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INTERLEUKIN-10 INHIBITS CYTOKINE – MEDIATED SYNERGISITC RELEASE OF INTERLEUKIN-6 IN ASTROCYTOMA CELLS

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ABSTRACT

Interleukin-10 Inhibits Cytokine-mediated Synergistic Release of Interleukin-6 in Astrocytoma Cells

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Astrocytes respond to pro-inflammatory cytokines such as, interleukin-1 (IL-1β) and tumor necrosis factor (TNF-α). However, the mechanisms in which IL-1β and TNF-α mediate cell's signaling need further investigation.

In previous research, the effects of γ-aminobutyric acid (GABA) on IL-1 and TNF-α signaling pathway were studied. GABA was unable to suppress IκB-α degradation and the phosphorylation of p38 by IL-1β and TNF-α. However, it was suggested that GABA may be able to inhibit IL-6 release by reducing the rate of IκB-α degradation.

Another cytokine, IL-10, which is well known in literature to have anti-inflammatory effects, is investigated in this study. This study presents IL-10 effects on the NF-κB and p38 signaling pathways as well as IL-10 inhibition of IL-1 and TNF synergistic induction of IL-6 release. The effect of other signaling molecules, believed to act as antagonists for p38 activation, were also investigated and presented in this study. Previous studies indicate that both IL-1β

and TNF-α were able to stimulate the phosphorylation of p38 and the degradation of NF-κB inhibitor, IκB-α, with no change in IκB-β. While IL-10 is unable to suppress the phosphorylation of p38 by IL-1β or TNF-α or degradation of IκB-α, inhibitor of NF-κB, it is also suggested that IL-10 may inhibit cytokine-mediated synergistic induction of IL-6 in mechanistically similar way to GABA.

Inflammatory cytokines IL-1β and TNF-α have repeatedly shown to phosphorylate p38 and activate NF-κB pathway. We also have shown their synergistic effect on release of extracellular IL-6 as well as synergistic increase in transcriptional activation of IL-6 mRNA. Anti-inflammatory effects of IL-10, GABA and p38 inhibitor SB203580 were also investigated. Experimental results suggest that neither IL-10 nor GABA can reverse p38 or NF-κB activation induced by IL-1β and TNF-α. However, they inhibit synergistic release of IL-6 but have no effect on IL-6 transcriptional activation. Further, SB203580, inhibitor of p38, decreased synergistic mRNA transcript after stimulation with IL-1β and TNF-α. Our findings may postulate that anti-inflammatory molecules such as GABA and IL-10 may have similar mode of action supposedly affecting post-translational mechanisms.

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ABBREVIATIONS

Aβ – β-Amyloid
AD – Alzheimer's Disease
APP – Amyloid Precursor Protein
β-ME – 2-mercaptoethanol
BSA – Bovine Serum Albumin
CNS – Central Nervous System
DMSO – Dimethyl Sulfoxide
ECL – Enhanced Chemiluminescence
EDTA – Ethylenediaminetetraacetic Acid
ELISA – Enzyme-Linked Immunosorbent Assay
ERK – Extracellular-Related Kinase
EtBr – Ethidium Bromide
FBS – Fetal Bovine Serum
GABA – γ-Aminobutyric Acid
HEPES – 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HRP – Horseradish Peroxidase
5-HT – 5-Hydroxytryptamine
IκB – Inhibitor of κB
IKK – IκB Kinase
IL – Interleukin
IL-1i – IL-1 Inhibitor
IL-1ra – IL-1 Receptor Antagonist
INF – Interferon
IPT - Isoprotenolol
JNK – c-Jun-N-Terminal Kinase
kDa – Kilodalton
LA – lipoic acid
MAPK – Mitogen Activated Protein Kinase
NaCl – Sodium Chloride
NF-κB – Nuclear Factor-κB
NFT – Neurofibrillary Tangle
PAGE – Polyacrylamide Gel Electrophoresis
PBS – Phosphate-buffered Saline
PDL – Poly-D-Lysine
PHF – Paired Helical Filament
RPMI – Roswell Park Memorial Institute
RT-PCR – Semi-Quantitative Reverse Transcription Polymerase Chain Reaction
SDS – Sodium Dodecyl Sulfate

TBST – Tris-Buffered Saline containing Tween
TI – Tumor Necrosis Factor-α and Interleukin-1β
TNF – Tumor Necrosis Factor
Tris – Tris-(hydroxylmethyl)-amino methane

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CHAPTER 1

INTRODUCTION

Biochemical Basis of the Central Nervous System

The biochemical basis of the central nervous system (CNS) must be discussed to better understand the nature of Alzheimer's disease (AD). The CNS consists of two types of cells: glia and neurons. Neurons process and transmit information by receiving electrochemical impulses and conducting action potentials and glia protect and support the neurons. Neurons are organized in complex networks to perform the function of the nervous system. Once stimulated, they transmit the action potentials to other neurons or effector organs (Seely et. al 2000)

Neurons consist of a cell body also called soma and two types of processes: dendrites and axons. The soma contains one nucleus which is centrally located and contains extensive rough endoplasmic reticulum and Golgi apparatus which indicates that this is the main site of protein synthesis. Dendrites are cytoplasmic extensions that branch off the neuronal cell body and can be stimulated to generate electric currents transmitted to the soma (Seely 2000). Axons are the extension of the neuronal soma. They have a constant diameter but can vary in length from few millimeters to more than one meter. At their

terminal extensions axons form presynaptic terminals where many small vesicles containing neurotransmitters are also present. Action potentials are conducted along the axon to the presynaptic terminal, where they can stimulate neurotransmitter release.

Neuroglia, on the other hand, are far more numerous than the neurons. They provide support, protection and nutrition for neurons. They maintain homeostasis, form myelin and participate in signal transduction in the nervous system. Glial cells are divided into two subgroups according to their structure and function: macroglia and microglia. Oligodendrocytes and astrocytes are macroglia. Oligodendrocytes have cytoplasmic extensions that surround and wrap around the axons and produce the myelin sheet to protect the neuron, except at the nodes of Ravier. Astrocytes possess extensions which spread out to occupy areas close to the blood vessels and provide nutrients to the neurons nearby. Astrocytes regulate the extracellular composition of brain fluid by releasing chemicals which promote the formation of tight junctions between endothelial cells and capillaries (Seely 2000). They also regulate the concentration of ions and gases and absorb and recycle neurotransmitters. Microglia provide the immune defense for the CNS and are specialized macrophages found around the brain and blood vessels. They become activated in response to injury or inflammation and release cytokines and other signaling molecules to repair the damage, phagocytize microorganisms or foreign substances.

Neurotransmission

Since glial cells lack an excitable membrane, they cannot transfer information. In order to transmit information in the CNS, neurons generate action potentials that reach chemical or electrical synapses and finally post synaptic receptor proteins. Presynaptic neurons receive signals causing them to release either excitatory or inhibitory neurotransmitters. These signaling molecules eventually reach and bind receptors on a postsynaptic neuron, causing an action potential to be generated. Subsequently, the postsynaptic neuron will propagate either excitatory or inhibitory signal.

Neurotransmitter vesicles in the pre-synaptic neuron undergo exocytosis in to the synaptic cleft in the wake of an action potential. Once in the synapse, the neurotransmitter can then bind the postsynaptic receptors on the postsynaptic neuron and continue the propagation of information in the neuronal net.

Molecular Pathology of Alzheimer's disease

Alzheimer's disease (AD) affects many elderly people and is one of the leading causes of death in developed nations. It is a slow but progressive terminal disorder that is characterized by deterioration of cognitive and functional abilities as well as psychiatric and behavioral symptoms (Yates et.al 2008). It was first described by Alois Alzheimer in 1907 as a 'peculiar disease of the cortex' that affects the CNS (Alzheimer, 1907). Nowadays, a century later, there is still no cure for the disease and its cause is still unknown but there are various

hypotheses that have been developed based on experimental data (Behl, C.,1999). Pathologists use three main biochemical features to define AD – extracellular senile plaques, intracellular tau-rich (τ-rich) neurofibrillary tangles (NFT) (Glenner and Wong, 1984) and inflammatory processes that contribute to deposition and formation of senile plaques (Rogers et. Al, 1996).

Kang et al. reported in 1987 that amyloid plaques (Aβ) or develop from amyloid precursor protein (APP). APP is a ubiquitous Type 1 membrane glycoprotein and is generated throughout life in all mammalian cells (Haass, C. *et al.*, 1992). APP is processed by β-secretase (BACE) and the presenilin-containing γ-secretase complex (Haas, 2004). β-secretase cleaves APP to produce a soluble version of APP (β-APP) and a 99-residue COOH-terminal fragment that remains bound to the membrane. This 99-rsidue fragment is a substrate for γ-secretase which performs the unusual cleavage in the middle of the transmembrane domain to produce a 4-kDa Aβ (Yoo-Hun Suh and Frederic Checler, 2002). It is also worth noting that the APP gene is located on the chromosome 21, an extra copy of which characterizes Down Syndrome. Patients suffering from both of these diseases exhibit increase amounts of Aβ proteins which leads to the formation of plaques (Mann and Esiri 1989). As suggested by Wisniewski et al. (1985) overprexpression of APP gene may accelerate the development of these neurodegenerative diseases although the cause is still not understood.

It is generally believed that the abnormal processing of APP leads to the aggregation and deposition of Aβ and ultimately leads to the pathogenesis of AD.

This understanding is generally referred to as 'amyloid cascade hypothesis' and is supported by a large number of studies but is not entirely accepted (J. Hardy, 2006).

The intracellular τ-rich NFT's are another feature of AD. Like Aβ plaques, NFT's are formed by the aggregation of proteins that are on the inside of the neuron and gradually fill the intracellular space. The main component of these neurofibrillary tangles is microtubule-associated protein tau (C. Ballatore, 2007). Under normal conditions tau is bound to the microtubules comprising the neuronal skeleton and stabilizes their structure. In AD tau becomes detached from the microtubules and aggregates to form NFT's. This process is also associated with phosphorylation of tau proteins which may be the onset of NFT formation. Thus, destabilized neuronal microtubules and NFT's inclusions seriously impair neuronal function even before cell death. Tau aggregates as paired helical filaments (PHF) to form the NFT's and in addition to that it is also found around senile plaques (Duyckaerts C, Dickson DW., 2003). There are several kinases that are involved in tau phosphorylation and formation of PHF's in AD, such as stress activated c-Jun N-terminal kinase (SAPK/JNK) and p38 kinase (p38), cyclin-dependent kinase-5 (CDK-5), glycogen synthase kinase-3β (GSK-3β) (Lovestone, Reynolds, 1997). Moreover, there two to three phosphate groups per tau molecule in normal adult brain vs. hyperphosphorylation, which results in 7-8 mol phosphates in AD (Ksiezak-Reding et al., 1992; Kopke et al., 1993).

Inflammatory responses may also be associated with AD onset. Documented occurrences of inflammatory processes in traumatic injuries to the CNS and in chronic diseases such as AD, suggest that inflammatory cytokines may be involved in neurodegenerative process (Schultzberg et al., 2007). Cytokines are small, multifunctional proteins that respond to injuries and infection and are produced by the glial cells when an inflammatory response is initiated. Some of the pro-inflammatory cytokines are interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α) and are involved in the initiation of the inflammatory process. There are reciprocal interactions between proinflammatory cytokines such as IL-1β and IL-6, and APP/β-amyloid (Aβ) peptide (Buxbaum JD,1992 and Del Bo R, 1995). Cytokines stimulate the synthesis and metabolism of APP and Aβ peptide induces the production and secretion of cytokines (Del Bo R, 1995). Depending on the Aβ peptide species and their aggregated forms the secretion of cytokines from rat neonatal microglia proceeds via different mechanisms. For instance, freshly dissolved Aβ peptides are more successful in initiating cytokine release than other larger forms (Lindberg C., 2005). Increasing release of proinflammatory cytokines regulates the processing and expression of APP which further leads to deposition of more Aβ fragmants (Goldgaber D., 1989). Microglial cells eventually become unsuccessful in removing these plaques and lyse, releasing the collected fragments (Nagele et al., 2004). This recursive system continues leading to the expression of AD.

Mitogen-Activated Protein Kinases

Mitogen-Activated Protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities. In mammalian cells the MAPK family consists of three main kinase subfamilies, the c-Jun-N-terminal kinases (JNKs), the p38 kinases, and the extracellular signal-regulated kinases (ERKs). The MAPK's phosphorylate on specific serine and threonine residues of their target proteins. In addition, they propagate and amplify external stimuli coming from the membrane, going to the cytoplasm and finally reaching the nucleus by phosphorylating and dephosphorylating with the help of phosphatases. The c-Jun-N-terminal kinases regulate gene transcription and are able to influence degenerative processes that depend on *de novo* protein synthesis. The ERK subfamily is composed of ERK1 and ERK2 and they are primarily involved in cell division, differentiation and proliferation. The p38 MAP kinases are encoded by four different genes (p38α,β,γ,δ). They get activated in times of stress by inflammatory cytokines. MAPK's are ubiquitously expressed and are present in nervous tissue. Growing research suggests that p38 may be involved in neurodegenerative diseases such as AD by contributing to neuronal death (Bendotti, C, 2006). P38 is activated by double phosphorylation of Thr 180 and Tyr 182 in a Thr-Gly-Tyr motif near the active site in response to inflammatory stimuli such as TNF and IL-1β (Ono, K. and Han, J., 2000). Interestingly, a substrate for p38 MAPK is tau protein. Once phosphorylated, tau can accumulate to form NFT which is one of the hallmarks of AD (Reynolds et al., 1997). P38 contributes to the neurotoxicity of Aβ.

Cytokines – Interleukin Family

Cytokines are low-molecular weight signaling proteins that are involved in many cell processes such as differentiation, proliferation and cell death. Cytokines can be divided according to their function. Some can be classified as growth factors, pro- or anti-inflammatory molecules or just aiding in the progression of immune response to an antigen. Cytokines are signaling molecules such as the interleukin family (IL), tumor necrosis factor (TNF) and many other growth factors. Cytokines can be classified in different families based on structure and activity. The TNF family includes more than 20 different members, encoded by different genes but with overlapping functions. The IL family includes 33 members, identified by a number 1-33. Some of the members of IL-1 subfamily include cytokines IL-1β and IL-1α which function as pro-inflammatory molecules (Dinarello, C.A., 2007). The IL-6 family includes members such as IL-6, leukemia inhibitory factor (LIF), and IL-11. The IL-10 family includes cytokines IL-10, IL-4 and IL-22 which are involved in inhibiting the immune response (Dinarello, C.A. ,2007).

The IL-1 family of cytokines consists of three members: IL-1α, IL-1β and their negative regulator (IL-1ra). All three are encoded by a different gene but are similar on a structural level. IL-1α and β are agonist molecules because they induce cellular responses. IL-1ra, however, binds to the same receptor but inhibits the signaling of IL-1 and is therefore considered an antagonist (Dinarello C A. 1994). IL-1α and β have similarities both in gene sequences and structurally, being mainly composed of 12 to 14 folds of β-pleated sheets (Murzin

A G, Lesk A M, Chothia C., 1992). In addition, the crystallographic structure of these cytokines reveals an Arg residue at the same position that is necessary for their biological function (Nanduri V B. et al., 1991). Inflammatory and stress processes stimulate the transcription of IL-1α and β. In fact, Nawroth P. et al. (1986) reported that TNF-α can stimulate the production of IL-1 in endothelial cells. In addition, IL-1 or TNF-α were also found to induce release of IL-1β *in vivo* (Dinarello C A., 1989). The IL-1ra antagonist is primarily secreted in the extracellular environment and is composed of β-pleated sheets, similar to IL-1α and β (Vigers G P. et al. 1994). Interestingly, IL-1ra has only one binding site for its receptor, similar to IL-1β, which was confirmed in a directed mutagenesis study by Evans et al. in 1995. This fact also explains the interruption of signal transmission in IL-1ra receptor antagonist.

