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REGULATION OF RESISTANCE TO HIV-VPR INDUCED APOPTOSIS

By

Sasmita Mishra

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Abstract

Apoptosis of CD4+ T cells and their eventual depletion constitute a hallmark of HIV infection and disease progression. However, monocytes/macrophages unlike CD4+ T cells survive HIV replication and hence represent a major reservoir of virus. The mechanism underlying this resistance to HIV-mediated apoptosis in monocytic cells is not clear. It is believed that TNF-α which is produced during HIV infection may play a major role in inducing resistance in monocytes. In this study I investigated the molecular mechanisms involved in regulation of resistance to HIV-Vpr induced apoptosis in monocytic cells.

LPS and LPS induced TNF-α are known to induce resistance to monocyte apoptosis. However, the molecular mechanism regulating this process is not known. c-IAP2, one of the anti-apoptotic genes is known to be induced in monocytes in response to LPS. First of all, I demonstrated that LPS and TNF-α induced survival of human monocytic cells through the induction of the c-IAP2 gene. Further, I studied the involvement of MAPKs and PI3K signaling pathways in c-IAP2 induction by using specific pharmacological inhibitors. My results suggest that neither MAPKs nor PI3K is involved in c-IAP2 induction. Since calcium signaling pathway is considered to be one of the upstream signaling molecules in signaling cascade, I investigated its involvement in c-IAP2 induction in monocytic cell line. I showed that binding of LPS/TNF-α to their respective receptors induces calcium influx. Calcium is also known to activate calmodulin (CaM) which in turn activates various kinases and phosphatases. My results revealed that activation of CaM and CaM kinase II (CAMKII) is involved in c-IAP2 induction, whereas calcineurin, the protein phosphatase activated by CaM is not involved in this process. I have shown for the first time that LPS/TNF-α induced c-IAP2 and associated anti-apoptotic activity is regulated by CaM/CAMKII through the activation of NFκB.

Since TNF-α is believed to be involved in inducing resistance to HIV induced apoptosis in monocytes, further I used Vpr, one of the HIV proteins to induce apoptosis in monocytes. My results showed that HIV-Vpr induces apoptosis in monocytes and pretreatment of cells with either LPS/TNF-α induces resistance to Vpr mediated apoptosis. To determine the molecular mechanisms involved in Vpr induced apoptosis, I used the C-terminal synthetic peptide (Vpr52-96) which mimics the whole Vpr protein for induction of apoptosis. I also used the N-terminal (Vpr1-45 aa) peptide as control. I demonstrated that both the C-terminal and N-terminal peptides phosphorylate all the three MAPKs such as p38, ERK, and JNK MAPK; however, only C-terminal (Vpr52-96) peptide induced apoptosis is regulated selectively by JNK MAPK. To determine the involvement of pro- and anti-apoptotic genes in Vpr induced apoptosis, RNAse protection assay was performed. My results revealed that in response to Vpr52-96, there was downregulation of Bcl2, whereas other pro and anti-apoptotic genes of Bcl2 family remained unchanged. I also demonstrated the involvement of c-IAP1, one of the inhibitor of apoptotic proteins (IAPs) in Vpr induced apoptosis. My results show that Vpr peptide-

induced apoptosis is mediated by down regulation of anti-apoptotic Bcl2 and c-IAP1 genes through JNK MAPK activation. Deregulation of the JNK pathway has been implicated in cancer and other diseases. Therefore, investigation of the molecular mechanisms that govern the role of the JNK pathway in apoptosis should provide insight into its biological functions and strategies to target this pathway for prevention and treatment of human diseases and cancer.

Finally, I demonstrated that pretreatment of cells with LPS or TNF-α induced resistance to Vpr52-96 mediated apoptosis. My results suggest that LPS induced resistance is mediated by endogenous production of TNF-α in monocytes. I also demonstrated that LPS/TNF-α mediated resistance is regulated by induction of c-IAP2 through NFκB by activation of CAMKII. However, pretreatment with Vpr followed by LPS or TNF-α stimulation no longer gave protection to Vpr induced apoptosis. Vpr peptide also inhibited LPS/TNF-α-induced calcium influx, activation of CAMKII, and c-IAP2 induction. Taken together, my results suggest that the c-IAP2 gene plays a critical role in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. Since the calcium/CAMKII pathway is involved in LPS/TNF-α induced resistance to Vpr mediated apoptosis, strategies based on manipulation of these molecules, which would suppress c-IAP2 induction may be useful in clearing virus reservoirs in monocytes.

Dedicated to

To my son, whose unbelievable strong wishes enable me to finish my work;

To my husband for his constant inspiration and cooperation throughout the study;

&

To my supervisor (Dr. Ashok Kumar), whose constructive suggestion and advice never made me look back.

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List of Abbreviations

$\Delta\psi m$	Transmembrane potential
$[Ca^{2+}]_i$	Intracellular calcium ions
aa	Amino acid
Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocator
Apafl	Apoptotic protease activating factor 1
ATP	Adenine triphosphate
Bad	Bcl2 associated death promoter
Bak	Bc-2 antagonistic killer
Bax	Bcl2-associated X protein
bp	Base pair
Bcl2	B cell follicular lymphoma
BclXL	Bcl2-X long form
Bid	Bcl2 interacting domain
Bim	Bcl2 interacting mediator
BIR	Baculoviral inhibitory repeat
BH	Bcl2 homology
CAD	Caspase activated deoxyribonuclease
CaM	Calmodulin
CAMKII	Calmodulin kinase II
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruitment domain
Caspase	Cysteine aspartate proteases
Cdc2	Cyclin dependent kinase
cGMP	Cyclic guanosine monophosphate
c-IAP1	Cellular inhibitor of apoptosis1
c-IAP2	Cellular inhibitor of apoptosis2
cDNA	Complementary DNA
cpm	Counts per minute
CTL	Cytotoxic T lymphocyte
Cyt-c	Cytochrome-c
DD	Death domain
DED	Death effector domain
DXM	Dexamethasone
Endo G	Endonuclease G
ERK	Extracellular-related kinase
Fas	Fibroblast associated
FADD	Fas associated death domain
GM-CSF	Granulocyte macrophage – colony stimulating factor
GTP	Guanosine triphosphate
HAX1	Hematopoietic lineage cell specific protein 1- associated protein X-1

HIV	Human immunodeficiency virus
HRPO	Horseradish peroxidase
IAPs	Inhibitors of Apoptotic Proteins
ICAD	Inhibitor of caspase activated deoxyribonuclease
IFN	Interferon
IL	Interleukin
IM	Inner membrane
JAK	Janus associated kinase
JNK	c-jun N-terminal kinase
Kb	Kilobase
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	Monoclonal antibody
MALT	Mucosa associated lymphoid tissues
MAPK	mitogen-assiociated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MAVS	Mitochondrial anti viral signaling protein
Mcl1	Myeloid leukemia cell differentiation protein
MEK	MAP kinase/ERK kinase
MMP	Mitochondrial membrane permeabilization
mRNA	Messenger RNA
NFκB	Nuclear factor κB
NK	Natural killer
NOXA	PMA-induced protein
NPC	Nuclear pore complex
nt	Nucleotides
OM	Outer membrane
PARP	Poly-(ADP-ribose)-polymerase
PBMC	Peripheral blood mononuclear cells
PI3K	Phosphatidylinositol 3 kinase
PIC	Preintegration complex
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinase
PTPC	Permeability transition pore complex
PUMA	p53- upregulated modulator of apoptosis
RPA	RNase protection assay
RT-PCR	Reverse transcription-polymerase chain reaction
SH2	Src-homology 2 domain
SEK	Stress activated protein/ERK kinase
Smac	Secondary mitochondrial activator of caspases
SOCS	Suppressor of cytokine signaling
SODD	Silencer of death domain
Th	T helper
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-β

TRADD	TNF receptor-associated death domain
TRAF	TNF-receptor associated factor
TRAIL	TNF-related apoptosis inducing ligand
VDAC	Voltage dependent anion channel
Vpr	Viral protein R
Vpr52-96	Vpr peptide containing 52-96 amino acids
Vpr1-45	Vpr peptide containing 1-45 amino acids
WHO	World health organization
XIAP	X linked inhibitor of protein
ZVAD	Z-Val-Ala-Asp-CH_2F

Chapter I

General Introduction

Introduction:

Acquired Immunodeficiency syndrome (AIDS) is caused by human immunodeficieny virus (HIV), one of the viruses in the Retrovirus family [1]. It has been 25 years since AIDS was first identified and the latest report from WHO states that around 42 million people are living with HIV throughout the world (WHO, 2002). The main clinical features in HIV include an acute infection followed by a period of clinical latency which may last for 3-15 years before AIDS develop [2;3]. HIV primarily infects and replicates in immune cells such as T cells and monocytes/macrophages, which accounts for many of the major aspects of HIV pathogenesis [4].

The hallmark of AIDS is that HIV infects and kills CD4+ T cells, resulting in depletion of CD4+ T cells and eventually the patient dies of multiple infection and/or malignancies [5;6]. The major mechanism for CD4+ T cell depletion is apoptosis which can be induced by multiple pathways by various viral proteins [7]. Unlike T cells, monocytes/macrophages can harbor large quantities of the virus without being killed [8]. The factors involved in inducing resistance to HIV induced apoptosis in monocytes/macrophages are largely unknown. However, it is known that there is production of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) during HIV infection [9;10]. Since lipopolysaccharide (LPS) and LPS induced TNF-α prevent apoptosis in monocytes [10;11] and also induce anti-apoptotic genes such as cellular inhibitor of apoptotic protein (c-IAP2) [11], it is most likely that TNF-α is one of the factors inducing resistance to HIV induced apoptosis in monocytes/macrophages. Among various proteins involved in inducing apoptosis in HIV infection, Vpr plays an important role being packaged in great quantities into the virion nucleocapsid and is also expressed

late in the infection suggesting the importance of this protein throughout the viral life cycle [12;13]. Therefore, elucidating the molecular mechanisms involved in inducing resistance to HIV induced apoptosis in monocytes/macrophages may have broad implications related to host-pathogen interactions, viral persistence and survival of monocytic cells not only in HIV infection but also in infectious diseases caused by intracellular pathogens such as tuberculosis. For my Ph.D. research project, I have investigated the signaling pathways involved in HIV-Vpr induced apoptosis and the resistance induced by LPS in human monocytic cells. My results suggest that HIV-Vpr-induced apoptosis is mediated by down regulation of anti-apoptotic Bcl2 and c-IAP1 genes through JNK MAPK activation. Further, I demonstrated that the c-IAP2 gene plays a critical role in LPS and LPS induced TNF-α mediated resistance to HIV-Vpr-induced apoptosis through activation of calcium signaling pathways, particularly calmodulin kinase II (CAMKII) in human monocytic cells.

Background literature:

HIV and apoptosis:

The exact mechanism by which CD4+ T cells are destroyed from HIV infected patients remains unclear. Several hypotheses have been proposed to explain this decline including 1) direct infection of T cells and apoptosis [3]; 2) bystander cell killing by viral proteins [14;15]; and 3) dysregulated cytokine production [16].

1) Direct infection of T cells by HIV leads to apoptosis which accounts only a small percentage of affected cells [14;17]. There are several viral proteins including structural protein such as Env, and accessory proteins such as Tat, Vpr, Vpu, and Nef are involved in infected cell death [18;19]. HIV has the property of inducing membrane

fusion that leads to syncitia formation [20]. Syncitia have short life span resulting in release of live virus potential for further infection. Besides that, continuous budding is also another feature of HIV infection which disrupts the plasma membrane [21]. Vpu induced membrane permeabilization [22], whereas Tat [23], Nef [24] and Env [25] specifically enhanced Fas mediated killing. Vpr is considered to have pro- and anti-apoptotic effects in the cell. The anti-apoptotic effect which includes upregulation of Bcl2 plays a key role during early infection resulting in productive infection [26]. However, in the late stage of infection which is associated with more quantity of Vpr production induces apoptosis in T cells [27]. CD4+ T cells are also killed by HIV specific cytotoxic T lymphocytes (CTL) [28].

2) HIV infected cells are killed by various means. However, more number of uninfected cells are killed than infected cells during HIV infection [14;15]. There are two mechanisms which induce cell death in uninfected bystander cells 1) apoptosis by HIV proteins; and 2) activation induced cell death [29]. HIV proteins such as gp120, Tat, Nef, Vpr, and Vpu released into the intracellular space have been shown to induce apoptosis in uninfected cells [29]. Soluble and membrane bound gp120 induces cross linking of the CD4 molecules followed by apoptosis by Fas dependent and independent manner [30]. Tat is found to be secreted from infected cell and has been shown to induce apoptosis in uninfected neurons and Th cells by upregulating caspase 8, and FasL [31;32]. Vpr is found to be present in serum and CSF [33] and induces apoptosis in T cells, and neurons [34;35]. It is mentioned earlier that some of the uninfected cells are killed and eliminated by activation induced cell death. It refers to high induction of Fas and FasL by HIV

infection on the uninfected cell surface resulting in apoptosis [36;37]. However, induction of apoptosis by HIV in monocyte is not clear.

3) Cytokines produced during HIV infection also play an important role in pathogenesis by modulating apoptosis. There is a shift in cytokine response predominantly type I cell mediated (IFN-γ, TNF-α, and IL-12) to type II humoral immune response (IL-10) while HIV progresses [16]. It is presumed that type 1 cytokine is essential for cell survival in the early stage of infection whereas in the late stage of infection, type II cytokine helps in disease progression by inducing apoptosis.

Apoptosis in monocytes:

During the last several years, the main interest was focused on the tendency of peripheral blood lymphocytes to undergo apoptosis following HIV infection. However, other immune cell such as monocyte has received much less attention. This is because of the belief that monocytes survive during HIV infection which acts as a major reservoir of virus. However, there are few reports stating about monocyte apoptosis and their impaired function during HIV infection. Normally, during the process of apoptosis, monocytes/macrophages induce more production of anti-inflammatory cytokine IL-10 and decrease the production of inflammatory cytokines such as TNF-α and IL-12. This pattern of cytokine enhances monocyte/macrophage apoptosis [9]. However, in HIV infection, monocytes/macrophages do not undergo apoptosis most likely due to cytokines such as TNF-α, and nerve growth factor produced endogenously by HIV infection. It has been reported that TNF-α which is produced during HIV infection by monocytes and lymphocytes activates NFκB which in turn induces high level of HIV and TNF-α transcription [38]. This is supplemented with the evidence made by Wahl *et al.* where

they observed that coinfection of monocytes/macrophages with HIV and *Mycobacterium avium* facilitates HIV infection by multiple pathways [10]. *M. avium* activated NFκB which in turn activated TNF-α known to induce anti-apoptotic genes and confers cell survival. This hypothesis was supported by a recent study in which nerve growth factor was shown to act as an autocrine survival factor that rescued monocytic cells from the cytopathic effects of HIV [39]. Papasavvas *et al.* (2005) also demonstrated that exposure of monocytic cells to IL-13 significantly decreased spontaneous apoptosis [40].

Further, there is evidence to suggest that monocytic cells from HIV infected patients and *in vitro* infection of monocytic cell line U937 with HIV exhibit enhanced propensity to spontaneous apoptosis [41-43]. It has been observed that overexpression of BclXL, one of the anti-apoptotic genes induced resistance to HIV-1 mediated apoptosis and HIV-1 replication in U937 cells. Similarly, monocyte-derived macrophages obtained from HIV-infected patients express high levels of FasL that was correlated with high degree of apoptosis under *in vitro* as well as *in vivo* conditions in the lymphoid tissues [44]. It happened *in vivo* most probably due to switch in cytokine production. However, the mechanism involved in cytokine induced protection to HIV-Vpr mediated apoptosis is not clear. Manipulating these signaling molecules involved in resistance may help in inducing apoptosis in monocytes leading to clearance of HIV from reservoir.

Pathways of apoptosis:

Apoptosis (In Greek meaning falling leaves), a self directed process of cell death was first described by Kerr, Wyllie, and Currie in 1972 [45]. It is a strictly regulated and conserved mechanism that is used to remove unwanted cells during embryonic development, morphogenesis, immune system function and maintenance of tissue

homeostasis [46;47]. Dysregulation of cell death pathways has been implicated in various pathological conditions including cancer, autoimmune and immunodeficiency diseases and neurodegenerative disorders [48;49]. The morphological changes that occur in cells during apoptosis are loss of cellular contact from matrix, cytoplasmic condensation, chromatin compaction, cell shrinkage leading to apoptotic body formation (plasma membrane blebbing) and absence of inflammatory response [45;46]. Apoptotic cells also expose phosphatidyl serine to their cell surface which is otherwise hidden in the plasma membrane resulting in phagocytosis of apoptotic cells [45;46]. The biochemical changes include activation of specific kind of proteases such as cysteine aspartate proteases known as caspases and activation of endonucleases leading to fragmentation of DNA [50-52]. Although a number of stimuli appear to trigger this apoptotic process, there are two major pathways of apoptosis: extrinsic or the death receptor pathway and the intrinsic or mitochondrial pathway [53-55] (Fig.1.1). There is also evidence to suggest that these two pathways cross talk in certain cell types [56;57]. Both the pathways lead to the activation of a complex array of caspases. Initiator caspases are caspase 2, 8, 9, and 10; whereas effector caspases are caspases 3, 7, and 6 [57;58]. Since initiator caspases are able to self activate and activate the downstream effector caspases, single apoptotic trigger is sufficient to cause apoptosis.

Extrinsic Pathway of Apoptosis:

The receptor mediated pathways are initiated by binding of death receptors such as fibroblast associated (Fas), tumor necrosis factor receptor (TNF-R) and TNF-related apoptosis inducing ligand receptor (TRAIL-R) to their respective ligands [53;57;59]. These proteins are integral membrane proteins with their receptor domains exposed at the

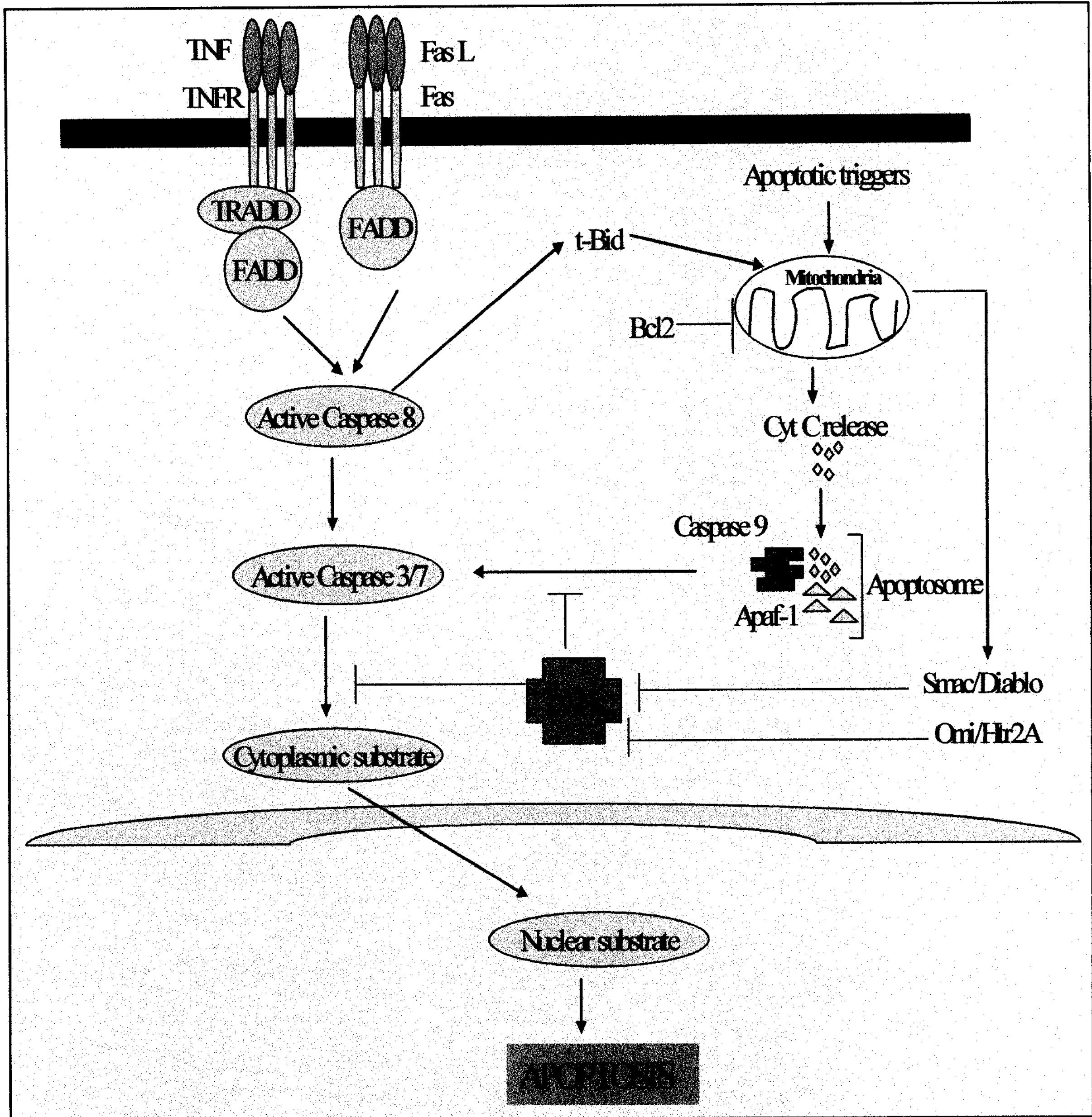
cell surface. The binding of complimentary death receptor to their respective ligands recruit procaspase 8 associated with its cofactor, FADD (Fas-associated protein with death domain) on the cytoplasmic side which becomes activated by proteolysis [60;61]. Further, activated caspase 8 promotes the cleavage of downstream effector caspases (caspases 3, 6, and 7) [62]. The effector caspases in turn activate a number of cytoplasmic and nuclear substrates including lamin B present on nuclear membrane, ICAD (inhibitor of caspase activated deoxyribonuclease) and PARP [poly (ADP-ribose) polymerase] [63]. The CAD-ICAD complex is located in the cytoplasm. After cleavage of ICAD, CAD translocates into the nucleus and degrades the chromosomal DNA leading to DNA fragmentation and cell death. PARP is a highly conserved protein that recognizes DNA strand break and implicated in DNA repair. It is a polypeptide of 118 kD and in the process of apoptosis it is cleaved into two fragments of 89 and 24 kD. PARP cleavage occurs early in the apoptotic process as a result of activity of caspase 3 and it is considered as one of the marker of apoptosis [64;65].

Intrinsic Pathway of Apoptosis:

The intrinsic pathway is initiated by various extracellular and intracellular stresses such as binding of glucocorticoids to nuclear receptors, physical agents such as heat, radiation, hypoxia, growth factor withdrawal, and oncogene induction [66-68]. Mitochondria are considered as the major organelles involved in this process. Mitochondria have two well defined compartments: the matrix surrounded by inner membrane (IM) and the intermembrane space surrounded by outer membrane (OM). The inner membrane contains ATP synthetase, and ANT (adenine nucleotide translocator), whereas the OM contains voltage dependent anion channel protein (VDAC), and

Fig. 1.1: Pathways of apoptosis:

Broadly, apoptosis is induced by two different pathways: receptor mediated and mitochondrial pathway. As shown in the figure, Fas ligands, which usually exist as trimers, bind to its receptor Fas on cytoplasmic membrane and induce receptor trimerization. Activated receptors recruit adaptor molecules such as FADD, which recruit procaspase 8 to the receptor complex, where it undergoes autocatalytic activation. Caspase 8 activates caspase 3, which leads to activation of various cytoplasmic and nuclear substrates responsible for DNA fragmentation. Caspase 8 also cleaves Bcl2 interacting protein (Bid) to truncated Bid (t-Bid) which translocates to mitochondria where it triggers cytochrome c release. However, Bcl2, one of the antiapoptotic proteins located in the mitochondrial membrane inhibits the release of cytochrome C. The released cytochrome c activates caspase 9 by binding to apoptotic protease activating factor-1 (Apaf-1) which in turn activates caspase 3. TNF also binds to TNFR and activate caspase 8 via recruitment of adapter molecules TNF receptor associated death domain (TRADD) and FADD. Caspase 8 in turn activates caspase 3. Stress such as drugs, irradiation, and growth factor withdrawl activates mitochondrial pathway. In response to death signal, mitochondria release cytochrome C and activate caspase 3 as described above. As shown in the figure, IAPs inhibit caspases, and Smac and Omi released from mitochondria block the activity of IAPs [69].


TNF
TNFR
Fas L
Fas
TRADD
FADD
FADD
Apoptotic triggers
t-Bid
Mitochondria
Bcl2
Active Caspase 8
Cyt C release
Caspase 9
Active Caspase 3/7
Apoptosome
Apaf-1
Smac/Diablo
Omi/Htr2A
Cytoplasmic substrate
Nuclear substrate
APOPTOSIS

Bcl2 [54;70]. The intermembrane space contains cytochrome c, certain procaspases and apoptosis inducing factors (AIF), endonuclease G (Endo G) and secondary mitochondrial activator of caspases (Smac/DIABLO) [71;72]. IM permeabilization (MMP) leads to dissipation of the proton gradient, loss of transmembrane potential ($\Delta\psi m$), matrix swelling and rupture of outer membrane. On the other hand, OM permeabilization (MMP) leads to release of cytochrome c (Cyt C), AIF, Endo G, and Smac/DIABLO to cytoplasm [54;57;64;70]. However, this total process is very tightly regulated and controlled by a variety of Bcl2 (B cell follicular lymphoma) family of proteins [73;74].

Basically, the Bcl2 family of proteins are divided into 2 main groups: one group consists of anti-apoptotic proteins that share structural and functional homology with Bcl2 designated as BH (Bcl2 homology region) domains (BH1-4), the other one includes pro-apoptotic proteins that share less homology to Bcl2 (BH domains) [74;75]. Again the pro-apoptotic proteins are subdivided into two subgroups: the Bcl2-associated X protein (Bax)-like death factors and the BH3 only proteins. Mammalian anti-apoptotic proteins Bcl2, BclXL (Bcl2-X large form), Bclw, Mcl1 (myeloid leukemia cell differentiation protein) and A1 share 3 or 4 regions (BH1-4) of sequence similarity, whereas pro-apoptotic proteins, Bak (Bcl2 antagonistic killer), and Bax share 3 regions (BH1-3) [74;75]. The short BH3 only domain (9-16 aa) is present in Bad (Bcl2 associated death promoter), Bid (Bcl2 interacting domain), Bim (Bcl2 interacting mediator), Noxa (PMA-induced protein) and PUMA (p53- upregulated modulator of apoptosis) proteins and they are referred as BH3 only protein [74;76]. The BH3 only proteins and Bax like proteins can promote apoptosis by interacting with anti-apoptotic Bcl2 family of proteins. The anti-apoptotic proteins are normally localized in the mitochondrial outer membrane to

prevent release of Cyt C, in contrast pro-apoptotic proteins are localized either in the cytosol or in the cytoskeleton [75]. In response to death signals, there is induction of pro-apoptotic BH3 only proteins such as Bid, Bim, Bad, Noxa, and PUMA. During apoptosis, Bax which is present in monomer form in the cytoplasm is translocated into the OM along with Bak to form pore [70;77]. Depending on the trigger, Bim, which is present in microtubules is translocated into the OM [78]. Bad is normally sequestrated in the cytoplasm in its phosphorylated form [79]. During the process of apoptosis, it is also dephosphorylated and translocates into the OM of mitochondria. NOXA and PUMA are activated by induction of p53 protein in response to DNA damage [75;80;81].

Based upon the trigger, all of these pro-apoptotic proteins interact with anti-apoptotic Bcl2 family of proteins such as Bcl2, BclXL and Mcl1 present on the mitochondrial membrane, triggers MMP, and dissipate $\Delta\psi m$. The induction of apoptosis by these pro-apoptotic factors occurs by interacting and opening the permeability transition pore complex (PTPC) which includes ANT and VDAC of IM and OM of mitochondria, respectively [70]. Caspase 8 from receptor mediated pathway also cleaves one of the pro-apoptotic Bcl2 family of proteins Bid in certain cell types, which is translocated in its truncated form (tBid) into OM of mitochondria. Bid provides the mechanism of linking receptor mediated pathway to the mitochondrial pathway [82]. Due to MMP there is release of Cyt C from mitochondria that forms a heptameric complex known as apoptosome in association with caspase 9 and Apaf1 (apoptotic protease activating factor-1) in the presence of binding and hydrolyzing of dATP/ATP [83]. Activated caspase 9 is released from the apoptosome complex and cleaves the downstream effector caspases such as caspase 3, 6, and 7 through its protease activity

which leads to digestion of structural proteins in the cytoplasm and degradation of chromosomal DNA, the hallmark of apoptosis [82]. MMP is also associated with release of AIF and Endo G. AIF and Endo G not only activate caspases, but also act directly on nuclei to induce peripheral chromatin condensation, and DNA fragmentation [84].

In addition to all of these factors, there is also release of Smac/DIABLO to the cytoplasm that interacts and inhibits the activity of the inhibitor of apoptotic proteins (IAPs). IAPs suppress apoptosis by binding to caspases 3, 7, and 9 [85]. By inhibiting IAPs, Smac/DIABLO enhances the process of apoptosis through caspase activation [71]. It is well accepted that Bcl2 protein controls MMP, however, susceptibility of the cell to various apoptotic triggers depends on the relevant interaction between the death promoting and death inhibiting proteins.

It is well known that apoptosis plays an important role in various disease conditions such as autoimmune diseases, neurological disorders, and human acquired immunodeficiency syndromes. Either of the pathways is important based on the condition of the cell and the type of apoptotic trigger. Since my project is on HIV-Vpr (HIV-Viral protein R), one of the proteins of HIV-AIDS; further I will discuss more in details how HIV-Vpr induces apoptosis *in vtro* specifically in monocytic cells.

HIV-Vpr (HIV-Viral protein R):

Several HIV proteins including one structural protein Env, and four accessory proteins Nef, Tat, Vpu, and Vpr have been implicated in apoptosis induction [86;87]. Vpr is one of the HIV proteins packaged in great quantities into the virion nucleocapsid through specific interaction with the p6 domain of p^{55gag} precursor protein and help in transportation of virus into the nucleus [12;13;88]. Vpr is also expressed late in the

infection suggesting the importance of this protein throughout the viral life cycle [27]. It has been suggested that Vpr induced apoptosis in CD4+ T cells [34] and other cell types including neuronal [89], neutrophils and promonocytic U937 cells [90]. However, the mechanism by which HIV may cause apoptosis of monocytic cells is not clear.

HIV-1 contains in its genome three structural *gag, pol* and *env* genes and six accessory genes namely *tat, rev, vif, vpr, vpu* and *nef* [90]. Vpr, a 14 kDa 96 amino acid protein, is the most conserved and multifunctional regulatory protein [90-92]. The Vpr protein is characterized by three well-defined α-helices: 17-33, 40-48 and 55-83 amino acid (aa) surrounded by flexible negatively charged N-terminal and basic C-terminal domains [93]. Furthermore, the amphipathic α-helix (55-83 aa) overlaps with a leucine rich domain that contains a short leucine zipper-like motif [93-96]. Mapping studies performed on lymphocytes or isolated mitochondria revealed that the N-terminal 1-51 aa Vpr protein (Vpr1-51) is required for virion incorporation and nuclear localization, whereas the C-terminal domain encompassing 52-96 aa (Vpr52-96) is essential for protein stability, induction of cell cycle arrest and apoptosis [97-100]. There is evidence to suggest that Vpr induces apoptosis by directly targeting the mitochondrial permeability transition pore complex (PTPC), a polyprotein complex organized around the two most abundant proteins of the inner and outer mitochondrial membranes, causing permeabilization of the mitochondrial membranes [101;102]. The NMR structure of the Vpr domain critical for its mitochondrial effects is comprised of 11 amino acid helical 71-HFRIGCRHSRI-82 peptide, with three positively charged R residues clustered on one side of the helix. Substitution of these R residues was shown to abolish Vpr-mediated

apoptotic potential [95;102;103]. Various domains of Vpr and their functions are detailed in Fig.1.2.

There are several activities ascribed to the Vpr protein in causing HIV-AIDS. 1) It plays a key role in virus replication by causing nuclear translocation of the HIV-1-preintegration complex [88]. 2) Vpr also induces cell cycle arrest at the G2/M phase suggesting the enhancement of virus replication by increasing transcription from HIV long terminal repeats (LTRs) [104]. 3) It induces apoptosis in variety of cell types including T cells, neutrophils, macrophages, and neuronal cells following infection with Vpr-expressing HIV isolates or exposure to the extracellular Vpr protein [34;105]. 4) Vpr protein also acts as a transcriptional activator of HIV promoter [106].

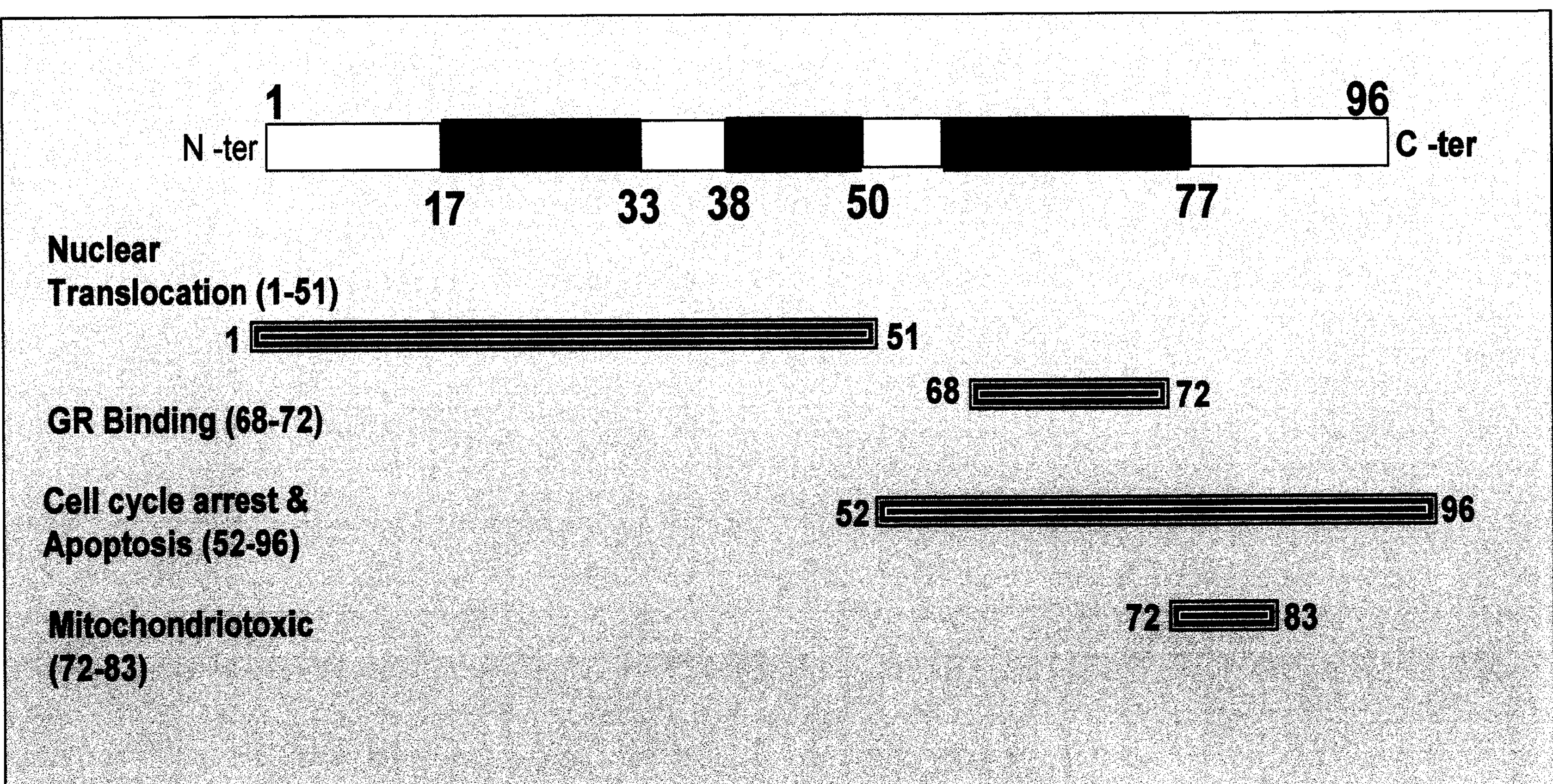
1) Vpr and nuclear transport of preintegration complex (PIC):

In HIV infection, the important target for viral replication is the non replicating T cell population, and macrophages [107]. Normally, it is easy for the HIV PIC to get access into the nucleus of the dividing cell, but the entry into non-dividing cell is questionable. HIV has evolved multiple redundant karyophilic proteins to facilitate nuclear uptake of this PIC [12]. Proteins that are targeted for transport through nuclear pore complex (NPC) possess a nuclear localization signal (NLS). Although HIV-Vpr has been characterized as a nuclear protein, yet possesses no sequence homology with NLS [108]. However, it has been reported by several groups of workers that Vpr contains multiple and diffuse nuclear entry signals [109]. Furthermore, Vpr is small enough to diffuse through the NPC and is considered to contribute to the infection of monocytes/macrophages by facilitating nuclear uptake of PIC. Vpr also causes disruption in the nuclear envelope, thus facilitate the entry of PIC into the nucleus [109].

Figure 1.2: Various domains of HIV-Vpr:

HIV-Vpr is 96 amino acid long protein and it has various domains. As shown in the figure, N terminal domain containing 1-51 amino acid is involved in nuclear translocation, whereas C terminal 52-96 amino acid domain induces cell cycle arrest and apoptosis in various cell types. The domain containing 68-72 amino acid binds to glucocorticoid receptor (GR) and 72-83 amino acid containing domain is mitochondriotoxic [110].

Domains of Vpr

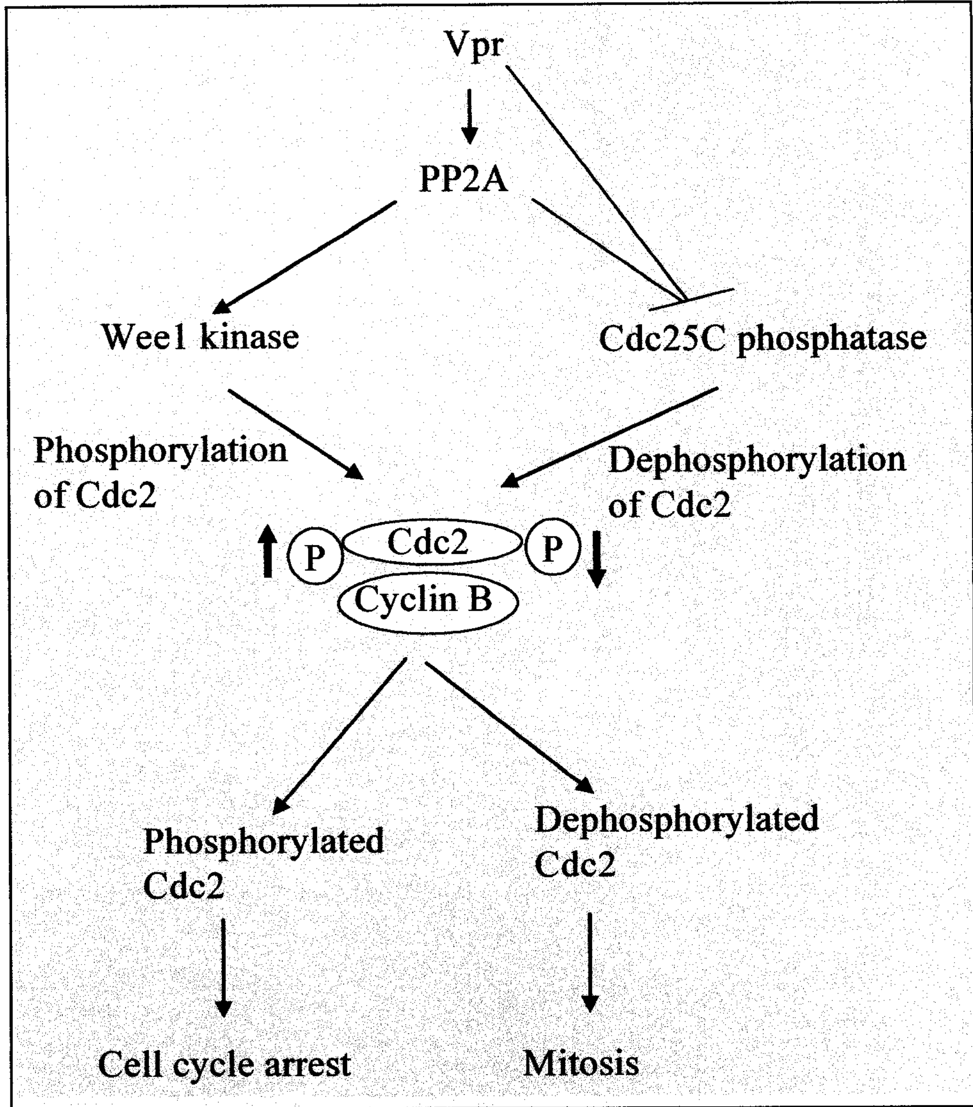


2) Vpr and cell cycle arrest:

Vpr inhibits cell proliferation and causes cell cycle arrest at G2/M phase in many human cells including primary CD4+ T lymphocytes and T cell lines. Transcription for HIV-LTR has been reported to increase 4-fold when cells are arrested in the G2/M phase of the cell cycle. The complex of cyclin dependent kinase 2 (Cdc2) and cyclin B1 is the key regulator for the check point between G2 and M phase [111;112]. This process is regulated mainly by Wee1 kinase and cell division cycle 25C (Cdc25C) phosphatase and detailed in the Fig. 1.3. During interphase, Wee1 kinase and Cdc2 are localized in the nucleus, whereas Cdc25C phosphatase and cyclin B1 are in the cytoplasm. Normally during that time, Cdc2 kinase is hyperphosphorylated by Wee1 kinase and transported to cytoplasm where it makes complex with cyclin B1 [104;113]. This complex is the target for Cdc25C phosphatase before it enters into the nucleus. Vpr mediated cell cycle arrest is regulated by activating protein phosphatase 2A (PP2A) which in turn activates Wee1 kinase and dephosphorylates Cdc25C phosphatase (inactive form), so that Cdc2 in a hyperphosphorylated form is attached to cyclin B1 and remains in the cytoplasm [114]. There is other evidence to suggest that Vpr may mediate cell cycle arrest by activating the ATR DNA damage response pathway. The Rad17 and Hus1 protein involved in the ATR pathway are required for cell cycle arrest by Vpr which involves the phosphorylation of Chk1 and histone 2A variant X, eventually leading to dephosphorylation of Cdc25C phosphatase and its inactivation [115]. Vpr was also shown to bind Cdc25C directly and inhibit Cdc25C phosphatase activity, perhaps blocking cyclin-B1-Cdc2 redundantly with the ATR pathway [109].

Fig. 1.3: HIV-Vpr induces cell cycle arrest:

Vpr mediated cell cycle arrest is regulated by activating protein phosphatase 2A (PP2A), which in turn activates Weel kinase and dephosphorylates Cdc25C phosphatase (inactive form). Weel kinase phosphorylates cyclin dependent kinase (Cdc2); and Cdc2 binds to cyclin B in its hyperphosphorylated form and remains in the cytoplasm. Cdc25C phosphatase in its active form dephosphorylates Cdc2 and helps cyclin B to enter into nucleus to participate in cell cycle. Vpr also binds to Cdc25C phosphatase directly and inhibit its activity [116;117].



Vpr
PP2A
Wee1 kinase
Cdc25C phosphatase
Phosphorylation
of Cdc2
Dephosphorylation
of Cdc2
P
Cdc2
P
Cyclin B
Phosphorylated
Cdc2
Dephosphorylated
Cdc2
Cell cycle arrest
Mitosis

3) Vpr and apoptosis:

Vpr induced apoptosis was first documented by Stewart *et al.* (1997) in human fibroblast, T cells, and peripheral blood lymphocytes [34]. They reported that although apoptosis occurred following G2 cell cycle arrest, maintenance of cell cycle arrest is not essential for the cells to undergo apoptosis. However, the exact mechanism of Vpr induced apoptosis is not yet clear. There are several lines of thought supporting the involvement of mitochondria as the major pathway for induction of apoptosis.

Role of Mitochondria in Vpr induced apoptosis: Vpr has been shown to induce apoptosis by modifying mitochondrial activity. Macreadie *et al.* (1997) has shown mitochondrial dysfunction in constitutively Vpr expressed cells [118]. They also observed that C terminal domain of Vpr which is involved in apoptosis is involved in mitochondrial dysfunction. The intact Vpr protein (1-96 aa) as well as its C terminal (52-96 aa) peptide target the mitochondrial apoptotic pathway resulting in its destabilization. When Vpr is added to the intact cell or purified mitochondria, it causes loss of mitochondrial membrane potential ($\Delta\psi m$), leading to release of Cyt C and AIF. Mitochondrial membrane potential is normally maintained by PTPC by asymmetric distribution of ions on both sides of the inner mitochondrial membrane. The C terminal moiety of Vpr specifically interacts with the ANT or to the VDAC/ANT complex, thereby creating a composite ion channel (PTPC) resulting in loss of $\Delta\psi m$ and induce apoptosis [102]. Muthumani *et al.* also reported loss of $\Delta\psi m$ in the viral delivered (adCMV-Vpr) Vpr in Jurkat cells [119]. Critical amino acids for interaction with ANT were identified as R73, R77, and R80. Vpr induced release of Cyt C interacts with Apaf-1 and procaspase 9 to create apoptosome, the caspase activation complex that causes

activation of other effector caspases such as caspase 3, 6 and 7 and downstream effects of apoptosis [120;121].

In addition to caspase 9, caspase 8 activation has also been reported by Vpr in NT2 neuron cell and Jurkat T cells [95;122]. The findings of both reports suggest that Vpr can be oligomerized and act as a ligand for any of the cell surface death receptor. However, another report contradicts this result [121] and it needs further investigation.

Although caspases play important role in Vpr induced apoptosis, in one report, Vpr induced apoptosis in caspase independent manner. Roumier *et al.* showed that either Apaf-1-/-, caspase9-/- or AIF-/- cells failed to overcome Vpr induced apoptosis [123]. However, overexpression of Bcl2 decreased the apoptotic activity of Vpr [123]. This suggests that Vpr induces apoptosis through a mitochondrial dependent but caspase independent manner.

Indirect role of Vpr in apoptosis: Vpr induced mitochondrial apoptosis is not only activated by interaction of Vpr with ANT of PTPC, but also Vpr indirectly influences other signaling molecules to be involved in this pathway. Vpr is reported to increase the expression levels of procaspase 9/caspase 9 in adCMV-Vpr infected cells [124;125]. It also suppresses the activity of NFκB transcription factor [126;127] which is involved in transcriptional modification of several survival factors such as TRAF1, TRAF2, c-IAP1, c-IAP2, and XIAP. NFκB also regulates Bcl2 family of anti-apoptotic proteins such as Bcl2, BclXL and A1 [128]. The suppressive effect of Vpr on NFκB is by interaction of Vpr with glucocorticoid receptor (GR) through Vpr interacting protein (VIP-1). Vpr mediated activation of GR leads to inhibition of NFκB by upregulation of IκB [127].

Extracellular Vpr and apoptosis: The effect of extracellular Vpr by using synthetic peptide was first observed by Macreadie *et al.* (1995) [103]. They found the C terminal domain of Vpr as cytotoxic domain which causes osmotic sensitivity, cell membrane permeabilization and cell death in yeast cell and these effects were dependent on the sequence HFRIGCRHSRIG containing two H(S/F)RIG motifs [118;129].

Recently, Vpr has been detected in the sera and cerebrospinal fluid of HIV-infected subjects at levels similar to those of p24 antigen [33;97;130]. Since extracellular Vpr is detected in CSF, it is presumed that Vpr is released from macrophages and microglia infiltrated into brain during HIV infection [33]. Astrocytes are also one more source from where Vpr is released [131]. Furthermore, circulating Vpr was found to be biologically active in inducing virion production from latently infected cells, induce apoptosis and depletion of bystander cells in lymphoid tissues during HIV infection [33;97;130]. Piller *et al.* conducted a number of experiments in neurons and their findings suggested that recombinant Vpr exhibited channel forming capacity in the living cell [89]. Extracellular Vpr either in the form of recombinant protein or synthetic peptide induces apoptosis in variety of other cell types including T cells. Finally, the interaction of Vpr with ANT of mitochondria was shown by using extracellular synthetic Vpr resulting in induction of MMP, rapid dissipation of $\Delta\psi m$, and release of several apoptogenic factors such as Cyt C and AIF [101;102]. It is generally accepted that extracellular Vpr enters into the cell independent of any of the cell surface receptor, most probably by endocytosis [98].

Vpr as negative regulator of apoptosis: Vpr not only causes apoptosis, but also inhibits apoptosis induced by other molecules depending on the cell condition and dose

of Vpr. Several studies showed that low level of Vpr protects cells from apoptosis induced by either cyclohexamide, TNF, anti-Fas antibody, serum starvation through upregulation of Bcl2 and downregulation of Bax [26;132;133]. One group of investigator suggested that Vpr enhances the expression of survivin, one of the IAPs after inducing G2/M cell cycle arrest, resulting in the inhibition of apoptosis [134]. On the other hand, several studies showed that high level expression of Vpr results in induction of apoptosis [34;35]. Based on all these findings, it appears that during early stage of infection, when Vpr is expressed at low level, it protects infected cells from external apoptotic stimuli and HIV induced cell death contributing to the destruction of host immune system and pathogenesis of AIDS. However, at the later stage, when high level of Vpr is expressed, Vpr spontaneously induced apoptosis in the infected cells [27].

Role of anti-apoptotic genes in Vpr induced apoptosis: It has been observed that the PTPC induced by Vpr is prevented by coculturing cells with recombinant Bcl2 by inhibiting ANT/Vpr interaction [101]. However, in one occasion, Bcl2 expression did not prevent the infected Jurkat cells to undergo Vpr induced apoptosis [124]. In addition to Bcl2, HAX1 (hematopoietic lineage cell specific protein 1- associated protein X-1), an anti-apoptotic factor is found to be the new mitochondrial target for Vpr [125]. The role of other pro-apoptotic proteins such as Bak and Bax in Vpr mediated apoptosis remains undetermined. IAPs are also known to inhibit apoptosis by interacting with caspases, however, their role in Vpr induced apoptosis is not known.

Signaling pathways involved in Vpr induced apoptosis:

The mitogen-activated protein-kinase (MAPK) family is a well characterized signaling pathway that transmit extracellular signal into the nucleus and regulate diverse

biological functions including cell proliferation, differentiation and survival/death [135]. The activation of c-Jun-N-terminal kinase (JNK) MAPK is most frequently associated with inhibition of cell growth and induction of apoptosis, whereas p38 and extracellular signal regulated kinase (ERK) MAPK are for cell survival [135;136]. Very little is known about the intracellular signaling molecules activated by Vpr and their involvement in Vpr-induced biological effects, particularly in apoptosis. Recently, the Vpr52-96 peptide was shown to activate JNK MAPK and was implicated in enhanced HIV-1 replication in chronically infected U1 promonocytic cells [137]. Also, Yoshizuka *et al.* demonstrated that Vpr-induced cell cycle arrest was associated with the down regulation of the ERK MAPK pathway in the 293 fibroblast cells [138]. The signaling pathway involved in Vpr52-96 induced-apoptosis in human monocytes is not known and will be investigated in this research project.

Role of LPS in apoptosis:

LPS, a structural component of the outer membrane of Gram-negative bacteria is a potent inducer of wide variety of proinflammatory cytokines (TNF-α, IL-1β) and prevents monocyte/macrophage apoptosis [139;140], however, in the absence of appropriate stimulation, monocytes are programmed to undergo cell death [141]. The mechanism by which LPS confers anti-apoptotic signals in monocytic cells is poorly understood. LPS may rescue monocytic cells from apoptosis directly through the activation of the CD14/TLR complex or indirectly via the induction of cytokines such as TNF-α in an autocrine manner [140;141], suggesting a key role for TNF-α in cell survival. It has also been seen that in vitro infection of purified monocytes with low doses of *Mycobacterium tuberculosis* markedly reduced spontaneous apoptosis of monocytes

by releasing TNF-α [142]. TNF-α is known to play an important role in inflammation, however, it also induces apoptosis when NFκB signal is blocked.

In general, pro- and anti-apoptotic proteins are involved in inducing apoptosis and also mediate resistance following apoptotic triggers. By now, there are two major families of proteins; Bcl2 and IAP involved in this process. Further, I will discuss more in details about these two families of proteins.

Bcl2 and its regulation:

Bcl2, one of the anti-apoptotic gene was first identified at the chromosomal translocation breakpoint between chromosomes 14 and 18 (t 14;18) in non Hodgkin's follicular B cell lymphoma. It plays an important role in apoptosis by preventing release of Cyt C from the intermembrane space of mitochondria [143]. There are various studies conducted on Bcl2 to determine its anti-apoptotic activity in various cell types, however, little is known about its transcriptional regulation and post translational modification. Bcl2 is expressed in a wide variety of fetal tissues, whereas in adult, it is restricted to more proliferating and differentiating cells. It is a 26 kD protein encoding 237 amino acids and contains all four BH (BH1-4) domains [73;74]. This gene can be upregulated by IL-7 [144] and tumor suppressor p53 [145]. Bcl2 is tissue specific and it undergoes distinct type of phosphorylation catalysed by several kinases. Recent studies have shown that phosphorylation of Bcl2 at Ser 70 residue by its agonist confer its full anti-apoptotic potential, at least in murine IL3 dependent myeloid cell line [146;147]. However, the antimitotic drugs such as paclitaxel, and taxane are found to inhibit anti-apoptotic effect of Bcl2 and triggers cell death by phosphorylating Bcl2 at multiple sites including Thr69, Thr74, Ser70, and Ser87 [147-149].

The Bcl2 gene is unusually large and contains three exons, extending over 200 kb [150]. Two promoter regions have been identified in the 5' untranslated region of Bcl2 gene: P1 and P2. P1, which is present in most cell types and located 1386-1423 bp upstream of translation start site [150] and displays multiple start sites and includes several consensus binding sites for Sp1 transcription factor [151]. There is also one cAMP responsive element (CRE) site in P1 promoter of t(14;18) translocated cells as well as in normal cells which controls Bcl2 levels via the activation of a transcription factor CREB [152]. There are several NFκB binding sites in the P1 promoter of Bcl2 in B cell lymphoma cell line and in t(14;18) lymphoma cells [151;153]. P2, which is the predominant promoter in neuronal cell is located 1.3 kb downstream of P1 promoter and displays two discrete initiation sites. There is evidence to suggest that Bcl2 gene is transcriptionally regulated by NFκB and a myb family of transcription factor present in its P2 promoter region [151;154;155]. Between P1 and P2, multiple non overlapping negative regulatory elements exist in Bcl2 promoter [156].

Many of the Bcl2 family of anti-apoptotic and pro-apoptotic genes are differentially regulated in various malignancies, autoimmune diseases, and neurodegerative disorders and some of them are used as diagnostic marker. It has also been reported that HIV induced apoptosis of T helper cells by downregulation of Bcl2 protein and simultaneous upregulation of bax [75;157]. Deregulation of Bcl2 family members has been tightly linked to tumorigenesis [158]. Anti-apoptotic Bcl2 protein is upregulated in B cell lymphoma and contributes to tumorigenesis by preventing cell death rather than by causing cell proliferation. Overexpression of Bcl2 is also associated with distinct type of tumors including prostate, colon, lungs, breast, gastric cancer, renal

cancer, neuroblastoma, non-hodgkin's lymphoma, leukemia, and skin cancer [75]. In most cases overexpression of Bcl2 is associated with resistance to chemotherapeutic and irradiation treatment.

IAPs:

The prototype IAP was first discovered in the baculoviruses by Lois Miller and colleagues in 1993 [159]. So far, there are eight mammalian homologous of IAPs: XIAP, NAIP, c-IAP1 (HIAP2), c-IAP2 (HIAP1), Livin (ML-IAP, KIAP), Survivin, Ts-IAP and Apollon (Bruce) [85;160-163]. The IAP family of proteins is distinguished by the presence of one to three baculovirus inhibition repeats (BIR) domains that are positioned at the N terminal region and most of them also possess a carboxyl terminal ring finger motif [163;164] (Fig 1.4). The BIR domain is characterized by its cysteine and histidine-rich protein folding domain and forms a compact globular structure. The ring zinc finger domain is found at the carboxy terminus of most of the IAPs that functions as E3 ubiquitin ligase. The first mammalian IAP homologue to be identified was neuronal apoptosis inhibitor (NAIP) protein, which was isolated during a positional cloning effort to identify the causative gene for neurodegenerative disease spinal muscular atrophy [162]. Subsequently, c-IAP1, c-IAP2, and XIAP were identified with 3 BIR domains and a carboxy terminal zinc finger domain [160;161;165]. The IAP family of protein further continued to expand with the identification of survivin, Livin, Ts-IAP and Apollon [85;163].

