

CANNABINOID RECEPTOR 2-SELECTIVE LIGANDS  
AS IMMUNOSUPPRESSIVE COMPOUNDS:  
UTILITY IN GRAFT REJECTION

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## ABSTRACT

Cannabinoids are known to have anti-inflammatory and immunomodulatory properties. Cannabinoid receptor 2 (CB2) is expressed mainly on leukocytes and is the receptor implicated in mediating many of the effects of cannabinoids on immune processes. The capacity of  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) and of two CB2-selective agonists to inhibit the murine Mixed Lymphocyte Reaction (MLR), an in vitro correlate of graft rejection following skin and organ transplantation was tested. Both CB2-selective agonists and  $\Delta^9$ -THC significantly suppressed the MLR in a dose dependent fashion. The inhibition was via CB2, as suppression could be blocked by pretreatment with a CB2-selective antagonist, but not by a CB1 antagonist, and none of the compounds suppressed the MLR when splenocytes from CB2 deficient mice were used. The CB2 agonists were shown to act directly on T-cells, as exposure of CD3<sup>+</sup> cells to these compounds completely inhibited their action in a reconstituted MLR and proliferation of purified T-cells by anti-CD3 and anti-CD28 antibodies was inhibited. Treatment of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells with a CB2-selective agonist inhibited the MLR, though significantly less than when both cell types were treated. T-cell function was decreased by CB2 agonists, as an ELISA of MLR culture supernatants revealed IL-2 release was significantly reduced in the cannabinoid treated cells. Further, treatment with O-1966 dose-dependently decreased levels of the active nuclear forms of the transcription factors NF- $\kappa$ B and NFAT in wild-type T-cells, but not T-cells from CB2 knockout (CB2R k/o) mice. Additionally, a gene expression profile of purified T-cells from MLR cultures, generated using a PCR T-cell activation array, showed that O-1966 decreased mRNA expression of

CD40 ligand and CyclinD3, and increased mRNA expression of Src-like-adaptor 2 (SLA2), Suppressor of Cytokine Signaling 5 (SOCS5), and IL-10. The increase in IL-10 was confirmed by measuring IL-10 protein levels in MLR culture supernatants. An increase in the percentage of regulatory T-cells (Tregs) was observed in MLR cultures and pretreatment with anti-IL-10 resulted in a partial reversal of the inhibition of proliferation and blocked the increase of Tregs. Additionally, O-1966 treatment caused a dose-dependent decrease in the expression of CD4 in MLR cultures from wild-type, but not CB2R k/o, mice. The ability of O-1966 treatment to block rejection of skin grafts in vivo was also tested. Mice received skin grafts from a histoincompatible donor, and the time to graft rejection was analyzed. Compared to mice that received the vehicle, mice that received O-1966 treatment had significantly prolonged graft survival and increased Tregs in the spleen. The spleen cells from O-1966-treated mice had reduced proliferation in an MLR and an increased percentage of Tregs. Together, these data support the potential of this class of compounds as useful therapies to prolong graft survival in transplant patients and possibly as a new class of immunosuppressive drugs.

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## LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AP-1	Activator protein-1
APC	Antigen-presenting cell
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
cDNA	Complementary DNA
CNI	Calcineurin inhibitor
CNS	Central nervous system
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
$\Delta^9$ -THC	$\Delta^9$ - tetrahydrocannabinol
DAG	1,2-diacyl- glycerol
DC	Dendritic cell
DEPC	Diethyl pyrocarbonate
dH <sub>2</sub> O	Deionized H <sub>2</sub> O
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate

EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveoretinitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Foxp3	Factor forkhead box P3
GPCR	G protein-coupled receptor
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T-cell co-stimulator
IFN $\gamma$	Interferon-gamma
IL-	Interleuken
i.p.	Intraperitoneal
k/o	Knockout
LPS	Lipopolysaccharide
LT	Lymphotoxin
MHC-I	Major Histocompatibility Complex class 1
MHC-II	Major Histocompatibility Complex class 2
MLR	Mixed lymphocyte reaction
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T-cells

OPTN	Organ Procurement and Transplantation Network
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RBC	Red blood cells
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute medium 1640
SCID	Severe combined immunodeficiency
SRTR	Scientific Registry of Transplant Recipients
TCR	T-cell receptor
TGF- $\beta$	Transforming growth factor beta
Th	T helper cell
TNF	Tumor necrosis factor
Treg	Regulatory T-cell
VCAM-1	Vascular cell adhesion protein 1
WT	Wild-type

# **CHAPTER 1**

## **INTRODUCTION**

### **Transplantation**

The first reported successful human organ transplantation occurred in 1954 when a healthy kidney was transplanted from a healthy donor to the donor's identical twin (Merrill et al. 1956). Today, over 30,000 organ transplantations are performed annually in the United States and about 52,000 worldwide, including those of the kidney, heart, lung, pancreas, liver, and intestine (SRTR/OPTN 2011). The Organ Procurement and Transplantation Network (OPTN), operated under contract with the U.S. Department of Health and Human Services, and the Scientific Registry of Transplant Recipients (SRTR) collect medical data from healthcare providers on all transplants nationwide and jointly release annual reports of the data. Previously, the obstacle to achieving graft survival was the inability to prevent immunologic organ rejection between allogeneic donors and recipients. This obstacle was overcome by two critical breakthroughs (Morris 2004). First, the development of tissue typing permitted assessment of the relatedness of the major histocompatibility complex (MHC) of the prospective donor and recipient to determine compatibility. Second was the discovery of immunosuppressive compounds that could be administered chronically to the recipient to prevent rejection. These advancements led to organ transplantation as a feasible treatment option for end-stage organ failure, although several problems must be solved to improve patient outcome,

including the loss of graft from rejection and complications associated with the available immunosuppressive drugs.

### **Allorecognition**

The ability of transplant recipients to recognize donor-derived antigens is called allorecognition and is responsible for the initiation of allograft rejection. The major histocompatibility complex is known as the human leukocyte antigens (HLA) and is comprised of two classes. MHC class I and MHC class II. MHC class I is expressed by all nucleated cells and presents peptides to CD8<sup>+</sup> T-cells cells, while MHC class II is expressed predominantly by antigen presenting cells (APCs) and presents peptides to CD4<sup>+</sup> T-cells (Neefjes et al. 2011). The T-cells, which respond to the foreign tissue peptides, are the mediators of graft rejection (Heeger 2003). According to the U.S. Department of Health & Human Services guidelines, donor and recipient MHC should be matched at seven polymorphic loci of the MHC class I and class II genes to prevent a strong immune response elicited by a MHC mismatch of allogeneic tissues, (*"Histocompatibility Laboratory Testing Requirements"*), thereby drastically reducing this severe trigger of allograft rejection.

However, even stringent MHC matching cannot prevent differences in minor histocompatibility antigens. The likelihood of inducing an anti-graft response is great due to the large number of proteins that can be presented on donor MHC that differ from the recipient. Typically, the magnitude of these reactions is less severe than grafts containing MHC-mismatches, but over time can decrease the function and viability of the graft

(Simpson et al. 2002). In spite of good matches at the major histocompatibility loci, after 5 years, only 40% of intestine, 50% of lung and pancreas, and 75-80% of heart and kidney transplants are viable (OPTN/SRTR 2011). Both CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells specific for minor antigens have been isolated from humans and animals rejecting grafts (Akatsuka et al. 2003, Simpson et al. 1997, Pietra et al. 2000, Haskova et al. 2003). In fact, even before transplantation, up to 10% of an individual's T-cell repertoire can react to alloantigens (Ashwell et al. 1986, Suchin et al. 2001). The high frequency of alloreactive T-cells can be attributed to the diversity of the T-cell repertoire, the large number of donor MHC/peptide complexes on the graft to which a potential recipient has not been tolerized, and the relatively low affinity of the TCR for its MHC/peptide ligand, which, could potentially result in a T-cell recognizing more than one MHC/peptide complex (Garcia et al. 1999, Brock et al. 1996, Kaye & Hedrick 1988, Matzinger & Bevan 1977a). Further, molecular mimicry can occur, causing T-cells specific for a foreign peptide to crossreact with alloantigens (Ashwell et al. 1986, Ben-Nun et al. 1983, Pantenburg et al. 2002).

### ***Direct Priming***

Allorecognition can occur by two separate mechanisms, depending on the source of the APC. Direct priming occurs when recipient T-cells directly interact with intact MHC on donor cells, resulting in a response nearly identical to T-cells that recognize a pathogen peptide bound on self-MHC (Reiser et al. 2000). Direct priming can occur when MHC molecules between donor and recipient are similar enough to interact, which

occurs often since transplants only occur between closely matched donors and recipients and the majority of MHC polymorphisms occur within the peptide binding grooves (Pease et al. 1991). Thus, recipient TCR and donor MHC ligation occur, and trigger subsequent T-cell activation (Kaye & Hedrick 1988, Daniel et al. 1998). In addition to this type of T-cell interaction with the peptide/MHC complex, which is well classified (Berkowitz & Braunstein 1992, Catipovic et al. 1992, Bluestone et al. 1992), there is also increasing evidence indicating some alloreactive T-cells can recognize allo-MHC molecules, irrespective of the peptide presented (Schneck et al. 1989, Villadangos et al. 1994), though this is still thought to be dependent on polymorphisms in the peptide binding region (Lombardi et al. 1991).

Dendritic cells (DCs) are the main antigen-presenting cells (APCs) that provide the major immunogenic stimulus to directly activate recipient T-cells (Lechler & Batchelor 1982). DCs are generally required for robust activation from direct priming (Fernandes et al. 2011). Graft rejection in recipients closely matched in MHC alleles have significantly delayed rejection if grafts from DC-depleted donors are used (Wang et al. 2004), a delay which can be reversed with the transfer of donor DCs to the recipient post-transplantation (Lechler & Batchelor 1982). During direct priming, recipient T-cells encounter donor DCs expressing MHC/peptide that have migrated to secondary lymphoid organs (Larsen et al. 1990, Ingulli et al. 1997, Steinman et al. 1997). T-cell priming and graft rejection do not occur when secondary lymphoid organs are absent (Lakkis et al. 2000, Barker & Billingham 1968) or if donor DCs are not able to migrate out of the graft (McKay et al. 2006). Donor DCs are predominantly responsible for the direct priming of

both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, as many alloreactive CD8<sup>+</sup> T-cells need helper signals from CD4<sup>+</sup> T-cells (Schuler & Steinman 1985, Schoenberger et al. 1998).

Direct T-cell priming likely only mediates early rejection and is time-limited, as donor APCs are destroyed during the priming process and die over time (Saiki et al. 2001). However, the initial high frequency of directly primed T-cells may result in the formation of allogenic memory T-cells (Zhai et al. 2002), although several studies have shown patients exhibiting renal allograft dysfunction several years post-transplantation do not have any evidence of CD4<sup>+</sup> or CD8<sup>+</sup> T-cells activated by the direct pathway (Baker et al. 2001a, Baker et al. 2001b). The induction of directly primed T-cells can be significantly attenuated if the graft is first transplanted into an intermediate host receiving immunosuppression for a sufficient time period to allow for the depletion of donor DCs (Lechler & Batchelor 1982, Roussey-Kesler et al. 2005), though rejection can still be mediated by other mechanisms.

### ***Indirect Priming***

The second mechanism of allorecognition is by indirect priming, in which recipient APCs process and present donor antigen to recipient T-cells (Lechler & Batchelor 1982, Auchincloss et al. 1993, Benichou et al. 1992). Therefore, all proteins not endogenous to the recipient have the potential to elicit an alloreactive response. Recipient APCs may endocytose donor antigen at the graft, migrate to secondary lymphoid organs, and prime recipient T-cells (Denton et al. 1999). Similar to direct priming, many of the presented peptides are from the polymorphic regions of the donor

MHC molecules (Benichou et al. 1992, Benichou et al. 1994, Watschinger et al. 1994). Recipient APCs in secondary lymphoid organs may present donor MHC antigens after the endocytosis and processing of donor cells that express MHC, including donor APC (Lechler & Batchelor 1982, Benichou et al. 1992), or soluble donor MHC molecules shed from the graft (Demaria & Bushkin 2000). In addition to priming CD4<sup>+</sup> T-cells via the MHC II processing pathway, DCs have been shown to interact with and prime CD8<sup>+</sup> T-cells, in a process called cross-priming (Matzinger & Bevan 1977b). DCs have been shown to be capable of priming alloreactive CD8<sup>+</sup> T-cells both in vitro and in vivo (Benichou et al. 1992, Albert et al. 1998, Yewdell et al. 1999, Brossart & Bevan 1997).

While direct priming is usually time limited, indirect priming could occur indefinitely because recipient APCs can migrate through the graft and endocytose donor antigen at any time post-transplantation. While the frequency of indirectly primed T-cells is approximately only 5-10% of the alloimmune repertoire, it is thought to be the main contributor to chronic allograft dysfunction (Benichou et al. 1999, Liu et al. 1993). Detection of indirectly primed T-cells correlates to episodes of rejection (Ciubotariu et al. 1998, Vella et al. 1997a). However, in the clinical setting, where MHC types are closely matched, it is often difficult to discern between direct and indirect donor antigen presentation.

### **Allograft Destruction**

Graft destruction that occurs by an alloimmune response is carried out by various effector cells, often with redundant mechanisms, that can result in antigen-specific cell

injury or the interruption of the physiological function of the graft through non-specific inflammatory response. Graft rejections are classified as hyperacute, acute, and chronic, but these classifications are based on the time post-transplantation and how long the graft remains viable after the start of rejection, not on the mechanism of rejection. Hyperacute rejection occurs minutes to hours after transplantation, while acute rejection can occur one week to years post-transplantation and develops quickly. Chronic rejection develops slowly over the course of months to years (SRTR/OPTN 2011). The pathogenesis of allograft rejection is caused by two interrelated effector elements: lymphocyte mediated cytotoxicity and alloantibodies. However, it is important to note that allograft rejection in human transplant recipients is complex and destruction of the graft may be by combinations of effector mechanisms that vary between patients, depend on the type of graft, the nature of the MHC disparity between the recipient and donor, and the type of immunosuppression medications used (Heeger 2003, Saleem et al. 1996, VanBuskirk et al. 1996, Schulz et al. 1995).

### ***Lymphocyte-Mediated Cytotoxicity***

Lymphocyte-mediated cytotoxicity has long been implicated in the destruction of allografts. T-cells can mediate rejection starting at just one week post-transplantation and can occur throughout the life of the graft (Heeger 2003). T-cells are vital to the alloimmune response, as T-cell deficient mice were unable to reject mismatched allografts, but rejection was reestablished if alloreactive T-cells were transferred to the mice (Rocha et al. 2003). CD8<sup>+</sup> T-cells, also called Cytotoxic T-Lymphocytes (CTLs),

are often involved in rejection (Arakelov & Lakkis 2000). The importance of CTLs in graft rejection was indicated in studies showing donor-specific CTLs could be found in human renal allografts undergoing rejection (Strom et al. 1975). In mice, the adoptive transfer of CTLs sensitized to the donor alloantigens was shown to be able to induce the rejection of MHC-I mismatched skin grafts (Rosenberg et al. 1987, Halamay et al. 2002). As stated previously, CD8<sup>+</sup> T-cells are able to be primed directly by donor APC and indirectly by recipient APC (Benichou et al. 1992, Yewdell & Bennink 1999), as well as cross-primed by recipient DCs (Albert et al. 1998, Yewdell et al. 1999, Brossart & Bevan 1997). In addition, CTLs can be induced by antigen presentation on MHC-I on activated donor vascular endothelium (Kreisel et al. 2002).

After CTLs are primed, the surface molecules CD62L, a lymph node homing receptor, is down-regulated and CD44, an activation/adhesion receptor, is up-regulated, allowing the circulation of activated CTLs in the periphery, where they can re-encounter their specific antigen on graft cells (Oehen & Brduscha-Riem 1998). 1-3 days after activation, cytotoxic granules containing perforin and granzymes can be detected within CTLs, and upon specific interaction with a target cell expressing the specific ligand complexed to MHC-I, the cytotoxic granules fuse with the cell membrane and release the contents into the immunological synapse (Oehen & Brduscha-Riem 1998). Perforins assemble into polyperforins and insert into the target cell membrane to facilitate the uptake of granzyme B, which activates caspases to induce apoptosis (Metkar et al. 2002). The significance of the caspase pathway in allograft rejection has been recently studied and levels of capase-3 were found to correlate with the rejection status in human cardiac

allografts (Narula et al. 2001). Further, caspase inhibitors were found to block rejection in rat cardiac allografts (Barr et al. 2000, Shim et al. 2002).

CTLs can also induce target cell apoptosis via the Fas pathway (Ju et al. 1994), though studies suggest this mechanism is secondary to the granzyme pathway (Krupnick et al. 2002, Wever et al. 1998). FasL can be packaged into cytotoxic granules and released into target cells or expressed on the CTL surface to interact with Fas expressed on the target cell membrane, both of which result in caspase-dependent apoptosis (Ju et al. 1994). Fas and FasL expression are detected during rejection, but their presence alone is not indicative of rejection (Xu et al. 2001, Jain et al. 2008). In fact, high expression of FasL on donor cells has been shown to inhibit rejection (Porter et al. 2000, Bellgrau et al. 1995) by inducing apoptosis in effector cells expressing Fas (Tourneur et al. 2001).

In addition to targeting cells of the graft, CTLs may mediate rejection by inducing the apoptosis of graft-associated endothelial cells, resulting in vasculitis (Kreisel et al. 2002, Valujskikh et al. 2002). The rejection of grafts that demonstrate pathologic evidence of vasculitis can exhibit endothelialitis, arteriosclerosis, vasculopathy, or perivascular inflammation (Krupnick et al. 2002). Damage to the graft-associated endothelium, as in cases of vasculitis, can impede blood supply, thus resulting to ischemic damage of the graft (Kreisel et al. 2002, Valujskikh et al. 2002). Indeed, CTLs have been shown to directly mediate vasculopathy in transplanted heart vessels in mice, resulting in graft destruction (Ensminger et al. 2000).

CD8<sup>+</sup> CTLs are not the sole mediators of cytotoxicity in graft rejection, as CD4<sup>+</sup> T-cells also have a well-documented role in cytotoxicity (Krieger et al. 1996). In fact,

there is some evidence that the priming of some CD8<sup>+</sup> CTLs requires help from CD4<sup>+</sup> T-cells (Schoenberger et al. 1998, Ridge et al. 1998). Primed CD4<sup>+</sup> T-cells can, under the proper stimulation, differentiate into Th1 cells which release IL-2, interferon gamma (IFN- $\gamma$ ), and tumor necrosis factor alpha and beta (TNF- $\alpha$  and TNF- $\beta$ ) to stimulate the growth, differentiation and survival of T-cells and to activate neutrophils and macrophages, all of which are known to infiltrate grafts during rejection (Mason et al. 1984, Auchincloss et al. 1993, Lee et al. 1994, Vella et al. 1997b, Valujskikh & Heeger 2000). The recruitment and activity of neutrophils and macrophages in mediating graft rejection has been shown in animal models of rejection, where inhibiting macrophages (Wyburn et al. 2005) or neutrophils (Surquin et al. 2005, Hirayama et al. 2006) delayed rejection. These cytokines also alter vascular permeability and decrease vascular integrity, which weakens the graft (Carrodeguas et al. 1999, Sirak et al. 1997). A study by Dalloul et al. showed that CD8-deficient mice were completely able to reject allogeneic skin grafts. Further, when the responding CD4<sup>+</sup> T-cells were transferred to T-cell deficient skin graft recipient mice that were previously unable to reject skin grafts, the grafts were promptly rejected (Dalloul et al. 1996). Further, transfer of an alloreactive Th1 cell line was shown to mediate rejection (Valujskikh et al. 1998). Further, in human transplant recipients and mouse models of transplantation, long-term graft acceptance is associated with low CD4<sup>+</sup> T-cell responses (VanBuskirk et al. 2000, Bickerstaff et al. 2001).

In addition to promoting the activation and effector function of CD8<sup>+</sup> T-cells, CD4<sup>+</sup> T-cells can directly mediate rejection independently of CD8<sup>+</sup> T-cells. Analogous to

the mechanism of CD8<sup>+</sup> CTL-mediated cytotoxicity, CD4<sup>+</sup> T-cells can induce apoptosis via the perforin/granzyme pathway and the Fas pathway (Williams & Engelhard 1996, Tateyama et al. 2000). Rejection was significantly delayed in murine allogeneic heart transplants when recipients expressed FasL-deficient CD4<sup>+</sup> T-cells (Grazia et al. 2010). Several studies have shown that CD4<sup>+</sup> T-cells can exhibit cytotoxicity when primed either directly (Pietra et al. 2000) or indirectly (Lee et al. 1994, Braun et al. 2001, Valujskikh et al. 1998). There is some evidence indicating the action of CD4<sup>+</sup> T-cells is dependent on the frequency of primed cells; high frequencies result in acute graft rejection, while low frequencies induce vasculopathy and/or fibrosis (Pietra et al. 2000, Le Moine et al. 1998). However, the development of fibrosis is a multiphase process and is generally considered a hallmark pathology of chronic rejection. Fibrosis begins with inflammatory cells, primarily macrophages and T-cells, and activation of fibroblasts and myofibroblasts follows (Racusen & Regele 2010). Several studies have concluded that damage of grafts mostly occurs in situations where there is a combination of fibrosis and ongoing immune injury (Racusen & Regele 2010).

### ***Alloantibody-Mediated Rejection***

Hyperacute rejection is the most immediate and robust alloimmune response, occurring only minutes to days post-transplantation (SRTR/OPTN 2011). This reaction occurs when allogeneic antibodies recognize donor MHC or blood group antigens from prior exposure due to pregnancy, blood transfusion, or previous transplantations (Rocha et al. 2003). Organ function is rapidly diminished from antibody deposition, complement

activation, and vascular destruction (Rosenberg et al. 1971). Highly vascularized grafts, such as kidneys, are most susceptible to ischemic damage from the obstruction of small vessels due to hemagglutination and platelet-agglutination that occur during the reaction (Rosenberg et al. 1971). However, hyperacute rejection is now rare because cross-matching and testing for allogeneic antibody is common (SRTR/OPTN 2011).

Alloantibodies also mediate both acute and chronic graft rejection, in many clinical cases of rejection, the presence of donor-specific antibodies correlates with rejection (Solez et al. 2008, Przybylowski et al. 1999, Qian et al. 1998). Additionally, infiltrating B-cells in graft biopsies correspond to episodes of rejection (Hippen et al. 2005, Lehnhardt et al. 2006). Recent studies have shown heart transplants in B-cell-deficient mice had delayed rejection compared to WT mice (Wasowska et al. 2001). This delay was abrogated when alloantibodies that activate complement were administered following transplantation, but not when alloantibodies that did not activate complement were administered (Murata et al. 2007, Rahimi et al. 2004). However, the initiation of the alloantibody response is still thought to require T-cell help. Experiments in mice suggest that in order to induce B-cell help, CD4<sup>+</sup> T-cells must be indirectly primed, probably because MHC-II on B-cells must interact with CD4 on the T-cells (Pettigrew et al. 1998). CD4<sup>+</sup> T-cell stimulation helps drive naïve B-cell proliferation and differentiation into antibody-secreting plasma cells and memory B-cells (Lanzavecchia et al. 2006).

