Cannabinoid signalling in TNF-α induced IL-8 release

Maria E Mormina, Shori Thakur, Areles Molleman, Cliff J Whelan

and [1]Anwar R Baydoun

School of Life Sciences, University of Hertfordshire,

College Lane, Hatfield, Herts AL10 9AB

[1]Author for correspondence: Professor A.R. Baydoun
University of Hertfordshire
School of Life Sciences
Faculty of Health and Human Sciences
College Lane
Hatfield
AL10 9AB.
United Kingdom

Tel: 01707 285 120
Fax: 01707 285 046
e-mail: a.baydoun@herts.ac.uk

Abstract

The molecular events mediating the immunomodulatory properties of cannabinoids have remained largely unresolved. We have therefore investigated the molecular mechanism(s) through which *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-napthanlenyl) methanone (WIN55212-2) modulate production of interleukin-8 (IL-8) in HT-29 cells. Release of IL-8 induced by tumor necrosis factor-α (TNF-α) was determined by enzyme-linked immunosorbent assay (ELISA). Changes in expression of inhibitory kappa B (IκB) were monitored by Western blotting and activation of nuclear factor-kappa B (NF-κB) was determined in electrophoretic mobility shift assay (EMSAs). TNF-α induced release of IL-8 was inhibited by WIN55212-2 which also blocked the degradation of IκB-α and activation of NF-κB induced by TNF-α. These data provide strong evidence that WIN55212-2 may modulate IL-8 release by negatively regulating the signaling cascade leading to the activation of NF-κB. These findings highlight a potential mechanism for the immunomodulatory properties of cannabinoids and contribute towards acquiring a clear understanding of the role of cannabinoids in inflammation.

Key Words: WIN55212-2, IL-8, IκB , NF-κB, CB_2 Receptors, HT-29.

1. Introduction

Pro-inflammatory cytokines are pivotal for the development of an inflammatory response and therefore constitute a pharmacological target for the development of anti-inflammatory therapies. In particular, the chemoattractant cytokine interleukin-8 (IL-8; now referred to as CXCL8) is essential for the recruitment of immune cells to the site of inflammation and for a coordinated immune response (Miller and Krangel, 1992; Baggiolini et al., 1997).

Because of their immunomodulatory properties, therapeutic use of cannabinoids could provide new approaches for the development of anti-inflammatory therapies. However, although many immunosuppressive as well as some immunostimulatory effects are documented (Croxford and Yamamura, 2005), and cannabinoids historically have been used as anti-inflammatory agents, the molecular mechanisms underlying these effects are poorly understood. Cannabinoids may however modulate immune responses by regulating the cytokine system (Klein et al., 1998), and this may be due in part to their ability to regulate signal transduction pathways associated with gene transcription and subsequent expression of immune cytokines. In this regard, cannabinoids have been reported to down regulate the adenylate cyclase (AC) cascade, which is thought to be the main signal transduction pathway implicated in the immune response (Schatz et al., 1992; Diaz et al., 1993; Kaminski et al.,1994; Kaminski, 1998). Inhibition of AC and thus of cAMP production may consequently inhibit protein kinase A (PKA), and hence phosphorylation of several downstream targets including the cAMP-response element binding protein/activation transcription factor (CREB/ATF) and NF-κB (Herring et al., 1998; Herring and Kaminski, 1999), thus suppressing the inflammatory process.

Epithelial cells are an important component of the mucosal immune system, performing a variety of immune functions, including the expression of adhesion molecules and the secretion of cytokines (Schuerer-Maly et al., 1994). This central role and their proximity to the intestinal lumen make these cells easily accessible to pharmacological agents, and modulation of their activities is of major therapeutic importance in inflammation. In this report, we have therefore used the human HT-29 colon epithelial cell line to examine the molecular mechanism by which cannabinoids may regulate cytokine release. The studies have focused on the potent cannabinoid CB_1/CB_2 receptor agonist *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-napthanlenyl) methanone mesylate (WIN55212-2) which has previously been shown to inhibited tumor necrosis factor-α (TNF-α) induced release of IL-8 via an action on the cannabinoid CB_2 receptors. The latter conclusion was based partly on functional studies where WIN55212-2 induced inhibition of IL-8 release was reversed by the cannabinoid CB_2 receptor antagonist [*N*-[(1*S*)-*endo*-1,3,3,-trimethyl-bicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide] (SR144528), but not by the CB_1 receptor antagonist [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorphenyl)-4-methyl-*H*-pyrazole-3 carboxyamidehydrochloride] (SR141716A) and partly on data from Western blot analysis using a polyclonal antibody raised against the amino terminus of the cannabinoid CB_2 receptor (Ihenetu et al., 2003a).

Since IL-8 release may require *de novo* synthesis and thus gene transcription, our studies have focused on establishing whether NF-κB may be involved in this process and, more importantly, whether cannabinoids may be able to modulate IL-8 release through regulation of the activity of this ubiquitous transcription factor which has been shown to play a crucial role in mediating cytokine-induced gene expression (Tak and Firestein, 2001). In light of current trends

in cannabinoid research, addressing the transcriptional component of this process may contribute towards acquiring a clear and valuable understanding of the inflammatory process as well as of the role of cannabinoids and cannabinoid receptors in the function of the immune system. The elucidation of such complex events may lead ultimately to new therapeutic approaches that take advantage of an intersection not yet fully exploited.

2. Materials and Methods

2.1. Drugs and Reagents

Tissue culture reagents were obtained from Gibco (Paisley, UK). Other reagents used were: WIN55212-2 (Tocris Cookson, Bristol, UK); TNF-α (PeproTech, Ltd, London, UK); *Z*-Leu-Leu-Leu-CHO (MG132; Calbiochem, Beeston, Nottingham, UK) and [γ^{32}P]dATP (Amersham, UK). 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), aprotinin, benzamidin, chymostatin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), sodium vanadate, actinomycin D and cycloheximide were purchased from Sigma-Aldrich (Pool, UK). The IκB-α rabbit affinity purified polyclonal antibody and anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) conjugated secondary antibody were from Biotechnology, UK. Biotinylated mouse anti-human IL-8 monoclonal antibody, streptavidin-HRP conjugate secondary antibody, tetramethylammonium-benzidine (TMB) and 96-well Nunc-immunoplates (maxisorpF96) for ELISA detection were from Pharmingen BD (Oxford, UK). DNA oligomers containing the NF-κB (GGG GAC TTT CCC) consensus sequences were purchased from Promega (Southampton, UK). All other drugs and chemicals were obtained from Sigma or BDH and were of the highest analytical grade obtainable.

2.2. Cell culture

The HT-29 colon epithelial cell line was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, Wiltshire, United Kingdom). Cells were grown in McCoy's 5A medium supplemented with 10% foetal bovine serum and penicillin/streptomycin (50U/ml and 50µg/ml respectively) plus amphotericin B (0.5µg/ml). Cultures were maintained in 75cm^2 culture flasks at 37ºC in a 5% CO_2 humidified tissue culture incubator.

