


2006

Gp120 Up-Regulation of the Mu Opioid Receptor in TPA Differentiated HL-60 Cells

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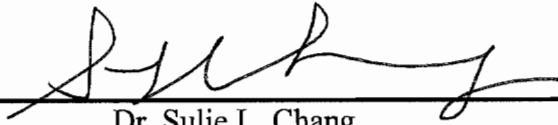
**Gp120 Up-regulation of the Mu Opioid Receptor
in TPA Differentiated HL-60 Cells**

Anitha Pallur

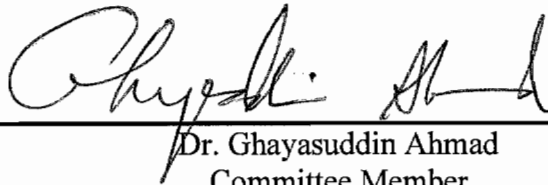
Submitted in partial fulfillment of the requirements for the
degree of Master of Science in Microbiology from the
Department of Biology of Seton Hall University

April 28, 2006

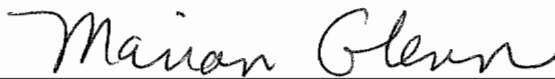
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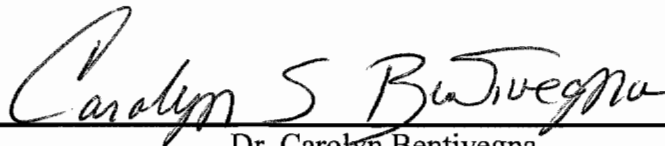
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ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Sulie L. Chang, who gave me the opportunity to work in her laboratory. She dedicated a tremendous amount of her personal time and effort through out the duration of my laboratory research. Her continuous guidance gave me enough strength to finish this project. I always remember Dr. Chang as a dedicated, strong and enthusiastic professor.

I would like to thank Dr. Heping Zhou, who introduced me to the Dr. Chang's laboratory.

I greatly appreciate love and support that I received from my beloved husband and my parents throughout my time spent at Seton Hall University. It would not have been successful without all the support I received from them.

I would like thank all my lab members for their guidance and support whenever I needed most. Especially I want to thank Mr. Jose. A. Beltran for the technical help and support he gave me in this project.

I want to express my gratitude to Dr. Ghayasuddin Ahmad and Dr. Marian Glenn, for their valuable time in reviewing my thesis paper and also participating in my defense committee.

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ABSTRACT

Opioid abuse acts as a potential cofactor and is also involved in the exacerbation of immuno pathogenesis of HIV infection to AIDS as it modulates the immune functions. Opiate actions, mediate through mu opioid receptors present on the immune cells that modulate the immune systems; alter the expression of cytokine receptors and secretion of the cytokines. In the present study, we investigated whether or not the HIV-1 gp120 upregulates the mu opioid receptors at transcriptional level in the TPA differentiated HL-60 cells. Results show that HIV-1 gp120 significantly upregulates the MOR mRNA in TPA-HL-60 cells and results were confirmed by real time PCR. The functionality of the mu opioid receptor was confirmed based on the morphine inhibition of forskolin induced cAMP, which was again reversible by mu opioid receptor antagonist, naloxone. Gp120 induces the secretion of the proinflammatory cytokines TNF-alpha and IL-1 beta in the TPA-HL-60 cells. By neutralizing the secreted TNF-alpha and the TNF-alpha receptors type II (TNFR II) through TNF-alpha neutralizing antibody and anti-TNF-alpha receptor type II antibody has shown the diminished gp 120 induced upregulation of the MOR mRNA levels in TPA-HL-60 cells. This clearly indicates that TNF-alpha involved in the regulation of the MOR mRNA level and its actions are solely mediated through TNF-alpha receptor type II. Therefore we concluded that HIV-1 gp120 is involved in the upregulation of mu opioid receptors and induces the TNF-alpha secretion which then mediates the upregulation of mu opioid receptors in autocrine/paracrine fashion. This would suggest that opiate exacerbation of the HIV disease progression is a result of the altered immune function and upregulation of its receptors.

INTRODUCTION

The opioid receptors are G-inhibitory-protein-coupled, seven-transmembrane receptors that have been further categorized into three classes, mu, delta and kappa (Kieffer *et al.*, 1992; Chen *et al.*, 1993; Li *et al.*, 1993; Chen *et al.*, 2005). Functional studies suggests that Immune cells such as macrophages and T-cells express mRNA for opioid receptors and their presence on these cells has been confirmed using binding analysis (Wybran *et al.*, 1979; Chuang *et al.*, 1993; Chuang *et al.*, 1995; Wick *et al.*, 1996). Furthermore, the receptors present on the immune cells share common characteristic features such as molecular size, immunogenicity and intracellular signaling pathways (Carr, 1991). Most of the opiates actions are mediate primarily through the mu opioid receptors. It has been postulated that opioids also directly effects the immune cells via brain-like opioid receptors (Chuang *et al.*, 1995). These opioid receptors play significant role in analgesic properties, addiction and also involved in the organism's response to the stress, infection and malignant transformation (Makman, 1994).

In a national sample of HIV patients, nearly one third were found to be addicted to opioids and abuse opioids (Steele *et al.*, 2003). Intravenous opioid drug users have a higher chance of progression of HIV disease as the opioids potentiate these retroviruses (Donahoe and Vlahov, 1998). Its been proved that these drugs of abuse stimulate in the central nervous system in such a way that HIV patients will have severe neurocognitive dysfunctions as these drugs may synergize with the neurotoxic substances released during the HIV infection (Nath *et al.*, 2002). Even though opiates have the

affinity acts through the other opioid receptors such as kappa and delta, but neurotoxic effects are mediated through mu opioid receptors only (Hauser *et al.*, 2005). Moreover opiates modulate the immune system and have been confirmed by both *in vitro* and *in vivo* studies (McCarthy *et al.*, 2001). The effects of opiates are mediated indirectly by the activation of the hypothalamic-pituitary-adrenal-(HPA) axis and directly by Opioid receptors which are expressed mainly in the central nervous system (Kraus *et al.*, 2001; Roy *et al.*, 2001; Ammon-Treiber and Holtt, 2005).

The effects of opioids through opioid receptors on the immune response are mediated at several levels. Some of the well known effects are modulation of the inflammatory response, inhibition of antibody responses, induction of chemotaxis in mononuclear cells, alteration of macrophage functions, and modulation in the cytokine production. Moreover, activation of the mu opioid receptors inhibits the adenylyl cyclase, stimulation of potassium-channel conductance and inhibition of calcium-channel conductance (Wybran *et al.*, 1979; Peterson *et al.*, 1987; Williams *et al.*, 1988; Yu and Sadee, 1988; Rojavin *et al.*, 1993).

The mechanism of HIV entry and infection of macrophages can demonstrate the possible alteration in the immune system and regulation of opioid receptors. HIV-1, Human immunodeficiency virus type-1, is the responsible causative agent for the commonly known acquired Human deficiency virus syndrome (AIDS), and also the most pandemic critical health crisis factor throughout the world now. Researchers are putting a lot of effort in developing drugs and vaccines to kill or completely eradicate HIV/AIDS.

HIV-1 virus causes profound immunodeficiency in the infected people which causes HIV patients to be more prone to opportunistic pathogenic infections as a result of progressive decrease of CD4+ T lymphocytes and malignancies, which are clinical hallmarks of HIV disease (Kessler *et al.*, 1992). An appropriate immune response results in lack of protection and even contributes to disease severity. The internal dynamics and the mechanism of the virus that it infects the cells have been well studied.

HIV is a retrovirus belonging to the family of lentiviruses, which commonly binds the CD4+ cells such as T helper lymphocytes and macrophages. The extra cellular portion of HIV virus exists as virion and consists of three important genes Gag, Pol and Env proteins which are considered mainly structural proteins. This virus has two copies of single stranded genomic RNA inside the protein core. This core contains additional material necessary for viral replication such as reverse transcriptase, integrase enzyme for integration into the genome and protease. The core is contained in an envelope derived from the previous host cell's original cell membrane. Before budding, however, viral proteins were inserted into this membrane (Center *et al.*, 2002).