The infiltration of B-cells and other immune cells mediating rejection into grafts can become severe over time. Human kidney allografts undergoing chronic rejection have a 50-fold increase of lymphatic-vessel density compared to regular kidneys and exhibit

nodular infiltrates of DCs, T-cells, B-cells, and plasma cells (Kerjaschki et al. 2004, Sarwal et al. 2003). This evidence has led to the idea that grafts can potentially become neo-lymphoid organs that have organized lymphoid tissue. Following the induction of a B-cell response, long-lived plasma cells migrate to the bone marrow or graft where they produce antibody chronically, even without T-cell help (Shapiro-Shelef & Calame 2005).

The main targets of alloantibodies are MHC-I molecules, which are expressed on all nucleated cells, including endothelial cells, and MHC-II molecules, which are expressed on APCs and microvascular endothelial cells in humans (Halloran et al. 1992, Erlich et al. 2001). The secretion of IFN- $\gamma$  by activated T-cells may increase MHC expression in donor cells, thus providing more potential antigenic targets for alloantibodies (Hidalgo & Halloran 2002). Graft destruction by alloantibodies is mainly attributed to deposition of donor-specific antibodies to graft endothelium and subsequent complement activation (Zhang & Reed 2009). The binding of antibodies and complement to endothelial cells stimulates production of chemokines, which attract T-cells, macrophages, monocytes, and neutrophils, which mediate graft injury (Magil & Tinckam 2003). This mechanism was tested in animal transplant models, which showed antibody and complement accumulation on graft endothelium (Jooste et al. 1981). If antibodies specific for donor endothelial cells were transferred to mice several weeks post-transplantation, graft rejection did not occur because the donor graft vessels had been replaced with recipient endothelium (Jooste et al. 1981).

Alloantibodies have been associated with chronic rejection since studies showed a strong correlation between the numbers of circulating donor-specific antibodies and the

presence of arterial lesions (Solez et al. 2008, Kaczmarek et al. 2008, Jeannet et al. 1970). Animal studies revealed that mice depleted of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells that received heart allografts did not reject the graft, but still eventually developed obstructive coronary lesions (Russell et al. 1994). Further, B-cell-deficient mice did not develop lesions in chronic rejection (Russell et al. 1997), but the transfer of alloantibodies restored this pathology (Hancock et al. 1998). Antibody-mediated chronic rejection can occur even without complement or inflammatory cells. MHC-I-specific antibodies have been shown to induce proliferation of endothelial cells (Jin et al. 2002, Smith et al. 2000) and bronchial epithelial cells (Jaramillo et al. 2003).

Other mechanisms by which B-cells or alloantibodies contribute to rejection have been postulated. B-cells can increase allogeneic T-cell response by infiltrating grafts and presenting graft-derived antigen to alloreactive T-cells via MHC-II (Alegre et al. 2007). Evidence against the importance of this pathway is that B-cell deficient mice do not exhibit decreased T-cell-mediated rejection (Epstein et al. 1995). Additionally, in mouse models of heart and skin transplantation, alloantibodies facilitate T-cell activation through the opsonization of graft cells, which are then taken up and presented more efficiently by APCs (Burns & Chong 2011).

## **Graft Tolerance**

### ***Regulatory T-cells***

Following transplantation, most patients require lifelong anti-rejection drug therapy. Though very rare, there are instances of recipients accepting grafts without immunosuppression, often occurring when immunosuppressive drugs must be discontinued for medical reasons (Kawai et al. 2013). Additionally, there have been some studies where liver transplant recipients were weaned off immunosuppressive medications and did not reject their grafts (Takatsuki et al. 2001). Early experiments in animals and humans revealed that the acceptance of allografts involved more than simply deleting donor-reactive immune cells (Kawai et al. 2013, Billingham et al. 2010). Subsequent studies revealed that rats that maintained allograft survival without immunosuppression had T-cells that could actively promote long-term allograft survival due to the induction of a tolerogenic immune response (Hall et al. 1984). These cells were later shown to be CD4<sup>+</sup> T-cells that express CD25, the  $\alpha$ -chain of the IL-2 receptor (Hall et al. 1984, Quigley et al. 1989) and were consistent with findings of cells with a similar phenotype that were involved in immune regulation and the development of autoimmunity (Sakaguchi 2000). These cells are now known as regulatory T-cells (Tregs). It has been subsequently shown that these cells can be characterized by the expression of the transcription factor forkhead box P3 (Foxp3) (Hori et al. 2003).

Tregs that can inhibit allograft rejection in vivo develop from two different mechanisms. Natural Tregs are selected in the thymus and function in the periphery to

suppress the immune response to self-antigen, while induced Tregs arise from CD4<sup>+</sup> T-cells that encounter antigen in a tolerogenic environment (Piccirillo & Shevach 2004, Chen et al. 2003). Both types of Tregs can recognize and respond to graft antigens, but induced Tregs are more likely to significantly contribute to the overall Treg response due to the persistent presence of donor antigen on the graft (Wood et al. 2011). Induced Treg populations include T regulatory 1 (Tr1) cells, which secrete IL-10, and T helper 3 (Th3) cells, which secrete TGF- $\beta$  (Apostolou et al. 2002, Akbar et al. 2003, Fantini et al. 2007). Tregs can also suppress effector T-cells by excess IL-2 consumption and the induction of apoptosis in target cells (Shevach 2009). Tregs also indirectly inhibit T-cell activation through the suppression of APC function (Shevach 2009). The production of IL-10 and TGF- $\beta$  then creates the tolerogenic environment to favor the induction of more Tregs (McMurchy et al. 2011). This increased anti-inflammatory signal may be essential for the induction of tolerance, since there are generally a high number of alloreactive T-cells in the recipient's immune repertoire, which may otherwise overwhelm the relatively small number of Tregs (Wood et al. 2012). Following transplantation in both humans and animals, the presence of Tregs in the spleen, draining lymph nodes, and at the site of the allograft, closely correlated with graft acceptance (Li et al. 2004, Brouard et al. 2007, Martinez-Liordella et al. 2007). Experiments in mice using T-cell receptor (TCR)-transgenic T cells and adoptive transfer have suggested that T cell deletion and regulation can together induce tolerance in mice (Wells et al. 1999, Li et al. 2001). Further, murine studies show Tregs stimulated in vitro with alloantigens can induce tolerance to skin and cardiac allografts (Joffre et al. 2008).

### *IL-10 and TGF- $\beta$*

IL-10 is a pleiotropic cytokine that is mainly involved in limiting and terminating inflammatory responses (Moore et al. 2001). IL-10 regulates growth and/or differentiation of cytotoxic and helper T-cells, B-cells, DCs, endothelial cells, natural killer (NK) cells, mast cells, granulocytes and keratinocytes (Moore et al. 2001). IL-10 is essential for the induction and maintenance of Tregs in vitro and in vivo (Wood et al. 2012). Most hematopoietic cells including macrophages, DCs, B-cells, and T-cells can produce IL-10, which induces Tregs, which then overproduce it (Groux et al. 1997). IL-10 is crucial for tolerance to grafts, as blocking the activity of IL-10 in vivo in transplantation models diminishes the survival of grafts (McMurchy et al. 2011, Kingsley et al. 2002). Likewise, TGF- $\beta$  is a regulatory cytokine that supports tolerance by the regulation of lymphocyte proliferation, differentiation, and survival and inhibits migration of lymphocytes, DCs, macrophages, NK cells, mast cells, and granulocytes (Li et al. 2006). In vitro, when naïve CD4<sup>+</sup>T-cells encounter antigen in the presence of TGF- $\beta$ , it induces Foxp3 expression, and thus regulatory capacity (Chen et al. 2003, Zheng et al. 2004). Similar to IL-10, TGF- $\beta$  is required for the induction of tolerance in murine models of transplantation (Daley et al. 2007, Cobbold et al. 2004).

Many studies have implicated the increased production of both IL-10 and TGF- $\beta$  in transplant tolerance. Th1-mediated responses following an allograft transplant was found to be markedly suppressed in mice that had accepted grafts, but the injection of neutralizing antibodies to IL-10 and TGF- $\beta$  restored the rejection (Bickerstaff et al. 2000). Similarly, when peripheral blood mononuclear cells (PBMCs) from human

transplant recipients, who were not undergoing immunosuppressive therapy and were not rejecting their grafts, were co-injected with donor antigen into the footpad of SCID mice, the inflammatory response was suppressed, which was reversed by antibodies to IL-10 and TGF- $\beta$  (VanBuskirk et al. 2000). Together, these studies show graft “acceptance” is an active process that is mediated, at least in part, by IL-10, TGF- $\beta$ , and Tregs.

### *Costimulation and tolerance*

T-cell activation is central to graft rejection and, consequently, is central to the efforts to promote graft acceptance. Stimulation through CD28, a molecule constitutively expressed on T-cells, can provide a potent co-stimulatory signal when it interacts with the B7 molecules expressed on APCs, leading to enhanced T-cell survival, NF-kB activation and IL-2 production (Sharpe & Freeman 2002). Since the lack of costimulation can induce T-cell anergy, antigen-nonspecific second signals to T-cells from accessory cells (Jenkins et al. 1991), particularly B7 on APCs, have become a target to induce immunosuppression (Greenwald et al. 2001, Wells et al. 2001).

Manipulation of the costimulatory signals preferentially produce anergic and/or Tregs. Blocking CD28 signaling on T-cells has been shown to prevent rejection in rats (Laskowski et al. 2002, Haspot et al. 2005). The CD28 pathway has also been disrupted by methods that utilize cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is expressed on activated T-cells and also binds to B7 (Walunas et al. 2011). CTLA-4 engagement has the opposite effect of CD28 and reduces IL-2 secretion and blocks proliferation (Krummel & Allison 1995). Antibodies that activate CTLA-4 (CTLA-4-Ig) have been

protective in models of transplantation (Lenschow et al. 1992, Pearson et al. 1994, Glysing-Jensen et al. 1997, Newell et al. 1999). Another costimulation target is inducible T-cell costimulatory (ICOS), which is also expressed on activated T-cells (Hutloff et al. 1999). ICOS blockade was found to prolong allograft survival in mice (Ozkaynak et al. 2001), although another study revealed only delayed blockade increased graft survival (Harada et al. 2003). While CD4<sup>+</sup> T-cell expansion and alloantibody levels were decreased by both early and late blockade, only delayed ICOS blockade resulted in the suppression of effector CD8<sup>+</sup> T-cells and significantly prolonged graft survival (Harada et al. 2003). This is supported by recent findings that ICOS signaling is involved in regulating effector responses (Coyle & Gutierrez-Ramos 2001). However, the complexities of the immune response in transplantation may diminish the universal application of costimulatory blockades. The frequency of alloreactive precursors has been shown to influence requirements of costimulation in allograft rejection in mice. Low frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells required costimulation and were thus sensitive to a costimulatory blockade, whereas when high frequencies of alloreactive precursors were present, costimulation was less crucial and the mice rejected grafts even with costimulatory blockade (Ford et al. 2007). Also, different transplanted tissues may contain different types of APCs, which could subsequently activate different T-cell subsets. This is an important factor because there is considerable evidence that the requirements of costimulation vary between CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, especially in transplantation (Gao et al. 2000). While both CD4<sup>+</sup> and CD8<sup>+</sup> have been shown to

mediate rejection in certain conditions, the effect of blockade of CD28 or the activation of CTLA-4 on CD8<sup>+</sup> T-cells is not always beneficial (Newell et al. 1999).

As previously discussed, CD4<sup>+</sup> T-cells can stimulate B-cells to proliferate and differentiate into antibody-secreting plasma cells (Lanzavecchia et al. 2006), which provides another potential target for anti-rejection therapy. B-cell activation by CD4<sup>+</sup> T-cells is thought to be dependent on CD40L (Steele et al. 1996). CD40L is expressed predominantly on activated CD4<sup>+</sup> cells and induces an activating response when it binds to its receptor, CD40, which is constitutively expressed on B-cells, but also on DCs, endothelial cells, and epithelial cells (van Kooten & Banchereau 2000). CD40L expression is increased 4-fold in cases of acute rejection, and antibodies against CD40L have been found to be protective in mouse and monkey models of transplantation, including renal, pancreas, and skin allografts (Daoussis et al. 2004).

## Current Treatments

The current anti-rejection treatments are listed in Table 1 and are discussed in further detail below.

<b>Table 1</b>			
<b>Current Anti- Rejection Therapies</b>			
	<b>Name(s)</b>	<b>Function</b>	<b>Reference</b>
<b>Induction Therapy</b>			
Antithymocyte globulins	Thymoglobulin® Atgam®	Deplete T-cells	(Cosimi et al. 1976)
IL-2 receptor blockers	Basiliximab (Simulect®) Daclizumab (Zenapax ®)	Block formation of high-affinity IL-2 receptors	(Maes & Vanrenterghem 1999, Do et al. 2004)
<b>Maintenance Therapy</b>			
Calcineurin inhibitors	FK506 (Prograf®) Cyclosporine	Block T-cell transcription	(Flanagan et al. 1991)
anti-metabolites	azathioprine (Azasan®) mycophenolate (CellCept®, Myfortic®)	Inhibit DNA synthesis	(Maltzman & Koretzky 2003) (Ransom 1995)
Corticosteroids	Prednisone and methylprednisolone	Inhibit transcription	(Northrop et al. 1992)
mTOR inhibitors	rapamycin (Rapamune®)	Block proliferation of B- and T-cells	(Sehgal 2003)

## *Induction Therapy*

Recipients of allogeneic transplants must maintain lifelong immunosuppressive therapies to prevent allograft rejection. Most transplant recipients receive induction therapy, which is intense immunosuppression for a short period before and/or after the transplantation to avoid early acute rejection. Though the drugs used in the initial period may be continued for the first 30 days after transplant, they are usually not used long-term for immunosuppressive maintenance. The current medications used in induction therapy are listed in Table 1.

T-cell depleting agents were given to 54% of transplant recipients in 2011 (SRTR/OPTN 2011). The T-cell depleting agents given most often are polyclonal anti-thymocyte globulins, raised in rabbits (Thymoglobulin®) or horses (Atgam®). Anti-thymocyte globulins usually have antibodies against many different T-cell surface markers including CD2, CD3, CD4, CD8, CD25, CD44, and CD45, and some known to interact with T-cells such as MHC-I and MHC-II (Cosimi et al. 1976).

Agents that block the IL-2 receptor (CD25) were given to 24% of transplant recipients 2011 (SRTR/OPTN 2011). Two of the most frequently used drugs in this class are Basiliximab (Simulect®) and Daclizumab (Zenapax®)(SRTR/OPTN 2011). Both biological are humanized mouse monoclonal antibodies that bind to the p55 unit of the  $\alpha$ -chain of the IL-2 receptor (CD25), which then blocks the binding of IL-2 by interfering with the formation of the high-affinity IL-2 receptor (Maes & Vanrenterghem 1999, Do et al. 2004).

## *Maintenance Therapy*

Following the induction therapy, allograft recipients continue lifelong immunosuppressive therapies to prevent allograft rejection. This phase is called maintenance therapy, and is critical for protecting allograft function while minimizing side effects. Generally, the successful upkeep of maintenance therapy is responsible for the long-term survival of the graft. Current anti-rejection regimens are listed in Table 1 and include calcineurin inhibitors, anti-metabolites, steroids, and mammalian target of rapamycin (mTOR) inhibitors (SRTR/OPTN 2011).

The OPTN and SRTR report that in 2011, more than 80% of transplant recipients of all types (kidney, heart, lung, pancreas, intestine, and liver) were receiving calcineurin inhibitors (CNIs) (SRTR/OPTN 2011). The theory behind the activity of these drugs is that when T-cells recognize their cognate antigen through the T-cell antigen receptor, they are activated and begin to divide. Investigation has shown that following T-cell activation, the expression of over 200 genes is transcriptionally altered, as T-cells prepare for cell division (Verweij et al. 1990). Signal transduction after TCR engagement elevates levels of intracellular calcium, which activates the calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin (Fruman et al. 1992). Calcineurin is composed of a catalytic subunit, the A chain, and two calcium-binding regulatory subunits, the B chain and the calmodulin protein (Hubbard & Klee 1989). The activation of calcineurin results in the nuclear translocation of cytosolic components of NFAT, NFATc1 and NFATc2 to the nucleus to participate in the transcription of many genes, including IL-2, IL-4, IFN  $\gamma$ , TNF, FasL, CD40L (Clipstone & Crabtree 1992). CNIs prevent this process

and inhibit NFAT transcription several hundred- to thousand-fold (Flanagan et al. 1991). 70% of patients using calcineurin inhibitors take tacrolimus (FK506, Prograf®), while the other 30% take cyclosporine (SRTR/OPTN 2011). Tacrolimus and cyclosporine are structurally unrelated, but both compounds bind to prolyl isomerases, cyclophilin and FKBP (FK506 binding protein), to block the phosphatase activity of calcineurin (Ho et al. 1996). Additionally, other targets possibly contribute to the overall suppression induced by these compounds, as the transcriptional activity of NF- $\kappa$ B is decreased by about two-fold and certain AP-1 sites are inhibited three- to fourfold, though they do not interact with calcineurin (Emmel et al. 1989).

More than 50% of transplant recipients are also given the anti-metabolites azathioprine (Azasan®) and mycophenolate (CellCept® or Myfortic®) in addition to a calcineurin inhibitor (SRTR/OPTN 2011). Azathioprine inhibits DNA synthesis by blocking amidophosphoribosyltransferase, an enzyme involved in purine synthesis, thus interfering with DNA replication (Maltzman & Koretzky 2003). Lymphocytes do not have a nucleotide synthesis salvage pathway, so azathioprine has relative specificity for lymphocytes (Maltzman & Koretzky 2003). Mycophenolate has a similar mechanism of action. It blocks DNA synthesis by inhibiting another enzyme involved in purine synthesis, inosine monophosphate dehydrogenase (IMPDH), which significantly lowers guanine pools in lymphocytes (Ransom 1995).

Corticosteroids, such as prednisone and methylprednisolone, comprise additional immunosuppressive treatment modalities for more than 50% of patients (SRTR/OPTN 2011). These drugs are nonspecific and suppress the overall immune response.

Corticosteroids enter the cytoplasm and bind to glucocorticoid receptors, which form complexes with *jun* transcription factors, resulting in the inactivation of the activator protein 1 (AP-1) (Ho et al. 1996). This results in the reduction of many pro-inflammatory cytokines, including IL-1, IL-2, IL-6, IFN- $\gamma$  and TNF- $\alpha$  (Northrop, Crabtree & Mattila 1992). In some cases, such as IL-2, repression requires an active glucocorticoid response element (GRE) while in other cases such an element does not seem to be necessary (Northrop et al. 1992).

mTOR inhibitors are used in about 5-10% of transplant patients (SRTR/OPTN 2011). The most commonly used mTOR inhibitor is rapamycin (Rapamune®). Rapamycin is structurally similar to tacrolimus, but does not block the transcription of pro-inflammatory cytokines despite binding to FKBP (Ho et al. 1996). Rather, the rapamycin and FKBP complex blocks the activation of the cell-cycle-specific serine/threonine kinase target of rapamycin (TOR) to block progression from G1 to S phase of the cell cycle (Sehgal 2003). Additionally, rapamycin has been found to be a potent inhibitor of B-cell proliferation and antibody production (Ho et al. 1996).

### ***Problems with Current Protocols***

The use of immunosuppressive drugs by transplant recipients results in marked morbidity and is a main limitation in the transplantation field. Adverse effects from non-specific immunosuppression or from off-target pharmacological activity are common. Suppressed immune systems leave patients more susceptible to infections and the activation of latent viral infections, particularly in the first year post-transplantation when

the immunosuppression regimen is the most intense (Chong & Alegre 2012). Transplant recipients also have increased development of malignancies. Epidemiological data show a direct correlation between the length and intensity of immunosuppressive therapy to increased incidence of post-transplant risk of malignancy (Gutierrez-Dalmau & Campistol 2007). Further, once cancer had developed, more intense immunosuppression can result in accelerated growth and metastasis of tumors, with lower patient survival (Gutierrez-Dalmau & Campistol 2007). Post-transplant malignancy is generally attributed to impaired immune surveillance and decreased regulation of oncogenic viruses (Ho et al. 1996).

Many transplant recipients suffer side effects from off-target pharmacological activity of immunosuppressants. Both CNIs and corticosteroids have been implicated in the development of diabetes mellitus. CNIs have been shown to have a direct toxic effect on pancreatic beta cells, causing decreased insulin secretion, and have also been linked to increased insulin resistance (Taylor et al. 2005). Corticosteroids have also been shown to increase incidences of diabetes from altered carbohydrate metabolism and induction of insulin resistance (Taylor et al. 2005). Another side effect that occurs often in transplant recipients is hypertension. CNIs activate the renal sodium chloride cotransporter, resulting in hypertension, hyperkalaemia, and hyperuricaemia (Hoorn et al. 2011). Corticosteroids cause fluid retention and hypertension (Taylor et al. 2005).

Levels of CNI are highest in the kidney and brain, so many side effects are due to nephrotoxicity and neurotoxicity (Ho et al. 1996). Up to 50% of transplant recipients have renal dysfunction in the 5 years after transplantation (SRTR/OPTN 2011).

Treatment with CNI causes severe vasoconstriction of the renal afferent arteriole, causing reduced renal blood flow and, further, induces progressive and permanent interstitial fibrosis and fibrous intimal thickening (Naesens et al. 2009). CNI can also cause progressive neurotoxicity, the severity of which is dose-dependent (Ho et al. 1996). The most common neurological side effects are headache and tremor, although agitation, convulsions, psychosis, and hallucinations have also been reported (Taylor et al. 2005). Thus, improved therapeutic agents with decreased toxicity are needed for use alone, or in combination with existing therapies given at reduced doses.

## **Cannabinoids**

### **The Endocannabinoid System**

Marijuana is known to have been used for centuries recreationally and medicinally (Vincent et al. 1983). However, its medicinal value was not reported to the western scientific community until 1843, when a physician working in India observed and researched its use there (O'Shaughnessy 1843). Later, the major psychoactive ingredient of cannabis was found to be  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) (Gaoni, Mechoulam 1964). Our understanding of the cannabinoid system greatly increased with the discovery of the cannabinoid receptors and endogenous cannabinoid ligands. The endogenous cannabinoid ligands, known as endocannabinoids, were only discovered recently, with arachidonylethanolamide (AEA) discovered in 1992, 2-

arachidonoylglycerol (2-AG) in 1995, and noladin ether in 2001 (Devane et al. 1992, Mechoulam et al. 1995, Hanus et al. 2001).

