Critical role of c-IAP-2 in mediating mechanisms of resistance to HIV-Vpr-induced apoptosis in human monocytic cells

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Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

In partial fulfillment of the requirements

For the PhD degree in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology

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ABSTRACT

Monocytic cells survive HIV replication and consequent cytopathic effects because of their decreased sensitivity to HIV-induced apoptosis. However, the mechanism underlying this resistance to apoptosis remains poorly understood. I hypothesized that exposure to microbial products, translocated from the gut, may confer anti-apoptotic properties in human monocytic cells through interaction with their corresponding Toll-like receptors (TLRs). Using HIV-Vpr(52-96) peptide as a model apoptosis-inducing agent, I demonstrated that unlike monocyte-derived macrophages, undifferentiated primary human monocytes and pro-monocytic THP-1 cells are highly susceptible to Vpr(52-96)-induced apoptosis. Interestingly, monocytes and THP-1 cells stimulated with TLR-9 agonists, CpG and E.coli DNA, induced almost complete resistance to Vpr(52-96)-induced apoptosis albiet via a TLR-9 independent signaling pathway. Moreover, CpG and E.coli DNA selectively induced the anti-apoptotic Inhibitor of Apoptosis Protein-2 (c-IAP-2) and inhibition of the c-IAP-2 gene by either specific siRNAs or synthetic second mitochondrial activator of caspases (Smac) mimetic reversed CpG-induced resistance against Vpr(52-96)-mediated apoptosis. I demonstrated that c-IAP-2 was regulated by the c-Jun N terminal kinase (JNK) and the calcium signaling pathway in particular the calmodulin-dependent protein kinase-II (CaMK-II). Furthermore, inhibition of JNK and the calcium signaling including CaMK-II by either pharmacological inhibitors or their specific siRNAs reversed CpG-induced protection against Vpr(52-96)-mediated apoptosis. I also showed that CpG-induced JNK phosphorylation through activation of calcium signaling pathway.

In an attempt to understand the mechanism of protection induced by CpG/bacterial DNA-induced c-IAP-2, I determined that c-IAP-2 regulates Vpr-induced release of mitochondrial apoptotic factors AIF and cytochrome c. Interestingly Vpr(52-96) was found to induce mitochondrial depolarization in these cells by activation of pro-apoptotic proteins caspase 8, Bid and Bax. Moreover this apoptosis cascade was regulated by Vpr(52-96)-mediated degradation of signaling molecules, TRAF-1 and TRAF-2. I showed that bacterial DNA-induced c-IAP-2 bestowed enhanced survival in monocytic cells by protecting TRAF-1/2 from Vpr(52-96)-induced degradation, thereby inhibiting activation of caspase 8, Bid and Bax. This is the first report demonstrating involvement of bacterial DNA and c-IAP-2 in protecting mitochondrial viability.

Overall, these findings suggest a novel and key role for bacterial DNA-induced c-IAP-2 in preventing Vpr-mediated mitochondrial depolarization and apoptosis through prevention of TRAF-1/2 degradation and sequential inhibition of caspase 8, Bid and Bax. Furthermore, strategies based on suppressing c-IAP-2 by targeting CaMK-II or JNK; or targeting c-IAP-2 or TRAF-1/2 for degradation, may be helpful in controlling reservoir formation in HIV infected individuals.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude towards my advisor Dr. Ashok Kumar for the continuous support of my PhD study and research. I truly appreciate his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me navigate and learn both the basics and the intricacies of science and research. I could not have imagined having a better advisor and mentor for my PhD study. I would also like to thank my thesis advisory committee, Dr. Angel, Dr. Holcik and Dr. McBride for their expert opinions and invaluable advice. I am thankful to the entire group at Apoptosis Research Center andd members of HIV journal club for all their help and feedback.

I will forever be grateful to all my lab mates, Liz, Aurelia, Maria, Shifawn, Danylo, Jay and Niranjala for all their support and comfort but most of all for being my closest friends.

My deepest most heartfelt gratitude towards my family cannot be expressed in words. I thank them for patiently supporting me through my ups and downs and for strengthening me with their love. This thesis is a direct result of their unswerving belief in me. Above all I would like to thank my husband, Prashant, for everything and more.

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LIST of ABBREVIATIONS

AICD	Activation Induced Cell Death
AIDS	Acquired Immunodeficiency Syndrome
AIF	Apoptosis Inducing Factor
ANT	Adenosine Nucleotide Translocator
Apaf-1	Apoptotic Protease Activating Factor 1
Bad	Bcl-2 Associated Death Promoter
Bak	Bcl-2 Antagonistic Killer Protein
Bax	Bcl-2 Associated X Protein
Bcl-XL	Bcl-2-X Large Form Protein
BH	Bcl-2 Homology
Bid	Bcl-2 Interacting Domain
Bim	Bcl-2 Interacting Mediator Protein
BIR	Baculovirus Inhibitor Of Apoptosis Protein Repeat
Ca2+	Calcium Ions
CaM	Calmodulin
CaMK-II	Calmodulin Kinase-II
CARD	Caspase Activation And Recruitment Domain
c-IAP-2	Cellular-Inhibitor Of Apoptosis Protein-2
CpG	Cytosine Guanosine
Da	Dalton
DC	Dendritic Cells
DCAF-1	DDB1-And Cullin 4A-Associated Factor -1
DDBI	Damaged-DNA Specific Binding Protein 1
DIABLO	Direct IAP-Binding Protein With Low Pi
DISC	Death-Inducing Signaling Complex
DN	Dominant Negative
DR	Death Receptor
E.coli	Escherichia Coli
EMSA	Electrophoretic Mobility Shift Assay
EndoG	Endonuclease G
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinases
FADD	Fas Associated Death Domain
FasL	Fas Ligand
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
IAP	Inhibitor Of Apoptosis Protein
IBM	IAP Binding Motif
IKK	Inhibitory Kappa B Kinase
IL	Interleukin
IMM	Inner Mitochondrial Membrane
IRAK4	Interleukin-1 Receptor-Associated Kinase4

IRF	Interferon Regulatory Factor
JNK	Janus N-Terminal Kinase
KO	Knock Out
LPS	Lipopolysaccharides
MAPK	Mitogen Activated Protein Kinase
Mcl-1	Myeloid Leukemia Cell Differentiation Protein
MDM	Monocyte Derived Macrophages
MEFs	Mouse Embryonic Fibroblasts
MOMP	Mitochondrial Outer Membrane Potential
MyD88	Myeloid Differentiation Factor 88
NFκB	Nuclear Factor Kappa B
NIAP	Neural IAP
NIK	NFκb Inhibitory Kinase
NLS	Nuclear Localization Signal
ODN	Oligodinucleotide
OMM	Outer Mitochondrial Membrane
PAMP	Pathogen Associated Molecular Patters
PARP	Poly (ADP-Ribose) Polymerase
PBMC	Peripheral Blood Mononuclear Cell
PCD	Programmed Cell Death
PIC	Pre-Integration Complex
PTPC	Permeability Transition Pore Complex
RING	Really Interesting New Gene
RIP-1	Receptor Interaction Protein-1
SD	Standard Deviation
SIV	Simian Immunodeficiency Virus
Smac	Second Mitochondrial Activator Of Caspases
tBID	Truncated BID
TIR	Toll/IL1R
TIRAP	TIR Domain Containing Adaptor Protein
TLR	Toll Like Receptor
TNFR	TNF-α Receptor
TNF-α	Tumor Necrosis Factor-α
TRADD	TNFR-I-Associated Death Domain Protein
TRAF	TNFR Associated Factor
TRAIL	TNF-α-Related Apoptosis-Inducing Ligand
TRIF	TIR Domain-Containing Adaptor Inducing-IFN-β
UBA	Ubiquitin-Associated
UNG-2	DNA Glycosylase-2
VDAC	Voltage Dependant Anion Channel
Vpr	Viral Protein, Regulatory
WT	Wild Type
X-IAP	X-Linked IAP
Z-IETD-FMK	Z-Leu-Glu-His-Asp-Fluoromethyl Ketone

Chapter: 1

1.1 General introduction

Acquired Immunodeficiency Syndrome (AIDS) is advanced stage of the disease caused by human immunodeficiency virus (HIV), a lentivirus belonging to the Retroviradae family (1, 2). Since the first case of HIV/AIDS was documented in 1981 more than 25 million lives have been claimed by this disease worldwide (UNAIDS 2009). The clinical features of HIV/AIDS can be broadly classified in three phases following primary infection: first is the acute stage characterized by increased plasma viral load and decreased CD4 T cell count, second is the clinically asymptomatic period of latency characterized by lowering of plasma viral load and stabilization of CD4 T cell count and the final stage of AIDS (1). Fortunately owing to almost three decades of relentless research and development, modern highly active antiretroviral therapy (HAART) is now remarkably efficient in restoring the health and maintaining below detectable levels of viral burden in HIV infected individuals for prolong periods of time. However, even prolonged HAART is unable to eradicate the virus and completely cure HIV/AIDS (3, 4). One major roadblock to achieving sterile HIV cure is the maintenance of viral reservoirs with in the infected hosts. These reservoirs, for example, cells of the monocytic lineage, are rendered resistant to cell death by HIV infection and become highly efficient instruments for maintaining viral viability even in the face of host antiviral immunity and HAART administration (5-8). The main aim of this thesis was to determine how HIV-1 induces enhanced survivability in otherwise susceptible human monocytic cells.

Rationale for the project was based on earlier findings that activation of monocytic cells by microbial components may confer anti-apoptotic survival signals (9, 10). Moreover,

HIV-1 infection was shown to cause translocation of microbial products from gut into the systemic circulation (11, 12), insinuating that these leaked microbial products may be responsible for making monocytic cells more apoptosis resistant.

Because it is difficult to achieve productive HIV infection in monocytes in vitro, HIV-Viral protein Regultaory (HIV-Vpr) was used as a model apoptosis inducing agent to delineate how monocytic cells develop resistance to apoptotic stimuli. Vpr, is a HIV accessory protein that plays important roles in viral life cycle (13). Interestingly, one of the properties of HIV-Vpr is its capacity to promote apoptosis in various cell types including monocytes (14-16).

In this thesis I investigated whether and how human monocytic cells could become resistant to HIV-Vpr-induced apoptosis if they were first stimulated with various microbial components like LPS and bacterial DNA. I also determined the anti-apoptotic proteins involved in this process, the signaling mechanisms involved in their induction and the mechanism by which these proteins protected the cells against apoptosis. My results suggest that Toll like Receptor (TLR) -2, 4 and -9 agonists induce resistance to Vpr-mediated apoptosis, with CpG-oligodioxynucleotide (ODN) inducing maximum resistance in primary human monocytes and THP-1 cells. Furthermore, this protection is mediated by cellular-Inhibitor of Apoptosis Protein-2 (c-IAP-2) induction through the calcium/Calmodulin kinase-II (CaMK-II) activated Jun-N terminal kinase (JNK) pathway in a TLR-9 independent manner. Moreover, c-IAP-2, via TRAF-1 and TRAF-2, inhibited Vpr-induced activation of pro-apoptotic caspase 8, Bid and Bax thereby protecting against Vpr-mediated mitochondrial membrane permeabilization and consequential apoptosis.

1.2 Overview of Apoptosis

The term apoptosis, meaning “falling of leaves” in Greek, was first coined by Kerr, Wyllie and Currie (17) to describe an “active and inherently controlled” form of cell death. Apoptosis is a well conserved cellular process that plays important roles in various physiological functions like maintaining tissue homeostasis, embryogenesis and clearing defective or infected cells (18-20). Aberration in this tightly regulated process may result in lethal disorders like autoimmune diseases, cancers, developmental defects, etc (21). Some of the typical characteristics of apoptosis include phosphatidylserine exposure, blebbing of the cellular membrane, shrinking of the cell, chromatin condensation and DNA fragmentation, eventually ending in phagocytosis by antigen presenting cells like macrophages to avoid enhanced inflammatory response in surrounding cells (22, 23). Note that apoptosis is different from necrosis, another form of cell death, as during necrosis the cells swell causing cell lysis and uncontrolled spilling of the cellular contents leading to inflammation in the surrounding tissue (24).

In 1993 Yuan and Horvitz *et al* described the role of nematode *Caenorhabditis elegans* protease CED-3 in apoptosis and suggested that the mammalian homologue of CED-3, interleukin-1β-converting enzyme (later termed caspase1), may serve a similar function in mammalian cell apoptosis. This seminal finding paved the way for discovery of the entire family of mammalian cystine proteases called caspases and delineation of apoptotic pathways observed in mammalian cells (25). Depending upon the nature of the death inducing signals, two major pathways of apoptosis are recognized, the intrinsic pathway and the extrinsic pathway.

Caspase activation is a common feature in both these pathways, although, apoptosis is known to occur in caspase independant manner as well. All caspases are expressed in the inactive “pro” zymogen form and need to be cleaved to become active (26). Based on their structure and function, apoptotic caspases can be broadly classified as either “initiator” or “effector”. Initiator caspases like caspase 8 and 9 are the first caspases to be cleaved and activated upon receiving the death stimuli. These caspases then activate other caspases down stream setting into action a caspase activation cascade. Effector caspases like caspase 7 and 3 are the last caspases to be activated in the cascade and ultimately lead to activation of other proteases and nucleases that execute the programmed cell death (PCD) (27-29). Caspases orchestrate PCD by cleaving substrates involved in several cellular processes. These substrates include; a) proteins mediating apoptosis, eg. pro-apoptotic Bcl-2 family protein Bid (30, 31), b) structural proteins that account for the characteristic morphological changes accompanying apoptosis, eg, filamentous proteins like keratins and vimentin (32) and nuclear lamins (33), c) DNA repair proteins like poly(ADP-ribose) polymerase (PARP) (34), which is rendered inactive by cleavage and d) cell cycle regulators like inhibitors of cyclin dependant kinase2 (Cdk2), $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. Cdk2 activation has been found to be very important for apoptosis and cleavage of Cdk2 inhibitors directly results in enhanced induction and activation of Cdk2 (35).

1.2.1 Extrinsic apoptotic pathway:

This pathway involves extrinsic signals like binding of death inducing ligands to their cell surface death receptors like fibroblast associated receptor (Fas), tumor necrosis factor (TNF) receptors and TNF-related apoptosis inducing ligand receptor (TRAIL-R) (36, 37). Engagement of death ligands with their cognate receptors induces stimulation and aggregation of the receptors followed by formation of the death inducing signaling complex (DISC) comprising of death receptors, procaspase 8 and Fas associated death domain (FADD), the death adaptor protein of the extrinsic pathway (38). DISC facilitates dimerization and activation of procaspase 8. Activation of caspase 8 can mediate apoptosis in several different ways (39). Active caspase 8 may mediate apoptosis via inducing direct activation of downstream effector caspases3 and 7, (28) or induce activation of Bid. Upon activation by cleavage, pro-apoptotic truncated Bid (tBid), may either directly translocate to the mitochondrial surface and induce mitochondrial outer membrane permiabilization (MOMP). tBid may also recruit another pro apoptotic Bcl-2 family member, Bax, causing it to translocate to the mitochondria, inducing MOMP (30, 40). In either case MOMP causes loss of mitochondrial potential and release of mitochondrial apoptogenic factors like apoptosis inducing factor (AIF) and cytochrome c.

1.2.2 Intrinsic apoptotic pathway

This pathway is initiated in response to various internal stress stimuli like DNA fragmentation caused by irradiation, chemicals, or genotoxic stress. MOMP is the pivotal event in the intrinsic pathway (19). Mitochondria are structurally composed of an inner matrix, an inner mitochondrial membrane, an outer mitochondrial membrane (OMM) and an inter-membrane chamber. The intrinsic pathway involves complicated interactions between

members of Bcl-2 family of proteins (40) which lead to MOMP allowing release of mitochondrial apoptogenic factors and metabolic failure. Mitochondrial apoptotogenic factors, cytochrome c (41), second mitochondrial activator of caspases (Smac) (42, 43), AIF (44) and Endonuclease G (45) are pro-apoptotic proteins that are contained and compartmentalized within the inter-membrane space in the mitochondria. Upon releasing into the cytosol, cytochrome c forms multimeric complexes with Apoptosis protease activating factor-1 (Apaf-1), a cytosolic death adaptor protein in the intrinsic pathway containing caspase activation and recruitment domain (CARD) (Fig 1.1). This multimeric complex termed, apoptosome, facilitates aggregation of pro caspase 9 instigating caspase activation cascade eventually leading to activation of caspase 3 (27). Intrinsic apoptosis may also be achieved in a caspase independent manner by nucleases like AIF and Endonulcease G. Once released from the mitochondria these nucleases translocate directly to the nucleus and cause DNA fragmentation (46, 47).

Cellular apoptosis is a finely tuned mechanism with several redundant pathways, regulatory checkpoints and counter checkpoints. A complex array of pro-survival, anti-apoptotic molecules like anti-apoptotic members of the Bcl-2 family and members of inhibitor of apoptosis protein (IAP) family (48), closely monitor the apoptotic mechanism to maintain a steady state environment.

Fig 1.1: Diagramatic representation of the mitochondrial membranes and the inter membrane space.

The mitochondria consist of two membranes, the inner mitochondrial membrane and the outer mitochondrial membrane. The outer mitochondrial membrane interfaces with the cytosol and contains the VDAC, a channel allowing transport of ions and small molecules to and from the mitochondria. The inner mitochondrial membrane interfaces with the mitochondrial matrix and is comparably less permeable and is the primary site of cellular respiration. ANT is located in the inner mitochondrial membrane. VDAC and ANT, along with several other mitochondrial transmembrane proteins comprise the PTPC. The intermembrane space between IMM and OMM contain the mitochondrial apoptogenic factors, cytochrome c, SMAC, AIF and EndoG. (OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; VDAC, voltage dependant anion channel; ANT, adenine nucleotide translocator; PTPC; permeability trasition pore complex)

Mitochondrial structure

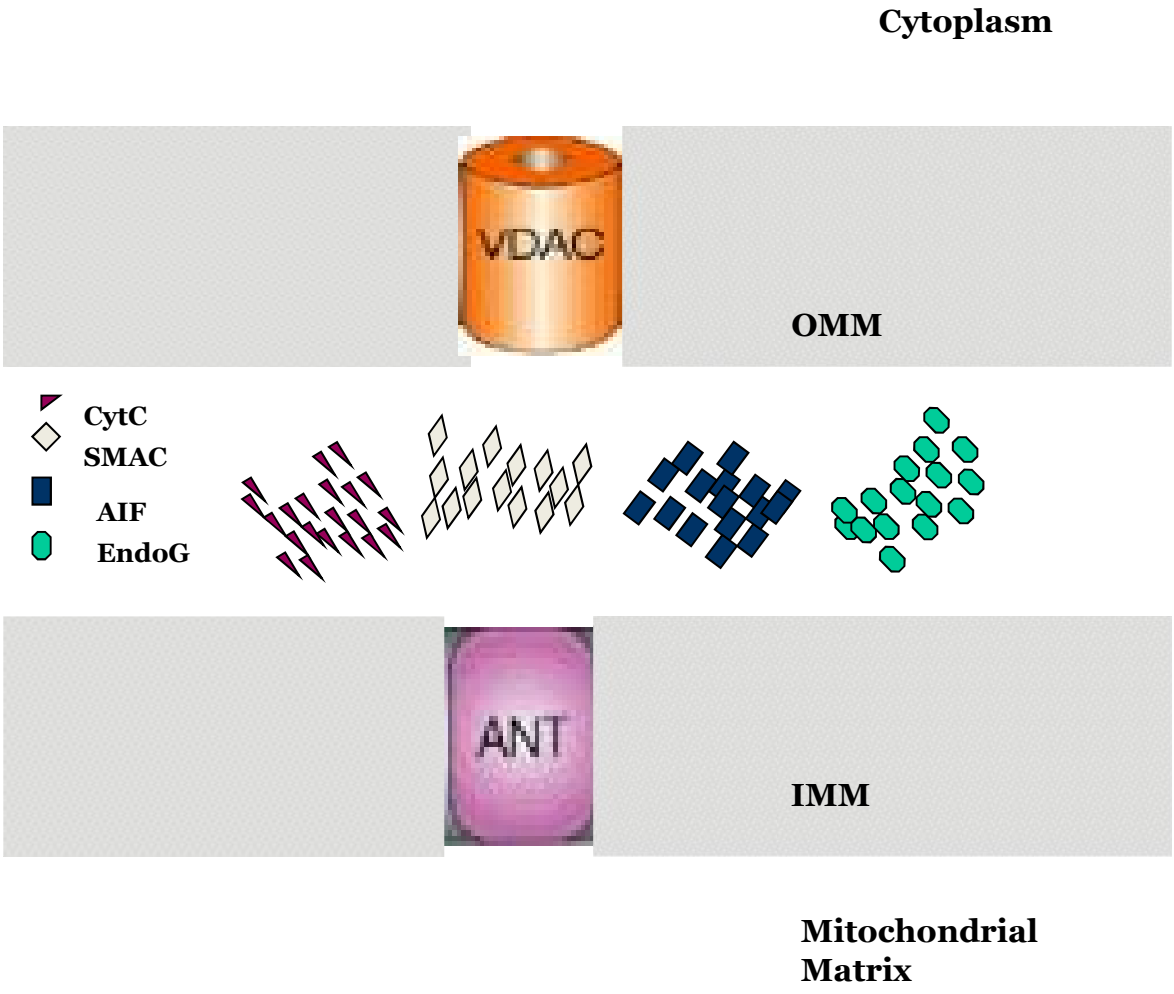


Figure 1.1

1.3 Bcl-2 family of proteins

Bcl-2 protein, the mammalian homologue of *C.elegans* anti-apoptotic protein CED-9 (49) derives its name from the site where it was first identified, the B cell lymphomas ((50). Over the years several homologs of Bcl-2 have been recognized in vertebrates forming the Bcl-2 family of proteins. This family includes anti-apoptotic proteins like Bcl-2, Bcl-X_L, Mcl-1, A1 and pro-apoptotic proteins like Bax, Bak, Bid, Bad, Bim etc (31, 50-57). Dynamic equilibrium between pro and anti-apoptotic members of this family arbitrates succumbing of the cell to the death inducing signals. All the Bcl-2 family members share sequence homology with a conserved region in Bcl-2 called Bcl-2 homology (BH) domain (58, 59). Each member can contain up to four BH domains designated BH1-BH4. Generally speaking the pro-survival proteins like Bcl-2, Bcl-X_L, Mcl-1 and A1 contain all four BH domains whereas the pro-apoptotic proteins like Bax and Bak are lacking in BH4 domain. Interestingly several pro-apoptotic members contain only the BH3 domain underscoring the pro-apoptotic properties of this domain. The proteins solely containing BH3 domain like Bid, Bim, Bad, NOXA and PUMA are referred to as BH3 only proteins (60-62)**.** The importance of pro-apoptotic Bax and Bak proteins is indicated by the observation that double knockout (KO) mice lacking both Bax and Bak either die as neonates or survive with developmental abnormalities (63). In addition, loss of Bax and Bak renders mouse embryonic fibroblasts (MEFs) resistant to a wide variety of apoptotic cues (64) purporting proteins with BH1-3 domains Bax and Bak, to be the executioners of MOMP. How these proteins execute the MOMP has been widely studied. Briefly, Bax normally exists as a monomer in the cytosol or lightly attached to the OMM. Upon induction of apoptosis it is translocated to the mitochondria and undergoes conformational changes that allow it to get

embedded within the OMM. Once integrated it oligomerizes creating pores in the mitochondrial membrane causing MOMP (58, 59, 65). The central issue of how BH3 only and the pro-survival Bcl-2 proteins interrelate to either control or induce Bax and Bak activation still remains to be resolved. Earlier studies had revealed that several BH3 only proteins including Bad displayed high binding affinity for pro-survival Bcl-2 proteins (51) where as Bid exhibited marked affinity for both pro-apoptotic Bax/Bak as well as anti-apoptotic Bcl-2 and Bcl-X_L (31) suggesting that BH3 only proteins may be mediating apoptosis either by occupying the anti-apoptotic Bcl-2 proteins or by activating Bax and Bak. Letai *et al* proposed the "direct activation" hypothesis to explain the duality in the nature of BH3 proteins. Using synthetic BH3 peptides from various BH3 only proteins, and isolated murine mitochondria, they showed that BID-like BH3 domains, termed "activators", alone were sufficient to activate Bak and Bax to induce MOMP as measured by cytochrome c release. They further determined that certain Bad-like BH3 domains did not directly activate Bak and Bax, instead these domains, termed "sensitizers", competitively bound with the Bcl-2 thereby freeing the Bid-like domains to activate Bax or Bak (66). An alternate "indirect activation" hypothesis was suggested by Willis *et al* according to which the Bcl-2 anti-apoptotic members primarily functioned by binding with and neutralizing Bax and Bak while the BH3 only proteins served to neutralize the pro-survival protein. They showed that in human embryonic kidney 293T cells, BH3 only Bid and Bim displayed marked affinity for Bcl-2 pro-survival proteins and very weak affinity for native Bax or Bak. Moreover, to further underscore this theory mutations were induced in Bid and Bim that completely abrogated their binding with Bax and Bak without altering their affinity for the pro-survival proteins. The mutant and wild type (WT) Bid and Bim induced comparable MOMP in

mouse embryo fibroblasts suggesting that BH3 proteins instigate apoptosis by releasing Bax and Bak from anti-apoptotic proteins and not by directly interacting with Bax or Bak (67).

1.4 IAPs

The IAPs, as the name suggests, are key regulators of apoptosis and are over expressed in many cancer cells (68). The first iap gene was identified and sequenced by Crook, Clem and Miller in *Cydia pomonella* granulovirus by its ability to rescue *Spodoptera frugiperda* (fall armyworm) SF21 cells from apoptosis induced by p53 mutant *Autographa californica* nuclear polyhedrosis virus and actinomycin D (69). c-IAPs were initially discovered as a part of TNFR signaling complex (70). There are now eight known mammalian homologs of IAPs: X-IAP (hILP), NIAP, c-IAP-1 (HIAP2), c-IAP-2 (HIAP1), livin (ML-IAP/KIAP), survivin, Ts-IAP (hILP2) and Apollon (Bruce) (68, 71).

Structurally, highly conserved, protein binding, baculovirus IAP repeat (BIR) domains are the primary distinguishing characteristic of IAPs and have been shown to be indispensible for the anti-apoptotic functions of these proteins (72, 73). IAPs may carry up to three BIR domains. Of these, BIR2 and 3 are primarily involved in caspase neutralization whereas BIR1 is involved in regulating the NFκB signaling (70, 71, 74). Besides BIR domains certain IAPs also contain the really interesting new gene (RING) domain and the ubiquitin associated (UBA) domain. The RING and UBA domains bestow ubiquitin ligase activity and ability to interact with ubiquitylated proteins respectively (75-77). Interestingly c-IAP-1 and c-IAP-2 also harbour a caspase-recruitment domain, CARD (71, 74, 78).

Functionally, X-IAP and c-IAPs mediate cell survival via fundamentally distinct mechanisms. X-IAP functions by blocking both effector capases3 and 7 and initiator caspase 9 (79). The peptide sequences with preferential affinity for BIRs are termed IAP-

binding motifs (IBMs). Proteins like caspase 3, 7 and 9 all carry IBMs in their substrate binding pockets (80). Analysis of the crystal structures of X-IAP and caspases revealed that the linker region between BIR1 and BIR2 domain in X-IAP directly bound and occupied the substrate binding pocket of caspase 3 and 7 thereby effectively occluding the catalytic activity of said caspases (81-84). On the other hand mutational and structural analysis determined that the BIR2 domain of X-IAP bound the active site on cleaved caspase 9 while BIR3 domain helped interrupt caspase 9 dimerization, an essential step in caspase 9 activation (82, 85, 86).

Conflicting data make it hard to determine if c-IAPs function in a similar fashion to X-IAP, ie, by negatively regulating distinct caspase activity. For example, using in vitro ubiquitination assay Huang *et al* observed that c-IAP-2 ubiquitinated caspases3 and 7 via its RING domain suggesting that c-IAPs may help target caspases for proteolytic degradation (87). Similarly, Deveraux *et al* also demonstrated that X-IAP, c-IAP-1 and c-IAP-2 significantly inhibited cytochrome c mediated cleavage/activation of initiator caspase 9 and effectively blocked caspase 8-induced apoptosis by inactivating cleaved caspase 3 (48, 88). Conversely, Eckleman *et al* used cell free extracts and recombinant c-IAPs to test the binding and inhibitory activity of c-IAPs over caspases. They concluded that even though both c-IAP-1 and c-IAP-2 bound caspases3, 7 and 9 no inhibition of the caspase activity was observed (89).

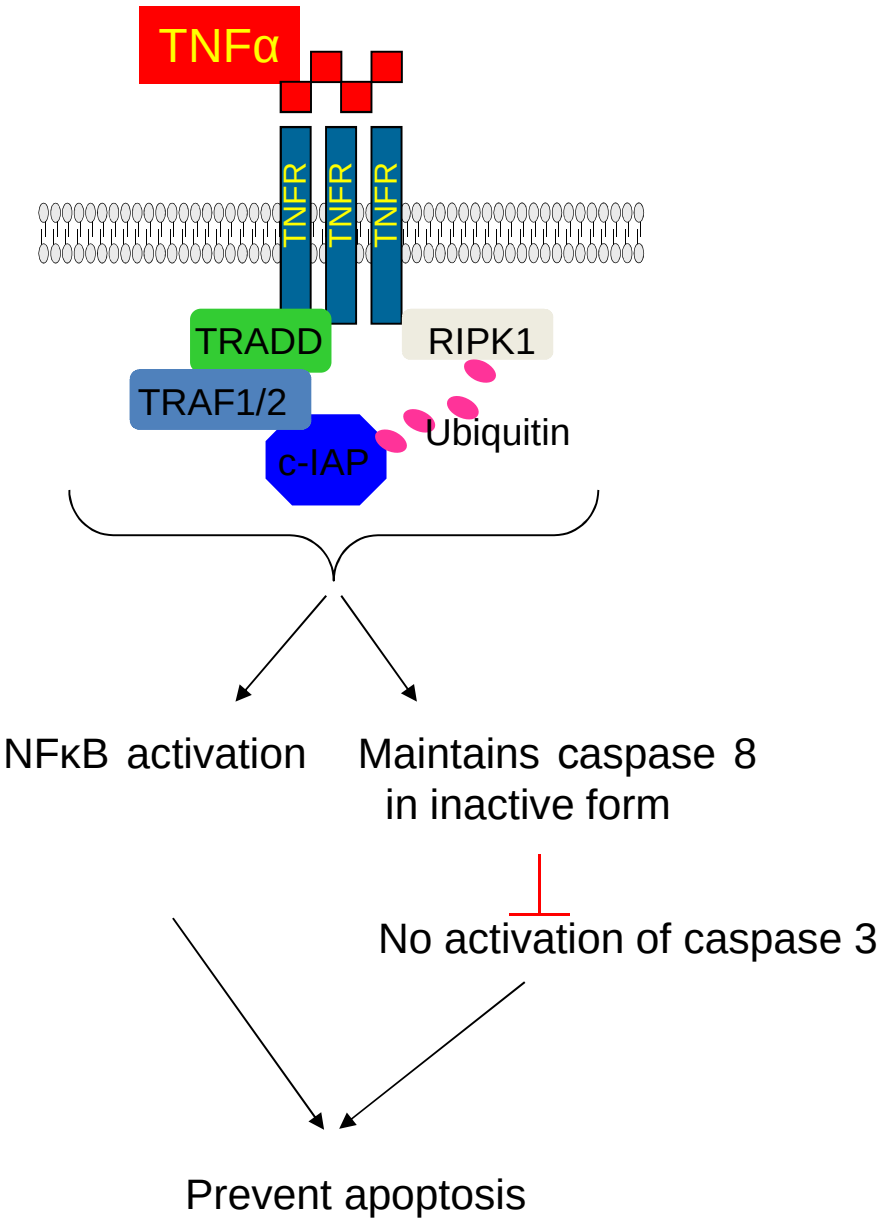
Although c-IAPs have been reported to affect caspases in some cell types, they have mainly been described to play a particularly important role in modulating the survival mechanism by regulating caspase 8 activation and inducing canonical NFκB pathway in response to TNF-α (Fig 1.2) (90, 91). The role of canonical NFκB activation in c-IAP

mediated resistance to apoptosis was underscored by the report that MEFs lacking both c-IAP-1 and c-IAP-2 failed to activate canonical NFκB pathway in response to TNF-α and consequently underwent apoptosis (92).

Fig 1.2: Schematic representation of c-IAP-2-mediated protection against TNF-α-induced apoptosis.

Upon interaction of TNF-α with the TNFRs, the TNFRs aggregate and induce formation of death complex by recruiting TRADD and RIP-1. c-IAP-2 gets recruited to this complex by TRAF-1 and TRAF-2. Being an E3 ligase, c-IAP-2 serves to ubiquitylate RIP-1 thereby inhibiting activation of upstream pro-caspase 8 and downstream caspase 3. Moreover, c-IAP-2 also targets inhibitors of canonical NFκB for degradation thereby enabling transcriptional induction of NFκB dependent anti-apoptotic genes.

c-IAP-2-mediated protection



The same group further ascertained that c-IAP dependant receptor interacting protein 1 (RIP-1) ubiquitination was the crucial factor in this process. Mechanistically, binding of TNF-α to TNFR-I instigates formation of a plasma membrane bound complex comprising of TNFR-I –associated DEATH domain protein (TRADD), TRAF-1/2, c-IAP-1/2 and receptor interacting protein 1 (RIP-1) (93). TRAF-1/2 and c-IAPs are all ubiquitin ligases but in this scenario TRAF-1/2 mainly bind with the BIR1 on c-IAPs and serve as adaptors for recruiting c-IAPs to the complex to regulate caspase 8 activation(90). c-IAPs functions as primary ubiquitin ligases (70) and along with ubiquitilating RIP-1 also facilitate enlisting of signaling proteins like inhibitory kappa B kinase (IKK) to the complex (77). IκKβ phosphorylates and targets NFκB inhibitor for proteasomal degradation allowing NFκB to translocate to the nucleus and initiate gene expression. Notably IAPs are transcriptionaly regulated by NFκB, hence activation of NFκB results in enhanced c-IAP production setting forth a positive feedback loop (74). It is interesting to note that c-IAPs have also been known to negatively regulate the alternate NFκB signaling pathway by targeting a central kinase in non-canonical pathway, NFκB inducing kinase (NIK), for proteasomal degradation (94).

1.5 IAP antagonists

Smac, also known as the direct IAP binding protein with low pI (DIABLO), is a well defined IAP antagonist that functions in a similar manner to its functional equivalents in Drosophila: Grim, Reaper and HID, by binding with IAPs and disengaging IAP-induced inhibition of caspase activation (42, 43, 95, 96). Cellular Smac is an endogenous antagonist of IAPs that is normally sequestered between the inner and outer mitochondrial membrane (97). Newly synthesized Smac contains a mitochondrial targeting sequence at the N terminal

which is proteolitycally cleaved in the mature Smac exposing the tetratpeptide, Ala-Val-Pro-Ile (AVPI) at its N terminal. This tetrapeptide represents the founding member of a family of IBMs. As mentioned earlier IBMs are necessary requisites for binding with the BIRs on IAPs (98). Upon reception of apoptotic stimuli Smac is released from the mitochondria along with other apoptogenic factors and interacts with caspase binding grooves in BIR2 and 3 in IAPs thereby disrupting caspase-IAPs interaction (43, 71). This modus operandi applies primarily to X-IAP mediated inhibition of caspases since c-IAP-1 and 2 have been shown to bind but not significantly inhibit caspases (89).

Since Smac was found to be such a potent antagonist of X-IAP-induced inhibition of caspases, efforts were made to developed small synthetic molecules that mimicked Smac activity and increased cell death in a variety of cancer cells. These small molecules are called Smac mimetic compounds (SMCs) (99-101). Interestingly, although SMCs were primarily designed to inactivate X-IAP it soon became apparent that SMCs were very efficient at regulating c-IAP-1 and 2 mediated repression of apoptosis as well via autoubiquitylation and rapid proteasomal degradation of c-IAP-1 and 2 (94). A strong body of research supports the autocrine role of TNF-α in SMC mediated apoptosis. Petersen and colleagues reported that certain human cancer cells with high TNF-α secretion profiles, were extremely sensitive to apoptosis induced by SMC treatment alone without requiring exogenous apoptotic stimuli. Furthermore SMC-mediated cell death in these cells was regulated by caspase 8, TNFR1, and TNF-α. The same group also determined that certain cancer cells resisted SMC-mediated apoptosis by enhancing c-IAP-2 expression and preventing excess TNF-α secretion via non-canonical NFκB signaling pathway (102, 103). Moreover c-IAP-1 was identified as the primary target of SMCs as c-IAP-1 degradation

induced enhanced stability of RIP-1 and NIK and increased secretion of TNF-α via NFκB signaling pathway (94, 104). The emerging model of SMC-induced apoptosis suggests a two pronged mechanism. Briefly, first SMCs induce conformational changes in IAPs causing them to auto-ubiquitilate and get proteasomaly degraded and so that they can no longer maintain RIP-1 in a non-apoptotic ubiquitilated form (105). Thereafter, TNF-α secreted from the cells activates TNFRI signaling mediated RIP-1 and caspase 8 dependant apoptosis. On the other hand absence of c-IAP-1 and 2 allows for stable expression of NIK and execution of NFκB activation leading to further secretion of TNF-α (106). Several aspects of SMC signaling are still unclear, for example, certain cancer cell lines like THP-1 cells are completely insensitive to SMC-mediated apoptosis even when provided with exogenous TNF-α (107, 108). Even though considerable information is available regarding the binding partners and signaling mechanisms of SMCs, further research in required to precisely delineate the pathways of SMC-mediated cell death.

1.6 HIV-induced apoptosis

HIV has been shown to induce apoptosis via direct and indirect mechanisms in various cells types, for example, CD4 T cells, hepatocytes, cardiomyocytes, renal cells, neurons, etc (109). HIV-induced syncytial dependent apoptosis is mainly governed by viral glycoprotein (gp) complex Env (109-114). This mode of apoptosis involves binding of gp120 on the virion surface with the CD4 receptor and a co-receptor like CXCR4 and CCR5, on the target cell (115-122), leading to formation of giant multi-nucleated cells, called synctia, formed by multi-cell fusion (123-125). The relevance of fusion-induced apoptosis to HIV pathogenesis is indicated by several *in vivo* and *in vitro* studies indicating a

strong correlation between emergence of syncytia-inducing HIV strains, viral load and rapid decline in circulating T cells (126-135).

Several lines of investigation support the hypothesis that activation induced cell death (AICD) caused due to excessive activation by consistent and prolonged exposure to HIV is the primary cause for lymphopenia in HIV/AIDS. Moreover, the enhanced activation and apoptosis was found to be independent of the viral load and disease stage and has been purported to be regulated by Bcl-2 down regulation mediated by constant activation by exposure to viral antigens (136-145). Along with lowered expression of anti-apoptotic Bcl-2, Fas-FasL and TNFR mediated cell death have also been demonstrated to be involved in HIV-elicited AICD (137, 141, 146-159). Another facilitator of extrinsic apoptosis, TRAIL and its receptors, death receptor4 and 5 (DR4 and5) have been implicated in HIV driven cell death (160, 160-166).

In a landmark study Finkel *et al* reported that in agreement with the HIV pathogenesis dogma, the degree of apoptosis in CD4 T cells correlated significantly with viral burden in the lymph nodes of humans and macaques harboring HIV and SIV infection respectively. However, most of apoptosis was observed in uninfected or non-productively infected cells whereas the infected cells were markedly non-apoptotic, implying the relevance of bystander apoptosis in disease progression (164, 167). In parallel, HIV infection was shown to upregulate expression of FasL on monocytic cells facilitating bystander apoptosis of FasR expressing uninfected CD4 T cells (168-170). As discussed above Env-mediated synctial apoptosis is also another mode of bystander apoptosis wherein HIV infected cells express gp120 on their cell surface promoting formation of apoptotic synctia with the surrounding uninfected cells (171).

All in all HIV-induced apoptosis and lymphocyte depletion is a product of simultaneous execution of several cell death mechanisms. HIV accessory proteins serve as another efficient medium for HIV induced cell death in different cell types and are responsible for several HIV/AIDS associated medical conditions.

1.7 HIV accessory protein: Vpr

The genetic structure of HIV-1 is composed of nine genes; *gag, pol, rev, env, tat, nef, vpr, vpu and vif* , encoded by two copies positive strand RNA (172). Of these nine genes *gag, pol* and *env* termed core/structural genes are necessary for virion formation as these encode structural proteins for progeny virus. Of the remaining six genes *tat* and *rev* are particularly essential for viral replication (173) whereas, *nef, vpr, vpu and vif* genes serve to produce accessory proteins that maybe dispensable for viral replication but represent crucial virulence factors with important roles in combating host immune surveillance via various mechanisms including but not limited to apoptosis (174). Of these Vpr, is particularly important in both early and late phases of viral life cycle. During the early phase of cellular infection, Vpr packaged in the virion is released in cytoplasm following uncoating of the capsid while *de novo* synthesis replenishes it during the late phase (175,176). Further evidence for the significance of Vpr in viral pathogenesis is indicated by several *in vitro* studies reporting a significant slump in the growth kinetics of infectious HIV clones with mutational disruption of functional Vpr genes (177, 178). Moreover, both human and chimpanzees infected with HIV_{IIIB} stock, harboring unstable and non-functional Vpr gene, spontaneously restored the truncation in Vpr open reading frame within two years indicating high positive selection pressure for Vpr function *in vivo* (179). The physiological significance of virion free Vpr is indicated by detection of extracellular Vpr in the sera and

cerebrospinal fluid of HIV infected subjects at levels similar to those of p24 antigen (180, 181). Furthermore, circulating Vpr was found to be biologically active as it induced virion production from latently infected cells, as well as apoptosis and depletion of bystander cells in lymphoid tissues during infection (180).

Vpr is a small 96 amino acid long, 14kDa protein that is highly conserved among various lentiviruses including HIV-1 and SIV (182). Tertiary structure of Vpr consists of a α-helix-turn-α-helix motif in the 1-51aa half of the peptide and an α-helix in the 51-96aa half enriched with leucine residues which may facilitate Vpr's interaction with other proteins (183-186). Although Vpr has been suggested to be dispensable for HIV replication in cell culture, it has multiple biological functions. It participates in the nuclear translocation of the HIV-1 preintegration complex (PIC), transcriptional regulation of the HIV-1 long-terminal repeat, apoptosis and induces cell cycle arrest at the G2/M phase (187-192). Multiple functions of Vpr have been attributed to its different domains. Mapping studies performed on lymphocytes and isolated mitochondria revealed that the N-terminal Vpr protein (1-51aa) is required for virion incorporation and nuclear localization, whereas the C-terminus (52-96aa) induces cell cycle arrest and apoptosis (184).

1.7.1 Role of Vpr in HIV reverse transcription

Reverse transcription is an essential initial step in HIV replication following viral entry into the target cell (183, 193-196). Vpr has been shown to play a very important role in maintaining fidelity of HIV reverse transcription so as to ensure progeny survival while maintaining genetic diversity (193, 194, 197-199). It has been suggested that Vpr influences accuracy of reverse transcription by binding and recruiting uracil DNA glycosylase (UNG2), a nuclear form of DNA repair enzyme (193, 194, 197, 200, 201). On the other hand certain

reports indicate that Vpr may actually induce proteasomal degradation of UNG2 to facilitate generation of highly genetically diversified virions (202, 203). However, the matter of Vpr's role in supporting or antagonizing recruitment of UNG2 is complicated and warrants further investigation.

1.7.2 Vpr and nuclear import of PIC

Vpr has been demonstrated to be instrumental in aiding translocation of viral PIC containing reverse transcribed viral DNA into the host's nucleus (13, 174, 204-207). HIV clones with loss of function mutations in Vpr gene failed to achieve efficient nuclear import of PIC and displayed poor viral replication in primary macrophages (189, 208). Interestingly Vpr does not seem to sport any traditional nuclear localization signals (NLS) (209) making it difficult to identify mechanism of Vpr-mediated nuclear import of PIC. (205, 210, 210-214). It has also been speculated that Vpr may only serve to bring the PIC to the nuclear envelope where other viral proteins in the PIC may help carry the viral DNA cargo across the nuclear pore complex using traditional mechanism (183, 204, 215).

1.7.3 Vpr and cell cycle arrest

Cell cycle arrest by checkpoint activation is one of the pathways used by cells' to protect the integrity of their genome. Generally speaking the checkpoint activation pathway is instigated when certain DNA-damage-sensing proteins like ataxia telangiectasia-mutated and Rad3-related (ATR) are alerted to abnormal lesions in DNA. Once activated ATR induces phosphorylation of several cellular proteins that eventually lead to cell cycle arrest (216). Certain viral components facilitate hijacking of this cell cycle arrest mechanism to promote viral persistence and survival. Indeed, Vpr-induced cell cycle arrest at the G2/M phase has been shown to be highly conserved among primate immunodefieciency viruses

indicating that this property of Vpr has important bearing on viral replication (217). Lending credence to this hypothesis is the observation that cells from HIV positive individuals do indeed display an abnormal G2/M phase arrested cell cycle profile (218). Furthermore, Goh *et al* demonstrated that exogenous addition of G2 cell cycle arresting agents rescued viral replication in Jurkat T cells infected with Vpr-negative HIV-1 clones (179) indicating that Vpr-induced cell cycle arrest creates a favorable environment for maximal virus production (179, 219, 220). It is well established that cell cycle arrest can be caused by interfering with ATR mediated activity of the Cdc2-cyclin B1 complex, a central regulator of the transition from G2 to mitosis. Interestingly, C-terminal half of Vpr (221) has been shown to cause cell cycle arrest by recruiting ATR to the nucleus and hyper-phosphorylating Cdc2 while dephosphorylating Cdc25 (222-227). In parallel, Yuan *et al* reported that depletion of a Cdc2 inhibitory kinase, Wee1, released Vpr-mediated hold on the cell cycle (228). Also, Vpr-induced cell cycle arrest was found to be associated with the down regulation of the ERK MAPK pathway in the 293 fibroblast cells (229). Exploitation of the host ubiquitin proteasome system is postulated to be another important mechanism of Vpr-mediated cell cycle arrest (14). Vpr binding protein, later identified as being associated with damaged-DNA specific binding protein 1 (DDB1) and renamed DDB1-and Cullin 4A-associated factor -1 (DCAF-1), was found to facilitate Vpr-mediated cell cycle arrest. Although no direct evidence was reported, it is hypothesized that DCAF-1-Vpr interaction may be allowing subversion of cell's ubiquitin ligase machinery for targeting yet unknown regulator of cell cycle progression for proteasomal degradation (230, 231). Elucidation of this yet unknown cellular target of Vpr is a subject requiring further investigation and its discovery may help in identification of a unifying pathway for Vpr-mediated cell cycle arrest.

1.7.4 Vpr and apoptosis

The first indication of Vpr's involvement in inducing cell death was provided by Macreadie *et al*, who showed that treating yeast cells with exogenous Vpr induced apoptosis in these cells. Furthermore they demonstrated that constitutive expression of Vpr by yeast cells lead to gross mitochondrial dysfunction implicating mitochondrial depolarization as a mechanism of Vpr induced cell death (232-234). The relevance of mitochondrial dysfunction in Vpr-mediated cell death was validated in human cells by Arunagiri *et al* who showed that both recombinant Vpr and Vpr C-terminal peptide caused significant loss of mitochondrial membrane potential and eventual apoptosis in human CD4 T cells (235).

Vpr is now known to induce apoptosis in various cell types including lymphocytes (187), monocytic cells (107, 108, 236) and neurons (191) either upon infection with Vpr-expressing HIV isolates (187, 190) or following exposure of cells to the purified Vpr protein (191, 237). It is interesting to note that genetic analysis of HIV strains isolated from long term non-progressors revealed mutations in Vpr genes that abrogated its apoptotic functions suggesting that apoptosis by Vpr may be a key determinant of viral pathogenesis (238, 239).