Initially the envelope glycoprotein gp120 existed in trimeric form with gp41 collectively called as gp160 polypeptide chain. After binding to the uninfected target cell, this gp160 cleaved into two fragments known as gp120 and gp41. Evidence supports the idea that gp120 recognizes the CD4 receptor on CD4+ cells. Though the binding of gp120 protein to CD4 is necessary, it is not sufficient for infection (Popik and Pitha, 1996). It was widely accepted that HIV-1 virus infects the macrophages through an affinity toward the CCR5 receptor and also gp120 binds to the other receptor known as

CXCR4 receptors, which are also found on the CD4+ T cells (O'Brien *et al.*, 1990). By applying the soluble CD4 proved that gp120 has got the high affinity towards its co-receptors which was clearly confirmed through in vitro studies (Myszka *et al.*, 2000), demonstrating that CCR5 binding site on gp120 is formed by conformational changes induced after binding to CD4 (Wyatt *et al.*, 1998). CCR5 and CXCR4 are the chemokine receptors belong to the super family of Seven- transmembrane G-protein coupled receptors. The virus enters into the cell and is incorporated into the genome of the cells by reverse transcriptase mediated pathway. This further causes the suppression of cell mediated immunity. HIV-1 gp120 binding to the CD4 cells and involvement of the chemokine receptors are critical for infection.

Human immunodeficiency virus-1 affects many cells in the central nervous system and also infects the immune cells. HIV can latently infect mononuclear phagocytes and tissue macrophages. Macrophages serve as the first line of defense for any invading pathogens as well as a reservoir for the virus and also are involved in the rebound of viral replication (Stefano *et al.*, 2001). Therefore monocytes and macrophages are very important during all the stages of HIV infection and also serve as transmitters of the virus to other CD4+ T cells. Human promyelocytic leukemic cells (HL-60) are widely used to investigate the differentiation of immune cells since they are immortal, pluripotent cells capable of differentiating into different cell lineages upon treatment with differentiating agents (Collins, 1987). Differentiation of monocytes to macrophages is very important for the permissiveness of HIV infection and its expression

(Goletti *et al.*, 2002). HL-60 cells are differentiated into monocyte-macrophage like cells when treated with 12-o-tetradecanoyl-phorbol-13-acetate (TPA).

HIV-1 surface viral glycoprotein known to be the potent modulator of the immune system and infection to macrophages mainly leads to alteration of its functions, resulting in phagocytosis, tumoricidal activity, nitric oxide production, superoxide formation and including the cytokine expression (Koff and Dunegan, 1985; Tomei and Renaud, 1997; Stefano *et al.*, 2001; Sacerdote, 2003; Bhat *et al.*, 2004) such as modulation of the secretion of many pro-inflammatory cytokines including the TNF-alpha and IL-1 beta (Nakajima *et al.*, 1989; Herbein *et al.*, 1994). These pro-inflammatory cytokines are secreted from macrophages by various responses like inflammatory stimuli and HIV infection. TNF-alpha and IL-1 beta play a significant role in the AIDS related dementia complex and also known to involve in the opioid-immunomodulatory effects. Studies suggest that modulation of cytokine secretion also alters the anti-HIV-1 reactivity. This would give us a possible clue in understanding the immunotherapy strategies for HIV-1 infection (Nath *et al.*, 2002; Connolly *et al.*, 2005). Moreover, Cytokines may induce or inhibit the secretions of other cytokines. Cytokines are known to play in an autocrine or paracrine fashions on the cells, and involved in the functional changes at the tissue locations (Benveniste and Benos, 1995).

TNF-alpha is a major pro-inflammatory cytokine secreted from activated macrophages during the HIV infection and also causes the profound disturbance to the cytokine network (Rimaniol *et al.*, 1997). TNF-alpha exists as homotrimer of 157 amino acid subunits (Hestdal *et al.*, 1997). TNF-alpha is a pleotropic cytokine secreted from

various cells, has beneficial effects such as neuro-protection role against toxicity, prevention of calcium accumulation in the cytosol of neurons (Gonzalez-Scarano and Martin-Garcia, 2005). TNF-alpha also has detrimental effects such as tissue damage, apoptosis and also enhancement of viral replication which leads to AIDS and HAD (Popik and Pitha, 1996). Studies have demonstrated that secretion of TNF-alpha has been involved in the activation of many down stream signaling pathways and enhances the HIV replication and disease progression.

It has been well studied that the opioid receptors can be modulated in both immune cells and neuronal cells by immune derived cytokines. It is also evident that TNF-alpha involved in the alteration of the opioid receptors and levels of endogenous opioid peptides and their receptors (Pol *et al.*, 2001; Buzas *et al.*, 2002; Cahill *et al.*, 2003). TNF-alpha up regulates the mu-opioid receptors in the neuronal cells and increases the transcription of the mu-opioid receptors in the immune effector cells (Kraus *et al.*, 2003).

TNF-alpha exists as mTNF-alpha and sTNF-alpha. It mediates its biological responses through the cell surface receptors p55 and p75 TNF receptors (TNFRs) after binding to it. It has been further evident that TNF-alpha mediates most biological responses such as induction of cell death to cytokine production through these TNFRs. Recent studies have shown that p75 TNF receptors also mediates the distinct cellular responses such as T and B cell proliferation, activation of NF-kB and induction of cytotoxicity (Hestdal *et al.*, 1997). Studies confirmed that the TNF-alpha can trigger the mu- opioid receptor regulation during the inflammatory process through the p75 TNF

receptor. In immune cells, the Mu opioid induction by TNF-alpha could be a possible feed back regulation in which the cytokine secretion is modulated by endogenous opioids that can in turn increase secretion of the same or other cytokines (Kraus *et al.*, 2003).

In the current study, we looked at the MOR m RNA in the TPA-differentiated HL-60 cells upon gp120 treatment. Functionality of expressed MOR gene was checked by using the agonist, morphine and antagonist, naloxone after treatment with gp120 and subsequent inhibition of forskolin induced intracellular cAMP as a result of activation of the MOR gene. We concentrated on the major proinflammatory cytokines TNF-alpha and IL-1 beta after gp120 treatment the TPA-HL-60 cells. We checked the regulation of the MOR at transcriptional level by the TNF-alpha and also role of the TNF-alpha receptors in the regulation. By taking all the above considerations, we hypothesized that gp120 up-regulation of the mu opioid receptor (MOR) in TPA-HL-60 cells may be mediated via the autocrine/paracrine actions of TNF-alpha released from the TPA- HL-60 cells in response to treatment with gp120.

MATERIALS & METHODS

Materials:

Morphine sulfate, Naloxone, and 12-o- tetradecanoyl –phorbol-13-acetate (TPA), were obtained from Sigma-Aldrich (St. Louis, MO). TRIzol reagent for RNA extraction was obtained from Invitrogen (Carlsbad, CA). Radioimmunoassay (RIA) Kit for determination of cAMP accumulation was obtained from Amersham (Piscataway, NJ). ELISA kits for detection of the cytokines TNF-alpha, IL-1 β , recombinant human TNF-alpha, anti-TNF-alpha receptor type I and anti- TNF-alpha receptor type II antibodies were obtained from R & D Systems (Minneapolis, MN). T- 75 cm² Flasks and 6- well plates were obtained from VWR International (Bridge Port, NJ). Oligonucleotide primers to detect MOR, GADPH for Real- time PCR were obtained from Integrated DNA Technologies, Inc (Coralville, IA). Rabbit-polyclonal Anti-human TNF-alpha antibody was obtained from Biosource (Camarillo, CA).

Virus Protein:

Full length Recombinant HIV-1 IIIB gp120 glycoprotein produced in the CHO Expression System obtained from Immunodiagnostic, Inc (Woburn, MA) obtained the stock of 50 ug of 0.5ml. In order to protect the stability, gp120 protein was aliquoted and frozen immediately in -80⁰ C freezer until further use.

Cell line and Cell Culture conditions

Human promyelocytic leukemic (HL-60) cells from ATCC (Rockville,MD) were propagated in RPMI-1640 media (GIBCO-BRL, Gaithersburg, MD) supplemented with 20% FBS (GIBCO-BRL, Gaithersburg,MD) and 1% penicillin-streptomycin (GIBCO-BRL, Gaithersburg, MD) in T- 75 cm² Flask (VWR International, Bridge port, NJ). Cells were maintained in the 5% CO₂ humidified cell culture incubator at 37 °C. Cells were observed every day for the density and cells were passaged two or three times per week.