To date, two cannabinoid receptors have been identified, designated CB1 and CB2. The receptors share 48% amino acid sequence identity and are typical G protein coupled receptors (GPCRs), which have 7 transmembrane spanning domains that are coupled to adenylyl cyclase and mitogen-activated protein kinase through G proteins (Felder et al. 1995). The CB1 receptor is found on neurons in the central and peripheral nervous systems (Matsuda et al. 1990, Herkenham et al. 1991, Galiegue et al. 1995), and to a lesser extent, on cells of the immune system and testes (Galiegue et al. 1995, Daaka et al. 1996, Waksman et al. 1999). The endocannabinoid system modulates several biological activities through CB1 in the CNS. CB1, located on presynaptic neurons, binds endocannabinoids that are synthesized and released from postsynaptic neurons, with the predominant effect of receptor engagement being a decrease in the release of neurotransmitters (Katona & Freund 2008). This retrograde signaling system regulates pain initiation, appetite, psychomotor behavior, memory, thermogenesis, and wake/sleep cycles (Ohno-Shosaku et al. 2012). Conversely, CB2 is primarily expressed on cells of the immune system, including B-cells, NK cells, monocytes, polymorphonuclear cells, T-cells, and activated microglia (Galiegue et al. 1995, Munro et al. 1993, Murikinati et al. 2010) and only sparsely in the CNS (Gong et al. 2006, Van Sickle et al. 2005). The primary endogenous ligand for CB2 is 2-AG, which is a full agonist and expressed highly in the bone marrow and spleen (Sugiura & Waku 2002), while anandamide is only a partial agonist and not readily detected outside of the brain (Pertwee 1999). The

production of 2-AG from the hydrolysis of 1,2-diacyl- glycerol (DAG), a minor component of the cell membrane, is rapidly induced in the presence of several stimuli, including lipopolysaccharides, endothelin, platelet-activating factor, ionomycin, and thrombin (Bisogno et al. 2005). CB2 is mainly thought to be involved in immunoregulation, and its activation is generally immunosuppressive (Howlett et al. 2002, Basu & Dittel 2011).

### **$\Delta^9$ -THC and the Immune System**

$\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) has been reported to have effects on immune responses since the 1970s (Gupta et al. 1974, Johnson & Wiersema 1974, Nahas et al. 1974, Neu et al. 1970). There are numerous reports on the suppression of macrophages by  $\Delta^9$ -THC, primarily by decreasing their antigen-presenting abilities. Macrophages exposed to  $\Delta^9$ -THC resulted in the inhibition of phagocytosis (Friedman et al. 1986), antigen processing of certain proteins (McCoy et al. 1996, McCoy et al. 1999, Matveyeva et al. 2000), capacity for co-stimulation (Clements et al. 1996), nitric oxide production (Waksman et al. 1999), pro-inflammatory cytokine release and production in macrophages (Fischer-Stenger et al. 1993, Chang et al. 2001) and microglia (Klein et al. 2006), as well as the migration of activated microglia (Fraga et al. 2011).

In addition, many reports have shown profound effects on lymphocytes, at least partly due the inhibition of adenylate cyclase and cyclic AMP (Schatz et al. 1992, Condie 1996). Treatment with  $\Delta^9$ -THC was found to suppress lymphocyte proliferation induced by mitogens or IL-2 (Klein et al. 1985, Kawakami 1988), as well as to reduce the

antibody-forming cell response to sheep red blood cells in vivo (Zimmerman et al. 1977) and in vitro (Baczynsky et al. 1983, Eisenstein et al. 2007).  $\Delta^9$ -THC also decreased the cytolytic function of NK cells in vitro and in vivo (Specter et al. 1986, Klein et al. 1987) and the induction and cytolytic function of cytotoxic T-cells in vitro and in vivo (Klein et al. 19921). In addition,  $\Delta^9$ -THC was found to induce a shift from T helper 1 (Th1) to T helper 2 (Th2) following *Legionella pneumophila* infection (Klein et al. 2000, Newton et al. 2007) and from activation by allogeneic dendritic cells (Yuan et al. 2002). Recent studies have shown  $\Delta^9$ -THC can also inhibit the co-stimulatory capability of T-cells by blocking the upregulation of inducible co-stimulator (ICOS) (Lu et al. 2009) and of CD40 ligand following cell activation (Ngaoteprutaram et al. 2012).

### **Therapeutic Use of Cannabinoids**

Increased research and understanding of the cannabinoids has led to greater interest in their use from a therapeutic perspective. The development of CB1-selective antagonists (Rinaldi-Carmona et al. 1994) and CB1-deficient (knock-out) mice (Zimmer et al. 1999) has provided tools to further dissect the function of this receptor and spurred interest in the therapeutic potential of cannabinoids as anxyolitics, antiemetics, analgesics, and for treatment for Tourette's Syndrome (Breivogel & Sim-Selley 2009), with the possibility of new applications still forthcoming.

The understanding of CB2-mediated effects has likewise been furthered by the development of selective antagonists (Rinaldi-Carmona et al. 1998), CB2 knockout mice (Buckley et al. 2000), and selective agonists (Huffman et al. 2005, Huffman et al. 1999,

Huffman et al. 1996, Marriott et al. 2006). As effects of exogenous and endogenous cannabinoids have been mostly reported to be immunosuppressive (Croxford & Yamamura 2005, Basu & Dittel 2011, Robinson et al. 2013, Eisenstein et al. 2007), their use as immunomodulatory and anti-inflammatory agents has been investigated (Nagarkatti et al. 2009). Further, it was recognized that the anatomically disparate expression of CB1 and CB2 would allow for the use of compounds selective for CB2, without the unwanted psychoactive effects from CB1 activation, while maintaining the anti-inflammatory and immunosuppressive properties. Until recently CB2 agonists were thought to have little or no function in the brain, but recent reports show that CB2 agonists affect cocaine's rewarding and locomotor stimulating effects (Xi et al. 2011), food and alcohol consumption (Onaivi et al. 2008a), and symptoms of depression (Onaivi et al. 2008b), suggesting a function in the central nervous system for this class of compounds.

The administration of CB2-selective agonists has been found to be effective in the attenuation of several inflammatory and autoimmune disease models in rodents, including Experimental Autoimmune Encephalitis (EAE), which is a mouse model of multiple sclerosis, (Zhang et al. 2009b, Maresz et al. 2007), ischemic/perfusion injury following an induced stroke (Ni et al. 2004, Zhang et al. 2009a, Zhang et al. 2007), rheumatoid arthritis (Sumariwalla et al. 2004), inflammatory bowel disease (Storr et al. 2009, Storr et al. 2008), Crohn's disease (Wright et al. 2008), inflammatory autoimmune diabetes (Li et al. 2001), spinal cord injury (Baty et al. 2008, Adhikary et al. 2011), sepsis

(Tschop et al. 2009), autoimmune uveoretinitis (Xu et al. 2007), osteoporosis (Ofek et al. 2006) and systemic sclerosis (Servettaz et al. 2010).

### **CB2-Selective Agonists and Graft Rejection**

Organ transplantation and skin grafts are conditions in which activated immune responses greatly hinder the success of the transplant. Because CB2 signaling has been shown to induce potent immunosuppression, this class of compounds is an attractive possibility for an anti-rejection therapeutic (Nagarkatti et al. 2010). Further, CB2-selective agonists have been shown to inhibit nearly every type of immune cell, so several aspects of rejection may be blocked by cannabinoid treatment (Basu & Dittel 2011).

### ***Cell Migration***

Cell migration is critical to the allogeneic immune response. Immune cells must migrate throughout the body, to secondary lymphoid organs for activation, and to the graft for destruction (Larsen et al. 1990, Ingulli et al. 1997, Steinman et al. 1997). Treatment with CB2-selective agonists has been shown to inhibit the chemotaxis of various immune cells to inflammatory stimuli in vitro. Treatment with CB2-selective agonists blocked the chemotaxis of primary human and Jurkat T-cells to CXCL12 (Ghosh et al. 2006), murine macrophages to CCL5 (Raborn et al. 2008), human monocytes to CCL2/CCL3 (Montecucco et al. 2008), neutrophils to CXCL2 (Murikinati et al. 2010), and DCs to CCL19 (Adhikary et al. 2012). Further, treatment of murine macrophages

with a CB2-selective cannabinoid reduced expression of the adhesion molecules VCAM-1 and ICAM-1 (Zhao et al. 2010).

The administration of CB2-selective agonists has been shown to reduce the migration of inflammatory cells in models of inflammation or disease. CB2-deficient mice had significantly increased number of encephalitogenic T-cells in the CNS during EAE (Maresz et al. 2007). Treatment with a CB2-selective agonist resulted in decreased leukocyte/endothelial interactions in EAE (Zhang et al. 2007) and in LPS-induced encephalitis (Ramirez et al. 2012). CB2 agonists decreased the number of peripheral CD34<sup>+</sup> hematopoietic cells that migrated into the brain in EAE (Palazuelos et al. 2008), and decreased the number of neutrophils in the brain during stroke (Murikinati et al. 2010) and in the lung during sepsis (Tschop et al. 2009). Dendritic cell migration from the mouse footpad into the draining lymph nodes was also inhibited during inflammation (Adhikary et al. 2012).

CB2-selective agonists can inhibit migration by down-regulating expression of adhesion molecules, chemokines and chemokine receptors in vivo. Mice treated with a CB2-selective agonist during experimental autoimmune uveoretinitis (EAU) had decreased leukocyte trafficking in the inflamed retina and T-cells down-regulated expression of the adhesion molecule CD11a (Xu et al. 2007). CB2 agonists decreased expression of the chemokines CCL2, CCL3, CCL5, and the chemokine receptors CCR1 and CCR2 in the spinal cord and bone marrow of mice with EAE and decreased expression of the chemokines CXCL-9 and CXCL-11 in the spinal cord during injury (Adhikary et al. 2011).

### ***CB2 and Endothelial Cells***

Endothelial cells also play an important role in graft rejection. Activated endothelial cells produce cytokines, express adhesion molecules, upregulate MHC molecules, and have decreased integrity allowing for increased leukocyte extravasation (Hunt, Jurd 1998). CB2 is expressed on endothelial cells, including brain, liver, pulmonary and coronary endothelium (Ramirez et al. 2012, Golech et al. 2004, Rajesh et al. 2007a, Rajesh et al. 2007b, Zoratti et al. 2003) and is highly up-regulated during inflammation and cell activation (Ramirez et al. 2012).

Treatment with CB2-selective agonists had significant effects on endothelial cell function. A CB2 agonist blocked endothelial cell activation by inhibiting activation of NF- $\kappa$ B and RhoA by TNF- $\alpha$  (Rajesh et al. 2007a). CB2 agonist treatment decreased the adhesion molecule P-selectin on endothelial cells during EAU (Xu et al. 2007) and decreased ICAM-1 and VCAM-1 expression on brain endothelium in LPS-induced encephalitis (Ramirez et al. 2012), human coronary artery endothelial cells activated with TNF- $\alpha$  (Rajesh et al. 2007a) and liver endothelium during hepatic ischemia and reperfusion injury (Rajesh et al. 2007b).

Additionally, CB2 mediates leukocyte extravasation by influencing the integrity of endothelium. CB2-deficient mice had accelerated vascular leukocyte infiltration in atherosclerotic plaques, and the treatment of WT mice with a CB2-selective agonist decreased lesions and improved endothelial function (Hoyer et al. 2011). Further studies confirmed cannabinoid treatment and augmented endothelial tightness and decreased permeability (Ramirez et al. 2012, Blazquez et al. 2003).

### ***CB2 and T-cells***

The capacity of CB2-selective cannabinoids to inhibit T-cells is paramount to their use as anti-rejection therapy, since alloreactive T-cells mediate tissue and organ rejection (Heeger 2003).  $\Delta^9$ -THC, given in vivo to mice, has been reported to inhibit ex vivo reactivity of spleen cells from treated animals when exposed to histoincompatible spleen cells in vitro in the mixed lymphocyte reaction (MLR), an in vitro correlate of graft rejection (Zhu et al. 2000). Similarly,  $\Delta^9$ -THC decreased proliferation of human T-cells when stimulated with allogeneic DCs (Yuan et al. 2002). However, whether the effect of  $\Delta^9$ -THC was via CB1 or CB2 receptors was not explored.

Nevertheless, CB2-selective cannabinoids have been shown to inhibit T-cells in many other experimental conditions. CB2 agonists caused decreased expression of the pro-inflammatory cytokines IL-2, IL-6, TNF- $\alpha$ , IFN- $\gamma$  and inhibited proliferation during ex vivo restimulation in mouse models of EAE (Maresz et al. 2007), EAU (Xu et al. 2007), and sepsis (Tschop et al. 2009). In vitro treatment of CB2 agonists also inhibited T-cell proliferation (Borner et al. 2009, Cencioni et al. 2010), IL-2 release (Borner et al. 2009, Cencioni et al. 2010), and number of T-cells producing TNF- $\alpha$  (Cencioni et al. 2010). Further, a CB2-selective agonist reduced transcription by NF- $\kappa$ B and NFAT in human primary and Jurkat T-lymphocyte lines expressing CB2 that were activated with anti-CD3/CD28 antibodies (Borner et al. 2009).

## **CHAPTER 2**

### **OBJECTIVES**

There is now a significant body of research showing that activation of cannabinoid 2 (CB2) receptors largely suppresses the action of leukocytes (Basu & Dittel 2011), including previous work from our laboratory which demonstrated that in vitro antibody production by mouse spleen cells in the plaque-forming cell assay was suppressed by  $\Delta^9$ -tetrahydrocannabinol and by anandamide through action at the CB2 receptor (Eisenstein et al. 2007). Administration of CB2-selective agonists has been found to be effective in the attenuation of several inflammatory and autoimmune disease models in rodents (Basu & Dittel 2011, Nagarkatti et al. 2009). Advancement of therapeutics to prevent graft rejection following organ transplantation is highly desirable, as a large number of grafts are eventually rejected (SRTR/OPTN 2011) and many graft recipients have considerable morbidity from current anti-rejection medications (Jose 2007). The hypothesis to be tested is that CB2-selective agonists are immunosuppressive in models of graft rejection. A positive outcome would suggest that CB2 agonists be pursued as a novel class of compounds suitable for anti-rejection therapy. This hypothesis will be explored by the following:

1. To evaluate the effects of cannabinoid agonist treatment on the mixed lymphocyte

reaction (MLR), an in vitro correlate of graft rejection. Proliferation of allogeneic T-cells will be measured by the uptake of [<sup>3</sup>H]-thymidine of T-cells in the presence of splenocytes with mismatched MHC molecules.

- a. The receptor mediating this reaction will be determined using CB1- and CB2-selective antagonists and splenocytes from CB2-deficient mice.
  - b. The cell type mediating this reaction will be ascertained by treating individual cell types separated by flow cytometry.
2. To evaluate the mechanisms by which CB2 agonists inhibit cells in the MLR.
- a. Assay levels of selected cytokines by ELISA and quantitative PCR.
  - b. Assess changes in activation status of cells by CB2-selective agonists using quantitative PCR and transcription factor assays.
  - c. Assess alterations of cell populations by flow cytometric analysis.
3. To determine whether the effects of CB2-selective agonists in vitro translate to prolongation of graft survival in vivo.
- a. The capacity of a CB2-selective agonist administered systemically to prolong allogeneic skin grafts between MHC-mismatched mouse strains will be tested.
  - b. Effects on administration of a CB2 selective-agonist on splenocyte composition will be assessed by flow cytometry.
  - c. The capacity of splenocytes from mice treated with a CB2 agonist in vivo to respond to allogeneic re-stimulation in an in vitro MLR will be measured.

## CHAPTER 3

### MATERIALS & METHODS

#### Compounds

$\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) was provided by The National Institute on Drug Abuse (NIDA, Rockville, MD).  $\Delta^9$ -THC was supplied as a solution of 50 mg/ml in absolute ethanol and stored at 4°C. JWH-015 (CB2-selective agonist) was purchased from Tocris Biosciences (Bristol, UK). O-1966 (CB2-selective agonist) was a generous gift from Anu Mahadevan (Organix, Woburn, MA). SR141716A (CB1-selective antagonist) and SR144528 (CB2-selective antagonist) were obtained from Research Triangle Institute (Research Triangle Park, NC). Each of these compounds was supplied as crystals and stored at -20°C. Before each use, JWH-015, SR141716A, and SR144528 were dissolved in absolute ethanol and O-1966 was dissolved in DMSO. The solutions were added drop-wise to the medium used for the assay (RPMI-1640) to obtain the desired concentration.

#### Mice

Six week-old, specific pathogen-free C3HeB/FeJ and C57BL/6J female mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Founder CB2 receptor deficient (CB2R k/o) mice, on a C57BL/6J background (Buckley et al. 2000) were obtained from the National Institutes of Health (Bethesda, MD) and bred in the Animal

Core of the Center for Substance Abuse Research, P30 Center for Excellence, at Temple University School of Medicine Central Animal Facility. Mice with a genetic disruption of the IL-17A gene (IL-17 k/o) mice were obtained from Jay Kolls (LSU School of Medicine), with the permission of Yoishiro Iwakura (Institute of Medical Science, the University of Tokyo, Japan), who originally developed these animals (Nakae et al. 2002).

### **One-way Mixed Lymphocyte Reaction (MLR)**

Mice were sacrificed and their spleens aseptically removed. Single cell suspensions were obtained by passing spleens through nylon mesh bags (Sefar Inc., Depew, NY) in RPMI-1640 with 5% fetal bovine serum (FBS) containing 50  $\mu$ M 2-mercaptoethanol (2-Me), and 100 U/ml penicillin and streptomycin sulfate. All reagents were purchased from Gibco Life Technologies (Carlsbad, CA), with the exception of FBS, which was purchased from HyClone Laboratories (Logan, UT). Red blood cells were lysed by hypotonic shock for 10 seconds with sterile water. Responder spleen cells from C57BL/6 mice were resuspended in RPMI with 10% FBS, 50  $\mu$ M 2-Me, and 100 U/ml penicillin and streptomycin sulfate. Splenocytes from C3HeB/FeJ were similarly prepared to serve as the stimulator cells, but they were inactivated by treatment with 50  $\mu$ g/ml of mitomycin C for 20 min at 37°C. The cells were washed 3 times to remove mitomycin C from the medium and resuspended to the desired concentration using a Beckman Coulter Z1 Dual Cell and Particle Counter (Beckman Coulter Inc., Indianapolis, IN). Responder cells ( $8 \times 10^5$ ) and stimulator cells ( $8 \times 10^5$ ) were co-cultured in 200  $\mu$ l in 96 well plates for 48 h at 37°C in 5% CO<sub>2</sub>. In wells where it was desired, 50  $\mu$ l of cannabinoid was added to 100  $\mu$ l responder cells 3 h prior to mixing with 50  $\mu$ l stimulator cells. If antagonists were used, 50  $\mu$ l were added to 50  $\mu$ l responder cells for 2 h prior to adding the agonist, followed by a 3 h incubation with 50  $\mu$ l agonist,

before mixing with 50  $\mu$ l stimulator cells. After a 48 h incubation period, cultures were pulsed with 1  $\mu$ Ci/well [ $^3$ H]-thymidine and harvested 18 h later onto glass fiber filters (Packard, Downers Grove, IL) using a Packard multichannel harvester, and placed in vials in liquid scintillation solution (Cytoscint, MP-Biomedical, Irvine, CA). [ $^3$ H]-thymidine incorporation on the filters was measured using a Packard 1900 TR liquid scintillation counter. Data were corrected for background by subtraction of [ $^3$ H]-thymidine incorporation in the absence of stimulator cells. Results are expressed as a Suppression Index (SI), where untreated spleen cells are given a value of 1.00 (100%), and responses of cultures receiving treatment with cannabinoids are calculated as:

$$SI = \frac{\text{Mean counts per minute of cannabinoid treated cultures}}{\text{Mean counts per minute of untreated cultures}}$$

## Flow Cytometry

MLR cultures were harvested at various time points and washed with staining buffer, (PBS containing 1% BSA, Sigma, St. Louis, MO).  $1 \times 10^6$  cells in 1 ml of PBS were added to Falcon<sup>TM</sup> polystyrene round-bottom tubes (BD Biosciences) and stained with 1  $\mu$ l of LIVE/DEAD<sup>®</sup> Dead Cell Stain (Molecular Probes, Inc) for 30 min on ice. The cells were washed twice with staining buffer and resuspended in 50  $\mu$ l of staining buffer. To prevent nonspecific binding, the cells were incubated with 1  $\mu$ g of 2.4G2 antibody specific for Fc $\gamma$  III/II receptor (BioLegend, San Diego, CA) at 4 $^{\circ}$ C for 5 minutes. Cells were then incubated with 0.5  $\mu$ g of fluorophore conjugated rat anti-mouse CD3 $\epsilon$  (BioLegend), rat anti-mouse CD4 (BioLegend), or isotype control for 30 min on ice, washed twice with staining buffer and resuspended in PBS with 2% (w/v) paraformaldehyde (Sigma) on ice for 15 min. The cells were washed 3 times with PBS and resuspended in 1 ml PBS with 0.5% (v/v) Tween 20 (Sigma), washed 3 times with

staining buffer and resuspended in 100  $\mu$ l staining buffer containing 0.5  $\mu$ g rat anti-mouse Foxp3 or isotype control (BioLegend) at room temperature for 30 min. The cells were washed 3 times with staining buffer, resuspended in 400  $\mu$ l staining buffer, and analyzed immediately on the LSRII (BD Biosciences, San Jose, CA) and analyzed using FACSDiva software (BD Biosciences) and post-analyzed with FlowJo (Tree Star, Inc., Ashland, OR).

### **Fluorescence Activated Cell Sorting (FACS)**

Splenocytes were resuspended in staining buffer: PBS containing 1% BSA (Sigma). Cells were incubated with 1  $\mu$ g/ $10^6$  cells of 2.4G2 antibody specific for Fc $\gamma$  III/II receptor at 4°C for 5 minutes to prevent nonspecific binding. Cells were then incubated with 0.5  $\mu$ g/ $10^6$  cells of fluorophore-conjugated rat anti-mouse CD11b, fluorophore-conjugated rat anti-mouse CD3 $\epsilon$ , fluorophore-conjugated rat anti-mouse CD4, or fluorophore-conjugated rat anti-mouse CD8 (BioLegend), for 30 min on ice. Cells were then washed twice with sorting buffer: PBS containing 0.1% BSA (Sigma). Cells were resuspended in sorting buffer (PBS containing 0.1% BSA) to a concentration of 40 x  $10^6$  cells/ml, and then sorted using the FACS Aria™ system (BD Biosciences). Purity of sorted cells was checked by analyzing a sample from each sorted population (CD3<sup>+</sup> and CD11b<sup>+</sup>) on the flow cytometer at the completion of sorting. Cell purity was 99% for all experiments.

### **Cell Viability**

Cell viability was assessed using cell cultures that were run in parallel with each experimental MLR. Viability from experiments was measured by flow cytometry using the LIVE/DEAD® Fixable Dead Cell Stain Kit from Molecular Probes, Inc. (Eugene,

OR).  $1 \times 10^6$  cells from cultures were resuspended in 1 ml FCM Staining Buffer and incubated for 30 min at room temperature with 1  $\mu$ l Dead Cell Stain. Cells were washed twice and resuspended in FCM staining buffer and analyzed using LSRII (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences). In addition, cell viability in several experiments was also checked with Trypan blue exclusion test. Cultures run in parallel with the experimental MLR were diluted to  $1.6 \times 10^6$  cells/ml and 0.2% Trypan Blue was added. The cells were scored for viability using a hemocytometer.

