

AN INVESTIGATION INTO THE ROLE OF
NEUROTRANSMITTER RECEPTORS IN THE
FUNCTION OF HUMAN IMMUNE CELLS

by

SARAH ELIZABETH MILTON

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Department of Neuroscience
School of Clinical and Experimental Medicine
College of Medical and Dental Sciences
University of Birmingham

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ABSTRACT

The interaction between the nervous and immune system is well documented, although is still not fully understood - particularly the impact of neurotransmitter receptors on immune cell function. 5-HT_{3A} receptor expression was identified on activated regulatory T cells (Treg) but not on effector T cells. Incubation of human peripheral blood mononuclear cells (PBMC) with the 5-HT₃ receptor agonist DDP733 and the positive allosteric modulator 5-chloroindole increased the percentage of CD25⁺FoxP3⁺ lymphocytes (Treg phenotype). Proliferation of PBMC was inhibited by DDP733 plus 5-chloroindole indicating functional impact by the 5-HT₃ receptor. The GPR55 receptor was also expressed by human T cells. The GPR55 agonist lysophosphatidylinositol increased cell viability by preventing apoptosis. However, the induced response was not blocked by the GPR55 receptor antagonist cannabidiol casting doubt over the GPR55 receptor mediating the response. Cannabidiol was demonstrated to have a pro-apoptotic effect in its own right, although whether this effect is mediated by GPR55 or the CB₂ cannabinoid receptor is unknown. Further experiments are required to elucidate the role of the 5-HT_{3A} receptor in lymphocyte function and the mechanism responsible for the immunoprotective role of lysophosphatidylinositol.

DEDICATION

Firstly, my thanks go to Professor Nicholas Barnes, who offered me the wonderful opportunity of joining his lab. With supervision also from Professor John Gordon, I am grateful to them both for the time and effort they have invested in me.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ANOVA	analysis of variance
APC	antigen presenting cell
CBD	cannabidiol
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein succinimidyl ester
CB ₁	cannabinoid receptor type 1
CB ₂	cannabinoid receptor type 2
CD	cluster of differentiation
DAPI	diamidino-2-phenylindole
FACS	Fluorescent automated cell sorting
FSC	forward scatter
GPR55	G protein coupled receptor 55
HEK293	human embryonic kidney cell line
IFN	interferon
LPI	lysophosphatidylinositol
mCPBG	m-chlorophenylbiguanide
MFI	median fluorescence intensity
mRNA	messenger ribonucleic acid
nACh	nicotinic acetylcholine receptor
PCR	polymerase chain reaction
PMA	phorbol myristate acetate
PBMC	peripheral blood mononuclear cells
PHA	phytohaemagglutinin
SSC	side scatter
SEM	standard error of the mean
SERT	serotonin transporter
TNF	tumour necrosis factor
TM	transmembrane

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CHAPTER 1

INTRODUCTION

Section 1.1 Serotonin

Section 1.1.1 History of serotonin

Serotonin was initially discovered in 1948 in bovine serum and was named due to its physiological properties in the vasculature (Rapport *et al.*, 1948); a year later its structure was determined and hence additionally became known as 5-hydroxytryptamine (5-HT) (Rapport, 1949). It was later identified in the CNS (Amin *et al.*, 1954) and its role has been well established as a neuromodulator. Serotonin is found systemically and within peripheral cells such as those of the immune-inflammatory axis (Tracey, 2002).

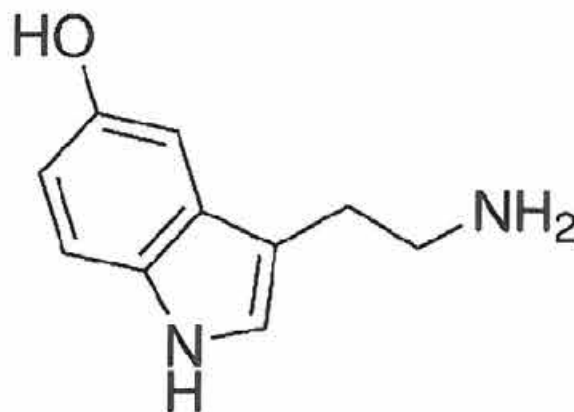


Figure 1.1: Structure of the monoamine neurotransmitter serotonin.

Section 1.1.2 Synthesis of serotonin

5-HT is stored in serotonergic neurones located in the CNS and myenteric plexus, enterochromaffin cells and in blood platelets (Lesch *et al.*, 1993). Serotonergic neurones and enterochromaffin cells synthesise 5-HT from the essential amino acid precursor L-tryptophan using the enzyme tryptophan hydroxylase (TPH), a phenotypic marker bestowing the cell's ability to synthesise 5-HT. Two isoforms of TPH exist which display near exclusive expression in the periphery (TPH1) and neurones (TPH2). Platelets however, rely upon the serotonin transporter (SERT) for the uptake of 5-HT (Mossner and Lesch, 1998).

Section 1.1.3 Serotonin receptors

In 1957, when Gaddum and Picarelli were investigating the contraction of smooth muscle in guinea pig ileum, it was noted that the effect of 5-HT could be partially antagonised by both dibenzyline and morphine; therefore it was suggested that the receptors be named 5-HT_D and 5-HT_M respectively (Gaddum and Picarelli, 1957). In the years that followed, more subtypes of receptor were identified and thus a new method of classification was required. Current nomenclature of the 5-HT receptors is based upon structural, physiological and pharmacological properties outlined by the International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (IUPHAR; <http://www.iuphar-db.org/index.jsp>). There are now 14 known subtypes of serotonin receptor, which fall into seven distinct families (Table 1.1).

Section 1.1.3.1 G protein-coupled 5-HT receptors

Receptor subtype	5-HT _{1A}	5-HT _{1B}	5-HT _{1D}	5-HT _{1E}	5-HT _{1F}
Transduction mechanism	G _i /G _o family	G _i /G _o family	G _i /G _o family	G _i /G _o family	G _i /G _o family
Effector/response	AC –, PLC +	AC –	AC –	AC –	AC –
CNS distribution	DRN, hippocampus, septum, thalamus, hypothalamus, amygdala, medulla, cerebral cortex	Raphe nucleus, cortex, caudate, putamen, substantia nigra, globus pallidus, amygdala, hippocampus	Globus pallidus, frontal cortex, putamen	Cortex, putamen, caudate, globus pallidus, amygdala	Cortex, striatum, hippocampus, thalamus, hypothalamus, cerebellum
Immune expression	PBMC, T cells	PBMC, dendritic cells		PBMC, monocytes, dendritic cells	

Receptor subtype	5-HT _{2A}	5-HT _{2B}	5-HT _{2C}
Transduction mechanism	G _i /G _o family G _q /G ₁₁ family	G _q /G ₁₁ family	G _i /G _o family G _q /G ₁₁ family
Effector/response	AC –, PLC +	PLC +	AC –, PLC +
CNS distribution	Cortex, hippocampus, nucleus accumbens, caudate, putamen, pontine nuclei, motor cranial nerve nuclei	Cerebellum, hypothalamus, amygdala, lateral septum	Cortex, hippocampus, subthalamic nucleus, amygdala, substantia nigra, raphe nuclei, brainstem
Immune expression	PBMC, monocytes, dendritic cells	Dendritic cells	

Receptor subtype	5-HT ₄	5-HT _{5A}	5-HT ₆	5-HT ₇
Transduction mechanism	G _i /G _o family	G _i /G _o family	G _i /G _o family	G _i /G _o family
Effector/response	AC + Rap1-rac + Rho +	AC –, PLC +	AC +, PLC +	AC +
CNS distribution	DRN, hippocampus, septum, thalamus, hypothalamus, amygdala, medulla, cerebral cortex	Raphe nucleus, cortex, caudate, putamen, substantia nigra, globus pallidus, amygdala, hippocampus	Globus pallidus, frontal cortex, putamen	Cortex, putamen, caudate, globus pallidus, amygdala
Immune expression	PBMC, monocytes, dendritic cells		PBMC	monocytes, dendritic cells, spleen

Table 1.1: G protein-coupled 5-HT receptor subtypes based on the IUPHAR committee on receptor nomenclature and drug classification, based on human studies, modified from (Hoyer *et al.*, 2002, Barnes and Sharp, 1999).

Although a putative 5-HT_{5B} receptor has been identified in rodent, the human homologue contains a stop codon and therefore is not expected to be functional (Grailhe *et al.*, 2001).

Section 1.1.3.2 Ligand gated 5-HT₃ receptors

The 5-HT₃ receptor is the only 5-HT receptor that is a ligand gated ion channel (LGIC). The 5-HT₃ receptor belongs to the cys-cys loop LGIC superfamily which also includes the nicotinic acetylcholine, γ -aminobutyric acid and glycine receptors, which are comprised of five subunits which surround a central, cation permeable channel (Figure 1.2). Each subunit is characterised by a long extracellular N-terminus, four transmembrane (TM) domains, and a short extracellular C-terminus (Figure 1.2). It is TM2 of each of the five subunits which forms the channel pore. cDNAs for five distinct 5-HT₃ receptor subunits have been cloned. The architecture of the 5-HT₃A, 5-HT₃B, 5-HT₃C and 5-HT₃E subunits is relatively similar, although the 5-HT₃D receptor subunit differs in that it lacks the majority of the large extracellular N-terminus, including the cys-loop region (Niesler *et al.*, 2003). The 5-HT₃A receptor subunit is thought to be the only subunit able to form functional homomeric 5-HT₃A receptors in mammalian cells which may be due to the lack of a particular tryptophan residue which is crucial for ligand binding (Niesler *et al.*, 2007). 5-HT₃B, C, D and E can form heteromeric receptors when expressed with the 5-HT₃A receptor subunit.

The nomenclature of the 5-HT₃ receptor was approved by the International Union of Basic and Clinical Pharmacology (IUPHAR) (Hoyer *et al.*, 1994). Homomeric 5-HT₃A receptors are distinguished from 5-HT₃A receptor subunits by the use of a subscript 3. Heteromeric receptors comprised of both 5-HT₃A and 5-HT₃B receptor subunits are known as 5-HT₃AB receptors.

The arrangement of the 5-HT₃A and 5-HT₃B receptor subunits in recombinant 5-HT₃AB receptors expressed by HEK293 cells is thought to be in the configuration B-

B-A-B-A (Figure 1.2) (Barrera *et al.*, 2005). This has been investigated more recently and it was suggested that ligand binding occurs at an A-A interface, which would insinuate the stoichiometry of the 5-HT₃AB receptors is different to the stoichiometry suggested previously (Lochner and Lummis, 2010). The stoichiometry of native heteromeric 5-HT₃ receptors is yet to be elucidated.

Data reported on the function of 5-HT₃ receptors describes either homomeric 5-HT₃A receptors or heteromeric 5-HT₃AB receptors. 5-HT₃AB receptors have a higher single channel conductance (Davies *et al.*, 1999), lower Ca²⁺ permeability, faster activation and deactivation kinetics and lower 5-HT potency than homomeric 5-HT₃A receptors (Walstab *et al.*, 2008, Dubin *et al.*, 1999). Studies using transfected cells expressing 5-HT₃A receptor subunits and one of either 5-HT₃C, 5-HT₃D or 5-HT₃E receptor subunits reveal similar pharmacological characteristics to homomeric 5-HT₃A receptors, although these subunits may impact the level of receptor expression at the cell membrane (Niesler *et al.*, 2007).

5-HT₃ receptors are heterogeneously expressed throughout the central nervous system. Importantly, 5-HT₃ receptor expression has been demonstrated in brain regions involved in the integration of the vomiting reflex, pain processing, cognition and the control of anxiety (Barnes *et al.*, 1989, Parker *et al.*, 1996), which makes them a target for the treatment of emesis, migraine and psychiatric disorders including addiction and neurodegenerative disorders.

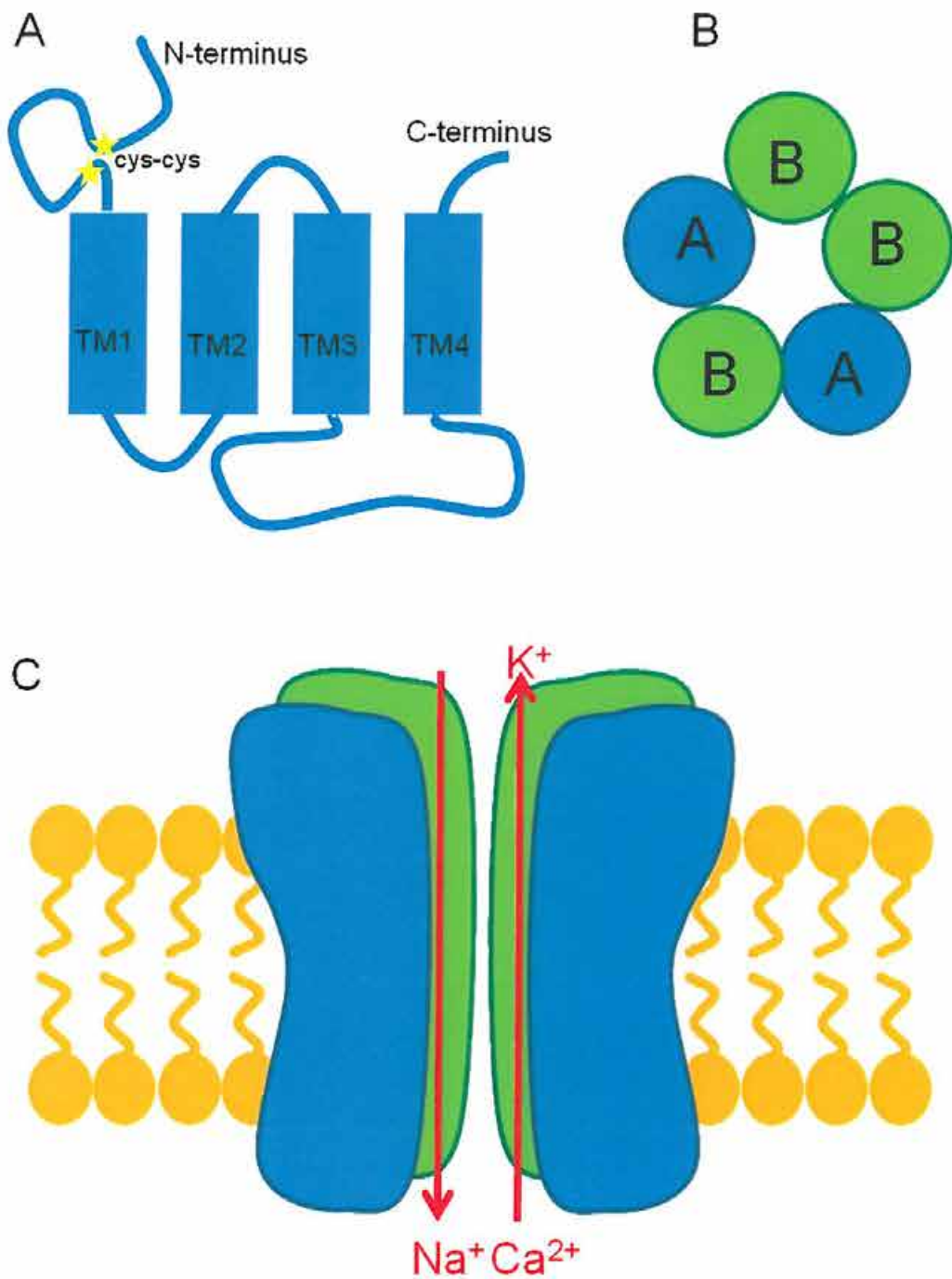


Figure 1.2: Schematic structure of the 5-HT₃ receptor. A) Simplified structure of the 5-HT_{3A} receptor subunit, with the cys-loop and long intracellular loop between TM3 and TM4. B) Arrangement of the 5-HT_{3A} and 5-HT_{3B} receptor subunits in a heteromeric 5-HT_{3AB} receptor as viewed from above. C) 3D view of a 5-HT_{3AB} receptor located in the cell membrane; one subunit has been removed to reveal the channel lumen, red arrows show the direction of cation movement through the channel.

5-HT₃ receptors are also expressed in the peripheral nervous system. 5-HT₃ receptors are expressed on vagal afferent neurones in the heart where their activation mediates the Bezold-Jarisch reflex (Malinowska *et al.*, 1995). Expression studies using human mRNA revealed that *HTR3A*, *HTR3B*, *HTR3C* and *HTR3D* gene transcripts were ubiquitously expressed, although *HTR3E* was restricted to gastrointestinal tissues for example the colon, intestine and stomach (Niesler *et al.*, 2003). 5-HT₃ receptors have also been identified in cells of the immune system which is discussed in Section 1.4.1.

Selective 5-HT₃ receptor agonists such as meta-chlorophenylbiguanide (mCPBG) are useful tools for investigating the function of 5-HT₃ receptors, yet due to their emetogenic and anxiogenic effects, 5-HT₃ receptor agonists have limited therapeutic potential. The specific 5-HT₃ receptor partial agonist DDP733 was initially developed for the treatment of irritable bowel syndrome with constipation (Evangelista, 2007). Besides ligands binding to the orthosteric binding site of the 5-HT_{3A} receptor, there are numerous positive allosteric modulators of the 5-HT_{3A} receptor. Most of these compounds (including alcohols, anaesthetics and cannabinoids) exert effects at other receptors (Davies, 2011). Data from our own lab has demonstrated 5-chloroindole specifically modulates 5-HT_{3A} receptors, significantly increasing the intrinsic activity of 5-HT_{3A} receptor agonists (unpublished, Butler *et al.*).

5-HT₃ receptor antagonists such as ondansetron and granisetron are used for the treatment of chemotherapy- and post-operative- induced nausea and vomiting (Russell and Kenny, 1992).

Section 1.1.4 The serotonin transporter and selective serotonin reuptake inhibitors

The bi-directional serotonin transporter (SERT) is a twelve-transmembrane domain protein (Blakely *et al.*, 1991) which is responsible for controlling the availability of 5-HT for neurotransmission in the synaptic cleft. SERT transports 5-HT back into presynaptic neurones where it is metabolised by monoamine oxidase.

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine are used in the treatment of anxiety and depression to increase the availability of 5-HT to facilitate serotonergic neurotransmission hereby alleviating the symptoms of clinical depression and anxiety disorders (Fuller *et al.*, 1991).

Due to its widespread distribution, 5-HT *via* its receptors and transporter, has important roles in many physiological processes; appetite, emotion, learning, memory, libido, thermoregulation and circadian rhythm (Barnes and Sharp, 1999). Unsurprisingly, dysfunction of the 5-HT system is implicated in many diseased states such as depression, anxiety, schizophrenia, attention deficit disorder, tinnitus and inflammatory bowel conditions (Marriage and Barnes, 1995, De Ponti and Tonini, 2001, Hawi *et al.*, 2002).

Section 1.2 Cannabinoids

Section 1.2.1 History of Cannabis Sativa

The therapeutic use of cannabis dates back 5000 years to the Chinese Emperor Shen-Nung, who assessed the medicinal value of hundreds of plants. The psychoactive properties of cannabis were also documented by the Greek historian Herodotus in the 5th Century BC. Cannabis was first introduced to Western medicine in 1839 by Dr. William Brooke O'Shaughnessy who administered preparations of cannabis extract to tetanus patients and reported its analgesic and sedative properties (Gorman, 1984). Inflammatory conditions were first treated with cannabis preparations in the mid nineteenth century. Despite the variation of responses in humans (most probably due to cannabis being insoluble in water making consistent preparation of the drug difficult), it was noted that cannabis was superior to opiate treatment in several ways: long-term use of cannabis caused minimal dependence or tolerance, had a relatively low toxicity level and few intolerable side effects (McMeen, 1860).

Section 1.2.2 Cannabinoids

Classic cannabinoids are compounds which are structurally similar to tetrahydrocannabinol (Δ^9 -THC), the psychoactive component of Cannabis Sativa (Figure 1.3). It was previously thought that due to the lipid-soluble, hydrophobic nature of Δ^9 -THC, its effects were produced by perturbing neuronal cell membranes. However, the development of analogues of Δ^9 -THC and investigations into the structural and spatial arrangement of the compounds suggested the involvement of specific receptors. Devane and colleagues demonstrated that saturable, high affinity membrane binding sites for cannabinoids existed using radioligand-binding

techniques (Devane *et al.*, 1988). Endogenous cannabinoids, known as endocannabinoids such as anandamide and 2-arachidonylglycerol (2-AG) are released from depolarised hippocampal neurones in a calcium dependent manner (Di Marzo *et al.*, 1994) and have an important role in neuronal homeostasis by acting as retrograde signalling molecules in the CNS (Wilson and Nicoll, 2001, Ohno-Shosaku *et al.*, 2001, Kreitzer and Regehr, 2001a, Kreitzer and Regehr, 2001b). The endogenous cannabinoid system mediates both depolarisation-induced suppression of inhibition (DSI) and depolarisation-induced suppression of excitation (DSE) by feedback inhibition of GABA and glutamate respectively (Ohno-Shosaku *et al.*, 2001, Kreitzer and Regehr, 2001a, Kreitzer and Regehr, 2001b). Endocannabinoids also mediate cellular effects via the transient receptor potential vanilloid 1 receptor (TRPV1), a non-selective cation channel, agonised by capsaicin (Zygmunt *et al.*, 1999).

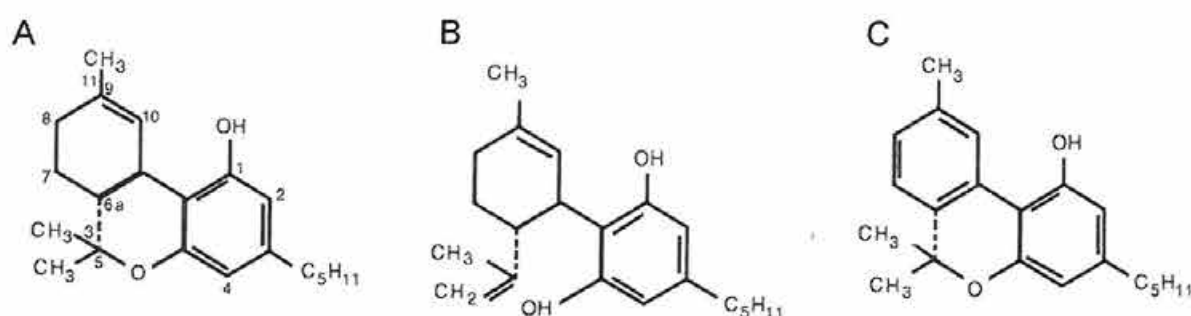


Figure 1.3: Chemical structures of the main cannabinoid compounds found in *Cannabis Sativa*: A) tetrahydrocannabinol (Δ^9 -THC), B) cannabidiol (CBD) and C) cannabinol (CBN). The psychoactive properties of Δ^9 -THC and (the less potent) CBN may be attenuated by non-psychoactive CBD (Ashton, 2001).

Section 1.2.3 Cannabinoid receptors

Two types of cannabinoid receptors have been characterised by molecular cloning techniques (Table 1.2). The CB₁ receptor was first identified in rat brain (Devane *et al.*, 1988) and cloned in 1990 (Matsuda *et al.*, 1990). CB₂ receptors were later identified in the promyelocytic leukemic line HL60, and cloned by homology screening (Munro *et al.*, 1993). Both CB₁ and CB₂ cannabinoid receptors belong to the large superfamily of G-protein coupled receptors (GPCRs), possessing the characteristic extracellular N-terminus, seven TM domains and intracellular C-terminus. CB₁ and CB₂ receptors couple to the G_i G-protein signal transduction pathway causing downstream inhibition of adenylate cyclase and activation of MAP kinase. The CB₁ receptor gene, CNR1 is located on chromosome 6q14-q15. Alternative splicing has identified long (472 amino acids) and short (411 amino acids) transcript variants of the CB₁ receptor. CNR2, the gene encoding the CB₂ receptor is located on chromosome 1p35-p36, encoding a receptor of 360 amino acids in length. Surprisingly, CB₁ and CB₂ receptors share little sequence homology (68 % in the transmembrane domains and 44 % overall) although remarkably, the pharmacology of the two receptors is very similar.

Receptor subtype	CB ₁	CB ₂
Transduction mechanism	G _i family	G _i family
Effector/response	AC -	AC -
CNS distribution	Cerebellum, basal ganglia, amygdala, cerebral cortex, hippocampus	Microglia (Benito <i>et al.</i> , 2003)
Immune system expression	Malignant B cells (Islam <i>et al.</i> , 2003)	Spleen, thymus, tonsils, B, T and NK cells, monocytes (Galiegue <i>et al.</i> , 1995) haematopoietic cells (Munro <i>et al.</i> , 1993)

Table 1.2: G protein-coupled cannabinoid receptor subtypes based on the IUPHAR committee on receptor nomenclature and drug classification, based on human studies. CB₂ receptor expression is found at levels 10 to 100 times higher than CB₁ receptor mRNA (Galiegue *et al.*, 1995).

Section 1.2.3.1 Expression and pharmacology of the CB₁ receptor

Commonly known as the central cannabinoid receptor, the CB₁ receptor is thought to be the most widely expressed GPCR in the human central nervous system (Herkenham *et al.*, 1990). More specifically, CB₁ receptors are found in the cerebellum, basal ganglia, extended amygdala, cerebral cortex and hippocampus (Herkenham *et al.*, 1990, Glass *et al.*, 1997, Tsou *et al.*, 1998, Wang *et al.*, 2003). In contrast, the CB₁ receptor is largely absent in the medulla oblongata, thus cannabinoids do not influence centrally controlled respiration or cardiovascular functions. CB₁ receptors are coupled to the inhibitory G-protein G_i, CB₁ receptors impacting adenylate cyclase activity, are also capable of modulating ion channels, inhibiting voltage gated calcium channels and activating inwardly rectifying potassium channels (Howlett *et al.*, 2004, Demuth and Molleman, 2006). CB₁ receptor-mediated modulation of GABA release has implicated CB₁ receptor antagonists for the treatment of depression (Witkin *et al.*, 2005b, Witkin *et al.*, 2005a). Rimonabant, an

inverse agonist of the CB₁ receptor was used in the treatment of obesity (Van Gaal *et al.*, 2005) and smoking cessation (Cahill and Ussher, 2007) until it was withdrawn due to an increased risk of psychotic episodes (Ugur *et al.*, 2008, Christensen *et al.*, 2007).

In the periphery, pre-synaptic CB₁ receptors are found on sympathetic neurones innervating blood vessels, contributing to the neurogenic vasopressor response (Godlewski *et al.*, 2004). Although the CB₁ receptor is also known as the central cannabinoid receptor, several tissues in the periphery express CB₁ receptors, such as the liver, where it is thought they mediate *do novo* lipogenesis (Osei-Hyiaman *et al.*, 2005). The CB₁ receptor is also present in human reproductive systems. In males, CB₁ receptors are expressed on Leydig cells of the testis (Wenger *et al.*, 2001) and human sperm (Rossato *et al.*, 2005). Activation of CB₁ receptors in sperm was shown to reduce motility of sperm (Rossato *et al.*, 2005). In females, CB₁ receptors are present on the ovaries and (during pregnancy) the placenta (Park *et al.*, 2003). Pharmacological blockade of CB₁ receptor function using SR141716 (rimonabant) in mice was associated with an alteration in the progesterone/estrogen ratio during pregnancy, subsequently leading to an increase in the incidence of premature birth (Wang *et al.*, 2008).

Section 1.2.3.2 Expression and pharmacology of the CB₂ receptor

The CB₂ receptor is also known as the peripheral cannabinoid receptor, located mainly on tissues of the immune system such as the spleen, tonsils, thymus (Galiegue *et al.*, 1995) and haematopoietic cells (Munro *et al.*, 1993). In the normal human immune system, CB₂ receptor mRNA is expressed, in decreasing rank order, by: B cells, natural killer cells, monocytes, PMNs, and T cells (Galiegue *et al.*, 1995). Studies using CB₂^{-/-} mice demonstrate that cannabinoid-induced immunomodulation is primarily attributed to the CB₂ receptors (Buckley *et al.*, 2000). Specifically, antigen-presenting dendritic cells produce large quantities of the CB₂ ligand, 2-AG (Matias *et al.*, 2002) and activated B cells express high levels of CB₂ receptor mRNA, as opposed to inactive B cells, which do not (Stratz *et al.*, 2002). On non-malignant T cells, CB₂ receptor expression is low, however in T-cell non-Hodgkin's lymphoma, high CB₂ protein expression has been reported (Schneider *et al.*, 1977).

Great attention has been paid to CB₂ receptors and their role in neuroinflammation associated with degenerative disease. Microglia cells, which many envisage as the macrophages of the CNS, are capable of expressing CB₂ receptors in response to antigens (Zhang *et al.*, 2003) and upon activation, release pro-inflammatory mediators such as IL-2, TNF- α , IL-6 and nitric oxide and anti-inflammatory cytokines such as IL-4 and IL-10. The therapeutic potential of targeting CB₂ receptors in diseased states is encouraging; however, the lack of selective CB₂ receptor ligands is limiting this research.

Section 1.2.4 Evidence for novel cannabinoid receptors

There is mounting evidence that some ligands for CB₁ and/or CB₂ target additional receptors. Several cannabinoid compounds such as naturally occurring oleylethanolamide and palmitoylethanolamide (OEA and PEA respectively) and phytocannabinoids (CBN and CBD) lack affinity at CB₁ and CB₂ cannabinoid receptors, yet still evoke pharmacological effects. Furthermore, studies performed using CB₁^{-/-} and CB₂^{-/-} mice have identified several non-CB₁/CB₂ sites (Ohno-Shosaku *et al.*, 2002, Kaplan *et al.*, 2003, Rao and Kaminski, 2006).

In the mesenteric vasculature of the rat, vasodilatation occurs in the resistance arteries in response to anandamide (arachidonylethanolamine; AEA) and analogues, but not synthetic cannabinoid receptor agonists (Wagner *et al.*, 1999). This response is prevented by endothelial denudation, and is sensitive to antagonism by rimonabant, a CB₁ receptor inverse agonist, but not by another CB₁ receptor inverse agonist AM251, although chemically similar. The authors proposed the receptor mediating this response be called the 'endothelial-anandamide receptor', highlighting the receptor's insensitivity to derivatives of cannabis (Wagner *et al.*, 1999). The concentration of rimonabant needed to inhibit vasodilatation is higher than that required at CB₁ receptors. Vasodilatation is also caused in response to abnormal cannabidiol (abn-cbd), which is thought to be a selective agonist at this non-CB₁/CB₂ endothelial site, having little activity at CB₁ or CB₂ receptors (Jarai *et al.*, 1999). Non-CB₁/CB₂ sites have been reported in the CNS, in particular the hippocampus. The phenomenon of depolarisation-induced suppression of inhibition, or excitement (DSI and DSE, respectively) was previously attributed to the CB₁ receptors, yet it has been demonstrated that both DSI and DSE can be induced in rat

hippocampus slices from wild-type $CB_1^{-/-}$ rats (Ohno-Shosaku *et al.*, 2002). This putative 'non- CB_1 hippocampal cannabinoid receptor' displays a similar pharmacological profile to the putative non- CB_1/CB_2 endothelial cannabinoid receptor. Cannabinoid-induced reduction of glutamatergic neurotransmission in the $CB_1^{-/-}$ mice can be antagonised by rimonabant, but not by AM251 (Hajos and Freund, 2002). Both the non- CB_1 hippocampal cannabinoid receptor and the non- CB_1/CB_2 endothelial cannabinoid receptor are sensitive to pertussis toxin, suggesting an involvement of a G_i/G_o G protein coupled receptor (Ohno-Shosaku *et al.*, 2002, Wagner *et al.*, 1999).

In immune cells, cannabinoid activity at non- CB_1/CB_2 receptor sites have been identified (Kaplan *et al.*, 2003). Splenocytes isolated from both wild-type and $CB_1^{-/-}/CB_2^{-/-}$ mice demonstrate elevations of intracellular calcium ($[Ca^{2+}]_i$) in response to Δ^9 -THC, CBN and HU210 but not CP55940. Both SR141716A and SR144528 (inverse agonists at CB_1 and CB_2 receptor respectively) blocked this effect, although only at concentrations higher than their reported affinities for CB_1 and CB_2 receptors (Rao and Kaminski, 2006).

Further studies using cannabinoid receptor knockout mice indicate other GPCRs as potential cannabinoid targets (Begg *et al.*, 2005, Mackie and Stella, 2006). Orphan receptor GPR119 was reported to be a receptor for OEA (Overton *et al.*, 2006) and orphan receptor GPR55 has demonstrated activation by a variety of cannabinoids, implicating them as candidates for novel cannabinoid receptors.

Section 1.2.5 Structure of the GPR55 receptor

The orphan G-protein coupled receptor GPR55 was identified in 1999 (*Sawzdargo et al., 1999*). The human gene maps to chromosome 2q37 and encodes a 319 amino acid protein. GPR55 receptors share low sequence homology with either CB₁ or CB₂ receptors (10-15 %), and the GPR55 receptor lacks the putative 'cannabinoid binding pocket' present in both CB₁ and CB₂ cannabinoid receptors (*Petit et al., 2006*). However, dot-plot homology identified particular regions with conserved sequences between GPR55 receptors and the classical cannabinoid receptors CB₁ and CB₂. The majority of conserved amino acids are contained within the putative TM domains of which domains I, II and III are thought to be binding sites for some cannabinoid receptor ligands (*Barnett-Norris et al., 2002*).

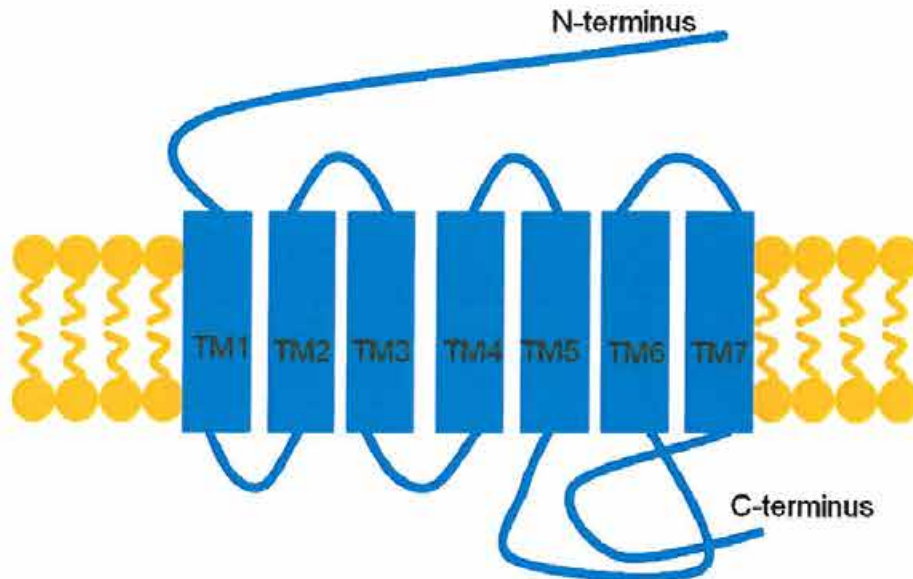


Figure 1.4: Schematic structure of the GPR55 receptor. Simplified structure of the GPR55 receptor located in the cell membrane, with a long extracellular N-terminus and intracellular loop between TM5 and TM6.

Section 1.2.6 GPR55 receptor signalling

A patent published by AstraZeneca reported that GPR55 receptors expressed by HEK293 cells were coupled to G_i or G_s (Drmoto *et al.*, 2004), yet the patent published by GlaxoSmithKline reported that GPR55 receptors expressed by yeast coupled to $G_{\alpha 12}$ or $G_{\alpha 13}$ (Brown and Wise, 2001).

A study investigating the G-protein coupling of GPR55 receptors was performed by (Ryberg *et al.*, 2007). The authors have reported that membranes from HEK293 cells expressing GPR55 receptors were still able to respond to GPR55 receptor agonists after treatment with pertussis toxin. Although the data was not shown, the authors suggest G_i G-proteins were not involved in GPR55 receptor signalling (Ryberg *et al.*, 2007). Using FLIPR, GPR55-expressing HEK293 cells were tested for calcium signalling, indicative of G_q coupling: no agonist-induced calcium signalling was detected. To investigate other G-protein coupling mechanisms, peptides corresponding to the last 112 amino acids for $G_{\alpha 1/2}$, $G_{\alpha 3}$, $G_{\alpha s}$, $G_{\alpha 13}$ were incubated with GPR55 receptor-expressing membranes for 15 minutes prior to GTP γ S assays. $G_{\alpha 1/2}$, $G_{\alpha 3}$, and $G_{\alpha s}$ failed to have an effect upon GTP γ S binding, consistent with the lack of effect from pertussis toxin. However, the peptide corresponding to $G_{\alpha 13}$ concentration dependently inhibited GTP γ S binding suggesting GPR55 receptor interacts with $G_{\alpha 13}$ specifically. For verification, antibodies were raised against the C-terminal peptides of the different G-proteins. Consistent with the peptide experiments, anti- $G_{\alpha 13}$ prevented GTP γ S binding concentration-dependently; which may support the earlier experiments implicating a role of $G_{\alpha 13}$ in GPR55 receptor activation (Figure 1.5) (Ryberg *et al.*, 2007).

Activation of GPR55 receptors expressed by HEK293 cells and the epithelial cell line EA.HY926 resulted in an increase in intracellular calcium release from inositol trispohosphate stores thought to be mediated by GPR55 coupling to $G_{\alpha q}$ (Waldeck-Weiermair *et al.*, 2008, Lauckner *et al.*, 2008). This research contributes to the understanding of GPR55 receptor signalling in *in vitro*, although the mechanism/s of GPR55 signalling has yet to be confirmed in native GPR55 receptors.

Section 1.2.7 Expression of GPR55 receptors

The expression of GPR55 receptor mRNA has been reported in the caudate nucleus and putamen (Ryberg *et al.*, 2007) but not in the hippocampus, thalamus, pons, cerebellum or frontal cortex of the brain (Sawzdargo *et al.*, 1999). In the periphery, GPR55 receptors are found in the ileum, spleen, tonsil, testis (Brown and Wise, 2001), on osteoclasts generated from bone marrow macrophages (Whyte *et al.*, 2009), adipose tissue and breast tissue (Drmota *et al.*, 2004), but not in the liver (Sawzdargo *et al.*, 1999). The functional significance of this varied distribution is yet to be elucidated, however the recent identification of GPR55 receptor expression on dorsal root ganglion (DRG) neurons in the mouse (Lauckner *et al.*, 2008) has fuelled speculation that GPR55 receptors could play an important role in the regulation of neuropathic pain, supported by data using GPR55^{-/-} animals (Staton *et al.*, 2008).

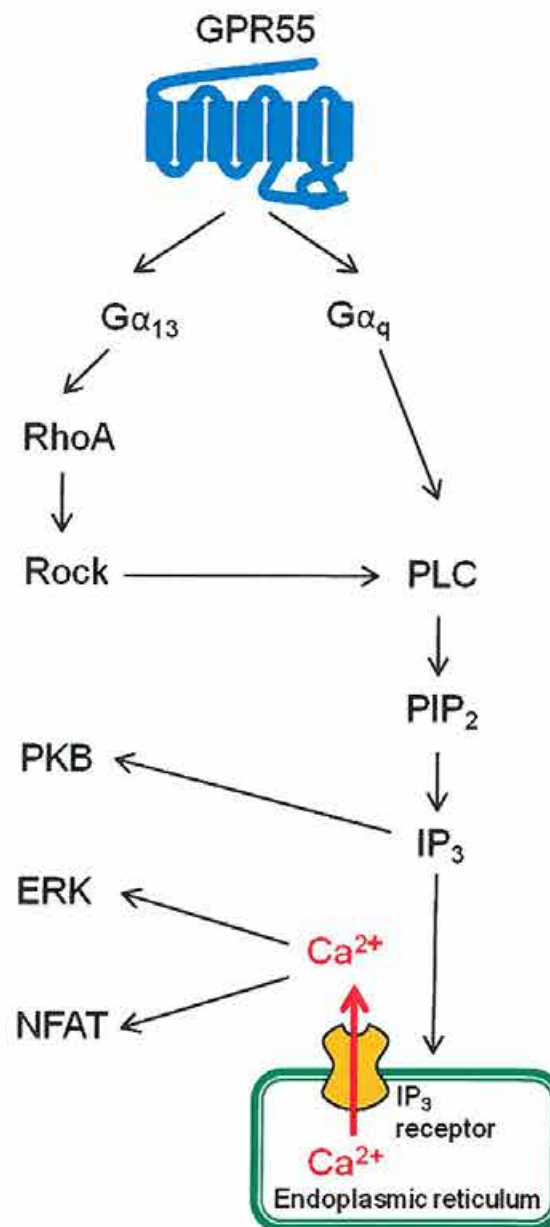


Figure 1.5: Downstream signalling cascades initiated by GPR55 receptor activation *in vitro*. Both $G_{\alpha 13}$ and $G_{\alpha q}$ have been implicated as the G protein responsible for the downstream effects of GPR55 receptor activation (Ryberg *et al.*, 2007, Oka *et al.*, 2010, Anavi-Goffer *et al.*, 2012, Waldeck-Weiermair *et al.*, 2008, Lauckner *et al.*, 2008). PLC, phospholipase C; PIP_2 , Phosphatidylinositol 4,5-bisphosphate; IP_3 ; inositol 1,4,5-trisphosphate; RhoA, Ras homolog gene family, member A; Rock, Rho-associated protein kinase; PKB, Protein Kinase B; ERK, extracellular-signal-regulated kinases; NFAT, Nuclear factor of activated T-cells.

Section 1.2.8 Pharmacology and function of GPR55 receptors

Two patents filed by GlaxoSmithKline (Brown and Wise, 2001) and AstraZeneca (Drmota *et al.*, 2004) independently reported cannabinoid activity at recombinant GPR55 receptors. The CB₁ receptor inverse agonist AM251 also acts as an agonist at GPR55 receptors (Brown and Wise, 2001). Δ^9 -THC, anandamide (AEA), arachidonoylglycerol (2-AG) and palmitoylethanolamide (PEA) are also potent agonists at GPR55 receptors, abnormal-cannabidiol (abn-cbd) produced only a weak activation of GPR55 receptors and the CB₂ receptor agonist JWH-133 and the CB₁/CB₂ receptor agonist WIN-55212-2 failed to stimulate GPR55 in yeast (Drmota *et al.*, 2004). In contrast, further research from GlaxoSmithKline reported that GPR55, when expressed in HEK293 cells, is activated by low nanomolar concentration of abn-cbd and the CBD analogue O1602 (Johns *et al.*, 2007). Many reports support the notion that the GPR55 receptor is a novel receptor for cannabinoid analogues, with a distinct pharmacological profile to that of CB₁ and CB₂ cannabinoid receptors (Tables 1.3 and 1.4).

Although GPR55 has been identified in vascular smooth muscle (Baker *et al.*, 2006) through their work using GPR55^{-/-} mice, Johns *et al.*, have demonstrated that GPR55 does not mediate the vascular responses of abn-cbd in the mouse mesenteric artery, despite being a potent agonist at GPR55 expressed in recombinant systems (Johns *et al.*, 2007). Even taking species differences into account, it seems unlikely that GPR55 is the endothelial anandamide receptor identified in the rat mesenteric artery. GPR55 is also distinct from the putative non-CB₁ hippocampal cannabinoid receptor, both in expression and G-protein coupling. Whether the putative endothelial

anandamide receptor and the non-CB₁ hippocampal cannabinoid receptor are the same class of cannabinoid receptor is also yet to be elucidated.

Ligand	GPR55 EC50 (nM)	GPR55 E _{max} (%)	CB ₁ EC50 (nM)	CB ₁ E _{max} (%)	CB ₂ EC50 (nM)	CB ₂ E _{max} (%)
Anandamide	18 ± 3	73 ± 5	31 ± 6	66 ± 4	27 ± 6	58 ± 5
Δ ⁹ THC	8 ± 1	92 ± 5	6 ± 1	61 ± 5	0.4 ± 0.1	67 ± 3
CBD	antagonist		>30,000		>30,000	
AM251	39 ± 3	88 ± 4	antagonist		antagonist	
O1602	13 ± 2	99 ± 4	>30,000		>30,000	

Table 1.3: Pharmacological profile of cannabinoid agonists at GPR55 receptors, CB₁ and CB₂ cannabinoid receptors. Cannabinoid ligands were assessed for their ability to stimulate GTPyS binding in HEK293 cells expressing GPR55 receptors (Ryberg *et al.*, 2007).

Ligand	GPR55 IC50 (nM)	CB ₁ IC50 (nM)	CB ₂ IC50 (nM)
CBD	445 ± 67	>30,000	>30,000
AM251	agonist	8 ± 1	2915 ± 102

Table 1.4: Pharmacological profile of cannabinoid antagonists at GPR55 receptors, CB₁ and CB₂ cannabinoid receptors. Cannabinoid ligands were assessed for their ability to inhibit CP55940-induced increases in GTPyS binding in HEK293 cells expressing GPR55 receptors (Ryberg *et al.*, 2007).

The bioactive lipid lysophosphatidylinositol (LPI) has been identified as an endogenous agonist at GPR55 receptors expressed by HEK293 cells (Oka *et al.*, 2007). Treatment of HEK293 cells, stably expressing GPR55 receptors, with LPI induced GPR55 receptor internalisation and a sustained oscillatory calcium release resulting in activation of nuclear factor of activated T cells (NFAT) (Henstridge *et al.*, 2009).

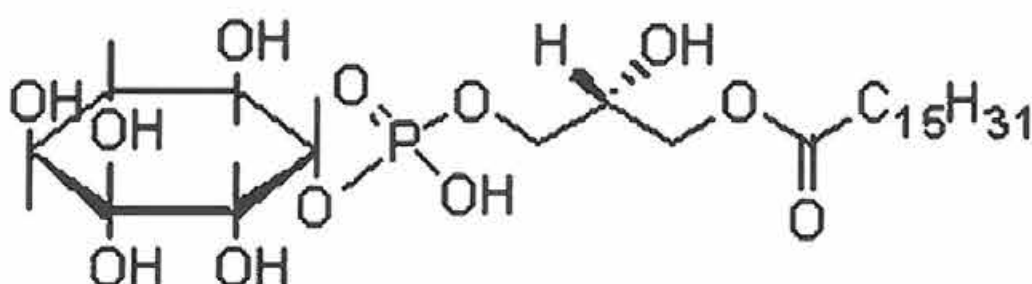


Figure 1.6: Chemical structure of lysophosphatidylinositol (Cho *et al.*, 2008).

Osteoclasts (generated from bone marrow macrophages) have been shown to express the GPR55 receptor (Whyte *et al.*, 2009). Treatment with GPR55 ligands LPI and O1602 resulted in inhibition of mouse osteoclast formation, an effect which was evident in both CB₁^{-/-} and CB₂^{-/-} knockout but not GPR55^{-/-} knockout mice. Furthermore, male GPR55^{-/-} mice show increased bone density compared to male wild-type animals (Whyte *et al.*, 2009). Several studies have reported GPR55 receptor signalling may be important in the proliferation of cancer cells (Hu *et al.*, 2011, Pineiro *et al.*, 2011), the authors of one report even suggested GPR55 receptor expression be used as a biomarker for malignancy (Pineiro *et al.*, 2011).

Section 1.3 The immune system

The immune system is the body's internal defence system against infection and uncontrolled cell division. It is dependent upon the function of several organs and immune cells, which communicate through direct cell-cell contact and soluble molecules such as cytokines and chemokines. Understanding the immune system has meant better treatment of autoimmune conditions and organ rejection following transplantation and led to the development of immunisation programmes.

The innate immune system is rapidly activated upon recognition of common constituents of infectious pathogens such as proteins or lipid structures, termed pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). Upon binding of PAMPs to receptors expressed by macrophages, activated macrophages secrete cytokines and chemokines, causing the recruitment of other neutrophils and monocytes from the blood stream. The inflammatory response is also aided by the activation of complement on the bacterial cell surface. Complement activates a cascade of proteolytic reactions which amplify the inflammatory response (Zhang *et al.*, 2009), in turn causing the dilation and increased permeability of local blood vessels, recruiting more neutrophils and monocytes to the site of infection.

Although slower than an innate immune response, adaptive immunity tends to be a more effective defence, which improves upon repeated exposure to the same pathogen, known as immunological memory (Welsh *et al.*, 2004). Adaptive immune responses involve the activation and differentiation of antigen specific CD4⁺ or CD8⁺ T cells. CD8⁺ T cells recognise class I major histocompatibility (MHC) antigens on the surface of our own cells, whereas CD4⁺ T cells recognise MHC class II

presented to them by antigen presenting cells (APCs). As such, CD8+ T cells are responsible for the removal of virally infected cells and CD4+ T cells destroy bacterial, fungal or parasitic pathogens. The adaptive immune system must be able to discriminate between 'self' and 'non-self' antigens. Self-tolerance is important for the prevention of autoimmune disorders and is achieved by several mechanisms: 1) clonal deletion of self-reactive T cells in the thymus; 2) the generation of naturally occurring, and inducible regulatory T cells; and 3) T cell anergy whereby T cells only proliferate upon stimulation of the T cell receptor (Sakaguchi, 2004).

Section 1.3.1 T cell development

Lymphoid progenitor cells migrate into the corticomedullary junction of the thymus (Lind *et al.*, 2001) attracted by chemokines such as stromal cell-derived factor (SDF α) released by epithelial cells lining the thymic cortex (Takahama, 2006). Here, T cell receptor (TCR) genes in double negative (DN) thymocytes rearrange to create two distinct lineages: $\gamma\delta$ and $\alpha\beta$ T cells (Robey and Fowlkes, 1994). For the development of $\alpha\beta$ T cells, signalling through the $\alpha\beta$ TCR switches off $\gamma\delta$ genes, therefore committing itself to the $\alpha\beta$ T cell lineage. DN thymocytes rearrange the β -chain locus and begin to express the pre-TCR- α (von Boehmer and Fehling, 1997). The β chain of CD25 assembles with the α -chain (pre-T-cell- α) to form a pre-TCR ($\beta:pT\alpha$) (Aifantis *et al.*, 1999). Expression of the pre-TCR is then lost, resulting in low-level surface expression of mature $\alpha\beta$ -TCR assembled with CD3 protein (van Oers *et al.*, 1995). DN thymocytes begin to express CD4 and CD8 becoming double positive (DP) thymocytes (Robey and Fowlkes, 1994). Subsequently, DP thymocytes encounter self-major histocompatibility complex (MHC) molecules on cortical thymic epithelial cells (cTEC; Figure 1.7) in the thymic cortex (Bousso *et al.*, 2002). DP

thymocytes failing to recognise self-MHC are destined for apoptosis by neglect, whilst those recognising self-MHC too effectively undergo apoptosis as these lymphocytes are deemed self-reactive and could contribute to autoimmune disorders. This process is known as negative selection. Of the DP thymocytes remaining (positively selected thymocytes which have an appropriate level of MHC recognition) those recognising MHC class I are destined to become 'cytotoxic' CD8⁺ T cells and those which recognise MHC class II become 'helper' CD4⁺ T cells (Teh *et al.*, 1988). It is at this stage that DP thymocytes cease to express either CD4 or CD8 respectively, becoming single positive (SP) thymocytes. Upregulation of CCR7 by SP thymocytes allows them to relocate to the medulla attracted by CCR7 ligand released by medullary thymic epithelial cells (mTEC) (Ueno *et al.*, 2004). In the medulla, SP thymocytes undergo another round of selection by interaction with the autoimmune regulator AIRE. Expression of AIRE by mTEC allows self-reactive T cells to be eliminated by negative selection, ensuring T cells are tolerant of self-antigen in the periphery (Anderson *et al.*, 2005).

A mere 2 % of DP thymocytes complete positive and negative selection to become exported from the thymus to create peripheral CD4⁺ and CD8⁺ T cells (Egerton *et al.*, 1990).

