IκB kinase after interaction with the Transforming growth factor β (TGF-β)–activated kinase 1 (TAK1) / TAK1 binding protein

(TAB) complex. Also, the transcription factor activator protein 1 (AP-1) is activated as a result of activation of mitogen-actived protein kinases (MAPK). These two transcription factors drive the expression of a host of genes, including pro-inflammatory genes including interleukin (IL) 1, IL-6, IL-12 p40, and tumor necrosis factor alpha (TNFα) [45, 46, 73, 74]. In addition, activation of TRL3 and TLR4 results in MyD88-independent increase of the transcriptional activity of interferon regulatory factor (IRF) 3 and IRF7, which are required primarily for the production of type I IFNs IFN-β and IFN-α, respectively. Instead of MyD88, Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) serves as the adapter protein. TRIF either signals through TRAF6, or TRAF3 [75, 76]. Activation through TRL7 and TLR9 triggers IRF7 activity entirely dependent on MyD88 through IRAK4/IRAK1, leading to the production of IFNα [73]. Activation of the NLRs is best understood in NLRP3 and results in inflammasome formation, containing the adapter protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) together with caspase-1 (CASP-1), thereby activating IL-1 and IL-18 by proteolytic cleavage [77]. Complete NLRP3 activation requires TLR signaling as well as cell damage signals which increase the transcriptional activation of inflammatory mediators into their pro-form. In the case of IL-1, for example, the transcription of pro-IL-1 is induced by TLR agonists, but the conversion and bioactivation of pro-IL-1 into IL-1 is performed by caspase 1 [78, 79]. The signals that directly activate NLRs include, but are not limited to, bacterial flagella, anthrax lethal toxin, uric acid, asbestos, silica and saturated fatty acids [80-83]. In addition, the NLRP3 inflammasome has been implicated in the recognition of influenza ssRNA and thus is part of the host response to influenza and also has been found to play a role in the tissue healing response [58, 84]. Also, as with the TLRs, there are endogenous ligands for the inflammasome pathway, such as misfolded proteins [85, 86]. RIG-I, MDA5, and PKR activate IRF3 and thus

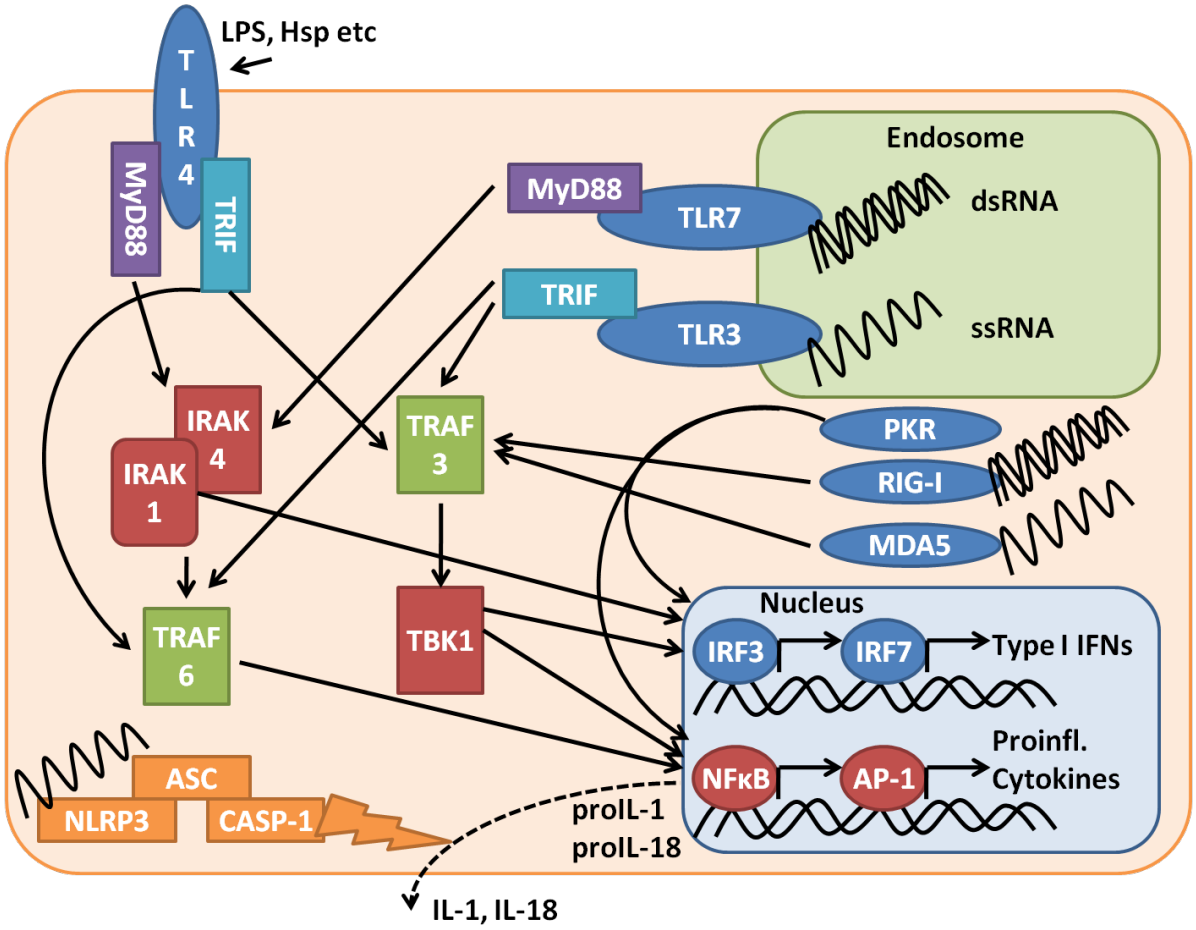


Figure 1. Simplified TLR Signaling.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

contribute to IFNβ production. Furthermore, PKR activates the NFκB pathway, upregulating pro-inflammatory genes in response to dsRNA [87]. Overall, the activation of PAMP receptors leads to the increase in production of pro-inflammatory cytokines and type I IFNs, which are essential in the immune response to pathogenic infection of the respiratory tract including influenza virus. The signaling described above is summarized in Figure 1.

I) Interplay between Epithelial and Immune Cells in the Lung

Interactions between the respiratory epithelium and resident, as well as recruited immune cells in the lung are an important part of immune defense [88]. In addition to the cells of the respiratory epithelium, resident immune cells in the lung include AM and DC. Intraepithelial DC were found to be CD11b+CD11c+ and thus are phenotypically closer to cDC than pDC [14, 89-91]. As a result, the main function of intraepithelial DC is antigen presentation rather than proinflammatory cytokine release. Under homeostatic conditions, AM are involved in impairing adaptive immunity by the release of nitric oxide, prostaglandins, IL-10 and transforming growth factor-β (TGFβ) [92]. In the event of PAMP activation, proinflammatory immune responses are initiated by epithelial cells, AM and DC in the lung and dictate the anteceding immune response. As a result, soluble mediators comprised of cytokines, chemokines, lipids and reactive oxygen species (ROS) cause an influx of immune cells to the airways first via the circulation and later via the afferent lymphatics [26]. For example, migration of cDC to the airways depends on airway epithelial cell produced Chemokine (C-C motif) ligand 20 (CCL20), which is enhanced during influenza infection [93, 94]. In addition to attracting immune cell populations to the lung, the respiratory epithelial cells and airway associated immune cells contribute to the licensing of the immune cells attracted to the lung by the release of cytokines [95]. The plasticity of DC and

their ability to elicit adaptive immune responses according to the environment encountered in tissues is well documented and is discussed in a separate section below [96]. Taken together, epithelial and resident immune cells in the lung control immune homeostasis and initiate proinflammatory responses in the lung to recruit immune cells involved in defense against pathogens.

II. Influenza Virus

A) Human Health Impact

Influenza virus is a respiratory virus and infects ciliated (avian origin) and non-ciliated (human origin) cells of the respiratory tract. Influenza causes on average about 40,000 deaths due to infection and immunopathology or infection with a secondary pathogen and is believed to be responsible for 200,000 hospitalizations per year in the US [97, 98]. The estimated total US economic cost of an annual influenza virus season is close to $90 billion, which includes cost of hospitalization, reduced productivity due to absence from the workplace and other costs including those associated with deaths [99].

B) Classification

Influenza A, B and C viruses belong to the orthomyxoviridae family of RNA viruses. The genome is composed of negative single stranded RNA ((-)ssRNA). Among the subtypes of influenza viruses, the most common is influenza A virus, which is of greatest concern due to its virulence and its potential to give rise to pandemics or epidemics. For the sake of brevity only

influenza A virus will be discussed. Based on antibody responses generated to the surface proteins hemagglutinin (H) and neuraminidase (N), the influenza A virus has been subdivided into several serotypes, for example H1N1 (human origin), the cause of the Spanish flu in 1918 and H5N1 (avian origin), responsible for the recent bird flu pandemic [100-102]. To identify isolates, the following nomenclature is used: type of influenza (A, e.g.)/origin/isolate number/year isolated. For example a common strain of virus used in laboratories and in the experiments comprising this dissertation is A/PR/8/34, where PR stands for "Puerto Rico", the origin of this isolate.

C) Virology – Influenza Virus Infection and Replication

The genome of influenza A viruses consists of eight segmented RNA strands that encode 11 proteins: envelope glycoproteins H and N, matrix protein (M1), the ion channel protein (M2), nucleoprotein (NP), three polymerase subunits (PB1, PB2, PA), and non-structural proteins NS1 and NS2 [103, 104]. Due to the virus' small genome it lacks the machinery necessary for autonomous replication and its propagation relies on a plethora of host factors [105]. To attach to host cells the influenza virus recognizes the monosaccharide N-acetylneuraminic (sialic) acid on the host cell surface via its hemagglutinin surface protein [106]. Entry of the influenza virus into the host cell is usually mediated by clathrin-coated pits, but clathrin-independent pathways also exist [107]. After endocytosis, viral uncoating occurs in the endosomal compartment due to a reduction in pH that allows for a conformational change in hemagglutinin and the fusion of the viral envelope with the endosomal compartment, thereby releasing the contents of the viral capsid into the cytoplasm [108, 109]. After release from the capsid, the ribonucleoprotein complex containing the viral genome is shuttled into the nucleus via nuclear localization signals

[110]. Inside the host cell nucleus, negative strand viral RNA (vRNA) is translated into positive strand vRNA by the viral polymerases from the capsid for viral protein synthesis and complimentary RNA, the intermediate of more negative sense genomic RNA. After nuclear export, mRNA is translated into protein via membrane bound ribosomes in the endoplasmic reticulum and modified in the Golgi apparatus. These post-translational modifications in the Golgi direct assembly of viral protein into a virion at the cell membrane, where incorporation of RNA segments into the virions occurs [101, 104]. After assembly at the plasma membrane, the virion remains attached to the sialic acid residues on the membrane (as described before) and the virions release depends on the sialidase activity of neuraminidase [111-113]. Due to the low fidelity of the viral polymerases frequent mutations occur in the viral genome [114], which is the underlying mechanism for antigenic drift (discussed later). In addition, in the case of dual infection by two non-identical influenza viruses in the same host, the genomes of the viruses may undergo a shuffling event, creating virus progeny with drastically altered RNA genome and amino acid sequences compared to the parent virions. This is called antigenic shift (also discussed in more detail later) [104, 115].

D) Mouse Model of Influenza: A/PR/8/34

Unlike ferrets, for example, *mus musculus* (laboratory mouse) cannot be infected with human isolates of influenza virus. Furthermore, *m. musculus* is not a natural host species for influenza virus. However, repeated passage of a human influenza virus isolates increases virulence in mice, thereby adapting the virus to its new host. Compared to other species, the mouse provides a relatively inexpensive and well characterized model of influenza infection and the ensuing immune response. After intranasal instillation with influenza virus, mice develop

lesions of the upper and lower respiratory tract with similar histopathology to humans [116, 117]. Over many years, influenza mouse models have greatly contributed to the understanding of the immune response to influenza [116]. One of the most commonly used experimental models to study influenza infection employs the mouse-adapted A/PR/8/34 strain and murine host challenge has been previously established and characterized in this laboratory [118-120].

III. Immune Responses to Influenza Virus

Influenza virus infection activates both innate and adaptive immune responses. The immune response to influenza optimally remains within a response magnitude window, limiting excessive immune responses, while at the same time inducing a sufficiently robust immune response to clear the virus. The antigen-specific adaptive immune responses provide the host with protective immunity against further infections from the same pathogen. By cell depletion studies it became evident that several effector lymphocytes of the adaptive immune system are important in the clearance of an influenza virus infection. Depletion of B cells, CD4+ T helper (Th) cells or CD8+ cytotoxic T lymphocytes (CTL) delayed viral clearance and depletion of more than one population resulted in almost complete virus-induced mortality with otherwise non-lethal challenge [121]. The immune response to influenza remains localized to the site of infection including the airways and lung associated lymph nodes, but is only found after viral clearance in the spleen [122]. The innate immune responses were discussed in detail above and include PAMP recognition by epithelial and resident immune cells in the lung. The recognition by PAMPs induces innate immune responses and results in the release of chemokines and proinflammatory cytokines and lung. Further discussion on innate responses will focus on the

contribution to inflammation, pathology, and the adaptive immune response. The responses of immune cells important in the immune defense against influenza are discussed in more detail below.

A) Chemotactic Responses during Influenza Virus Infection

Influenza virus induces the release of several chemokines at the site of infection: macrophage inflammatory proteins (MIP) 1α, 1β, 2, and 3α, monocyte chemotactic protein 1, interferon-inducible protein and Regulated on activation, normal T cell expressed and secreted (RANTES). Cytokines and chemokines induced by IFN-γ and IL-8 appear to be important for the recruitment of antigen presenting cells, neutrophils, T cells and NK cells to the lung after infection [93, 94, 123]. These chemokines are produced by the respiratory epithelium, monocytes/macrophages, T cells, DC and neutrophils [124]. Thus recruited immune cells contribute to the amplification of chemotactic signals generated initially by epithelial and resident immune cells.

B) Responses of DC to Influenza Virus

APC are involved in relaying stress information from TLR signaling or inflammasome activation and elicit appropriate lymphocyte responses depending on the stress signal encountered. Unlike lymphocytes their response to an immune stimulus does not depend on clonal expansion. DC are the most effective APC and are thus most effective at eliciting lymphocyte immune responses. As a result, they link the innate to the adaptive immune system by relaying information from innate stimuli and elicting effector cell responses accordingly [96]. DC are found in the airways and even in the alveoli in the absence of an immune stimulus. After an immune stimulus, such as influenza infection, DC migrate from the site of infection to the

draining lymph node and interact with and elicit effector immune cell populations [14]. Recruited DC populations display a mature phenotype based on the surface expression of T cell co-stimulating clusters of differentiation (CD) 80, CD86, and major histocompatibility complex II (MHC II), which is involved in antigen presentation to CD4+ T cells and constituitively express MHC I as all other nucleated cells for antigen presentation to CTL [125]. After infection, among APC, DC harbor the greatest amount of influenza virus as demonstrated by cellular analysis of the lung immune cell populations after infection with a green fluorescent protein (GFP) expressing virus [126]. Although these data do not differentiate between viral capture and active viral infection of DC, DC are recognized for their superior ability to acquire and process antigen [96]. Similar to effector lymphocyte populations, successful clearance of influenza virus depends on several DC populations, however the distinct roles of these DC subpopulations are poorly defined [127, 128]. There are two main types of DC, pDC and cDC. These cells are identified by their surface expression of CD11c, CD11b, and Gr-1. cDC are CD11c+, CD11b+, and Gr-1-, while pDC are CD11c+, CD11b-, Gr-1+ [127, 129-133]. Although cDC and pDC arise from distinct immediate committed precursors, further upstream a common precursor is shared [134, 135]. While the main function of cDC seems to be antigen capture, antigen presentation, and the elicitation of effector immune cells, pDC produce great amounts of type I IFNs and retain T cell elicitation functions, although are less apt at T cell elicitation than cDC [136, 137]. Both cDC and pDC express the maturation markers CD80, CD86, and MHC II and secrete an overlapping profile of cytokines, however the amount released varies depending on the stimulus and can differ between cDC and pDC [128-130, 138, 139]. Due to their abilities to present antigen and secrete cytokines, DC polarize effector lymphocyte responses, especially CD4+ T helper (Th) cells. There are at least four different ways to polarize Th cells which is

dependent on the presence of certain types of cytokines: Th1 (induced by IL-12, primarily secretes IFNγ), Th2 (induced by IL-4, primarily secretes IL-4, IL-5, IL-13), Th17 (induced by TGF-β and IL-6, primarily secretes IL-17), Treg (induced by TGF-β and IL-10 or IL-2, primarily secretes IL-10 and suppresses immune function) [140, 141]. Although mainly thought of as producers of type I IFNs, pDC can also contribute to Th polarization [142]. Overall, DC are central mediators of immune function and important for proper immune responses against viruses and maintaining immune homeostasis in the absence of a pathogen.

C) Responses of AM to Influenza Virus

AM are resident immune cells in the lung and are identified as CD11c+CD11b-Gr-1- [133]. Alveolar macrophages are important as a first line of defense against viral infection and produce type I IFNs in response to viral recognition [143]. The mechanism by which type I IFNs are induced involved intracellular (PKR) and extracellular receptors (TLR) as discussed above. Although AM might not have profound effects on the adaptive immunity, they can capture antigen and are among the first cells to enter the draining lymph nodes after pathogen infection of the lung [144, 145]. Furthermore, AM seem to play a role in maintaining tolerance in the lung thereby suppressing inflammation in the absence of a pathogenic stimulus [146].

D) Responses of Other APC to Influenza Virus

In addition to DC and AM, the presence of pathogens in the lung recruits other immune cell populations. These include be blood-derived macrophages and monocytes (CD11bloCD11clo) and myeloid-derived suppressor cells (MDSC) (CD11b+Gr-1+) [133]. Blood-derived macrophages are among other cells associated with inducing inflammatory conditions by release

of pro-inflammatory mediators including ROS and cytokines that can contribute to a robust adaptive immune response, yet excessive function might result in pathology [147]. MDSC on the other hand are known for their ability to suppress immune function. After influenza infection, MDSC are thought to be induced to ameliorate the inflammation associated with the infection and require cell-cell contact for their suppressive activity, which might be mediated by ROS [148-150]. Furthermore, blood derived macrophages are involved in the response to influenza virus. There are the classically activated macrophages (M1) and the alternatively activated macrophages (M2). While M1 play a role in inflammation and mainly produce proinflammatory cytokines as a result of PAMP activation in Th1 environment such as influenza infection, M2 are often found in Th2 environments, such as helminth infections and some forms of asthma [151]. However, unlike DC, macrophages do not produce IL-12 and IL-23 in response to influenza infection, which are the canonical cytokines driving the Th1 and Th17 lineages, respectively [152].

E) Responses of CD4+ T helper cells to Influenza Virus

Naïve CD4+ T cells exhibit high plasticity to environmental cues, which allows them to accept a phenotype according to the instructions received from APC, most importantly DC. Unlike APC, T cells need to clonally expand and the milieu of cytokines encountered drives the T cell clones towards a certain Th phenotype. T helper cells are elicited by DC via the interaction with peptide antigens of usually 12-15 amino acids presented in the context of MHC II and the T cell receptor (TCR) (signal 1) and by receiving a second signal from co-stimulatory molecules such as CD80 or CD86 on DC via the T cell co-stimulatory receptor CD28 (signal 2). Th contribute to the immune response to influenza by production of cytokines necessary for optimal

NK, CTL, and B cell responses but can also induce maturation in APC. In addition, they are able to mediate direct lysis of virus infected target cells by Fas-Fas ligand interaction [121, 153]. While on the one hand, the cytokines produced by Th cells are necessary for appropriate response of other immune cells as mentioned above, their non-discriminating action can contribute to immunopathology [154]. During an influenza infection the main Th types found are Th1 (producing mainly IFNγ) and Th17 (producing mainly IL-17) [155]. While IL-17 promotes pro-inflammatory responses [156-158], IFNγ exerts potent antiviral activity [159, 160], with both cytokines contributing to the immunopathology associated with influenza infection [161-163]. However, similar to APC, Th cells can contribute to a mechanism by which excessive inflammation is reduced, such that in acute infection IL-10 is produced by Th cells, a cytokine with potent immunosuppressive properties as well as important in the differentiation of B cells [164].

F) Responses of CD8+ CTL to Influenza Virus

CTL are elicited by DC in an antigen-specific manner by interaction with 8-10mer peptides presented in the context of MHC I, and like CD4+ T cells also depend on a co-stimulatory signal from DC received through CD28 on the T cell's surface. Differentiated CTL directly lyse target cells presenting cognate peptides on MHC I (present on all nucleated cells) by release of a cytolytic granules containing perforin and granzyme [154, 165]. Most of the CTL response is directed towards a select few peptides of the proteasome-processed influenza proteome, which are strong ligands of the MHC I binding pocket [166]. In addition to their lytic activity, CTL can produce IFNγ and IL-17, the latter of which occurs at the expense of lytic activity [167]. As

observed with Th cells, CD8+ T cells also produce IL-10 to dampen excessive inflammation during acute viral infections [164].

G) Responses of NK cells to Influenza Virus

NK cells are immediate responders in the immune response to influenza. They do not require previous activation and elicitation in order to lyse target cells by binding of polymorphic and non-polymorphic MHC I-like proteins via release of cytotoxic granules containing perforin and granzyme. Furthermore, NK cells produce cytokines including IFNγ and IL-17. NK cells can also be directly activated by TLRs or by interaction with DC [168-171]. In addition, NK cells contribute to responses of other immune cells, such as CTL, by creating a cytokine milieu that favors generation of these cells [172]. Also, NK cells have receptors for antibodies that allow NK cells to bind to B cell-produced Ig in order to mediate their cell lysis [173].

H) Responses of Granulocytes to Influenza Virus

Among the granulocyte population, neutrophils have been predominantly associated with playing a role in influenza infection. Early presence of neutrophils is required for optimal viral clearance; however, excessive presence and function of neutrophils has been associated with immunopathology [174, 175]. In addition to activation in response to the presence of cytokines, TLR activation of neutrophils occurs during influenza infection [176]. Their functions include the release of oxidants, proteases, and antimicrobial peptides, which contribute to host defense, however may also induce adverse tissue damage in the host [177]. In addition, neutrophils are involved in the removal of necrotic cells and produce chemokines that attract other immune cells [178].

I) Responses of B cells to Influenza Virus

B cells ultimately provide long lasting protection against influenza virus by production of antibodies that recognize surface antigens H and/or N [179-181]. B cells rely on stimulation by CD4+ T cells or DC for their optimal function, thus the B cell response is delayed compared to other leukocytes [182]. Initially B cells produce IgM, and after affinity maturation produce IgG2a, IgG2b, and IgA to neutralize influenza virions [183, 184]. TLR receptors on B cells and the products of TLR receptor ligation from other immune cells are important for achieving the appropriate isotype switching of B cells in the immune response to influenza [185].

J) Antigenic Drift and Shift

Although prior influenza virus infections provide the host with protective immunity in the event of an anteceding infection, this is only true if the repertoire of viral antigens does not differ significantly between infections. Of the estimated 40,000 casualties due to influenza virus, there are more deaths associated with virus infections after drastic changes in viral surface antigens. These can result from minor antigenic differences (antigenic drift) as a result of error prone replication or gene reassortment from concurrent infections of two non-identical viruses (antigenic shift) in a carrier host and are both based on surface H and N [104]. The host immune system is unable to recognize modified or completely new viral antigen due to lack of immune memory, such that primary immune responses are generated. This is one of several ways that viruses evade the immune system and creates the need for annual influenza vaccinations which primes the host's immune system with the projected viral antigens of the season allowing for an accelerated and robust immune response [115, 186].

IV. Immunotoxicology of Cannabinoids

Immunotoxicology is defined as the study of xenobiotics and organisms that adversely affect the function of the immune system. These toxic responses include suppression or enhancement of immune function, loss of tolerance resulting in autoimmunity, and the initiation of immune responses to neoantigens [187].

A) Cannabis Use

Cannabis or marijuana is the most commonly abused illicit drug with about 6% of the US population above the age of 12 and more than 20% in the 18-20 year old group reporting monthly use [188, 189]. Although human exposure to cannabinoids is primarily through smoking or ingestion of parts of the marijuana plant, consumption of synthetically produced cannabinoid compounds exist. Despite federal regulations, marijuana as well as other cannabinoids are used for recreational and medical purposes [190]. Also, there are several states in the US, including Michigan in 2008, that have decriminalized marijuana use for medical purposes [191]. Medical uses include, but are not limited to, ameliorating pain associated with cancer, as an antiemetic in chemotherapy, and as treatment for glaucoma, epilepsy and the autoimmune disorder multiple sclerosis. Also marijuana use is common among acquired immunodeficiency syndrome (AIDS) patients and was previously used as an appetite stimulant in patients with AIDS-associated wasting syndrome before the emergence of highly active antiretroviral therapy (HAART) [192]. Due to the high prevalence of cannabis use in the US population and the potential of cannabis

use to contribute to adverse health outcomes, there is a public health interest in studying the effects of cannabinoid compounds on various organ systems including the immune system.

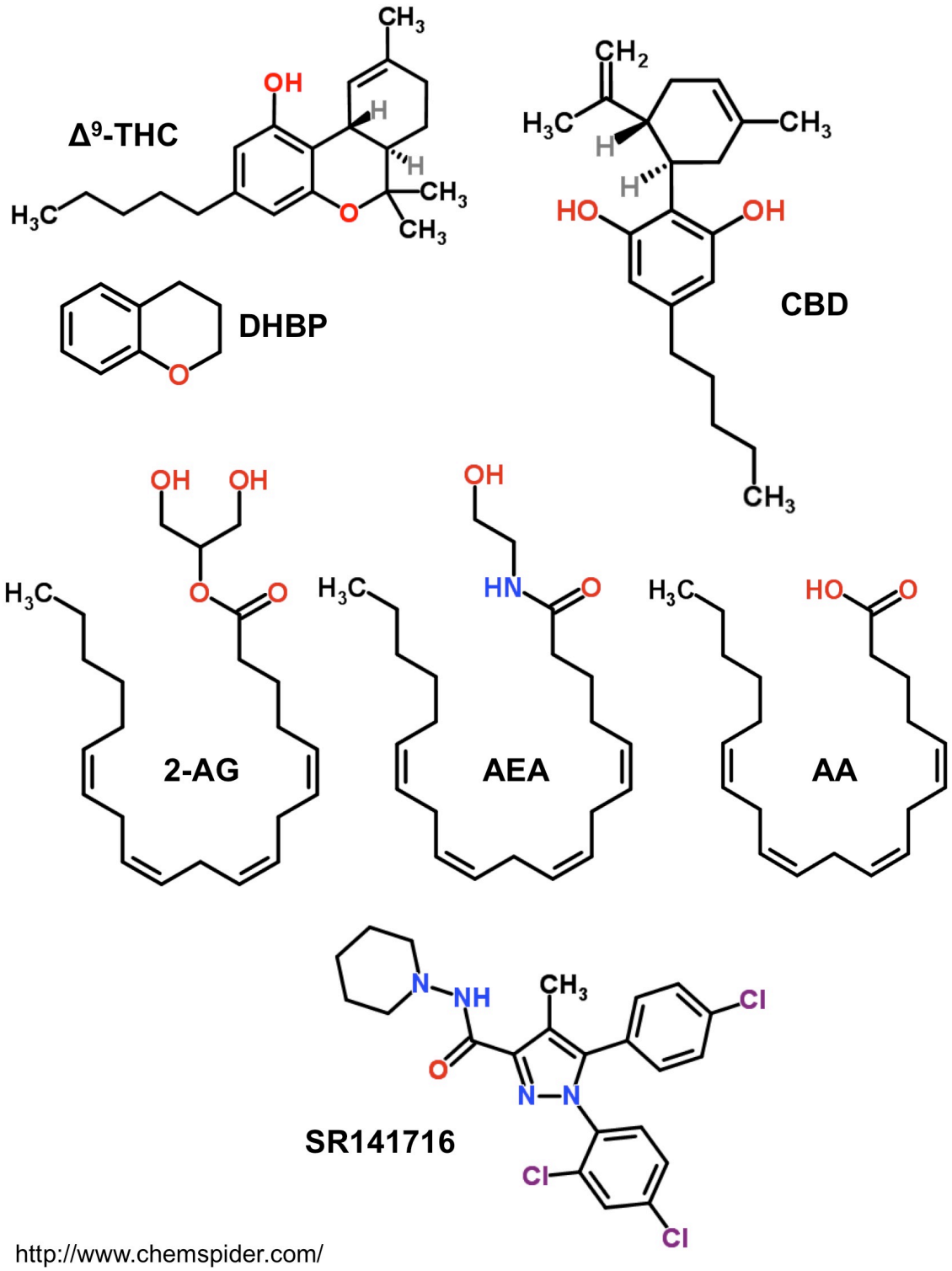
B) Cannabinoid Compounds

The first cannabinoid compound identified for its psychoactive activity was Δ9-tetrahydrocannabinol (Δ9-THC) in 1964 from *Cannabis sativa* or marijuana and is also the plant's main psychoactive component [193]. The family of plant-derived cannabinoids consists of more than 60 structurally-related tricyclic phytochemicals. The production of plant-derived terpenophenolic compounds occurs in the glandular trichomes predominantly of the female marijuana plant [194]. These phytocannabinoids exibit varying binding affinity at their identified target receptors, cannabinoid receptor 1 (CB1) and 2 (CB2). For example, while Δ9-THC is a non-selective agonist of CB1 and CB2, cannabidiol (CBD), a phytocannabinoid with similar structure to Δ9-THC, lacks binding activity at CB1 and CB2 [195]. Important for the binding activity of phytocannabinoids is the dihydrobenzopyran (DHBP) structure as well as a hydroxyl at the C-1 aromatic position and an alkyl group at the C-3 aromatic position. Furthermore, only plant compounds in cis configuration act as ligands of CB1 and/or CB2 [196]. Besides CB1 and CB2, other receptors have been proposed as targets for cannabinoids and receptors for cannabinoids will be discussed in a separate section.

Endogenously-produced cannabinoids, also termed endocannabinoids, have been identified and are synthesized within the cell from membrane lipids. Endocannabinoids were identified based on their ability to bind to CB1 and/or CB2 [197]. The most studied and thus best characterized endocannabinoids are 2-arachindonoylglycerol (2-AG) and N-arachidonoylethanolamine or anandamide (AEA), which structurally resemble arachidonic acid.

The production of AEA from membrane lipids requires two enzymatic steps. First, the membrane lipid phosphatidylethanolamine is cleaved by *N*-acyltransferase into N-arachidonoyl-phosphatidylethanolamine, which is then converted by the enzyme phospholipase D into AEA. The enzyme *N*-acyltransferase is dependent on Ca2+ and cAMP for optimal activity. The production of 2-AG is mediated by two possible pathways. The first pathway involves the production of 1,2-diacylglycerol (DAG) from the membrane lipid phosphatidylinositol (PI) by phospholipase C (PLC). DAG is then converted into 2-AG by DAG lipase. Alternately, PI is converted into lyso-PI by phospholipase A1 and hydrolized into 2-AG by lyso-PLC [198]. To elicit binding activity on CB1 and CB2, it is important that these endocannabinoids have a monosubstituted amide with an alkyl group [196].

In addition, other non-*cannabis*-derived non-classical cannabinoids are emerging and synthetic ligands for CB1 and CB2 have been developed. Many of the synthetic cannabinoids were developed as pharmacological tools, such as receptor-specific agonists and antagonists or as potential therapeutics [199]. One synthetic ligand developed for therapeutic purposes was the CB1 antagonist SR141716, also known as Rimonabant. An important aspect of the antagonist's structure-activity relationship is the amide group, which, if substituted, results in loss of activity [196]. The pharmaceutical company Sanofi-Aventis developed Rimonabant as an anti-obesity and smoking cessation drug. Despite pre-clinical successes of Rimonabant, the FDA halted further progress due to reports of alterations in mood, increases in episodes of depression, and a 2-fold increased risk of suicide in patients participating in the clinical trial. The studies suggested that cannabinoids are inherently involved in the body's reward system and caution should be



http://www.chemspider.com/

Figure 2. Structures of Select Cannabinoids.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

observed using cannabinoids as therapeutics [200-202]. In addition, consumption of synthetic cannabinoids has become a recent trend among adolescents in industrialized countries including the US. Disguised and sold as powdered bath salts or herbal mixes for incense, these synthetic cannabinoids are not labeled for consumption. These compounds tend to be stable derivatives of endogenous cannabinoids and unless identified do not fall under the substance abuse laws. Due to their synthetic nature, their effects on biological systems are unpredictable and largely unexplored at this point [203]. For comparative purposes, some of the chemical structures of the cannabinoid compounds mentioned above are shown in Figure 2.

C) Pharmocokinetics and Metabolism

Depending on the route of administration, inhalation or oral consumption for example, different pharmacokinetics are observed for cannabinoids. Cannabinoids are readily absorbed after inhalation or oral consumption and rapidly enter the bloodstream. Bioavailability of Δ9-THC is on average 30% after inhalation and is much lower and more variable after oral consumption, found between 4-12% [204, 205]. Peak levels of Δ9-THC in the plasma reach about 200 ng/mL after smoking of a marijuana cigarette but are lower after oral administration [206]. Initially after consumption, Δ9-THC is rapidly distributed to vascularized tissues including the brain. Due to Δ9-THC's lipophilic nature it is widely distributed inside the body and especially found in adipose tissue, resulting in a two compartment (bloodstream and tissues) distribution. Also the spleen was identified as a long-term storage site for Δ9-THC [204, 206].

Metabolism of Δ9-THC occurs in a multiphasic manner and the parent compound can be detected at low levels more than one week after dosing [204, 205]. After entering the blood stream, Δ9-THC is subject to first pass metabolism by the liver, which results in rapid clearance

of Δ9-THC from the plasma. Depending on the route of exposure, major sites for Δ9-THC metabolism might include the lung, especially AT-II and Clara cells after inhalation as well as intestinal epithelial cells and microbiota after ingestion [204, 207]. Due to its lipophilic properties and redistribution to fat-containing tissues, a second much slower phase of Δ9-THC clearance is observed [206]. Δ9-THC and other phytocannabinoids are metabolized by the P450 enzymes CYP2C9 and CYP3A4. The most common metabolites are the partially active 11-hydroxy-Δ9-THC, which is further hydroxylated into 8-alpha-11-dihydroxy-Δ9-THC, the inactive 11-nor-9-carboxy-Δ9-THC or glucoronidated by UDP-glucuronosyltransferase into the *O*-ester glucuronide of THC-COOH, the main metabolite found in urine and can also be exreted from the feces [204, 205, 208-211]. While it is easier to determine the pharmacokinetics in pure formulations of Δ9-THC, it has been reported that CBD interferes with the metabolism of Δ9-THC and thereby maintaining its plasma levels and potentiating its physiological effects [212]. Thus metabolism of cannabinoid compounds after marijuana consumption is likely slower than after administration of pure compounds.

The production of endocannabinoids is limited to the site of cellular synthesis and in the immune system, the main sources of endocannabinoids are activated immune cells. Thus, endocannabinoids are thought to signal in an autocrine and paracrine fashion. Different pathways for the metabolism of endocannabinoids exist compared to those of phytocannabinoids. AEA is readily metabolized by fatty acid amide hydrolyse (FAAH) into arachidonic acid and ethanolamide and 2-AG is enzymatically cleaved by monoglyceride lipase (MGL) into arachidonate and glycerol [198]. As a consequence, inhibitors and knockout mice of FAAH or MGL have elevated endocannabinoid levels [213-215].

D) Receptors for Cannabinoids

1) Cannabinoid Receptors 1 and 2

Initially there were two identified targets for cannabinoids, the G-protein coupled receptors (GPCR) CB1 and CB2. The two receptors share limited homology (48% conserved amino acid identity) and have different expression patterns based on cell type [196, 216]. CB1 is translated from a single exon, while the 5' region of the mRNA transcript contains untranslated sequence from a second exon. Also a rare splice variant of CB1 exists, which misses part of the coding sequence of exon 1 [196, 217]. CB2 is also translated from a single exon and also contains an exon in the 5' untranslated region [196]. A number of residues in the extracellular loops of CB1 and CB2 were found to be important for agonist binding from studies performed using human sequences of the receptors. Specifically, by using site-directed mutational analysis, lysine 192 and glycine 195 of helix three and valine 282 of helix five were found to be important for ligand binding in CB1 and serine 119 of helix three and phenylalanine 197 of helix 5 in CB2 [196]. CB1 was first discovered in cells and tissues associated with the central nervous system, while CB2 was discovered in splenocytes and then identified for its immunomodulatory activity in macrophages [216, 218, 219]. Expression of CB1 and CB2 is observed throughout the body, including almost every organ system. Highest expression of CB1 is observed in the central nervous system including the brain, while only glial cells (microgial cells and astrocytes) of the brain express CB2 [196, 220-222]. On the other hand, highest CB2 expression is observed in cells of the immune system [196]. In the immune system the expression of CB1 and CB2 varies between the different cell types. CB1 and CB2 steady state mRNA expression levels follow a similar pattern, but CB1 expression is lower than CB2 in the immune system. Highest steady state mRNA levels are observed in B cells followed by NK cells, monocytes, neutrophils, CD8+

T cells, and CD4+ T cells [223, 224]. In addition, the expression of CB1 and CB2 can change as a result of cellular activation, resulting in increases in CB1 in T cells, for example [225, 226].

2) Alternate Cannabinoid Receptors

In addition to CB1 and CB2, there are several other receptors that have been proposed as targets of cannabinoid ligands. Difficulties in studying cannabinoid pharmacology have occurred due to off-target effects present in mixtures of putative ligands and antagonists for CB1 and/or CB2. Ligands might produce off-target effects that are not blocked by available antagonists, or the antagonists might produce off-target effects by themselves leading to data misinterpretations. An important model to prove the existence of cannabinoid receptors other than CB1 and CB2 is the CB1/CB2 double knockout (CB1-/-CB2-/-) mouse. Effects by cannabinoids in CB1-/-CB2-/- mice and cells isolated from these mice can be attributed to alternate cannabinoid receptors [227-231]. Among the alternate cannabinoid receptors are the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), the Ca2+ ion channel transient receptor potential channel 1 (TRPC1), and the orphan receptors G-protein coupled receptor 18 (GPR18), GPR55 and GPR119 [232]. In this laboratory, the cytoplasmic Ca2+ influx in T cells was induced by Δ9-THC [233] and this and other Ca2+ associated signaling have been demonstrated to be independent of CB1 and CB2 but associated with TRPC1 [229, 231, 234]. Furthermore, using compounds such as CBD, which does not bind CB1 or CB2, suppression of T cell activation and cytokine production including IL-2 and IFNγ was observed [235]. The suppression of nuclear factor of activated T cells (NFAT)-dependent production IL-2 by 2-AG in T cells was found to be a consequence of a putative COX-2 metabolite of 2-AG, 15-deoxy-

Compound	CB1 Affinity	CB2 Affinity
Δ9-THC	21–80.3	3.13–75.3
CBD	4350–10,000+	2399–10,000+
2-AG	58.3, 472	145, 1400
AEA	61–543	279–1940
SR141716	1.8–12.3	514–13,200

Table 1. Dissociation Constants (Ki) of Select Cannabinoids for CB1 and CB2.

Shown are dissociation Ki (nM) values of CB1/CB2 receptor ligands for the displacement of a tritiated compound ([3H]CP55940, [3H]R-(+)-WIN55212, or [3H]HU-243) from rat, mouse, or human CB1 and CB2 receptors *in vitro*. A low dissociation constant value reflects dissociation of the tritiated compounds by low concentrations of the indicated compound. This table is adapted fromHowlett et al. 2002 and Pertwee et al 2010.

Δ12,14-prostaglandin J2-glycerol ester binding to the nuclear receptor PPARγ [236]. GPR18, GPR55 and GPR119 are recent additions to alternate CB, thus the least is known about these orphan receptors. Endogenous lipid ligands including endocannabinoids, but also non-classical cannabinoids activate these receptors. For example, agonists for GPR55 include the lipid lysophosphatidylinositol (LPI) and the CB1 inverse agonist AM251 and induce neutrophil chemotaxis [237]. GPR18 is a target for Δ9-THC and endogenous lipid mediatiors causing migration in a human endometrial cell line [238]. Binding affinities of select cannabinoids for CB1 and CB2 are summarized in Table 1.

VII. Intracellular Signaling by Receptors for Cannabinoids

A) Signaling by Gαi- protein coupled receptors: Cannabinoid Receptors 1 and 2

Both CB1 and CB2 belong to the GPCR family and associate with Gαi/o [239]. The following paragraph describes the signaling initiated by CB1 and CB2, what is common among Gαi/o-protein coupled receptors, and what might play a role in CB1 and CB2 signaling. Due to coupling to Gαi/o, ligation of these receptors inhibits adenylate cyclase activity, resulting in lower cytosolic cAMP levels, for example after concurrent treatment with a pharmacologic agent such as forskolin that will induce cAMP levels [240, 241]. The βγ subunits are important in recruiting G protein-coupled receptor kinases (GRK) which recruit β-arrestin to the receptor and initiate β -arrestin-mediated internalization of the GPCR [242]. After ligation of CB1, for example, β-arrestin-mediated internalization leads to tolerance to psychotropic effects after repeated exposures to cannabinoid ligands [243]. Furthermore, the GPCR subunits βγ couple

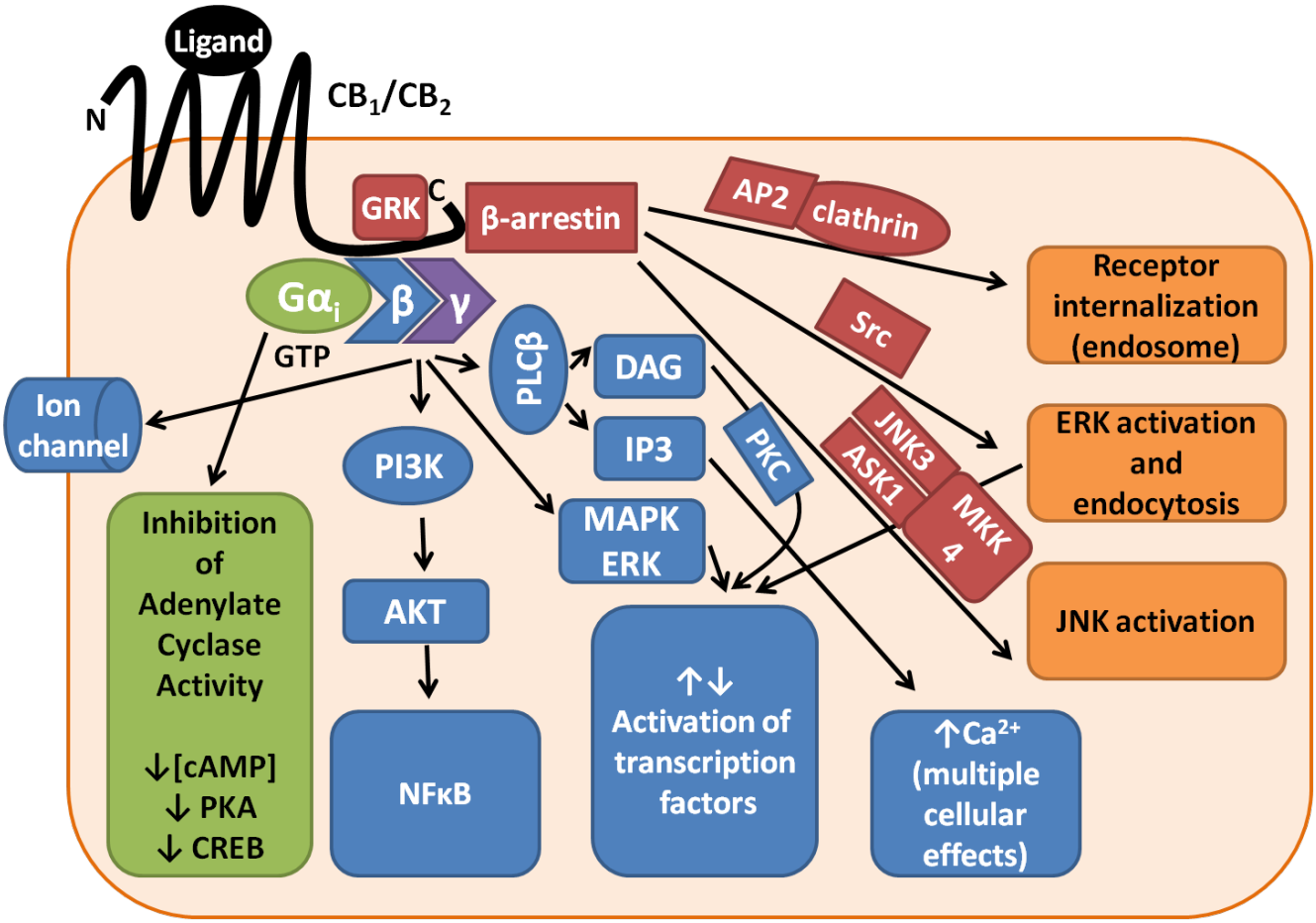


Figure 3. Simplified $G\alpha_i$ GPCR Signaling.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

CB1 and CB2 to the mitogen activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK) pathways [244]. Also activation of PI3K and PLCβ by Gβγ is possible, which activates downstream effectors AKT/NFκB and cause the enzymatic cleavage of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP3), thereby inducing intracellular Ca2+ levels from the endoplasmic reticulum stores and releasing DAG, which activates protein kinase C [245-247]. In addition to their role in targeting GPCRs for endocytosis, β-arrestins can form complexes with other signaling proteins thereby activating ERK1/2 and MAPK pathways. While adapter-protein 2 and clathrin mediate the endocytosis of GPCRs after binding to β-arrestin, interaction of β-arrestin with members of the Src family of kinases results in MAPK activation and/or internalization of the GPCR. Also, interaction of β-arrestin with apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase 3 (JNK3), and mitogen-activated protein kinase kinase 4 (MKK4) leads to the activation of the JNK pathway [248, 249]. While this has been demonstrated for other Gαi-coupled GPCRs, it is likely that the same occurs for CB1 and/or CB2 although direct evidence is lacking [250]. Also, regulator of G protein signalling (RGS) proteins exist and as their name suggests, RGS are involved in the deactivation (or regulation) of GPCR signaling by facilitating the hydrolysis of guanosine triphosphate to guanosine diphosphate. More recent findings have demonstrated that in addition to regulating GPCR signaling at the receptor, the RGS proteins are able to act on downstream targets of the GPCR cascade to regulate GPCR signaling [251]. The putative signaling pathways activated by CB1/CB2 are summarized in Figure 3. Despite the information on the downstream signaling of CB1 and CB2 it is currently unknown to what extent and how each pathway leads to the suppression of immune responses associated with cannabinoid treatment. To add further complexity to the signaling by CB1 and CB2, heterodimerization with

other GPCRs has been reported with CB1 and is common among the GPCR family and thus likely to occur with CB2 as well [252, 253]. It is currently unknown what the heterodimerization partners of CB1 and CB2 are and what putative changes in signaling result from heterodimerization. There are reports that for some GPCRs, up to 80% of exist as homodimers and as a result reduced activation of receptor-mediated downstream signaling occurs [253, 254].

