SUPPRESSION OF INTERLEUKIN-2 SECRETION BY 15 DEOXY-PROSTAGLANDIN J_2-GLYCEROL, A PUTATIVE CYCLOOXYGENASE-2 METABOLITE OF 2-ARACHIDONYL GLYCEROL, INVOLVES THE ACTIVATION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR γ

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ABSTRACT

SUPPRESSION OF INTERLEUKIN-2 SECRETION BY
15 DEOXY - PROSTAGLANDIN J_2 - GLYCEROL, A PUTATIVE CYCLOOXYGENASE-2
METABOLITE OF 2-ARACHIDONYL GLYCEROL, INVOLVES THE ACTIVATION OF
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2-Arachidonyl glycerol (2-AG) is an endogenous arachidonic acid derivative capable of suppressing interleukin (IL)-2 production by activated T cells. 2-AG-mediated IL-2 suppression is dependent on cyclooxygenase-2 (COX-2) metabolism and peroxisome proliferator activated receptor γ (PPARγ) activation. The overall goal of this project was to determine the mechanism of IL-2 suppression by 2-AG by testing the following hypothesis: 15d-PGJ_2-G, a putative COX-2 metabolite of 2-AG, suppresses IL-2 secretion, in part, by the activation of PPARγ and involves modulation of nuclear factor of activated T cells (NFAT) activity. NFAT is a critical transcription factor in IL-2 gene transcription. Because COX-2 metabolism of 2-AG is important for IL-2 suppression, the effect of 2-AG on COX-2 and PPARγ mRNA expression was investigated. In both activated Jurkat cells and primary murine splenocytes, COX-2 protein expression was upregulated (as early as 2 h and 4 h respectively after activation). 2-AG treatment decreased the upregulation of COX-2 mRNA following T cell activation which suggests negative feedback limiting COX-2 mediated metabolism of 2-AG. PPARγ mRNA expression was increased upon activation and 2-AG treatment produced a modest decrease in PPARγ mRNA expression. 15d-PGJ_2-G bound PPARγ-LBD in a PPARγ competitive binding assay. 15d-PGJ_2-G treatment suppressed IL-2 production by activated Jurkat cells, which was

partially attenuated when pretreated with T0070907. Concordant with IL-2 suppression, 15d-PGJ_2-G treatment decreased nuclear factor of activated T cells (NFAT) transcriptional activity in transiently transfected Jurkat cells. Interestingly, T0070907 alone markedly increased NFAT reporter activity suggesting the existence of endogenous PPARγ activation and modulation of NFAT. 15d-PGJ_2-G treatment decreased PMA/I_o-stimulated NFAT DNA binding to the human IL-2 promoter and nuclear NFAT2 accumulation. Interestingly, 15d-PGJ_2-G treatment increased nuclear active HDM2 (human homolog of the oncoprotein and E3 ubiquitin ligase murine double minute 2 – MDM2) expression whereas there was no change in the expression of glycogen synthase kinase 3 β (GSK-3β), both of which regulate NFAT. 15d-PGJ_2-G and other PPARγ agonists, such as rosiglitazone (RGZ) and ciglitazone (CGZ), decreased PMA/I_o-mediated elevation in intracellular calcium in activated Jurkat cells. Surprisingly, the PPARγ antagonists, T0070907 and GW9662, also decreased the PMA/I_o-mediated elevation in intracellular calcium in activated Jurkat cells. In addition, the presence of T0070907 plus 15d-PGJ_2-G (and other PPARγ agonists such as RGZ and CGZ) produced an additive decrease in PMA/I_o-mediated elevation of intracellular calcium, suggesting that 15d-PGJ_2-G effects on calcium might be PPARγ independent. Collectively, our findings suggest that 15d-PGJ_2-G activates PPARγ to decrease nuclear NFAT2 accumulation (via active HDM2) leading to decreased NFAT transcriptional activity eventually causing IL-2 suppression in activated T cells.

I dedicate my dissertation to the following:

To my beloved parents, Dr. Raman Nanjian and Mrs. Swarna Raman, for teaching me persistence and perseverance. With their continued support, I have been able to achieve what I have today. To my husband's family, for their unwavering love and support. To my husband, Sai, for his encouragement and his relentless love. To my brother, Gowrishankar Raman, and my sister-in-law, Meera Parthasarathy, for the encouragement and support when in grad school.To the rest of my family as the popular saying goes "It takes a village to raise a kid".

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LIST OF ABBREVIATIONS

12-HpETE-G	12(S)-hydroperoxyeicosatetraenoic acid glycerol ester
15d-PGJ_2-G	15-deoxy-$\Delta^{12,14}$-PGJ_2-glycerol ester
2-11,12-EG	2-(11,12-epoxyeicosatrienoyl)glycerol
2-14,15-EG	2-(14,15-epoxyeicosatrienoyl)glycerol
2-AG	2-Arachidonyl glycerol
2-EG	2-epoxyeicosatrienoylglycerols
AA	Arachidonic acid
ABHD12	serine hydrolase α-β-hydrolase domain 12
ABHD6	serine hydrolase α-β-hydrolase domain 6
AEA	Anandamide
AMT	Anandamide membrane transporter
AP-1	Activator protein-1
aP2	Adipocyte protein
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CBN	Cannabinol
CGZ	Ciglitazone
CNS	Central nervous system

COX-2	Cyclooxygenase 2
CRAC	Calcium Release Activated Ca^{2+} channels
CsA	Cyclosporin A
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
EAE	Experimental allergic encephalomyelitis
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
Foxp3	Forkhead box protein 3
GSK-3β	Glycogen synthase kinase - 3β
HDM2	Human homolog of the oncoprotein and E3 ubiquitin murine double minute 2 – MDM2
HETE-G	Hydroxyeicosatetraenoic acid glycerol ester
HPB-ALL	Peripheral blood acute lymphoid leukemia human T cell line
IFNγ	Interferon γ
IL	Interleukin
IL-2R	IL-2 receptor
IP_3	1.4.5-inositol trisphosphate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LBD	Ligand binding domain

LOX	Lipooxygenase
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear factor of the κ-enhancer in B cells
NGF	Nerve growth factor
NO	Nitric oxide
PAF	Platelet-activating factor
PG	Prostaglandin
PGH_2-G	Prostaglandin H_2 – glycerol ester
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLCγ	Phospholipase C - γ
PMA/I_o	Phorbol 12-Myristate 13-Acetate/Ionomycin
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	PPAR response elements
RXR	Retinoid X receptor
STAT	Signal transducer and activator of transcription

TGFβ	Transforming growth factor β
THC	Δ^9-tetrahydrocannabinol
TNF	Tumor necrosis factor

LITERATURE REVIEW

I. Cannabinoids

The term cannabinoids comprises a family of structurally similar compounds derived from the marijuana plant (*Cannabis sativa*) and their synthetic counterparts. There are over 60 plant-derived cannabinoids, of which Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN) and cannabidiol (CBD) are the most extensively characterized (Figure 1, on page 3) [1]. Marijuana has been suggested as a treatment for a variety of medical ailments such as nausea associated with chemotherapy, pain, migraine, epilepsy, glaucoma and hypertension [2]. The Food and Drug Adminstration has approved Marinol, an oral preparation of synthetic Δ^9-THC, in the treatment of nausea associated with chemotherapy. In addition, several states in the United States have passed propositions allowing the medical use of marijuana that has led to renewed interest in the therapeutic potential of cannabinoids. Several other synthetic cannabinoids including CP55,940 and WIN55,212-2 are used in the characterization of the cannabinoid receptors to which these compounds bind and act as agonists (Figure 1, on page 3) [3]. In addition to the synthetic agonists, cannabinoid receptor antagonists such as SR141716A, SR144528, AM251 and AM630 are also used to characterize the cannabinoid receptors. 2-Arachidonyl glycerol (2-AG) and anandamide (AEA) serve as major endogenous agonists of cannabinoids receptors. Consequently, the well studied 2-AG and AEA are referred to as endocannabinoids (Figure 1, on page 3) [4, 5]. Other lesser-studied endocannabinoids include 2-arachidonyl glyceryl ether (noladin ether), N-arachidonyl-dopamine and O-arachidonyl ethanolamine (virodhamine) [6].

A. Cannabinoid Receptors

Two cannabinoid receptors have been identified and cloned to date, CB1 and CB2 [7-9]. Two alternative splice variants, hCB1a and hCB1b, have also been discovered, although little is known about their physiological significance [10, 11]. CB1 receptors are found primarily at the terminals of central and peripheral neurons, where they usually mediate inhibition of release of various excitatory and inhibitory neurotransmitters. CB2 is located predominantly in immune cells and upon activation can modulate cell migration and cytokine release [12, 13]. In addition to immune cells, CB2 has also been detected in astrocytes, rat oligodendrocytes, C6 glioma cells, rat retina, embryonic rat, liver, rat placenta and rat uterus [14-17]. Within the immune system, CB2 has been detected in B cells, natural killer cells, monocytes, macrophages, neutrophils, cytotoxic T cells, helper T cells, mast cells, dendritic cells and microglial cells [18-25]. However, low levels of CB1 receptors are expressed by non-neuronal cells such as immune cells [13] and CB2 receptors are expressed by some neurons, both within the brain and elsewhere [26-28]. The role of neuronal CB2 is poorly understood.

Figure 1. Structures of plant-derived and synthetic cannabinoids.

THC, CBN and CBD are plant-derived cannabinoids. CP55,940 and WIN-55,212-2 are synthetic agonists of both CB1 and CB2.

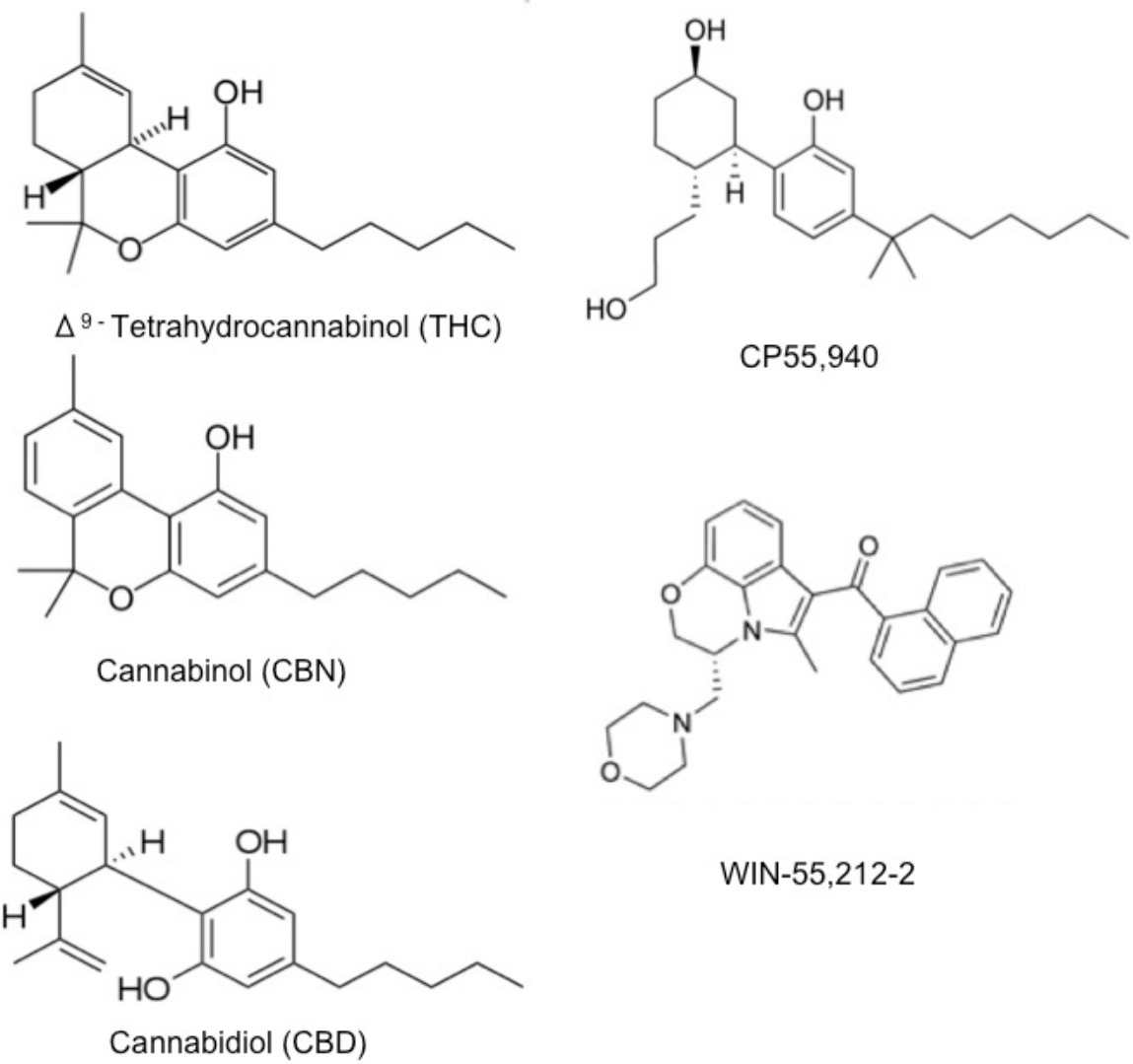
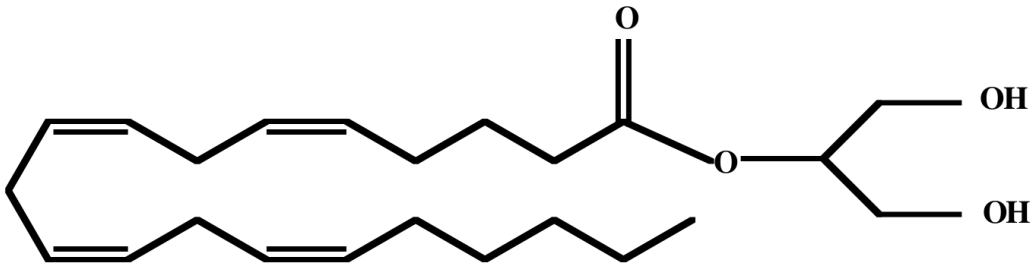
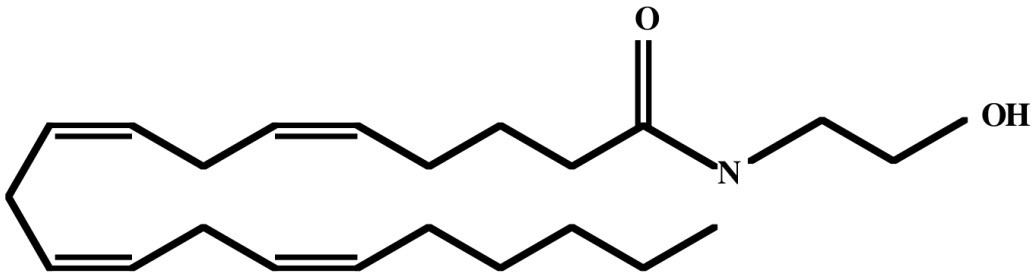


Figure 2. Structures of AA and the well-studied endocannabinoids, 2-AG and AEA.

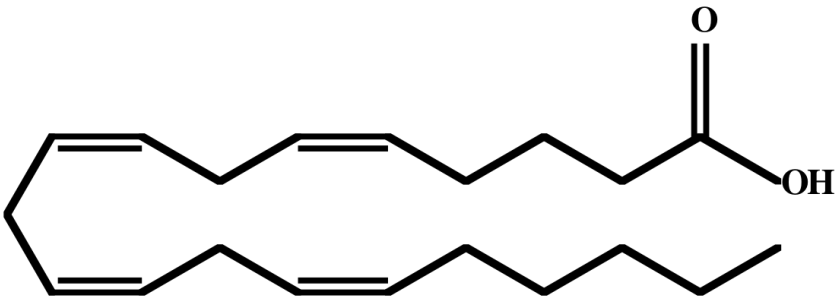
The structure of AA is provided to show the structural similarity between AA and the discussed endocannabinoids.



2-Arachidonoylglycerol (2-AG)



N-Arachidonoylethanolamide (AEA)
Anandamide



Arachidonic Acid (AA)

B. Cellular signaling of cannabinoid receptors

CB1 and CB2 are both G-protein coupled receptors and agonist-induced activation of both CB1 and CB2 inhibits adenylate cyclase leading to a decrease in cyclic adenosine monophosphate (cAMP) in a variety of different model systems. Inhibition of adenylate cyclase by both CB1 and CB2 receptors is pertussis toxin-sensitive, indicating that these receptors associate with $G_{i/o}\alpha$ proteins [29, 30]. It has been demonstrated that both CB1 and CB2 have high affinity for $G_i\alpha$ whereas the affinity of CB1 for $G_o\alpha$ is ten times higher than that of CB2 [31]. In addition, CB1 receptors can also signal through $G_s\alpha$ proteins to cause increased activation of adenylate cyclase resulting in increased cAMP in pertussis-toxin treated cells, suggesting that CB1 can associate with $G_s\alpha$ in the absence of $G_{i/o}\alpha$ proteins [32]. It has been hypothesized that CB1 and CB2 receptors can couple differently to various isoforms of adenylate cyclase and the result of cannabinoid activation depends on which $G\alpha$ subunits and adenylate cyclase isoforms are available [33].

CB1 activation has been shown to mediate activation of A-type potassium channels by modulating cAMP levels in rat hippocampal cells [34]. When CB1 is exogenously expressed in AtT-20 pituitary cells, CB1 can induce inwardly rectifying potassium currents [35]. In addition to potassium channels, CB1 activation can also modulate calcium channel activity. CB1 receptor activation has been associated with inhibition of L- and N- type calcium channels [36, 37]. Similarly, in AtT-20 pituitary cells expressing recombinant CB1 and in rat cortical or cerebellar brain slices, CB1 activation has been demonstrated to inhibit Q-type and P/Q-type calcium currents respectively [38, 39]. CB1 activation has also been demonstrated to induce a rapid, transient increase in intracellular calcium in some cell types. Activation of CB1 receptors in

NG108-15 neuroblastoma/glioma cells induces rapid, transient elevation of intracellular free Ca^{2+} [40]. This increase in intracellular calcium has been attributed to the activation of PLCβ by β/γ subunit (derived from $G_{i/o}$) through CB1 activation leading to the release of inositol-1,4,5-triphosphate (IP_3) that subsequently results in calcium influx [41, 42]. Similarly, cannabinoids augment depolarization-induced calcium influx in cultured cerebellar granule neurons [43]. CB2 activation has also been associated with the induction of transient calcium influx in HL60 cells [44].

In primary murine splenocytes, CBN causes a transient calcium influx that is attenuated when pretreated with both CB1 and CB2 antagonists suggesting that both CB1 and CB2 can modulate calcium channel activity [45]. In addition, Δ^9-THC robustly elevates intracellular calcium in primary murine splenocytes and in peripheral blood acute lymphoid leukemia (HPB-ALL) human T cell line. The removal of extracellular calcium severely attenuated the Δ^9-THC-mediated elevation in intracellular calcium in primary murine splenocytes and HPB-ALL cells, suggesting that Δ^9-THC-mediated increase in intracellular calcium is due to the influx of the extracellular calcium. Δ^9-THC-mediated increase in intracellular calcium in primary murine splenocytes and in HPB-ALL cells is also attenuated when pretreated with both CB1 and CB2 antagonists, SR141716A and SR144528 [46]. It is interesting to note that Δ^9-THC and CBN-mediated elevation in intracellular calcium was observed in both wild-type and $CB1^{-/-}/CB2^{-/-}$ murine splenocytes. Moreover both CB1 and CB2 antagonists, SR141716A and SR144528, attenuated the Δ^9-THC and CBN-mediated elevation in intracellular calcium in wild-type as well

as CB1/CB2 null mice [47]. The abovementioned results suggested that Δ^9-THC induces an influx of extracellular calcium in resting T cells in a CB1/CB2-independent manner and indicated the existence of another receptor that is activated by Δ^9-THC and is inhibited by CB1 and CB2 antagonists.

Both CB1 and CB2 have also been associated with the activation of mitogen activated protein (MAP) kinase in various cell types [48-50]. In C6 glioma cells and primary astrocytes, cannabinoid-mediated activation of MAP kinase (MAPK) is inhibited by pertussis toxin and a CB1 antagonist [51, 52]. CB1-mediated MAPK activation has been correlated with the expression of various immediate early genes, such as c-fos, c-jun and krox-24 [53-56]. Likewise, CB2 activation has been associated with MAPK activation and upregulation of krox-24 [57]. By contrast, CBN has been demonstrated to inhibit PMA/I_o-induced phosphorylated ERK1/2 MAP kinase and cause a decrease in nuclear expression of c-fos and c-jun proteins via their posttranslational modifications in PMA/I_o-activated murine splenocytes [58]. ERK MAPK activation was found to be important for IL-2 production as evident from the observation that the MAPK (ERK1/2) inhibitor PD098059 modestly decreased the nuclear expression of both c-fos and c-jun proteins and caused a decrease in IL-2 secretion in PMA/I_o-activated murine splenocytes [59]. A possible explanation for the differential activation or inhibition of ERK MAPK is the requirement for cellular activation. In the absence of cellular activation, splenocytes express very low levels of phosphorylated ERK MAPK and as result produce no IL-2. The studies in which CBN inhibited ERK MAPK [45, 58], the splenocytes were activated, whereas in the studies that demonstrated cannabinoid-induced activation of ERK MAPK, either the cells were treated directly with cannabinoid compounds [49-51, 56, 57] or the mice were

treated with only cannabinoids [53-55] without activation stimulus. In addition, CB1 and/or CB2 have also been linked to the activation of other secondary messengers such as cyclic guanosine monophosphate (cGMP), focal adhesion kinase, ceramide, and nitric oxide (NO) [60-63].

C. Physiological effects of CB1 and CB2 activation

CB1 activation by administering cannabinoids in mice elicits a characteristic "tetrad" of physiological effects: depression of locomotion, antinociception, hypothermia and catalepsy [64]. CB1 activation inhibits the release of various excitatory or inhibitory neurotransmitters both in the brain and peripheral nervous system [65, 66] indicating that CB1 plays a role in modulating synaptic transmission.

In the cardiovascular system, CB1 activation causes bradycardia and vasorelaxation, which is not observed in CB1 knockout mice [67, 68]. Chronic use of cannabis in humans as well as both acute and prolonged administration of Δ^9-THC to experimental animals produces a long-lasting reduction in blood pressure and heart rate [69, 70] suggesting that modulation of CB1 activity may be novel therapeutic approach in the treatment of hypertension. CB1 has also been implicated to play an important role in reproduction as CB1 is highly expressed in the mouse uterus and increased cannabinoids in the uterus have been associated with inhibition of embryo implantation, increased numbers of stillbirths and increased tubal ectopic pregnancies [71-74].

Within the gastrointestinal system, CB1 activation has been demonstrated to prevent emesis [75, 76] and indeed, Δ^9 THC is prescribed in the U.S. and other countries for this purpose. In addition, various CB1 agonists such as WIN 55,212-2, CP55,940 and ACEA

inhibited gastrointestinal motility in rodents *in vivo* and in isolated ileum and colon from both experimental animals and human, whereas a selective CB2 agonist, JWH-133, did not inhibit gastrointestinal motility [77-82]. The ability of cannabinoids to inhibit gastrointestinal motility coupled with their anti-inflammatory properties suggests that the modulation of the cannabinoid receptor system could offer significant lead in the treatment of various gastrointestinal pathological conditions, including inflammatory bowel disease.

CB1 receptor knockout mice develop severe progressive experimental allergic encephalomyelitis (EAE) upon subcutaneous injection of an emulsion of lyophilized spinal cord homogenate reconstituted in phosphate-buffered saline, and Mycobacterium-supplemented Freund's incomplete adjuvant. EAE is an animal model of multiple sclerosis and these studies indicated that CB1 is involved in protecting against neurodegeneration that results in multiple sclerosis [83]. In addition, CB1 activation provides significant neuroprotection against inflammatory CNS disease in an experimental allergic uveitis model [84]. Thus, there is interest in the clinical use of cannabinoids to slow neurodegenerative processes and for the treatment of multiple sclerosis.

In addition, CB1 activation has been correlated with certain immunological effects, although CB1 is usually expressed at lower levels than CB2 in immune cells. CB1 activation inhibits NO in primary murine astrocytes as well as in rat microglial cells and feline macrophages stimulated with interferon (IFN)γ/lipopolysaccharide (LPS) [62, 85, 86]. Activation of CB1 has been correlated with inhibition of interleukin (IL) – 12 and tumor necrosis factor (TNF) α and induction of the immunosuppressive cytokine, IL-10, in LPS-treated mice [87, 88] suggesting that CB1 activation may be involved in immune homeostasis by the inhibition of IL-12 which is implicated in autoimmunity and by the induction of the anti-

inflammatory cytokine, IL-10. By contrast, CB1 activation has also been associated with immunostimulatory effects, such as induction of IL-6 in mouse astrocytes [87].

Ligation of CB2 by Δ^9-THC significantly inhibited T cell proliferation, IFNγ secretion and shifted the balance of T helper 1 (Th1)/T helper 2 (Th2) cytokines in a co-culture system containing human T cells and dendritic cells and these effects were abrogated in the presence of the CB2 receptor antagonist, SR144528 [89]. This observation suggests that CB2 activation can regulate the activation and balance of human Th1/Th2 cells. By contrast, CB2 activation by CP55,940 ligation enhances the proliferation of CD40-stimulated B cells, which was attenuated in the presence of the CB2 receptor antagonist, SR144528 [90] supporting the involvement of CB2 receptors during B cell differentiation. Δ^9-THC produced a dose-dependent suppression of primary humoral responses to the T cell-dependent antigen, sheep RBC, as evidenced by the antibody forming response with no inhibitory effect on humoral responses to T cell-independent antigen, DNP-Ficoll or the polyclonal B-cell activator, lipopolysaccharide (LPS) [91]. This observation suggests that Δ^9-THC selectively suppresses T cell dependent humoral responses through direct inhibition of accessory T cell function.

CB2 activation has also been demonstrated to inhibit a number of proinflammatory cytokines such as IL-6, IL-12 and TNFα [92-94]. Δ^9-THC produced a marked inhibition of inducible NO synthase transcription and NO production by RAW 264.7 cells that express CB2 transcripts [95] suggesting a role for CB2 in immune homeostasis. CB2 activation by Δ^9-THC reduced the capacity of macrophages to process antigens that are necessary for the activation of $CD4^+$ T cells. This decrease in antigen processing is due to the ability of Δ^9-THC to suppress

the processing of intact lysozyme in a dose-dependent manner and this suppression was blocked by a CB2 selective antagonist [96, 97]. CBN treatment caused a concentration-dependent suppression of IL-2 in PMA/I_o-stimulated thymocytes [98] and caused a decrease in IL-2 production and steady state mRNA expression in PMA/I_o-stimulated primary mouse splenocytes and the murine T cells line, EL4.IL2 [99]. Conversely, CBN enhanced IL-2 gene expression in EL4.IL2 when suboptimally stimulated with PMA (2-10 nM) and this enhancement was attributed to an increase in NFAT-DNA binding activity to IL-2 distal NFAT site [100]. These observations suggest that CBN can possess either inhibitory or stimulatory activity depending on the intensity of cellular activation. More recently, it was demonstrated that Δ^9-THC and CBD suppressed or enhanced IFNγ and IL-2 production by murine splenocytes and human PBMC under optimal or suboptimal cellular activation using PMA/I_o respectively (manuscript submitted – [101]). Similar differential effects of cannabinoids were also observed on nuclear translocation of NFAT although both Δ^9-THC and CBD elevated intracellular calcium regardless of the stimulation level with PMA/I_o (manuscript submitted – [101]). These observations suggest that the cannabinoid-induced calcium elevation provides an adequate signal for activation in suboptimally stimulated T cells leading to an enhancement of cytokine production, whereas in optimally stimulated T cells, the cannabinoid-induced calcium elevation may provide an anergic-like signal due to excessive calcium eventually leading to suppression of cytokine production.

In addition, CB2 activation has been correlated with induction of cell migration in various immune cell types, such as microglia, myeloid precursor cells, differentiated HL-60 cells

and human peripheral blood monocytes [102-105]. Furthermore, CB2 has been correlated with diminished overall antitumor immunity, which is due to the augmentation of immune inhibitory cytokines, IL-10 and transforming growth factor β (TGFβ) [106].

Lymphocyte recruitment to the pulmonary airways is decreased in Δ^9-THC-treated mice challenged with influenza virus A/PR/8/ 34 (PR8) when compared with mice challenged with PR8 alone. In the same model, targeted deletion of CB1 and CB2 receptors produced enhanced inflammatory responses to influenza PR8 in the absence and presence of Δ^9-THC, suggesting involvement of CB1/CB2-dependent and -independent mechanisms in Δ^9-THC effects [107]. In addition, CB2 activation has been related to inhibition of pain responses leading to the speculation that CB2-specific agonists may be of therapeutic benefit in the management of pain and inflammation without CNS side effects [108].

II. Endocannabinoids

A. Identification of the endogenous cannabinoids

The discovery of the cannabinoid receptors lead to a hunt for the endogenous ligands of these receptors. The first endogenous cannabinoid discovered was anandamide (AEA), which was isolated from the porcine brain [4]. Shortly after the discovery of AEA, a second endocannabinoid, 2-arachidonyl glycerol (2-AG) was isolated from canine gut by Mechoulam's laboratory [5] and from rat brain by Sugiura's group [109]. AEA and 2-AG were characterized for biological activity by the Martin and Kaminski labs respectively [110, 111]. Both AEA and 2-AG are arachidonic acid derivatives and are structurally different from the plant-derived and synthetic cannabinoids (Figure 2, on page 4). Other less studied endocannabinoids are noladin

ether, virodhamine and N-arachidonyl dopamine [112]. Noladin ether is also known as 2-arachidonyl glyceryl ether and acts as an agonist at both CB1 and CB2 receptors [113, 114]. Virodhamine is also known as O-arachidonyl ethanolamine and acts as a partial agonist/antagonist at CB1 and full agonist at CB2 [115].

B. Tissue levels of 2-AG

Physiological concentrations of 2-AG are generally higher than the AEA in most cell types. The rat brain contains 3.25 nmol/g tissue of arachidonyl glycerol (2-AG + 1(3)-AG), which is about 800 times greater than the levels of AEA in the brain [109]. It has been reported that 2-AG is present in various rat brain regions such as cortex, limbic forebrain, hippocampus, hypothalamus, diencephalon, striatum, mesencephalon, cerebellum, brainstem, medulla and pituitary [116]. In addition, it has also been reported that the amount of 2-AG in the brain varied during development in rats [117]. Moreover, diurnal variation in the amounts of 2-AG in rat brain has also been observed [118].

In addition to the nervous system, 2-AG has also been detected in various rat tissues such as liver (1.15 nmol/g tissue), spleen (1.17 nmol/g tissue), lung (0.78 nmol/g tissue) and kidney (0.98 nmol/g tissue) [119]. Additionally, 2-AG has been detected in rat plasma (12 nM), human sera (10 nM) and human sera from patients with endotoxic shock (30 nM) [120]. It is not known if there are any differences in the tissue levels of 2-AG in various rat strains. It is possible for the 2-AG levels to vary between various rat strains depending on the expression levels of phospholipase C, the key enzyme involved the production of 2-AG and also on the expression levels of monoacyl glycerol lipase, the enzyme involved in the degradation of 2-AG. The accuracy of the reported values is ambiguous due to various confounding factors. It has also

been reported that the commonly used techniques for the detection of 2-AG are inefficient [120]. Furthermore, 2-AG is both metabolically and chemically highly unstable leading to isomerization and hydrolysis of 2-AG [121, 122].

C. Biosynthesis of 2-AG

2-AG is rapidly formed in various tissues and cells upon activation including ionomycin stimulated N18TG2 cells [123], electrically stimulated rat hippocampal slices, and ionomycin stimulated neurons [124], A23187-stimulated human umbilical vein endothelial cells [125], picrotoxin stimulated rat brain [126], LPS stimulated rat platelets [127], LPS stimulated rat macrophages, and LPS or ionomycin stimulated J774 macrophage-like cells [128], platelet-activating factor (PAF) stimulated human platelets and P388D1 macrophages [129] and PAF stimulated RAW264.7 cells [130]. Unlike neurotransmitters which are stored in vesicles, 2-AG is produced from membrane precursors on demand [6]. There are several enzymatic pathways proposed for the biosynthesis of 2-AG. The pathway that is considered physiologically most important is the one involving phospholipase C (PLC) and diacylglycerol lipase (DAG). This pathway involves the rapid hydrolysis of phosphatidylinositol bisphosphate by PLC to produce DAG, which is then cleaved by DAG lipase to produce 2-AG (Figure 3, on page 19) [124, 131, 132]. There is also evidence to suggest that combined actions of phospholipase A1 (PLA_1) and PLC on phosphatidylinositol bisphosphate can also result in 2-AG formation [132]. 2-AG can also be formed from arachidonic acid-containing lysophosphatidic acid through the action of a phosphatase [132]. Yet another pathway for 2-AG biosythesis has been suggested in which arachidonic acid-containing phosphatidic acid can be hydrolyzed by phosphatidic acid

phosphohydrolase to form DAG, which can then subsequently be cleaved by DAG lipase to form 2-AG [133, 134].

