

EFFECTS OF CANNABINOID RECEPTOR DELETION ON BONE MARROW-DERIVED
DENDRITIC CELL SUBTYPE DEVELOPMENT, MATURATION AND ANTIGEN
LOADING ON MHC CLASS I

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment for the requirements
for the degree of

Comparative Medicine and Integrative Biology- Master of Science

2016

ABSTRACT

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Cannabis is the most frequently consumed illicit drug in the world. Mammals express at least two types of cannabinoid receptors (CBRs), cannabinoid receptor 1 (CB₁) and cannabinoid receptor 2 (CB₂). Previous studies demonstrated that peptide pulsed bone marrow DCs (BMDCs) from CB₁^{-/-}CB₂^{-/-} mice induced a CD8⁺ T cell response in the absence lipopolysaccharide (LPS)-induced DC maturation. DCs are professional antigen presenting cells indispensable in linking the innate and adaptive immune response. Each distinct DC subset has its unique set of surface markers and capabilities to respond to environmental stimuli and process antigen. The objective of the present study is to characterize the role of CBRs on the development and function of DC subsets from mouse bone marrow (BM). Our results demonstrate CB₁^{-/-}CB₂^{-/-} mice have a higher percent of BMDCs in freshly isolated BM and after 24 hours in culture in comparison to WT. Freshly isolated BM cells isolated from CB₁^{-/-}CB₂^{-/-} mice elicited a CD8⁺ T cell response in the absence of LPS stimulation. Interestingly, there were no differences in MHC I or antigen-bound MHC I complexes on the surface of DCs that can account for this exacerbated activity. CD83, a DC marker implicated in maturation and stimulation of T cells, had a lower expression in DCs from CB₁^{-/-}CB₂^{-/-} mice. Taken together the results from this investigation suggest that CBRs are involved in DC bone marrow development and maturation.

TABLE OF CONTENTS

LIST OF FIGURES.....	v
KEY TO ABBREVIATIONS.....	vii
CHAPTER 1: LITERATURE REVIEW.....	1
1.1 Purpose of this research.....	1
1.2 Dendritic cell origin and subpopulations.....	2
1.2.1 Hematopoietic origin of DC.....	2
1.3 Transcriptional regulation of dendritic cell development.....	4
1.3.1 Cytokine regulation of DC development.....	4
1.3.2 Transcriptional regulation at early stage DC development.....	5
1.3.3 Transcriptional regulation at late stage DC development.....	6
1.4 Cannabinoids, cannabinoid receptors, their signaling and function in the immune system.....	7
1.4.1 Endogenous cannabinoids.....	7
1.4.2 Phytocannabinoids.....	8
1.4.3 Synthetic cannabinoids.....	8
1.4.4 Cannabinoid receptors.....	12
1.4.5 Cannabinoid receptor signaling.....	12
1.4.6 Immunomodulatory effects of cannabinoids on humoral and cell mediated immunity: contributions from our lab.....	14
1.5 Dendritic cell antigen presentation during pathogen infection.....	15
1.5.1 Activation and maturation of DCs.....	16
1.5.2 MHC class I antigen presentation.....	17
1.5.3 Antigen cross-presentation by MHC I.....	18
1.5.4 MHC class II antigen presentation.....	19
1.5.5 Rationale.....	19
CHAPTER 2: MATERIAL AND METHODS.....	21
2.1 Mice.....	21
2.2 Bone marrow cell isolation and culture.....	21
2.3 Surface antibody staining and flow cytometry.....	22
2.4 Co-culture of bone marrow cells and OT-I cells <i>in vitro</i>	23
2.5 Intracellular antibody labeling for flow cytometry.....	23
2.6 Statistical analysis.....	24
CHAPTER 3: EXPERIMENTAL RESULTS.....	25
3.1 Naïve bmDCs from CB ₁ ^{-/-} CB ₂ ^{-/-} mice elicit a CD8 ⁺ T cell response.....	25
3.2 Characterization of dendritic cell precursors and subpopulations in mouse bone marrow cells.....	25
3.2.1 Identification of CDP and MDP in bone marrow cells.....	25
3.2.2 Identification of DC subpopulations and characterization of MHC I, MHC II	

and CD86 expression.....	29
3.2.3 Identification of B cell and macrophages in bone marrow cells and their CD83 and CD86 expression.....	37
CHAPTER 4: DISCUSSION.....	48
4.1 Characterization of DC subpopulations in murine bone marrow cells and their ability to induce a CD8⁺ T cell response.....	48
4.2 Expression of MHC and co-stimulatory molecules as well as surface antigen loading capabilities on DC subpopulations.....	52
BIBLIOGRAPHY.....	57

LIST OF FIGURES

Figure 1.	The structure of four phytocannabinoids Δ^9 -THC, cannabindiol, Δ^8 -THC and cannabinol.....	10
Figure 2.	Chemical structures of synthetic cannabinoid receptor agonist.....	11
Figure 3.	Naïve bone marrow cells from $CB_1^{-/-} CB_2^{-/-}$ mice elicited a $CD8^+$ T cell response.....	27
Figure 4.	Percent of lineage positive, lineage negative and precursor cells in mouse bone marrow cells.....	28
Figure 5.	Composition of DC subpopulations in freshly isolated bone marrow cells and 24 hours in culture.....	32
Figure 6.	MHC I expression in DC subpopulations of freshly isolated bone marrow cells and 24 hours in culture.....	33
Figure 7.	MHC II expression in DC subpopulations of freshly isolated bone marrow cells and 24 hours in culture.....	34
Figure 8.	CD86 expression in DC subpopulations of freshly isolated bone marrow cells and 24 hours in culture.....	35
Figure 9.	Percent of DC subpopulations that are $MHC II^+ CD86^+$ in freshly isolated bone marrow cells and 24 hours in culture.....	36
Figure 10.	Percent composition of DCs , B cells, macrophages and $CD11b^+$ cells gated within $MHC I^+$ bone marrow cells.....	39
Figure 11.	Percent of mature DCs in bone marrow cells.....	40
Figure 12.	Percent $CD83^+$ expression on DCs , B cells, macrophages and $CD11b^+$ cells gated within $MHC I^+$ bone marrow cells.....	41
Figure 13.	$CD83$ MFI expression of DCs , B cells, macrophages and $CD11b^+$ cells gated within $MHC I^+$ bone marrow cells.....	42
Figure 14.	Percent $CD86$ expression of DCs , B cells, macrophages and $CD11b^+$ cells gated within $MHC I^+$ bone marrow cells.....	43

Figure 15.	CD86 MFI expression of DCs, B cells, macrophages and CD11b ⁺ cells gated within MHC I ⁺ bone marrow cells.....	44
Figure 16.	Expression of MHC I-SIINFEKL in bone marrow cells.....	46
Figure 17.	Peptide Internalization is not necessary for antigen loading on the surface of DCs.....	47

KEY TO ABBREVIATIONS

2-AG	2-arachidonoylglycerol
ACEA	arachidonoyl-2-chloroethylamide
ACPA	arachidonoyl-ciclopropylamide
AEA	arachidonylethanolamide or anandamide
Ag	antigen
AM356	R-methanandamide
APC	antigen presenting cell
bmDCs	bone marrow dendritic cells
cAMP	cyclic adenosine monophosphate
CB ₁	cannabinoid receptor 1
CB ₂	cannabinoid receptor 2
CBD	cannabidiol
CBR	cannabinoid receptor
CDP	common DC progenitors
CLIP	class II-associated invariant chain peptide
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
CREB	cyclic AMP response-element binding
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ER	endoplasmic reticulum

ERAP	endoplasmic reticulum aminopeptidases
Ets	E26 transformation specific
FLT3L	FMS-related tyrosine kinase 3 ligand
Gfi1	growth factor independent-1
GM-CSF	granulocyte and macrophage colony stimulating factor
HSC	hematopoietic stem cells
Id2	DNA binding protein 2
IFN γ	interferon γ
Ii	invariant chain
IL-23	interleukin 23
IL-6	interleukin 6
Irf7	interferon regulatory factor 7
Irf8	interferon regulatory factor 8
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
M-CSF	macrophage stimulating-colony factor
MDP	macrophage-dendritic cell progenitors
MHC	major histocompatibility complex
MHC I	major histocompatibility complex class I
MHCII	major histocompatibility complex class II
MIIC	MHC II- containing compartments
NADA	arachidonoyl dopamine
noladin ether	2-arachidonylglyceryl ether

PAMPs	pathogen associated molecular patterns
pDC	plasmacytoid dendritic cell
PI3k	phosphatidylinositol 3-kinase
PKA	protein kinase A
TAP	transporter associated with antigen processing
TCR	T cell receptor
THC	Delta-9-tetrahydrocannabinol
TLR4	toll like receptor 4
TLR9	toll like receptor 9
TNF α	tumor necrosis factor α
TRPV1	vanilloid receptor

CHAPTER 1: LITERATURE REVIEW

1.1 Purpose of this research

Cannabinoids are a group of highly lipophilic compounds derived from the plant *cannabis sativa* that produce a wide variety of effects in mammals. The receptors that cannabinoid compounds bind, termed cannabinoid receptors (CBRs), are present in almost all nucleated cells. Delta-9-tetrahydrocannabinol (THC) is the most extensively characterized ligand of CBRs and is also the main psychoactive constituent in the marijuana plant {1}. THC alters many different aspects of the immune response, including cell activation, proliferation and cytokine production by leukocytes. In some cases THC can act as an enhancer or repressor of immune responses, depending on the dose and type of immune challenge. Multiple findings, including from our lab highlight the regulatory effects of cannabinoids on the cell-mediated (T cell-dependent) and antibody-mediated (B cell-dependent) immune response. Deletion of CBRs in a mouse model has demonstrated an exacerbated immune response upon influenza challenge. The underlying reason for the heightened immune response in $CB_1^{-/-} CB_2^{-/-}$ mice is not well understood, but is thought, in part, to be the result of the absence of immune regulation by CBRs. This dissertation aims to test the hypothesis that bone marrow DC from $CB_1^{-/-} CB_2^{-/-}$ mice elicit an antigen specific $CD8^+$ T cell response in the absence of lipopolysaccharide (LPS)-induced maturation signal through (1) increased production of DCs, (2) co-stimulatory capabilities and (3) antigen surface loading.

1.2 Dendritic cell origin and subpopulations

The adaptive immune response is activated when an infection bypasses the body's first line of defense, the innate immune system. Key to the activation of the adaptive immune

response are dendritic cells (DCs) that are activated as part of the innate immune response and direct the primary cell-mediated immune response. DCs are a heterogeneous population of sentinels called antigen presenting cells (APC) that, in an immature state, are constantly sampling their surrounding environment in search for foreign antigens. DCs can reside in many different tissues throughout the body or circulate in the blood and respond to cues in the environment through pathogen associated molecular patterns (PAMPs). PAMPs produce changes in DCs that increase their ability to migrate to local lymph nodes and more effectively capture, process and present antigen in the context of major histocompatibility complex (MHC) to naïve T cells and direct their differentiation into several subsets {2}. DCs can arise from myeloid and lymphoid precursors in the bone marrow as well as from circulating monocytes {3}.

1.2.1 Hematopoietic origin of DC

During hematopoiesis, hematopoietic stem cells (HSC) give rise to common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), with monocytes, macrophages, megakaryocytes, granulocytes and erythrocytes originating from CMP and T cells, B cells and natural killer cells, originating from CLP. Both CLP and CMP give rise to DCs as indicated by studies where irradiated mice were injected with CLP and CMP {4}. Under steady state conditions DCs are derived from the myeloid lineage {5}. From CMP arise the macrophage-dendritic cell progenitors (MDP) which are the common precursor of macrophages, monocytes and DCs. MDPs are classified as $\text{Lin}^- \text{CX3CR1}^+ \text{CD11b}^- \text{CD115}^+ \text{cKit}^+ \text{CD135}^+$ {6}. MDP differentiate into common DC progenitors (CDP) that exclusively give rise to DCs. MDP and CDP reside in the bone marrow. Whereas pre DCs, immediate precursors to DCs derived from CDPs, can circulate in the blood. Pre DCs can migrate to lymph nodes through the blood, proliferate and differentiate into DCs. Although MDP are known to be the common precursors of

DCs from bone marrow, studies have shown that under certain conditions DCs originate from CLPs. Activation of toll like receptor 9 (TLR9) with CpG DNA and TLR4 signaling via lipopolysaccharide (LPS) leads to the generation of DCs from CLPs {7,8}.

All DCs express the integrin CD11c, routinely used in conjunction with other integrins (e.g., CD11b, CD103) and markers in their identification {9}. DCs are classified into two broad categories, conventional DC and non-conventional DCs. Conventional DCs are comprised of DC subsets derived from CDP and pre-DCs and can be further divided into migratory and lymphoid DCs. Migratory DCs reside in different tissues and have the capability to migrate to local lymph nodes upon antigen recognition. Among them are CD11b⁺ DC (CD11c⁺ CD11b⁺) and CD103⁺ DCs (CD11c⁺CD11b^{-/+} CD103⁺) that have been identified in the lung, skin and intestine. As the name implies, lymphoid DCs are found in lymphoid organs such as lymph nodes, spleen and thymus and have been further sub-divided depending on the varied expression of CD4 and CD8 {3}. Non-conventional DCs include plasmacytoid DCs (pDCs), which, although derived from CDP, are unique in their ability to secrete high amounts of IFN α . Additionally, there are several DC subsets derived from monocytes.

Monocytes are leukocytes known to play a role in inflammation. In human and mouse, monocytes comprise approximately 4 to 10% of nucleated cells. Monocytes have been demonstrated to be particularly dynamic in their ability to differentiate into several DC subsets under steady state and inflammatory conditions {10}. They are subdivided by the expression of the monocyte marker Ly6C in combination with other surface markers {11}. Ly6C^{low} monocytes have been shown *in vitro* to differentiate into DCs in the presence of granulocyte and macrophage colony stimulating factor (GM-CSF) and IL-4 {12}.

1.3 Transcriptional regulation of dendritic cell development

Identification of DC populations is possible by the proteins they express on their cell surface. The complex subpopulations comprising DC's are defined by their origin of development and tissue (lymphoid or non-lymphoid) of residence. Differentiation of DCs from hematopoietic progenitors is regulated by a myriad of transcription factors and soluble regulatory factors termed cytokines. Of special importance are three key cytokines that regulate DC development: FMS-related tyrosine kinase 3 ligand (FLT3L), macrophage stimulating-colony factor (M-CSF) and GM-CSF. For an overview of the developmental pathway and its regulating factors in each stage, Belz and Nutt elegantly summarized the complex pathway in their review {13}.

1.3.1 Cytokine regulation of DC development

FLT3L, M-CSF and GM-CSF control the initial lineage ramifications of DCs from hematopoietic progenitors. There are two well-established *in vitro* culture systems using FLT3L {14, 18} or GM-CSF {18} that have provided us with insight on the extrinsic effect of factors in DC transcriptional network. Bone marrow precursors cultured in the presence of FLT3L lead to the production of pDCs and lymphoid tissue resident conventional DCs {14, 16}. In accordance with the *in vitro* culture models, mice with FLT3L knocked out display a reduced number of lymphoid conventional DCs and pDCs{17,18}. Mice deficient in STAT3, a signaling molecule activated downstream of FLT3, displayed deficiency in lymphoid conventional DCs and pDCs and was shown to be essential for FLT3L-induced differentiation {19}. Later studies by Ginhoux and colleagues, using FLT3L^{-/-} mice revealed the importance of FLT3 in other DC populations, including migratory DCs, pre-DCs and CD103⁺ DCs while development of LC and CD11b⁺ DCs is predominantly independent of FLT3L {20}.