B) Signaling by Alternate Cannabinoid Receptors

While GPR18, GPR55, GPR119 and TRPC1 are surface receptors, PPARγ is cytosolic and requires its ligands to cross the cell membrane. Ligation of GPR55 results in activation of Rho-family GTPases, also activation of MAPK/ERK pathways has been demonstrated. As a result of Rho activation, intracellular Ca2+ release was observed, which led to activation and nuclear translocation of NFAT [255]. GPR119 is a Gαs-coupled receptor and induces intracellular cAMP levels upon ligation. In addition, Ca2+ levels are increased as a result of downstream effects of cAMP on ATP-sensitive potassium channels and voltage-dependent calcium channels [256]. Currently, no experiments have been performed to address the intracellular signaling downstream of GPR18. TRPC1 participates in the store operated calcium entry and thereby is involved in cell activation as a result of Ca2+ influx, for example occurring during activation of T cells [257]. The receptor responds to depletion of intracellular Ca2+ stores and allows for influx of extracellular Ca2+ to allow for sustained Ca2+ levels for optimal activation [258]. Upon ligand binding, PPARγ heterodimerizes with retinoid X receptor alpha (RXRα) and translocates to the nucleus to induce the transcription of its target genes. In addition, PPARγ interacts with intracellular signaling directly, for example to reduce NFAT-mediated induction of IL-2 expression in T cells [236]. Also cross-talk between PPARγ and Janus tyrosine

kinase-signal transducer and activator of transcription (JAK-STAT) signaling exists and ligation of PPARγ results in the reduction of transcriptional activity of STAT5b [259].

VI. Effects of Cannabinoids on Immune Function

A) Effects on Antigen Presenting Cells

It has been demonstrated that Δ9-THC inhibits APC function including antigen processing and presentation, and cytokine production [260]. The induction of TNFα by LPS and IFN-γ was suppressed by Δ9-THC in human and murine macrophages. Notably, culture serum concentrations of 5% abrogated the effect of Δ9-THC on induced TNFα production [261]. To the same extent LPS-induced NO production by the RAW264.7 macrophage cell line and peritoneal macrophages was decreased by Δ9-THC after treatment *in vitro* and was attributed to the suppression of NFκB/Rel and CREB/ATF binding and reduction of intracellular cAMP levels [262]. In addition, chemotaxis of macrophages to RANTES was reduced by Δ9-THC in a CB2-dependent manner by the use of a specific antagonist [263]. Furthermore, depending on the activation state of macrophages, different levels of induction of CB2 transcript were observed. The highest induction was observed in macrophages with inflammatory functions, suggesting a role for CB2 in a negative feedback loop to down-regulate inflammation in macrophages [264].

It has been observed that both CB1 and CB2 contribute to the modulation of DC function by Δ9-THC with the use of CB1- and CB2–specific antagonists [265] and that CB1 specifically diminishes K+ outward currents through Kv channels to suppress the ability of DC to elicit T cell resonses [266]. Furthermore, suppression of DC function by Δ9-THC might, in part, be independent of the G-protein coupled receptors as evidenced by Δ9-THC-mediated suppression

of maturation after in bmDC generated from CB1-/-CB2-/- mice. In support of this argument, pertussis toxin, which blocks the Gαi subunit necessary for CB1 and CB2 signaling [267, 268], did not completely abrogate the suppression of IL-12p40 production in bmDC [269]. Also selective antagonists to either CB1 and CB2 were not able to block the suppression of IL-12p40 by Δ9-THC [270]. This suggests that although CB1 and CB2 are involved in Δ9-THC modulation of DC, this might not be the exclusive mechanism. Δ9-THC has also been found to induce suppressive MDSC populations, providing another putative mechanism by which Δ9-THC suppresses immune function [271].

B) Effects on Granulocytes

Most of the studies of the effects of cannabinoids on granulocytes have been focused on neutrophils. There are conflicting reports about the involvement of CB1 and CB2 and the modus of the outcome of neutrophil function. It has been demonstrated that cannabinoids impair neutrophil chemotaxis independent of CB1 and CB2 [272]. Others argue that CB2 plays an important role in positively regulating the migration of neutrophils [273]. Furthermore, neutrophil superoxide generation was suppressed by the non-selective CB1 and CB2 agonist CP 55,940 independent of CB1 and CB2, suggesting alternate targets [274]. In contrast, others have shown a CB2-dependent stimulation of neutrophil function with endogenous and synthetic cannabinoids [275].

C) Effects on CD4+ T cells

Due to the critical role of proliferation in CD4+ T cell responses and the requirement of IL-2 for proliferation, which is rapidly and robustly induced after T cell activation, much of the

research on the immunomodulator action of cannabinoids have focused on proliferation, IL-2 production and their upstream signaling pathways. The first evidence for effects mediated by cannabinoids on T lymphocytes came from *in vivo* sheep erythrocyte assays in rats, where Δ9-THC treatment impaired the T cell dependent antibody formation against erythrocytes [276]. In 1977, Δ9-THC and its primary metabolite 11-OH-Δ9-THC were shown to suppress the proliferation as measured by radioactive thymidine incorporation of phytohemagglutinin-activated human T cells *in vitro*, which was confirmed by a second group [277, 278]. After confirmation of the previous results it was also noted that no cytotoxicity was observed by Δ9-THC on T cells with concentrations as high as 26 μM, but higher serum concentrations (20% of culture media) reduced the suppressive effect of Δ9-THC on proliferation, suggesting neutralization of Δ9-THC by serum proteins [278]. While not changing the percentage of CD4+ T cells in the spleen, Δ9-THC attenuated proliferation after pharmacological activation with PMA/Io and in allogeneic mixed lymphocyte responses as well as IL-2 and IFNγ production after PMA/Io activation [279]. In agreement with the Δ9-THC-mediated attenuation of proliferation, Δ9-THC decreased the surface expression of IL-2 receptors with high affinity, while increasing IL-2 receptors with low affinity [280]. The mechanism by which Δ9-THC reduced IL-2 expression in T cells was explored in the T cell line EL-4.IL2 and it was reported that Δ9-THC reduced the binding of AP-1 to the proximal promoter region of IL-2 [281]. Using cannabinol, a weaker selective CB2 agonist than Δ9-THC, suppressed AP-1 binding by decreasing nuclear presence of its protein components c-fos and c-jun. In addition, reduced NFAT binding was observed as a result of cannabinol treatment leading to reduced production of IL-2 [282, 283]. Moreover, the phosphorylation of c-fos and c-jun and the phosphorylation of ERK1 of the MAPK/ERK pathway were reduced by cannabinol [283]. In light of the critical role

of Ca2+ in the activation of NFAT, the focus shifted to modulation of intracellular Ca2+ by cannabinoids. Studies in this laboratory demonstrated that Ca2+ influx was temporally dysregulated by Δ9-THC in T cells through the TRPC1 receptor [231, 233, 284]. More recently, it has been demonstrated by this laboratory that Δ9-THC suppresses the induction of the co-activating molecule ICOS on CD4+ T cells after activation and it was suggested that this occurs in part via the modulation of NFAT signaling [285]. Furthermore, it has been argued that with certain stimulations, Δ9-THC shifts the polarization of the Th response to a Th2 phenotype [286]. Moreover, there is evidence that the effects of cannabinoids on T cells are independent of CB1 or CB2 which is in agreement with low expression levels for these receptors [231, 233, 234, 287]. In contrast, it has been demonstrated in the Jurkat T cell line that expression of CB1 increases after immune stimulation, suggesting that CB1 may act to suppress activity in activated T cells [288].

D) Effects on CD8+ T cells

In CD8+ cytotoxic T lymphocytes (CTL), it was first noticed that proliferation was suppressed in T cell subsets by Δ9-THC [289]. Cytolytic activity was attenuated *in vitro* by Δ9-THC in CTL. The mechanism was proposed to be bimodal: 1. during differentiation and proliferation of pre-cursor CTL and 2. during cytolytic activity, inhibiting target cell lysis [290]. Furthermore, *in vivo* treatment of Δ9-THC reduced CTL activity from mouse splenocytes in a model of herpes simplex virus type-1 (HSV-1). Using microscopy, it was observed that polarization towards, but not binding, to virus-infected target cells was inhibited [291].

E) Effects on NK cells

There is evidence that cannabinoids impair NK cell activity. Both Δ9-THC and the primary metabolite 11-OH-Δ9-THC were able to suppress target cell lysis by NK cells [292]. Also, it was observed that while the lytic activity was suppressed by Δ9-THC the binding to target cells was not affected [293]. The migration of NK cells was induced by the endocannabinoid 2-AG in a CB2-dependent manner, while Δ9-THC failed to induce migration [294]. The production of the proinflammatory cytokine TNF-α was suppressed by Δ9-THC in NK cells activated with *C. albicans* [295].

F) Effects on B cells

Among the immune cells B cells express higher levels of CB2, suggesting that this receptor mediates some of the effects associated with cannabinoid treatment. Cannabinoids have differential effects on B cells. B cell growth is enhanced at low nanomolar concentrations of Δ9-THC and synthetic cannabinoids and was abrogated by pertussis toxin, suggesting a GPCR-dependent mechanism, which the authors attributed to CB2 expression in B cells [296]. Futhermore, CD40-induced proliferation was enhanced in a CB2-dependent manner in B cells by the use of a CB2-specific antagonist. Also upregulation of CB2 occurred in B cells 24 hours after CD40 ligand-induced proliferation [225]. Studies from this laboratory demonstrated that endocannabinoids enhanced LPS-induced B cell proliferation at high cell densities, but suppressed proliferation at low cell densities [297]. Moreover, the antibody forming cell response to sheep red blood cell antigen was suppressed by Δ9-THC and the suppression was abrogated by the addition of the cell permeable cAMP analog dibutyryl-cAMP, suggesting a mechanism involving a Gαi-coupled GPCR, putatively CB2 [267]. Using the B cell line SRIH-B, it was demonstrated that Δ9-THC enhanced IL-8, but suppressed MIP-1β production [298].

G) Effects on Immune Outcome in Host Resistance Models

Although the effects of Δ9-THC on isolated immune cells *in vitro* have been extensively investigated, the effects of Δ9-THC on the immune system *in vivo* are more complex. Factors contributing to this complexity include the type of immune stimulus, tissue location, as well as the interplay between, and kinetics of, the cellular arms of immune response during an immunological insult, none of which can be completely capitulated *in vitro*. To date, few studies have focused on the effects of Δ9-THC on multiple immune cell populations using a pathogen challenge model [119, 120, 286, 299-305].

The first studies of the effects of cannabinoids on host resistance were performed using mouse models of infection with HSV-2 and the intracellular pathogen *Listeria monocytogenes* [302]. Both pathogens induce strong Th1 immune responses in the host resulting in control (HSV-2) or clearance (*L. monocytogenes*) of the pathogen by CTL when infected with lower innoculums [306]. Δ9-THC decreased the host resistance to both pathogens as evidenced by mice succumbing to lower innoculums of HSV-2 and *L. monocytogenes* compared to vehicle (VH) treated controls. In a follow-up study using the HSV-2 model, Δ9-THC suppressed host immune function to increase host mortality and viral titers after HSV-2 infection and Δ9-THC treatment resulted in lower serum complement fixing and antibody neutralization titers compared to VH [299]. Using the same model, it was subsequently determined that Δ9-THC reduced the type I IFN response and previous studies in mice were confirmed in guinea pigs [300, 305].

In studies using a *Legionella pneumophila* model, Δ9-THC suppressed primary and secondary immunity to the pathogen. *L. pneumophila*-induced serum levels of Th1 cytokines IFNγ and IL-12 and splenic steady state mRNA levels of IL-12 receptor beta were attenuated by

Δ9-THC treatment. The effects by Δ9-THC were attributed in part to a shift from Th1 to Th2 immunity as increased serum levels of IL-4 and steady state mRNA levels of GATA-3, a key Th2 transcription factor, were observed in the spleen. Furthermore, it was noted that both CB1 and CB2 contributed to the effects of Δ9-THC as determined by the use of receptor antagonists and knockout mice [286, 303, 304].

Using a lung cancer model, Δ9-THC impaired immune responses to tumor implants, which underwent accelerated growth as a result of Δ9-THC treatment. While the Th1 cytokine IFNγ was suppressed by Δ9-THC, the immunosuppressive cytokines IL-10 and TGF-beta were enhanced at the tumor site and in the spleens in mice receiving Δ9-THC treatment. In these studies a CB2 antagonist was effective in blocking the suppressive effects of Δ9-THC, thus leading the authors to conclude a CB2-dependent mechanism of suppression of anti-tumor immunity [301].

Most recently, studies were performed to determine changes in host resistance due to Δ9-THC treatment using a murine influenza virus host challenge model characterized in this laboratory [118]. While Δ9-THC reduced the lung tissue pathology as well as the influx of macrophages, CD4+, and CD8+ T cells into the airways, higher lung viral burden was observed [119]. Using CB1-/-CB2-/- mice, it was observed that these mice had drastically lower lung viral burden and greater protein levels of TNFα in the BALF. Furthermore, the Δ9-THC-mediated reduction in lung tissue pathology was dependent on CB1 and/or CB2. While the immune response to influenza was broadly characterized, it was unclear to which extent different immune cell populations contributed to the observed endpoints.

H) Phenotype of the Immune System as a Result of Loss or Lack of CB1 and/or CB2

There are a multitude of studies examining the immune system of mice after genetic ablation of CB1 and/or CB2 and humans with identified single nucleotide polymorphisms (SNPs) in CB1 or CB2. Consistent among these studies is an enhanced immune response phenotype with extinguished or reduced signaling through CB1 and/or CB2, either resulting in exacerbation of the disease state in mouse models or increased propensity for developing autoimmune disease in humans. Mice with a single deletion of CB1 or CB2 or CB1-/-CB2-/- mice are healthy, fertile and of similar size and weight compared to WT mice. However, increased mortality was observed in mice lacking CB1 [228, 307].

Much of the work of studying the phenotype of CB1-/- mice in the immune system has focused on intestinal inflammation models [308, 309]. Intrarectal infusion of 2,4-dinitrobenzene sulfonic acid and oral administration of dextrane sulfate sodium was used to induce colitis in WT and CB1-/- mice, intestinal inflammation was exacerbated in CB1-/- compared to WT mice. The authors attributed the exacerbation to the lack of signaling through CB1 myenteric neurons and lack of CB1-mediated control of inflammation-induced irriation of intestinal smooth muscle cells [309]. This study highlights the potential involvement of CB1 via the central nervous system affecting the peripheral organ systems. Signaling at the nerve terminal of the input neuron is believed to be reduced by postsynaptic release of endocannabinoids and ligation of CB1 on the input neuron, thereby reducing the action potential of the input neuron [310]. Thus ablation of CB1 could potentially lead to an overactive nervous system, which can lead to enhanced immune function as is experienced with angiotensin II release after sympathetic nervous system stimulation [311]. However no further characterization of the effects of the nervous system on the immune system was performed. In addition, reduced endotoxin hypotension was observed in

CB1-/- or CB1-/-CB2-/- mice compared to WT mice, suggesting a stronger response to LPS in the absence of CB1-/- [227].

While the lack ofCB2 alone or lack of both CB1 and CB2 in mice has an effect on the percent composition of splenic immune populations [279, 312], CB2 deletion resulted in abnormal formation of subsets of B and T cells compared to WT mice [313]. In particular, CB2-/- mice showed a deficiency by percent composition of intestinal intraepithelial NKT cells and a reduction in the percentages of marginal zone B cells and memory CD4+ T cells in the spleen. Furthermore, peritoneal B1a B cells, a subset of B cells that do not contribute to memory formation, was slightly reduced [313]. Additionally, deletion of CB2 resulted in accelerated age-related loss of bone mass compared to WT, which was characterized by a phenotype similar to osteoporosis in humans. The authors speculated that the loss of CB2 might have resulted in an increase in proresorptive cytokines such as IL-1 and TNF-α, as these are reduced by CB2 ligation [314].

CB1-/-CB2-/- mice were generated by crossbreeding previously generated CB1-/- [307] and CB2-/- [312] mice and the use of CB1-/-CB2-/- mice in experiments has been limited to a few studies [120, 227-229, 231, 279, 315, 316]. In studies from this laboratory CB1-/-CB2-/- mice were used mostly as a tool to demonstrate that cannabinoid-mediated suppression of T cell responses was independent of CB1 and CB2. Only few studies have described immune related endpoints in CB1-/-CB2-/- mice that offer insights into phenotypic differences compared to WT mice, with three of the studies coming from this laboratory. First, Kaplan *et al.* showed a trend towards increased release of IFNγ from pharmacologically stimulated CB1-/-CB2-/- compared to WT splenocytes, albeit not evaluated statistically [229]. Karsak *et al.* described the involvement of the endocannabinoid system in the amelioration of hypersensitivity responses to contact

sensitizers. In addition to greater ear swelling and more pronounced lesions in ear tissue, predominantly chemokines and chemokine receptors were found to be upregulated in CB1-/-CB2-/- mice compared to WT mice [315]. Springs *et al.* demonstrated that there was no difference in the percent composition of immune cells in the spleens of WT and CB1-/-CB2-/- mice. Moreover, although not deemed statistically significant, slightly greater lymphocyte proliferation as a result of pharmacological stimulation as well as enhanced IL-2 production after pharmacological activation was observed in splenocytes from CB1-/-CB2-/- mice compared to WT mice *in vitro*. Also the response to sheep red blood cell antigen was enhanced *in vivo* in CB1-/-CB2-/- compared to WT mice [279]. Most recently, a study from this laboratory by Buchweitz *et al.* showed that the viral lung burden is reduced in CB1-/-CB2-/- compared to WT mice after influenza infection. Furthermore, increases in BALF CD4+ T cell counts were observed in CB1-/-CB2-/- compared to WT mice [120]. On the other hand, in an adjuvant-free ovalbumin model of airway hypersensitivity, there were no overt differences in the airway responses to ovalbumin. However, the presence of neutrophil populations was enhanced at 96 h post challenge. Due to the absence of an inflammatory stimulus such as adjuvant in this model, it suggested the necessity for an inflammatory stimulus to observe enhanced immune reactivity in CB1-/-CB2-/- compared to WT mice [317]. Taken together, these studies demonstrate an inherent role for CB1 and CB2 in the regulation of the magnitude of the immune response after an inflammatory stimulus.

In humans, SNPs are often associated with a loss or lack of function of the target gene product and have been observed in CB1 and CB2 [318, 319]. A population was identified with a SNP in the CB1 transcript *Cnr1*, 1359 A/A to G/A, resulting in a silent mutation in CB1 at position 454 threonine. The G/A population and a control (A/A) population were evaluated for

ulcerative colitits (UC) and Crohn's disease. There was a significant association of the G/A population with UC susceptibility, demonstrating a potential role for CB1 in UC. Due to the apparent effect as a result of a silent mutation, the authors argued that CB1 splicing or RNA stability could have been altered due to the SNP resulting in the observed phenotype [319]. In a second study, a mutation in CB2 cDNA at position 188-189 from AA/AA to GG/GG resulted in the substitution of glutamine into arginine at amino acid position 63 of the CB2 protein. As a functional consequence, the inhibition of T cell proliferationin the CB2 GG/GG variant by the cannabinoids 2-AG and N-arachidonlyglycine was reduced. Furthermore the GG/GG genotype was significantly overrepresented in individuals diagnosed with autoimmune disease including multiple sclerosis, rheumatoid arthritis, systemic lupus, and myasthenia gravis [318]. Overall, these studies suggest that the cannabinoid system might be involved in preventing autoimmune disease by modulation of immune function through the cannabinoid receptors.

VI. Objectives

The objective for the studies presented as part of this dissertation was to test the hypothesis that Δ9-THC suppresses immune responses generated by influenza virus by perturbing an endogenous signaling pathway relying in part on the presence of the GPCRs CB1 and/or CB2. While CB1 and/or CB2 were deemed important targets of endocannabinoids and Δ9-THC in the impairment of APC-mediated immune responses, suppression of CTL responses was independent of CB1 and CB2. First, the effect of Δ9-THC on the generation of CTL and its CB1 and CB2 dependence was examined. These studies included the characterization of functional consequences of Δ9-THC on CTL effectors as well as the involvement of early

signaling of T cells in an attempt to shed light on a putative mechanism by which Δ9-THC perturbed the generation of CTL. Second, a kinetic study of influenza infection spanning including 1, 3, 5, and 7 dpi in WT and CB1-/-CB2-/- mice was undertaken to: 1. determine the peak time of the inflammatory immune response in WT and CB1-/-CB2-/- mice and 2. investigate the cellular events leading to greatly reduced viral burden in CB1-/-CB2-/- compared to WT mice [120]. Included in these studies were global analyses of steady state mRNA level changes in the lung, analyses of lung tissue for pathology, and extensive *ex vivo* and *in vitro* studies to identify immune cells potentially involved in the disparity of the viral burden in CB1-/-CB2-/- compared to WT mice. Third, the effect of Δ9-THC on the generation of an immune response to influenza infection was evaluated in WT and CB1-/-CB2-/- mice at 3 dpi, the peak day of the inflammatory and highly APC-involved immune response. Similar approaches to the previous studies were used, including evaluating steady state mRNA levels and extensive immune cell response characterization using *ex vivo* analysis and *in vitro* models. The results of these studies indicate that signaling through CB1 and/or CB2 is important in maintaining immune homeostasis and in response to an immune stimulus, such as influenza virus, preventing excessive immune reactivity. Specifically CB1 and/or CB2 are involved in regulating APC function and loss of signaling through these receptors leads to increased inflammatory responses and immunopathology after influenza infection. In particular DC, including cDC and pDC, are regulated by signaling through these two receptors as their immune functions are exacerbated in the absence of CB1 and CB2 signaling. Likewise, DC are very sensitive to Δ9-THC-mediated immunosuppression via CB1 and/or CB2 as observed in the suppression of maturation and the ability to elicit T cells *in vitro*. Moreover, T cell responses including CTL, are suppressed by Δ9-

THC independent of the expression of CB1 and CB2, suggesting alternate mechanisms for immunomodulation by Δ9-THC for T cells.

MATERIALS AND METHODS

I. Mice.

Mice were housed at Michigan State University (MSU) in pathogen-free animal housing facilities at 21 to 24°C and 40 to 60% relative humidity with a 12 hour (h) light/dark cycle. Water and Lab Chow (Nestlé Purina, St. Louis, MO) were provided *ad libitum*. All protocols and procedures were performed in accordance with guidelines set forth by the MSU Animal Care and Use Committee. CB1-/-CB2-/- mice on a C57Bl/6 genetic background were a kind gift of Dr. Andreas Zimmer at the Universität Bonn, Germany [315] and were maintained at MSU and C57Bl/6 (WT) mice were ordered from NCI. Mice were used for experiments between 8-12 weeks of age. CB1-/-CB2-/- mice were genotyped to ensure the targeted deletion of both receptors. It is notable that primers from Applied Biosystems were not able to differentiate *cnr2* gene expression between WT and CB1-/-CB2-/- mice, and therefore custom primers were used [320]. T cell receptor transgenic mice with CD8+ T cells recognizing the peptide OVA257-264 (OT-1) were purchased from Jackson Laboratory (Bar Harbor, ME) and used at 8 weeks of age.

II. Chemicals and Reagents

Δ9-THC was obtained from the National Institute on Drug Abuse (Bethesda, MD). Ethanol was purchased from Decon Labs (King of Prussia, PA). Ionomycin (Io) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI media was obtained from Gibco Invitrogen (Carlsbad, CA), and 51Cr as sodium chromate was obtained from Perkin Elmer (Waltham, MA).

III. T Cell Elicitation for Generation of Functional CTL

C57Bl/6 (WT) and CB1-/-CB2-/- mice were euthanized, the spleens isolated in a sterile environment, and splenocytes (SPLC) enumerated using a Coulter Counter (Beckman Coulter, Brea, CA). Cells from the mouse lymphoblast-like mastocytoma cell line P815 were irradiated with 3000 rads to prevent proliferation, washed 3 times with RPMI and counted using a hemacytometer. SPLC and irradiated P815 cells were combined at 1x10^6 and 1x10^5 cells, respectively, in RPMI 1640 supplemented with 5% bovine calf serum (BCS) in a total volume of 200 µL in a round bottom 96 well plate. The plates were incubated in a humidified incubator with 5% CO2 at 37°C for the indicated amounts of time.

IV. Drug Treatment *in vitro*

At the time of co-culture of SPLC and irradiated P815, Δ9-THC (1, 5, 10 µM), vehicle (VH, 0.1% ethanol) or RPMI (NA) was added. All Δ9-THC treatments had the same ethanol content (0.1%) as vehicle control.

V. 51Cr Release Assay

After elicitation, cells were harvested and washed twice with RPMI 1640 media without serum. P815 cells were washed once and 1x10^6 cells were incubated in the presence of Na251CrO4 for 1 h in 10% BCS supplemented RPMI 1640 media in a volume of less than 50 µL. After incubation P815 cells were washed 3 times using RPMI 1640 media without serum. 51Cr-labelled P815 cells were adjusted to 1x10^5 cells/mL in 2% BCS RPMI media. Elicited CTL were adjusted in 2% BCS complete RPMI media to ratios ranging from 50 (5x10^5) to 1 (1x10^4 cells): 1 (1x10^4) P815 cells, depending on the experimental design, in a volume of 200

μL. After co-culture, elicited CTL and P815 were added to a 96-well round bottom plate and centrifuged at 200g for 1 min to force cellular interactions. Control wells for spontaneous release (200 μL of P815 only) and total release (1% Triton-X 100 in 200 μL of P815 cells in RPMI) were used to determine the range of experimental release. After 5 h of co-culture in a humidified incubator with 5%CO2 at 37°C, cell lysis was assessed by aliquoting 100 μL of supernatant from each well, which represents the experimental release. The cytolytic activity was calculated as follows: % Release = (experimental release – spontaneous release) / (total release – spontaneous release) x 100.

VI. IFNγ T Cell Functional Analysis

CTL were elicited as described above. After 5 days, cells were harvested and co-cultured with P815 at a ratio of 10:1 (see above) for 12 h in the presence of brefeldin A to prevent IFNγ release and allow for detection by fluorescently labeled antibody. After co-culture, cells were prepared for fluorescent antibody staining (described below).

VII. Proliferation Assay

Prior to elicitation, SPLC were incubated with Cell Trace carboxyfluorescein succinimidyl ester (CFSE) dye (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. CTL were elicited as described above for generation of CTL. Dilution of dye staining is indicative of proliferation and staining profiles from elicited samples were compared to a 24 h SPLC only control, incubated without P815.

VIII. T Cell Activation Assays

CTL were elicited as described for generation of CTL; however, RPMI supplemented with 2% BCS was used instead of 5%. For direct stimulation, 0.5 μM ionomycin (Io) dissolved in 0.01% DMSO in RPMI was used as the Ca2+ ionophore to induce intracellular Ca2+ levels and thereby increasing CD69 expression. After 6 h, CD69 surface staining was performed using antibodies specific for CD69 and after 24 h for CD25 (staining described in detail below).

IX. Procedure for Immunofluorescent Staining of CD4+ and CD8+ Cells for Flow Cytometry

CTL were elicited as described above. Cells were washed in 96-well round bottom plates with HBSS once and incubated with Near IR LIVE/DEAD (Invitrogen) dye to assess viability according to manufacturer's instructions. This dye stains cells that have lost membrane integrity. Cells were washed twice with FACS buffer (1X HBSS, 1% bovine serum albumin (BSA), 0.1% sodium azide). Subsequently, Fc receptors were blocked using Purified Rat Anti-Mouse CD16/CD32 (BD Pharmingen, San Diego, CA). To stain surface proteins, cells were incubated for 20 min with 0.25-0.5 μg of CD4 (clone RM4-5, Biolegend, San Diego, CA) and CD8 (53-6.7, Biolegend). For the T cell activation experiments, cells were additionally stained with CD69 (H1.2F3, Biolegend) and CD25 (PC61, Biolegend). Cells were then washed twice and fixed with Cytofix (BD, Franklin Lakes, NJ). After re-stimulation (described above), on the day of flow cytometric analysis, cells were washed once, then permeabilized with Cytoperm (Becton, Dickinson and Company) by incubation for 20 min and stained with IFNγ (XMG1.2, Biolegend) for 30 min. All experiments included single stain controls to compensate for fluorescence overlap between detectors. Samples were individually analyzed for percent positive staining as

defined by a gate to perform statistical analysis and concatenated by treatment group for graphing using FlowJo v.8.8.6. for Macintosh.

X. Influenza instillation.

On the day of instillation, murine-adapted influenza virus A/PR8/34 (a kind gift of Dr. Allen Harmsen, Montana State University) was diluted in 0.9% NaCl (saline) solution (SAL) (Hospira, Lake Forest, IL) to 1 plaque forming unit (pfu) per µL. 25 µL were instilled per nostril resulting in a total inoculum of 50 pfu per mouse. Mice (n=5 each genotype) received either saline or A/PR8/34 under isofluorane anesthesia.

XI. Necropsy and lung tissue collection.

Mice were anesthetized by intraperitoneal injection of 250 mg/kg pentobarbital (Fatal-Plus, Vortech, Dearborn, MI) at 1, 3, 5, and/or 7 days post infection (dpi) and euthanized by exsanguination of the abdominal aorta. Lungs were excised *en bloc*.

XII. BALF analysis and differential cell counts.

Saline (2 x 0.9 mL) was used to lavage lungs and collect the broncho-alveolar lavage fluid (BALF). Total cell counts were obtained by counting an aliquot of the total obtained BALF using a hemacytometer. Differential cell counts were prepared by centrifuging BALF onto glass slides using a Shandon Cytospin 3 centrifuge (Block Scientific, Nutley, NJ) at 600 rpm for 10 minutes. The slides were stained using the Diff-Quik kit (Dade Behring, Newark, DE) following manufacturer's instructions. The stained slides were counted for at least 200 discernable cell

entities including lymphocytes, eosinophils, macrophages and neutrophils, and percentages were calculated. The total cell count was used to enumerate differential cell populations.

XIII. Histological and immunohistochemical staining of lung tissue sections.

After *en bloc* excision, left lung lobes were fixed and inflated under constant pressure (30 cm H2O) with 10% phosphate buffered formalin for one hour. After transverse cuts at airway generation five, tissue sections were paraffin embedded and cut into 4 μM sections. Tissue sections were either histochemically stained with hematoxylin and eosin (H&E) using a standard procedure or were immunohistochemically stained with antibody against clara cell secretory protein (CCSP). Glass slides (Fisher, Waltham, MA) were coated with 2% 3-aminopropyltriethoxysilane and tissue sections were mounted onto slides and dried at 56°C overnight. Xylene was used to deparaffinize the slides, which were hydrated with decreasing concentrations of ethanol to distilled water. Subsequently, the pH was adjusted by soaking slides in Tris Buffered Saline (TBS) pH 7.4 (Scytek Labs, Logan, UT) for 5 min. Following pH adjustment for CCSP staining, standard avidin-biotin complexing for staining was performed at room temperature using a DAKO Autostainer. All staining steps were performed using TBS with Tween 20 (Scytek Labs) for rinses. In short, non-specific protein was blocked using Normal Goat Serum (Vector Labs, Burlingame, CA) for 30 min followed by incubation with Avidin/Biotin blocking system for 15 min each (Avidin D, Vector Labs / d-Biotin, St. Louis, MO). The goat serum blocked slides were incubated with primary polyclonal rabbit anti-CCSP antibody diluted 1:1,600 (Seven Hills, Cincinnati, OH) in Normal Antibody Diluent (Scytek). Biotinylated goat anti-rabbit IgG (H + L) was diluted to 11.0μg/ml in NAD and incubated for 40 min followed by incubation with R.T.U. Alkaline Phosphatase ABC Reagent (Kirkegaard-Perry

Labs, Gaithersburg, MD) for 60 min. For the chromatic reaction Vector Substrate Kit I and counter stain in Gill Hematoxylin (Thermo Fisher, Kalamazoo, MI) for 15 seconds was used. Differentiation, dehydration, clearing and mounting was performed with synthetic mounting media.

XIV. Isolation of alveolar macrophages from BALF.

BALF was obtained as described above from naïve WT and CB1-/-CB2-/- mice. Cells were washed twice and counted with a Coulter Counter and incubated at 0.5x10^5 cells/mL in 2 mL in a 12 well dish. BALF cells were stimulated with and without (naïve, NA) LPS at 1 μg/mL for 24 hours. To assess maturation, MHC I, MHC II, and CD86 staining was performed in addition to CD11b and CD11c to gate on macrophage populations. Analysis of fluorescence intensity of maturation markers was exclusively performed on the alveolar macrophage populations (CD11bmidCD11chi), which constituted the majority of cells obtained from the BALF.

XV. Flow Cytometry on lung isolated cells.

From a second set of mice, lung tissue from left and right lung lobes was homogenized using a cell dissociation sieve kit (CD-1, Sigma, St. Louis, MO), connective tissue was removed and cells were immersed in RPMI media. For cytokine production analyses, cells were restimulated *in vitro* with phorbolmyristate acetate (PMA) and Io (both from Sigma) at 40 nM and 0.5 μM, respectively, for 5 h in the presence of Brefeldin A (Biolegend) in 2% BCS RPMI media supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (all from GIBCO

Invitrogen, Carlsbad, CA). Cells isolated from lung or restimulated *in vitro* were washed 3 times with FACS buffer (1 X Hank's Buffered Saline Solution, 1% bovine serum albumin, 0.1% sodium azide, pH adjusted to 7.6) and Fc receptors were blocked using purified rat anti-mouse CD16/CD32 (BD Pharmingen, San Diego, CA). Surface markers were stained with specific antibodies, using concentrations of 0.25-0.5 µg per 1x10^6 cells. FACS antibodies used were all from Biolegend (San Diego, CA): CD4 (clone RM4-5), CD8 (53-6.7), CD69 (H1.2F3) and NK1.1 (CD161, PK136). For lung APC populations the following antibodies (all from Biolegend) were used: CD11b (M1/70), CD11c (N418), CD80 (16-10A1), CD86 (GL-1), Gr-1 (RB6-8C5), MHC I (H-2Kb/H-2Db, 28-8-6), and MHC II (I-A/I-E, M5/114.15.2). Cells were fixed with Cytofix (BD, Franklin Lakes, NJ) before flow cytometric analysis. Intracellular staining for IFNγ (XMG1.2) and IL-17 (TC11-18H10.1) was performed using 1 X Perm/Wash (BD) for washes and incubation according to manufacturer's instructions. Samples were prepared in 96 well round-bottom plates and were analyzed using a BD Biosciences FACSCanto II flow cytometer. Obtained data were analyzed individually and concatenated for graphical representation with FlowJo v8.8.6 (Treestar Ashland, OR) for Mac.

XVI. mRNA extraction and cDNA synthesis.

Lung tissues from right lung lobes were minced with a dounce tissue grinder set (Sigma) and RNA extracted from right lung tissue using TRIzol reagent (Sigma) according to manufacturer's instructions. Extracted mRNA was reverse transcribed into cDNA using 2.5 µg total RNA in a 50 µL reaction volume with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

XVII. Mouse immune panel – low density microarray.

Mouse low density microarray immune panels were obtained from Applied Biosystems andcontained primers for 93 immune-related and three housekeeping genes using the TaqMan PCR system. The panels were loaded with 50 ng of cDNA/well and centrifuged two times for 1 minute at 1100 rpm in a Sorvall Legend T (Thermo Fisher Scientific, Waltham, MA). 18S gene expression was used to confirm consistent loading (all Ct values between 8-9) and was also used to normalize gene expression changes. Data were analyzed with an Applied Biosystems 7900HT Real Time PCR machine using the range of 2-5 cycles for 18S and 2-12 cycles for other genes to set a background level in order to determine a threshold for the fluorescence emission of the TaqMan probes. Fold expression levels were generated using ΔΔCt method with respect to WT SAL control samples set to a fold change level of 1. Genes were normalized with blom transformations and statistical analysis was performed with Statistical Analysis System (SAS) 9.1.3 (Cary, NC). Gene expression data were log-transformed, mean centered and normalized using Cluster version 2.11 and visualized using Treeview version 1.60 [321].

XVIII. Generation and stimulation of bone marrow-derived dendritic cells (bmDC) *in vitro*.

The isolation and generation of bmDC were performed as adopted from Lutz, *et.al.*,[322] and Brandt, *et.al.*,[323]. In short, mice were euthanized, femurs and tibias were removed aseptically, and bone marrow was flushed with sterile RPMI. Single cell suspensions were obtained and connective tissue was removed with a cell strainer (BD Falcon, Franklin Lakes, NJ). Cells were cultured at 2.5 x 10^5 cells/mL in 10% BCS RPMI supplemented with 20 ng/mL GMCSF (Peprotech, Rocky Hill, NJ). After exchange of 50% of media with fresh media and

removal of supernatant cells on days 3, 6, and 8 after start of culture, bmDC were used on day 9 for treatments. Lipopolysaccharide (LPS) (*salmonella typhimurium*, Sigma),1 μg/mL, and single stranded RNA (ssRNA) (Invivogen, San Diego, CA), 5 μg/mL,were used to stimulate bmDC for 24 hr in 2% FBS RPMI. Subsequently, cells were stained with LIVE/DEAD cell viability dye (Invitrogen) according to manufacturer's instructions and anti-mouse I-A/I-E (MHC II, M5/114.15.2), CD11c (N418), CD11b (M1/70), CD80 (16-10A1), CD86 (GL-1), H2Kb/H2Db (MHC I, 28-8-6) as described above.

XIX. bmDC co-culture with OT-1 cells.

bmDC, generated as described above, were seeded at 2 x 10^5 cells per well in a 96 well round bottom plate and pulsed with OVA257-264 (SIINFEKL) (Anaspec, San Jose, CA) for 2 h in 200 μL of RPMI media. Subsequently, bmDC were washed 3X with RPMI. Spleens from OT-1 mice were aseptically removed and washed in RPMI. OT-1 cells were labeled with CellTrace (Invitrogen) proliferation dye according to manufacturer's instructions. OT-1 cells were added into wells with SIINFEKL-pulsed and –unpulsed bmDC at a final concentration of 5 x 10^6 cells/mL and co-cultured for 4 days. After 4 days in culture, OT-1 cells were restimulated with addition of SIINFEKL at 5 ug/mL for 5 h in the presence of Brefeldin A in 2% BCS RPMI. Staining was done with LIVE/DEAD cell viability dye and antibodies to CD8, IFNγ as described above.

XX. Isolation and activation of naïve CD4+ T cells.

Naïve CD4+ T cells were isolated from splenocyte preparations using a naïve CD4+ T cell isolation kit according to manufacturer's instructions (Miltenyi, Auburn, CA). To

immobilize αCD3 (145-2C11, BD Biosciences, San Jose, CA), flat bottom 96-well plates were coated with 80 µL of αCD3 at 5 µg/mL overnight and washed 3 times with sterile PBS before addition of cells. After purification, 0.5 x 10^6 naïve CD4+ T cells were added per well in a total volume of 200 µL of RPMI supplemented with 5 % BCS and 1 % penicillin/streptomycin (Sigma). To elicit Th0, αCD28 (37.51, BD Biosciences) was added at a final concentration of 1 ug/mL. For T helper 17 (Th17)-polarizing conditions, additional supplementation with TGF-beta (5 ng/mL) (R&D Systems, Minneapolis, MN), IL-6 (20 ng/mL) (Jena Bioscience,Jena, Germany), and IL-2 (40 U/mL) (RocheApplied Science, Indianapolis, IN) as well as αIFNγ (10 µg/mL) (BD Pharmingen, San Diego, CA) and αIL-4 (10 µg/mL) (BD Pharmingen) was necessary [324]. Four days after elicitation, cell cultures were restimulated with PMA/Io and stained as described above.

XXI. Δ9-THC treatment *in vivo*

The National Institute on Drug Abuse (NIDA, Bethesda, MD) provided Δ9-THC for experimental use. For *in vivo* experiments, mice received corn oil (CO) or Δ9-THC by oral gavage at 0.1 ml/g body weight for five consecutive days (-2 to 2 dpi) surrounding the instillation of A/PR/8/34. Δ9-THC was dissolved in corn oil (CO) and administered at a final dose of 75 mg/kg mouse/day.

XXII. Quantitative Real-time PCR for type I IFN, CB1, CB2, and H1 mRNA

cDNA was obtained as described above and was analyzed for IFNα2 and IFNβ1 expression by specific Taqman primer probe sets (Applied Biosystems) according to manufacturer's instructions. Each target gene (IFNa, IFNβ, CB1, CB2, and H1) was multiplexed

with an 18S loading control. Baseline levels for the loading control and the target genes were set as described above and fold change levels were calculated as previously described. Data were analyzed and graphed using GraphPad Prism (La Jolla, CA) version 4.03.