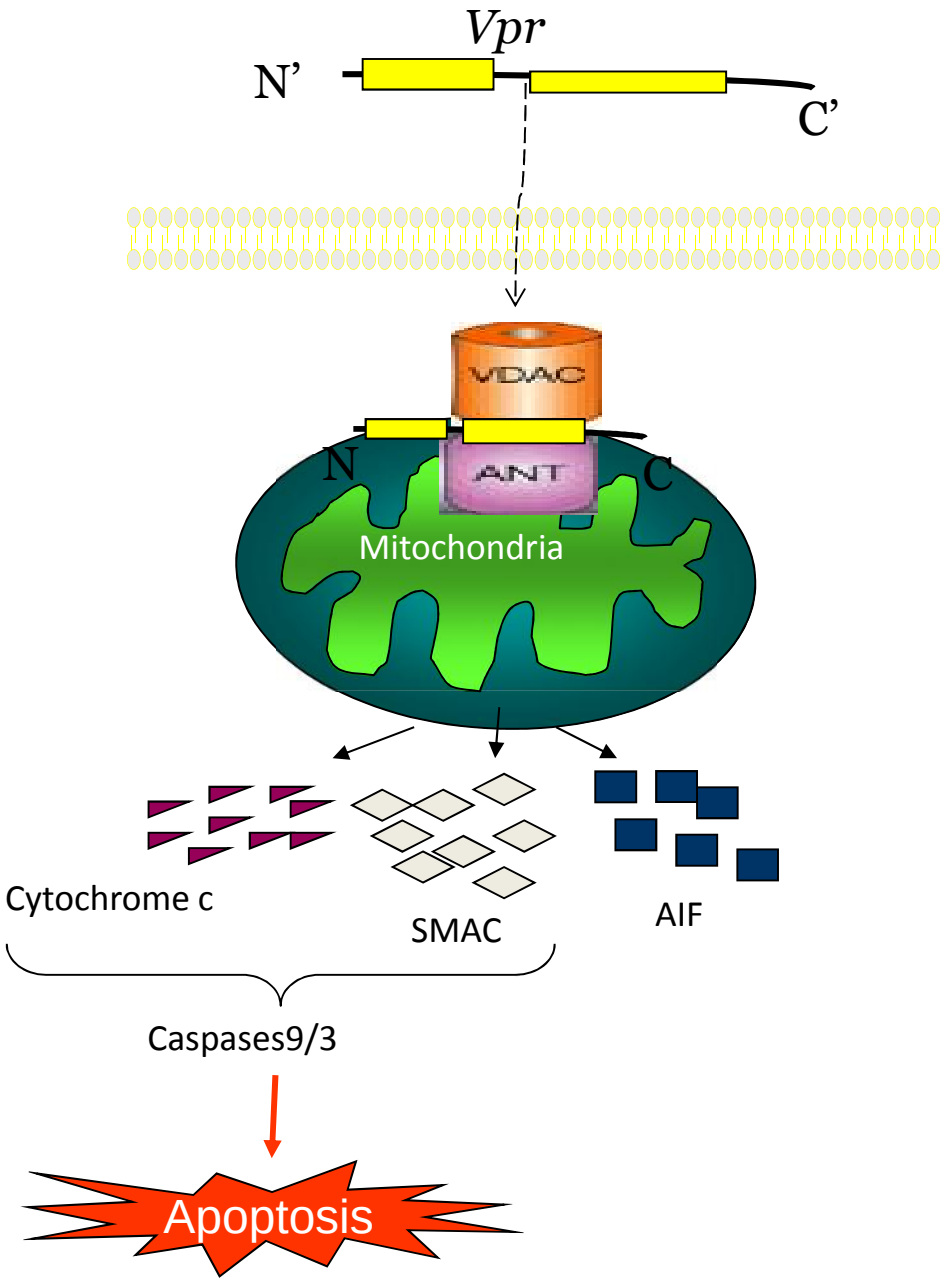
a) HIV-Vpr and its interaction with mitochondria:

Experiments performed by Jacotot *et al*, in intact cells, on isolated mitochondria and on proteins reconstituted into artificial membranes, revealed that amino acid residues in the C terminal half of Vpr peptide induced loss of mitochondrial potential and release of cytochrome c and AIF by interacting directly with a component of inner mitochondrial membrane, ANT (Fig 1.3) (133, 240-243). ANT is part of a cluster of mitochondrial membrane proteins, PTPC which spans across the inner and out mitochondrial membranes and consists of VDAC and Bcl-2 on the OMM and ANT in the IMM (242).

Fig 1.3: Schematic representation of Vpr-mediated apoptosis via mitochondrial permeabilization.

Upon entry into the cell, Vpr interacts with with a component of PTPC, ANT, and induced release of mitochondrial apoptogenic factors cytochrome c, AIF and SMAC nto the cytosol via VDAC. Once in the cytosol, SMAC serves to neutralize anti-apoptotic X-IAP, where as cytochrome c forms an apoptosome activating caspase 9 and caspase 3, with lead to apoptosis. Released endonuclease, AIF causes apoptosis by directly causing DNA damage.

Vpr-mediated apoptosis



The Vpr peptide is characterized by three well defined α-helices: 17-33, 40-48 and 55-83 (183-186). Three specific residues, positively charged Arginine amino acids, 73, 77 and 80 on one of the α-helices were identified to be essential for mitochondrial dysfunction as mutations in these residues was shown to abrogate Vpr-ANT interactions (184, 244), cause osmotic matrix swelling in the mitochondria and loss of membrane potential. Even though early reports provided compelling evidence for the role of Vpr-ANT interaction in Vpr-mediated mitochondrial depolarization, recent studies suggest otherwise. Andersen *et al* showed that siRNA-mediated knockdown of ANT did not appreciably prevent Vpr-mediated mitochondrial damage or apoptosis in HeLa cells. However, Bax knockdown significantly reduced Vpr-mediated mitochondrial depolarization and apoptosis. Moreover, they also showed that Vpr-induced cell death was dependant on cell cycle arrest as Bax activation by Vpr was regulated by cell cycle related proteins. These results suggest that Vpr-ANT interaction may be dispensable for Vpr-mediated apoptosis (16).

Jacotot and colleagues also demonstrated that pre-incubation of isolated mitochondria with anti-apoptotic Bcl-2 could rescue mitochondria from the toxic effect of Vpr as Bcl-2 interfered with Vpr-ANT interaction (240). Muthumani *et al*, developed a novel adenovirus based system, adCMV-Vpr, for delivering Vpr into various tumor cells. Their experiments with adCMV-Vpr demonstrated that Vpr-mediated cell death was indeed dependant on mitochondrial dysfunction and release of cytochrome c. However, no significant difference was observed in apoptosis between wild type and Bcl-2 over expressing Jurkat cells infected with ad-CMV-Vpr, suggesting that in whole cells Bcl-2 may not be an important modulator of Vpr-induced mitochondrial permiabilization (245). Several mitochondrial proteins have been implicated as targets or binding partners for Vpr. For

instance Vpr has been shown to physically interact with and dislodge mitochondrial anti-apoptotic protein HS-associated protein-X1 (HAX1) from the OMM leading to mitochondrial depolarization (246). Vpr has also been shown to cause proteasomal degradation of a mitochondrial fusion protein, mitofusin2 important for maintaining mitochondrial integrity (247).

Further exploring the cell death mechanism of Vpr, Muthumani *et al* and others determined that caspase 9 and caspase 3 were essential regulators of Vpr-mediated cell death downstream of cytoschrome c release, where as caspase 8, Fas-FasL or p53 apoptotic pathways may not be involved in this process (245, 248-251). Interestingly, it was reported that besides activating caspase 9, infection with Vpr expressing vectors also promoted transcriptional upregulation of caspase 9 (250). However, caspase 8 and Bid activation has been reported to be crucial for Vpr-mediated cell death in human neuronal cells, renal tubular epithelial cells and Jurkat cells (238, 252, 253). Since along with cytochrome c, Vpr-mediated mitochondrial injury also causes release of endonucleases like AIF and Endonuclease G, it is conceivable that caspases may be altogether dispensible for Vpr-mediated cell death. Indeed, Roumier *et al* demonstrated that HeLa cells were susceptible to Vpr-induced cell death even when pretreated with a pan caspase inhibitor ZVAD-FMK. Note that in experiments performed by Roumier *et al* Vpr was able to mediate cell death even in the absence of both AIF and caspase activation, suggesting that another yet unknown mechanism for Vpr-induced apoptosis may exist (244).

b) HIV-Vpr direct effect on the nucleus:

Another possible mechanism for Vpr-mediated cell death may be direct effect of Vpr on the nucleus. Vpr has been found to be colocalized at the nucleus (13). Moreover, cell cycle regulating protein ATR has been shown to regulate Vpr-mediated Bax activation (14, 16, 254). Vpr's direct interaction with the glucocortocoid receptor (255) has been implicated in inhibiting NFκB transcription by recruiting PARP to the nucleus (256). Since NFκB controls transcription of several anti-apoptotic genes, inhibiting NFκB helps create a pro-apoptotic environment in the cell (257). Moreover, treatment with glucocorticoid receptor antagonist prevented transcriptional upregulation of caspase 9 by Vpr (253, 258). All in all available data suggests that Vpr-induced apoptosis is a complicated and multi pronged mechanism. Further investigation into this mechanism is likely to reveal novel target for therapeutic intervention.

1.8 Resistance to HIV-mediated apoptosis and formation of reservoirs

Anti retroviral drugs and therapies have been successful in curtailing HIV-mediated T cell loss, lowering viral load to below detectable levels and reconstituting the immune system yet the goal of complete HIV cure remains elusive due to another interesting aspect of HIV pathogenesis that is reservoir formation. HIV has been shown to establish anatomical and cellular reservoirs that serve as shelters, supporting accumulation of latent yet potentially replication competent virus. Indeed these sanctuaries are refractory to anti-viral drug penetration and allow for low levels of viral replication, even during and after HAART, while being shielded against host immune surveillance (4, 259-262). Anatomical reservoirs are immune privileged sites that are compartmentalized from other lymphoid systems by a barrier, for example, the central nervous system serves as an reservoir

guarded by the blood brain barrier which limits the accessibility of drugs and pathogens alike to this site (263). Other anatomical reservoirs include the male genital tract, the gastrointestinal system, lymph nodes etc (264, 265). Cellular reservoirs are cells that can support latent viral persistence after initial infection without succumbing to apoptosis. Such cells, like the memory CD4 T cells and monocytic cells (262), are generally naturally armored with robust survival mechanisms and HIV takes advantage of these pre-existing mechanisms to insure its long term persistence and latency.

Latency is defined by evidence of amplifiable viral DNA but absence of viral mRNA in the cells. Two types of latency mechanism have been identified. Pre-integration latency is achieved when after infecting the cells; the virus does not undergo nuclear transport and fails to integrate with the host DNA. Such latency can be reversed if the cell is activated before the provirus in the cytoplasm gets degraded. The more stable and long lived formed of latency is the post-integration latency. Post-integration latency, as the name suggests occurs when the cells revert to a quiescent stage with minimal transcriptional activity post viral integration (266, 267).

Latently infected resting memory CD4 T cells harboring replicable virus have been found in HIV infected individuals during all stages of disease including post prolonged HAART administration when the viral load falls to below detectable (259, 263, 268, 268-272). The biological makeup of memory T cells allows these cells to survive for several years bestowing immunological memory to the host and facilitating rapid response to recall antigens. However, in case of latently infected memory T cells, the virus exploits this capacity of memory cells to prolong their life span, which can last from a few weeks to a few years, making these reservoirs a major impediment to achieving HIV eradication (273).

Since T cell depletion was so intricately linked with HIV/AIDS pathogenesis most of the early efforts were concentrated in deciphering the mode and mechanism of T cell cytopathy and reservoir formation (124, 145, 274). However, it is conceivable that besides CD4 T cells other vital sources of viral production also exist as viral replication continues even when CD4 T cell counts become too low to support significant viral load (275). Furthermore with increasing efficiency of antiviral drugs in suppressing viral replication in T cells alternative viral reservoirs have gained prominence in efforts to eradicate HIV. Cells of the monocytic lineage, especially macrophages, have been known to be highly permissive to HIV productive infection (276) and are considered a major HIV reservoir contributing actively to AIDS pathogenesis (277). Monocytic cells including macrophages and monocytes, being professional antigen presenting cells are naturally armed to withstand spontaneous and induced apoptosis to aid them in mounting an immune response to help clear infections (278). Therefore along with being permissive to productive HIV infection like CD4 T cells, monocytic cells are also considerably more resistant to apoptosis both *in vivo* and *in vitro* making these an ideal site for reservoir formation (279).

1.8.1 Monocytes as HIV reservoirs

Monocytic cells, like macrophages and CD16hi monocytes represent a significant source of *in vivo* viral reservoir (6). Analysis of this issue has been complicated since productive HIV infection in monocytes appears to be a function of their differentiation. *In vitro,* macrophages have been shown to be more susceptible to infection as opposed to undifferentiated monocytes (280, 281). However, the importance of circulating monocytes in replenishing viral progeny and maintaining chronic disease is underscored by the observation that replication competent HIV-1 could be detected in blood monocytes of HIV

positive patients who had been on HAART and had sustained undetectable viral load (282-284). Likewise several human monocytic cell lines such as THP-1 cells, U937 cells, MonoMac cells and HL60 have been successfully and productively infected with different strains of HIV *in vitro* (279). Evidence for *in vivo* HIV infection of monocytes was provided by Crowe *et al* and Zhu *et al* who described significant number of HIV-1 infected monocytes in blood of HIV positive patients (5, 285, 286). Crowe *et al* identified a subset of monocytes, phenotypically defined as $CD14^{low}$ and $CD16^{hi}$ (287), that displayed a higher propensity for HIV-1 infection (5, 285, 286). Furthermore $CD14^{low}$ and $CD16^{hi}$ monocyte subset has been found to be expanded in therapy naïve HIV-infected individuals (288) and in AIDS patients suffering from HIV associated dementia (289). Notably prevalence of $CD14^{low}$ and $CD16^{hi}$ subset was independent of viral load, CD4 T cell count and disease timeline (288, 290). A unique characteristic of $CD14^{low}$ and $CD16^{hi}$ monocytes, besides expression of CD16, is the altered expression of chemokine receptors on these cells including increased CCR5 expression as compared to $CD14^{hi}$ monocytes (291) which may account for higher infectivity of this cell type. Recently, differentiation of infected bone marrow progenitor cells into monocytes has been suggested to be another mechanism of seeding monocytes with HIV (286).

Along with being important sites of HIV infection, monocytes, (290) as demonstrated by Fulcher *et al*, are capable of supporting compartmentalization and nonparallel viral evolution with important implications for giving rise to viral heterogeneity and possible drug resistance (292). Similarly Llewellyn *et al*, showed that due to limited penetrability of antiretroviral drugs in monocytes (293), ART was substantially less effective in suppressing viral evolution in blood monocytes of seropositive patients as

compared to viral evolution in CD4 T cells (294). Another notable aspect of monocytes, their migratory property, makes these cells a key player in HIV pathogenesis. Monocytes are one of the few immune cells that have direct access to the immunology restricted sites like the central nervous system. As infected monocytes migrate across the blood brain barrier they serve as carriers of HIV into this immune privileged site resulting in serious HIV associated disorders of the central nervous system (295-297). Moreover, besides constituting an independent viral reservoir infected monocytes differentiate into tissue macrophages thereby contributing towards expanding the pool of macrophage reservoirs as well (277).

1.8.2 Monocytes and resistance to HIV-induced apoptosis

The cellular mechanism, through which a fraction of infected memory CD4 T cells and monocytic cells survive despite extreme cytopathic effect of HIV on CD4 T cells, is a remarkable and significant factor in HIV persistence (298). Therefore understanding how resistance to HIV-induced apoptosis is achieved in these cells is crucial to finding a cure for HIV/AIDS.

It is interesting to note that monocytes are relatively less resilient to apoptotic stimuli but gain anti-apoptotic properties during their differentiation into macrophages (299). However, it is generally believed that monocytic cells, unlike T cells, survive HIV replication without major signs of HIV-induced cytopathic effects (6, 300). It appears that monocytic cells can undergo apoptosis but may escape HIV cytopathic effects as a result of certain factors reducing their sensitivity to apoptosis (107, 108, 299, 301). In fact Pinti *et al* demonstrated that chronically infected U1 cells, derived from promonocytic U937 cells, displayed a marked down regulation of death receptor, Fas, on its cell surface as compared to the parent cell line and this down modulation of Fas was correlated with enhanced

resistance to FasL-mediated apoptosis in U1 cells as compared to uninfected U937 cells (302, 303). Same results were corroborated by Okamoto *et al*, who showed that uninfected promonocytic cell lines were more sensitive to receptor mediated cell death as opposed to their respective HIV infected clones (304). Moreover, recent evidence obtained by Fernandez-Larossa *et al*, from chronically HIV infected cell lines, indicates that differential regulation of apoptosis-related genes, Bcl-2 and Bax, by HIV may, at least in part, be responsible for endowing enhanced survival in monocytic cells against H_2O_2 and staurosporine-induced apoptosis (305). In accordance, pretreatment with HIV Tat induced resistance to TRAIL-mediated apoptosis via induction of anti-apoptotic Bcl-2 in human monocytes (306). Similarly distinct and consistent upregulation of a set of anti-apoptotic gene,s and downregulation of pro-apoptotic genes, in monocytes from seropositive individuals has been reported (307). Taken together this data strongly argues for yet unknown mechanisms via which HIV either directly or indirectly bestows resistance to apoptosis in human monocytic cells.

1.9 TLRs

TLRs are germline encoded pattern recognition receptors that recognize conserved molecular signatures in microbial pathogens commonly referred to as pathogen associated molecular patterns (PAMPs) (308, 309). The immune modulatory properties of toll gene was first identified in *Dorsophila melanogaster* (310, 311) whereas the human homologue of *Dorsophila* toll protein was cloned and characterized by Medzhitov *et* al (312). Today in mammals, at least 10 TLRs have been identified (313). Different TLRs bind diverse bacterial components, viral single stranded nucleic acids and small anti-viral or immunomodulatory molecules. TLR-1, TLR-2 and TLR-4 recognize various components of

bacterial and yeast cell walls and are expressed on the cell surface while TLR-3, TLR-7, TLR-8 and TLR-9 recognize microbial nucleic acid signals from endosomal compartments (314). Structurally TLRs are characterized by their cytoplasmic domain, termed toll/IL1R (TIR) domain since it bears homology with the IL1 receptor's intracellular tail. Other distinguishing features of TLRs are the leucine rich repeat containing ectodomains and transmembrane domains (309). TLRs are known to form hetero and homodimers when ligated with their cognate ligands allowing for a greater variety of PAMPs to be recognized (315). Death domain containing protein, MyD88 is the common adaptor used by all TLRs with the exception of TLR3. Upon activation MyD88 interacts with the TLRs through their TIR domain and recruits IL-1R-associated kinase 4 (IRAK4) via its death domain. IRAK4 in turn activates another downstream adapter TRAF6; leading to activation of JNK mediated interferon regulatory factors and NFκB signaling pathways (315, 316). TLR3 exclusively engages an alternate signaling cascade regulated by TIR domain-containing adaptor inducing-IFN-β (TRIF) instead of MyD88 (317). Interestingly TLR4 can use either MyD88 or TRIF to induce distinct immune responses depending upon the nature of PAMP, although it requires additional adaptors TIRAP and TRAM to engage MyD88 and TRIF respectively (308, 318-320). Several signaling pathways are activated following TLR stimulation, ultimately leading to activation of transcription factors like NFκB and interferon regulatory factor which modulate immune responsiveness and induction of pro-inflammatory cytokines (316). Two of the primary signaling cascades induced by TLR activation are calcium and JNK.

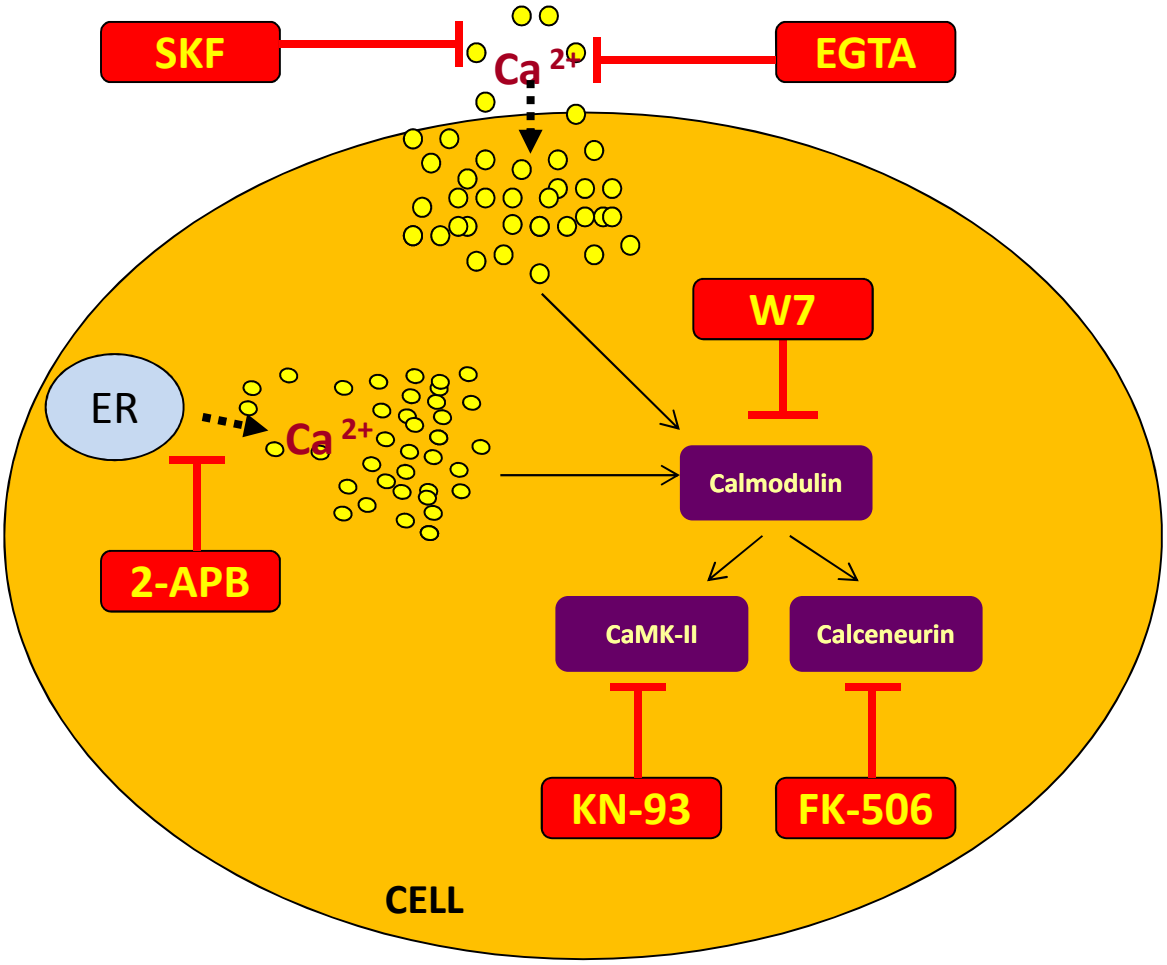
1.9.1 Calcium signaling

Calcium ions are prominent intracellular messengers and control a wide variety of cellular processes (321). The calcium signaling is instigated when concentration of calcium ions in the cytoplasm increases beyond a certain threshold. This increase can be caused by influx of calcium ions from the extracellular medium through voltage gated or receptor mediated calcium channels like the stores operated channels (SOCs) on the cell surface (321-323). Conversely release of calcium ions from intracellular calcium stored like endoplasmic reticulum (ER) or the mitochondria through the inositol-1,4,5-trisphosphate receptor (IP3R) and ryanodine receptor (RYR) families, may also induce calcium signaling (322, 324, 325). Calcium sensing proteins like calmodulin (CaM) (326, 327) bind calcium ions and undergo a conformational change, which enables them to modulate downstream regulators of calcium signaling like calcineurin and CaMKs (328-332). Once activated these modulators regulate a variety of cellular functions including cell survival, TLR signaling, metabolism and gene transcription (Fig 1.4) (321, 322).

Fig 1.4: Schematic representation of calcium signaling pathways and their specific inhibitors.

Calcium ions can enter the cells either through voltage gated calcium channels on the cell surface, (inhibited by SKF-96365), or through receptor gated calcium channels on the ER (inhibited by 2-APB). EGTA being a calcium chelator inhibits calcium entry into the cell. Upon accumulating inside the cell, calcium ions, activated CaM (inhibited by W-7). Activated Ca Minturn may activate downstream signal transducers, CaMK-II (inhibited by KN-93) or Calcineurin (inhibited by FK-506).

Overview of calcium signaling pathways



1.9.2 JNK signaling

JNK is one of the three members of MAPK family in mammals, the other two members being p38 kinase and ERK (333). It is a serine/threonine kinase encoded by three genes *jnk1, jnk2 and jnk3. jnk1 and 2* are expressed ubiquitously and *jnk3* is mainly expressed in heart, testis and brain (334). JNK is a multifunctional kinase involved in various cellular processes ranging from cell survival and embryogenesis to cytokine production (108, 335-339). However the role of JNK activation in apoptosis is difficult to assess as it is dependent on the cell type and nature of stress stimuli (339). JNK is activated in response to various stress stimuli and involves dual phosphorylation by MAPKK which are in turn activated by other upstream kinases (340). Several MAPKKs regulate JNK phosphorylation at distinct sites, in response to discrete signals thereby bestowing specificity of response on JNK activation (341). MAPKK4 and 7 phosphorylate JNK on its tyrosine and threonine residues, respectively, in the activation loop within the kinase domain (342). Furthermore targeted gene disruption studies in mouse reveal that MAPKK4 and 7 may phosphorylate JNK redundantly or discretely depending upon the nature of stimulus. For instance both mapkk4 and 7 genes were required for JNK activation in response to UV radiation where only mapkk7 gene was required to induce JNK activation in response to inflammatory cytokine treatment (343).

1.9.3 CpG/bacterial DNA mediated TLR signaling

Immunostimulatory properties of bacterial DNA in mammalian systems are derived from frequent occurance of unmethylated CpG motifs (344), which have a relatively small low frequency of occurrence in mammals and are largely methylated. (345). The mammalian receptor for bacterial DNA as well as synthetic CpG ODNs, TLR-9, was

discovered by Hemmi *et al*, who showed that TLR-9-/- mice failed to mount an inflammatory response even to lethal doses of bacterial DNA (346, 347). TLR-9 is originally expressed in the ER and traffics, via the Golgi apparatus, to the lysoendosomes, where it interacts with its DNA ligands (348, 349). Furthermore activation of TLR-9 requires acidification of endolysosomes since agents that block endosome and lysosome acidification adversely affect TLR-9 signaling (350). Barton *et al* demonstrated that chimeric TLR-9 expressed on the cell surface was able to induce signaling in response to self DNA (351). Similarly Yasuda *et al* showed that vertebrate DNA delivered endosomaly triggers TLR-9 activation (352). These observations suggest that the endosomal localization of TLR-9 is a means of safeguarding against activation by self DNA.

CpG-induced TLR-9 signaling has been shown to induce a strong Th1 response resulting in secretion of IL6, IL12, TNF-α and IFN-γ (346, 353). It has also been reported to prevent spontaneous apoptosis in mouse B cells and dendritic cells and via PI3K signaling pathway and up regulation of anti apoptotic Bcl-2, BclXl, c-IAP-1 and c-IAP-2 (10, 354). Plasmacytoid dendritic cells (pDCs) in particular are extremely responsive to CpG-mediated induction of type I IFN genes and production of copious amounts of type I IFN (355). Furthermore growing body of evidence suggests the TLR-9-CpG signaling induces class switching, proliferation and differentiation in B cells (356). The strong immunomodulatory properties of CpG ODNs make them a strong candidate for vaccine adjuvants (357).

Based on nucleotide sequence and effect on the target cells, CpG ODNs have been loosely classified into 5 classes. The five classes are Class A (Type D), Class B (Type K), Class C, Class P, and Class S (358). Class B ODNs or ODN 2006, first characterized by Krieg *et al*, are potent inducers of human pDCs maturation and B cell activation (344). Krug

et al first defined the Class A ODNs or ODN 2216, as strong inducers of IFNα from pDCs while having little effect of B cell of pDC maturity or proliferation (359).

1.9.4 TLR-9 independent CpG signaling

A growing body of evidence indicates that bacterial genomic DNA-induced signaling in mammalian cells may be TLR-9 independent as well. For instance *E.coli*-DNA was shown to induce Type 1 IFN and chemokine secretion in both WT and TLR knockout mice. The same group also demonstrated that in human embryonic kidney cells, bacterial DNA induced IFNβ and NFκB activation independently of TLR-9 (360). Similarly Geffner *et al* described activation of human neutrophils by both methylated and non-methylated CpG ODN (361). Furthermore CpG-mediated Akt activation in mouse bone marrow derived macrophages was shown to be dependent on DNA-dependent protein kinases instead of TLR-9 (362). On the other hand Smad phosphorylation and not TLR-9 activation was suggested to be responsible for CpG-mediated anti apoptotic effects in malignant plasma cells (363). The functional relevance of TLR-9 independent signaling is further underscored by the observation that non-TLR-9 expressing glomerular endothelial are able to secrete IL-6, chemokines and IFNα/β in response to CpG treatment (364). These observations suggest that TLR-9 may not be the only receptor for recognizing pathogenic and self DNA and yet unknown factors may be important in DNA-mediated signaling.

1.9.5 Role of TLRs in HIV-induced apoptosis and pathogenesis

TLR expression and activation has been purported to be important to various aspects of HIV pathogenesis including: systemic immune activation (11), HIV gene expression (365) and viral replication (366). Brenchley *et al* postulate that gut injury sustained by the hosts due to acute and chronic HIV infection leads to leakage of microbial products like LPS

from the gut into the systemic circulation causing activation of various cells including blood monocytes (11, 12). Furthermore HIV infection has been shown to alter TLR expression and activation profile in both *ex vivo* and *in vitro* infection models (367-370). Interestingly mass spectrometric analysis of protein profile in HIV infected and uninfected human promonocytic THP-1 cells revealed that HIV infection significantly downregulates expression of a crucial TLR signaling modulator, IRAK4, thereby debilitating the TLR response in human monocytic cells (371). Decrease in TLR response was also documented by Jiang *et al*, who showed that human PBMCs isolated from blood of HIV positive donors were unable to undergo maturation or induce IFNα/β in response to CpG stimulation (372-374). Immune responses to several co-infections during HIV infection have been considered to be a crucial factor in HIV pathogenesis and disease progression. Several co-infections such as endotoxins, tuberculosis and bacterial DNA interact with their respective TLRs (TLR2, -4 and -9). Subsequent TLR signaling has been shown to induce HIV long terminalrepeat transactivation and HIV replication (375-378). However, opposing effects of TLR signaling on HIV replications have also been observed (379).

Chapter 2: Rationale and Hypothesis

2.1 Rationale

HIV is a retrovirus that replicates in cells of the immune system, primarily in CD4+ T cells and macrophages (259, 380). HIV infection is responsible for the severe loss of CD4+ T-cells, acute viremia and progressive depletion of immune system responses (257, 381). However, certain host cells, like cells of monocytic lineage survive the extremely cytopathic environment created by HIV infection and develop into reservoirs for HIV. These cells provide a safe sanctuary for the virus to remain latent and hidden against both the host's antiviral responses as well as against various antiretroviral drugs. Moreover, being antigen presenting cells, monocytic cells help in spreading the virus and aid in establishing infection in immunologicaly protected sites like the brain (259, 265, 277, 296, 382). It is interesting to note that *in vitro* human monocytic cells are indeed susceptible to HIV mediated cell death. Therefore the observation that the number of monocytic cells do not decline even during acute infection, lends itself to the hypothesis that HIV decreases the sensitivity of this cell type to apoptosis *in vivo*.

Determining how HIV induces resistance against apoptosis in monocytes and how this protection can be reversed is vital for designing AIDS treatments effective in clearing viral reservoirs.

Since primary monocytes are refractory to productive HIV infection *in vitro* (280, 281), HIV-1 accessory protein, Vpr (204), was used as an apoptosis causing agent in order to explore mechanisms of resistance to HIV induced apoptosis. Vpr is bestowed with some unique properties; a) It can cross plasma membranes in a receptor independent manner via clathrin coated pits (383), b) its functions are domain dependant, i.e, the 1-45aa half of Vpr

(N terminal) is involved in retro transcription and nuclear import while the 52-96aa half (C terminal) regulates apoptosis and cell cycle progression (184) and c) biologically active Vpr is secreted from infected cells has been isolated from cerebrospinal fluid and serum of infected patients (180, 181). Vpr-induced apoptosis has been shown to be primarily mediated by direct interaction with and depolarization of mitochondria, leading to release of apoptotic factors like cytochrome c and AIF, and activation of caspase 9/3 (14, 16). However, a few incidences of caspase 8 activation in Vpr-mediated cell death have also been reported (252, 253). Recent body of research suggests that HIV infection may impair host's gut immunity and cause translocation of gut microbes and microbial products into systemic circulation (12). This enhanced evidence of microbial products in the serum of HIV infected patients has been correlated with systemic immune activation (11, 384, 385). Moreover activation by LPS and CpG has been shown to induce pro-survival mechanisms in human monocytic cells (9, 10). Hence given the propensity of HIV infection to induce systemic microbial translocation and considering the anti-apoptotic properties of microbial ligands, it is tempting to speculate that interaction with microbial products, through their TLRs on monocytic cells may be rendering these cells resistant to HIV-mediated cell death.

2.2 Hypothesis:

HIV-Vpr-mediated apoptosis in primary human monocytes and promonocytic THP-1 cells can be prevented by pre-treatment with bacterial DNA and induction of c-IAP-2.

2.3 Aims and Objectives:

To determine if stimulation with TLR ligand/s may confer protection in human monocytic cells against Vpr(52-96)-induced apoptosis.

a. Investigate which TLR agonists induce this protection and which anti-apoptotic proteins are involved.

b. Determine the signaling pathways involved in induction of these anti-apoptotic genes.

c. Determine the mechanism of how these anti-apoptotic gene/s induce resistance to apoptosis in human monocytic cells.

Chapter 3: Material and Methods

3.1 Isolation of primary human monocytes, generation of monocyte-derived macrophages (MDMs), cell lines, and reagents

PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). PBMCs thus obtained were subjected to Automacs negative selection (Miltenyi Biotech, Auburn, CA) as per manufacturer's instructions. THP-1 cells, a pro-monocytic cell line derived from an acute monocytic leukemia patient (386), were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in IMDM-10 (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 (all from Sigma-Aldrich, St-Louis, MO). SMAC mimetic AEG-730 was a gift from Dr. Korneluk (Apoptosis Research Centre, Ottawa, ON). Imiquimod, LPS (Sigma-Aldrich, St-Louis, MO), and TNF-α (Invitrogen, Grand Island, NY), CpG-B ODN 2006 (Hycult Biotech, Plymouth Meeting, PA), Poly I:C, Lyovec *E.coli* DNA, TLR-9 antagonist ODN and GpC control ODN (Invitrogen, CA) were purchased. The following signaling inhibitors were used: Chloroquine and EGTA (Sigma-Aldrich, St-Louis, MO); FK-506 (AG Scientific Inc., San Diego, CA) (387); 2-APB, W-7 hydrochloride, KN-93, SKF-96365 hydrochloride (Calbiochem, San Diego, CA) (325, 388); caspase-8-specific inhibitor z-Leu-Glu-His-Asp-fluoromethyl ketone (z-IETD-FMK) (R&D Systems, Minneapolis, MN, USA), SP600125 (Enzo Life sciences, PA), cycloheximide and Bcl-2 inhibitor HA14-1 (Sigma-Aldrich, St-Louis, MO). All other chemicals used for

electrophoresis and immunoblot analysis were obtained from (Sigma-Aldrich, St-Louis, MO).

For generation of MDMs, monocytes were isolated by the adherence method. Briefly, PBMCs were resuspended in serum free medium (5×10^6/ml) and cultured in 12 well polystyrene plates (Beckton Dickinson and Co, Mississauga, ON) for 3 hr to adhere to the plate. The non-adherent cells were washed off and adherent cells were cultured for another 6 days in IMDM-10 supplemented with 10ng/ml macrophage colony-stimulating factor (107, 108) (R&D Systems, Minneapolis, MN).

3.2 Vpr peptides

The Vpr(52-96) peptide (Invitrogen, California) and Vpr(1-45) peptides (Genemed Synthesis Inc, San Francisco, CA, USA) were synthesized by automated solid-phase synthesis and was purified by reverse-phase HPLC (>95%). The amino acid sequence of Vpr peptide Vpr(52-96) is, ^{52}DTWAGVEAI IRILQQLLFI HF**R**IGC**R**HS**R** IGVTRQRRAR NGASRS^{96} and Vpr(1-45) is, ^{1}MEQAPE DQGP-QREPYNEWTL-ELLEELKSEA-VRHFP RIWLH-NLGQH^{45}. These sequences are derived from HIV-1 (NL_{4-3}) strain (240, 242). The mutant Vpr peptide with three Arginine to Alanine mutations at sites R73, R77 and R80 and indicated in bold letters in the above sequence was synthesized (Genemed Synthesis, Inc, Texas). Cells were cultured for 12 hr in serum free media before treatment with Vpr peptides, as described previously (107, 108, 235).

3.3 Ca^{2+} influx

Changes in the levels of intracellular calcium were measured by flow cytometry as described previously. Briefly, cells were washed with Ca^{2+}-free PBS 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 Fluo3/AM (Molecular Probes, Eugene, OR) for 45 min at 37°C. The reaction was stopped by adding equal volume of buffer B (buffer A containing 5% FBS, pH 7.4) for 15 min at 37°C. The cells were washed and resuspended in buffer B ($0.5X10^6$ cells/ml) and analyzed for Ca^{2+} levels by flow cytometery. Ca^{2+} ionophore A23187 (20 mM) and 5mM EGTA (Sigma-Aldrich) were used as positive and negative controls, respectively.

3.4 Analysis of cellular apoptosis by intracellular propidium iodide (PI) and Annexin-V/PI staining

Apoptotic cells exhibiting sub-G_0 DNA content were indentified and analyzed by flow cytometry using PI staining of permeabilized cells, as described previously (107). Briefly, cells ($1.0x10^6$/ml) were washed twice with PBS containing 1% FBS, fixed with methanol for 15 min at 4°C, treated with 1 μg/ml of RNAse A (Roche Applied Science, Laval, Quebec) followed by staining with 50 μg/ml of PI (Sigma-Aldrich, St-Louis, MO) at 4°C for 1 hr. The DNA content was then analyzed by flow cytometry (BD FACS Canto equipped with BD FACS Diva software v5.0.3). Apoptosis was also measured by staining cells ($1.0x10^6$/ml) with FITC-labeled Annexin-V (Molecular probes, Eugene, OR) for 15

min at room temperature in the dark followed by flow cytometry and data analysis using Win-MDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

3.5 Extraction of mitochondrial and cytosolic fractions

Cells ($3X10^6$) were suspended in 70 ul of cell lysis and mitochondria intact buffer (120 mM KCl, 1mM EDTA, 50 mg/ml digitonin in PBS) and incubated on ice for 5 min. Thereafter the cells were subjected to 3500 rpm for 5 min at 4^0C. The supernatant was stored as the cytosolic fraction. Remaining pellet was suspended in 30 µl of universal immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA and complete protease inhibitors) and incubated on ice for 10 min followed by centrifugation at 10,900 rpm for 10 min at 4^0C. The supernatant was collected as the mitochondrial fraction (389, 390).

3.6 Western blot analysis

Briefly, total proteins from cell lysates were subjected to SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad Laboratory, Hercules, CA). The membranes were probed with antibodies specific for XIAP, Bcl-2, c-IAP-1, c-IAP-2, CaMK-II, Bid, full length caspase 8, Bax, TRAF-1, TRAF-2, phospho-p38, phospho-Akt, Akt, Mcl-1, Bcl-X_L and GAPDH (all from Cell Signaling Tech, Inc., Danvers, MA), cleaved caspase 8, phospho-JNK, JNK, phospho-ERK, ERK, p38 (Santa Cruz Biotechnology, Inc, Santa Cruz, California), Vpr (gift from Dr. Eric Cohen, University of Montreal), ANT

(MitoSciences,Eugene, Oregon) followed by donkey anti-rabbit secondary polyclonal antibodies conjugated to horseradish peroxidase (Amersham Bioscience, Montreal, Quebec). All immunoblots were visualized by enhanced chemiluminescence (Amersham Bioscience), as described previously.

3.7 Immunoprecipitation

Mitochondrial fraction was prepared as mentioned above. 30-100μg of mitochondrial extract was incubated with anti-ANT antibodies for 2 hours at 4^0C on a shaker and then washed 3 times with lysis buffer at 2500 rpm.The pellet was then suspended in lysis buffer and incubated with 20 μl of protein A/G Agarose beads (Santa Cruz Inc.) for 30 min at 4^0C on a shaker followed by resuspension in 40 μl of 1X electrophoresis sample buffer and boiled for 5 min. 20 μl aliquots were analyzed by western blotting.

3.8 Transient transfection

THP-1 cells were transiently transfected with either dominant negative (DN)-CaMK-II or empty vector plasmid using transfection reagent FuGENE6 (Roche Applied Science, Laval, Quebec). For transfection, 5 μg of the test plasmid was incubated for 30 min at room temperature with 10 μl of FuGENE6 in 100 μl of OPTI-MEM1 (Invitrogen, CA) medium to allow formation of DNA-liposome complexes. These complexes were then added to the cell suspension ($1.0x10^6$/ml) for 24 hr followed by stimulation with CpG for 12 hr. The cells were treated with Vpr(52-96) overnight and the percentage of apoptotic cells was determined by staining with Annexin-V and flow cytometry.

The siRNAs against c-IAP-2, JNK-1, JNK-2 and CaMK-IIγ (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) were used to inhibit CpG-induced c-IAP-2 expression and protection. Briefly, the control non-silencing siRNA (Qiagen, Mississauga, ON) and c-IAP-2 siRNA were incubated with 3 µl of Fugene6 (Roche Applied Science, Laval, Quebec) at a 1:3 ratio (µg : µL) in 100 µl of serum free medium for 30 min at room temperature before adding to THP-1 cells ($0.25x10^6$/0.5ml). After 5 hr of transfection, cells were transferred into complete medium and stimulated with CpG (5 µM) for 48 hr. The non-silencing siRNA or JNK-1 and JNK-2 siRNA was used to transfect THP-1 cells ($0.25x10^6$/0.5ml) using TransMessenger (Qiagen, Mississauga, ON) transfection reagent at a ratio of 1:5 (µg: µL) as per the manufacturer's protocol. Similarly, CaMK-IIγ siRNA was transfected using Fugene6 (Roche Applied Science, Laval, Quebec) transfection reagent at a 1:3 ratio (µg : µL) as described above. After 5 hr of transfection, cells were transferred into complete medium and incubated for 24 hr followed by stimulation with CpG (5 µM) for 12 hr.

Mcl-1, Bcl-2, Bcl-X_L, TRAF-1 and TRAF-2 siRNAs (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) were transfected using Mirus TransIT-TKO transfection reagent (Mirus, Madison, WI) in 50 µl of serum free medium for 30 min at room temperature before adding to THP-1 cells suspended in 250 µL of complete medium in 24 well plates. After 5 hr of incubation additional 200 µl of complete medium were added to the wells followed by 48 hr incubation following which the cells were stimulated with CpG (5 µM) for 12 hr.

The caspase 8, Bid and Bax specific siRNAs (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) were transfected using the Mirus TransIT-TKO transfection reagent Mirus,

Madison, WI) as described above for 24-48 hr. Following transfections the cells were treated with 1.5 µM Vpr(52-96) for 5 hr (mitochondrial permeabilization measurement) or 24 hr (apoptosis measurement).

3.9 Confocal microscopy

Briefly, THP-1 cells and monocytes were washed with PBS and plated on Poly-L-lysine (Sigma-Aldrich, St-Louis, MO) coated coverslips in 12 well plates for 20 min at 37^0C. MDMs were generated as mentioned above directly on glass coverslips in 6 well plates. The adhered cells were fixed (4% PFA in PBS for 30 min at 37^0C) and quenched (10 min in 50 mM NH_4Cl in PBS). This was followed by permeabilization in 0.1% Triton in PBS (10 min).

The cells were then incubated overnight at 4^0C with primary antibodies conjugated with fluorescent probes that is, a) Alexa flour 488-conjugated cytochrome c antibodies, FITC-conjugated AIF specific antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), FITC-conjugated monoclonal TLR-9-specific antibody and FITC-conjugated mouse IgG_{2a} isotype control antibody (both from Hycult Biotech, Plymouth Meeting, PA).

After permeabilization the cells were incubated overnight at 4^0C with Tom20, Bax 6A7 and cleaved caspase 8 specific primary antibodies (all from Cell Signaling Tech, Inc., Danvers, MA). Thereafter cells were washed with blocking solution three times and incubated with the secondary antibody conjugated with Alexa flour 488 (following Bax 6A7 and cleaved caspase 8) or Alexa flour 688 (following Tom 20) (Invitrogen, CA) for 1 hr at room temperature.

Eventually the coverslips were washed with blocking solution three times and mounted using Prolong Gold Antifade reagent with DAPI nuclear stain (Invitrogen, CA). Confocal fluorescent images were obtained using a Zeiss LSM510 confocal scan head mounted on a Zeiss Axiovert 200M on an inverted-base microscope with a 63X objective. Images were analyzed by Zeiss software and ImageJ (NIH freeware).

3.10 Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed to determine activation of NF-κB DNA binding. Briefly, cells were stimulated with LPS or CpG ODN and nuclear and cytoplasmic proteins were extracted from cell pellets using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce), as per the manufacturer's instructions. Nuclear protein concentration was measured by the Bradford method.

EMSAs were performed using annealed 5’ biotin-labeled duplex probes specific for the NF-κB binding sites for induction of c-IAP-2, with the respective sequences as follows, NF-κB site 1: 5' TC GGG GAT TTC CAT 3' and NF-κB site 3: 5' AG GGG AAC TCC AGC 3' (Integrated DNA Technologies, USA) (391)**.** Binding reactions were performed using the Lightshift Chemiluminescent EMSA Kit (Thermo Scientific, Rockford, IL) as per the manufacturer's instructions. Briefly, all binding reactions included 12 μg of nuclear extract, 10× Binding Buffer (100 mm Tris, 500 mm KCl, 10 mm dithiothreitol), 50% glycerol, 100 mm $MgCl_2$, poly(dIdC), 1% Nonidet P-40, and 10 pm of nuclear extract. To determine the specificity of the proteins for the probe sequence, nuclear proteins were incubated for 30 min at room temperature with an excess (500 pm) of annealed, unlabeled, cold competitor probe. DNA-protein complexes were then resolved on a 5% non-denaturing

polyacrylamide gel, transferred to nylon membranes (Thermo Scientific, Rockford, IL). Chemiluminescent detection was performed using the Lightshift Chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL), as per the manufacturer's instructions.

3.11 Determination of mitochondrial membrane permeability

Loss of Rhodamine 123 (107) and MitoTracker Green FM (392, 393) (both from Invitrogen Molecular Probes) flouresence was used as an indicator of mitochondrial membrane depolarization. Briefly THP-1 cells and MDMs were loaded with 100 ng/mL of Rhodamine 123 and primary monocytes were loaded with 200 nM/mL, in serum free medium and incubated for 30 min at 37°C after treatment with Vpr(52-96). Subsequently the cells were washed with PBS and analyzed by flowcytometry for green fluorescence. Some drawbacks of this approach include interruption of the mitochondrial respiration by the dye itself, over staining other organelles like the endoplasmic reticulum, inability of the dye to enter the cellular plasma membrane and degradation of the dye within the cell (449). In view of these drawbacks, immunoflorescent staining for mitocondrial loss of AIF and cytochrome c was employed as an alternate means of confirming mitochondrial membrane permeabilization.

3.12 Caspase 8 detection assay

Caspase 8 activation was detected using FITC-IETD-FMK caspase 8 activation kit (Calbiochem, San Diego, CA) according to manufacturer's protocol. This kit provides a cell permeable FITC-conjugated caspase 8 inhibitor that binds with cleaved caspase 8. The measure of FITC fluorescence reflects the degree of caspase 8 activation (394). Briefly, monocytes and THP-1 cells, treated with 1.5 μM Vpr(52-96) for 2 hr and 5 hr, respectively,

suspended in 300 µl of medium and 1 µl of FITC-IETD-FMK (caspase-8 detection kit; Calbiochem) was added. After 1 h of incubation at 37°C, cells were washed and analyzed for caspase 8 activation by flow cytometry.

3.13 Transmission electron microscopy

Cells were centrifuged and precipitated in glutaraldehyde followed by standard double fixation using glutaraldehyde and osmium tetraoxide. The samples were processed using Leica EMTP and cut on Leica Ultra UC6 ultra microtome and counterstained with lead citrate and uranyl acetate. The images were visualized and captured using Jeol 1230 transmission electron microscope equipped with AMT digital software.

3.14 Statistical analysis

Data sets were analyzed by the two tailed Student's t-test using Microsoft Excel software. In every graph only two groups are analyzed making t-test an appropriate statistical marker. The protective effect of TLR-ligands was calculated as % apoptosis relative to the Vpr-induced apoptosis. Where no error bars are added the experiment was performed only once.

3.15 Ethics Statement

Blood was obtained from healthy volunteers after approval of the protocol by the ethics review committee of the Ottawa Hospital, Ottawa, Ontario, Canada. A written informed consent was obtained from the study participants.

Chapter 4: Bacterial DNA protects human monocytic cells against HIV-Vpr-induced apoptosis by cellular inhibitor of apoptosis-2 through the calcium-activated JNK pathway in a TLR-9-independent manner

4.1 Chapter 4: Introduction

Monocytic cells represent a key target of HIV and play a crucial role in disease progression (300). Monocytes, MDM and tissue macrophages can be productively infected by HIV (5, 6). 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 eradicating HIV infection. It is generally believed that monocytic cells, unlike T cells, survive HIV replication without major signs of HIV-induced cytopathic effects (5, 6, 300). However, MDMs were shown to undergo apoptosis following *in vitro* infection with HIV, which was mediated by down regulation of Akt-1 and the FOXO3a transcription factor (161, 395). It appears that monocytic cells can undergo apoptosis but may escape HIV cytopathic effects as a result of certain factors reducing their sensitivity to apoptosis (301). For example, nerve growth factor was shown to act as an autocrine survival factor that rescued monocytic cells from the cytopathic effects of HIV (396), whereas IL-13 decreased their spontaneous apoptosis from HIV-infected patients (397). However, the mechanism(s) underlying the development of resistance to HIV-induced apoptosis in monocytic cells are poorly understood and may result in viral persistence.

Viral protein R (Vpr), is a multifunctional regulatory protein of HIV, can induce cell cycle arrest at the G2/M phase of the cell cycle (184), and promotes apoptosis in T cells (222), monocytes (236) and neuronal cells (191). Vpr-induced cell cycle arrest has been

suggested to create a favorable environment for maximal virus production (222). Vpr is also secreted from HIV-infected cells and has been detected in the serum and cerebrospinal fluid of HIV-infected patients at levels similar to those of the HIV-p24 antigen (181). Notably, extracellular Vpr can also be efficiently taken up by cells and its transport appears to be independent of cellular receptors or ionic gradients (184, 398). Moreover, circulating Vpr is biologically active as it has been shown to induce virion production from latently-infected cells and apoptosis of uninfected bystander cells (181, 399).