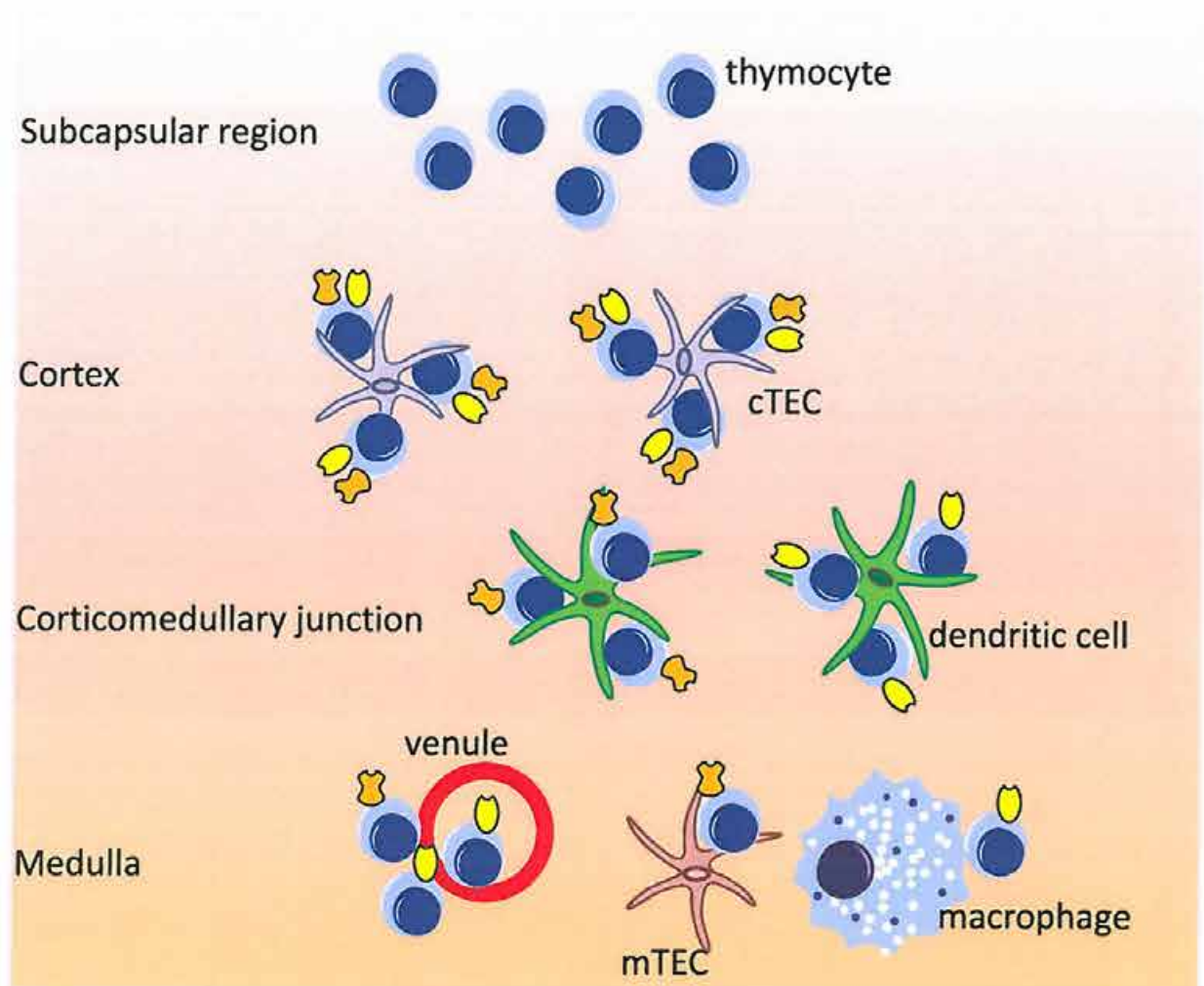




Figure 1.7: Developing thymocytes are found in distinct parts of the thymus. Immature DN thymocytes are localised to the subcapsular region. As they mature, thymocytes migrate through the thymus to the cortex; mature SP T cells leave the thymus via the circulation (CD4: , CD8: ).

Section 1.3.2 CD4+ T helper cell subsets

Section 1.3.2.1 Regulatory CD4+ T cells

CD4+ T cells constitutively expressing the surface marker CD25 are thought to mediate the suppressive qualities required for the maintenance of self-tolerance in the periphery and are therefore known as regulatory T cells (Itoh *et al.*, 1999). Sakaguchi *et al.* first showed that CD4+CD25+ T cells make up 5-10 % of CD4+ T cells and could prevent susceptibility to autoimmune disease (Sakaguchi and Sakaguchi, 2005, Thompson and Powrie, 2004), which is supported by studies showing the severity of autoimmune disorders is correlated to a reduction in the number of regulatory T cells (Barath *et al.*, 2007). Regulatory T cells are anergic to stimulation yet are able to suppress proliferation and production of IL-2 from activated CD4+ T cells in a contact-dependent manner (Takahashi *et al.*, 1998) although suppression of T cell responses are independent of antigen specificity (Marie *et al.*, 2005). CD25 is a component of the α -chain of the IL-2 receptor, and hence CD25 expression is essential for the correct function of regulatory T cells due to their dependency upon exogenous IL-2 for survival *in vivo* (Setoguchi *et al.*, 2005). Regulatory T cells are now more commonly defined by the expression of the intracellular transcription factor FoxP3.

The importance of FoxP3 was clarified in 2001 when it was shown that FoxP3 regulated T cell activation (Schubert *et al.*, 2001) and that FoxP3 expression is required for the development and function of naturally occurring regulatory T cells (Bettelli *et al.*, 2005, Fontenot *et al.*, 2005). FoxP3 expression can be triggered by TGF- β in activated CD4+CD25- T cells (Fu *et al.*, 2004), generating adaptive CD4+CD25+FoxP3+ regulatory T cells with suppressive properties similar to natural

regulatory T cells generated in the thymus (Tang and Bluestone, 2008). This inducible population of regulatory T cell is thought to be important in the clearance of an immune response after an infection, possibly to prevent secondary autoimmunity and inflammation (Figure 1.9). A genetic deficiency of FoxP3 in human causes the condition Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) which is characterised by neonatal onset of severe autoimmune disorders such as diabetes, eczema and hypothyroidism (Powell *et al.*, 1982). An *in vivo* model of the FoxP3 deficiency has been developed and as such, 'scurfy' mice have become an invaluable tool used in the research of regulatory T cells (Godfrey *et al.*, 1991, Bennett *et al.*, 2001).

Section 1.3.2.2 Mechanisms of regulatory T cell suppression

Since the discovery of regulatory T cells, much work has been achieved to identify the mechanisms by which regulatory T cells suppress activated immune cells. Experiments using transwell culture plates have shown that, certainly *in vitro*, regulatory T cell mediated suppression requires cell-cell contact (Thornton and Shevach, 1998). Regulatory T cell-mediated suppression declines in an age-dependent manner, although there are no gender-related differences in the suppressive capacity of regulatory T cells (Tsaknaridis *et al.*, 2003). The plethora of suppressive mechanisms exploited by regulatory T cells highlights the importance of their role in controlling immune responses. Direct and indirect mechanisms of suppression are characterised, although none of the mechanisms identified thus far has been shown to be completely essential for mediating suppression (Figure 1.8).

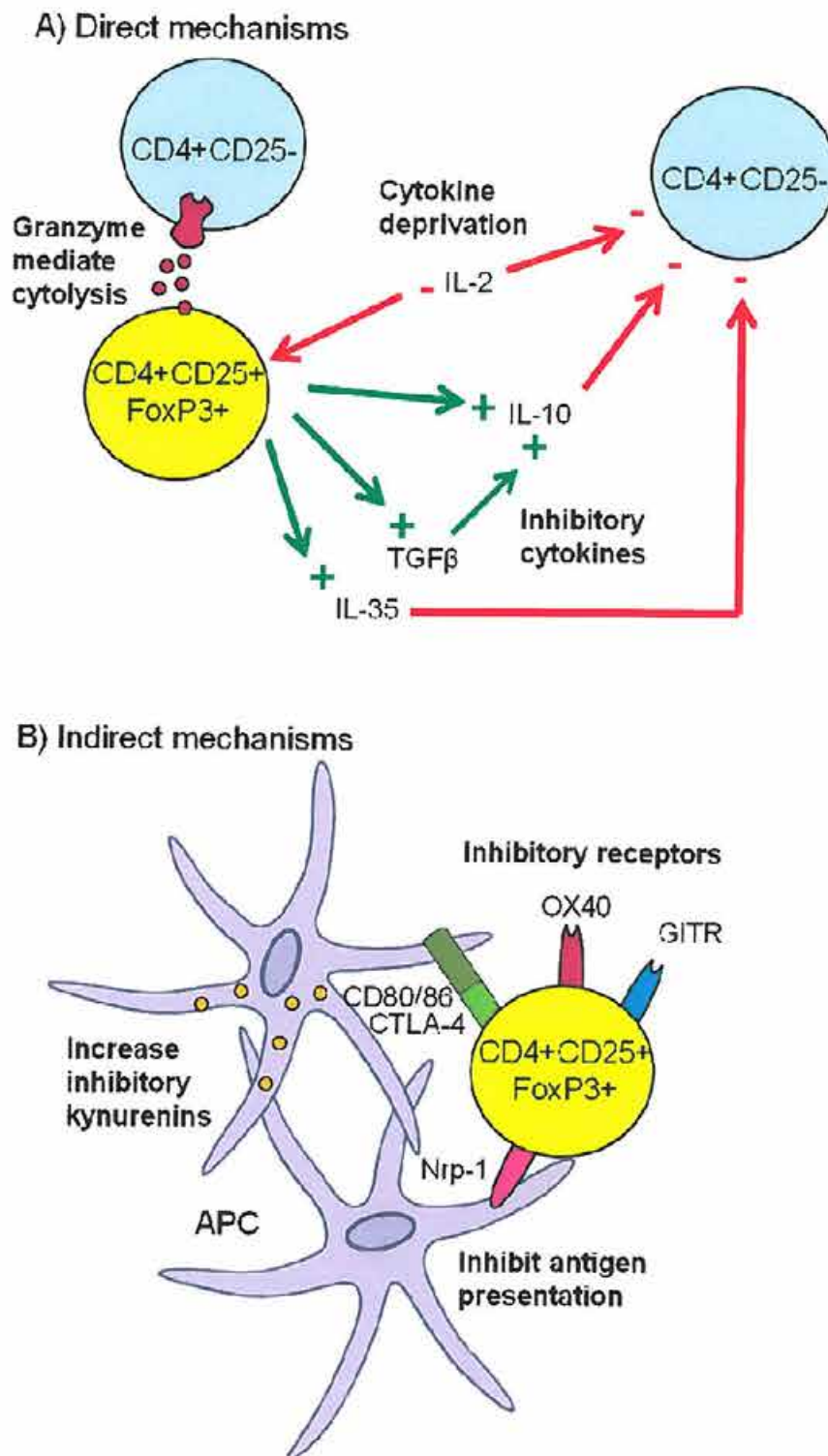


Figure 1.8: Schematic diagram of the strategies employed by regulatory T cells to mediate suppression of activated immune cells. Human CD4+CD25+FoxP3 regulatory T cells mediate direct suppression by A) cytokine deprivation, granzyme mediated cytotoxicity and inhibitory cytokines and B) indirect suppression by inhibitory receptors, increased inhibitory kynurenins via the breakdown of tryptophan by IDO and by shielding APCs from effector T cells.

Direct mechanisms of suppression

Regulatory T cells release inhibitory cytokines including IL-10, IL-35 and TGF- β which can directly inhibit activation of effector T cells. IL-10 released from activated regulatory T cells inhibits the release of TNF- α from macrophages and limiting the expression of MHC class II by dendritic cells (Moore *et al.*, 2001). IL-10 itself enhances the production of IL-10 by regulatory T cells (Barrat *et al.*, 2002). IL-35 is a member of the IL-12 family of cytokines and is a heterodimer of Epstein-Barr virus-induced gene 3 (*Ebi3*) and the p35 subunit of IL-12. IL-35 is preferentially expressed by CD4⁺CD25⁺FoxP3⁺ T cells, possibly a result of *Ebi3* being a downstream target for FoxP3. The contribution of IL-35 to the suppressive nature of regulatory T cells has been described by Collison *et al.*, who observed that IL-35 expression by HEK293 cells resulted in the suppression of CD4⁺CD25⁻ T cell proliferation (Collison *et al.*, 2007). TGF- β exerts its immunosuppressive effects by inhibiting IL-2 production by immune cells (Kehrl *et al.*, 1986) and stimulating the production of IL-10 by CD4⁺ regulatory T cells (Kitani *et al.*, 2003).

Regulatory T cells mediate cytotoxicity by a perforin-dependent mechanism (Grossman *et al.*, 2004a); natural, and adaptive regulatory T cells predominantly express granzyme A and granzyme B respectively (Grossman *et al.*, 2004b). Granzymes are serine proteases which enter the target cell through perforin pores and initiate apoptosis of the target cell (Bots and Medema, 2006).

The concept of IL-2-deprivation-mediated apoptosis is a controversial mechanism of regulatory T cell-mediated suppression. The gene for IL-2 was the first T cell growth factor to be molecularly cloned (Smith, 1988), and is commonly used for *in vitro*

culture of CD4+CD25+ T cells (Nelson, 2004) but is particularly important in the peripheral maintenance of CD4+CD25+ T cells *in vivo* (Malek *et al.*, 2002). A high level of IL-2 receptor chains (CD25, CD122 and CD132) are expressed by regulatory T cells which enables secreted IL-2 to be 'mopped up', reducing local IL-2 concentration during inflammation and effectively starving effector T cells of IL-2, limiting their proliferation (de la Rosa *et al.*, 2004). Studies have also shown that regulatory T cells can cause apoptosis by the absorption of other cytokines from cultures of CD4+CD25- T cells, which might describe a 'polyclonal' mechanism of suppression, rather than a 'clonal' deletion strategy (Pandiyan *et al.*, 2007).

Indirect mechanisms of suppression

Expression of inhibitory receptors such as CTLA-4, GITR and OX40 contribute to the suppressive function of regulatory T cells by suppressing antigen presentation.

Upon activation by antigenic stimulation, CTLA-4 expressed by regulatory T cells, transduces a negative signal which abrogates the regulatory T cell response resulting in autoimmune disease and inducing tumour immunity *in vivo*. Fallarino *et al.* have demonstrated that the interaction of CTLA-4 on regulatory T cells and CD80/86 on dendritic cells initiated the expression of IDO by dendritic cells (Fallarino *et al.*, 2003). IDO is the rate-limiting enzyme involved in the L-tryptophan-kynurenine pathway, converting the essential amino acid tryptamine to N-formylkynurenine which causes apoptosis of the target cell. Although constitutively expressed by activated regulatory T cells, CTLA-4 expression is not a pre-requisite for suppression. Regulatory T cells from CTLA-4^{-/-} mice also exhibit a normal suppressive response *in vitro* (Kataoka *et al.*, 2005).

Glucocorticoid-induced tumour necrosis factor (TNF) receptor-related protein (GITR, TNFSF18) and its ligand, GITR-L are thought to play an important role in the activity of regulatory T cells, in particular in their contribution to autoimmunity (Ji *et al.*, 2004). Polyclonal antibodies specific to GITR expressed by resting and activated CD4+CD25+ T cells and activated CD4+CD25- T cells, has been shown to abrogate the suppressive function of regulatory T cells in *in vitro*. The same group clarified this effect is mediated by CD4+CD25+, not CD4+CD25- T cells and depends upon pre-activation by the TCR (McHugh *et al.*, 2002). Experiments from another group have abrogated CD4+CD25+-mediated suppression by anti-GITR monoclonal antibody in CD4+ helper and CD8+ cytotoxic T cells. The authors also demonstrated that a depletion of GITR^{high} T cells induces autoimmune gastritis (Shimizu *et al.*, 2002).

OX40 is a member of the TNF family of receptors, expressed on T cells after activation (Gramaglia *et al.*, 1998). Mice deficient in OX40 or OX40-ligand are less susceptible to irritable bowel disease and allergic asthma (Jember *et al.*, 2001, Takeda *et al.*, 2004). Inhibition of suppression by regulatory T cells was achieved by agonistic antibodies to OX40, however anti-OX40 was unable to inhibit suppression mediated by pre-stimulated regulatory T cells, suggesting OX40 can only modulate suppression by naïve T cells (Valzasina *et al.*, 2005).

More recently it has been suggested that regulatory T cells can suppress activation of effector T cells by clustering around APCs which limits the opportunity for effector T cells to associate with the APC and become activated (Onishi *et al.*, 2008). To do this, the interaction between regulatory T cells and antigen presenting cells (APCs) must be enhanced and neuropilin (Nrp-1) has been implicated as mediating this effect on dendritic cells (Sarris *et al.*, 2008).

Section 1.3.2.3 Other CD4+ T helper cell subsets

Th1 helper T cells

Th1 cells are considered responsible for cell-mediated immunity. IFN- γ has been described as the principle cytokine of Th1 cells. IFN- γ activated macrophages and stimulates the production of IgG (London *et al.*, 1998). Cytokines released by Th1 T cells promote the differentiation of CD8+ T cells and stimulate neutrophils and NK cells (Figure 1.9). These actions are largely pro-inflammatory uncontrolled Th1-mediated responses may be involved in the onset of autoimmune disorders.

Th2 helper T cells

Cytokines produced by Th2 T cells such as IL-4 and IL-5 stimulate the production of IgE and the differentiation of eosinophils therefore primarily mediate the immune response to parasitic infections (MacDonald *et al.*, 2002). Th2 cells also produce and secrete IgM, providing extra assistance for B cell-mediated immunity.

Th17 helper T cells

Th17 cells were first defined in 2005 (Park *et al.*, 2005), and were named according to their hallmark cytokine IL-17, although the Th17 subset release other proinflammatory cytokines, including but not limited to IL-17F, IL-21, IL-22 and TNF- α (Figure 1.9) (Kaiko *et al.*, 2008, Dong, 2008).

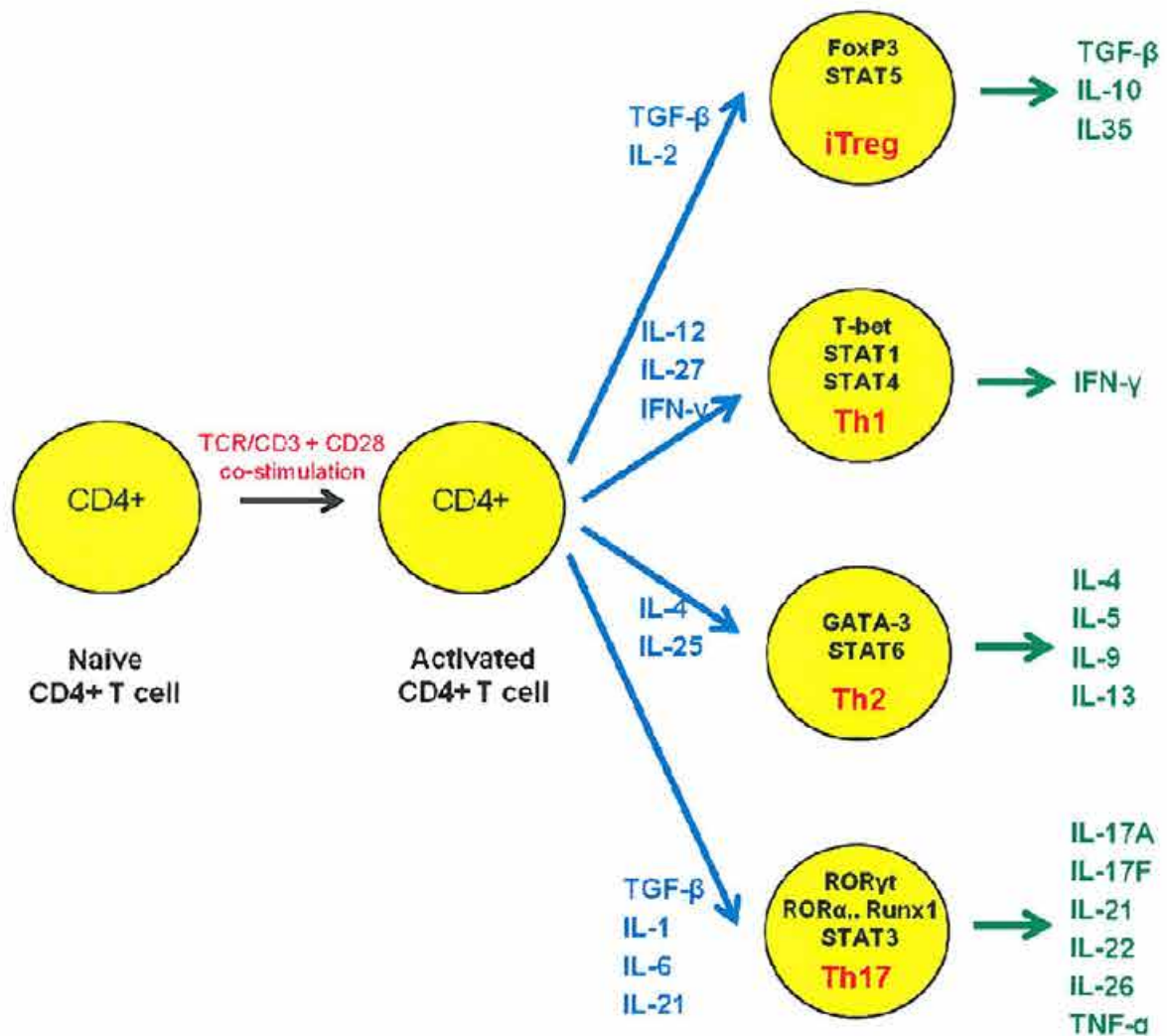


Figure 1.9: Summary of the distinguishing features of Treg, Th1, Th2 and Th17 T helper cell subsets (Dong, 2008, Brand, 2009) Cytokines influencing the development of CD4⁺ helper subsets are shown in blue, effector cytokines are shown in green. FoxP3, forkhead box P3; GATA, GATA binding protein; Runx, runt-related transcription factor; STAT, signal transducer and activator of transcription; T-bet, T box expressed in T cells.

Section 1.3.2.4 CD4+ helper T cell subsets and disease

Autoimmune diseases affect approximately 5 % of people in the Western world (Bach, 2003), and although more common in women, males are more severely affected. Although it still remains unclear precisely why, it is thought to be related to gender-related differences in levels of steroid hormones (Whitacre *et al.*, 1999). Traditionally, autoimmune disorders have been treated with immunosuppressant drugs. Not only does this treatment require continual use, patients have an increased susceptibility to infection and in particular a reduced ability to recognise tumour growth (Yang and Yang, 2005). Some studies have shown some immunosuppressant drugs such as cyclosporine can increase cell division, thus stimulating tumour cell growth (Freise *et al.*, 1999).

There are many studies which report a reduced capacity of CD4+ regulatory T cells isolated from patients with autoimmune conditions to suppress the proliferation of CD4+ effector T cells (Lindley *et al.*, 2005, Sugiyama *et al.*, 2005, Balandina *et al.*, 2005, Viglietta *et al.*, 2004). Besides autoimmune conditions, studies have investigated the role of CD4+ regulatory T cells in neurodegeneration. Depletion of regulatory T cells was shown to improve neuronal survival following an optic nerve crush injury (Kipnis *et al.*, 2002), suggesting that regulatory T cells help to sustain the balance between autoimmunity required for neuroprotection and repair, and excess autoimmunity contributing to disease. In an animal model of Parkinson's disease, adoptive transfer of CD3-activated CD4+CD25+ regulatory T cells attenuated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced microglial inflammatory responses (Reynolds *et al.*, 2007). Neurotoxic responses to α -synuclein were reduced, namely production of ROS and NO by microglia, which contribute to

oxidative stress (Reynolds *et al.*, 2007). Most remarkably, the transfer of just 3.5×10^6 regulatory T cells was sufficient to prevent the loss of approximately 90 % of the dopaminergic cell bodies (Reynolds *et al.*, 2007). Similar results from the adoptive transfer of regulatory T cells in a murine model of HIV-1-associated neurodegeneration have been documented (Liu *et al.*, 2009). Importantly in both studies, CD4+CD25⁻ effector T cells were ineffective compared to CD4+CD25⁺ regulatory T cells in mediating neuroprotection, highlighting the importance of regulatory T cells in attenuating neuroinflammation and their potential therapeutic use in adoptive transfer techniques.

Th1 and Th2 cell lineages represent polarised subsets of a heterogeneous CD4⁺ T helper immune response. Th1 and Th2 cells regulate each other's development in order to maintain a balanced and well controlled immune response. Crohn's disease and ulcerative colitis are two divisions of inflammatory bowel disease (Brand, 2009). Increased levels of Th1 cytokines (IFN- γ and IL-12) are found in Crohn's disease compared to an increase in Th2 cytokines (IL-13) found in ulcerative colitis. (Parronchi *et al.*, 1997, Berrebi *et al.*, 1998, Heller *et al.*, 2005). Clearance of helminth infections is regulated by a Th2 response (MacDonald *et al.*, 2002) whereas infections caused by bacteria such as *helicobacter pylori* are primarily controlled by Th1 immune responses (Lohoff *et al.*, 2000).

An increase in the expression of Th1 cytokines has been reported in lupus, lymphoma and psoriasis patients (Kagami *et al.*, 2010, Calvani *et al.*, 2005, Riedel *et al.*, 2001). Th2 immune responses are responsible for the formation of allergic responses to inert allergens and the pathophysiology of asthma (Colavita *et al.*, 2000). Allergy affects approximately 1 in 5 adults in the UK (Lee, 2003) therefore

represents a huge target market for the development of pharmacotherapies however the clinical effectiveness of anti-Th2 therapies has so far proved limited (Bosco *et al.*, 2006).

Th17 cells have been implicated in the pathophysiology of various inflammatory diseases such as rheumatoid arthritis, asthma, systemic lupus erythematosus, multiple sclerosis and Crohn's disease due to the increased expression of IL-17 mRNA or protein in tissues from patients (Spolski and Leonard, 2008, Kolls and Linden, 2004). An increased frequency of Th17 cells have been found in the gut of Crohn's disease patients (Annunziato *et al.*, 2007). IL-17 neutralising antibodies have been shown to improve disease severity in psoriasis and are the subject of ongoing clinical trials (Hueber *et al.*, 2010, Papp *et al.*, 2012b, Papp *et al.*, 2012a, Leonardi *et al.*, 2012).

Section 1.3.3 Markers of immune cell activation

Lymphocyte activation is a complex and integrated process. Physiological activation of the T cell receptor (TCR)/CD3 complex and co-stimulatory molecules such as CD28 cause protein kinase C activation and an increase in intracellular calcium. *In vitro* this process can be mimicked by CD3/CD38 activation beads. The cascade of events which leads to the upregulation of surface markers and cytokine production is well controlled. Several surface markers have been identified as indicators of lymphocyte activation, including CD25; others include CD69, CD71 and CD27 (Ferenczi *et al.*, 2000, Reddy *et al.*, 2004).

CD69 is one of the earliest activation cell surface markers expressed by activated leukocytes (Sancho *et al.*, 2005, Testi *et al.*, 1994), once presumed to imply, similar to CD25 expression, that CD69 had immunostimulatory properties, stimulating the release of IL-2 by T cells (Testi *et al.*, 1989). Once expressed, CD69 acts as a co-stimulatory molecule for T cell activation and proliferation (Ziegler *et al.*, 1994). More recently, CD69 expression has been associated with immune regulation *via* its role in the release of TGF- β . CD69^{-/-} mice develop a more severe form of collagen-induced arthritis, and T and B cells become hyper-responsive to collagen correlating with a reduction of TGF- β in inflamed joints (Sancho *et al.*, 2003). In tumour-bearing mice, the percentage of CD4+CD69+ T cells was found to be increased (Han *et al.*, 2009). Following subsequent analysis, it was shown that CD4+CD69+ T cells are potent inhibitors of T cell proliferation both *in vitro*, and *in vivo* mediated by membrane bound TGF- β 1, despite expressing very little CD25 and FoxP3 (Han *et al.*, 2009).

CD71 (transferrin receptor 1) is required for homeostasis of $[\text{Fe}^{3+}]_i$, and is expressed by all nucleated cells (Brekelmans *et al.*, 1994). CD71 expression is upregulated by activated lymphocytes (Salmeron *et al.*, 1995). Expression levels of CD71 remain elevated 72 hours post stimulation (Reddy *et al.*, 2004) highlighting the dependency of proliferating lymphocytes on Fe^{3+} (Lum *et al.*, 1986).

CD27 is a member of the TNF family, where transient expression is upregulated upon TCR activation (Hintzen *et al.*, 1994). Expression of CD27 in CD4+CD25+ regulatory T cells, has been associated with a highly suppressive phenotype, compared to CD4+CD25+CD27- regulatory T cells (Koenen *et al.*, 2005). Identification of enriched expression of FoxP3 in CD4+CD25+CD27+ T cells compared to their CD27- counterparts (Ruprecht *et al.*, 2005) have made the expression of CD27 a useful tool for the distinction between naturally occurring regulatory T cells, and activated effector T cells (Mack *et al.*, 2009).

Section 1.4 Neurotransmitters in the immune system

Immune cells have been shown to express a plethora of neurotransmitter receptors and transporters suggesting synaptic neurotransmission may also occur outside the blood brain barrier, which can directly affect immune cells. The close proximity of immune cells to each other may imply that the function of neurotransmitters in immune function may also result from volumic neurotransmission, where neurotransmitters act at extra-synaptic receptors. Similar to neuronal synapses, immune synapses also require the engagement of cell surface receptors to provide a stable link in order to facilitate effective secretion of cytokine or neurotransmitter. Recent research suggests common scaffolding proteins and signalling molecules are involved in the formation of both immune and neural synapses (Yamada and Nelson, 2007).

Neural input contributes to the clinical symptoms of a local acute-phase inflammatory response such as mild pain and reddening of the skin (Sternberg, 2006). Initiation of systemic acute-phase responses such as fever are mediated by the hypothalamus (Shibata, 1990). Sympathetic nerve fibres are found around blood vessels innervating immune tissues as well as adjacent to lymphocytes in the white pulp of the spleen (Felten and Olschowka, 1987). Heightened sympathetic nerve activity during stress or injury, can inhibit the synthesis of tumour necrosis factor (TNF) (Molina, 2001), and the concomitant release of adrenaline and noradrenaline stimulate release of the anti-inflammatory cytokine IL-10 from macrophages (van der Poll *et al.*, 1996).

In 1995, criteria were established to determine whether or not a neurotransmitter is capable of a neuroimmune interaction: 1) the association of neurotransmitter-specific nerve terminals within primary or secondary lymphoid tissue; 2) the release of a neurally derived substance and its availability to immune cells; 3) is the expression of specific neurotransmitter receptors on immune cells; and 4) the identification of immunomodulatory effects of the neurotransmitter. These criteria have been met for several neurotransmitters of the autonomic nervous system including acetylcholine noradrenaline and substance P, among others (Ader *et al.*, 1995, Borovikova *et al.*, 2000).

In the last few years, much attention has been paid to the immunomodulatory effects of acetylcholine (Kawashima and Fujii, 2004). It has been reported that acetylcholine dose-dependently inhibited the release of proinflammatory cytokines (TNF, IL-1 β , IL-6 and IL-18) from lipopolysaccharide-stimulated macrophage cultures, which was primarily mediated by α -bungarotoxin-sensitive nicotinic acetylcholine receptors (Borovikova *et al.*, 2000). The authors also reported serum TNF is significantly increased in vagotomised rats (Borovikova *et al.*, 2000) suggesting that stimulation of the efferent vagus nerve is involved in the regulation of TNF production and hence the phrase 'cholinergic anti-inflammatory pathway' was coined (Pavlov and Tracey, 2006). Further data from this group have specifically implicated the $\alpha 7$ subunit of the nicotinic acetylcholine receptor as a key regulator of TNF release, mediated by the vagus nerve during systemic inflammation (Daly *et al.*, 2010).

Section 1.4.1 The role of 5-HT in the immune system

The availability of 5-HT to immune cells is thought to come primarily from platelets (Mossner and Lesch, 1998). Immune cells near the gut mucosa would be open to 5-HT released from enterochromaffin cells (Yang and Lackner, 2004). 5-HT may also originate from mast cells (Kushnir-Sukhov *et al.*, 2007) located within the vascular system and those innervated by noradrenergic nerve terminals in the thymus (Artico *et al.*, 2002).

In experimental animal studies, the immune response can be assessed using an immunisation of sheep red blood cells (SRBC). T cells which recognise and encompass the SRBC are known as rosette-forming cells (RFC) and the number of plaque-forming cells (PFC) becomes an indicator of IgM-producing cells. There are many *in vivo* reports of experimentally-induced stress mediating immunosuppression (Villasenor-Garcia *et al.*, 2001, Zhang *et al.*, 1998). Due to the involvement of 5-HT in the pathophysiology of anxiety and stress disorders (Ressler and Nemeroff, 2000), the role of 5-HT in immunosuppression has been investigated by measuring the immune response to SRBC during stress. P-chlorophenylalanine (pCPA), an inhibitor of tryptophan hydroxylase, which therefore inhibits the rate-limiting step in the production of 5-HT from its precursor tryptophan hydroxylase, has been used to deplete 5-HT in experimental models (Sjoerdsma *et al.*, 1970). Increased intensity of the immune response was seen in non-stressed animals treated with pCPA (the number of both PFC and RFC increased) consistent with the notion that 5-HT contributes to immunosuppression (Idova *et al.*, 1997). Stress-induced immunosuppression was not evident in stressed 5-HT-depleted mice compared to vehicle treated non-stressed controls (Idova *et al.*, 1997) suggesting either that the

lack of 5-HT immunosuppression was sufficient to reverse the effect of stress, or perhaps 5-HT itself was responsible for stress-induced immunosuppression evident in non-pCPA-treated rats.

5-HT has been shown to be important for optimal T cell activation. By using CD25 as a marker of activation, depletion of endogenous 5-HT in pCPA-treated mice resulted in a reduced percentage of CD4⁺CD25⁺ T cells at rest, whereas the CD8⁺ subset was unaffected, indicating a requirement of 5-HT for normal proliferation of T cells (Young and Matthews, 1995). Similarly, the percentage of T cells expressing CD25 in proliferated (concanavalin A-induced) splenocytes was significantly reduced (both CD4⁺ and CD8⁺ subsets), which was restored by exogenous 5-HT (Young and Matthews, 1995). In rats, inhibition of 5-HT synthesis during embryonic development caused by pCPA also caused a significant decrease in concanavalin A-induced proliferation of thymocytes from three-day old rats; however at 18 days old, proliferation of thymocytes was statistically increased compared to control animals (Afanas'eva *et al.*, 2009).

Pro-inflammatory cytokines such as IFN, TNF and IL-1 β are able to modulate the availability of 5-HT; by the upregulation of IDO expression (Taylor and Feng, 1991). As mentioned previously, IDO catalyses the conversion of L-tryptophan to N-formylkynurenine, hereby limiting the availability of L-tryptophan used in the production of 5-HT by tryptophan hydroxylase. Interestingly, there is an increased incidence of depression in patients undergoing IFN- α -based immunotherapy for the treatment of hepatitis C (Bonaccorso *et al.*, 2002), providing yet more evidence of the bidirectional effect of central 5-HT and inflammatory mediators. Limiting 5-HT production by induction of IDO in the immune system, may contribute to the control of

immune cell activation, preventing uncontrolled immune responses. Conversely, the anti-inflammatory cytokines IL-4 and IL-10 reduce IDO expression and therefore limit its metabolism of L-tryptophan (Chiarugi *et al.*, 2001).

In humans, expression of the 5-HT_{3A} receptor subunit has been identified in monocytes, and T cells, but not dendritic cells (Fiebich *et al.*, 2004b). Tropisetron, a 5-HT₃ receptor antagonist was shown to inhibit the release of TNF and IL-1 β from activated monocytes (Fiebich *et al.*, 2004a). Furthermore, IL-2 released from human PBMC was inhibited by tropisetron, which may have been responsible for a reduction in the proliferation of stimulated PBMC (de la Vega *et al.*, 2005). Interestingly, ondansetron was only partially as effective as tropisetron at inhibiting IL-2 release; granisetron was ineffective. Granisetron was however, effective in limiting the total number of accumulated leukocytes in an air-pouch model of inflammation (Maleki-Dizaji *et al.*, 2010).

Section 1.4.2 SERT expression and function in immune cells

At sites of inflammation, aggregated platelets release 5-HT, causing high local concentrations of up to 100 μ M 5-HT (Benedict *et al.*, 1986). Activated lymphocytes expressing functional SERT (Section 1.1.4) could act to reduce the amount of 5-HT available for further 5-HT receptor activation (Meredith *et al.*, 2005a). Research has shown SERT expressed by platelets is identical to brain-derived SERT (Lesch *et al.*, 1993), although SERT protein identified in several Burkitt's lymphoma B cell lines appears at approximately 70 kDa, slightly smaller than the 90 kDa neuronal SERT (Serafeim *et al.*, 2002). This discrepancy may be a result of posttranscriptional modifications, which have been reported previously (Qian *et al.*, 1995). Pro-inflammatory cytokines have been shown to upregulate SERT expression (Morikawa *et al.*, 1998, Ramamoorthy *et al.*, 1995). TNF- α has been shown to enhance SERT-mediated 5-HT reuptake in the JAR cell line (Mossner *et al.*, 1998). Therefore enhanced SERT function by pro-inflammatory cytokines released by immune cells during an immune response, may create a negative feedback loop important in regulating the increased concentration of 5-HT released from aggregated platelets or lymphocytes for example, during inflammation. SERT protein was expressed by Burkitt's lymphoma cells and as a consequence of 5-HT entering these cells, there was an increase in apoptosis which was subsequently reversed by the SSRI fluoxetine (Serafeim *et al.*, 2002). Further studies have suggested that the pro-apoptotic effect of SSRIs is independent of their ability to block SERT (Meredith *et al.*, 2005b, Schuster *et al.*, 2007). SERT is also present on macrophages (Rudd *et al.*, 2005) and dendritic cells (O'Connell *et al.*, 2006) which are involved in the initiation of an adaptive immune response by their specialised activation of naïve T cells. Maturation of dendritic cells is correlated to increased expression of SERT

cDNA whilst the opposite is true following signalling *via* the 'inhibitory' receptor CTLA-4 expressed by regulatory T cells (O'Connell *et al.*, 2006).

SERT can also be responsible for the alteration of downstream signalling pathways. Once intracellular, 5-HT can become covalently bonded to glutamine residues by the enzyme transglutaminase; a process known as serotonylation (Walther *et al.*, 2003). Serotonylation is important in the blood clotting process where serotonylated proteins bind to 5-HT binding sites on clotting factors, contributing to thrombus formation (Dale *et al.*, 2002). Lymphocytes express both SERT and transglutaminase (Novogrodsky *et al.*, 1978), therefore SERT-trafficked 5-HT has the potential to alter lymphocyte signalling by modifying small GTPases although much more investigation is needed to characterise this phenomenon in detail.

In rodents, mast cells are a plentiful source of 5-HT, yet in humans, SERT and tryptophan hydroxylase expression by mast cells is associated with disease (mastocytosis) (Morishima, 1970). However, human mast cells also express tryptophan hydroxylase allowing production of their own 5-HT, albeit at much lower concentrations than the mouse (Kushnir-Sukhov *et al.*, 2007), which could be responsible for the increased concentration of 5-HT in the plasma of mastocytosis patients (Kushnir-Sukhov *et al.*, 2008).

Section 1.4.3 Expression of cannabinoid receptors in lymphocytes

T cells have been shown to express both CB₁ and CB₂ receptors (Bouaboula *et al.*, 1993, Galiegue *et al.*, 1995, Cencioni *et al.*, 2010). CB₂ receptors are expressed at much higher levels than CB₁ receptors (Galiegue *et al.*, 1995), and it has been reported that there was no upregulation in protein expression of either receptor in CD3+ T cells upon activation (Cencioni *et al.*, 2010).

Research into the role of cannabinoids in immune function was initiated by the identification of highly specific cannabinoid binding sites expressed by mouse splenocytes (Kaminski *et al.*, 1992), which prompted further investigation of cannabinoid-induced immunomodulation. In 1993, the expression of cannabinoid receptor mRNA was detected in (in order of expression level from highest to lowest) human B cells, NK cells, neutrophils, CD8+ T cells, monocytes and CD4+ T cells, although at much lower levels than that found in the brain (Bouaboula *et al.*, 1993). Cloning of the second cannabinoid receptor was also achieved that same year (Munro *et al.*, 1993), and as a result the cannabinoid receptor identified by Bouaboula *et al.* to be expressed by human leukocytes is now known as the CB₁ receptor. Subsequent studies by the same research group identified expression of the CB₂ receptor in (in order of expression level from highest to lowest) human NK cells, B cells, macrophages, neutrophils, CD4+ T cells and CD8+ T cells, although in all samples, the CB₂ receptor was expressed at a greater level than the CB₁ receptor (Galiegue *et al.*, 1995). For immune organs, both CB₁ and CB₂ receptor mRNA transcripts have been identified in the spleen (CB₁ receptor at very low levels), and the CB₂ receptor was identified in the thymus, although at much lower levels than that found in the spleen (Galiegue *et al.*, 1995).

GPR55 receptors are expressed by human neutrophils (Balenga *et al.*, 2011). Activation of GPR55 receptors was shown to limit CB₂ receptor-mediated immune responses in neutrophils (degranulation and production of reactive oxygen species (ROS) (Balenga *et al.*, 2011). The atypical cannabinoid O1602, which has been shown to act as an agonist of GPR55 receptors (Ryberg *et al.*, 2007), was shown to inhibit neutrophil recruitment and subsequently protect from experimentally-induced colitis (Schicho *et al.*, 2011). Interestingly however, the action of O1602 was found to be independent from cannabinoid receptors, as protection from colitis was still evident in CB_{1/2}^{-/-} and GPR55^{-/-} mice (Schicho *et al.*, 2011).

Section 1.5 Hypothesis and aims

In evolutionary terms, the ancient 5-HT_{3A} receptor has been adopted for multiple roles. In the present study, I hypothesised that the 5-HT_{3A} receptor is expressed by human immune cells and implicated in the function of human immune cells. Expression will be investigated using biochemical and molecular biological techniques. Where evident, selective ligands for the 5-HT_{3A} receptor will be used to assess the impact of 5-HT_{3A} receptor activation on immune cells activity and function.

The modulatory role of cannabinoid receptors and endocannabinoids in immune function prompts speculation that the novel cannabinoid receptor GPR55 receptor may contribute to immune responses to antigenic stimulation. Lysophosphatidylinositol (LPI) and cannabidiol (CBD) will be used as a GPR55 receptor agonist and antagonist (respectively) in an attempt to elucidate a role for the GPR55 receptor in human PBMC.

CHAPTER 2

METHODS

Section 2.1 Preparation of immune cell subsets

Section 2.1.1 Preparation of peripheral blood mononuclear cells (PBMC)

Buffy coats (National Blood Service, Bristol UK), diluted 1:1 with PBS and loaded onto 20 ml Ficoll-Paque (GE Healthcare, Buckingham UK) were centrifuged at 1200 rcf for 25 minutes with no brake. Using a sterile pipette, PBMC were removed from the interface and washed several times in PBS. PBMC were counted using a haemocytometer and resuspended in supplemented RPMI to a concentration of $\sim 1 \times 10^6$ /ml. Freshly isolated human immune cells were maintained in RPMI-1640 (Roswell Park Memorial Institute) supplemented with 10 % heat-inactivated foetal calf serum (HI-FCS) and 1 % penicillin/streptomycin, this will be referred to as supplemented RPMI.

All cell culture was completed in a class 2, type A laminar flow cabinet (GELAIRE, BSB-4A), preventing contamination with bacteria, fungi and viruses. Media was warmed to 37°C in an incubator (Lab Impex Research) prior to use. All cells were maintained at 37°C in an atmosphere of 95 % air, 5 % CO₂, 100 % relative humidity (LEEC, Mk II Proportional Temperature Controller).

RPMI-1640 and penicillin/streptomycin were all purchased from Sigma (Dorset UK). HI-FCS was bought from Invitrogen (Paisley UK). Phosphate buffered saline (10x; PBS was bought from Severn Biotech Ltd (Kidderminster UK). Falcon tubes (15 ml and 50 ml) were purchased from Sarstedt (Leicester UK).

Section 2.1.2 Preparation of human CD4+CD25+ regulatory T cells from PBMC

Isolation of CD4+CD25+ T cells was achieved using the commercially available Dynabeads® Regulatory CD4+CD25+ T cell kit and a DynaMag™-15 magnet (Invitrogen, Paisley UK; Figure 2.1). PBMC ($\sim 1 \times 10^8$), resuspended in 500 μ l isolation buffer and 200 μ l FCS and 200 μ l Antibody Mix Human CD4 were incubated for 20 minutes at 4°C. After washing in 10 ml cold isolation buffer, cells were resuspended in 2 ml isolation buffer and 1 ml Depletion MyOne Dynabeads® and incubated at room temperature for 20 minutes with gentle rotation. Resuspension of the bead-bound cells using a narrow-opening pipette tip prior to the addition of 3 ml isolation buffer ensured a single cell suspension and thus the efficient removal of the non-CD4+ PBMC during magnetic separation. After 3 minutes in the magnet, supernatant containing bead-free CD4+ T cells is removed to a fresh tube. Once washed with isolation buffer, the CD4+ T cells were counted and resuspended to $\sim 1.5 \times 10^7$ cells/ml. 200 μ l Dynabeads® CD25 was added per $\sim 1.5 \times 10^7$ CD4+ T cells and incubated at 4°C with gentle rotation for 25 minutes. Placed in the magnet for 1 minute, supernatant was removed containing the CD4+CD25- effector T cells. Bead-bound cells were carefully washed in 5 ml isolation buffer and the supernatant was removed. Bead-bound cells were resuspended in 500 μ l supplemented RPMI and 80 μ l of DETACHaBead® was added and incubated at room temperature for 45 minutes with gentle rotation. Cells were vortexed to ensure thorough detachment of cells from the beads. Using the magnet, CD4+CD25+ regulatory T cells were removed in the supernatant. Prior to counting, CD4+CD25+ regulatory T cells were placed in the magnet for 1 minute to ensure complete removal of Dynabeads®. Isolated T cell subpopulations were resuspended to $\sim 5 \times 10^6$ cells/ml supplemented RPMI until use. To increase the purity of the isolated CD4+CD25+ regulatory T cells, anti-human

CD127 (eBioscience, Hatfield UK) was added to the anti-CD4 antibody cocktail used above. Supernatant containing the non-CD4+CD25+ T cells was discarded due to contamination by activated T cells. A separate isolation was used to isolate CD4+CD25- effector T cells; purity was increased by the addition of anti-CD25 (BD Biosciences, Oxford UK) to the anti-CD4 antibody cocktail.

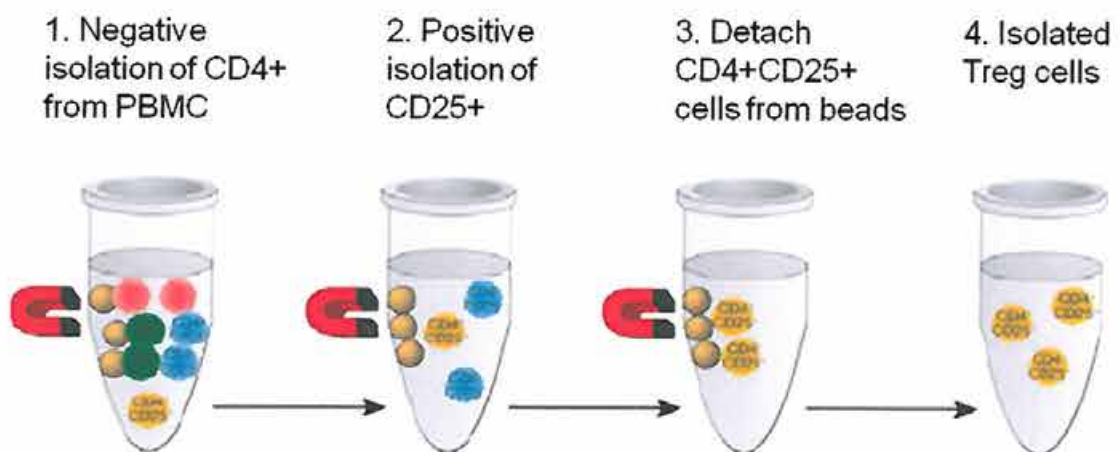


Figure 2.1: Isolation of regulatory T cells from PBMC. Non-CD4+ PBMC are labelled with Antibody Mix Human CD4. Depletion MyOne Dynabeads are added to remove the non-CD4+ PBMC, thus negatively isolating the CD4+ T cells. CD4+CD25+ cells are positively isolated from the CD4+ T cells using Dynabeads CD25. DETACHaBead® are used to remove CD4+CD25+ Regulatory T cells from the Dynabeads CD25. Diagram adapted from Dynabeads® Regulatory CD4+CD25+ T Cell Kit product literature available at: www.invitrogen.com/ivgn/product/11363D.

Section 2.1.3 *In vitro* polarisation of Th17 T helper cells

Th17 T helper cells were kindly prepared by Miss Siobhan Restorick. CD4⁺ T cells (5×10^5) were cultured in each well of a 96-well round bottomed plate in the presence of CD3/CD28 activation beads (1:32 bead:cell ratio) and 10 ng/ml IL-23 in supplemented RPMI in a humidified atmosphere at 37°C, 5 % CO₂. IL-23 (20 ng/ml) was reapplied after 72 hour. After 4 days of culture, cells were re-stimulated with 10 ng PMA (10 ng), ionomycin (250 ng) and brefeldin A (4 µg/ml) for 3 hours at 37°C prior to labelling for intracellular cytokines.

Section 2.1.4 Preparation of mouse splenocytes and thymocytes

Mouse splenocytes and thymocytes were kindly prepared by Dr Annelise Soulier. Briefly, male C57BL/6 mice were killed by dislocation of the neck. The spleen and thymus were removed immediately and mashed using a cell strainer. Splenocytes and thymocytes were washed in 1 ml RPMI. Splenocytes were incubated in 1 ml red blood cell lysis buffer for approximately 30 seconds at room temperature followed by a wash in 10 ml supplemented RPMI. Splenocytes and thymocytes were resuspended in 1 ml PBS and strained through a 30 µm sterile filter. Cells were counted and resuspended to 2×10^6 cells/ml supplemented RPMI and kept on ice until use.

Section 2.2 Assessment of neuronal receptor expression using molecular biological techniques

Section 2.2.1 RNA isolation

Successful isolation of RNA using the commercially available kit SV total RNA isolation system[®] (Promega, Southampton UK) requires 4 steps: disruption of cells, denaturation of nucleoprotein complexes, inactivation of endogenous ribonucleases (RNases) released from membrane-bound organelles and removal of contaminating DNA and protein. β -Mercaptoethanol inactivates RNases and guanidine thiocyanate (GTC) with sodium dodecyl sulphate (SDS) disrupts nucleoprotein complexes, allowing RNA to be isolated, in solution and free of protein. Ethanol is used to selectively precipitate RNA and binds to the silica membrane of the spin basket. DNase-I is applied to digest contaminating genomic DNA. Bound RNA is purified further by washing and finally eluted from the membrane by nuclease-free H₂O. Isolated RNA was quantified and stored at -80 °C until use.

Contamination by exogenous ribonucleases must be avoided. Prior to RNA isolation, an RNase-free environment was achieved by treatment of pipettes, gloves and worktops with RNaseZAP[®] (Sigma, Dorset UK) and using RNase-free filter tips throughout (Starlab, Milton Keynes UK).

Section 2.2.2 cDNA synthesis

Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) is a recombinant reverse transcriptase expressed in *E.coli*. Similar to other DNA polymerases, AMV RT transcribes single-stranded RNA into double-stranded DNA. The reverse transcriptase reaction is primed with Oligo(dT)15, hybridising to the 3' poly (A) tail of mRNA, initiating first-strand cDNA synthesis. cDNA synthesis was performed using the Reverse Transcription System (Promega, Southampton UK). Total RNA was heated at 70°C for 10 minutes, and kept on ice whilst the reaction was set up (Table 2.1).

Section 2.2.3 Quantification of DNA

The concentration of DNA in samples was calculated using a spectrophotometer (Thermo Spectronic Biomate 3; Thermo Fisher, Epsom UK), which measures the amount of UV irradiation absorbed by the nucleotide bases in the sample. Absorbance of a sample at a wavelength of 260 (A_{260}) is an indicator of the quantity of DNA or RNA in the sample. An Optical Density (OD) of 1.0 corresponds roughly to 50 µg/ml double-stranded DNA, 40 µg/ml single-stranded DNA or RNA and 20-33 µg/ml for single stranded oligonucleotides. Absorbance of UV at 280 nm gives the amount of protein in the sample. Purity of the sample is estimated by comparison of the ratio of absorbance at $OD_{260}:OD_{280}$ (DNA:protein). An $OD_{260}:OD_{280}$ greater than 1.8 is considered relatively pure and concentration of the DNA is accurate (Sambrook and Russell, 2001).

A) Reverse Transcription reaction	
MgCl ₂	5 mM
10x RT buffer	1x
10mM dNTP mix	1 mM
RNasin	1 unit/ μ l
AMV RT	15 unit/ μ g
Oligo(dT) 15	0.5 μ g
Total RNA	50 ng/ μ l
Nuclease free H ₂ O	Up to 20 μ l

B) PCR reaction	
2x reaction buffer	5 μ l
cDNA template	~10 ng
Forward primer	1 μ g
Reverse primer	1 μ g
dNTP (each)	200 μ M
DNA polymerase	2.5 unit
ddH ₂ O	Up to 50 μ l

C) RT cycling parameters	
42 °C	15 min
95 °C	5 min
4 °C	5 min
cDNA was stored at -20 °C	

D) PCR cycling parameters			
Step		Time	Temp
Initial denaturation		2 min	95 °C
Denaturation	35 cycles	1 min	95 °C
Annealing		1 min	55 °C
Extension		2 min	72 °C
Final extension		15 min	72 °C
Reaction held at 4 °C until use			

Table 2.1: Components of a typical reverse transcription reaction (A) and PCR reaction (B) with example parameters for cDNA synthesis (C) and PCR cycling conditions (D).