Differentiation of HL-60 cells

HL-60 cells were grown in T- 75 cm² Flasks and cultured at 10⁶ cells/ml. For the experiments, HL-60 cells were cultured in 6 well plates in a concentration of 1 x 10⁶ cells in 3 ml of RPMI medium per well and treated with 16 nM TPA (12-o-tetradecanoyl -phorbol-13-acetate (TPA) in 0.1% ethanol for 4 days. After the treatment, HL-60 cells were differentiated into macrophages/monocytic cells. The cell culture medium containing TPA was changed every 48 hours until the cells were fully differentiated into macrophage/monocytic cells.

Treatment of TPA-HL60 cells with HIV-1 gp120 protein

HL-60 cells were cultured and differentiated with TPA in 6-well plates. Once the cells were fully differentiated and were treated with different dose concentrations of 250

pM and 500 pM, 1 nM and 40 nM of HIV-1 gp120 protein for 24 hours, and the cells were harvested for RNA isolation to study the MOR mRNA expression through real time- PCR. After 24 hours of incubation, Supernatant was collected from the treatment and saved in -80° C for the cytokines induction study through ELISA.

Isolation of RNA

At the end of the TPA differentiation and/or following various treatments, HL-60 cells were harvested for total RNA isolation from the differentiated HL-60 cells with the help of TRIzol reagent from Invitrogen (Carlsbad, CA).

One mL of TRIzol reagent was added to each well of TPA-HL-60 cells. This was pipetted to remove and break up cells, and the cells were incubated for five minutes at 15 to 30° C to permit the complete dissociation of nucleoprotein complexes, and then the solution was transferred to 1.5 ml sterilized eppendorf tubes. This TRIzol was mixed with 0.2mL chloroform per 1 ml of TRIzol reagent. The tubes were shaken vigorously by hand for 15 seconds and incubated at 15 to 30° C for 2 to 3 minutes. After the incubation the samples were centrifuged at 12,000x g for 15minutes at 4° C.

After centrifugation, the mixture separated into lower red and an inter phase of phenol and chloroform and upper colorless aqueous phase. The top aqueous layer was removed into a new eppendorf tube and mixed with 0.5ml of isopropyl alcohol per 1ml of TRIzol. Samples were incubated for 10 minutes at 15 to 30° C. After the incubation the samples were centrifuged at 12,000 x g for 10 minutes at 4° C. The RNA was precipitated in the bottom of the tube after centrifugation.

Supernatant was removed/decanted and pellet was washed with 1ml 75% ethanol per 1 ml of TRIzol. The 75% ethanol was prepared in DEPC-treated water. The sample was mixed by vortexing and then they were centrifuged at 7,500 x g for 5 minutes at 4⁰ C.

After centrifugation the ethanol was removed and the obtained pellet was air-dried for 5 to 10 minutes. RNA was then resuspended in 25 to 30 µl of DEPC H₂O. The concentration of the RNA was determined by using a spectrophotometer set at 260nm and 280nm and 320nm as background. The 260/280 nm ratio determined the quality of the RNA. The 260 nm reading alone could be used to calculate the concentration of RNA. The integrity of the RNA of the samples was assessed using the electrophoresis of a 0.8% agarose gel containing the ethidium bromide (EtBr) and the bands were visualized by the UV transilluminator. The RNA was then stored at -80° C until further analysis.

Reverse Transcriptase Reaction (RT)

Initially, RNA was isolated, quantified, and stored as described above. Reverse transcription reaction was performed by using 1µg/µl of RNA could then be combined with 1µl of 200 U/ µl of Moloney murine leukemia virus Reverse Transcriptase enzyme (Invitrogen), 4 µl of 5X buffer standard (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen), 4µl of dNTP's (Invitrogen) of 0.5 mM concentration, 1µl of random Primers (Invitrogen) with 10ng/ µl, 6 µl of DEPC H₂O. Total RT reaction was carried in a final volume of 20µl by using the GeneAmp 2400 Thermocycler (Perkin-Elmer, Foster City, CA) for 1 hour at 37 °C, ten minutes at 67⁰C and samples were allowed to cooled down at 4⁰C. This procedure yields cDNA translated from the original RNA.

RT-real-time polymerase chain reaction

TPA- differentiated HL-60 cells were treated with gp120 with 250 pM, 500 pM and 1 nM, 40 nM concentrations and incubated for 24 hours for MOR expression. Untreated TPA-differentiated HL-60 cells were used as a control.

First the RNA isolation performed and quantified, then it was reverse transcribed into cDNA. 2 µl aliquot of the cDNA was amplified by real-time PCR in a 50 µl PCR master mix (25 µl of 1X PCR Mastermix, 400 nM of each primer and TaqMan probe, and 24.7 µl of water). Real-time PCR was performed using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The PCR primers for MOR amplification were (sense: 5'-TCA-TCA-TTA-CCG-TGT-GCT-ATG-GA-3' and antisense: 5'-TCC-TTT-TCT-TTG-GAG-CCA-GAG-A-3'). The TaqMan probe used for the MOR was (5'-CTT-GCG-CCT-CAA-GAG-TGT-CCG-CA-3'), was labeled with a fluorophore, 6-carboxyfluorescein (FAM), at the 5' end, and a quencher, 4-(4'-dimethylaminophenyl)benzoic acid (DABCYL), at the 3' end. Human GAPDH primers were used for the normalization for the MOR mRNA levels. The PCR primers for GAPDH amplification were (sense: 5'-GGA-AGC-TCA-CTG-GCA-TGGC-3' and antisense: 5'-CCC-CAC-TGC-CAA-CGT-GTC-AGTG-3'). The TaqMan probe used for the GAPDH was (5'-CCC-CAC-TGC-CAA-CGT-GTC-AGTT-3') was labeled with a fluorophore, 6-carboxyfluorescein (FAM), at the 5' end, and a quencher, 4-(4'-dimethylaminophenyl)benzoic acid (DABCYL), at the 3' end. Thermal cycling conditions were set at an initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 18 s, and at 60°C for

1 min. At the end of each PCR run, the data were automatically analyzed by the system, and amplification plots were obtained. A MOR RNA standard curve was generated to quantify the MOR mRNA; known amounts of the MOR RNA standard were serially diluted 10-fold, and amplified in the same plate under the same conditions. The quantity of MOR mRNA in the samples was calculated with the help of the standard curve. All amplification reactions were performed in duplicate. For normalization of the MOR mRNA levels in gp120 treated TPA-HL60 cells, a GAPDH mRNA fragment in these cells was also amplified for normalization, and the MOR mRNA copy numbers per microgram of total RNA was calculated.

cAMP accumulation assay

The cAMP accumulation assay was carried out in order to confirm the functionality of the MOR expressed in the TPA-differentiated HL-60 cells after HIV-gp120 protein treatment. For this study, HL-60 cells were cultured in 6-well plates with TPA-induced differentiation. At the end of the TPA treatment, the differentiated HL-60 cells were treated with or without gp120 treatment of 1 and 40 nM and the cells were incubated for 24 hours. After 24 hours of incubation, the supernatant was removed and collected in the respective labeled tubes, and the supernatant was saved at -80°C for the cytokine analysis. The monolayer of the cells was washed with 1ml of phosphate buffer saline and this was aspirated. Then each well of TPA-HL-60 cells were pretreated with 1 ml of 0.5mM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, to block the breakdown of cAMP; the cells were then incubated for 30 min at 37°C .

Then the culture medium with IBMX was aspirated and rinsed with 1 ml of Phosphate buffer saline and replaced in a medium containing either 10 μ M of morphine, 75 μ M of Forskolin, 100 μ M naloxane of alone and the cells were also treated with morphine and forskolin (75 μ M), morphine and naloxane or morphine plus naloxane (100 μ M) in the presence of forskolin. These treated cells were incubated for 10 min at 37°C. The medium was then removed, and the cells were washed, lysed with 0.1 N of HCL, and frozen at -20⁰ C. After 24 hours, this mixture was defrosted for the analysis of the levels of intracellular cAMP and for the protein concentration of the mixture was determined using the Bio-Rad Bradford reagent in order to normalize the data.