2.3. Plating of cells for experimentation

Confluent monolayers were subdivided and plated at a density of 5 x 10^5cells/ml in T75 tissue culture flasks. To detach the monolayer from the flasks, cells were washed once with sterile phosphate buffered saline (PBS) and incubated for approximately 5 min in the presence of 0.25% trypsin/ethylene diamine tetracetic acid (EDTA). Once detached, the action of trypsin was stopped by adding 5 ml of McCoy's 5A containing 10% foetal bovine serum. Cells were diluted to the appropriate density, plated and incubated as described.

Prior to each experiment cells were washed twice with warm sterile PBS and the incubation medium replaced with fresh serum-free McCoy's medium. All experiments were carried out in the absence of serum.

2.4. Enzyme linked immunosorbent assay for IL-8 release

TNF-α-induced IL-8 release from HT-29 cells was determined by ELISA. Confluent cells grown in 24-well plates as described above were pre-incubated in the presence of appropriate drugs for varying time periods depending on the experimental protocol. Cells were then activated with TNF-α (100ng/ml) for 24h. At the end of the incubation time supernatants were collected and stored at -20ºC until the assay was performed. 96-well Nunc-immunoplates were coated overnight with 1μg/ml anti-human IL-8 monoclonal capture antibody. Plates were subsequently washed three times with washing buffer (phosphate buffered saline plus 0.05% Tween 20) and blocked with assay diluent (phosphate buffered saline plus 10% foetal bovine serum) for 1h at room temperature. After three washes, standards and samples (supernatants diluted 1/10 in serum-free medium) were added and incubated at room temperature for 2h. Standard and samples were discarded and plates were washed five times with washing buffer. Detection was

performed in a single step by incubating the plates in the presence of a biotinylated anti-human IL-8 antibody and streptavidin linked peroxidase conjugate (each at 0.5µg/ml respectively). After incubation for 1h plates were washed seven times and TMB was used as a substrate to quantify the amount of bound conjugate by colorimetric measurement. Absorbance was read at a wavelength of 450 nm using a Multiskan II plate reader and the values obtained converted using an IL-8 standard curve constructed in parallel.

2.5. Western blot analysis of IκB-α expression

Lysates for Western blot analysis were prepared from cells following various experimental conditions. Protein concentrations were determined using bicinchoninic acid (BCA) reagents according to the instructions provided by the manufacturer. Western blotting was carried out as described by Cirino et al. (1996). Briefly, 2µl of 2% bromophenol blue and 5µl of stock β-mercaptoethanol were added to the samples and heated at 95ºC prior to loading 20µg of protein onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel (8%). After separation, proteins were transferred for 2h at 0.8 mA cm^{-2} onto a polyvinyldene difluoride (PVDF) membrane (Amersham, UK). Membranes were blocked for 1h at 37º in 10mM Tris, 100mM NaCl, 0.1% (v/v) Tween-20 (STT) buffer (pH 7.4) containing 5% (w/v) of dry non-fat milk and incubated with a polyclonal anti-IκB-α antibody for a further 1h at 37ºC. This was followed by 3 washes with STT buffer prior to incubating with a secondary anti-rabbit polyclonal IgG conjugated with horseradish peroxidase (dilution 1:5000 in STT buffer containing 5% (w/v) dry non-fat milk). Membranes were subsequently washed with STT buffer and the immunoreactive bands visualized using the enhanced chemiluminescence detection system.

2.6. Electromobility shift assay (EMSA)

Changes in the activation of NF-κB under different experimental conditions were determined by electromobility shift assays (EMSAs). Confluent HT-29 monolayers in T75 tissue cultures flasks were treated as required. Cells were then washed with ice-cold PBS and lysed in 1ml of lysis buffer consisting of: Tris-HCl (10mM, pH 7.5), $MgCl_2$ (1.5mM), KCl (10mM) and NP-40 (0.5%) supplemented with 1.5µg/ml of aprotinin, benzamidine, chymostatin, leupeptin and pepstatin, plus phenylmethylsulphonylfluoride (PMSF; 0.2mM), DTT (0.5mM) and sodium vanadate (10µM). Lysates were transferred into 1.5ml eppendorff tubes and centrifuged at 4ºC for 5 min at 3290xg. Nuclear fractions were subsequently treated with 0.5ml of the above lysis buffer. The lysate was centrifuged at 4ºC for 5 min at 3290xg and the supernatant discarded. The nuclear pellet was further lysed in 50µl buffer containing Tris-HCl (20mM, pH 7.5), MgCl (1.5mM), NaCl (420mM), EDTA (0.2mM), Glycerol (10%) plus the cocktail of protease inhibitors described above. After incubation on a platform rotator for 45 min lysates were centrifuged at 13147xg for 15 min at 4ºC and the supernatants diluted in 50µl buffer containing Tris-HCl (20mM, pH 7.5), MgCl (1.5mM), EDTA (0.2mM), Glycerol (10%) plus the cocktail of protease inhibitors. Samples were stored at –80ºC for subsequent use in EMSAs.

DNA oligomer containing the NF-κB (GGG GAC TTT CCC) consensus sequence was end-labeled with [γ^{32}P]dATP. Typically, 10µCi radioactive nucleotide (1µl of 10 mCi/ml) was used to label 3.5 pM of oligonucleotide (2µl of 1.75pmol/µl stock) by incubation at 37ºC in the presence of 1µl of T4 polynucleotide kinase (T4 PNK) in 1µl of 10X T4 Polynucleotide Kinase Buffer (Tris HCl (pH 7.6, 700mM), $MgCl_2$ (100mM) and DTT (50 mM)). The final volume was made up to 10µl with 5µl autoclaved H_2O. Nuclear extracts (10µg) were incubated with 1µl of the labeled probe in 2µl of binding buffer (20% glycerol, 5mM $MgCl_2$, 2.5mM EDTA, 2.5mM

DTT, 250mM NaCl, 50mM Tris-HCl (pH 7.5), and 0.25mg/ml poly(dI-dC)●poly(dI-dC)) for 40 min on ice. Samples were subsequently mixed with 1µl of 10X loading buffer (250mM Tris-HCl (pH 7.5), 0.2% bromophenol blue and 40% glycerol) before loading onto a 4% acrylamide non-denaturing gels and run at 130V for 2h. Gels were dried and autoradiographed for analysis.

2.7. Cell viability assay

Cells in 96-well plates were treated under corresponding experimental conditions and incubated with 100µg MTT for 2h at 37ºC, 5% CO_2, in standard culture medium. The medium was subsequently aspirated and purple formazan crystals solubilised in 100µl of isopropanol. The extent of reduction of MTT to formazan within cells was quantified by measurement of OD_{550} using a Multiskan II plate reader.

2.8. Statistical analysis

Unless specified, all results are expressed as percentage of IL-8 released by TNF-α (100ng/ml) treated cells and are the mean ± S.E.M. of at least 3 separate experiments. Statistical significance was calculated by analysis of variance (ANOVA) followed by Dunnett's post hoc test. Differences were taken to be statistically significant when $P<0.05$.