The second method of producing DCs *in vitro*, culturing bone marrow precursors with GM-CSF, is the most commonly used approach to generate DCs. Monocytes have been demonstrated to develop into DCs in this *in vitro* culture system with the addition of IL-4 {14}. Despite the broad use to produce large numbers of DCs *in vitro*, GM-CSF is not essential for differentiation in steady state. Studies using GM-CSF receptor knockout mice demonstrated almost no reduction in DC numbers {21}. On the other hand, recent studies indicated GM-CSF to be key for normal counts of CD103⁺ CD11b⁺ DCs in the intestine {22, 23}. Furthermore, GM-CSF may help the survival and activations of macrophages, as well as DC maturation {23}. Under inflammatory conditions, colony-stimulating factors (GM-CSF, M-CSF) can mirror their *in vitro* effects. M-CSF is a cytokine important in the survival, proliferation and differentiation of macrophages and monocytes. M-CSF receptor knockout studies suggest an important role of this receptor in Langerhan cell (LC) development not observed in M-CSF null mice due to the existence of another M-CSF receptor ligand (IL-34) {24,25}.

1.3.2 Transcriptional regulation at early stage DC development

Three transcription factors have been described that specify DC lineage in early progenitors: Ikaros, Pu.1 and Growth factor independent-1 (Gif-1). Ikaros is a member of zinc finger DNA-binding proteins transcription factors. It is a critical transcription factor in early hematopoiesis and in priming the lymphoid transcriptional program {26, 27, 28}. In both Ikaros null and Ikaros dominant negative mice, expression of FLT3 is absent on hematopoietic progenitors {26}. DCs need FLT3 signaling by its ligand, FLT3L, in order to differentiate from hematopoietic progenitors. Thus, the findings reported suggest a role in immune cell populations, especially DCs.

Pu.1, a member of the ETS family of transcriptional regulators, regulates commitment of hematopoietic progenitors to DC lineage. Pu.1 is an appealing transcriptional regulator since it is expressed by all DC subsets and precursors {29, 30, 31}. Studies by Carotta used conditional gene deletion in hematopoietic progenitors to show that Pu.1 is essential for conventional DC and pDC development {30}. Pu.1^{-/-} mice have profound impairment in hematopoiesis and die either in late gestation or shortly after birth. Pu.1 regulates other important targets such as M-CSF receptor {32} and GM-CSF {33}.

The transcription factor Gfi-1 is a zinc finger repressor. Deletion of Gfi-1 has demonstrated a reduction of DC precursors, conventional DC and pDC subsets in bone marrow, spleen, and lymphoid organs {34}. Hematopoietic progenitors from Gfi-1 knockout mice treated with FLT3 or GM-CSF did not restore DC development suggesting a role of Gfi-1 in DC versus macrophage development {35}.

1.3.3 Transcriptional regulation at late stage DC development

Plasmacytoid DC development is highly dependent on the basic helix-loop-helix transcription factor (E protein) E2-2 expression. Once pDCs reach a mature phenotype, constant expression of E2-2 is required in order to maintain the mature pDC phenotype {36, 37}. E proteins belong to the basic helix-loop-helix transcription factor family {38, 39}. E2-2 directly binds to the promoter regions of pDC-expressed genes and controls the expression level of transcription factors such as *Spi-B*, interferon regulatory factor 8 (Irf8), and interferon regulatory factor 7 (Irf7), which are important for pDC development {37, 40}. *Spi-B* is an Ets (E26 transformation specific) family member closely related to Pu.1. *Spi-B* expression is restricted to pDCs within the DC compartment. Knockdown of *Spi-B* inhibited pDC generation from CD34⁺ precursor cells confirming an essential role for *Spi-B* in human pDC development {40,

41}. CD8 α^+ and CD8 α^- lineages have their own set of diverse transcription factors. Some of them like DNA binding protein 2 (Id2) and Irf8 are well studied and there is a fair understanding of their function, but other transcription factors lack in depth research {42, 43, 44, 45}.

1.4 Cannabinoids, cannabinoid receptors, their signaling and function in the immune system

Cannabinoids are a group of highly lipophilic chemicals derived from the plant, *Cannabis Sativa*, which produce a wide variety of effects in mammals. Their receptors, termed cannabinoid receptors, are present in almost all nucleated cells. Advancement in the understanding of cannabinoid signaling has been made possible by the identification of two main receptors and the synthesis of ligands selective for each receptor. Below the three main classification of Cannabinoid compounds are will be discussed: (1) endocannabinoids (2) phytocannabinoids and (3) synthetic cannabinoids

1.4.1 Endogenous cannabinoids

The two best studied endocannabinoids are arachidonylethanolamide or anandamide (AEA) and 2-arachidonoylglycerol (2-AG). AEA was isolated in 1992 and was the first isolated lipid to bind cannabinoid receptors with relative high affinity {49}. Anandamide behaves as a partial or full agonist to cannabinoid receptor 1 (CB₁) but can also bind cannabinoid receptor 2 (CB₂), and vanilloid (TRPV1) receptors {48}. 2-AG, isolated in 1995, acts as an agonist at both CB₁ and CB₂ receptors {50, 51}. Additional endocannabinoids are noladin ether (2-arachidonylglycerol ether) {52}, arachidonoyl dopamine (NADA) {53}, and virodhamine {54}. The biological relevance of these additional endocannabinoids is still unknown.

1.4.2 Phytocannabinoids

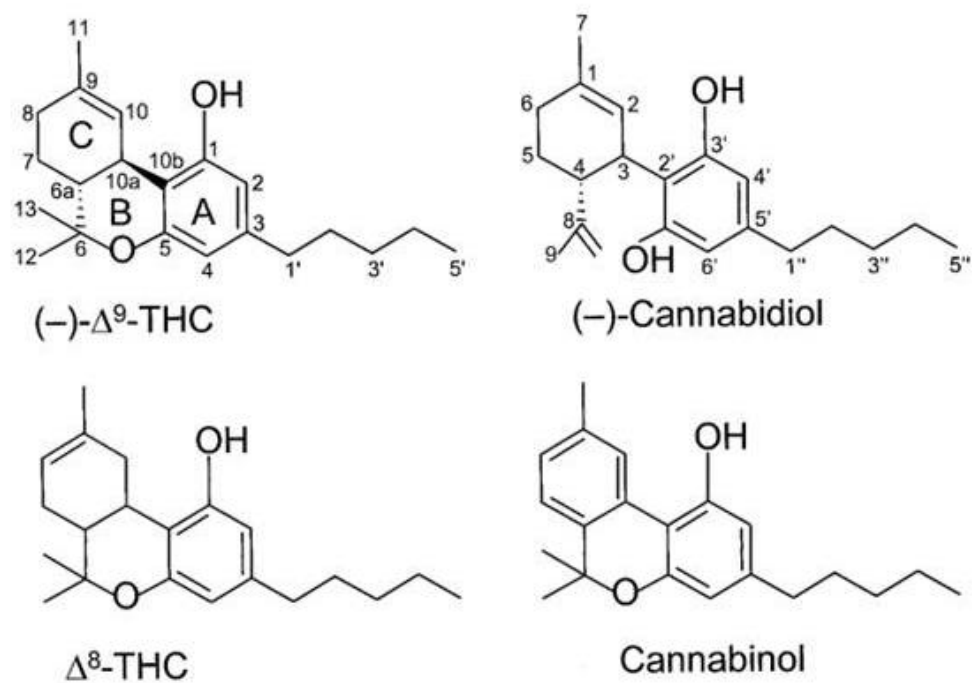
Phytocannabinoids are all cannabinoid compounds derived from *Cannabis Sativa*. Δ^9 -tetrahydrocannabinol (THC) is the most widely studied and characterized of these compounds. First identified in 1964 {55}, THC is the most prevalent cannabinoid in the plant and exhibits the most potent mental altering effects. THC acts as an agonist at both CB₁ and CB₂. Not only does THC produce typical neurological effects associated with the plant's abuse like behavioral effects, cognitive impairment and disruption of short-term memory, but also produces whole body effects like antinociception, hypothermia, hypomobility, and catalepsy. It also alters many different aspects of the immune response, including cell activation, proliferation and cytokine production by leukocytes (further discussed in a section below) {46, 47, 56}. A phytocannabinoid of interest, cannabidiol (CBD), intriguingly demonstrated cannabimimetic effects in leukocytes while possessing low affinity for CBRs {57}. Recent studies by Kaplan demonstrated that CBD suppressed IL-2 secretion in stimulated splenocytes from wild type and CB₁^{-/-} CB₂^{-/-} mice in a concentration dependent manner. In addition to its non-psychoactive effects independent of CB₁ and CB₂, CBD is one of the most abundant cannabinoids in the plant, making it very attractive for medical use {58}.

1.4.3 Synthetic cannabinoids

Synthetic cannabinoid's displays tremendous diversity with respect to their chemical structure. They are classified into: classical cannabinoids, nonclassical cannabinoids, aminoalkylindoles, and eicosanoids {59}.

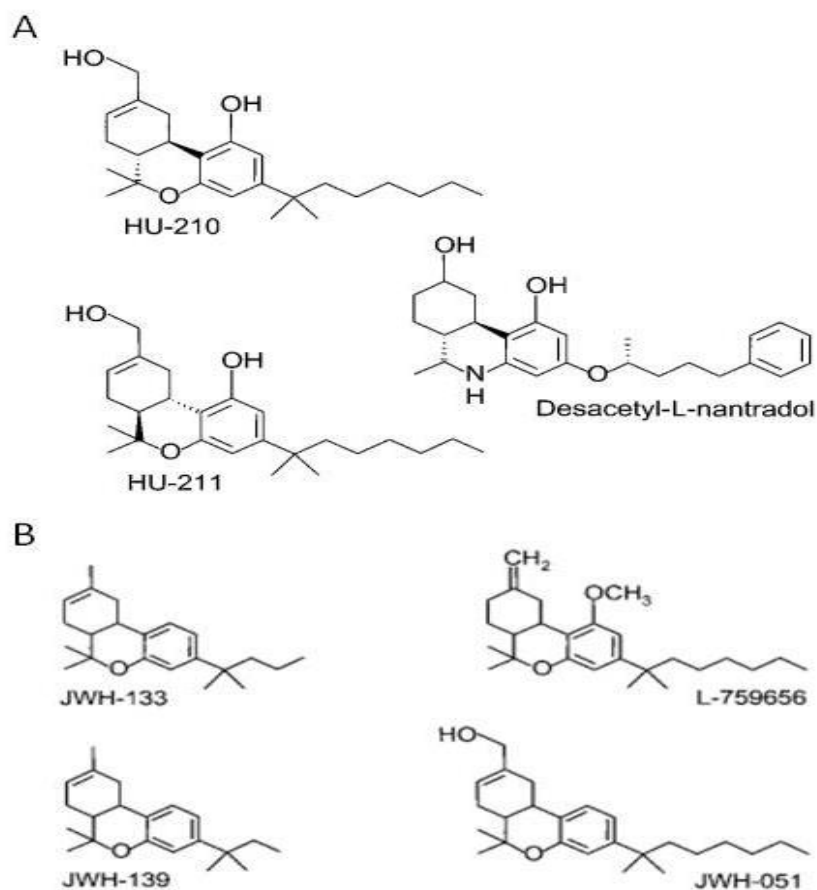
The classical cannabinoid has the tricyclic diterpene structure characteristic of THC and acts on both CBRs. Examples of this category are HU-210 and nabilone {60}. Classical cannabinoids selective for CB₂ receptors: include compounds such as JWH-133, JWH-51 and L-759656 {48}.

In nonclassical cannabinoid agonists, one of the rings of the tricyclic THC structure is open. Among them are CP55940, CP47497, CP55244, and the CB₂ selective agonist HU-308 {59, 61}. The aminoalkylindoles include compounds like WIN 55,212-2 {62}. Eicosanoids are predominantly derived from AEA, but they are metabolically more stable and selective. Examples of these analogs are R-methanandamide (AM356), arachidonoyl-2-chloroethylamide (ACEA) and arachidonoyl-cyclopropylamide (ACPA) {59}.



Adapted from Howlett et. al. Pharmacol Rev, 2002. **54**:161–202

Figure 1. The structure of four phytocannabinoids Δ^9 -THC, cannabidiol, Δ^8 -THC and cannabinol



Adapted from Howlett et. al. Pharmacol Rev, 2002. 54:161–202

Figure 2. Chemical structures of synthetic cannabinoid receptor agonist. A) The structures of the synthetic classical cannabinoid receptor agonists, HU-210 and desacetyl-L-nantradol, and of HU-211, the (+)-enantiomer of HU-210. B) The structures of the CB₂-selective cannabinoid receptor agonists JWH-133, JWH-139, L-759656 and JWH-051.

1.4.4 Cannabinoid receptors

Cannabinoid receptors (CBRs) are seven transmembrane domain G protein coupled receptors. CBRs contain an N-terminal extracellular domain that contains glycosylation sites, a C-terminal intracellular domain coupled to a G protein complex, and 7 hydrophobic transmembrane segments connected by alternating extracellular and intracellular loops {46,59}. The two major cannabinoid receptors: cannabinoid receptor 1 (CB₁) and cannabinoid receptor 2 (CB₂). The CB₁ receptor was first isolated from rat cerebral cortex in 1990 {63}. CB₁ receptors are expressed at high levels in the central nervous system and were later found also in a number of peripheral tissues, such as the cardiovascular and reproductive systems as well as the gastrointestinal tract {46}. On the other hand, CB₂ receptors are highly expressed on immune cells, but have also been detected on the central nervous system of mammals such as rodents, monkeys and humans under normal conditions {64}. CB₂ was discovered in the human promyelocytic leukemic cell line HL60 in 1993. The gene encoding the human CB₂ receptor shares 44% amino acid sequence identity with CB₁ throughout the total protein {65}.

CB₁ and CB₂ receptor ligands have been suggested to target non-CB₁ and non-CB₂ receptors. One such receptor worth noting is the human GPR55. Originally isolated as an orphan G protein coupled receptor, GPR55 is a member of the rhodopsin-like (class A) receptors {66,67}. This “orphan receptor” shares very little sequence identity with CB₁ (13.5%) and CB₂ (14.4%) receptors. Studies on the pharmacology of GPR55 are inconsistent but have determined that it preferentially binds to other isoforms of the G protein that are not G_{i/o} or G_s proteins {59}.

1.4.5 Cannabinoid receptor signaling

CBRs signal via G_i or G_o protein and lead to inhibition of adenylate cyclase, followed by reduction of cyclic adenosine monophosphate (cAMP) production and inhibition of cAMP-

dependent protein kinase A (PKA). Inhibition of PKA leads to a downregulation in the phosphorylation of cyclic AMP response-element binding (CREB) protein and gene transcription of important factors in cell growth, transformation and apoptosis {64, 68, 69, 70}. A CBR-mediated decrease in cAMP and PKA activity was confirmed when treatment with forskolin reversed the decrease in cAMP {68}. In addition to the cAMP pathway, CBRs can signal through other pathways. CB₁ mediates signaling through A-type K⁺ channels, inhibits N- and P/Q-type calcium currents, and hyperpolarizes of the membrane. This regulation of ion channels and hyperpolarization is thought to underlie the cannabinoid-induced inhibition of neurotransmitter release at axon terminals by causing a decrease in the depolarization stimuli. In addition, under certain conditions CB₁ can signal through G_s instead of G_i proteins leading to the accumulation of cAMP {59, 71, 72, 73, 74, 75}. Signaling through CB₁ has also been associated with the mitogen-activated protein kinase (MAPK) pathway. The mechanism by which MAPK regulates key cellular functions involves the activation of a tyrosine kinase-linked receptor, followed by activation of the intracellular G protein Ras and eventually phosphorylation and activation of MAPK {69}. Just like CB₁ receptors, CB₂ receptors are G_{i/o} protein coupled receptors that inhibit adenylyl cyclase activity and additionally signal through p42/p44 MAP kinase in a phosphatidylinositol 3-kinase (PI3k)-Akt manner {69, 76, 77}. There is also evidence that the CB₂ receptor induces the expression of genes through a PKC-dependent activation of MAP kinase {78}. Furthermore CB₂ receptors can increase intracellular calcium levels through the activation of phospholipase C {79}.

1.4.6 Immunomodulatory effects of cannabinoids on humoral and cell mediated immunity: contributions from our lab

The immunomodulatory properties of cannabinoids are well established. A large portion of studies demonstrate inhibitory effects on immune cell function. These effects can occur via CB₁/CB₂-dependent and -independent mechanisms. On the other hand, some recent studies show the endocannabinoid system containing immune stimulatory effects. This biphasic response is possibly due to cannabinoid ligand concentrations. It has been reported that inhibitory effects of cannabinoids are in the micromolar range, while stimulatory effects are in the nanomolar range {80}.