Tumor necrosis factor-alpha (TNF-α) is a wide spread cytokine with various functions, such as promoting growth and inhibition, angiogenesis, inflammation and cytotoxicity. Many cells have been reported to produce and secrete TNF-α in response to infection or tumor growth. Immune cells are primarily involved in the release of this cytokine (Aggarwal B B, Natarajan K., 1996). TNF-α is initially synthesized as a 26 kDa membrane-bound protein which later is cleaved to a mature 17 kDa form while its prosequence part remains associated with the membrane (Jue D M., 1990). Being one of the main pro-inflammatory cytokines, TNF-α plays a central role in initiating and regulating inflammatory processes. Bacterial lipopolysaccharide (LPS), injury, and viruses can stimulate the expression of TNF (Vassalli P., 1992). Notably, TNF along with

interferon gamma (IFN-γ), potently stimulates other pro-inflammatory cytokines such as IL-1 and IL-6 which in turn activate T and B cells and stimulate lepatic acute phase protein synthesis. Interestingly, TNF is observed to be elevated in the serum, CSF and cerebral cortex in AD patients (H. Fillit, W.H. Ding, L. Buee *et al.*, 1991). In 2001 Lucia et al. demonstrated that Aβ induces production of TNF in rat microglia. In addition, TNF as well as IL-1β were also found to exert both neurotrophic or neurotoxic effects, depending on their concentration and site of synthesis (Rothwell NJ, Hopkins SJ., 1995). This shows that TNF directly contributes to AD through subsequent neuroinflammation.

Interleukin-6 is another pleiotropic cytokine that is involved in inflammation processes in CNS. It is produced following viral or pathogen invasion as well as during inflammation (Van Snick J., 1990). The major species isolated from human T-cell line was reported to be an unglycosylated peptide with molecular mass between 19 to 21 kD (Hirano et al., 1985). Moreover, Santham et al. (1989) reported that secreted IL-6 is glycosylated and its molecular mass ranges from 23 to 30 kD. Previous studies show that IL-6 acts through gp130 receptor and affects the functioning of B and T cells, neuronal cells and many others (Kishimoto T, Akira S, Narazaki M, Taga T., 1995). It is interesting to note that IL-6 has anti-inflammatory functions as well as a pro-inflammatory role. It induces the production of IL-1ra, which is a receptor antagonist of IL-1 and also decreases production of TNF (Tilg H, Dinarello CA, Mier JW., 1997). By decreasing the amount of pro-inflammatory cytokines such as IL-1 and TNF, IL-6 actually shows an anti-inflammatory role. However, it is found in high

concentrations in tissues under stress and is able to take part in inflammatory processes. In addition, astrocytes and microglial cells in the CNS secrete IL-6 in times of infection or increased concentrations of other inflammatory cytokines. Moreover, it has been shown that IL-1β was able to activate the NFκB transcription factor and thus stimulate IL-6 promoter transcriptional activity (Zetterstrôm M. et al., 1998).

Signal Transduction Pathway of Interleukin -1β

The IL-1 family consists of at least three proteins – the agonist IL-1α and β and the receptor antagonist IL-1ra. IL-1α and β are synthesized precursor proteins. While IL-1α is active in its precursor form, IL-1β needs further cleavage by its converting enzyme (ICE; caspase-1), (Howard A D. et al., 1991). IL-1ra exists in three forms – one secreted and known to block IL-1 activity, and two additional intracellular forms which are all encoded by the same gene (Muzio M. et al., 1995). There are two membrane bound receptors – type I (IL-1RI) and type II (IL-1RII). The signal is initiated upon binding of IL-1β to the type I receptor. The IL-1RII is believed to be a non signaling receptor, serving as a decoy and trap for IL-1β. There is also an accessory protein involved in IL-1 signaling. The IL-1RAcP accessory protein, exhibits structural similarities to IL-1R subtypes but is unable to bind IL-1 alone. Instead, it forms a complex with IL-1RI and with any of the IL-1 agonists (Greenfeder S A. et al., 1995). IL-1 binds to its receptor and its accessory protein and this trimeric complex induces binding of myeloid-differentiation primary binding protein to the formed complex. This allows the

recruitment of IRAK-4 and IRAK-1. Phosphorylation of IRAK-1 results in the binding of TRAF (TNF receptor associated factor)-6 to the initial complex. This on the other hand, allows the dissociation of a part of the complex (TRAF-6/phopho-IRAK-1) which binds to another membrane bound protein complex TAK-1. The phosphorylatioon of TAK-1 results in the degradation of IRAK-1, releasing the TRAF-6-TAK-1 from the protein complex associated with the plasma membrane (Akira, 2003). The fully activated TAK-1 protein activates various signaling molecules that activate many genes through transcription factors such as NF-κB. Interestingly, TAK-1 is also able to induce IL-1β mediated activation of MAPK signaling.

Nuclear Factor – κB

Transcription nuclear factor –κB (NF-κB) belongs to the Rel family of proteins. It is a dimeric protein with each chain containing an N-terminal 300 amino acids conserved region called the rel homology domain (RHD). This region is responsible for DNA binding and interaction with the NF-κB inhibitors, IκB (Ghosh, 1998). In the cytoplasm, NF-κB is associated with its inhibitors IκB preventing its migration to the nucleus for activated gene transcription. IκB is a large family of inhibitors which include IκB-α, β, ε and γ. The best studied and characterized inhibitor is IκB-α, primarily due ti its initial discovery (Davis N., 1991). In order to be activated, IκB-α needs to be phospshorylated on its N-terminal domain in response to chemical signals. IκB-β is less studied because it was characterized after IκB-α. However, they both bind the same Rel subunits

but respond differently to chemical signals. The difference in activity may be due to strength or duration of signal. (Thompson J, 1995). Activated NF-κB promotes the transcription of IκB-α for feedback supression. On the other hand, NF-κB, once released from its inhibitor, does not promote transcription of IκB-β. Interestingly, one can correlate this permanent activation of NF-κB to inflammatory processes in AD which further exacerbate neurodegeneration.

According to Spiecker et al., (2000), IκB-ε, which is much less studied than the previously discussed inhibitors, and its functional relationship with NF-κB is not well understood. Finally, previous research suggests that IκB-γ inhibits NF-κB by preventing it from binding to DNA by associating with different DNA binding residues (Bell et al., 1996).

NF-κB is initially associated with its inhibitor IkB which in times of inflammation is phosphorylated by IκB kinase (IKK), a type of transferase which usually transfers a phosphate group. The cell marks the phosphorylated molecule for further degradation in the proteosome by ubiquitination. The other product of the action of IKK is the released NF-κB which can translocate to the nucleus and initiate transcription of various genes. These genes can encode other pro-inflammatory cytokines such as IL-1, TNF and IL-6, which can further stimulate the inflammatory process. Thus, the activation of NF-κB can be assessed indirectly by measuring the degradation of its inhibitors.

γ-Aminobutyric acid and catecholamines

γ-Aminobutyric acid (GABA) is one of the most important neurotransmitters in the CNS because it mediates membrane hyperpolarization and depolarization. It synthesis is accomplished by decarboxylation of glutamate by glutamic acid decarboxylase (GAD). GABA is then further modified and metabolized by the citric acid cycle. There are two classes of GABA receptors which are divided according to their function: the ionotropic receptor $GABA_A$ and the metabotropic receptor, $GABA_B$. $GABA_A$ receptors are chloride ion channels which open following GABA binding. $GABA_A$ receptors are primarily composed of five different subunits each belonging to a different subclass (Sarto-Jackson I., 2008). The $GABA_B$ receptor is a metabotropic G protein coupled receptor which initiates slow inhibitory responses. Activation of $GABA_B$ receptors increases potassium conductivity and decreases calcium currents which may be responsible for the amelioration of neurotransmission (Kerr and Ong 2001). Previous studies suggest that GABA can inhibit IL-1β mediated IL-6 release (Spangelo et al., 2004).

Other compounds that increase the intracellular level of cAMP also induce IL-6 release. Catecholamines are derived from tyrosine and contain catechol and amine groups. The catecholamines include epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine. Catecholamines are hormones secreted form the adrenal gland in response to not prolonged stress. Their mode of action is through elevating cAMP intracellular levels. Other drugs which elevate cAMP include cholera toxin and Bu_2cAMP. In fact, previous results

showed that Bu_2cAMP increases IL-6 mRNA accumulation in and IL-6 release from anterior pituitary cells (Spangelo et al., 1990). Interestingly, catecholamines are also able to stimulate the synergistic release of IL-6 in the presence of another cytokine, IL-1β (Zumwalt et al., 1999). This is important in disease processes in the CNS where the level of cytokine production is elevated in response to stress. Since AD is characterized by neurofibrillary tangles and senile plaques, production of IL-1 and IL-6 may further exacerbate the pathogenesis of this condition. Increasing levels of IL-1β may also increase levels of catecholamines which can further increase production of IL-6 and stimulate neuronal degradation.

Interleukin-10: An Anti-Inflammatory Cytokine

Astrocytes and microglial cells are activated in response to cellular injury and brain trauma. Glia function in regulating the inflammatory reaction and neuronal tissue repair in the CNS (Ridet et al., 1997). Astrocytes secrete cytokines and they also express membrane cytokine receptors suggesting that they can be targets of pro-inflammatory cytokines like IL-1β, TNF-α, or IL-6 (Ban et al., 1993). IL-1 is well known to cause cell death of neurons and to induce astrogliosis by the production of stress molecules such as nitric oxide (NO), arachidonic acid derivatives, or cytokines (Lee et al., 1995). To counter this process, the organism uses different tactics including the expression of IL-1ra type II receptor 'decoy' and production of the anti-inflammatory cytokines IL-10 and IL-4. Moore et al., 1993 showed that IL-10 inhibits the production of the pro-

inflammatory cytokines IL-1β, TNF-α and IL-6. Interleukin-10 was first described by Fiorentino et al. in 1989 as a cytokine synthesis inhibitory factor (CSIF). First described as an inhibitor of cytokine synthesis, it was classified as a Th-1-immunosupressive cytokine similar to interferon gamma and tumour necrosis factor-alpha. CSIF was later cloned from human cDNA (Vieira et al., 1991) and renamed IL-10. The human IL-10 gene encodes a protein of 178 amino acids which is cleaved to produce a mature sequence of about 18kDa, containing four cysteine residues (Windsor et al., 1993). It is noteworthy to mention that IL-4 is another anti-inflammatory cytokine with similarities to IL-10. For instance, IL-10 is synthesized in the normal brain (Wong et al., 1996) and in times of inflammation and brain injury both cytokines are expressed (Woodroofe et al., 1993). Glial cells were proposed to be major sources of IL-10 as well as IL-4 (Mizuno et al., 1994). Pousset et al., 1999 proposed that IL-4 and IL-10 might be active at different stages of the inflammatory response. Previous research investigated the effects of these anti-inflammatory cytokines on IL-1β induced IL-6 production by mouse primary astrocytes and reported that IL-4 and IL-10 differentially regulate IL-6 levels in activated astrocytes. They further suggested that IL-10 has anti-inflammatory properties while IL-4 represents stimulating effects depending on the cell type and cytokine environment (Pousset et al., 2000).

IL-10 activates the Jak/stat signaling pathway. It is suggested that IL-10 interferes with the Jak family tyrosine kinases by inducing tyrosine phosphorylation and activating transcription of latent transcription factors stat3 and Stat1 (Finbloom DS, Winestock KD., 1995). In addition, it was also reported

that IL-10 inhibits NF-κB activation in response to stimuli. This accomplished by two plausible mechanisms. The first is by inhibiting the activation of IκB kinase, and the second, by inhibiting NF-κB DNA binding activity (Schottelius et al., 1999). Furthermore, Pousset et al., 2000 reported that intracellular signaling pathways such as NFκB and Akt may be connected and downregulated by IL-4 and IL-10. Previous results also suggested that these anti-inflammatory cytokines may block the inflammatory effects of IL-1β through these pathways in astrocytes.

The anti-inflammatory properties of interleukin-10 need to be further investigated in light of its known functions of macrophage activation and T cell cytokine synthesis (Moore et al., 1993). Also, by further investigation of anti-inflammatory cytokines and chemokines, inflammatory processes in neurodegenerative diseases such as AD can be better understood.

Hypothesis Statement

Although IL-10 has been studied as an anti-inflammatory cytokine, its effects on astrocytes and in particular C6 glioma cell line, need to be further investigated. As reported previously, IL-10 interferes with NFκB signaling pathway (Pousset et al., 2000). Since NFκB signaling pathway as well as p38 can also be activated by IL-1β and TNF-α to induce the release of IL-6, we hypothesize that : [1] IL-10 inhibits the synergistic induction of IL-6 mediated by IL-1β and TNF-α; and [2] IL-10 may also interfere with IL-6 transcription induced by TNF-α and IL-1β.

CHAPTER 2

MATERIALS AND METHODS

Chemicals, Reagents and Equipment

γ-Aminobutyric acid (GABA), glycine, sodium chloride (NaCl), potassium chloride, 4-(2-hydroxyethyl)-monosodium salt (HEPES), sodium dodecyl sulphate (SDS), tris-(hydroxymethyl)amino-methane (Tris), tris-(hydroxymethyl)amino-methane hydrochloride, Tween-20, dimethyl sulfoxide (DMSO), 2-mercaptoethanol (β-ME), and bromophenol blue were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest grade possible. High molecular weight poly-D-lysine (PDL) hydrobromide was obtained from BD Biosciences (Bedford, MA). The prestained protein ladder was obtained from Bio-Rad (Hercules, CA). Roswell Park Memorial institute (RPMI)-1640, PSN antibiotic mix, trypsin-ethylenediaminetetraacetic acid (EDTA), heat-inactivated fetal bovine serum (FBS), phosphate buffer saline (PBS pH 7.2), RNeasy MINi Kit, quantiTec Reverse Transcription Kit and Qlashredder were obtained from QIAGEN, USA. Trypan blue was obtained from Invitrogen (Carlsbad, CA). Methanol was obtained from EMD Chemicals (Gibbstown, NJ). Bovine serum albumin (BSA) was obtained from Gemini Bioproducts (Woodland, CA). Recombinant rat IL-1β, TNF-α, IL-10, IL-4 and anti rat IL-6 antibodies were

obtained from PeproTech Inc. (Rocky Hill, NJ). Rabbit anti-rat IκB-α, IκB-β, phosphorylated p38 (T180/Y182), total p38, rabbit anti-rat IL-6 antibody, β-Actin and the biotinylated protein ladder was obtained from Cell Signaling (Danvers, MA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from KPL (Gaithersdurg, MD). The rat C6 Glioma cell lines were obtained from the American Tissue Type culture Collection (Rockville, MD). Rat IL-6 sandwich enzyme-linked immunosorbent assay (ELISA) kits, M-PER®, precasted 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels, protease and phosphatase inhibitor cocktails, and the microBCA® [rotein assay kit were all obrained from Pierce Biotechnology (Rockford, IL). Hoefer SE 260 minivertical gel electrophoresis unit, EPS 2A200 Power supply, Hoefer TE 22 tank transfer unit, MultiTemp III thermostatic circulator, Enhanced Chemilminescence (ECL)-Plus® detection reagent was obtained from Amersham (Piscataway, NJ). Schleicher & Schuell Optitran® brand supported 0.45 um nitrocellulose membranes were obtained from ISC Bioexpress (Kaysville, UT). Rabbit anti-rat IL-6 antibody was purchased from Cell Sciences (Canton, MA). All small molecule inhibitors dissolved in sterile solvent, aliquoted and stored at -20°C until use. The final concentrations of DMSO never exceeded 0.1% of the final solution.

C6 Glioma Cell Culture

Rat C6 glioma cells were maintained in continuous culture in a humidified atmosphere containing 95% air and 5% CO_2 in complete medium (RPMI-1640/

phenol red, medium containing 10% heat-inactivated FBS, 25mM HEPES pH 7.4, and PSN antibiotic mix). Cells were grown either in 25 cm^2 or 75 cm^2 area flasks. After 3-4 days in culture, cells were removed from the tissue culture flasks with 2 mL (for the 25 cm^2 area flask) or 6 mL (for 75 cm^2 area flask) of 0.25% trypsin/ 0.05% EDTA in Hanks-buffered salt solution. Trypsin was inactivated upon the addition of 8-24 ml of complete medium, cells were centrifuged, pelleted, supernatant removed, and the pelleted cells resuspended in complete medium. Cell suspension densities and cellular viability were determined via trypan blue exclusion. The cells were then placed (passed) back into continuous culture ($1.25x10^6$ cells/25 cm^2-area flask, BD Falcon) or were seeded into tissue culture plastic-ware at the plating densities noted for experiments. In all experiments presented, C6 glioma cells were used between passages 5-35.

Cytokine Quantification via ELISA

Rat C6 cells were plated ($0.125x10^6$/well) on a 96-well plate using Poly-D-Lysine (PDL) in complete medium and allowed to incubate 24 h to ensure attachment. For the experiment, cells were washed twice with 2mll serum-free RPMI 1640 and incubated serum or serum-free RPMI-1640 in the absence or presence of agents for the times and doses indicated for the experiments. IL-6 release was then assayed via rat IL-6 ELISA (Pierce Biotechnology) according to the manufacturers' instructions.