## **Enzyme-Linked Immunosorbance Assay (ELISA)**

### **IL-2**

IL-2 levels in the MLR culture supernatant were determined using the Quantikine<sup>®</sup> Mouse IL-2 Immunoassay (R&D Systems, Inc., Minneapolis, MN). 96 well microplates were obtained pre-coated with a polyclonal antibody specific for mouse IL-2. The supernatant was incubated for 2 h at room temperature, after which any unbound antigen was removed by five washes. Enzyme-linked polyclonal antibody for mouse IL-2 was added and incubated at room temperature for 2 h. Following five washes to remove unbound antibody, a stabilized hydrogen peroxide and chromogen substrate solution was added and incubated for 30 min at room temperature protected from light, followed by addition of dilute hydrochloric acid stop solution. The optical density was determined using a POLARstar Omega microplate reader (BMG LABTECH, Offenburg, Germany).

## **IL-10**

IL-10 levels in the MLR culture supernatant were determined using the Ready-Set-Go!<sup>®</sup> reagent set (eBioscience, San Diego, CA). Costar<sup>®</sup> 96 well flat bottom high affinity protein binding microplates (Corning Inc. Life Sciences, Tewksbury, MA) were coated overnight at 4°C with capture antibody specific for mouse IL-10. The rest of the assay and incubations were performed at room temperature. After 24 h in culture, the MLR supernatant was harvested and 100 µl/well was added into the prepared microplate and incubated for 2 h. After incubation, the wells were washed 5 times with wash buffer, PBS with 0.05% (v/v) of Tween-20 (Sigma), to remove unbound antigen. Then 100 µl/well of capture biotin-conjugated antibody against mouse IL-10 was added and incubated for 1 h. The wells were then washed 5 times to remove unbound antibody and an Avidin- horseradish peroxidase (HRP) solution was added and incubated for 30 min. The wells were washed 5 times and 100 µl/well of Tetramethylbenzidine (TMB) substrate solution was added and incubated for 15 min, followed by addition of 50 µl/well of dilute hydrochloric acid stop solution. The optical density was determined using a POLARstar Omega microplate reader (BMG LABTECH, Offenburg, Germany).

## **Apoptosis Assays**

### **TUNEL Assay**

The presence of apoptotic cells was examined using the APO-BrdU<sup>™</sup> TUNEL Assay Kit from Molecular Probes, Inc (Eugene, OR).  $2 \times 10^6$  cells per sample in MLR culture were collected 0, 24, and 48 h after stimulator cells were added. The cells were fixed with 1% (w/v) paraformaldehyde in PBS for 15 min on ice and then permeabilized by adding 3 mL ice-cold 70% ethanol in PBS. The cells were stored in this solution at -

20°C until day 3 of the experiment. The TUNEL assay was then performed by following the protocol provided by the manufacturer.

### **Caspase Activation Assay**

Activation of caspases was measured using the Vybrant<sup>®</sup> FAM Poly Caspases Assay Kit (Molecular Probes, Inc.).  $1 \times 10^6$  cells per sample in MLR culture were collected at 0, 24, and 48 h after stimulator cells were added, and the assay performed by following the protocol provided by the manufacturer.

### **In Vitro T-cell Activation**

96 well microplates were coated with 0.5 µg/well of LEAF<sup>™</sup> Purified anti-mouse CD3ε (BioLegend) and incubated for 2h at 37°C. Wells were washed to remove unbound antibody. C57BL/6J splenocytes were sorted by flow cytometry as described above and CD3<sup>+</sup> T-cells were negatively selected.  $2 \times 10^5$  cells were added to each well and incubated at 37°C for 30 min. Following incubation, 0.4 µg soluble LEAF<sup>™</sup> Purified anti-mouse CD28 (BioLegend) was added to each well for a final concentration of 2 µg/mL. Cultures were incubated for 48 hr at 37°C, pulsed with 1µCi/well [<sup>3</sup>H]-thymidine, and harvested 18 hr later, and radioactive uptake measured by liquid scintillation counting using a Packard 1900 TR liquid scintillation counter.

### **Transcription Factor Analysis**

Splenocytes from CB2R k/o mice or wild-type mice were treated for 3 h with O-1966 before activation with anti-CD3 and anti-CD28 antibodies, and then incubated for 18 hours.  $8.8 \times 10^6$  cells per treatment group were harvested and nuclear protein was extracted using a Nuclear Extract Kit (Active Motif, Carlsbad, CA), following the

provided protocol. Protein levels were quantified using the Bradford reagent (Sigma) and absorbance read on a POLARstar Omega microplate reader (BMG LABTECH, Offenburg, Germany). Protein levels were adjusted to 2.5  $\mu\text{g}/\mu\text{l}$  and stored at  $-80^{\circ}\text{C}$  until use. Levels of activated NFAT were measured using a TransAM™ NFATc1 Transcription Factor Assay Kit (Active Motif) according to manufacturer's protocol. Briefly, 20 pmol (in 2  $\mu\text{l}$ ) of oligonucleotide containing the consensus sequence for NFAT and 50  $\mu\text{l}$  containing 5  $\mu\text{g}$  of nuclear extract were used in the assay in the provided 96-well assay plate. 100  $\mu\text{l}$  mouse anti-NFATc1 antibody was added to the bound transcription factor followed by the addition of 100  $\mu\text{l}$  of anti-mouse horse radish peroxidase (HRP)-conjugated antibody and the provided reagents for a colorimetric reaction. The optical density was determined using a POLARstar Omega microplate reader (BMG LABTECH). NF- $\kappa$ B levels were measured using a TransAM™ Flexi NF $\kappa$ B p50 Transcription Factor Assay Kit (Active Motif) according to manufacturer's protocol. The protocol was the same as described above, except using 1 pmol (in 1  $\mu\text{l}$ ) of biotinylated oligonucleotide containing the consensus sequence for NF $\kappa$ B combined with 50  $\mu\text{l}$  containing 5  $\mu\text{g}$  of nuclear extract. Bound transcription factor was detected with 100  $\mu\text{l}$  rabbit anti- NF $\kappa$ B p50 and 100  $\mu\text{l}$  anti-rabbit HRP-conjugated antibody.

### **mRNA Expression Analysis**

Cells were harvested from the MLR 18 hr into culture and total RNA was extracted using an Rneasy® Mini Kit (Qiagen, Valencia, CA) according to the provided protocol. RNA concentration and purity was checked with a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA). RNA was then reverse transcribed to cDNA using the RT<sup>2</sup> First Strand Kit (Qiagen) following the provided protocol. cDNA was then analyzed using the RT<sup>2</sup> Profiler PCR Array for Mouse T-cell and B-cell Activation (Qiagen) on the

Mastercycler ep realplex2 (Eppendorf, Hamburg, Germany). The changes in expression of several genes that showed  $\geq 4$ -fold changes were confirmed by individual quantitative PCR (qPCR) with gene-specific primers (200 nM forward primer and 200 nM reverse primer) (Invitrogen, Grand Island, NY) and 10  $\mu$ l *Power SYBR*<sup>®</sup> Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) on the Mastercycler ep Realplex2 (Eppendorf). The relative quantification of experimental genes in comparison to the reference gene,  $\beta$ -Actin, was determined. The relative expression ratio was calculated based on the qPCR efficiency and the crossing points for the experimental genes and  $\beta$ -Actin transcripts.

<b>Table 2</b>		
<b>Quantitative PCR Primers</b>		
<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
<i>CD40L</i>	ACGCCCATTCATCGTCGGCCT	ACTGCTGCTCGCAAAGCTGGG
<i>Cyclin D3</i>	TGTGGGGGTGGACACTCGCT	CACAGCAGCTCCATCCACTGCC
<i>Sla2</i>	GGGCCTGAGATGCCGACTACCT	CCAAGATGCAGGGCGGCTGA
<i>SOCS5</i>	GACGGCTTAGTATCGAAGAA	GCTTATAACAATGGGTTGACC
<i>IL-10</i>	CCCTGGGTGAGAAGCTGAAG	CACTGCCTTGCTCTTATTTTCACA
<i><math>\beta</math>-Actin</i>	AGCTTCTTTGCAGCTCCTTCGTTGC	ACCAGCGCAGCGATATCGTCA

### **Skin Grafts**

Skin grafts were performed by Dr. Senthil Jayarajan, MD. Full-thickness trunk skin was harvested from sacrificed donor C3HeB/FeJ female mice. The skin was cut into 1-cm<sup>2</sup> grafts and placed in sterile PBS until use for transplantation (<30 min). Recipient

mice were anesthetized and shaved around the chest and abdomen. The skin allograft was placed in a slightly larger graft bed prepared over the chest of the C57BL/6J female recipient and secured using 5-0 non-absorbable monofilament sutures. Silk cloth bandages were wrapped over the graft and around the abdomen for 7 days. Mice were administered intraperitoneal injections of O-1966 (dissolved in 0.03% ethanol and 0.03% cremophor in saline) or vehicle (0.03% ethanol and 0.03% cremophor in saline). After day 7, mice were monitored daily for rejection status. Grafts were considered rejected when > 90% was necrotic.

## **Statistics**

Statistical analyses for Figures 1-7 and 9-11 were performed by Dr. John Guaghan (Biostatistics Consulting Center, Temple University School of Medicine). Data were transformed to normalized ratios, to accommodate non-normality of the data. Comparisons between groups were tested using ANOVA with vertical group comparisons at each dose. Least square means were used for horizontal and vertical comparisons between groups and doses. No adjustment was made for multiple comparisons. Statistical significance was defined as p values < 0.01 or 0.001.

Dr. Daohai Yu (Biostatistics, Temple Clinical Research Center, Temple University School of Medicine) performed statistical analyses on Figures 8 and 12- 17. Boxplots are produced to visualize potential differences across treatment groups. Mean differences and their confidence intervals in outcome variables of interest between any two independent or paired (e.g., when comparing post- vs. pre-treatment measurements as for the gene fold-change in Figure 13C) groups are always provided as well. Two-sample (independent or paired) t-test is employed to compare two treatment groups or post vs. pre-treatment within one sample. In case that there are more than two treatment

groups involved, ANOVA is utilized for between group comparisons, where multiple testing p-values are adjusted using the Dunnett method. Normality assumption of the study endpoints is empirically examined and transformations such as log or square-root are explored if evidence from the data does not support normality. Non-parametric tests will be considered in case of non-normal data. Specifically, the Wilcoxon rank-sum/signed rank test will be used in this situation to compare the differences between any two experimental groups or post vs. pre-treatment measurements. The two-way ANOVA is also employed to test differences in outcome measurements across different groups when there are two treatment conditions involved as well as possible interactions between them. Such is the case, for instance, when both cell types (wild-type and CB2 knockout) and treatment groups with various doses of O-1966 in addition to no treatment and vehicle were used to test their effects on NFkB concentration and NFAT OD in Figure 12. Similarly in Figures 16A and 16C, there were both antibody treatment and cannabinoid treatment conditions employed to detect their combined effects on SI and Tregs, and therefore a two-way ANOVA is appropriate for testing their individual as well as possible interaction effects on each of the two outcome variables. A p-value of 0.01 or less is considered to be statistically significant. SAS© software (version 9.3, Cary, NC) was used for all the data analyses reported here.

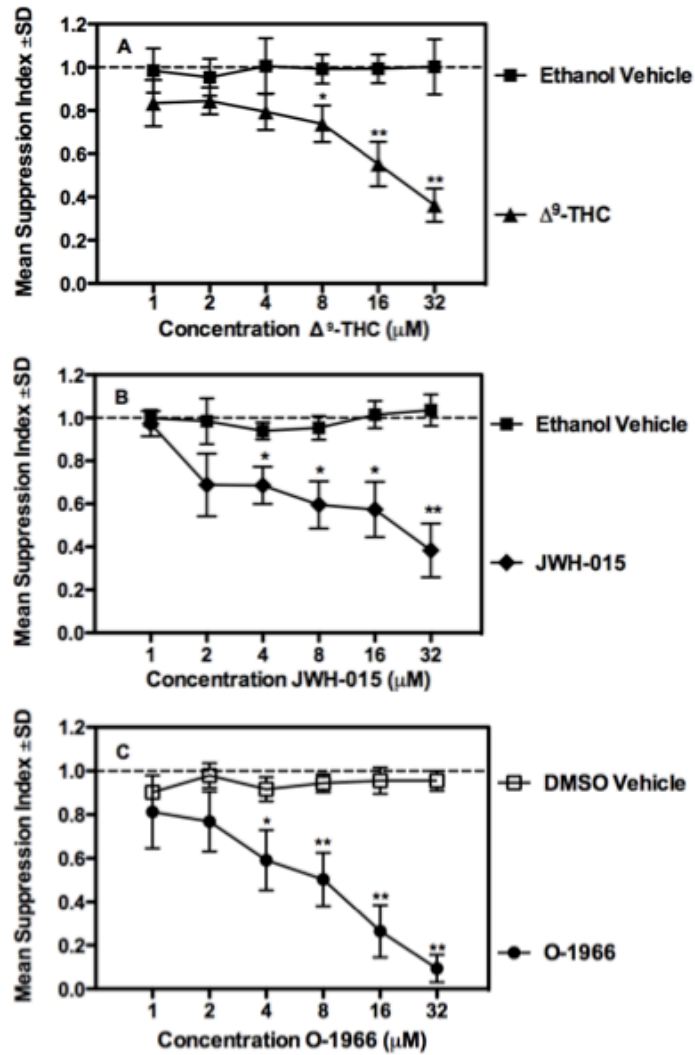
Figures 18-22 were analyzed using GraphPad InStat® (GraphPad Software, Inc. La Jolla, CA). Comparisons between groups were tested using ANOVA with vertical group comparisons at each dose. Least square means were used for horizontal and vertical comparisons between groups and doses. Skin graft rejection data was analyzed using the Log-rank (Mantel-Cox) test. Statistical significance was defined as p values < 0.01 or 0.001.

## CHAPTER 4

### RESULTS

#### **Cannabinoids Inhibit the MLR via the CB2 Receptor.**

To determine the effect of  $\Delta^9$ -THC, JWH-015 and O-1966 on the MLR,  $8 \times 10^5$  C57BL/6 responder splenocytes were pretreated for 3 h with cannabinoid or vehicle before addition of  $8 \times 10^5$  mitomycin C inactivated C3HeB/FeJ splenocytes. Figure 1 shows that pretreatment with all three cannabinoids inhibited the MLR in a dose-dependent manner, with suppression observed between 8 and 32  $\mu\text{M}$  compared to vehicle controls. For the CB2-selective agonists, significant suppression was observed at 4  $\mu\text{M}$ . Using a Live/Dead stain, cell viability was assessed and no difference observed in the number of dead cells between control and cannabinoid treated groups. For example, a representative group from data collected for Figure 1, showed cells from MLR cultures that received no treatment were 88.7% live, cells that were treated with ethanol vehicle were 86.9% live, and cells treated with 32  $\mu\text{M}$   $\Delta^9$ -THC were 87.9% live (data not shown). Similarly, cells from other cultures that were treated with 32  $\mu\text{M}$  JWH-015 or O-1966 were 88.6% and 88.5% live, respectively (data not shown). Viability was checked in each experiment hereafter, and cells were 85-90% live in all experiments.

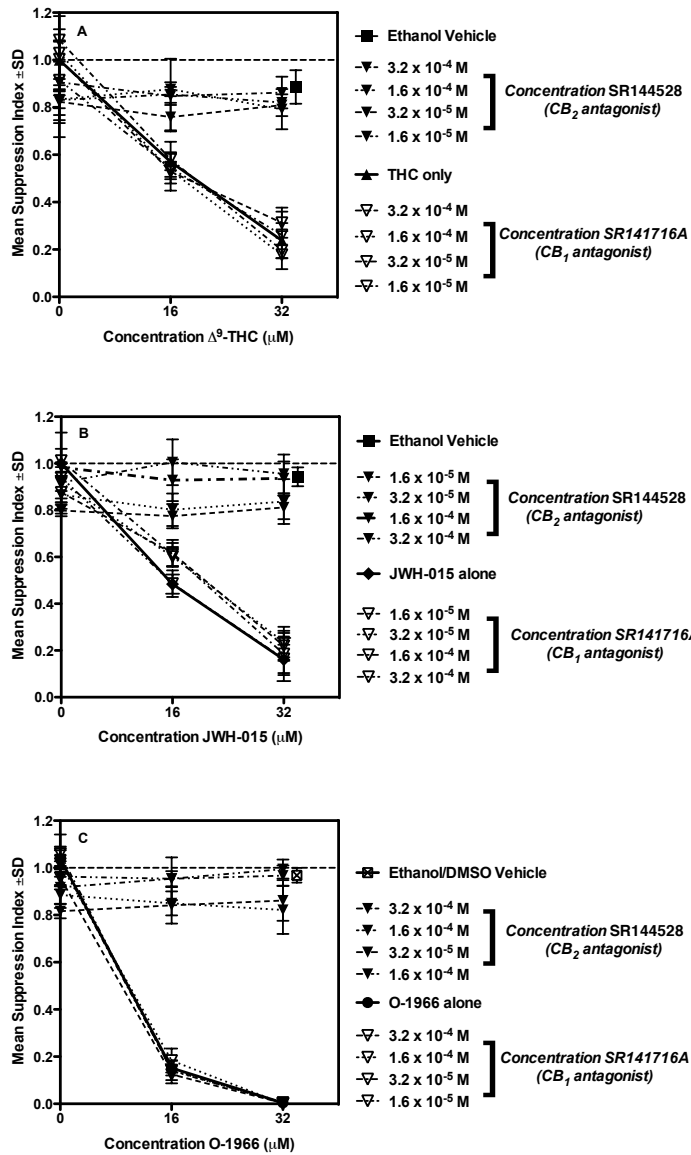


**Figure 1. The cannabinoids  $\Delta^9$ -THC, JWH-015 and O-1966 inhibit the MLR in a dose-dependent manner.** C57BL/6 responder splenocytes were pretreated for 3 h with: Panel A:  $\Delta^9$ -THC ( $\blacktriangle$ ) or ethanol vehicle ( $\blacksquare$ ), Panel B: JWH-015 ( $\blacklozenge$ ) or ethanol vehicle ( $\blacksquare$ ) or Panel C: O-1966 ( $\bullet$ ) or DMSO vehicle ( $\square$ ). Concentrations of ethanol (A and B) or DMSO (C) vehicle correspond to the amount needed to dissolve each concentration of cannabinoid from 1-32  $\mu$ M ( $1.25 \times 10^{-3}\%$  to 0.4% v/v). The  $\Delta^9$ -THC experiment was repeated 3 times, and the JWH-015 and O-1966 experiments were repeated 4 times each, with quadruplicate wells for each treatment in all experiments. Data are the mean  $\pm$  S.D. \* $p < 0.01$ , \*\* $p < 0.001$  (ANOVA, versus vehicle) Values for vehicle are not significantly different from 1.0 (no treatment) at any concentration.

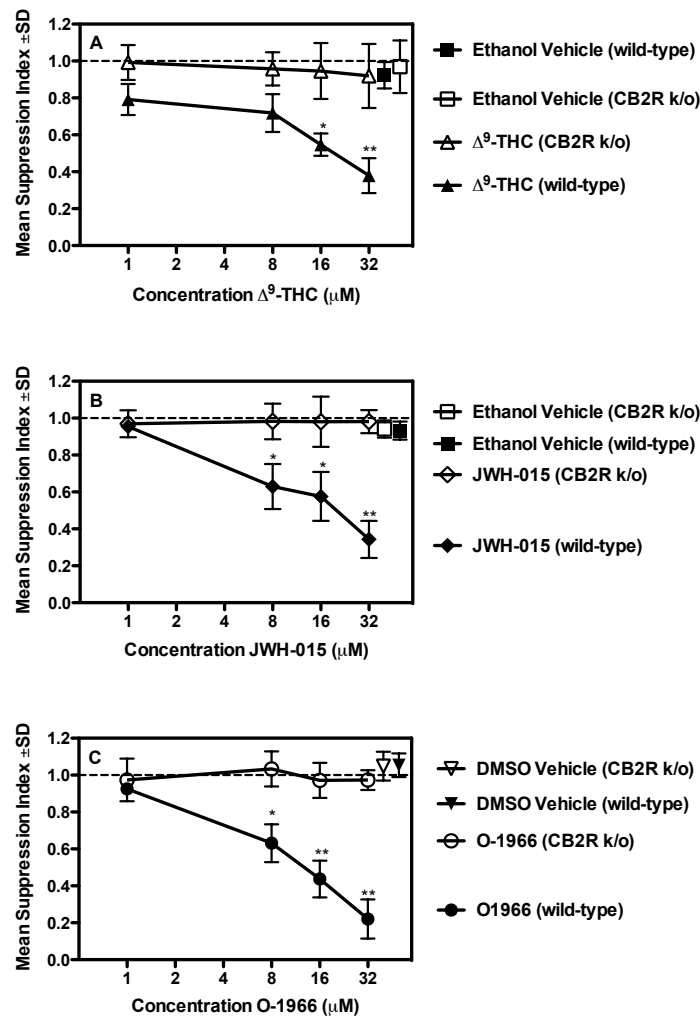
To verify whether the cannabinoids were inducing suppression of the MLR via CB1 or CB2 receptors, CB1- and CB2-selective antagonists were used. C57BL/6 responder splenocytes were pretreated for 2 h with the CB1-selective antagonist SR141716A, the CB2-selective antagonist SR144528, or ethanol vehicle. The cells were then treated for 3 h with  $\Delta^9$ -THC, JWH-015, O-1966, or vehicle controls, before mitomycin C inactivated C3HeB/FeJ splenocytes were added to each well. As shown in Figure 2, pretreatment with the CB2-selective antagonist significantly blocked suppression by  $\Delta^9$ -THC, JWH-015 and O-1966, while pretreatment with the CB1-selective antagonist had no effect on the suppression induced by any of the three cannabinoids.

To corroborate the pharmacological evidence that  $\Delta^9$ -THC, JWH-015, and O-1966 act via the CB2 receptor, splenocytes from CB2 receptor knockout (CB2R k/o) mice were treated with these compounds and tested in the MLR. As shown in Figure 3, pretreatment with  $\Delta^9$ -THC, JWH-015 or O-1966 inhibited the MLR when cells from wild-type mice were used, but not in cultures containing splenocytes from CB2R k/o mice. No difference in viability was observed between cultures from wild-type or CB2R k/o mice, with all treatments yielding viability between 85-90% viable cells.

Together, these results support the conclusion that  $\Delta^9$ -THC, JWH-015, and O-1966 are suppressing the MLR via the CB2 receptor.