Our lab and others have provided findings supporting CBR-dependent and independent effects. Kaplan demonstrated that CBD suppressed IL-2 secretion in stimulated splenocytes from wild type and CB₁^{-/-} CB₂^{-/-} mice in a concentration-dependent manner {81}. Later studies investigating pulmonary inflammation in bronchial alveolar lavage fluid after mice were instilled with lipopolysaccharide (LPS) showed that treatment with CBD, mainly attributed to be anti-inflammatory, induced a higher infiltration of neutrophils to the lungs as early as 6 hours post LPS instillation. Additionally, CBD induced higher secretion of LPS-induced proinflammatory cytokines like tumor necrosis factor α (TNF α), interleukin 6 (IL-6) and IL-23 {82}. These studies suggest that CBR effects, whether enhancing or repressive, are dependent on the target organ, type of stimulation and dose. Characterization of CB₁^{-/-} CB₂^{-/-} mice in several immune competence assays found mainly an increase in the background response in the CB knockout mice in comparison to wild type. However, THC treatment suppressed the anti-sheep red blood cell IgM antibody-forming cell response in wild-type but not in CB₁^{-/-} CB₂^{-/-} mice. Also, CD40-ligand-induced IgM responses were suppressed by THC in WT B cells only. These findings

suggest a role of CBRs in humoral immune response {83}. Additionally, Buchweitz et. al. demonstrated that following influenza challenge, CB null mice had increased CD4⁺ T cells and interferon γ (IFN γ) in bronchial alveolar lavage fluid and greater pulmonary inflammation, as compared to WT. Moreover, THC treated CB₁^{-/-} CB₂^{-/-} mice challenged with influenza virus had higher mucous cell metaplasia (84, 85). Collectively, these findings demonstrate an important role of CBRs in regulating the magnitude of the cell-mediated (T cell-dependent) and antibody-mediated (B cell-dependent) immune responses.

Antigen presentation by DCs is essential for the start of the adaptive immune response. Karmaus et al. reported that THC significantly suppressed the percentage of IFN- γ -producing CD4⁺ and interleukin-17-producing NK1.1⁺ cells. In addition, an influx of APC, including inflammatory myeloid cells and monocytes/macrophages, into the lung in a CB₁- and/or CB₂-dependent manner was observed (86, 87). *In vitro* studies showed that THC suppressed LPS-induced maturation of bone marrow dendritic cells (bmDCs). Interestingly, naïve bmDCs from CB₁^{-/-} CB₂^{-/-} mice induced an antigen-specific CD8⁺ T cell response and produced IFN- γ as robustly as LPS-stimulated WT bmDC (87). Also, CB₁^{-/-} CB₂^{-/-} mice cleared the influenza virus from the lungs significantly faster than WT mice but at the expense of aggravated lung tissue damage (87). The underlying reason for the exacerbated immune response in CB₁^{-/-} CB₂^{-/-} mice is not well understood, but is thought, in part, to be a result of the absence of immune regulation by CBRs on DCs.

1.5 Dendritic cell antigen presentation during pathogen infection

Dendritic cells are a heterogeneous population of APCs indispensable in linking the innate and adaptive immune system. The key features of the adaptive immune system are: 1) the

creation of antigen (Ag) specific clones by the rearrangement of immunoglobulin genes; and 2) immunological memory. APCs provide guidance and regulation to this system {88, 89}. DCs are uniquely qualified among APCs to instruct the adaptive immune system since they are activated along with the innate immune response, hence allowing the development of immunological memory {1, 2, 90}. DCs can reside in many different tissues throughout the body or circulate in the blood and respond to cues in the environment through pathogen-associated molecular patterns (PAMPs). PAMPs produce changes in immature DCs that increase their ability to migrate to local lymph nodes and more effectively capture, process and present antigen to activate naïve T cells in the context of MHC class I (MHC I) for CD8⁺ and MHC class II (MHC II) for CD4⁺ T cells {91, 92}. Although all DCs are important in adaptive immunity, not all DCs have the same ability to migrate, process and present antigen.

DCs possess unique characteristics that make them potent inducers of T cell-mediated immunity. First, DCs have a vast array of pattern recognition receptors to recognize molecular patterns in microorganism permitting them to phagocytize, process and present antigen very efficiently {92, 93, 94}. Second, DCs express chemokine receptors, such as CCR7, that allow them to migrate from sites of infection in peripheral tissues to the secondary lymphoid organs {93}. Third, during migration DCs go through maturation that results in the up regulation of membrane molecules (i.e. CD40, CD80, CD86) and cytokines (i.e. IL-12), leading to strong stimulation of T cell growth and differentiation {90, 1, 2, 94, 95}.

1.5.1 Activation and maturation of DCs

Upon stimulation with pathogen-derived products, immature DCs transiently increase their migratory capabilities and begin their activation program termed “maturation”. There are several steps in this phenotypic switch such as: (1) loss of endocytic/ phagocytic capabilities; (2)

upregulation of costimulatory molecules like CD40, CD80, and CD86; (3) change in morphology; (4) shift in lysosomal compartments; and (5) MHC II compartments. Morphological changes accompanying DC maturation include a loss of adhesive structures, cytoskeleton reorganization, and acquisition of high cellular motility. Once DCs reach the local lymphoid organ, they complete their maturation process by expressing antigen bound MHC molecules on their surface and activating naïve T cells. {90,92, 94, 96}. DCs provide the naïve T cell with two signals required for their activation. The first signal is the antigen-specific signal presented to the T cell receptor in the context of peptide bound MHC molecule. The second signal is provided by costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) triggering survival and proliferation through CD28 expressed on naïve T cells. Additionally, soluble regulatory factors like cytokines and chemokines help shape the immunological response by promoting or inhibiting differentiation, maturation, migration and survival signals {97}.

1.5.2 MHC class I antigen presentation

Dendritic cells once infected with a virus, rapidly upregulate MHC class I and II molecules together with co-stimulatory molecules and very efficiently present viral peptides leading to potent T cell stimulatory activity. The MHC class I antigen presentation pathway allows the immune system to detect transformed or infected cells. All nucleated cells express MHC I molecules, which present peptides derived from endogenous proteins that are degraded in the cytosol by the proteasome. The degraded endogenous protein products are transported to the endoplasmic reticulum (ER) by a transporter associated with antigen processing (TAP) to be loaded onto newly formed MHC class I molecules {98}. Once in the ER lumen, peptides too long to be loaded onto MHC I molecules can be trimmed by ER aminopeptidases (ERAP) {99}. Correct size peptides are loaded onto MHC I molecules with the help of the chaperones tapasin,

calnexin and calreticulin {100}. Finally, MHC I-peptide complexes are exported to the cell surface through the Golgi apparatus. Naive antigen specific CD8⁺ T cells must be activated into effector cytotoxic T lymphocytes (CTLs) in order to eliminate infected cells {98}. Only APCs, specially DCs, are capable of this by presenting the specific MHC I-antigen to a CD8⁺ T cell bearing a T cell receptor (TCR) capable of recognizing the complex thus allowing the CTL to detect and kill the infected cell. When the APCs are not directly infected, they need to acquire exogenous antigens from the infectious agent and present them on MHC class I molecules, by a mechanism known as cross-presentation {98,101}.

1.5.3 Antigen cross-presentation by MHC I

DCs, specifically mouse CD8⁺ lymphoid organ-resident DC (CD8⁺ DC), play a dominant role in cross-presentation, despite the fact that other APCs have been demonstrated to have the capability to cross-present *in vitro* {101, 102, 103, 104}. There are two ways exogenous antigens can be internalized: 1) micro or macro pinocytosis; and 2) endocytosis or phagocytosis. Once internalized, these antigens go through the same steps as endogenously produced peptides where they are degraded by proteasomes, loaded onto MHC I molecules on the ER and transported to the surface as MHC I-peptide complex. Two major pathways have been described for cross-presentation {105}. The first is the endocytic pathway where the degradation and loading occurs in the endosomal compartment of the APC. This pathway loads the correct size peptide onto MHC I molecules recycled from the plasma membrane, which are then transported back to the surface. In the cytosolic pathway, antigens are transported to the cytosol for processing. Then peptide loading occurs in the ER and subsequently transported to the surface of the plasma membrane. The literature suggests that cross-presentation may occur as combination of both the endocytic and the cytosolic pathway {100}. Toll like receptor signaling enhances

cross-presentation on class I MHC molecules at the level of antigen capture and processing in DCs. Stimulation with LPS showed enhanced uptake of soluble antigen and immune complexes, modest increases in proteasome and TAP activity and enhanced translocation of antigen to the cytosol {106, 107}.

1.5.4 MHC class II antigen presentation

MHC II molecules are composed of two chains, α and β . Both chains associate with Invariant chain (Ii) in order to facilitate the assembly MHC II and its exit from the endoplasmic reticulum. Ii plays also the role of preventing premature binding of peptides and transport to the endosomal system {108,109}. Within the MHC II- containing compartments (MIIC) Ii is degraded leaving MHC II bound to Class II-associated invariant chain peptide (CLIP) {110}. At the same time Ii is being degraded, proteases are degrading internalized proteins producing antigens for MHC II ligation. Then CLIP dissociates and the peptide binds to the MHC II binding pocket {111, 112}. MHC-II: peptide complexes are then delivered to the cell surface by mechanisms not well understood {113, 114, 115} and stimulate antigen-specific CD4⁺ T cells with cognate receptors.

1.5.5 Rationale

Cannabinoids are a group of highly lipophilic natural (over 60 compounds in *Cannabis sativa*) and synthetic ligands with similar chemical structure. The receptors for these groups of compounds, CBRs, are expressed in most nucleated cells hence their activation can lead to a wide range of effects. There are two well-studied receptors, CB₁ found mainly in the central nervous system and CB₂ restricted to the periphery, especially at high levels in immune cells. THC, the main psychoactive constituent in the marijuana plant, alters many different aspects of the immune response, including cell activation, proliferation and cytokine production by

leukocytes. Cannabinoids possess well-established immunomodulatory properties demonstrated to be CBR dependent and independent. Studies from our lab have described an influx of APCs including inflammatory myeloid cells and monocytes/macrophages, into the lung of influenza challenged mice in a CB₁- and/or CB₂-dependent manner. *In vitro* studies showed that THC suppressed LPS-induced maturation of bmDCs. Interestingly, naïve bmDCs from CB₁^{-/-} CB₂^{-/-} were able to mount an antigen-specific CD8⁺ T cell response and produce IFN-γ as robustly as LPS stimulated WT bmDC (86,87). The underlying reason for the exacerbated immune response in CB₁^{-/-} CB₂^{-/-} mice is not well understood, but is thought, in part, to be a result of the absence of immune regulation by CBRs. DCs are the most potent APCs making them essential to the adaptive immune response. Hence the goal of this thesis was to characterize the role of CBRs in DC hematopoietic development, antigen presenting function and maturation. Specifically, I am testing the **hypothesis** that bone marrow DC from CB₁^{-/-} CB₂^{-/-} mice elicit an antigen specific CD8⁺ T cell response in the absence of LPS-induced maturation signal through increased production of DCs , co-stimulatory capabilities and antigen surface loading.

CHAPTER 2: MATERIAL AND METHODS

2.1 Mice

Eight- to twelve-week-old C57BL/6 wild type (WT) mice, $CB_1^{-/-}CB_2^{-/-}$ mice bred on a C57BL/6 background and OT-I transgenic mice were used for *in vitro* experiments. WT mice were purchased from Charles River (Portage, MI), transferred to plastic cages containing sawdust bedding (five mice per cage), and quarantined for 1 week. $CB_1^{-/-}CB_2^{-/-}$ mice were a generous gift from Dr. Andreas Zimmer from the University of Bonn {116}. OT-I mice breeders were purchased from Jackson Laboratories (Bar Harbor, ME). OT-I mice are transgenic for T-cell receptor (Tcr) α and Tcr β , generating $CD8^+$ T cells specific for chicken ovalbumin (OVA₂₅₇₋₂₆₄; amino acid sequence: SIINFEKL). Animals were housed and bred at the Michigan State University animal care facilities at a relative humidity of 40-60% with a room temperature of (21°C - 24 °C) and a 12 h light/dark cycle. Food (Purina Certified Laboratory Chow) and spring water were provided *ad libidum*. Age-matched female mice were exclusively used for experiments primarily due to the dominant behavior exhibited by caged male mice (i.e. fighting) and the potential effects in immune competence this stress may bring. All animal housing, handling, and procedures were approved by and performed following the guidelines of the Institutional Animal Care and Use Committee at Michigan State University.

2.2 Bone marrow cell isolation and culture

Femurs and tibia from hind legs of female C57BL/6 and $CB_1^{-/-}CB_2^{-/-}$ mice 8- to 12-week-old were surgically removed, separated from muscle tissue and sterilized. Bone tip ends were snapped and marrow was flushed using a 25-gauge syringe with RPMI 1640 media (Invitrogen, Carlsbad, CA). Bone marrow cells were cultured at 5×10^5 cells/mL in 6-well flat bottom plates

(Corning, NY) with RPMI 1640 medium supplemented with 10 % charcoal/dextran treated Fetal Bovine Serum (HyClone Laboratories, Logan, UT), penicillin (100units/mL)/streptomycin (100µg/mL) for 24 h at 37°C and 5% CO₂. The cells were either left unstimulated (naïve) or were stimulated with LPS (1 µg/mL) for the duration of the incubation.

2.3 Surface antibody staining and flow cytometry

All staining protocols were performed in 5 mL or 15 mL round bottom polypropylene tubes (BD Falcon, Franklin Lakes, NJ). Bone marrow cells at 1×10^6 cells/mL were washed with $1 \times$ HBSS and stained with live/dead fixable near infra red dead cell stain (Invitrogen) for 20 min at 4°C. Dead cell stain was washed with FACS buffer (1x HBSS, 1% bovine serum albumin, 0.1% sodium azide, pH 7.6) and surface Fc receptors were blocked with anti-mouse CD16/CD32 unconjugated antibody (BD Biosciences, Franklin Lakes, NJ) for 15 min at 4°C. Cells were labeled for 20 min at 4°C with cocktail antibodies purchased from Biolegend aimed at identifying surface markers either important for dendritic cell effector function or identification of the specific precursors and subpopulations. See individual figure legends for a list of markers used in the specific experiments. Subsequent to fluorescent antibody labeling, cells were washed twice with FACS buffer, fixed with cytofix (BD Biosciences) for 15 min, and resuspended in FACS buffer. Fluorescent staining was analyzed using BD Biosciences FACSCanto II flow cytometer. Compensation and voltage settings of fluorescent parameters were performed using single color staining controls. Samples were either analyzed immediately or stored at 4°C.

2.4 Co-culture of bone marrow cells and OT-I cells *in vitro*

Splenocytes from OT-1 transgenic mice were isolated and labeled with cell trace (Invitrogen) proliferation dye to track loss of fluorescent staining as an indicator of proliferation. Naive or LPS-stimulated bone marrow cells were incubated with OT-1 TCR-specific peptide SIINFEKL for 1 h in RPMI supplemented with 2% FBS at 37°C. Then, peptide-pulsed cells were washed three times with serum-containing media. Peptide-pulsed cells (2.5×10^6 cells/mL) were cultured in a 96-well round bottom plate with cell trace-labeled OT-1 splenocytes (5×10^6 cells/mL) in 10% serum RPMI for three days at 37°C. At day three of co-culture cells were restimulated with SIINFEKL peptide and Brefeldin A for 4 h in 2% FBS RPMI. Subsequently, cells were stained with live/dead dye, CD8 α and IFN- γ (intracellular staining). Cells were analyzed by flow cytometry as described in a previous section. The gating was performed in the following order: singlets, size, live cells, and CD8 α for graphs showing proliferation or proliferation and IFN- γ production.