Colletion of Cellular Protein Lysates

Rat C6 glioma cells were plated ($3.0x10^6$/dish) on PDL-coated 35x10mm dishes in complete medium and allowed 24 h to attach. Following the attachment period, the cells were washed twice with 2ml serum-free RPMI-1640 and incubated in serum or serum-free RPMI-1640 in the absence or presence of stimulating agents for the times and concentrations. After treatments cells were washed twice in 2ml ice cold PBS and scraped in 200µL of M-PER® (Pierce), containing both phosphatase and protease inhibitors (each from Pierce). Protein- containing solutions were kept on ice and vortexed in 10 min intervals for a total time of 30 min on ice. Lysates were then centrifuged at 14,000 x g for 10 min at 4°C, and 175µL of the supernatant was recovered. Protein concentrations were determined with Micro-BCA protein assay kit (Pierce) using BSA as a standard. Protein lysates were diluted by 20% using a 2X SDS-PAGE loading buffer (10% w/v SDS, 2% v/v glycerol, 0.1% w/v bromophenol blue, 0.5M Tris pH 6.8, and 5% v/v β-ME), and boiled for 2 min. Protein lysates were subsequently separated by SDS-PAGE Western blot analysis or at -80°C for future analysis.

RNA Extraction and Semi-Quantitative RT-PCR Analysis

Rat C6 glioma cells were plated (3.0×10^6/dish) on PDL-coated 35 X 10mm dishes in complete medium, and allowed 24 h to attach. Following the attachment period, the cells were washed twice with 2 ml serum-free RPMI-1640 and incubated in 10% FBS RPMI-1640 in the presence or absence of stimulating agents for the times and doses indicated for the experiments. After treatments

cells were washed twice in 2ml ice cold PBS. Total RNA was then extracted using QIAGEN RNeasy MINI Kit purification system.

To detect the presence of mRNA encoding IL-6 and β-Actin, semi-quantitative RT-PCR analysis were performed as described by Elsawa et al., 2004 and Bost et al., 1995. One µg of the total RNA was reverse transcribed using QuantiTec Reverse Transcription Kit (QIAGEN) and apportion of the total cDNA was amplified using 94°C denaturation, 55°C annealing and 72°C extension temperatures, for 30 cycles with the first four cycles having extended times. Positive and negative strand primers used for the amplification of each mRNA species were obtained from QIAGEN, USA. Specific sequences were not supplied because of company propriety rules. Amplified products were electrophoresed on 0.5µg/ml ethidium bromide stained gels and visualized under UV illumination. Analysis of cDNA samples were corrected for expression of β-Actin.

SDS-PAGE Western Blot Analysis

Protein lysates were separated on a SDS-PAGE gel. Poly-acrylamide gles were purchased precasted (Pierce). The gels were subsequently transferred to a Hoffer (0.1 M HEPES, 0.1 M Tris, 0.1% w/v SDS) running buffer. Equal amounts of protein were loaded into each lane of the precast gel. Biotinylated and prestained protein ladders were also loaded onto the stacking gel to determine the molecular weight mobility and transfer efficiency, respectively. The proteins were electrophoretically separated at 4°C, using a constant voltage of 100V.

Following electrophoresis, the proteins were transferred (Hoefer TE22 Mighty Small TransPhor Tank, Amersham) to 0.45 µm nitrocellulose membranes at 4°C, using constant amperage of 360 mA for 90 min in Western Transfer buffer (25 mM Tris, 192 mM Glycine, and 20% v/v methanol, note: pH was not adjusted). Following the transfer period, protein-containing membranes were washed once for 5 min in tris-buffered saline containing tween (TBST: 50mM Tris pH 7.4, 150 mM NaCl, and 0.1 % v/v Tween-20) for 5 minutes. After the washing period the membranes were blocked with TBST containing 5% nonfat milk (Nestle Carnation) for 1 hour, then washed three times in 5 min intervals in TBST. The membrane was incubated 12 h with primary antibodies at 4°C. After the antibody incubation period, the antibody solution was removed and the membranes washed in TBST three times for 5 min intervals. Membranes were then incubated with a secondary antibody conjugated to a horseradish peroxidase (HRP) and anti-biotin. Both secondary and anti-biotin antibodies were diluted using the manufacturers' recommendation, of 1:5000 and 1:1000, respectively. All antibodies were diluted in TBST containing 5% BSA. The incubation period for secondary antibody and anti-biotin was1 h and afterwards both were removed and washed in TBST three times for 5 minute intervals. Proteins were visualized on the Typhoon multipurpose imager using the ECL-plus® detection reagent (Amersham).

Statistical Analysis

Statistical analyses consisted of analysis of variance (ANOVA), with significance confirmed using the Bonferroni test for multiple comparisons (GraphPad Instat, version 3.0). A *P*-value of ≤ 0.05 was considered significant. Where appropriate, data expressed as the mean ± SEM of groups consisting of three to four observations and each experiment was performed at lest three times.

CHAPTER 3

RESULTS

Other inhibitory modulators and their effects on IL-1β signaling

We investigated the effects of various signaling molecules, both inhibitors and enhancers of two signaling pathways: NF-κB and p38 MAPK. The effects of serotonin (5-HT) and lipoic acid (LA) on IL-1β mediated phosphorylation of p38 were investigated in a Western format. As suggested in literature, these inhibitory molecules are potent anti-inflammatory mediators. IL-1β successfully phosphorylates the total p38, but 5-HT was unable to reverse the effects of this cytokine **(Figure 2)**. Moreover, different concentrations of 5-HT were not able to reduce the p38 phosphrylation induced by IL-1β (**Figure 3**).

Modulators affecting cAMP

Bu_2cAMP increases cAMP and therefore the phosphorylation of p38 however, no synergistic effect was observed with IL-1β and Bu_2cAMP co-treatments (**Figure 2**).The effects of cholera toxin (CT), norepinephrine (NE) and isoprotrenolol (IPT) were investigated and compared to that of 5-HT by Western blot format. CT and NE were postulated to synergise IL-1β mediated phosphorylation of p38. NE acts through the β- adrenergic receptor to increase intracellular levels of cAMP but

showed no synergistic induction in p38 phosphorylation when applied together with IL-1β. However, IPT showed decrease in p38 phosphorylation (**Figure 4**). We further investigated the effects of IPT on IL-1β signaling. Our results showed no further proof of IPT decreasing IL-1β mediated phosphorylation of p38 (**Figure 5**).

Effects of lipoic acid on IL-1β signaling

We investigated the effects of LA and its potential in inhibiting the signaling of IL-1β and TNF-α. We observed that both IL-1β and TNF-α induced p38 phosphorylation and IκB-α degradation with no effect on total p38 and IκB-β. Lipoic acid was not able to reverse the activation of either pathway mediated by IL-1β or TNF-α (**Figure 6, 7**).

Effects of serum vs. serum free medium on IL-6 ELISA assay

We next investigated the effects of serum (10% FBS, RPMI - 1640) versus serum free (RPMI - 1640) in ELISA format. Both methods showed good standard curves with high R values (results not shown). A robust synergistic release of extracellular IL-6 due to IL-1β and TNF-α was observed in both experiments (**Figure 8 – Panel [A] and [B]**). Both GABA and IL-10 were able to suppress the synergistic induction of IL-6, suggesting that they may have the same mode of anti-inflammatory action. In addition, serum conditions showed larger fold suppression of IL-6 induction than serum free (RPMI – 1640) (**Figure 8,**).

Interestingly, serum free samples had more IL-6 in pg/well than 10% FBS RPMI - 1640 alone.

Effects of concentration of IL-10 and GABA on extracellular IL-6 release

Having determined that serum containing medium allows for better fold induction suppression of IL-6, we characterized the dose response effects of IL-10 and GABA on IL-6 suppression. In serum containing medium, we showed the highest concentrations of GABA and IL-10, 1 mM and 100 ng/ml, respectively, were able to best suppress the synergistic induction of IL-6 due to IL-1β and TNF-α. We also showed that even lower concentrations of GABA (0.2 mM) and IL-10 (10 ng/ml) induced some suppression of release of IL-6 (**Figure 9**).

Extended dose response of IL-10 on extracellular IL-6 release

Once we determined that the IL-10 reverses the synergistic induction of IL-6 due to IL-1β and TNF-α, we investigated its effects in an extended dose response. Groups were treated with 1, 5, 10, 25, 50 and 100ng/ml of IL-10. Highest concentration of IL-10 (100ng/ml) most efficiently suppressed the IL-6 release. Furthermore, concentrations as low as 1 and 5 ng/ml IL-10 reduced the synergistic release of IL-6 (**Figure 10**).

Effects of IL-4 compared to IL-10 and GABA in ability to suppress extracellular IL-6 release

We decided to test the test the effects of IL-4, a similar cytokine to IL-10, believed to have the same effects on IL-6 suppression. IL-4 was able to suppress

IL-1β and TNF-α synergism in inducing IL-6 release almost the same fold as GABA and IL-10. further, SB203580, an inhibitor of p38 activity completely wiped out the signal of IL-6 release, suggesting involvement of p38 MAP kinase in this pathway (**Figure 11**).

Effects of serum vs. serum free in Western format

The effects of serum vs. serum free medium in the ELISA assays suggested that serum medium was to be our working medium. We had to standardize this procedure for our Western format, so we investigated the same effects in a Western blot. Both IL-1β and TNF-α were able to induce the phosphorylation of total p38 and the degradation of IκB-α (inhibitor of NF-κB), but not IκB-β. We also showed synergy of the two cytokines in their ability to induce those two signaling pathways. IL-10 (100 ng/ml) was unable to reverse the effects from IL-1β and TNF-α. Also, we once again showed that serum conditions (10% FBS in RPMI - 1640) allowed greater induction of p38 phosphorylation, IκB-α degradation, and cellular accumulation of IL-6 (**Figure 12**). Furthermore, we performed separate experiments involving serum vs. serum free conditions and compared the effects of GABA and IL-10 on IL-1β and TNF-α signaling (**Figures 13, 14**).

Effects of different concentration of IL-1β on p38 phosphorylation

After having determined that serum rich conditions are optimal for our experiments, we investigated the role of IL-10 on IL-1β and TNF-α signaling. We further explored the idea that IL-10 may affect IL-1β signaling at low

concentrations for this pro-inflammatory cytokine. We found that IL-1β was able to stimulate phosphorylation of p38 even at concentrations as low as 10 ng/ml. In addition, IL-10 did not inhibit the p38 activation due to low concentrations of IL-1β. Furthermore, IL-10 did not induce any activation of p38 when administered alone (**Figure 15**).

Effects of different concentrations TNF-α on p38 phosphorylation

Since we determined that IL-10 had no effect on IL-1β induced p38 activation, we decided to test another hypothesis that IL-10 may affect the TNF-α mediated phosphorylation of p38. We found that TNF-α, like IL-1β induces activation of p38 even at low concentrations of 10 ng/ml. We observed a dose response of TNF-α on p38 activation. IL-10 did not reverse the effects of TNF-α on p38 at any concentrations of the co-treatments (**Figure 16**).

Effects of different concentration of IL-1β on IκB degradation

We further investigated the effects of IL-10 on IL-1β mediated degradation of IκB-α in NF-κB signaling. As expected, IL-1β was able to initiate degradation of IκB-α, inhibitor of NF-κB, with no change in IκB-β. However, IL-10 was not able to reverse IκB-α degradation even at lowest concentrations of IL-1β of 10 ng/ml. Moreover, IL-10 alone had no effect on IκB-α degradation **(Figure 17).**

Dose response effects of TNF-α on degradation of IκB-α: role of IL-10

We also investigated the hypothesis that IL-10 may have a role on TNF-α mediated NF-κB signaling. TNF-α activated the degradation of the NF-κB inhibitor IκB-α at 10 ng/ml. We also observed a dose response of TNF-α in NF-κB signaling. Some decrease in the intensity of the bands was observed when co-treatments with IL-10 were administered. This suggested that IL-10 may have a modest effect on TNF-α mediated IκB-α degradation. IL-10 had no effect on degradation of IκB-α, as shown in previous experiments and neither of these cytokines showed any change in IκB-β, as expected (**Figure 18**).

Effects of IL-10 and IL-4 on IL-1β and TNF-α mediated synergistic p38 activation and IκB-α degradation

We previously showed in an ELISA format that IL-10 and IL-4 inhibited the cytokine synergistic release of IL-6. We decided to further investigate the hypothesis that these two anti-inflammatory cytokines may decrease intracellular levels of IL-6 by reversing p38 phosphorylation and IκB-α degradation induced by TNF-α and IL-1β. As expected, both cytokines, TNF-α and IL-1β, induced p38 phosphorylation and IκB-α degradation in a synergistic fashion. IL-10 and Il-4, however, did not have any effect on these two signaling pathways. There was no change in IκB-α or total p38, as shown before by any of the cytokines (**Figure 19**). In addition, we also included the effects in JNK phosphorylation where IL-10 was able to reduce the phosphorylation of JNK mediated by TNF-α and IL-1β.

Time course of TNF-α and IL-1β synergism on IL-6 release

We decided to investigate the optimal time response for IL-6 induced by TNF-α and IL-1β. Interestingly, both cytokines induced IL-6 intracellular levels at their optimal concentrations, 100 ng/ml and 50 ng/ml, respectively. Moreover, differences in time course were not observed for any of the treatment groups and IL-6 remained unchanged for 1 h to 6 h (**Figure 20**).

Effects of IL-10 and GABA on TNF-α and IL-1β signaling

We investigated the effects of longer intervals of TNF-α and IL-1β signaling (in, 4 h and 6 h). Previously, we found that even at an extended time response both cytokines are able to stimulate the degradation of IκB-α and release of IL-6 (results not shown). On the contrary, IL-10 and GABA, did not reverse the effects of inflammatory cytokines on NF-κB signaling or change intracellular IL-6. Moreover, levels of β-actin and IκB-β remained unchanged. In addition, levels of IL-6 stimulation remained the same at both 4 h and 6 h treatment groups (**Figure 21**).

Effects of TNF-α and IL-1β on short time treatment in accumulation of intracellular IL-6

Previous results suggested that accumulation of intracellular IL-6 remained unchanged in treatments from 1 h to 6 h. We first expanded the time response from 2, 4 to 24 hours (**Figure 22**). Surprisingly, IL-6 content remained the same throughout the time response. Total p38, IκB-β and β-actin remained also

unchanged as expected. The phosphrylation of p38 however, showed stimulation by both TNF-α and IL-1β, and interestingly, was decreased by IL-10 at 2 and 4 h treatment. We decided to constrict the time frame of measuring IL-6 at 15 min and 30 min. We also included the effects of GABA, IL-4 and IL-10 on TNF-α and IL-1β signaling (**Figure 23**). All anti-inflammatory modulators showed decrease in p38 phosphorylation after stimulation with TNF-α and IL-1β at 30 min. No effect was observed at 15 minute treatments and no change was observed in IL-6 content throughout. Total p38, IκB-β or β-actin were not altered as expected.

Synergistic transcriptional activation of IL-6 mediated by TNF-α and IL-1β.

Effects of IL-10 and GABA

We then proceeded to investigate the transcriptional activation of IL-6 mRNA. Interestingly, TNF-α and IL-1β are able to synergistically stimulate the transcription of IL-6. GABA and IL-10, however, were not able to reverse this synergistic response. We also tested SB203580 compound, known to interfere and inhibit p38 activity. In fact, SB203580 was able to completely suppress IL-6 transcriptional activation mediated by TNF-α and IL-1β, suggesting its involvement in this signaling pathway (**Figure 24**).

Effects of IL-10 on JNK MAP kinase activation

Since IL-10 did not interfere with p38 or NF-κB signaling, we decided to explore the possibility of its interference with JNK kinase, another MAP kinase. Surprisingly we found that IL-10 reversed the phosphorylation of JNK induced by

TNF-α and IL-1β suggesting a plausible interference point of IL-10 in our pathway of investigation (**Figure 25, 26**).

