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REVIEW

The peripheral cannabinoid receptor knockout mice: an update

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This review gives an overview of the CB_2 receptor (CB_2R) knockout ($CB_2R^{-/-}$) mice phenotype and the work that has been carried out using this mutant mouse. Using the $CB_2R^{-/-}$ mice, investigators have discovered the involvement of CB_2R on immune cell function and development, infection, embryonic development, bone loss, liver disorders, pain, autoimmune inflammation, allergic dermatitis, atherosclerosis, apoptosis and chemotaxis. Using the $CB_2R^{-/-}$ mice, investigators have also found that this receptor is not involved in cannabinoid-induced hypotension. In addition, the $CB_2R^{-/-}$ mice have been used to determine specific tissue CB_2R expression. The specificity of synthetic cannabinoid agonists, antagonists and anti-CB_2R antibodies has been screened using tissues from $CB_2R^{-/-}$ mice. Thus, the use of this mouse model has greatly helped reveal the diverse events involving the CB_2R, and has aided in drug and antibody screening.
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Keywords: CB_2R; $CB_2R^{-/-}$ mice; antifibrogenic; pain; allergic dermatitis; atherosclerosis; chemotaxis; osteoporosis; multiple sclerosis

Abbreviations: 2-AG, 2-arachidonoylglycerol, endogenous cannabinoid; AM1241, synthetic cannabinoid, CB_2R selective agonist; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride, synthetic cannabinoid, CB_1R selective antagonist; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicycle [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide, synthetic cannabinoid, CB_2R selective antagonist; THC, Δ-9-Tetrahydrocannabinol; WIN 55,212-2, (*R*)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone, synthetic cannabinoid agonist

Introduction

The peripheral cannabinoid receptor (CB_2R) was the second cannabinoid receptor discovered (Munro *et al*., 1993), after the central cannabinoid receptor (CB_1R) (Matsuda *et al*., 1990). Both cannabinoid receptors are G-protein-coupled seven transmembrane (TM) receptors. Human CB_1R and CB_2R share 44% overall homology and 68% at the TM level (Munro *et al*., 1993). Human CB_1R and mouse CB_1R share 96% homology (Chakrabarti *et al*., 1995), while human CB_2R and mouse CB_2R share 82% homology (Shire *et al*., 1996b). Mouse CB_1R and CB_2R share 66% overall homology and 78% at the TM level (Shire *et al*., 1996a). CB_1R is expressed at high levels in brain tissue and to a lesser extent in peripheral tissues such as the adrenal glands, reproductive organs and on immune cells (Matsuda *et al*., 1990; Bouaboula *et al*., 1993; Galiegue *et al*., 1995). In contrast, CB_2R is mainly expressed in cells of haematopoietic origin. CB_2R expression has been demonstrated in spleen and thymus (Schatz *et al*., 1997), lymph nodes, Peyer's patches (Lynn and Herkenham, 1994) and immune system-derived cell lines. Human blood cell populations were reported to have different degrees of CB_2R expression with the following rank order: B cells > natural killer cells > monocytes > polymorphonuclear neutrophil cells > $CD8^+$ T cells > $CD4^+$ T cells (Galiegue *et al*., 1995; Carayon *et al*., 1998). The expression level of CB_2R in lymphocytes and macrophages has been shown to vary in relation to cell differentiation and activation state (reviewed in Klein *et al*., 2003). Thus, Carayon *et al*. (1998) reported that CB_2R expression varied depending on the stage of B-cell differentiation with virgin and memory B cells, expressing the highest levels of CB_2R mRNA followed by germinal-centre B cells and centroblasts (Carayon *et al*., 1998). Carlisle *et al*. (2002) showed that while resident macrophages lack CB_2R expression, thioglycollate-elicited and interferon-γ (IFN-γ)-primed macrophages have high-CB_2R levels (Carlisle *et al*., 2002). The expression of CB_2R gene in immune tissues has been reported to be 10–100 times that of CB_1R (Galiegue *et al*., 1995). More recently, CB_2R expression in osteoblasts,

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osteocytes and osteoclasts was observed (Ofeck *et al*., 2006). CB_2R expression has also been found in preimplantation embryos (Paria *et al*., 1995), and more recently in the normal central nervous system (CNS) (Ross *et al*., 2001; Van Sickle *et al*., 2005; Ashton *et al*., 2006; Gong *et al*., 2006; Onaivi, 2006; Onaivi *et al*., 2006). The existence of a putative third cannabinoid receptor, GPR55, has been reported. GPR55 is an orphan G-protein-coupled receptor that has low-sequence homology (10–15%), compared to that of CB_1R or CB_2R, and is expressed in the testis at approximately a 15-fold higher level than in the brain (Baker *et al*., 2006). However, full characterization of this receptor is lacking, and it cannot be concluded that it is a true cannabinoid receptor (Petitet *et al*., 2006). Recently, Sugiura *et al*. (2007) were unable to stimulate GPR55 activation using three different cannabinoids (Sugiura *et al*., 2007). To investigate the role of CB_1R and CB_2R, mutant mice with deletions in these receptors have been developed. Thus far, there are three lines of CB_1R knockout ($CB_1R^{-/-}$) mice (reviewed in Valverde *et al*., 2005), and two CB_2R knockout ($CB_2R^{-/-}$) mice, one developed by Buckley *et al*. (2000) and the one recently developed by Deltagen (San Mateo, CA, USA) and commercially available through Jackson Laboratory (Bar Harbor, ME, USA) (http://jaxmice.jax.org/strain/005786.html#genes). The goal of the present review is to provide an update on the multiple findings investigators have achieved with the use of the $CB_2R^{-/-}$ mice generated by Buckley *et al*. (2000).