2.5 Intracellular antibody labeling for flow cytometry

Intracellular staining protocol must be performed on the same day the samples will be analyzed on the flow cytometer to prevent signal degradation. After surface labeling and fixation steps described above, cells were washed twice with 1 $\times$ Perm/Wash (BD Biosciences) and incubated with 1 $\times$ Perm/Wash for 30 min at room temperature in 96-well round bottom plates. Then, cells were incubated with fluorescently labeled IFN γ antibody for 20 min at room temperature. After the incubation period cells were washed once with 1x Perm/wash and twice with FACS buffer. Cells were analyzed the same day by flow cytometry and cytokine-secreting cells were identified by gating on the negative population in unstimulated control samples.

2.6 Statistical analysis

The mean $\pm$ standard error was determined for each treatment group. Flow cytometry data analysis was performed using Flowjo and/or Kaluza software and graphed using Graphpad Prism. Statistical significance was determined using either one way or two-way ANOVA with a Bonferroni's or Tukey's post hoc test to compare select groups of samples. Statistical analyses were performed using GraphPad Prism v5.0, GraphPad Software (San Diego, CA).

CHAPTER 3: EXPERIMENTAL RESULTS

3.1 Naïve bmDCs from $CB_1^{-/-}$ $CB_2^{-/-}$ mice elicit a $CD8^+$ T cell response

Our initial goal was to determine whether bone marrow cells from $CB_1^{-/-}$ $CB_2^{-/-}$ could successfully elicit a $CD8^+$ T cell response in the absence of GM-CSF stimulation for 9 days per methods established by Karmus et al. [82]. Bone marrow cells were isolated from femurs and tibia of WT and $CB_1^{-/-}$ $CB_2^{-/-}$ mice and cultured for 24 hours at 37°C. Cells were then pulsed with SIINFEKL peptide for 2 hours and subsequently cultured with proliferation dye (cell trace) stained OT-I mouse splenocytes for 3 days. As observed previously in the *in vitro* differentiation model with GM-CSF, WT bone marrow cells were only able to elicit $CD8^+$ T cell proliferation and IFN γ secretion upon stimulation with LPS. By contrast the naïve unstimulated $CB_1^{-/-}$ $CB_2^{-/-}$ bone marrow cells strongly induced $CD8^+$ T cell proliferation and IFN γ secretion. LPS stimulation of $CB_1^{-/-}$ $CB_2^{-/-}$ bone marrow cells did not increase the response. (Figure 3)

3.2 Characterization of dendritic cell precursors and subpopulations in mouse bone marrow cells

3.2.1 Identification of CDP and MDP in bone marrow cells

One putative explanation for the exacerbated $CD8^+$ T cell response observed in naïve $CB_1^{-/-}$ $CB_2^{-/-}$ bone marrow cells (Figure 3) might be a change in the composition of immune cell populations and their precursors in bone marrow cells, compared to WT. To explore this possibility, a characterization of the composition of DC precursors and subpopulations in bone marrow was conducted in WT and $CB_1^{-/-}$ $CB_2^{-/-}$ mice. Toward this end a cocktail of antibodies was utilized to identify MDPs and CDPs, the direct precursors to all DC populations (Figure 4A). The anti-mouse lineage cocktail antibody is designed to identify hematopoietic progenitors

by targeting terminally differentiated cells such as T lymphocytes, B lymphocytes, monocytes/macrophages, granulocytes, NK cells, and erythrocytes using markers for surface receptors widely expressed in these cells. The percentage of lineage positive cells were statistically significantly higher in $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells in comparison to WT. Meanwhile the percentage of hematopoietic progenitors or lineage negative cells was decreased in $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells (Figure 4B). MDPs composed approximately 8% of total bone marrow in WT and 9% in $CB_1^{-/-}CB_2^{-/-}$ bone marrow. CDPs composed less than 1% and pre-DCs less than or equal to 2% of total bone marrow cells in WT and $CB_1^{-/-}CB_2^{-/-}$ mice. $MDP\Delta$ are another subdivision of MDPs that could be easily identified by their size in the scatter plots and demonstrated no difference between both mice bone marrow genotypes (Figure 4C).

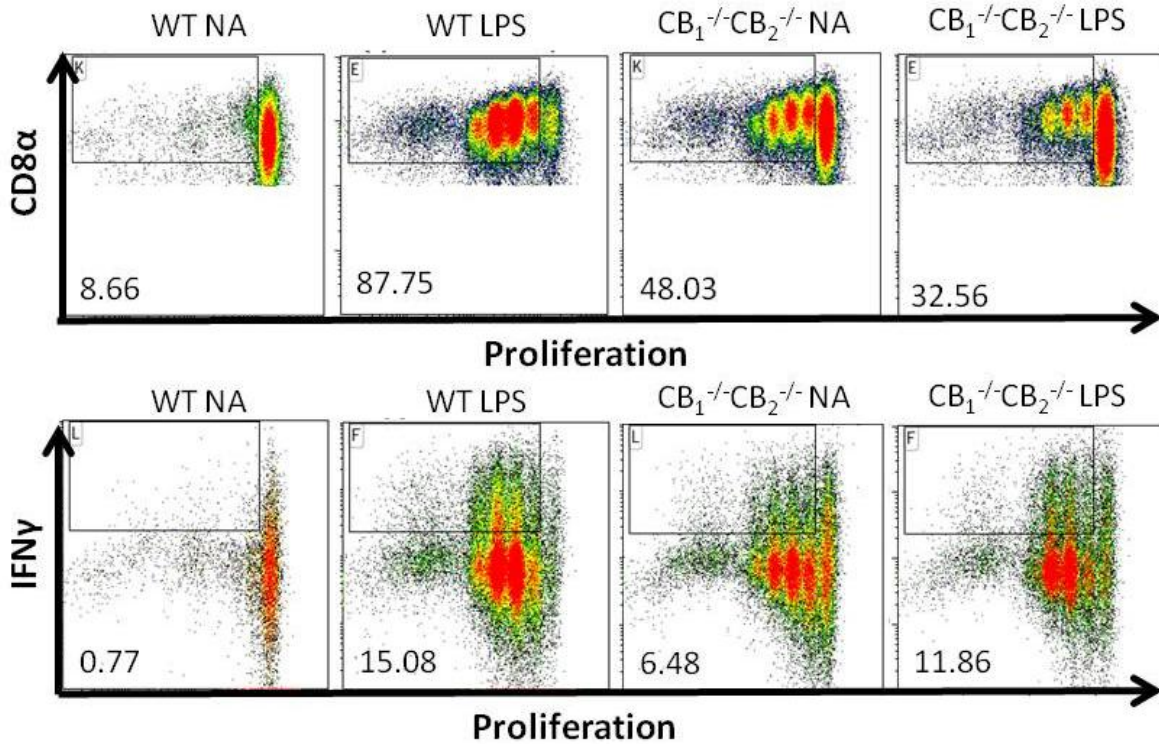


Figure 3. Naïve bone marrow cells from CB₁^{-/-}CB₂^{-/-} mice elicited a CD8⁺ T cell response.

Representative graphs of 4 separate experiments. Isolated bone marrow cells from C57Bl/6 (n=3) and CB₁^{-/-}CB₂^{-/-} (n=3) mice were stimulated with LPS (1μg/mL) and cultured at 5 x10⁵cells/mL for 24 h. Cells were then pulsed with OT-1 TCR-specific peptide SIINFEKL for 1h and washed three times prior to incubation with cell trace-labeled OT-1 splenocytes for 3 days. On the last day of incubation cells were restimulated with SIINFEKL peptide and Brefeldin A for 4 h in 2% FBS RPMI media, stained and analyzed by flow cytometry. Representative dot plots of three separate experiments for A) CD8 vs. proliferation and B) IFN-γ vs proliferation.

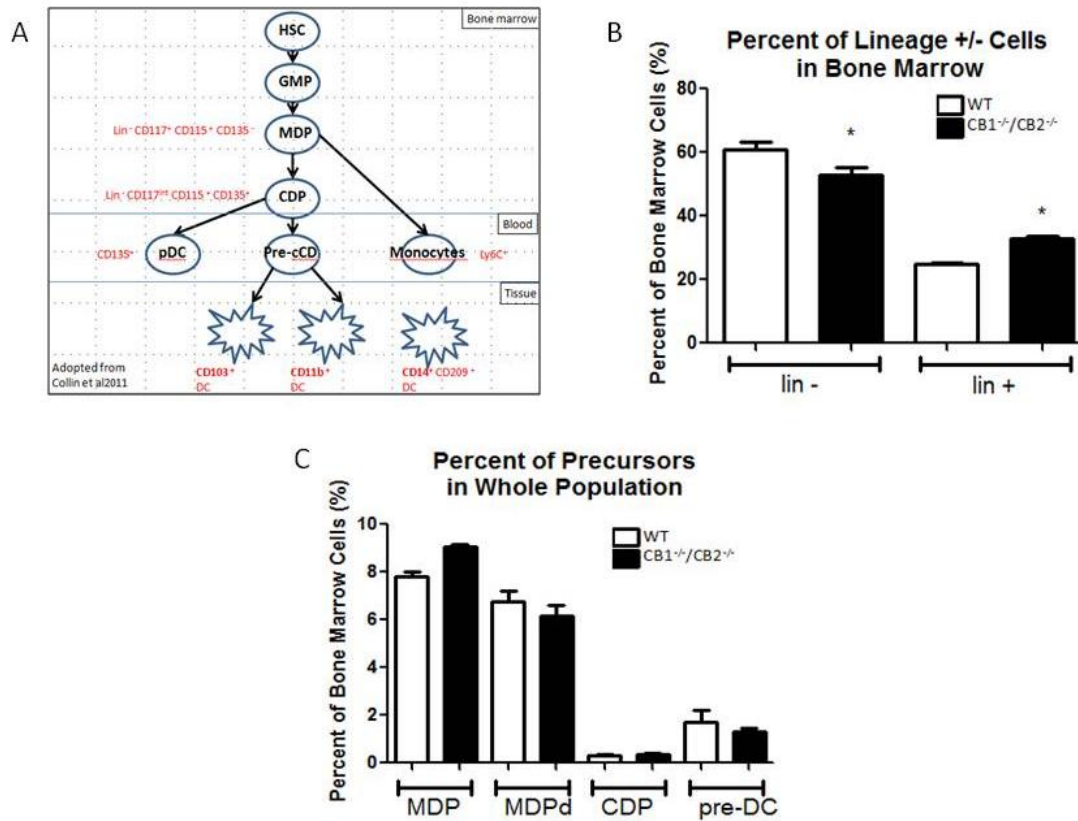


Figure 4. Percent of lineage positive, lineage negative and precursor cells in mouse bone marrow cells. Representative of two separate experiments. A) Scheme of mouse DC differentiation from hematopoietic stem cells in the bone marrow. B, C) Freshly isolated bone marrow cells isolated from C57Bl/6 (n=3) and CB1^{-/-}CB2^{-/-} (n=3) mice were labeled at concentration of 1 x 10⁶ cells/well with lineage cocktail along with precursor cell markers: CD117, CD115 and CD135 and accessed by flow cytometry. B) Percent of lineage positive and negative cells in freshly isolated bone marrow cells. C) Percent of dendritic cell precursors identified in freshly isolated bone marrow cells. MDP = Macrophage and DC precursor, Lin⁻ CD117⁺ CD115⁺ CD135⁻. CDP= common DC precursor, Lin⁻ CD117^{+/+} CD115⁺ CD135⁺ * p<0.05 as compared to WT.

3.2.2 Identification of DC subpopulations and characterization of MHC I, MHC II and CD86 expression

Our finding that $CB_1^{-/-}CB_2^{-/-}$ mice possess a significantly larger percentage of lineage positive cells in bone marrow led us to explore the percent composition of several DC subpopulations that may account for this observation as well as contribute to the exacerbated $CD8^+$ T cell responses. Bone marrow cells cultured were stained at day 0 and after 24 hours with or without LPS stimulation with CD11c, CD11b, $CD8\alpha$, CD103 antibodies to identify all $CD11c^+$ cells, $CD11b^+$ DCs, $CD103^+$ DCs, pDCs and $CD8\alpha^+$ DCs. The percent of $CD11c^+$ cells in freshly isolated bone marrow cells is slightly higher in $CB_1^{-/-}CB_2^{-/-}$ in comparison to WT (Figure 5A). As observed in Figures 5 A, B and C, the major DC population identified are the $CD11b^+$ DCs and presents a significant difference in freshly isolated bone marrow (Figure 5B) from $CB_1^{-/-}CB_2^{-/-}$ mice in comparison to WT. The rest of the DC subpopulations $CD8\alpha^+$, $CD103^+$ and pDCs comprise less than 5% of the total $CD11c^+$ cells with pDCs demonstrating a trend toward decreased percent in $CB_1^{-/-}CB_2^{-/-}$ (Figure 5A). After 24 hours in culture the percent of $CD11c^+$ cells is significantly higher in $CB_1^{-/-}CB_2^{-/-}$ (~60%) in comparison to WT (~50%). The percent of $CD11b^+$ DCs is higher in $CB_1^{-/-}CB_2^{-/-}$ and pDCs, $CD103^+$ and $CD8\alpha^+$ DCs are all under 2% of total bone marrow cells (Figure 5B). The percent composition of DC populations remains similar after 24 hour LPS stimulation. $CD103^+$ and $CD8\alpha^+$ DCs slightly increased their percent composition with no difference between WT and $CB_1^{-/-}CB_2^{-/-}$ (Figure 5C).

MHC I surface expression on the DC subpopulations identified above was assessed to determine any differences between WT and $CB_1^{-/-}CB_2^{-/-}$ mice. APCs process and load antigen onto MHC I molecules and induce $CD8^+$ T cell activation. In freshly isolated bone marrow cells the overall surface fluorescent intensity of $CD11c^+$ cells and all DC subpopulations is higher in

CB₁^{-/-} CB₂^{-/-} mice. CD103⁺ and CD8α⁺ DCs have significantly higher expression (Figure 6A). Interestingly, after 24 hours in culture in the presence or absence of LPS stimulation, MHC I expression inverts to a trend of decreased expression in CB₁^{-/-} CB₂^{-/-} in comparison to WT (Figure 6 B and C).

This phenomenon of decreased MHC expression on the surface on DCs after 24 hours in culture was repeated when we assessed surface MHC II expression on DC subpopulations. Although gating on all CD11c⁺ cells and CD11b⁺ DCs did not demonstrate any significant differences on freshly isolated bone marrow, other marginal DC subpopulations (pDCs, CD103⁺ and CD8α⁺ DCs) showed a significant increase in MHC II expression on their surface (Figure 7A). After 24 hours in culture with or without LPS stimulation, the significant increase in surface MHC II is no longer present (Figure 7B and C) and only a trend towards increased expression remains (Figure 7B). MHC I expression in CD8α⁺ DCs remains high in comparison to other subpopulations and upon LPS stimulation becomes significantly decreased in CB₁^{-/-} CB₂^{-/-} mice (Figure 7C).

Three signals are required for successful DC-T cell interaction. The first signal is antigen bound MHC interacting with the appropriate T cell receptor. The second signal is termed co-stimulation and requires the interaction of B7 (CD80, CD86) molecules on DCs with CD28, a co-receptor on the surface of T cells. The third signal derives from cytokines and aid in directing T cell differentiation and survival. The co-stimulatory signal is very important since it is key in maintaining DC- T cell interaction, survival of the cells involved and prevents the T cell from becoming anergic {2}. CD86 expression in DC populations from bone marrow cells was assessed to determine if there was an increase in co-stimulation that may aid in the exacerbated CD8⁺ T cell response observed in Figure 1. On freshly isolated bone marrow cells CD103⁺ DCs

and CD8 α ⁺ DCs express CD86 at a higher level than the other subpopulations identified. CD8 α ⁺ DCs demonstrated a significantly higher expression on CB₁^{-/-} CB₂^{-/-} in comparison to WT (Figure 8A). CD86 surface expression on bone marrow cells cultured for 24 hours without (Figure 8B) and with LPS stimulation (Figure 8C) showed a trend towards lower expression of CD86 on CB₁^{-/-} CB₂^{-/-} on all subpopulations and in general but not statistically significant.