The lysed monolayered frozen samples were taken out and kept on a shaker for thawing. The cAMP buffer of 0.05M, and cAMP standards from 25 to 1600 fmoles were taken out and kept at room temperature. For the labeled glass tubes the cAMP buffer, primary antibody and I ¹²⁵ tracer and 10 μ l of sample of interest were added, and standards of known concentration were also prepared by adding the serially diluted prepared standars, primary antibody and I ¹²⁵ tracer. The samples were mixed by shaking the rack, and were incubated for over night at room temperature. After overnight incubation, 250 μ l of secondary antibody was added and mixed the samples by shaking the rack and incubated for 10 minutes. The tubes were spun down at 1500 g for 15minutes. Afterwards, the centrifugation liquid was decanted into the radioactive labeled bottle. The obtained precipitate was counted in a Wallace Wizard I470 automatic gamma counter in order to study the intracellular c AMP levels from the cell lysates. The

results were normalized with protein values determined by Bio-Rad Bradford reagent assay.

Protein determination by Bio-Rad Bradford assay

The protein concentrations in the supernatants were determined by a commercial Bradford assay (Bio Rad). The Bradford reagent solution added to water was used as a zero standard, and solutions of known protein concentrations were used as appropriate additional standards. Bovine serum albumin of $0.5\mu\text{g}/\mu\text{l}$ was used as a standard known concentration protein. This protein determination was carried in a 96-well plate. Water, Bradford reagent and the samples/standards were added according to the protocol.

The standards were loaded in triplicates, and the actual samples were loaded in doubles. When the loading was done, the plate was kept on an orbital shaker for even mixing. The optical densities were measured on a microplate 96-well Biokinetics reader (EL312e , Bio Tech instrument) at a single wavelength of 595 nm. The optical density values of the known standard samples were plotted in order to calculate the unknown sample concentrations.

Enzyme linked immunosorbant assay (ELISA)

Supernatants were removed after the complete 24-hour gp 120 treatment. Samples were assayed immediately or stored at -80°C . The assay was performed as

instructed in the protocol provided by the manufacturer. The ELISA kits for TNF- alpha and IL-1 β were used for this assay.

Following the manufacturer's instructions, the standards were prepared from the stock standard solution. The other standards were prepared by serial dilutions and pipetted into Duplicate wells. This serial dilution of the standard would give a series of specific concentrations of the cytokine of interest. The supernatants of the gp120 dose dependent treated samples were pipetted into the remaining wells. After that, the ELISA plate was incubated for two hours at room temperature to allow binding between the cytokine of interest and the attached antibody to the well.

After the 2-hour incubation, the wells were emptied of their contents with the help of an aspirator or squirt bottle. The wash buffer was added to each well with the help of a multi channel pipette, and clearly rinsed out. This process was repeated three to four times (based on the assay). This washing will remove the unbound material and whatever remained will be the protein that was bound to the attached antibody to the well.

When the wash was complete, 200 μ l of conjugate solution was added to each well, which consisted of a secondary antibody. The secondary antibody had an oxidative enzyme which attaches to the Fc portion. The binding of the protein to the variable region of the cytokine results in 1:1 ratio of protein to oxidative enzyme. The plates were incubated at room temperature for one hour, and, again, a series of 3 to 4 washes (based on the assay) was used to remove all unbound secondary antibodies.

After complete washing, the substrate solution of 200 μ l was added. It reacts with the enzyme on the secondary antibody to create a colored product. The extent of

color change was directly proportional to the amount of the cytokine present in the wells. In order to stop the reaction at the end, the 50 μ l stop solution was added to the substrate solution.

The optical density of the colored products were measured on a microplate 96-well Biokinetics reader (EL312e , Bio Tech instrument) at a wavelength specific for that colored product (450 nm) and also a correction wavelength of 540nm. By reading the products in the serial dilution wells, a standard curve could be developed from which the unknown sample concentrations were determined. The unknown concentrations of the cytokines were normalized with protein values determined through Bio-rad Bradford assay.

Neutralization of secreted TNF-alpha and its receptors

TPA-differentiated HL-60 cells were treated with anti- TNF antibody and antibodies to TNFR1 (p55, TNFRSF1A) and TNFR2 (p75, TNFRSF1B) and conducted in separate experiments to neutralize the secretion of TNF-alpha and its receptors from TPA-HL-60 cells. Originally 1×10^6 cells/well were seeded in 6-well plates and treated with 16nM TPA for differentiation into macrophage like cells and end of the treatment TPA-HL-60 cells were treated as described below. No treatment TPA-HL-60 cells were kept as a control. There were other individual treatments gp120 1nM alone, TNF-alpha antibody alone, TNF-alpha and gp120 1nM concentration, incubated for 24 hours for the TNF-alpha neutralization experiment. For the TNF-alpha receptors type I & II neutralization experiment, both the antibodies were used at a concentration of 5 μ g/ml

from the stock solution. TPA-HL-60 cells were treated with TNFR1 antibody alone, TNFR2 antibody alone, antibodies to TNFR1 and TNFR2, gp120 1nM alone, gp120 + antibody to TNFR1, gp120 + antibody to TNFR2, gp120 + antibodies to both TNFR's respectively. After 24 hours incubation, the supernatant was collected and cells were harvested for RNA isolation and RT-real time PCR to check the MOR expression as described previously.

Statistical analysis

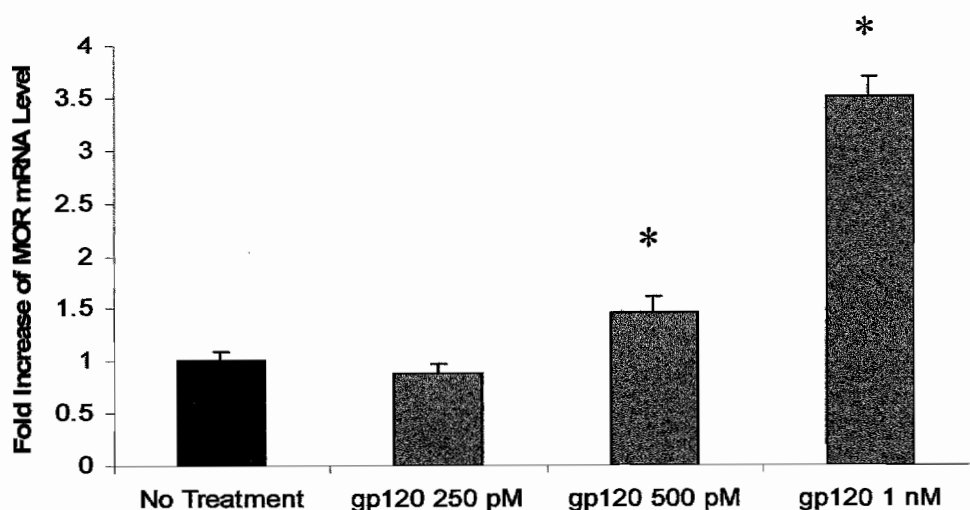
Data are presented as the mean \pm SE. The data was analysed by prism soft ware. Comparisons of data were made by one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test. Significance was assessed at the either the 95% ($p < 0.05$) or 99% ($p < 0.01$) confidence level. Significance was defined as a probability of 0.05 or less.

RESULTS

Gp-120 up-regulation of MOR mRNA in TPA- HL-60 cells

Real time Reverse transcriptase polymerase chain reaction was used to determine the presence of the MOR mRNA in TPA-differentiated HL-60 cells with and without treatment of gp120 protein. In order to observe the MOR mRNA levels, mu-opioid receptor specific primers and a TaqMan probe were designed to amplify a fragment of the coding region of the mu opioid receptor. Using the RT-real-time PCR, a standard curve plot was generated for the known concentration of the mu-opioid receptor gene and unknown treated samples were analyzed through the standard curve. Real time RT-PCR analysis of 1 nM gp120 treated TPA-differentiated HL-60 cells demonstrates a significant increase relatively 4 folds, in mu receptor gene expression when compared to the control treatment. 500 pM gp120 also showed significant 1.3 folds increased MOR expression when compared to the control. However the TPA-HL-60 cells treated with 250 pM gp120 didn't show significant difference compared to the control one (Figure 1a). In order to see the upregulation of MOR was concentration dependent, we treated the TPA-HL-60 cells with 40nM gp120. We observed that the upregulation of MOR was increased significantly 5.5 folds higher than control (Figure 1b).