3. Results

3.1. TNF-α dependent up-regulation of IL-8 release

Previous studies have shown that unstimulated HT-29 cells express low basal levels of IL-8 which can be increased in a time-dependent manner following stimulation of cells (Ihenetu et al., 2003a). Consistent with this observation, our initial studies confirm that treatment of HT-29 cells with TNF-α (10-1000ng/ml) for 24h results in a concentration-dependent increase in the levels of IL-8 released into the culture medium (Fig. 1). At the submaximal concentration of 100ng/ml, TNF-α significantly increased IL-8 release from a basal value of 890 ± 91pg/ml to 12250 ± 147pg/ml ($P<0.05$). This concentration was therefore used in all subsequent experiments to avoid using high cytotoxic concentrations of this cytokine which may be observed following prolonged incubations with 1000ng/ml.

3.2. Effects of MG132, cycloheximide and actinomycin D on TNF-α-induced IL-8 release

In order to establish whether the stimulatory effects of TNF-α on IL-8 release was due to the activation of NF-κB, induced gene transcription and *de novo* protein synthesis, experiments were carried out examining the effects of the proteasomal inhibitor MG132, the inhibitor of gene transcription actinomycin D and the protein synthesis inhibitor cycloheximide on TNF-α induced IL-8 release. In these studies, cells were pre-treated with each drug for 30 min prior to stimulation with TNF-α. The data obtained (Fig. 2) show that TNF-α induced IL-8 release was significantly attenuated by MG132 (3μM), actinomycin D (10μM) and by cycloheximide (10μM) which respectively inhibited IL-8 release by 76 ± 0.7%, 95 ± 3% and 80 ± 1.6%.

3.3. Degradation of IκB-α and activation of NF-κB by TNF-α

The data with MG132 strongly suggests that activation of NF-κB by TNF-α may be critical for the induction of IL-8 release. To substantiate this hypothesis, additional experiments were carried out examining the effects of TNF-α on both IκB-α degradation and on NF-κB-DNA binding activity. Treatment of HT-29 cells with TNF-α (100ng/ml) resulted in a time-dependent reduction in IκB-α protein which was evident after 10 min, reaching a maximum at 15 min and staying at this depleted level over 30 min before returning to pre-stimulated basal levels after 1h (Fig. 3).

In parallel studies, NF-κB-DNA binding was found to be minimal in nuclear extracts from control cells but increased after 10 min of exposure to TNF-α and remained at this elevated level for up to 1h after activation. This increase in NF-κB-DNA binding was significantly inhibited by a 10-fold excess of unlabelled NF-κB-specific oligonucleotide indicating the specificity of binding (Fig. 4).

3.4. Effects of the cannabinoid receptor agonist WIN55212-2 on TNF-α induced IL-8 release

To confirm that cannabinoids regulate IL-8 release, HT-29 cells were pre-incubated with the potent cannabinoid CB1/CB2 receptor agonist WIN55212-2 (10^{-10} to 10^{-4}M) for 2 h prior to activation with TNF-α (100ng/ml) for 24 h. As shown in Fig. 5, WIN55212-2 exerted a concentration-related inhibition of TNF-α induced secretion of IL-8, completely abolishing the latter at 10^{-5}M and inhibiting by approximately 70% at 10^{-6} M ($P<0.01$, n=6).

In parallel experiments, addition of WIN55212-2 (10^{-6} M) either simultaneously with or at different intervals after activation of cells with TNF-α (100ng/ml) failed to cause any significant change in TNF-α-induced IL-8 release. In these studies, levels of IL-8 remained unaltered with respect to the control TNF-α activated cells (Fig. 6).

3.5. Effects of WIN55212-2 on TNF-α induced NF-κB activation and IκB-α degradation

Effects of WIN55212-2 on the activation of NF-κB was examined in samples prepared from cells pretreated for 2h with increasing concentrations of WIN55212-2 (10^{-9} to 10^{-4} M) prior to activation with TNF-α (100ng/ml) for 1h. In these series of experiments, there were very little detectable NF-κB-DNA complexes formed by nuclear extract from control non-activated cells. In contrast, incubation of nuclear extracts from TNF-α activated cultures with the ^{32}P labeled κB oligonucleotide exhibited increased DNA binding activity which, as shown previously in Fig. 4, was virtually abolished by a 10-fold excess of unlabelled NF-κB-specific oligonucleotide. More importantly, WIN55212-2 significantly inhibited TNF-α induced NF-κB-DNA binding, virtually abolishing the latter at 10^{-5}M. A significant inhibition was also observed with 10^{-6} M WIN55212-2 with lower concentrations ($\leq 10^{-7}$ M) having little or no effect (Fig. 7). In parallel with the changes in NF-κB-DNA binding activity, TNF-α-induced degradation of IκB-α was also inhibited in a concentration-dependent manner by WIN55212-2. As shown in Fig. 8, WIN55212-2 completely restored IκB-α protein expression back to basal levels at 10^{-5} M with 10^{-6} M showing partial inhibition of the degradation of IκB.

4. Discussion

The molecular mechanisms by which cannabinoids exert their broad range of biological effects have still not been fully elucidated. One key area of progress in this field is the common understanding that regulation of adenylate cyclase may be critical for cannabinoid-mediated immunosuppression (Schatz et al., 1992; Diaz et al., 1993; Kaminski et al.,1994; Kaminski, 1998). Although, the precise role of cAMP signaling in immune regulation is still not well defined, there are indications that this may occur via down regulation of protein kinase A (PKA) through inhibition of cAMP production which may in turn suppress binding of CREB/ATF transcription factors to the cAMP response element (CRE) consensus motif (Koh et al., 1997; Herring et al., 1998; Herring and Kaminski, 1999). Additionally, cannabinoids acting in part through suppression of PKA may inhibit activation of NF-kB (Jeon et al., 1996; Herring et al., 1998; Herring and Kaminski, 1999). It is worth noting however that these actions may be cannabinoid and/or cell specific since in mouse thymocytes, cannabinol-induced inhibitions of CREB/ATF-1 phosphorylation and NF-κB-DNA binding activity could not be reversed by the membrane permeable analogue of cAMP, dibutyryl cAMP (DBcAMP), nor by the potent PKA inhibitor N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H89; Herring et al., 2001). Moreover, in mast cells, CB_1 ligands cause an accumulation rather than inhibition in cytosolic cAMP levels (Small-Howard et al., 2005) while in mouse dendritic cells, exposure to Δ^9-tetrahydrocannabinol (Δ^9-THC) induced phosphorylation of IκB-α and enhanced NF-κB activation (Do et al., 2004).

Despite the vast literature on the immunosuppressive actions of cannabinoids, there is currently little data on the molecular mechanisms that may account for the actions of these

compounds in modulating IL-8 release by other pro-inflammatory mediators. Attempts were therefore made in the current studies to elucidate the molecular mechanism responsible for the suppression of TNF-α-induced IL-8 release by WIN55212-2 in the human colon epithelial cell line HT-29. Stimulation of these cells with TNF-α for 24h caused a concentration-dependent increase in IL-8 production which was significantly inhibited by WIN55212-2. This finding is consistent with the inhibition by WIN55212-2 of IL-8 release in human astrocytes (Curran et al., 2005) and further supports our previous study that demonstrated the greater efficacy of WIN55212-2 over other cannabinoid receptor agonists in suppressing TNF-α induced IL-8 release in HT-29 cells (Ihenetu et al., 2003a).