$CB_2R^{-/-}$ mice

The $CB_2R^{-/-}$ mice were generated by homologous recombination. Upon homologous recombination, the last 341 bp of the CB_2R-coding exon in the gene was replaced by the neomycin gene, effectively removing bp 1186 onwards from the coding sequence (see accession U21681 in GenBank). The CB_2R deletion results in a gene lacking the coding region for part of the intracellular loop 3, TM regions 6 and 7 and the C terminus (Buckley *et al*., 2000). This deletion renders the CB_2R nonfunctional as macrophages derived from $CB_2R^{-/-}$ mice are unable to respond to THC (Buckley *et al*., 2000; Chuchawankul *et al*., 2004). However, these mice are responsive to the psychotropic effects of cannabinoids (Buckley *et al*., 2000). The $CB_2R^{-/-}$ mice display no gross morphological differences from their wild-type counterparts, but at the cellular level, the $CB_2R^{-/-}$ mice are deficient in splenic marginal zone B cells, peritoneal B1a ($CD5^+CD11b^+CD23^{lo}B220^{lo}$) cells, splenic memory $CD4^+$ T cells, and intestinal natural killer cells and natural killer T cells (Ziring *et al*., 2006). Furthermore, $CB_2R^{-/-}$ mice have a decreased number of diaphyseal osteoblast precursors, but have an increased osteoclast number and increased activity of their trabecular osteoblasts. This phenotype causes $CB_2R^{-/-}$ mice to undergo a greater age-related bone loss than their wild-type counterparts, a loss of bone mass that is apparent by 8 weeks of age (Ofeck *et al*., 2006).

$CB_2R^{-/-}$ mice and reproduction

The $CB_2R^{-/-}$ mice are fertile, care for their young and have litter sizes comparable to their wild-type counterparts (Buckley *et al*., 2000). CB_2R is present in the preimplantation embryo, but not in the oviduct or uterus (Das *et al*., 1995; Paria *et al*., 1995, 2001; Wang *et al*., 2004). CB_2R is expressed from the one-cell through the blastocyts embryonic stage, restricted to blastocyts inner cell mass, but not in the trophectoderm-derived trophoblast stem cells, which are directly involved in implantation (Paria *et al*., 1995). On the other hand, CB_1R expression is present in the trophectoderm of the preimplantation embryo (Paria *et al*., 1995; Yang *et al*., 1996). Congruent with this, was the finding that $CB_1R^{-/-}$ and $CB_1R^{-/-}/CB_2R^{-/-}$, but not $CB_2R^{-/-}$ embryos, were trapped in the oviduct (Wang *et al*., 2006). Furthermore, it has been shown that the number of uterine implantation sites and oviductal embryo transport in $CB_2R^{-/-}$ pregnant female mice was comparable to that of wild-type pregnant female mice (Wang *et al*., 2006). Interestingly, embryonic development in $CB_2R^{-/-}$ mice is retarded (Paria *et al*., 2001). It was found that on day 3 of pregnancy, only 41.5% of $CB_2R^{-/-}$ embryos were at the eight-cell stage as compared to the 84% wild-type embryos. There were 24% $CB_1R^{-/-}$ embryos at the eight-cell stage. On day 4 of pregnancy, 71.3% $CB_2R^{-/-}$ embryos were blastocysts and 25.7% morulas compared to the 97.5% wild-type blastocysts and 2.5% morulas. There were 61.5% $CB_1R^{-/-}$ embryos at the blastocyst stage and 37% at the morula stage (Paria *et al*., 2001). However, $CB_2R^{-/-}$ mice breeding pairs have normal litter sizes (Buckley *et al*., 2000), suggesting that the asynchronous $CB_2R^{-/-}$ embryonic development at days 3 and 4 of pregnancy does not hinder embryonic implantation at day 4 or 5. Taken together, these findings indicate that CB_1R, and not CB_2R, is mostly responsible for successful embryonic implantation, and that both receptors have a role in synchronizing embryonic development. The function of CB_2R in the embryonic stem cells is unknown, but may implicate this receptor in specifying pluripotent inner cell mass cell lineage during blastocyst formation (Paria *et al*., 2001; Wang *et al*., 2006).

$CB_2R^{-/-}$ mice and disease models

$CB_2R^{-/-}$ mice and bone loss

Cannabinoid receptors have been implicated in bone mass, bone loss and osteoclast activity (Idris *et al*., 2005; Ofeck *et al*., 2006). Unregulated osteoclast (bone-resorbing cells) or osteoblast (bone-forming cells) activity can cause bone loss, resulting in diseases such as osteoporosis (Helfrich, 2003). The involvement of CB_1R on bone mass has been reported previously (Idris *et al*., 2005). CB_2R is expressed in osteoblasts, osteocytes and osteoclasts (Ofeck *et al*., 2006). Osteoclasts are derived from cells in the myeloid lineage (Boyle *et al*., 2003; Xing *et al*., 2005). Osteoblasts, the precursors of osteocytes, are derived from precursor cells in the stromal element of bone marrow (Rickard *et al*., 1996). $CB_2R^{-/-}$ mice showed accelerated age-related trabecular bone loss and cortical expansion, although cortical thickness remained the same. $CB_2R^{-/-}$ osteoclast number and trabecular osteoblast activity were increased. However, there was a significant decrease in the number of diaphyseal osteoblast precursors. In wild-type mice, the CB_2R-specific agonist

HU-308 enhanced endocortical osteoblast number and activity and restrained trabecular osteoclastogenesis, apparently by inhibiting proliferation of osteoclast precursors. These findings suggest that CB_2R has a role in bone homeostasis and that this receptor is a potential drug target for the treatment of osteoporosis (Ofeck *et al*., 2006).

