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The Expression of Inflammatory Genes in Human Brain Endothelial Cells Stimulated by Beta-Amyloid Peptides is Mediated by JNK-AP1 Signaling Pathway

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ABSTRACT

The Expression of Inflammatory Genes in Human Brain Endothelial Cells Stimulated by Beta-Amyloid Peptides is Mediated by JNK-AP1 Signaling Pathway

Vanja Vukic
University of Ottawa, 2008

Advisor:
Professor Wandong Zhang

Alzheimer's disease (AD) is characterized by accumulation and deposition of beta-amyloid (Aβ) peptides in the brain. Deposition of Aβ peptides in cerebrovascular system results in chronic vascular inflammation, neurovascular uncoupling and insufficiency, contributing to neurodegeneration. The purpose of this study was to investigate the inflammatory response in human brain endothelial cells (HBEC) induced by aggregated $A\beta_{1-40}$ and the molecular mechanism of the Aβ-stimulated inflammatory gene expression. Primary or immortalized HBEC (iHBEC) were treated with aggregated $A\beta_{1-40}$, control peptides (scrambled $A\beta_{1-40}$ or poly-asparagine) or 2mM NaOH (vehicle), in the presence or absence of a specific JNK inhibitor (SP600125, 30 μM) at different time points, and inflammatory gene expression was analyzed by reverse-transcriptase polymerase-chain reaction (RT-PCR). TranSignal Protein/DNA Array was used to profile the activities of transcription factors (TF) in Aβ-stimulated HBEC. Nuclear extracts were isolated to determine interaction of AP-1 with DNA by electrophoretic mobility shift assay (EMSA) and supershift assay. The levels of total c-Jun and phosphorylated c-Jun at Ser73/Ser63 were analyzed through Western blotting, and AP-1 transcriptional activity was evaluated by reporter gene assays. In addition, to test indirect effects of Aβ on iHBEC, conditioned media from $A\beta_{1-42}$-stimulated primary microglia

was used to treat iHBEC at different time points in the presence of SP600125. The results obtained in this study have shown that aggregated $A\beta_{1-40}$ peptides strongly stimulated the expression of monocyte chemoattractant protein 1 (MCP-1), interleukin 8 (IL-8), interleukin 6 (IL-6) and growth-related oncogene (GRO). TF profiling has revealed that Aβ treatment strongly activated AP-1 in the cells. Electrophoretic migration shift assay (EMSA) and supershift assay have confirmed that AP-1 was strongly activated in HBEC at 2 and 4 h post-Aβ-treatment and physically interacted with AP-1-binding DNA sequence and that c-Jun is a component of the activated AP-1 dimeric complex. Aβ-stimulated AP-1 activity was further confirmed by reporter gene assay. Western blot has shown that c-Jun was activated as a result of phosphorylation at Ser73, suggesting that c-Jun N-terminal kinase (JNK) is involved in c-Jun phosphorylation and subsequent AP-1 activation. A specific JNK inhibitor was therefore selected to pre-treat the cells before Aβ-treatment. As a result, AP-1 activation and binding to DNA and c-Jun phosphorylation at Ser-73 was completely inhibited. The inhibitor also strongly inhibited Aβ-stimulated MCP-1 expression and AP-1 reporter gene activity. These results suggested that Aβ peptides interacted with cell membrane and activated JNK-AP1 signaling pathway to up-regulate the expression of inflammatory genes in the cells. Confocal imaging analysis has shown that Aβ-peptides interact with HBEC membrane at 30 min post-addition and then got internalized. Western blot and co-immunoprecipitation have demonstrated that RAGE is expressed in HBEC and interacts with Aβ-peptides. Pre-incubation of the cells with an anti-RAGE antibody significantly reduced Aβ-binding to cell membrane and its internalization and AP-1 activity. These

results suggested that interaction of Aβ peptides with RAGE may be responsible for triggering JNK-AP1 signaling cascade in HBEC.

To demonstrate indirect influence of Aβ-triggered inflammation in microglia on HBEC, conditioned media from human primary microglia stimulated with $A\beta_{1-42}$ was used to treat the cells. The conditioned media strongly stimulated the expression of IL-6, MCP-1, IL-8 and IL-1β in HBEC at various time points when compared to controls. Conditioned media induced-IL-6 expression was decreased in the presence of the JNK inhibitor; while other kinase inhibitors had no effect. These results suggested that secreted factors from Aβ-stimulated microglia also affected the inflammatory response in HBEC.

In summary, these experiments have demonstrated that binding and internalization of Aβ peptides at HBEC cellular membrane, possibly through RAGE, stimulated JNK signaling cascade pathway in HBEC which resulted in increased phosphorylation of c-Jun at Ser-73 and that activated AP-1 was responsible for increased expression of inflammatory genes in the cells. Aside from direct action on HBEC, Aβ peptides also stimulated microglia to release pro-inflammatory factors, which in turn, stimulated the expression of inflammatory genes in HBEC. The JNK-AP1 signaling pathway and inflammatory factors characterized in this study may potentially serve as therapeutic targets to relieve Aβ-induced inflammation in Alzheimer's disease.

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I. INTRODUCTION

Alzheimer's Disease and Beta-Amyloid Hypothesis

Alzheimer's disease (AD) is a multi-factorial neurodegenerative disorder characterized by progressive synaptic loss and neuronal death with gradual cognitive decline. However, the pathogenic factors and mechanisms of Alzheimer's disease are still not fully understood. The pathological characteristics of Alzheimer's disease include accumulation and deposition of amyloid beta (Aβ) peptides in brain parenchyma (senile plaques) and cerebrovessels and neurofibrillary tangles (NFTs). One of the main hypotheses about Alzheimer's disease is beta-amyloid hypothesis, which is supported by a number of epidemiological, genetic and experimental studies (Sisodia and Price, 1995). Aβ peptide is derived from a large transmembrane beta-amyloid precursor protein (APP). Sequential cleavage of APP by β-and γ-secretases yields either $A\beta_{1-40}$ or $A\beta_{1-42}$ peptide; while sequential cleavage of APP by α-and γ-secretases does not yield Aβ peptides (Selkoe, 2001). It is well established that familial Alzheimer's disease (FAD) results from genetic mutations in genes encoding APP (Swedish double mutation) or γ-secretase components presenilin-1 (PS1) or PS2. A large amount of Aβ peptides is produced and deposited in the brain resulting in early onset of Alzheimer's disease (Selkoe, 1999). APP gene is located on chromosome 21. Middle-aged trisomy 21 (e.g., Down's syndrome) individuals, who possess three copies of APP gene, develop neuropathology indistinguishable from that of AD patients (Giaccone *et al.*, 1989; Mann, 1989), which may be responsible for mental retardation of the syndrome. Transgenic mice carrying mutant human APP or/and PS-1 genes develop Alzheimer's Aβ pathology and display

cognitive deficits (Duyckaerts *et al.*, 2008; Blanchard *et al.*, 2003). Active Aβ immunization in Alzheimer's mouse models or in AD patients showed reversible Aβ pathology and improved cognitive performance. Experimental studies have shown that Aβ oligomers are toxic to neuronal cells and cause synaptic dysfunction (Lacor *et al.*, 2004; Morishima *et al.*, 2001). Aβ plaques are formed with neuronal death resulting in neuroinflammation (Craft *et al.*, 2006). Microglia are activated by deposited Aβ peptides and macrophages are attracted to the inflammatory sites. These cells release inflammatory cytokines. Aβ-stimulated neuroinflammation is known to contribute to neurodegeneration in Alzheimer's disease.

The amyloid cascade/neuroinflammation hypothesis has originated from the observations of histopathologic studies that show clustering of activated microglia at sites of amyloid deposition in AD brain, mainly at advanced senile plaques of the compact neuritic type (Haga *et al.*, 1989; Itagaki *et al.*, 1989; Rozemuller *et al.*, 1989; Matiacce *et al.*, 1990; Perlmutter *et al.*, 1992; Rogers *et al.*, 1992). Further studies have demonstrated that many cytokines and complement proteins can be detected in AD brain in association with microglia (Rogers *et al.*, 1992 Griffin *et al.*, 1995). Thus, amyloid cascade/neuroinflammation hypothesis states that amyloid peptide act on microglia and causes the cells to produce a variety of neurotoxic substances, such as reactive oxygen and nitrogen species, pro-inflammatory cytokines, complement proteins, and other inflammatory mediators that bring about neurodegenerative changes in Alzheimer's disease (Akiyama *et al.*, 2000; Eikelenbloom *et al.*, 2002; McGeer and McGeer, 2001; Rogers *et al.*, 2002).

Alzheimer's Disease and Cerebral Amyloid Angiopathy

A large number of Alzheimer's patients are associated with deposition of Aβ peptides in cerebral vessels and develop cerebral amyloid angiopathy (CAA) (Zlokovic, 2005). CAA involves both small and large brain vessels, including microvessels and capillaries. Aβ deposition to cerebrovascular structures induces significant damage to endothelial cells and may alter cellular redox, which triggers down-stream kinase cascades, leading to neurovascular inflammation (Malinski, 2007). In addition, in AD pathology the thickness of the smooth muscle layer due to Aβ deposition is often reduced, which results in rupture of vessel wall and intracerebral bleeding in many cases (Van Nostrand *et al.*, 2001). Neurovascular inflammation results in neurovascular uncoupling and vascular insufficiency and contributes to neurodegeneration in Alzheimer's disease. A faulty clearance of Aβ across blood-brain barrier (BBB) due to aberrant angiogenesis associated with low levels of Aβ clearance receptors (i.e., LRP) or increased level of its influx receptors (i.e., RAGE) and inefficient Aβ proteolysis, would lead to increased levels of soluble Aβ in brain ISF and lead to elevated fibrillar Aβ levels (Zlokovic, 2005). Dutch/Iowa mutant forms of Aβ, which are vasculotropic and rich in β-sheets (Davies *et al.*, 2004), are associated with impaired vascular clearance of Aβ across the BBB and increase in Aβ brain capillary deposition and formation of amyloid lesions (Deane *et al.*, 2004; McComb and Zlokovic, 2000). The relatively short distance between the adjacent human brain capillaries (~ 40 μm) enables rapid diffusion-mediated transport of nutrients and oxygen within brain interstitial fluid from blood to neurons, and rapid diffusion of neurotransmitters and metabolic waste products from synapses and neurons back to circulation. In AD, there is a distorted cytoarchitecture of the capillary

units and thus there is a dysregulated nutrient and/or oxygen transport and impaired elimination of metabolic waste products caused by dysfunction in transport clearance systems at the BBB, which contributes to neuronal dysfunction (Bailey *et al.*, 2004; Vinters and Farag, 2003; Farkas and Luiten, 2001). In addition to this process, endothelial wall degenerates as a result Aβ and amyloid deposits on the outer side of the basement membrane, which promotes neuroinflammatory vascular responses. Activated cells at the BBB site secrete pro-inflammatory cytokines, reinforcing vasoactive substances and amplifying inflammatory response. However, little is known about Aβ-induced neurovascular inflammation and the relevant molecular signaling mechanism.

Neuroinflammation and Neurovascular Inflammation in Alzheimer's Disease

All major pro-inflammatory cytokines and chemokines have been detected in AD brain, which strongly suggests that inflammatory reaction plays a crucial role in the pathogenesis of AD (Ho *et al.*, 2005; Tuppo and Arias, 2005; Neuroinflammation working group, 2000). A considerable body of *in vitro* evidence indicates that the inflammatory response of glial cells is reduced by non-steroidal anti-inflammatory drugs (NSAIDs). For example, indomethacin reduces inflammatory response of rat microglia to β-amyloid *in vitro* (Gotschall, 1996) and *in vivo* (Netland, 1998) and blocks the release of interleukin-6 (IL-6) in cultured human astrocytes stimulated with interleukin-1β (IL-1β) (Bloom *et al.*, 1997). In isolated brain microvessels obtained from AD patients, there were high levels of soluble IL-1β, IL-6, tumor necrosis factor-α (TNF-α) as well as cell-associated IL-1β and monocyte chemoattractant protein-1 (MCP-1) (Grammas and Ovase, 2001). *In vitro* studies show that treatment of cultured microglia with low doses

of Aβ peptides significantly induced secretion of IL-1β, TNF-α, MCP-1 and macrophage inflammatory protein-1α (MIP-1α) (Walker *et al.*, 2001, 2006). Many studies showed that microglia were closely associated with senile plaques and have substantially increased expression of many inflammatory markers, including major histocompatibility complex-I (MHC-I) and–II, IL-1 and TNF-α as well as receptors for a variety of cytokines and inflammatory reagents (Walker, 1998). A number of studies have shown that microglia stimulated by Aβ peptides play a crucial role in Aβ-induced chronic inflammation in AD, which contributes to neurodegeneration. However, little is known about the effects of Aβ on brain endothelial cells which compose the blood-brain barrier.

At a basal state, endothelial cells (EC) are known to perform crucial functions for vascular homeostasis. Once activated, EC participate in various vascular processes, such as angiogenesis, inflammation, and atherogenesis. Even though NF-κB has been reported to be involved in EC activation during inflammatory response, increasing evidence shows also involvement of activator protein-1 (AP-1). It has been shown that AP-1 is activated in EC in response to various stimuli, such as cytokines (Dixit *et al.*, 1989), bacterial endotoxin, antioxidants (Munoz *et al.*, 1996), hypoxia (Bandyopadhyay *et al.*, 1995), fluid shear stress (Chien *et al.*, 1998), LDL (Zhu *et al.*, 1998), etc. Furthermore, studies of sequence analyses in various genes encoding for chemokines and adhesion molecules show that AP-1-binding sites are commonly found in their promoter regions, such as human MCP-1 gene.

Activator Protein-1 and Its Signaling Activation Pathways

AP-1 is a transcription factor that regulates the expression of inflammatory genes and growth factor-inducible genes. AP-1 complex is biologically active as homodimer or heterodimer composed of Fos (v-Fos, c-Fos, Fos-B, Fra-1, fra-2), Jun (v-Jun, c-Jun, JunB, JunD) and activating transcription factor (ATF) subunits (Karin *et al.*, 1997). Jun-ATF or ATF homodimers preferentially bind to cAMP-responsive element (CRE) TGACGTCA (Hai and Curran, 1991).

In order to analyze AP-1 activation and its function, it is crucial to understand the upstream signaling of certain kinases known to be essential for induction of transcription factors. It is known that AP-1 dimer is part of MAPK (mitogen-activated protein kinases) signaling pathways, including ERK (extracellular signal-related kinase), JNK (c-Jun N-terminal kinase) and p38 kinases. The regulation of AP-1 activity occurs at the levels of transcription and protein stability as well as the level of post-translational modification by abundance of co-activators. C-Jun is a common component of AP-1 protein complex. The activity of c-Jun is regulated by multiple mechanisms, including phosphorylation by JNK. Phosphorylation of c-Jun at amino-terminal serine and threonine residues prevents its ubiquitination and degradation. However, phosphorylation of c-Jun at serine 73 or serine 63 activates AP-1 for binding to target genes and activate transcriptional expression of the genes (Thakur *et al.*, 2006). In addition, c-Jun expression is elevated in response to growth factors, cytokines and UV irradiation (Karin, 1995) as a result of activation of JNK and p38 groups of MAPKs (Hibi *et al.*, 1993; Raingeaud *et al.*, 1995; Derijard *et al.*, 1994).

Among three JNKs (JNK1-3), JNK2 shows the highest affinity for c-Jun since it contains the putative loop region which interacts with JNK-docking site on c-Jun. JNK is a stress-activated protein kinase that is activated by environmental insults, such as, osmotic shock, ultraviolet (UV) irradiation, pH changes and reactive oxygen species (ROS), inflammatory stimuli (antigens, cytokines and infection), and growth factors (Waetzig et al., 2005). Once phosphorylated, c-Jun has enhanced ability to dimerize or heterodimerize and bind to the transcription activation machinery. In the peripheral immune system, JNKs co-regulate immune responses, such as proliferation and maturation of T-lymphocytes (Arbour *et al.*, 2002; Conze *et al.*, 2002). Giri and colleagues (Giri *et al.*, 2003) showed that Aβ peptides at physiological concentration (Kuo *et al.*, 1999) trigger cellular signaling pathway in THP-1 monocytes and increase the gene expression of specific proinflammatory factors, such as TNF-α, IL-1, IL-8, and MCP-1. This signaling pathway involves activation of tyrosine kinase and extracellular signal-regulated kinase (ERK-1 and ERK-2), but not p38, that resulted in increased expression of transcription factors such as Egr-1 and AP-1 but not CREB and NF-κB. JNKs are also involved in mediating pro-inflammatory actions in microglia (Waetzig *et al.*, 2005). Waetzig and colleagues stimulated primary rat microglia with LPS (lipopolysaccharide) and found strong increase in the expression AP-1 target genes, such as cyclooxygenase 2 (Cox-2), MCP-1, TNF-α and IL-6. These authors also looked at the expression of different nuclear JNK isoforms upon LPS stimulation and found that JNK2 was much more increased as compared to other isoforms, suggesting that JNK2 isoform is most likely to be involved in activation of c-Jun. Similarly, experiments performed in our laboratory showed that treatment of primary human brain endothelial cells (HBEC)

with aggregated $A\beta_{1-40}$ peptides resulted in two-fold increase in AP-1 and CREB when compared to controls. This has been confirmed in AD brains as compared to age-matched non-demented brain (ND) samples (Callaghan *et al.*, 2007). In order to study the roles of AP-1 in transcriptional regulation of inflammatory genes stimulated by Aβ peptides in HBEC, it is crucial to understand the upstream signaling pathways essential for induction of AP-1 transcription factor and to examine the molecular mechanism underlying AP-1 signaling as result of Aβ-stimulated expression of inflammatory genes in HBEC.

The trigger for the mechanism of action for Aβ peptides at cellular membrane in HBEC cells still remains to be determined. By the virtue of its structure, Aβ is capable of binding to a variety of biomolecules, including lipids, proteins, and proteoglycans. Numerous studies have demonstrated Aβ interactions with various membrane receptors, such as receptor for advanced glycation end product (RAGE), insulin receptor, serpin-enzyme complex complex receptor (SEC-R), integrins, formyl-peptide receptor-like 1 (FPRL1), CD36, scavenger receptor A, and α-7 nicotinic acetylcholine receptor (α7nAChR) (Verdier *et al.*, 2004). *In vitro* studies have shown that RAGE interacts with Aβ peptide eliciting neuronal expression of macrophage–colony stimulating factor (M-CSF) through the activation of NF-κB attributing to inflammatory pathway (Du Yan *et al.*, 1997). The release of M-CSF by neurons stimulates receptors on microglial cells, inducing increased expression of ApoE and macrophage scavenger receptor and enhancing microglial cell proliferation and migration. Immunohistochemistry has revealed that Aβ, advanced glycosylation end products (AGE), and RAGE are co-localized in granules of astrocytes from AD brains (Sasaki *et al.*, 2001). This finding

suggests that glycated Aβ is taken up in the cells via RAGE and is degraded through lysosomal pathway in astrocytes. Aβ interaction with RAGE expressed on EC, neurons and microglia initiates cellular signaling leading to generation of oxidant stress as determined by the formation of lipid peroxides and activation of transcription factor NF-κB (Yan *et al.*, 1996). The oxidant stress in EC further up-regulated RAGE, and thereby augments further Aβ-RAGE interaction. Oxidant stress generated from Aβ-RAGE interaction may activate JNK signaling pathway. Similarly, Giri and colleagues demonstrated that $A\beta_{1\text{-}40}$ interaction with RAGE on cultured human brain microvascular endothelial cells (HBMVEC) initiates cellular signaling resulting in adhesion and several-fold increase in the migration of monocyte-like cells (HL-60 and THP-1) and peripheral blood monocytes across the confluent monolayer of HBMVEC (Giri *et al.*, 2000).