DCs upon encountering antigen must mature into MHC II⁺ CD86⁺ cells in order to efficiently activate T lymphocytes. In order to determine whether the exacerbated CD8⁺ T cell response observed in the co-culture system was due to an increase in mature DC, the percent of DCs subpopulations that co-expressed MHC II and CD86 on their surface was quantified. Overall only about 5% of all of the CD11c⁺ cells had a mature phenotype coming straight out of the bone marrow. Interestingly, over 50% of CD103⁺ DCs and 40% of CD8 α ⁺ DCs had a mature phenotype, with CD103⁺ DCs being significantly higher in CB₁^{-/-} CB₂^{-/-} (~65%) in comparison to WT (~55%) (Figure 9A). After 24 hour in culture the percent of mature DCs within all CD11c⁺ cells increases to around 15% and CD103⁺ DCs and CD8 α ⁺ DCs continue to have the highest percent of cells with a mature phenotype. The percent of mature CD103⁺ and CD8 α ⁺ DCs in CB₁^{-/-} CB₂^{-/-} mice bone marrow is lower than WT, with the decrease being statistically significant in CD8 α ⁺ DCs (Figure 9B). Upon LPS stimulation of WT and CB₁^{-/-} CB₂^{-/-} bone marrow cells, a significant decrease in the mature population of CD11c⁺ cells in general, pDCs, CD103⁺ DCs and CD8 α ⁺ DCs was observed in CB₁^{-/-} CB₂^{-/-} mice bone marrow cells (Figure 9C).

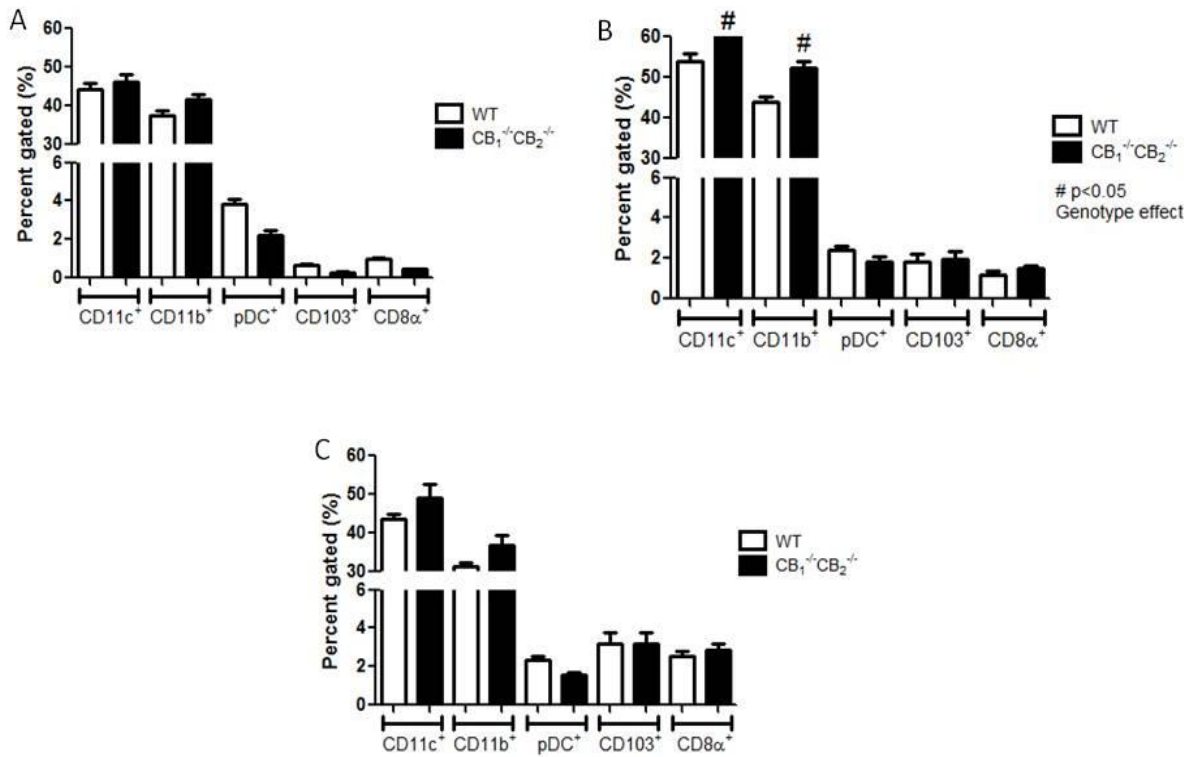


Figure 5. Composition of DC subpopulations in freshly isolated bone marrow cells and 24 hours in culture. Representative graphs of 3 separate experiments. Bone marrow cells flushed from femurs and tibia of C57Bl/6 (WT, n=4) and CB₁^{-/-}CB₂^{-/-} (n=4) mice were cultured in 6 well flat bottom plates at 5 x10⁵ cells/mL for 24 hours with or without LPS (1μg/mL) stimulation. Bone marrow cells were stained the same day of isolation (A), and 24 h in culture without (Naïve, B) and with LPS stimulation (C) with the following antibodies: CD11c, CD11b, CD8α, CD103 and analyzed in a flow cytometer to identify the percent makeup of several DC populations. CD11b⁺ DC= CD11c⁺ CD11b⁺, CD103⁺ DC= CD11c⁺CD11b^{-/+} CD103⁺, pDC= CD11c⁺ CD11b⁻ CD103⁻, CD8α⁺ DC= CD11c⁺ CD8α⁺

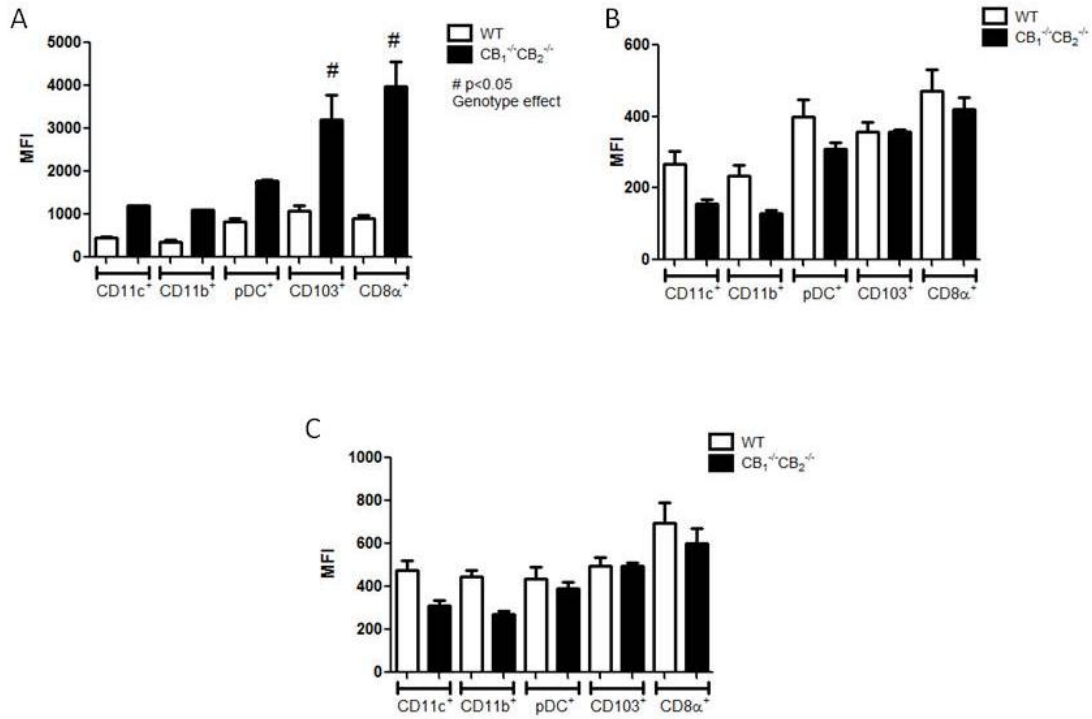


Figure 6. MHC I expression in DC subpopulations of freshly isolated bone marrow cells and 24 hours in culture. Representative graphs of 3 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and CB₁^{-/-}CB₂^{-/-} (n=4) mice were cultured in 6 well flat bottom plates at 5 x10⁵ cells/mL for 24 h with or without LPS (1μg/mL) stimulation. Surface mean fluorescent intensity (MFI) expression was determined by flow cytometry in several DC subpopulations the same day of bone marrow isolation (A), and 24 h in culture without (Naïve, B) and with LPS stimulation (C) with the following antibodies: CD11c, CD11b, CD8α, CD103. CD11b⁺ DC= CD11c⁺ CD11b⁺ , CD103⁺ DC= CD11c⁺CD11b^{-/+} CD103⁺, pDC= CD11c⁺ CD11b⁻ CD103⁻, CD8α⁺ DC= CD11c⁺ CD8α⁺

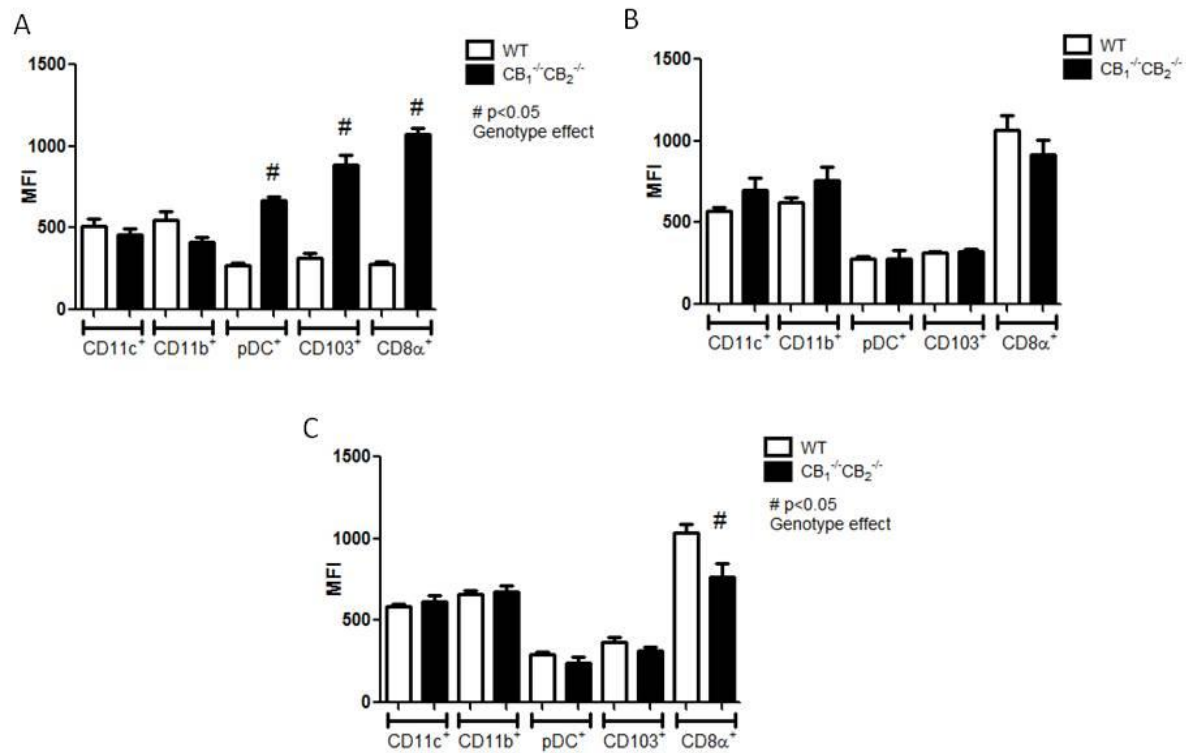


Figure 7. MHC II expression in DC subpopulations of freshly isolated bone marrow cells and 24 hours in culture. Representative graphs of 3 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and CB₁^{-/-}CB₂^{-/-} (n=4) mice were cultured in 6 well flat bottom plates at 5 x10⁵ cells/mL for 24 hours with or without LPS (1μg/mL) stimulation. MHC II surface MFI expression was determined by flow cytometry in several DC subpopulations the same day of bone marrow isolation (A), and 24 h in culture without (Naïve, B) and with LPS stimulation (C) with the following antibodies: CD11c, CD11b, CD8α, CD103. CD11b⁺ DC= CD11c⁺ CD11b⁺, CD103⁺ DC= CD11c⁺CD11b^{-/+} CD103⁺, pDC= CD11c⁺ CD11b⁻ CD103⁻, CD8α⁺ DC= CD11c⁺ CD8α⁺.

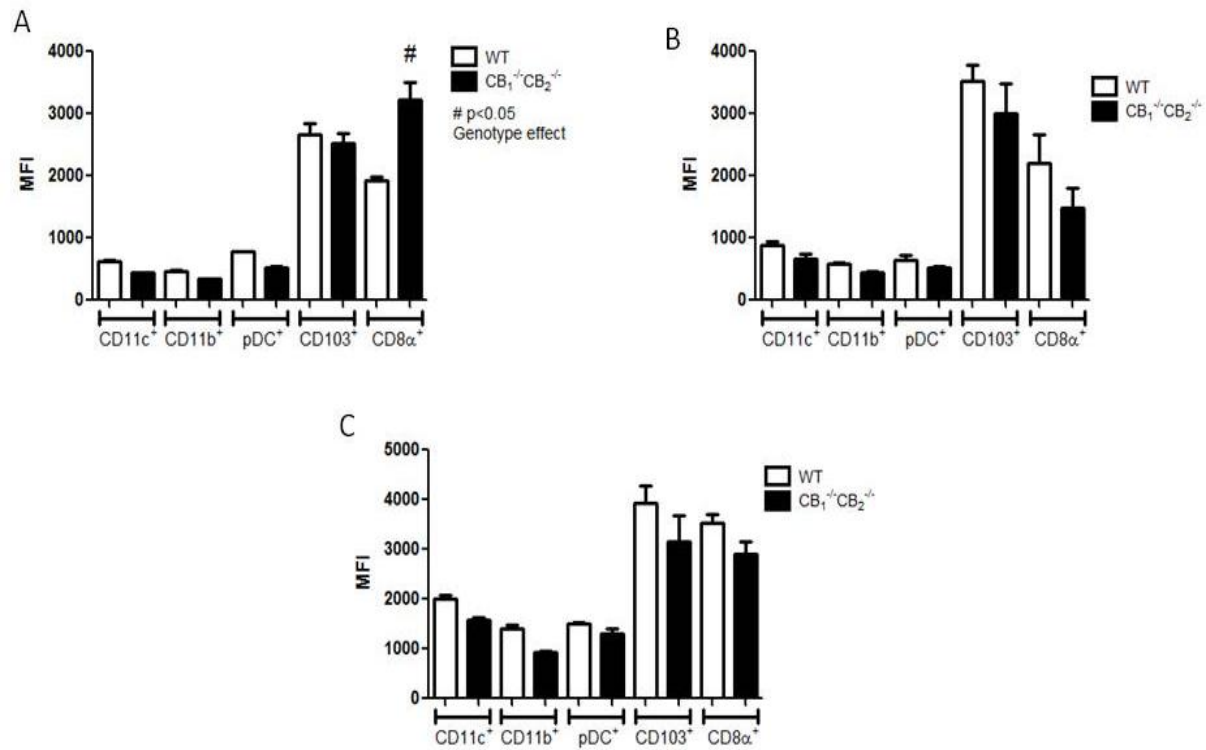


Figure 8. CD86 expression in DC subpopulations of freshly isolated bone marrow cells and 24 hours in culture. Representative graphs of 3 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and CB₁^{-/-}CB₂^{-/-} (n=4) mice were cultured in 6 well flat bottom plates at 5 x10⁵ cells/mL for 24 h with or without LPS (1μg/mL) stimulation. CD86 surface MFI expression was determined by flow cytometry in several DC subpopulations at the same day of bone marrow isolation (A), and 24 h in culture without (Naïve, B) and with LPS stimulation (C) with the following antibodies: CD11c, CD11b, CD8α, CD103. CD11b⁺ DC= CD11c⁺ CD11b⁺, CD103⁺ DC= CD11c⁺CD11b^{-/+} CD103⁺, pDC= CD11c⁺ CD11b⁻ CD103⁻, CD8α⁺ DC= CD11c⁺ CD8α⁺