Section 2.2.4 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a technique exploited by molecular biologists to replicate specific regions of DNA. Two synthetic oligonucleotides are needed to prime DNA synthesis by DNA polymerase. Appendix 1 shows oligonucleotide sequences used throughout the study. In addition to template DNA, also required are the four deoxynucleotide triphosphates (dNTPs), monovalent and divalent cations (usually Mg^{2+}) and buffer to maintain pH.

Each PCR cycle is comprised of 3 stages (Table 2.3). First, denaturation of the double-stranded DNA template is required. Secondly, annealing of the oligonucleotides to the single-stranded DNA molecule must occur before finally, extension of the product. The temperature needed for denaturation (T_m) to occur fully is calculated by the equation: $T_m = 0.41 \times \% GC + (64.9 - (600 - n))$, where % GC is the percentage of guanine and cytosine residues in the oligonucleotide and n is the expected product size. Extension of the annealed product will occur at an optimum temperature for each particular DNA polymerase. *Taq*, because of its thermo-stable properties, has a high optimum temperature of 75-80°C. Extension is performed for 1-2 minutes per 1000 base pairs of template DNA. Denaturation, annealing and extension make up one PCR cycle, and many cycles are continually repeated. After the last cycle, a final extension step will be used to fully extend the DNA products. PCR cycling was performed using a Techne automated thermocycler (Fisher, Loughborough UK).

Section 2.2.5 Agarose gel electrophoresis

Separation of DNA through a solid agarose matrix can be employed to purify DNA products. DNA possesses a net negative charge, allowing it to migrate towards the anode of an electrophoresis tank. The rate of migration is directly proportional to the size of the DNA fragment. DNA produced as a result of a PCR reaction, fragments from a restriction digest, or to characterise the restriction map of cDNA clones can be separated by this method.

2 % agarose in 1x Tris-acetate-EDTA (TAE) buffer was heated in a microwave until fully dissolved. Once cooled, 10 mg/ml ethidium bromide was added to a final concentration of 0.05 ng/ml. Gels were poured using an appropriate comb to cast wells and allowed to set at room temperature. Gels were immersed in 1x TAE in an electrophoresis tank (Thistle Scientific, Glasgow UK). Once samples had been loaded alongside a DNA molecular weight marker (Hyperladder IV; Bionline, London UK), electrophoresis was conducted at 100 V, 400 mA for approximately 1 hour. Ethidium bromide binds between double-stranded DNA to allow visualisation under a UV Transilluminator (Genetic Research Instrumentation Ltd, Essex UK). Photographs were taken using a MCID image capture system.

50x Tris-acetate-EDTA	
Tris	2 M
Glacial acetic acid	50 mM
EDTA pH 8	5 % (v/v)
pH to 7.6	

Table 2.2: Constituents of 50x Tris-acetate-EDTA; stored at room temperature.

Section 2.3 Immunofluorescent detection techniques

In order to investigate the expression and cellular localisation of a specific protein, antibodies can be used to label the protein or molecule of interest. Immunofluorescently-labelled cells can then be analysed using a fluorescent microscope or by flow cytometry. Anti-5-HT_{3A} and the corresponding peptide were bought from Abcam (Cambridge UK). Relevant secondary antibody (anti-rabbit IgG (H+L) 488) was purchased from Molecular Probes (Invitrogen, Paisley UK). Antibodies against immune cell surface markers, intracellular cytokines and FoxP3 were all purchased from BD Biosciences (Oxford UK).

Section 2.3.1 Adherent cell immunocytochemistry

Confluent cells were split 1:2 and seeded on sterile glass coverslips in 24-well cell culture plates. Following incubation for 24 hours in humidified atmosphere, 37°C, 5 % CO₂, culture media was aspirated from wells and coverslips washed 3 times with ice-cold PBS. Fixation was achieved using 2 % formaldehyde for 20 minutes and subsequently cells were washed 3 times with PBS. Cells were incubated with blocking buffer (10 % FCS-PBS) for 1 hour. Anti-5-HT_{3A} or peptide blocked anti-5-HT_{3A} (0.5 µg/ml diluted in blocking buffer) was applied to cells and incubated overnight at 4°C. For peptide blocking, anti-5-HT_{3A} was incubated with immunising peptide (100x concentration of antibody) for 1 hour at room temperature. Cells were washed thoroughly with PBS prior to incubation for 2 hours with anti-rabbit IgG (H+L) Alexa Fluor 488 (0.2 µg/ml diluted in blocking buffer). Finally, cells were washed extensively with PBS. To visualise the nuclei, Vectorshield Hardset mounting media with DAPI (Vector Laboratories, Peterborough UK) was applied to glass slides.

Coverslips were inverted onto the glass slides and left to air dry in the dark. Slides were kept at 4°C in the absence of UV light until use. Images were captured using a light microscope at 20x magnification.

Section 2.3.2 Suspension cell immunocytochemistry

Immune cells ($\sim 1 \times 10^7$) were washed with cold PBS to remove culture medium. Cells were then fixed with 2 % formaldehyde for 20 minutes. Cells were washed twice in PBS before incubation in blocking buffer for 30 minutes. Cells were incubated with anti-5-HT_{3A} (0.5 µg/ml diluted in blocking buffer) for 1.5 hours. Cells were washed twice in PBS, and incubated with anti-rabbit IgG (H+L) Alexa Fluor 488 (0.2 µg/ml diluted in blocking buffer) for 1.5 hours. Cells were washed three times with PBS, centrifuged and final pellets were resuspended in 500 µl PBS. A 20 µl aliquot of cells were spotted onto glass slides and left to air-dry. To visualise the nuclei, a drop of Vectorshield® Hardset mounting media with DAPI (Vector Laboratories, Peterborough UK) was applied to each slide and left to air-dry in the dark. Slides were kept at 4°C in the absence of UV light until use. Images were captured using a confocal microscope at 63x magnification.

Blocking buffer	
10x PBS	1x
FCS	10 %

Table 2.3: Constituents of blocking buffer required for immunocytochemistry. Reagents stored at 4°C.

Section 2.4 Flow cytometry

Flow cytometry (FC) is a high-throughput method of counting and analysing individual cells in a heterogeneous population. Immune cells can be labelled with fluorescent antibodies, relevant to phenotypic surface markers, which upon passing through a laser in a fine stream of fluid, allows the fluorescence associated with each cell to be quantified. In addition to the detection of fluorescence associated with antibodies labelling markers of the cell, scattering of the laser light detected by a detector in line with the laser is known as 'forward' scatter and is directly related to the size of the cell. Detectors perpendicular to the laser detect 'side' scatter caused by the granularity and the structural complexity of the cell in question. Forward and side scatter plots can be used to characterise cell types, which enables certain populations of cells of interest to be electronically 'gated' in order to analyse subpopulations of cells in a heterogeneous population (Figure 2.2).

FC data was collected using a Becton Dickinson FACSCalibur (BD Biosciences, Oxford UK) using CellQuest software (BD Biosciences, Oxford UK) and analysed using FlowJo software (TreeStar Inc, Ashland USA).

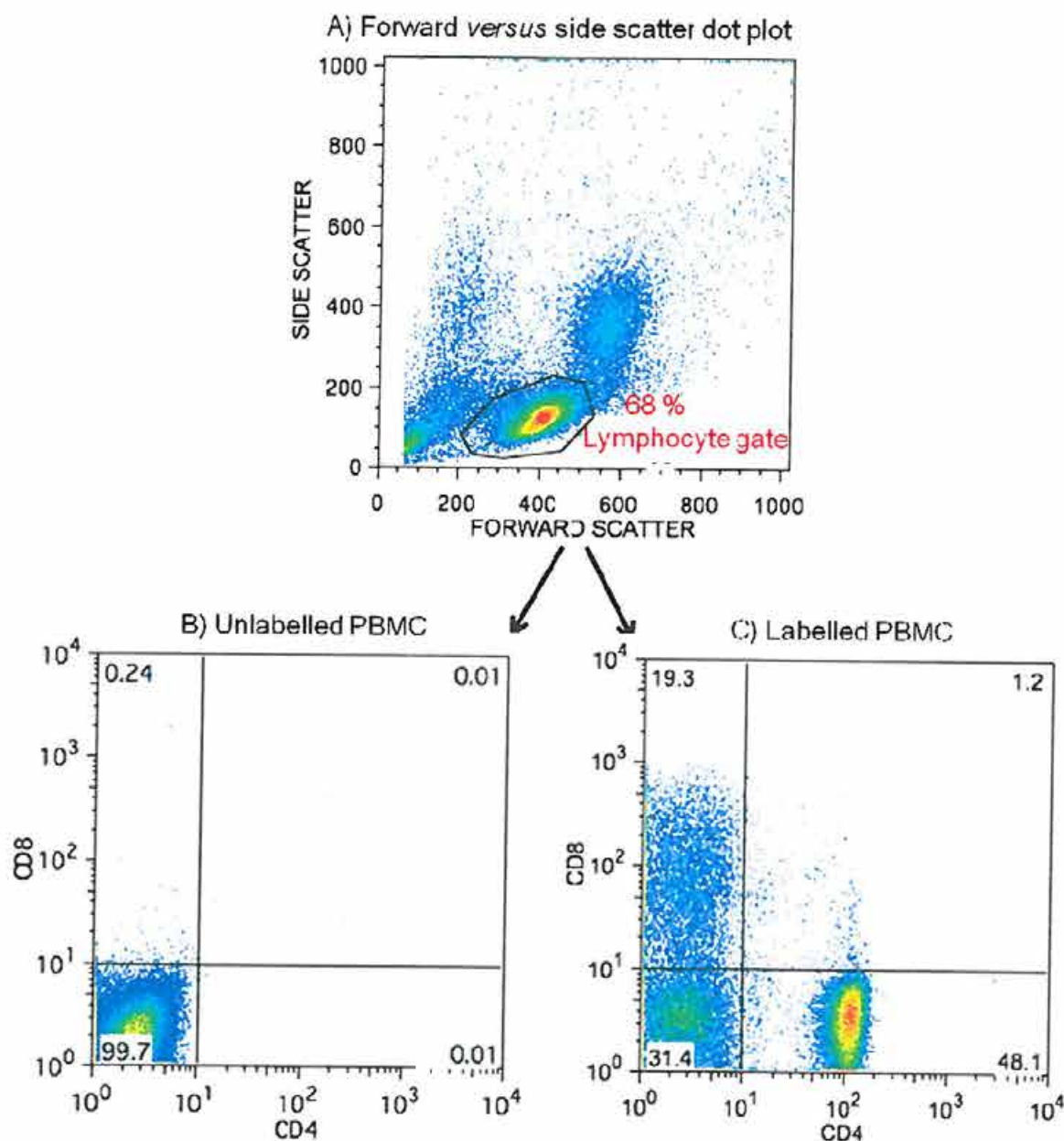


Figure 2.2: Interpretation of flow cytometry data. A) Forward scatter (FSC; x axis) and side scatter (SSC; y axis) dot plot of freshly isolated human PBMC. Lymphocytes are gated according to their physical characteristics using the gating tool on FlowJo data analysis software. In this example, 68 % of PBMC fall within the lymphocyte gate. The lymphocyte gate is then analysed using the parameters relative to the fluorescently-conjugated antibody of interest. In this example, PerCP immunofluorescence associated with CD4 labelling appears on the x axis, whilst FITC immunofluorescence associated with CD8 labelling appears on the y axis. B) Unlabelled cells are confined to the first log phase of fluorescence and are used to set the fluorescence gate which shows the percentage of cells in each quadrant. C) For this example donor, 48.1 % of gated lymphocytes are CD4+ and 19.3 % are CD8+.

Section 2.4.1 Labelling of immune cells for surface markers and intracellular cytokines

Cells were washed in PBS, resuspended in staining buffer and transferred to a 96-well round bottomed plate and pelleted by centrifugation. Cells were resuspended in a total volume of 50 μ l staining buffer (Table 2.4) containing appropriate antibody (Appendix 2; BD Biosciences, Oxford UK) and incubated at 4°C for 20 minutes. Cells were washed twice in 200 μ l staining buffer before fixation for 20 minutes at 4°C in 100 μ l fixation solution (BD Biosciences, Oxford UK). Samples were washed twice in stain buffer before being resuspended in 300 μ l PBS. Cells were kept at 4°C, in the absence of fluorescent light until analysis by flow cytometry.

Staining buffer	
10x PBS	1x
FCS	2 % (v/v)

Table 2.4: Constituents of staining buffer required for labelling of immune cells for surface markers. Reagents stored at 4°C.

For the detection of intracellular cytokines, brefeldin A (10 ng/ml), PMA (10 ng/ml) and ionomycin (250 ng/ml) were applied to samples for the last 4 hours of culture, prior to fixation and permeabilisation.

For intracellular staining, cells were fixed and permeabilised for 20 minutes at 4°C using 100 μ l fix permeabilisation solution (BD Biosciences, Oxford UK). Samples were washed twice in permeabilisation wash buffer (BD Biosciences, Oxford UK). Before staining for FoxP3 or cytokines and incubated for 20 minutes at 4°C. If necessary, antibodies were diluted in permeabilisation wash buffer (total volume of

50 μ l). After two more washes in permeabilisation wash buffer, and one in stain buffer, cells were resuspended in 300 μ l PBS and kept at 4°C, in the absence of fluorescent light until analysis by flow cytometry.

All centrifugation steps were performed at 300 rcf for 5 minutes.

Section 2.4.2 Measurement of immune cell proliferation using CFSE

CFSE (carboxyfluorescein succinimidyl ester) is a commonly used fluorescent dye, exploited by immunologists to monitor the proliferation of lymphocytes. Fluorescence intensity of CFSE-labelled cells progressively halves with each round of cell division. Although CFSE can be toxic at too high a concentration, CFSE can also be used *in vivo* to trace the migration of CFSE-labelled cells.

CFSE labelling was carried out in aseptic conditions, protected from fluorescent light. An equal volume of 2 μ M CFSE was added to 2×10^7 cells/ml sterile PBS. Cells were thoroughly vortexed to ensure uniform labelling and incubated for 10 minutes at room temperature with periodic shaking. Addition of an equal volume of supplemented RPMI for 1 minute quenches CFSE staining. Cells were washed twice in PBS and once in supplemented RPMI and allowed to rest for 1 hour at room temperature. Cells were washed once more and finally resuspended in supplemented RPMI.

Section 2.4.3 Measurement of the suppressive capacity of regulatory T cells

The function of CD4+CD25+ regulatory T cells can be analysed *in vitro* by a suppression assay.

CD4+CD25- effector T cells and CD4+CD25+ regulatory T cells were isolated using the Regulatory CD4+CD25+ T cell kit (Section 2.1.2). Purity of isolated regulatory T cells were assessed using the expression of CD4 and CD25 surface markers. CD4+CD25- effector T cells were labelled with CFSE (1 μ M; Section 2.4.2) and resuspended to 5×10^5 cells/ml in supplemented RPMI. Unlabelled CD4+CD25- effector T cells and unlabelled CD4+CD25+ regulatory T cells were also resuspended to 5×10^5 cells/ml in supplemented RPMI. The suppression assay is performed using a dilution series ranging from a ratio of 1:1 to 32:1 of CD4+CD25- effector T cells to CD4+CD25+ regulatory T cells. Each ratio was performed in duplicate. T cells were stimulated using CD3/CD28 activation beads (Invitrogen, Paisley UK) at a bead to cell ratio of 1:1. Cells were cultured in a total volume of 210 μ l supplemented RPMI in a humidified atmosphere, 37°C, 5 % CO₂. Cells were washed in PBS prior to data collection using a BD FACSCalibur (BD Biosciences, Oxford UK).

Activated lymphocytes were gated according to physical characteristics using FSC SSC plots using FlowJo software. Proliferation gates were set between the fluorescent peaks arising from unstimulated CFSE-labelled CD4+CD25- T cells (CFSE^{high}) and unlabelled CD4+CD25+ regulatory T cells (CFSE^{low}). Percentage proliferation was expressed as a percentage of the total number of proliferated CFSE-labelled CD4+CD25- effector T cells.

Section 2.4.4 Detection of apoptotic and necrotic cells using flow cytometry

Apoptosis, known as programmed cell death, is an intracellular process important in controlling immune challenges and normal cellular growth and differentiation (for example development of fingers and toes during embryogenesis). Caspases (cytosine-aspartate proteases) are a group of proteins which are responsible for apoptosis. Activation of caspase 3 leads to chromatin condensation and DNA fragmentation. The fluorogenic protease substrate Phiphilux® can cross intact cell membranes and detect intracellular caspase 3 activity. Another process of cell death is necrosis, which is initiated by extracellular factors. Necrotic cells cannot be recognised by phagocytes for removal, therefore damaged cell membranes result in the spillage of intracellular contents, resulting in inflammation. Propidium iodide (PI) is a fluorescent nucleic acid dye which can only enter cells with damaged cell membranes. Phiphilux® in conjunction with PI staining can be used to discriminate between live, necrotic, early apoptotic or late apoptotic cells.

PBMC ($\sim 3 \times 10^5$) in supplemented RPMI were aliquoted into 96-well round bottomed plates and cultured for 4 days in the presence of drug, stimulated with CD3/CD28 activation beads. After 4 days, samples were washed in sterile PBS and incubated with 30 μ l Phiphilux® (OncoImmunin, Inc, Gaithersburg USA) for 1 hour at 37°C. Cells were washed in PBS and transferred to a FACS tube. The cell suspension was made up to 300 μ l with PBS. 2 μ g/ml PI was added to cells and vortexed to ensure thorough mixing. After 5 minutes incubation, cells were analysed by flow cytometry. Data was collected by a BD FACSCalibur (BD Biosciences, Oxford UK) and analysed by FlowJo (TreeStar Inc, Ashland USA).

Section 2.5 Data analysis

Prior to statistical analyses, data were tested to determine whether or not the values followed a Gaussian (normal) distribution using the Kolmogorov-Smirnov test. Data which is normally distributed is said to be parametric, whilst data which does not follow the Gaussian distribution is non-parametric data. Parametric and non-parametric data were then analysed according to the table below:

	Parametric	Non-parametric
2 data sets	T Test	Wilcoxon test
More than 2 data sets	ANOVA	Friedman test

Table 2.5: Statistical tests used for the analysis of parametric and non-parametric data.

In particular, a two-tailed paired T test was used when data was comprised of two matched conditions of which either may have a larger mean than the other. Repeated measures ANOVA and Friedman's tests were used when data contained more than two different, matched conditions. Where necessary, data was also tested for outliers using Grubb's test. Outliers are data points which deviate from others in the same sample group. Grubb's test identifies single outliers, in data which is assumed to follow an approximately normal distribution (Grubbs, 1969). As no outliers were identified in data contained in this thesis, data that was not normally distributed was subsequently analysed using a non-parametric statistical test.

All statistical analysis was performed using GraphPad version 5.

CHAPTER 3

CHARACTERISATION OF THE EXPRESSION OF THE 5-HT_{3A} RECEPTOR SUBUNIT IN HUMAN T CELL SUBSETS, AND THE IMPLICATIONS OF 5-HT_{3A} RECEPTOR ACTIVATION ON THE FUNCTION OF HUMAN IMMUNE CELLS

Section 3.1 Introduction

The rationale for studying the expression of the 5-HT_{3A} receptor in immune cells is an extension of previous research identifying the expression of the serotonin transporter (SERT) in lymphocytes. Uptake of 5-HT into lymphocytes was associated with apoptosis (Serafeim *et al.*, 2002), which was inhibited by selective serotonin reuptake inhibitors (SSRIs). It is possible that SERT expression by immune cells may act to control the amount of 5-HT released by platelets or lymphocytes during inflammation, where it can be stored intracellularly ready for subsequent release when required.

Expression of 5-HT receptors has previously been reported in the immune system of the rat (Stefulj *et al.*, 2000). 5-HT_{1A} receptors have received much attention for their role in immune cell function and their expression is reported in human T cells (Aune *et al.*, 1994) although studies have primarily focussed on their role in rodent immune systems (Abdouh *et al.*, 2001, Iken *et al.*, 1995, Freire-Garabal *et al.*, 2003). Expression of the 5-HT₃ receptor in immune tissues varies across rodent species (Stefulj *et al.*, 2000, Leon-Ponte *et al.*, 2007). Expression of transcripts encoding the 5-HT_{3A} receptor subunit have been reported previously in human monocytes, chondrocytes, T cells and synovial fluid (Fiebich *et al.*, 2004b).

The research described herein contributes to the characterisation of 5-HT_{3A} receptor expression in the human immune system and identifies a functional impact of 5-HT_{3A} receptor activation upon human PBMC.

Section 3.2 Expression of 5-HT receptors in human T cell subsets

Using molecular biological techniques (Section 2.2), T cell subsets were assessed for expression of mRNA transcripts encoding 5-HT receptors. CD4+CD25⁻ and CD24+CD25⁺ T cell subsets were isolated from human PBMC (Section 2.1.1) and cultured for 5 days \pm CD3/CD28 activation beads. cDNA was used as template in PCR reactions containing gene-specific primers. For each sample, positive and negative cDNA was synthesised with and without (respectively) reverse transcriptase. cDNA and its negative control was used in PCR reactions containing oligonucleotide primers specific for the housekeeping gene β -actin. The more PCR cycles performed, the more chance of detecting non-specific products resulting from genomic contamination, therefore β -actin PCR was performed for 40 cycles. β -actin products were detected in all positive cDNA samples and not in negative cDNA samples, suggesting that cDNA samples were free from genomic contamination (Figure 3.1). As a positive control for β -actin, cDNA from HEK293 cells was used.

Subsequent PCR was performed using oligonucleotide primers specific for the 5-HT_{3A} receptor subunit (Figure 3.2). After 35 PCR cycles, PCR products were evident in the activated CD4+CD25⁺ T cell subset, whereas other T cell subsets were negative of mRNA transcripts. HEK293 cells stably expressing the 5-HT_{3A} receptor (HEK-3A cells) were used as a positive control. Detection of an appropriately sized PCR product (approximately 192 base pairs) in HEK-3A cells implied correct amplification of the correct target sequence.

5-HT₇ receptor mRNA transcripts were detected in resting CD4+CD25⁻ and resting CD4+CD25⁺ T cells, although activated CD4+CD25⁻ and activated CD4+CD25⁺ T

cell subsets do appear to express mRNA transcripts, albeit at lower levels than resting T cell subsets (Figure 3.3). cDNA from a neuroblastoma cell line was used as a positive control for 5-HT₇ receptor mRNA expression. mRNA transcripts were not detected in T cell subsets for 5-HT_{2A}, 5-HT_{2C} or 5-HT₄ receptors at 35 PCR cycles (Figure 3.4). PCR was repeated for 40 cycles to confirm mRNA transcripts were not expressed in T cell subsets.

For detection of 5-HT_{3A} receptor protein in human CD4+CD25+ T cells, an unconjugated anti-5-HT_{3A} antibody raised in rabbit was used for immunocytochemistry (Section 2.3). Specificity of anti-5-HT_{3A} was determined using the immunising peptide to block specific 5-HT_{3A} receptor immunofluorescence generated by HEK-3A cells (Figure 3.5). Immunofluorescence was only identified in activated CD4+CD25+ T cells, consistent with the detection of 5-HT_{3A} receptor subunit mRNA transcripts in activated CD4+CD25+ T cells.

The possibility of detecting 5-HT_{3A} receptor protein in immune cells using flow cytometry was explored next. The same anti-5-HT_{3A} antibody used for immunocytochemistry was used for flow cytometry, however specific 5-HT_{3A} receptor immunofluorescence was not detected (fluorescence was not greater than isotype controls). Six other unconjugated anti-5-HT_{3A} antibodies were tested, although none detected specific 5-HT_{3A} receptor immunofluorescence.

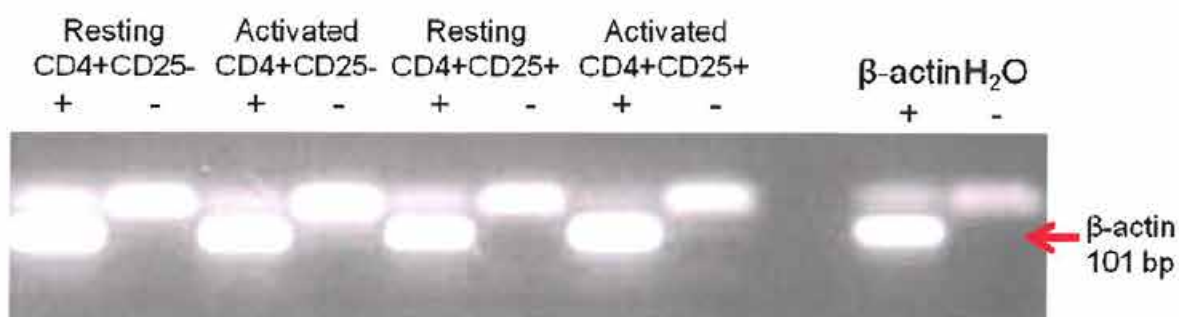


Figure 3.1: Expression of mRNA transcripts for β -actin in human T cell subsets. Oligonucleotide primers specific for the housekeeping gene β -actin (101 base pairs) were used in PCR performed for 40 cycles. HEK293 cells were used as a positive control for β -actin, and H₂O was used as no template negative control. cDNA made without RT enzyme. β -actin PCR was performed once per isolation (following DNase I treatment); '+' denotes positive cDNA whilst '-' denotes control cDNA. PCR shown is typical of each individual donor used for PCR screening (n>10).

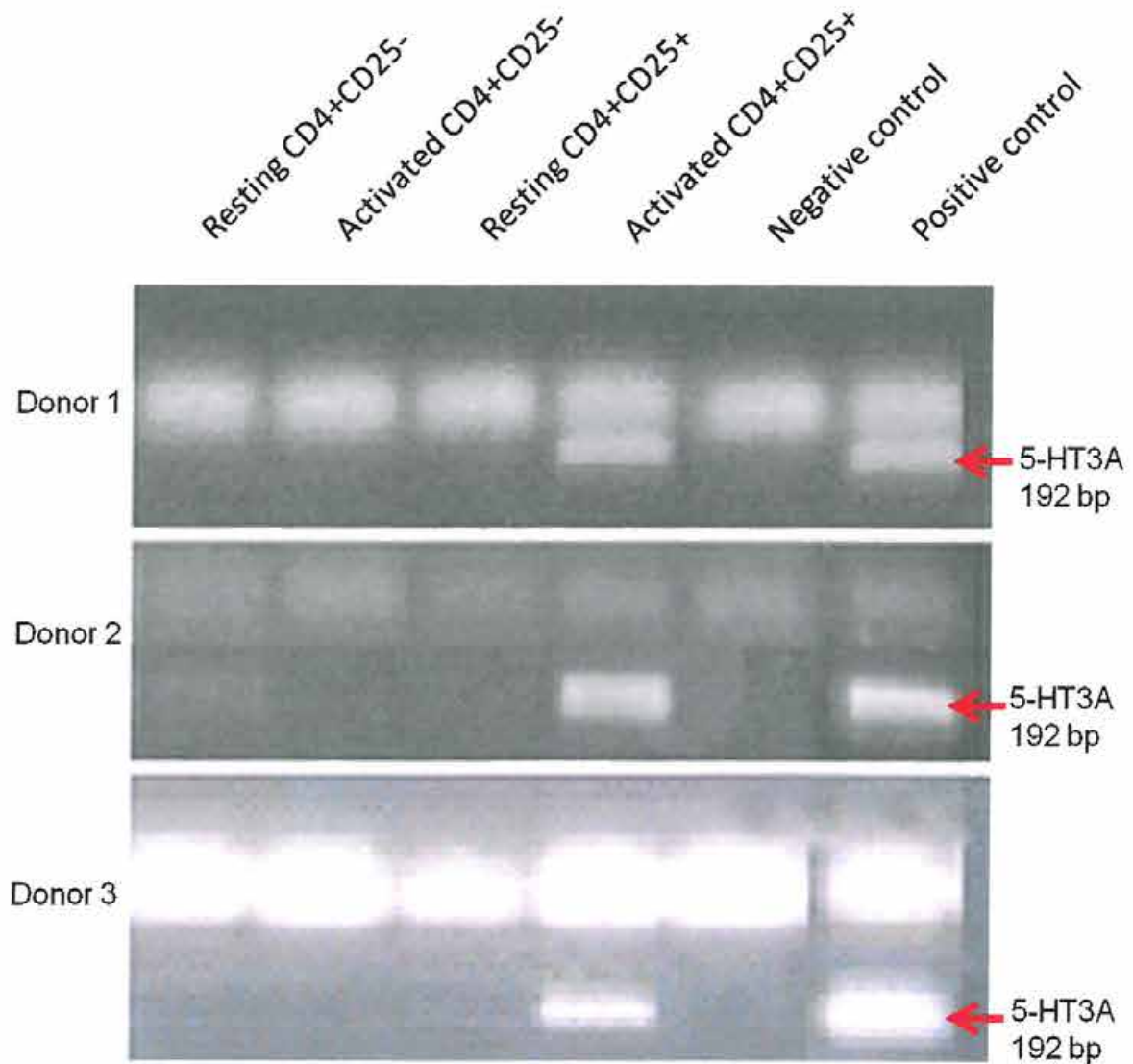


Figure 3.2: Detection of 5-HT3A receptor subunit mRNA transcripts in human T cell subsets. Oligonucleotide primers specific for the 5-HT3A receptor subunit (192 base pairs) were used in PCR performed for 35 cycles. HEK293 cells stably expressing the 5-HT3A receptor subunit were used as a positive control, and H₂O was used as no template negative control. PCR was performed at least twice for each donor; PCR shown is representative of each individual donor (n=3).

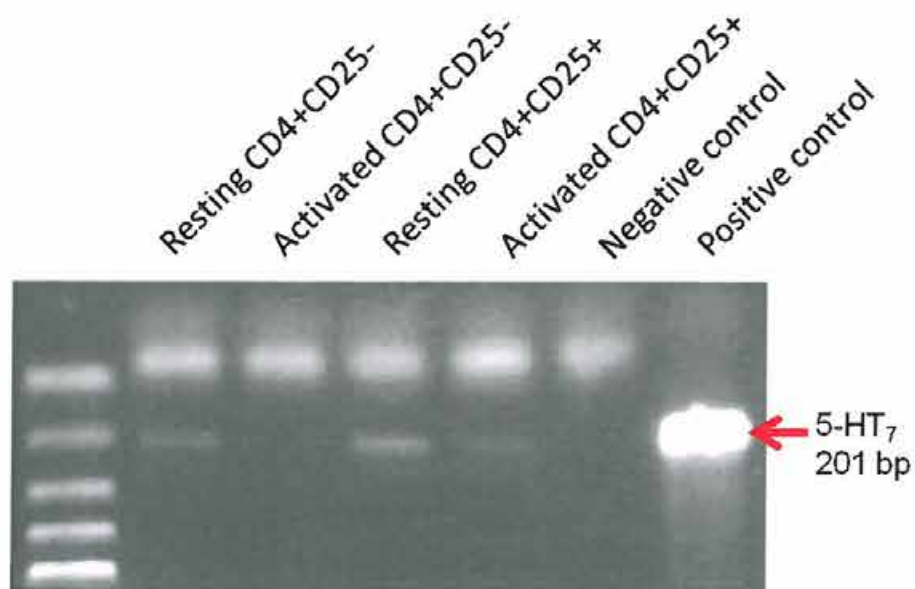


Figure 3.3: Detection of 5-HT₇ receptor subunit mRNA transcripts in human T cell subsets. Oligonucleotide primers specific for the 5-HT₇ receptor subunit were used in PCR performed for 35 cycles. A neuroblastoma cell line was used as a positive control, and H₂O was used as no template negative control. PCR is typical of 3 individual donors.

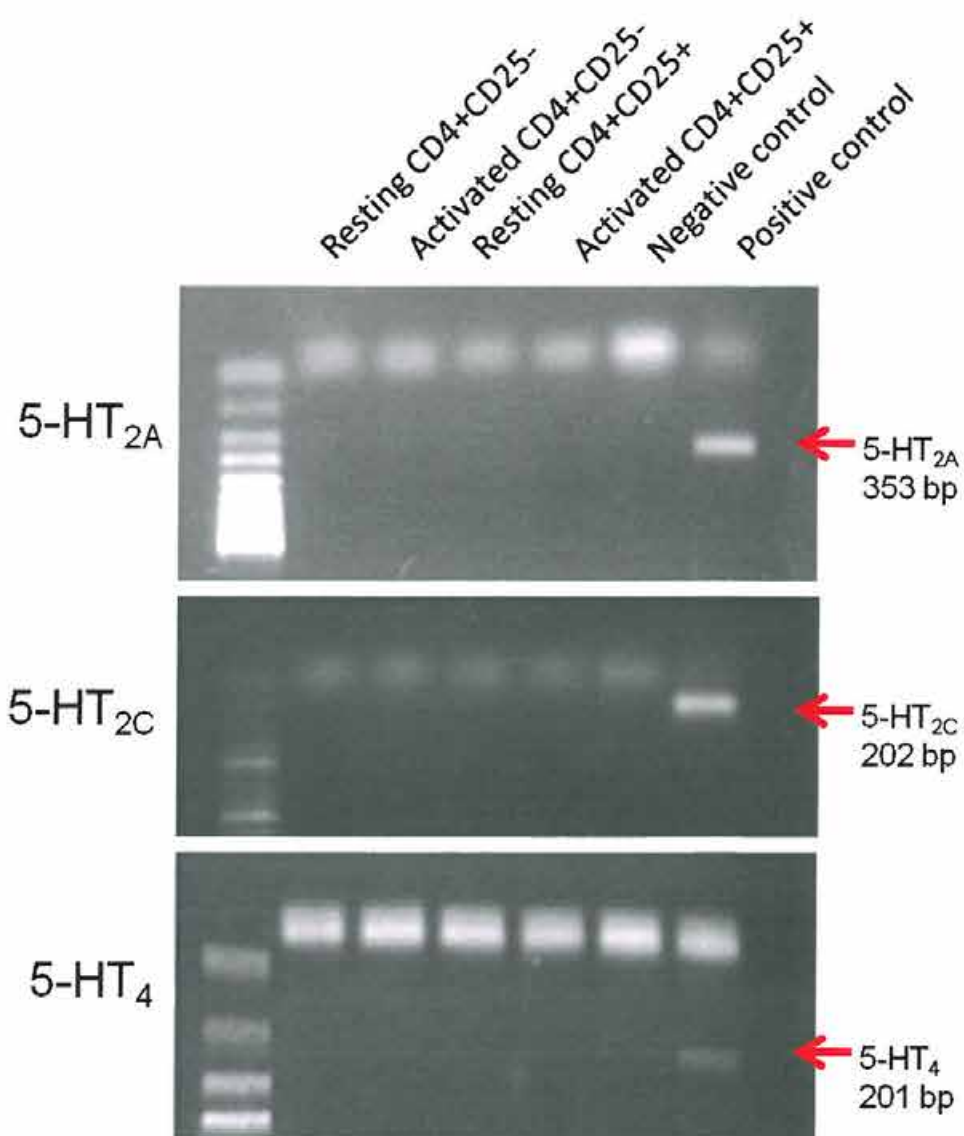


Figure 3.4: mRNA transcripts encoding the 5-HT_{2A}, 5-HT_{2C} and 5-HT₄ receptors were not detected in human T cell subsets. Oligonucleotide primers specific for 5-HT_{2A} (353 base pairs), 5-HT_{2C} (202 base pairs) and 5-HT₄ receptors (201 base pairs) were used in PCR. PCR was performed for 40 cycles; PCR shown is typical of 3 individual donors.

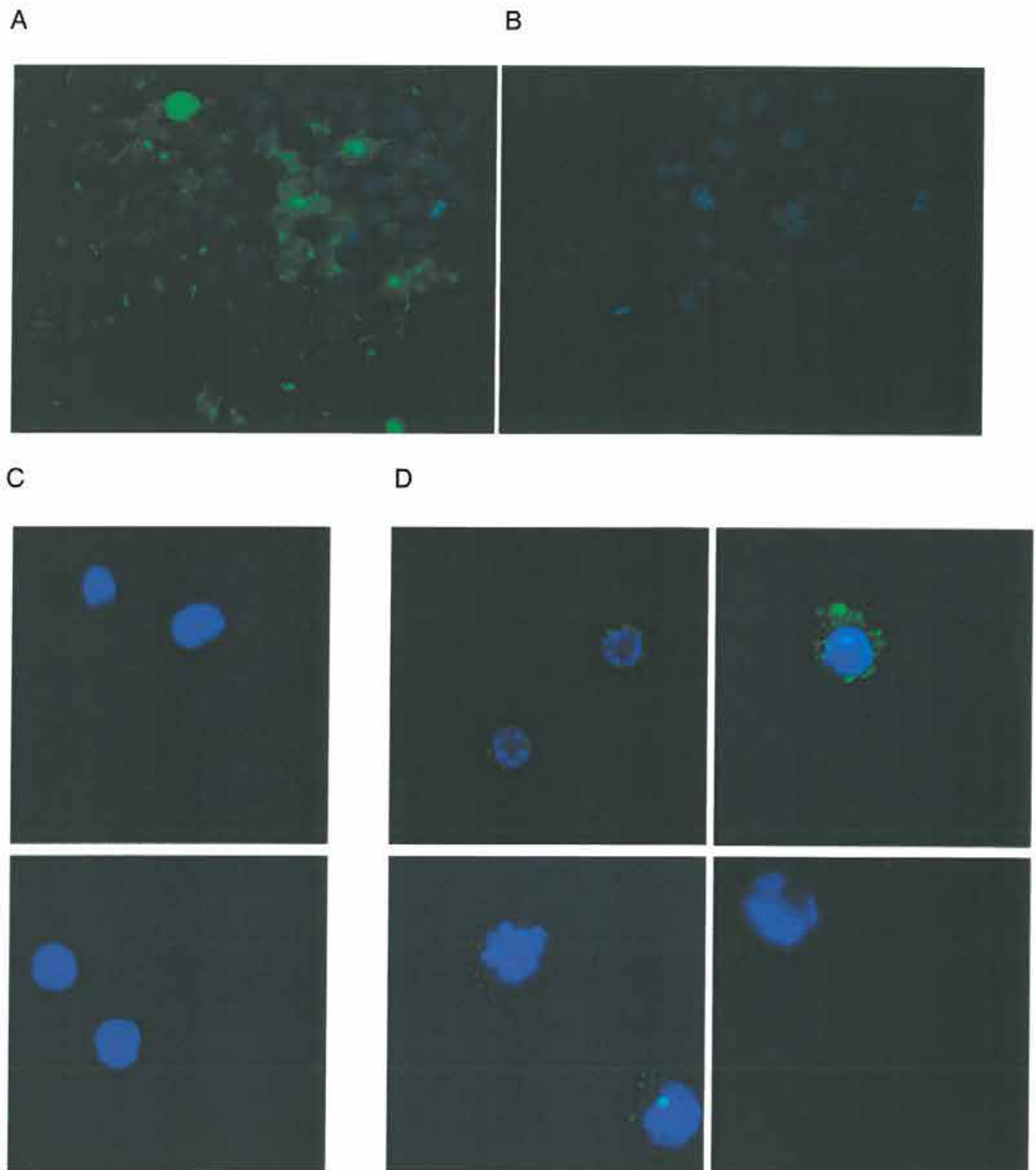


Figure 3.5: Upregulation of 5-HT_{3A} receptor protein in activated CD4+CD25+ T cells. h5-HT_{3A} receptor immunoreactivity appears in green, whereas blue fluorescence arises from DAPI staining of individual cell nuclei. A) HEK-3A cells labelled with anti-5-HT_{3A} and B) HEK-3A labelled using peptide blocked-anti-5-HT_{3A}. Images were taken using a fluorescent microscope at 20x magnification. C) resting CD4+CD25+ T cells and D) activated CD4+CD25+ T cells both labelled with anti-5-HT_{3A}. Images were taken using a confocal microscope at 63x magnification.

Section 3.3 Impact of 5-HT₃A receptor activation on the suppressive capacity of CD4+CD127^{low}CD25+ regulatory T cells

The function of regulatory T cells was assessed by suppression assays, where the inhibition of proliferation by regulatory T cells can be investigated. CD4+CD127^{low}CD25+ regulatory T cells and CD4+CD25- effector T cells were isolated from freshly prepared PBMC from human buffy coats (Section 2.4.3). Purities of isolated cell subtypes from one representative donor are shown in Figure 3.6.

Abrogation of suppression mediated by regulatory T cells can be achieved *in vitro* by exogenous IL-2. Application of IL-2 partially abrogated the suppression caused by CD4+CD127^{low}CD25+ regulatory T cells at each ratio examined, confirming CD4+CD127^{low}CD25+ regulatory T cells isolated using the protocol described are suppressors of activated CD4+CD25- effector T cells (Figure 3.7.1).

When the impact of 5-HT₃A receptor activation was assessed in these cells using a suppression assay there appeared to be no significant alteration in the suppression imparted by CD4+CD127^{low}CD25+ regulatory T cells at any ratio examined, despite the combined application of DDP733 and the positive allosteric modulator, 5-chloroindole (Figure 3.7.2). For example, in 2:1 T_{eff}:T_{reg} cultures, the total number of proliferated cells treated with both DDP733 (100 nM) and 5-chloroindole (10 µM) was 71691 ± 18074 cells (mean ± SEM; n=4), compared to 91494 ± 15752 vehicle-treated cells (mean ± SEM; n=4). Treatment with 5-chloroindole (10 µM) alone did not appear to affect proliferation, consistent with its function as an allosteric modulator. The 5-HT₃A receptor partial agonist DDP733 (100 nM) and positive allosteric modulator 5-chloroindole (10 µM) successfully evoke calcium responses in HEK293 cells expressing the h5-HT₃A receptor subunit (Manning *et al.*, 2011),

therefore it is assumed that the concentrations of DDP733 and 5-chloroindole are sufficient to activate native 5-HT_{3A} receptors expressed by human CD4+CD25+ T cells in PBMC.

Antagonism of the 5-HT_{3A} receptor by granisetron (1 μ M) did not affect the total number of proliferated cells either (92661 ± 10870 cells; mean \pm SEM; n=4). This may indicate that the expression of the 5-HT_{3A} receptor subunit on CD4+CD25+ T cells does not contribute to the suppression of CD4+CD25- T cell proliferation.

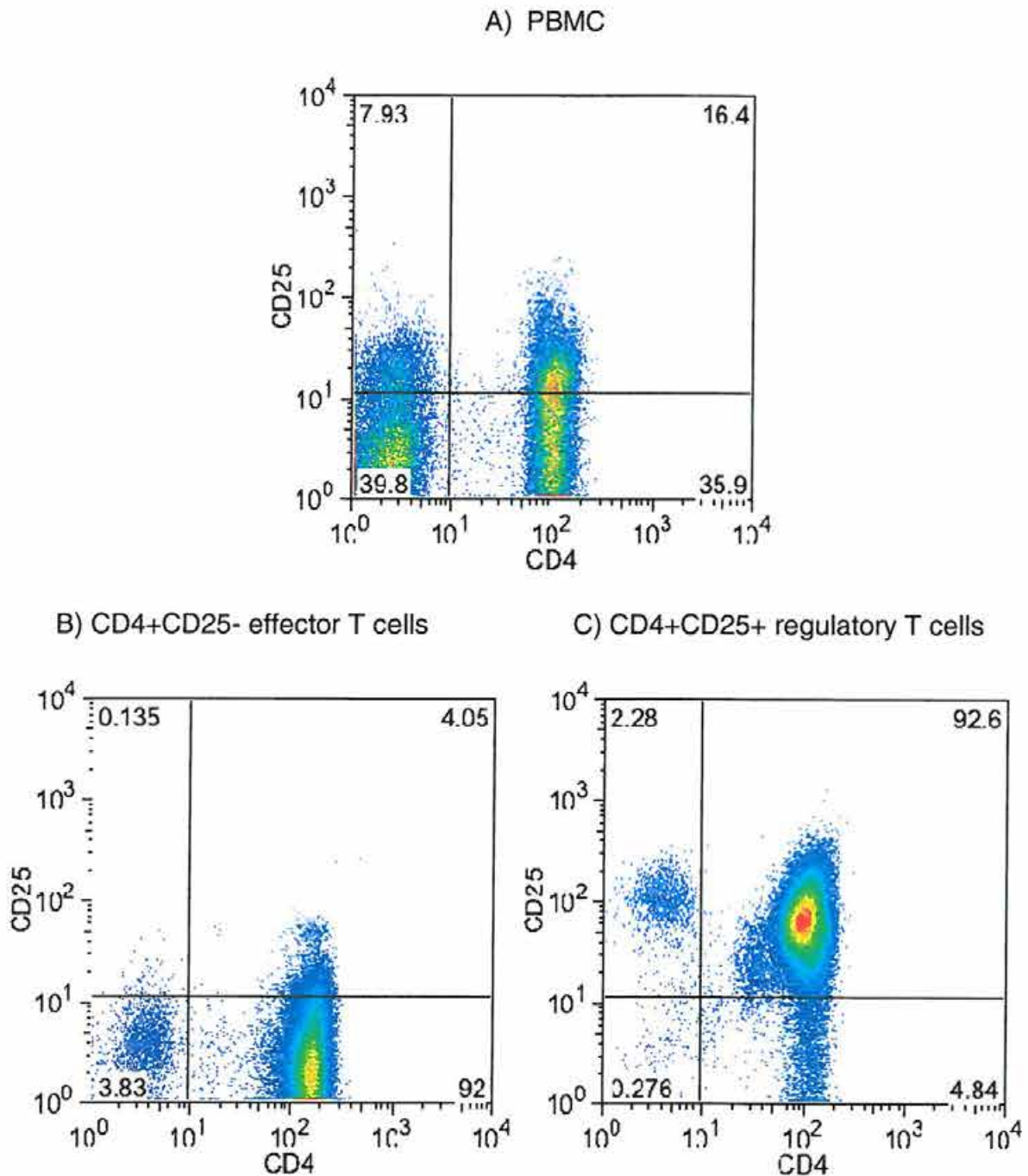


Figure 3.6: Phenotype of isolated regulatory and effector T cell populations based on CD4 and CD25 surface expression. A) freshly isolated PBMC, B) CD4+CD25⁻ effector T cells, C) CD4+CD127^{low}CD25⁺ regulatory T cells. Data is representative for cell populations used for suppression assays (n=4), where purity of each population was greater than 90 %.

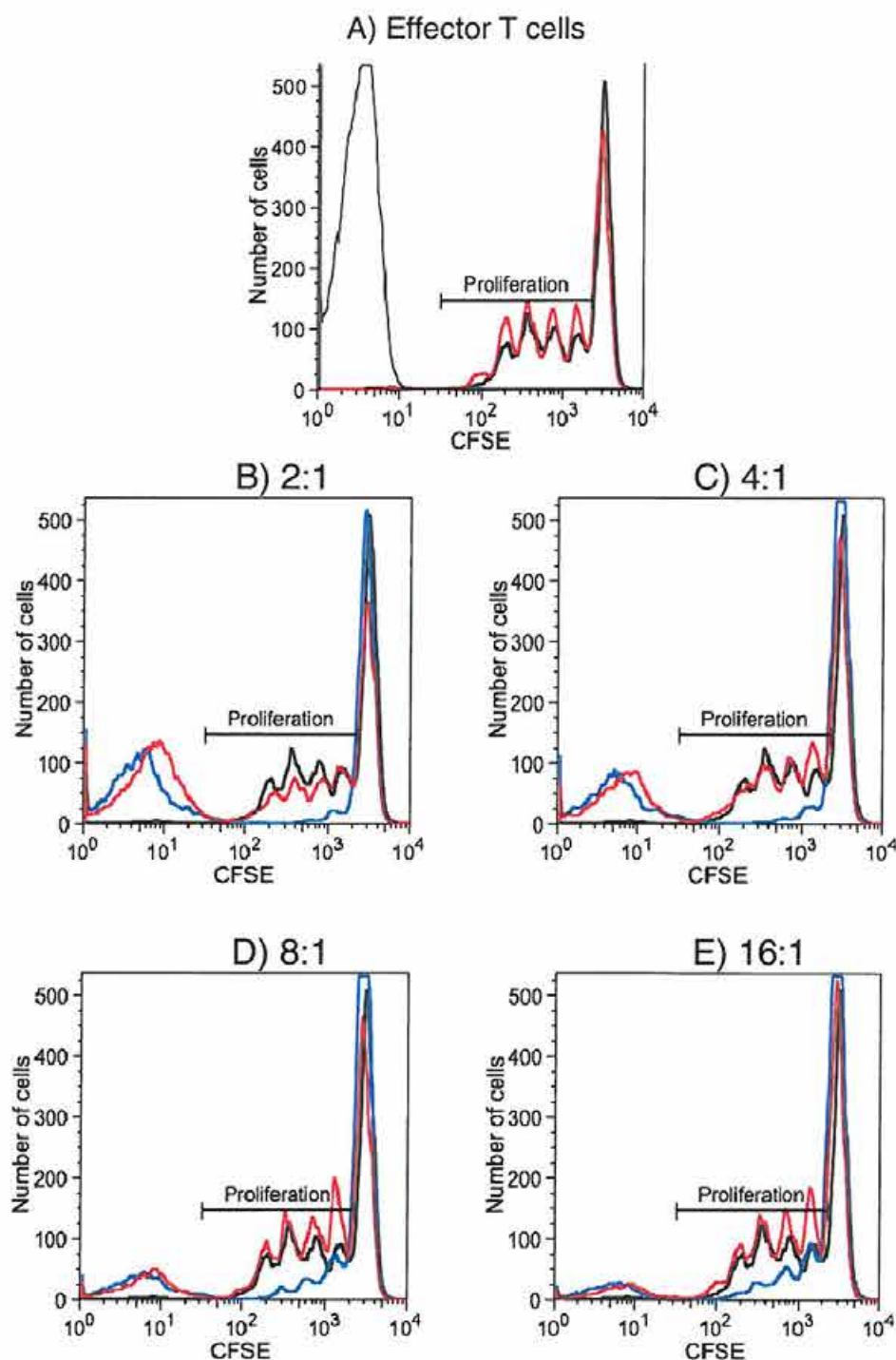


Figure 3.7: The function of regulatory T cells is not modulated by 5-HT_{3A} receptor activation.

Figure 3.7.1: Confirmation of the suppressive potential of isolated CD4+CD127^{low}CD25⁺ regulatory T cells. A) proliferation gate set between unstimulated CFSE-labelled CD4+CD25⁻ effector T cells and unlabelled CD4+CD127^{low}CD25⁺ regulatory T cells. Proliferation histograms of B) 2:1, C) 4:1, D) 8:1 and E) 16:1 ratio of T_{eff}:T_{reg} where proliferation of CD4+CD25⁻ effector T cells are shown in black, mixed T_{eff}:T_{reg} in blue and mixed culture treated with IL-2 (1000 units) shown in red.

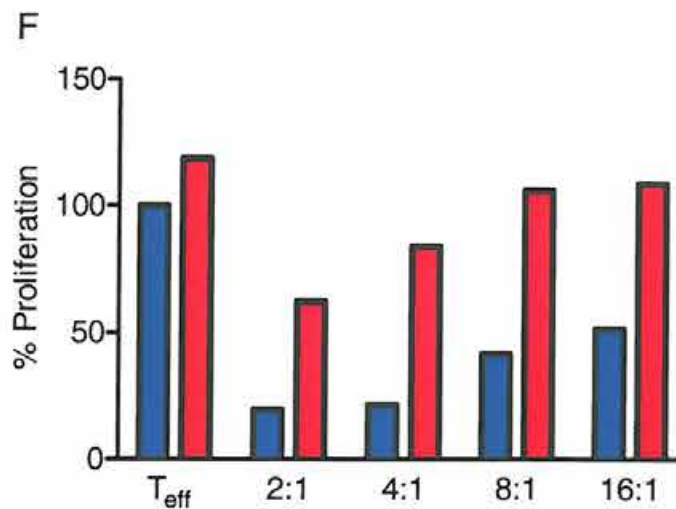


Figure 3.7.1 (continued): Confirmation of the suppressive potential of isolated CD4+CD127^{low}CD25+ regulatory T cells. F) proliferation expressed as a percentage of stimulated CFSE-labelled T_{eff} . Vehicle treated cells shown in blue and those treated with IL-2 (1000 units) are shown in red; $n=1$. Purity of isolated CD4+CD127^{low}CD25+ regulatory T cells was 93.6 % CD4+CD25+ for the experiment shown.