CHAPTER 4

DISCUSSION

Synergistic interaction between Interleukin-1β and catecholamines

After extensive literature review we decided to study the effects of catecholamines and other signaling molecules increasing intracellular level of cAMP on IL-1β signaling pathway. J. Zumwalt et al., 1999 previously showed synergistic release of IL-6 mediated by IL-1β and Bu_2cAMP, cholera toxin, norepinephrine and isoprotenolol. Our lab previously showed that IL-1β stimulates p38 phosphorylation and IκB-α degradation which ultimately leads to release of IL-6 (data not shown). We tested the synergistic effects of IL-1β together with Bu_2cAMP, cholera toxin, norepinephrine and isoprotenolol on p38 phosphorylation (**Figure 4, 5**). Optimal treatment times for stimulation of p38 by IL-1β was determined previously to be 15 min. Co-treatments with catecholamines were not able to increase activation of p38 in a synergistic manner. We determined that optimal concentrations of Bu_2cAMP (1 mM), CT (250 ng/ml), NE (100 μM) and IPT (10 μM) as suggested by literature, were not able to increase p38 phosphorylation. This suggests that their synergism in IL-6 release may go through a different pathway other than p38 MAPK. Moreover, catecholamines did not stimulate p38 phosphorylation when administered alone.

We also investigated the effects of other inhibitors and modulators such as Serotonin (5-HT) on p38 activation mediated by IL-1β. Previous research suggested that 5-HT was able to reverse IL-6 mRNA expression in MC-3 cells (C. Mahe et al., 2005). Our results concluded that 5-HT was not able to inhibit and reverse the phosphorylation of p38 induced by IL-1β and therefore may not inhibit IL-6 gene transcription through this pathway (**Figure 2, 3**).

We further investigated the effects of lipoic acid on IL-1β stimulated p38 and NF-κB pathways. We expected that lipoic acid would be a potent inhibitor of IL-1β mediated activation. However, our results showed that lipoic acid was not able to reverse the effects of IL-1β stimulation (**Figure 6, 7**).

Interleukin-10 inhibition of the synergistic release of IL-6 by TNF-α and IL-1β

Our lab has previously established that TNF-α and IL-1β combined induce a synergistic effect on IL-6 release (Roach et al., in press). Previous research also introduced the effects of GABA on the inhibition of IL-6 release induced by TNF-α and IL-1β. We investigated the effects of the anti-inflammatory cytokine, IL-10, on the synergistic release of IL-6 in ELISA format. Pousset et al. (2000) showed that infalmmatory cytokine IL-1β can induce NF-κB activation in primary mouse astrocytes. Furthermore, they showed that anti-inflammatory cytokines IL-10 and IL-4 were able to block the IL-1β induced NF-κB activation. We further investigated this idea and studied the effects of IL-10 on IL-1β and TNF-α mediated p38 phosphorylation, NF- κB activation and release of IL-6 in rat C6

astrocytes. In order to further understand the fold induction of IL-6 release and its suppression, we explored the effects of serum containing medium (10% FBS, RPMI - 1640) vs. serum free medium (RPMI - 1640). Our results indicated that serum rich containing medium induced better fold suppression of synergistic induction of IL-6 by GABA and IL-10 (**Figure 8**). Synergistic extracellular IL-6 release was best suppressed at optimal concentrations for GABA (1 mM) and IL-10 (100 ng/ml) in serum. We also observed that serum free treatments induced larger release of IL-6 but we achieved greater fold suppression when treatments were made in serum rich medium. We were also able to generate a dose response of GABA and IL-10 in ELISA format showing dose dependence of inhibitory modulators over inflammatory cytokines, IL-1β and TNF-α (**Figure 9**). Lowest doses of GABA and IL-10, 0.2 mM and 10 ng/ml, respectively, just slightly suppressed IL-1β and TNF-α synergism. However, highest doses of GABA (1 mM) and IL-10 (100 ng/ml) suppressed IL-6 induction by approximately 50%. In addition, when GABA and IL-10 were administered alone, no change in IL-6 release was measured.

To determine the minimal and maximal effective doses of IL-10 in reducing IL-6 in ELISA, we performed treatments with six different doses (1 - 100ng/ml). Co-treatments of IL-1β and TNF-α together with IL-10 inhibited IL-6 release at 100 ng/ml IL-10. Moreover, 1 ng/ml IL-10 of also slightly suppressed IL-6 release (**Figure 10**).

Further, we compared inhibition of IL-6 release by IL-10 and GABA to that of IL-4 and SB203580 in ELISA. As suggested by the literature, IL-4 has anti-

inflammatory properties similar to IL-10 (F. Pousset et al., 2000). We expected to observe IL-4 inhibition of IL-6 similar to that of IL-10. In fact, IL-4 suppressed IL-1β and TNF-α synergistic induction of IL-6 similarly to IL-10 and GABA (**Figure 11**). In addition, SB203580, a p38 inhibitor, completely reversed the effects of IL-1β and TNF-α on IL-6 release. Our results show that each of these anti-inflammatory cytokines and inhibitors in the CNS may have a similar mode of action. J. Roach, et al., (2008) suggested that GABA may inhibit IL-6 translation by blocking IL-6 transcript release form the nucleus or by inhibiting translation directly. With these results we can prove our first hypothesis that IL-10 inhibits synergistic induction of IL-6, due to TNF-α and IL-1β. From the data presented, we decided to investigate further the effects of IL-10 on p38 and NF-κB activation by Western.

Effects of Interleukin-10 inhibition on IL-1β and TNF-α mediated induction of IL-6

Since accelerated effect of p38 phosphorylation and NF-κB activation, induced by IL-1β and TNF-α may be the cause for synergistic induction of IL-6 as shown in our ELISA data, we needed to investigate IL-10 activation of these two pathways. First, we had to determine our optimal working conditions in serum rich or serum free medium. Results indicated that synergistic activation of p38 and NF-κB was better expressed in serum containing medium (**Figure 12**). We showed that IL-10 did not consistently reverse p38 phosphorylation or IκB-α degradation induced by IL-1β and TNF-α. We observed the expected synergism

of the two inflammatory cytokines as expected. However, IL-10 100 ng/ml and 15 min co-treatments was unable to show any effect. In addition, we observed no change in total p38, IκB-β, β-actin, as expected. Moreover, IL-10 did not reduce intracellular accumulation of IL-6. We once again confirmed that treatment with 100 ng/ml TNF-α and 50 ng/ml IL-1β resulted in IL-6 transcription and synergistic induction of p38 and NF-κB. However, IL-10 did not interfere with p38 and IκB-α activation.

We also demonstrated that GABA showed little or no difference in the IL-1β or TNF-α stimulation of p38 and NF-κB in serum free or serum rich medium (**Figure 13, 14**). The reason for this difference of GABA to IL-10 experiment in serum vs. serum free conditions may be due to the fact of different cell split number or other experimental conditions.

We also illustrated dose dependence activation of p38 pathway by IL-1β. Lowest doses of IL-1β at 10 ng/ml were just slightly able to induce phosphorylation. On the other hand, highest concentrations of 100 ng/ ml of IL-1β showed a great induction of p38. Moreover, IL-10 was not able to inhibit IL-1β activation of p38 at any dose of IL-1β. Maximal concentrations of IL-10 of 100 ng/ml were used for co-treatments (**Figure 15**). We investigated TNF-α dose dependence on p38 activation and IL-10 effects. Results show that TNF-α stimulated p38 phosphorylation at 10 ng/ml as well as at 100 ng/ml. Once again, IL-10 did not reverse TNF-α effects on p38 phosphorylation (**Figure 16**). These results indicate that IL-10 has no effect on stimulated p38 pathway and therefore

may not have an effect on intracellular accumulation of IL-6 message, which may be stimulated by acceleration of phosphorylation of p38.

The activation of NF-κB on IL-6 accumulation was also studied. Dose dependence of both IL-1β and TNF-α was observed to activate IκB-α degradation with no change in IκB-β. In either scenario, IL-10 did not reverse IL-1β or TNF-α stimulated degradation of IκB-α (**Figure 17, 18**).

Our ELISA data shown before suggested that IL-10 inhibits synergistic release of IL-6 mediated by IL1-β and TNF-α. Therefore, we decided to investigate further this response by Western. NF-κB and p38 activation was stimulated synergistically by IL1-β and TNF-α at maximal concentrations of 50 ng/ml and 100 ng/ml, respectively. Both anti-inflammatory cytokines, IL-10 and IL-4 had no effect on IκB-α degradation or phosphorylation of p38 (**Figure 19**). It was able to inhibit extracellular release of IL-6 induced by IL1-β and TNF-α but could not influence two of the main pathways involved in IL1-β and TNF-α signaling.

We further decided to measure the IL-6 cellular content when stimulated with IL1-β and TNF-α and observe any possible effects of IL-10. First, we wanted to observe the time dependence of IL-6 stimulation mediated synergistically by IL1-β and TNF-α. **Figure 20** represents the IL-6 synergistic accumulation by IL1-β and TNF-α. Interestingly, we observe the same amount of IL-6 after stimulation of 1, 2, 4 and 6 h. Cellular IL-6 remained the same throughout the time treatment and suggested no dependence on time. We further observed the effects of GABA and IL-10 on cellular IL-6 content stimulated by IL1-β and TNF-α. Neither GABA

nor IL-10 was able to reverse IL-6 accumulation at 4 and 6 hours. Furthermore, we see that both IL-1β and TNF-α induced IκB-α degradation, although very slightly. No change was observed in IκB-β or β-actin, as expected (**Figure 21**). Since no difference in IL-6 stimulation was observed with 6 h of stimulation with cytokines, we decided to design an experiment where IL-6 accumulation was observed after overnight treatment with inflammatory cytokines. Again, we observed no change in IL-6 content even after 24 h. The inhibitor IκB-α was slightly degraded even after extended times. IκB-β, β-actin and total p38 remained unchanged as expected. Interestingly, IL-1β and TNF-α induced phosphorylation in p38 at all time points. Moreover, IL-10 slightly reversed this effect at 2 and 4 hours compared to GABA (**Figure 22**). We therefore decided to reduce the time frame for cytokine stimulation. C6 cells were stimulated for 15 and 30 min with IL-1β and TNF-α and effects of GABA, IL-10, IL-4 and SB203580 were observed. Intracellular IL-6, β-actin and p38 remained the same. However, phosphorylation of p38 was apparently reversed at 30 min by GABA, IL-10, IL-4 and SB203580 compared to 15 minutes treatments (**Figure 23**). We were not able to deduce with complete accuracy the optimal time frame for maximal p38 inhibition by IL-10 or any other anti-inflammatory cytokine. There are many variables in the experiments including cell split number or time of treatment when cells are most responsive.

Finally, we investigated the effects of IL-10 on IL-6 transcription. We observed synergistic activation of IL-6 transcription induced by IL-1β and TNF-α. IL-10 had no effect and was not able to reverse the stimulation and neither could GABA.

SB203580 was a positive control and inhibited IL-6 transcription after stimulation with inflammatory cytokines (**Figure 24**). In addition, we decided to test another MAP kinase and its involvement in our pathway of investigation, c-Jun-N-terminal kinase (JNK). We found that when phosphorylation of JNK is stimulated either with TNF-α or IL-1β, IL-10 reduced pJNK (**Figures 25, 26**). This suggests that inhibition of extracellular IL-6 release may be through a mechanism involving a MAP kinase. Results in this thesis and the research performed led us to propose a plausible point of inhibition by IL-10, represented by **Figure 27**.

These results suggested that IL-10 may be interfering at a post translational level with IL-6 transcript. There are two plausible locations where IL-10 can inhibit or prevent IL-6 from leaving the cell. One potential model for IL-10 inhibition of IL-6 translation may be through interfering with the ribosomal assembly and thus reducing the amount of mature IL-6 transcript. Another model may be similar to the mode of action of brefeldin – A (BFA), a drug that disrupts the Golgi apparatus and secretory mechanism. In mammals the target of BFA is the GTP/GDP exchange factor required for ARF (ADP-ribosylation factor) binding (Helms and Rothman, 1992;). BFA changes the structure of Golgi apparatus and block the transport of secretory proteins from the endoplasmic reticulum (ER) to the Golgi (Klausner et al., 1992). Therefore, IL-10 may be preventing glycosylation and formation of mature IL-6 from leaving the cell by interfering with the Golgi apparatus.

In summary, our results presented in this thesis show the synergistic effect of IL-1β and TNF-α on the release of extracellular IL-6. We also showed that both of

these cytokines are able to stimulate and activate p38 and NF-κB signaling. Moreover, IL-10 may be involved in post-translational mechanism to prevent IL-6 release based on the following: [1] IL-10 did not prevent the activation of p38 or NF-κB, [2] IL-10 did not reduce transcription of IL-6 [3] IL-10 showed no change in intracellular IL-6 levels after stimulation with IL-1β and TNF-α [4] IL-10 reduced phosphorylation of JNK, induced by TNF-α and IL-1β, suggesting a possible mechanism involving MAP kinase in inhibiting IL-6 release. IL-10 mechanism of action is very similar to that GABA, another inhibitory neurotransmitter in the CNS which may suggest a whole new class of inhibitory modulators interfering at post-translational level.

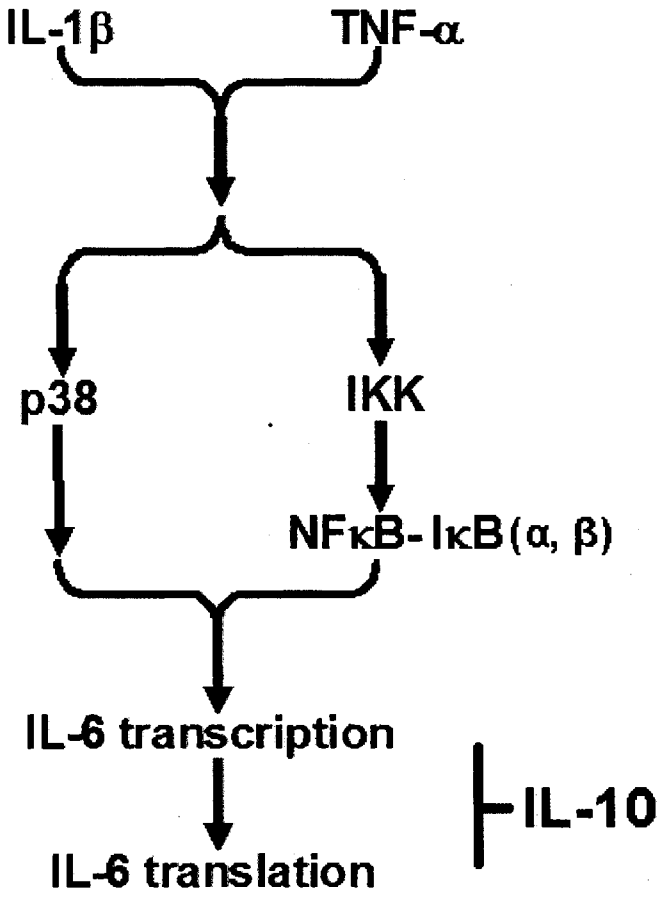


Figure 1: Schematic representation of p38 and NF-κB signaling induced by IL-1β and TNF-α and possible IL-10 interference point.