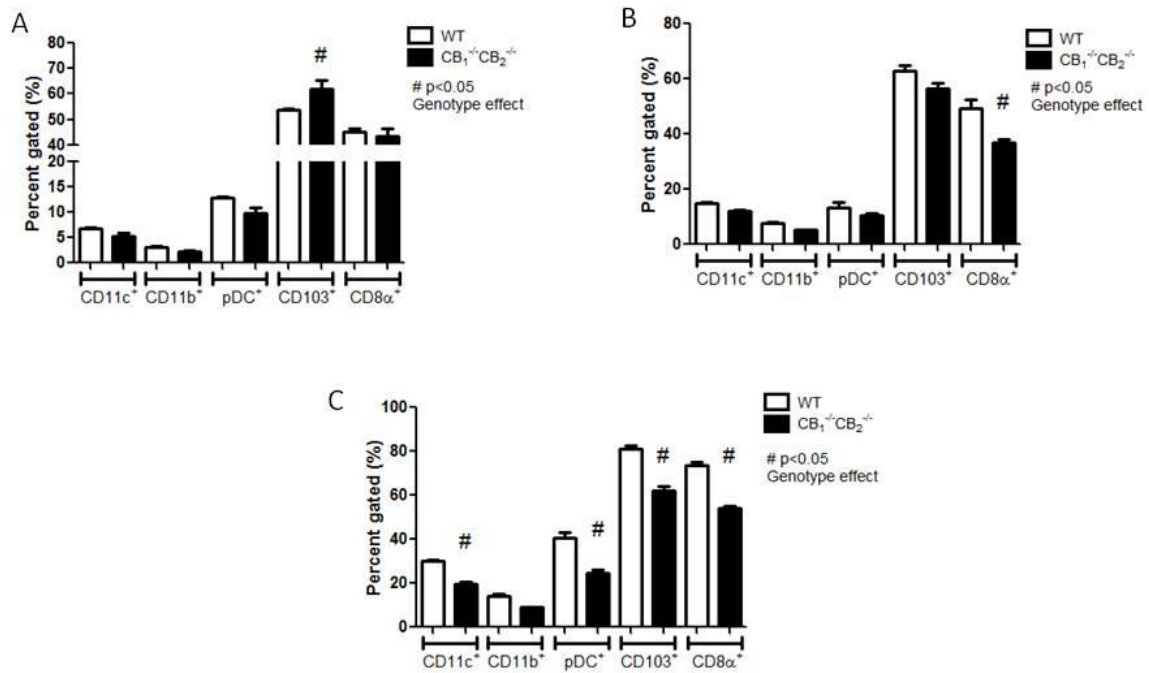


Figure 9. Percent of DC subpopulations that are MHC II⁺ CD86⁺ in freshly isolated bone marrow cells and 24 hours in culture. Representative graphs of 3 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and CB₁^{-/-}CB₂^{-/-} (n=4) mice were cultured in 6 well flat bottom plates at 5 x10⁵ cells/mL for 24 h with or without LPS (1μg/mL) stimulation. The percent of MHC II and CD86 double positive expression (indicative of a mature phenotype on DCs) was determined by flow cytometry in several DC subpopulations the same day of bone marrow isolation (A), and 24 h in culture without (Naïve, B) and with LPS stimulation (C) with the following antibodies: CD11c, CD11b, CD8α, CD103. CD11b⁺ DC= CD11c⁺ CD11b⁺, CD103⁺ DC= CD11c⁺CD11b^{-/+} CD103⁺, pDC= CD11c⁺ CD11b⁻ CD103⁻, CD8α⁺ DC= CD11c⁺ CD8α⁺

3.2.3 Identification of B cell and macrophages in bone marrow cells and their CD83 and CD86 expression

In addition to DCs, bone marrow cells contain other APCs such as B cells and macrophages that may possibly play a role in the exacerbated immune response observed with $CB_1^{-/-} CB_2^{-/-}$ mouse bone marrow cells (Figure 3). Our goal was to identify macrophages and B cells along with DCs and other population of cells that express MHC I and may be involved in $CD8^+$ T cell activation. Classical DCs, $CD11c^+ CD11b^+$, comprise less than 2% of the total MHC I⁺ bone marrow cells at day 0. After 24 hours in culture $CB_1^{-/-} CB_2^{-/-}$ bone marrow cells have a higher percent of classical DCs expressing MHC I in comparison to WT. Additionally, the percent of $CD11c^+ CD11b^-$ cells is higher on $CB_1^{-/-} CB_2^{-/-}$ in comparison to WT in freshly isolated bone marrow and after 24 hours in culture (Figure 10A). $CD11b^+$ DCs had the highest percent of MHC I expression among all the immune cells identified. No significant difference in the percent MHC I expression between both mice genotypes was detected for $CD11b^+$ DCs, B cells and macrophages (Figure 10B).

Mature DCs can be identified by the positive surface expression of MHC II and CD86. Classical DCs from $CB_1^{-/-} CB_2^{-/-}$ have a significant decreased in the percent of mature DCs on freshly isolated bone marrow. After 24 hours in culture the percent of mature DCs increased but demonstrated no significant difference between WT and $CB_1^{-/-} CB_2^{-/-}$ mice. The percent of mature $CD11c^+ CD11b^-$ cells were below 10% and had no significant difference between both phenotypes (Figure 11).

CD83 is a maturation marker found on APCs and implicated in T cell activation. After culturing bone marrow cells for 24 hours, classical DCs increase their percent of CD83 expression being significantly lower in $CB_1^{-/-} CB_2^{-/-}$ (Figure 12A). Similar to classical DCs,

CD11c⁺CD11b⁻, CD11c⁻CD11b⁻ and B cells had a modestly higher percent of CD83 expression after 24 hours in culture, albeit not statistically significant between WT and CB₁^{-/-} CB₂^{-/-}. Macrophages had no difference in percent expression between both mouse genotypes in freshly isolated bone marrow cells and after 24 hours in culture. (Figure 12B). The MFI expression of CD83 on classical DCs is lower on CB₁^{-/-} CB₂^{-/-} after 24 hours in culture (Figure 13A), supporting the significant decrease in percent in CD83 expression observed on figure 9A. CD83 MFI expression was lower on CD11c⁺CD11b⁻ cells in fresh bone marrow cells and had no difference at 24 hours between WT and CB₁^{-/-} CB₂^{-/-} (Figure 13A). While B cells had a decrease in MFI expression from 0 to 24 hours, macrophages increased their CD83 MFI expression after 24 hours in culture. No significant difference in CD83 MFI expression was observed in B cells and macrophages (Figure 13B).

The second signal necessary in the activation of T cells by APCs termed co-stimulation involves the interaction of B7 molecules (CD80, CD86) on APCs with CD28 on T cells {2,89, 90, 91, 92, 93, 94}. Classical DCs from CB₁^{-/-} CB₂^{-/-} mouse bone marrow cells demonstrated a significantly lower percent of CD86 expressing cells in comparison to WT. After 24 hours the percent of classical DCs expressing CD86 increases with a tendency to lower expression on CB₁^{-/-} CB₂^{-/-}. The percent of CD11b⁺ DCs, CD11c⁺CD11b⁻ and CD11c⁻CD11b⁻ expressing CD86 is markedly less when compared to classical DC's expression. No significant difference in the percent of CD86 expression was detected between WT and CB₁^{-/-} CB₂^{-/-} mice (Figure 14). The MFI expression of CD86 overall had no significant difference between the two mice genotypes. Classical DCs and CD11c⁺ CD11b⁻ cells had an increase in CD86 MFI expression after 24 hours in culture while CD11b⁺DCs and CD11c⁻CD11b⁻ cells had no increase in CD86 expression (Figure 15).

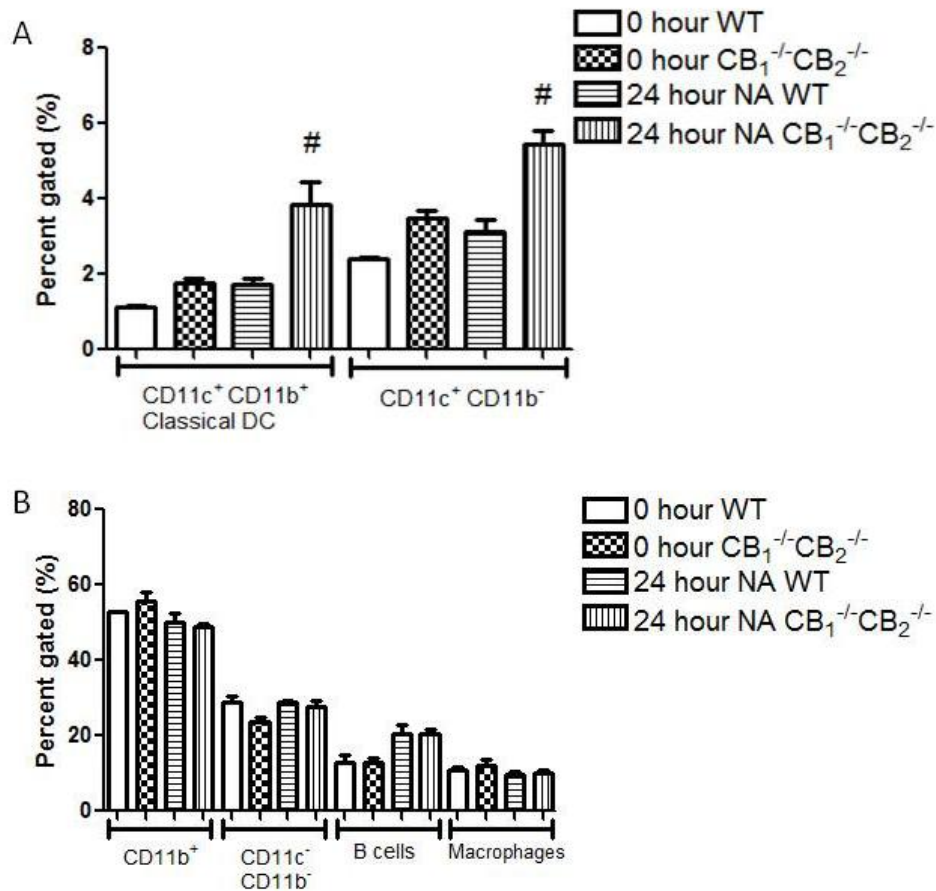


Figure 10. Percent composition of DCs , B cells, macrophages and CD11b⁺ cells gated within MHC I⁺ bone marrow cells. Representative graphs of 2 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and $CB_1^{-/-}CB_2^{-/-}$ (n=4) mice were cultured in 6 well flat bottom plates at a 5×10^5 cells/mL for 24 h with or without LPS (1 μ g/mL) stimulation. Treatment groups were assessed by flow cytometry for the percent composition of A) DCs (CD11c⁺ CD11b⁺ , CD11c⁺ CD11b⁻) and B) CD11b⁺, macrophages, B cells and CD11c⁻ CD11b⁻ cells within MHC I⁺ cells . The following antibodies were used: CD11c, CD11b, CD19, F4/80. B cells= CD19⁺ CD11c⁻ CD11b⁻ Macrophages= F4/80⁺ CD11c⁻ CD11b⁺. # = p<0.05 genotype effect

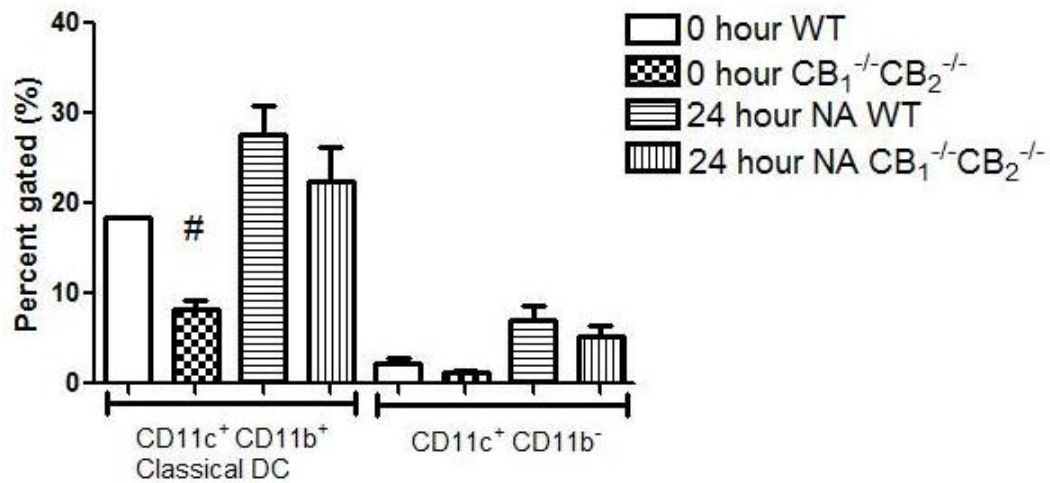


Figure 11. Percent of mature DCs in bone marrow cells. Representative graphs of 2 separate experiments . Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and CB₁^{-/-}CB₂^{-/-} (n=4) mice were cultured in 6 well flat bottom plates at a 5 x10⁵ cells/mL for 24 h with or without LPS (1μg/mL) stimulation. Treatment groups on CD11c⁺ CD11b⁺ and CD11c⁺ CD11b⁻ DCs were assessed by flow cytometry for the percent of MHCII⁺ CD86⁺. # = p<0.05 genotype effect

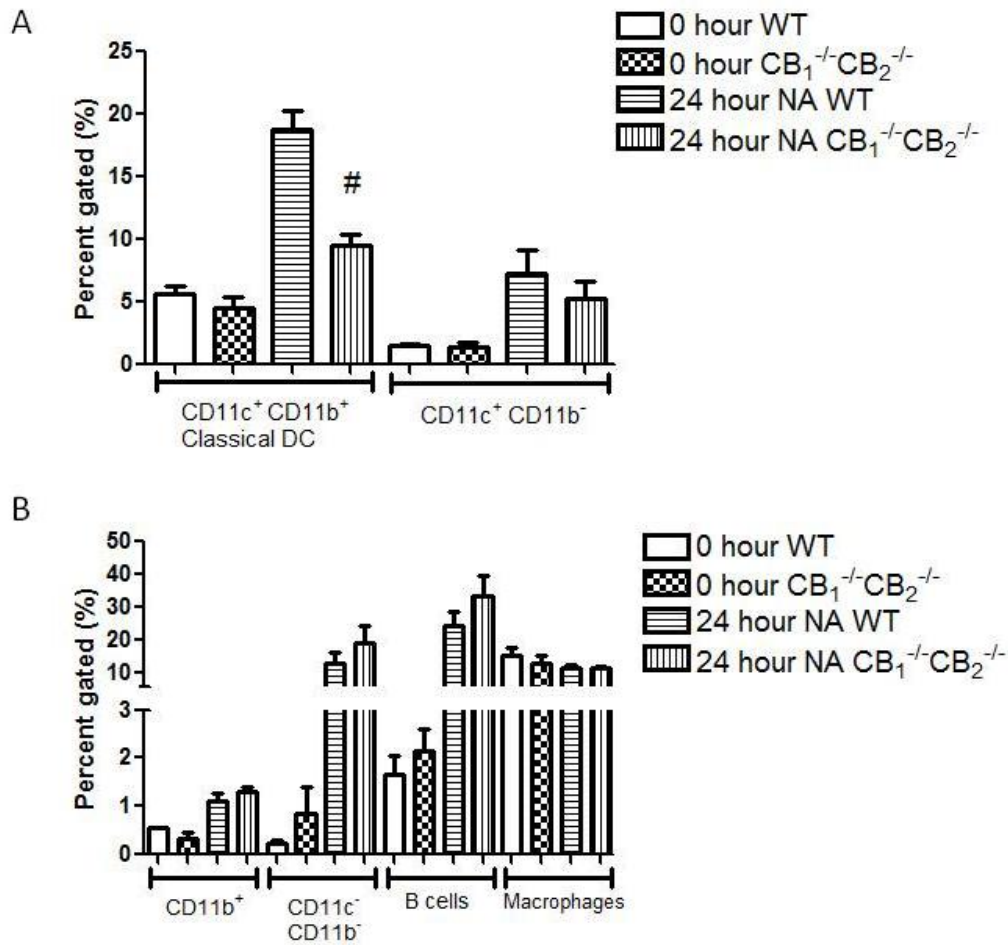


Figure 12. Percent CD83⁺ expression on DCs , B cells, macrophages and CD11b⁺ cells gated within MHC I⁺ bone marrow cells. Representative graphs of 2 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and $CB_1^{-/-}CB_2^{-/-}$ (n=4) mice were cultured in 6 well flat bottom plates at a 5×10^5 cells/mL for 24 h with or without LPS (1 μ g/mL) stimulation. Treatment groups were assessed by flow cytometry for the percent of CD83 expression of A) DCs (CD11c⁺ CD11b⁺, CD11c⁺ CD11b⁻) and B) CD11b⁺, macrophages, B cells and CD11c⁻ CD11b⁻ cells within MHC I⁺ cells. The following antibodies were used: CD11c, CD11b, CD19, F4/80. B cells= CD19⁺ CD11c⁻ CD11b⁻ Macrophages= F4/80⁺ CD11c⁻ CD11b⁺. # = p<0.05 genotype effect