Interestingly, the above data contrast with reports in the promyelocytic cell line HL60 where (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol (CP-55940; Jbilo et al., 1999; Kishimoto et al., 2004) and 2-Arachidonoylglycerol (Kishimoto et al., 2004) were shown to enhance IL-8 mRNA and protein expression in a CB_2 dependent manner. The precise reasons for these paradoxical actions of different cannabinoids on IL-8 release are unclear and may be governed by several factors, including the cannabinoid in question; the cell types used and even the status of the cells being experimented upon. In this context, it has been reported that cannabinol may induced IL-2 production in partially activated T-cells but decrease the same process when the cells are optimally activated (Jan and Kaminski, 2001). This highlights the complex nature of the cellular actions of cannabinoids and may warrant caution in interpreting results from one system to the next. It is important to emphasising however that the immunosuppressive action of WIN55212-2 is a widely reported phenomenon which is not restricted to the inhibition of IL-8 alone. WIN55212-2 has also been shown to

reduce LPS-induced TNF-α production in human peripheral blood mononuclear cells (Germain et al., 2002), rat cortical microglial cells (Facchinetti et al., 2003), bronchoalveolar lavage fluid (Berdyshev et al., 1998), and inhibits interleukin-2 (IL-2) release from human peripheral blood mononuclear cells (Ihenetu et al., 2003b) and from mouse splenocytes (Kaplan et al., 2003). Moreover, such actions are common with other cannabinoids including Δ^9-THC, which like WIN55212-2 also suppressed LPS-induced TNF-α levels in bronchopulmonary inflammation in mice (Berdyshev et al., 1998) and reduced LPS-induced mRNAs for IL-1 and TNF-α in neonatal rat microglial cells (Puffenbarger et al., 2000). Similarly, suppression of IL-2 release has been reported with anandamide (Rockwell and Kaminski, 2004), cannabinol (Herring and Kamisky, 1999; Hering et al., 2001) and 2-arachidonyl-glycerol (Ouyang et al., 1998).

The inhibitory effects of WIN55212-2 in our studies were critically dependent on the time of addition of the compound prior to activation of cells. Maximal effects were observed when WIN55212-2 was added before TNF-α and allowed to equilibrate with cells before they were being activated. In contrast, application of WIN55121-2 together with or up to 9h after TNF-α failed to cause any significant change in induced IL-8 production. This may be due to the combination of a slow action of WIN55212-2 and a quick activation by TNF-α of the NF-κB signaling cascade. It is possible that in this case, WIN55212-2 maybe acting through mechanisms that trigger events such as new protein synthesis which take time to occur. Activation of the cascade may well be initiated after a brief exposure to WIN55212-2 but the changes may not occur for hours as is usual for events dependent on changes in cellular and/or nuclear signal transduction pathways. Thus it is not surprising that without pre-treatment, WIN55212-2 was not able to modify TNF-α induced IL-8 release. These findings are not unique

to our studies since other reports have indicated that WIN55212-2 may require prolonged incubation with tissue to produce its maximal effect. For instance in a study by Tyler et al. (2000) WIN55212-2 required between 50–70 min to exert maximal inhibitory effects on mucosal transport of fluids and electrolytes across the gastrointestinal tract of the rat. Similar observations were also made for other cannabinoids acting on the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine (Pertwee et al., 1992), and in a study on human astrocytes, WIN55212-2 was incubated with the cells for 24 h prior to initiating induction of NO production with IL-1-β (Sheng et al., 2005).

Previous studies showed that the IL-8 promoter contains consensus sequences for NF-κB (Casola et al., 2002). To determine the involvement of this transcription factor in the regulation of TNF-α induced IL-8 release in HT-29 cells, the profiles of activation of NF-κB was determined. Two approaches were taken, the first being to monitor changes in basal IκB-α levels by Western blotting and the second to directly measure changes in NF-κB-DNA activity using EMSAs. Under normal physiological conditions, NF-κB is sequestrated in the cytoplasm in an inactive form as a NF-κB-IκB complex which may be activated through phosphorylation of the inhibitory IκB subunit by IκB kinases (IKK; Mercurio et al., 1997; Regnier et al., 1997; Zandi et al., 1997). Phosphorylation of IκB results in its ubiquitination and subsequent degradation by the ATP-dependent proteolytic 26S proteasome (Palombella et al., 1994) with the consequential release of NF-κB which then translocate to the nucleus. As a result, basal levels of IκB are usually depleted by agents that activate NF-κB. Consistent with this notion, exposure of HT-29 cells to TNF-α in our studies resulted in a time-dependent degradation of the IκB-α which was relatively rapid in onset, taking approximately 15 min to reach a maximum and remained

depleted for up to an hour before returning to the pre-stimulated levels. This profile is consistent with the rapid but transient activation of the NF-κB pathway due to an auto-regulatory feedback loop that allows activated NF-κB to mediate upregulation of IκB-α and in turn switch off any signal production (Ghosh et al., 1998)

The decreases in IκB levels observed in our studies correlated well with the activation of NF-κB which, once in the nucleus, binds to its corresponding response element on the promoter of its target gene and initiates transcription. Electromobility shift assays revealed that NF-κB-DNA binding was prominent after 10 min reaching a peak at 15 min and sustained for up to an hour. Thus, it is clear that TNF-ą may stimulate signaling cascades that lead to the activation of NF-κB and this may subsequently result in IL-8 release. Confirmation of this being the case was obtained in studies employing the potent proteasomal inhibitor MG132 which is known to block the degradation of IκB (Palombella et al., 1994). When employed, MG132 caused a distinct inhibition of TNF-α-induced IL-8 release from HT-29 cells indicating that the latter process requires activation of NF-κB. Consistent with the concept of induced gene transcription and *de novo* protein synthesis are the additional data obtained with actinomycin D and with the protein synthesis inhibitor cycloheximide. Pre-treatment with either compound prior to stimulation with TNF-α produced a significant inhibition of IL-8 release, indicating that the release of IL-8 requires *de novo* protein synthesis and not a mere release of the protein from pre-existing intracellular stores.

To address whether WIN55212-2 was able to suppress induced IL-8 release through inhibition of NF-κB, studies were carried out examining the effects of this cannabinoid on TNF-

α-induced activation of the NF-κB/IκB pathway in HT-29 cells. In these experiments, Western blot analysis of IκB-α expression and NF-κB-DNA binding studies showed that WIN55212-2 was able to inhibited TNF-α-induced degradation of IκB-α and subsequently NF-κB-DNA binding. Moreover, these effects occurred over the same concentration range that inhibited IL-8 release and were both maximal at the highest concentrations of used (10^{-5} M), with significant inhibition of both processes seen at 10^{-6} M WIN55212-2.