XXIII. Statistical analyses

Statistical analysis was performed with SAS and GraphPad Prism 4.03 (La Jolla, CA) software. To determine statistical significance of the effect of treatment (A/PR/8/34 or SAL) with respect to time, One Way ANOVAs with comparison to WT 1 dpi SAL were performed. Factorial ANOVA with a Tukey's post-hoc analysis was chosen for the statistical test with a 2 by 2 design including interactions for comparison between each genotype at each dpi. The independent variables were genotype (WT or CB1-/-CB2-/-) and treatment (A/PR/8/34 or SAL) and the dependent variable was the obtained experimental value. For studies using Δ9-THC and CO, a 2x2x2 (genotype, immune stimulation, drug treatment) design was used. A p-value of 0.05 or less was deemed statistically significant. In scenarios where data were non-parametric, such as data represented as percentages, Friedman's test was used as a substitute for ANOVA. To determine a statistical significance of a Δ9-THC treatment effect, T-tests or non-parametric Mann-Whitney U tests were performed.

EXPERIMENTAL RESULTS

I. Δ9-THC Suppresses CTL Responses Independent of CB1 and CB2

A) Kinetics of CTL Response

In order to determine the kinetics of the P815-induced CTL response, CTL were elicited for 3, 5, 7, and 9 days after co-culture with irradiated P815 cells. Splenocytes from WT and CB1-/-CB2-/- mice were used as the source of naïve CD8+ T cells. Elicited CTL were assayed for CTL activity by 51Cr release assay using 51Cr-labelled P815 target cells. A ratio of 50:1 CTL:P815 cells was used to maximize release, thereby increasing sensitivity on non-peak days. 51Cr release is a result of CTL-dependent lysis of P815 cells and the peak day of CTL activity occurred at day 5 post elicitation (Figure 4). At the peak day of CTL elicitation, CB1-/-CB2-/- splenocytes had a statistically significant reduction in CTL activity, when compared to WT ($p \leq 0.01$). The same trend was observed for non-peak days, although it was not deemed statistically significant. Subsequent experiments determining CTL effector activity under various conditions were performed at day 5 post elicitation.

B) Δ9-THC Suppressed CTL Responses during Elicitation but not during the Effector Response

To determine the sensitivity of CTL function to Δ9-THC treatment in the P815 allogeneic model, Δ9-THC and/or vehicle (0.1% ethanol) were added directly to culture during either the elicitation or effector phase of the CTL response. A concentration of 10 μM Δ9-THC was used to determine sensitivity of CTL to immune modulation, a concentration that historically impaired T cell immune responses in the absence of direct cytotoxicity [279]. Δ9-THC suppressed CTL

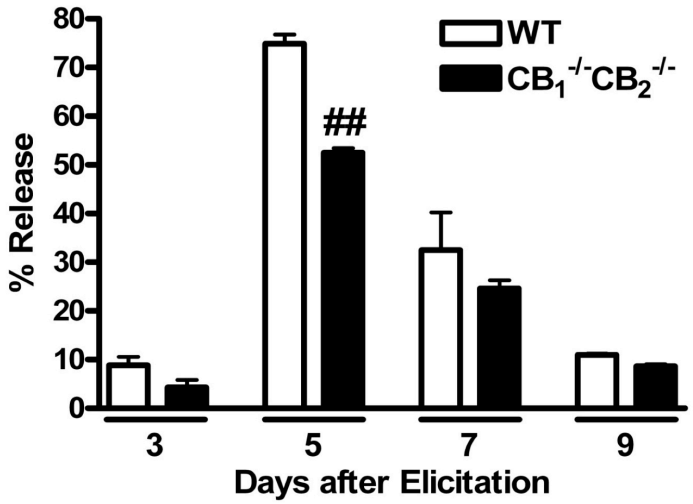


Figure 4. CTL Activity Peaks at Day 5 After Elicitation. To determine the peak day of CTL activity, SPLC from WT and CB1-/-CB2-/- mice were co-cultured with irradiated P815 cells to elicit an allogeneic CTL response. At varying days after elicitation, CTL were harvested and assayed for target (P815) lysing activity by reincubation at a ratio of 50:1 (Effector CTL : P815 Targets) with 51Cr labeled P815 cells. Percent release was calculated as described in Methods (n=4). The experiment was performed once, while more replicate experiments were performed for WT only. ## $p \leq 0.01$ indicates difference between WT and CB1-/-CB2-/-.

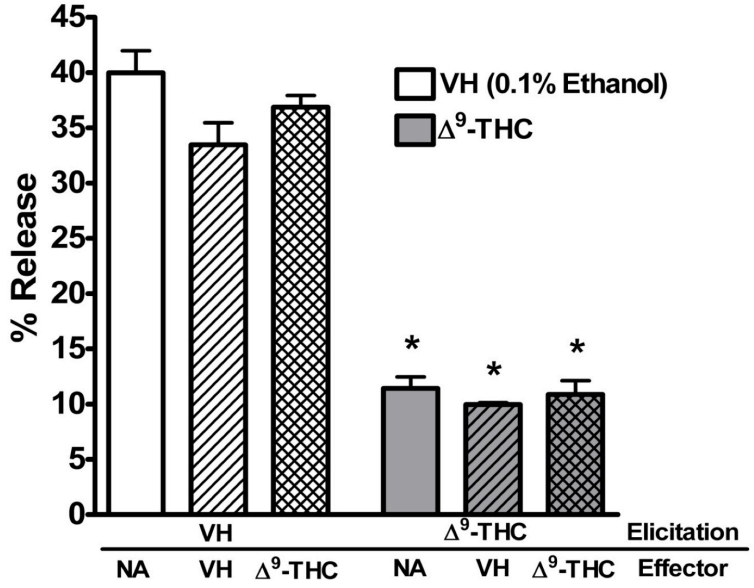


Figure 5. Δ9-THC Suppresses CTL Activity During Elicitation But Not Effector Phase. SPLC were treated with Δ9-THC and/or VH (0.1% ethanol) and stimulated with irradiated P815 cells for 5 days. After harvest, CTL were reincubated at a 10:1 ratio with 51Cr labeled P815 cells in the presence of Δ9-THC and appropriate controls (NA – naïve: no treatment; VH – 0.1 % ethanol) (n=4). Data shown are representative of two repeat experiments. * $p \leq 0.05$ indicates difference as compared to VH.

activity only when added prior to, but not after, the elicitation phase (Figure 5). Likewise, repeated addition of Δ9-THC during elicitation and effector phases did not further reduce CTL activity below single addition of the drug during the elicitation phase (Figure 5).

C) Concentration-dependent Suppression of CTL Effector Function

Next, we assessed whether CTL responses are suppressed by Δ9-THC in a concentration-dependent manner. At E:T ratios ranging from 10:1 to 1:1, Δ9-THC suppressed CTL responses in a concentration-dependent manner with statistically significant suppression at concentrations as low as 1 μM (Figure 6 A). Also, lower E:T ratios resulted in lower CTL activity, demonstrating that the elicited cell populations are responsible for the cytolytic activity and that Δ9-THC suppressed CTL activity independent of the E:T ratio (Figure 6 A). Regression analysis was performed on CTL activity results and a statistically significant difference was observed from zero regression values for E:T ratios 10:1 ($r^2=0.8650$, $p<0.0001$), 5:1 ($r^2=0.7246$, $p=0.0004$), 2.5:1 ($r^2=0.7461$, $p=0.0003$), 1:1 ($r^2=0.7692$, $p=0.0002$). Next, we assessed whether the effect by Δ9-THC was dependent on the presence of CB1 and/or CB2. Δ9-THC suppressed CTL activity in a concentration-dependent manner in CTL elicited from CB1-/-CB2-/- splenocytes with significantly reduced CTL activity at concentrations of 5 μM or more as in WT (Figure 6 B). Regression analysis revealed a statistically significant difference from zero regression of E:T ratio of 10:1 in WT ($r^2=0.6893$, $p=0.0008$) and showed the same trend in CB1-/-CB2-/- albeit not significant ($r^2=0.2905$, $p=0.0706$) . Consistent with previous observations, CTL activity of CB1-/-CB2-/- splenocytes was lower in magnitude compared to WT, although differences in genotypes were not deemed statistically significant.

Figure 6. Δ9-THC Suppresses CTL Activity in a Concentration-Dependent Manner. SPLC from WT were treated with 1, 5 and 10 μM of Δ9-THC and/or VH (control) and co-cultured for 5 days with irradiated P815 cells. After harvest, CTL were reincubated with 51Cr labeled P815 cells at indicated ratios of 10:1 to 1:1 (A) (n=4). In a second experiment SPLC from WT and CB1-/-CB2-/- were elicited and restimulated with 51Cr labeled P815 cells at a ratio of 10:1 (B) (n=4). Data shown are representative of two repeat experiments. * $p \leq 0.05$, ** $p \leq 0.01$ indicate differences as compared to VH.

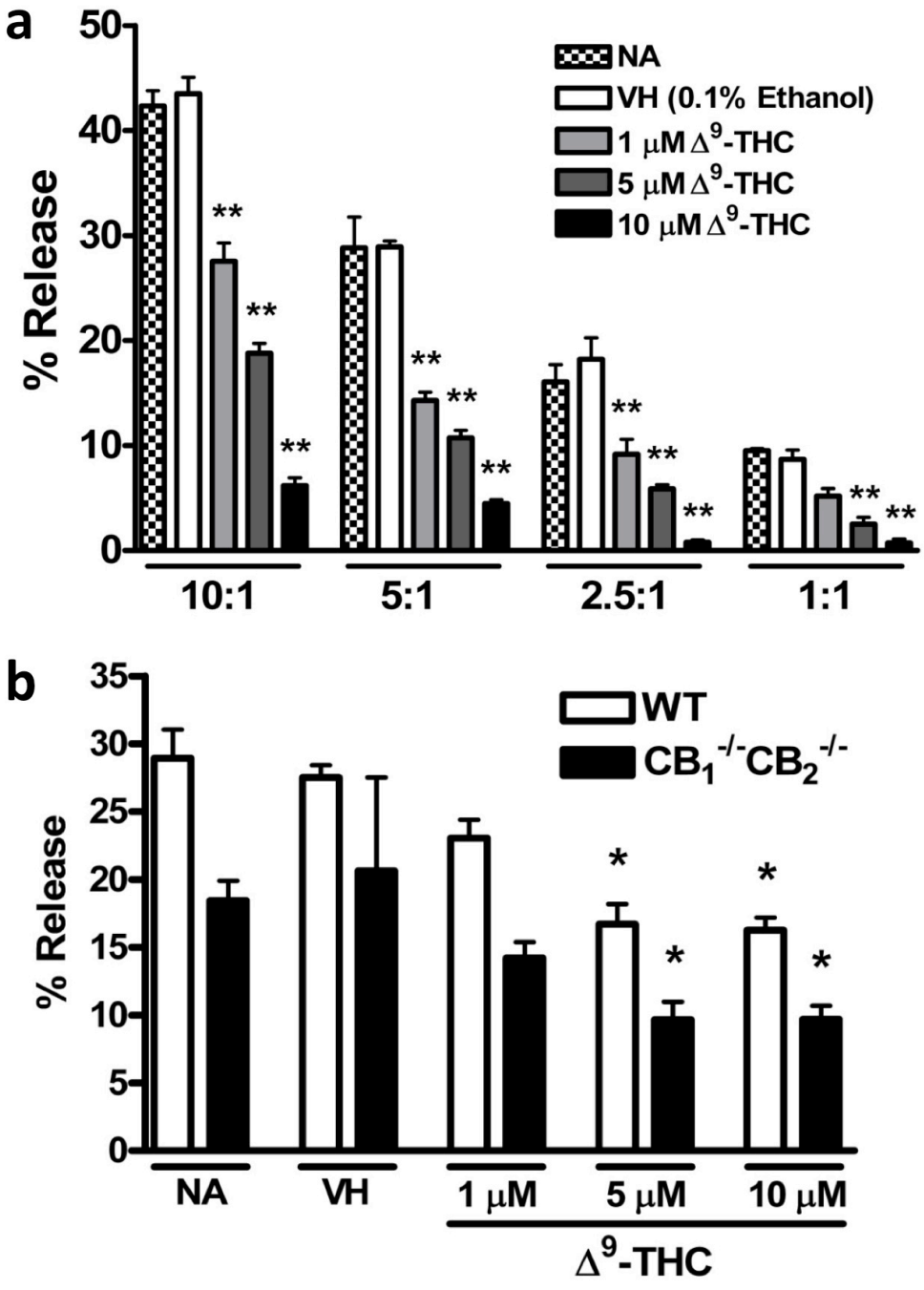


Figure 6. Δ9-THC Suppresses CTL Activity in a Concentration-Dependent Manner.

D) Δ9-THC-Mediated Suppression of CTL Generation

Although MHC I mismatch is a strong stimulus for driving the expansion of differentiated CD8+ T cell effectors, the overall number of viable T cells within control cultures (NA or VH treatment groups) on day 5 of co-culture with P815 allogenic target cells was modest. This demonstrated that T cell proliferation and clonal expansion in response to MHC I-restricted alloantigens in this assay system induced a relatively small number of T cells from the spleen. To assess the effects of Δ9-THC on its ability to impair alloantigen-induced expansion of T cell effectors, the number of viable CD4+ and CD8+ T cells was enumerated on Day 5 of the elicitation phase of the CTL response. Viability was determined using LIVE/DEAD dye (Invitrogen), which permeates dead cells and thus low staining is observed in viable cell populations. The percentage of viable CD4+ (Figure 7 A, B) and CD8+ (Figure 7 C, D) was markedly reduced by Δ9-THC treatment 5 days after elicitation with concentrations as low as 1 μM in both WT and CB1-/-CB2-/- samples. No significant differences were found in the percent of viable CD8+ cells between CB1-/-CB2-/- and WT splenocytes (Figure 7 C, D); however, a greater number of viable CD4+ T cells was observed in CB1-/-CB2-/- compared to WT splenocytes (Figure 7 A, B).

IFNγ production by CD8+ T cells is often used as a surrogate for CTL activity. After gating for single live cells (Figure 8 A), CD4+ cells did not produce significant amounts of IFNγ as P815 cells are MHC I-mismatched to C57Bl/6 cells and do not express significant amounts of MHC II (Figure 8 B). However, significant IFNγ production was detected by CD8+ cells, which was enhanced in CTL elicited from WT compared to CB1-/-CB2-/- (Figure 8 C-E). In the presence of Δ9-THC, enhanced IFNγ production was observed in CD8+ T cells derived from

Figure 7. Lower Viability of CD4+ and CD8+ Cells Following Δ9-THC Treatment.

CTL were elicited from SPLC of WT and CB1-/-CB2-/- mice in the presence or absence of VH (0.1 % ethanol) or Δ9-THC (1, 5, and 10 μM). After 5 days in culture, CD4 and CD8 surface staining was performed and viability was assessed using LIVE/DEAD staining. Viability was determined within CD4+ (A, B) and CD8+ (C, D) cells after singlet and lymphocyte size gating (n = 4). Data shown are representative of four repeat experiments. * $p \leq 0.05$, ** $p \leq 0.01$ indicate differences as compared to VH, ## $p \leq 0.01$ as compared to WT.

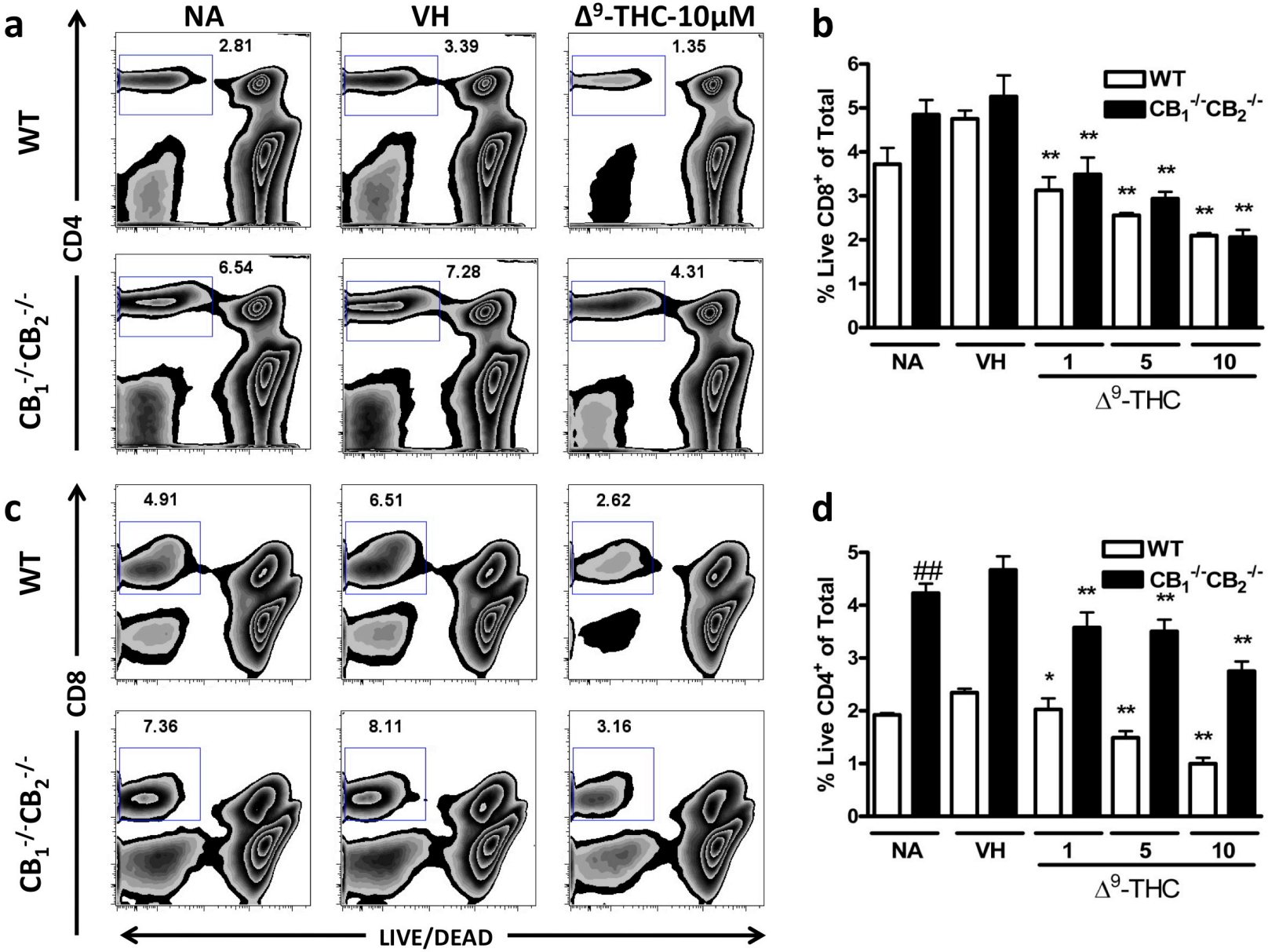


Figure 7. Lower Viability of CD4+ and CD8+ Cells Following Δ9-THC Treatment.

Figure 8. Increased IFNγ Production in Live But Not Total Cells After Δ9-THC treatment.

CTL of WT and CB1-/-CB2-/- were elicited as before with and without VH (0.1% Ethanol) or Δ9-THC (1, 5, 10 μM) treatment for 5 days. Cells were restimulated for 12 h in the presence of Brefeldin A and stained for CD4+, CD8+, LIVE/DEAD and IFNγ. Gating scheme is shown (A) for populations of CD4+ (B) and CD8+ (C) cells and dot plots indicate concatenated samples (n=4). Bar graph for CD8+ cells are shown within live populations % (D), MFI (E), and in % of total populations (F) (n=4). Data shown are representative of three repeat experiments. ** $p \leq 0.01$ indicate differences as compared to VH, ## $p \leq 0.01$ as compared to WT.

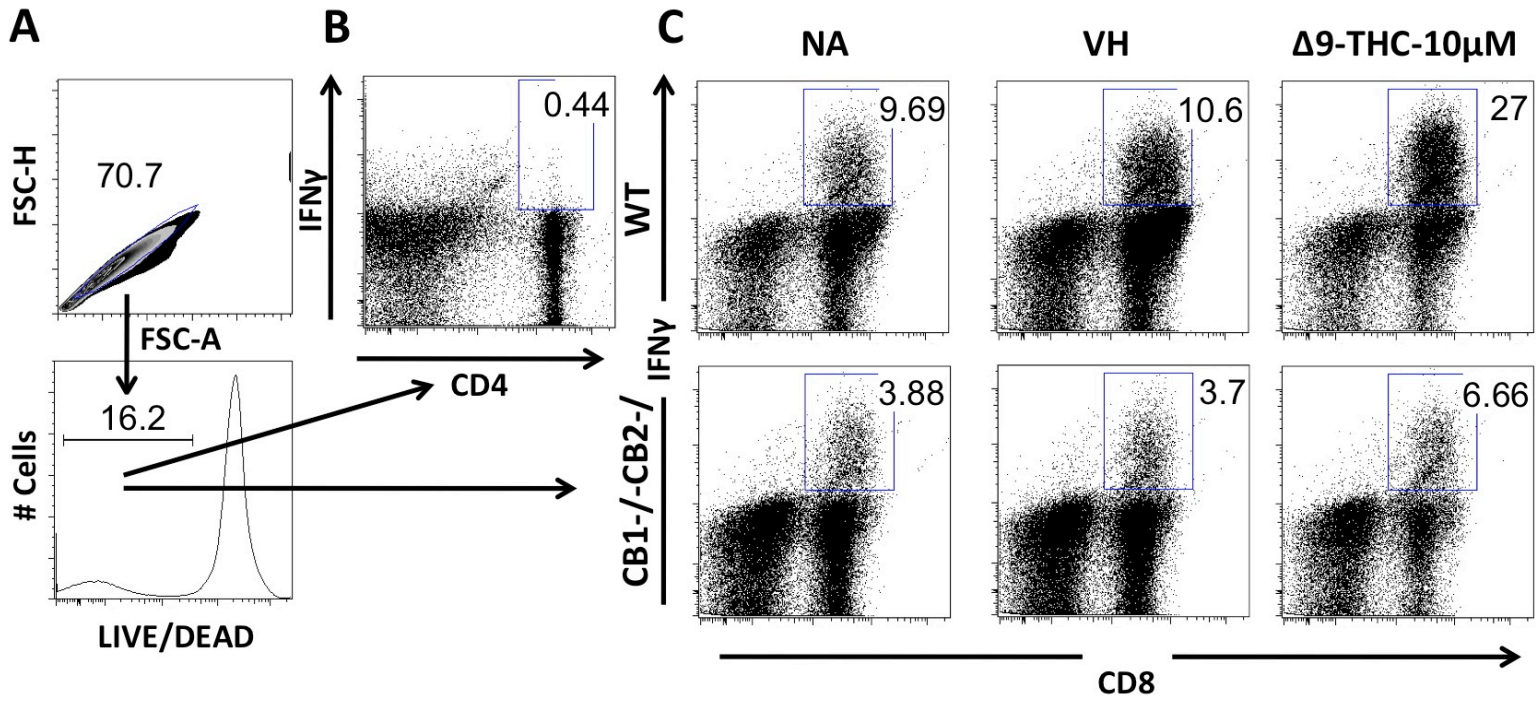


Figure 8. Increased IFNγ Production in Live But Not Total Cells After Δ9-THC treatment.

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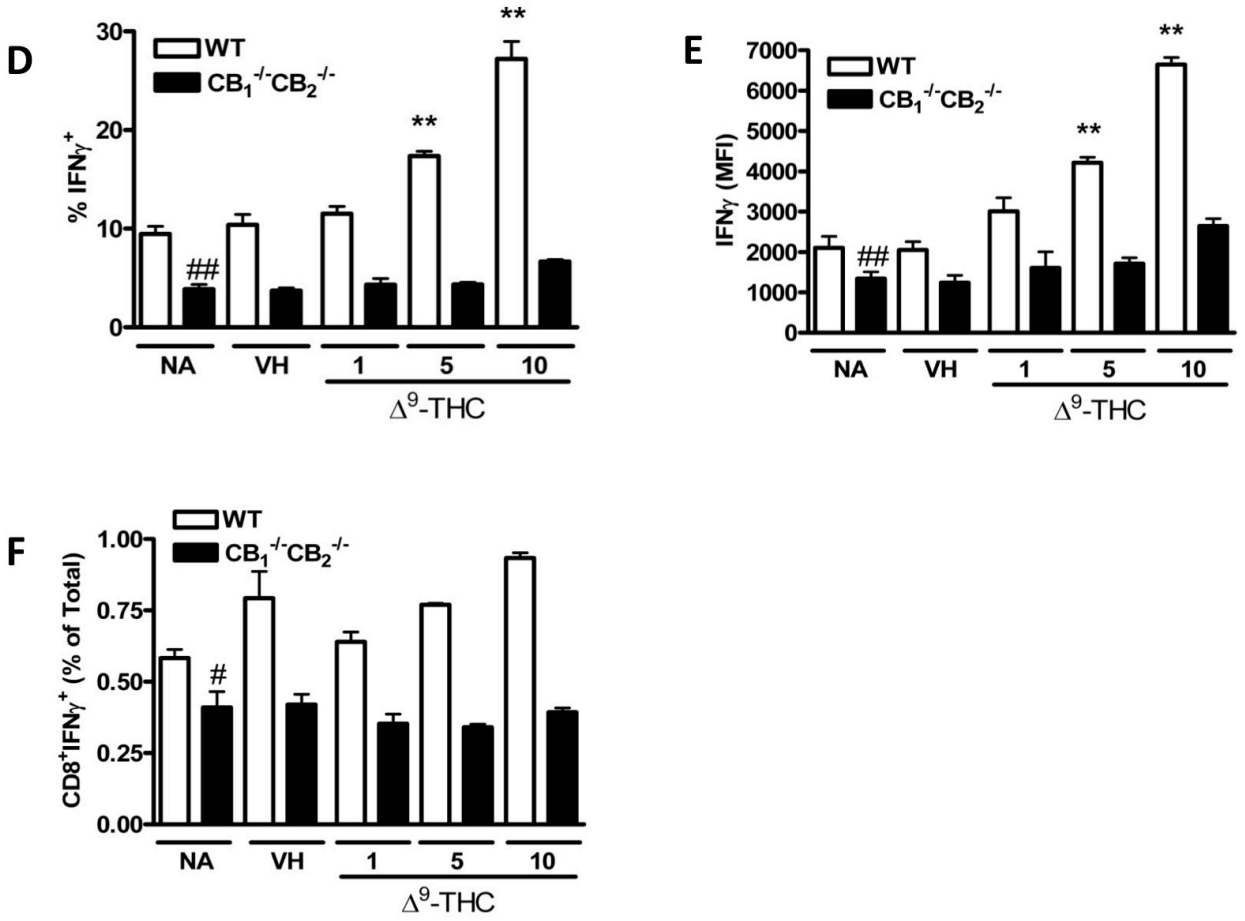


/-CB2-/- mice, although not deemed statistically significant (Figure 8 C-E).

Also, IFNγ production was reduced in samples from CB1-/-CB2-/- compared to WT mice as previously observed in 51Cr release assays (Figure 8 C-E). Analysis of CD8+ IFNγ-secreting cells, as a percentage of viable cells, takes into account the reduced viability as a result of Δ9-THC addition (see Figure 7). However, while there was an enhancement of IFNγ-secreting cells with Δ9-THC treatment, there were also fewer viable cells after Δ9-THC treatment, thus overall the percentage of CD8+ cells secreting IFNγ within the total population did not change (Figure 8 F).

E) Δ9-THC Does Not Affect CD8+ T cell Proliferation Induced by P815 Co-culture

Prior to becoming functional CTL, proliferation occurs in T cells to expand the effector pool. Thus, we focused on proliferation as a potential endpoint leading to decreased viability and decreased CTL response. Whole splenocytes were labeled with CFSE dye to determine proliferation by dye dilution. No statistically significant differences in proliferation were observed between VH and Δ9-THC-treated groups in CD8+ or in CD4+ cells from WT or CB1-/-CB2-/- mice (Figure 9 A-D). In comparison to WT, CD4+ cells obtained from CB1-/-CB2-/- spleens underwent much greater proliferation. CD4+ T cells were not directly stimulated in this model, suggesting that greater proliferation in CB1-/-CB2-/- CD4+ cells might either be due to higher basal proliferation in CB1-/-CB2-/- cells or a result of the cytokines produced by CD8+ cells. In contrast, notably more CD8+ cells were elicited in samples from WT compared to CB1-/-CB2-/- spleens (Figure 9 C, D).

Figure 9. No Effect of Δ9-THC on CTL Proliferation.

SPLC were isolated from WT and CB1-/-CB2-/- mice and labeled with Cell Trace CFSE according to manufacturer's instructions. Four days after co-culture with irradiated P815 in the presence or absence of VH (0.1 % ethanol) and Δ9-THC (1, 5, 10 μM), surface staining with CD4 and CD8 were performed and CFSE fluorescence was assessed by FACS. Shown are concatenated samples (n=4) of CFSE staining as a result of proliferation in CD4+ (A, B) and CD8+ cells (C, D). Data shown are representative of two repeat experiments. * $p \leq 0.05$ indicate differences as compared to VH, ## $p \leq 0.01$ as compared to WT.

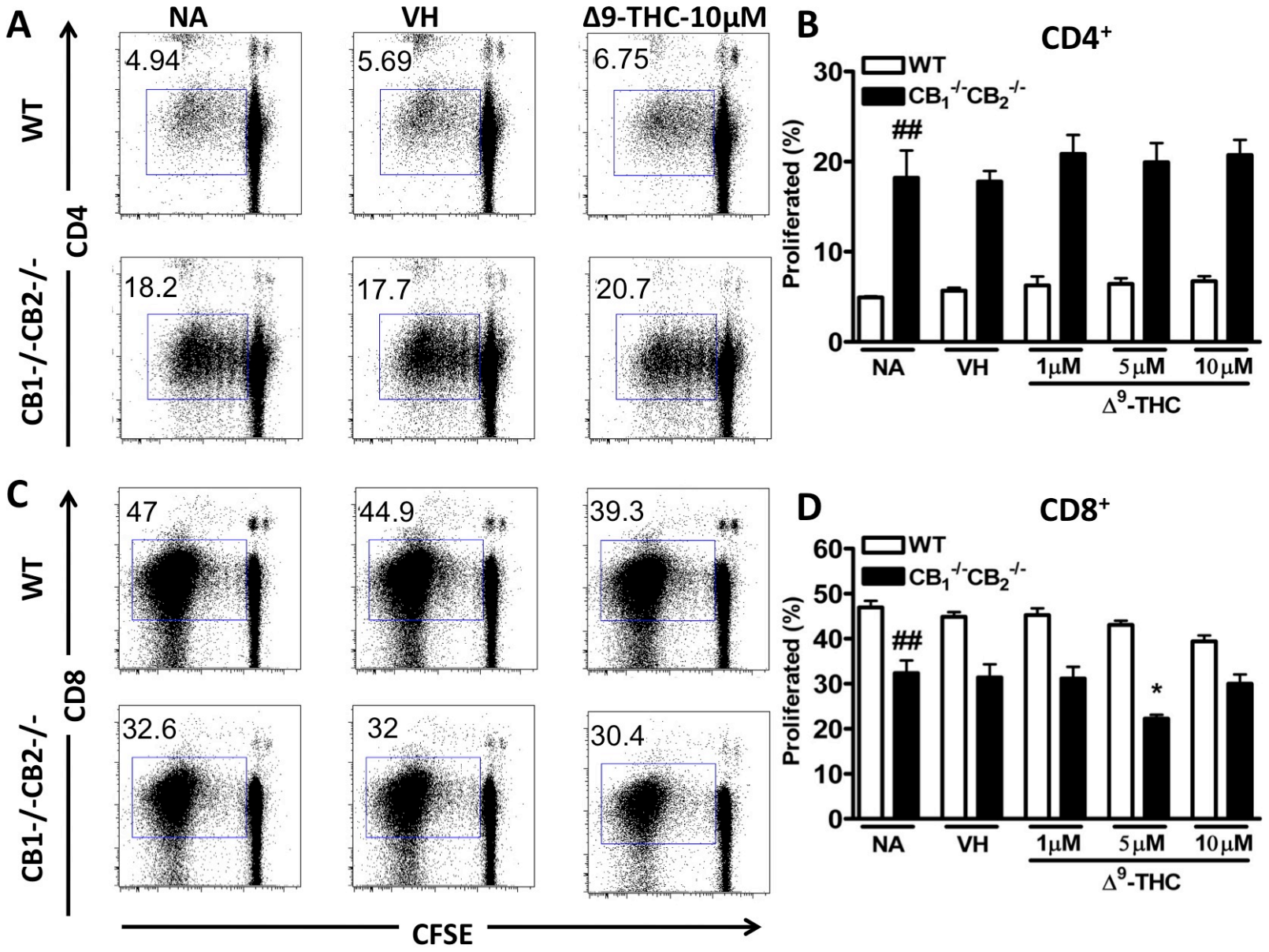


Figure 9. No Effect of Δ9-THC on CTL Proliferation.

F) Increase of CD69 Expression on CD8+ Cells with Δ9-THC Treatment

In an effort to determine events leading to the Δ9-THC-mediated reduction in cell viability and CTL activity seen at day 5, without any apparent effects on proliferation, we focused on T cell activation. Two markers of cellular activation found on both CD4+ and CD8+ T cells are CD69 and CD25. Whereas CD69 is thought to be one of the earliest markers of activation and is rapidly upregulated, CD25 expression increases gradually on activated T cells and is observed later than CD69 on activated T cells [325, 326]. We focused on CD69 expression at 6 h and CD25 expression at 12 h after co-culture with irradiated P815 cells. Cells were gated on singlets, by size, and then on CD4 or CD8 (Figure 10 A). In CD4+ cells, CD69 expression was unaffected by co-culture with P815 cells; however, cells from CB1-/-CB2-/- mice showed higher basal expression compared to WT mice (Figure 10 B, C). In addition, no effect with Δ9-THC treatment was observed on CD69 expression on CD4+ cells (Figure 10 B, C). In contrast, the expression of CD69 on CD8+ was increased by co-culture with P815 cells and CD8+ T cells from CB1-/-CB2-/- expressed higher levels of CD69 than WT CD8+ T cells (Figure 10 D, E). CD69 expression was increased in a concentration-dependent manner by Δ9-THC in CD8+ T cells from both WT and CB1-/-CB2-/- spleens, which was significant at concentrations as low as 5 μM in CB1-/-CB2-/- -derived cells (Figure 10 D, E).

CD25 expression on CD4+ T cells was increased as a result of stimulation with P815 cells and increased levels of CD25 expression were observed in naïve unstimulated samples from CB1-/-CB2-/- compared to WT mice (Figure 11 A, B). There was a trend towards a Δ9-THC-dependent increase in CD25 expression in both WT and CB1-/-CB2-/- CD4+ cell populations, but this trend was not statistically significant (Figure 11 B). In CD8+ cells, the expression of CD25

Figure 10. Δ9-THC Increases CD69 Expression on CD8+ cells in a Concentration-Dependent Manner

SPLC from of WT and CB1-/-CB2-/- mice were incubated with irradiated P815 cells for 6 h to induce CD69 surface expression, in the presence or absence of VH (0.1 % ethanol) or Δ9-THC (1,5, and 10 μM). Cells were gated on singlets and lymphocyte populations by size and then on CD4 and CD8 (A). CD4+ (B, C) and CD8+ (D, E) cells positive as defined by the box gate for CD69 in % are shown (n=4). Data shown are representative of three repeat experiments. Difference due to stimulation by P815 is indicated by ++ $p \leq 0.01$, between genotypes is indicated by ##, $p \leq 0.01$ due to Δ9-THC by *, $p \leq 0.05$** $p \leq 0.01$.

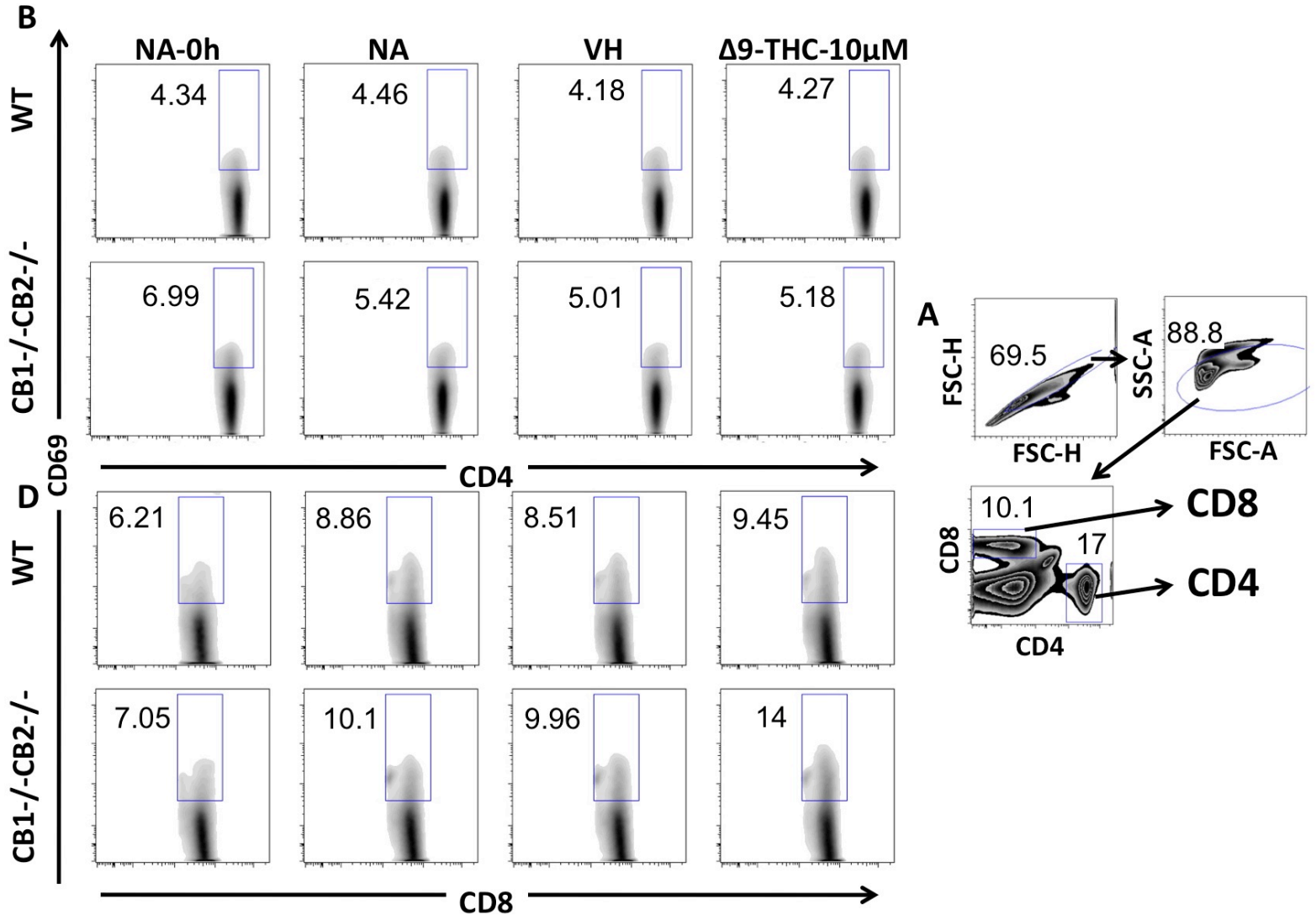


Figure 10. Δ9-THC Increases CD69 Expression on CD8+ cells in a Concentration-Dependent Manner

Figure 10. cont'd.

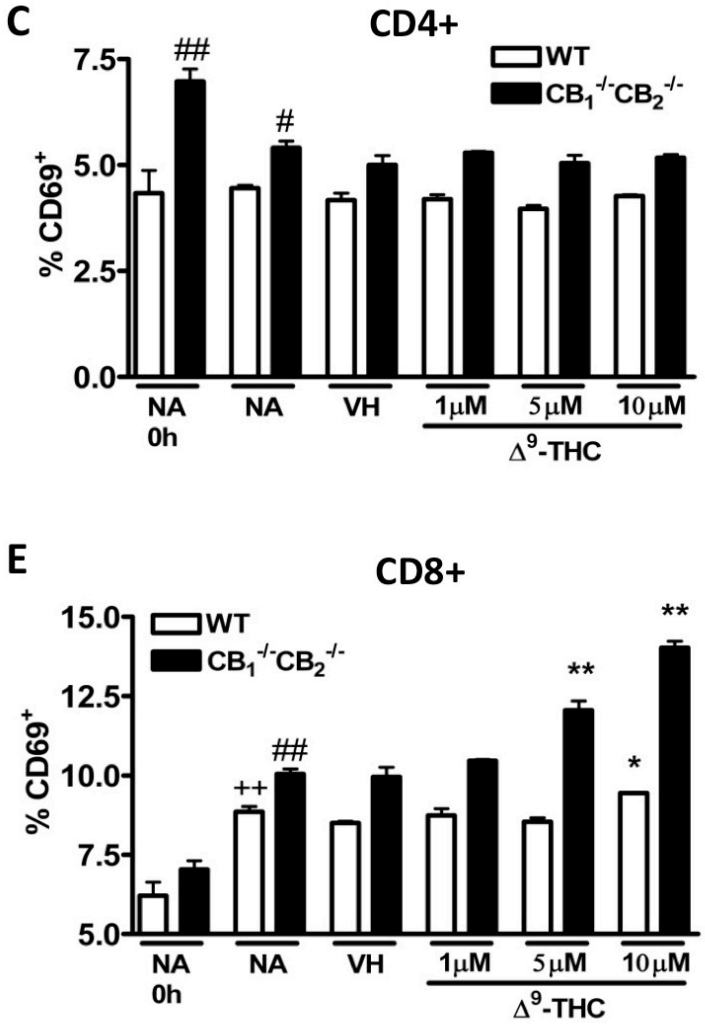


Figure 11. Δ9-THC Decreases CD25 Expression on CD8+ Cells from CB1-/-CB2-/- mice in a Concentration-Dependent Manner

SPLC from of WT and CB1-/-CB2-/- mice were co-cultured with irradiated P815 cells for 12 h to induce CD25 levels, in the presence or absence of VH (0.1 % ethanol) or Δ9-THC (1,5, and 10 μM). Cells were gated on singlets and lymphocyte populations by size and cells positive for CD25 in % are shown (n=4). Data shown are representative of two repeat experiments. Difference due to stimulation by P815 is indicated by ++ $p \leq 0.01$, between genotypes is indicated by ##, $p \leq 0.01$ due to Δ9-THC by *, $p \leq 0.05$.