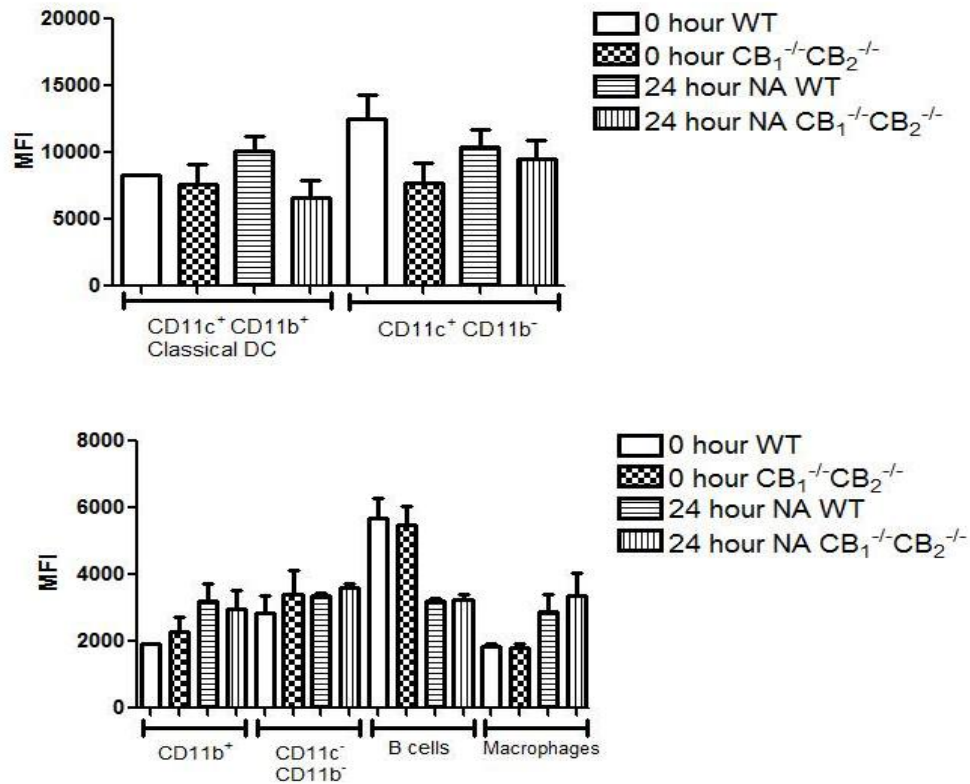


Figure 13. CD83 MFI expression of DCs , B cells, macrophages and CD11b⁺ cells gated within MHC I⁺ bone marrow cells. Representative graphs of 2 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and $CB_1^{-/-}CB_2^{-/-}$ (n=4) mice were cultured in 6 well flat bottom plates at a 5×10^5 cells/mL for 24 with or without LPS (1 μ g/mL) stimulation. Treatment groups were assessed by flow cytometry for the MFI expression of CD83 on A) DCs (CD11c⁺ CD11b⁺ , CD11c⁺ CD11b⁻) and B) CD11b⁺, macrophages, B cells and CD11c⁻ CD11b⁻ cells within MHC I⁺ cells . The following antibodies were used: CD11c, CD11b,CD19, F4/80. B cells= CD19⁺ CD11c⁻ CD11b⁻ Macrophages= F4/80⁺ CD11c⁻ CD11b⁺

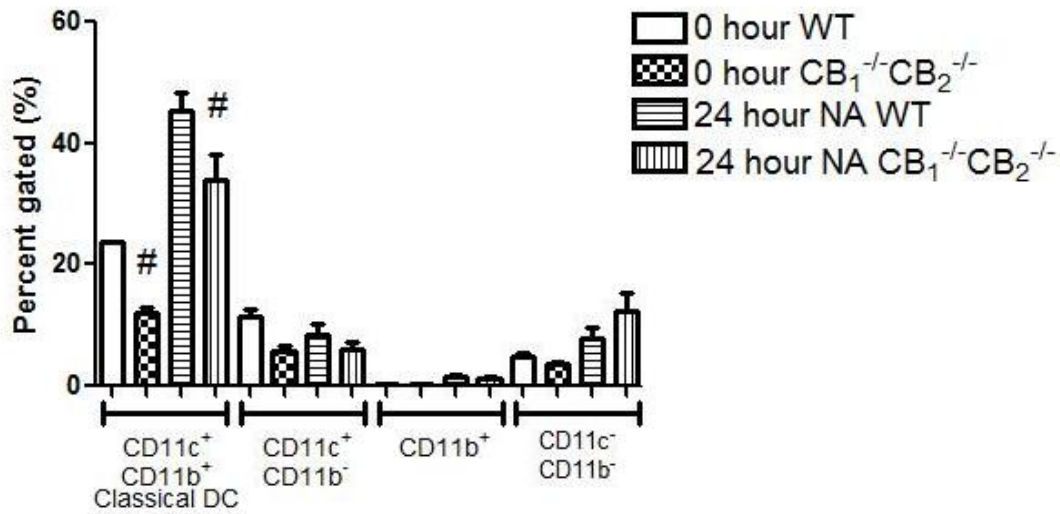


Figure 14. Percent CD86 expression of DCs , B cells, macrophages and CD11b⁺ cells gated within MHC I⁺ bone marrow cells. Representative graphs of 2 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and CB₁^{-/-}CB₂^{-/-} (n=4) mice were cultured in 6 well flat bottom plates at a 5 x10⁵ cells/mL for 24 h with or without LPS (1µg/mL) stimulation. Treatment groups were assessed by flow cytometry for the percent expression of CD86 on CD11c⁺ CD11b⁺ , CD11c⁺ CD11b⁻ , CD11b⁺, CD11c⁻ CD11b⁻ cells within MHC I⁺ cells. # = p<0.05 genotype effect

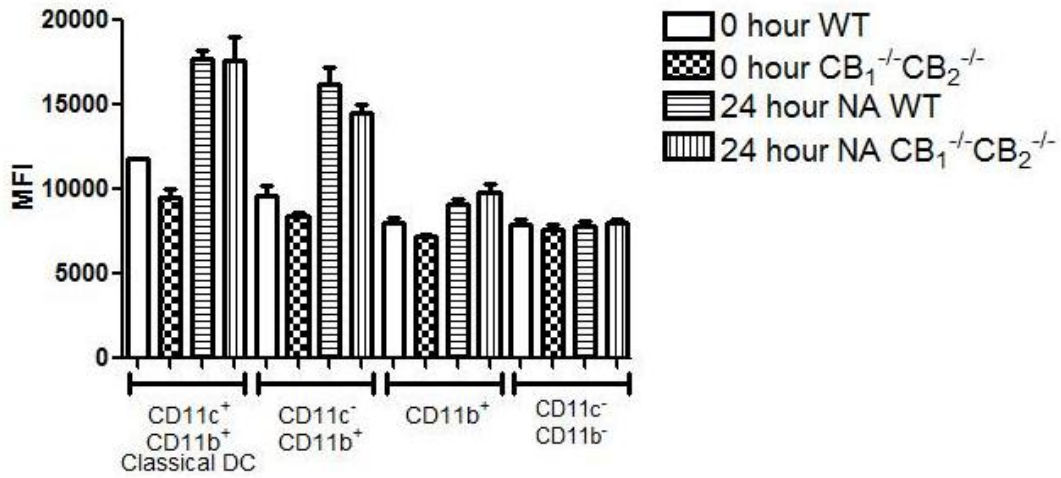


Figure 15. CD86 MFI expression of DCs , B cells, macrophages and CD11b⁺ cells gated within MHC I⁺ bone marrow cells. Representative graphs of 2 separate experiments. Bone marrow cells isolated from femurs and tibia of C57Bl/6 (WT, n=4) and $CB_1^{-/-}CB_2^{-/-}$ (n=4) mice were cultured in 6 well flat bottom plates at a 5×10^5 cells/mL for 24 h with or without LPS (1 μ g/mL) stimulation. Treatment groups were assessed by flow cytometry for CD86 MFI expression on CD11c⁺ CD11b⁺ , CD11c⁺ CD11b⁻ , CD11b⁺ , CD11c⁻ CD11b⁻ cells within MHC I⁺ cells .

DCs must efficiently process an antigen, load the correct size peptide on to the MHC molecules and present it on the surface. The expression of MHC I-SIINFEKL complex expression was assessed on the surface of bone marrow cells in the presence or absence of LPS. There was no significant difference in the expression of MHC I-SIINFEKL complex on naïve bone marrow cells from WT and $CB_1^{-/-} CB_2^{-/-}$. Upon LPS stimulations there was an increase in the MHC I-SIINFEKL complex detected in both WT and $CB_1^{-/-} CB_2^{-/-}$. Additionally, $CB_1^{-/-} CB_2^{-/-}$ mice bone marrow cells stimulated with LPS have a significant increase in the MFI expression of MHC I-SIINFEKL complex in comparison to WT (Figure 16).

Subsequent studies to determine if the antigen used in the co-culture experiments, SIINFEKL, is capable of binding to the MHC I pocket without being internalized first. Bone marrow cells isolated from WT and $CB_1^{-/-} CB_2^{-/-}$ mice were fixed immediately after isolation and pulsed with SIINFEKL for 2 hours then washed and stained with MHC I-SIINFEKL complex antibody. MHC I bound SIINFEKL complexes were detected on the surface of fixed bone marrow cells, suggesting that SIINFEKL peptide did not need to be internalized in order to bind surface MHC I molecules.

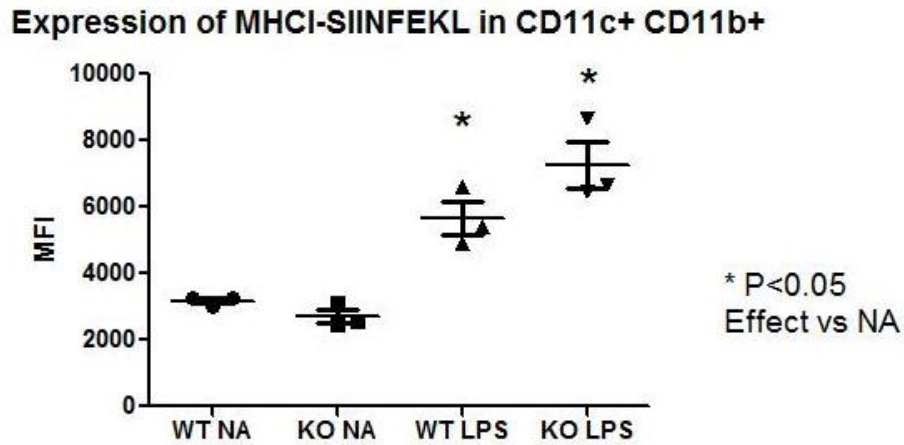


Figure 16. Expression of MHC I-SIINFEKL in bone marrow cells . Representative graphs of 5 separate experiments. Bone marrow cells isolated from WT (n=3) and $CB_1^{-/-}CB_2^{-/-}$ (n=3) mice were cultured in 6 well flat bottom plates at 5×10^5 cells/mL for 24 h with or without LPS (1 μ g/mL) stimulation. Antigen loading on the surface of DCs was determined using a MHC I-SIINFEKL antibody that specifically reacts with ovalbumin-derived peptide SIINFEKL bound to H-2Kb of MHC class I, but not with unbound H-2Kb or H-2Kb bound with an irrelevant peptide. Representative of 4 separate experiments. # p<0.05 effect vs Naïve.

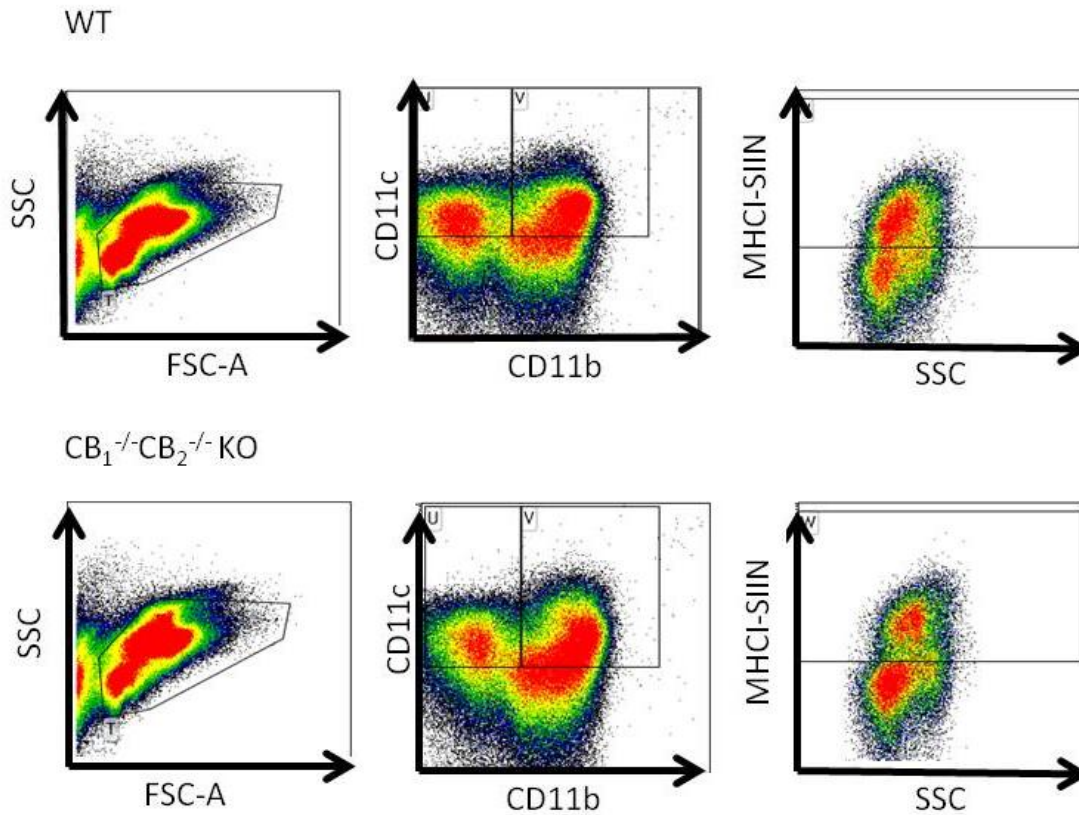


Figure 17. Peptide Internalization is not necessary for antigen loading on the surface of DCs. Representative graphs of 2 separate experiments. Bone marrow cells isolated from WT (n=3) and CB₁^{-/-}CB₂^{-/-} (n=3) mice were fixed with Brefeldin A immediately after isolation and pulsed with SIINFEKL for 2 hours. Then cells were washed and stained with CD11c, CD11b and MHC I-SIINFEKL complex antibody. BMDCs were identified by their size and CD11c and CD11b expression. MHC I-SIINFEKL expression was determined on CD11c⁺CD11b⁺ cells.