In summary, our data provide strong evidence that the cannabinoid WIN55212-2 is able to regulate IL-8 released by inhibiting the activation of NF-κB in HT-29 cells. This effect may be mediated via the cannabinoid CB_2 and not the CB_1 receptors since our previous studies have demonstrated that inhibition of TNF-α-induced IL-8 release by WIN55212-2 could be prevented by the potent CB_2 receptor antagonist SR144528 but not by the CB_1 receptor antagonist SR141716A (Ihenetu et al., 2003a). At present, we cannot rule out that other novel receptors or indeed effects independent of cannabinoid receptors may be involved in the actions of WIN55212-2. This is particularly pertinent in light of recent reports showing that WIN55212-2 could inhibit IL-1 signalling in human astrocytes independently of cannabinoid receptor activation (Curran et al., 2005). Although this issue remains to be clarified, our data addressing the molecular mechanisms for some of the immunomodulatory properties of cannabinoids have a number of implications. Firstly our findings contribute towards a better understanding of the molecular mechanism of actions of cannabinoids and would help unfold the signaling events implicated in the immune response. Secondly, our data may provide the basis for the development of novel therapeutic strategies that may be beneficial in inflammatory disease states associated with the overproduction of cytokines, including IL-8.

References

Baggiolini, M., Dewald, B., Moser, B., 1997. Human Chemokines:An Update. Ann. Rev. Immunol. 15, 675-705.

Berdyshev, E., Boichot, E., Corbel, M., Germain, N., Lagente, V., 1998. Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice. Life Sci. 63, PL125-PL129.

Casola, A., Garofalo, R.P., Crawford, S.E., Estes, M.K., Mercurio, F., Crowe, S.E., Brasier, A.R., 2002. Interleukin-8 Gene Regulation in Intestinal Epithelial Cells Infected with Rotavirus: Role of Viral-Induced IkB Kinase Activation. Virology 298, 8–19.

Cirino, G., WheelerJones, C.P.D., Wallace, J.L., DelSoldato, P., Baydoun, A.R., 1996. Inhibition of inducible nitric oxide synthase expression by novel nonsteroidal anti-inflammatory derivatives with gastrointestinal-sparing properties. Brit. J. Pharmacol. 117, 1421-1426.

Croxford, J.L., Yamamura, T., 2005. Cannabinoids and the immune system: Potential for the treatment of inflammatory diseases? J. Neuroimmunol. 166, 3-18.

Curran, N.M., Griffin, B.D., O'Toole, D., Brady, K.J., Fitzgerald, S.N., Moynagh, P.N., 2005. The synthetic cannabinoid R(+)WIN 55,212-2 inhibits the interleukin-1 signaling pathway in human astrocytes in a cannabinoid receptor-independent manner. J. Biol. Chem. 280, 35797-35806.

Diaz, S., Specter, S., Coffey, R.G., 1993. Suppression of lymphocyte adenosine 3':5'-cyclic monophosphate (cAMP) by delta-9-tetrahydrocannabinol. Int. J. Immunopharmacol. 15, 523-532.

Do, Y., McKallip, R.J., Nagarkatti, M., Nagarkatti, P.S., 2004. Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. J. Immunol. 173, 2373-2382.

Facchinetti, F., Del Giudice, E., Furegato, S., Passarotto, M., Leon, A., 2003. Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide. Glia 41, 161-168.

Germain, N., Boichot, E., Advenier, C., Berdyshev, E.V., Lagente, V., 2002. Effect of the cannabinoid receptor ligand, WIN 55,212-2, on superoxide anion and TNF-α production by human mononuclear cells. Int. Immunopharmacol. 2, 537-543.

Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-kappa B and rel proteins: Evolutionarily conserved mediators of immune responses. Ann. Rev. Immunol. 6, 225-260.

Herring, A.C., Kaminski, N.E., 1999. Cannabinol mediated inhibition of nuclear factor kB, cAM response element binding protein, and interleukin-2 secretion by activated thymocytes. J. Pharmacol. Exp. Ther. 291, 1156-1163.

Herring, A.C., Faubert Kaplan, B.L., Kaminski, N.E., 2001. Modulation of CREB and NF-kB signal transduction by cannabinol in activated thymocytes. Cell Signal. 13, 241-250

Herring, A.C., Koh, W.S., Kaminski, N.E., 1998. Inhibition of the cyclic cAMP cascade and nuclear factor binding to CRE and kB elements by cannabinol, a minimally CNS-active cannabinoid. Biochem. Pharmacol. 55, 1013-1023.

Ihenetu, K., Molleman, A., Parsons, M.E., Whelan, C.J., 2003a. Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. Eur. J. Pharmacol. 458, 207-215.

Ihenetu, K., Molleman, A., Parsons, M.E., Whelan, C.J., 2003b. Pharmacological characterisation of cannabinoid receptors inhibiting interleukin 2 release from human peripheral blood mononuclear cells. Eur. J. Pharmacol. 464, 207-215.

Jan, T.R. and Kamanski, N.E., 2001. Role of mitogen-activated protein kinases in the differential regulation of interleukin-2 by cannabinol. J. Leukoc. Biol. 69, 841-849.

Jbilo, O., Derocq, J.M., Segui, M., Le,Fur, G., Casellas, P., 1999. Stimulation of peripheral cannabinoid receptor CB2 induces MCP-1 and IL-8 gene expression in human promyelocytic cell line HL60. FEBS Lett. 448, 273-277.

Jeon, Y.J., Yang, K-H., Pulaski, J.T., Kaminski, N.E., 1996. Attenuation of inducible nitric oxide synthase gene expression by D^9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor-kB/Rel activation. Mol. Pharmacol. 50, 334–341.

Kaminski, N.E., 1998. Inhibition of the cAMP signaling cascade via cannabinoid receptors: a putative mechanism of immune modulation by cannabinoid compounds. Toxicol. Lett. 102–103, 59–63.

Kaminski, N.E., Koh, W.S., Yang, K.H., Lee, M., Kessler F.K., 1994. Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylate cyclase by a pertussis toxin-sensitive G-protein coupled mechanism. Biochem. Pharmacol. 48, 1899–1908.

Kaplan, B.L., Rockwell, C.E., Kaminski, N.E., 2003. Evidence for cannabinoid receptor-dependent and independent mechanisms of action in leukocytes. J. Pharmacol. Exp. Ther. 306, 1077-1085.

Kishimoto, S., Kobayashi, Y., Oka, S., Gokoh, M., Waku, K., Sugiura, T., 2004. 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces accelerated production of chemokines in HL-60 cells. J. Biochem. 135, 517-524.

Klein, T., W., Newton, C., Friedman, H., 1998. Cannabinoid receptors and immunity. Immunol. Today 19, 373-381.

Koh, W.S., Crawford, R.B., Kaminski, N.E., 1997. Inhibition of protein kinase A and cyclic AMP response element (CRE)-specific transcription factor binding by delta9-tetrahydrocannabinol (delta9-THC): a putative mechanism of cannabinoid-induced immune modulation. Biochem. Pharmacol. 53, 1477-1484.