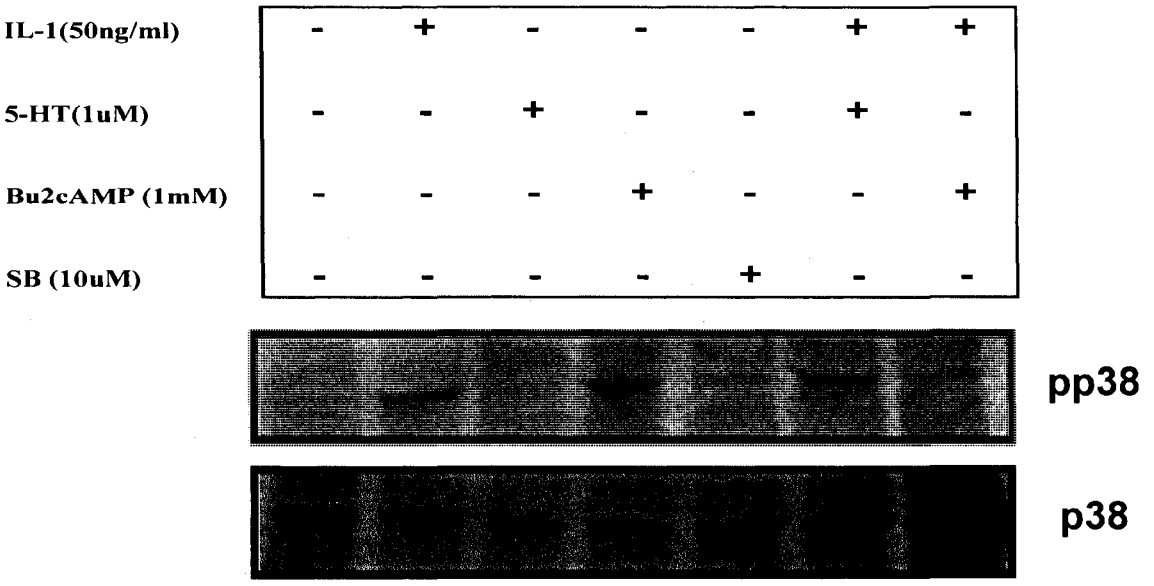


Figure 2: Effects of srotonin (5-HT) on IL-1β signaling. Rat C6 cells were plated (3.0×10^6 cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 1uM of 5-HT and 1mM of Bu2cAMP for 1 hour and co-treated with 1uM of 5-HT, 1mM of Bu2cAMP and IL-1β (50ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 µg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

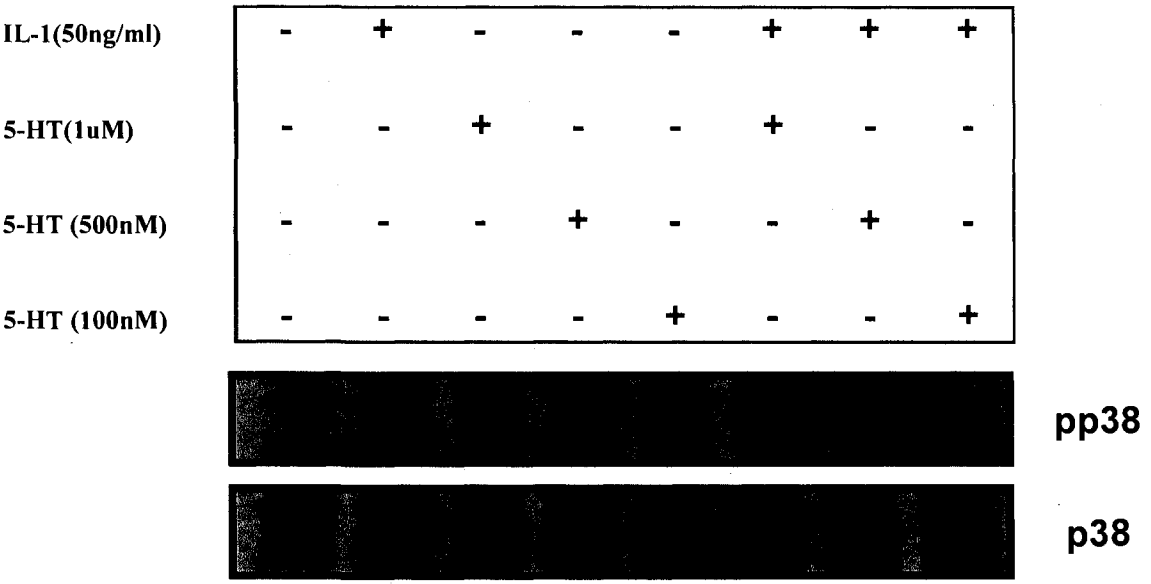


Figure 3: Effects of concentration of 5-HT on IL-1β signaling. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100nM, 500nmM and 1μM of 5-HT for 1 hour and co-treated with 100nM, 500nmM and 1μM of 5-HT and IL-1β (50ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

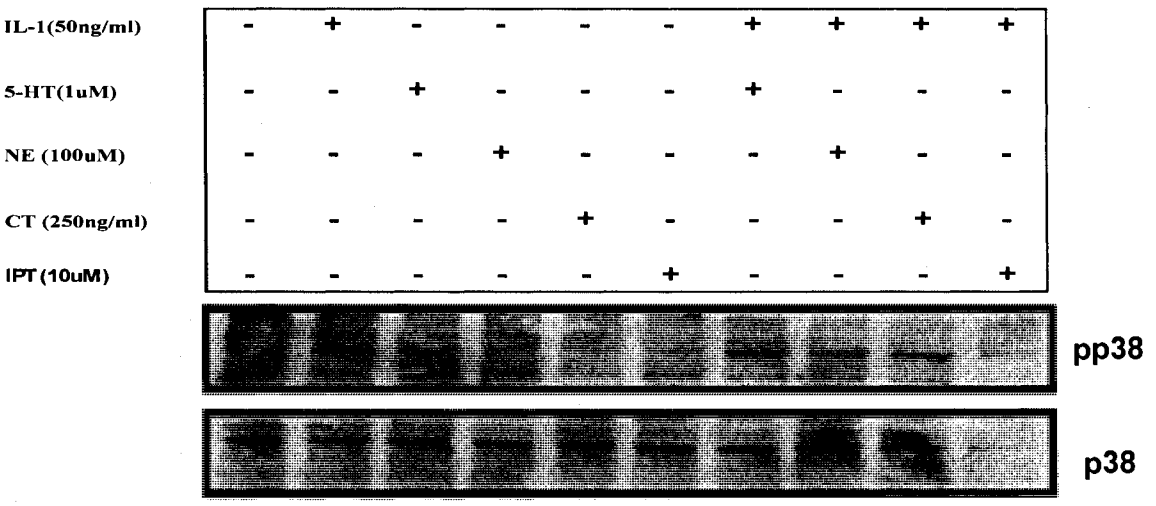


Figure 4: Effects of other inhibitors/modulators on IL-1β mediated phosphorylation of p38. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 1μM 5-HT, 100μM NE,250ng/ml Cholera Toxin and 10μM IPT for 1 hour and co-treated with 1μM 5-HT, 100μM NE, 250ng/ml Cholera Toxin, 10μM IPT and IL-1β (50ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

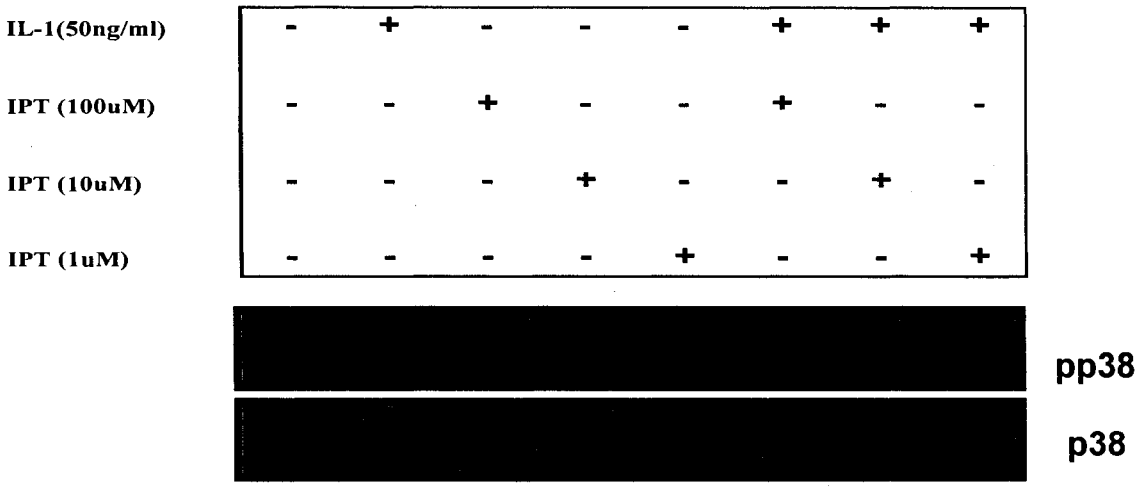


Figure 5: Concentration response of IPT on IL-1β mediated p38 signaling pathway. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 1μM, 10μM and 100μM IPT for 1 hour and co-treated with 1μM, 10μM and 100μM IPT and IL-1β (50ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

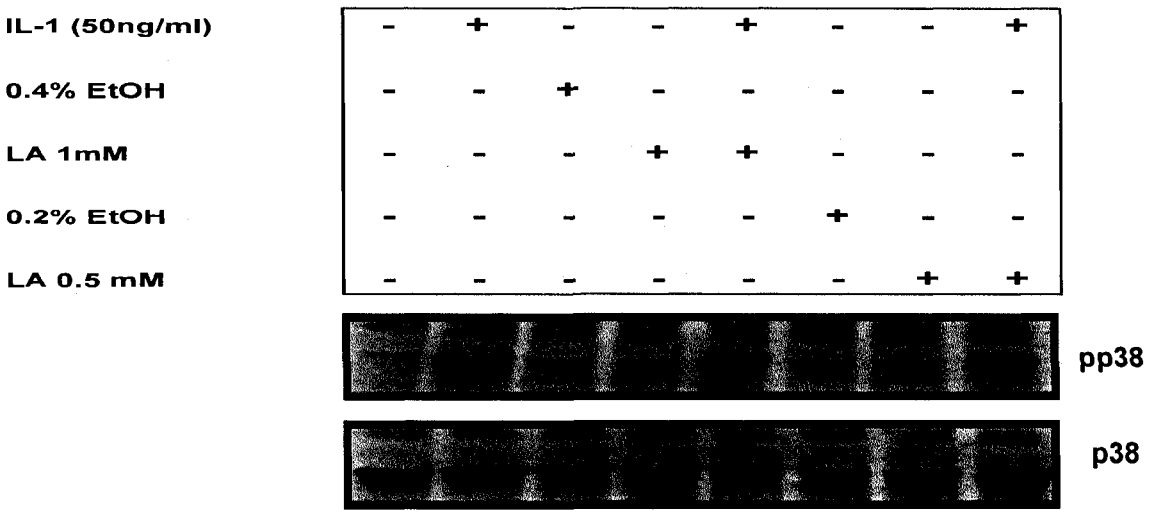


Figure 6: Lipoic acid as an inhibitor of IL-1 mediated p38 pathway. Rat C6 cells were plated (3.0 X10^6 cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 0.5mM and 1mM lipoic acid for 1 hour and co-treated with 0.5mM and 1mM lipoic acid and IL-1β (50ng/ml) for 15 minutes. 0.4% and 0.2% EtOH control points were showed in order to exclude possible interference with cell death. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

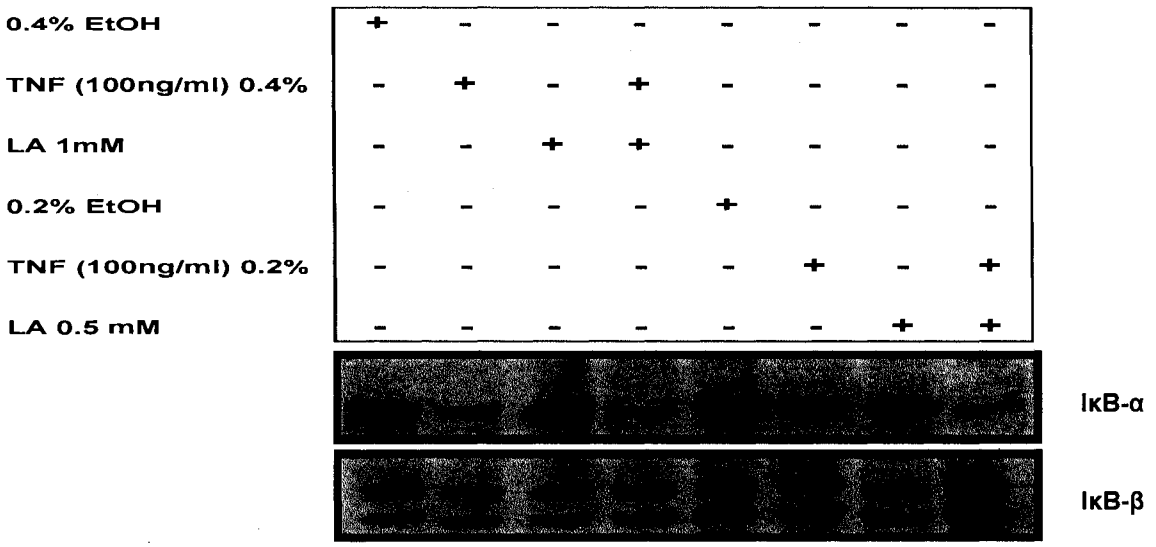
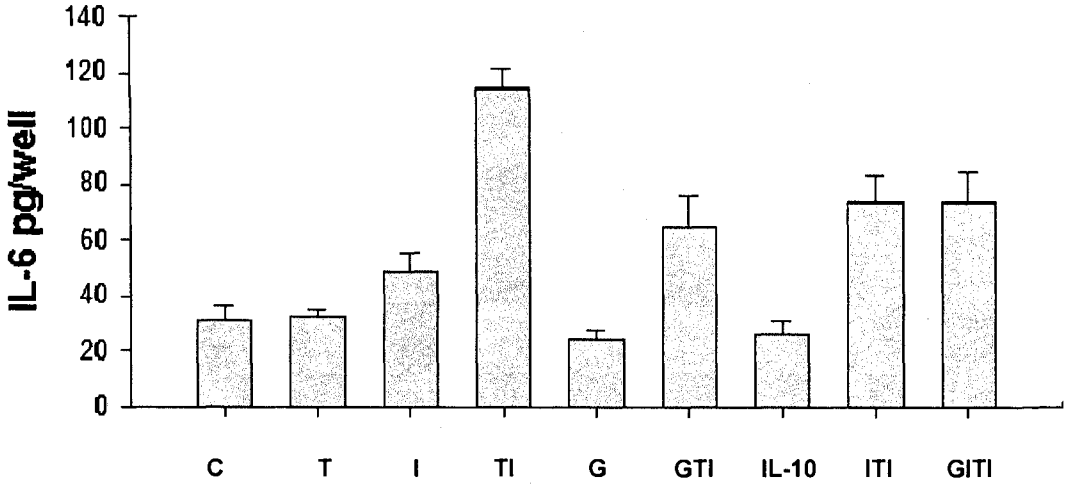


Figure 7: Lipoic acid as an inhibitor of TNF-α mediated NF-κB pathway. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 0.5mM and 1mM lipoic acid for 1 hour and co-treated with 0.5mM and 1mM lipoic acid and IL-1β (50ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

Panel [A]



Panel [B]

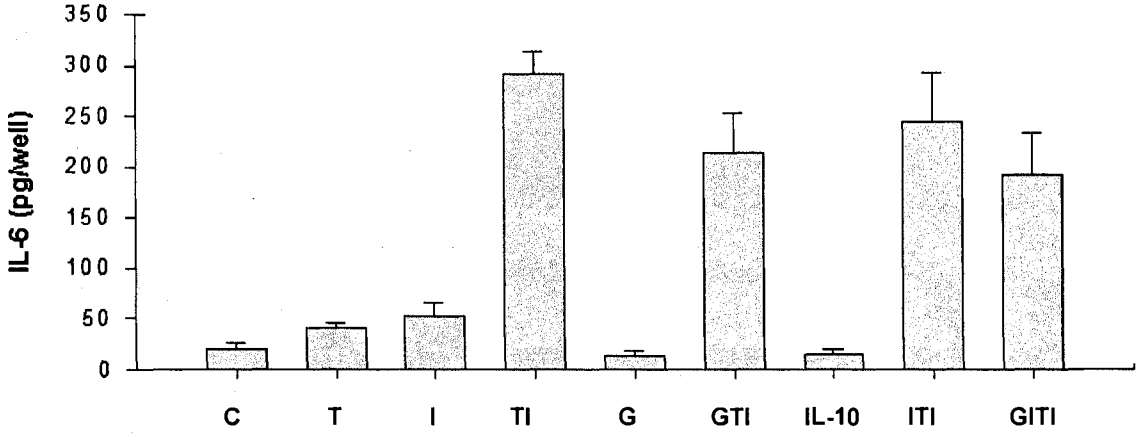


Figure 8: Effects of serum containing medium (Panel [A]) vs. serum free (Panel [B]) on IL-1β and TNF-α mediated synergistic release of IL-6 in ELISA. Rat C6 cells were plated (125 X 10^3 cells/well in a PDL-coated 96-well plate) and were pretreated with inhibitors (1mM GABA, 100ng/ml IL-10) alone for 1h and co-treated with inhibitors alone, 50ng/ml IL-1β and 100ng/ml TNF-α for 24 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer's specifications. T = TNF-α, I = IL-1β, G = GABA, IL-10 = interleukin 10. The data are represented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment.

