Selective induction of programmed-cell death in HIV-infected macrophages

By

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Abstract

In order to achieve cure for HIV-1 infection in patients undergoing suppressive antiretroviral therapy, eradication of all latently infected reservoirs of the virus is required. The focus of HIV cure is predominantly centred on the elimination of latently infected memory T cells, while information on possible elimination of infected macrophages is lacking. Macrophages support continuous virus replication without succumbing to cytopathic effects of HIV-1. Recently, our laboratory has shown a protective role for cellular inhibitor of apoptosis proteins (IAPs) 1/2 in macrophages against Vpr-induced apoptosis. Depletion of cIAP1/2 by Smac mimetics (SM) reverse the IAP-mediated protection and sensitize macrophages to Vpr-induced cell death. My research aims to understand the role IAPs play in apoptotic resistance of HIV-infected macrophages. I hypothesized that ablation of cIAP1/2 by SM may induce apoptosis in HIV-infected macrophages. My results show that SM does not induce cell death in uninfected or healthy macrophages, but induces cell death in chronically infected U1 cells, *in vitro* infected monocyte-derived macrophages, and *ex vivo* derived HIV-infected macrophages from HIV-infected individuals. SM induce cell death of infected myeloid cells through apoptosis and not through necroptosis. Moreover, SM-induced apoptosis is independent of TNFα and other endogenously secreted cytokines. *In vitro* infection of monocyte-derived macrophages leads to the downregulation of RIPK1, RIPK3, and TRAF-1. Interestingly, necrostatin-1-mediated RIPK1-inhibition does not affect viability of healthy macrophages, but in combination with IAP degradation by SM leads to significant induction of apoptosis. This suggests a key role for RIPK1 in SM-induced apoptosis of HIV-infected macrophages. Altogether, the results from this project suggest that modulation of the IAP-associated signalling pathways by SM may be a potential strategy for selective killing of HIV-infected macrophages.

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List of abbreviations

AIDS	acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Apaf	apoptosis protease activating factor
ARV	antiretroviral
ASK	apoptosis signal-regulating kinase
Bcl2	B-cell lymphoma 2
BIR	Baculoviral IAP repeat
BLT	bone-marrow/liver/thymus
cART	combinatorial antiretroviral Therapy
Caspases	cysteine-dependent aspartate proteases
CD	cluster of differentiation
Cdk	Cyclin-dependent kinase
cIAP	Cellular inhibitor of apoptosis
CNS	central nervous system
CRA	chemokine receptor antagonist
DISC	death inducing signalling complex
FADD	Fas-associated death domain protein
FI	fusion inhibitors
FLICE	Fas-associated death domain (FADD)-like IL-1β converting enzyme
FLIP	FADD-like IL-1β converting enzyme (FLICE) inhibitory protein
HIV	Human immunodeficiency virus
IAP	Inhibitor of apoptosis
IFNγ	Interferon gamma
IL	interleukin
INSTI	integrase inhibitors
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MoM	myeloid-only mice
MPTP	mitochondrial permeability transition pore
NIK	NFκB inducing kinase
NFκB	Nuclear factor kappa-light-chain enhancer of activated B cells
NRTI	nucleoside reverse transcriptase
NNRTI	non-nucleoside reverse transcriptase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PI	Propidium iodide

PIs	protease inhibitors
PMA	Phorbol myristate acetate
RING	Really interesting new gene
RIP1	Receptor interacting protein 1
SM	Smac mimetic
SMAC	second mitochondrial activator of caspases
SHIV	simian immunodeficiency virus/HIV-1 chimera
TNF-α	Tumor necrosis factor alpha
TNFR	TNF receptor
TRAIL	TNF-related apoptosis inducing ligand
TRAF	TNF receptor-associated factor
TRADD	TNF receptor associated death domain protein
TREM	triggering receptor expressed on myeloid cells 1
VCC	virus-containing compartments
XIAP	X-chromosome-linked IAP

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Chapter 1: Introduction

1.1 General Overview

The human immunodeficiency virus (HIV)-1 poses a challenge in the modern scientific and medical communities due to its seemingly incurable nature. After more than 30 years of vigorous and intensive research, much progress has been made in understanding the pathogenesis of the virus, developing combinatorial drug therapy, and bettering the well-being of HIV patients. Despite these advancements, there is no cure for the infection because of the major roadblock that comes in the form of latently infected cells which act as viral reservoirs and hinder the complete eradication of the virus from the host. T cells and macrophages are the prime targets of HIV-1[1,2]. Whereas the number of CD4 T cells exhibit rapid decline during the course of infection, populations of monocytes and macrophages largely remain stable[1,3]. The focus of developing cure is aimed toward eradication of resting $CD4^+$ T memory cells, as very little is known about the elimination of macrophage reservoirs.

To better understand how HIV-1 establishes reservoirs in macrophages, it is important to identify anti-apoptotic genes responsible for the development of resistance to cell death in infected macrophages. We recently showed that inhibitor of apoptosis proteins (IAPs), particularly cellular IAP1/2 have protective role against HIV-Vpr induced apoptosis in monocytes and macrophages. Degradation of IAPs by a class of compound called second-mitochondrial activator of caspases (SMAC)-mimetics (SM) rendered macrophages susceptible to cell death induced by HIV-Vpr protein[4]**.** Thus, targeting IAPs during HIV-infection may be a possible therapeutic strategy for clearing infected macrophage reservoirs.

1.2 Overview of HIV-1

1.2.1 HIV/AIDS global pandemic

HIV-1 epidemic arose after zoonotic infections with simian immunodeficiency viruses from African primates and has now become a worldwide health concern[5]**.** The disease progression of HIV-1 infection can be classified into three phases. The first phase is the acute infection characterized by the rampant virus production and aberrant decrease in the number of $CD4^+$ T helper cells. It is followed by chronic infection during which the virus exhibits latency with continuous viral production and gradual decrease of $CD4^+$ T cells. Acquired immunodeficiency syndrome (AIDS)[6] is the final manifestation of HIV infection where the immune system has been significantly compromised to the point that the host becomes susceptible to various opportunistic infections such as cytomegalovirus, *mycobacterium tuberculosis*, *Cryptococcus neoformans*, Hepatitis B virus, as well as developing cancer malignancies such Kaposi Sarcoma, non-Hodgkin lymphoma[7–12].

Since the identification of HIV-1 three decades ago, more than 75 million people have contracted the virus and about 35 million have died from AIDS-related illnesses[13]**.** Currently, there are approximately 36.7 million people living with HIV/AIDS and about 20.9 million people have access to combinatorial antiretroviral therapy to treat the infection. Despite major advancements in controlling the infection and provision of treatment, HIV/AIDS remains an incurable disease.

1.2.2 HIV life cycle

HIV is composed of two copies of single-stranded viral RNA that are enclosed in a nucleocapsid along with three essential enzymes for virus replication: protease, reverse transcriptase, and integrase[14–16]. The principal cell targets of HIV *in vivo* are CD4 expressing cells such as T-cells and macrophages[17]**.** Cell tropism of HIV is facilitated by the presence of co-

receptors on the cell surface; CXCR4 is highly expressed in T-cells, while CCR5 is highly expressed in T-cells and macrophages. Viral fusion is mediated by the engagement of virally encoded gp120 with the CD4 surface receptor on the target**.** This initial interaction causes gp120 to undergo structural change and exposes the binding sites for the CXCR4 or CCR5 co-receptors on the cell surface and allows for the host cell entry of the virus. Following host entry, the enzyme reverse transcriptase uses the viral RNA as a template to generate double stranded viral DNA, which is subsequently trafficked into the nucleus and integrated into the host genome by the enzyme integrase [18,19] Upon integration, the fate of the proviral DNA will be determined by the virus' exploitation of the host's cellular machinery. The life cycle of HIV will either proceed with the transcription of viral RNA, translation of viral proteins, virus assembly, and release of new virions, or the virus will enter a state of latency[16]**.**

1.3 Combinatorial Antiretroviral therapy

Relentless research on the pathogenesis of HIV has dramatically improved our knowledge of the replication cycle of the virus, which in turn led to the development and approval of more than 25 antiretroviral (ARV) drugs designed to treat HIV infection[20]**.** Mechanistically, there are six classes of anti-retroviral drugs: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INSTIs), fusion inhibitors (FIs), and chemokine receptor antagonists (CRA antagonists)[21]**.** Combinatorial antiretroviral therapy (cART) is a powerful therapy that reduces viral burden down to undetectable levels (50 copies/mL), which leads to reduced HIV-associated morbidity[22–24] prolonged survival, and equally importantly – prevention of transmission[25–27]**.** Although highly effective in suppressing viremia, the therapy may be associated with additive intolerable drug toxicities and it is not able to completely eradicate the virus which leaves patients to be on

antiretroviral for the duration of their lifetime[28,29]. Nonetheless, the therapy has dramatically changed our perspective on curing HIV infection- from treating an acute fatal infection to providing remedy to a chronic non-fatal disease.

1.4 HIV reservoirs as impediment to curing HIV infection

The disease progression to AIDS can be prevented with the use of combinatorial antiretroviral drugs; however, the successful cure towards the eradication of HIV-1 has been hampered by the ability of the virus to establish reservoirs[20,30,31]. Viral reservoirs are sites where replication competent virus accumulates and stably persists[32] **.** Latency is a dormant stage of reversibly non-productive infection where viral DNA can be detected, but not viral RNA[33]**.** Reservoirs shield the virus from host immune surveillance; leaving it undetectable in this state of latency[32,34,35]**.** HIV can exist within the reservoir in a pre-integrated latent form or post-integrated latent provirus. Upon treatment interruption, these reservoirs become the source of active virus replication to replenish and re-establish another state of HIV replication within the host[36–39]**.**

HIV has been recognized to colonize different tissues and cellular targets in which reservoirs can be established. Tissue reservoirs are classified as sites that are immunologically protected or separated by a barrier from the blood and lymphoid system which include the central nervous system, gastrointestinal tract, liver, and the genital tract[32] **.** Cellular reservoirs are cells that permit latency of the virus after surviving the initial infection without undergoing cell death. Typically, cellular reservoirs have long life spans, which contribute to the persistence of the virus within the host [30]. The best described cellular reservoir of HIV-1 is the resting memory $CD4^+$ T cell subset. Latency is established in resting $CD4^+$ T cells when activated T cells successfully revert to quiescent resting stage without succumbing to apoptosis and becoming memory cells[40]**.** The

longevity and quiescence of memory T cells plays an important part in the persistence of HIV despite of ART[41].

Because the hallmark of HIV-1 infection is the aberrant decline of $CD4^+$ T cells leading to AIDS, much of the research on HIV has been focused on elucidating the mechanisms of T cell decline, establishment of latency, as well as the elimination of latently infected memory T cells. Over time, it became increasingly recognized that viral reservoirs of HIV could also be established in other cellular targets of the virus. Among them were macrophages that were described more than 30 years ago to harbour replication competent HIV-1 *in vivo*[42]**.** The mechanism of reservoir formation and virus persistence within macrophages are largely unknown. Thus, it is imperative to understand the contribution of macrophages in the pathogenesis of HIV-1.

1.5 HIV infection of monocytes and macrophages

Myeloid progenitor cells in the bone marrow give rise to monocytes that transiently circulate in the blood stream, eventually moving into tissues to terminally differentiate into either macrophages or dendritic cells in response to chemotactic or inflammatory signalling[43,44]**.** Cells of the monocytic lineage such as monocytes and macrophages are important players in the initial and late stages of HIV infection[45]. These cells express CD4, chemokine receptor type4 (CXCR4), and chemokine receptor type 5 (CCR5) receptors that HIV utilizes for viral entry[46]**.** Monocytes are divided into three groups: $CD14^{++}CD16^-$ (classical), $CD14^{++}CD16^+$ (intermediate), and $CD14^+CD16^{++}$ (non-classical)[47]**.** Phenotypical comparative studies reveal that intermediate and non-classical monocyte subsets are more susceptible to HIV-1 infection *in vitro* than the classical subset[48,49]**.** In addition, *ex vivo* analyses of peripheral monocytes from HIV patients undergoing ART show that $CD16^+$ subset preferentially harbour HIV-1[48,50]**,** although less than 1% of total circulating monocytes have integrated HIV-1[51]**.** Furthermore, $CD16^+$ monocyte subset is -

reportedly expanded in HIV-1 infected individuals treated or untreated with ARV[48,50,52]**,** whereas it is only about 10% of the total circulating monocytes in healthy controls[53]**.** More importantly, $CD16^+$ monocytes bear the same immunophenotypic characteristics as infected macrophages [54] as they promote highly efficient viral replication upon differentiation[49].

Monocyte-colony stimulating factor (MCSF) mediates the differentiation of monocytes into macrophages which upregulates the surface expression of CCR5 and CXCR4, and allows macrophages to be susceptible to HIV-1 infection[55]. The tropism of HIV-1 has been described in two ways: X4 and R5 tropics in which the virus uses CXCR4 and CCR5 as its co-receptor along with CD4, respectively[56]**.** Although both CXCR4 and CCR5 co-receptors are expressed in macrophages, most strains of R4 tropic HIV-1 fail to establish productive infection in macrophages[57]. In addition, it has recently been shown by Baxter *et al.* (2014) that macrophages participate in a very efficient mode of viral spread by selectively phagocytosing infected T cells which lead to their own infection[58]**.** Altogether, these findings conclusively implicate monocytes and macrophages in the pathogenesis of HIV-1.

1.6 Macrophages as HIV reservoirs

Although macrophages have often been described to be secondary to resting memory T cells in functioning as reservoirs, there exists a strong body of evidence from recent years which provide support that macrophages are equally important. Macrophages sequester infectious virions within specialized vacuolar subcellular structures called virus-containing compartments (VCCs)[59,60]**.** Engulfed virus particles are stored and remain infectious for extended period within VCCs[61–63] where they are protected from effector molecules and inaccessible to anti-viral drugs[63–65]**.** Furthermore, infected macrophages have been shown to evade host-mediated immune response

as $CD8^+$ T cells are not able to clear these cells[66]**.** HIV-1 efficiently establishes reservoirs within macrophages because it can hijack the functional nature of macrophages to its own advantage.

Monocytes have poor longevity *in vivo* and *in vitro,* and undergo spontaneous apoptosis unless activated by pro-inflammatory cytokines or growth factors[67]**.** As monocytes undergo terminal differentiation into macrophages, they acquire resistance to various apoptotic stimuli to fulfill their roles as part of the innate immunity[4]**.** This change in functional phenotype favours the establishment of virus reservoirs within macrophages. Compared to activated $CD4^+$ T cells, macrophages are more resistant to the cytopathic effects of lentiviral replication[68–70]**.** The number of macrophages does not decline during acute infection, and they play a role in the continuous virus replication towards the later stage of infection when the $CD4^+$ T cells have been depleted[45]**.** This is supported by a study where *rhesus macaques* infected with Simian immunodeficiency virus/HIV type 1 chimera (SHIV) had macrophages that supported viremia in the late stage of infection in the absence of $CD4^+$ T cells[70]**.** Moreover, by employing Myeloid-only-mice (MoM), a humanized mouse model for HIV-1, *Honeycutt et al.* showed that macrophages were capable of sustaining HIV replication in the absence of T cells and that replication-competent virus could be rescued *ex vivo* from these cells[71]. Even in the face of suppressive ART, HIV continued to persist in tissues of humanized MoM mice. Upon treatment cessation, plasma viral load would quickly rebound in the mice models, which directly shows the relevance of macrophages as important viral reservoir[72]**.**

Macrophages have lifespan that extend from weeks to several months depending on their tissue localization[73,74]**.** For instance, microglia which are the resident macrophages within the central nervous system can survive for up to two decades[75]**.** One of the central problems in curing HIV is the persistence of the virus within the CNS which is known for being an immunologically protected

site with poor drug penetrance[65]**.** During acute infection, HIV-1 invades the CNS with notable infection of macrophages, microglia, and astrocytes[76–79]. Infected macrophages that reside within the CNS are faced with sub-optimal drug pressure that is inefficient in fully suppressing viral replication. As shown by Avalos and colleagues, virally suppressed macaques have brain macrophages that harbour replication competent virus[80]. Moreover, due to limited drug penetrance in the CNS, virus mutations may arise that can potentially increase the fitness of HIV-1[81]**.** In fact, mutant variants with drug-resistant phenotypes have already been identified in the cerebrospinal fluid of HIV-1 infected patients[82,83]**.**

1.7 Apoptosis overview

Apoptosis is an evolutionary conserved programmed cell death that serves crucial homeostatic functions in tissue development, viral infection, and autoimmunity[84–87]**.** Apoptosis features distinct morphological and biochemical hallmarks such as protein cleavage, chromatic condensation, fragmentation of DNA, cytoplasmic shrinking, and membrane blebbing[84,85]. The commitment of a cell to undergo apoptosis is determined by the activation of cysteine-dependent aspartate proteases (Caspases) which are widely distributed in the cytosol as inactive procaspases[88]**.** Activated caspases can often activate other procaspases and lead to the amplification of apoptotic signalling through proteolytic cascade initiation[89]. Currently, there are 11 caspases that have been identified and they are broadly classified into initiator (caspase-2, -8, -9, and -10), effector or executioners (caspase-3,-6, and -7), and inflammatory caspases (caspase-1, -4, and -5) and others (caspase-11, -12, -13, and -14) that do not belong in the general category[89,90]**.** Upon the detection of cell death inducing signals, engagement of initiator caspases leads to the activation of effector caspases that act directly on cellular substrates to dismantle the cell[88,91]**.** The induction of apoptotic

pathway is mediated through extrinsic and intrinsic signalling which are summarized below **(Figure A)**.

1.7.1 Death receptor - Extrinsic pathway

Ligation of death inducing ligands such Fas, TNFα, and TRAIL to their cognate death receptors activate the extrinsic apoptotic pathway[10]**.** The extrinsic pathway of apoptosis is best characterized with FasL/Fas and tumor necrosis factor alpha and receptor 1 (TNFα/TNFR1) models [89,92]. Following ligand-receptor interaction (FasL/Fas and TNF/TNFR1) adaptor molecules (Fas-associated death domain protein (FADD), TNF receptor associated death domain protein (TRADD))[93,94] are recruited in the cytosolic tail of the receptor which leads to the formation of death inducing signalling complex (DISC)[95]**.**The DISC platform, consisting of TRADD, FADD, and RIP[89] is responsible for the activation of caspase-8[95,96]**.** Activated caspase-8 may induce apoptosis by targeting downstream effector caspase-3, and -7 or through the activation of Bid. Caspase-8 targets Bid for proteosomal cleavage and produces truncated Bid which localizes in the mitochondria membrane, and oligomerizes with Bax/Bak to form a channel that depolarizes the mitochondria to release apoptogenic factors leading to induction of cell death[97–100]**.** A key regulatory molecule of the extrinsic pathway is cellular FADD-like interleukin-β converting enzyme (FLICE) inhibitory protein (cFLIP). Following the ligation of death inducing ligand with its cognate receptor, cFLIP binds to FADD and caspase-8 in DISC and prevents the activation of caspase-8 which ultimately limits the signal transduction of the extrinsic pathway of apoptosis [89,101]**(Figure A)**.

1.7.2 Mitochondrial-Intrinsic pathway

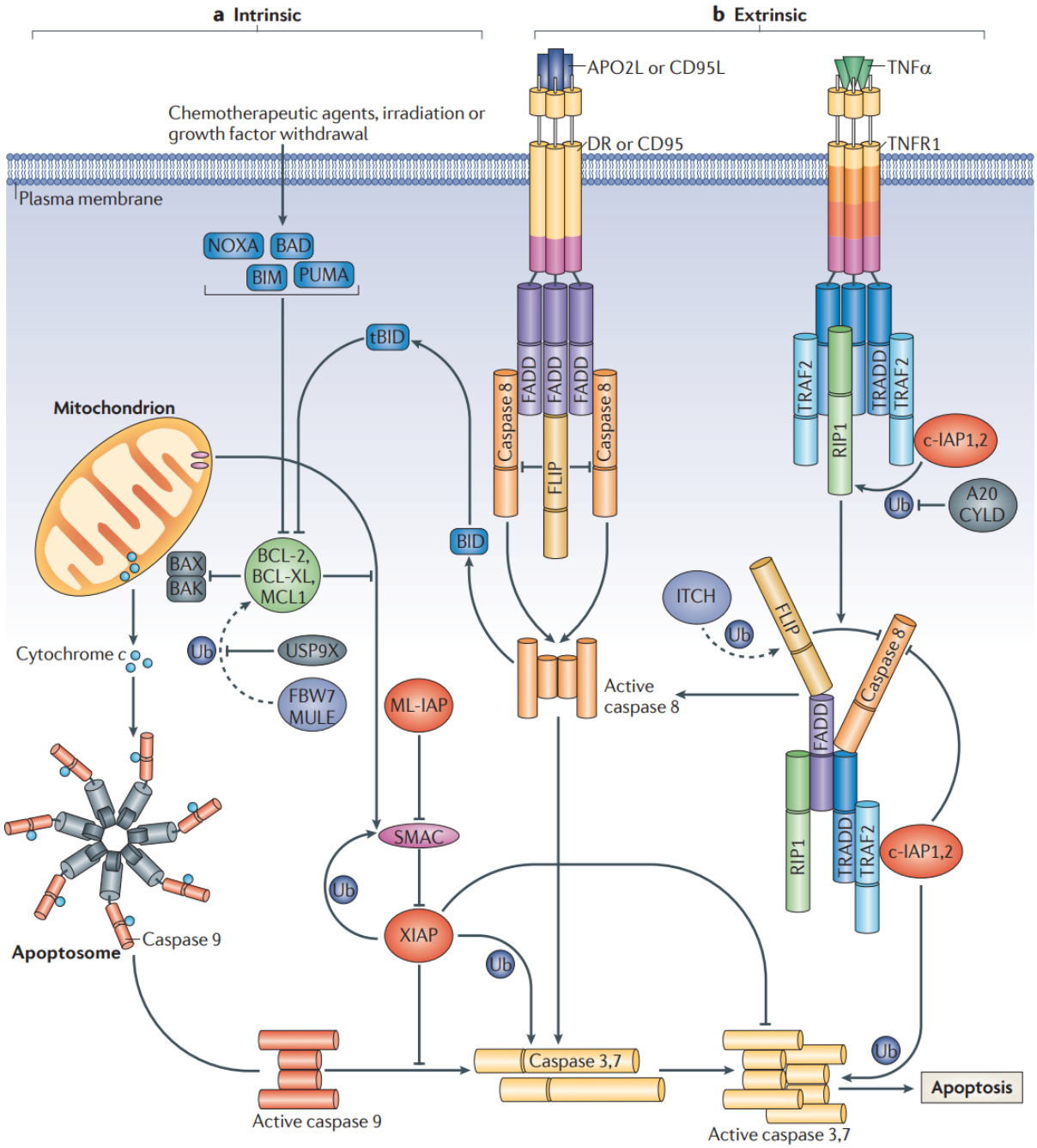
The intrinsic pathway of apoptosis is initiated by internal stressors caused by an array of non-receptor stimuli that produce intracellular signals which disrupts the integrity of the

mitochondria[89]. Stimuli such as radiation, hyperthermia, genotoxic stress, and free radicals, loss of apoptosis suppression and growth hormones can lead to opening of a high conductance channel called mitochondrial permeability transition pore (MPTP)[102]. Opening of MPTP depolarizes the mitochondria and releases two group of apoptogenic factors that are usually sequestered in the mitochondria in steady state[89]. The first group of apoptogenic factors have the capacity to directly activate the mitochondrial-dependent pathway. For instance, second-mitochondrial activator of caspases (SMAC) binds to Inhibitor of apoptosis proteins (IAPs) and disrupts their anti-apoptotic properties to allow the cell to undergo apoptosis[99]. In addition, cytochrome c can bind and activate apoptosis protease activating factor-1 (Apaf-1) as well as caspase-9 to form an "apoptosome" complex which directly targets procaspase-3 for proteolytic activation[103]. The second group of apoptogenic factors released upon mitochondrial depolarization include apoptosis inducing factor (AIF), endonuclease G, caspase-activated DNAse. These proteins translocate into the nucleus and mediate the fragmentation and degradation of chromatin and DNA[89]**.** The intrinsic pathway is most commonly regulated through the maintenance of the mitochondrial integrity[10]. The Bcl2 family of proteins include anti-apoptotic Bcl-xL and Bcl2 which prevent mitochondrial dysfunctions, as well as pro-apoptotic Bak and Bax which promote the release of sequestered apoptogenic factors from the mitochondria into the cytosol[104]**.** The balance between the anti-apoptotic and pro-apoptotic Bcl2 members dictate fate of a cell to undergo apoptosis or survival **(Figure A).**

1.8 Structure and function of inhibitor of apoptosis proteins

Apoptosis is an important cellular process that has severe repercussions if not regulated properly. There are numerous pathological conditions that exhibit aberrant apoptosis such as neurodegenerative diseases and autoimmune deficiency syndrome (AIDS)[105]. Conversely, lack of

Figure A. Induction and regulation of extrinsic and intrinsic pathways of apoptosis. Ligation of death inducing stimuli with cognate receptors engages the activation of extrinsic pathway of apoptosis leading to the activation of caspase-8 which targets downstream effector caspase-3, and -7. Release of cytochrome C or SMAC (second-mitochondrial activator of caspases) from the mitochondrial engages the intrinsic pathway of apoptosis. Cytochrome C mediates the formation of apoptosome which is responsible for activation of initiator caspase-9.



Vucic *et al.* 2011[106]

apoptosis in cancer cells and cells infected with some virus make these conditions hard to treat. Therefore, apoptosis must be finely tuned to prevent the induction of disease phenotype. The extrinsic and intrinsic pathways of apoptosis have their own respective regulatory mechanisms that come in the form of FADD-like IL-1β converting enzyme (FLICE) inhibitory protein (FLIP) and Bcl2 family of proteins, respectively[107,108]**.** However, the last line of defense against apoptotic self-destruction is the family of proteins called inhibitor of apoptosis proteins (IAPs)[109].There are 8 members of the IAP family in humans; however, cellular IAP1 and 2 (cIAP1/2) and x-linked IAP (XIAP) are the members most studied [110]. These proteins feature baculovirus IAP repeat domain (BIR), a 70-residue large zinc binding domain that mediates binding of IAPs with adaptor molecules[110]**.** BIR domains are characterized by the presence of peptide-binding grooves and are divided into two groups: type-1 and type-2 BIR. Type-2 BIR is characterized by a distinctive deep hydrophobic cleft through which they bind certain IAP-binding motifs (IBMs) on target proteins such as SMAC, caspases, or analogous peptides[111], while Type-1 BIR has shallow groove and incapable of binding such proteins. Instead, Type-1 BIR use distinct sites to interact with their target proteins [110,112]**.** Moreover, IAPs have C-terminal RING finger domain which mediates their E3 ubiquitin ligase activity[113–115]**.** In addition to BIR and RING domains, another conserved region in IAPs is the caspase recruitment domains (CARD) which plays a role in protein-protein interaction[114,116–119] **(Figure B)**.