Multiple functions of Vpr have been attributed to its different domains. Mapping studies performed on isolated mitochondria revealed that the N-terminal 1-51 aa of Vpr, Vpr(1-51), are vital for virion incorporation and nuclear localization, whereas the C-terminal 52-96 aa, Vpr(52-96), induce cell cycle arrest and apoptosis (184, 185, 239, 398). Vpr can cause apoptosis either upon infection with Vpr-expressing HIV isolates or following exposure of cells to the purified protein (191, 235, 236). We and others have shown that this apoptotic effect is mimicked by Vpr(52-96) but not by the Vpr(1-51) moiety (242).

Activation of monocytic cells by microbial components has been shown to confer anti-apoptotic survival signals (9, 10). Recently, elevated levels of microbial products, as a consequence of their translocation from the gut, were found in the serum of persons with chronic HIV infection and this was linked to systemic immune activation and immunodeficiency (11, 385). Moreover, immune responses to several co-infections, like those causing endotoxaemia and tuberculosis, have been considered to be a crucial factor in HIV pathogenesis and disease progression (376, 400). I hypothesized that monocyte activation by such microbial products interacting with their corresponding TLRs may confer anti-apoptotic survival signals against HIV-induced cytopathic effects in these cells. My

results show for the first time that among various TLR agonists examined, pre-treatment with TLR-9 ligand, CpG ODN, afforded maximum protection in monocytic cells against HIV-Vpr-induced apoptosis. Subsequently, I investigated the mechanism underlying CpG-mediated protection against HIV-induced cytopathic effects in monocytic cells by using Vpr(52-96) peptide as a model apoptosis causing agent. Results presented in this study further suggest that this resistance to apoptosis was mediated through TLR-9-independent signaling via induction of the c-IAP-2 gene and involved the selective activation of CaM/CaMK-II and JNK MAPK.

4.2 Chapter 4: Results

4.2.1 *Monocytic cells are susceptible to Vpr(52-96)-induced apoptosis before differentiation into macrophages*

Vpr-containing retroviruses have been shown to induce apoptosis in human monocytic cells (236, 250). To precisely delineate the signaling pathways involved in Vpr(52-96)-induced apoptosis, synthetic Vpr peptides were utilized as these are free of contaminating bacterial products present in recombinant Vpr proteins and certain non-specific or undefined factors present in retroviral supernatants which may be capable of non-specifically and/or transiently activating various signaling pathways. Moreover, Vpr(52-96) peptide mimics the apoptotic activity of full length Vpr-peptide (250)**.** Even though Vpr(52-96) induced significant apoptosis in undifferentiated primary monocytes and THP-1 cells (Fig 4.1A and B), differentiated MDMs displayed a marked resistance to Vpr(52-96)-induced apoptosis (Fig 4.1C). Three Vpr arginine residues at sites 73, 77 and 80 have been shown to be essential for Vpr-mediated mitochondrial permeabilization and apoptosis (238, 239). The control mutant Vpr(52-96) peptide with arginine to alanine mutations at sites 73, 77 and 80 did not induce cell death either in MDMs or THP-1 cells (Fig 4.1C and D). Interestingly both Vpr(52-96) and mutant Vpr(52-96) were able to enter the cell indicating that apoptosis abolishing mutations in Vpr(52-96) did not affect the ability of Vpr to penetrate the cell wall (Fig 4.1E).

Fig 4.1: Vpr(52-96) induces apoptosis in undifferentiated monocytic cells but not in differentiated macrophages.

Cells ($1.0x10^6$/ml) were treated with various concentrations of Vpr(52-96) for 4 hr in monocytes **(A)** or for 24 hr in THP-1 cells **(B)** followed by Annexin-V staining and flow cytometry for analysis of apoptosis. **C**. MDMs ($1.0x10^6$/ml) were treated with 1.5 μM Vpr(52-96) or mutant Vpr for 24 hr followed by intracellular PI staining and measurement of apoptosis by flow cytometry. **D.** THP-1 cells ($1.0x10^6$/ml) were also treated with varying concentrations of mutant Vpr for 24 hr and then analyzed by intracellular PI staining and flow cytometry. **E**. Mitochondrial extracts harvested from THP-1 cells ($3.0x10^6$/ml) treated with 1.5 μM Vpr(52-96) or mutant Vpr for 1 hr were analyzed by western blotting using antibodies against full length Vpr. Results in A, B, C and D are expressed as mean ± SD of three independent experiments. $*(p < 0.05)$, ** $(p < 0.005)$ The p values were calculated against media/cells alone. No loading control for (E) was performed as the purpose of this experiment was to determine, qulitatively and not quantitavely, if both wild type and mutant Vpr had access to the mitochondria.

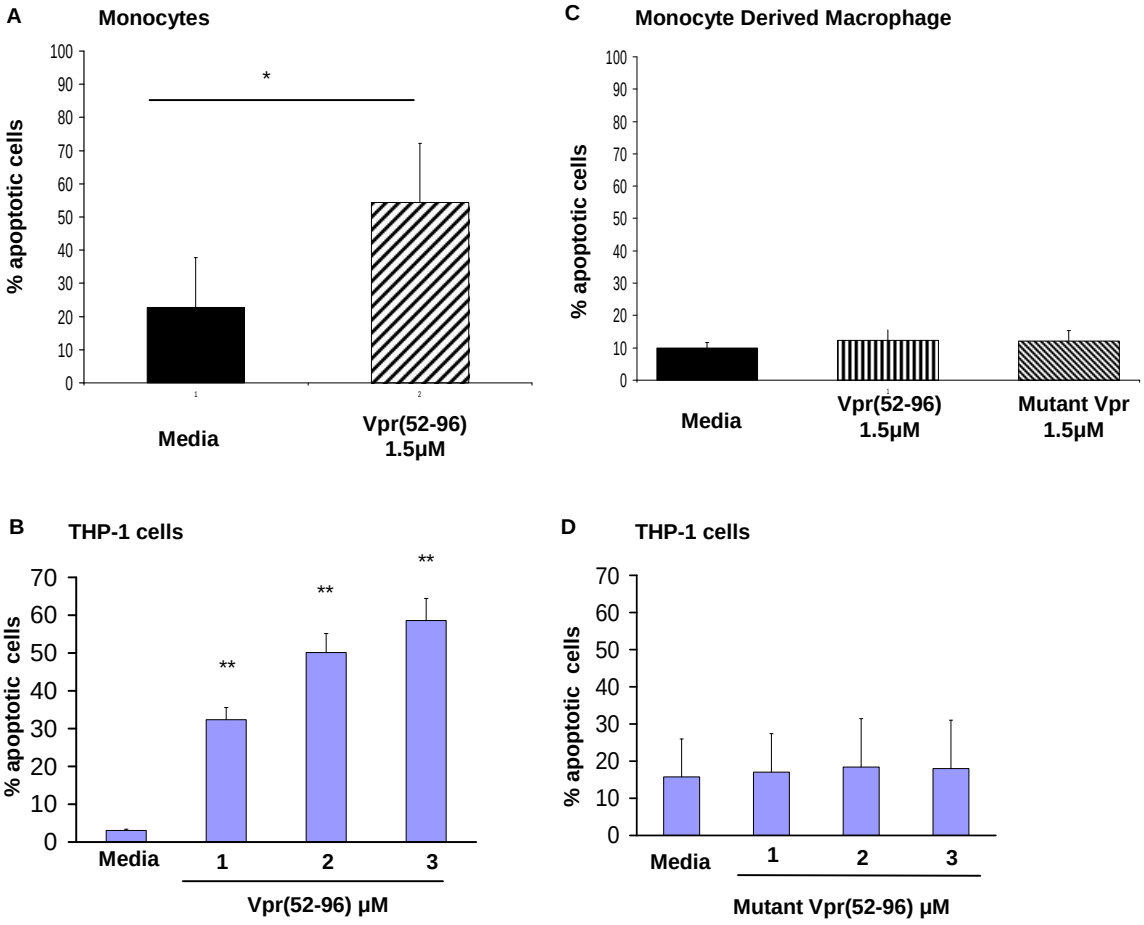

A
Monocytes
% apoptotic cells
Media
Vpr(52-96)
1.5μM
C
Monocyte Derived Macrophage
% apoptotic cells
Media
Vpr(52-96)
1.5μM
Mutant Vpr
1.5μM
B
THP-1 cells
% apoptotic cells
Media
Vpr(52-96) μM
D
THP-1 cells
% apoptotic cells
Media
Mutant Vpr(52-96) μM
E
THP-1 cells: Mitochondrial fraction
Media
mVpr
Media
Vpr(52-96)
Vpr

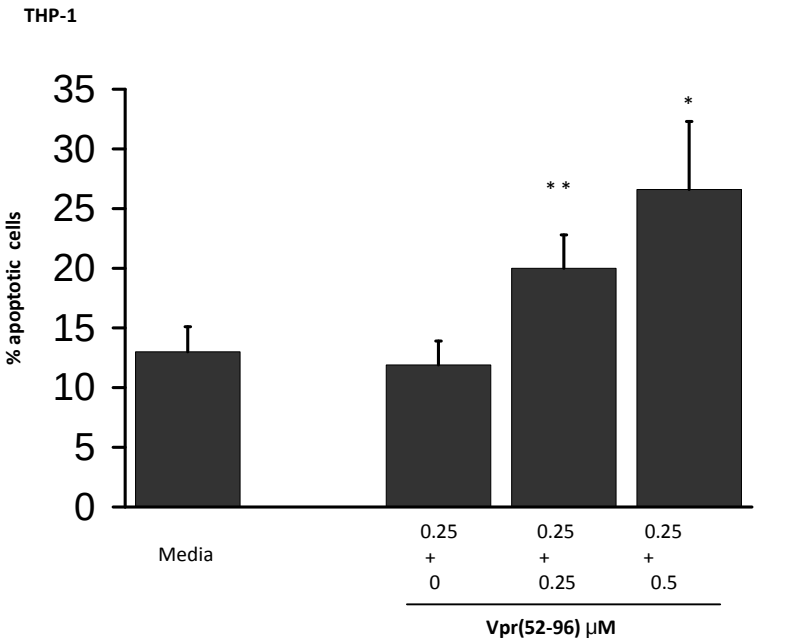
Figure 4.1

4.2.2 *Sequential exposure of undifferentiated monocytic cells to low non-apoptotic concentrations of Vpr causes apoptosis*

Despite the apoptogenic affect of Vpr at high concentrations, low concentrations of Vpr have been shown to protect CD4+ T cells against apoptotic agents like cyclohexamide, TNF-α and sorbitol (401, 402). To determine if low concentrations of Vpr protected monocytic cells against Vpr-mediated cytopathic effects, we sequentially treated monocytic cells with two low, non-apoptogenic concentrations of Vpr(52-96) at intervals of 24 hr in THP-1 cells and 2 hr in primary monocytes followed by measurement of apoptosis. It was observed that cells that received two treatments with low doses of Vpr(52-96) exhibited significantly higher apoptosis compared to cells receiving single dose of Vpr (Fig 4.2). These results suggest that low concentrations of Vpr(52-96) instead of conferring protection enhanced apoptosis upon subsequent exposures to a low non-apoptotic dose of Vpr(52-96). Additionaly both low and high doses of Vpr(52-96) induced comparable phosphorylation of all three MAPkinases, that is, JNK, p38 and ERK (Fig 4.3). Taken together this data indicates that low non-apoptotic concentrations of Vpr present in the serum of HIV-infected individuals (181) may be apoptotic for primary monocytes and thus may have biological relevance as an apoptosis-inducing agent under *in vivo* conditions.

Fig 4.2: Sequential exposure of undifferentiated monocytic cells to low non-apoptotic concentrations of Vpr causes apoptosis.

THP-1 cells and primary monocytes ($1.0x10^6$/ml) were treated with a first non-apoptotic dose of 0.25 μM Vpr(52-96) for 24 hr in THP-1 cells and for 2 hr in primary monocytes. Cells were treated with a second low dose of either 0.25 or 0.5 μM Vpr(52-96) for another 24 hr in THP-1 cells and for 2 hr in primary monocytes. Cells were then analyzed by Annexin-V staining and flow cytometry for the measurement of apoptosis. Results are expressed as a mean ± SD of three independent experiments. ∗($p < 0.05$), ** ($p < 0.005$) The p values were calculated against media/cell alone.


THP-1
% apoptotic cells
35
30
25
20
15
10
5
0
* *
*
Media
0.25 + 0
0.25 + 0.25
0.25 + 0.5
Vpr(52-96) μM

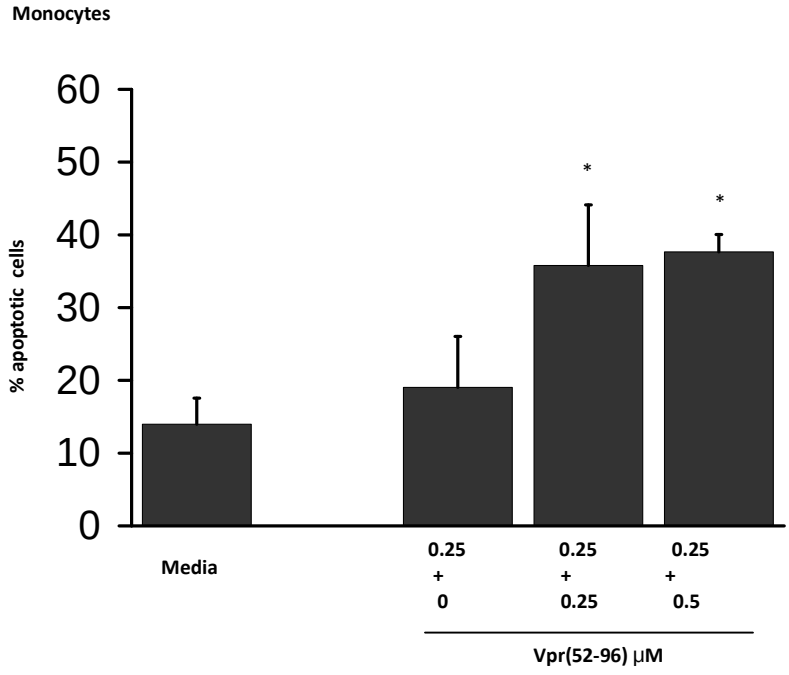

Monocytes
% apoptotic cells
60
50
40
30
20
10
0
*
*
Media
0.25 + 0
0.25 + 0.25
0.25 + 0.5
Vpr(52-96) μM

Figure 4.2

Fig 4.3: Exposure to both low and high doses of Vpr(52-96) induces comparable activation of MAPK signaling pathway.

THP-1 cells ($1.0x10^6$/ml) were treated with either low, non-apoptotic dose of 0.25 μM Vpr(52-96) or with high, apoptosis inducing dose of 1.5 μM Vpr(52-96) for 10-60 min. Total cellular proteins were subjected to immunoblotting and the membranes were sequentially probed with antibodies specific for phospho-JNK, phosphor-p38 and phopho-ERK. The membranes were probed with anti-total JNK, p38 and ERK antibodies to serve as a loading control.

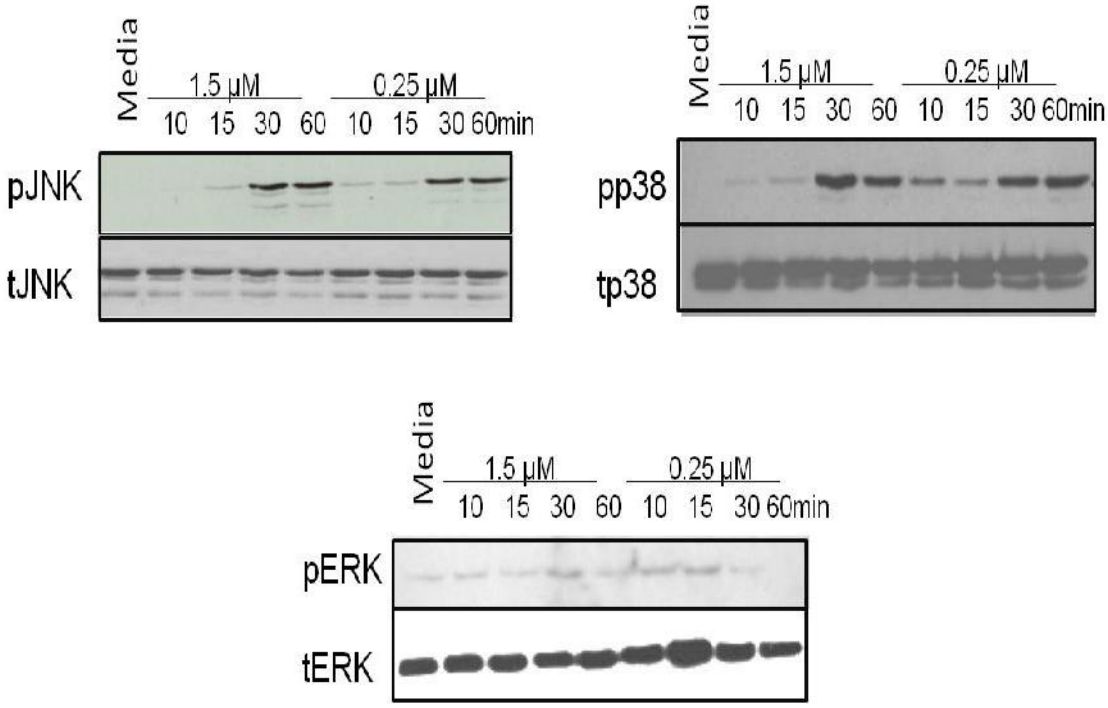

Media
1.5 μM
0.25 μM
10 15 30 60 10 15 30 60min
pJNK
tJNK
Media
1.5 μM
0.25 μM
10 15 30 60 10 15 30 60min
pp38
tp38
Media
1.5 μM
0.25 μM
10 15 30 60 10 15 30 60min
pERK
tERK

Figure 4.3

4.2.3 *TLR-9 ligand CpG, induces protection against Vpr(52-96)-mediated apoptosis in primary monocytes and THP-1 cells*

Recently, translocation of various microbial products, like LPS, from the gut have been implicated in causing systemic immune activation, TLR stimulation (11, 385) and upregulation of programmed death receptors-1 on monocytes (384) in HIV-infected individuals. Therefore, I hypothesized that stimulation with various TLR ligands may protect monocytic cells against Vpr(52-96)-induced apoptosis. To determine the effect of TLR-ligands on Vpr(52-96)-induced apoptosis, THP-1 cells and monocytes, were stimulated initially with LTA, Poly I:C, LPS, imiquimod and CpG-B ODN, the ligands for TLR-2, TLR-3, TLR-4, TLR-7 and TLR-9, respectively, followed by treatment with Vpr(52-96) peptide and analysis for apoptosis. Pretreatment with LTA decreased apoptosis by 60% in THP-1 cells (Fig 4.4A) and about 50% in monocytes (Fig 4.5A) whereas LPS pretreatment exhibited a modest protection (Fig 4.4A). In contrast, imiquimod and Poly I:C did not induce protection against Vpr(52-96)-induced apoptosis (Fig 4.4A). Interestingly, pretreatment with TLR-9 agonists, synthetic CpG-B ODN and *E. coli* DNA at concentrations recommended by the manufacturer reduced apoptosis by more than 80% and 50%, respectively, in THP-1 cells in a dose-dependent manner (Fig 4.4). CpG ODNs with small differences in the nucleotide sequences have been classified into separate classes loosely based on the cell specific response elicited by each ODN. Type A ODNs have been described to induce cytokines in pDCs whereas type B ODNs have been shown to activate both B cells and pDCs. Type C ODNs have been depicted to have some properties of both type A and B ODNs (344, 358, 359). Interestingly stimulation of THP-1 cells with different types of CpG ODNs, ie, type A, B and C, prior to Vpr(52-96) treatment yielded comparable

protection from apoptosis, indicating that protection afforded by CpG ODNs against Vpr(52-96)-mediated apoptosis in independent of the nucleotide sequence of CpG ODNs in human monocytic cells (Fig 4.4C). Where not mentioned the experiments were performed with type B CpG ODN, here on referred to as CpG. Similar results were obtained in primary monocytes following CpG and *E. coli* DNA stimulation (Fig 4.5B and C). Notably, CpG failed to induce protection if cells were treated with Vpr(52-96) prior to CpG stimulation (Fig 4.5D).

Fig. 4.4: TLR-2, TLR-4 and TLR-9 agonists induce resistance against Vpr(52-96)-mediated apoptosis in THP-1 cells.

A. THP-1 cells ($1.0x10^6$/ml) were stimulated with either 5 µg/ml LTA, 50 µg/ml Poly I:C, 1µg/ml LPS, 10 µg/ml imiquimod or 5 µM CpG B for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 24 hr. **B**. THP-1 cells ($1.0x10_6$/ml) were stimulated with increasing concentrations of CpG-B for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 24 hr. **C**. THP-1 cells ($1.0x10^6$/ml) were stimulated with 5 µM CpG A, B and C for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 24 hr. **D**. THP-1 cells ($1.0x10^6$/ml) were stimulated with increasing concentrations of *E. coli* DNA for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 24 hr. Cells were analyzed by Annexin-V staining for the measurement of apoptosis. Results in A and C are expressed as a mean ± SD of three independent experiments. Results in B and D show a representative experiment. The protective effect of TLR-ligands was calculated as % apoptosis relative to the Vpr-induced apoptosis following normalization against apoptosis in cells cultured in media alone. ∗($p<0.05$) ** ($p<0.005$). The p values were calculated against Vpr(52-96) treated cells.

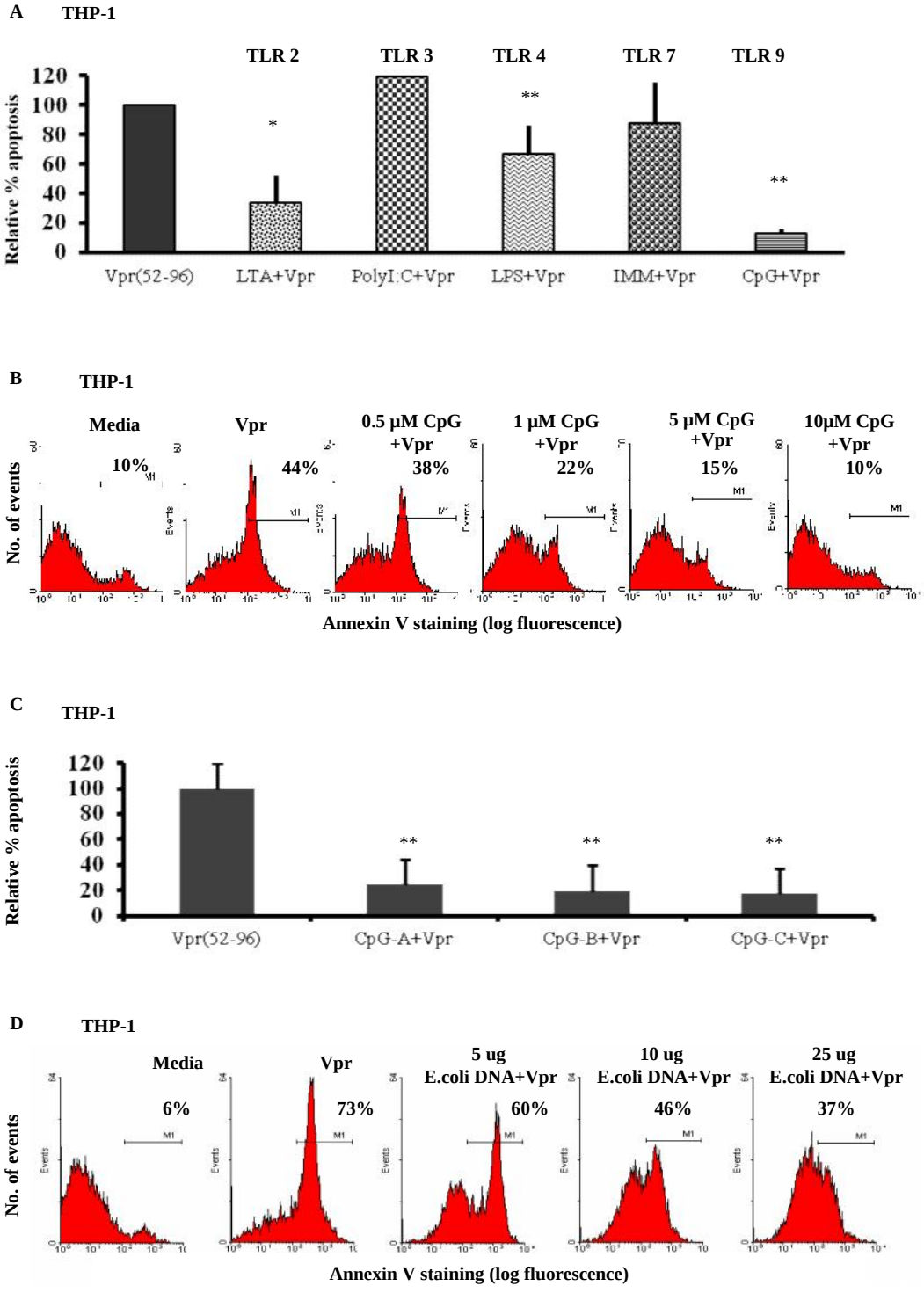

A
THP-1
TLR 2
TLR 3
TLR 4
TLR 7
TLR 9
Relative % apoptosis
120
100
80
60
40
20
0
*
**
**
Vpr(52-96)
LTA+Vpr
PolyI:C+Vpr
LPS+Vpr
IMM+Vpr
CpG+Vpr
B
THP-1
Media
10%
Vpr
44%
0.5 µM CpG +Vpr
38%
1 µM CpG +Vpr
22%
5 µM CpG +Vpr
15%
10µM CpG +Vpr
10%
No. of events
Annexin V staining (log fluorescence)
C
THP-1
Relative % apoptosis
120
100
80
60
40
20
0
**
**
**
Vpr(52-96)
CpG-A+Vpr
CpG-B+Vpr
CpG-C+Vpr
D
THP-1
Media
6%
Vpr
73%
5 ug E.coli DNA+Vpr
60%
10 ug E.coli DNA+Vpr
46%
25 ug E.coli DNA+Vpr
37%
No. of events
Annexin V staining (log fluorescence)

Figure 4.4

Fig. 4.5: TLR-9 agonists induce resistance against Vpr(52-96)-mediated apoptosis in primary human monocytes.

A. Primary monocytes ($1.0x10^6$/ml) were stimulated with 5 µg/ml LTA for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 4 hr. **B**. Primary monocytes ($1.0x10^6$/ml) were stimulated with 5 µM CpG for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 4 hr. **C**. Primary monocytes ($1.0x10^6$/ml) were stimulated with 25 µg *E. coli* DNA for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 4 hr. **D**. Primary monocytes ($1.0x10^6$/ml) were treated with Vpr(52-96) for 2 hr followed by stimulation with 5 µM CpG for 12 hr. Cells were analyzed by Annexin-V staining for the measurement of apoptosis. Results in A, C, D and right panel in B are expressed as a mean ± SD of three independent experiments. Results in left panel in B show a representative experiment. The protective effect of TLR-ligands was calculated as % apoptosis relative to the Vpr-induced apoptosis following normalization against apoptosis in cells cultured in media alone. $*p < 0.05$) ** ($p < 0.005$). The p values were calculated against Vpr(52-96) treated cells.

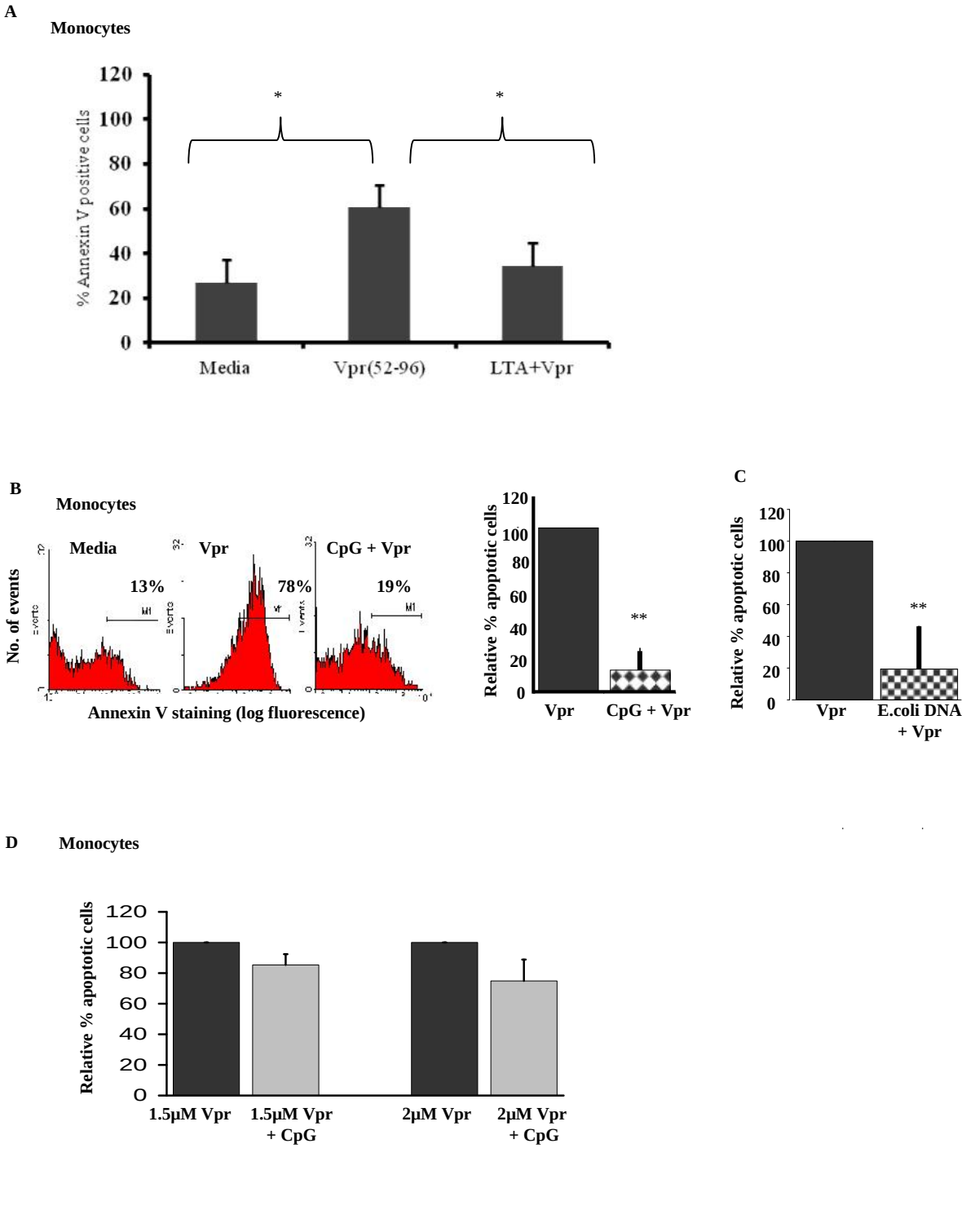

A
Monocytes
% Annexin V positive cells
120
100
80
60
40
20
0
*
*
Media
Vpr(52-96)
LTA+Vpr
B
Monocytes
Media
Vpr
CpG + Vpr
13%
78%
19%
No. of events
Annexin V staining (log fluorescence)
Relative % apoptotic cells
**
Vpr
CpG + Vpr
C
Relative % apoptotic cells
**
Vpr
E.coli DNA + Vpr
D
Monocytes
Relative % apoptotic cells
1.5μM Vpr
1.5μM Vpr + CpG
2μM Vpr
2μM Vpr + CpG

Figure 4.5

4.2.4 *CpG-induced protection against Vpr(52-96)-mediated apoptosis is regulated by anti-apoptotic c-IAP-2 gene*

Cell survival is regulated by distinct anti-apoptotic genes in different cell types (68). To identify anti-apoptotic gene(s) involved in CpG-induced protection against Vpr(52-96)-mediated apoptosis, I first analyzed the induction of pro-/anti-apoptotic genes, c-IAP-1, c-IAP-2, XIAP, Bcl-2, Mcl1 and Bax in CpG-stimulated THP-1 cells and primary monocytes. The results show that both CpG and *E. coli* DNA upregulated c-IAP-2 expression in THP-1 cells in a dose-dependent manner. Similar results were obtained in primary monocytes following CpG treatment as well. However, no significant change was observed in the expression of c-IAP-1, XIAP, Bcl-2, Mcl1 or Bax genes in either cell type following CpG treatment (Fig 4.6A). In accordance with the above results showing that pretreatment with Vpr(52-96) abrogated CpG-induced resistance against Vpr(52-96)-mediated apoptosis (Fig 4.5D), monocytes did not exhibit c-IAP-2 induction in response to CpG if they had been pretreated with Vpr(52-96) (Fig 4.6B). It is interesting to note that both TLR-4 and TLR-2 ligands, LPS and LTA respectively, also induced enhanced expression of c-IAP-2 (Fig 4.6C).

Since CpG selectively induced c-IAP-2 expression, I hypothesized that CpG-induced protection may be regulated by c-IAP-2. Therefore, THP-1 cells were transfected with siRNA against c-IAP-2 prior to stimulation with CpG. c-IAP-2 specific siRNA significantly inhibited CpG-induced c-IAP-2 expression compared to the cells transfected with non-silencing control siRNA (Fig 4.7A). Significantly, prior stimulation with CpG failed to inhibit Vpr(52-96)-induced apoptosis in cells transfected with c-IAP-2 siRNA compared to the cells transfected with control siRNA (Fig 4.7B). These results suggest that the protective

effect of CpG against Vpr(52-96)-mediated apoptosis may be regulated via c-IAP-2 induction in monocytic cells.

To confirm the role of CpG-induced c-IAP-2 in conferring protection against Vpr(52-96)-mediated apoptosis, I used SMAC mimetic (SMC) AEG-730. These are synthetically produced novel small molecules that mimic the activity of cellular SMAC and target IAPs for rapid degradation (106). THP-1 cells and primary monocytes were treated with SMC and CpG. Consistent with the results observed with c-IAP-2 siRNA (Fig 4.7), stimulation with CpG failed to inhibit Vpr(52-96)-induced apoptosis in THP-1 cells or primary monocytes treated with SMC (Fig 4.8A and B). Significantly, treatment with SMC also inhibited CpG-induced c-IAP-2 expression (Fig 4.8C). Please note that treatment with SMC alone did not cause apoptosis in either cell type (Fig 4.8D and E). These results combined with the siRNA data support a protective role for c-IAP-2 in CpG-induced resistance to apoptosis caused by Vpr(52-96) in human monocytic cells.

Fig. 4.6: **CpG selectively up regulates anti-apoptotic c-IAP-2 protein in THP-1 cells and primary monocytes.**

A. Monocytes ($2x10^6$/ml) and THP-1 cells ($1.0x10^6$/ml) were stimulated with increasing concentrations of CpG (0-10 µM) and *E. coli* DNA (0-25 µg) for 12 hr. **B**. Monocytes ($2x10^6$/ml) were treated with 1-2 µM Vpr(52-96) for 2 hr followed by stimulation with 5 µM CpG for 12 hr. **C**. THP-1 cells ($1.0x10^6$/ml) were stimulated with 1 µg/ml of LPS or with increasing concentrations of LTA (0-100 µg) for 12 hr. Total cellular proteins were subjected to immunoblotting and the membranes were sequentially probed with antibodies specific for c-IAP-1, c-IAP-2, Bcl-2, BcL XL, McL1, Bax and XIAP proteins. The membranes were probed with anti-GAPDH antibodies to serve as a loading control. The results shown are a representative of three independent experiments.

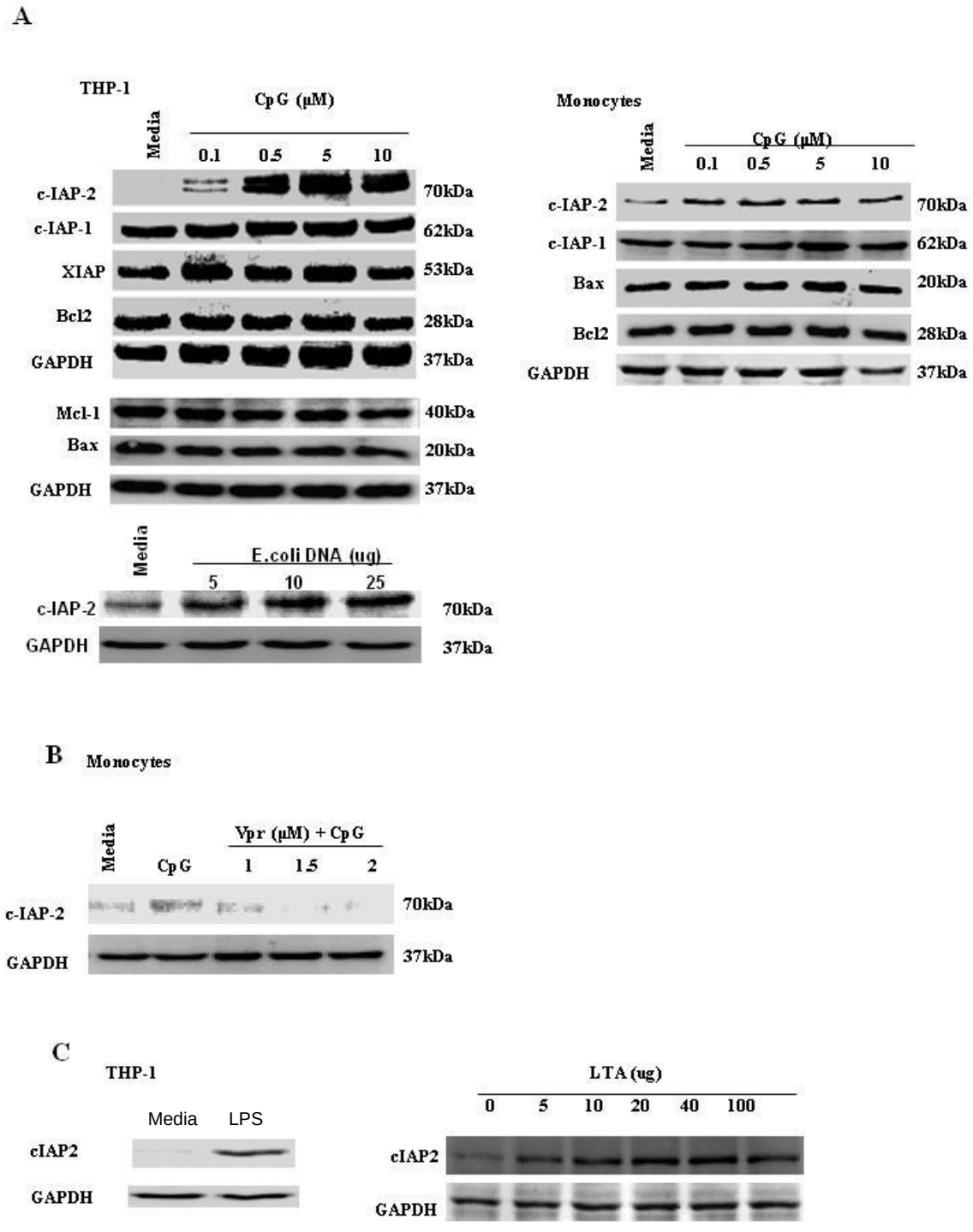

A
THP-1
CpG (μM)
Media
0.1
0.5
5
10
c-IAP-2
70kDa
c-IAP-1
62kDa
XIAP
53kDa
Bcl2
28kDa
GAPDH
37kDa
Mcl-1
40kDa
Bax
20kDa
GAPDH
37kDa
Media
E.coli DNA (ug)
5
10
25
c-IAP-2
70kDa
GAPDH
37kDa
Monocytes
Media
CpG (μM)
0.1
0.5
5
10
c-IAP-2
70kDa
c-IAP-1
62kDa
Bax
20kDa
Bcl2
28kDa
GAPDH
37kDa
B
Monocytes
Media
CpG
Vpr (μM) + CpG
1
1.5
2
c-IAP-2
70kDa
GAPDH
37kDa
C
THP-1
Media
LPS
cIAP2
GAPDH
LTA (ug)
0
5
10
20
40
100
cIAP2
GAPDH

Figure 4.6

Fig. 4.7: siRNA-mediated knockdown of c-IAP-2 abrogates CpG-induced protection from Vpr(52-96)-mediated apoptosis.

A. THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 1 µg of either c-IAP-2 or non-silencing control siRNA for 5 hr followed by stimulation with CpG (5 µM) for 48 hr. Subsequently total cell proteins were analyzed for c-IAP-2 expression by immunoblotting. **B.** Transfected cells were stimulated with CpG (5 µM) for 48 hr followed by treatment with 1.5 µM Vpr(52-96) for 24 hr and measurement of apoptotic cells by Annexin-V staining. Results in A and lower panel in B are representative of three independent experiments. The results in the top panel in B are expressed as a mean ± SD of three independent experiments. ∗($p < 0.05$) ** ($p < 0.005$).

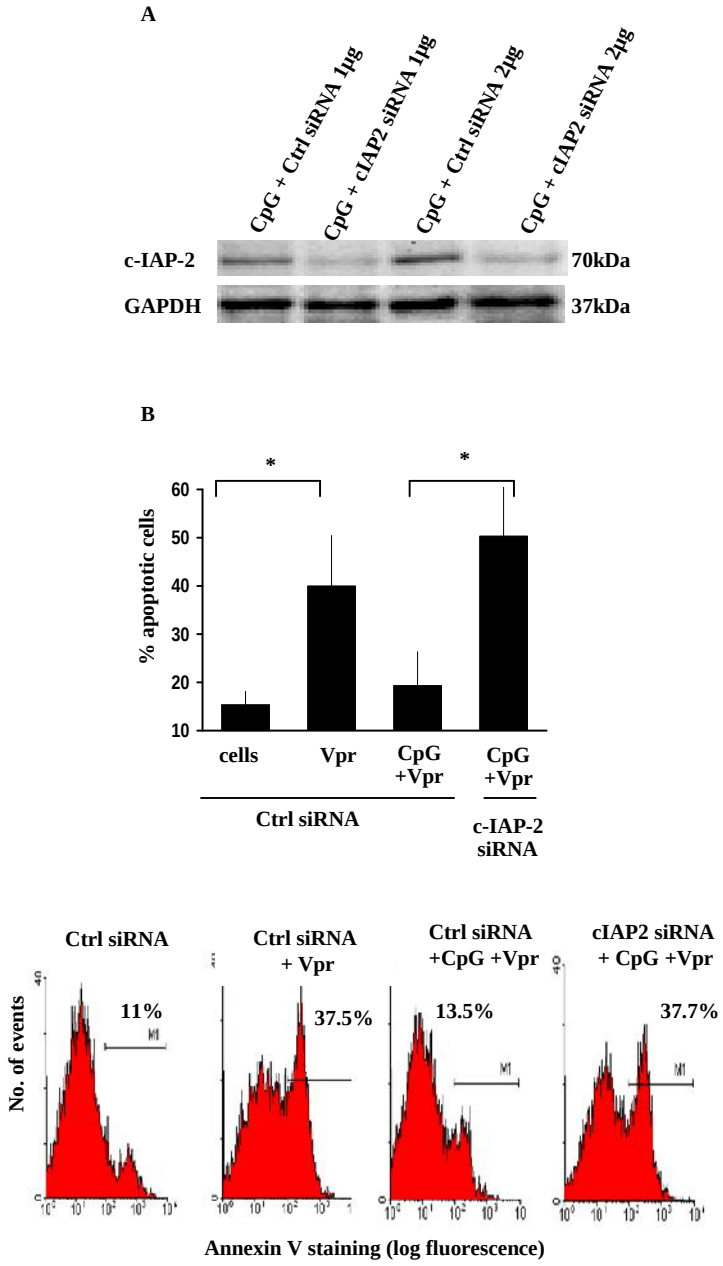
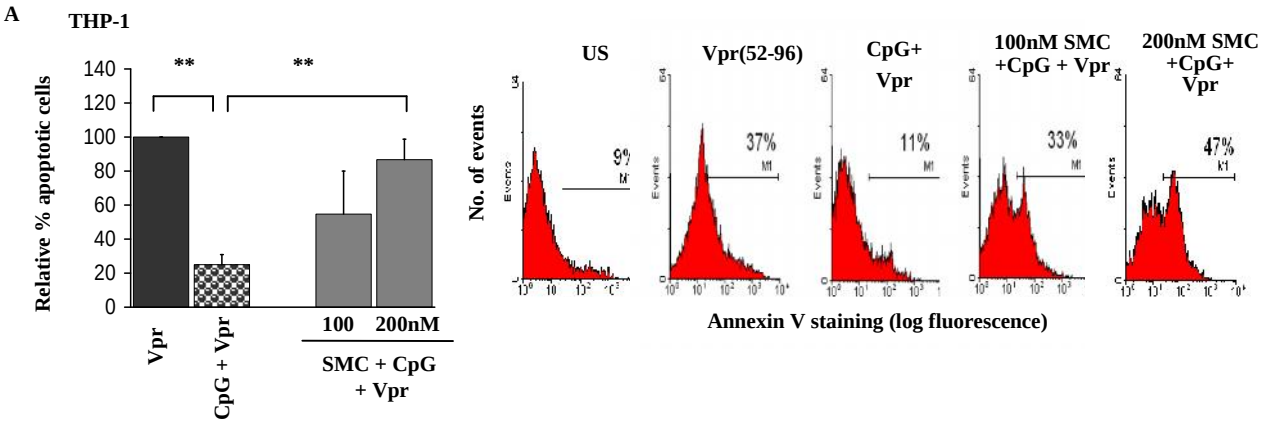
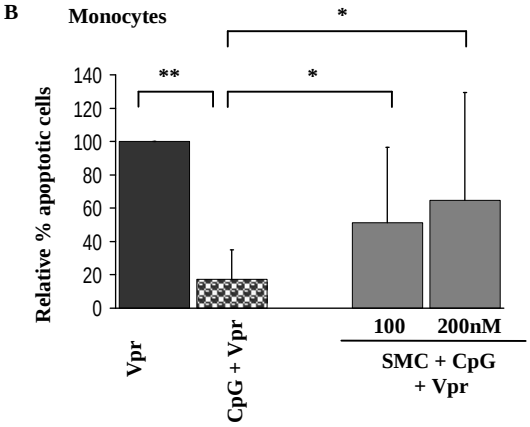

A
CpG + Ctrl siRNA 1μg
CpG + cIAP2 siRNA 1μg
CpG + Ctrl siRNA 2μg
CpG + cIAP2 siRNA 2μg
c-IAP-2
70kDa
GAPDH
37kDa
B
% apoptotic cells
60
50
40
30
20
10
*
*
cells
Vpr
CpG +Vpr
CpG +Vpr
Ctrl siRNA
c-IAP-2 siRNA
Ctrl siRNA
Ctrl siRNA + Vpr
Ctrl siRNA +CpG +Vpr
cIAP2 siRNA + CpG +Vpr
No. of events
11%
37.5%
13.5%
37.7%
M1
Annexin V staining (log fluorescence)

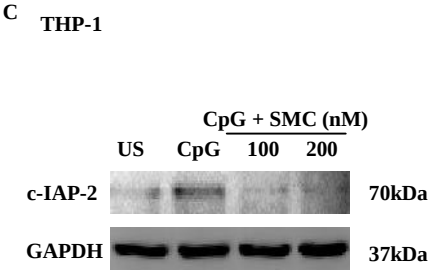
Figure 4.7

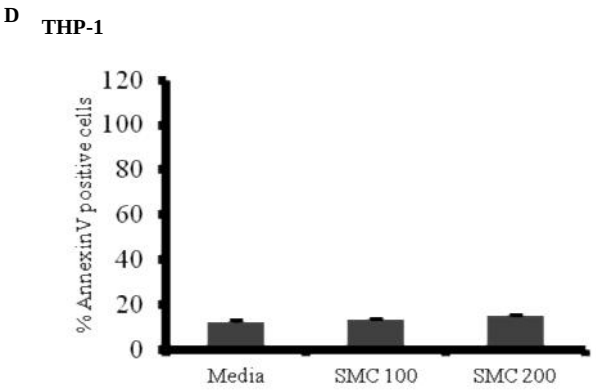
Fig. 4.8: SMC-mediated knockdown on c-IAP-2 abrogates CpG-induced protection from Vpr(52-96)-mediated apoptosis in THP-1 cells and primary monocytes.