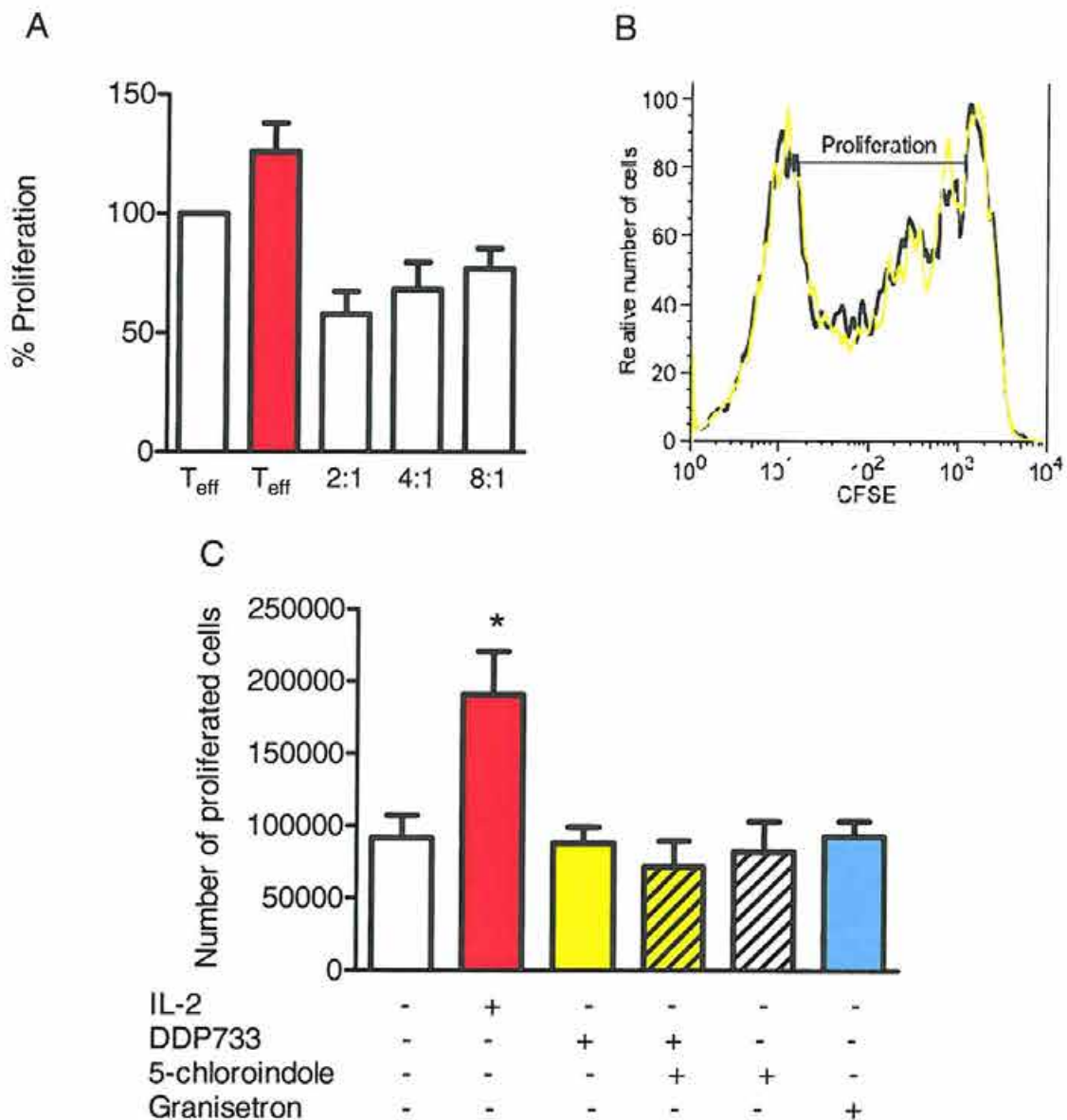


Figure 3.7.2: Modulation of 5-HT_{3A} receptor activation does not impact the suppressive capacity of isolated CD4⁺CD127^{low}CD25⁺ regulatory T cells. A) Suppression assay expressed as a percentage of the proliferation of T_{eff} treated with vehicle. Proliferation of T_{eff} treated with IL-2 (1000 units) shown in red as a positive control for proliferation. 2:1, 4:1 and 8:1 ratios of T_{eff}:T_{reg}; n=4. B) Histogram of the proliferation of T_{eff}:T_{reg} at a 2:1 ratio of T_{eff}:T_{reg}; shown for vehicle and DDP733 + 5-chloroindole treated cells (black and yellow histogram, respectively); data is representative of four individual experiments. C) Total number of proliferated cells at 2:1 ratio of T_{eff}:T_{reg} treated with IL-2 (1000 units), DDP733 (100 nM) and 5-chloroindole (30 μ M) or granisetron (1 μ M); n=4. Friedman test P=0.0543 followed by Dunn's multiple comparison test where P<0.05*.

Section 3.4 Impact of 5-HT_{3A} receptor activation on IFN- γ and TNF- α production and CD4/CD8 ratio in PBMC

Interferon (IFN- γ) and tumour necrosis factor (TNF- α) are important cytokines involved in the regulation of the immune response. IFN- γ is an immunomodulatory molecule released by activated lymphocytes, and is particularly crucial during viral infections. TNF- α is an immunostimulatory cytokine, able to prime and recruit immune cells during an immune response. Regulatory T cells suppress activated effector T cells, hereby inhibiting the release of IFN- γ and TNF- α from activated lymphocytes during an immune response. Therefore, due to the identification of both 5-HT_{3A} receptor subunit mRNA and protein in activated CD4⁺CD25⁺ T cells, the impact 5-HT_{3A} receptor activation on expression of IFN- γ and TNF- α was investigated in human PBMC.

PBMC were isolated (Section 2.5.1) and stimulated using phytohaemagglutinin (PHA; 5 μ g/ml). Cells were incubated with mCPBG (1 μ M), with and without 5-chloroindole (10 μ M) for 5 days. A sample of cells was labelled for CD4 and CD8 surface markers (Section 2.4.1). Prior to intracellular labelling for IFN- γ and TNF- α , brefeldin A (10 μ g/ml) was added to cultures for 4 hours, to inhibit the transport (and consequently the release of) intracellular cytokines. Phorbol myristate acetate (PMA; 10 ng/ml) and ionomycin (250 ng/ml) were also added to cultures to stimulate cytokine production, hereby resulting in the accumulation of cytokines which can be detected by intracellular staining and FACS analysis. Lymphocytes were gated according to physical characteristics using FSC SSC plots.

The percentage of either IFN- γ ⁺ or TNF- α ⁺ cells present in drug treated cultures were expressed as a percentage of vehicle control (Figure 3.8 and 3.9, respectively).

No difference was observed in the percentage of either IFN- γ + or TNF- α + cells as a result of 5-HT_{3A} receptor activation by mCPBG (1 μ M; 104.97 ± 10.82 % and 89.08 ± 9.18 % of vehicle; mean \pm SEM; n=5 and n=7, respectively) despite combined application of mCPBG (1 μ M) and 5-chloroindole (10 μ M; 111.64 ± 13.35 % and 90.60 ± 8.95 % of vehicle; mean \pm SEM; n=5 and n=7, respectively). Treatment of PBMC with the positive allosteric modulator 5-chloroindole (10 μ M) alone did not affect either IFN- γ or TNF- α production (106.63 ± 14.85 % and 106.49 ± 8.48 % of vehicle; mean \pm SEM; n=5 and n=7, respectively).

There was no change in the percentage of lymphocytes expressing either the surface marker CD4 or CD8 as a result of treatment with mCPBG with or without 5-chloroindole. Consequently, there was no change in the CD4/CD8 ratio (Figure 3.10).

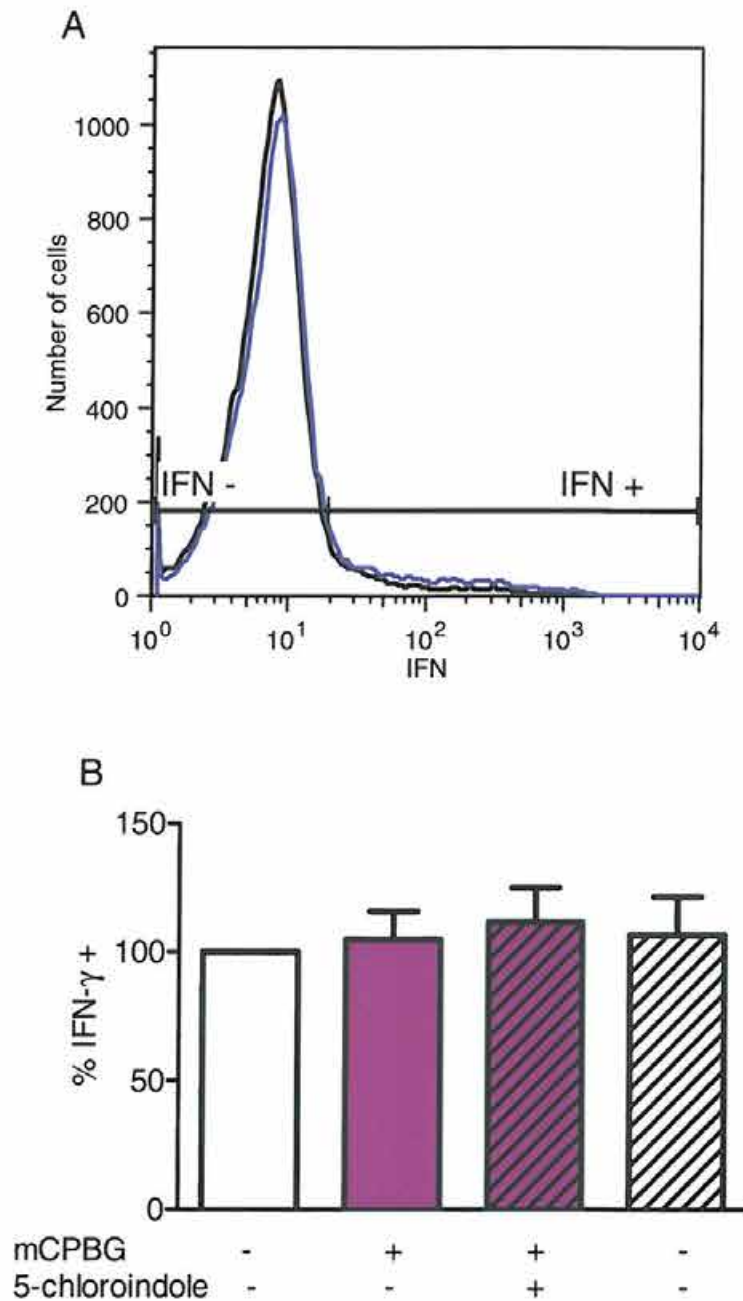


Figure 3.8: Activation of the 5-HT_{3A} receptor does not affect levels of IFN- γ production in lymphocytes. PHA-stimulated PBMC were incubated with mCPBG (1 μ M) \pm 5-chloroindole (10 μ M). Cells were treated with brefeldin A (10 μ g/ml), PMA (10 ng/ml) and ionomycin (250 ng/ml) for 4 hours prior to intracellular labelling for IFN- γ . A) IFN- γ histogram from a representative donor showing vehicle in black and mCPBG + 5-chloroindole treatment in purple. B) IFN- γ + cells expressed as a percentage of vehicle. Data represents the mean + SEM; n=5.

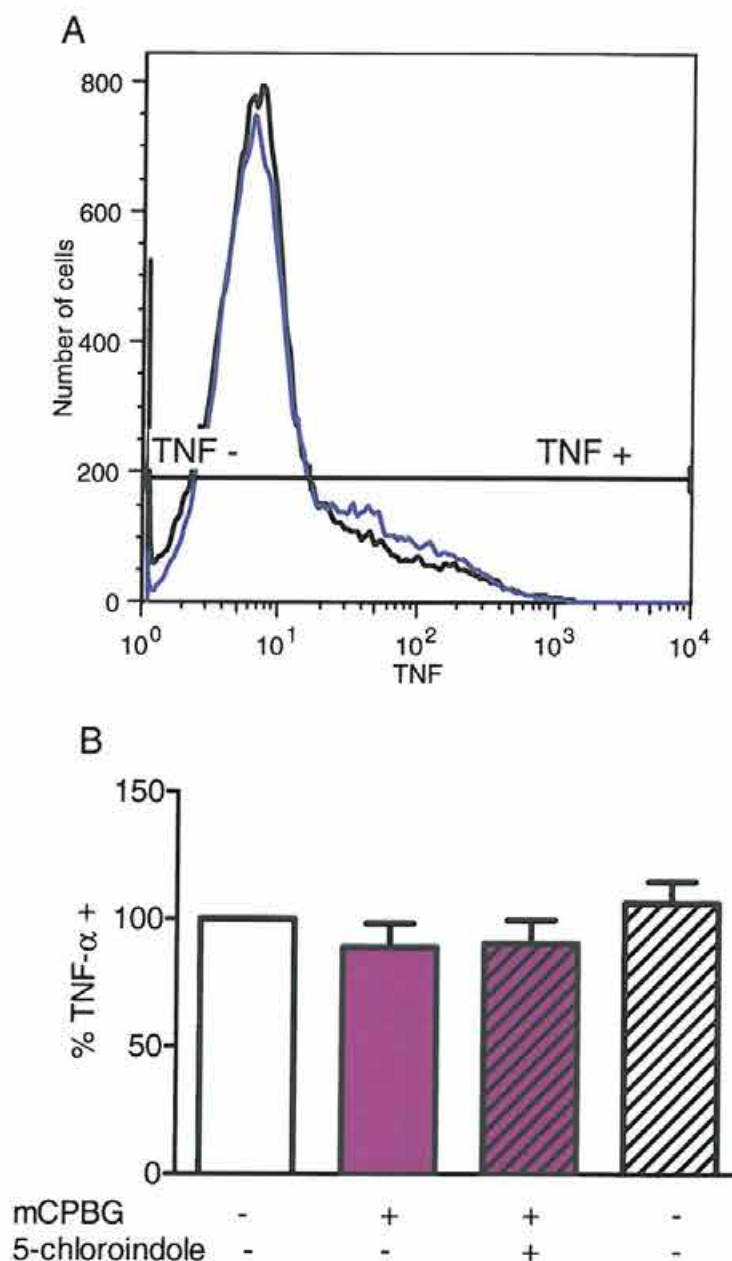


Figure 3.9: Activation of the 5-HT_{3A} receptor does not affect levels of TNF- α production in lymphocytes. PHA-stimulated PBMC were incubated with mCPBG (1 μ M) \pm 5-chloroindole (10 μ M). Cells were treated with brefeldin A (10 μ g/ml), PMA (10 ng/ml) and ionomycin (250 ng/ml) for 4 hours prior to intracellular labelling for TNF- α . A) TNF- α histogram from a representative donor showing vehicle in black and mCPBG + 5-chloroindole treatment in purple. B) TNF- α + cells expressed as a percentage of vehicle. Data represents the mean + SEM; n=7.

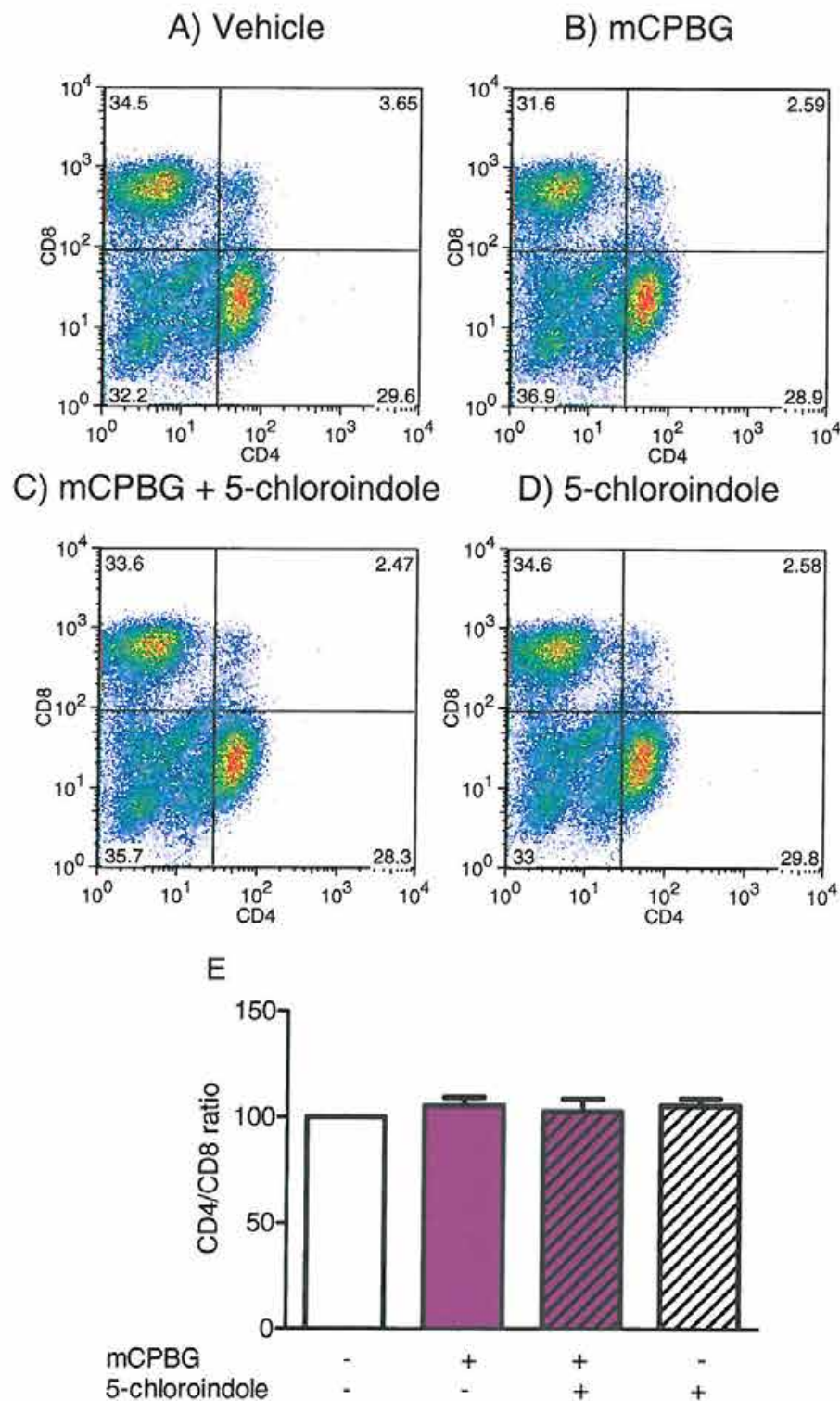


Figure 3.10: CD4/CD8 ratio is not affected by activation of the 5-HT₃A receptor. PBMC were incubated for 5 days with PHA and were labelled for CD4 and CD8 expression prior to FACS analysis. Representative dot plots of CD4 *versus* CD8 expression in lymphocyte-gated PBMC treated with A) vehicle B) 5-chloroindole (10 μ M) C) mCPBG (1 μ M) D) mCPBG +5-chloroindole. E) CD4/CD8 ratio was calculated by the percentage of CD4⁺ lymphocytes divided by the percentage of CD8⁺ T cells. Data represents the mean + SEM; n=4.

Section 3.5. Impact of 5-HT₃A receptor activation on the generation of CD25+FoxP3+ cells

Given the presence of 5-HT₃A receptor subunit mRNA transcripts in activated CD4+CD25+ T cells, and the expression of 5-HT₃A receptor protein at the surface of activated CD4+CD25+ T cells, it was asked next whether 5-HT₃A receptor activation might impact the generation of cells with a regulatory phenotype (CD25+FoxP3+) within stimulated cultures of PBMC. This was investigated using the 5-HT₃A receptor agonists DDP733 and mCPBG. PBMC were isolated from human buffy coats (Section 2.1.1) and stimulated with PHA (5 µg/ml) in the presence of DDP733 (100 nM ± 30 µM 5-chloroindole), mCPBG (1 µM ± 10 µM 5-chloroindole). After 5 days of culture, cells were stained at the surface for CD25 and intracellularly for the transcription factor FoxP3 before analysis by FACS (Section 2.4.1). Cells were gated for lymphocytes according to their physical characteristics using FSC SSC plots.

The percentage of lymphocytes with a regulatory phenotype (expressing both CD25 and FoxP3) was increased after treatment with DDP733 (100nM; 139.76 ± 13.45 % of vehicle; mean ± SEM; n=9, although not significant, Figure 3.11). This increase is exacerbated in cells treated with both DDP733 (100 nM) and 5-chloroindole (30 µM), which reaches statistical significance (221.62 ± 25.87 % of vehicle; mean ± SEM; P<0.01, n=9). This effect of 5-HT₃A receptor activation appears to be replicated by treatment with mCPBG (1 µM) and 5-chloroindole (10 µM) although is not statistically significant (219.52 ± 59.90 % of vehicle; mean ± SEM; n=9).

Treatment with 5-chloroindole (10 µM) alone does not impact the percentage of CD25+FoxP3+ cells in PBMC, consistent with the accepted definition of an allosteric

modulator. Despite binding to the 5-HT₃A receptor's putative allosteric binding site, 5-chloroindole has no effect without occupation of the 5-HT₃A receptor's orthosteric site (in this case DDP733; 100 nM or mCPBG; 1 μ M).

Expression of both CD25 and FoxP3 are often cited as a hallmark of a regulatory T cell. Classic regulatory T cells are frequently associated with CD4⁺ T cells (Fontenot *et al.*, 2003) therefore the effect of 5-HT₃A receptor activation in CD4⁺ T cells were investigated. CD4⁺ T cells were negatively isolated from freshly prepared PBMC (Section 2.1.2) and stimulated with CD3/CD28 activation beads in the presence of 5-HT₃A receptor agonists (DDP733; 100 nM or mCPBG; 1 μ M) with and without 5-chloroindole (10 μ M).

Treatment with the 5-HT₃A receptor agonists DDP733 (100 nM) or mCPBG (1 μ M) do not influence the expression of CD25 and FoxP3 in CD4⁺ T cells (84.78 ± 1.59 % and 82.50 ± 4.29 % of vehicle; mean \pm SEM; n=3, respectively; Figure 3.12) irrespective of the presence of 5-chloroindole (91.46 ± 7.34 % and 91.77 ± 3.57 % of vehicle; mean \pm SEM; n=3, respectively; Figure 3.12).

It is evident from the CD25 *versus* FoxP3 dot plots shown in Figure 3.11, that there is a shift in the fluorescence intensity of FoxP3 in PBMC, which is not so evident for CD25 expression. Therefore the median fluorescence intensity (MFI) was obtained using FlowJo FACS analysis software and expressed as a percentage of the MFI of stimulated PBMC treated with vehicle.

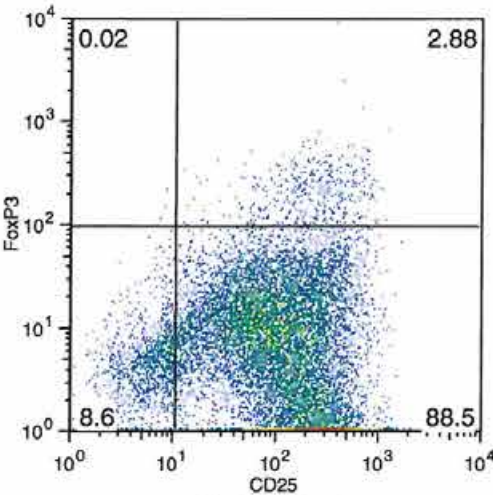
From this analysis, it can be noted that the MFI of FoxP3 expression in gated lymphocytes appears to be increased (though not reaching significance) following

application of DDP733 or mCPBG to PBMC (Figure 3.13; 276.13 ± 103.58 % and 348.18 ± 95.96 % of vehicle; mean \pm SEM; n=3, respectively). Potentiating 5-HT_{3A} receptor activation by mCPBG (but not DDP733) using 5-chloroindole (10 μ M) significantly increases the MFI of FoxP3 expression in lymphocytes (673.74 ± 107.92 % of vehicle; mean \pm SEM; $P < 0.05^*$; n=3; Figure 3.13).

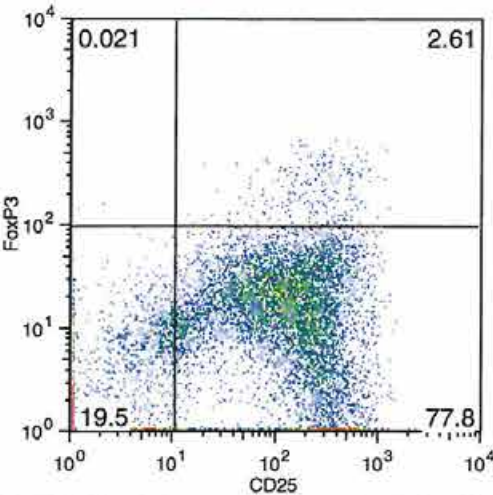
There was no change in the MFI of CD25 expression in gated lymphocytes from PBMC cultures treated with DDP733 (100nM; 100.73 ± 4.76 % of vehicle; mean \pm SEM; n=3) or mCPBG (1 μ M; 85.12 ± 3.77 % of vehicle; mean \pm SEM; n=3) with or without co-incubation with 5-chloroindole (10 μ M; 106.66 ± 15.49 % and 121.91 ± 3.03 % of vehicle; mean \pm SEM; n=3, respectively).

To confirm there is no similar effect of FoxP3 expression in isolated CD4⁺ T cells, MFI of CD25 and FoxP3 expression was analysed in the same way as PBMC (Figure 3.14). Consistent with Figure 3.12, there is no alteration in CD25 or FoxP3 expression in isolated CD4⁺ T cells determined using MFI.

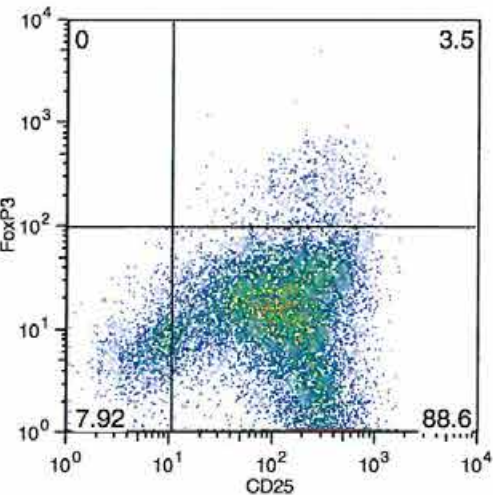
A) Vehicle



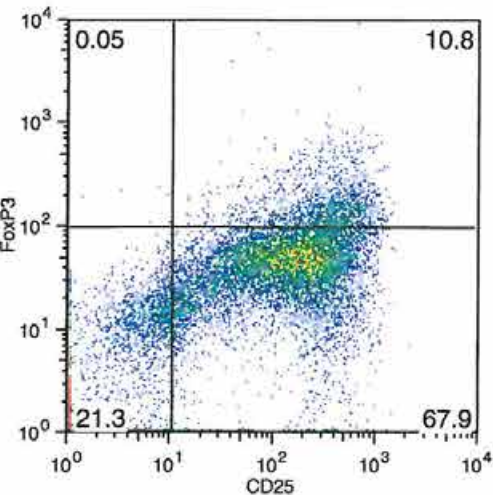
B) 5-chloroindole (30 μ M)



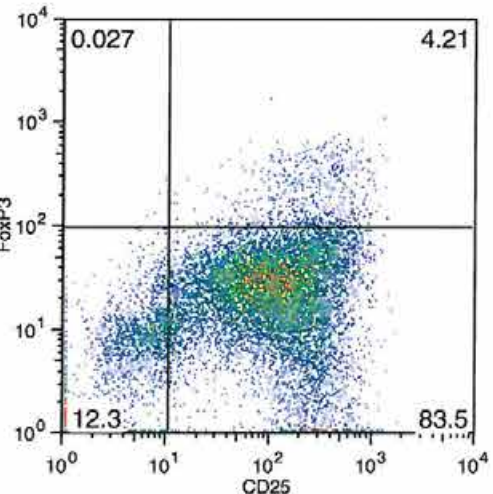
C) DDP733



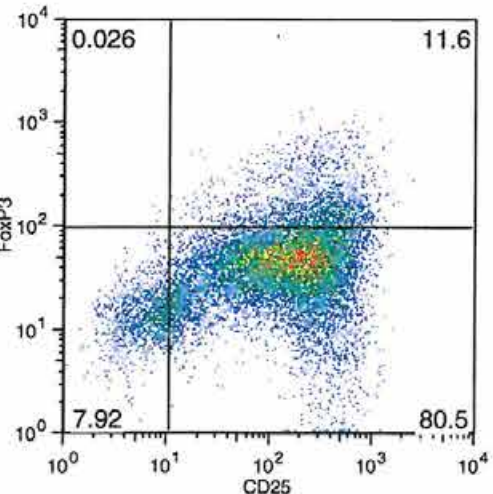
D) DDP733 + 5-chloroindole (10 μ M)



E) mCPBG



F) mCPBG + 5-chloroindole (30 μ M)



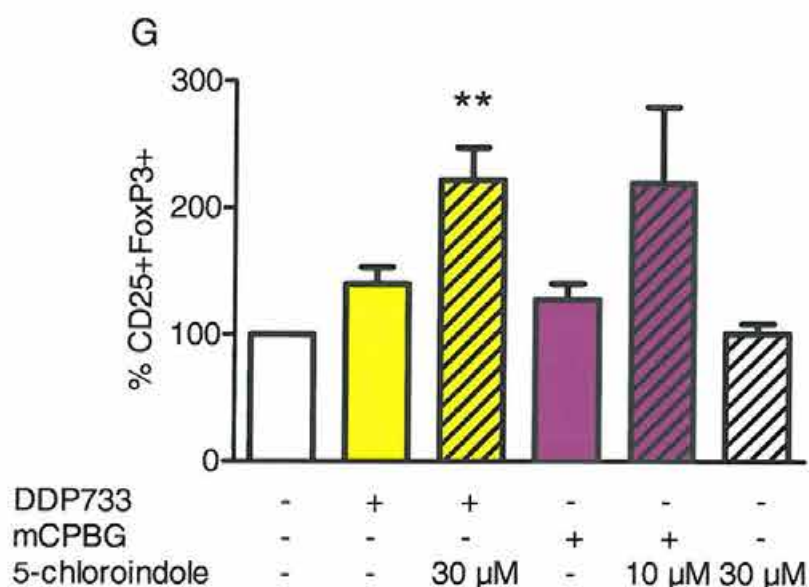
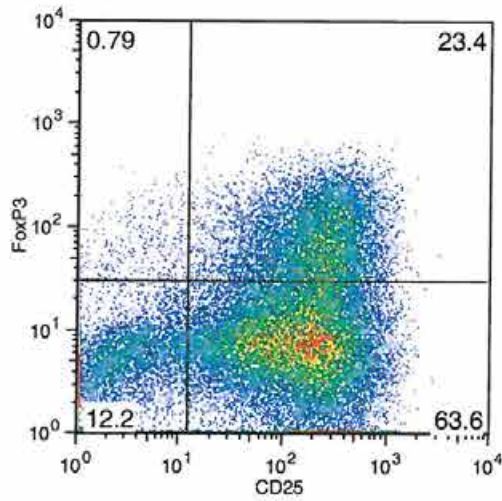
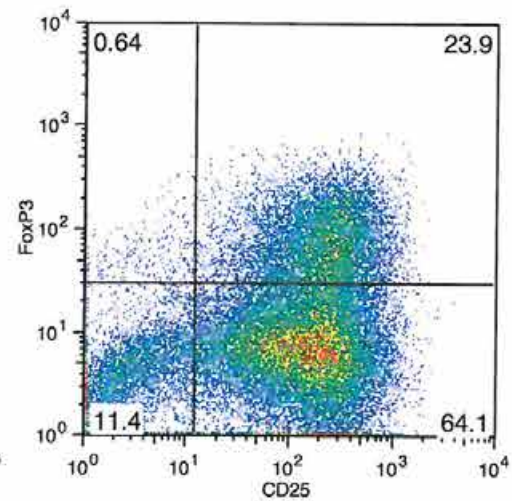
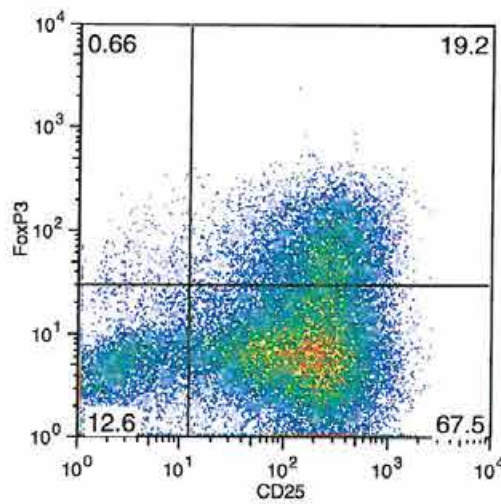
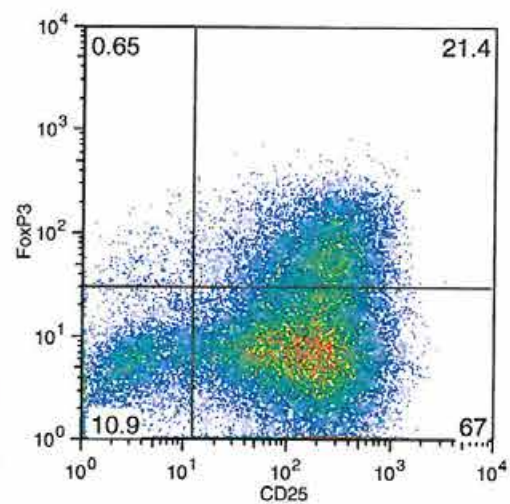


Figure 3.11: The percentage of CD25+FoxP3+ cells in PBMC increases as a consequence of 5-HT_{3A} receptor activation, potentiated by the positive allosteric modulator 5-chloroindole. PBMC were incubated for 5 days with PHA and DDP733 (100 nM), mCPBG (1 μ M) or 5-chloroindole. Cells were labelled for CD25 and intracellular FoxP3 expression prior to FACS analysis. Representative dot plots of CD25 *versus* FoxP3 expression in lymphocyte-gated PBMC treated with A) vehicle B) 5-chloroindole (30 μ M) C) DDP733 D) DDP733 + 5-chloroindole (10 μ M) E) mCPBG F) mCPBG + 5-chloroindole (10 μ M). G) Percentage of CD25+FoxP3+ lymphocytes in PBMC expressed as a percentage of vehicle. Friedman test $P=0.00005^{***}$ followed by Dunn's multiple comparison test where $P<0.01^{**}$. Data represents the mean + SEM; $n=9$.

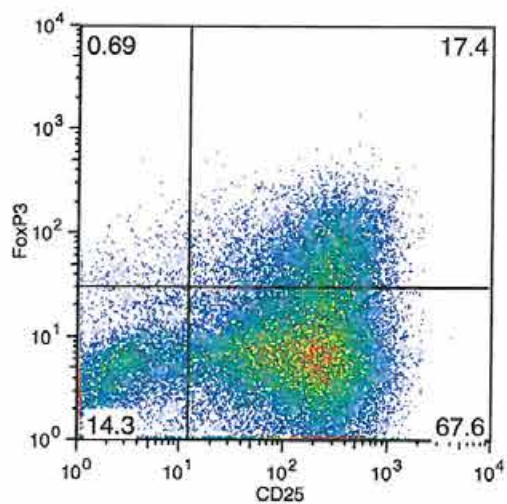
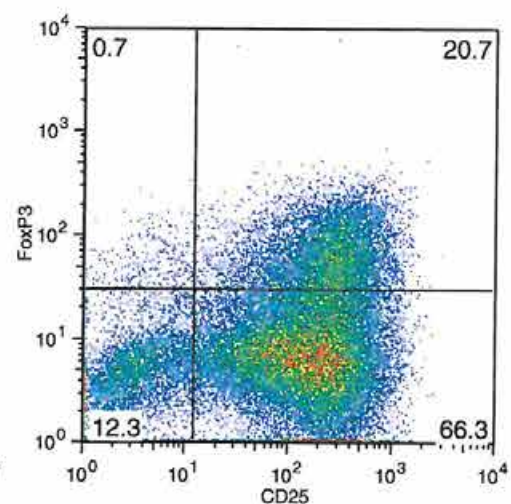
A) Vehicle

B) 5-chloroindole (30 μ M)

C) DDP733

D) DDP733 + 5-chloroindole (10 μ M)

E) mCPBG

F) mCPBG + 5-chloroindole (30 μ M)

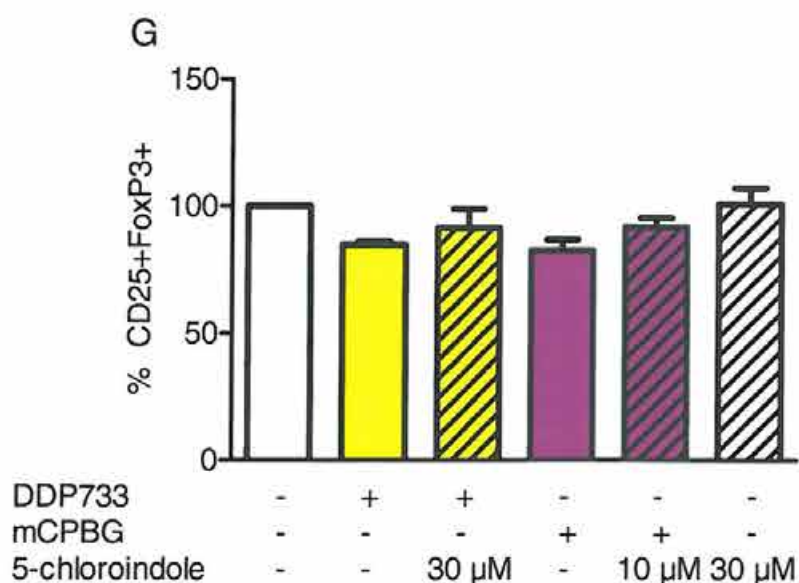


Figure 3.12: The percentage of CD25+FoxP3+ cells in isolated CD4+ T cells does not change as a consequence of 5-HT_{3A} receptor activation, potentiated by the positive allosteric modulator 5-chloroindole. CD4+ T cells were isolated from fresh PBMC and incubated for 5 days with CD3/CD28 activation beads and either vehicle, DDP733 (100 nM) or mCPBG (1 μ M) both \pm 5-chloroindole. Cells were labelled for CD25 and intracellular FoxP3 expression prior to FACS analysis. Representative dot plots of CD25 *versus* FoxP3 expression in CD4+ T cells treated with A) vehicle B) 5-chloroindole (30 μ M) C) DDP733 D) DDP733 + 5-chloroindole (10 μ M) E) mCPBG F) mCPBG + 5-chloroindole (10 μ M). G) Percentage of CD25+FoxP3+ lymphocytes in isolated CD4+ T cells expressed as a percentage of vehicle. Data represents the mean + SEM; n=3.

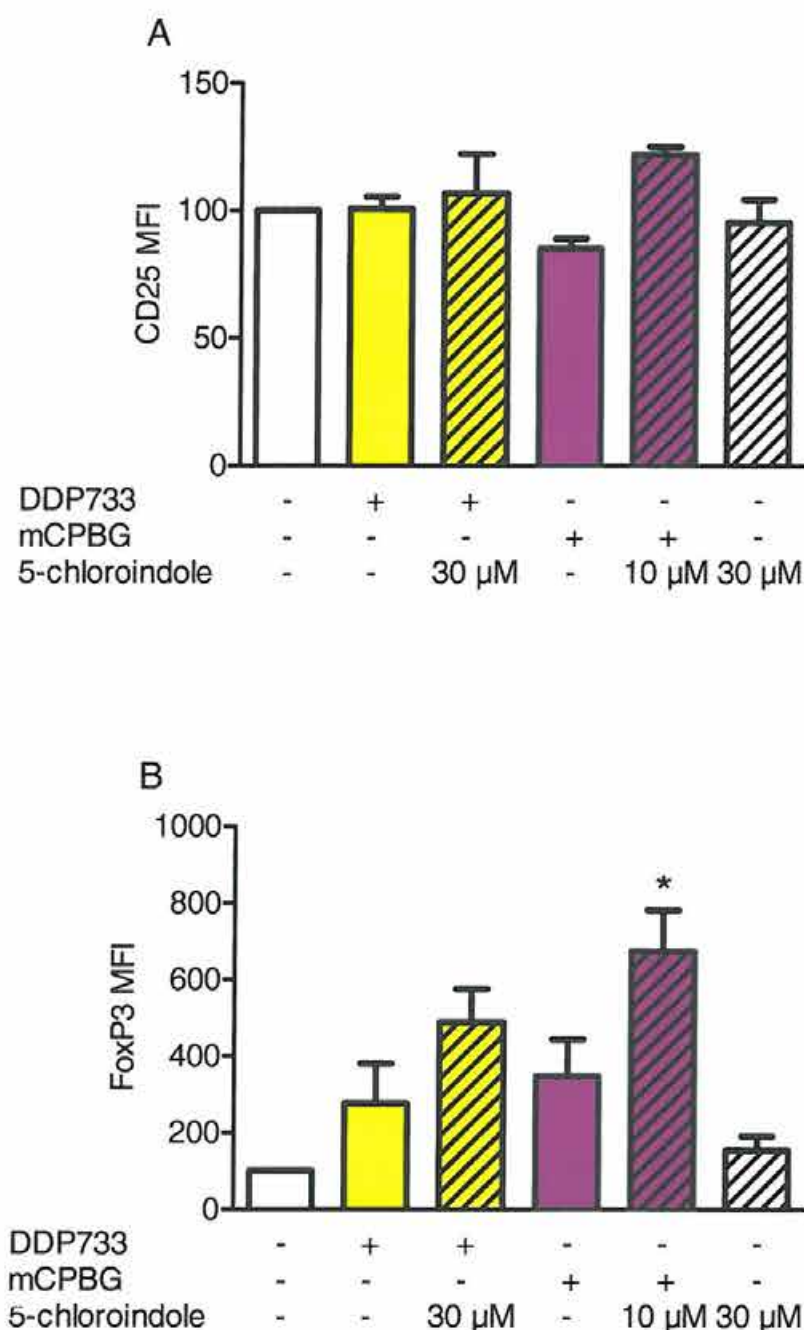


Figure 3.13: MFI of FoxP3 expression in PBMC increases upon activation of the 5-HT_{3A} receptor potentiated by the positive allosteric modulator 5-chloroindole, yet CD25 MFI does not. PBMC were incubated for 5 days with CD3/CD28 activation beads and either vehicle or DDP733 (100 nM) or mCPBG (1 μ M), both \pm 5-chloroindole. Cells were labelled for CD25 and intracellular FoxP3 expression prior to FACS analysis. MFI is expressed as a percentage of vehicle for A) CD25 and B) FoxP3 expression in PBMC. Friedman's test $P=0.0117^*$ followed by Dunn's multiple comparison test where $P<0.05^*$. Data represents the mean + SEM; $n=3$.

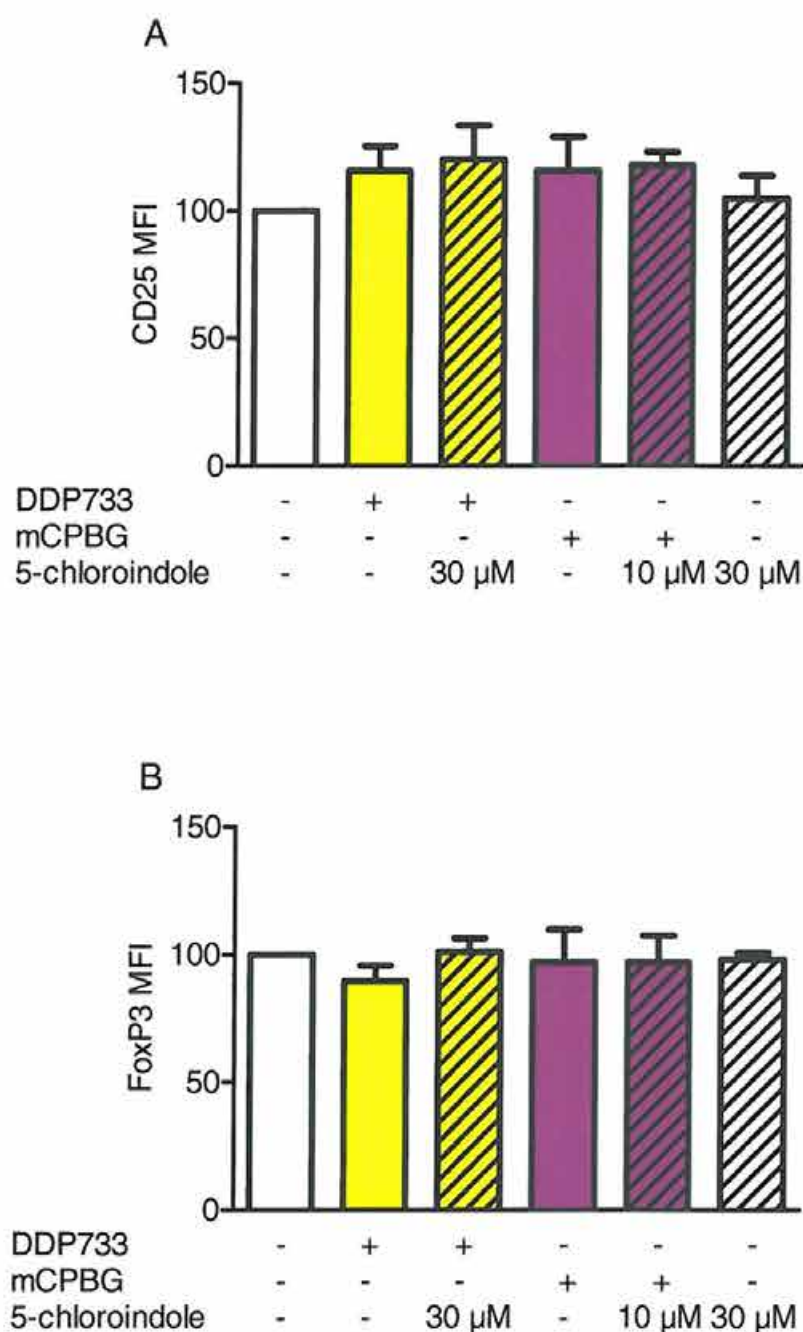


Figure 3.14: MFI of FoxP3 and CD25 expression in isolated CD4⁺ T cells is not affected by 5-HT₃A receptor activation despite potentiation by 5-chloroindole. CD4⁺ T cells were incubated for 5 days with CD3/CD28 activation beads and either vehicle or DDP733 (100 nM) or mCPBG (1 μ M), both \pm 5-chloroindole. Cells were labelled for CD25 and intracellular FoxP3 expression prior to FACS analysis. MFI is expressed as a percentage of vehicle for A) CD25 and B) FoxP3 expression in isolated CD4⁺ T cells. Data represents the mean \pm SEM; n=3.

Section 3.6 Impact of 5-HT₃A receptor activation on the proliferation and expression of activation markers in human PBMC

Given the observed increase in the percentage of CD25+FoxP3+ cells in stimulated PBMC cultures as a consequence of DDP733 plus 5-chloroindole, the effect of DDP733 plus 5-chloroindole on the proliferation of lymphocytes was investigated.

PBMC isolated from human buffy coats (Section 2.1.1) were labelled with CFSE (1 μ M; Section 2.4.2) and stimulated with PHA (5 μ g/ml). Cells were incubated with DDP733 (100 nM) \pm 5-chloroindole (30 μ M) for 5 days. Proliferation (defined as CFSE^{mid-low}) is expressed as a percentage of the proliferation of vehicle treated cells (Figure 3.15).

Activation of the 5-HT₃A receptor by DDP733 (100 nM) did not significantly reduce proliferation of PBMC (81.23 \pm 9.93 % of vehicle; mean \pm SEM; n=8). However, the combined application of DDP733 (100 nM) plus 5-chloroindole (30 μ M), resulted in a significant inhibition of proliferation (65.58 \pm 8.17 % of vehicle; mean \pm SEM; P<0.001; n=8). 5-chloroindole alone had no effect on proliferation (96.13 \pm 3.24 % of vehicle; mean \pm SEM; n=8).

To further investigate the impact of 5-HT₃A receptor activation on lymphocyte proliferation, the expression of early activation markers were investigated at 24 hours post stimulation. Treatment of PBMC with DDP733 (100 nM) did not alter the percentage of CD3+CD69+ (104.05 \pm 3.07 % of vehicle; mean \pm SEM; n=5; Figure 3.16), neither did the combined addition of 5-chloroindole (30 μ M, and DDP733; 100 nM; 110.68 \pm 6.13 % of vehicle; mean \pm SEM; n=5; Figure 3.16). Similarly, the

expression of CD3+CD71+ cells was not modulated by the 5-HT_{3A} receptor agonist DDP733 (100 nM; 102.85 ± 4.73 % of vehicle; mean \pm SEM; n=5; Figure 3.17) even when combined with 5-chloroindole (30 μ M; 106.79 ± 8.65 % of vehicle; mean \pm SEM; n=5; Figure 3.17). Treatment with the allosteric modulator alone (5-chloroindole; 30 μ M) did not affect the percentage expression of CD3+CD69+ (113.24 ± 6.38 % of vehicle; mean \pm SEM; n=5; Figure 3.16) or CD3+CD71+ T cells (103.14 ± 4.83 % of vehicle; mean \pm SEM; n=5; Figure 3.17).

The percentage of CD25+CD27+ cells is statistically increased in PBMC treated with both DDP733 and 5-chloroindole (117.09 ± 7.09 % of vehicle; mean \pm SEM; n=5; Figure 3.18), though there is no increase in expression with DDP733 alone (100.11 ± 3.22 % of vehicle; mean \pm SEM; n=5). The percentage of CD25+CD27+ cells is also statistically increased when cultures are treated with 5-chloroindole alone (120.45 ± 8.18 % of vehicle; mean \pm SEM; n=5). The percentage of CD25+CD27+ in PBMC treated with 5-chloroindole, with and without DDP733 was similar (117.09 ± 7.08 % and 120.45 ± 8.18 % of vehicle, respectively).

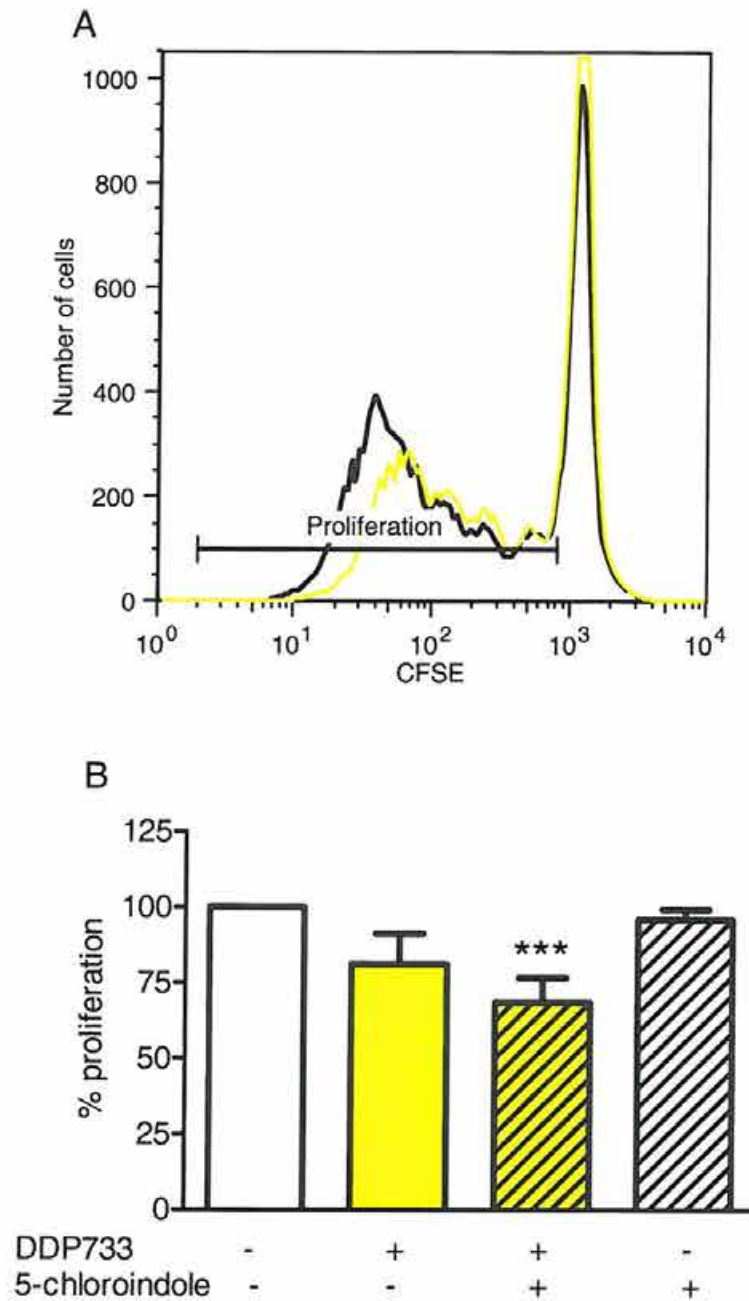


Figure 3.15: Proliferation of PBMC is inhibited as a consequence of 5-HT₃A receptor activation potentiated by the positive allosteric modulator 5-chloroindole. CFSE-labelled PBMC were incubated for 5 days with PHA and DDP733 (100 nM) ± 5-chloroindole (30 μM). Events were gated for stimulated lymphocytes according to physical characteristics using FSC *versus* SSC plots. A) CFSE histogram plot from a representative donor where proliferation of vehicle-treated cells is shown in black, and DDP733 + 5-chloroindole in yellow. B) Proliferation (CFSE^{mid-low}) expressed as a percentage of vehicle-treated cells. Data represents the mean + SEM; n=8. Friedman Test P=0.0015** followed by Dunn's multiple comparison test where P<0.001***.