Aside from its direct action on brain endothelial cells (BEC), Aβ peptide is likely to have an indirect influence on BECs by primarily triggering activation of microglia and astrocytes that surround BECs at physiological settings, and this, in turn, resulted in production of a wide plethora of inflammatory chemokines and cytokines. These pro-inflammatory factors can elicit their effects on BECs to further augment the process of inflammation. Cytokines such as IL-1β and TNF-α have been shown to elicit induction of inflammatory genes in human endothelial cells by activating NF-κB and AP-1 transcription factors (Goebler *et al.*, 2001; Klampfer *et al.*, 1994). Also, it has been shown that Aβ peptide potentiated cytokine secretion in IL-1β-activated human astrocytoma cells (Gitter *et al.*, 1995).

In the current study, I have examined the effects of aggregated $A\beta_{1\text{-}40}$ on human brain endothelial cells to determine if Aβ-stimulated HBECs respond through increase in

inflammatory gene expression. I have also investigated the signaling events that take place prior to inflammatory gene induction in Aβ-stimulated HBEC. This *in vitro* experimental model allowed me to study inflammatory mechanisms that could take place in AD vascular pathology. My results show that treatment of HBEC with aggregated $A\beta_{1\text{-}40}$ results in peptide binding to cellular membrane receptors and internalization, possibly through RAGE receptor. This Aβ interaction with HBEC induces JNK signaling pathway, which, in turn, phosphorylated Ser73 residue of c-Jun, a component of AP-1, and then activates AP-1, leading to an increase in inflammatory gene expression in HBEC cells.

II. MATERIALS AND METHODS

Chemical and biochemical reagents

Dullbecos modified Eagles's medium (DMEM) was purchased from Invitrogen Inc., (N.Y., US). EBM-2 media (Clonetics Co., Lonza), growth factor and supplements were purchased from BioWhittaker Inc. Fetal bovine serum (FBS) was purchased from Hyclone, Inc. Synthetic $A\beta_{1-40}$ peptide at 1mg/vial was purchased from Biopeptides Co., Inc. (San Diego, CA, USA) and prepared in 2mM NaOH to a final concentration of 231μM. Dissolved Aβ peptide was sonicated for 5 min, aggregated overnight at 37°C and stored at -80°C. The stock solution was diluted to a desired concentration immediately before the use in serum-free media (DMEM). AP-1 promoter luciferase construct was obtained from Panomics Inc. Lipofectamine™ 2000 reagent and SuperFect Transfection Reagent were purchased from Invitrogen Inc., and QIAGEN Inc., respectively. SB600125, an anthrapyrazolone inhibitor of c-Jun N-terminal kinase (JNK), was obtained from Calbiochem Inc. PhosphoPlus(R) c-Jun (Ser63) II and c-Jun (Ser73) Antibody kit (Cat# 9260) was obtained from Cell Signaling Technology (Danvers, MA, USA). .

Conditioned microglial media were prepared by Dr. Douglas Walker at the Sun Health Research Institute (Sun City, Arizona). Primary human microglia cultures were prepared from autopsy brain tissues in Dr. Walker's laboratory and stimulated with either 2 μM or 5 μM $A\beta_{1-42}$ for 24 hr. The media were harvested, shipped on dry ice and stored in -80°C. The media were centrifuged at 10, 000 g for 10min at 4°C before use.

Cell Cultures

Primary human brain endothelial cell (HBEC) cultures were generously provided by Dr. Alexander Prat at the Montreal Neurological Institute (Montreal, CA). The cultures were prepared from in-house surgical brain samples of patients with epilepsy. The cells express an endothelial marker Factor-VIII related antigen. Passages 4 to 6 were used in the experiments of this study. HBEC cultures were maintained as described by Zhang *et al* (Zhang *et al.*, 1999) in DMEM supplemented with 5% human serum, 10% FBS, 20 % MCM, 1.5 mg/ml ECGS, 1000x ITS and 2500 units/ml heparin. Cells were plated on 0.5% gelatin-coated culture dishes. The medium was changed every second day and cells were plated at an appropriate density according to each experimental scale.

An immortalized HBEC (iHBEC) cell line was provided by Dr. Pierre-Olivier Couraud (Paris, France) at passage 29 (Weksler *et al.*, 2005) and maintained in EBM-2 media supplemented with 2.5 % FBS, hydrocortisone, VEGF, hFGF, R3-IGF-I, ascorbic acid, heparin and gentamycin. iHBEC were plated on rat tail collagen type I-coated culture dishes (1:30 dilution) and media was changed every second day. Human embryonic kidney epithelia 293 cells (HEK293) were maintained in 10% FBS in DMEM. No coating required on culture dishes and media was changed every second day.

RNA Isolation, Reverse transcription-polymerase chain reaction (RT-PCR) and Agarose Gel Electrophoresis

Total RNA was isolated from HBEC plated on 35 mm Petri dishes, using TRIzol reagent (Invitrogen Inc.) following manufacturer's instructions. Cells were lysed in TRIzol reagent in culture dishes, and the lysates were transferred to 1.5ml tubes.

Chloroform was added and the samples were centrifuged at 14, 000 rpm for 15 min at 4°C followed by addition of one volume isopropanol. After the subsequent centrifugation at 10, 000 g for 10 min, RNA was precipitated as a pellet in the tube. The pellet was washed twice with 70% ethanol in DEPC-treated dH_2O. RNA Pellets were resuspended in DEPC-treated H_2O and heated to 55°C for 10 min. RNA concentration was determined in DEPC-treated H_2O and reading at 260 OD values for each sample using Beckman DU 530 Life Science UV/Vis Spectrophotometer. For reverse transcription, 2 μg of RNA was pre-heated with oligo (dT) primers at 70°C and then chilled on ice. This was followed by addition 5x first strand buffer, 10mM dNTP, 0.1 M DTT and MLV reverse transcriptase (Invitrogen Inc.) to reaction mix and incubated at 42°C for 1h according to manufacturer's instructions. Specific DNA sequences were amplified using PCR master reaction mixture, including 10x PCR buffer, 10mM dNTP, 1.5mM $MgCl_2$, 10μM primer cocktail, 1.25 units/reaction Taq polymerase and RNase/DNase-free dH_2O. PCR primer sequences used in this study are listed in Table 1, and all primers were synthesized by Alpha DNA Inc. (Montreal, Quebec). Six-time loading buffer was added to amplified products and samples were loaded (15μl/lane) on 1.5 % agarose gel in 1x TBE buffer. PCR products were resolved on 1.5% agarose gel by electrophoresis at 150V for 1h. The gels were stained with 5μg/ml ethidium bromide followed by exposing gels to UV light using Kodak EDAS 290 Transilluminator (UVP, Inc., San Gabriel, CA, USA) for 1s and taking an image with Kodak DC290 Zoom Digital Camera.

To quantify DNA bands on gels from RT-PCR, densitometry analysis was performed for β-actin and MCP-1 PCR products. This was achieved by taking the numerical values of the β-actin and MCP-1 bands taken from the gel images using Kodak

1D 3.6, Version program. The obtained values for each band represented number of dark spots in a given area found in each band from the gel photographs. Final values used to plot bars on graph were determined by taking numerical value of MCP-1/β-actin ratios and setting Aβ-stimulated samples as ratio of 100%.

Table 1: Primers used in the RT-PCR reactions

Genes	Primer sequences	
β-actin	S primer	5'- GGCTACAGCTTCACCACCAC - 3'
	AS primer	5'-TACTTCCGCTCAGGAGGAGC - 3'
MCP-1	S primer	5'- GCTCGCTCAGCCAGATGCAAT - 3'
	AS primer	5'- TGGGTTGTGGAGTGAGTGTTC - 3'
IL-6	S primer	5'- CCAGGAGCCCAGCTATGAAC - 3'
	AS primer	5'- GCAGCCACTGGTTCTGT GCC - 3'
IL-8	S primer	5'- ATGACTTCCAAGCTGGCCGTG - 3'
	AS primer	5'- CTCCA CAACC CTCTGCACCC - 3
IL-1β	S primer	5'- TCCCTTCATCTTTGAAGAAGA - 3'
	AS primer	5'- GAGGCCCAAGGCCACAGG - 3'
GRO	S primer	5'- CAATCCCCGGCTCCTGCG - 3'
	AS primer	5'- CTTCCCATTCTTGAGTGTGGC - 3'
TNF-α	S primer	5'- GCCACCACGCTCTTCTG - 3'
	AS primer	5'- GGTGTGGGTGAGGAGCAC - 3'

Western blot analyses

Confluent iHBEC, grown in 60mm culture dishes, were treated with 20-40μM $A\beta_{1-40}$ peptides for 4 and 8h. The cells were collected and washed 2 times in 1x phosphate-buffered saline (PBS). Cells were lysed in buffer A mix containing buffer A (100mM HEPES; pH 7.9; 100mM KCl and 100mM EDTA), (Panomics, Inc.), 100 mM DTT, protease inhibitor cocktail and 10 % IGEPAL. After 10 min lysis on ice platform, cells/lysates were scraped off and centrifuged at 10,000 g for 3 min at 4°C. Supernatant was separated and stored at -80°C until the use, while nuclear pellet was dissolved in buffer B mix containing buffer B (100 mM HEPES; ph 7.9; 2 M NaCl; 5mM EDTA; 50% glycerol), protease inhibitor cocktail and 100 mM DTT. The pellet was lysed for 2h on shaking platform at 200 rpm, and the lysate was centrifuged at 10,000 g for 5 min at 4°C. Due to the concern of high salt concentration, buffer C mix [20mM Tris, ph 7.9; 20% (w/v) glycerol, 0.1M KCl, 0.2mM EDTA and 0.5mM DTT] was added to collected supernatant from the nuclear extract. Protein concentration was determined using BioRad DC Protein Assay kit (BioRad Laboratories Inc., CA). Protein assay was preformed on 96-well plate format by initially establishing a standard curve using 2mg/ml BSA and serially diluting it in H_2O and spiking it with buffer B mix/buffer C mix (1:1) buffer while samples were diluted with H_2O. Each well received initially prepared mixture of Reagent A and S, followed by addition of reagent B obtained from the kit. Plate was incubated for 10 min until bluish color developed and was subsequently read by Spectra MAX 340 spectrophotometer under 750nm (Molecular Devices Inc.) using SoftMax PRO program. Loading buffer was added to 10 μg of nuclear extract protein samples which were run on 10% SDS-polyacrylamide gel at 150V for 1h and 30 min.

Proteins were transferred from the gel to nitrocellulose membrane sandwiched in Western blot transfer unit and ran at 450mA for 2hr. The blots were blocked for 1h at room temperature in fresh blocking buffer [0.1 % Tween 20 in Tris-buffered saline (TBST), pH 7.4, containing 5% skimmed milk]. Primary polyclonal rabbit IgG antibodies of anti-c-Jun and anti-Ser73 or anti-Ser63-Phospho-c-Jun were diluted at 1:500 and added to 0.1% TBST containing 1% skimmed milk, and the blots were incubated overnight at 4°C. Following three washes in 0.1% TBST, transferred proteins were incubated with a secondary antibody (1:2000 dilution) conjugated with horseradish peroxidase (donkey anti rabbit IgG) for 1 h at room temperature. Blots were washed three times with 0.1% TBST and were subsequently developed using ECL Plus substrate solution (Amersham Pharmacia Biotech, Montreal, Quebec) for 1 min according to manufacturer's instructions and exposed to X-ray films.

Co-immunoprecipitation

iHBECs were plated in 60 mm dishes and grew to confluence. Media was aspirated and cells were washed in 1x PBS buffer 3 times. Panomics buffer A mix was used to lyse the cells in the dishes for 10 min on shaking ice bucket at 250 rpm. Cells were scraped and place into 1.5ml-ultracentrifuge tubes and centrifuged at 40,000 g for 30 min at 4°C. Supernatant was placed into separate Eppendorf tubes and this represented the cytosolic fraction, while the pellet, which represented membrane fraction, was dissolved in Panomics buffer B mix/Glycomics buffer (1:1 ratio). Protein concentration in both cytosolic and membrane fractions was determined by using DC BioRad protein assay kit as described above. Protein lysate (500 μg) from each fraction

was placed in 1.5 ml-tube, diluted in 1x PBS buffer to 1mg/ml final concentration. Five micrograms of $A\beta_{1-40}$ peptide was added to each sample followed by rotation overnight at 4°C. Two tubes were prepared, and one of them was used as a negative control. Ten micrograms of Aβ antibody (6E10, mouse monoclonal Aβ IgG antibody, Signet Inc.) was added to one of the two tubes and not to the negative control. The samples were rotated 2-3 hrs at 4°C. Then, 50 µl of Protein A/G beads (Santa Cruz Biotechnology, USA) was added, and the samples were rotated for another 2 h at 4°C. Tubes were centrifuged at 15, 000 rpm for 30 sec and supernatant was removed. Beads were washed 5 times with 1ml 1x PBS and spun down each time for 30 sec at 4°C. At last, the buffer was removed and the pellet was resuspended in 50 µl of 2x Western loading buffer. Protein samples were prepared according to general Western blot protocol as described earlier and the blots were probed using polyclonal rabbit RAGE antibody IgG (1:500).

TranSignal™ Protein/DNA Array

Primary HBEC were plated on 60 mm culture dishes and grown to confluence. Cells were then treated with 5 µM $A\beta_{1-40}$, 5 µM scrambled $A\beta_{1-40}$ or 2 mM NaOH (vehicle) for 8h, and nuclear extracts were prepared from the cells as described above. Nuclear extract proteins and TranSignal probe mix (TF-Probe mix) were mixed and incubated for 30 min at 15°C. TF-Probe mix was passed through a spin column previously washed with 1x Column Incubation Buffer, and centrifuged at 5, 000 g for 30 sec at 4°C. The spin column was washed with wash buffer for three times, and the column was spun at 5,000 g after each wash. Elution buffer was added to spin column to elute TF-bound probe. The probes were denatured at 95°C for 3 min, chilled on ice and

subsequently added to array membranes, previously pre-warmed at 42°C water bath, and hybridized at 42°C overnight in rotating hybridization membrane bottles. On next day, the membranes were washed twice with pre-warmed hybridization wash buffer for 20 min in rotating hybridization oven. Using forceps each membrane was carefully taken out from a hybridization bottle and placed into a container filled with 1x blocking buffer, in which it incubated at room temperature for 15 min with gentle shaking. For signal detection, membranes were incubated with Streptavidin-HRP conjugate 1:1000 made in 1x blocking buffer for 15 min at room temperature. This was followed by washing the membrane for three times with wash buffer. Detection buffer was then added to each membrane and they were incubated at room temperature for 5 min. The membrane was exposed to an x-ray film.

Electrophoretic mobility shift assay (EMSA) and Supershift Assay

Primary HBEC cultures were grown to confluence in 100 mm dishes and treated with 5 μM $A\beta_{1\text{-}40}$, 5 μM scrambled $A\beta_{1\text{-}40}$ or 2mM NaOH (vehicle). Nuclear extraction kit from Panomics Inc. was used to prepare nuclear extracts from the cells. Protein concentration was determined by BioRad DC protein assay reagents. Ten micrograms of protein sample was used in the reaction. Synthetic double-strand nucleotides containing AP-1-binding site was labeled with 50 μCi [γ-^{32}P]-ATP using T4 polynucleotide kinase and separated from free [γ-^{32}P]-ATP by gel filtration using a G-25 sephadex column (Armesham Pharmacia Biotech). Double-strand nucleotide sequences used for EMSA were as follows: wild-type AP-1(2): 5'-CGC TTG ATG ACT CAG CCG GAA– 3' and AP-1 mutant: 5'-CGC TTG ATG ACT TGG CCG GAA- 3', purchased from Alpha DNA

(Montreal, Quebec). Prior to addition of [γ-^{32}P]-ATP-labeled nucleotide (25,000 cpm), 10 μg of nuclear extract was mixed with DNA binding buffer [4% glycerol, 1mM EDTA, 1mM DTT, 100 mM NaCl, 10mM Tris-HCl (pH 7.5)], herring sperm DNA and poly (dI-dC), mixed and kept at room temperature for 10 min. Subsequently, [γ-^{32}P]-ATP-labeled nucleotides were added to nuclear extract reaction mix, and the reaction was incubated for 20 min at room temperature. Gel loading buffer was added to the reaction, and the samples were loaded to 5% polyacrylamide gel in 1x Tris-Glycine buffer. Gel was run at 200V for 2 hr, then dried for 1h under vacuum and exposed to X-ray film overnight for radiography.

Transient transfection and AP-1 luciferase reporter gene assay

HEK293 cells were used for plasmid transfection and reporter gene assays due to low transfection efficiency in iHBEC cells (<15%). HEK293 cells were grown to 80-90% confluence and were transiently transfected with AP-1 luciferase reporter gene vector that contains either AP-1(2)-binding insert (Panomics Inc. Redwood, CA) or AP-1-binding insert cloned from human MCP-1 gene, respectively, using Lipofectamine transfection reagent (at 2:1 ratio of reagent in μl to plasmid in μg) or SuperFect transfection reagent (at 5:1 ratio of reagent in μl to plasmid in μg). After 48-h recovery period at 37°C, transfected cells were treated with 5 or 10 μM $A\beta_{1-40}$ peptide, control peptides or vehicle for 2 and 4h. Luciferase assay was preformed using a Promega kit (Cat# E1500, Promega Inc, Madison, WI) and luminescence units were determined using FLUOstar OPTIMA Software (BMG Laboratories Inc). Luminescence units were normalized to protein in μg per sample using BioRad DC protein assay reagents (Bio-Rad

Laboratories, Hercules, CA). Each reaction was duplicated, and the experiments were repeated at least three times.