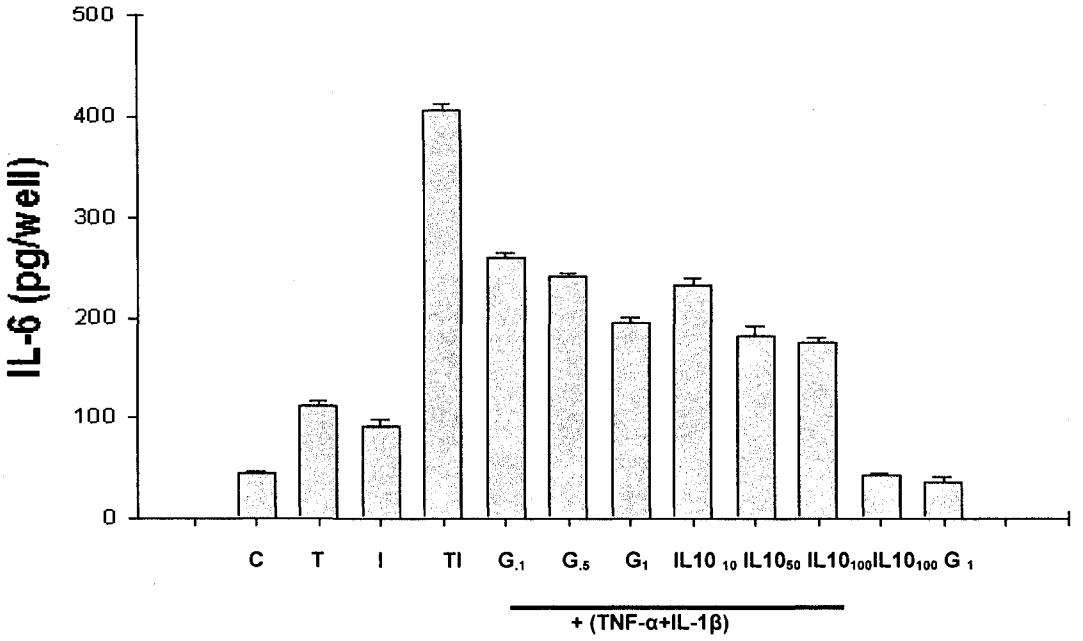


Figure 9: Effects of concentration of GABA and IL-10 on IL-1βand TNF-α mediated synergistic release of IL-6 in ELISA. Rat C6 cells were plated (125 X 10^3 cells/well in a PDL-coated 96-well plate) and were pretreated with inhibitors (0.1, 0.5 and 1mM GABA, 10, 25, 50 and 100ng/ml IL-10) alone for 1h and co-treated with inhibitors alone, 50ng/ml IL-1β and 100ng/ml TNF-α for 24 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer's specifications. T = TNF-α, I = IL-1β, G = GABA, IL-10 = interleukin 10. The data are represented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment.

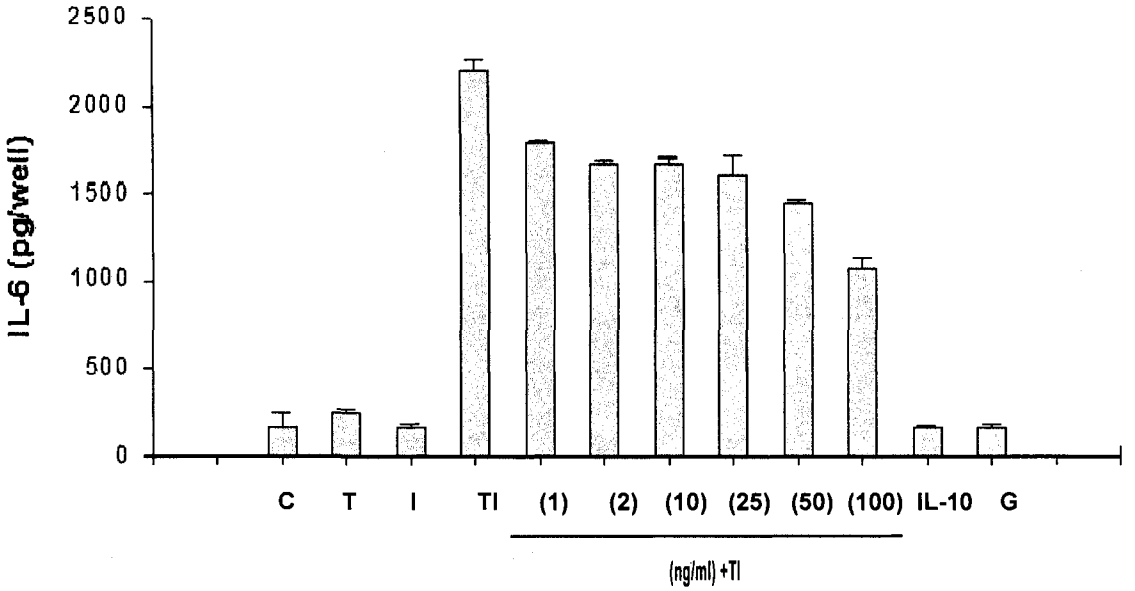


Figure 10: Evaluation of extended dose response of IL-10 on IL-1βand TNF-α mediated synergistic release of IL-6 in ELISA. Rat C6 cells were plated (125 X 10^3 cells/well in a PDL-coated 96-well plate) and were pretreated with inhibitors (1mM GABA, 1, 2,10,25,50 and 100ng/ml IL-10) alone for 1h and co-treated with inhibitors alone, 50ng/ml IL-1β and 100ng/ml TNF-α for 24 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer's specifications. T = TNF-α, I = IL-1β, G = GABA, IL-10 = interleukin 10. The data are represented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment.

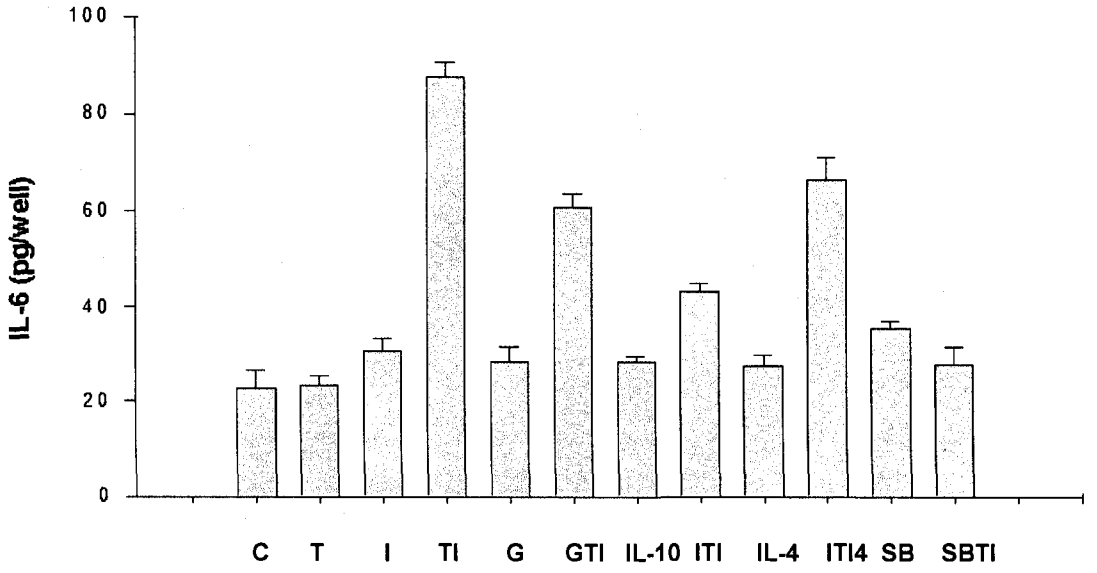


Figure 11: Effects of IL-4 vs. IL-10 and GABA on IL-10 on IL-1βand TNF-α mediated synergistic release of IL-6 in ELISA. Rat C6 cells were plated (125 X 10^3 cells/well in a PDL-coated 96-well plate) and were pretreated with inhibitors (1mM GABA, 100ng/ml IL-10, 100ng/ml IL-4, 10μM SB203580) alone for 1h and co-treated with inhibitors alone, 50ng/ml IL-1β and 100ng/ml TNF-α for 24 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer's specifications. T = TNF-α, I = IL-1β, G = GABA, IL-10 = interleukin 10, IL-4 = Interleukin 4, SB = SB 203580. The data are represented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment.

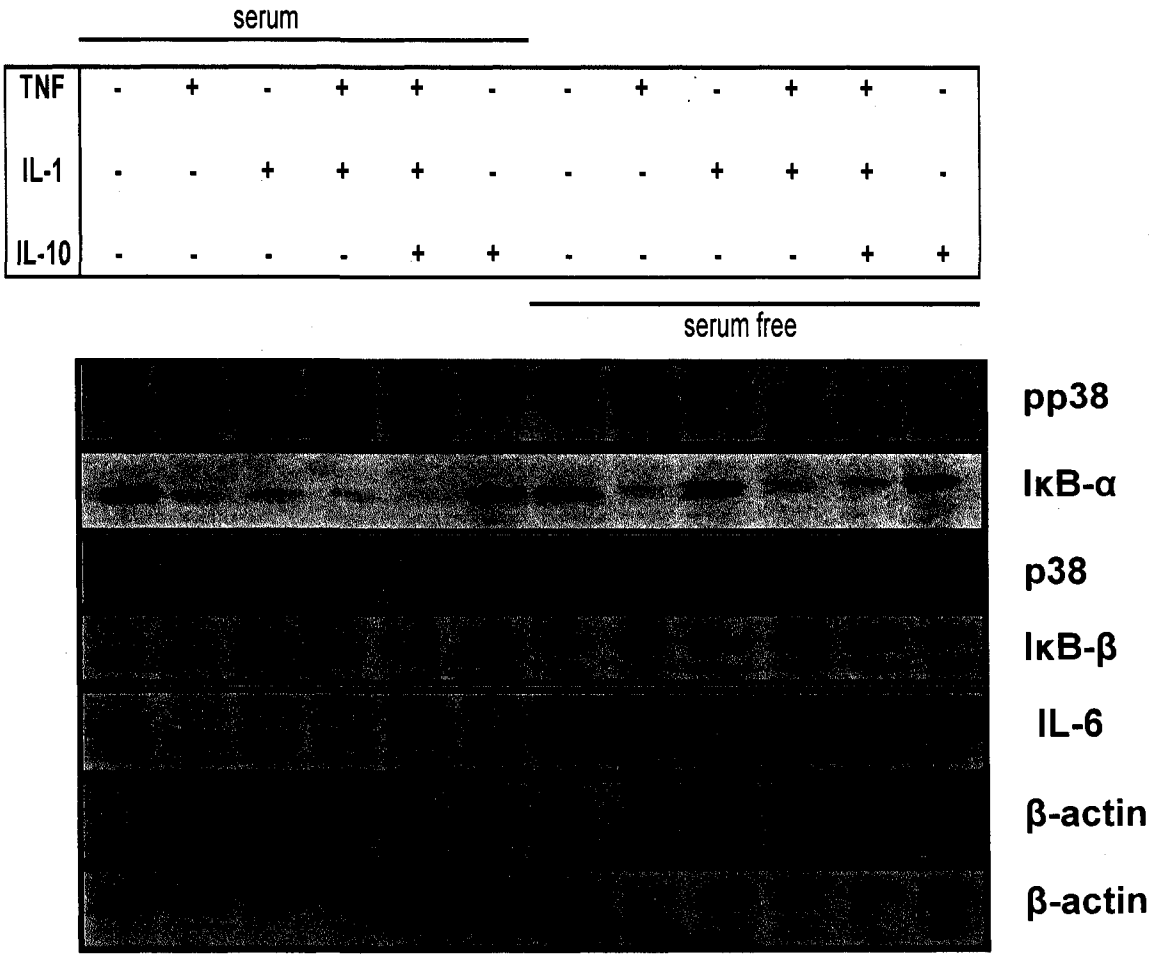


Figure 12: Effects of IL-10 in serum containing medium vs. serum deficient medium on IL-1β and TNF-α mediated NF-κB degradation and phosphorylation of p38. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 for 1 hour and co-treated with 100ng/ml IL-10 and IL-1β (50ng/ml) and TNF-α for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein, IκB-α and IκB-β. IL-6 and β-actin were obtained by stripping the membranes by blocking with non-fat milk overnight and then probing with primary and secondary antibodies (1:1000 for all). The data are represented as the observations obtained from a single experiment.

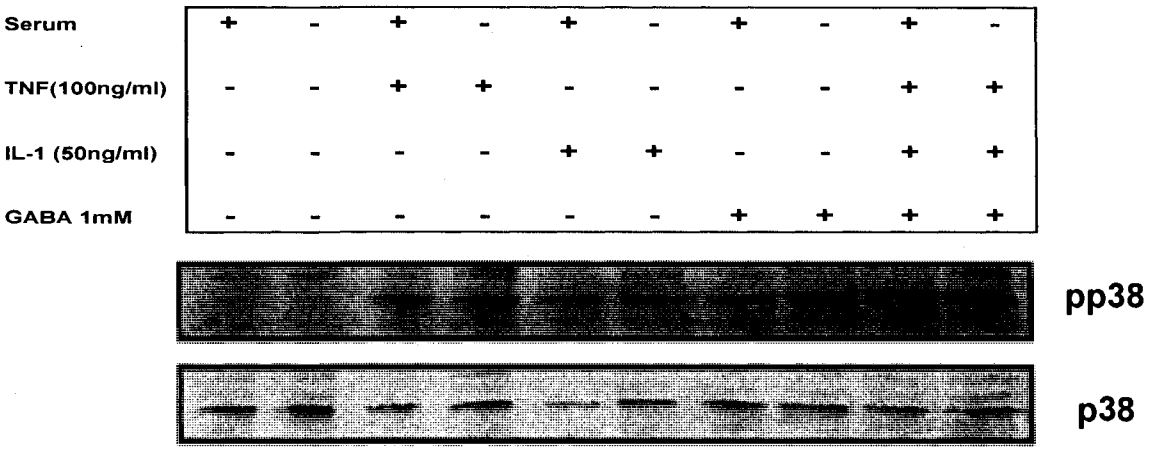


Figure 13: Effects of GABA in serum vs. serum deficient medium on phosphorylation of p38. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 1 mM GABA for 1 hour and co-treated with 1 mM GABA, IL-1β (50ng/ml) and TNF-α (100ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

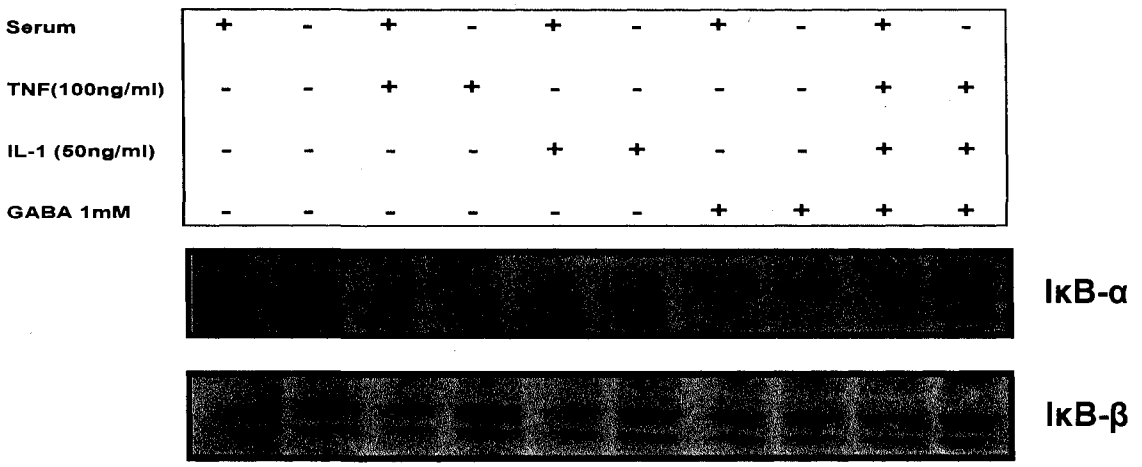


Figure 14: Effects of GABA in serum vs. serum deficient medium on degradation of IκB inhibitor of NF-κB. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 1 mM GABA for 1 hour and co-treated with 1 mM GABA, IL-1β (50ng/ml) and TNF-α (100ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α and IκB-β protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