$CB_2R^{-/-}$ mice and liver disorders

The CB_2R has been implicated to have an antifibrogenic role in the liver (Julien *et al*., 2005). Liver fibrosis occurs due to chronic liver injury that can eventually lead to cirrhosis and its complications. Julien *et al*. (2005) found that while normal liver does not express CB_2R, human cirrhotic liver does. In this study, it was found that CB_2R activation had potent antifibrogenic effects, including hepatic myofibroblast growth inhibition and increased apoptosis. Furthermore, when liver fibrosis was induced in wild-type and $CB_2R^{-/-}$ mice using carbon tetrachloride (CCl_4), they found that $CB_2R^{-/-}$ mice developed enhanced liver fibrosis compared to their wild-type counterparts. That is, they observed a higher collagen deposition as determined by histological examination and hydroxyproline measurement. In addition, smooth muscle α-actin mRNA (α-SMA) was higher for CCl_4-treated $CB_2R^{-/-}$ mice compared to CCl_4-treated wild-type or vehicle-treated $CB_2R^{-/-}$ mice (Julien *et al*., 2005). Hepatocyte proliferation was also impaired in $CB_2R^{-/-}$ mice compared to wild-type mice (Deveaux *et al*., 2007). These findings provide evidence for a protective role of CB_2R in liver injury.

The CB_2R also seems to play a protective role in liver ischaemia/reperfusion (I/R) injury (Batkai *et al*., 2007). The I/R model of organ ischaemia followed by reperfusion mimics events that may develop in common disease such as myocardial infarction and stroke, coronary bypass surgery and organ transplantation. In the I/R model, liver ischaemia was induced for 60 min by clamping the hepatic artery and portal vein. Reperfusion was then allowed for 90 min or 24 h (Batkai *et al*., 2007). Liver damage to I/R was greater in $CB_2R^{-/-}$ mice compared to wild-type mice as determined by increased serum transaminase AST/ALT and myeloperoxidase activities, malondialdehyde formation as an indicator or lipid peroxidation, and an increase in proinflammatory cytokines and chemokines. Histological examination also revealed much more extensive liver injury and neutrophil infiltration in the liver from $CB_2R^{-/-}$ mice than that from wild-type mice. Interestingly, the hepatic levels of the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) were also substantially elevated upon I/R (Batkai *et al*., 2007).

Taken together, the findings on the involvement of CB_2R in the liver fibrosis model and in the I/R model implicate the CB_2R in the liver repair mechanism.

$CB_2R^{-/-}$ mice and pain

It is widely recognized that cannabinoids reduce pain in humans and animals. This effect by cannabinoids is thought to be largely mediated by the CB_1R. More recently, however, the implication of CB_2R in pain modulation is being recognized. CB_2R has been shown to modulate acute pain, chronic inflammatory pain, post-surgical pain, cancer pain and pain associated with nerve injury (reviewed in Whiteside *et al*., 2007). The CB_2R agonist, AM1241, has been shown to exert its antinociceptive effects locally without producing CNS effects (Malan *et al*., 2001). Furthermore, using three different assays of nociception, it was demonstrated that $CB_2R^{-/-}$ mice have a lower threshold of pain compared to wild-type mice in the presence of cannabinoids. This was determined using the hot-plate, paw-withdrawal and tail-flick assays. While the hot-plate assay requires supraspinal responses, the paw-withdrawal and tail-flick assays require spinal reflexes. Thus, pain induced by these methods can be inhibited by drugs acting at these peripheral sites. In the absence of cannabinoids, using the Hargreaves' method (Hargreaves *et al*., 1988), the paw-withdrawal assay showed reduced latency in $CB_2R^{-/-}$ mice compared their wild-type counterparts. Taken together, these findings provide evidence that CB_2R is involved in acute nociception, and thus, is a likely target to treat acute pain (Ibrahim *et al*., 2006).

$CB_2R^{-/-}$ mice and experimental autoimmune encephalomyelitis, a multiple sclerosis model

Cannabinoids have been shown to alleviate spasticity associated with experimental autoimmune encephalomyelitis (EAE) in rodents (Baker *et al*., 2000; Pryce and Baker, 2007). To determine whether CB_2R is involved in the regulation of autoimmunity, EAE was induced in wild-type and $CB_2R^{-/-}$ mice on a B10.PL background (Maresz *et al*., 2007). It was found that $CB_2R^{-/-}$ mice exhibited a higher incidence of disease, a significantly increased clinical score and a reduced recovery rate than their wild-type counterparts. Furthermore, when EAE was induced in wild-type mice with $CB_2R^{-/-}$ encephalitogenic T cells by adoptive transfer, there was a more severe clinical disease. The disease was characterized by a higher mortality rate and the presence of increased numbers of infiltrating mononuclear cells. Examination of the lesions revealed that there was an increase in the number of $CB_2R^{-/-}$ T cells in and around the lesions when EAE was induced with $CB_2R^{-/-}$ T cells as compared to that induced with wild-type T cells. This increase in T-cell number in the CNS was due to more proliferation and decreased apoptosis of the $CB_2R^{-/-}$ T cells. Furthermore, the CNS $CB_2R^{-/-}$ encephalitogenic T cells secreted more proinflammatory cytokines than the wild-type encephalitogenic T cells. This explains the more severe disease in mice receiving the $CB_2R^{-/-}$ T cells (Maresz *et al*., 2007). It is known that endocannabinoids are synthesized in the CNS (Salzet *et al*., 2000) and that endocannabinoids have immune suppressive effects such as induction of apoptosis and inhibition of lymphocyte proliferation (Salzet *et al*., 2000; Klein *et al*., 2003), thus these findings provide evidence that the CNS actively suppresses T-cell function through the CB_2R (Maresz *et al*., 2007).