Bacterial transformation and plasmid DNA preparation

AP-1-binding insert cloned from human MCP-1 gene into pGL3 reporter vector was verified by restriction digestion with BamHI and HindIII for the presence of correct sizes of fragments (please see Appendices). pGL3 reporter vectors (2 ng of plasmid DNA) were incubated with TOP10 competent bacterial cells for 30 min on ice. The cells were heat-shocked for 30 sec at 42°C and immediately transferred to ice. SOC medium, equilibrated at RT, was added to the cells, and the cells were then incubated at 37°C on a shaking platform set to 250 rpm for 1h. Sterile Petri dishes were plated with LB agar containing 100μg/ml ampicillin, and 50 μl of transformed cells were spread on Petri dishes followed by overnight incubation at 37°C. On the next day, well-isolated colonies from each plate were picked up and placed in 100-ml Erlenmeyer flasks containing LB broth and 100μg/ml ampicillin, followed by incubation overnight at 37°C on shaking platform set to 250 rpm. Plasmid DNA purification was performed using QIAprep Spin Miniprep Kit (QIAGEN Inc). Cells in LB broth were transferred to 50-ml tubes and spun at 4,000 g for 10 min. Supernatant was discarded. Bacterial cell pellet was resuspended in RNase A lysis buffer under alkaline conditions (Buffer P1). After resuspension, the cells were lysed in NaOH/SDS (Buffer P2). The lysate was neutralized and adjusted to high salt conditions by addition of Buffer N3 where high salt concentrations caused denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate; while smaller plasmid DNAs renatured correctly and stayed in solution. Plasmid DNA was

flown through QIAprep spin column, precipitated with isopropanol and resuspended in TE buffer. To determine concentration of plasmid DNA, 1:20 dilution of plasmid DNA was diluted in Mili-Q H_2O and OD260/280 values were recorded by Beckman DU 530 Life Science UV/Vis spectrophotometer. The quality of plasmid DNA was examined by agarose gel electrophoresis.

Immunocytochemistry and Confocal Imaging

iHBEC were grown to 70% confluence on 25-mm glass slips placed in 35-mm culture dishes, media was aspirated and cells were fixed in 10% formalin solution in neutral buffer (Sigma-Aldrich, USA) for 10 min. Cells were washed in 1x PBS, blocked with 5% normal goat serum for 2 h and incubated with 5μM $A\beta_{1\text{-}40}$ conjugated to FITC at 1:100 dilution for different time periods (5, 15, 30, 45 and 60 min). Counterstaining with wheat germ agglutinin conjugated to rhodamine (RH) at 1:10000 was done for visualization of cell membrane. After washing four times with 1x PBS buffer, cells were mounted on glass slides with DAKOCytomation Fluorescent mounting medium (DakoCytomation Inc.) for confocal imaging analysis. Confocal images were taken under a 63x objective through z-series at same settings using Zeiss LSM confocal microscope. Individual z-series and composite images were further grouped in Photoshop (version 6.0, Adobe).

Statistical analysis

Data were presented as mean ±SD. Statistical analysis for single comparison was performed by unpaired Student's *t*-test where each experiment was done at least 3 times (n=3). The criterion for statistical significance was $p<0.05$.

III. RESULTS

$A\beta_{1\text{-}40}$ induces strong inflammatory gene expression in primary HBEC

Treatment of primary HBEC with 5 μM $A\beta_{1\text{-}40}$ resulted in a strong increase in the expression of MCP-1, IL-6, IL-8 and GRO genes as compared to control treatments over 2, 4 and 8 h post treatment **(Fig. 1)**. β-actin was used to normalize the inflammatory gene expression. The increase in inflammatory gene expression in Aβ-treated HBEC seems to be more pronounced at 2 and 4h than at later times. This finding is in agreement with experiments previously conducted in our laboratory where Aβ-stimulated HBEC resulted in significant increase in protein levels of MCP-1, MCP-2, GRO, IL-6 and IL-8 (Callaghan *et al.*, 2007). HBEC media alone (lane# 11) as well as 0.0025% DMSO in HBEC media (lane# 12), representing internal controls (DMSO was used to dilute poly-asparagine), did not have any effect on inflammatory gene expression in HBEC.

Knowing that differential activation and binding of inducible transcription factors (such as AP-1 and NF-κB to the promoter regions of chemokine genes) provides a critical regulatory mechanism by which the chemokines can be selectively expressed in a cell type-specific and stimulus-specific manner, we have preformed TranSignal™ Protein/DNA Array blot analyses which profiled 54 different transcription factors after HBEC were stimulated with 5 μM $A\beta_{1\text{-}40}$, 5 μM scrambled $A\beta_{1\text{-}40}$, and vehicle (2mM NaOH) for 8 h. The Arrays demonstrated that Aβ treatment of HBEC resulted in two-fold increase in transcription factors AP-1, CREB, NFATc, GRE and GATA when compared to control peptide and vehicle treatments **(Fig. 2)**. Similar finding, by our laboratory, has shown an increase in AP-1 and CREB in human AD brain when

compared to age-matched non-demented brain samples (data not shown). The data indicate that Aβ peptides stimulate the activation of AP-1 and CREB in HBEC cells as well as in Alzheimer's brain. These activated transcription factors may potentially be involved in the regulation of inflammatory gene expression in HBEC cells and AD brain.

Activation of JNK-AP1 signaling pathway in Aβ-stimulated HBEC cells

I have demonstrated by EMSA that there is a strong induction and binding of AP-1 to AP-1-binding DNA sequence in HBEC stimulated with 5 μM $A\beta_{1-40}$ when compared to 5 μM scrambled $A\beta_{1-40}$ and vehicle at 2 and 4h post-treatment (**Fig. 3A**). AP-1 induction and binding was greatly reduced at 8 h post Aβ treatment. MAPK pathways have been suggested to be closely involved in AP-1 activation. There are at least three MAPK signaling pathways, including ERK (extracellular signal-related kinase), JNK (c-Jun N-terminal kinase), and p38 kinases, which can activate AP-1. Since AP-1 is known to exist as homodimer or heterodimer of c-Jun and c-Fos proteins, supershift assay was preformed using c-Jun antibody by incubating with Aβ-induced HBEC nuclear samples for 30 min. The binding of c-Jun antibody to AP-1/DNA complex shifted the band upward in the gel (**Fig. 3A**). This analysis has confirmed that c-Jun is a component of AP-1 protein complex which is activated in response to Aβ peptides in HBEC **(Fig. 3 A)**. CREB is also activated in Aβ-stimulated HBEC when compared to peptide control and vehicle at 4h post-treatment, however at much less degree as compared to AP-1. Thus, CREB could be potentially contributing, at a lesser degree, to Aβ-induced inflammatory response in HBEC **(Fig. 3 B)**. From both EMSA gel analyses, it is clear that AP-1 is a more critical transcription factor (TF) of the two potential TF candidates. A specific JNK

inhibitor (SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase) has been used to test whether JNK and c-Jun are involved in AP-1 activation. HBEC was pre-incubated with 30 μM SP600125 followed by Aβ-induction for 2h. This result has shown that AP-1 induction and binding in EMSA assay was completely inhibited by SP600125, suggesting that JNK signaling pathway is involved in Aβ-induced inflammatory gene expression in HBEC.

Due to rare availability of primary HBEC cultures, an immortalized HBEC (iHBEC) was obtained from Dr. P-O. Couraud and used in the following experiments. The biological properties of iHBEC cells were well characterized and similar to those of primary HBEC cultures (Weksler *et al.*, 2005); however, higher concentrations of $A\beta_{1-40}$ peptides (20-40μM) were needed to stimulate the cells to express inflammatory genes as compared to primary HBEC cells.

C-Jun expression and phosphorylation at Ser-73 in immortalized HBEC treated with $A\beta_{1-40}$

To further analyze the effects of Aβ peptides on c-Jun expression and JNK-mediated c-Jun phosphorylation, iHBEC cells have been treated with Aβ peptides in the absence or presence of the JNK inhibitor, SP600125, which prevents phosphorylation of JNK substrates by blocking ATP-binding domain of JNKs. As the dual phosphorylation motif of JNK remains unaffected, the inhibitory effects of SP600125 can only be seen by reduction of phosphorylation of JNK substrates, i.e., c-Jun (Duyckaerts *et al.*, 2008). Western blot analyses have demonstrated that there was a moderate increase in total c-Jun protein in iHBEC treated with 40 μM $A\beta_{1-40}$ at 8 h when compared to vehicle **(Fig. 4A)**.

This Aβ-induced increase in c-Jun protein was completely eliminated by SP600125 **(Fig. 4A)**. It is known that AP-1 is activated as a result of c-Jun phosphorylation. There are two serine residues in c-Jun (Ser-63 and Ser-73) for phosphorylation by JNK. The Western blot showed that phosphorylated Ser-73 c-Jun was strongly increased in Aβ-stimulated iHBEC at 4h as compared to vehicle (**Fig. 4B**). This increase in phosphorylation at Ser-73 was completely inhibited by the JNK inhibitor SP600125 **(Fig. 4B)**. Furthermore, the JNK inhibitor completely inhibited the binding to Aβ-activated AP-1 to AP-1-binding DNA sequence in the EMSA assay (**Fig. 3A**). Phosphorylation at Ser-63 residue of c-Jun was not detected in Aβ-stimulated iHBEC and controls **(Fig. 4C)**. These results suggested that phosphorylation of c-Jun at Ser-73 is responsible for AP-1 activation and validated the direct involvement of JNK signaling pathway in response to Aβ peptides in iHBEC cells.

JNK inhibitor SP600125 significantly reduces MCP-1 gene expression in iHBEC cells-treated with $A\beta_{1-40}$ peptides

To further test the involvement of JNK signaling pathway in AP-1-mediated regulation of inflammatory gene expression in iHBEC-induced by Aβ peptides, semi-quantitative RT-PCR analysis has been preformed on MCP-1 gene expression in iHBEC in the absence or presence of the JNK inhibitor SP600125. The cells were pre-incubated with 30 μM SP600125 and then treated with 20 μM $A\beta_{1-40}$ or 2 mM NaOH (vehicle) for 2, 4 and 8 h. RT-PCR analysis shows that MCP-1 gene expression was increased in Aβ-treated iHBEC when compared to vehicle at 4 and 8 h post-treatment. The increase of Aβ-stimulated MCP-1 gene expression in iHBEC was inhibited by SP600125 **(Fig. 5A)**.

Densitometry analysis of RT-PCR results showed that the expression of MCP-1 gene expression in iHBEC-treated with Aβ for 4h was significantly increased as compared to vehicle ($p<0.009$) and that SP600125 significantly reduced Aβ-stimulated MCP-1 gene expression ($p<0.004$) **(Fig. 5B)**.

To validate this finding, I took advantage of a more sensitive method for detection of transcription factor activity, the luciferase reporter gene assay. There are two typical AP-1 binding sites (TPA-response elements, TREs) in the promoter region of human MCP-1 gene. This part of the promoter region was previously cloned into a reporter gene vector pGL3 promoter vector in our laboratory. Bacterial plasmids were isolated, transformed into bacteria followed by purification of plasmid DNAs. In order to verify the accuracy of AP-1 binding sites in the vector, restriction digestion and sequencing analysis of the plasmid were performed. The luciferase-based reporter gene vectors were transiently transfected into HEK293 cells, the cells were recovered overnight and subsequently treated with 5 μM $A\beta_{1-40}$, 5 μM control peptide or 2 mM NaOH (vehicle) for 2 and 4 h. My results show that Aβ peptides significantly induced AP-1 activity in HEK293 cells when compared to vehicle at 2 h post treatment ($p< 0.05$). No significant effect was seen at 4 h post treatment **(Fig. 6)**. When transfected HEK293 cells were pre-incubated with 30μM SP600125 and then treated with Aβ peptides, AP-1 reporter gene activity was significantly reduced in ($p<0.05$) **(Fig. 7)**. All of these data strongly suggests that JNK-AP1 signaling pathway is involved in Aβ-induced inflammatory gene expression in HBEC cells.

$A\beta_{1\text{-}40}$ interaction with cell membrane through RAGE in iHBEC cells

Little is known how Aβ peptides interact with cellular membrane and trigger the signaling pathway to activate AP-1 and the expression of inflammatory genes in cells. In literature, a membrane receptor RAGE has been shown to be expressed on endothelial cells and that this receptor is responsible for Aβ-induced toxicity in EC by production of reactive oxygen species mediated by NF-κB (Yan *et al.*, 1996). Since Aβ peptides bind to RAGE and RAGE is involved in initiating inflammatory response in cells (Giri *et al.*, 2000), it was interesting to see whether this receptor interacts with Aβ peptides and if this interaction could be responsible for activation of JNK-AP1 signaling pathway. And so, the interactions of Aβ peptides with iHBEC cell membrane were studied by confocal microscopy and co-immunoprecipitation and the effect of RAGE antibody on AP-1 activity by reporter gene assay. My Western blot confirms that the 46kDa RAGE is expressed in iHBEC (**Fig. 8A**). A RAGE isomer was also found in both fractions, which was not surprising, as shown by other studies that demonstrated existence of different RAGE isomers **(Fig. 8A)**. As expected, RAGE is present in both membrane and cytoplasmic fractions of iHBEC cells (**Fig. 8B**). To test whether Aβ and RAGE interact with each other and co-immunoprecipitate together, $A\beta_{1\text{-}40}$ peptides were mixed with membrane and cytoplasmic fractions of iHBEC cells and the complex was precipitated with Aβ antibody. The complex was resolved on 10 % polyacrylamide gel and probed with the RAGE antibody (**Fig. 8B**). Western blot confirms that RAGE was detected in the complex precipitated by the Aβ antibody (**Fig. 8B**). These results demonstrate direct interaction of Aβ peptides with RAGE in iHBEC cells.

Confocal image analyses show that fluorescent $A\beta_{1-40}$ peptides (green) start binding to cell membrane and possibly get internalized into iHBEC cells at 30, 45 and 60 min post-Aβ treatment. It appears that after Aβ binding, peptides get internalized and are located in close proximity to nucleus **(Fig. 9)**. To test whether RAGE would be involved in Aβ binding to cell membrane, iHBEC were pre-incubated with the RAGE antibody for 30 min and then treated the cells with fluorescent $A\beta_{1-40}$ for 30 min. Confocal image analysis shows that RAGE antibody greatly decreased $A\beta_{1-40}$ binding and internalization **(Fig. 10A).** In order to determine if RAGE is involved in activating JNK-AP-1 signaling pathway, HEK293 cells were transfected with the AP-1 reporter gene vector, pre-incubated the cells with the RAGE antibody for 60 min and then treated the cells with 10 μM $A\beta_{1-40}$ peptides. Reporter gene assay shows that AP-1 reporter gene activity stimulated by Aβ peptides was significantly reduced in HEK293 cells pre-incubated with the RAGE antibody ($p<0.00001$) **(Fig. 10B)**. All of these results suggest that RAGE is involved in the activation of Aβ-induced AP-1 activity for the up-regulation of inflammatory gene expression in cells.

Indirect effects of Aβ peptides on inflammatory gene expression in iHBEC cells via microglia

Senile plaques enriched with Aβ peptides are found in close proximity to activated microglia in human AD brain (Haga *et al.*, 1989; Itagaki *et al.*, 1989; Perlmutter *et al.*, 1992). Activated microglia are shown to release a variety of inflammatory factors to the environment, possibly affecting surrounding brain cells and altogether augmenting the inflammatory process. To test whether activated microglia-released factors would

affect HBEC cells, iHBEC were treated with conditioned media from $A\beta_{1-42}$-stimulated microglia (2μM or 5μM $A\beta_{1-42}$ for 24h) and control media from unstimulated microglia or iHBEC media. Primary human microglial cultures were generated from autopsy brain samples and the conditioned media were prepared by Dr. Douglass Walker at the Sun Health Research Institute, Arizona. The conditioned media was used in dilution with iHBEC media at 1:1 ratio. iHBEC cells were treated with the conditioned media and control media for 2, 4, and 8h. RNA samples were isolated from the cells. RT-PCR analysis shows that the expression of inflammatory genes MCP-1, IL-8, IL-1β and IL-6 was strongly up-regulated in iHBEC treated with microglia-conditioned media when compared to control media over 2 and 4h post-treatment (**Fig. 11A**). IL-8 gene expression was strongly induced even at 8 h post treatment when compared to controls **(Fig. 11 A)**. Pre-incubation of iHBEC cells with 30 μM SP600125 reduced IL-6 gene expression, while MCP-1 and IL-8 gene expression was not affected by SP600125. The inhibitors for p38, ERK1/2, and PI-3 kinase pathways had no effect on the expression of MCP-1, IL-6 or IL-8 induced by the conditioned media **(Fig 11B)**. A NF-κB inhibitor MG-132 also did not significantly affect the expression of the inflammatory genes induced by the conditioned media (data not shown). The findings suggest that some inflammatory factors or a group of inflammatory mediators, acting in concert, found in microglia-conditioned media, induced the expression of inflammatory genes in iHBEC and that IL-6 gene expression induced by the conditioned media may partially involve JNK signaling pathway. These results demonstrate that there was an indirect effect of Aβ peptides on endothelial cells via activated microglia during neuroinflammation evoked by Aβ peptide deposition in AD brain.

Many studies have shown that the expression and secretion of IL-1β and TNF-α are increased in AD brains (Goebler *et al.*, 2001; Klampfer *et al.*, 1994; Gitter *et al.*, 1995). It is likely that these two cytokines are potentially present in the microglia-conditioned media and responsible for the induced expression of inflammatory genes in iHBEC. Dr. D. Walker's ELISA has confirmed that TNF-α is present in the conditioned media (personal communication). To verify the effects of TNF-α and IL-1β cytokine on IL-6 gene expression and to test whether JNK signaling pathway is also involved in the regulation of IL-6 expression by TNF-α and IL-1β, iHBEC was pre-treated with SP600125 as well as p38, ERK1/2 and PI-3 kinase inhibitors followed by treatment with human TNF-α and IL-1β recombinant cytokines. RT-PCR analysis shows that both TNF-α and IL-1β induced strong expression of IL-6 and that this IL-6 stimulation was reduced by SP600125 (**Fig. 12**). These results suggest that either a synergy of TNF-α and IL-1β action or an individual cytokine action resulted in up-regulation of IL-6 expression, and that JNK signaling pathway may be partially involved in IL-6 gene expression-induced by TNF-α and IL-1β in iHBEC cells.

To verify whether IL-1β is involved in MCP-1 expression induced mciroglia-conditioned media in iHBEC, microglia-conditioned media or recombinant IL-1β peptide was pre-incubated with an IL-1β antibody for 60 min and then used them to treat iHBEC for 2 and 4 h. RT-PCR analysis shows that IL-1β-induced expression of MCP-1 was blocked by IL-1β antibody, but IL-1β antibody did not prevent the induction of MCP-1 gene expression by microglia-conditioned media (**Fig. 13**). In contrary, IL-1β antibody-treated microglia-conditioned media strongly stimulated the expression of MCP-1 (**Fig.**

13). The data suggest that IL-1β may not be responsible for MCP-1 expression in iHBEC stimulated by microglia-conditioned media.