**Figure 2.  $\Delta^9$ -THC and the CB2-selective agonists, JWH-015 and O-1966, inhibit the MLR via the CB2 receptor.** C57BL/6 responder splenocytes were pretreated with varying concentrations of SR141716A, a CB1 antagonist, ( $\nabla$ ), SR144528, a CB2 antagonist ( $\blacktriangledown$ ), or ethanol vehicle ( $\blacksquare$ ) for 2 h. The cultures were then treated with one of three cannabinoids. Panel A:  $\Delta^9$ -THC ( $\blacktriangle$ ) or ethanol vehicle ( $\blacksquare$ ). Panel B: JWH-015 ( $\blacklozenge$ ) or ethanol vehicle ( $\blacksquare$ ). Panel C: O-1966 ( $\bullet$ ) or DMSO vehicle ( $\boxtimes$ ). Vehicle controls were the amount of ethanol needed to dissolve the antagonist (0.05% v/v) plus 0.4% v/v of ethanol (A,B) ( $\blacksquare$ ) or the amount of DMSO (C) ( $\boxtimes$ ), needed to dissolve 32  $\mu$ M  $\Delta^9$ -THC, JWH-015 or O-1966. Data are mean  $\pm$  S.D. of 3 separate experiments, with quadruplicate wells for each treatment.  $p < 0.001$  (ANOVA, agonist + CB1 antagonist versus agonist alone, agonist + CB2 antagonist versus vehicle)



**Figure 3.**  $\Delta^9$ -THC, JWH-015, and O-1966 do not suppress the MLR when splenocytes from CB2R k/o are used. Splenocytes from CB2-deficient mice (open symbols) or wild-type mice (closed symbols) were treated for 3 h. Panel A:  $\Delta^9$ -THC (WT: ▲, k/o: △) or ethanol vehicle (WT: ■, k/o: □). Panel B: JWH-015 (WT: ◆, k/o: ◇) or ethanol vehicle (WT: ■, k/o: □). Panel C: O-1966 (WT: ●, k/o: ○) or DMSO vehicle (WT: ▼, k/o: ▽). Concentrations of ethanol (A,B) or DMSO (C) vehicle correspond to the amount needed to dissolve the highest concentration of cannabinoid (0.4% v/v). Each experiment was repeated 3 times, with quadruplicate wells for each treatment. \* $p < 0.01$ , \*\* $p < 0.001$  (ANOVA, WT versus CB2R k/o). Values for vehicle are not significantly different from 1.0 (no treatment).

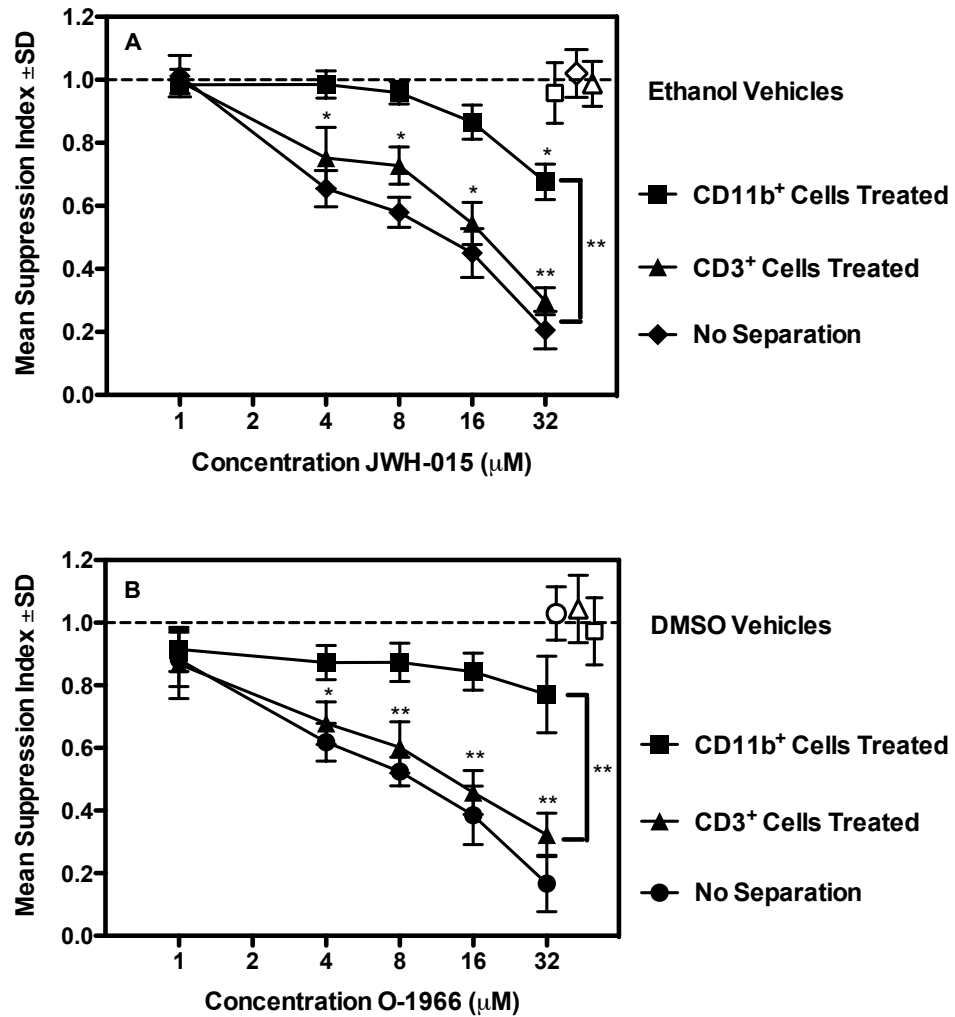
## **CB2-Selective Cannabinoids Directly Inhibit T-cells**

The question was addressed as to whether the cannabinoids act directly on the T-cells, on accessory cells, or on both types of cells. Splenocytes from wild-type C57BL/6 mice were sorted into highly purified subpopulations using flow cytometry. Specifically, CD3<sup>+</sup> (T-cells) and CD11b<sup>+</sup> (myeloid derived cells) populations were selected and individually treated with JWH-015 or ethanol vehicle, or O-1966 or DMSO vehicle, before being added back to the remainder of the untreated spleen cells, which were either CD3 or CD11b depleted, to restore the normal spleen population. The reconstituted cells were then incubated with mitomycin C inactivated C3HeB/FeJ stimulator splenocytes. Figure 4 shows that complete inhibition of the MLR was observed only in cultures containing CD3<sup>+</sup> cells that had been treated with a cannabinoid. In cultures that received cannabinoid treated CD11b<sup>+</sup> cells, significant inhibition was only observed for CD11b<sup>+</sup> cells that were treated with 32  $\mu$ M JWH-015 ( $p < 0.01$ ), however the inhibition was statistically different from that observed when unfractionated cells were treated. Treatment of CD11b<sup>+</sup> cells with any dose of O-1966 did not reach statistical significance. Further, CD11b<sup>+</sup> cells treated with 8  $\mu$ M, 16  $\mu$ M, or 32  $\mu$ M of JWH-015 or O-1966 were significantly less inhibited than unsorted cells treated with the same dose of cannabinoid, indicating that the observed effect of CB2 agonists can be attributed primarily to a direct effect on the T-cells.

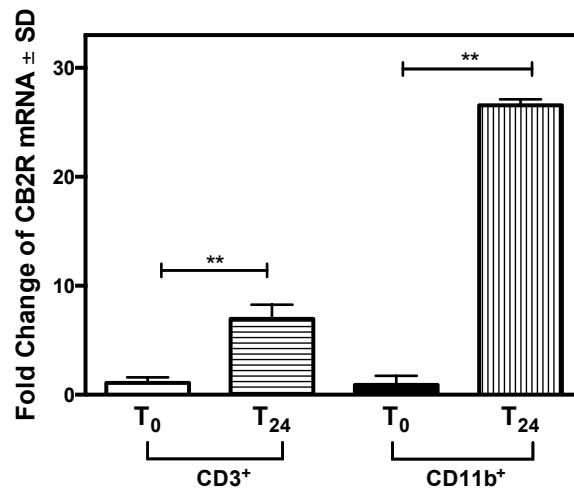
To rule out the possibility that cannabinoids were inhibiting CD3<sup>+</sup> cells to a greater extent than CD11b<sup>+</sup> cells from disproportionate expression of CB2 receptors, quantitative PCR was performed to measure CB2 receptor (CB2R) RNA expression

levels in purified CD3<sup>+</sup> cells and CD11b<sup>+</sup> cells immediately after spleens were removed (T<sub>0</sub>) and after 24 h in the MLR (T<sub>24</sub>). CB2R expression was not significantly different between these cell populations at T<sub>0</sub>. By T<sub>24</sub>, both populations had significantly increased CB2R expression, with a 26.6-fold increase in CD11b<sup>+</sup> cells and a 6.9-fold in CD3<sup>+</sup> cells (Fig. 5). Thus, the data do not support the hypothesis that the reason for the increased inhibition by CD3<sup>+</sup> cells was due to a greater expression of CB2R.

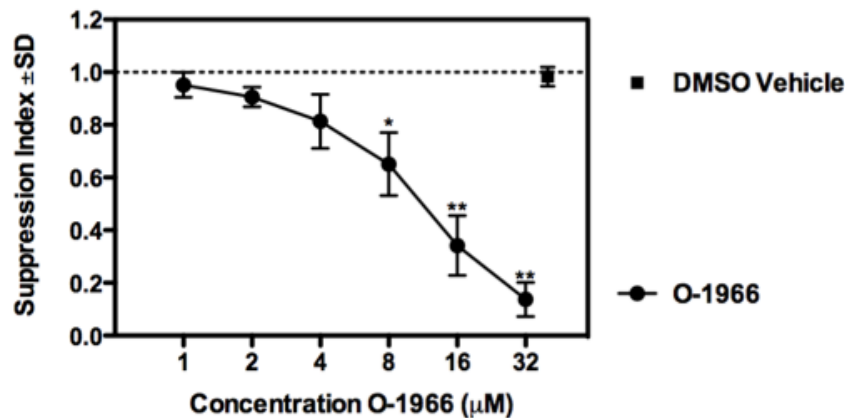
To further verify that the cannabinoids can directly suppress T-cells, splenocytes were sorted into a CD3<sup>+</sup> population. Purified T-cells were treated for 3 hours with O-1966 or DMSO vehicle and then activated with the anti-CD3 and anti-CD28 antibodies. In the presence of O-1966 (Figure 6), there was a dose-dependent, marked decrease in proliferation. This experiment shows conclusively that the cannabinoids can act directly on T-cells, as proliferation could be inhibited by the cannabinoid in the absence of accessory cells.



**Figure 4. JWH-015 and O-1966 directly inhibit T-cells.** C57BL/6 splenocytes were sorted by flow cytometry into CD3<sup>+</sup>, CD11b<sup>+</sup>, or CD3<sup>-</sup>CD11b<sup>-</sup> fractions. CD3<sup>+</sup> fractions (▲) or CD11b<sup>+</sup> fractions (●) were treated for 3 h with the desired cannabinoid (closed symbols) or vehicle (open symbols). Panel A: JWH-015 or ethanol vehicle. Panel B: O-1966 or DMSO. CD3<sup>+</sup> treated populations were combined with untreated CD11b<sup>+</sup> and CD3<sup>-</sup>CD11b<sup>-</sup> FACS sorted cell subsets to reconstitute the normal splenocyte population for carrying out the MLR. Likewise, CD11b<sup>+</sup> treated populations were combined with untreated CD3<sup>+</sup> and CD3<sup>-</sup>CD11b<sup>-</sup> populations. Data are the mean of 3 separate experiments, with quadruplicate wells for each treatment. \*p < 0.01, \*\*p < 0.001 (ANOVA, treated fraction versus vehicle, CD3<sup>+</sup> treated versus CD11b<sup>+</sup> treated)



**Figure 5. CB2 receptor (CB2R) expression increases in the MLR.** C57BL/6 splenocytes were sorted by flow cytometry immediately after harvest (T<sub>0</sub>) or after 24 h in the MLR (T<sub>24</sub>). CD3<sup>+</sup> and CD11b<sup>+</sup> populations were collected, RNA was extracted, reverse transcribed, and analyzed by quantitative PCR. The fold change of CB2R expression in CD3<sup>+</sup> cells from T<sub>0</sub> (□) to T<sub>24</sub> (▨) and in CD11b<sup>+</sup> cells from T<sub>0</sub> (■) to T<sub>24</sub> (▩) is shown. Levels of CB2R were normalized to the reference gene β-Actin. Data are the mean of 2 experiments. \*\*p < 0.001 (Two sample t-test)

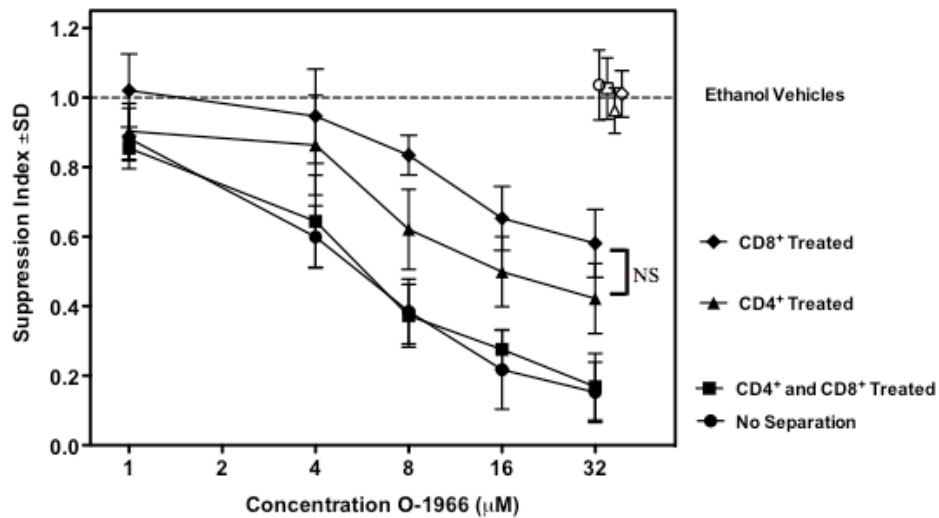


**Figure 6. O-1966 inhibits T-cell proliferation in response to activation by anti-CD3 and anti-CD28 antibodies.** Purified C57BL/6 T-cells (CD3<sup>+</sup>) were treated for 3 h with O-1966 (●) or DMSO vehicle (■). The T-cells were added to a plate coated with 25 µg anti-CD3 antibody/well, and soluble anti-CD28 antibody (0.4 µg/well) was added to each well. Data are the average of 3 separate experiments with quadruplicate wells for each treatment. \*p < 0.01, \*\*p < 0.001. (ANOVA, versus vehicle)

### CB2-Selective Agonists Inhibit Both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells

To determine whether O-1966 suppresses CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, or both, splenocytes from wild-type mice were sorted by flow cytometry and CD4<sup>+</sup> and CD8<sup>+</sup> cells were selected. The populations were individually treated with O-1966 or ethanol vehicle, before being added back to the remainder of the untreated spleen cells, which were either CD4 or CD8 depleted, to restore the normal spleen population for the MLR. Figure 7A shows complete inhibition of the MLR was observed only in unsorted cultures and cultures containing cannabinoid treated CD4<sup>+</sup> and CD8<sup>+</sup> cells. In cultures that received cannabinoid treated CD4<sup>+</sup> cells, the maximum suppression was 65% of the unsorted maximum and treatment of CD8<sup>+</sup> cells was 50% of the maximum unsorted suppression, indicating O-1966 suppressed both CD4<sup>+</sup> and CD8<sup>+</sup> cells. O-1966 treatment did not alter the CD4:CD8 ratio during the course of the MLR (Table 3).

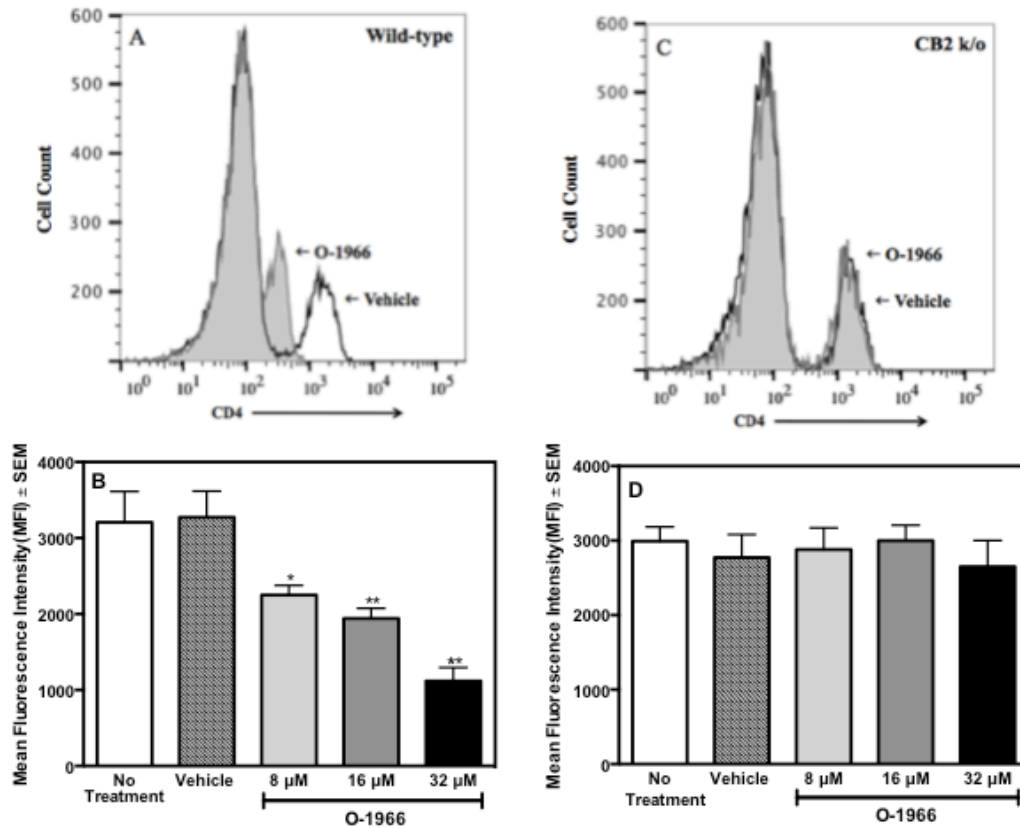
<b>Table 3.</b> Percent CD3 <sup>+</sup> CD4 <sup>+</sup> and CD3 <sup>+</sup> CD8 <sup>+</sup> in the MLR after 72 h in culture.		
	<b>CD4<sup>+</sup></b>	<b>CD8<sup>+</sup></b>
No Treatment	53.1 ± 3.2	42.6 ± 2.1
Ethanol Vehicle	55.2 ± 2.9	40.4 ± 2.2
1 μM O-1966	54.5 ± 3.1	41.9 ± 2.4
8 μM O-1966	55.3 ± 2.8	41.4 ± 2.1
16 μM O-1966	56.6 ± 3.3	40.5 ± 1.9
32 μM O-1966	58.7 ± 3.2	38.2 ± 2.1



**Figure 7. O-1966 inhibits both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells.** C57BL/6 splenocytes were sorted by flow cytometry into CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, or CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> fractions. CD4<sup>+</sup> fractions were treated with O-1966 (▲) or ethanol vehicle (△). CD8<sup>+</sup> fractions were treated with O-1966 (◆) or vehicle (◇). Both CD4<sup>+</sup> and CD8<sup>+</sup> fractions were treated with O-1966 (■) or vehicle (□). Unseparated populations were treated with O-1966 (●) or vehicle (○), for 3 h. Treated CD4<sup>+</sup> or CD8<sup>+</sup> populations were combined with untreated CD8<sup>+</sup> or CD4<sup>+</sup>, respectively, and then with the CD4<sup>-</sup>CD8<sup>-</sup> cell subset to reconstitute the normal splenocyte population for carrying out the MLR. Data are the mean of 2 separate experiments, with quadruplicate wells for each treatment. (ANOVA, CD4 treated versus CD8 treated)

The expression of CD4 on the cell surface of CD3<sup>+</sup> cells was measured. MLR cultures were started using splenocytes from wild-type or CB2R k/o mice. The cells were pretreated for 3 h with O-1966 or ethanol vehicle and harvested 48 h into the assay, stained, and analyzed by flow cytometry. Figure 8A is a representative comparative histogram of the fluorescent intensity of CD4 in cultures treated with 32 µM O-1966 or ethanol vehicle control. While the percentage of CD4<sup>+</sup> cells in the CD3<sup>+</sup> population did not change, O-1966 treatment caused a negative shift of fluorescence intensity of these cells. Figure 8B shows that treatment with 8, 16, and 32 µM of O-1966 resulted in a dose

dependent decrease of the mean fluorescence intensity of CD4 expression on the cell surface. Further, when splenocytes from CB2R k/o mice were used, there was no change in CD4 fluorescence intensity when treated with similar doses of O-1966, demonstrating that the decreased expression in wild-type mice is CB2 mediated.



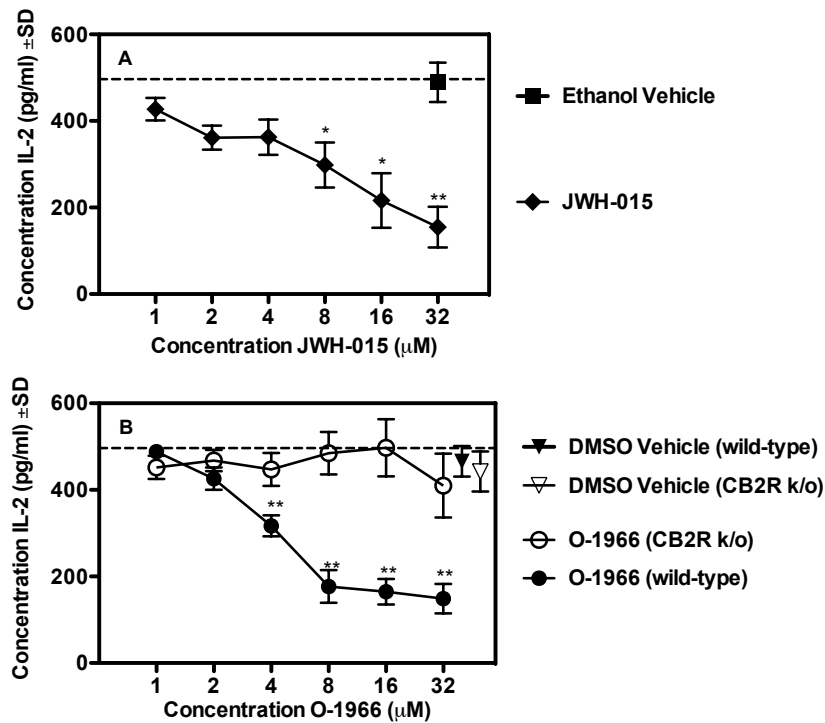
**Figure 8. O-1966 treatment decreases CD4 expression in vitro.** Wild-type (panels A and B) or CB2R k/o (panels C and D) C57BL/6 responder splenocytes were pretreated for 3 h with O-1966 or ethanol vehicle. MLR cultures were harvested at 48 hr and analyzed for CD4 expression on CD3<sup>+</sup>CD4<sup>+</sup> populations by flow cytometry. An equal number of CD3<sup>+</sup>CD4<sup>+</sup> cells were analyzed for each treatment group. Representative histograms of CD3<sup>+</sup> cells from cultures treated with 32 μM O-1966 (gray filled) compared to vehicle treated cells (white filled) with responder cells from wild-type (Panel A) and CB2R k/o (Panel C). Mean Fluorescence Intensity (MFI) of CD4 in CD3<sup>+</sup>CD4<sup>+</sup> populations from MLR cultures that received no treatment (□), ethanol vehicle (▨), 8 μM O-1966 (▩), 16 μM O-1966 (▤), or 32 μM O-1966 (■). Data are mean ± S.E.M. of 3 separate experiments. \* p < 0.01, \*\* p < 0.001 (ANOVA, O-1966 versus vehicle). Values for vehicle are not significantly different from no treatment.