Irrespective of its exact mechanism of biosynthesis, it has been demonstrated that increased intracellular calcium and glutamate receptor activation results in neuronal 2-AG production [124, 135]. Similarly, high-frequency stimulation of the schaffer collaterals in freshly dissected hippocampal slices produced a Ca^{2+}-dependent increase in tissue 2-AG content [124]. Notably, there was no effect on the concentrations of non-cannabinoid monoacylglycerols such as 1(3) palmitoyl glycerol, indicating that 2-AG formation is not due to generalized increase in the rate of lipid turnover [124]. Likewise, the activation of $P2X_7$ purinergic receptors has been associated with 2-AG production in murine astrocytes and microglial cells [136, 137]. 2-AG production upon activation has been reported in several immune cells; 2-AG has been shown to be produced in LPS-stimulated rat platelets [127], in LPS-stimulated rat macrophages and LPS- or ionomycin-stimulated J774 macrophage-like cells [128], in platelet-activating factor (PAF)-stimulated human platelets [129], in PAF-stimulated P388D1 macrophages [129] and in PAF-stimulated RAW264.7 cells [130].

D. Hydrolytic degradation of 2-AG

2-AG is rapidly metabolized by various cell types to yield arachidonic acid and glycerol (Figure 4, on page 20) [132, 138]. The most common mechanism of degradation of 2-AG involves a monoacylglycerol lipase (MAGL) [139, 140]. Northern blot and *in situ* hybridization analyses showed that MAGL mRNA is expressed in various regions of rat brain, the highest levels being in the regions where the CB1 receptor is abundant [141]. Furthermore, RNA interference-mediated silencing of the expression of MAGL greatly enhanced the accumulation

of 2-AG in HeLa cells [142] and treatment with inhibitors of MAGL increased 2-AG levels in the rat hippocampus [143]. In addition to MAGL, fatty acid amide hydrolase (FAAH) is known to hydrolyze 2-AG to arachidonic acid and glycerol to a lesser extent [144, 145]. Repeated intraperitoneal administration of FAAH inhibitors modestly increased the level of 2-AG in the rat brain [146] whereas systemic adminstration of a selective MAGL inhibitor, JZL184, significantly increased 2-AG, but not AEA, levels in whole brain and elicited CB1 receptor-mediated analgesic, hypothermic, and locomotor suppressant effects [147]. Thus far, FAAH-mediated hydrolysis appears to be a less important pathway compared to MAGL-mediated hydrolysis of 2-AG.

Recently, two additional enzymes, serine hydrolase α-β-hydrolase domain 6 (ABHD6) and serine hydrolase α-β-hydrolase domain 12 (ABHD12), were identified as 2-AG hydrolases [148, 149]. These novel 2-AG α/β hydrolases make up for most of the non-MAGL-dependent 2-AG hydrolysis found in mouse brain [150]. Because MAGL, ABHD6 and ABHD12 have different subcellular localizations, they might be able to control independent pools of 2-AG and, thus, signaling events.

E. Enzymatic oxidative metabolism of 2-AG

Apart from hydrolysis, 2-AG is also reported to undergo enzymatic oxidative metabolism (Figure 5, on page 24). It has been demonstrated that 2-AG can be oxygenated by COX-2 to yield PGH_2 glycerol ester (PGH_2-G) and to a lesser extent, hydroxyeicosatetraenoic acid glycerol esters (HETE-G) [151, 152]. In addition, 2-AG was also shown to be metabolized to PGH_2-G and then to PGD_2-G in RAW264.7 cells. This metabolism was demonstrated to be COX-2 dependent whereas COX-1 did not participate in the metabolism of 2-AG. Importantly,

human COX-2 and murine COX-2 metabolized 2-AG as efficiently as arachidonic acid (AA). It is interesting to note that a COX-2 metabolite of AA, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), acts as a ligand for peroxisome proliferator activated receptor γ (PPARγ) (Figure 5, on page 24) [153]. Among the various arachidonyl esters, 2-AG is the most preferred substrate for metabolism by COX-2 [121]. It has been demonstrated that PGE_2-G and PGI_2-G are generated in zymosan-stimulated mouse peripheral macrophages [154]. These results suggest that 2-AG may act as a natural COX-2 substrate in mammalian tissues. Oxygenated derivatives of 2-AG are demonstrated to be metabolically stable compared with PGs derived from free arachidonic acid and may have long half-lives [155]. Interestingly, 2-AG-mediated IL-2 suppression in activated Jurkat cells is attenuated in the presence of a COX-2 specific inhibitor suggesting that a COX-2 metabolite of 2-AG is responsible for the observed IL-2 suppression [156].

In addition to metabolism by COX-2, 2-AG can also be metabolized by lipooxygenases (LOXs) *in vitro*. 2-AG when incubated with soybean 15-LOX produced 15-HETE-G, which has been demonstrated to be a ligand for PPARα [157]. 2-AG was metabolized by leukocyte-type 12-LOX to yield 12(S)-hydroperoxyeicosatetraenoic acid glycerol esters (12-HpETE-G) whereas platelet-type 12-LOX did not metabolize 2-AG effectively [158]. Recently, two endogenous cytochrome P450 metabolites of AA were identified - 2-(11,12-epoxyeicosatrienoyl) glycerol (2-11,12-EG) and 2-(14,15-epoxyeicosatrienoyl) glycerol (2-14,15-EG), which are both found in the rat kidney and rat spleen, whereas 2-11,12-EG is also detected in the rat brain [159]. These metabolites are termed 2-epoxyeicosatrienoylglycerols (2-EG) and interestingly, both 2-11,12-EG and 2-14,15-EG activated the two cannabinoid receptor subtypes, CB1 and CB2, with high affinity and elicited biological responses in cultured cells expressing CB receptors and elicited

hypomotility and hypothermia in intact mice in intact animals [159]. Understanding if 2-EG or its analogues can be produced from 2-AG by the action of cytochrome P450 requires further investigation.

Figure 3. Schematic representation of the major pathway for 2-AG biosynthesis.

Rapid hydrolysis of phosphatidylinositol (PI) by PLC produces diacylglycerol (DAG), which is then cleaved by DAG lipase (DGL) to produce 2-AG (taken from Piomelli. The molecular logic of endocannabinoid signalling. Nat Rev Neurosci (2003) vol. 4 (11) pp. 873-84). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 3. (cont'd)

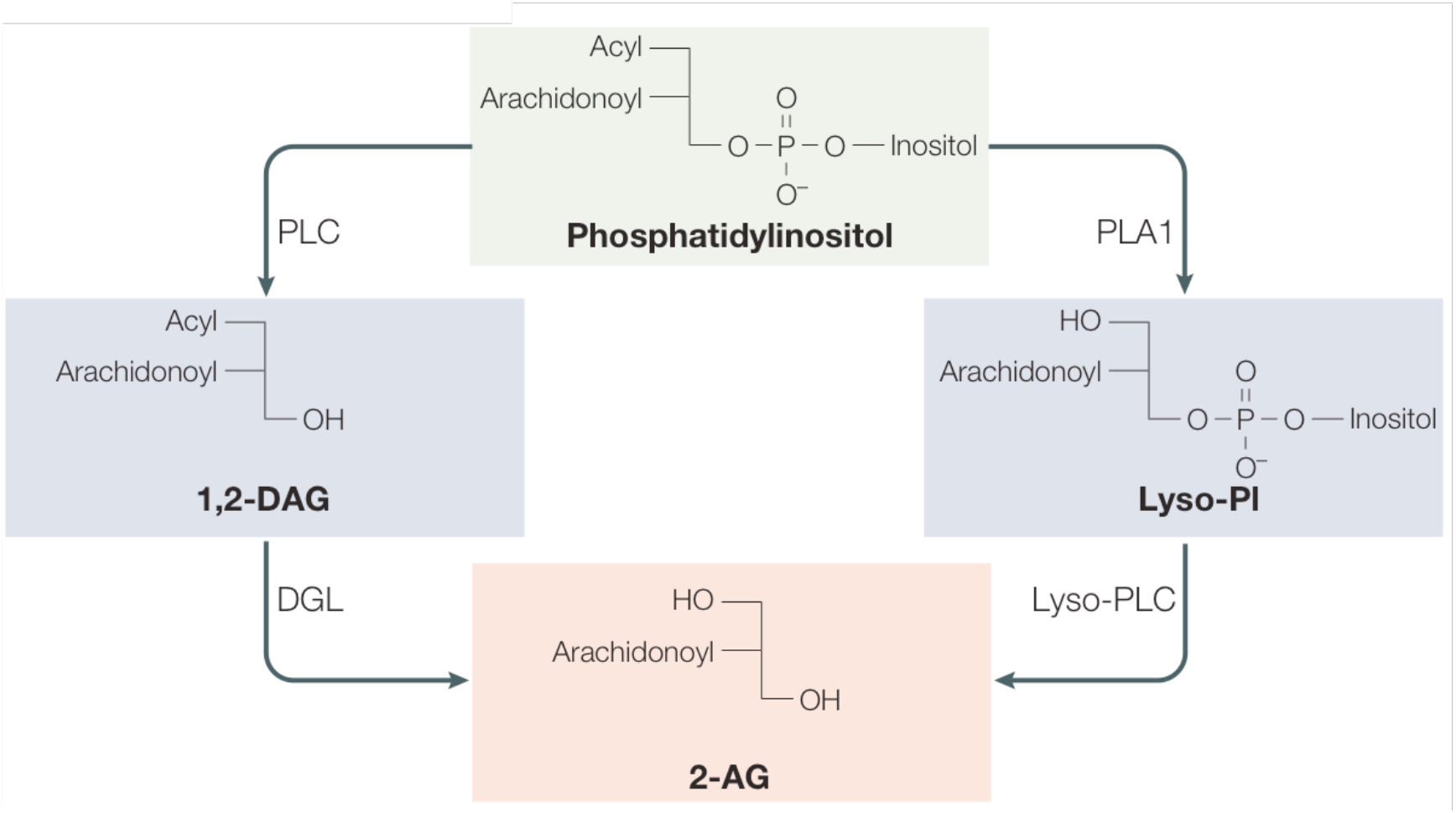
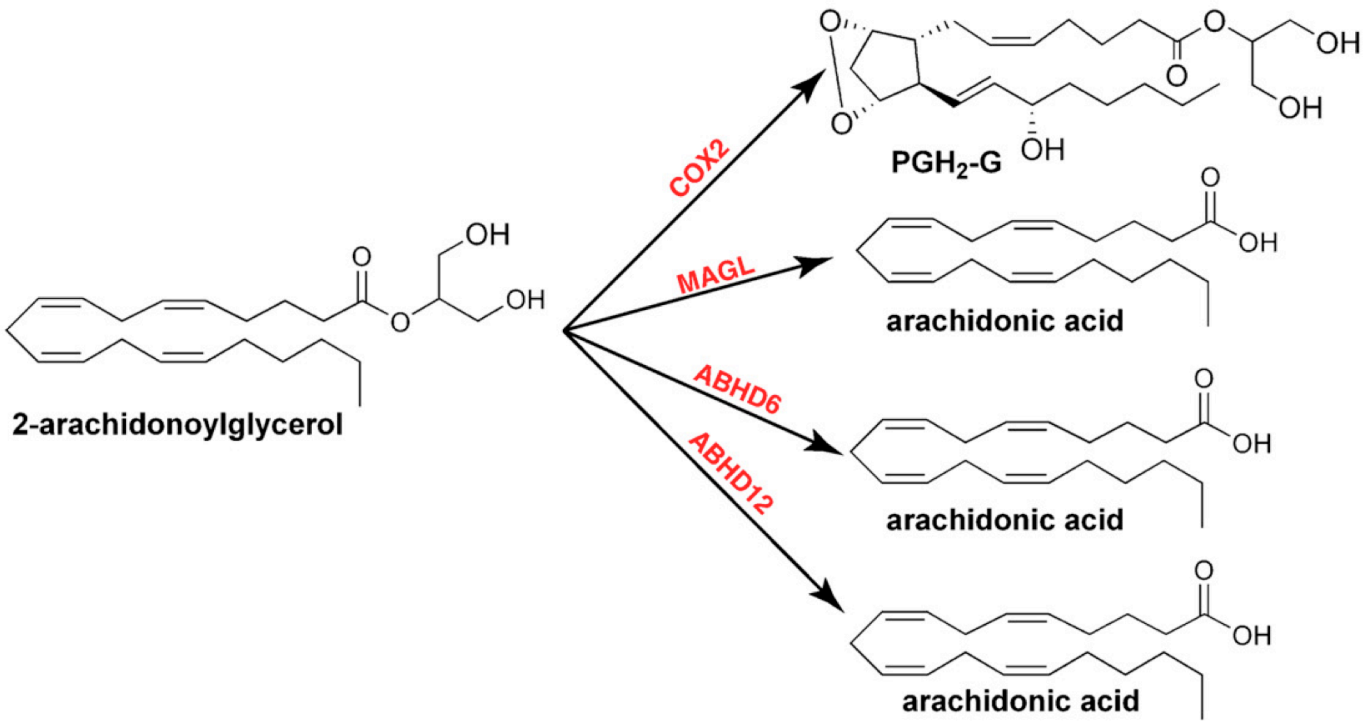


Figure 4. Schematic representation of the pathways of 2-AG degradation.

Various enzymes such as MAGL, ABHD6 and ABHD12 are involved in the degradation of 2-Arachidonyl glycerol. COX-2 is responsible for the oxidative metabolism of 2-AG (taken from Muccioli. Endocannabinoid biosynthesis and inactivation, from simple to complex. Drug Discov Today (2010) vol. 15 (11-12) pp. 474-83).

Figure 4. (cont'd)



F. Transport of AEA and 2-AG

Several investigators have postulated the presence of an AEA membrane transporter (AMT) on the basis of temperature dependency, saturability, substrate specificity and selective inhibition [160-162]. The carrier-mediated transport of AEA does not require intracellular ATP or an ion gradient and hence, is presumed to be occurring through facilitated diffusion [163]. However, the AMT has not yet been cloned and controversy exists over its existence. In human astrocytoma CGF-STTG1 cells, it was demonstrated that [^{3}H]-2-AG accumulated through a Na^{+}- and energy-independent process and that non-radiolabelled 2-AG, AEA or the AMT inhibitor AM404 inhibited [^{3}H]-2-AG uptake [164]. These results suggested that 2-AG and AEA are internalized through a common carrier-mediated mechanism. In addition, the uptake of [^{3}H]-2-AG and [^{14}C]AEA by rat C6 glioma cells was inhibited by AMT inhibitors, AM404 and linvanil with the same potency [165]. Yet, whether one single transporter mediates the transport of both 2-AG and AEA or two distinct molecules mediate the transport of each endocannabinoid or whether either endocannabinoid is transported via a transporter is not known.

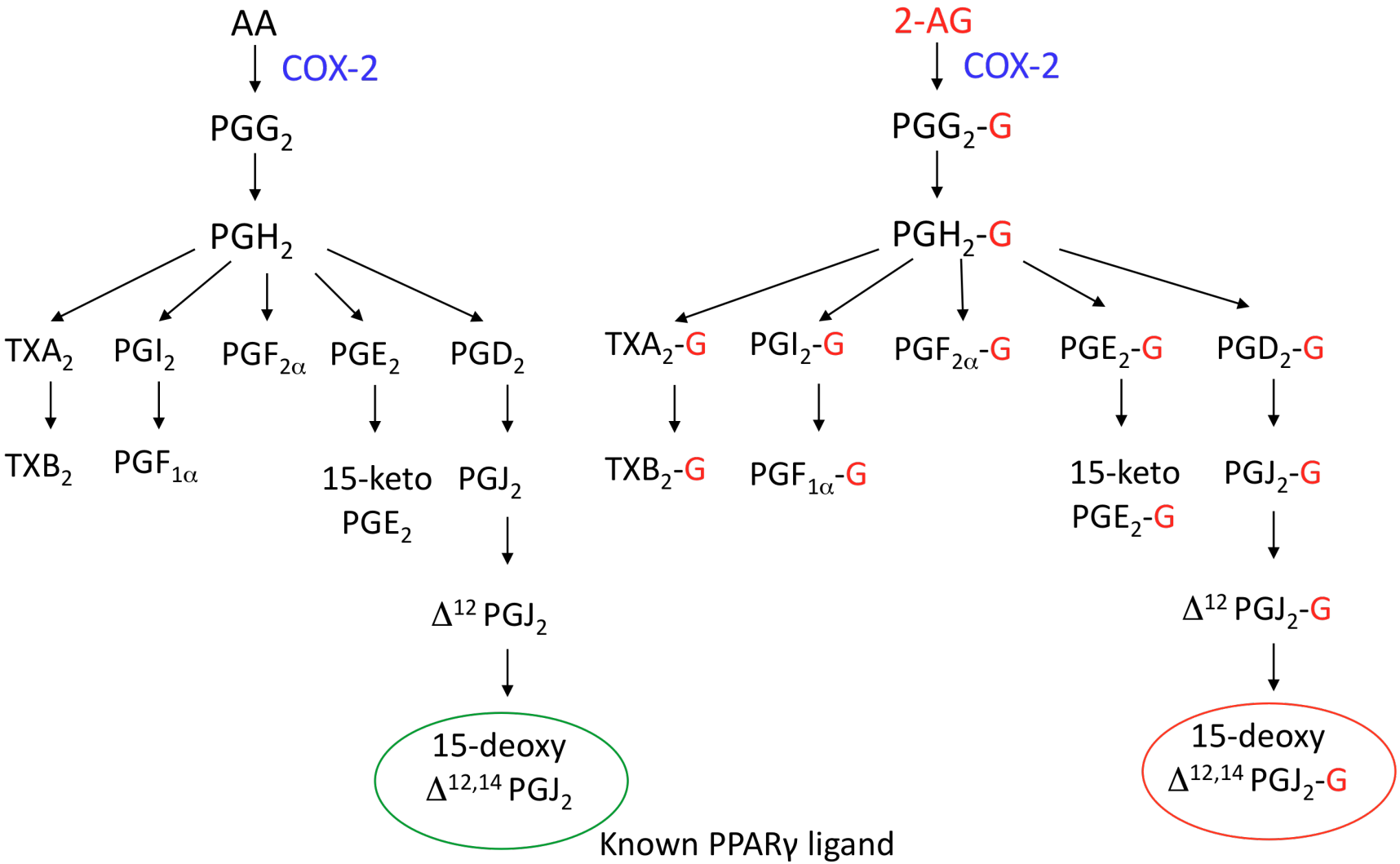
G. Receptor targets and cellular signaling

2-AG has been demonstrated to bind and activate both CB1 and CB2 receptors [5]. Initially, when 2-AG was identified as a ligand for cannabinoid receptors, it was reported to have lower affinity for both CB1 (K_i = 472 nM) and CB2 (K_i = 1400 nM) compared to AEA (K_i = 52 nM as assessed in brain synaptosomal membranes) [4, 5].

Figure 5. Comparison of the oxidative metabolism of AA and 2-AG.

COX-2 is the rate-limiting enzyme that results in the conversion of AA and 2-AG into various prostaglandins, thromboxanes and their respective glycerol esters. 15d-PGJ_2 is a known endogenous ligand of PPARγ.

Figure 5. (cont'd)



However, later studies have demonstrated that the affinity of 2-AG for CB1 (K_i = 58 nM) and CB2 (145 nM) is higher than the earlier studies [166]. Unlike AEA, 2-AG acts as a full agonist for CB1 and CB2 receptors in a variety of assay systems [41, 44]. 2-AG has been demonstrated to activate both G_i and G_o as well as inhibit cAMP accumulation through CB1 activation [31, 167]. Similarly, 2-AG also produced maximal inhibition of cAMP accumulation through CB2 activation [167].

2-AG mediates a transient elevation in intracellular calcium in neuroblastoma NG108-15 cells, which is inhibited with a CB1 antagonist [168, 169]. Likewise, 2-AG induces rapid transient increases in intracellular free Ca^{2+} in HL-60 cells that naturally express CB2 receptor and this increase in intracellular Ca^{2+} is blocked with CB2 antagonist and pertussis toxin [44]. In rat sympathetic neurons, 2-AG has been demonstrated to inhibit L-type calcium channels and inwardly rectifying potassium channels (Girk 1 and 4) through CB1 activation. In resting murine splenocytes 2-AG did not induce an elevation in intracellular calcium whereas 2-AG inhibited thapsigargin-induced elevation of intracellular calcium by 30 – 40 % [170]. These studies suggest that 2-AG may be capable of altering various calcium-dependent signaling pathways thus modulating neurotransmitter release and T cell activation.

MAP kinases are involved in all aspects of immune responses, from the initiation phase of innate immunity, to activation of adaptive immunity, and to cell death when immune function is complete. 2-AG also modulates MAP kinase pathways in a number of different models. 2-AG was shown to activate ERK1 and ERK2 in HL-60 cells through CB2 activation [171]. In murine hippocampal slices, 2-AG has been demonstrated to activate p38 MAP kinase, which was absent in CB1 null mice [172]. Similarly, 2-AG also activates ERK1 in microglial cells in a

CB2-dependent manner [134]. These observations implicate a pivotal role for 2-AG in the proper functioning of T cells as T cells utilize MAPKs for proper regulation of their development, activation and function. 2-AG has been demonstrated to inhibit hormone-induced breast cancer cell proliferation by down-regulating prolactin receptors [173]. In addition, 2-AG has been shown to reduce nerve growth factor (NGF)-induced breast cancer cell proliferation by down-regulating the levels of *trk* NGF receptor [174]. These studies suggest an important role for 2-AG in cancer therapy.

Although CB1 and CB2 have been implicated in many of the effects of 2-AG, there is mounting evidence for CB1- and CB2- independent effects of 2-AG, AEA and other plant derived cannabinoids [45] [175]. It has been demonstrated that 2-AG suppresses IFNγ production in phorbol ester/ionomycin-activated mouse splenocytes independent of CB1 and CB2 [170]. Interestingly and of particular significance to the studies in this dissertation, it has been demonstrated that IL-2 suppression by 2-AG in activated Jurkat T cells is mediated through PPARγ and is independent of both CB1 and CB2 [176].

H. Physiological significance of 2-AG

The proposed physiological role of 2-AG in the synapse is attenuation of neurotransmission. In neuronal cells, 2-AG suppresses the depolarization-induced rapid elevation of intracellular-free Ca^{2+} concentration $[Ca^{2+}]_i$ [168]. 2-AG, generated through increased inositol phospholipid metabolism induced upon synaptic transmission, is rapidly released in to the synaptic cleft which then binds to the cannabinoid receptor CB1 and stimulated $G_{i/o}$ to inhibit the voltage-gated Ca^{2+} channels leading to decreased intracellular Ca^{2+} concentration, thus dampening the subsequent neurotransmitter release [177]. In addition, 2-AG

has been reported to act as a retrograde messenger in the synaptic transmission of the brain thereby playing important roles in the induction of depolarization-induced suppression of inhibition in the hippocampus and depolarization-induced suppression of excitation in the cerebellum [178-180]. Concordant with its proposed neuroprotective role, the level of endogenous 2-AG was significantly elevated in the mouse brain after closed head injury and 2-AG administration caused a significant reduction in brain edema, infarct volume and hippocampal cell death leading to improved recovery after closed head injury [181]. In addition to the roles in the central nervous system, 2-AG has been implicated to play essential roles in the cardiovascular system. Intravenous administration of 2-AG into rats induces hypotension suggesting that 2-AG may be involved in the regulation of vascular tone [182]. Human umbilical vein endothelial cells stimulated with thrombin or A23187 generated 2-AG [125] and human monocytes treated with 2-AG induced the generation of nitric oxide [183] suggesting vasomodulatory functions for 2-AG.

Intravenous injection of 2-AG into mice induced analgesia suggesting that 2-AG is involved in analgesia [5]. 2-AG inhibited the proliferation of the human breast cancer cell lines MCF-7 and EFM-19 [173]. In addition, 2-AG suppressed the proliferation of prostate cancer DU-145 cells and nerve growth factor-induced proliferation of human breast cancer cells [174]. 2-AG has also been reported to varying effects on the immune system, which are outlined in the following section.

I. Effects of 2-AG on the immune system

2-AG has been shown to inhibit cytokine production such as TNFα release from both LPS-stimulated mouse macrophages and LPS-stimulated rat microglial cells [184, 185], IL-6

production by J774 macrophage-like cells [186] and IL-2 production by murine T lymphocytes and Jurkat T cells [176, 187]. In contrast, 2-AG increased production of IL-8 and MCP-1 by HL-60 cells [188]. 2-AG has been reported to induce calcium influx in HL-60 cells [44], increase NO production in human monocytes [189] and enhance antibody formation in murine splenocytes [111]. 2-AG has also been reported to induce the migration of human peripheral blood monocytes and HL-60 cells [105]. It has been reported that 2-AG induces rapid phosphorylation and activation of the extracellular signal-regulated kinase (ERK) in HL-60 cells in a CB2 receptor- and $G_{i/o}$-dependent manner [171] and ERK has been demonstrated to play a crucial role in 2-AG-induced cell migration [105]. It has been reported that through ERK activation, 2-AG stimulates AP-1 dependent transcriptional activity and thus enhances the epidermal growth factor-induced cell transformation in mouse epidermal JB6 P+ Cl41 cells [190]. In addition, 2-AG has been shown to induce the activation of p38 MAPK and JNK [191].

2-AG-mediated suppression of IL-2 secretion in murine splenocytes and Jurkat cells has been demonstrated to be independent of CB1 and CB2 receptors [176]. Importantly, IL-2 suppression by 2-AG treatment has been shown to be COX-2 dependent and mediated through PPARγ activation [156, 176].

III. Regulation of IL-2

A. Role of IL-2 and clinical importance

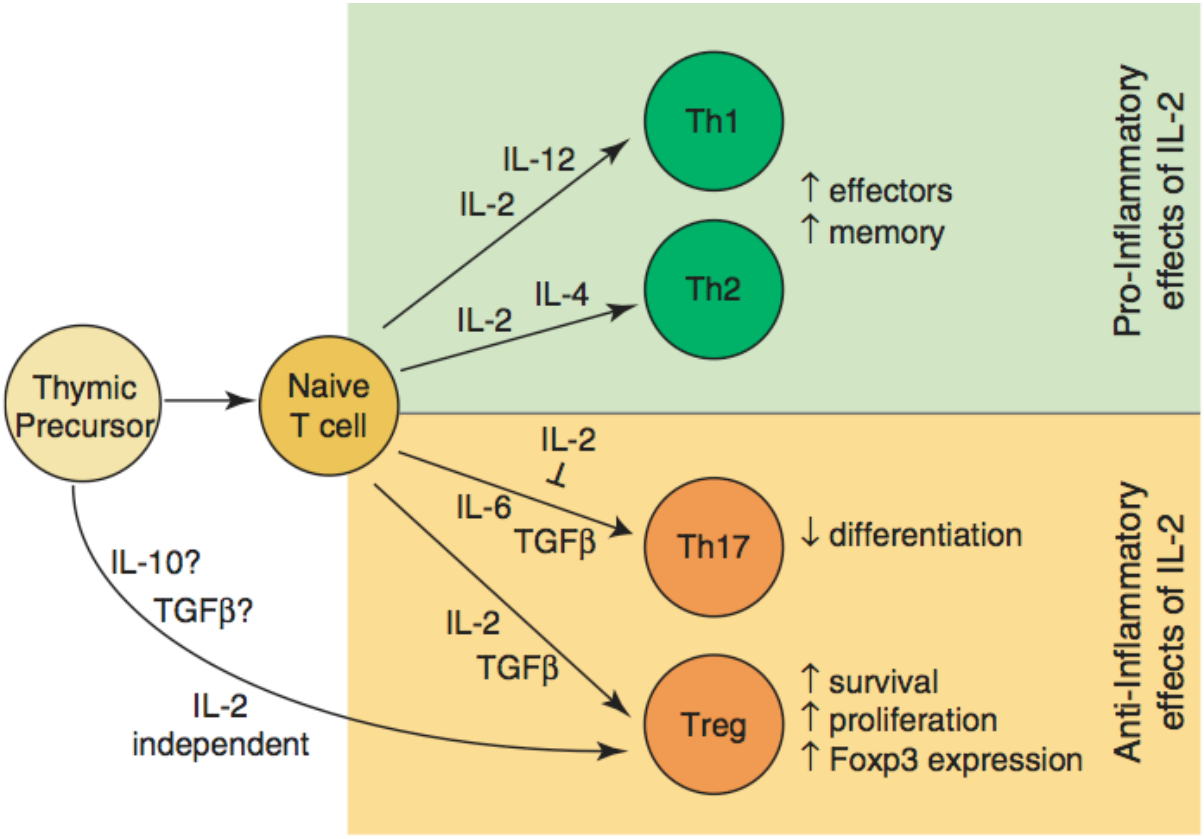
IL-2, a 15kDa α-helical cytokine, is produced almost exclusively by activated T cells. It is a hallmark of T cell activation as there is virtually no basal expression, but is rapidly secreted upon T cell activation [192]. The IL-2 receptor (IL-2R) consists of three membrane-bound subunits: α (CD25), β (CD122) and common γ (γc) (CD132) chains. In T cells, IL-2 binding to

the IL-2R activates the Janus kinase (JAK)/signal transducer and activator of transcription 5 (STAT5) pathway, as well as MAPK and phosphoinositide 3-kinase signaling, leading to the transcription of proinflammatory cytokine, survival and cell cycle genes [192-194]. IL-2 plays an important role as an autocrine and paracrine growth factor during the first 48 to 72h of T cell activation and is also important for T cell survival, proliferation and in some cases, differentiation [195, 196]. IL-2 is important for the differentiation of $CD4^+$ T cells into Th1 and Th2 effector subsets [192] and is required for the development of memory T cells [197, 198]. T regulatory cells (Tregs) comprise 5-15% of the peripheral $CD4^+$ T cell population and are characterized by the expression of CD25 and the transcription factor forkhead box protein 3 (Foxp3). Natural Tregs are generated in the thymus and are considered to be specific for self-antigens. In addition, peripheral Tregs can be generated in response to foreign or self-antigens. Tregs function to suppress effector T cell proliferation and cytokine production [199]. IL-2 is important for the maintenance of immune homeostasis as evident from inflammatory autoimmune disease in IL-2 and IL-2 receptor knockout mice [200-202]. Although there is conflicting evidence for a requirement for IL-2 in thymic generation of $CD25^+$ Tregs [201, 203-207], it has been recently shown that IL-2 is critical for the generation and survival of peripheral Tregs [208, 209]. The inflammatory autoimmune disease in IL-2 and IL-2R knockout mice may be a result of failure to control inflammatory responses due to the decreased Tregs [206]. Th17 effector subset, producing IL-17, is considered to be the major mediator of tissue inflammation in several autoimmune inflammatory diseases [210]. IL-2 causes an inhibition of Th17 differentiation via STAT5 signaling [211]. Thus, IL-2 blocks inflammation by preventing the generation and hence the expansion of the inflammatory Th17 cells, at the same time increasing the number of suppressive Tregs (Figure 6, on page 32). IL-2 (along with IL-15) is also a growth

factor for natural killer cells [212-214] and promotes production of natural killer cells derived cytokines such as TNFα, IFNγ and granulocyte macrophage colony stimulating factor [215]. In addition, IL-2 has been known to act synergistically with IL-12 to enhance natural killer cytotoxic activity [216]. In IgM-expressing B cells, IL-2 along with IL-5 upregulates expression of heavy and light chain genes as well as induces de novo synthesis of immunoglobulin J chain gene which is required for oligomerization of the IgM pentamer (a tightly controlled stage in B cell activation) [217, 218]. IL-2 increases expression of the high affinity IL-2Rα in both B and T cells, thus enhancing responsiveness to IL-2 in these cells [219, 220]. IL-2 is highly regulated both transcriptionally through various transcription factors (to be discussed) and post-transcriptionally through the stabilization of IL-2 mRNA [192]. There is limited data on serum IL-2 levels in humans, both in healthy and disease states. However, a correlation exists between elevated IL-2 levels and progression of gastric and non-small cell lung cancer [221, 222]. High serum levels of IL-2 are linked with progression of autoimmune conditions such as scleroderma and rheumatoid arthritis [223, 224]. Whether this elevated IL-2 aids to increase peripheral Tregs in these disease states is yet to be understood. In HIV infection, it has been reported that there is a selective deficiency in the production of IL-2 by surviving $CD4^+$ and $CD8^+$ T cells in addition to the decrease in total number of $CD4^+$ T cells [225]. The lack of IL-2 results in the decreased ability of the immune system to activate antigen-specific $CD8^+$ cytotoxic T lymphocytes. IL-2 is being tested in large-scale clinical trials for the treatment of HIV infection [226]. Furthermore, IL-2 is currently approved by the Federal Drug Administration for the treatment of renal cell carcinoma and melanoma [227] [228].

Figure 6. Roles of IL-2 in inflammatory immune responses.

IL- 2 promotes inflammatory responses through the generation of Th1 and Th2 effector cells. IL-2 also blocks the differentiation of T cells into Th17 effectors and promotes the development and the maintenance of peripheral Tregs (taken from Hoyer et al. Interleukin-2 in the development and control of inflammatory disease. Immunol Rev (2008) vol. 226 pp. 19-28).



