# Antiapoptotic proteins in human macrophage survival, differentiation, innate immunity and protection from HIV-induced apoptosis

by

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## Abstract

Macrophages represent long lived immune cells that are remarkably resistant to apoptosis, which allows them to perform in highly stressful environments. Apoptosis resistance is a characteristic that develops during the differentiation process from monocytes to macrophages. However, the signaling pathways that mediate the development of macrophage antiapoptotic phenotype during differentiation remain mostly unknown. Because of their decreased susceptibility to cell death, macrophages are also key viral reservoirs during HIV infection. My research aims to understand the molecular mechanisms and signaling pathways that mediate cell survival during and after monocyte to macrophage differentiation and the involvement of the main families of antiapoptotic proteins, IAPs (inhibitors of apoptosis) and Bcl2 in this process. HIV accessory protein Vpr was used as an apoptotic stimulus, due to its death inducing abilities in other cell types.

My results show that survival of macrophages is distinctively regulated during and after differentiation. I have identified a signaling pathway consisting of PI3K/Akt activation of NFκB that is important in survival of differentiating macrophages by specifically sustaining antiapoptotic Bcl-xL expression. However, once differentiated, Mcl-1, but not Bcl-xL is dependent on PI3K/Akt activation. Moreover, differentiated macrophages are resistant to the effect of HIV-Vpr, which is highly apoptotic for monocytes. In contrast, resistance to HIV-Vpr induced apoptosis of human macrophages is specifically mediated by antiapoptotic IAP proteins, with no involvement of the Bcl2 family, which maintains macrophage viability in the absence of any apoptotic stimuli.

In addition to their antiapoptotic properties, IAPs are also important regulators of macrophage function. By using chemical compounds (SMAC mimetics) that target IAPs for degradation, I have shown that IAPs positively modulate LPS-induced IL10, IL-27 and MIG (monokine induced by IFNγ) production in human macrophages, by promoting TRAF2, JNK and p38 signaling and NFκB activation. In addition, IAPs also contribute to LPS-induction of CD80/CD86 costimulatory molecules.

Overall, my results suggest that both IAPs and Bcl2 families contribute to survival of human macrophages and that IAPs are also involved in innate immune responses. Unraveling the mechanisms that control macrophage survival and function in various settings would provide therapeutic strategies aimed at eliminating cells when their survival is no longer beneficial for the host, as in the case of HIV infection or autoimmune diseases.

## Acknowledgements

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I am grateful to my parents for their unconditional love, constant support and encouragement that got me this far. I only hope that one day I can be just as great a role model for my children.

To my husband, his patient love and encouragement through long and sometimes failed experiments enabled me to complete this work.

To my beautiful infant daughter Alexandrina, for being my inspiration throughout the process of thesis writing and all the work that will follow from now on.

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## List of abbreviations

| | |
|---|---|
| AIDS | Acquired immune deficiency syndrome |
| AIF | Apoptosis inducing factor |
| ANT | Adenine nucleotide translocator |
| Bcl2 | B-cell lymphoma 2 |
| BIR | Baculoviral IAP repeat |
| Cdc | Cell division cycle |
| Cdk | Cyclin-dependent kinase |
| cIAP | Cellular inhibitor of apoptosis |
| ERK | Extracellular signal-regulated kinases |
| FLICE | Fas-associated death domain (FADD)-like IL-1β converting enzyme |
| FLIP | The FADD-like IL-1β converting enzyme (FLICE) inhibitory protein |
| G-CSF | Granulocyte colony-stimulating factor |
| HIV | Human immunodeficiency virus |
| IAP | Inhibitor of apoptosis |
| IL | Interleukin |
| IFNγ | Interferon gamma |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| Mcl-1 | myeloid cell leukemia 1 |
| M-CSF | Macrophage colony-stimulating factor |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NIK | NFκB inducing kinase |

| | |
|---|---|
| NFκB | Nuclear factor kappa-light-chain enhancer of activated B cells |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PI | Propidium iodide |
| PI3K | Phosphatidylinositol-3-kinase |
| PMA | Phorbol myristate acetate |
| PTPC | Permeabilization transition pore complex |
| RING | Really interesting new gene |
| RIP1 | Receptor interacting protein 1 |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| siRNA | Small-interfering ribonucleic acid |
| SMAC | Second mitochondria-derived activator of caspases |
| SMM | Smac mimetic |
| STAT | Signal transducers and activators of transcription |
| Tat | Trans-activator of transcription |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor alpha |
| TRAIL | TNF-related apoptosis inducing ligand |
| TRAF | TNF receptor-associated factor |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| TIR | The Toll/Interleukin-1 receptor |
| XIAP | X-chromosome-linked IAP |

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## General introduction:

Monocytes are a very plastic cell type originating from myeloid precursors that circulate in the bloodstream in steady state or during infection. Depending on the local inflammatory milieu and pathogen-associated pattern-recognition receptors, monocytes leave the bloodstream and migrate to various sites, where they differentiate into macrophages, dendritic cells and osteoclasts programmed to perform tissue specific functions (1). Macrophages are key players in innate immunity due to their ability to internalize and digest extracellular bacteria and apoptotic cells during infection; they also produce inflammatory mediators that directly kill microorganisms or indirectly activate other cell types (2, 3). Processing and presentation of antigens also contribute to T cell activation, with direct consequences on the development of both the humoral and cell-mediated immunity.

Because of their crucial role in immunity, regulation of monocyte/macrophage life span is important in both physiological and pathological processes. Resting macrophages have a long half life of months, compared to monocytes which undergo spontaneous apoptosis within days (4). Inflammation can prolong the macrophage life span due to increased secretion of survival factors (5). However, activated macrophages can undergo activation induced apoptosis (6) at the inflammation site, which can also contribute to the pathogenesis of inflammatory conditions, such as atherosclerosis (7). Macrophage survival is of particular interest in HIV infection, because they are not susceptible to the HIV cytopathic effects typical of infected CD4+ T lymphocytes and they survive the active viral replication, continuing to be sources of viral progeny during the late phases of infection, when CD4+ T cells numbers are low (8).

Bcl2 (B cell lymphoma 2) and IAPs (inhibitors of apoptosis) are the main classes of antiapoptotic proteins that act at different stages throughout caspase activation to counteract death inducing signals and prevent over-activation of the apoptotic machinery (9, 10). IAPs are also implicated in regulation of innate immunity, as they mediate TNF-α-induced NFκB activation (11).

The role of these two families in macrophage survival and function is the focus of my project. Understanding apoptosis regulation has a direct impact on therapeutic strategies - IAP and Bcl2 antagonists are currently being developed as cell death inducing therapies in cancer cells (12, 13). The results of this study may indicate the use of these antagonists as a new strategy of eliminating macrophages when their survival is no longer beneficial, due to their impact on the apoptotic machinery.

**Apoptosis overview**

Cellular demise can take the form of three broad types of programmed cell death (PCD) that is genetically encoded and leads to cell suicide. Type I cell death is known as apoptosis and is morphologically characterized by chromatin condensation and cell shrinkage, without extracellular release of cytoplasmic material. Autophagy or type 2 PCD is distinguished by the formation of double membrane vacuoles inside the cell and can result in either cell survival or demise. Necroptosis or type 3 PCD is a more rapid form of cell death that results in loss of membrane integrity and release of intracellular contents, which can promote an immune response (14). The recently described pyroptosis is another form of cell death that includes caspase-1 dependent release of pro-inflammatory cytokines IL-1β, IL-18, and IL-33, in addition to DNA condensation (15).

Apoptosis is a self-destructive cellular process important in tissue development and immune regulation that generally culminates with the sequential activation of caspases, the cysteine proteases responsible for cleavage of specific proteins that ultimately results in cellular demise (16). Classically, there are two pathways that can result in apoptosis depending on the origin of the apoptotic signal. The extrinsic pathway is initiated by ligand binding to the molecules of the death receptor family, whereas the intrinsic pathway gets activated when damage of the mitochondrial membrane causes release of cytochrome-c in the cytosol and subsequent caspase activation. Both pathways converge with the activation of effector caspases-3 and -7 (17).

There are three classes of antiapoptotic proteins that act at different stages throughout caspase activation to counteract death-inducing signals and prevent over-activation of the apoptotic machinery (Fig. 1).

1. The FADD-like IL-1β converting enzyme (FLICE) inhibitory protein (FLIP) is the main antiapoptotic mechanism in the extrinsic pathway. It prevents activation of caspase-8 (also known as FLICE) following ligation of death receptors like Fas and the TNF-related apoptosis inducing ligand (TRAIL) receptor and subsequent cleavage of effector caspases (e.g. caspase-3) (18). Due to structural homology with caspase-8, FLIP is able to bind and form FLIP-FLICE heterodimers that prevent subsequent activation of FLICE (19).

2. The antiapoptotic proteins of the Bcl2 family (e.g. Bcl-xL and Bcl2) maintain the integrity of the mitochondrial membrane and prevent activation of caspases due to cytochrome-c release (20, 21). The Bcl2 family also contains proapoptotic members such as Bax and Bak that promote apoptosis by binding and inactivating their antiapoptotic counterparts (22). The balance between the two groups ultimately dictates cell fate.

**Figure 1. Overview of apoptosis and the main antiapoptotic molecules**

Binding of death receptors (Fas, TNF-R, TRAIL-R) to their ligands (Fas-L, TNF, TRAIL) initiates the extrinsic apoptotic pathway. Association of adaptor molecules (FADD - Fas-associated death domain protein, TRADD–TNF receptor associated death domain protein) induces the formation of DISC (death inducing signaling complex), which activates caspase-8 and subsequently caspase-3. FLIP is the main antiapoptotic molecule of this pathway, as it prevents caspase-8 activation. Release of cytochrome-c from the mitochondria in response to cellular stress initiates the intrinsic apoptotic pathway**.** Cytochrome-c associates with Apaf-1 and procaspase-9 to form the apoptosome, a multimeric protein complex that induces cleavage of inactive caspase-9 to its active form. Both pathways converge with activation of caspase-3, the main effector caspase. The two pathways are also connected by the ability of active caspase-8 to activate Bid (BH3-interacting domain death agonist). tBid (truncated Bid) is a proapoptotic molecule that can induce release of cytochrome-c from the mitochondria and thus initiate the intrinsic pathway. The two main antiapoptotic families are Bcl2 and IAPs. Antiapoptotic Bcl2 members maintain mitochondrial integrity, while IAPs can inactivate caspases. IAP activity is antagonized by second mitochondria-derived activator of caspase (Smac), a proapoptotic molecule released from the mitochondria.

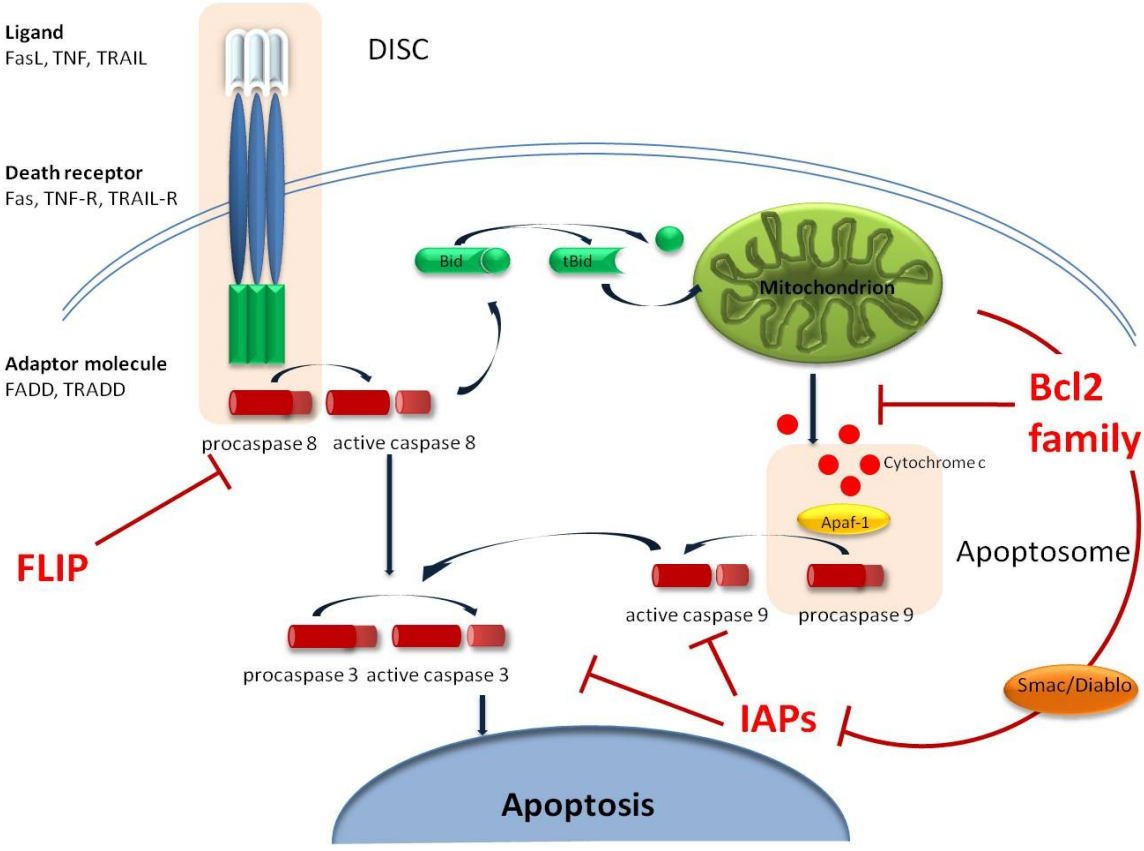

Ligand
FasL, TNF, TRAIL
DISC
Death receptor
Fas, TNF-R, TRAIL-R
Adaptor molecule
FADD, TRADD
Bid
tBid
Mitochondrion
Bcl2 family
procaspase 8
active caspase 8
Cytochrome c
Apaf-1
Apoptosome
FLIP
active caspase 9
procaspase 9
procaspase 3
active caspase 3
Smac/Diablo
IAPs
Apoptosis

**Figure 1**

3. The third group comprises the family of inhibitors of apoptosis (IAPs) proteins. These proteins are major regulators of cell survival because they act on caspases activated either through the extrinsic or the intrinsic pathway. Initially discovered in baculoviruses as an *iap* gene (23), there are now eight mammalian IAPs: cellular IAP1 (cIAP1), cIAP2, X-chromosome-linked IAP (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin, livin, IAP-like protein 2 (ILP2) and baculovirus inhibitor of apoptosis repeat containing ubiquitin-conjugating enzyme (BRUCE) (24). IAPs share variable numbers of baculoviral IAP repeat (BIR) motifs, structural domains that are important for binding and inactivation of both initiator and effector caspases (9, 25).

**Bcl2 family – overview and role in apoptosis**

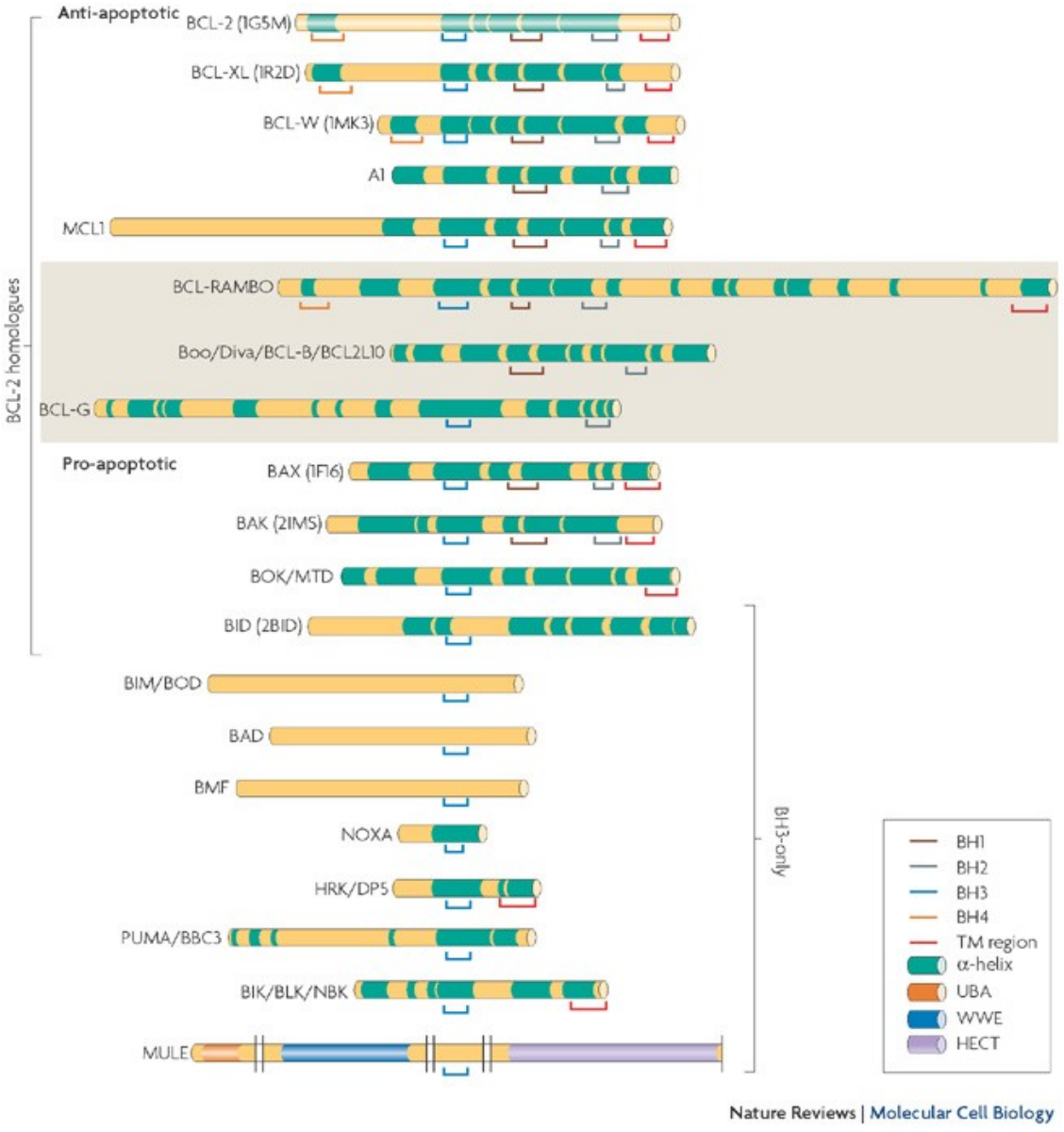
Bcl2 (B-cell lymphoma-2) gene was the first member of the family to be discovered in B-cell follicular lymphomas over 25 years ago (26). Soon after its discovery, it was reported that it promoted carcinogenesis by inhibition of apoptosis, rather than induction of proliferation, like other oncogenes (27). Apart from its importance in tumor progression, Bcl2 roles also include physiological programmed cell death, organogenesis and host defense (28). There are currently 20 members of this family, with common structural motifs called Bcl2 homology (BH) domains (Fig 2). Functionally, the family comprises three groups of proteins: one that inhibits apoptosis, another that promotes cell death and a regulatory group that indirectly induces cellular demise (the BH3 only proteins). The main antiapoptotic proteins (Bcl2, Bcl-xL, Bcl-W, A1 and Mcl-1) contain up to four BH domains (BH1-4), whereas the BH3 group (Bad, Bid, NOXA, PUMA) have one conserved BH3 domain used for protein interaction (28).

Proapoptotic Bcl2 members (Bax, Bak, Bok) are important in activating the intrinsic pathway of apoptosis, due to their ability to form pores in the mitochondrial membrane, thus causing its permeabilization and subsequent release of cytochrome-c and caspase activation. BH3 only proteins also promote mitochondrial permeabilization. There are two mechanisms that explain their effect: they either directly activate proapoptotic Bax and Bak or they bind and inactivate antiapoptotic molecules, thus indirectly de-repressing Bax and Bak pore-forming action (29). Membrane permeabilization is initiated by proapoptotic Bcl2 members. Located in the cytosol of healthy cells, Bax undergoes major conformational changes and translocates to the mitochondria upon induction of apoptosis. These changes include an epitope exposure of the N-terminus than can be detected by a specific activation antibody (30) and insertion into the mitochondrial outer membrane in such a way that transmembrane domains of multiple Bax monomers make up an oligomeric pore (31). Therefore, Bax oligomerization and pore formation happen after membrane insertion.

Classically, antiapoptotic Bcl2 proteins protect against cell death by binding and inactivating proapoptotic Bax or Bak, thus maintaining mitochondrial integrity (32). In order to perform this function, Bcl2 and Bcl-xL also undergo conformational changes, in a manner similar to their proapoptotic counterparts. Unlike Bax, Bcl2 is anchored in the mitochondrial membrane, with the rest of the peptide situated on the cytoplasmic side. When apoptosis is triggered, it changes into a multispanning conformation that includes insertion of multiple domains into the lipid bilayer. Once in the transmembrane conformation, the pore-forming regions of Bax and Bcl2 interact and thus Bax oligomerization is prevented (33). These protein interactions have different specificities, with Mcl-1 and Bcl-xL, and not Bcl2 binding to Bak (34) and all antiapoptotic proteins targeting Bax.

**Figure 2. Bcl2 family of proteins**

Green bars depict α-helical segments. Red lines label transmembrane (TM) domains. Sequence homologies of the BH1 (brown lines), BH2 (grey lines), BH3 (blue lines) and BH4 (orange lines) regions are shown. The upper five proteins (BCL-2, BCL-XL, BCL-W, A1 and MCL1) are generally antiapoptotic. The three proteins in the shaded area are less well studied and cannot be categorized at this time. The lower 12 proteins are considered to be proapoptotic. MULE contains a ubiquitin-associated domain (UBA), the Trp-Trp-Glu interaction module (WWE) and a HECT ubiquitin ligase domain. BID has a unique role as both a BCL-2 homologue and a BH3-only protein and links the intrinsic and extrinsic apoptosis pathways. BIM (also known as BOD), BAD and BMF are unstructured proteins.




Nat Rev Mol Cell Biol. 2008 Jan;9(1):47-59.

**Figure 2**

Bcl-xL antiapoptotic protein is translated from the larger mRNA that results from the alternative splicing of the *bcl-x* gene. The shorter product gives rise to Bcl-xS, a protein with proapoptotic functions (35). Bcl-xL is located in the cytosol as homodimers in healthy cells. Upon induction of apoptosis, Bcl-xL translocates to the mitochondrial membrane, where it interacts with Bax via its hydrophobic domain that binds BH3 fragment of Bax. Thus, heterodimer formation prevents Bax pore-forming function (36).

Mcl-1 was initially identified in a myeloid leukemia cell line during PMA differentiation along the monocytic lineage (37). Structurally, it has BH 1-3 domains and a C-terminus transmembrane domain that serves as a localization signal to the mitochondrial membrane, similarly to Bcl2 and Bcl-xL. Unlike these two, it lacks BH4 in the N-terminus (38). Mcl-1 expression is rapidly induced by a number of cytokines and survival signals (39), but it has a short half-life (of hours), following which it is degraded via the proteosomal machinery. Its rapid increase is thought to confer early protection against apoptosis of hematopoietic precursors (40) and in the early stages of embryogenesis (41). Its mechanism of action involves heterodimer formation with proapoptotic Bcl2 proteins such as Bak to prevent mitochondrial permeabilization (42). Caspase-3 can also cleave Mcl-1 to produce a proapoptotic C-terminus domain, therefore creating a positive regulatory loop that promotes cell death (43).

A1 was discovered as an early response gene induced by GM-CSF in murine hematopoietic cell lineages, including myeloid precursors and macrophages (44). A1 is expressed in multiple cell types, such as granulocytes (45), plasmacytoid dendritic cells (46), activated T cells (47) and endothelial cells (48), where it displays antiapoptotic properties. Macrophages have a constitutive level of A1 expression that confers steady state survival

(49), but A1 expression is also induced during inflammatory responses following infection with intracellular pathogens (50, 51) or LPS stimulation (44).

Antiapoptotic Bcl-w protein is present in multiple organs, in myeloid cell lines and few lymphoid lines (52). However, Bcl-w knockout mice display normal hematopoiesis but defective spermatogenesis (53) and sensory neuropathy (54), indicating the involvement of Bcl-w in maintaining viability of germ cells and sensory neurons respectively. The mechanism of Bcl-w induced protection is complex formation with proapoptotic factors Bax and Bak (55).

Due to their effect on cell death inhibition, overexpression of antiapoptotic Bcl2 proteins has been established as a major mechanism of tumor formation, metastasis and chemoresistance in cancer research (56). With the discovery of small BH3-like molecules that have the ability to bind and inhibit various Bcl2 members, a novel therapeutic opportunity has emerged. Bcl2 inhibitors (57) and the recently discovered Bcl-xL inhibitors (58) prevent protein-protein interactions by binding and inactivating antiapoptotic Bcl2 and Bcl-xL molecules, which allows for apoptosis induction in the absence of direct mitochondrial damage (13).

**Role of antiapoptotic Bcl2 proteins in hematopoiesis**

Antiapoptotic members of the Bcl2 family are differentially implicated in hematopoiesis of the myeloid lineage. Granulocytes and monocytes/macrophages are two distinct lineages that originate from a common myeloid precursor. *In vitro* studies with CD34+ progenitor cells (59) and the promonocytic cell line HL60 (60) induced to differentiate by chemical agents revealed an increased expression of Bcl-xL in cells committed to the monocyte/macrophage lineage, but not when cells were induced to

differentiate to granulocytes. Bcl-xL upregulation throughout the monocytic lineage is accompanied by downregulation of antiapoptotic Bcl2 protein (61-63) suggesting divergent roles among antiapoptotic members of this family in determining the enhanced lifespan of monocytes over granulocytes.

Differential involvement of Bcl2 and Bcl-xL in hematopoiesis is also illustrated in mouse model studies. Bcl-xL knockout mice die during embryogenesis with massive apoptosis of cells of the hematopoietic and central nervous systems (64). In contrast, Bcl2 knockout mice are born with organ malformations but they survive without major disruptions in hematopoiesis (65). These studies suggest that while Bcl2 is necessary for normal morphogenesis, Bcl-xL is vital for hematopoiesis. Interestingly, when macrophages are obtained from immature bone marrow precursors cultured in the presence of M-CSF, Bcl2 expression shows a different pattern, being upregulated in both human (66) and mouse models (66, 67). Although the expression of Bcl-xL was not examined in these studies, one possible explanation for these divergent results would be that immature bone marrow precursors are highly susceptible to apoptosis and require M-CSF for survival, which may trigger a different pattern of antiapoptotic genes expression in order to overcome higher susceptibility to apoptosis.

Antiapoptotic Mcl-1 has been linked with survival of hematopoietic precursors and lymphocyte development. Studies using mice with conditional deletions for Mcl-1 in various populations have established a requirement for Mcl-1 in survival of hematopoietic stem cells (68), B and T lymphocytes (69). Mcl-1 is also involved in myelopoiesis, by promoting survival of granulocytes (70) and monocytes (71). Mouse data have also confirmed the importance of Mcl-1 in neutrophil survival, with no impact on macrophage numbers in vivo

(72); however, macrophage effector function was defective, with impaired phagocytic activity during bacterial infection (73).

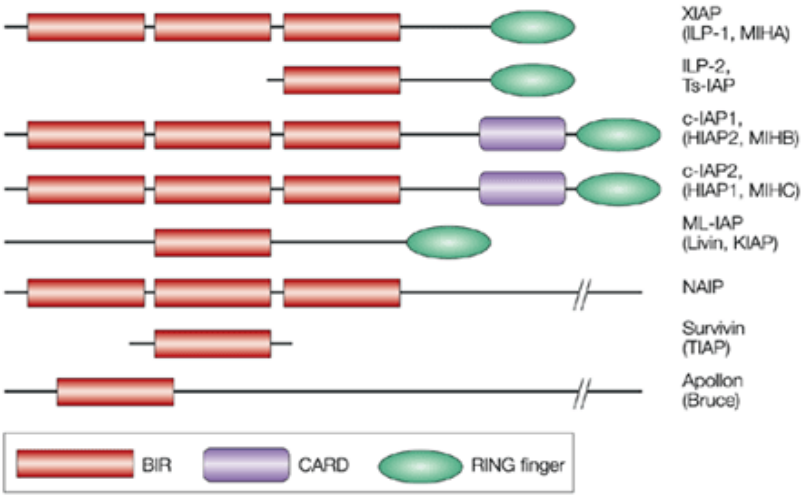
**IAP family - overview and role in apoptosis**

IAP gene was initially discovered in baculovirus infected insect cells, were it was able to prevent actinomycin D-induced apoptosis (74). IAPs contain up to three common structural motifs named Baculoviral IAP Repeat (BIR) in their N-terminus. BIRs are 70 aa long domains with three anti-parallel beta-sheet and four alpha helices that contribute to protein-protein interactions (75). Their binding specificities vary, with BIR3 of XIAP inhibiting caspase-9 whereas BIR1 and 2 inactivate caspases-3 and -7 (76). More specifically, a groove on BIR2 binds to a terminal region generated at the N-terminus of caspase-3 following its cleavage and activation (77). In addition to BIRs, there is also a CARD (caspase-associated recruitment domain) and a RING domain in the C-terminus, with the roles of protein interaction and ubiquitin ligase activity respectively (24).

XIAP, cIAP1, and cIAP2 are the most studied of all eight mammalian IAPs (Fig.3). Overexpression studies have shown that IAPs main function is prevention of apoptosis. Initial reports have shown that all three IAPs bind and effectively inhibit executioner caspases-3,-7 and -9 (76, 78). However, subsequent experiments demonstrated that XIAP is the only IAP that can effectively inhibit caspase enzymatic activity (79). Although cIAP1 and 2 can bind caspases, the physiological consequence of this effect is unknown and the mechanism by which they protect against apoptosis is still unclear (80). Two mechanisms of caspase inactivation have been described: direct binding and inhibition of the enzymatic active site, which has been described for XIAP, and polyubiquitination and subsequent caspase proteosomal degradation, as in the case of XIAP (81) and cIAP2 (82).

**Figure 3. IAP family of proteins**

The X-linked IAP (XIAP), cIAP1 and cIAP2 are the best-characterized members of this family. BIR, baculovirus IAP repeat; CARD, caspase-recruitment domain; ILP, IAP-like protein; MIHA, mammalian IAP homologue A; NAIP, neuronal apoptosis inhibitory protein.




Nat Rev Mol Cell Biol. 2002 Jun;3(6):401-10.

**Figure 3**

Caspase ubiquitination via the RING domain may explain the antiapoptotic properties of cIAP1/2 in the absence of direct caspase inhibition.

**Smac and Smac mimetics**

Another effect that promotes cell survival is the ability of IAPs to bind and inactivate mitochondrial proapoptotic molecules, such as Smac (second mitochondria-derived activator of caspase). Smac is an IAP binding protein released from the mitochondria in response to apoptotic stimuli that promotes cell death by binding and inactivating IAPs (83). Smac binds to BIR3 motif of XIAP in a manner that displaces caspase-9 from XIAP interaction and relieves caspase inhibition. More specifically, the last N-terminal four residues (Ala-Val-Pro-Ile or AVPI) of Smac bind to a surface groove on BIR3 of XIAP (84). Smac can also bind to XIAP in a dimeric form, binding concomitantly to BIR2 and 3 and releasing caspases-3 and -7 in addition to caspase-9 (85).

Recent studies have also uncovered a role of Smac in receptor-mediated apoptosis. TRAIL and Fas-induced complete activation of caspase-3 seems to require two signals: one is cleavage and activation of caspase-8, the initiator caspase of the extrinsic pathway, and another is a mitochondrial signal, independent of cytochrome-c or caspase-9. This second signal, required to relieve caspase-3 from binding XIAP, has been identified as Smac (86, 87). This model is in agreement with earlier reports that caspase-8 activation is amplified by the mitochondria (88).

Due to their apoptosis inhibition properties, IAPs levels have been implicated in carcinogenesis, with high levels expressed in malignancies such as leukemias and lymphomas. Their expression has been correlated with poor prognosis or resistance to chemotherapy (89). In view of the mechanistic insight into XIAP interaction with the AVPI

domain of Smac, there has been great interest in designing chemical compounds that would prevent this interaction and relieve caspases from XIAP control, in order to promote cell death. Smac mimetics are small molecules that mimic the AVPI tetrapeptide in its binding to XIAP BIR3 domain, thus inducing cell death in the absence of mitochondrial release of apoptotic factors (12). Both Smac mimetics (90-94) and Bcl-2 family inhibitors (95-97) are intensely studied as potential antineoplasic drugs.

These XIAP inhibitors promote caspase activation and induce cell death as single agents of multiple tumor cell lines (92, 93, 98), they can sensitize resistant cell lines to chemotherapy agents (99, 100), or suppress tumor growth in various xenograft mice models (98). Since Smac mimetics had been designed to target XIAP-caspase-9 interaction and induce cell death via caspase-9 activation, the mechanism of Smac mimetics-induced cell death was surprisingly shown to depend on caspase-8 activation, instead of caspase-9. Three independent reports have demonstrated that Smac mimetics induced a rapid and dramatic proteosomal degradation of cIAP1/2 and activation of caspase-8 in addition to caspases-3 and -9. In agreement with extrinsic pathway activation, these experiments demonstrated that Smac inhibition caused TNF-α dependent cell death, since both caspase-8 knockdown and TNF-α blockade were able to prevent this effect and protect cells from Smac mimetics-induced cell death (91-93).

**Role of IAPs in signal transduction**

cIAP1/2 were initially identified as part of the TNF receptor signaling complex, as binding partners of adaptor proteins TRAF1 and TRAF2 (101). cIAPs regulate signaling through the TNF receptor 1 (TNF-R1), which can promote both cell death and cell survival. Ligation of TNF to TNF-R1 leads to recruitment of TRADD (TNF receptor associated death

domain), RIP1 (receptor interacting protein 1) and TRAF2 (TNF associated factor 2). The association of these molecules makes up complex I, which further activates NFκB and promotes cell survival. A second signaling complex, complex II, is formed when TRADD, TRAF2 and RIP1 associate instead with FADD (Fas associated death domain) and caspase-8 to promote caspase activation and cellular demise. Formation of complex II is prevented by the antiapoptotic molecule FLIP (102, 103).

Being bound to TRAF2, cIAPs are also engaged during complex I assembly and TNF-α induced activation of NFκB. Their role is to maintain RIP1 in an ubiquitinated state and thus prevent complex II formation. RIP1 ubiquitination promotes recruitment of the NFκB regulatory protein NEMO to the TNF-R1 complex, with the final outcome of IκBα phosphorylation and NFκB activation through the classical pathway (103). As a result, downregulation of both cIAP1 and 2 leads to a severe impairment of NFκB activation in response to TNF-α. Moreover, cells become sensitive to TNF-α-induced apoptosis (11, 104).

cIAPs are also negative regulators of non-canonical NFκB signaling. Through their RING domains they serve as ubiquitin ligases responsible for proteosomal degradation of NIK, the kinase responsible for non-classical NFκB activation (91). As a consequence of cIAP1/2 degradation, IAPs antagonist treatment leads to NIK accumulation and to increased p100 processing to its mature form p52, the active subunit of non-canonical NFκB signaling (91, 92).

Since cIAP1 and 2 are positive regulators of the canonical pathway and inhibitors of the alternative NFκB signaling, it was expected that Smac mimetics would have the opposite effect, in the absence of cIAPs. Surprisingly, IAP antagonists were shown to activate both pathways of NFκB signaling, with a requirement for canonical pathway for TNF-α secretion

and cell death (91, 92). It would seem that in the absence of cIAPs, NFκB turns from a pro-survival pathway into one that promotes cell death via TNF-α. Therefore, the mechanism of cell death induced by Smac mimetics is an indirect effect of cIAP1/2 downregulation, which leads to TNF-α secretion and receptor mediated apoptosis, rather than de-repression of caspases from XIAP blockage.

**Role of IAPs in innate immunity**

IAPs involvement in innate immunity has been addressed in knockout mouse models. Thus, macrophages of cIAP2 null mice display an impaired inflammatory response following LPS treatment, which renders these mice resistant to LPS-induced endotoxic shock (105). In the absence of cIAP2, macrophages become sensitive to the apoptotic effect of LPS-induced proinflammatory environment (105). Similarly, XIAP null mice have a diminished cytokine production and NFκB activation following infection with *Listeria monocytogenes* (106) and cIAP1 was involved in innate immune responses that control *Chlamydia pneumoniae* infection (107).

cIAP1/2 are also associated with TLR4 and CD40L signaling complexes, where they serve as ubiquitin ligases that promote degradation of adaptor proteins such as TRAF3 (108, 109). As a result, cIAP ablation via Smac mimetic treatment results in diminished cytokine production in response to LPS, with no effect on interferon responses, indicating a selective involvement of IAPs in cytokine production and not in type I interferon secretion (108).

cIAPs have also been implicated in adequate antiviral response via RIG-I signaling. RIG-I and MDA5 are cytoplasmic pattern recognition receptors that recognize viral RNA via their RNA helicase domains. Viral RNA recognition leads to the recruitment of the mitochondrial adaptor protein MAVS, which plays a central role in type I interferon

responses via activation of NFκB and interferon regulatory factor (IRFs) 3 (110). MAVS interaction with TRAF3 is required for optimal antiviral effects (111). cIAPs role in this signaling cascade is to ubiquitinate and activate TRAF3 during viral infection, an effect that is essential for IRF3 activation (112).