(a)



(b)

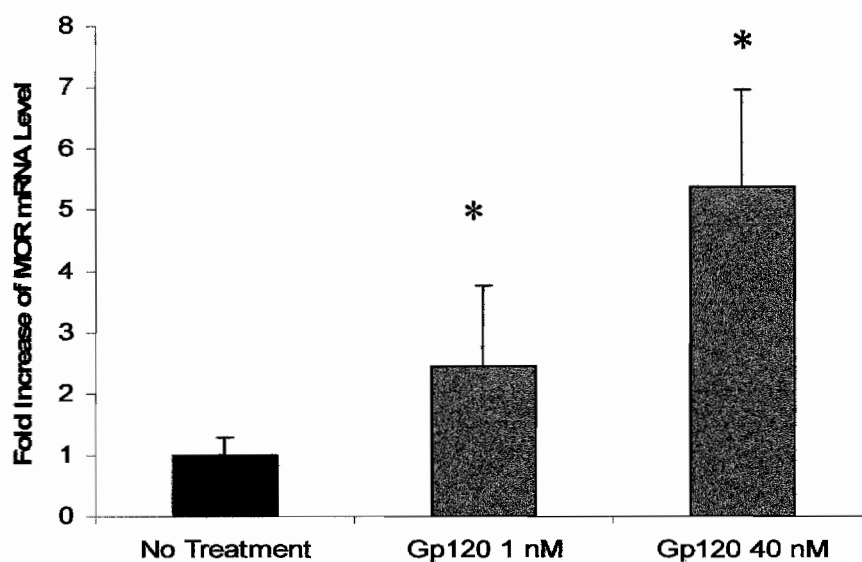


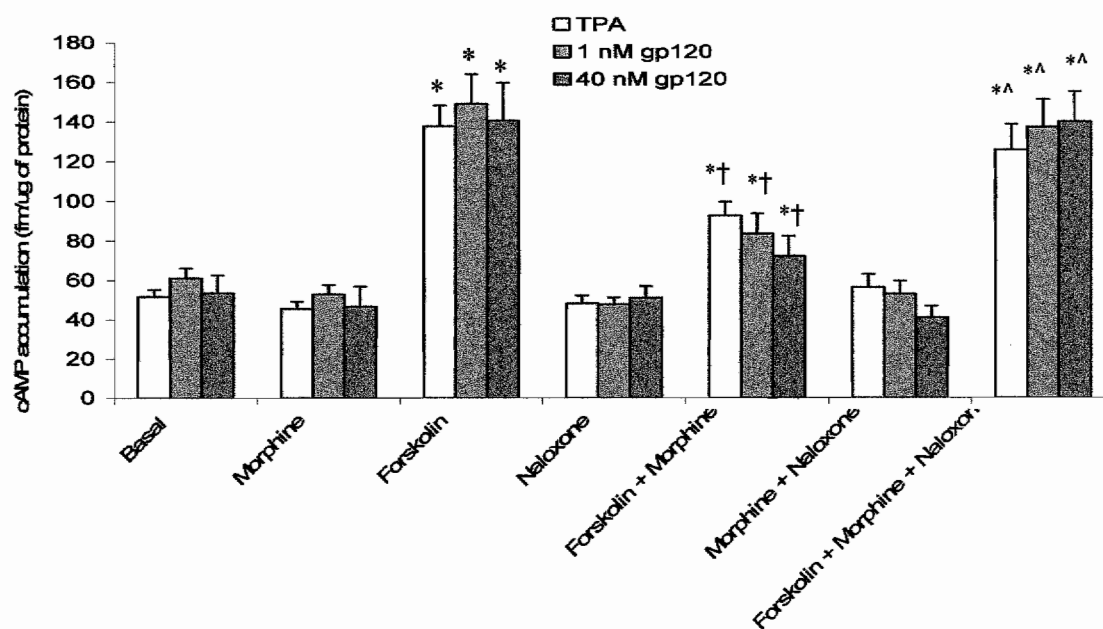
Figure 1: (a) Effect of HIV-1 gp120 protein on mu-opioid receptor expression in TPA-differentiated HL-60 cells. Mu-opioid receptor expression was determined by real-time RT-PCR. TPA-HL-60 cells were treated with 250 pM, 500 pM, 1 nM gp120 for 24 hours and cDNA was amplified through real-time-PCR and the results were normalized with internal control GAPDH and data was analysed statistically. There was significant increase in the MOR expression in 500 pM and 1 nM gp120 treatments compared to the control. (b) Up-regulation of the MOR in TPA-HL-60 cells is gp120 concentration dependent. TPA-HL-60 cells have shown MOR mRNA upregulation with 40 nM gp120 treatment which 5.5 fold significant difference with the control.

Morphine inhibition of forskolin induced cAMP levels in gp120 treated TPA-HL-60 cells.

The opioid receptors have been shown to stimulate an inhibitory G protein that suppresses the activity of adenylyl cyclase whenever they get activated, further resulting in the decreased amount intracellular cAMP levels. The MOR gene contains a cAMP response element; therefore, induction of intracellular levels of cAMP would result in an alteration in MOR mRNA expression (Lee *et al.*, 2005). To determine if there was a change in adenylyl cyclase activity which catalyzed *de novo* synthesis of cAMP in gp120 treated TPA-differentiated HL-60 cells, forskolin-stimulated cAMP accumulation experiments were performed to measure intracellular cAMP levels by using the morphine, MOR agonist.

Gp120 treated TPA-HL 60 cells has shown increased cAMP levels with forskolin treatment compared to basal levels, whereas morphine alone and Naloxone alone did not alter cAMP levels. However, exposure to morphine down-regulated forskolin-stimulated cAMP production and this down-regulation was reversible as shown by co-treatment with morphine and naloxane in the presence of forskolin, a morphine antagonist. MOR gene was functional in gp120 treated TPA-HL-60 cells. Naloxone reversed the morphine inhibition of cAMP levels in gp120 concentration dependent manner. The percentage inhibition of forskolin to forskolin with morphine has significant difference in gp120 1 nM and 40 nM when compared to the basal (TPA-HL-60 cells) [Figure 2a and Figure 2b]

(a)



(b)

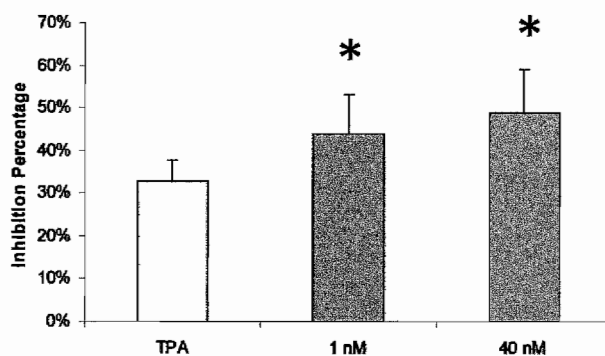


Figure 2: (a) cAMP assay was performed to test the functionality of the MOR gene by incubating the TPA-HL-60 cells with morphine, forskolin and naloxone alone, and forskolin with morphine, morphine with naloxone and forskolin, morphine and Naloxone for 10 minutes. One way ANOVA was performed. ** $p < 0.001$ forskolin alone compared with basal level ; †† $p < 0.001$ forskolin with morphine compared with forskolin alone; ^^ $p < 0.001$ compared with forskolin and Morphine followed by naloxone treatment. (b) Percentage inhibition of cAMP in forskolin and morphine treated sample when compared to TPA-HL-60 cells alone. Inhibition was significant in 1 nM and 40 n M gp120 treated TPA-HL-60 cells.

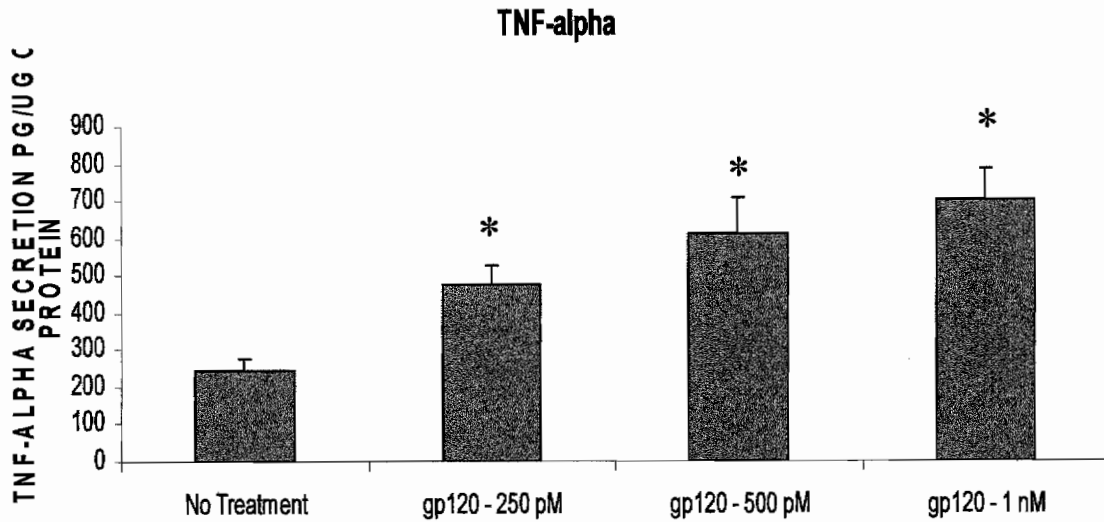
Cytokine induction in gp120 treated TPA-differentiated HL-60 cells

TPA-differentiated HL-60 cells were treated with different concentration of gp120 250, 500 pM, and 1 nM for 24 hours, and supernatant was collected and assayed for Pro-inflammatory cytokines TNF-alpha and IL-1 β .