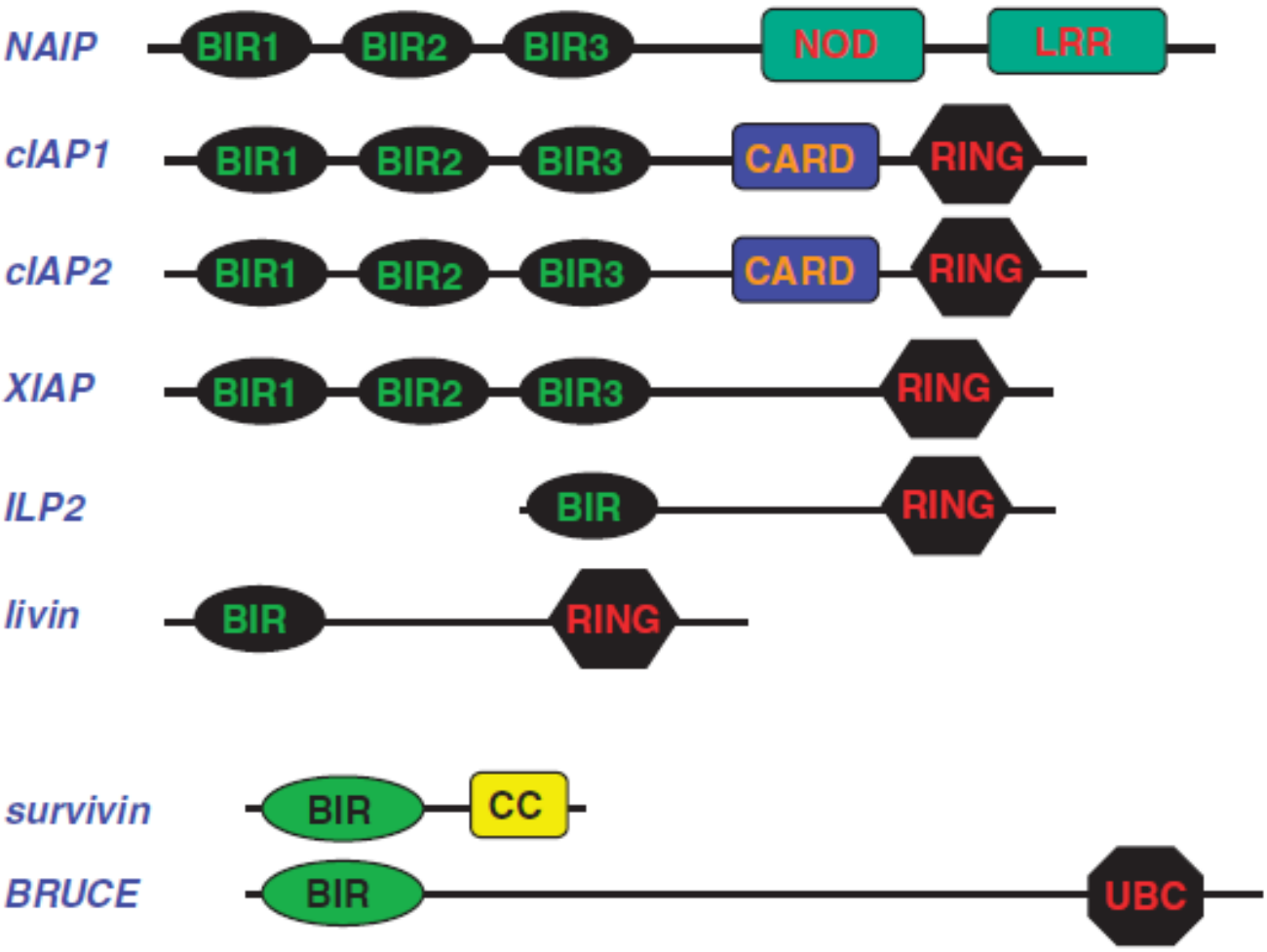
1.9 Regulation of apoptosis by IAPs

Intricate studies on the roles of human XIAP, cIAP1, and cIAP2 reveal that these IAPs vary in the way they regulate apoptosis. XIAP has been extensively characterized as a natural potent antagonist of caspases in a "lock-and-key" mechanism [112,120,121]**.** XIAP contains BIR2 and

BIR3 that bind to the IBM motifs of the catalytically active forms of caspase-3, 7, and 9 [120,122,123]**.** BIR2 domain selectively binds to the surface above the active sites of caspase-3 and 7, thereby prevents substrate entry and limits their enzymatic activity[120,124–126]. On the other hand, BIR3 binds to active caspase-9 and traps the protease in a incompetent monomeric conformation[115,122].

In human, cIAP1 and cIAP2 are the closest paralogues of XIAP[127]**;** however, compared to the role of XIAP in suppressing apoptosis, there is controversy surrounding the anti-apoptotic function of these members of IAP-family of proteins. Because of the high degree of homology with XIAP, cIAPs have been often considered to function as direct caspase inhibitors[128]**,** but several studies show that cIAPs do not function the same way. Much like XIAP, cIAP1/2 have been shown to bind to caspase-3, -7, and -9; however, these proteins have been reported to unsuccessfully ablate the activity of these caspases due to the differences in the in IBM-interacting sites on their BIR domains[110,120,129]**.** Nevertheless, various studies have shown that cIAPs grant cells resistance against apoptotic stimuli,[130,131]**,** facilitate tumorigenesis [132], and survival of cancer cells [133,134]**.** The antiapoptotic activity of these proteins are dependent on their E3 ubiquitin ligase activity[133,135–137]**.** The RING finger domains on the C-terminal of cIAPs grants these proteins the capacity for catalysing the transfer of ubiquitin to the target caspase-3 and 7 [137,138]**.** Upon being ubiquitinated, caspases are sent for proteosomal degradation which effectively prevent the induction the apoptotic pathway [137,138] **(Figure A).**

Figure B. Visual representations of IAP family of proteins.
Of the IAP family of proteins identified, XIAP (X-linked IAP), cIAP1, and cIAP2 are best-described. BIR baculovirus IAP repeat, NOD nucleotide-binding and oligomerization domain, LRR leucine-rich repeat, CARD caspase-recruitment domain, RING really interesting new gene, CC coiled-coil, UBC ubiquitin-conjugating domain.



LaCasse *et al.* 2008[139]

1.10 Role of IAPs in signal transduction

cIAPs have functions that go beyond protecting cells from apoptotic stimuli. The family members of the tumor necrosis factor (TNF) and TNF receptor (TNFR) are conducive to the regulation of immune function, and aberrations in this system have been implicated in several genetic diseases [140]. Upon binding of the TNF ligand with cognate TNF receptor, the assembly of receptor-associated complexes is triggered which mediates the activation of signalling pathways such as nuclear factor kB (NF-kB) and mitogen-activated protein kinase (MAPK) and even apoptosis. cIAP1 and cIAP2 play a crucial role in the activation of NF-kB and MAPK through their ubiquitination functionality. cIAPs poly-ubiquitinate the K11 and K63 of receptor-interacting protein 1 (RIP1) at the TNFR-1, which allows for the recruitment several kinase proteins, one of which is the inhibitor of kinase kinase beta (IKK-β) [141]. NF-κB transcription factor is sequestered by IKB-α in the cytosol. IKK-β phosphorylates IKB-α and facilitates its degradation which in turn lifts the sequestration of NF-κB to allow it to enter the nucleus and regulate the transcription of targeted genes[131,141]. In addition to RIP1, cIAPs also target apoptosis signal-regulating kinase 1 (ASK1) for proteosomal degradation to activate c-Jun N-terminal kinase (JNK) and p38 MAPK which are important for blocking apoptosis by promoting cell survival [141,142].

1.11 Second-mitochondrial activator of caspases (SMAC) and SMAC-mimetics (SM)

In response to apoptotic stimuli, one of the apoptogenic factors that is released from the mitochondria is second-mitochondria-derived activator of caspases (SMAC)/direct-IAP binding protein with low PI (DIABLO) which functions as an endogenous inhibitor of IAP molecules [115,143,144]. Fully matured SMAC has an exposed hydrophobic tetrapeptide motif composed of Ala-Val-Pro-Ile that to its pro-apoptotic functions[143,145]. The tetrapeptide allows SMAC/DIABL0 to bind to BIR domains of XIAP to directly relieve the inhibition of activated caspase-3, -7, and -9

[115,143], while SMAC/DIABLO promotes the auto-ubiquitination and subsequent degradation of these cIAP1/2[146]. Since a variety of human cancer cells have overexpressed levels of IAPs, manipulation of the IAP-associated signalling pathways has been proposed to be a target for cancer therapeutics[147,148]. Based on the interaction of SMAC with XIAP, a class of compounds that mimicked the activity of SMAC/DIABLO called SMAC-mimetics (SM) was developed [149,150]. SM has been shown to be well-tolerated in *in vivo* settings[151,152] and monomeric SM such as LCL161 is currently being tested in phase 1/2 clinical trials in patients with advanced stages solid tumors and lymphomas[147,151,152].

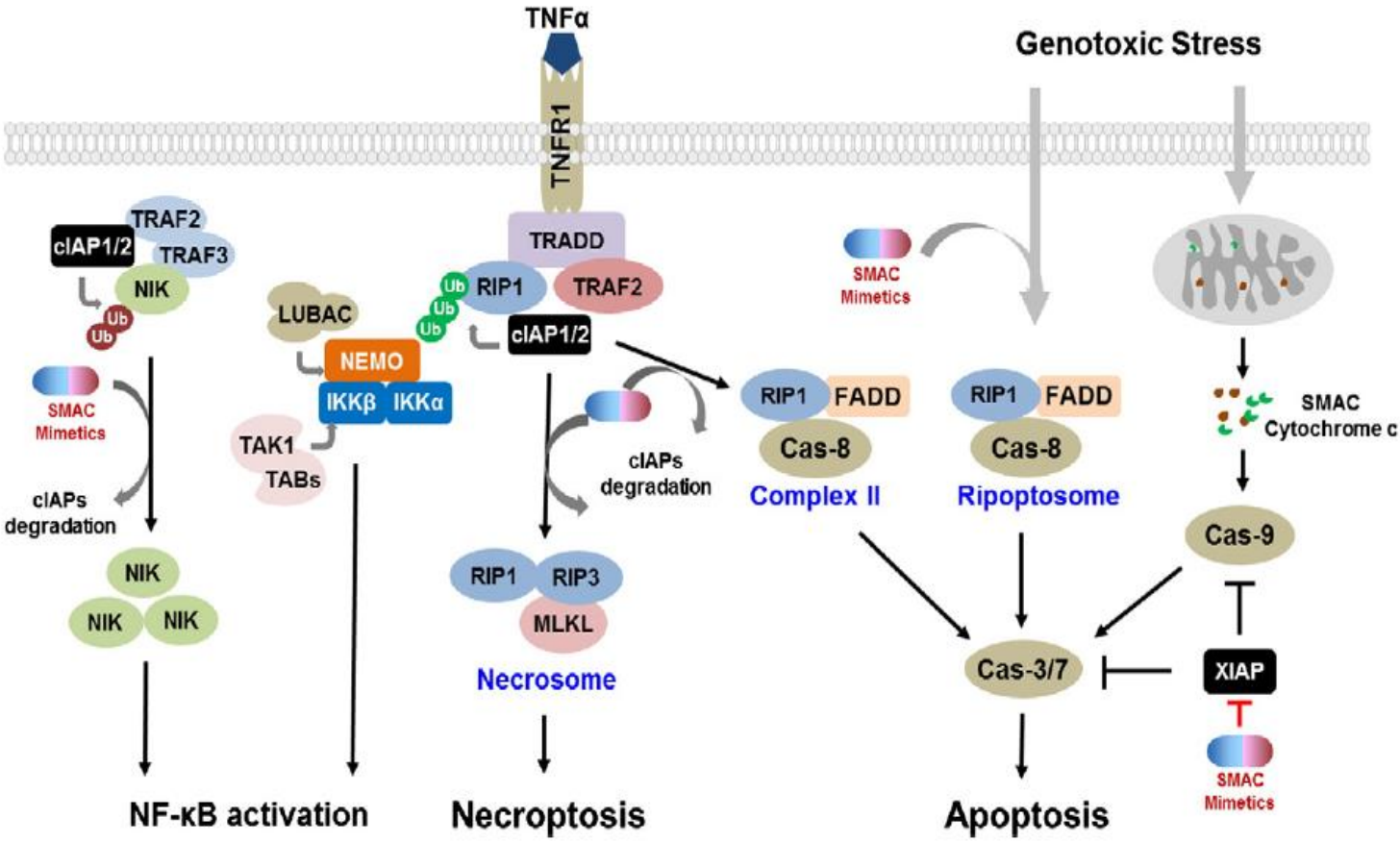
1.12 SM mechanism of action to induce apoptosis

In the absence of mitochondrial release of apoptogenic factors, SM can induce cell death by disrupting the interaction of BIR domains of XIAP with caspase-3, -7, and -9, leading to cell death induction [153,154]. In addition, SM stimulates the E3 ubiquitin ligase activity of cIAPs which promotes their autoubiquitination and targets themselves for proteosomal degradation[150,155,156] The absence of cIAPs, not only disrupts the activation of canonical NF-κB pathway, but subsequently activates the non-canonical NF-κB. cIAP1/2 degradation destabilizes NF-kB-inducing kinase (NIK) and as a result, leads to the transcriptional activation of NF-kB genes such as TNFα [157]. Thereafter, the secreted TNFα triggers the induction of cell death pathway in SM-treated cells in an autocrine or paracrine fashion via the TNFR1 ligation[136,158] **(Figure C).**

A strong body of evidence shows that as a single agent, SM mimetic can induce cell death in several cancers such as acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML)[159–161]. In SM monotherapy, the degradation of cIAPs is an early event in inducing apoptosis in cancer initiated by the autocrine/paracrine secreted TNFα leading to apoptosis of SM-sensitive cells[159,161]. Accordingly, ablation of the TNFα/TNFR1 signalling via siRNA[159,161] or blocking

antibodies has been shown to abrogate the therapeutic effect of SM [135,136]**.** Interestingly, most tumour cells are not sensitive to SM-induced cell death due to failed secretion of TNFα and even though most of the required mediators for apoptosis execution such as caspase-8, RIP1, and FADD are present[162]**.** However, upon the addition of exogenous TNFα, rapid induction of cell death can be observed in SM-treated cells[160,162,163].

Figure C. Schematic representation of the known function of cIAP1/2 and its regulation by SM. cIAP1/2 ubiquitinates RIP1 allowing for the recruitment of signalling complex for activation of NF-κB. Loss of cIAPs by SM allows for the formation necrosome complex composed of RIP1, RIP3, and MLKL to induce necroptosis. Alternatively, apoptosis can be induced through the formation of complex II and ripoptosome from RIP1, FADD, and caspase-8.



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1.13 Increased resistance to HIV-induced cell death

Apoptosis is an important branch of host immunity against invading intracellular pathogens because it serves to limit pathogen viability, prevents dissemination, facilitates presentation of pathogenic antigens, and in the purpose of HIV infection- prevents the formation of viral reservoirs [10]**.** Therefore, how apoptosis is regulated could dictate the result of the host-pathogen interaction. Pathogens of human macrophages have developed novel strategies to counteract the induction of apoptosis by dysregulating the balance between pro- and anti-apoptotic machineries as an attempt to persist within the host [165–167]**.** Similarly, there are evidence to suggest that HIV-1 possess such capabilities.

Initial findings supporting the notion of HIV-1 mediated increase in apoptotic resistance came from cell line models. Infection with HIV-1 disrupts the CD95/CD95L death receptor pathways which renders U1 cell line, a promonocytic cell line chronically infected with HIV-1, less susceptible to Fas-mAB mediated apoptosis compared to its non-infected parental cell line, U937[168,169]**.** Moreover, susceptibility to various apoptotic stimuli such as TNFα, γ-irradiation, cycloheximide, and staurosporine was also observed to be reduced in chronically infected U1 than in U937[170]**.** The increase in Bcl2/Bax ratio in the mitochondria of chronically infected U1 granted this cell line an anti-apoptotic phenotype which rendered these cells less susceptible to staurosporine- and H_2O_2-induced apoptosis[171]. Moreover, Berro and colleagues reported the that x-linked inhibitor of apoptosis protein (XIAP) was significantly active in infected U1 than uninfected U937[172]**.** Admittedly, these reports have important experimental and physiological limitations. Although the mechanisms for increased apoptotic resistance in monocytic lineage infected with HIV-1 is unclear, these findings indicate that the virus may be modulating the balance between pro- and anti-apoptotic factors to its own advantage.

The analysis of apoptosis susceptibility between non-infected and infected cells has been hampered by the inefficient infection rate of monocytes and macrophages *in vitro*; however, emerging findings from primary monocytic cell lineage provide support that infected monocytes and macrophages exhibit increase resistance to apoptosis. Circulating monocytes from untreated chronically HIV-seropositive viremic patients have anti-apoptotic gene signature associated with metallothionein, p53, CD40L, TNF-α, and MAPK survival pathways[173,174]. Even in the absence of productive infection, monocytes from viremic patients exhibit resistance to FasL-mediated apoptosis induction *ex vivo*, which corroborates the previous observations in chronically infected monocytic cell lines[173,175].

Macrophages are naturally resilient cells that can resist a battery of apoptotic stimuli and HIV infection further promotes the anti-apoptotic phenotype of these cells[10]. It was reported that *in vitro* HIV-1 infection of monocyte-derived macrophages resulted in the upregulation of telomerase activity, leading to reduced oxidative stress damage which could be considered as a strategy for maintaining longevity of resistant viral reservoir[176]. Furthermore, anti-apoptotic Bcl2 family of proteins such as Bcl-2 and Bcl-xL are upregulated in the levels of mRNA and proteins upon HIV-infection which prevent mitochondrial depolarization by maintaining mitochondrial integrity[177,178]. Moreover, primary macrophages infected with HIV have increased secretion of MCSF which not only prolonged longevity, but also reduced TRAIL receptors which rendered Mϕ less susceptible to TRAIL-induced cell death[69]. Additionally, triggering receptor expressed on myeloid cells 1 (TREM-1), a transmembrane glycoprotein receptor that mediates mitochondrial integrity in macrophages through Bcl-2 induction, has also been shown to be upregulated upon HIV infection and prolong survival of MDMs[179,180]. Although the mechanisms for the increased resistance to apoptosis were not fully elucidated in these earlier studies, it is reasonable to

hypothesize that it is predicated on the differential regulation of pro- and anti-apoptotic proteins in infected monocyte/macrophages.

1.14 Selective killing of HIV-infected macrophages as a therapeutic approach to HIV cure

Since macrophages are resistant to a battery of apoptotic stimuli, and HIV-1 modulates and prolongs survival of these cells, developing a therapeutic strategy capable of inciting cell death in HIV-infected MDMs (Mϕ) while sparing the uninfected ones becomes a challenge. The most common theme in selectively inducing cell death of infected macrophages is to take advantage of the factors upregulated or associated with HIV infection[181–183]. For instance, HIV viral envelope was reported to prolong survival of infected macrophages by upregulating the secretion of pro-survival MCSF leading to TRAIL receptor downregulation and rendering the cells less susceptible to TRAIL-induced cell death[69]. Cunyat and colleagues showed that blockade of MCSF signalling pathway by selective antagonist of MCSF receptor PLX3397/PLX5622 abrogated the HIV-env-induced protection and allowed infected macrophages to succumb to TRAIL-induced apoptosis[184]**.** A finding from another group showed that TREM-1 induced anti-apoptotic Bcl-2 in response to lipopolysaccharide which prolonged survival of macrophages during inflammatory response[179]**.** Interestingly, TREM-1 was found to be upregulated in HIV-infected macrophages and knockdown of TREM-1 by siRNA rendered infected macrophages susceptible to cytopathic effects of HIV-1 [179]**.**

Recently, we have demonstrated a protective role for cIAPs against apoptogenic HIV-Vpr induced cell death in monocytes and macrophages[4,185,186]**.** HIV-Vpr is an accessory protein produced early during HIV-infection and plays a role in infection of non-dividing cells such as macrophages[187,188]. Monocytes are susceptible to Vpr-induced cell death but gain resistance upon differentiation into mature macrophages through increased expression of anti-apoptotic cIAP1/2.

Additionally, CpG- and bacterial DNA-induced cIAP1/2 were shown to protect monocytes against HIV-Vpr-induced cell death[185]. Degradation of cIAP1/2 by SM rendered monocytes and macrophages susceptible to apoptosis via HIV-Vpr. Thus, targeting IAPs is an attractive strategy for clearing infected macrophages. In fact, there have been other groups that pursued the role of IAPs in selectively inducing cell death of HIV infected cells. Berro and colleagues demonstrated that XIAP downregulation by flavopiridol cyclin-dependent kinase 9 (CDK-9) inhibitor increased apoptosis of latently infected ACH2 cell line compared to the uninfected parental cell line counterpart[172]. Furthermore, GDC-0152, a XIAP antagonist was demonstrated to induce cell death of latently infected primary $CD4^+$ T cells[189]**.** Interestingly, Birinipant, a dimeric SM was demonstrated to clear hepatitis B-infected cells both *in vitro* and in immunocompetent mouse model which suggested that SM may have efficacy in treating intracellular infections[190,191]**.** The role that IAPs play during HIV infection of macrophages remains largely unknown and therefore warrant intensive investigation.

Chapter 2: Rationale and Hypothesis

2.1 Rationale

The hallmark of HIV disease progression is the rampant depletion of $CD4^+$ T cells, but not of monocyte/macrophages. Macrophages can support productive infection without succumbing to cytopathic effects of HIV-1. As a result, the number of these cells does not decline during acute or chronic stage of infection. In the face of suppressive cART, macrophages serve as cellular reservoirs that ubiquitously reside in tissues throughout the body, including the poorly drug-accessible CNS. To fully eradicate HIV-1, it is imperative to eradicate all type of reservoirs. However, most of HIV cure research is focused on the elimination of infected memory $CD4^+$ T cells reservoir, whereas limited knowledge about the possible elimination of macrophage reservoirs exists.

In order to eliminate macrophage viral reservoirs, it is of paramount importance to understand the mechanisms that mediate the resistance of HIV-infected macrophages to apoptosis. Preliminary reports on chronically infected cell lines and *in vitro* infection of macrophages with HIV show that the antiapoptotic phenotype of infected monocytes/macrophages may be predicated on the differential regulation of pro- and anti-apoptotic genes such as the Bcl-2 family of proteins and IAPs. In our laboratory, we have reported for the first time a protective role of antiapoptotic cIAP1/2 in macrophages against HIV-Vpr protein. Depletion of cIAP1/2 by SM renders macrophages susceptible to HIV-Vpr-induced apoptosis. Thus, elucidation of possible mechanisms of SM-mediated killing of macrophages during infection warrant further investigation.

2.2 Hypothesis

SMAC-mimetics (SM) induce cell death of HIV-infected macrophages by apoptosis

2.3 Research Aims

Aim 1: Assess the effect of SM on the viability of HIV-infected myeloid cells

1A. Assess the effect of SM on non-infected/chronically infected U937(U1)

1B. Assess the effect of SM on *in vitro* mock/HIV-infected macrophages

Aim 2: Elucidate the underlying mechanism of selective SMAC-induced killing of HIV-infected macrophages

2A. Determine the involvement of TNFα in SM-induced cell death

2B. Assess the cytokine profile of HIV-infected macrophages

2C. Determine the regulation of IAP-related signalling molecules in HIV-infected MDMs before and after SM treatment.

Chapter 3: Material and methods

3.1 Isolation of primary monocytes, generation of monocyte-derived macrophages, cell lines, chemical reagents

Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll Paque (GE Healthcare Life Sciences Buckingmshire, UK) from the blood of healthy donors. Human monocyte-derived macrophages were generated from monocytes via adherence methods. Quickly, $2.0x10^6$ PBMCs/well were plated 2-3 hours. Monocytes were allowed to adhere for 3 hours and non-adherent cells were washed off. Adherent monocytes were cultured for 7 days in DMEM (Wisent Inc., St. Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, Missouri, USA) and 10ng/mL MCSF (R&D Systems, Minneapolis, MN, USA). MCSF-containing media was replaced every 2 days until the 7^{th} day at which point the monocytes would have differentiated into macrophages. Purity of macrophages were assessed by measuring CD14 expression by flow cytometry.

U937 and U1 cells were obtained from NIH AIDS reagent program and were cultured in DMEM media supplemented with 10% FBS, penicillin and streptomycin. $5.0*10^5$ cells/well were used for all the chemical treatments. For differentiation, $5.0*10^5$ U937 and U1 cells were differentiated using 20nM PMA (Sigma Aldrich, St. Louis, Missouri, USA) for 2 days.

The following chemicals were used: LCL161 (Active Biochem, Hongkong), necrostatin-1 (ApexBio, Houston, TX, USA), staurosporine (ApexBio, Houston, TX, USA), lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, Missouri, USA).

3.4 Assessment of cell death by intracellular PI staining and IncuCyte® live cell imaging, and quantification of caspase activation

Apoptotic cells were assessed by the levels of intracellularly stained sub-G_0 DNA content, as previously described[185]**.** Briefly, cells ($1.0x10^6$/well) were washed with PBS and were fixed with cold methanol for 15 minutes at 4ºC. The cells were then treated with 1µg/mL RNAse A (Roche Applied Science, Laval, Quebec, Canada) and 1µg/mL propidium iodide (PI) stain (Sigma Aldrich, St. Louis, Missouri, USA) for 1 hour at 4ºC. Activation of caspase-3, -8, and -9 was measured as per Abcam's Caspase staining kit protocol (Abcam, Toronto, ON, Canada). Quickly, after 48 hours of SM treatment of both mock and HIV-infected Mϕ, the cells were gently harvested, transferred into a flow tube, and washed twice with PBS. 100uL of cell permeable caspase-3, -8, and -9 FITC-conjugated specific inhibitors, provided in the kit, were added to each tube and the cells were placed in the incubator for 1 hour. The cells were then washed and resuspended in 300µL PBS. Specific inhibitors of caspases bind irreversibly to active caspases in apoptotic cells. After washing, FITC labelled cells were measured by flowcytometry (BD Fortessa X-20, BD Bioscience, San Jose, CA, USA)) and the results were analyzed by FlowJO v.10 software.

Kinetics of SM-induced cytotoxicity was measured through IncuCyte® live cell imaging technology (Essen Bioscience, Michigan, USA). $1.0x10^6$/well were washed twice with PBS and fresh media was added. 50nM of Yoyo-1 (ThermoFisher Scientific, Waltham, Massachusetts, USA), a nonpermeable fluorescent dye which stains DNA of cells that have reduced or lost cell membrane integrity. The cells were then treated with increasing concentration of SM (0.5- 4.0 µM) as well as staurosporine (1µM) for positive induction of cell death. The plate was then placed in the plate tray holder into IncuCyte® ZOOM (Essen Bioscience, Michigan, USA). Yoyo-1 fluorescence was acquired by the machine every two hours for the period of 48 hours and increase

in Yoyo-1 reuptake, and therefore fluorescence was indicative of apoptotic cells. Analysis of cell death was then conducted through IncuCyte® ZOOM 2016A software.

3.5 Extraction of cytosolic protein contents and protein quantification by Bradford assay

Cell pellets ($1.0x10^6$/well) were lysed using lysis buffer (1% Triton X-100, 150 mM NaCl, 10mM Tris, 1mM EDTA, 1mM EGTA, 0.5% NP-40) for 1 hour at 4°C. The lysed cells were subjected to 14,000 rpm centrifugation for 30 minutes and the supernatants collected. Protein content was quantified using Bradford assay. Quickly, 1:5 dilution of protein assay dye concentrate (BioRad Laboratory, Hercules, CA, USA) was added to a cytosolic protein solution (1:200 dilution) and the 595nm absorbance of the Coomasie® Brilliant Blue G-250 was measured with a spectrophotometer.

3.6 Western immunoblot analysis and antibodies

Quantified proteins were subjected to SDS-PAGE electrophoresis and were transferred onto polyvinylidene difluoride (PVDF) membrane (BioRad Laboratory, Hercules, CA). For 10-well gels, 70 ug or proteins was loaded, and for 50 ug of proteins for a 15-well gel. The membranes were probed using primary antibodies specific for cIAP1, cIAP2, XIAP, caspase-3, caspase-8, caspase-9, beta actin, PARP, BcL-xL, Bcl-2, Bax, Bak, TRA1, TRAF2, RIP1, RIP3 (Cell Signalling Tech, Inc., Danvers, MA), followed by goat anti-rabbit or anti-mouse secondary polyclonal antibodies conjugated to horseradish peroxidase (BioRad Laboratory, Hercules, CA). Proteins were visualized by enhanced chemiluminescence (Amersham Bioscience).

3.8 Virus Production

Dual tropic HIV-CS204 was gifted by Dr. Jonathan Angel (Ottawa General Hospital, Ottawa, ON, Canada). HIV_{CS204} stocks were produced in $CD8^+$ depleted PBMCs from healthy

blood donors. Stocks of mock virus were produced in the same condition, but with the absence of the virus. Virus growth was confirmed indirectly by measuring the amounts of HIV p24 proteins in the supernatants. Samples of mock and HIV stocks were inactivated for 1 hour with 1% TRITON X-100 (Sigma Aldrich, St. Louis, MO). p24 proteins were measured by HIV-1 $p24^{CA}$ capture kit as per the instructions of the manufacturer (AIDS & Cancer Virus Program, National Cancer Institute, Fredrick, MD).