In addition to RIG-I and TLR signaling, cIAPs have been linked to regulation of immune responses downstream of the nucleotide-binding oligomerization domain (NOD) receptors. NOD1 and NOD2 are pattern recognition receptors that recognize bacterial peptidoglycan. NOD oligomerization leads to NFκB and MAPK activation and ultimately to production of inflammatory mediators (113). RICK (also known as RIP2) is a critical downstream mediator of NOD signaling that is required for NFκB activation (114). Similarly to their role in promoting RIP1 ubiquitination in TNF signaling (94), cIAPs are required for ubiquitination of RIP2, which serves to recruit IKK complex and activate NFκB. As a result, cIAP depletion inhibited chemokine and cytokine secretion in response to NOD stimulation (115).

Most studies involving Smac mimetics and their impact on innate immunity and cell signaling have been carried out in cell lines or mouse models, therefore it is yet to be determined whether they affect non-cancerous human cells in the same manner. I will show that in primary human macrophages cIAPs downregulation does not have a major impact on cell survival in a steady state, but that it sensitizes them to apoptosis induced by HIV accessory protein Vpr. In contrast, Smac mimetic treatment does influence macrophage function and intracellular signaling in response to LPS and CD40L, two major macrophage activators.

**Antiapoptotic proteins and signaling pathways involved in macrophage differentiation**

As monocytes differentiate into macrophages, they also increase their resistance to spontaneous and induced apoptosis, a beneficial mechanism during immune responses against pathogens. Enhanced survival of macrophages is even more important in various pathological conditions in which cells of the monocytic lineage are key players, such as infections with intracellular viral and bacterial pathogens, inflammatory conditions and monocytic malignancies, where the enhanced survival of this cell type is no longer beneficial and becomes a main factor in disease pathogenesis. Apoptosis is a very important weapon of host immunity against intracellular pathogens like Human Immunodeficiency Virus (HIV) and *Mycobacterium tuberculosis*.

Apoptosis of infected cells serves several purposes: 1) killing or reducing the viability of intracellular pathogens, 2) preventing their dissemination 3) providing other antigen presenting cells (APCs) with microbial antigens in apoptotic bodies and 4) preventing persistence and formation of reservoirs (116). There is evidence to suggest that intracellular pathogens may evade apoptosis of infected monocytic cells by up regulating various host antiapoptotic genes that dysregulate both extrinsic and intrinsic apoptotic pathways in these cells (117).

Monocyte to macrophage differentiation is controlled by hematopoietic growth factor macrophage colony stimulating factor (M-CSF) and its receptor CSF-1R (also known as c-fms, M-CSFR, and CD115), expressed in monocytes, macrophages and mononuclear phagocyte precursors (118). Under the effect of M-CSF, monocytes stop their cell cycle progression, they become adherent and have an elongated, spindle-like appearance. They

gain antigen presenting functions and have increased secretory abilities, mainly by modulating genes involved in lipid and fatty acid metabolism (119, 120). However, overactivation of the cellular innate immune system can lead to inflammation and damage of the host tissue, which over time and in the presence of genetic susceptibility leads to inflammatory diseases, such as atherosclerosis and arthritis.

The differentiation process affects not only macrophage function, but also their susceptibility to apoptosis. Monocytes have a short lifespan when cultured *in vitro*. Even in the presence of growth factors, these cells survive only for a few days (121). However, differentiated macrophages become resistant to both spontaneous and induced apoptosis (122), a characteristic necessary for them to perform their functions in a stressful microenvironment while fighting invading pathogens.

Although an enhanced resistance to apoptosis of monocyte-derived macrophages (MDMs) is known for many years (123), the precise molecular mechanisms are still not fully elucidated. Unless activated by various stimuli such as inflammatory cytokines or growth factors, monocytic cells undergo spontaneous apoptosis when cultured *in vitro* (123-126). Activated monocytes are more resistant to various apoptotic stimuli such as death receptor ligands (Fas-L, TNF) (127, 128), reactive oxygen species (129), DNA damage (130), and proteasome inhibitors (131, 132). While the extrinsic apoptotic pathway was responsible for spontaneous apoptosis of monocytes through activation of the Fas-Fas ligand pathway, this interaction did not seem to be operating in macrophages, suggesting that protection is the result of events downstream of the death receptors (121).

To unravel the mechanisms of protection in activated monocytes, the apoptotic pathway affected by monocyte activation has been investigated. Perera *et al*. showed that

LPS activation induced the expression of antiapoptotic *Bfl-1* gene of the Bcl2 family and decreased caspase-8 expression, suggesting their role in enhanced survival of activated monocytes in response to various apoptotic stimuli (130). Subsequently, Perlman *et al*. demonstrated that the resistance of MDMs to spontaneous and Fas-FasL mediated apoptosis was attributed to the upregulation of FLIP antiapoptotic protein throughout differentiation (122). An enhanced transcription of the *FLIP* gene was detected on day 3 of differentiation and its role was confirmed using antisense oligonucleotides that were able to abrogate the resistance to Fas-L apoptosis by inhibiting the activation of caspase-8. Furthermore, differentiated MDMs require constitutive activation of NFκB (49, 133) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways (134) in order to sustain their viability. These two pathways independently induce the sustained expression of two different genes of the Bcl2 family, *A1* for NFκB (49) and *Mcl-1* for PI3K/Akt (134) pathway, both of which are responsible for maintaining mitochondrial integrity.

M-CSF binding to its tyrosine kinase receptor CSF-1R (also known as c-fms) induces its autophosphorylation and subsequent phosphorylation of downstream molecules (135). Other signaling pathways activated downstream of CSF-1R include PI3K/Akt (136, 137) and ERK (137). PI3K/Akt is required to protect against apoptosis rather than to promote proliferation in response to M-CSF (136, 138). Oscillations in PI3K/Akt signaling are necessary in the early differentiation process of monocytes treated with M-CSF and these early oscillations are required for non-apoptotic caspase activation (139). The mechanism of Akt induced survival during M-CSF treatment has not been yet investigated. As shown in Objective I, my results indicate that PI3K/Akt signaling confers resistance to apoptosis by controlling Bcl-xL expression during differentiation and Mcl-1 expression after

differentiation. Both extracellular-signal-related kinase (ERK) (140) and NFκB (66) activation have been reported downstream of M-CSF in monocytes undergoing differentiation. I have expanded these observations by studying the interplay between PI3K/Akt and NFκB signaling in response to M-CSF. As shown in Objective I, my results indicate that Akt-induced NFκB activation maintains Bcl-xL expression in monocytes undergoing differentiation.

In addition to *FLIP* and genes of the *Bcl2* family, monocyte to macrophage differentiation has also been shown to affect the third group of antiapoptotic genes, *IAPs*. XIAP has been shown to be upregulated in many experimental models of monocyte differentiation: human (66) or mouse (67) bone marrow-derived macrophages, human macrophages differentiated from promonocytic cell lines in the presence of PMA (62, 141) or from THP1 cells in the presence of bryostatin-1 (132), its upregulation being responsible for the enhanced resistant phenotype of macrophages.

Recently, a new mechanism of antiapoptotic gene involvement in monocyte differentiation has been proposed. Plenchette *et al*. demonstrated that cIAP1 translocates from the nucleus to the Golgi apparatus in both mouse and human monocytes induced to differentiate into macrophages. There was no nucleo-cytoplasmic redistribution of cIAP1 in cells whose differentiation was prevented, such as in Bcl2 overexpressing cells and monocytes from patients with chronic myelomonocytic leukemia (142). The functional nuclear export signal was restricted to its caspase recruitment domain (CARD), a motif that is classically involved in protein interactions and caspase aggregation (143). Although caspase activation was not evaluated during cIAP1 translocation, the same group also showed that monocytic cells undergoing macrophage differentiation require a basal level of

caspase-3 and -9 activation that does not result in apoptosis, but is nevertheless essential for optimal differentiation (144-146), thus extending our knowledge about the key role of caspases in monocyte differentiation (147). The contribution of antiapoptotic genes in the regulation of caspase activation during monocyte differentiation is yet to be determined. However, these observations suggest a relationship between various levels of antiapoptotic genes that are known to prevent caspase activity and the activation status of caspases in monocytes undergoing differentiation. Overexpression of the Bcl2 gene has been shown to inhibit differentiation of U937 monocytic cell line in the presence of phorbol ester (144) or bleomycin (148), so it is tempting to speculate that the mechanism of this inhibition would be prevention of caspase activation.

These observations suggest the existence of multiple seemingly redundant mechanisms directed at preventing the release of cytochrome-c from the mitochondria, a crucial event of apoptosis that is controlled by Bcl2 proteins. However, the mechanism responsible for the differential involvement of some members of the Bcl2 family over others in regulating macrophage survival remains unknown. Modulation of antiapoptotic genes that act at various stages of the apoptotic pathway seems to be the main mechanism by which differentiation confers macrophages enhanced surviving abilities. This complex process does not involve just one key player or antiapoptotic molecule, but multiple mechanisms that vary with the nature of the stress signal, a situation that is to be expected for a cell type that is often the first line of defense in immune responses.

## Survival of monocytes/macrophages in HIV infection

### HIV background

HIV is a retrovirus with a capsid consisting of two copies of viral RNA in complex with nucleocapsid protein and the enzymes protease, reverse transcriptase and integrase (149). T cells and macrophages are the main targets of HIV. Fusion with target cells requires binding of the envelope protein gp120 to a cellular receptor (CD4) and of gp41 to a coreceptor (CCR5 or CXCR4). Following cellular entry, the reverse transcriptase uses the viral genome as a template to generate DNA, which is subsequently transported to the nucleus and integrated to the host genome by viral integrase. New viral copies are produced as the virus hijacks the host's cellular machinery (150). The viral genome encodes three structural proteins (Gag, Pol and Env), three viral enzymes (protease, reverse transcriptase and integrase) and six accessory proteins (Nef, Rev, Tat, Vpr, Vpu, Vif) (149). I have used extracellular Vpr peptide as a model of apoptosis, due to its ability to cause cell death in a multitude of cell types, in the absence of direct infection, as will be discussed below.

### Monocytic cells as viral reservoirs

Enhanced survival of the monocytic lineage is particularly important during HIV infection. Along with CD4+ T cells, monocytic cells represent major viral reservoirs (151, 152). However, there are important differences between virus biology within these two cellular systems: viral replication in T cells is highly cytopathic and as the virus replicates and the disease state advances, the number of CD4+ T cells is ultimately reduced. The formation of viral reservoirs in T cells is correlated with the antigen-induced T cell activation and proliferation. The infected lymphocytes that survive the viral cytolytic effect return to a resting state which corresponds to memory T cell establishment (153) which is

incapable of supporting active viral replication. Since these cells contain integrated virus, viral replication can be reactivated by stimulation with the cognate antigen (154-156). It seems that the virus adapts itself perfectly for long-term survival by hijacking the normal mechanism of long-lived memory cell establishment. Hence, there is no clear evidence to suggest that latency in CD4+ T cells has evolved as a viral mechanism to promote persistence in this cell type.

On the other hand, macrophages get infected early during infection and survive active viral replication (8, 157), serving as a continuous source of viral progeny especially during the late phases of infection, when CD4+ T cells are lost (158). Macrophages also have a higher level of constitutive NFκB transcriptional activity compared to monocytes (159), which also contributes to optimal HIV replication, given the presence of an NFκB - responsive element in the HIV LTR (160).

Macrophages represent a very heterogeneous population and HIV susceptibility also varies with their anatomical location, such as lung, brain, intestinal tract and genital tract. Thus, subepithelial macrophages of the vaginal, but not intestinal tract, support replication of an R5 HIV strain, due to variable expression of CCR5 coreceptor (161). Perivascular macrophages are the main cell type infected by HIV in the central nervous system (162, 163) and alveolar macrophages in the lung (164). HIV replication is stimulated during opportunistic infections and one of the mechanisms involves NFκB activation via cytokine production and direct T cell-macrophage contact (165). Infected macrophages are also responsible for bystander cell apoptosis of uninfected T cells through the apoptosis extrinsic pathway of Fas-FasL interactions (166, 167).

Investigations on viral reservoir formation have received a lot of attention, most of it being focused on memory T cells and the regulation of viral transcription (168). However, it is clear that different mechanisms operate in T cells and monocytic cells, susceptibility to the virus cytopathic effect being one of them.

As mentioned, monocyte to macrophage differentiation induces an increased resistance to a multitude of apoptotic stimuli. However, comparative studies of monocyte and macrophage susceptibility to apoptosis in the context of HIV infection have been hampered by the monocytes' lack of susceptibility to *in vitro* HIV infection (169, 170). Monocyte differentiation increases their permissiveness to viral replication, with macrophages making up a primary source of HIV reservoirs (171). Viral replication is blocked before reverse transcription in monocytes (169), although they bind the virus and support viral entry (172). The difference in viral permissivity between the two cell types has been attributed to the differential expression of APOBEC3, a deoxycytidine deaminase with antiviral effects that induces C to U mutations in the DNA formed during HIV reverse transcription (173). However, persistently-infected monocytes have also been isolated from the peripheral blood of HIV-infected patients, including those under antiretroviral treatment (174-176), suggesting that monocytes' lack of susceptibility to infection is not absolute *in vivo*.

### Signaling pathways and antiapoptotic proteins involved in resistance to apoptosis in HIV infection

**Evidence from cell lines:** It is now clear that HIV affects the apoptotic pathways differently in monocytic and lymphocytic cell lines chronically infected with HIV. Chronically infected monocytic U1 cells were found to be more resistant to apoptosis

induced by γ-irradiation or TNF-α plus cycloheximide compared to the chronically infected lymphocytic cell line ACH-2. At the same time, the Fas/FasL death receptor pathway was less functional in both cell lines compared to their uninfected counterparts (177). Although the mechanisms responsible for the discrepancies between the two cell types were not investigated, these results confirmed earlier observations on the decreased sensitivity to death receptor-induced apoptosis of HIV-infected monocytic cells (178, 179). Even though the mechanism underlying increased apoptosis resistance of persistently infected monocytic cells is not clear, it is reasonable to hypothesize that differential expression of antiapoptotic molecules may contribute to this resistance. Recently, Fernandez-Larrosa *et al* (180) showed that the increased resistance to staurosporine- and $H_2O_2$- induced apoptosis in chronically HIV-infected monocytic cell lines was modulated through the mitochondrial pathway, with an increased Bcl2/Bax ratio in the infected cells favoring an antiapoptotic phenotype. More importantly, this resistance to apoptosis was independent of the magnitude of viral replication, which suggests that controlling the apoptotic pathway is a major factor that influences viral persistence beyond continuous replication.

**Evidence from primary cells:** The involvement of Bcl2 family in increased survival of macrophages during HIV infection was also confirmed in studies that used *in vitro* infection of MDMs (181, 182). In this model, HIV infection increased the expression of antiapoptotic Bcl2 and Bcl-xL and decreased proapoptotic Bax and Bad proteins (182). A recent study has shown that spontaneous cell death and IFN-γ-induced monocyte cell death was elevated in HIV+ patients compared to HIV- controls (183). However, monocytes from HIV+ patients under different conditions ($CdCl_2$ and Fas) are more resistant to cell death compared to uninfected controls (184), even in the absence of productive infection. Even

though the expression of Bcl2 and IAP families was not evaluated, the enhanced resistance of monocytes isolated from HIV+ donors may be linked with the virus ability to bind and activate CCR5, the main coreceptor for macrophage tropic viruses (185). These observations indicate that the effects of viral infection may extend beyond infected cells, and highlight the complicated role of apoptosis in HIV pathogenesis.

Several HIV proteins such as Tat, Nef and Vpr can modulate the survival of monocytic cells depending on their stage of differentiation and through the expression of a variety of cytokines such as MIP-1 α, β and other proinflammatory TNF-α or IL-6 cytokines. The effect of various accessory HIV proteins on the survival of monocytic cells will be briefly discussed below.

**HIV Tat**

Macrophages not only resist the cytopathic effect of HIV (186), but also contribute to an increased cell death of uninfected bystander CD4 T cells by upregulating the expression of FasL and interacting with Fas-expressing lymphocytes (187, 188) or by increasing TRAIL secretion (189). The ability to contribute to lymphocyte apoptosis has been demonstrated for monocytes too. Consistent with bystander cell apoptosis, treatment of monocytes with HIV-Tat resulted in an increased secretion of TRAIL in the culture media which is more cytotoxic for uninfected than HIV-infected CD4+ T cells (190). Interestingly enough, treatment of monocytes with HIV Tat was also shown to increase expression of Bcl2 which was able to protect monocytes against TRAIL-induced apoptosis (191). The ability of Bcl2, a protein associated with maintaining mitochondrial membrane integrity and antiapoptotic activity through the intrinsic pathway, to protect against receptor-mediated cell death (extrinsic

pathway) shows the complexity of cell survival regulation in this cell type. This may be the reason why the virus has evolved pathways to target this protein family to its advantage.

**HIV Nef**

The Bcl2 family has also been implicated in the enhanced survival of macrophages in response to HIV-Nef (192, 193), an accessory protein known to protect lymphocytes from HIV cytopathic effects by inducing phosphorylation and inactivation of proapoptotic Bad (194). Although other antiapoptotic genes were not evaluated and a direct cause and effect relationship was not established, Nef expression was correlated with increased Bad phosphorylation and cell survival in a VSV-G HIV pseudovirus infection model of MDMs (192). Similarly, Nef expression increased the survival of a TF-1 macrophage precursor cell line after cytokine removal by enhancing Bcl-xL gene expression (193). Recently, the ability of HIV-infected macrophages to resist TRAIL-mediated apoptosis was found to be associated with induction of the Bcl2 gene family (195). Increased Mcl-1 and Bfl-1 levels as a result of HIV infection protected infected macrophages against TRAIL-induced apoptosis. Moreover, this resistance was found to be dependent on secretion of M-CSF, a cytokine known to stimulate HIV replication (196). In addition, this resistance to apoptosis was not detected in HIV strains deficient for the envelope gene (195).

Overall, it seems that Tat and Nef have overlapping activities in terms of promoting survival of infected cells especially by targeting Bcl2 family of proteins (results summarized in Table 1). Although results depend on different experimental settings and the models used to deliver viral proteins, it is reasonable to conclude that modulation of the apoptotic pathway contributes to maintenance of viral reservoir status. The implications may also be

extended to bystander uninfected cells, given the ability of infected cells to secrete viral proteins such as Tat in the extracellular medium (197).

HIV infection can also modulate the signaling pathways and transcription factors implicated in the expression of antiapoptotic proteins to promote cell survival. HIV long terminal repeat (LTR) has NFκB binding sites, which allows for HIV transcription after NFκB stimulation (160). As a result, NFκB inhibition leads to impaired HIV replication (198). In monocytic cells HIV infection maintains a constitutive level of NFκB activation (199), which contributes to their enhanced survival (200). There is evidence that NFκB promotes cell survival in both HIV infected (199, 200) and uninfected macrophages (49, 133, 141, 201). NFκB is known to induce the expression of XIAP (66, 132, 141) and Bcl-2 (66) in uninfected cells, yet in HIV infected macrophages, Bcl-xL and not Bcl-2 expression was dependent on NFκB (182), indicating that HIV infection may have redundant survival pathways.

PI3K/Akt signaling pathway is also a target of HIV, given its role in cell survival (134). However, there are conflicting reports on the effect of HIV infection on PI3K/Akt signaling. Thus, one study showed that HIV infection decreased Akt phosphorylation, an effect that was potentiated further by TNF-related apoptosis-inducing ligand (TRAIL) exposure and ultimately induced TRAIL-dependent apoptosis of infected cells (202). However, other reports indicate that HIV increases Akt kinase activity by decreasing PTEN protein expression, the negative regulator of this pathway (203). The activatory effect on Akt has been attributed to Tat accessory protein (203-205). Although the impact of Akt activation on the expression levels of antiapoptotic proteins was not investigated, infected cells were sensitized to PI3K/Akt inhibitors, which indicates that PI3K/Akt inhibitors may

**Table 1. The effect of HIV infection and viral proteins on the expression levels of pro/anti-apoptotic genes in monocytic cells.**

The impact on cell survival or death, the molecular mechanisms implicated (when investigated) and the references are also indicated.

| Experimental setting | Cell type | Molecules involved | Effect | | Mechanism | Ref |
|---|---|---|---|---|---|---|
| **in vitro HIV infection, HIV Tat protein** | human monocyte derived macrophages | Bcl2 | upregulation | | | (181) |
| **in vitro HIV infection** | human monocyte derived macrophages | Bcl2<br>Bcl-xL | upregulation | increased survival of infected cells | increased TNFα secretion in culture media | (182) |
| | | Bax<br>Bad | downregulation | | | |
| **expression of HIV-Nef by retroviral transduction** | TF1 macrophage precursor cell line | Bcl-xL | upregulation | increased survival of Nef expressing cells to cytokine removal | ERK-/MAPK signaling | (193) |
| **VSV pseudotyped-HIV infection, *Δnef* clones** | human monocyte derived macrophages | Bad | increased phosphorilation | protection of infected cells against spontaneous apoptosis | TNF independent | (192) |
| **Vpr synthetic peptide** | primary monocytes, THP1 cells | Bcl2<br>cIAP1 | downregulation | apoptosis | JNK signaling pathway | (206) |
| **TNFα, TLR3-, TLR4-, TLR9-agonists** | primary monocytes, THP1 cells | cIAP2 | upregulation | protection against Vpr-induced apoptosis | | (206) |
| **in vitro HIV infection** | human monocyte derived macrophages | Bfl-1<br>Mcl-1 | upregulation | protection against TRAIL-induced apoptosis | - increased M-CSF production<br>- downregulation of TRAIL-R1 | (195) |
| **HIV Tat protein** | primary monocytes | Bcl2 | upregulation | protection against TRAIL-induced apoptosis | | (191) |

be a novel therapy aimed at eliminating long-living HIV reservoirs, such as macrophages and microglia (203-205, 207).

TRAIL upregulation on the surface of immune cells is one of the mechanisms by which HIV causes depletion of CD4+T cells. TRAIL expression in HIV infected cells is regulated by type I interferon responses in macrophages (208), CD4+ T cells (209) and monocytes (210). HIV infection activates the signaling molecules involved in type I interferon pathway, including IRF-1, IRF-7 (208) and STAT1 (208, 210), thus promoting TRAIL expression and death receptor mediated apoptosis.

### HIV Vpr

#### Structure

Vpr is a small (96 aa and 14 kDa) accessory protein of HIV, highly conserved among viral strains, that is produced late in the viral life cycle and incorporated into newly formed virions by interaction with the structural Gag protein. It is also secreted extracellularly from infected cells, therefore its effects extend to bystander uninfected cells as well (211). Its structure includes an N-terminal half (1 to 51 aa) with two α helices and a C-terminal half (52 to 96 aa), with an α helix rich in leucine residues that allows the peptide to dimerize in solution (212). NMR structure of the entire protein has shown that the three α helices fold around a hydrophobic core (213).

#### Role in apoptosis

Vpr's main function is to induce apoptosis and this effect has been documented in several cell types, including lymphocytes (214, 215), neurons (216-218) and cell lines (219, 220). The mechanism of Vpr-induced apoptosis has been investigated in T cells and PBMCs

and includes activation of the intrinsic pathway, with mitochondrial permeabilization, cytochrome-c release and caspase activation (214). C-terminus Vpr specifically targets the adenine nucleotide translocator (ANT), a component of the permeabilization transition pore complex (PTPC), to induce mitochondrial membrane permeabilization. Vpr-ANT interaction leads to formation of pores into the lipid bilayer and this effect is prevented by Bcl2 protein (221).

Vpr has also been shown to promote caspase-independent but mitochondria-dependent cell death. Roumier et al have demonstrated that Apaf negative or caspase-3 negative cells are still able to undergo cell death in response to C-terminus Vpr peptide (222), which is indicative of apoptosis inducing factor (AIF) activity. Once released into cytosol, AIF translocates to the nucleus and induces DNA fragmentation and chromatin condensation in a manner independent of caspase activation (223). The cytotoxic effect of Vpr was narrowed down to the H(S/F)RIG motif in the C-terminus (224). Vpr peptides with this motif were successful in inducing mitochondrial permeabilization and apoptosis in CD4+ T cells (225).

Vpr cytotoxic effect is of particular interest for its potential effect on the central nervous system (226). The current paradigm is that HIV gets access to the central nervous system by entering monocytes that cross the blood-brain barrier and become productively infected once they differentiate into macrophages (227). HIV associated dementia is a long term complication of HIV infection and its pathogenesis includes neuronal overactivation and cell death, in the absence of viral infection (228). Extracellular Vpr has been detected in the sera and cerebrospinal fluid of HIV positive patients (229, 230) and given the ability of free Vpr to enter the cells (230) in the absence of direct infection, it may also contribute to

neuronal loss. In vitro studies have documented Vpr cytotoxic effect on cultured rat neurons (231), human neuronal precursors and mature neurons (217). Vpr expression in brain monocytes of a transgenic mouse model was also associated with neuronal death and in vivo neurodegeneration (232).

By using a synthetic peptide encoding the C-terminal 52-96 amino acid sequence of Vpr, which contains the apoptosis inducing domain (221), our group has shown that Vpr causes apoptosis in primary human monocytes and in the THP1 promonocytic cell line (206). Our results also show that the Vpr-induced apoptotic effect could be inhibited by pretreatment with Toll-like receptor (TLR) 9 agonist, CpG, via induction of antiapoptotic cIAP2 gene (206).

The effect of Vpr on viability of human macrophages has not been yet investigated. My results indicate that differentiated MDMs are no longer responsive to the Vpr-induced apoptotic effect, which further emphasizes the fact that differentiated macrophages possess an enhanced survival phenotype during HIV infection. My results also suggest that resistance to apoptosis is linked to Vpr's inability to downregulate antiapoptotic molecules in macrophages (233). If this is the case, it is tempting to hypothesize that targeting antiapoptotic genes would provide a valuable therapeutic tool in eliminating this viral reservoir.

Other functions of Vpr include transactivation of HIV long terminal repeat (LTR), transport of the preintegration complex to the nucleus and cell cycle arrest in the G2 stage, which will be discussed briefly below.

**Role in nuclear transport**

Although it lacks a putative nuclear localization signal, Vpr can shuttle between cytoplasm and nucleus (234). This function allows for delivery of HIV preintegration complex (Vpr, matrix protein, integrase and DNA) through the nuclear membrane in the absence of nuclear membrane breakdown during mitosis (235). Importins are soluble proteins that mediate transport to the nucleus of the cargo proteins by binding to nuclear localization signals in their structure (236). The mechanism of Vpr entry to the nucleus involves binding to importin-α (237). Regions 17 to 34 aa (first α helix) and 46 to 74 aa (second α helix) are crucial for the nuclear localization of Vpr and its interaction with importin-α (238). Nuclear export to the cytoplasm has also been described for Vpr, since this is required for successful Vpr incorporation into newly formed virions (239).

The functional consequence of Vpr's nucleophylic property is the ability to infect non-dividing cells that are terminally differentiated and no longer progress through the cell cycle, such as macrophages. Similar results were obtained with extracellular Vpr in experiments that used a synthetic Vpr peptide which was able to stimulate HIV replication of acutely infected macrophages (240) or rescue infectability of Vpr-deficient HIV strains (241).

**Role in cell cycle arrest**

One of the earliest Vpr functions observed was cell cycle arrest (242). Arrest at the G2 stage of the cell cycle has been attributed to Vpr ability to influence cyclin-dependent kinases (Cdks) and cyclins, proteins that normally regulate cell cycle progression in a cell. In addition to mitosis (M), cell cycle phases also include G1, S and G2, with G1 and G2 constituting "gaps" between DNA synthesis (S phase) and cell division (M) (243). Vpr

causes arrest the G2 stage and subsequent progression towards mitosis by interfering with cyclin B-cdk1 complex, which is responsible for controlling the G2/M checkpoint. Cdk1 activity is tightly regulated by phosphorylation/dephosphorylation and its regulatory proteins include Wee kinase (inhibitory) and Cdc25 phosphatase (activatory) (244). Vpr promotes cdk1 phosphorylation and subsequent inactivation by inhibiting Cdc25 phosphatase (245) and activating Wee kinase (245, 246).

G2 arrest is a physiological response in cells with DNA damage and its purpose is to allow time for DNA repair and protect the integrity of the cell genome. The early sensors of the DNA damage pathway are ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR). Earlier reports considered that the effect of Vpr was independent of this pathway (247). However, more recent data indicates that ATR is required for Vpr-induced G2 arrest via Cdk1phosphorylation, since siRNA depletion of ATR relieved Vpr-induced cell cycle arrest and LTR transactivation (248). Zimmerman et al have shown that ATR is also required for HIV infection to cause G2 arrest in CD4+ T cells (249).

In vitro studies on the functional consequences of Vpr-induced cell cycle arrest have shown that G2 arrest results in increased transcription from the viral promoter LTR, with increased viral replication (242). This result was also confirmed in vivo, with a study showing that infected lymphocytes from HIV infected patients were arrested in the G2 phase (249).

The link between G2 arrest and apoptosis has long been a matter of debate in the literature, with evidence supporting both the requirement of G2 arrest for apoptosis and also their independence. More specifically, some of the earlier studies have shown that prolonged G2 arrest leads to apoptosis (219, 220, 250), but that these two functions can also occur

separately (251-253). G2 arrest and apoptosis inter-relationship is also supported by a study showing that cell cycle arrest due to ATR activation by Vpr also led to Bax activation and apoptosis and that ATR knockdown prevented these effects (254). While the different model systems or the mode of Vpr delivery (endogenously expressed or extracellularly added protein) used in these reports may account for the seemingly opposing results, it is also possible that Vpr activates multiple pathways simultaneously, such as DNA damage pathway via ATR activation or the intrinsic apoptosis pathway via ANT.

## Rationale

Macrophages are cells of the innate immune system that display a remarkable resistance to a variety of apoptotic stimuli, a characteristic that allows them to perform in stressful environments. This resistant phenotype is acquired during the process of differentiation from the monocytic precursors, yet the signaling pathways and molecular mechanisms of this process are not established. Enhanced resistance to apoptosis is also the main reason why macrophages survive viral replication during HIV infection, thus becoming long lived viral reservoirs.

Members of the Bcl2 and IAP families have been shown to contribute to cell survival in a variety of cell types, but their specific involvement in conferring resistance during macrophage differentiation or HIV infection is yet to be determined. I have studied resistance to apoptosis in the context of HIV infection by using accessory protein Vpr as a model, due to its cell death inducing abilities in other cell types, including monocyte precursors.

Recent studies using cell lines and mouse models have indicated a role of IAP family in innate immunity, due to IAPs involvement in major signaling pathways, such as NFκB activation and TLR signaling. However, the role of IAPs in the immune function of primary human macrophages is yet to be determined.

## Overall hypothesis

Antiapoptotic Bcl2 and IAP proteins play an important role in human macrophages survival, differentiation, innate immunity and protection from HIV-Vpr induced apoptosis**.**

## Specific objectives

My specific objectives are as follows:

**Objective 1**: To investigate the antiapoptotic proteins and signaling pathways responsible for development of resistance to apoptosis during monocyte to macrophage differentiation.

**Objective 2**: To evaluate the sensitivity of human macrophages to the effect of HIV-Vpr

**Objective 3:** To investigate the role of IAPs in human macrophage function.

## Materials and methods

### Cells and reagents

THP1, a promonocytic cell line derived from a human acute monocytic leukemia patient, was obtained from the American Type Culture Collection (Manassas, VA) and maintained in IMDM-10 (Sigma-Aldrich, St-Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY), 100 U/ml penicillin and 100 g/ml gentamicin (both from Sigma-Aldrich). THP1 cells ($5\times10^5$/ml) were differentiated with 20 ng/ml PMA (Sigma-Aldrich) for 2 days to obtain THP1-derived macrophages (THP1-MACs) (31). THP1-MACs had the following phenotype CD14+, CD11a+, CD11b+, CD11c+ and CD80-, CD83-, CD16- (Supplementary fig. C).

Blood was obtained from healthy volunteers according to a protocol approved by the Ethics Review Committee of The Ottawa General Hospital. Donors gave written, informed consent. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque (GE Healthcare Life Sciences Buckinghamshire, UK) density centrifugation and the cell layer consisting mainly of mononuclear cells was collected.

To generate monocyte-derived macrophages (MDMs), monocytes were isolated by the adherence method. PBMCs were resuspended in serum free media ($5\times10^6$/ml) and plated in 12 well polystyrene plates (Becton Dickinson, Franklin Lakes, NJ USA). After being allowed to adhere for 3 h, non-adherent cells were washed off and adherent cells were cultured for 6 days in IMDM supplemented with 10% fetal bovine serum and 10 ng/ml M-CSF (R&D Systems, Minneapolis, MN USA). Media containing M-CSF was replenished every 2 days. MDMs thus obtained were 98% CD14+ (Supplementary fig. A) and had the

following phenotype CD14+, CD16+, CD80+, CD11a+, CD11b+, CD11c+, HLA-DR+ and CD83- (Supplementary fig. B).

For experiments performed before differentiation, adherent monocytes were treated with chemical inhibitors for 2h or transfected with siRNA for 5h before induction of differentiation with M-CSF as described.

Primary monocytes used for Vpr and Smac mimetics experiments were obtained from PBMCs by using Automacs negative selection (Miltenyi Biotech, Auburn, CA) as per manufacturer's instructions.

Dendritic cells were generated by obtaining monocytes by the adherence method as described above, followed by culture for 6 days in IMDM supplemented with 10% fetal bovine serum and 50 ng/mL GM-CSF and 50 ng/mL IL-4 (both from R&D Systems).

Bcl2 inhibitor HA14-1 was purchased from Sigma-Aldrich. LPS (Sigma-Aldrich) was dissolved in complete medium, aliquoted, and stored at −80°C. CD40L was obtained from Cell Signaling Technology, Danvers, MA. Second mitochondria-derived activator of caspases (Smac) mimetics LN730 and SM164 were a generous gift from Dr. Korneluck (Children's Hospital of Eastern Ontario Research Institute, Apoptosis Research Centre).

### NFκB and MAP kinases inhibition

Chemical inhibitors LY294002 (PI3K/Akt inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD 098059 (ERK inhibitor), CAPE and parthenolide (both NFκB inhibitors) were purchased from Calbiochem and used to treat THP1 cells or monocytes for 2h before inducing differentiation or MDMs before LPS stimulation. For experiments involving the broad caspase inhibitor Zvad-fmk (Biomol), cells were treated with inhibitor for 2h before adding LY294002.

**Cell treatment with Vpr**

C terminus Vpr (52–96 aa) was synthesized by Invitrogen, Carlsbad, California. Mutant Vpr peptide was synthesized by Genemed Synthesis Inc., San Francisco, CA. Peptides were obtained by automated solid-phase synthesis using 9-fluorenylmethoxycarbonyl and purified by reverse-phase high pressure liquid chromatography (>95%) followed by analysis with electrospray mass spectrometry. The amino acid sequence is as follows: Vpr (52–96): DTWAGVEAIIRILQQLLFIHFRIGCRHSR IGVTRQRRARNGASRS.

Mutant Vpr (mVpr) has the same sequence as Vpr (52–96) except for R to A substitutions at positions 73, 77 and 80 (underlined in the sequence above), which are important for Vpr interaction with the mitochondria and subsequent apoptosis (32, 33). Because of the high propensity of Vpr to bind to proteins, cells were treated with Vpr peptides in an isotonic buffer for 30 min (12), followed by addition of culture medium. Unless otherwise specified, primary monocytes were treated with Vpr for 4 h, whereas THP1-MACs and MDMs were collected after 24 h of Vpr treatment.

**Cell treatment with Smac mimetics**

MDMs were treated with 50 nM Smac mimetics LN or SM for 24 h, followed by LPS 100 ng/ml or CD40L 400 ng/ml for another 24 h. After LPS or CD40L treatment supernatants were collected and frozen at -80°C until evaluated for cytokine secretion. Cell pellets were used to extract protein and perform Western blots evaluating p100/52, cIAP1, cIAP2, TRAF1, TRAF2, TRAF6 and MyD88 expression. For experiments involving MAPKs and classical NFκB signaling, cells were stimulated with LPS for 30 min following Smac mimetics treatment of 24 h.

**Apoptosis analysis by intracellular propidium iodide (PI) or annexin-V staining**

Apoptosis in primary monocytes and THP1 cells was measured by staining cells with FITC-labelled Annexin-V (Invitrogen Molecular probes, Eugene, OR) for 15 min at room temperature in the dark. Annexin-V positive cells were quantified by flow cytometry. Since annexin-V staining is not appropriate for adherent cells, adherent macrophages were evaluated for cell death by using intracellular PI staining. Following Vpr treatment cells were washed with PBS and fixed with methanol for 15 min at 4°C. The methanol was washed away with PBS and cells were treated with 25 µl of 10 µg/ml RNase A, followed by staining with 25 µl of 1 mg/ml PI solution (Sigma-Aldrich) at 4°C for 1 h. The DNA content was analyzed using a FACSCanto flow cytometer (BD Biosciences) and the FACSDiva software. The subdiploid DNA peak (<2N DNA), immediately adjacent to the G0/G1 peak (2N DNA), represents apoptotic cells and was quantified by histogram analyses. Cells with minimal light scatter were gated out from the analysis. PI histograms figures were obtained with WinMDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

**Cytokine measurement by flow cytometry**

Cytokine secretion in culture supernatants was measured using the FlowCytomix Multiple Analyte Detection System from eBioscience. The FlowCytomix is a bead-based multiplex immunoassay that allows simultaneous detection of multiple analytes in the same sample volume. Briefly, antibody-coated beads were added to 25 µl of supernatant, followed by biotin-conjugated antibodies against various analytes. Streptavidin-PE was used to detect biotin-conjugated antibodies. Flow cytometry was used to differentiate bead populations according to bead size and varying intensities of an internal fluorescent dye that emits at 690 nm. Streptavidin-PE bound to the biotin conjugate emitting at 578 nm allowed quantification

of the analyte. Beads coated with antibodies against MIG, MIP-1α, MIP-1β, IL-8, IL-10, IL-1β, IL-5, IL-6, IL-18, IL-23 and IFN-γ were used. Fluorescence intensities were analyzed using a FACSCanto flow cytometer (BD Biosciences) and the FACSDiva software. Cytokine quantification was performed using the FlowCytomix Pro 3.0 Software. The detection limit was 0.9 pg/mL for MIG and 1.9 pg/mL for IL-10.