THP-1 cells **(A)** and primary monocytes **(B)** ($1.0x10^6$/ml) were stimulated with 100 and 200 nM AEG-730 SMC and 5 µM CpG for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 24 hr in THP-1 cells and for 4 hr in monocytes before measurement of apoptosis by Annexin-V staining. **C**. THP-1 cells ($1.0x10^6$/ml) were treated with 100 and 200 nM AEG-730 SMC and 5 µM CpG for 12 hr. Cell lysates were analyzed for c-IAP-2 expression by immunoblotting. THP-1 cells **(D)** and primary monocytes **(E)** ($1.0x10^6$/ml) were stimulated with 100 and 200 nM AEG-730 SMC for 40 hr followed by measurement of apoptosis by Annexin-V staining. Results in C and the right panel in A are representative of three independent experiments. The results in B, D, E and the left panel in A, are expressed as a mean ± SD of three independent experiments. The protective effect of CpG was calculated as % apoptosis relative to the Vpr-induced apoptosis after normalization against apoptosis in cells alone. ∗($p < 0.05$) ** ($p < 0.005$). US: unstimulated


A
THP-1
Relative % apoptotic cells
140
120
100
80
60
40
20
0
**
**
Vpr
CpG + Vpr
100
200nM
SMC + CpG
+ Vpr
No. of events
US
Vpr(52-96)
CpG+
Vpr
100nM SMC
+CpG + Vpr
200nM SMC
+CpG+
Vpr
9%
37%
11%
33%
47%
Annexin V staining (log fluorescence)


B
Monocytes
Relative % apoptotic cells
140
120
100
80
60
40
20
0
**
*
*
Vpr
CpG + Vpr
100
200nM
SMC + CpG
+ Vpr


C
THP-1
CpG + SMC (nM)
US
CpG
100
200
c-IAP-2
70kDa
GAPDH
37kDa


D
THP-1
% AnnexinV positive cells
120
100
80
60
40
20
0
Media
SMC 100
SMC 200

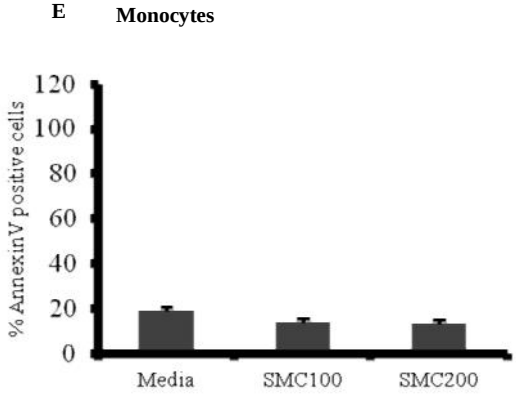

E
Monocytes
% AnnexinV positive cells
120
100
80
60
40
20
0
Media
SMC100
SMC200

Figure 4.8

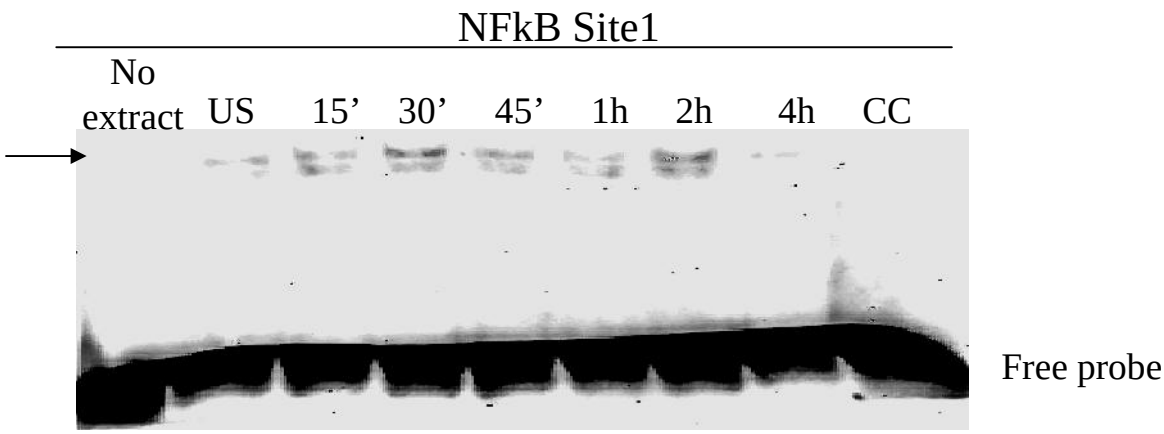
4.2.5 *Induction of c-IAP-2 by CpG is regulated by NFκB transcription factor in THP-1 cells*

Induction of c-IAP-2 by various stimuli has previously been shown to be regulated by NFκB, cAMP response element or glucoccorticoid response element binding sites in the c-IAP-2 promoter region in T cells, lung cancer cells and colon cancer cells, respectively (391, 403-405). LPS has specifically been reported to induce c-IAP-2 by inducing translocation of cytosolic NFκB into the nucleus and binding with NFκB site 1 on c-IAP-2 promoter region where as TNF-α-mediated c-IAP-2 induction has been shown to require both NFκB binding sites 1 and 3 (391). In order to determine whether transcriptional regulation of CpG-induced c-IAP-2 was also mediated via NFκB, nuclear extracts harvested from THP-1 cells treated with CpG were analyzed by EMSA for binding of NF-κB to NF-κB oligonucleotide probes corresponding to NFκB binding site 1 and site 3 in the c-IAP-2 promoter. Nuclear extract from cells treated with LPS was used as a positive control. The results showed significant shift in CpG and LPS treated samples as compared to cells cultured in media alone indicating involvement of NFκB binding site 1 and NFκB in CpG and LPS-mediated c-IAP-2 induction in human monocytic cells (Fig 4.9A and B). Interestingly, similar to LPS and unlike TNF-α, CpG-mediated regulation of c-IAP-2 was found to be independent of NFκB binding site 3. The specificity of NF-κB binding was demonstrated by competition with unlabeled oligonucleotides (Fig 4.9A and B).

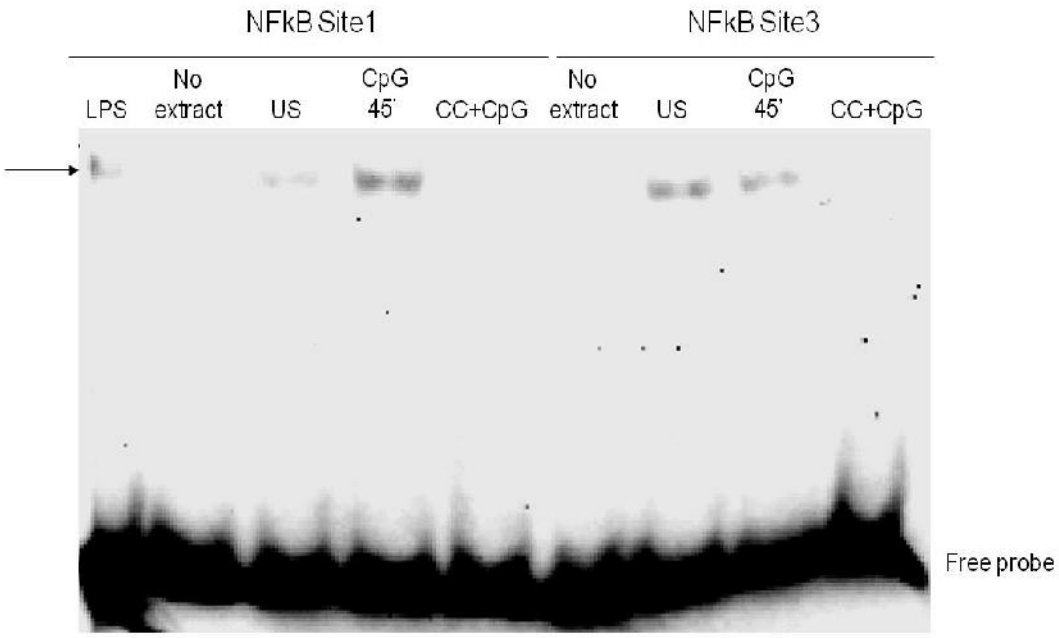
Fig. 4.9: NF-κB transcription factor binds to the NF-κB binding site 1 on the c-IAP2 promoter in CpG-stimulated cells

A. THP-1 cells ($2.0x10^6$/ml) were treated with CpG (5 µM) for 15 min to 4 hr. **B**. THP-1 cells ($2.0x10^6$/ml) were treated with LPS (1 µg/ml) or CpG (5 µM) for 30 min and 45 min respectively. Cell pellets were harvested and nuclear protein extracts were subjected to EMSA as described in *Materials and Methods* to measure DNA binding activity of NF-κB. Cold competitor (*CC*) lanes contained 50× excess of unlabeled probes compared with biotin-labeled probes. US: unstimulated

A **THP-1**



B **THP-1**



CC= cold competition

Figure 4. 9

4.2.6 *CaM/CaMK-II activation regulates CpG-induced c-IAP-2 expression and protection against Vpr(52-96)-mediated apoptosis in primary monocytes and THP-1 cells*

CaM/CaMK-II pathway has previously been demonstrated to regulate response to TLR-ligands in monocytic cells (406, 407). Therefore, I hypothesized that in addition to the JNK pathway, CpG-induced protection against Vpr-mediated apoptosis may also be regulated by the calcium signaling pathway in monocytic cells. Consistent with my hypothesis, CpG treatment was found to cause rapid calcium influx in THP-1 cells that was inhibited upon addition of calcium chelator EGTA in THP-1 cells (Fig 4.10A). The biological activity of various pharmacological inhibitors of calcium signaling pathway namely, SKF, W-7, EGTA and KN-93 was confirmed by their ability to inhibit CpG-induced CaMK-II phosphorylation in a dose-dependent manner in THP-1 cells (Fig 4.10B). Notably, more than 24 hr treatment of THP-1 cells and primary monocytes with the highest concentrations of all the inhibitors used in this study did not cause significant apoptosis (Fig 4.10C). Elevated cytoplasmic calcium concentrations occur in response to stimuli that activate voltage or ligand-gated calcium channels in the plasma membrane or following the release of calcium mainly from the endoplasmic reticulum (ER) (408). Interestingly, treatment with calcium chelator, EGTA, prior to stimulation with CpG reversed the CpG-mediated protection against Vpr(52-96)-induced apoptosis in both THP-1 cells and primary monocytes (Fig 4.11A). The role of receptor-mediated entry of extracellular Ca^{2+} was studied by using SKF-96365 (388). To determine whether calcium release from ER regulates CpG-induced resistance to Vpr-mediated apoptosis, I used 2-APB, which inhibits the release of calcium from ER by blocking IP3 receptor-gated channels (325). Unlike 2-APB, SKF-96365 treatment prior to stimulation with CpG reversed the CpG-mediated

protection against Vpr(52-96)-induced apoptosis in both cell types (Fig 4.11A). Calmodulin, 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. One major family of calcium/CaM effectors is CaMK which includes multifunctional kinases, CaMK-II and calcineurin (321, 409). The role of CaM, CaMK-II and calcineurin was determined by using their inhibitors W-7, KN-93 and FK-506, respectively. Unlike FK506, pretreatment with both W-7 and KN93 significantly reversed the CpG-mediated protection against Vpr(52-96)-induced apoptosis in both cell types (Fig 4.11A). These inhibitor studies suggest that protection induced by CpG is mediated via influx of extracellular calcium and CaM/CaMK-II activation whereas ER calcium stores and calcineurin do not play a significant role in CpG-induced protection from apoptosis caused by Vpr(52-96) treatment. In addition, pretreatment with EGTA, SKF, KN-93 and W-7 also prevented CpG-induced expression of c-IAP-2 in THP-1 cells and monocytes (Fig 4.11B) suggesting that c-IAP-2 induced by CpG was instrumental in protecting cells from apoptosis caused by Vpr(52-96).

The role of CaMK-II was confirmed by transfecting THP-1 cells with CaMK-II specific siRNA as well as a DN-CaMK-II construct followed by stimulation with CpG and subsequent determination of c-IAP-2 expression and Vpr(52-96)-induced apoptosis. Transfection with both CaMK-II siRNA and DN-CaMK-II construct significantly inhibited both, the c-IAP-2 expression induced by CpG (Fig 4.12A and C) and the protective effects of CpG (Fig 4.12B and D) compared to the cells transfected with control siRNA or control vector. These results suggest that CpG-induced protection against Vpr(52-96)-mediated

apoptosis is regulated, at least in part, by c-IAP-2 expression through the activation of JNK and CaMK-II in human monocytic cells.

Given the similarity in transcriptional regulation of c-IAP-2 by LPS and CpG (Fig 4.6C left panel and 4.9B) and the significance of calcium signaling for CpG-mediated c-IAP-2 induction (Fig 4.10-4.12), I wanted to explore whether the CaM/CaMK-II-mediated signaling was involved in LPS-mediated c-IAP-2 regulation. To this end SKF-96365 and EGTA were used to prevent influx of calcium ions through the voltage gated calcium channels on the cell surface (388). Furthermore inhibitors of CaM and CaMK-II, W-7 and KN-93, respectively, were used to determine the role of these modulators in LPS-mediated c-IAP-2 induction (321, 409). Similar to CpG, prior treatment with EGTA, SKF-96365, W-7 and KN-93 inhibited c-IAP-2 expression in LPS treated cells (Fig 4.13).

Fig. 4.10: CpG treatment induces calcium influx and CaMK-II phosphorylation in THP-1 cells.

A. THP-1 cells ($1.0X10^6$/ml) were loaded with Fluo3/AM. Cells were then stimulated with 5 µM CpG or 5mM of ionophore A23187 in the presence and the absence of EGTA. The resulting Ca^{2+} influx was measured by flow cytometry. **B.** THP-1 cells ($1.0x10^6$/ml) were treated with various concentrations of EGTA, W-7, SKF and KN-93 for 2 hr before stimulation with 5 µM CpG for 15 min. Cell lysates were analyzed for CaMK-II phosphorylation by immunoblotting. Protein loading was normalised by reprobing the membranes with total anti-CaMK-II antibodies. **C**. 20 mM EGTA, 25 µM W-7, 50 µM SKF and 50 µM KN-93 were added to THP-1 cells ($1.0x10^6$/ml) for 40 hr followed by analysis of apoptosis by Annexin-V staining. US: unstimulated

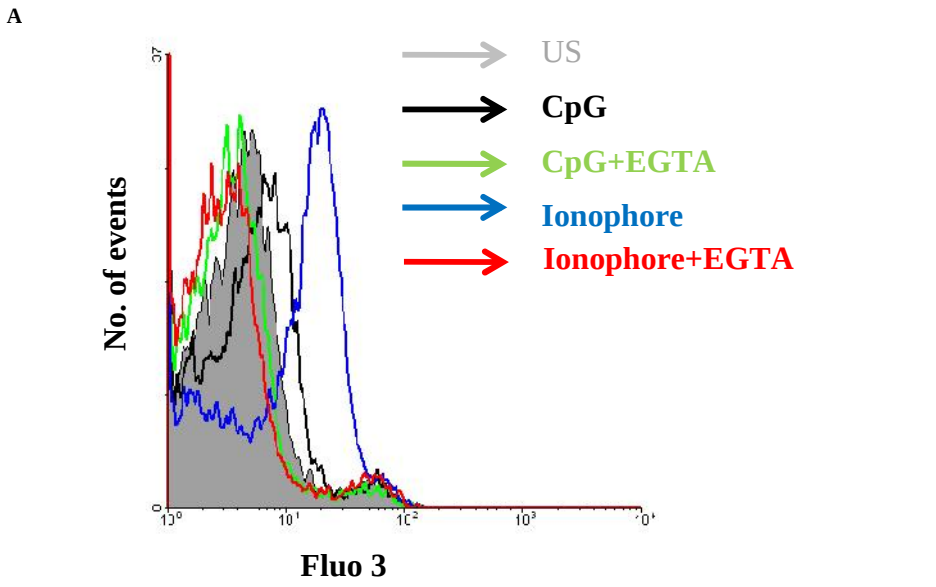

A
US
CpG
CpG+EGTA
Ionophore
Ionophore+EGTA
No. of events
Fluo 3
B
Media
CpG
CpG+EGTA (mM)
5 10 20
p-CaMKII
t-CaMKII
50kda
50kda
CpG+KN93 (μM)
10 20 50
CpG+SKF (μM)
10 20 50
CpG+W7 (μM)
20 30
C
% AnnexinV positive cells
120
100
80
60
40
20
0
Media
EGTA
SKF
KN-93
W-7

Figure 4.10

Fig. 4.11: CpG-mediated resistance to Vpr(52-96)-induced apoptosis is regulated through the activation of CaM/CAMK-II.

A.THP-1 cells and primary monocytes ($1.0x10^6$/ml) were treated with various concentrations of EGTA, W-7, SKF, KN-93, APB and FK-506, for 2 hr prior to stimulation with 5 µM CpG for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) for 24 hr in THP-1 cells and 4 hr in monocytes, followed by analysis of apoptosis by Annexin-V staining. Results are expressed as a mean ± SD of three independent experiments. **B**. THP-1 cells ($1.0x10^6$/ml) and primary monocytes ($2.0x10^6$/ml) were treated with increasing concentrations of EGTA, W-7, SKF and KN-93 for 2 hr followed by stimulation with 5 µM CpG for 12 hr. Cell lysates were analyzed for c-IAP-2 expression by immunoblotting. The results shown in B are representative of three independent experiments. The protective effect of CpG was calculated as % apoptosis relative to the Vpr-induced apoptosis after normalization against apoptosis in cells alone. $p < 0.05$, ** $p < 0.005$. The p values calculated for EGTA, SKF, KN93 and W7 were against cells treated with CpG and Vpr.

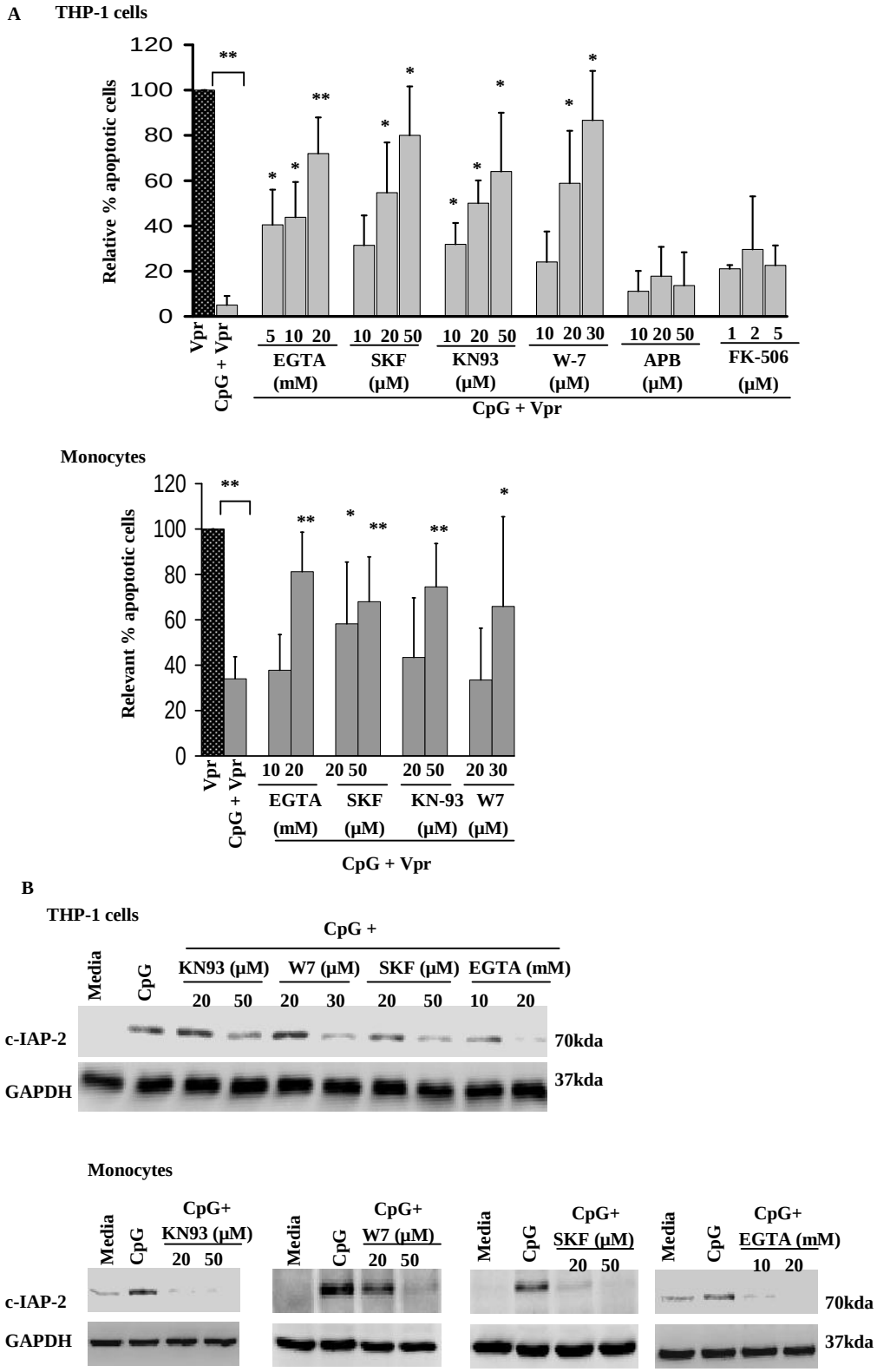
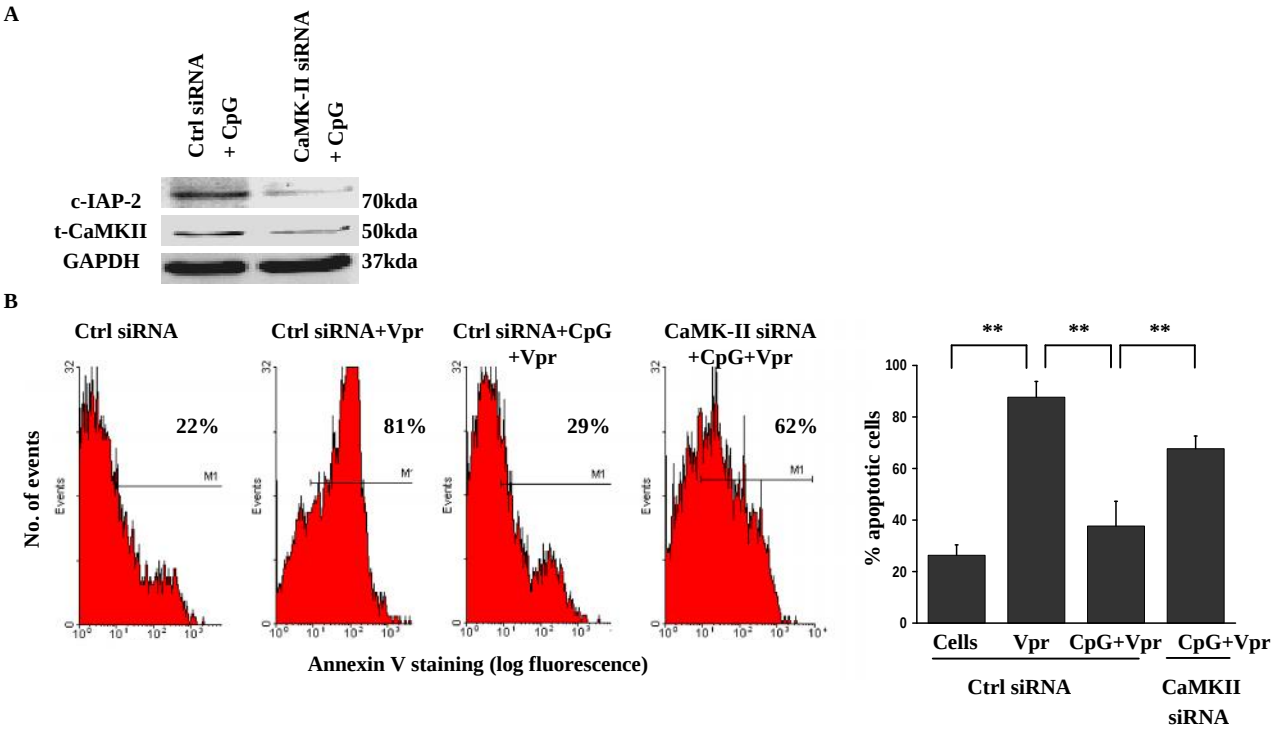

A
THP-1 cells
Relative % apoptotic cells
120
100
80
60
40
20
0
Vpr
CpG + Vpr
5 10 20
EGTA
(mM)
10 20 50
SKF
(μM)
10 20 50
KN93
(μM)
10 20 30
W-7
(μM)
10 20 50
APB
(μM)
1 2 5
FK-506
(μM)
CpG + Vpr
Monocytes
Relevant % apoptotic cells
120
100
80
60
40
20
0
Vpr
CpG + Vpr
10 20
EGTA
(mM)
20 50
SKF
(μM)
20 50
KN-93
(μM)
20 30
W7
(μM)
CpG + Vpr
B
THP-1 cells
CpG +
Media
CpG
KN93 (μM)
20 50
W7 (μM)
20 30
SKF (μM)
20 50
EGTA (mM)
10 20
c-IAP-2
70kda
GAPDH
37kda
Monocytes
Media
CpG
CpG+
KN93 (μM)
20 50
Media
CpG
CpG+
W7 (μM)
20 50
Media
CpG
CpG+
SKF (μM)
20 50
Media
CpG
CpG+
EGTA (mM)
10 20
c-IAP-2
70kda
GAPDH
37kda

Figure 4.11

Fig. 4.12: CaMK-II siRNA and DN-CaMK-II vector reverse CpG-mediated resistance to Vpr(52-96)-induced apoptosis.

A. THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 1 μg of either CaMK-II siRNA or non-silencing siRNA for 24 hr, followed by stimulation with 5 μM CpG for 12 hr. Cell lysates were analyzed for c-IAP-2 and CaMK-II expression by immunoblotting. **B**. THP-1 cells transfected with CaMK-II or non-silencing siRNA as above, were stimulated with 5 μM CpG for 12 hr prior to treatment with 1.5 μM of Vpr(52-96) for 24 hr followed by measurement of apoptotic cells by Annexin-V staining. Results are expressed as a mean ± SD of three independent experiments. The results shown in A and left panel in B are representative of three independent experiments. **C**. THP-1 cells ($1x10^6$/ml) were transfected with DN-CaMK-II or control vector for 24 hr, followed by stimulation with 5 μM CpG for 12 hr. Cell lysates were analyzed for c-IAP-2 expression by immunoblotting. **D**. THP-1 cells transfected with DN-CaMK-II or control vector as above, were stimulated with 5 μM CpG for 12 hr prior to treatment with 1.5 μM of Vpr(52-96) for 24 hr followed by measurement of apoptotic cells by Annexin-V staining. The results shown are representative of two independent experiments. *$p < 0.05$, ** $p < 0.005$.


A
Ctrl siRNA + CpG
CaMK-II siRNA + CpG
c-IAP-2
t-CaMKII
GAPDH
70kda
50kda
37kda
B
Ctrl siRNA
Ctrl siRNA+Vpr
Ctrl siRNA+CpG +Vpr
CaMK-II siRNA +CpG+Vpr
22%
81%
29%
62%
M1
No. of events
Events
Annexin V staining (log fluorescence)
% apoptotic cells
**
Cells
Vpr
CpG+Vpr
CpG+Vpr
Ctrl siRNA
CaMKII siRNA

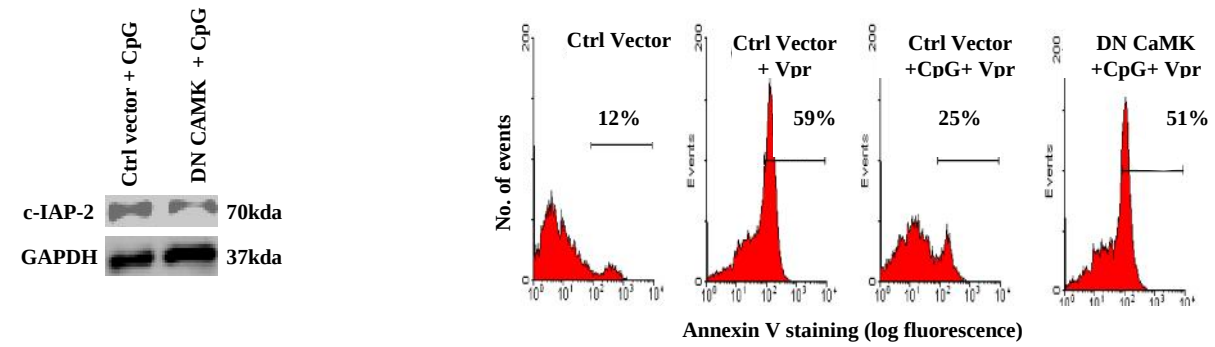

C
Ctrl vector + CpG
DN CAMK + CpG
c-IAP-2
GAPDH
70kda
37kda
D
Ctrl Vector
Ctrl Vector + Vpr
Ctrl Vector +CpG+ Vpr
DN CaMK +CpG+ Vpr
12%
59%
25%
51%
No. of events
Events
Annexin V staining (log fluorescence)

Figure 4.12

Fig. 4.13: Pre-treatment with CaM/CaMK-II inhibitors abrogates LPS-mediated c-IAP-2 expression in THP-1 cells.

THP-1 cells ($1.0x10^6$/ml) were treated with increasing concentrations of EGTA, W-7, SKF and KN-93 for 2 hr followed by stimulation with 1 µg/ml LPS for 12 hr. Cell lysates were analyzed for c-IAP-2 expression by immunoblotting. The results shown are representative of three independent experiments.

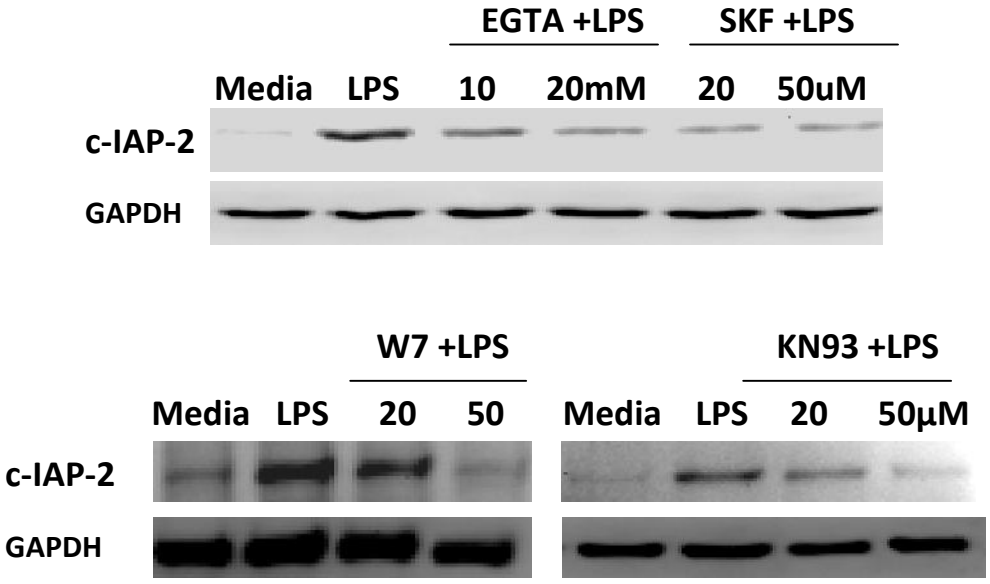

EGTA +LPS
SKF +LPS
Media
LPS
10
20mM
20
50uM
c-IAP-2
GAPDH
W7 +LPS
KN93 +LPS
Media
LPS
20
50
Media
LPS
20
50µM
c-IAP-2
GAPDH

Figure 4.13

4.2.7 *JNK activation regulates CpG-induced c-IAP-2 expression and protection from Vpr(52-96)-mediated apoptosis in primary monocytes and THP-1 cells*

The c-IAP-2 gene has been shown to be regulated by the extracellular signal-regulated kinase (ERK) and p38 MAPKs in human colon epithelial cells and by phosphoionositide-3 kinase (PI3K) pathway in human breast cancer MCF-7 cells (403, 410). To determine the role of MAPK and PI3K pathways in CpG-induced protection against Vpr(52-96)-induced apoptosis, specific pharmacological inhibitors of these pathways were used. First, the biological activity of SB203580, SP600125, PD98059 and Ly294002, the specific inhibitors for p38, JNK, ERK and Akt, respectively, were confirmed for their ability to inhibit phosphorylation of corresponding kinases in response to CpG stimulation (Fig 4.14A). Thereafter, THP-1 cells were treated with SP600125, PD98059, SB203580 and Ly294002 before stimulation with CpG followed by treatment with Vpr(52-96) and analysis for apoptosis. Interestingly, pretreatment with JNK inhibitor, SP600125 significantly prevented protection afforded by CpG in THP-1 cells. In contrast, inhibitors for p38, ERK or PI3K pathways did not affect CpG-mediated protection in these cells (Fig 4.14B). Similar results were obtained in primary monocytes with respect to JNK inhibition (Fig 4.14C). Notably more than 24 hr treatment with highest dose of SP600125 did not induce apoptosis in the cells (Fig 4.14D). In accordance with the protection results, pretreatment of THP-1 cells and primary monocytes with SP600125 prevented CpG-induced c-IAP-2 expression whereas inhibiting p38, ERK or Akt phosphorylation did not affect c-IAP-2 induction (Fig 4.14E).

The involvement of JNK in CpG-induced protection was further confirmed by transfecting THP-1 cells with either control non-silencing siRNA or JNK siRNAs for 48 hr

and stimulating with CpG for 12 hr followed by Vpr(52-96) treatment. Consistent with the JNK inhibitor studies (Fig 4.14), cells transfected with JNK siRNAs displayed a marked lack of protection against Vpr(52-96)-induced apoptosis compared to control siRNA transfected cells (Fig 4.15A). Moreover, cells transfected with JNK siRNA failed to upregulate CpG-induced c-IAP-2 expression compared to control siRNA transfected cells (Fig 4.15B).

Fig. 4.14: JNK MAPK regulates CpG-induced expression of c-IAP-2 and protection from Vpr(52-96)-mediated apoptosis.

A. THP-1 cells ($1.0x10^6$/ml) were treated with various concentrations of SP600125 (SP), SB203580 (SB), PD98059 (PD) and Ly294002 (Ly), for 2 hr before stimulation with 5 μM CpG for 15 min. Cell lysates were analyzed for JNK, p38, Akt or ERK phosphorylation by immunoblotting. Protein loading was normalised by reprobing the membranes for total JNK, p38, Akt and ERK antibodies, respectively. THP-1 cells (**B**) and primary monocytes (**C**) ($1.0x10^6$/ml) were treated with various concentrations of SP600125, SB203580, PD98059 and Ly294002 for 2 hr prior to stimulation with 5 μM CpG for 12 hr. Subsequently cells were treated with 1.5 μM Vpr(52-96) for 24 hr in THP-1 cells and 4 hr in monocytes, followed by analysis of apoptosis by Annexin-V staining. **D**. THP-1 cells ($1.0x10^6$/ml) were treated 50 μM SP for 40 hr followed by analysis of apoptosis by Annexin-V staining. **E**. THP-1 cells ($1.0x10^6$/ml) and primary monocytes ($2x10^6$/ml) were treated with increasing concentrations of SP600125, SB203580, PD98059 and Ly294002, for 2 hr followed by stimulation with 5 μM CpG for 12 hr. Cell lysates were analyzed for c-IAP-2 expression by immunoblotting. The results shown in A and E are representative of three independent experiments. Results in B and C are expressed as a mean ± SD of three independent experiments The protective effect of CpG was calculated as % apoptosis relative to the Vpr-induced apoptosis after normalization against apoptosis in cells alone. *p <0.05, **p <0.005. The p values calculated for SP600125 were against cells treated with CpG and Vpr.

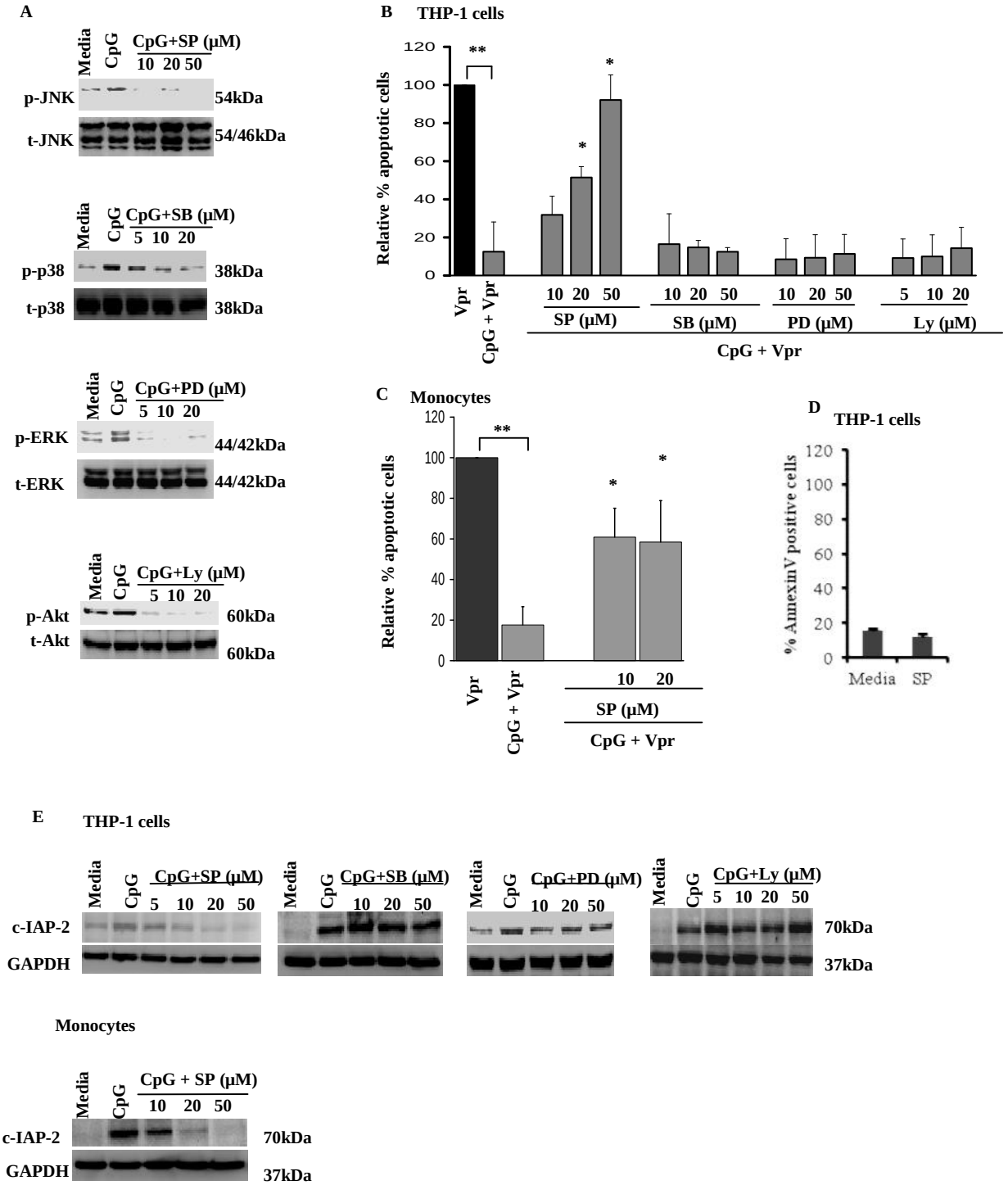
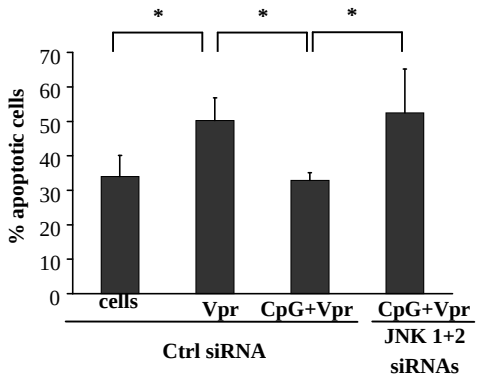

A
Media
CpG
CpG+SP (μM)
10 20 50
p-JNK
54kDa
t-JNK
54/46kDa
Media
CpG
CpG+SB (μM)
5 10 20
p-p38
38kDa
t-p38
38kDa
Media
CpG
CpG+PD (μM)
5 10 20
p-ERK
44/42kDa
t-ERK
44/42kDa
Media
CpG
CpG+Ly (μM)
5 10 20
p-Akt
60kDa
t-Akt
60kDa
B
THP-1 cells
Relative % apoptotic cells
120
100
80
60
40
20
0
**
*
*
Vpr
CpG + Vpr
10 20 50
SP (μM)
10 20 50
SB (μM)
10 20 50
PD (μM)
5 10 20
Ly (μM)
CpG + Vpr
C
Monocytes
Relative % apoptotic cells
120
100
80
60
40
20
0
**
*
*
Vpr
CpG + Vpr
10 20
SP (μM)
CpG + Vpr
D
THP-1 cells
% AnnexinV positive cells
120
100
80
60
40
20
0
Media
SP
E
THP-1 cells
Media
CpG
CpG+SP (μM)
5 10 20 50
Media
CpG
CpG+SB (μM)
10 20 50
Media
CpG
CpG+PD (μM)
10 20 50
Media
CpG
CpG+Ly (μM)
5 10 20 50
c-IAP-2
GAPDH
70kDa
37kDa
Monocytes
Media
CpG
CpG + SP (μM)
10 20 50
c-IAP-2
GAPDH
70kDa
37kDa

Figure 4.14

Fig 4.15: JNK specific siRNAs reverse the protective effect of CpG against Vpr(52-96)-induced apoptosis.

A. THP-1 cells ($0.25x10^6$/0.5ml)) were transfected with 0.4 µg JNK-1 and JNK-2 specific siRNAs or non-silencing control siRNA for 48 hr and then stimulated with CpG (5 µM) for 12 hr. Cells were then treated with 1.5 µM Vpr(52-96) for 24 hr followed by measurement of apoptosis by Annexin-V staining. The results are expressed as a mean ± SD of three independent experiments. *$p < 0.05$, ** $p < 0.005$. **B.** Cells transfected with siRNAs were stimulated with CpG as above. Cell lysates were analyzed for c-IAP-2 and JNK expression by immunoblotting. The results shown are a representative of three independent experiments.

A



B

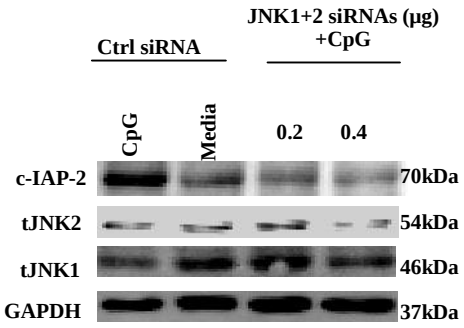


Figure 4.15

4.2.8 *Calcium signaling pathways regulate JNK activation in response to CpG*

The above results suggest that CpG-induced c-IAP-2 expression was regulated by both, activation of JNK and calcium pathways. Therefore, it was interesting to determine if these two pathways cross talked and regulated each other. For this, I inhibited either calcium signaling using EGTA or JNK activation by SP600125 followed by stimulation with CpG. Blocking calcium signaling inhibited not only phosphorylation of CaMK-II but also prevented JNK activation (Fig 4.16A). In contrast, blocking JNK activation did not prevent CaMK-II activation (Fig 4.16B) suggesting that JNK activation takes place downstream of CaMK-II phosphorylation in response to CpG stimulation.

Fig. 4.16: CpG-induced JNK activation is regulated by the calcium signaling pathway in THP-1 cells.

THP-1 cells ($1.0x10^6$/ml) were treated with various concentrations of either calcium pathway inhibitor, EGTA **(A)** or JNK inhibitor SP600125 (SP) **(B)** for 2 hr followed by CpG stimulation for 15 min. Cell lysates were analyzed for p-CaMK-II and p-JNK expression by immunoblotting. The membranes were stripped and reprobed with either anti-CaMK-II or anti-JNK antibodies to control for protein loading. The results shown are representative of three independent experiments.

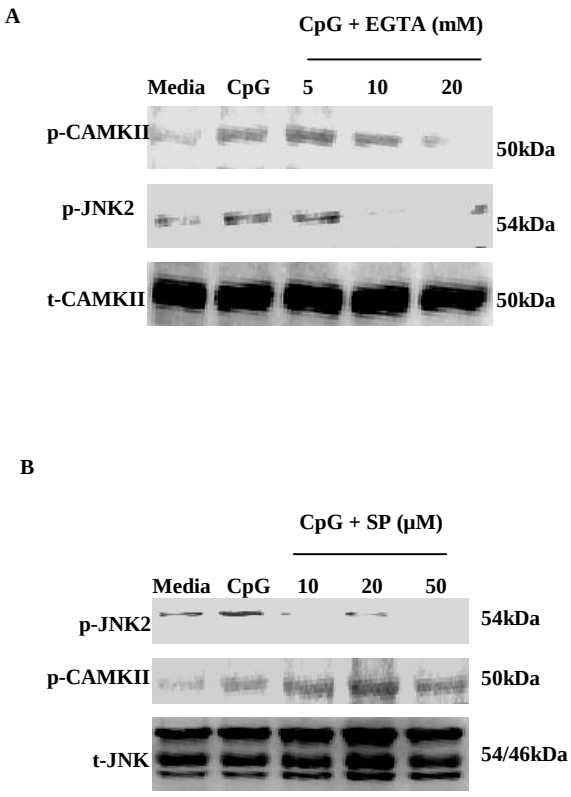

A
CpG + EGTA (mM)
Media
CpG
5
10
20
p-CAMKII
50kDa
p-JNK2
54kDa
t-CAMKII
50kDa
B
CpG + SP (μM)
Media
CpG
10
20
50
p-JNK2
54kDa
p-CAMKII
50kDa
t-JNK
54/46kDa

Figure 4.16

4.2.9 *CpG-induced c-IAP-2 expression and protection from Vpr(52-96)-mediated apoptosis in human monocytic cells is mediated via TLR-9 independent mechanisms*

Human monocytes have been reported to express low levels of TLR-9 mRNA (411). Keeping in view the above mentioned results, I determined if CpG-mediated protection from Vpr(52-96)-induced apoptosis is indeed mediated through TLR-9 signaling. For this, I first demonstrated that THP-1 cells and monocytes expressed TLR-9 (Fig 4.17A). Chloroquine has been shown to inhibit endocytic maturation and subsequent TLR-9 activation (350). Therefore, to further confirm the involvement of TLR-9 signaling, THP-1 cells and monocytes were treated with chloroquine followed by CpG stimulation and Vpr(52-96) treatment. Cells pretreated with chloroquine at concentrations of 25 µM failed to significantly reverse CpG-induced protection (Fig 4.17B) suggesting that CpG, under these experimental conditions, may exert its effects through TLR-9 independent mechanisms (361, 363, 364, 412). In support of this, I found that stimulation with non-TLR-9 activating GpC control ODNs prior to Vpr(52-96) treatment were equally capable of inhibiting Vpr(52-96)-induced apoptosis (Fig 4.17C). Similarly pre-incubation with TLR-9 antagonising ODNs did not abrogate CpG-induced protection (Fig 4.17D). Additionaly prior treatment with CaM/CaMK-II inhibitors significantly reduced the protection afforded by GpC control ODNs against Vpr(52-96)-mediated apoptosis (Fig 4.18) suggesting that similar to CpG-induced protection, GpC-mediated cell survival was also regulated by the CaM/CaMK-II signaling. Thus, these results suggest that CpG-induced protection in human monocytes from Vpr(52-96)-mediated apoptosis may be regulated by the inhibitor of apoptosis c-IAP-2 through the calcium-activated JNK pathway but in a TLR-9 independent manner (Fig 4.19).

Fig. 4.17: TLR-9 independent signaling for CpG-induced c-IAP-2 expression and protection against Vpr(52-96)-mediated apoptosis in human monocytic cells.

A. THP-1 cells and monocytes ($1.0x10^6$/ml) were stained with FITC conjugated anti-TLR-9 or anti-IgG isotype control antibodies and visualized using confocal microscope with a 63X lens at 4x magnification (THP-1 cells) and 9x magnification (monocytes). **B**. THP-1 cells and monocytes ($1.0x10^6$/ml) were treated with 25 µM chloroquine for 2 hr prior to stimulation with 5 µM CpG for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) for 24 hr in THP-1 cells or 4 hr in monocytes followed by analysis of apoptosis by Annexin-V staining. **C**. THP-1 cells ($1.0x10^6$/ml) were treated with 5 µM GpC control ODNs for 12 hr before stimulation with 1.5 µM Vpr(52-96) for 24 hr followed by analysis of apoptosis by Annexin-V staining. **D**. THP-1 cells ($1.0x10^6$/ml) were incubated with 5, 10 or 25 µM TLR-9 antagonist ODNs for 1 hr before being treated with 5 µM CpG for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) for 24 hr followed by analysis of apoptosis by Annexin-V staining. Results in B and C are expressed as a mean ± SD of three independent experiments. The protective effect of CpG in B and C was calculated as % apoptosis relative to the Vpr-induced apoptosis after normalization against apoptosis in cells alone.*$p < 0.05$, ** $p < 0.005$.

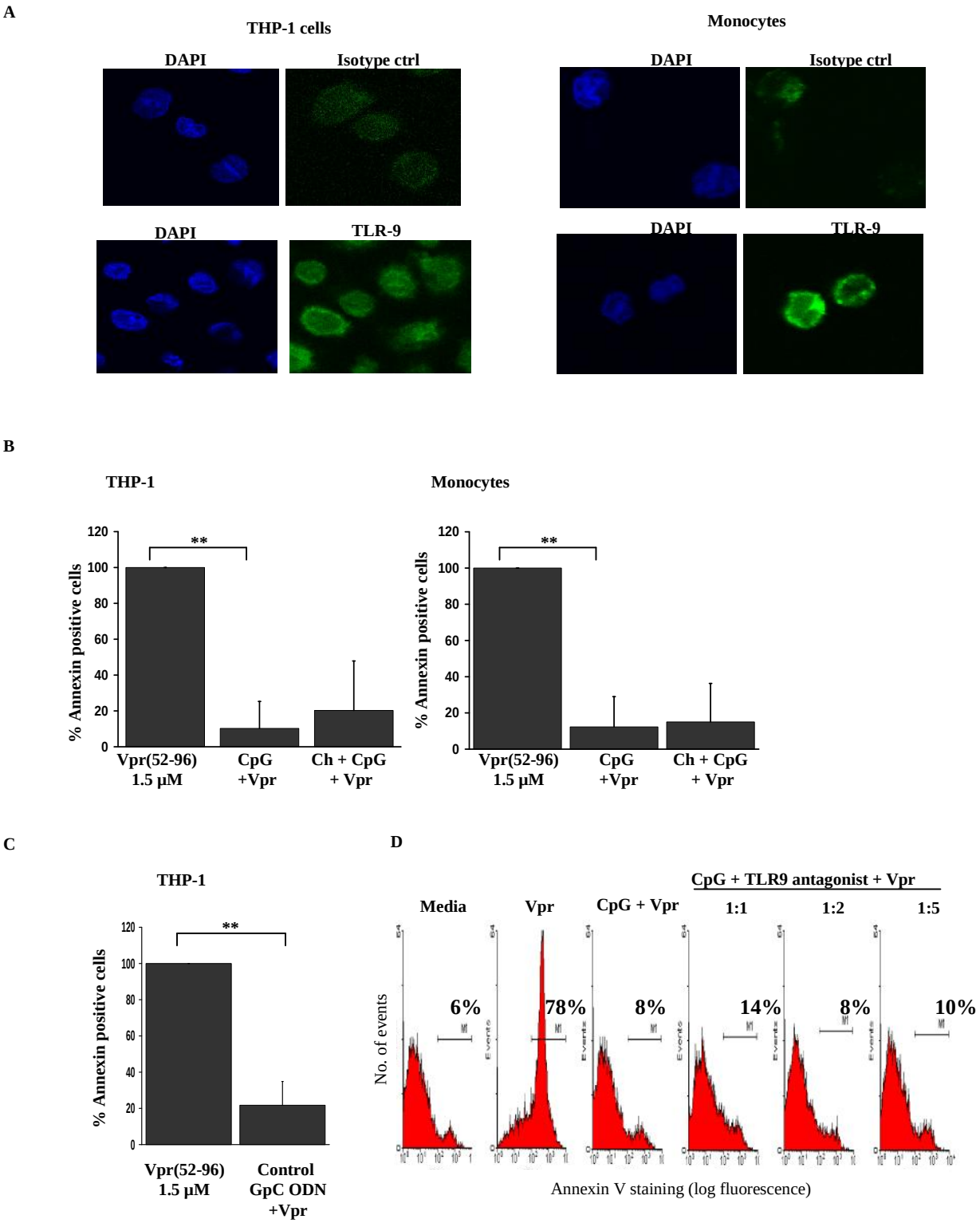

A
THP-1 cells
Monocytes
DAPI
Isotype ctrl
DAPI
Isotype ctrl
DAPI
TLR-9
DAPI
TLR-9
B
THP-1
Monocytes
% Annexin positive cells
120
100
80
60
40
20
0
**
Vpr(52-96) 1.5 µM
CpG +Vpr
Ch + CpG + Vpr
% Annexin positive cells
120
100
80
60
40
20
0
**
Vpr(52-96) 1.5 µM
CpG +Vpr
Ch + CpG + Vpr
C
THP-1
% Annexin positive cells
120
100
80
60
40
20
0
**
Vpr(52-96) 1.5 µM
Control GpC ODN +Vpr
D
CpG + TLR9 antagonist + Vpr
Media
Vpr
CpG + Vpr
1:1
1:2
1:5
No. of events
6%
78%
8%
14%
8%
10%
Annexin V staining (log fluorescence)

Figure 4.17

Fig. 4.18: TLR-9 independent signaling for GpC ODN-induced protection against Vpr(52-96)-mediated apoptosis is mediated via CaM/CaMK-II signaling in THP-1 cells.