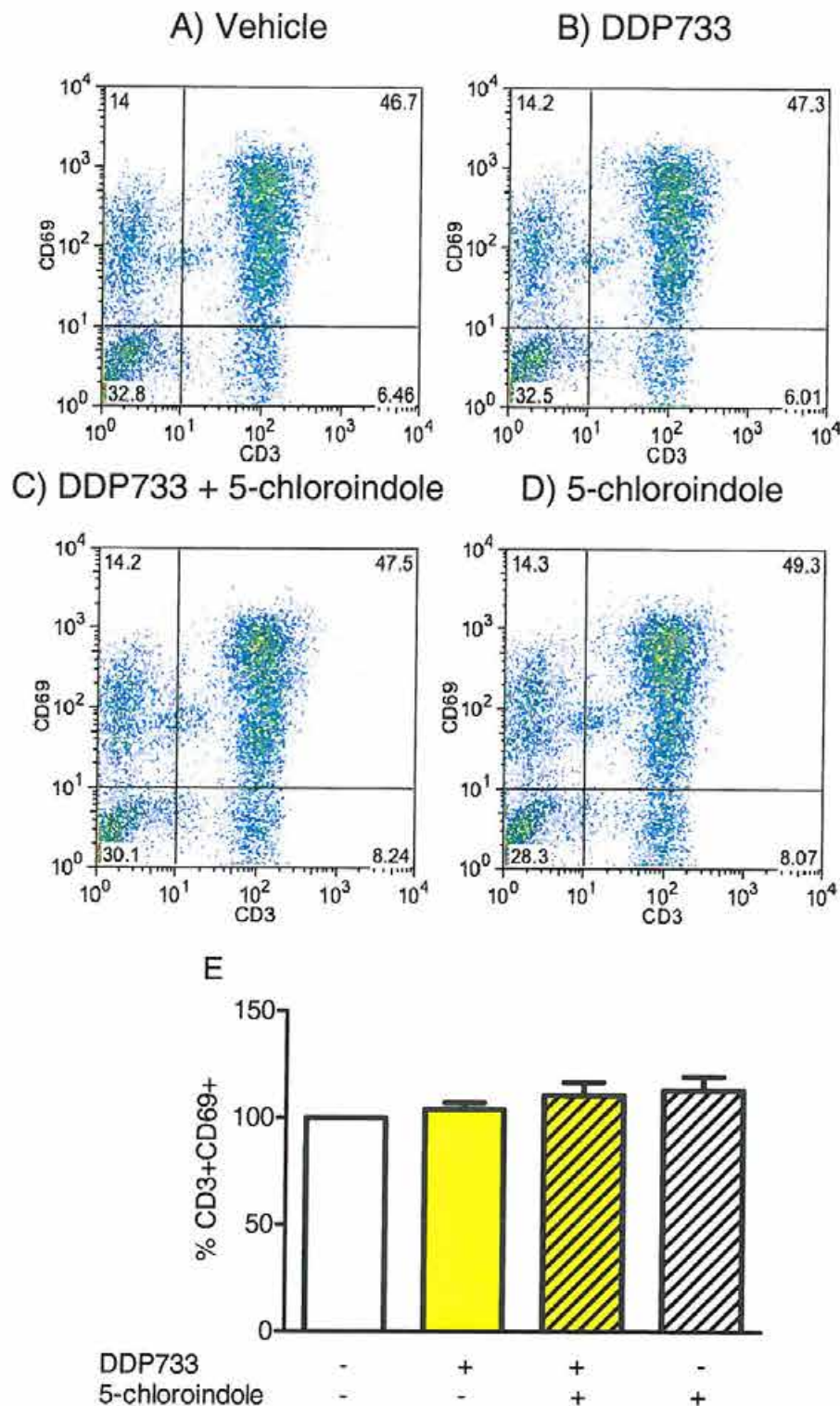


Figure 3.16: 5-HT_{3A} receptor compounds do not affect the percentage of CD3+CD69+ lymphocytes. PBMC were incubated for 24 hours with CD3/CD28 activation beads with DDP733 (100 nM) ± 5-chloroindole (30 μM) and labelled for CD3 and CD69 prior to FACS analysis. CD3 *versus* CD69 dot plots from a representative donor A) vehicle B) DDP733 C) DDP733 + 5-chloroindole D) 5-chloroindole. E) Frequency of CD3+CD69+ lymphocytes expressed as a percentage of vehicle. Data represents the mean + SEM; n=5.

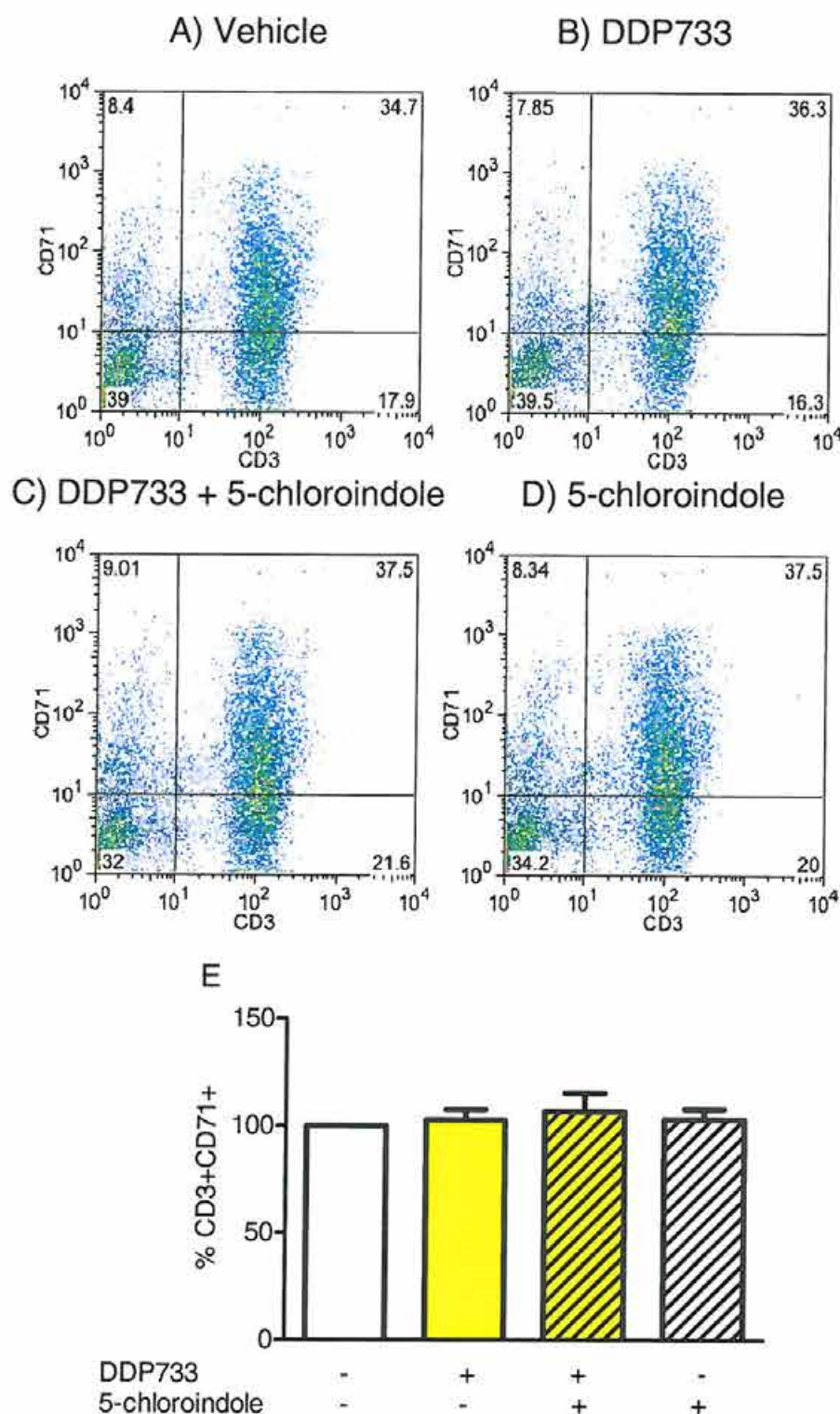


Figure 3.17: 5-HT₃A receptor compounds do not affect the percentage of CD3+CD71+ lymphocytes. PBMC were incubated for 24 hours with CD3/CD28 activation beads with DDP733 (100 nM) ± 5-chloroindole (30 μM) and labelled for CD3 and CD71 prior to FACS analysis. CD3 *versus* CD71 dot plots from a representative donor A) vehicle B) DDP733 C) DDP733 + 5-chloroindole D) 5-chloroindole. E) Frequency of CD3+CD71+ lymphocytes expressed as a percentage of vehicle. Data represents the mean + SEM; n=5.

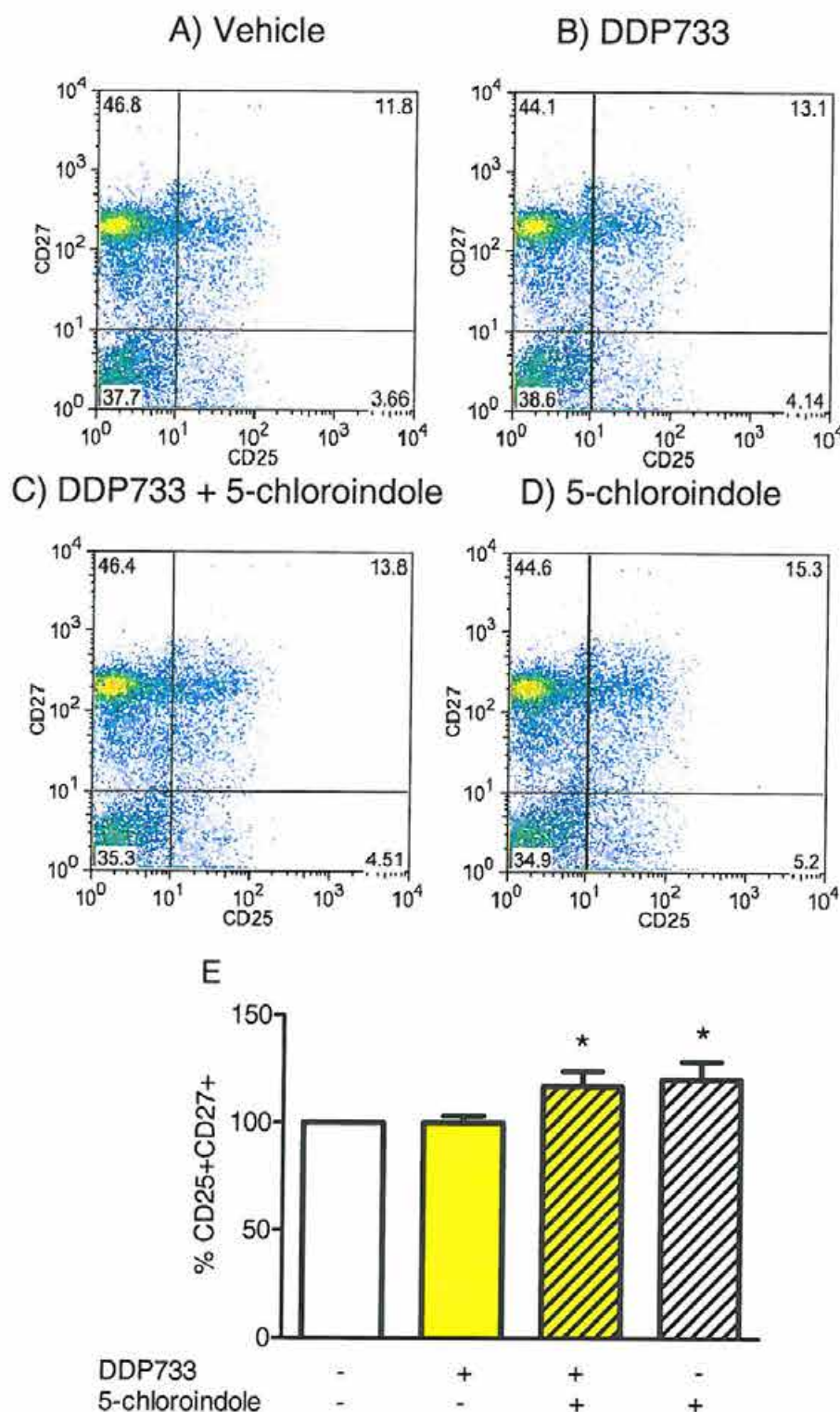


Figure 3.18: Impact of 5-HT_{3A} receptor compounds on the percentage of CD25+CD27+ lymphocytes. PBMC were incubated for 24 hours with CD3/CD28 activation beads with DDP733 (100 nM) \pm 5-chloroindole (30 μ M) and labelled for CD25 and CD27 prior to FACS analysis. CD25 *versus* CD27 dot plots from a representative donor A) vehicle B) DDP733 C) DDP733 + 5-chloroindole D) 5-chloroindole. E) Frequency of CD25+CD27+ lymphocytes expressed as a percentage of vehicle. Repeated measures ANOVA $P=0.0101^*$ followed by Dunnett's multiple comparison test where $P<0.05^*$. Data represents the mean + SEM; $n=5$.

Section 3.7 Impact of 5-HT₃A receptor antagonism on human lymphocytes

Potential of 5-HT₃A receptor activation in PBMC resulted in an increase in the percentage of CD25+FoxP3+ lymphocytes, which correlated with a reduction in overall lymphocyte proliferation. Subsequently, the effect of 5-HT₃A receptor antagonism on the percentage of cells with a regulatory phenotype (CD25+FoxP3+) was investigated using the selective 5-HT₃A receptor antagonist granisetron (1 μ M).

PBMC were isolated from human buffy coats (Section 2.1.1) and stimulated with PHA (5 μ g/ml) and either vehicle or granisetron (1 μ M). After 5 days of culture, cells were stained for CD25 and the intracellular transcription factor FoxP3 (Section 2.4.1) before analysis by FACS. Lymphocytes were gated according to their physical characteristics using FSC SSC dot plots. The percentage of lymphocytes expressing both CD25 and FoxP3 in cultures treated with granisetron was expressed as a percentage of vehicle.

There is a statistically significant increase in lymphocytes with a CD25+FoxP3+ regulatory phenotype in the granisetron treated cultures (1 μ M; 134.51 ± 6.48 % of vehicle; mean \pm SEM; $P < 0.01$; $n = 6$; Figure 3.19). The MFI of both CD25 and FoxP3 expression in PBMC was not altered by granisetron treatment (1 μ M; 90.15 ± 8.62 % and 104.91 ± 8.33 % of vehicle; mean \pm SEM; $n = 3$, respectively; Figure 3.21). Granisetron (1 μ M) did not cause an increase in the percentage of CD25+FoxP3+ cells (93.18 ± 6.19 % of vehicle; mean \pm SEM; $n = 3$) when applied to isolated CD4+ T cells (stimulated with CD3/CD28 activation beads; Figure 3.20). Similarly, there was no change in the MFI of both CD25 and FoxP3 expression in CD4+ T cells treated

with granisetron (1 μ M; 98.49 ± 1.52 % and 98.61 ± 4.98 % of vehicle; mean \pm SEM; n=3, respectively; Figure 3.21).

Given the increase in the percentage of CD25+FoxP3+ cells appearing in PBMC cultures, the effect of granisetron on the proliferation of CFSE-labelled PBMC was investigated. Cells were stimulated with PHA and incubated with granisetron (1 μ M) for 5 days. Cells were gated for lymphocytes according to their physical characteristics using FSC SSC plots and proliferation was determined by CFSE intensity (CFSE^{mid-low}). Proliferation of granisetron treated cells was expressed as a percentage of vehicle treatment.

Despite the increase in the percentage of CD25+FoxP3+ cells in PBMC, proliferation of PBMC was not affected by granisetron treatment (1 μ M; 94.30 ± 9.79 % of vehicle; mean \pm SEM; n=11; Figure 3.22).

Subsequent studies using granisetron (1 μ M) have so far revealed no other functional effect of antagonism of the 5-HT_{3A} receptor in human PBMC (Figure 3.23). Granisetron (1 μ M) treatment did not affect the percentage expression of CD3+CD69+ T cells (106.70 ± 3.52 %; mean \pm SEM; n=5), CD3+CD71+ T cells (98.83 ± 2.20 %; mean \pm SEM; n=5) or CD25+CD27+ T cells (101.90 ± 4.78 % of vehicle; mean \pm SEM; n=5) in activated PBMC.

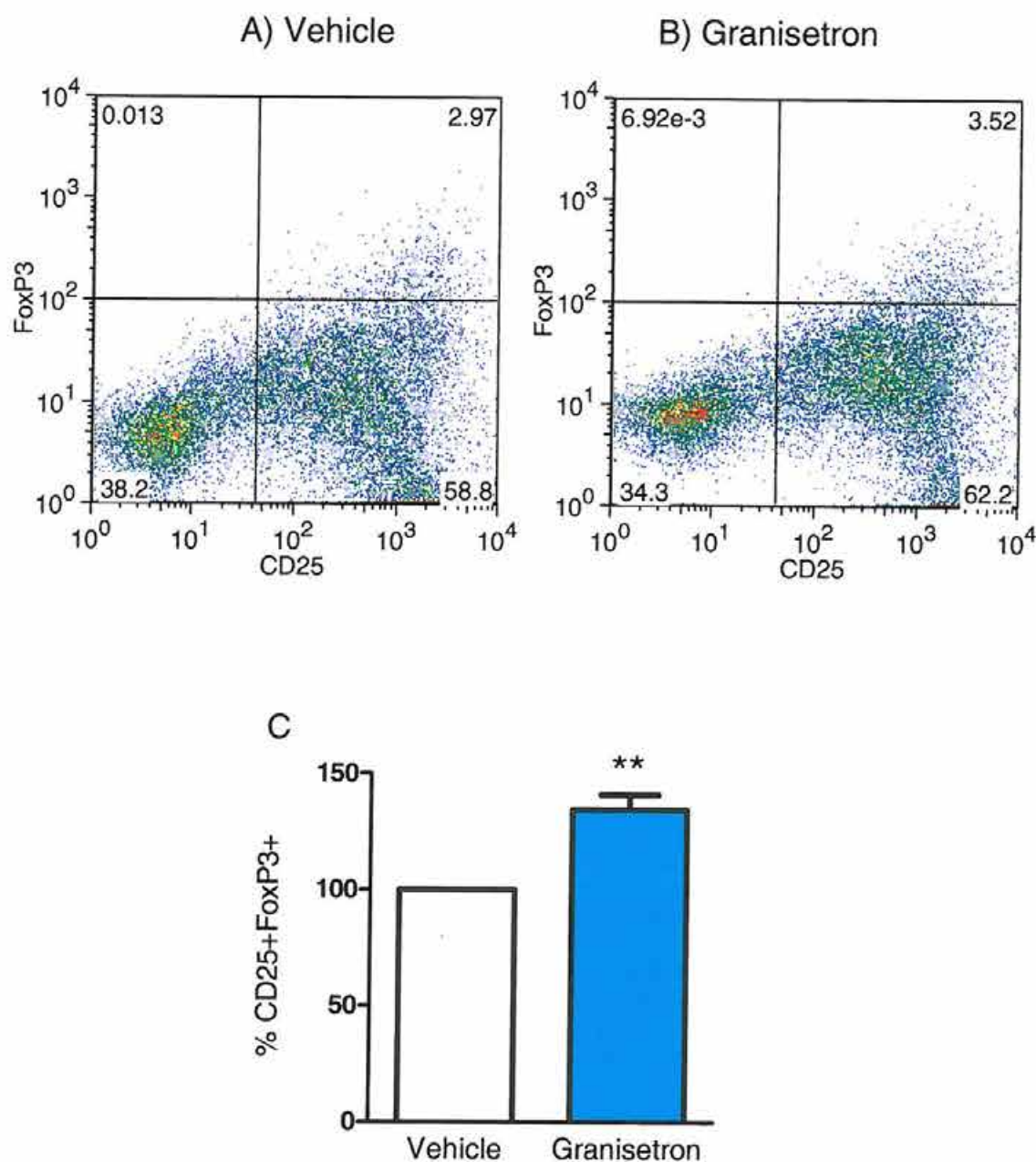


Figure 3.19: Antagonism of the 5-HT_{3A} receptor increases the percentage of CD25+FoxP3+ cells in PBMC. Isolated PBMC were incubated for 5 days with CD3/CD28 activation beads and either vehicle or granisetron (1 μ M). Cells were labelled for CD25 and intracellular FoxP3 expression prior to FACS analysis. Representative dot plots of CD25 *versus* FoxP3 expression in lymphocyte-gated PBMC treated with A) vehicle and B) granisetron. C) Percentage of CD25+FoxP3+ lymphocytes in PBMC expressed as a percentage of vehicle. Two-tailed paired T test where $P=0.0031^{**}$. Data represents the mean + SEM; $n=6$.

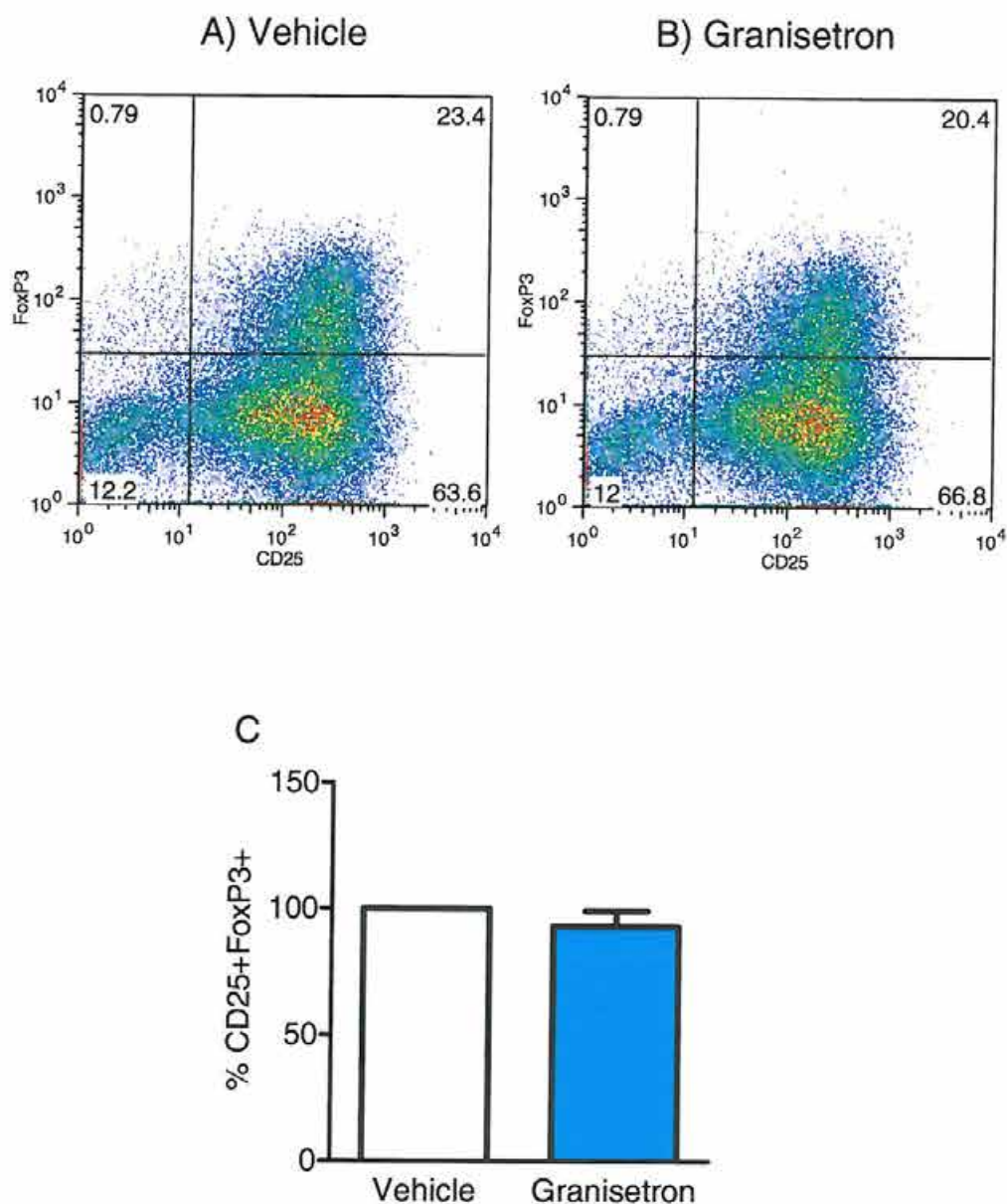


Figure 3.20: Antagonism of the 5-HT_{3A} receptor does not change the percentage of CD25+FoxP3+ cells in isolated CD4+ T cells. CD4+ T cells were negatively isolated from freshly prepared PBMC. Cells were incubated for 5 days with CD3/CD28 activation beads and either vehicle or granisetron (1 μ M). Cells were labelled for CD25 and intracellular FoxP3 expression prior to FACS analysis. Representative dot plots of CD25 *versus* FoxP3 expression in CD4+ T cells treated with A) vehicle and B) granisetron. C) Percentage of CD25+FoxP3+ lymphocytes in isolated CD4+ T cells expressed as a percentage of vehicle. Data represents the mean + SEM; n=3.

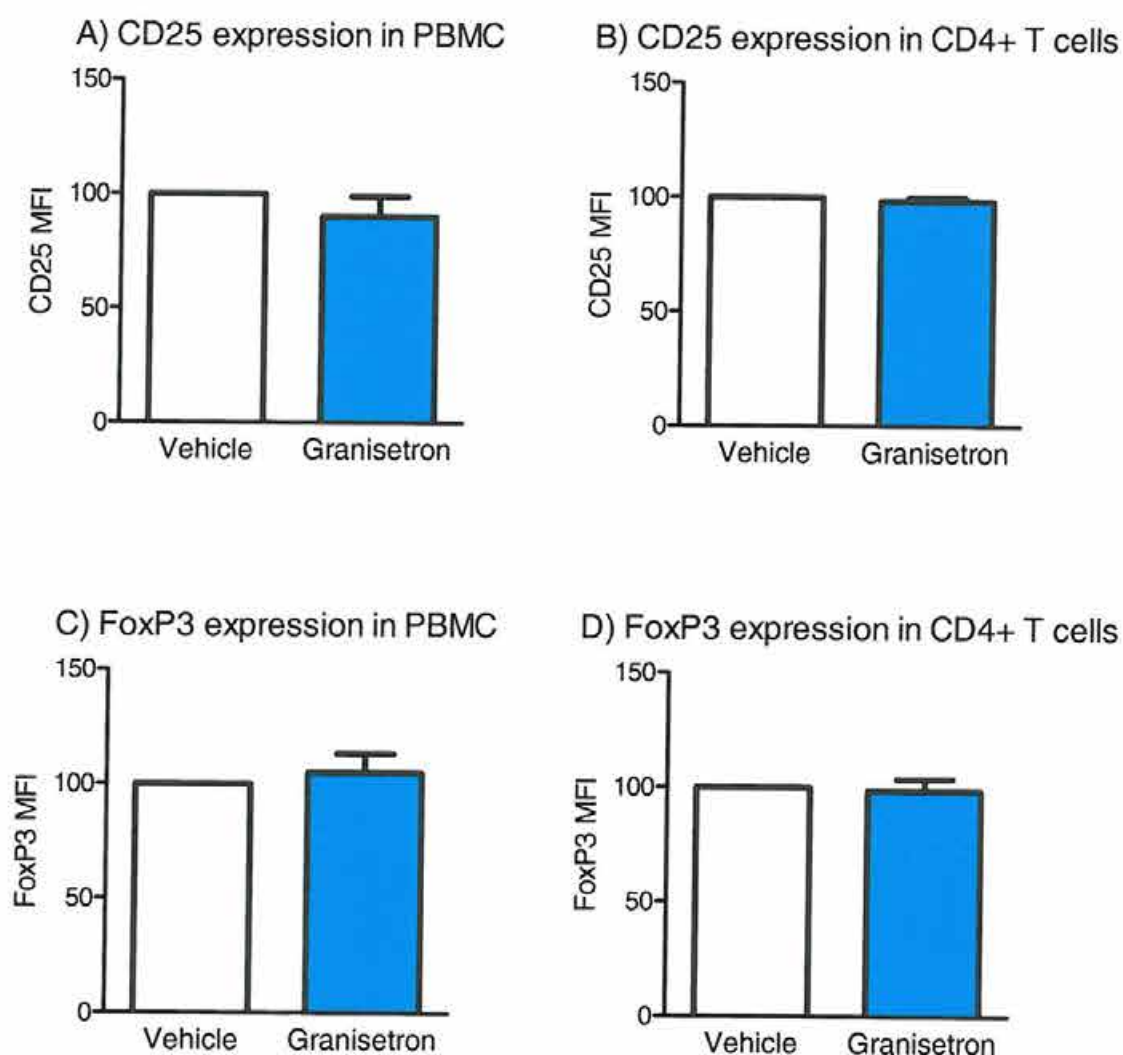


Figure 3.21: MFI of CD25 and FoxP3 expression in PBMC and isolated CD4+ T cells is not increased as a result of granisetron treatment. PBMC and isolated CD4+ T cells were incubated for 5 days with CD3/CD28 activation beads and either vehicle or granisetron (1 μ M). Cells were labelled for CD25 and intracellular FoxP3 expression prior to FACS analysis. Events were gated for stimulated lymphocytes according to physical characteristics using FSC SSC plots. MFI is expressed as a percentage of vehicle. Data represents the mean + SEM; n=3.

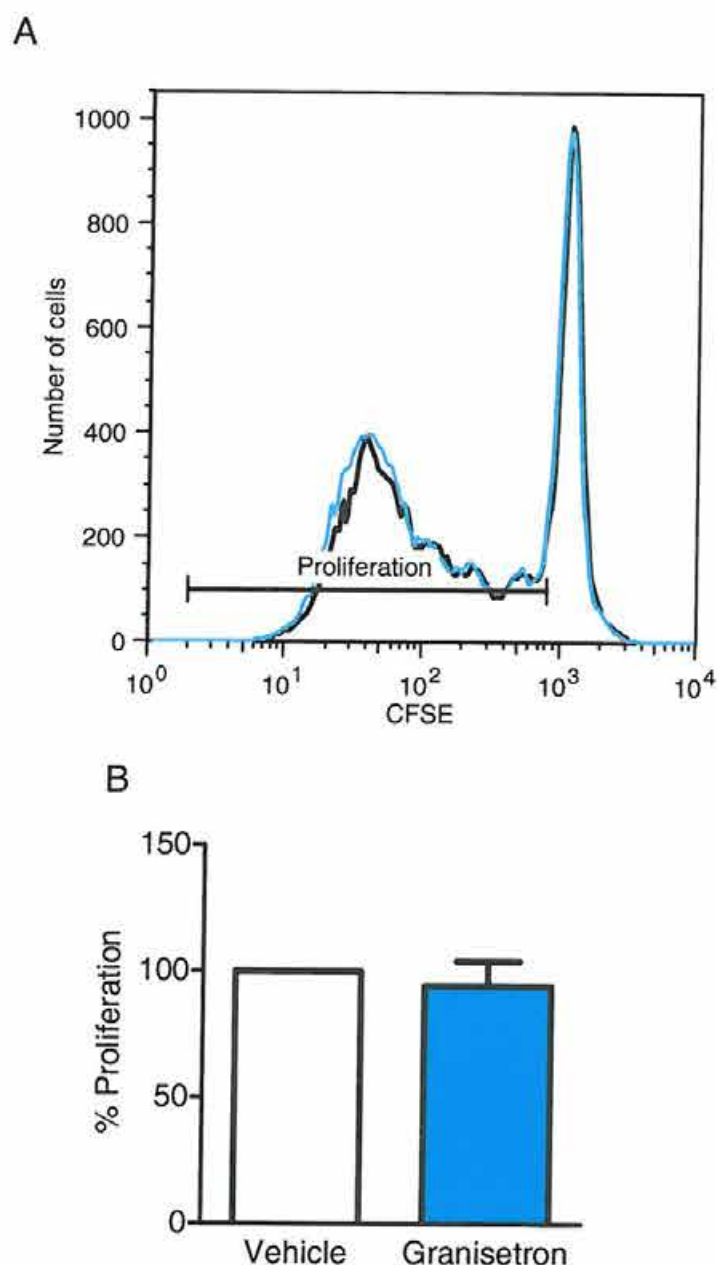


Figure 3.22: Proliferation of PBMC is not inhibited despite increased CD25+FoxP3+ expression in PBMC as a consequence of 5-HT₃A receptor activation potentiated by the positive allosteric modulator 5-chloroindole. CFSE-labelled PBMC were incubated for 5 days with PHA and either vehicle or granisetron (1 μ M). Events were gated for stimulated lymphocytes according to physical characteristics using FSC SSC plots. A) CFSE histogram plot from a representative donor where proliferation of vehicle-treated cells is shown in black, and granisetron in blue. B) Proliferation (CFSE^{mid-low}) expressed as a percentage of vehicle-treated cells. Data represents the mean + SEM; n=11.

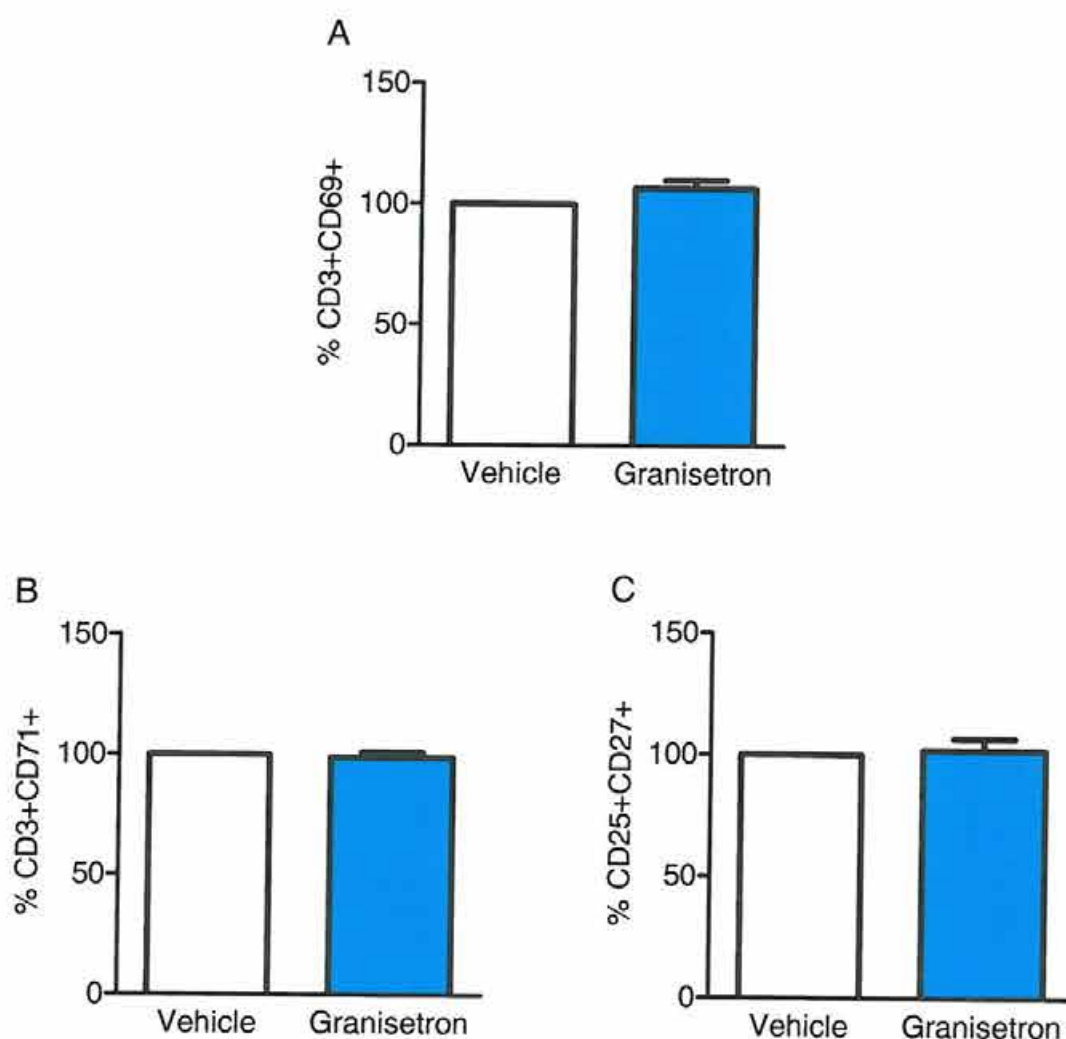


Figure 3.23: 5-HT_{3A} receptor antagonism does not affect the percentage expression of activation markers in lymphocytes. PBMC were incubated for 24 hours with CD3/CD28 activation beads and granisetron (1 μ M). Cells were labelled for activation markers prior to FACS analysis. Events were gated on stimulated lymphocytes according to physical characteristics using FSC SSC plots. Expression of A) CD3+CD69+ B) CD3+CD71+ C) CD25+CD27+ lymphocytes expressed as a percentage of vehicle. Data represents the mean + SEM; n=5.

Section 3.8 Discussion

Section 3.8.1 Expression of 5-HT₃A receptors in human immune cells

5-HT₃A receptors have been identified in human T cells (Fiebich *et al.*, 2004b), but the authors did not specify the isolation method used for their T cell preparation and are therefore most likely to contain a wide variety of T cell subtypes. In the current study, the expression of 5-HT₃A receptor mRNA transcripts was assessed in CD4⁺ T cell subsets, based on the expression of CD25. Transcripts encoding the 5-HT₃A receptor subunit were found expressed only in activated CD4⁺CD25⁺ T cells (Figure 3.2). Restriction of the expression of 5-HT₃A receptor subunit may indicate a specific role of the 5-HT₃A receptor in activated CD4⁺CD25⁺ regulatory T cells. Expression of 5-HT₃A receptor subunit protein was also identified on the surface of activated CD4⁺CD25⁺ T cells (Figure 3.5). Although it is quite possible that the 5-HT₃A receptor subunit is expressed in other non-CD4⁺ T cells subsets, it is evident that the distribution of the 5-HT₃A receptor subunit is confined to definite subpopulations of CD4⁺ T cell, rather than be ubiquitously expressed by all CD4⁺ T cells. Although it should be noted that expression of functional 5-HT₃ receptors is dependent on the correct arrangement of five subunits, of which the 5-HT₃A receptor subunit is an essential component, the potential for both functional homomeric 5-HT₃A receptors and heteromeric 5-HT₃ receptors would contribute to the diverse repertoire of the immune system.

The 5-HT₃A receptor conducts primarily Na⁺, K⁺ and Ca²⁺ and upon activation causes depolarisation and the release of neurotransmitters in neurones. In the immune system, extracellular Ca²⁺ stabilises binding of the TCR to antigen, while intracellular

Ca^{2+} is an important second messenger (Vig and Kinet, 2009). Therefore, due to its expression in the immune system, activation of the 5-HT_{3A} receptor may contribute to the control of $[\text{Ca}^{2+}]_i$, which ultimately may impact T cell activation.

In PCR expression studies, the expression of 5-HT_{3A} receptor subunit transcripts were found restricted to the activated CD4+CD25+ subset only. CD4+CD25+ regulatory T cells play an important role in the homeostasis of the immune system and in curbing inflammatory responses during an immune response once the invading pathogen has been defeated, through the suppression of activated immune cells. The expression of the transcription factor FoxP3 is widely cited as the 'master regulator' and has become a hallmark of naturally occurring regulatory T cells (Feuerer *et al.*, 2009). However, FoxP3 is located intracellularly, thus making the isolation of functional cells based solely on FoxP3 expression impossible. To complicate matters further, the expression of both CD25 and FoxP3 is upregulated generally upon activation, which has made the distinction between naturally occurring CD4+CD25+FoxP3+ regulatory T cells difficult. Induction of FoxP3 in activated T cells, which are subsequently capable of suppression are known as peripherally-induced regulatory T cells. However, the surface marker CD127 had been shown to be simultaneously downregulated upon activation of FoxP3. The mean percentage expression of FoxP3 in CD4+CD127^{low}CD25+ T cells in PBMC from ten healthy donors was >85 % (Liu *et al.*, 2006) making CD127 a useful surface marker for the discrimination between naturally occurring regulatory T cells (CD4+CD127^{low}CD25+FoxP3+) and activated helper T cells (CD4+CD127+CD25+FoxP3+), therefore enabling the isolation of purer naturally occurring regulatory T cells.

	Mean % of FoxP3	Range
CD4+CD127 ^{low} CD25+	86.6	67.4-93.6
CD4+CD127 ^{low} CD25-	25.5	14.8-39.5
CD4+CD127+CD25+	22.9	11.5-39.2

Table 3.1: Mean percentage of FoxP3 expression in CD127 T cell subsets assessed using flow cytometry. PBMC were stained for cell surface expression of CD4, CD25 and CD127 and subsequently fixed, permeabilised and stained intracellularly for FoxP3. Lymphocytes were gated based on FSC SSC profiles. Data shown is from Liu *et al.*, 2006.

Section 3.8.2 Impact of 5-HT₃A receptor activation on the function of regulatory T cells and lymphocyte phenotypes

Activation of the 5-HT₃A receptor by DDP733 plus 5-chloroindole did not impact regulatory T cell-mediated suppression of effector cells (Figure 3.7.2). DDP733 is a partial 5-HT₃A receptor agonist, therefore may have acted as an antagonist at 5-HT₃ receptors should 5-HT have been present. A possible source of 5-HT may have been the serum used to supplement the growth media, however heat-inactivated FCS was used in experiments using human immune cells, therefore 5-HT would have been degraded by high temperatures (56 °C for 30 minutes). Should this trend be a result of antagonism of the 5-HT₃A receptor by DDP733 and 5-chloroindole, it may appear contradictory that treatment with granisetron, a 5-HT₃ receptor antagonist, did not result in a similar trend (a reduction in the number of proliferated cells). SR57227A is almost a full agonist at the 5-HT₃ receptor, therefore could be used in future experiments to eliminate the possibility of partial agonists acting as antagonists. Antagonism of the 5-HT₃A receptor by granisetron also did not modulate the level of suppression compared to vehicle treated cells.

The expression of the 5-HT_{3A} receptor may represent a novel subset of regulatory T cells, however these data may indicate that the function of the 5-HT_{3A} receptor expressed by activated CD4⁺CD25⁺ T cells may not be involved in the modulation of the suppressive capacity of regulatory T cells but rather impact the expression of other lymphocyte phenotypes.

mCPBG, a selective 5-HT_{3A} receptor agonist was assessed for its effect on cytokine production and the percentage of CD4⁺ and CD8⁺ T cells present in lymphocyte-gated PBMC.

There was no indication of an alteration in the production of either IFN or TNF as a result of 5-HT_{3A} receptor activation by mCPBG (1 μ M), regardless of the presence or absence of 5-chloroindole (10 μ M; Figure 3.8 and 3.9, respectively), contrary to another study showing 5-HT inhibited production of TNF- α (by approximately a third) in PBMC (Cloezy-Tayarani *et al.*, 2003).

While a previous study reports the expression of the 5-HT_{3A} receptor in human T cells (Fiebich *et al.*, 2004b), there is little data with regards to its function in this context. mCPBG, either alone or with the positive allosteric modulator 5-chloroindole, did not impact the percentage of either CD4⁺ helper T cells, or CD8⁺ cytotoxic T cells, therefore the CD4/CD8 ratio was unaffected by activation of the 5-HT_{3A} receptor (Figure 3.10). Expression of the 5-HT_{3A} receptor subunit was found to be restricted to a T cell subset (CD4⁺CD25⁺), which make up only a very small percentage of T cells. The impact of 5-HT_{3A} receptor activation by mCPBG may be insignificant when analysing a large, heterologous population of immune cells.

There was a trend for PBMC treated with 5-HT₃ receptor agonists plus 5-chloroindole to express a greater percentage of CD25+FoxP3+ lymphocytes (Figure 3.11). Upon analysis of the mean fluorescence intensity (MFI) of FoxP3 expression, a similar trend was observed in cells treated with 5-HT₃ receptor agonists plus 5-chloroindole (Figure 3.13). There was no increase in either the percentage of CD25+FoxP3+ T cells or the MFI of CD25 or FoxP3 expression in isolated CD4+ T cells treated 5-HT₃ receptor agonists plus 5-chloroindole (Figures 3.12 and 3.14). Therefore these data may suggest that non-CD4+ lymphocytes are responsible for the alteration of FoxP3 expression caused by 5-HT₃ receptor activation in PBMC.

Considering there was no increase in CD25 MFI, the increase in the percentage of CD25+FoxP3+ cells in PBMC treated with DDP733 plus 5-chloroindole, may imply that this was a result of an increase in the percentage of FoxP3+ cells, rather than CD25+ cells (Figure 3.24). It is also possible that the density of FoxP3 expression was altered by treatment with DDP733 plus 5-chloroindole, although it is not possible to determine the density of FoxP3 expression using MFI alone. Although MFI is calculated using the same analysis gate, the size of the cells may have been affected by drug treatment. Cell size should be analysed to determine whether there is an absolute change in the density of FoxP3 expression in PBMC. It is also plausible that 5-HT_{3A} receptor activation plus 5-chloroindole may increase the total number of FoxP3-expressing cells in PBMC.

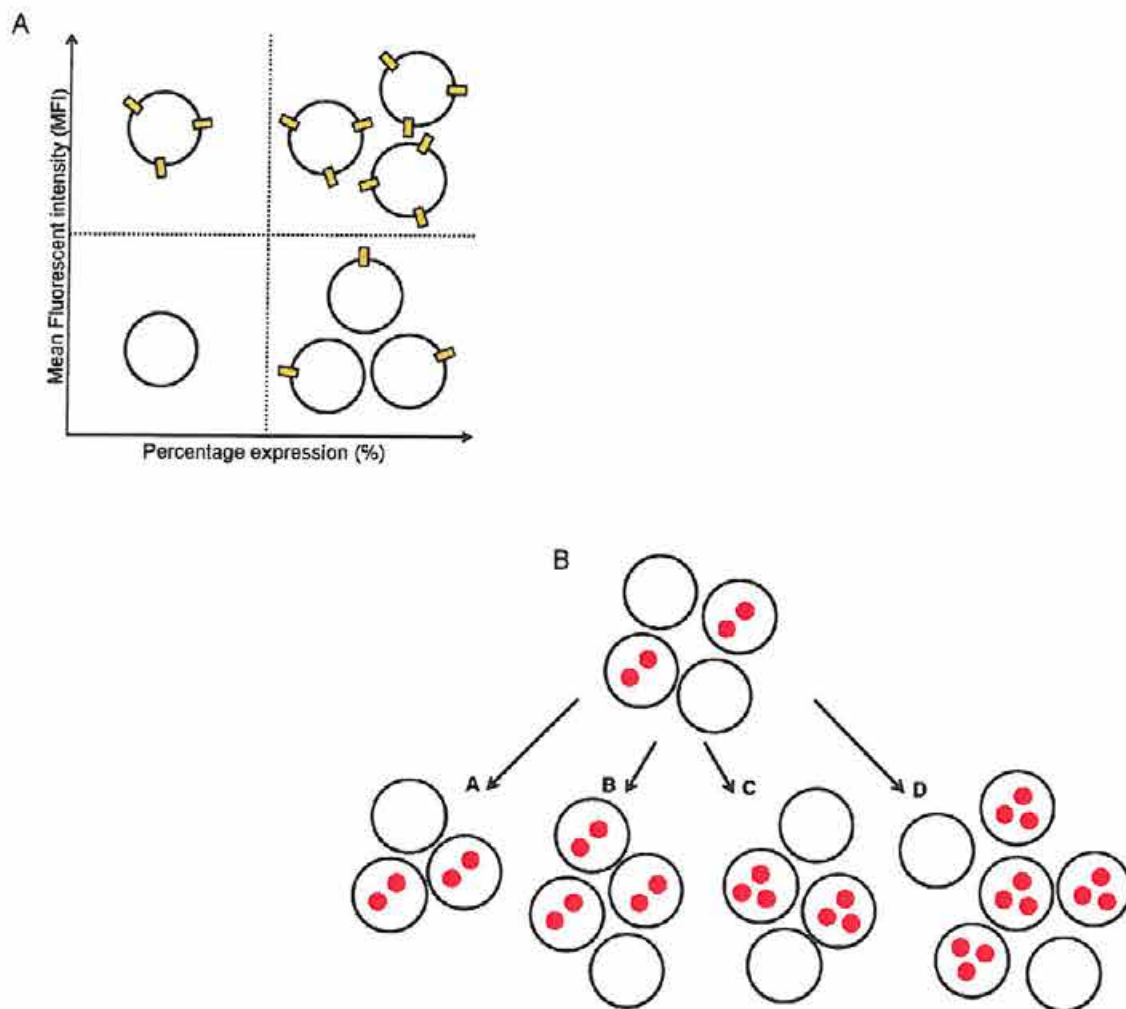


Figure 3.24: Diagram showing the difference between MFI and percentage expression used in FACS analysis. A) Many cells may express very little receptor protein (percentage^{high}, MFI^{low}) or few cells may express very high levels of receptor (percentage^{low}, MFI^{high}). B) Potential causes of an increase in MFI. A) There is a reduction in the total number of cells, yet FoxP3 expression remains the same. Where total cell number is not affected, there may either be an increase in B) the number of FoxP3+ cells or C) the density of FoxP3 expression, although these may not be mutually exclusive (D).

Section 3.8.3 Impact of 5-HT₃A receptor activation on proliferation and the expression of early activation markers in human PBMC

An early study which identified 5-HT₃ receptors in fish PBMC, observed that the 5-HT₃ receptor agonist 2-methyl-5-HT caused a reduction in PHA-induced stimulation, although a substantial reduction (approximately 40 %) was only seen using a high dose (50 µM) and above (Meyniel *et al.*, 1997). Contradictorily, in the Jurkat T cell line, 2-methyl-5-HT was seen to have an immunostimulatory effect on PHA-induced stimulation (Khan and Poisson, 1999) although it should be noted that Jurkat cells, although from human origin, are an immortalised T cell line originating from a patient with leukaemia (Schneider *et al.*, 1977), therefore may not faithfully represent the situation in healthy individuals. Therefore, although modulation of proliferation by 5-HT₃ receptor agonists has been reported previously, the impact of 5-HT₃A receptor activation in human immune cells using more pharmacologically relevant concentrations of 5-HT₃A receptor agonist, was investigated.

The CD3/CD28-induced proliferation of CFSE-labelled PBMC was reduced by the 5-HT₃A receptor agonist DDP733, which became statistically significant upon potentiation of 5-HT₃A receptor activation by 5-chloroindole (Figure 3.15). It is possible that proliferation was inhibited by the increase in FoxP3 expression in non-CD4⁺ lymphocytes as a result of treatment with 5-HT₃A receptor agonists and 5-chloroindole, as seen in previous experiments.

The role of calcium in the activation of lymphocytes should also be considered being a key mediator of lymphocyte activation (Whitney and Sutherland, 1972, Kay, 1971), particularly in the early initiation phase of stimulation (Diamantstein and Odenwald, 1974). Proliferation of CD4⁺ T cells stimulated with CD3/CD28 activation beads has

been shown to increase $[Ca^{2+}]_i$ to 220 nM from a baseline of 40 nM, although was most Ca^{2+} sensitive between 90 nM and 120 nM (Schwarz *et al.*, 2007). Apoptosis of activated T cells is required to limit an uncontrolled immune response, although apoptosis was relatively constant (Schwarz *et al.*, 2007). A reduction in proliferation was observed in PBMC treated with the calcium channel blockers nifedipine and mibefradil, and the intracellular calcium antagonist TMB-8 (Lijnen *et al.*, 1998). Subsequent studies demonstrated that although expression of the IL-2 receptor remained unchanged, the release of IL-2 was inhibited by treatment with calcium antagonists, and proliferation was restored by the addition of exogenous IL-2 (Petrov and Lijnen, 2000).

Stimulation of T cells in the presence of both DDP733 and 5-chloroindole may result in a reduction in proliferation due to desensitisation of the 5-HT₃ receptor. Desensitisation would limit Ca^{2+} entry into activated T cells, reducing proliferation. This may explain the increase in percentage of CD25+FoxP3+ cells in PBMC treated with 5-HT_{3A} receptor agonists (in the presence of 5-chloroindole). Proliferation of stimulated cells expressing the 5-HT₃ receptor, may be limited by desensitisation induced by DDP733 and 5-chloroindole. This may result in a relative increase in anergic or regulatory T cells (as characterised by CD25+FoxP3 expression).