**Cytokine measurement by ELISA**

Culture supernatants of TNF-α, IL-12p40, IL-23 and IL-27 levels were determined by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. Ready-Set-Go IL-23 ELISA kit (eBiosciences, San Diego, CA) was used to detect IL-23 and IL-27 ELISA kit was purchased from R&D Systems, Minneapolis, MN.

Reactions were performed in Costar high binding 96 well ELISA plates (Corning Incorporated, Corning, NY), using 0.05% Tween 20 (Biorad) in phosphate buffered saline (PBS) for washing. Microplates were coated overnight at 4 °C in ELISA coating buffer (eBioscience, San Diego, CA) with the following capture antibodies: 4 μg/mL of IL-12 p40 antibody (Invitrogen, Burlington ON, CA), 2 μg/mL of IL-23 p19 antibody (eBioscience, San Diego, CA), 0.4 μg/mL of IL-27 antibody (R&D systems, Minneapolis, MN) and 0.2 μg/mL of TNF-α antibody (Invitrogen, Burlington ON, CA). The next day plates were washed, blocked for 2 h with 10% FCS in PBS, followed by addition of standards and sample supernatants for another 24 h. On the third day biotinylated secondary mouse monoclonal anti-human antibodies were added, followed by streptavidin-HRP. Visualization was carried out using 3,3',5,5'-tetramethylbenzibidine (TMB) one component horse radish peroxidase (HRP) microwell substrate solution and 450 nm liquid stop solution for TMB microwell substrates (BioFX Laboratories, Owings Mills, MD). Absorbance was read using

Bio-Rad iMark microplate reader and data was processed using Micro Plate Manager 6 software.

**Determination of mitochondrial membrane permeability**

Mitochondrial dysfunction was assessed by utilizing Rh123 cationic lipophilic green fluorochrome rhodamine (Invitrogen Molecular Probes). Disruption of the mitochondrial membrane potential is associated with decreased Rh123 retention and lower fluorescence. After Vpr treatment, cells were incubated with 100 ng/ml of Rh123 for 30 min at 37°C (23) followed by flow cytometry analysis. Fluorescence intensities were analyzed using a FACSCanto flow cytometer (BD Biosciences) and the FACSDiva software.

**Flow cytometry analysis of surface expression**

PE or FITC conjugated antibodies against CD3, CD11a, CD11b, CD11c, CD14, CD16, CD19, CD80, CD83, CD86, HLA-DR were purchased from BD Biosciences Pharmingen (San Diego, CA). For FACS staining, single cell suspensions were washed in PBS then stained with specific antibodies for 30 min at 4°C. Cells were washed to remove excess antibodies and analyzed by flow cytometry using a FACSCanto flow cytometer (BD Biosciences) and the FACSDiva software.

**Confocal laser scanning fluorescence microscopy**

For experiments involving MDMs and Vpr entry, monocytes were initially adhered and differentiated on round glass coverslips in 12 well plates. After Vpr treatment cells were fixed in 4% paraformaldehyde for 15 min, rinsed in PBS and then permeabilized with 0.1% triton X for another 10 min. The coverslips were incubated with anti-Vpr rabbit primary antibody at 4°C overnight. On the second day coverslips were rinsed with 5% BSA in PBS

and incubated in the same buffer with Alexa Fluor 680 conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Coverslips were mounted on microscopy slides using ProLong Gold antifade mounting media with DAPI (Invitrogen) and examined with a Zeiss LSM 510 Meta confocal microscope using a red HeNe (633 nm) laser. The objective used was Plan-Apochromat 63x.

For experiments involving measurement of apoptosis THP1 cells were initially transfected with Akt or Bcl-xL siRNA in 12 well plates with round glass coverslips. Similarly, primary monocytes were initially adhered on round glass coverslips, then transfected as described below. After transfection cells were treated with PMA (to generate THP1-MACs) or with M-CSF (to generate MDMs). Following differentiation cells were rinsed with PBS, resuspended in annexin-binding buffer and then stained with FITC-labelled Annexin-V (Invitrogen Molecular probes, Eugene, OR) for 30 min at room temperature in the dark.

Coverslips were then rinsed and mounted on microscopy slides using ProLong Gold antifade mounting media with DAPI (Invitrogen) and examined with a Zeiss LSM 510 Meta confocal microscope using a using a 488 nm (green) and a 405 nm (blue) laser. Fluorescent images were acquired with ZEN 2009 software and analyzed with Image J software.

**Western blot analysis**

Total cell proteins obtained after lysis of cell pellets were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with antibodies against cellular IAP1 (cIAP1), cIAP2, XIAP, TRAF1, TRAF2, TRAF6, MyD88, RIP1, Bcl2, Bcl-xL, Mcl-1, Bax, poly-ADP ribose polymerase (PARP), caspase-3, caspase-9 (all from Cell Signaling Technology, Danvers,

MA). Anti - p100/p52 antibody was from Sigma Aldrich. For experiments involving signaling pathways, antibodies against IκBα, P-Akt, Akt, P-p38, p38, (all from Cell Signaling) and P-JNK, JNK, P-ERK, ERK (all from Santa Cruz Biotechnology) were used. The anti-Vpr antibody was kindly provided by Dr Eric Cohen, University of Montreal, Montreal, Canada. Membranes were probed with primary antibodies at 4°C overnight, followed by goat secondary antibodies conjugated to horseradish peroxidase (Bio-Rad). To control for total protein loading, membranes were stripped of the primary antibodies and reprobed with anti-GAP-DH (Sigma Aldrich) or β actin (Cell Signaling Technology) antibodies. Immunoblots were visualized using the Amersham ECL (Enhanced Chemiluminescence) Western blotting detection system. The images were obtained with the Chemigenius Bio-imaging system and the GeneSnap software (both from Syngene). Quantification of band intensities was performed using GeneTools software (Syngene).

**Transfection with siRNA**

All specific siRNAs were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Before differentiation THP1 cells ($5\times10^5$/ml) and adherent monocytes were treated with Akt siRNA, Bcl-xL siRNA and control siRNA (Qiagen, Venlo, the Netherlands) using TransIT-TKO transfection reagent (Mirus Bio) according to the manufacturer's instructions. This protocol allowed siRNA delivery to cells in complete media, without the need to maintain monocytic cells in serum free conditions, given their high susceptibility to cell death after serum withdrawal. Briefly, 40 pmoles siRNA were mixed with 4 µl transfection reagent in 100 µl of serum free media to allow formation of RNA-complexes. After 30 min, the siRNA mixtures were added to cells in complete media. After 5 h, transfected cells were induced to differentiate with either PMA or M-CSF.

A similar transfection protocol was used with Akt siRNA and Mcl-1 siRNA in differentiated MDMs and THP1-MACs. Bax siRNA was used to transfect THP1 cells for 5h before adding LY294002 for another 2h. Following transfection and inhibitor treatment, cells were induced to differentiate with PMA and collected after 2 days to measure apoptosis and efficiency of protein knockdown.

For experiments involving Vpr, THP1-MACs ($5\times10^5$/ml) were treated with cIAP1 siRNA, cIAP2 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) and control siRNA (Qiagen, Venlo, the Netherlands) in serum free media, using Fugene 6 (Roche, Basel, Switzerland) as a transfection reagent, according to the manufacturer's instructions. Initially, 250 and 500 ng siRNA were mixed with Fugene 6 in 100 µl media to allow formation of RNA-complexes. The proportion of Fugene 6 to siRNA was 3 µl:1 µg siRNA. After 45 min, the siRNA mixtures were added to cells in serum free media, which was replaced with complete media after 5 h. Mcl-1 and Bcl-xL siRNAs along with the transfection reagent were purchased from Santa Cruz Biotechnology and used as per the manufacturer's instructions. Briefly, 40 pmoles siRNAs were preincubated for 30 min with 4 µl transfection reagent in 100 µl serum free media before addition to the cells. Serum free media was replaced with complete media after 5 h. Following transfection, cells were either collected after 24 h for evaluation of knockdown efficiency or treated with Vpr 2.5 µM for another 6 h (for PARP cleavage and caspase-3 cleavage) or 24 h (for apoptosis measurement).

For experiments involving LPS stimulation, differentiated MDMs were transfected with RIP1 siRNA, TRAF2 siRNA, TRAF3 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) or control siRNA (Qiagen, Venlo, the Netherlands) using TransIT-TKO transfection reagent (Mirus Bio) as described above. The following day after transfection, cell were

treated with LPS for another 24h, then supernatants and cell pellets were collected for cytokine evaluation and Western blotting respectively.

**Electromobility shift assay (EMSA)**

Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). Non-radioactive EMSA was performed using biotin-labeled oligonucleotides and LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific). Briefly, nuclear proteins (6 μg) were incubated for 30 min at 4 °C with 10 pmole of a biotin-labeled oligonucleotide and 6 μg poly IC, 1% NP-40, 100 mM MgCl2 and 50% glycerol. The samples were loaded on a 5% nondenaturing polyacrylamide gel, run at 100 V, then transferred onto a nylon membrane, UV crosslinked, probed with streptavidin-HRP conjugate and visualized using the Amersham ECL (Enhanced Chemiluminescence) detection system. The probes consisting of double-stranded oligonucleotides corresponding to the NFκB -binding motif on the *Bcl-xL* gene promoter (5'-AGTGGGGGCGGGGGGGACTGCCCCCTCTCCTT-3') (255) with a 5' biotin tag were purchased from Eurofins MWG Operon (Huntsville, AL). For the cold competition sample protein extracts were incubated with a 100-molar excess of the unlabeled probe to verify signal specificity.

**Statistical analyses**

Data was plotted using Windows Excel 2010 (Microsoft) and GraphPad Prism 5. Significance was determined using Student t test or Anova, followed by Tukey test. A *p* value of less than 0.05 was considered significant. * indicates $p<0.05$. Unless otherwise specified, plotted data represent the mean ± standard deviation of at least three experiments.

## Results

## Objective 1: To investigate the antiapoptotic proteins and signaling pathways responsible for development of resistance to apoptosis during monocyte to macrophage differentiation.

### Introduction

The development of the mononuclear phagocyte system is controlled by hematopoietic growth factor macrophage colony stimulating factor (M-CSF) (118). Under the effect of M-CSF, monocytes undergo multiple functional and morphological changes during differentiation towards the macrophage lineage. One of the most striking features that macrophages acquire as a result of differentiation is increased resistance to apoptosis (1). Monocytes are susceptible to death receptor ligand apoptosis and have a very short life span of 1-2 days (121). However, this process can be prevented during infection by activation with pro-inflammatory cytokines (124, 125) or during differentiation (122). In the context of HIV infection, we have recently shown that monocytes are highly susceptible to the apoptotic effect of HIV accessory protein Vpr, but they develop resistance once they undergo differentiation towards the macrophage lineage (233) or following treatment with TLR-9 agonist CpG (206). While increased resistance to apoptosis is documented in macrophages, the exact molecular mechanisms and signaling pathways that mediate this effect in the early stages and throughout the differentiation process are still unclear.

Phosphatidylinositol-3 kinase (PI3K) and its downstream serine-threonine kinase, protein kinase B (PKB) or Akt constitute a major signal transduction pathway that mediates cell survival in a variety of cells, such as monocytes (117), macrophages (134), dendritic cells (256) and osteoclasts (257). PI3K/Akt can activate transcription factors to promote synthesis of antiapoptotic molecules or phosphorylate and inactivate proapoptotic Bad (258).

In M-CSF-induced differentiation of monocytes into macrophages, early oscillations in PI3K/Akt signalling were required to cause activation of caspases-8 and -3 necessary to drive the differentiation process towards macrophages (139). However, the contribution of this pathway to the development of resistant phenotype that macrophages acquire during differentiation is yet to be determined.

Bcl2 family proteins are key regulators of apoptosis that can function either as cell death antagonists (Bcl2, Bcl-xL and Mcl-1) or agonists (Bax and Bak) (32). Antiapoptotic Bcl2 members such as A1 (49) and Mcl-1 (134, 233) are known to contribute to steady state survival in differentiated macrophages. The data from animal models indicate a predominant role of Bcl-xL over Bcl2 in hematopoiesis. Bcl-xL knockout mice exhibit a lethal phenotype due to massive apoptosis of neurons and hematopoietic cells (64), whereas Bcl2 knockout mice are born with organ malformations and normal myeloid hematopoiesis (65). Bcl-xL has been shown to contribute to survival of monocytes (59-61, 259), erythroid differentiation (260, 261) and thrombocyte survival and differentiation (262, 263). However, the contribution of Bcl2 proteins in conferring resistance during macrophage differentiation is not yet established.

In this study, I examined the signaling pathways that confer resistance to apoptosis during the process of monocyte to macrophage differentiation. Specifically, I investigated the role of PI3K/Akt signaling and its relationship with various antiapoptotic proteins in this process. I also compared the mechanism of PI3K/Akt induced resistance to apoptosis during and after differentiation, since the signaling pathways may be different and PI3K may support survival via multiple mechanisms. As models of differentiation I used monocyte derived macrophages (MDMs) generated from primary human monocytes in the presence of

M-CSF and THP1 macrophages (THP1-MACs) generated by stimulation of promonocytic THP1 cells with phorbol myristate acetate (PMA). I show for the first time that PI3K/Akt differentially regulates macrophage survival during and after differentiation by maintaining Bcl-xL expression during differentiation, in contrast to the Akt-dependent Mcl-1 expression after differentiation.

## Results

### PI3K pathway regulates apoptosis in human macrophages during and after differentiation

To identify the signaling pathways responsible for resistance to apoptosis during monocyte to macrophage differentiation, PI3K/Akt and mitogen-activated protein kinases (MAPK) were investigated as potential candidates due to their implication in apoptosis resistance in other cell types (134, 264, 265). I initially evaluated the activation status of Akt and MAPKs (extracellular signal-related kinases, ERK; p38 and the c-Jun amino-terminal kinase, JNK) during the early stages of differentiation following treatment of THP1 cells with PMA and M-CSF stimulation of primary monocytes. As expected, all tested signaling pathways were activated following PMA (Fig. 4A) or M-CSF treatment (Fig. 5A), as indicated by their phosphorylation status. To determine the signalling pathways involved in cell survival during differentiation, I used a series of chemical inhibitors: LY294002 (PI3K/Akt inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and PD 098059 (ERK inhibitor) that were able to inhibit their specific pathways (Fig. 4A). THP1 cells and primary monocytes were treated with the above mentioned inhibitors for 2 h before inducing differentiation with PMA and M-CSF, respectively. Cells were collected after the differentiation period and evaluated for apoptosis. Although all the evaluated signaling

**Figure 4. Blocking PI3K/Akt pathway before and after differentiation causes apoptosis in THP1-MACs**

**A.** THP1 cells ($1x10^6$/2ml) were pretreated with the indicated inhibitors at various concentrations before adding PMA 20 ng/ml for 30 min. Following PMA treatment total cell proteins were subjected to Western blotting and the membranes were probed with anti-p-Akt, p-JNK, p-p38, p-ERK antibodies to evaluate the biological activity of the inhibitors. Total levels of unphosphorylated Akt and MAPKs were used as a loading control.
**B**. THP1 cells ($0.5x10^6$/ml) were treated with the indicated concentrations of LY294002 (PI3K/Akt inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD 098059 (ERK inhibitor) for 2 h before adding PMA 20 ng/ml for another 2 days to generate macrophages. Following differentiation, THP1-MACs were evaluated for apoptosis by flow cytometry using intracellular PI staining. The numbers represent percentage apoptotic cells with subdiploid DNA content. Upper panel shows the mean of % apoptotic cells ± SD of at least three experiments. * indicates $p<0.05$. Lower panel histograms show data from one experiment with LY294002 were THP1 controls (no PMA added) were included.
**C**. THP1-MACs ($0.5x10^6$/ml) were treated with the indicated concentrations of LY294002 for 48 h before collection and apoptosis evaluation using intracellular PI staining.

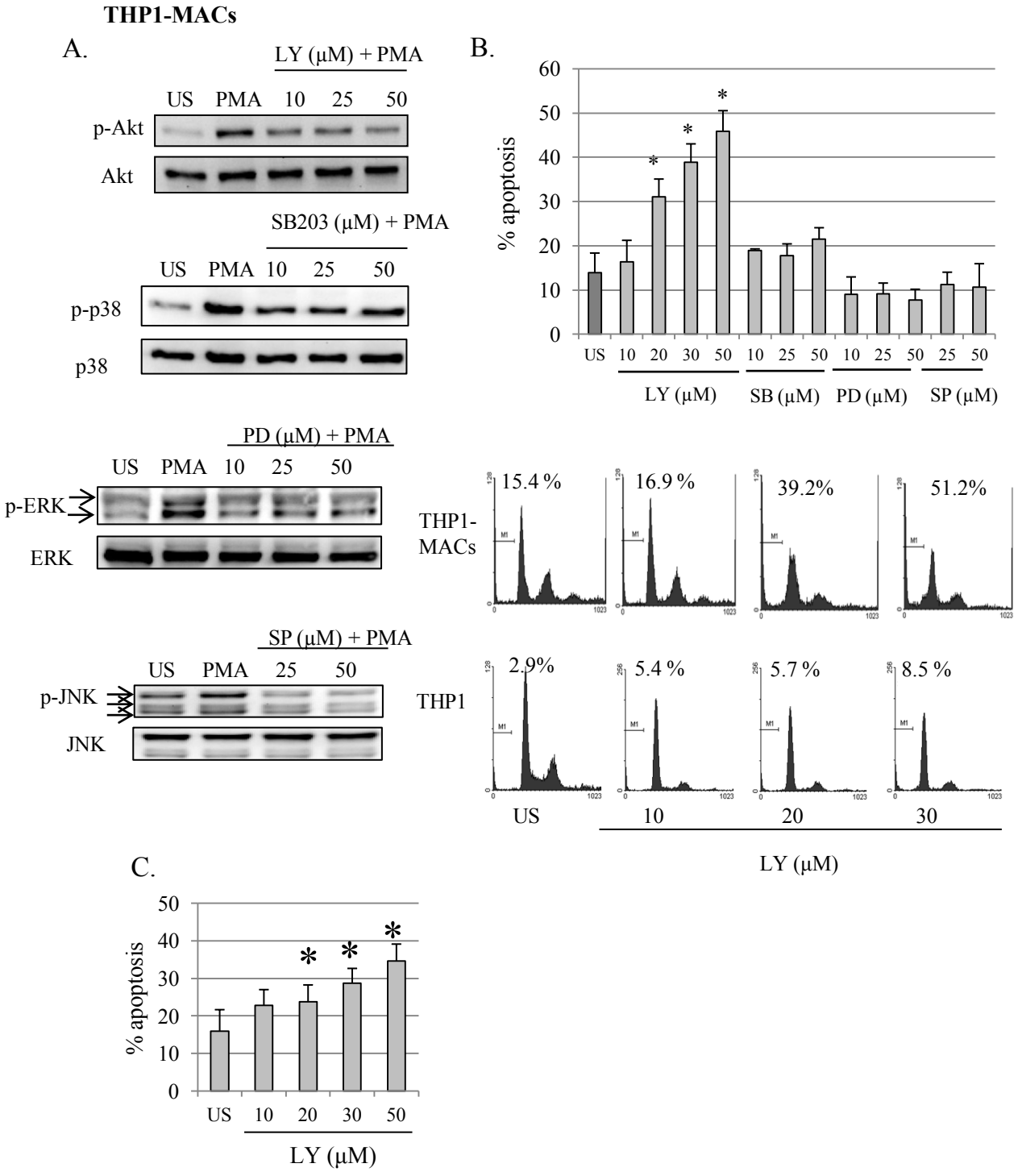

THP1-MACs
A.
LY (μM) + PMA
US PMA 10 25 50
p-Akt
Akt
SB203 (μM) + PMA
US PMA 10 25 50
p-p38
p38
PD (μM) + PMA
US PMA 10 25 50
p-ERK
ERK
SP (μM) + PMA
US PMA 25 50
p-JNK
JNK
B.
% apoptosis
US 10 20 30 50 10 25 50 10 25 50 25 50
LY (μM) SB (μM) PD (μM) SP (μM)
THP1-MACs
15.4 %
16.9 %
39.2%
51.2%
THP1
2.9%
5.4 %
5.7 %
8.5 %
US 10 20 30
LY (μM)
C.
% apoptosis
US 10 20 30 50
LY (μM)

**Figure 4**

**Figure 5. Blocking PI3K/Akt pathway before inducing differentiation causes apoptosis in MDMs**

**A.** Adherent monocytes obtained as described in Materials and methods were treated for the indicated times with M-CSF 10 ng/ml, following which total cell proteins were subjected to Western blotting. Membranes were probed with anti-p-Akt, p-JNK, p-p38, p-ERK antibodies to evaluate Akt and MAPKs activation in response to M-CSF. Membranes were stripped and reprobed with antibodies against total levels of unphosphorylated JNK, p38 and ERK and against GAP-DH.

**B.** Primary monocytes were treated with the indicated concentrations of LY294002 (PI3K/Akt inhibitor) for 2 h before adding M-CSF 10 ng/ml for another 6 days to generate macrophages. Following differentiation, MDMs were evaluated for apoptosis by flow cytometry using intracellular PI staining. The numbers represent percentage apoptotic cells with subdiploid DNA content. The bar graph shows the mean of % apoptotic cells ± SD of three experiments with cells from different donors. * indicates $p<0.05$.

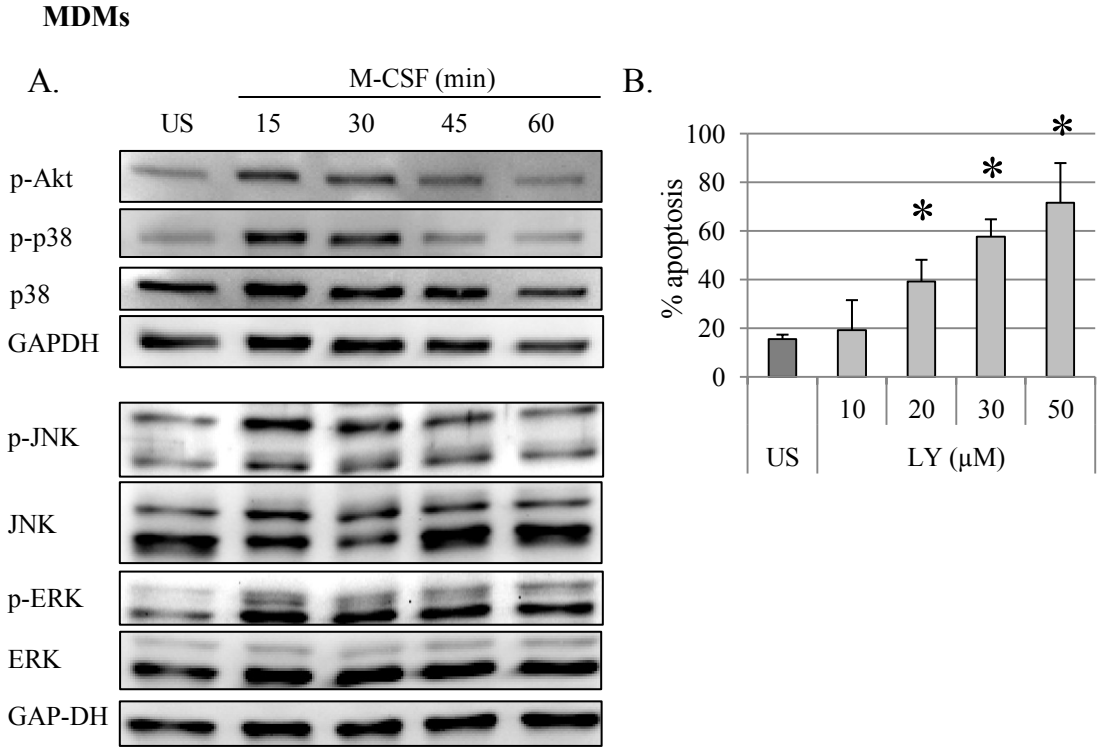

MDMs
A.
M-CSF (min)
US
15
30
45
60
p-Akt
p-p38
p38
GAPDH
p-JNK
JNK
p-ERK
ERK
GAP-DH
B.
% apoptosis
100
80
60
40
20
0
10
20
30
50
US
LY (μM)

**Figure 5**

pathways including p38, ERK, JNK MAPKs and PI3K were activated during differentiation, blocking of PI3K/Akt pathway only was able to induce apoptosis following PMA treatment of THP1 cells (Fig. 4B) and M-CSF stimulation of monocytes (Fig. 5B). Moreover, inhibition of MAPKs did not impact survival of differentiating THP1-MACs (Fig. 4B) or MDMs (data not shown), suggesting that this effect was specific for PI3K/Akt.

Importantly, LY294002 did not induce cell death in THP1 cells that were cultured for the same amount of time in the absence of PMA (Fig. 4B, lower panel), suggesting that the observed effect is not due to inhibitor toxicity, but is specific to cells undergoing differentiation. Similar to the results obtained with macrophages undergoing differentiation, differentiated THP1-MACs exhibited apoptosis following treatment with the PI3K inhibitor, LY294002 (Fig 4C). These results suggest that blocking of the PI3K pathway induces apoptosis in macrophages undergoing differentiation as well as differentiated macrophages. Furthermore, PI3K pathway plays a key role in conferring resistance to apoptosis during macrophage differentiation as well as after differentiation.

**LY294002 induced apoptosis during differentiation of human macrophages involves caspase-3 but not caspase-9**

To characterize the mechanism of LY294002-induced apoptosis during differentiation into macrophages, I employed Zvad-fmk as a broad caspase inhibitor. THP1 cells were treated with Zvad-fmk and LY294002 prior to inducing differentiation with PMA. Zvad-fmk treatment significantly reduced LY294002-induced apoptosis in THP1-MACs (Fig. 6A), suggesting that caspases mediate apoptosis induced following PI3K blockade in differentiating macrophages. Since both caspase-3 and -9 have been shown to mediate LY294002-induced cell death in differentiated macrophages (134), I investigated

**Figure 6. LY294002 induced apoptosis involves cleavage of caspase-3**

**A.** THP1 cells ($0.5x10^6$/ml) were pretreated for 2 h with the broad caspase inhibitor Zvad-fmk (25 μM) before adding LY294002 at the indicated concentrations for another 2 h. Cells were then induced to differentiate in the presence of PMA 20 ng/ml for another 2 days. THP1-MACs were collected and stained with PI to evaluate apoptosis by flow cytometry. The numbers represent percentage apoptotic cells with subdiploid DNA content. The bars show the mean ± SD of three experiments. * indicates $p<0.05$.
**B** and **C** Following LY294002 treatment before inducing differentiation, total cell proteins were subjected to Western blotting. Membranes were probed with anti-PARP, anti-caspase-3 or anti-caspase-9 antibodies to evaluate their cleavage as markers of apoptosis. GAP-DH was used as a loading control and staurosporine treatment (1 μM for 6h) was used as a positive control for caspase-9 cleavage in THP1-MACs. THP1-MACs were treated as in A (B) and primary monocytes were treated with the indicated concentrations of LY294002 for 2 h before adding M-CSF 10 ng/ml for another 6 days to generate macrophages (C).

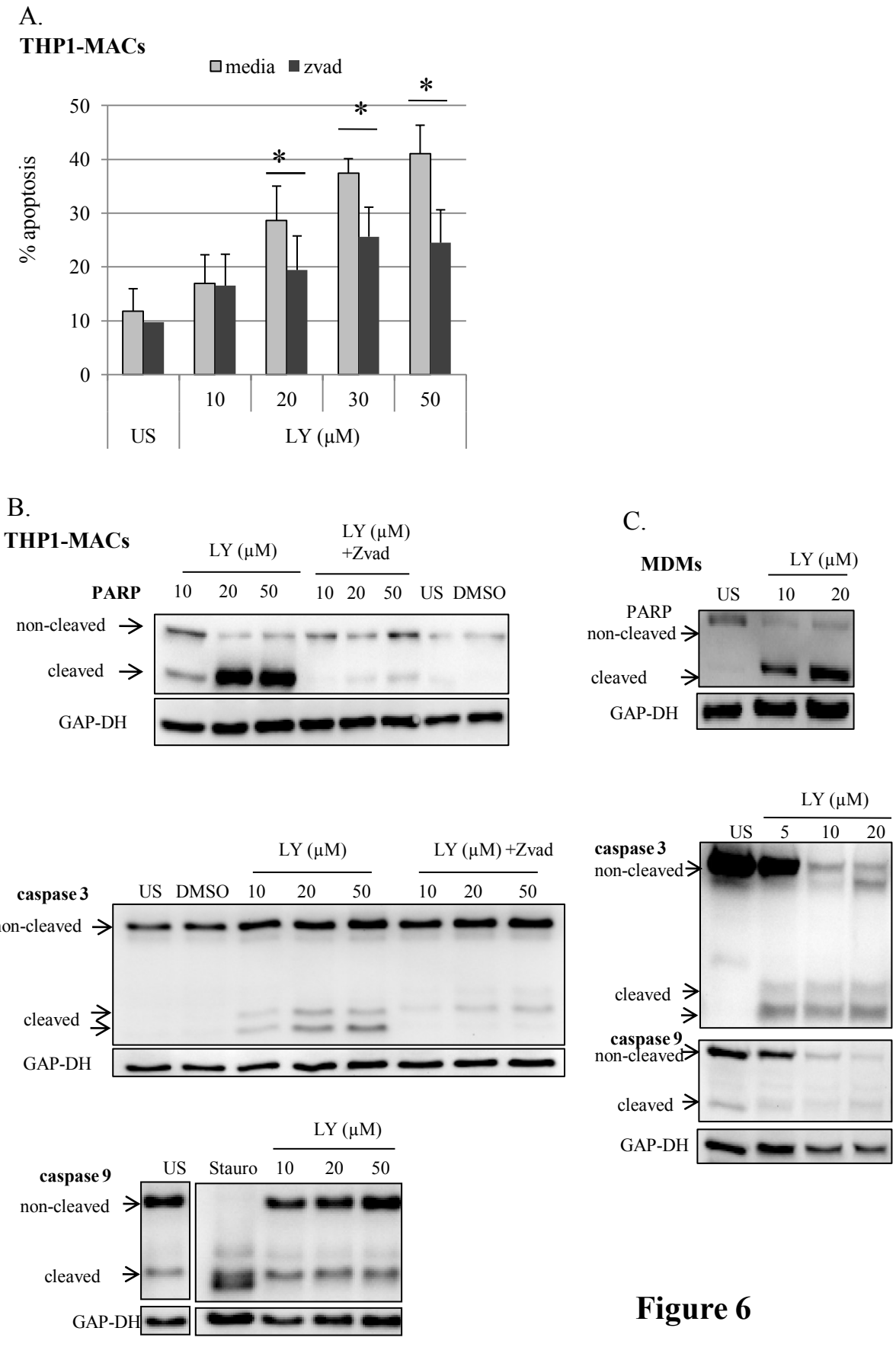

A.
THP1-MACs
media
zvad
% apoptosis
50
40
30
20
10
0
*
*
*
US
10
20
30
50
LY (μM)
B.
THP1-MACs
LY (μM)
LY (μM) +Zvad
PARP
10 20 50 10 20 50 US DMSO
non-cleaved
cleaved
GAP-DH
LY (μM)
LY (μM) +Zvad
caspase 3
US DMSO 10 20 50 10 20 50
non-cleaved
cleaved
GAP-DH
LY (μM)
caspase 9
US Stauro 10 20 50
non-cleaved
cleaved
GAP-DH
C.
MDMs
LY (μM)
US 10 20
PARP
non-cleaved
cleaved
GAP-DH
LY (μM)
US 5 10 20
caspase 3
non-cleaved
cleaved
caspase 9
non-cleaved
cleaved
GAP-DH

**Figure 6**

their involvement in LY294002-induced cell death during differentiation. My results show that both caspase-3 and its downstream substrate PARP were cleaved in LY294002-treated THP1-MACs (Fig. 6B) and MDMs (Fig. 6C) and their cleavage was prevented by Zvad (Fig. 6B). However, I did not detect caspase-9 cleavage in response to LY294002 in THP-1 cells (Fig. 6B) and MDMs (Fig. 6C). These results suggest that apoptosis induced following blockage of the PI3K pathway during differentiation is mediated by caspase-3 and not caspase-9.

**Akt maintains Bcl-xL expression during differentiation and Mcl-1 expression after differentiation in human macrophages**

We have previously shown that the levels of antiapoptotic Bcl-xL and Mcl-1 proteins are being upregulated during monocyte to macrophage differentiation (233). Since LY294002 pretreatment before differentiation induced cell death, I investigated if LY294002 treatment of human monocytic cells prior to differentiation impacted the expression of anti- or proapoptotic proteins. PMA treatment of THP1 cells upregulated the expression of antiapoptotic Bcl-xL, Mcl-1, cIAP1 and decreased the expression of proapoptotic Bax, with no effect on XIAP or Bcl2 (Fig. 7A, compare THP1 lanes with PMA lanes). However, LY294002 treatment prior to PMA stimulation attenuated the expression of Bcl-xL (Fig. 7A, left panel) and enhanced the expression of proapoptotic Bax protein (Fig. 7A, right panel) in a dose-dependent manner. In contrast, LY294002 pretreatment did not impact the expression of other antiapoptotic proteins tested including XIAP, cIAP1, Bcl2, and Mcl-1. As a control, treatment of THP1 cells with SB230185 prior to differentiation with PMA did not affect the expression of XIAP, cIAP1 and Bcl-xL (Fig. 7A, left panel).

**Figure 7. Akt maintains Bcl-xL expression during differentiation of human macrophages**

**A.** THP1 cells ($0.5x10^6$/ml) were treated with the indicated concentrations of LY294002 or SB203580 for 2 h before adding PMA 20 ng/ml for another 2 days to generate macrophages. THP1-MACs were collected after differentiation and whole cell protein extracts were subjected to Western blotting. Membranes were probed with specific antibodies against various members of the Bcl2 and IAP families. GAP-DH was used as a loading control. Images shown are representative for at least three experiments with similar results.
**B.** Primary monocytes were treated with the indicated concentrations of LY294002 (PI3K/Akt inhibitor) for 2 h before adding M-CSF 10 ng/ml for another 6 days to generate macrophages (left panel). Differentiated MDMs were treated with LY294002 for 48 h (right panel). Protein extracts were subjected to Western blotting and membranes were probed with specific antibodies against Bcl-xL, Bax and p-Akt. GAP-DH was used as a loading control. Images shown are representative for at least three experiments with similar results.
**C.** THP1 cells and primary monocytes were transfected with Akt siRNA or control siRNA as described in Materials and methods before inducing differentiation with PMA and M-CSF respectively. Protein extracts were subjected to Western blotting and membranes were probed with specific antibodies against Akt and various members of the Bcl2 family. GAP-DH was used as a loading control. Based on the densitometric analysis, the bar graph in the right lower panel shows the mean ± SD of relative Akt and Bcl2 proteins expression from three experiments with different donors.