IV. DISCUSSION

Aβ peptides stimulate inflammatory gene expression in HBEC via JNK-AP1 signaling pathway

My research work has demonstrated that aggregated $A\beta_{1-40}$ peptides can up-regulate the expression of inflammatory genes in HBEC and that this Aβ-stimulated up-regulation of inflammatory gene expression in HBEC is mediated by the JNK-AP1 signaling pathway. This is supported by the following evidence: 1) aggregated $A\beta_{1-40}$ peptides bind to RAGE on plasma membrane of HBEC and trigger JNK signaling pathway resulting in c-Jun phosphorylation at serine 73 residue; 2) c-Jun is a component of dimeric AP-1 transcription factor complex; 3) activated AP-1 mediated by JNK signaling pathway up-regulates the expression of inflammatory genes in the cells; and 4) a JNK-specific inhibitor SP600125 completely inhibited JNK-mediated c-Jun phosphorylation at serine 73 and thus AP-1 activation in HBEC-treated with Aβ peptides, leading to down-regulation of inflammatory gene expression in the cells.

AD is a multifaceted neurodegenerative disease. One of the important mechanisms for neurodegeneration is neuroinflammation, including neurovascular inflammation. Up-regulation of inflammatory factors has been found in AD brain (McGeer and McGeer, 2004). However, the molecular mechanisms of the inflammation in AD brain still remain largely uncertain. Accumulation and deposition of Aβ in the brain is a hallmark of the disease. Aβ peptides aggregate to form fibriliar deposits, the principal component of senile plaques, which triggers inflammatory response in AD brain. *In vitro* and *in vivo* studies have suggested that the resident phagocytes, microglia,

are the major players of Aβ-triggered inflammation in AD brain. Microglia can be activated by small doses of aggregated $A\beta_{1-42}$ *in vitro* and secrete inflammatory cytokines, such as MCP-1, TNF-α, IL-8 and IL-1β (Murphy *et al.*, 1998; Araujo and Cotman, 1992; Meda *et al.*, 1995; Chao *et al.*, 1994, 1998). Similarly, activated microglia are consistently associated with senile plaques in patients with AD (Mackenzie *et al.*, 1995). Microglia also respond to Aβ deposits in brain through activation of tyrosine kinase-based intracellular signal transduction cascade involving Lyn, Syk, FAK, and Pyk2 (McDonald *et al.*, 1997, 1998; Combs *et al.*, 1999) activation of multiple independent signaling pathways leading to induction of pro-inflammatory gene expression, such as TNF-α and IL-6 (Combs *et al.*, 2000; Davies, 2000), and production of reactive oxygen and nitrogen species. As a result, these inflammatory products, acting in concert, produce neuronal toxicity and death (Bamberger and Landreth, 2001). *In vitro* study showed that Aβ peptides generate oxidant stress in neurons by activating NF-κB and inducing expression of macrophage-colony stimulating factor (M-CSF). Released M-CSF by neurons stimulate its receptor c-fms on microglia inducing activation of macrophage scavenger receptor and ApoE (Du Yan *et al.*, 1997). $A\beta_{1-42}$ peptides also activate astrocytes *in vitro* and *in vivo*, resulting in activation of NF-κB and production of iNOS (Davies, 2004). Astrocytes in AD brains can also secrete IL-1, IL-6 and transforming growth factor β (TGF-β) (Ata *et al.*, 1997; Del Bo *et al.*, 1995). It appears that NF-κB and the relevant signaling pathway are activated by Aβ peptides in cultured microglia, neuronal cells and astrocytes and responsible for inflammatory response in these cells. However, the study conducted in our laboratory has revealed that NF-κB is not activated in Alzheimer's brains (data not shown).

My research work suggests that brain endothelial cells, like microglia and astrocytes, are also involved in the inflammation observed in AD (Griffin and Stanley, 1993). However, little is done on characterization of brain endothelial cells for their involvement, if any, in the inflammatory response. Suo and colleagues (Suo *et al.*, 1998) have attempted to study the effect of Aβ peptides on brain endothelial cells by using a cell line from human aortic endothelial cells and manipulating it with different factors such as bovine brain extract to mimic brain environment. This model has many variable parameters and limitations to validate the true effect of $A\beta_{1-40}$ on brain endothelial cells (BEC). Instead, I have used primary HBEC cultures as an *in vitro* model and treated it with aggregated Aβ peptides to closely mimic the environment in experimental AD pathology. These HBEC cultures have been well characterized as described previously (Zhang *et al.*, 1999, 2000, 2003). My finding that Aβ-stimulates the expression of IL-1β, MCP-1, GRO, and IL-6 in cultured HBEC has been confirmed in Alzheimer's brains by real-time quantitative RT-PCR analyses conducted in our laboratory (data not shown). These genes are up-regulated over two-fold in AD brain as compared to ND brain samples (data not shown).

It is unknown which signaling pathway and transcription factors are responsible for Aβ-triggered inflammatory response in human brain endothelial cells. Understanding and characterization of the molecular mechanism involved in Aβ-evoked inflammatory response in HBEC may uncover therapeutic targets for Alzheimer-related neurovascular inflammation. To study transcriptional regulation of the inflammatory genes, TranSignal DNA/Protein array blot was employed to profile the activities of 54 different transcription factors (TF) in HBEC-treated with $A\beta_{1-40}$ peptides. Transcription factor is

known to be located at the end of a signaling pathway and, once activated, it binds to the promoter regions of the target genes and thus regulates their expression in response to various stimuli by either increase or decrease in their transcription. The TF array analyses show that activities of several TFs, including AP-1, CREB (cyclic-AMP response element binding protein), NFATc (nuclear factor of activated T-cells), GRE and GATA, were increased over two folds in Aβ-treated HBEC as compared to controls. However, the activity of NF-κB was not changed. Among the five activated TFs, AP-1 is considered to be one of the most common TFs involved in the regulation of inflammatory gene expression. CREB is known to be activated by various extracellular stimuli and regulate the expression of genes important to cell proliferation, differentiation, adaptation, and survival in many cell types. Some of the genes involving inflammatory process (such as COX-2) are regulated by CREB. Further work conducted in our laboratory has provided convincing evidence that both AP-1 and CREB are also activated in AD brains as compared to ND brains but not NF-κB. My EMSA validated the physical binding of AP-1 and CREB to their cognitive binding sites in Aβ-induced HBEC. A greater increase in AP-1 binding was observed in Aβ-induced HBEC but a smaller increase in CREB binding was seen in Aβ-stimulated HBEC. This led to the conclusion that AP-1 is more crucial among the two transcription factors involved in Aβ-induced response in HBEC. This novel finding that AP-1 is activated in both AD brain and HBEC-treated with Aβ suggests that AP-1, rather than NF-κB, is the main transcription factor involved in the regulation of inflammatory genes in Alzheimer's neuroinflammation and/or neurovascular inflammation.

Various studies show the importance of AP-1 in inflammatory responses: 1) AP-1 is induced in human umbilical vein endothelial cells (HUVEC) by *orientia tsutsughamushi,* a virus which causes vasculitis in humans by replicating in macrophages and endothelial cells, and results in increased expression of MCP-1 (Cho *et al.*, 2002); 2) Adenovirus-mediated overexpression of c-Jun and c-Fos in HUVEC induces expression of ICAM-1 and MCP-1 (Wang *et al.*, 1999); and 3) TNF-α induces increase in AP-1/c-Jun proto-oncogene transcription (Dixit et al., 1989); and 4) Many of inflammatory genes contain functional c-Jun/AP-1 consensus sequences, and JNKs have been shown to control their expression in peripheral immune cells (Neff *et al.*, 2001; Swantek *et al.*, 1997; Tyt *et al.*, 1999). AP-1 is a dimeric protein complex. Multiple signaling pathways are known to activate AP-1 in cells, including ERK-1/2, JNK, p38 kinase, and PI-3 kinase pathways. One approach to study the upstream signaling pathway for AP-1 activation is to identify its subunit components. My supershift assay with c-Jun antibody demonstrates the presence of c-Jun in AP-1 complex. C-Jun is recognized as a most potent transcriptional activator in its group (Angel and Karin, 1991) and can be activated by JNK-mediated phosphorylation. My observation from supershift assay is further supported by the use of a JNK-specific inhibitor, SP600125. Pre-treatment of HBEC with the compound completely inhibited AP-1 activation and binding to AP-1-binding DNA sequence. The involvement of JNK signaling pathway in Aβ-stimulated expression of inflammatory genes, such as MCP-1, in HBEC was also confirmed by my RT-PCR and reporter gene assay.

Several lines of evidence indicate the involvement of JNK in Alzheimer's disease: 1) JNK can phosphorylate tau protein in a manner similar to that of paired helical

filaments (PHF)-tau in AD (Goedert *et al.*, 1997; Reynolds *et al.*, 2000); 2) Aβ peptides induce JNK signaling which mediates Aβ toxicity and adverse effects on long-term potential in hippocampus (Bozycko-Coyne *et al.*, 2001; Morishima *et al.*, 2001; Troy *et al.*, 2001; Wei *et al.*, 2002; Minogue *et al.*, 2003); 3) JNK is found to translocate from the cytoplasm to nucleus in mild AD; however, it transclocates from nucleus to cytoplasm in more advanced stages of AD (Zho *et al.*, 2001a; Zho *et al.*, 2003c); 4) JNK's upstream activator JKK1 is activated in vulnerable neurons in AD (Zho *et al.*, 2003a). However, there is no indication in the literature that JNK and AP-1 are involved in Alzheimer's neuroinflammation. Activation of JNK results in phosphorylation of c-Jun on residues Ser63 and Ser73 (Whitmarch and Davies, 1996), which enhances the activity of c-Jun. Phosphorylation on these sites also leads to inhibition of c-Jun ubiquitination and degradation. My results show an increase in total c-Jun protein level at 8 h post Aβ treatment and an increase in phosphorylation at Ser73 residue of c-Jun in iHBEC-treated with Aβ, but phosphorylation at Ser63 residue is not observed. The increased *de novo* synthesis of c-Jun observed in iHBEC-treated with Aβ at 8h might occur due to the fact that AP-1 heterodimer complex composed of c-Jun/ATF2 subunits has with high-binding affinity to the sequence 5'-TTACCTCA-3' found in the promoter of c-Jun gene (Van Dam *et al.*, 2003). Therefore, increased AP-1 activity in Aβ-stimulated HBEC regulates not only inflammatory gene expression but also c-Jun expression. This is supported by my observation that the expression of total c-Jun and phosphorylation of c-Jun (at Ser 73) are completely inhibited by the JNK inhibitor. Differential phosphorylation of serine residues on c-Jun protein in response to various stimuli was also observed by Thakur and colleagues (Thakur *et al.*, 2006). These authors found that Ser73-phosphorylated c-Jun is

more prominent than Ser63-phosphorylated c-Jun in human AD brains; but others reported that Ser63 and not Ser73 is phosphorylated in AD brains (Pearson *et al.*, 2006). These studies looked at cell death but not inflammation. My data indicate, at least, that JNK-mediated phosphorylation of c-Jun occurs at Ser73 in Aβ-stimulated HBEC.

The involvement of JNK in the regulation of pro-inflammatory factors in HBEC as demonstrated in my work extends therapeutic relevance to JNK inhibitors for an anti-inflammatory strategy, ie. neuroprotection by targeting inflammatory pathways. As for the peripheral immune system, inhibition of JNKs has already been shown to partially block the inflammation, such as colitis (Hommes *et al.*, 2002) and in experimental arthritis (Han *et al.*, 2001).

RAGE-Aβ interaction in iHBEC cells may be responsible for activation of JNK signaling pathway

My work suggests that RAGE-Aβ interaction may be responsible for activation of JNK-AP1 signaling pathway. This is supported by the evidence in literature that RAGE-Aβ interaction generates oxidative stress, which is one of the main stimuli to activate JNK. Recent studies show interactions of Aβ peptides with neuronal and glial cell membrane, and a subset of membrane proteins binds aggregated form of Aβ, including α7-nicotinic acetylcholine receptor, integrins, RAGE, FPRL1 (formyl peptide receptor like 1), NMDA-R (N-methyl-Daspartate receptor), P75 neutrophin receptor, scavenger receptors (A, BI and CD36) (Verdier *et al.*, 2004). RAGE has been also shown to mediate interaction of Aβ with endothelial cells and neurons resulting in cellular oxidative stress and cytokine production and chemotaxis. There is also increased RAGE

expression in microglia and vasculature of AD brain (Verdier *et al.*, 2004). My work shows that RAGE is expressed in iHBEC cells and that RAGE co-immunoprecipitates with $A\beta_{1\text{-}40}$ peptides. Furthermore, my reporter gene assay shows decreased AP-1 activity in the presence of RAGE antibody. This interaction is further confirmed by my confocal imaging analysis that RAGE antibody reduced the binding of Aβ peptides to HBEC and internalization. It was postulated by Giri and colleagues that $A\beta_{1\text{-}40}$ interaction with RAGE expressed on endothelium of BBB initiates cellular signaling to allow monocytes to transmigrate through cell-cell- junctions. This group demonstrated that $A\beta_{1\text{-}40}$–RAGE interaction on a confluent monolayer of HBMVEC triggers PKC signaling pathway and results in increased adherence and transmigration of monocytic cells (THP-1) (Giri *et al.*, 2000). RAGE is involved in inflammatory mechanism in AD pathology; however, due to different RAGE isoforms and the specificity of RAGE ligands, the signaling events triggered by RAGE may be cell-type and stimulus-type specific (Ding and Keller, 2005). The intracellular domain of RAGE, which serves as a scaffolding for initiation of signal transduction, is crucial for many forms of RAGE signaling, including NF-κB, MAPK, and JNK, which may involve Rho-family of GTP-ases as well as Ras (Schmidt *et al.*, 2000, 2001; Thornall, 1998; Yen *et al.*, 2001; Li *et al.*, 2004; Dukic-Stefanovic *et al.*, 2003). Taken all together, RAGE interaction with Aβ peptides on cells may be one of the mechanisms responsible for induction of JNK signaling pathway and inflammatory response seen in HBEC cells.

Indirect effects of Aβ peptides on iHBEC cells via microglia

Aβ peptides not only have direct effects on iHBEC cells but also have indirect effects on these cells. Microglia are the primary group of phagocytic cells in brain that respond to inflammatory stimuli and secrete many inflammatory mediators into the microenvironment, such as TNF-α and IL-1β (Ishihara *et al.*, 2003). These secreted inflammatory mediators may have direct effects on surrounding HBEC, augmenting inflammation. To test this, I have used conditioned media from microglia stimulated with $A\beta_{1-42}$ peptides to treat iHBEC. As expected, the conditioned media strongly induced the expression of IL-6, IL-8, MCP-1 and IL-1β in iHBEC cells. IL-6 gene expression induced by the conditioned media was reduced in the presence of the JNK inhibitor, suggesting that JNK signaling pathway may be partially involved in the process. Since $A\beta_{1-42}$-stimulated human microglia secretes IL-1β and TNF-α and increased IL-1β-expressing microglia are associated with AD progression, I have selected recombinant TNF-α and IL-1β to treat iHBEC in the presence or absence of different kinase inhibitors. The expression of MCP-1, IL-6 and IL-8 was strongly increased in iHBEC treated with TNF-α or IL-1β. However, only IL-6 expression was reduced by JNK inhibitor. Thus, IL-6 expression induced by microglia-conditioned media, recombinant TNF-α or IL-1β is partially mediated through JNK signaling pathway. In support of this finding, many other studies describe IL-6 to be an inflammatory mediator and to be causally involved in AD pathology: 1) A strong IL-6 immunoreactivity was found to be associated in early stages of amyloid deposition in the cortex and hippocampus of AD patients (Bauer *et al.*, 1991; Hull *et al.*, 1996; Strauss *et al.*, 1992; Wood *et al.*, 1993); 2) IL-6 positive plaques also show elevated levels of acute phase proteins including α2-

macroglobulin (Bauer *et al.*, 1991; Strauss *et al.*, 1992; Van Gool *et al.*, 1993; Wood *et al.*, 1993); 3) IL-6 induces α2-macroglobulin synthesis in human neuronal cell line (Ganter *et al.*, 1991); 4) IL-6, as well as IL-1β, modulate APP expression in neurons and astrocytes (Brugg *et al.*, 1995; Del Bo *et al.*, 1995); 5) cytokines such as IL-6 are elevated in cerebrospinal fluid (CSF) of AD patients (Blum-Degen *et al.*, 1995) To test if IL-1β is present in microglia-conditioned media, I incubated the conditioned media with IL-1β antibody. IL-1β antibody did not inhibit MCP-1 gene expression in iHBEC induced by the conditioned media, but completely inhibited MCP-1 gene expression-induced by recombinant IL-1β. This suggests that IL-1β alone, if present in microglial-stimulated media, was not responsible for inflammatory gene expression in iHBEC, but most likely it was acting in concert with other cytokines in order to activate signaling pathways leading to inflammatory response.

Taken all together, my study has yielded several important findings: 1) AP-1, not NF-κB, is activated in Aβ-treated HBEC and in Alzheimer's brain; 2) AP-1 is activated as a result of c-Jun phosphorylation at Ser73; 3) c-Jun phosphorylation at Ser73 is mediated by JNK; 4) RAGE-Aβ interaction may activate JNK in HBEC; and 5) activated microglia stimulated by Aβ peptides releases secreted factors that affect the expression of inflammatory genes in HBEC (**Fig. 14**). These findings provide critical insights into the molecular mechanism of neuro-and neurovascular inflammation in Alzheimer's disease and potential therapeutic targets in controlling the progression of the disease.

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VI. APPENDICES

PROTOCOLS

Experimental methods and Buffer recipes:

1. ***Reverse transcriptase poly chain reaction (RT-PCR)***
2. ***Western blotting***
3. ***Electrophoretic mobility shift assay (EMSA)***
4. ***Reporter gene assay***
5. ***Co-immunoprecipitation***
6. ***Immunocytochemistry***

RT-PCR

Total RNA was extracted from iHBEC in 35 mm Petri dishes using Trizol reagent (500 ml/dish) and was purified using protocol provided by manufacturer (Gibco-BRL, Burlington, Ontario): Dishes were incubated at room temperature (RT) for 10 min, on a shaking platform (250rpm). Cells were scraped and pipette was used to break the clumps. Cells in Trizol were transffered into 1.5 microcentrifuge tubes and 100 μl choloroform was added. Tubes were gently inverted 5 times for solutions to mix. Then samples were centrifuged at 10, 000 g for 15 min at 4°C. After centrifugation, there are three visible layers present in sample; we take the top layer carefully without disturbing middle layer which contains proteins and DNA. We place the top supernatant to new tubes and add to it same volume (approximately 250 ml) of isopropanol. Tubes are inverted gently 10

times and incubated for 15 min at -20°C followed by 10 min incubation at RT. Samples are subsequently centrifuged at 10, 000 g for 10 min at 4°C so pellets could be visible. Supernatant was discarded and pellet was washed 2 times with cold 75% ethanol (diluted in DEPC-treated dH_2O). After the last ethanol wash, pellets were left to dry for 5-10 min until they appeared gel like. Pellets were resuspended in 40 µl DEPC-treated dH_20 and heated at 55°C for 10 min after which samples were placed on ice. RNA concentration was determined afterward by recording OD260 values in diluted 1:20 samples which were prepared in DEPC-treated dH_20 buffer.