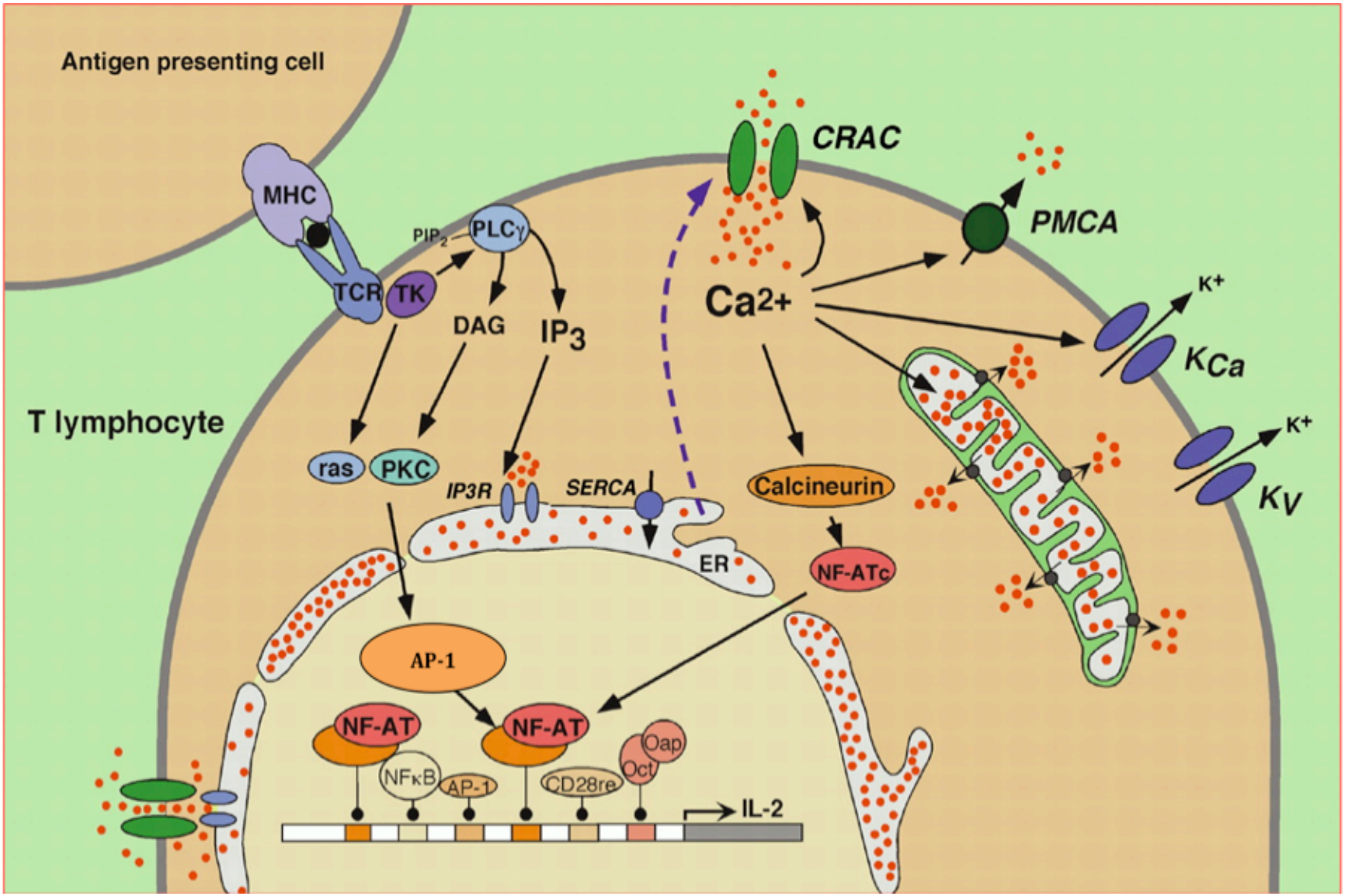
B. T cell activation and IL-2 secretion

When an antigen-presenting cell presents antigen to the T cell receptor, various tyrosine kinases are activated following T cell receptor engagement. One of those tyrosine kinases in turn activates phospholipase C-γ (PLCγ). PLCγ cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane and generates diacylglycerol (DAG) and 1.4.5-inositol trisphosphate (IP_3). DAG activates protein kinase C (PKC) and ras – dependent pathways. The Ras-Raf-Erk pathway leads to the production of c-fos, which along with c-jun that is produced from JNK pathway form activator protein 1 (AP-1). AP-1 cooperates with multiple transcription factors in composite DNA binding sites, including nuclear factor of activated T cells (NFAT). IP_3 releases calcium from the endoplasmic reticulum and the resulting depletion of the calcium stores activates Calcium Release Activated Ca^{2+} channels (CRAC). Activation of the CRAC causes the influx of the extracellular calcium in to the cell to increase intracellular calcium, which activates calcineurin, a calcium dependent phosphatase. Activated calcineurin dephosphorylates NFAT and allows its translocation in to the nucleus. NFAT binds to its response element within the IL-2 promoter to initiate transcription of the IL-2 gene (Figure 7, on page 34) [229]. In addition to NFAT and AP-1, other transcription factors are also involved in the regulation of IL-2 and are discussed below.

Figure 7. Schematic representation of T cell activation

This figure represents the signaling cascade involved in IL-2 gene transcription following T cell activation. TCR: T cell receptor, TK: tyrosine kinase, PLCγ: phospholipase C γ, DAG: diacylglycerol, IP3: Inositol trisphophate, CRAC: Calcium release activated Ca^{2+} channels, PKC: Protein kinase C, AP-1: activator protein 1, NFAT: nuclear factor of activated T cells, NFκB: nuclear factor κB, IL-2: interleukin-2(adapted from Acuto and Cantrell. T cell activation and the cytoskeleton. Annu Rev Immunol (2000) vol. 18 pp. 165-84).

Figure 7. (cont'd)



C. IL-2 minimal essential promoter region

The IL-2 minimal essential promoter possesses various response elements, such as NFAT, AP-1, NFκB, Oct and CD28RE sites (Figure 8, on page 40). IL-2 promoter contains two well-characterized sites for NFAT, referred to as the distal and proximal NFAT binding sites, and it has been demonstrated that both sites are occupied in stimulated T cells [230]. In addition to these well-characterized sites, at least three other putative NFAT binding sites have been identified within the IL-2 promoter [231, 232]. The binding of NFAT to the IL-2 promoter is essential for IL-2 gene transcription [233]. The specific NFAT proteins involved in IL-2 gene regulation are NFAT 2 (NFATc1, NFATc) and NFAT1 (NFATp, NFATc2) [234].

The NF-κB family consists of p50, p52 , p65 (also known as RelA) , Rel and RelB, which all share an amino-terminal REL homology domain. Dimers of NFκB proteins are regulated by eight IκB family members through sequestration. In resting T cells, NFκB family members exist in association with inhibitory IκB in the cytoplasm. T cell activation results in the phosphorylation of IκB by IκB kinase, which causes it to be ubiquitinated and targeted for degradation. Consequently, NFκB is released and its nuclear localization signal is exposed, resulting in rapid nuclear translocation [235]. There are two NFκB sites within the IL-2 promoter and one of them is a composite element containing an AP-1 site (termed as CD28RE/AP site) [236].

AP-1 protein is a heterodimer consisting of jun and fos family members [237, 238]. AP-1 cooperates with multiple transcription factors in composite DNA binding sites including NFAT, NFκB and Oct [239]. AP-1 binds to the IL-2 promoter at two different sites, distal and proximal binding sites [240]. It has been demonstrated that AP-1 binding at the distal site enhances IL-2 transcription whereas AP-1 binding at the proximal site is essential for IL-2

transcription [240, 241]. The IL-2 promoter contains two binding sites for the Oct family proteins, Oct-1, which is constitutively expressed in T cells, and Oct-2, which is upregulated after T cell activation [233, 242-244]. The Oct-1 site is a composite NFAT:AP-1:Oct site, as cooperative binding is observed with both NFAT and AP-1 [231]

CBN caused a robust and sustained inhibition of NFAT binding to distal NFAT site of the IL-2 promoter up to 240 min whereas CBN produced a transient inhibition of AP-1 binding to proximal AP-1 site of the IL-2 promoter that was partially recovered by 240 min in PMA/I_o-stimulated the murine thymoma cells, EL4.IL2 cells [245]. CBN treatment in EL4.IL2, decreased PMA/I_o-mediated CREB/c-fos/c-jun binding to the AP-1 proximal site of the mouse IL-2 promoter [99]. In addition, CBN treatment resulted in robust inhibition of NFAT promoter activity and modest inhibition of Oct promoter activity whereas had no effect of NFκB promoter activity [245]. In murine primary splenocytes, CBN inhibited NFAT binding to distal NFAT site of the IL-2 promoter and AP-1 binding to proximal AP-1 site of the IL-2 promoter [58]. The decrease in AP-1-DNA binding was partly due to the decreased nuclear expression of c-fos and c-jun [58]. CBN decreased DNA-binding activity of NFκB/c-Rel protein in forskolin-stimulated or PMA/I_o-stimulated primary murine splenocytes and thymocytes to κB consensus motif [98, 246]. These studies suggest that CBN treatment in optimally activated cells leads to decreased binding of various important transcription factors required for IL-2 gene transcription. By contrast, CBN increased NFAT binding to distal NFAT site from the IL-2 promoter and enhanced NFAT transcriptional activity along with IL-2 secretion when EL4.IL2 cells were suboptimally stimulated with PMA (2-10 nM) or when splenic T cells were suboptimally stimulated with soluble anti-CD3 monoclonal antibody [100]. Other cannabinoids such as

CP55940, Δ^9-THC and CBD also exhibited concentration-dependent enhancement of IL-2 secretion in suboptimally activated EL4.IL2 cells [100]. Enhanced NFAT transcriptional activity along with enhanced IL-2 secretion in suboptimally activated EL4.IL2 cells was abrogated by calcium/calmodulin-dependent protein kinase inhibitor, KN93, but not by a CB2 receptor antagonist, SR144528 [100] suggesting that there may be other molecular targets involved in enhanced NFAT transcriptional activity in suboptimally activated cells.

D. Glycogen synthase kinase - 3β (GSK-3β)

Numerous serine/threonine protein kinases have been identified as regulators of NFAT activity and are classified into maintenance and export kinases, which function to retain NFAT in the cytoplasm or by promoting its export from the nucleus, respectively. Glycogen synthase kinase 3β (GSK-3β), acts as an export kinase by phosphorylating the SP2 and SP3 motifs of NFAT1 and NFAT2, an event that requires prior phosphorylation of NFAT by the priming kinase protein kinase A (PKA) [247, 248] thus leading to the nuclear export of NFAT. In addition to being an export kinase, GSK-3β also acts as a maintenance kinase by keeping the cytosolic NFAT phosphorylated thus retaining NFAT in the cytosol. GSK-3β is a constitutively active kinase that is phosphorylated and inactivated by PI3-K and Akt signaling, one of the most frequently deregulated pathways in human tumors [249].

E. Human homolog of the oncoprotein and E3 ubiquitin ligase MDM2 (HDM2)

While intracellular calcium levels control NFAT translocation in to the nucleus, HDM2, the human homolog of the oncoprotein and E3 ubiquitin ligase MDM2 (murine double minute 2), has been reported to cause ubiquitination of NFAT. Phosphorylation of HDM2 by Akt has

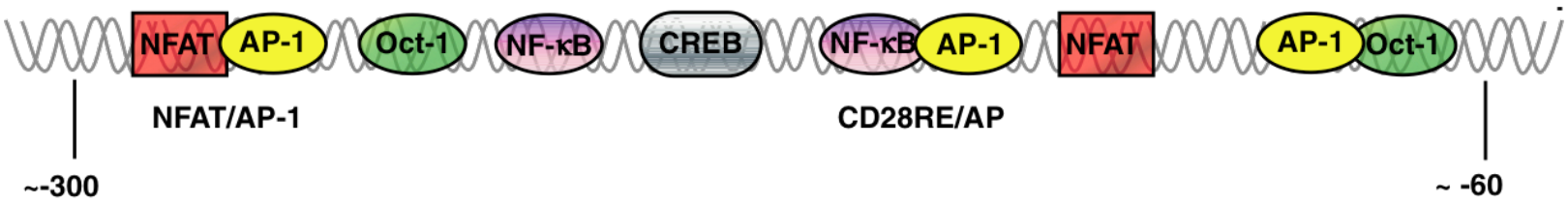
been implicated in the degradation of a number of proteins including p53 and NFAT1 [250]. MDM2 plays a critical role in promoting p53 (tumor suppressor protein) ubiquitination and subsequent destruction [251, 252]. NFAT1 is also ubiquitinated by the E3 ubiquitin ligase MDM2 downstream of Akt and GSK-3 signaling in breast cancer cells [250, 253].

F. Effect of 2-AG on transcription factors involved in IL-2 production

2-AG has been reported to suppress IL-2 production in both activated murine splenocytes and activated Jurkat T cells [176, 187]. 2-AG decreased both the NFAT binding to NFAT site from IL-2 promoter and NFAT promoter activity in a concentration-dependent manner in murine primary splenocytes [187]. In addition, 2-AG decreased NFκB/Rel binding to NFκB consensus sequence and promoter activity along with a modest inhibition of AP-1 promoter activity without an effect on AP-1, Oct and cAMP response element binding activity to IL-2 promoter [187]. In PMA/I_o-stimulated Jurkat cells, 2-AG decreased transcriptional activity of NFAT and NFκB in the absence but not in the presence of a PPARγ antagonist, T0070907 and also produced a modest inhibition of AP-1 promoter activity [176]. Interestingly, it has been demonstrated that 2-AG-mediated IL-2 suppression is COX-2 dependent and is mediated through PPARγ, independently of CB1 and CB2 receptors [156, 176]. COX-2 is the rate-limiting enzyme in the metabolism of AA to produce various prostaglandins and thromboxanes. It is important to note that 15d-PGJ_2, a COX-2 metabolite of AA is the known ligand for PPARγ. 15d-PGJ_2-G, a glycerol ester of 15d-PGJ_2 and a putative metabolite of 2-AG, due to its structural similarity may act as a ligand for PPARγ.

Figure 8. Schematic representation of the proximal promoter of the human IL-2 gene

This figure represents the various transcription factors that bind to proximal promoter of human IL-2 gene. NFAT: nuclear factor of activated T cells, AP-1: activator protein 1, Oct-1: octamer-1, NFκB: nuclear factor κB, CREB: cAMP response element binding protein (taken from Gaffen and Liu. Overview of interleukin-2 function, production and clinical applications. Cytokine (2004) vol. 28 (3) pp. 109-23).



IV. Peroxisome Proliferator Activated Receptors (PPARs)

A. PPAR subtypes

The PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily [254, 255]. Three different members of the PPAR family have been identified, each encoded by a different gene: PPARα, PPARβ/δ and PPARγ [256]. These receptors exhibit distinct patterns of tissue distribution and functions and, to a certain extent, different ligand specificities [254, 257]. PPARs usually exist as a heterodimer with retinoid X receptor (RXR). In the resting state, the PPAR:RXR complex is present on PPAR response elements (PPRE) that are present in the regulatory regions of various target genes and PPAR:RXR complex may or may not exist in association with corepressors depending on the promoter. Agonist binding within the ligand-binding domain (LBD) of PPARs results in conformational change promoting the dissociation of corepressors (if associated) and the recruitment of coactivators such as SRC1 and CBP/p300. Coactivator recruitment facilitates the integration of histone acetyl transferases (HAT) and thus helps in transcriptional activation of target genes [258]. In contrast, antagonist binding to the LBD results in a conformational change that is favorable for the binding of corepressors [259, 260]. Physiologically, PPAR-RXR may bind to PPREs in the absence of a ligand. The occupancy of PPRE by unliganded PPAR-RXR has varying effects depending on the promoter context and cell type even though ligand-bound PPAR-RXR is required for transcriptional activation [258]. In adipocytes, for some PPARγ target genes, unliganded PPARγ-RXR recruit corepressors complexes, which results in active repression, whereas for other genes, PPAR-RXR does not recruit corepressors [261]. There are many potential endogenous ligands for the PPARs – fatty acids, specifically unsaturated fatty acids, several eicosanoids and their metabolites [260].

B. PPARγ

PPARγ plays an important role in the differentiation of adipocytes where it is abundantly expressed. PPARγ also promotes the storage of lipids in adipose tissues and is involved in lipid metabolism [262, 263]. PPARγ is the receptor target of thiazolidinediones, a class of drugs widely used in the treatment of type 2 diabetes [264]. Differential promoter usage and alternative splicing produce four PPARγ variants of which PPARγ1 and PPARγ2 are the two major forms of the protein [265]. PPARγ1 is expressed in a variety of tissues such as liver, skeletal muscle, adipose tissue and bone. PPARγ2 contains 30 additional amino acids in its N-terminus compared to PPARγ1 and is mainly expressed in adipogenic cells [266]. In addition, PPARγ has been found in other cell types such as fibroblasts, myocytes, breast cells, the white and red pulp of rat spleen, human bone-marrow precursors, macrophages/monocytes and T cells [266-268]. PPARγ1 expressed in immune cells, such as B cells, macrophages and T cells seems to regulate a number of immune functions [269].

A known endogenous ligand for PPARγ is an arachidonic acid derivative, 15-deoxy prostaglandin J_2 (15d-PGJ_2) [153]. In addition to transactivation by binding to PPRE, ligand-activated PPARγ/RXR also participates in transrepression by physical association with, and sequestration of transcription factors including NFAT, NFκB and AP-1, leading to subsequent inhibition of their functions in gene transcription [270, 271].

C. Effect of PPARγ on the immune system

There is increasing evidence implicating a role for PPARγ in regulating inflammatory responses [270, 272, 273]. PPARγ-specific ligands have been demonstrated to suppress the production of various inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-10 and IL-12

[270, 272, 274] and other cytokines such as IL-2, IL-4 and IFNγ [176, 275, 276]. However, PPARγ activation has also been linked with upregulation of CD14, CD11b/CD18 and CD36 [277, 278]. It is not entirely clear if all these effects can be ascribed to PPARγ as troglitazone, ciglitazone and 15d-PGJ_2 suppress TNFα and IL-6 production in both wild-type and PPARγ-null macrophages [279].

The presence of rosiglitazone during LPS- or CD40 ligand-induced dendritic cell maturation has been reported to cause the cell-surface expression of class-B scavenger receptor CD36 and the co-stimulatory molecule CD80 [280]. 15d-PGJ_2 and other synthetic PPARγ ligands had an antiproliferative and cytotoxic effect on both normal and malignant B cells [281, 282]. PPARγ is present at lower levels in resting T cells but its expression is upregulated during T cell activation [276, 283]. It has been reported that activation of PPARγ could inhibit the expression of IL-2 after T cell activation as a result of physical interactions by the ligand-bound PPARγ with NFAT thus blocking IL-2 gene transcription [271, 283].

Although the synthetic PPARγ agonist rosiglitazone (Avandia) is currently approved for restricted use in the management of diabetes, there is mounting evidence that PPARγ activation is involved in immune regulation and in moderating inflammatory responses. PPARγ in T cells has been demonstrated to prevent gut inflammation in mice with experimental inflammatory bowel disease [284]. Similarly, PPARγ selectively suppressed human and mouse Th17 differentiation (T helper cells secreting IL-17, which play a crucial role in autoimmune diseases such as multiple sclerosis) [285]. Likewise, PPARγ has been shown to negatively regulate allergic rhinitis in mice [286]. PPARγ agonist treatment appears to suppress T cell function in EAE, which is an animal model of multiple sclerosis [287]. Interestingly, 2-AG has also been

shown to improve the symptoms of EAE [288]. Furthermore, PPARγ also plays an essential role in the control of inflammatory responses by acting on various immune cells such as T cells [283], dendritic cells [289], macrophages [270] and mast cells [290]. Collectively, the aforementioned findings suggest that PPARγ may play an important function in maintaining immune homeostasis and in preventing autoimmune diseases.

V. Rationale

2-AG has been reported to modulate various immune responses such as suppression of cytokine secretion, inhibition of immunoglobulin production and suppression of $CD8^+$ T cell migration. Specifically, 2-AG-mediated suppression of IL-2 secretion in activated T cells has been demonstrated to be dependent on COX-2 metabolism of 2-AG. Interestingly, 15d-PGJ_2, a COX-2 metabolite of AA, is a known endogenous ligand for PPARγ, which is also implicated in attenuating various inflammatory responses including suppression of IL-2 secretion. Since AA and 2-AG are structurally similar, it is possible for 2-AG to undergo COX-2 metabolism to yield a glycerol ester of 15d-PGJ_2 that may act as a ligand for PPARγ. Since the endocannabinoid system has been implicated in immune homeostasis, COX-2-dependent metabolism of 2-AG may translate into increased activity of the endocannabinoid system, leading to increased immune regulation by those metabolites. In addition, previous studies from this laboratory have determined that 2-AG-mediated IL-2 suppression occurs through the inhibition of NFAT activity thus decreasing IL-2 gene transcription. **The overall goal of this project was to determine the mechanism of IL-2 suppression by 2-AG by testing the following hypothesis: 15d-PGJ_2-G, a putative COX-2 metabolite of 2-AG, suppresses IL-2 secretion, in part, by the activation**

of PPARγ and involves modulation of NFAT activity which is a transcription factor critical in IL-2 gene transcription.

The aforementioned hypothesis will be tested by investigating the following specific aims:

Specific aim 1: To characterize the expression levels of COX-2 mRNA and protein in activated T cells. Since COX-2 is the rate-limiting enzyme in the metabolism of 2-AG, the expression levels of COX-2 will be measured. In addition, the effect of 2-AG on COX-2 expression levels will be investigated.

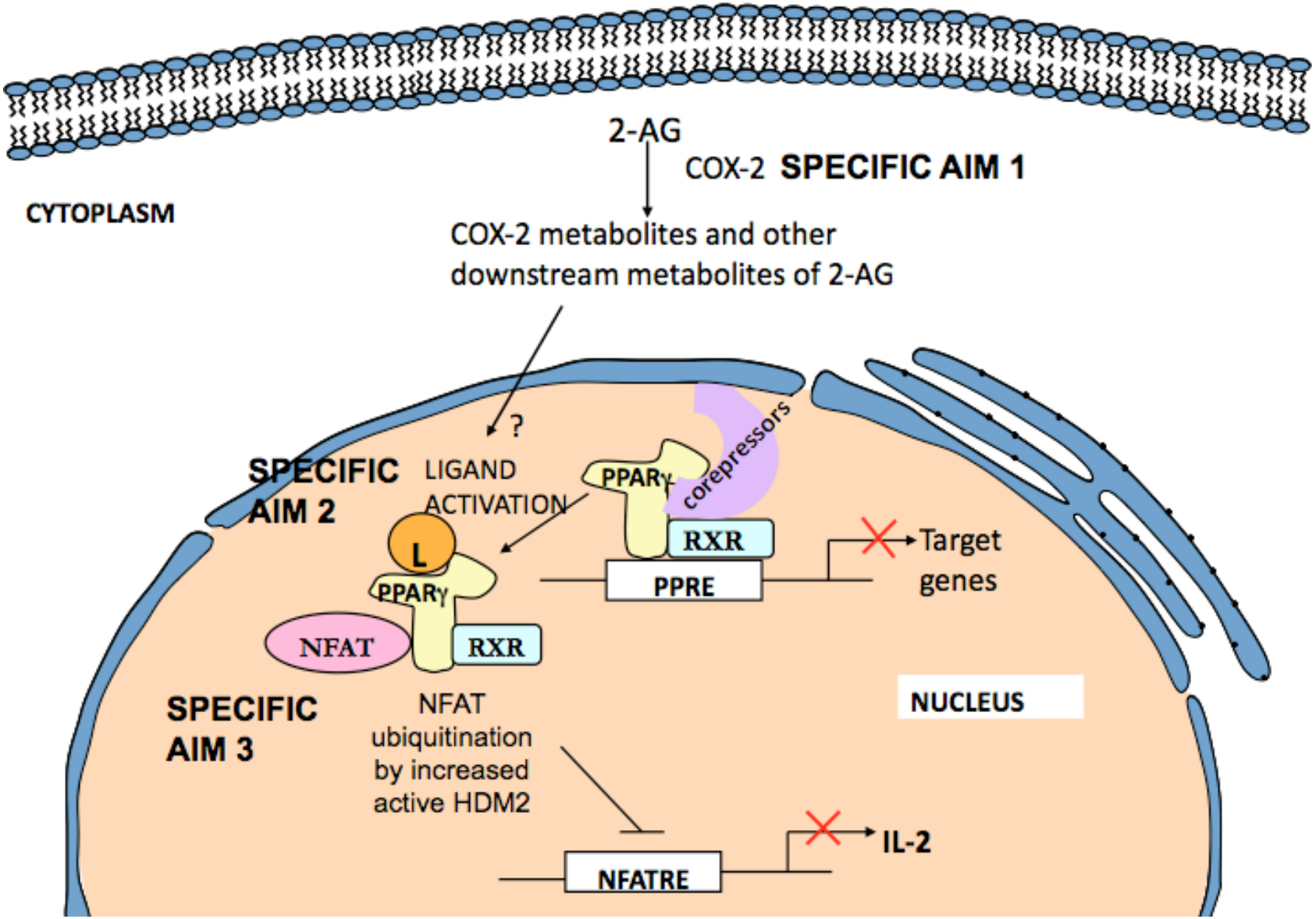
Specific aim 2: To characterize the PPARγ ligand binding activity and PPARγ activation by 15d-PGJ_2-G, a putative COX-2 metabolite of 2-AG. In addition, the effect of 15d-PGJ_2-G on IL-2 secretion by activated T cells will be investigated.

Specific aim 3: To characterize the effect of 15d-PGJ_2-G on NFAT activity and to investigate the molecular mechanisms involved in the modulation of NFAT activity in 15d-PGJ_2-G treated cells.

To test this hypothesis, Jurkat cells were used as the model system. It is an immortalized line of human T cells that has been widely used in the characterization of T cell signaling. In addition, Jurkat cells lack CB1 receptors and the CB2 receptors present in this cell line does not couple to adenylate cyclase. Hence, it is a great model system to study the effect of 15d-PGJ_2-G on IL-2 secretion without the involvement of CB1 and CB2 receptors. Furthermore, Jurkat cells were

found to be more sensitive to IL-2 suppression by 2-AG and AEA when compared to murine splenocytes [156].

Figure 9. Schematic representation of the hypothesis.



MATERIALS AND METHODS

I. Reagents

2-AG and AEA was provided by the National Institute on Drug Abuse. Ciglitazone (CGZ), rosiglitazone (RGZ), 15d-PGJ_2, 15d-PGJ_2-G and T0070907 were purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated.

II. Cell culture and animals

Jurkat cells (clone E6-1, ATCC, Manassas, VA) and Jurkat T-Ag cells (Jurkat T cells stably transfected with large T antigen - a generous gift from Dr. Arthur Weiss, UCSF) were maintained in RPMI 1640 medium supplemented with 10% Bovine Calf Serum (BCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 1X solutions of non-essential amino acids and sodium pyruvate (Invitrogen, Carlsbad, CA). HEK 293T (293T) cells were purchased from Open Biosystems and maintained in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 3.7 g/L sodium bicarbonate.

Female B6C3F1 mice, 6 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). Studies requiring animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Spleens were isolated aseptically and processed into single-cell suspensions (2×10^6

cells/ml) in RPMI 1640 supplemented with 2% bovine calf serum for calcium determination assays.

III. IL-2 ELISA

Jurkat cells were cultured in triplicate (5 x 10^5 cells/ml) in 48-well culture plates (1 ml/well). The supernatants were collected 24 h after phorbol 12-myristate 13-acetate plus calcium ionophore (PMA/I_o - 40 nM/0.5 μM) stimulation, and IL-2 protein was quantified by a sandwich ELISA method as described previously [45]. The IL-2 standard (human recombinant IL-2), mouse anti-human IL-2 antibody and biotinylated anti-human IL-2 antibody were purchased from BD Pharmingen (San Diego, CA).

IV. Lanthascreen™ TR-FRET PPARγ competitive binding assay

PPARγ binding was assessed according to the manufacturer's instructions (Invitrogen catalog # PV4894). The assay was performed in 384 black microwell plates (Matrical # MP101-1-PP). Briefly, a 20 μl total reaction mixture contained 0.5 nM PPARγ-LBD (GST), 5 nM of TB-anti-GST-tagged antibody, 5 nM of Fluormone™ Pan-PPAR Green, 5 mM DTT (dithiothreitol) and varying concentrations of RGZ, CGZ, 2-AG, AEA and 15d-PGJ_2-G (1 pM - 100 μM). The negative control was devoid of the agonist but contained everything else contained in the agonist wells. Following 3 h incubation in the dark, TR-FRET measurements were made in the SPECTRAmax GEMINI XS spectrofluorometer using the following settings: optical module - LanthaScreen™, delay time - 100 μsec, and integration time - 200 μsec. The ratiometric emission (520/490) was plotted against varying agonist concentrations. The data was

analyzed using Graph Pad Prism software from GraphPad Software, Inc. (La Jolla, CA) using the sigmoidal curve equation with variable slope to obtain IC_{50} values. The assay quality/robustness score – Z' was calculated and was found to be 0.68 (a value above 0.5 indicates a robust assay).

V. Plasmids

Human PPARγ-LBD and pFR-luc reporter gene plasmids were a generous gift from Dr. John P. Vanden Heuvel (Pennsylvania State University). The LBD of human PPARγ was fused to the DNA-binding domain of the yeast transcription factor Gal4 under the control of the SV40 promoter. This plasmid was cotransfected with pFR, a luciferase reporter under the control of the Gal4 DNA response element [291]. NFAT-luc reporter gene plasmid was purchased from Clontech (Mountain View, CA).

VI. Transient Transfection Assays

For PPARγ reporter assay in HEK293T cells, 2.5 X 10^4 cells per well were preseeded in a 96 well plate in growth medium for 16 to 20 h. The cells were then incubated with the transfection reagents [25 ng of hPPARγ-LBD, 25 ng of pFR-luc and 0.125 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for every well] for 4 h in serum-free medium. After the 4 h incubation, the medium containing the transfection reagents was removed, replaced with complete growth medium, and the cells were allowed to recover for 1 h. Hence, 5 h after transfection, the cells were cultured in the absence or presence of either vehicle (0.02% DMSO) or T0070907 for 30 min, followed by the addition of either 15d-PGJ_2 or 15d-PGJ_2-G (0.1-10 μM). Treatments were performed in triplicate.

For Jurkat T-Ag, the cells (5 X 10^5 c/ml) were incubated with transfection reagents (0.75 μg of hPPARγ-LBD, 0.75 μg of pFR-luc and 3 μl of Lipofectamine 2000 for every 5 X 10^5 cells) for 4 h in RPMI 1640 medium with 2% BCS. After the 4 h incubation, the cells were treated with either CGZ (positive control), 15d-PGJ_2 or 15d-PGJ_2-G. Treatments were performed in triplicate.

For studies with Jurkat, the cells (5 X 10^5 c/ml) were incubated with transfection reagents (1.5 μg of NFAT-luc and 3 μl of Lipofectamine 2000 for every 5 X 10^5 cells) for 4 h in RPMI 1640 medium with 2% BCS. After the 4 h incubation, the cells were cultured in the absence or presence of either vehicle (0.02% DMSO) or T0070907 for 30 min, followed by the addition of cyclosporin A (CsA), CGZ, 15d-PGJ_2 or 15d-PGJ_2-G for 30 min followed by PMA/I_o stimulation (40 nM/0.5 μM). Twenty-four hours after transfection (for HEK293T, Jurkat T-Ag cells and Jurkat cells), luciferase activity was determined using the Luciferase Assay System and Reporter Lysis Buffer (RLB) from Promega (Madison, WI). Protein determinations were performed using a Bicinchoninic Acid Assay (BCA; Sigma, St. Louis, MO).

VII. Whole cell protein isolation

Jurkat cells (2.5 x 10^7 cells) or splenocytes (2 x10^7 cells) were treated with PMA/I_o for 2, 4, 8 or 12 h. At the end of the culture period, the cells were centrifuged and suspended in 100 μl of RIPA buffer (phosphate buffered saline containing 1% Igepal, 0.5% sodium deoxycholate and 0.1% SDS) for 5 min at ambient temperature. The suspension was then sonicated and incubated on ice for 30 min. The cells were then centrifuged at 10000 x g for 20 min at 4˚C and the

supernatant was retained. The protein concentration was quantified by BCA assay (Sigma, St. Louis, MO).

VIII. Gel electrophoresis and Western blot analysis

Protein (50 μg Jurkat protein; 30 μg splenocyte protein) was diluted with loading buffer (0.0625M Tris, 2% SDS 10% glycerol, 0.01% bromophenol blue, and 1% 2-ME), loaded into an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and incubated with blocking buffer (4% dry non-fat milk in 0.05% TBS-Tween 20 for Jurkat protein; also added 1% bovine serum albumin for splenocyte protein). Three different primary COX-2 antibodies were utilized (monoclonal and polyclonal obtained from Cayman Chemical, Ann Arbor, MI, were compared with an antibody obtained from Dr. David Dewitt, Michigan State University). Secondary antibodies were HRP-linked (Amersham Biosciences, Piscataway, NJ). The blots were developed using Supersignal West Femto maximum sensitivity substrate (Pierce Biotechnology, Rockford, IL). Bands were quantified using a densitometer visual imaging system (Bio-Rad, Hercules, CA).