$CB_2R^{-/-}$ mice and allergic dermatitis

A model of cutaneous contact dermatitis was used to study the allergic response in mutant mice. It was found that, compared to wild-type mice, $CB_1R^{-/-}$, $CB_2R^{-/-}$ and

$CB_1R^{-/-}/CB_2R^{-/-}$ mice exhibit enhanced allergic responses to 2,4-dinitrofluorobenzene (DNFB) (Karsak *et al*., 2007). In this model, mice were sensitized with DNFB on the shaved abdomen. On days 5, 13 and 21, the mice were challenged with DNFB on the right ear. Swelling was then measured on the right ear and compared to the left ear within the same animal. While swelling was increased in the right ear of wild-type mice, swelling was significantly higher in $CB_1R^{-/-}$, $CB_2R^{-/-}$ and $CB_1R^{-/-}/CB_2R^{-/-}$ ears. Furthermore, upon injection of the CB_1R and CB_2R antagonists, SR141716A and SR144528, respectively, the swelling was enhanced in wild-type animals. Swelling, however, was decreased by THC, but enhanced by HU-308. Moreover, the levels of the endogenous cannabinoids 2-AG and anandamide were elevated in $CB_1R^{-/-}/CB_2R^{-/-}$ mice (Karsak *et al*., 2007). Interestingly, using a slightly different approach to induce a cutaneous reaction, another group reported a decrease, not an increase in the ear swelling of $CB_2R^{-/-}$ mice. These investigators passively sensitized the mice by i.v. injection of 1 μg of monoclonal anti-dinitrophenol IgE followed by a topical challenge with DNFB (Ueda *et al*., 2007). Furthermore, this group reported that oral administration of SR144528 suppressed ear swelling (Ueda *et al*., 2005, 2007). Although both groups find that the CB_2R is involved in the cutaneous allergic response, their findings, using the $CB_2R^{-/-}$ mice as well as the CB_2R antagonist, are opposite. While Karsak *et al*. (2007) implicate the endocannabinoid system in the attenuation of the inflammatory response, Ueda *et al*. (2005, 2007) propose that CB_2R participate in the induction of the cutaneous reaction. The discrepancy in their findings, and hence conclusions, may be due to the different approaches used to induce the allergic response in wild-type and $CB_2R^{-/-}$ mice, and in delivering the SR144528 compound.

$CB_1R^{-/-}/CB_2R^{-/-}$ and $CB_2R^{-/-}$ mice in hypotension

Prolonged and profound hypotension is associated with diverse forms of shock, such as haemorrhagic (Wagner *et al*., 1997), endotoxaemic (Varga *et al*., 1998) and cardiogenic (Wagner *et al*., 2001) shock and the hypotension occurring in advanced liver cirrhosis (Batkai *et al*., 2001). THC, anandamide and 2-AG are known to cause long-lasting hypotension and bradycardia in most animal models (Benowitz and Jones, 1975; Varga *et al*., 1995; Jarai *et al*., 2000; Cohen *et al*., 2002). The cannabinoids anandamide and abnormal cannabidiol are known to induce vasodilation of mesenteric arteries in wild-type and $CB_1R^{-/-}/CB_2R^{-/-}$ mice. This vasodilation is inhibited by SR141716A, but not by other CB_1R antagonists (Jarai *et al*., 1999). A newly discovered endocannabinoid-like molecule, *N*-arachidonoyl-L-serine (ARA-S) has also been shown to produce endothelium-dependent vasodilation that is not reversed by SR141716 nor by SR144528. ARA-S binds very weakly to CB_1R and does not bind to CB_2R. ARA-S decreases LPS-induced plasma TNF-α levels in wild-type, $CB_1R^{-/-}/CB_2R^{-/-}$ and $CB_2R^{-/-}$ mice (Milman *et al*., 2006). This LPS-induced hypotension and cardiac contractility were prevented by SR141716. However, LPS-induced hypotension and its inhibition by SR141726 were similar in wild-type, $CB_1R^{-/-}$ and $CB_1R^{-/-}/CB_2R^{-/-}$ mice (Batkai *et al*., 2004). These findings prove that these cannabinoids cause hypotension via a non-CB_1R/CB_2R mechanism.

$CB_2R^{-/-}$ mice and atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the large arteries, and is the primary cause of heart disease and stroke in Western countries (Libby, 2002). It has been found that oral administration of THC significantly reduced the progression of atherosclerosis in the apolipoprotein mouse model ($ApoE^{-/-}$), an animal model for atherosclerosis. The CB_2R antagonist SR144528 reversed the effect of THC, suggesting the involvement of the CB_2R. Splenocytes derived from $ApoE^{-/-}$ mice treated with THC showed reduced concanavalin A (Con A)-induced proliferation and IFN-γ secretion. THC was also found to decrease $ApoE^{-/-}$ and wild-type thioglycollate-induced macrophage migration in response to the monocyte chemoattractant protein-1, but not the migration of $CB_2R^{-/-}$ macrophages (Steffens *et al*., 2005). Apoptosis of macrophages is an important event in the pathophysiology of atherosclerosis (Liu *et al*., 2005). Oxidized low-density lipoproteins (OxLDL) are a major lipid component of atherosclerotic lesions and endocytosis of OxLDL is a potent inducer of apoptosis in cultured macrophages (Reid *et al*., 1993; Hardwick *et al*., 1996). Several macrophage processes associated with ongoing atherogenesis are regulated by CB_2R (Zhu *et al*., 1998; Maccarrone and Finazzi-Agro, 2003; Steffens *et al*., 2005). It has been found that wild-type peritoneal macrophages treated with OxLDL and its cholesterol derivative 7-ketocholesterol readily undergo apoptosis. OxLDL- and 7-ketocholesterol-induced apoptosis in $CB_2R^{-/-}$ peritoneal macrophages was significantly decreased compared to wild-type macrophages. Staurosporine-induced apoptosis was similar in wild-type and $CB_2R^{-/-}$ peritoneal macrophages, indicating that apoptotic mechanism are intact in the $CB_2R^{-/-}$ macrophages. These findings suggest that CB_2R influences the development and progression of atherosclerotic lesions by mediating the apoptotic response of macrophages to OxLDL (Thewke *et al*., 2007). Taken together, these observations suggest that drugs targeting the CB_2R may be valuable tools to treat atherosclerosis.

CB_2R and macrophage chemotaxis

A crucial event occurring early in an inflammatory response is the migration of macrophages towards the chemostimulant. It has been found that peritoneal macrophage response to the chemokine RANTES/CCL5 is significantly inhibited by THC, CP55,940 and by the CB_2R-specific agonist O-2137, but not by the CB_1R agonist, ACEA. Moreover, the inhibition by THC was reversed by SR144528 but not by SR141716. THC treatment had a minimal effect on the chemotactic response of $CB_2R^{-/-}$ peritoneal macrophages (Raborn *et al*., 2007). These findings implicate the CB_2R in the modulation of macrophage migration in response to chemoattractants.