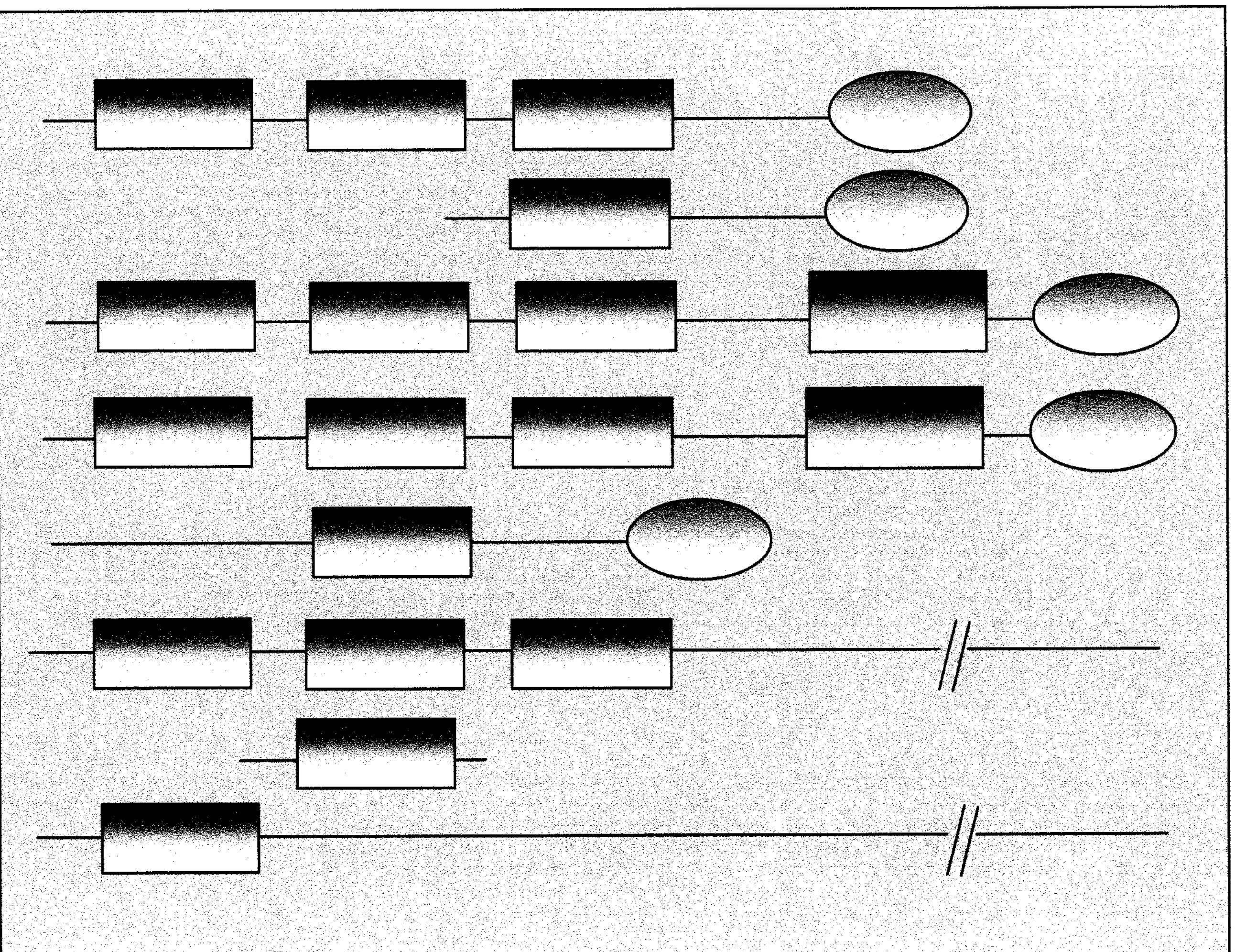
When compared with other anti-apoptotic proteins, IAPs were found to protect cells against broad spectrum of apoptotic signals by inhibiting the terminal effector caspases 3, 7, and 9 [166-168] (Fig. 1.1). XIAP, c-IAP1 and c-IAP2 are shown to bind

and potently inhibit caspases. Caspase 3 and 7 are inhibited by BIR2 linker and BIR2 domain, whereas caspase 9 is inhibited by BIR3 domain [169]. In low apoptotic stress, the IAPs containing ring finger domains trigger ubiquitination of caspase 3, 7, and 9 which leads to degradation of IAP/caspase complex [170]. On the other hand, in response to lethal apoptotic stress, endogenous IAPs are saturated by interaction with Smac and/Omi which will lead to cell death by proteosome mediated degradation of IAP/Smac or IAP/Omi complexes [163;170]. In addition to caspase inhibition, XIAP, and c-IAP1 are reported to be degraded by caspases making these proteins pro-apoptotic and it happens at the late stage of apoptosis [170]. Unlikely in XIAP, c-IAP1 and c-IAP2 contain a caspase recruitment domain (CARD) located between the BIR and RING finger domains [85;171]. The CARD domain may help in recruitment of c-IAP1 and c-IAP2 to the TNFRII signaling complex. However, the function of CARD domain is not clearly known.

c-IAP2 is 8.7 kb length (70 kD) and is contained within eight coding and two non coding (5' UTR) exons [172]. The 4.5 kb c-IAP1 (68 kD) message is contained within eight coding region exons and a single 5' UTR exon [172]. The c-IAP1 and c-IAP2 genes lie in tandem at chromosome location 11q22-23 with the intrinsic distance being approximately 7 kb, a locus associated with development of leukemia and lymphoma [173]. The c-IAP1 and c-IAP2 share homology of greater than 75% at both the nucleotide (coding region) and amino acid level. It suggests that the c-IAP1 and c-IAP2 genes originate from a gene duplication event. The c-IAP1 mRNA is expressed high in adult skeletal muscle, pancreas, thymus, and testis, whereas expression of c-IAP2 is high specifically in lymphoid tissues.

Fig. 1.4: IAPs and their domains:

The IAP family of proteins contains one to three baculovirus inhibition repeat (BIR) domains that are positioned at the N terminal region. The BIR domain is characterized by its cysteine and histidine-rich protein folding domain and forms a compact globular structure. The ring zinc finger domain is found at the carboxy terminus of some of the IAPs that functions as E3 ubiquitin ligase. Only c-IAP1 and c-IAP2 contains CARD domain and its function is not well known [163].


XIAP
TS-IAP
c-IAP1
c-IAP2
ML-IAP
NAIP
Survivin
Apollon
BIR domain
CARD domain
RING Finger domain

Role of IAPs in diseases:

The existence of multiple IAP proteins and their expression pattern suggests specific roles in promoting cell survival during development and tissue homeostasis. In addition to that, the induction of IAPs plays a key role in the regulation of cells susceptibility to death in cancer therapeutic [170;171;174]. Among all the IAPs, XIAP (57 kD, 9.0 kb in length) is probably the best characterized IAP and is a potent inhibitor of cell death (review) [169;175]. There is also mounting biochemical and genetic data to suggest that c-IAP1 and c-IAP2 are key regulators of apoptosis within the cell and are overexpressed in many cancer cell lines [176].

Chromosomal amplification of 11q21-q23 region which encompasses both c-IAP1 and c-IAP2 has been observed in variety of malignancies including medulloblastomas, renal cell carcinoma, glioblastoma, gastric carcinomas, and non small cell lung carcinoma [85;177;178]. In esophageal sqamous cell carcinoma, these genes are amplified and c-IAP1 is constantly overexpressed. There is also genetic evidence linking c-IAP2 to disease states comes from discovery that the t(11;18) (q21;q21) translocation is responsible for 50% of the mucosa associated lymphoid tissues lymphoma (MALT lymphoma) where they encode a chimeric protein containing c-IAP2 BIR domain and carboxy terminus of MALT1 [179-181]. The majority of gastric MALT lymphomas also display c-IAP2-MALT1 translocation. Overexpession of IAP proteins including c-IAP1 and c-IAP2 have been shown to suppress apoptosis in different disease model such as sciatic nerve axotomy, optic nerve axotomy, and Parkinson's disease.

Regulation of IAPs by immunoregulatory molecules:

There is evidence to suggest that LPS and TNF-α induce c-IAP1 and c-IAP2 in a variety of cell types and inhibit apoptosis [139;182-186]. After TNF-α stimulation, steady state level of c-IAP2 transcripts are markedly elevated in Jurkat T cells, whereas expression of c-IAP1 transcripts are unaffected [182]. However, XIAP, c-IAP1 and c-IAP2 are strongly upregulated in TNF-α/IL-1β/LPS stimulated human primary endothelial cells, which are resistant to variety of apoptotic death stimuli [186]. In neutrophils, c-IAP1, c-IAP2, and XIAP are expressed in various levels. However, in response to G-CSF, c-IAP2 is upregulated selectively and it is associated with G-CSF mediated antiapoptosis. There is also remarkable overexpression of c-IAP2 mRNA in chronic neutrophilic leukemia (CNL) patient and that raises the possibility of prolonged survival of neutrophils in CNL patients. Besides LPS and cytokines, other immunoregulatory molecules [187], stress [188;189] or bacterial infection also induce c-IAP2 expression. However, regulation of IAP genes by either cytokines or LPS (bacterial infection) in monocytes is not well understood. There is at least one report demonstrating the upregulation of c-IAP2 in LPS stimulated monocytes and macrophages, suggesting that LPS may protect macrophages from apoptotic cell death through induction of c-IAP2 and provide a mechanism to maintain immune function of macrophages [190].

Signaling molecules involved in the regulation of IAPs:

It is believed that activation of NFκB leads to upregulation of many TNF responsive genes including c-IAP2 and XIAP [186;191]. There is also strong evidence to suggest the involvement of NFκB transcription factor in upregulation of IAP proteins in a

number of cell types in response to LPS, TNF-α and IL-1 [182;186]. In addition, there are reports to suggest that stress (polyamine depletion or by X-ray induction) [188;189] and bacterial infection (*C .pneumoniae* and *H. pylori*) [192;193] induced c-IAP2 through activation of NFκB pathway

Recently, several reports have appeared which suggests that c-IAP2 and XIAP can act as upstream signaling molecules to regulate the activation of NFκB. Furthermore, two members of the IAP gene family (c-IAP1 and c-IAP2) have been shown to be involved in TNF-α signaling [161]. XIAP has also been shown to act as a potent activator of NFκB in endothelial cells. The mechanism by which XIAP target NFκB involves the activation of MAP3 kinase TAK1 [194]. On similar lines, selective activation of JNK in human embryonic kidney cells was found necessary for the anti-apoptotic activity of XIAP but not that of c-IAP1 and c-IAP2 [194-196].

Expression of c-IAP2 has been suggested to be regulated by multiple regulatory elements in its promoter region [182;186;191]. In addition to NFκB, c-IAP2 induction was recently shown to be regulated through a putative glucocorticoid response element in A549 human lung cancer cells in response to stimulation with dexamethasone [197]. In another study, the cAMP responsive element was shown to be vital for c-IAP2 induction in T84 colon cancer cells [198]. Recently, c-IAP2 expression was found to be regulated by ERK MAPK, p38 MAPK, and protein kinase C-δ (PKC-δ) in human colon cancer cells [198;199]. In addition, phosphatidylinositol 3-kinase (PI3K) and ERK MAPK were implicated in endoplasmic reticulum (ER) stress-induced cell death, whereas the Janus Kinase2 (Jak2)-signal transducers and activators of transcription-3 (STAT-3) pathway was suggested to regulate G-CSF-stimulated c-IAP2 expression in human neutrophils

[200]. However, the molecular signaling mechanism by which c-IAP2 is induced in monocytes in response to various stimuli such as TNF-α and endotoxin (LPS) is not well understood.

Signaling pathways involved in response to cytokines:

Signal transduction involves the transmission of biochemical information from external environment into the nucleus of cell. Cytokines are small secreted proteins which act by binding to specific receptors expressed on the target cells and then signal the cell via second messengers, most often by tyrosine kinases to alter the expression of variety of genes [201]. Cytokines may act on the cells by autocrine manner, or on the nearby cells by paracrine manner [202].

Cytokines are basically divided based on the structure of their receptors [203]. The major subset of cytokine receptor (Class I receptor) family includes receptor for most of the interleukins, colony stimulating factors, growth hormone, and prolactine. The class II cytokine receptor family includes receptors for IFNs and IL10 [204;205], whereas Class III is for TNF and related family of proteins [206]. The receptors for IL1 and related family of proteins come under class IV receptor [207]. Immunoglobulin superfamily includes receptor for SCF, M-CSF, PDGF, and VEGF [208]. Upon binding of cytokines to their respective receptors transmit the signals into the cell that is associated with protein-protein interactions and the generation of second messenger molecules (Fig. 1.5). Two major mechanisms play significant role as molecular switches in cytokine receptor signaling. One is guanine nucleotide binding proteins (G proteins) such as Ras, and Rac which oscillates between inactive (GDP bound) forms to active (GTP bound) form [209;210]. The signal is terminated when GTPase hydrolyzes and

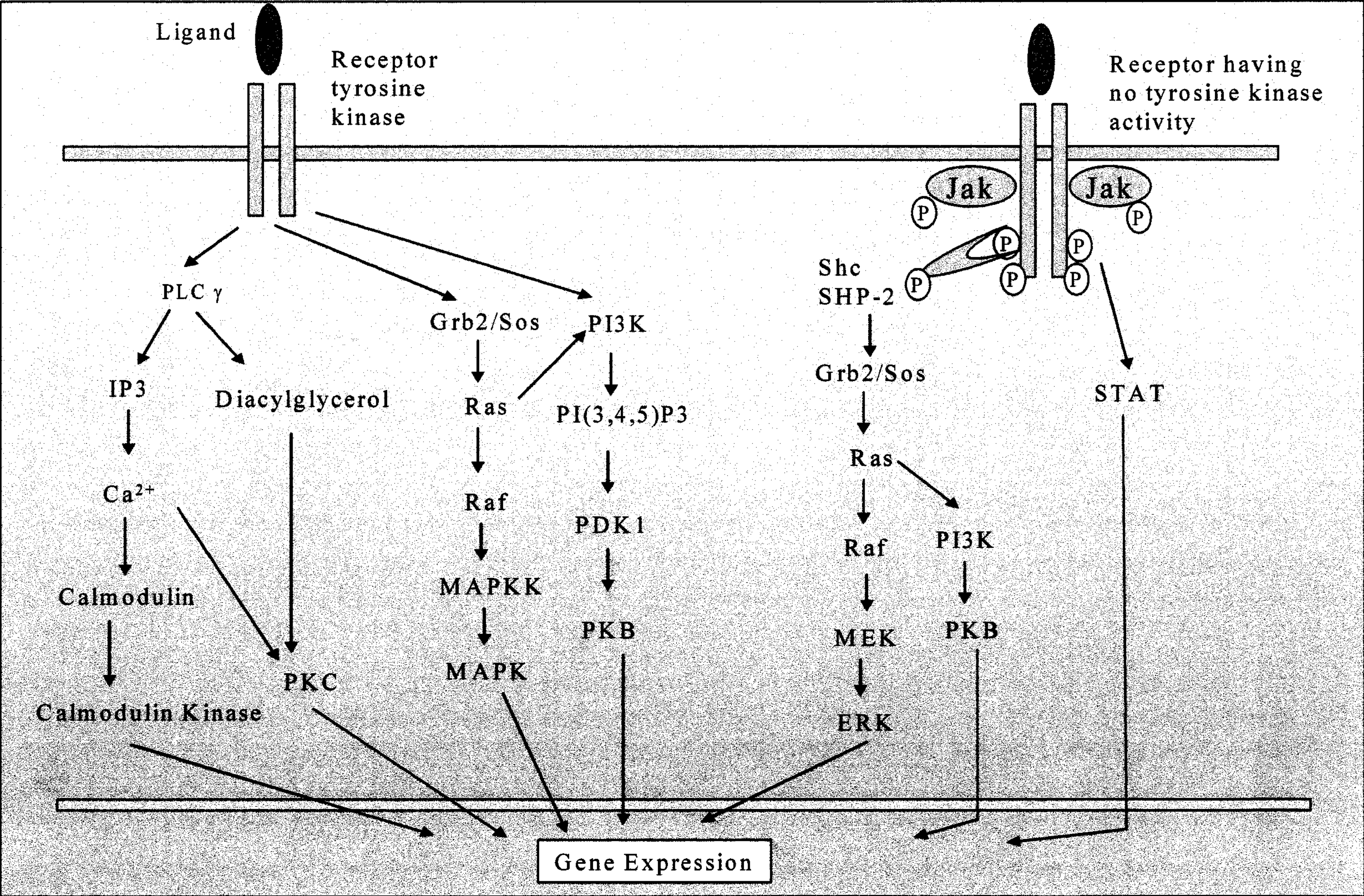
converts GTP to GDP [210]. Other mechanism is protein phosphorylation which is classified into two types: serine/threonine and tyrosine phosphorylation. Phosphorylation state of the molecule is determined by the activity of protein kinase and it also creates docking site for other signaling molecules. Type I and II receptors do not have any known enzymatic activity such as kinases or phosphatases. Therefore, cytoplasmic tyrosine kinases such as Jak associated with the cytoplasmic domain of type I and II receptors play a critical role in transmitting signals [211-213].

The immunoglobulin family of receptors has intrinsic tyrosine kinase activity. Ligand binding induces autophosphorylation of tyrosine residues of these receptors and this in turn creates docking sites for multiple signaling molecules with SH2 and PTB domains [214].

Small G protein Ras is activated by several growth factors and also by type I receptor family of cytokines [215]. In general, ligand binding to receptor induces phosphorylation of tyrosine. The phosphorylation serves as a docking site for Grb2/SOS (son of sevenless) complex which will lead to recruitment of SOS to the plasma membrane, where it can encounter Ras [216;217]. Activated Ras has several effectors of which serine/threonine kinase Raf and its downstream MEK (MAPK-ERK) cascade is activated [216;218]. It also binds directly to the p110α catalytic subunit of PI3K and increase the lipid kinase activity of PI3K [216]. Ras mediated signaling is not only confined to plasma membrane but also occurs on golgi apparatus, endoplasmic reticulum and mitochondria [219;220].

Fig. 1.5: Generalized intracellular signal transduction pathways:

Upon binding of ligands to their respective receptors, signal is transmitted into the cell. As shown in the figure, there are two different mechanisms involved in this process, one is through the receptors having tyrosine kinase activity and the other one is without tyrosine kinase activity. Ligand binding induces autophosphorylation of tyrosine residues of these receptors and this in turn creates docking sites for multiple signaling molecules with SH2 and PTB domains. Grb2/SOS complex binds to the docking sites which will lead to recruitment of SOS to the plasma membrane, where it encounters Ras. Activated Ras has several effectors including Raf and its downstream MEK (MAPK-ERK). It also binds directly to the p110α catalytic subunit of PI3K and increases the lipid kinase activity of PI3K. Phosphorylated receptor also activates phospholipase Cγ (PLCγ) which in turn activates the PKC pathway and the calcium signaling pathway. The receptors having no known enzymatic activity are activated by phosphorylation of Jak which is associated with the cytoplasmic domain of the receptors. As shown in the figure, the receptor then creates docking sites for various signaling molecules to transduce the signal inside the cell. Jak also activates STAT, one of the transcription factors for gene expression.


Ligand
Receptor tyrosine kinase
Receptor having no tyrosine kinase activity
Jak
Jak
P
Shc
SHP-2
PLC γ
IP3
Diacylglycerol
Ca2+
Calmodulin
Calmodulin Kinase
PKC
Grb2/Sos
Ras
Raf
MAPKK
MAPK
PI3K
PI(3,4,5)P3
PDK1
PKB
Grb2/Sos
Ras
Raf
MEK
ERK
PI3K
PKB
STAT
Gene Expression

MAPK pathway:

The MAPK signaling pathway is the evolutionally well conserved signaling pathway activated by a variety of extracellular stimuli such as growth factor stimulation and plays a major role in cell differentiation, cell division and cell death and survival [221]. The signaling pathway can be schematically divided into membranous and cytoplasmic phases. The first one occurs in close proximity to the growth factor receptors and involves the activation of a small GTP binding protein (Ras) via the adapter molecule Grb2 [217]. The MAPK family is grouped into three subfamilies: ERK, p38 MAPK and JNK [222]. The MAPK cascade is a three tier system which is initiated by activation of MAPK kinase kinase (MAPKKK) [135;223]. The activated MAPKKK is a protein kinase and acts in turn on MAPK kinase (MAPKK) which follows the activation of specific MAPK [135;222]. MAPK pathway is detailed in Fig 1.7 along with the specific inhibitors mentioned in separate box.

ERK: The ERK pathway is activated by a number of extracellular stimuli including growth factors, LPS, and chemotherapeutic agents [223]. ERK activation is associated with cell proliferation, activation and differentiation [224]. In most cases, ERK activation protects cells from drug induced cell death, but in others it contributes to cell death. The outcome of ERK activation solely depends on the type of cell and stimuli. ERK is made up of two isoforms, ERK1 and ERK2 (commonly called as ERK1/2) [218;225]. The activation of ERK is initiated by activation of Raf (MAPKKK) followed by activation of MEK1/2 (MAPKK) which in turn activates ERK1/2 [224]. Raf is a highly conserved kinase that is activated by the G protein Ras in association with Grb2-

SOS complex [224]. ERK1/2 has been shown to activate transcription factors such as Elk-1 and STAT3 following translocation into the nucleus [226;227].

p38 MAPK: p38 is activated primarily by cellular stresses, endotoxin and ultraviolet exposure [228]. In some instances, activation of p38 is associated with cell death, in others as cell protection [229]. There are five different isoforms of p38 namely p38α (SAPK2), p38β, p38β2, p38γ (SAPK3), and p38δ [228;230]. Expression of different isoforms of p38 is tissue specific. p38α is expressed in leukocytes and bone marrow, p38β is expressed in heart and brain, and p38γ is expressed predominantly in skeletal muscle [230;231]. External stimuli initiate activation of small G protein (RAC1 and CDC42) followed by activation of MAPKKK including MEKK4, ASK1, and TGFβ activated protein kinase 1 (TAK1) [232]. These serine/threonine kinases phosphorylate and activate the MAPKK3, MKK4, and MKK6 which in turn phosphorylate p38 on Thr 180 and Tyr 182 [232]. Major substrates for p38 include MAPK activated protein kinase-2 (MAPKAP-K2) and MAPKAP-K3 [233]. p38 also phosphorylates and activates the transcription factors ATF-2 and SP1 [234].

JNK: JNK is preferably activated by various environmental stresses such as UV irradiation, hyperthermia, oxidative stress, protein synthesis inhibitors, inflammatory cytokines, and chemotherapeutic agents [235]. It is also induced by cytotoxic drugs and is associated with cell death. The JNK group of MAPK is encoded by three genes: *jnk1* and *jnk2* genes which are ubiquitously expressed, and the *jnk3* gene, expressed primarily in the heart, testis, and brain [235]. The immediate upstream kinases for JNK activation are two MAPKKs, MKK4 and MKK7 (SEK1) which possess dual specificity on Thr 183 and Tyr 185 [235]. Upstream of MAPKK, several MAPKKKs have been identified including

MEK kinase 1 (MEKK1), MEK kinase 4 (MEKK4), apoptosis signal regulated kinase (ASK1)/ MKKK5, and MUK/DLK/ZPK [235]. Activated JNK phosphorylates ATF-2, Elk-1 and c-jun [236;237]. Thus, JNK plays an important role in activation of the transcription factor AP-1 which is made up of either c-jun and c-fos heterocomplex or c-jun homodimer [236;237]. Activation of JNK plays an important role in T cell proliferation, differentiation and IL-2 production [235].

PI3 Kinase Pathway:

PI3 kinase plays an important role in cell survival and deregulation of this process influences numerous cellular responses which are associated with cancer phenotypes [238]. This family of enzymes is activated by growth factor receptor or by small GTPase Ras and produces 3' phosphoinositide lipids [238;239]. PI3 kinase is involved in phosphorylation of phosphatidylinositol (Ptdins) on the third carbon of the inositol ring [238;240]. The phosphorylation of Ptdins to Ptdins-3-P and Ptdins-3,4,5-P is a part of many signal cascade in a cell. The lipid products of PI3K act as second messengers by binding to and activating diverse cellular target proteins which leads to cell proliferation, differentiation, chemotaxis, survival, and trafficking [241-243]. Ptdins (3, 4) P2 and Ptdins (3, 4, 5) P3 lipids produced by PI3K are able to bind Akt recruiting it to the plasma membrane where PDK1 and PDK2 phosphorylate it to its active form [238;243]. Phosphorylated Akt targets the proteins involved in apoptosis/cell death including Bcl2 and caspase 9 and protects cell from undergoing apoptosis. The PI3 kinase is divided into 4 different classes, namely Class Ia, Ib, II, and III based on their structure and substrate specificity [238]. All of them contain one catalytic domain and one regulatory domain. Class Ia enzymes consists of any one of the catalytic unit (p110α, p110β, and p110δ)

complexed with any one of the regulatory subunits (p85α, p85β, p55γ) [242]. There is only one Class Ib enzyme exists and that contains p110 γ catalytic unit and p101 regulatory subunit. Class II and III enzymes are not yet well characterized. Class Ia enzymes are activated by tyrosine kinases (e.g. growth factor receptor, antigen receptors), while the class Ib enzyme is activated by G protein coupled receptor [242].

Ca^{2+} signaling pathway:

Ca^{2+} is a highly versatile intracellular signal that can regulate many different cellular functions [244]. It may also synergize with other signaling pathways [245;246]. Fig.1.6 describes the calcium signaling pathway along with the specific pharmacological inhibitors highlighted in different color. One of the experimental evidence suggest the involvement of three cellular sensors of the cytosolic Ca^{2+} levels such as calmodulin (CaM), PKC and the $p21^{ras}$ /PI3K/Akt pathway simultaneously links the Ca^{2+} second messenger to NFκB activity [247]. PKC may integrate two types of signals, one coming from membrane receptors acting through PLC [248] and the second elicited by Ca^{2+} for Ca^{2+} sensitive PKCs [249]. The ras pathway acts similarly, integrating membrane receptor signals through G proteins and Ca^{2+} signals through ras-GRF and it may activate PI3K [250;251]. Since ras activate MAPK cascade, I believe that Ca^{2+} can activate MAPK through ras by ras GRF. CaM, a Ca^{2+} activated protein regulates phosphatases and kinases, among which calcineurin and calmodulin kinaseII (CAMKII) have been reported to be involved in activation of NFκB in neuron cells [247]. Induction of NFκB by various stimuli has already been shown to require Ca^{2+} for proper signal transduction [247;252;253]. Since Ca^{2+} is upstream of all the major kinase signaling pathways, I

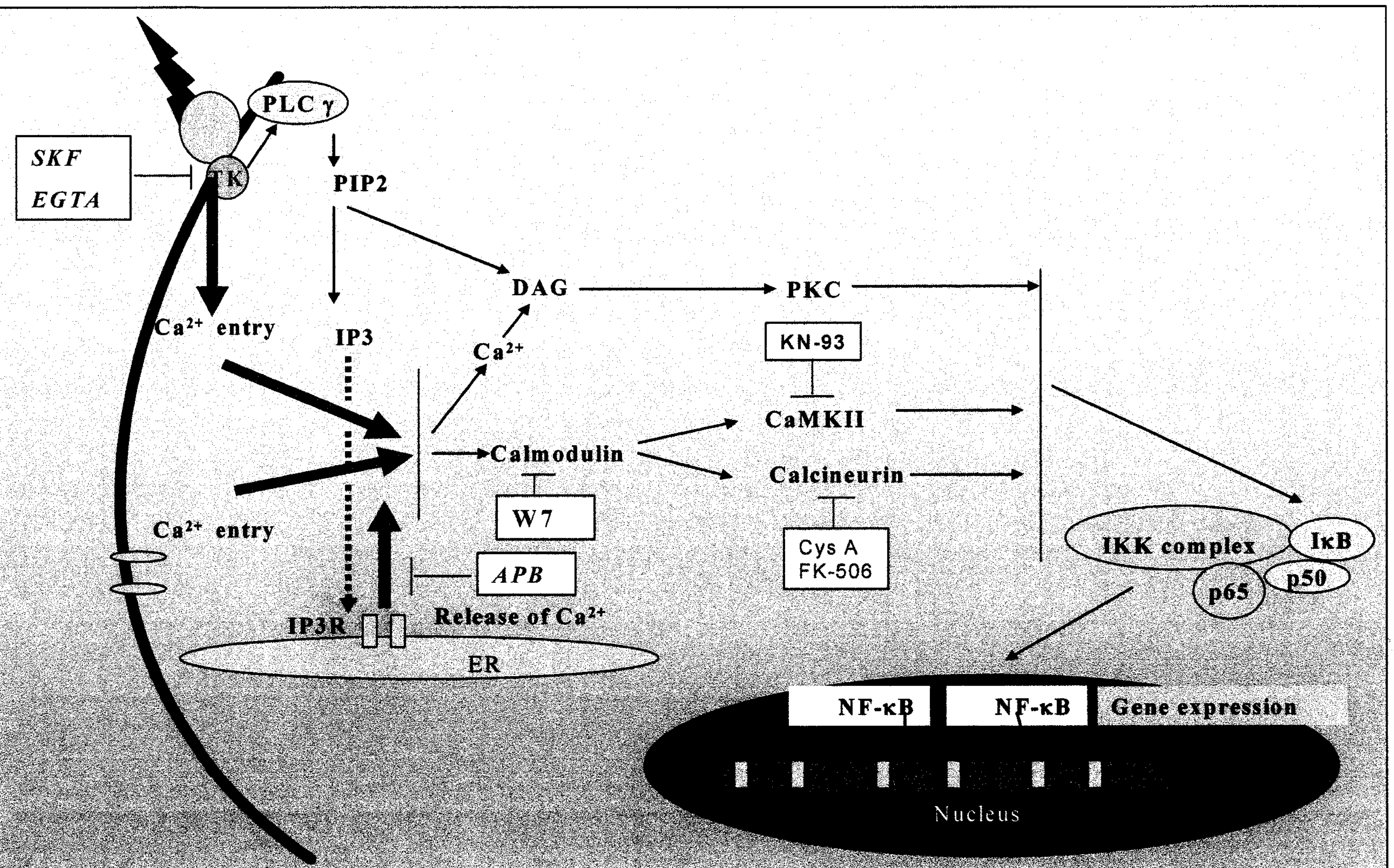
investigated the involvement of calcium signaling pathway in inducing resistance to HIV-Vpr mediated apoptosis in human monocytes.

LPS Signaling Pathways:

LPS is the principal component of the outer membrane of gram negative bacteria. It binds to the cell surface receptor CD14 present mostly on the myeloid cells including monocytes [254]. The interaction of LPS with CD14 is mediated by LPS binding protein (LBP) [255] which is present in plasma at 3-10 ng/ml, but level rises dramatically after an acute phase response. CD14 is also present in plasma in soluble form (sCD14) that helps in transducing signal mediated by LPS to the CD14 negative cells [256;257]. CD14 is expressed on the surface of myeloid cells via a glycosylphosphatidylinositol (GPI) tail, which anchors the protein to the membrane without a transmembrane segment responsible for transduction of signals into the cell [258;259]. It suggests that CD14 is not the LPS signaling receptor. The signal is mediated by toll like receptor 4 (TLR4) with leucine rich motif to the extracellular domain for interaction with the ligand such as LPS [260] (Fig. 1.7). In addition to TLR4, MD2 is associated with LPS signaling in monocytes. MD2 is also required for LPS activation of MAPK pathways [261;262]. LPS induced NFκB signaling is mediated by MyD88, IL-1 receptor associated kinase (IRAK), and TNF-α receptor associated factor (TRAF6) [263;264]. TRAF6 activates either MEKK1 or TAK1 that leads to phosphorylation of IκB kinase (IKKs). Normally, NFκB remains in the cytoplasm in association with IκB in a complex. The phosphorylated IκB kinase activates IκB and NFκB is released and translocated into the nucleus for further gene expression. Binding of LPS to TLR4 also induces PI3K activation without

Fig. 1.6: Calcium signaling pathway:

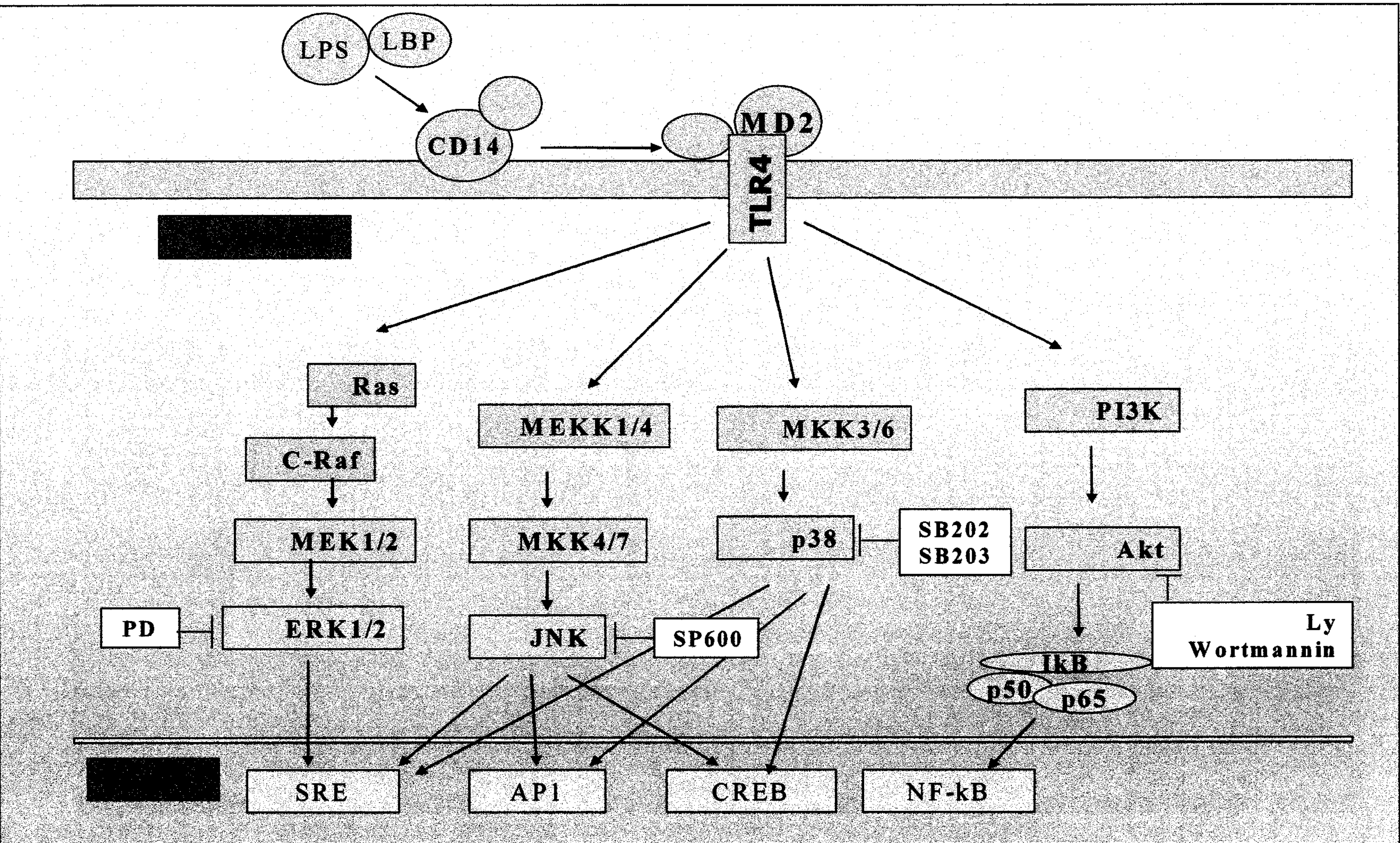
Binding of ligand to respective receptor induces calcium influx either through receptor mediated entry or through voltage dependent ion channel entry from external source. There is also release of calcium from endoplasmic reticulum through binding of IP3 to its receptor on endoplasmic reticulum. As shown in the figure, influx of calcium activates calmodulin followed by CAMKII and calcineurin which leads to activation of NFκB resulting in expression of various genes. Calcium also activates PKC pathway in association with diacylglycerol leading to NFκB regulated gene expression. The pharmacological inhibitors used to block the activity of specific signaling molecules are highlighted [247].



SKF
EGTA
TK
PLC γ
PIP2
Ca^{2+} entry
IP3
DAG
Ca^{2+}
PKC
KN-93
CaMKII
Calmodulin
Calcineurin
W7
Cys A
FK-506
Ca^{2+} entry
APB
IP3R
Release of Ca^{2+}
ER
IKK complex
IκB
p50
p65
NF-κB
NF-κB
Gene expression
Nucleus

Fig. 1.7: LPS signaling pathway:

As shown in the figure, LPS, the principal component of the outer membrane of Gram-negative bacteria binds to LPS binding protein (LBP) which helps transport LPS to CD14. LPS then comes into contact both with TLR4 and MD-2, a small protein associated with the TLR4 ectodomain. The signal is then transmitted to various signaling molecules including MAPKs and PI3K. MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli. The specific pharmacological inhibitors used in this study are mentioned in the yellow boxes [265].



LPS
LBP
CD14
MD2
TLR4
Ras
C-Raf
MEK1/2
ERK1/2
PD
MEKK1/4
MKK4/7
JNK
SP600
MKK3/6
p38
SB202
SB203
PI3K
Akt
Ly
Wortmannin
IkB
p50
p65
SRE
AP1
CREB
NF-kB

involvement of MyD88, IRAK, and TRAF6 [265]. PI3K activates NFκB pathway [265], however the intermediate molecules are not so far identified.

In addition to PI3K and NFκB pathways, LPS also induces PKC and MAPKs signaling pathways in monocytes [266-268]. In response to LPS, tyrosine kinase in the cytoplasmic domain of receptor is phosphorylated which leads to activation of Ras-Raf-MEK1/2 followed by phosphorylation of ERK1/2 [269]. However, there is also evidence for LPS activation of ERK1/2 pathway in a c-Raf independent manner [270]. The substrates for ERK1/2 include Elk1, and SRF [271]. LPS also activates JNK pathway via activation of MEKK1/4 (MAPKKK) followed by MKK4/7 (MAPKK) [272]. The downstream signaling molecules in JNK pathway include c-Jun, ATF2 and Elk-1 transcription factors [273]. The upstream signaling molecules in LPS induced p38 signaling pathway include CdC42, PAK, and Rac1 [263]. The MAPKKKs involved in that cascade are protein kinase RNA regulated (PKR), ASK1, and TAK1 and the MAPKKs include dual specificity kinase MKK3/6 [274]. The p38 phosphorylates and activates transcription factors that include ATF2, Elk1, CHOP, and MEF2C [265].

TNF Signaling Pathway:

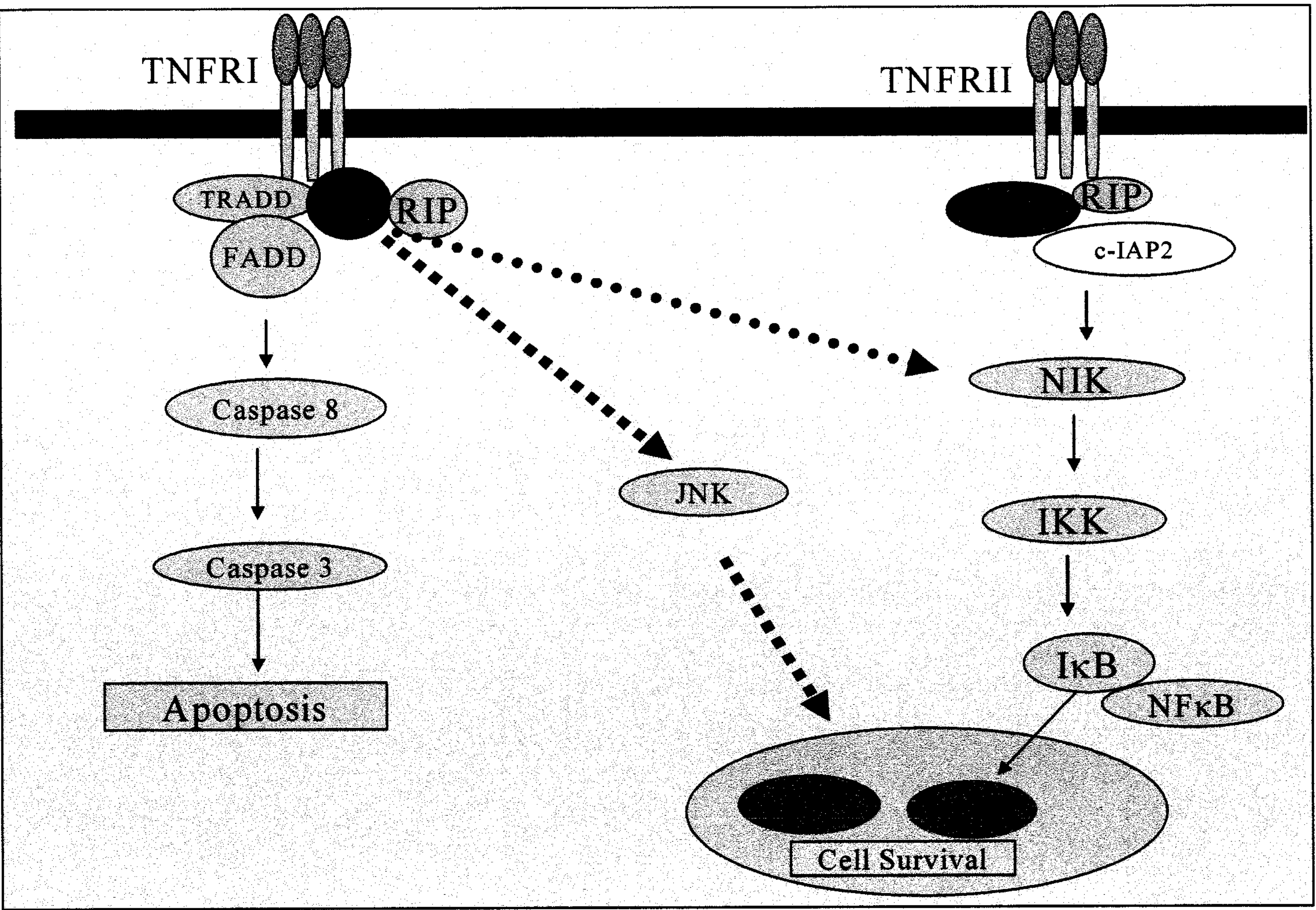
The class III cytokine receptor family mainly includes TNFRI, TNFRII, Fas, LTβR, CD27, CD30, and CD40 [275;276]. The cytoplasmic part of the receptors in TNF receptor family has no known enzymatic activities. The activation of receptor involves trimerization of receptor following binding of trimeric ligands such as TNF-α, TNF-β or Fas ligand. TNF-α is produced by monocytes, T and B lymphocytes [275;276]. It can also be produced by wide range of other cells. On the other hand, the main producers of

TNF-β are only Th1 lymphocytes and some stages of B lymphocytes. TNFRI is expressed in all cell types, whereas TNFRII is quite restricted and expressed in only monocytes and lymphocytes. Both TNFRI and TNFRII are activated by TNF-α, and TNF-β. In general, TNFRI and Fas induce apoptosis via caspase activation in response to their respective ligand binding in the cells where RNA or protein synthesis is blocked [277]. Fig. 1.8 describes the TNF signaling pathways in details. TNFRI and Fas possess an 80 amino acid death domain (DD) in the cytoplasmic portion of the receptor which transduces death signal inside the cell. The DD of TNFRI [277] interacts with DD of TRADD (TNFRI associated death domain), that results in recruitment of DD of FADD (Fas associated death domain) to this complex [278]. Then the death effector domain (DED) of FADD interacts with DED of caspase 8 and activates the caspase cascades to induce apoptosis. TNF not only induce cell death, but also activate NFκB pathway (for cell survival) by TNFRI and TNFRII receptors. Induction of NFκB by TNFRI involves the recruitment of TNF receptor associated factor2 (TRAF2) to TRADD, whereas TNFRII interacts directly with TRAF2 followed by recruitment of TRAF1 to that complex. TNF-α is shown to activate various signaling pathway including MAPKs, PI3K, PKC, and calcium signaling pathways and then from this cascade activation of AP1 and NFκB has been demonstrated.

I demonstrated that HIV-Vpr induces apoptosis in human monocytes by using the C-terminal domain of Vpr protein. I also showed first time that pretreatment of monocytes with LPS/TNF-α reversed back the process of apoptosis indicating the role of LPS/cytokines in monocyte survival in HIV infection. Further, I dissected the signaling pathways involved in HIV-Vpr induced apoptosis and determined the pro and anti-

Fig. 1.8: TNF signaling pathway:

Binding of TNF to TNFRI and TNFRII exerts two different kinds of signals. In TNFRI signaling, involvement of TRADD and FADD leads to activation of caspases and cells undergo apoptosis. On the contrary, when TRAF and RIP are involved, there is activation of JNK or NFκB pathway and cell survive. In TNFRII signaling, mostly there is involvement of TRAF and RIP which leads to NFκB signaling [275].


TNFRI
TNFRII
TRADD
RIP
FADD
RIP
c-IAP2
Caspase 8
NIK
JNK
Caspase 3
IKK
Apoptosis
IκB
NFκB
Cell Survival

apoptotic genes involved in this process. Subsequently, I investigated the molecular mechanism involved in LPS/TNF-α induced resistance in Vpr mediated apoptosis along with the regulation of anti-apoptotic genes in survival of monocytic cells.

Rationale:

Monocytes/macrophages are productively infected by HIV as are CD4+ activated T cells, however, unlike T cells, monocytic cells survive HIV replication without major signs of HIV-induced cytopathic effects. Persistently infected monocytic cells serve as a major reservoir of HIV in lymphoid tissues at all stages of disease and represent a key challenge to eradicate HIV infection by eliminating virus reservoirs. It is believed that one of the reasons by which monocytic cells escape HIV cytopathic effects is the capacity of HIV to decrease the sensitivity of this cell type to apoptosis. This hypothesis was supported by a recent study demonstrating that nerve growth factor (NGF) produced following HIV infection can rescue monocytic cells from the cytopathic effects of HIV. Vpr, one of the accessory proteins of HIV induces apoptosis in monocytic cells. How monocytic cells develop resistance to apoptosis following HIV infection specifically by Vpr remains poorly understood. In my research work, I employed a synthetic peptide corresponding to the C-terminal 52-96 amino acid sequence of Vpr (Vpr52-96) as a model, because the apoptotic effect of Vpr has been shown to be mimicked by the C-terminal Vpr52-96 but not by the N-terminal Vpr1-51 moiety. My preliminary results suggest that Vpr52-96 peptide interestingly induced apoptosis in normal human monocytic cells as well as in the promonocytic THP-1 cells. Furthermore, this Vpr52-96-induced apoptotic effect in both cell types could be reversed by pretreatment of cells with LPS. These observations suggest that LPS or the cytokine produced endogenously by

LPS such as TNF-α, may be one of the critical factors responsible for protecting monocytic cells from the apoptotic effects of HIV-Vpr and consequently promoting monocytic cells to act as potent viral reservoirs. However, the molecular mechanism underlying the Vpr induced apoptosis and resistance mediated by LPS is not known and remains to be investigated.

Hypothesis:

Vpr induces apoptosis through the activation of intracellular signaling pathways and by modulation of pro-or anti-apoptotic genes such as members of the Bcl2 and IAP family of genes. Furthermore, LPS or cytokines produced endogenously by LPS such as TNF-α protect human monocytic cells from HIV-Vpr-induced apoptosis by interfering with the intracellular signaling pathways and/ or the expression of pro-apoptotic/anti-apoptotic genes.

Aims and objectives:

The main aim of this research project is to elucidate the intracellular signaling pathways involved in HIV-Vpr mediated apoptosis and the resistance induced by LPS in normal human monocytic cells and promonocytic THP-1 cell line using Vpr1-45 and Vpr52-96 peptides as models. The specific objectives of this research project are as follows:

1) To elucidate the signaling pathways involved in regulation of LPS mediated resistance to apoptosis in human monocytic cells.

2) To elucidate the signaling pathways involved in Vpr induced apoptosis and to identify the pro/anti-apoptotic genes involved in its regulation in human monocytic cells.

3) To identify the anti-apoptotic genes involved in LPS induced resistance in HIV-Vpr mediated apoptosis in human monocytic cells and to determine the intracellular signaling pathways involved in their regulation.

Chapter II

Materials and Methods

Cell line and cell culture:

THP-1, a promonocytic cell line derived from a human acute lymphocytic leukemia patient, was obtained from the American Type Culture Collection (Manassas, VA) [279]. Cells were cultured in IMDM (Sigma-Aldrich, St-Louis, MO), supplemented with 10% FBS (Invitrogen, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml gentamicin, 10 mM HEPES, and 2 mM glutamine. The cells were cultured in a T-75 cm^2 flask at 37° C in CO_2 incubator (5% CO_2) untill reaching confluency. Cells were washed in PBS followed by splitting into new flasks at 1 X 10^6 cells/ml in IMDM media. To avoid cells undergoing too many passages, cells were frozen in 10% DMSO in cryovials in liquid nitrogen storage and whenever required, cells were thawed following the standard procedure of cell culture.

Reagents:

LPS derived from *Escherichia coli* 0111:B4 (Sigma-Aldrich Canada Ltd., Oakville, ON), rTNF-α (Biosource, Montreal, Quebec, Canada), and anti-TNF-αR1 Ab (R & D Systems, Minneapolis, MN) capable of neutralizing TNF-α activity were purchased. The source and dose of inhibitors used in this study are detailed in table 2.1. The MAPK inhibitors used in this study are as follows: PD98059 (2′-Amino-3′-methoxyflavone), an inhibitor of MAP/ERK kinase-1, which selectively blocks the activity of ERK MAPK and has no effect on the activity of other serine threonine protein kinases including Raf1, p38 and JNK MAPK, was also purchased [135;280]. The pyridinyl imidazole SB202190 [FHPI, 4- (4- Fluoro phenyl) -2-(4-hydroxy phenyl)- 5-(4-pyridyl)1H-imidazol], a potent inhibitor of p38 MAPK, has no significant effect on the

activity of ERK or JNK MAPK subgroups [135;281]. SP600125 (Anthra(1,9-cd)pyrazol-6(2H)-one 1,9-Pyrazoloanthrone), a specific JNK inhibitor, is a reversible ATP competitive inhibitor with more than 300 fold selectivity versus related MAPK including ERK1 and p38 [282]. Dexamethasone [(DXM; (9α-fluoro-16α-methylprednisolone)] and curcumin were used as JNK inhibitors as well. The PI3K inhibitors Ly294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], a potent inhibitor that acts on the ATP binding site; and Wortmannin, a fungal metabolite that blocks the catalytic activity of PI3K without affecting the upstream signaling events were purchased from Calbiochem. The following calcium signaling inhibitors were employed: EGTA (Ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid), a calcium chelating agent; SKF-96365 hydrochloride [1-[b-[3-(4- Methoxyphenyl) propoxy] -4 -methoxyphenethyl]-1H-imidazole,HCl] specifically inhibits receptor-mediated Ca^{2+} entry [283]; 2-APB (2-Aminoethoxydiphenylborate) inhibits inositol (1,4,5) triphosphate (IP3) induced Ca^{2+} release from the ER [284]; W-7 hydrochloride [N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl] is a calmodulin antagonist; KN-93 [2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methyl benzylamine] is a specific cell permeable inhibitor of CAMKII; FK-506 (Tacrolimus) interacts with FK506-binding protein (FKBP), forming a FK506–FKBP complex, which binds to and blocks calcineurin; and cyclosporine A binds to cyclophilin and inhibits the Ca^{2+} dependent phosphatases. ZVAD-fmk (Z-Val-Ala-Asp-CH_2F), the broad spectrum caspase inhibitor was obtained from Calbiochem. Caffeic Acid Phenethyl Ester (CAPE) used in this study has been shown to act as a potent and specific inhibitor of NFκB

activation mediated by reactive oxygen species. All other chemicals used for electrophoresis and immunoblot analysis were obtained from Sigma-Aldrich.

Isolation of monocytes:

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) by automacs negative selection (Miltenyi Biotech Inc. Auburn, CA). Blood was obtained for isolation of PBMCs from healthy volunteers after approval of the protocol by the ethics review committee of the Children's Hospital of Eastern Ontario, Ottawa, Canada. PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) as previously described [285]. Briefly, the cell layer consisting mainly of mononuclear cells was collected and washed twice in PBS containing 2% EDTA (ethylenediaminetetraacetic acid) followed by incubation with automacs FcR blocking reagent along with biotin antibody cocktail for 10 min at 4° C. Following incubation, cells were treated with anti biotin microbeads for 15 min at 4° C. Cells were then washed once and subjected to automacs negative selection separation as per the manufacturer's instructions. Cell populations thus obtained contained more than 95% CD14+ monocytes. Cells were cultured in IMDM (Sigma-Aldrich, St-Louis, MO) supplemented with 10% FBS (Invitrogen, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml gentamicin, 10 mM HEPES, and 2 mM glutamine.

Stimulation of cells with Vpr peptides:

Vpr C-terminal (52-96 aa) and Vpr N-terminal (1-45 aa) peptides were synthesized by Genemed Synthesis Inc, San Francisco, California, USA by automated solid-phase synthesis using 9-fluorenylmethoxycarbonyl and were purified by reverse-

Table 2.1: Detailed description of pharmacological inhibitors, their source, and doses used in this study:

Name of the Inhibitors	Source	Used Concentration	Cellular Targets
SB202190	Calbiochem, San Diego, CA	5-50 μM	P38 MAPK
PD98059	Calbiochem	5-50 μM	ERK MAPK
SP600125	Calbiochem	5-50 μM	JNK MAPK
Dexamethasone	Sabex, Boucherville, Quebec	2-100 nM	JNK MAPK
Curcumin	Calbiochem	100-750 nM	JNK MAPK
EGTA	Calbiochem	2-10 mM	Calcium chelator
SKF96365 HCl	Calbiochem	20-100 μM	Receptor mediated calcium entry
2-APB	Calbiochem	10-50 μM	IP3 induced calcium release
W-7-HCl	Calbiochem	10-50 μM	Calmodulin
KN-93	Calbiochem	5-50 μM	CAMKII
FK 506	AG Scientific, San Diego, CA	0.5-5 μM	Calcineurin
Cyclosporine A	Sigma-Aldrich	0.5-5 μM	Calcineurin
Ly 294002	Calbiochem	1-20 μM	PI3K
Wortmannin	Calbiochem	5-50 nM	PI3K
ZVAD-fmk	Calbiochem	10-50 μM	Caspases
CAPE	Calbiochem	10-100 μM	NFκB

phase HPLC (>95%) followed by analysis with electrospray mass spectrometry. The sequence of C-terminal (52-96 aa) and N-terminal end (1-45 aa) peptides are as follows: Vpr (52-96) peptide, DTWAGVEAI IRILQQLLFI HFRIGCRHSR IGVTRQRRAR NGASRS; Vpr (1-45) peptide, MEQAPEDQGP QREPYNEWTL ELLEELKSEA VRHFPRIWLH NLGQH. Because of high propensity of Vpr peptides to bind to proteins, cells ($1x10^6$ cells/ml) were treated with peptides in an isotonic buffer (13 mM HEPES, 2.4% glucose, 68 mM NaCl, 1.3 mM KCL, 4 mM Na_2HPO_4, 0.7 mM KH_2PO_4, pH 7.2) for 30 min followed by addition of cell culture medium as described [286]. Cells were also stimulated with either LPS or TNF-α before or after treatment of Vpr peptides wherever necessary.

Ca^{2+} influx:

THP-1 cells were washed with Ca^{2+}-free PBS for 5 min at room temperature and resuspended in Buffer A (RPMI 1640 containing 20 mM HEPES, pH 7). The cells were washed again and resuspended in Buffer A containing 1 mM calcium binding dye Fluo3/AM (Molecular Probes, Eugene, OR) in 1 mM DMSO and 3.75% Pluronic F-127 solution (Sigma) followed by incubation in dark for 45 min in a 37°C shaking water bath. The reaction was stopped by adding equal volume of Buffer B (Buffer A containing 5% FBS, pH 7.4) followed by incubation for 15 min in a 37°C water bath. The cells were washed and resuspended in Buffer B at a final concentration of 0.5 X 10^6 cells/ml and analyzed for Ca^{2+} levels by the FACScan flow cytometer (Becton–Dickinson, Franklin Lakes, NJ) equipped with CellQuest software, Version 3.2.1fl. Cell samples were maintained at 37°C during acquisition of data. Intracellular Ca^{2+} levels at baseline and following stimulation with LPS/TNF-α were measured. Ca^{2+} ionophore A23187 (20 mM)

and 5 mM EGTA (Sigma) were used as positive and negative controls, respectively. To determine the effect of Vpr, cells were treated with different doses of Vpr peptides for 2 hr prior to load the dye.

Analysis of cellular apoptosis:

Propidium iodide (PI) staining for determination of cell cycle arrest: Apoptotic cells with DNA fragmentation were analyzed by flow cytometry with PI staining in permeabilized cells. Briefly, cells (1 x 10^6/ml) were washed twice with PBS containing 1% FBS, fixed with chilled methanol for 15 min at 4°C, treated with 1 μg/ml of RNAse A followed by staining with 50 μg/ml of PI (Sigma) at 4°C for 1 hr. The DNA content was analyzed by flow cytometry (FACScan, BD Bioscience, Mississauga, Canada).

Annexin-V and PI staining for detection of apoptotic cells: Early stage of apoptosis is determined by annexin V which binds to phosphatidyl serine exposed on the cell surface of apoptotic cell. Following stimulation, cells were collected, washed with PBS and then stained with FITC labeled annexin-V and PI (Molecular probes, Eugene, OR) for 15 min at room temperature in the dark and analyzed by flow cytometry [287]. Annexin- positive cells were plotted as histograms.

JC-1 staining for determination of mitochondrial membrane potential (MMP): JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) is a lipophilic carbocyanin that exists in a monomeric form and is able to accumulate in mitochondria. In the presence of high $\Delta\psi$, JC-1 can reversibly form aggregates that after excitation at 488 nm emit in the orange/red channel (FL-2). The collapse in $\Delta\psi$ provokes the decrease in the number of JC-1 aggregates, and consequent increase in JC-1

monomers (FL-1). Cells were stained with 10 μg/ml Δψ-sensitive probe JC-1 (Molecular probes) in IMDM containing 10% FCS for 15 min at room temperature in dark and analyzed by flow cytometry, as described [288]. Apoptotic cells with depolarized mitochondria are present in the lower right quadrant of the panel. JC-1 monomers and aggregates were plotted as dot plot. All the histograms and dot plots were generated by using Win-MDI version 2.8 software.

Cell stimulation and Western blot analysis:

Cells (1 x 10^6 cells/ml) were treated with the indicated concentration of inhibitors for 2 hr followed by stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 15-60 min for detection of kinases activation and for 24 hr to determine c-IAP2 expression by Western blot analysis as described earlier [285]. Cells (1 x 10^6/ml) were also treated with the inhibitors for 2 hr followed by stimulation with Vpr peptide(s) for various time points for analysis of MAPKs activation, and Bcl2/c-IAP1 expression by Western blot analysis as described earlier [285]. Briefly, cell lysates were prepared by treating the cell pellets with lysis buffer for 1 hr (50 mm HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 100 mM NaF, 100 mM sodium orthovandate, and 1 mM EGTA pH 7.7), followed by centrifugation for 15 min at 20,000 x g at 4°C. The protein concentration of the supernatants was determined using the Bio-Rad protein determination assay (Bio-Rad Laboratories, Hercules, CA). Total proteins (30 μg) were subjected to SDS-PAGE followed by transfer onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratory, Hercules, CA). The membranes were probed with either anti-c-IAP1/c-IAP2 (RIAP-1) polyclonal antibody, anti-Bcl2, or with anti-phospho Bcl2 antibodies. To detect MAPKs, membranes were probed either with anti-phospho-JNK,

anti-phospho-ERK or with anti-phospho-p38 antibodies overnight at 4°C. Following incubation, membranes were washed with TBST and then incubated with donkey anti-rabbit polyclonal antibody conjugated to horseradish peroxidase or goat anti-mouse polyclonal antibody wherever necessary. Detailed information about the species, and source of antibodies are presented in table 2.2. To control for total protein loading, the membranes were stripped of the primary antibodies and reprobed with rabbit polyclonal antibodies specific for the total p38, ERK, or JNK MAPKs or with mouse monoclonal antibodies specific for β-actin. All immunoblots were visualized by enhanced chemiluminescence (Santa Cruz).