Viral construct NL4-3-Bal-IRES-HSA was gifted by Dr. Jean Tremblay (Centre de Recherche en Infectiologie, Centre Hospitalier de l'Université Laval, and Faculté de Médecine). The plasmid was amplified using One Shot® Stbl3™ competent *E. coli* **(**Invitrogen, Carlsbad, CA, USA) and was isolated using endotoxin-free plasmid DNA isolation mega kit (Thermofisher, Waltham, Massachusetts, USA). The plasmid was transfected into 293T cells to generate HIV-$^{NL4.3\text{-}Bal\text{-}IRES}$-HSA strain. Virus production was confirmed by p24 ELISA as using HIV-1 $p24^{CA}$ antigen capture assay kit (Leidos, Frederick National Laboratory for Cancer Research, Frederick, MD, USA).

3.9 Infection of macrophages with HIV

After seven days of differentiation, macrophages were washed twice with PBS and were infected with 100ng p24 per $1.0*10^6$ cells supplemented with 5ug/mL polybrene (Sigma Aldrich, St. Louis, Missouri, USA) and incubated overnight (~16 hours). Cells were then washed and infected with clinical isolate HIV_{CS204} or $HIV_{NL4.3\text{-}IRES\text{-}Bal}$-HSA and incubated for 7 days post-infection before chemical treatment. Supernatants were then collected and levels of p24 were assessed using p24 ELISA kit as stated above.

3.10 Isolation of HIV-HSA-infected macrophages

PBMCS were seeded in a 15 cm^3 petri dish plate and the protocol for generation of monocyte-derived macrophages was followed. After 7 days of differentiation, cells were infected with $HIV_{NL4.3\text{-}IRES\text{-}Bal}$-HSA. Nine days post-infection incubation, cells were subjected to HSA-CD24 magnetic sorting (MACS Miltenyi Biotec, Auburn, CA, USA)) through column separation, as previously described[192]**.** Briefly, infected macrophages were washed thoroughly with PBS and detached with Accutase (Innovative Cell Technologies, San Diego, CA, USA) before staining. FcRγII receptors on cells were then blocked with FcR blocker (MACS Miltenyi Biotec, Auburn, CA, USA) and cells were then stained with primary CD24-Biotin conjugated antibody and incubated with anti-biotin ultra pure microbeads (MACS Miltenyi Biotec, Auburn, CA, USA). HSA-expressing cells were collected by positive selection in LS columns (MACS Miltenyi Biotec, Auburn, CA, USA). Cells not expressing HSA (negative fraction) were collected after passing through the labelled cells through the column for the first time. Positively labelled cells were collected by detaching the column from the magnet and plunged out from it. Purity of the HSA-infected macrophages was assessed by staining the CD24-Biotin labelled cells with anti-Biotin PECy7 antibody and analyzed by flow cytometry.

3.11 Cytokine analysis by ELISA and cytokine array

Human TNF-α and TRAIL duo set (R&D System, Minneapolis, MN, USA) were used to quantify the levels of secreted TNF-α and TRAIL in supernatants. ELISA was conducted based on the lot-specific protocol of the kit. The 96-well plate was preincubated with TNF-α/TRAIL capture antibody in PBS overnight (~16 hours) at room temperature. The following day the plate was washed with wash Buffer (0.01% tween PBS) using Elx50 ELISA washer (Biotek, Winooski, VT) and the plate was blocked with 1% FBS in PBS buffer for 2 hours at room temperature. Following

the blocking incubation, the TNFα/TRAIL standard with highest dilution of 1000pg/mL, was added to the plate as well as the undiluted/diluted samples and the plate was left to incubate at 4^0 overnight (~16 hours). On the third day, the plate was washed as previously described, and the plate was incubated with detection antibody for two hours at room temperature. The plate was aspirated as previously described, and 100uL/well of substrate solution was added and the enzymatic reaction was allowed to occur until the reaction in the lowest diluted standard was visible. 50uL/well of stop solution (BioFX Labs, Owing Mills, MD) was added to stop the reaction. Lastly, the plate was read at 490nM using iMark Microplate reader (BioRad, Mississauga, ON, Canada) and the output absorbances were analyzed using microplate manager 6 software.

Levels of secreted cytokines were measured as per protocol provided in Milliplex map kit (cat. # HTH17MAG-14K, Millipore, Etobicoke, ON, Canada). IL-17F, GM-CSF, IFNγ, IL-10, CCL20/MIP3a, IL-12p70, IL-13, IL-15, IL-17a, IL-22, IL-9, IL-1β, IL-33, IL-21, IL-23, IL-5, IL-6, IL-17ε/IL-25, IL-27, IL-31, TNFα, TNFβ, and IL-28A were detected using antibody-immobilized magnetic beads and were quantified by MAGPIX® multiplex with xPONENT® software by Luminex Corporation.

3.12 Statistical analysis

Data was plotted using GraphPad Prism 5. Statistical significance was calculated using student T test, One-way Anova, followed by Dunnett post test, or Mann-Whitney U test. Plotted data represent the mean±standard deviation of at least n=3, unless otherwise indicated.

3.13 Ethics statement

Healthy participants involved in the study gave informed written consent and the protocol for obtaining blood samples was approved by the Review Ethics Board of the Ottawa General Hospital.

Chapter 4: Results

Aim 1: Assess the effect of SM on the viability of HIV-infected myeloid cells

Optimization of the dose of SM on Mϕ and myeloid cell lines

cIAPs are important known regulators of the NF-κB pathway, a pathway which plays a role in protecting cells from programmed cell death[116,193,194]. A class of synthetic compound called SM have been developed as cancer therapeutic agents to disrupt the functionality of overexpressed IAPs in cancer cells and induce cell death[149]**.** SM have also been reported to be associated with dose-limiting toxicity *in vivo*[152]. Therefore, it was imperative to optimize the concentration of SM to be used in future experimentation. Mϕ were treated with increasing concentration of LCL161 (SM hereafter) and the kinetics of cell death induction over the period of two days was determined by Incucyte® live cell analysis. The green fluorescent signal was indicative of the yoyo-1 reuptake, and therefore apoptotic cells. Treatment with SM caused a minimal induction of cell death compared to the untreated control; however, all five concentrations (0.5 to 4.0 μM) of SM that were used showed similar levels of apoptosis induction **(Figure 1A).** Staurosporine (STS) was used to induce significant apoptosis as a positive control.

SM bind to cIAP1/2 and promote their E3 ligase activity which leads to the autoubiquitination and subsequent proteosomal degradation of these proteins [150,155,156]**.** To verify the functional activity of SM, Mϕ were treated with increasing concentration of SM for 48 hours and levels of cIAP1/2 were detected by Western immunoblotting. Both cIAP1 and cIAP2 were targeted for proteosomal degradation with the treatment of SM; however, cIAP2 showed greater degree of downregulation compared to cIAP1 **(Figure 1B)**. For determination of optimal dose for treatment of Mϕs, M1-polarized Mϕs were treated with increasing concentration of SM (0.5, 1.0, 2.0, and 4.0 μM) as positive control, and cell death was assessed through intracellular PI staining

after 48 hours of treatment. Previously in our laboratory, we have shown that M1-polarized Mϕ show the most susceptibility to SM-induced cell death amongst the *in vitro* generated subsets of Mϕ. Consistent with our previous results, M1-polarized Mϕ were highly susceptible to SM. Significant induction of cell death (**p=0.0065, *p= 0.0103) was observed between all the concentrations and DMSO vehicle control in the M1-polarized group **(Figure 1C)**. On the other hand, SM did not show toxicity in Mϕ as no significant differences in cell death between the chemical treatments and vehicle control were observed. Representative histograms of the intracellular PI staining assay are shown in **figure 1D**. In view of this information, I used SM at concentrations of 0.5, 1.0, 2.0, and 4.0 μM for the treatments of primary Mϕ in my study depending on various experimental conditions.

Figure 1. Determination of optimal concentration of SM for the treatment of Mϕ. (A). Mϕ were treated with increasing concentration of LCL161 and levels of cell death was assessed by quantifying the yoyo-1 fluorescent green signal marker using Incucyte® live cell imaging technology. **(B)** Cytosolic fractions from Mϕ that were treated with increasing concentration of SM LCL161 for 48 hours were collected and subjected to western immunoblotting. The membranes were probed with antibodies specific for human cIAP1/2. **(C)** Mϕ were polarized with IFNγ (20ng) for two days to obtain M1 phenotype and the cells treated with increasing concentration of SM LCL161 for two days (n=3). Cell death was assessed by intracellular PI staining and flow cytometry and **(D)** shows representative histograms for the treatment. p-values were calculated using Mann-Whitney U test.

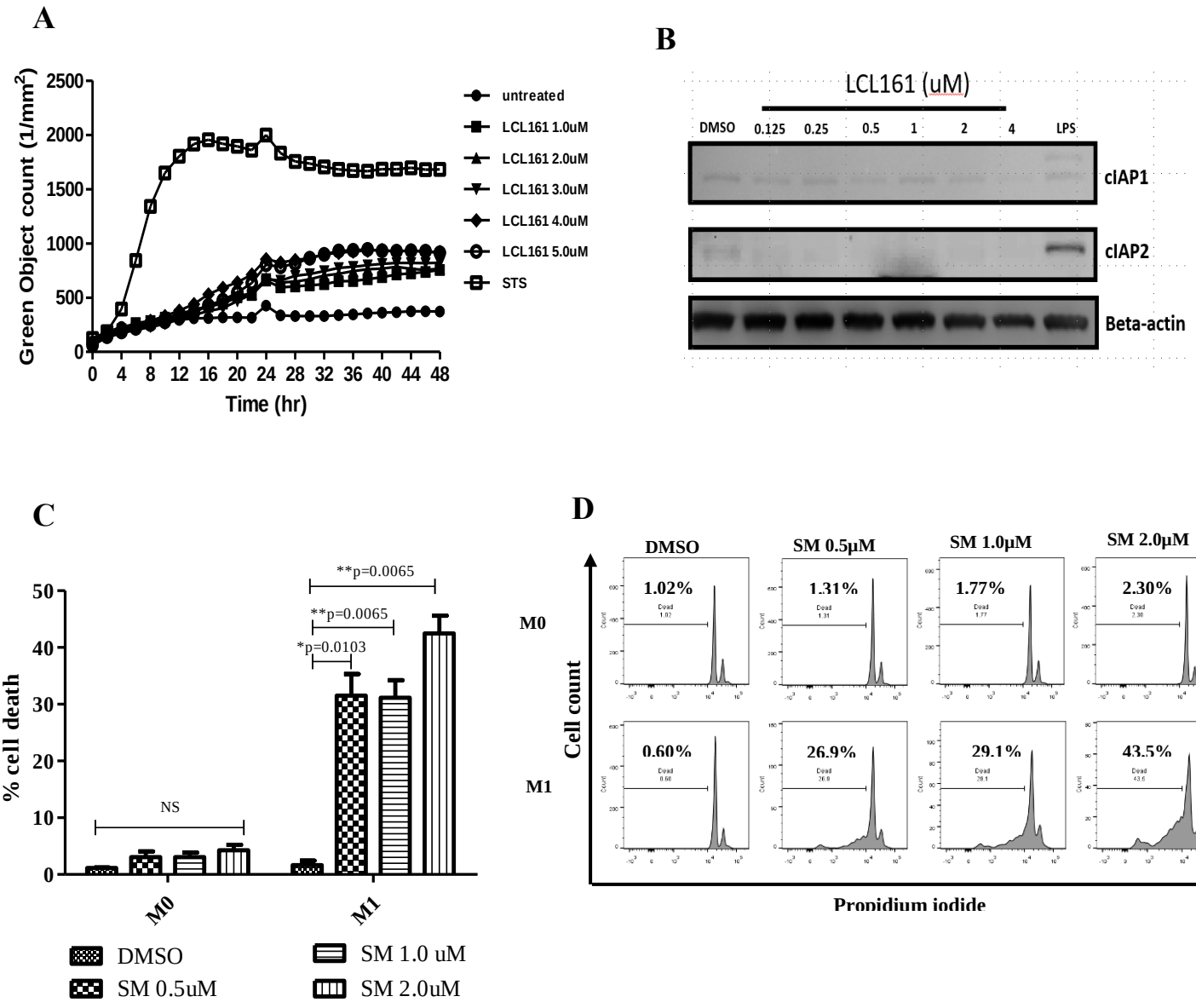

A
Green Object count (1/mm²)
2500
2000
1500
1000
500
0
0 4 8 12 16 20 24 28 32 36 40 44 48
Time (hr)
untreated
LCL161 1.0uM
LCL161 2.0uM
LCL161 3.0uM
LCL161 4.0uM
LCL161 5.0uM
STS
B
LCL161 (uM)
DMSO 0.125 0.25 0.5 1 2 4 LPS
cIAP1
cIAP2
Beta-actin
C
% cell death
50
40
30
20
10
0
NS
**p=0.0065
**p=0.0065
*p=0.0103
M0
M1
DMSO
SM 0.5uM
SM 1.0 uM
SM 2.0uM
D
DMSO
SM 0.5μM
SM 1.0μM
SM 2.0μM
M0
M1
1.02%
1.31%
1.77%
2.30%
0.60%
26.9%
29.1%
43.5%
Cell count
Propidium iodide

Figure 1

SM induce apoptosis in HIV-infected U1 cells, but not in corresponding uninfected U937 cells.

The IAP-signalling pathway has been implicated in conferring macrophages resistance against HIV-Vpr induced apoptosis[4,186,195]. cIAP1/2 mediate the survival of macrophages in response to HV-Vpr peptide which as independent of Bcl-xL and Mcl-1. In addition, degradation of IAPs by SM abrogated Vpr-induced apoptosis in Mϕ[4]**.** Therefore, it is conceivable that in the actual presence of HIV within the cell, SM may selectively target the infected cells for apoptosis induction. U937 and U1 cells are established model systems for studying chronicity of HIV infection. U1 harbors a single copy of integrated HIV-1 genome [196], and this cell line is derived from U937 cells, a human promonocytic cell line generated from a patient with generalized histiocytic lymphoma [196]**.** To explore the possibility of SM-induced killing of HIV-infected cells, monocytic U937 and its chronically infected counterpart, U1 cells, were treated with increasing concentration of SM LCL161 for 48 hours and the levels of cell death was assessed with intracellular PI staining and flow cytometry. SM treatment of undifferentiated U937 did not induce significant cell death **(Figure 2A)**; however, significant cell death was observed in the undifferentiated U1 cells **(Figure 2B).** To determine whether differentiation of U937 will render these cells susceptible to SM-induced apoptosis, promonocytic U937 and U1 cells were differentiated into macrophage-like phenotype using PMA. Similar to the effect of SM on undifferentiated U1 cells, SM LCL161 induced significant cell death in differentiated version of U1 cells **(Figure 1D),** and no differences were observed in the cell death of differentiated U937 (Figure 1C). Notably, the basal levels of apoptosis in undifferentiated U937 was higher compared to U1 cells; however, upon differentiation, differentiated U1 cells exhibited higher degree of basal cell death.

Figure 2. Preferential induction of cell death by SM LCL161 in promonocytic and differentiated U1 cells, but not in non-infected counterpart U937. (A) & **(B)** Promonocytic U937 and U1 cells were treated with SM LCL161 at 1, 2, and 4μM for 48 hours and cell death was assessed by intracellular PI staining (n=9, n=10, respectively). **(C)** & **(D)** differentiated U937 and U1 cells were treated with SM LCL161 at 1, 2, and 4μM for 48 hours and cell death was assessed by intracellular PI staining (n=7, n=11, respectively). p-values were calculated using Mann-Whitney U test.

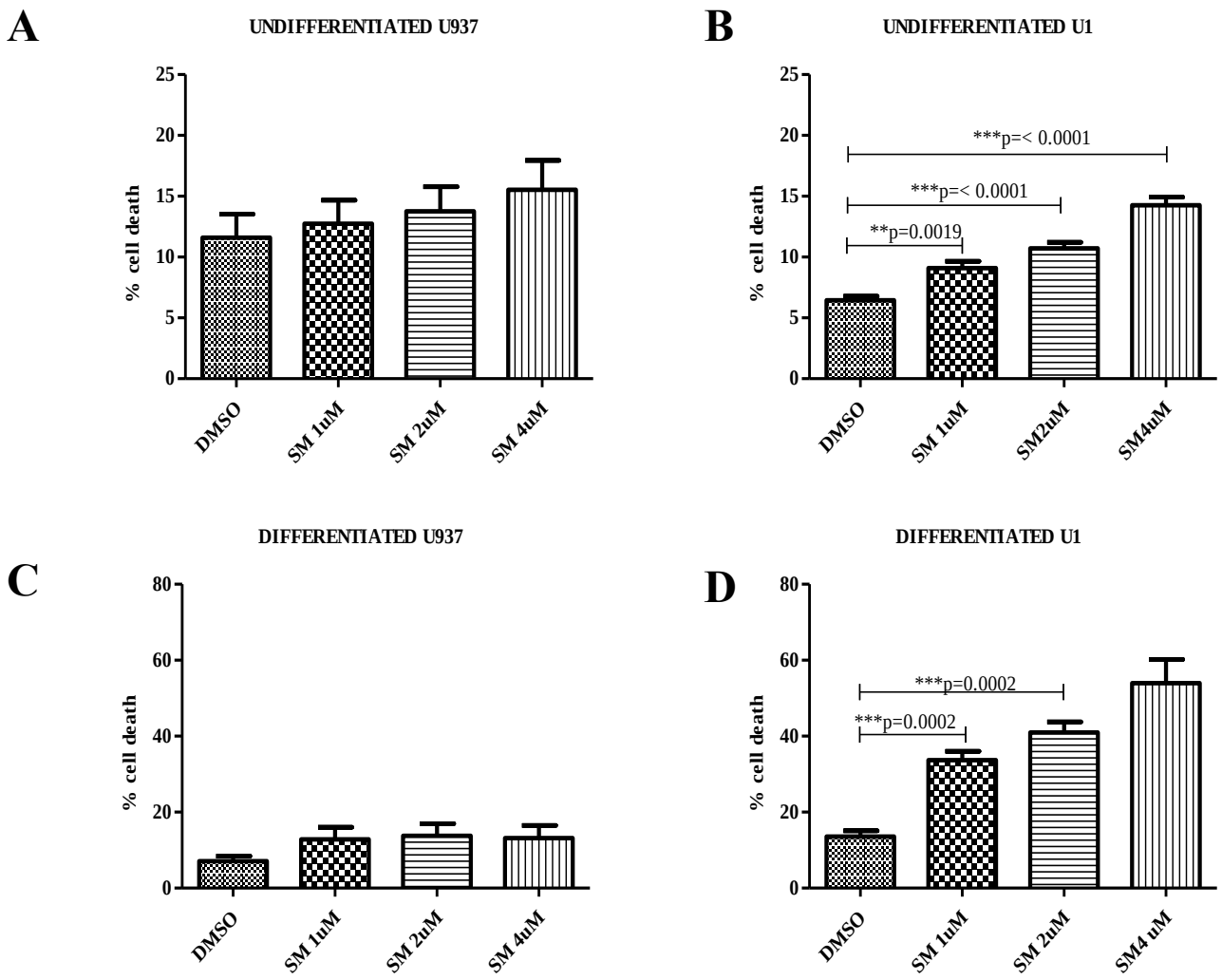

A
UNDIFFERENTIATED U937
% cell death
25
20
15
10
5
0
DMSO
SM 1uM
SM 2uM
SM 4uM
B
UNDIFFERENTIATED U1
% cell death
***p=< 0.0001
***p=< 0.0001
**p=0.0019
DMSO
SM 1uM
SM2uM
SM4uM
C
DIFFERENTIATED U937
% cell death
80
60
40
20
0
DMSO
SM 1uM
SM 2uM
SM 4uM
D
DIFFERENTIATED U1
% cell death
***p=0.0002
***p=0.0002
DMSO
SM 1uM
SM 2uM
SM4 uM

Figure 2

SM selectively induce cell death in *in vitro* HIV-infected Mϕ and Mϕ derived from HIV+ patients

As previously mentioned, we have demonstrated for the first time that cIAP/2 genes play a protective role in mediating survival of Mϕ in response to HIV-Vpr induced cell death which is independent of anti-apoptotic Bcl-xL and Mcl-1[4]**.** Degradation of cIAP1/2s by SM sensitized Mϕ to apoptosis after the addition of HIV-Vpr [4,185,186]. In addition, above results show the selectivity of SM to induce cell death in HIV-infected U1 cells, but not in U937 cells. To determine whether SM has an impact on apoptosis in HIV-infected Mϕ, Mϕ were *in vitro* infected with HIV^{CS204} for 7-days, after which the supernatants were collected, and the presence of the virus was detected by p24 ELISA **(Figure 3A).** Next, the *in vitro* mock and HIV infected Mϕ for 7 days were treated with increasing concentration of SM for 48 hours and cell death was assessed through intracellular PI staining and flow cytometry. SM selectively induced cell death of *in vitro* HIV-infected Mϕ, but not in mock-infected control **(Figure 3B)**. At concentration of 1.0 and 2.0 µM LCL161, significant cell death was observed in HIV-infected Mϕ (**p=0.0026, *p=0.0209, respectively). Statistical analysis between media control and 4.0 µM of LCL161 in the HIV-infected group could not be conducted due to lack of sample size at this concentration; however, the trend toward increased cell death was still followed. Representative histograms of the intracellular PI staining are shown in **figure 3C**.

To determine whether Mϕ derived from HIV+ individuals would have similar susceptibility to SM-induced cell death, PBMCs from ART treated and naïve HIV-infected patients were isolated, and Mϕ were *ex vivo* generated by the established adherence-differentiation protocol in the laboratory[4,186,195] . HIV-infected, *ex vivo* derived Mϕ were subjected to similar SM treatment and cell death was assessed through intracellular PI staining and flow cytometry, as

previously described[4,186,195]. Similar to the results from *in vitro* infected Mϕ, *ex vivo* derived Mϕ from infected patients showed increased susceptibility to SM in a dose-dependent fashion **(Figure 4)**. Interestingly, the degree of SM-induced cell death between these groups varied, with the ART treated group showed higher degree of cell death compared to the naïve untreated group. Overall, these results suggest that modulation of the IAP-signalling pathway may be a possible target for specific killing of HIV-infected Mϕ.

Figure 3. SM induces cells death of HIV-infected Mϕs. (A) Human PBMC-derived macrophages were *in vitro* infected with mock or HIV-CS204 for 2 hours followed by 7- days post-infection incubation. HIV infection was verified by p24 ELISA. (B) The cells were then treated with SM LCL161 at 0.5, 1, 2, and 4 μM for 48 hours and cell death was assessed by intracellular PI staining and panel (C) shows representative histogram (**p=0.026) and *p=0.0209). p-values were calculated using Mann-Whitney U test.

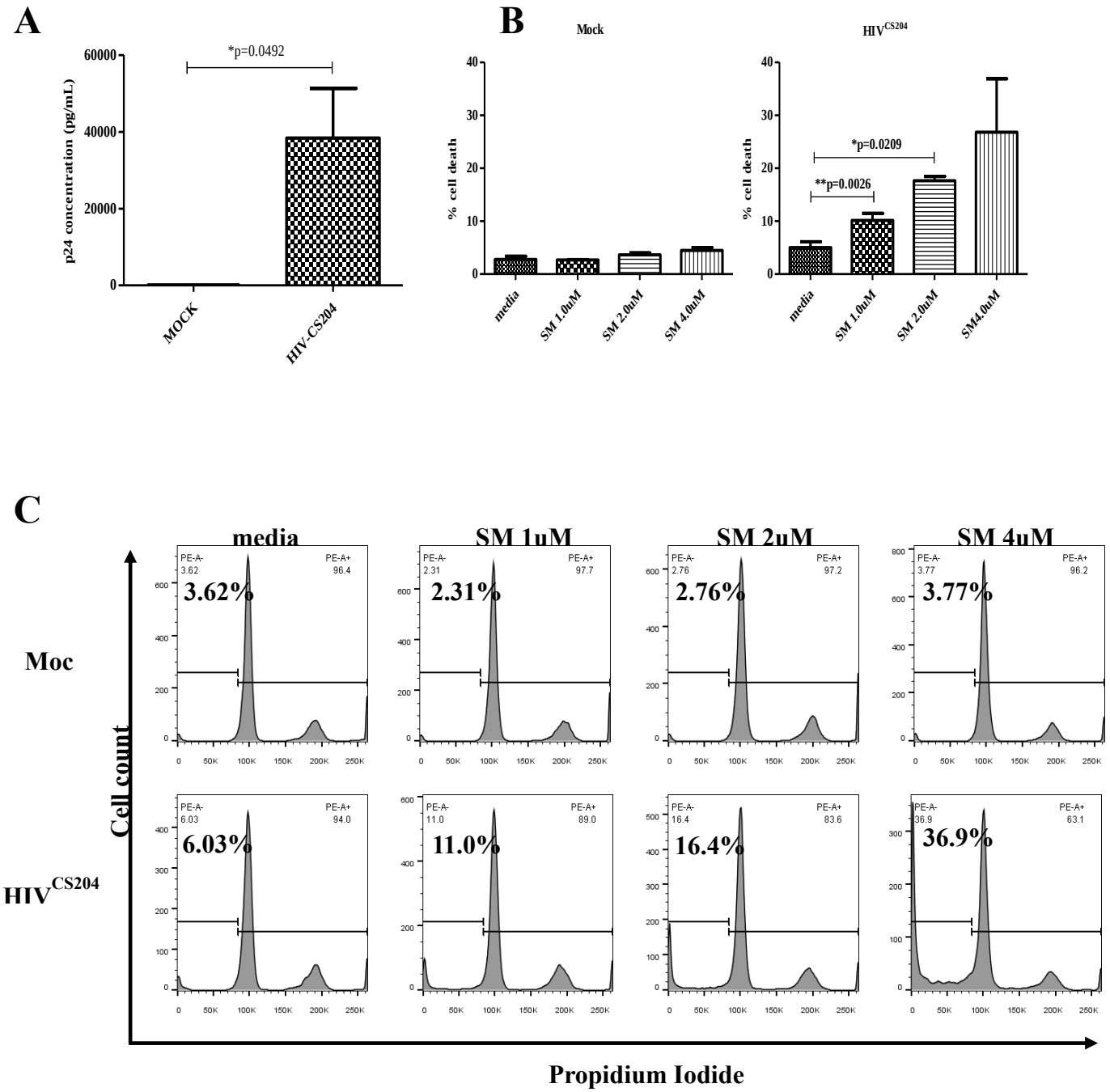

A
*p=0.0492
p24 concentration (pg/mL)
60000
40000
20000
0
MOCK
HIV-CS204
B
Mock
HIV^CS204
% cell death
40
30
20
10
0
media
SM 1.0uM
SM 2.0uM
SM 4.0uM
*p=0.0209
**p=0.0026
SM4.0uM
C
media
SM 1uM
SM 2uM
SM 4uM
Moc
HIV^CS204
Cell count
PE-A- 3.62
PE-A+ 96.4
3.62%
PE-A- 2.31
PE-A+ 97.7
2.31%
PE-A- 2.76
PE-A+ 97.2
2.76%
PE-A- 3.77
PE-A+ 96.2
3.77%
PE-A- 6.03
PE-A+ 94.0
6.03%
PE-A- 11.0
PE-A+ 89.0
11.0%
PE-A- 16.4
PE-A+ 83.6
16.4%
PE-A- 36.9
PE-A+ 63.1
36.9%
0 50K 100K 150K 200K 250K
Propidium Iodide

Figure 3

Figure 4. *Ex vivo* derived HIV-infected Mϕ from HIV+ patients are susceptible to SM-induced cell death. Monocytes from untreated or treated HIV+ patients were differentiated into mature macrophages and the cells were treated with increasing concentration of monomeric SM LCL161 for 48 hours. Cell death was assessed through intracellular PI staining and flow cytometry. p-values were calculated using Mann-Whitney U test (n=4).