Mercurio, F., Zhu, H.Y., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J.W., Young, D.B., Barbosa, M., Mann, M., 1997. IKK-1 and IKK-2: Cytokine-activated I kappa B kinases essential for NF-kappa B activation. Science 278, 860-866.

Miller, M.D., Krangel, M.S., 1992. Biology and biochemistry of the chemokines: A family of chemotactic and inflammatory cytokines. Crit. Rev. Immunol. 12, 17–46.

Ouyang, Y., Hwang, S.G., Han, S.H., Kaminski, N.E., 1998. Suppression of interleukin-2 by the putative endogenous cannabinoid 2-arachidonyl-glycerol is mediated through down-regulation of the nuclear factor of activated T cells. Mol. Pharmacol. 53, 676-683.

Palombella, V.J., Rando, O.J., Goldberg, A.L., Maniatis, T., 1994. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 78, 773-785.

Pertwee, R.G., Stevenson, L.A., Elrick, D.B., Mechoulam, R., Corbett, A.D., 1992. Inhibitory Effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. Br. J. Pharmacol. 105, 980–984.

Puffenbarger, R.A., Boothe, A.C., Cabral, G.A., 2000. Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. Glia. 29, 58-69.

Regnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z.D.,Rothe, M., 1997. Identification and characterization of an I kappa B kinase. Cell 90, 373-383.

Rockwell, C.E. and Kaminski, N.E., 2004. A cyclooxygenase metabolite of anandamide causes inhibition of interleukin-2 secretion in murine splenocytes. J. Pharmacol. Exp. Ther. 311, 683-690.

Schatz, A.R., Kessler, F.K., Kaminski, N.E., 1992. Inhibition of adenylate cyclase by delta 9-tetrahydrocannabinol in mouse spleen cells: a potential mechanism for cannabinoid-mediated immunosuppression. Life Sci.51, PL25-PL30.

Schuerer-Maly, C.-C., Eckmann, L., Kagnoff, M.F., Falco, M.T., Maly, F.E., 1994. Colonic epithelial cell lines as source of interleukin-8: stimulation by inflammatory cytokines and bacterial lipopolysaccharides. Immunol. 81, 85- 91.

Sheng, W.S., Hu, S., Min, X., Cabral, G.A., Lokensgard, J.R., Peterson, P.K., 2005. Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta-stimulated human astrocytes. Glia. 15, 211-219.

Small-Howard, A.L., Shimoda, L.M., Adra, C.N., Turner, H., 2005. Anti-inflammatory potential of CB1-mediated cAMP elevation in mast cells. Biochem, J. 388, 465-473.

Tak, P.P., Firestein, G.S., 2001. NF-kappa B: a key role in inflammatory diseases. J. Clin. Invest. 107, 7-11.

Tyler, K., Hillard, C.J., Greenwood-Van Meerveld, B., 2000. Inhibition of small intestinal secretion by cannabinoids is CB1 receptor-mediated in rats. Eur. J. Pharmacol. 409, 207-211.

Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., Karin, M., 1997. The I kappa B kinase complex (IKK) contains two kinase subunits, IKK alpha and IKK beta, necessary for I kappa B phosphorylation and NF-kappa B activation. Cell 91, 243-252.

Figure Legends

Fig. 1. TNF-α induced release of IL-8 from HT-29 cells. Confluent monolayers of cells were stimulated with increasing concentrations of TNF-α (10-1000ng/ml) for 24h. IL-8 release was measured by ELISA as described in methods. Data represent means ± S.E.M. of three separate experiments. * denotes $P<0.01$.

Fig. 2. Effect of MG132, actinomycin D and cycloheximide on TNF-α induced IL-8 release in HT-29 cells. Confluent monolayers of cells were treated with either MG132 (3μM), actinomycin D (10 μM) or cycloheximmide (10 μM) for 30 min prior to stimulation with TNF-α (100ng/ml) for 24h. Data are expressed as percentage of inhibition with respect to the TNF-α activated controls and represent means ± S.E.M. of three separate experiments.

Fig. 3. Time course of TNF-α induced IκB degradation in HT-29 cells. Confluent monolayers of cells were stimulated with TNF-α (100ng/ml) at 37ºC for the indicated times. Levels of IκB were determined by Western blotting using a specific monoclonal antibody as described in methods. The blot is representative of three separate experiments.

Fig. 4. Time course of TNF-α induced NF-κB activation in HT-29 cells. Nuclear extracts (10μg per lane) were prepared from cells treated with TNF-α (100ng/ml) as described in methods. Nuclear proteins were probed in EMSAs with ^{32}P labeled κB oligomer and autoradiographed. The blot is representative of three separate experiments. CC: extract from cold competition with unlabelled probe; C: extract from control non-activated cells.

Fig. 5. Effect of WIN55212-2 on TNF-α induced IL-8 release in HT-29 cells. Confluent monolayers of cells were incubated for 2h in the presence of increasing concentrations of WIN55212-2 prior to stimulation with TNF-α (100ng/ml) for 24h. IL-8 release was measured by ELISA as described in methods. Data represent means ± S.E.M. of six separate experiments. *denotes $P<0.01$.

Fig. 6. Effect of WIN55212-2 on IL-8 release in TNF-α pre-stimulated HT-29 cells. Confluents monolayers of cells were activated with TNF-α (100ng/ml) and WIN55212-2 (10^{-6} M) added at the time points indicated post-activation. Supernatants were collected 24h after TNF-α activation and analyzed by ELISA for IL-8. Data represent means ± S.E.M. of three separate experiments.

Fig. 7. Effect of WIN55212-2 on TNF-α induced NF-κB activation in HT-29 cells. Nuclear extracts were prepared from confluent cultures pre-treated for 2h with WIN55212-2 prior to activation with TNF-α (100ng/ml) for 1h. Nuclear proteins were probed with ^{32}P labeled κB oligomer and autoradiographed. C: non-activated control; A: TNF-α activated control. The blot is representative of three separate experiments.

Fig. 8. Effect of WIN55212-2 on TNF-α induced IκB degradation in HT-29 cells. Cell lysates were prepared from cultures pre-treated with WIN55212-2 for 2h prior to activation with TNF-α (100ng/ml) for 30 min. Levels of IκB were determined by Western blotting using a specific monoclonal antibody as described in methods. C: non-activated control; A: TNF-α activated control. The blot is representative of three separate experiments.