Measurement of CAMKII activity:

The CAMKII assay was performed using a CAMKII kit (Upstate Biotechnology Inc., Mississauga, Ontario, Canada) as per the manufacturer's instructions. Cells were pretreated with either inhibitors or Vpr peptides for 2 hr followed by stimulation of cells with either LPS or TNF-α for 30 min. Similarly cells were also transfected with DN-CAMKII or control vector before stimulation with LPS/TNF-α. Cell pellets were lysed and cytoplasmic extracts were collected as described previously [289]. CAMKII activity was assayed from total cellular proteins utilizing a peptide substrate (KKALRRQETVDAL) specific for CAMKII. Total proteins (200 μg) were added to 10 μl of CAMKII substrate, 0.4 μM each of peptide inhibitors for PKA and PKC, and 100 μCi of $MgCl_2$-[γ-^{32}P] ATP in ADB II buffer (20 mM MOPS, pH 7.2, 2.5 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT, and 1 mM $CaCl_2$). The reaction

Table 2.2: Detailed descriptiption of antibodies and their source:

Name of the antibodies	Species	Source
α-phospho-p38 MAPK	Rabbit	Cell Signaling, Danvers, MA
α-total p38-MAPK	Rabbit	Santa Cruz Biotechnology, CA
α-phospho-JNK MAPK	Rabbit	Cell Signaling
α-total JNK-MAPK	Rabbit	Santa Cruz Biotechnology
α-phospho-ERK MAPK	Mouse	Santa Cruz Biotechnology
α-total ERK-MAPK	Rabbit	Santa Cruz Biotechnology
α-c-IAP1	Rabbit	In house raised*
α-c-IAP2	Rabbit	In house raised*
α-phospho-Bcl2	Rabbit	Cell Signaling
α-total-Bcl2	Rabbit	Cell Signaling
α-rabbit-HRPO	Donkey	Amersham Bioscience, Montreal, Canada
α-mouse-HRPO	Goat	Bio Rad, CA
α-p50 (EMSA)	Rabbit	Santa Cruz Biotechnology
α-p65 (EMSA)	Rabbit	Santa Cruz Biotechnology
α-Sp1 (EMSA)	Rabbit	Santa Cruz Biotechnology
α-CREB (EMSA)	Rabbit	Santa Cruz Biotechnology

* (Holcik *et al,* 2002)[290]

was incubated at 30°C for 10 min, and the phosphorylated substrate was separated from the residual [γ-^{32}P]-ATP using P81 phosphocellulose paper. The papers were washed twice in 0.75% H_3PO_4 and once in acetone for 2 min, and radioactivity was measured by Microbeta counter (Wallac, Turko, Finland). Blanks to correct for nonspecific binding of [γ-^{32}P]-ATP and its breakdown products to the phosphocellulose paper and controls for phosphorylation of endogenous proteins in the sample were performed. CAMKII activity was expressed as cpm/μg of protein.

RNA isolation and Real time PCR for c-IAP2:

c-IAP2 mRNA levels was measured using real time quantitative RT-PCR as per the Taqman method. Total RNA was isolated using RNeasy mini-spin columns combined with DNAse I treatment (Qiagen, Mississauga, Ontario, Canada). The reverse transcribed RNA was amplified by PCR using the Taqman EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA). All the RT-PCR steps were performed on an ABI Prism 7700 sequence Detector and quantified using the cycle threshold method, and normalized to GAPDH mRNA using PE-ABI supplied primers (600 nM) and probe (200 nM, JOE-labeled). The thermal cycling conditions for the RT step were: 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min followed by 45 PCR cycles at 94°C for 20 sec and 60°C for 1 min per cycle.

Semiquantitative RT-PCR analysis for Bcl2, c-IAP1, c-IAP2, and β actin:

Total RNA was isolated from cells as described earlier (Qiagen, Mississauga). Total RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse

transcriptase (Perkin Elmer) as described previously [285]. Equal aliquots (5 μl) of cDNA were subsequently amplified for Bcl2, c-IAP1, c-IAP2, and β-actin. The oligonucleotide primer sequences used for Bcl2 (Biomol), c-IAP1, c-IAP2, and β-actin (Stratagene, La Jolla, CA) are described in table 2.3. The amplification conditions for Bcl2, c-IAP1, c-IAP2 and β-actin were as follows: denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. After 30 cycles, the amplified Bcl2, c-IAP1, c-IAP2, and β-actin fragments were resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Plasmid construction and mutagenesis:

The full length c-IAP2 promoter (3.5 kb) was amplified by PCR (with Pfu turbo™ enzyme) from a previously characterized bacterial artificial chromosome containing the genomic region encompassing both c-IAP1 and c-IAP2 genes (Gene Bank accession # AF070674) [172;191]. The primers used were as follows: sense-5'-GAT GGT ACC ACT AGT ACT AGA ATA ATG C-3'; and antisense 5'-GCT GAA TTC GCA TGC ACC AGC AAG GAC-3'. The underlined bases indicate restriction sites for cloning that together with the preceding bases do not correspond to sequences in the promoter. The amplified promoter fragment was cloned into pCR2.1 TOPO, sequenced and then subcloned into the pGL3B vector. Since two NF-κB sites (#1 and #3) are critical in induction of c-IAP2, site specific mutagenesis of these two sites was performed with the Quick Change™ multisite-directed mutagenesis kits (Stratagene, La Jolla, CA) as per the manufacturer's protocol with 5' phosphorylated primers: site # 1, 5'-CTT TTG GGT CAT GGA AAT **AG**C CGA GTG GGT TTG CCA G-3', site # 3, 5'-GGT TAT TAC

Table 2.3: Sequence of primers used in PCR and oligonucleotide probes in EMSA:

Oligonucleotides/Primers	Sequences
Bcl2 Oligonucleotides (EMSA)	
CREB sense	5'-GAA CCG TGT GAC GTT ACG CA-3'
CREB antisense	5'-TGC GTA ACG TCA CAC GGT TC-3'
NFκB sense	5'-TGC CAA GAG GGA AAC ACC AGA ATC AA-3'
NFκB antisense	5'-TTG ATT CTG GTG TTT CCC TCT TGG CA-3'
Sp1 sense	5'- CAG AGG AGG GCT CTT TCT TTC- 3'
Sp1 antisense	5'- GAA AGA AAG AGC CCT CCT CTG- 3'
c-IAP1 Oligonucleotides (EMSA)	
CREB sense	5'- GGG CGC GCT GAC GTC ATC GTG CGT- 3'
CREB antisense	5'- ACG CAC GAT GAC GTC AGC GCG CCC- 3'
NFκB sense	5'- GAG AAA GGC TAG TCC CTT TTC-3'
NFκB antisense	5'-GAA AAG GGA CTA GCC TTT CTC- 3'
c-IAP2 Oligonucleotides (EMSA)	
NFκB (site# 1) sense	5'-ATG GAA ATC CCC GA-3'
NFκB (site# 1) antisense	5'-TCG GGG ATT TCC AT-3'
NFκB (site# 3) sense	5'-GCT GGA GTT CCC CT-3'
NFκB (site# 3) antisense	5'-AGG GGA ACT CCA GC-3'
Primers for RT PCR	
Bcl2 sense	5'- TTC TTT GAG TTC GGT GGG GTC-3'
Bcl2 antisense	5'- TGC ATA TTT GTT TGG GGC AGG -3'
c-IAP1 sense	5'-AGC TGT TGT CAA CTT CAG ATA CCA CT-3'
c-IAP1antisense	5'-TGT TTC ACC AGG TCT CTA TTA AAG CC-3'
c-IAP2 sense	5'- ACT TGA ACA GCT GCT ATC CAC ATC-3'
c-IAP2 antisense	5'-GTT GCT AGG ATT TTT CTC TGA ACT GTC-3'
β-actin sense	5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'
β-actin antisense	5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'

CGC TGG AGT T**<u>AA</u>** CCT AAG TCC TAA AAG G-3'. The mutagenized bases are indicated (underlined and bold) which created convenient EcoR1 restriction site for analysis. The primers were used together in the mutagenesis reactions to create the double mutant. Successful mutants were identified by EcoR1 digests and sequencing.

Transient transfection with c-IAP2 luciferase construct and luciferase assay:

Cells were transiently transfected with plasmids containing the c-IAP2 promoter by Lipofectamine 2000 (Invitrogen) as described previously [279;291]. Briefly, 5 μg of the test plasmid and 3 μg of pSV-β galactosidase vector (Promega, Madison, NJ) were incubated for 30 min at room temperature with 16 μl of Lipofectamine reagent in 200 μl of OPTI-MEM1 medium to allow formation of DNA-liposome complexes. These complexes were then added to the cell suspension (2 x 10^6 cells/ml) for 24 hr followed by stimulation either with LPS or TNF-α in the presence or the absence of the indicated inhibitors. Cells were also treated with Vpr peptide(s) for 2 hr following which cells were stimulated with LPS/TNF-α overnight. The cells were harvested and assayed for luciferase and β galactosidase activity by using luciferase assay and β galactosidase assay kits (both from Promega) in a Bio Orbit 1250 Luminometer (Fisher, Pittsburgh, PA) and spectrophotometer , respectively.

Transient transfection with antisense c-IAP2 oligonucleotides:

THP-1 cells were also transfected with either antisense oligonucleotides for c-IAP2 (5' GAU GTT TTG GTT CTT CUU C 3') or control oligonucleotides (5' CUU CTT CTT GGT TTT GUA G 3'). Briefly, 5 μg of the either antisense oligonucleotides or control oligonucleotides was incubated for 30 min at room temperature with 10 μl of

Lipofectamine reagent in 100 μl of OPTI-MEM1 medium to allow formation of DNA-liposome complexes. These complexes were then added to the cell suspension (2 x 10^6 cells/ml) for 24 hr followed by stimulation either with LPS or TNF-α for 24 hr. Cells were collected, lysed and the expression of c-IAP2 is determined by Western blotting. Wherever it is required, cells were treated either with staurosporine or with Vpr peptides to induce apoptosis followed by detection of apoptotic cells either by PI staining or by annexin/PI staining.

Transient transfection with dominant negative (DN)-CAMKII:

The dominant negative mutant for the human CAMKII γ isoform in pSRα vector (pCAMKII-γ) was kindly provided by Drs. Alain Lilienbaum and Alain Israel from the Institute Pasteur, Paris [292]. The control vector pSRα was generated from pCAMKII-γ by digesting with EcoRI. Endotoxin--free preparations of pCAMKII-γ and control vector were used to transfect cells. Briefly, 5 μg of the either DN-CAMKII plasmid or control vector were incubated for 30 min at room temperature in the presence of 10 μl of Lipofectamine reagent in 100 μl of OPTI-MEM1 medium. Following incubation, the complexes were added to the cell suspension (2 x 10^6 cells/ml) for 24 hr followed by stimulation either with LPS or TNF-α. To detect CAMKII activity, CAMKII assay was conducted by using cell extracts from these transfected and stimulated cells, as described earlier. Transfected cells were also treated with either staurosporine or Vpr peptides after stimulation either with LPS/TNF-α followed by detection of apoptotic cell by PI or annexin/PI staining.

Transient transfection with stealth JNK siRNA:

THP-1 cells were transiently transfected with stealth siRNA (Invitrogen) specific for either JNK1 or JNK2 using FuGENE 6 transfection reagent (Roche Diagnostics, Quebec, Canada) as described previously [285]. Briefly, 50 pM of either stealth JNK or control RNA were incubated for 45 min at room temperature with 3 μl of FuGENE 6 in 100 μl of serum free IMDM to allow formation of RNA-liposome complexes. These complexes were then added to the cell suspension (10^6/ml) for 24 hr followed by treatment with Vpr peptide(s) for either 2 or 24 hr following which cells were analyzed for JNK phosphorylation by Western blot analysis or for apoptosis, cell cycle arrest and for mitochondrial membrane potential by annexin/PI, PI, and JC-1 stain, respectively.

Electrophoretic mobility shift assays (EMSA):

EMSAs were performed as per the standard technique and as described earlier [279;291]. Briefly, cells were stimulated either with LPS or TNF-α for 45-60 min. in the presence or the absence of the indicated inhibitors or Vpr peptides. Cells were also treated with Vpr peptide(s) for 3 hr in the presence or in the absence of indicated inhibitors. Cells were harvested in Tris-EDTA-saline buffer (pH 7.8) and centrifuged at 200Xg for 5 min. at 4°C. Subsequently, cells were lysed for 10 min at 4°C with buffer A (10 mM of HEPES, 10 mM of KCl, 1.5 mM of $MgCl_2$, 0.5 mM of PMSF, pH 7.9) containing 0.1% Nonidet P-40. The lysates were centrifuged at 20,000 x g for 10 min at 4°C. The pellet containing the nuclei was suspended in buffer B (20 mM of HEPES, 420 mM of NaCl, 1.5 mM of $MgCl_2$, 0.2 mM of EDTA, and 25% glycerol) at 4°C for 15 min. Both buffer A and B contained the protease inhibitors including DTT, PMSF, and

spermidine at concentrations of 0.5 mM each, as well as 0.15 mM of spermine, and 5 μg/ml each of aprotonin, leupeptin and pepstatin. The supernatant containing the nuclear proteins was collected. Protein concentration was determined using the Bio-Rad protein determination assay kit (Bio-Rad, Hercules, CA) and frozen at –80°C untill further use. The nuclear proteins (5 μg) were mixed with ^{32}P-labeled NFκB oligonucleotide probes for 20 min and the resulting complexes were separated on a 5% nondenaturing gel. The oligonucleotide probes contained sequences corresponding to the CREB, NFκB and Sp1 sites in the Bcl2 promoter; CREB and NFκB sites in the c-IAP1 promoter (Gene Bank Accession No. AF070674); and NFκB site # 1 and # 3 in the c-IAP2 promoter are described in table 3. To determine specificity of transcription factor binding, parallel EMSA reactions were incubated with 100-200 fold excess of unlabelled specific and non-specific oligonucleotide probes for 20 min prior to the addition of corresponding labeled probe. Supershift experiments were also performed by using rabbit anti-NFκB p50 and p65, anti-CREB, and anti-Sp1 polyclonal antibodies (Table 2). The gel was dried and exposed to x-ray film (Eastman Kodak).

RNAse protection assay (RPA):

Cells were treated with various concentrations of Vpr peptide(s) for 0-6 hr followed by isolation of total RNA. RPA was performed by using BD Riboquant package kit (BD Bioscience) with a multi probe template set of anti- and pro-apoptotic genes of the Bcl2 family as well as GAPDH and L32 as internal controls as per the manufacturer's protocol [293]. Briefly, ^{32}P-labeled riboprobes of defined length were generated using T7 RNA polymerase and 50 ng of DNA template in the presence of 150 μCi of [α-^{32}P] UTP

(Amersham Bioscience). Template DNA was digested with RNAse free DNAse followed by precipitation of labeled RNA. Five μg of total cellular RNA was mixed with 6.2×10^5 CPM of the ^{32}P-labeled riboprobe in hybridization buffer (40 mM PIPES, 1mM EDTA, 0.4 M NaCl, and 80% formamide) and incubated for 5 min at 90°C followed by 12 hr at 56°C. The hybridized RNA duplex were then digested with RNAse and proteinase K followed by extraction with phenol and precipitation by the addition of equal volumes of 4 M ammonium acetate and 2 volumes of ethanol. Labeled RNA samples were resolved on 6% urea denaturing gels, dried and visualized by autoradiography.

Statistical analysis: Means were compared by the two tailed Student's t test. The results are expressed as mean ± SD.

Chapter III

Regulation of LPS mediated resistance to apoptosis in human monocytic cells

Introduction:

Activation of macrophages by cytokines or a mild bacterial infection is shown to confer anti-apoptotic survival signal, however, in the absence of appropriate stimulation, monocytes are programmed to undergo apoptosis. Macrophage survival may thus influence inflammatory and immune responses, and susceptibility to microbial pathogens. Recently, LPS has been shown to confer survival signals in monocytic cells [190], however, the molecular mechanism by which LPS confers anti-apoptotic survival signals in monocytic cells is poorly understood.

LPS may rescue monocytic cells from apoptosis directly through the activation of the CD14/Toll like receptor complex or indirectly via the induction of cytokines such as TNF-α in an autocrine manner suggesting a key role for TNF-α in monocytic cell survival. TNF-α generates two opposing signals: one that triggers apoptosis and the other that inhibits apoptosis [276]. The outcome of TNF-α-mediated effects is determined by the balance between these two signals [275]. It has been suggested that TNF-α-induced cell death is mediated by the TNF-receptor-1 (TNF-RI) whereas both TNF-RI and II are required to transduce signals for anti-apoptotic activity primarily through NFκB activation [276]. The protective role of NFκB against apoptosis is believed to be mediated by the induction of anti-apoptotic genes including c-IAP2 [186;191]. It has been suggested that TNF-α stimulation of Jurkat T cells induced the expression of the c-IAP2 through the activation of NFκB and c-IAP2 exerts its anti-apoptotic activity by directly binding and inhibiting the downstream protease caspases-3, 7 and 9 [186].

Expression of c-IAP2 has been suggested to be regulated by multiple regulatory elements in its promoter region. In addition to NFκB, c-IAP2 induction was recently shown to be regulated through a putative glucocorticoid response element and cAMP responsive element in A549 human lung cancer cells and T84 colon cancer cells [197;294], respectively. However, the upstream signaling molecules involved in the activation of these transcription factors regulating c-IAP2 expression are not well understood. Recently, c-IAP2 expression was shown to be regulated by ERK MAPK, p38 MAPK, PI3K, JAK2-STAT3 and PKC-δ in various cell lines [198-200].

LPS was shown to induce the expression of the anti-apoptotic gene, c-IAP2, in PMA-differentiated human monocytic U-937 cells [190], however, its role in suppressing apoptosis in monocytic cells is not clear [190]. ***Therefore, I hypothesize that LPS may inhibit apoptosis or enhance survival of macrophages, at least in part, through the induction of c-IAP2. However, the molecular mechanism by which LPS induce the expression of c-IAP2 is not well understood. Therefore, the present study is designed to determine the role and regulation of c-IAP2 in the development of LPS mediated resistance to apoptosis in human monocytic cell.***

Herein, I investigated the molecular mechanism by which LPS and the proinflammatory cytokine TNF-α induce anti-apoptotic survival signals in human monocytic cells by employing promonocytic THP-1 cells as a model system. I demonstrate for the first time that both LPS and TNF-α induce survival of human monocytic cells through the induction of the c-IAP2 gene. Furthermore, LPS induced c-IAP2 expression, at least in part, through the endogenous production of TNF-α in an autocrine manner. The molecular mechanism involved in the upregulation of c-IAP2

following stimulation of monocytic cells either with LPS or TNF-α is not known. I investigated the role of upstream signaling molecules including the members of the MAPK, PI3K, and the calcium signaling pathways involved in the regulation of c-IAP2 expression and consequent survival of monocytic cells. My results suggest that LPS- and TNF-α-induced c-IAP2 expression in THP-1 cells and their survival are regulated selectively by CAMKII through the activation of NFκB.

Results:

LPS-induced c-IAP2 expression is mediated by endogenously produced TNF-α in THP-1 cell:

LPS and TNF-α induce variety of anti-apoptotic genes including c-IAP2 in different cell types. To determine the expression of LPS induced c-IAP2 in monocytic cell, THP-1 cells were stimulated with 1 μg/ml of LPS for various period of time and c-IAP2 expression was determined in protein as well as in mRNA level by Western blotting (Fig. 3.1A) and real time RT-PCR (Fig. 3.1B), respectively. c-IAP2 expression was detectable as early as 2 hr and maximum induction to the extent of 20 fold was observed 24 hr following LPS stimulation compared with the unstimulated cells. Since TNF-α is produced in response to LPS stimulation in THP-1 cells [295], I investigated whether LPS-induced c-IAP2 expression is mediated by endogenously produced TNF-α. To determine the role of endogenous TNF-α, I employed neutralizing antibodies specific for TNFR1 (10-20 μg/ml). This antibody is capable of neutralizing the biological activity of TNF-α, as described previously and recommended by the manufacturer (R & D System). The results show that anti-TNFR1 antibodies inhibited LPS-induced c-IAP2 expression in a dose dependent manner (Fig. 3.1C). Furthermore, exogenous TNF-α induced c-IAP2

expression in THP-1 cells as determined by both Western blot and real time PCR analysis (Fig 3.1A & B). Similar to the results obtained with LPS, c-IAP2 protein expression in response to TNF-α was detectable as early as 2 hr, and maximum induction to the extent of 15 fold was observed at 24 hr compared with the unstimulated cells. It may be noted that cells expressed c-IAP1 constitutively that was not inducible by either LPS or TNF-α.

c-IAP2 prevents staurosporine induced apoptosis in THP-1 cells:

THP-1 cells are resistant to the Fas/FasL-induced apoptosis since this cell line does not express Fas on its surface [296]. Therefore, staurosporine has been used in this study to induce apoptosis in these cells [198;294]. To examine the role of c-IAP2 in LPS- and TNF-α-induced inhibition of apoptosis, I determined if LPS and TNF-α stimulation could prevent staurosporine-induced apoptosis in THP-1 cells. LPS and TNF-α-stimulated cells were treated with staurosporine for 4 hr followed by determination of apoptosis by PI staining. LPS/TNF-α alone had no effect on cell viability, whereas treatment of unstimulated THP-1 cells with staurosporine resulted in ~30% cell death. Stimulation of cells with either LPS or TNF-α in the presence of staurosporine resulted in significant inhibition of staurosporine-induced apoptosis from ~30% to 10% (Fig 3.1E).

To determine if c-IAP2 is involved in LPS- or TNF-α-induced anti-apoptotic cell survival, cells were transfected with c-IAP2 antisense (AS) or control oligonucleotides prior to stimulation with either LPS or TNF-α for 24 hr followed by determination of c-IAP2 expression and staurosporine-induced apoptosis. Antisense c-IAP2 oligonucleotides significantly decreased LPS- and TNF-α-induced expression of c-IAP2 by 4 fold as compared to the cells treated with control oligonucleotides (Fig. 3.1D). To delineate the

role of c-IAP2 in apoptosis, we induced apoptosis with staurosporine after stimulation with either LPS/TNF-α. Flow cytometric analysis by propidium iodide reveals that treatment of LPS- and TNF-α-stimulated cells with antisense c-IAP2 oligonucleotides enhanced staurosporine-induced apoptosis compared to the cells treated with control oligonucleotides (Fig 3.1E). However, antisense c-IAP2 oligonucleotides did not affect staurosporine induced apoptosis in unstimulated cells compared with the cells treated with control oligonucleotides (data not shown). It may be noted that abrogation of c-IAP2 expression by antisense oligonucleotides may not be possible because of low transfection efficiency in monocytic cells. These results suggest that stimulation with either LPS or TNF-α enhanced monocytic cell survival that was mediated by c-IAP2 induction.

The MAPKs and PI3 kinase/Akt pathways are not involved in upregulation of LPS/ TNF-α induced c-IAP2 expression:

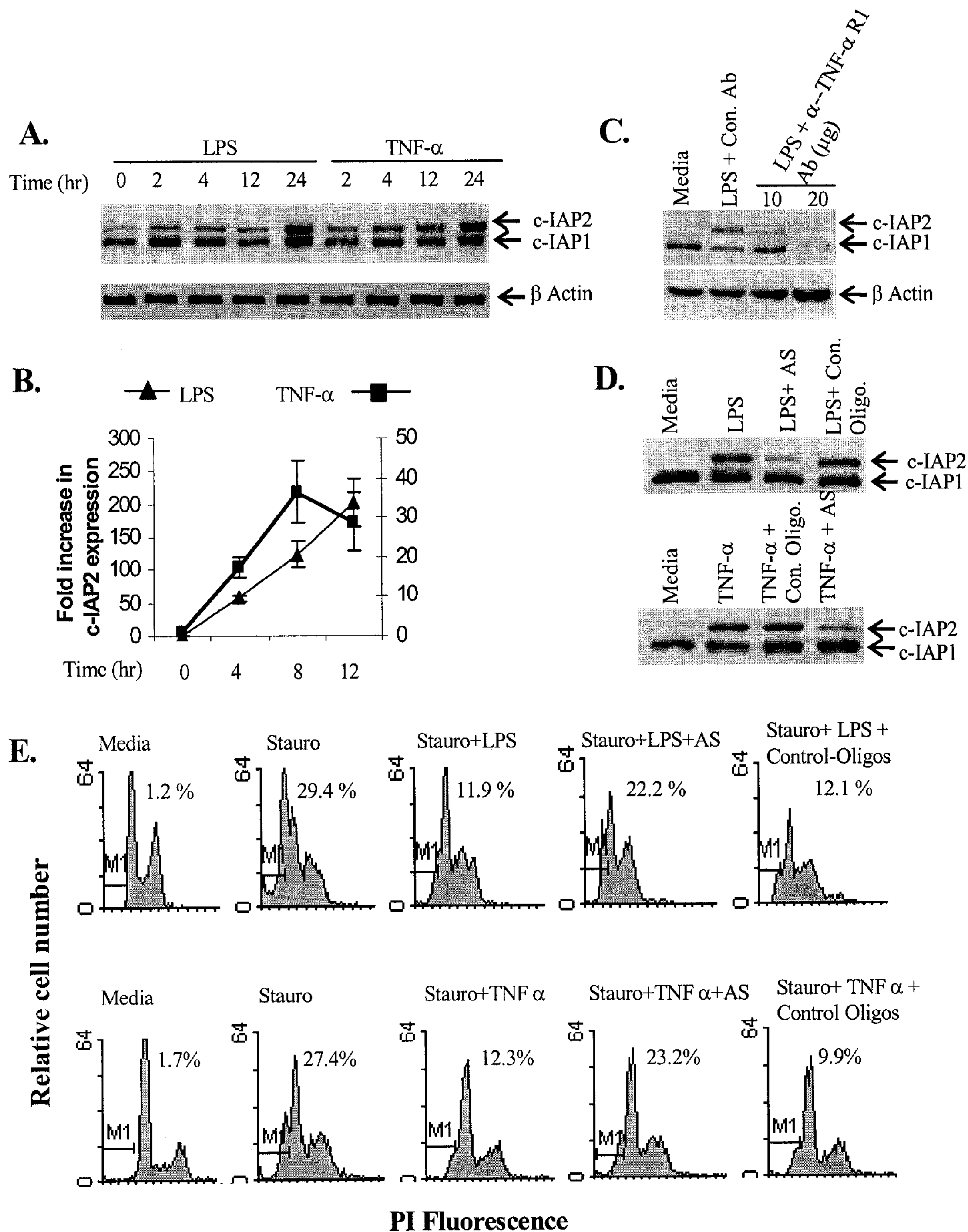
MAPK and PI3K have been shown to be involved in apoptosis in epithelial and leukemic cell systems. Recently, JNK was shown to mediate the anti-apoptotic activity of XIAP whereas p38 and ERK MAPKs were shown to regulate c-IAP2 expression in colon cancer cell lines [196;198]. In addition, PI3K and ERK MAPK have been demonstrated to be involved in endoplasmic reticulum (ER) stress-induced cell death [297]. To elucidate the signaling pathways involved in the regulation of c-IAP2 expression, I first investigated the role of MAPKs in LPS and TNF-α-stimulated THP-1 cells. To delineate the role of MAPKs, I first examined whether LPS and TNF-α stimulation induced the activation of JNK, ERK and p38 MAPKs and their activation can be inhibited by the specific inhibitors, namely, SP600125 for JNK, PD98059 for p42/44 ERK, and

Fig. 3.1: LPS and TNF-α-induced inhibition of apoptosis is mediated by c-IAP2 expression in THP-1 cells.

A, B. LPS and TNF-α induce c-IAP2 expression. Cells (1 X 10^6/ml) were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 0-24 hr. c-IAP2 expression was determined by Western blot **(A)** and real time-RT-PCR analysis **(B).** The experiments shown are representative of three different experiments.

C. LPS-induced c-IAP2 expression is mediated by endogenously produced TNF-α. Cells (1 X 10^6/ml) were stimulated with LPS (1 μg/ml) in the presence and the absence of anti-TNF-α-R1 (10-20 μg/ml) or isotype matched control antibodies followed by determination of c-IAP2 expression by Western blot analysis. The experiments shown are representative of three different experiments.

D, E. LPS- and TNF-α-induced inhibition of apoptosis is mediated by c-IAP2 expression. Cells (10^6/ml) were transfected with either antisense (AS) c-IAP2 or control oligonucleotides followed by stimulation with LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 hr followed by determination of c-IAP2 expression by Western blot analysis **(D).** Stimulated cells were also treated with staurosporine (Stauro) (2 μM) for 4 hr and DNA content of cells were analyzed by PI staining **(E).** The experiments shown are representative of three different experiments.


A.
LPS
TNF-α
Time (hr) 0 2 4 12 24 2 4 12 24
c-IAP2
c-IAP1
β Actin
C.
Media
LPS + Con. Ab
LPS + α--TNF-α R1 Ab (μg)
10 20
c-IAP2
c-IAP1
β Actin
B.
LPS
TNF-α
Fold increase in c-IAP2 expression
Time (hr) 0 4 8 12
D.
Media
LPS
LPS+ AS
LPS+ Con. Oligo.
c-IAP2
c-IAP1
Media
TNF-α
TNF-α + Con. Oligo.
TNF-α + AS
c-IAP2
c-IAP1
E.
Media
1.2 %
Stauro
29.4 %
Stauro+LPS
11.9 %
Stauro+LPS+AS
22.2 %
Stauro+ LPS + Control-Oligos
12.1 %
Media
1.7%
Stauro
27.4%
Stauro+TNF α
12.3%
Stauro+TNF α+AS
23.2%
Stauro+ TNF α + Control Oligos
9.9%
M1
Relative cell number
PI Fluorescence

SB202190 for p38 MAPKs. Cells were stimulated with LPS or TNF-α for 15 min. and subjected to Western immunoblotting for phosphorylation of JNK, ERK and p38 MAPKs by using anti-phospho-JNK, anti-phospho-ERK and anti-phospho-p38 specific antibodies, respectively. The same blots were stripped and reprobed with anti-JNK, anti-p42/44 and anti-p38 Abs to ensure equal protein loading. The results show that both LPS and TNF-α induced the phosphorylation of JNK, p42/44 ERK and p38 MAPKs, whereas total protein of respective kinases remained same. Furthermore, LPS and TNF-α-induced phosphorylation of JNK, p42/44 ERK and p38 MAPKs was inhibited by their specific inhibitors in a dose dependent manner (Fig. 3.2A, B & C). To determine the role of JNK, p42/44 ERK and p38 MAPKs in LPS- and TNF-α-induced c-IAP2 expression, THP-1 cells were treated with SP600125, PD98059, or SB202190 for 2 hr prior to stimulation with either LPS or TNF-α for 24 hr. The results show that c-IAP2 expression was not inhibited by any of these inhibitors at any concentration (Fig. 3.3A, B & C). Doses higher than 50 μM for these inhibitors were not used because of their cytotoxic effect as determined by the trypan blue exclusion test.

To determine the role of PI3K pathway, I first examined whether LPS and TNF-α induce phosphorylation of Akt, the downstream substrate for PI3K, and whether this phosphorylation can be inhibited by PI3K specific inhibitors, LY294002 and wortmannin. The results show that LPS and TNF-α induced Akt phosphorylation at 30 and 15 min post-stimulation, respectively (Fig 3.4A and B Left panel), and this phosphorylation was inhibited by both LY294002 and wortmannin in a dose dependent manner (Fig. 3.4A & B Right panel). To determine the role of PI3K in LPS- and TNF-α-induced c-IAP2 expression, THP-1 cells were treated with Ly294002 or wortmannin for

Fig. 3.2: JNK, p42/44 ERK and p38 MAPKs inhibitors down regulate the expression of phospho JNK, p42/44 ERK and p38 MAPKs in LPS/TNF-α stimulated THP-1 cells:

Cells (1.0 X 10^6/ml) were pretreated with either A) SP600125, B) SB202190 or C) PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 0-120 min. Crude protein extracts (30 μg) were subjected to SDS-PAGE followed by Western blot analysis using either anti-phospho-JNK (indicated by arrows as pp46/54), anti-phospho-p42/44 (indicated by arrows as pp42/44) or anti-phospho-p38 (pp38) Abs. To ensure equal loading of protein, the membranes were stripped and reprobed either with anti-JNK, anti-p42/44 or anti-p38 Abs, respectively (indicated by arrows as p46/54, p42/44 and p38). The experiments shown are representative of three different experiments.

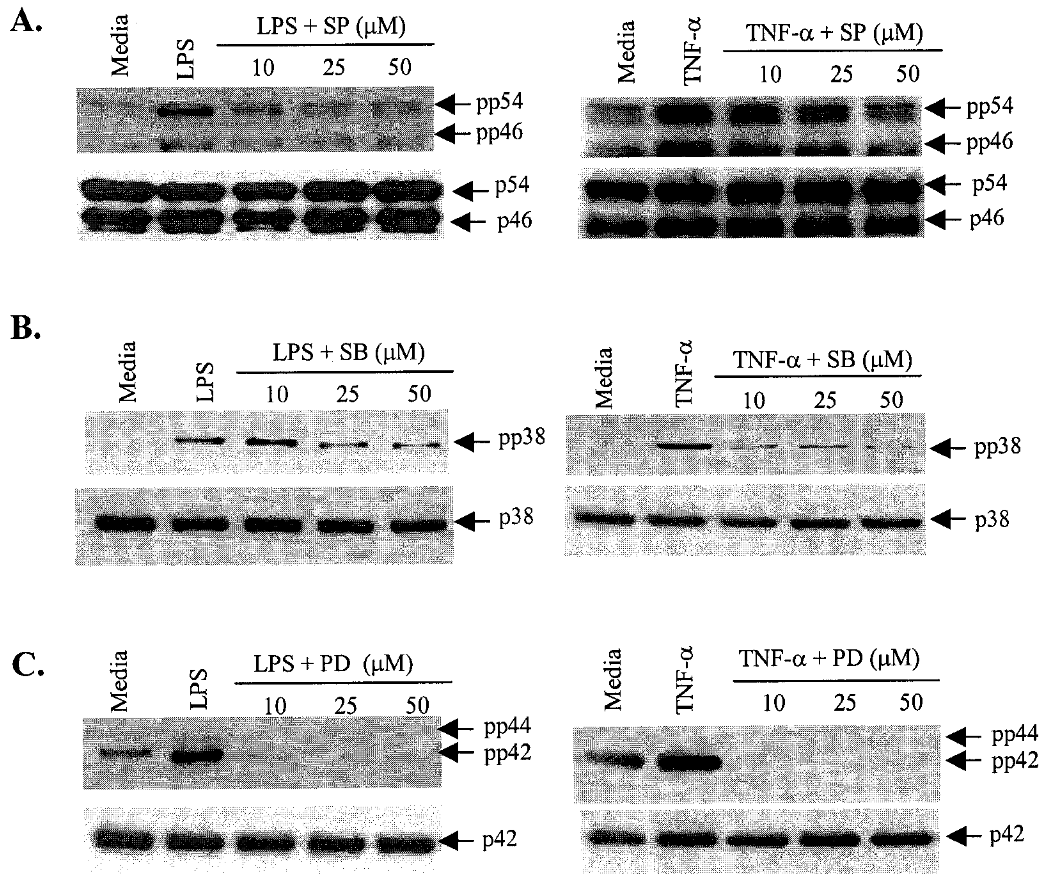
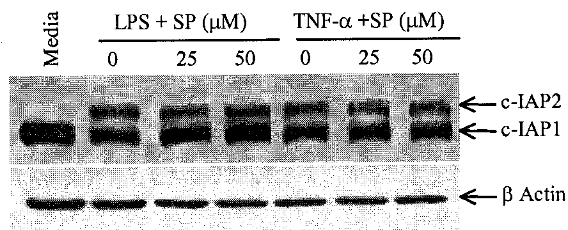

A.
Media
LPS
LPS + SP (μM)
10
25
50
pp54
pp46
p54
p46
Media
TNF-α
TNF-α + SP (μM)
10
25
50
pp54
pp46
p54
p46
B.
Media
LPS
LPS + SB (μM)
10
25
50
pp38
p38
Media
TNF-α
TNF-α + SB (μM)
10
25
50
pp38
p38
C.
Media
LPS
LPS + PD (μM)
10
25
50
pp44
pp42
p42
Media
TNF-α
TNF-α + PD (μM)
10
25
50
pp44
pp42
p42

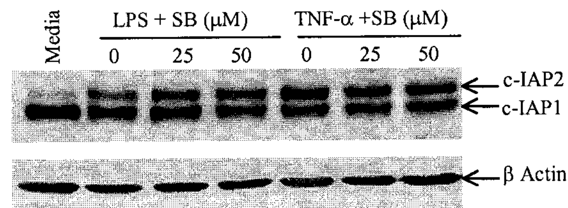
Fig. 3.3: JNK, p42/44 ERK and p38 MAPKs inhibitors do not affect c-IAP2 expression in LPS/TNF-α stimulated THP-1 cells:

THP-1 cells (1.0 X 10^6/ml) were pretreated with either A) SP600125, B) SB202190 or C) PD98059 at varying concentrations ranging from 0 to 50 μM for 2 h prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 24 hr. Crude protein extracts (30 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with c-IAP2 Abs. To ensure equal loading of protein, the membranes were stripped and reprobed with anti-β actin Abs. The experiments shown are representative of three different experiments.

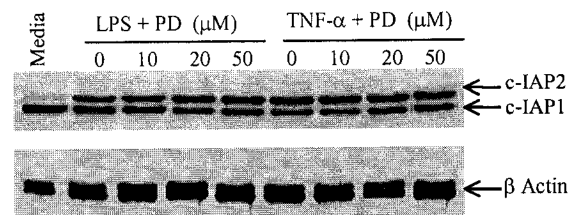
A.



B.



C.



2 hr followed by stimulation with either LPS or TNF-α for 24 hr. c-IAP2 expression was not inhibited by either Ly294002 or wortmannin at any concentration (Fig. 3.4C). These results suggest that LPS- and TNF-α-induced c-IAP2 expression in THP-1 cells does not involve the activation of either MAPK or PI3K.

LPS- and TNF-α- induced c-IAP2 expression is selectively regulated by calcium signaling pathway in monocytic cell:

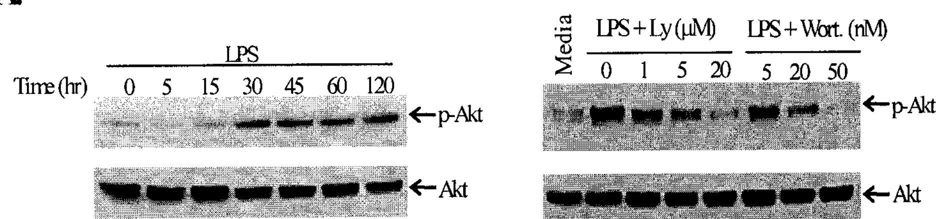
The changes in intracellular Ca^{2+} concentrations play a major role in transcription, protein synthesis and apoptosis [244]. Calcium signaling pathway also acts at the upstream of many other major signaling pathways. To determine the role of Ca^{2+} in LPS- and TNF-α-induced c-IAP2 expression, I first determined whether LPS and TNF-α activated calcium signaling by examining calcium influx by flow cytometry using Fluo-3 as a Ca^{2+} binding dye. The results show that LPS and TNF-α both induced calcium influx at 12 and 8 min poststimulation, respectively. To delineate the role of calcium in the regulation of LPS induced c-IAP2 expression, EGTA (2-10 mM), one of the calcium chelator was used. The results revealed that EGTA inhibited influx of calcium to the basal level (Fig 3.5A). To determine the role of Ca^{2+} in the regulation of c-IAP2 expression, I analyzed c-IAP2 expression in THP-1 cells treated with EGTA (2.5-10 mM) for 2 hr prior to stimulation with either LPS or TNF-α for 24 hr. LPS- as well as TNF-α-induced c-IAP2 expression was inhibited by EGTA in a dose dependent manner (Fig. 3.5B). EGTA at concentrations of 10 mM decreased the expression of c-IAP2 to undetectable levels following LPS stimulation and by 6 fold following TNF-α stimulation suggesting the involvement of Ca^{2+} signaling pathway in LPS- and TNF-α-induced expression of c-IAP2.

Fig. 3.4: PI3K inhibitors do not inhibit the expression of c-IAP2 expression in LPS/TNF-α stimulated THP-1 cells:

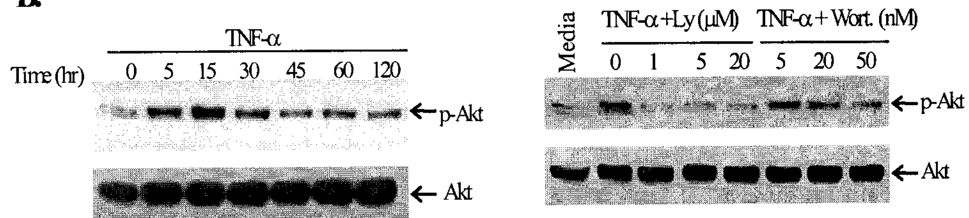
A & B. Left panel: THP-1 cells (1.0 X 10^6/ml) were stimulated with either LPS or TNF-α for a period of 0-120 minutes and cell extracts were subjected to Western blot analysis using anti-phospho-Akt Abs. To normalize equal loading of protein, the membranes were stripped and reprobed with anti-Akt Abs. **Right panel**: THP-1 cells (1.0 X 10^6/ml) were pretreated with either Ly294002 (1-20 μM) or wortmannin (5-50 nM) for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 15-30 min. Crude protein extracts (30 μg) were subjected to SDS-PAGE followed by Western blot analysis using anti-phospho-Akt Abs. To normalize equal loading of protein, the membranes were stripped and reprobed with anti-Akt Abs.

C. THP-1 cells (1.0 X 10^6/ml) were pretreated with either Ly29420 (1-20 μM) or wortmannin (5-50 nM)) for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 24 h. Crude protein extracts (30 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with c-IAP2 Abs. To ensure equal loading of protein, the membranes were stripped and reprobed with anti-β actin Abs. The experiments shown in A, B, and C are representative of three different experiments.

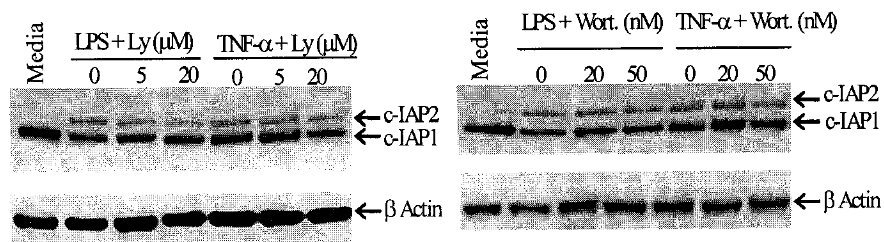
A.



B.



C.



Increase in cytoplasmic Ca^{2+} concentration occurs via diverse stimuli which activate voltage ligand-gated Ca^{2+} channels in the surface membrane or by release of Ca^{2+} contained in intracellular stores, mainly in endoplasmic reticulum (ER), and followed by extracellular Ca^{2+} entry [244;298]. To check the possibility that Ca^{2+} release from intracellular store could regulate c-IAP2 expression, I used one of the inositol 1,4,5-triphosphate receptor (IP_3R) inhibitor, 2-APB that inhibits the release of Ca^{2+} from ER [284]. Our results show that 2-APB even at high concentration (100 μM) did not inhibit either LPS- or TNF-α-induced c-IAP2 expression (Fig. 3.5C). In the next series of experiments, I investigated the role of receptor-mediated entry of extracellular Ca^{2+} following LPS or TNF-α stimulation by employing SKF-96365, a specific inhibitor for receptor-mediated Ca^{2+} entry [283]. Cells were pretreated with SKF-96365 for 2 hr prior to stimulation with either LPS or TNF-α. SKF-96365 treatment significantly reduced both LPS- and TNF-α-induced c-IAP2 expressions in a dose dependent manner (Fig. 3.5D). SKF-96365 and 2-APB were biologically active as both of these agents inhibited TNF-α-induced CD44 expression (data not shown). Taken together, these results suggest that receptor-mediated Ca^{2+} entry rather than the Ca^{2+} release from the ER may be involved in LPS- and TNF-α-induced c-IAP2 expression.

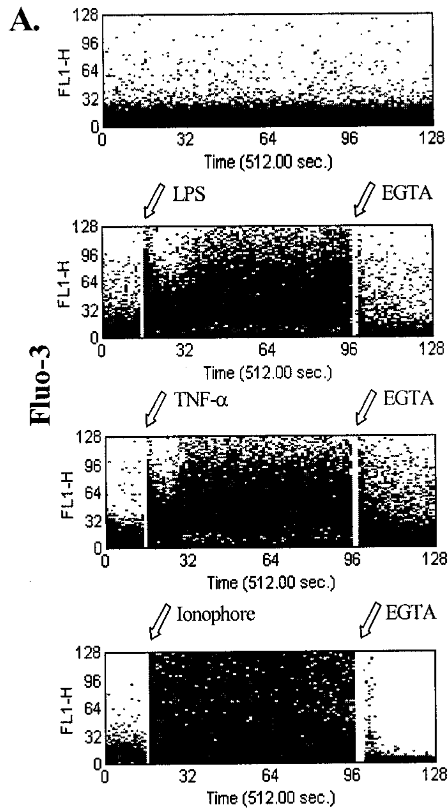
CaM and CAMKII regulate LPS- and TNF-α-mediated c-IAP2 expression:

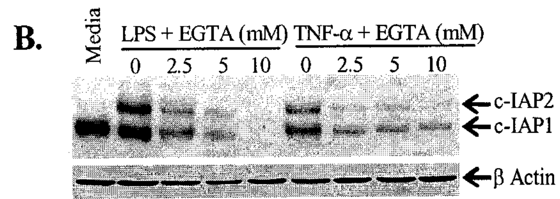
CaM, a 17 kDa protein that serves as a major Ca^{2+} receptor present in cytoplasmic and nuclear compartments, regulates several downstream targets such as protein kinases and protein phosphatases [299]. To understand the role of CaM, I examined whether its

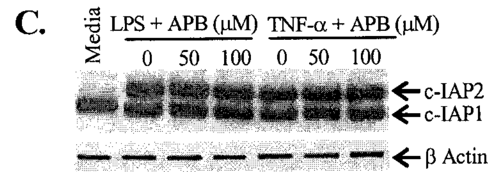
Fig. 3.5: Involvement of receptor-mediated Ca^{2+} entry rather than the Ca^{2+} release from endoplasmic reticulum in LPS- and TNF-α-induced c-IAP2 expression in THP-1 cells:

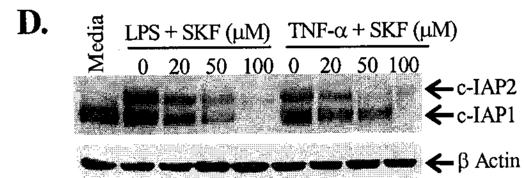
A. Stimulation of THP-1 cells with either LPS or TNF-α induces Ca^{2+} influx. THP-1 cells (0.5 x 10^6/ml) loaded with Fluo3/AM were stimulated with either LPS or TNF-α and the resulting Ca^{2+} influx was measured by flow cytometric analysis. **Top panel**: Baseline Ca^{2+} levels in unstimulated cells. **2nd panel:** Stimulation with LPS followed by the addition of EGTA. **3rd panel:** Stimulation with TNF-α followed by the addition of EGTA; **Bottom panel:** Stimulation with the Ca^{2+} ionophore A23187 followed by the addition of EGTA.

B,C, & D. Cells (1 X 10^6/ml) were pretreated with either **(B)** EGTA, **(C)** 2-APB, or **(D)** SKF-96365 for 2 hr prior to stimulation with either LPS (1 µg/ml) or TNF-α (10 ng/ml) for 24 h followed by analysis of c-IAP2 expression by Western blot analysis. To ensure equal loading of protein, the membranes were stripped and reprobed with anti-β-actin antibodies. The experiments shown in A, B, C, and D are representative of three different experiments.


A.
Fluo-3
FL1-H
Time (512.00 sec.)
LPS
EGTA
TNF-α
Ionophore


B.
Media
LPS + EGTA (mM)
TNF-α + EGTA (mM)
0 2.5 5 10 0 2.5 5 10
c-IAP2
c-IAP1
β Actin


C.
Media
LPS + APB (μM)
TNF-α + APB (μM)
0 50 100 0 50 100
c-IAP2
c-IAP1
β Actin


D.
Media
LPS + SKF (μM)
TNF-α + SKF (μM)
0 20 50 100 0 20 50 100
c-IAP2
c-IAP1
β Actin

specific inhibitor are able to downregulate c-IAP2 expression in response to stimulation with LPS and TNF-α. The results show that W-7-Hydrochloride, a specific CaM antagonist [247], inhibited LPS- and TNF-α-induced c-IAP2 expression in a dose dependent manner (Fig. 3.6A Left panel). To further gain insight into the role of CaM pathway, I examined the involvement of CAMKII which is activated subsequently to the binding of Ca^{2+} to CaM by employing its specific inhibitor KN-93 [247;299]. To determine the role of CAMKII in c-IAP2 induction, cells were treated with KN-93 for 2 h followed by stimulation with either LPS or TNF-α for 24 hr. KN-93 inhibited both LPS- and TNF-α-induced c-IAP2 expressions by 7- and 11- fold respectively and in a dose dependent manner (Fig. 3.6A Middle & Right panel). I also demonstrated that stimulation of cells with either LPS or TNF-α for 30 min induced CAMKII activity. To determine the biological activity of KN-93 and W-7, cells were treated with these inhibitors at varying concentrations for 2 hr followed by stimulation with LPS or TNF-α for 30 min. The results show that both W-7 and KN-93 inhibited LPS- and TNF-α-induced CAMKII activity in a dose dependent manner (Fig. 3.6B).

To confirm the involvement of CAMKII in LPS and TNF-α-induced c-IAP2 expression, cells were transfected with a DN CAMKII plasmid or a control vector. c-IAP2 expression was significantly inhibited by 4 fold in cells transfected with the DN CAMKII plasmid following LPS as well as TNF-α stimulation compared to the cells transfected with the control vector (Fig 3.6C). In addition, LPS- and TNF-α-induced CAMKII activity was inhibited by transfecting cells with DN CAMKII plasmid. Following transfection with the DN CAMKII plasmid, CAMKII activity was observed as 3 ± 1.0 and 2.8 ± 1.0 pM following stimulation with either LPS or TNF-α, respectively

compared to the activity of 6.5 ± 1.0 pM in LPS- and 5.5 ± 1.0 pM in TNF-α-stimulated cells transfected with the control vector.

Calcineurin is also activated by the binding of Ca^{2+} to CaM, which dissociates the two components and allows the catalytic site of calcineurin to become accessible [247;300]. To determine the role of calcineurin, cells were treated with cyclosporine A (Cyclo) or FK-506, the inhibitors of calcineurin, prior to stimulation with either LPS or TNF-α. Neither cyclosporine A nor FK-506 inhibited LPS- or TNF-α-induced c-IAP2 expression at any concentration (Fig. 3.6D). The biological activity of cyclosporine A and FK506 was determined by analysis of NFAT4 expression in Jurkat T cells, as described [301;302]. PMA and ionomycin are potent activators of calcineurin and cause dephosphorylation of NFAT proteins. Cyclo and FK506 are potent inhibitors of calcineurin phosphatase activity and restore PMA- and ionomycin induced NFAT dephosphorylation and thus, NFAT mediated gene induction. My results show that NFAT4 phosphorylation was significantly reduced after stimulation of Jurkat T cells with PMA and ionomycin. Pretreatment of cells with either Cyclo or FK506 prior to stimulation with PMA and ionomycin restored NFAT4 phosphorylation (Fig. 3.6E). These results suggest that calcineurin is not involved in c-IAP2 induction, however, LPS and TNF-α-induced c-IAP2 expression is regulated by calmodulin through the activation of CAMKII. It should be noted that none of these inhibitors were found to be apoptotic at the concentrations used as determined by PI staining (data not shown). Based on all these findings, it is concluded that c-IAP2 expression is regulated by activation of CAMKII through influx of calcium via CaM.

Fig. 3.6: CaM and CAMKII regulate LPS- and TNF-α-induced c-IAP2 expression:

A. LPS- and TNF-α-induced c-IAP2 expression is mediated by CaM and CAMKII. THP-1 cells (1 X 10^6/ml) were treated with either W-7 or KN-93 for 2 h prior to stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 h followed by determination of c-IAP2 expression by Western blot analysis.

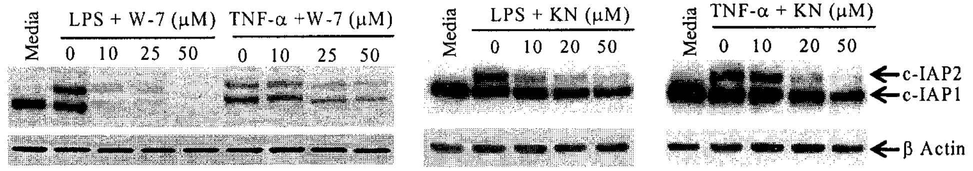
B. LPS- and TNF-α-induced CAMKII activity is inhibited by W-7 and KN-93. THP-I cells were pretreated with inhibitors for 2 h followed by stimulation of cells with either LPS or TNF-α for 30 min. CAMKII activity was assayed from total cell proteins utilizing a peptide substrate (KKALRRQETVDAL) specific for CAMKII. The results shown represent the mean ± S.D. of three independent experiments (* $p<0.01$).

C. Cells were transfected with either DN CAMKII or control vector and cultured for 24 h, followed by stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for another 24 h. c-IAP2 expression was determined by Western blot analysis.

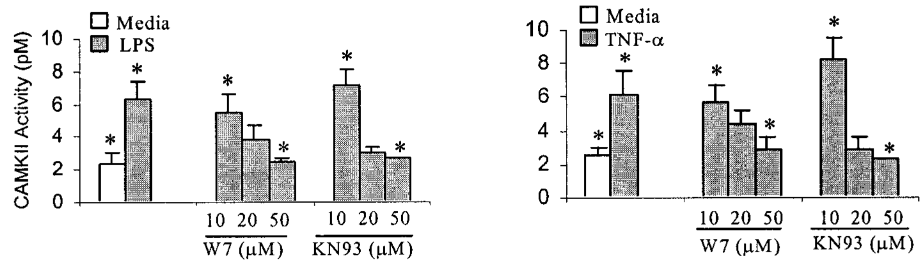
D. Cyclo and FK506 do not induce LPS- or TNF-α-mediated c-IAP2 expression. THP-1 cells (1 X 10^6/ml) were treated with various concentrations of either Cyclo or FK506 for 2 h prior to stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 h followed by determination of c-IAP2 expression by Western blot analysis.

E. Jurkat T cells (1 X 10^6/ml) were treated with either Cyclo or FK506 for 2 h prior to stimulation with PMA (*P*) and ionomycin (*I*) for 5 min followed by determination of expression of NFAT4 by employing anti-NFAT4 antibodies by Western blot analysis. To ensure equal loading of protein, the membranes were stripped and reprobed with anti-β-actin antibodies. All of the experiments shown above are representative of three different experiments.

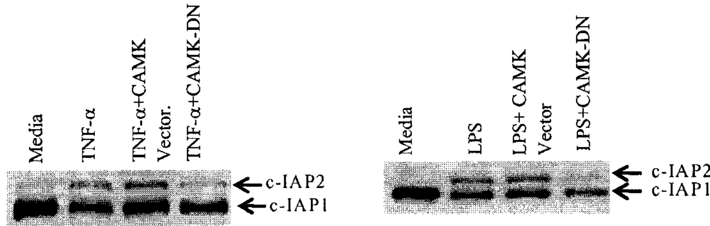
A



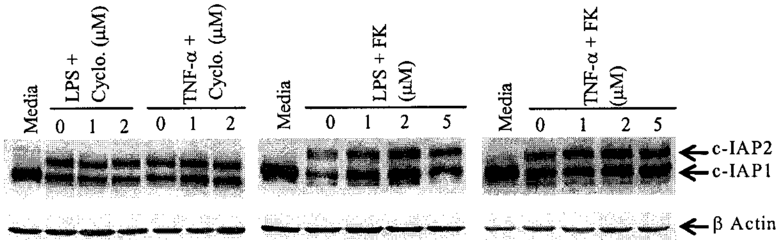
B.



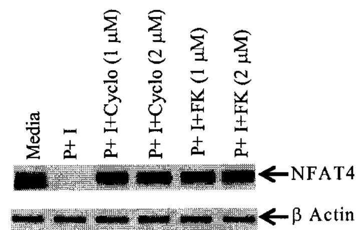
C.



D.



E.