We have used 2 µg of RNA to make total of 40 µl cDNA samples. To prepare cDNA, 2 µg RNA was mixed with oligo (dT) primers and DEPC-treated water to initially make 25.5 µl volume and samples were incubated at 70°C for 10 min, after which they were put on ice. In separate tube, master mix was prepared involving 0.1 M DTT, 10 mM dNTP, MLV (reverse transcriptase) and 5x first strand buffer. 14.5 µl of master mix was added to each sample and those were gently mixed by pipetting up and down. Samples were then incubated for 60 min at 42°C and then reverse transcriptase was inactivated by heating the reaction mixture to 70°C for 10 min. At this step, cDNA is formed and was subsequently used in 25 µl PCR reaction. In PCR reaction mix we add the following: 10x PCR reaction buffer, 1.5 mM $MgCl_2$, 10 mM dNTP mix, 10mM of primer's cocktail mix (sense and anti-sense primer), 5 µl of cDNA (for β-actin) and 10 µl of cDNA for all other genes, commercial RNA-ase/DNA-ase treated H_2O and 1.25 units/reaction Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, New Jersey, USA). The amplification reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA) in 0.2 ml PCR tubes (Rose Scientific Ltd., Alberta, CA). All

amplifications were then performed using denaturation step at 94°C for 30 sec, an annealing step at 55°C for 45 sec and a polymerization step at 72°C for 40 sec. B-actin required 30 cycles, MCP-1 required 35 cycles while IL-6, IL-8, GRO, IL-1β and TNF-α required 40 cycles for amplification. After PCR reactions were done, 6x loading buffer was added to tubes and samples were ready to load on an Agarose gel. We used 1.5 % agarose gel prepared in 1x TBE buffer to which we added EtBr for visualization of bands. Ten microliters of sample for β-actin and 15 μl of sample for other genes was loaded on gel and ran for 1 hr at 150 V. Subsequently images were taken by exposing gels to UV light for 1s using Kodak EDAS 290 Transilluminator (UVP, Inc., San Gabriel, CA, USA) and taking an image with Kodak DC290 Zoom Digital Camera.

5x 1st Strand buffer: 375 mM KCl, 250 mM TRIS and 15 mM $MgCl_2$

10x PCR buffer: 150 mM Tris-HCl, pH 8.0, 500 mM KCl

10x TBE buffer: 0.89M Tris, 0.02M EDTA-Na2-salt and 0.89 M Boric acid

TE buffer: 10 mM Tris, pH 7.5, 1mM EDTA

Western blotting

Cells were grown in 60 or 100 mm Petri dish and nuclear isolation was performed using the protocol from nuclear extraction kit (Panomics, Redwood City, CA, USA). Before the start of the protocol, we needed to prepare:1x Buffer A from the 10x Buffer A stock; 1x Buffer B from 5x Buffer B stock; 1x PBS from 20x PBS stock. Media was aspirated and plates were washed twice with 1x PBS. In clean, sterile 1.5 ml-Eppendorf tube we prepare Buffer A mix including: 1x Buffer A, 100 mM DTT, Protease inhibitor

cocktail and 10% IGEPAL. This buffer A mix is added to each plate and plates are put on ice in ice bucket and left to incubate for 10 min on shaking platform. Cells were scraped using sterile scraper and pipetted up/down several times to disrupt the cell clumps. Then reaction mixture was placed in clean 1.5 ml tubes and centrifuged at maximum speed (10, 000 g) for 3 min at 4°C. Tubes are immediately placed on ice and supernatant (cytosolic fraction) was used for later if needed. In meantime, buffer B mix is prepared including: 1x buffer B, protease inhibitor cocktail and 100 mM DTT. Pellet is resuspended in 150 µl buffer B mix, tubes are placed horizontally and shaken on ice bucket for 2 hrs at 200 rpm. This was followed by centrifugation of samples at maximum speed fro 5 min at 4°C. Due to concern with high salt concentration in sample buffers we added buffer C mix which included: 20 mM Tris, pH 7.9, 20% (w/v) glycerol, 0.1 M KCl, 0.2 mM EDTA and 0.5 mM DTT. Then, tubes are centrifuged again at maximum speed for 20 min at 4°C and supernatant (nuclear extract) is collected. Protein concentration was measured in each sample using DC BioRad protein assay reagents. Samples are kept at -80°C until use.

To run the samples we prepare 10% acrylamide based gels. At first we make the separating gel pour it in a western blot gel cast and put a layer of isopropanol over it. Leave the gel to polymerize for 30 min. then remove isopropanol and wash the gel 1x with mili Q H_20. Stacking gel is poured over it and immediately well combs are inserted before stacking gel polymerizes which takes approximately 15 min. After, remove the combs and wash it with running buffer. Samples are prepared by mixing protein with 5x loading buffer and heating it at 100°C for 10 min. Samples are chilled on ice for 5 min and centrifuged at maximum speed for 5 min. 50 µg of protein per lane is loaded as well as 5 µl of prestained molecular weight marker in first lane. Gel is run at 150V until dye

front reaches bottom of gel. Gel is transferred to PVDF membrane in transfer sandwich composed of wet PVDF membrane soaked previously in methanol, Whatman papers, sponges and gel in transfer buffer. This sandwich apparatus is stacked in a way so that proteins from the gel migrate to membrane in orientation of negative to positive electrode. Transfer is done at 450 mA for 1h 45 min. After transfer is complete membrane is place in 5% skim milk powder in TBS-T, as a blocking solution for 1 h at RT. Primary antibody is prepared at desired dilution (1:500-1:1000) in 1% skim milk powder in TBS-T and membrane is incubated in it overnight at 4°C. After incubation with primary antibody, membrane is washed 3 times with TBS-T for 5 min and then was immersed in secondary antibody mixture for 45 min. Secondary antibody is made in 5% skim milk powder in TBS-T at 1:5000 dilution. Membrane is washed 3 times with TBS-T for 10 min and Enhanced ChemiLuminescence (ECL Plus) method was used for detection of bands on gel. While membrane is still left in a dish ECL reagents are mixed in 1:1 ratio. 2 ml of ECL mix was used per membrane and placed on top of saran wrap onto which membrane is place facing up-side down for 1-2 min, away from light. Membrane is removed from saran wrap and transferred to another saran wrap, sealed and exposed to x-ray film. Exposure varied for different antibodies (5 sec-30 min).

10x Buffer A: 100 mM HEPES, pH 7.9, 100 mM KCl and 100 mM EDTA

5x Buffer B: 100 mM HEPES, pH 7.9, 2 M NaCl, 5 mM EDTA and 50 % glycerol

10x Tris-Glycine: 60 g Tris and 288 g Glycine in 2.0L H_20.

1x Transfer buffer: 400 ml of 10x Tris-Glycine and 800 ml Methanol in 4.0 L H_20

10x TBS-T buffer: 24.2g Tris, 175g NaCl , pH 8.0, 10ml Tween-20 in 2.0 L H_20

10x Running buffer: 60g Tris, 288g Glycine, 20g SD in 2.0 L H20

2x Separating buffer: 0.75M Tris-HCl, pH 8.8, 0.2% SDS

2x Stacking buffer: 0.25M tris-HCl, pH 6.8, 0.2% SDS

5x Loading buffer: 25% Glycerol, 25 % βME, 15% SDS, 0.25% BPB, 50 mM Tris-HCl, pH 6.8

10% Separating gel: 5 ml of 40%/0.40% Acryl/Bis, 10 ml 2x Separating buffer, 5 ml H_2O, 100 µl of 10% APS and 12.5 µl TEMED.

Stacking gel: 1.5 ml of 40%/0.40% Acryl/Bis, 5 ml of 2x Stacking buffer, 3.5 ml H_2O, 100 µl of 10 % APS and 25 µl of TEMED

EMSA

Nuclear extraction is performed as described above. Probes are being labeled as following per reaction tube: 2 µl 10x kinase buffer, 2 µl T4PNK (Poly Nucleotide Kinase), 20 ng oligo, 50 µCi γ-^{32}P ATP and bring it up to 20 µl with H_2O. Mix this reaction and incubate at 37oC for 1 hr. Add another 30 µl H_2O and mix again. Purify this reaction mix on a G-25 sephadex column by centrifuging it for 1 min at 6000 g then add labeled probe and spin again for 1 min at 6000 g. This needs to be further diluted to 200 µl by adding to it: 98 µl H_2O, 40 µl 5x WCE, 10 µl 2M KCl and 2 ml 50 mM DTT. Count 2 µl of reaction min which should give at least 100,000 cpm/µl. Keep this labeled probe at -20°C in shielded container. To prepare samples for EMSA, place 10 µg of nuclear extract in microtubes and add to it 5 µl DNA binding buffer and 2 µg of poly(dI-dC) and mix gently incubating it at RT for 10 min. ((For the supershift assay, we added antibody (c-Jun antibody, 1/10 of sample) and incubate it at RT for 25 min)). Add 0.2 ng

of 5'-end radiolabelled probe to each tube and incubate it at RT for 20 min., followed by addition of 5 µl of loading buffer and subsequently loading wells.

We make 5 % native polyacrylamide gel in 1x Tris-Glycine composed of following ingredients: 6 ml of 10x tris-Glycine, 10 ml of Acrylamide/Bis (30:0.5), 43.4 ml H_2O, 600 µl of 10% APS and 30 µl of TEMED. Let it polymerize for 1 h, remove combs and rinse wells with 1x Tris-Glycine. Pre-run gel for 1 h at 150V before loading samples. Run gel at 150V until dye front or probe runs off in 1x Tris-Glycine buffer. Dry gel for 1 h under vacuum and expose it to film overnight for autoradiography.

Acrylamide/Bis (30:0.5): 30g Acrylamide, 0.5g Bis in 100ml H_20 (filter through 0.45µm and store in dark bottle)

Loading buffer: 400 µl Glycerol, 350 µl 0.5% BPB, 200 µl 5x WCE, 50 µl KCl.

5x DNA binding buffer: 100 mM HEPES, 1 mM EDTA, 1 mM EGTA, 250 mM KCL, 5 mM MgCl2, 25% Glycerol and 5 mM DTT (add just before use).

10x Kinase buffer: 500 mM Tris-HCl pH 7.6, 100 mM MgCl2 and 50% Glycerol.

5x WCE buffer: 100 mM HEPES pH 7.9, 2.5 mM EDTA, 2.5 mM EGTA and 50% Glycerol.

Reporter gene assay

HEK 293 cells are grown to 70-90 % confluence in 24 well plate format and were transiently transfected with reporter gene vectors for 48 hrs after which cells are treated with 5-10 µM $A\beta_{1-40}$, 5-10 µM control peptide (Poly Asparagine) or vehicle (2 mM NaOH) LPS or TPA was used as positive control. Each reporter gene vector had control

(the internal control) which did not possess the insert of interest. In this project we used two different but similar vectors. First vector we used had AP-1 binding sites inserted into pGl3 vector just upstream of luciferase gene. These AP-1 sites were cloned from human MCP-1 gene. In order to transfect this vector into the cells we used Superfect transfection reagent and followed the protocol by SuperFect Transfection Reagent Handbook. We used 1 μg of DNA and 5 μl of Superfect reagent in serum free media, mixed it and incubated it at RT for 15 min. Just prior to addition to cells we added to reaction mixture a warm HEK 293 media and then added it to cells.

The second reporter gene vector had AP-1 (2) sequence (5'-TGACTAATGACTAATGACTAATGACTAATGACTAATGACTAATGACTAATGA CTAA-3') inserted into pTransLucent reporter vector (4.8 kb) upstream of luciferase gene (Panomics, Redwood City, CA, USA) and for this transfection we used Lipofectamine reagent. To transfect cells with this vector we used 1 μg of DNA and 8 μl of Lipofectamine reagent in serum free media and added it directly to cells in the wells. Both reporter gene assays had same protocol after 48 transient transfection period. After cells were treated for 2 or 4 hr time periods, 100 μl of lysis buffer was added to wells and cells were lysed for 10 min on 250 rpm shaker at RT. Cells were pipetted up/down to disrupt the clumps and samples were transferred to tubes for 2 min spin at 10,000 g at 4°C. 20 μl of cell lysate was placed in 96-well plate for each sample and mixed with 100 μl of Luciferase substrate. Plate was immediately read using FLUOstar OPTIMA Software. Luminescence units were normalized to protein concentration determined by DC BioRad protein assay reagents.

Sequence analysis of pGL3 vectors with AP-1 inserts cloned from human MCP-1 gene (please see following).

Clone 12b:

CGATAGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTCCCT

TCNCCCTTGATTTAACAGCCCACTTATCACTCATGGAAGATCCCTCCTCCTGC

TTGACTCCGCCCTCTCTCCCTCTGCAAGGGCGAATTCTGCAGATATCCATCAC

ACTGGCGGCCGCTCGAGATCTGCGATCTGCATCTCAATTAGTCAGCAACCAT

AGTTCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCC

ATTCTCCGCCCCATCGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCC

GCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCT

AGGCTTTTGCAAAAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGG

AAGACGCCAAAAACATAAAGAAGGCCGGCGCCATTCTATCCGCTGGAAGAT

GGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGGTT

CTGGGACAATTGCNTTTAACAGATGCACATATCGAGGNGGGNATCCNTTACC

TGANTACNTCNAAATGTCCGTTCGGGTNGGANAAACTATGNAAANA

Clone 21a:

CGGATCCNCTAGTAACGGCCGCCAGTGCNCCCCNNNNNNCCCTTGATTTAAC

AGCCCACTTATCACTCATGGAAGATCCCTCCTCCTGCTTGACTCCGCCCTCTC

TCCCTCTGCAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCG

AGATCTGCGATCTGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACT

CCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATCG

CTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGC

TATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAA

GCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGACGCCAAAAACA

TAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGA

GCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAAACAATTGC

TTTTTACAGATGCACATATCGAGGTGGACATCACTTTACCTGANTACTTCCAA

ATGGTCCGNTCGGTTGGCAAAAACTATGNA

Co-immunoprecipitation

Cells were plated in 60 mm dishes and once they reached a confluence, media was aspirated and cells were washed in 1x PBS buffer 3 times. 1 ml of Panomics buffer A mix was added to each plate and cells were lysed for 10 min on shaking ice bucket. Cells were scraped and place into 1.5 ml-ultracentrifuge tubes and centrifuged at 40,000 g for 30 min. Supernatant represents the cytosolic fraction and pellet gets dissolved in Panomics buffer B mix/Glycomics buffer (1:1 ratio). Protein concentration is determined from both fractions using DC BioRad protin assay reagents.

500 μg of protein lysate from each fraction is placed in new 1.5 ml- Eppendorf tubes diluted in 1x PBS buffer to make up 1mg/ml final concentration. We rotated these samples with 5 μg $A\beta_{1-40}$ peptide overnight at 4°C. Two tubes are prepared, so one can be used as negative control. To these samples we add 10 μg of Aβ antibody (6E10, mouse monoclonal Aβ IgG, Signet Inc.), (add antibody only to one tube-control), and rotate it 2-3 hrs at 4°C. Then, add 50 μl of Protein A/G beads (Santa Cruz Biotechnology, USA) and rotate for 2 h at 4°C. Tubes are centrifuged at 15, 000 rpm for 30 sec and supernatant is removed. Beads are washed 5 times with 1ml 1x PBS and spun down each time for 30 sec at 4°C. At last, all buffer is removed and pellet is resuspended in 50 μl of 2x Western

loading buffer. From this step on, general protocol for Western blotting is used (as described above).

Immunocytochemistry

Cells are grown to 70% confluence on 25 mm glass cover slips places in 35 mm Petri dishes and fixed in 10 % formalyn in neutral buffer solution for 10 min. This follows 4 x washes with HBSS where on the last wash dishes can be sealed with paraffin and placed in fridge until ready to use. Cells' integrity is verified under microscope to make sure they are not lifting of. Cells are then permeablized with 0.1% Triton X-100 in HBSS using 500 μl/dish for 5 min at RT. This is followed by 3 times cell washing with HBSS. Blocking solution of 4% normal goat serum in HBSS is added to cells and incubated at RT for 3 h on gently rocking platform. Before adding the primary antibody, again make sure cells integrity is good. Primary antibody is applied in 1:100 dilution prepared in 1% normal goat serum (diluted with HBSS) and left overnight at 4°C. Cells are washed 2 times with HBSS and secondary antibody is applied in 1:500 dilution (in HBSS) for 30 min at RT, away from light. This follows washings 2 times in HBSS, then 2 times in ddH_2O and cells are ready to mount. Coverslips are mounted on slides using fluorescent DAKO Mounting medium and images were taken using 63x objective of Zeiss LSM confocal microscope.

Table 2, CFDA staining on iHBEC treated with 5 μM $A\beta_{1-40}$. iHBEC at passage 43.