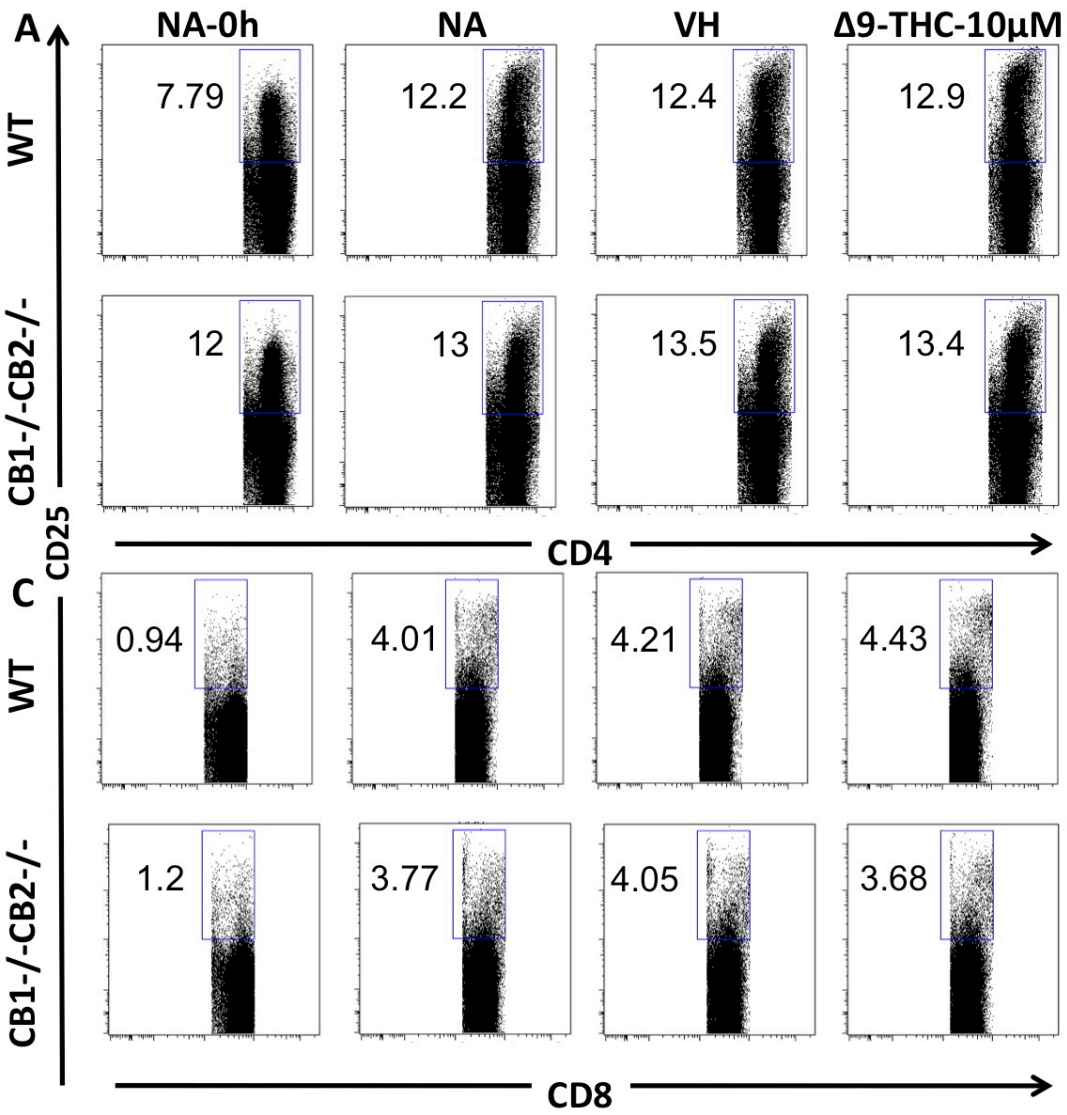
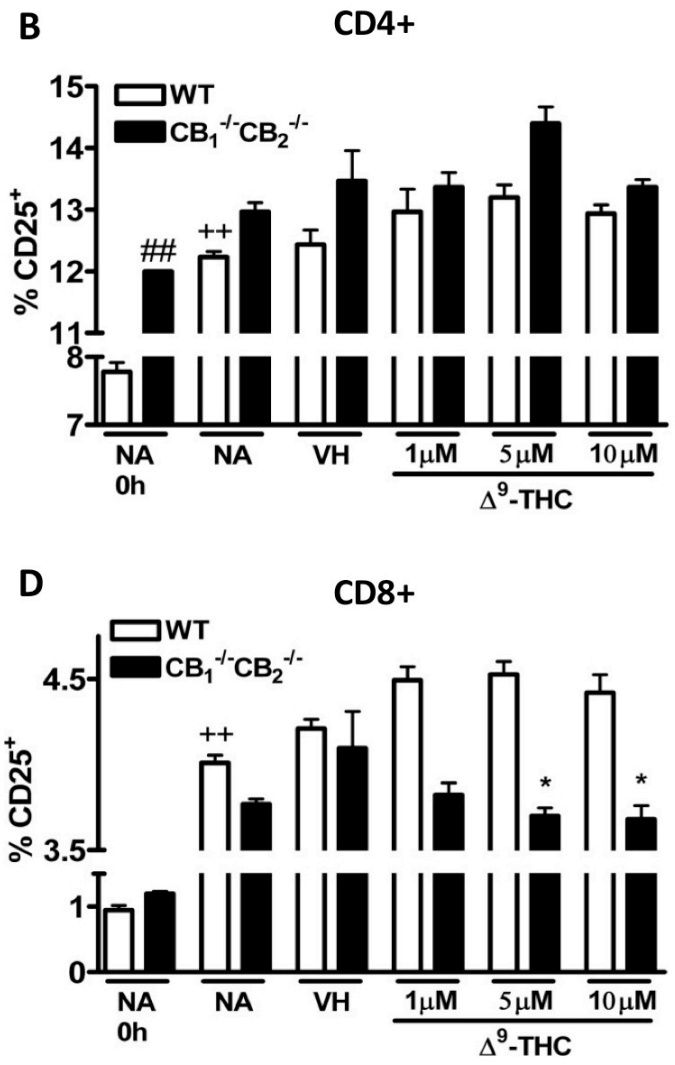


Figure 11. Δ9-THC Decreases CD25 Expression on CD8+ Cells from CB1-/-CB2-/- mice in a Concentration-Dependent Manner

Figure 11 cont'd



was much lower than in CD4+ cells (Figure 11 A-D). Expression of CD25 on CD8+ cells of both genotypes was increased by co-culture with P815 cells (Figure 11 C, D). In CD8+ cells from CB1-/-CB2-/- mice, a concentration-dependent decrease with Δ9-THC treatment was observed, while in WT mice Δ9-THC had no effect (Figure 11 C, D).

G) **Δ9-THC Synergizes with Io to Induce CD69 Expression**

In light of the rapid Δ9-THC-mediated increase in CD69 expression of CD8+ T cells and its inverse correlation with Δ9-THC-mediated suppression of CTL activity, we focused on intracellular signals leading to CD69 protein expression. Previously, it was determined that independent of CB1 and CB2, Δ9-THC partially mediated its effects through the TRPC1 channel, which causes an increase in intracellular Ca2+ levels [284]. Using the calcium ionophore, Io, we tested whether a rise in intracellular Ca2+ might contribute to the observed increase in CD69 expression. In addition, the effect of Δ9-THC on Io-induced CD69 expression was evaluated. Again, cells were gated as described above (Figure 10 A). In CD4+ cells, Δ9-THC increased expression of CD69 moderately in the absence of an activating stimulus when isolated from CB1-/-CB2-/-, but not WT mice (Figure 12 A, C). Δ9-THC increased CD69 expression in CD8+ splenocytes from both WT and CB1-/-CB2-/- mice in the absence of an activating stimulus (Figure 12 A, E). After stimulation with Io, CD69 expression was dramatically increased in CD4+ and CD8+ cells from both genotypes (Figure 12 B, D, F). While CD69 expression was similar between WT and CB1-/-CB2-/- CD8+ cells (Figure 12 B, F), a marked reduction in CD69 expression was observed in CB1-/-CB2-/- CD4+ cells, when compared to WT CD4+ cells (Figure 12 B, D). Most importantly, Δ9-THC synergized with Io to

Figure 12. Δ9-THC synergizes with Io to upregulate CD69 expression.

SPLC were incubated in the presence of Io (0.5 μM) or vehicle (0.01 % DMSO) in the presence or absence of VH (0.1 % ethanol) or Δ9-THC (1,5, and 10 μM). For NA-0 h staining was performed after isolation of a single cell suspension from the spleen otherwise 6 h after co-culture, CD69 staining was performed. Cells were gated on singlet, lymphocytes and within CD4 (A-D) or CD8 (A, B, E, F) positive populations. Data shown in histograms are concatenated (n=4) and are representative of two repeat experiments. Difference due to stimulation by Io is indicated by ++ $p \leq 0.01$, between genotypes is indicated by ##, $p \leq 0.01$ due to Δ9-THC by **, $p \leq 0.01$.

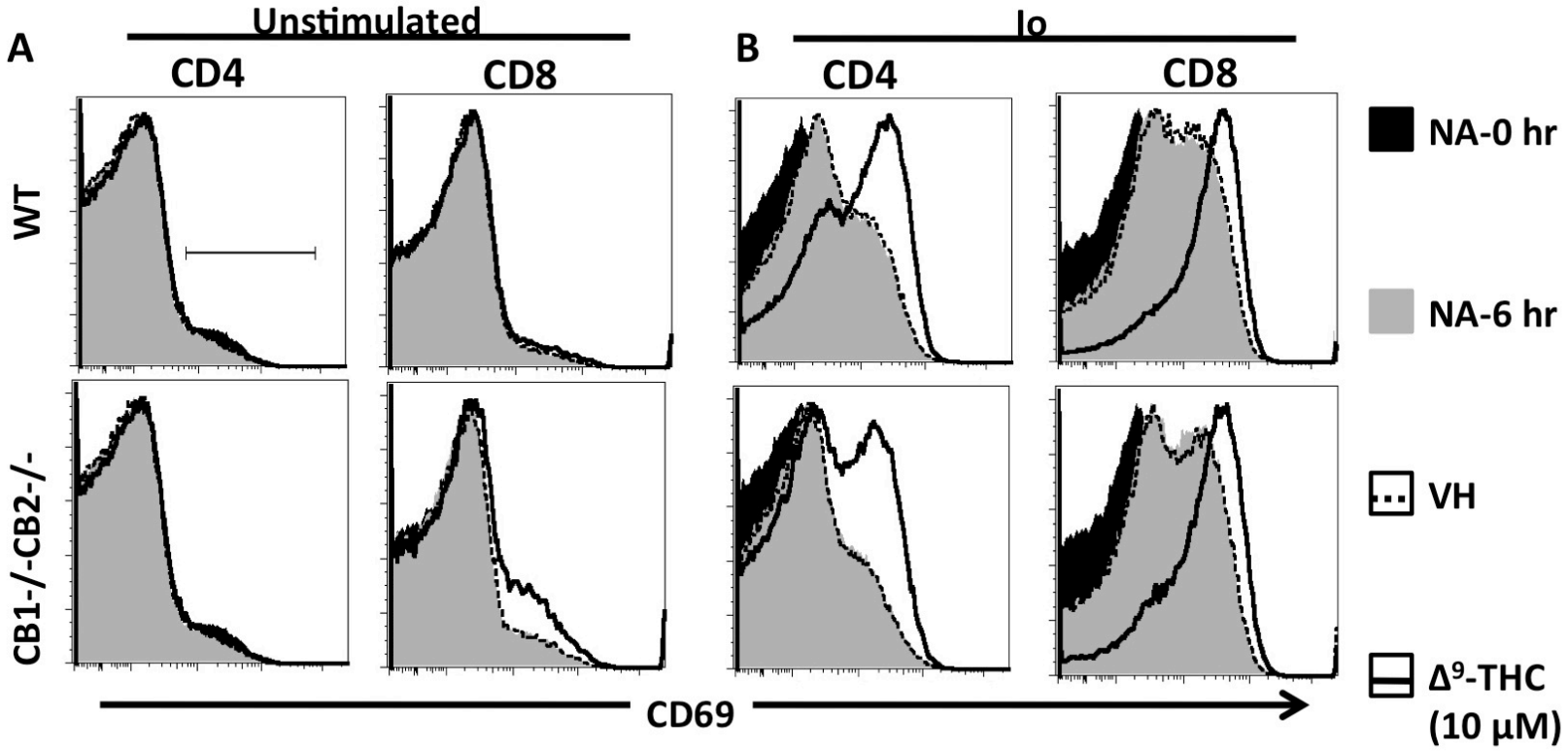
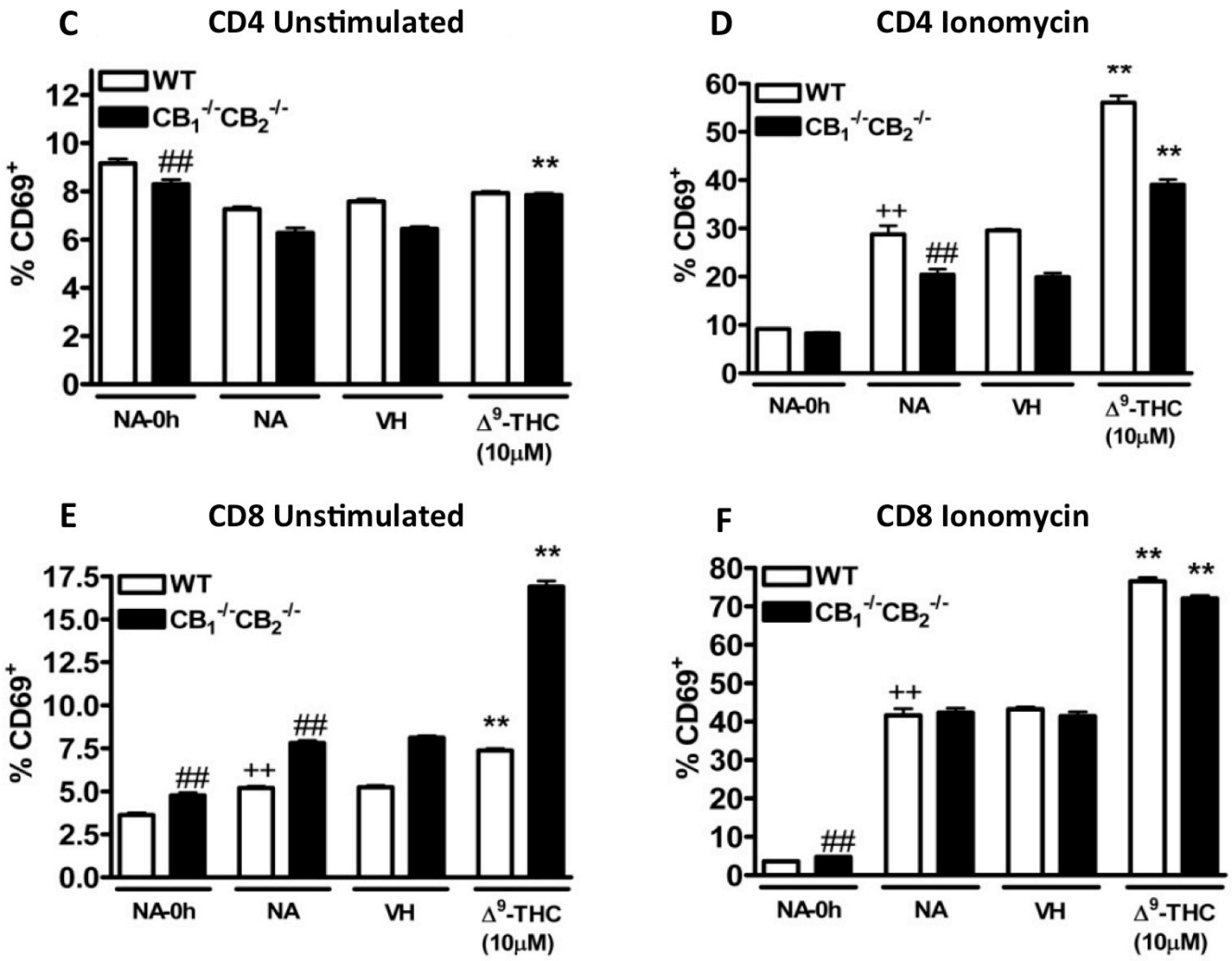


Figure 12. Δ9-THC synergizes with Io to upregulate CD69 expression.

Figure 12 cont'd



further increase CD69 surface expression in CD4+ and CD8+ cells derived from both genotypes (Figure 12 B, D, F).

II. *In Vivo* Influenza PR8 Immune Response Kinetics in WT and CB1-/-CB2-/- mice

A) Enhanced leukocyte infiltration and injury in airways of CB1-/-CB2-/- mice after influenza infection

Principal histopathology in the lungs of WT mice instilled with influenza virus was a necrotizing bronchiolitis of large-diameter axial and pre-terminal bronchioles (Figure 13 A). These airways had conspicuous necrosis and exfoliation of surface epithelium associated with a mixed inflammatory cell infiltration. Inflammatory cell influx along with edema was also present in peribronchiolar and perivascular tissues (Figure 13 A). CB1-/-CB2-/- mice instilled with influenza virus developed a similar, but more severe, necrotizing bronchiolitis. No lung lesions were present in WT and CB1-/-CB2-/- SAL-control mice (Figure 13 A). Not only were the epithelial and inflammatory responses in large-diameter bronchioles more marked, the virus-induced epithelial necrosis, exfoliation and accompanying inflammation extended to more distal, small-diameter, terminal bronchioles with some extension into adjacent alveolar ducts (Figure 13 A). This difference in severity and extent of viral bronchiolitis between CB1-/-CB2-/- and WT mice was also immunohistochemically demonstrated by a loss of anti-inflammatory CCSP in bronchiolar epithelium lining the terminal bronchioles of virus-treated CB1-/-CB2-/- mice (Figure 13 B). Influenza infection induced airway neutrophil and monocyte infiltration most strongly at 3 dpi as enumerated in the BALF (Figure 14). The magnitude of the cell infiltrate was enhanced in CB1-/-CB2-/- compared to WT mice 3 dpi, the peak day of inflammatory cell

Figure 13. More Severe Virus-Induced Necrotizing Bronchiolitis and Loss of Clara Cell Secretory Protein in CB1-/-CB2-/- compared to WT Mice 3 Days Post-infection.

Light photomicrographs of axial airways (AA; generation 5) and terminal bronchioles (TB; generation 5) from the left lung lobe of wild type and CB1-/-CB2-/- mice intranasally instilled with saline vehicle alone or PR8 influenza virus in saline at 3 dpi. The insets are low magnification of tissue sections of the left lung lobe taken perpendicular to the AA at generation 5 (see methods section for microdissection details). All tissues were stained with hematoxylin and eosin (A) or immunohistochemically stained for Clara Cell Secretory Protein (red chromagen) located in bronchiolar epithelium (arrows) at 3 dpi (B). Virus-induced necrotizing bronchiolitis is evident in the AA of WT and CB1-/-CB2-/- mice with greater necrosis and exfoliation (arrows) of the surface epithelium (e) and interstitial cellular inflammation and edema (asterisks) in the CB1-/-CB2-/- mouse compared to the WT mouse. Similar necrotizing bronchiolitis is evident in the terminal bronchiole (TB) of CB1-/-CB2-/- mouse, but not in the virus-treated WT mouse. Compared to saline-instilled control mice there was an apparent loss of CCSP staining in the bronchiolar epithelium (e) lining the large-diameter AA and smaller diameter preterminal and terminal bronchioles of virus-instilled CB1-/-CB2-/- mice. In virus-treated WT mice, there was a loss of CCSP only in large-diameter AA, but CCSP was still present in preterminal and terminal bronchioles.

Figure 13 Continued. More Severe Virus-Induced Necrotizing Bronchiolitis and Loss of Clara Cell Secretory Protein in CB1-/-CB2-/- compared to WT Mice 3 Days Post-infection (continued). All CCSP stained tissue sections were counter-stained with hematoxylin. AD, alveolar duct; ap, alveolar parenchyma; p, pleura; a, alveolus; bv, blood vessel; asterisk, necrotic cellular debris and inflammatory cells in airway lumen.Light microscopic examinations of histologic tissue sections were conducted using an Olympus BX41 clinical microscope with UPlanApochromat 10x, 20x, and 40x and Achromat 60x brightfield objectives (www.olympusamerica.com). A 5.0 Megapixel Qlympus Q-Color5™ camera system was used for all light photomicroscopy. This included a High Resolution FireWire™ digital CCD color camera, designed for publication and documentation, with a 2/3" Bayer color sensor and 2580 x 1944 pixel resolution. The camera was connected to a Dell Precision 380 computer and QCapture Suite™ software for Windows allowing for real-time viewing and full computer control. For interpretation of the references to *color* in this and all other *figures*, the reader is referred to the electronic version of this *dissertation.*

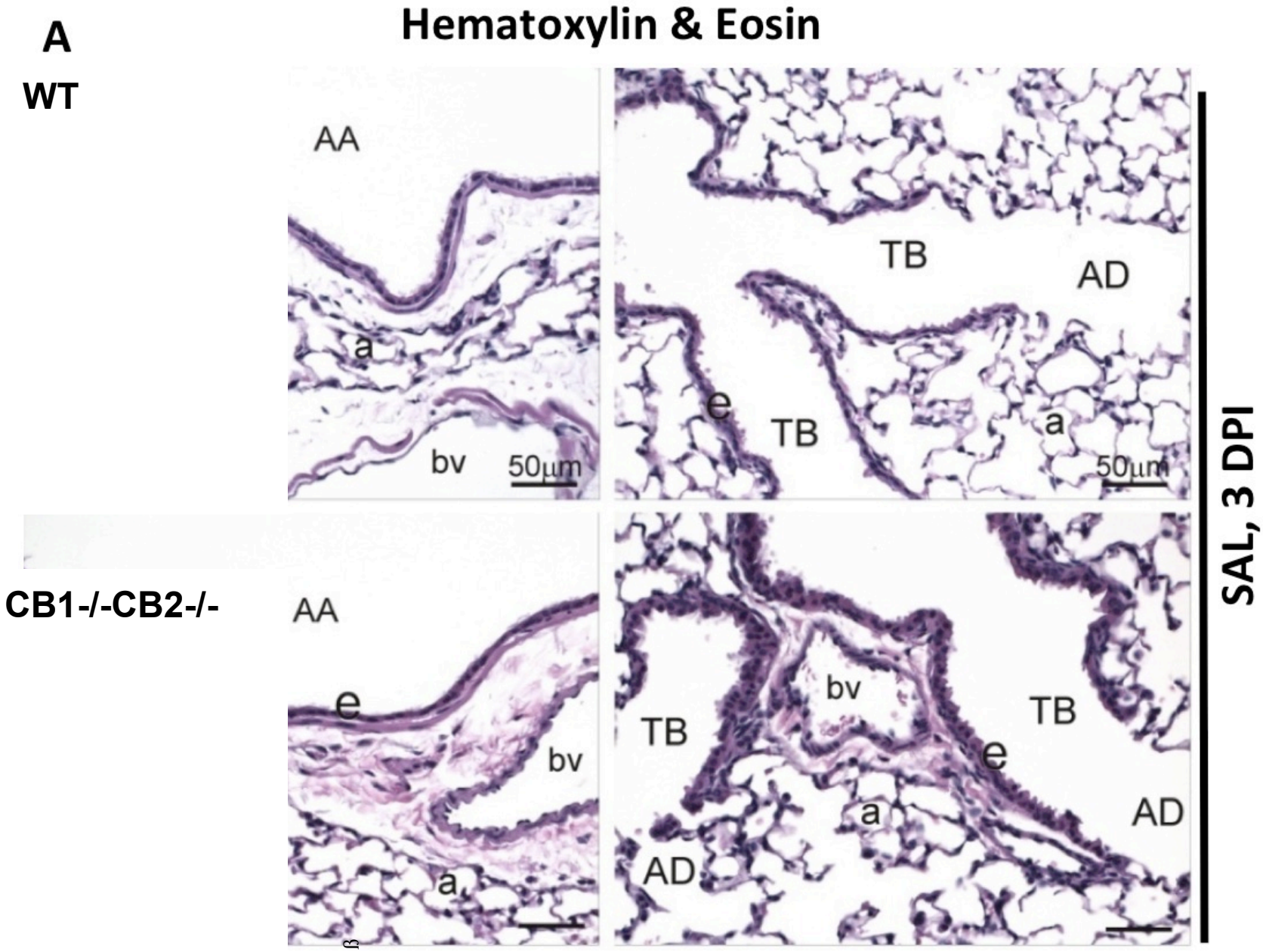
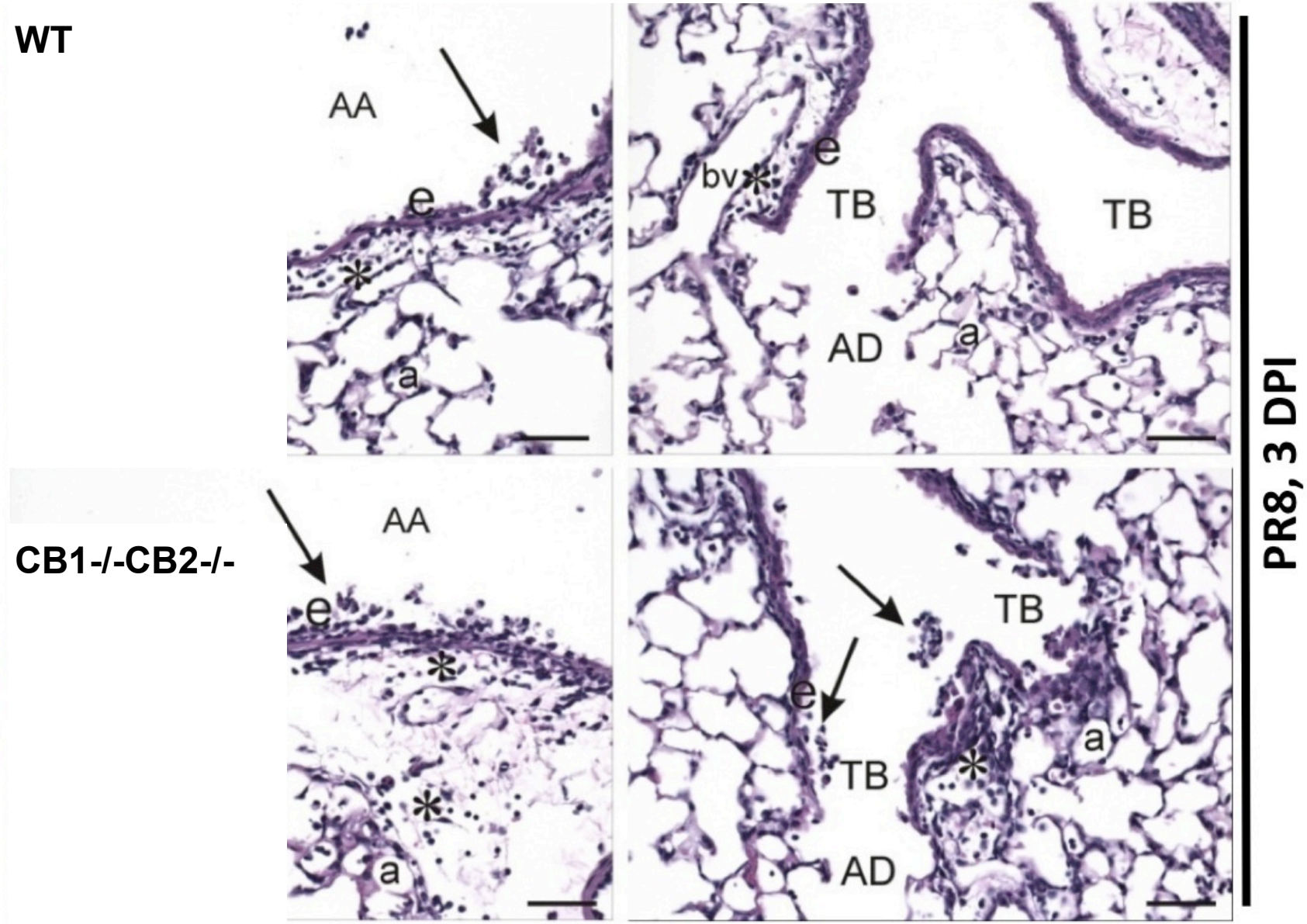


Figure 13. More Severe Virus-Induced Necrotizing Bronchiolitis and Loss of Clara Cell Secretory Protein in CB1-/-CB2-/- compared to WT Mice 3 Days Post-infection

Figure 13 cont'd.



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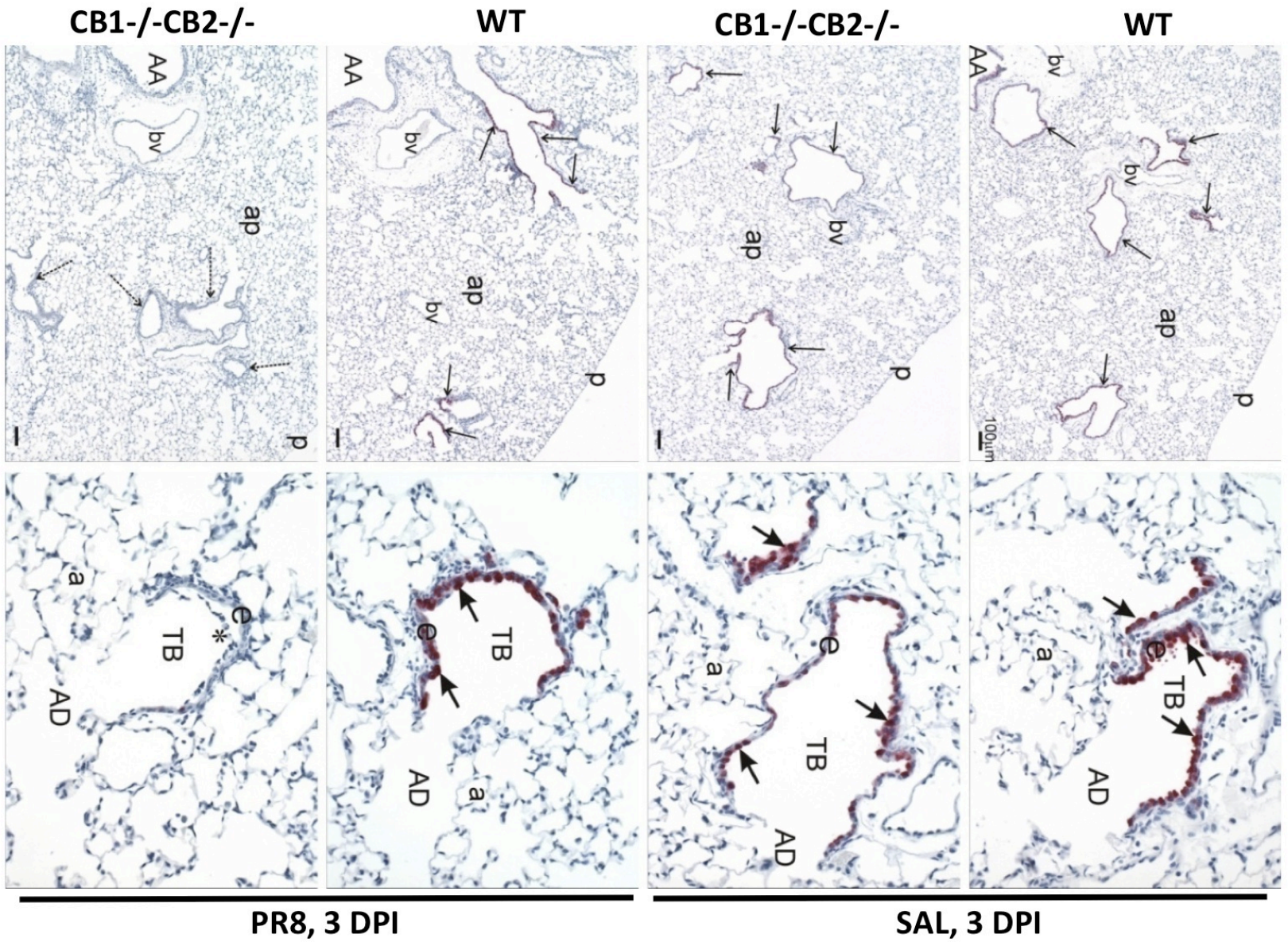

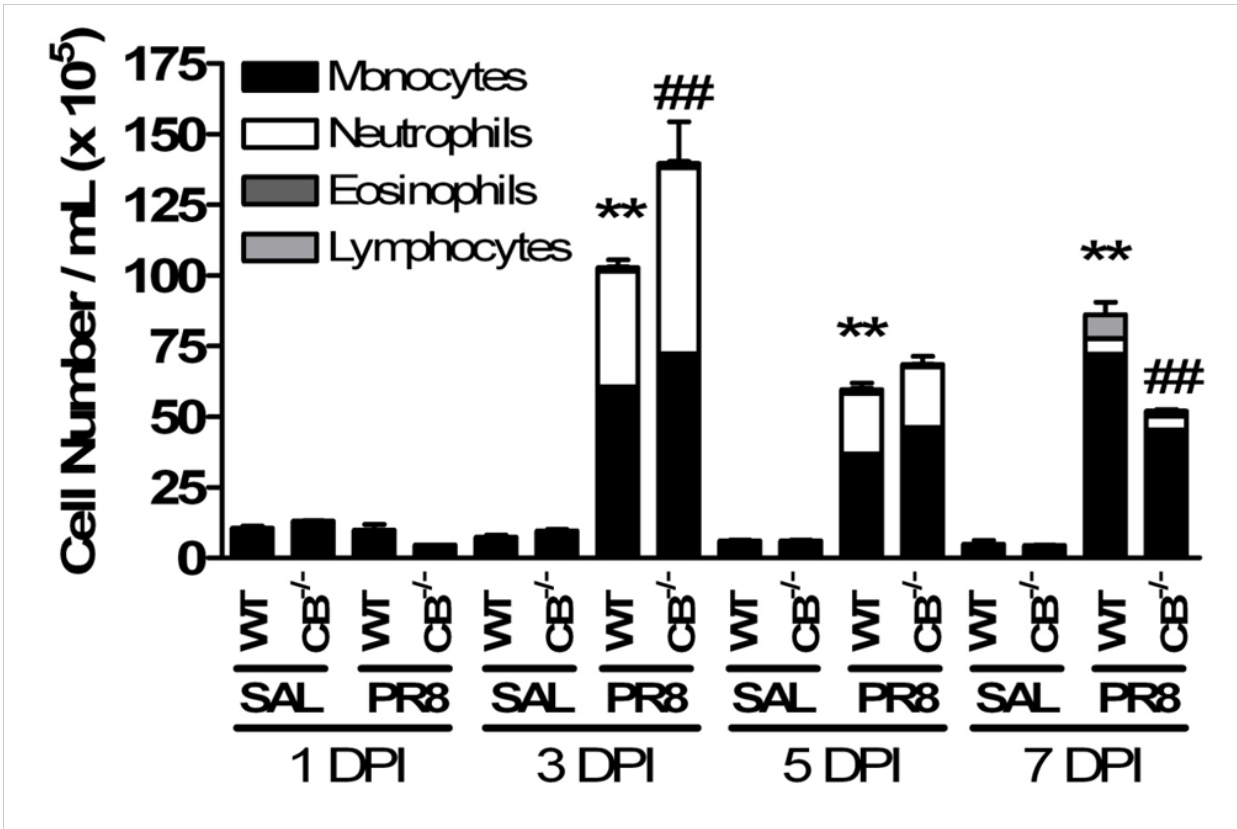

Figure 14. Greater Neutrophils and Macrophage Recruitment after Influenza Infection in CB1-/-CB2-/- mice.

Lungs of C57Bl/6 and CB1-/-CB2-/- mice (n=5) were lavaged with SAL to collect BALF. Total cell number was determined by counting of cells in BALF and DiffQuick staining was performed to distinguish between immune cell populations, which were enumerated by based on the percentage found on the slide using light microscopy. * ($p \leq 0.05$) ** ($p \leq 0.01$) marks a significant change induced by influenza within a timepoint, while # ($p \leq 0.05$) ## ($p \leq 0.01$) marks a significant difference between genotypes.

influx into the airways. At 7dpi, the immune cell infiltrates in the airways were lower in CB1-/-CB2-/- compared to WT mice.

B) Increased mRNA expression levels of pro-inflammatory immune mediators in lungs from CB1-/-CB2-/-mice

Based on the enhanced immune cell infiltration and increased tissue injury in influenza-infected lungs of CB1-/-CB2-/- mice 3 dpi, gene expression changes were assessed using a PCR-based immune panel with total lung RNA from 1 and 3 dpi (Figure 15). Differential gene expression was observed in the lungs of uninfected (SAL-instilled) CB1-/-CB2-/- compared to WT mice (Figure 15 A). Specifically, increased expression of transcripts for MHCII subunit *H2-Eb1*, cytokines *Il1a*, *IL1b*, chemokine *Ccl3*, chemokine receptor *Ccr4*, and enzyme prostaglandin synthase 2 *Ptgs2* were observed. In addition, decreases in chemokine *Ccl19* and transferrin receptor 1 *Tfrc* were evident (Figure 15 A).

In influenza-infected mice, most influenza-associated gene expression changes were not evident prior to 3 dpi (data not shown). For the purpose of this study, the focus was placed on genes that were differentially regulated between CB1-/-CB2-/- compared with WT mice. Exacerbated expression of cytokines, *Csf2* (GMCSF), *Il13*, *Il17*, and *Tnf*, negative regulator of cytokines, *Socs2*, chemokine receptor *Ccr7*, surface marker *Cd38*, and the previously mentioned *H2-Eb1* and *Ptgs2* was observed (Figure 15 B). Collectively, gene transcript levels indicating increased inflammation and maturation of antigen presenting cells and the presence of cellular responses involving cytokine secretion in CB1-/-CB2-/- mice defined the overall profile.

Figure 15. Relative transcript levels of genes associated with inflammation are increased in lungs of CB1-/-CB2-/- mice compared to WT mice basally and after influenza infection at 3 dpi.

RNA from lung samples (n=4) was converted to cDNA and analyzed using an Applied Biosystem's Mouse Immune Panel. Genes were included based on 1.5 fold up- or downregulation and fold changes in gene expression levels were analyzed using factorial ANOVAs for significance. Genes with significantly different expression between CB1-/-CB2-/- and WT mice were visualized in a heat map with red indicating an increase and blue a decrease in expression in a log2 relative expression scale as shown on the right side of the figure. The p-value of the factorial ANOVA is shown on the left side of each gene. Arrangement of the heat map is as follows: A – differentially regulated genes basally, without influenza infection between CB1-/-CB2-/- and WT mice, B – gene expression differentially expressed in CB1-/-CB2-/- mice after influenza infection. For interpretation of the references to *color* in this and all other *figures*, the reader is referred to the electronic version of this *dissertation.*

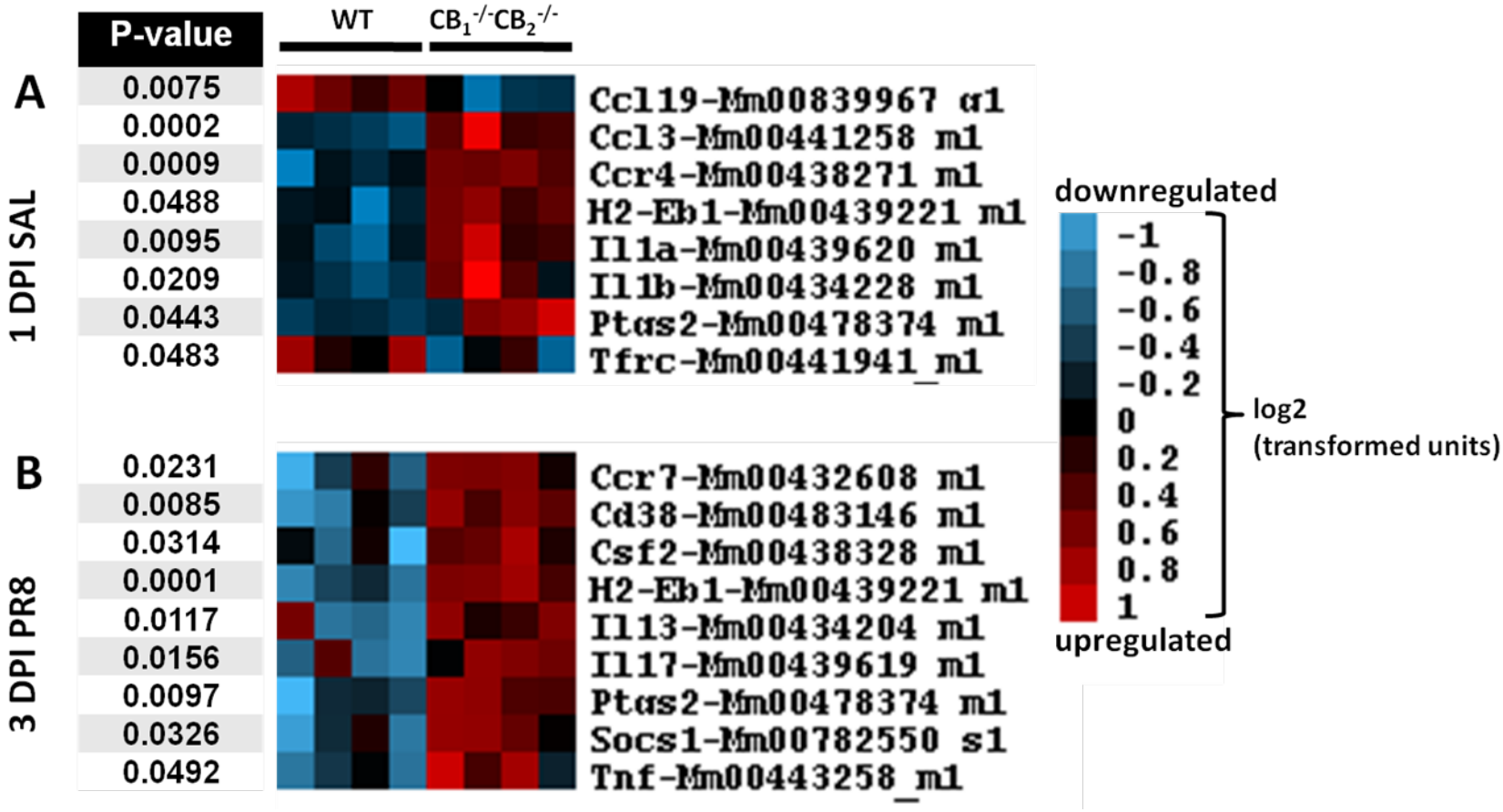

Figure 15. Relative transcript levels of genes associated with inflammation are increased in lungs of CB1-/-CB2-/- mice compared to WT mice basally and after influenza infection at 3 dpi.

Figure 16. Peak H1 mRNA Levels 3 dpi in CB1-/-CB2-/- and WT Mice and Increases in Steady-state CB1 Transcript Levels 7 dpi

RNA was isolated from whole lungs (n=5) and assessed for steady state levels of H1 RNA (A), CB1 (B) and CB2 (C) mRNA using 18S as a housekeeping gene. All fold changes are relative to WT SAL 1 dpi. Factorial ANOVA was used to determine significance for H1 RNA levels and One-Way ANOVA was used for CB1 and CB2 levels. ** $p = 0.01$ compared to WT SAL.