IX. Real-Time PCR

Jurkat T cells (5 X 10^6 cells) were left untreated, stimulated with PMA/I_o or treated with 2-AG (20 μM) for 30 min at 37°C, followed by PMA/I_o (40 nM/0.5 μM) stimulation for 2, 4, 8 and 12 h in complete medium containing 2% BCS in 60 mm X 15 mm cell culture dish (Corning Inc, NY). Cells were harvested and RNA isolation was performed using Promega SV Total RNA Isolation System (Promega, Madison, WI). Total RNA was reverse transcribed using random primers with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems,

Foster City, CA). cDNA was amplified with Taqman primers and probe sets (Applied Biosystems) and analyzed using a 7900 HT Fast Real-Time PCR System (Applied Biosystems).

X. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were isolated from naïve or PMA/I_o (40 nM/0.5μM)-activated (for 30 min) Jurkat cells (2.5 x 10^7 cells/ml) that were pre-treated with either vehicle (0.1% EtOH) or 15d PGJ_2-G (1, 5 and 10 μM) or Cyclosporin A (CsA; 0.01 μM – used as a positive control) for 30 min. After the culture period, the cells were pelleted and washed once with cold 1X PBS. The cell pellets were re-suspended in 5 ml of hypotonic buffer (10 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 1 mM dithiothreitol and 0.2 mM PMSF) and allowed to swell on ice for 15 min. The samples were centrifuged at 3000 rpm for 5 min at 4°C and the supernatants were discarded. The nuclear pellets were washed thrice with 2 ml of MDHS buffer (3 mM $MgCl_2$, 25 mM HEPES pH 7.5, 0.1M NaCl, 1 mM dithiothreitol and 0.2 mM PMSF) and pelleted at 3000 rpm for 5 min at 4° C. The nuclear pellets were re-suspended in 100 μl of cold buffer C (30 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 0.45 mM NaCl, 0.1% Igepal, 1 mM dithiothreitol and 0.2 mM PMSF) and rocked on ice for 30 min for high-salt extraction. Cellular debris was removed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatants were collected and combined with 100 μl of cold buffer D (30 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol and 0.2 mM PMSF). The protein concentration of the supernatant was quantified using the BCA assay (Sigma-Aldrich, St. Louis, MO). The binding reaction was performed by adjusting the final NaCl concentration to 25 mM

by the addition of Buffer D (Buffer C prepared as above, but devoid of NaCl) followed by incubation of 1 μg of nuclear protein with 62.5 ng of poly dI-dC (Roche, Indianapolis, IN) on ice for 10 min. Following incubation with poly dI-dC, the double stranded ^{32}P-labeled probe containing the NFAT site derived from human IL-2 promoter (5′-AGAAAGGAGGAAAAACTGTT-3′; 45,000 cpm per lane) [292] was added to the reaction and incubated at room temperature for another 20 min. To assess the specificity of DNA binding activity, the nuclear extracts were incubated with 100-fold excess of unlabeled probe prior to addition of the radiolabeled probe. The resulting protein–DNA complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA). The gel was dried on 3 mm filter paper (Whattman, Hillsboro, OR) and autoradiographed. Autoradiograph bands were quantified by densitometry using the image-processing software ImageJ, version 10.2 (http://rsbweb.nih.gov/ij). For the supershift assay, the nuclear proteins were incubated with anti-NFATc1 monoclonal (7A6) antibody (MA3-024, Thermo Fisher Scientific Inc., Rockford, IL) for 1 h at 4° C prior to the addition of radiolabeled probes.

XI. Cellular fractionation and Western blotting

Naïve or PMA/I_o-activated Jurkat cells (2.5 x 10^7 cells) pre-treated with vehicle or 15d-PGJ_2-G (10 μM) were subjected to cellular fractionation using the modified REAP method [293]. Briefly, after the culture period, the cells were harvested and the cell pellet was washed with 5 ml of ice cold PBS. After the wash, the cells were pelleted and re-suspended in 500 μl of buffer A (0.1% Igepal in 1X PBS, 1 mM dithiothreitol and 0.2 mM PMSF), and the samples were triturated 10 times using a p1000 micropipette. The samples were incubated on ice for 10

min and again triturated 10 times. An aliquot of the samples was observed under the compound microscope for the complete disruption of cell membranes and for the presence of intact nuclei. The nuclei were pelleted by centrifuging for 10 s (short setting) in the microfuge. Supernatant (300 μl) containing the cytosolic fraction was collected and 100 μl of 4X loading buffer (0.25 M Tris, 8% SDS, 40% glycerol, 0.04% bromophenol blue, and 4% 2-mercaptoethanol) was added. The remaining supernatant was discarded and the nuclear pellets were washed twice with ice cold buffer A. The nuclear pellet was re-suspended in 125 μl of 1X loading buffer (0.0625 M Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% 2-mercaptoethanol) and sonicated using microprobes at level 2, twice for 5 s. This resultant nuclear fraction is therefore 4X more concentrated than the cytosolic fraction. Both the cytosolic and the nuclear fractions were boiled for 3 min at 95°C. Five μl of the cytosolic fraction and 25 μl of the nuclear fraction was used for the detection of NFAT2, PPARγ, α-tubulin and Histone H1. Five μl of the cytosolic fraction and 10 μl of the nuclear fraction was used for the detection of GSK-3β, phospho GSK-3β, HDM2 and phospho HDM2. The samples were loaded into SDS-polyacrylamide gels (7.5% for NFAT2, PPARγ, GSK3β and phospho-GSK3β, HDM2, phospho-HDM2, 12% for α-tubulin and Histone H1), transferred to a nitrocellulose membrane and incubated with blocking buffer (5% dry non-fat milk in Tris buffered saline containing 0.05 % Tween 20). The following primary antibodies were used – NFAT2 (anti-NFATc1 (7A6), MA3-024, Thermo Fisher Scientific Inc., Rockford, IL), PPARγ (anti-PPARγ (H-100), sc-7196, Santa Cruz Biotechnology Inc., Santa Cruz, CA), GSK-3β (anti-GSK-3β (27C10), # 9315, Cell Signaling Technology, Danvers, MA), phospho GSK-3β (anti-phospho-GSK-3β (Ser9) (D3A4), Cat # 9322, Cell Signaling Technology), HDM2 (anti-HDM2-323, sc-56164, Santa Cruz Biotechnology Inc.), phospho-HDM2 (anti-phospho-HDM2 (Ser 166), sc-293105, Santa Cruz

Biotechnology Inc.), α-tubulin (anti-tubulin-alpha (10D8), Cat # 627901, Biolegend, San Diego, CA) and Histone H1 (anti-Histone H1 (AE-4), sc-8030, Santa Cruz Biotechnology Inc.). Secondary antibodies were HRP-linked (Amersham Biosciences, Piscataway, NJ). The blots were developed using Supersignal West Femto maximum sensitivity substrate (Pierce Biotechnology, Rockford, IL). Bands were quantified by densitometry using the image-processing software ImageJ, version 10.2 (http://rsbweb.nih.gov/ij).

XII. Calcium determination

Jurkat cells and splenocytes were discriminated by flow cytometric measurements of cellular forward scatter and right angle scatters, using a FACScalibur (BD Biosciences, San Jose, CA). Fluo 3 (Cat # F23915, Invitrogen, Carlsbad, CA) and Fura Red (Cat # F3021, Invitrogen) were excited at 488 nm. Fluo 3 emission was detected at 530/30 nm and Fura Red emission was detected at 670/LP nm. For experiments involving the addition of only the PPARγ agonists, the first 1 min of the analysis was considered as an initial baseline. Then, the vehicle/PPARγ agonist was added for a min followed by the addition of PMA/I_o (40 nM/0.5 μM) and the measurement was continued for another 3 min. For experiments involving the addition of PPARγ antagonists, the first 1 min of the analysis was considered as an initial baseline. At the end of 1 min, the vehicle/PPARγ antagonist was added. After another min, vehicle/PPARγ agonist was added for a min. Then, PMA/I_o (40 nM/0.5 μM) was added and the measurement was continued for another 3 min. For all experiments, the ratio intensity of Fluo3/Fura Red vs. time was calculated using FlowJo (Tree Star, Inc., Ashland, OR).

XIII. Statistical Analysis

The mean ± SE was determined for each treatment group in the individual experiments. Homogenous data were evaluated by one-way parametric analysis of variance. Dunnett's two-tailed t test was used to compare treatment groups to the vehicle control when significant differences were observed using Graph Pad Prism software (GraphPad Software, Inc., La Jolla, CA).

EXPERIMENTAL RESULTS

I. COX-2 expression in resting and activated human Jurkat T cells and freshly isolated murine splenocytes.

As COX-2 is the rate-limiting enzyme in the metabolism of 2-AG, the expression levels of COX-2 protein in Jurkat T cells were measured using Western blotting. Basal levels of COX-2 protein was readily detected in resting Jurkat cells and were further increased as early as 2 h following activation (Figure 10, on page 61). Additionally, modest increases in COX-2 expression were also detected in the absence of activation over the duration of the culture period. In accordance with the protein levels of COX-2 detected by Western blot analysis in Jurkat cells, increases in COX-2 mRNA were also detected at 2 h post-activation by real-time PCR (Figure 11, on page 63). For comparison, COX-2 expression in primary murine splenocytes was also determined. COX-2 protein levels were virtually undetectable in resting splenocytes but robustly induced upon activation. Increased COX-2 protein was observed as early as 4 h after activation and reached levels as high as 600-fold induction over naïve, untreated cells at 12 h post-activation (Figure 12, on page 65). Increased COX-2 protein upon PMA/I_o activation may play a role in generating more COX-2 metabolites of 2-AG including 15d-PGJ_2-G in Jurkat T cells.

II. 2-AG significantly decreases PMA/I_o-mediated increase in COX-2 mRNA expression, but produces only a modest decrease in PPARγ mRNA expression

COX-2 oxygenates 2-AG as effectively as AA and may lead to the generation of glycerol esters of prostaglandins [121]. We have previously demonstrated that PMA/I_o stimulation of

Jurkat cells caused an increase in both COX-2 mRNA and protein expression (Figure 10, on page 61) [156]. We now investigated the effect of the presence of 2-AG on COX-2 mRNA expression in activated Jurkat cells. PMA/I_o stimulation increased COX-2 mRNA expression at 2, 4, 8 and 12 h post stimulation as demonstrated earlier (Figure 11, on page 63) [156]. Interestingly, 2-AG treatment (20 μM) significantly decreased PMA/I_o-mediated increase in COX-2 mRNA expression at 2 and 8 h (Figure 13, on page 67). On the other hand, PMA/I_o stimulation of Jurkat cells caused an increase in PPARγ mRNA expression only at 4 h post stimulation and the expression decreased sharply at 8 h and 12 h (Figure 14, on page 69). 2-AG treatment modestly decreased the PMA/I_o-mediated increase in PPARγ mRNA expression at 4 h, although it was not statistically significant (Figure 14, on page 69). These results suggest the existence of a negative feedback loop to limit the generation of metabolites from 2-AG by COX-2 metabolism and it is tempting to speculate that this feedback inhibition may serve as a checkpoint to limit IL-2 suppression by PPARγ activation.

III. 15d-PGJ_2-G activates PPARγ-LBD in HEK293T and Jurkat T-Ag cells

A comparison of the eicosanoid pathway of AA and 2-AG suggested that both AA and 2-AG can be metabolized by COX-2 to form PGH_2 and PGH_2-G (glycerol ester), and depending on the various downstream enzymes, they can form various prostaglandins/thromboxanes and glycerol esters of prostaglandins/thromboxanes, respectively [121] (Figure 5, on page 24). One of the putative metabolites of 2-AG is 15d-PGJ_2-G, which is the glycerol ester of 15d-PGJ_2, a known agonist for PPARγ. 15d-PGJ_2-G treatment of HEK293T cells transfected with PPARγ

reporter plasmids induced PPARγ-LBD-driven luciferase activity in a concentration-dependent manner (Figure 15, on page 71). 15d-PGJ_2 was used as a positive control and also induced PPARγ-LBD-driven luciferase activity in a concentration-dependent manner. It is noteworthy that in the presence of the PPARγ antagonist, T0070907, PPARγ reporter activity was abolished confirming that 15d-PGJ_2-G activates PPARγ-LBD (Figure 15, on page 71). Since 15d-PGJ_2-G activates PPARγ-LBD in HEK293T cells and it has been demonstrated that 2-AG, other PPARγ agonists, and 15d-PGJ_2 suppress IL-2 in activated T cells [176], it was investigated if 15d-PGJ_2-G activates PPARγ-LBD in a T cell line. PPARγ reporter assays were performed in Jurkat T-Ag cells because constitutive expression of the large T antigen is essential for the robust expression of the PPARγ-LBD plasmid (Jurkat cells – clone E6-1 lacks large T-antigen). In Jurkat T-Ag cells transfected with PPARγ reporter plasmids, 15d-PGJ_2-G induced PPARγ-LBD-driven luciferase activity in a concentration-dependent manner. CGZ and 15d-PGJ_2, which are known PPARγ agonists [153], also activated PPARγ-LBD in Jurkat T-Ag cells (Figure 16, on page 73).

To determine if plant-derived cannabinoids activate PPARγ-LBD, the reporter activity assay was carried out in HEK293T cells in the presence of the plant-derived cannabinoids, CBD, CBN and THC. These compounds did not activate PPARγ-LBD as evidenced by the basal levels of luciferase activity comparable to the vehicle control whereas the positive control, CGZ, produced an increase in the luciferase activity (Figure 17, on page 75).

Figure 10. Western blot analysis of COX-2 protein levels in resting and activated human Jurkat T cells.

Jurkat human T cells were either left untreated (NA) or treated with 40 nM PMA and 0.5 mM ionomycin (P/I) for the indicated times (2, 4, 8, 12 h). The cells were then lysed and the lysates assayed for COX-2 protein by Western analysis. COX-2 protein levels were quantified by densitometric analysis and normalized to beta-actin protein levels. The results are expressed as fold induction over NA (average value of all NA samples). The results are representative of three separate experiments.

Figure 10. (cont'd)

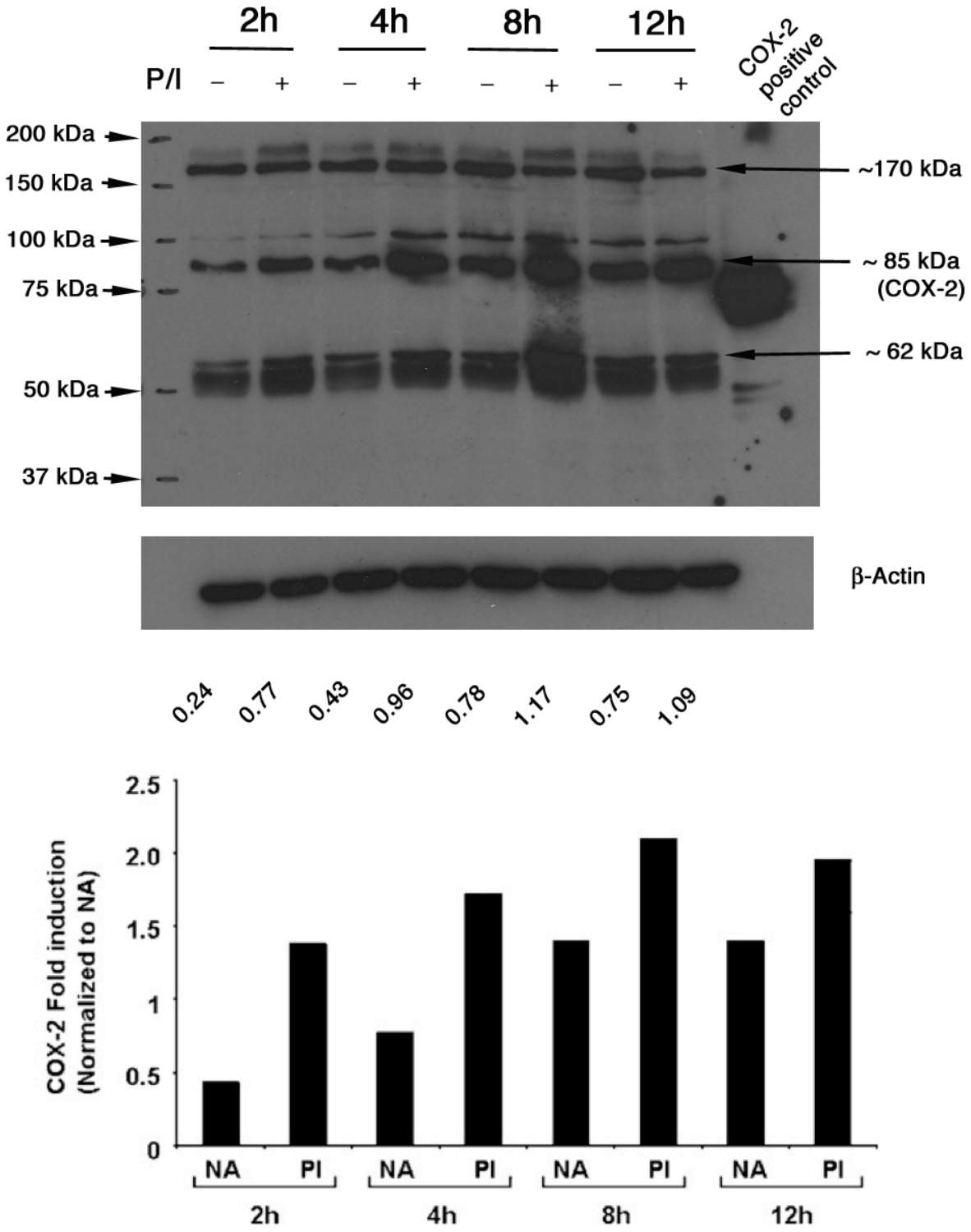


Figure 11. Real-time PCR analysis of COX-2 mRNA levels in resting and activated human Jurkat T cells.

Jurkat cells were either left untreated (NA) or treated with 40 nM PMA and mM ionomycin (P/I) for the indicated times (2, 4, 8, 12 h). The cells were then harvested and the RNA was isolated. COX-2 mRNA levels were detected by real-time PCR analysis and normalized to 18S mRNA levels. The results are expressed as fold induction over the time-matched NA samples. The results are the mean ± standard error of triplicate cultures. * denotes $p < 0.05$ compared to the time- matched NA group as determined by two-tailed Dunnett's analysis. The results are representative of three separate experiments.

Figure 11. (cont'd)

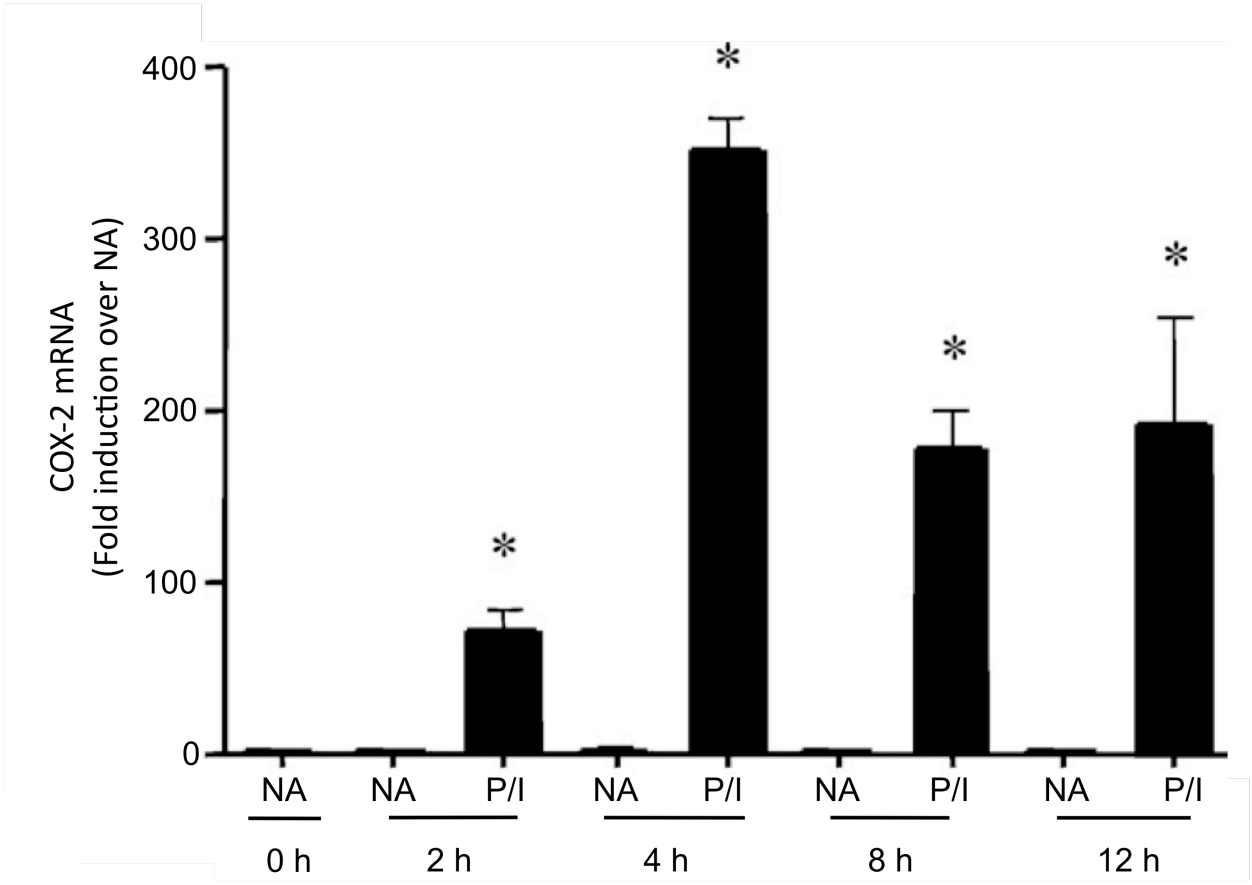


Figure 12. Western blot analysis of COX-2 protein levels in resting and activated primary splenocytes.

Freshly isolated murine splenocytes were either left untreated (NA) or treated with 40 nM PMA and 0.5 mM ionomycin (P/I) for the indicated times (0–12 h). The cells were then lysed and the lysates assayed for COX-2 protein by Western analysis. COX-2 protein levels were quantified by densitometric analysis and normalized to beta-actin protein levels. The results are expressed as fold induction over NA (average value of all NA samples). The results are representative of three separate experiments.

Figure 12. (cont'd)

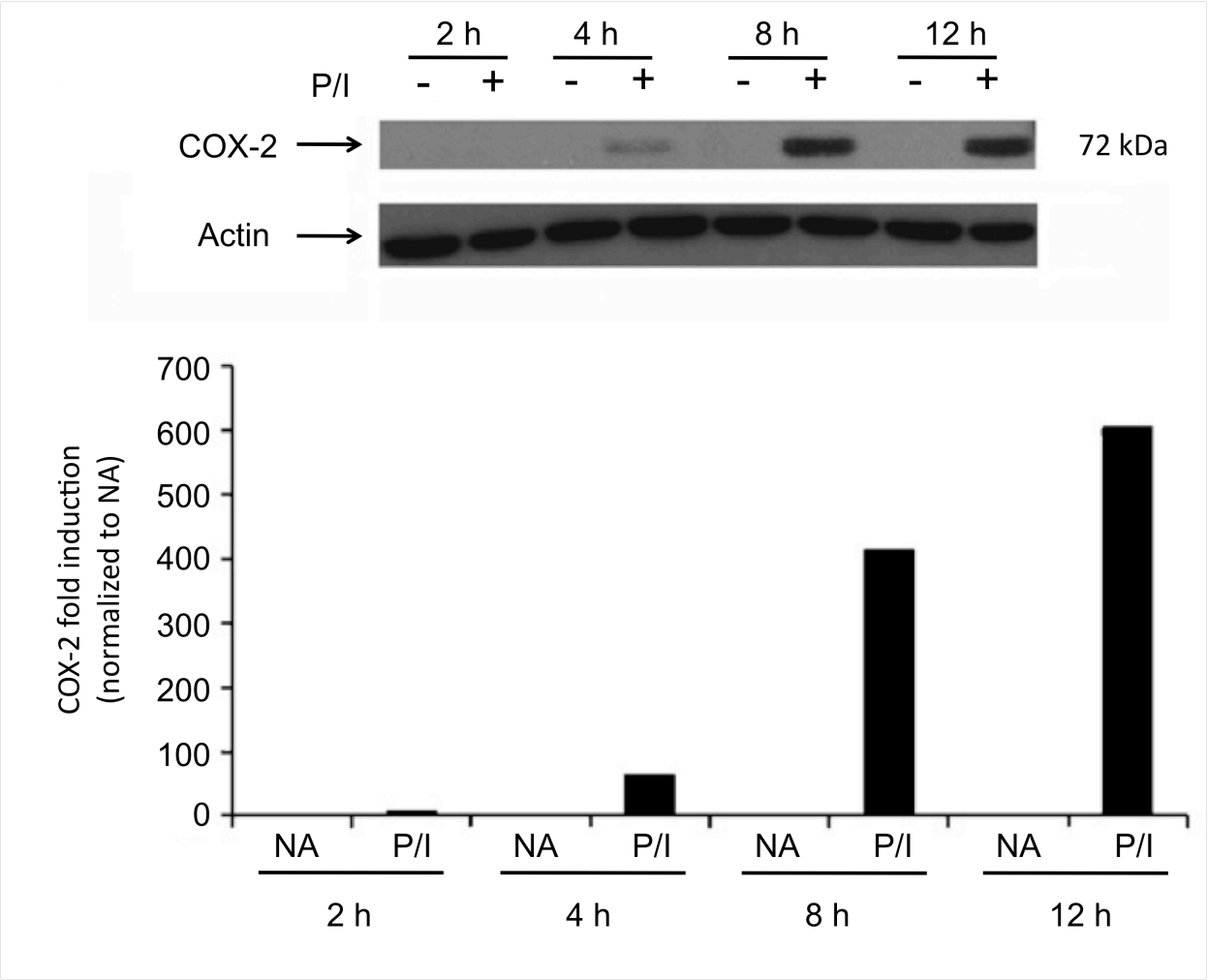


Figure 13. Real-time PCR analysis of COX-2 mRNA levels in resting and activated Jurkat T cells treated with 2-AG.

Jurkat cells were left untreated (NA-Naive), treated with vehicles (VEH of 2-AG - 0.1 % EtOH; VEH of PMA/I_o- 0.1 % DMSO) or 2-AG (20 μM) in the presence or absence of 40 nM PMA and 0.5 mM ionomycin (P/I) for the indicated times (2, 4, 8, 12 h) after which the RNA was isolated. COX-2 mRNA mRNA levels were detected by real-time PCR analysis and normalized to 18S mRNA levels. The results are expressed as fold induction over the NA samples at 0 h. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to the time-matched EtOH + P/I. The results are representative of three separate experiments.

Figure 13. (cont'd)

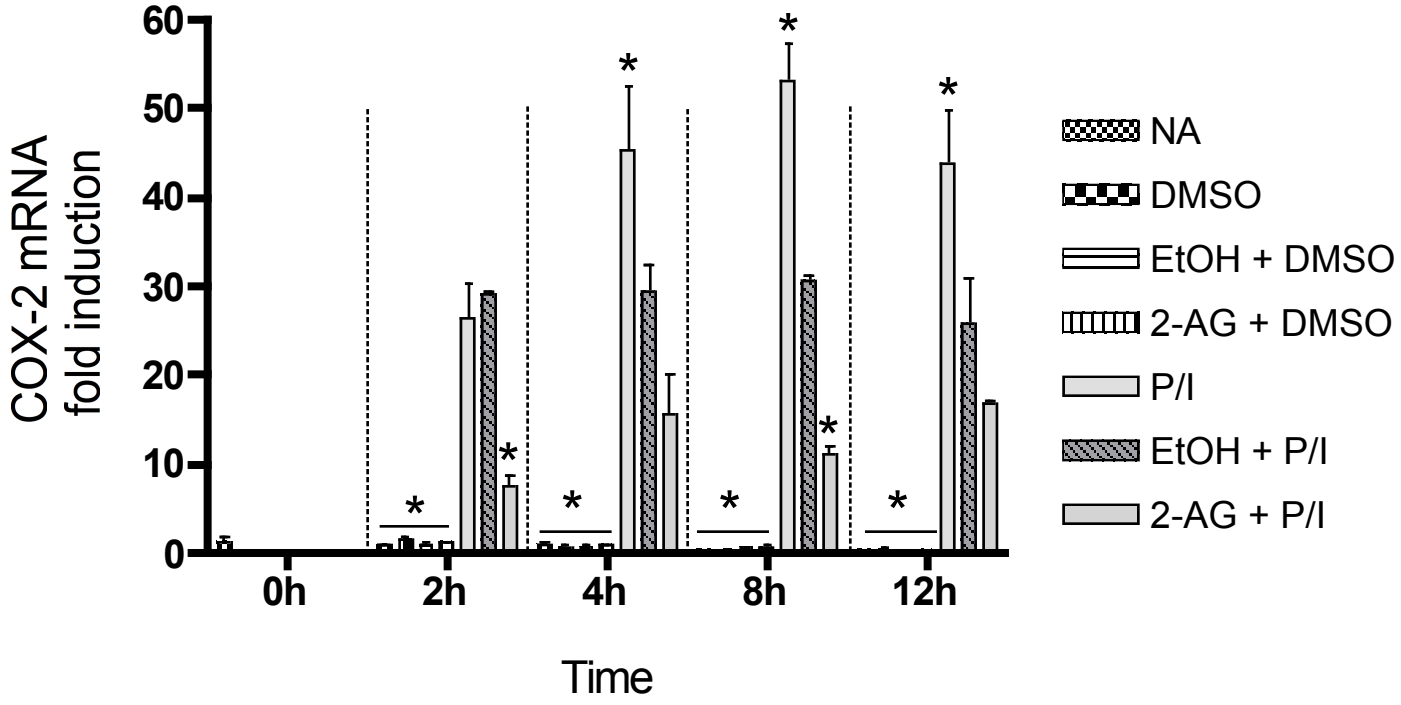


Figure 14. Real-time PCR analysis of PPARγ mRNA levels in resting and activated Jurkat T cells treated with 2-AG.

Jurkat cells were left untreated (NA- Naive), treated with vehicles (VEH of 2-AG - 0.1 % EtOH; VEH of PMA/I_o- 0.1 % DMSO) or 2-AG (20 μM) in the presence or absence of 40 nM PMA and 0.5 mM ionomycin (P/I) for the indicated times (2, 4, 8, 12 h) after which the RNA was isolated. PPARγ mRNA levels were detected by real-time PCR analysis and normalized to 18S mRNA levels. The results are expressed as fold induction over the NA samples at 0 h. The results are the mean ± S.E. of triplicate cultures. The results are representative of three separate experiments.

Figure 14. (cont'd)

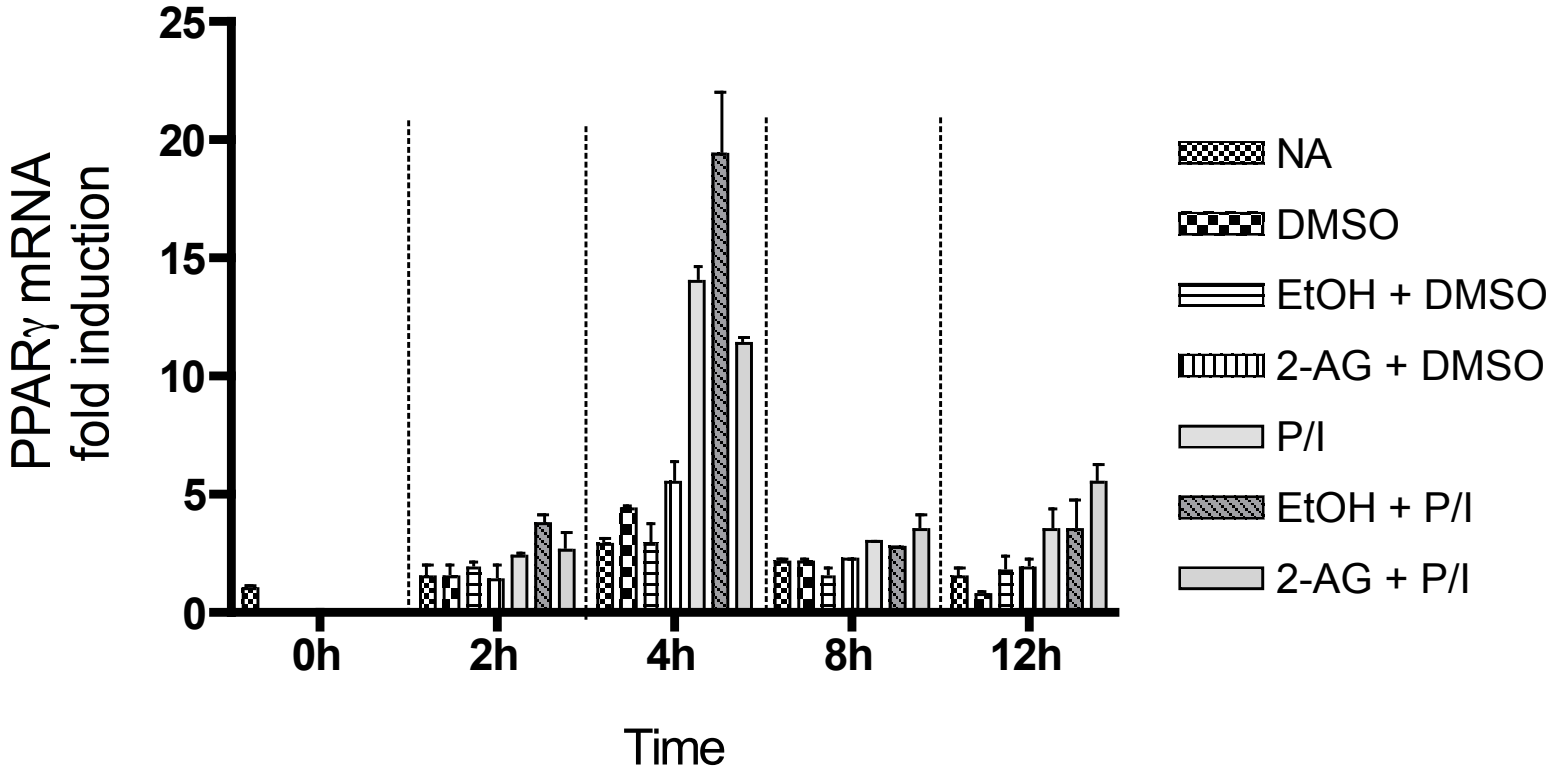


Figure 15. PPARγ-LBD reporter activity in HEK293T cells treated with 15d-PGJ_2-G.