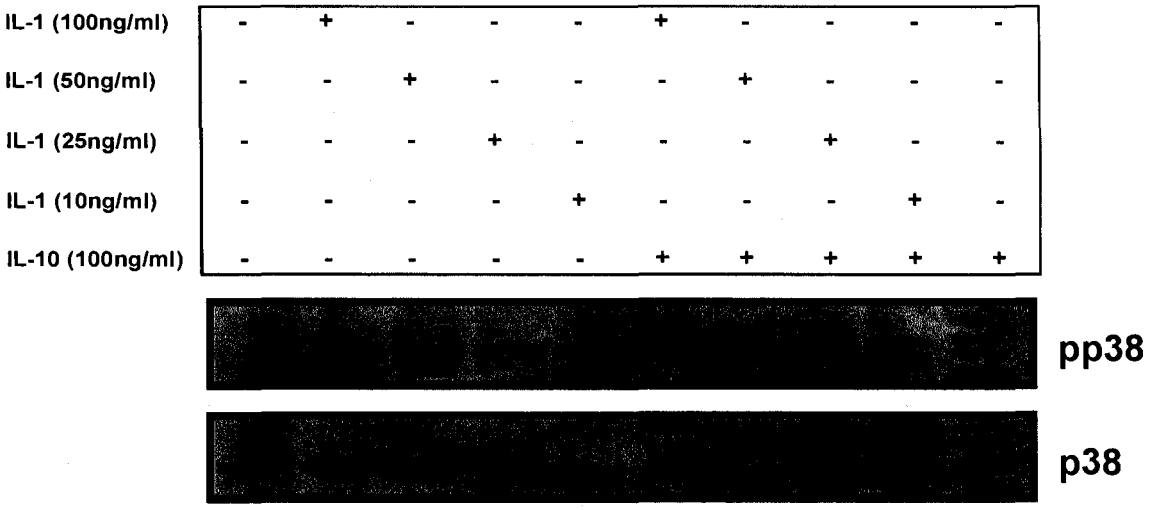


Figure 15: Effects of low concentration of IL-1β on p38 signaling pathway. Rat C6 cells were plated (3.0 X10^6 cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 for 1 hour and co-treated with 100ng/ml IL-10 and IL-1β (10 ng/ml, 25ng/ml, 50ng/ml and 100ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

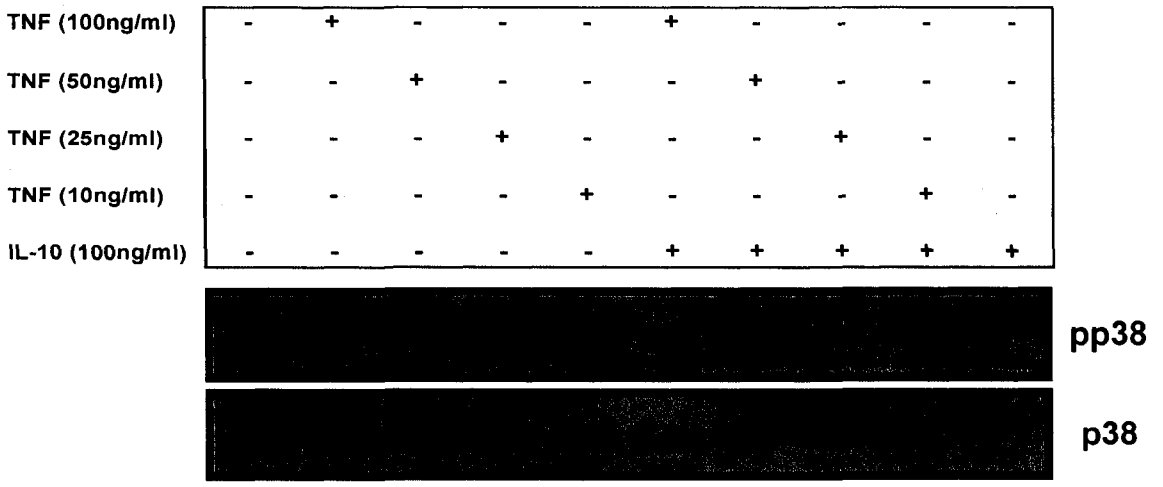


Figure 16: Effects of IL-10 on TNF-α-mediated phosphorylation p38. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 for 1 hour and co-treated with 100ng/ml IL-10 and TNF-α (10 ng/ml, 25ng/ml, 50ng/ml and 100ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

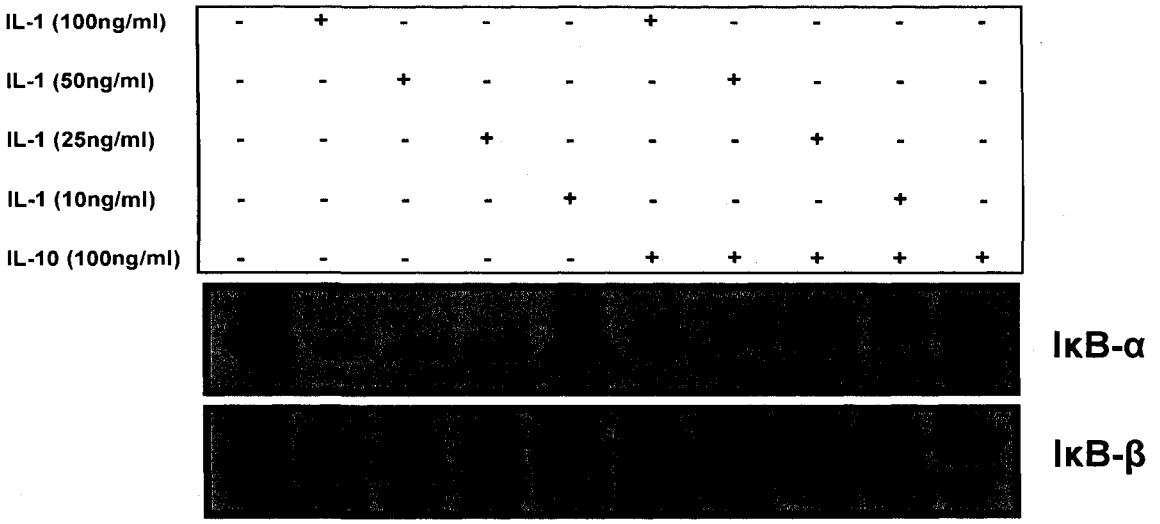


Figure 17: Effects of low concentration of IL-1β on NF-κB signaling pathway. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 for 1 hour and co-treated with 100ng/ml IL-10 and IL-1β (10 ng/ml, 25ng/ml, 50ng/ml and 100ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 µg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α and IκB-β protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

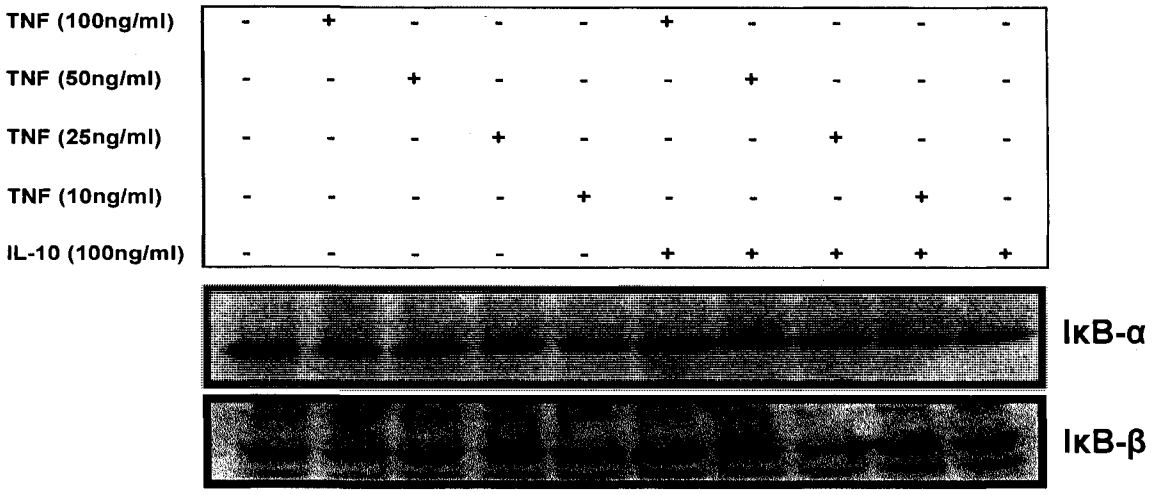


Figure 18: Effects of low concentration of TNF-α on NF-κB signaling pathway. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 for 1 hour and co-treated with 100ng/ml IL-10 and TNF-α (10 ng/ml, 25ng/ml, 50ng/ml and 100ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α and IκB-β protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

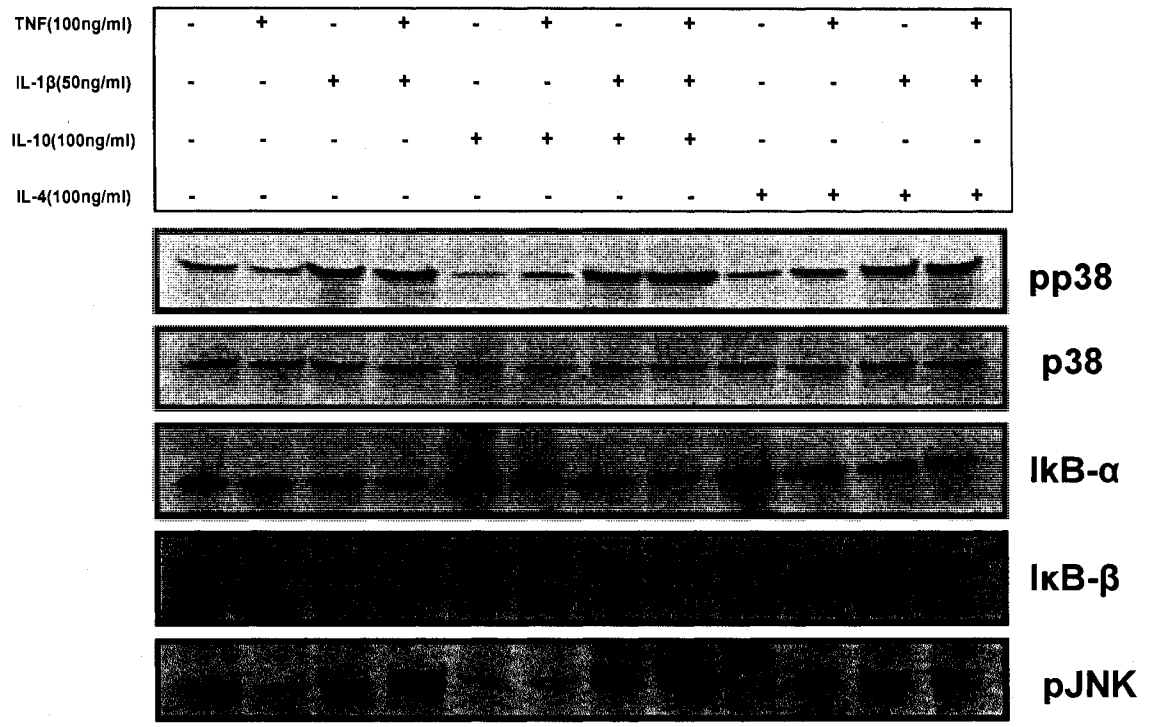


Figure 19: Effects of IL-4 vs. IL-10 on IL-1β and TNF-α signaling. Rat C6 cells were plated (3.0 X10^6 cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 and IL-4 for 1 hour and co-treated with 100ng/ml IL-10, 100ng/ml IL-4 and TNF-α (100ng/ml) + IL-1β (50ng/ml) for 15 minutes. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, total p38 and phosphorylated p38 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

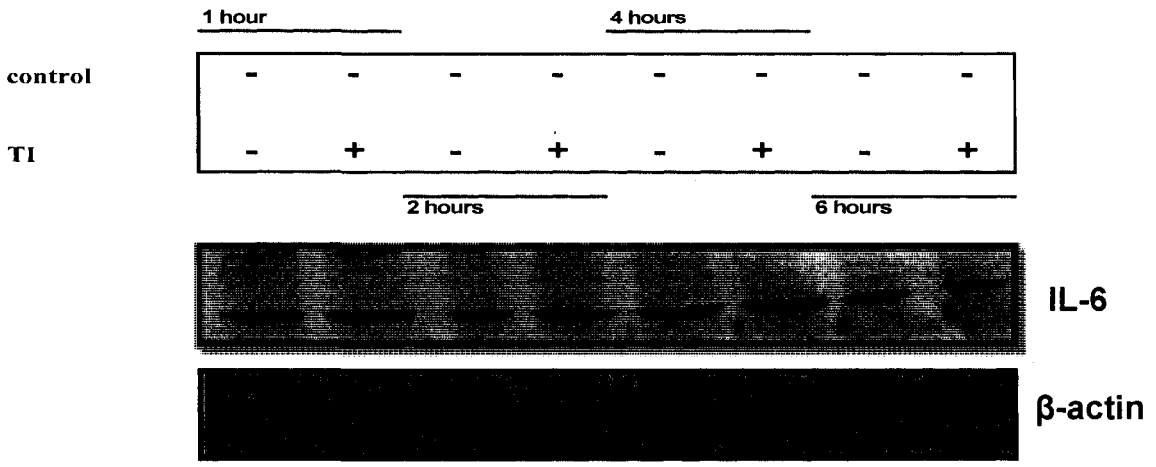


Figure 20: Time dependence of IL-1β and TNF-α mediated synergistic release of intracellular IL-6 in Western blot. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were treated with TNF-α (100ng/ml) and IL-1β (50ng/ml) for 1, 2, 4 and 6 hours. Post stimulation cellular protein was extracted and 25 μg of the total protein was subsequently separated via SDS-PAGE followed by Western analysis for IL-6 (Cell Sciences) protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

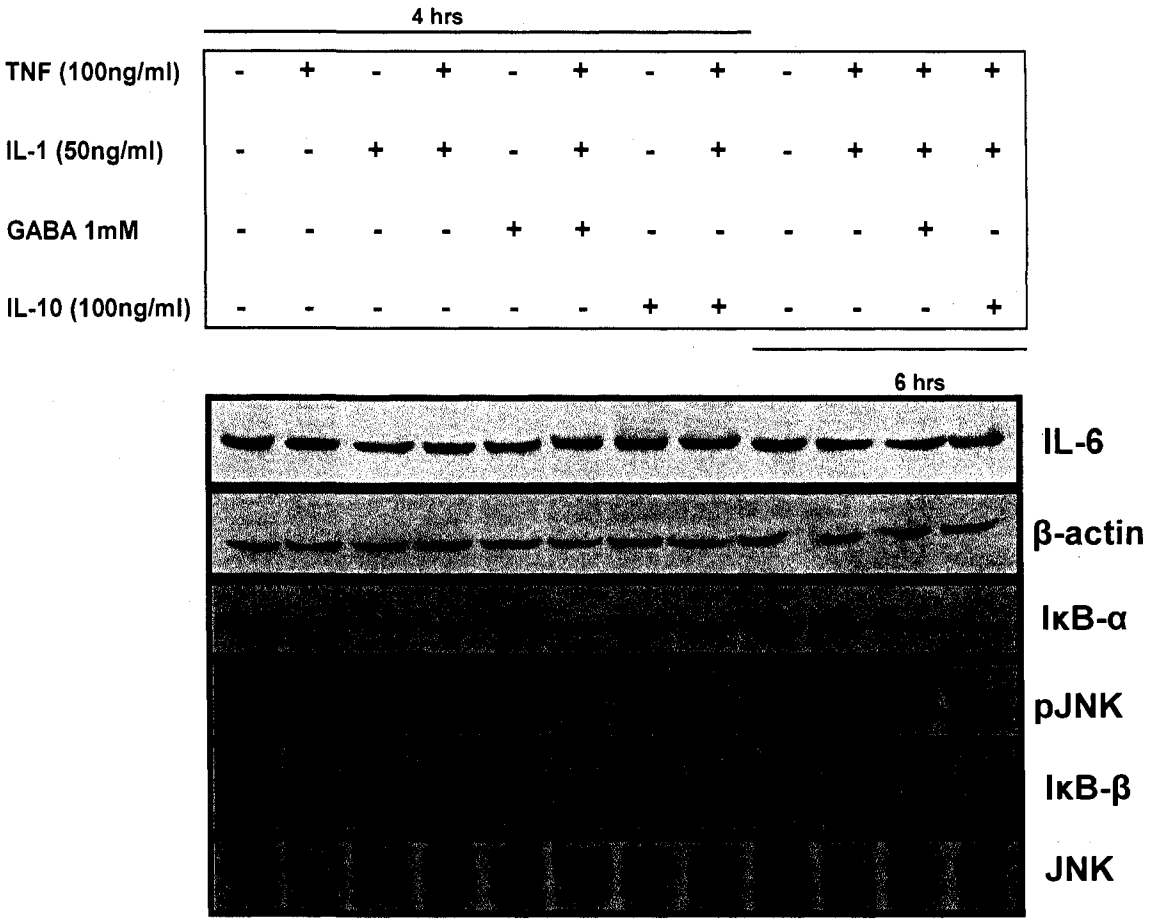


Figure 21: Time dependence of IL-1β and TNF-α mediated synergistic release of intracellular IL-6 of GABA vs. IL-10 in serum rich medium. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 and 1mM GABA for 1 hour and co-treated with 100ng/ml IL-10, 1mM GABA and TNF-α (100ng/ml) + IL-1β (50ng/ml) for 4 and 6 hours. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, β-Actin and IL-6 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