TNF-alpha secretion levels were increased by gp120 treatment when compared to the control, which were TPA-HL-60 cells. There was a significant increase in 250 pM ($p < 0.01$), 500 pM ($p < 0.001$), 1n M ($p < 0.001$) of gp120 treatments (Figure 3A). However there was significant difference in gp120 250 pM treatment with that of 500 pM and 1 nM gp120. How ever, there was no significant difference in between 500 pM and 1 nM gp120 treatments.

IL-1beta cytokine secretion levels also increased and there was a significant difference increase when compared to the control (No treatment) to gp120 250 pM ($p < 0.001$), gp120 500 pM ($p < 0.001$), gp120 1nM ($p < 0.001$) treated samples. So HIV-1 gp120 protein induces the cytokine secretion of TNF-alpha and IL-1beta from TPA-differentiated HL-60cells.

(a)



(b)

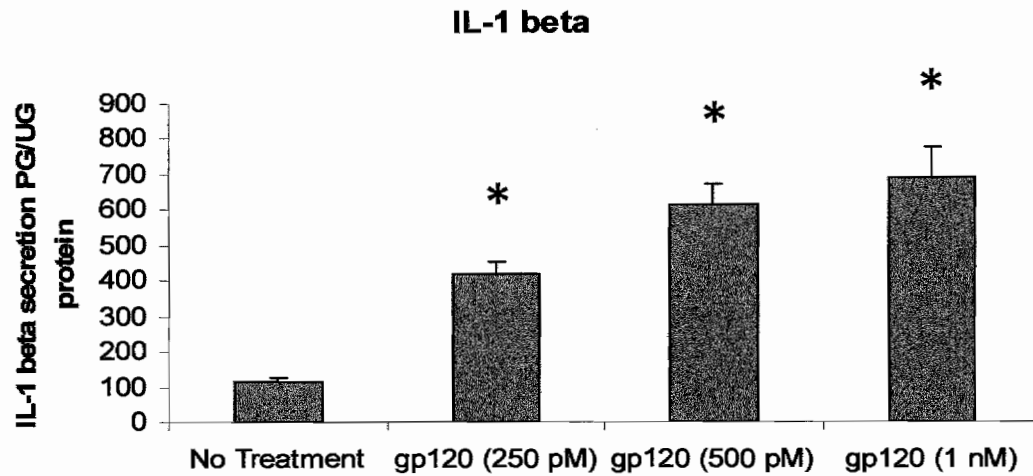


Figure 3 a & b: Effect of dose dependent concentrations of gp120 treatment on TNF-alpha and IL-1beta cytokine level secretions. TPA-HL-60 cells(1×10^6 /well) were cultured in 6 well plates with 20% FBS and 1% penicillin and after the differentiation the TPA-HL 60 cells were treated with gp120 250 pM, 500 pM and 1 nM incubated for 24 hours and supernatant were assayed for TNF-alpha and IL-1beta cytokine levels through ELISA. Gp120 treated (250 pM, 500 pM and 1 nM) TPA-HL-60 cells has shown the significant increase in the TNF-alpha cytokine levels secretion ($p < 0.002$) and IL-1 beta ($p < 0.001$) when compared to the control

TNF-alpha induces the up-regulation of the MOR mRNA in TPA-HL-60 cells

According to the Kraus et al, 2003, it was evident that TNF-alpha involved in regulation of mu opioid receptor gene in various immune effector cells. So we tested the MOR upregulation at mRNA levels by treating the TPA-HL-60 cells with TNF-alpha alone with different concentration of 50, 100, 200 µg/ml and cells were incubated for 24 hours. Next day cells were harvested for total RNA isolation and processed for RT and Real time PCR for MOR mRNA regulation levels as described in the methods section. The untreated TPA-HL-60 cells served as a control. The TPA-HL-60 cells were treated with TNF-alpha, significantly increased MOR mRNA levels in a dose dependent fashion when compared to the control (Figure 4).

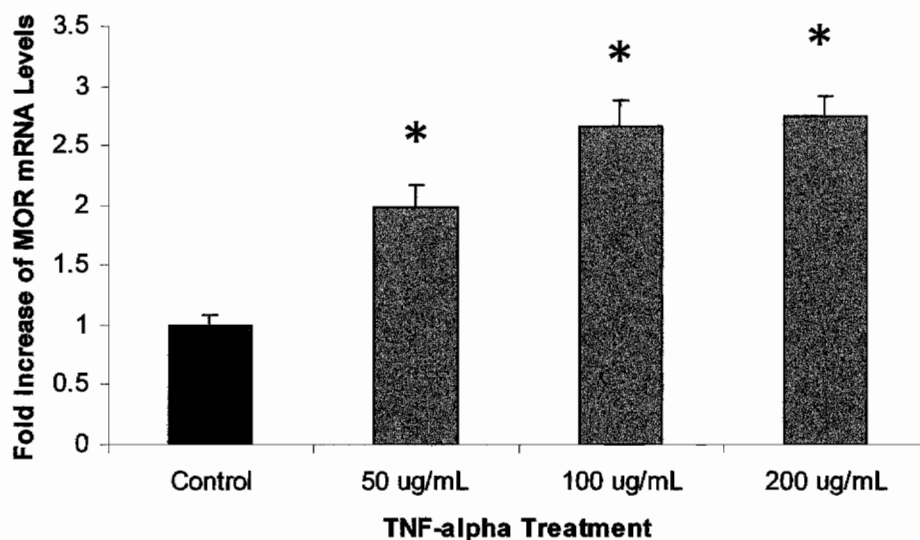


Figure 4: Up-regulation of the MOR is TNF-alpha concentration dependent. TNF induces mu-opioid receptor transcription in gp120 treated TPA-HL-60 cells. TPA-HL-60 cells were treated with 50, 100 and 200 $\mu\text{g/ml}$ concentration of TNF-alpha and cells were incubated for 24 hours and tested for MOR mRNA regulation levels with and without the treatment of TNF-alpha. RNA and cDNA were prepared and subjected to real-time RT-PCR. The relative amounts of mu-opioid receptor mRNA at the different dose dependent treatment was plotted after normalization to equal GAPDH mRNA levels.

Neutralization of secreted TNF-alpha and its effects on MOR regulation

We observed that TNF-alpha alone can regulate the MOR mRNA levels at dose dependent treatment, in TPA-HL-60 cells which was analysed through real time PCR . Therefore we tested the MOR regulation when the TPA-HL-60 cells were treated with TNF-alpha antibodies to neutralize its secretion and the further consequence results in MOR regulation. TPA-HL-60 cells were treated with gp120 1n M alone, TNF-alpha antibody alone and gp120 1nM and TNF-alpha antibody to see the MOR regulation at mRNA level. Data was analysed and Normalized with GADPH internal control values and results were plotted. When the cells were treated with gp120 1n M alone has increased the MOR mRNA upregulation when compared to the untreated TPA-HL-60 cells and TNF-alone was not able to upregulates the MOR. However, there was a significant decrease in the MOR mRNA upregulation of TPA-HL-60 cells treated with both gp120 and TNF-alpha antibodies when compared to the gp120 1nM. This result indicates that neutralization of secreted TNF-alpha diminishes gp120 up-regulation of the MOR in TPA-HL-60 cells (Figure 5)