HEK293T cells (2.5 X 10^4 cells per well) were preseeded in a 96 well plate in growth medium for 16 to 20 h. The cells were then transiently transfected with hPPARγ-LBD and pFR-luc. After transfection, the cells were cultured in the absence or presence of either vehicle (0.02% DMSO) or T0070907 for 30 min, followed by the addition of vehicle (VEH of agonist - 0.1% EtOH), 15d-PGJ_2 or 15d-PGJ_2-G (0.1, 1 or 5 μM). Twenty-four hours after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist alone and †, $p < 0.05$ compared to VEH for T0070907 within each treatment group. The results are representative of three separate experiments.

Figure 15. (cont'd)

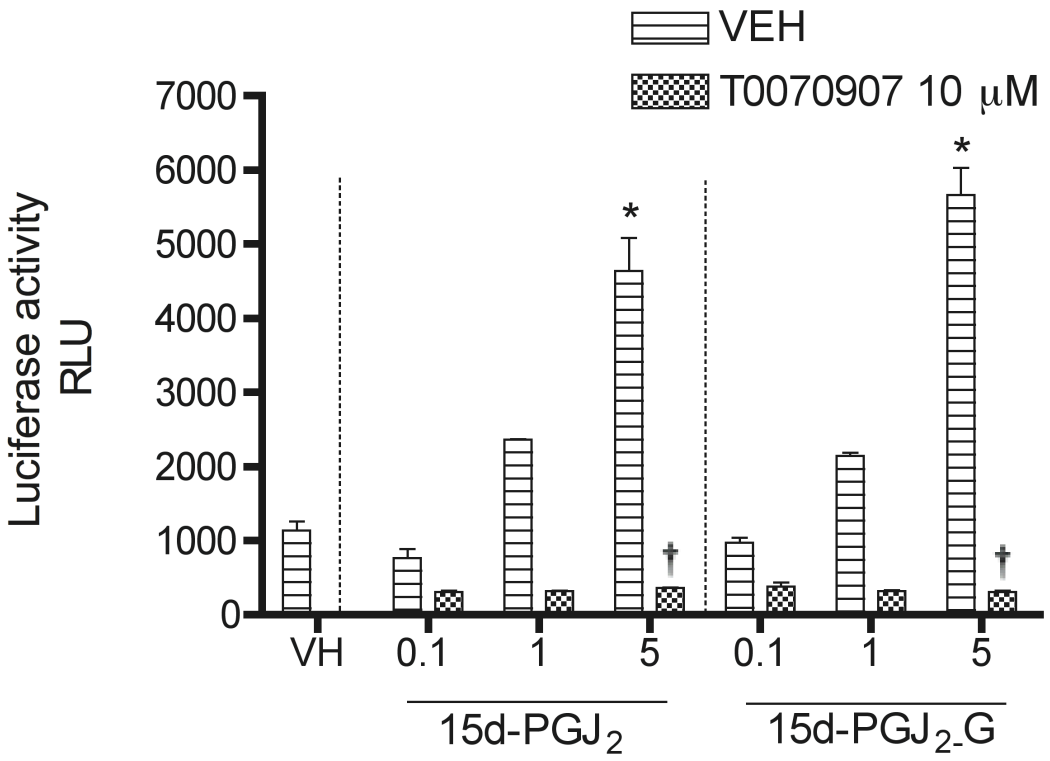


Figure 16. PPARγ-LBD reporter activity in Jurkat T-Ag cells treated with 15d-PGJ$_2$-G.

Jurkat T-Ag cells (5 X 10^5 c/ml) were transiently transfected with hPPARγ-LBD and pFR-luc using Lipofectamine 2000 for 4 h in RPMI 1640 medium with 2% BCS in a 48 well plate. After the 4 h incubation, the cells were either left untreated (NT – No treatment) or treated with CGZ, 15d-PGJ$_2$, 15d-PGJ$_2$-G or vehicle (VEH of agonist - 0.1% EtOH). Twenty-four hours after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist. The results are representative of three separate experiments.

Figure 16. (cont'd)

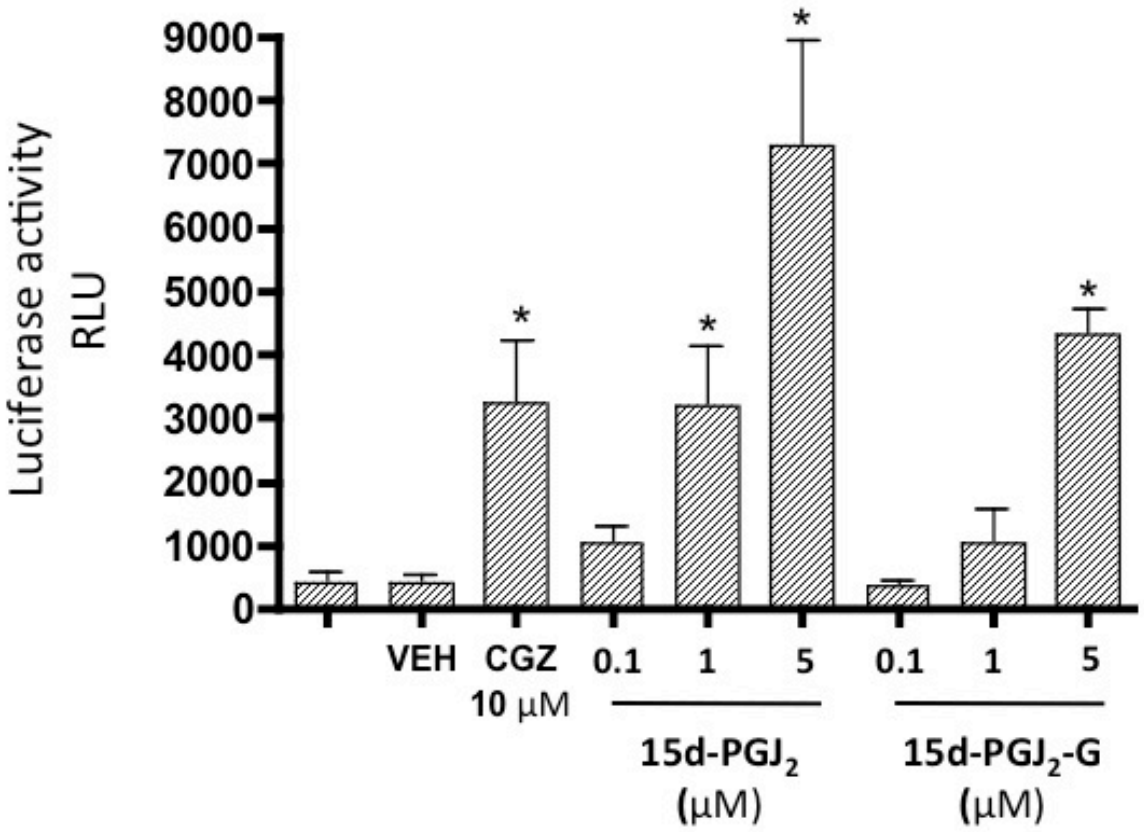
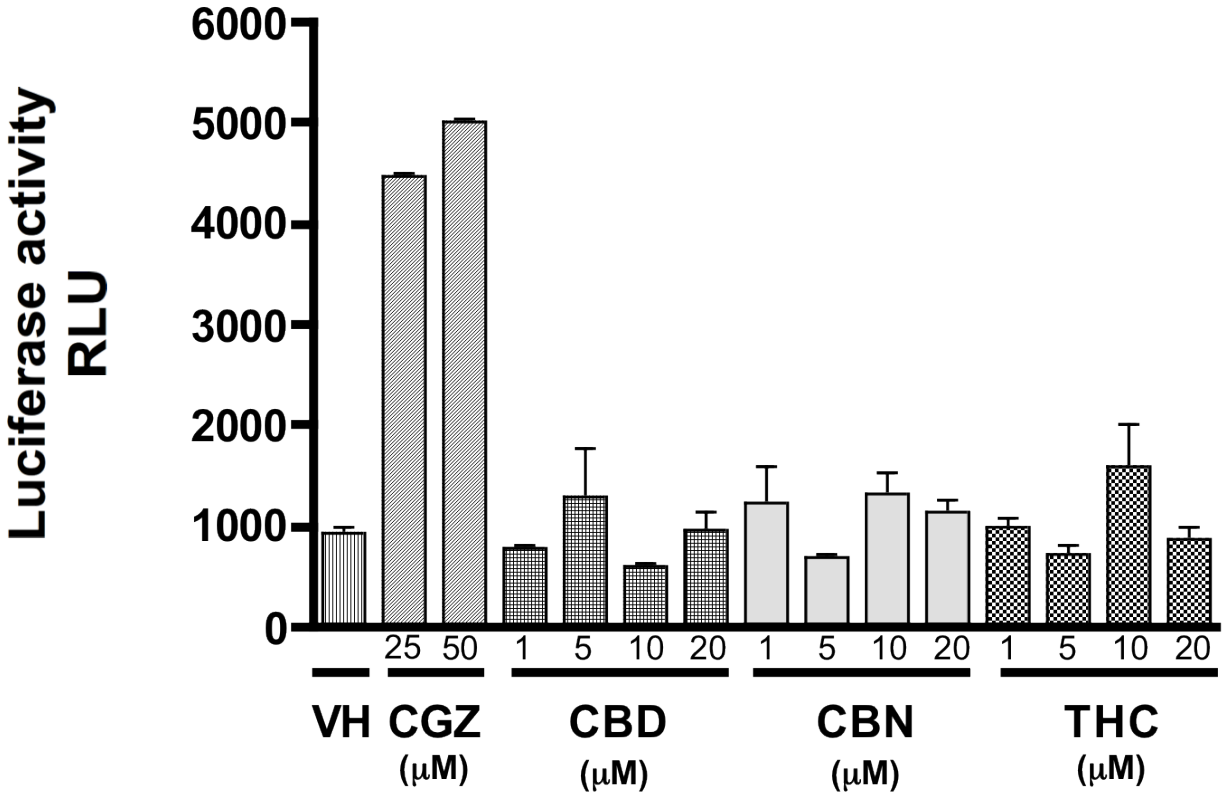


Figure 17. PPARγ-LBD reporter activity in HEK293T cells treated with plant-derived cannabinoids, CBD, CBN and THC.

HEK293T cells (2.5 X 10^4 cells per well) were preseeded in a 96 well plate in growth medium for 16 to 20 h. The cells were then transiently transfected with hPPARγ-LBD and pFR-luc. After transfection, the cells were cultured in the presence of vehicle (VH of agonist - 0.1% EtOH), CGZ (25 and 50 μM), CBD (1, 5, 10 and 20 μM), CBN (1, 5, 10 and 20 μM) or THC (1, 5, 10 and 20 μM). Twenty-four hours after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. The results are representative of three separate experiments.

Figure 17. (cont'd)



IV. 15d-PGJ$_2$-G binds to PPARγ-LBD in a PPARγ competitive binding assay

In order to verify direct binding of 15d-PGJ$_2$-G to PPARγ, a Lanthascreen^{TM} TR-FRET PPARγ competitive binding assay was performed. PPARγ agonists, RGZ and CGZ, were used as positive controls. 15d-PGJ$_2$-G bound PPARγ-LBD by competitively displacing the Fluormone^{TM} Pan-PPAR Green and this is evidenced by the decrease in the 520/490 ratio (Figure 18, on page 79). The IC_{50} values were calculated from these concentration response curves and are as follows: RGZ – 74.6 nM, 15d-PGJ$_2$-G – 367.5 nM, and CGZ – 3.45 μM. These results show that 15d-PGJ$_2$-G binds to PPARγ-LBD with lower affinity than RGZ but with higher affinity than CGZ. In addition, the endocannabinoids 2-AG and AEA were also assayed for binding to PPARγ-LBD (Figure 19, on page 81). The determined IC_{50} values were 2-AG – 13.5 μM and AEA – 26.8 μM. The rank order of IC_{50} is RGZ > 15d-PGJ$_2$-G > CGZ > 2-AG > AEA. These results further show that 2-AG itself is a relatively low affinity ligand for PPARγ, especially when compared to 15d-PGJ$_2$-G.

V. 15d-PGJ$_2$-G suppresses IL-2 secretion in a concentration-dependent and time-dependent manner

Since 15d-PGJ$_2$-G bound to and activated PPARγ, the effect of 15d-PGJ$_2$-G-mediated PPARγ activation on IL-2 secretion was investigated in PMA/I_o-stimulated Jurkat T cells. 15d-PGJ$_2$-G treatment produced a robust concentration-dependent suppression of IL-2 secretion

compared to the vehicle control (VEH – 0.1% EtOH) (Figure 21, on page 85). Treatment with increasing concentrations of 15d-PGJ_2 (comparative control) produced a concentration-dependent suppression of IL-2 secretion [176]. CGZ, which was used as a synthetic agonist of PPARγ, also suppressed IL-2 secretion [283] (Figure 20, on page 83). Further, we investigated the effect of the presence of 15d-PGJ_2-G at various times in relation to stimulation of Jurkat T cells. Time of addition studies in activated Jurkat T cells with 15d-PGJ_2-G demonstrated that the presence of 15d-PGJ_2-G either 30 min before or after stimulation of Jurkat cells produced pronounced IL-2 suppression whereas when 15d-PGJ_2-G was added at later time points, 15d-PGJ_2-G-mediated IL-2 suppression was attenuated (Figure 22, on page 87). A similar profile was obtained with the presence of CGZ at varying time points. Overall, these results suggest that 15d-PGJ_2-G has to be present early during T cell activation for robust IL-2 suppression.

VI. A PPARγ antagonist, T0070907, attenuates 15d-PGJ_2-G-mediated IL-2 suppression

Since 15d-PGJ_2-G activated PPARγ and caused IL-2 suppression in activated Jurkat cells, we investigated if a PPARγ antagonist, T0070907, attenuated the IL-2 suppression. Pretreatment of Jurkat cells with T0070907 partially attenuated the IL-2 suppression caused by 15d-PGJ_2-G and CGZ. Interestingly, T0070907 (1 μM) in the presence of VEH (0.1% EtOH) produced an increase in IL-2 secretion, suggesting an intrinsic endogenous activity through PPARγ (Figure 23, on page 89).

Figure 18. Lanthascreen™ TR-FRET PPARγ competitive binding assay - Effect of 15d-PGJ$_2$-G, CGZ and RGZ.

The reaction mixture contained 0.5 nM PPARγ-LBD (GST), 5 nM of TB-anti-GST-tagged antibody, 5 nM of Fluormone™ Pan-PPAR Green, 5 mM DTT (dithiothreitol) and varying concentrations of RGZ, CGZ and 15d-PGJ$_2$-G (1 pM - 100μM). Following 3-h incubation in the dark, TR-FRET measurements were made in the SPECTRAmax GEMINI XS spectrofluorometer. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to the TR-FRET ratio of respective vehicle control (VEH of RGZ – 5% EtOH – 0.43063, VEH of CGZ and 15dPGJ$_2$-G – 1% EtOH – 0.44179). The results are representative of three separate experiments.

Figure 18. (cont'd)

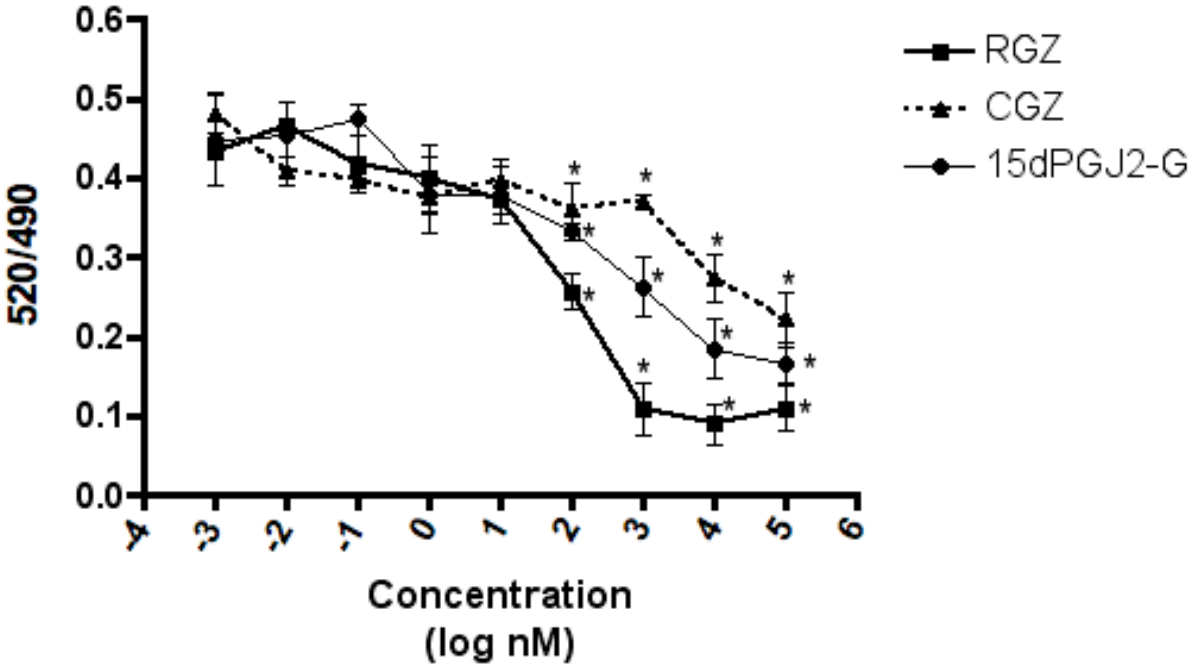


Figure 19. Lanthascreen™ TR-FRET PPARγ competitive binding assay - Effect of the endocannabinoids, 2-AG and AEA.

The reaction mixture contained 0.5 nM PPARγ-LBD (GST), 5 nM of TB-anti-GST-tagged antibody, 5 nM of Fluormone™ Pan-PPAR Green, 5 mM DTT (dithiothreitol) and varying concentrations of RGZ, 2-AG and AEA (1 pM - 100μM). Following 3-h incubation in the dark, TR-FRET measurements were made in the SPECTRAmax GEMINI XS spectrofluorometer. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to the TR-FRET ratio of respective vehicle control (VEH of RGZ – 5% EtOH – 0.43063, VEH of 2-AG and AEA – 1% EtOH – 0.43342). The results are representative of three separate experiments.

Figure 19. (cont'd)

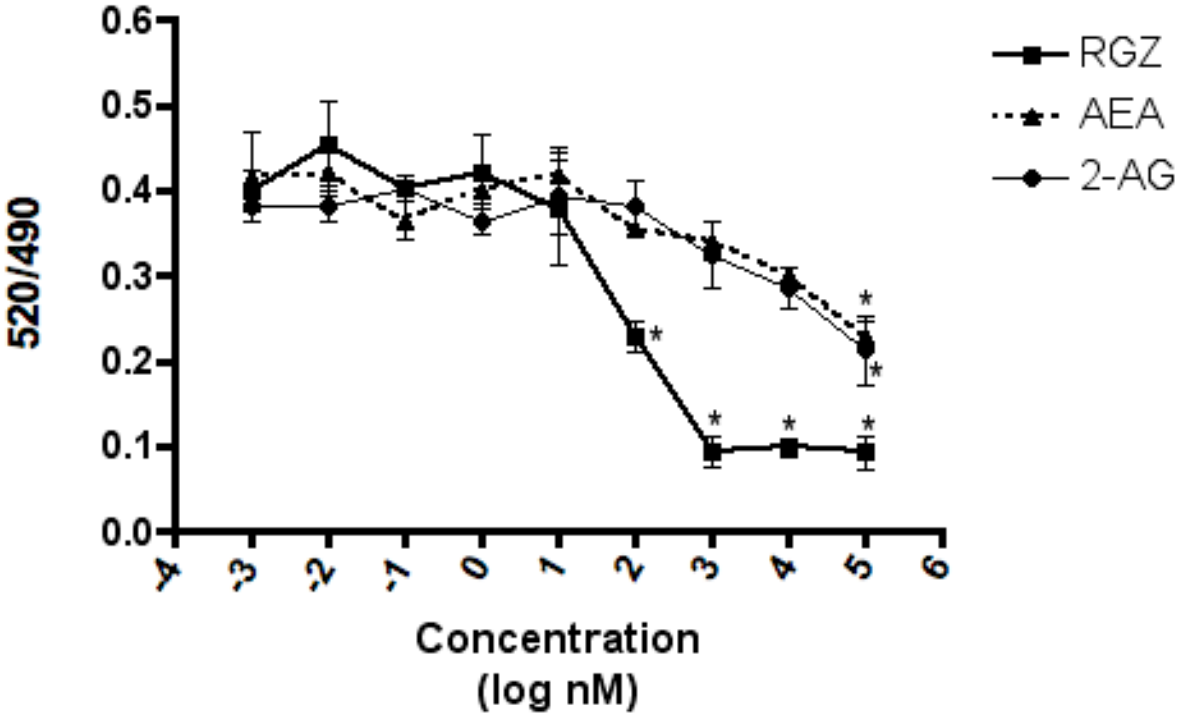


Figure 20. Effect of CGZ, a synthetic PPAR agonist, on IL-2 secretion.

Jurkat cells (5 x 10^5 cells/ml) were either left untreated (NA – Naive) or treated with CGZ (5-50 μM) and vehicle (VH – 0.1% EtOH) for 30 min. Cells were then stimulated with 40 nM PMA/0.5 μM ionomycin (P/I) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VH. The results are representative of three separate experiments.

Figure 20. (cont'd)

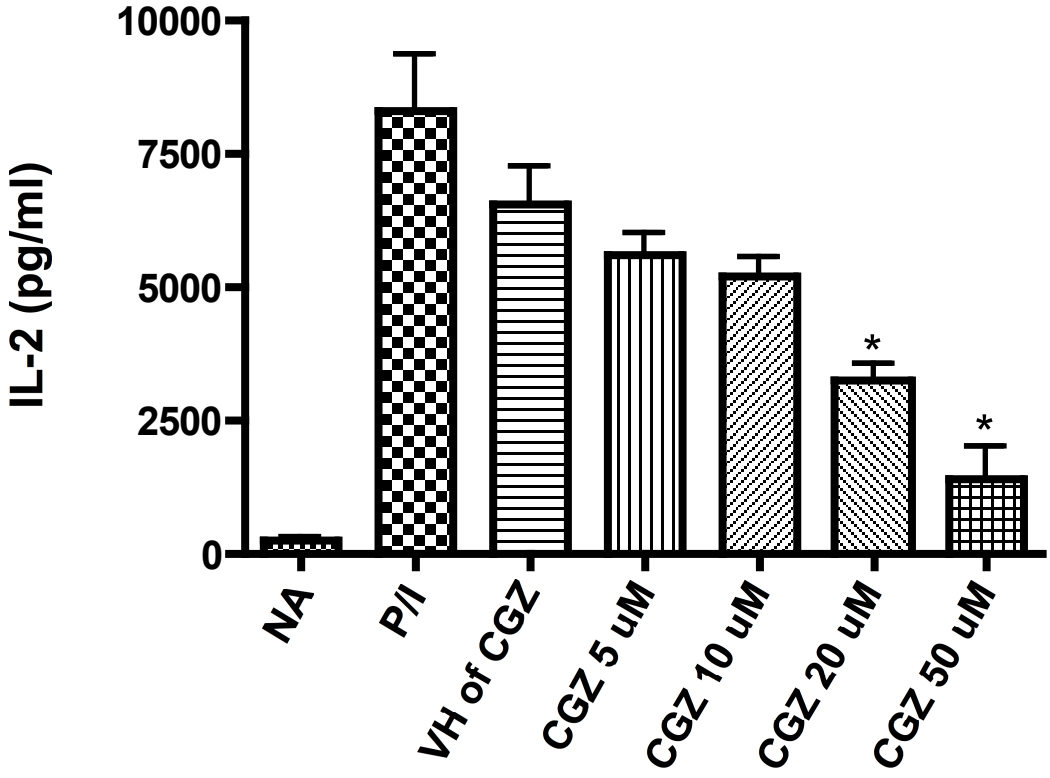


Figure 21. Effect of 15d-PGJ_2-G upon IL-2 secretion by activated Jurkat cells.

Jurkat cells (5 x 10^5 cells/ml) were either left untreated (NT – No treatment) or treated with CGZ (50 μM), 15d-PGJ_2 (0.1 – 10 μM), 15d-PGJ_2-G (0.1 – 10 μM) or vehicle (VEH of agonist – 0.1% EtOH) for 30 min. Cells were then stimulated with 40 nM PMA/0.5 μM ionomycin (P/I) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist. The results are representative of three separate experiments.

Figure 21. (cont'd)

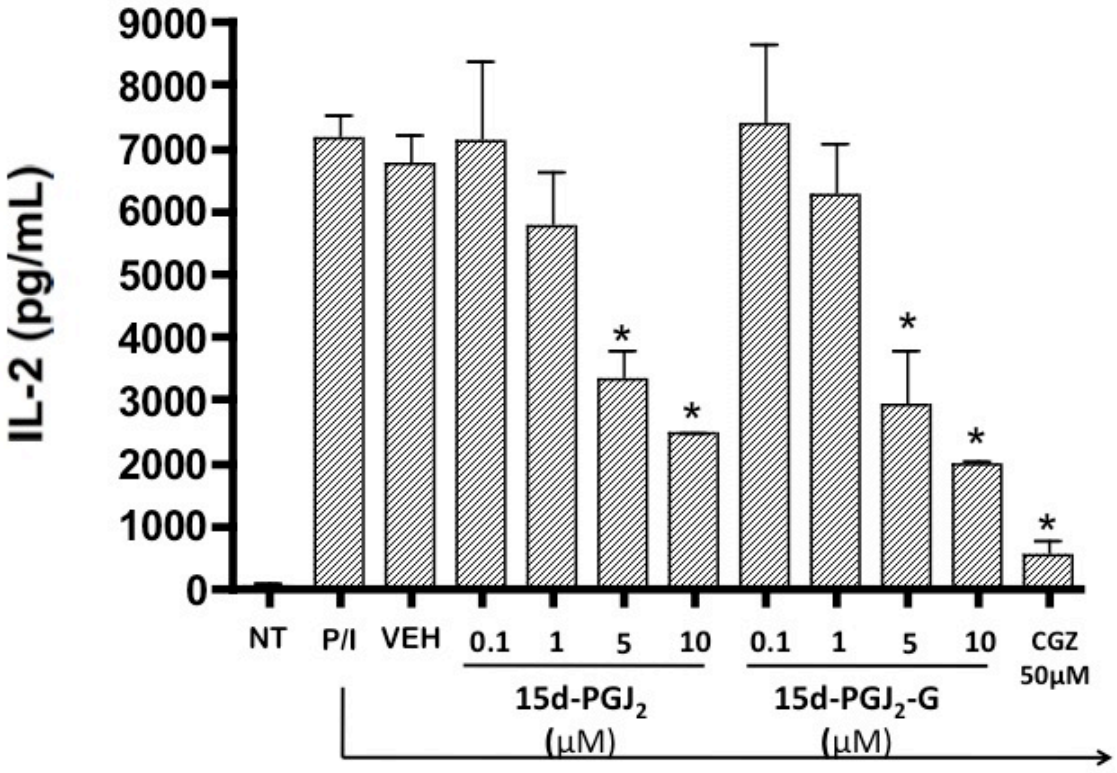


Figure 22. Time of addition studies with 15d-PGJ_2-G in activated Jurkat cells.

At time -30 min to 8 h post stimulation with PMA/I_o (P/I), Jurkat cells (5 x 10^5 cells/ml) were treated with CGZ (50 μM) or 15d-PGJ_2-G (5 μM) or vehicle (VEH of agonist – 0.1% EtOH). Twenty-four hours after stimulation, the supernatants were harvested and IL-2 production was measured by ELISA. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to the time-matched VEH control and †, $p < 0.05$ compared to the respective – 30 and + 30 min treatment. The results are representative of three separate experiments.

Figure 22. (cont'd)

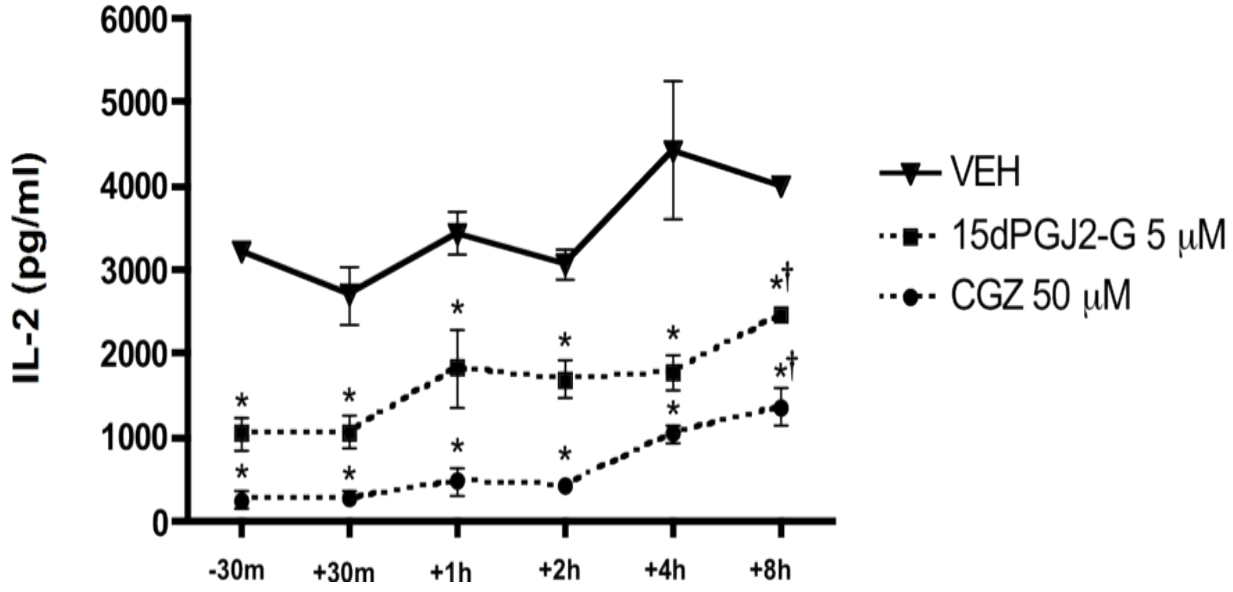
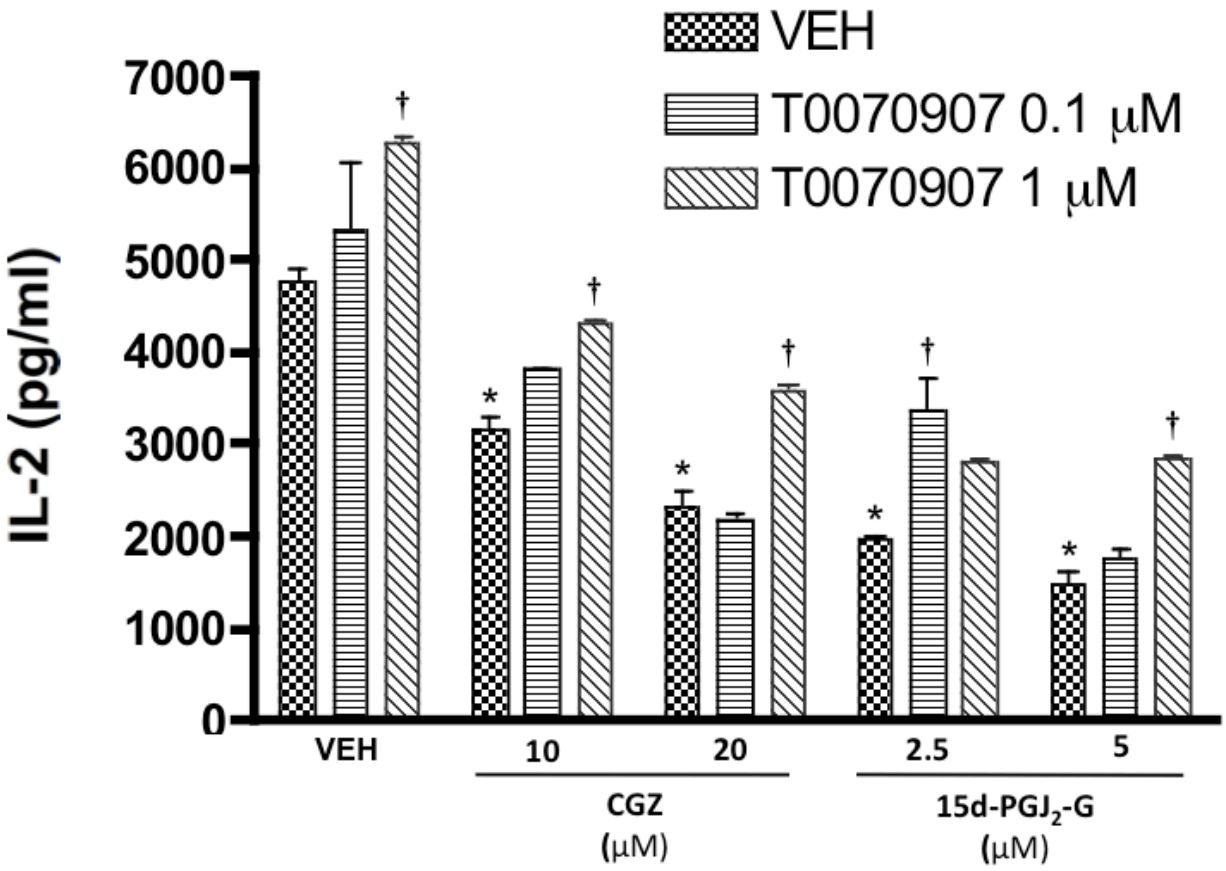


Figure 23. Effect of T0070907, a PPARγ antagonist, on 15d-PGJ$_2$-G-mediated IL-2 suppression in activated Jurkat cells.