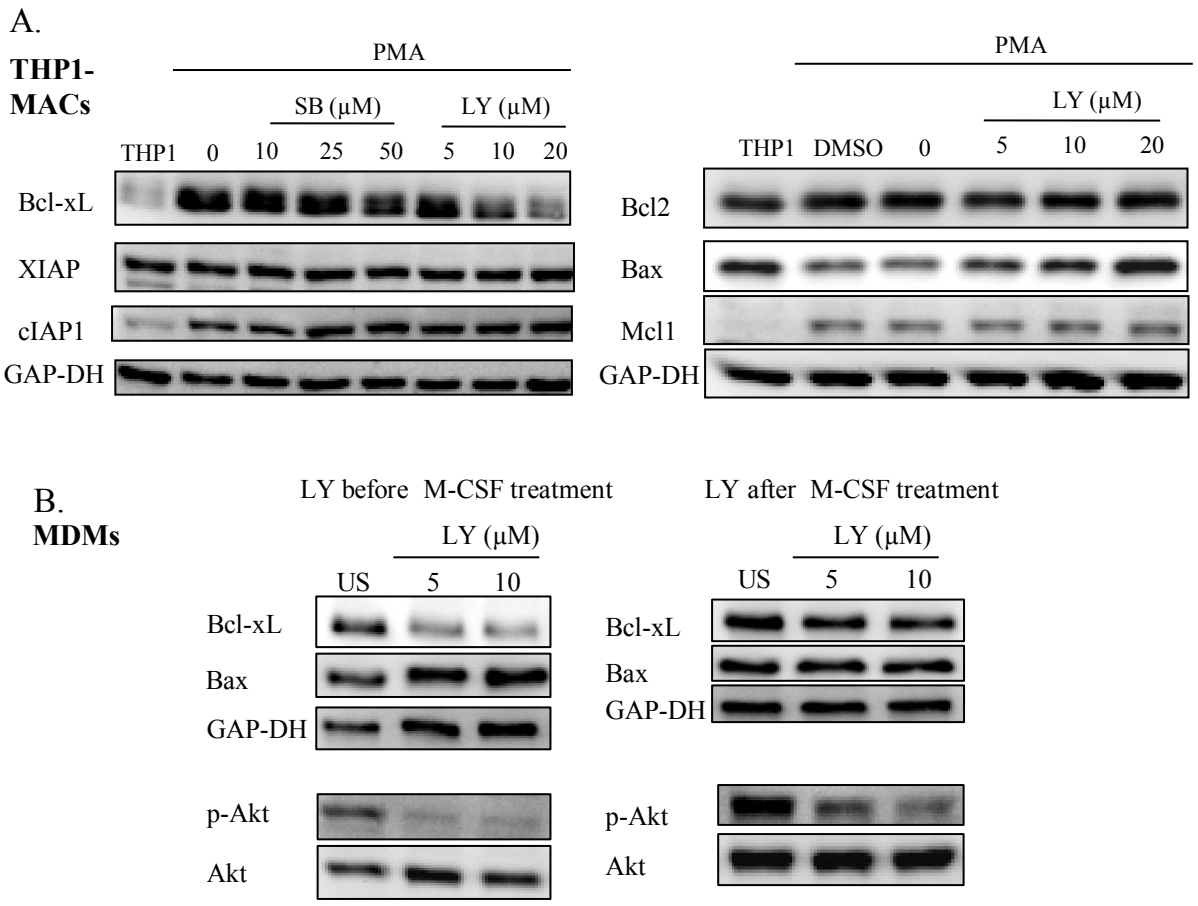

A.
THP1-MACs
PMA
SB (μM)
LY (μM)
THP1 0 10 25 50 5 10 20
Bcl-xL
XIAP
cIAP1
GAP-DH
PMA
LY (μM)
THP1 DMSO 0 5 10 20
Bcl2
Bax
Mcl1
GAP-DH
B.
MDMs
LY before M-CSF treatment
LY (μM)
US 5 10
Bcl-xL
Bax
GAP-DH
p-Akt
Akt
LY after M-CSF treatment
LY (μM)
US 5 10
Bcl-xL
Bax
GAP-DH
p-Akt
Akt
C.
THP1-MACs BEFORE
contr siRNA
Akt siRNA
Akt
100 55
Bcl-xL
100 76
Mcl-1
100 109
Bcl2
100 94
GAP-DH
MDMs BEFORE
contr siRNA
Akt siRNA
Akt
100 52
Bcl-xL
100 59
GAP-DH
Mcl-1
100 113
Bcl2
100 117
GAP-DH
Relative expression
140
120
100
80
60
40
20
0
*
*
contr siRNA
Akt siRNA
Akt expression
Bcl-xL expression
Mcl1 expression
Bcl2 expression

**Figure 7**

Similar to the results obtained with THP1 cells, LY294002 treatment of monocytes prior to differentiation into MDMs inhibited Bcl-xL expression (Fig. 7B, left panel). In contrast, there was no change in the expression of Bax (Fig. 7B, left panel). These results suggest that cell death observed following the blockage of the PI3K pathway in human monocytic cells during differentiation may be attributed to the inhibition of Bcl-xL or upregulation of Bax expression.

To confirm that the downregulation of Bcl-xL following LY294002 treatment is due to Akt inhibition, siRNA for Akt was employed. Monocytes and THP1 cells were transfected with Akt siRNA and then differentiated with M-CSF and PMA, respectively, followed by evaluation of antiapoptotic Bcl2 proteins levels. Akt siRNA successfully knocked down Akt protein levels in both THP1-MACs (Fig. 7C, left panel) and MDMs (Fig. 7C, right upper panel). Similar to the results obtained with LY294002, transfection of THP1 cells and monocytes with Akt siRNA prior to induction of differentiation resulted in a significant decrease in the expression of Bcl-xL but not of Mcl-1 and Bcl2 levels compared to cells transfected with control siRNA (Fig. 7C).

Inhibition of PI3K/Akt pathway in differentiated macrophages was shown to induce loss of antiapoptotic Mcl-1 protein, with no effect on Bcl-xL expression (134). Since my results suggest that inhibition of the PI3K pathway prior to differentiation decreased the expression of Bcl-xL without impacting the expression of Mcl-1 in both MDMs and THP1-MACs, I determined whether the inhibitory effect of LY294002 on Bcl-xL expression was specific to cells undergoing macrophage differentiation. For this, MDMs differentiated with M-CSF for 6 days were treated with LY294002 followed by evaluation of Bcl-xL expression after 48 hr. LY294002 treatment efficiently down regulated Akt phosphorylation in MDMs

(Fig. 7B, right panel). However, loss of Bcl-xL expression was detected only when cells were treated with LY294002 before M-CSF treatment (Fig. 7B, left panel) and this effect was not observed when cells were allowed to differentiate and then treated with LY294002 (Fig. 7B, right panel).

To confirm that Akt inhibition does not affect expression of Bcl-xL in differentiated macrophages, THP1-MACs and MDMs were transfected with Akt siRNAs followed by analysis of Bcl2 proteins expression. Although Akt siRNA effectively decreased Akt levels, it did not affect Bcl-xL levels, but significantly decreased the expression of Mcl-1 in both THP1-MACs (Fig. 8 left panel) and MDMs (Fig. 8 right panel). Following Akt inhibition in MDMs, there was also an increase in Bcl2 levels (Fig. 8 right panel), most likely as a compensatory mechanism for Mcl-1 loss. These results suggest that Bcl-xL and Mcl-1 may differentially regulate macrophage survival during and after differentiation.

**Bax increase following PI3K/Akt inhibition does not play a role in LY294002-induced apoptosis in human macrophages**

Since inhibition of PI3K pathway by LY294002 resulted in an increase in proapoptotic Bax in THP1-MACs (Fig. 7A) along with a loss of Bcl-xL expression in both MDMs and THP1-MACs (Fig. 7A and B), I investigated the involvement of Bax in LY294002-induced apoptosis by employing Bax siRNA in THP1-MACs. THP1 cells were transfected with Bax siRNA followed by LY294002 treatment prior to differentiation with PMA and subsequent analysis of apoptosis. My results show that despite successful knock-down of Bax protein levels by Bax siRNA, LY294002 induced comparable levels of apoptosis in control siRNA and Bax siRNA transfected cells (Fig. 9). These results suggest that upregulation of Bax following PI3K inhibition may not play a significant role in LY294002-induced apoptosis.

**Figure 8.Akt maintains Mcl-1 and not Bcl-xL expression in differentiated human macrophages**

THP1-MACs and MDMs were allowed to differentiate first with PMA and M-CSF respectively, then transfected with Akt siRNA or control siRNA for 48 h as described in Materials and methods. Protein extracts were subjected to Western blotting and membranes were probed with specific antibodies against Akt and various members of the Bcl2 family. GAP-DH was used as a loading control. Based on the densitometric analysis, the bar graph in the right lower panel shows the mean ± SD of relative Akt and Bcl2 proteins expression from three experiments with different donors.

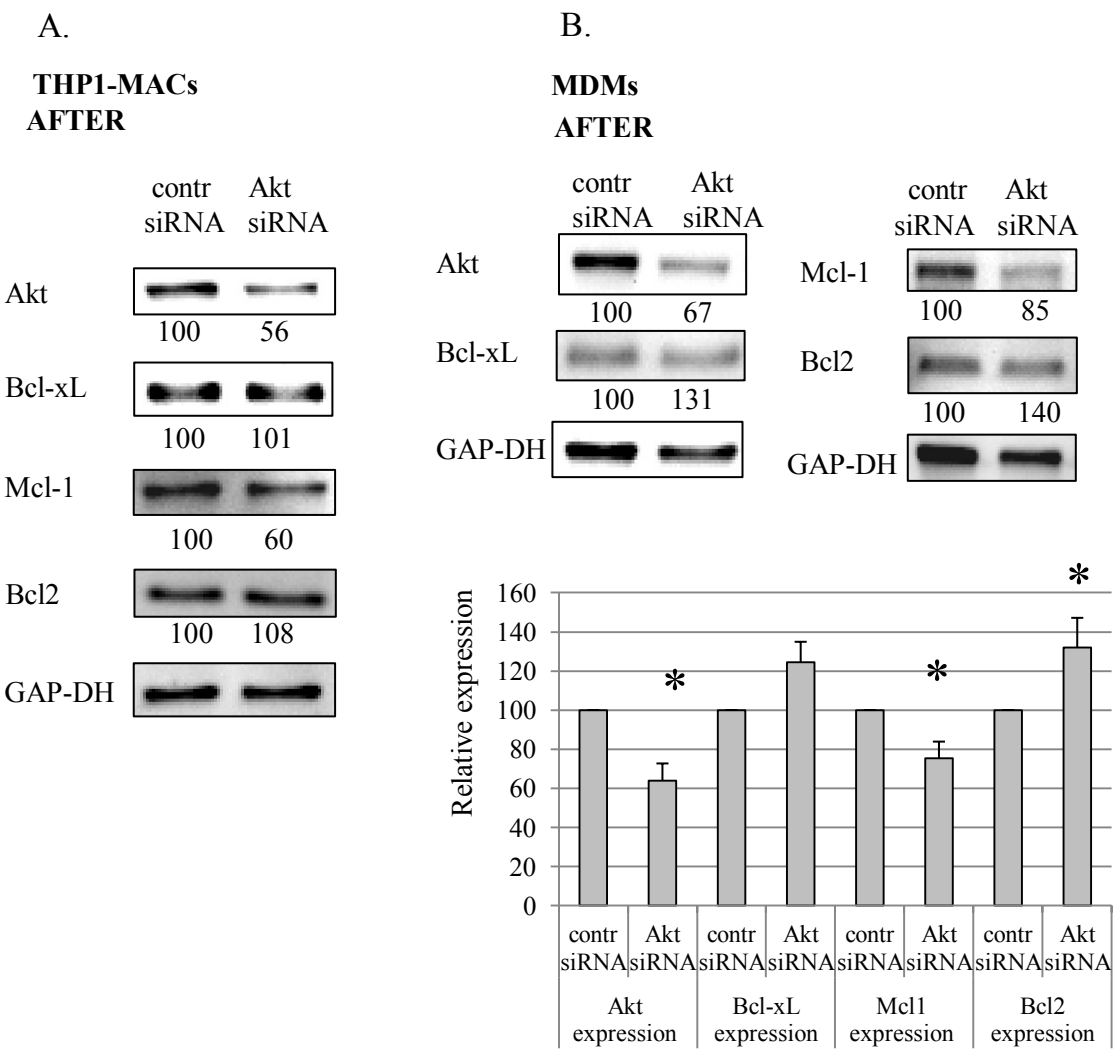

A.
THP1-MACs
AFTER
contr siRNA
Akt siRNA
Akt
100
56
Bcl-xL
100
101
Mcl-1
100
60
Bcl2
100
108
GAP-DH
B.
MDMs
AFTER
contr siRNA
Akt siRNA
Akt
100
67
Bcl-xL
100
131
GAP-DH
contr siRNA
Akt siRNA
Mcl-1
100
85
Bcl2
100
140
GAP-DH
Relative expression
160
140
120
100
80
60
40
20
0
*
*
*
contr siRNA
Akt siRNA
Akt expression
Bcl-xL expression
Mcl1 expression
Bcl2 expression

**Figure 8**

**Figure 9. Bax does not play a role in LY294002-induced apoptosis in human macrophages**

THP1 cells ($0.5x10^6$/ml) were transfected with Bax siRNA or control siRNA for 5 h before adding LY294002 for another 2 h. PMA 20 ng/ml was then added to induce differentiation and cells were collected another 2 days later. THP1-MACs were evaluated for efficiency of protein knock-down by Western blotting (left panel) or stained with PI to evaluate apoptosis by flow cytometry (right panel).

**THP1-MACs**

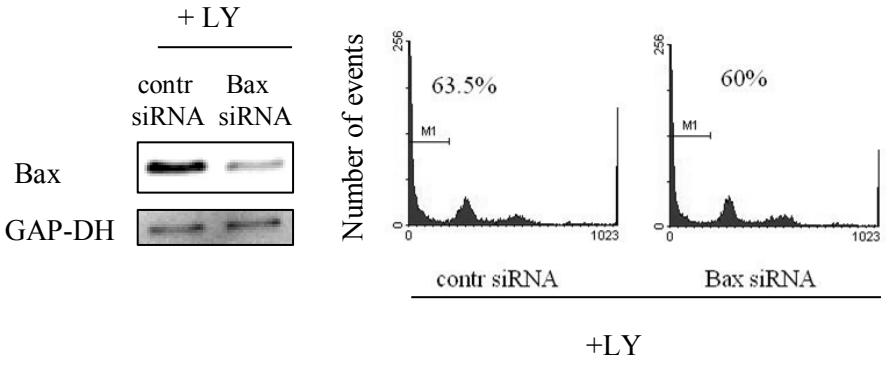



**Figure 9**

**Downregulation of Akt and Bcl-xL before differentiation causes apoptosis and caspase-3 cleavage in human macrophages.**

To confirm the roles of Akt and Bcl-xL in acquiring resistance to apoptosis during differentiation, THP1 cells and primary monocytes were transfected with Akt or Bcl-xL siRNAs before induction of differentiation with PMA or M-CSF, respectively. Transfection with Bcl-xL siRNAs effectively down regulated Bcl-xL levels in both differentiating THP1 cells and monocytes, without affecting the expression of other members of the Bcl2 family, including Mcl-1 and Bcl2 (Fig. 10A, left panel). Transfected THP1 cells and monocytes were allowed to differentiate with PMA and M-CSF, respectively, and then evaluated for caspase-3 cleavage and/or annexin-V staining as a measure for apoptosis. In both MDMs and THP1-MACs, siRNA inhibition of Bcl-xL resulted in higher degree of caspase-3 cleavage compared to control siRNA transfected cells (Fig. 10A, right panel). There was also higher annexin-V staining in both Bcl-xL and Akt siRNA transfected cells compared to control siRNA transfected cells, in both MDMs and THP1-MACs (Fig. 10B). These results confirm above observations obtained with the inhibitors that blockage of the PI3K pathway during differentiation induces apoptosis through the downregulation of Bcl-xL expression in human macrophages.

**Downregulation of Akt and Mcl-1 after differentiation causes apoptosis and caspase-3 cleavage in human macrophages.**

The above results show that treatment of differentiated macrophages with LY294002 resulted in the downregulation of Mcl-1 (Fig. 8) and subsequent apoptosis (Fig. 4B and 5B). To confirm the role of Mcl-1 in LY294002-induced apoptosis in differentiated macrophages,

**Figure 10. Downregulating Akt and Bcl-xL before differentiation causes apoptosis and caspase 3 cleavage in human macrophages**

A. THP1 cells and primary monocytes were transfected with Bcl-xL siRNA or control siRNA as described in Materials and methods before inducing differentiation with PMA and M-CSF respectively. Protein extracts were subjected to Western blotting and membranes were probed with specific antibodies against Bcl-xL, Mcl-1 and Bcl2 to evaluate knock-down efficiency (left panel) or anti-caspase-3 antibody to evaluate its cleavage as a marker of apoptosis (right panel). GAP-DH was used as a loading control. Results shown are representative of three different experiments with similar results.
B. Following adherence on coverslips, cells were treated as in A. After differentiation cells were stained with annexin-V and DAPI according to the protocol described in Materials and methods. Images were acquired with a Zeiss LSM 510 Meta confocal microscope using a 63X objective. Total magnification 189X.

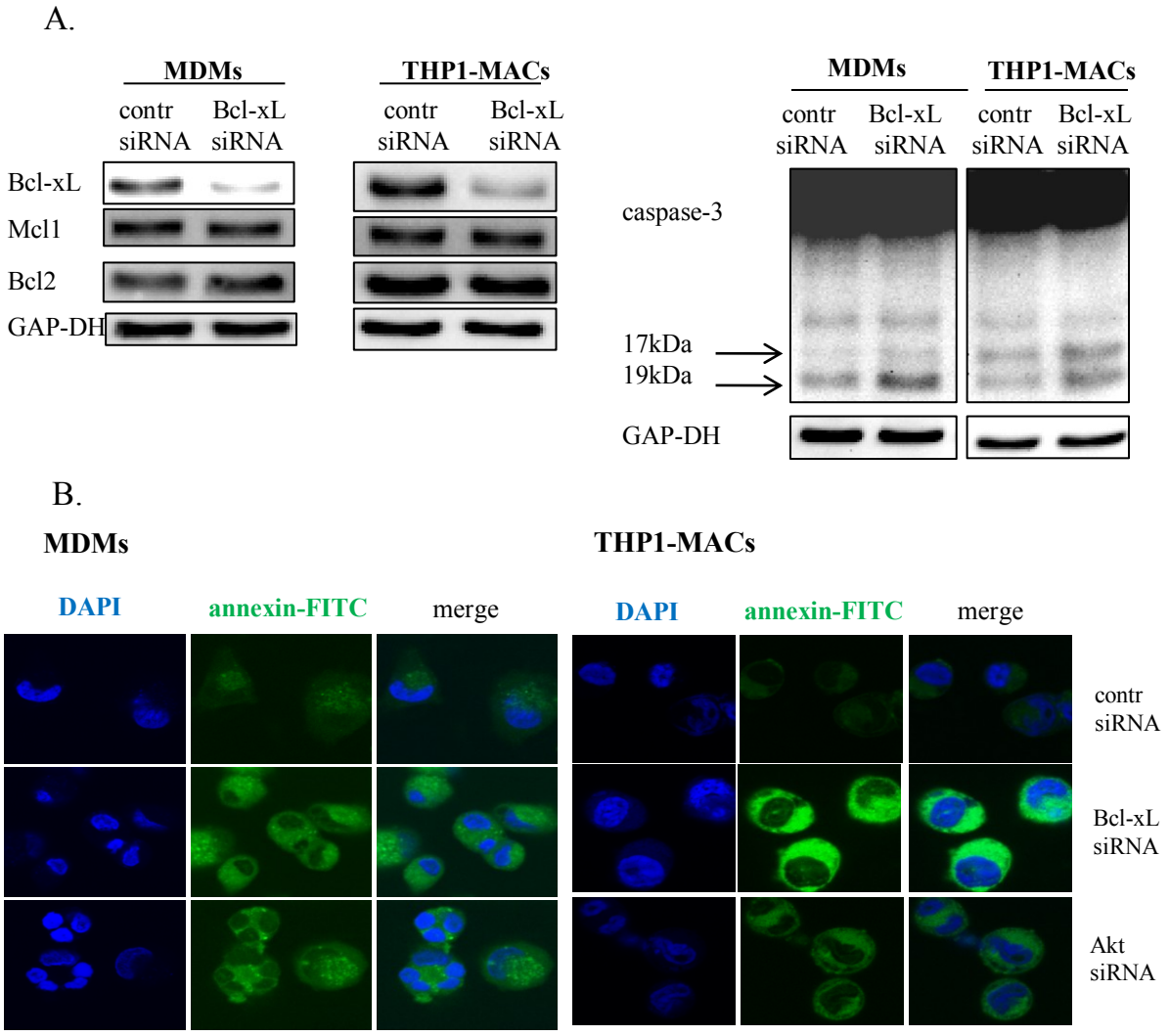

A.
MDMs
THP1-MACs
contr siRNA
Bcl-xL siRNA
Bcl-xL
Mcl1
Bcl2
GAP-DH
caspase-3
17kDa
19kDa
B.
DAPI
annexin-FITC
merge
contr siRNA
Bcl-xL siRNA
Akt siRNA

**Figure 10**

I transfected THP1-MACs with Akt- or Mcl-1-specific siRNAs followed by analysis of caspase-3 cleavage and apoptosis. Transfection with Mcl-1 resulted in the inhibition of Mcl-1 expression (Fig. 11A), cell death (Fig. 11B) and higher levels of caspase-3 cleavage (Fig. 11C) compared to the cells transfected with control siRNAs. Similarly, transfection of THP1-MACs with Akt siRNA resulted in a significant increase in caspase-3 cleavage that was comparable to that observed in Mcl-1 siRNA transfected cells (Fig. 11C). These results confirm above observations that PI3K/Akt distinctively regulates cell survival during and after macrophage differentiation, with a specific involvement of antiapoptotic Bcl-xL during the differentiation process, since its expression is no longer dependent on Akt in terminally differentiated macrophages. In contrast, PI3K/Akt maintains survival of in differentiated macrophages through the expression of antiapoptotic Mcl-1.

**NFκB regulates macrophage survival during differentiation through Bcl-xL expression**

Since NFκB has been shown to contribute to survival of differentiated macrophages (49, 133) and Bcl-xL levels are inducible via NFκB in other cell types, such as T (266) and B cells (267), I determined if PI3K-induced Bcl-xL contributes to macrophage survival during differentiation via NFκB activation. NFκB dimers are located in the cytoplasm, bound by IκB proteins and thus prevented from entering the nucleus. Upon activation, IκB proteins are phosphorylated and targeted for degradation, which allows the NFκB to translocate to the nucleus and initiate transcription of dependent genes (268). To study the role of NFκB in mediating survival of differentiating macrophages, I used CAPE (269) and parthenolide (270) as NFκB specific chemical inhibitors. Similar to LY294002,

**Figure 11. Downregulating Akt and Mcl-1 causes apoptosis and caspase 3 cleavage in differentiated macrophages**

THP1 cells were allowed to differentiate first with PMA for 48h, then transfected with Akt siRNA, Mcl-1 siRNA or control siRNA for 48 h as described in Materials and methods. Protein extracts were subjected to Western blotting and membranes were probed with specific antibodies against Mcl-1 (A) and caspase-3 (C). GAP-DH was used as a loading control. Mcl-1 transfected cells were also evaluated for apoptosis by PI staining and flow cytometry (B). Images shown are representative for at least three experiments with similar results.

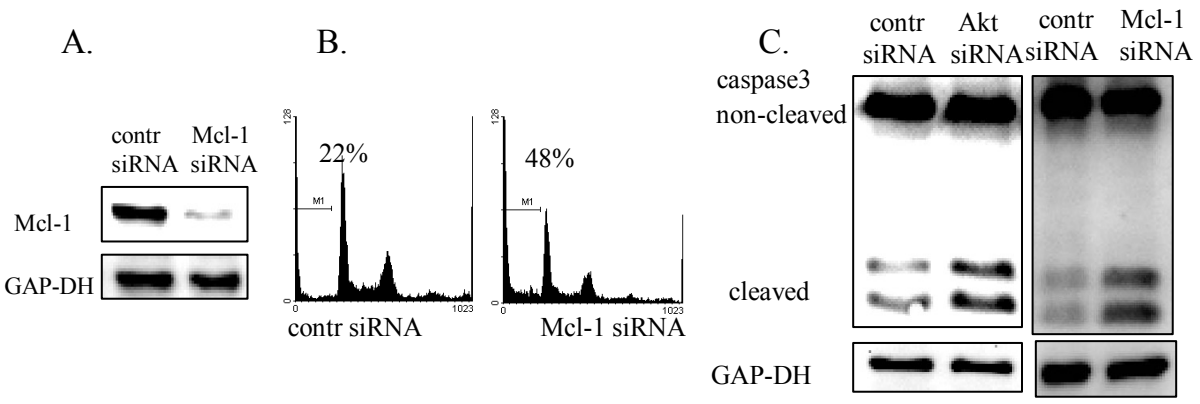

A.
contr siRNA
Mcl-1 siRNA
Mcl-1
GAP-DH
B.
22%
48%
M1
contr siRNA
Mcl-1 siRNA
C.
contr siRNA
Akt siRNA
contr siRNA
Mcl-1 siRNA
caspase3
non-cleaved
cleaved
GAP-DH

**Figure 11**

treatment of THP1 cells and primary monocytes with CAPE and parthenolide prior to induction of differentiation with PMA and M-CSF induced cell death in both MDMs (Fig. 12A, left panel) and THP1-MACs (Fig. 12A, right panel). Moreover, parthenolide or CAPE treatment alone, in the absence of PMA, did not cause cell death in undifferentiated THP1 cells (Fig. 12A, right panels), suggesting that NFκB activity was specifically necessary for survival during the differentiation process. CAPE activity was confirmed in MDMs by its ability to increase the levels of IκBα (Fig. 12A, left lower panel).

Since Bcl-xL expression was required for survival during macrophage differentiation, I determined if NFκB regulated Bcl-xL expression during differentiation. Results show that treatment of monocytes or THP1 cells with either CAPE or parthenolide prior to differentiation with M-CSF or PMA, respectively, inhibited the expression of Bcl-xL protein in both MDMs and THP1-MACs (Fig. 12B), in addition to inducing apoptosis. These results suggest that NFκB-mediated Bcl-xL expression may regulate macrophage survival during differentiation.

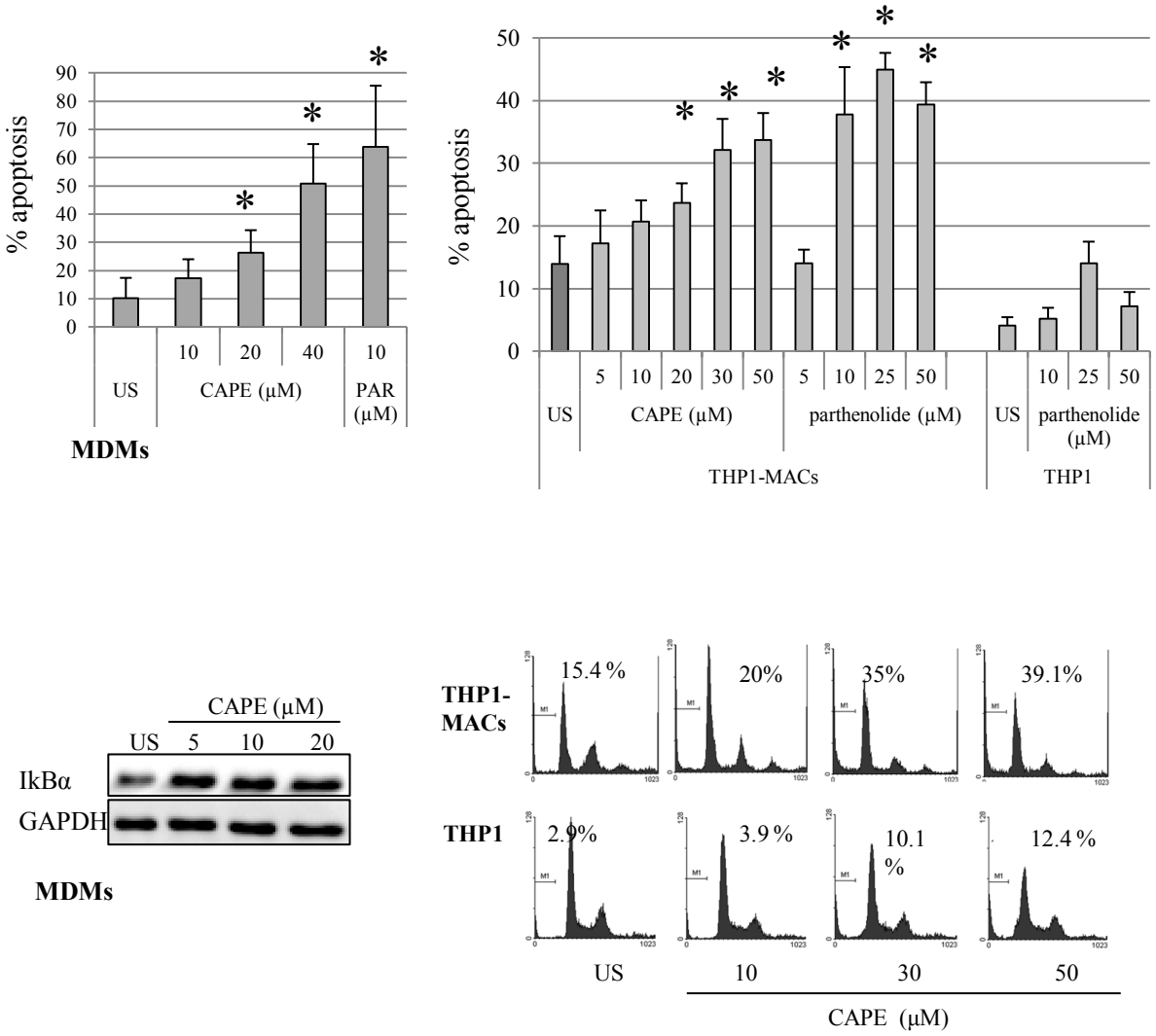
**PI3K/Akt mediates Bcl-xL induction via NFκB activation during macrophage differentiation**

Akt kinase can contribute to NFκB activation by promoting phosphorylation and degradation of IκB proteins (271). However, PI3K/Akt and NFκB have also been reported to function as separate survival pathways in differentiated macrophages (134). Since PI3K/Akt and NFκB mediate survival and Bcl-xL expression of differentiating macrophages, I determined if PI3K contributes to macrophage survival during differentiation by inducing NFκB dependent Bcl-xL expression. To understand if PI3K/Akt contributes to NFκB activation during macrophage differentiation, I measured the effect of PI3K inhibition on

**Figure 12. NFκB inhibition before initiation of macrophage differentiation causes apoptosis and loss of Bcl-xL expression in differentiating macrophages**

**A.** Primary monocytes (left panel) and THP1 cells (right panel) were treated with the indicated concentrations of CAPE and parthenolide before inducing differentiation with M-CSF or PMA respectively. THP1 cells treated with inhibitor alone (no PMA) were included as controls. Following differentiation cells were evaluated for apoptosis by flow cytometry using intracellular PI staining. The numbers represent percentage apoptotic cells with subdiploid DNA content. The bar graphs show the mean of % apoptotic cells ± SD of four experiments with each cell type. * indicates $p<0.05$. Lower panel histograms show data from one experiment with CAPE were THP1 controls (no PMA added) were included. Left lower panel: MDMs were collected after CAPE treatment and differentiation and protein extracts were subjected to Western blotting. Membrane was probed with anti-IκBα antibody to test the biological activity of CAPE. GAP-DH was used as a loading control.
**B.** Cells treated as in A were collected and evaluated by Western blotting for Bcl-xL expression. GAP-DH was used as a loading control.

A.


% apoptosis
US
CAPE (μM)
PAR (μM)
MDMs
parthenolide (μM)
THP1-MACs
THP1
IkBα
GAPDH
THP1-MACs
THP1
15.4 %
20%
35%
39.1%
2.9%
3.9 %
10.1 %
12.4 %
CAPE (μM)

B.

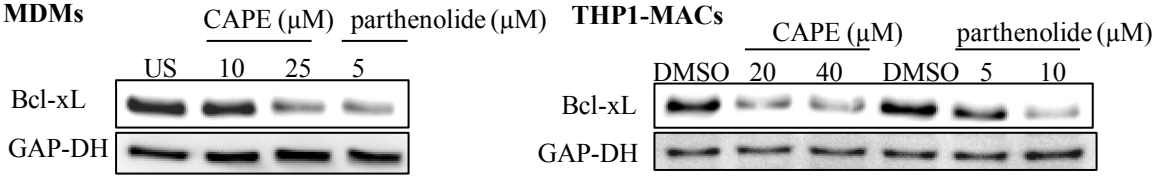

MDMs
CAPE (μM)
parthenolide (μM)
THP1-MACs
DMSO
Bcl-xL
GAP-DH

**Figure 12**

IκBα expression during the early stages of PMA-induced differentiation in THP1 cells. Treatment of THP-1 cells with PMA led to a gradual increase of IκBα degradation, indicative of NFκB activation (Fig. 13A). However, treatment of THP1 cells with LY294002 prior to PMA stimulation prevented PMA-induced IκBα degradation, resulting in significant accumulation (Fig. 13B), suggesting that PI3K/Akt blockage in THP1 cells resulted in NFκB inhibition. Similar results were obtained in MDMs, where blockade of PI3K pathway by LY294002 prevented IκBα degradation following differentiation with M-CSF (Fig. 13C).

I have also analyzed whether NFκB contributes to Bcl-xL expression following PMA stimulation and whether PI3K regulates NFκB activity in THP1 cells by EMSA. Oligonucleotides containing NFκB binding site of the Bcl-xL promoter were used as probes. THP1 cells were treated with LY294002 prior to stimulation with PMA followed by analysis of NFκB activation by EMSA. My results show that PMA activation enhanced binding of NFκB to the probes and that this effect was inhibited in cells pretreated with LY294002 (Fig. 13D). Parthenolide was used as a positive control for NFκB inhibition. Overall, these results suggest that PI3K/Akt-mediated NFκB activation regulates survival of human macrophages during differentiation through Bcl-xL expression.

**Figure 13. PI3K/Akt is required for NFκB activation during macrophage differentiation**

THP1 cells were treated with PMA 20ng/ml for the indicated times. TNF-α treatment (10 ng/ml for 30 min) was used as a positive control for IκBα downregulation (NFκB activation) **(A).** THP1 cells **(B)** and primary monocytes **(C)** were treated with the indicated concentrations of LY294002 before inducing differentiation. Cells were then collected and protein extracts were subjected to Western blotting. Membranes were probed with anti-IκBα antibody and GAP-DH was used as a loading control. THP1 cells were treated with LY294002 or parthenolide for 2 h, followed by PMA for 30 min. **(D)** Nuclear proteins were used to perform EMSA using oligonucleotides corresponding to the NFκB binding site on the human Bcl-xL gene promoter. For the cold competition sample unlabeled oligonucleotides were used at a 100-fold molar excess and the negative control has no protein.

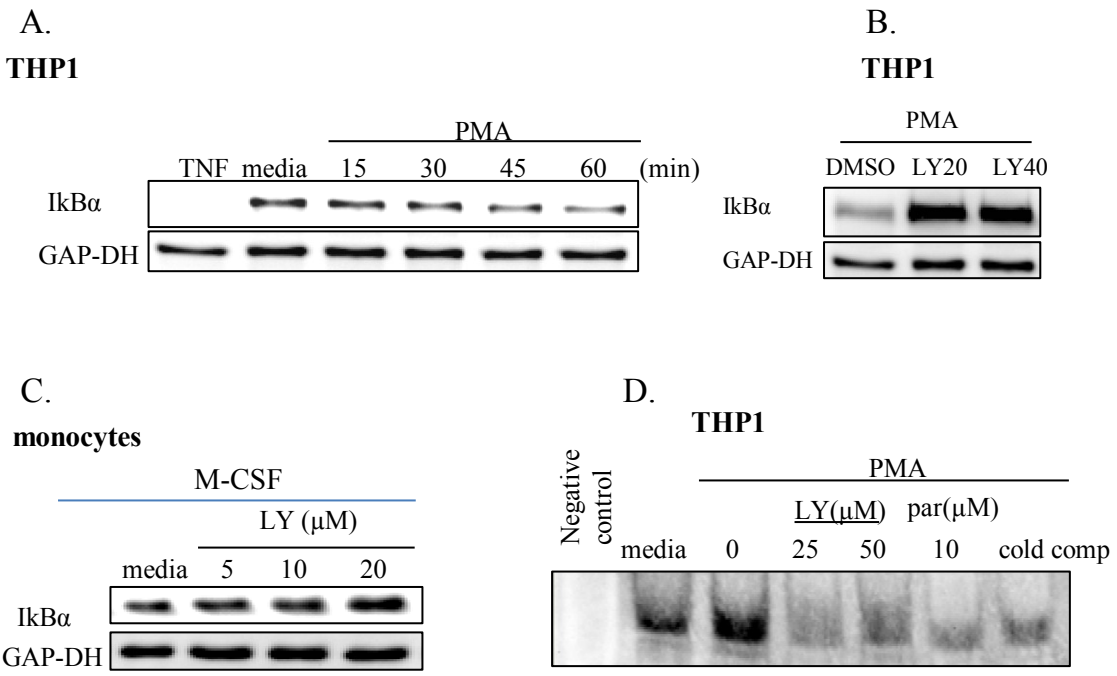

A.
THP1
PMA
TNF media 15 30 45 60 (min)
IkBα
GAP-DH
B.
THP1
PMA
DMSO LY20 LY40
IkBα
GAP-DH
C.
monocytes
M-CSF
LY (μM)
media 5 10 20
IkBα
GAP-DH
D.
THP1
Negative control
PMA
LY(μM) par(μM)
media 0 25 50 10 cold comp

**Figure 13**

## Discussion

My results suggest for the first time that PI3K/Akt pathway differentially regulates macrophage survival by maintaining the expression of Bcl-xL during the differentiation process and of Mcl-1 after differentiation. Although Akt and NFκB are known independent survival pathways in differentiated cells, I show that in monocytes undergoing differentiation, Akt-activated NFκB constitutes one survival pathway that promotes Bcl-xL expression during the differentiation process. Bcl-xL expression, however, does not remain dependent on PI3K/Akt signalling in the differentiated macrophages, wherein survival is regulated by the PI3K/Akt-activated Mcl-1 expression.