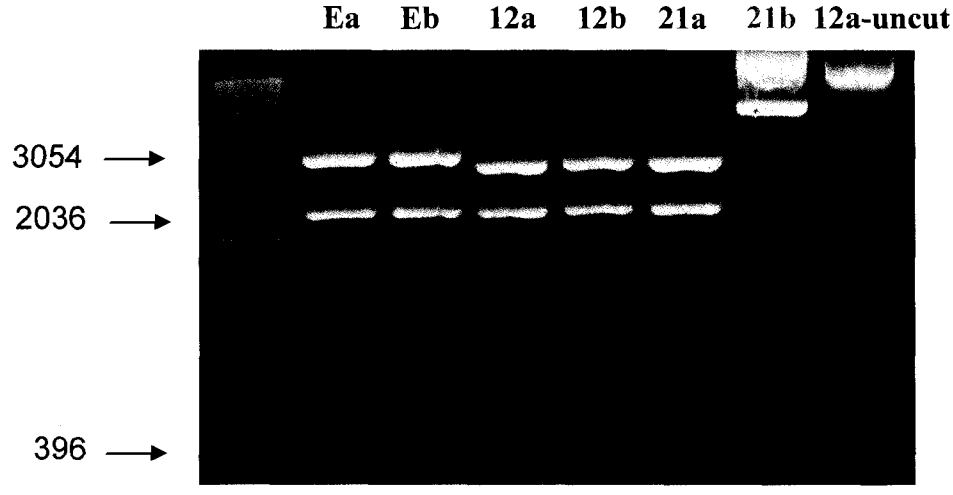
TREATMENT	FLOURESCENCE	FLUORESCENCE (AVERAGE)	SD	%VIABILITY
Control	2847	2936	114	100
(media)	3064			
	2896			
Media	2249	2667	372	91
($A\beta_{1-40}$)	2789			
	2963			
Media	2756	2856	87	107
(NaOH)	2914			
	2899			
EBM-2	2643	2711	118	95
	2847			
	2643			
EBM-2	2625	2764	145	102
($A\beta_{1-40}$)	2753			
	2914			
EBM-2	2701	2709	119	98
(NaOH)	2594			
	2832			
6 mM H_2O_2	214	231	18	8.5
	250			
	229			

CFDA staining on iHBEC treated with 5 μM $A\beta_{1-40}$. iHBEC at passage 43, were treated with 5 μM $A\beta_{1-40}$ in iHBEC media or serum free media (EBM-2) and vehicle (2mM NaOH) for 24 h. Cells were stained with CFDA compound and luminescent readings were taken with 485 Excitation and 530 nm Emission wavelengths, at sensitivity scale of 50. Six milimolar H_2O_2 was used as a positive control for cells that are not viable. $A\beta_{1-40}$ and EBM-2 media did not have toxic effect on iHBEC cells.

Table 3. Transfection efficiency in HEK 293 cells using different transfection reagents.

	FUGENE HD	(3:2) Transfection reagent: μg of DNA	%	
	Number of cells	Transfected	Transfection	
	598	10		
	683	29		
	697	12		
Average	659	17	2.7	
	LIPOFECTAMINE	(2:1) Transfection reagent: μg of DNA		
	Number of cells	Transfected	Transfection	
	850	658		
	852	701		
	769	692		
Average	824	684	75	X
	SUPERFECT	(5:1) Transfection reagent: μg of DNA		
	Number of cells	Transfected	Transfection	
	784	480		
	805	395		
	815	405		
Average	801	427	58	

EGFP-reporter gene plasmid was transfected into HEK 293 cells for 48 h and cells were observed under flouorescent microscope and transfeceted cells were counted. Cells transfected with LipoFectamine reagent had the highest transfection efficiency.



pGL3-promoter vector containing AP-1-binding sites cloned from human MCP-1 gene. Restriction enzymes: HindIII and BamHI were used to cut the vectors carrying cloned AP-1 binding sites from human MCP-1 gene, clone 12 and clone 21. The inserts were cloned into pGL3-promoter vector (5010bp in lenght). The expected fragments after restriction enzyme reaction are: 3054, 2036, and 396bp. Following are sequencing results from each clone and highlighted bases are TPA response element (TRE) regions (e.g., AP-1-binding site).

Clone#12:
CAGGCTCGGCCAGAACATTTCTCTATCGATAGGTACCGAGCTCGGATCCACTAGTA
ACGGCCGCCAGTGTGCTGGAATTCGCCCTTGATTTAACAGCCCACTTA**TCACTCA**T
GGAAGATCCCTCCTCCTGNT**TGACTCC**GCCCTCTCTCCCTCTGNAAGGGCGAATTC
TGCAGATATCCATCACACTGGCGGCCGCTCGAGATCTGCGATCTGCATCTCAATTA
GTCAGCAACCATAGTCCC.

Clone#21:
AGCCGGCAGGCCGGCCAGAAATTTCTCTATCGATAGGTACCGAGCTCGGATCCACT
AGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTGATTTAACAGCCCACTTA**TCAC**
TCATGGAAGATCCCTCCTCCTGGT**TGACTCC**GCCCTCTCTCCCTCTGGAAGGGCGA
ATTCTGNAGATATCCATCACACTGGCGGNCGCTCGAGATCTGCGATCTGCATCTCA
ATTANTCAGCAACCATAGNCCCGCCCCTAACTCCGNCCATNCCGCCCCTAACTCCG
CCCAGNTCCGCCCNTTN.

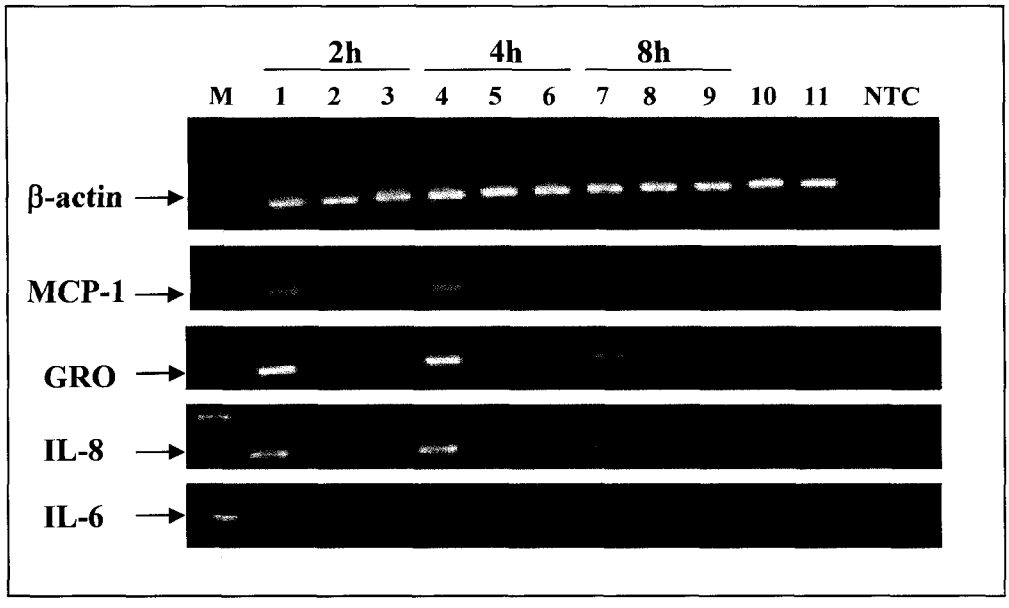
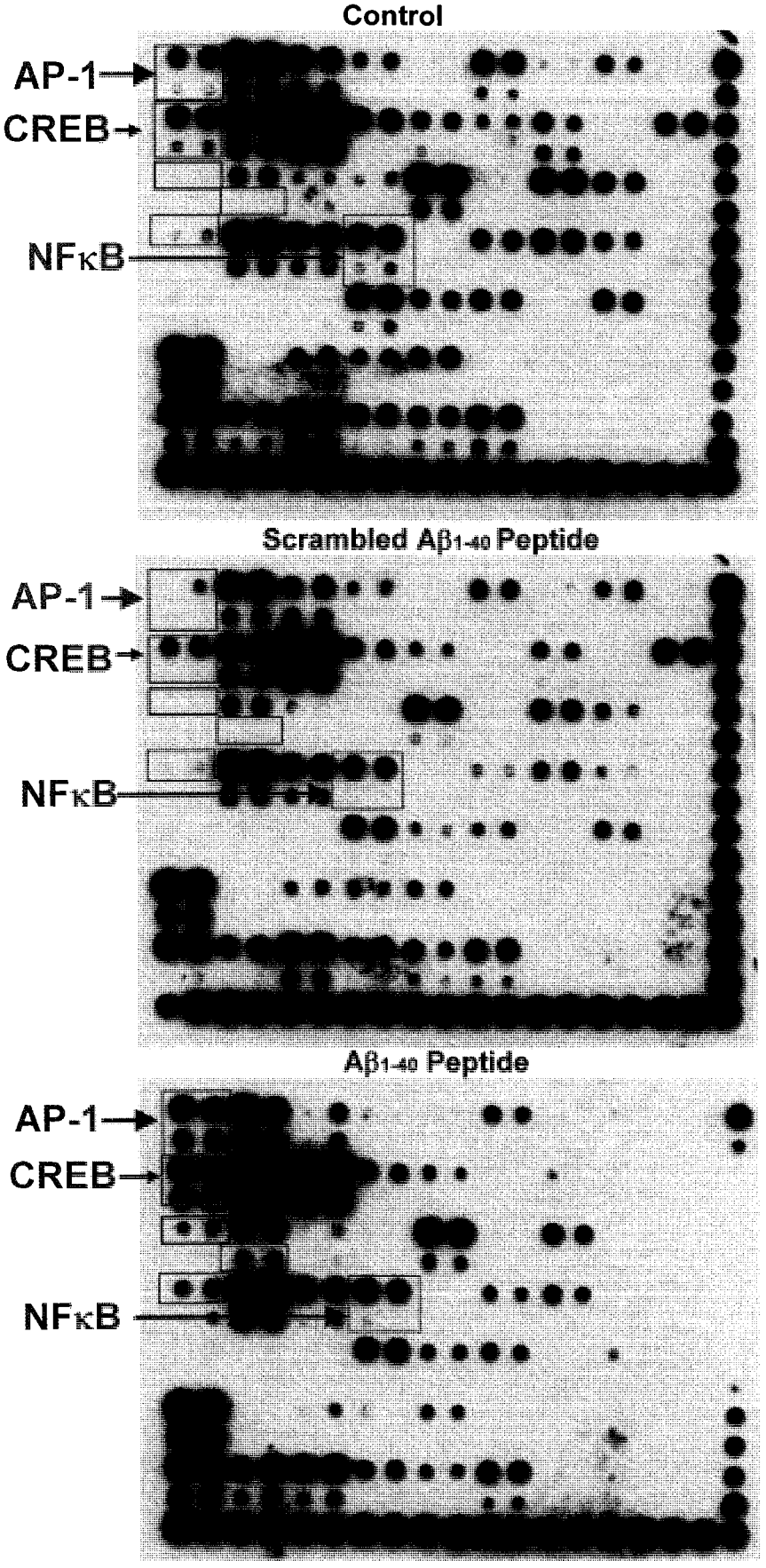


Figure 1. Aggregated $A\beta_{1-40}$ peptides induce the expression of inflammatory genes in HBEC. Primary HBEC cultures were treated with 5μM $A\beta_{1-40}$, 5μM control peptides or 2mM NaOH (vehicle) over 2, 4 and 8 h, and RNA was isolated from the cells. The expression of the genes MCP-1, GRO, IL-8 and IL-6 was analyzed by RT-PCR and normalized to β-actin. The experiment was repeated three times with consistent results. Lanes# 1, 4, and 7 were treated with 5μM $A\beta_{1-40}$. Lanes# 2, 5, and 8 were treated with 5μM control peptide. Lanes# 3, 6, and 9 were treated with 2mM NaOH. Lane# 10 was treated with stale HBEC media. Lane# 11 was treated with dimethyl sulfoxide (DMSO) in which control peptide was resuspended. NTC: negative control for PCR.



TF two- fold increase in Aβ-treated HBEC

- AP-1
- CREB
- GATA
- GRE
- NFATc

Figure 2. Aβ1-40 activates AP-1 in HBEC. Primary HBEC cultures were treated with 5μM Aβ1-40, 5μM scrambled Aβ peptides or 2mM NaOH (vehicle) for 8h. Nuclear protein was isolated from the cells. Transcription factor (TF) profiling was conducted using TranSignal Protein/DNA Array blot (Panomics Inc) as described in the Materials & Methods. The levels of five transcription factors AP-1, CREB, GATA, GRE, and NFATc were increased over two-fold in Aβ-treated HBEC as compared to control and scrambled Aβ-treated cells. NFκB was not activated (the experiment was conducted by Debbie Callaghan with help from Vanja Vukic).

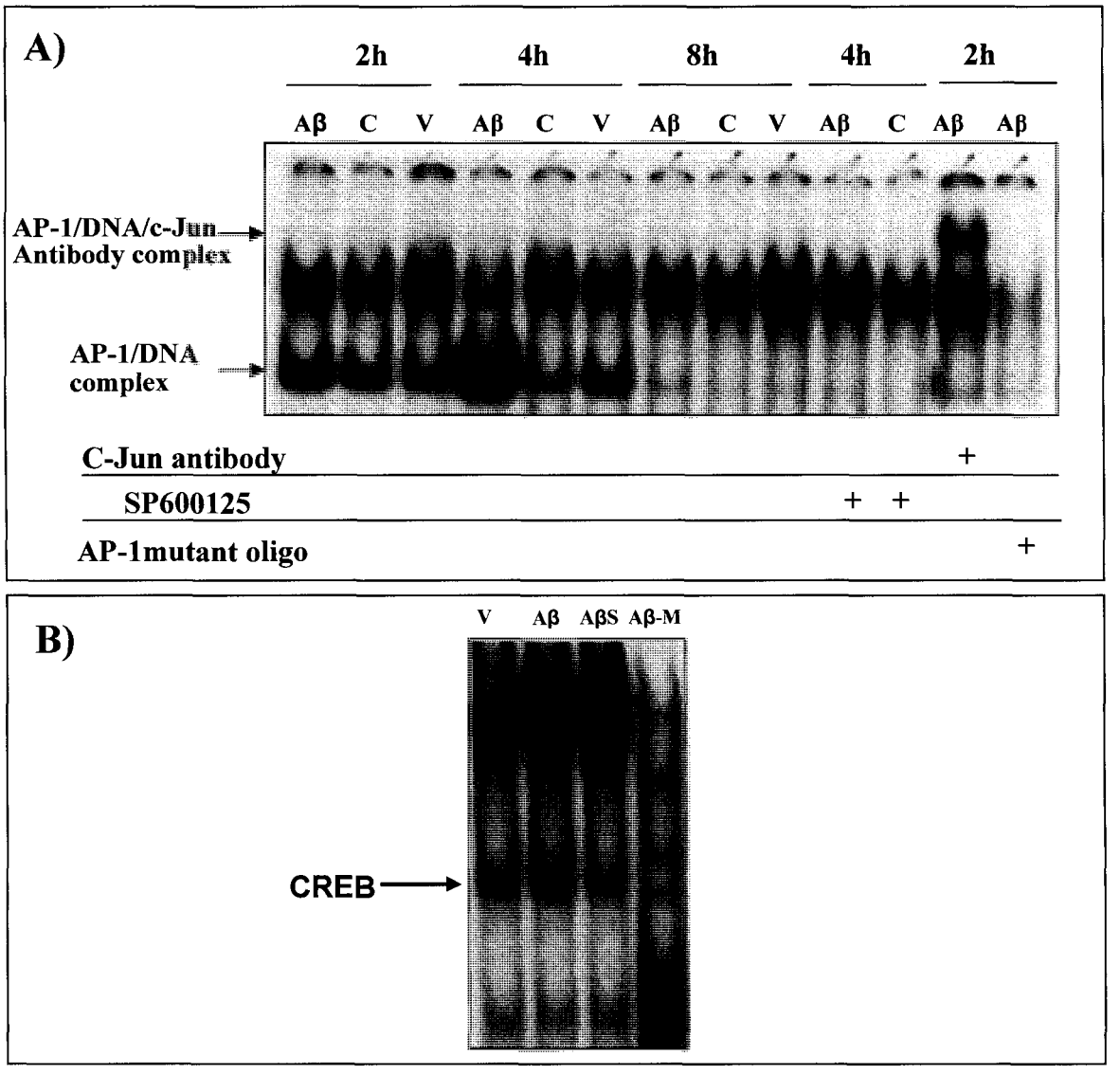


Figure 3. Aβ peptides activate AP-1 and CREB in HBEC. Panel A shows EMSA for physical binding of activated AP-1 protein complex to AP-1-binding DNA sequence (e.g., TPA-response element, TRE). Primary HBEC were treated with 5 μM $Aβ_{1-40}$, 5 μM control peptide or vehicle (2 mM NaOH) for 2, 4 and 8h. Some of the HBEC were pre-incubated with 30 μM JNK inhibitor (SP600125) for 60 min before adding Aβ. EMSA was then performed in the presence of wild-type AP-1 or mutant AP-1 oligonucleotides. Supershift was performed using a c-Jun antibody (1:100) to validate the presence of c-Jun in AP-1 protein complex. AP-1 was activated in Aβ-treated HBEC at 2 and 4h. AP-1 activation was inhibited by SP600125. c-Jun is a component of AP-1 transcription factor complex. **Panel B** shows EMSA for CREB. HBEC were treated with vehicle (V), $Aβ_{1-40}$ or scrambled $Aβ_{1-40}$ (AβS) for 4h. EMSA was performed in the presence of wild-type CREB or mutant CREB (Aβ-M) DNA sequences. More CREB binding to wild-type DNA sequence was found in Aβ-treated HBEC.