$CB_2R^{-/-}$ mice and infectious models

$CB_2R^{-/-}$ mice and bacterial infections

Δ-9-Tetrahydrocannabinol treatment of mice has been shown to suppress the immune response to *Legionella pneumophila*, an intracellular bacterium that causes Legionnaires' disease. In this model, THC suppresses Th1 immunity while enhancing Th2 response (Newton *et al*., 1994; Klein *et al*., 2000). The Th2-biasing effect of THC involves cannabinoid receptors, suppression of IL-12 and IL-12 receptor and IFN-γ along with an increase in the Th2-biasing transcription factor GATA 3 (Klein *et al*., 2004). Dendritic cells are potent antigen-presenting cells that upon maturation produce co-stimulatory molecules and cytokines such as IL-12 that bias helper T cells towards Th1 immunity (Kapsenberg, 2003). Since THC treatment significantly suppressed IL-12 serum levels in infected mice, dendritic cells were isolated from wild-type, $CB_1R^{-/-}$ and $CB_2R^{-/-}$ mouse bone marrow, they were infected *in vitro* with *L. pneumophila* and treated with THC. THC was able to suppress IL12p40 production in dendritic cells derived from all three mice genotypes. When SR141716 was used on $CB_2R^{-/-}$ dendritic cells and SR144528 on $CB_1R^{-/-}$ dendritic cells, the antagonists only partially attenuated the THC-induced suppression of IL-12 production by the cells. These findings suggest partial participation of CB_1R and CB_2R in the dendritic response to THC in this infectious model (Lu *et al*., 2006).

$CB_2R^{-/-}$ mice and parasitic infections

Infection of wild-type mice with the *Plasmodium berghei* is a model for cerebral malaria. This infection leads to 100% mortality after 6–7 days of infection. It was found that $CB_2R^{-/-}$ mice are resistant to cerebral disease after being infected with this parasite. Infection leads to comparable microglial migration in the CNS of wild-type and $CB_2R^{-/-}$ mice. However, there is an increase in the number of $CD11b^+$ cells in spleens of infected $CB_2R^{-/-}$ mice compared to infected wild-type mice. The reason for the resistance of $CB_2R^{-/-}$ mice to cerebral malaria is still unknown (Alferink *et al*., 2007).

$CB_2R^{-/-}$ mice, lymphocyte proliferation and cytokine production

It is known that cannabinoids and cannabinoid receptors modulate lymphocyte proliferation and cytokine production (reviewed by Klein *et al*., 2003; Massi *et al*., 2006). To investigate whether the endogenous cannabinoid 2-AG modulates cytokine production via CB_1R and CB_2R, Ouyang, Kaplan and colleagues used the $CB_1R^{-/-}/CB_2R^{-/-}$ double knockout mice. They found that 2-AG suppressed interleukin-2 (IL-2) and IFN-γ production in phorbol myristate 13-acetate/ionomycin (PMA/Io)-treated wild-type mouse splenocytes (Ouyang *et al*., 1998; Kaplan *et al*., 2005). In addition, 2-AG suppressed IFN-γ production in splenocytes derived from $CB_1R^{-/-}/CB_2R^{-/-}$ mice (Kaplan *et al*., 2005). 2-AG ether, a nonhydrolysable analogue of 2-AG, also suppressed IL-2 expression in wild-type and $CB_1R^{-/-}/CB_2R^{-/-}$ splenocytes (Rockwell *et al*., 2006). Our findings are consistent with these. We isolated splenocytes and $CD4^+$ T cells from wild-type and $CB_2R^{-/-}$ mice, and treated them *in vitro* with the T-cell mitogen Con A or with anti-CD3 and anti-CD28 antibodies. The cells were cultured in the presence or absence of 2-AG or WIN 55,212-2. We found that 2-AG and WIN 55,212-2 inhibited the secretion of IL-2 and IFN-γ in wild-type and $CB_2R^{-/-}$ splenocytes stimulated with Con A (Figures 1 and 2). Furthermore, WIN 55,212-2 inhibited the secretion of IL-2 and IFN-γ in wild-type and $CB_2R^{-/-}$ and $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 (Table 1). However, WIN 55,212-2 did not alter the secretion of TGF-β in wild-type or $CB_2R^{-/-}$ splenocytes and $CD4^+$ T cells (data not shown). In addition, 2-AG and WIN 55,212-2 did not alter proliferation in wild-type or $CB_2R^{-/-}$ splenocytes stimulated with Con A. However, WIN 55,212-2 inhibited proliferation in wild-type and $CB_2R^{-/-}$ $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies. This suggests that the effect of WIN 55,212-2 on cell proliferation may be responsible for decrease in cytokine production seen with this cannabinoid (Buranapramest, 2006). Taken together, these findings suggest that CB_1R and CB_2R are not involved in the inhibition of splenocyte proliferation and IL-2 and IFN-γ secretion by 2-AG and WIN 55,212-2, respectively.

$CB_2R^{-/-}$ mouse cells and intracellular Ca^{2+} levels

The $CB_1R^{-/-}/CB_2R^{-/-}$ double knockout mice have also been used to show that the effects of cannabinoids on intracellular calcium increase is not dependent on CB_1R and CB_2R. Rao and Kaminski (2006) found that THC (12.5 μM), cannabinol (20 μM) and HU-210 (20 μM) induced a rise in intracellular calcium in wild-type and $CB_1R^{-/-}/CB_2R^{-/-}$ splenocytes. Interestingly, SR141716A and SR144528 (1 μM) attenuated the rise in calcium elicited by the cannabinoids. The partial attenuation of the cannabinoid-induced calcium increase in wild-type and $CB_1R^{-/-}/CB_2R^{-/-}$ splenocytes, suggest that the antagonists used may be acting via mechanism distinct from those involving CB_1R and CB_2R.