Jurkat cells (5 x 10^5 cells/ml) were treated with vehicle of T0070907 (VEH of antagonist - 0.02% DMSO) or (0.1 and 1 μM) for 30 min, followed by the addition of CGZ (10 or 20 μM), 15d-PGJ$_2$-G (2.5 or 5 μM) or vehicle (VEH of agonist – 0.1% EtOH) for 30 min. Cells were then stimulated with 40 nM PMA/0.5 μM ionomycin (P/I) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to (VEH of agonist + VEH of antagonist) and †, $p < 0.05$ compared to VEH of T0070907 within each treatment group. The results are representative of three separate experiments.

Figure 23. (cont'd)



VII. 15d-PGJ_2-G decreases transcriptional activity of NFAT and T0070907 alone markedly increases NFAT reporter activity in activated Jurkat cells

One of the most important transcription factors involved in IL-2 gene transcription is NFAT and the increased availability of nuclear NFAT following activation of T cells is responsible for increased IL-2 gene transcription [294]. Ligation of PPARγ with 15d-PGJ_2 decreased DNA binding of NFAT and caused sequestration of NFAT to PPARγ in human peripheral blood T cells [271]. Since 15d-PGJ_2-G activated PPARγ and decreased PMA/I_o-stimulated IL-2 secretion, we evaluated the effect of 15d-PGJ_2-G on NFAT transcriptional activity. 15d-PGJ_2-G treatment of activated Jurkat cells transiently transfected with NFAT-luc caused a concentration-dependent decrease in PMA/I_o-stimulated luciferase activity (Figure 24, on page 93). CsA, an inhibitor of calcineurin that prevents NFAT dephosphorylation and entry in to the nucleus, was used as a positive control [294]. Importantly, CGZ also decreases the luciferase activity, suggesting that activation of PPARγ causes a decrease in the transcriptional activity of NFAT (Figure 24, on page 93). Since PPARγ agonists such as troglitazone and 15d-PGJ_2 decreased the transcriptional activity of NFAT [271], and we also showed that 15d-PGJ_2-G and CGZ decreased the transcriptional activity of NFAT, we investigated whether a PPARγ antagonist can attenuate the 15d-PGJ_2-G-mediated decrease in NFAT transcriptional activity in activated Jurkat cells. Interestingly, NFAT reporter activity was greatly enhanced with T0070907 (5 and 10 μM) in the presence of the vehicle of agonist (Figure 25, on page 95). The T0070907-mediated enhanced NFAT reporter activity was attenuated in the presence of 15d-

PGJ_2-G. It has also been demonstrated earlier that 2-AG decreases NFAT reporter activity in activated Jurkat cells [176]. Importantly, the 2-AG-mediated decrease in NFAT reporter activity was attenuated in the presence of the PPARγ antagonist, T0070907 [176]. These data suggest that the NFAT reporter activity observed after PMA/I_o stimulation is a balance between the reporter activity caused by PMA/I_o stimulation and the suppression of reporter activity caused by the endogenous agonist(s) for PPARγ. In the presence of T0070907, the suppression of the reporter activity caused by PPARγ activation (by the endogenous agonist(s)) may be relieved and hence an increase in NFAT reporter activity was observed.

Figure 24. NFAT reporter activity in Jurkat cells treated with 15d-PGJ$_2$-G.

Jurkat cells (5 X 10^5 c/ml) were transiently transfected with NFAT luciferase reporter using Lipofectamine 2000 for 4 h in RPMI 1640 medium with 2% BCS in a 48 well plate. After the 4 h incubation, the cells were either left untreated (NA – Naïve) or treated with vehicle (VEH of agonist - 0.1% EtOH), CsA (0.01 μM), CGZ (50 μM), or 15d-PGJ$_2$-G (5 and 10 μM) for 30 min followed by PMA/I_o stimulation (P/I - 40 nM/0.5 μM). Twenty-four hours after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist. The results are representative of three separate experiments.

Figure 24. (cont'd)

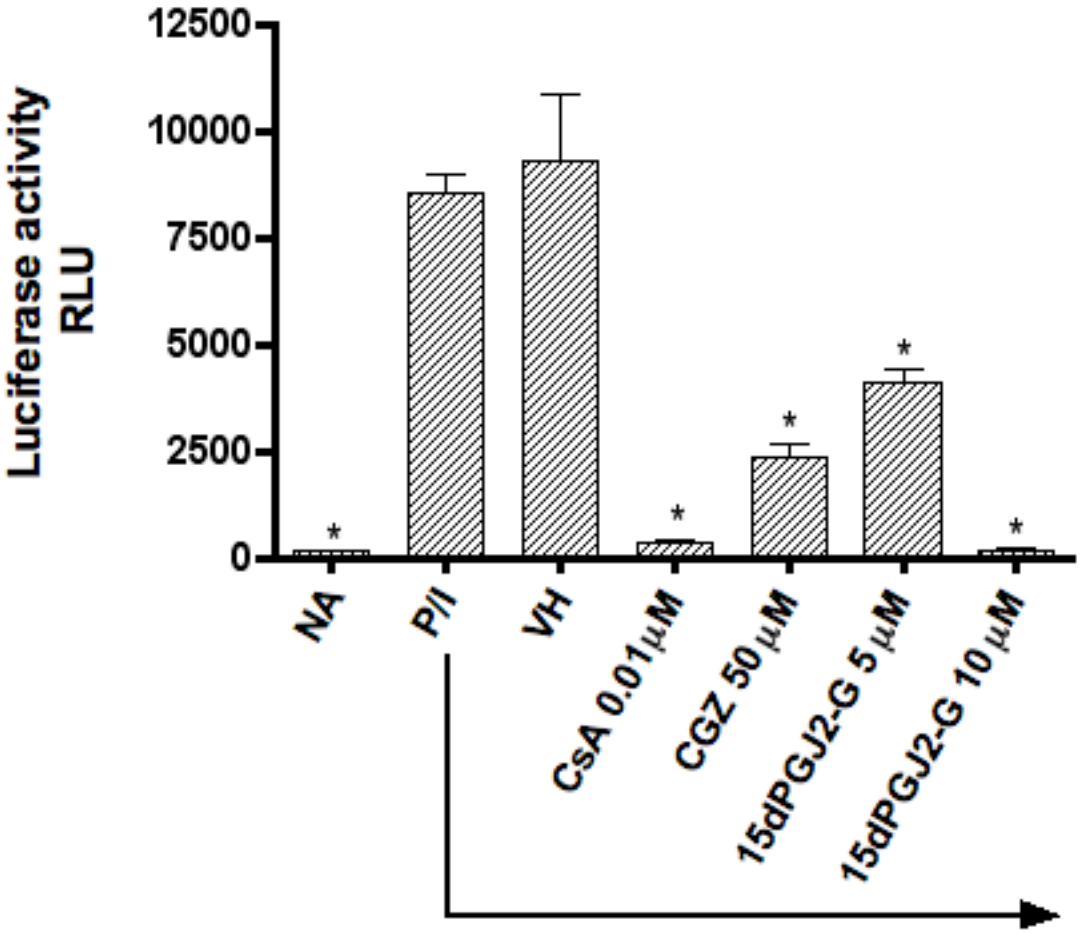
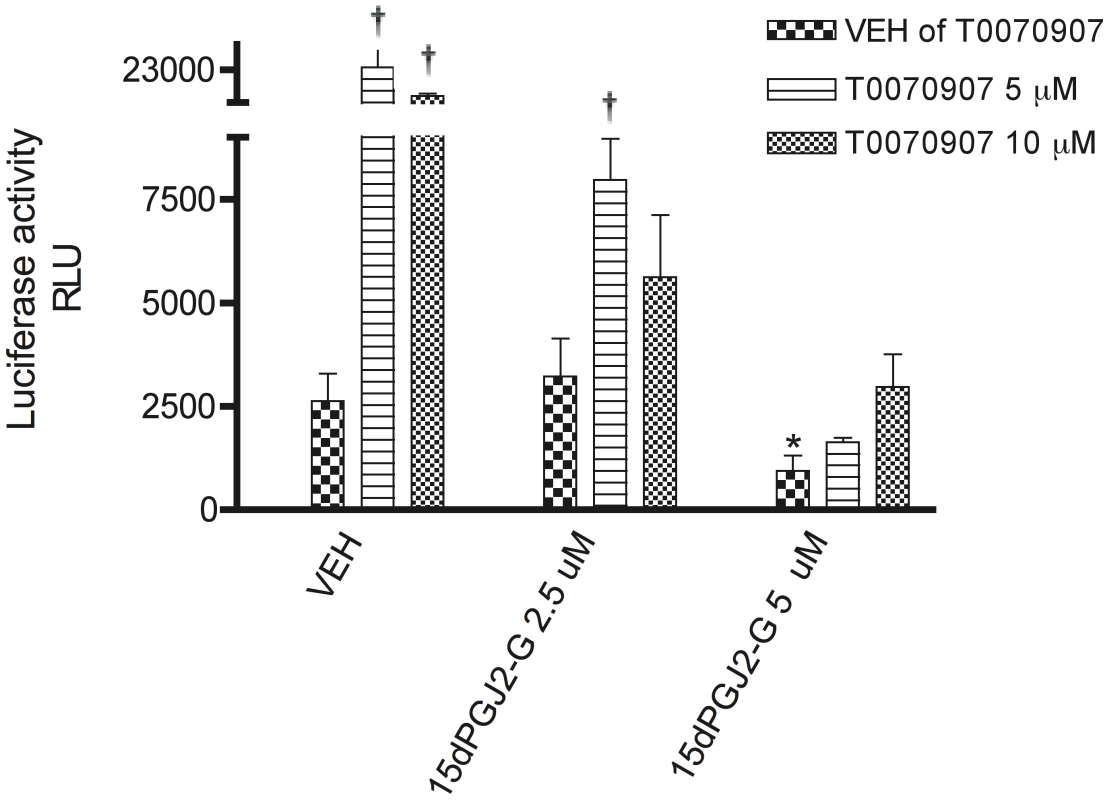


Figure 25. NFAT reporter activity in Jurkat cells treated with 15d-PGJ$_2$-G in the presence of a PPARγ antagonist, T0070907.

Jurkat cells (5 X 10^5 c/ml) were transiently transfected with NFAT luciferase reporter using Lipofectamine 2000 for 4 h in RPMI 1640 medium with 2% BCS in a 48 well plate. After the 4 h incubation, the cells were treated with vehicle of the antagonist (0.02% DMSO) or T0070907 (5 and 10 μM) for 30 min. Then the cells were treated with vehicle (VEH of agonist - 0.1% EtOH), or 15d-PGJ$_2$-G (2.5 – 5 μM) for 30 min followed by PMA/I$_o$ stimulation (P/I - 40 nM/0.5 μM). Twenty-four hours after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to (VEH of agonist + VEH of T0070907) and †, $p < 0.05$ compared to VEH for T0070907 within each treatment group. The results are representative of three separate experiments.

Figure 25. (cont'd)



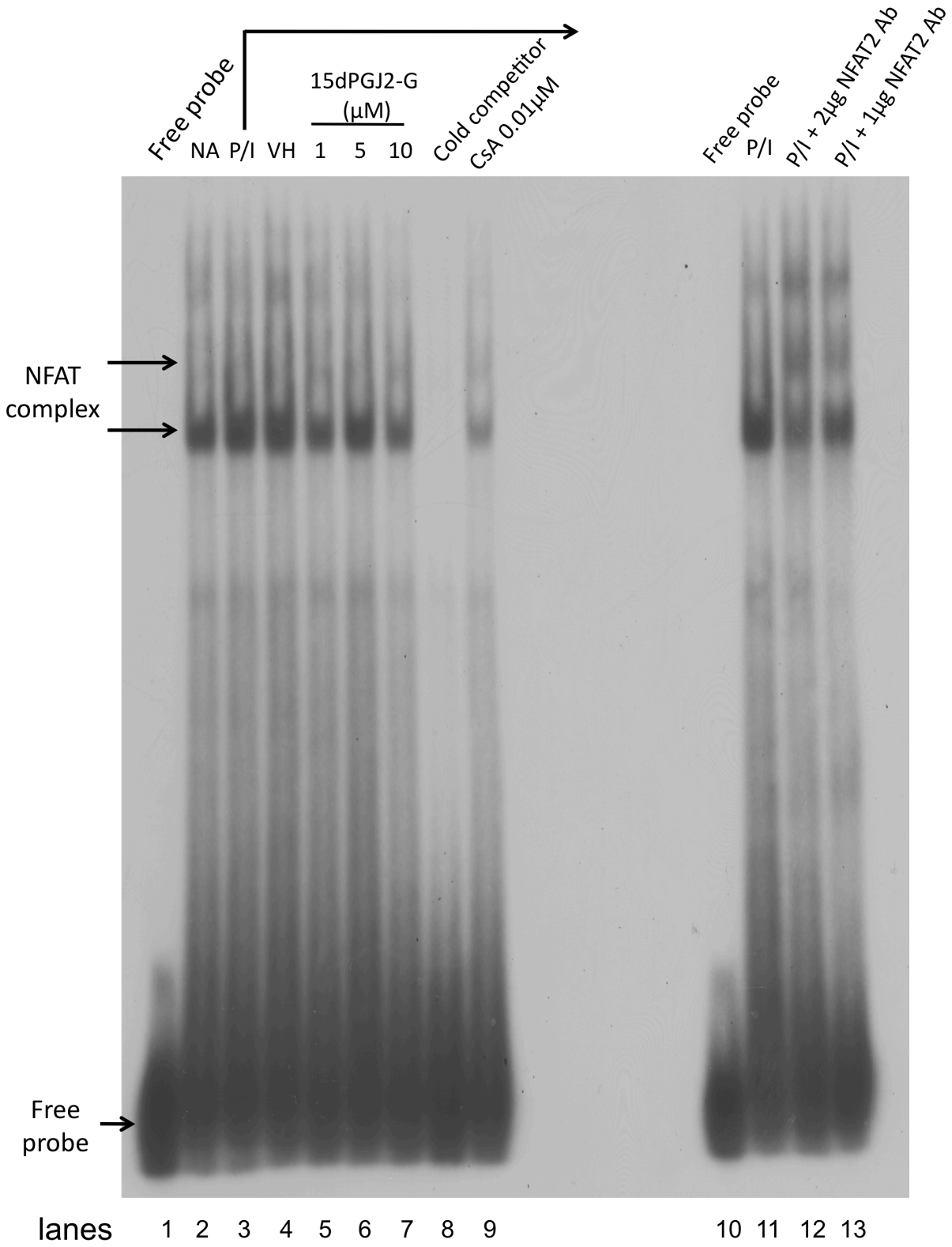
VIII. 15d-PGJ_2-G decreases NFAT-DNA binding activity in activated Jurkat cells

Since 15d-PGJ_2-G decreased IL-2 secretion and NFAT transcriptional activity in activated Jurkat cells [295], the effect of 15d-PGJ_2-G on NFAT-DNA binding in activated Jurkat cells was investigated. An NFAT oligonucleotide derived from human IL-2 promoter was utilized for the electrophoretic mobility shift assay. Two NFAT-DNA complexes were detected upon autoradiography (Figure 26, on page 98). Upon PMA/I_o stimulation for 30 min (lane 3), NFAT-DNA binding activity was increased as compared to resting cells (naive; NA-lane 2). Vehicle treatment (VH-lane 4) did not alter the PMA/I_o-stimulated NFAT-DNA binding. Pretreatment with 15d-PGJ_2-G (1, 5, 10 μM-lanes 5, 6 and 7) followed by PMA/I_o stimulation decreased NFAT-DNA binding. When a cold competitor (100 fold molar excess-lane 8) was included in the binding reaction, all the three bands corresponding to NFAT-DNA complexes disappeared, indicating specificity. CsA is a calcineurin inhibitor that causes decreased NFAT translocation in to the nucleus [294] and here demonstrated inhibition of PMA/I_o-stimulated NFAT-DNA binding activity as expected. To confirm the identity of the observed NFAT-DNA complexes, a super shift assay was performed. When the PMA/I_o-stimulated sample was preincubated with anti-NFAT2 antibody (2 μg and 1 μg – lanes 12 and 13 respectively) before the binding reaction, there was a shift in the lower band and the upper and middle bands intensified, confirming that the detected complexes contain NFAT.

Figure 26. NFAT2 DNA binding activity at human IL-2 promoter in activated Jurkat cells when treated with 15d-PGJ_2-G.

Jurkat cells (2.5×10^7) were pretreated with VH (0.1% EtOH –lane 4), 15d-PGJ_2-G (1, 5 and 10 μM – lanes 5, 6 and 7) or CsA (0.01 μM – lane 9) for 30 min and then stimulated with PMA/I_o (40 nM/0.5 μM) for 30 min at 37°C. In addition, the basal level of NFAT DNA binding was measured in stimulated cells (lane 2). The nuclear proteins (1 μg) were resolved by EMSA as described in the Materials and Methods. Arrows, NFAT/DNA complex or free probe, respectively. Lane 1, radiolabeled probe alone, Lane 3, PMA/I_o treated cells and lane 8, included 100-fold molar excess of the unlabeled NFAT oligonucleotide as a competitor using the same protein as loaded in lane 3. Lanes 10-13, supershift analysis of NFAT-DNA binding activity at human IL-2 promoter. Lane 10, radiolabeled probe alone, Lane 11, PMA/I_o treated cells. Lane 12 and 13, preincubated anti-NFAT2 antibody (2 μg and 1 μg respectively) for 1 h along with the same protein as loaded in lane 11. The data are representative of three independent experiments.

Figure 26. (cont'd)



IX. 15d-PGJ_2-G causes a decrease in NFAT2 and PPARγ localization in the nucleus of activated Jurkat cells

Since a decrease in the NFAT-DNA binding was observed with 15d-PGJ_2-G, NFAT2 localization was further investigated. The localization of PPARγ was also investigated since 15d-PGJ_2-G acts as a ligand for PPARγ [295] (Figure 27a, on page 102). α-Tubulin was used as the cytosolic loading control and histone H1 was used as the nuclear loading control. The mutually exclusive identification of α-tubulin and histone H1 in cytosolic and nuclear fractions suggests there is little cross contamination. Ponceau staining of bands corresponding to histone also suggest the same. Interestingly, in resting cells (NA), PPARγ was predominantly localized in the cytosol compared to nucleus (Figure 27a, on page 102). It is important to note that 20X more protein was loaded per lane in the nuclear fraction. PMA/I_o stimulation alone or VH treatment followed by PMA/I_o stimulation did not significantly alter the PPARγ localization either in the nucleus or in the cytosol (Figure 27a & b, on page 102). When Jurkat cells were treated with 15d-PGJ_2-G prior to PMA/I_o stimulation, there was a decrease in the localization of PPARγ in both the nuclear and the cytosolic fraction (Figure 27a & b, on page 102).

With respect to NFAT2 localization, there was very little NFAT2 in the nucleus and high expression of NFAT2 in the cytosol in resting cells (NA). Upon PMA/I_o stimulation, there was increased NFAT2 in the nucleus and a corresponding decrease in the cytosolic NFAT2. Pretreatment with vehicle (VH) followed by PMA/I_o stimulation did not significantly alter the increased NFAT2 in the nucleus or the corresponding decrease in the cytosolic NFAT2 (Figure

27a & c, on page 102). Upon pretreatment with 15d-PGJ_2-G followed by PMA/I_o stimulation, there was a significant decrease in the nuclear and cytosolic NFAT2 (Figure 27a & c, on page 102).

Figure 27. Western blot analysis of NFAT2 and PPARγ protein levels in resting and activated Jurkat T cells pretreated with 15d-PGJ_2-G.

Jurkat T cells were either left untreated (NA) or treated with VH (0.1% EtOH) or 15d-PGJ_2-G (J2G 10 μM) for 30 min followed by treatment with PMA/I_o (P/I-40 nM/0.5 μM) for 30 min. The nuclear and cytosolic fractions from these treatment groups were obtained and analyzed by Western analysis (a). α-Tubulin was used a loading control for cytosolic fraction and histone H1 was used as a loading control for nuclear fraction. In addition, ponceau staining (corresponding to histone bands) also suggested very little cross contamination between the fractions. NFAT2 protein levels (b) and PPARγ protein levels (c) within each fraction were quantified by densitometric analysis and normalized to the loading controls. The results are expressed as fold change normalized to NA within each fraction. Statistical analysis was performed using Dunnett's test for each fraction. *, $p < 0.05$ when compared to NA within the respective fraction. †, $p < 0.05$ when compared to VH within the respective fraction. The data are representative of three independent experiments.

Figure 27. (cont'd)

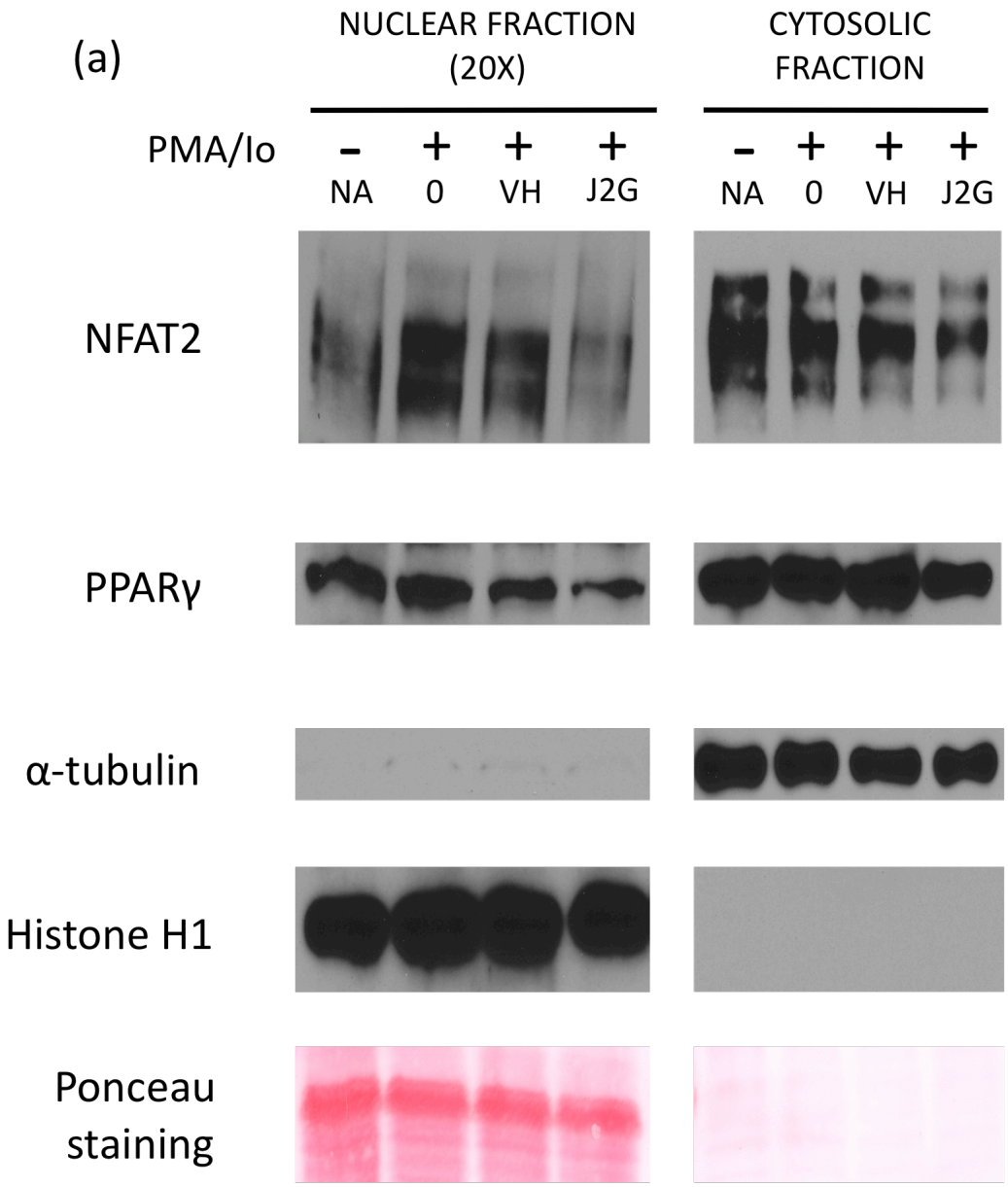
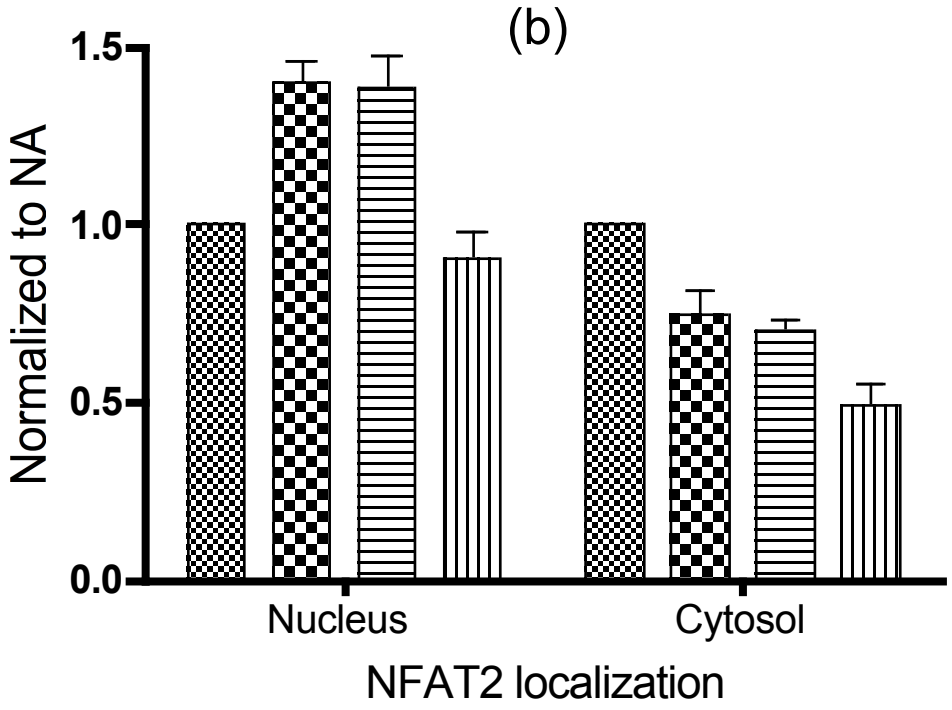
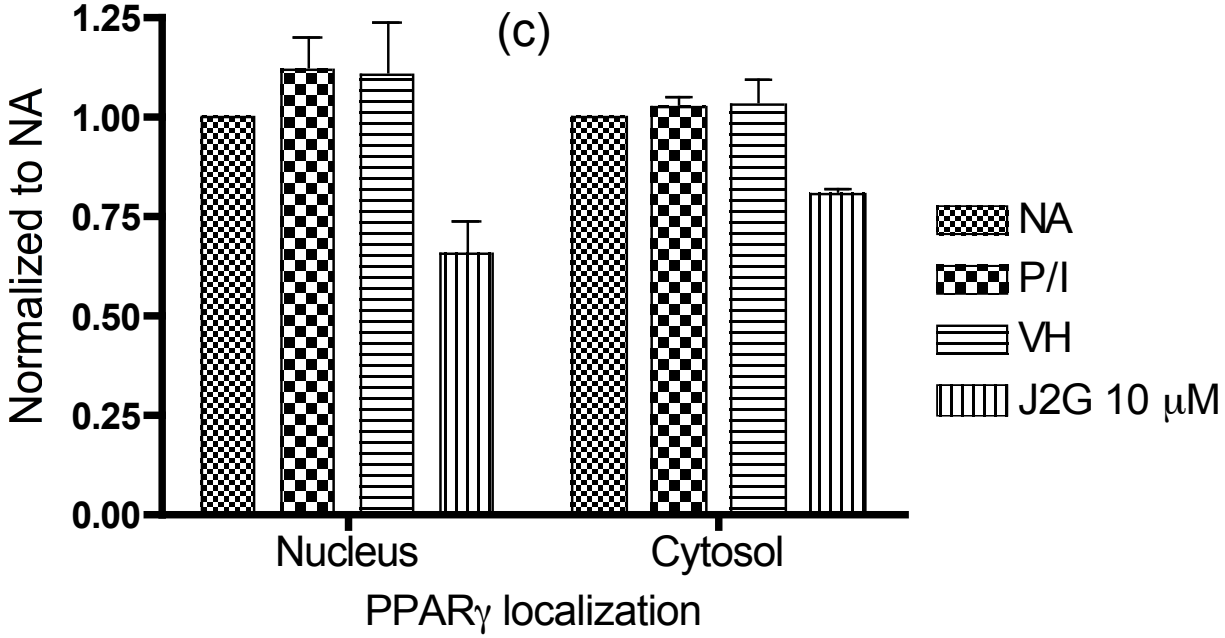


Figure 27. (cont'd)





X. 15d-PGJ$_2$-G does not alter the levels of the active form of GSK-3β in PMA/I$_o$-stimulated Jurkat cells

Expression levels of total GSK-3β and phospho GSK-3β (inactive form) were investigated to understand if GSK-3β was responsible for the observed decrease in nuclear NFAT2 accumulation in PMA/I$_o$-stimulated Jurkat cells pretreated with 15d-PGJ$_2$-G (Figure 27a & c, on page 102). Active GSK-3β acts as a maintenance kinase (keeps NFAT phosphorylated in the cytosol to retain it in the cytosol) as well as an export kinase (phosphorylate NFAT in the nucleus to cause its export in to the cytosol). In resting cells (NA), there was very little inactive GSK-3β (phospho GSK-3β) in the nucleus suggesting that GSK-3β was predominantly in the active form, thus facilitating NFAT2 export in to the cytosol (Figure 27a, on page 102 and Figure 28a & b, on page 107). Upon PMA/I$_o$ stimulation, there was an increase in the inactive GSK-3β (phospho GSK-3β) in the nucleus, suggesting that there was a decrease in the active GSK-3β, thus facilitating NFAT2 accumulation in the nucleus (Figure 27a, on page 102 and Figure 28a & b, on page 107). Pretreatment with vehicle (VH) or 15d-PGJ$_2$-G (J2G) followed by PMA/I$_o$ did not significantly alter the expression of either total GSK-3β or phospho GSK-3β, suggesting that there was no change in active GSK-3β compared to PMA/I$_o$ treated cells (Figure 27a, on page 102 and Figure 28a & b, on page 107). In addition, the nuclear to cytoplasmic ratio (N:C ratio) of total GSK-3β and phospho GSK-3β did not change with 15d-PGJ$_2$-G treatment (Figure 28c, on page 107).

XI. 15d-PGJ_2-G increases the levels of total HDM2 and the active form of HDM2 in the nucleus of activated Jurkat cells

There was decreased nuclear and cytosolic NFAT2 levels in the PMA/I_o-stimulated Jurkat cells that were pretreated with 15d-PGJ_2-G with no changes in the nuclear and cytosolic expression of GSK-3β (one of the important kinases that regulates nuclear NFAT accumulation). This observation suggested that ubiquitination might play a role in the decreased NFAT2 levels. The expression levels of both total HDM2 and phospho HDM2 (active form) were assessed as active HDM2 (E3 ubiquitin ligase) has been implicated in the ubiquitination of NFAT [250] (Figure 29a, on page 110). The expression of total HDM2 and active HDM2 (phospho form) was increased in the nucleus with a corresponding decrease in the cytosol upon 15d-PGJ_2-G pretreatment followed by PMA/I_o stimulation (Figure 29a & b, on page 110). This increase in the total and active HDM2 in the nucleus was further evident with the N:C ratio, suggesting that there was increased active HDM2 in the nucleus of 15d-PGJ_2-G treated activated Jurkat cells (Figure 29c, on page 110). This increased expression of HDM2 may be responsible for the ubiquitination of NFAT2 and might lead to decreased localization of NFAT2 in the nucleus of 15d-PGJ_2-G treated PMA/I_o-stimulated cells (Figure 27a, on page 102).