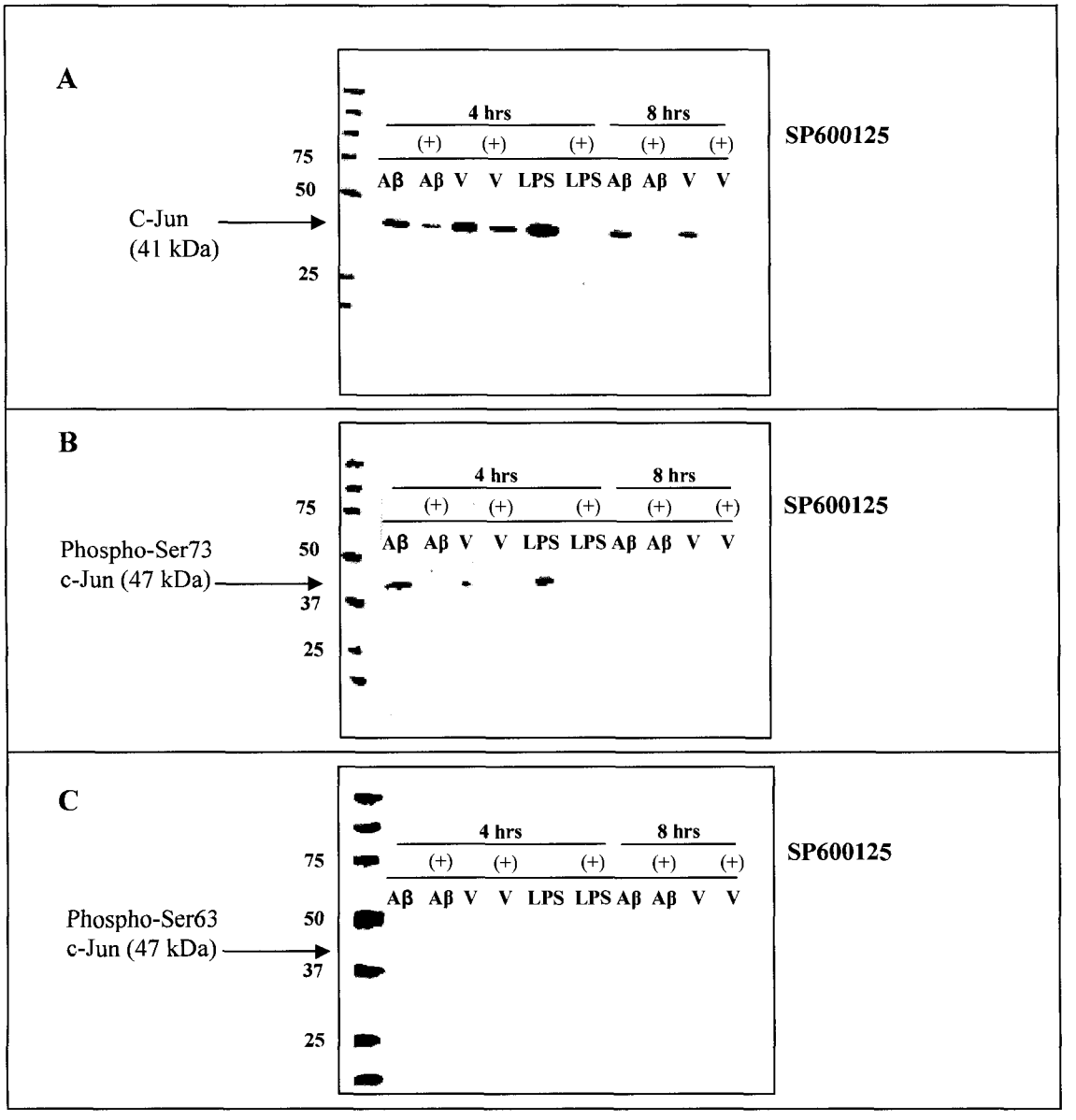


Figure 4. c-Jun expression and phosphorylation in Aβ-treated iHBEC. iHBEC were pre-incubated with 30μM SP600125 for 60 min and then treated with $A\beta_{1-40}$, vehicle (2mM NaOH) or LPS (1:500, positive control) for 4 and 8h. Nuclear extracts were prepared and used in Western blots. Blots were visualized using ECL reagents. **Panel A**: Polyclonal rabbit c-Jun antibody (1:500 dilution) was used to detect total c-Jun. Total c-Jun was increased in Aβ-treated iHBEC when compared to vehicle at 8h. **Panel B:** Polyclonal rabbit antibody was used to detect phosphorylated c-Jun at serine 73 (1:500 dilution). Ser73 phosphorylated c-Jun was remarkably increased in Aβ-treated iHBEC at 4h as compared to control. JNK inhibitor SP600125 strongly inhibited c-Jun phosphorylation at ser73. **Panel C:** Polyclonal rabbit antibody (1:500 dilution) was used to detect c-Jun phosphorylation at ser 63. Phosphorylation at c-Jun ser 63 was not detected in Aβ-induced iHBEC.

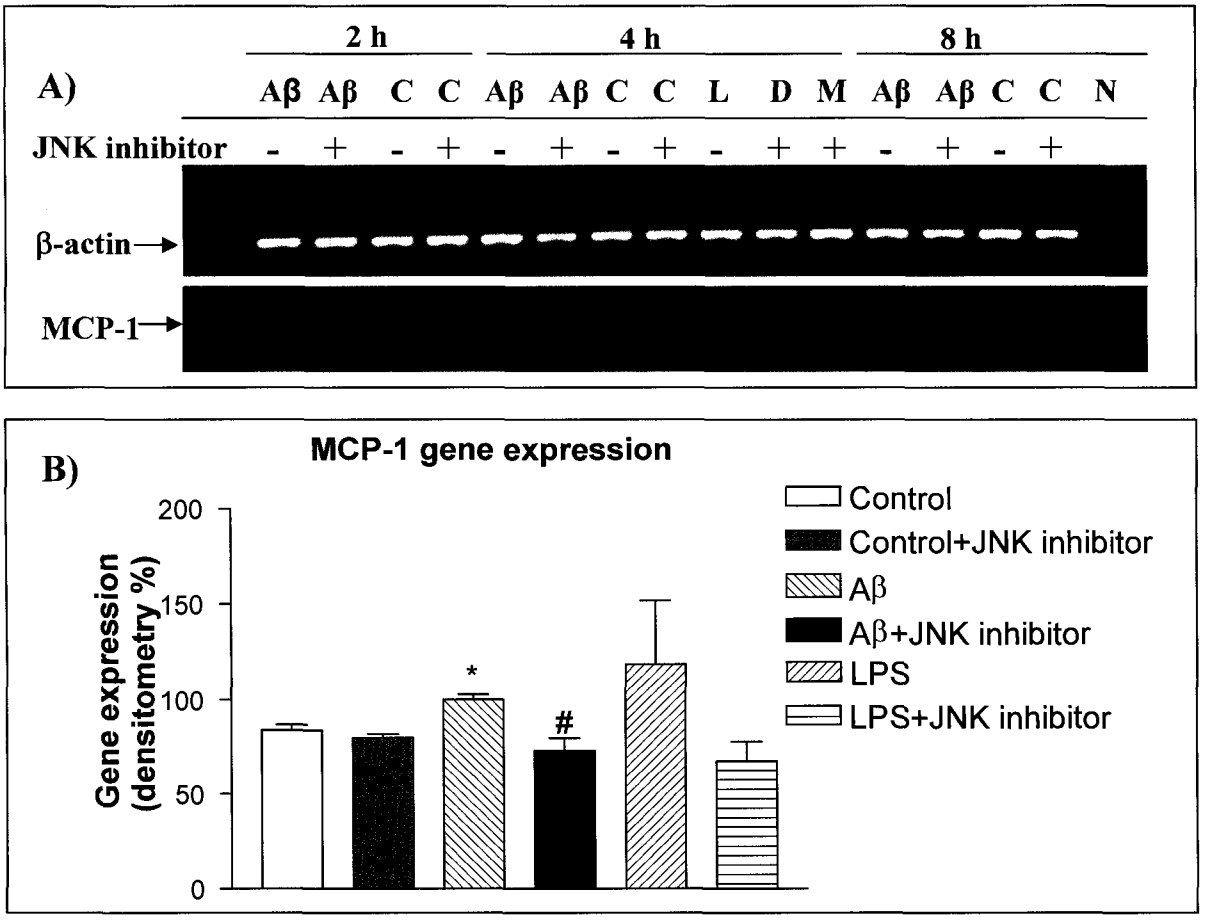


Figure 5. JNK inhibitor SP600125 decreases the expression of MCP-1 in Aβ-treated immortalized HBEC. iHBEC cultures were treated with 20 μM $Aβ_{1-40}$ or vehicle 2mM NaOH (C) over 2, 4, and 8 h in the presence or absence of an JNK inhibitor SP600125 (30μM). Lipopolysaccharide (L) was used as a positive control. **Panels A:** RT-PCR analysis of MCP-1 expression in the presence or absence of SP600125 compared to controls. **Abbreviations**: Aβ, $Aβ_{1-40}$; C, vehicle (2 mM NaOH); L, LPS; D, DMEM (serum free media, in which treatments were prepared in); M, media for iHBEC. **Panel B:** Densitometry analysis for RT-PCR data at 4 h post-treatment with Aβ peptides with or without SP600125. The experiment was repeated 4 times with consistent results. Student *t*-test is used for statistical analysis (*$p<0.009$; #$p<0.004$). Aβ-treatment of iHBEC resulted in an increase of MCP-1 expression when compared to vehicle. MCP-1 expression induced by Aβ was inhibited by SP600125.

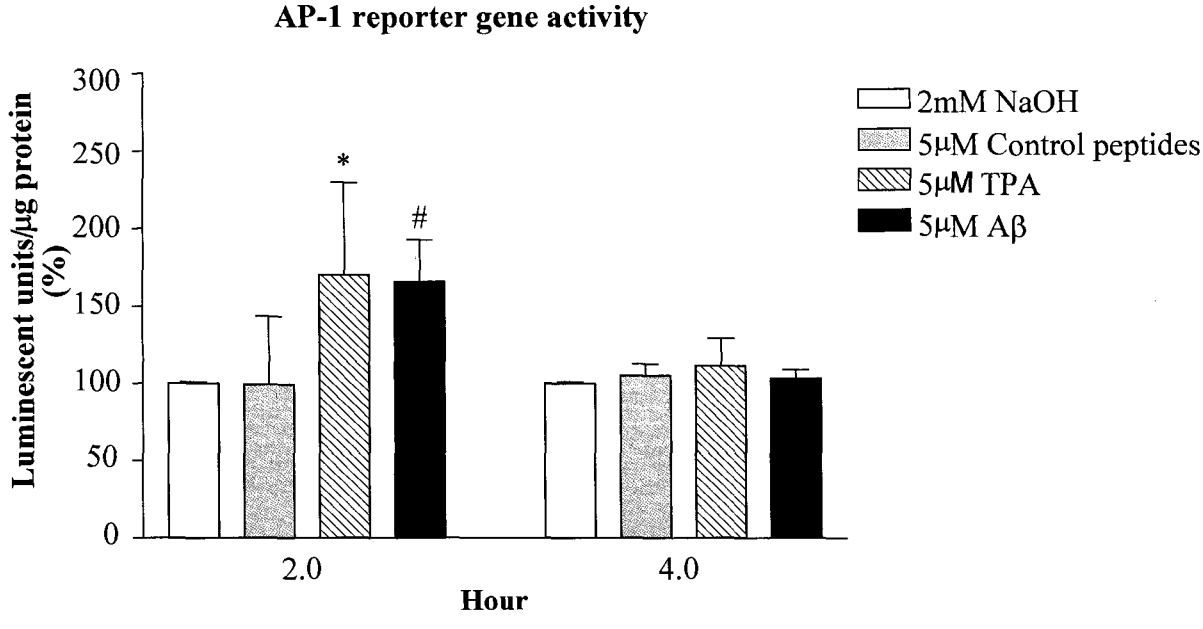


Figure 6. $A\beta_{1-40}$ stimulates AP-1 reporter gene activity in HEK293 cells. HEK293 cells were transfected with a reporter gene vector carrying AP-1-binding element cloned from human MCP-1 gene. The transfected cells were recovered overnight and then treated with 5μM $A\beta_{1-40}$, 20 nM TPA (positive control), 5μM control peptides or 2 mM NaOH (vehicle) over 2 and 4 hr. Luminescence units were normalized to protein level in μg. Experiment was repeated at least 3 times and student *t*-test analysis was performed (*$p<0.05$ and #$p<0.05$ as compared to controls). Both TPA and Aβ strongly stimulated AP-1 reporter activity in transfected HEK 293 cells.

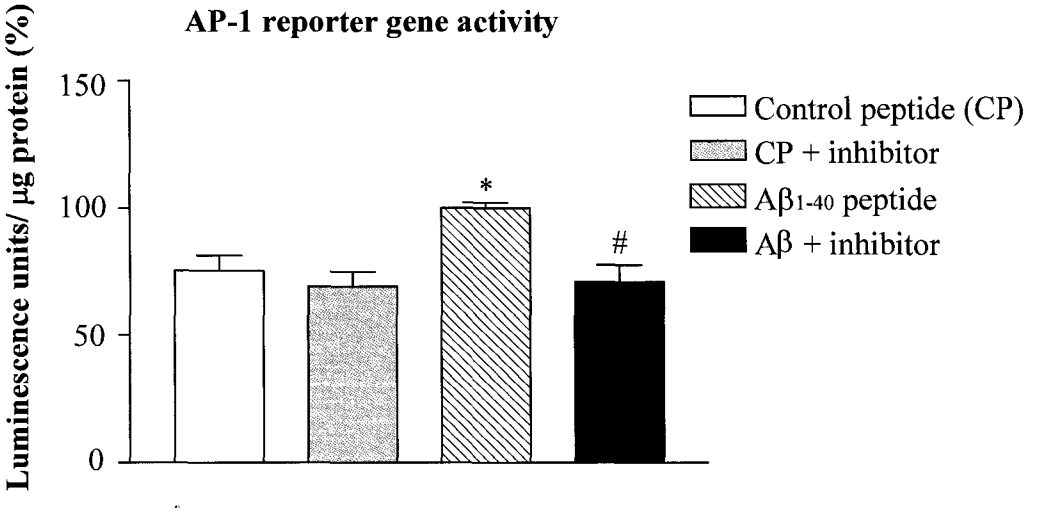


Figure 7. JNK inhibitor SP600125 inhibits AP-1 reporter gene activity in Aβ-treated HEK 293 cells. HEK 293 cells were transiently transfected with the reporter gene vector carrying AP-1-binding sequence. The cells were recovered overnight and then treated with 10µM $A\beta_{1-40}$ or control peptide for 2h in presence or absence of 30µM SP600125. Luciferase assay was performed where AP-1 activity is directly proportional to luciferase expression and chemiluminescence. Experiment was repeated 3 times and student *t*-test analysis was performed. Aβ strongly stimulated AP-1 reporter gene activity (*$p<0.05$) as compared to control. The Aβ-stimulated activity of AP-1 reporter gene was significantly inhibited by the JNK inhibitor (#$p<0.05$).

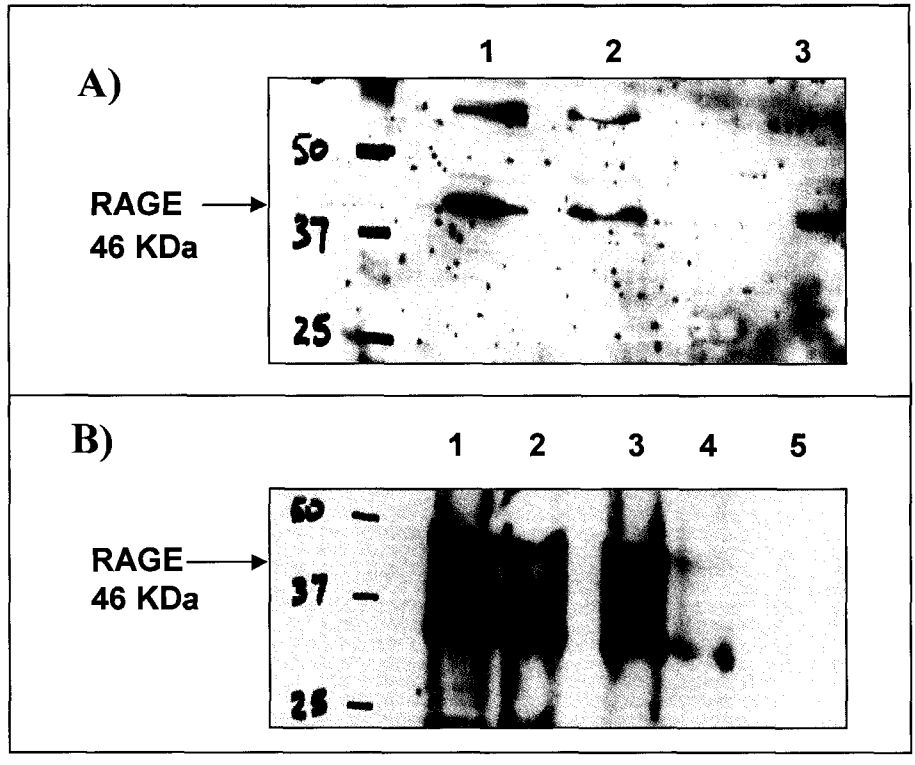


Figure 8. Expression of RAGE in iHBEC and co-immunoprecipitation of RAGE with Aβ peptides in iHBEC. **Panel A** shows the presence of RAGE (46 kDa) and its isoform in both cytoplasmic and membrane fractions of iHBEC cells. *Lanes# 1 & 2*: 50μg protein lysates from cytoplasmic or membrane fraction, respectively. *Lane# 3*: positive control -25 μg membrane fraction. **Panel B** shows co-immunoprecipitation of $A\beta_{1\text{-}40}$ with RAGE. iHBEC lysates (0.5mg protein) were pre-incubated with $A\beta_{1\text{-}40}$ peptides (5μg) overnight and then incubated with 10μg Aβ antibody (1:500) for 3h. The immune complex was precipitated by Protein-A/G beads (50μl) for 2 h and then used for Western blot analysis probed with a RAGE antibody. The RAGE antibody was used at 1:500 dilution and detected with a secondary antibody (HRP conjugated) at 1:2000 dilution. Lanes# *1 and 3*: 50μg cytoplasmic (lane #1) or membrane (lane #3) fraction was incubated with Aβ peptides, and Aβ antibody was then added to precipitate RAGE-Aβ complex. Lanes *# 2 and 4*: 50μg cytoplasmic (lane #2) or membrane (lane #4) fraction was incubated with Aβ peptides, but Aβ antibody was not added for precipitation. Lane# *5*: negative control-lysis buffer with Aβ peptides and Aβ antibody. RAGE was co-immunoprecipitated with Aβ peptides in both fractions by Aβ antibody and was detected in both membrane and cytoplasmic fractions by the RAGE antibody on Western blot.

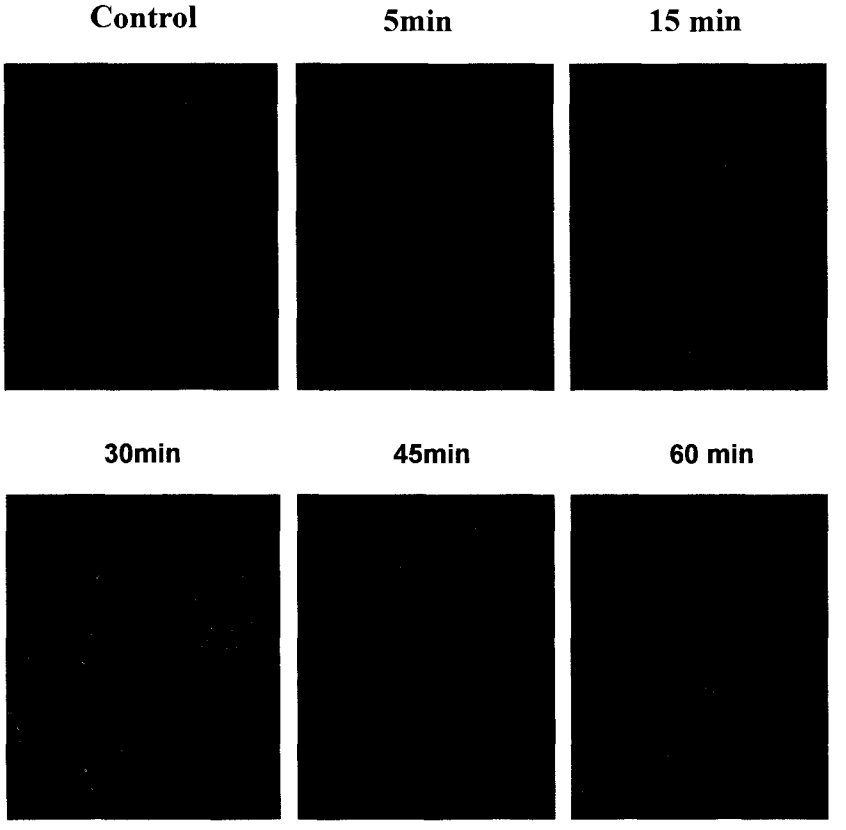


Figure 9. Aβ1-40 interaction with cell membrane in iHBEC. iHBEC were grown on 25mm cover slips to 70% confluence and then treated with 5μM Aβ1-40 conjugated to FITC (stains with green colour) for 5, 15, 30, 45 and 60 min. The cells were then fixed with 10% formalin in neutral buffer solution for 5 min, washed and stained with wheat germ agglutinin conjugated to rhodamine (RH) at 1:10000 dilution (stains with red color for membrane visualization) for 2 min. The iHBECs were mounted on glass slides using DAKO mounting media and 63x objective of LSM Zeiss confocal microsope was used to do Z-sectioning at 0.5 mm thickness per image. Photoshop (Adobe 6.0 version) was used to put sections together to get final image. Aβ peptides started to aggregate on cell membrane at 30min and then internalized.