The initial step in the Akt/NFκB/Bcl-xL signaling cascade is PI3K/Akt activation in response to stimulation of monocytes and THP-1 cells with M-CSF and PMA, respectively. I have observed a basal level of constitutive activation of PI3K/Akt in these cells probably due to isolation and culture conditions that promote plastic adherence and subsequent activation of monocytic cells (272). Nevertheless, the levels of phosphorylated Akt were enhanced above the basal level at early time points during differentiation. Oscillations of Akt activation have been linked to caspase activation that promotes monocyte differentiation in the absence of apoptosis (139). My results show that PI3K/Akt activation also promotes survival during differentiation by maintaining Bcl-xL expression. Using siRNA technology, I have shown that knocking-down Akt levels in monocytic cells prior to differentiation significantly inhibited the expression of Bcl-xL without affecting the expression of Bcl2 or Mcl-1. In contrast, knocking down Akt levels in differentiated macrophages did not impact Bcl-xL expression, but significantly decreased the levels of Mcl-1 antiapoptotic protein. The molecular mechanism governing this process by which Bcl-xL expression becomes

independent of PI3K/Akt signalling and Mcl-1 expression is dependent on Akt pathway in the differentiated macrophages needs to be investigated.

The chemical inhibitor and siRNA experimental results from monocytic cells prior to induction of differentiation suggest that both Akt and Bcl-xL are necessary to promote survival. However, the broad caspase inhibitor, Zvad-fmk, caused a significant albeit incomplete prevention of apoptosis following PI3K/Akt inhibition, despite its increased efficiency in inhibiting LY294002-induced caspase-3 and PARP cleavage. This suggests that both caspase-dependent and caspase-independent mechanisms are operative in cells undergoing apoptosis following PI3K/Akt blockage. The caspase-dependent cell death involved cleavage of caspase-3 and its downstream target PARP. Since these effects were amenable to Zvad-fmk inhibition, I speculate that caspases are responsible for about 50% of the LY294002-induced apoptosis (Fig. 6A). Caspase-3 is the main effector caspase that undergoes proteolytic cleavage and activation as a result of a cascade activation initiated by the upstream initiator caspases, such as caspase-9 (273). Interestingly, I did not detect caspase-9 cleavage in response to PI3K/Akt inhibition in both MDMs and THP1-MACs (Fig. 6B and C). As a result, the involvement of other caspases such as caspase-2 (274) or caspase-8 (275) in this process cannot be ruled out. Furthermore, how PI3K blockage activates caspase-3 or alternatively caspases-2 or 3 in differentiating macrophages needs to be investigated.

The caspase independent mechanisms that may contribute to cell death following PI3K/Akt inhibition may include release of mitochondrial apoptotic molecules such as AIF (apoptosis-inducing factor), endo-G (endonuclease G) and SMAC (second mitochondria-derived activator of caspases). Inhibition of Akt activity has been shown to facilitate AIF

dependent cell death in cancer cells (276) and endo-G and SMAC-mediated apoptosis in endothelial cells (277). Further studies are needed to address the potential implications of these caspase-independent mechanisms in macrophage cell death following PI3K/Akt inhibition.

The results of this study suggest that antiapoptotic Bcl-xL protein is paramount in survival of differentiating monocytic cells. While Bcl-xL has previously been shown to confer resistance to cell death in other hematopoietic lineages, such as erythrocytes (261) and thrombocytes (263), this is the first report showing its involvement in monocyte to macrophage differentiation. The molecular mechanism by which Bcl-xL prevents apoptosis and caspase-3 cleavage in differentiating macrophages has not been investigated, but it may involve prevention of mitochondrial membrane permeabilization and subsequent release of cytochrome-c. Apart from proapoptotic (Bax, Bak) and prosurvival proteins (Bcl2, Bcl-xL, Mcl-1), Bcl2 family also contains "BH3-only" members with regulatory function (Bad, Bim), that can initiate activation of Bax-like proapoptotic proteins (32). In this context, there are two main models that try to explain Bcl-xL activity: Bcl-xL can either directly bind activated Bax and thus prevent its pore forming action (278) or it can indirectly inhibit Bax by binding to a BH3-only activator such as tBid (279). My results show that LY294002 treatment of THP1-MACs before differentiation also leads to increased Bax expression, in addition to a loss of Bcl-xL expression. However, Bax siRNA experiments showed similar levels of cell death in response to LY294002 in the presence or absence of Bax (Fig. 9), suggesting that Bax presence is dispensable for cell death in this context. Moreover, there was no change in Bax levels following LY294002 treatment in differentiating MDMs, indicating that loss of Bcl-xL was sufficient to induce apoptosis in this cell type.

Activation of the Akt/NFκB signaling cascade has been reported to promote cell growth and metastasis of cancer cells (280, 281), survival of lymphocytes (282) and endothelial cells (283), and T cell activation (284). NFκB activation through the classical pathway involves proteosomal degradation of the sequestering proteins IκB, which allows the NFκB subunits to translocate to the nucleus and induce gene expression (285). Phosphorylation of IκBα at serine residues 32 and 36 is required to target it for subsequent degradation (286) and it is carried out by the upstream IκB kinase (IKK) complex, composed of kinases IKKα and IKKβ and the regulatory subunit, NFκB essential modulator (NEMO) (268). IKK activation is also regulated through phosphorylation and the serine–threonine kinase Akt has been reported to activate NFκB via this mechanism (271, 287). Regulation of *bcl-xL* gene via NFkB activation has been shown to confer anti-apoptotic properties in CD40-mediated survival signals in human B cells (267). Whether NFκB-activated Bcl-xL confers antiapoptotic signals in human monocytic cells remains unknown. In this study, I initially investigated if NFκB contributes to maintaining Bcl-xL expression during the process of macrophage differentiation. My results show that inhibition of NFκB before inducing differentiation of MDMs and THP1-MACs with M-CSF or PMA, respectively, caused cell death and loss of Bcl-xL expression. Subsequently, I evaluated if Akt contributes to activation of NFκB pathway and the impact of this effect on cell survival during monocyte to macrophage differentiation, given the fact that both PI3K/Akt and NFκB pathways were involved in maintaining Bcl-xL expression. Akt inhibition with LY294002 was able to inhibit the DNA binding activity of NFκB in response to PMA in THP1-MACs. Similarly, LY294002 treatment increased IκBα protein expression in both MDMs and THP1-MACs models of macrophage differentiation, which was indicative of NFκB inhibition.

Collectively, these results suggest that NFκB activation is PI3K/Akt dependent in differentiating macrophages. Interestingly, this is a different mechanism than observed in differentiated macrophages, wherein NFκB and PI3K/Akt constitute two distinctive survival pathways that seem to function independently (134).

The antiapoptotic role of Mcl-1 has been described for other cell types, including macrophages (134), neutrophils (72) and granulocytes (73). I have shown for the first time that Mcl-1 contributes to cell survival and its expression is regulated via PI3K/Akt pathway only in differentiated macrophages and not during the process of monocyte differentiation. Moreover, PI3K/Akt is a component of a signaling pathway that includes NFκB and Bcl-xL, and not Mcl-1. Currently, I am addressing the role of NFκB in Mcl-1 expression in differentiated cells. The molecular mechanism of Mcl-1 antiapoptotic activity involves binding to proapoptotic Bcl2 proteins such as Bak to prevent mitochondrial damage (42). The exact mechanism by which Mcl-1 promotes cell survival in differentiated macrophages and the involvement of other Bcl2 family members such as Bak needs to be addressed in future studies.

In summary, this study shows that PI3K/Akt pathway contributes to macrophage survival via different mechanisms before and after the differentiation process. Understanding the molecular pathways involved in monocyte survival throughout differentiation may allow targeting of key intracellular events in these cells, such as Akt activation or Bcl-xL induction, in order to reduce cell numbers. This could be accomplished at the differentiation timepoint by preventing formation of new macrophages when their presence is no longer advantageous. Targeted apoptosis of differentiating macrophages may prove beneficial in chronic inflammatory conditions such as rheumatoid arthritis (288) or Crohn's disease (289).

## Objective 2: To evaluate the sensitivity of human macrophages to the effect of HIV-Vpr

### Introduction

Persistence of latent viral reservoirs is the main barrier that prevents eradication and cure of HIV infection. Memory CD4+ T cells and macrophages represent the two major viral reservoirs. While CD4+ T cells ultimately decrease as a result of incessant viral replication leading to cell death and reach alarmingly low levels characteristic of AIDS associated lymphopenia, macrophages support viral replication without any cytopathic effects (8, 151). Moreover, infected macrophages contribute to viral dissemination and bystander cell apoptosis (187). Formation of viral reservoirs in memory T cells involves a very low number of infected lymphocytes that contain integrated provirus and support viral replication only when reactivated with their cognate antigen. However, the mechanisms that enable macrophages to become viral reservoirs are poorly understood, but may include an intrinsic resistance to apoptosis acquired during differentiation (121) or an indirect result of infection or specific viral proteins (181, 182, 192) .

One of the factors that contribute to HIV persistence in macrophages is their resistance to various apoptotic stimuli. This characteristic is acquired as a result of a complex differentiation process that results in increased phagocytic and secretory functions and enables macrophages to perform their function in stressful environments (1). Primary monocytes are not susceptible to *in vitro* HIV infection (169), but they become highly permissive after differentiation (171). Increased susceptibility to infection and a resistant phenotype acquired during differentiation are important factors that are believed to promote viral reservoir formation in macrophages (290).

Since resistance to apoptosis plays a key role in HIV persistence in cellular reservoirs, I investigated how monocyte to macrophage differentiation would impact resistance to apoptosis in the context of HIV infection. In order to circumvent the lack of productive *in vitro* infection of primary human monocytes, I used the viral protein R (Vpr) as an apoptosis inducing agent. Vpr is a 96 aa, 14kDa accessory protein of HIV, known for its ability to cause apoptosis in several cell types (221, 291) including lymphocytes (214, 215), monocytes (206) and neurons (216). It has been shown that the two halves of Vpr are functionally distinct: N-terminal 1-51 aa of Vpr, Vpr(1-51), is responsible for nuclear localization of the preintegration complex, whereas C-terminal 52-96 aa, Vpr(52-96), induces cell cycle arrest and apoptosis (292, 293). Moreover, Vpr is required for HIV to infect macrophages (215), and extracellular Vpr can rescue replication of Vpr-deficient HIV strains in macrophages (241).

The expression of the main antiapoptotic Bcl2 and IAP (inhibitors of apoptosis) family members has been linked to cell survival (294). Apoptosis induced through the intrinsic pathway is initiated by proapoptotic members of the Bcl2 family such as Bax when not bound and thus not inactivated by antiapoptotic members. Bax forms pores into the mitochondrial membrane through which apoptogenic factors are released into the cytosol (10). The basal levels of antiapoptotic Bcl2 proteins, namely A1 (49), Mcl-1 (134), Bcl-xL (59) and Bcl2 (66) gradually increase during macrophage differentiation and contribute to the development of resistance to apoptosis in different model systems. HIV infection has been shown to induce the expression of Bcl2 and Bcl-xL in human macrophages (182). These antiapoptotic proteins were also shown to play a role in HIV Tat- and HIV Nef-mediated resistance to apoptosis (181, 193). In contrast, members of the IAP family act on

caspases activated either through the extrinsic (death receptor) or the intrinsic (mitochondrial) pathway (273). X-linked-IAP (XIAP) upregulation during macrophage differentiation was linked to their enhanced survival (66, 67). However, the role of IAPs in enhanced survival of macrophages in HIV infection remains unknown.

Biologically active Vpr is released from infected cells and has been detected in the serum and cerebrospinal fluid of HIV-infected patients (229), but its effect on survival of human macrophages remains unknown. I show for the first time that differentiated macrophages, unlike primary monocytes, are resistant to the apoptotic effects of Vpr suggesting that differentiation may render macrophages resistant to the cytopathic effects of Vpr present in the circulation. To investigate the mechanism underlying the development of this resistance to Vpr-induced apoptosis, I show that Bcl-xL and Mcl-1 play a critical role in macrophage survival in the absence of apoptotic stimuli. In contrast, downregulation of IAPs render macrophages susceptible to the apoptotic effects of Vpr suggesting a critical role this family may play in acquiring resistance against Vpr-induced cell death.

## Results

### Macrophages are resistant to Vpr-induced apoptosis compared to undifferentiated monocytic cells

To determine whether macrophage differentiation influences susceptibility to Vpr-induced apoptosis, I measured apoptosis in response to Vpr in undifferentiated primary monocytes and THP1 cells and compared it with differentiated MDMs and THP1-MACs. I utilized synthetic C terminus Vpr(52-96) peptide, as it is free of contaminating bacterial products present in recombinant Vpr proteins and other factors present in retroviral supernatants which may non-specifically activate various signaling pathways. Moreover, Vpr(52-96) peptide mimics the apoptotic activity of full length Vpr peptide (221). The commercial synthesis of full length Vpr peptide (1-96) was not feasible for the purpose of apoptosis induction since its length may have caused stability and functionality issues. Unless otherwise specified, I will refer to the Vpr(52-96) peptide as Vpr. Primary monocytes, THP1 cells, THP1-MACs and MDMs were treated with Vpr for varying periods of time followed by measurement of apoptosis by intracellular PI and annexin-V staining. Vpr induced a high level of apoptosis in THP1 cells and primary monocytes in a dose-dependent manner (Fig. 14 upper and middle left panels). In contrast, the apoptotic effect of Vpr was completely abolished in MDMs (Fig. 14, upper left panel). Mutant Vpr (mVpr) was used as a negative control, as it does not induce apoptosis (Fig. 14, left lower panel).

Treatment of THP1-MACs with Vpr caused apoptosis to moderately low levels of two fold compared to the 12 fold increase seen in THP1 cells. THP1-MACs exhibited a significant reduction in cell death, with an apoptosis level of around 30% even with the highest dose of Vpr peptide (Fig. 14 left middle panel). Vpr-induced apoptosis was

**Figure 14. Monocyte differentiation confers resistance to Vpr induced apoptosis**

THP1 cells, MDMs and THP1-MACs ($0.5x10^6$/ml) were treated with the indicated concentrations of Vpr or mVpr for 24 h. Primary monocytes ($0.5x10^6$/ml) were treated with Vpr for 4 h. Apoptosis was measured by flow cytometry using intracellular PI and/or annexin-V staining. The numbers represent percentage apoptotic cells with subdiploid DNA content (MDMs and THP1-MACs) or annexin-V positive cells (THP1, monocytes). Left panels show the mean of % apoptotic cells ± SD of at least three experiments. Right panel histograms show data from one representative experiment with each cell type.
* indicates $p<0.05$.

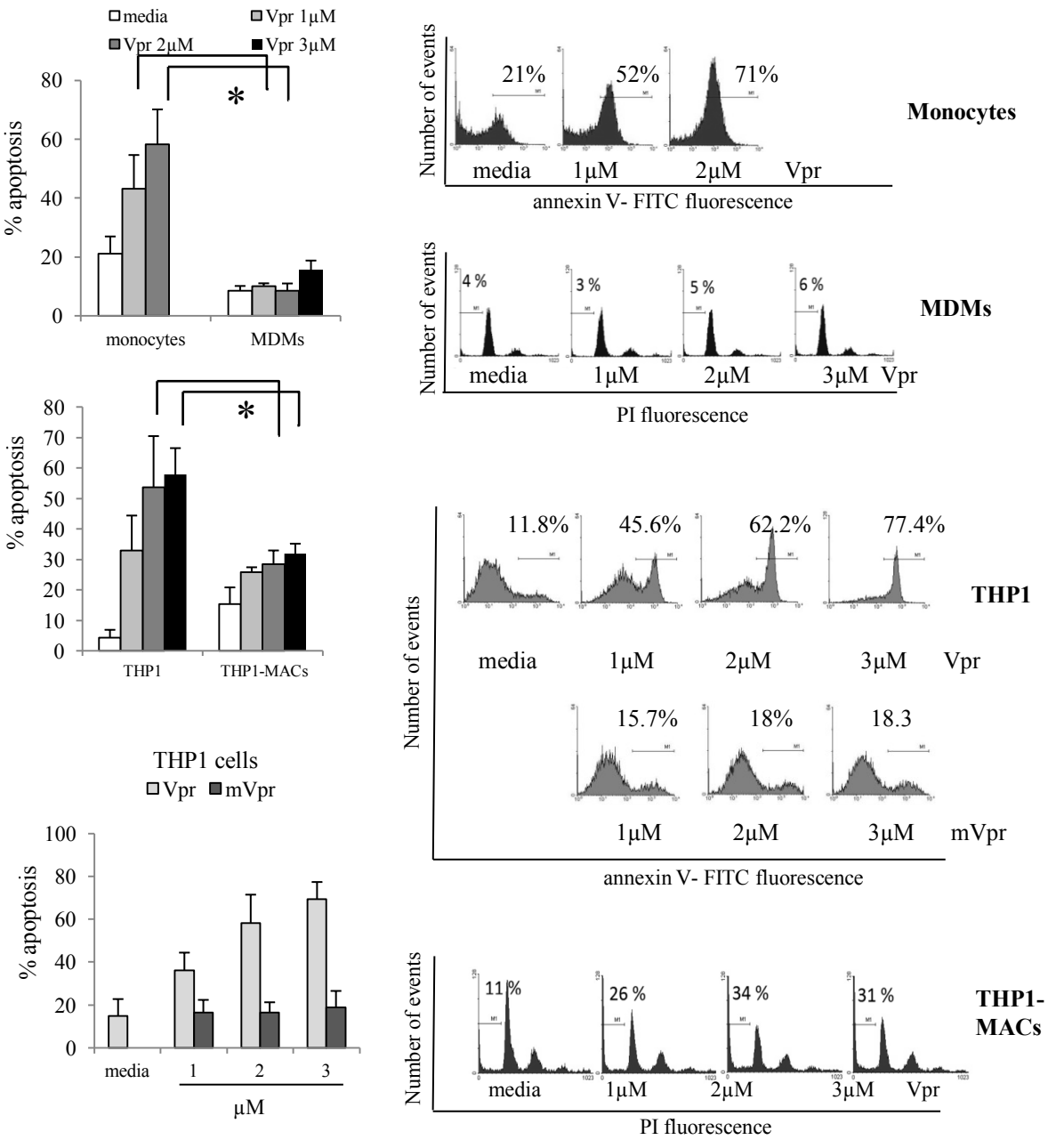

media
Vpr 1µM
Vpr 2µM
Vpr 3µM
% apoptosis
monocytes
MDMs
THP1
THP1-MACs
THP1 cells
Vpr
mVpr
µM
Number of events
21%
52%
71%
media
1µM
2µM
Vpr
annexin V- FITC fluorescence
Monocytes
4 %
3 %
5 %
6 %
3µM
PI fluorescence
MDMs
11.8%
45.6%
62.2%
77.4%
THP1
15.7%
18%
18.3
11 %
26 %
34 %
31 %
THP1-MACs

**Figure 14**

confirmed by analyzing caspase-3 cleavage and PARP cleavage as a marker of caspase-dependent apoptosis (295). Both PARP (Fig. 15A) and caspase-3 cleavage (Fig. 15B) were observed after 6 h of Vpr treatment in THP1 cells, whereas this effect was not seen in THP1-MACs and MDMs, in agreement with their lower sensitivity to Vpr-induced apoptosis. In correlation with the lack of apoptosis, mutant Vpr did not affect either caspase-3 or PARP cleavage (Fig. 15A and B). Since Vpr is known to induce apoptosis via the intrinsic pathway in sensitive cells (214), I determined if Vpr causes mitochondrial depolarization in MDMs by rhodamine staining. Mitochondrial depolarization was seen in Vpr-treated primary monocytes in a dose-dependent manner, whereas no difference was detected between the levels of rhodamine positive cells in Vpr-treated MDMs compared to the untreated cells. Mitochondrial depolarization of MDMs was easily detected after 4 h treatment with the Bcl2 family inhibitor HA14-1 as a positive control (Fig. 16A).

Vpr enters cells by a process that occurs independently of cellular receptors (241, 293). Since MDMs exhibited resistance to Vpr-induced apoptosis, I investigated the ability of Vpr to enter human macrophages. I was able to detect Vpr in whole cell extracts prepared from Vpr-treated MDMs using an anti-Vpr antibody (Fig. 16B, left panel). In order to confirm the intracellular location of Vpr following treatment, I also performed confocal microscopy on Vpr treated MDMs. Microscopy images indicate that Vpr localizes in the cytoplasm of MDMs (Fig. 16B, right panel), in agreement with previous findings that indicate the presence of a nuclear localization signal to the N-terminus of the peptide (296). These results suggest that the lack of cell death is not due to the inability of the peptide to enter the cells, since Vpr could be detected in the MDMs cytoplasm.

**Figure 15. Vpr causes PARP and caspase-3 cleavage in monocytic cells, but not in macrophages**

Cells were treated with Vpr, mVpr or staurosporine for 6 h following which total cell proteins were subjected to Western blotting and the membranes were probed with anti-PARP (A) or anti-caspase-3 (B) antibodies to evaluate their cleavage as markers of apoptosis. GAPDH was used as a loading control. Based on the densitometric analysis, the bar graphs in the lower panels show the mean ± SD of cleaved/uncleaved ratios from three experiments in THP1 cells.

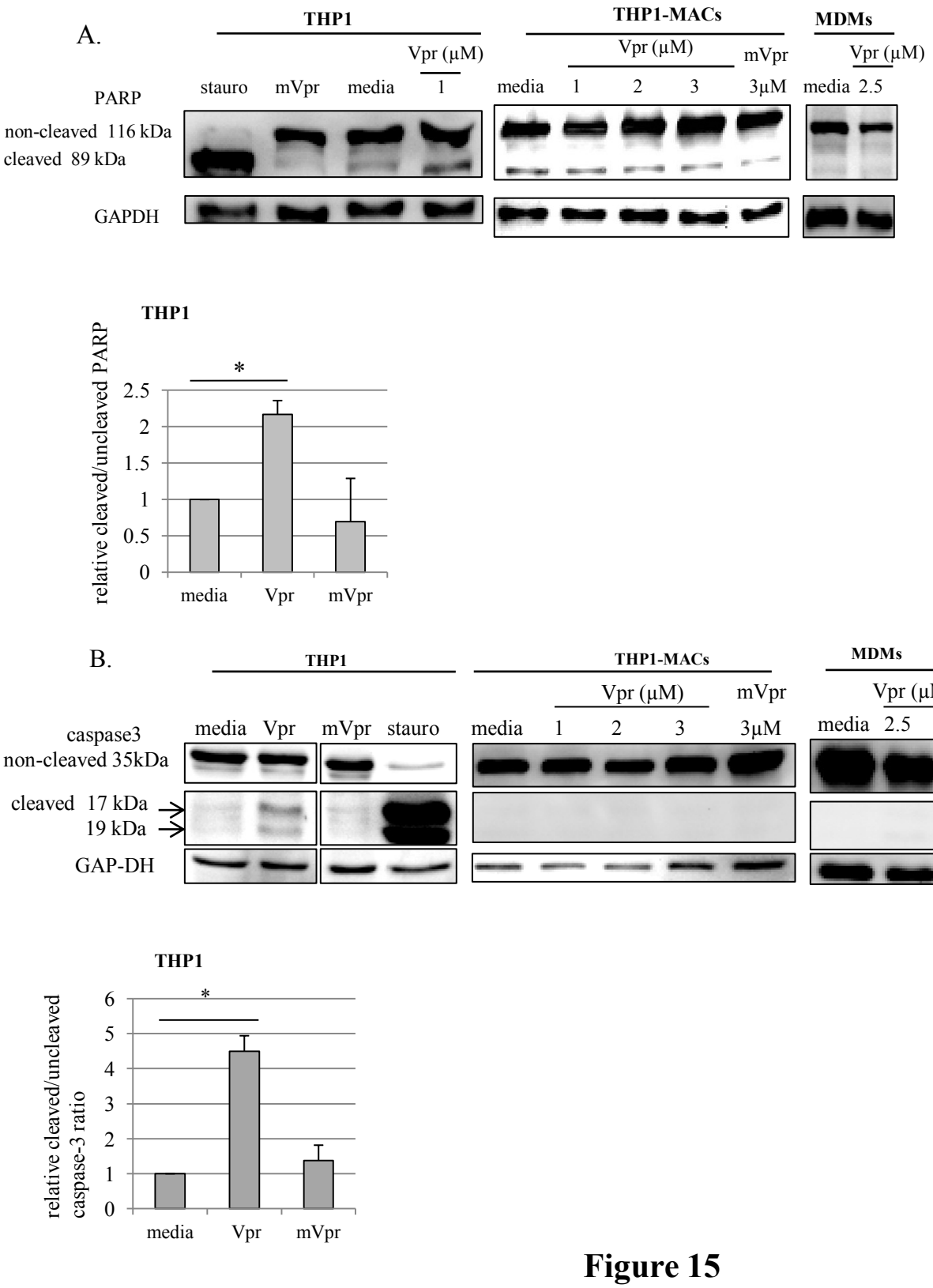

A.
THP1
THP1-MACs
MDMs
Vpr (μM)
Vpr (μM)
mVpr
Vpr (μM)
stauro
mVpr
media
1
media
1
2
3
3μM
media
2.5
PARP
non-cleaved 116 kDa
cleaved 89 kDa
GAPDH
THP1
relative cleaved/uncleaved PARP
*
2.5
2
1.5
1
0.5
0
media
Vpr
mVpr
B.
THP1
THP1-MACs
MDMs
Vpr (μM)
mVpr
Vpr (μM)
media
Vpr
mVpr
stauro
media
1
2
3
3μM
media
2.5
caspase3
non-cleaved 35kDa
cleaved 17 kDa
19 kDa
GAP-DH
THP1
relative cleaved/uncleaved caspase-3 ratio
*
6
5
4
3
2
1
0
media
Vpr
mVpr

**Figure 15**

Since THP1-MACs respond to the apoptotic effect of Vpr, albeit at lower levels compared to THP1 cells (Fig. 14 left middle panel), I have also compared the relative levels of Vpr in THP1-MACs and THP1 cells. I did not detect significant differences in Vpr expression in whole cell extracts prepared from Vpr-treated THP1 cells and THP1-MACs (Fig. 16C), suggesting that Vpr uptake may not explain the difference in sensitivity of these cells to the apoptotic effect of Vpr.

**Sequential exposure of monocytes to low non-apoptogenic concentrations of Vpr causes apoptosis**

Although the effect of Vpr has been described as mainly apoptogenic, there are also a few reports that describe the protective effect of low concentrations of Vpr on T cells (297, 298). Moreover, since Vpr concentrations used in these *in vitro* experiments are higher than the ones described in the serum of HIV infected patients (299), I wanted to determine if low concentrations of Vpr affect survival of monocytic cells. For this cells were exposed to low concentrations of Vpr sequentially in a way that it mimics the *in vivo* exposure to extracellular Vpr. Monocytes and THP1 cells were treated with two low, non-apoptogenic concentrations of Vpr followed by measurement of apoptosis. Our results indicate that two sequential doses of non-apoptogenic concentrations 0.5 μM or 0.25 μM Vpr will eventually cause higher apoptosis compared to a single dose (Fig. 17). These results indicate that monocytic cells are not protected from apoptosis by low doses of Vpr, but on the contrary, that repeated non-apoptogenic doses will eventually cause cell death. Furthermore, higher *in vitro* concentrations may mechanistically reflect *in vivo* continuous exposures to lower Vpr concentrations.

**Figure 16. Vpr enters MDMs without causing mitochondrial depolarization**

**A** MDMs and primary monocytes were treated with the indicated concentrations of Vpr, following which cells were stained with rhodamine for mitochondrial membrane potential evaluation as described in Materials and methods. Bcl2 inhibitor HA14-1 was used as a positive control and mVpr as a negative control for MDMs. Histograms show rhodamine positive cells, indicative of live cells. Histograms show one representative experiment for three similar results.
**B** Left panel: MDMs were treated with Vpr for 24 h, following which whole cell protein extracts were subjected to Western blotting. The membrane was probed with anti-Vpr antibody and anti-β actin antibody to control for protein loading. **B** Right panel: Following differentiation on coverslips, MDMs were treated with Vpr for another 24 h. Cells were stained according to the protocol described in Materials and methods and images were acquired with a Zeiss LSM 510 Meta confocal microscope using a 63X objective. Total magnification 157.5 X.
**C** THP1 cells and THP1-MACs were treated with Vpr for 6 h, following which total proteins were subjected to Western blotting. The membranes were probed with anti-Vpr antibody and anti-GAP-DH antibody to control for protein loading. Vpr expression level was quantified relative to GAP-DH and the bar graph shows the mean ± SD of four experiments.

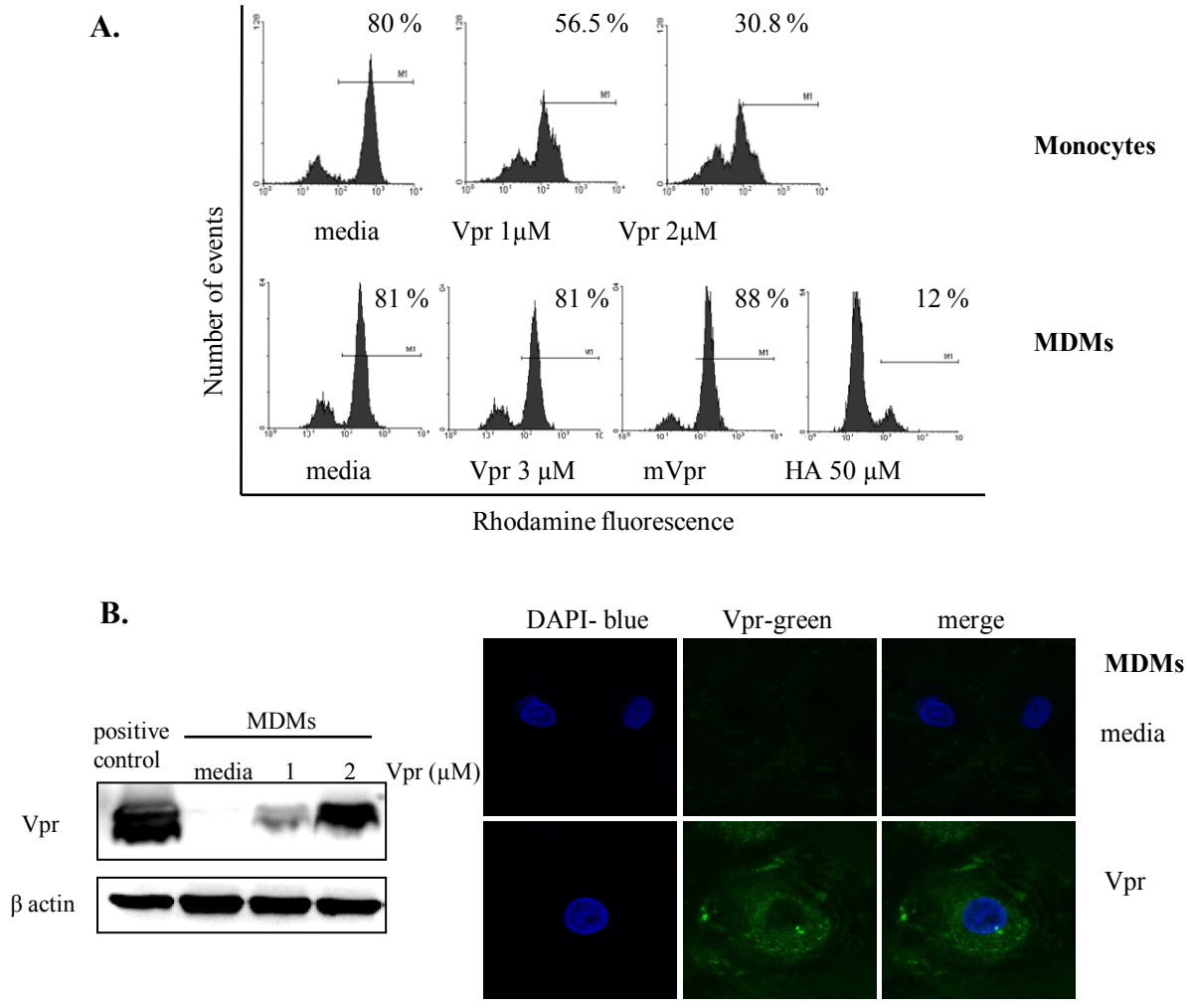

A.
80 %
56.5 %
30.8 %
Monocytes
media
Vpr 1µM
Vpr 2µM
81 %
81 %
88 %
12 %
MDMs
Number of events
media
Vpr 3 µM
mVpr
HA 50 µM
Rhodamine fluorescence
B.
DAPI- blue
Vpr-green
merge
MDMs
media
Vpr
positive control
MDMs
media
1
2
Vpr (µM)
Vpr
β actin
C.
THP1
THP1-MACs
media
1
2
media
1
2
Vpr (µM)
Vpr
GAP-DH
ns
ns
relative Vpr expression
4
3.5
3
2.5
2
1.5
1
0.5
0
THP1
THP1-MACs
THP1
THP1-MACs
1 µM Vpr
2 µM Vpr

**Figure 16**

**Figure 17. Sequential exposure of undifferentiated monocytic cells to low non-apoptotic concentrations of Vpr causes apoptosis**

Primary monocytes ($1.0x10^6$/ml) were treated first with a non-apoptotic dose of 0.5 μM Vpr for 2 hr, followed by a second low dose of 0.25 or 0.5 μM Vpr for another 2 hr. THP1 cells ($1.0x10^6$/ml) were treated first with a non-apoptotic dose of 0.5 μM Vpr for 24 hr, followed by a second low dose of 0.25 or 0.5 μM Vpr for another 24 hr. Cells were then analyzed by annexin-V staining and flow cytometry for the measurement of apoptosis. Right panels show the mean % apoptosis ± SD of three experiments. * indicates $p<0.05$. Histograms show one representative experiment.