### **CB2 Agonists Decrease IL-2 Release**

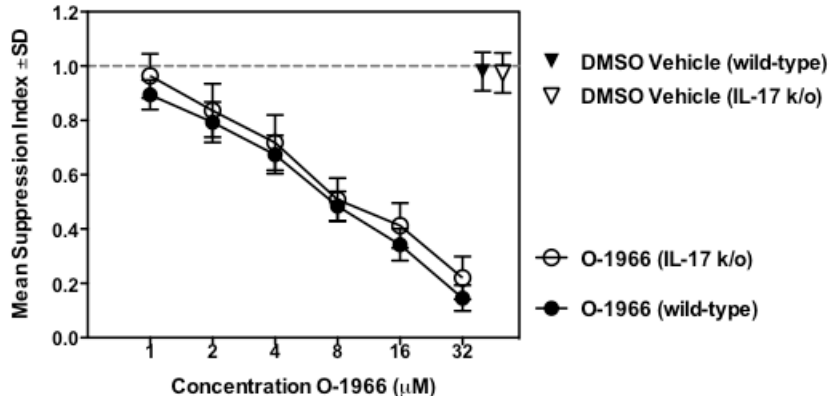
To examine the effect of cannabinoids on one aspect of T-cell function, the release of IL-2 in the MLR was examined. Culture supernatants from unfractionated spleen cells were collected 24 h after the start of the MLR incubation. Figure 9 shows that both JWH-015 and O-1966 inhibited IL-2 release in a dose-dependent manner, indicating that the cannabinoids inhibit this parameter of T-cell function. Furthermore, when splenocytes from CB2R k/o mice were used, O-1966 treatment did not inhibit IL-2 release, indicating this effect is CB2 receptor dependent.

### **Inhibition by CB2 Agonists is not IL-17-mediated**

Whether IL-17 was important in suppression by O-1966 was tested in the MLR using IL-17 k/o mice. Figure 10 shows inhibition by O-1966 in the MLR was the same with splenocytes from IL-17 k/o as with WT mice, indicating IL-17 modulation does not mediate suppression by CB2-agonists.



**Figure 9. JWH-015 and O-1966 inhibit the release of IL-2 in the MLR.** To determine the effect of CB2-selective cannabinoids on the release of IL-2, CB2R k/o responder splenocytes (open symbols) or wild-type littermates (closed symbols) were pretreated for 3 h with: Panel A: JWH-015 (◆) or ethanol vehicle (■). Panel B: O-1966 (WT: ●, k/o: ○) or DMSO vehicle (WT: ▼, k/o: ▽). The cultures were incubated for 24 h; supernatants were collected; and concentrations of IL-2 were assessed by ELISA. Concentrations of ethanol or DMSO vehicle correspond to the concentration needed to dissolve the highest concentration of cannabinoid. JWH-015 data are the mean of 3 separate experiments with triplicate wells for each treatment, and O-1966 are the mean of 2 separate experiments with triplicate wells for each treatment. \* $p < 0.01$ , \*\* $p < 0.001$  (ANOVA, A: JWH-015 versus vehicle; B: WT versus k/o). Values for vehicle are not significantly different from 1.0 (no treatment)

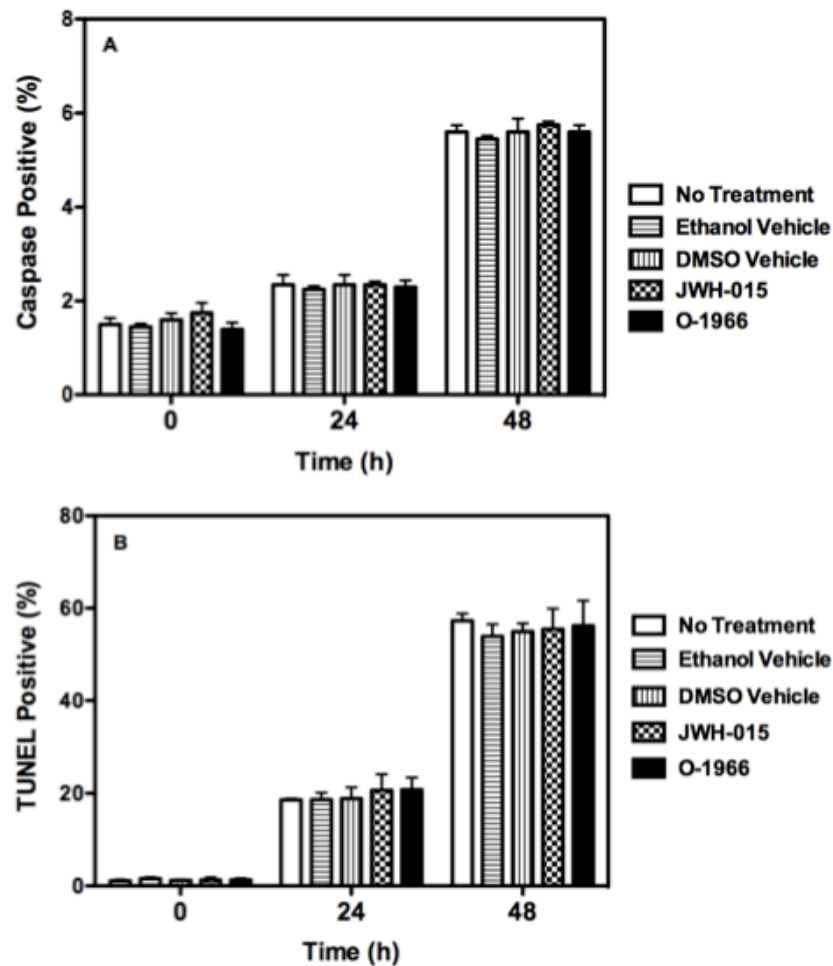


**Figure 10. O-1966 suppresses the MLR when splenocytes from IL-17 k/o are used.** Splenocytes from IL-17-deficient mice (open symbols) or wild-type mice (closed symbols) were treated for 3 h with O-1966 (WT: ●, k/o: ○) or DMSO vehicle (WT: ▼, k/o: ▽). Concentrations of DMSO vehicle correspond to the amount needed to dissolve the highest concentration of cannabinoid (0.4% v/v). Data are the mean of 2 separate experiments with quadruplicate wells for each treatment. Values between WT and k/o are not significantly different (ANOVA).

### JWH-015 and O1966 do not Induce Apoptosis

A possible mechanism that has been proposed for cannabinoid mediated immunosuppression is through the induction of apoptosis of activated immune cells (Lombard et al. 2007; McKallip et al. 2002). Experiments carried out in the present studies show that membrane integrity of the cells in the MLR was unchanged by cannabinoid treatment, as measured by LIVE/DEAD staining in each experimental condition. To verify our conclusion that the CB2 agonist did not induce apoptosis, more precise measurements were used to detect and measure apoptotic cells. To detect cells in the early stages of apoptosis, MLR cultures treated with JWH-015, O-1966, or vehicle were harvested at the start of the culture, and 24 and 48 h into the assay. Levels of caspases 1, 3, 4, 5, 7, 8, and 9 were measured by flow cytometry using a caspase assay kit

(Vybrant® FAM Poly Caspases Assay Kit, Molecular Probes, Inc., Eugene, OR). Figure 11A shows that, while the number of caspase positive cells increased as time in culture increased, there were no differences between cells that received no treatment or treatment with vehicle, as compared with treatment with a cannabinoid. Additionally, DNA fragmentation was measured using a terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (Fig. 11B), to test cells from MLR cultures that were harvested at the start of the assay ( $T_0$ ), and 24 or 48 h after culture initiation. At all time points tested, there was no difference between treatment groups, showing that apoptosis is not the mechanism by which JWH-015 and O-1966 are suppressing the MLR.



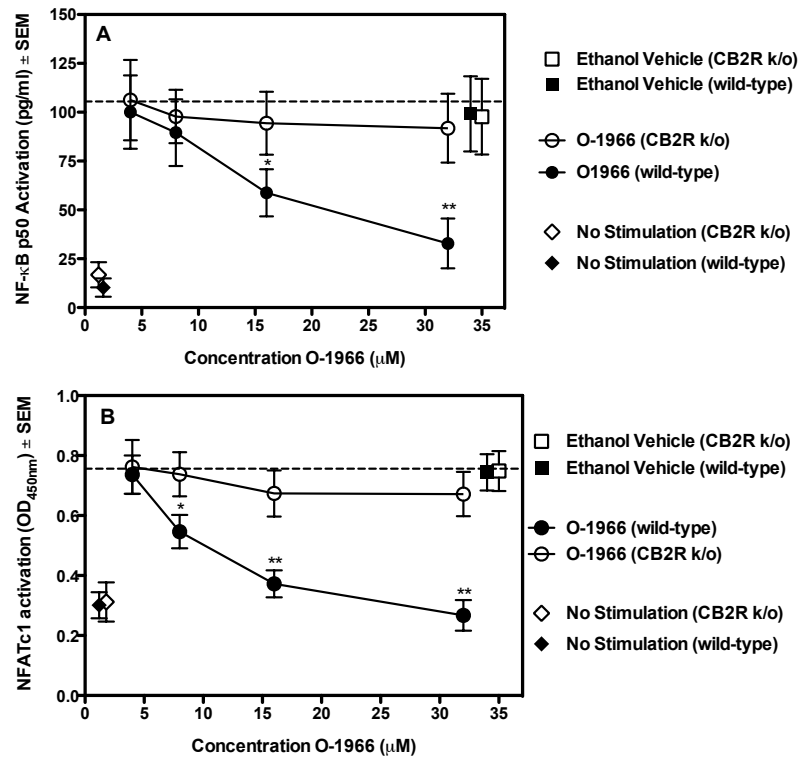
**Figure 11. JWH-015 and O-1966 do not induce apoptosis in the MLR.** To determine if the CB2-selective cannabinoids induce apoptosis in the MLR, cells were harvested at time zero, or after 24 or 48 hours in culture. Panel A: cells were stained for activation of caspases and analyzed by flow cytometry. Panel B: TUNEL assay. Cells received no treatment (□), 32 μM JWH-015 (▣), ethanol vehicle (▤), 32 μM O-1966 (■), or DMSO vehicle (▥). Concentrations of ethanol or DMSO vehicle correspond to the concentration needed to dissolve the respective cannabinoid. Data are mean of 3 separate experiments, with duplicate wells for each treatment. There was no significant difference in numbers of cells positive for activated caspases or TUNEL positive cells at any time points tested (ANOVA).

## O-1966 Alters Gene Expression in T-cells

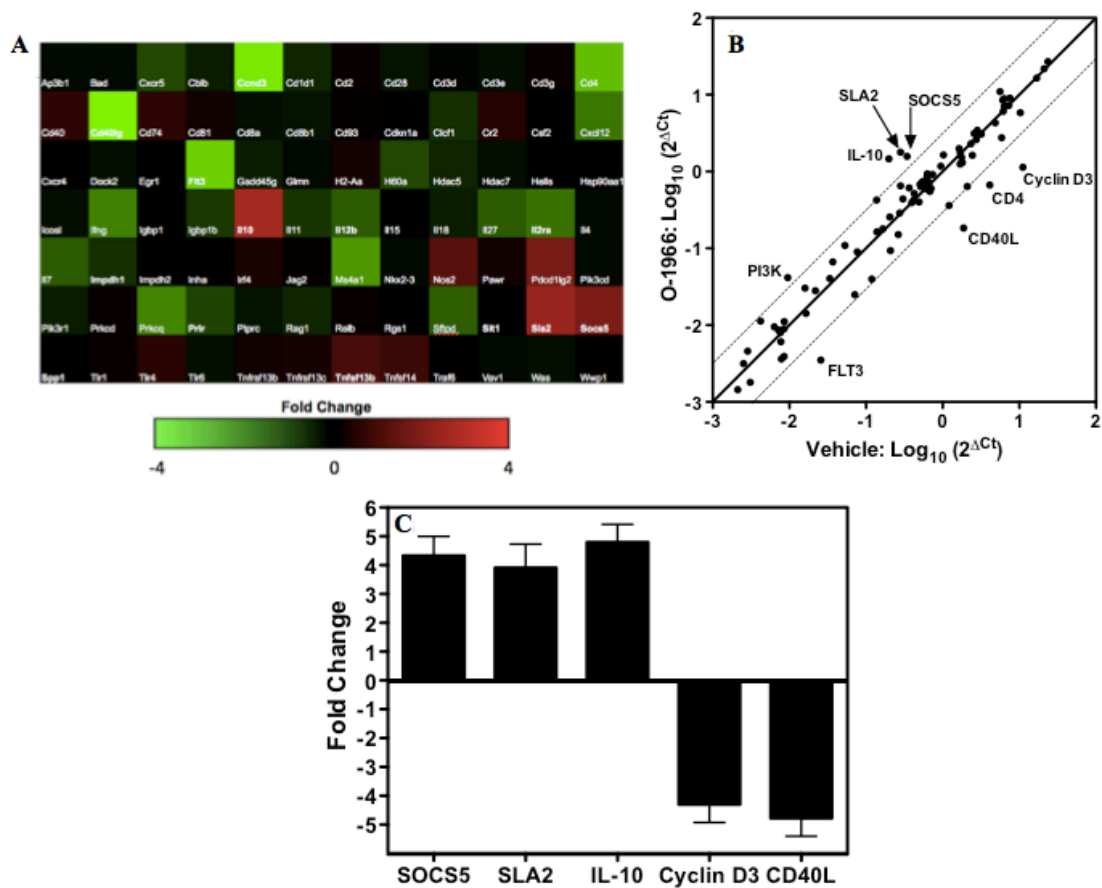
To examine a possible mechanism of suppression by O-1966, nuclear levels of the transcription factors NF- $\kappa$ B and NFAT were measured in the presence or absence of O-1966. Splenocytes from wild-type C57BL/6 mice or CB2 receptor knockout (CB2R k/o) mice were sorted by flow cytometry and T-cells were negatively selected (CD11b<sup>-</sup>B220<sup>-</sup>). The T-cells were treated for 3 hours with O-1966 or ethanol vehicle and then activated with anti-CD3 and anti-CD28 antibodies and incubated for 18 h. The cells were then harvested and nuclear proteins extracted. Levels of activated NF- $\kappa$ B and NFAT that were able to bind to their target promoters were measured using the TransAM<sup>®</sup> transcription factor ELISA kits for NF- $\kappa$ B p50 and NFATc1. Figure 12 shows that treatment of T-cells from wild-type mice with O-1966 significantly decreased levels of both transcription factors in a dose-dependent manner, with suppression observed at concentrations of the cannabinoid of 16 and 32  $\mu$ M for NF- $\kappa$ B, and of 8 to 32  $\mu$ M for NFAT, as compared to ethanol vehicle controls. Suppression of neither transcription factor was observed in cultures containing T-cells from CB2R k/o mice. Identical cultures run in parallel, were harvested at 18 h and tested to measure cell viability using a Live/Dead<sup>®</sup> dead cell stain. No difference was observed in the number of dead cells between control and cannabinoid treated groups (data not shown).

To examine the effect of cannabinoids on the expression of genes involved in T-cell activation, an RT<sup>2</sup> Profiler<sup>™</sup> PCR Array for T-cell and B-cell activation was used. C57BL/6 responder splenocytes were pretreated for 3 h with 32  $\mu$ M O-1966 or ethanol vehicle before the addition of mitomycin C inactivated C3HeB/FeJ splenocytes.

After 18 hours, the cultures were harvested and purified by flow cytometry. The CD3<sup>+</sup> T-cell population was collected and RNA extracted and analyzed. Figure 13 shows the differences in levels of gene expression between O-1966 and vehicle treated cells. Panel A shows a scatterplot of gene expression changes in T-cells of all the genes tested in the array. Genes that showed  $\geq 4$ -fold changes in O-1966 treated cells, indicated by lying outside the dotted lines, were subsequently tested in individual qPCR reactions. These data presented in Figure 13b show that O-1966 treated T-cells from the MLR had a 4.8-fold and a 4.3-fold reduction in the expression of CD40 ligand and CyclinD3, respectively. In addition, there was a 4.3-fold increase in Suppressor of Cytokine Signaling 5 (SOCS5), a 3.9-fold increase of Src-like-adaptor 2 (SLA2), and a 4.8-fold increase of IL-10 mRNA expression.



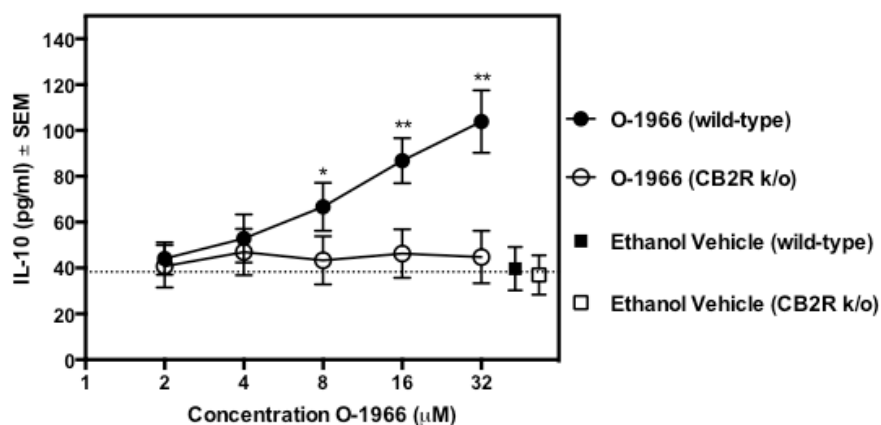
**Figure 12. O-1966 decreases levels of activated nuclear forms of NF-κB and NFAT in T-cells.** Splenocytes from CB2R k/o mice (open symbols) or wild-type mice (closed symbols) were treated for 3 h with O-1966 (WT: ●, k/o:○) or ethanol vehicle (WT: ■, k/o: □) and then added to a plate coated with 25 μg anti-CD3 antibody/well and soluble anti-CD28 antibody (0.4 μg/well) or left unstimulated ( WT: ◆, k/o: ◇). The cultures were incubated for 18 h and cultures were harvested and nuclear proteins extracted. Levels of activated nuclear NFκB (Panel A) and NFAT ( Panel B) were measured using a TransAM ® Transcription Factor ELISA. Data are the mean ± S.E.M. of 3 separate experiments with triplicate wells for each treatment. \*p < 0.01, \*\*p < 0.001. (ANOVA, WT versus k/o). Values for vehicle are not significantly different than no treatment.



**Figure 13. O-1966 treatment alters gene expression of T-cells in the MLR.** C57BL/6 responder splenocytes were pretreated for 3 h with 32  $\mu\text{M}$  O-1966 or 0.4 % ethanol vehicle. The cultures were incubated for 18 h and cells were harvested and sorted by flow cytometry for  $\text{CD3}^+$  T-cells. mRNA was extracted from this population and reverse transcribed. A Qiagen RT<sup>2</sup> qPCR T-cell activation array was used to generate a gene expression profile. Panel A: Heatmap showing up-regulation (red) and down-regulation (green) of genes in T-cells from O-1966 treated versus vehicle treated cultures. Panel B: Scatter plot showing gene expression in T-cells from vehicle treated cultures (x-axis) versus O-1966 treated cultures (y-axis). Points along the central line indicates unchanged expression and dotted lines designate 4-fold change cutoff of genes up-regulated in O-1966 treated cultures (left) and down-regulated in O-1966 treated cultures (right). Panel C: Genes showing  $\geq 4$ -fold changes in the array were confirmed by 2 individual qPCR assays for each gene. Data are the mean  $\pm$  S.E.M. of 2 qPCR reactions from 2 separate experiments.  $p < 0.01$  (Two-sample t-test, O-1966 versus vehicle)

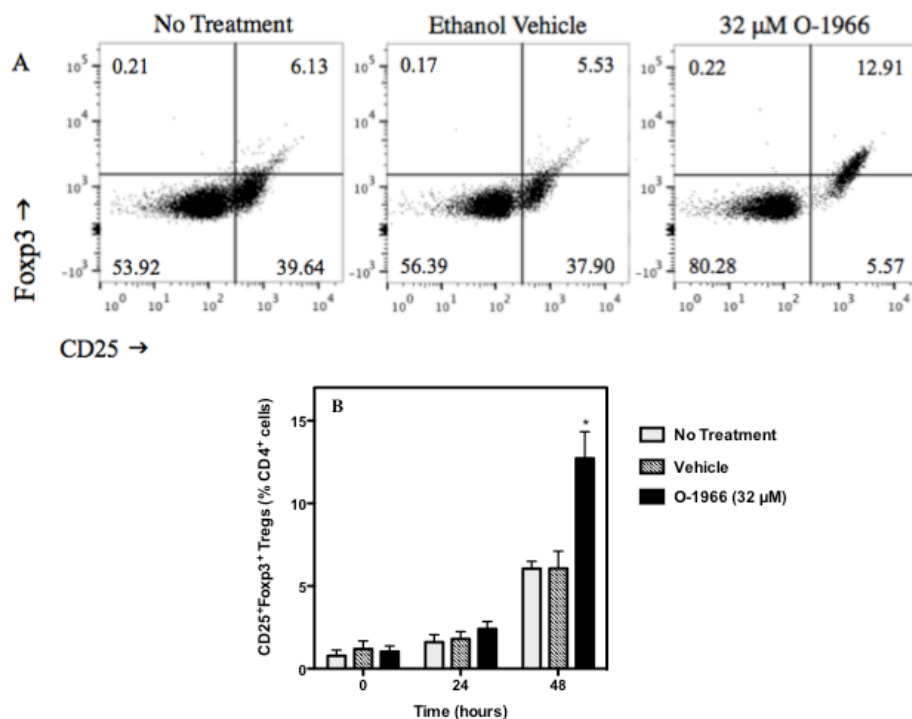
## O-1966 Induces IL-10 and Tregs

The release of IL-10 in the MLR was examined to support the observed increase of IL-10 mRNA expression detected in the array. Culture supernatants were collected 24 h after the start of the MLR. Figure 14 shows that O-1966, in doses ranging from 8 to 32  $\mu\text{M}$ , significantly increased IL-10 release in a dose-dependent manner, indicating that O-1966 promotes an increase in this anti-inflammatory cytokine. Furthermore, when splenocytes from CB2R k/o mice were used, O-1966 treatment did not increase IL-10 release, indicating that this effect is CB2 receptor dependent.