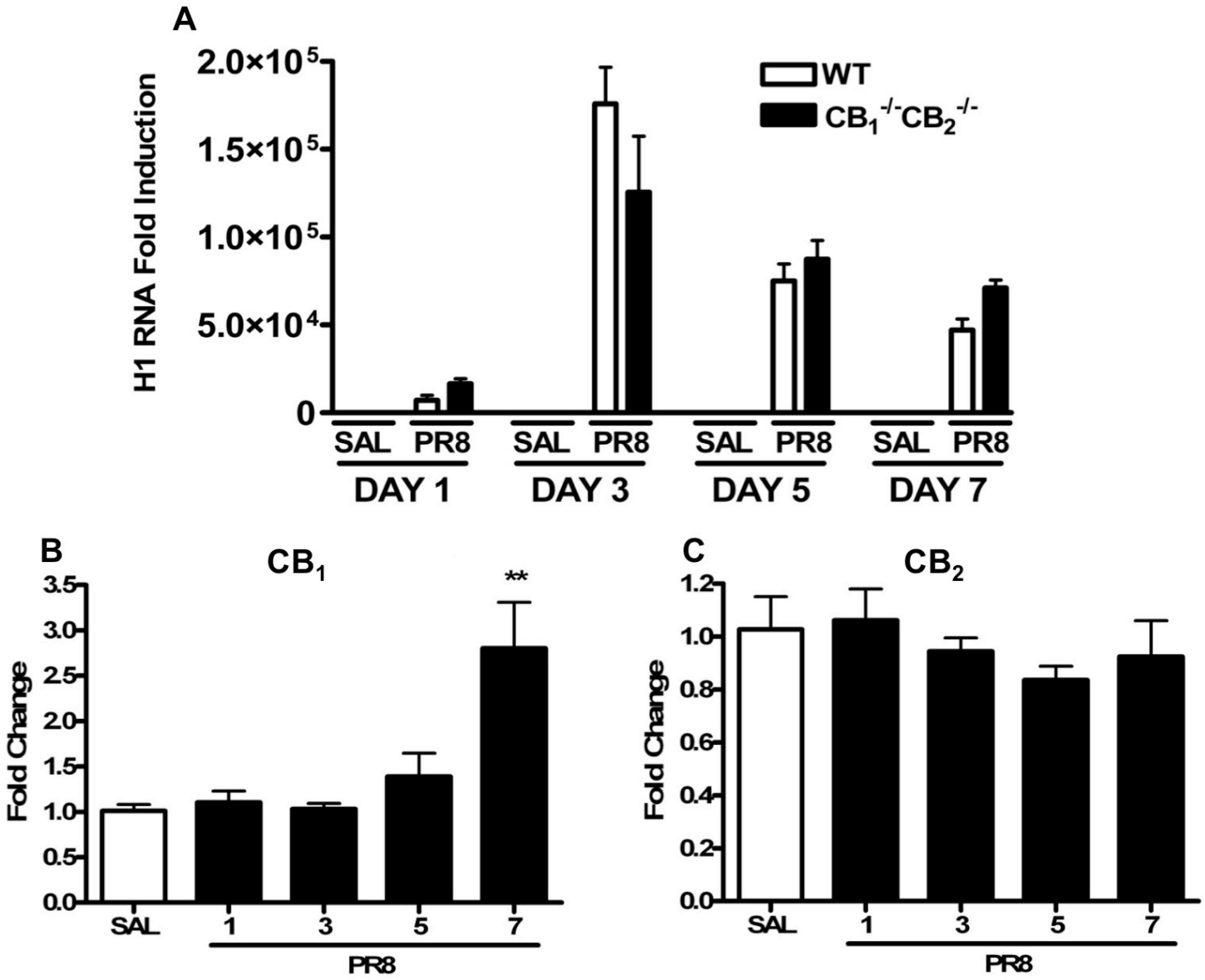

Figure 16. Peak H1 mRNA Levels 3 dpi in CB1-/-CB2-/- and WT Mice and Increases in Steady-state CB1 Transcript Levels 7 dpi

C) No difference in the H1 RNA Levels between WT and CB1-/-CB2-/- mice, But Increase in CB1 Transcript in WT Mice after Influenza Infection.

RNA was isolated and converted to cDNA as described before and H1 levels were assessed in lung RNA isolated from WT and CB1-/-CB2-/- mice CB1 and CB2 were assessed in WT samples only. The greatest levels of H1 RNA were observed at 3 dpi in both, WT and CB1-/-CB2-/- mice. In contrast to previous observations in this laboratory [120], there was no significant difference between the H1 RNA levels between WT and CB1-/-CB2-/- mice (Figure 16 A). While CB2 steady-state mRNA levels remained unchanged after PR8 infection (Figure 16 C), CB1 steady-state mRNA levels increased, reaching significance at 7 dpi compared to SAL (Figure 16B).

D) T cells from CB1-/-CB2-/- mice undergo accelerated T cell activation

To investigate the effect of gene expression changes on the T cell response in CB1-/-CB2-/- mice after influenza infection, CD69 expression was monitored on 1-7 dpi as a marker of T cell activation in whole lung preparations. In contrast to BALF, in which myeloid cells predominated (shown in Figure 14), whole lung preparations harbored cells of the lymphoid lineage. Induction of surface CD69 was observed 3 dpi in both CD4+ and CD8+ T cells, with greater staining intensity observed in CD4+ T cells (Figure 17). CD69 expression was greater in CD4+ T cells from CB1-/-CB2-/- compared to WT mice as early as 3 dpi (Figure 17 A). The same trend was observed in CD8+ T cells, yet to a lesser degree than in CD4+ T cells (Figure 17 B). Increased CD69 expression was observed in SAL-instilled CB1-/-CB2-/- compared to WT mice. At 7 dpi, WT and CB1-/-CB2-/- mice displayed similar levels of surface CD69 in both

Figure 17. More rapid T cells activation after Influenza infection in CB1-/-CB2-/- compared to WT mice.

At 1, 3, 5, and 7 dpi cells were isolated from lung tissue (n=5), Fc receptors were blocked on the surface and samples stained for surface markers CD4, CD8 and CD69. FlowJo software was used to analyze samples and create histograms shown. Gating was performed on single cell populations, size and expression of CD4 and CD8 (bottom left). Within CD4+ (A) and CD8+ (B) populations histograms depict the fluorescence intensity of CD69 staining. Due to bimodal expression of CD69, percent positively gated cells, as show in the first panel (top right, A), were enumerated (CD4: C, CD8: D). Friedman's tests for non-Gaussian data were performed and significance was indicated in figures as: # $p = 0.05$ and ## $p = 0.01$ in comparison of WT to CB1-/-CB2-/-.

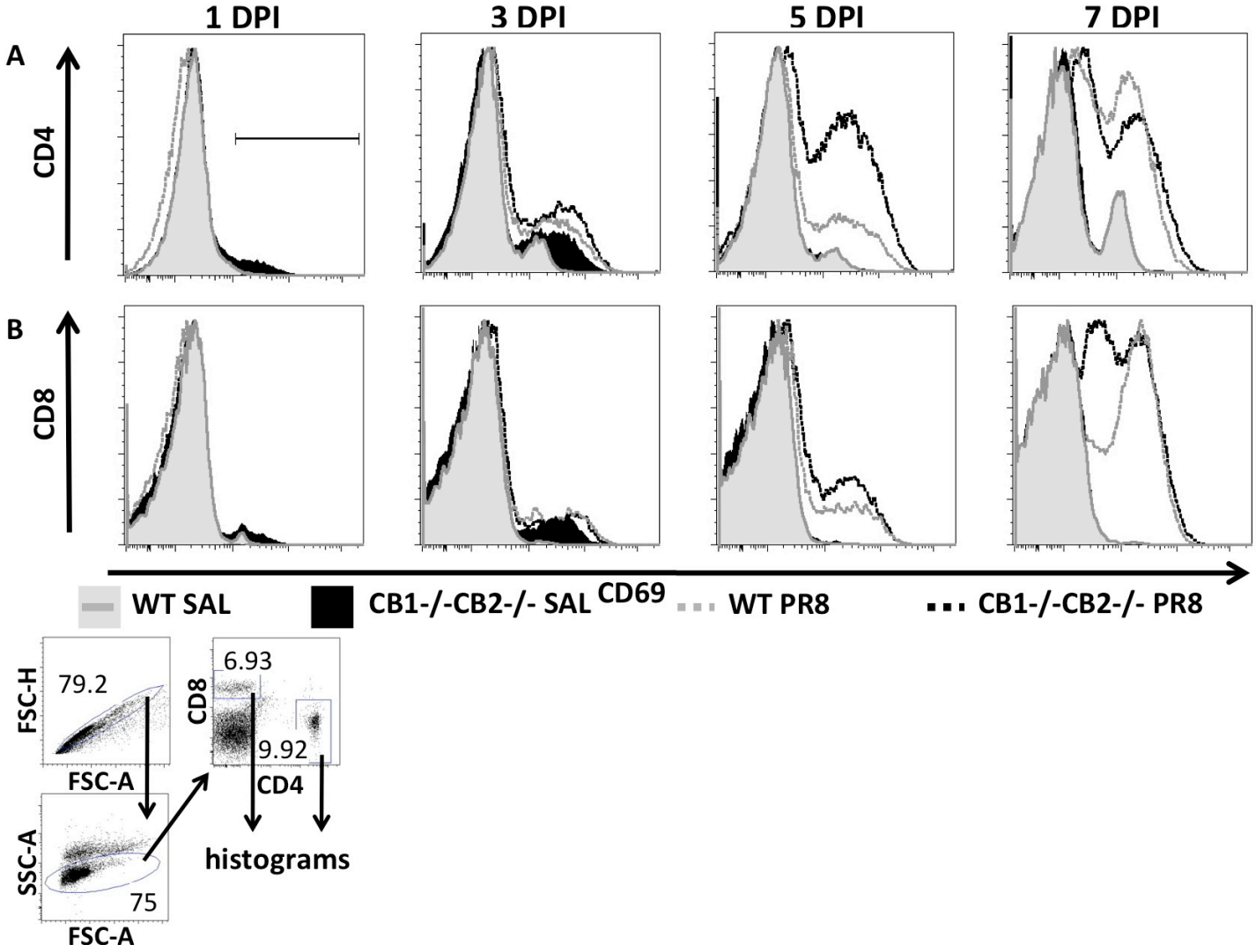

Figure 17. More rapid T cells activation after Influenza infection in $CB_1^{-/-}CB_2^{-/-}$ compared to WT mice.

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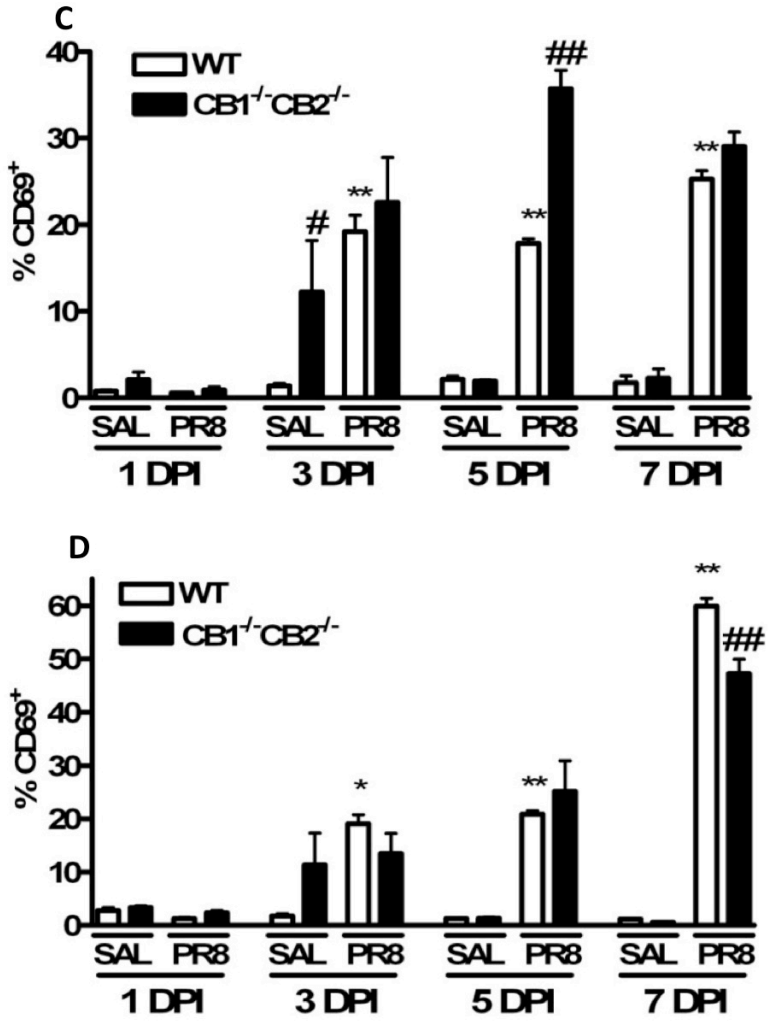

CD4+ and CD8+ T cells with CD69 expression starting to wane in cells from CB1-/-CB2-/- mice (Figure 17 A, B).

D) Enhanced IL-17 and IFNγ production in NK, and T cells isolated from lungs of CB1-/-CB2-/- mice in response to influenza

The observation of elevated steady state mRNA levels of IFNγ and IL-17, and more rapid T cell activation as assessed by CD69 in CB1-/-CB2-/-, when compared to WT mice, suggested a regulatory function by cannabinoid receptors in antiviral T cell immunity. In light of the increased transcriptional expression of the T cell-produced cytokines, IFNγ and IL-17, in the lung (Figure 15) and their pivotal roles in lung inflammation, identification of the cellular source(s) of IFNγ and IL-17 was important in determining the cell types affected by CB1 and CB2 deletion.

The gating scheme for the analysis of cytokine secretion from whole lung homogenates is shown in Figure 18 A. Minor basal IFNγ production, mainly from CD4+ and CD8+ cells, was observed in SAL control samples in WT (Figure 18 B). In CB1-/-CB2-/- mice, percentages of IFNγ producing cells in the absence of influenza challenge were markedly enhanced compared to WT mice, especially in CD4+ cells (Figure 18 B). The percentage of IFNγ-producting cells was modestly decreased 3 and 5 dpi in WT but not CB1-/-CB2-/- CD4+ cells, while increases in IFNγ-producing cells were observed in CD8+ and NK1.1+ populations (Figure 18 B).Overall, the percentage of IFNγ producing cells was greater in all three (CD4+, CD8+, NK1.1+) delineated effector cell types isolated from CB1-/-CB2-/-mice compared to WT mice.

On the other hand, basal IL-17 production was limited predominantly to NK1.1+ cells in WT mice (Figure 18 B). In contrast, both CD4+ and NK1.1+ cells from uninfected CB1-/-CB2-/-

Figure 18. Greater cytokine production by leukocytes from CB1-/-CB2-/- mice compared to WT mice.

Lungs from mice were mechanically disrupted and passed through a sieve (n=5). After removal of connective tissue, cells were counted and restimulated with PMA/Io for 5 h in the presence of Brefeldin A. Cells were blocked with Fc receptors and stained with CD4, CD8, and NK1.1 in addition to cytokine staining with IFNγ and IL-17. Flow cytometry samples were gated as depicted in A. Cytokine secretors were identified as percent of positive cells within surface delineation and unstimulated samples were used as controls (B). Immune cell populations were enumerated for percent cytokine expression in FlowJo (type of cytokine indicated on the left) within effector cell populations (surface marker indicated on the right) in FlowJo and bar graphs were generated using GraphPad Prizm (C). Friedman's tests for non-Gaussian data were performed and significance is indicated in figures as: # $p = 0.05$ and ## $p = 0.01$ in comparison of WT mice to CB1-/-CB2-/- mice.

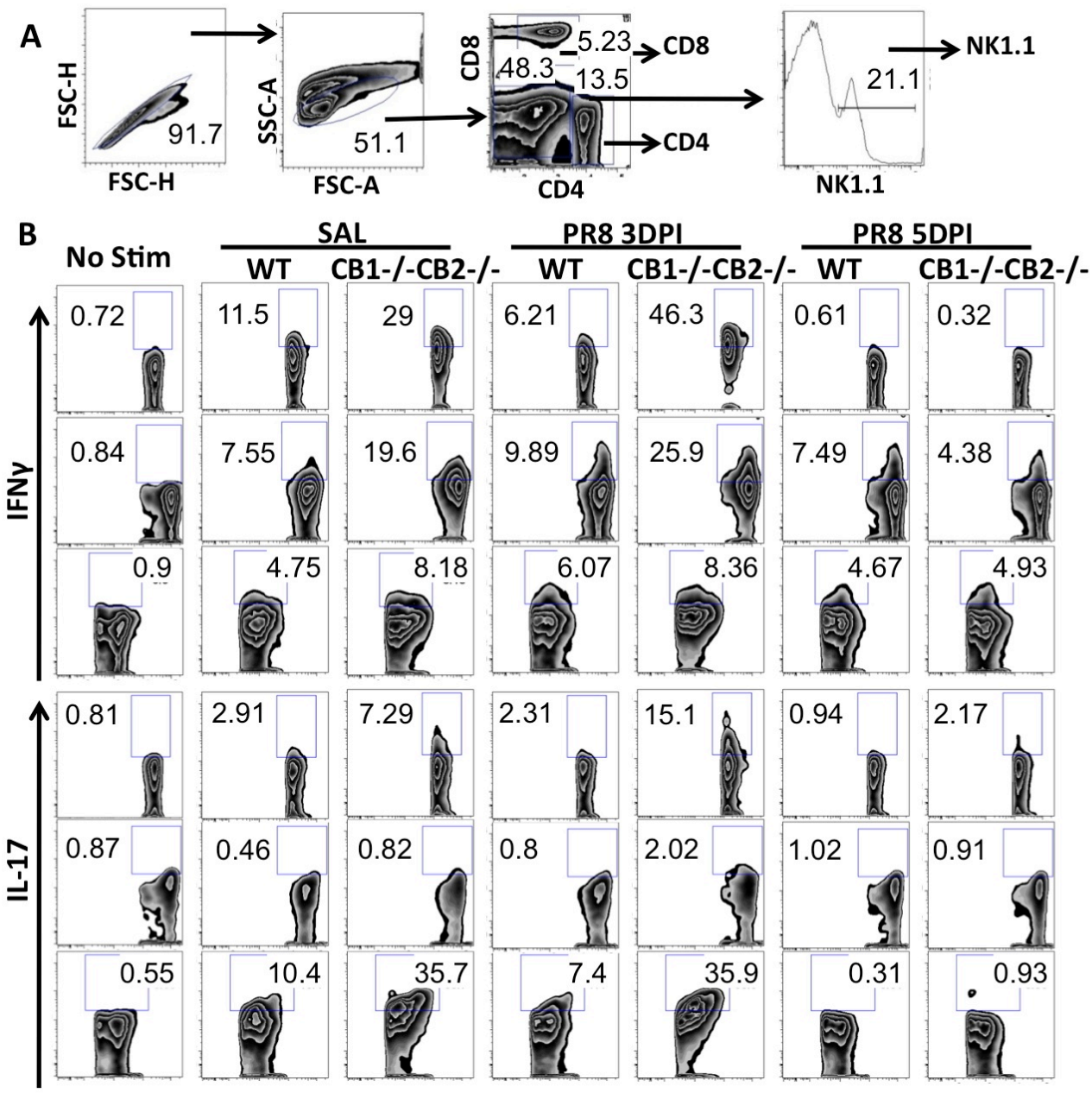

Figure 18. Greater cytokine production by leukocytes from CB1-/-CB2-/- mice compared to WT mice.

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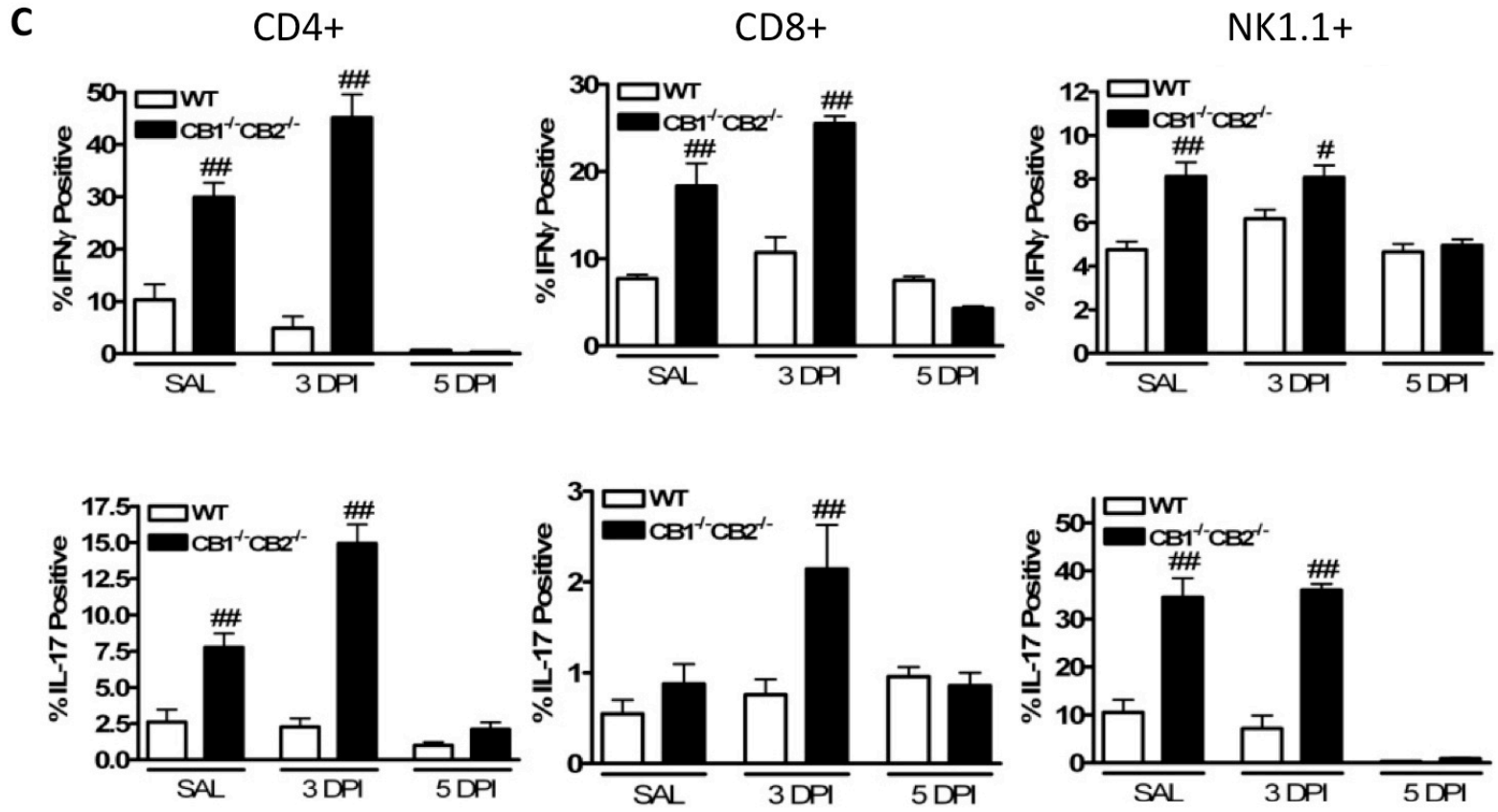


mice produced significant amounts of IL-17 (Figure 18 B). At 3 dpi, the IL-17 producing cell types did not change, but the percentage of detectable IL-17-producing cells increased, especially in CD4+, and to a lesser extent in CD8+ cells, with greater percentages of IL-17 positive cells detected in CB1-/-CB2-/- mice compared to WT mice (Figure 18 B). By 5 dpi, the percentage of cells staining positive for IL-17 was greatly reduced in NK and CD4+ cells in WT and CB1-/-CB2-/- mice and in CD8+ cells of CB1-/-CB2-/- but not WT mice. In summary, IL-17 and IFNγ hypercytokinemia in effector cells isolated from the lungs was evident in the absence of influenza infection and even more pronounced after infection in CB1-/-CB2-/- compared to WT mice.

E) Fewer Naïve CD4+ T cells in CB1-/-CB2-/- compared to WT mice

Since IFNγ- and IL-17-secreting CD4+ T cells were found in uninfected, SAL-instilled lungs of CB1-/-CB2-/- mice, we focused on the percent composition of naïve T cells (CD62L+CCR7+). After gating for CD4+ cells from splenocytes (Figure 19 A), CD62L-CCR7- and a CD62L-CCR7+ non-NA CD4+ T cells populations were identified and found in greater percentages in CB1-/-CB2-/- mice (Figure 19 B, C). To assess whether deletion of CB1 and/or CB2 had a direct effect on the ability of T cells to secrete cytokines, naïve CD62L+ CD4+ T cells were isolated from splenocytes and stimulated with immobilized αCD3 and soluble αCD28 under Th17 polarizing conditions to induce IL-17 secretion. Under these conditions, which favor IL-17 production, IL-17 was detected from both WT and CB1-/-CB2-/- non-naïve T cells (CD62L-) and naïve T cells (CD62L+) (Figure 19 D). No difference in the production of IL-17 was observed in CD4+ T cells from CB1-/-CB2-/- compared to WT (Figure 19 E-G). This

Figure 19. Lower number of NA CD4+ T cells and no difference in T cell stimulation *in vitro* in CB1-/-CB2-/-mice compared to WT mice.

Splenocytes (n=3) were obtained from WT and CB1-/-CB2-/- mice and analyzed by flow cytometry with gating shown in A. Non-NA T cells (CCR7-CD62L- or CD62L-), were gated for in B and analyzed statistically by Friedman's test in C. NA (CD62L+) (NA) and non-naïve (non-NA) CD4+ cells were isolated from splenocytes and driven towards a Th17 phenotype in the presence of TGF-β and IL-6 as described in methods. Cells were gated as shown in D and then analyzed for cytokine production in E. Results for Friedman's tests for non-Gaussian data were of experimental data in Th17 polarizing conditions from E are shown in F and G. The experiment is representative of two repeat *in vitro* studies. * $p \leq 0.05$, ** $p \leq 0.01$ comparing WT and CB1-/-CB2-/-.

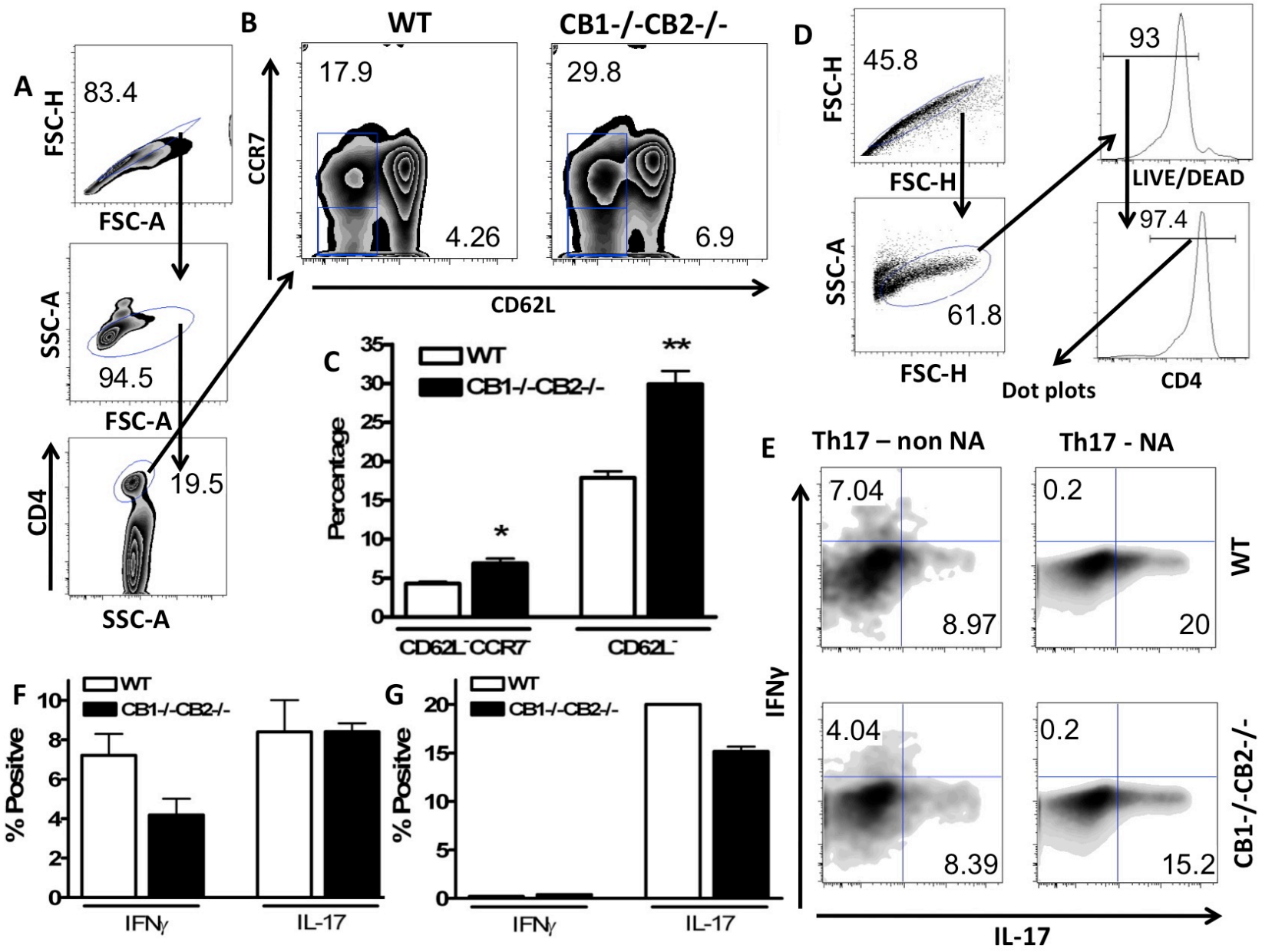


Figure 19. Lower number of NA CD4+ T cells and no difference in T cell stimulation *in vitro* in CB1-/-CB2-/-mice compared to WT mice.

Figure 20. Increased expression of DC maturation markers in bmDC from CB1-/-CB2-/- mice.

bmDC were generated as described in the methods. In short, bone marrow was isolated from femurs and tibias and cultured in the presence of GMCSF (20 ng/mL) for 9 days, then immature bmDC were washed and stimulated with TLR agonists (n=3). A, bmDCgated for singlets, LIVE (LIVE/DEAD dye), and high CD11b and CD11c expression. B, surface expression of maturation markers MHC I, MHC II, CD80, CD86 were analyzed after stimulation for 24 h with LPS (1 μg/mL) and ssRNA (5 μg/mL). C, due to the Gaussian distribution of the fluorescent staining, mean fluorescence intensity (MFI) was used to compare samples using statistics. The results shown are representative from four identical experiments.

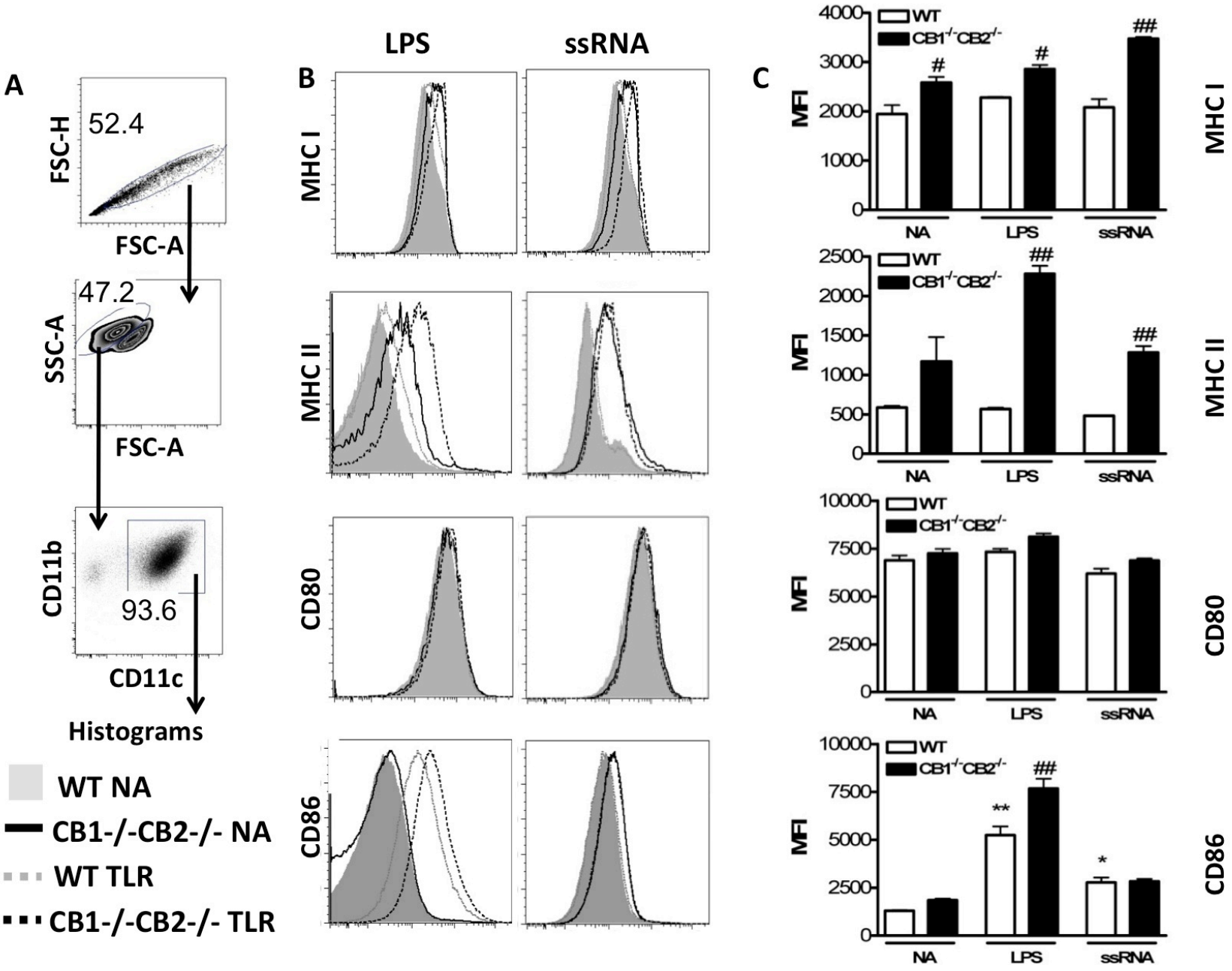


Figure 20. Increased expression of DC maturation markers in bmDC from CB1-/-CB2-/- mice.

suggests that hypercytokinemia experienced in CB1-/-CB2-/-mice *in vivo* might be a result of interaction with APCs since T cells intrinsically did not produce different level of cytokines.

F) Increased APC maturation and ability to induce proliferation and cytokine production in T cells in CB1-/-CB2-/-mice compared to WT mice

To determine whether APC responses are in fact deregulated in CB1-/-CB2-/-mice, as suggested by a lower percentage of NA T cells, an *in vitro* model was used to investigate DC, which link innate to adaptive immune responses based on their role in T cell elicitation. Immature bone marrow-derived dendritic cells (bmDC) were generated *in vitro* from femurs and tibias of CB1-/-CB2-/- and WT mice and stimulated with LPS (1 μg/mL) or ssRNA (5 μg/mL) for 24 h to induce a mature phenotype. Flow cytometric staining for CD11b and CD11c was performed to confirm conventional DC (cDC) phenotype (CD11b+CD11c+) [133] (Figure 20 A). Staining for MHCI, MHCII, CD80, CD86 was performed to evaluate the maturation status (Figure 20 B). Increased staining for MHCI, MHCII and CD86 was found in bmDC of CB1-/-CB2-/- compared to WT mice without TLR treatment (Figure 20 B). LPS upregulated surface expression of MHCII and CD86, while ssRNA increased CD86 expression in both genotypes. In addition, ssRNA treatment elevated MHCI in bmDC from CB1-/-CB2-/-mice only. In contrast, CD80 expression did not change with TLR treatment (Figure 20 C). Overall, bmDC generated *in vitro* from CB1-/-CB2-/- but not WT mice displayed a pre-activated phenotype. Upon TLR activation, maturation markers were induced with greater magnitude in CB1-/-CB2-/-compared to WT mice.

To determine whether the observed phenotypic changes on bmDC exerted an effect on T cell elicitation and function, bmDC pulsed with SIINFEKL were cultured with OT-1 splenocytes

Figure 21. bmDC from CB1-/-CB2-/-elicit OT-1 responses without requirement for maturation stimulus and more potently than WT.

OT-1 cells were elicited as described in the methods section. In short, bmDC were matured with different TLR agonists for 24 h and pulsed with SIINFEKL peptide for 2 h, washed, and incubated with OT-1 cells for 4 days, then restimulated for 5 h to induce cytokine secretion (n=3). A, gating scheme for OT-1 cells; singlet, size, and LIVE gating was applied before visualizing data. B, proliferation was assessed by Cell Trace dye dilution and a gate depicting proliferated populations was drawn according to WT-NA population shown in tinted grey. The legend for the other experimental groups is below the figure and the TLR agonist used is shown beside proliferation graph. C, proliferation and concurrent IFNγ secretion were visualized using dot plots. LPS (1μg/mL) and ssRNA (5μg/mL) were used to stimulate bmDC maturation as previously (Figure 6). Proliferation and IFNγ secretion gates were drawn according to a WT-NA group without SIINFEKL peptide, which showed no proliferation or IFNγ secretion. D, data from proliferation and cytokine plots are summarized using percentages obtained from shown gates. Statistical significance is indicated as # - $p \leq 0.05$ comparing WT to CB1-/-CB2-/- using Friedman's test for non-parametric data. The results shown are representative from three identical experiments.

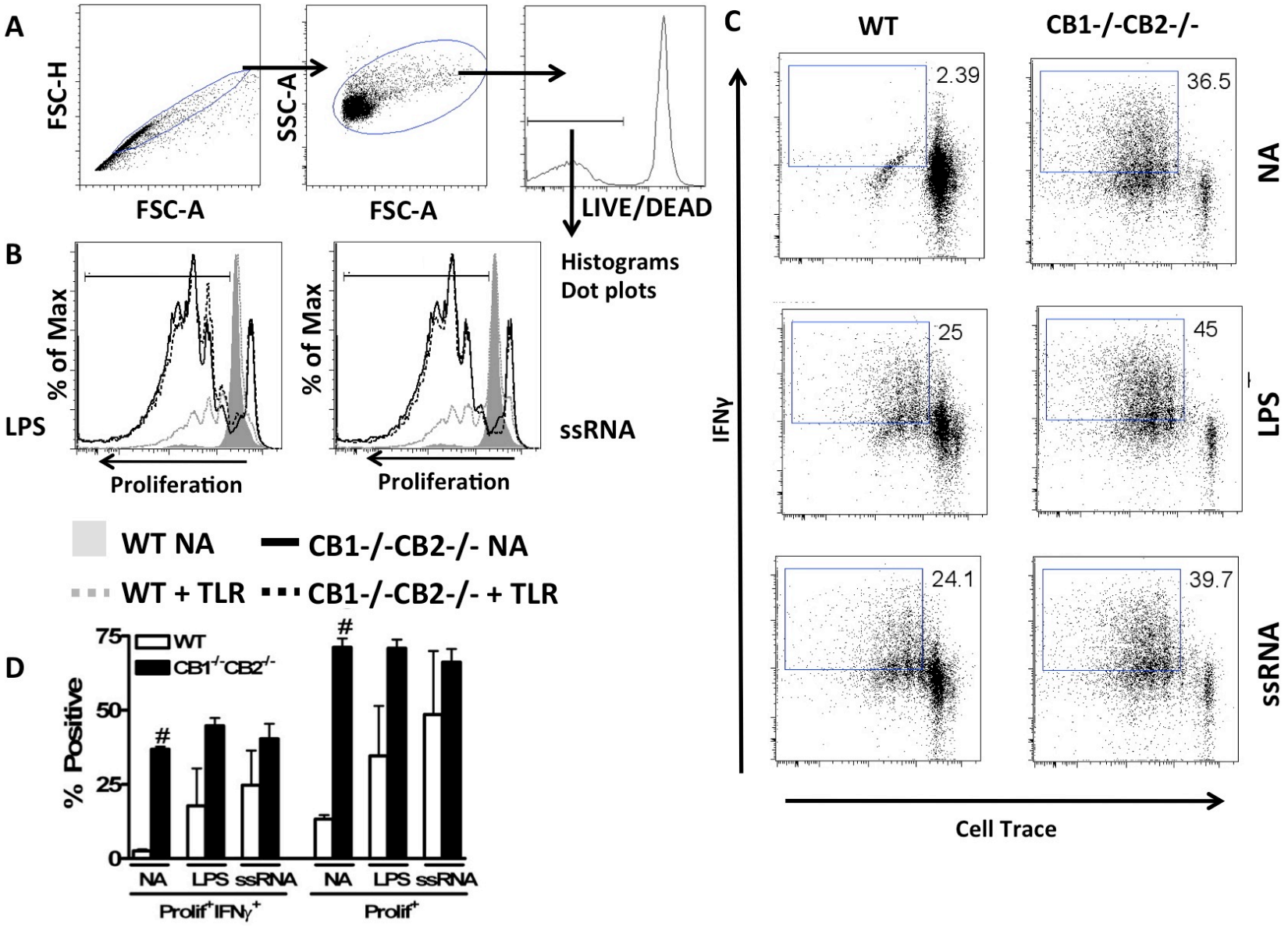


Figure 21. bmDC from CB1-/-CB2-/-elicit OT-1 responses without requirement for maturation stimulus and more potently than WT.

Figure 22. AM isolated from BALF are mature in CB1-/-CB2-/- but not WT.

AM were isolated by flushing the lungs with saline, washed and plated in the presence or absence of LPS (1 μg/mL) (n=3). Maturation was assessed by staining for MHC I, MHC II, and CD86 on the surface of AM. A, AM were gates as CD11b+CD11c hi cells. B, histograms depicting changes in fluorescence intensity of MHC I, MHC II, and CD86 in AM obtained from lavage fluid of WT and CB1-/-CB2-/- mice. C, summary of MFI data, statistical significance is indicated as ** $p \leq 0.01$ WT-NA vs. WT-LPS, # $p \leq 0.05$ and ## $p \leq 0.01$ WT vs CB1-/-CB2-/- within groups. The results shown are representative from two identical experiments.

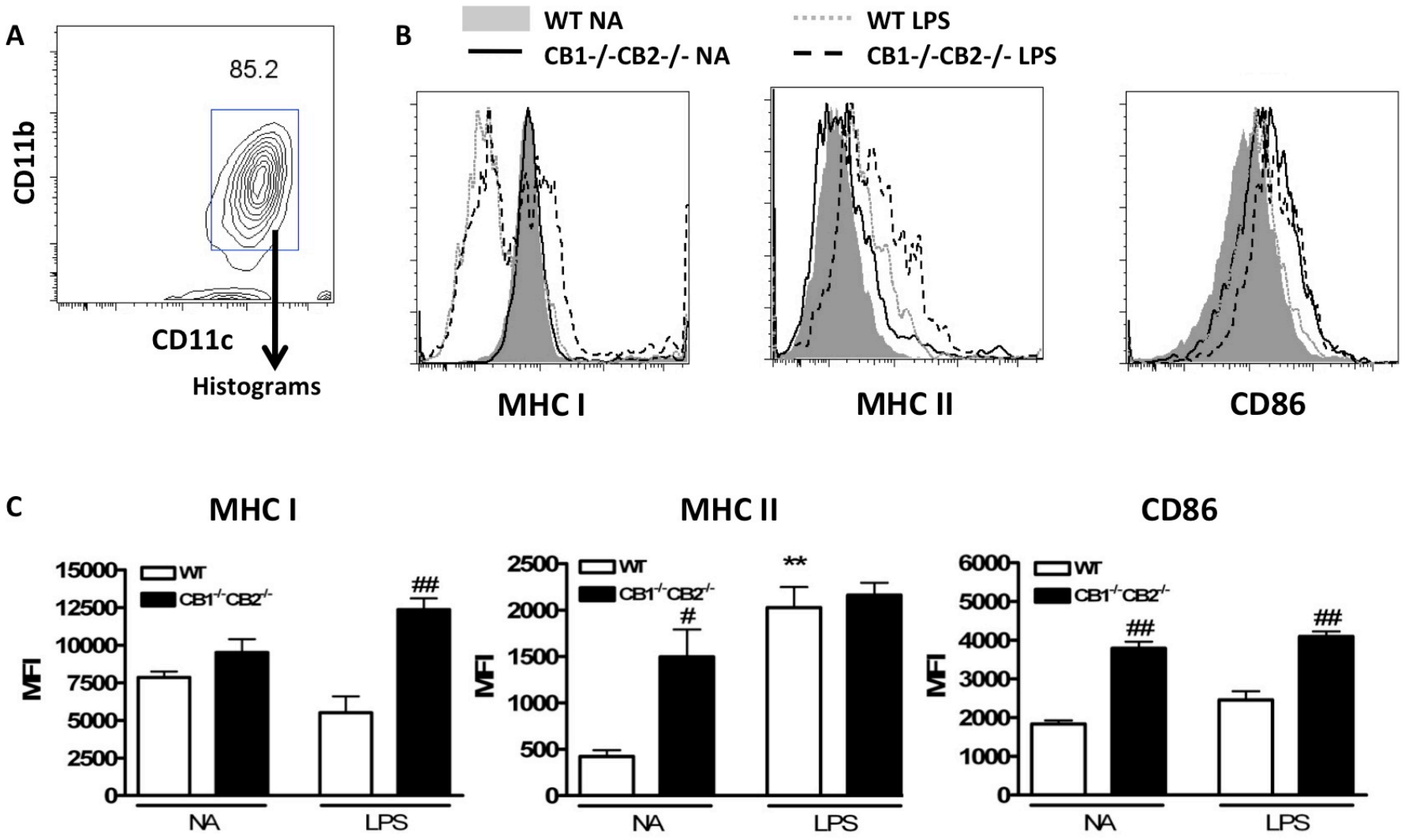


Figure 22. AM isolated from BALF are mature in CB1-/-CB2-/- but not WT.

and evaluated after gating on live cells (Figure 21 A). OT-1 splenocytes were also labeled with a dye for assessment of proliferation by dye dilution and stained for IFNγ to assess function after restimulation. No proliferation or IFNγ was detected in OT-1 splenocytes incubated with NA bmDC from WT mice (Figure 21 B, C). However, OT-1 splenocytes added to bmDC from CB1-/-CB2-/- mice were induced to proliferate and secreted IFNγ in the absence of a maturation stimulus such as LPS or ssRNA (Figure 21 B, C). LPS and ssRNA treatment induced maturation in bmDC from WT mice sufficiently to detect proliferation and IFNγ secretion in co-cultured OT-1 splenocytes; however, still far less than after co-culture with bmDC derived from CB1-/-CB2-/- mice with LPS or ssRNA treatment as evidenced by the percentage of proliferating cells or percentage of proliferating and IFNγ+ cells (Figure 21 D). Overall, the contribution of bmDC to elicit T cell responses is consistent with the overall phenotypic difference observed between WT and CB1-/-CB2-/-mice observed after influenza infection *in vivo*.