Fig 1

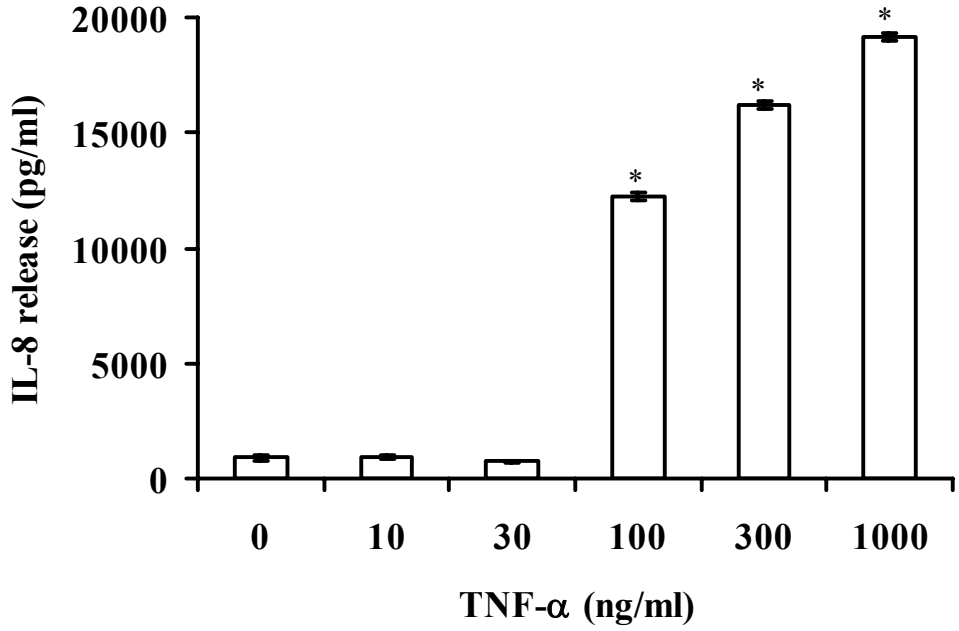
20000
15000
10000
5000
0
IL-8 release (pg/ml)
*
*
*
0
10
30
100
300
1000
TNF-α (ng/ml)

Fig 2

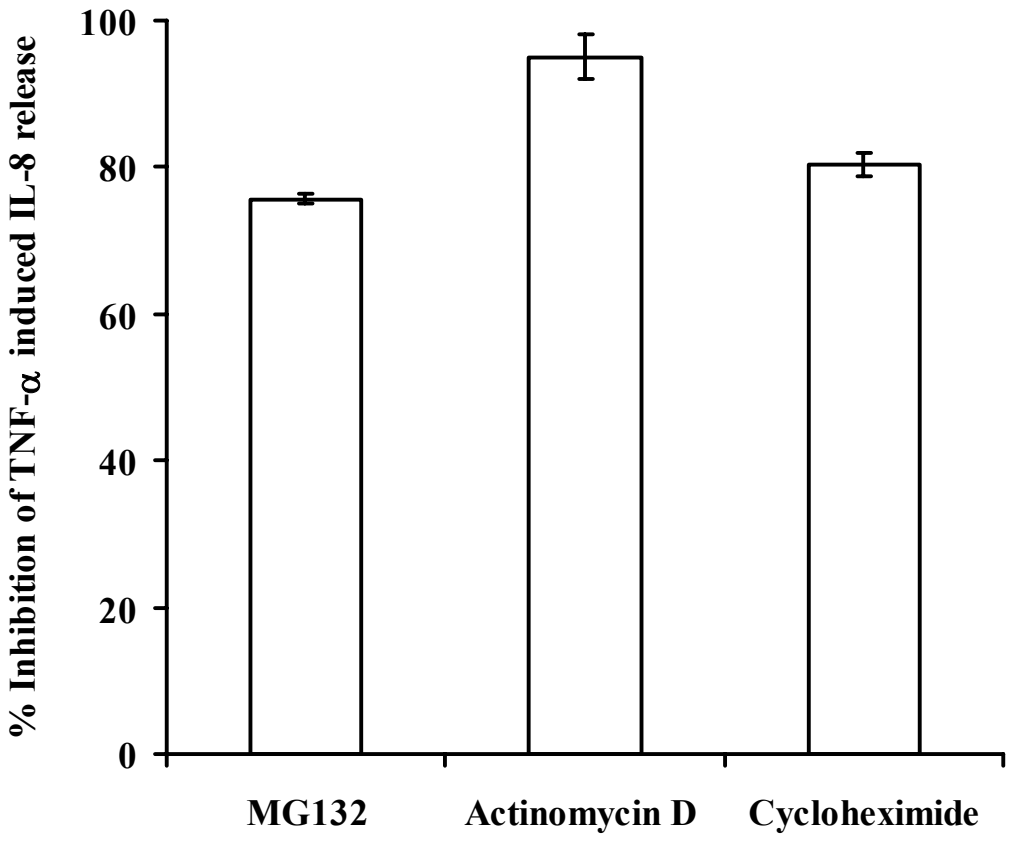
% Inhibition of TNF-α induced IL-8 release
100
80
60
40
20
0
MG132
Actinomycin D
Cycloheximide