Involvement of the NFκB binding sites within the c-IAP2 promoter in the regulation of LPS- and TNF-α-induced c-IAP2 expression:

c-IAP2 gene transcription has been shown to be regulated by the activation of NFκB transcription factor. NFκB is a heterodimer of rel proteins consisting of a DNA binding subunit (p50) and a transactivation domain (relA/p65) [303;304]. NFκB is normally present in the cytoplasm in association with IκB. IκB is degraded by phosphorylation by NFκB inducing kinase (NIK) and allow the NFκB to enter into the nucleus for further transcription [303]. To understand the role of NFκB in LPS- and TNF-α-induced c-IAP2 expression, THP-1 cells were transfected with c-IAP2 promoter (+121 to -606 bp) linked to the luciferase reporter construct (pc-IAP2Pr-GL3B) followed by stimulation with either LPS or TNF-α. The results show that in response to LPS as well as TNF-α, significant six to ten fold increase in luciferase activity was observed at 24 hr following stimulation compared to the unstimulated cells (Fig 3.8A). A computer aided analysis of the c-IAP2 promoter sequence between +1 and -606 bp revealed the existence of 3 NFκB consensus sequences (Fig. 3.7). However, sites 1 and 3 have been shown to be important for c-IAP2 gene transcription. Therefore, NFκB sites 1 and 3 were mutated by site directed mutagenesis followed by cloning into the pGL3B vector. THP-1 cells transfected with the c-IAP2 promoter construct containing the NFκB mutant sites and linked to the luciferase reporter vector (pc-IAP2Pr-mNFκB-GL3B) were stimulated with either LPS or TNF-α. The results show that luciferase activity was significantly decreased in cells transfected with pc-IAP2Pr-mNFκB-GL3B compared to cells transfected with the wild type pc-IAP2Pr-GL3B (Fig. 3.8B). To further confirm the role of NFκB, cells were cotransfected with either IκB super repressor plasmid or the IkB

control vector along with the wild type pc-IAP2Pr-GL3B following which the cells were stimulated with either LPS or TNF-α. Results show that LPS or TNF-α stimulation of cells cotransfected with IκB super repressor and pc-IAP2Pr-GL3B plasmids resulted in a significantly decreased luciferase activity compared to the cells cotransfected with control IκB vector and the wild type pc-IAP2Pr-GL3B (Fig. 3.8B). The role of NFκB was also confirmed by treating THP-1 cells transfected with the wild type pc-IAP2Pr-GL3B with CAPE, the broad spectrum NFκB inhibitor for 2 hr prior to stimulation with either LPS or TNF-α followed by detection of c-IAP2 expression by luciferase reporter assay. The results show that treatment of cells with CAPE inhibited LPS- and TNF-α-induced c-IAP2 expression in a dose dependent manner as determined by luciferase activity (Fig. 3.8C). Treatment of THP-1 cells with CAPE prior to stimulation with either LPS or TNF-α also resulted in downregulation of c-IAP2 expression as determined by Western blot analysis. CAPE at a concentration of 100 μM decreased the expression of c-IAP2 to undetectable levels following LPS stimulation and by 5 fold following TNF-α stimulation (Fig. 3.8D). Taken together, these results suggest that NFκB activation plays a key role in LPS- and TNF-α-induced c-IAP2 expression in THP-1 cells.

CaM and CAMKII regulate c-IAP2 expression in LPS- and TNF-α-stimulated THP-1 cells through the activation of NFκB:

It has been previously demonstrated that CAMKII acts as a mediator of IKK activation specifically in response to T cell receptor/CD3 and phorbol ester stimulation [305;306]. The above results suggest that LPS and TNF-α-induced c-IAP2 expression is regulated by CAMKII through the activation of CaM and Ca^{2+} influx. To gain further insights into signaling pathways involved in c-IAP2 expression, we investigated whether

Fig. 3.7: Nucleotide sequence of the first exon and 5' flanking promoter region of c-IAP2 gene (GeneBank accession No. AF070674). NFκB binding sites are boxed. Mutation is underlined and in bold type.

-606 at ctttaaaatg gtaaaaataa actgcaaagg agaactgcat gattttttc acatacccct

-544 acatttcctt tcacctctta ctttcttgat cagaacaaaa agtaaaaata aatagaaata

-484 tttcacaaag tttcgatttt ttttttttta aatgctggac ttctgcagct atagtagaag

-424 attgaaaaac ctaacctttt tacgtgtaaa gtgtatggcg gatggagggt ggagaacagg

-364 gcatattgac cttttccagg caggctaagc aatgatcgtc ctctctatat gggttgttat

-304 caagatttcc tctgacccac gagcaatgaa gcaaatgtct ttcagtaaat gccgcgaaga

NFkB site# 1

-244 tatgccacgg ttaagagtca tgcttttggg **tcatggaaat c**^{A}**c**^{G}**ccga**gtgg gtttgccagg

NFkB site# 3

-184 ccactgatta agaggaagtg tgtgtggtta ttacc**gctgg agttc**^{A}**c**^{A}**ccta** agtcctaaaa

-124 ggaaagcacc agtgcacatg caaaccactg ggaggagtgc ggaacgcctg gtacagatag

-64 gggtggggat ttgggtgacg ca**tttaaaa**g acagcgtgag actcgcgccc tccggcacgg

-4 aaa**a** ggccaggcga caggtgtcgc ttgaaaagac tgggcttgtc cttgctggtg catgcgtcgt

+61 cggcctctgg gcagcaggtt tacaaaggag gaaaacgact tcttctagat ttttttttca

+121 gtttcttcta taaatcaaaa catctcaaaa tggagaccta aaatccttaa agggacttag

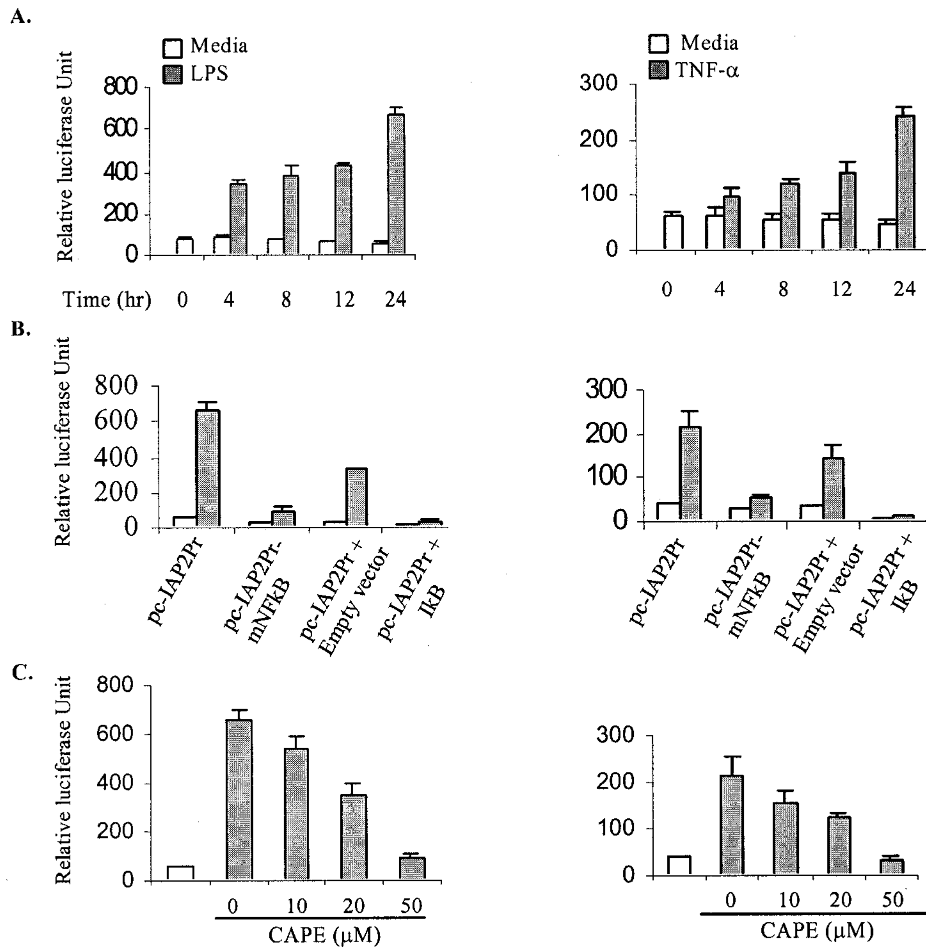
Fig. 3.8: Involvement of the NFκB binding sites within the c-IAP2 promoter in the regulation of LPS and TNF-α-induced c-IAP2 expression.

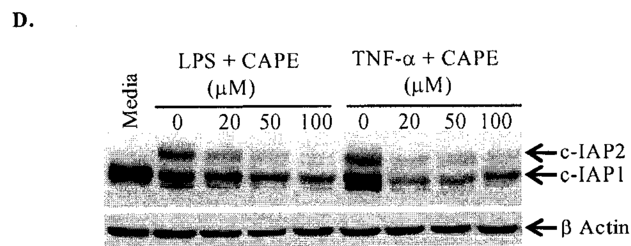
A. Kinetics of c-IAP2 promoter activity following LPS and TNF-α stimulation. THP-1 cells (10^6/ml) were transiently cotransfected with 5 μg of pc-IAP2pr-GL3B and 3 μg of β-galactosidase plasmid. After 24 h, cells were stimulated with either 1 μg/ml LPS or 10 ng/ml TNF-α for 0–24 h, followed by determination of luciferase and β-galactosidase activities. Luciferase activity was normalized for β-galactosidase activity to give relative luciferase units (RLU).

B. NFκB regulates c-IAP2 promoter activity. Cells (10^6/ml) were cotransfected with 5 μg of either WT pc-IAP2pr or NFκB mutant pc-IAP2pr-mNFκB, and 3 μgof β-galactosidase plasmid. Cells were also transfected with either the IκB superrepressor gene in pcDNA3 or vector alone. After 24 h, cells were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml), followed by determination of luciferase activity.

C. Cells (10^6/ml) cotransfected with 5 μg of pc-IAP2pr and 3 μg of β-galactosidase plasmid were pretreated with varying doses of CAPE (10–50 μM) for 2 h followed by stimulation with either 1 μg/ml of LPS or 10 ng/ml of TNF-α for 24 h. Cell lysates were analyzed for luciferase activity (relative luciferase units) as described for *A*. All of the results shown above are a mean ± S.D. of three experiments performed in triplicate.

D. Cells (10^6/ml) were pretreated with various concentrations of CAPE (20–100 μM) for 2 h prior to stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 h followed by determination of c-IAP2 expression by Western blot analysis. The experiment shown is representative of three different experiments.


A.
Media
LPS
Relative luciferase Unit
800
600
400
200
0
Time (hr) 0 4 8 12 24
Media
TNF-α
300
200
100
0
0 4 8 12 24
B.
Relative luciferase Unit
800
600
400
200
0
pc-IAP2Pr
pc-IAP2Pr-mNFkB
pc-IAP2Pr + Empty vector
pc-IAP2Pr + IkB
300
200
100
0
pc-IAP2Pr
pc-IAP2Pr-mNFkB
pc-IAP2Pr + Empty vector
pc-IAP2Pr + IkB
C.
Relative luciferase Unit
800
600
400
200
0
0 10 20 50
CAPE (μM)
300
200
100
0
0 10 20 50
CAPE (μM)


D.
Media
LPS + CAPE (μM)
0 20 50 100
TNF-α + CAPE (μM)
0 20 50 100
c-IAP2
c-IAP1
β Actin

CAMKII and CaM regulate LPS- and TNF-α-induced c-IAP2 expression through the activation of NFκB. To address this issue, I examined the effect of pharmacological inhibitors of CaM and CAMKII as well as of other inhibitors of calcium signaling pathway such as EGTA, APB, SKF-96365, W-7, KN-93, FK506, and Cyclo on the luciferase activity of cells transfected with the wild type pc-IAP2Pr-GL3B following stimulation with either LPS or TNF-α. THP-1 cells transfected with the full length pc-IAP2Pr-GL3B plasmid were treated for 2 hr with the above mentioned inhibitors of calcium signaling pathway prior to stimulation with either LPS or TNF-α for 24 hr followed by measurement of luciferase activity. As expected, pretreatment of cells with either EGTA or SKF-96365 reduced the LPS- or TNF-α-induced luciferase activity in a dose dependent manner (Fig. 3.9A, & B)). Similar to the results observed above, the involvement of CaM and CAMKII in the regulation of c-IAP2 was confirmed as CaM antagonist W-7 and CAMKII inhibitor KN-93 significantly reduced the luciferase activity in cells in a dose dependent manner (Fig 3.9A, & B). In contrast, pretreatment of cells with high concentrations of either 2-APB, FK506 or Cyclo under similar experimental conditions did not have any effect on luciferase activity as compared with LPS/TNF-α stimulated cells (Fig. 3.9A, & B). These results further suggest that CaM and CAMKII regulate LPS- and TNF-α-induced c-IAP2 transcription through the activation of NFκB.

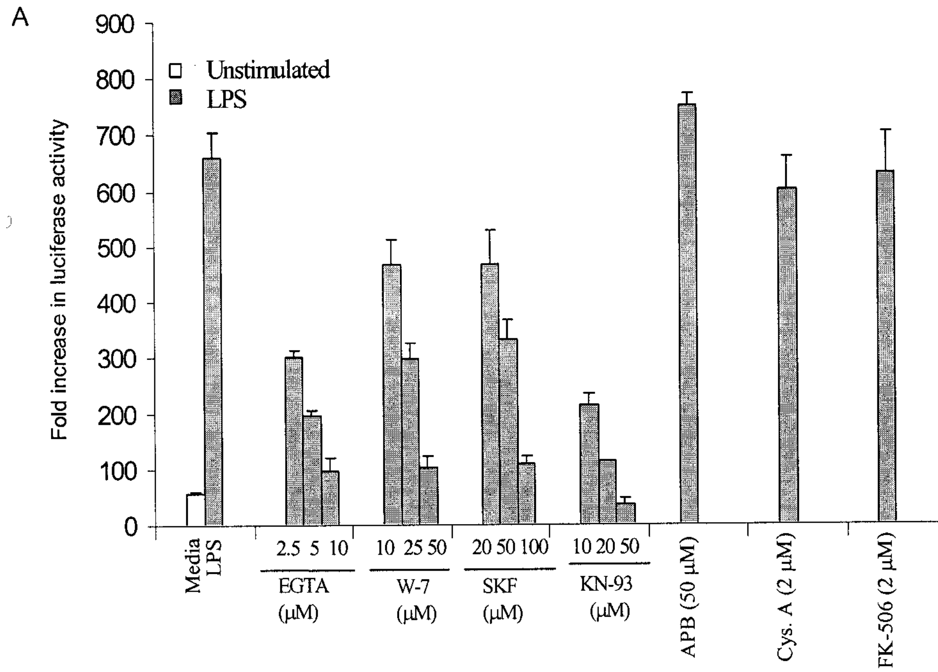
CaM and CAMKII regulate binding of transcription factor NFκB to NFκB binding sites of c-IAP2 promoter in LPS- and TNF-α-stimulated THP-1 cells:

To further confirm the role of calcium signaling pathway and in particular the role of CaM and CAMKII in the regulation of c-IAP2 gene transcription through NFκB activation, I investigated whether LPS and TNF-α-stimulation of THP-1 cells induced the

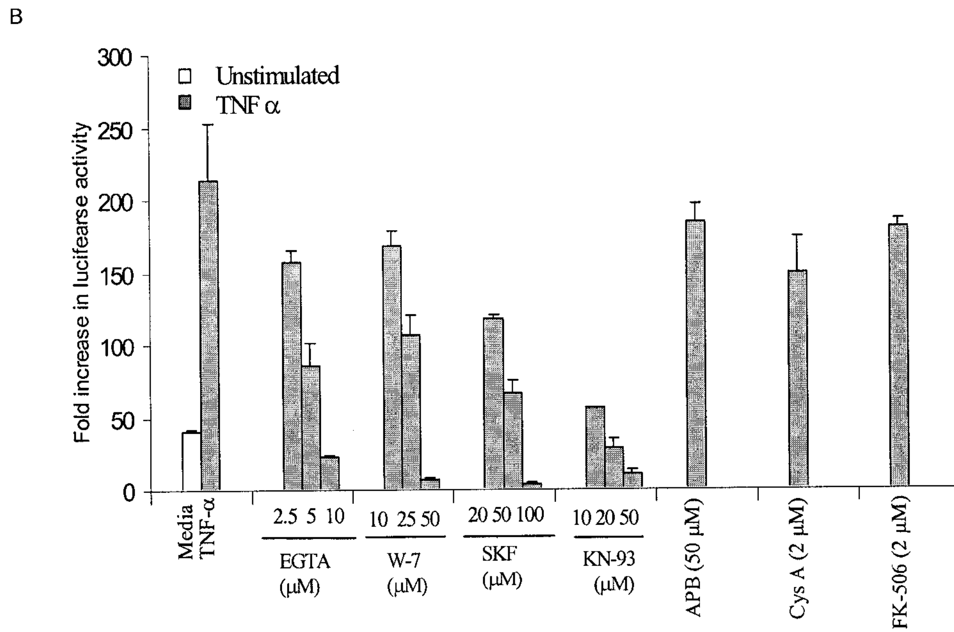
Fig. 3.9: CaM and CAMKII regulate c-IAP2 expression in LPS- and TNF-α-stimulated THP-1 cells through NFκB activation.

Cells (10^6/ml) were cotransfected with 5 μg of wild type pc-IAP2pr and 3 μg of β-galactosidase plasmid. After 24 h, the transfected cells were pretreated with varying concentrations of either EGTA, W-7, SKF-96365, KN-93, APB, Cyclosporin A or FK-506 for 2 h followed by stimulation with 1 μg/ml LPS or 10 ng/ml TNF-α for 24 h. Cell lysates were assessed for luciferase activities (relative luciferase units; RLU) as described above in the legend of 3.7. The results shown are a mean ± S.D. of three experiments performed in triplicate.

A



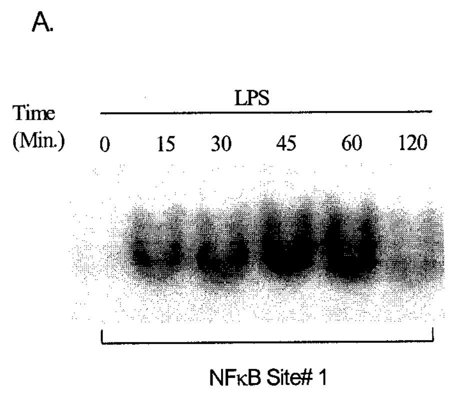
B

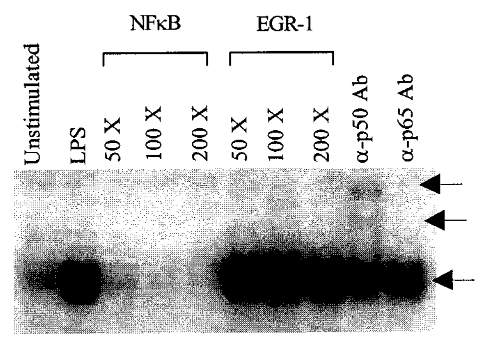


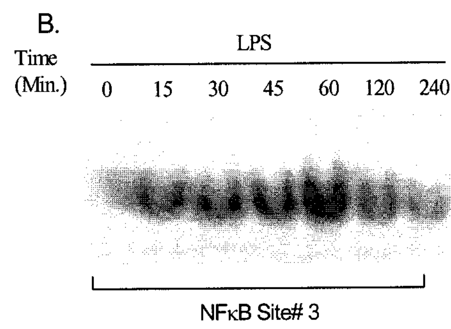
binding of NFκB to its binding sites 1 and 3 present in the c-IAP2 promoter. THP-1 cells were stimulated with LPS or TNF-α over a period of time ranging from 0-240 min. The nuclear extracts thus harvested from cells stimulated with either LPS or TNF-α for 0-4 hr were analyzed by electrophoretic mobility shift assay for binding of NFκB to NFκB oligonucleotide probes corresponding to their sites in the c-IAP2 promoter. The results show a significant binding of NFκB to site # 1 and # 3 in 45-60 min following stimulation of cells with either LPS or TNF-α (Fig. 3.10, left panels). The specificity of NFκB binding was demonstrated by cold competition experiments employing specific and non-specific oligonucleotides and by super shift analysis with anti NFκB p50 and p65 antibodies (Fig. 3.10, right panels). To determine whether binding of NFκB to the NFκB binding sites # 1 and # 3 in the c-IAP2 promoter was regulated by the pharmacological inhibitors of the calcium signaling pathway, THP-1 cells were treated with pharmacological inhibitors of CaM and CAMKII as well as of other inhibitors of calcium signaling pathway such as EGTA, APB, SKF-96365, FK506, and Cyclo for 2 hr before stimulation with either LPS or TNF-α for 60 and 45 min, respectively, followed by analysis of NFκB binding to its corresponding oligonucleotide probes. The results suggest that both EGTA and SKF-96365 inhibited binding of NFκB to NFκB oligonucleotide probes corresponding to both NFκB sites #1 and # 3, in LPS and TNF-α stimulated THP-1 cells (Fig. 3.11A, & B). The involvement of CaM and CAMKII in c-IAP2 transcription was similarly demonstrated by showing that pretreatment of cells with either W-7 or KN-93 inhibited the binding of NFκB to its sites on c-IAP2 promoter in both LPS and TNF-α-stimulated THP-1 cells (Fig. 3.11A, B, & C). Taken together, the

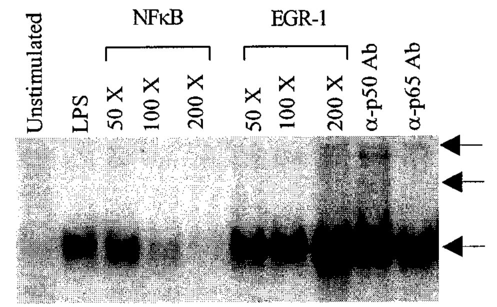
Fig. 3.10: A, B, C, & D. NFκB transcription factor binds to the site 1 and 3 of NFκB on the c-IAP2 promoter in LPS- and TNF-α-stimulated cells.

Cells were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 0-240 min for time course and cells were also stimulated with LPS for 60 min and TNF-α for 45 min for rest of the experiments. Nuclear proteins (5 μg) were incubated with ^{32}P-labeled oligonucleotide probes corresponding to NFκB site 1 and 3 sequences derived from the c-IAP2 promoter. The specificity of NFκB binding was determined by incubating nuclear proteins with unlabeled NFκB or nonspecific Egr-1 oligonucleotides. The supershift analysis was performed by treating the nuclear proteins with oligonucleotide probes in the presence or the absence of anti-p50 or anti-p65 NFκB antibodies. The supershifted bands are indicated by arrows. The results shown are representative of three different experiments.


A.
LPS
Time
(Min.)
0
15
30
45
60
120
NFκB Site# 1


NFκB
EGR-1
Unstimulated
LPS
50 X
100 X
200 X
50 X
100 X
200 X
α-p50 Ab
α-p65 Ab


B.
LPS
Time
(Min.)
0
15
30
45
60
120
240
NFκB Site# 3


NFκB
EGR-1
Unstimulated
LPS
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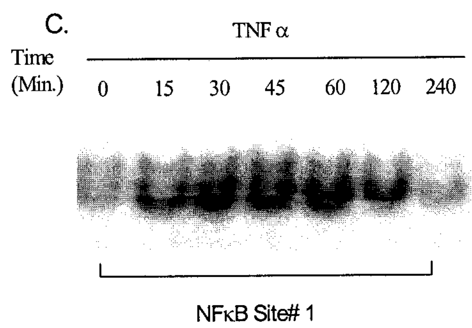
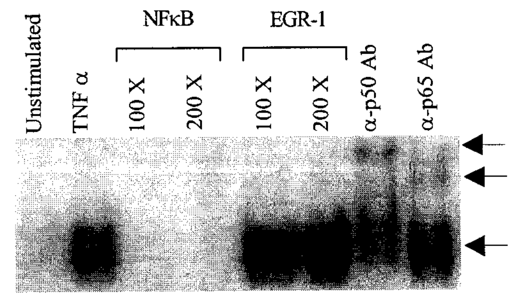
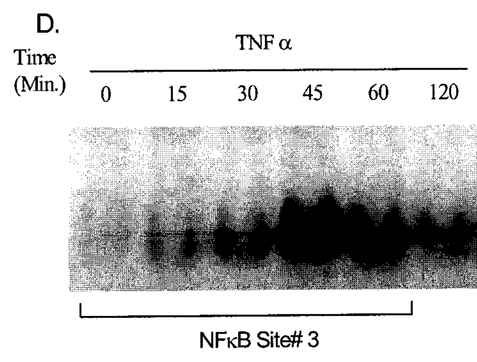
100 X
200 X
50 X
100 X
200 X
α-p50 Ab
α-p65 Ab


C.
TNF α
Time
(Min.)
0
15
30
45
60
120
240
NFκB Site# 1


NFκB
EGR-1
Unstimulated
TNF α
100 X
200 X
100 X
200 X
α-p50 Ab
α-p65 Ab


D.
TNF α
Time
(Min.)
0
15
30
45
60
120
NFκB Site# 3

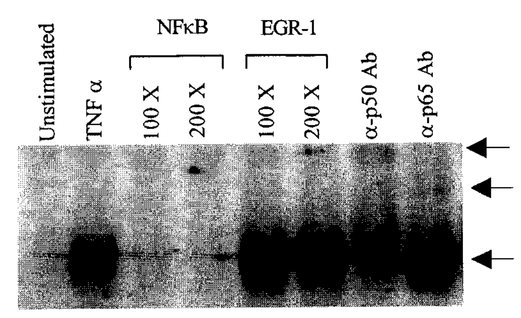
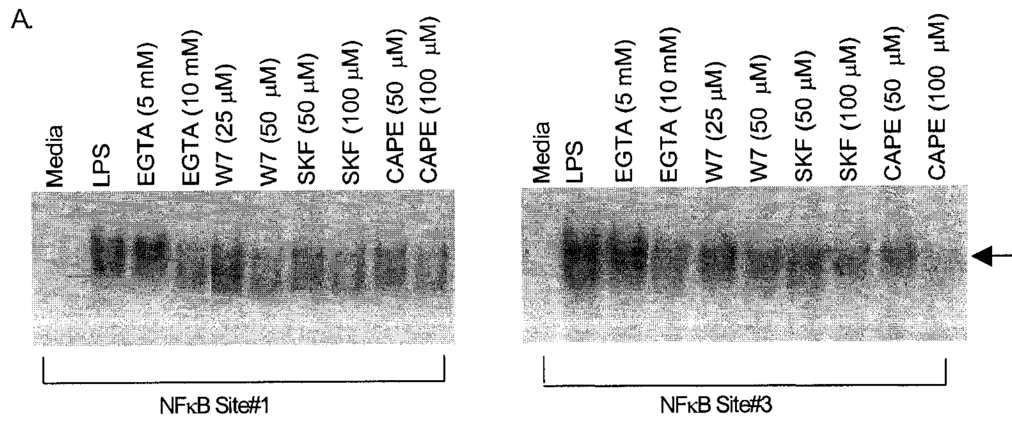

NFκB
EGR-1
Unstimulated
TNF α
100 X
200 X
100 X
200 X
α-p50 Ab
α-p65 Ab

Fig 3.11: A, B, & C. CaM and CAMKII regulate binding of NFκB to its binding sites on the c-IAP2 promoter in LPS- and TNF-α-stimulated cells.

THP-1 cells were pretreated with either EGTA, W-7, SKF-96365, CAPE, or KN-93, for 2 h followed by stimulation with 1 μg/ml LPS or 10 ng/ml TNF-α for 60 and 45 min, respectively. Nuclear proteins (5 μg) were analyzed for NFκB binding by ^{32}P-labeled oligonucleotide probes corresponding to site 1 and 3 sequences. The results shown are representative of three different experiments.

A.



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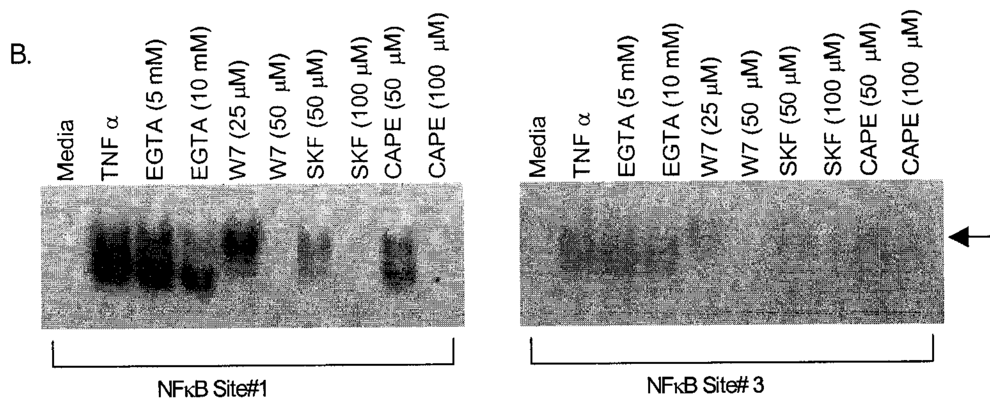
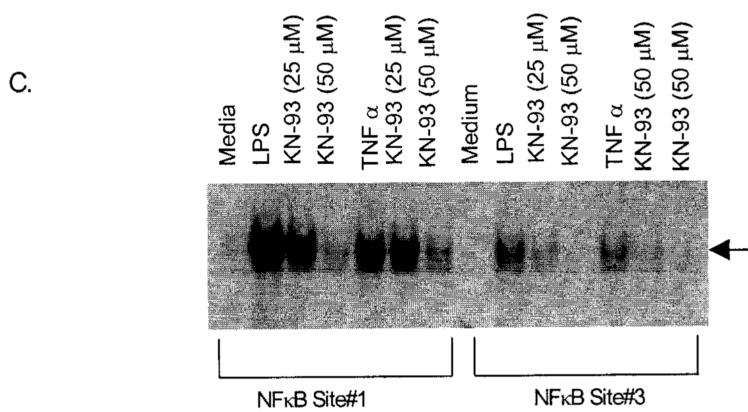


C.



results suggest that LPS- and TNF-α-induced c-IAP2 gene transcription may be selectively regulated by CaM and CAMKII through the activation of NFκB.

CaM and CAMKII regulate LPS- and TNF-α-mediated inhibition of apoptosis in THP-1 cells:

In view of the above results suggesting the involvement of CaM and CAMKII in c-IAP2 expression, I determined whether CaM and CAMKII also regulated LPS- and TNF-α-mediated inhibition of apoptosis in THP-1 cells. Cells were treated with various inhibitors of the calcium signaling pathway for 2 hr before stimulation with either LPS or TNF-α followed by analysis of staurosporine-induced apoptosis by PI staining. As expected, stimulation with either LPS or TNF-α inhibited staurosporine-induced apoptosis in a dose dependent manner. Significantly, prior treatment of cells with EGTA, SKF-96365, W-7, and KN-93 reversed the LPS- and TNF-α-mediated inhibition of staurosporine induced apoptosis, whereas inhibitors of MAPK, PI3K, and calcineurin did not have any effect (Fig. 3.12A).

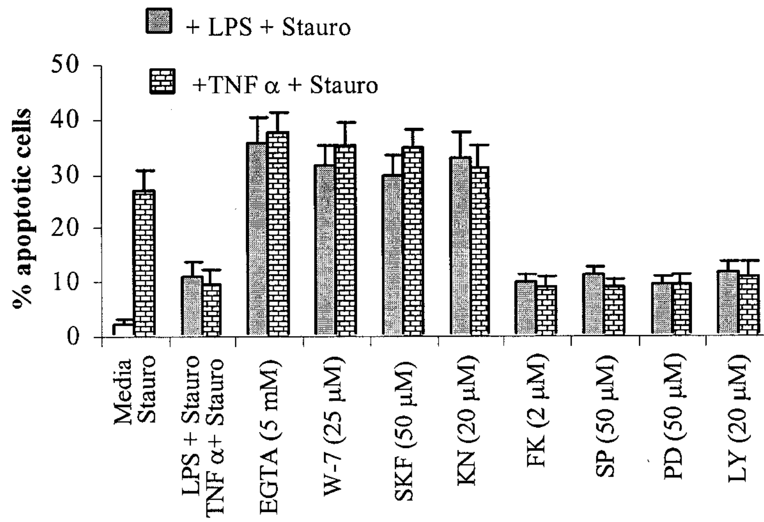
To further confirm the involvement of CAMKII in LPS- and TNF-α-mediated inhibition of apoptosis, THP-1 cells were transfected with a DN CAMKII mutant construct. After 24 hr, transfected cells were stimulated with either LPS or TNF-α followed by analysis of staurosporine-induced apoptosis. Stimulation with either LPS or TNF-α of cells transfected with control vector inhibited staurosporine-induced apoptosis. In contrast, transfection of cells with DN CAMKII mutant construct reversed the LPS- and TNF-α-mediated inhibition of staurosporine-induced apoptosis (Fig 3.12B).

Fig. 3.12: A. CaM and CAMKII regulate LPS- and TNF-α-mediated inhibition of apoptosis in THP-1 cells.

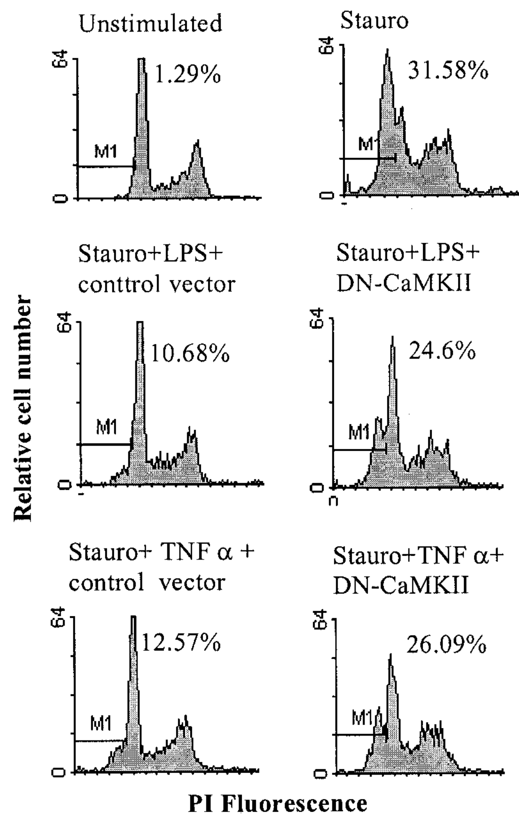
A. Cells (1 X 10^6/ml) were pretreated with indicated doses of either EGTA, W-7, SKF-96365, KN-93, FK506, SP600125, PD98059, or LY294002 for 2 h prior to stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml). After 24 h, cells were treated with staurosporine (2 μM) for 4 h followed by analysis of apoptotic cells by PI staining and flow cytometry. The results shown are mean ± S.D. of three different experiments.

B. DN CAMKII reverses LPS- and TNF-α-mediated inhibition of staurosporine-induced apoptosis in THP-1 cells. Cells (1 X 10^6/ml) were transfected with either 5 μg of DN-CAMKII plasmid or control vector and cultured for 24 h followed by stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for another 24 h. Stimulated cells were treated with staurosporine (Stauro)(2 μM) for 4 h, following which DNA content of cells was analyzed by PI staining. The values shown in the histograms indicate percentage of apoptotic cells. The experiment shown is representative of three different experiments.

A.



B.



Overall, my findings suggest that c-IAP2 is induced by LPS through the endogenous production of TNF-α and it is regulated selectively by CAMKII through activation of NFκB. c-IAP2 also plays a key role in monocytic cell survival.

Discussion:

Monocytes/macrophages play a central role in orchestrating the immune response to infection and tumors. In the absence of appropriate stimulus, monocytes are programmed to undergo a sequence of events leading to apoptosis. Modulation of monocyte apoptosis by either endotoxin or pro-inflammatory cytokines may be closely associated with the outcome of inflammation. Both LPS and TNF-α have been shown to induce the expression of various anti-apoptotic proteins including members of the Bcl2 and IAP families in various cell types such as Jurkat T cells, primary endothelial cells and human monocytic cells [182;189-191;197;294]. However, the molecular mechanism by which LPS and TNF-α induce anti-apoptotic activity in monocytic cells remains unknown. I have demonstrated that LPS- and TNF-α-induced c-IAP2 expression and its associated anti-apoptotic survival signals in THP-1 cells are regulated selectively by CaM/CAMKII through NFκB activation.

It is well known that cytokines produced during inflammation affect monocyte survival. LPS, a component of bacterial endotoxin and a potent inducer of proinflammatory cytokine TNF-α, has been shown to prevent apoptosis. TNF-α has also been shown to prevent apoptosis in *Mycobacterium tuberculosis* infected monocytes. I have investigated the role of IAP family of anti-apoptotic genes in LPS-/TNF-α-induced resistance to apoptosis. My results revealed that c-IAP1 is constitutively expressed in THP-1 cells, whereas, the level of expression of c-IAP2 is very low, even sometimes non-detectable. It is interesting to observe that c-IAP1 and c-IAP2 are differentially induced following stimulation with LPS and TNF-α in THP-1 cells suggesting a cell type

specific role for these molecules. Specific inhibition of c-IAP2 by antisense oligonucleotides reversed LPS- and TNF-α-mediated protection from staurosporine-induced apoptosis suggesting a key role for c-IAP2 in this anti-apoptotic effect. Furthermore, LPS-induced c-IAP2 expression was regulated by the endogenous production of TNF-α. The IAP family of proteins has been shown to suppress apoptosis induced by a variety of stimuli including TNF-α, Fas, and growth factor withdrawal by inhibiting activation of procaspase-9, and active caspase-3 and caspase-7 [164;170]. In addition, c-IAP2 appears to be a part of the signaling complex recruited to the cytoplasmic domain of TNFR by binding to the TRAF-1/TRAF-2 heterocomplexes and functions to suppress caspase-8 activation [161;164;168]. These observations suggest that LPS- and TNF-α-induced c-IAP2 may inhibit apoptosis by acting on caspases in THP-1 cells.

LPS exerts its effects following its association with the plasma-LPS-binding protein and the binding of this complex with the CD14/TLR-4 complex. Therefore, regulation of LPS-induced c-IAP2 expression in monocytic cells may involve a combination of signals delivered by the interaction of LPS with the CD14/TLR complex as well as by the interaction of endogenously produced TNF-α with its cognate receptors TNFRI and TNFRII. Although TNF-induced cell death is mediated primarily by TNFRI, both TNF receptors can transduce signals leading to the activation of transcription factors including NFκB that help cells to survive. It is believed that NFκB activation leads to the upregulation of many TNF responsive genes such as c-IAP2. The molecular signaling pathways particularly the upstream signaling molecules involved in upregulation of c-

IAP2 in monocytic cells in response to stimulation with either LPS or TNF-α are not known.

Apoptosis has been shown to be regulated by a number of upstream protein kinases activation including MAPK and PI3K in epithelial and leukemic cells [168;196;297;297;307;308]. Recently, JNK was shown to mediate the anti-apoptotic activity of XIAP [196], whereas p38 and ERK MAPKs were shown to regulate c-IAP2 expression in colon cancer cell lines [198]. I first investigated the role of MAPK and PI3K in LPS- and TNF-α-induced c-IAP2 expression. Although both LPS- and TNF-α-induced the activation of p38, ERK, and JNK MAPK, and PI3K in this study, none of these kinases were involved in LPS- or TNF-α-induced c-IAP2 expression or anti-apoptotic activity in monocytic cells. Specific MAPKs and PI3K inhibitors, even when used at very high concentrations failed to inhibit either LPS- or TNF-α-induced c-IAP2 expression.

Because of the lack of involvement of these major signaling pathways, I investigated the role of upstream Ca^{2+} signaling proteins, which are important intracellular messengers in many biological processes including apoptosis [244;298]. Influx of Ca^{2+} through ligand and voltage gated calcium channels in the plasma membrane together with Ca^{2+} release from ER stores, results in complex calcium signaling cascades [244;298]. Several mechanisms may control Ca^{2+} entry in response to external stimuli including membrane depolarization, activation of intracellular messengers, and depletion of intracellular calcium storage [299]. The release of Ca^{2+} from internal stores (ER) is controlled by Ca^{2+} itself or by an expanding group of messengers. For example, the inositol 1,3,4 triphosphate (IP3), produced in response to a

signal from the membrane lipid phosphatidyl inositol, triggers Ca^{2+} release from the ER after binding to the IP3 receptor [299].

Calcium signaling has been shown to play a key role in LPS- and TNF-α-induced regulation of several genes [309-315]. Both LPS and TNF-α have been shown to induce calcium flux by several laboratories [309-311;316-319]. Furthermore, lipid A component of LPS has been suggested to markedly enhance free intracellular calcium [316]. There is also evidence to suggest that the sources of increased cytosolic calcium are extracellular calcium as well as stored calcium from the endoplasmic reticulum [320]. To determine the role of Ca^{2+} signaling pathway, we first employed EGTA, the Ca^{2+} chelator, which is known to block the binding of Ca^{2+} to any other molecules. EGTA inhibited the expression of c-IAP2 to its basal level in a dose dependent manner in both LPS- and TNF-α-stimulated cells. I further examined the source of Ca^{2+} (extracellular/intracellular or both) involved in regulation of c-IAP2. To understand the role of extracellular Ca^{2+}, I employed SKF-96365, a Ca^{2+} channel blocker that prevents the entry of extracellular Ca^{2+} from the plasma membrane into the cytosol. SKF-96365 inhibited the induction of c-IAP2 in both LPS and TNF-α stimulated THP-1 cells. It is known that entry of extracellular Ca^{2+} is activated itself or by release of Ca^{2+} from intracellular stores (ER). To check the involvement of intracellular Ca^{2+} release, I used 2-APB, one of the IP3 receptor blockers on ER. I demonstrated that blocking of intracellular release of Ca^{2+} did not alter the c-IAP2 induction. The results of this study suggest that LPS- and TNF-α-mediated c-IAP2 expression and anti-apoptotic activity required extracellular influx of Ca^{2+} into the cytosol. However, calcium influx observed after LPS or TNF-α stimulation was observed several minutes after stimulation compared to some highly potent agents

such as ionomycin in which calcium influx is observed within seconds. Although LPS has been found to increase (Ca^{2+}) ion, and that the lipid A component has been specifically found to trigger calcium flux [316;321], the precise mechanism by which LPS and TNF-α impact calcium signaling is still not understood.

Calmodulin, a key signaling protein responsible for integrating the Ca^{2+} signal with transcription factor activation, is known to regulate cell cycle and related cytoskeletal functions and ion channel activity [299;322;323]. CaM is highly conserved among different species with 148 aa residues and a M.W. of 17 kD. Following binding to Ca^{2+}, CaM undergoes a conformational change that renders it active and able to recognize and bind target proteins with high affinity. To determine the role of CaM, I employed CaM antagonist, W-7, which inhibited c-IAP2 expression in both LPS- and TNF-α-stimulated THP-1 cells [299;323]. Amongst the possible downstream targets of CaM are calcineurin and CAMKII [324-326]. Since one of the targets of CaM is calcineurin, I further used two pharmacological inhibitors, Cyclo and FK-506 to demonstrate whether CaM regulates c-IAP2 expression via calcineurin activation. Neither inhibitor affected c-IAP2 expression suggesting that calcineurin is not involved in c-IAP2 regulation in both LPS- and TNF-α-stimulated THP-1 cells.

Another kinase known to be activated by CaM is CAMKII. Ca^{2+}/CAMKII was found to have relatively broad substrate specificities with respect to endogenous protein substrate in nervous system. CAMKII from rat brain is a large multimeric enzyme composed of two related subunits with 9α (50 kDa) and 3β (60 kDa), respectively. Like other kinases, it undergoes an autophosphorylation that seems to be an intramolecular process. The autophosphorylation of Ca^{2+}/CAMKII on a threonine residue contained in a

phosphopeptide common to α and β subunits converts it into a Ca^{2+}/CaM independent enzyme [191]. To determine the role of CAMKII, I used KN-93, a selective inhibitor of CAMKII. KN-93 selectively and directly binds to CaM binding site of CAMKII or its vicinity and prevents the association of CaM with CAMKII. My results show that KN-93 significantly inhibited c-IAP2 expression in a dose dependent manner. Furthermore, I confirmed the involvement of CAMKII by using DN CAMKII construct in c-IAP2 expression suggesting that CAMKII acts as a key link for CaM activation and c-IAP2 expression. I also demonstrated that LPS and TNF-α induced CAMKII plays an important role in mediating protection to staurosporine induced apoptosis. Taken together, my results clearly suggest that LPS- and TNF-α-induced c-IAP2 expression and its associated anti-apoptotic activity are regulated distinctly by calmodulin/CAMKII.

NFκB plays an important role in cell development, cell survival and oncogenesis and mediates its function through homodimer or heterodimers formed by NFκB/Rel family members (RelA/p65, RelB, C-Rel, NFκB1/p50 and NFκB2/p52) [303]. The activity of NFκB is strictly regulated by one of the IκB-inhibiting proteins such as IκBα or IκBβ, which form a complex with NFκB and keeps NFκB sequestrated in cytoplasm. Upon activation, IκB becomes phosphorylated, ubiquitinated and subsequently degraded by proteosome, enabling nuclear translocation of NFκB and participation in transactivation of a variety of genes [303;304]. Inhibition of NFκB, one of the upstream signaling pathways during TNF-α stimulation, has been shown to induce apoptosis in a variety of cell types originally resistant to TNF-α- induced apoptosis.

Intracellular mobilization of Ca^{2+} triggered by various stimuli is known to act as a key second messenger necessary for the induction of NFκB activity [247;324;326].

Furthermore, the protective role of NFκB against apoptosis is believed to be mediated by the induction of various anti-apoptotic genes including IAPs. NFκB has been shown to regulate c-IAP2 gene transcription in Jurkat T cells in response to stimulation with TNF-α. In this study, I demonstrate for the first time that LPS- and TNF-α-induced c-IAP2 expression is regulated by NFκB. It has been shown that two NFκB elements are required for promoter activity and they function cooperatively in mediating TNF-α-induced c-IAP2 promoter activity [191]. The involvement of both NFκB binding sites in the upregulation of LPS- and TNF-α-induced c-IAP2 expression was demonstrated by analysis of c-IAP2 promoter activity and gel shift assays. The involvement of NFκB was confirmed by employing its specific physiological inhibitor, CAPE, and by co-transfection of cells with IκB superrepressor and c-IAP2 promoter constructs.

Having shown the role of calcium and NFκB in LPS- and TNF-α-induced c-IAP2 expression, I examined whether LPS- and TNF-α-induced c-IAP2 expression is regulated by NFκB through intracellular calcium mobilization and subsequent activation of calmodulin/calmodulin kinase signaling pathway. My results clearly show that prevention of receptor-mediated Ca^{2+} entry by specific inhibitor SKF 96365 inhibited LPS- and TNF-α-induced c-IAP2 expression, NFκB activity in the c-IAP2 promoter and the binding of NFκB transcription factor to its binding sites on the c-IAP2 promoter. In contrast, blocking of Ca^{2+} release from ER failed to have any effect on the NFκB activity or NFκB binding to its promoter. This identified a calcium triggered signaling cascade which may stimulate p50/p65 NFκB transactivating potential eventually leading to c-IAP2 gene transcription. I therefore investigated if calmodulin and calmodulin kinase

could establish a link between receptor-mediated intracellular mobilization of Ca^{2+} on one hand and p50/p65 NFκB on the other. Surprisingly, I found that CaM and the CAMKII, one of the pathways linking calcium signal to downstream transcription factors, regulated c-IAP2 expression through the activation of NFκB. W-7 and KN-93, the CaM and CAMKII inhibitors, respectively, inhibited LPS- and TNF-α-induced c-IAP2 expression, NFκB activity in the c-IAP2 promoter and the binding of NFκB transcription factor to its binding sites on the c-IAP2 promoter. In contrast, calcineurin, the other pathway linking calcium with the downstream transcription factor, did not influence NFκB activity in the c-IAP2 promoter. Intracellular mobilization of calcium triggered by various stimuli is known to induce NFκB activity in different cell types suggesting that Ca^{2+} acts as a key second messenger necessary for the induction of NFκB activity. CAMKII has also been previously demonstrated to act as a mediator of IKK activation specifically in response to T cell receptor/CD3 and phorbol ester stimulation [305;306]. A recent report has also demonstrated that CAMKII transactivates p65 subunit of NFκB. In conclusion, in the present work, I show that intracellular mobilization of Ca^{2+} selectively activates CaM/CAMKII kinase pathway to induce NFκB phosphorylation and eventually c-IAP2 expression.

At present, the role of c-IAP1 in LPS- and TNF-α-mediated anti-apoptotic effect is not clear. Both c-IAP1 and c-IAP2 have been suggested to contribute towards apoptotic resistance of different cancers [199]. The genes encoding c-IAP1 and c-IAP2 share 75% homology at the level of nucleotide and amino acid sequence and are thought to have arisen from a gene duplication event [172]. The molecular mechanism involved in the regulation of c-IAP1 expression is not clear at present. Herein, I show that c-IAP1 is

expressed constitutively, and is not induced by either LPS or TNF-α. Furthermore, constitutive expression of c-IAP1 may be regulated selectively by the calcium pathway as its expression levels were down-regulated by EGTA, SKF, and W-7. However, c-IAP1 does not seem to be regulated by the CAMKII pathway as its inhibitor KN-93 inhibited LPS/TNF-α-induced expression of c-IAP2 alone. Since expression levels of c-IAP1 are not affected by antisense c-IAP2 oligonucleotides, LPS- and TNF-α-induced anti-apoptotic activity may not be regulated by c-IAP1. It is likely that c-IAP1 may have a role in spontaneous survival of THP-1 cells. Furthermore, the role of other inhibitory proteins including Bcl2 and other members of the IAP family in LPS- and TNF-α-induced anti-apoptotic activity can not be ruled out and needs further investigation.

In summary, this is the first study that demonstrates the involvement of c-IAP2 in LPS and TNF-α-induced anti-apoptotic survival of human monocytic cells. My results clearly suggest that LPS- and TNF-α-induced c-IAP2 expression and its associated anti-apoptotic activity are regulated distinctly by CaM/CAMKII through the activation of NFκB pathway. Since c-IAP2 is one of the important anti-apoptotic genes in cell apoptosis and recruited to TNFR signaling complex, understanding the regulation of this gene and characterization of signal transduction pathways may help in providing insight into general TNF signaling in regulation of NFκB, one of the prosurvival transcription factors in various cell types. In addition to that, modulation of signaling molecules involved in c-IAP2 upregulation may be helpful in controlling infection with pathogenic organisms and inflammatory responses.

Chapter IV

Molecular mechanism involved in induction of resistance by LPS to Vpr-mediated apoptosis in monocytes

Introduction:

It is well known that cells of the myelomonocytic lineage play a crucial role in HIV pathogenesis and disease progression [3;327;328]. Monocytic cells which play a central role in both innate and acquired immunity are productively infected by HIV as are $CD4^+$ activated T cells, however, unlike CD4+ T cells, monocytic cells survive HIV replication without major signs of HIV-induced cytopathic effects [8]. Persistently infected monocytic cells serve as a major reservoir of HIV in lymphoid tissues at all stages of disease [329-331] and represent a key challenge to eradicate HIV infection by eliminating virus reservoirs inspite of use of antiretroviral therapy [332-336]. It is believed that one of the reasons by which monocytic cells escape HIV cytopathic effects is the capacity of HIV to decrease the sensitivity of this cell type to apoptosis. This hypothesis was supported by a couple of studies demonstrating that cytokines such as nerve growth factor [39] and IL-13 can rescue monocytic cells from the cytopathic effects of HIV [40]. However, there is evidence to suggest that monocytic cells or monocyte derived macrophages from HIV infected patients and monocytic cell line U937 infected *in vitro* with HIV exhibit enhanced propensity to apoptosis [41-43].

The first part of my research work was to investigate the molecular mechanisms involved in resistance to apoptosis in human monocytic cells. I demonstrated that monocytic cells exhibit resistance to staurosporine induced apoptosis by upregulating anti-apoptotic c-IAP2 gene [11]. However, staurosporine is not considered as a physiological trigger to cause apoptosis. In an attempt to understand protection against apoptosis in response to physiological stimuli, I employed Vpr, the accessory protein of HIV-1, which is believed to interfere with cell proliferation and causes growth arrest in

the G2/M phase of the cell cycle and apoptosis in several cell types including CD4+ T cells and neuronal cells [35;117;120;337;338]. More specifically, I employed a synthetic peptide corresponding to the C-terminal 52-96 amino acid sequence of Vpr (Vpr52-96). The apoptotic effect of Vpr has been shown to be mimicked by the C-terminal Vpr52-96 but not by the N-terminal Vpr1-51 moiety [101;102]. My preliminary results suggest that this Vpr52-96 peptide interestingly induced apoptosis in normal human monocytic cells as well as in the promonocytic THP-1 cells. Furthermore, Vpr-induced apoptotic effect in both cell types could be reversed by pretreatment of cells with LPS. Based on these observations, I hypothesize that TNF-α, the cytokine known to be produced in abundance in HIV infection may be one of the critical factors responsible for protecting monocytic cells from the apoptotic effects of Vpr and consequently promoting monocytic cells to act as potent viral reservoirs [339-341]. However, the mechanism responsible for the resistance developed by the monocytes to undergo apoptosis in HIV infection is not known. ***Therefore, in the second part of my research work I studied the intracellular signaling pathways responsible for inducing apoptosis in response to Vpr52-96 peptide as a model. In addition, I also studied the signaling molecules and/or the anti-apoptotic/pro-apoptotic genes responsible for inducing LPS mediated protective anti-apoptotic signals in monocytic cells.***

First of all, I investigated the signaling pathways in particular the role of MAPKs, and the transcription factors involved in the regulation of genes mediating Vpr52-96 induced apoptosis in primary human monocytes and human promonocytic THP-1 cells. My results suggest that Vpr1-45 and Vpr52-96 peptides induced phosphorylation of all the three p38, ERK and JNK MAPKs, however, Vpr52-96 induced apoptosis was regulated

selectively through JNK activation. Furthermore, Vpr induced apoptosis was mediated by downregulation of anti-apoptotic genes Bcl2 and c-IAP1 through activation of upstream JNK MAPKs.

Further, I demonstrated for the first time that LPS-/TNF-α-induced resistance to Vpr52-96 mediated apoptosis. I also investigated the involvement of c-IAP2 and its regulation by calcium signaling pathway in this process. Pretreatment of cells with Vpr52-96 peptide inhibited LPS-/TNF-α-induced calcium influx, activation of CAMKII, and c-IAP2 induction as determined by Western blot and luciferase assays. Furthermore, Vpr peptide inhibited the binding of NFκB to its binding site on the c-IAP2 promoter as determined by gel shift analysis. Taken together, my results suggest that c-IAP2 gene plays a critical role in LPS- and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells.

Objective # 1

Signaling pathways involved in Vpr-induced apoptosis.

Results:

Vpr52-96 peptide induces apoptosis in THP-1 cells:

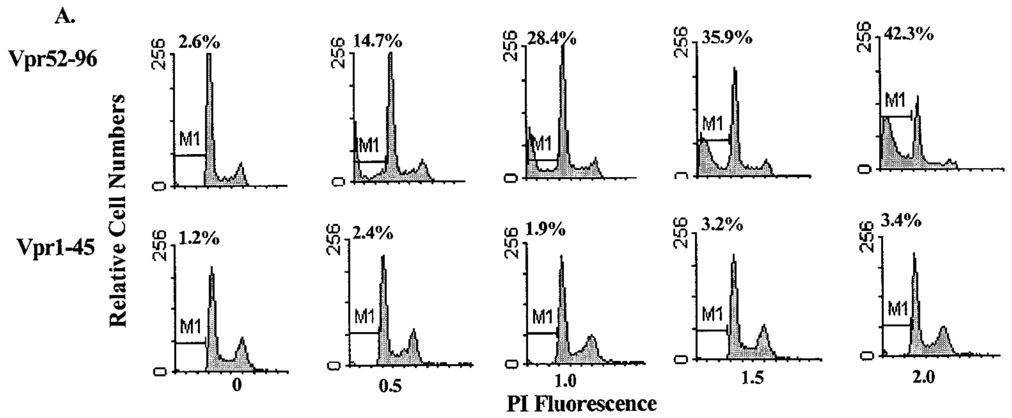
C-terminal region of Vpr containing highly conserved amino acid sequences was shown to cause cell cycle arrest and apoptosis in T cells [102;123;286]. To determine if monocytic cells transduced with Vpr peptides undergo a similar cell cycle arrest, THP-1 cells were treated with various concentrations of Vpr52-96 or Vpr1-45 peptides for 24 hr followed by cell cycle analysis and apoptosis as determined by PI and annexin-V staining and flow cytometry. As shown in Fig. 4-1.1A, & B, Vpr52-96 in contrast to Vpr1-45 peptide induced apoptosis in a dose dependent manner, with approximately 40% cells showing apoptosis at a concentration of 2 μM. These results suggested that Vpr52-96 peptide selectively arrested cells at G2/M phase of the cell cycle. To further confirm, stimulated cells were also analysed by annexin/PI staining. Annexin detects the phosphatidyl serine positive cells, which is exposed to cell surface in apoptotic cell in the very early stage of apoptosis. Similar kind of results were obtained in annexin/PI staining with 45% of apoptosis detected in Vpr52-96 treated cells as compared to 7% in Vpr1-45 treated cells (Fig. 4-1.1B).

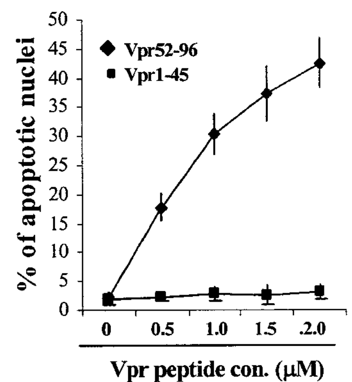
In the apoptotic signal transduction pathway, mitochondria play a key role by releasing caspase-activating apoptotic factors such as cytochrome C released during mitochondrial membrane potential loss [342]. To elucidate further the mechanism

Fig. 4-1.1: Vpr52-96-induced apoptosis in THP-1 cells:

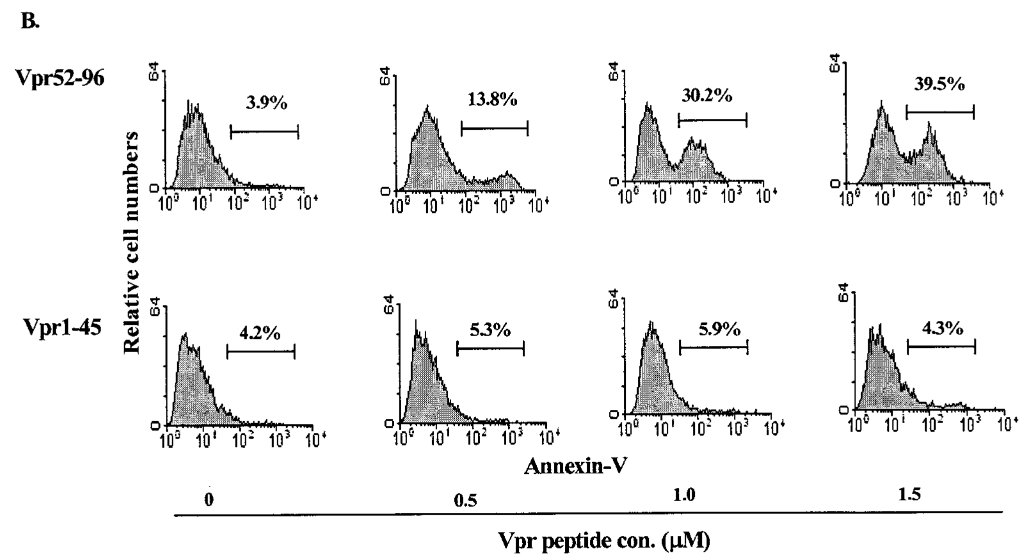
Cells (0.5 x 10^6/ml) were stimulated with various concentrations of Vpr52-96 or Vpr1-45 peptide for 24 h followed by staining with intracellular PI for DNA content **(A)** and annexin-V/PI for measurement of apoptosis **(B).** The graphs shown are a mean ± SD of three experiments performed in triplicate.