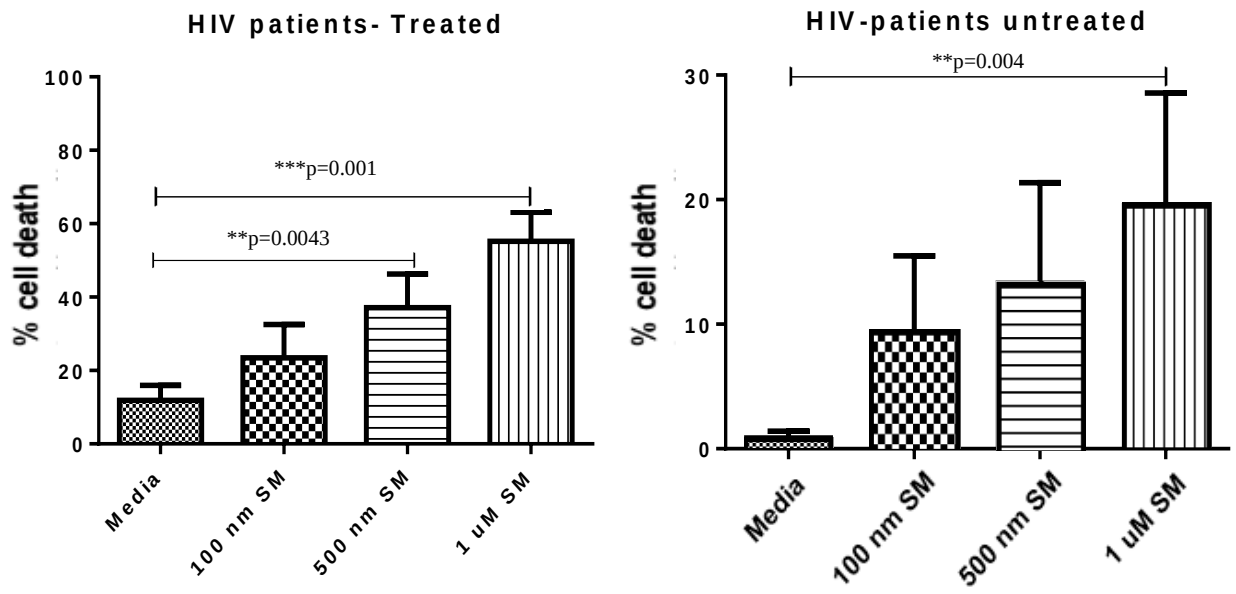

HIV patients- Treated
% cell death
100
80
60
40
20
0
***p=0.001
**p=0.0043
Media
100 nm SM
500 nm SM
1 uM SM
HIV-patients untreated
% cell death
30
20
10
0
**p=0.004
Media
100 nm SM
500 nm SM
1 uM SM

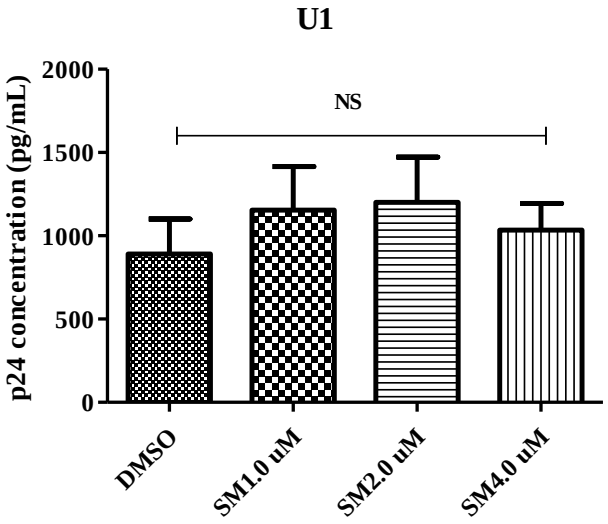
Figure 4

Virus replication is not affected by the treatment of SM

The above results show that SM can selectively induce cell death of HIV-infected myeloid cells. Recently, it was reported that apoptosis can induce viral activation and replication[197]. In addition, Pache and colleagues have demonstrated that SM can affect viral transcription in infected $CD4^+$ T cells via NF-κB dependent signalling [198]**.** The high constitutive NF-κB transcriptional activity in macrophages[199] allows for continuous virus replication because of the presence of the NF-κB responsive elements found in the LTR region of the integrated HIV provirus[198]. Given that SM induces degradation of cIAP1/2, which are critical regulators of many intracellular signalling pathways including NF-κB, I hypothesized that treatment with SM would have a negative impact on virus transcription in HIV-infected myeloid cells. To address this issue, U1 cells were treated with increasing concentration of SM for 48 hours and levels of p24 antigen in the supernatants were assessed by p24 ELISA. Interestingly, virus replication in U1 cells was not affected by SM treatment as there was no significant differences in the levels of p24 between the vehicle control and SM treatments **(Figure 5A).** Similarly, SM treatment of *in vitro* HIV-infected Mϕ had no effect on the virus replication as there was no observable difference in the amount of p24 in the supernatants **(Figure 5B).** Overall, these results suggest cIAP1/2 may not play a role in the HIV replication in infected Mϕ.

Figure 5. SM treatment of U1 and *in vitro* HIV-infected Mφ does not affect virus replication. (A) $5*10^5$ U1 cells (n=6) and (B) *in vitro* HIV-infected Mφ (n=5) were treated with increasing concentration of LCL161 for 48 hours after which supernatants were collected and inactivated with TritonX for 1hr at 37^0C. Thereafter, p24 antigen was quantified through p24 ELISA. p-values were calculated using Mann-Whitney U test.

A


U1
p24 concentration (pg/mL)
2000
1500
1000
500
0
NS
DMSO
SM1.0 uM
SM2.0 uM
SM4.0 uM

B

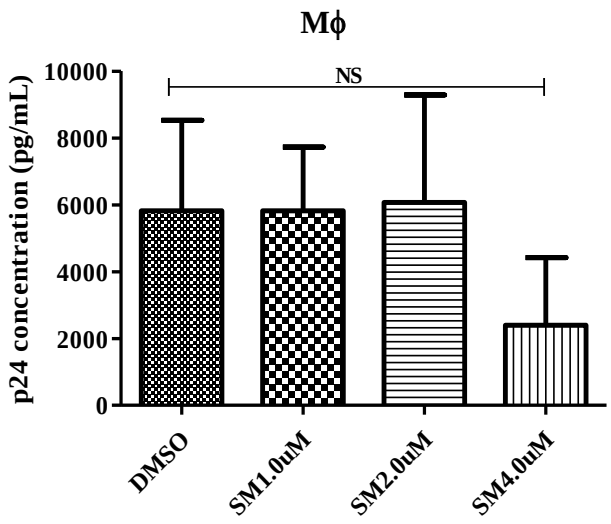

Mϕ
p24 concentration (pg/mL)
10000
8000
6000
4000
2000
0
NS
DMSO
SM1.0uM
SM2.0uM
SM4.0uM

Figure 5

Aim 2: Elucidate the underlying mechanism of SM-induced killing of HIV-infected macrophages

SM preferentially induce cell death of HIV-infected myeloid cells by activating apoptosis, and not due to necroptosis

IAPs are targeted by SM for autoubiquitination and subsequent proteosomal degradation, which relieves the inhibition on caspase activation [115,143,146]. To determine if SM causes cell death by apoptosis, Western immunoblotting was employed to determine the cleavage and activation of caspases in LCL161-treated U937 and U1 cells. U937 treated with SM showed no cleavage of caspase-3 and caspase-8 **(Figure 6A),** while caspase-3, but not caspase-8, was cleaved in U1 cells with the treatment of LCL161 **(Figure 6B).** These results indicate that SM-induced cell death of U1 cells is mediated by apoptosis.

To determine the involvement of caspases in SM-mediated cell death of *in vitro* HIV-infected macrophages, similar treatment protocol as described above was followed, and caspase activation was quantified based on the fluorescent signal of cleaved caspase substrates. In mock-infected MDMs, treatment with SM LCL161 did not induce activation of caspases- there was no significant differences in the activation of caspase-3, 8, and 9 compared to media control **(Figure 7A-C).** However, clear activation of caspases 3, 8, and 9 were observed in the SM treatment of *in vitro* HIV-infected Mϕ **(Figure 8A-C)**. Moreover, prior treatment with zVAD-FMK, a caspase inhibitor, reduced the activation of caspase-8, and caspase-9 after SM treatment **(Figure 8B-C).**

An alternative form of cell death is necroptosis [200]. The key regulator of necroptosis is RIPK1, which can be specifically inhibited by the chemical inhibitor necrostatin-1. To determine whether necroptosis, in addition to apoptosis, play a role in SM-induced killing of HIV-infected Mϕ, *in vitro* HIV-infected Mϕ were treated with necrostatin-1 alone or prior to treatment with increasing SM concentration. Consistent with above results, SM treatment led to cell death of HIV-

infected macrophages in a dose-dependent manner. Treatment with necrostatin-1 alone did not have an affect on the level of cell death. In combination with SM, necrostain-1 failed to reduce the percentage of dying cells, indicating that the cell death induced by SM was not due to necroptosis **(Figure 9).** Overall, these results suggest that SM-mediated cell death of HIV-infected myeloid cells occurs through apoptosis.

Figure 6. SM induces the cleavage and activation of caspase-3 in U1. Promonocytic U937 (A) and U1 (B) cells were treated with SM LCL161 at 1, 2, and 4.0 μM for 48 hours. Cell pellets were collected, lysed, and proteins were quantified by Bradford assay. Western Immunoblotting was conducted to detect the cleavage of caspase-3 and caspase-8.

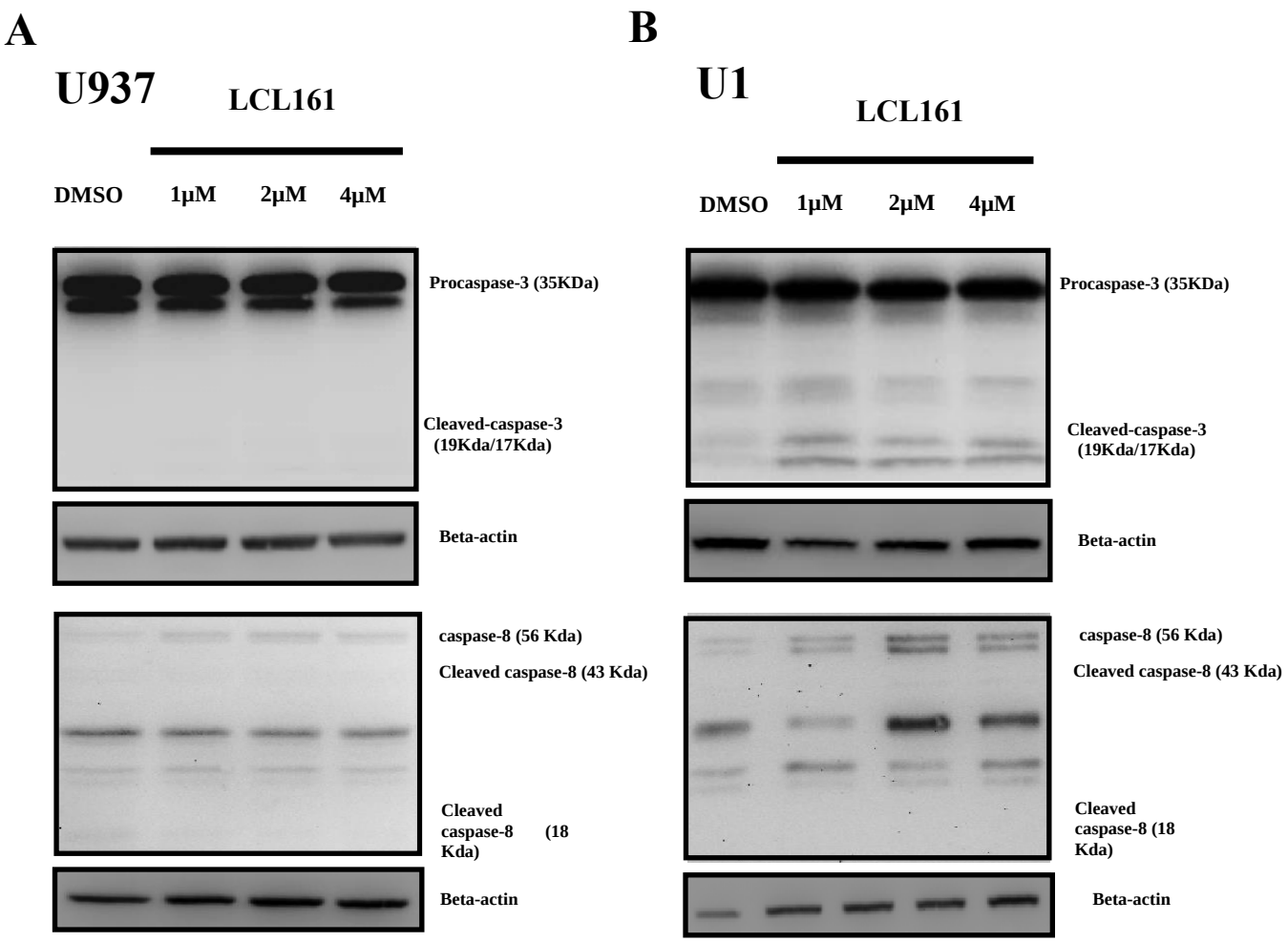

A
U937
LCL161
DMSO
1μM
2μM
4μM
Procaspase-3 (35KDa)
Cleaved-caspase-3 (19Kda/17Kda)
Beta-actin
caspase-8 (56 Kda)
Cleaved caspase-8 (43 Kda)
Cleaved caspase-8 (18 Kda)
Beta-actin
B
U1
LCL161
DMSO
1μM
2μM
4μM
Procaspase-3 (35KDa)
Cleaved-caspase-3 (19Kda/17Kda)
Beta-actin
caspase-8 (56 Kda)
Cleaved caspase-8 (43 Kda)
Cleaved caspase-8 (18 Kda)
Beta-actin

Figure 6

Figure 7. SM does not activate caspases in *in vitro* mock-infected macrophages. *In vitro* mock-infected Mϕ were treated with LCL161 for 48 hours and the activation of caspases was detected by fluorescent caspase-substrate and flow cytometry. (caspase-3 n=3, caspase-8 n=3, caspase-9 n=2). Representative histograms of the untreated control and highest SM concentration are shown. p value was calculated using Mann-Whitney U test

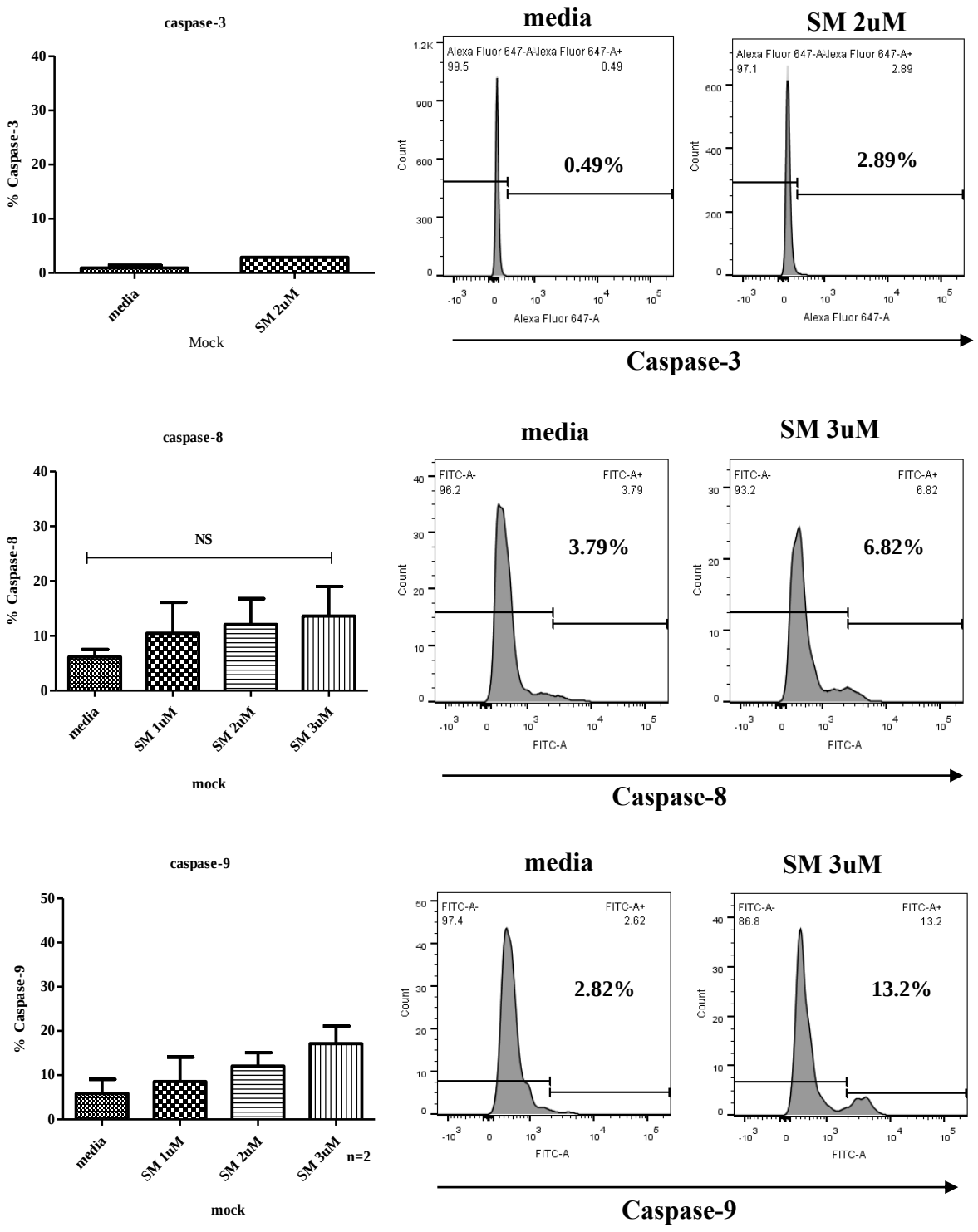

caspase-3
% Caspase-3
media
SM 2uM
Mock
media
SM 2uM
Alexa Fluor 647-A-Jexa Fluor 647-A+
99.5
0.49
0.49%
97.1
2.89
2.89%
Count
Alexa Fluor 647-A
Caspase-3
caspase-8
% Caspase-8
NS
media
SM 1uM
SM 2uM
SM 3uM
mock
media
SM 3uM
FITC-A-
FITC-A+
96.2
3.79
3.79%
93.2
6.82
6.82%
FITC-A
Caspase-8
caspase-9
% Caspase-9
media
SM 1uM
SM 2uM
SM 3uM
n=2
mock
media
SM 3uM
97.4
2.62
2.82%
86.8
13.2
13.2%
Caspase-9

Figure 7

Figure 8. SM activates caspases in *in vitro* HIV-infected macrophages. *In vitro* HIV-infected Mϕ were treated with LCL161 for 48 hours and the activation of caspases was detected by fluorescent caspase substrate (caspase-3 n=3, caspase-8 n=4, caspase-9 n=6). Representative histograms of the untreated control and highest SM concentration are shown. p value was calculated using Mann-Whitney U test

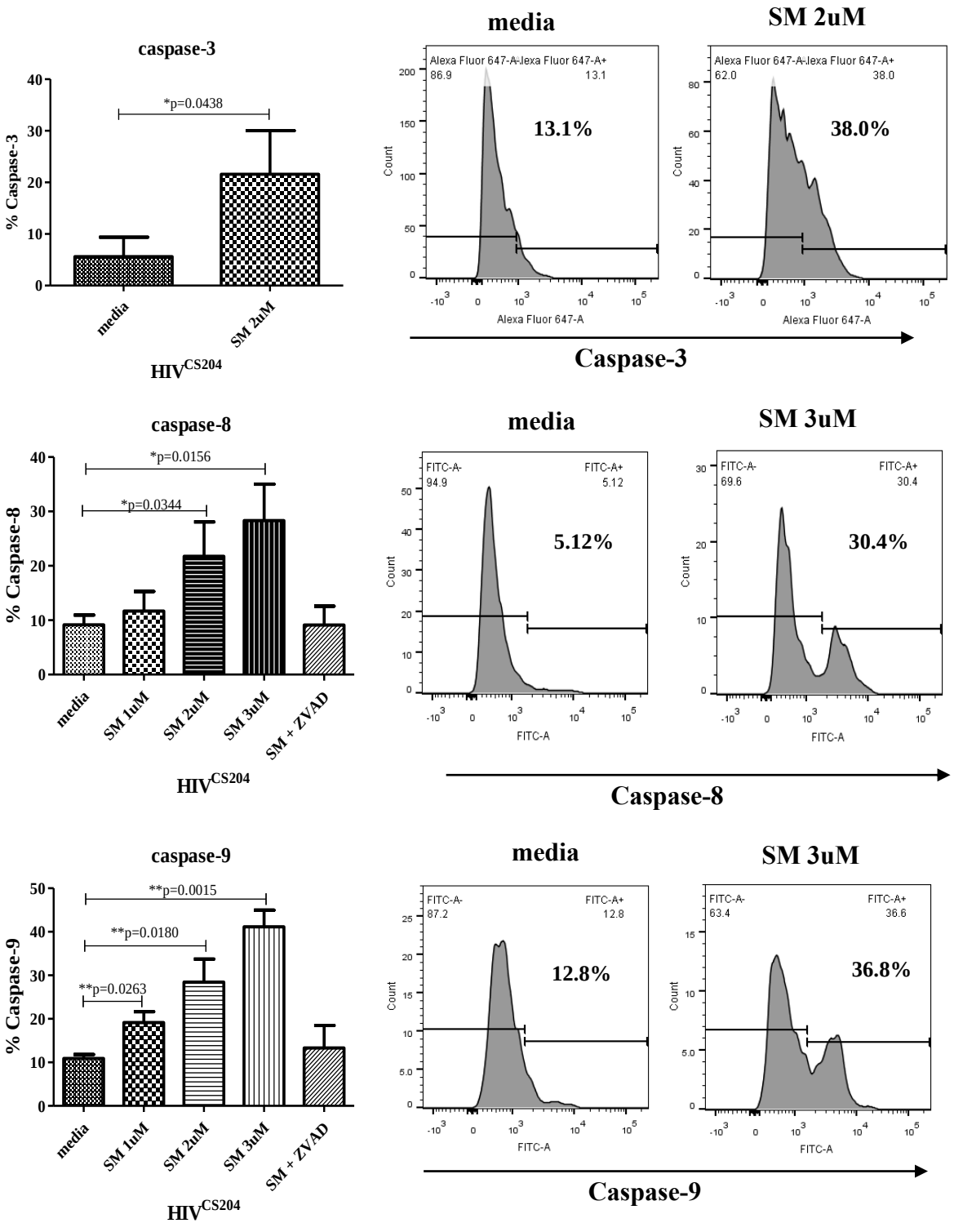

caspase-3
% Caspase-3
*p=0.0438
media
SM 2uM
HIV^CS204
media
SM 2uM
Alexa Fluor 647-A-Jexa Fluor 647-A+
86.9
13.1
13.1%
62.0
38.0
38.0%
Count
Alexa Fluor 647-A
Caspase-3
caspase-8
% Caspase-8
*p=0.0156
*p=0.0344
media
SM 1uM
SM 2uM
SM 3uM
SM + ZVAD
HIV^CS204
media
SM 3uM
FITC-A-
FITC-A+
94.9
5.12
5.12%
69.6
30.4
30.4%
FITC-A
Caspase-8
caspase-9
% Caspase-9
**p=0.0015
**p=0.0180
**p=0.0263
media
SM 1uM
SM 2uM
SM 3uM
SM + ZVAD
HIV^CS204
media
SM 3uM
87.2
12.8
12.8%
63.4
36.6
36.8%
Caspase-9

Figure 8

Figure 9. SM-induced cell death of HIV-infected macrophages is not due to necroptosis. *In vitro* mock-infected Mϕ were treated with RIPK1 inhibitor, necrostatin-1 alone or with combination of increasing concentration of LCL161 for 48 hours. The degree of cell death was assessed by intracellular PI staining.

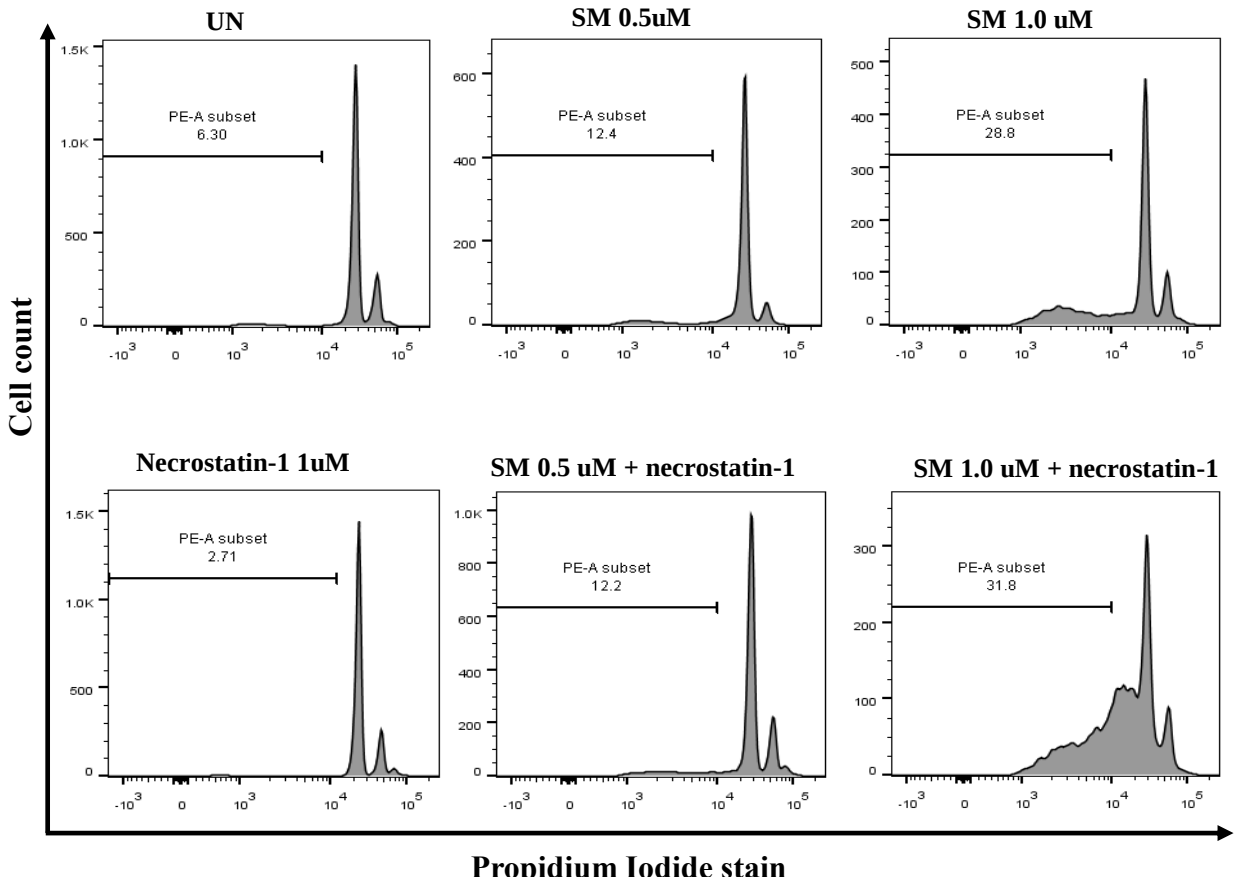

UN
PE-A subset
6.30
SM 0.5uM
PE-A subset
12.4
SM 1.0 uM
PE-A subset
28.8
Necrostatin-1 1uM
PE-A subset
2.71
SM 0.5 uM + necrostatin-1
PE-A subset
12.2
SM 1.0 uM + necrostatin-1
PE-A subset
31.8
Cell count
Propidium Iodide stain

Figure 9

SM induces the secretion of TNFα in U937 and U1 cell lines

There exists strong body of evidence that SM treatment results in the activation of the non-canonical NF-κB pathway and leads to pro-inflammatory cytokine production, especially TNFα in a variety of tumour cell lines [135,136,160,161]. With SM treatment of certain tumour cells, cIAP degradation is the early stage in the apoptosis induction when the autocrine-secreted TNFα activates the extrinsic death pathway by ligating to its cognate TNFR1 [160]. Therefore, I hypothesized that SM-induced apoptosis in HIV-infected macrophages is due to TNFα induction following treatment that subsequently leads to apoptosis. To determine whether SM treatment of U937 and U1 cells would lead to TNF-α secretion, supernatants were collected after 48-hour treatment with LCL161 and TNFα levels were quantified by ELISA. The basal level of TNFα secreted by the undifferentiated U937 and U1 cells fell in the range of 20-30pg/mL; however, after treatment with LCL161 for 48 hours, the levels of TNFα was significantly increased in a dose-dependent manner **(Figure 10A & B)** (***p=0.0001), as analyzed by One-way analysis of variance. In the treatment of differentiated U937 and U1 cells with LCL161, similar trend was observed. The basal level of TNFα produced by differentiated U937 cells was higher **(Figure 10C)** (~130 pg/mL) than the basal TNFα of the differentiated U1 cells **(Figure 10D)**. After LCL161 treatment however, TNFα was significantly upregulated in a dose-dependent manner (***p=0.0001). Collectively, these results suggest SM induced significant production of TNFα in U937 and U1 undifferentiated and differentiated cells.