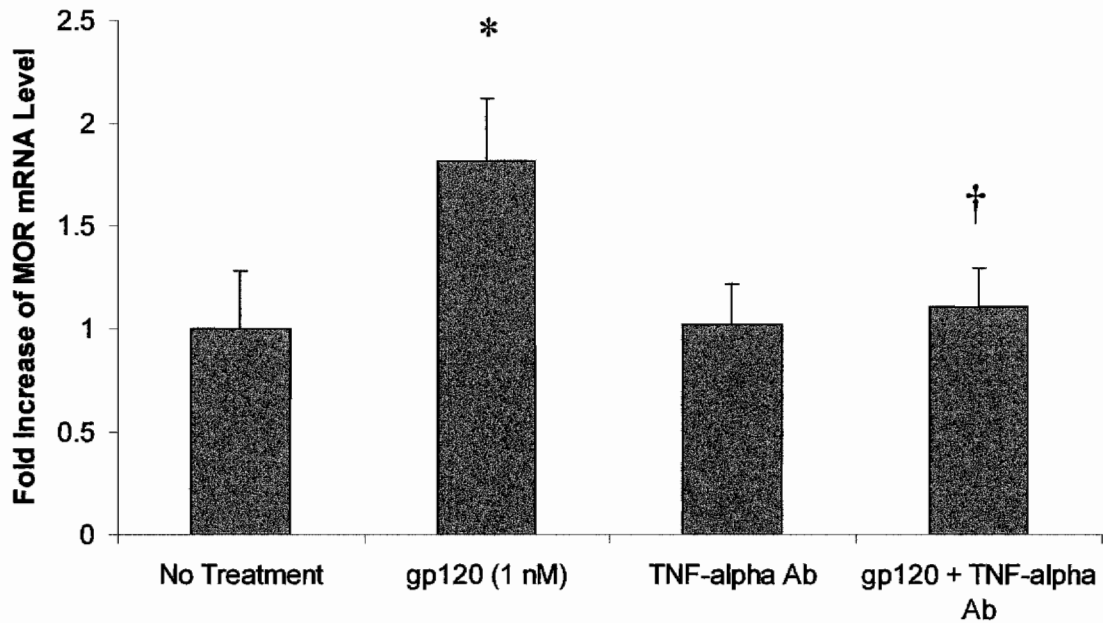


Figure 5: Neutralization of secreted TNF-alpha diminished the upregulation of the MOR expression at mRNA level in the gp120 treated TPA-HL-60 cells. TPA-HL-60 cells treated with gp120 1 n M alone, TNF-alpha antibody alone (1:500 dilution from the original stock solution) and gp120 along with antibody to TNF-alpha and incubated for 24 hours. After the incubation cells were processed for RT and real time PCR for MOR expression. * >Gp120 treated cells alone have shown upregulation the MOR at transcriptional level. Antibody to TNF-alpha did not show any significant difference in the expression when compared to control. † > indicates that gp120 and antibody to TNF-alpha has shown diminished expression of MOR when compared to the gp120 treatment alone.

TNFR2 mediates induction of mu-opioid receptor transcription in TPA-HL-60 cells.

Transcriptional induction of mu-opioid receptor gene was mediated through cytokine tumor necrosis factor alpha and was proved through the neutralization of TNF-alpha experiment. It is known that TNF-alpha exerts through its action by binding to the two cell surface receptors p55 and p75 TNF-receptors. It is also evident that TNF-alpha triggers the MOR regulation through TNFR-II during the inflammatory process (Kraus *et al.*, 2003). We wanted to see through which receptors the TNF-alpha exerting its effects.

Neutralizing antibodies against TNFR1 (p55, TNFRSF1A) and TNFR2 (p75, TNFRSF1B) were used to determine the TNFR by which the effect on mu-opioid receptor gene transcription is mediated (Fig. 6). TPA-HL-60 cells were treated with gp120 1nM alone, TNF-alpha receptor 1 antibody and TNF-alpha receptor 2 antibody alone, TNFR I plus II, gp120 with TNFR-1 antibody, gp120 with TNFR-II antibody and gp120 with both receptor antibodies. Gp120 treated TPA-HL-60 cells has shown MOR mRNA upregulation and antibodies to TNF-receptors alone did not have any effect on the upregulation of the MOR. Moreover, the addition of TNFR1 antibodies to the cells treated with gp120 1nM had no diminished effect on MOR upregulation. In contrast, mu-opioid receptor upregulation at the transcriptional level was diminished by TNFR2 antibodies when the cells were treated with gp120, suggesting that the TNF effect is mediated solely via the TNFR2 (Figure 6).

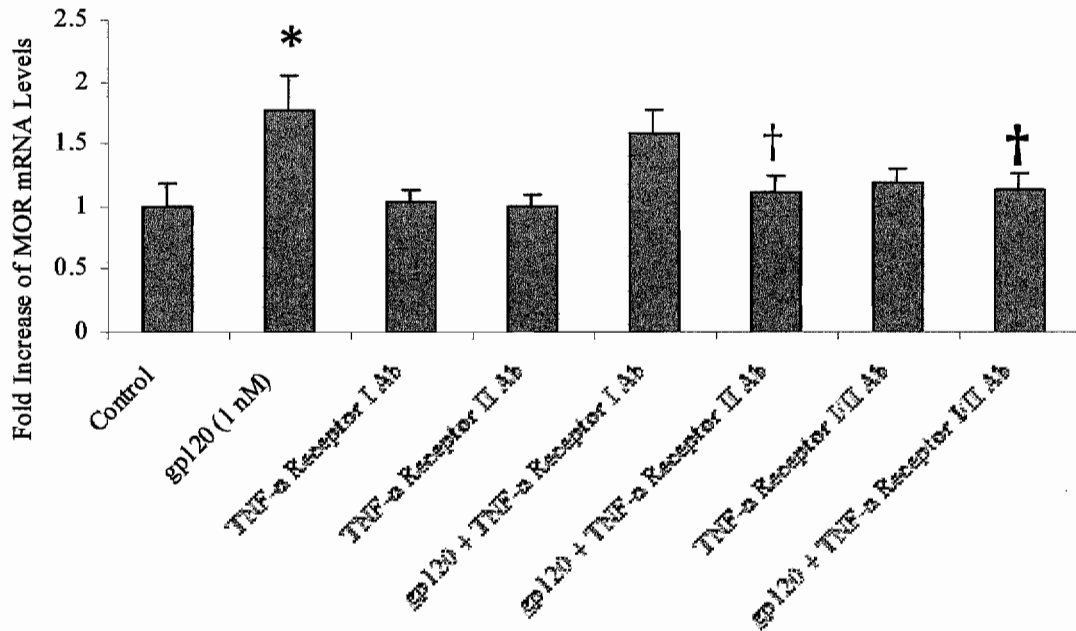


Figure 6: Neutralization to TNFRII diminished gp120 up-regulation of the MOR in TPA-HL-60 cells. TNF-induction of mu-opioid receptor transcription in TPA-HL-60 cells was mediated by TNFR2. TNFR1 antibodies have no effect on TNF-induced Mu-opioid receptor transcription. TPA-HL-60 cells were treated with TNFR1 and II, incubated for 24 hours with the indicated amounts of TNFR antibodies. Cells were harvested for RNA extraction and RT-real time PCR. The results were plotted after normalization with GADPH. TNFR2 antibodies block TNF-alpha induced mu-opioid receptor transcription in gp120 treated TPA-HL-60 cells.

DISCUSSION

Although Opioids are known to possess analgesic properties, they were abused for many recreational reasons. Mu opioid receptors (MOR) are high affinity binding sites for the opioids such as morphine and methadone and may alter the immune functions through mu-opioid receptors on immune cells. In total, one third of the HIV patients abuse the opioids and the chances disease progression will be higher in these patients as the immune functions are much more altered and these people are at a greater risk of developing opportunistic pathogenic infections than non-opiate users of HIV (Mahajan *et al.*, 2005).

In this study, we first showed that there was increased MOR expression in TPA-differentiated HL-60 cells. TPA differentiates the Human promyelocytic leukemic cells (HL-60) into macrophage like cells. Even though undifferentiated HL-60 cell lines have CD4 expression, but no change in the HIV infection productivity without differentiation into macrophages (Kitano *et al.*, 1990). Previous studies indicated that chronically infected promonocytic U1 cell line used was a model for HIV expression and this function mainly involved with synergistic functions of the cytokines and macrophage differentiating agent such as phorbol- 12 myristate- 13 acetate (PMA) (Goletti *et al.*, 2002). Differentiating agents mimic the several features of the infected macrophages such as accumulation of the virions intracellular budding like that of infected macrophages. It had been evident that differentiated macrophage cells have mu opioid receptors (Makman, 1994). In the present study it has been established that gp120 dose

dependent concentration study i.e 250 pM, 500 pM and 1 nM and 40 nM had upregulated the mu opioid receptor at mRNA level significantly 3 to 5.5 folds when compared to the control, indicating that gp120 involved in the upregulation of the mu opioid receptors.