Considering the impact of 5-HT_{3A} receptor activation on $[Ca^{2+}]_i$, it seems unlikely that the reduction in proliferation of PBMC treated with 5-HT_{3A} receptor agonists and 5-chloroindole, is a direct result of the increase on Ca^{2+} . Subsequently, Ca^{2+} is an important second messenger, regulating several downstream pathways. One such signalling pathway, the calcineurin/NFAT pathway, can control the transcription of FoxP3.

The statistical significance of 5-chloroindole (30 μ M) increasing the percentage of CD25+CD27+ lymphocytes might imply that 5-chloroindole acts at a novel target expressed by CD25+CD27+ lymphocytes. These experiments should be repeated in order to verify this data. Should this data in fact be reproducible, further experiments would be warranted in order to investigate the impact of 5-chloroindole in the CD25+CD27+ lymphocyte subset.

Section 3.8.4 Impact of 5-HT₃A receptor antagonism on human lymphocytes

The highly selective 5-HT₃ receptor antagonist granisetron (1 μ M) was found to increase the percentage of CD25+FoxP3+ cells in lymphocyte-gated PBMC (Figure 3.20) although the MFI of CD25 and FoxP3 staining (Figure 3.22) remained unchanged. This may indicate there is a reduction in CD25 and FoxP3 expression per cell although proliferation of PBMC was unaffected by granisetron treatment (Figure 3.24), suggesting that the impact of granisetron was not sufficient to impact proliferation. As mentioned previously, the relative size of the stimulated lymphocytes has not been analysed, therefore from these data, the density of CD25 and FoxP3 expression cannot be determined.

The concentration of 5-HT in blood plasma, released from platelets and lymphocytes and from enterochromaffin cells in the gut, is reported to be less than 100 nM (Mossner and Lesch, 1998). The influence of 5-HT on T cell activation is thought to be mediated by 5-HT₁ (Aune *et al.*, 1994), 5-HT₂ receptors (Young *et al.*, 1993) and 5-HT₇ receptors (Leon-Ponte *et al.*, 2007) although there are several reports of tropisetron, a 5-HT₃ receptor antagonist, having anti-inflammatory effects in the

immune system (Fiebich *et al.*, 2004a, Mousavizadeh *et al.*, 2009, de la Vega *et al.*, 2005, Hrycaj, 2004).

Other immune cells such as human monocytes have been shown to express 5-HT₃ receptors (Fiebich *et al.*, 2004b), and subsequently, it was reported that tropisetron inhibited the release of TNF and IL-1 β from lipopolysaccharide-activated monocytes (Fiebich *et al.*, 2004a), prompting the authors to suggest the anti-inflammatory effect of tropisetron be used for the treatment of chronic inflammatory joint disease (Mousavizadeh *et al.*, 2008). Although this study used a range of tropisetron concentrations, the lowest concentration (5 μ g/ml) was very high for specific targeting of the 5-HT₃ receptor, which has an affinity for the 5-HT₃ receptor in nanomolar concentrations. A previous study found a local injection of tropisetron (5 mg) was well tolerated and effective at reducing pain associated with tendinopathies, although was no more effective than the current treatment protocol using corticosteroids (Stratz *et al.*, 2002). More recently, a reduction in colonic damage caused by acetic acid-induced colitis in mice was observed following treatment with tropisetron (Mousavizadeh *et al.*, 2009). In human PBMC, tropisetron was shown to inhibit the release of IL-2, presumed to be responsible for the large reduction in proliferation caused by pre-treatment with tropisetron (de la Vega *et al.*, 2005). Although both responses to tropisetron were concentration dependent, the doses used (50, 25 and 10 μ g/ml), were very high (the authors quote the IC₅₀ of tropisetron in reducing IL-2 production and proliferation as approximately 50 μ M). Another 5-HT₃ receptor antagonist, ondansetron (50 μ g/ml) only partially inhibited IL-2 release, and had no effect at lower doses (25 μ g/ml). Although the authors do not report the concentration of granisetron used, it was noted that neither levels of IL-2 nor proliferation were affected by pre-treatment with granisetron (de la Vega *et al.*, 2005). Granisetron has

also been investigated for its anti-inflammatory potential in an air-pouch model of inflammation. In this study, the total number of leukocytes was reduced upon granisetron treatment (50 µg/pouch), although there was no reduction in the overall pouch volume (Maleki-Dizaji *et al.*, 2010). However even at the lowest dose, the concentration of granisetron (approximately 25 µM) greatly exceeds that required to saturate 5-HT₃ receptors (highest granisetron dose used was 200 µg/pouch), which may indicate that the impact of granisetron in this model was independent of 5-HT₃ receptors (Maleki-Dizaji *et al.*, 2010).

Despite the similar potency of granisetron and tropisetron at the 5-HT₃ receptor, (Hope *et al.*, 1996, Dubin *et al.*, 1999), the varying differences in the physiological responses to granisetron and tropisetron may suggest the anti-inflammatory effects of 5-HT₃ receptor antagonists are not mediated *via* inhibition of 5-HT₃ receptor function. Tropisetron is known to act as an antagonist at 5-HT₄ receptors, as a partial agonist at nicotinic α7 acetylcholine receptors (Cui *et al.*, 2009, Papke *et al.*, 2005), and as an antagonist at non-α7 acetylcholine receptors (Papke *et al.*, 2004). CD4⁺ T cell subsets were investigated for the expression of 5-HT₄ receptors, although mRNA transcripts were not detected by PCR (Figure 3.4). Interestingly however, expression of nicotinic α7 receptor mRNA transcripts was identified in T cell subsets during the course of these studies (Appendix 3). Therefore, it may be suggested that nicotinic acetylcholine receptors mediate the anti-inflammatory effect of tropisetron; although considering the high concentrations of tropisetron and granisetron used in the aforementioned studies the mechanism of action of these compounds may well be receptor-independent.

Clearly a lot more work is required to investigate the anti-inflammatory potential of 5-HT₃ receptor antagonists. These may well prove to have great therapeutic potential, particularly for treatment of patients with chronic inflammatory conditions, who suffer from the side effects associated with long-term corticosteroid use (Hrycaj, 2004).

CHAPTER 4

CHARACTERISATION OF GPR55 RECEPTOR EXPRESSION IN HUMAN T CELLS AND THE IMPACT OF PUTATIVE GPR55 RECEPTOR LIGANDS ON THE FUNCTION OF HUMAN IMMUNE CELLS

Section 4.1 Introduction

The GPR55 receptor has been cited as a novel cannabinoid receptor. Despite lacking the putative cannabinoid binding pocket (Petitet *et al.*, 2006), GPR55 receptors are activated by a variety of cannabinoid compounds and endocannabinoids. In the immune system, non CB₁/CB₂ receptor sites have been identified in CB₁^{-/-}CB₂^{-/-} mice therefore GPR55 receptors may mediate some of the immunomodulatory effects of endocannabinoids. The identification of a specific agonist for GPR55 (lysophosphatidylinositol) (Ryberg *et al.*, 2007, Oka *et al.*, 2007) has triggered much research into the role of GPR55 in various physiological processes since its identification in 1999 (Sawzdargo *et al.*, 1999, Whyte *et al.*, 2009).

GPR55 receptor expression has previously been identified in the spleen (Brown and Wise, 2001) which may suggest GPR55 receptor activation could influence the function of the immune system. The CB₂ receptor is also expressed in human immune cells, although many of its ligands also target central CB₁ receptors, which although not expressed in the immune system, limits their therapeutic use due to psychological side effects. As such, GPR55 may represent a novel target for the manipulation of immune cell function.

Section 4.2 Expression of GPR55 mRNA transcripts in human T cells

PCR using cDNA prepared from CD3/CD28-stimulated CD4⁺CD25⁻ and CD24⁺CD25⁺ T cell subsets was performed using oligonucleotide primers specific for the classic cannabinoid receptors CB₁ and CB₂, and the novel cannabinoid receptor GPR55 (Section 2.2). cDNA had previously been assessed for genomic contamination using primers specific for the housekeeping gene β -actin (Figure 3.1). Appropriately sized products were evident for CB₂ and GPR55 PCR, indicating the presence of mRNA transcripts for both the peripheral cannabinoid receptor and the novel cannabinoid receptor in CD4⁺ T cell subsets (Figure 4.1). There appeared to be an upregulation of the expression of CB₂ receptor and GPR55 receptor transcripts upon activation with CD3/C28 activation beads, compared to those subsets at rest. mRNA transcripts for the CB₁ receptor were not detected in CD4⁺ T cell subsets.

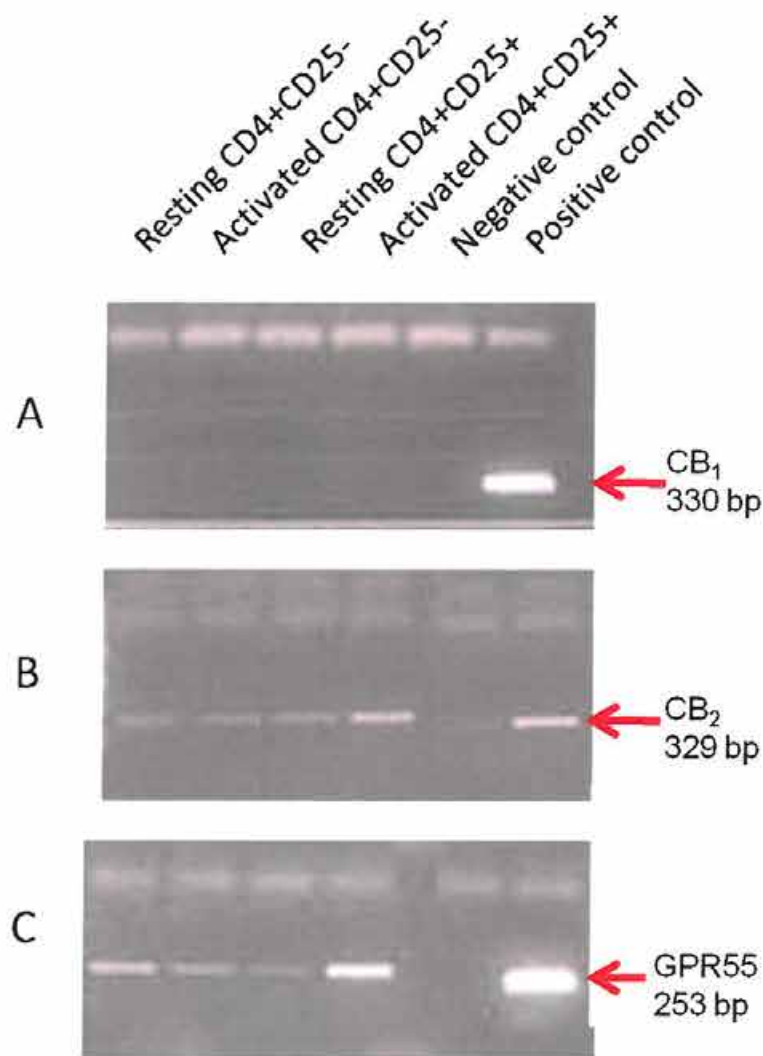


Figure 4.1: Detection of cannabinoid receptor mRNA transcripts in human T cell subsets. Products are the result of 35 PCR cycles containing oligonucleotide primers specific for cannabinoid receptors: A) CB₁ receptor, B) CB₂ receptor and C) GPR55. HEK293 cells transiently expressing either CB₁, CB₂ or GPR55 receptors were used as positive controls for PCR. PCR data shown is from the same donor, and is typical of four individual donors.

Section 4.3 Impact of GPR55 receptor activation on human immune cells

The function of GPR55 receptors expressed by human T cells was investigated using freshly isolated PBMC from healthy human donors. LPI has recently been identified as an endogenous ligand for GPR55 (Oka *et al.*, 2007). Cannabidiol (CBD) is an 'atypical' cannabinoid, having little or no activity at either CB₁ or CB₂ cannabinoid receptors, however, CBD has been used in many studies as an antagonist at GPR55 (Whyte *et al.*, 2009, Lauckner *et al.*, 2008, Ryberg *et al.*, 2007).

Freshly isolated PBMC (Section 2.1.1) were incubated with LPI (3 μ M) or CBD (10 μ M). After four days, cells were analysed by flow cytometry. Using the flow cytometry analysis software FlowJo, analysis gates were drawn on FSC SSC plots based upon physical characteristics of cell populations to estimate the percentage of live, or apoptotic lymphocytes. As shown in Figure 4.2, the percentage of resting PBMC falling within a live lymphocyte gate is increased (68.97 ± 3.80 % compared to 52.52 ± 4.26 % in vehicle treated cells; mean \pm SEM; n=6), whilst the percentage of cells falling within a 'dead' cell gate is significantly reduced in the presence of 3 μ M LPI (2.24 ± 0.52 % compared to 18.73 ± 4.11 % with vehicle treatment; mean \pm SEM; n=6). No effect on either live or dead cell percentage was seen after treatment with the GPR55 receptor antagonist CBD (10 μ M; 50.12 ± 5.18 % and 19.47 ± 4.79 % respectively; mean \pm SEM; n=6). The reported GPR55 receptor antagonist CBD does not reverse the effects of LPI in these experiments. A higher concentration of CBD (30 μ M) was used in an attempt to inhibit the inhibition of apoptosis achieved using LPI (3 μ M), however this concentration proved highly apoptotic to PBMC cultured *in vitro* (data not shown).

These experiments were repeated using O1602, another GPR55-selective agonist (Figure 4.3.1 and 4.3.2). In five individual donors, the percentage of events within live, or dead cell gates was not affected by O1602 (500 nM; 60.88 ± 9.86 % and 10.23 ± 2.14 % respectively; mean \pm SEM; n=5). The above noted effect of LPI was once more observed with respect to both live and dead cell gates (70.02 ± 7.63 % and 2.20 ± 0.60 % respectively; mean \pm SEM; n=5).

Propidium iodide (PI) is a membrane-impermeable dye, which intercalates between DNA base pairs in cells with a compromised cell membrane, therefore is a more accurate method of identifying apoptotic cells by flow cytometry. Cells were labelled with PI (2 ng/ml) prior to FACS analysis.

Concentration response curves for the effect of LPI in promoting cell survival, and the inhibition of apoptosis in resting cells were determined using human PBMC (Figure 4.4). Cells were cultured for 4 days with increasing concentrations of LPI. For analysis, cells were gated for lymphocytes, including live and apoptotic cells (but not debris) according to FSC SSC plots. Cells staining positive for PI are designated as apoptotic cells. LPI increases the percentage of live cells ($EC_{50} = 1.30$ nM; n=5) and reduces the percentage of apoptotic cells ($EC_{50} = 0.54$ nM; n=5).

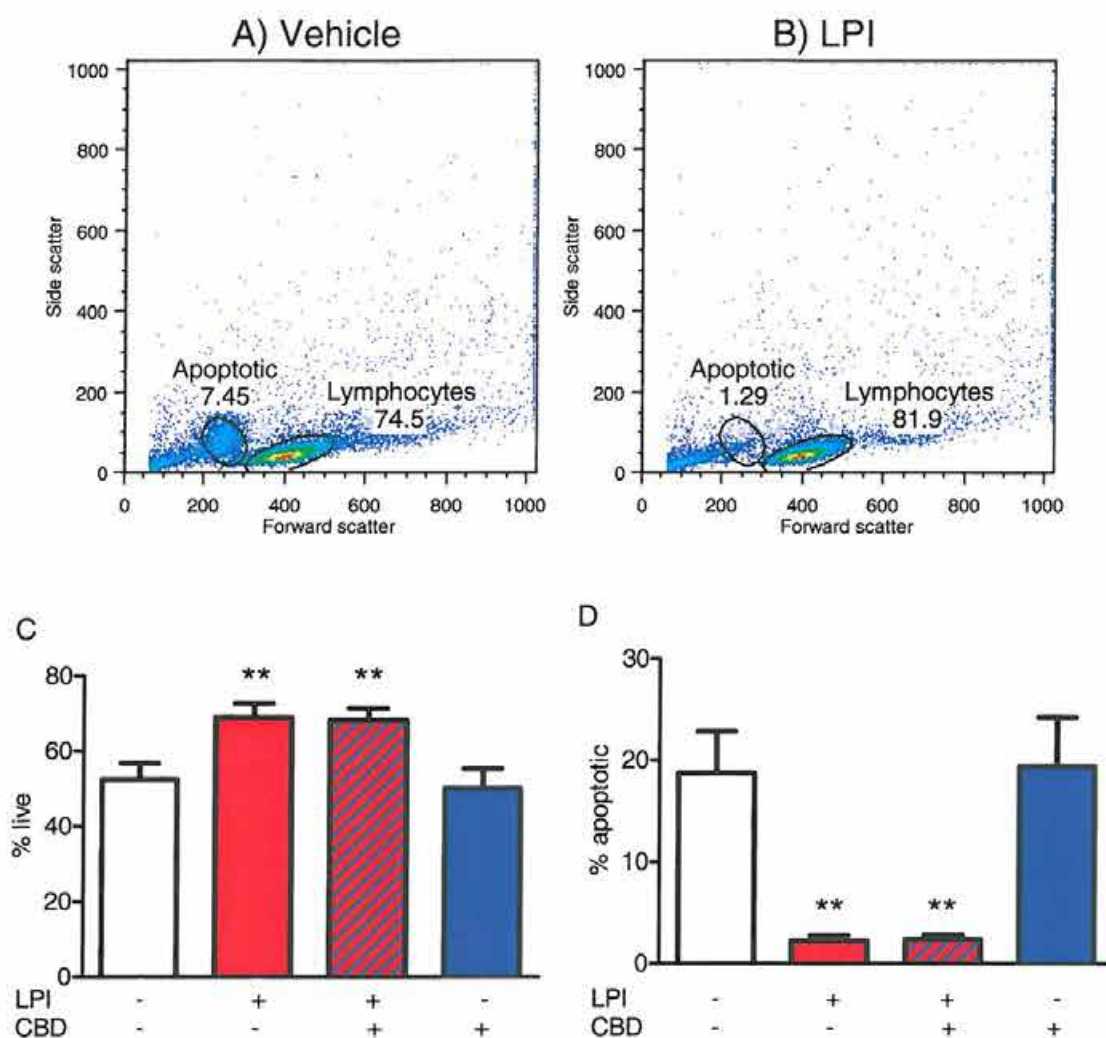


Figure 4.2: Effect of GPR55 receptor activation on cell survival in resting PBMC. Isolated PBMC were incubated for 4 days with LPI (3 μ M), CBD (10 μ M) or both. Live and apoptotic cell gates were drawn according to physical characteristics using FSC SSC plots. FSC SSC plots from a representative donor of resting human PBMC treated with A) vehicle and B) LPI. C) percentage of events within lymphocyte gate. Repeated measures ANOVA $P=0.0006$; Dunnett's multiple comparison test $P<0.01^{**}$. D) percentage of events within dead cell gate. Repeated measures ANOVA $P=0.0002$; Dunnett's multiple comparison test where $P<0.01^{**}$. Data represents mean + SEM; $n=6$.

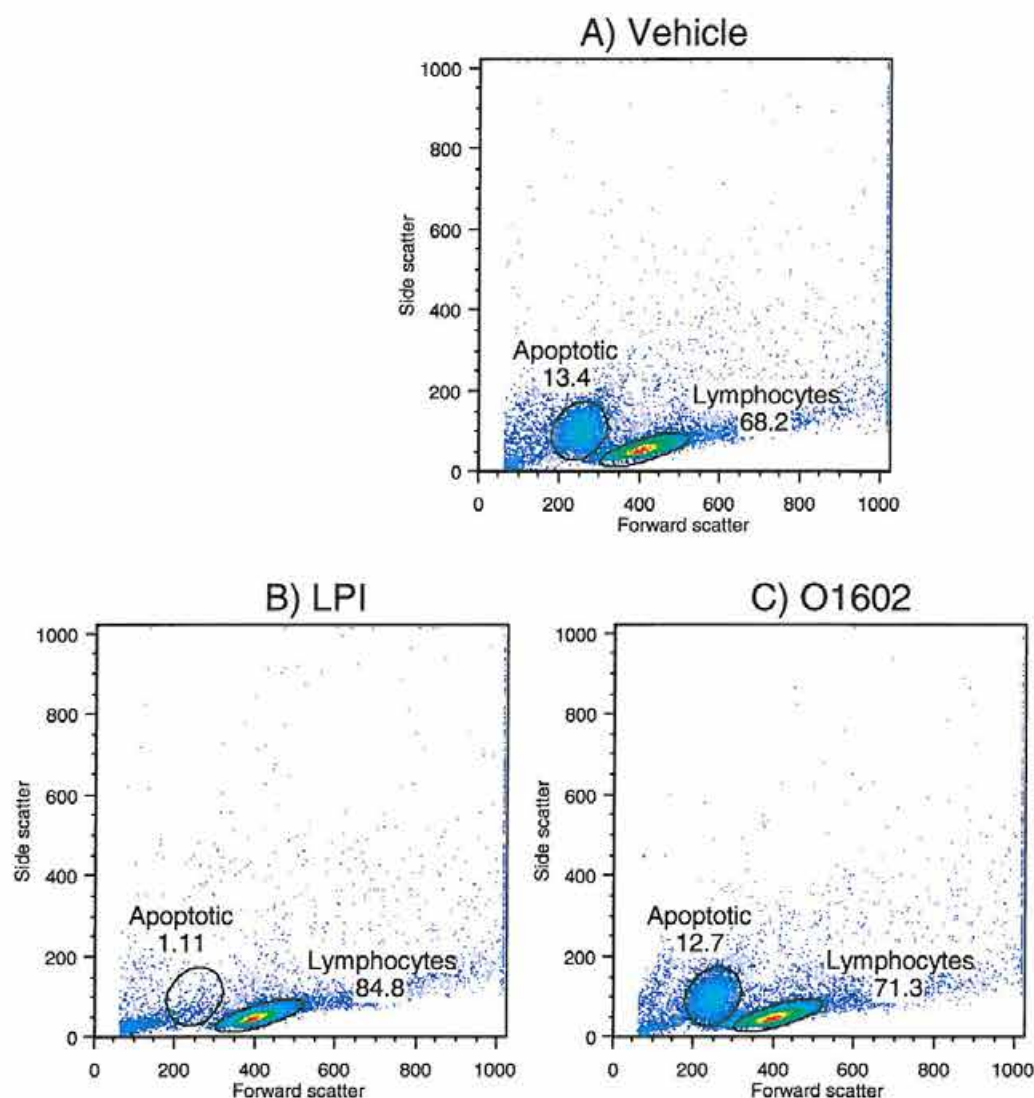


Figure 4.3: Reduction in the percentage of dead cells may not be mediated by the GPR55 receptor.

Figure 4.3.1: Impact of GPR55 receptor agonists on FSC SSC profiles. Isolated PBMC were incubated for 4 days with GPR55 agonists LPI (3 μ M) or O1602 (500 nM). Live and apoptotic cell gates were drawn according to physical characteristics using FSC SSC plots. Representative FSC SSC dot plots of PBMC treated with A) Vehicle B) LPI and C) O1602.

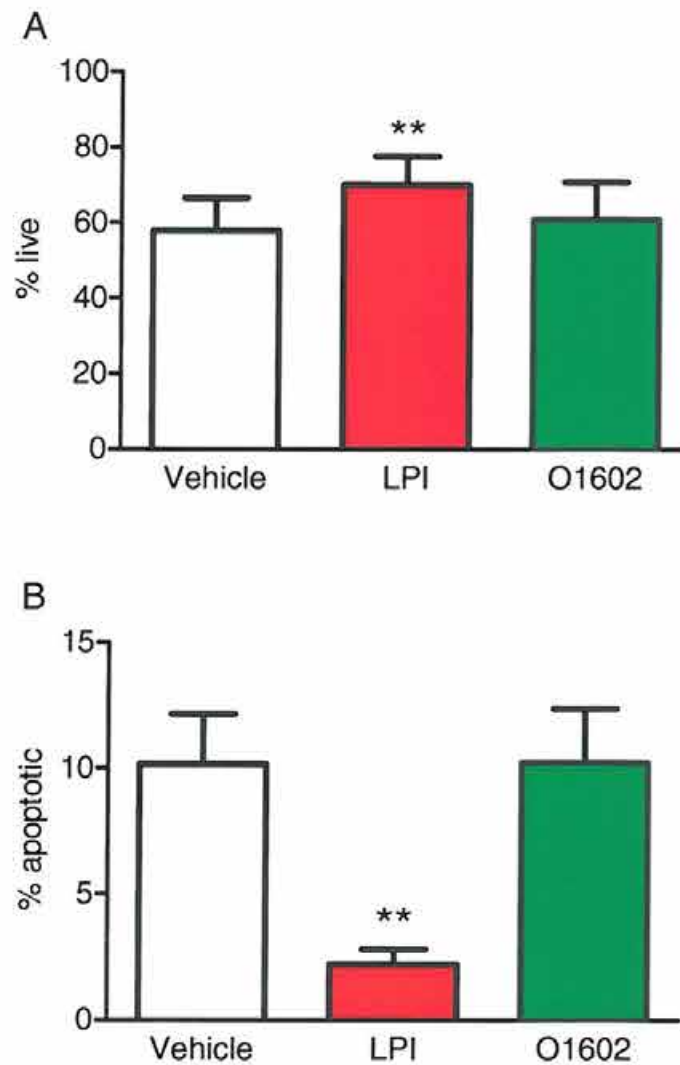


Figure 4.3.2: O1602 does not reduce the percentage of apoptotic cells. Isolated PBMC were incubated for 4 days with GPR55 agonists LPI (3 μ M) or O1602 (500 nM). Live and apoptotic cell gates were drawn according to physical characteristics using FSC SSC plots A) Percentage of events within live cell gate. Repeated measures ANOVA $P=0.0039^{**}$ followed by Dunnett's multiple comparison test where $P<0.01^{**}$, $n=5$. B) Percentage of events within apoptotic cell gate. Repeated measures ANOVA $P=0.0017$ followed by Dunnett's multiple comparison test where $P<0.01^{**}$, $n=5$.

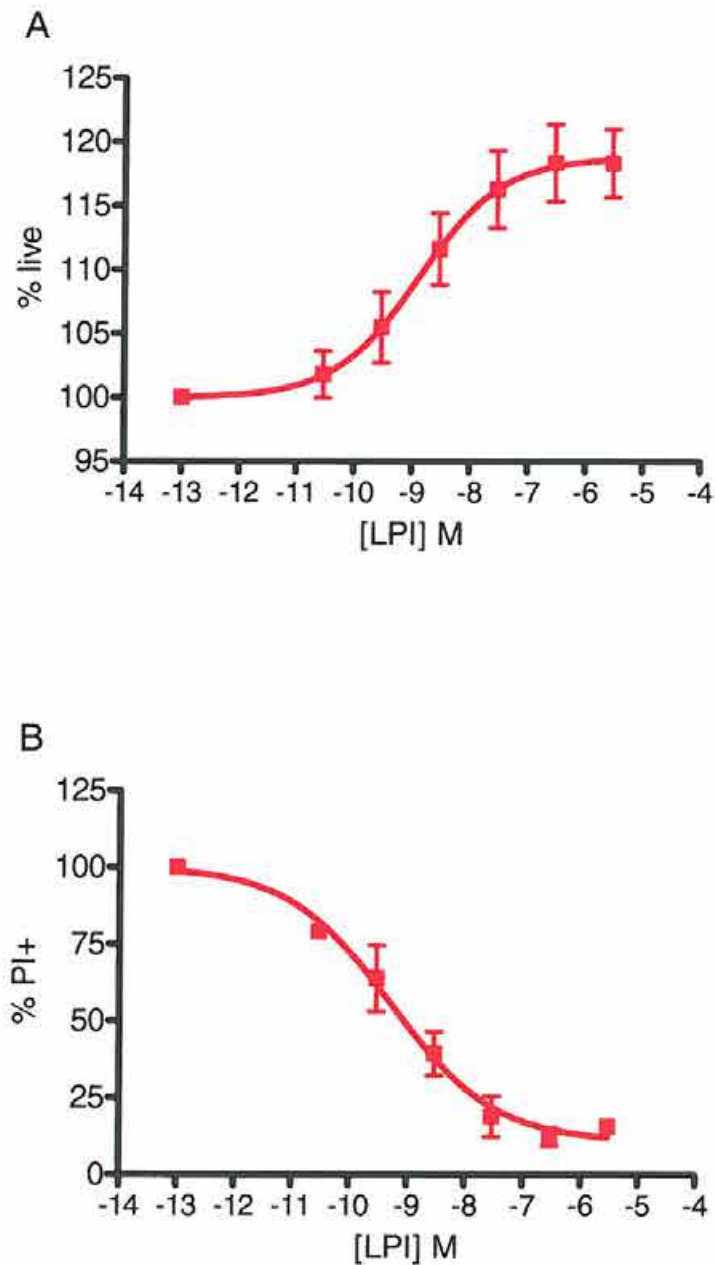


Figure 4.4: Concentration response curves for LPI in resting PBMC. Isolated PBMC were incubated for 4 days with increasing concentrations of LPI. A lymphocytes gate was drawn containing live and apoptotic cells (but not debris), according to physical characteristics using FSC SSC plots. Percentage of A) live cells; $n=5$, $EC_{50}=1.30$ nM and B) PI+ cells, $EC_{50}=0.54$ nM, within gated lymphocytes. Data represents mean percentage plus SEM, $n=5$.

Section 4.4 Impact of LPI on the absolute number of lymphocyte-gated PBMC

Previous data has been analysed using percentage expression, generated by using gating parameters based on the FSC SSC plots from a pre-determined total number of events to be collected by FACS (usually 25,000 total events were analysed). This method does not take into consideration any change in the total number of cells in each sample. Therefore counting beads were used to calculate the total number of cells by flow cytometry.

Resting, and CD3/CD28 bead-activated PBMC (Section 2.1.1) were incubated with three increasing concentrations of LPI (3 pM, 3 nM and 3 μ M) for 4 days. PBMC were gated for lymphocytes based on FSC SSC plots, including apoptotic cells (but excluding debris). Apoptotic cells were distinguished from live cells by propidium iodide staining (Section 2.4.4).

In unstimulated cells, the absolute number of total lymphocytes is not affected by LPI, even at the highest concentration used (3 μ M; 81.74 ± 11.50 % of vehicle; mean \pm SEM; n=3; Figure 4.5). However, the total number of resting PI+ cells in gated lymphocytes was dose-dependently reduced by increasing concentrations of LPI (3 pM and 3 nM; 68.79 ± 30.81 % and 35.54 ± 12.27 % of vehicle, respectively; mean \pm SEM; n=3). The reduction in total number of PI+ cells is statistically reduced in cultures treated with the highest dose of LPI (3 μ M; 10.88 ± 4.80 % of vehicle; mean \pm SEM; n=3).

In CD3/CD28 bead-activated cultures (Figure 4.6), LPI treatment did not affect the total number of lymphocytes (3 μ M; 98.78 ± 15.01 % of vehicle; mean \pm SEM; n=3)

which is consistent with unstimulated cells. However, LPI did not affect the total number of PI+ cells in activated lymphocytes (3 μ M; 84.08 ± 33.52 % of vehicle; mean \pm SEM; n=3) which is considerably different to the effect of LPI (particularly at 3 μ M) in resting lymphocytes.

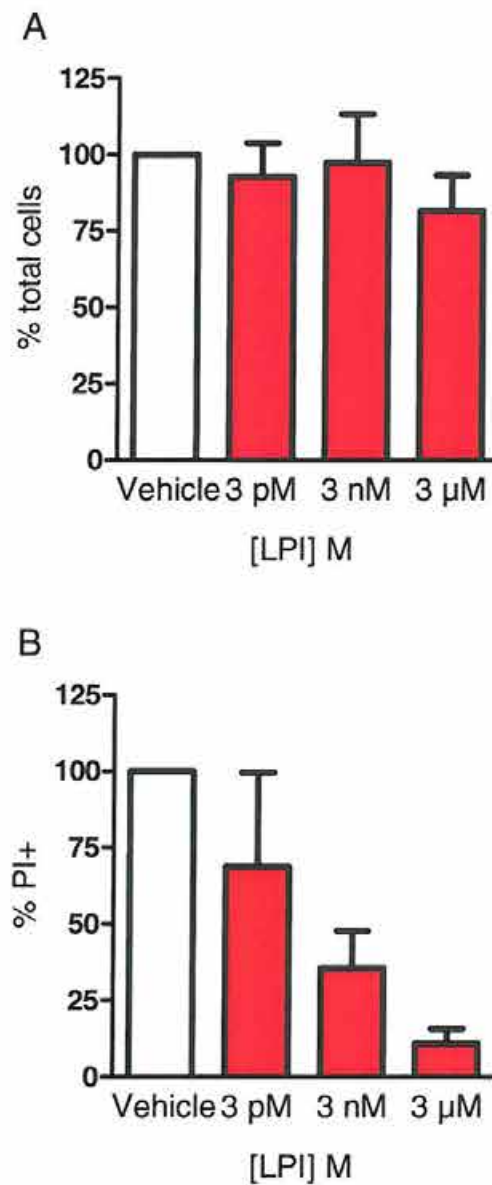


Figure 4.5: Treatment with LPI reduces the percentage of PI+ cells in unstimulated PBMC, but does not affect total cell number. Isolated PBMC were incubated for 7 days with increasing concentrations of LPI. Cells were stained with propidium iodide (2 ng/ml) prior to FACS analysis. Absolute cell numbers were calculated using counting beads. Events were gated for resting lymphocytes according to physical characteristics determined using FSC SSC plots. A) total number of cells and B) percentage of total cells PI+ cells per sample. Data bars represent the mean + SEM; n=3.

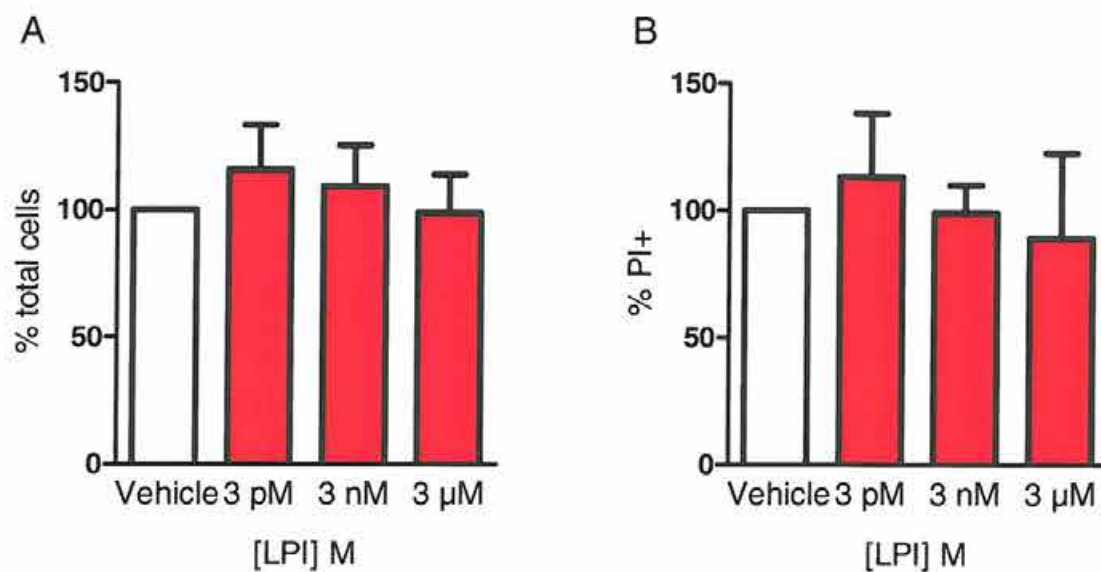


Figure 4.6: Treatment with LPI does not affect total cell number or the percentage of PI+ cells in activated cultures. Isolated PBMC were incubated for 7 days with CD3/CD28 activation beads in the presence of increasing concentrations of LPI. A) total number of cells and B) percentage of total cells PI+ cells per sample. Data bars represent the mean + SEM; n=3.

Section 4.5 Impact of LPI on mouse immune cells

GPR55 receptors are also expressed by rodents. Therefore, the effect of LPI on cell viability in the mouse was assessed using isolated splenocytes and thymocytes. Splenocytes and thymocytes were kindly isolated from wild-type mice by Dr Annelise Soulier (Section 2.1.4). Cells were incubated for 24 hours in the presence of increasing concentrations of LPI (3 pM, 3 nM and 3 μ M). Live and dead cell gates were drawn according to physical characteristics from FSC SSC plots.

The percentage of events in a dead cell gate appeared to be reduced in splenocytes treated with 3 μ M LPI (8.50 ± 0.22 % compared to 42.98 ± 0.23 % in vehicle; mean \pm SEM; n=2; Figure 4.7.1). However, this was not a dose dependent effect and treatment with lower doses of LPI (3 nM and 3 pM) did not reduce the percentage of cells in a dead cell gate (38.78 ± 1.38 % and 40.50 ± 0.95 %, respectively; mean \pm SEM; n=2). Consequently, the percentage of cells in a live cell gate appeared to increase only when splenocytes were treated with the highest dose of LPI (3 μ M; 38.30 ± 2.60 % compared to 24.75 ± 1.45 % in vehicle; mean \pm SEM; n=2). There was no effect of LPI on increasing the number of live cells using lower doses.

The highest concentration of LPI (3 μ M) appeared to reduce the total number of splenocytes (44.77 ± 4.13 % of vehicle; mean \pm SEM; n=2). There appeared to be no reduction in the total number of cells by lower doses of LPI. The total number of thymocytes was reduced by the highest concentration of LPI (3 μ M; 71.52 ± 2.02 % of vehicle; mean \pm SEM; n=2), although the total number of cells were not affected by lower doses of LPI. Therefore it would appear that mouse thymocytes are less sensitive to LPI (3 μ M) than mouse splenocytes (Figure 4.7.3).

Similar to splenocytes, the percentage of thymocytes contained by a dead cell gate was reduced when treated with 3 μ M LPI (8.04 ± 0.43 % compared to 26.35 ± 1.60 % in vehicle; mean \pm SEM; n=2; Figure 4.7.2). No effect was seen with lower doses of LPI. The percentage of thymocytes in a live cell gate appeared to increase upon treatment with 3 μ M LPI (59.58 ± 3.18 % compared to 49.78 ± 1.88 % in vehicle; mean \pm SEM; n=2) although there was no effect on the number of live cells using lower doses of LPI (3 nM and 3 pM; 49.15 ± 3.00 % and $46.73 \pm 0.97.8$ %, respectively; mean \pm SEM; n=2).

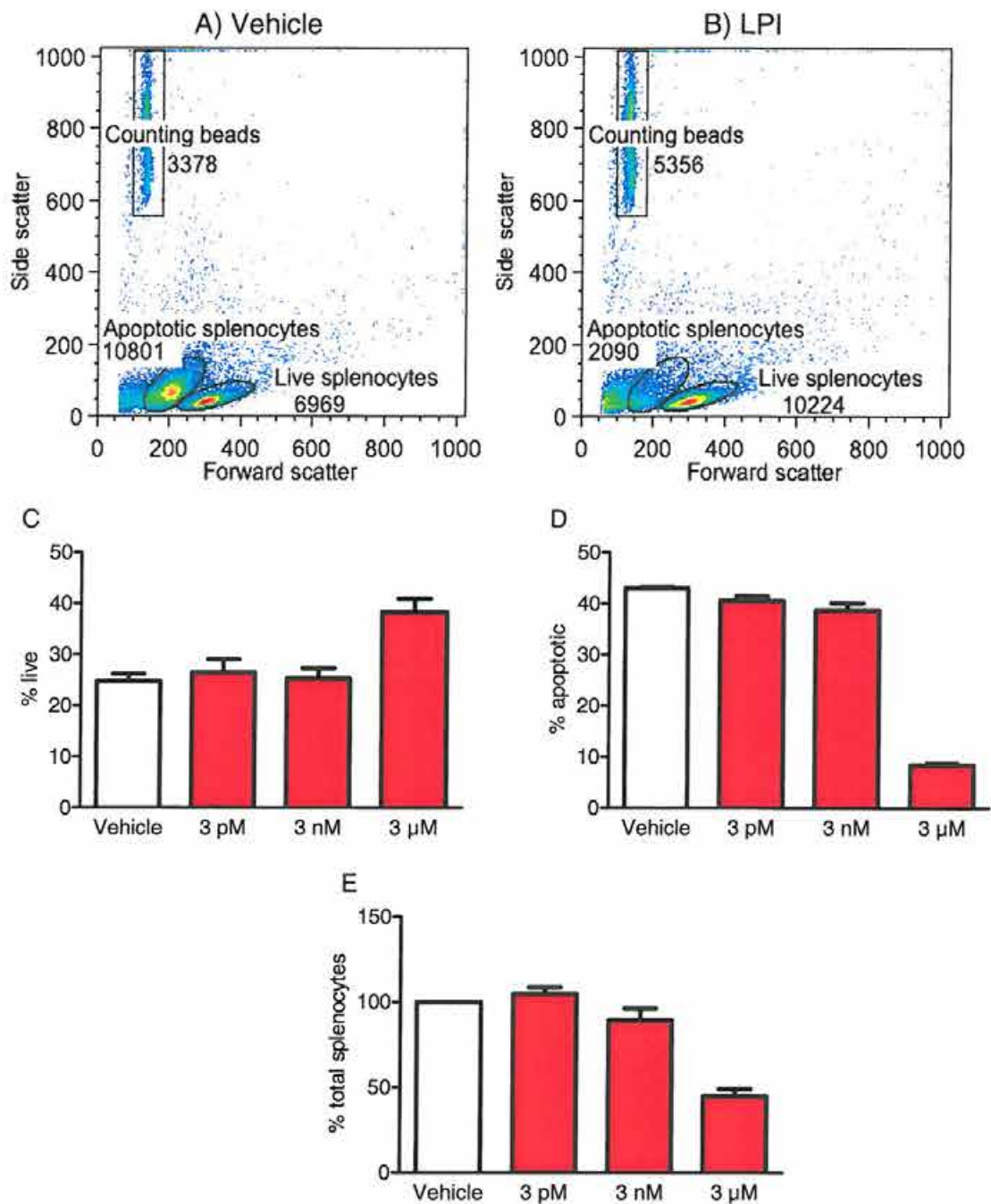


Figure 4.7: Impact of LPI on mouse immune cells. Cells were incubated for 24 hours with various concentrations of LPI; n=2.

Figure 4.7.1: LPI reduces apoptosis in resting splenocytes. FSC SSC plot of resting splenocytes treated with A) vehicle and B) 3 μ M LPI. Percentage of total events within FSC SSC gates: C) live splenocytes, D) apoptotic splenocytes and E) total number of splenocytes, expressed as a percentage of vehicle; n=2.

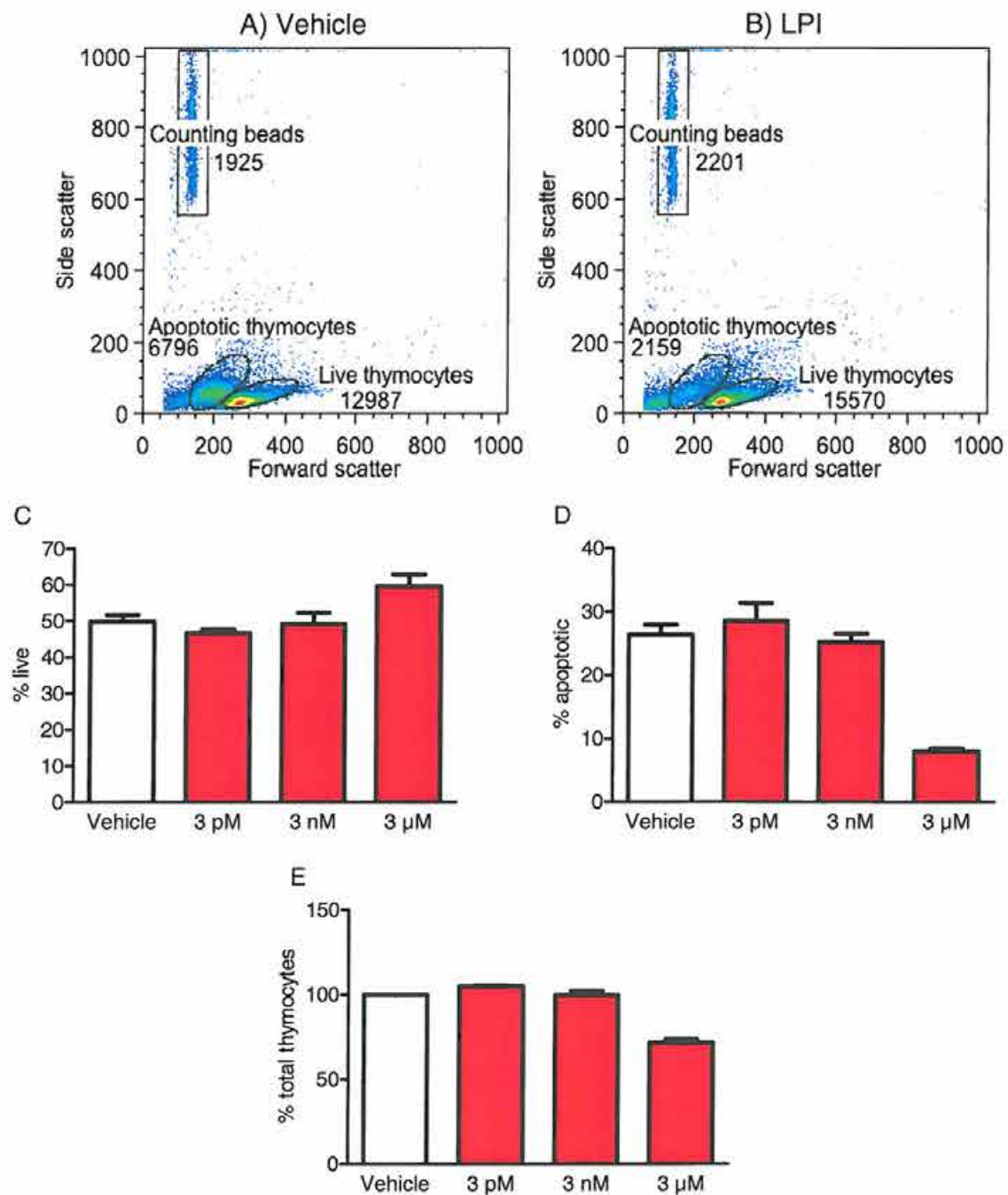


Figure 4.7.2: LPI reduces apoptosis in resting thymocytes. FSC SSC plot of resting thymocytes treated with A) vehicle and B) 3 μ M LPI. Percentage of total events within FSC SSC gates: C) live thymocytes, D) apoptotic thymocytes and E) total number of thymocytes, expressed as a percentage of vehicle; n=2.

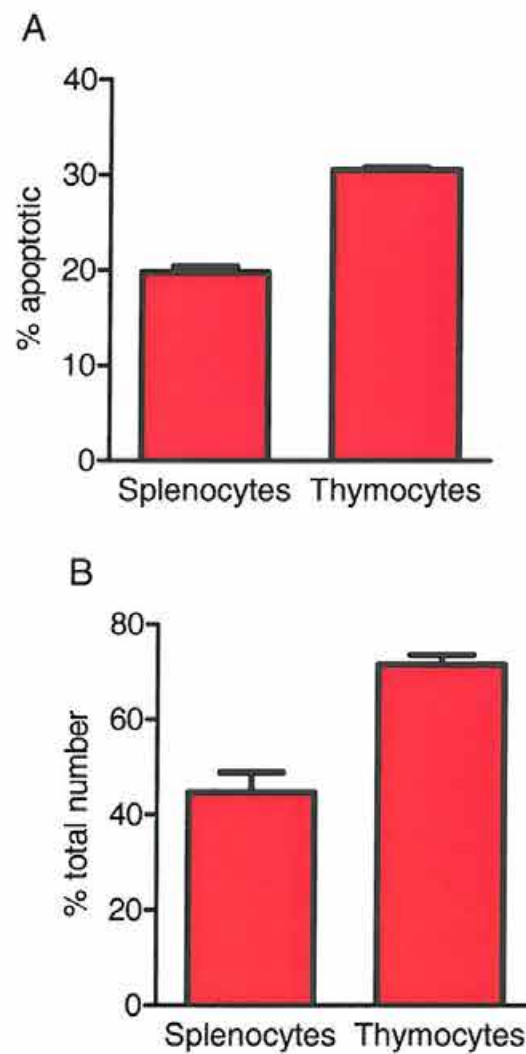


Figure 4.7.3: Splenocytes are more sensitive to LPI than thymocytes. A) percentage of apoptotic cells B) total number of cells as a result of treatment with LPI (3 μ M), both expressed as a percentage of vehicle, n=2.

Section 4.6 Impact of GPR55 receptor activation on proliferation

Next, the effect of LPI on the proliferation of CD3/CD28 bead-stimulated PBMC was investigated. For the detection of proliferation, PBMC were labelled with CFSE (1 μ M; Section 2.4.2) prior to incubation for 4 days with the T cell stimulant. Cells were treated with vehicle, or agonists of the GPR55 receptor, LPI (3 μ M), O1602 (500 nM) or AM251 (1 μ M; also a CB₁ receptor inverse agonist). Counting beads were used to determine absolute cell number. For analysis, PBMC were gated for activated lymphocytes based on FSC SSC plots. The absolute number of total lymphocytes was determined using counting beads. Total proliferation was also determined by counting beads to calculate the absolute number of proliferated cells (defined as CFSE^{mid-low}). Both were expressed as a percentage of vehicle-treated cells (Figure 4.8).

Treatment of stimulated CFSE-labelled PBMC with LPI (3 μ M) increased the total number of proliferated lymphocytes (135.06 ± 25.93 % of vehicle; mean \pm SEM; n=4), although this was not statistically significant. Consistent with earlier data (Figure 4.6), the total number of activated PBMC was not affected by LPI (3 μ M; 113.45 ± 16.10 % of vehicle; mean \pm SEM; n=4). O1602 (500 nM) a selective GPR55 receptor agonist, did not increase either the absolute number of total lymphocytes (131.98 ± 41.27 % of vehicle; mean \pm SEM; n=4) or the total number of proliferated cells (130.96 ± 31.12 % of vehicle; mean \pm SEM; n=4). The GPR55 receptor agonist/CB₁ receptor inverse agonist AM251 (1 μ M) appeared to inhibit the total number of proliferated lymphocytes (73.45 ± 7.91 % of vehicle; mean \pm SEM; n=4) and also reduce the absolute number of total cells (85.55 ± 8.39 % of vehicle; mean \pm SEM; n=4) although neither of these results were statistically significant.

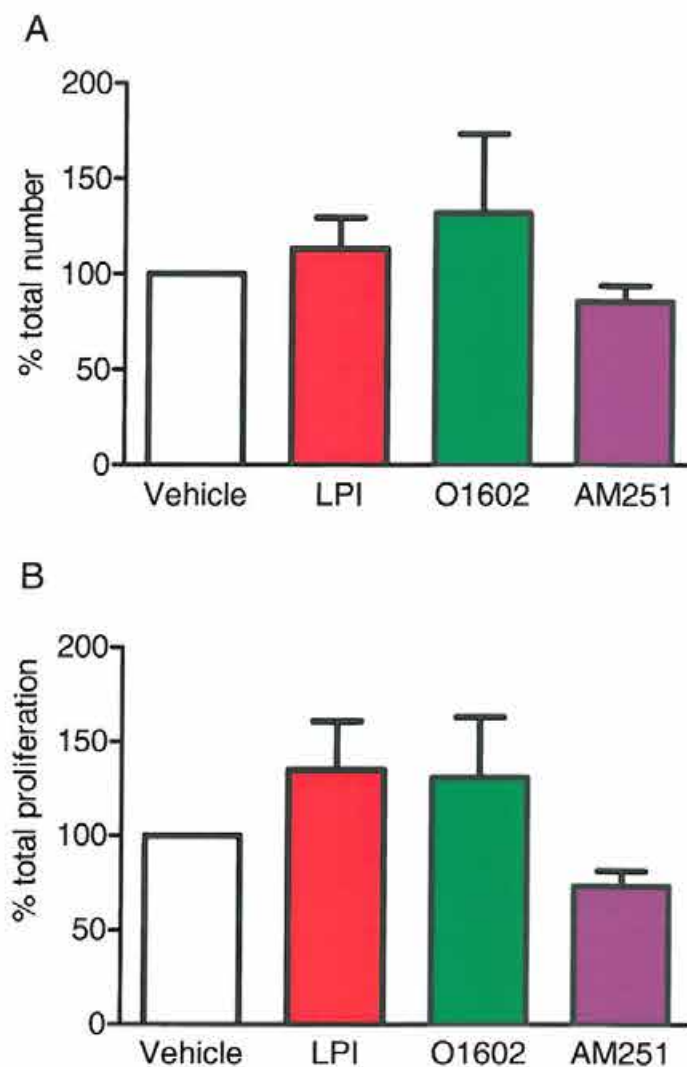


Figure 4.8: Treatment with GPR55 agonists does not affect the total number or proliferation of activated lymphocytes. Isolated PBMC were incubated for 4 days with LPI (3 μ M), O1602 (500 nM) or AM251 (1 μ M). Cells were gated for stimulated lymphocytes according to physical characteristics using FSC SSC plots. Absolute cell numbers were determined using counting beads. A) total number of lymphocytes, expressed as a percentage of vehicle. B) total proliferation expressed as a percentage of the proliferation of vehicle treated cells. Data represents the mean + SEM; n=4.