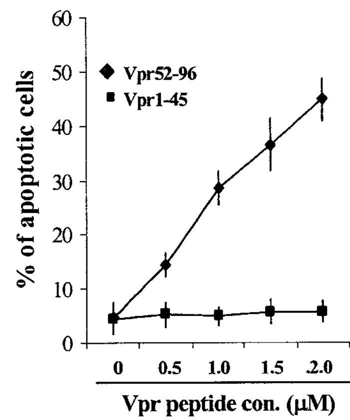
A.





B.





underlying Vpr-induced apoptosis with respect to the loss of mitochondrial membrane potential, Vpr52-96 and Vpr1-45 peptides treated cells were stained with JC-1 and analyzed by flow cytometry. JC-1 is a lipophilic cation agent that can form an orderly arrangement in an electric field generating aggregates revealed as orange red fluorescent color around normally functioning mitochondria in healthy cells in contrast to the exaggerated monomeric green form in the apoptotic cells. The results show that Vpr52-96, in contrast to Vpr1-45 peptide, induced loss of mitochondrial membrane potential in a dose dependent manner, with approximately 40% cells showing monomeric green color at a concentration of 2 μM (Fig 4-1.2A, & B). These results suggest a distinct involvement of mitochondria in Vpr52-96-induced apoptosis.

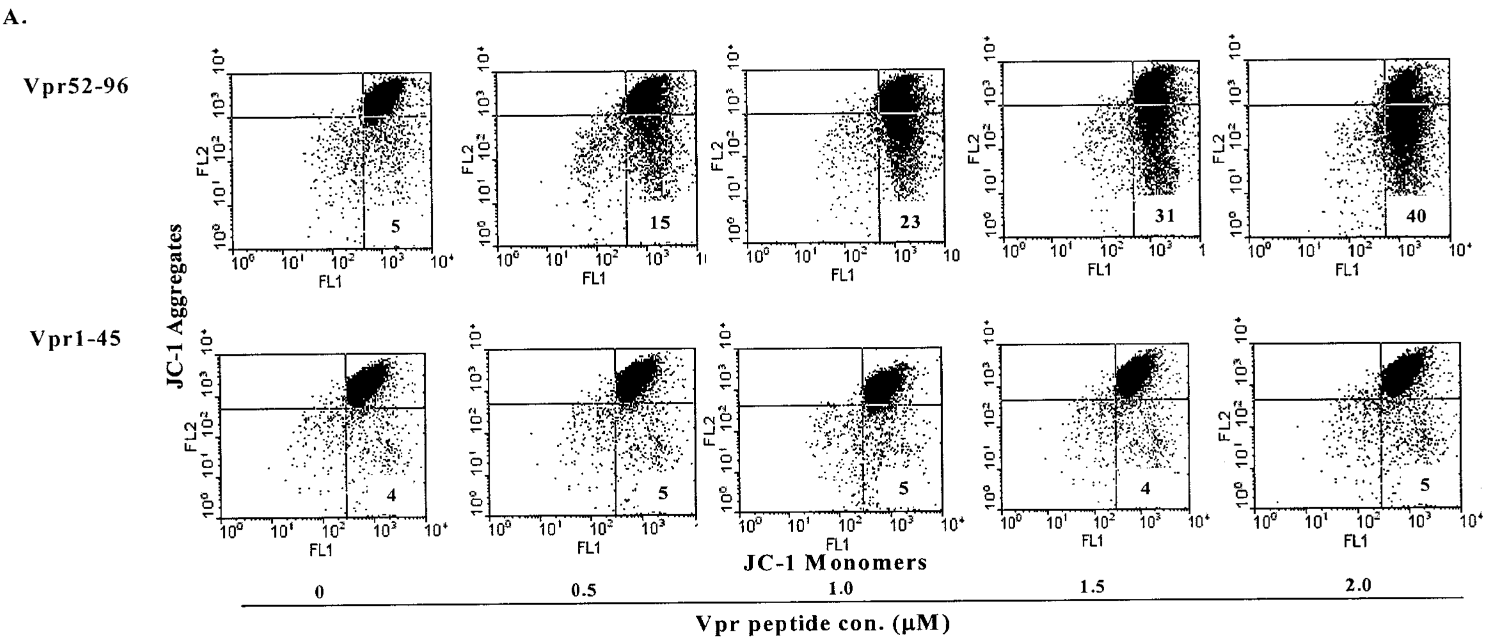
Vpr52-96 peptide-induced apoptosis is caspase-dependent:

Cytochrome-C release from mitochondria results in sequential procaspase activation and ultimately apoptosis [342]. To determine the involvement of caspase in Vpr52-96-induced apoptosis, the effect of broad spectrum caspase inhibitor ZVAD-fmk was examined. Our results showed that pretreatment of cells with ZVAD-fmk resulted in a significant reduction of apoptosis in a dose dependent manner as determined by PI and annexin/PI staining (Fig. 4-1.3A, & B). In contrast, ZVAD-fmk pretreatment did not affect Vpr52-96-mediated loss of mitochondrial membrane potential in THP-1 cells (Fig. 4-1.3C). Since the effect of caspase inhibitors is limited to block nuclear fragmentation and not the mitochondrial damage, the results suggest that loss of mitochondrial potential is regulated independently of ZVAD-fmk inhibitable caspases in monocytic cells as observed in T cells [343].

Fig. 4-1.2: Vpr52-96-induced apoptosis is mediated by the mitochondrial pathway:

Cells (0.5 x 10^6/ml) were stimulated with various concentrations of Vpr52-96 or Vpr1-45 peptide for 24 h followed by staining with JC-1 for mitochondrial membrane potential. The values shown in the lower right quarter in **C** indicate percentage of apoptotic cells. The graphs shown are a mean ± SD of three experiments performed in triplicate.

A.



B.

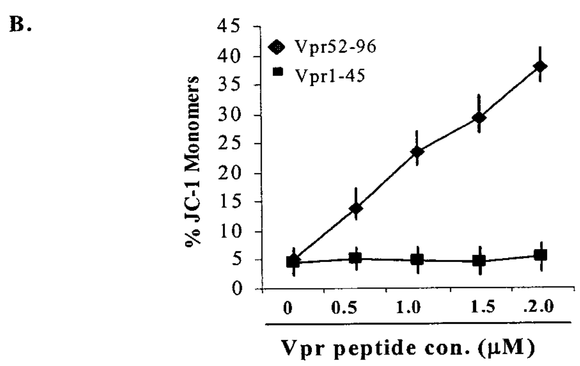
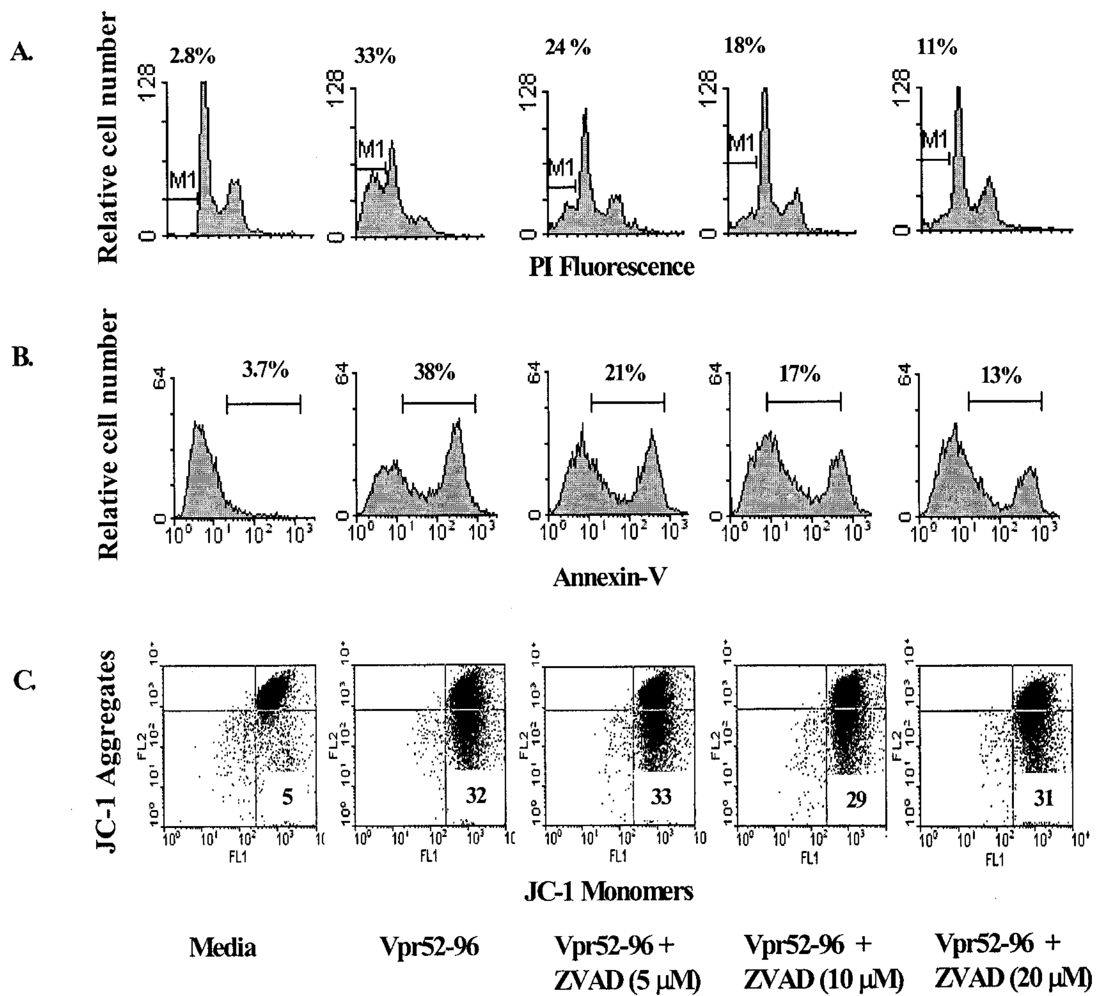


Fig. 4-1.3: Vpr52-96-induced apoptosis is caspase-dependent:

THP-1 cells (0.5 x 10^6/ml) were treated with 1.5 μM of Vpr 52-96 peptide in the presence or the absence of various concentrations of ZVAD-fmk for 24 h followed by staining with intracellular PI **(A)**, annexin/PI **(B)** or JC-1 **(C).** The values shown in the lower right quarter in **C** indicate percentage of apoptotic cells. The results shown are a representative of three different experiments.


A.
B.
C.
Relative cell number
2.8%
33%
24 %
18%
11%
M1
PI Fluorescence
Relative cell number
3.7%
38%
21%
17%
13%
Annexin-V
JC-1 Aggregates
5
32
33
29
31
JC-1 Monomers
Media
Vpr52-96
Vpr52-96 + ZVAD (5 μM)
Vpr52-96 + ZVAD (10 μM)
Vpr52-96 + ZVAD (20 μM)

Vpr52-96 peptide activated JNK selectively induces apoptosis in THP-1 cells:

Because MAPKs play a key role in cell proliferation and apoptosis [135], I investigated the role of MAPK pathway in Vpr-induced apoptosis. To understand the MAPKs pathway involved in Vpr52-96-induced apoptosis, I first investigated if Vpr peptides activated either of the p38, ERK, or JNK MAPKs in THP-1 cells. Cells were treated with either 1.5 μM of Vpr52-96 or Vpr1-45 peptide for various times and subjected to Western immunoblotting for p38, ERKs and JNK MAPKs activation by using anti-phospho-p38, anti-phospho-p42/44 ERK and anti-phospho-JNK specific antibodies, respectively. The same blots were stripped and reprobed with anti-p38, anti-p42/44 and anti-JNK antibodies to ensure equal protein loading. The results show that treatment of cells with Vpr52-96 peptide resulted in strong activation of ERK and JNK MAPKs. Activation of both MAPKs became apparent at about 1 hr following treatment and was sustained over the next 4 hr (Fig.4-1.4A, & C). In contrast, p38 MAPK was phosphorylated as early as 10 min after incubation with Vpr52-96 peptide and the phosphorylation was sustained for 4 hr (Fig. 4-1.4B). Similarly, treatment of cells with Vpr1-45 peptide induced phosphorylation of p38, p42/44 ERK and JNK MAPKs in a similar manner (Fig. 4-1.4A, B, & C). Prior treatment of cells with specific inhibitors of MAPK family member such as PD98059 (ERK), SB202190 (p38), SP600125 (JNK) or DXM (JNK) [282] inhibited Vpr52-96- and Vpr1-45 peptides-induced phosphorylation of ERK, p38 and JNK MAPKs, respectively (Fig. 4-1.4A, B, & C).

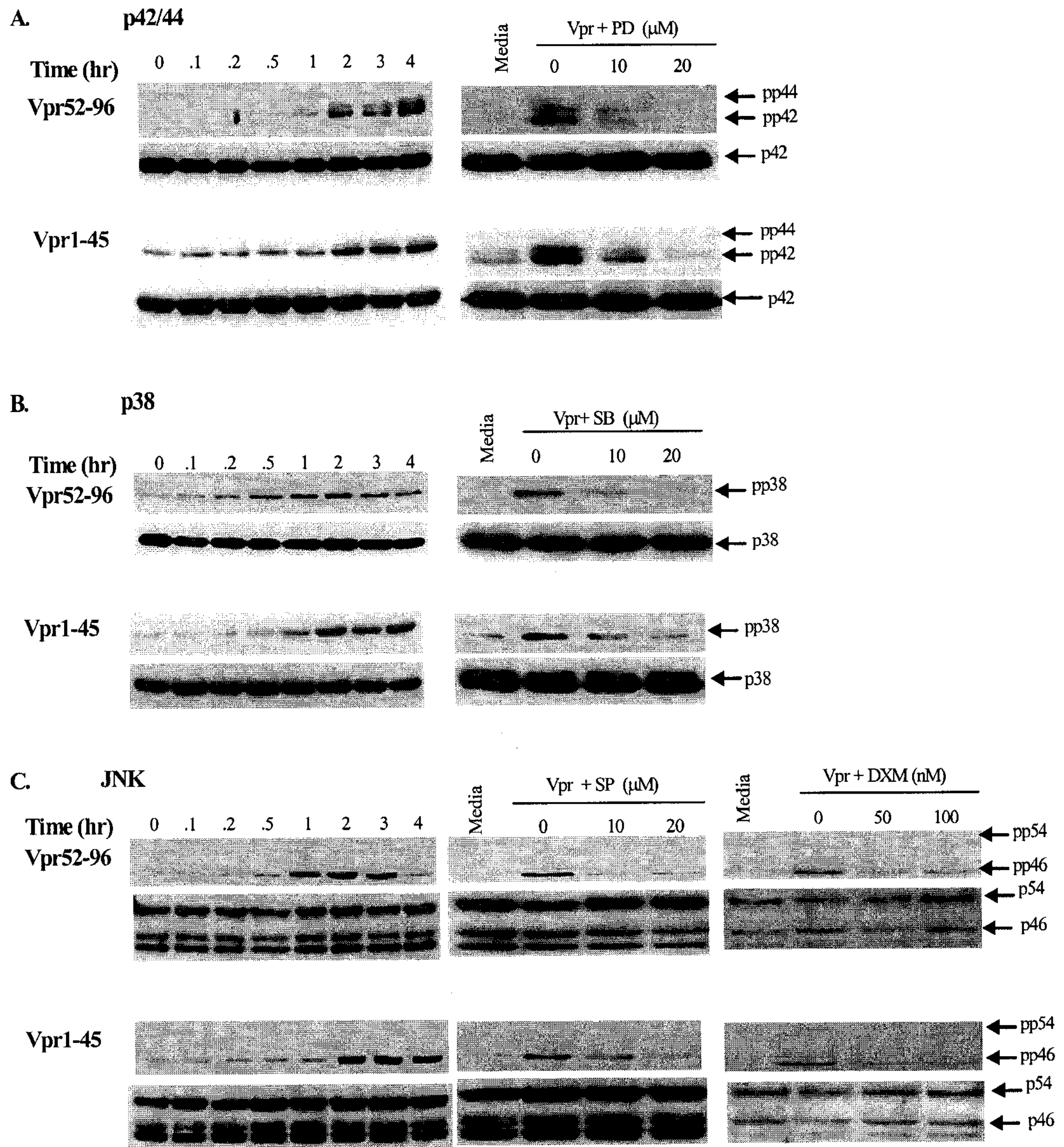
To determine the possible involvement of MAPKs in the regulation of Vpr52-96 peptide-induced apoptosis, cells were treated with inhibitors specific for p38, ERK and

Fig. 4-1.4: Vpr52-96 and Vpr1-45 peptides activate p38, p42/44 ERK and JNK MAP kinases:

Left panel (A, B and C).

Left panel (A, B and C). THP-1 cells (1.0 x 10^6/ml) were treated with 1.5 μM of either Vpr52-96 or Vpr1-45 peptides for 0-240 min and cell extracts were subjected to Western blot analysis using anti-phospho-p42/44 (indicated by arrows as pp42/44), anti-phospho-p38 (pp38) or anti-phospho-JNK (pp46/54) antibodies. To normalize protein loading, the membranes were stripped and reprobed either with anti-p42/44, anti-p38 or anti-JNK antibodies, respectively.

Right panel (A, B and C). Cells (1.0 x 10^6/ml) were pretreated with indicated concentrations of either **(A)** PD98059, **(B)** SB202190, **(C)** SP600125 or DXM for 2 hr prior to stimulation with 1.5 μM of Vpr52-96 or Vpr1-45 peptides. Total proteins (30 μg) were subjected to SDS-PAGE followed by Western blot analysis using either anti-phospho-p42/44 (pp42/44), anti-phospho-p38 (pp38) or anti-phospho-JNK antibodies (pp46/54). To ensure equal protein loading, the membranes were stripped and reprobed either with anti-p42/44, anti-p38 or anti-JNK antibodies, respectively. The results shown are representative of three different experiments.



A.
p42/44
Time (hr)
0
.1
.2
.5
1
2
3
4
Vpr52-96
Vpr1-45
Media
Vpr + PD (μM)
0
10
20
pp44
pp42
p42
B.
p38
Vpr+ SB (μM)
pp38
p38
C.
JNK
Vpr + SP (μM)
Vpr + DXM (nM)
50
100
pp54
pp46
p54
p46

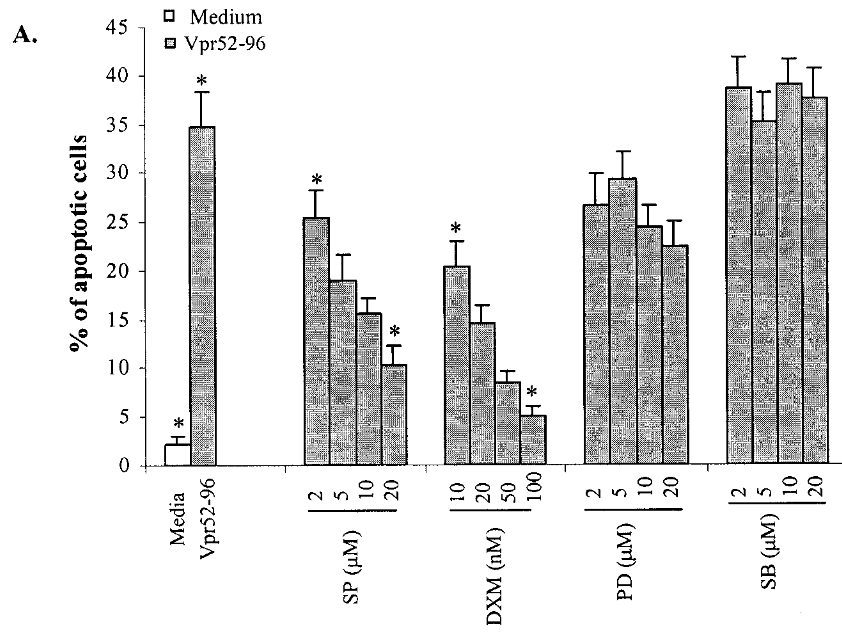
JNK MAPKs for 2 hr followed by stimulation with 1.5 μM of either Vpr52-96 or Vpr1-45 peptides for 24 hr following which cells were analyzed for apoptosis. In the absence of Vpr52-96 peptide, pretreatment with any inhibitor at the concentration used did not affect cell growth as determined by PI staining over the course of experiment (data not shown). As observed earlier, Vpr52-96 peptide treatment significantly enhanced apoptotic cell population as determined by PI staining. Interestingly, pretreatment of cells with SP600125 and DXM significantly reversed the Vpr52-96-induced apoptosis in a dose dependent manner (Fig. 4-1.5A). Similar results were obtained by another broad spectrum JNK inhibitor, curcumin (data not shown). In contrast, neither SB202190 nor PD98059 affected Vpr52-96-induced apoptosis significantly even when used at the highest concentration (Fig. 4-1.5A). Similar observations were made when cells were analyzed by staining with either annexin V/PI or JC-1 (data not shown).

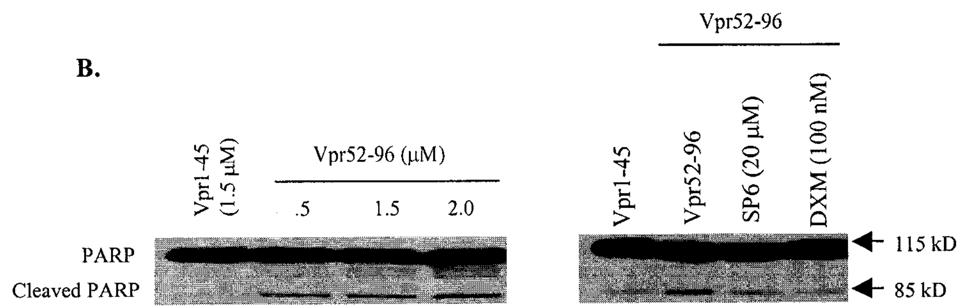
Poly (ADP-ribose) polymerase (PARP), a 118 kD nuclear enzyme, recognizes DNA strand breaks and is implicated in DNA repair and apoptosis [344]. On activation, PARP gets cleaved into two fragments of 89 kD and 24 kD which occurs early in apoptotic response as a result of the activity of caspase 3. PARP cleavage has been used as a marker of apoptosis [344]. Therefore, it was interesting to determine whether Vpr52-96 peptide-induced JNK phosphorylation and associated apoptosis involves PARP activation and cleavage. The results show that treatment of THP-1 cells with Vpr52-96 induced cleavage of PARP suggestive of apoptosis. Furthermore, pretreatment of cells with JNK inhibitor SP600125 and DXM inhibited PARP cleavage indicative of reversion of apoptotic process (Fig. 4-1.5B).

Fig. 4-1.5: Vpr52-96-activated JNK selectively induced apoptosis in THP-1 cells:

A. Cells (0.5 x 10^6/ml) were pretreated with indicated concentrations of either SP600125, DXM, PD98059, or SB202190 for 2 h prior to stimulation with 1.5 μM of Vpr52-96 peptide for 24 h followed by intracellular PI staining and analyzed by flow cytometry. The results shown are mean ± SD of three different experiments (*$p<0.001$).

B. Cells (1 x 10^6/ml) were pretreated with indicated concentrations of SP600125 or DXM for 2 h prior to stimulation with 1.5 μM of Vpr52-96 peptide for 12 h. Cell lysates were subjected to SDS-PAGE and Western blot analysis for PARP expression by using anti-PARP antibodies. The results shown are a representative of three different experiments.


A.
Medium
Vpr52-96
% of apoptotic cells
45
40
35
30
25
20
15
10
5
0
Media
Vpr52-96
SP (μM)
DXM (nM)
PD (μM)
SB (μM)

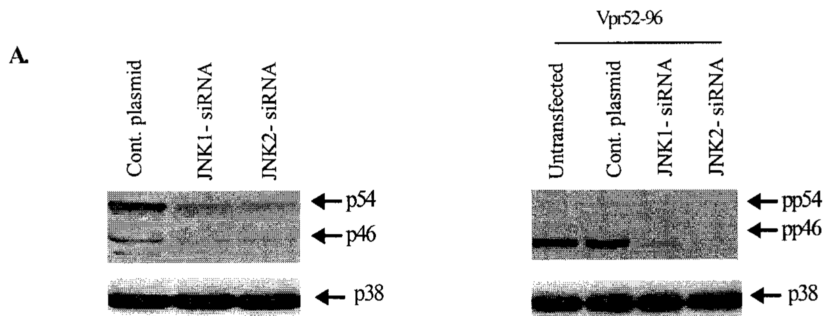

B.
Vpr1-45 (1.5 μM)
Vpr52-96 (μM)
.5
1.5
2.0
PARP
Cleaved PARP
Vpr52-96
Vpr1-45
Vpr52-96
SP6 (20 μM)
DXM (100 nM)
115 kD
85 kD

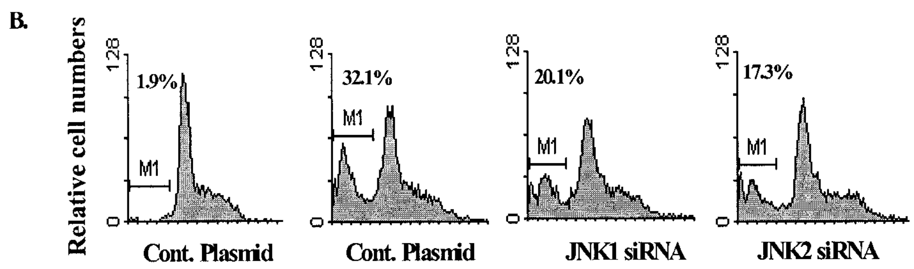
To confirm that Vpr52-96 peptide induced apoptosis involves JNK MAPK activation, I used stealth siRNA specific for JNK1 and JNK2 to knockdown endogenous and Vpr52-96 induced JNK activation and determined its effect on apoptosis. THP-1 cells were transfected either with siRNA for JNK1, JNK2 or control siRNA followed by treatment with Vpr peptides for another 2 hr. Transfection of cells either with siJNK1 or siJNK2 RNAs significantly inhibited endogenous expression of JNK1 and JNK2, respectively (Fig. 4-1.6A, left panel). Vpr52-96 peptide stimulation of cells transfected with either siRNAs specific for JNK-1 or JNK-2 failed to induce phosphorylation of JNK-1 and JNK-2, respectively (Fig 4-1.6A, right panel) compared to cells transfected with control siRNA. Cell cycle analysis of Vpr52-96 peptide treated cells transfected with either JNK-1 or JNK-2 siRNA revealed a significant decrease in apoptotic cell population from 32% to 17% as determined by PI staining and flow cytometry (Fig. 4-1.6B). Similar results were obtained when cells were analyzed for apoptosis by staining with annexin/PI or JC-1. Percentage of annexin-V positive apoptotic cells drops down from ~40 to ~20% in JNK1/JNK2 stealth RNA treated cells. Similarly, JC-1 monomers also declined from ~50 to ~25% in the siRNA treated cells (Fig. 4-1.6C). Overall, the results suggest that Vpr52-96 peptide induced apoptosis through the selective activation of JNK. In contrast, Vpr1-45 peptide treatment although induced JNK activation yet it did not affect apoptosis.

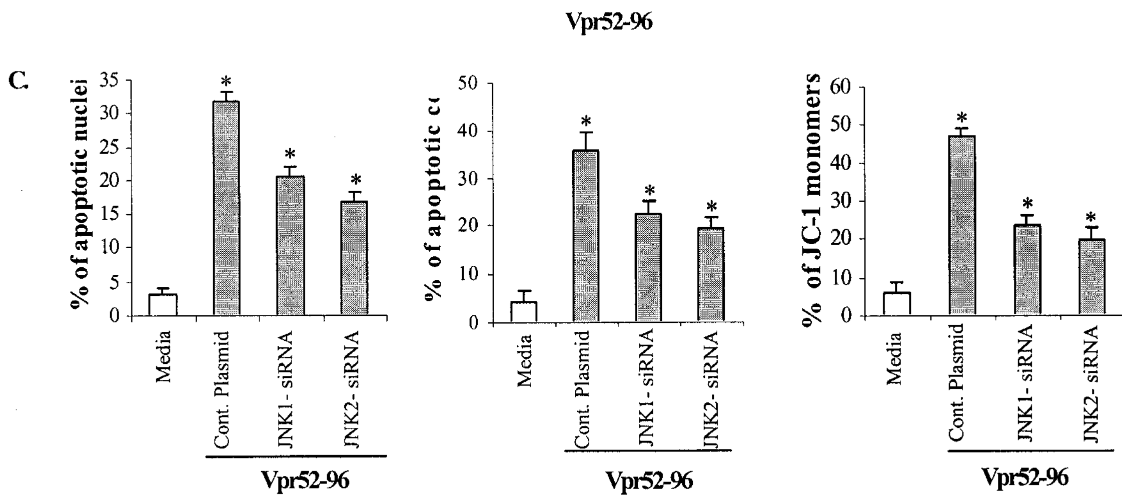
Fig. 4-1.6: JNK specific siRNAs reverse Vpr52-96-induced apoptosis in THP-1 cells:

A. JNK specific siRNA reduced endogenous and Vpr52-96-stimulated phospho-JNK: Cells (1 x 10^6/ml) were transfected with either JNK1, JNK2 or control stealth siRNA for 24 h **(left panel)** followed by stimulation with 1.5 μM of Vpr52-96 peptide for 2 h (right panel). Cell lysates were analyzed by Western blot analysis using anti-JNK **(left panel)** or anti-phospho JNK antibodies **(right panel)**. To ensure equal protein loading, the membranes were reprobed with total anti-p38 antibodies. The results shown are a representative of three different experiments.

B & C. JNK specific siRNAs reversed Vpr52-96-induced apoptosis: THP-1 cells (1 x 10^6 /ml) were transfected with either JNK1, JNK2 or control stealth siRNA for 24 h followed by stimulation with 1.5 μM of Vpr52-96 peptide for 24 h and staining with intracellular PI **(B and left panel of C),** annexin/PI **(middle panel, C)** or JC-1 **(right panel, C).** The results shown in C represent a mean ± SD of three independent experiments (*$p<0.01$).


A.
Vpr52-96
Cont. plasmid
JNK1- siRNA
JNK2- siRNA
Untransfected
Cont. plasmid
JNK1- siRNA
JNK2- siRNA
p54
p46
p38
pp54
pp46
p38


B.
Relative cell numbers
128
0
1.9%
32.1%
20.1%
17.3%
M1
Cont. Plasmid
Cont. Plasmid
JNK1 siRNA
JNK2 siRNA
Vpr52-96


C.
% of apoptotic nuclei
Media
Cont. Plasmid
JNK1- siRNA
JNK2- siRNA
Vpr52-96
% of apoptotic c
% of JC-1 monomers

Vpr52-96 peptide-induced apoptosis is mediated via down regulation of anti-apoptotic Bcl2 and c-IAP1 genes:

Vpr52-96 peptide may cause apoptosis either by upregulating the expression of pro-apoptotic genes or by down regulating the anti-apoptotic genes. This was investigated by determining the effect of Vpr52-96 peptide on the expression of pro-apoptotic and anti-apoptotic genes by RNAse protection assay by employing the Riboquant kit (BD Biosciences). This kit contained probes for the Bcl2 family of pro- and anti-apoptotic genes (BclW, BclX, BikB, Bad, Bax, Bak, and Bcl2 in addition to that of Mcl1). THP-1 cells were treated with either Vpr52-96 or Vpr1-45 peptides for 1-6 hr followed by analysis of gene expression by RNAse protection assay. Results suggest that Vpr52-96 peptide significantly inhibited selectively the expression of Bcl2 gene. Decreased expression of Bcl2 mRNA was detected at 3 hr following treatment of cells with Vpr52-96 peptide and the level of mRNA expression was decreased significantly by 6 hr of treatment as compared to the cells treated with the Vpr1-45 peptide (Fig. 4-1.7A). Surprisingly, there was no significant change in the level of apoptotic genes (Bak and Bax) and anti-apoptotic genes (BclXl, and Mcl1). The densitometric analysis of all the bands were performed by using Image J software and normalized with house keeping genes such as L32 and GAPDH (Fig. 4-1.7B). The effect of Vpr52-96 peptides on the expression of Bcl2 was further confirmed by RT-PCR and Western blot analysis (Fig 4-1.7C & E).

I also investigated the effect of Vpr peptides on the expression of another IAP family of anti-apoptotic genes by RT-PCR analysis. Treatment of cells with the Vpr52-96 and not the Vpr1-45 peptide significantly reduced the expression of c-IAP1 gene. Neither

of these peptides significantly affected the expression of c-IAP2 genes in THP-1 cells as determined by RT-PCR (data not shown) and Western blot analysis (Fig. 4-1.7D & E).

Vpr52-96 peptide-induced apoptosis is mediated by down regulation of anti-apoptotic Bcl2 and c-IAP1 genes through JNK MAPK activation:

Because Vpr52-96 peptide induced JNK activation, and blockage of JNK phosphorylation by JNK inhibitors inhibited Vpr52-96- induced apoptosis, it was interesting to determine if Vpr-induced apoptosis is regulated by Bcl2 and c-IAP1 through the activation of JNK MAPKs. Further to assess the involvement of JNK MAPK in regulation of Bcl2 mediated Vpr52-96 peptide-induced apoptosis, I performed RT-PCR. THP-1 cells were treated with Vpr52-96 peptide in the presence or absence of JNK MAPK inhibitors. Vpr52-96 peptide significantly reduced the expression of Bcl2 in THP-1 cells as compared to Vpr1-45 peptide treated cells. JNK MAPK inhibitors SP600125 and dexamethasone restored the expression of Bcl2 as well as c-IAP1 as determined by RT-PCR and Western blot analysis (Fig 4-1.7C, D, & E). Pretreatment of cells with the p38 inhibitor SB202190 did not affect Vpr52-96 peptide induced expression of either Bcl2 or c-IAP1 (Fig 4-1.7C, D, & E).

Since Bcl2 is post-translationally modified by phosphorylation, it is believed that expression of Bcl2 alone is not enough to exert its anti-apoptotic effects [345]. IL-3 and erythropoietin have been shown to induce their anti-apoptotic effects through phosphorylation of Bcl2 at S70 [148]. In contrast, Bcl2 phosphorylation at T69, S70 and S87 has been proposed to inactivate Bcl2-mediated anti-apoptotic effects. Therefore, I determined if Vpr52-96 peptide modulated Bcl2 phosphorylation at S70 and whether

Fig. 4-1.7: Vpr52-96-activated JNK induces down regulation of anti-apoptotic Bcl2 and c-IAP1 genes:

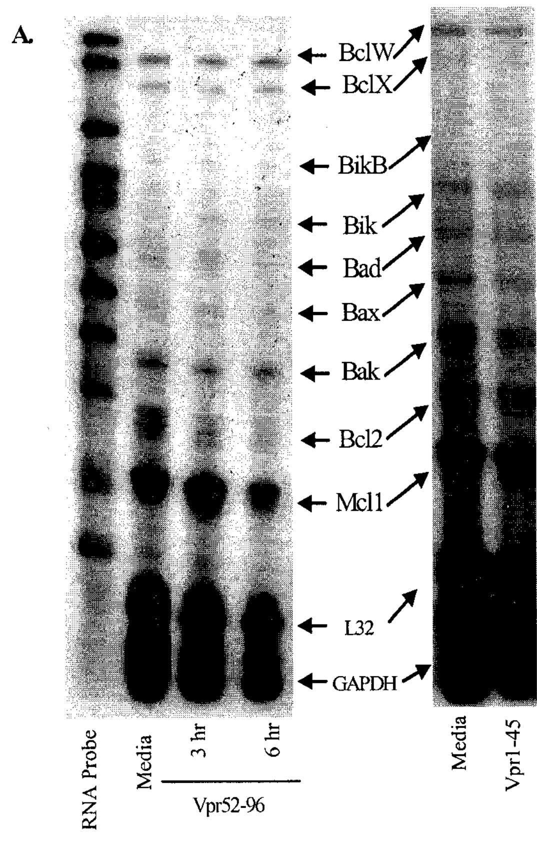
A. Vpr52-96 peptide inhibited Bcl2 expression as determined by RPA: THP-1 cells (5 x 10^6/ml) were stimulated with 1.5 μM of Vpr52-96 or Vpr1-45 peptides for 0-6 h. RNA was extracted as described in materials and methods. RPA was performed by using BD Riboquant package kit with a multi-probe template set of anti- and pro-apoptotic genes of the Bcl2 family, and GAPDH and L32 as internal controls. The results shown are a representative of three different experiments.

B. Densitometric analysis of Bcl2 genes following Vpr52-96 treatment from the results shown in 6A.

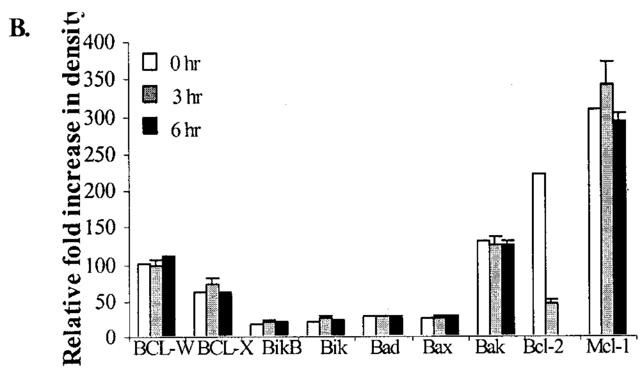
C. Vpr52-96 peptide induced down regulation of anti-apoptotic Bcl2 and c-IAP1 genes through JNK activation: Cells (5 x 10^6/ml) were stimulated with 1.5 μM of Vpr52-96 peptides for 3 hr in the presence or the absence of JNK (SP600125 and DXM) or p38 inhibitors (SB202190) followed by RNA extraction and semiquantitative RT-PCR analysis for Bcl2 and c-IAP1 expression.

D & E. Cells (1 x 10^6 /ml) were pretreated with either JNK (SP600125 and DXM) or p38 inhibitors (SB202190) for 2 hr followed by stimulation with 1.5 μM of Vpr52-96 peptides for 24 hr. Cell lysates were subjected to Western blot analysis for expression of c-IAP1 **(D)**, and Bcl2 and phospho-Bcl2 **(E)** using anti RIAP1, anti-Bcl2, and anti-phospho-Bcl2 antibodies, respectively. To ensure equal protein loading, the membranes were reprobed with anti-β-actin antibodies. The results shown are representative of three different experiments.

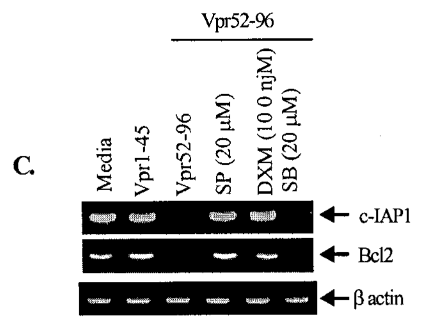
A.



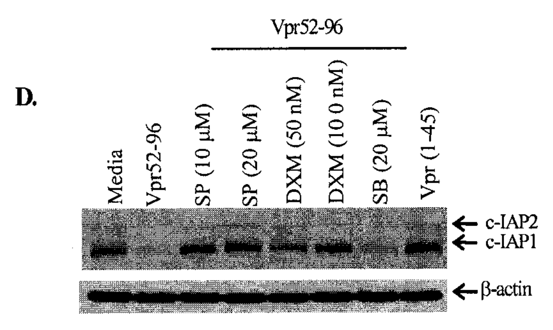
B.



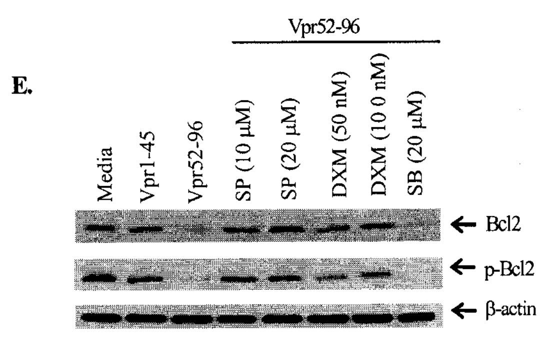
C.



D.



E.



JNK inhibitors reversed Bcl2 phosphorylation at S70 corresponding to its enhancing effects on cell survival. THP-1 cells exhibited high basal level of Bcl2 phosphorylation at the serine 70 residues, and their treatment with Vpr52-96 peptide abrogated S70 phosphorylation. Furthermore, pretreatment with JNK inhibitors SP600125 and DXM restored the Vpr52-96-mediated down regulation of S70 phosphorylation (Fig 4-1.7E). As observed above with total Bcl2 expression, treatment of cells with the p38 inhibitor SB202190 did not affect the Vpr52-96-mediated down regulation of Bcl2 phosphorylation. The results suggest the involvement of S70 phosphorylation of Bcl2 in Vpr52-96-mediated apoptosis and cell survival of THP-1 cells following inhibition of JNK activation.

Vpr52-96 peptide-activated-JNK MAPK inhibits Bcl2 transcription by down regulating the binding of NFκB, Sp1 and CREB transcription factors to their respective binding site in the Bcl2 promoter in THP-1 cells:

Vpr52-96 peptide-induced apoptosis may be regulated by Bcl2 and/or c-IAP1 at the level of transcription through JNK activation. Bcl2 gene is regulated by multiple transcription factors namely NFκB, CREB and Sp-1 [151;346]. It is likely that Vpr52-96 peptide activated JNK MAPK may down regulate Bcl2 transcription by inhibiting the activities of either NFκB, CREB and/or Sp-1. Therefore, I investigated whether Vpr52-96 peptide-activated transcription factors NFκB, CREB and Sp-1 to their binding sites present in the Bcl2 promoter by gel shift assay. Nuclear extracts harvested from Vpr52-96 treated and untreated THP-1 cells were analyzed for binding of all these three transcription factors to the oligonucleotide probes corresponding to their binding sites in the Bcl2 promoter. In the untreated THP-1 cells, significant constitutional binding of NFκB, CREB and Sp-1 to their respective oligonucleotide probes was observed (Fig. 4-

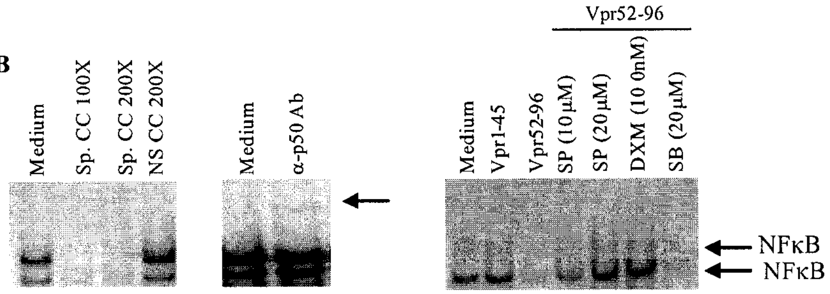
1.8A, B & C, left panels). The specificity of these transcription factors binding was demonstrated by competition with specific and non specific oligonucleotides and supershift analysis with respective rabbit polyclonal antibodies (Fig 4-1.8A, B, & C, left and middle panels). Interestingly, treatment of THP-1 cells with the Vpr52-96 resulted in the inhibition of binding of NFκB, CREB and Sp-1 to their respective oligonucleotide probes (Fig 4-1.8A, B, & C, right panels). To determine if Vpr52-96 peptide activated JNK MAPK caused the inhibition of NFκB, CREB and Sp-1 binding, cells were pretreated with JNK inhibitors SP600125, DXM, or SB202190 for 2 hr followed by stimulation with either Vpr52-96 or Vpr1-45 peptides. Surprisingly, SP600125 and DXM pretreatment restored the binding of all the three transcription factors namely NFκB, Sp-1 and CREB to their respective probes whereas SB202190 pretreatment did not have any effect on their binding (Fig 4-1.8A, B, & C, right panels).

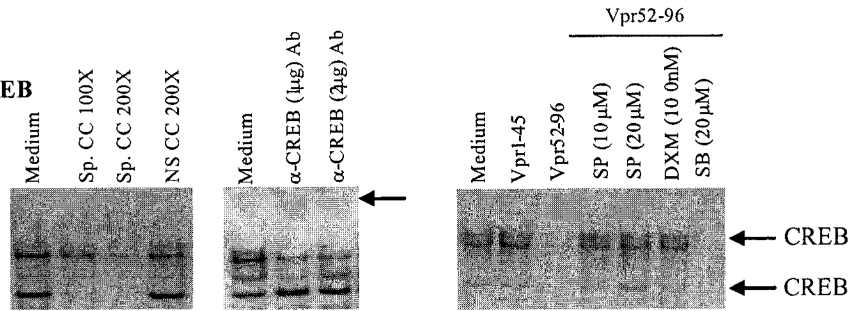
Vpr52-96 peptide-activated-JNK MAPK inhibits c-IAP1 transcription by down regulating the binding of NFκB and CREB transcription factors to their respective binding site in the c-IAP1 promoter in THP-1 cells:

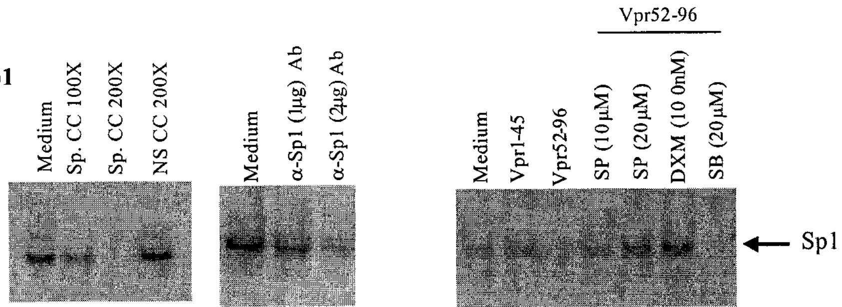
Since the above results also suggest that Vpr52-96 peptide induced apoptosis may be attributed to the inhibition of another anti-apoptotic gene, c-IAP1, I investigated whether Vpr-induced apoptosis is also regulated by c-IAP1 at the level of transcription through JNK activation. c-IAP1 transcription has been shown to be regulated by NFκB and CREB-1 [199;347]. Experiments conducted on similar lines suggest that untreated THP-1 cells exhibited constitutional binding of NFκB and CREB transcription factors to their oligonucleotide probes with sequences corresponding to their binding site in the

Fig. 4-1.8: Vpr52-96-activated JNK inhibits Bcl2 transcription by down regulating the binding of NFκB, CREB or Sp1 transcription factors:

Cells (5 x 10^6/ml) were treated with 1.5 μM of Vpr52-96 or Vpr1-45 peptides for 2 h in the presence or the absence of JNK (SP600125 and DXM) or p38 inhibitors (SB202190). Nuclear proteins (5 μg) were incubated with ^{32}P-labelled oligonucleotide probes corresponding to NFκB **(A)**, CREB **(B)** or Sp1 **(C)** sequences derived from the Bcl2 promoter. The specificity of NFκB, CREB and Sp1 binding was determined by incubating nuclear proteins with unlabelled NFκB, CREB and Sp1 or non-specific oligonucleotides. The supershift analysis was performed by treating the nuclear proteins with oligonucleotide probes in the presence or the absence of anti-p50, anti-p65 NFκB, anti-CREB or anti-Sp1 antibodies. The results shown are representative of three different experiments.


A. Bcl2-NFκB
Medium
Sp. CC 100X
Sp. CC 200X
NS CC 200X
Medium
α-p50 Ab
Vpr52-96
Medium
Vpr1-45
Vpr52-96
SP (10μM)
SP (20μM)
DXM (10 0nM)
SB (20μM)
NFκB
NFκB


B. Bcl2-CREB
Medium
Sp. CC 100X
Sp. CC 200X
NS CC 200X
Medium
α-CREB (1μg) Ab
α-CREB (2μg) Ab
Vpr52-96
Medium
Vpr1-45
Vpr52-96
SP (10μM)
SP (20μM)
DXM (10 0nM)
SB (20μM)
CREB
CREB


C. Bcl2-Sp1
Medium
Sp. CC 100X
Sp. CC 200X
NS CC 200X
Medium
α-Sp1 (1μg) Ab
α-Sp1 (2μg) Ab
Vpr52-96
Medium
Vpr1-45
Vpr52-96
SP (10μM)
SP (20μM)
DXM (10 0nM)
SB (20μM)
Sp1

c-IAP1 promoter. The specificity of NFκB and CREB binding was demonstrated by competition with specific and non specific oligonucleotides and supershift analysis with respective rabbit polyclonal antibodies (Fig 4-1.9A, left and middle panel; Fig 4-1.9B, left panel). Surprisingly, Vpr52-96 peptide inhibited the binding of NFκB and CREB to their corresponding oligonucleotide probes. Similarly, pretreatment of Vpr52-96-stimulated cells with JNK inhibitors, SP600125 and DXM, restored the binding of both NFκB and CREB to their respective probes. However, pretreatment of Vpr52-96 stimulated cells with the control p38 inhibitor, SB202190 did not influence the binding of any of these transcription factors (Fig 4-1.9A and B, right panels).

These results suggest that Vpr52-96 peptide activated JNK MAPKs selectively inhibit the transcription of Bcl2 as well as c-IAP1 by inhibiting the binding of responsible transcription factors to the respective binding sites in the Bcl2 and c-IAP1 promoters, respectively.

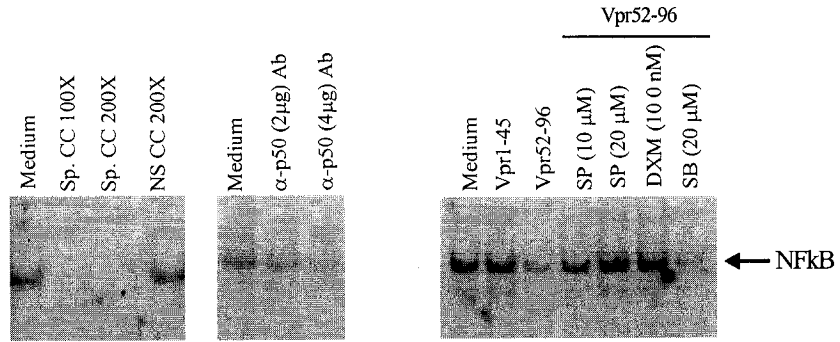
Vpr52-96 peptide induced apoptosis is mediated by JNK MAPK activation in CD14+ normal human monocytes:

I next investigated if normal human monocytes transduced with Vpr52-96 undergo a similar cell cycle arrest and whether Vpr52-96 peptide induced apoptosis through the selective activation of JNK. CD14+ purified human monocytes were treated with various concentrations of Vpr1-45 and Vpr52-96 peptides for 12 hr followed by cell cycle analysis and apoptosis as determined by PI, annexin-PI and JC staining. Similar to the results obtained with THP-1 cells, Vpr52-96 peptide in contrast to the Vpr1-45 peptide induced apoptosis in a dose dependent manner as demonstrated by PI (Fig 4-1.10A). Furthermore, Vpr52-96 peptide induced apoptosis in normal monocytes was

Fig. 4-1.9: Vpr52-96-activated JNK inhibits cIAP-1 transcription by down regulating the binding of NFκB or CREB transcription factors:

Cells (5 x 10^6/ml) were treated with 1.5 μM of Vpr52-96 or Vpr1-45 peptides for 2 h in the presence or the absence of JNK (SP600125 and DXM) or p38 inhibitors (SB202190). Nuclear proteins (5 μg) were incubated with ^{32}P-labelled oligonucleotide probes corresponding to NFκB **(A)**, or CREB **(B)** sequences derived from the c-IAP1 promoter. The specificity of NFκB, and CREB binding was determined by incubating nuclear proteins with unlabelled NFκB, and CREB or non-specific oligonucleotides. The supershift analysis was performed by treating the nuclear proteins with oligonucleotide probes in the presence or the absence of anti-p50, anti-p65 NFκB, or anti-CREB antibodies. The results shown are representative of three different experiments.

A. c-IAP1-NFκB



B. c-IAP1-CREB

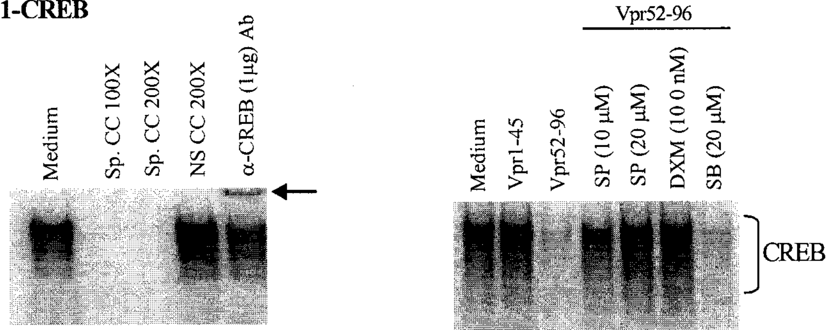
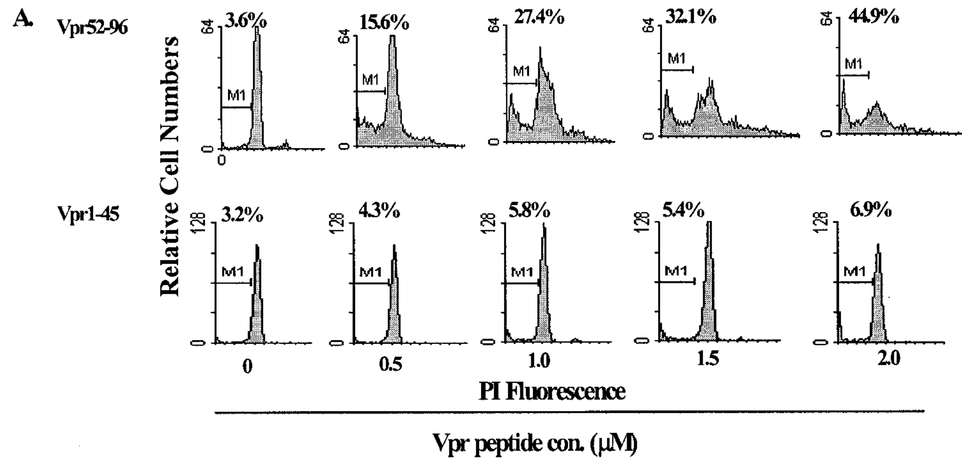


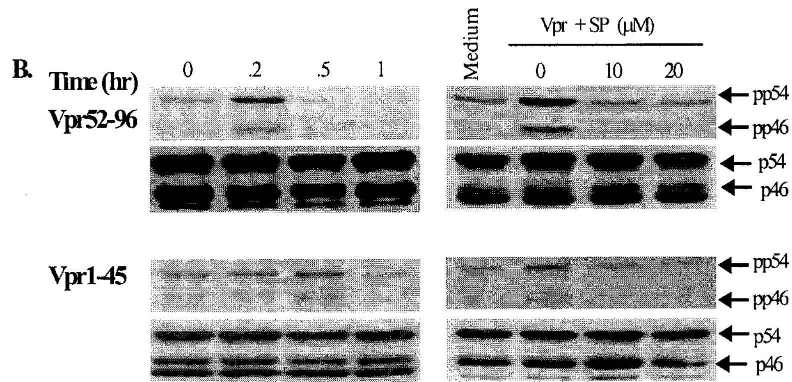
Fig. 4-1.10: Vpr52-96-activated JNK induces apoptosis in normal human monocytes:

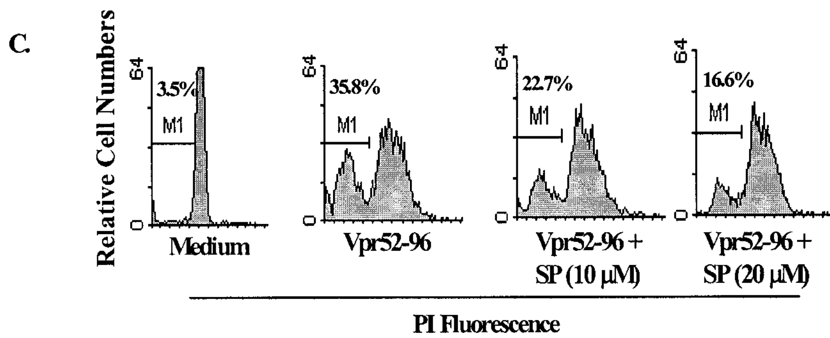
(A). Vpr52-96-induced apoptosis in human monocytes: Monocytes (0.5 x 10^6 /ml) were stimulated with various concentrations of either Vpr52-96 or Vpr1-45 peptides for 12 h followed by staining with intracellular PI and analyzed by flow cytometry. The results shown are representative of three different experiments.

(B). Vpr52-96 and Vpr1-45 peptides activate p38, p42/44 and JNK MAPKs in normal human monocytes: **Left panel:** Monocytes (3.0 x 10^6/ml) were treated with 1.5 μM of either Vpr52-96 or Vpr1-45 peptides for 0-60 min. **Right panel:** Monocytes (1.0 X 10^6/ml) were pretreated with SP600125 (0-20 μM) for 2 h prior to stimulation with Vpr52-96 or Vpr1-45 peptides. Total protreins (30 μg) were subjected to SDS-PAGE followed by Western blot analysis using anti-phospho-JNK antibodies. To ensure equal protein loading, the membranes were reprobed with anti-JNK antibodies. The results shown are representative of three different experiments.

(C). Vpr52-96-activated-JNK induces apoptosis in human monocytes: Cells (0.5 x 10^6/ml) were pretreated with SP600125 (0-20 μM) for 2 h prior to stimulation with 1.5 μM of Vpr52-96 peptide. After 12 h, cells were analyzed for apoptosis by intracellular PI staining and flow cytometry. The results shown are representative of three independent experiments.


A.
Vpr52-96
Vpr1-45
Relative Cell Numbers
3.6%
15.6%
27.4%
32.1%
44.9%
3.2%
4.3%
5.8%
5.4%
6.9%
M1
0
0.5
1.0
1.5
2.0
PI Fluorescence
Vpr peptide con. (μM)


B.
Time (hr)
0
.2
.5
1
Medium
Vpr + SP (μM)
0
10
20
Vpr52-96
Vpr1-45
pp54
pp46
p54
p46


C.
Relative Cell Numbers
3.5%
35.8%
22.7%
16.6%
M1
Medium
Vpr52-96
Vpr52-96 + SP (10 μM)
Vpr52-96 + SP (20 μM)
PI Fluorescence

found to be caspase dependent as prior treatment of cells with caspase inhibitor ZVAD-fmk resulted in a significant reduction of apoptosis (data not shown).