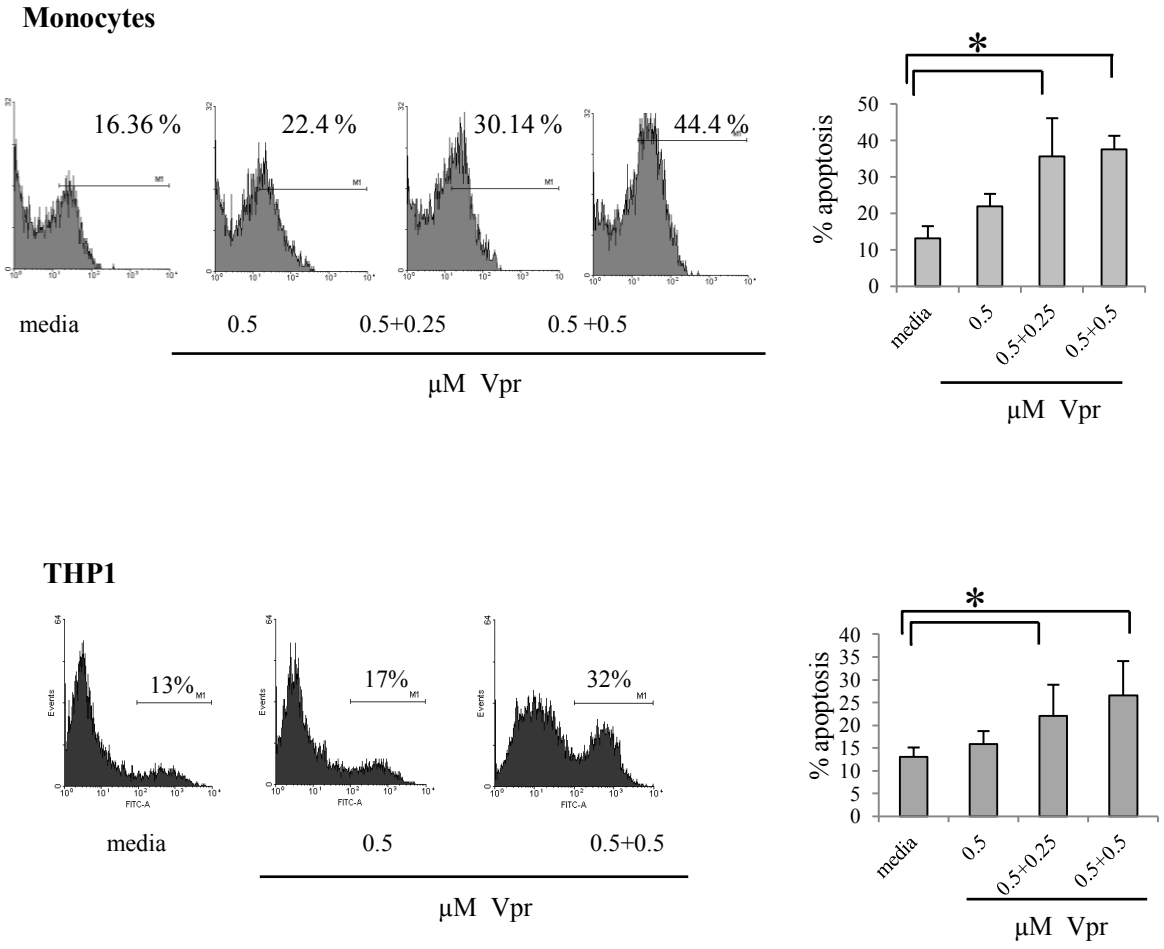

Monocytes
16.36 %
22.4 %
30.14 %
44.4 %
media
0.5
0.5+0.25
0.5 +0.5
µM Vpr
% apoptosis
50
40
30
20
10
0
*
media
0.5
0.5+0.25
0.5+0.5
µM Vpr
THP1
13%
17%
32%
Events
FITC-A
media
0.5
0.5+0.5
µM Vpr
% apoptosis
40
35
30
25
20
15
10
5
0
*
media
0.5
0.5+0.25
0.5+0.5
µM Vpr

**Figure 17**

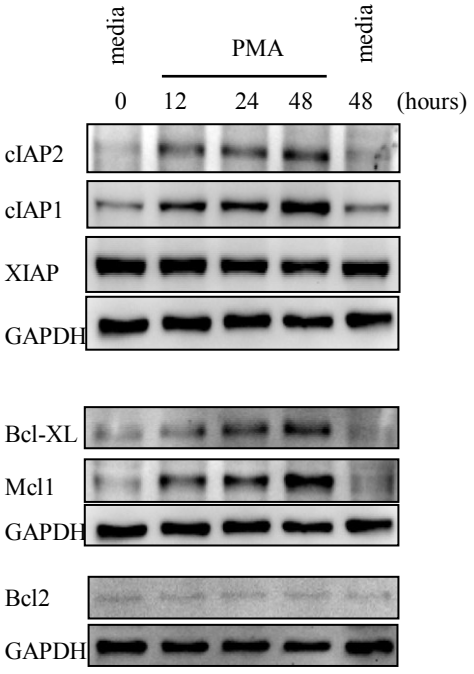
**Analysis of antiapoptotic proteins profile following differentiation in MDMs and THP1-MACs**

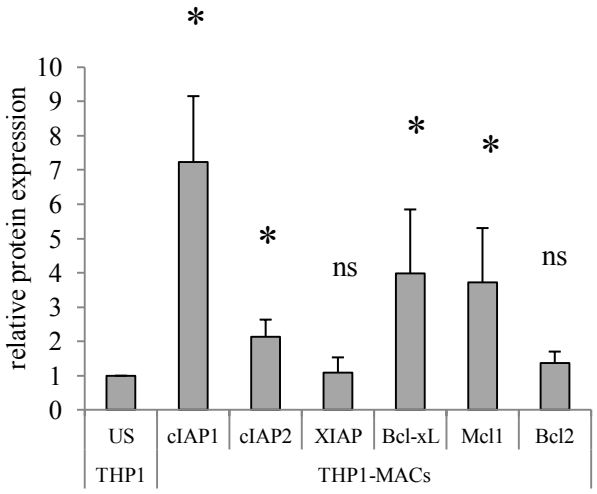
To understand the molecular mechanism governing resistance of human macrophages to Vpr-induced apoptosis, I have also employed THP1 cells as a model of differentiation, as these cells are more committed towards a monocyte/macrophage lineage than other monocytic cell lines (300). In addition, the expression profile of antiapoptotic proteins in these two cell types (THP1-MACs and MDMs) is not known. Therefore, I initially determined the expression of Bcl2 and IAPs antiapoptotic family members in both models of differentiation. There was a gradual increase in expression of Bcl-xL and Mcl-1 (Fig. 18A) in THP1-MACs. During monocyte differentiation with M-CSF, there was a similar gradual increase in Bcl-xL and Mcl-1 expression. However, I observed a constant decrease in the levels of Bcl2, in agreement with observations from studies performed in CD34+ progenitor cells (Fig. 18B) (63). Among the IAP family members, cIAP1 and cIAP2 expression was increased while XIAP levels remained unchanged following THP1 cells differentiation with PMA (Fig. 18A). In MDMs expression of cIAP1 remained unchanged, whereas cIAP2 and XIAP levels showed a bimodal distribution, with a peak on day 3 after M-CSF treatment (Fig. 18B). Overall these results show induction of Bcl-xL and Mcl-1 following differentiation in both models. In contrast, induction of cIAP1/2, XIAP and Bcl2 was not similar in the two models, which may be attributed to the distinct cell types used (cell line versus primary cells) and to the agents employed for differentiation (PMA for THP1-MACs versus M-CSF for MDMs). Therefore, these results suggest that both IAPs and Bcl2 family proteins may be responsible for the acquisition of a resistant phenotype towards Vpr-induced apoptosis in both models of macrophage differentiation. However, I cannot

**Figure 18. Expression of antiapoptotic proteins during macrophage differentiation**

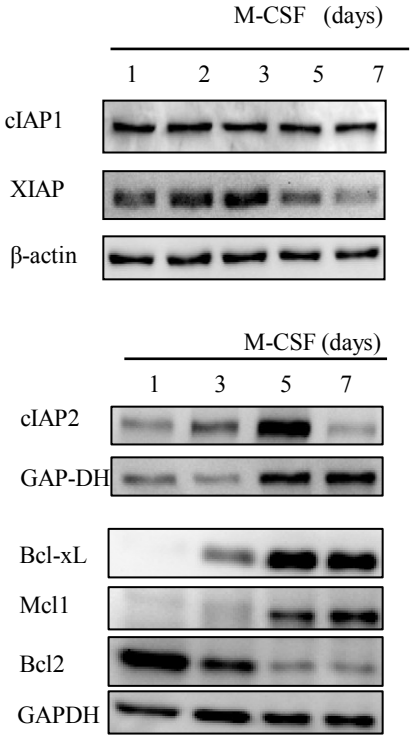
THP1 cells ($0.5x10^6$/ml) were differentiated with 20 ng/ml of PMA **(A).** Monocytes isolated by adherence were differentiated with 10 ng/ml of MCSF **(B).** Cells were collected at the indicated times (12 h, 24 h and 48 h for THP1-MACs and days D1, D3, D5, D7 for MDMs) throughout differentiation and whole cell proteins extracts were subjected to Western blotting. Membranes were probed with specific antibodies against various members of the Bcl2 and IAP families and the expression level of antiapoptotic proteins was quantified to control for equal protein loading. Right panels show the mean relative protein expression ± SD of at least three experiments for each cell type at the indicated timepoints for MDMs (right lower panel) and at 48h for THP1-MACs (right upper panel) .

**A. THP1-MACs**




**B. MDMs**



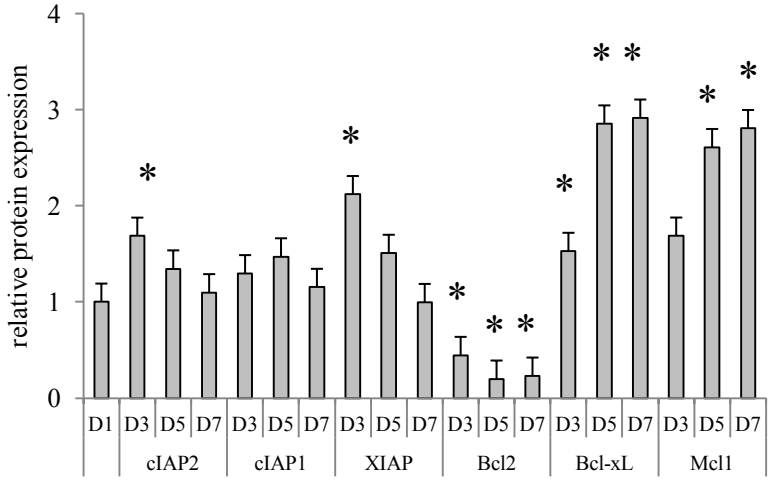




**Figure 18**

exclude the possibility that the existing basal levels of antiapoptotic proteins that did not change or decreased during differentiation in both models may be enough to protect cells from Vpr-induced cell death.

**Vpr does not affect Bcl2 and cIAP1 expression in macrophages**

To elucidate the mechanisms underlying macrophage resistance to Vpr-induced apoptosis, I examined the effect of Vpr on the expression of apoptosis-related proteins of the Bcl2 and IAPs families. We have recently shown that cIAP2 induction by TLR-9 ligand CpG pretreatment can protect primary monocytes from Vpr-induced apoptosis (206). Treatment with Vpr for 24 h downregulated cIAP1 and Bcl2 expression in primary monocytes (Fig. 19A, upper left panel) and cIAP1, Bcl2 and Mcl-1 expression in THP1 cells (Fig. 19A, upper right and lower panels). Similarly, downregulation of cIAP1 and Bcl2 was also observed at an earlier timepoint, following 12 h of Vpr treatment in THP1 cells (Fig. 19B). However, there was no effect on the levels of XIAP (Fig. 19C), indicating that Vpr targets cIAP1 and Bcl2 selectively in order to induce cell death in sensitive cells. However, Vpr did not affect the expression of antiapoptotic cIAP1, cIAP2, XIAP, Bcl2 in MDMs (Fig. 20A) or of cIAP1, cIAP2, XIAP, Bcl2, Bcl-xL and Mcl-1 proteins in THP1-MACs (Fig. 20B), suggesting that both members of Bcl2 and IAP families may play a role in macrophage protection against Vpr-induced apoptosis.

**Figure 19. Vpr downregulates the expression of Bcl2 and cIAP1 in monocytic cells**

Primary monocytes ($2x10^6$/ml) **(A)** and THP1 cells ($1x10^6$/ml) were treated with the indicated concentrations of Vpr or mVpr for 24h **(B)** and 12h **(C)**. Total cell proteins were subjected to Western blotting and the membranes were probed with the indicated antibodies. In THP1 cells the expression level of antiapoptotic proteins was quantified to control for equal protein loading. Based on the densitometric analysis, the bar graphs in the lower panels show the mean ± SD of at least three experiments.

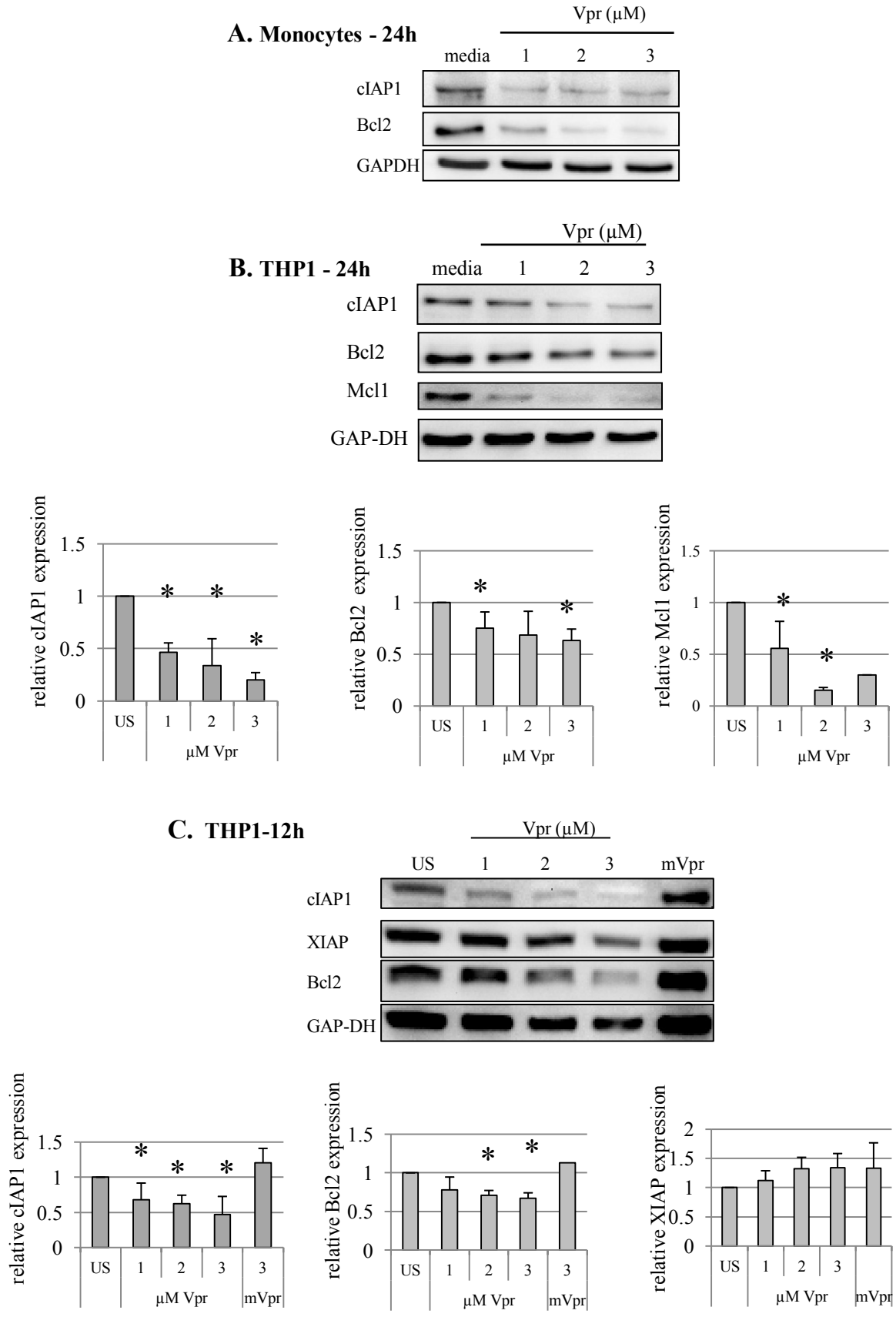

A. Monocytes - 24h
Vpr (μM)
media
1
2
3
cIAP1
Bcl2
GAPDH
B. THP1 - 24h
Vpr (μM)
media
1
2
3
cIAP1
Bcl2
Mcl1
GAP-DH
relative cIAP1 expression
relative Bcl2 expression
relative Mcl1 expression
1.5
1
0.5
0
US
1
2
3
μM Vpr
C. THP1-12h
Vpr (μM)
US
1
2
3
mVpr
cIAP1
XIAP
Bcl2
GAP-DH
relative cIAP1 expression
relative Bcl2 expression
relative XIAP expression
2
1.5
1
0.5
0
μM Vpr
mVpr

**Figure 19**

**Figure 20. Vpr does not affect the expression of Bcl2 and cIAP1 in macrophages**

MDMs **(A)** and THP1-MACs **(B)** were treated with the indicated concentrations of Vpr or mVpr for 24h. Total cell proteins were subjected to Western blotting and the membranes were probed with specific antibodies against various members of the Bcl2 and IAP families. The results shown are representative of three separate experiments with similar results.

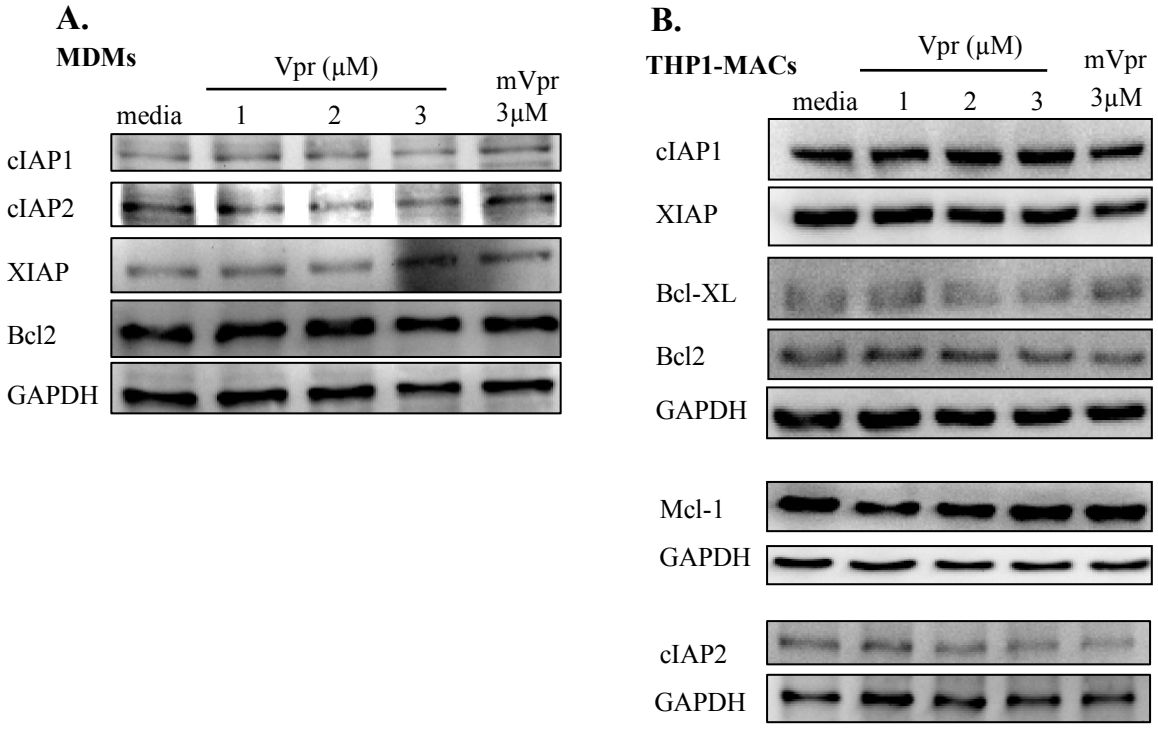

A.
MDMs
Vpr (μM)
media
1
2
3
mVpr 3μM
cIAP1
cIAP2
XIAP
Bcl2
GAPDH
B.
THP1-MACs
Vpr (μM)
media
1
2
3
mVpr 3μM
cIAP1
XIAP
Bcl-XL
Bcl2
GAPDH
Mcl-1
GAPDH
cIAP2
GAPDH

**Figure 20**

**Bcl-xL and Mcl-1 play a role in macrophage survival but not in resistance to Vpr-induced apoptosis**

The apoptotic effect of Vpr is associated with mitochondrial depolarization and activation of the intrinsic apoptotic pathway culminating in caspase-3 activation in T cells (301). Since the Bcl2 family members are primarily responsible for maintaining mitochondrial membrane potential (10), I investigated if Bcl2 proteins mediate resistance of macrophages against Vpr-induced cell death. To dissect the role of the Bcl2 family members I employed HA14-1, a small molecule that mimics the Bcl2-homology 3 domain of Bcl2 proteins. The inhibitory mechanism of HA14-1 involves binding and inactivating antiapoptotic Bcl2 and Bcl-xL proteins (57). THP1-MACs were treated with various concentrations of HA14-1 for 4 h followed by a second treatment with 2.5 μM Vpr for 24 h. The biological activity of HA14-1 was tested in NIH 3T3 cells (data not shown), where it caused caspase-dependent cell death (57). HA14-1 induced apoptosis on its own in THP1-MACs, indicating the importance of Bcl2 proteins in sustaining THP1-MACs viability (Fig. 21A). However, Vpr treatment of THP1-MACs previously exposed to HA14-1 did not enhance the levels of apoptosis beyond those observed with the HA14-1 inhibitor alone (Fig. 21A). These results suggested that blocking Bcl2 proteins does not impact on susceptibility to Vpr-induced apoptosis.

The inhibitory effect of HA14-1 is limited to Bcl2 and Bcl-xL members of the Bcl2 family (57). The differentiation experiments results indicated that only Bcl-xL expression was upregulated, whereas Bcl2 expression remained unaffected following THP1 differentiation with PMA (Fig. 18A). Following differentiation with M-CSF in MDMs,

**Figure 21. Bcl-xL contributes to macrophage survival, but is not involved in resistance to Vpr-induced apoptosis**

**A** THP1 cells ($1x10^6$/ml) were treated for 2 h with the indicated concentrations of Bcl2 inhibitor HA14-1 prior to Vpr (2.5 μM) treatment for another 24 h following which cells were stained with PI and evaluated for apoptosis by flow cytometry. The numbers represent percentage apoptotic cells with subdiploid DNA content. The bar graph shows the mean ± SD of three experiments. * indicates $p<0.05$.
**B** After differentiation, THP1-MACs and MDMs were treated with Bcl-xL or control siRNA as described in Materials and methods section. Efficiency of protein knockdown and siRNA specificity were evaluated by Western blotting after 24 h and 48 h of siRNA treatment.
**C** At the indicated times following transfection, cells were evaluated for apoptosis by flow cytometry (left panel) or for caspase-3 cleavage by Western blotting (right panel, 48h post-transfection). The numbers in the left panel represent percentage apoptotic cells with subdiploid DNA content. The bar graph shows the mean % apoptosis ± SD of three experiments. * indicates $p<0.05$.
**D** THP1-MACs were initially transfected with Bcl-xL and control siRNA for 24h, after which Vpr (2.5 μM) was added for another 24h. Following Vpr treatment, cells were collected and stained with PI for flow cytometry evaluation. The numbers represent percentage apoptotic cells with subdiploid DNA content. The graph bar shows the mean ± SD of three experiments (upper panel). Histograms show one representative experiment for three similar results (lower panel).

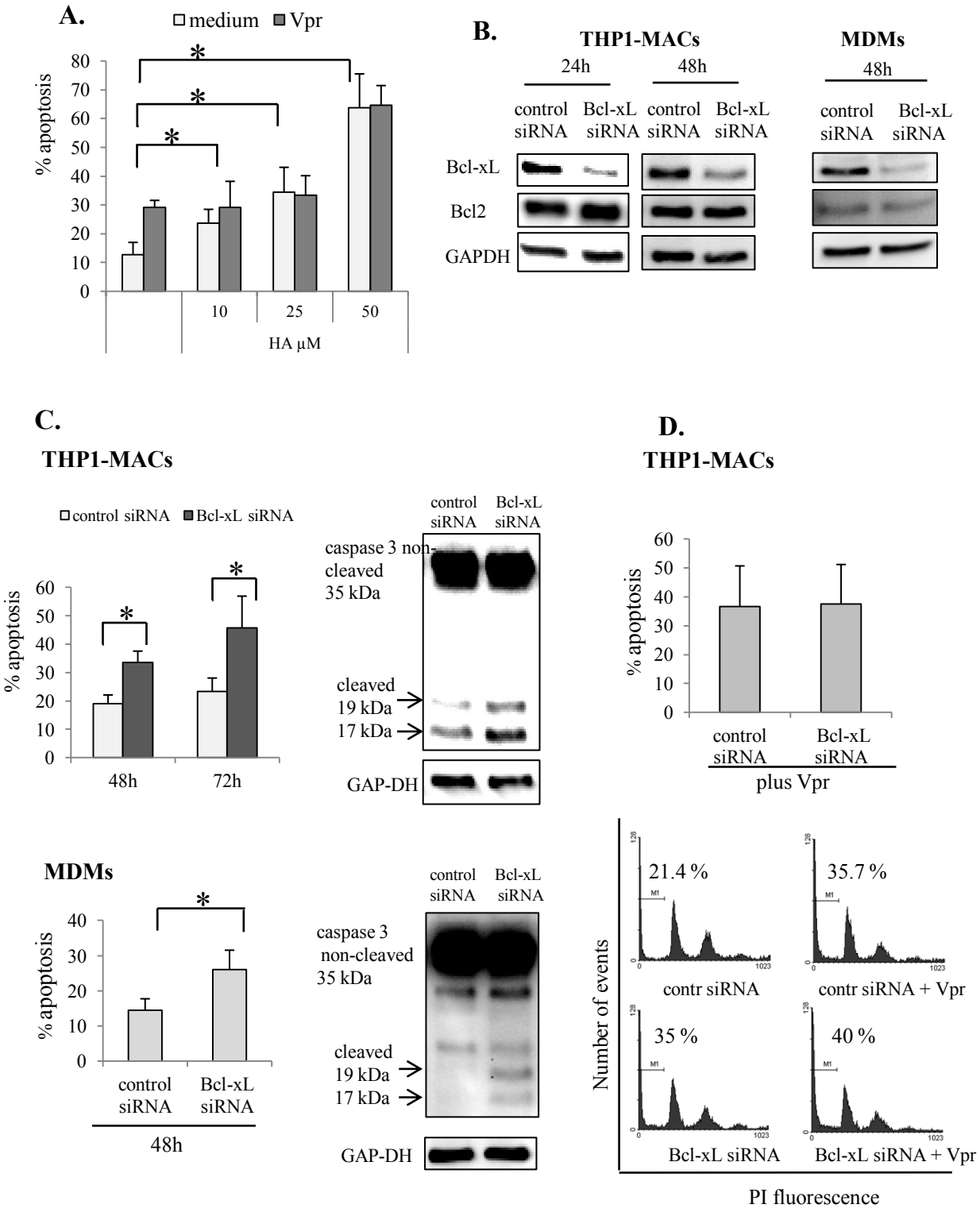

A.
medium
Vpr
% apoptosis
HA µM
B.
THP1-MACs
MDMs
24h
48h
control siRNA
Bcl-xL siRNA
Bcl-xL
Bcl2
GAPDH
C.
THP1-MACs
control siRNA
Bcl-xL siRNA
% apoptosis
48h
72h
caspase 3 non-cleaved 35 kDa
cleaved 19 kDa
17 kDa
GAP-DH
MDMs
D.
THP1-MACs
plus Vpr
21.4 %
35.7 %
35 %
40 %
contr siRNA
contr siRNA + Vpr
Bcl-xL siRNA
Bcl-xL siRNA + Vpr
Number of events
PI fluorescence

**Figure 21**

Bcl-xL expression was enhanced and Bcl2 was downregulated (Fig. 18B). Since Bcl-xL upregulation was common in both models of macrophage differentiation, I confirmed the role of Bcl-xL in cell survival by using Bcl-xL specific siRNA to inhibit its expression in both THP1-MACs and MDMs. Transfection of THP1-MACs and MDMs with Bcl-xL siRNA decreased the basal expression of Bcl-xL (Fig. 21B). In contrast, Bcl2 expression was not affected, which controlled for the siRNA specificity. Decreased Bcl-xL expression was associated with a significant increase in cell death of both cell types (Fig. 21C, left panels). This increase in apoptosis was associated with enhanced caspase-3 cleavage in cells transfected with Bcl-xL siRNA as compared to the cells transfected with control siRNA (Fig. 21C, right panels). To determine the effect of Bcl-xL inhibition on resistance to Vpr-induced apoptosis, THP1-MACs transfected with Bcl-xL siRNA for 24 h were treated with Vpr for another 24 h, following which apoptosis was measured by flow cytometry. Notably, knocking down Bcl-xL levels did not change susceptibility of macrophages to Vpr-induced apoptosis (Fig. 21D).

The expression of antiapoptotic Mcl-1 was also increased in THP1-MACs and MDMs (Fig. 18A and B). Moreover, Vpr decreased the expression of Mcl-1 in THP1 cells (Fig. 19A) but not in THP1-MACs (Fig. 19B) suggesting that Mcl-1 upregulation during differentiation may contribute to the resistant phenotype of THP1-MACs. Since the inhibitory effect of HA14-1 does not affect Mcl-1 activity (57), I analyzed the role of Mcl-1 by using specific siRNA. Mcl-1 siRNA significantly decreased Mcl-1 expression at both 24 h and 48 h post-transfection of THP1-MACs, with no effect on Bcl2 (Fig. 22A).

**Figure 22. Mcl-1 contributes to macrophage survival, but is not involved in resistance to Vpr-induced apoptosis**

**A.** After differentiation, THP1-MACs ($5\times10^5$/ml) were transfected with Mcl-1 or control siRNA as described in Materials and methods section. Efficiency of protein knockdown and siRNA specificity were evaluated by Western blotting after 24 h and 48 h of transfection.
**B** Following transfection, cells were stained with PI for apoptosis assessment. The numbers represent percentage apoptotic cells with subdiploid DNA content. The bar graph shows the mean ± SD of three experiments. * indicates $p<0.05$
**C** Following transfection cells were cultured for 24 h and Vpr was added to the cells for another 24 h. Subsequently, cells were stained with PI for flow cytometry. Y axis shows the percentage increase in apoptotic cells following Vpr treatment, calculated by subtracting the percentage of apoptotic cells in transfected samples from the transfected and Vpr treated samples. The bar graph shows the mean ± SD of three experiments.
**D**. Cells treated as in C were collected and evaluated for caspase-3 and PARP cleavage by Western blotting.

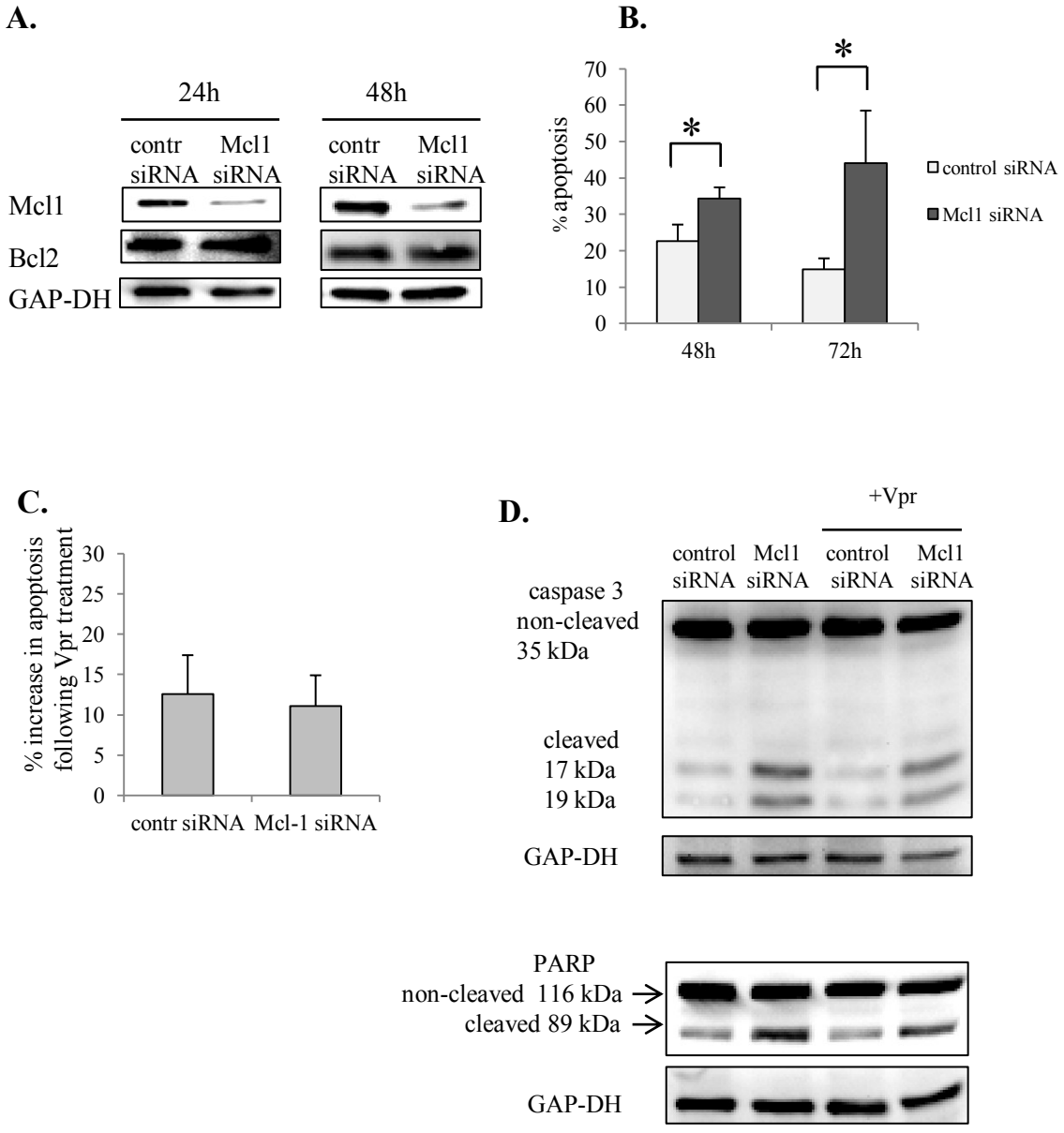

A.
24h
48h
contr siRNA
Mcl1 siRNA
contr siRNA
Mcl1 siRNA
Mcl1
Bcl2
GAP-DH
B.
% apoptosis
70
60
50
40
30
20
10
0
*
*
48h
72h
control siRNA
Mcl1 siRNA
C.
% increase in apoptosis following Vpr treatment
30
25
20
15
10
5
0
contr siRNA
Mcl-1 siRNA
D.
+Vpr
control siRNA
Mcl1 siRNA
control siRNA
Mcl1 siRNA
caspase 3
non-cleaved
35 kDa
cleaved
17 kDa
19 kDa
GAP-DH
PARP
non-cleaved 116 kDa
cleaved 89 kDa
GAP-DH

**Figure 22**

Decreased Mcl-1 expression was associated with a significant increase in apoptosis 48 h after transfection that was even more pronounced at 72 h post-transfection (Fig. 22B). In order to address the effect of Mcl-1 inhibition on susceptibility to Vpr-induced apoptosis, THP1-MACs transfected with Mcl-1 siRNA for 24 h were treated with Vpr for another 24 h, following which apoptosis was measured by flow cytometry. Similarly to the Bcl-xL siRNA results, apoptosis in response to Vpr treatment did not increase following transfection of THP1-MACs with Mcl-1 siRNA (Fig. 22C). Also, Vpr did not increase PARP and caspase-3 cleavage in either control or Mcl-1 siRNA transfected cells as compared to the control or Mcl-1 siRNA transfected cells, respectively (Fig. 22D, lane 1 versus 3 and lane 2 versus 4). Overall, these results suggest that while Bcl2 and Mcl-1 are important in maintaining macrophage steady state viability, they do not contribute to protection against Vpr-induced apoptosis.

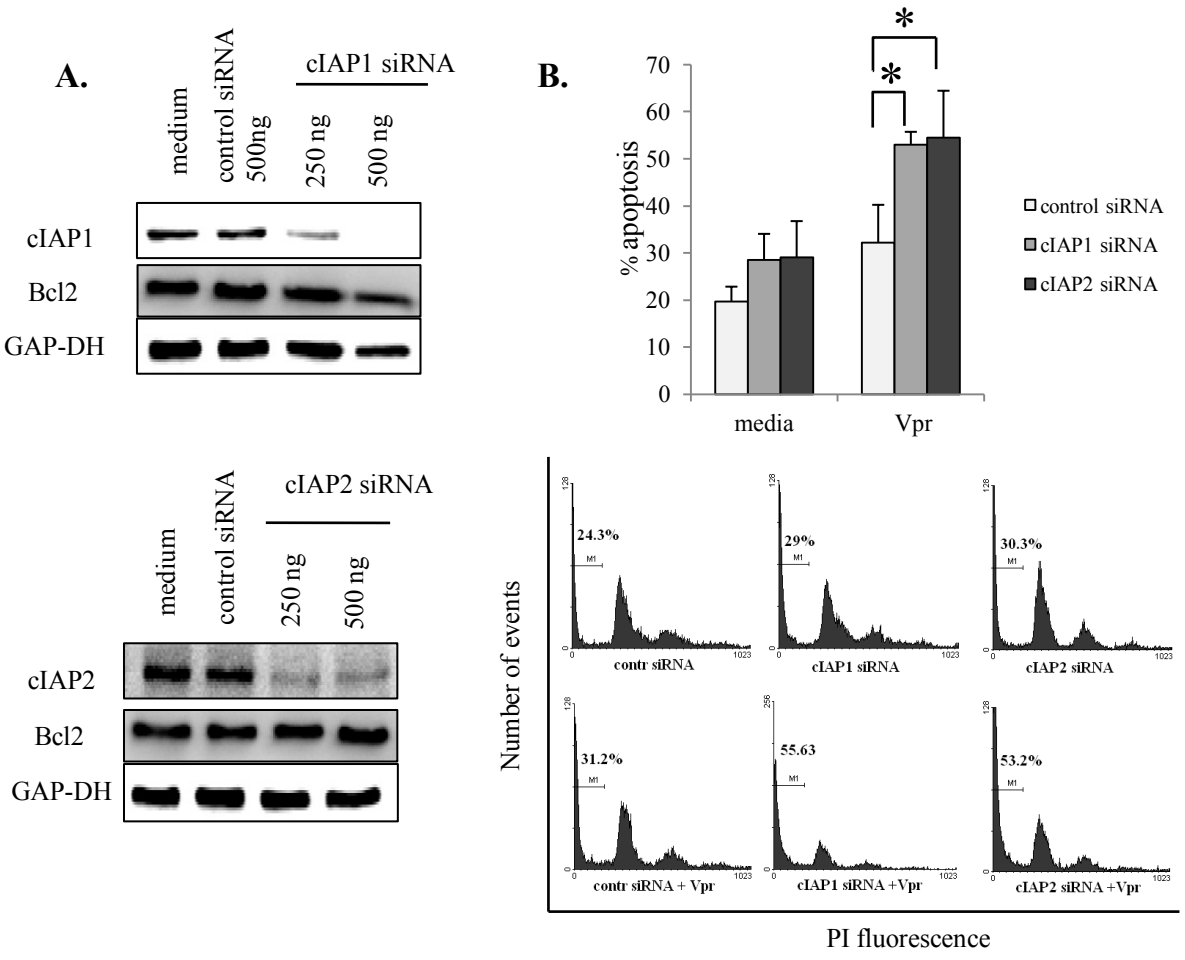
**IAPs protect macrophages against Vpr-induced apoptosis**

We have recently shown that cIAP2 plays a critical role in protecting undifferentiated monocytic cells from Vpr-induced apoptosis (206). The increased expression levels of cIAP1 and cIAP2 in macrophages (Fig. 18A), along with the discrepancy between the ability of Vpr to downregulate cIAP1 in monocytes (Fig. 19), but not in macrophages (Fig. 20), prompted us to determine if cIAP1 or cIAP2 mediate decreased macrophage sensitivity to Vpr-induced apoptosis.