THP-1 cells ($1.0x10^6$/ml) were treated with various concentrations of EGTA, W-7, SKF, KN-93, APB and FK-506, for 2 hr prior to stimulation with 5 µM GpC for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) for 24 hr in THP-1 cells followed by analysis of apoptosis by Annexin-V staining. Results are expressed as a mean ± SD of three independent experiments. The protective effect of GpC was calculated as % apoptosis relative to the Vpr-induced apoptosis after normalization against apoptosis in cells alone. *p <0.05, ** p <0.005. The p values calculated for EGTA, SKF, KN93 and W7 were against cells treated with GpC and Vpr.

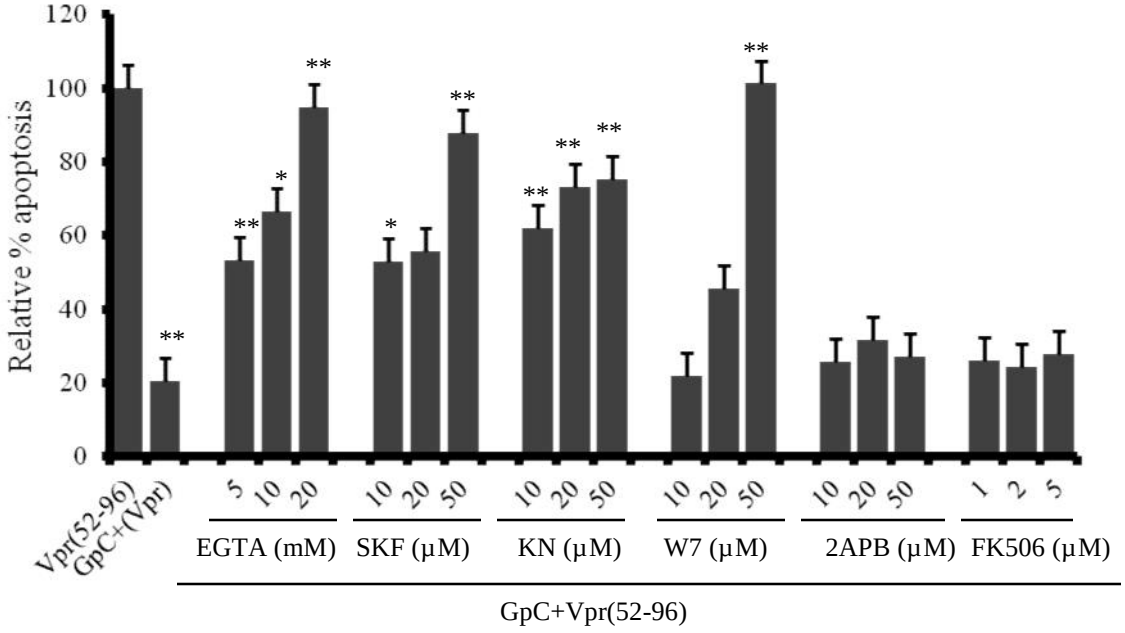

Relative % apoptosis
120
100
80
60
40
20
0
Vpr(52-96)
GpC+(Vpr)
5 10 20
EGTA (mM)
10 20 50
SKF (µM)
10 20 50
KN (µM)
10 20 50
W7 (µM)
10 20 50
2APB (µM)
1 2 5
FK506 (µM)
GpC+Vpr(52-96)

Figure 4.18

Fig. 4.19: Schematic representation of TLR-9 independent CpG-induced protection against Vpr(52-96)-mediated apoptosis in human monocytic cells.

Upon entering the cell, CpG induces calcium influx which activates CaM. CaM activates CaMK-II which in-turn induces JNK phosphorylation leading to c-IAP-2 expression. c-IAP-2 serves to protect cells against apoptosis caused by HIV-Vpr.

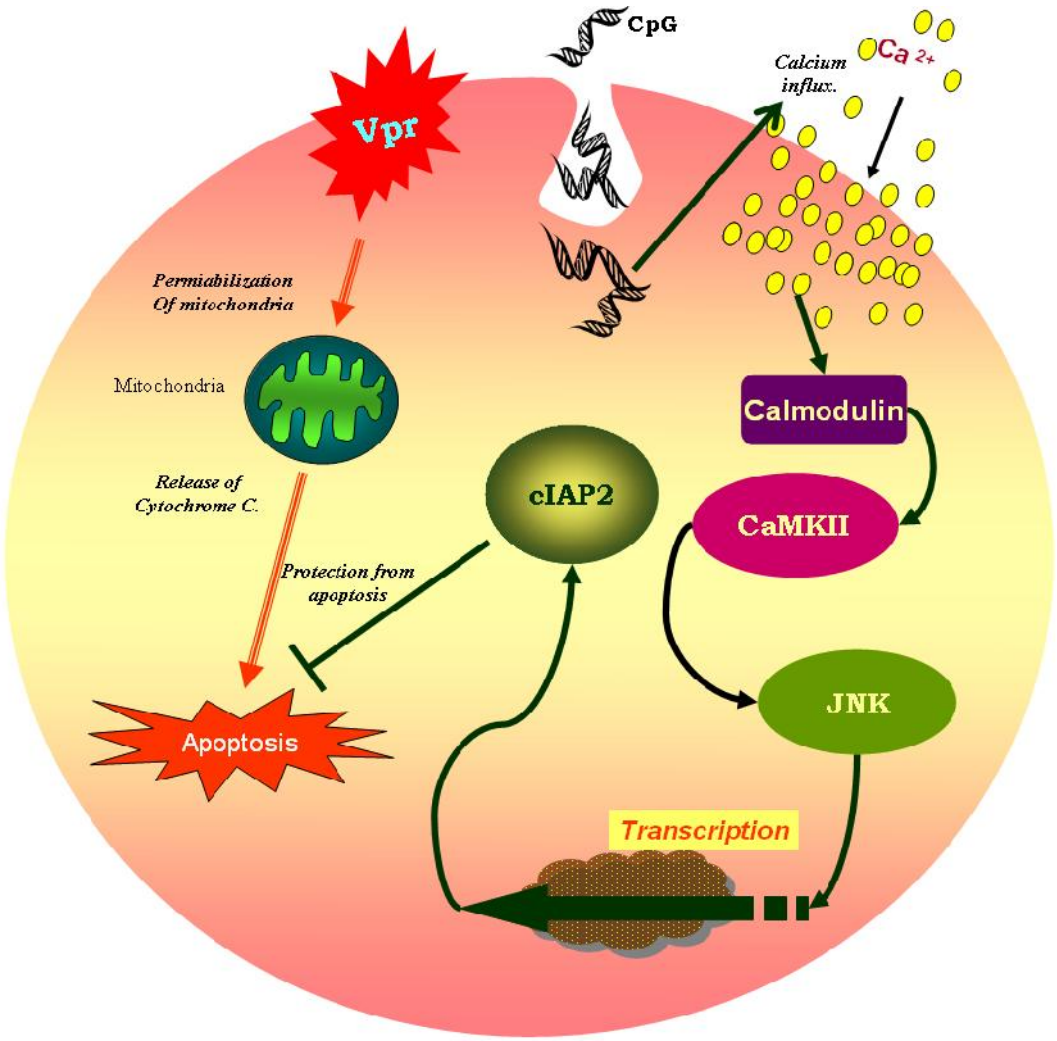

CpG
Vpr
Calcium
influx.
Ca 2+
Permiabilization
Of mitochondria
Mitochondria
Calmodulin
Release of
Cytochrome C.
cIAP2
CaMKII
Protection from
apoptosis
JNK
Apoptosis
Transcription

Figure 4.19

4.3 Chapter 4: Discussion

Recent developments indicating translocation of microbes or their components from the gut into the circulation during chronic HIV infection suggest a critical role for TLR ligands in HIV pathogenesis. In fact, high levels of bacterial LPS and DNA have been found in the plasma of persons infected with HIV. Moreover, LPS levels in plasma have also been correlated with indices of T cell activation, IFN-γ plasma levels and CD4+ T cell restoration following antiretroviral therapy (11, 385). Besides, chronic HIV-1 infection was associated with increased TLR expression and immune responsiveness (370). Since LPS and CpG DNA have been implicated in enhanced cell survival (10, 301, 413), I determined whether monocytic cells' stimulation by various TLR agonists can modulate their survival in response to apoptogenic Vpr(52-96) peptide. Herein, I show for the first time that TLR-2, TLR-4 and TLR-9 agonists induce resistance to Vpr(52-96)-mediated apoptosis, with CpG inducing maximum resistance in primary human monocytes and THP-1 cells. Furthermore, this protection is mediated by c-IAP-2 induction through the calcium/CaMK-II activated JNK pathway in a TLR-9 independent manner.

Synthetic/bacterial DNA rich in unmethylated CpG motifs has previously been shown to enhance cell survival by preventing spontaneous apoptosis in mouse B cells and dendritic cells via PI3K signaling and upregulation of anti apoptotic Bcl-2, BclXl, c-IAP-1 and c-IAP-2 genes (10, 354). CpG DNA is a classical TLR-9 ligand (346) and has been shown to induce potent Th1 responses resulting in secretion of IL12 and IFN-γ via MyD88-dependent signaling pathway involving MyD88, IL-1 receptor associated kinases and TNF-receptor-associated factor-6 (313, 317). In the recent past, a growing body of evidence suggests that DNA can also be recognized by the host cells independent of TLR-9 leading to

inflammatory events such as activation of a set of genes encoding type-I interferons, chemokines, histocompatibility complex, costimulatory molecules, transcription factors STAT1 and IRF and protein kinase R (361, 363, 364, 412, 414, 415). The precise mechanism by which CpG exerts its effects via TLR-9-independent pathways is not well understood. Several TLR-9-independent CpG signaling cascades have been suggested including activation of two Src family kinases, Hck and Lyn (412), activation of transcription factor IRF-3 and IFN-β-promotor stimulator-1 through the signaling pathway requiring the kinases TANK-binding kinase-1 and inducible-κ-B-kinase-i (360), DNA-dependent protein kinases-induced Akt activation in murine macrophages (362) and inhibition of Smad proteins-regulated signaling in human osteoblasts (363).

Calcium (Ca2+), a pervasive intracellular second messenger, plays a key role in mediating the transcription of several cellular genes (321, 416). In general, binding of a ligand to its receptor induces influx of extracellular Ca2+ and/or release of intracellular Ca2+ stores from the ER. Many of the effects of Ca2+ are mediated via CaM. CaM is a highly conserved 17kD protein associated either with membrane, cytoplasm or nucleus. It binds up to four calcium ions and undergoes a conformational change that renders it active to bind its target proteins with high affinity. CaMK-II is one of the key targets for CaM (408). It is activated by increases in intracellular calcium levels and is essential for the translation of calcium signals to enable gene transcription. CaMK-II can phosphorylate several downstream effectors including transcription factors involved in diverse cellular functions such as T cell activation and cytokine production (321, 408).

Ca2+ influx in various cell types including monocytic cells in response to TLR-4 ligands has been shown (416, 417). However, very little is known about the involvement of

Ca2+ signaling and its major downstream kinases, CaMKs, in CpG-mediated activation of cellular genes. In the present study, I provide evidence that CpG can trigger elevation of intracellular Ca2+ by inducing influx of extracellular Ca2+ and activation of CaMK-II in monocytic cells. These observations clearly suggest that calcium signaling and in particular CaMK-II activation may serve as a key pathway in TLR-9-independent CpG signaling leading to the development of resistance to apoptosis in human monocytic cells.

JNK, a serine/threonine kinase, plays a critical role in cell survival as it has been shown to be both pro- and anti-apoptotic depending upon the cell type and the apoptosis-inducing agent (339). For example, thymocytes from JNK null mice were shown to suffer from increased Fas/CD95 and CD3 mediated apoptosis (418). In contrast, $jnk1^{-/-}$ and $jnk2^{-/-}$ murine embryonic fibroblasts were found to be resistant to UV induced apoptosis (419). Herein, I report an anti-apoptotic role of JNK in CpG-induced protection against apoptosis caused by Vpr(52-96) in human monocytic cells via upregulation of c-IAP-2. In accordance with previous studies showing cross interaction between MAPK and Ca+ signaling (321), my results demonstrate that CaM/CaMK-II activation is a prerequisite for JNK phosphorylation in response to TLR-9-independent CpG signaling.

The IAP genes are key regulators of apoptosis and are over expressed in many cancer cells (68). The results of this study suggest that CpG selectively up regulates the expression of c-IAP-2. Moreover, inhibition of c-IAP-2 expression by CaM/CaMK-II or JNK antagonists reversed CpG-mediated protection against Vpr(52-96)-induced apoptosis. The expression of other anti-apoptotic gene products such as c-IAP-1, XIAP and Bcl-2 was not upregulated in this study suggesting these genes may not play a significant role in development of resistance to Vpr-induced apoptosis in monocytic cells following CpG

stimulation. However, determination of the precise role of these genes in the development of resistance to Vpr-induced apoptosis needs further investigation.

Vpr has been shown to induce apoptosis by directly affecting the mitochondrial permeability transition pore complex and by specifically acting on the mitochondrial adenine nucleotide translocator, a component of the permeability transition pore complex. This event enhances permeability of the outer mitochondrial membrane with consequent release of apoptogenic factors such as apoptosis inducing factor and cytochrome c (242, 250). 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 (420). The precise mechanism by which c-IAP-2 prevents Vpr-induced apoptosis will be discussed in Chapter 5.

Others and I have used relatively high concentrations of Vpr as apoptosis inducing agents compared to the physiologically relevant low concentrations present in the plasma of HIV-infected individuals (181). Moreover, low concentrations of Vpr have been suggested to be protective rather than apoptosis-inducing agents in T cells (401, 402). My results clearly suggest that undifferentiated primary monocytes and THP-1 cells, when exposed sequentially to low non-apoptogenic concentrations of Vpr, undergo enhanced apoptosis. Furthermore both low and high concentrations of Vpr induced similar activation of MAPkinases further challenging the notion that Vpr has different biological effects at low and high concentrations. Therefore, primary monocytes exposed persistently to low concentrations of Vpr present in serum / lymph nodes of HIV-infected individuals under *in vivo* conditions may infact undergo apoptosis. However, these cells may be protected from the apoptogenic effects of Vpr following differentiation in tissues or exposure to various

microbial products present in the circulation of HIV-infected individuals. In addition, interesting parallels can be drawn between resistance of MDMs and CpG-stimulated monocytes to Vpr(52-96) driven apoptosis. Since CpG has previously been reported to induce macrophage like characteristics in monocytes, such as increased expression of co-stimulatory and antigen presenting molecules (345), it is possible that CpG induces resistance against Vpr-mediated apoptosis by propelling undifferentiated monocytes into a more mature and macrophage-like state of differentiation. The molecular mechanism responsible for the development of resistance to Vpr-induced apoptosis will be addressed in Chapter 5.

Highly purified monocytes depleted of pDCs do not express TLR-9 or express low levels of TLR-9 and do not respond to TLR-9 agonists unless reconstituted with the plaSmacytoid DCs (411). However, other reports indicate that monocytes isolated by adherence method or positive selection respond to CpG and express TLR-9 (421-425) suggesting that the expression of TLR-9 may depend on the method of isolation, cell activation, and the presence of non-monocytic cells in the preparation. I have shown that both THP-1 cells and monocytes do express TLR-9 and respond to CpG although the monocytes used in this study may contain non-monocytic cells (2-5%) including pDCs. My results also suggest that TLR-9-independent mechanisms may be operative in CpG-induced protection from Vpr(52-96) mediated apoptosis, as chloroquine was unable to prevent CpG-induced protection. Concordantly, GpC control ODNs consistently prevented Vpr(52-96)-induced apoptosis whereas TLR-9 antagonist ODNs failed to inhibit CpG-induced protection. Moreover both CpG and GpC induced protection via the calcium signaling pathway, further suggesting the role of TLR-9-independent mechanisms. These observations are in

accordance with several studies which describe TLR-9 independent CpG signaling in neutrophils, DCs, mouse embryonic stem cells, macrophages and THP-1 cells (361, 363, 364, 412, 414, 415).

In summary, this is the first study that demonstrates a CpG/bacterial DNA-dependent mechanism for the resistance of human monocytic cells to a HIV accessory protein, Vpr. Moreover, TLR-9-independent CpG-mediated resistance to Vpr-induced apoptosis occurred via expression of c-IAP-2 regulated by activation of CaM/CaMK-II and JNK signaling pathways (Fig 4.19).

Chapter 5: Bacterial DNA-induced c-IAP-2 protects human monocytic cells against HIV-Vpr-mediated mitochondrial damage.

5.1 Chapter 5: Introduction

Cells of the monocytic lineage, including macrophages and monocytes, serve as a major source of HIV reservoir contributing actively to AIDS pathogenesis (6, 277, 282, 283, 290). Infected macrophages act as shelters for HIV, supporting accumulation of latent virus. Interestingly primary monocytes are generally believed to be rather susceptible to apoptotic stimuli (108, 299). However despite being susceptible to apoptosis induced by HIV *in vitro*, monocytes survive HIV-induced cytopathic environment *in vivo* (5, 285, 381). Therefore it is reasonable to hypothesize that HIV infection induces mechanism/s that allow monocytes to survive HIV-mediated cytopathic effects. Several factors have been implicated in induction of resistance to HIV-mediated apoptosis in monocytes, including down regulation of cell surface death receptors and differential regulation of apoptosis related genes (107, 108, 303-305). However the exact mechanism enabling monocytes to withstand cytopathic effects of HIV still remain to be determined.

Synthetic Vpr(52-96) peptide, an accessory protein of HIV (204), was used in this study to induce apoptosis in monocytic cells so as to circumvent lack of productive *in vitro* HIV infection in human monocytic cells (280, 281) and to avoid interference from endotoxins often found in recombinant proteins.

Recent body of research suggests that HIV infection impairs host's gut immunity and causes translocation of gut microbes and microbial products into systemic circulation thereby causing systemic immune activation (11, 12, 384, 385). Moreover interaction with

microbial products was shown to bestow pro-survival properties on monocytic cells indicating that HIV-induced microbial translocation from the gut could render monocytes resistant to apoptosis (9, 10). As demonstrated in Chapter 4, CpG or *E.coli* DNA prevented Vpr(52-96)-mediated apoptosis by induction of anti apoptotic c-IAP-2 gene in human monocytic cells.

c-IAP-2 belongs to a family of anti-apoptotic proteins called, IAPs. IAPs are key regulators of apoptosis and are found over expressed in many cancer cells (68). Although some reports have demonstrated a role for c-IAP-2 in neutralizing caspases 9 and 3 directly, the principle mode c-IAP-2-mediated protection is considered to be via prevention of caspase 8 activation and transcriptional up regulation of anti-apoptotic proteins via induction of canonical NFκB signaling (90, 91). Mechanistically, binding of TNF-α to TNFR-I instigates formation of a plasma membrane bound complex comprising of TRADD, TRAF-1/2, c-IAP-1/2 and RIP-1 (90, 93). TRAF-1/2 help recruit c-IAP-1/2 to the signaling complex where c-IAP-1/2 function as primary ubiquitin ligases (70) and serve to maintain RIP-1 in a ubiquitilated state thereby preventing activation of pro-caspase 8 (74).

I have previously demonstrated that bacterial DNA-induced c-IAP-2 reversed the apoptosis caused by Vpr(52-96) in human monocytic cells (**Chapter 4**). In this study the precise mechanism by which c-IAP-2 protects against Vpr-induced apoptosis is investigated. Herein, I show that Vpr(52-96)-induced apoptosis is caused by mitochondrial depolarization which is mediated by TRAF-1/2 degradation and subsequent activation of pro-apoptotic caspase 8, Bid and Bax. Furthermore, bacterial DNA-induced c-IAP-2 protected against Vpr(52-96)-mediated mitochondrial membrane permeabilization and prevented release of AIF and cytochrome-c through CaMK-II activation. Overall, these results suggest a novel

and key role played by bacterial DNA-induced c-IAP-2 in protection against Vpr-mediated mitochondrial depolarization and apoptosis through prevention of TRAF-1/2 degradation and sequential inhibition of caspase 8, Bid and Bax.

5.2 Chapter 5: Results

5.2.1 *Bacterial DNA induces protection against Vpr(52-96)-mediated mitochondrial depolarization in primary monocytes and THP-1 cells*

Several studies performed in intact cells as well as on isolated mitochondria have indicated that Vpr regulates apoptosis by interacting with the proteins on the mitochondrial membrane and causing loss of mitochondrial potential (**Chapter 1 Fig 1.3**) (16, 235, 240, 242). Given my previous observations indicating that pretreatment with bacterial DNA reversed Vpr(52-96)-mediated apoptosis in human monocytic cells (**Chapter 4 Fig 4.4 and 4.5**), and since Vpr is believed to be a potent inducer of gross mitochondrial dysfunction I hypothesized that protection induced by bacterial DNA pretreatment may be modulated through prevention of Vpr(52-956)-mediated mitochondrial membrane permeabilization in human monocytic cells.

Mitochondrial membrane potential is a key indicator of mitochondrial health or injury. It is defined as a proton motive force accumulated due to reductive transfer of electrons through the electron transfer chain from complex I to IV, forcing the electrons against gradient and creating a polarized environment. Rhodamine 123 is a lipophillic cationic dye that accumulates in the mitochondrial membrane matrix in proportion to mitochondrial membrane potential (449). To determine whether bacterial DNA pretreatment interfered in Vpr(52-96)-mediated physical and functional mitochondrial damage, THP-1 cells and primary human monocytes were initially stimulated with E.Coli DNA or CpG followed by treatment with Vpr(52-96) peptide and analysis for gross changes in mitochondrial structural morphology and loss of membrane potential.

Pretreatment with CpG /*E.coli* DNA prevented Vpr(52-96)-mediated damage to mitochondrial structure (Fig 5.1A) and also prevented loss of mitochondrial potential by almost 60% in both THP-1 cells (Fig 5.2A and B) and primary monocytes (Fig 5.2C). Furthermore cells pretreated with CpG were rescued from Vpr(52-96)-induced release of mitochondrial cytochrome c (Fig 5.3A) and AIF (Fig 5.3B) into the cytosol. Interestingly, in accordance with the previously described observation regarding the TLR-9 independent nature of bacterial DNA-induced protection in human monocytic cells (**Chapter 4 Fig 4.17 and 4.18**), pretreatment with non-TLR-9 stimulating GpC control ODN exhibited significant protection against Vpr(52-96)-mediated physical injury and loss of membrane potential in the mitochondrial (Fig 5.1 and Fig 5.2B**).**

Similarly treatment with TLR-9 inhibiting drug, chloroquine (350), prior to CpG or *E.coli* DNA stimulation and Vpr(52-96) treatment failed to reverse CpG and *E.coli* DNA-mediated mitochondrial protection (Fig 5.2B and C). Three specific positively charged Vpr Arginine residues at sites, 73, 77 and 80 have been identified as being essential for Vpr-mediated mitochondrial dysfunction (184,244). Treatment with varying concentrations of mutant Vpr(52-96) peptide with Arg to Ala mutations at 73, 77 and 80 amino acids did not induce significant mitochondrial depolarization (Fig 5.2A). Apoptotic properties of Vpr have been mapped to C-terminal half of Vpr, that is, Vpr(52-96), where as the N-terminal half of Vpr, Vpr(1-45) has been described to be non-apoptotic (184). Consistent with the results obtained with mutant Vpr (Fig 5.2A), treatment with control Vpr(1-45) did not cause loss of mitochondrial cytochrome c or AIF (Fig 5.3).

Notably cells treated with CpG alone did not display signs of mitochondrial damage (Fig 5.1). Interestingly when equal number of mitochondria from each sample condition in 5.1 were analysed for diameter and size no significant difference in mitochondrial size was observed (data not shown). This may indicate that metabolic state of mitochondria do not change upon Vpr or bacterial DNA treatment. Conversly difference in microscopic fields make it hard to accurately quantify mitochondrial size differences between samples. For purposes of this study the presence of double membrane and occurance of dense, discernable cristae was taken as a measure of healthy respiring mitochondria.

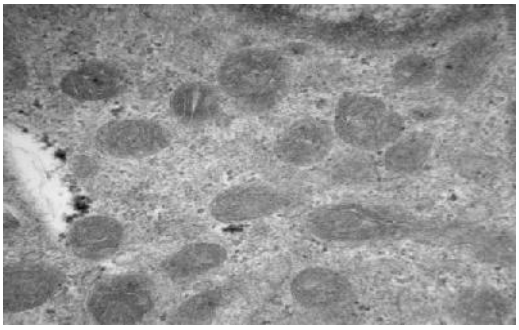
Fig 5.1: Pretreatment with bacterial DNA induces resistance against Vpr(52-96)-mediated changes to mitochondrial cristae as observed by transmission electron microscope.

THP-1 cells ($1.0x10^6$/ml) were stimulated with either with 5 μM CpG, 25 μg *E.coli* DNA or 5 μM GpC for 12 hr followed by treatment with 1.5 μM Vpr(52-96) for 5 hr. Cells were fixed and analyzed by transmission electron microscopy as described in *Materials and Methods* at the magnification of x 50,000.

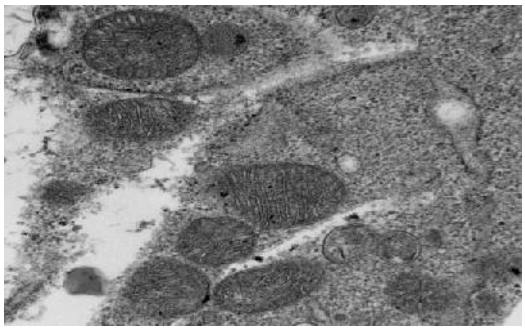
A

Media

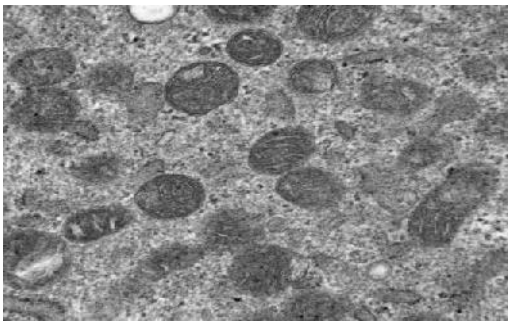
Vpr(52-96)



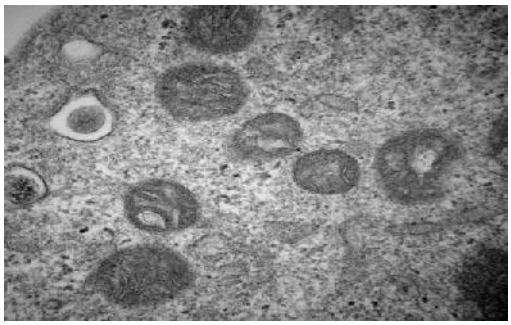
CpG + Vpr



E.coli DNA + Vpr



GpC + Vpr



CpG alone

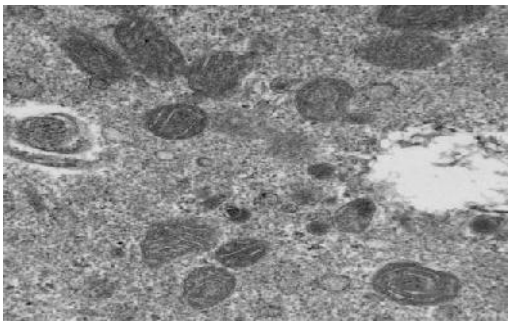
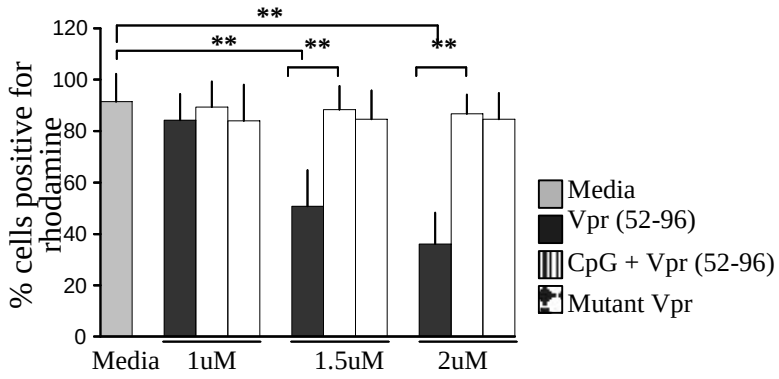


Figure 5.1

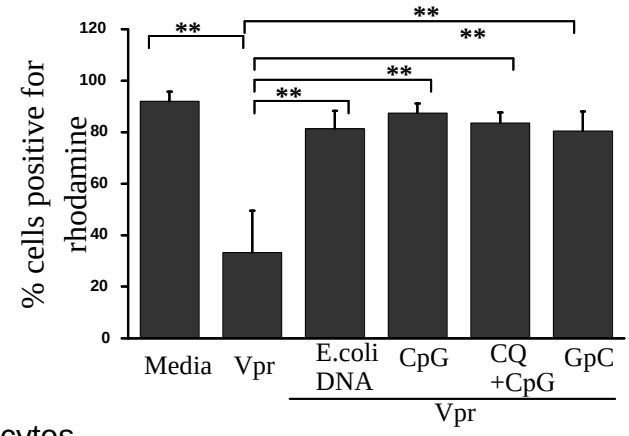
Fig 5.2: Pretreatment with bacterial DNA induces resistance against Vpr(52-96)-mediated loss of mitochondrial membrane potential.

(A) THP-1 cells ($1.0x10^6$/ml) were stimulated with CpG (5 µM) for 12 hr followed by treatment with 1-2 µM Vpr(52-96) or 1-2 µM mutant Vpr for 5 hr. Cells were then stained with Rhodamine 123 dye for mitochondrial membrane potential evaluation by flow cytometry. THP-1 cells (**B**) and monocytes (**C**) ($1.0x10^6$/ml) were treated 25 µM chloroquine (CQ) for 2 hr prior to stimulation with 5 µM CpG, 25 µg *E.coli* DNA or 5 µM GpC for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) for 5 hr (THP-1 cells) or 2 hr (monocytes) followed by staining with Rhodamine 123 (THP-1 cells) or MitoTracker Green (monocytes) for mitochondrial membrane potential evaluation by flow cytometry. Results are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

A THP-1



B THP-1



C Monocytes

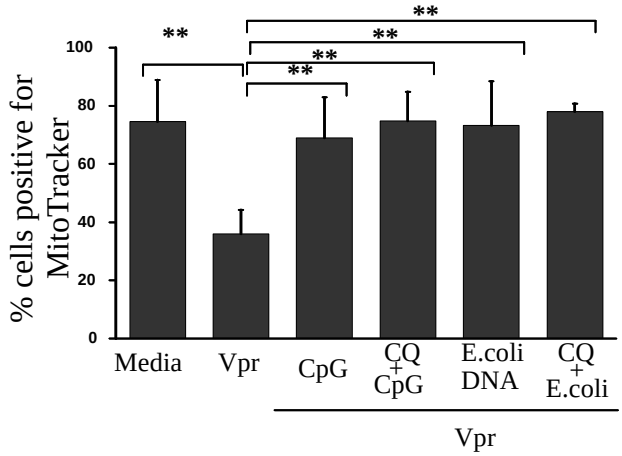


Figure 5.2

Fig 5.3: Pretreatment with CpG prevents Vpr(52-96)-mediated release of mitochondrial cytochrome c and AIF.

A.THP-1 cells ($3.0x10^6$/ml) were stimulated with 5 μM CpG for 12 hr followed by treatment with 1.5 μM Vpr(52-96) or Vpr(1-45) for 5 hr. Subsequently mitochondrial and cytoplasmic extracts were harvested and analyzed by immunoblotting using antibodies against cytochrome c. Membranes were reprobed with antibodies against SMAC and GAPDH to normalize loading. **B**. THP-1 cells ($3.0x10^6$/ml) were stimulated with 5 μM CpG for 12 hr followed by treatment with 1.5 μM Vpr(52-96) or Vpr(1-45) for 5 hr. Subsequently mitochondrial extracts were harvested and analyzed by immunoblotting using antibodies against AIF and SMAC.

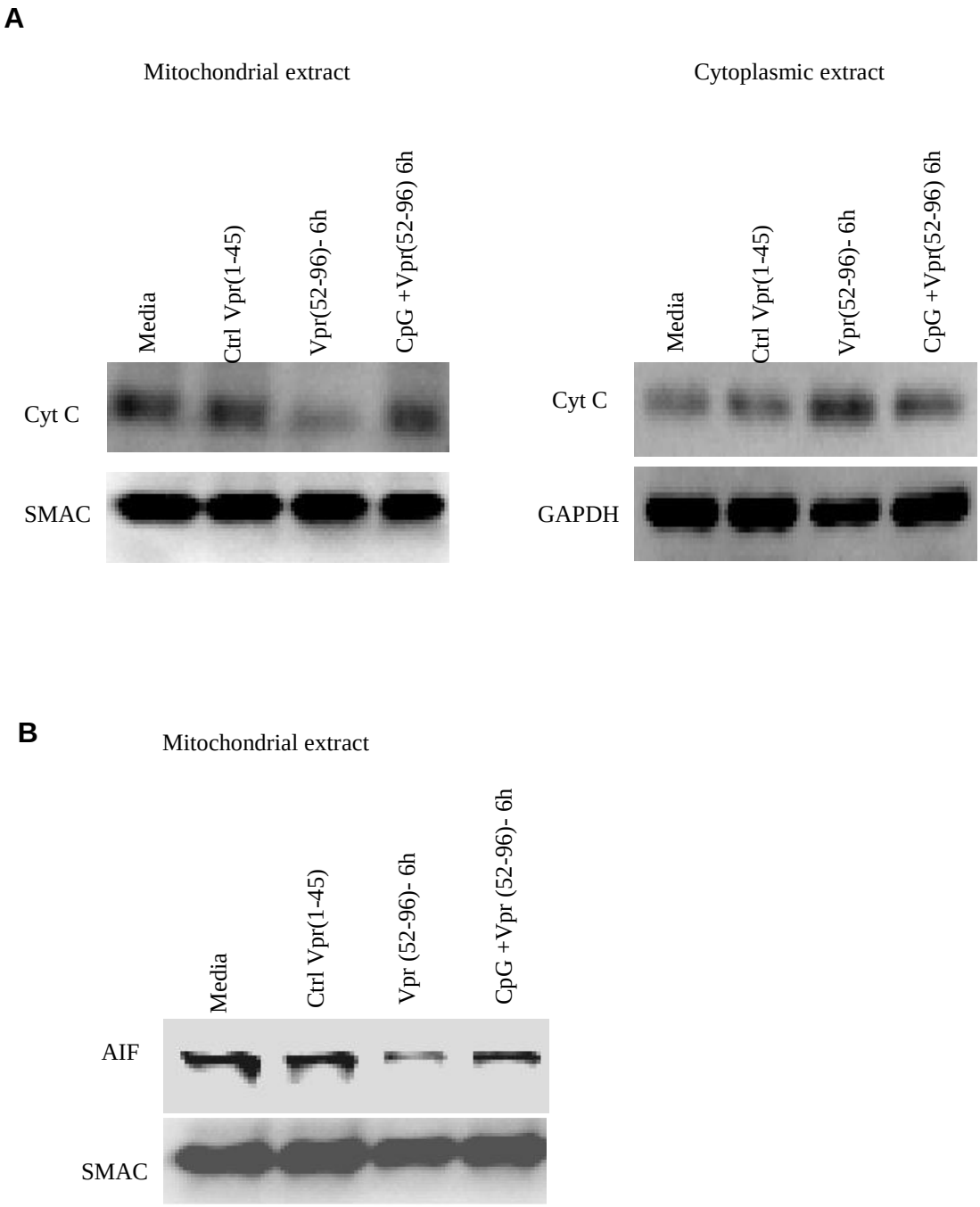

A
Mitochondrial extract
Cytoplasmic extract
Media
Ctrl Vpr(1-45)
Vpr(52-96)- 6h
CpG +Vpr(52-96) 6h
Cyt C
SMAC
GAPDH
B
Mitochondrial extract
Media
Ctrl Vpr(1-45)
Vpr (52-96)- 6h
CpG +Vpr (52-96)- 6h
AIF
SMAC

Figure 5.3

5.2.2 *Anti-apoptotic protein c-IAP-2 regulates bacterial DNA-mediated protection against Vpr(52-96)-induced mitochondrial depolarization*

c-IAP-2 has been shown to mediate cell survival via several mechanisms. These mechanisms include neutralizing pro-apoptotic activity of effector caspases 3, 7 and 9 activated by release of cytochrome c from permeabilized mitochondrian, inhibition of initiator caspase 8 activation and positive regulation of canonical NFκB pathway (48, 88, 91, 426, 427). I have previously shown that CpG and *E.coli* DNA stimulation induced enhanced expression of anti-apoptotic c-IAP-2 and prevention of c-IAP-2 expression negated CpG and *E.coli* DNA-induced protection against Vpr(52-96)-mediated apoptosis in monocytic cells (**Chapter 4**). Given the above mentioned observations, it was tempting to speculate that CpG and *E.coli* DNA-regulated protection from Vpr(52-96)-mediated mitochondrial damage may also be attributed to c-IAP-2 induction. Inorder to validate this hypothesis, c-IAP-2 was knocked down in THP-1 cells and primary monocytes by siRNA and SMC prior to stimulation with CpG and treatment with Vpr(52-96). THP-1 cells transfected with c-IAP-2 specific siRNA failed to induce c-IAP-2 in response to CpG and also displayed significantly higher mitochondrial depolarization as compared to cells transfected with non-silencing siRNA (Fig 5.4). Similarly, SMC treatment degraded c-IAP-2 (102, 107, 108) induced in response to CpG (Fig 5.5A) and significantly inhibited CpG-induced protection from Vpr(52-96)-induced mitochondrial depolarization (Fig 5.5B and C). In parallel, prior treatment with SMC significantly reduced *E.coli* DNA-mediated mitochondrial protection against Vpr(52-96)-induced damage in primary monocytes (Fig 5.5D).

It is interesting to note that monocytes and pro-monocytic THP-1 cells are susceptible to Vpr(52-96)-induced apoptosis (**Chapter 4 Fig 4.1**) but gain anti-apoptotic

properties during differentiation into MDMs due to up regulation of IAPs (108, 299). Therefore I used differentiated MDMs and THP-1 derived macrophages as c-IAP-2 over expression models to determine whether knocking down c-IAP-2 would render these cells sensitive to Vpr(52-96)-mediated mitochondrial damage. Treatment of MDMs with SMC prior to addition of Vpr(52-96) induced loss of c-IAP-2 expression (Fig 5.6A) rendering these cells responsive to Vpr(52-96)-mediated loss of mitochondrial membrane potential (Fig 5.6B). Moreover, prior treatment with SMC caused marked release of apoptogenic factors cytochrome c (Fig 5.7A) and AIF (Fig 5.7B) from the mitochondria as compared to cells treated with Vpr(52-96) alone in MDMs. Similarly, THP-1 derived macrophages transfected with c-IAP-2 specific siRNA exhibited lack of c-IAP-2 expression (Fig 5.8A) and release of mitochondrial AIF as compared to control siRNA transfected cells, in response to Vpr(52-96) treatment (Fig 5.8B). Note that treatment with SMC alone did not induce mitochondrial depolarization and apoptosis (Fig 5.5D and 5.6B).

Fig 5.4: c-IAP-2 siRNA abrogates CpG-induced resistance against Vpr(52-96)-mediated loss of mitochondrial membrane potential.

A. THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 1 µg of either c-IAP-2 or non-silencing control siRNA for 5 hr followed by stimulation with CpG (5 µM) for 48 hr. Thereafter cells were treated with 1.5 µM Vpr(52-96) for 5 hr followed by Rhodamine 123 staining and flow cytometry for mitochondrial membrane potential evaluation. **B**. Subsequently total cell proteins were analyzed for c-IAP-2 expression by immunoblotting. Results in B and bottom panel in A are representative of thee independent experiments. The results top panel of A are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

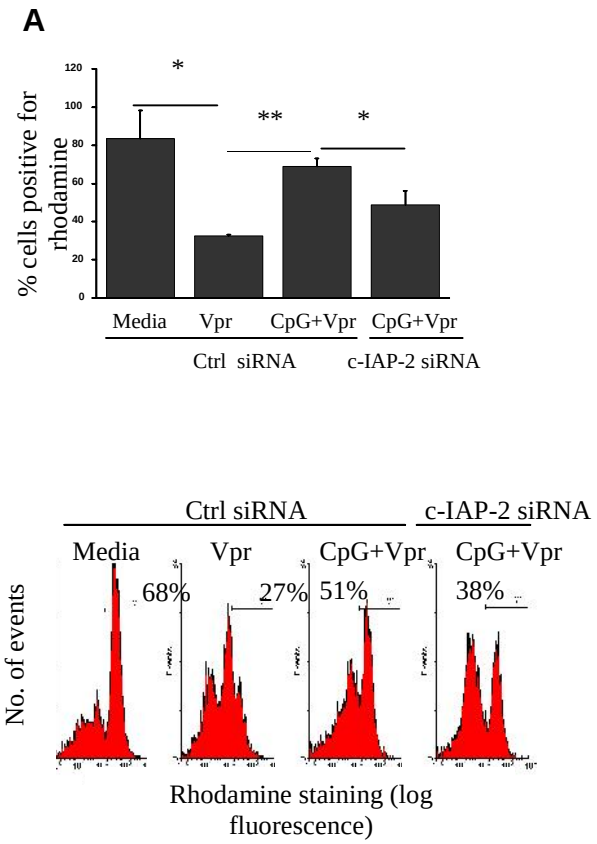
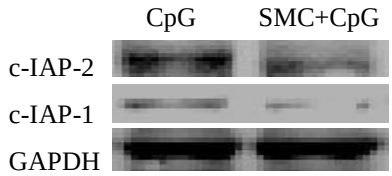

A
% cells positive for rhodamine
120
100
80
60
40
20
0
*
**
*
Media
Vpr
CpG+Vpr
CpG+Vpr
Ctrl siRNA
c-IAP-2 siRNA
Ctrl siRNA
c-IAP-2 siRNA
Media
Vpr
CpG+Vpr
CpG+Vpr
68%
27%
51%
38%
No. of events
Rhodamine staining (log fluorescence)
B
CpG + siRNA
Ctrl
cIAP2 1µg
cIAP2 2µg
c-IAP-2
c-IAP-1
GAPDH

Figure 5.4

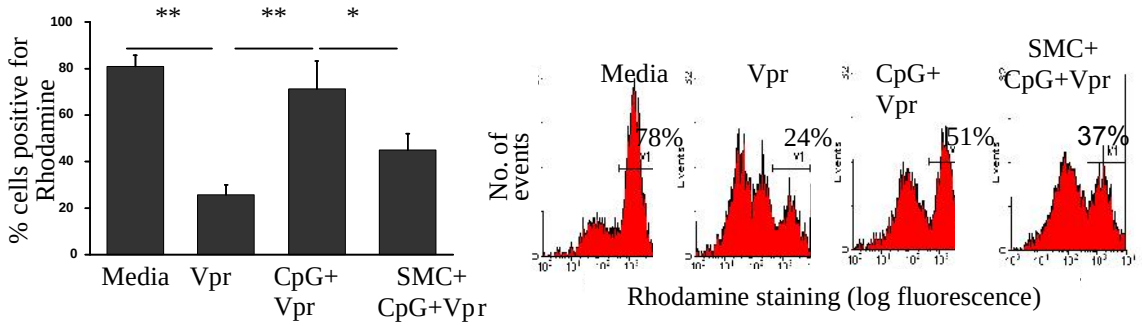
Fig 5.5: SMC-mediated degradation of c-IAP-2 abrogates bacterial DNA-induced resistance against Vpr(52-96)-mediated loss of mitochondrial membrane potential.

A. THP-1 cells ($1.0x10^6$/ml) were stimulated with 200 nM AEG-730 SMC and 5 µM CpG for 12 hr. Cell lysates were analyzed for c-IAP-2 and c-IAP-1 expression by immunoblotting. **B**. THP-1 cells ($1.0x10^6$/ml) were stimulated with 200 nM AEG-730 SMC and 5 µM CpG for 12 hr followed by 5 hr treatment with 1.5 µM Vpr(52-96). Monocytes ($1.0x10^6$/ml) were stimulated with 200 nM AEG-730 SMC and (**C**) 5 µM CpG or (**D**) 25 µg *E.coli* DNA for 12 hr followed by 2 hr treatment with 1.5 µM Vpr(52-96). Subsequently cells were stained with Rhodamine 123 (THP-1 cells) or MitoTracker Green (monocytes) for mitochondrial membrane potential evaluation by flow cytometry. Results in A, and right panels in B and C are representative of three independent experiments. The results in D and left panels in B and C are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

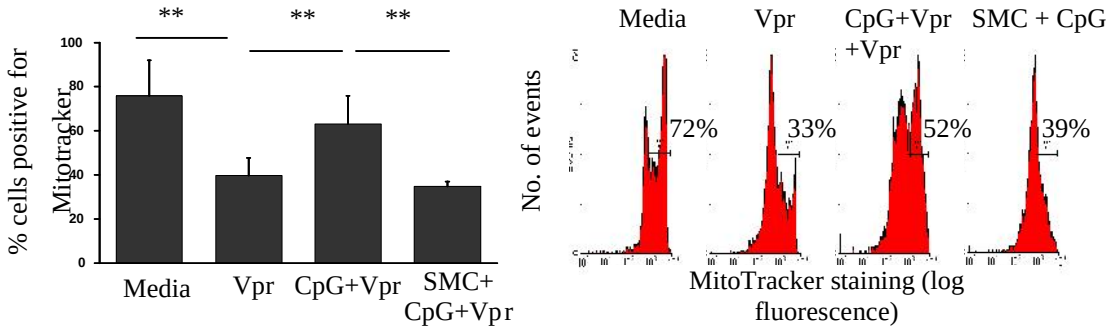
A THP-1



B THP-1



C Monocytes



D Monocytes

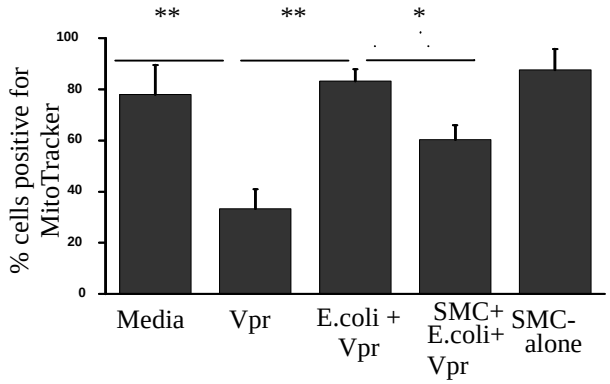
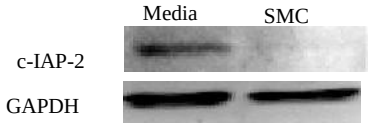


Figure 5.5

Fig 5.6: c-IAP-2 mediates resistance to Vpr(52-96)-induced mitochondrial loss of membrane potential in MDMs.

A. MDMs ($1.0x10^6$/ml) were treated with 200 nM AEG-730 SMC for 12 hr and the cell lysates were analyzed for c-IAP-2 expression by immunoblotting. **B**. MDMs ($1.0x10^6$/ml) were treated with 200 nM AEG-730 SMC for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 6 hr. Cells were stained with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. Results in A are representative of three independent experiments. The results in B are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

A MDM



B MDM

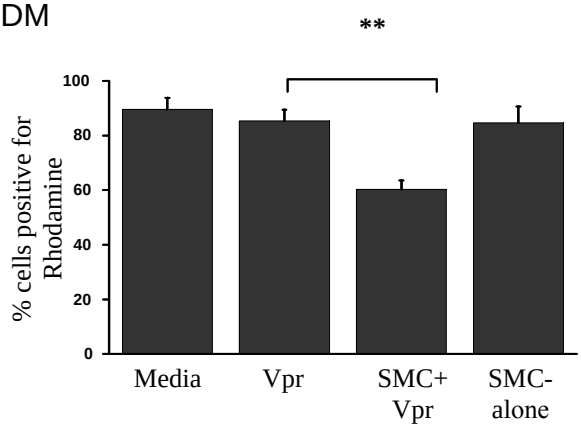


Figure 5.6

Fig 5.7: Pre-treatment with SMC renders MDMs sensitive to Vpr(52-96)-mediated loss of mitochondrial cytochrome c and AIF.