To determine that Vpr52-96 peptide-induced apoptosis is regulated through the activation of JNK MAPKs, I first demonstrated that both Vpr1-45 and Vpr52-96 peptides induced the phosphorylation of JNK MAPKs (Fig 4-1.10B). Subsequently I demonstrated that prior treatment of monocytes with the JNK MAPKs specific inhibitors SP600125 (Fig 4-1.10C), DXM and curcumin (data not shown) significantly reversed Vpr52-96 peptide-induced apoptosis in a dose-dependent manner as determined by PI, and annexin-PI staining (data not shown). These results suggest that Vpr52-96 peptide induces apoptosis in normal monocytes and similar to the results obtained with THP-1 cells, this apoptosis is mediated through JNK activation.

Vpr52-96 peptide-induced apoptosis is mediated by inhibition of anti-apoptotic Bcl2 and c-IAP1 genes through JNK MAPK activation in CD14+ normal human monocytes:

To further determine if Vpr52-96 peptide-induced apoptosis is regulated by Bcl2 and c-IAP1 genes through the activation of JNK MAPKs, freshly purified CD14+ monocytes were treated with the JNK inhibitor SP600125 for 2 hr followed by treatment with the Vpr52-96 peptide for 12 hr and 6 hr for protein (Western blotting) and mRNA (RT-PCR) analysis respectively. Unstimulated monocytes expressed constitutively both Bcl2 and c-IAP1 (Fig. 4-1.11A, B, & C). Treatment of cells Vpr52-96 peptide unlike Vpr1-45 peptide abrogated the expression of both Bcl2 and c-IAP1 in proteins and mRNA levels. Similar to the results obtained with THP-1 cells, exposure of monocytes with the JNK inhibitor SP600125 prior to treatment with the Vpr52-96 peptide restored the expression of Bcl2 and c-IAP1 genes to levels similar to that observed in

unstimulated cells (Fig. 4-1.11A, B, & C). In contrast, exposure of monocytes with the p38 inhibitor SB202190 prior to treatment with the Vpr52-96 did not affect the expression of Bcl2 and c-IAP1 genes (Fig. 4-1.11A, B, & C).

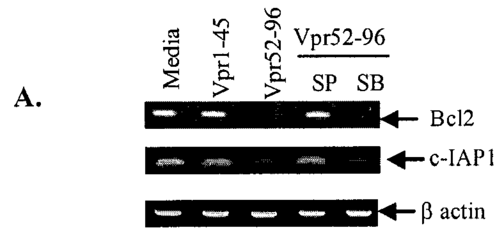
Based on all these findings, it is concluded that Vpr induced apoptosis in monocytes by downregulating anti-apoptotic Bcl2 and c-IAP1 genes through activation of JNK MAPK.

Fig. 4-1.11: Vpr52-96-activated JNK induces apoptosis by inhibiting the expression of Bcl2 and c-IAP1 in normal human monocytes:

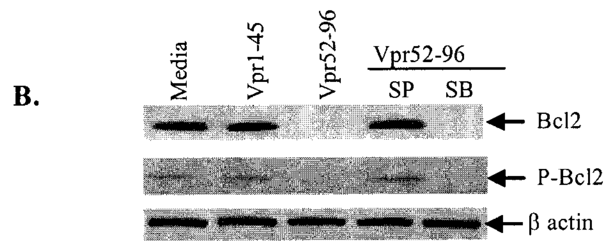
(A). Monocytes (5 x 10^6 /ml) were stimulated with 1.5 μM of Vpr52-96 peptides for 2 h in the presence or the absence of JNK inhibitor (SP600125) followed by RNA extraction and semiquantitative RT-PCR analysis for Bcl2 and c-IAP1 expression.

(B, & C). Monocytes (2 x 10^6/ml) were pretreated with SP600125 for 2 h followed by stimulation with 1.5 μM of Vpr52-96 for 12 h. Cell lysates were subjected to Western blot analysis for expression of Bcl2 and phospho-Bcl2 **(B)** and c-IAP1 **(C)** using anti-Bcl2, anti-phospho-Bcl2, and anti RIAP1 antibodies, respectively. To ensure equal protein loading, the membranes were reprobed with anti–β–actin Abs. The results shown are representative of three independent experiments.

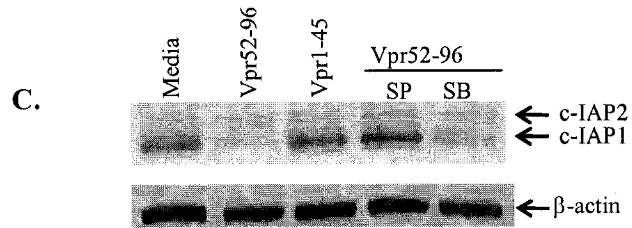
A.



B.



C.



Objective # 2

Signaling pathways involved in development of LPS- and TNF-α-mediated resistance to HIV-Vpr-induced apoptosis:

Results:

LPS-induced resistance to Vpr52-96 mediated apoptosis in human monocytic cell:

I already demonstrated that C terminal Vpr52-96 peptide induced apoptosis in a dose dependent manner in THP-1 cells, the promonocytic cell line and in normal human monocytes isolated from healthy donors, whereas N terminal Vpr1-45 peptide at similar concentrations failed to exert apoptotic effects in both the cell types. To determine the effect of LPS on Vpr52-96 induced apoptosis, cells were stimulated with LPS for 6 hr prior to treatment with Vpr52-96 or Vpr1-45 peptides for 24 hr followed by determination of apoptosis. The results show that pretreatment of cells with LPS significantly reduced apoptosis as determined by PI and annexin/PI staining in THP-1 cell and by PI staining in monocytes (Fig. 4-2.1A, B, & C). However, stimulation of cells with LPS for less than 4 hr did not induce protection against Vpr52-96-induced apoptosis (data not shown). In contrast, when THP-1 cells and primary monocytes were stimulated with LPS for 24 hr following treatment with Vpr52-96 or Vpr1-45 peptides for 2 hr, Vpr52-96 peptide abrogated LPS-mediated resistance to Vpr52-96-induced apoptosis (Fig. 4-2.1D & E). Overall, these observations suggest that Vpr52-96 peptide induced apoptosis in both cell types and prior stimulation with LPS induced resistance to apoptosis. Furthermore, Vpr52-96 peptide attenuated LPS-induced resistance to Vpr-induced apoptosis.

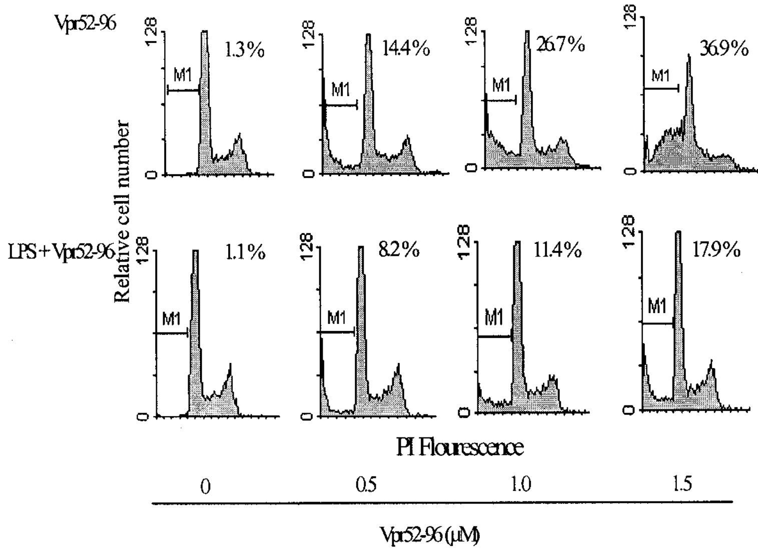
Fig. 4-2.1: LPS induced resistance to Vpr52-96 mediated apoptosis in primary human monocytes and THP-1 cells:

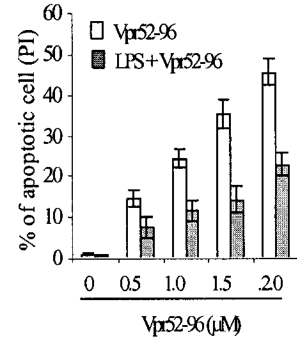
A & B. THP-1 cells (0.5 x 10^6/ml) were stimulated with LPS (1 μg/ml) for 6 h followed by treatment with various concentrations of Vpr52-96 peptide for 24 h. Cells were then stained with intracellular PI for DNA content **(A, & B),** annexin-V/PI for measurement of apoptosis **(B)**. The graphs shown are a mean ± SD of three experiments performed in triplicate.

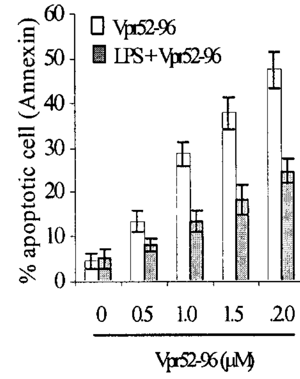
C. Human monocytes (1 x 10^6/ml) were also pretreated with LPS (1 μg/ml) for 6 h prior to stimulation with 1.5 μM of Vpr52-96 peptide for 12 h. Cells were then stained with intracellular PI for DNA content The graphs shown are a mean ± SD of three experiments performed in triplicate (* $p<0.005$).

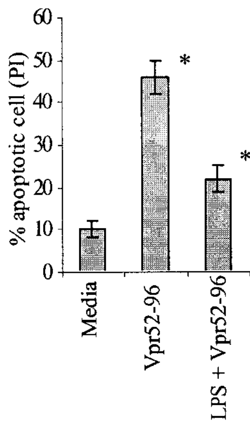
D. THP-1 cells (0.5 x 10^6/ml) were stimulated with various concentrations of Vpr52-96 for 2 h prior treatment with LPS (1 μg/ml) for 24 h. Cells were then stained with annexin-V/PI for measurement of apoptosis. The graphs shown are a mean ± SD of three experiments performed in triplicate.

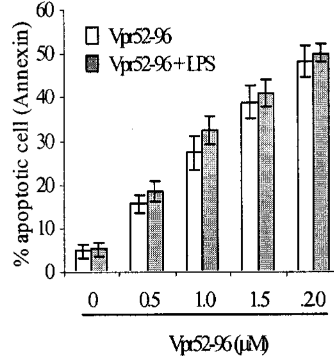
E. Monocytes (0.5 x 10^6/ml) were stimulated with 1.5 μM of Vpr52-96 for 2 h prior treatment with LPS (1 μg/ml) for 12 h. Cells were then stained with PI for measurement of apoptosis. The graphs shown are a mean ± SD of three experiments performed in triplicate.

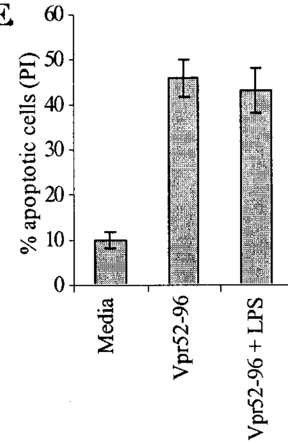

A.
Vpr52-96
LPS + Vpr52-96
Relative cell number
128
0
M1
1.3%
14.4%
26.7%
36.9%
1.1%
8.2%
11.4%
17.9%
PI Flourescence
0
0.5
1.0
1.5
Vpr52-96 (μM)


B.
Vpr52-96
LPS + Vpr52-96
% of apoptotic cell (PI)
0
0.5
1.0
1.5
.2.0
Vpr52-96 (μM)


Vpr52-96
LPS + Vpr52-96
% apoptotic cell (Annexin)
0
0.5
1.0
1.5
.2.0
Vpr52-96 (μM)


C.
% apoptotic cell (PI)
Media
Vpr52-96
LPS + Vpr52-96
*


D.
Vpr52-96
Vpr52-96 + LPS
% apoptotic cell (Annexin)
0
0.5
1.0
1.5
.2.0
Vpr52-96 (μM)


E.
% apoptotic cells (PI)
Media
Vpr52-96
Vpr52-96 + LPS

LPS induced resistance against Vpr52-96 mediated apoptosis is regulated by endogenous production of TNF-α:

Since TNF-α is produced in response to LPS stimulation in monocytic cells, I determined whether LPS-induced protection is mediated by endogenous production of TNF-α. THP-1 cells and primary human monocytes were treated with various concentrations of neutralizing anti-TNF-receptor (anti-TNFR-I) antibodies followed by stimulation of cells with LPS for 6 hr. Subsequently, cells were treated with Vpr52-96 peptides for 24 hr followed by determination of apoptosis. Treatment of cells with Vpr52-96 resulted in 40-45% apoptosis, whereas prior stimulation with LPS significantly reduced the percentage of apoptotic cells to approximately 22%. However, pretreatment of cells with anti-TNFR-I antibodies significantly inhibited the LPS induced protection in both cell types suggesting the involvement of endogenous TNF-α in LPS-induced resistance to Vpr-mediated apoptosis (Fig. 4-2.2A). This was further confirmed by determining the protective effect of recombinant TNF-α on Vpr52-96-induced apoptosis in monocytic cells. THP-1 cell and human monocytes were stimulated with TNF-α for 6 hr prior to treatment with Vpr52-96 peptide followed by determination of apoptosis. The results in Fig.4-2.2B show that pretreatment with TNF-α significantly reduced Vpr52-96-induced apoptosis, as determined by PI and annexin/PI staining. Similar to the study conducted in LPS induced resistance against Vpr52-96-induced apoptosis, THP-1 cells and monocytes were also treated with Vpr52-96 for 2 hr prior to stimulation with TNF-α followed by measurement of apoptosis. The results show that pretreatment of cells with Vpr52-96 abrogated the protective effect of TNF-α to Vpr-induced apoptosis (Fig. 4-2.2C & 4-2.2D).

Fig. 4-2.2: LPS induced resistance against Vpr52-96 mediated apoptosis is regulated by endogenous production of TNF-α:

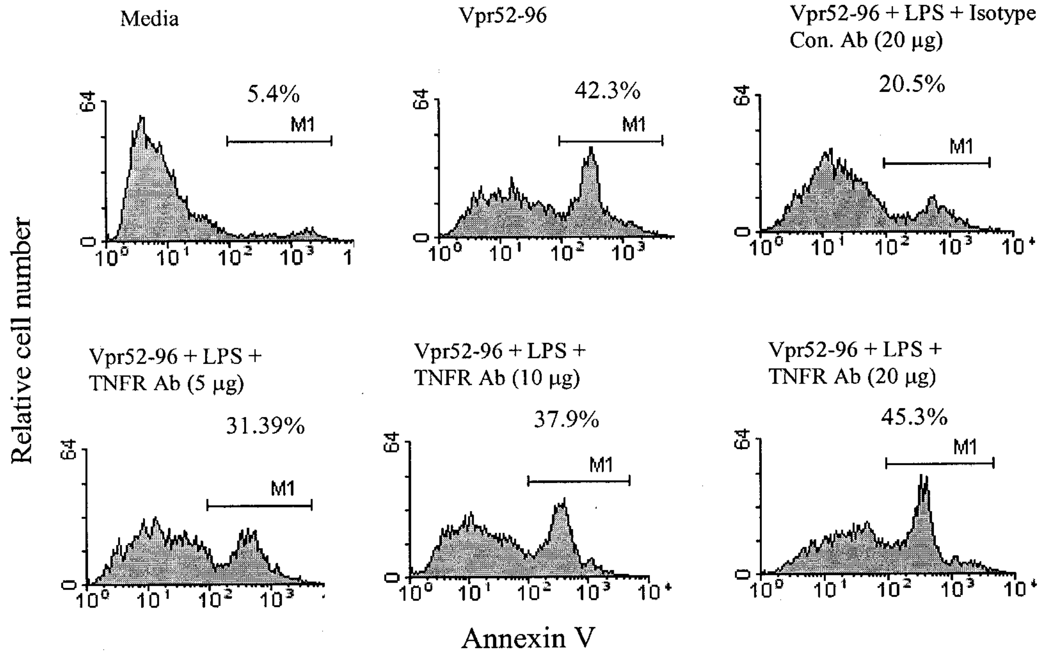
A. THP-1 cells or human monocytes (0.5 x 10^6/ml) were stimulated with LPS (1 μg/ml) for 6 h in the presence or absence of anti-TNF-α-R1 (10-20 μg/ml) or isotype matched control antibodies followed by treatment with 1.5 μM Vpr52-96 peptide for 24 h (THP-1 cell) or 12 h (monocytes). Cells were then stained with annexin-V/PI (THP-1) or PI (monocytes) for measurement of apoptosis. The results shown are representative of three different experiments (* $p < 0.01$).

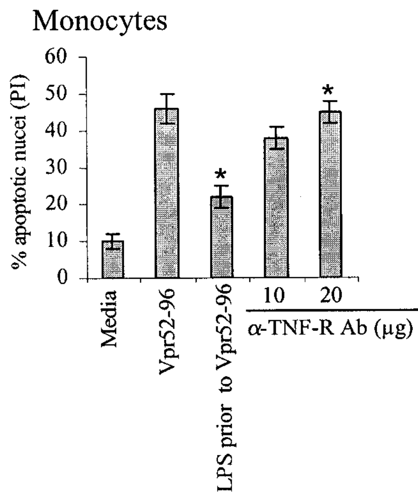
B. Cells (0.5 x 10^6/ml) were stimulated with TNF-α (10 ng/ml) for 6 h followed by treatment with various concentrations of Vpr52-96 peptide for 24 h. Cells were then stained with intracellular PI for DNA content **(Left panel),** annexin-V/PI for measurement of apoptosis **(Middle panel)**. The graphs shown are a mean ± SD of three experiments performed in triplicate. Human monocytes (1 x 10^6/ml) were also pretreated with TNF-α for 6 h prior to stimulation with 1.5 μM of Vpr52-96 peptide for 12 h. Cells were then stained with intracellular PI for DNA content **(Right panel)**. The graphs shown are a mean ± SD of three experiments performed in triplicate (* $p < 0.005$).

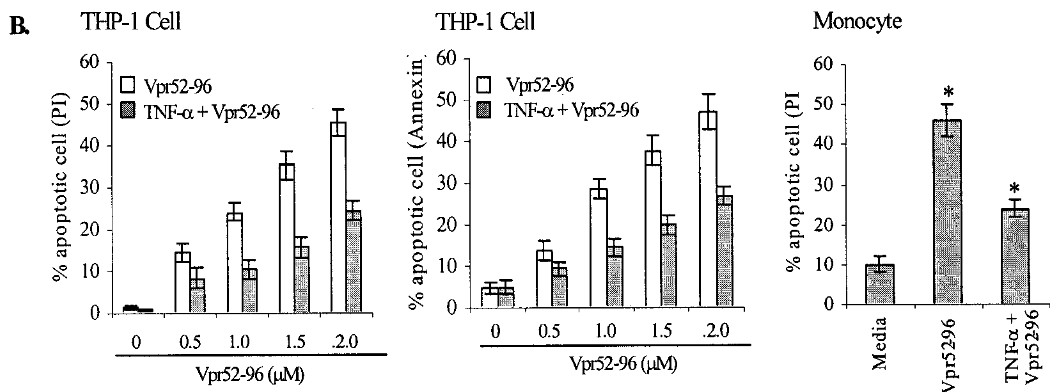
C & D. Cells (0.5 x 10^6/ml) were stimulated with various concentrations of Vpr52-96 for 2 h prior treatment with TNF-α for 24 h (THP-1 cell) or 12 h (monocytes). Cells were then stained with annexin-V/PI (THP-1) or PI (monocytes) for measurement of apoptosis. The graphs shown are a mean ± SD of three experiments performed in triplicate.

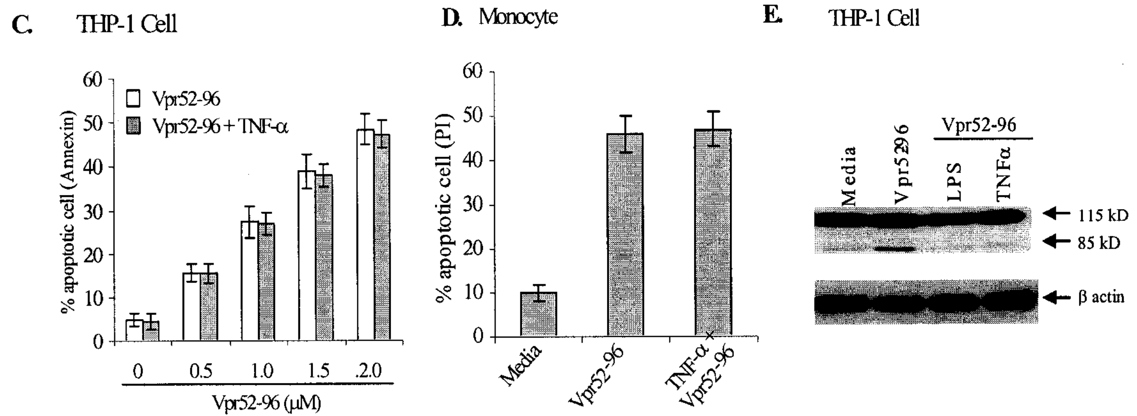
E. THP-1 cells (1 x 10^6/ml) were also pretreated with LPS/TNF-α for 6 h prior to stimulation with 1.5 μM of Vpr52-96 peptide for 12 h. Cell lysates were subjected to SDS-PAGE and Western blot analysis for PARP expression by using anti-PARP antibodies. The results shown are a representative of three different experiments.

A. THP-1 cells






B.
THP-1 Cell
% apoptotic cell (PI)
Vpr52-96
TNF-α + Vpr52-96
Vpr52-96 (μM)
THP-1 Cell
% apoptotic cell (Annexin
Vpr52-96
TNF-α + Vpr52-96
Vpr52-96 (μM)
Monocyte
% apoptotic cell (PI
Media
Vpr5296
TNFα + Vpr5296


C.
THP-1 Cell
% apoptotic cell (Annexin)
Vpr52-96
Vpr52-96 + TNF-α
Vpr52-96 (μM)
D. Monocyte
% apoptotic cell (PI)
Media
Vpr52-96
TNF-α + Vpr52-96
E. THP-1 Cell
Media
Vpr5296
Vpr52-96
LPS
TNFα
115 kD
85 kD
β actin

It is known that in response to apoptotic stimuli, PARP, one of the enzymes present in the nucleus that helps in repairing of DNA damage, is cleaved and it is considered as a marker of apoptosis. Our results show that treatment of cells with Vpr52-96 peptide cleaved PARP and prior treatment with LPS and/or TNF-α inhibited the fragmentation of PARP in THP-1 cells (Fig. 4-2.2E).

LPS/TNF-α induced protection in Vpr52-96-mediated apoptosis is regulated by c-IAP2:

My earlier results suggest LPS and TNF-α mediated protection against staurosporine-induced apoptosis was mediated through the anti-apoptotic gene c-IAP2 in THP-1 cells. Therefore, it is possible that c-IAP2 induction may regulate LPS and/or TNF-α-induced protection against Vpr52-96-mediated apoptosis. To address this question, I first demonstrated that LPS as well as TNF-α induced the expression of c-IAP2 gene in both primary monocytes as well as THP-1 cells. As observed earlier, low level of c-IAP2 mRNA and c-IAP2 proteins were detected in unstimulated THP-1 cells and monocytes and their expression levels increased significantly following stimulation with either LPS or TNF-α. To determine the involvement of c-IAP2 in LPS-/TNF-α-induced protection, THP-1 cells were transfected with antisense c-IAP2 or control oligonucleotides prior to stimulation with either LPS or TNF-α for 24 hr followed by determination of c-IAP2 expression by Western blotting and Vpr52-96 induced apoptosis. My earlier results showed that transfection of cells with antisense c-IAP2 oligonucleotides significantly inhibited the LPS- or TNF-α-induced expression of c-IAP2 compared to the cells transfected with control oligonucleotides (Fig. 3.1D). Similar to the results obtained with untransfected cells, LPS and TNF-α stimulation inhibited Vpr52-

96-induced apoptosis in cells transfected with control oligonucleotides (from 45-50% to 20-25%). In contrast, stimulation with either LPS or TNF-α failed to inhibit significantly Vpr52-96-induced apoptosis in cells transfected with anti-sense c-IAP2 oligonucleotides (45-50%) (Fig. 4-2.3A). These results suggest that LPS-/TNF-α-induced resistance to Vpr-mediated apoptosis is regulated through the induction of c-IAP2 gene in THP-1 cells. I further demonstrated that LPS induced protection against Vpr-mediated apoptosis is regulated by endogenously produced TNF-α through the induction of c-IAP2 gene. I already showed that prior treatment of LPS-stimulated THP-1 cells with anti-TNFR1 antibodies inhibited the induction of c-IAP2 gene compared to the cell pretreated with isotype control antibodies (Fig 3.1C). I also confirmed this finding in human monocytes (Fig. 4-2.3B).

Vpr52-96 inhibits LPS-/TNF-α-induced c-IAP2 expression in monocytic cells:

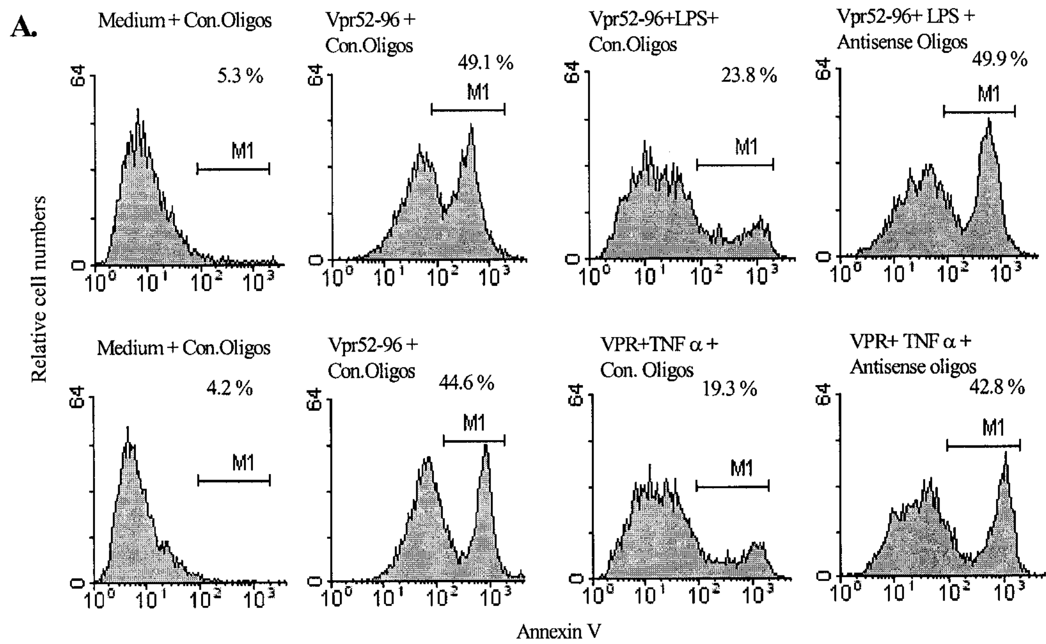
Because the above results suggest that c-IAP2 induction regulates LPS- and TNF-α-induced protection against Vpr52-96-mediated apoptosis and pretreatment of cells with Vpr52-96 peptide inhibited LPS- and TNF-α-induced protection against Vpr52-96-mediated apoptosis, it was of interest to determine if Vpr52-96 inhibits LPS- and TNF-α-induced expression of c-IAP2. As expected, stimulation of cells with either LPS or TNF-α induced the expression of c-IAP2 in both monocytes and THP-1 cells. However, prior treatment with Vpr52-96 peptide for 2 hr inhibited LPS- and TNF-α-induced expression of c-IAP2 in THP-1 cells and monocytes at protein level as determined by Western immunoblotting and at the RNA level in THP-1 cell by RT-PCR (Fig. 4-2.4A). It may be pointed out that pretreatment of cells with Vpr52-96 peptide also inhibited the basal

Fig. 4-2.3: LPS/TNF-α induced protection in Vpr52-96-mediated apoptosis is regulated by c-IAP2:

A. THP-1 cells (10^6/ml) were transfected with either antisense (AS) c-IAP2 or control oligonucleotides followed by stimulation with LPS (1 μg/ml) or TNF-α (10 ng/ml) for 6 h. Transfected and stimulated cells were treated with Vpr52-96 peptide for 24 h followed by determination of apoptotic cells by annexin/PI staining. The experiments shown are representative of three different experiments.

B. Monocytes (0.5 x 10^6/ml) were stimulated with LPS (1 μg/ml) for 6 h in the presence and the absence of anti-TNF-α-R1 (10-20 μg/ml) or isotype matched control antibodies followed by treatment with 1.5 μM Vpr52-96 peptide for 12 h. Cell extracts were prepared and c-IAP2 expression was determined by Western blot analysis. The results shown are representative of three different experiments.

A.



B.

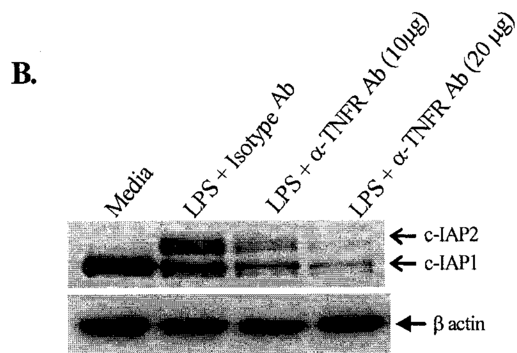


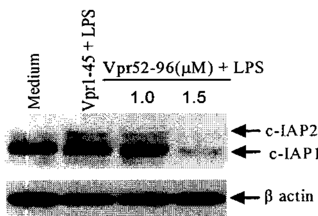
Fig. 4-2.4:Vpr52-96 inhibits LPS-/TNF-α-induced c-IAP2 expression in monocytic cells:

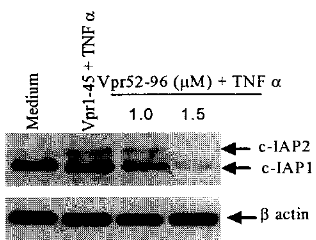
A. THP-I cells or monocytes (5 x 10^6/ml) were treated with various concentrations of Vpr52-96 or Vpr1-45 peptide for 2 h followed by stimulation with either LPS (1 μg/ml) or TNF-α. **Upper panel:** Cell extracts were prepared and c-IAP2 expression was determined by Western blot analysis. **Lower panel:** RNA was extracted from THP-1 cells and semi-quantitative PCR was conducted for c-IAP2. The results shown in A and B are representative of three different experiments.

B. THP-I cells or monocytes (5 x 10^6/ml) were stimulated with either LPS (1 μg/ml) or TNF-α for 6 h followed by treatment with various concentrations of Vpr52-96 or Vpr1-45 peptide. **Upper panel:** Cell extracts were prepared and c-IAP2 expression was determined by Western blot analysis. **Lower panel:** RNA was extracted from THP-1 cells and semi-quantitative PCR was conducted for c-IAP2.

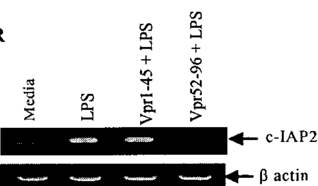
A. LPS after treatment (THP-1 cells)

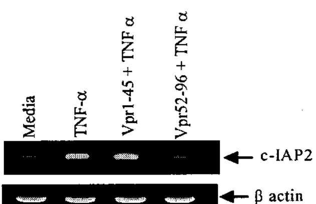
Western blotting



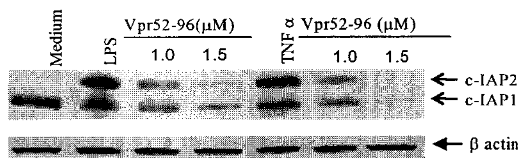


RT-PCR



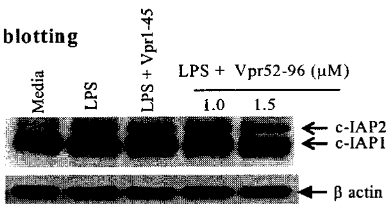


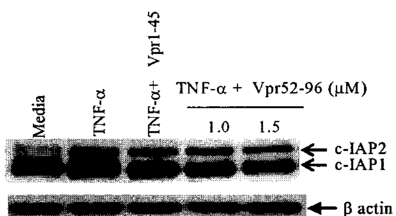
Monocytes



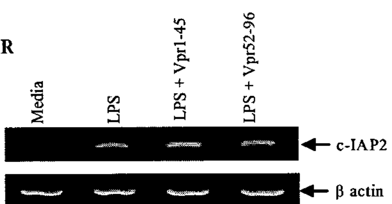
B. LPS prior treatment (THP-1 cells)

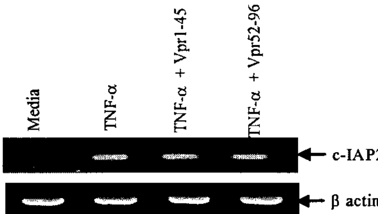
Western blotting



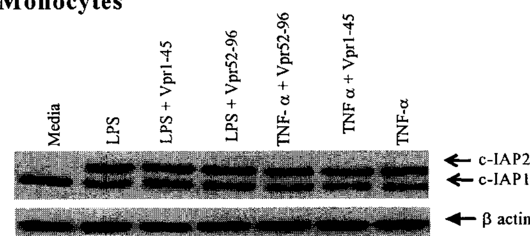


RT-PCR





Monocytes



levels of c-IAP1 in both monocytes and THP-1 cells. In contrast, if cells were treated with Vpr52-96 peptide following stimulation with either LPS or TNF-α, the expression of c-IAP2 was not inhibited at RNA (THP-1 cell) as well as protein level (monocyte and THP-1 cell) even when Vpr peptide was used at a highest concentration of 2 μM (Fig. 4-2.4B). These results suggested that LPS- and TNF-α-induced protection against Vpr52-96-mediated apoptosis may be regulated, at least in part, through the induction of anti-apoptotic c-IAP2 gene. Furthermore, Vpr52-96 peptide may inhibit LPS and TNF-α-induced protection by inhibiting c-IAP2 induction.

LPS/TNF-α induced activation of CAMKII confers protection to Vpr52-96 induced apoptosis:

I have previously demonstrated that CaM/CAMKII regulates LPS- and TNF-α-induced expression of c-IAP2 in THP-1 cells. To determine whether CaM/CAMKII also regulates c-IAP2 expression in primary human monocytes, pharmacological inhibitors specific for calcium influx (EGTA), calmodulin (W-7), and CAMKII (KN-93) were employed. The results show that EGTA, W-7 and KN-93 inhibited LPS- as well as TNF-α-induced expression of c-IAP2 in monocytes (Fig. 4-2.5C).

Since c-IAP2 induction has been shown to induce resistance to staurosporine-induced apoptosis, it was of interest to determine the role of calcium signaling and in particular the role of CaM/CAMKII in LPS- and TNF-α-induced resistance to Vpr52-96-mediated apoptosis in primary human monocytes and THP-1 cells. THP-1 cells and monocytes were treated with pharmacological inhibitors specific for the calcium signaling such as calcium chelator EGTA for 2 hr prior to stimulation with LPS and TNF-α for 6 hr. Subsequently cells were treated with various concentrations of Vpr52-96

or Vpr1-45 peptides for 24 hr followed by analysis for apoptosis. As expected, stimulation with either LPS or TNF-α inhibited Vpr52-96-induced apoptosis in both THP-1 cells (Fig. 4-2.5A) and monocytes (Fig. 4-2.5B). Significantly, prior treatment of cells with EGTA reversed the LPS/TNF-α mediated inhibition of Vpr52-96 induced apoptosis in both monocytes and THP-1 cells (Fig. 4-2.5A and 4-2.5B). Elevations in cytoplasmic calcium concentrations occur following stimulation by diverse stimuli that activate voltage or ligand-gated calcium channels in the plasma membrane or following release of calcium present in intracellular stores, mainly in the endoplasmic reticulum (ER). The role of receptor-mediated entry of extracellular Ca^{2+} was studied by employing SKF-96365. To determine whether calcium release from the ER regulates LPS- and TNF-α-induced resistance to Vpr-mediated apoptosis, we employed 2-APB, an inhibitor of the IP3 receptor which blocks the release of calcium from the ER by blocking IP3 receptor-gated channels. Treatment with SKF prior to stimulation with LPS or TNF-α reversed the LPS- as well as TNF-α- mediated inhibition of Vpr52-96-induced apoptosis in THP-1 cells (Fig. 4-2.5A). However, prior treatment with 2-APB did not affect Vpr52-96-induced apoptosis (Fig 4-2.5A).

Calmodulin (CaM), a major calcium receptor, is present in both cytoplasmic and nuclear compartments. The calcium/CaM complex regulates several downstream targets including protein kinases and phosphatases. To investigate the role of CaM, a specific calmodulin inhibitor, W-7, was employed. One major family of calcium/CaM effectors is the calmodulin-dependent protein kinases (CAMK), which includes a multifunctional kinase, CAMKII. To gain further insight into the role of CaM, we examined the involvement of CAMKII by employing the CAMKII-specific inhibitor, KN-93. Results

show that calmodulin and CAMKII inhibitors significantly reversed the protection induced by LPS- and TNF-α in THP-1 cells and human monocytes (Fig 4-2.5A and 4-2.5B). It may be pointed out that pretreatment of cells with inhibitors specific for p38 (SB202190), p42/44 ERK (PD98059), JNK MAPKs (SP600125), PI3K (Ly294002), (data not shown) and calcineurin such as FK506 and Cyclosporin A did not affect the level of protection induced by LPS and TNF-α in THP-1 cells (Fig 4-2.5A).

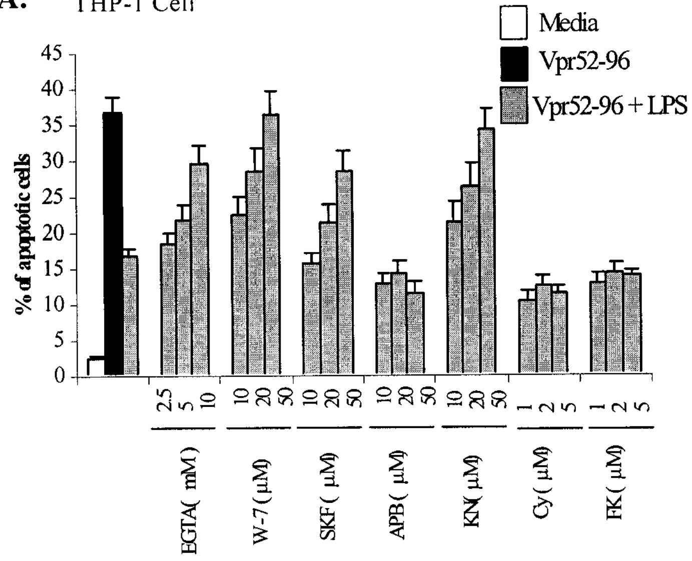
To confirm the involvement of CAMKII in LPS- and TNF-α-mediated inhibition of Vpr52-96-induced apoptosis, THP-1 cells were transfected with DN-CAMKII plasmid. After 24 hr, transfected cells were stimulated with either LPS or TNF-α followed by determination of c-IAP2 expression and analysis of Vpr52-96-induced apoptosis. My earlier results showed significant inhibition of c-IAP2 expression in cells transfected with DN-CAMKII plasmid following LPS as well as TNF-α stimulation compared to the cells transfected with the control vector (Fig. 3.6C). Stimulation with either LPS or TNF-α inhibited Vpr52-96-induced apoptosis in cells transfected with control vector. In contrast, transfection of cells with DN-CAMKII construct reversed the LPS- (from 21 to 43%) and TNF-α-mediated (from 20 to 40%) inhibition of Vpr52-96-induced apoptosis (Fig. 4-2.6). Overall, these results suggest that LPS and TNF-α induce protection against Vpr52-96-mediated apoptosis by c-IAP2 through CAMKII activation.

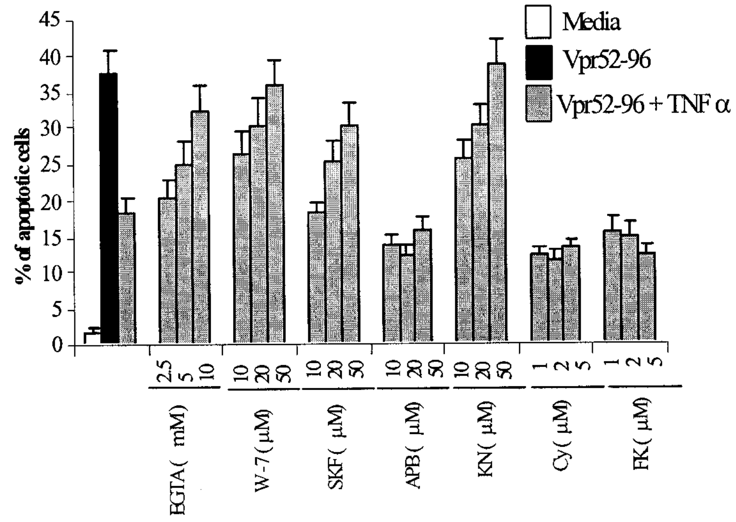
Fig. 4-2.5: LPS-/TNF-α-mediated resistance to Vpr52-96 induced apoptosis is regulated through the activation of CaM/CAMKII:

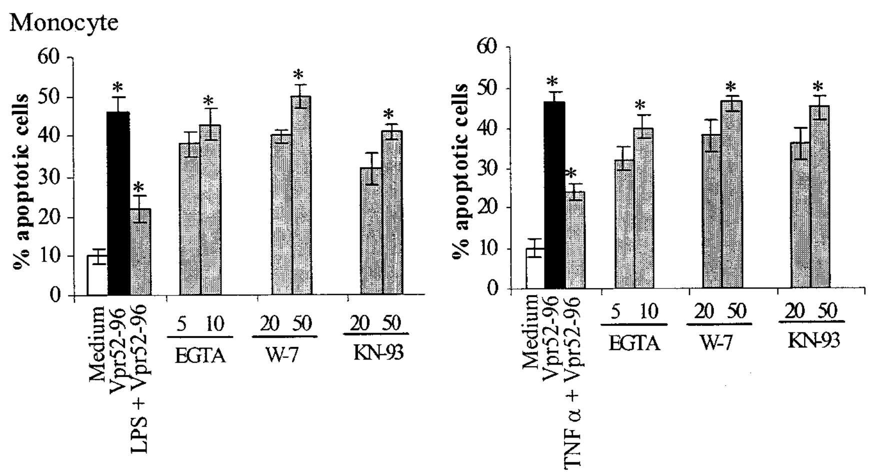
A. THP-1 cells (0.5 x 10^6/ml) were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 6 h in the presence or absence of various concentrations of calcium signaling inhibitors such as EGTA, W-7, SKF, APB, KN-93, Cyclo, and FK506 followed by treatment with 1.5 μM Vpr52-96 peptide for 24 h. Cells were then stained with intracellular PI for measurement of DNA content. The graphs shown are a mean ± SD of three different experiments.

B & C. Monocytes (0.5 x 10^6/ml) were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 6 h in the presence or absence of various concentrations of calcium signaling inhibitors such as EGTA, W-7, and KN-93 followed by treatment with 1.5 μM Vpr52-96 peptide for 12 h. Cells were then stained with intracellular PI for measurement of DNA content. The graphs shown are a mean ± SD of three different experiments (* $p < 0.001$). Cell extracts were used to detect c-IAP2 expression by Western blot. The experiments shown are representative of three different experiments.

A. THP-1 Cell






B.
Monocyte
% apoptotic cells
60
50
40
30
20
10
0
Medium
Vpr52-96
LPS + Vpr52-96
5 10
EGTA
20 50
W-7
20 50
KN-93
TNF α + Vpr52-96

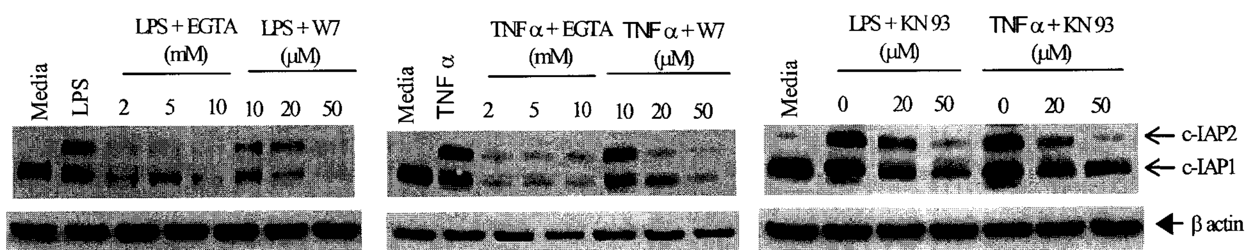
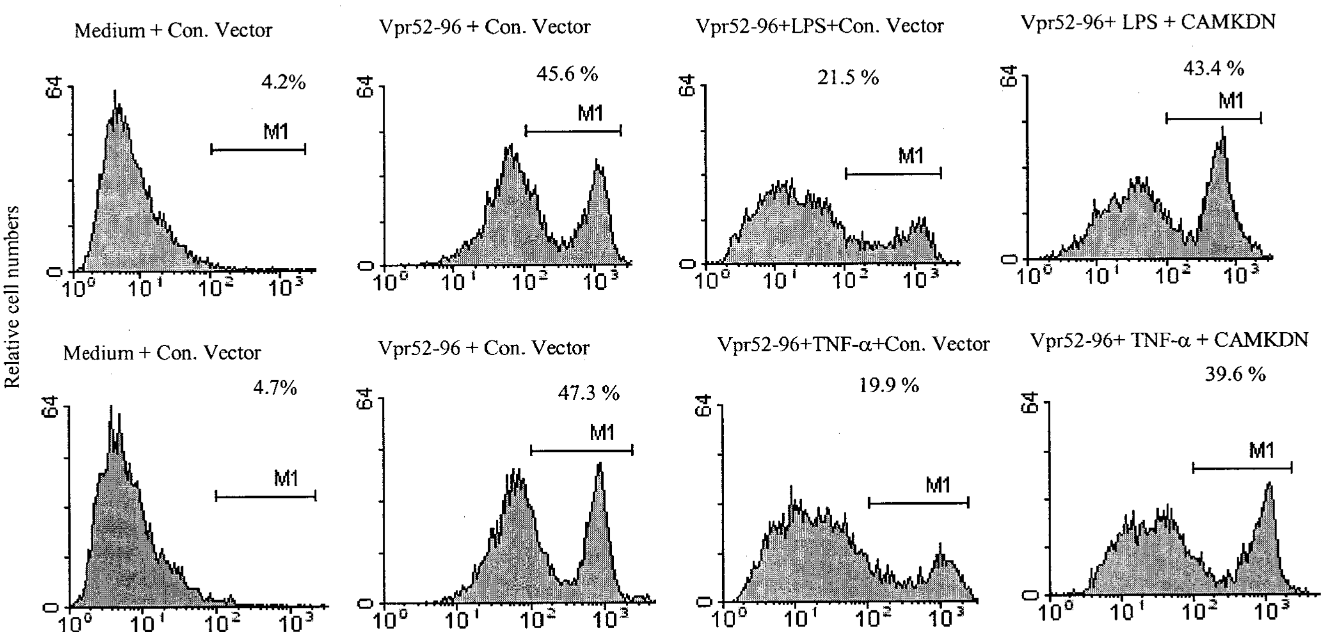

C.
Monocyte
Media
LPS
LPS + EGTA
(mM)
2 5 10
LPS + W7
(μM)
10 20 50
TNF α
TNF α + EGTA
(mM)
TNF α + W7
(μM)
LPS + KN 93
(μM)
0 20 50
TNF α + KN 93
(μM)
c-IAP2
c-IAP1
β actin

Fig. 4-2.6: LPS-/TNF-α- induced activation of CAMKII confers protection to Vpr52-96 induced apoptosis:

THP-1 cells were transfected with either DN-CAMKII or control vector and cultured for 24 h, followed by stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for another 6 h. Cells were then treated with Vpr52-96 for 24 h followed by determination of apoptotic cells by annexin/PI staining. The results shown are representative of three different experiments.


Medium + Con. Vector
4.2%
Vpr52-96 + Con. Vector
45.6 %
Vpr52-96+LPS+Con. Vector
21.5 %
Vpr52-96+ LPS + CAMKDN
43.4 %
Medium + Con. Vector
4.7%
Vpr52-96 + Con. Vector
47.3 %
Vpr52-96+TNF-α+Con. Vector
19.9 %
Vpr52-96+ TNF-α + CAMKDN
39.6 %
M1
64
0
Relative cell numbers
Annexin V

Vpr52-96 inhibits LPS- and TNF-α-induced resistance to Vpr52-96-mediated apoptosis by blocking LPS- and TNF-α-induced calcium influx, CAMKII phosphorylation and c-IAP2 transcription in human monocytic cells:

Vpr52-96 interferes with the LPS- and TNF-α-induced calcium influx and CAMKII phosphorylation:

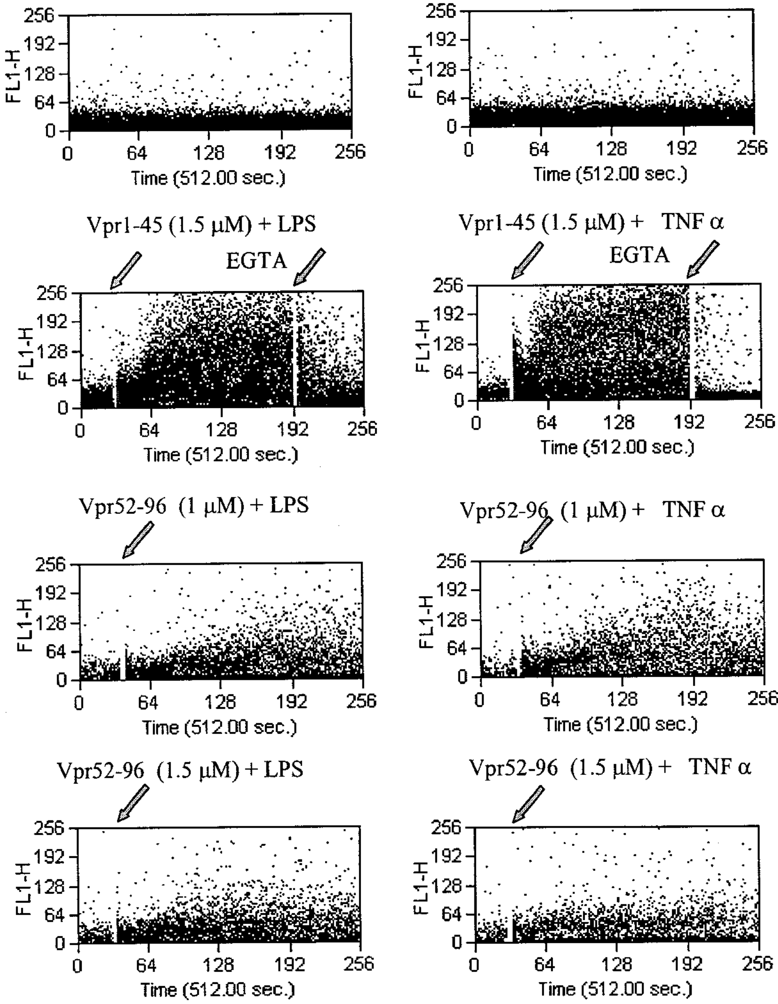
Since Vpr52-96 treatment prior to stimulation of monocytic cells with either TNF-α or LPS inhibited LPS- and TNF-α-mediated c-IAP2 expression (Fig 4-2.4A and 4-2.4B), it is likely that Vpr52-96 may interfere with the LPS- and TNF-α-induced CaM/CAMKII pathway to inhibit LPS-/TNF-α-mediated protection against Vpr52-96-induced apoptosis. I first determined if Vpr52-96 interferes with the LPS- and TNF-α-induced calcium pathway, and in particular the calcium influx, cells were treated with Vpr52-96 followed by stimulation with LPS or TNF-α and measurement of calcium flux at various times. Both LPS as well as TNF-α induced calcium influx at 12 and 8 min post stimulation, respectively, and treatment with EGTA inhibited LPS- and TNF-α-induced calcium influx to the basal level in THP-1 cells (Fig. 4-2.7A). LPS- and TNF-α-induced calcium influx was inhibited by Vpr52-96 in a dose dependent manner (Fig. 4-2.7A). In contrast, pretreatment of cells with Vpr1-45 did not have any effect on LPS- or TNF-α-induced calcium influx in THP-1 cells (Fig 4-2.7A). Similar results were obtained when monocytes were treated with Vpr peptides prior to stimulation with either LPS or TNF-α (data not shown). To determine if Vpr52-96 inhibited LPS- and TNF-α-induced CAMKII activity, THP-1 cells were treated with Vpr52-96 for 2 hr followed by stimulation with either LPS or TNF-α and measurement of CAMKII activity. Vpr52-96 inhibited LPS- and TNF-α- induced CAMKII activity (Fig 4-2.7B).

Fig. 4-2.7: Vpr52-96 interferes with the LPS- and TNF-α-induced calcium influx and CAMKII activity in human monocytic cells:

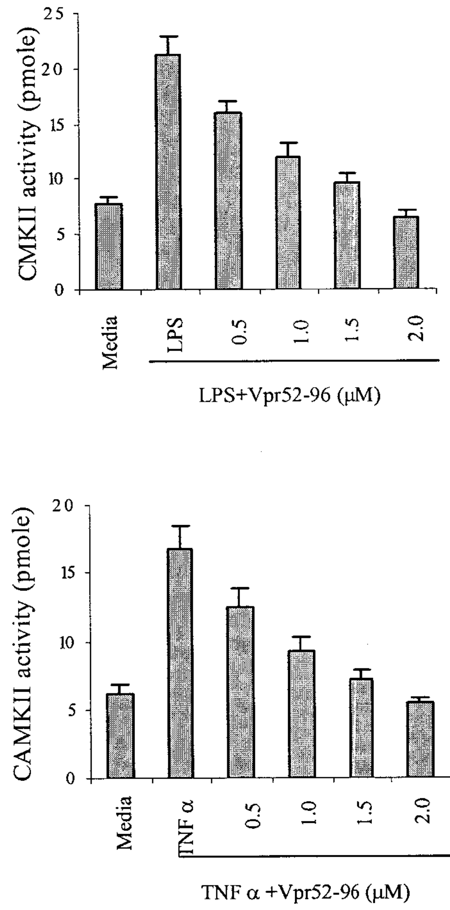
A. THP-1 cells (0.5 x 10^6/ml) treated with Vpr52-96 peptide for 2 h followed by loaded with Fluo3/AM. Cells were then stimulated with either LPS or TNF-α and the resulting Ca^{2+} influx was measured by flow cytometric analysis. **Top panel:** Baseline Ca^{2+} levels in unstimulated cells. **2nd panel:** Stimulation with LPS/TNF-α followed by the addition of EGTA. **3rd and 4th panels:** Treatment with Vpr52-96 peptide prior to stimulation with LPS/TNF-α. The results shown are representative of three different experiments.

B. THP-1 cells (2.0 x 10^6/ml) were pretreated with Vpr52-96 for 2 h followed by stimulation of cells with either LPS or TNF-α for 30 min. CAMKII activity was assayed from total cell proteins utilizing a peptide substrate (KKALRRQETVDAL) specific for CAMKII. The results shown represent the mean ± S.D. of three independent experiments.

A.



B.



Vpr52-96 inhibits LPS-induced c-IAP2 transcription in human monocytic cells:

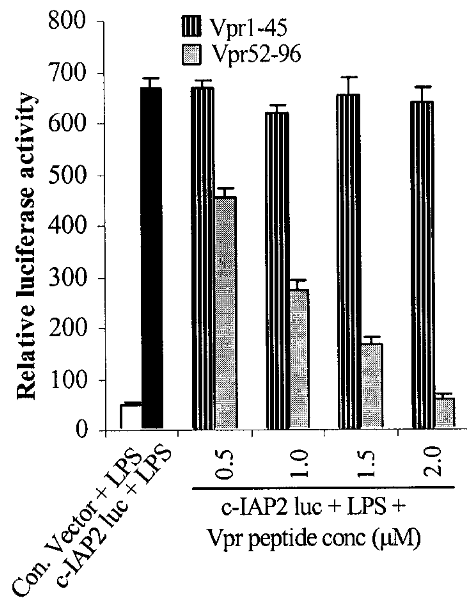
NFκB has been shown to play a critical role in c-IAP2 transcription following LPS and TNF-α stimulation in monocytic cells [11]. To determine whether Vpr52-96 inhibits c-IAP2 transcription by inhibiting NFκB activation, THP-1 cells were transfected with human c-IAP2 promoter (-606 to + 121 bp) linked to the luciferase reporter construct. Subsequently, transfected cells were stimulated with either LPS or TNF-α in the presence or absence of Vpr peptides. Stimulation of cells with either LPS or TNF-α significantly enhanced c-IAP2 promoter activity compared to the cells transfected with the control vector. (Fig 4-2.8). Vpr52-96 treatment prior to LPS or TNF-α stimulation significantly inhibited c-IAP2 promoter activity whereas pretreatment with Vpr1-45 peptide did not have any effect on luciferase activity (Fig. 4-2.8).

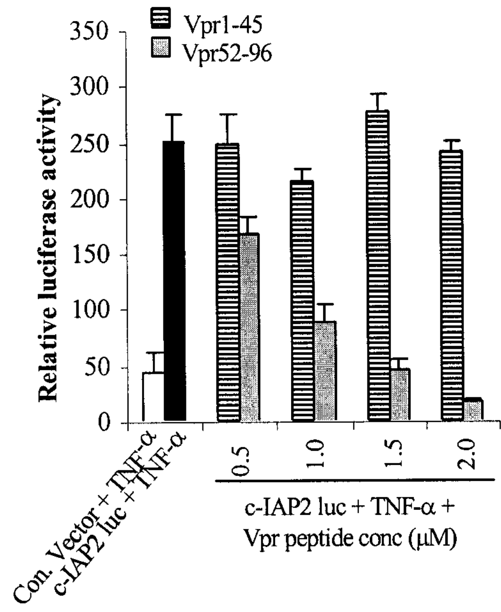
Vpr52-96 inhibits binding of NFκB to its binding sites in the c-IAP2 promoter in LPS- and TNF-α-stimulated monocytic cells:

To further confirm that Vpr52-96 inhibits LPS-/TNF-α-induced c-IAP2 gene transcription by inhibiting the binding of NFκB to the c-IAP2 promoter, the nuclear extract harvested from LPS- and TNF-α-stimulated cells in the presence and absence of Vpr peptides were analyzed for binding of NFκB to the oligonucleotide probes containing NFκB sequences corresponding to their sites in the c-IAP2 promoter. There are at least two NFκB binding sites in the c-IAP2 promoter that have been implicated in transcription. Stimulation of cells with either LPS or TNF-α induced the binding of NFκB to the oligonucleotide probes containing NFκB site # 1 as well as site # 3 (Fig. 4-2.9). I have already demonstrated in my earlier study the specificity of NFκB binding by

Fig. 4-2. 8: Vpr52-96 inhibits LPS-induced c-IAP2 transcription in human monocytic cells:

THP-1 cells (10^6/ml) were transiently cotransfected with 5 μg of pc-IAP2pr-GL3B (c-IAP2 luc) and 3 μg of β-galactosidase plasmids. After 24 h, cells were treated with either Vpr1-45 or Vpr52-96 peptides for 2 h followed by stimulation with either 1 μg/ml LPS or 10 ng/ml TNF-α for 24 h. Following incubation, luciferase and β-galactosidase activities were determined. Luciferase activity was normalized for β-galactosidase activity to give relative luciferase units (RLU).