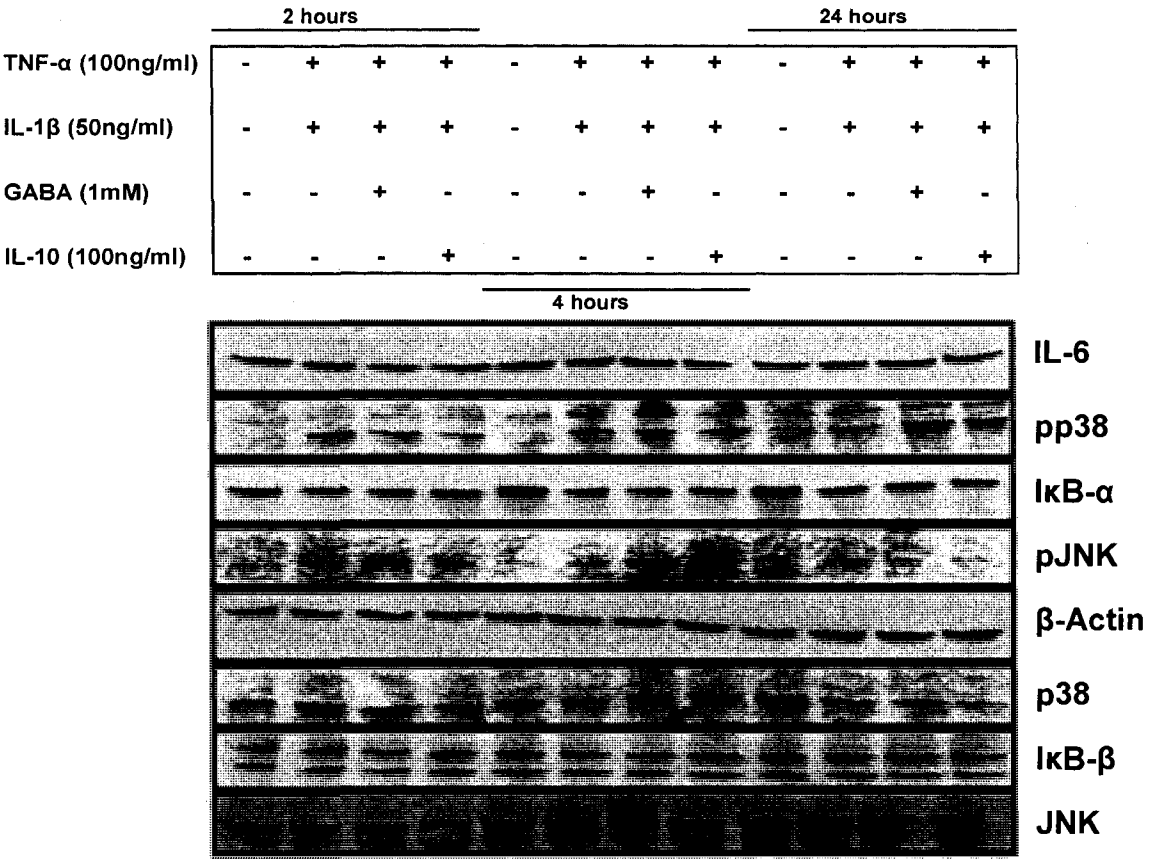


Figure 22: Extended time response of IL-1β and TNF-α mediated synergistic release of intracellular IL-6. Effects of GABA and IL-10 on p38 and NF-κB pathways. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 and 1mM GABA for 1 hour and co-treated with 100ng/ml IL-10, 1mM GABA and TNF-α (100ng/ml) + IL-1β (50ng/ml) for 2, 4 and 24 hours. Post stimulation cellular protein was extracted and 25 µg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for p38, pp38, IκB-α, IκB-β, β-Actin and IL-6 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

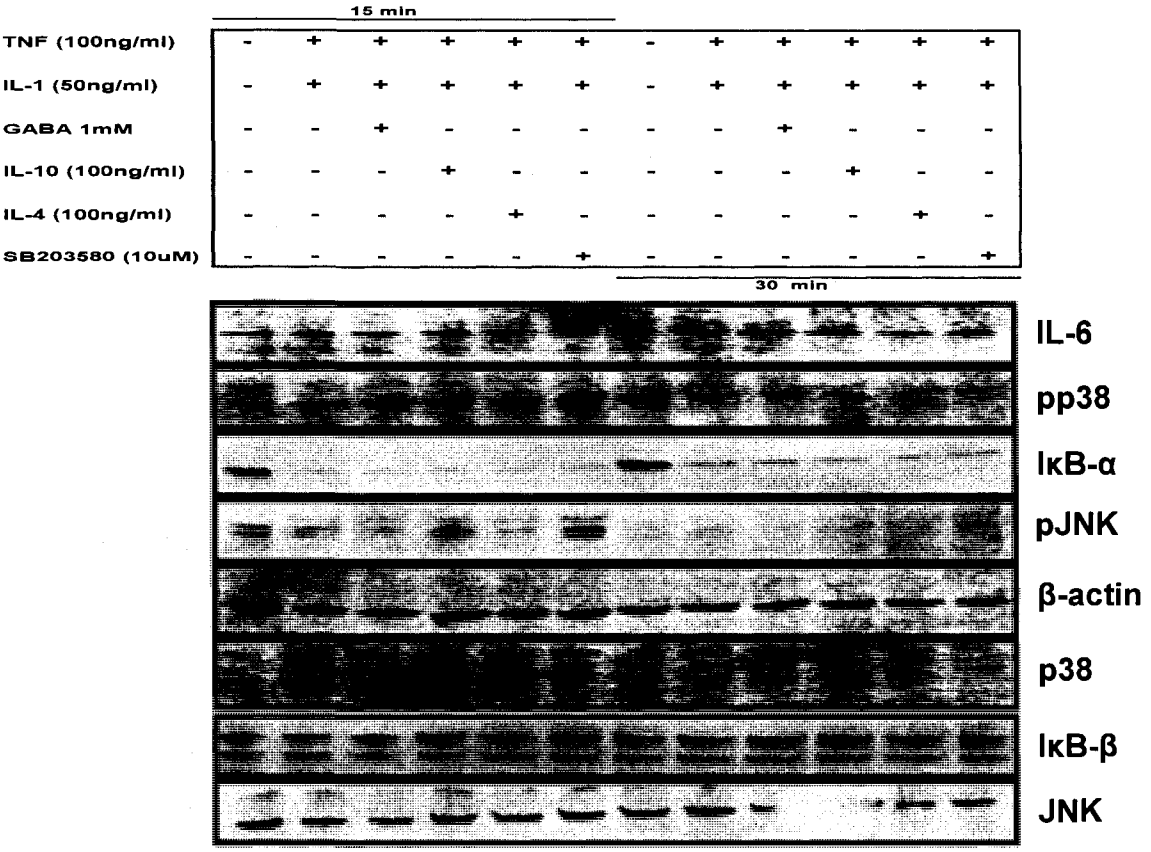


Figure 23: Time dependence intracellular IL-6 release. Rat C6 cells were plated (3.0 X10^6 cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10, 1mM GABA, 100ng/ml IL-4 and 10μM SB203580 for 1 hour and co-treated with 100ng/ml IL-10, 1mM GABA and TNF-α (100ng/ml) + IL-1β (50ng/ml) for 4 and 6 hours. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, β-Actin and IL-6 protein (1:1000 for all). The data are represented as the observations obtained from a single experiment.

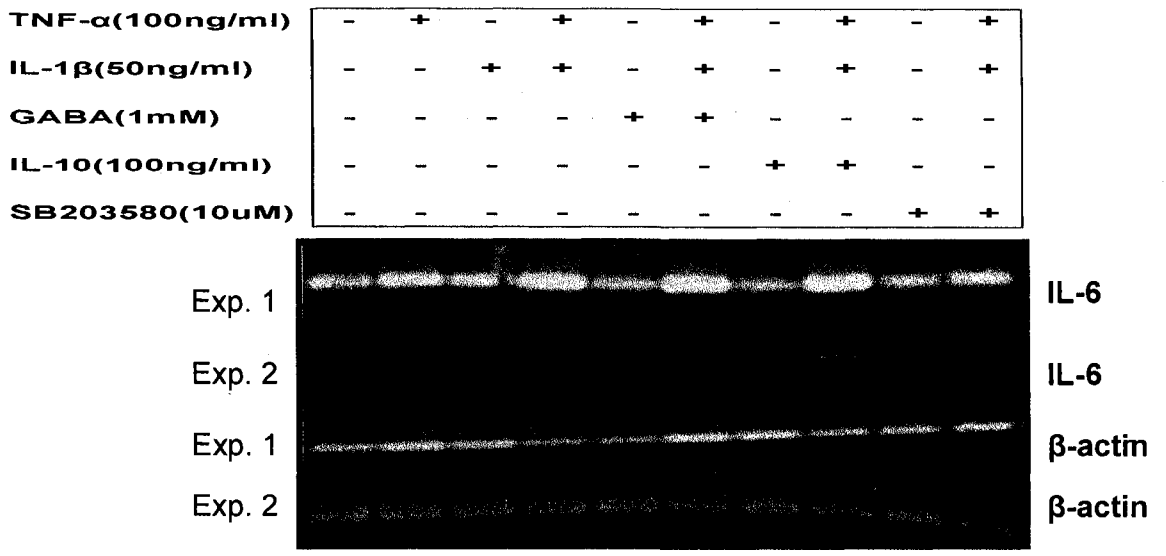


Figure 24: Relative expression of IL-6 transcript in cells treated with GABA, IL-10, TNF-α and IL-1β. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 and 1mM GABA for 1 hour and co-treated with 100ng/ml IL-10, 1mM GABA and TNF-α (100ng/ml) + IL-1β (50ng/ml) for 4 hours. Post stimulation cellular RNA was extracted and 1 µg of total ENA extract were subsequently separated and analyzed via semi-quantitative RT-PCR. The data are presented as observations obtained from two consecutive experiments.

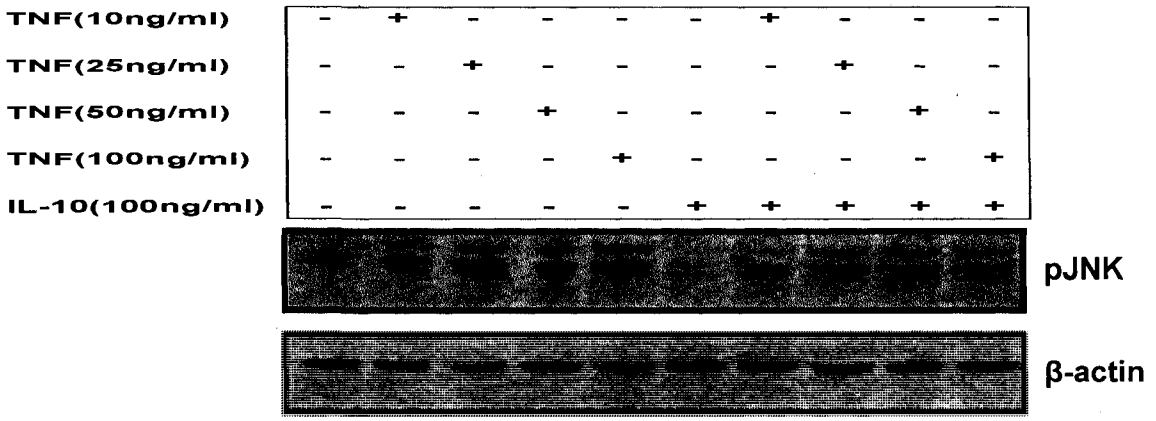


Figure 25: Effects of IL-10 on TNF-α-mediated phosphorylation of JNK. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 for 1 hour and co-treated with 100ng/ml IL-10 and TNF-α (100, 50, 25, 10ng/ml) for 15 min. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for pJNK and β-Actin (1:1000 for all). The data are represented as the observations obtained from a single experiment.

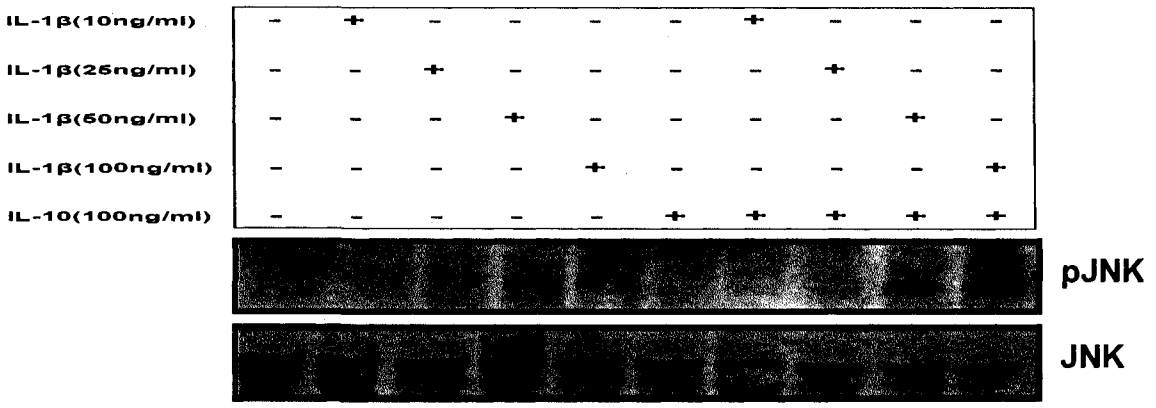


Figure 26: Effects of IL-10 on IL-1β-mediated phosphorylation of JNK. Rat C6 cells were plated (3.0 $X10^6$ cells/dish in a PDL-coated 35 X 10 mm-dishes). Cells were pretreated with 100ng/ml IL-10 for 1 hour and co-treated with 100ng/ml IL-10 and IL-1β (100, 50, 25, 10ng/ml) for 15 min. Post stimulation cellular protein was extracted and 25 μg of the total protein were subsequently separated via SDS-PAGE followed by Western analysis for pJNK and total JNK (1:1000 for all). The data are represented as the observations obtained from a single experiment.

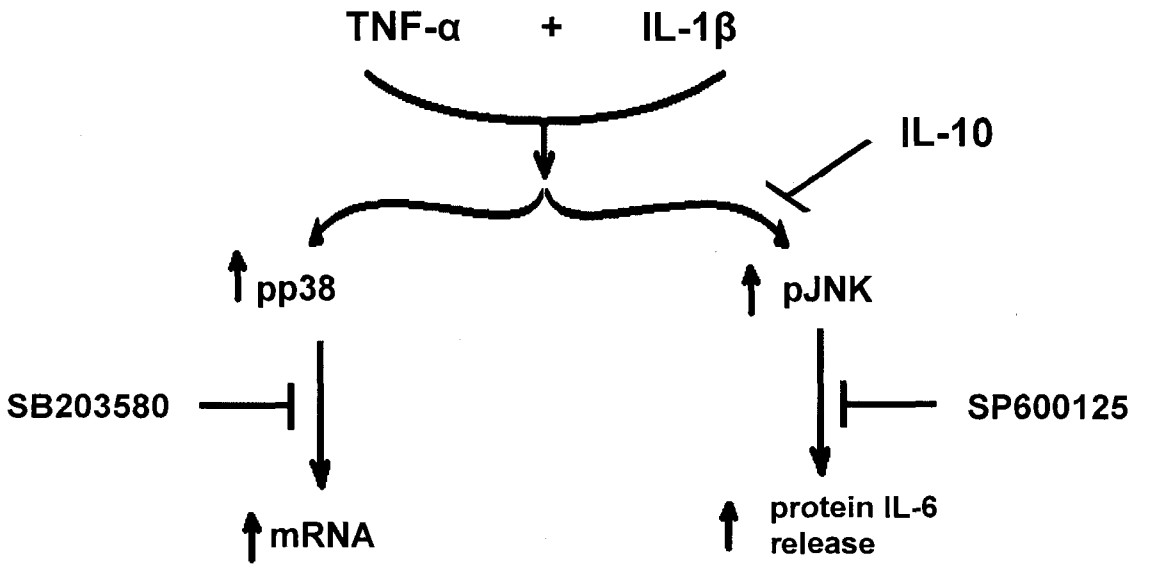


Figure 27: Postulated mechanism of IL-10 interaction with TNF-α and IL-1β activation of JNK and p38 MAP kinases.

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