AM isolated from BALF of NA WT and CB1-/-CB2-/- mice were used to extend the findings with bmDC. As LPS was much more potent and the amount of cells available in the BALF are limited, we reduced our study design to a single TLR agonist, especially since TLR-initiated signaling by ssRNA and LPS converges at MyD88 [327]. AM, as identified by CD11b-CD11c+ expression [133], were obtained from BALF and stimulated overnight with LPS (Figure 21). As previously seen in the bmDC experiments, AM isolated from CB1-/-CB2-/- mice exhibited a pre-activated phenotype compared to macrophages from WT mice based on the expression of MHCII and CD86 (Figure 22 B, C). LPS stimulation again increased maturation markers, especially MHC II (Figure 22 B, C) and AM from CB1-/-CB2-/- mice presented a more mature phenotype than WT after TLR stimulation. Overall, the increased maturation of APC

from CB1-/-CB2-/- mice suggests that dysregulation due to extinguished CB1 and CB2 signaling predominantly affects the functional regulation of APC such as DC and AM.

III. *In Vivo* Influenza Study 3 dpi to Study the Effect of Δ9-THC on Immune Endpoints of WT and CB1-/-CB2-/- Mice

A) Δ9-THC Reduced Steady-state mRNA Expression of Genes Induced by Influenza in the lungs of WT but not CB1-/-CB2-/- mice

Previously, effects of simultaneous deletion of CB1 and CB2 were assessed using the A/PR/8/34 influenza model and 3 dpi was determined to be the peak time of the inflammatory response. To further investigate the effects of Δ9-THC on the anti-influenza immune response in the lungs of WT and CB1-/-CB2-/- mice, a 96-gene immune panel was employed to assess gene expression changes. 18S rRNA was used as the loading control and all samples were normalized to 18S rRNA gene expression. In uninfected mice, the expression of mRNA of very few genes was altered by Δ9-THC (Figure 23 A). In contrast, PR8 induced broad steady-state mRNA expression level changes, many of which were suppressed by Δ9-THC in WT but not CB1-/-CB2-/- mice, suggesting CB1 and/or CB2-dependent modulation (Figure 23 B). In addition, mRNA levels for only a few genes were upregulated in CB1-/-CB2-/- mice as a result of Δ9-THC treatment, suggesting CB1 and CB2-independent mechanisms of Δ9-THC immune modulation.

Figure 23. Reduction of Gene Expression Associated with Influenza Infection as a Result of Δ9-THC Treatment.

Mice (n=4) were treated with corn oil vehicle (CO) or Δ9-THC (75 mg/kg) for 5 consecutive days surrounding the intranasal instillation of PR8 or SAL. Lung RNA was isolated 3 dpi and converted into cDNA and gene expression levels were analyzed using a Taqman low density gene array. Fold change values were normalized with blom transformation then log transformed, mean and median centered across genes with Cluster and visualized using Treeview. Shown are genes significantly different between CO and Δ9-THC samples, with a p-value of $p \leq 0.1$ in SAL (A) and PR8 instilled (B) groups 3 dpi. Data were analyzed using factorial ANOVA in SAS 9.1.3 and p-values are indicated in the left most column and represent statistical differences as a result of Δ9-THC treatment. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

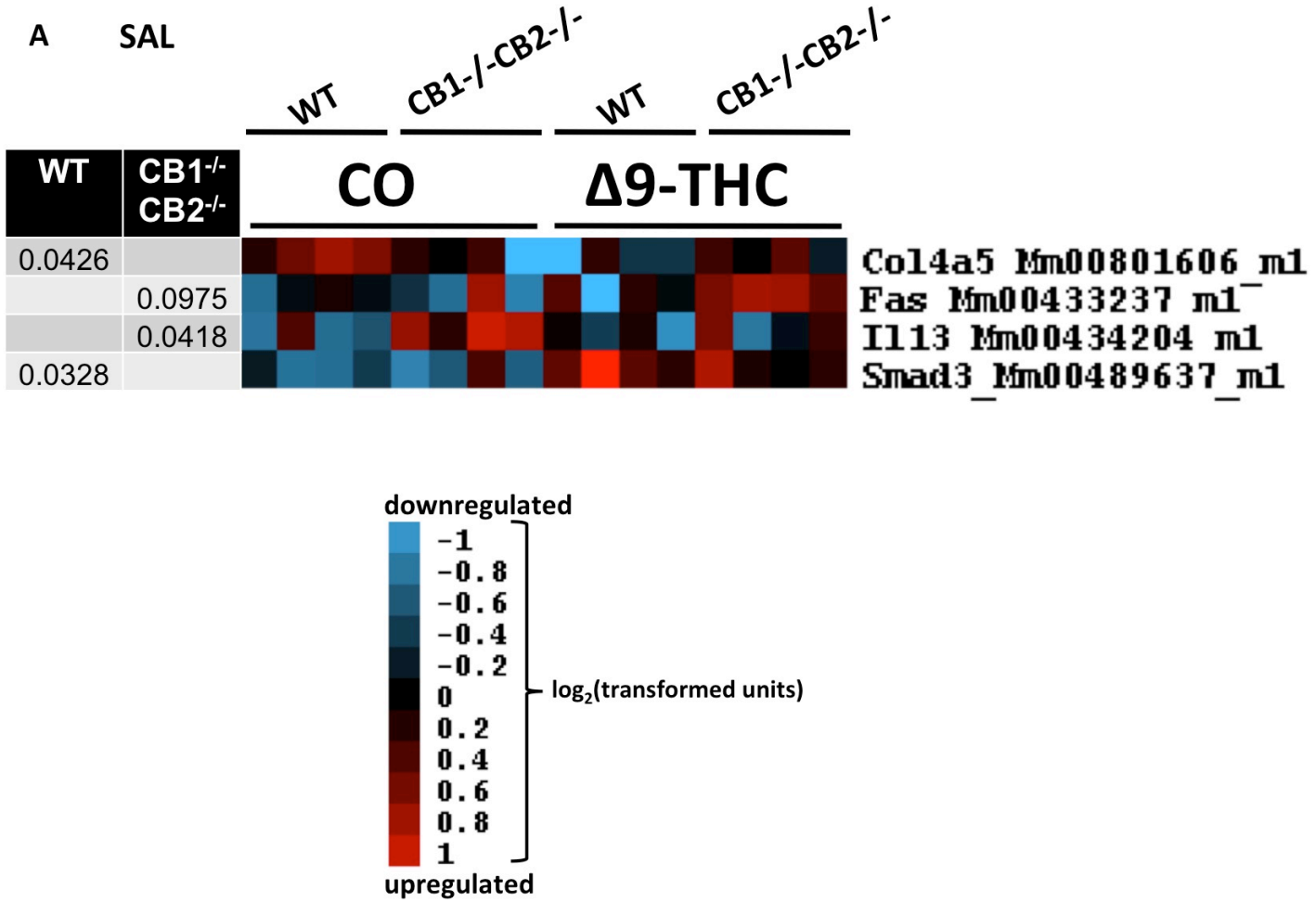
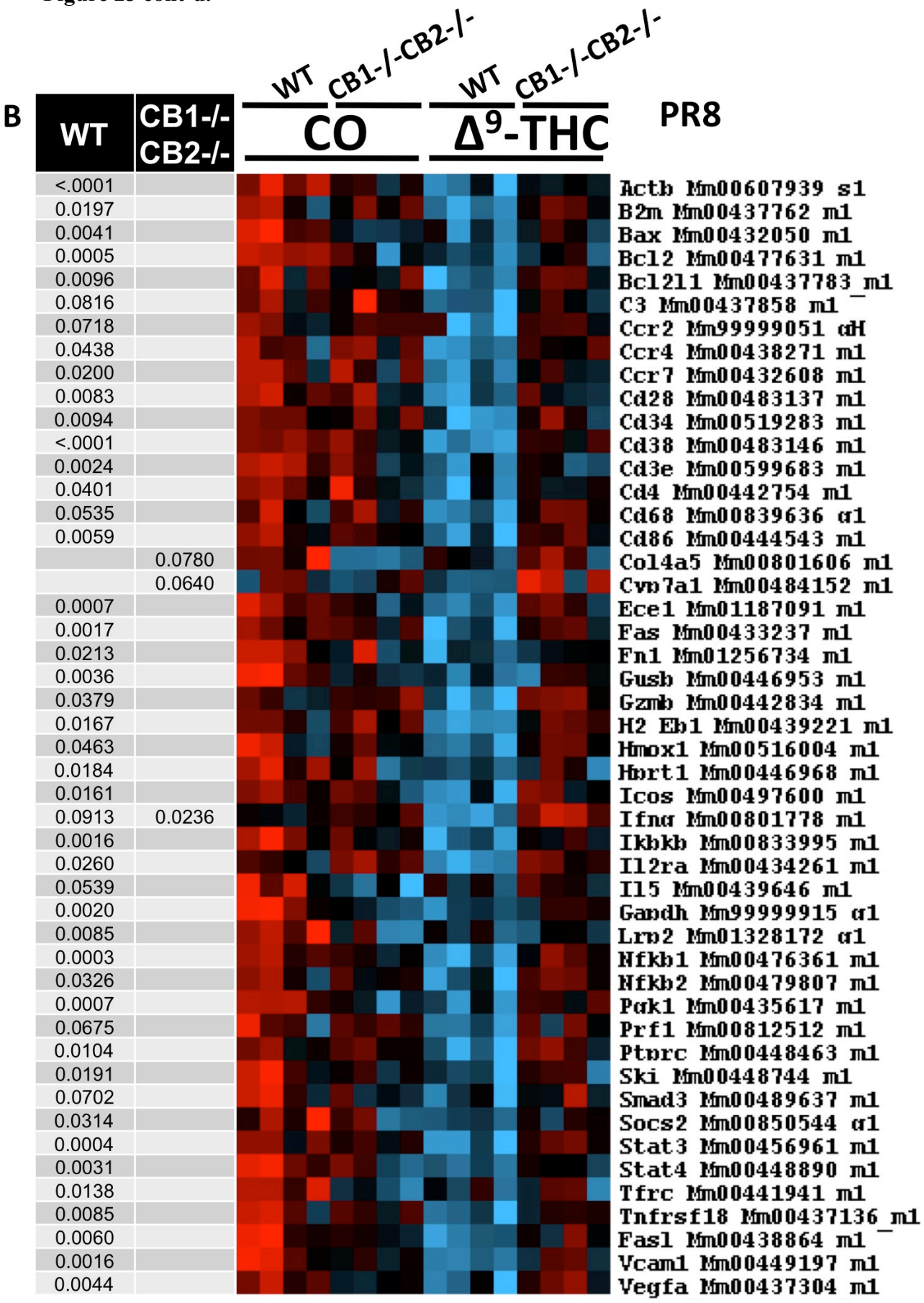


Figure 23. Reduction of Gene Expression Associated with Influenza Infection as a Result of Δ9-THC Treatment.

Figure 23 cont'd.



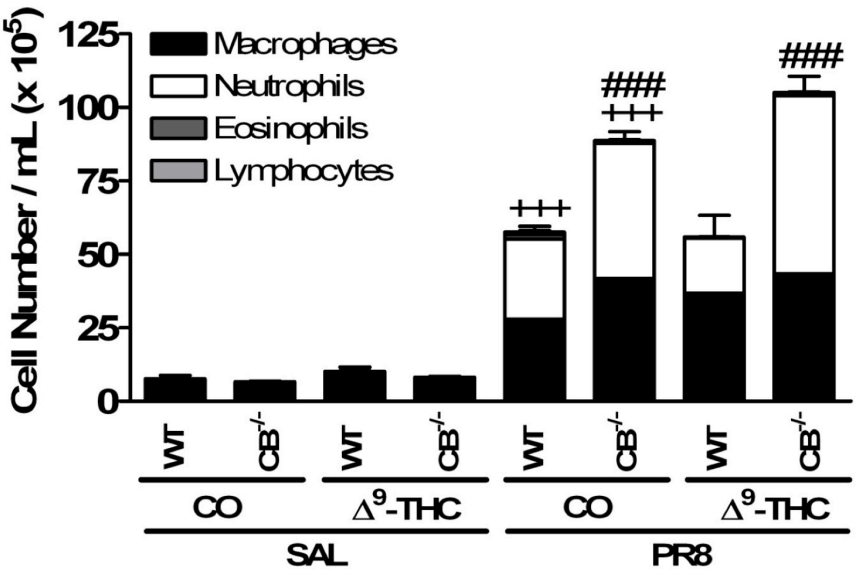


Figure 24. Δ9-THC does not Alter BALF Cell Counts or Composition After Influenza Infection.

BALF was isolated by flushing of lungs of mice 3 dpi twice with 0.9 mL 0.1% saline (n=5). Total cell numbers were counted using a hemacytometer and after Diff-Quick staining of cells centrifuged onto slides and differential cell counts were performed. Shown are the percent of differentially stained cells out of the total. Data were analyzed using an ANOVA: +++ ($p \leq 0.001$) difference between NA (SAL) and PR8; ### ($p \leq 0.001$), difference between WT and CB1-/-CB2-/-.

Figure 25. No Change in Percent Immune Effector Cell Composition in Lungs of Mice Treated with Δ9-THC.

Lungs were mechanically disrupted 3 dpi and single cell suspensions were obtained. Cells were stained with fluorescently labeled antibodies for surface markers CD4, CD8, NK1.1 and analyzed by flow cytometry. Cells were gated on single cells, size, CD4+, CD8+, or CD4-CD8-NK1.1+ (A). The percent of cells in the lung is shown for each treatment group (n=5) (B). Data were analyzed using Friedman's test for non-parametric data: +++ ($p \leq 0.001$) difference between NA (SAL) and PR8; # ($p \leq 0.05$), difference between WT and CB1-/-CB2-/-.

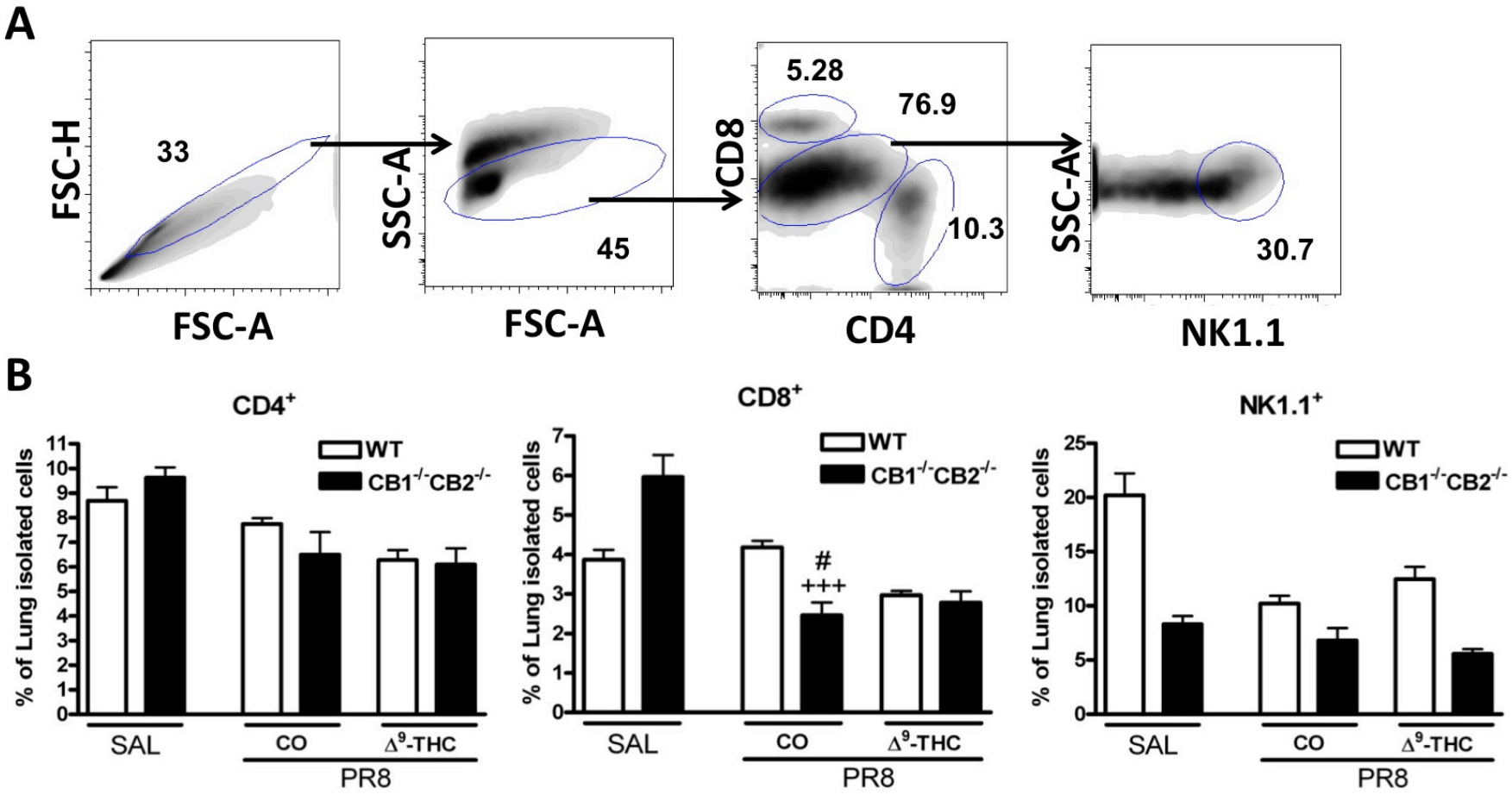


Figure 25. No Change in Percent Immune Effector Cell Composition in Lungs of Mice Treated with Δ9-THC.

Figure 26. Decreased IFNγ Production after Δ9-THC Treatment in Lung-Isolated Immune Cells from WT but not CB1-/-CB2-/- mice.

After mechanical disruption of lung tissue, single cell suspensions were restimulated *in vitro* with PMA/Io (40 nM/ 0.5 μM) to induce cytokine secretion and in the presence of Brefeldin A in 2% BCS RPMI for 5 hours to inhibit cytokine release. After restimulation, cells were stained for CD4, CD8, and NK1.1 surface expression. On the day of flow cytometric analysis, cells were stained for intracellular IFNγ and analyzed for fluorescence intensity (n=5). Data were analyzed using Friedman's test for non-parametric data: * difference between CO and Δ9-THC ++ ($p \leq 0.01$) difference between NA (SAL) and PR8; ## ($p \leq 0.01$), ### ($p \leq 0.001$) difference between WT and CB1-/-CB2-/-. Shown are samples concatenated (n=5) within treatment groups.

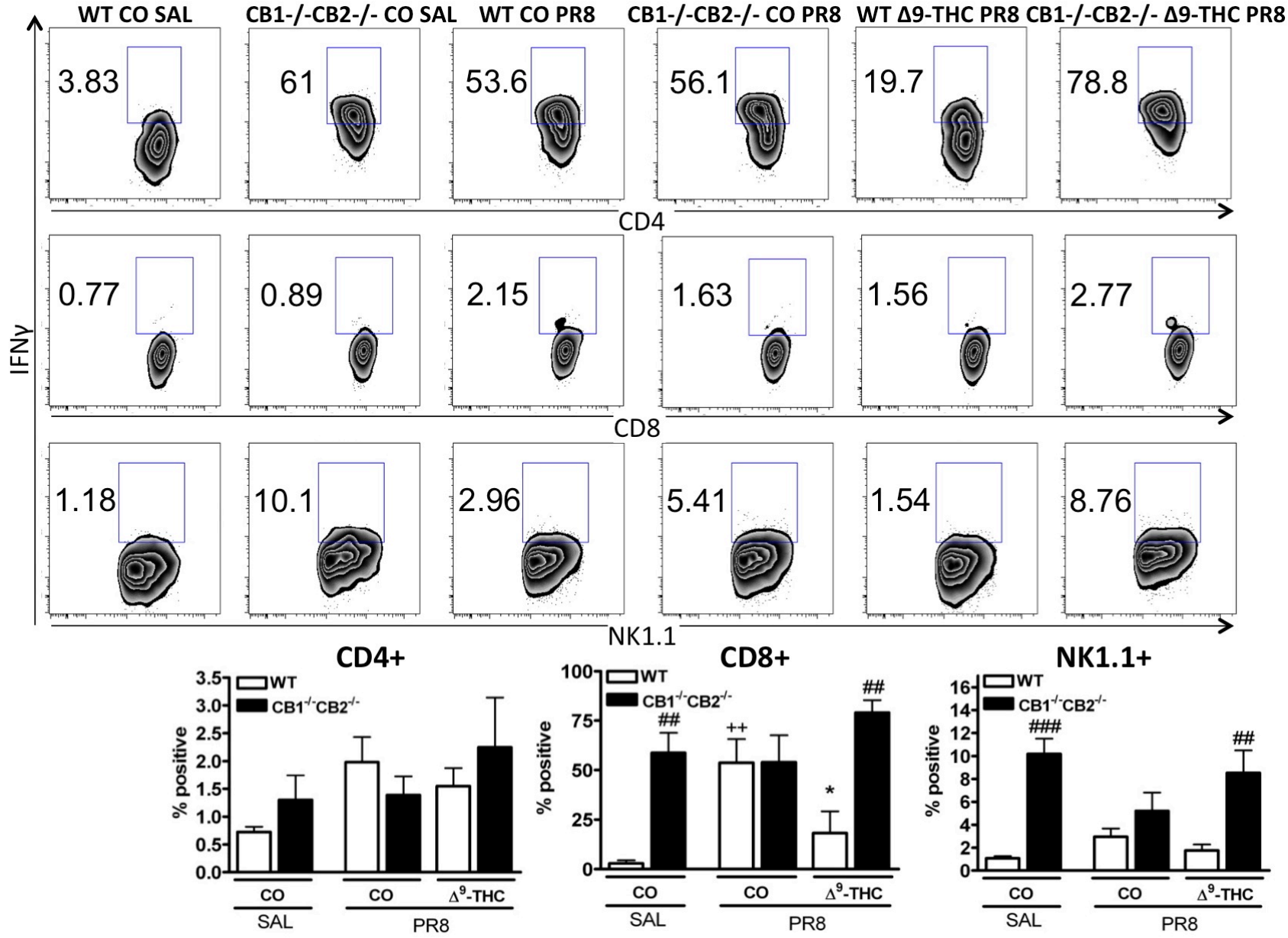


Figure 26. Decreased IFNγ Production after Δ9-THC Treatment in Lung-Isolated Immune Cells from WT but not CB1-/-CB2-/- mice.

Figure 27. Decreased IL-17 Production after Δ9-THC Treatment in Lung-Isolated Immune Cells from WT but not CB1-/-CB2-/- mice.

Lungs were mechanically disrupted and restimulated *in vitro* as described before. Markers CD4, CD8, and NK1.1 were stained on the surface of isolated cells and intracellular staining for IL-17 and flow cytometry was performed (n=5). Non-parametric percentage data were analyzed using Friedman's test: * difference between CO and Δ9-THC, ++ ($p \leq 0.01$) difference between NA (SAL) and PR8; # ($p \leq 0.05$), ## ($p \leq 0.01$), ### ($p \leq 0.001$) difference between WT and CB1-/-CB2-/-. Displayed are concatenated samples (n=5) of each treatment group.

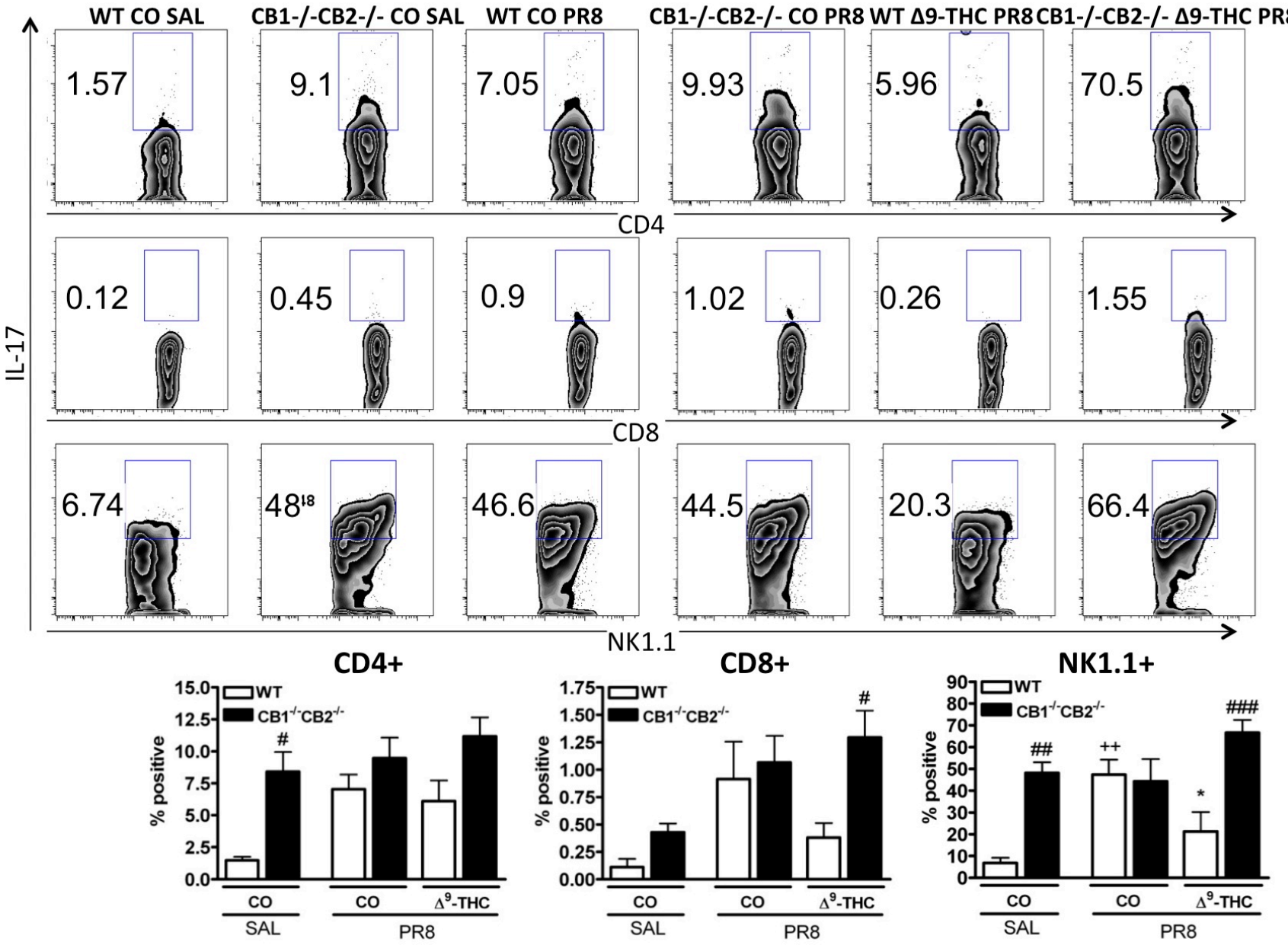


Figure 27. Decreased IL-17 Production after Δ9-THC Treatment in Lung-Isolated Immune Cells from WT but not CB1-/-CB2-/- mice.

B) Δ9-THC Suppressed Influenza-Induced Cytokine Production by Effector Lymphocyte Populations.

Recruitment of neither leukocytes into the BALF (Figure 24) nor effector cells in whole lung isolates was affected by Δ9-THC (Figure 25). However, greater numbers of neutrophils and monocyte/macrophages were found in the BALF and lower numbers of CD8+ cells were found in the lungs of CB1-/-CB2-/- compared to WT mice. As previously reported, these endpoints are consistent with enhanced inflammatory responses observed in CB1-/-CB2-/-mice. In light of the absence of altered cellular recruitment to the lungs by Δ9-THC, effector function was assessed in lymphocyte populations by measuring cytokine secretion in CD4+, CD8+, and NK1.1+ cells. As previously determined, percentages of cells staining positive for cytokine secretion were increased in uninfected lungs of CB1-/-CB2-/- compared to WT mice (Figure 26, 27). Influenza infection significantly induced the percentage of IFNγ-producing cells in WT CD4+ samples and there was a trend towards induction of the IFNγ positive percentage in CD8+ and NK1.1+ cells. Δ9-THC suppressed the percentage of IFNγ-producing populations in WT but not CB1-/-CB2-/- CD4+ cells, while CD8+ and NK1.1+ cells showed a similar trend, yet were not deemed statistically significant. The percentage of the IL-17-producing population was also induced by influenza infection in WT mice, especially in NK1.1+ cells. The percentage of IL-17-producing cells was suppressed by Δ9-THC significantly in NK1.1+ cells (Figure 27). Taking into account the percentage of cells positive for cytokine production, CD8+ T cells had almost negligible contributions to IFNγ and IL-17 production when compared to CD4+ and NK1.1+ cells (Figure 25, 26). Consistent with the histopathology as observed by H&E staining in previous experiments [120], Δ9-THC enhanced the presence of IFNγ-producing populations in CD4+ and NK1.1+ cells and percentages of IL-17-producing cells in CD8+ and NK1.1+ cells from CB1-/-

CB2-/- mice, which was significantly different from Δ9-THC-treated WT samples (Figure 26, 27). Collectively, Δ9-THC suppressed the percentages of functional effector CD4+ and NK1.1+ cells 3 dpi in a CB1 and/or CB2-dependent manner.

C) No Effect of Δ9-THC on Induction of the CD11b+Gr-1+ Population After Influenza Infection

One potential mechanism for a reduction in cytokine production by Δ9-THC in effector lymphocyte populations is the induction of immune suppressor populations, among which are the myeloid-derived suppressor cells (MDSC). MDSC are identified as CD11b+Gr-1+ and can be induced during infection and inflammation [150]. A slightly higher basal presence of MDSC was observed in CB1-/-CB2-/- compared to WT mice (Figure 28). Influenza infection drastically induced MDSC populations in WT and CB1-/-CB2-/-, but no significant effect of Δ9-THC on the induction of MDSC was observed. Albeit not statistically significant, higher percentages of MDSC were observed in CB1-/-CB2-/- compared to WT mice after influenza infection, consistent with previous reports of greater inflammatory responses in these mice. Thus these MDSC seem to be attracted to inflammatory environments and may be one of several mechanisms to ameliorate inflammation due to influenza infection. On MDSC, the expression of MHC I, MHC II and CD86 was greatly induced after influenza infection (Figure 29). Δ9-THC administration had no effect on the expression levels of MHC I and CD86 after influenza infection, but there were trends showing a decrease in MHC II in WT and an increase in MHC II in CB1-/-CB2-/- mice. Overall, there is no direct evidence for the contribution of MDSC to the Δ9-THC-mediated suppression of cytokine production in lymphocytes, but these cells might play a role in suppressing influenza-induced inflammation [148].

Figure 28. No Effect by Δ9-THC Treatment on Presence of Influenza Infection-Induced Gr-1+CD11b+ Lung-Isolated Cell Populations. Cells were isolated from lungs as described before. Cell surfaces were stained with fluorescently labeled antibodies to Gr-1 and CD11b and analyzed using a flow cytometer (n=5). Percentage data were analyzed using Friedman's test: +++ ($p \leq 0.001$) difference between NA (SAL) and PR8; ## ($p \leq 0.01$) difference between WT and CB1-/-CB2-/-. Shown are concatenated samples (n=5) within each treatment group.

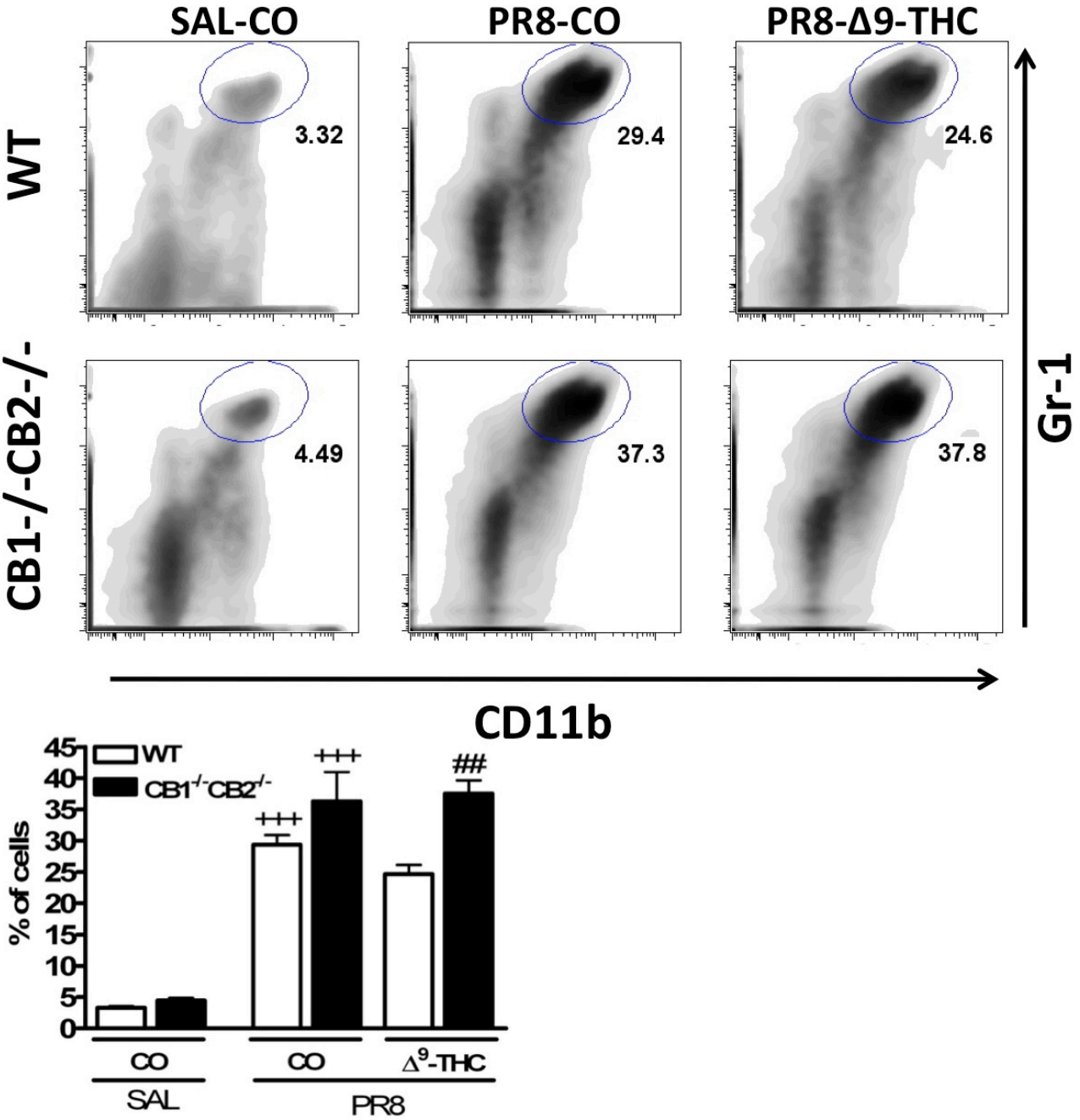


Figure 28. No Effect by Δ9-THC Treatment on Presence of Influenza Infection-Induced Gr-1+CD11b+ Lung-Isolated Cell Populations.

Figure 29. Δ9-THC Treatment does not Alter the Maturation Status of Gr-1+CD11c+ cells. Lung-isolated cells were gated on the Gr-1+CD11b+ population shown in Figure 6. Within the gated Gr-1+CD11b+ population, expression of maturation markers MHC I, MHC II, CD86 was assessed using fluorescence intensity after flow cytometric analysis (n=5). The data as shown was concatenated within each group. Data were analyzed using ANOVA: +++ ($p \leq 0.001$) difference between NA (SAL) and PR8.

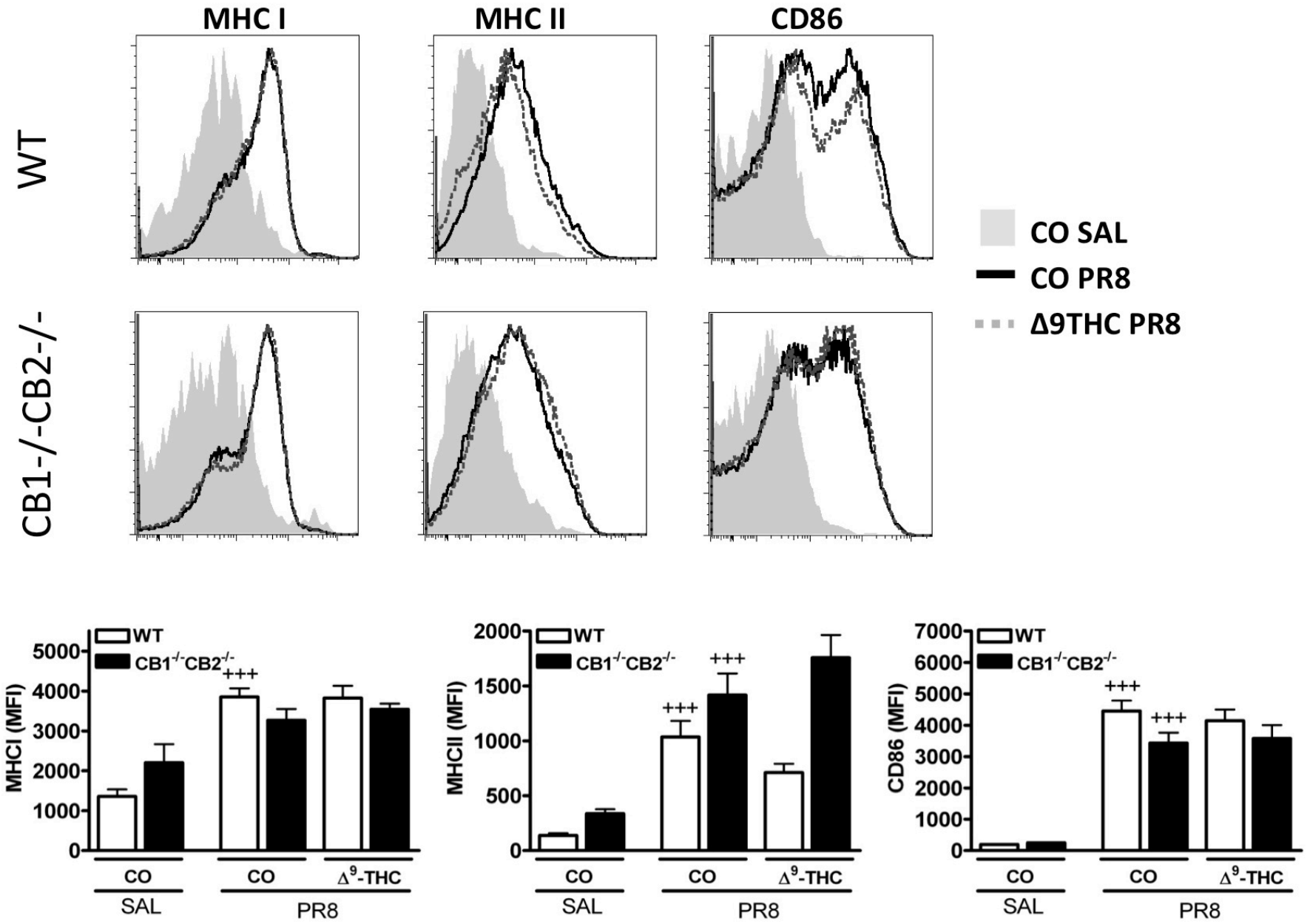


Figure 29. Δ9-THC Treatment does not Alter the Maturation Status of Gr-1+CD11c+ cells.

Figure 30. Δ9-THC Reduces Recruitment of pDC, cDC, and AM into the Lungs of PR8-Infected Mice Dependent of CB1 and/or CB2. Mice (n=5) were intranasally instilled with saline (NA) or 50 pfu of PR8 virus and 3 dpi lungs were excised *en bloc* and homogenized by mechanical disruption. Single cell suspensions obtained from the lung were stained for CD11b, CD11c, Gr-1, MHC I, MHC II, and CD86. CD11b, CD11c, and Gr-1 were used as markers to identify separate cell populations as shown in the gating scheme (A), which were then enumerated by percentage (B). Friedman's test was used to perform statistics on non-parametric percentage data: ** ($p \leq 0.01$), * ($p \leq 0.05$) difference between CO and Δ9-THC, ++ ($p \leq 0.01$), +++ ($p \leq 0.001$) difference between NA (SAL) and PR8; ## ($p \leq 0.01$) difference between WT and CB1-/-CB2-/-.