Vpr1-45
Vpr52-96
Relative luciferase activity
800
700
600
500
400
300
200
100
0
Con. Vector + LPS
c-IAP2 luc + LPS
0.5
1.0
1.5
2.0
c-IAP2 luc + LPS +
Vpr peptide conc (μM)

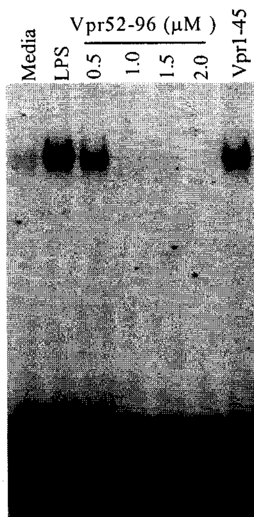

Vpr1-45
Vpr52-96
Relative luciferase activity
350
300
250
200
150
100
50
0
Con. Vector + TNF-α
c-IAP2 luc + TNF-α
0.5
1.0
1.5
2.0
c-IAP2 luc + TNF-α +
Vpr peptide conc (μM)

cold competition with specific and non specific oligonucleotides and by supershift analysis using anti NFκB-p50 and p65 antibodies (Fig. 3.10). Further, treatment of THP-1 cells with Vpr52-96 peptide for 2 hr prior to stimulation with either LPS or TNF-α inhibited the binding of NFκB to its oligonucleotide probes in a dose dependent manner (Fig. 4-2.9). In contrast, prior treatment of cells with Vpr1-45 peptide did not have any effect on NFκB binding (Fig. 4-2.9). Taken together, these results suggest that Vpr52-96 inhibits LPS- and TNF-α-induced resistance to Vpr52-96-mediated apoptosis by blocking LPS- and TNF-α-induced calcium influx, CAMKII phosphorylation and c-IAP2 transcription in human monocytic cells.

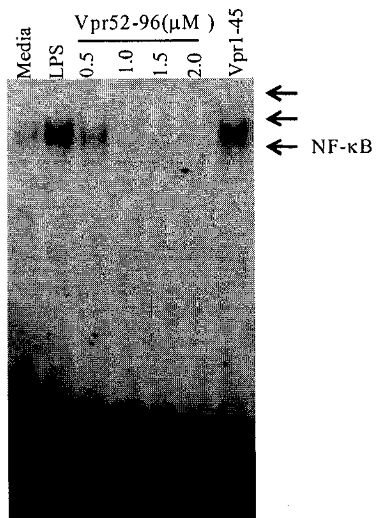
Based on all these findings, my results suggest that LPS-/TNF-α-induced resistance to Vpr52-96 mediated apoptosis is due to c-IAP2 upregulation via activation of CAMKII through NFκB.

Fig. 4-2. 9: Vpr52-96 inhibits binding of NFκB to its binding sites in the c-IAP2 promoter in LPS- and TNF-α-stimulated monocytic cells:

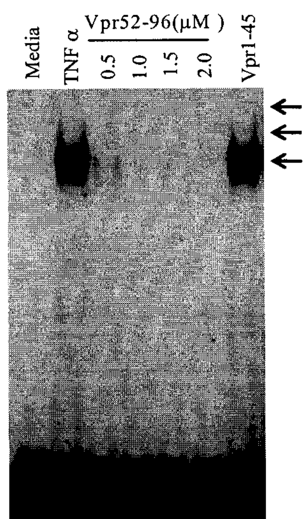
THP-1 cells (5 X 10^6/ml) were treated with either Vpr1-45 or Vpr52-96 peptides for 2 h followed by stimulation with either 1 μg/ml LPS or 10 ng/ml TNF-α for 60 and 45 min, respectively. Nuclear proteins (5 μg) were incubated with ^{32}P-labeled oligonucleotide probes corresponding to NF-κB site 1 and 3 sequences derived from the c-IAP2 promoter. The samples were run in 5% non denaturing polyacrylamide gel, dried and autoradiographed. The results shown are representative of three different experiments.



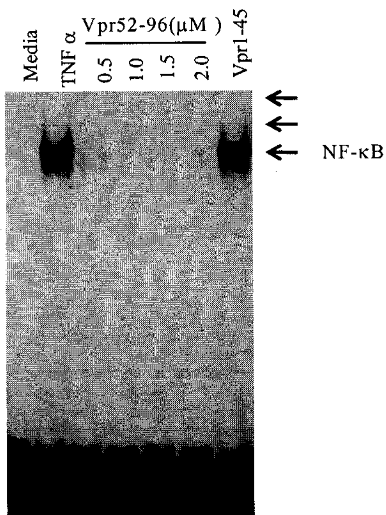
NF-κB site # 1



NF-κB site # 3



NF-κB site # 1



NF-κB site # 3

Discussion:

HIV is known to cause apoptosis in CD4+ T cells and other cell types including neuronal cells, and neutrophils and plays a key role in HIV pathogenesis [90]. However, monocytes/macrophages unlike CD4+ T cells survive HIV replication and serve as a major reservoir of HIV in lymphoid and extralymphoid tissues at all stages of the disease [8]. The inability to eliminate persistently infected monocytic cell reservoirs in HIV-infected patients despite highly active anti-retroviral therapy poses a major hurdle in curing the disease [332-336]. Based on my preliminary results, I believe that the pro-inflammatory cytokine, TNF-α which is known to enhance HIV replication and is produced following HIV infection may be a key to protect monocytic cells from the cytopathic effects of HIV. This may promote the development and maintenance of these cells as viral reservoirs.

Among various proteins involved in inducing apoptosis in HIV infection, Vpr plays an important role being packaged in great quantities into the virion nucleocapsid and is also expressed late in the infection suggesting the importance of this protein throughout the viral life cycle [12;13]. HIV Vpr is believed to cause cell death in several cell types including CD4+ T cells and neuronal cells [34;95;129]. In this study, I used C terminal Vpr peptide (Vpr52-96) which mimics the apoptosis inducing property of Vpr and the N terminal end (Vpr1-45) as control. First of all, I delineated the mechanism involved in Vpr induced apoptosis in monocytes and monocytic cell line. My results revealed that Vpr52-96 induced apoptosis in a caspase dependent manner by inducing mitochondrial membrane permeabilization. I also demonstrated that activation of JNK MAPK downregulates anti-apoptotic genes such as c-IAP1 and Bcl2 by inhibiting the

respective transcription factors which lead to apoptosis. Further, I have shown that LPS/TNF-α induces protection to Vpr52-96-mediated apoptosis and it is regulated by upregulation of one of the anti-apoptotic genes c-IAP2 through activation of CAMKII in monocytes and a monocytic cell line. These two important findings are discussed as below in separate sections.

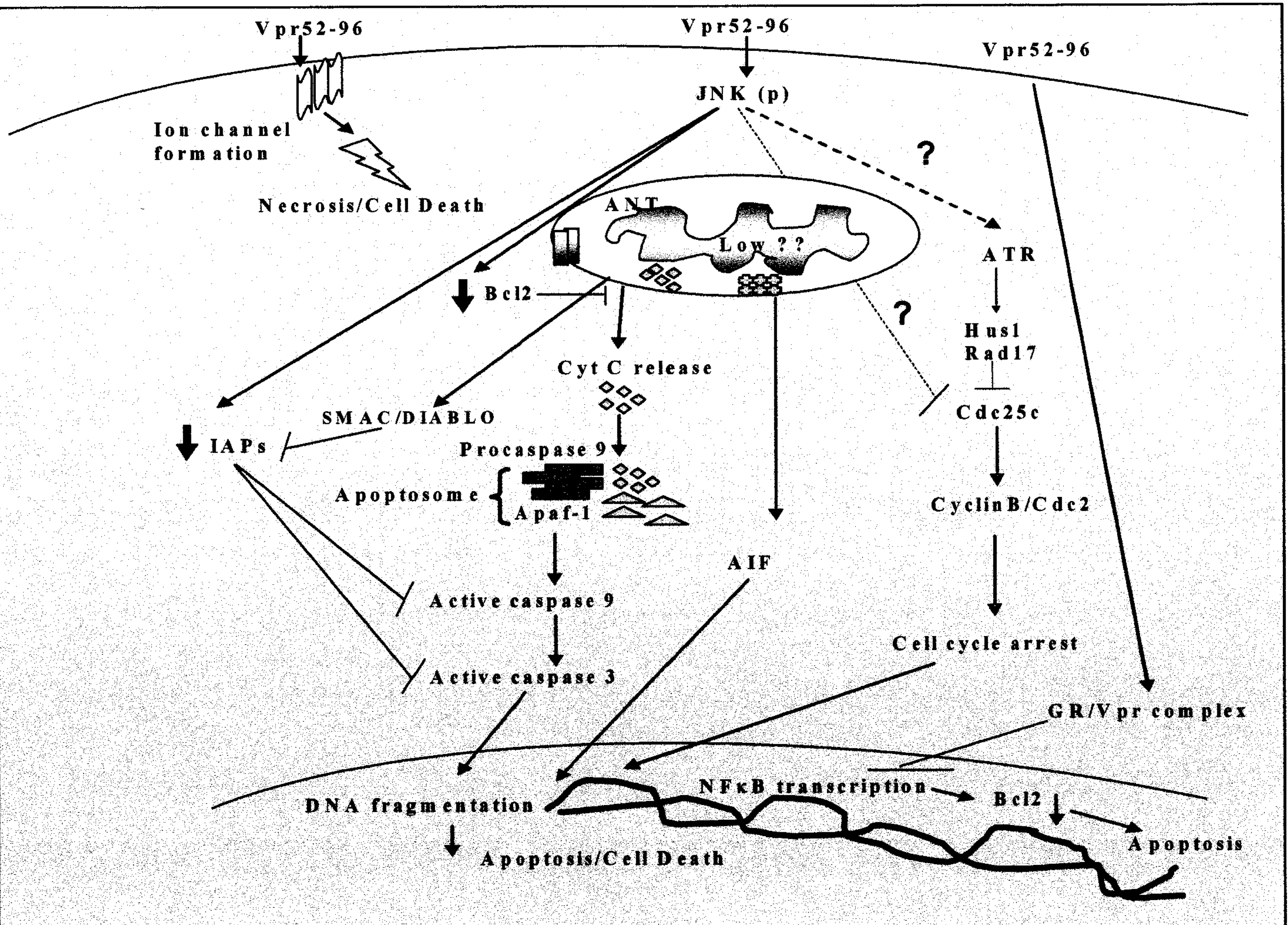
Signaling pathways involved in Vpr induced apoptosis:

The primary aim of this study was to investigate the signaling pathways, particularly the role of MAPKs, and the transcription factors involved in the regulation of Vpr-induced apoptosis in human monocytic cells. My results suggest that Vpr52-96-induced apoptosis in normal human monocytes and promonocytic THP-1 cells occurs in a caspase-dependent manner. Although both Vpr1-45 and Vpr52-96 peptides induced phosphorylation of p38, ERK, and JNK MAPKs, Vpr52-96-induced apoptosis was regulated selectively through JNK activation. Furthermore, Vpr-52-96-induced apoptosis was attributed to the down regulation of anti-apoptotic genes Bcl2 and c-IAP1 through the activation of upstream JNK MAPKs (Fig. 4-3.1).

It is well established that cell cycle arrest occurs by interfering with the activity of the Cdc2-cyclin B1 complex, a central regulator of the G2 to mitosis transition [348]. In general, this complex is inactivated by Weel kinase and activated by Cdc25C phosphatase [348]. Vpr mediates cell cycle arrest by phosphorylation and inactivation of Cdc25C phosphatase via activating the ATR DNA damage response pathway [115;349]. The Rad17 and Hus1 proteins involved in the ATR pathway were shown to be required for cell cycle arrest by Vpr [350]. Vpr was also shown to bind Cdc25C directly and

Fig. 4-3.1: HIV-Vpr induced apoptosis by JNK-MAPK in human monocytes:

Entry of Vpr into the monocytic cells induces JNK activation resulting in transcriptional inhibition of c-IAP1 and Bcl2 expression, activation of caspases, and apoptosis. Vpr is known to activate ATR DNA damage pathway that acts on Cdc25C through Hus1 and Rad17. This process leads to inactivation of cyclin B and cell cycle arrest [115;349-351]. Vpr is also known to bind glucocorticoid receptor and downregulates NFκB regulated anti-apoptotic gene expression and induces apoptosis [127;352]. Smac released from mitochondria inhibits the activation of c-IAPs which may result in the activation of caspase cascade and apoptosis.


Vpr52-96
Vpr52-96
Vpr52-96
JNK (p)
Ion channel formation
Necrosis/Cell Death
ANT
Low ??
?
?
ATR
Bcl2
Hus1
Rad17
Cyt C release
SMAC/DIABLO
Cdc25c
IAPs
Procaspase 9
Apoptosome
Apaf-1
CyclinB/Cdc2
AIF
Active caspase 9
Cell cycle arrest
Active caspase 3
GR/Vpr complex
NFκB transcription
Bcl2
DNA fragmentation
Apoptosis
Apoptosis/Cell Death

inhibit Cdc25C phosphatase activity [349;351], perhaps blocking cyclin-B1-Cdc2 redundantly with the ATR pathway. Recently, Jacotot *et al.* demonstrated that Vpr52-96-induced apoptosis may occur through a direct effect on the mitochondrial permeability transition pore complex (PTPC) and specifically on the mitochondrial adenine nucleotide translocator (ANT), a component of the PTPC [101]. This event leads to permeabilization of the outer mitochondrial membrane with consequent release of apoptosis inducing factor and cytochrome C [101;102]. Cytochrome C interacts with Apaf-1 and procaspase 9 to create an apoptosome, the caspase activation complex that causes activation of other caspases resulting in apoptosis [101;102;120;121;123]. My results suggest that Vpr52-96 peptide caused a dramatic reduction of mitochondrial membrane potential in monocytic cells in a caspase-dependent manner.

Although numerous studies have implicated the JNK pathway in pro-apoptotic as well as anti-apoptotic cellular functions [136;353], there is little information on the role of MAPKs in Vpr-mediated cell cycle arrest and apoptosis. Recently, Vpr-induced cell cycle arrest was shown to be associated with down regulation of ERK MAPK pathway in a 293 fibroblast cell line [138]. My results suggest that Vpr-induced apoptosis in normal human monocytes and THP-1 cells is regulated through the activation of JNK MAPKs. Pretreatment of cells with JNK inhibitors SP60025, dexamethasone, and curcumin or transfection with JNK-specific siRNAs significantly inhibited Vpr-induced apoptosis. Although MAPKs have been reported to regulate HIV replication in T cells and latently infected cells [137;354], this is the first report describing a novel pathway involving JNK activation in Vpr-induced apoptosis.

The JNK group of MAPK is encoded by three genes: *jnk1* and *jnk2* genes which are ubiquitously expressed, and the *jnk3* gene, expressed primarily in the heart, testis, and brain [235]. Targeted disruption of *jnk1* or *jnk2* genes results in mice that exhibit defects in apoptosis, development and functions of effector T cells, and cytokine production [136;355]. However, disruption of both *jnk1* and *jnk2* genes resulted in embryonic death [135;136;356]. Although a distinct role for JNK-1 and JNK-2 in apoptosis, immunological and developmental processes is not clear at present, some studies have suggested a differential involvement for JNK-1 and JNK-2 [135;136;357;358]. I employed siRNAs specific for JNK-1 and JNK-2, both of which prevented Vpr-mediated cell death to equal levels suggesting redundancy between the two JNK isoforms at least with respect to Vpr-induced apoptosis in monocytic cells.

I have also demonstrated that Vpr-induced apoptosis was mediated by the down regulation of anti-apoptotic Bcl2 gene. Bcl2 and its closest homologues, BclXL and Bclw, potently inhibit apoptosis in response to many signaling pathways [342;359;360]. Bcl2 has been shown to protect cells from Vpr-induced apoptosis most likely by regulating inner and outer MMP through interactions with major components of PTPC such as ANT in the inner membrane, and the voltage dependent anion channels in the outer membrane and/or through autonomous channel-forming activities [101;102;123;124]. Vpr was shown to physically and functionally interact with the ANT104-116 site in ANT, consequently converting ANT into a non-specific pore leading to inner MMP which triggers matrix swelling, outer membrane rupture, and permeable to cytochrome C [101;102].

There is substantial agreement that anti-apoptotic c-IAP1 and c-IAP2 genes endow cells with protection against a number of apoptotic stimuli [85;168]. In my earlier studies, I demonstrated a critical role for c-IAP2 in conferring resistance to staurosporine-induced apoptosis in human monocytic cells [11]. Herein, I show that Vpr-induced apoptosis in monocytic cells may be mediated by inhibition of c-IAP1 expression in addition to that of Bcl2. How IAPs confer anti-apoptotic signals has been recently investigated. IAPs regulate the caspase cascade by binding to caspase 3 and 7 and by inhibiting the activation of procaspase 8 and 9 [168]. c-IAP1 was also shown to inhibit apoptosis through noncaspase mechanisms such as via activation of NFκB and JNK MAPK [195]. In addition, c-IAPs were suggested to obstruct apoptosis through binding to the IAP antagonists such as SMAC [361] or interfering with the signaling pathway initiated by TNF-α by interacting with TRAF through the BIR1 (Baculoviral IAP Repeat 1) domain [362].

JNK can phosphorylate Ser/Thr-Pro motifs in the activation domains of various transcription factors such as c-Jun, Jun-B, Jun-D, activating transcription factor-2, PU.1, Sp-1, and Ets-2 [136;285;363;364]. My results suggest that Vpr52-96-activated JNK causes down regulation of transcription factors involved in the regulation of Bcl2 and c-IAP1 gene transcription. Bcl2 transcription is regulated by NFκB, Sp-1 and CREB [151], whereas c-IAP1 transcription was shown to involve activation of NFκB and CREB transcription factors [199]. Selective activation of JNK by Vpr52-96 but not Vpr1-45 peptides resulted in inhibition of binding of corresponding NFκB and CREB transcription factors to their binding sites, and inhibition of JNK activity restored this binding and thus transcription of Bcl2 and c-IAP1 genes. These observations suggest specific interaction

of Vpr52-96 peptide with member(s) of the JNK MAPKs family or with any of these transcription factors either alone or as a complex in the cytoplasm to prevent their translocation into the nuclei. The identity of the JNK family member and/or the transcription factor involved in binding with Vpr52-96 peptide remains to be investigated. Nonetheless, Vpr has been shown to engage with a number of host cellular proteins to promote cell cycle arrest such as VprBP, a protein believed to interfere with Vpr-mediated arrest by sequestering Vpr in the cytoplasm [365] and the human homologue of MOV34, a proteosomal subunit [366].

Recently, glucocorticoid receptor complex was identified as an intracellular target for Vpr [366-368] suggesting a possible involvement of these receptors in Vpr52-96-induced apoptosis. The observations that glucocorticoids and Vpr inhibited NFκB induction suggest a role for glucocorticoid receptors in Vpr-mediated apoptosis [127]. However, unlike glucocorticoids that are known to inhibit JNK activity [285], Vpr52-96 induced JNK phosphorylation in my study. Therefore, it seems unlikely that Vpr52-96 induces apoptosis in monocytic cells through glucocorticoid receptors, although I can not rule out this possibility at present.

Signaling pathways involved in development of LPS- and TNF-α-mediated resistance to HIV-Vpr-induced apoptosis:

Monocytes/macrophages survive HIV-induced cytopathic effects and acts as a major source of viral reservoir. However, the mechanism underlying the development of resistance to apoptosis remains unknown. In this study, I have demonstrated that HIV-Vpr induces apoptosis in human monocytes and monocytic cell lines. However, I also showed that bacterial endotoxin (LPS) and the pro-inflammatory cytokine, TNF-α that is produced in

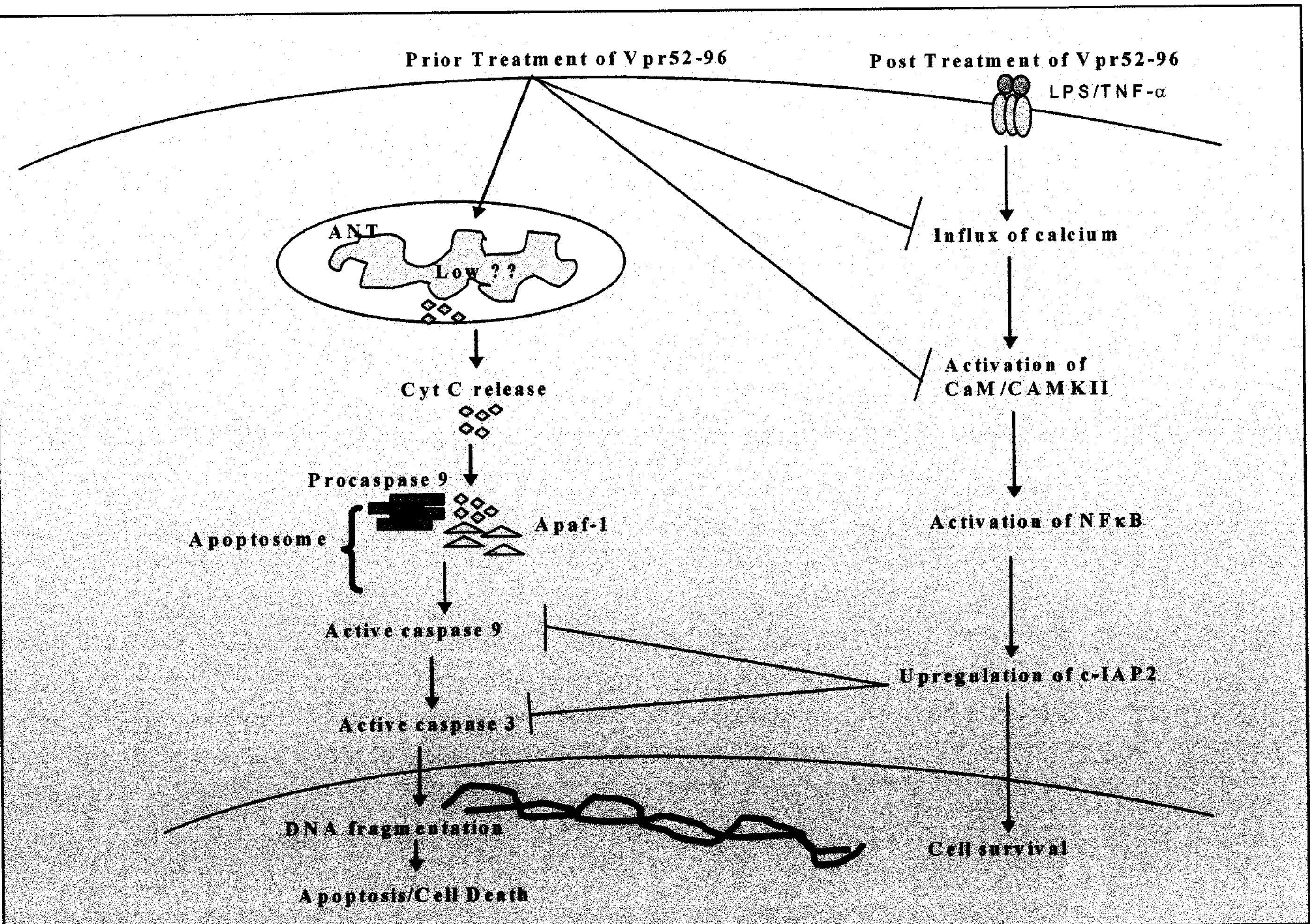
abundance during HIV infection, play a key role in inducing anti-apoptotic survival signals in human monocytic cells by inducing the expression of anti-apoptotic c-IAP2 gene through the activation of CaM/CAMKII and its down stream effector NFκB. In contrast, if cells are treated with Vpr52-96 prior to LPS or TNF-α stimulation, it abrogates LPS- and TNF-α-induced resistance to apoptosis by inhibiting calcium influx, activation of CAMKII and NFκB and eventually c-IAP2 expression (Fig. 4-3.2).

LPS, a structural component of the outer membrane of Gram–negative bacteria has been shown to prevent monocyte apoptosis [141;369]. There is evidence to suggest that LPS-induced resistance to apoptosis may be mediated by the c-IAP family of anti-apoptotic genes [190]. The c-IAP1 and c-IAP2 are key regulators of apoptosis and are overexpressed in many cancer cell lines. It is interesting to observe that c-IAP2 is selectively induced following stimulation of primary monocytes and THP-1 cells with either LPS or TNF-α. In this study, I show for the first time that specific inhibition of c-IAP2 by antisense oligonucleotides or the pharmacological inhibitors specific for CaM/CAMKII reversed LPS- and TNF-α-mediated protection from Vpr52-96-induced apoptosis. These results suggest a key role for c-IAP2 in the development of resistance to apoptosis in human monocytic cells.

c-IAP2 is known to inhibit apoptosis by interacting with initiator as well as effector caspases including caspases 3, 7, and 9 in various cell types [85]. In addition, c-IAP2 appears to be a part of the signaling complex recruited to the cytoplasmic domain of TNF-R by binding to the TRAF-1/TRAF-2 heterocomplexes and functions to suppress caspase-8 activation [161;164;168]. Since I have previously demonstrated that Vpr52-96-induced apoptosis in human monocytic cells is caspase-dependent, it is likely that c-

Fig. 4-3.2 LPS/TNF-α induced resistance to HIV-Vpr mediated apoptosis:

As shown in the figure, LPS- and TNF-α-mediated resistance to Vpr-induced apoptosis is regulated by induction of c-IAP2 through activation of calcium signaling pathway, particularly CAMKII. However, pretreatment of cell with Vpr peptide no more provide protection by blocking calcium influx, activation of CAMKII and NFκB, and induction of c-IAP2.


Prior Treatment of Vpr52-96
Post Treatment of Vpr52-96
LPS/TNF-α
ANT
Low ??
Influx of calcium
Activation of
CaM/CAMKII
Cyt C release
Procaspase 9
Apoptosome
Apaf-1
Activation of NFκB
Active caspase 9
Upregulation of c-IAP2
Active caspase 3
DNA fragmentation
Cell survival
Apoptosis/Cell Death

IAP2 is inducing resistance to Vpr52-96 mediated apoptosis by interacting with caspases. c-IAP2 is also known to interfere in the apoptosis process in a caspase-independent manner by interacting with TRAF-6. Therefore, it is possible that TNF-α-induced resistance to apoptosis may be mediated in a caspase-independent manner through c-IAP2 interactions with TRAF-6.

It is well established that calcium signaling plays a key role in mediating transcription of a variety of cellular genes [244]. In general, binding of a ligand to its receptor induces influx of calcium which in turn leads to release of calcium from endoplasmic reticulum [244;298]. CaM is highly conserved 17 kD protein associated either with membrane, cytoplasm or nucleus. It binds to four calcium ions and as a consequence of calcium binding, it undergoes a conformational change that renders it active to bind the target proteins with high affinity [299]. CAMKII is one of the key targets for CaM binding [247;299]. CaM is also an activator of Ser-Thr-phosphatase, calcineurin. It is activated by increase in intracellular calcium levels and is essential for translation of calcium signals into the nuclei and gene transcription [247;300]. In this study, I have demonstrated for the first time that LPS- and TNF-α-induced c-IAP2 expression is regulated by CaM/CAMKII in primary human monocytes in addition to the THP-1 cells described earlier. By employing specific inhibitors and the dominant negative mutants, my results suggest that LPS- and TNF-α-induced resistance to Vpr52-96-mediated apoptosis in primary human monocytes and THP-1 cells is regulated selectively by CaM and CAMKII activation. It is known that NFκB plays a key role in c-IAP2 transcription [191]. My results also show that NFκB activation plays a key role in LPS- and TNF-α-induced resistance to Vpr-mediated apoptosis in primary human monocytes as well as THP-1 cells.

The precise intracellular pathway responsible for inducing resistance to Vpr52-96-mediated apoptosis remains unclear at the present moment. As mentioned, Vpr52-96 interacts with mitochondrial protein, ANT [101], following which apoptogenic factors such as AIF, Smac, and Cyt-c, are released to deliver apoptotic signals [47;64;370]. It is possible that prior stimulation of cells with LPS or TNF-α may interfere with the process of Vpr internalization and/or its interaction with the mitochondrial protein ANT, and subsequent release of apoptotic factors. Since Vpr52-96-activated JNK was found to cause apoptosis in monocytic cells, it is possible that Vpr52-96 may not be able to activate JNK MAPK in TNF-α/LPS prestimulated monocytic cells.

My results also show that Vpr52-96 if treated prior to LPS/TNF-α stimulation, abrogated LPS- and TNF-α-induced c-IAP2 expression as well as protection against Vpr52-96-induced apoptosis. These results suggested that Vpr interfered with the LPS- and TNF-α-induced signaling pathway responsible for c-IAP2 induction and survival of monocytic cells. I have demonstrated for the first time that Vpr52-96 inhibited LPS- and TNF-α-induced calcium influx, activation of CaM and CAMKII, its down stream effector NFκB, and eventually c-IAP2 transcription. How Vpr blocks LPS-/TNF-α-induced calcium influx is not known. Since entry of Vpr is receptor independent, it is possible that internalization of Vpr through cell membrane may disturb the integrity of the cell surface receptors responsible for binding to either LPS or TNF-α, and consequent blocking of calcium entry in monocytic cells.

In summary, my results suggest for the first time that JNK activated selectively by the HIV-Vpr peptide plays a critical role in the induction of cell death in normal human monocytes and leukemic THP-1 cells. In view of studies suggesting the role of HIV

Vpr52-96 peptide as a potential therapeutic anti-proliferative agent against malignancies [119;371;372], my results showing inhibition of Bcl2 and c-IAP1 expression provide a broad basic mechanism for Vpr-induced apoptosis. Since deregulation of the JNK pathway has been implicated in cancer and other diseases including Alzheimer's and Parkinson's diseases [135;357;358], investigation of the molecular mechanisms that govern the role of the JNK pathway in apoptosis should provide insight into its biological functions and strategies to target this pathway for prevention and treatment of human diseases and cancer.

The results of my study demonstrate for the first time induction of resistance to apoptosis by a HIV accessory protein, Vpr, in primary human monocytes and a monocytic cell line following stimulation with LPS or endogenously produced TNF-α. Therefore, monocytes of HIV-infected patients may have a reduced propensity to undergo apoptosis at least in part due to the protective effect of TNF-α produced in the intracellular milieu following HIV infection or as a result of the inflammatory immune responses in the patients. Since TNF-α is known to enhance HIV replication, its production following HIV infection may enhance monocytic cell survival and provide an intracellular environment independent of HIV cytopathic effects to stimulate viral transcription and formation of potent viral reservoirs. I have also demonstrated that an anti-apoptotic gene, c-IAP2 plays a critical role in LPS and TNF-α-induced survival of human monocytic cells. Moreover, inhibition of c-IAP2 expression by antisense oligonucleotides or the pharmacological inhibitors specific for CaM/CAMKII reversed LPS- and TNF-α-mediated protection from Vpr52-96-induced apoptosis. These results further suggest that strategies based on suppression of c-IAP2 induction by agents known

to inhibit CaM/CAMKII/NFκB signaling pathways may be helpful in controlling HIV reservoir formation and in general prevent infection with pathogenic intracellular organisms and associated inflammatory responses.

Chapter V

Conclusion & Future direction

Monocytic cells are productively infected by HIV, however, they survive HIV infection and act as a major source of viral reservoir. The mechanism by which HIV enhances monocytic cell survival is largely unknown. The main goal of my study was to investigate the molecular mechanisms involved in regulation of resistance to apoptosis in human monocytic cells in general and in particular against HIV infection. My results suggest that Vpr, one of the accessory proteins of HIV induces apoptosis in monocytic cells and pretreatment of cells with LPS/TNF-α mediates protection to HIV-Vpr-induced apoptosis. Subsequently, I elucidated the intracellular signaling pathways involved in HIV-Vpr-mediated apoptosis and the resistance induced by LPS and TNF-α in normal human monocytic cells and promonocytic cells (THP-1) using Vpr1-45 and Vpr52-96 peptides.

The main aim of my first objective was to elucidate the intracellular signaling pathways involved in LPS-/TNF-α-induced resistance to apoptosis in human monocytes. I have demonstrated that c-IAP2, one of the anti-apoptotic genes is involved in inducing resistance to staurosporine mediated apoptosis in monocytic cells. c-IAP2 was shown to be induced in response to LPS and TNF-α, whereas the level of c-IAP1 remained unchanged in monocytic cells. It is likely that c-IAP1 plays a role in spontaneous survival of THP-1 cells and it may not be involved in LPS- and TNF-α-induced anti-apoptotic activity. The involvement of XIAP, one of the IAPs that provides protection was ruled out since it was not inducible in LPS/TNF-α stimulation of monocytic cells. The involvement of the Bcl2 family of anti-apoptotic proteins including Bcl2, BclXL, Mcl, and A1 could not be ruled out at this stage. It is likely that any one of them may also be involved in inducing resistance. However, there is at least one report demonstrating that

Bcl2 is not inducible in response to LPS. LPS/TNF-α induced resistance by other members of IAP and Bcl2 family of genes in monocytic cell needs further investigation.

In an attempt to understand the mechanism underlying LPS/TNF-α-induced expression of c-IAP2 and subsequent development of resistance to apoptosis in human monocytic cells, I investigated the role of MAPKs and PI3K. My results suggest that neither MAPKs nor PI3K is involved in LPS-/TNF-α-induced c-IAP2 expression. These results prompted me to investigate the upstream signaling molecules such as calcium in c-IAP2 induction and resistance to apoptosis. I have demonstrated that LPS-/TNF-α-induced c-IAP2 and its associated anti-apoptotic activity is regulated selectively by the calcium signaling pathway, particularly CaM/CAMKII protein kinase through activation of NFκB. To determine the involvement of transcription factors in c-IAP2 induction, gel shift assays were conducted. It will be of interest to perform chromatin immunoprecipitation (CHIP) assays to confirm the results. Further, it is to be determined whether the link between CAMKII and NFκB operates directly through activation of IκB or whether there are some other intermediate molecules involved in this activation process.

The molecular mechanism involved in the regulation of c-IAP1 expression is not clear at present. My results suggest that constitutive expression of c-IAP1 may be regulated selectively by the calcium pathway as its expression was down-regulated by EGTA, SKF, and W-7. However, c-IAP1 does not seem to be regulated by the CAMKII pathway as its inhibitor KN-93 inhibited LPS-/TNF-α-induced expression of c-IAP2 alone. The regulation of c-IAP1 in spontaneous survival of monocytes needs further investigation.

In my earlier studies, I used staurosporine to induce apoptosis in monocytic cells. Since staurosporine is not a physiological trigger, I used HIV-Vpr synthetic peptide to induce apoptosis in my second objective. I demonstrated that Vpr52-96-induced apoptosis in monocytic cells by activating JNK through the mitochondrial pathway in a caspase-dependent manner. However, the mechanism by which Vpr52-96-activated JNK induces apoptosis through the mitochondrial pathway is not known and needs further investigation. It is known that Vpr52-96 interacts with ANT in the inner mitochondrial membrane through which it has been suggested to release apoptogenic factor(s) such as AIF, Smac and/or Cyt-c to induce apoptosis. It is to be determined if JNK activation by Vpr influences Vpr-ANT interactions resulting in induction of apoptosis. Vpr peptides were recently shown to enter mitochondria. Keeping in view our results showing the involvement of Vpr-activated JNK through the mitochondrial pathway in inducing apoptosis, it is of interest to determine if Vpr-activated JNK enters into mitochondria and induces apoptosis by regulating the release of apoptogenic factor(s) from the mitochondria. Since Vpr induces apoptosis in T lymphocytes and neurons, I would also like to know if similar signaling pathways like monocytes are involved in Vpr-induced apoptosis.

I demonstrated that Vpr-induced apoptosis in monocytes was mediated by down regulation of anti-apoptotic Bcl2 and c-IAP1 genes through activation of JNK. IAPs are known to regulate the caspase cascade by binding to caspase 3, and 7 and by inhibiting the activation of procaspase 9. c-IAP1 has also been shown to inhibit apoptosis through non-caspase mechanisms such as via activation of NFκB and JNK MAPKs. It would be interesting to determine the non-caspase mechanism used by c-IAP1 in inducing

resistance to apoptosis. The possibility of XIAP and other anti-apoptotic factors involvement in Vpr-induced apoptosis also need to be investigated. How JNK activation downregualted Bcl2 and c-IAP1 is not known. It is speculated that downregulation may occur by direct interaction between these molecules or through involvement of any intermediate molecules.

Vpr has been shown to inhibit Cdc25 phosphatases to induce cell cycle arrest. It is likely that Vpr52-96-activated JNK may also induce cell cycle arrest by influencing cyclin-Cdc2 interactions and could be a subject of future investigations.

My third objective regarding the protective effect to Vpr induced apoptosis revealed that the Vpr-mediated apoptotic effect was reversed by pretreatment of cells with either LPS or TNF-α produced endogenously following LPS stimulation. These observations suggest that TNF-α, a cytokine that enhances HIV replication and is known to be produced following HIV infection, may be one of the critical factors responsible for protection of monocytic cells against the apoptotic effects of Vpr and thereby promotes viral reservoir formation and maintenance. Therefore, it would be interesting to determine if HIV-infected monocytes have a reduced propensity to undergo apoptosis at least in part due to TNF-α. Further, I demonstrated the involvement of c-IAP2 in LPS-/TNF-α-mediated resistance to Vpr-induced apoptosis through activation of CAMKII.

Interestingly, my results also demonstrate that Vpr interferes with the LPS-/TNF-α-induced resistance by inhibiting calcium influx, CAMKII activation, and translocation and activation of NFκB essential for c-IAP2 induction. However, the mechanism underlying the interference of Vpr in LPS-/TNF-α-induced resistance in apoptosis is not clearly known. It is most likely that Vpr disturbs cell surface receptors such as CD14 and

TLR4 which are essential for LPS binding and needs further investigation. Recently several groups have identified a novel mitochondrial anti-viral signaling protein (MAVS), which plays an important role in inducing NFκB stress response and antiviral type-I interferon response [373;374]. It is likely that Vpr may cause cleavage/disruption of MAVS protein which in turn may cause disruption of NFκB signaling and impaired expression of anti-apoptotic genes such as Bcl2/IAPs and apoptosis.

I have conducted my studies using HIV-Vpr peptides in monocytes. It will be of interest to reproduce these findings with recombinant Vpr protein or intracellularly expressed Vpr. It is to be determined if dendritic cells and monocyte derived macrophages which act as viral reservoir are protected against HIV-Vpr induced apoptosis in a manner similar to human monocytic cells. Further, to confirm the involvement of IAPs in inducing resistance, drugs acting specifically on IAP proteins may be used to determine the impact on survival of monocytic cells and eventual destruction of HIV carrying virus reservoir.

Overall, my findings suggest that LPS and the LPS induced proinflammatory cytokine TNF-α play an important role in HIV pathogenesis. Since LPS-/TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in monocytes is mediated by c-IAP2 upregulation, manipulating signaling molecules involved in c-IAP2 regulation such as calcium and CAMKII may help in clearing the viral reservoir from monocytes.

Reference List

[1] Emerman M, Malim MH. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. Science 1998 Jun 19;280(5371):1880-4.

[2] Fox R, Eldred LJ, Fuchs EJ, et al. Clinical manifestations of acute infection with human immunodeficiency virus in a cohort of gay men. AIDS 1987 May;1(1):35-8.

[3] Levy JA. Pathogenesis of human immunodeficiency virus infection. Microbiol Rev 1993 Mar;57(1):183-289.

[4] Hewson T, Lone N, Moore M, Howie S. Interactions of HIV-1 with antigen-presenting cells. Immunol Cell Biol 1999 Aug;77(4):289-303.

[5] McCune JM. The dynamics of CD4+ T-cell depletion in HIV disease. Nature 2001 Apr 19;410(6831):974-9.

[6] Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science 1996 May 24;272(5265):1167-70.

[7] Gottlieb MS, Groopman JE, Weinstein WM, Fahey JL, Detels R. The acquired immunodeficiency syndrome. Ann Intern Med 1983 Aug;99(2):208-20.

[8] Gorry PR, Churchill M, Crowe SM, Cunningham AL, Gabuzda D. Pathogenesis of macrophage tropic HIV-1. Curr HIV Res 2005 Jan;3(1):53-60.

[9] Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature 1997 Nov 27;390(6658):350-1.

[10] Wahl SM, Greenwell-Wild T, Peng G, et al. Mycobacterium avium complex augments macrophage HIV-1 production and increases CCR5 expression. Proc Natl Acad Sci U S A 1998 Oct 13;95(21):12574-9.

[11] Mishra S, Mishra JP, Gee K, McManus DC, LaCasse EC, Kumar A. Distinct role of calmodulin and calmodulin-dependent protein kinase-II in lipopolysaccharide and tumor necrosis factor-alpha-mediated suppression of apoptosis and antiapoptotic c-IAP2 gene expression in human monocytic cells. J Biol Chem 2005 Nov 11;280(45):37536-46.

[12] Lu YL, Spearman P, Ratner L. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. J Virol 1993 Nov;67(11):6542-50.

[13] Paxton W, Connor RI, Landau NR. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. J Virol 1993 Dec;67(12):7229-37.

[14] Finkel TH, Tudor-Williams G, Banda NK, et al. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med 1995 Feb;1(2):129-34.

[15] Embretson J, Zupancic M, Ribas JL, et al. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 1993 Mar 25;362(6418):359-62.

[16] Clerici M, Shearer GM. The Th1-Th2 hypothesis of HIV infection: new insights. Immunol Today 1994 Dec;15(12):575-81.

[17] Muro-Cacho CA, Pantaleo G, Fauci AS. Analysis of apoptosis in lymph nodes of HIV-infected persons. Intensity of apoptosis correlates with the general state of activation of the lymphoid tissue and not with stage of disease or viral burden. J Immunol 1995 May 15;154(10):5555-66.

[18] Herbein G, Van LC, Lovett JL, Verdin E. Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. J Virol 1998 Jan;72(1):660-70.

[19] Phenix BN, Badley AD. Influence of mitochondrial control of apoptosis on the pathogenesis, complications and treatment of HIV infection. Biochimie 2002 Feb;84(2-3):251-64.

[20] Lifson JD, Reyes GR, McGrath MS, Stein BS, Engleman EG. AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. Science 1986 May 30;232(4754):1123-7.

[21] Fauci AS. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science 1988 Feb 5;239(4840):617-22.

[22] Gonzalez ME, Carrasco L. Human immunodeficiency virus type 1 VPU protein affects Sindbis virus glycoprotein processing and enhances membrane permeabilization. Virology 2001 Jan 5;279(1):201-9.

[23] Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature 1990 May 3;345(6270):84-6.

[24] Zauli G, Gibellini D, Secchiero P, et al. Human immunodeficiency virus type 1 Nef protein sensitizes CD4(+) T lymphoid cells to apoptosis via functional upregulation of the CD95/CD95 ligand pathway. Blood 1999 Feb 1;93(3):1000-10.

[25] Tateyama M, Oyaizu N, McCloskey TW, Than S, Pahwa S. CD4 T lymphocytes are primed to express Fas ligand by CD4 cross-linking and to contribute to CD8 T-cell apoptosis via Fas/FasL death signaling pathway. Blood 2000 Jul 1;96(1):195-202.

[26] Conti L, Rainaldi G, Matarrese P, et al. The HIV-1 vpr protein acts as a negative regulator of apoptosis in a human lymphoblastoid T cell line: possible implications for the pathogenesis of AIDS. J Exp Med 1998 Feb 2;187(3):403-13.

[27] Conti L, Matarrese P, Varano B, et al. Dual role of the HIV-1 vpr protein in the modulation of the apoptotic response of T cells. J Immunol 2000 Sep 15;165(6):3293-300.

[28] Watanabe N, Yamaguchi T, Akimoto Y, Rattner JB, Hirano H, Nakauchi H. Induction of M-phase arrest and apoptosis after HIV-1 Vpr expression through uncoupling of nuclear and centrosomal cycle in HeLa cells. Exp Cell Res 2000 Aug 1;258(2):261-9.

[29] Alimonti JB, Ball TB, Fowke KR. Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. J Gen Virol 2003 Jul;84(Pt 7):1649-61.

[30] Banda NK, Bernier J, Kurahara DK, et al. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J Exp Med 1992 Oct 1;176(4):1099-106.

[31] Li-Weber M, Laur O, Dern K, Krammer PH. T cell activation-induced and HIV tat-enhanced CD95(APO-1/Fas) ligand transcription involves NF-kappaB. Eur J Immunol 2000 Feb;30(2):661-70.

[32] Bartz SR, Emerman M. Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. J Virol 1999 Mar;73(3):1956-63.

[33] Levy DN, Refaeli Y, MacGregor RR, Weiner DB. Serum Vpr regulates productive infection and latency of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A 1994 Nov 8;91(23):10873-7.

[34] Stewart SA, Poon B, Jowett JB, Chen IS. Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. J Virol 1997 Jul;71(7):5579-92.

[35] Yao XJ, Mouland AJ, Subbramanian RA, et al. Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells. J Virol 1998 Jun;72(6):4686-93.

[36] Badley AD, Parato K, Cameron DW, et al. Dynamic correlation of apoptosis and immune activation during treatment of HIV infection. Cell Death Differ 1999 May;6(5):420-32.

[37] Badley AD, Dockrell DH, Algeciras A, et al. In vivo analysis of Fas/FasL interactions in HIV-infected patients. J Clin Invest 1998 Jul 1;102(1):79-87.

[38] Han X, Becker K, Degen HJ, Jablonowski H, Strohmeyer G. Synergistic stimulatory effects of tumour necrosis factor alpha and interferon gamma on replication of human immunodeficiency virus type 1 and on apoptosis of HIV-1-infected host cells. Eur J Clin Invest 1996 Apr;26(4):286-92.

[39] Garaci E, Caroleo MC, Aloe L, et al. Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. Proc Natl Acad Sci U S A 1999 Nov 23;96(24):14013-8.

[40] Papasavvas E, Sun J, Luo Q, et al. IL-13 acutely augments HIV-specific and recall responses from HIV-1-infected subjects in vitro by modulating monocytes. J Immunol 2005 Oct 15;175(8):5532-40.

[41] Lum JJ, Pilon AA, Sanchez-Dardon J, et al. Induction of cell death in human immunodeficiency virus-infected macrophages and resting memory CD4 T cells by TRAIL/Apo2l. J Virol 2001 Nov;75(22):11128-36.

[42] Aquaro S, Panti S, Caroleo MC, et al. Primary macrophages infected by human immunodeficiency virus trigger CD95-mediated apoptosis of uninfected astrocytes. J Leukoc Biol 2000 Sep;68(3):429-35.

[43] Marshall WL, Datta R, Hanify K, Teng E, Finberg RW. U937 cells overexpressing bcl-xl are resistant to human immunodeficiency virus-1-induced apoptosis and human immunodeficiency virus-1 replication. Virology 1999 Mar 30;256(1):1-7.

[44] Dockrell DH, Badley AD, Villacian JS, et al. The expression of Fas Ligand by macrophages and its upregulation by human immunodeficiency virus infection. J Clin Invest 1998 Jun 1;101(11):2394-405.

[45] Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972 Aug;26(4):239-57.

[46] Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.

[47] Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell 1997 Feb 7;88(3):347-54.

[48] Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995 Mar 10;267(5203):1456-62.

[49] Bellamy CO, Malcomson RD, Harrison DJ, Wyllie AH. Cell death in health and disease: the biology and regulation of apoptosis. Semin Cancer Biol 1995 Feb;6(1):3-16.

[50] Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. Blood 2001 Nov 1;98(9):2603-14.

[51] Waterhouse NJ, Goldstein JC, von Ahsen O, Schuler M, Newmeyer DD, Green DR. Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. J Cell Biol 2001 Apr 16;153(2):319-28.

[52] Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 1993 Nov 19;75(4):641-52.

[53] Trauth BC, Klas C, Peters AM, et al. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 1989 Jul 21;245(4915):301-5.

[54] Green DR, Reed JC. Mitochondria and apoptosis. Science 1998 Aug 28;281(5381):1309-12.

[55] Kroemer G, Zamzami N, Susin SA. Mitochondrial control of apoptosis. Immunol Today 1997 Jan;18(1):44-51.

[56] Gupta S. Molecular steps of cell suicide: an insight into immune senescence. J Clin Immunol 2000 Jul;20(4):229-39.

[57] Gupta S. Molecular steps of death receptor and mitochondrial pathways of apoptosis. Life Sci 2001 Nov 9;69(25-26):2957-64.

[58] Los M, Stroh C, Janicke RU, Engels IH, Schulze-Osthoff K. Caspases: more than just killers? Trends Immunol 2001 Jan;22(1):31-4.

[59] Kasibhatla S, Genestier L, Green DR. Regulation of fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor kappaB. J Biol Chem 1999 Jan 8;274(2):987-92.

[60] Raisova M, Bektas M, Wieder T, et al. Resistance to CD95/Fas-induced and ceramide-mediated apoptosis of human melanoma cells is caused by a defective mitochondrial cytochrome c release. FEBS Lett 2000 May 4;473(1):27-32.

[61] Chinnaiyan AM, Tepper CG, Seldin MF, et al. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem 1996 Mar 1;271(9):4961-5.

[62] Muzio M, Chinnaiyan AM, Kischkel FC, et al. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell 1996 Jun 14;85(6):817-27.

[63] Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 1998 Jan 1;391(6662):96-9.

[64] Modjtahedi N, Giordanetto F, Madeo F, Kroemer G. Apoptosis-inducing factor: vital and lethal. Trends Cell Biol 2006 May;16(5):264-72.

[65] Plummer ER. Inhibition of poly(ADP-ribose) polymerase in cancer. Curr Opin Pharmacol 2006 Aug;6(4):364-8.

[66] Sellins KS, Cohen JJ. Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. J Immunol 1987 Nov 15;139(10):3199-206.

[67] Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980 Apr 10;284(5756):555-6.

[68] Osborne BA. Induction of genes during apoptosis: examples from the immune system. Semin Cancer Biol 1995 Feb;6(1):27-33.

[69] Hussein MR, Haemel AK, Wood GS. Apoptosis and melanoma: molecular mechanisms. J Pathol 2003 Mar;199(3):275-88.

[70] Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000 May;6(5):513-9.

[71] Martins LM, Iaccarino I, Tenev T, et al. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem 2002 Jan 4;277(1):439-44.

[72] van LG, Schotte P, van GM, et al. Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell Death Differ 2001 Dec;8(12):1136-42.

[73] Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci 2001 Jan;26(1):61-6.

[74] Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998 Aug 28;281(5381):1322-6.

[75] Thomadaki H, Scorilas A. BCL2 family of apoptosis-related genes: functions and clinical implications in cancer. Crit Rev Clin Lab Sci 2006 Jan;43(1):1-67.

[76] Marsden VS, Strasser A. Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. Annu Rev Immunol 2003;21:71-105.

[77] Griffiths GJ, Dubrez L, Morgan CP, et al. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J Cell Biol 1999 Mar 8;144(5):903-14.

[78] Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell 1999 Mar;3(3):287-96.

[79] Harada H, Becknell B, Wilm M, et al. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol Cell 1999 Apr;3(4):413-22.

[80] Oda E, Ohki R, Murasawa H, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 2000 May 12;288(5468):1053-8.

[81] Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001 Mar;7(3):683-94.

[82] Gross A, Yin XM, Wang K, et al. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 1999 Jan 8;274(2):1156-63.

[83] Beere HM. Stressed to death: regulation of apoptotic signaling pathways by the heat shock proteins. Sci STKE 2001 Jul 31;2001(93):RE1.

[84] Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999 Feb 4;397(6718):441-6.

[85] Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. Genes Dev 1999 Feb 1;13(3):239-52.

[86] Azad AA. Could Nef and Vpr proteins contribute to disease progression by promoting depletion of bystander cells and prolonged survival of HIV-infected cells? Biochem Biophys Res Commun 2000 Jan 27;267(3):677-85.

[87] Roshal M, Zhu Y, Planelles V. Apoptosis in AIDS. Apoptosis 2001 Feb;6(1-2):103-16.

[88] Heinzinger NK, Bukinsky MI, Haggerty SA, et al. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc Natl Acad Sci U S A 1994 Jul 19;91(15):7311-5.

[89] Piller SC, Jans P, Gage PW, Jans DA. Extracellular HIV-1 virus protein R causes a large inward current and cell death in cultured hippocampal neurons: implications for AIDS pathology. Proc Natl Acad Sci U S A 1998 Apr 14;95(8):4595-600.

[90] Wong-Staal F, Chanda PK, Ghrayeb J. Human immunodeficiency virus: the eighth gene. AIDS Res Hum Retroviruses 1987;3(1):33-9.

[91] Subbramanian RA, Cohen EA. Molecular biology of the human immunodeficiency virus accessory proteins. J Virol 1994 Nov;68(11):6831-5.

[92] Trono D. HIV accessory proteins: leading roles for the supporting cast. Cell 1995 Jul 28;82(2):189-92.

[93] Morellet N, Bouaziz S, Petitjean P, Roques BP. NMR structure of the HIV-1 regulatory protein VPR. J Mol Biol 2003 Mar 14;327(1):215-27.

[94] Mahalingam S, Ayyavoo V, Patel M, Kieber-Emmons T, Weiner DB. Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. J Virol 1997 Sep;71(9):6339-47.

[95] Lum JJ, Cohen OJ, Nie Z, et al. Vpr R77Q is associated with long-term nonprogressive HIV infection and impaired induction of apoptosis. J Clin Invest 2003 May;111(10):1547-54.

[96] Nie Z, Bergeron D, Subbramanian RA, et al. The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocation of proviral DNA in growth-arrested cells. J Virol 1998 May;72(5):4104-15.

[97] Sherman MP, Schubert U, Williams SA, et al. HIV-1 Vpr displays natural protein-transducing properties: implications for viral pathogenesis. Virology 2002 Oct 10;302(1):95-105.

[98] Coeytaux E, Coulaud D, Le CE, Danos O, Kichler A. The cationic amphipathic alpha-helix of HIV-1 viral protein R (Vpr) binds to nucleic acids, permeabilizes membranes, and efficiently transfects cells. J Biol Chem 2003 May 16;278(20):18110-6.

[99] Henklein P, Bruns K, Sherman MP, et al. Functional and structural characterization of synthetic HIV-1 Vpr that transduces cells, localizes to the nucleus, and induces G2 cell cycle arrest. J Biol Chem 2000 Oct 13;275(41):32016-26.

[100] Sawaya BE, Khalili K, Gordon J, et al. Transdominant activity of human immunodeficiency virus type 1 Vpr with a mutation at residue R73. J Virol 2000 May;74(10):4877-81.

[101] Jacotot E, Ferri KF, El HC, et al. Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein rR and Bcl-2. J Exp Med 2001 Feb 19;193(4):509-19.

[102] Jacotot E, Ravagnan L, Loeffler M, et al. The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. J Exp Med 2000 Jan 3;191(1):33-46.

[103] Macreadie IG, Castelli LA, Hewish DR, Kirkpatrick A, Ward AC, Azad AA. A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. Proc Natl Acad Sci U S A 1995 Mar 28;92(7):2770-4.

[104] Jowett JB, Planelles V, Poon B, Shah NP, Chen ML, Chen IS. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. J Virol 1995 Oct;69(10):6304-13.

[105] Huang MB, Weeks O, Zhao LJ, Saltarelli M, Bond VC. Effects of extracellular human immunodeficiency virus type 1 vpr protein in primary rat cortical cell cultures. J Neurovirol 2000 Jun;6(3):202-20.

[106] Cohen EA, Dehni G, Sodroski JG, Haseltine WA. Human immunodeficiency virus vpr product is a virion-associated regulatory protein. J Virol 1990 Jun;64(6):3097-9.

[107] Cohen OJ, Fauci AS. Current strategies in the treatment of HIV infection. Adv Intern Med 2001;46:207-46.

[108] Yao XJ, Subbramanian RA, Rougeau N, Boisvert F, Bergeron D, Cohen EA. Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. J Virol 1995 Nov;69(11):7032-44.

[109] Le RE, Benichou S. The Vpr protein from HIV-1: distinct roles along the viral life cycle. Retrovirology 2005;2(1):11.

[110] Deniaud A, Brenner C, Kroemer G. Mitochondrial membrane permeabilization by HIV-1 Vpr. Mitochondrion 2004 Jul;4(2-3):223-33.

[111] Zhao RY, Bukrinsky M, Elder RT. HIV-1 viral protein R (Vpr) & host cellular responses. Indian J Med Res 2005 Apr;121(4):270-86.

[112] Zhao Y, Cao J, O'Gorman MR, Yu M, Yogev R. Effect of human immunodeficiency virus type 1 protein R (vpr) gene expression on basic cellular function of fission yeast Schizosaccharomyces pombe. J Virol 1996 Sep;70(9):5821-6.

[113] He J, Choe S, Walker R, Di MP, Morgan DO, Landau NR. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J Virol 1995 Nov;69(11):6705-11.

[114] Elder RT, Yu M, Chen M, Zhu X, Yanagida M, Zhao Y. HIV-1 Vpr induces cell cycle G2 arrest in fission yeast (Schizosaccharomyces pombe) through a pathway involving regulatory and catalytic subunits of PP2A and acting on both Weel and Cdc25. Virology 2001 Sep 1;287(2):359-70.

[115] Roshal M, Kim B, Zhu Y, Nghiem P, Planelles V. Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R. J Biol Chem 2003 Jul 11;278(28):25879-86.

[116] Amini S, Khalili K, Sawaya BE. Effect of HIV-1 Vpr on cell cycle regulators. DNA Cell Biol 2004 Apr;23(4):249-60.

[117] Sherman MP, de Noronha CM, Williams SA, Greene WC. Insights into the biology of HIV-1 viral protein R. DNA Cell Biol 2002 Sep;21(9):679-88.