Figure 10. SM induces the secretion of TNFαin U937 and U1 cells. (A) & **(B)** Promonocytic and **(C)** & **(D)** differentiated U937 and U1 cells were treated with SM LCL161 at 1, 2, & 4 µM for 48 hours and levels of TNF-α was measured using ELISA. p-values were calculated using One-way ANOVA and Dunnett post-test was used to compare the difference between each treatment with DMSO negative control. Each experiment was conducted with sample size of n=4.

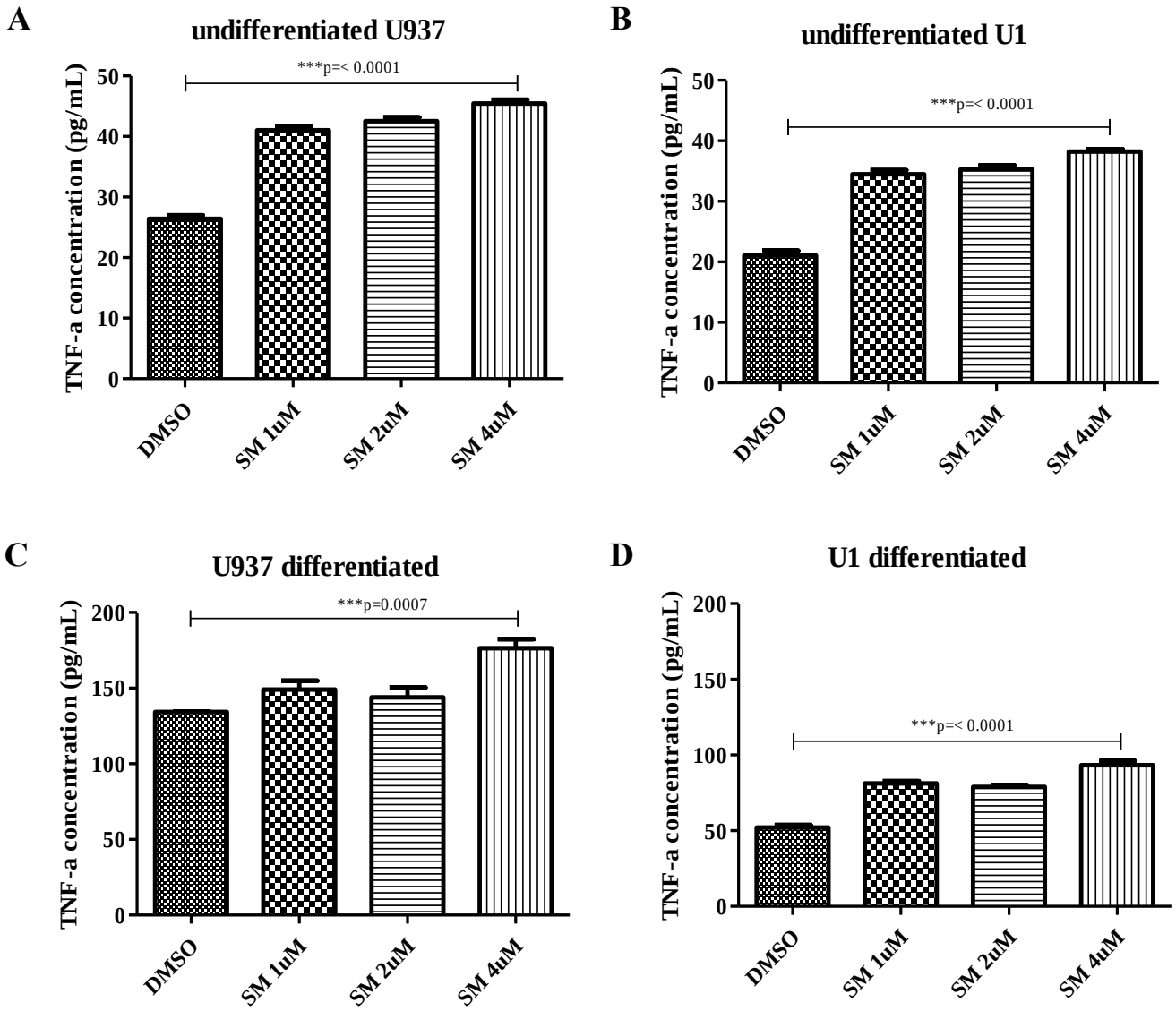

A
undifferentiated U937
***p=< 0.0001
TNF-a concentration (pg/mL)
0
10
20
30
40
50
DMSO
SM 1uM
SM 2uM
SM 4uM
B
undifferentiated U1
***p=< 0.0001
TNF-a concentration (pg/mL)
0
10
20
30
40
50
DMSO
SM 1uM
SM 2uM
SM 4uM
C
U937 differentiated
***p=0.0007
TNF-a concentration (pg/mL)
0
50
100
150
200
DMSO
SM 1uM
SM 2uM
SM 4uM
D
U1 differentiated
***p=< 0.0001
TNF-a concentration (pg/mL)
0
50
100
150
200
DMSO
SM 1uM
SM 2uM
SM 4uM

Figure 10

SM treatment of HIV-1 infected Mϕ does not affect the production of TNFα

HIV seropositive individuals exhibit higher plasma/serum levels of pro-inflammatory markers such as TNFα, CXCL10, sCD27, and IL-8 [201–204]**.** This observation begged the question whether macrophages, being one of the chief secretors of battery of cytokines, would secrete TNFα in response to HIV-infection. To address this question, Mϕ were *in vitro* infected with mock- and HIV^{CS204}, [as] previously described. After 7-days post infection incubation, the supernatants were subjected to ELISA to measure the amount of secreted TNFα. Infection of Mϕ with HIV^{CS204} resulted in significant secretion of TNFα compared to mock-infected control **(*p=0.0280)** (**Figure 11A)**. In addition, measurement of secreted TNFα was also conducted on the supernatants of e*x vivo* derived Mϕ from HIV+ individuals undergoing treatment. Likewise, *ex vivo* derived HIV-infected Mϕ secreted significantly higher level of TNFα compared to Mϕ derived from healthy patients(*p=0.0275) **(Figure 11B)**. Together, these results indicate that Mϕ infected with HIV-1 are capable of secreting TNFα.

In the context of SM monotherapy, the combination of TNF-α and SM is sufficient to induce cell death of some tumour cells[147]**.** Therefore**,** I hypothesized that SM-induced apoptosis of HIV-infected macrophages is mediated by secreted TNFα in supernatants. To this end, *in vitro* mock- and HIV-infected, as well as *ex vivo* derived HIV-infected Mϕ were thoroughly washed, replaced with fresh media and were subjected to SM treatment in increasing concentration for 48 hours. As measured through TNFα ELISA assay, both *in* vitro mock- and HIV-infected Mϕ produced similar basal levels of TNFα between 20 to 40pg/mL range. However, SM treatment did not alter the secretion of TNFα in both *in vitro* mock- and HIV-infected Mϕ, as no significant differences were calculated between the chemical treatments and negative control **(Figure 12A & B)**. When HIV-infected *ex vivo* derived Mϕ from HIV-infected patients were subjected to the same

SM treatment, similar results were obtained. SM did not alter the secretion of TNFα compared to the negative control **(Figure 12C).**

Given that TNFα was not significantly induced with the treatment of SM, it was possible that other proinflammatory cytokines may be involved in the selective killing of infected macrophages. TNF-related apoptosis-inducing ligand (TRAIL) is considered one of the most promising candidate for SM based combination treatments [205–208]**.** Since TRAIL has often been shown to have elevated levels in HIV-infected patients [209,210], it was of interest whether this cytokine would play a role in SM-induced killing of HIV-infected macrophages**.** To this end, TRAIL was measured in the supernatants of *in vitro* mock- and HIV-infected Mϕ after 7 days post-infection, as well as supernatants from *ex vivo* derived Mϕ from infected patients after 7-day post-differentiation incubation using TRAIL ELISA. Contrary to the upregulation of TNFα in response to HIV-infection, TRAIL was not significantly induced. In the *in vitro* mock- and HIV-infected Mϕ, TRAIL was secreted in the supernatants at similar levels **(Figure 13A).** For the *ex vivo* derived HIV-infected Mϕ, similar level of TRAIL was found with the healthy control **(Figure 13B).** Since TRAIL is not significantly upregulated with HIV-infection of macrophages, this cytokine may not play a role in the SM-induce selective killing of infected Mϕ**.**

Collectively, these results indicate that even though HIV-infected Mϕ can produce TNFα, SM has no affect on the secretion this proinflammatory cytokine which suggests that SM-induced killing of HIV-infected Mϕ may be independent of TNFα.

Figure 11. Mϕ show increased secretion of TNFα in response to HIV-infection (A) Mϕ were *in vitro* infected with mock of HIV-CS204 for 2 hours. Following the infection, the cells were washed and replaced with fresh media and were left to incubate for 7days. **(B)** Monocytes from HIV-infected patients were differentiated into Mϕ for 7 days, after which the cells were left to incubate in fresh media for 7 days**.** Supernatants after 7-days post infection incubation were subjected to ELISA to measure TNF-α. p-values were calculated using Mann-Whitney U test (n=6).

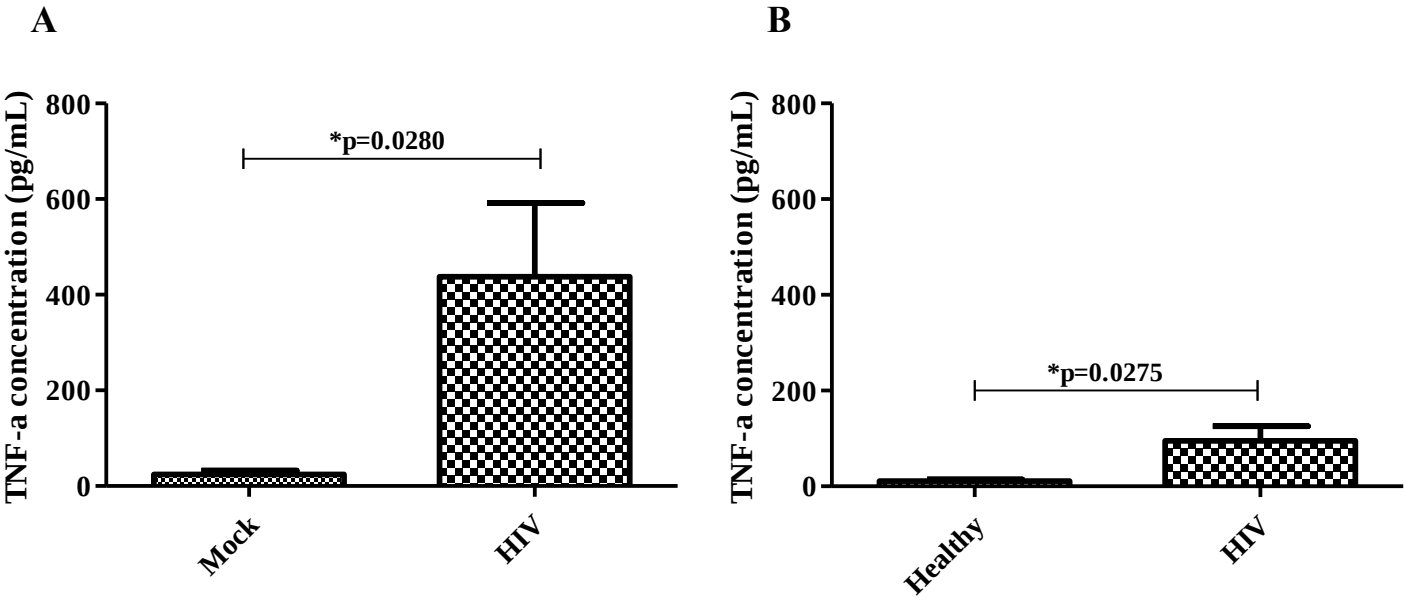

A
B
TNF-a concentration (pg/mL)
800
600
400
200
0
*p=0.0280
Mock
HIV
*p=0.0275
Healthy
HIV

Figure 11

Figure 12. SM does not affect the secretion of TNFα by *in vitro*-infected and HIV+ patient derived Mϕ. (A)PBMCs from healthy donors were differentiated into MDMs for 7 days followed by infection of mock or HIVCS204 for 2 hours. The cells were incubated for 7 days post-infection. MDMs were treated with SM LCL161 for 48 hours and supernatants were collected and levels of TNFα were measured through TNFα ELISA. (n=3). **(B)** MDMs derived from HIV-patients were treated with SM for 48 hours and supernatants were collected for TNFαα analysis through ELISA (n-=4). p values were calculated using Mann-Whitney U test.

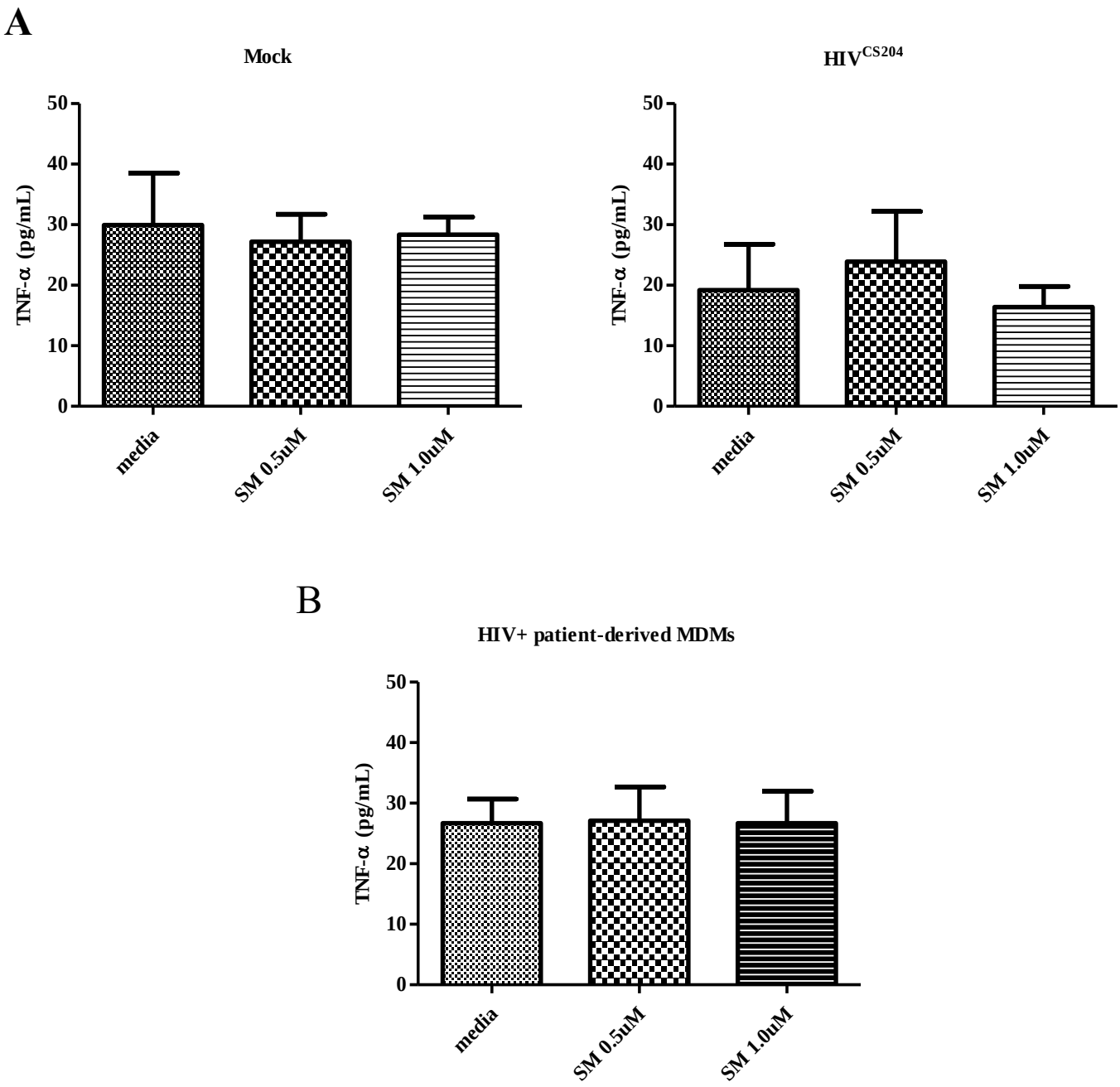

A
Mock
HIV^CS204
TNF-α (pg/mL)
50
40
30
20
10
0
media
SM 0.5uM
SM 1.0uM
B
HIV+ patient-derived MDMs

Figure 12

Figure 13. Mϕ do not show increased secretion of TRAIL in response to HIV-infection (A) Mϕ were *in vitro* infected with mock of HIV-CS204 for 2 hours. Following the infection, the cells were washed and replaced with fresh media and were left to incubate for 7days. **(B)** Monocytes from HIV-infected patients were differentiated into Mϕ for 7 days, after which the cells were left to incubate in fresh media for 7 days**.** Supernatants after 7-days post infection incubation were subjected to ELISA to measure TRAIL. p-values were calculated using Mann-Whitney U test (n=4).

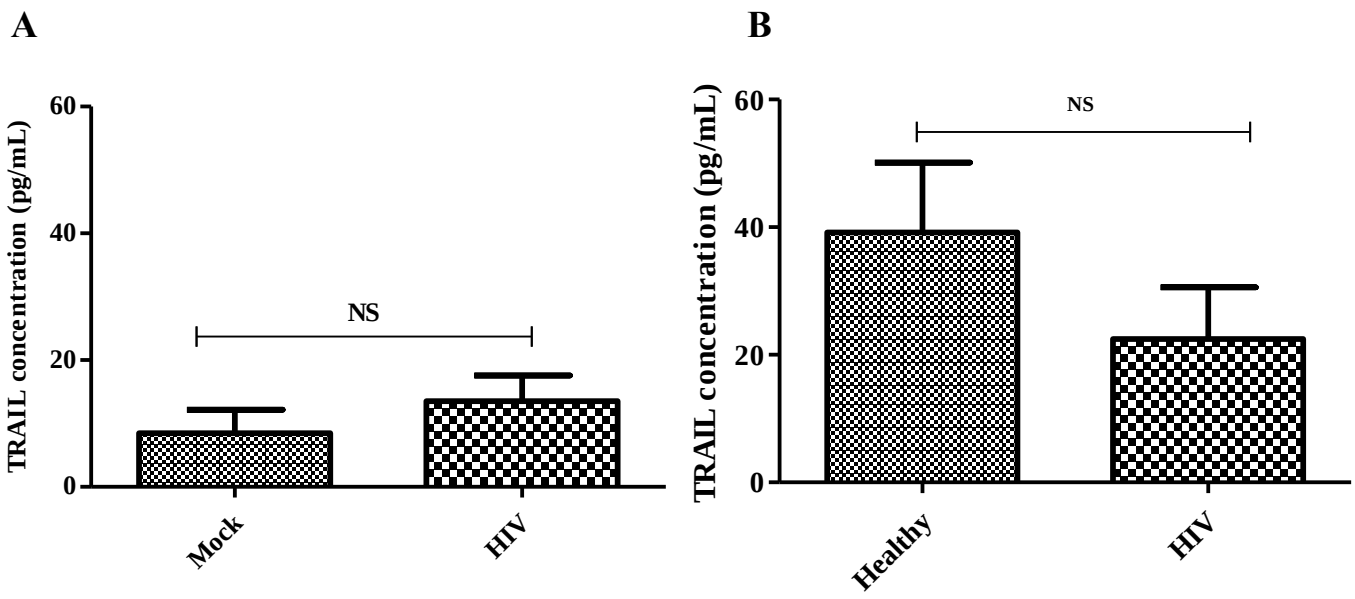

A
TRAIL concentration (pg/mL)
60
40
20
0
NS
Mock
HIV
B
TRAIL concentration (pg/mL)
60
40
20
0
NS
Healthy
HIV

Figure 13

SM sensitize U937 and U1 cells to TNF-α induced apoptosis, but not Mϕ

As previously stated, several tumour cells treated with SM fail to produce TNFα, and therefore not sensitive to SM monotherapy. However, when supplemented with exogenous TNF-α, resistant tumour cell lines quickly undergo apoptosis through the initiation of the TNFR1 death pathway [149,162]**.** To determine whether the addition of exogenous TNFα to SM treated cells would result in apoptosis induction, U937 and U1 cells were treated with constant concentration of LCL161, with or without the addition of increasing amount of recombinant TNFα for 48 hours followed by the assessment of cell death by intracellular PI staining and flow cytometry. Both U937 and U1 cells were susceptible to TNFα-induced cell death **(Figure 14A & B).** Treatment with rTNFα alone was able to induce significant cell death in both U937 and U1 cells compared to the media control (*p=0.0286) **(Figure 14)**. SM alone did not induce cell death in U937; however, when SM and rTNFα were used in combination, there was a marked increased in apoptotic cells compared to the sole treatment with rTNFα **(Figure 14A)**. Similarly, combination of rTNFα and SM induced synergistic effect and significant cell death of U1 cells; however, the effect of the combination treatment was comparatively lower than in the U937 **(Figure 4B)**. These results are in corroboration with previous observations in some tumour cell lines that exogenous TNFα synergizes with SM for the induction of cell death[211–213]**.**

To determine the impact of TNFα and SM on apoptosis of primary macrophages, Mϕ were pretreated with increasing concentration of SM for 2 hours, followed by the addition of the recombinant TNF-α (15ng/mL). The cells were cultured for two days and cell death was assessed by intracelullar PI staining and flow cytometry. Contrary to the myeloid cell lines, Mϕ did not undergo apoptosis with the rTNFα treatment alone. Moreover, pre-treatment with SM did not sensitize Mϕ to TNFα mediated cell death **(Figure 15B).** No significant differences in apoptotic

cells were observed between any concentration of SM alone and SM/rTNFα combination **(Figure 15A).** Representative histograms of the intracellular PI staining and flow cytometry are shown in **figure 15B**. It was possible that the rTNFα was biochemically inactive which would explain the lack of sensitization of Mϕ to TNFα-induced cell death with the treatment of SM. To determine whether the rTNFα was biochemically active, Mϕ were treated with rTNFα for 0, 10, 20, 30, and 40 minutes. The cells were harvested after the indicated time points, lysed, and cytosolic protein fractions were subjected to Western immunoblot to determine the degradation of IKBα, a complex that sequesters the NF-κB transcription factor downstream of the TNFα/TNFR1 signalling pathway. Addition of rTNFα to Mϕ resulted in the degradation of IKBα indicating that the rTNFα was biochemically active **(Figure 15C).**

Altogether, these results suggest that there is a differential effect of SM/TNFα between myeloid cells lines and primary macrophages. Given that TNFα was not upregulated after SM treatment of HIV-infected Mϕ and TNFα did not synergize with SM to induce cell death of Mϕ, the selective SM-mediated killing of HIV-infected macrophages may be independent of TNFα, suggesting that other factors may be involved in this process.

Figure 14. SM LCL161 and TNF-α synergize to induce cell death in undifferentiated U937 and U1. promonocytic U937 and U1 cells were treated with SM LCL161 and TNFα for 48 hours and the levels of cell death were assessed by intracellular PI staining (n=4). (C) was assessed through intracelullar PI staining (n=3). p values were calculated using Mann-Whitney U test.

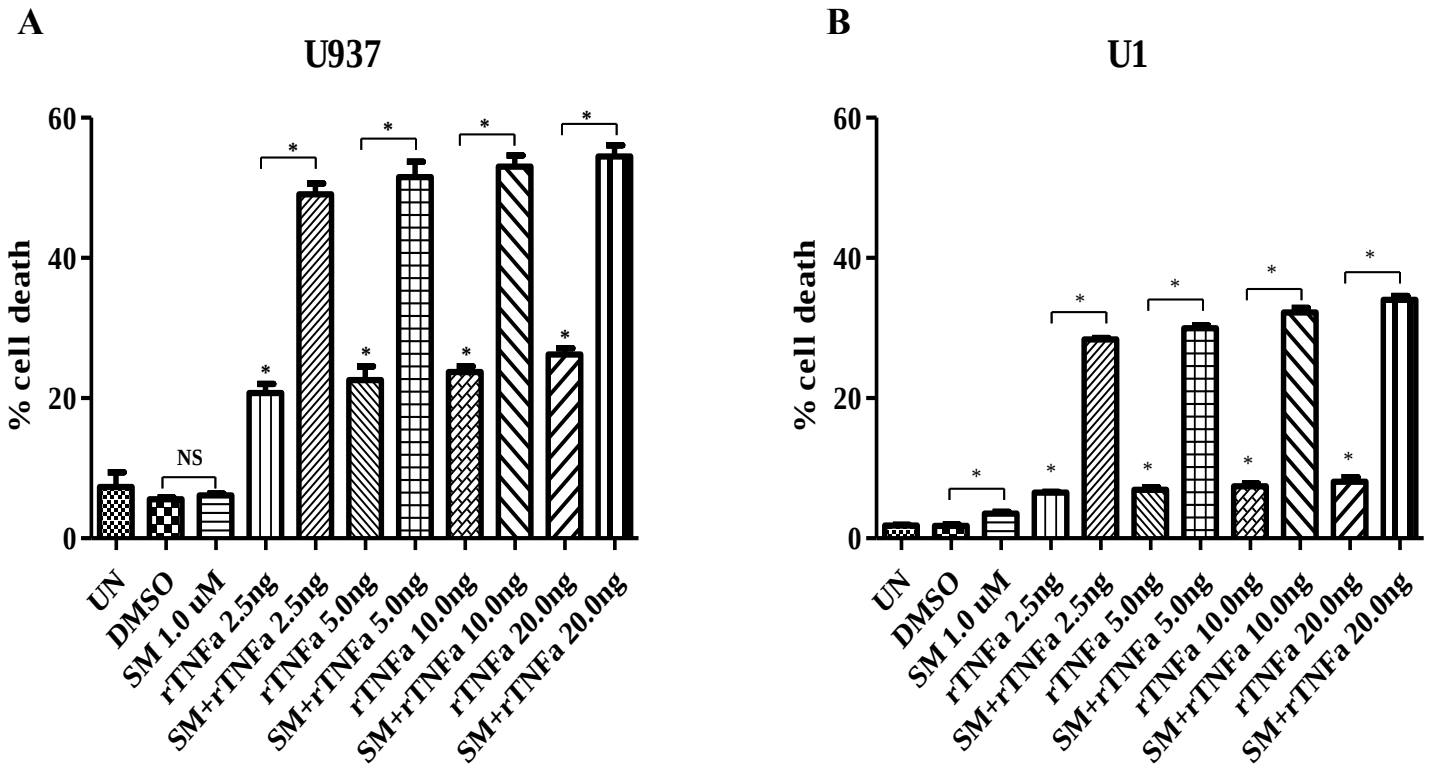
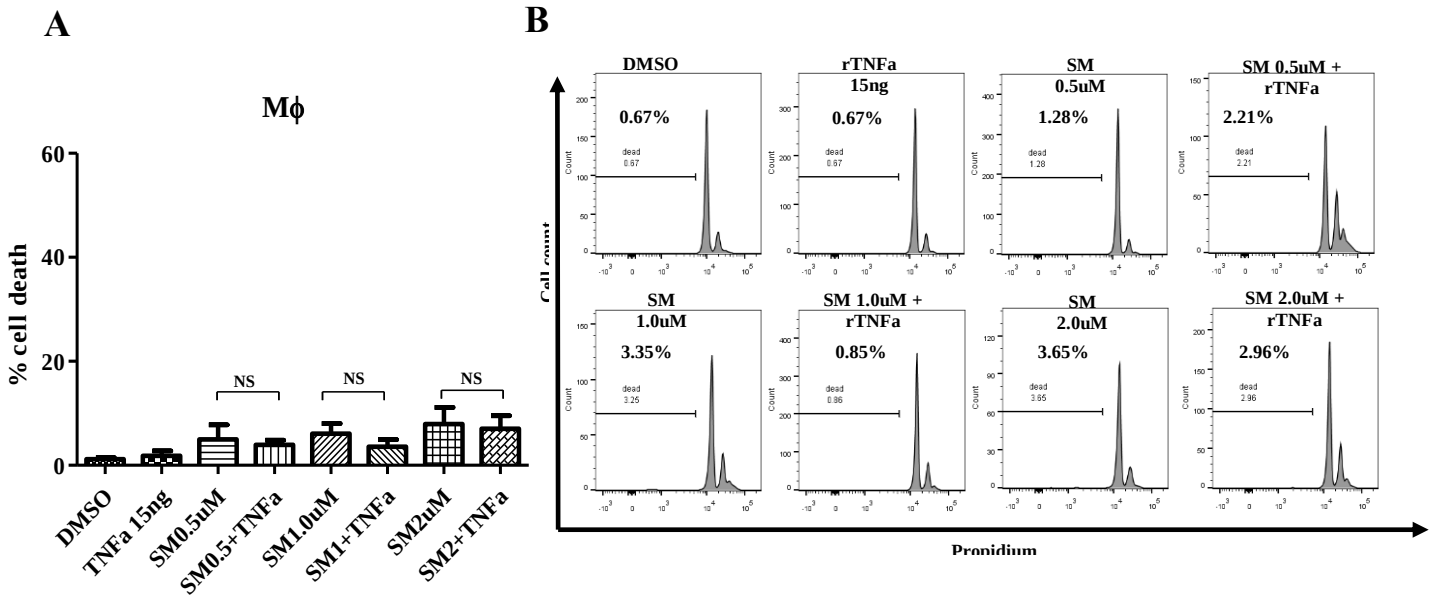
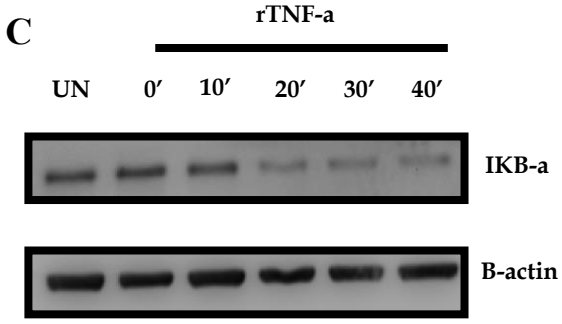

A
U937
% cell death
60
40
20
0
NS
*
UN
DMSO
SM 1.0 uM
rTNFa 2.5ng
SM+rTNFa 2.5ng
rTNFa 5.0ng
SM+rTNFa 5.0ng
rTNFa 10.0ng
SM+rTNFa 10.0ng
rTNFa 20.0ng
SM+rTNFa 20.0ng
B
U1
% cell death
60
40
20
0
*
UN
DMSO
SM 1.0 uM
rTNFa 2.5ng
SM+rTNFa 2.5ng
rTNFa 5.0ng
SM+rTNFa 5.0ng
rTNFa 10.0ng
SM+rTNFa 10.0ng
rTNFa 20.0ng
SM+rTNFa 20.0ng

Figure 14

Figure 15. SM does not sensitize Mϕ to TNFα cell death. (A)MDMs were treated with 15ng of rTNF-α and the cells were collected after 0, 10, 20, 30, and 40 minutes. Total proteins were subjected to western immunoblot and IKB-α degradation was monitored by anti-IKB-α antibody. **(B)** MDMs were preincubated with SM for 2 hours and followed by the addition of rTNF-α. Intracellular PI staining and flow cytometry were used to assessed levels of cell death and **(C)** shows representative histograms. p-values were calculated by students paired t test (n=3).