MDMs ($1.0x10^6$/ml) were treated with 200 nM AEG-730 SMC for 12 hr followed by treatment with 1.5 μM Vpr(52-96) for 6 hr. Cells were prepared for microscopy as described in Materials and Methods. A. Cells were co-stained for the nuclear stain DAPI (blue), mitochondrial marker Tom20 (red) and cytochrome c (green); and visualized using confocal microscope with a 40X lens at 1x magnification. B. Cells were co-stained for the nuclear stain DAPI (blue), Mitotracker (green) and AIF (red); and visualized using confocal microscope with a 40X lens at 4x magnification.Yellow/Orange represents colocalization of cytochrome c or AIF with the mitochondria. The staining for AIF and cytochrome c becomes undetectable in samples treated with SMC+Vpr, as enhanced permeabilization of mitochondrial outer membrane in these samples releases of concentrated AIF/cytochrome c into the cytosol, thereby diluting the dye and reducing the immunoflorescent signal .

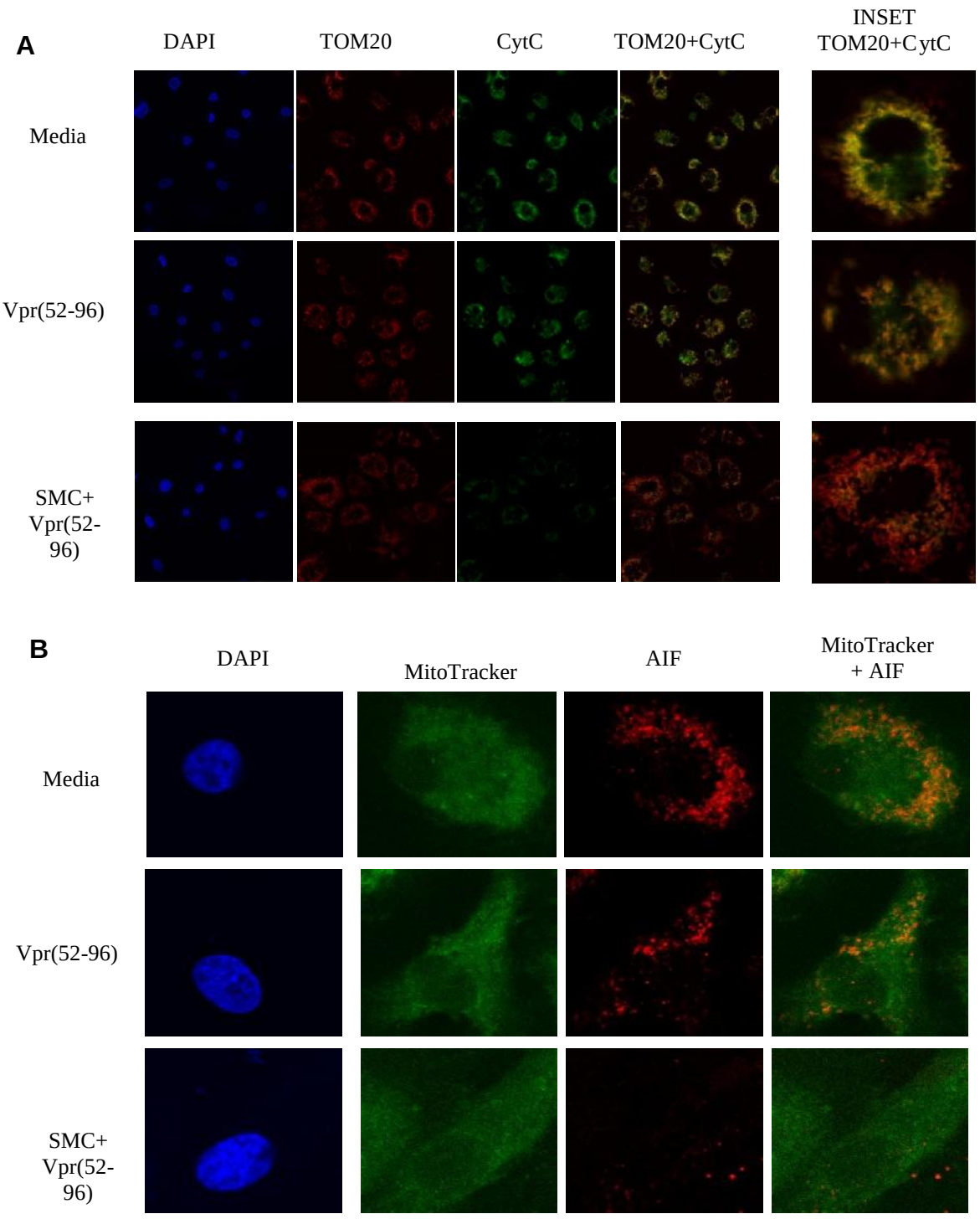
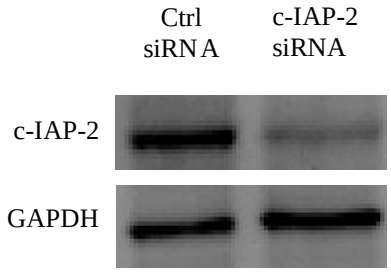

A
DAPI
TOM20
CytC
TOM20+CytC
INSET
TOM20+CytC
Media
Vpr(52-96)
SMC+
Vpr(52-
96)
B
DAPI
MitoTracker
AIF
MitoTracker
+ AIF
Media
Vpr(52-96)
SMC+
Vpr(52-
96)

Figure 5.7

Fig 5.8: c-IAP-2 mediates resistance to Vpr(52-96)-induced loss of mitochondrial apoptogenic factors in THP-1 derived macrophages.

Fully differentiated THP-1 macrophages ($5x10^5$/ml) were transfected with 1 μg of either c-IAP-2 or non-silencing control siRNA for 48 hr. **A**. Cell lysates were analyzed for c-IAP-2 expression by immunoblotting. **B**. Cells transfected as above were stimulated with 1.5 μM Vpr(52-96) for 6 hr and prepared for microscopy by staining for the nuclear stain DAPI (blue), MitoTracker (green) and AIF (red) followed by visualization using confocal microscope with a 40X lens at 4x magnification. Yellow represents colocalization of AIF with the mitochondria. The staining for AIF becomes undetectable in samples treated with SMC+Vpr, as enhanced permeabilization of mitochondrial outer membrane in these samples releases of concentrated AIF into the cytosol, thereby diluting the dye and reducing the immunoflorescent signal .

A THP-1 Macrophages



B THP-1 Macrophages

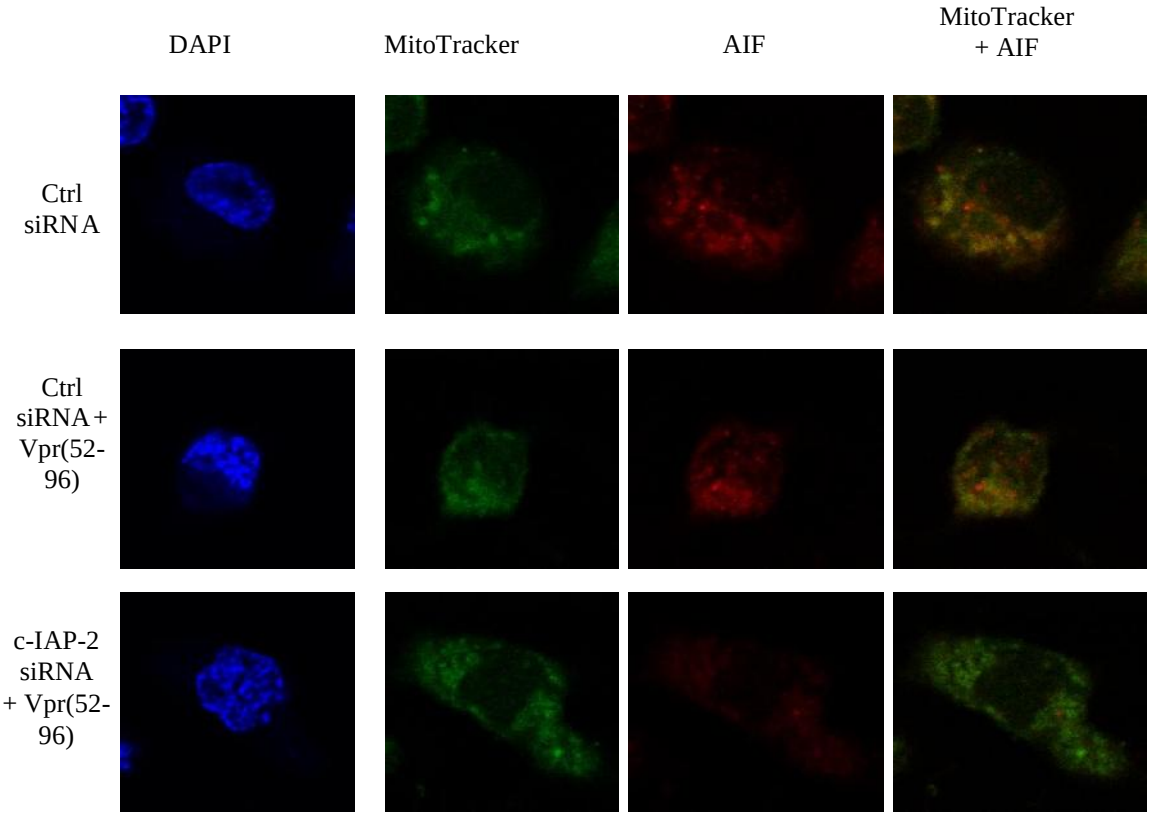


Figure 5.8

5.2.3 *CaM/CaMK-II activation regulates bacterial DNA-mediated protection against Vpr(52-96)-induced mitochondrial depolarization and release of mitochondrial apoptogenic factors*

My previous observations suggested that CpG-induced calcium influx, through the calcium channels on the cell surface and activation of CaM/CaMK-II signaling pathway regulated c-IAP-2 induction and associated protection against Vpr(52-96)-mediated apoptosis (**Chapter 4 Fig 4.10-4.12**). To further validate the novel role for c-IAP-2 in protecting against loss of mitochondrial viability, I hypothesized that inhibiting c-IAP-2 expression via blocking activation of CaM/CaMK-II should inhibit bacterial DNA-mediated rescue of mitochondrial loss of membrane potential caused by Vpr(52-96). Interestingly, prior treatment with pharmacological inhibitors of; a) influx of calcium ions through receptor gated calcium channels on the cell surface, SKF-96365 (388), b) inhibitor of CaM activation, W-7, c) inhibitor of CaMK-II phosphorylation, KN-93 and d) calcium chelator EGTA (321, 409), significantly reversed the CpG-mediated protection against Vpr(52-96)-induced release of mitochondrial cytochrome c (Fig 5.9) and AIF (Fig 5.10). Notably, 24 hr treatment of THP-1 cells with the highest concentrations of all the inhibitors used in this study did not cause significant release of mitochondrial AIF (Fig 5.11). The biological activity of EGTA, SKF-96365, W-7 and KN-93, was determined by inhibition of CpG-induced phosphorylation of CaMK-II (**Chapter 4 Fig 4.10B).** In accordance, THP-1 cells transfected with CaMK-II specific siRNA prior to CpG stimulation, exhibited lack of c-IAP-2 induction in response to CpG (Fig 5.12B) and displayed a marked decrease in protection afforded by CpG against Vpr(52-96)-mediated loss of mitochondrial membrane potential as compared to cells transfected with control non-silencing siRNA (Fig 5.12A).

Fig 5.9: Inhibitors of calcium signaling reverse bacterial DNA-mediated resistance to Vpr(52-96)-induced loss of mitochondrial cytochrome c.

THP-1 cells ($1.0x10^6$/ml) were treated with 10 mM EGTA, 25 μM SKF, 20 μM KN-93, and 20 μM W-7 and for 2 hr prior to stimulation with 5 μM CpG or 25 μ g E.coli DNA for 12 hr. Subsequently cells were treated with 1.5 μM Vpr(52-96) for 5 hr and prepared for microscopy by staining for the nuclear stain DAPI (blue), mitochondrial marker Tom20 (red) and cytochrome c (green) before being visualized by confocal microscopy with a 63X lens at 4x magnification. Results are representative of three independent experiments. The staining for cytochrome c becomes undetectable in samples treated with Vpr alone or pretreated with calcium inhibitors, as enhanced permeabilization of mitochondrial outer membrane in these samples releases of concentrated cytochrome c into the cytosol, thereby diluting the dye and reducing the immunoflorescent signal .

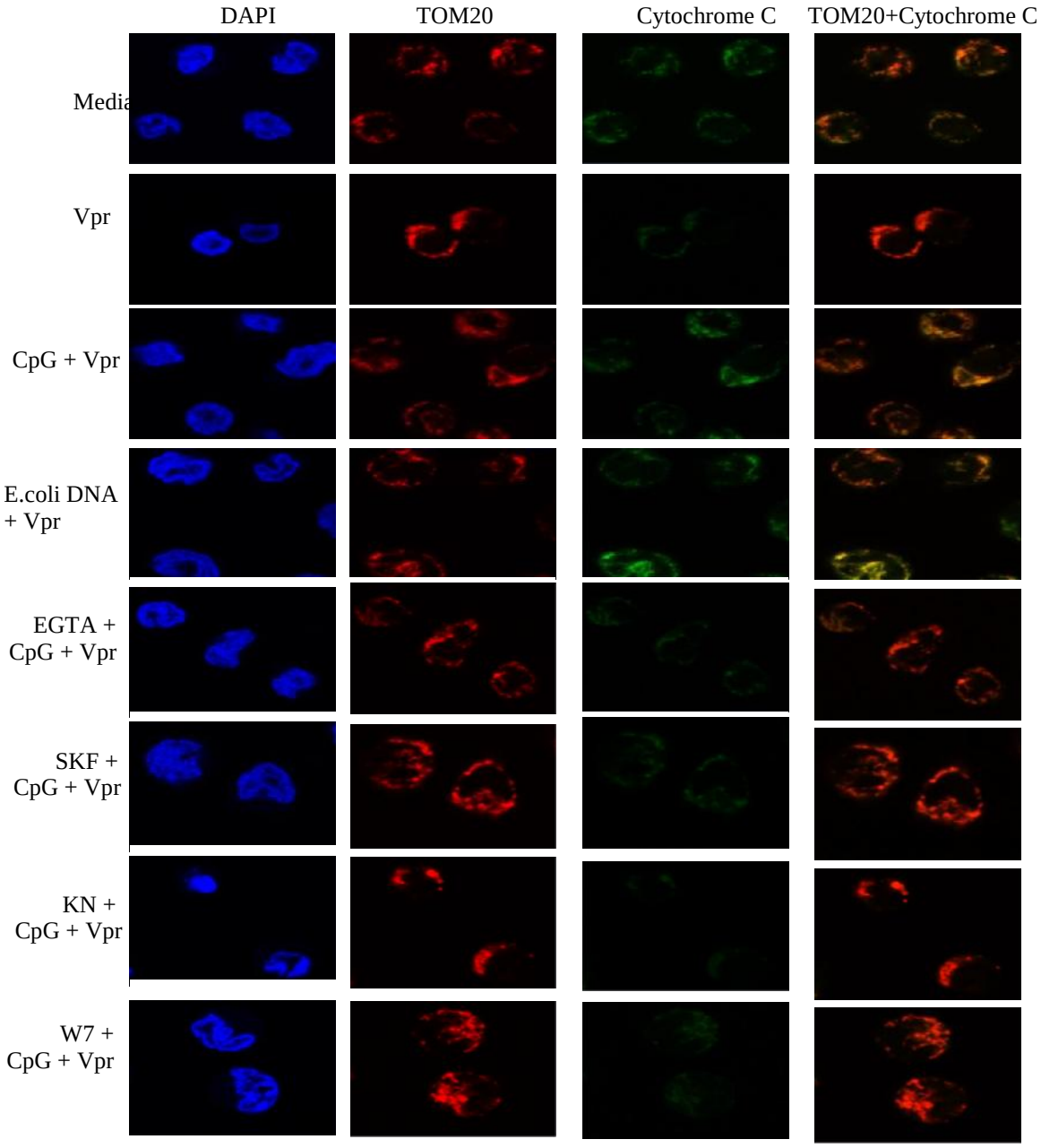

DAPI
TOM20
Cytochrome C
TOM20+Cytochrome C
Media
Vpr
CpG + Vpr
E.coli DNA + Vpr
EGTA + CpG + Vpr
SKF + CpG + Vpr
KN + CpG + Vpr
W7 + CpG + Vpr

Figure 5.9

Fig 5.10: Inhibitors of calcium signaling reverse bacterial DNA-mediated resistance to Vpr(52-96)-induced loss of mitochondrial AIF.

THP-1 cells ($1.0x10^6$/ml) were treated with 10 mM EGTA, 25 μM SKF, 20 μM KN-93, and 20 μM W-7 and for 2 hr prior to stimulation with 5 μM CpG or 25 μg *E.coli* DNA for 12 hr. Subsequently cells were treated with 1.5 μM Vpr(52-96) for 5 hr and prepared for confocal microscopy by staining for the nuclear stain DAPI (blue), mitochondrial marker Tom20 (red) and AIF (green) before being visualized using confocal microscope with a 63X lens at 4x magnification. Results are representative of three independent experiments. The staining for AIF becomes undetectable in samples treated with Vpr alone or pretreated with calcium inhibitors, as enhanced permeabilization of mitochondrial outer membrane in these samples releases of concentrated cytochrome c into the cytosol, thereby diluting the dye and reducing the immunoflorescent signal .

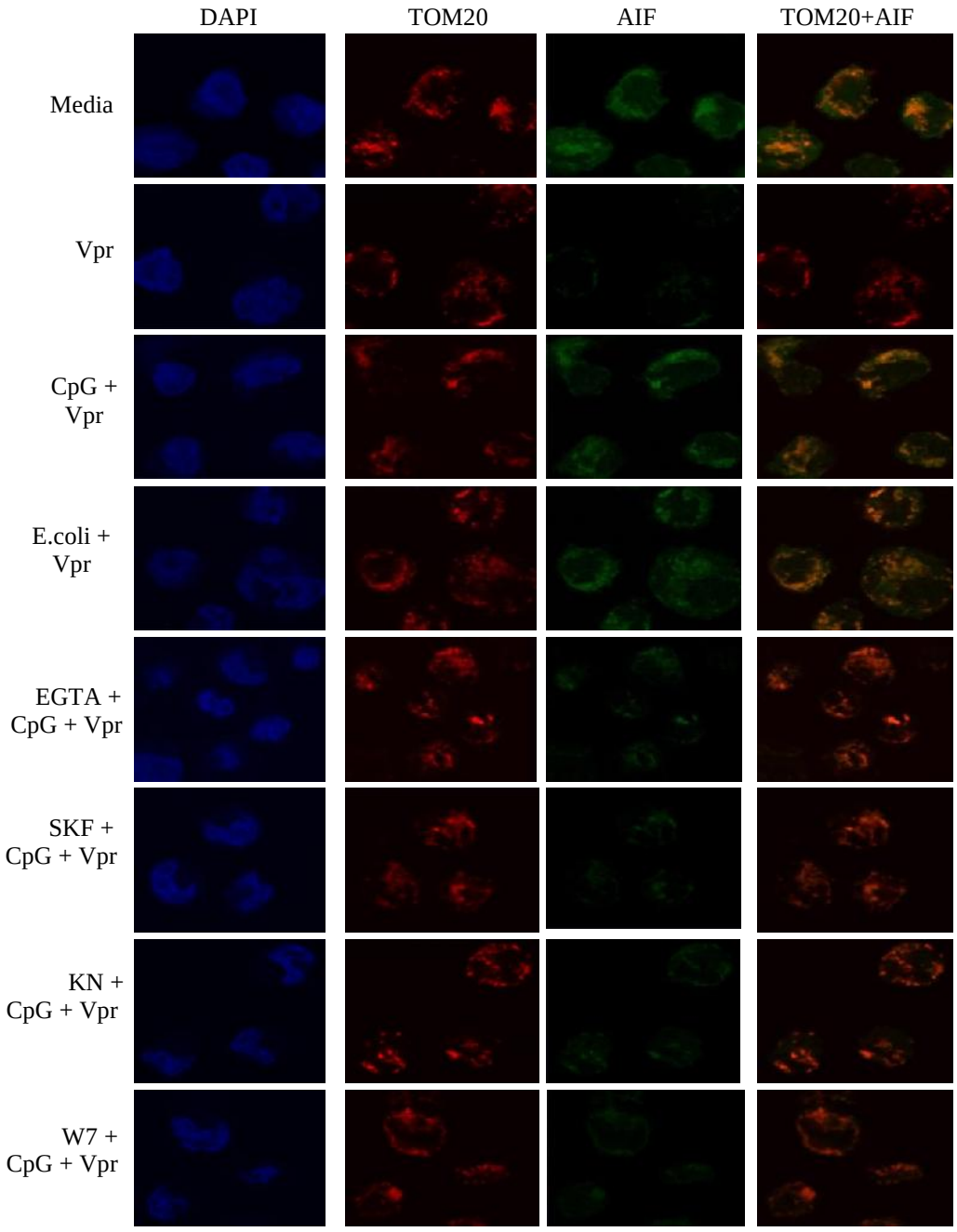

DAPI
TOM20
AIF
TOM20+AIF
Media
Vpr
CpG + Vpr
E.coli + Vpr
EGTA + CpG + Vpr
SKF + CpG + Vpr
KN + CpG + Vpr
W7 + CpG + Vpr

Figure 5.10

Fig 5.11: Treatment with inhibitors of calcium signaling alone does not cause loss of mitochondrial AIF.

THP-1 cells ($1.0x10^6$/ml) were treated with 20 mM EGTA, 50 μM SKF, 50 μM KN-93, and 50 μM W-7 and for 14 hr and prepared for microscopy by staining for AIF (red) before being visualized using a confocal microscope with a 63X lens at 4x magnification. Results are representative of three independent experiments.

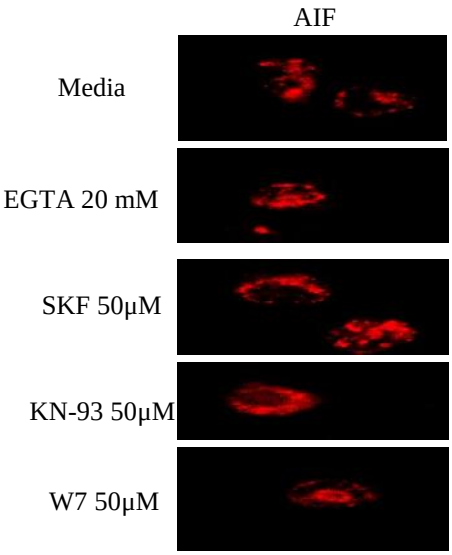
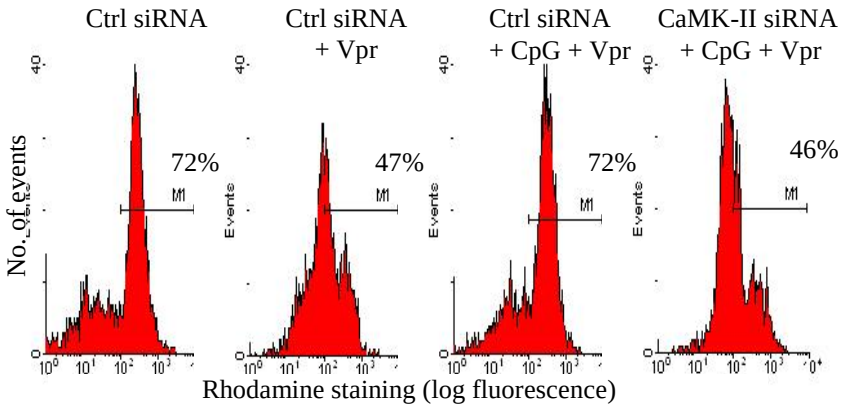


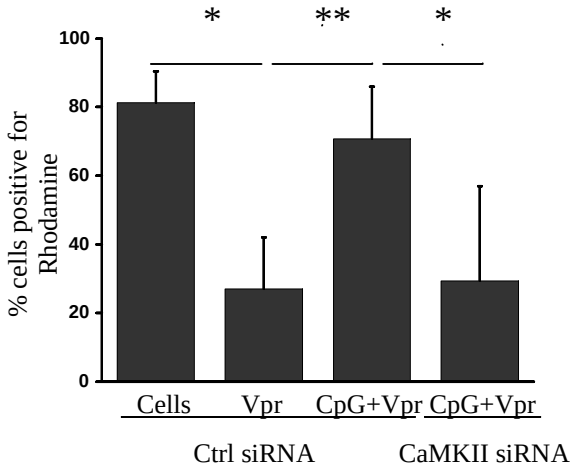
Figure 5.11

Fig 5.12: CaMK-II siRNA reverses CpG-mediated resistance to Vpr(52-96)-induced loss of mitochondrial membrane potential.

A. THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 1 µg of either CaMK-II siRNA or non-silencing siRNA for 24 hr, followed by stimulation with 5 µM CpG for 12 hr. Thereafter cells were treated with 1.5 µM of Vpr(52-96) for 5 hr followed by staining with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. **B.** Cell lysates were analyzed for c-IAP-2 and CaMK-II expression by immunoblotting. Results in B and top panel in A are representative of three independent experiments. The results in lower panel in A are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

A THP-1





B

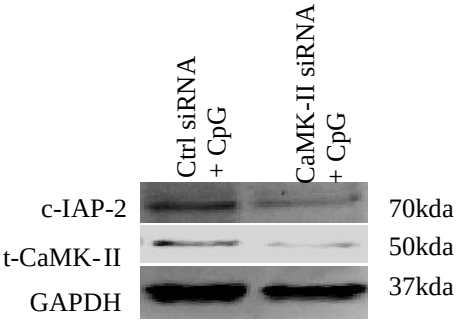


Figure 5.12

5.2.4 *Vpr(52-96)-mediated down regulation of TRAF-1 and TRAF-2 provides a mechanism for c-IAP-2-regulated protection against apoptosis and mitochondrial depolarization caused by Vpr(52-96)*

Several reports have emphasized the importance of TRAF-1 and TRAF-2 in c-IAP-2-mediated anti-apoptotic signaling as these proteins act as adaptors for physically interacting with and recruiting c-IAP-2 to the membrane bound signaling complex (90). Moreover, similar to c-IAP-2, TRAF-1 expression is significantly enhanced in several cancer cell types (428).

Given the importance of TRAF-1 and TRAF-2 for anti-apoptotic functions of c-IAP-2, I wanted to determine the functional relevance of TRAF-1 and TRAF-2 for c-IAP-2-mediated protection against apoptosis and mitochondrial membrane permeabilization caused by Vpr(52-96). Notably Vpr(52-96) induced rapid down regulation of both TRAF-1 and TRAF-2 in THP-1 cells (Fig 5.13A) which could be rescued by pretreatment with CpG, *E.coli* DNA or GpC (Fig 5.13B, C and D). However degradation of c-IAP-2 using SMC (Fig 5.5A) prior to stimulation with bacterial DNA abrogated the ability of CpG and *E.coli* DNA to protect TRAF-1 and TRAF-2 from Vpr(52-96)-mediated down regulation (Fig 5.13D). Note that the treatment with SMC alone did not alter TRAF-1 and TRAF-2 expression (Fig 5.13E). Furthermore, siRNA mediated knockdown of TRAF-1 and TRAF-2 (Fig 5.14C) induced significant loss of CpG-mediated protection against Vpr(52-96)-triggered apoptosis (Fig 5.14A) and mitochondrial depolarization (Fig 5.14B). These observations indicate that bacterial DNA-induced c-IAP-2 prevents Vpr(52-96)-mediated down regulation of TRAF-1 and TRAF-2 which play a central role in evading Vpr(52-96)-induced apoptosis and mitochondrial damage in human monocytic cells.

Fig 5.13: SMC pre-treatment protects against Vpr(52-96)-mediated down regulation of TRAF-1 and 2.

A. THP-1 cells ($2x10^6$/ml) were treated with 5 µM CpG for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 1 hr. **B**. THP-1 cells ($2.0x10^6$/ml) were treated with 25 µM chloroquine (CQ) for 2 hr prior to stimulation with 5 µM CpG, 25 µg *E.coli* DNA or 5 µM GpC for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) or Vpr(1-45) for 1 hr. **C**. THP-1 cells ($2x10^6$/ml) were treated with 5 µM CpG for 12 hr followed by treatment with 1.5 µM Vpr(52-96) or Vpr(1-45) for 1-2 hr. **D**. THP-1 cells ($2x10^6$/ml) were treated with 200 nM AEG-730 SMC and 5 µM CpG or 25 µg *E.coli* DNA for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 2 hr. **E**. THP-1 cells ($2x10^6$/ml) were treated with 0-200 nM SMC for 24 hr. Subsequently cell lysates were analyzed for TRAF-1 and TRAF-2 expression by immunoblotting. Membranes in A, B, D and E were reprobed with antibodies against GAPDH, where as membrane in C was probed with antibodies against Smac to normalize loading.

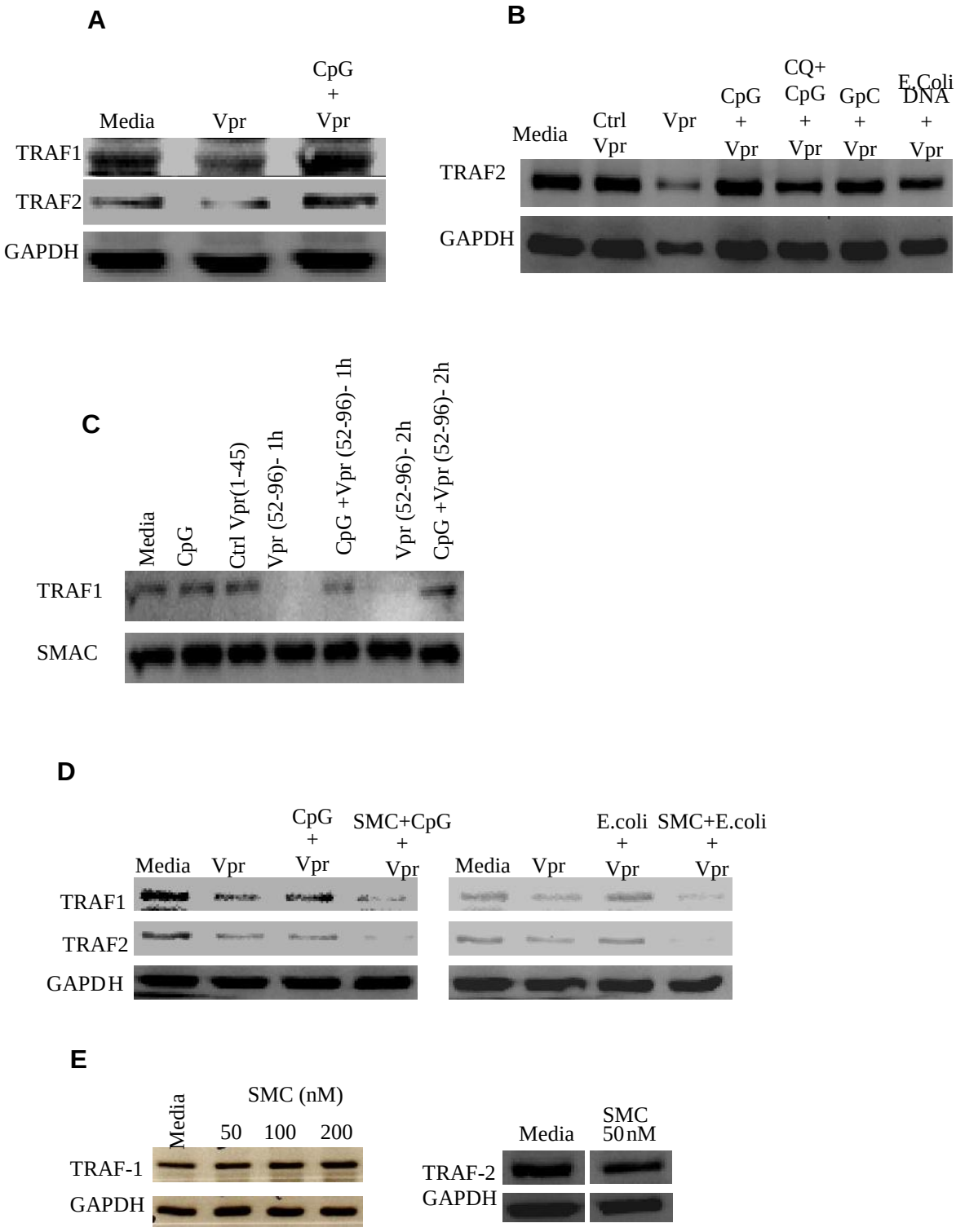
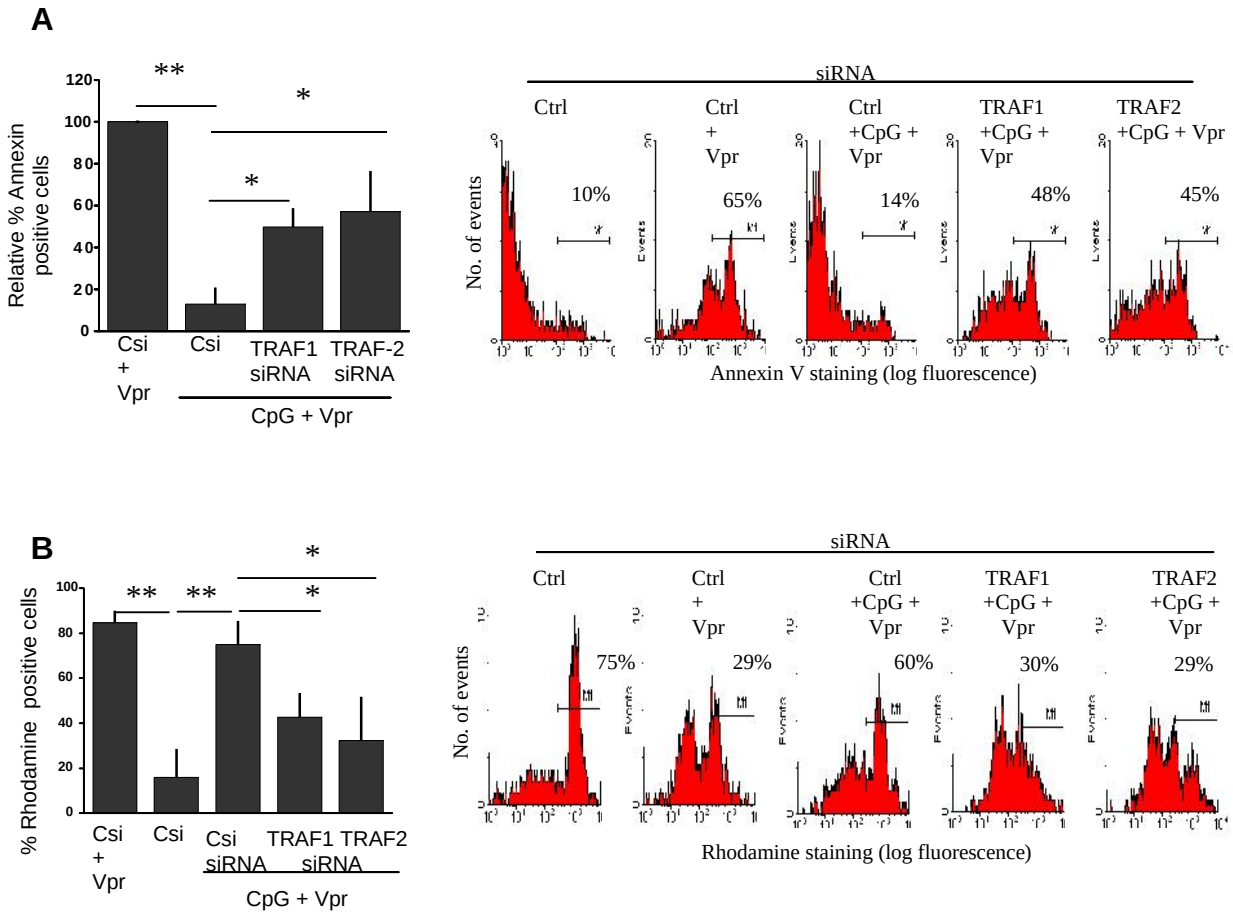

A
Media
Vpr
CpG + Vpr
TRAF1
TRAF2
GAPDH
B
Media
Ctrl Vpr
Vpr
CpG + Vpr
CQ+ CpG + Vpr
GpC + Vpr
E.Coli DNA + Vpr
TRAF2
GAPDH
C
Media
CpG
Ctrl Vpr(1-45)
Vpr (52-96)- 1h
CpG +Vpr (52-96)- 1h
Vpr (52-96)- 2h
CpG +Vpr (52-96)- 2h
TRAF1
SMAC
D
Media
Vpr
CpG + Vpr
SMC+CpG + Vpr
Media
Vpr
E.coli + Vpr
SMC+E.coli + Vpr
TRAF1
TRAF2
GAPDH
E
Media
SMC (nM)
50
100
200
TRAF-1
GAPDH
Media
SMC 50nM
TRAF-2
GAPDH

Figure 5.13

Fig 5.14: TRAF-1 and 2 siRNAs reverse the protective effect of CpG against Vpr(52-96)-induced apoptosis and loss of mitochondrial membrane potential.

THP-1 cells ($0.25x10^6$/0.5ml)were transfected with 20 nM TRAF-1 and TRAF-2 specific siRNAs or non-silencing control siRNA for 48 hr followed by stimulation with CpG (5 µM) for 12 hr. Cells were then treated with 1.5 µM Vpr(52-96) **(A)** for 24 hr followed by measurement of apoptosis by Annexin-V staining or **(B)** for 5 hr followed by staining with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. **C.** Cell lysates were analyzed for TRAF-1 and TRAF-2 expression by immunoblotting. Results in left panel in A and B are expressed as a mean ± SD of three independent experiments. The results shown in C and in the right panel in A and B are representatives of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).


A
Relative % Annexin positive cells
120
100
80
60
40
20
0
**
*
*
Csi + Vpr
Csi
TRAF1 siRNA
TRAF-2 siRNA
CpG + Vpr
siRNA
Ctrl
Ctrl + Vpr
Ctrl +CpG + Vpr
TRAF1 +CpG + Vpr
TRAF2 +CpG + Vpr
No. of events
10%
65%
14%
48%
45%
Annexin V staining (log fluorescence)
B
% Rhodamine positive cells
100
80
60
40
20
0
**
**
*
*
Csi + Vpr
Csi
Csi siRNA
TRAF1 siRNA
TRAF2 siRNA
CpG + Vpr
siRNA
Ctrl
Ctrl + Vpr
Ctrl +CpG + Vpr
TRAF1 +CpG + Vpr
TRAF2 +CpG + Vpr
No. of events
75%
29%
60%
30%
29%
Rhodamine staining (log fluorescence)

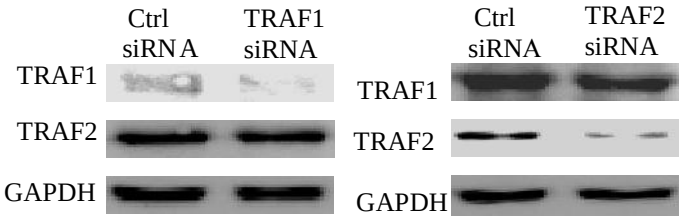

C
Ctrl siRNA
TRAF1 siRNA
TRAF1
TRAF2
GAPDH
Ctrl siRNA
TRAF2 siRNA
TRAF1
TRAF2
GAPDH

Figure 5.14

5.2.5 *Vpr(52-96)-mediated apoptosis and mitochondrial membrane permeabilization is regulated via activation of pro-apoptotic caspase 8 in THP-1 cells and primary human monocytes*

In order to identify which aspect of Vpr(52-96)-instigated apoptotic pathway was being interrupted by the c-IAP-2-TRAF-1-TRAF-2, it was first imperative to determine the mechanism of Vpr(52-96)-mediated apoptosis in human monocytic cells. Consistent with previously published data, Vpr(52-96) interacted physically with mitochondrial ANT in THP-1 cells as early as 30 min after treatment (Fig 5.15). Even though a large body of evidence implicates direct interaction with mitochondria as the primary mode of Vpr(52-96)-induced apoptosis, Vpr has been shown to activate caspase 8 in human neuronal cells, renal tubular epithelial cells and Jurkat cells (238, 252, 253). Therefore I wanted to explore whether caspase 8 activation was involved in Vpr(52-96)-mediated apoptosis and mitochondrial depolarization in monocytic cells. To this end it was first determined if Vpr(52-96) treatment could induce caspase 8 activation and whether treatment with bacterial DNA interfered in this process. Indeed it was observed that prior treatment with CpG, *E.coli* DNA and GpC (Fig 5.16 and 5.17) inhibited Vpr(52-96)-mediated caspase 8 activation. Consistent with these observations, treatment of primary monocytes with specific inhibitor of caspase 8 activation, ZVAD-IETD-FMK (429, 430), before addition of Vpr(52-96), significantly reduced Vpr(52-96)-mediated apoptosis (Fig 5.18A) and loss of mitochondrial membrane potential (Fig 5.18B). Cycloheximide and TNF-α have been widely shown to be strong inducers of caspase 8 mediated apoptosis (90, 426). Hence, the biological activity of caspase 8 specific ZVAD-IETD-FMK inhibitor was confirmed by its ability to inhibit cycloheximide and TNF-α-mediated apoptosis in THP-1 cells (Fig 5.19). Note that

prolonged treatment with ZVAD-IETD-FMK alone did not cause significant apoptosis or loss of mitochondrial membrane potential (Fig 5.18 and 5.19). Further evidence for functional importance of caspase 8 activation in Vpr(52-96)-induced cell death was obtained by knocking down caspase 8 expression using siRNA (Fig 5.20C). Cells transfected with caspase 8 siRNA exhibited significantly lower apoptosis (Fig 5.20A) and mitochondrial depolarization (Fig 5.20B) as compared to cells transfected with non-silencing siRNA, in response to Vpr(52-96)-treatment. RIP-1 had previously been shown to be an integral component of c-IAP-2-mediated inhibition of caspase 8 activation and cell survival (74, 92). However, contrary to previously published role of RIP-1, prior treatment with necrostatin, an inhibitor of RIP-1's biological activity, did not significantly alter CpG-induced cell survival in Vpr(52-96) treated cells (Fig 5.21), indicating that in human monocytic cells RIP-1 may not play a central role in bacterial DNA-mediated protection against Vpr(52-96)-induced apoptosis. Biological activity of Necrostatin was confirmed in the laboratory by caspase-independent cell death induced by the combined treatment of TNF-α/cycloheximide and ZVAD and identified by the lack of annexin V positivity in the presence of necrostatin. Taken together, these results suggest that stimulation with bacterial DNA serves to protect against Vpr(52-96)-induced apoptosis and mitochondrial depolarization by modulating caspase 8 activation in a RIP-1 independent manner.

Fig 5.15: Vpr(52-96) interacts physicaly with mitochondrial ANT.

THP-1 cells ($3x10^6$/ml) were treated with 1.5 µM HIV-Vpr(52-96) for 30 min to 8 hr. Mitochondrial fractions were harvested and subjected to immunoprecipitation against anti-ANT antibodies using A/G protein Agarose beads. Elutant was analyzed by immunoblotting for ANT, Vpr and Bax expression.

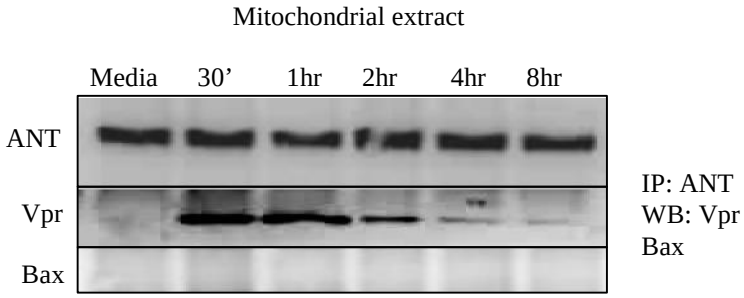
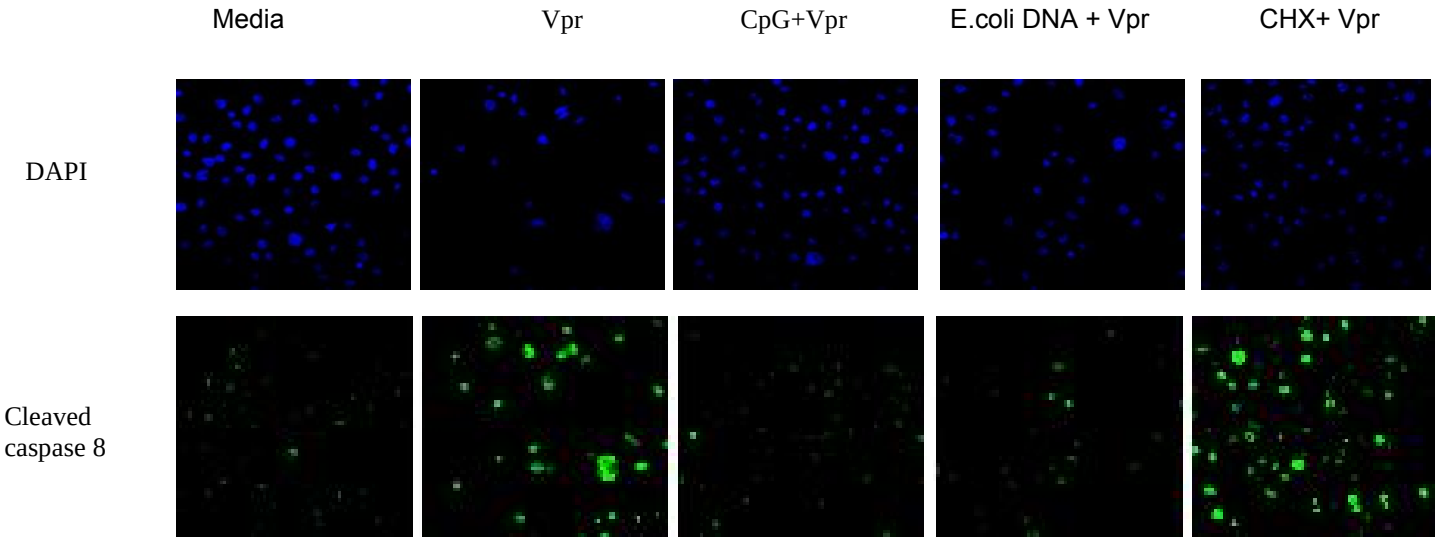

Mitochondrial extract
Media
30’
1hr
2hr
4hr
8hr
ANT
Vpr
Bax
IP: ANT
WB: Vpr
Bax

Figure 5.15

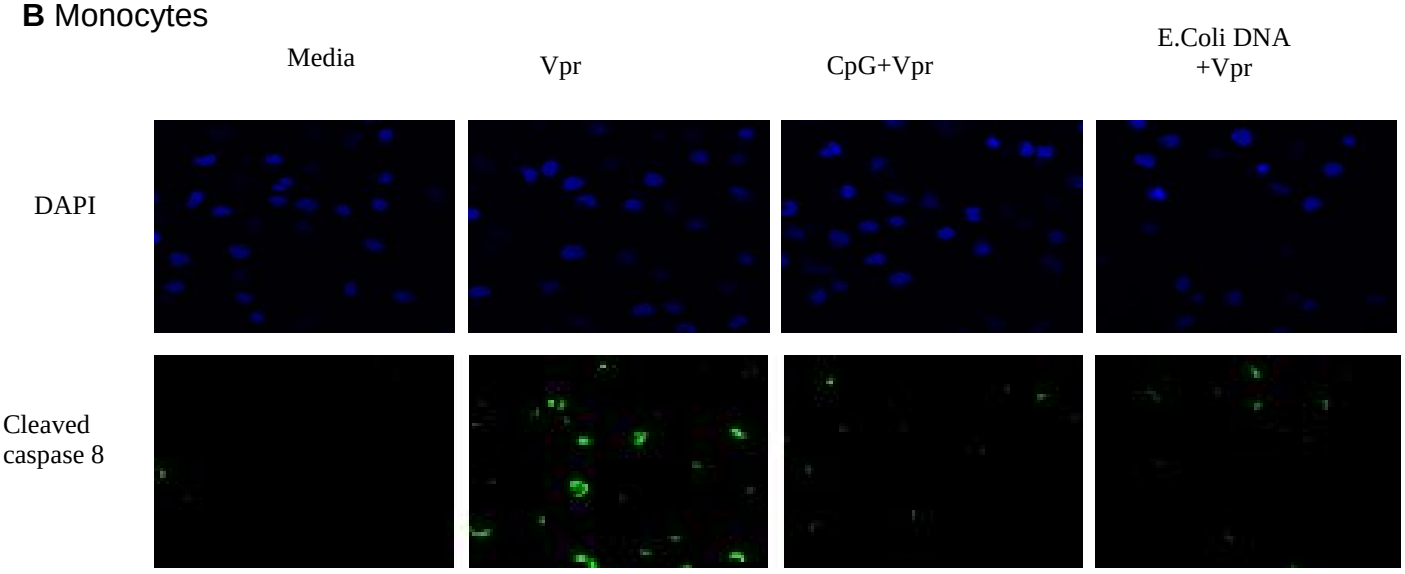
Fig 5.16: CpG and *E.coli* DNA pre-treatment prevents Vpr(52-96)-mediated caspase 8 activation.