[118] Macreadie IG, Thorburn DR, Kirby DM, Castelli LA, de Rozario NL, Azad AA. HIV-1 protein Vpr causes gross mitochondrial dysfunction in the yeast Saccharomyces cerevisiae. FEBS Lett 1997 Jun 30;410(2-3):145-9.

[119] Muthumani K, Zhang D, Hwang DS, et al. Adenovirus encoding HIV-1 Vpr activates caspase 9 and induces apoptotic cell death in both p53 positive and negative human tumor cell lines. Oncogene 2002 Jul 11;21(30):4613-25.

[120] Stewart SA, Poon B, Song JY, Chen IS. Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation. J Virol 2000 Apr;74(7):3105-11.

[121] Muthumani K, Hwang DS, Desai BM, et al. HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells. J Biol Chem 2002 Oct 4;277(40):37820-31.

[122] Patel CA, Mukhtar M, Pomerantz RJ. Human immunodeficiency virus type 1 Vpr induces apoptosis in human neuronal cells. J Virol 2000 Oct;74(20):9717-26.

[123] Roumier T, Vieira HL, Castedo M, et al. The C-terminal moiety of HIV-1 Vpr induces cell death via a caspase-independent mitochondrial pathway. Cell Death Differ 2002 Nov;9(11):1212-9.

[124] Muthumani K, Hwang DS, Desai BM, et al. HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells. J Biol Chem 2002 Oct 4;277(40):37820-31.

[125] Yedavalli VS, Shih HM, Chiang YP, et al. Human immunodeficiency virus type 1 Vpr interacts with antiapoptotic mitochondrial protein HAX-1. J Virol 2005 Nov;79(21):13735-46.

[126] Refaeli Y, Levy DN, Weiner DB. The glucocorticoid receptor type II complex is a target of the HIV-1 vpr gene product. Proc Natl Acad Sci U S A 1995 Apr 11;92(8):3621-5.

[127] Ayyavoo V, Mahboubi A, Mahalingam S, et al. HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B. Nat Med 1997 Oct;3(10):1117-23.

[128] Chen C, Edelstein LC, Gelinas C. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol 2000 Apr;20(8):2687-95.

[129] Macreadie IG, Arunagiri CK, Hewish DR, White JF, Azad AA. Extracellular addition of a domain of HIV-1 Vpr containing the amino acid sequence motif H(S/F)RIG causes cell membrane permeabilization and death. Mol Microbiol 1996 Mar;19(6):1185-92.

[130] Levy DN, Refaeli Y, Weiner DB. Extracellular Vpr protein increases cellular permissiveness to human immunodeficiency virus replication and reactivates virus from latency. J Virol 1995 Feb;69(2):1243-52.

[131] Brack-Werner R. Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. AIDS 1999 Jan 14;13(1):1-22.

[132] Fukumori T, Akari H, Iida S, et al. The HIV-1 Vpr displays strong anti-apoptotic activity. FEBS Lett 1998 Jul 31;432(1-2):17-20.

[133] Fukumori T, Akari H, Yoshida A, et al. Regulation of cell cycle and apoptosis by human immunodeficiency virus type 1 Vpr. Microbes Infect 2000 Jul;2(9):1011-7.

[134] Zhu Y, Roshal M, Li F, Blackett J, Planelles V. Upregulation of survivin by HIV-1 Vpr. Apoptosis 2003 Jan;8(1):71-9.

[135] Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. Annu Rev Immunol 2002;20:55-72.

[136] Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000 Oct 13;103(2):239-52.

[137] Varin A, Decrion AZ, Sabbah E, et al. Synthetic Vpr protein activates activator protein-1, c-Jun N-terminal kinase, and NF-kappaB and stimulates HIV-1 transcription in promonocytic cells and primary macrophages. J Biol Chem 2005 Dec 30;280(52):42557-67.

[138] Yoshizuka N, Yoshizuka-Chadani Y, Krishnan V, Zeichner SL. Human immunodeficiency virus type 1 Vpr-dependent cell cycle arrest through a

mitogen-activated protein kinase signal transduction pathway. J Virol 2005 Sep;79(17):11366-81.

[139] Flad HD, Grage-Griebenow E, Petersen F, et al. The role of cytokines in monocyte apoptosis. Pathobiology 1999;67(5-6):291-3.

[140] Manna SK, Aggarwal BB. Lipopolysaccharide inhibits TNF-induced apoptosis: role of nuclear factor-kappaB activation and reactive oxygen intermediates. J Immunol 1999 Feb 1;162(3):1510-8.

[141] Mangan DF, Wahl SM. Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. J Immunol 1991 Nov 15;147(10):3408-12.

[142] Durrbaum-Landmann I, Gercken J, Flad HD, Ernst M. Effect of in vitro infection of human monocytes with low numbers of Mycobacterium tuberculosis bacteria on monocyte apoptosis. Infect Immun 1996 Dec;64(12):5384-9.

[143] Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. Science 1985 Jun 21;228(4706):1440-3.

[144] von Freeden-Jeffry U, Solvason N, Howard M, Murray R. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. Immunity 1997 Jul;7(1):147-54.

[145] Silvestrini R, Veneroni S, Daidone MG, et al. The Bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. J Natl Cancer Inst 1994 Apr 6;86(7):499-504.

[146] May WS, Tyler PG, Ito T, Armstrong DK, Qatsha KA, Davidson NE. Interleukin-3 and bryostatin-1 mediate hyperphosphorylation of BCL2 alpha in association with suppression of apoptosis. J Biol Chem 1994 Oct 28;269(43):26865-70.

[147] Ruvolo PP, Deng X, May WS. Phosphorylation of Bcl2 and regulation of apoptosis. Leukemia 2001 Apr;15(4):515-22.

[148] Deng X, Gao F, Flagg T, May WS, Jr. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. Proc Natl Acad Sci U S A 2004 Jan 6;101(1):153-8.

[149] Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol Cell Biol 1999 Dec;19(12):8469-78.

[150] Seto M, Jaeger U, Hockett RD, et al. Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. EMBO J 1988 Jan;7(1):123-31.

[151] Heckman CA, Mehew JW, Boxer LM. NF-kappaB activates Bcl-2 expression in t(14;18) lymphoma cells. Oncogene 2002 May 30;21(24):3898-908.

[152] Wilson BE, Mochon E, Boxer LM. Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. Mol Cell Biol 1996 Oct;16(10):5546-56.

[153] Kurland JF, Kodym R, Story MD, Spurgers KB, McDonnell TJ, Meyn RE. NF-kappaB1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. J Biol Chem 2001 Nov 30;276(48):45380-6.

[154] Taylor D, Badiani P, Weston K. A dominant interfering Myb mutant causes apoptosis in T cells. Genes Dev 1996 Nov 1;10(21):2732-44.

[155] Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene 2001 Nov 1;20(50):7342-51.

[156] Young RL, Korsmeyer SJ. A negative regulatory element in the bcl-2 5'-untranslated region inhibits expression from an upstream promoter. Mol Cell Biol 1993 Jun;13(6):3686-97.

[157] Weiss RA. How does HIV cause AIDS? Science 1993 May 28;260(5112):1273-9.

[158] Harada K, Ishibashi S, Miyashita T, et al. Bcl-2 protein inhibits oxysterol-induced apoptosis through suppressing CPP32-mediated pathway. FEBS Lett 1997 Jul 7;411(1):63-6.

[159] Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol 1993 Apr;67(4):2168-74.

[160] Liston P, Roy N, Tamai K, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996 Jan 25;379(6563):349-53.

[161] Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 1995 Dec 29;83(7):1243-52.

[162] Roy N, Mahadevan MS, McLean M, et al. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell 1995 Jan 13;80(1):167-78.

[163] Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol 2002 Jun;3(6):401-10.

[164] Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998 Apr 15;17(8):2215-23.

[165] Duckett CS, Nava VE, Gedrich RW, et al. A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J 1996 Jun 3;15(11):2685-94.

[166] Chai J, Wu Q, Shiozaki E, Srinivasula SM, Alnemri ES, Shi Y. Crystal structure of a procaspase-7 zymogen: mechanisms of activation and substrate binding. Cell 2001 Nov 2;107(3):399-407.

[167] Huang Y, Park YC, Rich RL, Segal D, Myszka DG, Wu H. Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell 2001 Mar 9;104(5):781-90.

[168] Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 1997 Dec 1;16(23):6914-25.

[169] Holcik M, Gibson H, Korneluk RG. XIAP: apoptotic brake and promising therapeutic target. Apoptosis 2001 Aug;6(4):253-61.

[170] Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene 2003 Nov 24;22(53):8568-80.

[171] LaCasse EC, Baird S, Korneluk RG, Mackenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 1998 Dec 24;17(25):3247-59.

[172] Young SS, Liston P, Xuan JY, McRoberts C, Lefebvre CA, Korneluk RG. Genomic organization and physical map of the human inhibitors of apoptosis: HIAP1 and HIAP2. Mamm Genome 1999 Jan;10(1):44-8.

[173] Rajcan-Separovic E, Liston P, Lefebvre C, Korneluk RG. Assignment of human inhibitor of apoptosis protein (IAP) genes xiap, hiap-1, and hiap-2 to chromosomes Xq25 and 11q22-q23 by fluorescence in situ hybridization. Genomics 1996 Nov 1;37(3):404-6.

[174] Holeik M. Targeting endogenous inhibitors of apoptosis for treatment of cancer, stroke and multiple sclerosis. Expert Opin Ther Targets 2004 Jun;8(3):241-53.

[175] Holcik M, Korneluk RG. XIAP, the guardian angel. Nat Rev Mol Cell Biol 2001 Jul;2(7):550-6.

[176] Liston P, Young SS, Mackenzie AE, Korneluk RG. Life and death decisions: the role of the IAPs in modulating programmed cell death. Apoptosis 1997;2(5):423-41.

[177] Imoto I, Yang ZQ, Pimkhaokham A, et al. Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. Cancer Res 2001 Sep 15;61(18):6629-34.

[178] Imoto I, Tsuda H, Hirasawa A, et al. Expression of cIAP1, a target for 11q22 amplification, correlates with resistance of cervical cancers to radiotherapy. Cancer Res 2002 Sep 1;62(17):4860-6.

[179] Dierlamm J, Baens M, Wlodarska I, et al. The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21)p6ssociated with mucosa-associated lymphoid tissue lymphomas. Blood 1999 Jun 1;93(11):3601-9.

[180] Liu H, Ye H, Dogan A, et al. T(11;18)(q21;q21) is associated with advanced mucosa-associated lymphoid tissue lymphoma that expresses nuclear BCL10. Blood 2001 Aug 15;98(4):1182-7.

[181] Liu H, Ye H, Ruskone-Fourmestraux A, et al. T(11;18) is a marker for all stage gastric MALT lymphomas that will not respond to H. pylori eradication. Gastroenterology 2002 May;122(5):1286-94.

[182] Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc Natl Acad Sci U S A 1997 Sep 16;94(19):10057-62.

[183] O'Neill AJ, Doyle BT, Molloy E, et al. Gene expression profile of inflammatory neutrophils: alterations in the inhibitors of apoptosis proteins during spontaneous and delayed apoptosis. Shock 2004 Jun;21(6):512-8.

[184] Stehlik C, de MR, Binder BR, Lipp J. Cytokine induced expression of porcine inhibitor of apoptosis protein (iap) family member is regulated by NF-kappa B. Biochem Biophys Res Commun 1998 Feb 24;243(3):827-32.

[185] Xiao CW, Ash K, Tsang BK. Nuclear factor-kappaB-mediated X-linked inhibitor of apoptosis protein expression prevents rat granulosa cells from tumor necrosis factor alpha-induced apoptosis. Endocrinology 2001 Feb;142(2):557-63.

[186] Stehlik C, de MR, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J Exp Med 1998 Jul 6;188(1):211-6.

[187] Bureau F, Seumois G, Jaspar F, et al. CD40 engagement enhances eosinophil survival through induction of cellular inhibitor of apoptosis protein 2 expression: Possible involvement in allergic inflammation. J Allergy Clin Immunol 2002 Sep;110(3):443-9.

[188] Ueda T, Akiyama N, Sai H, et al. c-IAP2 is induced by ionizing radiation through NF-kappaB binding sites. FEBS Lett 2001 Feb 23;491(1-2):40-4.

[189] Zou T, Rao JN, Guo X, et al. NF-kappaB-mediated IAP expression induces resistance of intestinal epithelial cells to apoptosis after polyamine depletion. Am J Physiol Cell Physiol 2004 May;286(5):C1009-C1018.

[190] Cui X, Imaizumi T, Yoshida H, Tanji K, Matsumiya T, Satoh K. Lipopolysaccharide induces the expression of cellular inhibitor of apoptosis protein-2 in human macrophages. Biochim Biophys Acta 2000 Dec 15;1524(2-3):178-82.

[191] Hong SY, Yoon WH, Park JH, Kang SG, Ahn JH, Lee TH. Involvement of two NF-kappa B binding elements in tumor necrosis factor alpha -, CD40-, and epstein-barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene. J Biol Chem 2000 Jun 16;275(24):18022-8.

[192] Yanai A, Hirata Y, Mitsuno Y, et al. Helicobacter pylori induces antiapoptosis through buclear factor-kappaB activation. J Infect Dis 2003 Dec 1;188(11):1741-51.

[193] Wahl C, Maier S, Marre R, Essig A. Chlamydia pneumoniae induces the expression of inhibitor of apoptosis 2 (c-IAP2) in a human monocytic cell line by an NF-kappaB-dependent pathway. Int J Med Microbiol 2003 Nov;293(5):377-81.

[194] Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J, de Martin R. Activation of NF-kappa B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. J Biol Chem 2000 Jul 21;275(29):22064-8.

[195] Sanna MG, da Silva CJ, Ducrey O, et al. IAP suppression of apoptosis involves distinct mechanisms: the TAK1/JNK1 signaling cascade and caspase inhibition. Mol Cell Biol 2002 Mar;22(6):1754-66.

[196] Sanna MG, Duckett CS, Richter BW, Thompson CB, Ulevitch RJ. Selective activation of JNK1 is necessary for the anti-apoptotic activity of hILP. Proc Natl Acad Sci U S A 1998 May 26;95(11):6015-20.

[197] Webster JC, Huber RM, Hanson RL, et al. Dexamethasone and tumor necrosis factor-alpha act together to induce the cellular inhibitor of apoptosis-2 gene and

prevent apoptosis in a variety of cell types. Endocrinology 2002 Oct;143(10):3866-74.

[198] Nishihara H, Hwang M, Kizaka-Kondoh S, Eckmann L, Insel PA. Cyclic AMP promotes cAMP-responsive element-binding protein-dependent induction of cellular inhibitor of apoptosis protein-2 and suppresses apoptosis of colon cancer cells through ERK1/2 and p38 MAPK. J Biol Chem 2004 Jun 18;279(25):26176-83.

[199] Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998 Sep 11;281(5383):1680-3.

[200] Hasegawa T, Suzuki K, Sakamoto C, et al. Expression of the inhibitor of apoptosis (IAP) family members in human neutrophils: up-regulation of cIAP2 by granulocyte colony-stimulating factor and overexpression of cIAP2 in chronic neutrophilic leukemia. Blood 2003 Feb 1;101(3):1164-71.

[201] Cohen MC, Cohen S. Cytokine function: a study in biologic diversity. Am J Clin Pathol 1996 May;105(5):589-98.

[202] Aman MJ, Leonard WJ. Cytokine signaling: cytokine-inducible signaling inhibitors. Curr Biol 1997 Dec 1;7(12):R784-R788.

[203] Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci U S A 1990 Sep;87(18):6934-8.

[204] Moore KW, O'Garra A, de Waal MR, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993;11:165-90.

[205] Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. Annu Rev Immunol 1997;15:563-91.

[206] Lotz M, Setareh M, von KJ, Schwarz H. The nerve growth factor/tumor necrosis factor receptor family. J Leukoc Biol 1996 Jul;60(1):1-7.

[207] Oppenheim JJ, Gery I. From lymphodrek to interleukin 1 (IL-1). Immunol Today 1993 May;14(5):232-4.

[208] Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 1990 Jan 4;343(6253):76-9.

[209] Moore M, Marroquin BA, Gugliotta W, Tse R, White SR. Rho kinase inhibition initiates apoptosis in human airway epithelial cells. Am J Respir Cell Mol Biol 2004 Mar;30(3):379-87.

[210] Bhattacharya M, Babwah AV, Ferguson SS. Small GTP-binding protein-coupled receptors. Biochem Soc Trans 2004 Dec;32(Pt 6):1040-4.

[211] Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994 Jun 3;264(5164):1415-21.

[212] O'Shea JJ. Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? Immunity 1997 Jul;7(1):1-11.

[213] Stepkowski SM, Kirken RA. Janus tyrosine kinases and signal transducers and activators of transcription regulate critical functions of T cells in allograft rejection and transplantation tolerance. Transplantation 2006 Aug 15;82(3):295-303.

[214] Schlessinger J, Ullrich A. Growth factor signaling by receptor tyrosine kinases. Neuron 1992 Sep;9(3):383-91.

[215] Negishi M, Katoh H. Rho family GTPases and dendrite plasticity. Neuroscientist 2005 Jun;11(3):187-91.

[216] Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 2000 Oct 15;351 Pt 2:289-305.

[217] Scita G, Tenca P, Frittoli E, et al. Signaling from Ras to Rac and beyond: not just a matter of GEFs. EMBO J 2000 Jun 1;19(11):2393-8.

[218] Caunt CJ, Finch AR, Sedgley KR, McArdle CA. Seven-transmembrane receptor signalling and ERK compartmentalization. Trends Endocrinol Metab 2006 Sep;17(7):276-83.

[219] Wallace DC. Mitochondria and cancer: Warburg addressed. Cold Spring Harb Symp Quant Biol 2005;70:363-74.

[220] Choy E, Chiu VK, Silletti J, et al. Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. Cell 1999 Jul 9;98(1):69-80.

[221] Cowan KJ, Storey KB. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J Exp Biol 2003 Apr;206(Pt 7):1107-15.

[222] Zhang YL, Dong C. MAP kinases in immune responses. Cell Mol Immunol 2005 Feb;2(1):20-7.

[223] Dong C, Davis RJ, Flavell RA. Signaling by the JNK group of MAP kinases. c-jun N-terminal Kinase. J Clin Immunol 2001 Jul;21(4):253-7.

[224] Sugden PH, Clerk A. Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. Cell Signal 1997 Aug;9(5):337-51.

[225] Nishimoto S, Nishida E. MAPK signalling: ERK5 versus ERK1/2. EMBO Rep 2006 Aug;7(8):782-6.

[226] Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 1997 Nov;17(11):6508-16.

[227] Gille H, Kortenjann M, Thomae O, et al. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. EMBO J 1995 Mar 1;14(5):951-62.

[228] Ono K, Han J. The p38 signal transduction pathway: activation and function. Cell Signal 2000 Jan;12(1):1-13.

[229] Westra J, Limburg PC. p38 mitogen-activated protein kinase (MAPK) in rheumatoid arthritis. Mini Rev Med Chem 2006 Aug;6(8):867-74.

[230] Hale KK, Trollinger D, Rihanek M, Manthey CL. Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages. J Immunol 1999 Apr 1;162(7):4246-52.

[231] Jiang Y, Chen C, Li Z, et al. Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). J Biol Chem 1996 Jul 26;271(30):17920-6.

[232] Ashwell JD. The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat Rev Immunol 2006 Jul;6(7):532-40.

[233] Rouse J, Cohen P, Trigon S, et al. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 1994 Sep 23;78(6):1027-37.

[234] Zhang S, Liu H, Liu J, Tse CA, Dragunow M, Cooper GJ. Activation of activating transcription factor 2 by p38 MAP kinase during apoptosis induced by human amylin in cultured pancreatic beta-cells. FEBS J 2006 Aug;273(16):3779-91.

[235] Gupta S, Barrett T, Whitmarsh AJ, et al. Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 1996 Jun 3;15(11):2760-70.

[236] Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 1993 Nov;7(11):2135-48.

[237] Kyriakis JM, Banerjee P, Nikolakaki E, et al. The stress-activated protein kinase subfamily of c-Jun kinases. Nature 1994 May 12;369(6476):156-60.

[238] Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. Annu Rev Biochem 1998;67:481-507.

[239] Rodriguez-Viciana P, Warne PH, Dhand R, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 1994 Aug 18;370(6490):527-32.

[240] Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. Biochim Biophys Acta 1998 Dec 8;1436(1-2):127-50.

[241] Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol 2001;17:615-75.

[242] Koyasu S. The role of PI3K in immune cells. Nat Immunol 2003 Apr;4(4):313-9.

[243] Vanhaesebroeck B, Leevers SJ, Ahmadi K, et al. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem 2001;70:535-602.

[244] Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 2000 Oct;1(1):11-21.

[245] Steffan NM, Bren GD, Frantz B, Tocci MJ, O'Neill EA, Paya CV. Regulation of IkB alpha phosphorylation by PKC- and Ca(2+)-dependent signal transduction pathways. J Immunol 1995 Nov 15;155(10):4685-91.

[246] Trushin SA, Pennington KN, Algeciras-Schimnich A, Paya CV. Protein kinase C and calcineurin synergize to activate IkappaB kinase and NF-kappaB in T lymphocytes. J Biol Chem 1999 Aug 13;274(33):22923-31.

[247] Lilienbaum A, Israel A. From calcium to NF-kappa B signaling pathways in neurons. Mol Cell Biol 2003 Apr;23(8):2680-98.

[248] Courtney MJ, Akerman KE, Coffey ET. Neurotrophins protect cultured cerebellar granule neurons against the early phase of cell death by a two-component mechanism. J Neurosci 1997 Jun 1;17(11):4201-11.

[249] Jaken S. Protein kinase C isozymes and substrates. Curr Opin Cell Biol 1996 Apr;8(2):168-73.

[250] D'Mello SR, Borodezt K, Soltoff SP. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J Neurosci 1997 Mar 1;17(5):1548-60.

[251] Miller TM, Tansey MG, Johnson EM, Jr., Creedon DJ. Inhibition of phosphatidylinositol 3-kinase activity blocks depolarization- and insulin-like growth factor I-mediated survival of cerebellar granule cells. J Biol Chem 1997 Apr 11;272(15):9847-53.

[252] Jefferson KK, Smith MF, Jr., Bobak DA. Roles of intracellular calcium and NF-kappa B in the Clostridium difficile toxin A-induced up-regulation and secretion of IL-8 from human monocytes. J Immunol 1999 Nov 15;163(10):5183-91.

[253] Pahl HL, Baeuerle PA. Activation of NF-kappa B by ER stress requires both Ca2+ and reactive oxygen intermediates as messengers. FEBS Lett 1996 Aug 26;392(2):129-36.

[254] Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990 Sep 21;249(4975):1431-3.

[255] Schumann RR, Leong SR, Flaggs GW, et al. Structure and function of lipopolysaccharide binding protein. Science 1990 Sep 21;249(4975):1429-31.

[256] Bazil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 1991 Sep 1;147(5):1567-74.

[257] Pugin J, Schurer-Maly CC, Leturcq D, Moriarty A, Ulevitch RJ, Tobias PS. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci U S A 1993 Apr 1;90(7):2744-8.

[258] Simmons DL, Tan S, Tenen DG, Nicholson-Weller A, Seed B. Monocyte antigen CD14 is a phospholipid anchored membrane protein. Blood 1989 Jan;73(1):284-9.

[259] Ferrero E, Goyert SM. Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14. Nucleic Acids Res 1988 May 11;16(9):4173.

[260] Beutler B. Tlr4: central component of the sole mammalian LPS sensor. Curr Opin Immunol 2000 Feb;12(1):20-6.

[261] Yang H, Young DW, Gusovsky F, Chow JC. Cellular events mediated by lipopolysaccharide-stimulated toll-like receptor 4. MD-2 is required for activation of mitogen-activated protein kinases and Elk-1. J Biol Chem 2000 Jul 7;275(27):20861-6.

[262] Akashi S, Shimazu R, Ogata H, et al. Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J Immunol 2000 Apr 1;164(7):3471-5.

[263] Muller JM, Ziegler-Heitbrock HW, Baeuerle PA. Nuclear factor kappa B, a mediator of lipopolysaccharide effects. Immunobiology 1993 Apr;187(3-5):233-56.

[264] Zhang FX, Kirschning CJ, Mancinelli R, et al. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J Biol Chem 1999 Mar 19;274(12):7611-4.

[265] Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001 Feb;13(2):85-94.

[266] Herrera-Velit P, Knutson KL, Reiner NE. Phosphatidylinositol 3-kinase-dependent activation of protein kinase C-zeta in bacterial lipopolysaccharide-treated human monocytes. J Biol Chem 1997 Jun 27;272(26):16445-52.

[267] Shapira L, Takashiba S, Champagne C, Amar S, Van Dyke TE. Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNF-alpha and IL-1 beta production by human monocytes. J Immunol 1994 Aug 15;153(4):1818-24.

[268] Sweet MJ, Hume DA. Endotoxin signal transduction in macrophages. J Leukoc Biol 1996 Jul;60(1):8-26.

[269] Reimann T, Buscher D, Hipskind RA, Krautwald S, Lohmann-Matthes ML, Baccarini M. Lipopolysaccharide induces activation of the Raf-1/MAP kinase pathway. A putative role for Raf-1 in the induction of the IL-1 beta and the TNF-alpha genes. J Immunol 1994 Dec 15;153(12):5740-9.

[270] Buscher D, Hipskind RA, Krautwald S, Reimann T, Baccarini M. Ras-dependent and -independent pathways target the mitogen-activated protein kinase network in macrophages. Mol Cell Biol 1995 Jan;15(1):466-75.

[271] Watson DK, Robinson L, Hodge DR, Kola I, Papas TS, Seth A. FLI1 and EWS-FLI1 function as ternary complex factors and ELK1 and SAP1a function as ternary and quaternary complex factors on the Egr1 promoter serum response elements. Oncogene 1997 Jan 16;14(2):213-21.

[272] Hambleton J, Weinstein SL, Lem L, DeFranco AL. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. Proc Natl Acad Sci U S A 1996 Apr 2;93(7):2774-8.

[273] Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997 Apr;9(2):240-6.

[274] Goh KC, deVeer MJ, Williams BR. The protein kinase PKR is required for p38 MAPK activation and the innate immune response to bacterial endotoxin. EMBO J 2000 Aug 15;19(16):4292-7.

[275] Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003 Sep;3(9):745-56.

[276] Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001 Feb 23;104(4):487-501.

[277] Shen HM, Pervaiz S. TNF receptor superfamily-induced cell death: redox-dependent execution. FASEB J 2006 Aug;20(10):1589-98.

[278] Gupta S. Molecular mechanisms of apoptosis in the cells of the immune system in human aging. Immunol Rev 2005 Jun;205:114-29.:114-29.

[279] Ma W, Lim W, Gee K, et al. The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. J Biol Chem 2001 Apr 27;276(17):13664-74.

[280] Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A 1995 Aug 15;92(17):7686-9.

[281] Lee JC, Young PR. Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. J Leukoc Biol 1996 Feb;59(2):152-7.

[282] Bennett BL, Sasaki DT, Murray BW, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 2001 Nov 20;98(24):13681-6.

[283] Merritt JE, Armstrong WP, Benham CD, et al. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. Biochem J 1990 Oct 15;271(2):515-22.

[284] Peppiatt CM, Collins TJ, Mackenzie L, et al. 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. Cell Calcium 2003 Jul;34(1):97-108.

[285] Lim W, Gee K, Mishra S, Kumar A. Regulation of B7.1 costimulatory molecule is mediated by the IFN regulatory factor-7 through the activation of JNK in lipopolysaccharide-stimulated human monocytic cells. J Immunol 2005 Nov 1;175(9):5690-700.

[286] Arunagiri C, Macreadie I, Hewish D, Azad A. A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human CD4+ lymphocytes. Apoptosis 1997;2(1):69-76.

[287] Wilson DJ, Alessandrini A, Budd RC. MEK1 activation rescues Jurkat T cells from Fas-induced apoptosis. Cell Immunol 1999 May 25;194(1):67-77.

[288] Reers M, Smith TW, Chen LB. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 1991 May 7;30(18):4480-6.

[289] Lim W, Ma W, Gee K, et al. Distinct role of p38 and c-Jun N-terminal kinases in IL-10-dependent and IL-10-independent regulation of the costimulatory molecule B7.2 in lipopolysaccharide-stimulated human monocytic cells. J Immunol 2002 Feb 15;168(4):1759-69.

[290] Holcik M, Lefebvre CA, Hicks K, Korneluk RG. Cloning and characterization of the rat homologues of the Inhibitor of Apoptosis protein 1, 2, and 3 genes. BMC Genomics 2002;3(1):5.

[291] Ma W, Gee K, Lim W, et al. Dexamethasone inhibits IL-12p40 production in lipopolysaccharide-stimulated human monocytic cells by down-regulating the activity of c-Jun N-terminal kinase, the activation protein-1, and NF-kappa B transcription factors. J Immunol 2004 Jan 1;172(1):318-30.

[292] Nghiem P, Saati SM, Martens CL, Gardner P, Schulman H. Cloning and analysis of two new isoforms of multifunctional Ca2+/calmodulin-dependent protein kinase. Expression in multiple human tissues. J Biol Chem 1993 Mar 15;268(8):5471-9.

[293] Choi HJ, Smithgall TE. HIV-1 Nef promotes survival of TF-1 macrophages by inducing Bcl-XL expression in an extracellular signal-regulated kinase-dependent manner. J Biol Chem 2004 Dec 3;279(49):51688-96.

[294] Nishihara H, Kizaka-Kondoh S, Insel PA, Eckmann L. Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. Proc Natl Acad Sci U S A 2003 Jul 22;100(15):8921-6.

[295] Gee K, Kozlowski M, Kumar A. Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. J Biol Chem 2003 Sep 26;278(39):37275-87.

[296] Kim KM, Lee K, Hong YS, Park HY. Fas-mediated apoptosis and expression of related genes in human malignant hematopoietic cells. Exp Mol Med 2000 Dec 31;32(4):246-54.

[297] Hu P, Han Z, Couvillon AD, Exton JH. Critical role of endogenous Akt/IAPs and MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death. J Biol Chem 2004 Nov 19;279(47):49420-9.

[298] Kasri NN, Holmes AM, Bultynck G, et al. Regulation of InsP3 receptor activity by neuronal Ca2+-binding proteins. EMBO J 2004 Jan 28;23(2):312-21.

[299] Stull JT. Ca2+-dependent cell signaling through calmodulin-activated protein phosphatase and protein kinases minireview series. J Biol Chem 2001 Jan 26;276(4):2311-2.

[300] Aramburu J, Rao A, Klee CB. Calcineurin: from structure to function. Curr Top Cell Regul 2000;36:237-95.

[301] Feske S, Draeger R, Peter HH, Eichmann K, Rao A. The duration of nuclear residence of NFAT determines the pattern of cytokine expression in human SCID T cells. J Immunol 2000 Jul 1;165(1):297-305.

[302] Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunol Today 1992 Apr;13(4):136-42.

[303] May MJ, Ghosh S. Rel/NF-kappa B and I kappa B proteins: an overview. Semin Cancer Biol 1997 Apr;8(2):63-73.

[304] May MJ, Ghosh S. Signal transduction through NF-kappa B. Immunol Today 1998 Feb;19(2):80-8.

[305] Hughes K, Edin S, Antonsson A, Grundstrom T. Calmodulin-dependent kinase II mediates T cell receptor/CD3- and phorbol ester-induced activation of IkappaB kinase. J Biol Chem 2001 Sep 21;276(38):36008-13.

[306] Hughes K, Antonsson A, Grundstrom T. Calmodulin dependence of NFkappaB activation. FEBS Lett 1998 Dec 11;441(1):132-6.

[307] Schwenger P, Alpert D, Skolnik EY, Vilcek J. Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IkappaB alpha phosphorylation and degradation. Mol Cell Biol 1998 Jan;18(1):78-84.

[308] Yu C, Rahmani M, Dai Y, et al. The lethal effects of pharmacological cyclin-dependent kinase inhibitors in human leukemia cells proceed through a phosphatidylinositol 3-kinase/Akt-dependent process. Cancer Res 2003 Apr 15;63(8):1822-33.

[309] Mishra JP, Mishra S, Gee K, Kumar A. Differential involvement of calmodulin-dependent protein kinase II-activated AP-1 and c-Jun N-terminal kinase-activated EGR-1 signaling pathways in tumor necrosis factor-alpha and lipopolysaccharide-induced CD44 expression in human monocytic cells. J Biol Chem 2005 Jul 22;280(29):26825-37.

[310] Han H, Stessin A, Roberts J, et al. Calcium-sensing soluble adenylyl cyclase mediates TNF signal transduction in human neutrophils. J Exp Med 2005 Aug 1;202(3):353-61.

[311] Richter J, Ng-Sikorski J, Olsson I, Andersson T. Tumor necrosis factor-induced degranulation in adherent human neutrophils is dependent on CD11b/CD18-integrin-triggered oscillations of cytosolic free Ca2+. Proc Natl Acad Sci U S A 1990 Dec;87(23):9472-6.

[312] Amrani Y, Chen H, Panettieri RA, Jr. Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyper-responsiveness in asthma? Respir Res 2000;1(1):49-53.

[313] Sutterwala FS, Noel GJ, Clynes R, Mosser DM. Selective suppression of interleukin-12 induction after macrophage receptor ligation. J Exp Med 1997 Jun 2;185(11):1977-85.

[314] Lo CJ, Garcia I, Cryer HG, Maier RV. Calcium and calmodulin regulate lipopolysaccharide-induced alveolar macrophage production of tumor necrosis factor and procoagulant activity. Arch Surg 1996 Jan;131(1):44-50.

[315] McLeish KR, Dean WL, Wellhausen SR, Stelzer GT. Role of intracellular calcium in priming of human peripheral blood monocytes by bacterial lipopolysaccharide. Inflammation 1989 Dec;13(6):681-92.

[316] Prpic V, Weiel JE, Somers SD, et al. Effects of bacterial lipopolysaccharide on the hydrolysis of phosphatidylinositol-4,5-bisphosphate in murine peritoneal macrophages. J Immunol 1987 Jul 15;139(2):526-33.

[317] Qu JM, Leaver HA, Yap PL, Wilson NH. Kinetics of calcium mobilisation and inositol phosphate metabolism in endotoxin-stimulated HL-60 cells. Biochem Soc Trans 1991 Apr;19(2):95S.

[318] Schumann MA, Gardner P, Raffin TA. Recombinant human tumor necrosis factor alpha induces calcium oscillation and calcium-activated chloride current in human neutrophils. The role of calcium/calmodulin-dependent protein kinase. J Biol Chem 1993 Jan 25;268(3):2134-40.

[319] Wilkinson MF, Earle ML, Triggle CR, Barnes S. Interleukin-1beta, tumor necrosis factor-alpha, and LPS enhance calcium channel current in isolated vascular smooth muscle cells of rat tail artery. FASEB J 1996 May;10(7):785-91.

[320] Portoles MT, Ainaga MJ, Municio AM, Pagani R. Intracellular calcium and pH alterations induced by Escherichia coli endotoxin in rat hepatocytes. Biochim Biophys Acta 1991 Mar 19;1092(1):1-6.

[321] Letari O, Nicosia S, Chiavaroli C, Vacher P, Schlegel W. Activation by bacterial lipopolysaccharide causes changes in the cytosolic free calcium concentration in single peritoneal macrophages. J Immunol 1991 Aug 1;147(3):980-3.

[322] Bultynck G, Vermassen E, Szlufcik K, et al. Calcineurin and intracellular Ca2+-release channels: regulation or association? Biochem Biophys Res Commun 2003 Nov 28;311(4):1181-93.

[323] Colbran RJ, Brown AM. Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. Curr Opin Neurobiol 2004 Jun;14(3):318-27.

[324] Kim Y, Moon JS, Lee KS, et al. Ca2+/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF-kappaB activity in LPS-stimulated mouse peritoneal macrophages and RAW 264.7 cells. Biochem Biophys Res Commun 2004 Feb 13;314(3):695-703.

[325] Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D. NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci 2003 Oct;6(10):1072-8.

[326] Antonsson A, Hughes K, Edin S, Grundstrom T. Regulation of c-Rel nuclear localization by binding of Ca2+/calmodulin. Mol Cell Biol 2003 Feb;23(4):1418-27.

[327] Fauci AS. Host factors and the pathogenesis of HIV-induced disease. Nature 1996 Dec 12;384(6609):529-34.

[328] Badley AD, Pilon AA, Landay A, Lynch DH. Mechanisms of HIV-associated lymphocyte apoptosis. Blood 2000 Nov 1;96(9):2951-64.

[329] Mahlknecht U, Herbein G. Macrophages and T-cell apoptosis in HIV infection: a leading role for accessory cells? Trends Immunol 2001 May;22(5):256-60.

[330] Perno CF, Newcomb FM, Davis DA, et al. Relative potency of protease inhibitors in monocytes/macrophages acutely and chronically infected with human immunodeficiency virus. J Infect Dis 1998 Aug;178(2):413-22.

[331] Aquaro S, Calio R, Balestra E, et al. Clinical implications of HIV dynamics and drug resistance in macrophages. J Biol Regul Homeost Agents 1998;12(1-2 Suppl):23-7.

[332] Ciborowski P, Gendelman HE. Human immunodeficiency virus-mononuclear phagocyte interactions: emerging avenues of biomarker discovery, modes of viral persistence and disease pathogenesis. Curr HIV Res 2006 Jul;4(3):279-91.

[333] Lum JJ, Badley AD. Resistance to apoptosis: mechanism for the development of HIV reservoirs. Curr HIV Res 2003 Jul;1(3):261-74.

[334] Collman RG, Perno CF, Crowe SM, Stevenson M, Montaner LJ. HIV and cells of macrophage/dendritic lineage and other non-T cell reservoirs: new answers yield new questions. J Leukoc Biol 2003 Nov;74(5):631-4.

[335] Belmonte L, Bare P, de Bracco MM, Ruibal-Ares BH. Reservoirs of HIV replication after successful combined antiretroviral treatment. Curr Med Chem 2003 Feb;10(4):303-12.

[336] Crowe SM, Sonza S. HIV-1 can be recovered from a variety of cells including peripheral blood monocytes of patients receiving highly active antiretroviral therapy: a further obstacle to eradication. J Leukoc Biol 2000 Sep;68(3):345-50.

[337] Muthumani K, Choo AY, Hwang DS, et al. Mechanism of HIV-1 viral protein R-induced apoptosis. Biochem Biophys Res Commun 2003 May 9;304(3):583-92.

[338] Muthumani K, Choo AY, Premkumar A, et al. Human immunodeficiency virus type 1 (HIV-1) Vpr-regulated cell death: insights into mechanism. Cell Death Differ 2005 Aug;12 Suppl 1:962-70.:962-70.

[339] Kedzierska K, Crowe SM, Turville S, Cunningham AL. The influence of cytokines, chemokines and their receptors on HIV-1 replication in monocytes and macrophages. Rev Med Virol 2003 Jan;13(1):39-56.

[340] Schutze S, Wiegmann K, Machleidt T, Kronke M. TNF-induced activation of NF-kappa B. Immunobiology 1995 Jul;193(2-4):193-203.

[341] Kobayashi N, Hamamoto Y, Yamamoto N. Is TNF involved in the progression of AIDS? Virus Genes 1990 Jul;4(2):183-90.

[342] Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997 Feb 21;275(5303):1132-6.

[343] Sade H, Sarin A. IL-7 inhibits dexamethasone-induced apoptosis via Akt/PKB in mature, peripheral T cells. Eur J Immunol 2003 Apr;33(4):913-9.

[344] Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 1993 Sep 1;53(17):3976-85.

[345] Haldar S, Jena N, Croce CM. Inactivation of Bcl-2 by phosphorylation. Proc Natl Acad Sci U S A 1995 May 9;92(10):4507-11.

[346] Viatour P, tires-Alj M, Chariot A, et al. NF- kappa B2/p100 induces Bcl-2 expression. Leukemia 2003 Jul;17(7):1349-56.

[347] Dong Z, Nishiyama J, Yi X, et al. Gene promoter of apoptosis inhibitory protein IAP2: identification of enhancer elements and activation by severe hypoxia. Biochem J 2002 Jun 1;364(Pt 2):413-21.

[348] Takizawa CG, Morgan DO. Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. Curr Opin Cell Biol 2000 Dec;12(6):658-65.

[349] Kino T, Gragerov A, Valentin A, et al. Vpr protein of human immunodeficiency virus type 1 binds to 14-3-3 proteins and facilitates complex formation with Cdc25C: implications for cell cycle arrest. J Virol 2005 Mar;79(5):2780-7.

[350] Zimmerman ES, Chen J, Andersen JL, et al. Human immunodeficiency virus type 1 Vpr-mediated G2 arrest requires Rad17 and Hus1 and induces nuclear BRCA1 and gamma-H2AX focus formation. Mol Cell Biol 2004 Nov;24(21):9286-94.

[351] Goh WC, Manel N, Emerman M. The human immunodeficiency virus Vpr protein binds Cdc25C: implications for G2 arrest. Virology 2004 Jan 5;318(1):337-49.

[352] Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science 1995 Oct 13;270(5234):286-90.

[353] De SE, Zazzeroni F, Papa S, et al. Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. Nature 2001 Nov 15;414(6861):308-13.

[354] Muthumani K, Wadsworth SA, Dayes NS, et al. Suppression of HIV-1 viral replication and cellular pathogenesis by a novel p38/JNK kinase inhibitor. AIDS 2004 Mar 26;18(5):739-48.

[355] Nakano H. Signaling crosstalk between NF-kappaB and JNK. Trends Immunol 2004 Aug;25(8):402-5.

[356] Dong C, Yang DD, Tournier C, et al. JNK is required for effector T-cell function but not for T-cell activation. Nature 2000 May 4;405(6782):91-4.

[357] Brecht S, Kirchhof R, Chromik A, et al. Specific pathophysiological functions of JNK isoforms in the brain. Eur J Neurosci 2005 Jan;21(2):363-77.

[358] Conze D, Krahl T, Kennedy N, et al. c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(+) T cell activation. J Exp Med 2002 Apr 1;195(7):811-23.

[359] Vander Heiden MG, Thompson CB. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol 1999 Dec;1(8):E209-E216.

[360] Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 1997 Nov 28;91(5):627-37.

[361] Wilkinson JC, Wilkinson AS, Scott FL, Csomos RA, Salvesen GS, Duckett CS. Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs). A caspase-independent mechanism for apoptotic inhibition. J Biol Chem 2004 Dec 3;279(49):51082-90.

[362] Samuel T, Welsh K, Lober T, Togo SH, Zapata JM, Reed JC. Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. J Biol Chem 2006 Jan 13;281(2):1080-90.

[363] Bubici C, Papa S, Pham CG, Zazzeroni F, Franzoso G. NF-kappaB and JNK: an intricate affair. Cell Cycle 2004 Dec;3(12):1524-9.

[364] Smith JL, Schaffner AE, Hofmeister JK, et al. ets-2 is a target for an akt (Protein kinase B)/jun N-terminal kinase signaling pathway in macrophages of motheaten-viable mutant mice. Mol Cell Biol 2000 Nov;20(21):8026-34.

[365] Zhang S, Feng Y, Narayan O, Zhao LJ. Cytoplasmic retention of HIV-1 regulatory protein Vpr by protein-protein interaction with a novel human cytoplasmic protein VprBP. Gene 2001 Jan 24;263(1-2):131-40.

[366] Ramanathan MP, Curley E, III, Su M, Chambers JA, Weiner DB. Carboxyl terminus of hVIP/mov34 is critical for HIV-1-Vpr interaction and glucocorticoid-mediated signaling. J Biol Chem 2002 Dec 6;277(49):47854-60.

[367] Muthumani K, Choo AY, Zong WX, et al. The HIV-1 Vpr and glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. Nat Cell Biol 2006 Feb;8(2):170-9.

[368] Kino T, Gragerov A, Slobodskaya O, Tsopanomichalou M, Chrousos GP, Pavlakis GN. Human immunodeficiency virus type 1 (HIV-1) accessory protein Vpr induces transcription of the HIV-1 and glucocorticoid-responsive promoters by binding directly to p300/CBP coactivators. J Virol 2002 Oct;76(19):9724-34.

[369] Rietschel ET, Kirikae T, Schade FU, et al. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J 1994 Feb;8(2):217-25.

[370] Sabbah EN, Druillennec S, Morellet N, Bouaziz S, Kroemer G, Roques BP. Interaction between the HIV-1 Protein Vpr and the Adenine Nucleotide Translocator. Chem Biol Drug Des 2006 Feb;67(2):145-54.

[371] Stewart SA, Poon B, Jowett JB, Xie Y, Chen IS. Lentiviral delivery of HIV-1 Vpr protein induces apoptosis in transformed cells. Proc Natl Acad Sci U S A 1999 Oct 12;96(21):12039-43.

[372] Muthumani K, Choo AY, Hwang DS, Ugen KE, Weiner DB. HIV-1 Vpr: enhancing sensitivity of tumors to apoptosis. Curr Drug Deliv 2004 Oct;1(4):335-44.

[373] Li XD, Sun L, Seth RB, Pineda G, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci U S A 2005 Dec 6;102(49):17717-22.

[374] Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell 2005 Sep 9;122(5):669-82.

RESEARCH AND TEACHING EXPERIENCE (WORK HISTORY):

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- Teaching Assistant (Biochemistry), U of Ottawa — 2003-2006
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- Proctor (Faculty of Science), U of Ottawa — 2003-2006
- Proctor (Faculty of Management Studies), U of Ottawa — 2002-2006
- Research Assistant (Cangene Corporation, Toronto) — 2002
- Scientist (Indian council of Agricultural Research) — 1995-01
- Research Assistant (Indian Veterinary Research Institute) — 1990-95

PUBLICATION IN REFERRED JOURNAL:

- **Mishra S**, Mishra JP, and Kumar A. 2007. A critical role for anti-apoptotic c-IAP2 gene in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. **Journal of Biological Chemistry (Submitted)**

- Ma W*, **Mishra S***, Gee K, Mishra JP, Nandan D, Reiner NE, Angel JB, and Kumar A. 2007. Cyclosporin A and FK506 inhibit IL-12p40 production through the calmodulin/calmodulin-dependent protein kinase-activated PI3K in LPS-stimulated human monocytic cells. **Journal of Biological Chemistry. March 8, Epub ahead of print (* co-author)**

- **Mishra S**, Mishra JP, and Kumar A. (2007). Activation of JNK-dependent pathway is required for HIV viral protein R-induced apoptosis in human monocytic cells: involvement of antiapoptotic BCL2 and c-IAP1 genes. **Journal of Biological Chemistry. 282 (7): 4288-300**

- Gee K, Angel JB, **Mishra S**, and Kumar A (2007). IL-10 regulation by HIV-Tat in primary human monocytic cells: involvement of calmodulin/calmodulin-dependent protein kinase-activated p38 MAPK and Sp-1 and CREB-1 transcription factors. **J Immunol. 178(2):798-807**

- Gee K, Angel JB, Ma W, **Mishra S,** Gajanayak N, and Kumar A (2006). Intracellular HIV-Tat expression induces IL-10 synthesis by the CREB-1 transcription factor through Ser133 phosphorylation and its regulation by the ERK1/2 MAPK in human monocytic cells. **J Biol Chem. 281(42):31647-58**

- **Mishra S,** Mishra JP, Gee K, McManus DC, Lacasse EC, Kumar A (2005). Distinct role of calmodulin and calmodulin-dependent protein kinase-II in lipopolysaccharide and tumor necrosis factor-alpha -mediated suppression of apoptosis and antiapoptotic c-IAP2 gene expression in human monocytic cells. **J Biol Chem.280(45):37536-46**

- Mishra JP, **Mishra S**, Gee K, Kumar A. (2005). Differential involvement of calmodulin-dependent protein kinase II-activated AP-1 and c-Jun N-terminal kinase-activated EGR-1 signaling pathways in tumor necrosis factor-alpha and lipopolysaccharide-induced CD44 expression in human monocytic cells. **J Biol Chem. 280(29):26825-37**

- Lim W, Gee K, **Mishra S,** Kumar A. (2005). Regulation of B7.1 Costimulatory Molecule Is Mediated by the IFN Regulatory Factor-7 through the Activation of JNK in Lipopolysaccharide-Stimulated Human Monocytic Cells. **J Immunol. 175(9):5690-700**

- Rahimi AA. Gee K. **Mishra S,** Lim W, and Kumar, A. (2005). STAT-1 mediates the stimulatory effect of IL-10 on CD14 expression in human monocytic cells. **J Immunol. 15;174(12):7823-32**

- Ma, W.; Gee, K.; Lim, W.; **Mishra, S.** and Kumar, A. (2004). Dexamethasone inhibits IL-12p40 production in LPS stimulated human monocytes by down regualting the activity of JNK, AP-1 and NF-κB. **12th International Congress of Immunology and 4th Annual Conference of FOCIS. 239-42**

- **Mishra, S.;** Kataria, J.M.; Sah, R.L.; Verma, K.C. and Mishra, J.P. (2001). Studies on pathogenesis of Newcastle disease virus isolates in guinea fowl. **Trop. Anim. Hlth. Prod. 33: 313-320**

- **Mishra, S.;** Kataria, J.M.; Verma, K.C. and Sah, R.L. (2000). Response of chicken to guinea fowl isolate of Newcastle disease virus. **Trop. Anim. Hlth. Prod. 32(5): 276-284**

- **Mishra, S.**; Mishra, J.P.; Dimri, U.; Srivastava, M.K. and Bhagwan, P.S.K. (2001). Antitrypsin activity in pneumonic sheep. Ind. J. Anim. Sci. 71(9): 33-34.

- **Mishra, S.**; Kataria, J.M.; Verma, K.C. and Mishra, J.P. (2001). Comparison of protein profile of Newcastle disease virus isolated from different avian species. Ind. J. Anim. Sci. 71(2): 88-91

- Das, S.C.; Mishra, N. **Mishra, S.** and Bhagwan, P.S.K. (2000). An outbreak of enterotoxaemia in rabbit in an organised farm. Ind. J. Comp. Micro. Immun. Infec. Dis. 21(2): 155-156.

- **Mishra, S.**; Kataria, J.M.; Sah, R.L.; Verma, K.C. and Mishra, J.P. (2000). Pathogenesis of Newcastle disease virus isolates in pigeon. Ind. J. Anim. Sci. 70(11): 1125-1126.

- **Mishra, S.**; Mishra, J.P. and Bhagwan, P.S.K. (2000). Haematobiochemical studies on soviet chinchilla and white giant rabbits. Ind J. Anim. Hlth. 39(1): 69-71

- **Mishra, S.**; Kataria, J.M.; Verma, K.C. and Mishra, J.P. (2000). Serological response of chicken to various Newcastle disease virus isolates. Ind. J. Anim. Sci. 70 (6): 556-558

- Mishra, N; **Mishra, S.**; Pawaiya, R.V.S. and Bhagwan, P.S.K. (2000). Isolation and characterization of P. haemolytica strains from a field outbreak in sheep of Rajasthan. Ind. J. Anim. Sci. 70(5) : 443-445

- **Mishra, S.**; Kataria, J.M.; Sah, R.L.; Verma, K.C. and Mishra, J.P. (2000). Pathogenicity of chicken and pigeon isolates of Newcastle disease virus. Ind. J. Anim. Sci.70 (4): 343-345

- **Mishra, S.**; Kataria, J.M.; Verma, K.C. and Mishra, J.P. (2000). Strain differentiation of Newcastle disease virus by laboratory tests. Ind. J. Anim. Sci. 70 (4): 346-348

- **Mishra, S.**; Kataria, J.M.; Verma, K.C. and Mishra, J.P. (2000). Pathogenicity of Newcastle disease virus in guinea fowl. Ind. J. Anim. Sci. 70 (4) : 390-391

- **Mishra, S.**; Das, S.C.; Mishra, N. and Bhagwan, P.S.K. (1999). Development of indirect ELISA for study of immune response to P. multocida strains in sheep. Ind. J. Comp. Micro. Immun. Infec. Dis. 20 (1): 70-71

- Das, S.C. **Mishra, S.**; Mishra, N. and Bhagwan, P.S.K. (1999). Comparison of capsular antigens of P. multocida strains. Ind. J. Anim. Sci. 69(11) : 872-874

- **Mishra, S.**; Kataria, J.M.; Verma, K.C. and Mishra, J.P. (1998). Paramyxo virus infection in pigeon - a review. Ind. J. Virol. 14(2) :133-142

- Mishra, N.; Das, S.C.; **Mishra, S.** and Bhagwan, P.S.K. (1998). Use of indirect and dot ELISA in detecting prevalence of bluetongue virus antibodies in sheep. Ind. J. Virol. 14(2) : 151-153

- **Mishra, S.**; Kataria, J.M.; Verma, K.C. and Mishra, J.P. (1998). Biochemical changes in serum due to Newcastle disease virus strains. Ind. J. Anim. Sci. 68(10) :1009-1011

- **Mishra, S.** and Mehrotra, M.L. (1998). Evaluation of quick and precise diagnostic tests for bluetongue. Ind. J. Anim. Sci. 68 (3): 203-205

- Mishra, N.; **Mishra, S.** and Bhagwan, P.S.K. (1998). Usefulness of fecal examination in diagnosis of Johne's disease in sheep. Ind. J. Small Ruminants. 4(1): 37-39

- Das, S.C.; **Mishra, S**.; Mishra, N. and Bhagwan, P.S.K. (1997). Comparison of competitive ELISA, indirect ELISA and agar gel precipitation test for the detection of bluetongue virus antibodies in sheep. Ind. J. Anim. Sci. 67 (12). 1033-1035

- Mishra, N.; **Mishra, S** and Bhagwan, P.S.K. (1997). Occurrence of an outbreak of FMD in an organised goat farm. Ind. J. Comp. Micro. Immun. Infec. Dis. 18(2): 189

- **Mishra, S**.; Mishra, N and Bhagwan, P.S.K. (1997). Prevalence of E. coli serotypes in rabbits. Ind. J. Comp. Micro. Immun. Infec. Dis. 18(2): 196-197

- **Mishra, Sasmita** and Mehrotra, M.L. (1996). Physicochemical and biological characterization of an Indian isolate of bluetongue virus.Ind. J. Comp. Micro. Immun. Infec. Dis. 17(2):109-113

Abstracts:

- Ma, W.; Gee, K.; Lim, W.; **Mishra, S.** and Kumar, A. (2004). Dexamethasone inhibits IL-12p40 production in LPS stimulated human monocytes by down regualting the activity of JNK, AP-1 and NF-κB. **12th International Congress of Immunology and 4th Annual Conference of FOCIS, Montreal**

- Rahimi AA. Gee K. **Mishra S,** Lim W, and Kumar, A. (2005). STAT-1 mediates the stimulatory effect of IL-10 on CD14 expression in human monocytic cells. **25th Annual Conference of American Society for Reproductive Immunology, Providence, RI, US, June 16-18, 2005**

- Gee, K., J.B. Angel, W. Ma, **S. Mishra,** N. Gajanayaka, K. Parato, and A. Kumar. 2005. Intracellular HIV-tat expression induces IL-10 synthesis by serine 133 phosphorylation of the CREB-1 transcription factor through the activation of extracellular signal-related kinase 1/2 in human monocytic cells. **Ontario HIV Treatment Network Annual Conference. Toronto, Ontario, Canada, November 24-25, 2005.**

- Gee, K., J.B. Angel, W. Ma, **S. Mishra**, N. Gajanayaka, K. Parato, and A. Kumar. 2006. Intracellular HIV-tat expression induces IL-10 synthesis by serine 133 phosphorylation of the CREB-1 transcription factor through the activation of extracellular signal-related kinase 1/2 in human monocytic cells. **HIV Pathogenesis Keystone Symposia, Denver, Colorado, USA, March 26-April 2, 2006.**

- **Mishra S**, Mishra JP, and Kumar A. 2006. A critical role for anti-apoptotic c-IAP2 gene in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. **2006 AIDS conference, Toronto**

- **Mishra S**, Mishra JP, and Kumar A. (2006). A JNK-dependent pathway is required for HIV-Vpr induced apoptosis in human monocytic cells: Involvement of antiapoptotic Bcl2 and c-IAP1 genes. **Ontario HIV Treatment Network Annual Conference. Toronto, Ontario, Canada, November 27-28, 2006**

- **Mishra S**, Mishra JP, and Kumar A. 2006. A critical role for anti-apoptotic c-IAP2 gene in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. **Ontario HIV Treatment Network Annual Conference. Toronto, Ontario, Canada, November 27-28, 2006**

- Gee K, Angel JB, **Mishra S**, and Kumar A (2005). HIV-Tat induces IL-10 expression via calcium dependent p38 MAPK induecd CREB1 activation in primary human monocytic cells. **Ontario HIV Treatment Network Annual Conference. Toronto, Ontario, Canada, November 27-28, 2006**

- **Mishra S**, Mishra JP, and Kumar A. (2006). A JNK-dependent pathway is required for HIV-Vpr induced apoptosis in human monocytic cells: Involvement of antiapoptotic Bcl2 and c-IAP1 genes. **Molecular and cellular determinant of HIV Pathogenesis Keystone Symposia, Whistler, British Columbia, Canada, March 25-March 30, 2007**

- **Mishra S**, Mishra JP, and Kumar A. 2006. A critical role for anti-apoptotic c-IAP2 gene in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. **Molecular and cellular determinant of HIV Pathogenesis Keystone Symposia, Whistler, British Columbia, Canada, March 25-March 30, 2007**

- **Mishra S**, Mishra JP, and Kumar A. (2006). A JNK-dependent pathway is required for HIV-Vpr induced apoptosis in human monocytic cells: Involvement of antiapoptotic Bcl2 and c-IAP1 genes. **Experimental Biology Annual Meeting, Washington DC, US, April 28-May 2, 2007**

- **Mishra S**, Mishra JP, and Kumar A. 2006. A critical role for anti-apoptotic c-IAP2 gene in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. **Experimental Biology Annual Meeting, Washington DC, US, April 28-May 2, 2007**

- **Mishra S**, Mishra JP, and Kumar A. 2006. A critical role for anti-apoptotic c-IAP2 gene in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. **16th Annual Canadian conference on HIV/AIDS Research, Toronto,Canada, April 26-29, 2007**

- Ma W*, **Mishra S***, Gee K, Mishra JP, Nandan D, Reiner NE, Angel JB, and Kumar A. 2007. Cyclosporin A and FK506 inhibit IL-12p40 production through the calmodulin/calmodulin-dependent protein kinase-activated PI3K in LPS-stimulated human monocytic cells. **94th Annual Meeting of The American Association of Immunologist, Miami, US, May 18-22, 2007**

- Mishra JP, **Mishra S**, Kumar A. 2007. STAT-6, as a novel regulator of CD44 expression in human B cells. **94th Annual Meeting of The American Association of Immunologist, Miami, US, May 18-22, 2007**