$CB_2R^{-/-}$ mice and drug specificity

Recently, the $CB_2R^{-/-}$ mouse has been used to test drug specificity. The specificity of GW405833 for CB_2R has been tested using this mutant mouse. Inflammatory hyperalgesia was induced in wild-type or $CB_2R^{-/-}$ mice by an intraplanar injection of Freund's complete adjuvant and 24 h later GW405833 was given intraperitoneally. Tactile allodynia was developed by wild-type and $CB_2R^{-/-}$ mice in response to the Freund's complete adjuvant injection. While GW405833 was able to reverse this effect in wild-type mice, it did not produce a reduction in tactile allodynia in the $CB_2R^{-/-}$ mice (Valenzano *et al*., 2005; Whiteside *et al*., 2005). Furthermore, it was shown that the central effects of high dose of GW405833 were not mediated by CB_2R as determined by carrying out the hot-plate, tail-flick and rotarod tests in $CB_2R^{-/-}$ mice and finding similar responses to those found in wild-type mice (Whiteside *et al*., 2005). These findings

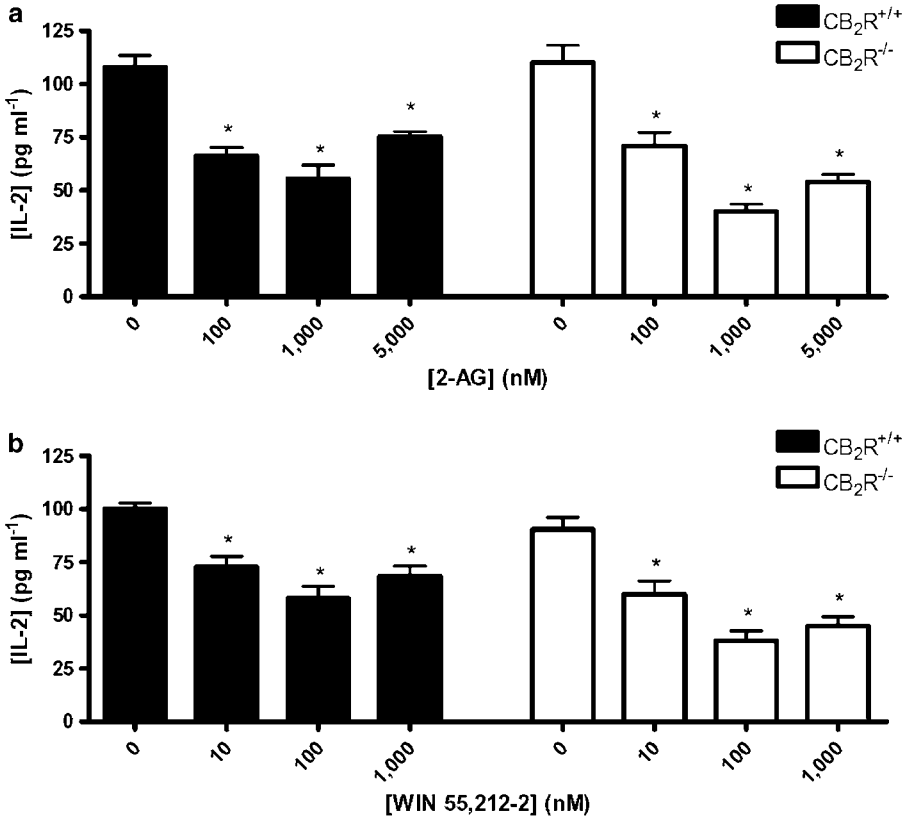

Figure 1 Cannabinoids inhibited the secretion of interleukin-2 (IL-2) in $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes stimulated with concanavalin A (Con A). $CB_2R^{+/+}$ or $CB_2R^{-/-}$ splenocytes (1×10^6 cells per ml per well) were stimulated with Con A (2.5 μg ml^{-1}) and treated with the indicated concentrations of 2-arachidonoylglycerol (2-AG) (**a**) or WIN 55,212-2 (**b**). IL-2 secretion levels were determined from 72-h cell culture supernatants by ELISA. Data are expressed as the mean of triplicate samples ± s.d. and are representative of three independent experiments. *Significantly different from untreated control, $P<0.05$.

show that GW405833 is an effective drug that, at low doses, targets the CB_2R.

The specificity of AM1241 and HU-308 in *in vivo* LPS-induced TNF-α and IL-10 production has also been tested using the $CB_2R^{-/-}$ mouse. AM 1241 (50 mg kg^{-1}, i.p.) and HU-308 (30 mg kg^{-1}, i.p.) inhibited LPS-induced TNF-α production. To test if this effect was mediated by the CB_2R, AM1241 and HU-308 were given to wild-type and $CB_2R^{-/-}$ mice. An hour later, mice were given LPS (0.15 mg kg^{-1}, i.p.). It was found that plasma TNF-α was significantly inhibited and IL-10 significantly elevated in wild-type and $CB_2R^{-/-}$ mice in response to these agonists. These findings indicate that *in vivo* administration of AM1241 and HU-308 alter LPS-induced TNF-α and IL-10 production independent of the presence of CB_2R (Huang *et al.*, 2007). However, another study revealed that some LPS-induced physiological events are mediated by the CB_2R. Recently, Duncan *et al.* (2007) have found that while wild-type animals are able to mount a febrile response to an *in vivo* LPS (100 μg kg^{-1}, i.p.) challenge, $CB_2R^{-/-}$ mice do not (Duncan *et al.*, 2007).

Thus, it can be concluded that the $CB_2R^{-/-}$ mouse model is a very useful tool in drug discovery.

$CB_2R^{-/-}$ mice and antibody specificity

Tissues from the $CB_2R^{-/-}$ mice have been used to test the specificity of antibodies raised against the CB_2R. The expression of CB_2R protein in wild-type and $CB_2R^{-/-}$ mice has been studied by immunohistochemistry in diverse tissues.