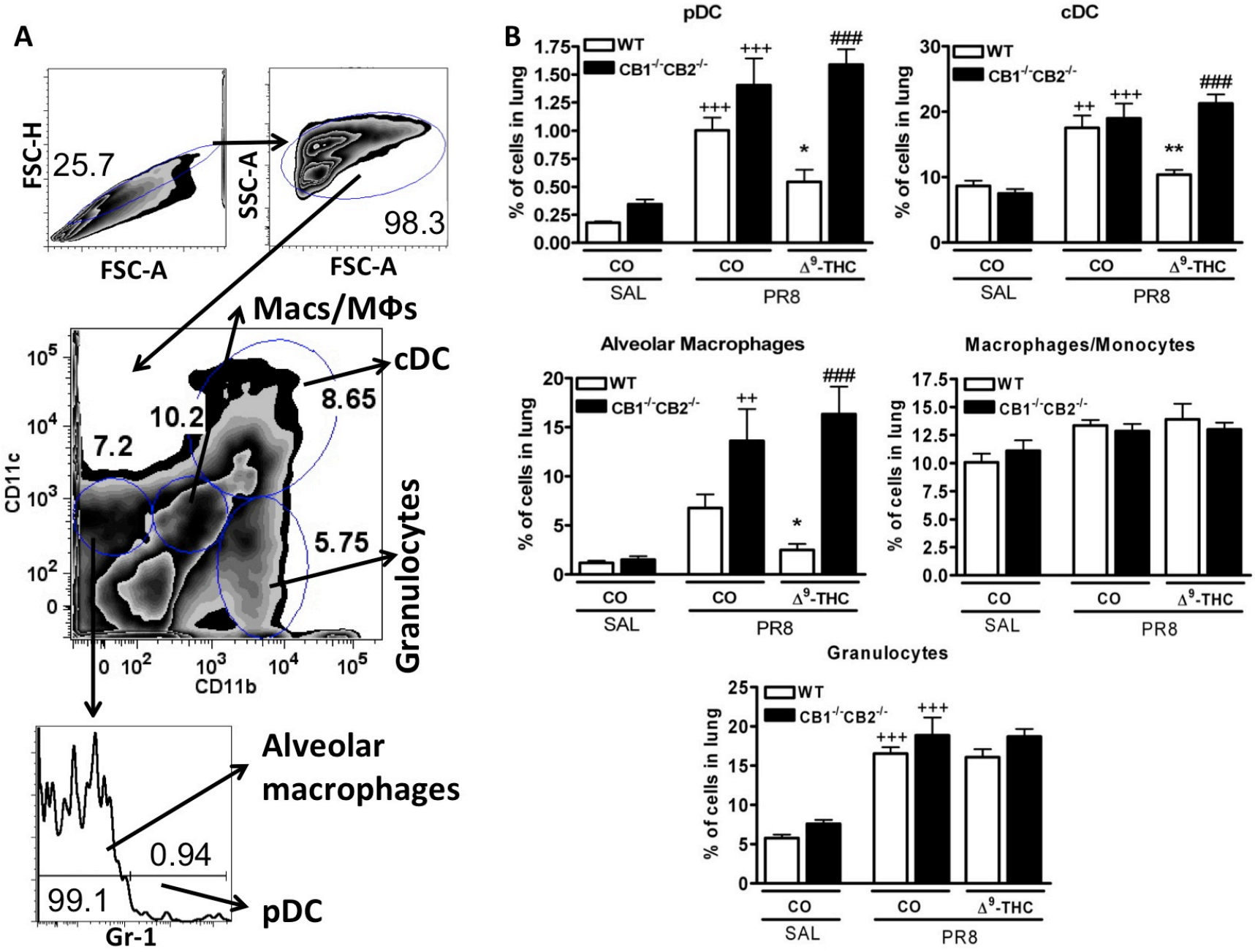


Figure 30. Δ9-THC Reduces Recruitment of pDC, cDC, and AM into the Lungs of PR8-Infected Mice Dependent of CB1 and/or CB2.

D) Reduced Presence of APC with Δ9-THC Treatment After Influenza Infection

To become functional effectors, lymphocytes depend on the presence of, and interaction with, APC. In the lung, several populations of immune cell populations including APC are present and can be identified according to their surface expression of CD11b, CD11c, and Gr-1 [131, 328] (Figure 30 A). Granulocytes are CD11b+CD11c-, macrophages are CD11bloCD11clo, cDC are CD11b+CD11c+, while AM are CD11c+CD11b- as well as Gr-1-. Similar to AM, pDC as also express CD11c and are identified as CD11b-CD11c+Gr-1+. The presence of pDC, cDC, and granulocytes was significantly increased in both WT and CB1-/-CB2-/- mice as a result of influenza infection, while there was no change in the macrophage/monocyte (Mac/MΦ) population (Figure 30 B). Furthermore, greater percentages of AM were found in lungs of CB1-/-CB2-/- compared to WT mice after influenza infection. The increase of pDC, cDC, and AM after infection was attenuated in Δ9-THC-treated WT but not CB1-/-CB2-/-mice, suggesting CB1- and/or CB2- dependence of the Δ9-THC effect (Figure 29 B). In summary, a reduction in lymphocyte effector function by Δ9-THC might be attributed to reduced numbers of APC, specifically, AM, cDC and pDC present in the lungs in Δ9-THC-treated WT mice with influenza.

A) Greater Maturation in APC from CB1-/-CB2-/- Compared to WT and CB1-and/or CB2-Dependent Suppression of Maturation by Δ9-THC after Influenza Infection.

Maturation markers MHC I, MHC II and CD86 were assessed in APC subpopulations: cDC (Figure 31), pDC (Figure 32), AM (Figure 33) and Mac/MΦ (Figure 34). No changes in expression of maturation markers as a result of influenza infection were observed in cDC or AM (Figure 31 and 33); however, in pDC and Mac/MΦ surface expression of MHC II and CD86 was

Figure 31. Δ9-THC does not Alter the Maturation Status of Lung-Isolated cDC after PR8 Infection. Within CD11b+CD11c+ population (gates shown in Figure 30), maturation markers MHC I, MHC II, CD86 were determined by flow cytometry (n=5) (A). The data as shown was concatenated within each treatment group. Bar graphs show mean fluorescence intensities (MFI) for indicated maturation markers (B) (n=5). Analysis was performed using ANOVA (B): # ($p \leq 0.05$), difference between WT and CB1-/-CB2-/-.

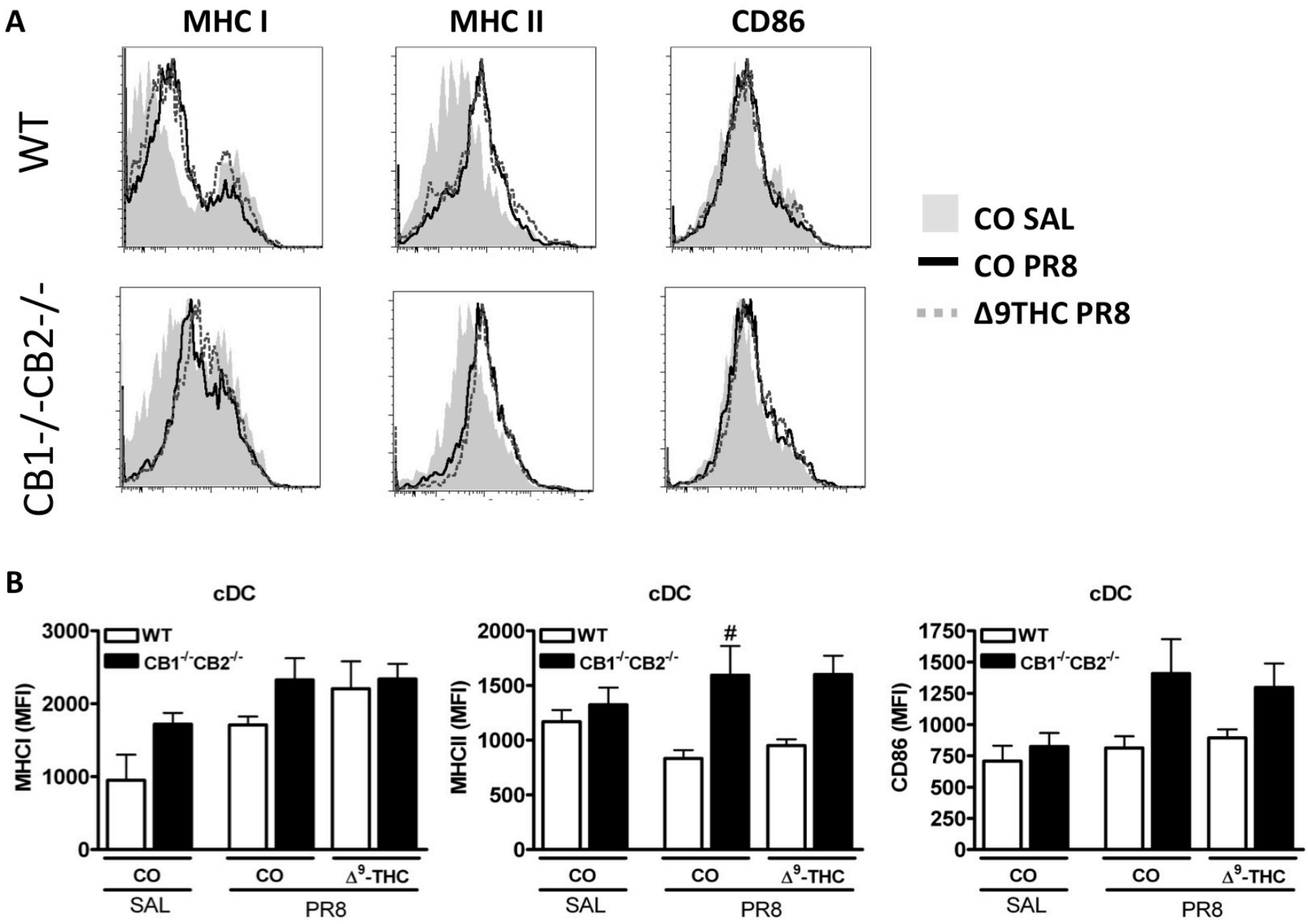


Figure 31. Δ9-THC does not Alter the Maturation Status of Lung-Isolated cDC after PR8 Infection.

Figure 32. Suppression of MHC II Expression in pDC by Δ9-THC after PR8 Infection. Within the CD11b-CD11c+ population (gates shown in Figure 30) the Gr-1+ pDC population (Figure 8 A) was assessed for expression of maturation markers MHC I, MHC II, and CD86 by flow cytometry (A) (concatenated, n=5). Bar graphs are indicating MFI (B) (n=5). Samples were analyzed using ANOVA (B): * ($p \leq 0.01$), difference between Δ9-THC and VH (CO); +++ ($p \leq 0.001$) difference between SAL and PR8; #, ($p \leq 0.05$), ### ($p \leq 0.001$), difference between WT and CB1-/-CB2-/-.

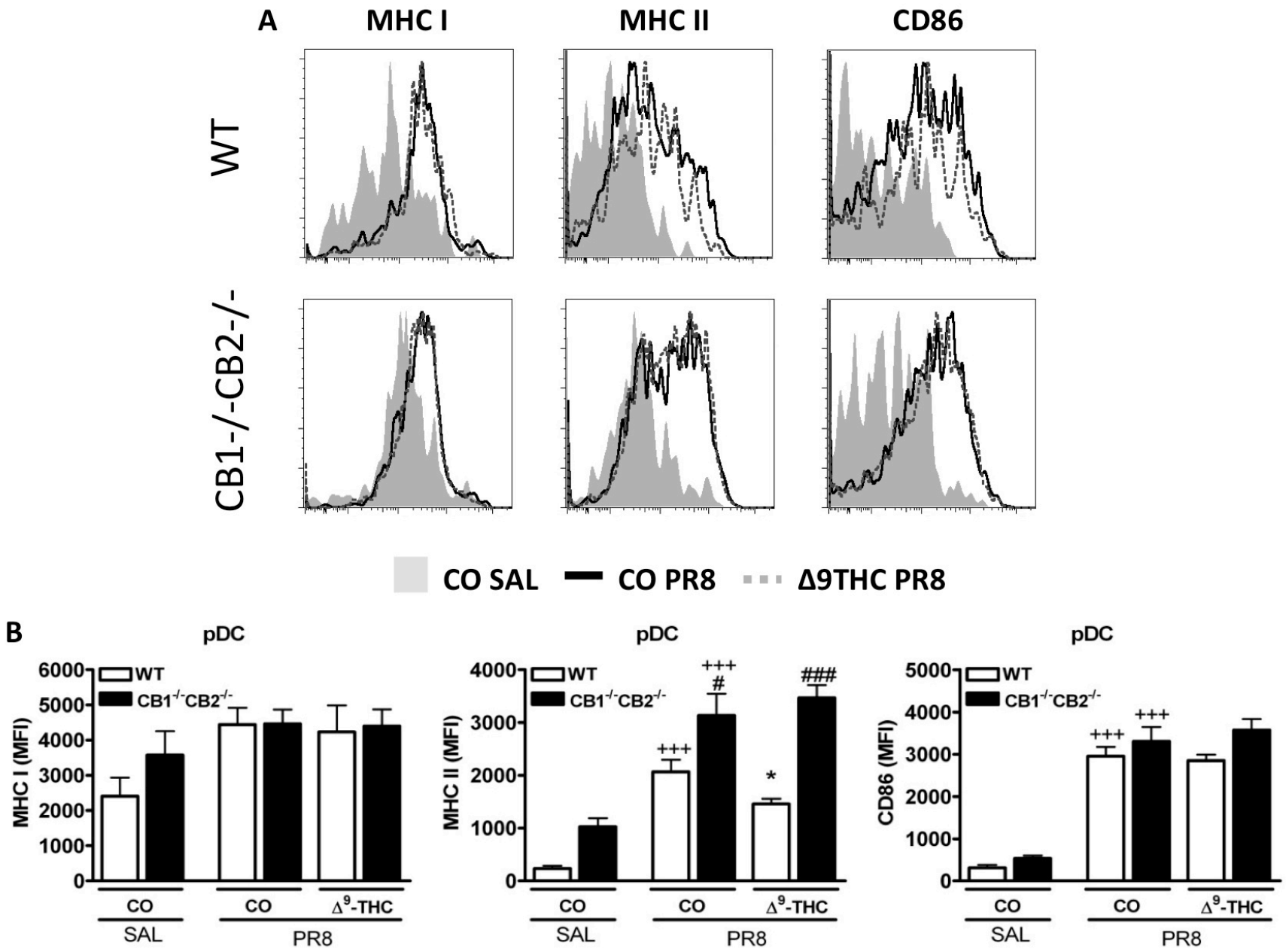


Figure 32. Suppression of MHC II Expression in pDC by Δ9-THC after PR8 Infection.

Figure 33. Δ9-THC does not Alter the Maturation Status of Lung-Isolated AM after PR8 Infection.

Within CD11b-CD11c+ population (gates shown in Figure 30) the Gr-1- AM population (Figure 8 A) was gated and expression of maturation markers MHC I, MHC II, and CD86 was assessed by flow cytometry (concatenated, n=5). Bar graphs indicate MFI (C) (n=5). Samples were analyzed using ANOVA.

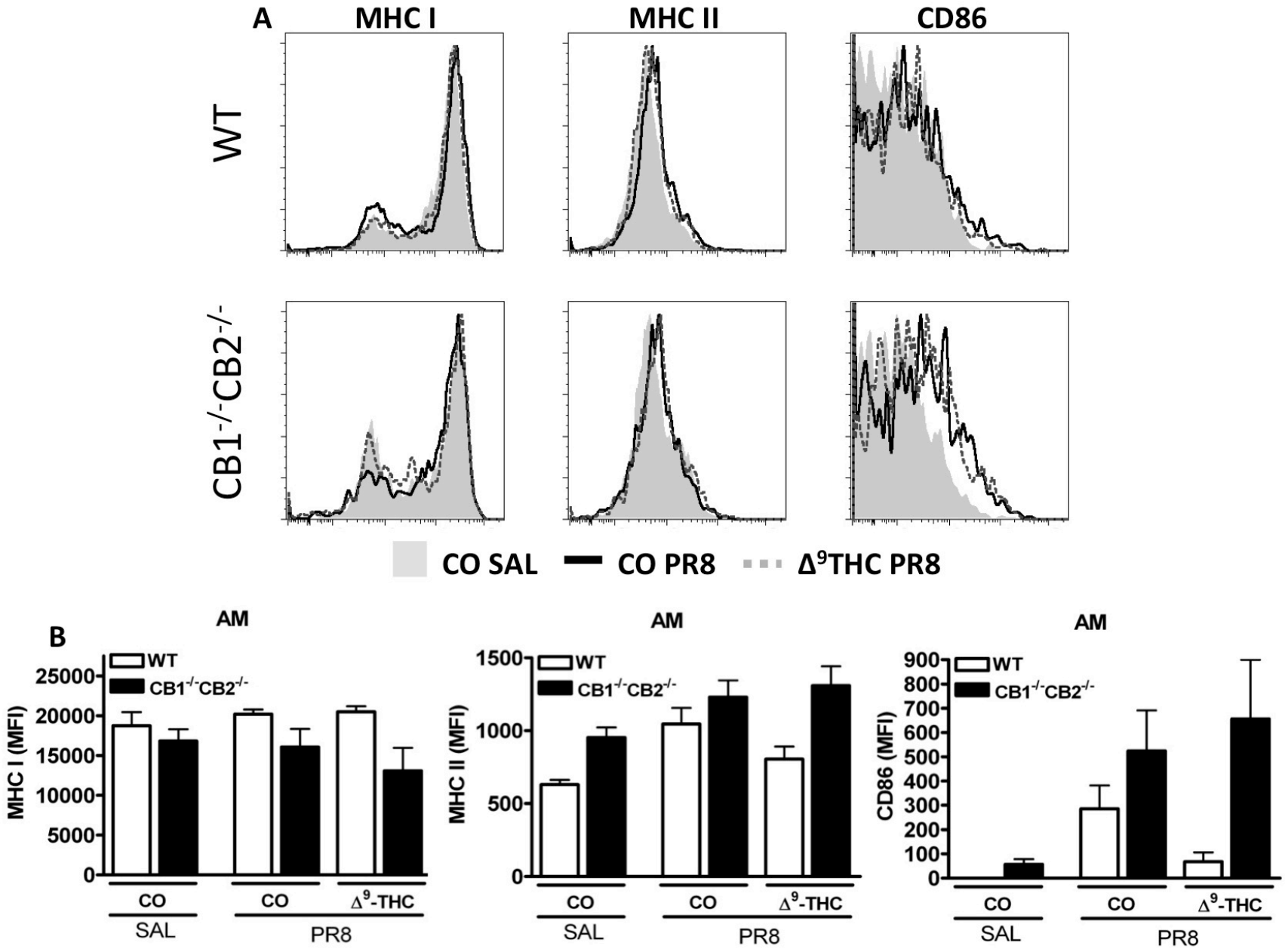


Figure 33. Δ9-THC does not Alter the Maturation Status of Lung-Isolated AM after PR8 Infection.

Figure 34. Δ9-THC does not Alter the Maturation Status of Lung-Isolated Macrophages/Monocytes after PR8 Infection.

Cells were gated on the CD11b lo CD11c lo population for macrophages and/or monocytes as shown in Figure 30. Expression of maturation markers MHC I, MHC II, and CD86 was assessed by FACS (shown are concatenated samples, n=5 per group) (A). Bar graphs were generated using MFI output of FACS data from (n=5) (B). Samples were analyzed using ANOVA: +++ ($p \leq 0.001$) difference between SAL and PR8; #, ($p \leq 0.05$), ### ($p \leq 0.001$), difference between WT and CB1-/-CB2-/-.

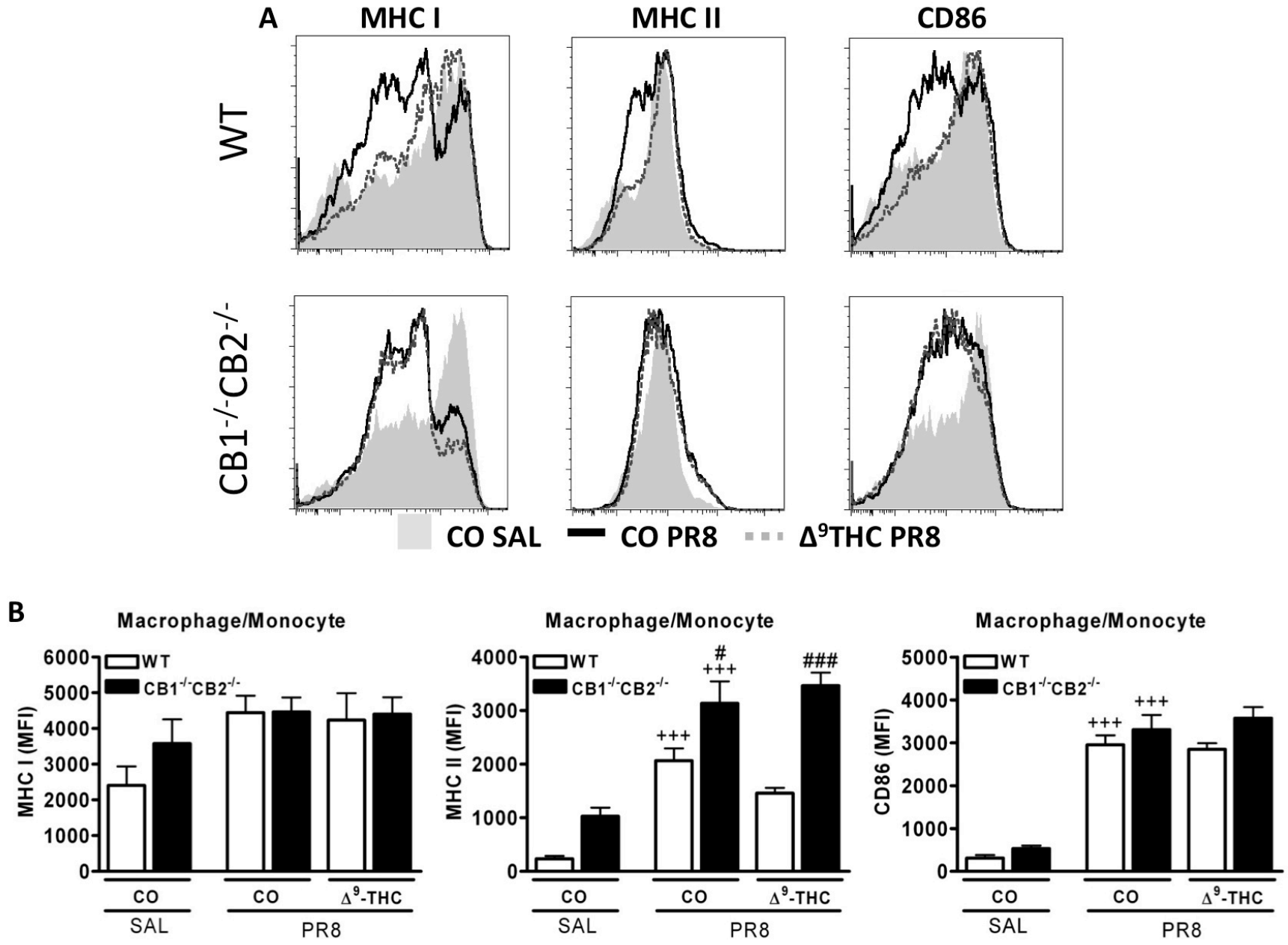


Figure 34. Δ9-THC does not Alter the Maturation Status of Lung-Isolated Macrophages/Monocytes after PR8 Infection.

increased as a result of influenza infection (Figure 32 A, B and Figure 34 A, B). More robust expression levels of MHC II were observed in cDC, pDC and Mac/MΦ from CB1-/-CB2-/- mice compared to WT mice after influenza infection (Figure 31, 32, and 34). Δ9-THC suppressed MHCII surface expression in pDC in a CB1- and/or CB2- dependent manner (Figure 32) although similar trends were observed in MHCII and CD86 in AM (Figure 33) and MHCII in Mac/MΦ (Figure 34) albeit not statistically significant. Taken together, these results suggest that to a great extent the suppression of lymphocyte effector function by Δ9-THC occurs by reducing the migration of APC into the lungs, rather than suppressing the maturation status on APC. Furthermore, greater maturation was observed in inflammatory APC such as pDC and Mac/MΦ in CB1-/-CB2-/- compared to WT mice.

B) Attenuation of bmDC Maturation by Δ9-THC *In Vitro*

An alternate explanation for the reduced presence of cDC, pDC and AM in the lungs as a result of Δ9-THC treatment without changes in maturation, could be that migration is dependent on the maturation status. Indeed, only mature DC are able to migrate, thus it is plausible that the site of infection is enriched for mature DC as immature DC are not able to migrate to the site of infection [329-331]. However, Δ9-THC might elicit its effects on the maturation of DC, thereby reducing their migration to the site of infection. An *in vitro* bmDC model was used to study the effects of Δ9-THC on DC maturation induced by TLR ligation. These bmDC were identified as CD11b+CD11c+ and thus similar to the cDC phenotype (Figure 35 A). LPS (1μg/mL), a TLR4 ligand [332] significantly induced maturation in WT and CB1-/-CB2-/- bmDC, as evidenced by MHC II, CD80 and CD86 expression (Figure 35 B, C). In contrast, R848 (5μg/mL), a TLR 7/8 agonist [333] did not induce bmDC maturation to the same extent as LPS, which was especially

Figure 35. Δ9-THC Suppresses TLR-Stimulated bmDC Maturation Independent of CB1 and CB2.

Bone marrow was used to generate bmDC in the presence of GMCSF (20 ng/mL) for 9 days. bmDC were washed and incubated for 24 hours in the presence or absence (NA) of LPS (1μg/mL) or R848 (5μg/mL) and co-treated with VH (0.1% ethanol) or Δ9-THC (10 μM). After incubation, staining for CD11b, CD11c (not shown), MHC I, MHC II, and CD86 were performed as previously described. Cells obtained after culture were CD11b+CD11c+ (A), indicating cDC phenotype. Graphs shown are concatenated samples (B, n=3). ANOVA tests were performed on samples from 2 A and B (C), ** ($p \leq 0.01$), difference between Δ9-THC and VH (CO); ++ ($p \leq 0.01$), +++ ($p \leq 0.001$) difference between SAL and PR8; ## ($p \leq 0.01$), difference between WT and CB1-/-CB2-/-. The experiment is representative of three identical repeat experiments.

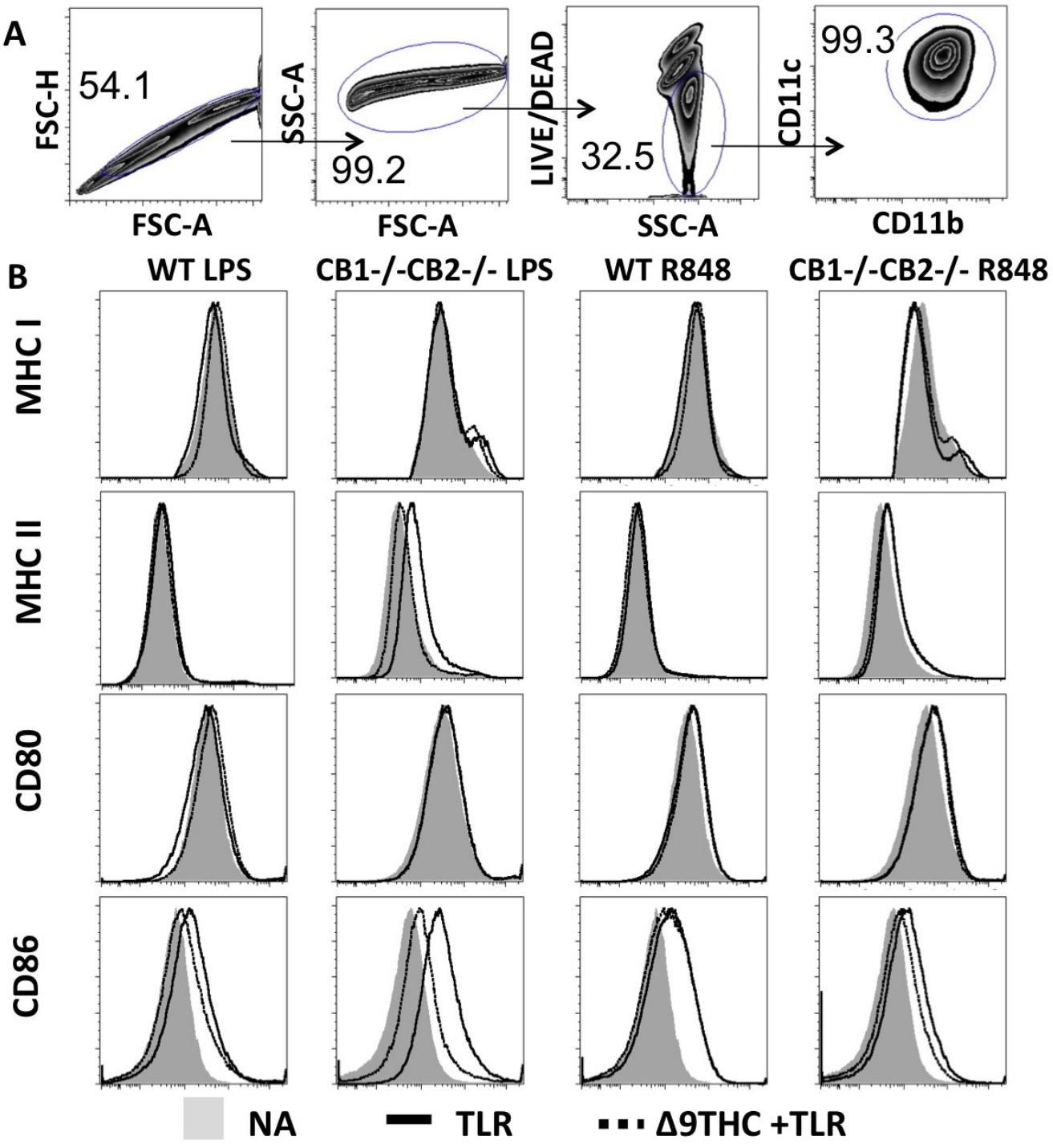


Figure 35. Δ9-THC Suppresses TLR-Stimulated bmDC Maturation Independent of CB1 and CB2.

Figure 35 cont'd.

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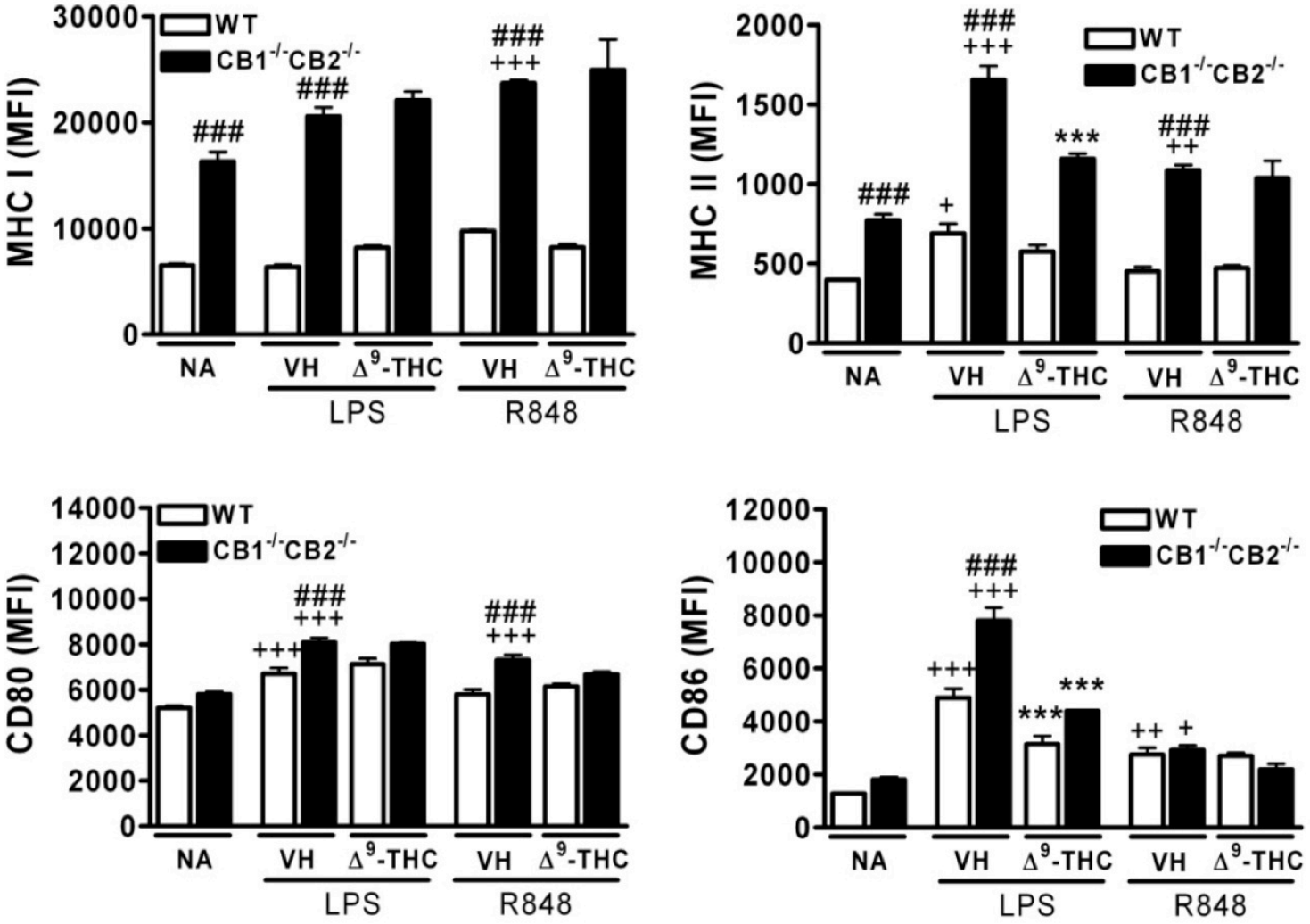


noticeable for CD86 expression (Figure 35 B, C). As previously observed, greater maturation levels as evidenced by MHCI and MHCII expression were observed in unstimulated bmDC generated from CB1-/-CB2-/- when compared to WT. Δ9-THC (10 μM) reduced the LPS- but not R848-induced maturation, as evidenced by lower MHC II in CB1-/-CB2-/- and CD86 expression in WT and CB1-/-CB2-/-. Collectively, bmDC maturation was suppressed by Δ9-THC independent of CB1 and/or CB2.

C) Impaired Elicitation of T cells after Δ9-THC Treatment of bmDC

To determine the functional consequences of Δ9-THC-mediated suppression of TLR-stimulated maturation of bmDC, a T cell co-culture model was used. Cells from OT-1 mice with a transgenic T cell receptor in the CD8+ T cell compartment specific to cognate epitope OVA257-264 (SIINFEKL) were incubated with bmDC pulsed with SIINFEKL. The ability of OT-1 CD8+ T cells to proliferate and secrete IFNγ is a direct result of the interaction with bmDC, and is thus a functional assay for the ability of bmDC to elicit T cells. Four days after co-culture, OT-1 cells were restimulated with SIINFEKL and after staining with fluorescently labeled antibodies were gated as described in methods to exclude any cells other than CD8+ T cells (Figure 36 A). No proliferation was observed in T cell co-cultures with NA WT bmDC in contrast to bmDC from CB1-/-CB2-/-, which readily elicited T cells without the need for prior stimulation as previously reported (Figure 36 B). While LPS- or R848-treated bmDC induced antigen-specific T cell proliferation in WT, Δ9-THC (10 μM) potently reduced proliferation. Due to the high response magnitude in naive bmDC from CB1-/-CB2-/- mice, TLR stimulation had little effect on T cell elicitation and Δ9-THC treatment of CB1-/-CB2-/- bmDC did not alter their ability to elicit T cells (Figure 36 B). In addition to proliferation, it is important to determine T

Figure 36. Δ9-THC Impairs Antigen-Specific bmDC-Elicited T cell responses.

bmDC, NA and TLR treated, with VH (0.1% ethanol) or Δ9-THC were pulsed with the OT-1 TCR-specific peptide SIINFEKL for 1 hour and washed three times prior to incubation with Cell Trace-labeled OT-1 splenocytes for 4 days. Cells were restimulated with SIINFEKL and stained for CD8 and IFNγ and gated as depicted in the scheme to obtain dot plots (A). OT-1 cells were co-cultured with bmDC and then gated as described in 3 A. Dot plots with Cell Trace loss indicating proliferation on the X-axis and CD8 staining on the Y-axis are shown. Gate shown indicates proliferation (loss of Cell Trace fluorescence compared to control) and CD8 staining (B) or IFNγ staining (C). Shown are concatenated samples of each group (n=3). Kruskal-Wallis tests were performed on samples from 3 B-E, * ($p \leq 0.05$), difference between Δ9-THC to VH (CO); ++ ($p \leq 0.01$), ## ($p \leq 0.01$), ### ($p \leq 0.001$) difference between WT and CB1-/-CB2-/-. The experimental data is representative of two identical repeat experiments.

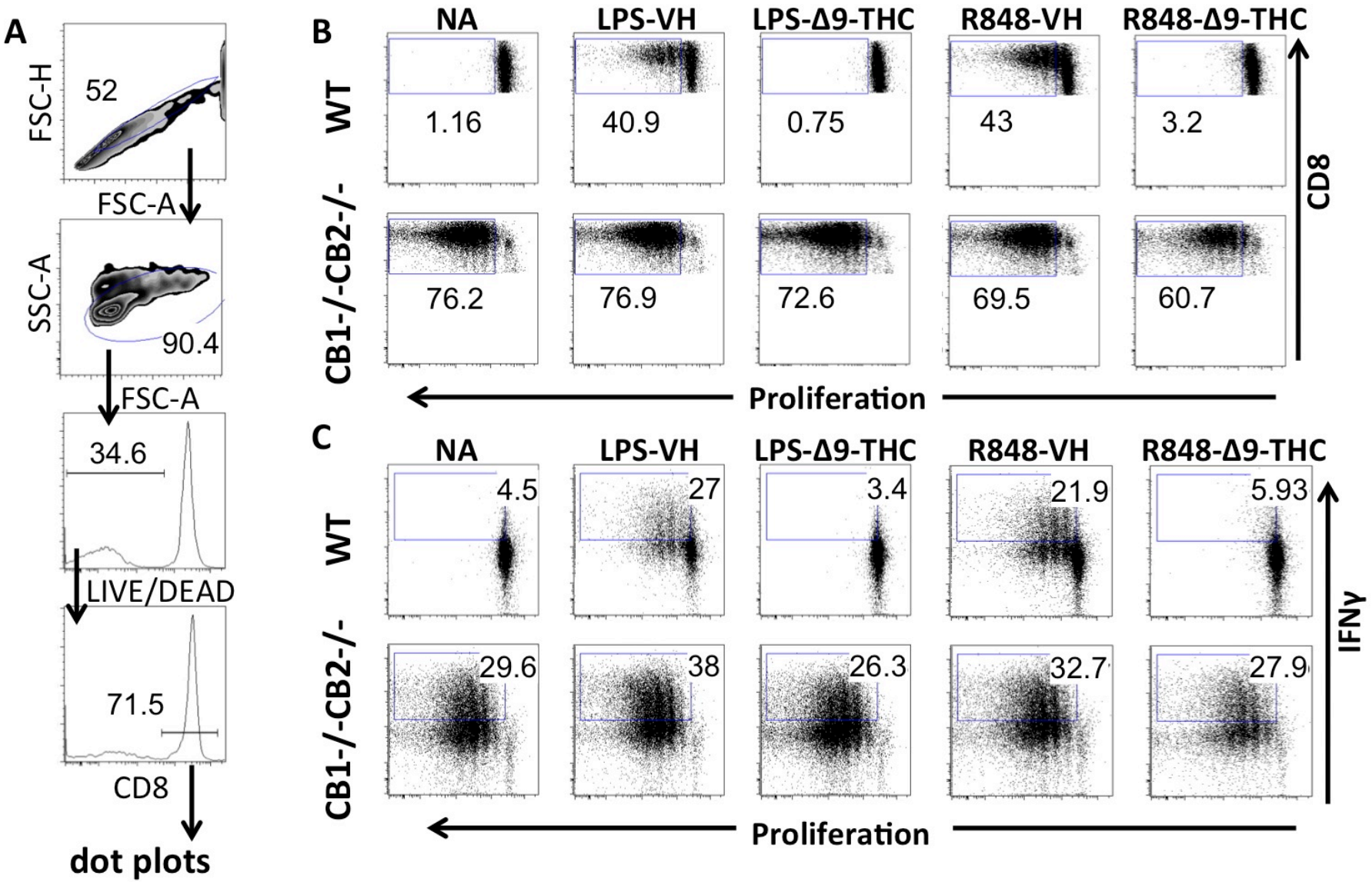
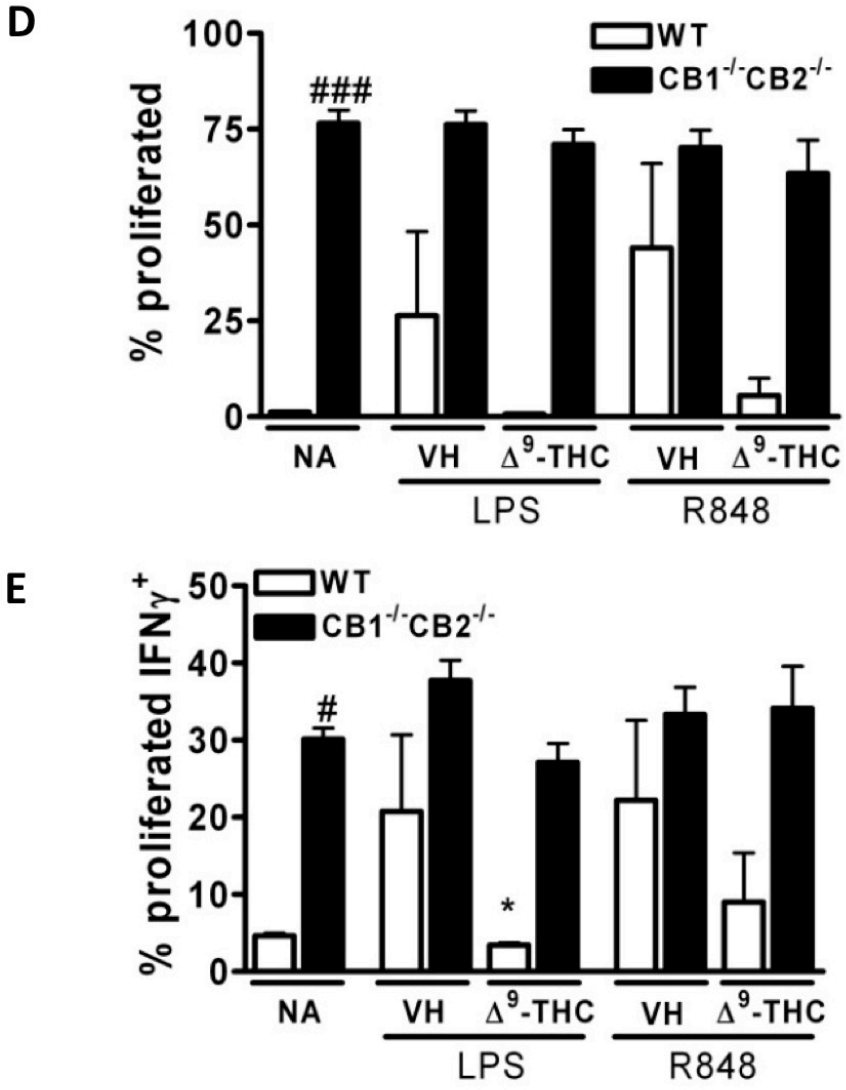


Figure 36. Δ9-THC Impairs Antigen-Specific bmDC-Elicited T cell responses.

Figure 36 cont'd.



secretion of cytokines. Concurrent analysis of IFNγ secretion and proliferation was performed after elicitation. Similar to the proliferation results, without TLR stimulation no cytokine release was observed in OT-1 cells elicited by co-culture with SIINFEKL-pulsed WT bmDC, but cytokine release was induced with TLR stimulation and significantly suppressed with Δ9-THC (10 μM) treatment (Figure 36 C). CB1-/-CB2-/- bmDC elicited OT-1 T cell responses of high magnitude without the need for prior stimulation and were resistant to Δ9-THC suppression (Figure 36 C). Overall, these results suggest that CB1 and/or CB2 are important in the suppression of DC-mediated T cells responses by Δ9-THC. Furthermore, in light of maturation-dependent migration, the impairment of DC maturation by Δ9-THC might consequentially attenuate DC migration, thereby reducing effector cell responses in the lung after influenza infection.

DISCUSSION

I. Suppression of the CTL Response by Δ9-THC

The observation that the influx of CD8+ T cell populations after influenza infection *in vivo* was greatly impaired in response to Δ9-THC treatment warranted further studies to characterize the effect of Δ9-THC on CD8+ T cells at the cellular level [119]. A model using P815 cells as an allogeneic stimulus to elicit CTL and cells isolated from CB1-/-CB2-/- and WT mice was employed to investigate the effect of Δ9-THC on the generation of of CTL *in vitro*. Collectively, these studies provide evidence that Δ9-THC-mediated suppression of CD8+ T cell function occurs independent of CB1 and CB2. Moreover, Δ9-THC alters early signaling events that involve putative changes in intracellular Ca2+ levels, eventually resulting in lower numbers of CTL effectors. Finally, this study introduces an inherent role for CB1 and/or CB2 in the elicitation of CTL, because elicitation and function of CTL was reduced in CB1-/-CB2-/- mice *in vitro*.