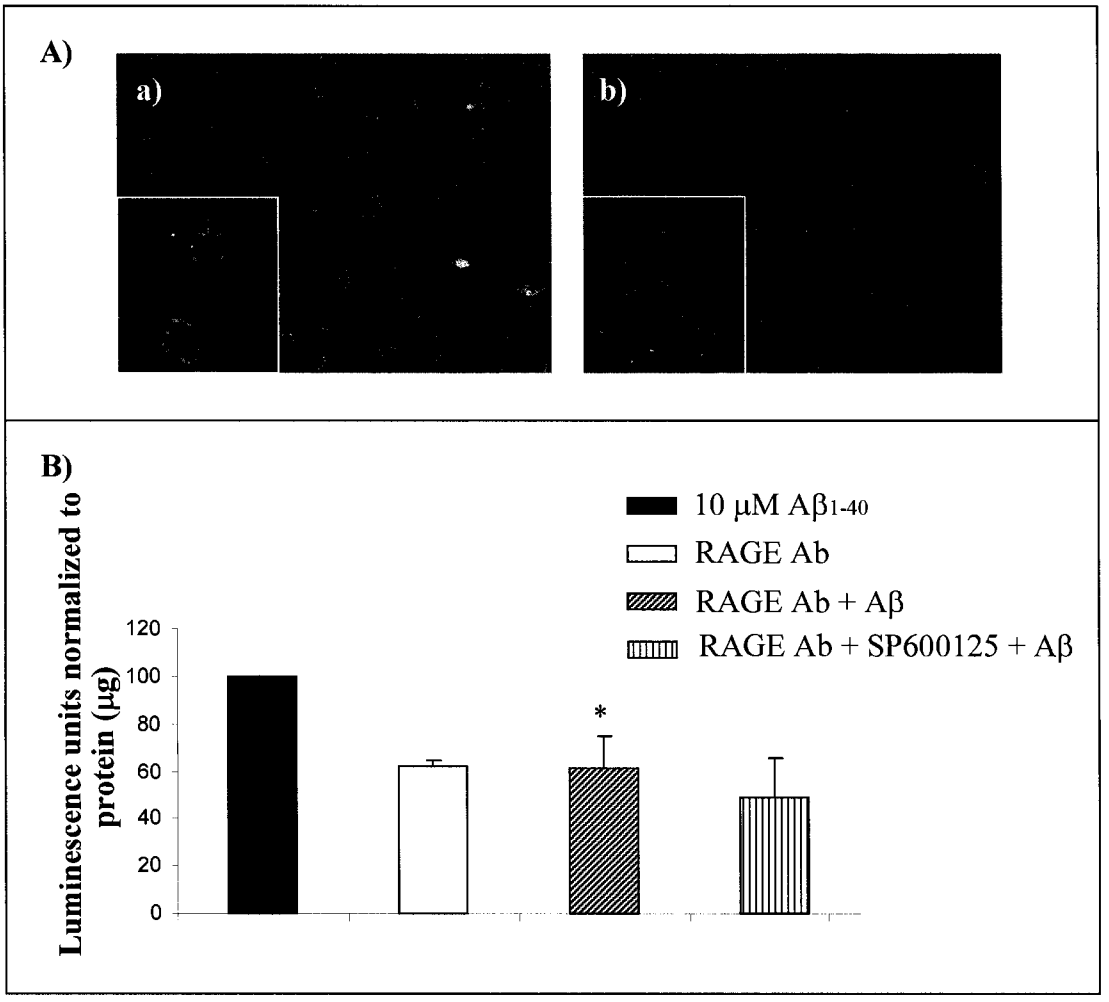


Figure 10. RAGE interacts with Aβ1-40 on iHBEC cells. Panel A shows confocal imaging analysis of RAGE interaction with Aβ peptides. **Image a)**: iHBEC cells were treated with 5µM Aβ1-40 conjugated to FITC (green color) for 30 min. Cell membrane was stained with germ wheat agglutinin conjugated to rhodamine (RH, red color) (1:10000). Aβ peptides were aggregated on cell membrane and entered the cells. The insert shows an enlarged image of Aβ aggregation and internalization. **Image b)**: iHBEC were pre-incubated with the RAGE antibody (1:100) for 30 min and then treated with 5 µM Aβ1-40 conjugated to FITC for 30 min. RAGE antibody reduced Aβ aggregation and entry into the cells. The insert shows an enlarged image of less Aβ interaction with cell membrane. **Panel B:** HEK293 cells were transiently transfected with the reporter gene vector containing AP-1-binding sites for 48 h. Cells were pre-treated with the RAGE antibody (RAGE Ab) for 30 min or/and pre-incubated with JNK inhibitor SP600125 for 60 min and then treated with 10 µM Aβ1-40 for 2 h. Luciferase assay was performed and readings were normalized to protein as µg/sample. Experiment were repeated 3 times and student *t*-test was performed (*$p<0.00001$). RAGE antibody significantly decreased Aβ-stimulated AP-1 reporter gene activity.

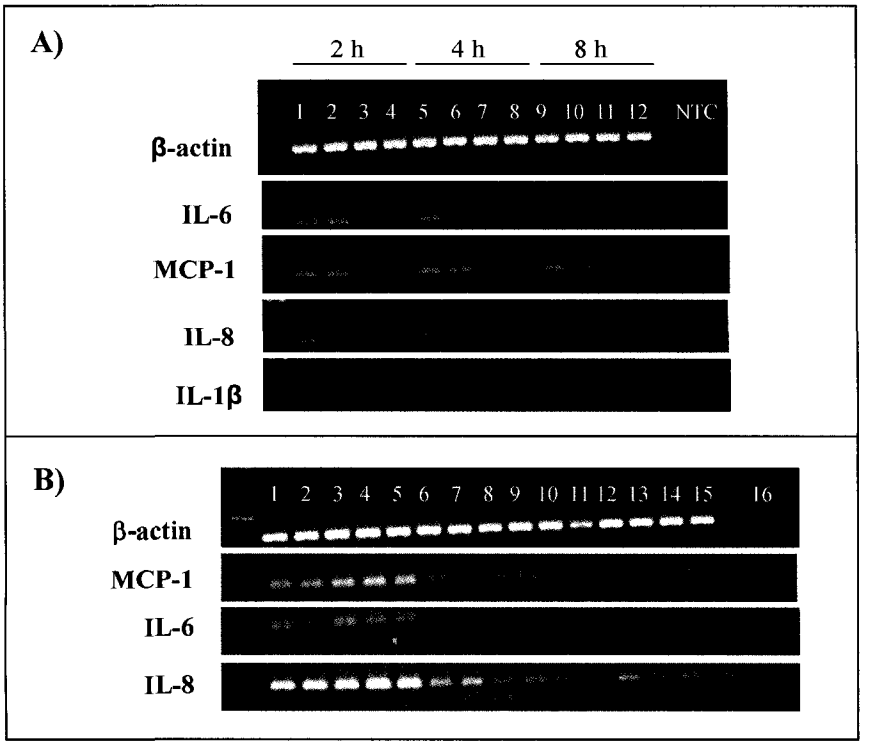


Figure 11. Stimulation of inflammatory gene expression in iHBEC by microglia-conditioned media. Panel A: iHBEC cells were treated with microglia-conditioned media or vehicle media at 1:1 ratio with iHBEC media for 2, 4 or 8 h. RT-PCR was used to analyze the expression of IL-6, IL-1β, IL-8, GRO, TNF-α and MCP-1 normalized to β-actin. Lanes # *1, 5, 9*: iHBEC treated with conditioned media (CM) from microglia stimulated with 5μM $A\beta_{1-42}$. Lanes *#2, 6, 10*: iHBEC treated with CM from microglia stimulated with 2μM $A\beta_{1-42}$. Lanes # *3, 7, 11*: iHBEC treated with CM from unstimulated microglia. Lanes #4, 8, and 12: iHBEC media. CM from Aβ-stimulated microglia induced the expression of IL-6, MCP-1, IL-8 and IL-1β in iHBEC as compared to controls. **Panel B:** Cells were pre-incubated with various MAP kinase inhibitors for 60 min then treated with Aβ-stimulated microglial CM, unstimulated microglial CM, and iHBEC media for 4h in order to determine what signaling pathway is responsible for increase in inflammatory gene expression. The inhibitors used are: 30μM SP600125 (JNK inhibitor), 10μM p38 inhibitor, 100nM wortmanin (ERK ½ inhibitor) or 10 μM PI-3 kinase inhibitor. RT-PCR analysis was done on MCP-1, IL-6 and IL-8, normalized to β-actin. Lane *#1*: iHBEC were treated with CM from microglia stimulated with 5μM $A\beta_{1-42}$. Lanes# *2, 3, 4, 5:* iHBEC were treated with Aβ-stimulated microglial CM in the presence of JNK, p38 kinase, ERK1/2 or PI-3 kinase inhibitor, respectively. Lane# 6:iHBEC were treated with unstimulated microglial CM. Lanes# 7, 8, 9, 10: iHBEC were treated with unstimulated microglial CM in the presence of JNK, p38 kinase, ERK1/2 or PI-3 kinase inhibitor, respectively. Lane# 11: iHBEC were treated with iHBEC media only. Lanes# 12, 13 14, 15: iHBEC were treated with iHBEC media in the presence of JNK, p38 kinase, ERK1/2 or PI-3 kinase inhibitor, respectively. NTC: negative control for PCR. Both experiments were repeated 3 times with consistent results. JNK inhibitor slightly reduced IL-6 expression induced by microglial CM.

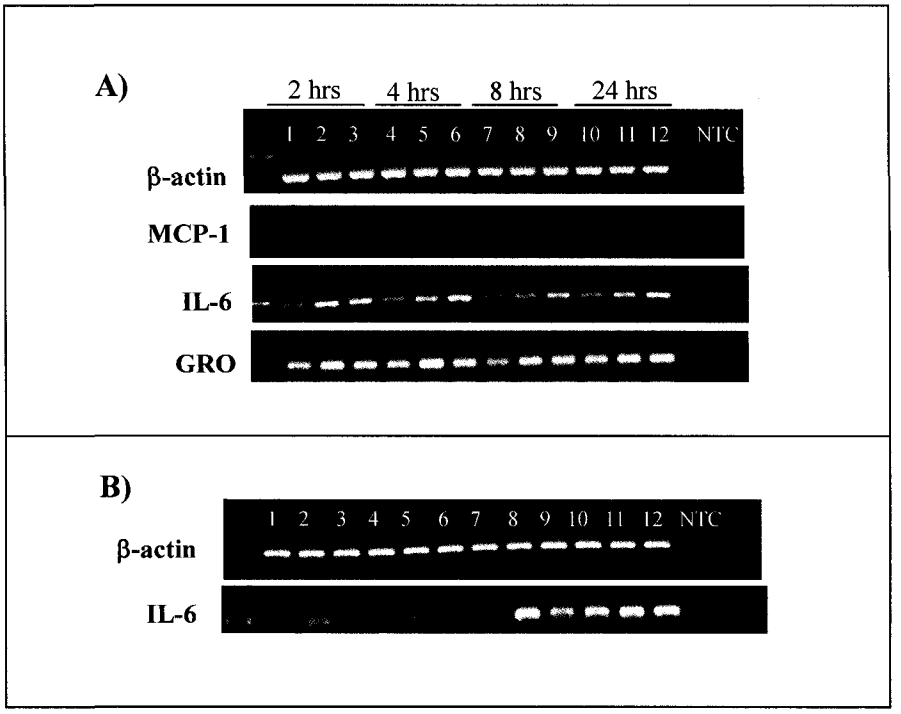


Figure 12. IL-1β or TNF-α treatments induce the expression of inflammatory gene in iHBEC cells. Panel A: iHBEC cells were treated with 50 units/ml TNF-α or IL-1β compared to vehicle for 2, 4, 8 and 24 h. RT-PCR is used to analyze the expression of MCP-1, IL-6, and GRO, and the results were normalized to β-actin. Lanes: *#1, 4, 7, 10*: Vehicle. Lanes *# 2, 5, 8, 11*: 50 units/ml TNF-α. Lanes *# 3, 6, 9, 12*: 50 units/ml IL-1β. **Panel B**: IL-6 gene expression analyzed through RT-PCR and normalized through β-actin. iHBECs were pre-incubated with MAP kinase inhibitors for 60 min and then treated with 50 units/ml IL-1β or TNF-α over 4h. Lanes# *1, 7*: vehicle (iHBEC media). Lanes# *2, 8*: iHBEC were treated with 50 units/ml IL-1β or TNF-α, respectively. Lanes# 3, 4, 5, 6: iHBEC were treated with IL-1β in the presence of JNK, p38 kinase, ERK1/2 or PI-3 kinase inhibitor, respectively. Lanes# *9, 10, 11, 12*: iHBEC were treated with TNF-α in the presence of JNK, p38 kinase, ERK1/2 or PI-3 kinase inhibitor, respectively. NTC: negative control for PCR. JNK inhibitor reduced IL-1β-induced and TNF-α-induced expression of IL-6. These experiments were repeated 3 times with consistent results.

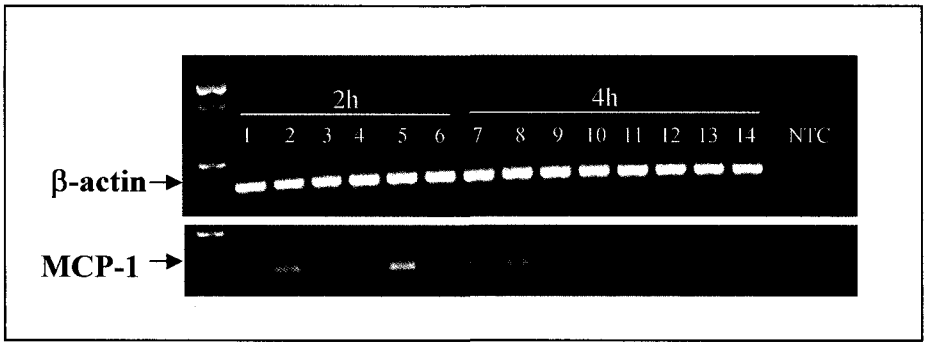


Figure 13. The effect of IL-1β antibody on MCP-1 expression in iHBEC induced by microglia-conditioned media or IL-1β. Microglia-conditioned media (MCM) was incubated with IL-1β antibody (1:100 dilution) for 60 min at 37°C and then used to treat iHBEC for 2 and 4h. Recombinant IL-1β cytokine (1μg/ml) was used as a positive control. MCP-1 gene expression was analyzed through RT-PCR and normalized to β-actin. Lanes# *1, 7*: MCM (2μM $A\beta_{1-42}$ stimulated). Lanes# *2, 8*: MCM incubated with IL-1β antibody. Lanes# *3, 11*: iHBEC media. Lanes# *4, 12*: iHBEC media with IL-1β antibody. Lanes# *5, 13*: 50 units/ml IL-1β. Lanes# *6, 14*: 50units/ml IL-1β + IL-1β antibody. Lane# *9*: unstimulated microglial media. Lane# *10:* unstimulated microglial media + IL-1β antibody. IL-1β antibody inhibited IL-1β-induced expression of MCP-1 but not MCM-induced expression of MCP-1 in iHBEC.

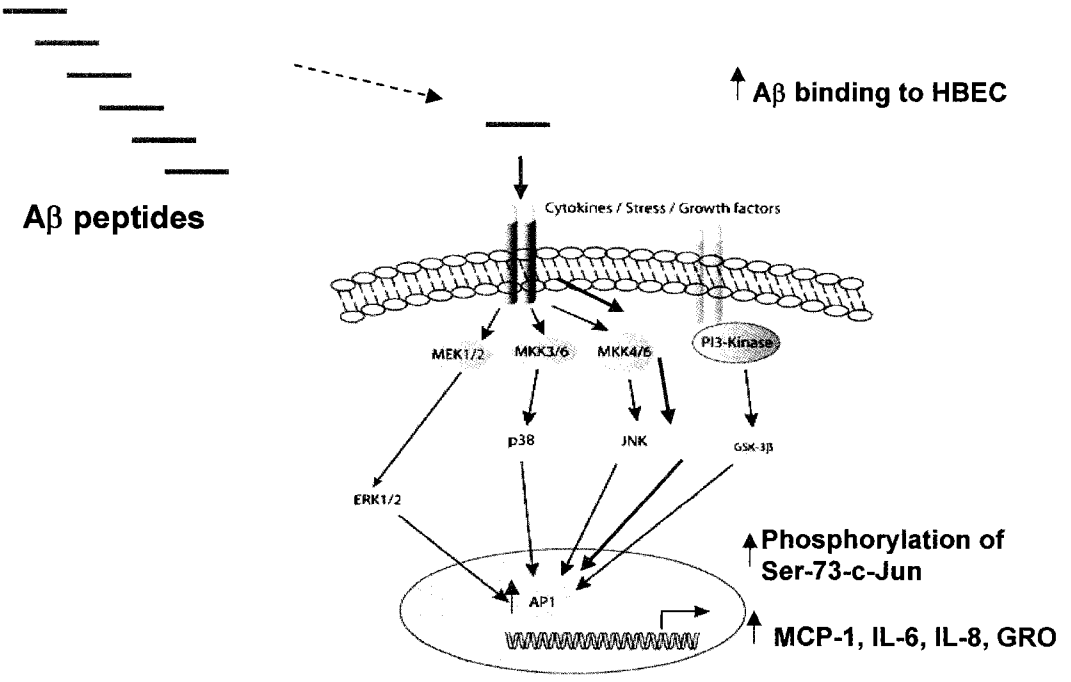


Figure 14. Aβ-evoked signaling cascade in HBEC. Accumulation and deposition of Aβ peptides on brain endothelial cells and interaction with RAGE activates JNK-AP1 signaling pathway and up-regulates the expression of inflammatory factors in cells.