Although several laboratories failed to detect CB_2R in the brain (Derocq *et al.*, 1995; Galiegue *et al.*, 1995; Schatz *et al.*, 1997; Carlisle *et al.*, 2002; Ibrahim *et al.*, 2003, 2006), recent studies have revealed the presence of this receptor in diverse brain regions. Using polyclonal antibodies generated against the C terminus of CB_2R from Alpha Diagnostics (San Antonio, TX, USA), Van Sickle *et al.* (2005) reported the expression of CB_2R protein in neurons of the brainstem from wild-type animals. To test the specificity of their antibody, they carried out immunostaining on the dorsal motor nucleus of the vagus from wild-type and $CB_2R^{-/-}$ mice. While they found CB_2R expression in the wild-type mice, there was no immunostaining in $CB_2R^{-/-}$ mice (Van Sickle *et al.*, 2005). Using rabbit anti-human CB_2R polyclonal antibody (Cayman Chemicals, Ann Arbor, MI, USA and Sigma, St Louis, MO, USA), Onaivi, Gong and colleagues have found CB_2R widely expressed in the brain. Unfortunately, the specificity of the Cayman anti-CB_2R antibody was not tested using the $CB_2R^{-/-}$ brain. Instead, using a different antibody, one raised against the C terminus of CB_2R (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), they showed that while CB_2R expression was found in the interpolar part of spinal 5th nucleus of the wild-type mouse brain, CB_2R expression was not found in the same brain region of $CB_2R^{-/-}$

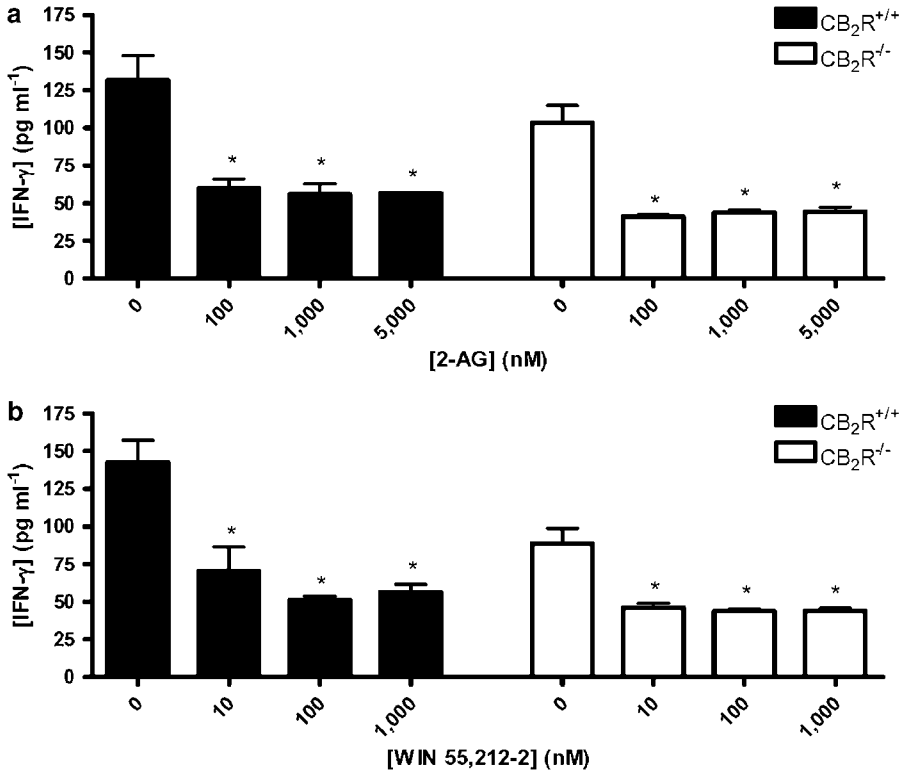

Figure 2 Cannabinoid inhibited the secretion of interferon-γ (IFN-γ) in $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes stimulated with concanavalin A (Con A). $CB_2R^{+/+}$ or $CB_2R^{-/-}$ splenocytes (1×10^6 cells per ml per well) were stimulated with Con A ($2.5\,\mu g\,ml^{-1}$) and treated with the indicated concentrations of 2-arachidonoylglycerol (2-AG) (**a**) or WIN 55,212-2 (**b**). IFN-γ secretion levels were determined from 72-h cell culture supernatants by ELISA. Data are expressed as the mean of triplicate samples ± s.d. and are representative of three independent experiments. *Significantly different from untreated control, $P<0.05$.

Table 1 IL-2 and IFN-γ secretion in anti-CD3 and anti-CD28 stimulated $CD4^+$ cells treated with WIN 55,212-2

WIN 55,212-2 (nM)	*$CB_2R^{+/+}$ cells, IL-2 ($pg\,ml^{-1}$ ± s.d.)*	*$CB_2R^{-/-}$ cells, IL-2 ($pg\,ml^{-1}$ ± s.d.)*	*$CB_2R^{+/+}$ cells, IFN-γ ($pg\,ml^{-1}$ ± s.d.)*	*$CB_2R^{-/-}$ cells, IFN-γ ($pg\,ml^{-1}$ ± s.d.)*
0	49.17 ± 9.42	158.20 ± 52.84	134.50 ± 17.05	139.00 ± 27.02
3	47.64 ± 5.20	115.60 ± 90.35	133.50 ± 32.23	124.40 ± 42.46
10	22.37 ± 15.19	77.04 ± 21.67	87.54 ± 6.35*	80.20 ± 2.86*
31	14.22 ± 2.42*	30.72 ± 9.90*	110.40 ± 23.10	101.50 ± 13.88
100	38.98 ± 27.10	64.98 ± 11.11	122.20 ± 5.86	123.80 ± 24.58
316	48.92 ± 45.70	72.78 ± 53.71	93.67 ± 18.04	96.37 ± 13.11
1000	7.65 ± 1.87*	45.38 ± 0.60*	96.85 ± 12.90	88.89 ± 15.90
3162	13.10 ± 8.50*	18.75 ± 5.36*	91.21 ± 5.21*	82.98 ± 10.50*