CHAPTER 4: DISCUSSION

This dissertation research aimed to characterize the involvement of cannabinoid receptors on DC development, maturation and antigen presenting function in the context of MHC I. For this purpose cannabinoid receptor null mice were used to evaluate the antigen specific CD8⁺ T cell response on bone marrow cells, the composition of DC progenitors and subpopulations on bone marrow cells and their specific expression level of MHC molecules, CD86 and CD83. We found an increase in the percent of lineage committed cells and DCs in CB₁^{-/-}CB₂^{-/-} mouse bone marrow cells but no significant difference in surface MHC I, MHCII and CD86 expression. We did not detect an increase in MHC I-antigen complex on the surface of DCs. Despite no significant increase in MHC I-antigen complex on their surface, naive bone marrow cells from CB₁^{-/-}CB₂^{-/-} mice elicited a CD8⁺ T cell response in an *in vitro* co-culture model. First, we'll begin by discussing the characterization of DC subpopulations in murine bone marrow cells.

4.1 Characterization of DC subpopulations in murine bone marrow cells and their ability to induce a CD8⁺ T cell response.

Essential for the initiation of the adaptive immune response is the DC's antigen presenting function. Karmaus et. al. described in an influenza challenge model an influx of APCs, including inflammatory myeloid cells and monocytes/macrophages, into the lungs in a CB₁- and/or CB₂-dependent manner. *In vitro* studies showed that THC suppressed LPS-induced maturation of bmDCs. Interestingly, naïve bmDCs from CB₁^{-/-}CB₂^{-/-} induced an antigen-specific CD8⁺ T cell response and produce IFN-γ as robustly as LPS stimulated WT bmDC. This response was impaired in WT bmDC upon THC treatment [82]. The bmDCs used for above studies were obtained by the *in vitro* differentiation of bone marrow cells with GM-CSF for 9

days. Culturing bone marrow precursors with GM-CSF is the most commonly used approach to generate DCs. Monocytes have been demonstrated to develop into DCs in this *in vitro* culture system with the addition of IL-4 (13). Despite the broad use to produce large numbers of DCs *in vitro*, GM-CSF is not essential for differentiation in steady state conditions. Studies using GM-CSF receptor knockout mice demonstrated almost no reduction in DC numbers (19). The initial objective was to determine if bone marrow cells from $CB_1^{-/-}CB_2^{-/-}$ could induce a $CD8^{+}$ T cell response without the additional culture with GM-CSF for 9 days. Similar to what Karmaus described, our studies demonstrated that naive bone marrow cells from $CB_1^{-/-}CB_2^{-/-}$ but not WT mice elicited an antigen specific $CD8^{+}$ T cells response in the absence of LPS stimulation.

The exacerbated $CD8^{+}$ T cell response may be partly due to an increase in DC precursors or subpopulation in the bone marrow. To test these possibilities we isolated bone marrow cells from both WT and $CB_1^{-/-}CB_2^{-/-}$ mice and compared the frequency of hematopoietic precursors and DC subpopulations. The lineage cocktail antibody was used to differentiate hematopoietic precursors from more terminally differentiated cells. The percent of lineage positive cells in $CB_1^{-/-}CB_2^{-/-}$ bone marrow was significantly lower in comparison to WT. On the other hand, the percent of lineage negative cells was significantly lower in $CB_1^{-/-}CB_2^{-/-}$ mice when compared to WT bone marrow cells. One possible interpretation is there is an increase in differentiation of hematopoietic precursors into terminally differentiated immune cells, which include DCs. In the same studies we identified MDPs, CDPs and pre DCs out of which only MDPs demonstrated a trend towards an increase in $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells. Surprisingly, no significant difference was detected in the percent of CDPs and pre-DCs between WT and $CB_1^{-/-}CB_2^{-/-}$ mice. When taking into consideration the decreased percent of lineage negative cells in $CB_1^{-/-}CB_2^{-/-}$ mouse bone marrow one would expect a decrease in precursors as well. The marked decrease in

lineage negative cells may be reflected in other hematopoietic cells not measured or identified in these experiments, as for example CLP or CMPs. Also, the notion of an expected decrease in either MDPs, CDPs or pre-DCs in $CB_1^{-/-}CB_2^{-/-}$ mouse bone marrow cells may be erroneous since a decrease in lineage negative cells in general does not necessarily mean a disproportion of a specific cell type. One alternative is that deletion of CBRs leads to a deregulation of transcription factors like Pu.1 and Ikaros or cytokines (e.g. FLT3L and GM-CSF) important in early stage DC development. In preliminary studies we attempted to measure the expression of GM-CSF receptor on the surface bmDCs but due to the unavailability of a fluorescently labeled antibody with high specificity precluded our ability to pursue this avenue of research. One important observation during these experiments is that the literature for the identification of hematopoietic cell populations is not conclusive and as the techniques become more refined more surface protein markers emerge and hence more cell subpopulations are discovered. Not all labs use the same surface markers to identify one specific cell population. Taking this into consideration, an exhaustive literature search was performed to choose the surface markers most commonly used to identify hematopoietic precursors and DC subpopulations as we will show further below.

After assessing the composition of precursor populations, DC subpopulations were identified in freshly isolated and in cultured bone marrow cells to determine if the increase in lineage positive cells correlated with an increase in DCs. We identified and quantified four distinct subpopulations: $CD11b^{+}$, $CD103^{+}$, $CD8\alpha^{+}$ DCs and pDCs at the day of bone marrow isolation and after 24 hours in culture. We found the percent of total $CD11c^{+}$ cells was higher in $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells after 24 hours in culture. The largest population of all of the DCs identified, $CD11b^{+}$ DCs, were significantly increased in naïve $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells after 24 hours of culture. The literature on DC subpopulations and their function notes that not

all DCs have the same antigen presenting capabilities. Among the two DC subpopulations with higher APC function are CD103⁺ and CD8 α ⁺ DCs. Both CD103⁺ and CD8 α ⁺ DCs have been demonstrated to present exogenous antigens on MHC I molecules to CD8⁺ T cells (115, 116, 117). Stimulation of TLR signaling induces secretion of IL12p70, an important inflammatory cytokine, in both CD103⁺ and CD8 α ⁺ DCs (118). Our studies show no difference in the percent of both DC subpopulations between WT and CB₁^{-/-}CB₂^{-/-} mice bone marrow cells but the percent of CD103⁺ and CD8 α ⁺ DCs does not seem to be negatively affected after 24 hours in culture. Previous data from the Kaminski lab in an influenza challenge model found an increased influx of myeloid cells and pDCs in the lung of CB₁^{-/-}CB₂^{-/-} mice (5). In support of the increased influx of cells observed by Karmaus et.al., we show an increase in lineage positive cells and subsequently an increase in CD11c⁺ cells. Only CD11b⁺ DCs were significantly increased in CB₁^{-/-}CB₂^{-/-} bone marrow cells. Since both CD103⁺ and CD8 α ⁺ DCs are specialized for antigen cross presentation, we expected to observe an increase their percent expression in CB₁^{-/-}CB₂^{-/-} mice bone marrow cells. All DCs, including pDCs, express the integrin CD11c, which is routinely used in conjunction with other markers in the identification of DCs (16). The DC subpopulations identified above reside throughout the body. For example CD103⁺ DCs reside in the skin, lungs and gut, while CD8 α ⁺ DCs reside in the spleen, thymus and lymph nodes (40). One possible reason we did not detect any differences in the bone marrow is that DCs like CD103⁺ and CD8 α ⁺ DCs quickly migrate to the organs of residency once differentiation is completed (115). Follow up studies would focus on identifying DC subpopulations within several organs like the spleen and lung.

4.2 Expression of MHC and co-stimulatory molecules as well as surface antigen loading capabilities on DC subpopulations

Essential to the viral immune response is the activation of $CD8^+$ T cells into Cytotoxic T cells (CTLs). In order for CTLs to acquire their ability to kill infected cells, $CD8^+$ T cells must first be primed in the context of MHC I by a professional antigen presenting cell, primarily mature DCs. We assessed MHC I expression in all of the DC subpopulations to determine if there was an increase in expression that may account for the exacerbated antigen specific $CD8^+$ T cell response observed. Interestingly, MHC I expression on all DC subpopulations from $CB1^{-/-}$ $CB2^{-/-}$ mouse bone marrow was higher on freshly isolated bone marrow cells. Although only $CD103^+$ DCs and $CD8\alpha^+$ DCs from $CB1^{-/-}$ $CB2^{-/-}$ mice bone marrow had a statistically significant increase in MHC I expression on freshly isolated bone marrow. After 24 hours in culture the expression is reversed and WT has a trend toward higher expression of MHC I in comparison to $CB1^{-/-}$ $CB2^{-/-}$ in all of the DC subpopulations measured. All nucleated cells express MHC I on their surface and are constantly displaying cellular protein peptides on their surface without inducing a CTL response. MHC I unbound to peptide is structurally unstable on the surface of cells and is targeted for internalization and degradation. The broken down components of the internalized unbound MHC I molecule will be used to form new MHC I molecules (63). Our results are in contrast to previous studies by Karmaus et. al., which showed that bmDCs differentiated for 9 day with GM-CSF had a higher expression of MHC I expression after 24 hours in culture. Over 90% of the cells in the 9 day differentiation model with GM-CSF were driven toward a monocyte DC phenotype while our culture system is comprised of a heterogeneous population of cells. In our studies we have confirmed the presence of DC precursors, several subsets, B cells and macrophages all of which will influence the cellular

environment either by cell-to-cell communication or secretory factors. The interaction between other cells types in our culture may influence the behavior of the cells. Additionally, DCs are not the only APCs present in bone marrow cells. Our studies identified macrophages and B cells that may influence the response to peptides presence and the cell culture microenvironment. Despite our contrasting findings of MHC I expression after 24 hours in culture, looking at the MHC I expression of freshly isolated bone marrow cells may be more indicative of the immune status of the mouse *in vivo*. We found an increased level of MHC I expression in all DC subtypes from the $CB_1^{-/-}CB_2^{-/-}$ mice which may suggest a heightened immune status.

DCs reside in tissues of the body as sentinels, inspecting their surrounding environment. In response to encountering a foreign pathogen, DCs engulf the pathogen, produce peptide and load onto MHC molecules to subsequently stimulate naive T cells (11, 23). Our results have demonstrated an increase in the frequency of DCs in $CB_1^{-/-}CB_2^{-/-}$ mouse bone marrow, but no increase in MHC I expression after 24 hours in culture. Our following goal was to determine if $CB_1^{-/-}CB_2^{-/-}$ DCs have an increased ability to present antigen on their surface. Bone marrow cells isolated from WT and $CB_1^{-/-}CB_2^{-/-}$ mice were cultured for 24 hours then pulsed with SIINFEKL peptide and stained with an antibody that recognizes surface MHC I molecules bound to SIINFEKL. No significant difference in quantity of surface MHC I-SIINFEKL complexes were found between WT and $CB_1^{-/-}CB_2^{-/-}$ mice. Initially we speculated that CBRs could influence antigen proteolysis and loading on to MHC molecules in the ER. Hence we expected that bone marrow DCs from $CB_1^{-/-}CB_2^{-/-}$ mice would have an increased quantity of MHC I-SIINFEKL complex expression on their surface. The antigen used in the co-culture experiments, SIINFEKL, is the correct size to bind surface unbound MHC I receptors and possibly circumvent the internalization and processing pathway. On follow up studies we detected MHC I bound

SIINFEKL complexes on the surface of fixed bone marrow cells. Suggesting that SIINFEKL peptide did not need to be internalized in order to bind surface MHC I molecules. We had already demonstrated no significant changes in MHC I surface expression after 24 hours in culture. Assuming that our Ag peptide used in these experiments does not need to be internalized, it would make sense that we did not observed any changes in MHC I-SIINFEKL complex expression on the surface of bone marrow cells.

Simultaneously while processing a foreign antigen, DCs begin a cellular process termed maturation that involves the upregulation of surface MHC II molecules as well as T cell co-stimulatory molecules such as CD86 (23). MHC II expression on pDCs, CD103⁺ DCs and CD8α⁺ DCs from CB1^{-/-}CB2^{-/-} demonstrated higher expression in freshly isolated bone marrow cells in comparison to WT. We found no significant difference in MHC II expression between WT and CB1^{-/-}CB2^{-/-} bone marrow cells on all four DC subsets mentioned above. CD103⁺ DCs and pDC's MHC II expression on CB1^{-/-}CB2^{-/-} cells decreased while CD8α⁺ DCs maintained their level of expression. WT CD8α⁺ DCs after 24 hours in culture increased their MHC II expression to the level of CB1^{-/-}CB2^{-/-} and demonstrated a significantly higher expression upon LPS stimulation. The percent of CD86 expression was significantly lower in classical DCs in fresh bone marrow cells and after 24 hours in culture. Previous studies by Karmaus demonstrated an increase in MHC II and CD86 on classical DCs on naïve and LPS stimulated groups. Our results showed similar results on freshly isolated bone marrow but in our culture system without GM-CSF we observed a decrease in MHC II and CD86.

Maturation in DCs is commonly determined by the double positive expression of MHC II and CD86 (23). Our results show that on fresh bone marrow cells from CB1^{-/-}CB2^{-/-} mice, CD103⁺ DCs had a higher percent of MHC II⁺ CD86⁺ expression. After 24 hours in culture the

percent of mature cells in all DC subpopulations trended to be lower on $CB_1^{-/-}CB_2^{-/-}$ cells. The percent of mature classical DC is significantly decreased in $CB_1^{-/-}CB_2^{-/-}$ on freshly isolated bone marrow cells. In addition our studies measured CD83 expression, a marker recently described as being involved in maturation and activation of naïve T cells. The percent CD83 expression in classical DCs found on $CB_1^{-/-}CB_2^{-/-}$ mice bone marrow cells was significantly decreased in comparison to WT after 24 hours in culture. Initially we hypothesized that naïve $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells elicited a $CD8^+$ T cells response due to an increase in mature DC providing a population of cells ready to respond. Immature DCs engulf pathogens and begin their breakdown while simultaneously beginning their maturation process. Mature DCs decrease their phagocytic capabilities hence they are unreactive to new pathogen (20). We found an increase in the percent DC populations in $CB_1^{-/-}CB_2^{-/-}$ mice bone marrow and no significant increase in $MHC\ II^+ CD86^+$ double positive expression in comparison to WT bone marrow cells. Our initial thought of an increased mature DC population was incorrect since having a reactive population of DCs will be beneficial for a prompt immune response. Our data suggest that an increase in the population of immature DCs in $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells is, in part, the cause of the exacerbated $CD8^+$ T cell response observed in $CB_1^{-/-}CB_2^{-/-}$ bone marrow cells.

In summary, our studies show that $CB_1^{-/-}CB_2^{-/-}$ mouse bone marrow contain an increased $CD11c^+$ cell population. When cultured for 24 hours naïve bone marrow cells from $CB_1^{-/-}CB_2^{-/-}$ mice elicit a $CD8^+$ T cell response in the absence of LPS stimulation. Several DC subpopulations: $CD11b^+$, $CD103^+$, $CD8\alpha^+$ DCs and pDCs were identified and their MHC I, MHC II and CD86 expression was not significantly increased after 24 hours in culture. MHC I expression on freshly isolated bone marrow cells was significantly higher in all of the DCs from $CB_1^{-/-}CB_2^{-/-}$ mice in comparison to WT. In addition to DCs, B cells and macrophages were

identified in murine bone marrow cells from both WT and $CB_1^{-/-}CB_2^{-/-}$ mice and demonstrated no significant difference in their CD83 expression. The data from this thesis suggest that CBRs influence the development of DC in the bone marrow, their surface marker expression and maturation status. Although an increase in DC numbers could potentially give $CB_1^{-/-}CB_2^{-/-}$ mice an advantage in the clearance of a pathogen, this alone does not explain their ability to circumvent the canonical LPS stimulation required for maturation of DCs.

Soluble regulatory factors like cytokines and chemokines help shape the immunological response by promoting or inhibiting differentiation, maturation, migration and survival signals. Future studies directed at exploring the levels of secreted regulatory factors like IL-12, IL-6, FLT3L and GM-CSF will help clarify which pathways or transcription factors are regulated by CBRs on DCs that affect their differentiations and APC function. A limitation to our studies is that the mouse bone marrow contains a heterogeneous population of undifferentiated and differentiated cells. Among the APCs, bone marrow cells contain B cells, macrophages and DCs. Future studies will have to address this issue by isolating pure DCs using negative selection magnetic bead isolation. Isolating DCs will confirm that co-culture results and cytokine profile are due to DC populations in the bone marrow.

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