A) Reduced CTL Response in CB1-/-CB2-/- Compared to WT mice

In contrast to other reports describing enhanced immune responses in CB1-/-CB2-/- mice [120, 315], in this *in vitro* model of alloantigen-induced CTL elicitation and effector function, CTL activity in cells from CB1-/-CB2-/- mice was reduced when compared to WT mice. Although this was consistent with our previous study in which we observed a reduction in the T cell-dependent humoral immune response to sheep erythrocytes *in vitro* in CB1-/-/CB2-/- as compared to WT mice [279], there are several studies demonstrating enhanced immune function in CB1-/-CB2-/- mice [120, 315]. In a previous study from this laboratory [120], it was

demonstrated that CB1-/-CB2-/- mice, in addition to responding with a greater magnitude to immune stimuli, have altered immune homeostasis compared to WT mice as evidenced by greater numbers of CD4+ T cells and higher levels of TNF-α in the BALF in the absence of influenza challenge. In addition, there appeared to be mechanisms in CB1-/-CB2-/- mice that might compensate for the loss of CB1 and/or CB2, as evidenced by greater levels of the endocannabinoids, 2-arachidonoylglycerol and anandamide [315], and increased transcripts of *cnr2* using primers specific for the undeleted portion of the gene [334]. Although CB1-/-CB2-/- mice seem to be particularly vulnerable to proinflammatory insult, there were no drastic differences observed in an adjuvant-free airway hypersensitivity model between CB1-/-CB2-/- and WT mice [317]. Reasons for reduced function of CTL from CB1-/-CB2-/- mice compared to WT mice, although not altogether clear, might be due to the present studies being conducted *in vitro*, and/or the nature in which the immune response was initiated, in this case by direct stimulation of CD8+ T cells with an alloantigen.

Greater proliferation was detected in CD4+, but lower proliferation in CD8+ cells, from CB1-/-CB2-/- compared to WT mice. This lower proliferation in CD8+ cells might contribute to the reduced CTL activity in CB1-/-CB2-/- compared to WT mice. Furthermore, higher percentages of CD4+CD25+ were observed in unstimulated splenocytes of CB1-/-CB2-/- compared to WT mice. These CD4+CD25+ cells are thought to be regulatory T cells (Treg) [335], which are involved in the suppression of the immune responses. It has been demonstrated that an increase in CD4+CD25+ cells leads to reduced CTL responses; thereby Tregs could potentially contribute to the reduced activity *in vitro* in CTL generated from CB1-/-CB2-/- mice. [336]. This *in vitro* model, due to direct stimulation of CTL, lacks a proinflammatory stimulus and does not produce IL-6, which has been implicated in overriding Treg responses, thus Treg

responses might be maintained during the *in vitro* culture [337]. As our focus was on the Δ9-THC-mediated suppression of CTL responses, we did not further characterize these CD4+CD25+ cells for expression of Foxp3 or other cellular markers of the Treg lineage [338]. Additional studies are required to determine the role of Tregs in CB1-/-/CB2-/-mice.

B) Sensitivity of CTL to Δ9-THC Immune Modulation

Addition of Δ9-THC during the elicitation and effector response showed that only naïve CD8+ cells, but not differentiated CTL, were sensitive to Δ9-THC-mediated suppression of CTL function as assessed by 51Cr release assay, suggesting Δ9-THC-mediated immune modulation during elicitation. Arguably, the concentrations of Δ9-THC at which altered CTL activity was observed in this investigation are higher than detected in the serum of recreational marihuana smokers. However, it is important to emphasize two points. The first is that in addition to Δ9-THC, marihuana smoke contains over 60 other structurally-related cannabinoids, a number of which are also well established immune modulators and the majority of which remain to be evaluated for immunomodulatory activity [339, 340]. Second, and equally important, the goal of this study was to relate the decrease in viral clearance observed after Δ9-THC treatment of animal with influenza-infected airways [119] and the possibility that this may be due to cannabinoid-mediated suppression in CTL activity. In the case of inhaled marihuana smoke, the cannabinoid concentrations attained in the lung are significantly higher than systemic serum Δ9-THC concentrations and may be more closely related to those used in the current study [341, 342]. The timeframe of sensitivity to Δ9-THC might reflect a high level of T cell plasticity prior to differentiation and reduced plasticity after differentiation [343]. Other immunomodulatory compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and glucocorticoids

exert their greatest effect on early stages of immune cell differentiation and tend to have a lower efficacy if added after the onset of the differentiation program [344, 345]. Specifically, cyclosporin A is very efficacious in suppressing *in vitro* culture of allogeneic cells only when added prior to elicitation [346]. Overall, this suggests a critical window of sensitivity for immunomodulatory compounds, including Δ9-THC, for immune suppression, specifically during the transition of naïve CD8+ T cells to CTL during elicitation.

Using IFNγ as a surrogate for CTL activity, it was demonstrated that CD8+ cells are the sole producers of IFNγ after allogeneic stimulation, suggesting that only CTL directly contribute to the 51Cr release from labeled target cells. Although it appeared that Δ9-THC increased IFNγ expression in CTL, the cells were gated on the live cell populations. Upon examination of the results from the total population, there was no effect of Δ9-THC on IFNγ, suggesting alternative interpretations of the data. Taken together, our results suggest that while there seemed to be a population of CTL whose activity cannot be suppressed by Δ9-THC, which become the IFNγ-secreting cells, the remaining cells are sensitive to Δ9-THC and were not capable of surviving the five-day culture period. Thus, the suppression by Δ9-THC does not appear to reduce the amount of IFNγ each cell produces, but rather impaired the differentiation of a select pool of naïve cells from becoming effector CTL.

C) Alterations in T cell Activation by Δ9-THC

No effects of Δ9-THC treatment on the proliferation of CD4+ or CD8+ cells WT or CB1-/-CB2-/- mice were observed. To link the functional outcome of Δ9-THC treatment to earlier T cell signaling, the expression of activation markers, CD25, forming the high affinity IL-2 receptor, and CD69, the earliest known lymphocyte activation marker [325] were assessed.

While CD25 expression was increased as a result of P815 co-culture, only CD8+ cells from CB1-/-CB2-/- mice had reduced CD25 expression as a result of Δ9-THC treatment. CD69 expression was also increased as a result of co-culture with P815 cells in both WT and CB1-/-CB2-/- CD8+ cells and Δ9-THC treatment further enhanced the P815-induced rise in CD69 expression in both genotypes. The ability of Δ9-THC to increase CD69 expression on the surface of CD8+ T cells from WT and CB1-/-CB2-/- after P815 cell stimulation provides further evidence for CB1 and/or CB2-independent mechanism(s) of Δ9-THC immune modulation. Notably, expression of CD69 was inversely correlated with CTL activity. For example, the highest CD69 expression and lowest CTL activity were observed in cells treated with the highest concentration of Δ9-THC (10 μM) from CB1-/-CB2-/- mice.

CD69 expression is controlled by several transcription factors, including AP-1 in the proximal promoter [347] as well as EGR, ATF/CREB [348], and NFκB [325]. However, the pathways regulating the expression of CD69 are different depending on the cell type [349]. It is not entirely clear what cellular signals contribute to CD69 induction in CD8+ T cells, but it is known that Ca2+ influx into T cells increases CD69 expression [350] and, at least in thymocytes, extracellular Ca2+ was found to be critical for CD69 induction [351]. In these studies Δ9-THC synergized with Io to increase CD69 expression suggesting that Ca2+ plays an important role in the Δ9-THC-mediated increase of CD69 expression. Previously, this laboratory reported that Δ9-THC strongly induced intracellular Ca2+ in T cells, which was mediated by TRPC1 [233, 284] independent of the requirement for T cell activation. These studies, taken together with previous results, suggest that modulation of an activation stimulus by concurrent induction of Ca2+ through Δ9-THC-mediated opening of TRPC1 channels contributes directly to the suppression of CTL activity. In addition, there are reports indicating that Δ9-THC has a high

binding affinity for GPR55 [352] and causes increases in intracellular Ca2+ levels via GPR55 [353], but there are also conflicting reports demonstrating that Δ9-THC possesses a low affinity for GPR55 [354]. The role of GPR55 in the present study is unknown; however, due to the expression of GPR55 in the spleen [352] and its ability to modulate Ca2+ currents, it is tempting to speculate that GPR55 contributes to alterations in Ca2+ signaling induced by Δ9-THC. Ca2+ is a ubiquitous intracellular signaling molecule and, in addition to activation, Ca2+ is central to the induction of anergy and cell death, which are seemingly separate processes, yet involve shared mechanisms and endpoints [355, 356]. Thus, due to the role of Ca2+ in the generation of CTL, it seems plausible that Δ9-THC-mediated alterations in Ca2+-signaling are involved in the suppression of CTL responses. An alternate explanation for the synergism of Δ9-THC with Io-induced CD69 levels might involve MAPK activation of AP-1. For example, it has been demonstrated that the βγ subunits of GPCRs as well as β-arrestin are responsible for MAPK activation [244, 248, 249]. Although plausible, this scenario is speculative but has been observed for Gαi-coupled receptors.

D) Summary and Conclusion of the Effect of Δ9-THC on CTL

Collectively, the reduced cytolytic activity, decreased number of CTL effectors, and the increase in CD69 expression, although temporally distinct, might be interrelated, and a consequence of early Δ9-THC-induced changes in Ca2+ signaling. Concordantly, immune cells appear to initially undergo greater activation (CD69), but become unresponsive to secondary stimulation; in essence, anergic. The critical role of calcium in leukocyte activation and subsequent differentiation and effector function is well established. Δ9-THC treatment prior to cellular activation significantly elevates intracellular Ca2+ levels, which in turn interferes with

either entry or execution of the T cell differentiation program. The specific downstream signaling pathways affected through the Δ9-THC-mediated elevation of intracellular Ca2+ levels affecting T cell function remain to be elucidated but likely involve p38 MAPK, JNK, and ERK. Indeed, prior studies by this laboratory demonstrated decreased ERK phosphorylation by cannabinol, another cannabinoid acting independently of CB1 and CB2 [357]. Thus, the immediate perturbation of intracellular Ca2+ levels by cannabinoids, including Δ9-THC, seems to be the initial event triggering a cascade of events, eventually resulting in immune suppression. Signaling by cannabinoids seems to alter the regulation of Ca2+ that is central to the initiation of a T cell response to impair immune function.

II. Influence of Cannabinoid Receptors 1 and 2 in on the Magnitude of the Immune Responses to Influenza

Although the experiments performed on CTL *in vitro* provided insights into the suppression of CTL function by Δ9-THC, the cellular mechanisms contributing to lower viral burden in CB1-/-CB2-/- compared to WT mice remained to be elucidated. Cannabinoid receptors and their endogenous ligands have been investigated extensively for their immunomodulatory activity; however, only a few studies have investigated the outcome of concomitant targeted deletion of both CB1 and CB2 on immune competence [120, 279, 315]. Based on a sizable literature demonstrating that plant-derived, synthetic and endogenous cannabinoids typically attenuate immune responses [196, 358], it has been long hypothesized that one role of CB1 and CB2 might be to contribute to immune homeostasis by preventing overstimulation of the immune system.

Our findings indicate that a principle function of cannabinoid receptors is to modulate APC function and a lack thereof is critically involved in the described hyper-responsive immune phenotype observed in CB1-/-CB2-/- mice. To assess the role of CB1 and CB2 in the immunopathology induced by influenza, the majority of this investigation was focused on events at 3 dpi, the peak day of the inflammatory response in this A/PR/8/34 influenza model . The inflammatory response is to a great extent responsible for the immunopathology observed as a result of influenza infection.

A) Greater Pathology in CB1-/-CB2-/- Compared to WT Mice after Influenza Infection

After influenza infection, immunopathology was evident in lungs of CB1-/-CB2-/- mice to a greater degree than in lungs of WT mice as observed by morphologic differences in H&E staining and the loss of CCSP. In addition, the influx of macrophages and neutrophils into the airways was more pronounced with a concomitant increase in necrosis and exfoliation of surface epithelium surface in CB1-/-CB2-/- compared to WT mice. Since the viral burden was not different between WT and CB1-/-CB2-/- mice, the damage to the respiratory epithelium likely resulted as a consequence of an exacerbated immune response. The inflammatory mediators of macrophages and neutrophils are likely responsible for damage to the airways [359, 360]. Within the airways, CCSP is thought to perform a protective function, especially involving the reduction of neutrophilia due to viral infections, such as respiratory syncytial virus [361]. Thus, the increase in neutrophils in the BALF of CB1-/-CB2-/- mice is consistent with the observed decrease in CCSP and increased airway injury in CB1-/-CB2-/- compared to WT mice. In WT mice CB2 levels did not change after PR8 infection, however CB1 levels were increased suggesting a potential role for CB1 in the amelerioration of immune responses in the lung.

B) Altered T cell Responses in CB1-/-CB2-/- Compared to WT Mice after Influenza *In Vivo* But not after T cell Stimulation *In Vitro*

Several unique gene expression changes were evident in CB1-/-CB2-/- compared to WT mice after infection at 3 dpi. In contrast to basal differences in transcription, several lymphocyte-specific cytokines (IL-13 and IL-17) and indices of lymphocyte activity (CD38, found on cytokine releasing T cells, especially Th17 [362, 363]) were upregulated after influenza infection. The presence of cytokine producing lymphocytes was confirmed by flow cytometry and greater percentages of IFNγ- and IL-17-producing CD4+, CD8+, and NK1.1+ were observed in CB1-/-CB2-/- mice compared to WT mice after influenza infection. These findings are in accordance with accelerated activation as evidenced by CD69 staining on the surface of CD4+ and CD8+ cells. Taken together, exacerbated lymphocyte responses were observed in CB1-/-CB2-/- compared to WT mice.

To investigate whether inherent differences in the function of WT and CB1-/-CB2-/- are present in the lymphocyte compartment, the ability of naïve (CD62L+) and non-naïve (CD62L-) CD4+ T cells to polarize toward a Th17 phenotype after αCD3/αCD28 stimulation was examined. The levels of IL-17 produced from CB1-/-CB2-/- CD4+ T cells were not significantly different from WT. Consistent with *in vitro* studies of mixed lymphocyte responses and PMA/Io stimulation [279], cytokine production was similar after *in vitro* stimulation of naïve CD4+ T cells from CB1-/-CB2-/- and WT mice, suggesting no inherent difference in T cell function between the two genotypes.

C) Dysregulation of APC Responses in CB1-/-CB2-/- Mice Compared to WT mice

During T cell isolation consistently lower numbers of naïve T cells were isolated from CB1-/-CB2-/- mice, and this phenomenon was confirmed by lower percentages of naïve (CD62L+ and CCR7+CD62L+) CD4+ T cells in spleens of CB1-/-CB2-/- compared to WT mice. Accordingly, CCL19 mRNA levels, the chemokine that binds to CCR7, were reduced in CB1-/-CB2-/- mice. In an IκB transgenic mouse model, enhancement of NFκB signaling reduced the naïve T cell compartments as well, while at the same time, greater IL-17 production was observed [364]. In the present study, a similar scenario was observed, in which increased inflammation resulted in the generation of more Th17 cells at the expense of the naïve T cell compartment. Collectively, with no observable difference of *in vitro* cytokine production from naïve CD4+ T cells, evidence for greater numbers of non-naïve T cells suggested involvement of APC in eliciting T cell responses rather than inherent differences in the T cell compartment due to loss of CB1 and CB2. Thus, the environment encountered and the ability of APC to elicit stronger lymphocyte responses provided a plausible explanation for the enhanced cytokine production by lymphocytes *in vivo* and reduction in the naive lymphocyte compartment *in vivo* observed in CB1-/-CB2-/- mice.

Indeed, profound differences in APC maturation of CB1-/-CB2-/- compared to WT mice were observed. Basal and TLR-inducible maturation of bmDC and AM was more robust in CB1-/-CB2-/- mice as assessed by expression of surface markers CD86, MHC I, and MHC II. Consistent with this was the observation of increased mRNA levels of *H2Eb1*, one of the MHC II subunits, in the lung. In CB1-/-CB2-/- mice, increased expression of CCL3, IL-1, and cyclooxygenase-2 (*Ptgs2*) genes were observed, which are often produced by APC and characteristic of an inflammatory response [365]. Concordantly, but less well understood and characterized, Tfrc, an iron transporting receptor has been reported to be downregulated in

macrophages during chronic inflammation as well as in response to inflammatory stimuli [366, 367]. Taken together, a very consistent profile of increased inflammation and maturation of APC exists in lungs of CB1-/-CB2-/- compared to WT mice.

The consequence of APC maturation was reflected in the marked differences in the ability of bmDC pulsed with SIINFEKL to stimulate proliferation in OT-1 cells and their ability to become functional effectors as assessed by IFNγ secretion. While this model did not investigate the role of CB1 and CB2 in antigen processing and presentation by DC, it has been previously demonstrated that cannabinoids including Δ9-THC, can suppress APC function [368, 369]. Furthermore, it has been previously reported that Δ9-THC-mediated suppression of co-stimulatory activity by macrophages was dependent on CB2 [312, 370, 371]. Most recently, CB1 was also shown to play a role in regulation of co-stimulation by DC [266]. Only after TLR stimulation were measurable OT-1 responses elicited by bmDC from WT, while the ability of bmDC from CB1-/-CB2-/- surpassed that of WT with the same TLR stimulation. These *in vitro* studies demonstrate, that at the cellular level, altered function of APC, especially DC, is critical in the observed phenotypic differences between CB1-/-CB2-/- and WT mice.

D) A Role For CB1 and/or CB2 in Regulating the Magnitude of Immune Response to Influenza to Reduce Pathology

While the phenotypic changes as a result of CB1 and CB2 deletion in the immune system described above appear to have improved resistance to virus infection as evidenced by a reduced lung viral burden after influenza challenge [120], our results indicate that this occurs with a concomitant increase in immunopathology. The drastic loss of CCSP staining and greater inflammation as evidenced by H&E staining in addition to cytokine production by NK and T

cells and lung mRNA transcript levels for proinflammatory mediators suggest that while CB1-/-CB2-/- mice have lower viral burden in the lung [120], the lung tissue damage and airway injury was more severe when compared to WT mice. Excessive immune activation can result in lethality from influenza virus infection [372, 373], although in the present study, a sublethal inoculum of influenza virus was administered to avoid lethality to the murine host [118]. Taken together, the loss of a mechanism to negatively regulate immune function, as shown in CB1-/-CB2-/- mice, resulted in increased immunopathology to a pathogenic stimulus, in this case influenza.

Our results suggest that CB1 and/or CB2, although reducing the magnitude of the influenza-induced immune response, provide protection against excessive airway injury in the lung from exacerbated immunopathology. In humans, single nucleotide polymorphisms (SNPs), which can result in a loss of function, have been identified in CB1 and were associated with ulcerative colitis [319]. Similarly a SNP in CB2 was associated with an increased overall risk of autoimmune disease [318]. In autoimmune diseases such as Crohn's disease and experimental autoimmune encephalomyelitis, a murine multiple sclerosis model, increased maturation of DC was observed [374, 375]. Furthermore, it has been demonstrated that DC can induce and contribute to maintaining autoimmunity [376]. Taken together with the present studies, CB1 and/or CB2 might be considered host factors that attenuate immune responses, in this case to influenza, in order to reduce tissue damage. In humans, who, unlike laboratory animals, are routinely exposed to pathogenic bacteria, viruses, fungi and their PAMPs, DCs confer immunity by acting as accessory cells in the elicitation of effector cell responses (e.g., T cells). However, exacerbated and/or unwanted DC responses can occur through a reduction or absence of signaling through CB1 and/or CB2 for example as a result of a loss of function SNP.

Consequently, in the absence of regulation by the cannabinoid system, DC-elicited effector responses might contribute to the pathologies described above including autoimmune disease. Overall, these findings provide evidence that the enhanced immune phenotype in CB1-/-CB2-/- mice after influenza infection influences a cascade of events that exist, in part, due to altered basal immune function and functional exacerbation of select immune populations after immune stimulation. As demonstrated with NA cells *in vitro*, our results suggest that the lymphocyte compartment is not inherently responsible for exacerbated cytokine production, but is dependent on dysregulation of APC function as a result of extinguished cannabinoid receptor signaling. Collectively, these results indicate that the cannabinoid receptors play a fundamental role in attenuating inflammation and immune responses orchestrated by APC.

III. Role of the Cannabinoid Receptors 1 and 2 in Δ9-THC-mediated Suppression of Immune Responses to Influenza Virus Challenge

The studies characterizing and comparing the kinetics of the immune response to influenza in WT and CB1-/-CB2-/- mice demonstrated a critical involvement of CB1 and/or CB2 expressed on APC in reducing the magnitude of immune response. The investigation was then extended to characterize which APC populations *in vivo* were affected by the loss of CB1 and CB2 in the anti-influenza response and to determine the effect of Δ9-THC on the maturation and recruitment of APC into the lung. Since the discovery of cannabinoids and their receptors CB1 and CB2, their effects on the immune system in health and disease have remained elusive. In part, it has been difficult to determine which immune cell types are regulated by CB1 and CB2 signaling

and the ensuing consequences on cell function. In this study, an influenza model was used to stimulate the immune system and the inflammatory response 3 dpi was investigated after co-treatment with the non-selective CB1 and CB2 agonist, Δ9-THC, in WT and CB1-/-CB2-/- mice. Lower inflammatory responses were observed in WT as compared to CB1-/-CB2-/- mice with Δ9-THC treatment as evidenced by reduced cytokine secretion by CD4+ T cells and NK cells, lower percentages of APC populations in the lung, and decreased expression of genes associated with the immune response to influenza as previously demonstrated. Furthermore, Δ9-THC reduced T cell responses through modulation of DC function in WT but not in CB1-/-CB2-/- mice. Thus, although other targets for Δ9-THC exist, CB1 and/or CB2 expressed on cDC are important in mediating the immunomodulatory effects of Δ9-THC on effector cell populations involved in the immune response to influenza virus infection.

A) Contributions of CB1 and CB2 to Cannabinoid Modulation of APC

It has been observed that both CB1 and CB2 contribute to the modulation of DC function by Δ9-THC with the use of CB1- and CB2–specific antagonists [265] and that CB1 specifically diminishes K+ outward currents through Kv channels to suppress DC function [266]. Furthermore, suppression of DC function by Δ9-THC might, in part, be independent of the G-protein coupled receptors as evidenced by Δ9-THC-mediated suppression of maturation after in bmDC generated from CB1-/-CB2-/- mice. In support of this argument, pertussis toxin, which blocks the Gαi subunit necessary for CB1 and CB2 signaling [267, 268], did not completely abrogate the suppression of IL-12p40 production in bmDC [269]. Also selective antagonists to either CB1 and CB2 were not able to block the suppression of IL-12p40 by Δ9-THC [270]. This suggests that although CB1 and CB2 are involved in Δ9-THC modulation of DC, this is not the

exclusive mechanism. In the present study, Δ9-THC suppressed LPS-induced maturation of bmDC generated from CB1-/-CB2-/- mice, however; this suppression of maturation did not alter elicitation of T cell responses in co-cultures. Collectively, these results suggest that the maturation level, although suppressed, was sufficient in CB1-/-CB2-/- bmDC to elicit T cell responses. Alternately, other factors aside from the maturation markers assessed on bmDC might be involved in the suppression of maturation independently of CB1 and CB2. In support of this argument, R848 did not induce maturation to the same extent as LPS in bmDC, yet R848-treated DC were able to elicit T cells in co-cultures. Moreover, cytokine production in lymphocytes was enhanced *in vivo* as a result of Δ9-THC treatment in CB1-/-CB2-/- further supporting the view that targets, in addition to CB1 and CB2, other receptors might contribute to the Δ9-THC-mediated effects on DC. Thus although CB1 and/or CB2 appear to play critical roles in the Δ9-THC-mediated suppression of DC maturation and impairment of their ability to elicit T cell responses, additional mechanisms are might be involved in the immue modulation of DC function by Δ9-THC.

Among the APC populations in the lung, greater sensitivity to Δ9-THC-mediated immunosuppression was observed in the reduced presence of cDC, pDC, and AM in the lung after influenza infection. Migration of these three APC populations is regulated by distinct chemokines and receptors [377-379]. Δ9-THC reduced steady-state mRNA levels of chemokine receptors but not of chemokines. Taken together with the reduced percentages of APC populations in the lung, the broad mRNA level changes might be a reflective of a reduced influx of immune cells into the lung.

B) Putative Mechanisms by Which Δ9-THC Might Alter APC Function

While not as clear in AM, it has been demonstrated that cDC and pDC migration is influenced by their maturation status [329-331, 378]. Aside from MHC II in pDC no overt changes in the maturation status of the DC subsets was observed as a result of Δ9-THC treatment. However, broad transcriptional suppression of influenza-induced genes associated with immune response by Δ9-THC was observed in RNA isolated from the lung. Especially proinflammatory cytokines are involved in the maturation of cDC, pDC, and AM and their attraction to the site of infection [380-383]. Thus, indirect effects of reduced tissue inflammation may be involved in the suppression of maturation and maturation-induced migration. *In vitro,* Δ9-THC treatment impaired the LPS-induced maturation of DC as evidenced by lower expression of CD86, for example. Taken together, it is plausible that reduced maturation of DC by Δ9-THC, as demonstrated *in vitro*, decreases the ability of DC to migrate *in vivo*, which could interfere with trafficking to or persisting in the lung, thus enriching for a DC population with a mature phenotype. Furthermore, leukocyte trafficking, to a large extent, is dependent on chemokine receptors which are part of the G-protein coupled receptor (GPCR) family [384]. It has been shown that Δ9-THC and other cannabinoids alter the migration of multiple immune cells. Although it is not clear under which conditions migration is augmented or impaired; however, involvement of phosphatidylinositol 3-kinase/Akt and ERK1/2 has been demonstrated [294, 385-388]. Moreover, crosstalk between GPCRs exists [389], and a role for Gαi has been suggested in CB2 signaling which in turn inhibited signaling by other GPCRs [390]. Thus, results in this investigation can be explained, in part, by a mechanism involving CB2-mediated diminution of signaling through chemokine receptors. However, the intracellular signaling pathways contributing to altered leukocyte trafficking by Δ9-THC remain to be elucidated and the putative involvement of CB2 at this time is therefore speculative.

In contrast, it has been proposed that ligation of CB1 and CB2 induces apoptosis in DC, which could account for the lower percentage of DC observed in the lung [265]. In the present study, differences in bmDC viability *in vitro* using the LIVE/DEAD stain were not observed (data not shown). It is noteworthy that there were several differences in the experimental conditions in our study and that used by Do *et. al.*. Differences include higher LPS concentrations (10 μg/mL), extended preincubation periods with Δ9-THC (2 hr preincubation), sequential instead of concurrent treatment with LPS and Δ9-THC, and use of serum free media [265], which by itself is a stressor to immune cells in culture [391].

It was previously observed that the synthetic CB1 and CB2 agonist, *R*(+)WIN55,212-2, suppressed proinflammatory cytokine production in a multiple sclerosis model [392] and other studies suggest that the mechanism by which cannabinoids suppress proinflammatory cytokine production might involve IRF3-dependent upregulation of IFNβ [393]. Both IRF3 activation and IFNβ and other type I interferon production are activated by influenza's innate signaling in separate pathways from pro-inflammatory cytokines [394-396]. While there were beneficial effects observed with *R*(+)WIN55,212-2 in an experimental autoimmune encephalomyelitis model, which mimics some aspects of multiple sclerosis by upregulation of type I IFN production, one could hypothesize that Δ9-THC produces similar events in the influenza model [393]. Although low in number, pDC greatly contribute to the inflammatory milieu and are involved in the early phases of the anti-influenza immune response and are known to produce large amounts of type I IFNs [397-399]. Indeed, IFNβ has been shown to suppress Th1 differentiation by DC when present prior to maturation [400, 401]. Furthermore, DC migration was reduced by IFNβ [402] and the differentiation of naïve CD4+ T cells into the Th1 phenotype was also suppressed [403, 404]. However, these studies suggest that pDC-produced IFNβ may be

beneficial in ameliorating inflammation. In this influenza model, pDC are one of the main producers of type I IFNs including IFNβ, and Δ9-THC suppressed their presence and maturation. Furthemore, steady-state mRNA levels of type 1 IFNs were not enhanced by Δ9-THC treatment (Figure 36). Thus it is unlikely that in the influenza model IFNβ plays a role in the reduction in proinflammatory cytokines.

C) Concluding Remarks on the Role of CB1 and CB2 on APC

Collectively, the present studies demonstrate that Δ9-THC suppressed the host immune response against influenza virus challenge. Furthermore, after influenza infection, cDC, pDC, and AM are sensitive to Δ9-THC-mediated immune suppression, which requires the presence of CB1 and/or CB2. *In vitro* it was demonstrated that cDC-like bmDC play a critical role in suppressing T cell responses after treatment with Δ9-THC, which was also found to be dependent on CB1 and/or CB2. In conclusion, signaling through CB1 and CB2 might present an avenue for therapeutic intervention in order to specifically attenuate APC function.

I. Significance, Relevance, and Future Studies

The work presented in this dissertation makes a significant contribution to our understanding of the role of CB1 and CB2 in the immune system. By characterizing the immune response in WT and CB1-/-CB2-/- mice after infection with the pathogen influenza *in vivo* and using immune cells isolated from these mice *in vitro*, the role of CB1 and CB2 in regulating immune function was further elucidated.

Figure 37 Δ9-THC Does Not Alter Steady-state Whole Lung mRNA Levels of Type I IFNs. RNA was isolated from whole lungs and reverse transcribed into cDNA (n=5). IFNα and IFNβ mRNA levels were assessed with respect to the WT-CO-SAL samples, set to 1 fold. Factorial ANOVA was used to analyze fold mRNA change: +++ ($p \leq 0.001$) difference between NA (SAL) and PR8; ### ($p \leq 0.001$) difference between WT and CB1-/-CB2-/-.

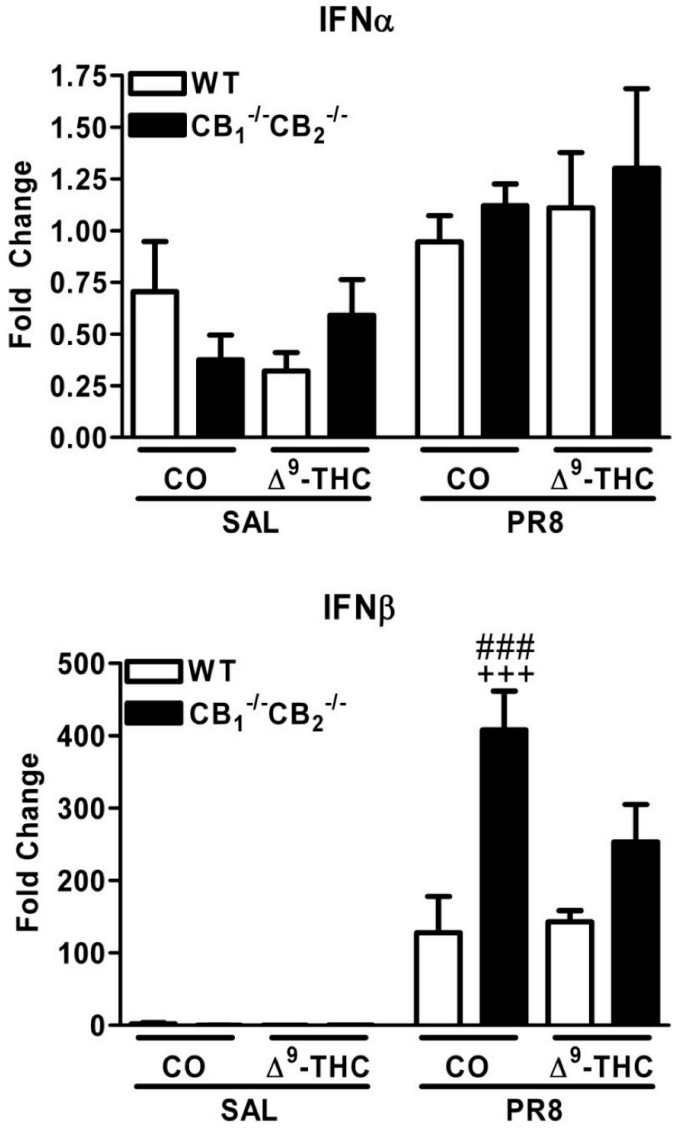


Figure 37. Δ9-THC Does Not Alter Steady-state Whole Lung mRNA Levels of Type I IFNs.

Since CD8+ T cells express relatively little transcript for CB1 and CB2, it was not surprising that the effects mediated by Δ9-THC were independent of CB1 and/or CB2, since non-CB1/CB2-mediated effects on T cells had been previously reported by this laboratory [231, 234, 284]. In addition, CD8+ T cells from CB1-/-CB2-/- mice upregulated the surface expression of CD69 in the presence of Δ9-THC alone, while CD8+ T cells from WT did not. It had been argued that there might be compensatory mechanisms in CB1-/-CB2-/- mice as evidenced by upregulation of the non-deleted portion of the CB2 gene transcript [334]. It is unknown what receptor(s), if any, caused the Δ9-THC-mediated increase in CD69 expression in the absence of stimulation. Studies identifying this unknown target and defining the transcriptional regulation of CB1 and CB2 will provide further information on the mechanisms by which cannabinoids alter immune function. Furthermore, the apparent increase in CD69 by Δ9-THC was inversely correlated with the magnitude of CTL activity, suggesting that signaling pathways leading to an increase in CD69 expression and suppression of CTL function might share a common mechanism of regulation.

A second novel finding is that the deletion of CB1 and CB2 mainly affects APC function, suggesting that the modulation of T lymphocyte function by cannabinoids is independent of CB1 and CB2 as described for CTL above. Although there were differences in the percent of cytokine-producing lymphocytes i*n vivo*, *in vitro* studies showed no difference in T cell cytokine production between CB1-/-CB2-/- and WT mice, suggesting involvement of APC populations, which elicit T cells. As a logical consequence, subsequent studies focused on APC populations such as bmDC and AM. In the presence and absence of TLR stimulation the magnitude of maturation in bmDC and AM CB1-/-CB2-/- mice exceeded the maturation observed in cells from WT mice under the same conditions. In addition, the ability of bmDC to elicit T cells was greater in bmDC from CB1-/-CB2-/- and did not require prior stimulation with a TLR ligand.

These results lead to the conclusion that not only the magnitude of response, but also the maintenance of an unresponsive state of APC is governed by CB1 and/or CB2.

A third unique contribution of these studies is the finding of dysregulated DC responses in the absence of immune stimulation in CB1-/-CB2-/- compared to WT mice after GMCSF expansion of DC precursors *in vitro*. This was evidenced by greater levels of maturation markers (e.g. MHC II) and the ability to elicit antigen-specific T cell responses. These results suggest CB1 and/or CB2 play an important role in maintaining DC homeostasis, potentially by providing a mechanism for self-regulation by endocannabinoids generated during the *in vitro* culture. The endocannabinoids likely signal in an autocrine or paracrine manner since GMCSF-expanded DC cultures are over 95% pure after 9 days. Cannabinoids might be one of many ways to fine tune DC homeostasis and provide negative feedback to reduce "leaky" transcription [405] of proinflammatory transcripts and other genes involved in DC homeostasis. While this idea is speculative, reduction of "leaky" transcription is thought to occur as a result of cAMP response element binding protein 2 activity, for example [406]. Furthermore, in the absence of negative feedback provided by CB1 and/or CB2 in DC, autocrine-amplifying loops might result in "leaky" transcription [405] leading to upregulation of CD86 and MHC II and expression of cytokines, eventually presenting itself as a mature phenotype. Although speculative, these autocrine-amplifying loops of soluble mediator release have been observed for complement protein 3 in keratinocytes before [407].

Despite these novel findings of DC immune regulation by CB1 and/or CB2, there is a data gap in the role of each receptor and its individual contribution to the phenotype observed. Furthermore, it is unknown whether there are existing differences in GMCSF receptor (GMCSFR) expression or the differences in the responses elicited as a result of GMCSFR

ligation between WT and CB1-/-CB2-/- DC precursors. In addition, there might be differences in the number and/or development of the DC precursors in the bone marrow of WT and CB1-/-CB2-/- mice. Although these studies are beyond the scope of this dissertation, another researcher might find interest in pursuing these experiments.

Among the APC populations, pDC, and AM were most sensitive to the loss of CB1 and CB2. Both cell types have a similar integrin profile defined by CD11c+CD11b- and consistently bmDC generated from CB1-/-CB2-/- showed greater CD11c levels than bmDC generated from WT (data not shown). Loss of integrin regulation including CD11c was implicated in adverse immune outcomes in mice, suggesting that loss of CB1 and CB2 results in a multi-faceted dysregulation in the phenotype of APC, most drastically in DC subpopulations including cDC and pDC [408]. Potentially, antagonists to CB1 and/or CB2 could be used to temporarily induce the generation and exacerbate the maturation of pDC and cDC populationsfor a therapeutic benefit. For example, in HIV positive patients an increase in the number or maturation of cDC populations might allow for the elicitation of HIV-specific CTL populations without the requirement of CD4+ T cell help. Moreover, correcting the loss of pDC populations as observed during HIV infection with antagonists to CB1 and/or CB2 might slow or halt disease progression in HIV positive patients as regulating the balance of pDC has been implicated in the successful control of HIV infection [409].

Since the dysregulation of APC immune function in CB1-/-CB2-/- compared to WT was found *in vivo* and *in vitro*, it is less likely that the cells other than those of the immune system significantly contribute to the immune exacerbation to influenza infection *in vivo*. However, to test this, bone-marrow chimeras using adoptive transfer of CB1-/-CB2-/- bone marrow into WT recipients and WT bone marrow into WT recipients would have to be generated. Differences in

immune reactivity between CB1-/-CB2-/- and WT bone marrow transfers would confirm that the immune exacerbation can be attributed to the immune cells of CB1-/-CB2-/- mice. Furthermore, using adoptive transfers of CB1-/-CB2-/- bmDC into WT donors and WT bmDC into WT donors in a T cell activation or influenza challenge model would determine whether these cDC-like cells are sufficient in inducing exacerbated immune responses. The described experiments are technically challenging and the adoptive transfer of bmDC was attempted, however, the data were equivocal as little to no T cell activation was observed and are not presented in this dissertation.

The prevalence of marijuana consumption in industrialized countries is a reason for concern regarding the findings presented in this dissertation. As a result of globalization the spread of influenza virus is very rapid and poses difficulties to national governments in preparing for a pandemic or epidemic. In years of accurate antigen prediction, annual vaccination to influenza virus is the most effective method in halting viral spread and limiting its mortality. The concept of herd immunity describes the idea that the vaccination of a significant portion of the population prevents viral spread within the entire population (including non-vaccinated), because vaccinated individuals do not transmit the pathogen. In a murine influenza challenge model the immune response magnitude was drastically reduced by Δ9-THC treatment. These results suggest that vaccinations might be rendered less effective in marijuana users and could thereby reduce herd immunity to influenza hence leading to reduced protection of a greater population from a viral pandemic or epidemic [410, 411].

The dysregulation of DC responses as a result of the loss of CB1 and CB2 are particularly alarming considering the important role that DC play in the orchestration of immune responses. DC can induce and maintain tolerance, as well as contribute to the elicitation of effector immune

cells. While DC elicited immune responses are important in producing successful vaccinations and protection from pathogens, unwanted DC immune responses can lead to autoimmune disease [96, 376, 412-414]. Due to the identification of SNPs in CB1 and CB2 in humans, the potential for reduced ability to regulate the magnitude of immune response by endocannabinoids exists potentially resulting in an exacerbated immune phenotype and manifesting itself as autoimmune disease. Given the association of SNPs in CB1 and CB2 with autoimmune disorders it is likely that DC can play a role in the development of the phenotypes of autoimmune disease [318, 319]. The system and cells isolated from humans with SNPs in either or both CB1 and CB2 need to be further characterized to determine a human correlate between the findings presented in this dissertation and human health. The concepts described in this section are summarized in Figure 37.

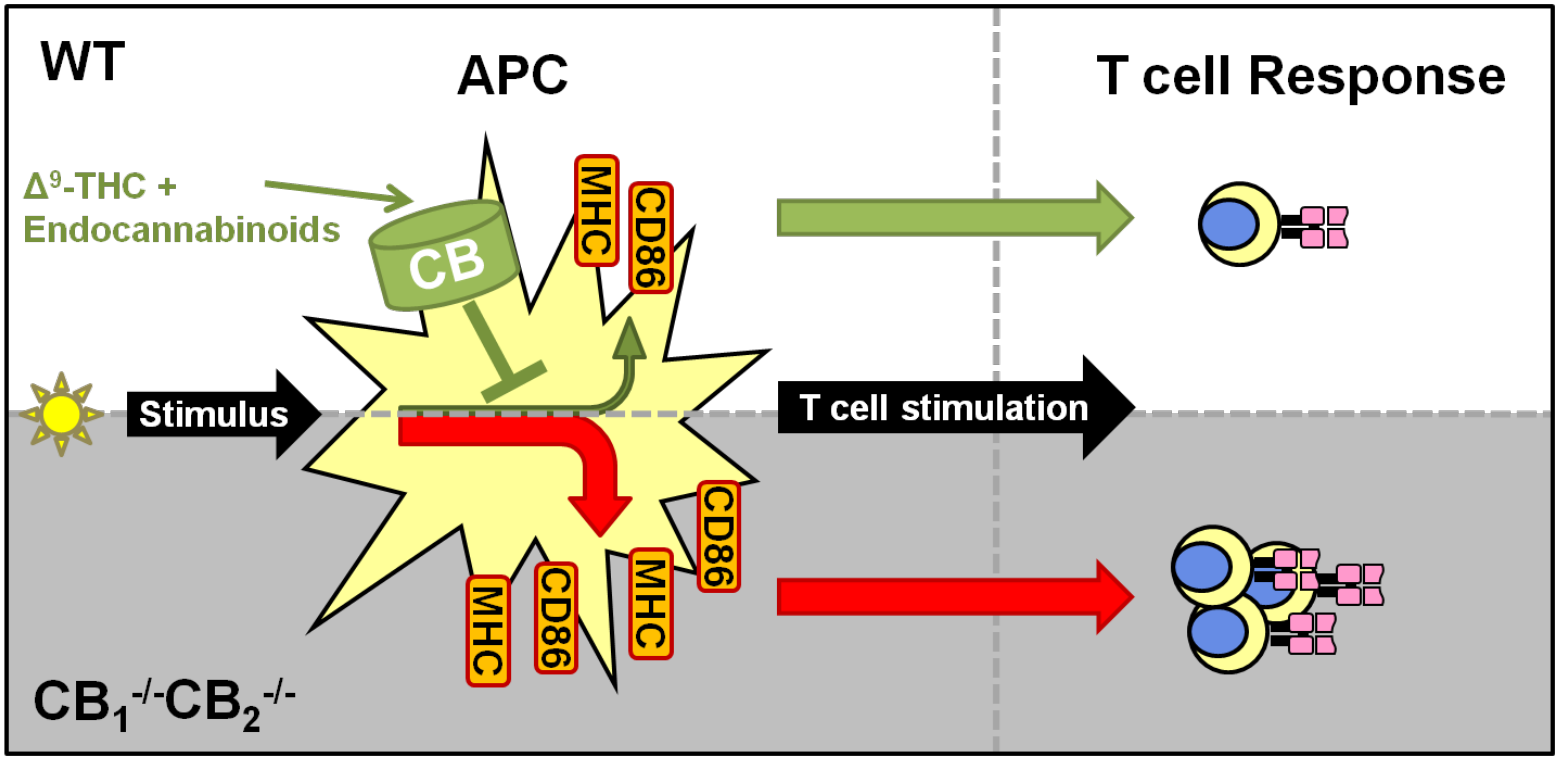


Figure 38. DC Cannabinoid Immune Modulation Model. For interpretation of the references to *color* in this and all other *figures*, the reader is referred to the electronic version of this *dissertation*.

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