M0s were treated with TNF-a (15 ng/mL) and cells were harvested at indicated times.

Figure 15

Cytokine profile of HIV-infected MDMs in response to SM treatment

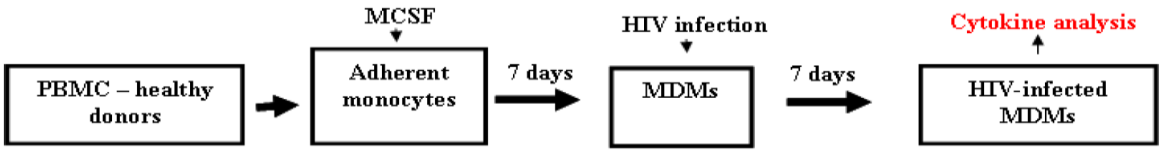
As shown above, Mϕ polarized with IFNγ assume an M1 phenotype which is highly susceptible to SM-induced cell death. To determine whether HIV-infected Mϕ have M1 phenotype before or after SM-treatment, cytokine array analysis was conducted to analyse markers of M1 phenotype in the supernatant of infected Mϕ such as IFNγ and GM-CSF[214–216]. Supernatants after 7-day post-infection incubation of *in vitro* mock- and HIV-infected Mϕ were collected and subjected to cytokine array analysis through Magpix technology **(Figure 16A)**. The Human Th17 magnetic bead panel (Millipore, Etobicoke, ON, Canada) array simultaneously quantified 21 different cytokines in the supernatants; namely: IL-17F, GM-CSF, IFNγ, IL-10, CCL20/MIP3a, IL-12p70, IL-13, IL-15, IL-17a, IL-22, IL-9, IL-1β, IL-33, IL-21, IL-23, IL-5, IL-6, IL-17ε/IL-25, IL-27, IL-31, TNFα, TNFβ, and IL-28A. Of the cytokines in the array panel, Mϕ secreted detectable quantities of CCL20/MIP3a, IL-6, IL-23, IL-10, IL-21, IL-13, and TNFα. However, significant difference was observed in the secretion of CCL20/MIP3a, IL-6, and TNFα between the *in vitro* mock- and HIV-infected Mϕ where infected Mϕ had upregulated secretion of these cytokines compared to the mock control. There was no significant difference in the secretion of IL-10, IL-21, IL-13, and IL-23 between the mock and HIV-infected control **(Figure 16B).** Therefore, HIV-infected Mϕ did not have an M1 phenotype due to the lack of IFNγ induction after HIV infection but exhibited a small degree of pro-inflammatory phenotype by the virtue of pro-inflammatory IL-6, TNFα, and CCL20/MIP3a.

To determine whether SM could modulate the cytokine profile of HIV-infected MDMs leading to polarization into M1 phenotype, *in vitro* mock and HIV-infected Mϕ were treated with SM for 48 hours, after which the supernatants were collected and subjected to similar cytokine array analysis as described above **(figure 17A)**. Treatment with SM did not modulate the secretion of cytokines as there were no significant differences between SM treatments and untreated control in both the mock and HIV-infected groups. **(Figure 17B)**. More importantly, IFNγ was not induced in response to SM-treatment of HIV-infected macrophages suggesting that the cells remained in unpolarized Mϕ phenotype. Next, *ex vivo* derived HIV-infected Mϕ from HIV+ individuals were subjected to the same SM treatment followed by

analysis of secreted cytokines **(Figure 17C)**. Similarly, *ex vivo* derived HIV-infected Mϕ secreted detectable amounts of CCL20/MIP3a, IL-6, IL-23, IL-10, IL-21, IL-13, and TNFα in the supernatants; however, SM had no affect on the secretion of these cytokines **(Figure 17D)**. Lastly, IFNγ nor GM-CSF was produced by these cells before of after SM treatment. Overall, these results suggest that SM-mediated apoptosis of HIV-infected Mϕ may be independent of M1-polarization due to endogenously produced cytokines.

Figure 16. Production of cytokines by Mϕ in response to HIV-infection. (A) Schematic diagram for the analysis of cytokines. (B) MDMs were *in vitro* infected with mock or HIV^{CS204} and supernatants were collected after 7 days of post-infection incubation. Levels of secreted cytokines were measured using Human Th17 magnetic panel cytokine array kit for 22 different cytokines. Seven cytokines could be detected by the kit, but only CCL20/MIP3, TNFα, and IL-6 showed significant secretion after HIV infection (n=6). P-values were calculated using Mann-Whitney U test

A


MCSF
HIV infection
Cytokine analysis
PBMC – healthy donors
Adherent monocytes
7 days
MDMs
7 days
HIV-infected MDMs

B

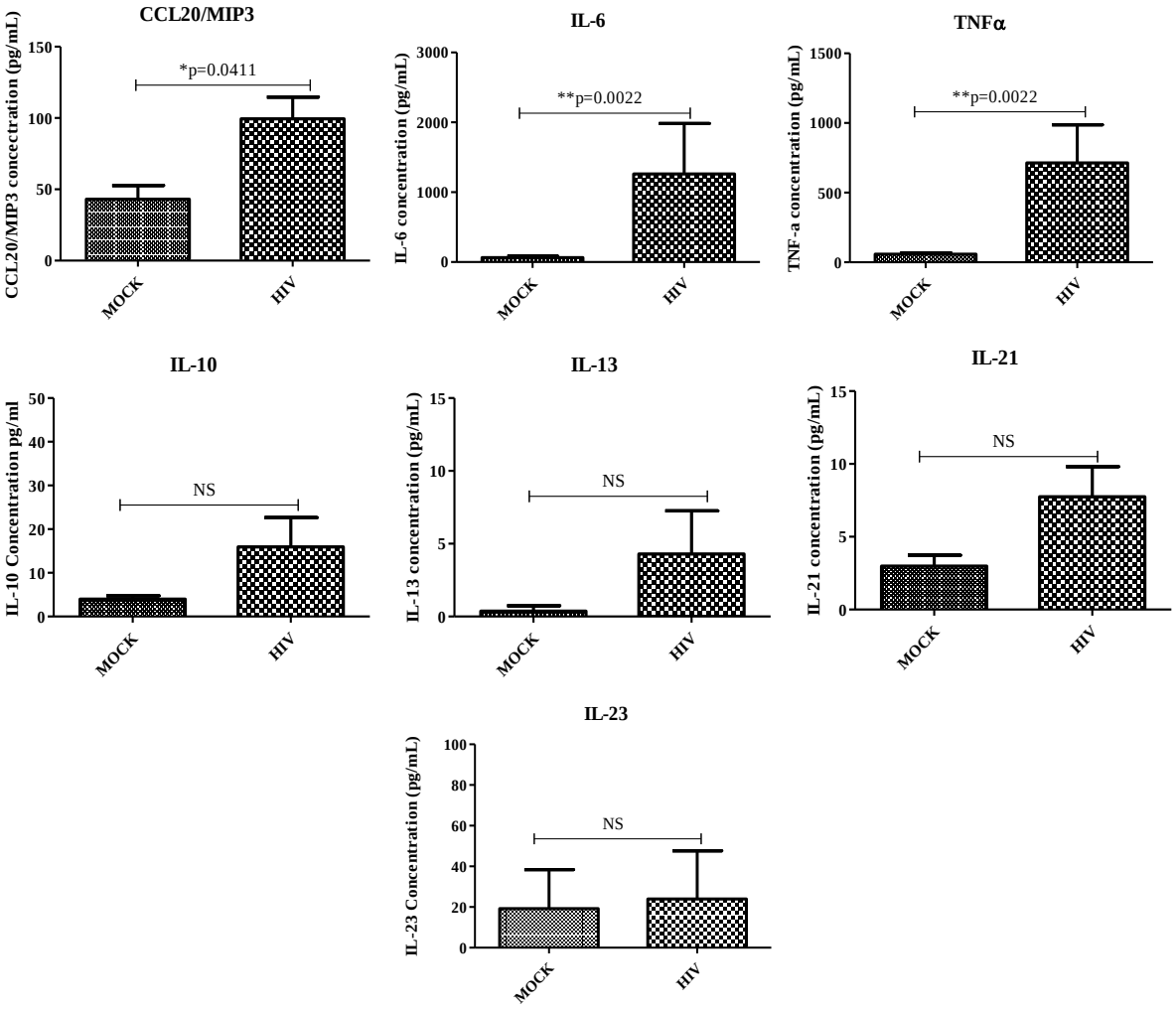
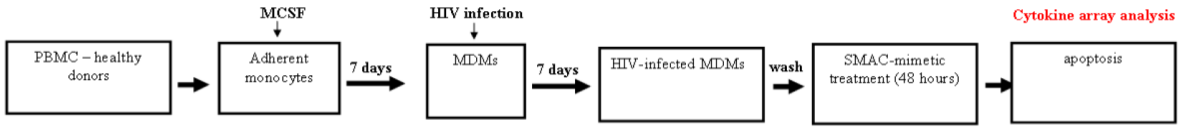

CCL20/MIP3
CCL20/MIP3 concectration (pg/mL)
*p=0.0411
MOCK
HIV
IL-6
IL-6 concentration (pg/mL)
**p=0.0022
TNFα
TNF-a concentration (pg/mL)
**p=0.0022
IL-10
IL-10 Concentration pg/ml
NS
IL-13
IL-13 concentration (pg/mL)
NS
IL-21
IL-21 concentration (pg/mL)
NS
IL-23
IL-23 Concentration (pg/mL)
NS

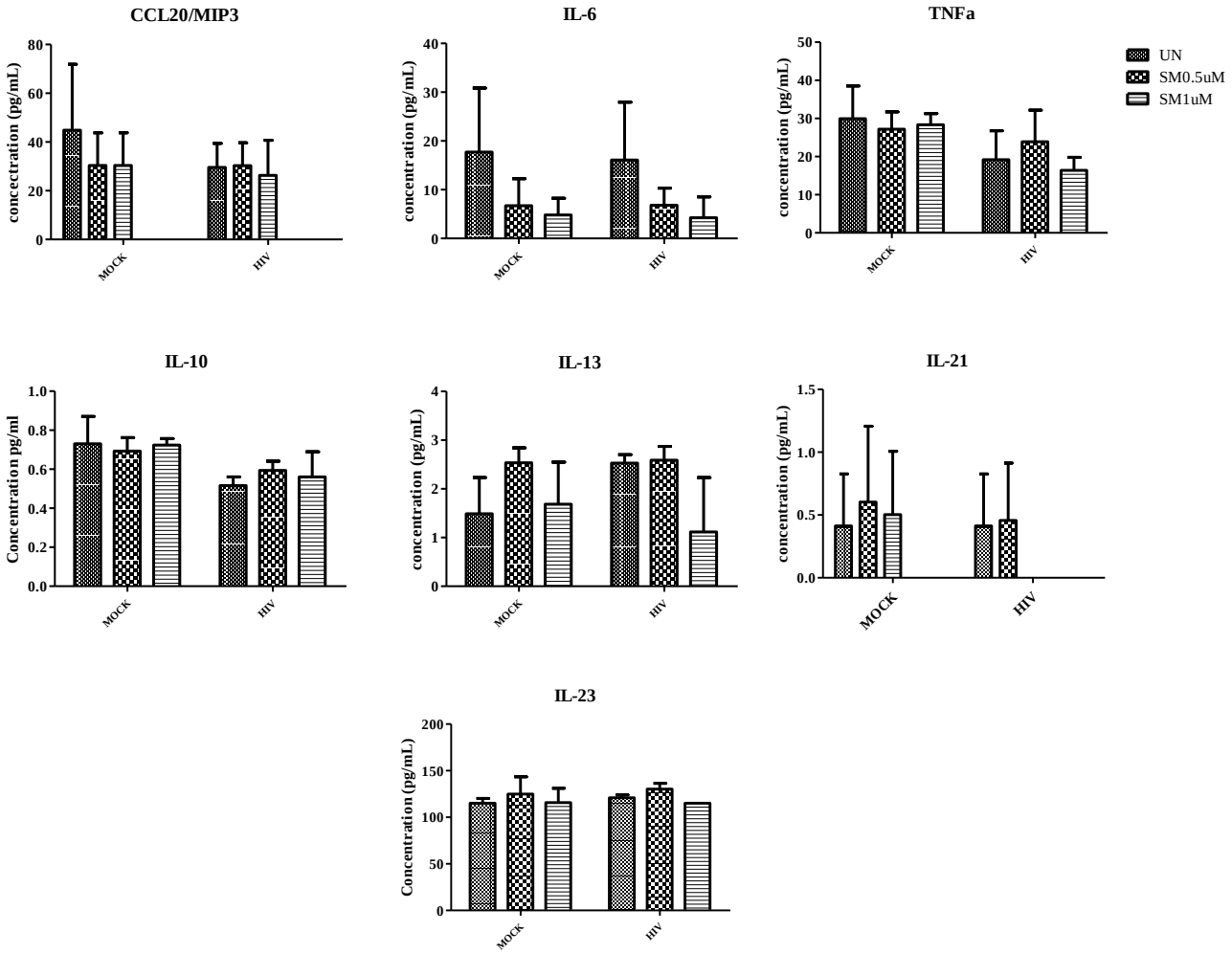
Figure 16

Figure 17. Secretion of cytokines by *in vitro* mock/HIV-infected Mϕ and *ex vivo* derived HIV-infected Mϕ in response to SM treatment. (A) Schematic diagram for the analysis of cytokines secreted by *in vitro* mock- and HIV-infected Mϕ. **C)** Schematic diagram for the analysis of cytokines secreted by *ex vivo* derived HIV-infected Mϕ. **(B)** & **(D)** *in vitro* mock and HIV-infected and *ex vivo* derived HIV-infected Mϕ were subjected were treated with SM LCL161 for 48 hours. Supernatants were collected, and cytokine profile was analyzed through Human Th17 magnetic panel cytokine array kit (n=3, n=4, respectively). p-values were calculated using paired student's t test.

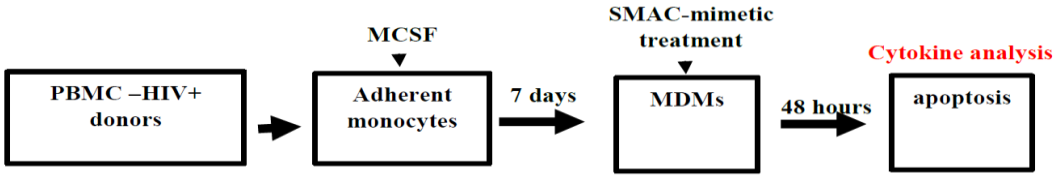
A


MCSF
HIV infection
Cytokine array analysis
PBMC – healthy donors
Adherent monocytes
7 days
MDMs
7 days
HIV-infected MDMs
wash
SMAC-mimetic treatment (48 hours)
apoptosis

B


CCL20/MIP3
IL-6
TNFa
UN
SM0.5uM
SM1uM
concecntration (pg/mL)
concentration (pg/mL)
MOCK
HIV
IL-10
IL-13
IL-21
Concentration pg/ml
IL-23
Concentration (pg/mL)

C


PBMC –HIV+ donors
MCSF
Adherent monocytes
7 days
SMAC-mimetic treatment
MDMs
48 hours
Cytokine analysis
apoptosis

D

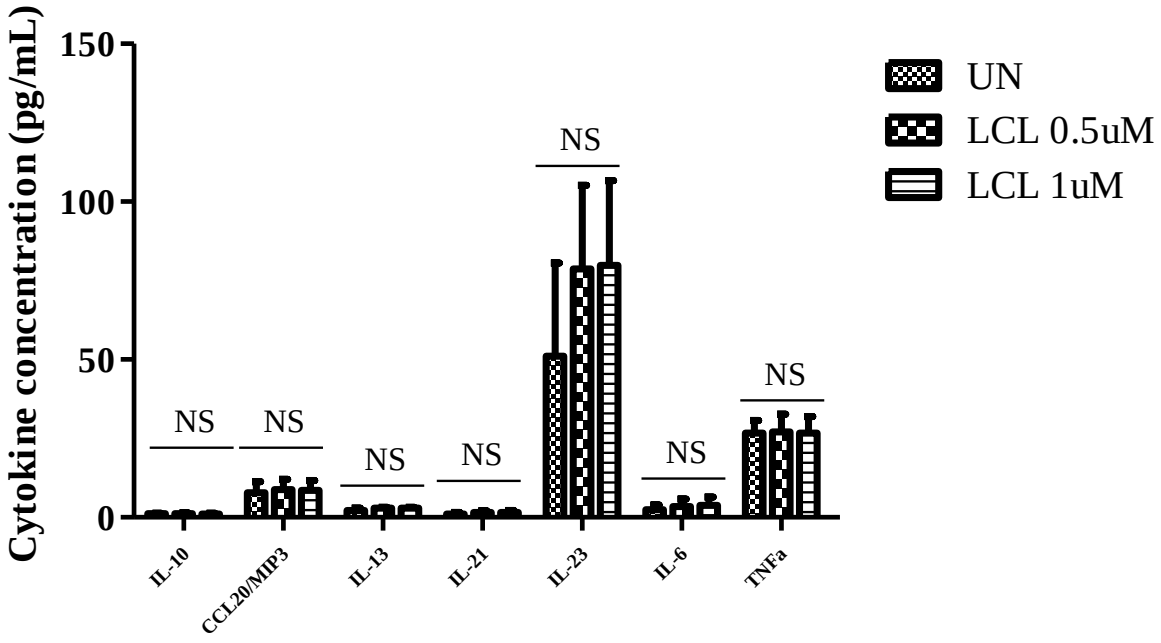

Cytokine concentration (pg/mL)
150
100
50
0
NS
NS
NS
NS
NS
NS
NS
IL-10
CCL20/MIP3
IL-13
IL-21
IL-23
IL-6
TNFa
UN
LCL 0.5uM
LCL 1uM

Figure 17

2C. Determine the regulation of IAP-associated signalling molecules in HIV-infected MDMs before and after SM treatment.

HIV-infection downregulates RIPK1 in MDMs

RIPK1is a member of receptor interacting protein kinase which is important in regulation of variety of cellular processes such NF-κB pathway, apoptosis, and necroptosis [217,218]**.** Previous report has demonstrated that RIPk1 Is a target substrate for HIV protease [219]**.** Protease that is synthesized late in the viral life cycle is responsible for the cleavage and inactivation of RIPK1 in primary CD4+ T cells infected with replication competent HIV-1 [219]. To explore the possibility that RIPK1 may be cleaved and therefore inactivated during HIV infection, Mϕ were infected with HIV^{CS204} for 2 hours after which the cells were replaced with fresh media and left to incubate for 8 days. At days 0, 2, 4, 6, and 8, cells were collected, and total proteins were subjected to Western immunoblot to check the expression level of RIP1. Infection with HIV-CS204 resulted in cleavage of RIPK1 over time, up to a period of 8 days. There was a relative decrease in the intensity full length RIPK1 while the cleaved RIPK1 product gradually increased over time **(Figure 18A).**

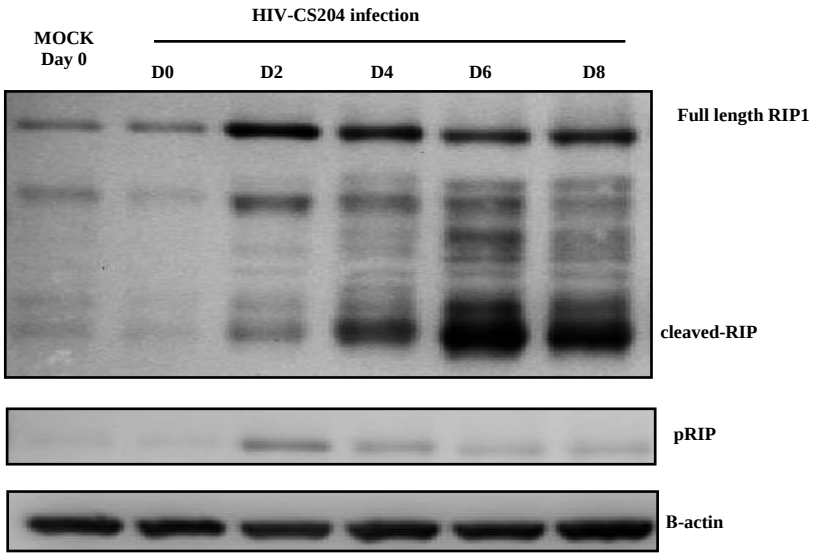
Expression of RIPK1 in purified HIV-HSA-infected Mϕ

To verify the downregulation of RIPK1 in HIV-infected Mϕ, I employed an R5 laboratory strain of HIV-1 called HIV-HSA [192]. Infection with this strain results in the expression of mouse CD-24 or mouse heat stable antigen (HSA) that can be used for positive selection of infected cells. Positive selection of HIV-HSA-infected Mϕ can be performed by staining the cells with anti-CD24-biotin conjugated antibody and subsequent addition of anti-biotin magnetic beads. After 9-days post-infection incubation, the cells were subjected to magnetic column separation. Negative fraction represents the cells that do not express HSA on their surface, and hence get eluted after

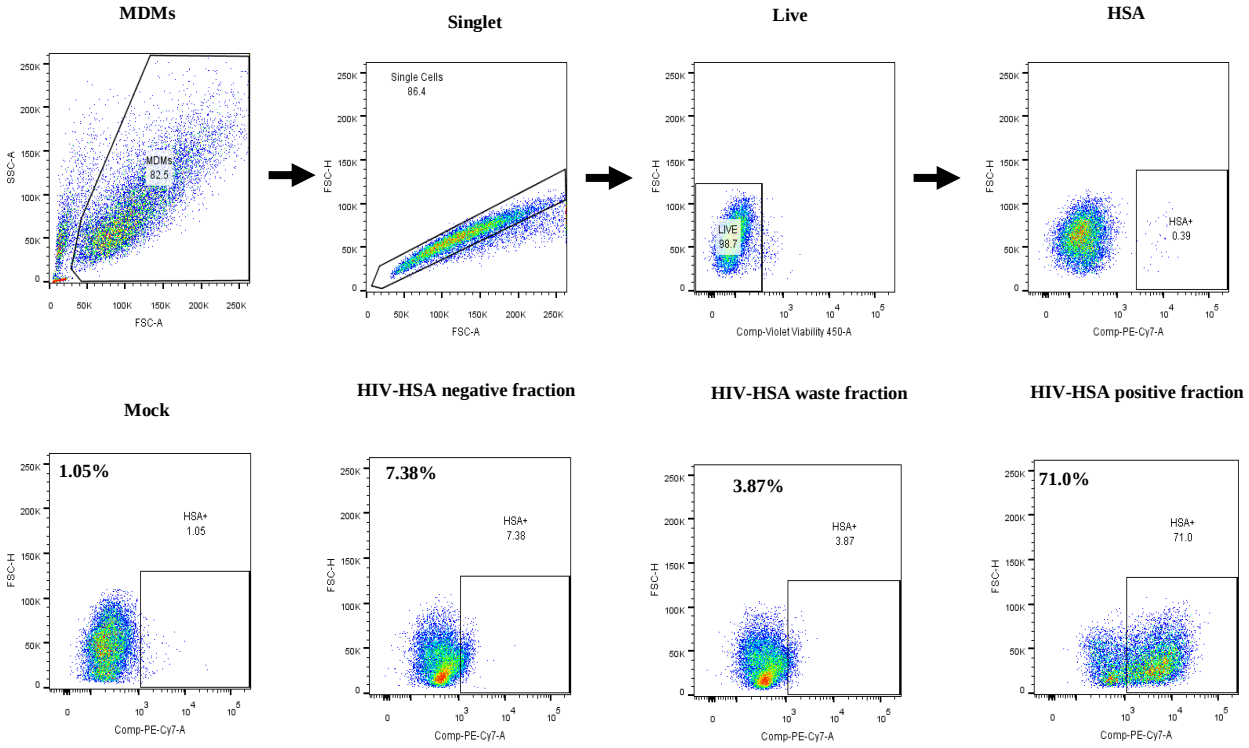
the first passing of the labelled cells through magnetic column. Waste fraction represents the cells that are eluted during the column wash prior to the collection of the HSA-selected Mϕ. Finally, positive fraction represents the HIV-infected HSA-labelled cells retained in the magnetic column that are eluted at the end of the HSA-selection protocol. The positively selected Mϕ infected with HIV-HSA showed ~71% purity while the negatively selected HIV-uninfected cells had ~7% HSA expressing Mϕ **(Figure 18B).** The isolated fractions from the column separation were lysed and subjected to Western immunoblot to detect the levels of RIPK1. In support of the previous finding in figure 18A, RIPK1 was downregulated in the positively selected HIV-HSA enriched fraction compared to the mock infected and negatively selected HIV-uninfected Mϕ fractions **(Figure 18C).** Taken together, these results suggest that RIPK1 degradation is a consequence of HIV infection in primary macrophages.