Figure 28. Western blot analysis of total GSK-3β and phospho GSK-3β protein levels in resting and activated Jurkat T cells pretreated with 15d-PGJ_2-G.

Jurkat T cells were either left untreated (NA) or treated with VH (0.1% EtOH) or 15d-PGJ_2-G (J2G 10 μM) for 30 min followed by treatment with PMA/I_o (P/I-40 nM/0.5 μM) for 30 min. The nuclear and cytosolic fractions from these treatment groups were obtained and analyzed by Western analysis (a). α-Tubulin (from Figure 27) was used a loading control for cytosolic fraction and histone H1 (from Figure 27) was used as a loading control for nuclear fraction. Total GSK-3β (b, top panel) and phospho GSK-3β (b, bottom panel) protein levels within each fraction were quantified by densitometric analysis and normalized to the loading controls (b). The results are expressed as fold change normalized to NA within each fraction. The nuclear to cytoplasmic ratio (N:C ratio) of total GSK-3β (c, top panel) and phospho GSK-3β (c, bottom panel) was calculated (c). The data are representative of two independent experiments.

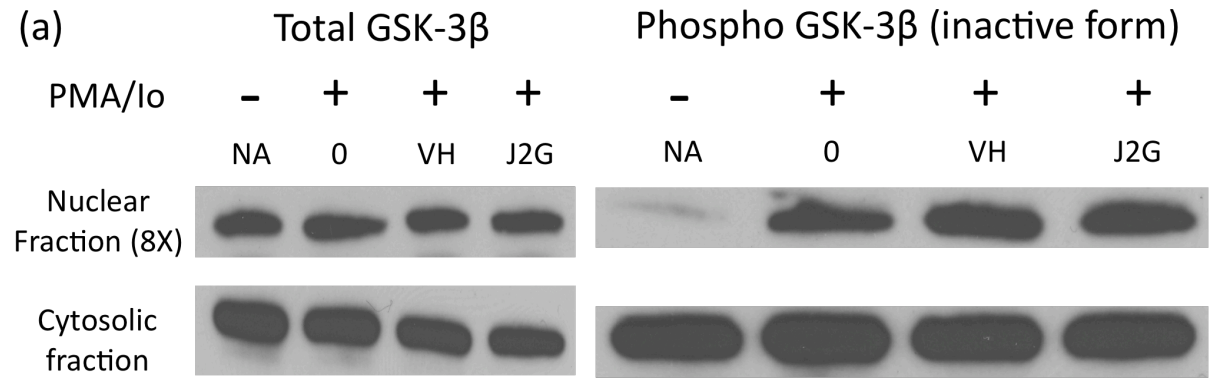
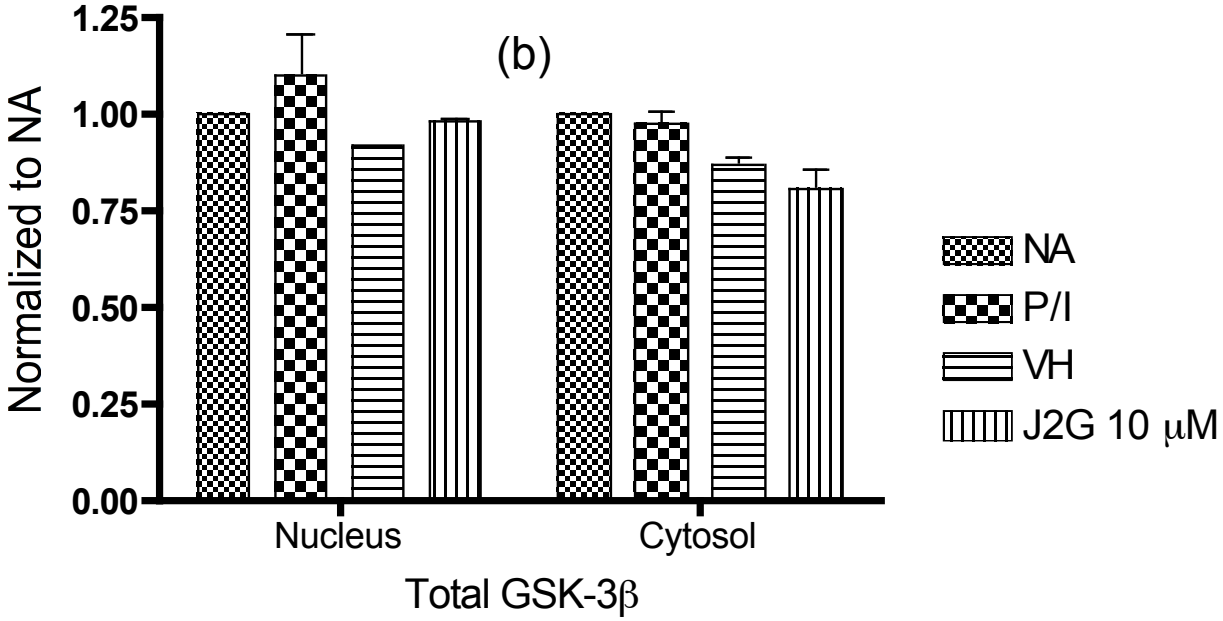


Figure 28. (cont'd)



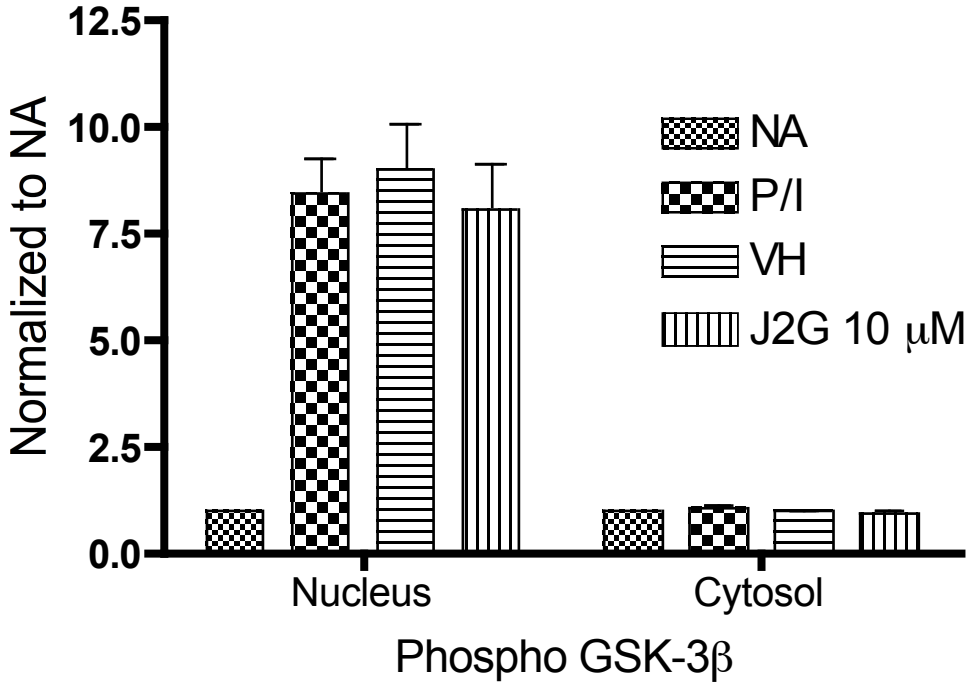


Figure 28. (cont'd)

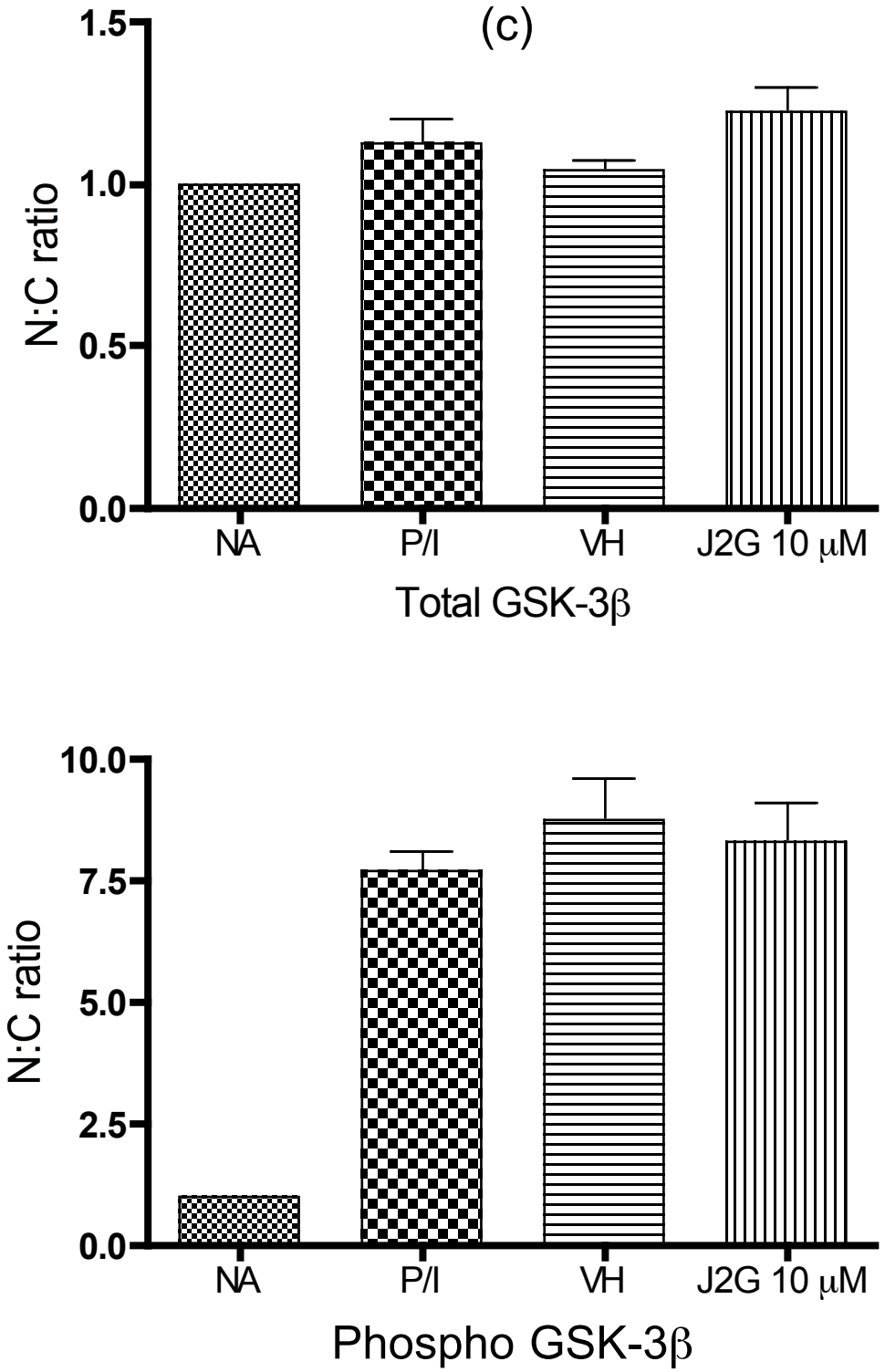


Figure 29. Western blot analysis of total HDM2 and phospho HDM2 protein levels in resting and activated Jurkat T cells pretreated with 15d-PGJ$_2$-G.

Jurkat T cells were either left untreated (NA) or treated with VH (0.1% EtOH) or 15d-PGJ$_2$-G (J2G 10 μM) for 30 min followed by treatment with PMA/I$_o$ (P/I-40 nM/0.5 μM) for 30 min. The nuclear and cytosolic fractions from these treatment groups were obtained and analyzed by Western analysis (a). α-Tubulin (from Figure 27) was used a loading control for cytosolic fraction and histone H1 (from Figure 27) was used as a loading control for nuclear fraction. Total HDM2 (b, top panel) and phospho HDM2 (b, bottom panel) protein levels within each fraction were quantified by densitometric analysis and normalized to the loading controls (b). The results are expressed as fold change normalized to NA within each fraction. The nuclear to cytoplasmic ratio (N:C ratio) of total HDM2 (c, top panel) and phospho HDM2 (c, bottom panel) was calculated (c). The data are representative of two independent experiments.

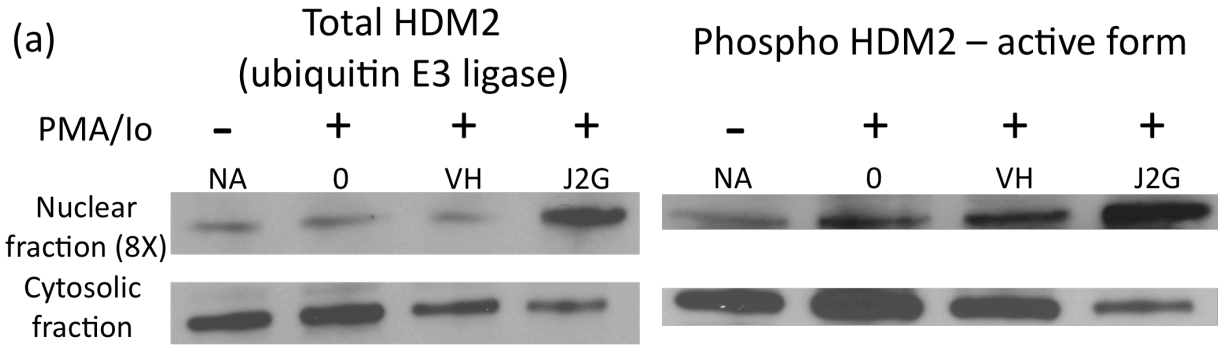


Figure 29. (cont'd)

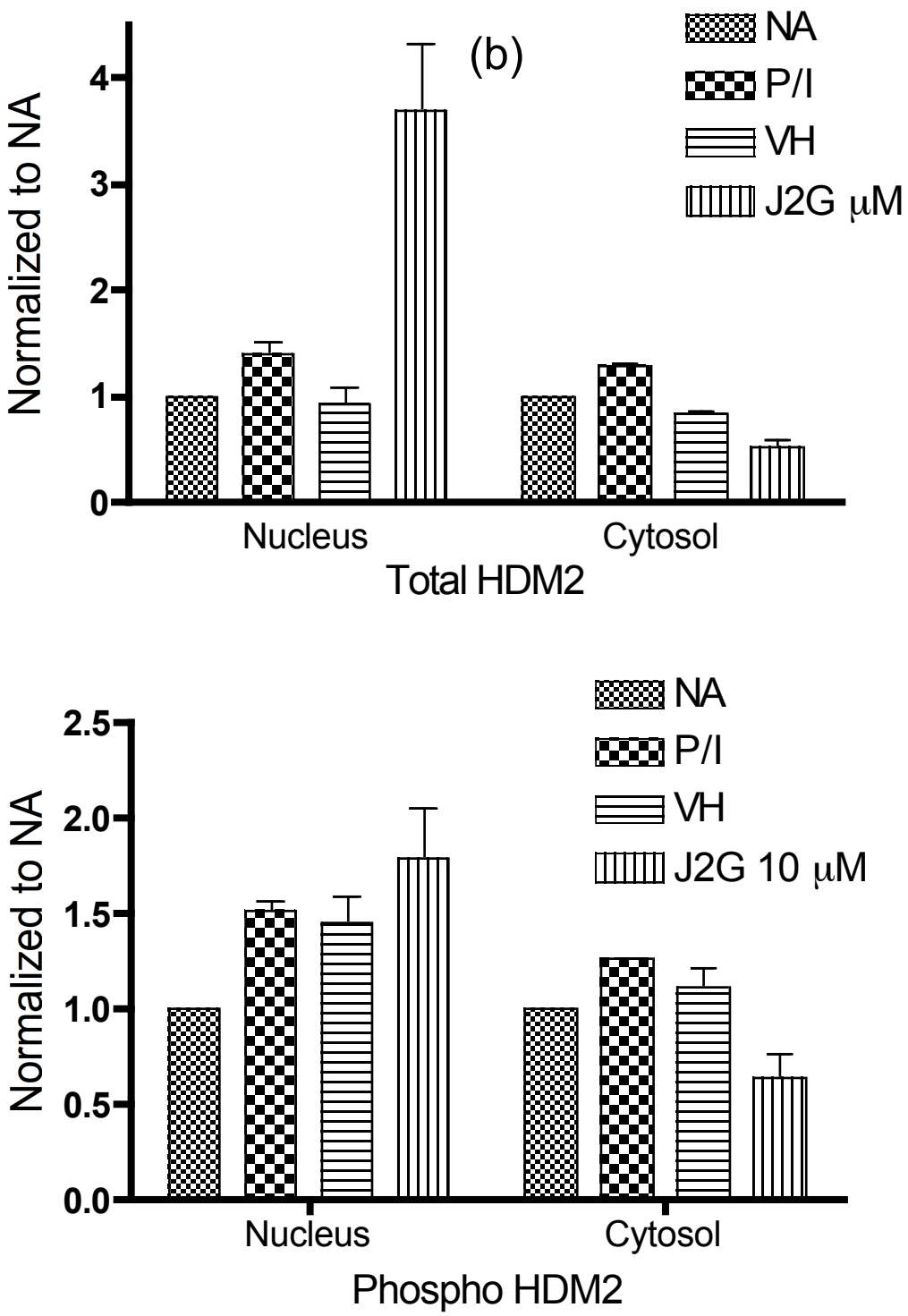
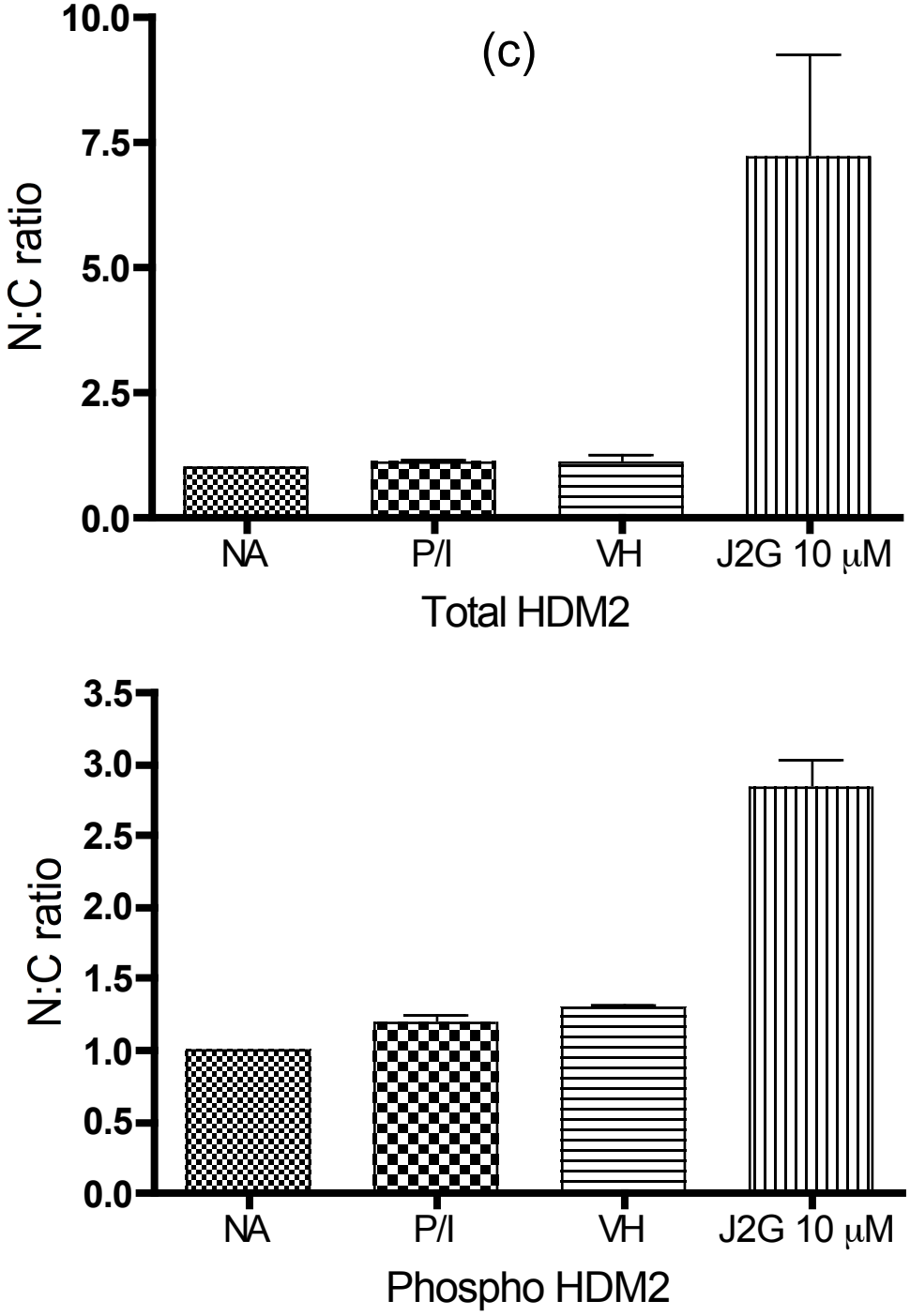


Figure 29. (cont'd)



XII. 15d-PGJ$_2$-G decreased PMA/Io-mediated increase in intracellular calcium in activated Jurkat cells and B6C3F1 splenocytes

Since changes in the level of intracellular calcium (by regulating calcineurin activation) is a contributing mechanism by which NFAT translocation into the nucleus is modulated, the effect of 15d-PGJ$_2$-G on PMA/I$_o$-mediated elevation in intracellular calcium in activated Jurkat cells was investigated. 15d-PGJ$_2$-G decreased PMA/I$_o$-mediated elevation in intracellular calcium in Jurkat cells (Figure 30a, on page 115). 15d-PGJ$_2$-G also decreased PMA/I$_o$-mediated elevation in intracellular calcium in mouse splenocytes, (Figure 30b, on page 115), confirming the results in primary cells. Two well-studied PPARγ agonists, CGZ and RGZ, also decreased PMA/I$_o$-mediated elevation in intracellular calcium in activated Jurkat cells (Figure 31a & b, on page 117).

XIII. PPARγ antagonists, T0070907 and GW9662, decreased PMA/Io-mediated increase in intracellular calcium in activated Jurkat cells

Since 15d-PGJ$_2$-G and the other known PPARγ agonists such as RGZ and CGZ decreased intracellular calcium in PMA/I$_o$-activated Jurkat cells, we investigated if this decrease was dependent on PPARγ using the antagonists, T0070907 and GW9662. Both the antagonists, T0070907 and GW9662, alone decreased PMA/I$_o$-mediated elevation in intracellular calcium in activated Jurkat cells (Figure 32a & b, on page 119). In addition, when activated Jurkat cells were pretreated with both T0070907 and 15d-PGJ$_2$-G, there seemed to be an additive effect

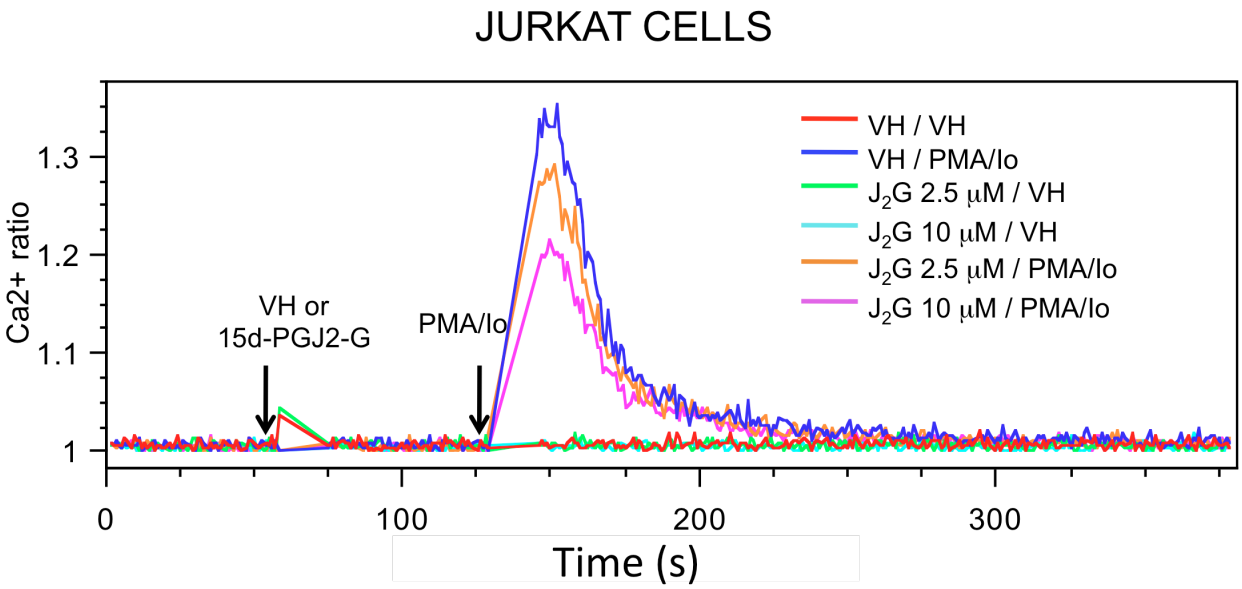
(decrease in intracellular calcium caused by both antagonist and agonist) on the decrease in PMA/I_o-mediated elevation in intracellular calcium (Figure 32c, on page 119). Furthermore, when activated Jurkat cells were pretreated with both T0070907 and RGZ/CGZ, there was an additive effect (decrease in intracellular calcium caused by both antagonist and agonist) on the decrease in PMA/I_o-mediated increase in intracellular calcium (Figure 33, on page 121).

Figure 30. The effect of 15d-PGJ$_2$-G, CGZ and RGZ on PMA/I$_o$-mediated increase in intracellular calcium.

(a) In Jurkat cells (b) B6C3F1 splenocytes. A 500 μl aliquot of fura red and fluo-3 loaded cells (2 x 10^6 cells/ml) was treated with VH (0.1% EtOH) or 15d-PGJ$_2$-G at 60 s and PMA/I$_o$ (40 nM/0.5 μM) (VH of PMA/I$_o$ – 0.1% DMSO) was added at 135 s and the intracellular calcium was measured up to 360 s. Arrows indicate the addition of PPARγ ligands and PMA/I$_o$. Intracellular calcium changes are presented as the ratio intensity of Fluo3/Fura Red vs. time. The calcium traces are representative of three independent experiments.

Figure 30. (cont'd)

(a)



(b)

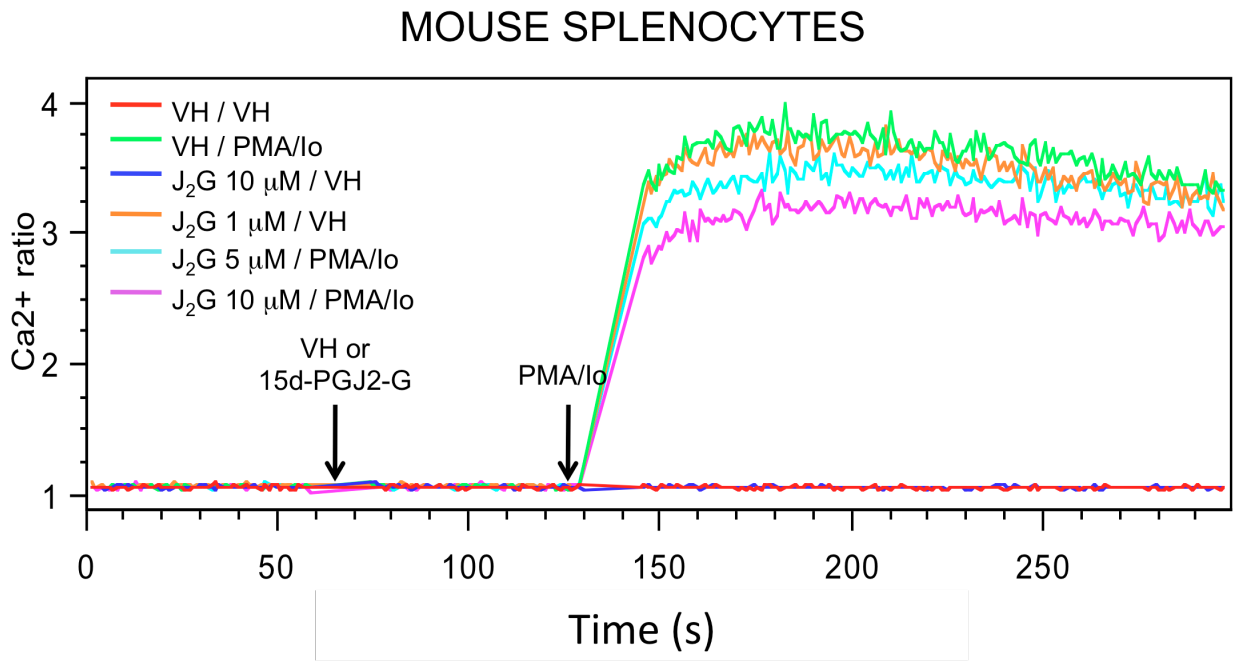
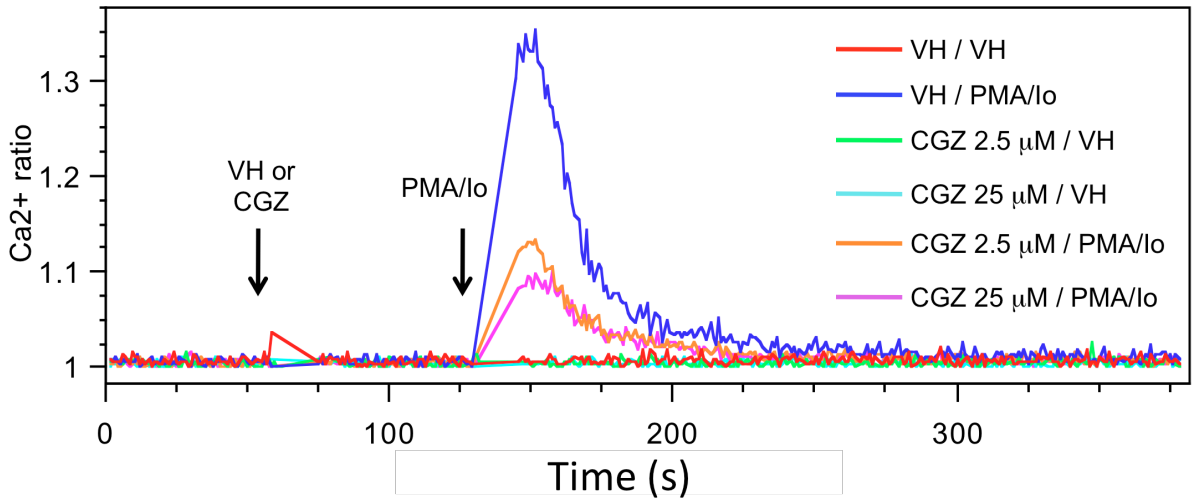


Figure 31. The effect of CGZ and RGZ on PMA/I_o-mediated increase in intracellular calcium in Jurkat cells.

A 500 μl aliquot of fura red and fluo-3 loaded Jurkat cells (2 x 10^6 cells/ml) was treated with VH (0.1% EtOH), (a) CGZ (2.5 and 25 μM) or (b) RGZ (2.5 μM) at 60 s and PMA/I_o (40 nM/0.5 μM) (VH of PMA/I_o – 0.1% DMSO) was added at 135 s and the intracellular calcium was measured up to 360 s. Intracellular calcium changes are presented as the ratio intensity of Fluo3/Fura Red vs. time. The calcium traces are representative of three independent experiments.

Figure 31. (cont'd)

(a)



(b)

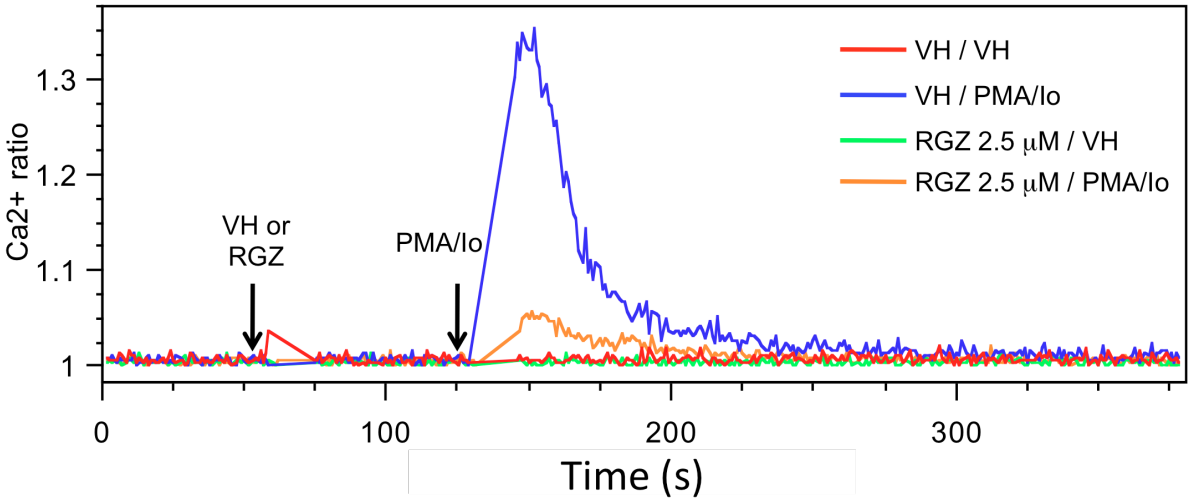


Figure 32. The effect of T00709707 (a) and GW9662 (b) on PMA/I_o-mediated increase in intracellular calcium in Jurkat cells and (c) the effect of T0070907 on 15d-PGJ_2-G-mediated decrease in PMA/I_o-activated Jurkat cells.