THP-1 cells (**A**) ($1.0x10^6$/ml) and monocytes (**B**) ($1.0x10^6$/ml) were treated either with 20 ng TNF-α and 25 μg/ml cyclohexamide (CHX) for 6 hr alone or with 5 μM CpG and 25 μg*E.coli* DNA for 12 hr followed by treatment with 1.5 μM Vpr(52-96) for 5 hr (in THP-1 cells) and 2 hr (in monocytes). Cells were prepared for microscopy by staining for the nuclear stain DAPI (blue) and activated caspase 8 (green) before visualization using confocal microscope with a 63X lens at 1x magnification (THP-1 cells) and 4x magnification (monocytes). C. THP-1 cells (1.0x106/ml) stimulated with 5 μM CpG for 12 hr were treated with 1.5 μM Vpr(52-96) for 5 hr.Subsequently cells were treated with 1 μl FITC-IETD- ZFMK and analyzed by flow cytometry for detection of activated caspase 8.The results shown are representatives of three independent experiments.

A THP-1 cells



B Monocytes



C THP-1

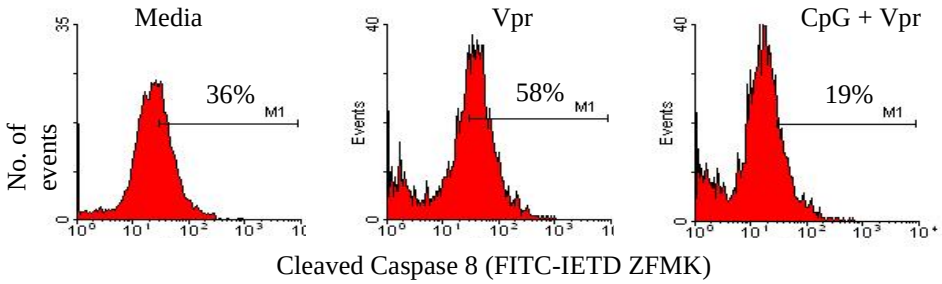


Figure 5.16

Fig 5.17: GpC pre-treatment prevents Vpr(52-96)-mediated caspase 8 activation.

A.THP-1 cells ($1.0x10^6$/ml) and monocytes ($1.0x10^6$/ml) were treated either with 5 µM GpC for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 5 hr (in THP-1 cells) and 2 hr (in monocytes). Cells were prepared for microscopy by staining for the nuclear stain DAPI (blue) and activated caspase 8 (green) before visualization using confocal microscope with a 63X lens at 1x magnification (THP-1 cells) and 4x magnification (monocytes).

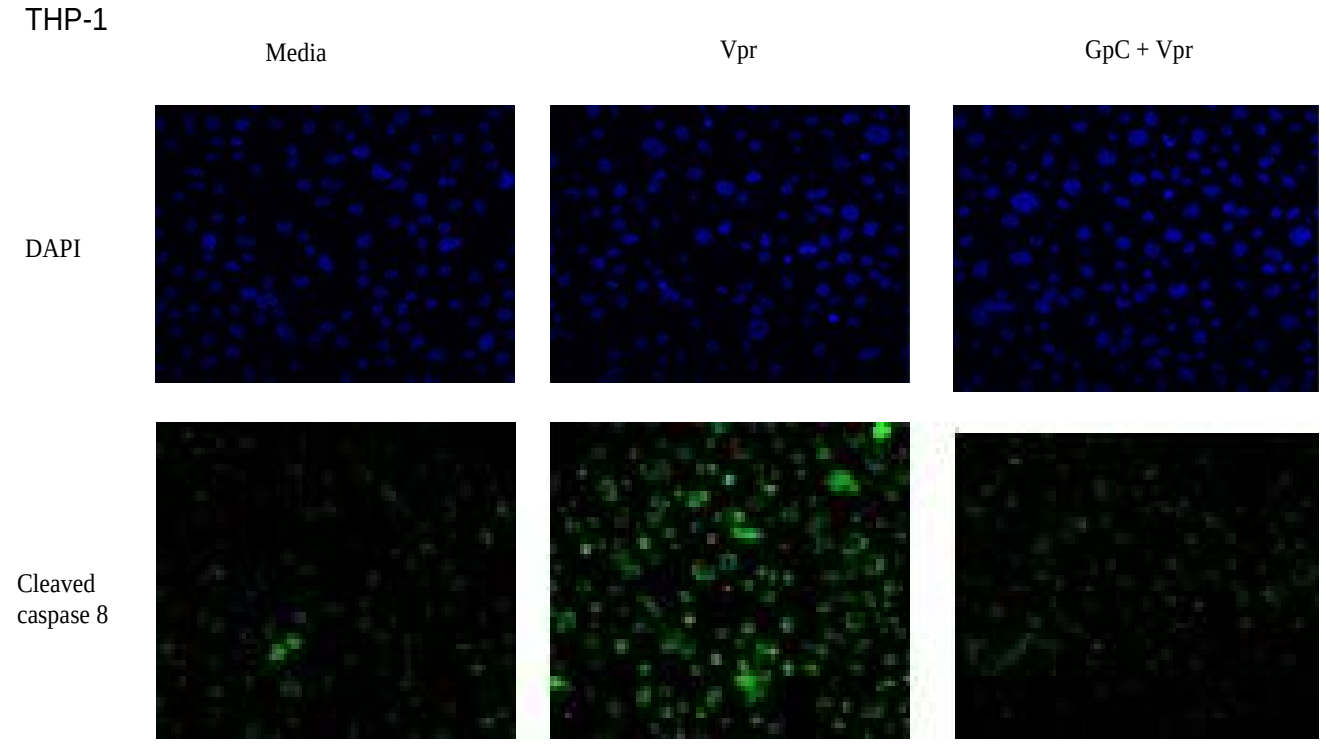

THP-1
Media
Vpr
GpC + Vpr
DAPI
Cleaved caspase 8
Monocytes
Media
Vpr
GpC + Vpr
DAPI
Cleaved caspase 8

Figure 5.17

Fig 5.18: Specific inhibitor of caspase 8 activation inhibits Vpr(52-96)-induced apoptosis and mitochondrial membrane permeabilization in primary human monocytes.

Monocytes ($1x10^6$/ml) were treated with 20 μM ZVAD-IETD-FMK for 2 hr before treatment with 1.5 μM Vpr(52-96); **(A)** for 4 hr followed by measurement of apoptosis by Annexin-V staining or **(B)** for 2 hr followed by staining with MitoTracker green for mitochondrial membrane potential evaluation by flow cytometry. Results in top panels in A and B are expressed as a mean ± SD of three independent experiments. The results shown in bottom panels in A and B are representatives of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

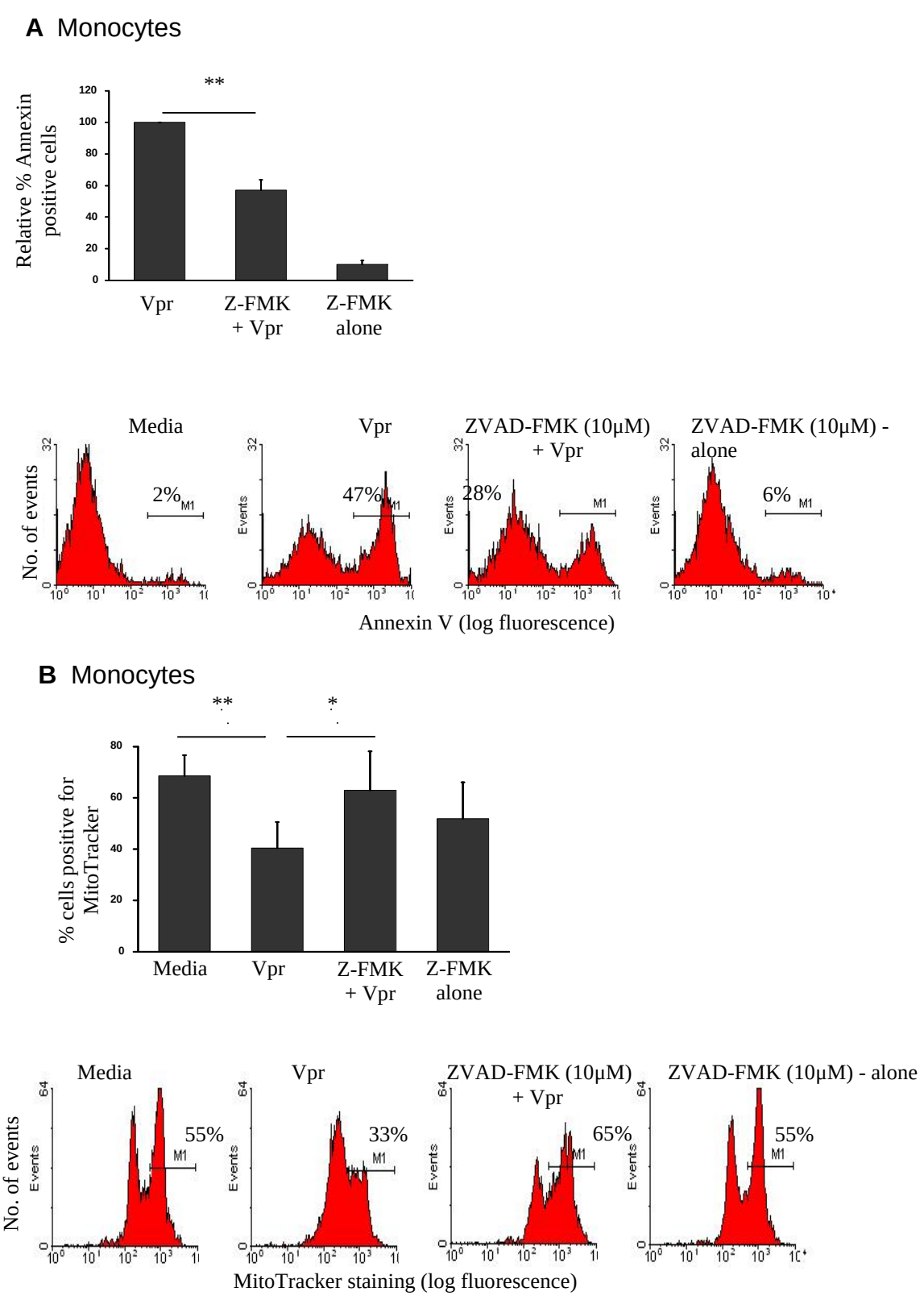

A Monocytes
**
Relative % Annexin positive cells
120
100
80
60
40
20
0
Vpr
Z-FMK + Vpr
Z-FMK alone
Media
Vpr
ZVAD-FMK (10μM) + Vpr
ZVAD-FMK (10μM) - alone
No. of events
Events
2%
47%
28%
6%
M1
Annexin V (log fluorescence)
B Monocytes
**
*
% cells positive for MitoTracker
80
60
40
20
0
Media
Vpr
Z-FMK + Vpr
Z-FMK alone
Media
Vpr
ZVAD-FMK (10μM) + Vpr
ZVAD-FMK (10μM) - alone
No. of events
Events
55%
33%
65%
55%
M1
MitoTracker staining (log fluorescence)

Figure 5.18

Fig 5.19: ZVAD-IETD-FMK reversed TNF-α and cyclohexamide-induced apoptosis.

THP-1 cells ($1x10^6$/ml) were treated with 20 μM ZVAD-IETD-FMK for 2 hr before treatment with 20 ng TNF-α and 25 μg/ml cylohexamide for 24 hr followed by measurement of apoptosis by Annexin-V staining. The results shown are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

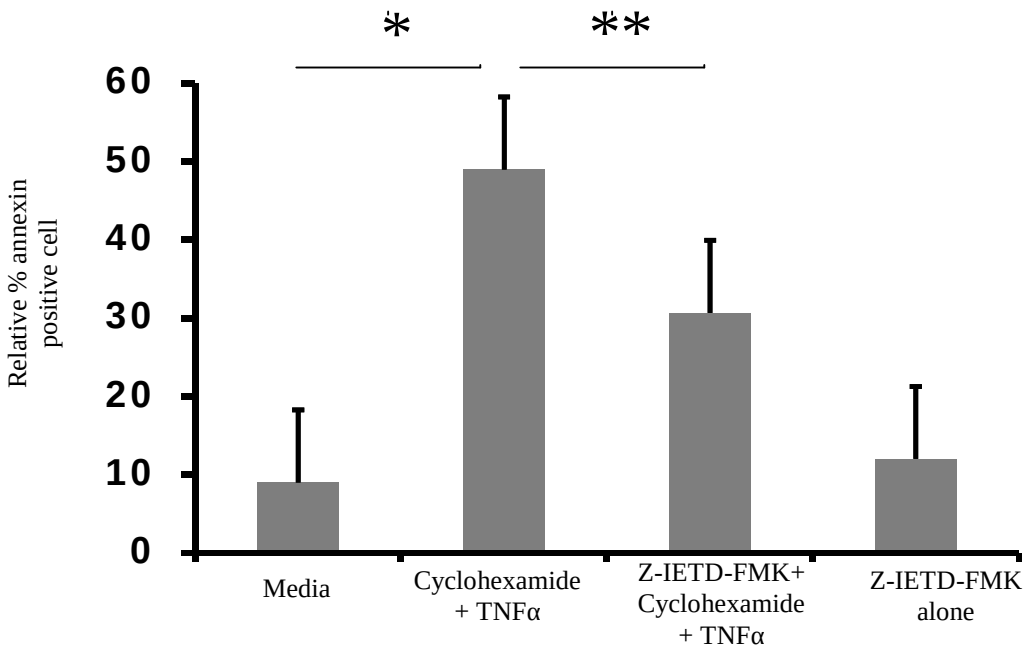

*
**
60
50
40
30
20
10
0
Relative % annexin positive cell
Media
Cyclohexamide + TNFα
Z-IETD-FMK+ Cyclohexamide + TNFα
Z-IETD-FMK alone

Figure 5.19

Fig 5.20: Vpr(52-96)-induced apoptosis and mitochondrial membrane permeabilization are dependent upon caspase 8 activation in THP-1 cells.

THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 10 nM caspase 8 specific siRNA or non-silencing control siRNA for 48 hr followed by treatment with 1.5 µM Vpr(52-96) **(A)** for 24 hr followed by measurement of apoptosis by Annexin-V staining or **(B)** for 5 hr followed by staining with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. **C** Cell lysates were analyzed for caspase 8 expression by immunoblotting. Results in left panels in A and B are expressed as a mean ± SD of three independent experiments. The results shown in C and right panels in A and B are a representative of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

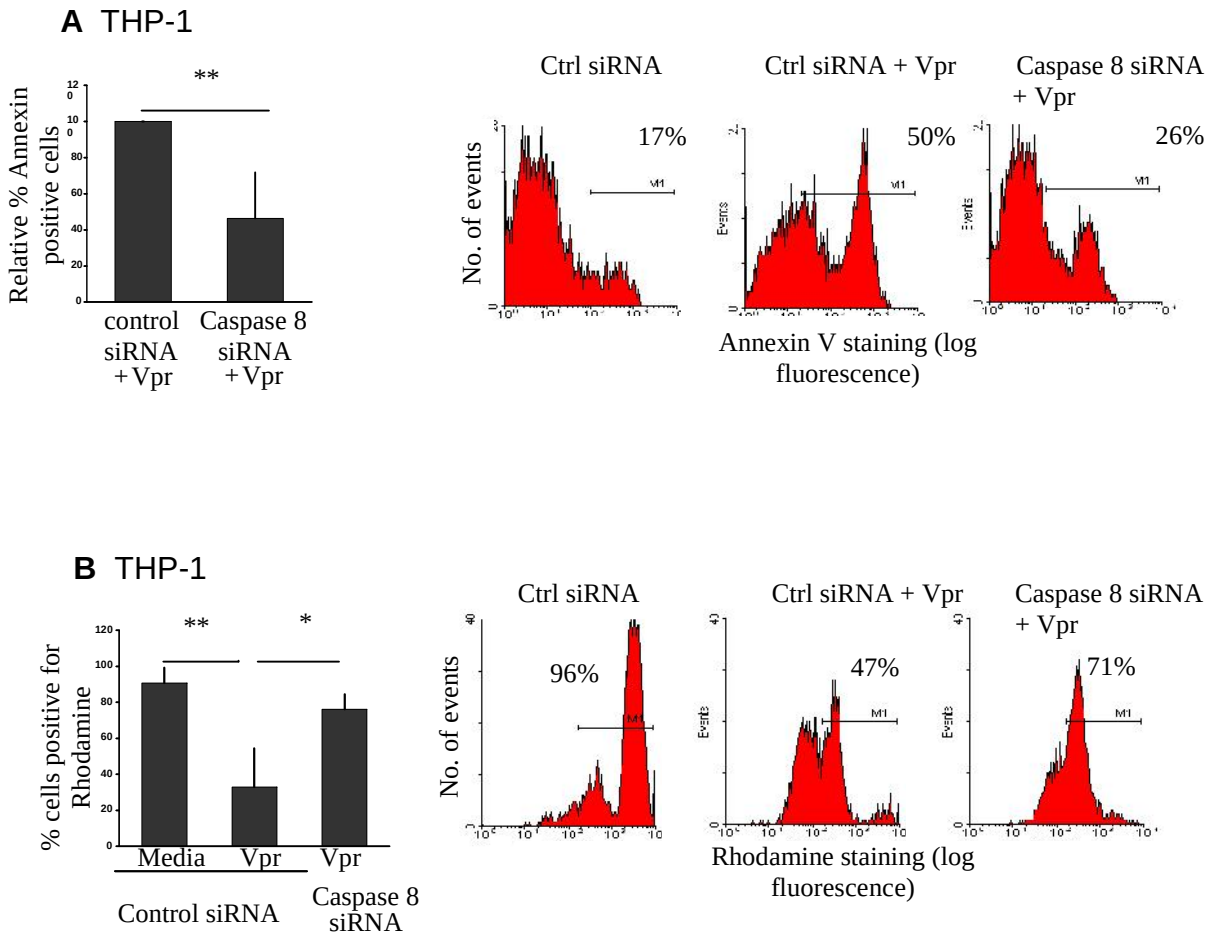
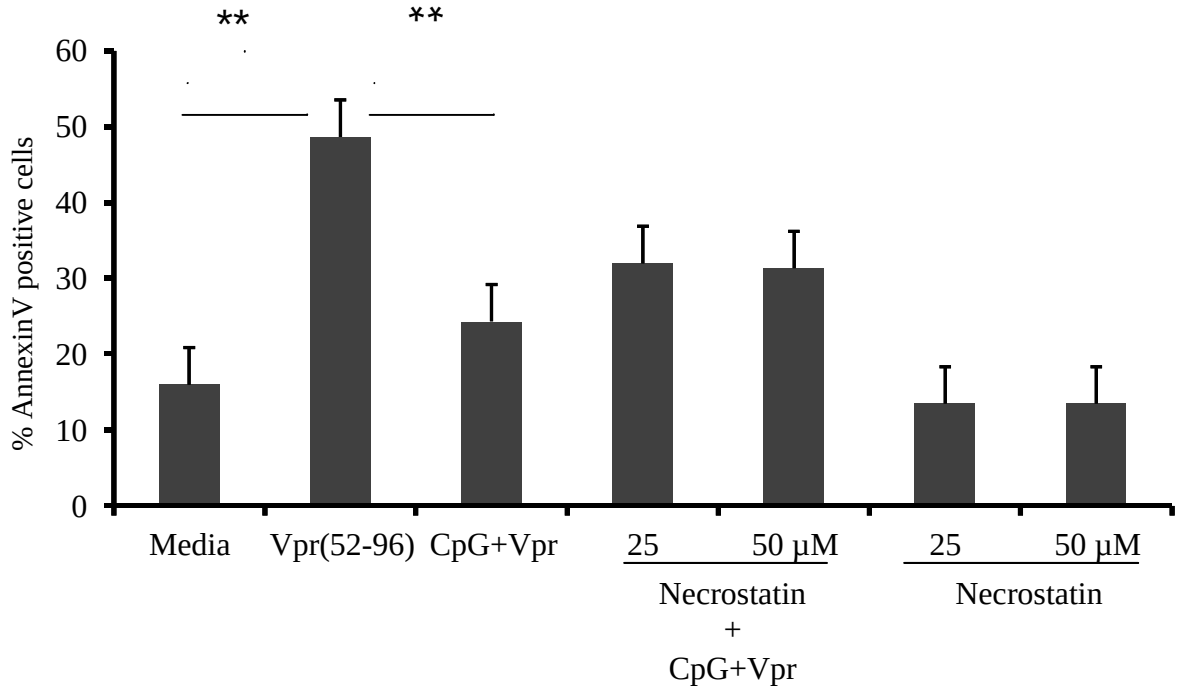

A THP-1
**
Relative % Annexin positive cells
control siRNA +Vpr
Caspase 8 siRNA +Vpr
Ctrl siRNA
Ctrl siRNA + Vpr
Caspase 8 siRNA + Vpr
17%
50%
26%
No. of events
Annexin V staining (log fluorescence)
B THP-1
**
*
% cells positive for Rhodamine
Media
Vpr
Vpr
Control siRNA
Caspase 8 siRNA
Ctrl siRNA
Ctrl siRNA + Vpr
Caspase 8 siRNA + Vpr
96%
47%
71%
No. of events
Rhodamine staining (log fluorescence)
C THP-1
Ctrl siRNA
Cas8 siRNA
Full length caspase 8
GAPDH

Figure 5.20

Fig 5.21: Necrostatin pretreatment does not interrupt CpG-induced protection against Vpr(52-96)-mediated apoptosis in human monocytic cells.

Monocytes (**A**) ($1.0x10^6$/ml) and THP-1s **(B)** ($1.0x10^6$/ml) were treated with 25-50 µM necrostatin prior to stimulation with 5 µM CpG-ODN for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 4 hr (monocytes) and 24 hr (THP-1 cells) followed by measurement of apoptosis by Annexin-V staining. The results in A are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).


A Monocytes
**
**
% AnnexinV positive cells
60
50
40
30
20
10
0
Media
Vpr(52-96)
CpG+Vpr
25
50 µM
Necrostatin
+
CpG+Vpr
25
50 µM
Necrostatin

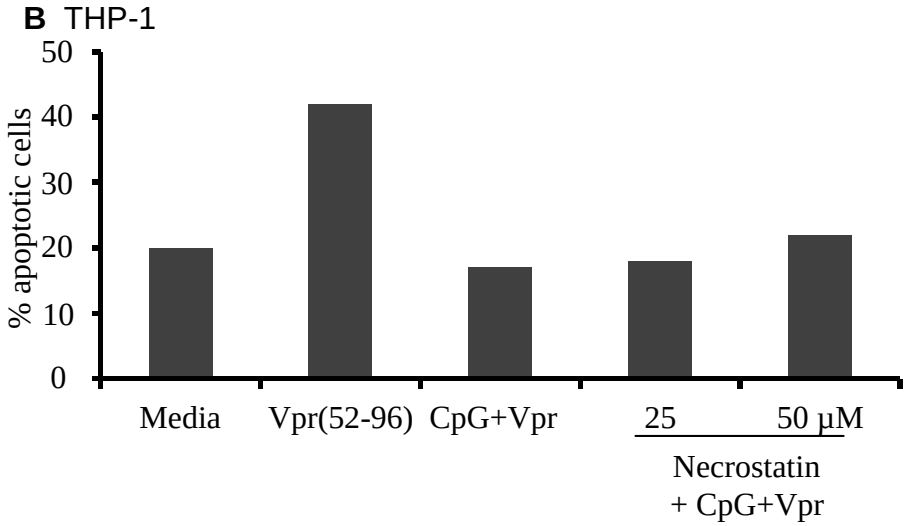

B THP-1
% apoptotic cells
50
40
30
20
10
0
Media
Vpr(52-96)
CpG+Vpr
25
50 µM
Necrostatin
+ CpG+Vpr

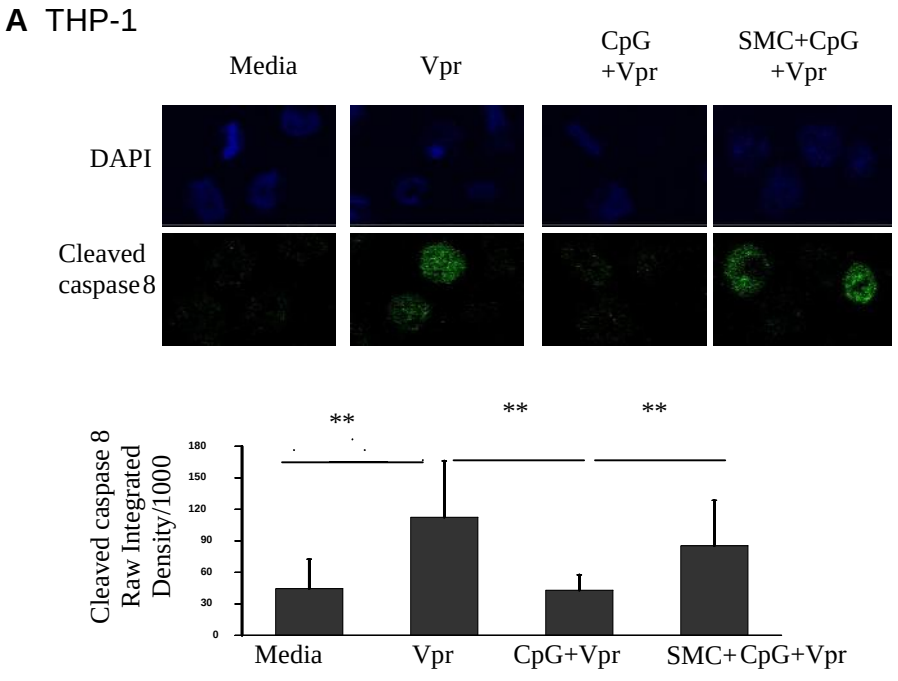
Figure 5.21

5.2.6 *Bacterial DNA-induced c-IAP-2 inhibits Vpr(52-96)-mediated caspase 8 activation in THP-1 cells and primary monocytes*

Taking into account: 1) The propensity of c-IAP-2 for inhibiting TNF-α-induced apoptosis by hindering caspase 8 activation (91, 103, 426), 2) the importance of caspase 8 activation for Vpr(52-96)-induced apoptotic affects in monocytic cells (Fig 5.16-5.18 and 5.20) and 3) the reversal of Vpr(52-96)-induced caspase 8 activation, mitochondrial depolarization and apoptosis by bacterial DNA pre-treatment, lead me to hypothesize that direct inhibition of Vpr(52-96)-mediated caspase 8 activation may be a key step in c-IAP-2 regulated anti-apoptotic mechanisms. Indeed it was observed that protection afforded by CpG against Vpr(52-96)-mediated caspase 8 activation was reversed if c-IAP-2 was degraded, using SMC, as observed by employing, a) antibodies against activated caspase 8 and visualizing caspase 8 activation with confocal microscopy (Fig 5.22A and 5.23A**)**, b) by incubating Vpr(52-96) treated cells with FITC-conjugated caspase 8 inhibitor that specifically binds to cleaved caspase 8 and allows for analysis of caspase 8 activation by flow cytometry (Fig 5.23B), and c) by analyzing cellular lysates from Vpr(52-96) treated cells by western blotting using antibodies against full length and cleaved caspase 8 (Fig 5.22B). Cells treated with cycloheximide and TNF-α were used as positive controls for caspase 8 activation (Fig 5.20C, 5.22B and 5.24). Notably treatment with SMC alone did not cause significant caspase 8 activation in THP-1 cells or primary monocytes (Fig 5.24). Moreover siRNA-mediated knockdown of TRAF-1 and TRAF-2 significantly abrogated the protection afforded by CpG against Vpr(52-96)-mediated caspase 8 activation in THP-1 cells (Fig 5.25). These observations indicate that TRAF-1 and TRAF-2 help inhibit caspase 8 activation by Vpr(52-96), once their expression is stabilized by c-IAP-2.

Fig 5.22: Pre-treatment with SMC reverses CpG-mediated rescue of caspase 8 activation induced by Vpr(52-96) in THP-1 cells.

A. THP-1 cells ($1.0x10^6$/ml) were treated with 200 nm AEG-730 SMC and 5 µM CpG for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) for 5 hr and prepared for microscopy by staining for nuclear stain DAPI (blue) and activated caspase 8 (green) before visualization using confocal microscope with a 63X lens at 4x magnification. Green fluorescence intensity from 50 randomly chosen cells from each sample was tabulated to obtain the bar graph. **B**. THP-1 cells ($2.0x10^6$/ml) were stimulated with either 20 ng TNF-α and 25 µg/ml cylohexamide alone; or with 200 nM AEG-730 SMC and 5 µM CpG for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 5 hr. Cell lysates were analyzed for full length and cleaved caspase 8 expression by immunoblotting. Results in bottom panel in A are expressed as a mean ± SD of three independent experiments. The results shown in B and top panel in A are representatives of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).


A THP-1
Media
Vpr
CpG +Vpr
SMC+CpG +Vpr
DAPI
Cleaved caspase 8
Cleaved caspase 8 Raw Integrated Density/1000
180
150
120
90
60
30
0
**
**
**
Media
Vpr
CpG+Vpr
SMC+CpG+Vpr

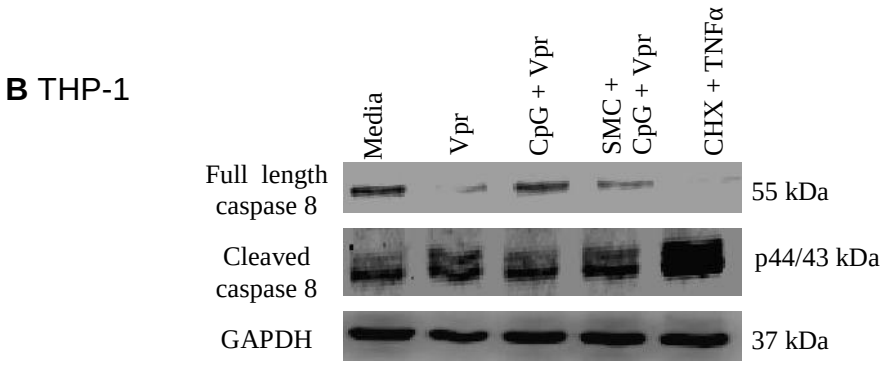
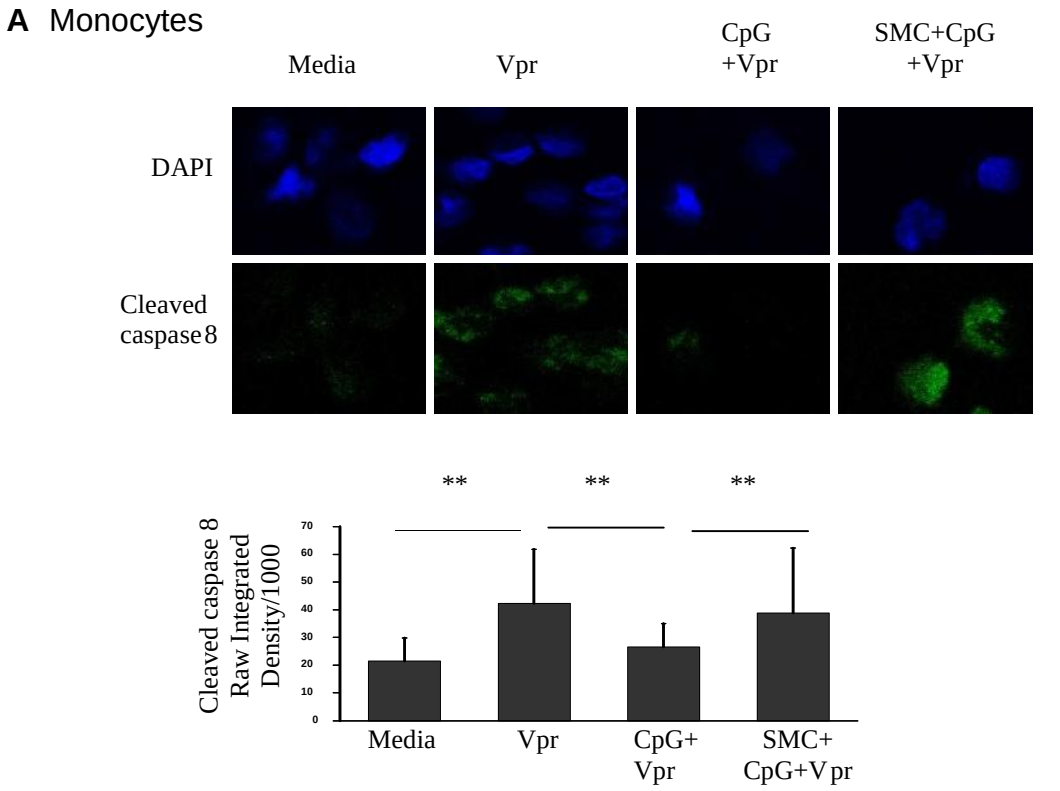

B THP-1
Media
Vpr
CpG + Vpr
SMC + CpG + Vpr
CHX + TNFα
Full length caspase 8
55 kDa
Cleaved caspase 8
p44/43 kDa
GAPDH
37 kDa

Figure 5.22

Fig 5.23: Pre-treatment with SMC reverses CpG-mediated prevention of caspase 8 activation induced by Vpr(52-96) in primary human monocytes.

A. Monocytes ($1.0x10^6$/ml) were treated with 200 nm AEG-730 SMC and 5 µM CpG-ODN for 12 hr. Subsequently cells were treated with 1.5 µM Vpr(52-96) for 2 hr and prepared for microscopy by staining for the nuclear stain DAPI (blue) and activated caspase 8 (green) before visualization using confocal microscope with a 63X lens at 9x magnification. Green fluorescence intensity from 50 randomly chosen cells from each sample was tabulated to obtain the bar graph. B. Monocytes (1.0x106/ml) were treated with 200 nm AEG-730 SMC and 5 µM CpG-ODN for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 2 hr. Subsequently the cells were treated with 1µl FITC-IETD-ZFMK and analyzed by flow cytometry for detection of activated caspase 8. The results shown in top panels in A and B are representatives of three independent experiments and the results bottom panels in A and B are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).


A Monocytes
Media
Vpr
CpG +Vpr
SMC+CpG +Vpr
DAPI
Cleaved caspase 8
**
**
**
Cleaved caspase 8 Raw Integrated Density/1000
70
60
50
40
30
20
10
0
Media
Vpr
CpG+ Vpr
SMC+ CpG+Vpr

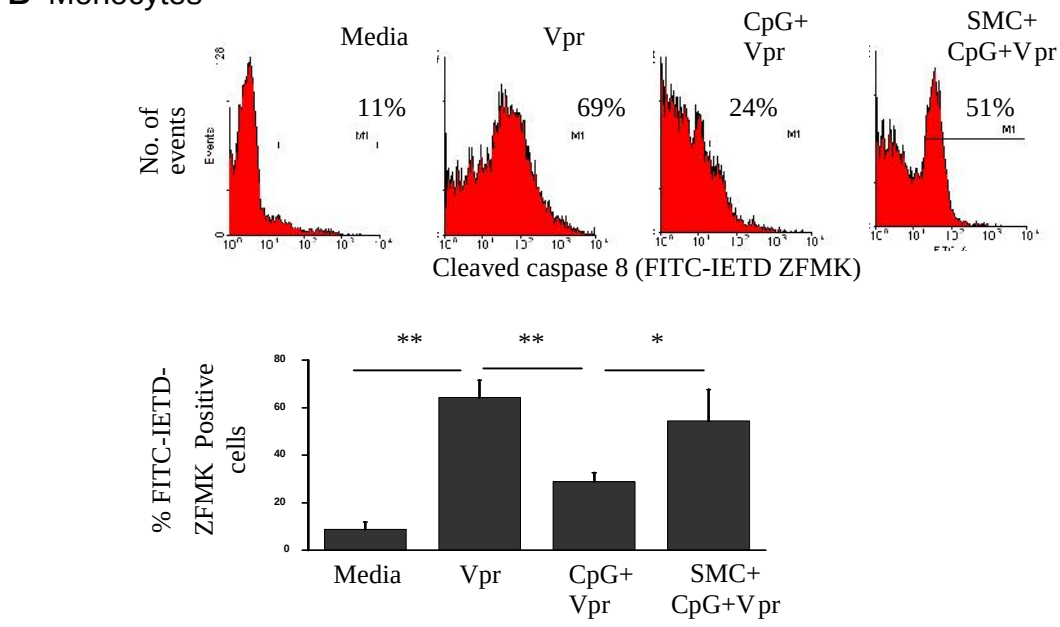
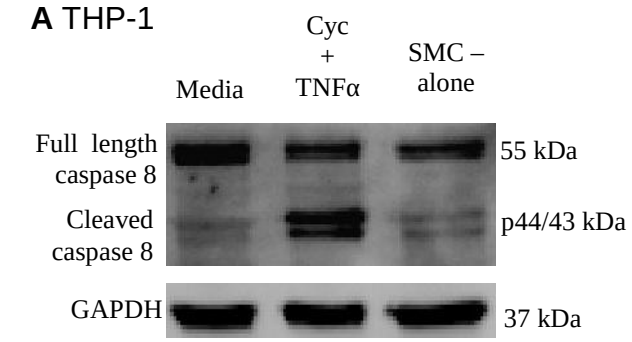

B Monocytes
Media
Vpr
CpG+ Vpr
SMC+ CpG+Vpr
No. of events
11%
69%
24%
51%
M1
Cleaved caspase 8 (FITC-IETD ZFMK)
**
**
*
% FITC-IETD-ZFMK Positive cells
80
60
40
20
0
Media
Vpr
CpG+ Vpr
SMC+ CpG+Vpr

Figure 5.23

Fig 5.24: Treatment with SMC alone does not cause caspase 8 activation.

A. THP-1 cells ($1.0x10^6$/ml) were stimulated with 200 nM AEG-730 SMC or 20 ng TNF-α and 25 μg/ml cyclohexamide for 12 hr. **B**. Monocytes ($1.0x10^6$/ml) were stimulated with 50-200 nM AEG-730 SMC or 20 ng TNF-α and 25 μg/ml cycloheximide for 12 hr. Cell lysates were analyzed for full length and cleaved caspase 8 expression by immunoblotting.


A THP-1
Media
Cyc + TNFα
SMC – alone
Full length caspase 8
Cleaved caspase 8
GAPDH
55 kDa
p44/43 kDa
37 kDa

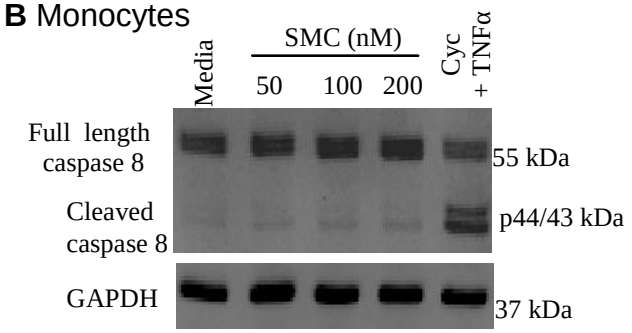

B Monocytes
Media
SMC (nM)
50
100
200
Cyc + TNFα
Full length caspase 8
Cleaved caspase 8
GAPDH
55 kDa
p44/43 kDa
37 kDa

Figure 5.24

Fig 5.25: CpG-induced protection from Vpr(52-96)-mediated caspase 8 activation is dependent on TRAF-1 and TRAF-2.

THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 20 nM TRAF-1 and TRAF-2 specific siRNAs or non-silencing control siRNA for 48 hr followed by stimulation with CpG (5 µM) for 12 hr. Cells were then treated with 1.5 µM Vpr(52-96) for 5 hr followed by treatment with 1µl FITC-IETD-ZFMK and analyzed by flow cytometry for detection of activated caspase 8. Results in top panel are representative of three independent experiments. The results in the bottom panel are expressed as a mean ± SD of three independent experiments. *($p < 0.05$), ** ($p < 0.005$). For evidence of effective knock down of TRAF-1 and TRAF-2 please see to Fig. 5.14.

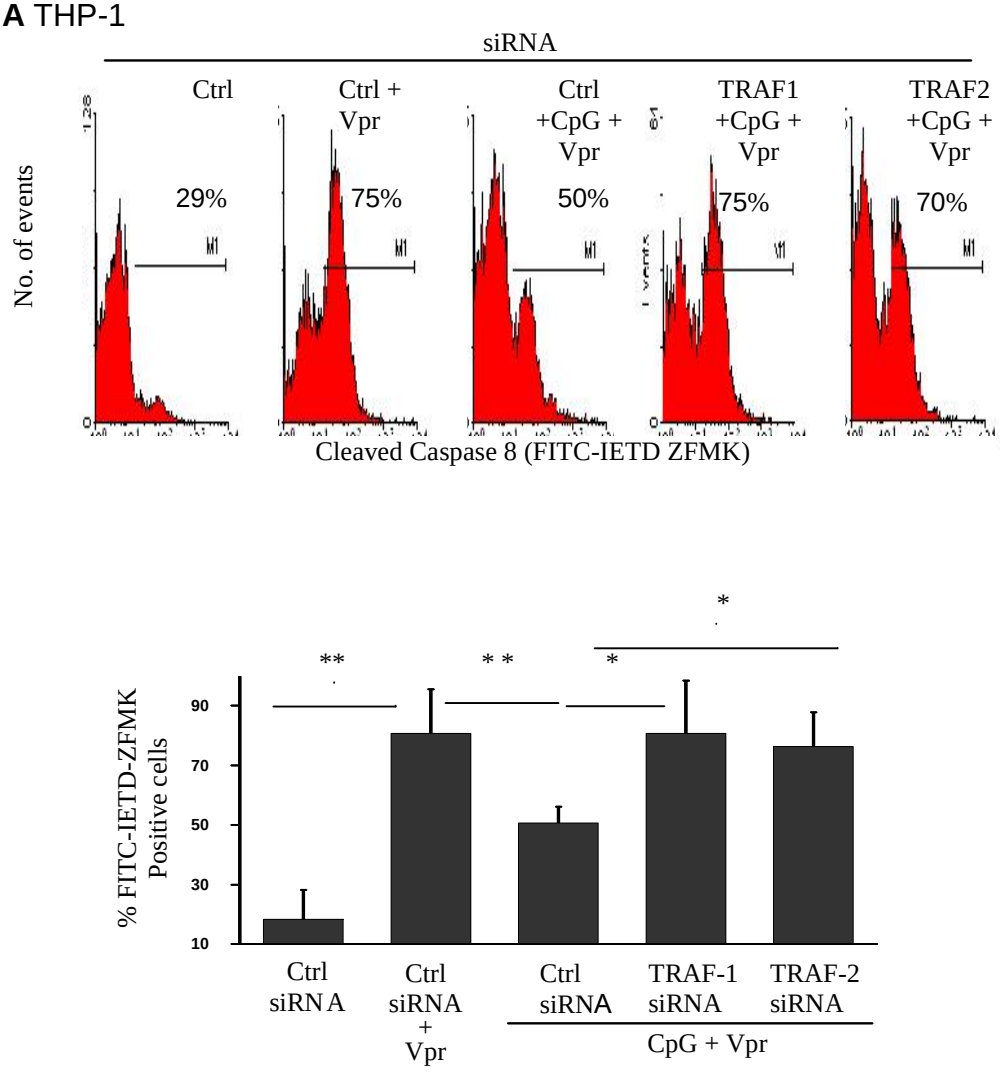

A THP-1
siRNA
Ctrl
Ctrl + Vpr
Ctrl +CpG + Vpr
TRAF1 +CpG + Vpr
TRAF2 +CpG + Vpr
29%
75%
50%
75%
70%
No. of events
Cleaved Caspase 8 (FITC-IETD ZFMK)
% FITC-IETD-ZFMK Positive cells
90
70
50
30
10
**
* *
*
*
Ctrl siRNA
Ctrl siRNA + Vpr
Ctrl siRNA
TRAF-1 siRNA
TRAF-2 siRNA
CpG + Vpr

Figure 5.25

5.2.7 *Pretreatment with bacterial DNA prevents Vpr(52-96)-mediated activation of pro-apoptotic Bid and Bax.*

Activated caspase 8 has been demonstrated to orchestrate apoptosis either by directly activating caspases 3/7 thereby bypassing the mitochondria completely (28) or by activating its downstream pro-apoptotic substrate Bid (431). Since mitochondrial depolarization was identified as a key component of Vpr-induced cell death (Fig 5.2) it was reasonable to hypothesize that Vpr(52-96)-activated apoptotic signaling via caspase 8 may be mediated by Bid activation. Inorder to investigate this hypothesis Bid, was knocked down using siRNAs (Fig 5.26C**)**. Samples transfected with Bid siRNA were less sensitive to apoptosis (Fig 5.26A) and mitochondrial potential loss (Fig 5.26B) in response to Vpr(52-96) treatment as compared to control siRNA transfected samples. The biological activity of Bid siRNA was confirmed by it ability to inhibit cycloheximide and TNF-α-mediated apoptosis in THP-1 cells (Fig 5.26D).

Bid mediated apoptosis can be regulated in several different ways (39). Upon activation by cleavage, pro-apoptotic tBid, may either directly translocate to the mitochondrial surface and induce mitochondrial membrane permeabilization or, tBid may activate another pro apoptotic Bcl-2 family member, Bax (30, 40). Activated Bax mediates apoptosis by embedding and oligomerizing within the mitochondrial membrane, causing loss of mitochondrial potential and apoptosis (58, 59, 65). siRNA and shRNA mediated knock down of Bax, was recently shown to abrogate Vpr-mediated cell death in HeLa cells and human epithelial cells (16, 252). Therefore I wanted to determine whether Vpr(52-96)-mediated caspase 8-dependant apoptotic mechanism involved Bax activation in human monocytic cells. Bax activation entails undergoing a conformational change revealing amino

terminal epitope recognized by the monoclonal antibody 6A7 (58, 432, 433). To detect Bax activation, in response to Vpr(52-96), cells were stained with 6A7 Bax antibody and analyzed by confocal microscopy. Prior treatment with CpG (Fig 5.27A), *E.coli* DNA (Fig 5.27B) and GpC (Fig 5.28) inhibited Vpr(52-96)-mediated Bax activation in both THP-1 cells and monocytes. Furthermore, THP-1 cells transfected with Bax siRNA (Fig 5.29C) exhibited significantly lower apoptosis (Fig 5.29A) and mitochondrial depolarization (Fig 5.29B) in response to Vpr(52-96) treatment as compared to cells transfected with non-silencing siRNA, suggesting the significance of Bax in Vpr(52-96)-mediated cell death in this cell type.

Fig 5.26:Vpr(52-96)-induced apoptosis and mitochondrial membrane permeabilization are dependent upon Bid activation in THP-1 cells.

THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 40 nM Bid specific siRNA or non-silencing control siRNA for 48 hr followed by treatment with 1.5 µM Vpr(52-96) **(A)** for 24 hr followed by measurement of apoptosis by Annexin-V staining or **(B)** for 5 hr followed by staining with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. **C.** Cell lysates were analyzed for Bid expression by immunoblotting. **D**. THP-1 cells were transfected with Bid siRNA as mentioned above and then treated with 20 ng TNF-α and 25 µg/ml cyloheximide for 24 hr followed by measurement of apoptosis by Annexin-V staining. Results in left panel in A is expressed as a mean ± SD of three independent experiments. The results shown in C and right panels in A are a representative of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

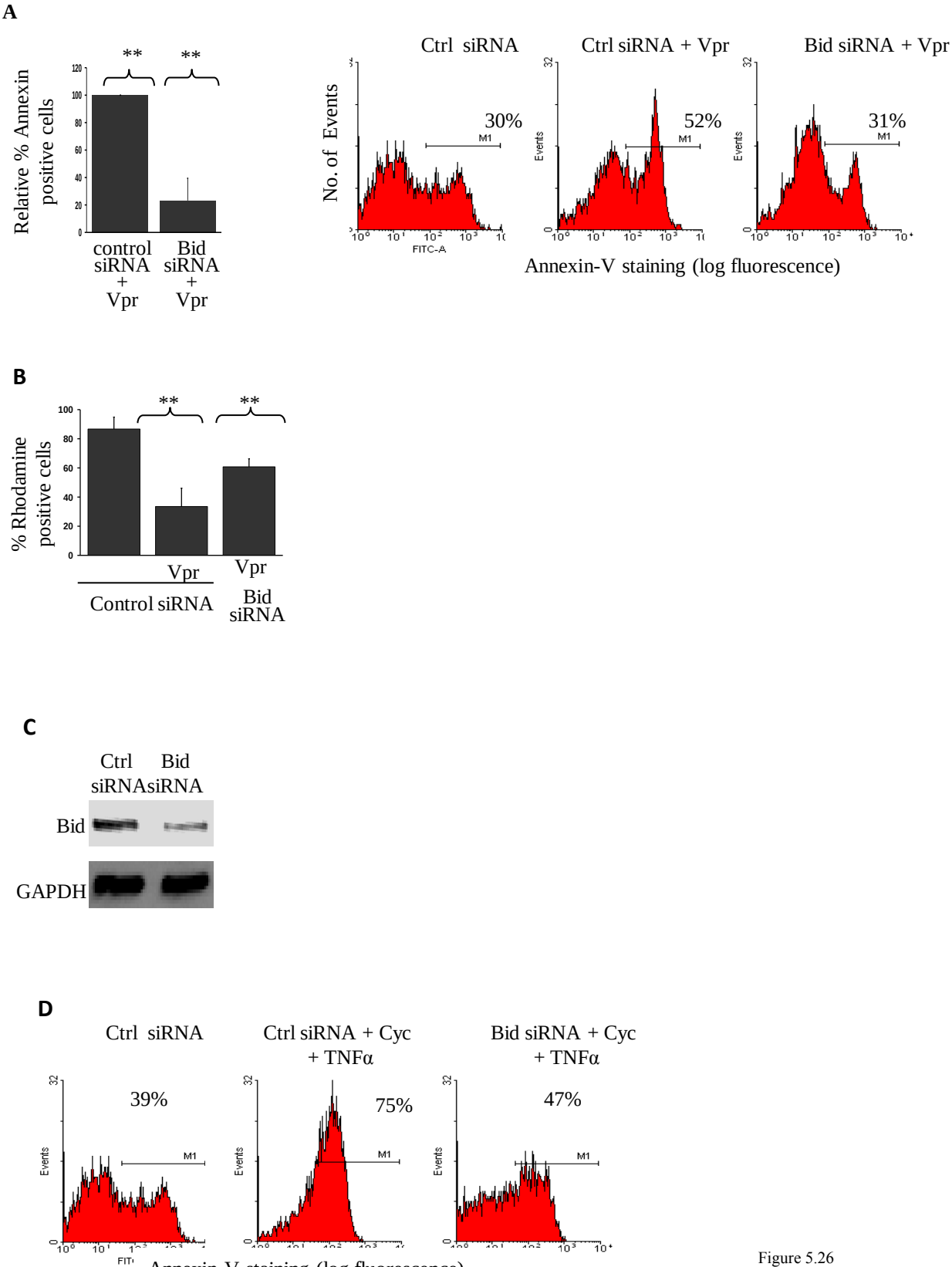
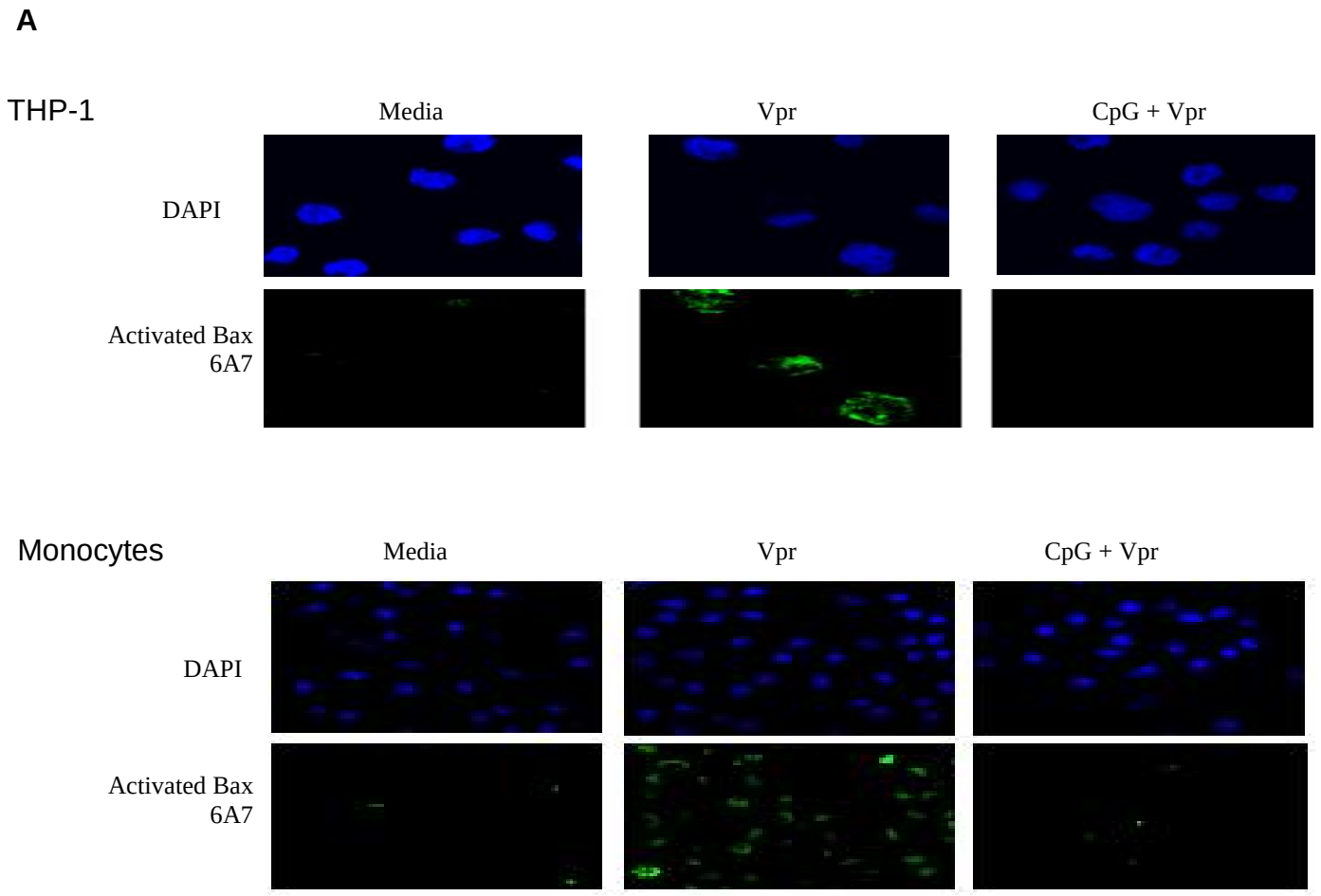

A
Relative % Annexin positive cells
**
**
control siRNA + Vpr
Bid siRNA + Vpr
Ctrl siRNA
Ctrl siRNA + Vpr
Bid siRNA + Vpr
No. of Events
30%
52%
31%
M1
Annexin-V staining (log fluorescence)
B
% Rhodamine positive cells
**
**
Vpr
Vpr
Control siRNA
Bid siRNA
C
Ctrl siRNA
Bid siRNA
Bid
GAPDH
D
Ctrl siRNA
Ctrl siRNA + Cyc + TNFα
Bid siRNA + Cyc + TNFα
39%
75%
47%
Events
M1
Annexin-V staining (log fluorescence)

Figure 5.26

Fig 5.27: Bacterial DNA pre-treatment inhibits Vpr(52-96)-induced Bax activation.

A. THP-1 cells ($1.0x10^6$/ml) and monocytes ($1.0x10^6$/ml) were treated with 5 µM CpG for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 5 hr (in THP-1 cells) and 2 hr (monocytes). **B**. Monocytes ($1.0x10^6$/ml) were treated with 5 µM CpG or 25 µg *E.coli* DNA for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 2 hr. Subsequently cells were prepared for microscopy by staining for the nuclear stain DAPI (blue) and activated Bax (green) and visualization using confocal microscope with a 63X lens at 1x magnification (THP-1 cells) and 4x magnification (monocytes). The results are representative of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).


A
THP-1
Media
Vpr
CpG + Vpr
DAPI
Activated Bax
6A7
Monocytes
Media
Vpr
CpG + Vpr
DAPI
Activated Bax
6A7

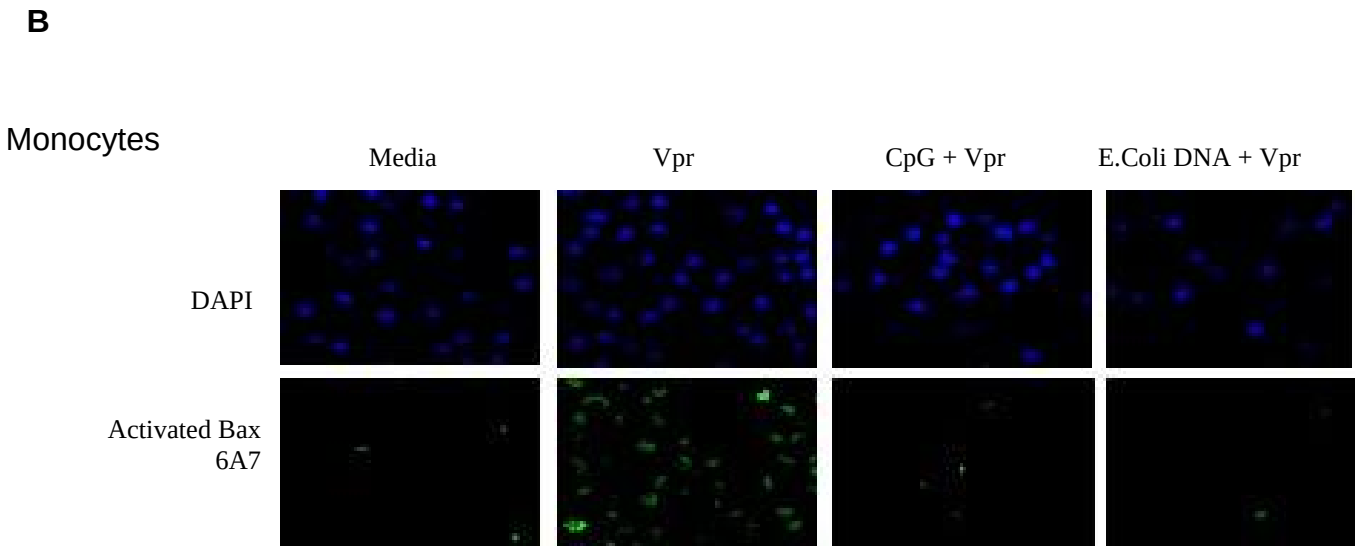
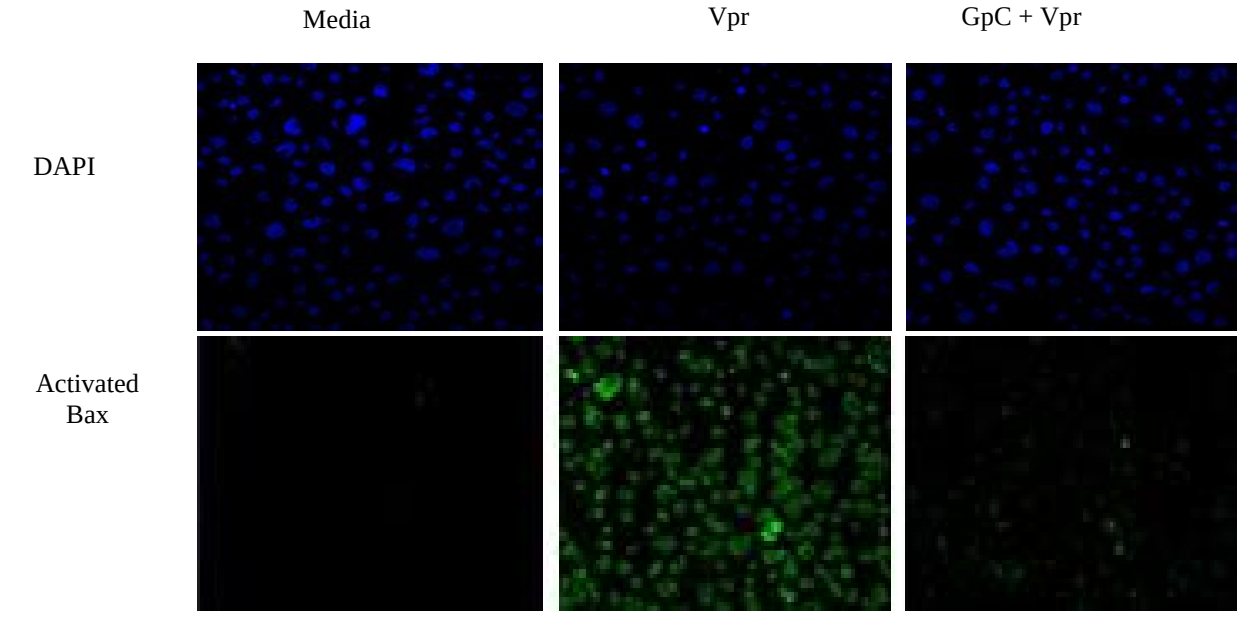

B
Monocytes
Media
Vpr
CpG + Vpr
E.Coli DNA + Vpr
DAPI
Activated Bax
6A7

Figure 5.27

Fig 5.28: GpC pre-treatment inhibits Vpr(52-96)-induced Bax activation.

THP-1 cells ($1.0x10^6$/ml) and monocytes ($1.0x10^6$/ml) were treated with 5 µM GpC for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 5 hr (in THP-1 cells) and 2 hr (monocytes). Subsequently cells were prepared for microscopy by staining for the nuclear stain DAPI (blue) and activated Bax (green) and visualization using confocal microscope with a 40X lens at 1x magnification (THP-1 cells) and 4x magnification (monocytes). The results are representative of three independent experiments.

THP-1



Monocytes

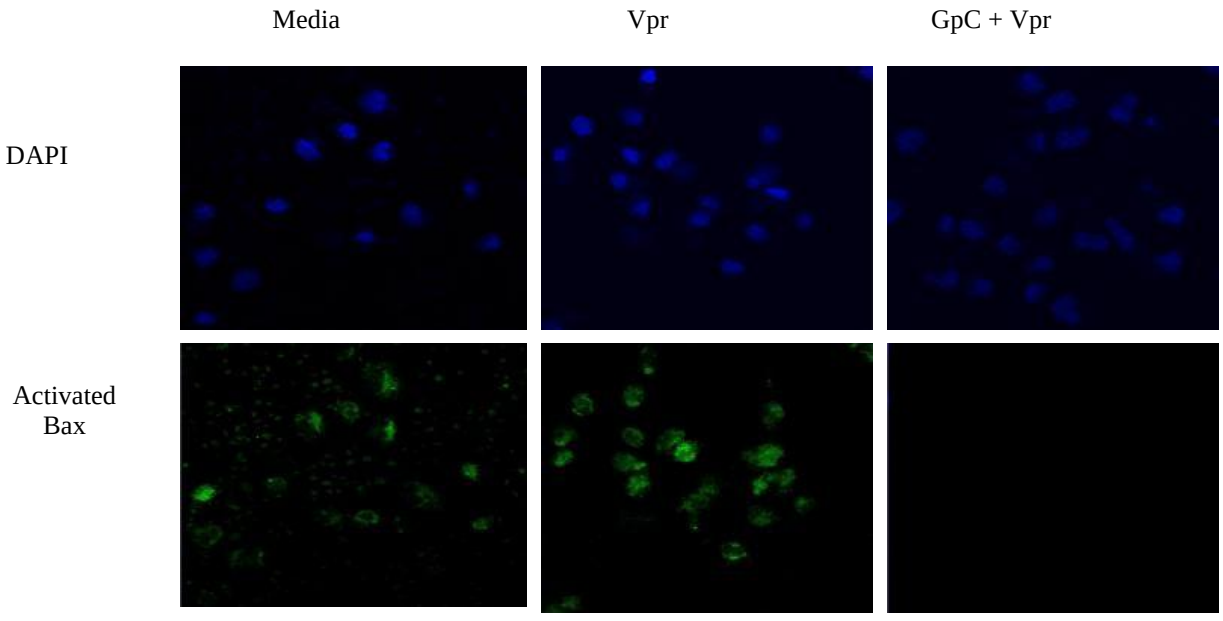
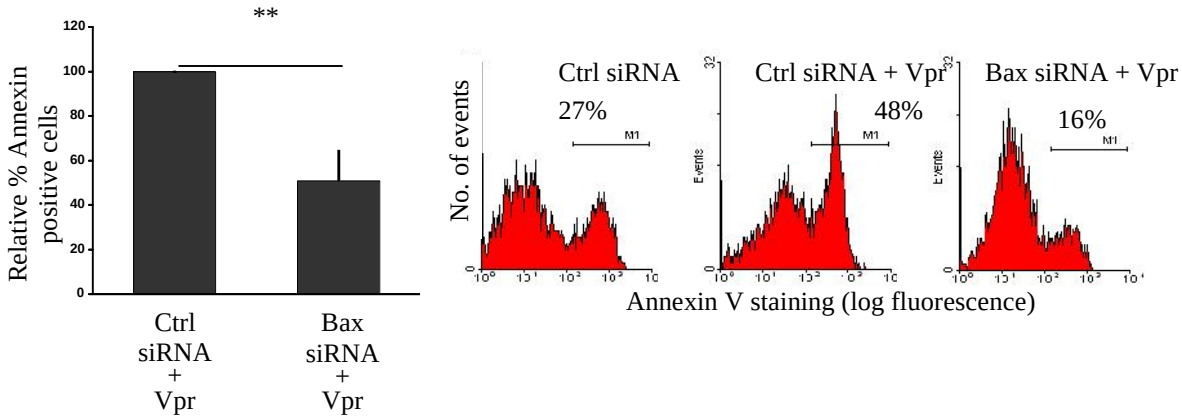


Figure 5.28

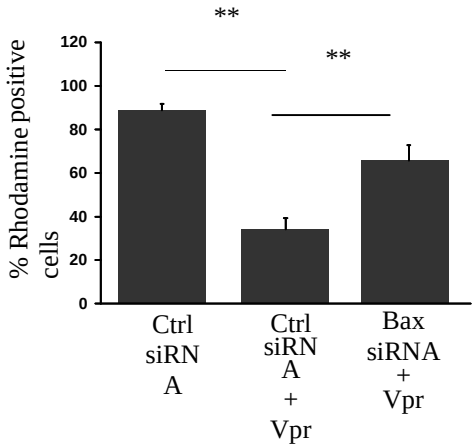
Fig 5.29: Vpr(52-96)-induced apoptosis and mitochondrial membrane permeabilization are dependent upon Bax activation.

THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 10 nM Bax specific siRNA or non-silencing control siRNA for 48 hr followed by treatment with 1.5 µM Vpr(52-96) for **(A)** 24 hr followed by measurement of apoptosis by Annexin-V staining or **(B)** for 5 hr followed by staining with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. **C** Subsequently cell lysates were analyzed for Bax expression by immunoblotting. Results in left panel in A is expressed as a mean ± SD of three independent experiments. The results in C and right panels in A are representative of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

A THP-1



B THP-1



C THP-1

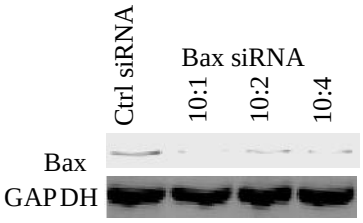


Figure 5.29

5.2.8 *Anti-apoptotic Bcl-2 proteins, Mcl-1, Bcl-X_L and Bcl-2 do not play a role in CpG-induced protection against Vpr(52-96)-mediated apoptosis in THP-1 cells*

Anti-apoptotic members of Bcl-2 family, Bcl-2, Bcl-X_L and Mcl-1 have been demonstrated to mediate cell survival by protecting the mitochondrial integrity through neutralizing pro-apoptotic Bcl-2 proteins like Bax and Bid (31, 66, 67). Interestingly CpG was shown to induce longetivity in murine DCs via induction of Bcl-2 and Bcl-X_L (10). Even though CpG treatment did not enhance expression of any of the Bcl-2 proteins tested for in human monocytic cells (**Chapter 4 Fig 4.6A**), I determined whether Mcl-1, Bcl-2 or Bcl-X_L, may play a role in CpG-induced prevention of Vpr(52-96)-mediated apoptosis. To this end, THP-1 cells were treated with HA14-1 prior to stimulation with CpG and Vpr(52-96). HA14-1 is a small synthetic compound that inhibits biological activities of Bcl-2 proteins, in particular Bcl-2 and Bcl-X_L without altering their expression (434). Interestingly, HA14-1 pretreatment did not reduce the protection afforded by CpG against Vpr(52-96)-mediated apoptosis (Fig 5.30). Induction of caspase-mediated apoptosis in NIH 3T3 cells was performed to confirm biological activity of HA14-1 (107). Furthermore, although siRNA-mediated knockdown of Bcl-X_L and Mcl-1 did inhibit Bcl-X_L and Mcl-1 expression (Fig 5.31D), it did not significantly reduce CpG-induced protection against Vpr(52-96)-mediated apoptosis (Fig 5.31A) or mitochondrial depolarization (Fig 5.31B). Note that Bcl-2 knockdown using siRNA (Fig 5.31D) induced spontaneous loss of mitochondrial membrane potential in THP-1 cells (Fig 5.31C), therefore this technique could not be used to determine relevance of Bcl-2 in CpG-mediated protection.

Overall, these results suggest that Vpr(52-96) mediates apoptosis in human monocytic cells by down regulating TRAF-1/TRAF-2 and activating caspse8, Bid and Bax,

eventually leading to mitochondrial membrane permeabilization, release of cytochrome c/AIF and apoptosis. However stimulation of the cells with bacterial DNA prior to Vpr(52-96) treatment, protects TRAF-1 and TRAF-2 via induction of anti-apoptotic c-IAP-2, thereby inhibiting instigation of caspase 8 mediated apoptotic pathway (Fig 5.32).

Fig 30: Pre-treatment with HA14-1 does not affect CpG-induced protection against Vpr(52-96)-mediated apoptosis.

THP-1 cells (1.0x106/ml) were treated with 5-20 µ M HA14-1 prior to stimulation with 5 µM CpG-ODN for 12 hr followed by treatment with 1.5 µM Vpr(52-96) for 24 hr. Subsequently cells were stained with AnnexinV for measurement of apoptosis.

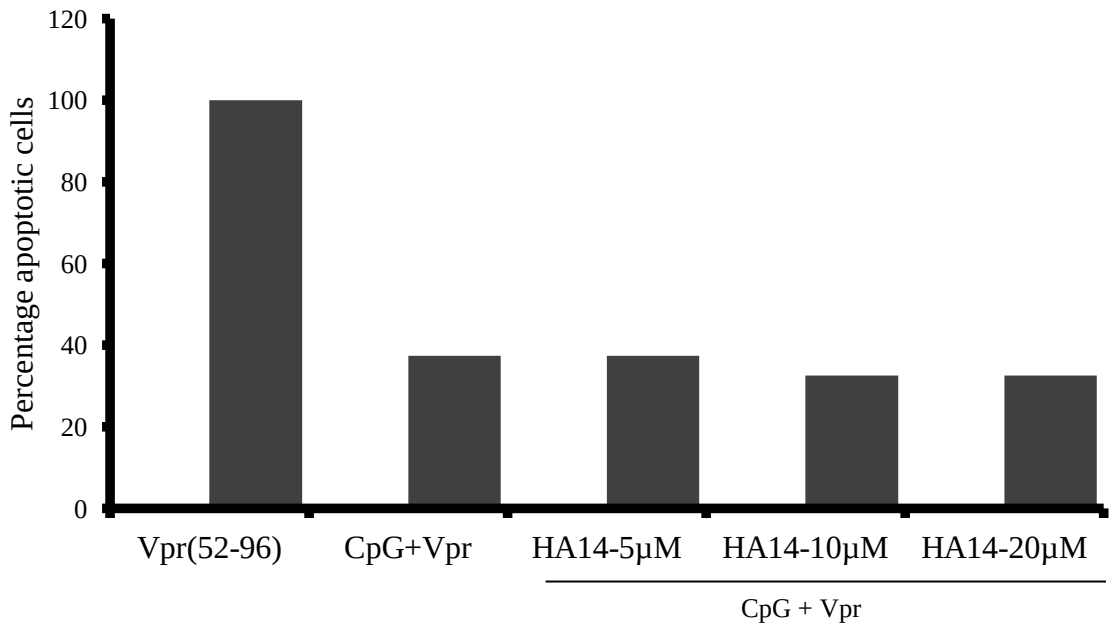

Percentage apoptotic cells
120
100
80
60
40
20
0
Vpr(52-96)
CpG+Vpr
HA14-5µM
HA14-10µM
HA14-20µM
CpG + Vpr

Figure 5.30

Fig 5.31: siRNA-mediated knockdown of Mcl-1 and Bcl-X_L does not abrogate CpG-induced protection against Vpr(52-96)-mediated apoptosis and mitochondrial injury in THP-1 cells.

THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 40 nM Bcl-X_L or Mcl-1 specific siRNAs or non-silencing control siRNA for 48 hr before by treatment with 1.5 µM Vpr(52-96) for **(A)** 24 hr followed by measurement of apoptosis by Annexin-V staining or **(B)** for 5 hr followed by staining with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. **C.** THP-1 cells ($0.25x10^6$/0.5ml) were transfected with 40 nM Bcl-2 specific siRNA or non-silencing control siRNA for 24 hr followed by staining with Rhodamine 123 for mitochondrial membrane potential evaluation by flow cytometry. **D**. Subsequently cell lysates were analyzed for Bcl-X_L, Mcl-1 and Bcl-2 expression by immunoblotting. Results in A and B are expressed as a mean ± SD of three independent experiments. The results in D are representative of three independent experiments. *($p < 0.05$), ** ($p < 0.005$).

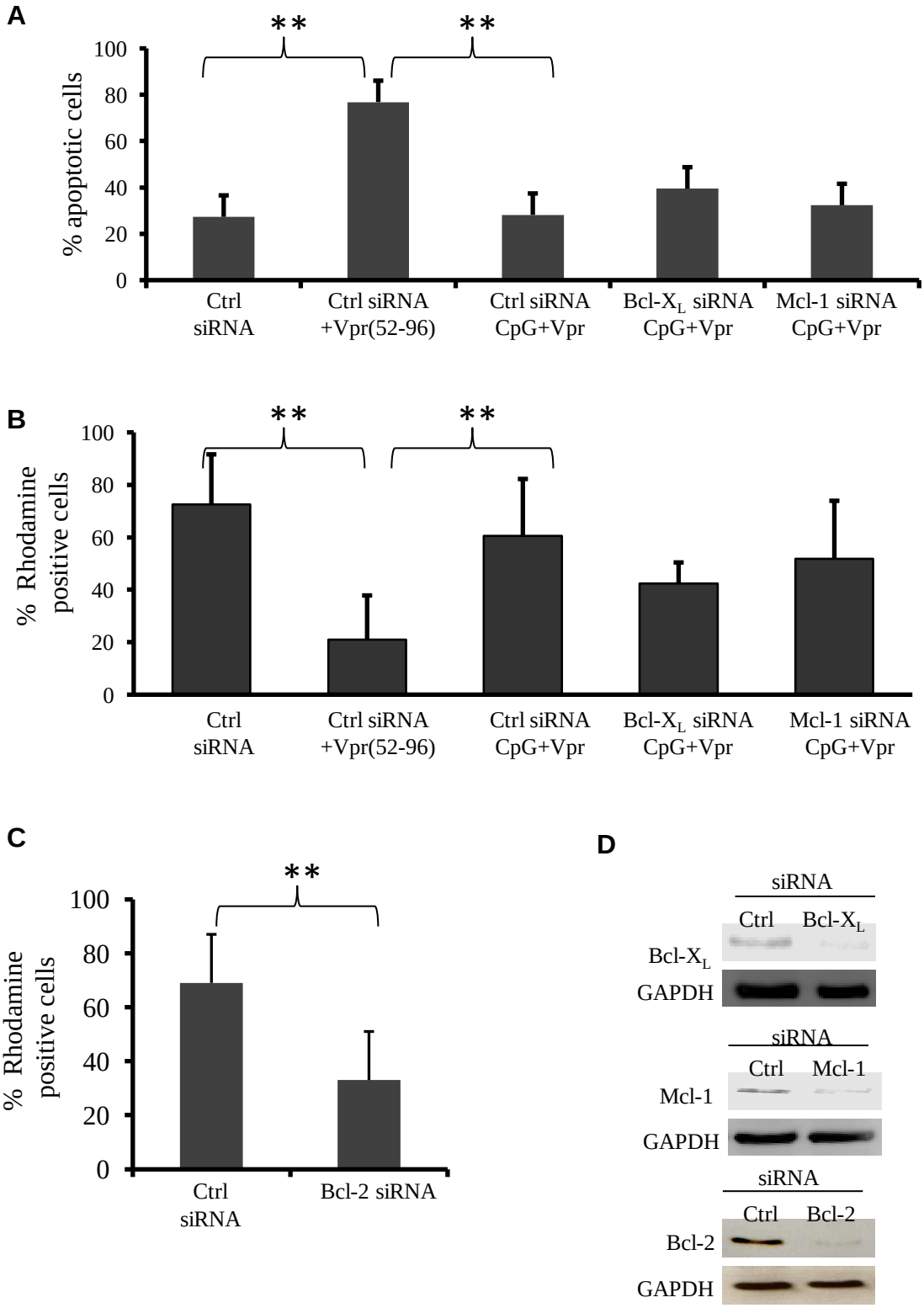

A
**
**
% apoptotic cells
100
80
60
40
20
0
Ctrl siRNA
Ctrl siRNA +Vpr(52-96)
Ctrl siRNA CpG+Vpr
Bcl-X$_L$ siRNA CpG+Vpr
Mcl-1 siRNA CpG+Vpr
B
**
**
% Rhodamine positive cells
100
80
60
40
20
0
Ctrl siRNA
Ctrl siRNA +Vpr(52-96)
Ctrl siRNA CpG+Vpr
Bcl-X$_L$ siRNA CpG+Vpr
Mcl-1 siRNA CpG+Vpr
C
**
% Rhodamine positive cells
100
80
60
40
20
0
Ctrl siRNA
Bcl-2 siRNA
D
siRNA
Ctrl
Bcl-X$_L$
Bcl-X$_L$
GAPDH
siRNA
Ctrl
Mcl-1
Mcl-1
GAPDH
siRNA
Ctrl
Bcl-2
Bcl-2
GAPDH

Figure 5.31

Fig 5.32: Schematic representation of bacterial DNA/CpG-mediated resistance against HIV-Vpr-induced apoptosis in human monocytic cells.

Stimulation with bacterial DNA or CpG ODN induces c-IAP-2 expression via CaMK-II mediated JNK activation. Induced c-IAP-2 stabilizes TRAF-1 and TRAF-2 thus inhibiting Vpr-mediated activation of pro-apoptotic caspase 8, Bid and Bax, thereby protecting against mitochondrial injury and apoptosis.

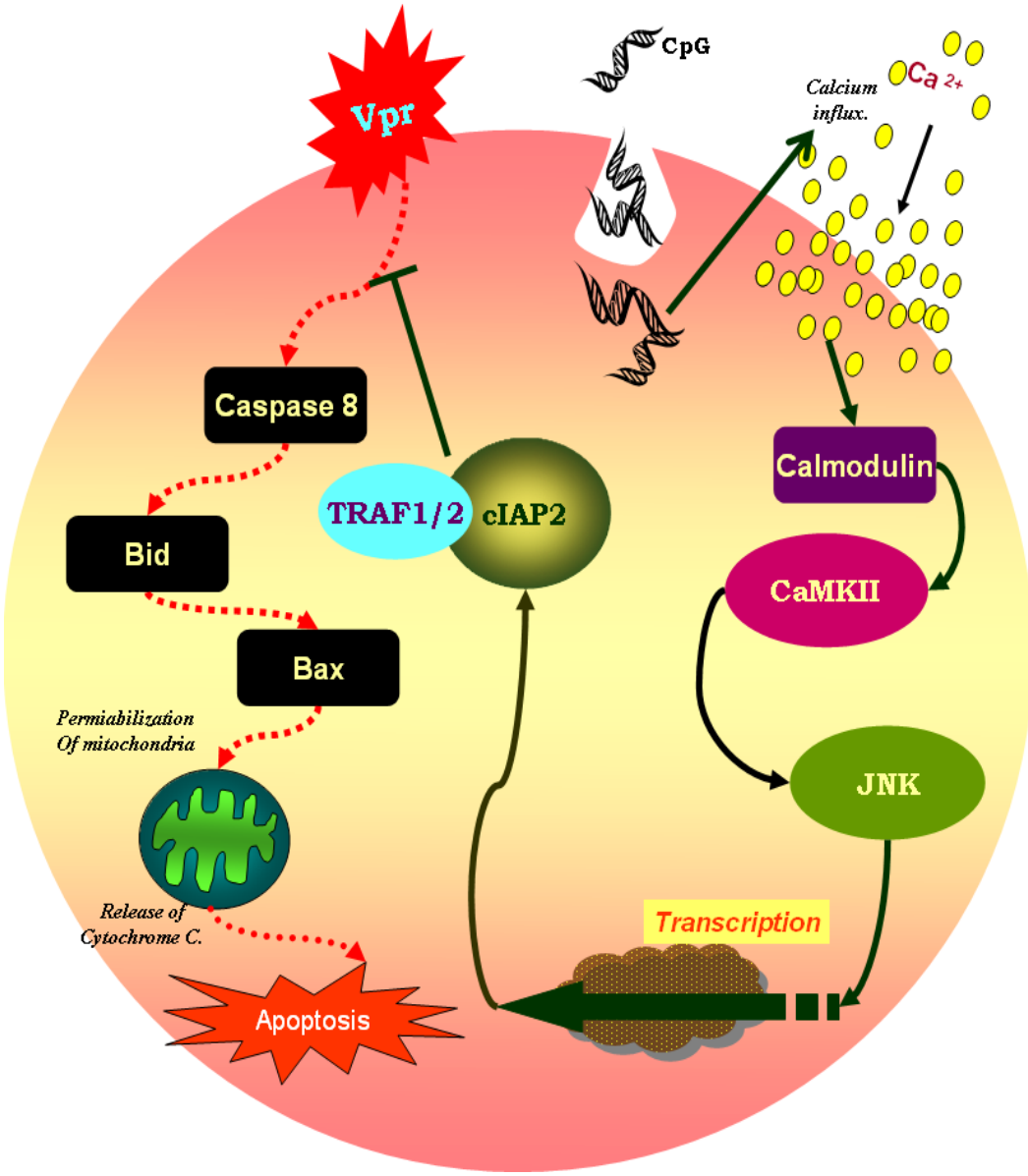

Vpr
CpG
Calcium
influx.
Ca 2+
Caspase 8
TRAF1/2
cIAP2
Calmodulin
Bid
CaMKII
Bax
Permiabilization
Of mitochondria
JNK
Release of
Cytochrome C.
Transcription
Apoptosis

Figure 5.32

5.3 Chapter 5: Discussion

I have previously shown a central role of bacterial DNA-induced c-IAP-2 in mediating resistance against HIV-Vpr–induced apoptosis in human monocytic cells (108). Herein, I provide evidence that Vpr-induced mitochondrial depolarization and cell death in human monocytic cells is mediated via sequential activation/cleavage of pro-apoptotic caspase 8, Bid and Bax culminating in release of mitochondrial apoptogenic factors AIF and cytochrome c. Furthermore Vpr-induced caspase 8 activation and apoptosis were found to be dependent upon rapid down regulation/degradation of signaling molecules TRAF-1 and TRAF-2. Bacterial DNA-induced c-IAP-2 endowed protection through CaMK-II activation against Vpr-induced mitochondrial depolarization by inhibiting TRAF-1/2 degradation, and caspase 8, Bid and Bax activation. With the exception of mitochondrial survivin (435), no other member of IAP family has been shown to exert anti-apoptotic effects by directly protecting mitochondria from depolarization and release of mitochondrial apoptotic factors. My results suggest a novel and key role played by c-IAP-2 in protection against Vpr-mediated mitochondrial depolarization.

Vpr has been shown to induce apoptosis by physically interacting with a component of mitochondrial permeability transition pore complex, ANT, and causing release of mitochondrial apoptogenic proteins like cytochrome-c, AIF and SMAC (133, 240-243). However, recently the role of Vpr-ANT interaction in apoptosis has been challenged as siRNA mediated knockdown of ANT was shown to not affect Vpr's ability to permeabilize mitochondria or cause apoptosis. Instead activation of pro-apoptotic Bax was suggested to be critical for Vpr's apoptotic functions (16). Vpr-induced release of cytochrome c into the cytosol facilitates aggregation of apoptosome, consisting of cytochrome-c, pro-caspase 9 and

Apaf-1, eventually leading to activation of caspase 9 followed by activation of caspase 3 which ultimately leads to apoptosis (19, 27). Interestingly there is a lack of consensus regarding the importance of caspase 8 activation for Vpr-mediated apoptotic effects. Caspase 8 has been shown to be dispensable (245, 248-251) as well as critical for Vpr-mediated mitochondrial depolarization and apoptosis (238, 252, 253). In this study, I have confirmed that Vpr induced gross impairment to mitochondrial morphology and caused mitochondrial membrane depolarization that was mediated via caspase 8 activation.

My results show that Vpr-induced mitochondrial damage, both structural and functional, can be restored by pretreating cells with TLR-9 ligand, CpG (344) or bacterial DNA. Role of TLR-9 in responsiveness to bacterial DNA in human monocytic cells has recently been questioned, as these cells express very little TLR-9 (359, 411). In fact, induction of cytokines by human monocytes in response to bacterial DNA or CpG has been attributed to contaminating plamacytoid dendritic cells (359). Moreover, experiments performed with TLR knock out mice or with non-TLR-9 stimulating ODNs, have challenged the overall TLR-9 dependence for bacterial DNA mediated signaling (360-363). I have shown that human monocytic cells express TLR-9 however; immunomodulatory effects of bacterial DNA were of TLR-9 independent nature (108). This study further supports the TLR-9 independent effects of bacterial DNA in protecting human monocytic cells against Vpr-mediated mitochondrial damage and apoptosis.

Differentiated macrophages exhibit enhanced resistance to apoptotic stimuli. Indeed MDMs and THP-1 macrophages were shown to become resistant to Vpr-mediated apoptosis via over-expression of c-IAP-1 and c-IAP-2 (107). The results of this study confirm the role of c-IAP-2 in rendering differentiated macrophages refractory to Vpr-mediated apoptosis by

showing that down regulation of c-IAP-2 made MDMs and differentiated macrophages sensitive to Vpr-mediated loss of mitochondrial membrane potential and release of cytochrome c and AIF. These results strengthen the notion that protection from mitochondrial injury is a key mechanism for c-IAP-2-mediated cell survival in both differentiated and undifferentiated macrophages.

I have previously shown that LPS and CpG/bacterial DNA-induced c-IAP-2 expression is regulated by the calcium signaling via CaMK-II (Chapter4) (108). Since c-IAP-2 was shown to prevent mitochondrial depolarization, inhibit Vpr-induced apoptosis and mediate resistance to apoptosis in differentiated macrophages, I reasoned that agents/signaling pathways involved in c-IAP-2 synthesis may also mediate such processes. In the current study, I provide evidence that similar to induction of resistance against apoptosis (108), c-IAP-2-mediated protection against mitochondrial damage and release of apoptogenic factors is regulated via activation of CaM/CaMK-II-mediated calcium signaling pathway in monocytic cells.

IAPs regulate apoptosis by binding and ubiquitinating various cellular proteins via their highly conserved BIR domains through their RING and UBA domains (75-77). Traditionally c-IAP-2 has been described to induce protection against TNF-α-induced apoptosis primarily by; a) modulating caspase 8 activation by controlling the ubiquitination state of RIP-1 via interaction with TRAF-1/2 and b) inducing canonical NFκB pathway (90, 91). Additionally, c-IAP-2 has been reported to prevent apoptosis by directly binding with and neutralizing caspases3, 7 and 9 in some cell types, however contradicting studies suggest that c-IAP-2 binds but does not inhibit caspase activity (89). My results suggest that Vpr-mediated down regulation of endogenously expressed TRAF-1/2 is essential for

caspase 8 activation and mitochondrial depolarization. Vpr has been reported to take advantage of the cells's proteasomal degradation mechanisms by enlisting E3 ubiquitin ligase cullin 4A via DCAF-1, to target various cellular substrates for degradation in order to induce cell cycle arrest or apoptosis (230, 231). Additionally TRAF-2 is an E3 ligase and contains a RING domain facilitating protein-protein interaction (436, 437). Hence given the rapid down regulation of TRAF-1/2 by Vpr, it is reasonable to hypothesize that exploitation of the host ubiquitin proteasome system may be involved in this process.

Since my results suggest a role for TRAF-1/2 down regulation in Vpr-mediated caspase 8 activation and because of the ability of TRAF-1/2 to bind c-IAP-2 and mediate cell survival, I hypothesized that c-IAP-2 may serve to inhibit Vpr-induced caspase 8 activation by preventing loss of TRAF-1/2. Indeed, siRNA mediated knockdown of TRAF-1/2 rendered the cells sensitive to Vpr-mediated caspase 8 activation, mitochondrial depolarization and apoptosis despite the induction of c-IAP-2. These observations clearly suggest that TRAF-1/2 and c-IAP-2 are required for optimal protection from Vpr-induced caspase 8 activation and subsequent apoptosis. Interestingly, individual knock down of TRAF-1/2 did not completely abrogate c-IAP-2-mediated protection suggesting redundancy in the functions of TRAF-1/2. It is possible that TRAF-1/2 exist as a complex with pro-caspase 8, serving to maintain caspase 8 in an inactivated form in monocytic cells. When the cells are treated with Vpr, TRAF-1/2 get targeted for ubiquitination and proteasomal degradation, leaving caspase 8 free for cleavage/activation. However, pre-exposure of cells to bacterial DNA causes induction of c-IAP-2 which binds with TRAF-1/2, making them refractory to Vpr-mediated degradation and thereby inhibiting caspase 8 activation and

apoptosis. However, further investigation is required to confirm the precise mechanism involved.

It is noteworthy that contrary to previously published mechanisms of c-IAP-2-mediated protection, pretreatment of human monocytic cells with RIP-1 inhibitor, necrostatin (438), did not inhibit the protective effect of c-IAP-2 against Vpr-mediated apoptosis. It is possible that in monocytic cells c-IAP-2-mediated protection may be modulated independently of RIP-1 via other yet unknown protein/s. Conversely necrostatin is a non-specific inhibitor of RIP-1 and has been described to only inhibit partial RIP-1 functions (439). However, due to inconsistent expression of RIP-1 in THP-1 cells and monocytes, siRNA-mediated knock down of RIP-1 could not be performed. Therefore, the precise role of RIP-1 in c-IAP-2-mediated cell survival in human monocytic cells needs to be investigated.

As no increase was observed in the expression of any IAP family member besides c-IAP-2 in response to CpG-ODN treatment, the remaining members of this family were not investigated for their role in protecting cells against Vpr-mediated cell death. My results showed that both SMC-induced c-IAP-2 degradation and siRNA-mediated c-IAP-2 knock down rendered both bacterial DNA treated monocytes and differentiated macrophages sensitive to Vpr-mediated cell death. However, since SMC degrades c-IAP-1 and X-IAP along with c-IAP-2, the role of c-IAP-1 and X-IAP in protecting monocytic cells against HIV-Vpr-mediated apoptosis cannot be ruled out. Further studies targeting c-IAP1, c-IAP-2 and X-IAP together and individualy will help delineate the functional and biochemical aspects of interplay between these proteins.

Anti-apoptotic Bcl-2 family of proteins such as Bcl-2, Bcl-Xl and Mcl-1 exert their protective effects by inhibiting mitochondrial permeabilization via neutralization of pro-apoptotic proteins like Bid and Bax (107, 440). Considering that protection of mitochondrial viability is critical for inhibiting Vpr-mediated apoptosis and taking into account the significance of Bid and Bax in causing Vpr-induced mitochondrial depolarization, it is possible that along with c-IAP-2; Bcl-2, Bcl-X_L and Mcl-1 may also play a role in bacterial DNA endowed protection against apoptosis caused by Vpr. However, neither siRNA-mediated knock down of these proteins nor the pharmacological inhibition of their biological activity was observed to cast significant difference in bacterial DNA-mediated protection against Vpr-induced apoptosis.

In summary, this is the first report, describing a role for bacterial DNA-induced c-IAP-2 in protecting against apoptosis by acting upstream of mitochondrial membrane permeabilization; through inhibition of HIV-Vpr-mediated caspase 8 activation and mitochondrial injury, with the help of TRAF-1 and TRAF-2. Furthermore, this study for the first time shows the involvement of TRAF-1/2 in Vpr-mediated cell death through the activation of caspase 8, Bid and Bax. Additionally, considering the significance of c-IAP-2, calcium signaling and TRAF-1/2 in establishing resistance to cell death, drugs like SMC, aimed at destroying this resistance by targeting c-IAP-2, CaM/CaMK-II or TRAF-1/2, may serve to prevent HIV reservoir formation in human monocytic cells.

Concluding remarks and future studies

The primary purpose of this study was to determine what aspects of HIV infection induced resistance to apoptosis in human monocytes allowing these cells to become viral reservoirs. For this 96 aa HIV accessory protein, Vpr, was used to facilitate examination of apoptotic mechanisms in monocytic cells in an HIV context.

The first order of this project was to ascertain if treating human monocytes and pro-monocytic THP-1 cells with various microbial products would make these cells resistant to Vpr-induced cell death. Components from both gram positive and gram negative bacteria were found to be potent inducers of protection from Vpr-induced cell death. However, CpG/bacterial DNA pretreatment endowed maximal protection. Furthermore, using knock down studies it was determined that CpG/bacterial DNA-induced protection was regulated through induction of anti apoptotic protein, c-IAP-2 via CaM/CaMK-II mediated calcium signaling and MAPK JNK signaling as summarized in Fig 4.19. Given the evidence presented in this study advocating the role of c-IAP-2 in inducing survival against Vpr-induced apoptosis, it would be interesting to determine if direct or bystander apoptosis induced by other HIV related factors like Tat and gp120 (441) could also be similarly countered by c-IAP-2 over expression. Furthermore, investigating whether stimulation with bacterial DNA bestows enhanced resistance to apoptosis in macrophages, in addition to the resistance imparted by the differentiation process, would provide meaningful insights into the pro-survival nature of these cells. A primary source of HIV reservoirs during all stages of the disease is memory T cells (259). It would be interesting to investigate if the mechanism of bacterial DNA-induced survival is applicable in memory T cells and other cellular reservoirs of HIV.

Interestingly, although CpG/bacterial DNA is recognized as a classical TLR-9 ligand (344), protection against Vpr-mediated apoptosis was found to be independent of TLR-9 stimulation. The precise mechanism of TLR-9 independent signaling by bacterial DNA in these cells remains to be elucidated and raises interesting possibilities for future research. These include questions regarding the mode of bacterial DNA entry in the cell and occurrence of a yet undiscovered receptor for this ligand in monocytes. Furthermore, unlike monocytes (411) dendritic cells (352, 421) and B cells (356, 372, 442), express TLR-9 and are potent responders of bacterial DNA stimulation. It would be interesting to investigate if bacterial DNA induces protection in these cells against apoptotic stimuli and whether this protection is dependent upon TLR-9 signaling. Moreover, given the observations that LPS and LTA stimulation also induced expression of c-IAP-2 and protection against apoptosis in monocytes it can be hypothesized that bacterial DNA may bypass TLR-9 and signal directly through a downstream modulator of TLR cascade, like MyD88, to activate NFκB and induce c-IAP-2 expression.

The second part of the study was dedicated to determining the mechanism of protection induced by CpG/bacterial DNA-induced c-IAP-2, against Vpr-mediated apoptosis. For this a two pronged approach was adopted which entailed identifying the exact mechanism of Vpr-induced cell death in monocytes and simultaneously investigating determinants of bacterial DNA-induced protection. In the present study Vpr-mediated mitochondrial depolarization and apoptosis were found to be mediated via sequential activation of pro-apoptotic caspase 8, Bid and Bax. Interestingly prior treatment with CpG significantly inhibited instigation of this Vpr-induced apoptotic cascade. Since activation of caspase 8 has historically been shown to be regulated by the TNFR signaling (38), future

studies exploring the possibilities that Vpr may be a) interacting with the TNFR complex or b) engaging the TNFR while entering the cell, will provide further insights into regulation of apoptosis by HIV. Bid and Bax were found to play important roles in Vpr-mediated cell death. However, role of other pro-apoptotic Bcl-2 proteins in this context was not determined. Therefore a comprehensive study investigating the role of each pro-apoptotic Bcl-2 family protein is warranted to define the precise mechanism of Vpr-induced apoptosis.

Having determined the mode of Vpr-mediated apoptosis I explored whether CpG-induced c-IAP-2 could counter these apoptotic mechanisms. Indeed, inhibition of c-IAP-2 expression by siRNAs or by use of SMC successfully reversed CpG-induced protection against Vpr-induced caspase 8 and Bax activation. Moreover, knocking down c-IAP-2 expression in c-IAP-2 overexpressing cells, MDMs and THP-1 derived macrophages (107), rendered these cells sensitive to Vpr-mediated loss of mitochondrial potential and release of cytochrome c/AIF.

TRAF-1/2 are recognized as crucial components of c-IAP-2-mediated protection against TNF-α-induced cell death as these adaptor proteins physically interact with c-IAP-2 (443). Vpr was shown to induce rapid down regulation of TRAF-1/2 which could be rescued by induction of c-IAP-2. Additionally, siRNA-mediated knockdown of TRAF-1/2 caused a significant decline in CpG-induced protection against Vpr-mediated loss of mitochondrial potential and apoptosis. These observations indicate that c-IAP-2 may primarily serve to prevent down regulation of TRAF-1/2 by Vpr.

The exact nature of Vpr-caspase 8-TRAF1/2-c-IAP-2 interaction remains to be delineated. It is possible that other Vpr-binding proteins may be crucial in this process.

Furthermore mechanism of Vpr-mediated down regulation of TRAF-1/2 remains to be determined. An interesting observation in this study was the RIP-1 independent nature of c-IAP-2-mediated survival. The exact contribution of RIP-1 to bacterial DNA-induced protection in human monocytes and macrophages will be instrumental in designing drug targets for controlling cell longitivity. Taken together these observations, as summarized in Fig 5.32, indicate a novel role for bacterial DNA induced c-IAP-2 in protecting against mitochondrial damage and apoptosis. Future studies exploring different functions of c-IAP-2 may bring to light novel and noteworthy roles for this protein in varied aspects of host immunity like regulation inflammatory responses.

The conclusions drawn from this project shed light on the functional importance of co-infections and microbial translocation in the process of HIV reservoir formation. It is reasonable to speculate that monocytic cells from HIV-infected patients may have a reduced propensity to undergo apoptosis at least in part due to the presence of microbial products including LPS and DNA present in chronically-infected HIV patients (11) via induction of c-IAP-2. Moreover reservoir forming capabilities of CpG/bacterial DNA-induced c-IAP-2 advocate caution for the use of CpG as an adjuvant for vaccines against infectious pathogens (444-446). Keeping in view my results implicating c-IAP-2 in bacterial DNA-induced survival against Vpr-mediated apoptosis, strategies based on suppression of c-IAP-2 (447, 448) by agents known to inhibit CaM/CaMK-II/JNK signaling pathways may prove helpful in controlling HIV reservoir formation and broadly prevent infection with intracellular pathogens and associated inflammatory responses

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