**Figure 14. O-1966 increases IL-10 release.** To determine the effect of O-1966 on the release of IL-10, CB2R k/o responder splenocytes (open symbols) or cells from wild-type littermates (closed symbols) were pretreated for 3 h with O-1966 (WT: ●, k/o: ○) or ethanol vehicle (WT: ■, k/o: □). The cultures were incubated for 24 h; supernatants were collected; and concentrations of IL-10 were assessed by ELISA. Concentrations of ethanol vehicle correspond to the concentration needed to dissolve the highest concentration of cannabinoid. Data are the mean  $\pm$  S.E.M. of 3 separate experiments with triplicate wells for each treatment. \* $p < 0.01$ , \*\* $p < 0.001$ . (ANOVA, WT versus k/o). Values for vehicle are not significantly different from no treatment.

Experiments were carried out to determine whether O-1966 treatment increased the presence of Tregs in the MLR cultures. To measure Tregs, MLR cultures were treated with 32  $\mu$ M O-1966 or ethanol vehicle. Cells in the culture were harvested from wells at the start of the culture ( $T_0$ ), and 24 and 48 h into the assay. The cells were then stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry. Figure 15 shows that cells harvested at 48h had a doubling in the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the live CD4<sup>+</sup> population, from 6.1% in untreated or vehicle treated cells to 12.7% in O-1966 treated cells.

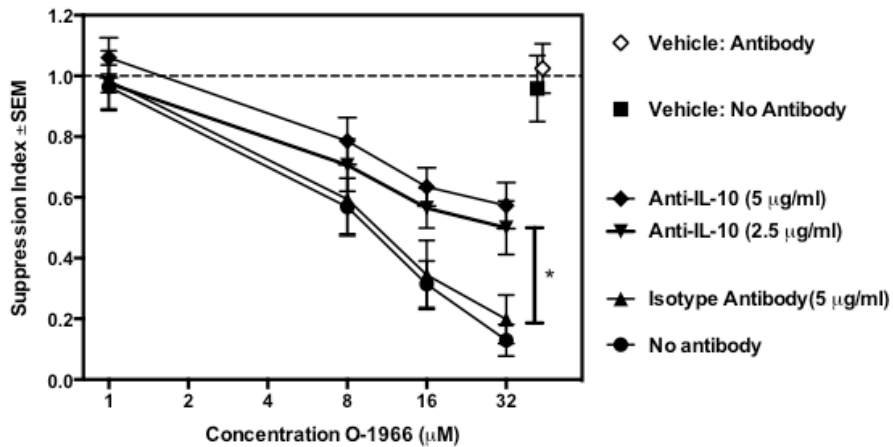


**Figure 15. O-1966 increases the percentage of Tregs in the MLR.** To determine if O-1966 induces Tregs in the MLR, cultures were analyzed by flow cytometry for live CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. Panel A shows representative scatterplots of MLR cultures left untreated or pretreated with 32  $\mu$ M O-1966 or ethanol vehicle, harvested at 48 hours, and stained with antibodies for CD25 and Foxp3. The cells were gated on live CD4<sup>+</sup> cells and are expressed as a percentage of total live CD4<sup>+</sup> cells. Panel B shows the average number of CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs as a percentage of total live CD4<sup>+</sup> from cultures left untreated (□) or pretreated with 32  $\mu$ M O-1966 (■) or ethanol vehicle (▨), and harvested at time zero, or after 24 or 48 hours in culture. Data are the mean  $\pm$  S.E.M. of 3 separate experiments. \* $p < 0.01$  (ANOVA, O-1966 versus vehicle)

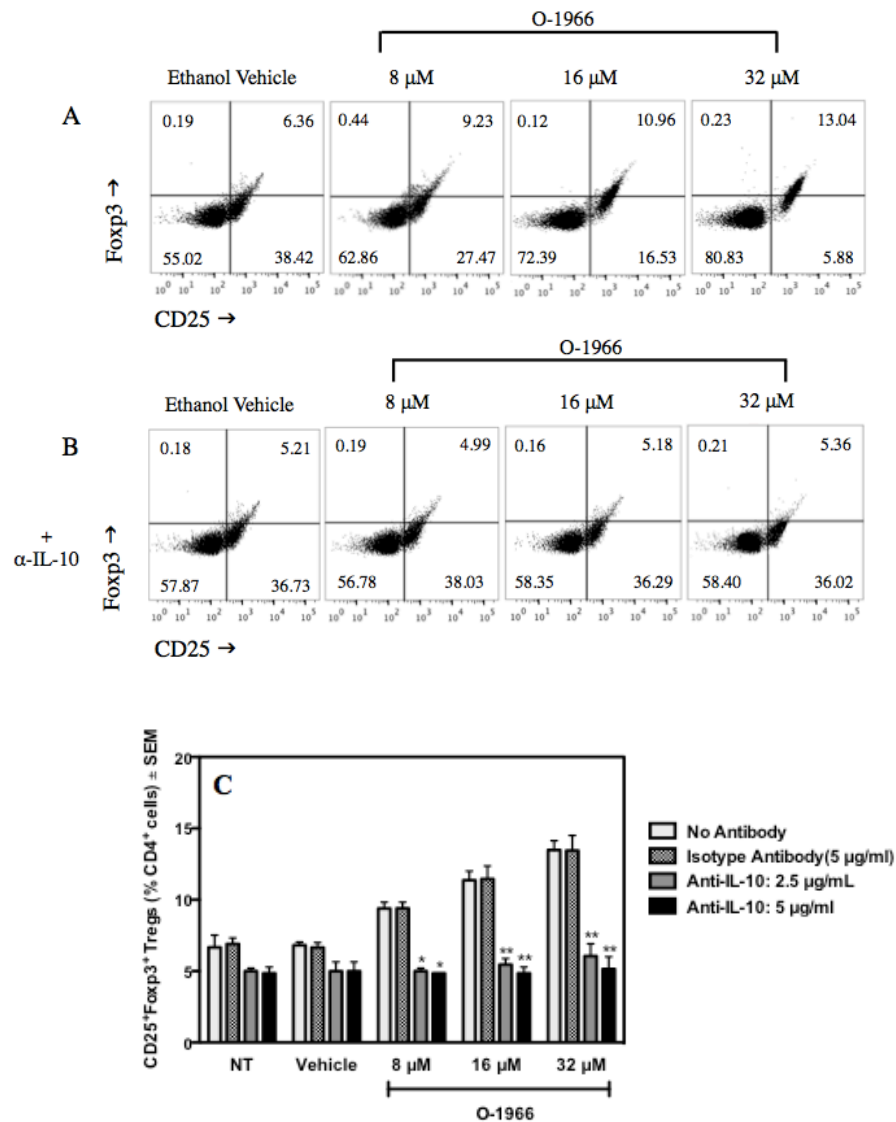
### Suppression by O-1966 is Partly Mediated by IL-10

To assess the importance of increased IL-10 levels in the suppression of the MLR, cultures were treated with 2.5 or 5  $\mu$ g/ml of anti-IL-10 antibody at T<sub>0</sub> and 24 h into the assay to deplete the released IL-10. Figure 16a shows that the addition of 5  $\mu$ g/ml anti-IL-10 antibody gave approximately 50% reversal of suppression induced by the 32  $\mu$ M dose of O-1966. As IL-10 is reported to up-regulate Treg cells (Groux et al., 1997), the effect

of IL-10 depletion on the increase of the percentage of Tregs in the MLR by O-1966 was also assessed. MLR cultures were treated with 2.5 or 5  $\mu\text{g/ml}$  anti-IL-10 at  $T_0$  and 24 h, and cells harvested 48 h into the assay were stained and analyzed by flow cytometry for Tregs. Figure 16d shows that the addition of both 2.5 and 5  $\mu\text{g/ml}$  anti-IL-10 antibody completely blocked the increase of  $\text{CD}25^+\text{Foxp}3^+$  Tregs in the  $\text{CD}3^+\text{CD}4^+$  population in cultures treated with 8 to 32  $\mu\text{M}$  O-1966. The addition of 5  $\mu\text{g/ml}$  of an isotype control antibody did not affect the suppression of proliferation or the increase of the percentage of Tregs at any dose of O-1966 tested. Together, these results indicate that the increased levels of IL-10 seen in O-1966 treated cultures is an important contributing mechanism for the suppression of proliferation and is essential for the increase of Tregs in the MLR.



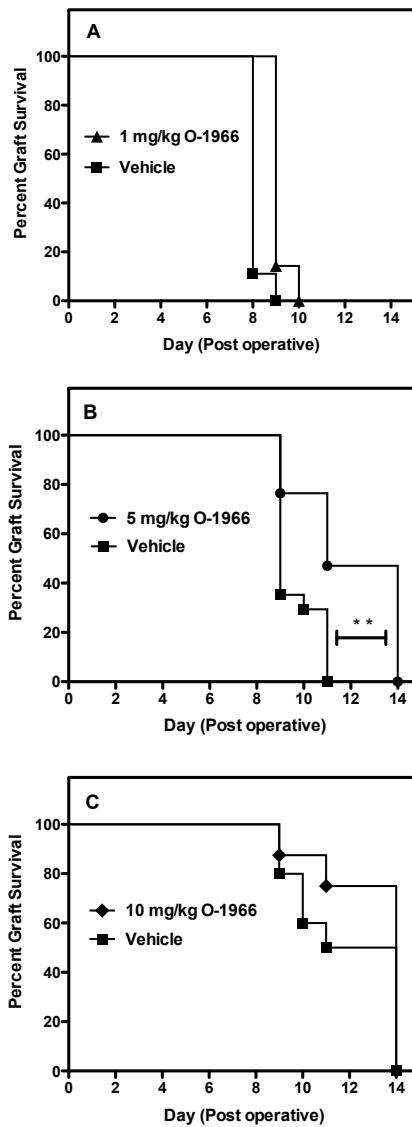
**Figure 16. Anti-IL-10 antibody partially reverses suppression of proliferation by O-1966.** Responder splenocytes were pretreated with O-1966 for 3 hr and with a neutralizing anti-IL-10 antibody 1 hr before and 24 hr after the start of assay. MLR response: Cultures were treated with O-1966 ( $\bullet$ ) or ethanol vehicle ( $\blacksquare$ ), O-1966 and 2.5  $\mu\text{g/ml}$  anti-IL-10 antibody ( $\blacktriangledown$ ), O-1966 and 5  $\mu\text{g/ml}$  anti-IL-10 antibody ( $\blacklozenge$ ), O-1966 and 5  $\mu\text{g/ml}$  isotype control antibody ( $\blacktriangle$ ), or O-1966 and antibody vehicle control ( $\diamond$ ) and progressed to complete MLR to measure proliferation. MLR data are mean  $\pm$  S.E.M. of 3 separate experiments. \*  $p < 0.01$  (two-way ANOVA, anti-IL-10 versus isotype antibody)



**Figure 17. Anti-IL-10 antibody blocks the increase of Tregs by O-1966.** Panels A and B: Representative scatterplots of MLR pretreated with ethanol vehicle or 8, 16, or 32  $\mu$ M O-1966. Panel B was treated with 5  $\mu$ g/ml anti-IL-10 antibody. Cells were stained with antibodies for CD25 and Foxp3 and are gated on live CD4<sup>+</sup> cells. Quadrants are expressed as a percentage of total live CD4<sup>+</sup> cells. Panel C: Average number of CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs as a percentage of total live CD4<sup>+</sup> cells from 3 experiments from cultures pretreated as indicated along with with no antibody ( $\square$ ), 5  $\mu$ g/ml isotype control ( $\square$ ), 2.5  $\mu$ g/ml anti-IL-10 antibody ( $\square$ ), or 5  $\mu$ g/ml anti-IL-10 antibody ( $\square$ ), harvested 48 hr into culture and analyzed by flow cytometry for percentage of Tregs. Data are mean percentage  $\pm$  S.E.M. CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs of total live CD4<sup>+</sup> in the culture and the average of 3 separate experiments. \* $p < 0.01$  \*\* $p < 0.001$  (two-way ANOVA, anti-IL-10 versus isotype antibody)

## **O-1966 Blocks Skin Graft Rejection in vivo**

The capacity of O-1966 treatment to inhibit rejection of a skin graft in vivo was tested. Flank skin was harvested from donor C3HeB/FeJ female mice and transplanted to the flank of recipient female C57BL/6J mice. The graft was sutured and bandaged for 7 days. Doses of O-1966 or vehicle (0.03% ethanol and 0.03% cremophor in saline) were administered by intraperitoneal injection (i.p.) every other day from 1 hour before transplantation to post-operative day 14. Bandages were removed on day 7 and the graft was monitored for rejection. An allograft was considered fully rejected when it was >90% necrotic. Treatment with 5 mg/kg O-1966 increased the mean survival time of the grafts to 11 days, though several grafts were viable for 14 days compared to a mean survival time of 9 days for vehicle treated mice, with some grafts remaining viable for only 11 days (Figure 18B). Treatment with 1 mg/kg or 10 mg/kg O-1966 did not affect graft survival (Figure 18A,C).



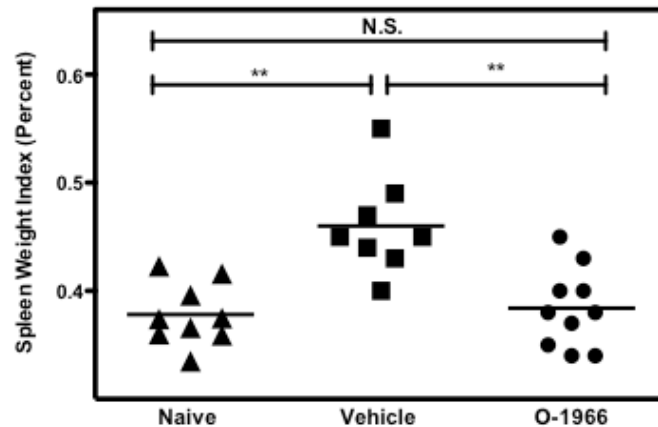
**Figure 18. O-1966 treatment prolongs skin graft viability.** Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 hour pre-op to 14 days post-op. On Day 7 bandages were removed and grafts were monitored for rejection. Percent graft survival of mice treated with A: 1 mg/kg O-1966 (▲), or vehicle (■), B: 5 mg/kg O-1966 (●) or vehicle (■), or C: 10 mg/kg O-1966 (◆) or vehicle (■). Panels A and C are results of a single experiment (n=8 per group), and data in Panel B are the mean of 2 experiments (n=17 per group). \*\* p < 0.001 O-1966 versus vehicle (Logrank [Mantel-Cox] test)

### **O-1966 Treatment Decreases Splenic Weight and Increases Tregs**

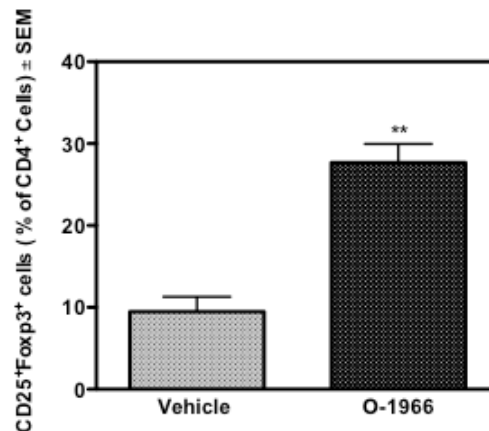
Allograft-recipient C57BL/6J mice were administered 5 mg/kg O-1966 or vehicle (0.03% ethanol and 0.03% cremophor in saline) i.p. every other day from 1 hour before transplantation to post-operative day 14. On day 14, the spleens of recipient mice were removed and weighed, and normalized to body weight. The spleen weight of mice treated with O-1966 were significantly decreased compared to vehicle treated mice (Figure 19). Spleen weight between O-1966-treated mice was not different from control mice that did not receive skin grafts.

The splenocytes were then stained for CD4, CD25 and Foxp3 and analyzed by flow cytometry. Figure 20 shows that mice treated with O-1966 had 27.7% CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the live CD4<sup>+</sup> population, while mice treated with vehicle had only 9.5% CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs.

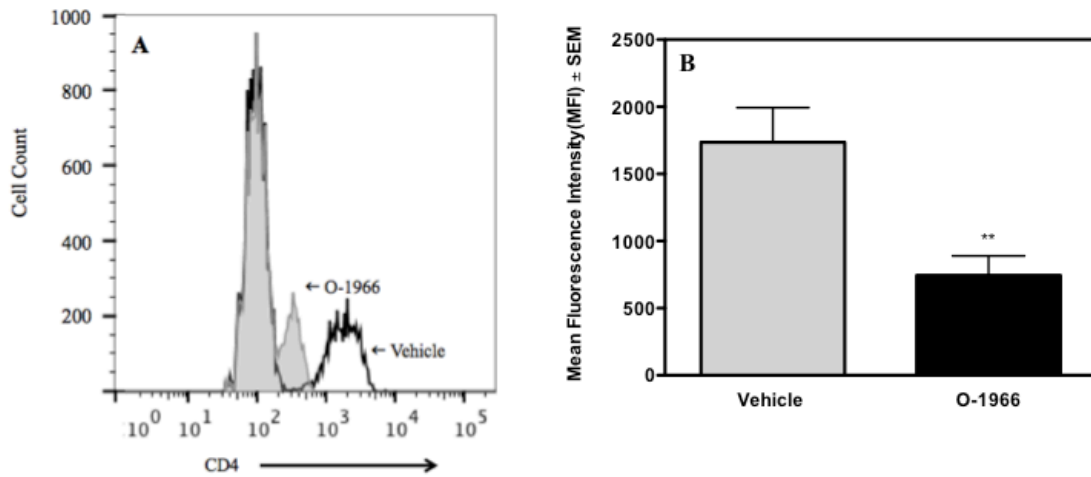
The expression of CD4 on the cell surface of CD3<sup>+</sup> cells was altered by O-1966 treatment in vivo. Figure 21A is a representative comparative histogram of the fluorescent intensity of CD4 on T-cells from mice treated with O-1966 or vehicle. O-1966 treatment caused a negative shift of fluorescence intensity of these cells. Figure 22B presents the mean fluorescence intensity of CD4 from all recipient mice (n=17 for each treatment group), and shows that the average intensity of CD4 expression on the cell surface is decreased.



**Figure 19. O-1966 decreases spleen weights in skin graft recipient mice.** Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 hour pre-op to 14 days post-op. On post-op day 14, animals were sacrificed and spleens were removed. The spleen weight to body weight ratio was calculated and is expressed as a percentage, of control mice ( $\blacktriangle$ ) (n=9) and grafted mice treated with 5 mg/kg O-1966 ( $\bullet$ ) (n=8) or vehicle ( $\blacksquare$ ) (n=10). \*\* p < 0.001 (ANOVA)



**Figure 20. O-1966 treatment increases percentage of splenic Tregs in skin graft recipient mice.** Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 hour pre-op to 14 days post-op. Splenocytes were harvested from grafted mice treated with O-1966 ( $\blacksquare$ ) or vehicle ( $\square$ ) on day 14 and were analyzed by flow cytometry for CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (n=17 for both groups). Data show number of Tregs as a percentage of total live CD4<sup>+</sup> cells (LIVE/DEAD® dead cell stain negative). Data are mean of 2 separate experiments. \*\* p < 0.001 (ANOVA, O-1966 versus vehicle).



**Figure 21. O-1966 treatment decreases CD4 expression in skin graft recipient mice.** Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 hour pre-op to 14 days post-op. Splenocytes were harvested on day 14, stained for CD4, and analyzed by flow cytometry. Panel A: Representative histograms of CD4 expression on CD3<sup>+</sup> cells from mice treated with O-1966 (gray filled) or vehicle (white filled). Panel B: Mean Fluorescence Intensity (MFI) of CD4 in CD3<sup>+</sup>CD4<sup>+</sup> populations from mice treated with O-1966 (■) or vehicle (□). Data are mean of 2 experiments (n=17 for both groups). \*\* p < 0.001 (ANOVA, O-1966 versus vehicle).

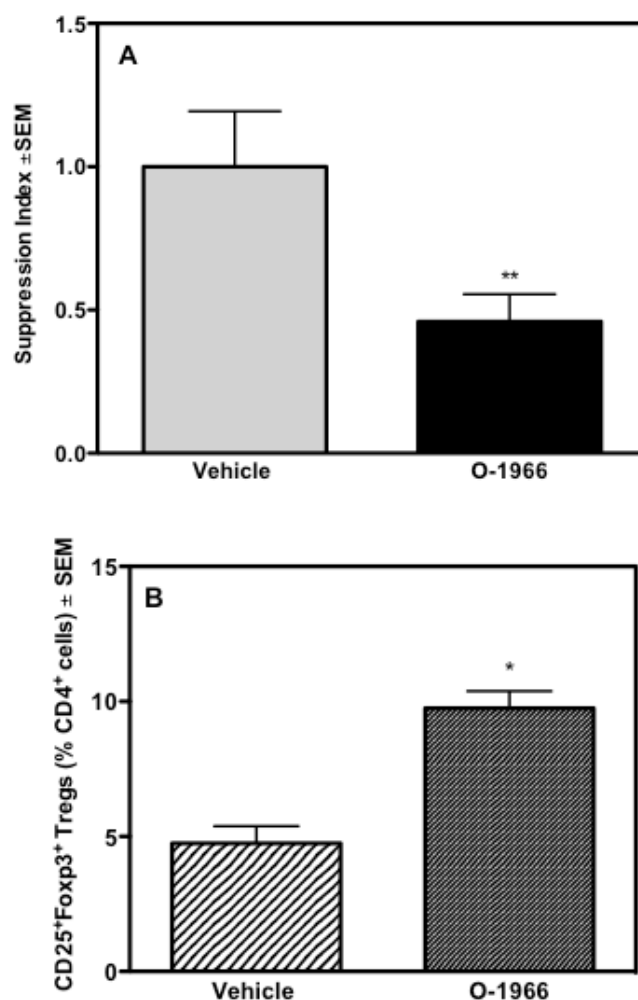
## In vivo O-1966 Treatment Suppresses Splenocytes

### Stimulated ex vivo in the MLR

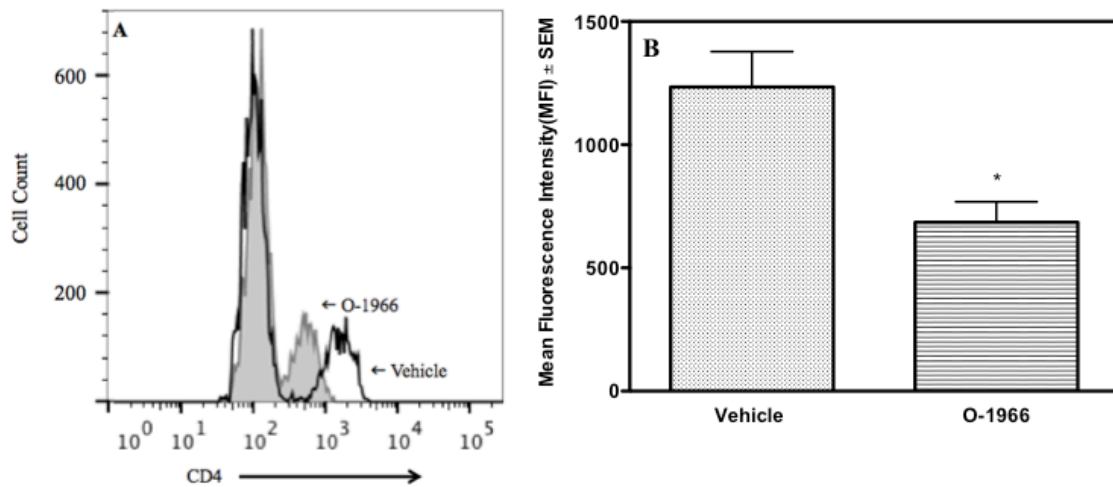
C57BL/6J mice received skin allografts from C3HeB/FeJ mice. Recipient mice were administered 5 mg/kg O-1966 or vehicle (0.03% ethanol and 0.03% cremophor in saline) i.p. every other day from 1 hour before transplantation to post-operative day 14. On day 14, the spleens of recipient mice were removed.  $8 \times 10^5$  splenocytes were restimulated with  $8 \times 10^5$  mitomycin C inactivated C3HeB/FeJ splenocytes for an MLR. Splenocytes from mice treated with O-1966 in vivo had significantly decreased proliferation in response to ex vivo stimulation (Figure 22A). Cultures were harvested 48 h after the start of the assay and stained for CD4, CD25 and

Foxp3 and analyzed by flow cytometry. Figure 22B shows that cultures from mice treated with O-1966 in vivo had double the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs compared to cultures using spleen cells taken from vehicle treated mice, with the percentage increasing from 4.7% to 9.8%.