Figure 18. RIP1 is downregulated by HIV-infection in Mϕ. **(A)** *In vitro* mock and HIV-infected MDMs were treated with LCL161 for 48 hours and the total cytosolic proteins were subjected to western immunoblot. **(B**) Mϕs were infected with HIV-CS204 and the cells were harvested at indicated times. Total proteins were subjected to western immunoblotting. (C) Mϕ were *in vitro* infected with R5 tropic HIV-HAS and the cells were subjected to magnetic column separation using CD24-Biotin conjugated antibodies. Purity of the isolated HIV-infected Mϕ were assessed by CD-24 (HAS) staining and flow cytometry. (D) Isolated fractions of HIV-HAS were lysed and cytosolic proteins were subjected to western immunoblotting. Membranes were probed with antibody specific to RIP1 and RIP3

A


HIV-CS204 infection
MOCK
Day 0
D0
D2
D4
D6
D8
Full length RIP1
cleaved-RIP
pRIP
B-actin

B


MDMs
Singlet
Live
HSA
MDMs
82.5
Single Cells
86.4
LIVE
98.7
HSA+
0.39
Mock
HIV-HSA negative fraction
HIV-HSA waste fraction
HIV-HSA positive fraction
1.05%
HSA+
1.05
7.38%
HSA+
7.38
3.87%
HSA+
3.87
71.0%
HSA+
71.0

C

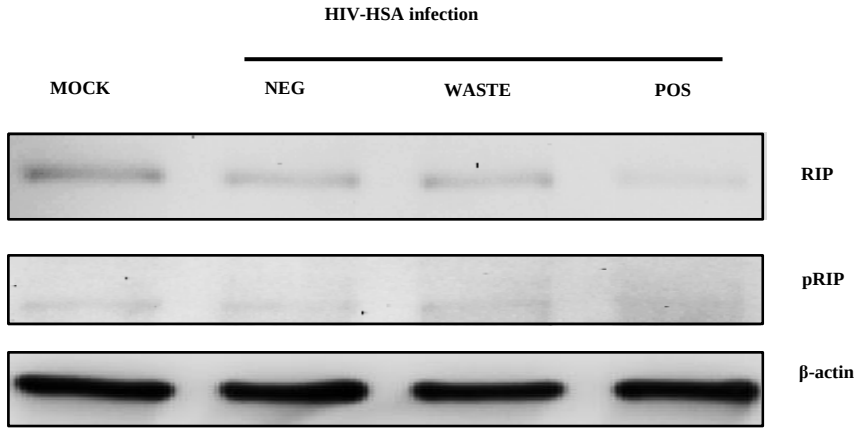

HIV-HSA infection
MOCK
NEG
WASTE
POS
RIP
pRIP
β-actin

Figure 18

SM treatment of HIV-infected Mϕ downregulated signalling molecules involved in apoptotic pathway

The process of apoptosis requires the fine-tuned functionality of several signalling molecules including RIPK1, RIPK3, and TRAF-1/2, as well as proteins that regulate homeostasis of mitochondria such as Bid and Bax [97,220–224]. I sought to determine how these signalling molecules are regulated in response to SM treatment of HIV-infected Mϕ. *In vitro* mock- and HIV-infected Mϕ were subjected to SM, as previously described and expressions of aforementioned signalling proteins were determined by Western immunoblotting. Treatment of HIV-infected Mϕ with SM resulted in the downregulation of RIP1 with increasing concentration of the compound **(Figure 19)**. Moreover, the increased intensity of the cleaved product of RIPK1 corresponded with the decrease intensity of the full-length RIPK1. In addition, RIPK3, an interacting protein that associates with RIPK1 was also found to be downregulated in HIV-infected Mϕ treated with SM. TRAF2 was not affected by SM treatment **(Figure 19A)**, while TRAF1was downregulated in SM treated HIV-infected Mϕ **(Figure 19B)**. Bax showed no significant change in expression in mock- and HIV-infected group after SM treatment **(Figure 19B)**. Bid did not show any change in expression with SM treatment in the mock-infected group; however, it was downregulated with increasing concentration of SM in the HIV-infected Mϕ **(Figure 19A)** Overall, these results indicate that SM dysregulates the expression of apoptosis-associated signalling molecules leading to cell death of HIV-infected macrophages.

Figure 19. Regulation of signalling molecules involved in apoptosis in response to SM treatment of Mφ. (A) *In vitro* mock and HIV-infected MDMs were treated with LCL161 for 48 hours and the total cytosolic proteins were subjected to western immunoblot probing for TRAF-2, TRAF-1, BID, BAX, and B-actin.

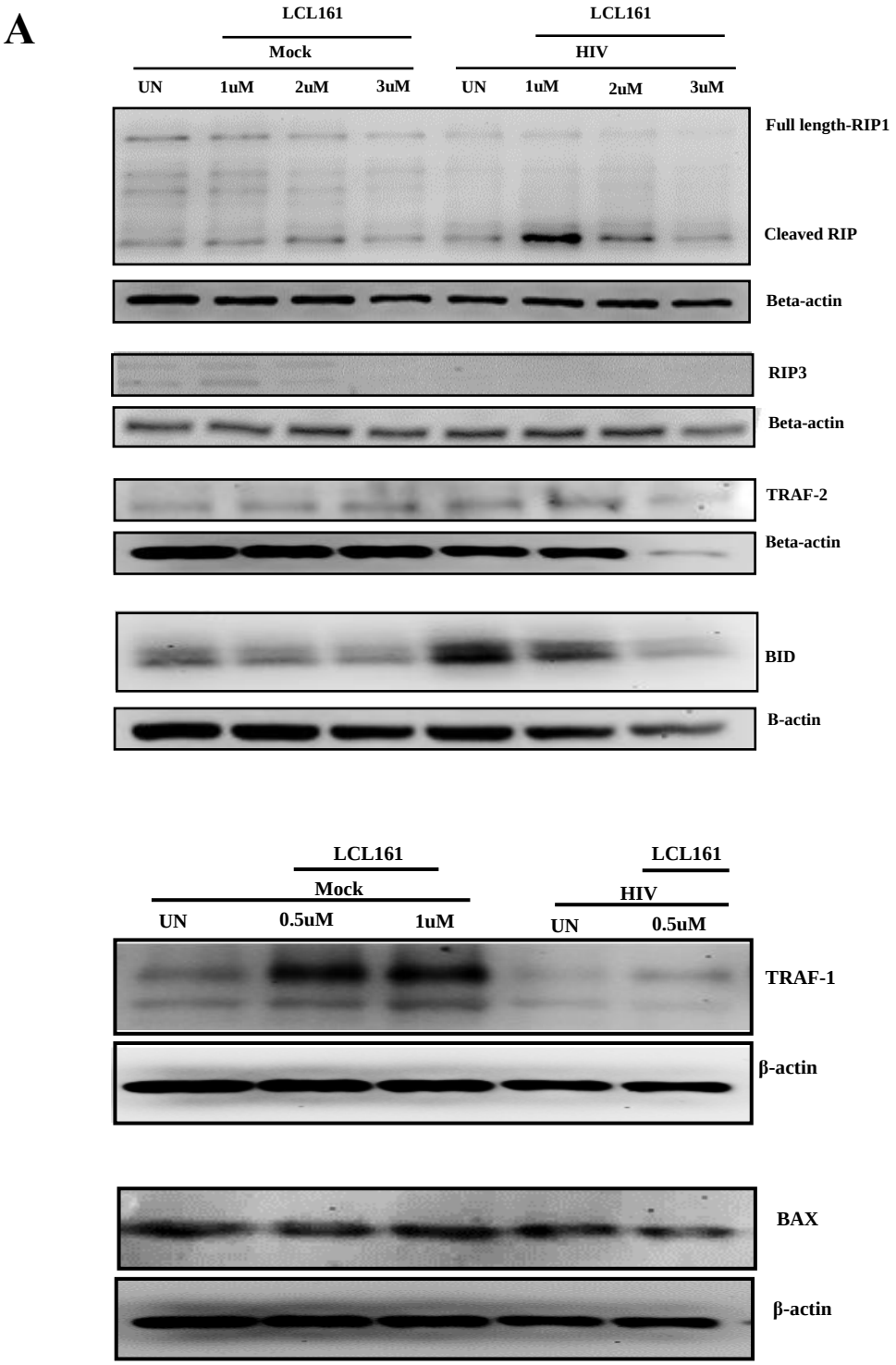

A
LCL161
Mock
LCL161
HIV
UN
1uM
2uM
3uM
UN
1uM
2uM
3uM
Full length-RIP1
Cleaved RIP
Beta-actin
RIP3
Beta-actin
TRAF-2
Beta-actin
BID
B-actin
B
LCL161
Mock
LCL161
HIV
UN
0.5uM
1uM
UN
0.5uM
TRAF-1
β-actin
BAX
β-actin

Figure 19

cIAP1/2 and RIPK1 are essential for survival of Mϕ

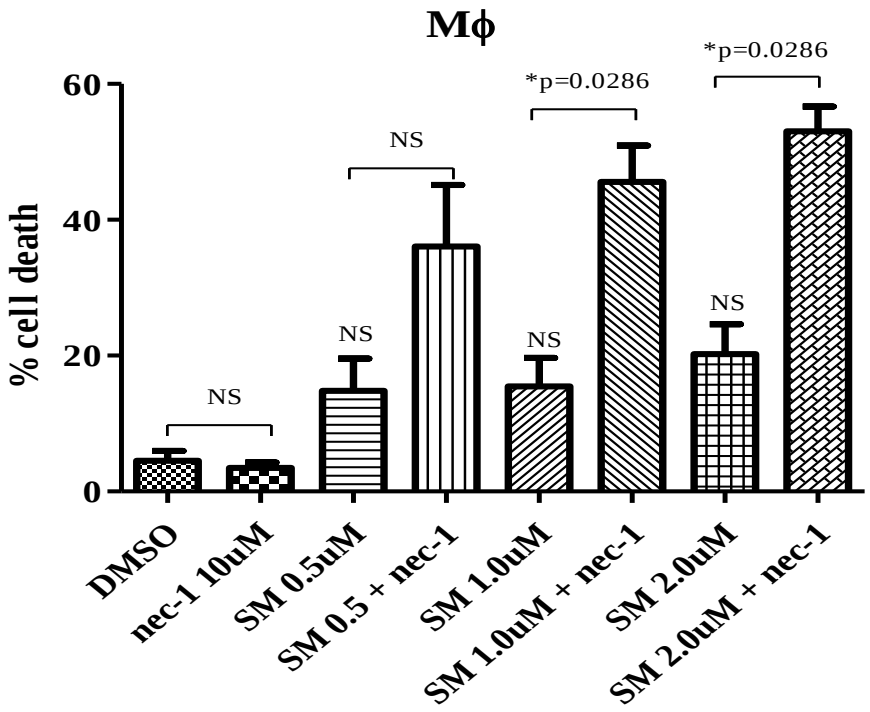
cIAPs are dispensable host factors for the survival of primary MDMs, and RIPK1 has been shown to be targeted for cleavage and inactivation by HIV protease during HIV replication [4,219]. It was therefore of interest whether inactivation of RIP1 in a situation where cIAPs were absent could affect the viability of macrophages. In uninfected macrophages, IAP degradation did not affect cell viability **(Figure 3A)**. Similarly, in HIV-infected Mϕ, loss of RIPK1 was evident and it had no effect on survival of the cells **(Figure 18A)**. I hypothesized that knockdown of both RIPK1 and cIAP1/2 in the same cell may cause cell death. To determine the impact of cIAPs and RIPK1 in the survival of Mφ, uninfected macrophages were pretreated with necrostatin-1, a specific RIP1 inhibitor, for 2 hours followed by the treatment of LCL161 in increasing concentration. After 24 hours, intracellular PI staining was employed to assess the level of cell death. Treatment with SM did not induce significant cell death of uninfected Mϕ similar to the vehicle control **(Figure 20A)**. Moreover, treatment of uninfected cells with necrostatin-1 alone had no effect on cell death. However, combination treatment of SM and necrostatin-1 resulted in significant increase in cell death of primary macrophages **(Figure 20A).** Figure 20B shows representative histograms of the intracellular PI stain.

To verify the induction of cell death of macrophages with the combination treatment of SM and necrostatin-1, I employed Western immunoblotting to check the activation of apoptosis markers. Treatment of necrostatin-1 alone showed no sign of apoptosis activation as cleaved PARP was not observed on the membrane nor the cleaved caspases. SM did not induce the cleavage of caspase-8 and 9 in macrophages, but caspase-3 showed minimum activation (17KDa, 19KDa) as their bands could be faintly observed **(Figure 20C**. However, with combination of necrostatin-1 and SM, the cleaved bands of the three caspases showed increase in intensity which indicated

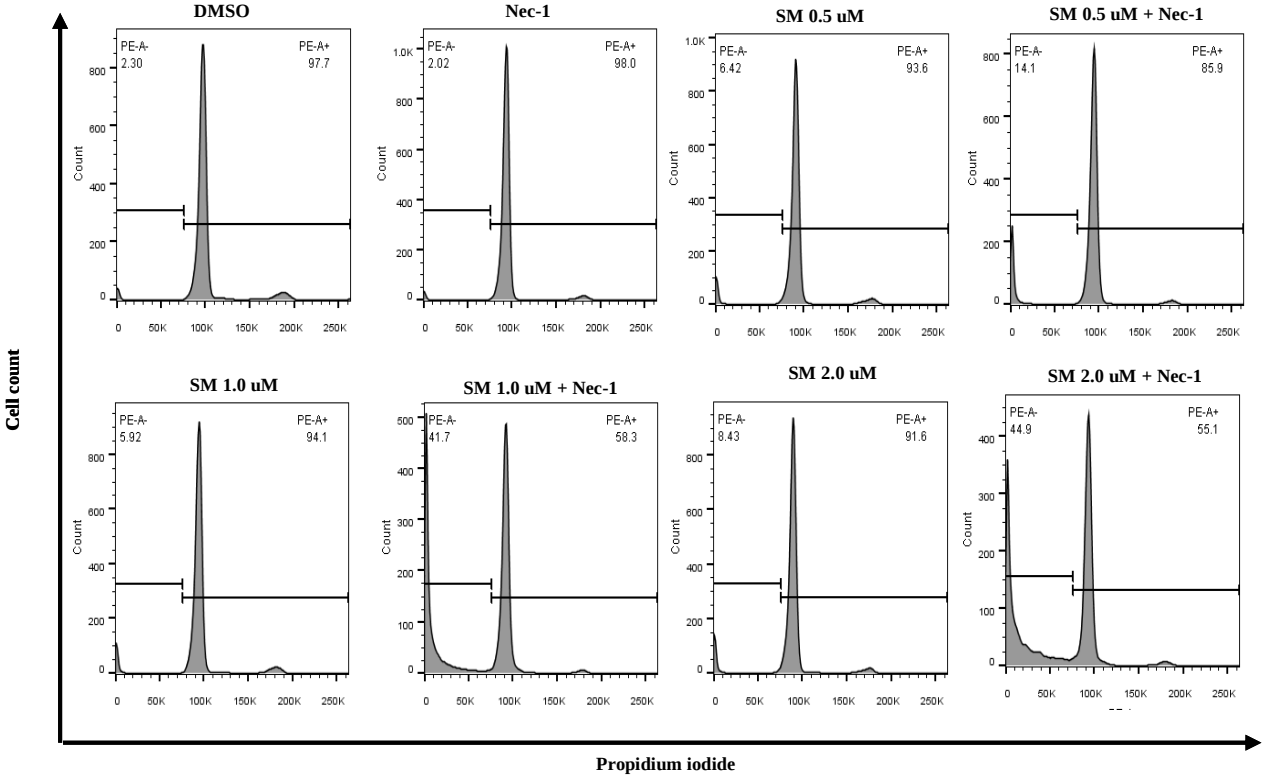
greater activation of apoptosis. Moreover, similar to the downregulation of TRAF1 by SM in HIV-infected Mϕ, combination treatment of nec-1 and SM resulted in the significant downregulation of TRAF1, while TRAF2 was minimally affected **(Figure 20C).** Lastly, anti-apoptotic Bcl-2 was also downregulated when the combination of necrostatin-1 and SM was used **(Figure 20C).** Overall, these results suggest cIAP1/2 and RIP1 play an important role in regulating viability of primary human macrophages and that RIPK1 plays a key role in SM-induced cell death of HIV-infected Mϕ.

Figure 20. cIAP1/2 degradation and RIPK1 inactivation lead to cell death of Mϕ. (A) Mϕ were pre-treated with 10 μM necrostatin-1 for 2 hours and were treated with or without increasing concentration of LCL161. Cell death was assessed after 48 hours through intracellular PI staining and flow cytometry. (**B)** Shows representative histograms**. (C)** Total proteins from the same treatment were subjected to western immunoblot and membranes were probed with antibodies specific for caspase-3, -8, -and 9, PARP, and beta-actin. p-values were calculated using Mann-Whitney U test (n=4).

A


Mϕ
% cell death
60
40
20
0
NS
NS
NS
*p=0.0286
NS
*p=0.0286
NS
DMSO
nec-1 10uM
SM 0.5uM
SM 0.5 + nec-1
SM 1.0uM
SM 1.0uM + nec-1
SM 2.0uM
SM 2.0uM + nec-1

B


DMSO
PE-A- 2.30
PE-A+ 97.7
Nec-1
PE-A- 2.02
PE-A+ 98.0
SM 0.5 uM
PE-A- 6.42
PE-A+ 93.6
SM 0.5 uM + Nec-1
PE-A- 14.1
PE-A+ 85.9
SM 1.0 uM
PE-A- 5.92
PE-A+ 94.1
SM 1.0 uM + Nec-1
PE-A- 41.7
PE-A+ 58.3
SM 2.0 uM
PE-A- 8.43
PE-A+ 91.6
SM 2.0 uM + Nec-1
PE-A- 44.9
PE-A+ 55.1
Count
Cell count
Propidium iodide

C

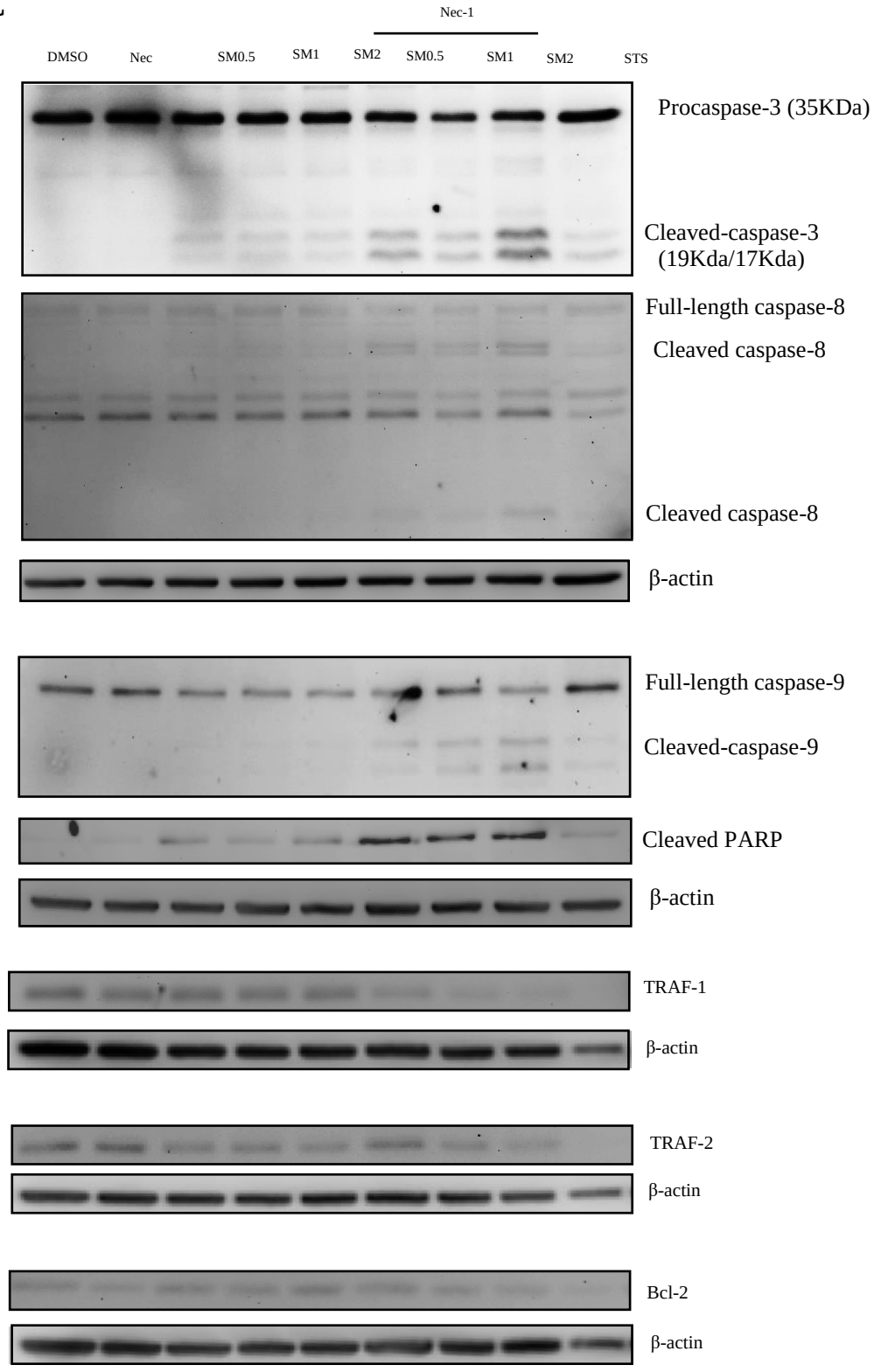

Nec-1
DMSO
Nec
SM0.5
SM1
SM2
SM0.5
SM1
SM2
STS
Procaspase-3 (35KDa)
Cleaved-caspase-3
(19Kda/17Kda)
Full-length caspase-8
Cleaved caspase-8
Cleaved caspase-8
β-actin
Full-length caspase-9
Cleaved-caspase-9
Cleaved PARP
β-actin
TRAF-1
β-actin
TRAF-2
β-actin
Bcl-2
β-actin

Figure 20

Chapter 5: Discussion

Resting memory $CD4^+$ T cells and macrophages represent the major HIV reservoirs in cART treated patients[2,225,226]**.** Cessation of treatment results in rapid rebound and further establishment of more reservoirs. The prime focus of HIV cure research is centered towards developing strategies to eradicate resting memory T cells infected with the virus; however, very little is known about the elimination of infected macrophage reservoirs. Macrophages are susceptible to HIV-1 infection and they play an integral role in virus pathogenesis, both during acute and chronic infections. Armed with intrinsic defense mechanisms against a battery of apoptotic stimuli, it is no wonder why macrophages are ideal sanctuaries for HIV-1. Unlike T cells, macrophages survive active HIV replication and are resistant to cytopathic effects of HIV-1. Therefore, in order to eliminate macrophage viral reservoirs of HIV-1, it is important to elucidate anti-apoptotic genes responsible for conferring apoptosis resistance in these cells.

The aim of my research project was to elucidate the role of IAP and IAP-associated signalling pathways involved in apoptotic resistance of HIV-infected myeloid cells. My results show that cIAP1/2 are dispensable host factors for the viability of myeloid cells in steady-state survival. However, cIAP1/2 are critical for survival of HIV-infected myeloid cells. My results reveal that SM selectively induce apoptosis of HIV-infected myeloid cells, both in cell lines and primary Mϕ. In contrast to several tumour cell lines, the SM-induced apoptosis of HIV-infected Mϕ is independent of TNF-α and other endogenously produced cytokines. Furthermore, I have found that RIPK1 may play an important role in SM-induced killing of HIV-infected Mϕ.

Primary Mϕ are resistant to SM-induced apoptosis.

IAPs contribute to the activation of the classical and inhibition of the non-canonical NF-κB pathways by variety of stimuli such as TNFα[157]. Given that NF-κB has an overwhelming importance in the regulation of immune signalling[227–229], it is important to address the impact of modulating this pathway by SM on immune cells. Preliminary reports from early phase clinical trials in various cancers reveal that administration of monomeric or dimeric SM is well tolerated [151,152,230]**.** However, the effect of SM on non-transformed human immune cells such as macrophages remains largely unknown. Reports show that primary monocytes are susceptible to SM BV6-induced necrotic cell death[231] while SM SM-164 treatment results in necroptotic death of bone marrow-derived mouse (BMDM) macrophages[232,233]**.** Contrary to these previous findings, our laboratory has demonstrated that dimeric SM AEG730, at nanomolar concentrations, does not incite cell death in primary macrophages[4,185,186]**.** In conformity with these observations, my results showed that micromolar concentrations of monomeric SM LCL161 did not induce cell death in primary Mϕ, with no significant differences in the degree of cell death between optimal concentration of SM treatments and the control. SM were developed with the intent to disrupt the function of overexpressed XIAP in tumour cells[149]; however several reports showed that SM mediated the degradation of cIAP1/2 by upregulating their autocatalytic ubiquitin functionality. By linking the BIR domains, the E3 ligase activity gets potentiated and targets cIAP1/2 autoubiquitination and subsequent proteosomal degradation[139,133,149,160,234]. Indeed, treatment of Mϕ with SM resulted in the degradation of cIAP1/2 **(Figure 21A)**. Overall, these results suggest that ablation of cIAP1/2 by SM does not affect the steady state survival of primary human Mϕ.

SM mediate the selective killing of HIV-infected myeloid cells by apoptosis

Cell lines: We have shown for the first time the protective role of cIAP1/2 against HIV-Vpr-induced cell death of monocytes and Mϕ[4,185]**.** Ablation of these proteins by SM negated the intrinsic resistance of Mϕ to apoptogenic HIV-Vpr[4]**.** These findings prompted us to ask whether SM could be applied as a possible strategy in clearing HIV-infected macrophages. As proof-of-concept experiments, I employed U937 and U1 cells**.** Several reports show that U1 cells are less susceptible to apoptotic stimuli compared to its uninfected U937 counterpart, due to the dysregulation of early and advanced apoptotic markers[169,171,172,196]**.** Similar to tumour cells, the role of IAPs in conferring resistance to cell death has been suggested in increased resistance of U1 cells to apoptotic stimuli[172]**.** Hence, U937 and U1 cells provide an ideal model for studying the involvement of SM in killing of HIV-infected cells. Cytolytic activity of SM have been reported in several tumour cell lines as well as in treatment regimens of *ex vivo* acute myeloid leukemia (AML) and chronic lymphoblastic leukemia (CLL)[235–237]. Herein, I demonstrated that treatment with SM LCL161 selectively induced cell death of U1 cells infected with HIV-1 as compared to its uninfected counterpart U937 cells. Contrary to U937, significant induction of cell death with increasing dosage of SM was observed in chronically infected U1**.** Although SM induced significant cell death in undifferentiated U1 cells compared to control U937 cells, the maximum induction was only 10% higher than in U937 cells. However, treatment after PMA-induced differentiation resulted in a dramatic increase in cell death in Mϕ-U1, while SM had no discernable effect in cell death of Mϕ-U937. The SM-mediated cell death of the U1 cells occurred through the induction of apoptosis. Western immunoblotting showed that caspase-3 and caspase-8 were distinctly activated and cleaved in the U1 cells after treatment with SM, which corroborates the results from the intracellular PI staining experiments. On the other hand, neither caspase-3 or

caspase-8 were cleaved and activated in U937 after SM treatment. The reason for this selective killing of U1 cells is not clear; it may be attributed to the uninduced low-level virus replication and inefficient synthesis of HIV-proteins that would otherwise synergize with SM to cause cell death, one of which is Vpr as previously described[186,195,238].The effect of SM in selectively killing HIV-infected myeloid cell line is not linked to the differentiation state of the cells. In addition to induction of cellular differentiation, PMA also elicits secretion of cytokines[239,240] including TNFα, which can enhance the effect of SM in selectively killing infected cells. However, the effect of TNFα can be disregarded in selectively killing U1 cells since undifferentiated and PMA-differentiated U1 produced significant levels of TNFα. Altogether, the results from these preliminary experiments provided reasonable incentives to further pursue the modulation of IAP-signalling associated pathway as a mean for elimination of HIV-infected primary macrophages.