Section 4.7 Impact of GPR55 receptor activation on apoptosis

The differential effect of LPI treatment on resting and activated human PBMC was investigated further (Figure 4.9). Phiphilux is a membrane-permeable protease substrate which detects intracellular caspase 3 activity, a characteristic marker of apoptotic cells. Phiphilux labelling, used in conjunction with propidium iodide, can differentiate between apoptotic cells (Phiphilux+) and necrotic cells (PI+). Cells were stained with Phiphilux (Section 2.4.4) and PI prior to FACS analysis.

Figure 4.9.1 schematically demonstrates the gating parameters used for the analysis of apoptosis by Phiphilux and PI staining. Dot plots of Phiphilux *versus* propidium iodide staining are shown in Figure 4.9.2, and are representative of four individual donors.

In unstimulated cultures (Figure 4.9.3), the percentage of late apoptotic cells (Phiphilux+PI+) in resting PBMC was statistically reduced when treated with LPI (3 μ M; 0.75 ± 0.20 %; mean \pm SEM; n=4) compared to vehicle treated cells (1.61 ± 0.25 %; mean \pm SEM; n=4). Treatment with the GPR55 antagonist CBD (10 μ M) resulted in an increase in the percentage of late apoptotic cells (2.87 ± 0.48 %; mean \pm SEM; n=4), but does not reverse the effect of LPI (0.88 ± 0.31 %; mean \pm SEM; n=4). A similar effect of LPI and CBD was evident in the percentage of early apoptotic cells (Phiphilux+PI-).

Interestingly, there is no significant reduction in the percentage of necrotic cells (Phiphilux-PI+) in PBMC treated with LPI (3 μ M; 1.98 ± 0.81 %; mean \pm SEM; n=4) compared to vehicle (2.53 ± 0.78 %; mean \pm SEM; n=4). Although CBD (10 μ M)

treatment resulted in an increase in the percentage of both early and late apoptotic cells, CBD did not result in a significant increase in necrotic cells (3.13 ± 0.49 %; mean \pm SEM; n=4).

The percentage of live cells (Phiphilux-PI-) was increased by LPI treatment ($3 \mu\text{M}$; 93.33 ± 1.18 %; mean \pm SEM; n=4) compared to the percentage in vehicle-treated cells (86.73 ± 0.71 %; mean \pm SEM; n=4) although this increase does not reach significance. CBD ($10 \mu\text{M}$) reduces the percentage of live cells (60.93 ± 17.44 %; mean \pm SEM; n=4) compared to vehicle, which although appears reasonably substantial, was not statistically significant.

In CD3/CD28 bead-activated PBMC (Figure 4.9.4), LPI significantly reduced (from 10.18 ± 1.53 % in vehicle, to 4.98 ± 0.95 %; mean \pm SEM; n=4), the percentage of early apoptotic cells. Although CBD ($10 \mu\text{M}$) significantly increased the percentage of early apoptotic cells (15.73 ± 0.29 %; mean \pm SEM; n=4), this was not sufficient to reverse the reduction in early apoptotic cells caused by LPI.

Contrary to what was observed with resting cells, the reduction in late apoptotic cells treated with LPI ($3 \mu\text{M}$; 4.85 ± 1.64 % from 6.77 ± 2.11 % in vehicle treated cells; mean \pm SEM; n=4) was not statistically significant amongst CD3/CD28 bead-activated PBMC. Again, CBD ($10 \mu\text{M}$) increased the percentage of late apoptotic cells (9.81 ± 0.64 %; mean \pm SEM; n=4) consistent with resting PBMC, as was the failure of CBD to reverse the effect of LPI. Consistent with Figure 4.8, LPI ($3 \mu\text{M}$) did not reduce the percentage of necrotic cells in activated PBMC. However, the percentage of necrotic cells in stimulated PBMC significantly increases from $2.05 \pm$

0.58 % to 4.55 ± 0.87 % (mean \pm SEM; n=4) when treated with CBD, which differs from the effect of CBD in necrotic cells in resting PBMC.

In activated cells, the percentage of live cells was increased (although not statistically so) by LPI treatment ($3 \mu\text{M}$; 88.20 ± 2.67 %; mean \pm SEM; n=4) compared to the percentage in vehicle-treated cells (81.04 ± 3.87 %; mean \pm SEM; n=4). Consistent with resting cells, CBD ($10 \mu\text{M}$) reduces the percentage of live cells (69.93 ± 1.21 %; mean \pm SEM; n=4) compared to vehicle, which is statistically significant.

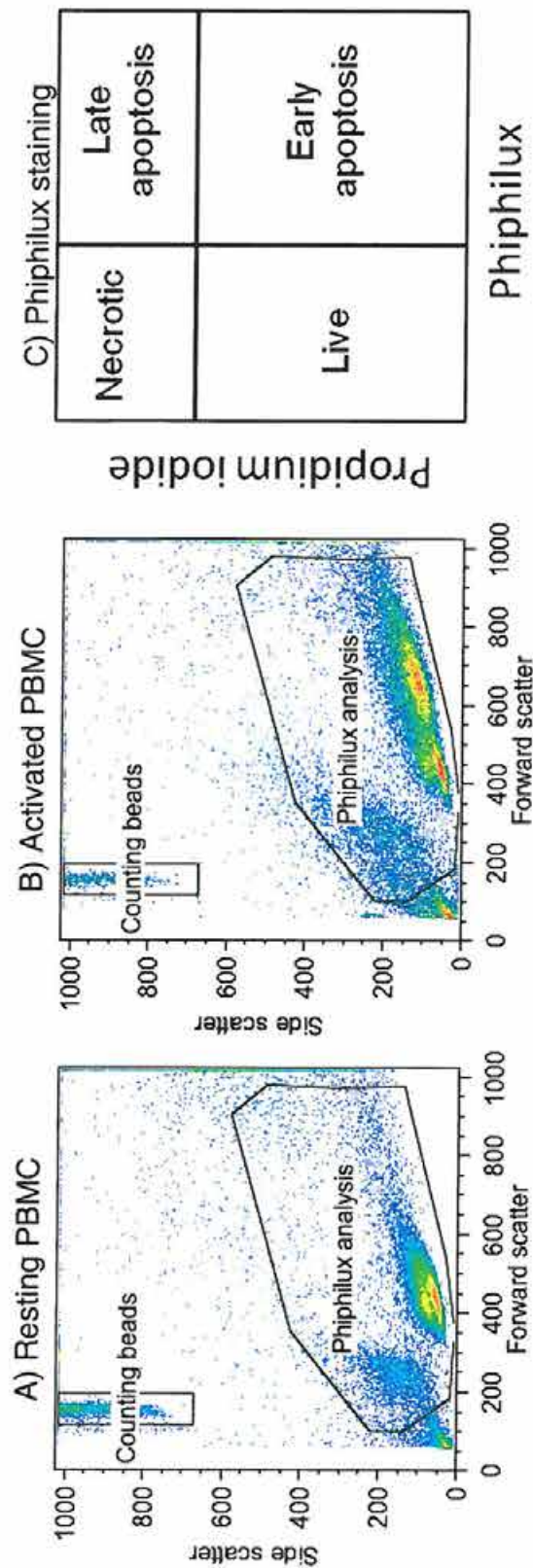
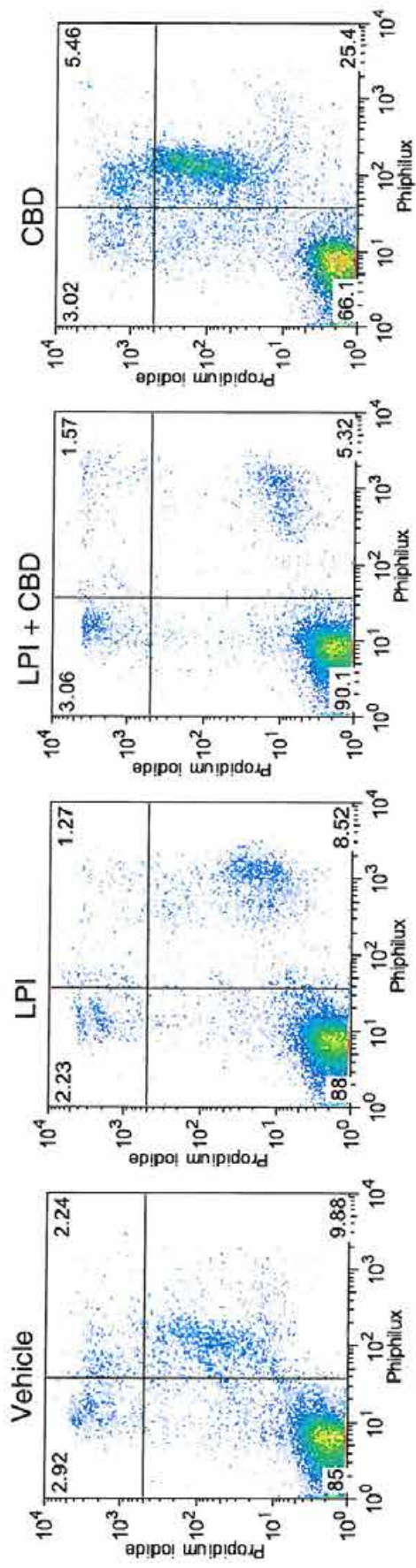


Figure 4.9: Effect of GPR55 receptor ligands on the induction of apoptosis.

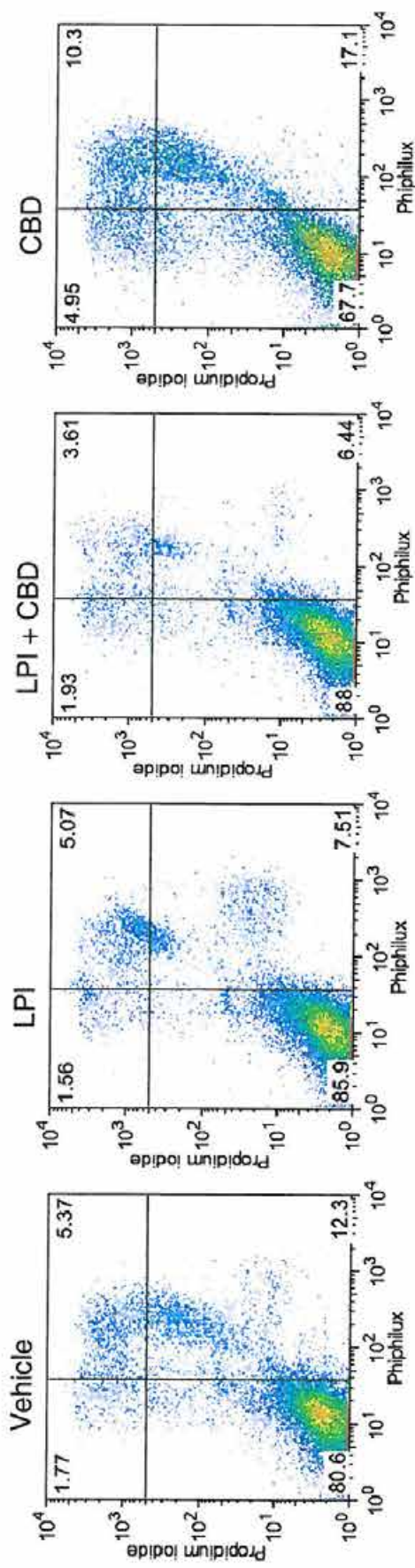
Figure 4.9.1: Analysis of apoptosis in PBMC labelled with Phiphilux and propidium iodide. Isolated PBMC were incubated for 4 days with, or without CD3/CD28 activation beads in the presence of LPI (3 μ M), CBD (10 μ M) or both. Cells were labelled with phiphilux and propidium iodide prior to FACS analysis. Representative FSC SSC plots are shown for A) resting and B) activated PBMC. Lymphocytes were gated according to physical characteristics determined using FSC SSC plots. C) Apoptotic and necrotic cells were assessed by phiphilux versus propidium iodide staining.

Figure 4.9.2: (following page) Phiphilux and propidium iodide staining in resting (top row) and activated (bottom row) PBMC. Percentage of necrotic, late apoptotic, early apoptotic and live lymphocytes are shown for each drug condition. Data shown is from the same donor, but representative of 4 individual donors.

Resting PBMC



Activated PBMC



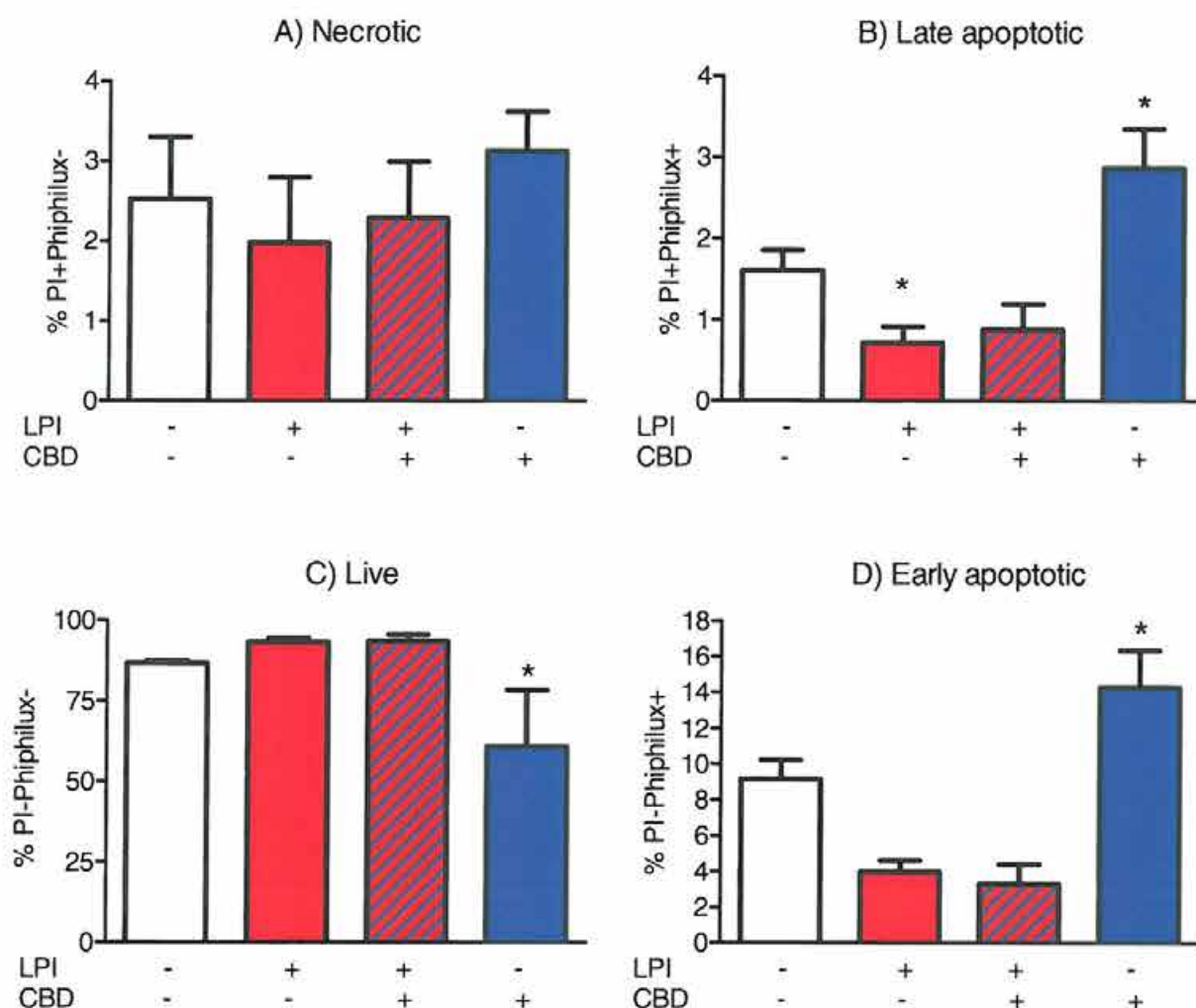


Figure 4.9.3: Effects of GPR55 ligands on the induction of apoptosis in unstimulated PBMC. Isolated PBMC were incubated for 4 days with LPI (3 μ M), CBD (10 μ M) or both. Cells were stained with phiphilux and propidium iodide prior to FACS analysis. Cells are gated for PBMC according to FSC SSC profile. Percentage of A) necrotic cells (phiphilux-PI+). B) late apoptotic cells (phiphilux and PI+; Friedman Test $P=0.001^{**}$) C) live cells (double negative; Friedman Test $P=0.0009^{***}$).and D) early apoptotic cells (phiphilux+PI-; Friedman Test $P=0.0009^{***}$). Data represents the mean of 4 experiments + SEM. Dunn's multiple comparison test where $P<0.05^*$.

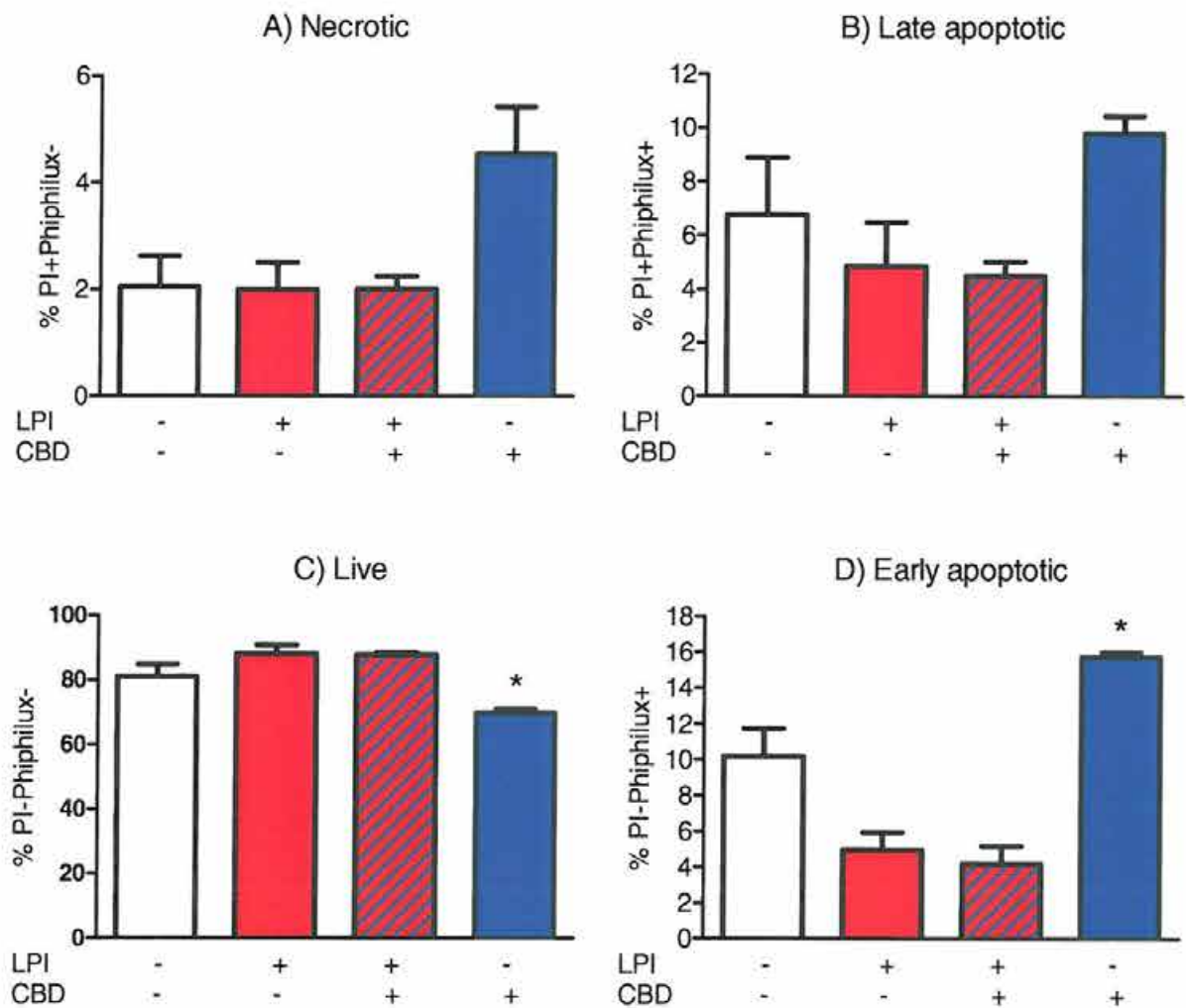


Figure 4.9.4: Effects of GPR55 ligands on the induction of apoptosis in activated PBMC. Isolated PBMC were incubated for 4 days in the presence of CD3/CD28 activation beads with LPI (3 μ M) or CBD (10 μ M) or both. Cells were stained with phipphilux and propidium iodide prior to FACS analysis. Cells are gated for PBMC according to FSC SSC profile. Percentage of A) necrotic cells (phipphilux-PI+). B) late apoptotic cells (phipphilux and PI+) C) live cells (double negative; Friedman Test $P=0.0016^{**}$) and D) early apoptotic cells (phipphilux+PI-; Friedman Test $P=0.0009^{***}$). Data represents the mean of 4 experiments + SEM. Dunn's multiple comparison test where $P<0.05^*$.

Section 4.8 Impact of lysophospholipids on human immune cells

In order to determine whether the anti-apoptotic property of LPI is a characteristic of other lysophospholipids, lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC) were investigated. Lysophospholipids (3 μ M) were applied to freshly isolated PBMC \pm CD3/CD28 activation beads. PI staining was used to discriminate apoptotic cells from live cells.

In unstimulated PBMC (Figure 4.10), LPI (3 μ M) treatment resulted in fewer PI+ cells (64.23 ± 3.10 % of vehicle; $n=3$) although this reduction is not significant. Neither LPC nor LPA treatment influenced apoptosis in resting PBMC.

In CD3/CD28 bead-stimulated PBMC (Figure 4.11), LPI (3 μ M) caused a significant reduction in the total number of PI+ cells (65.26 ± 5.56 % of vehicle; $P<0.01$, $n=5$). Opposite to LPI and its effect in resting PBMC, treatment of stimulated PBMC with LPC resulted in a significant increase in the total number of PI+ cells (3 μ M; 123.40 ± 7.97 % of vehicle; $P<0.05$, $n=5$) whereas LPA did not (3 μ M; 126.60 ± 2.57 % of vehicle; $n=3$).

To assess the impact of phospholipids on proliferation, LPI, LPC and LPA (all 3 μ M) were applied to CFSE-labelled PBMC stimulated with CD3/CD28 beads (Figure 4.12). LPI significantly enhances the total number of proliferated cells (3 μ M; 163.10 ± 13.58 % of vehicle; $P<0.05$, $n=3$). LPC also significantly increased the total number of proliferated lymphocytes (3 μ M; 155.50 ± 7.30 % of vehicle; $P<0.05$, $n=3$) although LPA did not (3 μ M; 129.50 ± 1.09 % of vehicle).

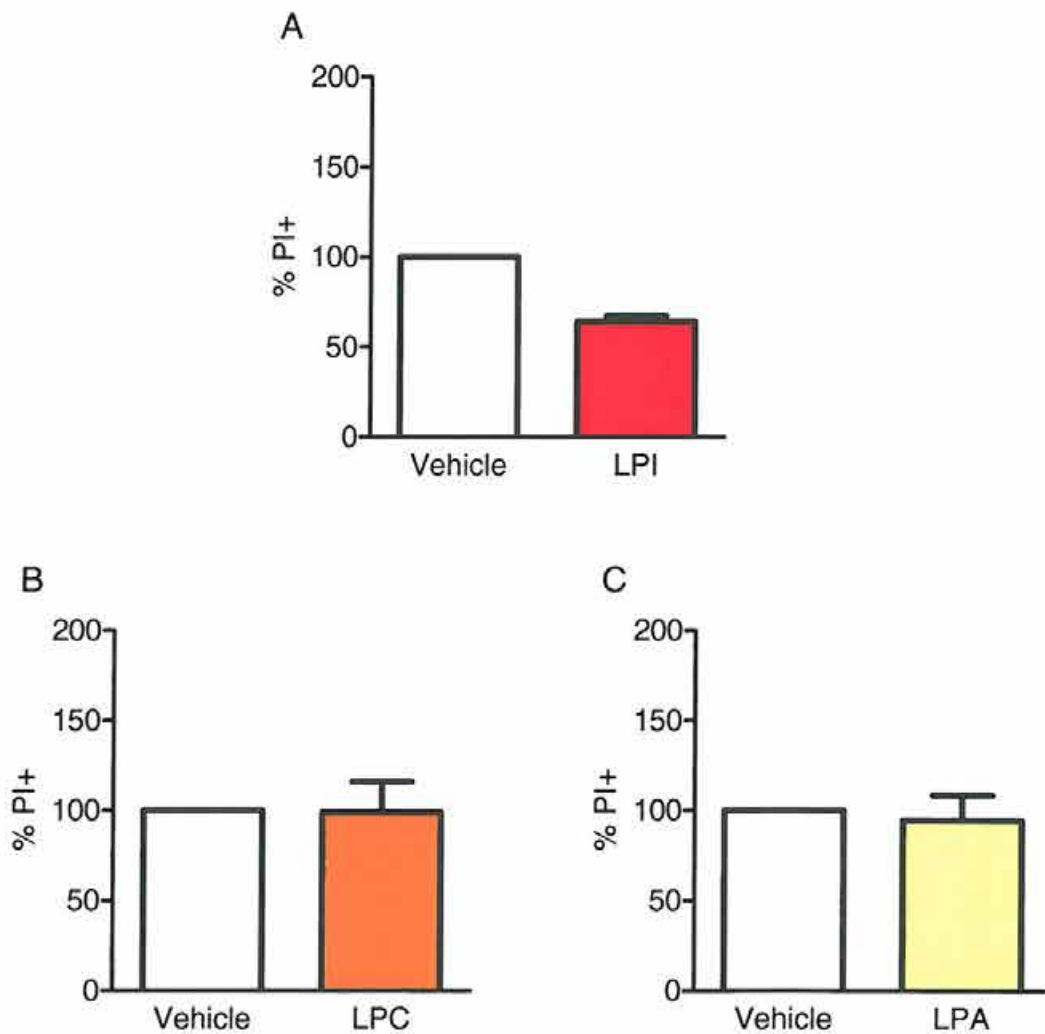


Figure 4.10: Effect of LPI in resting PBMC is not mimicked by treatment with other lysophospholipids. PBMC were incubated for 4 days with 3 μ M of each lysophospholipid. Cells were gated for resting lymphocytes according to physical characteristics using FSC SSC plots. Absolute cell numbers were determined using counting beads. Total number of PI+ cells in resting lymphocytes treated with A) LPI, B) LPC and C) LPA. Data (compared to their own vehicle) represents the mean + SEM; n=3.

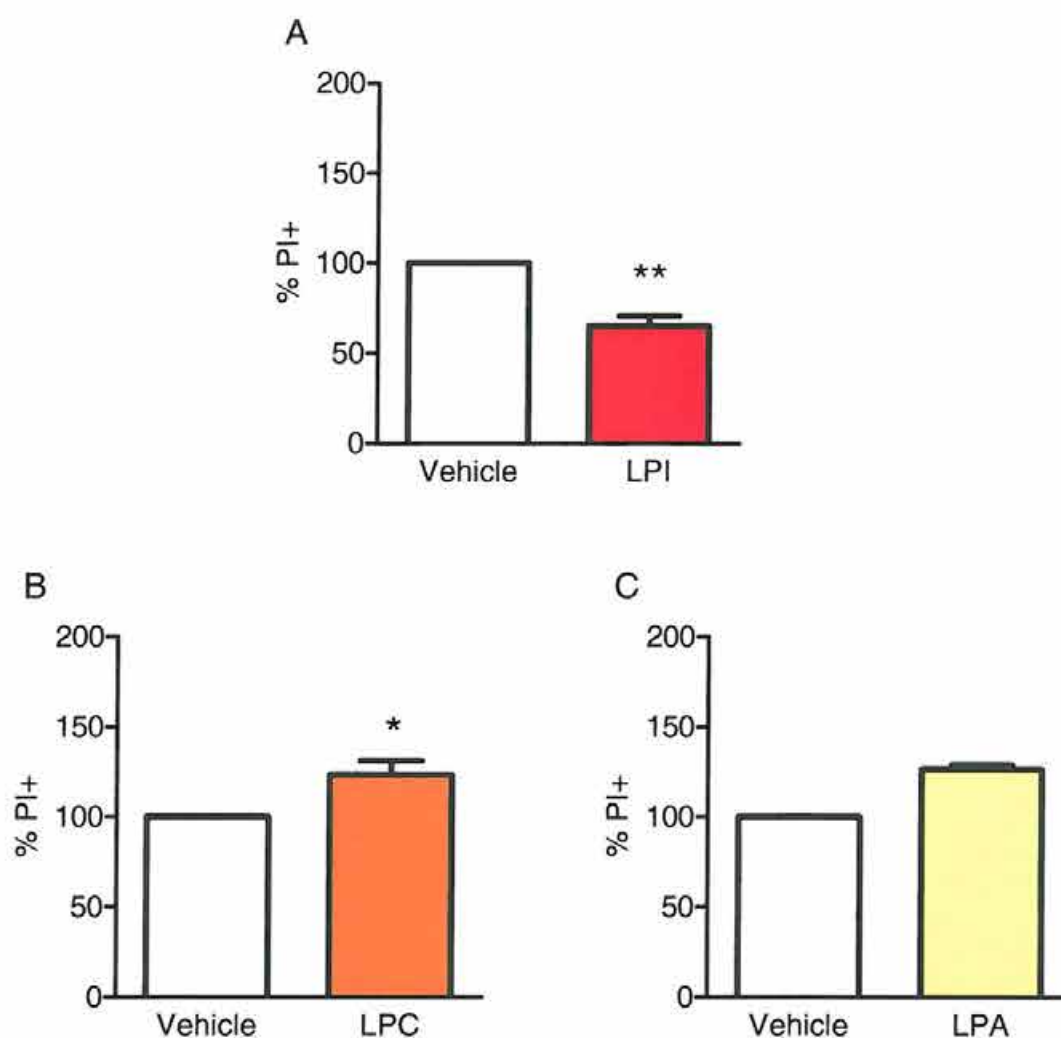


Figure 4.11: Effect of LPI in stimulated PBMC is not mimicked by treatment with other lysophospholipids. PBMC were incubated for 4 days with 3 μ M of each lysophospholipid. Cells were gated for activated lymphocytes according to physical characteristics using FSC SSC plots. Absolute cell numbers were determined using counting beads. Total number of PI+ cells in stimulated lymphocytes treated with A) LPI; $P=0.0033^{**}$, B) LPC; $P=0.0427^{*}$ or C) LPA. Two-tailed paired T test where $p<0.05^{*}$ and $P<0.01^{**}$. Data (compared to their own vehicle) represents the mean + SEM; LPI and LPC; $n=5$ and LPA; $n=3$.

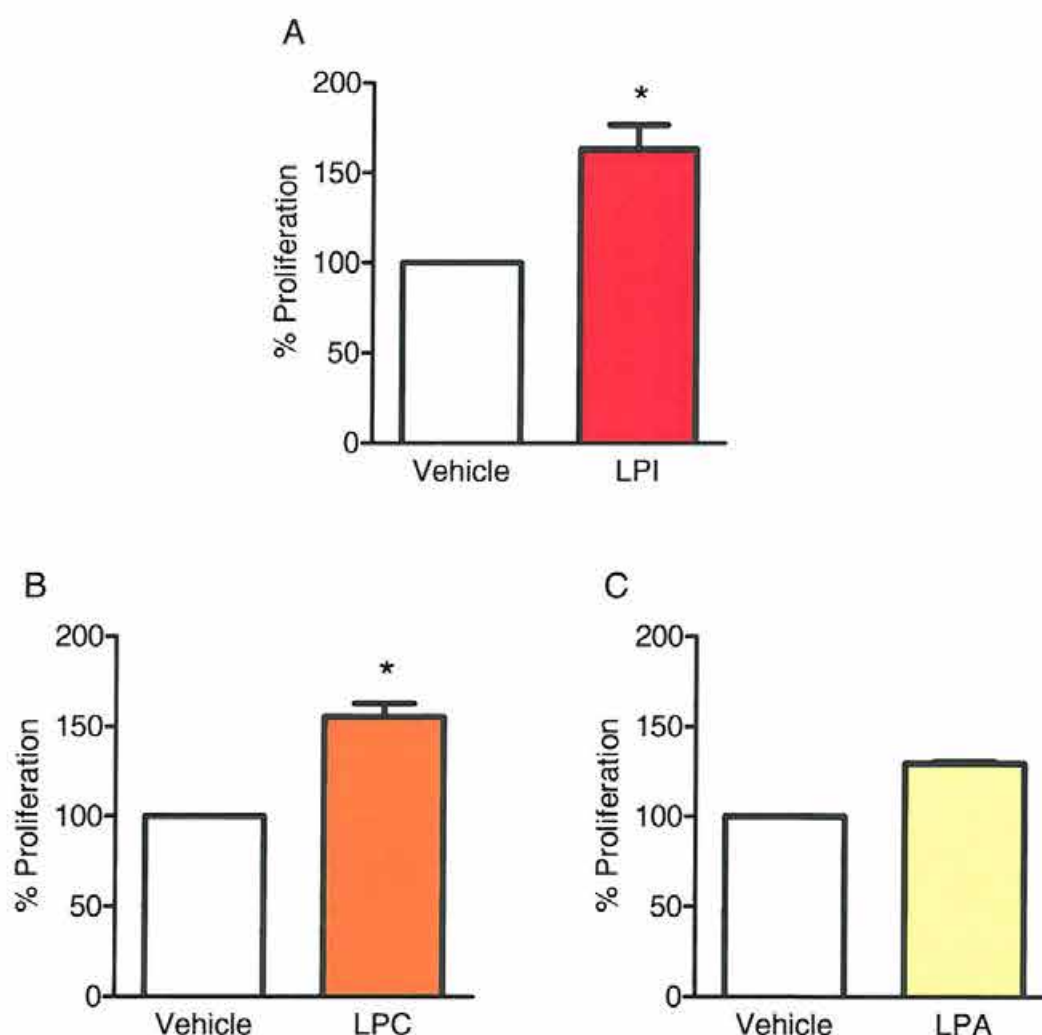


Figure 4.12: Lysophospholipids enhance the proliferation of stimulated PBMC. CFSE-labelled PBMC were incubated for 4 days with 3 μ M of each lysophospholipid. Cells were gated for activated lymphocytes according to physical characteristics using FSC SSC plots. Absolute cell numbers were determined using counting beads. Total number of proliferated cells (defined as CFSE^{mid-low}) in stimulated lymphocytes treated with A) LPI, $P=0.0434^*$, B) LPC; $P=0.0169^*$, or C) LPA. Two-tailed paired T test where $p<0.05^*$. Data (compared to their own vehicle) represents the mean + SEM; $n=3$.

Section 4.9 Impact of CBD on human immune cells

The CBD-induced reduction in the percentage of live cells in activated PBMC was also evident from the FSC SSC profile of CBD-treated PBMC. For the example donor shown in Figure 4.13, CBD (10 μ M) resulted in a 32.7 % reduction in the number of stimulated lymphocytes (25,000 bead events were collected for each sample).

To investigate this more precisely, counting beads were used to determine the absolute number of cells (within a lymphocyte gate) for each sample. By adding a known number of beads (25,000 beads) to a fixed volume of sample (300 μ l), the ratio of the number of cell events to the number of bead events was calculated and used to determine the absolute number of total cells.

Freshly isolated PBMC were incubated for 4 days in the presence of CD3/CD28 activation beads, with CBD (10 μ M) or vehicle. Counting beads were added to each cell sample immediately prior to analysis by flow cytometry. Bead events and PBMC were gated according to FSC SSC plots using FlowJo (Figure 4.14). The absolute number of PBMC was calculated for each sample, and expressed as a percentage of the total number of vehicle-treated cells.

CBD (10 μ M) treatment caused a reduction in the absolute number of lymphocytes (77.05 ± 3.77 % of vehicle; mean \pm SEM; $n=5$; $P<0.05$; Figure 4.14), which was not reversed by the GPR55 agonist LPI (3 μ M; 77.10 ± 10.19 % of vehicle; mean \pm SEM; $n=5$).

From FSC SSC plots, it was also noted that CBD appears to inhibit the proliferation of activated lymphocytes, compared to vehicle controls (Figure 4.13 and 4.14). Therefore, freshly isolated PBMC were labelled with CFSE (1 μ M) and cultured with activation beads with either CBD (10 μ M) or vehicle, for 4 days. Total proliferation was determined using counting beads to calculate the absolute number of proliferated cells (defined as CFSE^{mid-low}) and was expressed as a percentage of the total number of proliferated cells treated with vehicle (Figure 4.15).

CBD (10 μ M) caused a substantial inhibition in the total number of proliferated lymphocytes (44.21 ± 2.99 % as a percentage of vehicle; mean \pm SEM; $P < 0.05$, $n=5$). Consistent with previous data, LPI (3 μ M) was not sufficient to fully reverse this inhibition, only partially restoring proliferation (64.41 ± 13.80 % of vehicle; mean \pm SEM; $n=5$; Figure 4.15).

The impact of both CBD and LPI on the expression of the activation marker CD25 was assessed at 4 and 16 hours post simulation (Figure 4.16). The MFI of CD25 expression appears to be reduced by CBD (10 μ M) at 16 hours (67.27 ± 1.75 % of vehicle; mean \pm SEM; $n=3$; Figure 4.16) although no change is evident at 4 hours. The impact of LPI (3 μ M) on the MFI of CD25 expression was not significantly different to vehicle at either time point (4 hours; 100.38 ± 1.99 % and 16 hours; 110.52 ± 6.39 % of vehicle; mean \pm SEM; $n=3$).

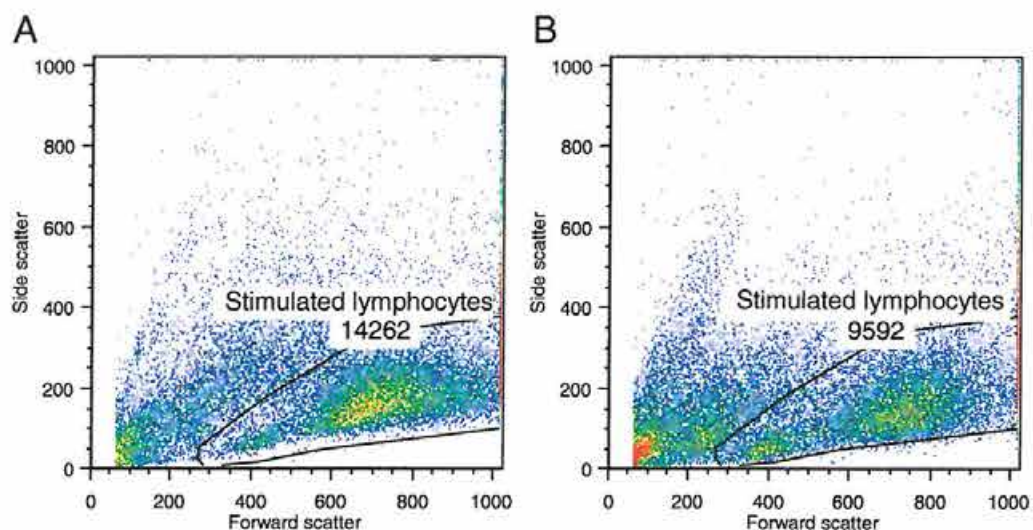


Figure 4.13: CBD appears to reduce the number of, and inhibits the proliferation of activated lymphocytes. PBMC were incubated for 4 days with 10 μ M CBD. Data was analysed by FACS where 25000 events were collected per sample. The number of PBMC contained within a stimulated lymphocyte gate is shown for A) vehicle and B) CBD treatment. Data is representative of more than 8 donors.

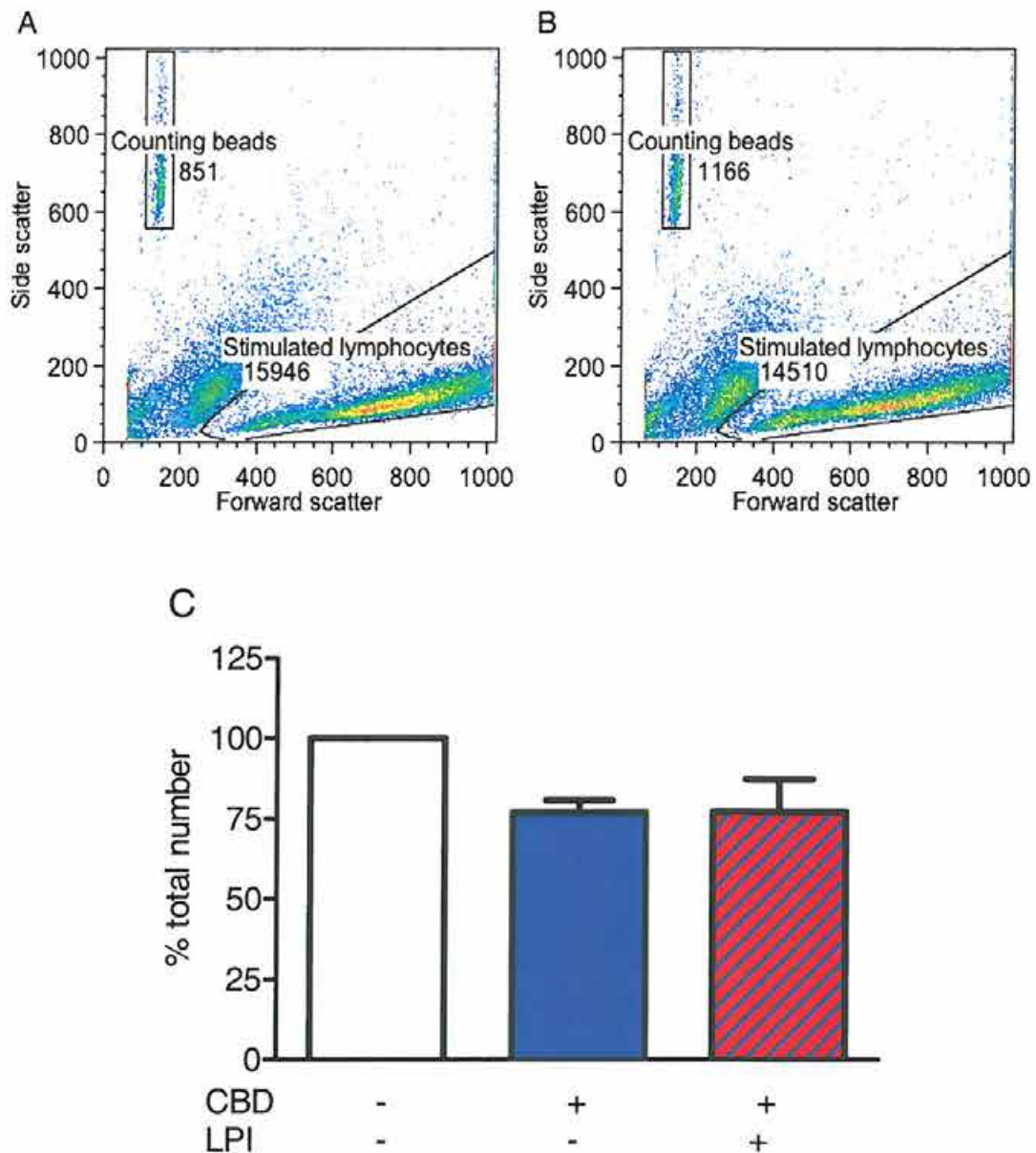


Figure 4.14: Treatment with CBD reduces the total number of lymphocytes in activated PBMC, but is not reversed by LPI. Isolated PBMC were incubated for 4 days with CD3/CD28 activation beads and either vehicle, CBD (10 μ M) or LPI (3 μ M) + CBD (10 μ M). Events were gated for stimulated lymphocytes according to physical characteristics determined using FSC SSC plots. A) FSC SSC plots from a representative donor where vehicle-treated cells are shown on the left, and CBD (10 μ M) on the right. B) Absolute cell numbers were calculated using counting beads and expressed as a percentage of the total number of vehicle-treated cells. Data represents the mean + SEM; n=5.

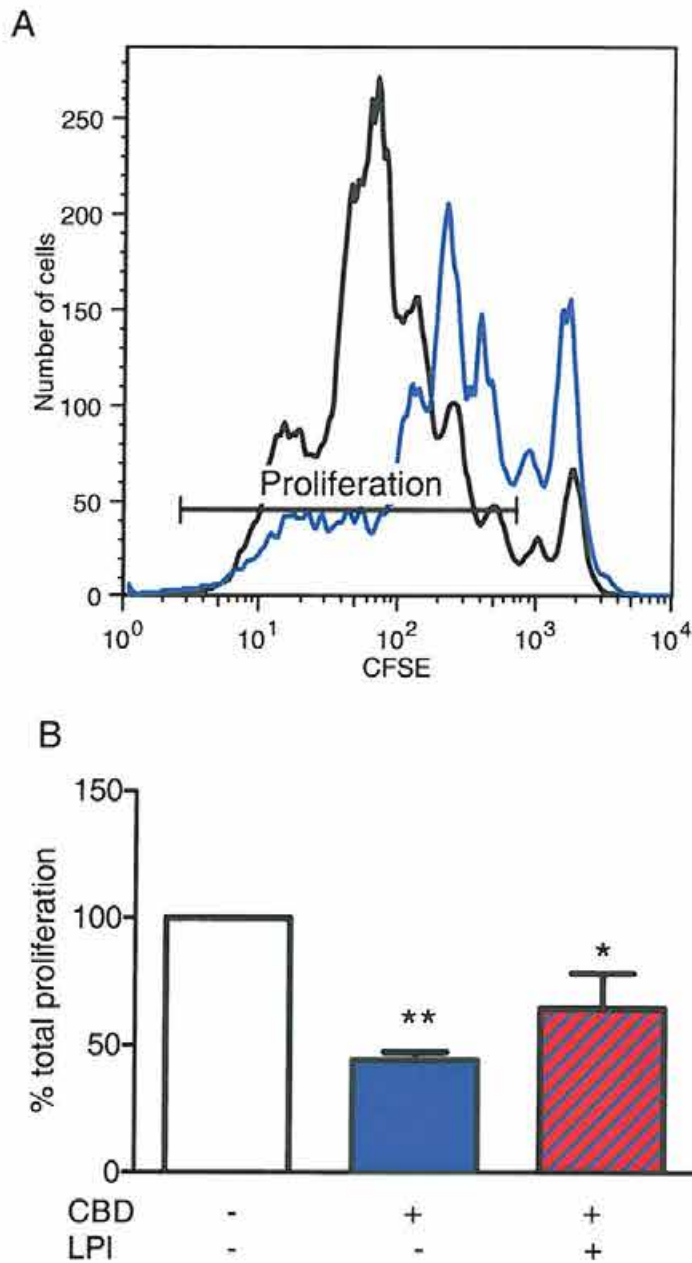


Figure 4.15: CBD inhibits the proliferation of lymphocytes in activated PBMC, which is not reversed by LPI. Isolated PBMC were CFSE-labelled and incubated for 4 days with CD3/CD28 activation beads \pm drug; CBD (10 μ M) or LPI (3 μ M). Events were gated for stimulated lymphocytes according to physical characteristics using FSC SSC plots. Proliferation was determined by the number of CFSE^{mid-low} cells. A) CFSE histogram plot from a representative donor where proliferation of vehicle-treated cells is shown in black, and CBD (10 μ M) in blue. B) Total proliferation expressed as a percentage of the proliferation of vehicle-treated cells. Data represents the mean of 5 individual donors \pm SEM. Repeated measures ANOVA $P=0.0038$ followed by Dunnett's multiple comparison test where $P<0.05^*$ and $p<0.01^{**}$.

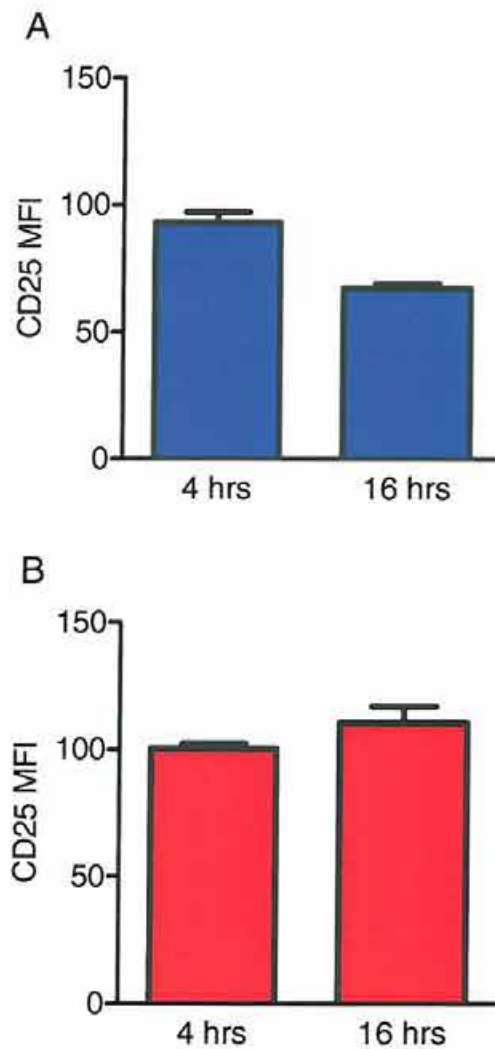


Figure 4.16: CBD reduces the MFI of CD25 expression in activated PBMC . Isolated PBMC were incubated with CD3/CD28 activation beads with either CBD (10 μ M) or LPI (3 μ M). Cells were labelled for CD25 expression at 4 and 16 hours post-stimulation. Events were gated for stimulated lymphocytes according to physical characteristics using FSC SSC plots. MFI of CD25 expression in lymphocyte gated PBMC, expressed as a percentage of vehicle treatment. A) CBD and B) LPI. Data represents mean + SEM; n=3.

The inhibitory effect of CBD on human immune cells was investigated further by characterising the effect of CBD on T cell subsets.

CD4⁺ helper T cell and CD8⁺ cytotoxic T cell subsets were investigated by staining for CD4 and CD8 surface markers (Section 2.4.1) in CD3/CD28 bead-activated PBMC after 4 days treatment with CBD (10 μ M). CBD inhibited, to a variable degree, the proliferation of both CD4⁺ and CD8⁺ lymphocytes (Figure 4.17). When analysed by a two-tailed paired T test, the reduction in CD4⁺ lymphocytes was not significant (51.13 ± 8.91 % proliferation compared to 60.10 ± 11.09 % in vehicle, $n=3$) but the inhibition of CD8⁺ proliferation by CBD was statistically significant (10 μ M; 33.73 ± 17.54 % proliferation compared to 44.97 ± 19.17 % in vehicle; $P=0.05$, $n=3$).

The differential effect of CBD on helper and cytotoxic T cell subsets was also evident when determined in non-CD4⁺ T cells and non-CD8⁺ T cells. The proliferation of non-CD4⁺ T cells was statistically reduced by CBD (10 μ M; 30.07 ± 8.23 % proliferation compared to 40.77 ± 9.84 % in vehicle; $P<0.05$, $n=3$), although the reduction in proliferation of non-CD8⁺ T cells was not.

A differential effect of CBD on CD4⁺ and CD8⁺ T cell subsets was also evident (Figure 4.18). There is a significant reduction in the percentage of CD8⁺ T cells in CBD-treated PBMC compared to vehicle (10 μ M; 26.58 ± 3.70 % and 36.24 ± 3.18 % CD8⁺, respectively; $p<0.05$, $n=4$). In comparison to vehicle treatment, the percentage of CD4⁺ T cells in PBMC were unaffected by CBD treatment. Consequently, there was a trend for the CD4/CD8 ratio to increase as a result of CBD treatment (10 μ M;

2.06 ± 0.40 ; $n=3$) compared to vehicle (1.57 ± 0.21) although this was not statistically significant.

The impact of CBD on other CD4⁺ T cell subsets was investigated using Th17 cells, polarised from isolated CD4⁺ T cells *in vitro* using IL-2 and IL-23 (Section 2.1.3; Figure 4.19). The percentage of Th17 cells (CD3⁺CD4⁺IFN- γ +IL-17⁺ T cells) appeared to be reduced when treated with a higher dose of CBD (10 μ M; 27.95 ± 10.54 % of vehicle; $n=2$) although there appeared to be no effect using a lower dose of CBD (3 μ M; 81.50 ± 14.65 %; $n=2$). IFN- γ -producing CD3⁺CD4⁺ T cells (IL-17⁻) do not appear to be as sensitive to CBD (10 μ M) as either IFN- γ +IL-17⁻ or IFN- γ -IL-17⁺ CD3⁺CD4⁺ T cells (60.66 ± 5.74 % and 40.43 ± 2.54 % of vehicle, respectively; $n=2$).