We then tested the functionality of the Mu opioid receptor which was upregulated in TPA- differentiated HL-60 cells after gp120 treatment. The MOR is a G-protein coupled seven transmembrane receptors and mediates the intracellular actions such as stimulation of potassium-channel conductance (Williams *et al.*, 1988) inhibition of calcium channel conductance (Hescheler *et al.*, 1987) and also inhibition of adenylyl cyclase activity. (Yu and Sadee, 1988). Based on the studies, it has been indicated that acute stimulation of opioid receptors activates the inhibitory protein, G α i, which results in the acute inhibition of adenylyl cyclase and reduction in the cyclic AMP production (Sharma *et al.*, 1975). This cAMP acts as second messenger for MOR agonists such as Morphine (Law *et al.*, 1981). Additionally the MOR gene also contains the various binding regions at the promoter regions of DNA including for cAMP responsive elements. This suggests that MOR gene transcription would be influenced by cAMP levels (Min *et al.*, 1994)

Intracellular c AMP was not easily detectable as it was degraded by phosphodiesterase enzyme(Daniel *et al.*, 1998). Therefore, we used IBMX and Forskolin, to prevent the hydrolysis of cAMP to AMP and an activation of adenylyl cyclase in order to increase the accumulation of cAMP in the cells respectively (Chowdhury *et al.*, 1993). In this study, we used the Morphine as a MOR agonist in order to see the reduced

cAMP production which was catalyzed by adenylyl cyclase and these effects could be reversed by the non selective mu opioid receptor antagonist, Naloxone. TPA-differentiated HL-60 cells (basal) did not show the increased cAMP levels. However forskolin treatment increased the cAMP levels and it was significant in 1 and 40 nM gp120 treated TPA-HL-60 cells, but Morphine and naloxone treatments alone did not alter the cAMP levels. Moreover, forskolin treated gp120 TPA-HL-60 cells and TPA-HL-60 cells has shown down regulated cAMP levels after exposure to morphine and this down regulation was reversed by co-treatment with naloxone. It was clearly established that morphine inhibition of cAMP levels was gp120 concentration dependent, and hence MOR gene was functional.

Human immunodeficiency virus type-1 virus causes major disturbances to the cytokine network because of the sustained immune activation of the immune cells and results in the production of major inflammatory cytokines including the TNF-alpha and IL-1beta from the monocyte/macrophages. TNF-alpha and IL-1 beta were selected for cytokine level secretion study because of the potential importance in relation to the the HIV- macrophage interaction. Moreover studies proved that multiple cytokines can affect the HIV replication in acute and chronic HIV infected T lymphocytes or monocytic cell lineage (Goletti *et al.*, 2002). We tested the presence of cytokine levels in the supernatant samples in a dose dependent fashion with gp120 treatment in TPA-HL-60 cells. The ELISA results showed that treatment of gp120 protein can induce differential cytokines including TNF-alpha and IL-1beta and we observed that there was significant increase in the secretion of cytokines in a dose dependent fashion when compared to the

control cells. Studies indicated that IL-1beta secretion involved in the upregulation of the mu opioid receptor expression (Vidal *et al.*, 1998) and also functions as a modulator for the chemokine expression which could lead to increase in the HIV-viral replication (Goletti *et al.*, 1996; Boven *et al.*, 2000). However the mechanism of modulation of the mu-opioid receptors at the transcriptional level by IL-1 beta is still unclear. But the studies indicated that mu opioid receptor gene has cytokine response element and the increased mu opioid expression is due to the increased stability of the RNA transcript (Ruzicka *et al.*, 1996). IL-1 beta is known to increase the severity of the CNS infection, trauma and injury. From this, it has been clearly understood that cytokines play important role in the modulation of the opioid receptors. Furthermore, we can study the possible synergistic effects of the pro-inflammatory cytokines such as TNF-alpha and IL-1 beta on the mu opioid upregulation. We can also delineate the possible effects of the IL-1 beta expression during the HIV-1 virus infection and its role in the AIDS pathogenesis.

Macrophages function as a first line of defense whenever there is an infection and also serve as a reservoir for HIV-1 virus. Macrophages synthesize and secrete the major inflammatory cytokine TNF-alpha. Studies indicate that MOR is involved in the modulation of TNF-alpha synthesis (Roy *et al.*, 2001). TNF-alpha is known to act through the autocrine or paracrine fashion once it is secreted after numerous stimuli to macrophages such as LPS and known to be involved in the regulation of NO release and also in the IFN-gamma production (Calder *et al.*, 2005). Studies also indicated that TNF-alpha involved in the regulation of most vital body functions including the modulation of the mu opioid receptor gene at transcriptional level during the inflammatory process in

neuronal cells (Kraus *et al.*, 2003). In this study, we observed that recombinant TNF-alpha involved in the regulation of potent physiological MOR gene at transcriptional level and results established that upregulation of MOR mRNA was seen at dose dependent treatment of TNF-alpha in the TPA-HL-60 cells. Figure 4, clearly presented that TNF-alpha involved in the modulation of MOR at transcriptional level which was again confirmed by RT-real time PCR.

According to the Kraus *et al.*, 2003, MOR upregulation was mediated through TNF-alpha secretion. Figure 5 suggested that neutralization of secreted TNF-alpha secretion diminished the upregulation of MOR mRNA level in gp120. However such diminished effects were not found in the gp120 treated TPA-HL-60 cells, indicating that TNF-alpha was involved in the modulation MOR at transcriptional level.

TNF-alpha mediates its effect through its receptors P55 TNFR1 and p75 TNFR2. Studies indicated that TNF-alpha mediates the regulation of MOR transcriptional level through TNFR2 during the inflammatory process. Therefore, we wanted see which receptor was really involved in the regulation of MOR process. In the present study, we applied the antibodies to both TNF-alpha receptors to see the diminished effects of the MOR regulation. However the antibody to TNFR2 blocked the TNF-alpha induced mu-opioid receptor transcription in gp120 treated TPA-HL-60 cells. But the antibodies to TNFR1 did not have any effect. These results clearly indicate that TNF-alpha mediates its effects through TNFR2. From this experiment, it has been clearly established that TNFR2 playing a critical role in the upregulation of the mu opioid receptor mRNA.

Blocking of the TNFR2 probably would help in controlling TNF-alpha associated pathogenesis also.

The present study could also give us a mechanism of TNF-alpha involved in the disease progression of HIV or AIDS by increasing the concentration of mu opioid receptors, especially in the HIV-opioid intravenous drug users (IVDUs) and opioid drug abusers.

SUMMARY

In summary, our study showed that recombinant HIV-1 virus glycoprotein gp120 increased the expression of MOR at mRNA level in TPA-differentiated HL-60 cells. The expressed MOR gene was also confirmed by the morphine inhibition of forskolin stimulated cAMP levels in a concentration dependent manner, which was reversible by naloxone, a MOR receptor non selective antagonist. Gp120 was also involved in the induction of pro-inflammatory cytokines including the TNF-alpha and IL-1 beta. TNF-alpha mediated the regulation of MOR gene at transcriptional level in dose dependent fashion. Neutralization of secreted TNF-alpha diminished the gp120 upregulation of MOR. TNF-alpha mediates its effects through its receptors expressed on the cell surface. Neutralization of the TNF-alpha receptors especially TNFR2 diminished the upregulation of gp120 induced MOR expression in TPA-HL-60 cells, suggesting that TNFR2 mediates the induction of MOR transcription in differentiated HL-60 cells. Taking all this into consideration, our study clearly demonstrated that gp120 up-regulation of the mu opioid receptor (MOR) in TPA-HL-60 cells may be mediated via the autocrine/paracrine actions of TNF-alpha released from the TPA- HL-60 cells in response to treatment with gp120. The present study also contributes to the understanding of the interaction between the TNF-alpha and its role of modulation in the mu opioid receptors, clearly suggesting the role of TNF-alpha in the physiological and pathological regulatory circuits.

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