Abbreviations: IFN-γ, interferon-γ; IL-2, interleukin-2.
Purified total T cells or $CD4^+$ T cells were isolated by negative selection using the Pan T Cell Isolation Kit or $CD4^+$ T cell Isolation Kit, respectively (Miltenyi Biotech, Auburn, CA, USA) from spleens. $CB_2R^{+/+}$ or $CB_2R^{-/-}$ $CD4^+$ T cells (1×10^5 cells per 0.1 ml per well) were stimulated with anti-CD3 ($5\,\mu g\,ml^{-1}$) and anti-CD28 ($0.5\,\mu g\,ml^{-1}$) antibodies and treated with the indicated concentrations of WIN 55,212-2. IL-2 and IFN-γ secretion levels were determined from 72-h cell culture supernatants by ELISA. Data are expressed as the mean of triplicate samples ± s.d. and are representative of three independent experiments. *Significantly different from untreated control, $P<0.05$.

mice (Gong *et al*., 2006; Onaivi, 2006; Onaivi *et al*., 2006). Coincident with these data is the observation that CB_2R is present in neural progenitors from late embryonic stages to adult brain. Moreover, activation of the CB_2R *in vitro* promoted neural progenitor cell proliferation, this was not seen in $CB_2R^{-/-}$ cells. In addition, i*n vivo* treatment with HU-308 increased hippocampal progenitor proliferation in wild-type but not in $CB_2R^{-/-}$ mice (Palazuelos *et al*., 2006). On the other hand, Wotherspoon *et al*. (2005) have not been able to find CB_2R expression in sections of naive mouse dorsal root ganglia or spinal cord using the anti-CB_2R antibody from Cayman. Although CB_2R immunoreactivity was seen following unilateral nerve damage, and was localized to the superficial laminae of the dorsal horn of the spinal cord, ipsilateral to the nerve damage. Antibody specificity was confirmed in $CB_2R^{-/-}$ spinal cord and spleen sections.

Using the anti-CB_2R antibody from Cayman, expression of CB_2R was also found in mouse atherosclerotic plaques (Steffens *et al*., 2005) and in osteoblasts, osteocytes and osteoclasts (Ofeck *et al*., 2006) of wild-type mice. CB_2R

antibody specificity was determined by using spleens and bone from $CB_2R^{-/-}$ mice.

When obtaining data using the currently available polyclonal anti-CB_2R antibodies, one should be cautious. At the moment, there are no CB_2R-specific monoclonal antibodies available. It must therefore be recognized that the fine specificity of such commercial polyclonal antisera may change over time, depending on the bleed and individual animals being immunized during antibody production. Thus, tissues derived from $CB_2R^{-/-}$ mice will continue to provide a very useful tool to elucidate the specificity of current and future anti-CB_2R antibodies.

Conclusion

The reports reviewed here emphasize the usefulness of the $CB_2R^{-/-}$ mouse. Using this mutant mouse, investigators have discovered, or confirmed, CB_2R tissue and/or cellular expression under normal or abnormal conditions. Because the CB_2R was originally cloned and reported to be present in immune cells (Munro *et al.*, 1993), it is not surprising that the CB_2R is found in cells of the haematopoietic lineage. The presence of this receptor in preimplantation embryonic stem cells (Paria *et al.*, 1995) is very interesting and may implicate this receptor in the early development of haematopoietic cells and perhaps other cells. We found that in the embryonic rat, CB_2R was expressed in the liver in cells that appeared to be Kuffer cells (Buckley *et al.*, 1998). The CB_2R has also been shown to be expressed in other normal adult cells. These include cells within the CNS (Van Sickle *et al.*, 2005; Gong *et al.*, 2006; Onaivi, 2006; Onaivi *et al.*, 2006) and bone (Ofeck *et al.*, 2006). In other instances, however, CB_2R expression is only evident under certain disease conditions. Thus, CB_2R expression is upregulated in pain models (Wotherspoon *et al.*, 2005), atherosclerosis (Steffens *et al.*, 2005), liver disorders (Julien *et al.*, 2005) and during inflammation (Maresz *et al.*, 2007) (Figure 3). On the basis of the findings using the $CB_2R^{-/-}$ mouse, investigators have speculated as to the function of this receptor. CB_2R activation is thought to be antinociceptive and anti-inflammatory. It is also thought to be involved in bone homeostasis and in protective mechanisms during atherosclerosis and liver injury. On the other hand, using this mutant mouse, investigators have found that the CB_2R is not involved in hypotension. The implication of CB_2R in so many events lends validity to the notion that CB_2R is biologically functionally relevant. Nevertheless, living organisms are complex and one can reason that CB_2R and its endocannabinoid ligands do not work alone and other important molecular players are equally necessary. Finally, the $CB_2R^{-/-}$ mouse has been a very useful tool to help determine drug and antibody specificity and promises to continue to be very useful in drug and antibody discovery.

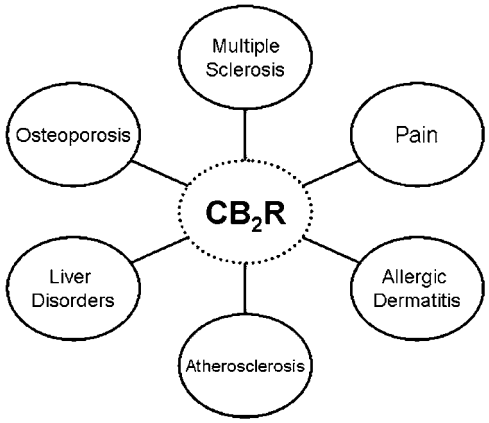

Figure 3 Using the $CB_2R^{-/-}$ mice, the CB_2R has been implicated in diverse diseases.

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Conflict of interest

The author states no conflict of interest.

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