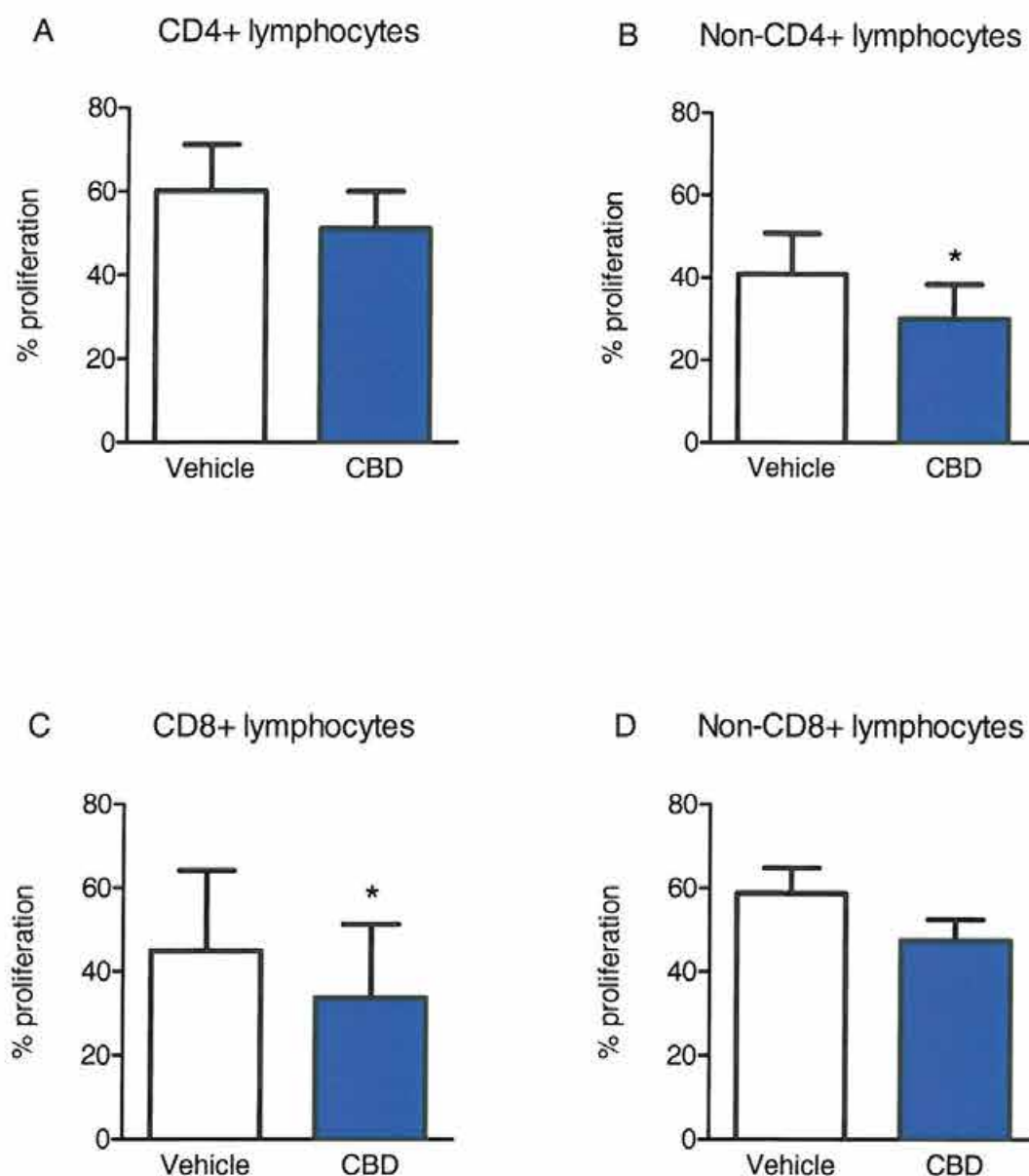


Figure 4.17: CD8+ T cells are more sensitive to CBD-induced inhibition of proliferation than CD4+ T cells. CFSE-labelled PBMC were incubated for 4 days with CD3/CD28 activation beads \pm CBD (10 μ M). Cells were labelled with surface markers prior to FACS analysis. Events were gated for stimulated lymphocytes according to physical characteristics using FSC SSC plots. Proliferation of A) CD4+ lymphocytes B) Non-CD4+ lymphocytes C) CD8+ lymphocytes and D) Non-CD8+ lymphocytes were determined by the percentage of CFSE^{mid-low} cells. Data represents the mean \pm SEM; n=3. Two-tailed paired T test, where $P < 0.05^*$.

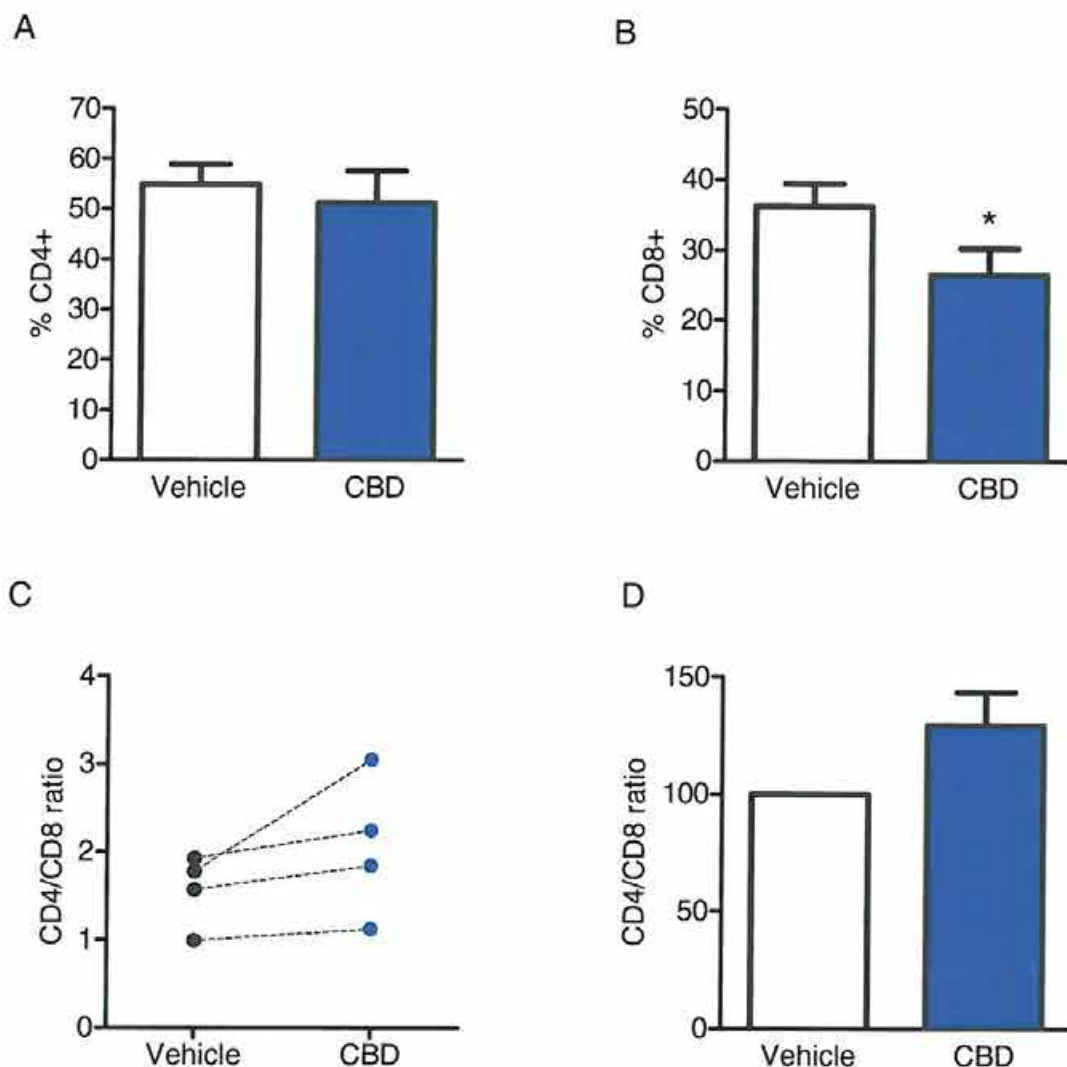


Figure 4.18: CBD reduces the percentage of CD8+, but not CD4+ T cells. PBMC were incubated for 4 days with CD3/CD28 activation beads \pm CBD (10 μ M). Cells were stained for either CD4 or CD8 prior to FACS analysis. Events were gated for stimulated lymphocytes according to physical characteristics using FSC SSC plots. Percentage of A) CD4+ lymphocytes and B) CD8+ lymphocytes. Two-tailed paired T test where $P < 0.05^*$. C) CD4/CD8 ratio was calculated by dividing the percentage of CD4+ lymphocytes by the percentage of CD8+ lymphocytes. Points are shown for 4 individual donors. D) CD4/CD8 ratio expressed as a percentage of vehicle treatment. Data represents the mean \pm SEM; $n=4$.

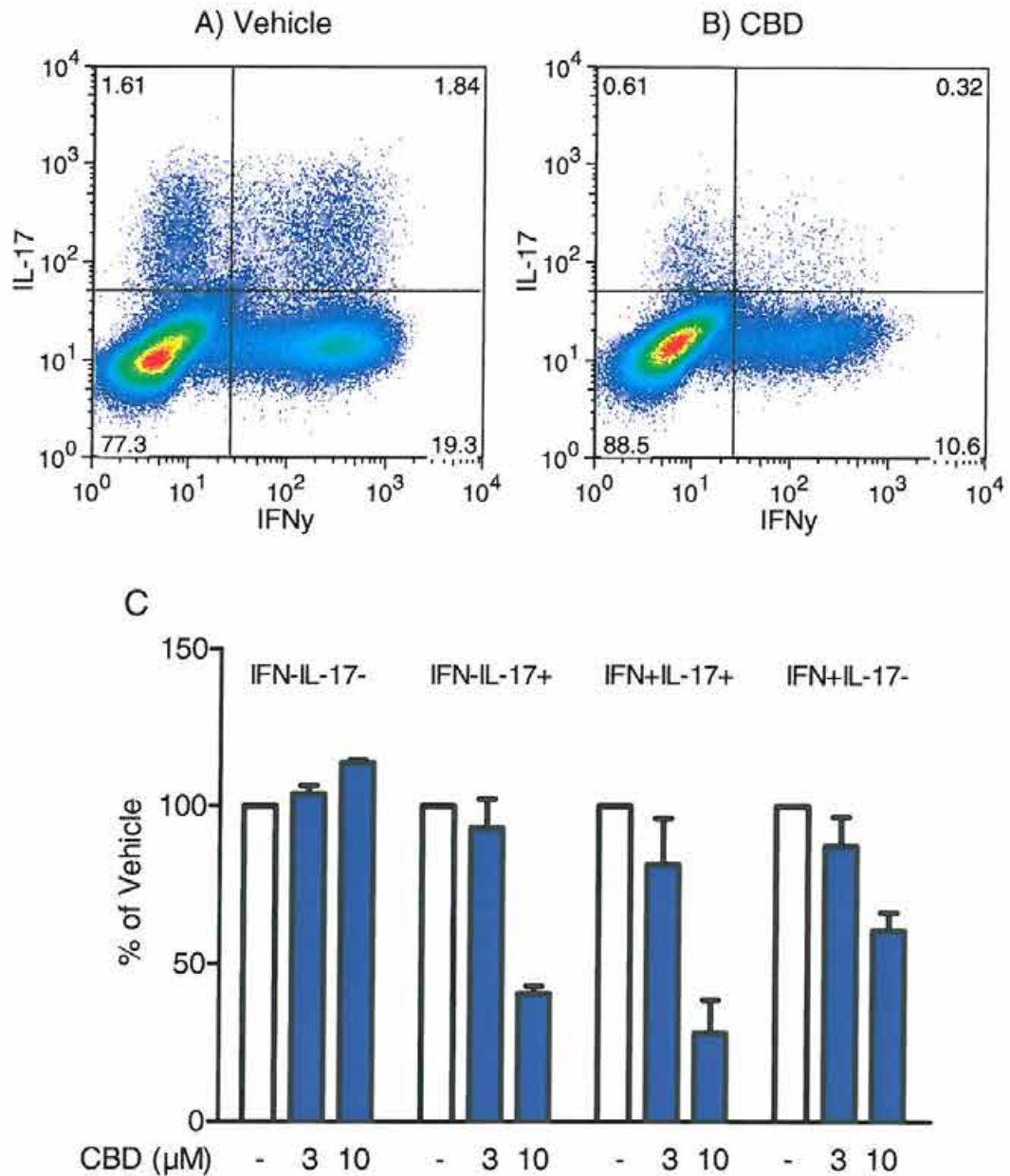


Figure 4.19: CBD inhibits the generation of Th17 lymphocytes. Negatively isolated CD4⁺ T lymphocytes were negatively isolated from fresh PBMC and incubated for 5 days with cytokines IL-2 and IL-23 with CD3/CD28 activation beads. Cells were stimulated with PMA/ionomycin/brefeldin A prior to intracellular labelling for IFN and IL-17. Dot plots are representative of two independent donors; A) vehicle and B) CBD (10 μ M) treated cells, C) frequency of IFN⁺ and IL-17⁺ subsets in CD3⁺CD4⁺ gated lymphocytes, expressed as a percentage of vehicle; n=2.

Section 4.10 The potential role of oxidative stress in CBD-induced immunosuppression

In an attempt to determine whether oxidative stress was responsible for the observed inhibitory effects of CBD on human PBMC, the antioxidant N-acetyl cysteine (NAC) was used as a reactive oxygen species (ROS) scavenger (Kim and Nel, 2005, Rosati *et al.*, 2004). CBD (10 μ M) plus NAC (5 mM and 10 mM) were applied to freshly prepared CFSE-labelled PBMC and stimulated with CD3/CD28 activation beads for 4 days. Counting beads were added prior to analysis using flow cytometry to determine absolute cell numbers.

CBD (10 μ M) caused a slight reduction in cell number (67.74 ± 5.64 % of vehicle; $n=4$), and an inhibition of proliferation (60.53 ± 9.76 % of vehicle; $n=4$) although these results are not statistically significant (Figure 4.20). The reduction in the number of lymphocytes caused by CBD appears to be reversed by 10 mM NAC (110.80 ± 13.72 % of vehicle; $n=4$). Similarly only the higher concentration of NAC (10 mM) appeared to partially reverse CBD-induced inhibition of proliferation (74.10 ± 20.09 % of vehicle). A lower dose of NAC (5 mM) was ineffective in either experiment.

Treatment of PBMC with NAC (10 mM), increases the total number of PBMC compared to vehicle treatment (150.50 ± 14.55 % of vehicle; $P<0.05$, $n=4$). NAC treatment alone did not impact proliferation. Taken together, these results may suggest that CBD causes oxidative stress in human PBMC, leading to a reduction in the total number of cells and an inhibition of proliferation.

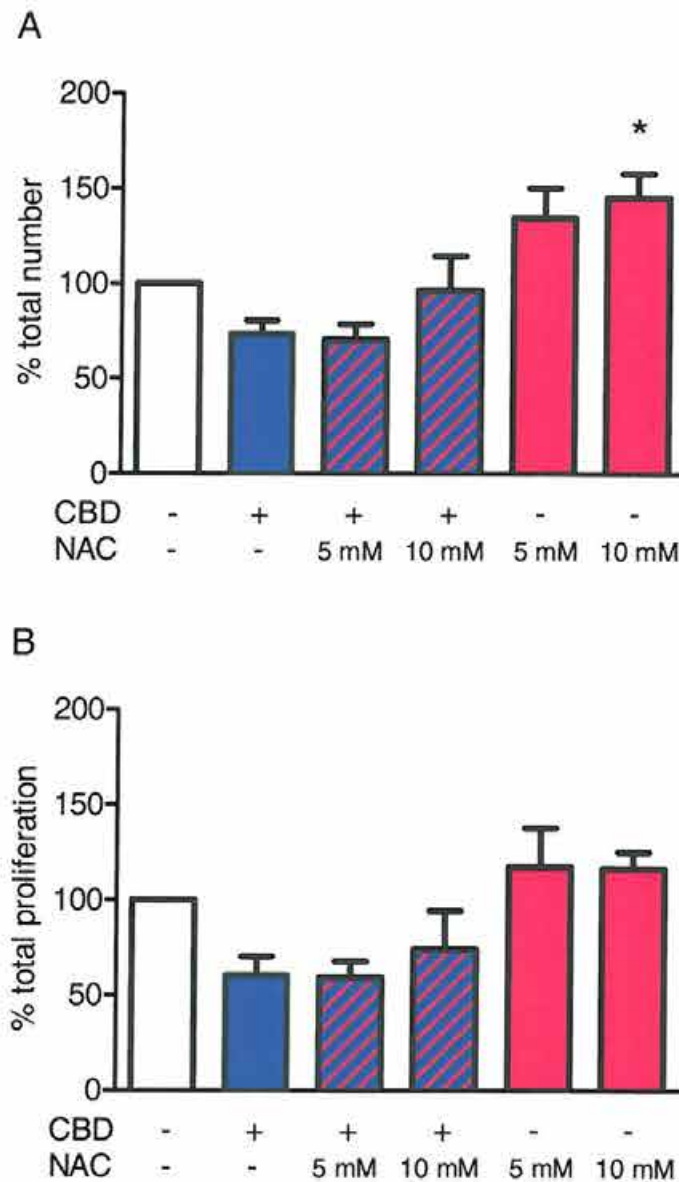


Figure 4.20: CBD may inhibit proliferation through oxidative stress. CFSE-labelled PBMC were incubated for 4 days with CD3/CD28 activation beads \pm CBD (10 μ M) \pm NAC. Events were gated for stimulated lymphocytes according to physical characteristics determined using FSC SSC plots. A) Absolute cell numbers were determined using counting beads, Friedman Test followed by Dunn's multiple comparison test; $P=0.0016^{**}$ where $P<0.05^*$. B) Proliferation was determined by the absolute number of CFSE^{mid-low} cells. Data represents the mean + SEM; $n=4$.

Section 4.11 Discussion

Section 4.11.1 Expression and function of GPR55 receptors in immune cells

The expression of GPR55 receptor mRNA in human tissues has recently been investigated using real-time PCR. Of the human tissues examined, spleen and thymus contained the highest levels of GPR55 receptor expression (spleen>thymus), much greater than the level of expression found in brain (Oka *et al.*, 2010). The data presented herein demonstrates CD4⁺ T cell subsets appeared to express GPR55 receptor mRNA transcripts at a similar level to CB₂ receptors.

Although GPR55 receptors are activated by some atypical cannabinoids, the phospholipid lysophosphatidylinositol (LPI) was identified as a selective endogenous agonist of GPR55 receptors (Oka *et al.*, 2007). Consistent with the evidence implicating GPR55 receptors in cancer biology, levels of LPI have also been linked with cancer pathologies (Sutphen *et al.*, 2004, Shen *et al.*, 2001). Cannabidiol (CBD), one of the major constituents of Cannabis Sativa has been used as a selective antagonist of the GPR55 receptor with an IC₅₀ of 455 nM at GPR55 receptors expressed by HEK293 cells (Ryberg *et al.*, 2007), having very little activity at either CB₁ or CB₂ receptors (IC₅₀ > 30,000 nM at each receptor).

The expression of both CB₂ and GPR55 receptors by human CD4⁺ T cells is interesting, as a recent publication also reported the expression of both CB₂ and GPR55 receptors by neutrophils, which upon activation with agonists for CB₂ and GPR55 receptors (2-AG and either LPI or AM251, respectively) resulted in a synergistic increase in neutrophil chemotaxis (Balenga *et al.*, 2011). However, contrary to this, an earlier study demonstrated the migration of neutrophils was

inhibited by virodhamine, an agonist of the GPR55 receptor and CB₁ receptor antagonist (McHugh *et al.*, 2008). Consistently, both studies have also reported a lack of CB₁ receptor expression by neutrophils (Balenga *et al.*, 2011, McHugh *et al.*, 2008), although the action of virodhamine, which was attributed to a 'non-CB1/CB2 receptor' (McHugh *et al.*, 2008) is opposite to the effect of GPR55 receptor agonists LPI and AM251 on neutrophil chemotaxis reported by Balenga *et al.* (2011). Another major contradiction between these two studies was the effect of LPI on neutrophil chemotaxis, in which McHugh's study reported LPI had no effect on chemotaxis (McHugh *et al.*, 2008). An alternative target for virodhamine may be the orphan GPR18 receptor, a putative abnormal-cannabidiol receptor (McHugh *et al.*, 2010), although this does not explain the difference in the effect of LPI between the two studies.

The impact of GPR55 receptor activation by LPI in PBMC was investigated. For preliminary experiments, PBMC were analysed for the percentage of live, and apoptotic cells based on FSC SSC profiles generated by flow cytometry. There was a marked reduction in the percentage of cells treated with LPI (3 μ M) falling within an apoptotic cell gate, and an increase in resting cells contained by a live cell gate (Figure 4.2), however there was no evidence of the reversal of either effect by the supposed GPR55 receptor antagonist CBD (Pineiro *et al.*, 2011, Ryberg *et al.*, 2007, Whyte *et al.*, 2009).

Although GPR55 receptor transcripts were identified across all CD4⁺ T cell subsets investigated, CD4⁺ T cells are only one subset of lymphocytes; others include CD8⁺ cytotoxic T cells and CD19⁺ B cells for example. Such a drastic effect of LPI in PBMC, may suggest that either expression of the GPR55 receptor is widespread

throughout lymphocytes, or that the effect of LPI, observed in these experiments, may be independent of GPR55 receptor activation. Application of the selective GPR55 receptor agonist O1602 (500 nM) to PBMC *in vitro* did not replicate the effect of LPI (Figure 4.3) suggesting the effect of LPI not be mediated *via* activation of GPR55 receptors. The maximum concentration of LPI (3 μ M) used throughout was sufficiently below the critical micellar concentration of approximately 75 μ M (Falasca and Corda, 1994) which would exclude any possibility of a detergent effect of LPI on immune cells. Concentration response curves for the percentage of live, and percentage apoptotic cells in human PBMC were plotted which indicate LPI is relatively potent (Figure 4.4), with an EC_{50} in the nanomolar range, similar to that reported previously (Ryberg *et al.*, 2007).

It is possible that LPI may be affecting the physical properties of the lymphocyte cell membrane, rendering lymphocytes more resilient to necrosis. In electrophysiological studies, the effect of LPI (10 μ M) on Ca^{2+} -dependent K^+ channels developed slowly, although was reversed after less than 15 minutes of wash-out (Bondarenko *et al.*, 2010), which may indicate that LPI may not have modified the physical structure of the cell membrane. However, the prolonged exposure of human PBMC to LPI over 4 days in these experiments may be sufficient for the intercalation of LPI into the plasma membrane increasing the resilience to necrosis. Although it is likely that LPI may be metabolised before being able to insert itself into lymphocyte cell membranes.

A similar effect to LPI in human PBMC was observed in splenocytes and thymocytes (Figure 4.7.1 and 4.7.2). Interestingly, there appears to be a different sensitivity of splenocytes and thymocytes to LPI (Figure 4.7.3), indicating that the sensitivity of

immune cell subsets to LPI may be different. In humans, GPR55 receptors have been shown to be expressed at much higher levels in the spleen, than in the thymus (Oka *et al.*, 2010). Determining the levels of GPR55 receptor expression in mouse splenocytes and thymocytes would help to clarify whether the differential effect of LPI on immune cells is due to the level of GPR55 receptor expression. Furthermore, to determine whether the effect of LPI is mediated by the GPR55 receptor, these experiments could be repeated using GPR55^{-/-} mice. Should the effect of LPI on cell viability still be evident in GPR55^{-/-} splenocytes and thymocytes then it may be deduced that GPR55 receptor activation is not responsible for the anti-necrotic properties of LPI. Unfortunately however, tissue from GPR55 receptor knockout animals was not available.

Of the GPR55 receptor agonists tested, there was no impact on the total number of proliferated cells. However, LPI (3 μ M) tended to enhance proliferation, whilst AM251 (1 μ M), a non-selective GPR55 receptor agonist and CB₁ receptor inverse agonist, appeared to inhibit proliferation. There is very limited data available on the impact of GPR55 agonists on proliferation, particularly immune cells. However, cholangiocarcinoma cells expressed similar levels of GPR55 receptor expression (both at the mRNA and protein level) as non-malignant cholangiocytes, however only the proliferation of malignant cells was inhibited by both anadamide and O1602 (Huang *et al.*, 2011, DeMorrow *et al.*, 2007).

Activation of the GPR55 receptor by O1602 (10 μ M) reduced cell viability (although only at the highest concentration used), which correlated with an increase in Annexin V staining (Huang *et al.*, 2011). Subsequently, GPR55 receptors were shown to be associated with non-lipid raft fractions of cell membranes, although upon receptor

activation with either anandamide or O1602, the GPR55 receptor migrated into lipid raft regions of the cell membrane, hence the anti-proliferative effect of O1602 was disrupted by destruction of lipid rafts in cholangiocarcinoma cell lines (Huang *et al.*, 2011). Previous to these studies, the same group found the anti-proliferative action of anandamide was mediated *via* an accumulation of ceramide, and the recruitment of Fas death receptor into lipids rafts (DeMorrow *et al.*, 2007). Interestingly, another endocannabinoid 2-arachidonylglycerol disrupted the organised structure or lipid rafts, coinciding with a pro-proliferative effect of 2-arachidonylglycerol in cholangiocarcinoma cell lines (DeMorrow *et al.*, 2007).

These data may implicate the role of the novel cannabinoid receptor GPR55 and its ligands in modulation of immune cell proliferation, mediated by their incorporation into lymphocyte cell membranes. However the opposing effects of endocannabinoids warrants further investigation in the future, including the impact of cannabinoids on the proliferation of defined immune cells subsets.

Using propidium iodide together with phiphilux, the impact of LPI on immune cells was dissected further (Figure 4.9.3). Necrotic cells (phiphilux-PI+) were not rescued by LPI treatment. However, the percentage of late apoptotic cells (phiphilux+PI+) in resting PBMC treated with LPI (3 μ M) was significantly reduced, which may represent the reduction noticed previously in total PI+ cells, where no distinction for necrosis or caspase 3 activity was made. The percentage of necrotic cells (phiphilux-PI+) in activated PBMC was not affected by LPI (3 μ M), consistent with previous experiments therefore it would appear that the anti-apoptotic effect of LPI is dependent upon the activation status of immune cells.

In addition to LPI, PBMC were treated with the putative GPR55 receptor antagonist, CBD (10 μ M) in an attempt to antagonise the effects mediated by LPI (3 μ M). CBD did not reverse the effect of LPI, which supports the notion that (certainly in these experiments) LPI does not mediate its immunoprotective effects specifically through the GPR55 receptor. It may be possible however that the function of CBD in these experiments was a result of antagonism of GPR55 receptor activation by endogenous LPI, preventing endogenous activation of GPR55 receptors.

Section 4.11.2 Impact of lysophospholipids on human immune cells

Although LPI may be acting independent of GPR55 receptors, LPA and LPC have previously been shown to be ineffective at activating GPR55 receptors (Oka *et al.*, 2007). Therefore, if the function of LPI in immune cells was not through activation of GPR55 receptors, then it is possible that other lysophospholipids had a similar impact on immune cells.

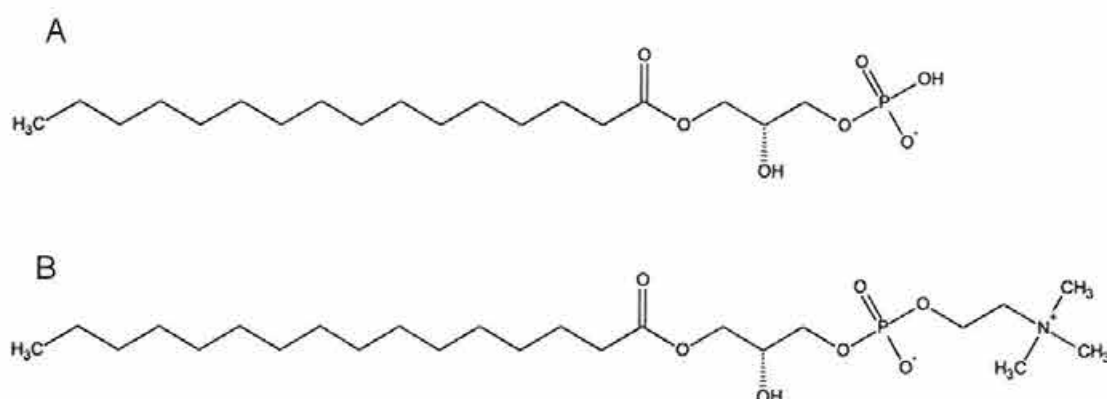


Figure 4.21: Structure of lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC). A) LPA can be produced by the hydrolysis of LPC to LPA by autotaxin. LPA, although can act as a signalling molecule, is most likely produced intracellularly for the production of other lysophospholipids (Guo *et al.*, 2009). B) LPC is produced by the hydrolysis of membrane phosphatidylcholine by phospholipase A₂ but can also be released by thrombin-stimulated endothelial cells (McHowat and Corr, 1993).

Lysophospholipids can be released from platelets (Motohashi *et al.*, 2000, Eichholtz *et al.*, 1993) and fibroblasts (Falasca *et al.*, 1998) and have been cited as important molecules in the cardiovascular (Karliner, 2002), nervous (Toman and Spiegel, 2002) and immune systems (Gendaszewska-Darmach, 2008). Although due to the increase in lysophospholipids during cerebral ischemia, inflammation and cancer (Sasagawa *et al.*, 1999, Shen *et al.*, 2001, Kinouchi *et al.*, 1990, Okita *et al.*, 1997), the role of lysophospholipids was presumed to be damaging (Table 4.1).

	LPI	LPC	LPA
Ovarian cancer	14.7 ± 9.7	37.3 ± 11.6	18.9 ± 14.7
Non-malignant	0.9 ± 0.7	23.8 ± 10.6	2.9 ± 2.0

Table 4.1: Mean concentration (μM) of lysophospholipids in human ascites fluid from ovarian cancer or non-malignant patients with hepatic cirrhosis (Xiao *et al.*, 2001).

The neuroprotective role of LPI has been assessed in both *in vitro*, and *in vivo* models of neurotoxicity. Administration of LPI (200 nmol/kg) was able to protect CA1 pyramidal neurones from cell death, up to 30 minutes after a 15 minute period of ischemia (Blondeau *et al.*, 2002). Cerebral granular cells were protected from glutamate-induced toxicity by both LPI. However, similar results were achieved by LPC, but not LPA, and the authors have suggested that LPI/LPC-mediated neuroprotection was attributed to their activity at 2P-domain K^+ channels (of which LPI and LPC are activators (Blondeau *et al.*, 2002).

In the immune system, 2P-domain K⁺ channels have been identified in human PBMC, spleen and thymus, although expressed at relatively low levels compared to the CNS (Lesage *et al.*, 2000). LPI reduced the total number of PI⁺ cells in unstimulated PBMC, whereas both LPC and LPA were ineffective (Figure 4.10), which may rule out the involvement of 2P-domain K⁺ channels as mediating the immunoprotective effect of LPI.

In our experiments, LPC (3 μ M) resulted in an increase in the total number of PI⁺ cells in activated PBMC, which is opposite to the effect of LPI (Figure 4.11). Similar to LPI, LPC enhanced the proliferation of stimulated PBMC, whereas LPI did not (Figure 4.12). These data would suggest that lysophospholipids have a diverse modulatory role in immune function.

Section 4.11.3 Impact of CBD on human immune cells

CBD has previously been investigated for its impact on immune function, although many studies have been performed using cell lines or primary cells from rodents, rather than freshly isolated human PBMC.

In *in vivo* experiments, continuous systemic administration of a relatively low dose of CBD (5 mg/kg) resulted in a reduction in the total number of B and T cells, where both CD4⁺ and CD8⁺ T cell subsets were affected similarly (Ignatowska-Jankowska *et al.*, 2009). Murine models of autoimmune conditions such as autoimmune arthritis and diabetes respond positively to CBD (5 mg/kg) (Weiss *et al.*, 2008, Malfait *et al.*, 2000), which may occur as a functional consequence of a reduction in effector T cells.

CBD (8 μ M) has previously been shown to activate both caspase 3 and 8 in mouse splenocytes (Wu *et al.*, 2008). *In vitro*, CBD (10 μ M) resulted in an increase in the percentage of early and late apoptotic cells in unstimulated PBMC, and necrotic, early apoptotic and late apoptotic cells (although this fraction was not significantly increased) in stimulated PBMC. Contradictory to LPI, CBD caused an inhibition in the percentage of live cells in both resting and activated PBMC. In resting cells however, this reduction was insignificant, although in activated PBMC was very significant despite a reduction of just 13.8 %. A reduction in the total number of activated lymphocytes was observed upon treatment with CBD (10 μ M; Figure 4.13 and 4.14), by approximately 23 %. CBD (10 μ M) caused a substantial and very significant inhibition of the total number of proliferated lymphocytes.

CBD has previously been shown to induce the generation of reactive oxygen species (ROS) contributing to apoptosis in glioma and leukaemia cell lines (Massi *et al.*, 2006, McKallip *et al.*, 2006). Induction of apoptosis was evident at concentrations of 2.5-10 μ M CBD, although Jurkat cells were more sensitive than MOLT-4 cells (McKallip *et al.*, 2006). These studies found that CBD-induced apoptosis was prevented by the selective CB₂ receptor antagonist SB144528 (5 μ M). However, not all CBD-mediated immune responses have been attributed to its impact on cannabinoid receptors, (Vaccani *et al.*, 2005) which may be due to the low potency of CBD at both CB₁ and CB₂ receptors. In CBD-treated CD4⁺ splenocytes, apoptosis was approximately six times that of vehicle treated cells, whereas CD8⁺ splenocytes were not so sensitive to CBD-induced apoptosis (8 μ M) (Wu *et al.*, 2008).

Differential effects of CBD on cell types and cytokine secretion have been reported previously (Hegde *et al.*, 2008, Ignatowska-Jankowska *et al.*, 2009, McKallip *et al.*,

2006). When taken together these data indicate that CBD inhibits Th1-associated cytokines and increases secretion of Th2-associated cytokines (Weiss *et al.*, 2006, Watzl *et al.*, 1991, Weiss *et al.*, 2008, Newton *et al.*, 1998). This may indicate that CBD may cause a shift in the balance from a Th1 to a Th2 mediated response. CBD reduced the proliferation of CD8+ cytotoxic T cells by approximately 25 % which was a greater reduction than in CD4+ T helper cells. Not only was the susceptibility to CBD-induced inhibition of proliferation different for CD4+ and CD8+ T cell subset, CBD impacted the percentage expression of CD8 in lymphocyte-gated PBMC, whereas CD4 expression remained unchanged.

Th17 T helper cells are a relatively newly characterised subset of T helper cell which have been implicated as having an important role in the pathogenesis of autoimmune conditions (Fouser *et al.*, 2008, Weaver *et al.*, 2006, Harrington *et al.*, 2006). The generation of Th17 T cells appeared to be inhibited by CBD (10 μ M; Figure 4.19). Interestingly, IL-17 producing cells appeared to be more sensitive to CBD than IL-17- T cells. The non-selective endocannabinoid anandamide was also recently shown to inhibit the production of IL-17, and IFN- γ in primary human CD3+ T cells, which was not associated with a reduction in cell viability (Cencioni *et al.*, 2010). This is a promising area of research as immunosuppression often results in a reduction of cell viability, which may leave the patient susceptible to opportunistic infections. The immunosuppressive effect of anandamide was attributed to CB₂ receptors due to the reversal of anandamide inhibition of proliferation by the CB₂ receptor antagonist SR144528, but not SR141617, a CB₁ receptor antagonist. The mechanism of action of CBD is yet to be clarified, however CBD is reported to act selectively at GPR55 receptors over both CB₁ and CB₂ cannabinoid receptors. Despite this, a non-receptor mediated effect should not be excluded at this stage. Irrespective of its action, CBD

may represent a novel method for the reduction of IL-17+ cells. Neutralisation of IL-17 in murine models of autoimmune conditions may provide a target for the prevention of organ rejection and severe autoimmune conditions (Sonderegger *et al.*, 2006, Itoh *et al.*, 2010).

Reactive oxygen species (ROS) are produced during normal cellular metabolism, where the generation of 'superactive' oxygen molecules can cause oxidation of DNA and proteins, a process known as oxidative stress (Davis *et al.*, 2001). The production of ROS has been implicated in the apoptosis of T cells (Tripathi and Hildeman, 2004). The balance of ROS is mediated by thiols which cause reduction of oxidised molecules, limiting oxidative damage. The thiol N-acetyl cysteine (NAC) is the precursor to glutathione, a reducing agent involved in the regulation of ROS. Consequently NAC has been shown to reduce apoptosis in both tonsillar B cells (Rosati *et al.*, 2004) and rat T cell lines (Sandstrom *et al.*, 1994).

Wu *et al.*, found that N-acetylcysteine (1 mM) was sufficient to reverse increased levels of caspase 8 activity caused by CBD (8 μ M) in mouse splenocytes (Wu *et al.*, 2008). In our experiments, only a higher concentration of NAC (10 mM) tended to reverse CBD-induced (10 μ M) lymphopenia in human PBMC, whereas a lower dose of NAC was ineffective (5mM; Figure 4.20). Although this concentration was much greater than that used by Wu *et al.*, for their experiments on mouse splenocytes (Wu *et al.*, 2008) and human monocytes (Wu *et al.*, 2010), it is however less than that used by the groups of Rosati and McKallip (up to 20 and 25 mM NAC, respectively). However, this may reflect the difference in sensitivity of experimentally transformed cell lines and primary immune cells to oxidative stress.

The inhibition of proliferation caused by CBD in human PBMC did not appear to be greatly affected by NAC (10 mM) which may suggest that CBD-induced inhibition of proliferation is not necessarily a direct consequence of oxidative stress.

CBD was initially used in this thesis as an antagonist of GPR55 receptors, which has been successful in other studies (Whyte *et al.*, 2009, Pineiro *et al.*, 2011). Although CBD has very low potency at CB₁ and CB₂ receptors (Ryberg *et al.*, 2007), one study has implied that CBD may act as an inverse agonist at CB₂ receptors (Thomas *et al.*, 2007). Besides a specific action at cannabinoid receptors, cannabinoids are lipophilic therefore the apoptotic role of CBD may be a result of its interaction with cell membranes directly, which has been indicated as a mechanism of action for other cannabinoids (Powles *et al.*, 2005).

CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Section 5.1 5-HT_{3A} receptors in human immune cells

The role of 5-HT in the immune system is thought to be both stimulatory and inhibitory, which may be achieved by the expression of a variety of 5-HT receptor subtypes (Cloezy-Tayarani *et al.*, 2003), although there is little evidence (i.e. studies using physiologically relevant concentrations of 5-HT) implicating the 5-HT_{3A} receptor as either pro- or anti-inflammatory. The data presented in this thesis suggests that the role of 5-HT_{3A} receptors may act to control inflammatory immune responses. The role of Ca²⁺ is potentially important in this phenomenon and an extension of this research should investigate this possibility.

Although regulatory T cells of the CD4⁺ lineage tend to attract the most attention, CD25 and FoxP3 expression are not restricted to CD4⁺ T cells. Regulatory cells from any lymphocyte lineage are important in controlling autoimmunity, yet under pathological conditions may contribute to the mechanisms by which tumours and cancerous cells avoid detection and elimination by the immune system (Karanikas *et al.*, 2008, Hinz *et al.*, 2007). For example, studies have implicated the role of CD8⁺FoxP3⁺ cells in colon cancer (Sobhani and Le Gouvello, 2009, Chaput *et al.*, 2009), where Chaput *et al.* found the percentage of CD8⁺CD25⁺FoxP3⁺ cells to be significantly higher in both the blood and colonic tissue of colon cancer patients compared to healthy individuals (Chaput *et al.*, 2009). Although there is relatively little evidence describing the role of FoxP3 in the function of regulatory B cells, previous research suggests a link between FoxP3 expression in B cells and malignancy (Ebert *et al.*, 2008). Expression of 5-HT_{3A} receptor subunits should be investigated in CD8⁺ regulatory T cells and CD19⁺ regulatory B cells to determine the selectivity of 5-HT_{3A} receptor subunit expression by cells with a suppressor function.

Furthermore, the expression of other 5-HT₃ receptor subunits should be examined, preferably by real-time PCR. Expression of heteromeric 5-HT₃ receptors in the immune system would add to the complexity of immune responses. From a therapeutic perspective, expression of 5-HT₃ receptors in human immune cells may offer a target for pharmacological modulation using specific ligands.

The generation of antibodies suitable for the detection of 5-HT_{3A}, 5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} receptor subunits by flow cytometry would be advantageous in progressing this research. Flow cytometry would be an efficient, high-throughput method of characterising the phenotype of cells expressing 5-HT₃ receptor subunits. Furthermore, the isolation of immune cell populations expressing the 5-HT₃ receptor subunits would be useful in investigating the consequence of 5-HT₃ receptor activation more directly. For example, cells expressing various 5-HT₃ receptor subunits could be isolated and cultured in the presence of 5-HT₃ receptor-specific ligands. An alteration of cytokine secretion as a result of 5-HT₃ receptor activation could be detected by ELISA. This would further characterise the functional impact 5-HT₃ receptor expression in immune cells.

Section 5.2 GPR55 receptors in human immune cells

We have identified GPR55 receptor transcripts in mRNA from healthy human CD4⁺ T cells. Due to the association of GPR55 receptor expression with malignancy and poor clinical outcome, the expression of GPR55 in T and B lymphocytes from patients with lymphoma, compared to healthy individuals would be a natural progression of this research, particularly as GPR55 receptor expression has been suggested to be useful as a marker for disease progression (Pineiro *et al.*, 2011). As an extension of

the data presented, the impact of CBD on PBMC isolated from patients with disease pathologies would be of particular interest.

There is evidence from one group which suggests that THC enriched suppressor cell populations such as regulatory T cells and myeloid-derived suppressor cells (MDSC), enhance the suppression of other immune cells directly (Hegde *et al.*, 2008, Hegde *et al.*, 2010). Therefore CBD may also enhance the number of regulatory T cells, contributing to the generalised immunosuppressive effect of CBD and could be investigated using human PBMC.

Although the use of controlled substances for medicinal purposes is controversial, the exploitation of the non-psychoactive CBD has successfully led to the approval of Sativex (although currently only in Canada), a THC- and CBD-containing spray used for the treatment of neuropathic pain caused by multiple sclerosis (Barnes, 2006). Autoimmune conditions affect vast numbers of people worldwide therefore the commercial potential of better therapies is attractive to pharmaceutical companies, of which CBD (and its derivatives perhaps) may be a valuable target.

Although unlikely to be mediated by GPR55 receptors, the immunoprotective role of LPI warrants further investigation as this characteristic was distinct from other lysophospholipids which were also investigated (albeit briefly). Metabolites of LPI should be explored as it is possible that LPI is not acting directly on immune cells, but rather is metabolised and a by-product is imparting protection from apoptosis.

Overall the research contained in this thesis furthers our current understanding and knowledge on the important role of neurotransmitter receptors and their ligands in

immune function. Both the serotonergic and cannabinoid systems which have been investigated offer novel targets for pharmacological modulation of the immune response which may benefit those suffering with conditions caused by dysfunction of the immune system.

CHAPTER 6

APPENDICES

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Appendix 1

A)		Oligonucleotide sequence	% GC	T _m °C	Product size bps	Annealing °C
HTR2A	F	TGA TGA CAC CAG GCT CTA CAG T	50.0	60.3	353	55.7
	R	GCC ACC GGT ACC CAT ACA G	63.2	61.0		
HTR2C	F	GCA GAT TCC AAG AGT TGC CG	55.0	59.4	202	55.5
	R	TGC TGT TCT TTC TCA CAC ACT GCT AA	42.3	61.6		
HTR3A	F	CAC CCT GGT TAT GCT CTG GT	55.5	59.4	192	51.3
	R	TGA GAT GAA TTG GCA TTG GA	40.0	53.2		
HTR4	F	ATG CTA ATG TGA GTT CTG AGG AGG	45.8	61.0	201	54.9
	R	GCA CCG AAA CCA GCA GAT C	57.9	58.8		
HTR6	F	TTT GTG GGC TAG GCT ATG CAA	50.0	57.6	218	53.2
	R	TGA GAG GTG CGC AGT GAT G	57.9	58.8		
HTR7	F	TTC TGT GGC TAG GCT ATG CAA	47.6	57.9	201	53.6
	R	CAG CAT CTT GTA GCA CAA ACT CAG	41.7	59.3		

B)		Oligonucleotide sequence	% GC	T _m °C	Product size bps	Annealing °C
CNR1	F	CAT CAT CAT CCA CAC GTC TG	50.0	57.3	330	52.3
	R	ATG CTG TTA TCC AGA GGC TG	50.0	57.3		
CNR2	F	TTT CCC ACT GAT CCC CAA TG	50.0	57.3	329	52.3
	R	AGT TGA TGA GGC ACA GCA TG	50.0	57.3		
GPR55	F	CGT CTT CAC CAT CTG CTT CA	50.0	57.3	253	52.3
	R	GAT CAT GGT GCT GGC GA	50.0	59.8		

C)		Oligonucleotide sequence	% GC	T _m °C	Product size bps	Annealing °C
nACh α 7	F	AAG TTT GGG TCC TGG TCT	50.0	60.3	450	55.1
	R	GAT CAT GGT GCT GGC GA	50.0	59.3		

Table 6.1: Oligonucleotide sequences and annealing properties of primers used for RT-PCR. A) 5-HT receptors B) cannabinoid receptors and C) α 7 nicotinic acetylcholine receptors. Primers were purchased from MWG Eurofins, Ebersberg Germany. Nuclease-free ddH₂O was used to reconstitute lyophilised primers which were immediately aliquoted into 5 μ l aliquots and stored at -20 °C.

Appendix 2

	Fluorochrome	Clone	Isotype
CD3	FITC, PE	HIT3a	Mouse IgG1κ
CD4	PerCP	SK3	Mouse IgG1κ
CD8	FITC, PE	HIT8a	Mouse IgG1κ
CD19	FITC, PE	HIB19	Mouse IgG1κ
CD25	FITC	2A3	Mouse IgG1κ
CD27	PE	M-T271	Mouse IgG1κ
CD31	PE	WM59	Mouse IgG1κ
CD56	PE	B159	Mouse IgG1κ
CD69	PE	FN50	Mouse IgG1κ
CD71	PE	M-A712	Mouse IgG1κ
FoxP3	PE	259D/C7	Mouse IgG1κ
IFN	FITC	B27	Mouse IgG1κ
TNF	FITC	MAb11	Mouse IgG1κ

Table 6.2: Fluorescent antibodies used throughout the project for phenotyping of human immune cells by flow cytometry. Antibodies were purchased from BD Biosciences, Oxford UK and stored at 4 °C in the absence of fluorescent light.

Appendix 3**Detection of nACh $\alpha 7$ receptor subunit mRNA transcripts in human T cell subsets**

PCR was performed using oligonucleotide primers specific for the nACh $\alpha 7$ receptor. At 35 PCR cycles, mRNA transcripts were identified for the $\alpha 7$ subunit of the nicotinic acetylcholine receptor in human T cell subsets (Figure 6.1). Specifically, mRNA transcripts were expressed in both resting and CD3/CD28 bead-activated CD4+CD25+ subsets. As a positive control for nACh $\alpha 7$ receptor PCR, human gut cDNA was used.

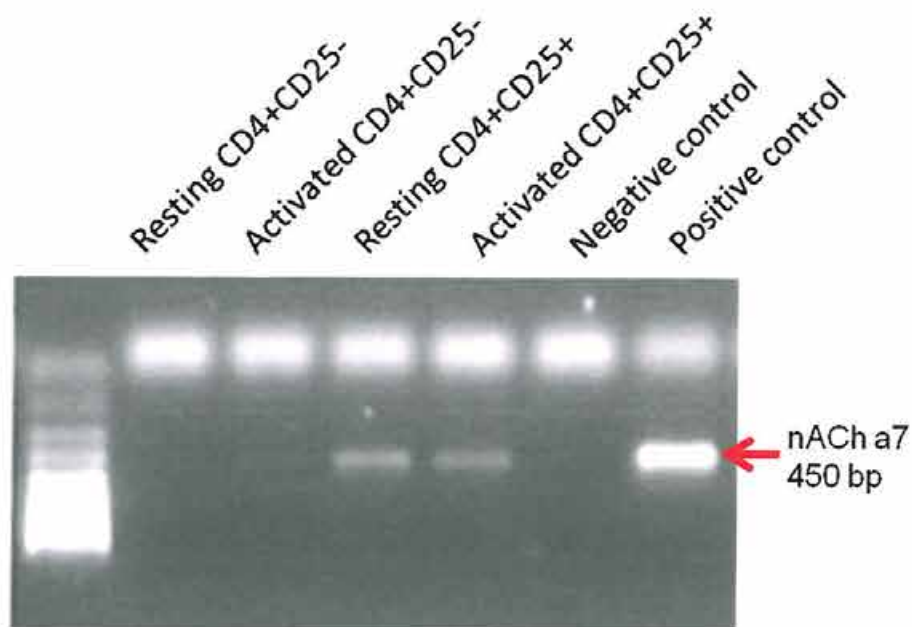


Figure 6.1: Detection of nACh $\alpha 7$ receptor subunit mRNA transcripts in human T cell subsets. Oligonucleotide primers specific for the nACh $\alpha 7$ receptor subunit were used in PCR performed for 35 cycles. A 'no template' (H_2O) control was used as a negative control, whilst human gut cDNA was used as a positive control. PCR is typical of 3 individual donors.

Appendix 4**Effects of CBD vehicle on proliferation of PBMC**

Vehicle used for the dissolution of CBD does not impact the proliferation of human immune cells.

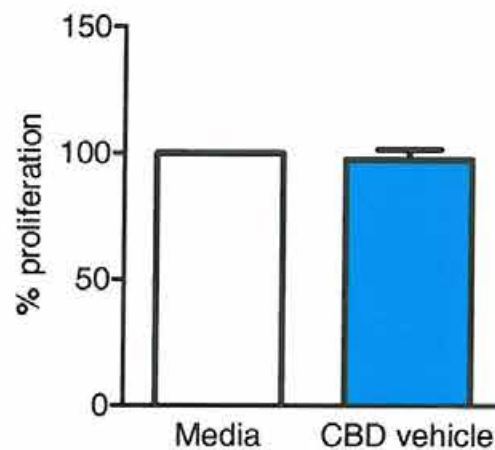


Figure 6.2: Effects of CBD vehicle on proliferation of PBMC. CFSE-labelled PBMC were incubated for 4 days with CD3/CD28 activation beads and with the equivalent concentration of ethanol (0.013 %) in CBD (10 μ M). Events were gated for stimulated lymphocytes according to physical characteristics determined using FSC SSC plots, proliferation was determined by the percentage of CFSE^{mid-low} cells. Data represent the mean + SEM; n=3.

Appendix 5

Published communications arising directly from this thesis and published prior to its completion:

MILTON S., BUTLER A., CURNOW J., GORDON J., BARNES N. 2010. Activation of 5-HT receptors by human immune cells: impact of physiological activation in Serotonin: the new wave. Proceedings of the 2010 Serotonin Club meeting. *Neuropharmacology* 61 (3) 347-549. **P14**. Serotonin Club, Montreal.

MILTON S., BUTLER A., CURNOW J., GORDON J., BARNES N. 2010. Activation of 5-HT receptors by human immune cells: impact of physiological activation. *Basic & Clinical Pharmacology and Toxicology* 107 (S1) 162-692. **P233**. WorldPharma, Copenhagen.

Published communications related to data not presented within this thesis:

MILTON SE., BUTLER AS., LAING JA., WANG S., TURVEY., GU Y., BARNES NM. 2008. Pharmacological comparison of the heteromeric h5-HT_{3A}3C receptor with the homomeric h5HT_{3A} receptor. *Fundamental & Clinical Pharmacology* 22 (Suppl. 2) 119–131. P053. Serotonin Club 2008, Oxford

MILTON S., LOBARINAS E., WEST N., MAWER P., BUTLER A., SALVI R., BARNES NM. 2008. Quantitative PCR analysis of 5-HT receptor expression in tissue from the inferior colliculus and cochlear nucleus of an animal model of tinnitus. *Proceedings of the British Pharmacological Society*. **P139**. British Pharmacological Society Winter meeting, Brighton.

CHAPTER 7

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