Fig 3

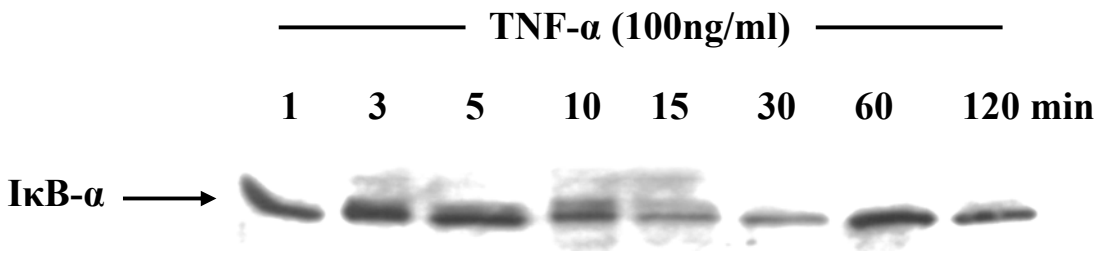

Fig 4

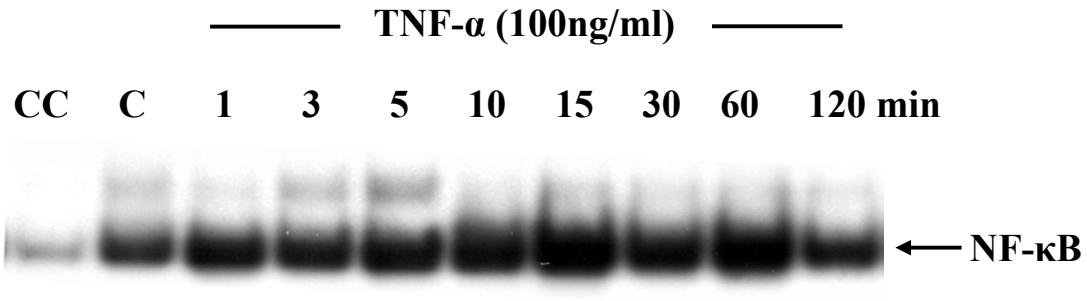

Fig 5

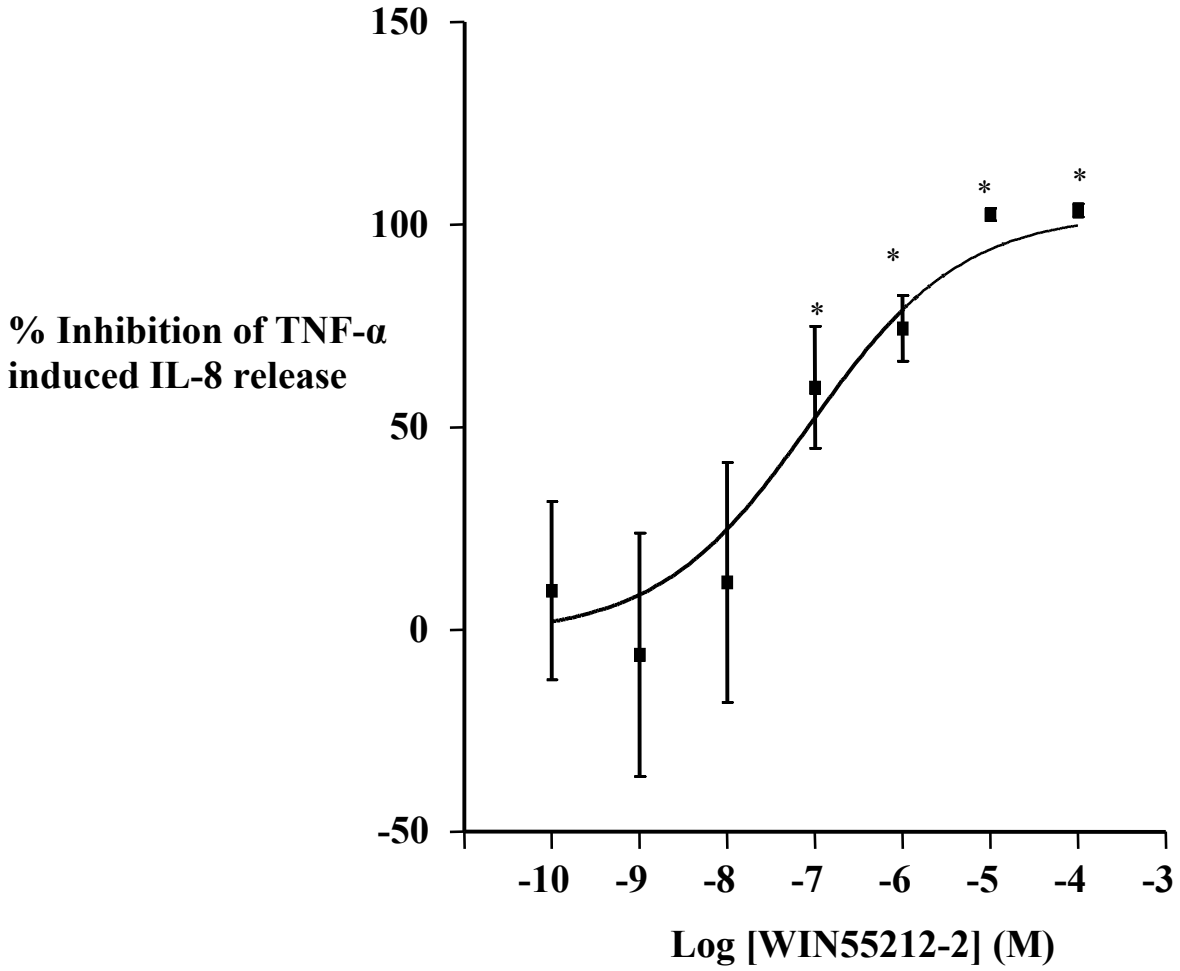
150
100
50
0
-50
% Inhibition of TNF-α induced IL-8 release
*
*
*
*
-10
-9
-8
-7
-6
-5
-4
-3
Log [WIN55212-2] (M)

Fig 6

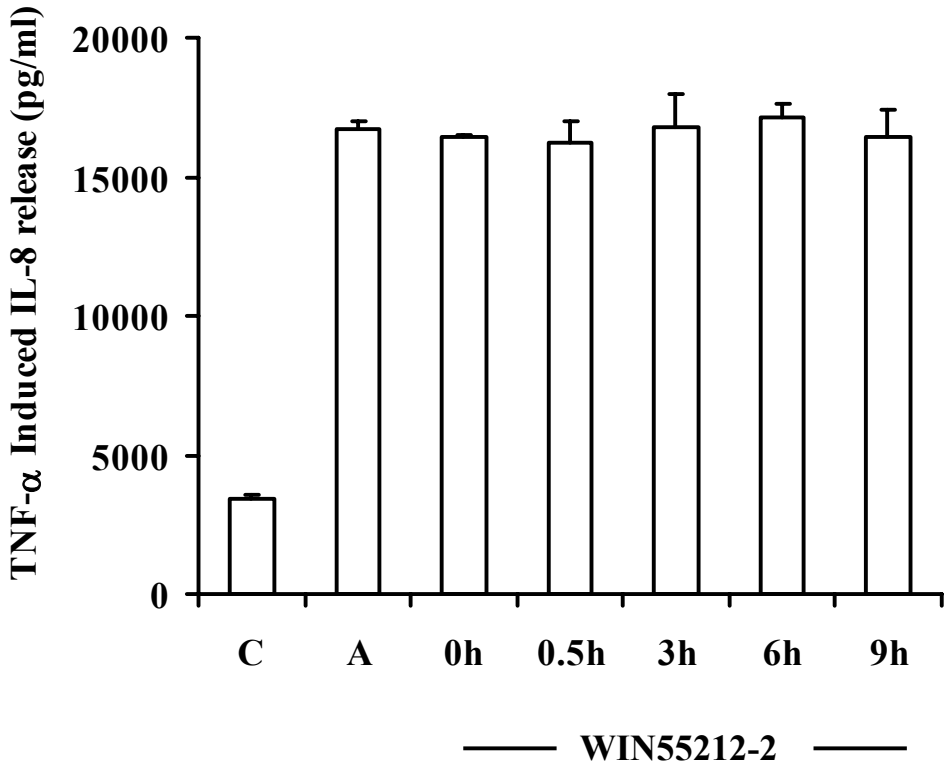
TNF-α Induced IL-8 release (pg/ml)
20000
15000
10000
5000
0
C
A
0h
0.5h
3h
6h
9h
WIN55212-2

Fig 7

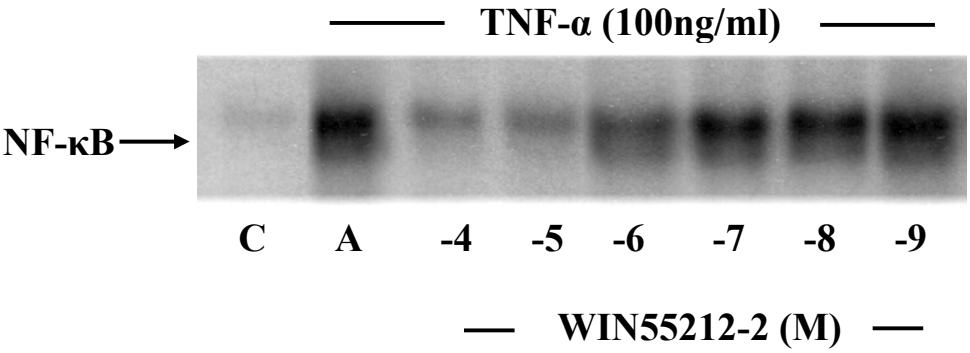

Fig 8