Primary macrophages: Recently several groups have explored the role of IAPs in killing of virally infected cells. Pache and colleagues demonstrated that SM could activate the non-canonical NF-κB pathway by the virtue of RelA:p50 and RelB:p52 transcription factors which bind to the HIV-1 LTR region and resulted in induction of virus transcription in latently infected JLat cell line model. Single agent activity of SM in reversing latency in JLat models via non-canonical NF-κB activation was also observed by different groups (Sampey, G. *et al.* 2017, Elbezanti, W. *et al.* 2017, Journal of Virus Eradication, poster presentations). This reversal in latency was potentiated with histone deacetylase inhibitor (HDACi) in latently infected primary memory $CD4^+$ T cell[198]. As a consequence of active viral replication, $CD4^+$ T cells succumb to the cytopathic effects of HIV leading to the elimination of these infected cells. Interestingly, XIAP down regulation by flavopiridol cyclin-dependent kinase 9 (CDK-9) inhibitor resulted in increased apoptosis of ACH2

cells compared to their untreated control[172]. Moreover, SM Birinapant has also been shown to induce cell death of latently infected primary memory $CD4^+$ T cells independent of latency reversal (G.R Campbell *et al*. 2015, CROI, conference)[189]. Interestingly, ablation of cIAP1/2 by SM cleared hepatitis B virus (HBV) from infected competent mouse models[190,191], which further supports the rationale behind IAP-modulation for clearing intracellular pathogens such as HIV-1 in macrophages and other cell types.

In order to achieve full eradication of HIV-1 in patients undergoing suppressive ART, it is imperative to study and understand the formation of viral reservoirs in other cell targets such as macrophages. Previously, we have shown that the resistance of monocyte-derived macrophages to apoptogenic HIV-Vpr was linked to cIAP1/2[4,185,186]. Thus, a possible strategy for clearing HIV-infected Mϕ in HIV-infected patients may be underscored by the mechanisms that underlie SM-induced cell death. In support of the hypothesis stated above, *in vitro* infected Mφ were indeed susceptible to SM-mediated cell death, while mock-infected and healthy controls had no significant induction after SM treatment. Similarly, *ex vivo* derived HIV-infected Mϕ generated from either ART-treated or naïve HIV-infected patients showed susceptibility to SM-mediated cell death. However, the degree of SM-mediated killing between the ART-treated and naive group differed. The treated Mϕ had higher percentage of mean apoptotic cells at the highest dose of LCL161 compared to the untreated Mϕ **(Figure 21C)**. This effect may be reflected by the fact that antiretroviral drugs come with detrimental consequences that may be potentiated by SM. Particularly, the most common ART-associated toxicities are reported to be associated to mitochondrial dysfunctions [241,242]**.** Given that the mitochondria play an important role in cell survival by sequestering apoptogenic factors within itself through regulation of Bcl2 family of proteins, the interplay between antiretroviral drugs and SM warrants further investigation.

The inhibition of IAP function not only induces apoptosis, but depending on the presence of key regulatory molecules, necroptosis may be elicited [243]. Inhibition of RIPK1, key regulator of necroptosis[200], by necrostatin-1 failed to reduce the degree of SM-induced cell death of HIV-infected Mϕ with combination of SM indicating that necroptosis was not involved in killing the cells. SM treatment of HIV-infected Mϕ resulted in the increase activation of initiator caspase-8 and caspase-9 as well as the effector caspase-3 which supported the above findings of increased cell death in HIV-infected Mϕ, as detected by PI staining.

Although Mϕ are susceptible to HIV-infection, the number of infected cells is very little due to the fact that Mϕ are non-dividing cells and they express a milieu of HIV restriction factors that limit the life cycle of the virus[192,244,245]**.** Studies reported in the literature show primary Mϕ infection rates of about 2-10% in culture[65,192]**.** However, there seems to be a discrepancy between the apoptotic cells and caspase activation in SM-treated HIV-infected Mϕ. Caspase-8- and caspase-9 activation in HIV-infected cells exceeded 30%, which could imply that SM caused non-specific killing of Mϕ. Since I showed that SM did not inhibit virus replication, as there was no change in HIV-p24 in response to the treatment, it is possible HIV proteins may have been expressed and secreted in the supernatants, particularly Vpr[246], which resulted in bystander killing of Mϕ with SM treatment. Moreover, results from Mr. Simon Dong, a PhD candidate in our laboratory show that HIV-HSA-infected Mϕ are specifically killed off by SM AEG relative to uninfected controls (data not shown). However, there seems to be killing of bystander cells at high concentration of SM as there is a fraction of uninfected Mϕ that undergo apoptosis in the HIV-negative population with high dose of SM (in submission). Alternatively, the additional increase in caspase activation does not necessarily have to correlate with cell death. As reviewed

somewhere else, caspases function not only in induction of apoptosis, but also include cell proliferation, migration, differentiation, and cellular fission/fusion [247,248].

SM treatment does not induce virus replication in HIV-infected Mϕ and U1 cells

Viruses such as Kaposi's sarcoma-associated herpesvirus (KSHV), human herpesvirus (HHV), Eppstein-Barr virus (EBV), and herpes simplex virus-1 (HSV-1) undergo rampant virus replication upon detection of host cell apoptosis through a process called alternative replication program (ARP)[197,249–252]. Recently, this phenomenon was also observed in HIV-1 in chronically infected cell line models, U1 and ACH2[197]. Treatment with cytotoxic drugs such as doxorubicin, etoposide, fludarabine phosphate, or vincristine resulted in virus reactivation which was correlated with the increased activity of caspases and hence, cell death[197]. Since SM treatment of U1 cells and HIV-infected Mϕ induced apoptosis, this begged the question whether the SM treatment would have affected virus replication. Previously, through a targeted siRNA screen, cIAP1 was found to be a negative regulator of LTR-dependent HIV-1 transcription in chronically infected JLat latency model and in latently infected primary memory T cells[198]. These findings suggest that SM may be amenable for "Shock-and-kill" approach of latently infected T cells. However, these observations are in direct contrast with my results as SM treatment of U1 cells and HIV-infected Mϕ had no affect on HIV-transcription as measured by HIV-p24 secretion in the supernatants. We have recently shown in our laboratory that SM treatment of Mϕ did not activate either classical or alternative NF-κB pathways[253].The differences in the effects of IAP ablation in our studies may be rooted in the type of cells (ie. Mϕ compared to the T cells used by Pache *et al.* (2015)[198]. Through the E3 ubiquitin-ligase activity, cIAP1 and cIAP2 are important regulators of NF-κB but appear to have redundancy in their roles[253]. It is possible that cIAP1 and cIAP2 may function differently

during HIV infection, and by disrupting their activity may have different consequences. The specific roles that cIAP1 and cIAP2 play in HIV infection requires close examination.

Differential requirement for TNFα in SM-induced killing of HIV-infected myeloid cells

TNFα signalling mediates cell survival or cell death and this functional dichotomy is regulated by two distinct protein complexes[160,254–257]. Following TNFα ligation to TNFR1, the pro-survival mechanism is initiated by the formation of a protein complex composed of TRADD, TRAF2, RIPK1, and cIAP1/2 which activates I-kappa kinases to activate the canonical NF-κB-dependent pathways[131,160,258]**.** On the other hand, the pro-death mechanism is activated when cIAP1/2 are absent which leads to the formation of death inducing complex DISC composed of TRADD, RIPK1, FADD, and caspase-8 leading to caspase-3 activation[257]**.** Moreover, the absence of cIAPs potentiates the non-canonical NF-κB pathway leading to higher TNFα production that promotes autocrine-mediated apoptosis[147,160,259,260]**.** Thus, IAP degradation by SM is a two-prong death inducing mechanisms. Based on these observations, I hypothesized that SM-mediated killing of HIV-infected myeloid cells is dependent on the autocrine signalling of TNF-α. SM treatment of undifferentiated and PMA-differentiated U937 and U1 cells resulted in the increased secretion of TNFα. It was not clear how SM exclusively induced apoptosis in U1 cells, and not in uninfected U937 counterpart even though both cell types had increased secretion of TNF-α. It is possible that the SM-induced TNFα is not sufficient to synergize with SM to induce cell death[261] in U937 cells. I showed that addition of recombinant TNFα at 2-20ng/mL, which was about 20-200X higher than the secreted concentration, was sufficient to induce a significant cell death in both U937 and U1 cells. U937 and U1 cells are myeloid leukemia cell line models so it was not surprising for the cells to undergo cell death after combination treatment of SM/TNFα since there are reports that show this effect on other myeloid leukemia cell line models such as MV4-11, Molm13,

MonoMac6, mixed lineage leukemia (MLL)[159,243]. Irrespective of the role TNFα, the results from undifferentiated and PMA-differentiated U937 and U1 cells clearly implicate the involvement of a component of HIV which acts in concert with SM to induce apoptosis. Indeed, HIV has been described to modulate the intrinsic and extrinsic pathway of apoptosis in U1 cells by increasing the antiapoptotic to pro-apoptotic Bcl2/Bax ratio and downregulating CD95/Fas, respectively, leading to reduced susceptibility to apoptosis[170,262]. As will be discussed below, HIV modulates the expression of host cell factors in primary macrophages such as RIPK1, RIPK3, TRAF1, and Bid. These modifications may be present in the U1 cells as well and may influence the cytotoxic potential of SM in this cell line and require further investigations.

Macrophages are amongst the main producers of TNFα and are highly responsive to this cytokine [263]**.** During HIV-1 exposure, Mϕ exhibit increased secretion of TNFα which can enhance viral transcription through the activation of NF-κB transcription factor-dependent mechanisms[178]. Given that Mϕ produce high amounts of TNFα, this pro-inflammatory cytokine may be involved in SM-mediated killing of HIV-1 infected Mϕ. Consistent with the literature, *in* vitro HIV-infected Mφ and *ex vivo* derived HIV-infected Mϕ produced copious amounts of TNFα compared to the mock-infected and healthy controls. However, after SM treatment of these Mϕ, no change in the TNFα secretion was observed in both mock/healthy and infected groups, suggesting that the mechanistic action of SM inducing cell death in Mϕ was independent on the autocrine-secreted TNFα. This was verified when exogenous TNFα was added to the SM-treated healthy macrophages. Even though the rTNFα was functionally active, as seen by TNFα-induced degradation of IKBα, combination with SM failed to induce cell death in primary Mϕ.

TRAIL was another cytokine of interest as it has been shown by multiple studies to act in concert with SM in inducing cell death[209,210]**.** However, with HIV-infection, this cytokine was not

upregulated and hence its role in SM-mediated killing of HIV-infected Mϕ was not further investigated. Overall, M1-induced apoptosis of primary Mϕ and leukemic cell lines via distinct mechanisms. Whereas, TNFα can confound the selectivity of SM in killing infected U1 cells, infected primary Mϕ remain resilient in the face of TNFα/SM combination.

SM does not modulate cytokine profile of HIV-infected Mϕ

Cytokines exert profound influence on the functional phenotype of Mϕ[214]**.** Depending on the cytokines present in the microenvironment, Mϕ can be activated and polarized towards extreme phenotype conventionally termed classical (M1) or alternative (M2)**,** with the M2 further subdivided into M2a, M2b, and M2c phenotypes[214,264,265]**.** HIV infection results in dysregulation of cytokine profile *in vivo* and *in vitro* and can possibly have an affect the polarization state of Mϕ[266]**.** Our laboratory has shown that M1 macrophages, generated by treating Mϕ with IFNγ, exhibit the most susceptibility to SM-mediated cell death amongst the *in vitro* generated macrophage subsets. Therefore, I hypothesized that *in vitro* HIV-1 infection polarizes Mϕ into M1 phenotype which makes these cells susceptible to SM- induced apoptosis. M1-polarized Mϕ can be characterized by the expression of prototypic proinflammatory responses and markers, most typically IFNγ, GM-CSF, TNF-α [214]**.** My results revealed that after infection, both *in vitro* mock and HIV-1^{CS204} infected Mϕs produced CCL20/MIP3, IL-6, IL-23, IL-10, IL-21, IL-13, and TNFα. However, between the detectable cytokines, only CCL20/MIP3, IL-6, and TNF-α showed increased secretion after infection with HIV. IFNγ and GM-CSF were not induced by *in vitro* HIV-1 infection, suggesting that the Mϕ were not polarized into M1 phenotype, even though the infected cells exhibited an upregulation of IL-6, CCL2/MIP3a, and TNFα.

Broad proinflammatory cytokine response triggered by SM treatments is widely described in cancer therapeutics[152,205,267]. Both *in vivo* and *in vitro* settings from different cancer models

show the upregulation of pro-inflammatory cytokines such as GM-CSF, IL-8, IL-6, TNFα in response to SM treatment[267–269]. Interestingly, besides TNFα, some of these proinflammatory cytokines such as IFNγ and TRAIL act in concert with SM to induce cell death of various cancer cells[270–272]**.** In addition, several *in vivo* reports have noted the reversion of functional phenotype of tumour-associated macrophages (TAMs) from being immunoregulatory to proinflammatory after treatment with SM[268,269,271]**.** For instance, Kim and colleagues have demonstrated that LCL161 could alter cytokine expression within tumour microenvironment toward pro-inflammatory profile which was supportive of anticancer immunity and was associated with the generation of M1-polarized macrophages[268]**.** In contrast, infection of mouse macrophages with *Chlamydia pneumoniae* led to M1 polarization, but after treatment with SM Birinapant resulted in reversion into M2 phenotype[273]. I sought to determine whether treatment of *in vitro* infected Mϕ and *ex vivo* derived Mϕ from infected individuals with SM LCL161 would lead to an aberrant secretion of pro-inflammatory cytokines that could potentially mediate the cell death of these Mϕ. My results revealed that after treatment with SM, the cytokine profile of both the *in vitro* infected and *ex vivo* derived Mϕ were not significantly altered. Notably, of the proinflammatory cytokines, only TNFα, IL-6, IL-23, and IL-13 were detected by the cytokine array, and that treatment with SM had no affect on the secretion of these cytokines. Additionally, TAMs have been shown to secrete IFNγ upon treatment with LCL161 which correlate with the state of their M1 polarization; however, the same was not observed in HIV-infected Mϕ. Altogether, these results suggest that SM-mediated killing of HIV infected Mϕ is independent of TNFα and that the induction of apoptosis may originate from the intrinsic pathway of apoptosis.

Involvement of RIPK1 in SM-induced killing of HIV-infected Mϕ

The pathways of apoptosis and necroptosis are regulated by RIPK1, which also plays an important role for the optimal activation of NF-κB and MAPK pathways[274]. For instance, in TNFα-induced cell survival, RIPK1 is recruited in a multiprotein complex I along with TRADD, TRAF2, and cIAP1/2 to activate NF-kB and promote transcription of genes with anti-apoptotic properties which include cIAP1/2, XIAP, MnSOD, and c-FLIP[221]**.** On other hand, RIPK1 may also be recruited in a protein complex composed of TRADD, FADD, and caspase-8, which depending on additional proteins recruited, can induce apoptosis or necroptosis of the cell[217,221]**.** A recent report showed that HIV infection of primary activated $CD4^+$ T cells led to the downregulation of RIPK1 [275]. It was found that RIPK1 was a *bona fide* substrate of HIV-1 protease in the infection of $CD4^+$ T cells [219]. With Mϕ being one of the major targets of HIV-1, it was of interest whether the same phenomenon could be observed. Indeed, infection with HIV-CS204 resulted in the downregulation and cleavage of RIPK1 over time. When Mϕ infected with HIV-HSA were isolated through HSA-biotin positive selection, the levels of RIPK1 in the enriched population (positive fraction) was significantly lower compared to the mock infected control or the uninfected population (negative fraction)**.** RIPK1 modification in response to virus infection has been investigated by several groups**.** Recently, Croft and colleague has shown that human rhinovirus 3C protease cleaves RIPK1 to generate a cleaved product which possessed regulatory activity that may limit apoptosis of the host cells[276]. Additionally, in Newcastle disease virus (NDV) infection, overexpression of RIPK1 reduced apoptosis, while depletion of this same protein promoted apoptosis[277]. By extrapolation, the cleavage of RIPK1 may be a possible mechanism that allows HIV-1 to persist within Mϕ. RIPK1 was also shown to be dispensable for proper development of dendritic cells[278]. Thus, it is likely that inhibition of RIPK1 function is not detrimental to Mϕ function during HIV-

infection. Indeed, necrostatin-1 inhibition of RIPK1 in healthy Mϕ did not impair survival of these cells in steady state **(Figure 21B)**. Another possible explanation for the downregulation of RIPK1 during HIV-infection is that it may prevent the induction of apoptosis. Since RIPK1 is an important adaptor protein that interacts with signalling molecules upon engagement of extrinsic pathway, its downregulation may ultimately modulate how caspase-8 is activated in the event death inducing ligands bind to cognate receptors of its host cells [276]. It is also possible that downregulation RIPK1 may prevent the induction of necroptosis[219]. A recent study showed that K45A mutation of RIPK1 attenuated necroptosis induction in response to TNFα, LPS, and IFNβ in the absence of caspase signalling[279]. HIV-1 ultimately wants to remain undetected from the host immune response, and proper functioning of necroptosis, a process that exposes and releases pathogen-associated molecular patters (PAMPS) and danger-associated molecular patterns (DAMPS) into the microenvironment[280,281], may limit the chances of the virus to achieve this goal.

TRAF1 is an important receptor interacting protein that forms a complex with TRAF2 to transduce TNFα-mediated activation of MAPK and NF-κB[282] and TRAF2 was reported to be an important determinant for SM-induced degradation of cIAP1/2[150]. We have shown previously that HIV-Vpr targets TRAF1 and TRAF2 for proteosomal degradation and the downstream effect ultimately leads to mitochondrial outer membrane polarization (MOMP) and apoptosis of monocytes[186]. Induction of cIAP1/2 protects monocytes from cell death by preventing Vpr-induced TRAF1/2 degradation, while SM-mediated degradation of cIAP1/2 abrogates this effect[186]. Therefore, I wanted to investigate the regulation of TRAF1 and TRAF2 in infected Mϕ after treatment with SM. HIV infection downregulated the expression of TRAF1, while TRAF2 was unaffected. Additionally, SM-treatment of HIV-infected Mϕ with SM resulted in the downregulation of TRAF1 while sparing TRAF2. Although these observed effects were exclusive

to TRAF1 and not TRAF2, it is likely that the SM-induced killing of HIV-infected Mϕ is similar to Vpr-induced killing of SM-treated Mϕ [185,238].Keeping in view of the previous finding that SM did not inhibit virus replication and that HIV-Vpr is one of the early genes expressed in virus life cycle, the interplay between Vpr and SM-mediated effect may lead to cell death of HIV-infected Mϕ. It is also of interest to determine how other accessory proteins such as Nef, Vif, and Vpu expressed by HIV-1 may influence the SM-induced killing of infected macrophages since these proteins have been described to play a role in ensuring survival of the virus[283].

Bid is a pro-apoptotic molecule localized in an inactive form in the cytosol which becomes activated by proteolytic cleavage of active caspase-8 [97]. The downregulation of Bid is consistent with the increase in caspase-8 activation in HIV-infected Mϕ after SM treatment. Upon activation, cleaved Bid translocates in the mitochondrial and forms a complex with Bax to disrupt the integrity of the organelle to release apoptogenic factors that would lead to caspase-3 activation as demonstrated in the results above. The functional relevance of the modulation of these apoptosis related genes in response to SM-treatment of HIV-infected Mϕ warrants further investigation. Whether SM-treatment of infected macrophages lead mitochondrial depolarization remain to be elucidated.

Given that SM LCL161 did not induce cell death in uninfected Mϕ, and HIV-infection of Mϕ resulted in downregulation and inactivation of RIP, I hypothesized that inactivation of RIPK1 and degradation of IAPs in the same cell would result in induction of apoptosis. Necrostatin-1 treatment of Mϕ did not induce significant cell death suggesting that RIPK1 is dispensable in steady-state survival of Mϕ. As per previous experiments, SM LCL161 had no significant difference in the levels of cell death compared to vehicle control**.** However, the inactivation of RIPK1 by necrostatin-1 following IAP degradation by SM resulted in a dramatic increase in cell

death of healthy macrophages. This suggests that RIP-1 plays a key role in SM-induced selective killing of HIV-infected Mϕ**.** Western immunoblots show that treatment with necrostatin-1 and SM lead to apoptosis of Mϕ, as main caspases involved in the process, caspase-3, 8, and 9 are activated. The role of RIPK1 degradation during HIV-1 infection of Mϕ needs further investigation. Overall, my results suggest that modulation of the IAP-associated signalling pathway may be a possible strategy for clearing HIV-1 infected Mϕ.

Figure 21. Proposed model for SM-induced apoptosis of HIV-infected Mϕ. (A) In steady state, RIPK1, TRAF1/2, cIAP1/2 form a complex that mediate survival signalling. cIAP1/2 and dispensable host factors for survival of Mϕ**. (B)** HIV- and necrostatin-1-mediated RIPK1 inhibition does not affect survival of Mϕ. **(C)** Inhibition of RIPK1 via necrostain-1 or HIV-infection coupled with SM treatment results in cell death of Mϕ.

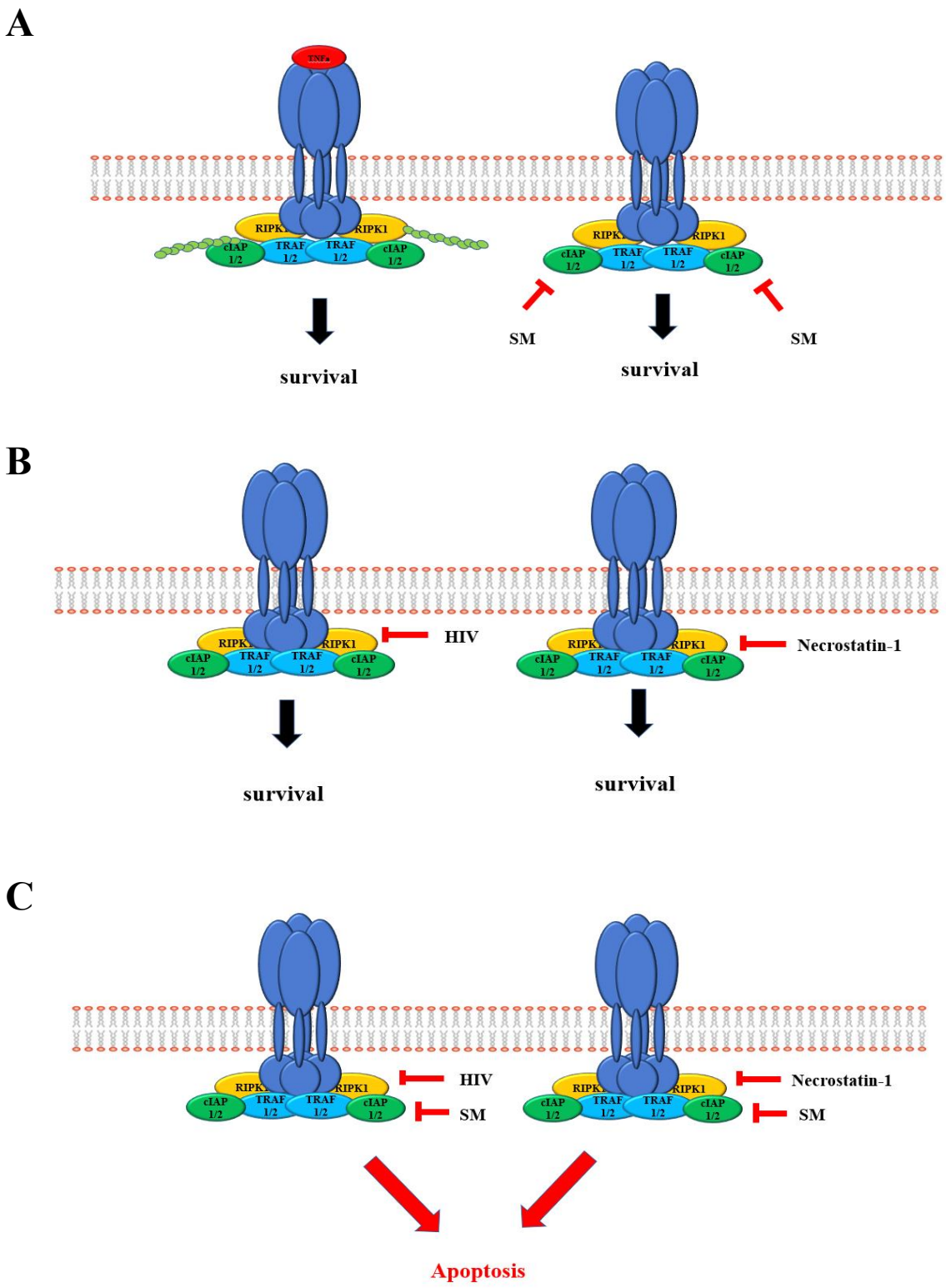

A
TNFα
RIPK1
RIPK1
cIAP 1/2
TRAF 1/2
TRAF 1/2
cIAP 1/2
survival
SM
SM
survival
B
HIV
Necrostatin-1
survival
survival
C
HIV
SM
Necrostatin-1
SM
Apoptosis

Figure 21

Chapter 6: Concluding remarks and future directions

Mϕ are important reservoirs of HIV-1 due to the intrinsic traits of these cell that benefit the virus for the establishment of latent infection. Infected Mϕ remain one of the major roadblocks that limit cure for the infection. Herein, I have demonstrated that IAPs may be a possible candidate for developing sterilizing cure of HIV-1, as targeting of these family of proteins by SM leads to the induction of infected Mϕ. The results from the first aim of the project demonstrated the selectivity of SM LCL161 in killing infected myeloid cells such as chronically infected U1 cell, *in vitro* infected Mϕ, and *ex vivo* derived HIV-infected Mϕ from HIV+ individuals compared to mock or healthy control. The second aim delved into the elucidation of mechanisms that underlie the SM-mediated killing of HIV-infected Mϕ. I showed that treatment of SM led to induction and activation of apoptosis through caspase activation, which is independent of TNFα. Moreover, the killing occurred solely through apoptosis and not necroptosis. In addition, the selectivity of SM in eliminating infected macrophages was independent of endogenously produced cytokines, suggesting that the observed induction of apoptosis was independent of extrinsic pathway of apoptosis. SM-induced killing may be linked to the dysregulation of anti- and pro-apoptotic genes and other signalling molecules such as TRAF1, Bid, and particularly RIPK1. The loss of RIPK1 activity coupled by degradation of IAPs by SM in healthy macrophages induced suggesting a role for RIPK1 in SM-induced cell death of HIV-infected Mϕ **(Figure 21)**.

Despite the progress that has been developed in this project, there are still important issues that must be addressed. HIV-1 codes for structural and regulatory proteins that influence the pathogenesis of the virus within the host[284]. HIV glycoproteins can induce cell death of both uninfected and infected T cells and monocytes/macrophages[69,285], while accessory protein such as

Nef protects Mϕ from HIV-induced apoptosis[286]. The interplay between the proteins coded by HIV-1 and SM are still unknown and warrant further investigation.

Another avenue for future investigation in this project is the elucidation of the downstream effect of RIPK1 loss in HIV-1 macrophages. Given that RIPK1 regulates a number of cellular processes related to immune signalling, it is possible that RIPK1 modulation may be a possible mechanism related to formation of reservoirs in macrophages. The function of HIV-1 protease was implied in the degradation of RIPK1[219] and inhibitor of this viral enzyme is available which can be used to further elucidate the downstream effect of RIPK1-loss in macrophages.

As mentioned above, over the recent years there have been increasing number of reports that show the potential of SM as latency reversal agents in cell lines and latently infected primary $CD4^+$ T cells. Although I have shown that SM did not induce virus replication in either U1 or HIV-infected macrophages, the mechanism the governed this effect was not elucidated. Therefore, it would be interesting to understand the discrepancy in the effect of SM in virus replication between $CD4^+$ T cells and macrophages.

Even though SM has well tolerated safety profile, it has often been associated with the generation of increase cytokine secretion that may limit its use as a therapeutic agent. Given that other important cytokines in response to virus infection was not assessed, such as IFNα and IFN β, both of which have been implicated in SM-induced killing of cancer cells, its important to determine whether there would be aberrant cytokine productions associated with the treatment *in vivo*. Most importantly, the efficacy of SM in clearing HIV-1 infection *in vivo* must be determined. One way to address this issue is to utilize humanized BLT mice that has been reconstituted with human immune cells that can support productive viral infection. It would be ideal to determine

whether SM could lower viral load of the virus as well as the number of HIV reservoirs within the humanized mouse model.

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Contributions of collaborators

The generation of *ex vivo* derived HIV-infected Mϕ and the experiments depicted on Figures 4, 7, 8, 9, and 19 were conducted and analyzed by Niranjala Gajanayaka.

Appendices

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Figure A. Induction and regulation of extrinsic and intrinsic pathways of apoptosis

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Figure B. Visual representation of IAP family of proteins

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Figure C. Schematic representation of the known function of IAP and its regulation by SM

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