A 500 μl aliquot of fura red and fluo-3 loaded Jurkat cells (2 x 10^6 cells/ml) was treated with VH (0.02% DMSO), (a) T0070907 (100 nM, 1 μM and 5 μM) or (b) GW9662 (100 nM, 1 μM and 5 μM) at 60 s and VH (0.1% EtOH) or 15d-PGJ_2-G (c) at 135 s followed by PMA/I_o (40 nM/0.5 μM) (VH of PMA/I_o – 0.1% DMSO) at 210 s and the intracellular calcium was measured up to 360 s. Intracellular calcium changes are presented as the ratio intensity of Fluo3/Fura Red vs. time. The calcium traces are representative of three independent experiments.

Figure 32. (cont'd)

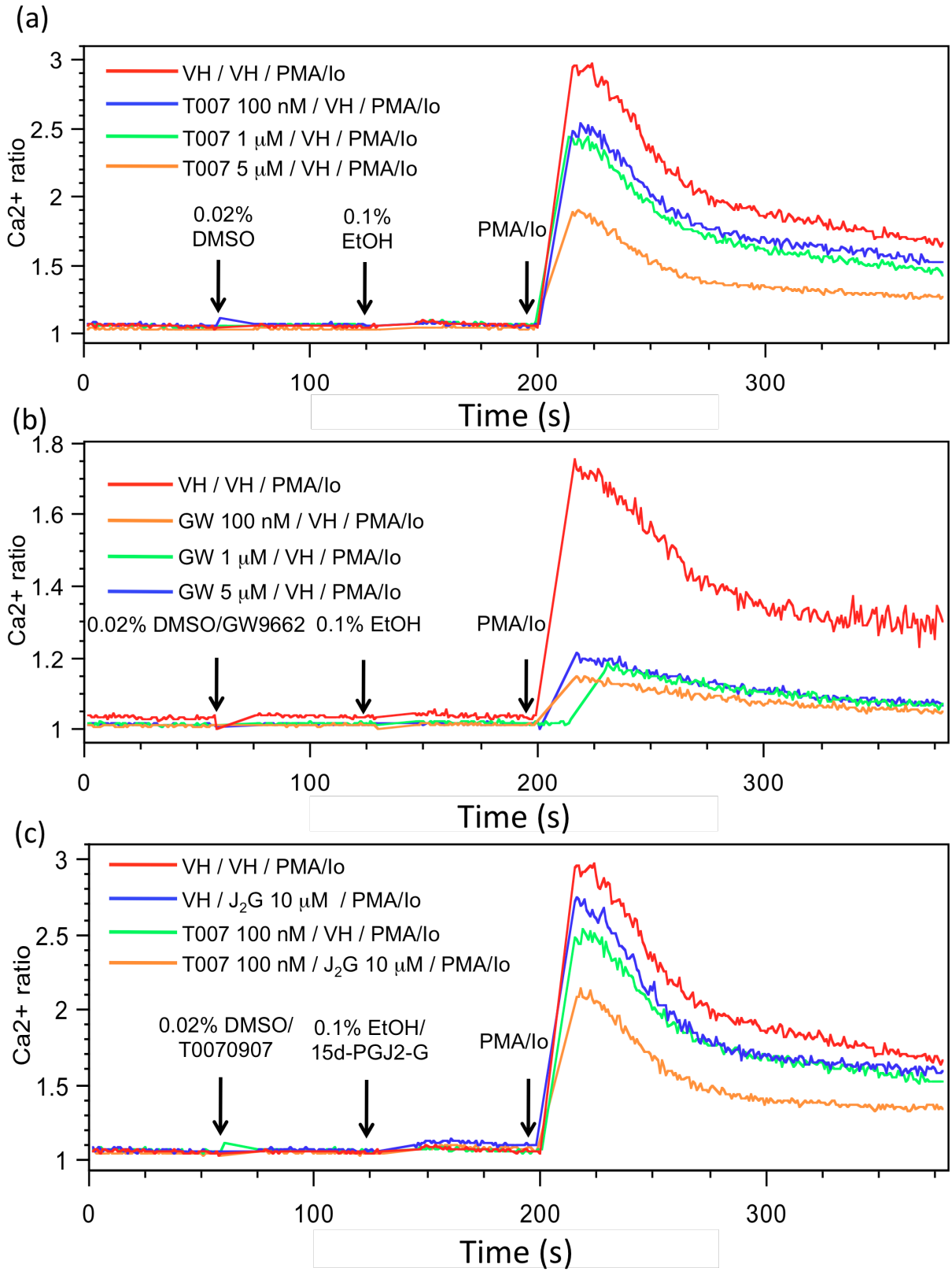
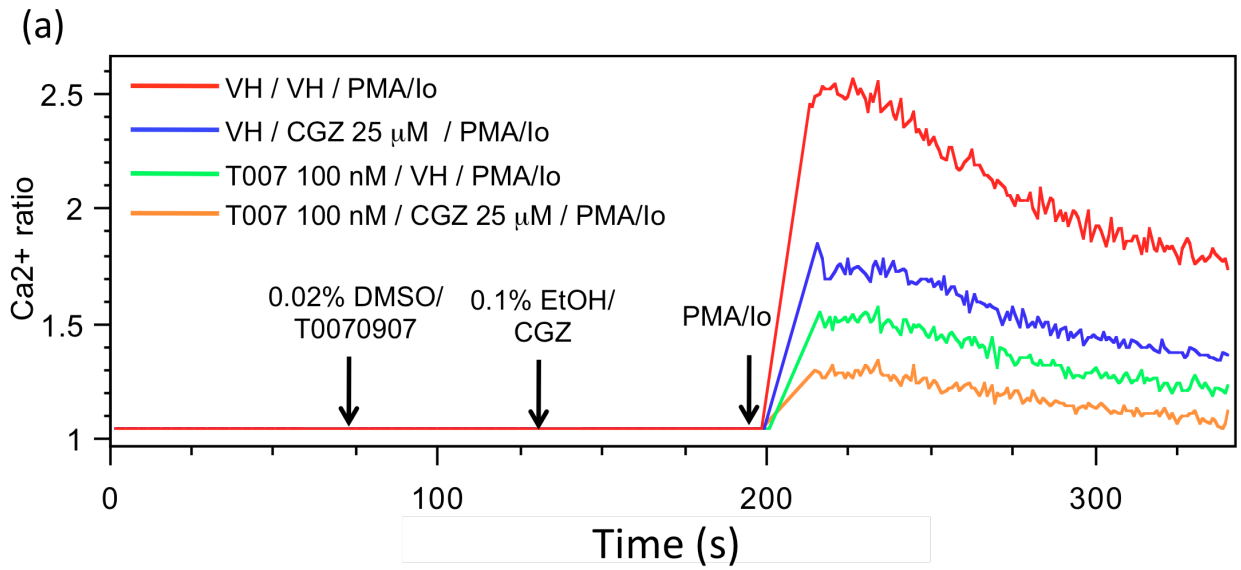
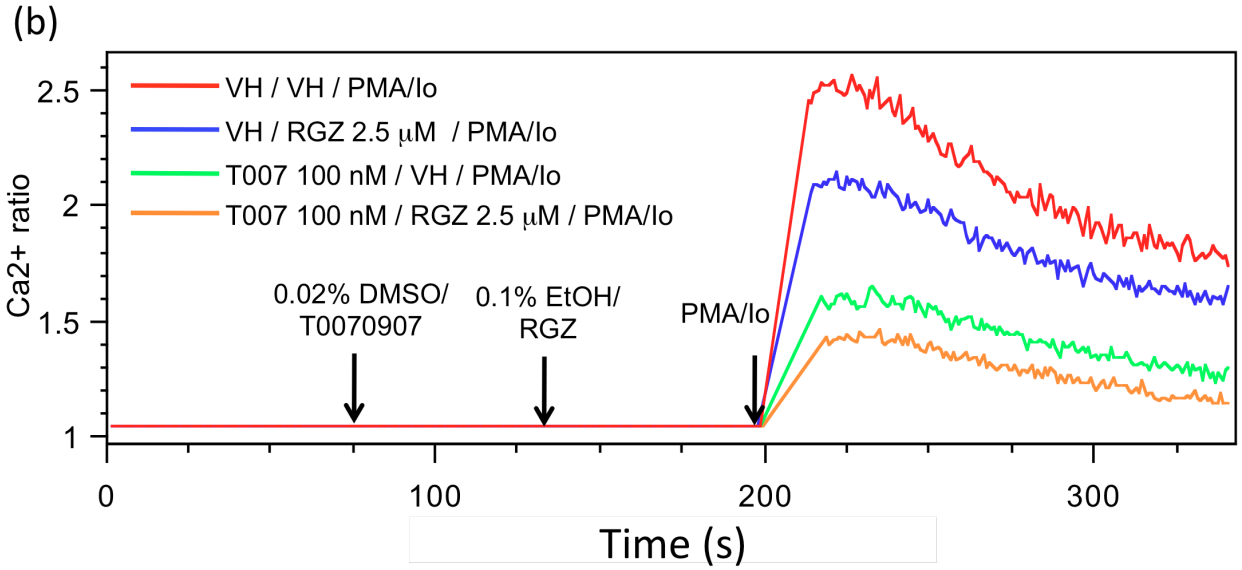


Figure 33. The effect of T0070907 on CGZ/RGZ-mediated decrease in PMA/I_o-activated Jurkat cells.

A 500 μl aliquot of fura red and fluo-3 loaded Jurkat cells (2 x 10^6 cells/ml) was treated with VH (0.02% DMSO) or T0070907 (100 nM, 1 μM and 5 μM) at 60 s and VH (0.1% EtOH), (a) CGZ or (b) RGZ at 135 s followed by PMA/I_o (40 nM/0.5 μM) (VH of PMA/I_o – 0.1% DMSO) at 210 s and the intracellular calcium was measured up to 360 s. Intracellular calcium changes are presented as the ratio intensity of Fluo3/Fura Red vs. time. The calcium traces are representative of three independent experiments.

Figure 33. (cont'd)





DISCUSSION

I. Role of COX-2 in 2-AG mediated IL-2 suppression

COX-2 is the rate-limiting enzyme in the metabolism of 2-AG. We demonstrated that there is a robust increase in COX-2 mRNA and protein expression upon T cell activation [156] suggesting that increased COX-2 expression may lead to increased 2-AG metabolism leading to increased production of COX-2 metabolites, including 15d-PGJ_2-G that binds to PPARγ. Treatment with 2-AG attenuated the increase in COX-2 mRNA caused by T cell activation implicating the existence of a negative feedback loop that decreases COX-2 expression after cellular activation. This decrease in COX-2 expression may lead to decreased generation of metabolites, and thus may be limiting IL-2 suppression that is mediated by the metabolite(s). The negative feedback loop may be either PPARγ dependent or independent. There is evidence for inhibition of COX-2 by PPARγ ligands in human cervical cancer cells and it appears to be mediated predominantly through impairment of AP-1 protein binding to the cAMP response element site in the COX-2 promoter [296]. In macrophage-like differentiated U937 cells treated with LPS, 15d-PGJ_2 treatment caused a decrease in COX-2 mRNA expression and COX-2 promoter activity by interfering with the NF-κB signaling pathway, implicating the existence of a negative feedback loop between PPARγ activation and COX-2 expression [297]. PPARγ ligands decreased PMA-mediated induction of COX-2 transcription in a concentration-dependent manner in human epithelial cells [298] and down-regulated COX-2 mRNA and protein expression in the human liver cancer cell line, HepG2 [299]. In contrast, there is also evidence that a prototypical peroxisome proliferator, WY-14,643, enhanced COX-2 expression in human mammary cells and colonic epithelial cells [300]. WY-14,643 is primarily an activator of

PPARα but it also activates PPARγ [301] and PPARβ to a lesser extent [302]. Perhaps the increase in COX-2 expression is due to the actions on PPARα and PPARβ. These results taken together suggest that PPARγ activation generally seems to inhibit COX-2 expression and this effect may be cell-type and/or tissue-type specific. In our study, COX-2 mRNA was decreased by 2-AG treatment beginning at 2 h, whereas PPARγ expression was upregulated only at 4 h. Therefore, the decrease in COX-2 mRNA expression may be independent of PPARγ at earlier time points and dependent or independent of PPARγ activation during the later time points. More studies are required to understand the molecular mechanisms involved in the negative feedback regulation of COX-2 by PPARγ.

2-AG is a substrate for COX-2 and there is considerable evidence suggesting the formation of glycerol esters of prostaglandins from 2-AG [121]). The diversity of prostaglandins obtained from AA and 2-AG may provide a unique repertoire of mediators customized for specific responses. It has been demonstrated that the glycerol esters of prostaglandins are in general more stable than the free acid prostaglandins, suggesting that the glycerol esters of prostaglandins may have a longer duration of action, albeit it is possible for 15d-PGJ_2-G to undergo hydrolysis to form 15d-PGJ_2 with time. However, these studies are novel in that they suggest that a metabolite rather than the parent molecule itself is responsible for aspects of the immunomodulatory activity mediated by 2-AG.

II. Role of PPARγ and NFAT in 2-AG-mediated IL-2 suppression in activated T cells

The expression of PPARγ1 has been established in T cells and its activation has been correlated with IL-2 suppression [283]. Many endogenous ligands of PPARγ have been

identified, such as 15d-PGJ_2 [153], 5-S-hydroxyeicosatetraenoic acid (15-HETE) [277], polyunsaturated fatty acids [303], 13-oxooctadecadienoic acid (13-OXO) [304], 2,4-dienone 13-oxooctadecadienoic acid (13-Oxo-ODE) [305] and components of oxidized low-density lipoprotein, such as 9-hydroxyoctadecadienoic acid (9-HODE) and 13-S-hydroxyoctadecadienoic acid (13-HODE) [277]. Of these ligands, 15-HETE is produced by the action of 15-lipooxygenase (15-LOX) on AA whereas 15d-PGJ_2 is produced by the action of COX-2 on AA. We have previously demonstrated that pretreatment with a COX-2 specific inhibitor, NS398, attenuated the 2-AG-mediated IL-2 suppression in activated Jurkat cells [156]. Therefore, we focused our studies on the putative COX-2 metabolite of 2-AG, 15d-PGJ_2-G, which is structurally similar to the known endogenous PPARγ agonist, 15d-PGJ_2.

The activation of a PPARγ-specific Gal4-responsive reporter by 15d-PGJ_2-G in HEK293T and Jurkat T-Ag cells suggests that this putative metabolite can act as a PPARγ agonist. Importantly, 15d-PGJ_2-G bound PPARγ-LBD with high affinity as evidenced by the IC_{50} value (367.5 nM) compared to that of 2-AG (13.5 μM). This observation implicates that 15d-PGJ_2-G is a higher affinity PPARγ agonist than the parent molecule, 2-AG. In addition, the rank order for the IC_{50} of PPARγ agonists is RGZ > 15d-PGJ_2-G > CGZ. These IC_{50} values are concordant with the reported EC_{50} values for RGZ (60 ± 4 nM) and CGZ (3.0 ± 0.7 μM) as evidenced by transactivation assays in CV-1 cells transfected with a chimera consisting of the PPARγ-LBD fused to the Gal4 DNA-binding domain, together with a reporter plasmid containing a GAL4-responsive promoter driving expression of chloramphenicol transferase

[306]. Furthermore, 15d-PGJ_2-G also produced a concentration-dependent decrease in IL-2 secretion and this decrease was pronounced if 15d-PGJ_2-G was present surrounding the time of T cell activation. 15d-PGJ_2-G-mediated IL-2 suppression was partially attenuated in the presence of T0070907, a PPARγ antagonist, suggesting the involvement of PPARγ in the IL-2 suppression. The present studies are the first to demonstrate that a putative metabolite of 2-AG that is downstream of COX-2 metabolism, 15d-PGJ_2-G, might be responsible for the IL-2 suppression observed by 2-AG. The ability of 15d-PGJ_2-G to bind, activate PPARγ-LBD and suppress IL-2 secretion in activated Jurkat cells strongly suggests that PPARγ activation is involved in IL-2 suppression. 15d-PGJ_2-G must be present early during T cell activation to robustly suppress IL-2 implicating that PPARγ activation interferes with molecular events active early during IL-2 gene transcription. It has been demonstrated that NFAT can associate with PPARγ when activated with PPARγ agonists, troglitazone and 15d-PGJ_2 [271]. Concordantly, NFAT reporter assays showed decreased reporter activity in activated Jurkat cells when treated with 15d-PGJ_2-G and 2-AG [176]. Importantly, CGZ also decreased NFAT reporter activity suggesting that PPARγ activation leads to a decrease in NFAT transcriptional activity. Together, these results show that PPARγ activation by 15d-PGJ_2-G impairs NFAT function in IL-2 gene transcription.

It is noteworthy that in the NFAT reporter system, T0070907 markedly enhanced reporter activity suggesting the existence of endogenous PPARγ activity in activated T cells. Presumably, upon treatment with T0070907, endogenous PPARγ activity is antagonized leading

to increased function/release of NFAT that is reflected as increased reporter activity. In agreement with these results, there is a modest increase in IL-2 production in activated Jurkat cells when treated with T0070907 alone at 1 μM. It is consistent with the observed increase in NFAT activity. In another study that is concordant with these results, activated EL4.IL2 T cells treated with the PPARγ antagonist, GW9662, showed increased interferon γ (IFNγ) mRNA levels and protein expression [275]. It is notable that NFAT is also critical for IFNγ gene transcription [307]. This T0070907-mediated induction of NFAT transcriptional activity is in contrast with our previous studies in which T0070907 enhanced IL-2 production, but had no significant effect on NFAT reporter activity in activated Jurkat cells [176]. The discrepancy might be due to the fact that in the previous studies, T0070907 was present in the culture for only 12 h, whereas in these studies, T0070907 was present for 20 h.

We investigated the mechanisms responsible for decreased NFAT transcriptional activity in 15d-PGJ_2-G treated activated T cells. It is evident that 15d-PGJ_2-G decreased PMA/I_o-mediated increase in NFAT-DNA binding at the human IL-2 promoter in activated Jurkat cells. Since DNA binding of the NFAT is required for robust IL-2 gene transcription, the 15d-PGJ_2-G–mediated decrease in NFAT-DNA binding activity can result in decreased NFAT transcriptional activity and might lead to decreased IL-2 gene transcription.

Because 15d-PGJ_2-G acts as a PPARγ ligand, PPARγ localization was investigated. Surprisingly, we found that, in Jurkat cells, PPARγ was predominantly localized in the cytosolic fraction compared to the nuclear fraction. It is noteworthy that the nuclear fraction was loaded at 20X more protein than the cytosolic fraction. Although PPARγ has been reported to be predominantly present in the nucleus, there is also an earlier report for predominant cytosolic

localization of PPARγ in Jurkat cells [308]. Interestingly, PPARγ expression in the nucleus decreased in PMA/I_o-stimulated Jurkat cells pretreated with 15d-PGJ_2-G. Next, we investigated if the 15d-PGJ_2-G-mediated decrease in NFAT-DNA binding was mediated by a decrease in NFAT accumulation in the nucleus. Increased NFAT2 accumulation in the nucleus upon PMA/I_o stimulation (compared to NA cells) indicates robust activation of the Jurkat cells. As expected, with PMA/I_o stimulation, there was a corresponding decrease in NFAT2 localization in the cytoplasm. With 15d-PGJ_2-G treatment, there was a decrease in PMA/I_o-mediated increase in NFAT2 accumulation in the nucleus without a corresponding increase in NFAT2 localization in the cytoplasm. This observation lead us to investigate if the decreased NFAT2 accumulation in the nucleus with 15d-PGJ_2-G treatment followed by PMA/I_o stimulation was caused by decreased NFAT2 translocation in to the nucleus or due to another mechanism such as ubiquitination that contributes to the decreased NFAT2 accumulation in the nucleus and in the cytoplasm.

GSK-3β is a maintenance kinase that regulates NFAT nuclear export by regulating the phosphorylation state of NFAT [234]. We investigated if the decreased nuclear NFAT2 accumulation in PMA/I_o-stimulated Jurkat cells pretreated with 15d-PGJ_2-G was caused by changes in GSK-3β expression. There were no significant changes in the expression of total and inactive GSK-3β in PMA/I_o-stimulated cells pretreated with 15d-PGJ_2-G compared to the PMA/I_o-stimulated cells, suggesting that GSK-3β does not contribute to the decreased NFAT2 accumulation in 15d-PGJ_2-G treated PMA/I_o-stimulated Jurkat cells. The expression patterns of

GSK-3β also confirm that GSK-3β is not involved in the decreased NFAT2 in the nucleus since NFAT2 was decreased in both the nuclear and cytoplasmic fraction upon 15d-PGJ_2-G treatment in activated T cells.

Since a role for GSK-3β was ruled out in the decreased NFAT2 accumulation after 15d-PGJ_2-G treatment, the role of HDM2 (an ubiquitin E3 ligase) was investigated. Phosphorylation of HDM2 has been implicated in the ubiquitination of NFAT. HDM2 is regulated by Akt phosphorylation at a number of residues that control HDM2 protein stability and its nuclear-cytoplasmic shuttling [250, 309]. When PMA/I_o-activated Jurkat cells were treated with 15d-PGJ_2-G, there was an increase in both total HDM2 and phospho HDM2 (active form) in the nucleus. This increase in active HDM2 may be responsible for the ubiquitin tagging of NFAT2 in the nucleus and once NFAT2 is exported in to the cytosol, it can be degraded by cytosolic proteasomes.

It is also of interest to note that in PMA/I_o-stimulated Jurkat cells pretreated with 15d-PGJ_2-G, there was decreased PPARγ in both the nucleus and the cytosol. Interestingly, in addition to the activation of the receptor, PPARγ ligands also lead to the ubiquitination of the receptor [310]. Since ligand-activated PPARγ has been known to interact with NFAT [271], it is possible that 15d-PGJ_2-G-activated PPARγ is in association with NFAT, eventually leading to the degradation of both PPARγ and NFAT. Considering the expression patterns of HDM2 and GSK-3β, it is tempting to speculate that 15d-PGJ_2-G-mediated PPARγ activation primarily causes an increase in the expression of active HDM2 (E3 ubiquitin ligase) without a significant

change in the expression of GSK-3β, resulting in ubiquitin tagging of PPARγ and/or PPARγ:NFAT complex, eventually leading to the degradation of both PPARγ and NFAT.

III. Role of calcium in 15d-PGJ_2-G-mediated IL-2 suppression

In addition to the above-mentioned mechanism, we also examined the effect of 15d-PGJ_2-G on PMA/I_o-mediated increase in intracellular calcium in activated Jurkat cells since calcium levels can modulate calcineurin, a calcium dependent phosphatase that regulates NFAT translocation in the nucleus. 15d-PGJ_2-G caused a decrease in PMA/I_o-mediated elevation in intracellular calcium in activated Jurkat cells and murine splenocytes. Other well-studied PPARγ agonists such as RGZ and CGZ also decreased PMA/I_o-mediated elevation in intracellular calcium in activated Jurkat cells. Interestingly, PPARγ antagonists such as T0070907 and GW9662 also decreased PMA/I_o-mediated elevation in intracellular calcium in activated Jurkat cells, suggesting that the decrease in intracellular calcium in PMA/I_o-stimulated Jurkat cells may be independent of PPARγ activation. In addition, when 15d-PGJ_2-G was added in the presence of T0070907, there was a further attenuation in the expected rise in intracellular calcium. On the other hand, since five PPARγ ligands (agonists and antagonists) caused a decrease in intracellular calcium, it is also tempting to speculate that the decrease in intracellular calcium may be dependent on the occupation of the ligand binding domain of PPARγ and which then causes a confirmational change, allowing PPARγ to couple with the calcium channels. Pioglitazone and other non-thiazolidinedione PPARγ agonists such as GI 262570, GW 7845 and GW 1929 have been reported to inhibit L-type voltage-dependent calcium channel in freshly

isolated smooth muscle cells from mesenteric arteries and cause a decrease in intracellular calcium [311]. More investigation is required to understand whether this decrease in intracellular calcium is independent/dependent of PPARγ and if dependent, how and which calcium channels are modulated by PPARγ activation. It is also important to investigate if 15d-PGJ_2-G-mediated decrease in intracellular calcium significantly contributes to decrease in nuclear NFAT2 accumulation and to the decrease in IL-2 secretion.

Although the contribution of 15d-PGJ_2-G and PPARγ to the decrease in intracellular calcium is not clearly understood, 15d-PGJ_2-G-mediated decrease in NFAT transcriptional activity is dependent on PPARγ as evidenced by the reversal of the decrease in NFAT transcriptional activity in the presence of a PPARγ antagonist, T0070907 [295]. In addition, 15d-PGJ_2-G-mediated IL-2 suppression in activated Jurkat cells was attenuated in the presence of the PPARγ antagonist, T0070907, although it was a partial reversal of IL-2 suppression (the presence of the PPARγ antagonist did not attenuate IL-2 suppression back to the levels of PMA/I_o stimulation alone). This partial reversal may be due to the fact that T0070907 alone decreases intracellular calcium significantly and in the presence of 15d-PGJ_2-G, there is an additive decrease in intracellular calcium. The partial attenuation of 15d-PGJ_2-G-mediated IL-2 suppression may be a net effect of PPARγ antagonism on 15d-PGJ_2-G-mediated decrease in nuclear NFAT accumulation (via active HDM2) and T0070907-mediated decrease in intracellular calcium.

IV. Summary

The studies reported in this dissertation suggest that 15d-PGJ_2-G, a putative COX-2 metabolite of 2-AG that binds PPARγ, mediates IL-2 suppression by the activation of PPARγ and by modulating NFAT activity. Since 2-AG-mediated IL-2 suppression was dependent on COX-2, we investigated the expression levels of COX-2 upon PMA/I_o stimulation. COX-2 expression was significantly higher upon PMA/I_o stimulation compared to resting Jurkat cells. In addition, 2-AG decreased the PMA/I_o-mediated increase in COX-2 mRNA levels whereas 2-AG did not change the PMA/I_o-mediated increase in PPARγ mRNA levels in Jurkat cells, suggesting that a negative feedback exists to limit the amount of COX-2 metabolites of 2-AG. 15d-PGJ_2-G, activated PPARγ-LBD in HEK293T and Jurkat T-Ag cells. In addition, 15d-PGJ_2-G bound to PPARγ-LBD in a PPARγ competitive binding assay along with other PPARγ agonists such as RGZ and CGZ and endocannabinoids such as 2-AG and AEA. The rank order of potency (based on IC_{50} values) was found to be RGZ > 15d-PGJ_2-G > CGZ > 2-AG > AEA suggesting that 15d-PGJ_2-G, the COX-2 metabolite of 2-AG, binds to PPARγ rather than the parent molecule (2-AG) itself. 15d-PGJ_2-G suppressed IL-2 secretion in activated Jurkat cells in a concentration and time-dependent manner, suggesting that 15d-PGJ_2-G has to be present surrounding the activation stimulus for robust IL-2 suppression. In addition, a PPARγ antagonist, T0070907, attenuated 15d-PGJ_2-G-mediated IL-2 suppression in activated Jurkat cells, suggesting that PPARγ is involved in 15d-PGJ_2-G-mediated IL-2 suppression. Further

investigation of 15d-PGJ_2-G-mediated IL-2 suppression revealed that 15d-PGJ_2-G caused a decrease in NFAT transcriptional activity in activated Jurkat cells, which was attenuated in the presence of PPARγ antagonist. Additionally, 15d-PGJ_2-G decreased NFAT-DNA binding activity in an electrophoretic mobility shift assay. 15d-PGJ_2-G decreased NFAT2 and PPARγ localization in the nucleus of activated Jurkat cells. Interestingly, 15d-PGJ_2-G treatment increases active HDM2 (E3 ubiquitin ligase) in the nucleus of activated Jurkat cells, whereas there was no change in the expression of GSK-3β. Increased active HDM2 in the nucleus could mediate increased ubiquitination of NFAT and/or NFAT:PPARγ complex (as liganded PPARγ has been known to interact with NFAT and ligand activation of PPARγ has been reported to increase PPARγ ubiquitination as well). Once the ubiquitinated proteins are exported out of the nucleus, it is possible for the cytosolic proteasomes to degrade them.

15d-PGJ_2-G decreased a PMA/I_o-mediated increase in intracellular calcium in both Jurkat cells and B6C3F1 splenocytes. In addition, other known PPARγ agonists such as RGZ and CGZ also decreased a PMA/I_o-mediated increase in intracellular calcium. But PPARγ antagonists such as T0070907 and GW9662 also decreased the PMA/I_o-mediated increase in intracellular calcium in Jurkat cells suggesting that 15d-PGJ_2-G-mediated decrease in intracellular calcium may be PPARγ independent. Since five PPARγ ligands (including three agonists and two antagonists) decrease PMA/I_o-mediated increase in intracellular calcium, it is difficult to exclude the possibility that this effect is mediated through PPARγ. It is also tempting to speculate that the decrease in intracellular calcium may be dependent on the occupation of the

ligand binding domain of PPARγ and which then causes a confirmational change, allowing PPARγ to couple with the calcium channels. Interestingly, cross-talk between PPARγ activation and calcium signaling in human colorectal cancer cells was inferred from bioinformatic analysis [312]. Further experiments are required to characterize the mechanism by which 15d-PGJ_2-G decreases intracellular calcium and if this decrease is intracellular calcium is relevant to IL-2 suppression.

The findings of this dissertation are relevant in that they suggest that 15d-PGJ_2-G, a putative metabolite of 2-AG, binds to PPARγ and activates it. Although 15d-PGJ_2 is known to act as a ligand for PPARγ, these studies are novel in that they demonstrate that 15d-PGJ_2-G, the glycerol ester of 15d-PGJ_2, is capable of binding and activating PPARγ. In addition, these studies indicate that 15d-PGJ_2-G, a putative COX-2 metabolite of 2-AG that binds to PPARγ, is involved in IL-2 suppression rather than the parent molecule (2-AG) itself. These studies also suggest that active HDM2 plays a critical role in the ubiquitination of NFAT and/or NFAT:PPARγ complex, leading to IL-2 suppression. Although PPARγ is thought to be localized predominantly in the nucleus, these studies have revealed the predominant cytosolic localization of PPARγ in Jurkat cells. These studies are also unique in that they illustrate that 15d-PGJ_2-G and other known PPARγ agonists and antagonists decreased the PMA/I_o-mediated elevation of intracellular calcium although it is yet to be determined if this effect is PPARγ dependent or independent. Although 2-AG carries out its biological function through CB1 and CB2, these studies implicate that metabolism of 2-AG does not result in termination of physiological

activity. 15d-PGJ_2-G, the putative COX-2 metabolite of 2-AG, can produce prolonged biological function by the activation of PPARγ. In addition, these studies provide evidence for cross-talk between PPARγ ligands and calcium signaling inferred earlier from bioinformatic analysis. Moreover, these studies suggest a novel mechanism by which NFAT accumulation in the nucleus may be decreased in activated T cells that are exposed to the increasing concentrations of 2-AG, eventually leading to IL-2 suppression.

In addition to IL-2 suppression, PPARγ has been shown to suppress the expression of proinflammatory cytokines such as tumor necrosis factor α, interleukin-1β and interleukin-6 [313, 314]. Further, PPARγ ligands can inhibit activation-induced production of the classical T_H1-cell cytokine, interferon γ, and IL-12 production by dendritic cells [276, 315] thus indicating that PPARγ might play a role during differentiation of naïve T cells into their effector subsets. Additionally, PPARγ blocks transforming growth factor-β/interleukin-6-dependent expression of the retinoid receptor-related orphan receptor γT (RORγT), the key transcription factor for Th17 differentiation [285]. Hence, PPARγ is a promising molecular target for precise immune intervention in Th17-mediated autoimmune diseases such as multiple sclerosis. Identification of the molecular mechanisms responsible for the anti-inflammatory actions of PPARγ is therefore likely to be of practical importance in the efforts to develop effective and safer treatment options for diseases associated with autoimmunity. It has been demonstrated that 2-AG levels are elevated in various immune cell types upon activation and that PPARγ activation, 2-AG and 15d-PGJ_2 (a known endogenous PPARγ ligand) treatments ameliorate the symptoms of numerous animal models of autoimmune diseases. Collectively, this suggests that

activation of PPARγ by 15d-PGJ_2-G may play a significant role in the control of exaggerated immune responses thereby facilitating immune homeostasis.

Overall, elevated 2-AG levels during inflammation can result in increased chemotaxis of immune cells to the site of infection through CB2 activation. Later, when COX-2 expression is upregulated during inflammation, 2-AG can be metabolized to form glycerol esters of various prostaglandins, including 15d-PGJ_2-G. Increased levels of 15d-PGJ_2-G can result in the activation of PPARγ, which has been suggested to have an anti-inflammatory role, thus contracting the immune responses after initiation. Therefore, 2-AG and its putative metabolite, 15d-PGJ_2-G play an important role in maintaining immune homeostasis. Understanding the molecular targets involved in this immune homeostasis can help in developing a therapy directed to decrease exaggerated immune responses in autoimmune diseases.

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