The expression level of CD4 expressed on T-cells taken from the mice given the cannabinoid in vivo was also measured by flow cytometry after 48 h in culture,. Figure 23A is a representative comparative histogram of the fluorescent intensity of CD4 on CD3<sup>+</sup> T-cells from mice treated with O-1966 and vehicle. O-1966 decreased the fluorescence intensity of CD4 on the cell surface. Figure 23B is the mean fluorescence intensity of CD4 expression on CD3<sup>+</sup>CD4<sup>+</sup> T-cells from MLR cultures.



**Figure 22. In vivo O-1966 treatment decreases proliferation and increases the percentage of Tregs following ex vivo stimulation.** Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 hour pre-op to 14 days post-op. On post-op day 14, animals were sacrificed and spleens were aseptically removed, restimulated with C3HeB/FeJ splenocytes and put into culture for MLR (Panel A) or harvested at 48 h and analyzed by flow cytometry (Panel B). Panel A: Proliferation of cultures with splenocytes from O-1966 treated mice (■) or vehicle treated mice (□). Panel B: Cultures harvested at 48 h from mice treated with O-1966 (▨) or vehicle (▩) and analyzed by flow cytometry for CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (n=17 for both groups). Data show number of Tregs as a percentage of total live CD4<sup>+</sup> cells (LIVE/DEAD® dead cell stain negative). Data are mean of 2 separate experiments. \*p < 0.05, \*\* p < 0.01 (ANOVA, O-1966 versus vehicle).



**Figure 23. In vivo O-1966 treatment decreases CD4 expression following ex vivo stimulation.** Donor C3HeB/FeJ flank skin was transplanted to the back of a recipient C57BL/6J mice, sutured, and bandaged. Doses of O-1966 (5 mg/kg) or vehicle (0.03% ethanol and 0.03% cremophor in saline) were injected i.p. every other day from 1 hour pre-op to 14 days post-op. On post-op day 14, animals were sacrificed and spleens were aseptically removed, restimulated with C3HeB/FeJ splenocytes and put into culture for 48 h and analyzed by flow cytometry CD4 expression. Panel A: Representative histogram of CD4 expression on CD3<sup>+</sup> cells in cultures from splenocytes from skin graft recipient mice treated with O-1966 (gray filled) or vehicle (white filled). Panel B: Mean Fluorescence Intensity (MFI) of CD4 in CD3<sup>+</sup> populations in cultures from O-1966 treated mice (▨) or vehicle treated mice (□). Data are representative of 2 experiments (Panel A) or mean of 2 separate experiments (Panel B). \*  $p < 0.01$  (ANOVA, O-1966 versus vehicle).

## CHAPTER 5

### CONCLUSIONS

1.  $\Delta^9$ -THC, a mixed CB1/CB2 agonist, and JWH-015 and O-1966, CB2-selective agonists, were shown to inhibit the murine MLR, an in vitro correlate of organ and skin graft rejection, in a CB2 receptor dependent manner.
2. CB2 selective agonists inhibited the MLR predominantly through a direct effect on T-cells in the mixed splenic leukocyte population.
3. Studies on the mechanisms by which CB2-selective agonists inhibited T-cells in the MLR showed that they decreased levels of activating factors and up-regulated an immunosuppressive cytokine and suppressor T-cells. Thus, CB2 compounds:
  - a. decreased activation of the transcription factors NFAT and NF- $\kappa$ B.
  - b. decreased IL-2 production
  - c. decreased CD40L and Cyclin D3 expression
  - d. increased SOCS5, SLA2, and IL-10
  - e. decreased CD4 expression
  - f. increased T-regulatory (Tregs) cells
4. IL-10 was increased in MLR cultures treated with a CB2 agonist. IL-10 was found to:
  - a. partially mediate the inhibition of proliferation of T-cells in the MLR
  - b. mediate the increase in Tregs.

5. Treatment with a CB2 agonist in vivo prolonged skin graft rejection, which was accompanied by:
  - a. decreased proliferation of splenocytes when tested ex vivo in the MLR
  - b. decreased splenic weights
  - c. increased numbers of Tregs in the spleen.
6. These studies demonstrate the potential utility of CB2 selective agonists as agents to prevent graft rejection, and potentially as a broader new class of immunosuppressive drugs.

## CHAPTER 6

### DISCUSSION

Recipients of allogeneic transplants must maintain lifelong immunosuppressive therapies to prevent rejection. Immunosuppressive agents most often target T-cells (Heeger 2003). Even with continuous anti-rejection therapy, eventually many grafts are ultimately rejected (OPTN/SRTR 2012). Further, many of the current anti-rejection medications have serious side effects, such as nephrotoxicity, post-transplant diabetes mellitus, hypertension, neurotoxicity, and hyperlipidemia (Jose 2007). Improved therapeutic agents with decreased toxicity are needed for use alone or in combination with existing therapies given at reduced doses.

Cannabinoids have been found to affect almost every type of cell in the immune system and have robust immunosuppressive and anti-inflammatory effects (Croxford & Yamamura 2005, Kaminski 1998, Klein & Cabral 2006, Klein 2005, Klein et al. 2003, Nagarkatti et al. 2009). CB2-selective cannabinoids have been proposed as possible candidates to block graft rejection (Nagarkatti et al. 2010). However, prior to the current studies (Robinson et al. 2013), there were no publications showing that this class of compounds is effective in this regard. The results presented here provide pre-clinical evidence that a CB2-selective agonist is a potential treatment to prevent graft rejection.

In the current studies,  $\Delta^9$ -THC, a mixed CB1/CB2 agonist, and two CB2-selective agonists were shown to inhibit the Mixed Lymphocyte Reaction (MLR), an in vitro correlate of organ and skin graft rejection. Suppression was observed in the MLR by  $\Delta^9$ -THC, JWH-015, and O-1966, in a dose dependent manner in the range of concentrations

from 4  $\mu$ M to 32  $\mu$ M, with the highest dose giving the greatest effects, between 62% and 90%. Based on the affinity of these compounds for the CB2 receptor ( $\Delta^9$ -THC: 3.9-75.2 nM (Howlett et al. 2002), JWH-015: 13.8 nM (Showalter et al. 1996), O-1966: 23 nM (Wiley et al. 2002) ), these doses would seem to be high by several orders of magnitude. However, other investigators have also reported effects on immune function by cannabinoids in this dosage range (Adhikary et al. 2012, Borner et al. 2009, Ngaoteprutaram et al. 2012). Previous research has shown that the concentration of  $\Delta^9$ -THC needed to suppress B- and T-lymphocytes in vitro must be increased proportionally to increases of serum levels in the culture media, (Nahas et al. 1977, Klein et al. 1993). MLR cultures were incubated in medium containing 10% fetal bovine serum, an amount that necessitated micromolar concentrations of cannabinoids in previous studies. It should be noted that in the present experiments, extensive controls were included to address any concerns about the possibility of cell toxicity, non-receptor mediated or off target effects, or conditions that are not physiological due to the concentrations of cannabinoids used. In every experiment, parallel cultures were stained with Invitrogen's LIVE/DEAD dead cell stain that was analyzed by flow cytometry. In preliminary experiments trypan blue exclusion was also used as a test of cell viability. In no experiment did any cannabinoid agonist, antagonist, vehicle control, or any combination of agonists, antagonists, and vehicle control increase the number of dead cells compared to cultures that received no treatment. These results show that exposure of the spleen cells to the doses of cannabinoids that resulted in suppression was not toxic, and thus, cell death can be excluded as the cause of the suppression of the MLR. As a further control, apoptosis was

also measured in cannabinoid treated MLR cultures by testing levels of several caspases, which are important mediators in the induction of apoptosis. Neither JWH-015 nor O-1966 were found to increase levels of apoptosis in the MLR. In addition, the TUNEL assay, which measures DNA fragmentation seen in apoptotic cells was also employed. Interestingly, all cultures showed marked increases in TUNEL positive cells by 48 hrs in culture, but there was no differential increase in the wells receiving the cannabinoids. The experiments presented here also show that the cannabinoid doses that resulted in immunosuppression, even though seemingly high, were exerting their effect through the CB2 receptor, as suppression was 100% blocked by the CB2-selective antagonist. Further, cells taken from mice lacking the CB2 receptor (CB2R k/o mice) were not suppressed when exposed to the cannabinoids, and the cannabinoids did not inhibit their IL-2 production, showing that these cannabinoids, at the doses tested in the micromolar range, did not have generalized toxicity. Another potential concern is whether the effect of the cannabinoids at these concentrations is physiologically relevant. Several studies have shown that  $\Delta^9$ -THC and CB2-selective agonists have in vivo immunosuppressive effects. For example, in mice,  $\Delta^9$ -THC inhibited antitumor immunity (5 mg/kg) (Zhu et al. 2000), and increased the Th2 phenotype following *Legionella pneumophila* infection (8 mg/kg) (Newton et al. 2009). O-1966 has been shown to be effective in treating spinal cord injury (Baty et al. 2008, Adhikary et al. 2011) and stroke (Zhang et al. 2007, Zhang et al. 2009) at doses from 1-10 mg/kg. In studies presented here, 5 mg/kg O-1966 successfully lengthened allogeneic skin graft survival in mice. JWH-133, another CB2-selective agonist, was shown to improve outcomes in models of atherosclerosis (Hoyer et

al. 2011), systemic sclerosis (Servettaz et al. 2010), colitis (Storr et al. 2009), stroke (Murikinati et al. 2010), autoimmune uveoretinitis (Xu et al. 2007), and inflammation following LPS challenge (Rajesh et al. 2007, Ramirez et al. 2012) at doses from 1-20 mg/kg. Thus, there seems to be a poor correlation between the doses needed in vitro to demonstrate efficacy of these cannabinoids in the immune system, and the in vivo doses that demonstrate anti-inflammatory and immunosuppressive effects.

The inhibition by  $\Delta^9$ -THC, JWH-015, and O-1966 in the MLR was shown to be CB2 dependent, as pretreatment with the CB2-selective antagonist, SR144528, completely reversed the suppression, while pretreatment with the CB1-selective antagonist, SR141716A, had no effect. Further, suppression did not occur in CB2 receptor knock-out (CB2R k/o) mice. In addition, the CB2-selective cannabinoids, JWH-015 and O-1966, decreased the release of IL-2 in the MLR, which did not occur when splenocytes from CB2R k/o mice were used.

The studies also explored which cell subset is targeted by the CB2 agonists. Evidence was presented showing that inhibition of the MLR occurred predominantly when the CD3<sup>+</sup> population, but not the CD11b<sup>+</sup> population, was treated with the CB2-selective compounds. This difference was not due to differential expression of the CB2 receptor because before activation, CD3<sup>+</sup> cells and CD11b<sup>+</sup> cells expressed comparable very low levels of CB2 mRNA, and after activation, CD11b<sup>+</sup> cells expressed CB2 mRNA levels many fold higher than CD3<sup>+</sup> cells. The CB2 agonists were also shown to inhibit proliferation of purified T-cell populations stimulated with antibodies. A CB2-selective agonist inhibited both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in the MLR. Treatment of either T-cell

population resulted in inhibition of the MLR, though the inhibition was significantly less than when both populations were treated.

Previous work investigating the suppressive effect of  $\Delta^9$ -THC on the MLR and T-cells, showed that in vivo administration of  $\Delta^9$ -THC (5 mg/kg) decreased ex vivo proliferation in the MLR (Zhu et al. 2000). Another group reported that treatment of splenocytes with  $\Delta^9$ -THC in vitro could inhibit proliferation in the MLR, but using CB1/CB2 k/o mice, this group concluded that the inhibition was CB1- and CB2-independent (Springs et al. 2008). Data presented here show very clearly that  $\Delta^9$ -THC added in vitro inhibits proliferation in the MLR in a CB2-dependent manner, based on both use of receptor specific antagonists and use of cells from CB2 receptor k/o mice. Several other laboratories have shown that the inhibition of T-cells by cannabinoids is CB2 mediated. Yuan et al. reported that  $\Delta^9$ -THC treatment decreased mRNA levels for IL-2 and IFN- $\gamma$  in T-cells activated with anti-CD3 and anti-CD28 antibodies, and that this suppression could be reversed by treatment with the CB2 antagonist SR144528 (Yuan et al. 2002). Other studies have shown CB2-selective cannabinoids significantly decreased proliferation of T-cells and IL-2 release in response to various methods of stimulation (Borner et al. 2009, Cencioni et al. 2010, Ihenetu et al. 2003, Maresz et al. 2007). Our data clearly demonstrate a direct effect of CB2-selective cannabinoids on T-cells in the context of graft rejection.

Ideally, the anatomically disparate expression of CB1 and CB2 would allow for the use of compounds selective for CB2, and thus eliminate the unwanted psychoactive effects from CB1 activation, while maintaining the anti-inflammatory and

immunosuppressive properties. While CB2 receptors have been found to have limited neuronal expression (Gong et al. 2006, Van Sickle et al. 2005), recent reports show that CB2 agonists affect cocaine's rewarding and locomotor stimulating effects (Xi et al. 2011), food and alcohol consumption (Onaivi et al. 2008a), and symptoms of depression (Onaivi et al. 2008b), suggesting a function in the central nervous system for this class of compounds. However, intravenous administration of O-1966 to mice in doses up to 30 mg/kg did not produce any effect in the tetrad test, a common series of behavioral analyses used to assess the psychoactive effects of cannabinoids (Zhang et al. 2007). More work should be done to evaluate the neuronal effects of CB2-selective agonists in order to fully understand the prospect of this class of compounds as potential immunomodulatory therapeutics.

The current studies also explored the mechanism of immunosuppression by the CB2-selective agonist, O-1966, on mouse T-cells. It was found that this CB2 agonist decreased levels of the active nuclear forms of NF- $\kappa$ B and NFAT in T-cells of wild-type mice, but not of CB2R k/o mice. In addition, O-1966 treatment significantly decreased mRNA expression for CD40L and Cyclin D3, a positive regulator for the transition from G<sub>1</sub> to S phase during cell division (Ando et al. 1993). This compound also caused a dose-dependent decrease of CD4 expression on wild-type T-cells in the MLR, but not on T-cells lacking CB2.

These findings are in accordance with recent literature which reported that  $\Delta^9$ -THC blocked similar aspects of T-cell activation. Treatment with  $\Delta^9$ -THC decreased NFAT transcriptional activity in a murine T-cell line activated by phorbol ester (PMA)

plus ionomycin (Lu et al. 2009). Another group reported that  $\Delta^9$ -THC and the CB2-selective agonist, JWH-015, reduced transcription by NF- $\kappa$ B and NFAT in human primary and Jurkat T-lymphocyte lines expressing CB2 that were activated with anti-CD3/CD28 antibodies (Borner et al. 2009). Ngaoteprutaram et al. showed that pretreatment with  $\Delta^9$ -THC decreased levels of CD40L mRNA and protein in mouse splenocytes activated by anti-CD3/CD28 antibodies (Ngaoteprutaram et al. 2012). In the present studies, we show that signaling through the CB2 receptor by a CB2-selective agonist is sufficient to achieve the same results.

There is ample evidence in the literature to suggest preventing T-cell activation would provide sufficient protection against graft rejection (Heeger 2003), including the mechanisms induced by O-1966 reported here. Blocking the transcriptional activity of NFAT or NF- $\kappa$ B abrogates allograft rejection (Ueno et al. 2011, Finn et al. 2001). The inhibition of calcineurin by tacrolimus and cyclosporine, the standard anti-rejection drugs, blocks the translocation of the cytosolic component of NFAT to the nucleus (Ho et al. 1996). NFAT has been shown to be involved in the regulation of expression of CD40L on T-cells (Peng et al. 2001). CD40L is expressed predominantly on activated CD4<sup>+</sup> cells and induces an activating response when it binds to its receptor, CD40, which is expressed on a variety of cell types (van Kooten & Banchereau 2000). CD40L expression is increased 4-fold in cases of acute rejection, and antibodies against CD40L have been found to be protective in mouse and monkey models of transplantation, including renal, pancreas, and skin allografts (Daoussis et al. 2004). Therefore, the 4-fold decrease in CD40L expression on T-cells treated with O-1966 in the MLR reported here may provide

significant protection against graft rejection. Additionally, O-1966 treatment reduced expression levels of CD4 on the cell surface. Recently, CD4 has been shown to regulate the sensitivity of T-cells to antigen through the recruitment of the leukocyte-specific protein tyrosine kinase (Lck) (Li et al. 2004). Lck is one of the most proximal signaling proteins downstream of the T cell receptor (TCR) and, after TCR activation, autophosphorylates and induces subsequent downstream TCR signaling (Straus & Weiss 1992). Because of this, Lck has been suggested as a target to reduce rejection. Lck knock-out mice have been shown to accept allogeneic skin grafts indefinitely (Wen et al. 1995) and treatment of wild-type rats with a Lck inhibitor prolonged the survival of heart allografts to a similar extent as treatment with the calcineurin inhibitor cyclosporine (Stachlewitz et al. 2005). Börner et. al showed that treatment with  $\Delta^9$ -THC or the CB2-selective agonist, JWH-015, blocked the dephosphorylation of an inhibitory region of Lck that prevents autophosphorylation and subsequent initiation of TCR signaling in primary human T-cells and Jurkat T-cells activated with anti-CD3/ CD28 antibodies (Börner et al. 2009). Whether CB2 agonists block T-cell activation in the MLR by reducing CD4 surface expression and thus dampening TCR signaling through Lck should be investigated further.

In addition to blocking T-cell activation, O-1966 also induced a potent suppressive response in the MLR, through enhanced IL-10 release, which has been shown to inhibit the MLR (Bejarano et al. 1992), and also by increasing the percentage of Tregs. It has previously been shown that IL-10 levels were enhanced in several experimental conditions by treatment with  $\Delta^9$ -THC (Zhu et al. 2000, Klein et al. 2000,

McKallip et al. 2005) and the synthetic nonselective CB1/CB2 agonists WIN55,212 and HU-210 (Smith et al. 2000). It has also been previously reported that Win55,212-2 increased Tregs in the CNS in a mouse model of multiple sclerosis (Arevalo-Martin et al. 2012) and  $\Delta^9$ -THC increased Tregs in the spleen and liver of a mouse model of hepatitis (Hegde et al. 2008). The current results extend the published observations by using CB2 selective compounds. The increased number of Tregs in O-1966 treated MLR cultures was completely blocked by the addition of neutralizing anti-IL-10 antibodies. This is consistent with a study by Groux et al. that showed IL-10 was able to induce Tregs, which then overproduced IL-10 and suppressed the proliferation of CD4<sup>+</sup> T-cells in response to antigen (Groux et al. 1997). The observation linking the action of a CB2 agonist with the interplay of IL-10 and Tregs is novel.

The induction of IL-10 and Tregs by a CB2-selective agonist make this class of compounds particularly promising, because the generation of Tregs could increase the likelihood of graft survival while decreasing the need for long-term immunosuppressive therapies. IL-10 has been shown to inhibit antigen presentation, antigen-specific T-cell proliferation, and decrease Th1 cytokine production (Mitra et al. 1995, Fiorentino et al. 1991). Blocking the activity of IL-10 in vivo in transplantation models diminished the survival of graft (Kingsley et al. 2002, McMurchy et al. 2011) and was essential for the induction and maintenance of Tregs (Wood et al. 2012). Indeed, following transplantation in both humans and animals, the presence of Tregs in the spleen, draining lymph nodes, and at the site of the allograft, closely correlated with graft acceptance (Wood et al. 2012).

In the present studies, proof of concept was obtained for the utility of CB2 agonists as potential therapies to prevent graft rejection. It was shown that O-1966 could inhibit rejection of allogeneic skin grafts *in vivo*. Mice treated with 5 mg/kg every other day had mean graft survival increased by 2 days compared to mice treated with vehicle. This prolongation of graft survival is comparable to the lengthened graft survival time in mice treated with calcineurin inhibitors (Lagodzinski et al. 1990). Further, the mice treated with O-1966 doubled the percentage of Tregs compared to mice treated with vehicle. The O-1966 treated animals also exhibited significantly decreased CD4 expression on their splenic T-cells. Further, the splenocytes of recipient mice treated *in vivo* with O-1966 had less proliferation and increased induction of Tregs after allogeneic restimulation in an *ex vivo* MLR, indicating O-1966 has lasting suppressive effects.

Together, the present data show that CB2 activation can suppress T-cells in the MLR by blocking T-cell function while favoring the induction of Tregs both *in vitro* and *in vivo*. The results support CB2-selective agonists as a new class of compounds to prolong graft survival in transplant patients, and potentially as a new class of immunosuppressive drugs to inhibit graft rejection. CB2-selective agonists could also be used in combination with calcineurin inhibitors to reduce the dose of the latter and decrease the cumulative side effects associated with their chronic use. Calcineurin inhibitors effectively inhibit the activity of effector T-cells by inhibiting transcription, but may negatively impact the generation and/or expansion of Tregs (Segundo et al. 2006, Zeiser et al. 2006, Segundo et al. 2007, Sewgobind et al. 2010). CB2 agonists increase IL-10 and Tregs, so a combination of the two classes of drugs may improve clinical

outcomes. Further studies to investigate the combination of CB2-selective cannabinoids and calcineurin inhibitors are certainly warranted.

In addition, these findings have wider significance. As noted in the Introduction and Discussion of this thesis, CB2 agonists have been tried in mouse models for a number of inflammatory diseases, many autoimmune in origin. The mechanisms elucidated in the present studies using the MLR and graft rejection extend our knowledge of how these compounds work to dampen immune responses. This knowledge can be extended and applied to the potential use of CB2 agonists for a variety of other conditions where the immune system is the cause of immunopathology due to uncontrolled up-regulation.

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