To study the role of cIAPs, THP1-MACs were transfected with specific siRNAs for cIAP1 and cIAP2 followed by treatment with Vpr peptide and analysis of apoptosis. The results suggest that transfection of THP1-MACs with cIAP1 and cIAP2 siRNA significantly downregulated the endogenous expression of cIAP1 and cIAP2 proteins, without affecting

**Figure 23. IAPs protect macrophages against Vpr-induced apoptosis**

**A.** After differentiation, THP1-MACs ($5\times10^5$/ml) were transfected with cIAP1, cIAP2 or control siRNA as described in Materials and methods section. Following transfection, cells were collected after 24 h for evaluation of protein knockdown. Total cell proteins were subjected to Western blotting and the membranes were probed with antibodies specific for cIAP1, cIAP2 and Bcl2 to ensure siRNA specificity.
**B** and **C** On the second day after transfection, cells were treated with Vpr (2.5 μM) for another 24 h and stained with PI for apoptosis measurement (B) or subjected to Western blotting for PARP and caspase-3 cleavage (C). Only 500 ng IAPs siRNA and control siRNA were used for these experiments. Bar graph in the upper panel of B show the mean % apoptosis ± SD of four separate experiments. * indicates $p<0.05$. Histograms in the lower panel of B show one representative experiment indicating percentage of cells with subdiploid DNA content.


A.
medium
control siRNA
500ng
cIAP1 siRNA
250 ng
500 ng
cIAP1
Bcl2
GAP-DH
cIAP2 siRNA
cIAP2
B.
% apoptosis
media
Vpr
control siRNA
cIAP1 siRNA
cIAP2 siRNA
Number of events
24.3%
29%
30.3%
31.2%
55.63
53.2%
contr siRNA
cIAP1 siRNA
cIAP2 siRNA
contr siRNA + Vpr
cIAP1 siRNA +Vpr
cIAP2 siRNA +Vpr
PI fluorescence

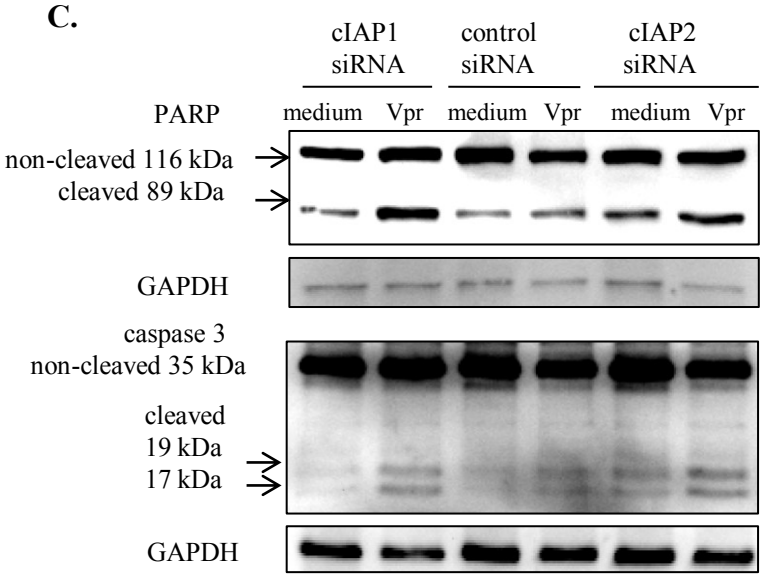

C.
cIAP1 siRNA
control siRNA
cIAP2 siRNA
PARP
medium
Vpr
non-cleaved 116 kDa
cleaved 89 kDa
GAPDH
caspase 3
non-cleaved 35 kDa
cleaved
19 kDa
17 kDa
GAPDH

**Figure 23**

**Figure 24. Smac mimetic AEG40730 sensitizes MDMs to Vpr-induced apoptosis**

**A.** MDMs from three different donors were treated with SMAC mimetic AEG40730 (200 nM) for 24 h and IAPs and Bcl2 expression was evaluated by Western blotting.
**B** and **C.** Following 24 h treatment with SMAC mimetic, MDMs were treated with Vpr (2.5 μM) for another 24 h. Cells were stained with PI for apoptosis evaluation (B) or they were used for Western blotting to evaluate PARP and caspase-3 cleavage (C). Dot plot in B shows flow cytometry data and the mean % apoptosis ± SD of six separate experiments with cells from different donors. * indicates $p<0.05$. Western blotting results shown in C are representative of three different donors.

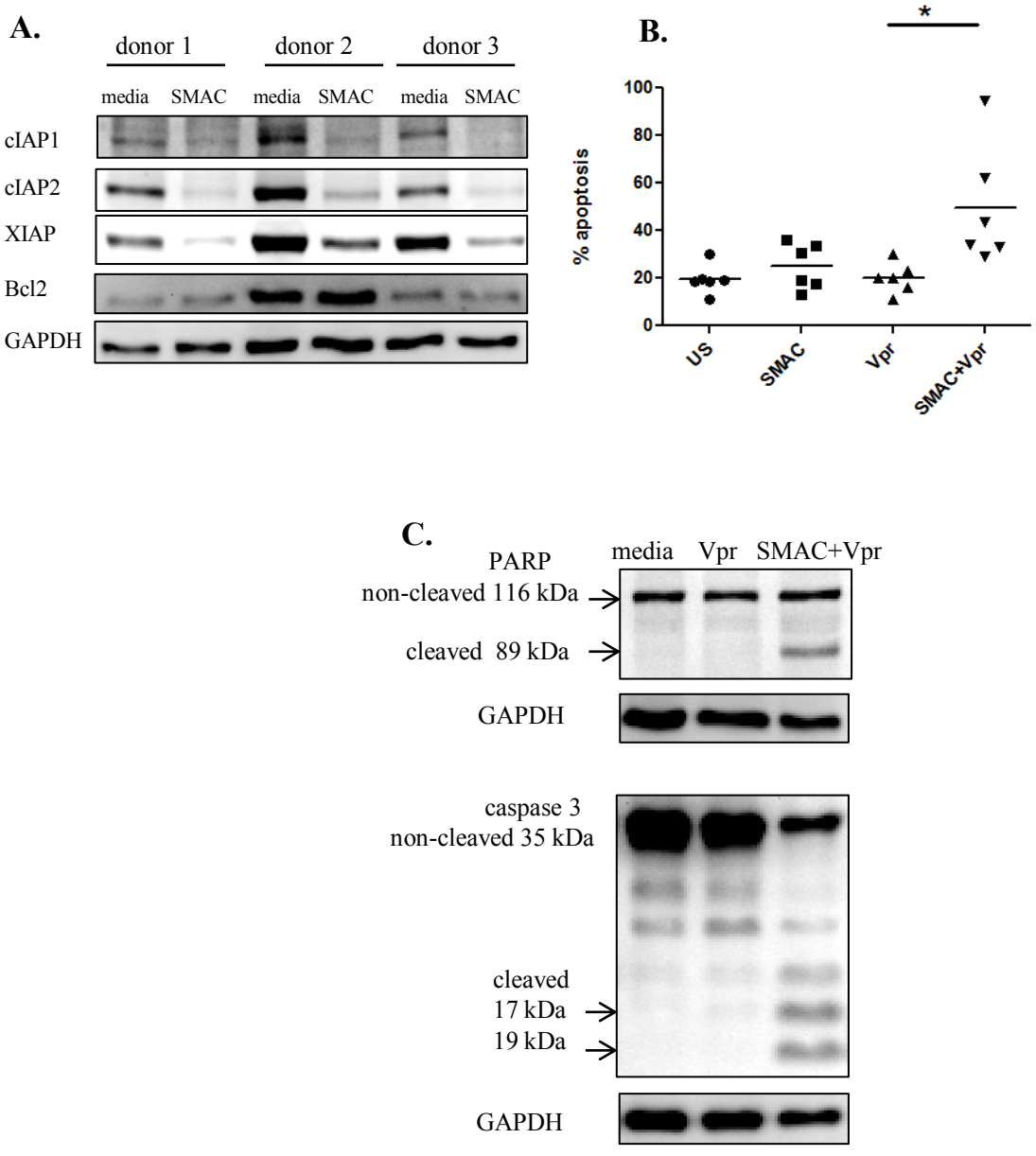

A.
donor 1
donor 2
donor 3
media
SMAC
media
SMAC
media
SMAC
cIAP1
cIAP2
XIAP
Bcl2
GAPDH
B.
*
% apoptosis
100
80
60
40
20
0
US
SMAC
Vpr
SMAC+Vpr
C.
PARP
media
Vpr
SMAC+Vpr
non-cleaved 116 kDa
cleaved 89 kDa
GAPDH
caspase 3
non-cleaved 35 kDa
cleaved
17 kDa
19 kDa
GAPDH

**Figure 24**

Bcl2 expression level (Fig. 23A). However, unlike Bcl-xL and Mcl-1 siRNA results, transfection of THP1-MACs with cIAP1 and cIAP2 siRNA did not affect the basal level of apoptosis between cells transfected with control and cIAPs siRNA (Fig. 23B). In contrast, Vpr significantly increased apoptosis of cIAP1 and cIAP2 siRNA-transfected cells compared to the cells transfected with control siRNA (Fig. 23B). This increase in Vpr-induced apoptosis in IAPs transfected cells was also associated with notable increase in PARP and caspase-3 cleavage following Vpr treatment as compared to the cells transfected with control siRNA (Fig. 23C). These results suggest that both cIAP1 and cIAP2 are involved in resistance to Vpr-induced apoptosis in THP1-MACs.

To study the involvement of IAPs in protection of MDMs, I employed SMAC mimetic AEG40730, designed to knock down IAPs protein levels by targeting them for ubiquitination and proteosomal degradation (302). Initially, I evaluated the effect of AEG40730 on the expression of various antiapoptotic proteins. Treatment of MDMs from three different donors with AEG40730 significantly decreased the expression levels of cIAP1, cIAP2 and XIAP, with no effect on Bcl2 (Fig. 24A), suggesting that the SMAC mimetic acts specifically on the IAP proteins. AEG40730 alone did not have a significant effect on apoptosis of MDMs (Fig. 24B). Similarly, Vpr alone, as expected, did not enhance apoptosis compared with untreated cells. However, pretreatment of MDMs with SMAC mimetic was able to sensitize MDMs to the apoptotic effect of Vpr. This increase in Vpr-induced apoptosis in SMAC mimetic-treated cells was associated with enhanced PARP and caspase-3 cleavage compared to the untreated cells (Fig. 24C). These results suggest that SMAC mimetic renders MDMs sensitive to caspase 3-dependent cell death. Overall, the

results suggest that both cIAP1 and cIAP2 do not influence survival in the absence of an apoptotic stimulus (spontaneous apoptosis), but that these antiapoptotic proteins contribute to macrophage protection against Vpr-induced apoptosis.

## Discussion

Macrophages represent major viral reservoirs in HIV infection because they are resistant to the viral cytopathic effects (8). Hijacking the apoptotic pathway in the initial phases of infection has been shown to promote survival of macrophages infected with either bacterial (303, 304) or viral pathogens (117). However, the mechanism underlying macrophage resistance to apoptosis and their establishment as HIV reservoirs remains unknown. In this study, I have investigated the apoptotic effect of HIV-Vpr on survival of undifferentiated and differentiated human monocytic cells. I demonstrate for the first time that although primary human monocytes are susceptible to Vpr-induced apoptosis, they develop resistance to this HIV peptide upon differentiation into macrophages, suggesting that differentiation provides a protective mechanism against the Vpr-mediated apoptotic effect. In determining the molecular mechanism governing the development of this resistance, my results suggest that resistance to Vpr-induced apoptosis is specifically mediated by cIAP1 and cIAP2 of the IAP family, independently of the Bcl-xL and Mcl-1 of the Bcl2 family, which play a critical role in maintaining viability in the absence of any apoptotic stimulus.

The mechanism by which Vpr induces apoptosis has been investigated in various cell types, including lymphocytes (214) and neurons (305). Vpr was shown to bind to the adenine nucleotide translocator of the mitochondrial membrane resulting in membrane permeabilization and release of apoptogenic factors such as cytochrome-c into the cytosol and culminating in caspase-3 activation and apoptosis (221). Furthermore, pretreatment of mitochondria with Bcl2 protein was able to prevent Vpr-induced cell death (221).

Since our results indicate that Vpr does not induce mitochondrial permeabilization or PARP and caspase-3 cleavage in macrophages, in contrast to primary undifferentiated monocytes, I initially investigated the role of antiapoptotic Bcl2 family of proteins in rendering macrophages resistant to Vpr-mediated apoptosis. Differentiation of THP1 cells was associated with increased expression of Bcl-xL and Mcl-1, with no change in Bcl2. In primary monocytes, Bcl2 expression was consistently downregulated following differentiation, while Bcl-xL and Mcl-1 expression were also increased. These differences are most likely due to the different stimuli used to induce differentiation (PMA and MCSF) as well as to the cell type that is being induced to differentiate (primary monocytes and leukemic THP1 cells, respectively). I also expect the apoptotic machinery to be tipped towards resistance to cell death in cell lines compared to primary cells, since enhanced resistance to cell death is an intrinsic feature of cancerous cells. Therefore, chemical inhibitors and siRNA technology were employed to evaluate the role of individual antiapoptotic proteins.

Based on these observations, I initially hypothesized that increased expression of antiapoptotic Bcl2 proteins may account for the inability of Vpr to induce apoptosis in macrophages. Both Bcl-xL and Mcl-1 are important for cell viability because of their ability to maintain mitochondrial integrity. Bcl-xL has been shown to contribute to survival of bone marrow derived macrophages in both murine (306) and human (59) models of differentiation. Mcl-1 was also implicated in survival of human macrophages, in either physiological (134) or pathological conditions (307). My results show that inhibition of Bcl2 activity by pretreatment of THP1-MACs with HA14-1 or by siRNAs specific for Bcl-xL and Mcl-1 do not impact on their susceptibility to Vpr-induced apoptosis. Instead, these

antiapoptotic proteins were responsible for maintaining viability in the absence of any apoptotic stimulus.

Since members of the IAP family directly bind and inactivate both initiator and effector caspases downstream of mitochondria (273), I evaluated their involvement in macrophage resistance to Vpr-induced apoptosis. IAPs are characterized by conserved structural baculoviral IAP repeat (BIR) domains that mediate protein-protein interactions. There are currently 8 members of the IAP family with cIAP1, cIAP2 and XIAP being the most studied (24). Inhibition of cIAP1 and cIAP2 by employing specific siRNAs restored susceptibility to Vpr-induced apoptosis in macrophages. Similarly, SMAC mimetic AEG40730 which induces degradation of IAPs was also able to sensitize cells to caspase-3 cleavage and apoptosis in response to Vpr treatment. Moreover, in contrast to Bcl-xL and Mcl-1, inhibition of IAPs did not cause cell death in the absence of Vpr, making these proteins more amenable to therapeutic approaches. These results also suggest a novel, distinct role for cIAP1 and cIAP2 in conferring protection of macrophages against Vpr-induced apoptosis. To our knowledge, this is the first report implicating cIAPs in protecting cells from apoptosis in the context of HIV infection.

The precise molecular mechanism and the signaling pathways by which IAPs induce resistance to Vpr in differentiated macrophages remain to be investigated. It is possible that IAPs expressed in these cells may bind caspase-3, thereby making it inaccessible to cleavage and activation following Vpr treatment. In human peripheral blood mononuclear cells, Vpr causes release of cytochrome-c from the mitochondria with subsequent activation of caspase-9 and caspase-3 (214). However, although caspase-3 was detected in macrophages pretreated with AEG40730 following Vpr treatment (Fig. 11C), I was unable to detect

caspase-9 cleavage (data not shown). This result suggests that sensitization of macrophages probably occurs downstream or independently of mitochondria. This is in agreement with the observations that inhibition of Bcl2 family proteins responsible for maintaining mitochondrial integrity did not render cells susceptible to Vpr treatment.

Another possibility is that Vpr may not inhibit NF-kB activity in macrophages. Both cIAP1 and Bcl2 are known NF-kB-dependent genes (308). This mechanism may be ineffective in macrophages that have constitutively active NF-kB (134) and may explain why Vpr was unable to decrease cIAP1 and Bcl2 expression levels in macrophages. Macrophages may need additional stimuli such as SMAC mimetic to bring down IAPs levels to a minimum threshold that would release caspase-3 from IAP blockage and make it available to Vpr cleavage in order to sensitize macrophages to Vpr-induced cell death.

The apoptotic effect of Vpr has been functionally linked to its ability to cause cell cycle arrest through the activation of the DNA damage-signaling protein ATR in T cells (249). This stress signaling pathway was defective in terminally differentiated macrophages due to absence of key proteins, thus explaining the inability of Vpr to induce cell cycle arrest in a cell type that no longer progresses through the cell cycle (249). Although cell cycle arrest may be a requirement for Vpr-induced apoptosis in lymphocytes, my results suggest that this requirement can be bypassed by downregulating IAPs in differentiated cells.

Although relatively low concentrations of Vpr have been detected in the blood of HIV-infected individuals (229), we and others have used relatively high concentrations of Vpr (1-3 μM) as an apoptosis inducing agent (221, 231). It has been suggested that low levels of Vpr are protective in T cells (297). Our results show that undifferentiated monocytes exposed sequentially to low non-apoptogenic concentrations of Vpr eventually

undergo apoptosis. These results suggest that primary monocytes exposed persistently to low concentrations of Vpr present in serum/lymph nodes of HIV-infected individuals under *in vivo* conditions may in fact undergo apoptosis.

Although the role of Vpr induced apoptosis has been investigated in vivo only in respect to neurons (232), our own results and previous reports suggest a complex model of Vpr effect in HIV infected cells of the monocyte/macrophage lineage. In a recent study Laforge et al. have reported an increased rate of monocytes undergoing apoptosis during the early stage of SIV infection in Rhesus macaques independently of viral replication (309). Although the role of specific HIV peptides was not investigated, the involvement of extracellularly released Vpr in apoptosis of monocytes cannot be ruled out. It was previously shown that despite the lack of productive infection, monocytes from HIV-infected individuals displayed higher levels of both spontaneous and IFNγ-induced cell death compared to uninfected controls (183). If monocytes are also infected, once differentiated they give rise to macrophages that display impaired functioning and are prone to apoptosis (309). However, if macrophages get infected with HIV after differentiation, these cells are protected from cell death. My results suggest that differentiated macrophages display high degree of resistance to Vpr-induced apoptosis. Furthermore, macrophages infected in vitro with HIV were also protected from cell death (data not shown). In non-dividing cells such as macrophages, Vpr is particularly important as it contributes to the localization of the pre-integration complex to the nucleus and allows for permissive HIV infection in the absence of mitosis (310). This property, along with their resistance to Vpr-induced apoptosis that we describe here, promote reservoir formation in this cell type.

Keeping in view our results, it will be interesting to determine if macrophages infected *in vitro* with HIV can be killed by extracellular Vpr in the presence of agents which inhibit IAP expression or activity. We were unable to induce apoptosis in *in vitro* HIV-infected macrophages with SMAC mimetic as a single agent, possibly because of other accessory HIV proteins such as Nef and Tat which have been shown to enhance cell survival (181, 193). However, our results suggest that Vpr peptide provides an excellent experimental setting to study intrinsic cellular factors that may explain macrophage resistance and eliminate indirect effects that other accessory HIV proteins may have on cell survival.

In summary, our results suggest for the first time that by downregulating cIAPs, macrophages could be sensitized to Vpr-induced cell death. Strategies aimed at eliminating HIV viral reservoirs have received considerable interest. Memory T cells have been reactivated with various agents that would drive them out of their resting state and thus sensitize them to conventional antiretroviral therapy (311). Because the mechanisms of viral persistence are different in memory T cells and macrophages, it is reasonable to assume that viral eradication would require different strategies in macrophages. Inducing cell death by manipulating the apoptotic machinery is a strategy currently explored in cancer treatment, where overexpression of antiapoptotic proteins is a major mechanism of chemo-resistance that can be counteracted by using Bcl2 inhibitors (312) or SMAC mimetics (313). Subsequent experiments are needed to indentify apoptotic stimuli that SMAC mimetics may sensitize infected macrophages to. Targeting cIAPs may thus potentially prove beneficial in purging the macrophage HIV reservoir and eventually in killing persistently infected cells.

## Objective 3: To investigate the role of IAPs in human macrophage function.

### Introduction

Macrophages are cells of the innate immune system that participate in inflammation, innate and adaptive immunity in response to various infectious agents (1). Activation of innate immune responses is mediated through pattern recognition receptors such as Toll-like receptors (TLRs) that recognize conserved bacterial or viral structures referred to as pathogen-associated molecular patterns (PAMPs). IAP proteins, which play a critical role in resistance to apoptosis (24), have recently emerged as important regulators of innate immune signaling (314). cIAP1 and cIAP2 associate with the TNF-α receptor complex I to mediate TNF-α induced activation of the classical NFκB pathway (11). In contrast, cIAPs inhibit the alternative NFκB pathway by promoting constant degradation of NFκB-inducing kinase (NIK), the regulatory kinase of non-canonical NFκB signaling (315). cIAP1/2 are also associated with TLR4 and CD40L signaling complexes, where they serve as ubiquitin ligases that promote degradation of adaptor proteins such as TRAF3 (108, 109). IAPs involvement in innate immunity has also been addressed in knockout mouse models. Thus, macrophages of cIAP2 null mice display an impaired inflammatory response in response to LPS, which renders these mice resistant to LPS-induced endotoxic shock (105). cIAP1 was shown to be involved in innate immune responses that control *Chlamydia pneumoniae* infection (107). Similarly, XIAP null mice have a diminished cytokine production and NFκB activation following infection with *Listeria monocytogenes* (106).

Recently, Smac mimetics (SMMs), the IAP antagonists, have been used to enhance our knowledge of the roles of IAPs in innate immunity. SMM are small chemical compounds that replicate the N-terminus of second mitochondria-derived activator of

caspases (SMAC), a mitochondrial protein released into the cytosol in response to apoptotic stimuli to counteract IAP activity. Although initially designed to relieve caspase blockage from XIAP interaction, the ability of SMM to induce apoptosis in cancer cells has been attributed to cIAP1 and 2 downregulation and sensitization to TNF-α-induced cell death, rather than to caspase de-repression from XIAP binding (103). In contrast to cancer cells, this mechanism does not seem to operate in primary cells, which display a remarkable resistance to SMM induced cell death. We have previously shown that downregulating IAPs via SMM treatment does not affect the viability of primary human monocytes (206) or macrophages (233). Similar results were reported for mouse lymphocytes (315).

SMMs induce auto-ubiquitination of IAPs, which leads to their rapid proteosomal degradation. This effect has lead several groups to conclude that cIAPs ablation promotes alternative NFκB activation (91, 92, 315) and inhibits cytokine gene transcription in response to TLR4 signaling (108) in various cell lines and mouse models. However, the impact of SMM treatment on primary cells and human macrophage function remains poorly understood and constitutes the focus of this study. I hypothesized that the activity of SMM extends beyond their effects of cell survival, given the roles of IAPs in NFκB signaling in mouse models and cell lines and the lack of cell death following SMM treatment of primary cells. In this study, I evaluated the effect of cIAPs depletion via SMM treatment on the ability of human macrophages to respond to classical activators such as LPS (TLR4) and CD40L (TNF receptor family) signaling. My results suggest for the first time that SMM treatment inhibits secretion of LPS-induced IL-10 and IL-27 due to its effect on TRAF2. SMMs also inhibit macrophage maturation and LPS-induced activation of MAPKs and classical NFκB pathways, while promoting non-classical NFκB activation.

## Results

### cIAPs selectively regulate LPS and CD40L-induced secretion of monokine induced by IFN-γ (MIG) and anti-inflammatory IL-27 and IL-10

Initially I tested the ability of two Smac mimetics, LN730 and SM164 to downregulate the expression of IAPs. LN730 significantly decreased basal levels of cIAP1, cIAP2 and XIAP (Fig. 25A), whereas SM 164 affected only cIAP1 and cIAP2 (Fig. 25B). Based on these results, I used 50 nM concentration for both compounds in subsequent experiments. Since macrophages are important sources of chemokines and cytokines during the first stages of an immune response (316), I evaluated the impact of SMM treatment on the ability of macrophages to secrete various chemokines and cytokines in response to stimulation with LPS and CD40L. My results indicate that IAPs downregulation via LN or SM treatment resulted selectively in impaired secretion of MIG in response to LPS (Fig. 26A and B) and following LN treatment in response to stimulation with CD40L (Fig. 26C). Although LPS induced higher levels of MIG production compared to CD40L (Fig. 26A and C, left panels), the inhibitory effect of LN and SM was similar for both stimulants, with about 70-80% inhibition of their effect (Fig. 26A and C, right panels). In contrast to the effect on MIG secretion, SMM treatment did not impact the production of macrophage inflammatory protein- 1α (MIP-1α), MIP-1β and IL-8 secretion in response to LPS (Fig. 27). Moreover, SMMs had no effect as single treatment on secretion of the above chemokines. These results indicate a selective effect of IAP ablation on the ability of macrophages to secrete chemokines in response to LPS.

To further understand the effects of SMMs on the immune system, I evaluated the impact of IAPs ablation on the ability of human macrophages to secrete both pro- and anti-

**Figure 25. Effect of Smac mimetics on IAPs levels in MDMs**

MDMs were treated with the indicated concentrations of Smac mimetic LN (A) or with 50 nM of LN and SM (B) for 24 h. Cells were collected and analyzed for IAPs protein expression by Western blotting.

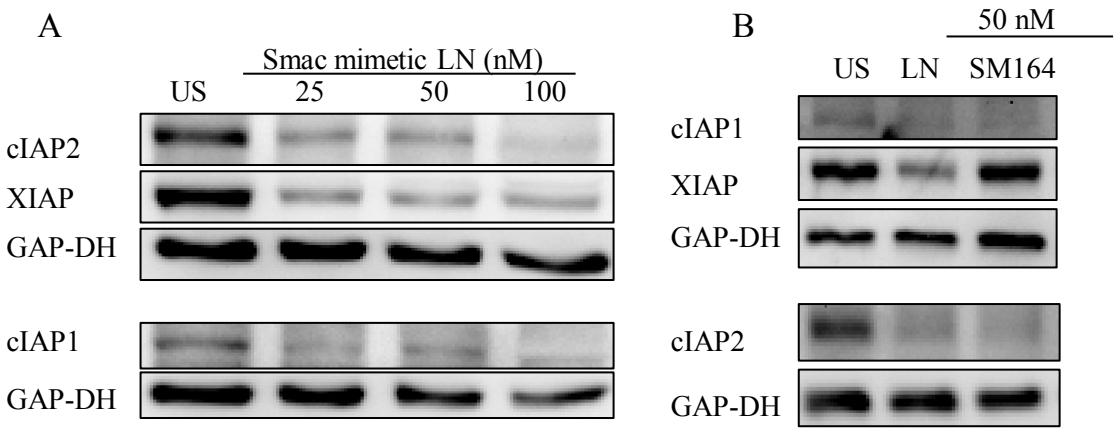

A
Smac mimetic LN (nM)
US
25
50
100
cIAP2
XIAP
GAP-DH
cIAP1
GAP-DH
B
50 nM
US
LN
SM164
cIAP1
XIAP
GAP-DH
cIAP2
GAP-DH

**Figure 25**

**Figure 26. Smac mimetics prevent LPS and CD40L-induced MIG secretion in MDMs**

MDMs from different donors were treated with 50 nM of Smac mimetic LN (A and B) or SM (C) for 24 h, followed by another 24 h of LPS 100 ng/ml (A and C) or CD40L (B). Supernatants were collected and evaluated for MIG expression by flow cytometry as described in Materials and methods. Each symbol on the left side dot plots represents an individual donor. Horizontal lines indicate mean values. Right side panels show % MIG secretion relative to maximal concentration induced by LPS/CD40L, which was considered 100%. Graph bars show the mean ±SD.* indicates $p<0.05$.

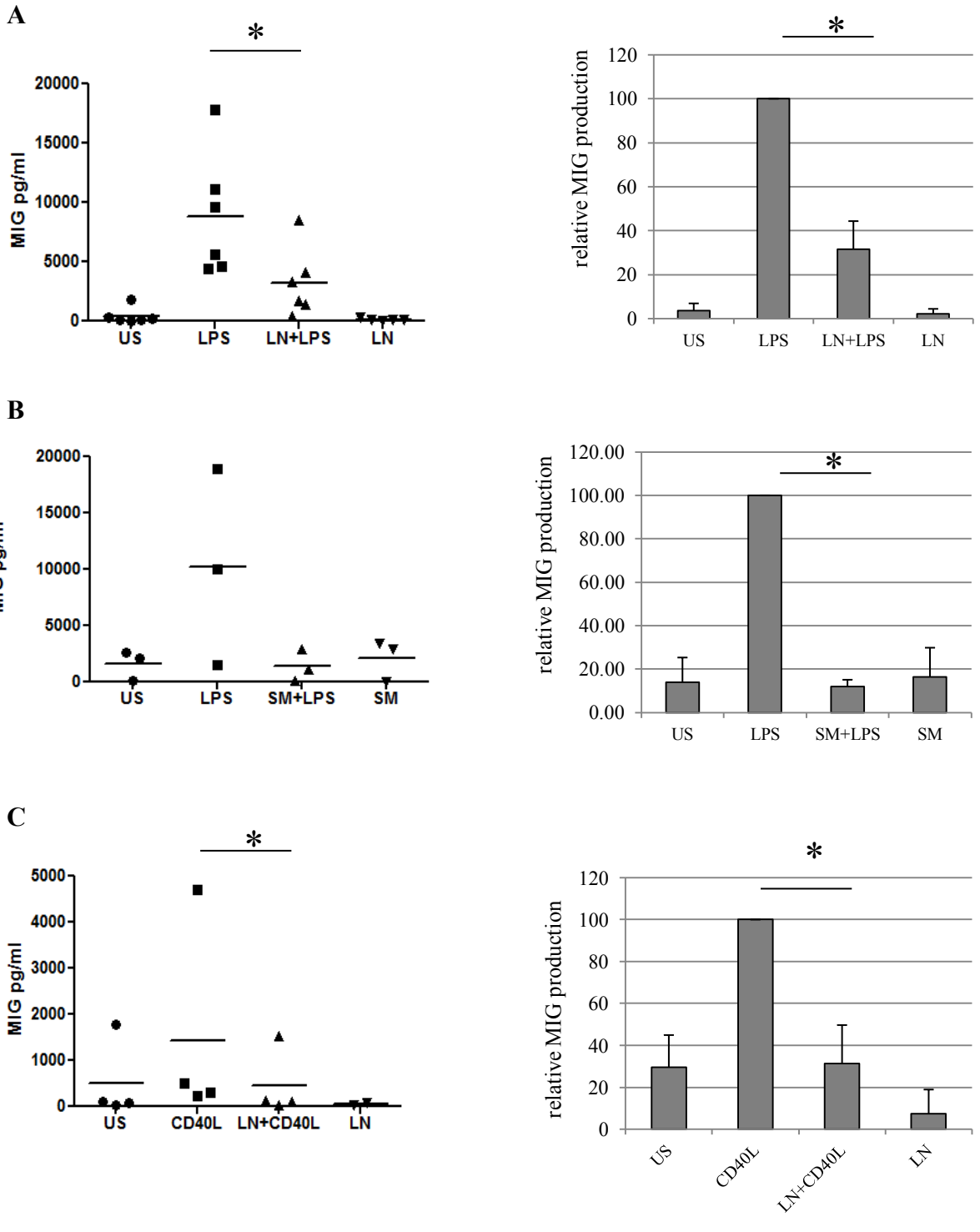

A
*
MIG pg/ml
20000
15000
10000
5000
0
US
LPS
LN+LPS
LN
relative MIG production
120
100
80
60
40
20
0
B
SM+LPS
SM
120.00
100.00
80.00
60.00
40.00
20.00
0.00
C
CD40L
LN+CD40L
5000
4000
3000
2000
1000

**Figure 26**

**Figure 27. Smac mimetic LN does not affect LPS-induced MIP-1α, MIP-1β and IL-8 secretion in MDMs**

MDMs were treated with 50 nM of LN for 24 h, followed by another 24 h of LPS 100 ng/ml. Supernatants were collected and evaluated by flow cytometry as described in Materials and methods for expression of MIP-1α, MIP-1β and IL-8. Each symbol represents an individual donor. Horizontal lines indicate mean values.

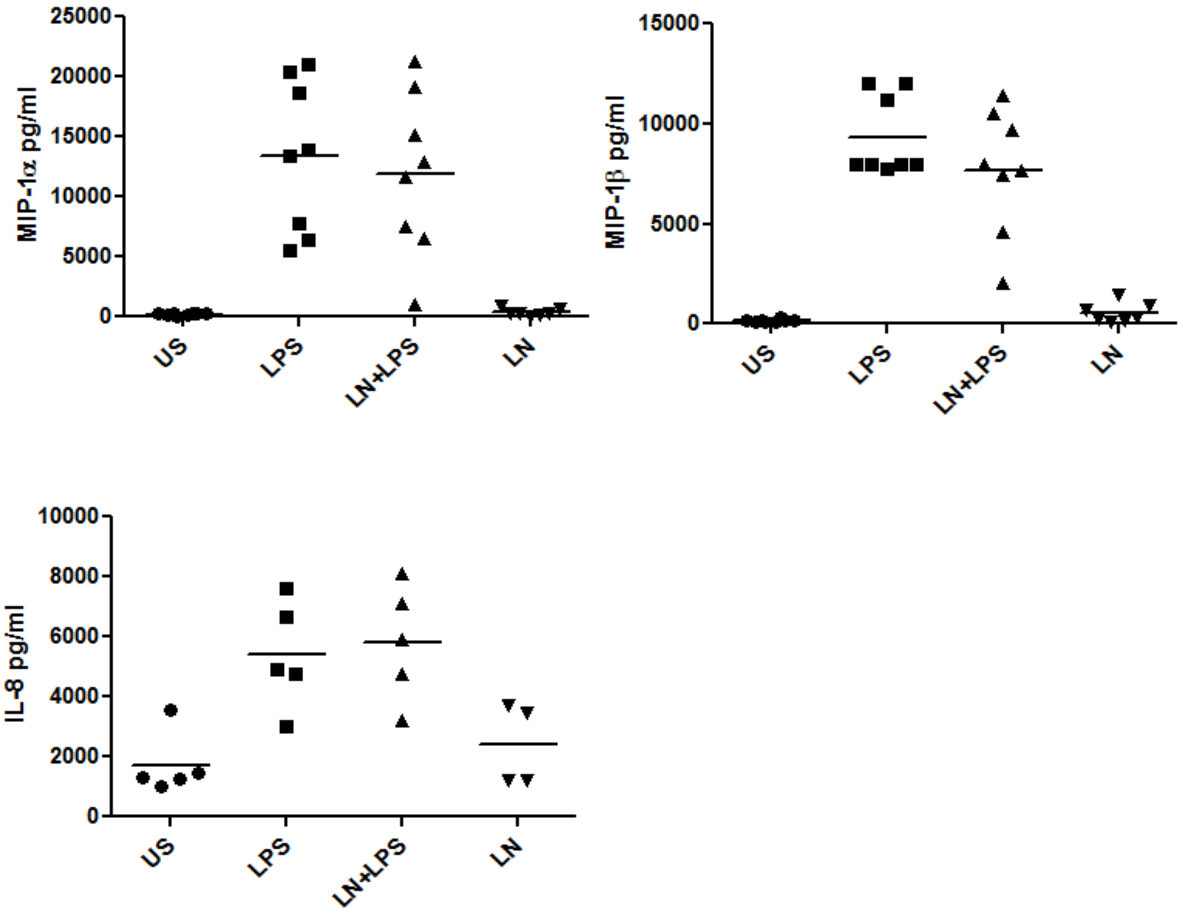

MIP-1α pg/ml
25000
20000
15000
10000
5000
0
US
LPS
LN+LPS
LN
MIP-1β pg/ml
15000
10000
5000
0
US
LPS
LN+LPS
LN
IL-8 pg/ml
10000
8000
6000
4000
2000
0
US
LPS
LN+LPS
LN

**Figure 27**

inflammatory cytokines. My results show that both LN and SM inhibited LPS-induced IL-10 secretion with similar efficiency of 60% (Fig. 28). Furthermore, IAPs downregulation by LN or SM inhibited secretion of IL-27 in response to LPS (Fig. 29A and B) and following LN treatment in response to stimulation with CD40L (Fig. 29C). Similar results showing inhibition of LPS-induced IL-10 and IL-27 production following SMMs treatment of primary monocytes were observed (Fig. 30) suggesting that this effect is not limited to macrophages. However, LN did not affect the expression of other proinflammatory cytokines including IL-1β, IL-5, IL-6, IL-18, IL-23, IFN-γ, TNF-α and IL-12p40 in response to LPS (Fig. 31).

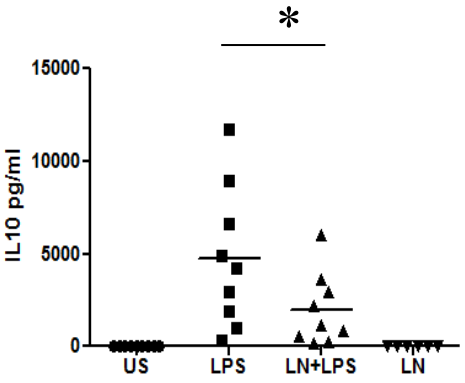
**cIAPs-mediated MAPKs signaling contributes to LPS-induced IL-10, IL-27 and MIG production**

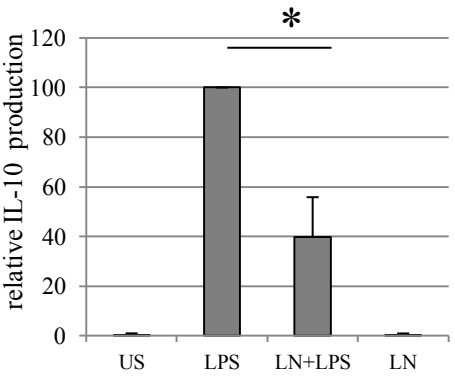
During an inflammatory response, TLR4 signaling in response to LPS leads to MAPKs and NFκB activation to promote cytokine synthesis (317). cIAPs are also required for MAPK activation in response to stimulation with LPS or CD40L ligation (109). To determine if Smac mimetics influence cytokine secretion by inhibition of MAPKs, I first measured the phosphorylation of p38, JNK and ERK in response to LPS in MDMs pretreated with Smac mimetics. Although MAPK activities in LN treated and unstimulated cells were comparable, inhibition of cIAPs by Smac mimetics significantly inhibited LPS-induced p38, ERK and JNK phosphorylation compared to LPS alone -stimulated cells (Fig. 32).

**Figure 28. Smac mimetics LN and SM inhibit LPS-induced IL-10 secretion in MDMs**

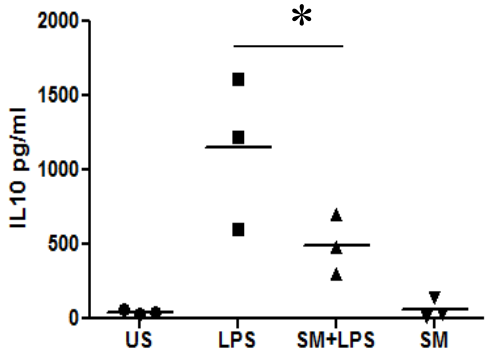
MDMs were treated with 50 nM of Smac mimetic LN (A) or SM (B) for 24 h, followed by another 24 h of LPS 100 ng/ml. Supernatants were collected and evaluated for IL-10 expression by flow cytometry as described in Materials and methods. Each symbol on the left side dot plots represents an individual donor. Horizontal lines indicate mean values. Right side panels show % IL-10 secretion relative to maximal concentration induced by LPS, which was considered 100%. Graph bars show the mean ±SD.* indicates $p<0.05$.

A


*
15000
10000
5000
0
IL10 pg/ml
US
LPS
LN+LPS
LN


*
120
100
80
60
40
20
0
relative IL-10 production
US
LPS
LN+LPS
LN

B


*
2000
1500
1000
500
0
IL10 pg/ml
US
LPS
SM+LPS
SM

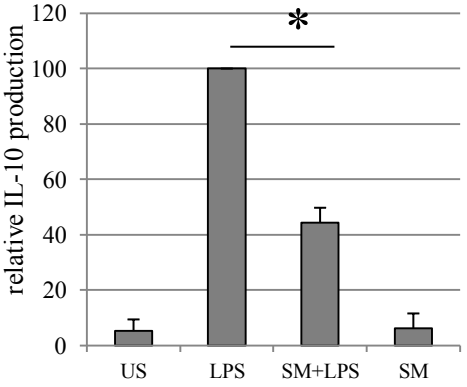
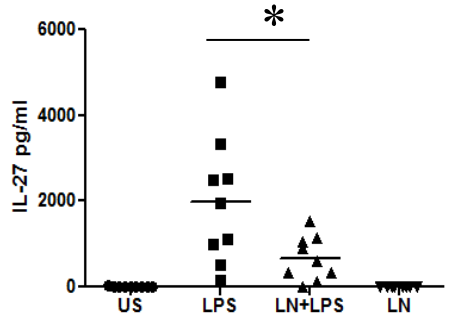

*
120
100
80
60
40
20
0
relative IL-10 production
US
LPS
SM+LPS
SM

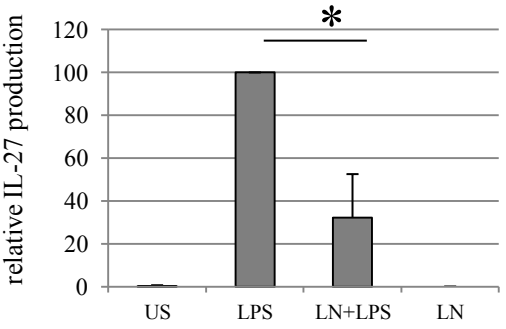
**Figure 28**

**Figure 29. Smac mimetics inhibit LPS and CD40L-induced IL-27 production in MDMs**

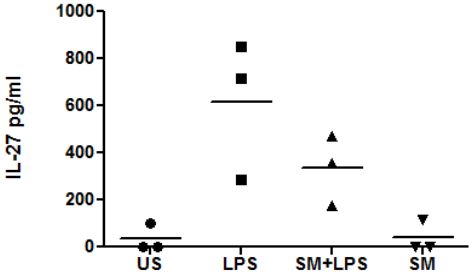
MDMs were treated with 50 nM of Smac mimetic LN (A and B) or SM (C) for 24 h, followed by another 24 h of LPS 100 ng/ml (A and C) or CD40L (B). Supernatants were collected and evaluated for IL-27 expression by ELISA as described in Materials and methods. Each symbol on the left side dot plots represents an individual donor. Horizontal lines indicate mean values. Right side panels show % IL-27 secretion relative to maximal concentration induced by LPS/CD40L, which was considered 100%. Graph bars show the mean ±SD.* indicates $p<0.05$.

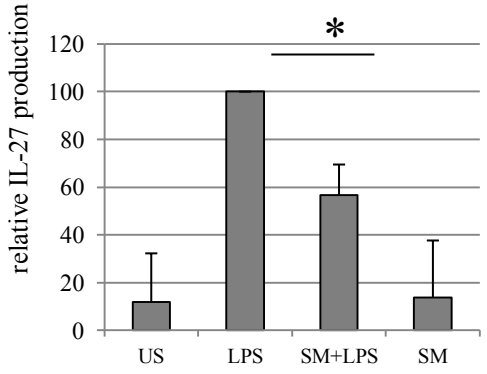
A.



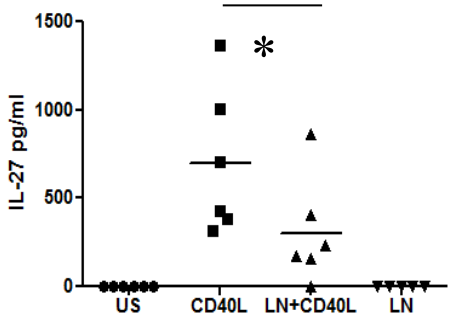




B.




C.



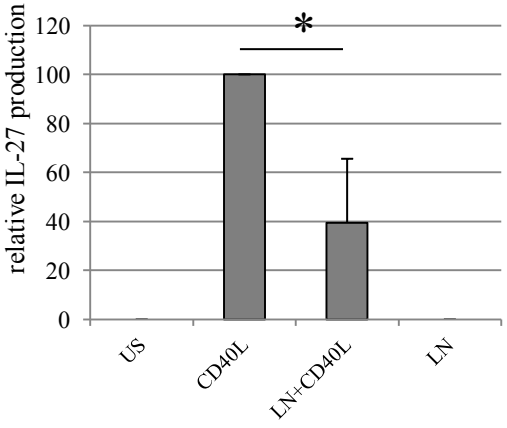




**Figure 29**

**Figure 30. Smac mimetics inhibit LPS-induced IL-27 and IL-10 production in primary human monocytes**

Following isolation, primary monocytes were treated with Smac mimetics and LPS (1 μg/ml) at the same time. The second day supernatants were collected and evaluated for IL-27 by ELISA and for IL-10 by flow cytometry as described in Materials and methods. Each symbol on the left side dot plots represents an individual donor. Horizontal lines indicate mean values. Right side panels show % secretion relative to maximal concentration induced by LPS, which was considered 100%. Graph bars show the mean ±SD.* indicates $p<0.05$.

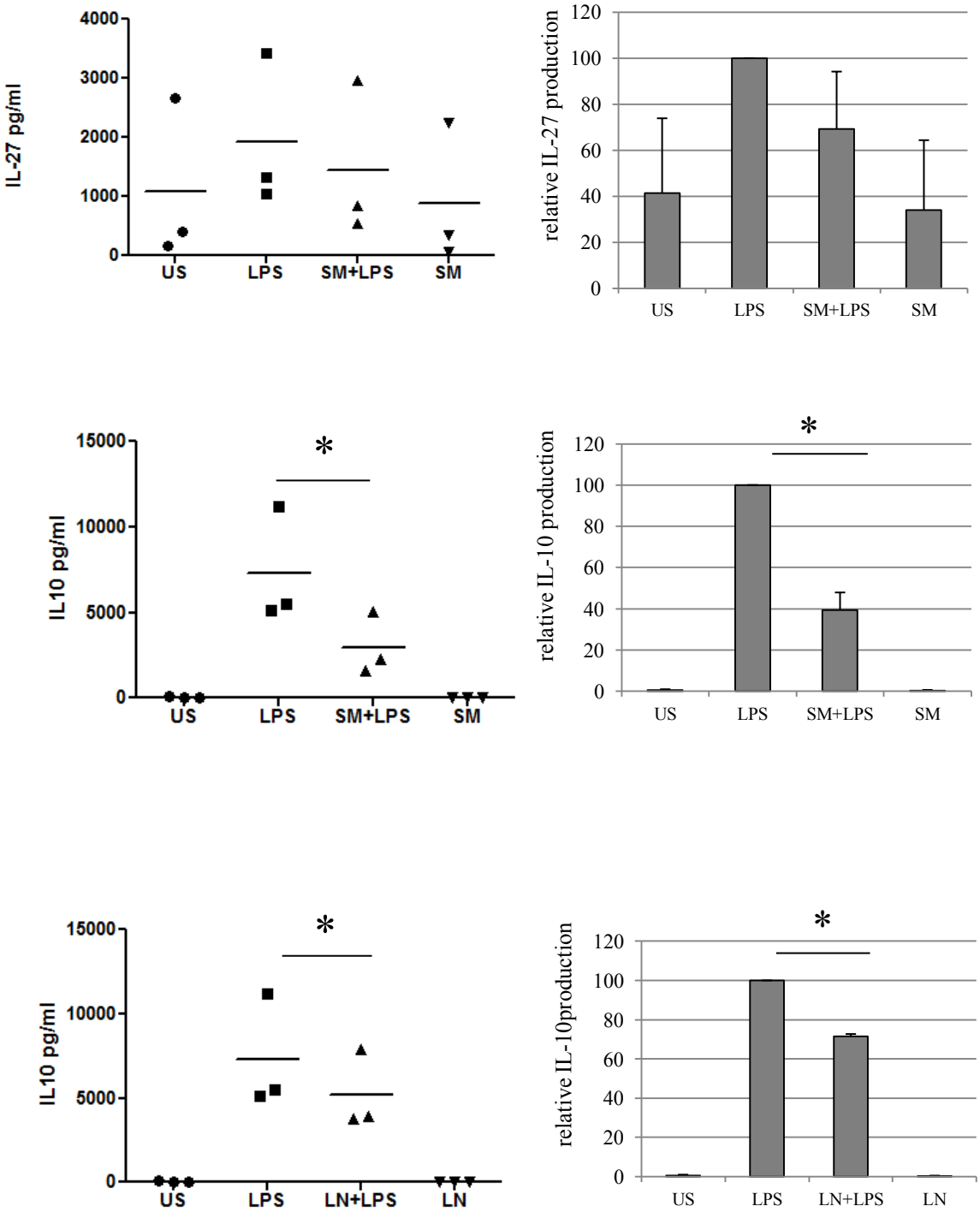

IL-27 pg/ml
4000
3000
2000
1000
0
US
LPS
SM+LPS
SM
relative IL-27 production
120
100
80
60
40
20
0
US
LPS
SM+LPS
SM
IL10 pg/ml
15000
10000
5000
0
*
US
LPS
SM+LPS
SM
relative IL-10 production
120
100
80
60
40
20
0
*
US
LPS
SM+LPS
SM
IL10 pg/ml
15000
10000
5000
0
*
US
LPS
LN+LPS
LN
relative IL-10production
120
100
80
60
40
20
0
*
US
LPS
LN+LPS
LN

**Figure 30**

**Figure 31. Smac mimetic LN does not affect LPS-induced IL-1β, IL-5, IL-6, IL-18, IL-23, IFN-γ, IL-12p40 and TNF-α production in MDMs**

MDMs were treated with LPS either in the presence or absence of LN pretreatment. After 24 h supernatants were collected and evaluated for the indicated cytokines by flow cytometry (IL-1β, IL-5, IL-6, IL-18, IL-23, IFN-γ) or ELISA (IL-12p40, TNF-α) as described in Materials and methods. Each symbol represents an individual donor. Horizontal lines indicate mean values.

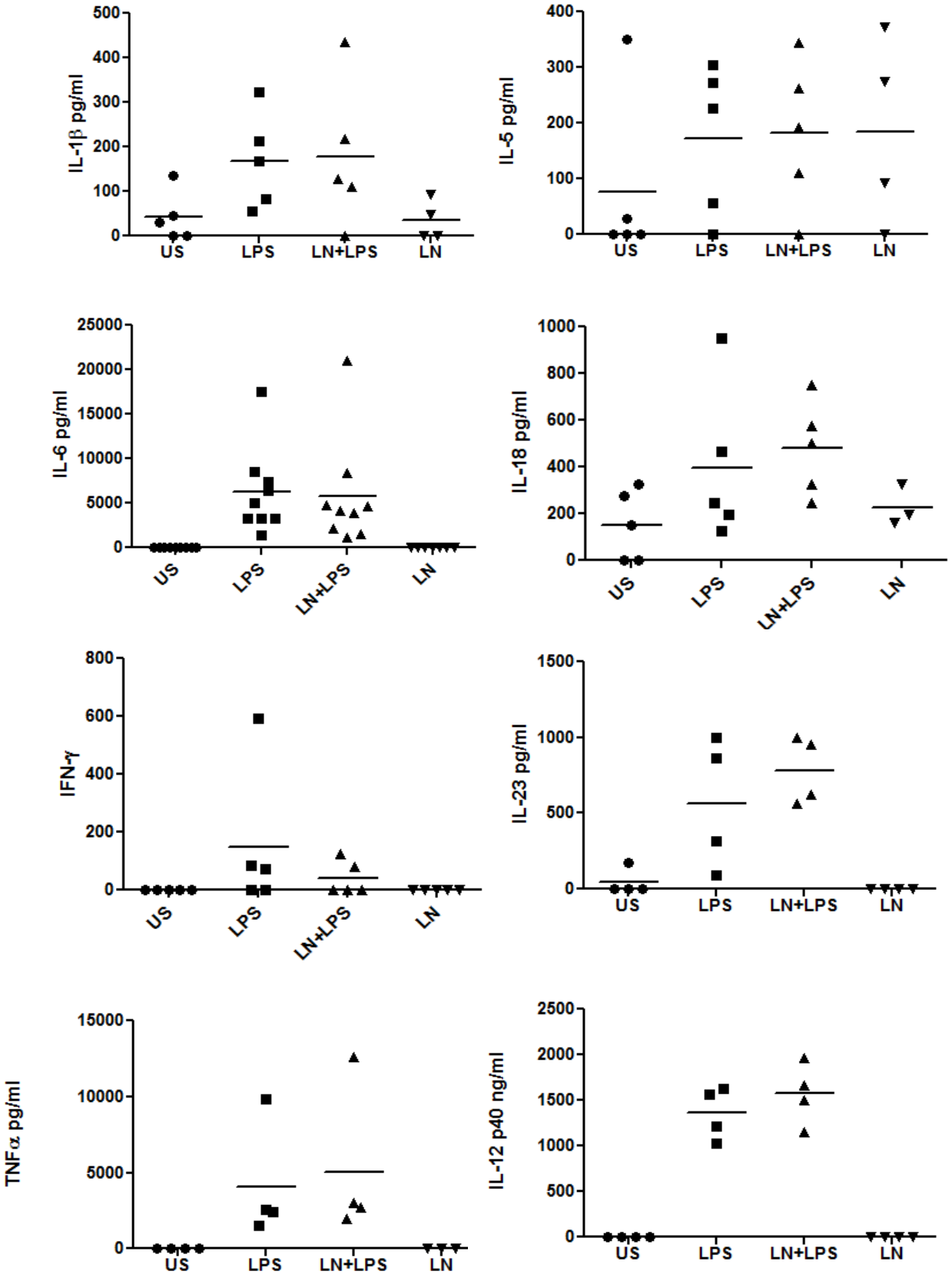

IL-1β pg/ml
500
400
300
200
100
0
US
LPS
LN+LPS
LN
IL-5 pg/ml
400
300
200
100
0
US
LPS
LN+LPS
LN
IL-6 pg/ml
25000
20000
15000
10000
5000
0
US
LPS
LN+LPS
LN
IL-18 pg/ml
1000
800
600
400
200
0
US
LPS
LN+LPS
LN
IFN-γ
800
600
400
200
0
US
LPS
LN+LPS
LN
IL-23 pg/ml
1500
1000
500
0
US
LPS
LN+LPS
LN
TNFα pg/ml
15000
10000
5000
0
US
LPS
LN+LPS
LN
IL-12 p40 ng/ml
2500
2000
1500
1000
500
0
US
LPS
LN+LPS
LN

**Figure 31**

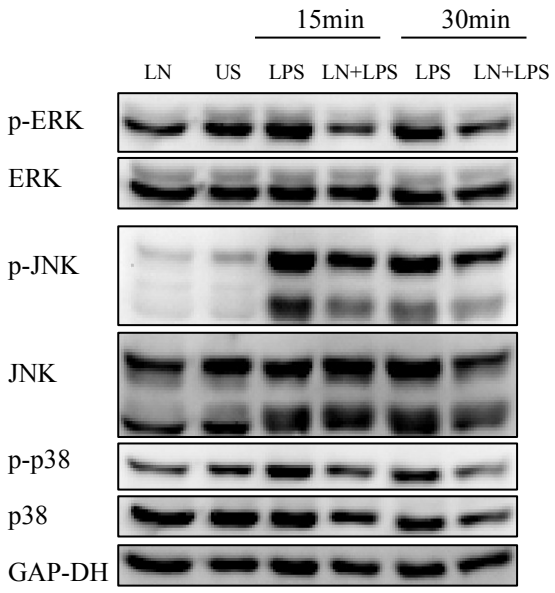
To determine if SMM effect on secretion of IL-10, IL-27 and MIG could be attributed to altered MAPKs signaling, I initially determined the requirement for these pathways in regulation of IL-10, IL-27 and MIG. The signaling pathways involved in IL-10 regulation have been investigated in various cell types including primary monocytes (318, 319). However, the mechanisms of regulation of IL-10, IL-27 and MIG in human MDMs remains unknown. For this purpose, I have used a set of specific chemical inhibitors: SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and PD098059 (ERK inhibitor). Inhibition of JNK and p38 at concentrations of 25 and 50 μM significantly reduced the ability of LPS to induce IL-27 (Fig. 33A). Similarly, NFκB and JNK inhibition resulted in diminished secretion of IL-10 (Fig. 33B) and MIG (Fig. 33C) at both of the tested concentrations. However, inhibition of ERK did not affect LPS-induced production of either MIG, IL-10 or IL-27 (Fig 33). In summary, JNK and p38 positively regulate LPS-induced IL-27, IL-10 and MIG production in human MDMs. These results also suggest that Smac mimetics may be influencing cytokine secretion at least in part by inhibition of MAPKs signaling.

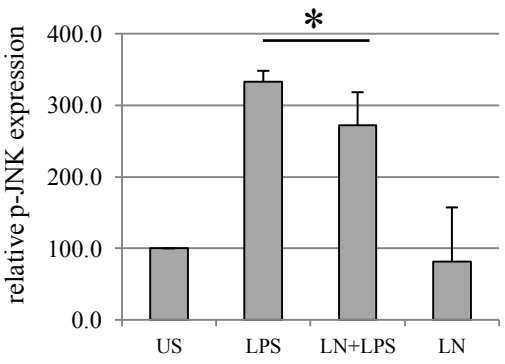
**cIAPs-mediated NFκB signaling contributes to LPS-induced IL-10, IL-27 and MIG production**

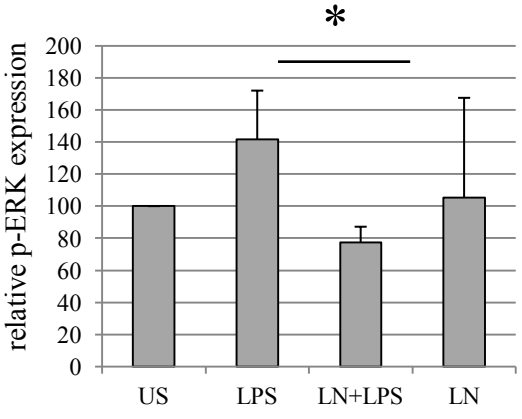
Since TLR4 and CD40L signaling culminate with activation of NFκB pathway, I evaluated if Smac mimetics affect NFκB signaling in MDMs. cIAPs ablation via Smac mimetics has been shown to promote NFκB activation of the classical pathway in cancer cells due to increased TNF-α secretion (91, 92), but the effect of SMMs in human macrophages is not known. Therefore, I evaluated the level of IκBα protein as an indirect

**Figure 32. LPS-induced MAPKs activation is dependent on IAPs.**

MDMs were treated with Smac mimetic LN for 24 h before stimulation with LPS for the indicated times. Cells were collected and evaluated for phosphorylation status of MAPKs by Western blotting. GAP-DH was used as a loading control. Based on the densitometric analysis, the bar graphs show the mean ± SD of relative expression from three experiments with different donors.


15min
30min
LN
US
LPS
LN+LPS
LPS
LN+LPS
p-ERK
ERK
p-JNK
JNK
p-p38
p38
GAP-DH


relative p-JNK expression
400.0
300.0
200.0
100.0
0.0
*
US
LPS
LN+LPS
LN


relative p-ERK expression
200
180
160
140
120
100
80
60
40
20
0
*
US
LPS
LN+LPS
LN

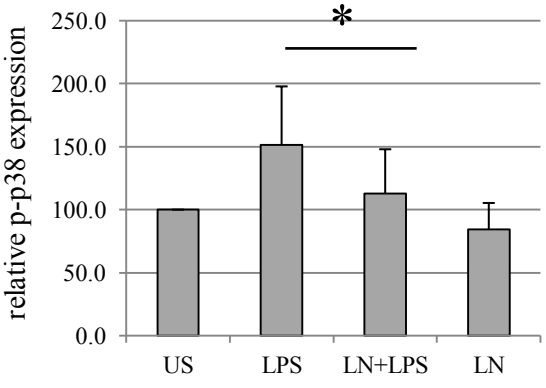

relative p-p38 expression
250.0
200.0
150.0
100.0
50.0
0.0
*
US
LPS
LN+LPS
LN

**Figure 32**

**Figure 33. The involvement of MAPKs signaling pathways in the regulation of IL-10, IL-27 and MIG**

MDMs were treated for 2 h with the indicated concentrations of pharmacological inhibitors SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and PD098059 (ERK inhibitor) before LPS stimulation. After 24 h of LPS treatment, supernatants were collected and evaluated for IL-27 by ELISA and for IL-10 and MIG by flow cytometry as described in Materials and methods. Graph bars show the mean ±SD of % cytokine secretion relative to maximal concentration induced by LPS, which was considered 100%. * indicates $p<0.05$. N=2-4

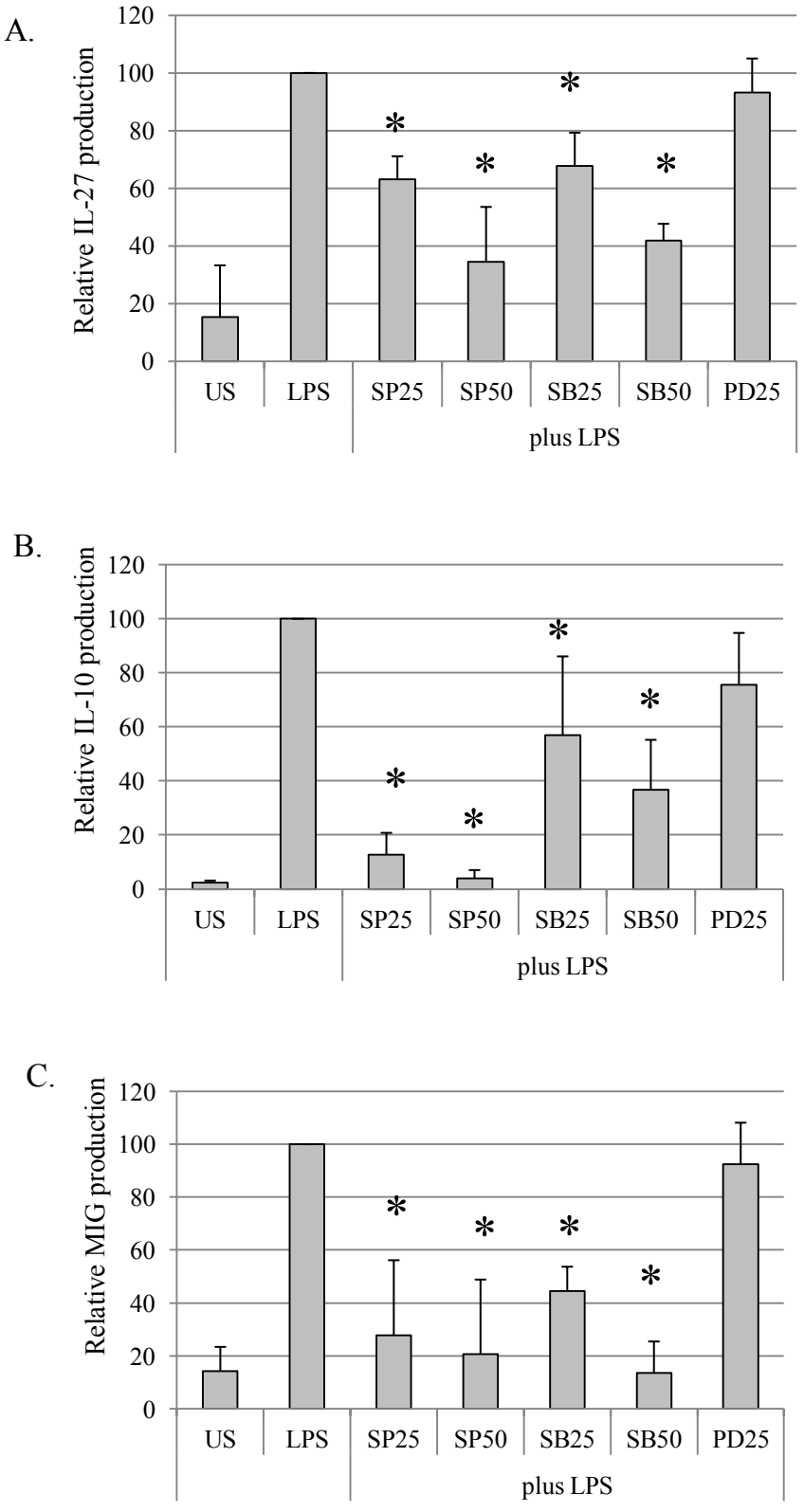

A.
Relative IL-27 production
120
100
80
60
40
20
0
US
LPS
SP25
SP50
SB25
SB50
PD25
plus LPS
B.
Relative IL-10 production
120
100
80
60
40
20
0
US
LPS
SP25
SP50
SB25
SB50
PD25
plus LPS
C.
Relative MIG production
120
100
80
60
40
20
0
US
LPS
SP25
SP50
SB25
SB50
PD25
plus LPS

**Figure 33**

indicator of the classical NFκB activity. LPS decreased IκBα levels, an effect that was attenuated in macrophages primed with either LN or SM (Fig. 34A). The Smac mimetics alone did not affect IκBα expression.

Non-canonical regulation of NFκB depends on NFκB-inducing kinase (NIK), which promotes partial processing of the p100 subunit to its p52 mature form (285). In resting cells this pathway is subdued via continuous NIK degradation performed by cIAP1/2 (315). Smac mimetic treatment leads to NIK accumulation and subsequent activation of non-canonical NFκB pathway in cancer cells (91, 92) and mice (315). The effect of Smac mimetics on alternative NFκB signaling in human macrophages is currently unknown. My results suggest that Smac mimetics augment both LPS and CD40L-induced p100 processing to p52 (Fig. 34B). Moreover, SMMs also increased p52/p100 ratio compared to untreated cells (Fig. 34B). In conclusion, SMMs promote alternative NFκB signaling in contrast to the inhibitory effect on the classical pathway. Overall, these results suggest a dual effect on NFκB, both inhibitory (classical pathway) and activatory (alternative pathway).

To determine if SMM effect on secretion of IL-10, IL-27 and MIG could be attributed to altered NFκB signaling, I next determined the requirement for NFκB in regulation of IL-10, IL-27 and MIG. For this purpose, I have used the chemical inhibitor CAPE to block NFκB pathway. Similarly to JNK and p38 results, inhibition of NFκB at concentrations of 25 and 50 μM significantly reduced the ability of LPS to induce IL-27 (Fig. 34C), IL-10 (Fig. 34D) and MIG (Fig. 34E). Given the requirement for classical NFκB in regulating IL-27, IL-10 and MIG and the inhibitory effect of SMMs on this pathway, I can speculate that IAPs contribute to cytokine secretion at least in part by promoting NFκB signaling.

**Figure 34. Effect of Smac mimetics treatment on NFκB pathway and the involvement of NFκB in the regulation of IL-10, IL-27 and MIG**

MDMs were treated with Smac mimetics for 24 h, followed by LPS treatment for 30 min (A). Both LPS and CD40L were used for another 24 h to activate alternative NFκB signaling (B). Cells were collected and evaluated for the expression of IκBα (A) and p100 to p52 processing (B) by Western blotting. Results shown are representative for at least three different donors. For cytokine production MDMs were treated for 2 h with the indicated concentrations of CAPE (NFκB inhibitor), followed by another 24 h of LPS stimulation (C-E). Supernatants were collected and evaluated for IL-27, IL-10 and MIG production as described. Graph bars show the mean ±SD of % cytokine secretion relative to maximal concentration induced by LPS, which was considered 100%. * indicates $p<0.05$. N=2-4

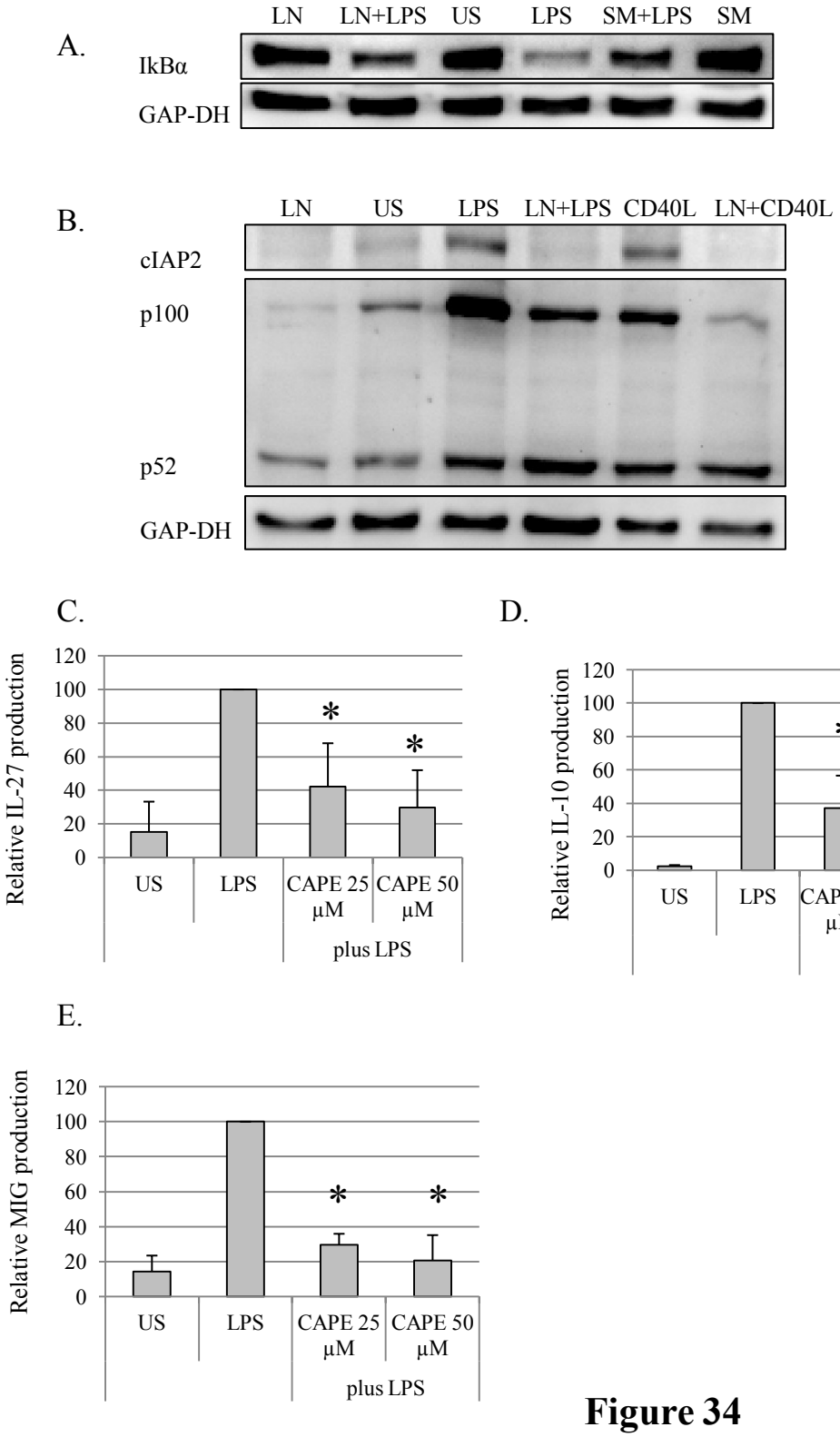

A.
LN
LN+LPS
US
LPS
SM+LPS
SM
IkBα
GAP-DH
B.
LN
US
LPS
LN+LPS
CD40L
LN+CD40L
cIAP2
p100
p52
GAP-DH
C.
Relative IL-27 production
120
100
80
60
40
20
0
US
LPS
CAPE 25 µM
CAPE 50 µM
plus LPS
D.
Relative IL-10 production
120
100
80
60
40
20
0
US
LPS
CAPE 25 µM
CAPE 50 µM
plus LPS
E.
Relative MIG production
120
100
80
60
40
20
0
US
LPS
CAPE 25 µM
CAPE 50 µM
plus LPS

**Figure 34**

**cIAPs regulate RIP1 induction independently of their effect on LPS-induced IL-10, IL-27 and MIG**

To further dissect the molecular mechanism of SMM inhibition of LPS and CD40L signaling, I investigated the role of receptor interacting protein 1 (RIP1), a death domain containing kinase involved in TNF-R1 signaling (320). RIP1 acts as an adaptor protein that specifically mediates p38 MAPK activation in response to TNF-α (321) and NFκB activation via TLR3 and TLR4 (322). RIP1 is required for the apoptotic effect of Smac mimetics on cancer cells, since RIP1 knockdown protected cells from cell death following SMM treatment (93, 94). Taking into consideration the importance of RIP1 in pro-inflammatory signaling and its requirement for SMM-induced in apoptosis, I determined if it was also involved in SMM effect on cytokine signaling.

Initially, I tested the ability of SMM to influence RIP1 levels in human macrophages. Similar to the effect on cIAP2, which was used as a control for SMM activity, RIP1 levels were increased following LPS or CD40L treatment and this effect was prevented when cells were pretreated with SMM (Fig. 35A). To determine the role of RIP1 in SMM-mediated inhibition of LPS-induced IL-10, IL-27 and MIG production, I have used siRNA to knockdown RIP1 levels and measure the ability of macrophages to secrete IL-10, IL-27 and MIG in response to LPS. Despite successful knockdown of RIP1 (Fig. 35B), LPS ability to secrete IL-10, IL-27 or MIG remained unchanged (Fig. 35C). These results suggest that SMM-mediated inhibition of LPS-induced IL-10, IL-27 and MIG production does not involve RIP1 induction.

**Figure 35. RIP1 is not involved in the regulation of LPS induced IL-10, IL-27 and MIG production**

**A**. MDMs were treated with LPS/CD40L in the presence or absence of LN pretreatment. Whole protein lysates were evaluated for RIP1 expression by Western blotting. GAP-DH was used as a loading control and cIAP2 was used as a positive control for LN activity. **B** and **C**. MDMs were transfected with RIP1 siRNA or control siRNA for 24 h as described in Materials and methods before adding LPS for another 24 h. After LPS treatment whole protein lysates were evaluated for RIP1 knockdown by Western blotting (B) and supernatants were used to measure IL-27 by ELISA and IL-10 and MIG by flow cytometry (C). Graph bars show the mean ±SE of three experiments with different donors.

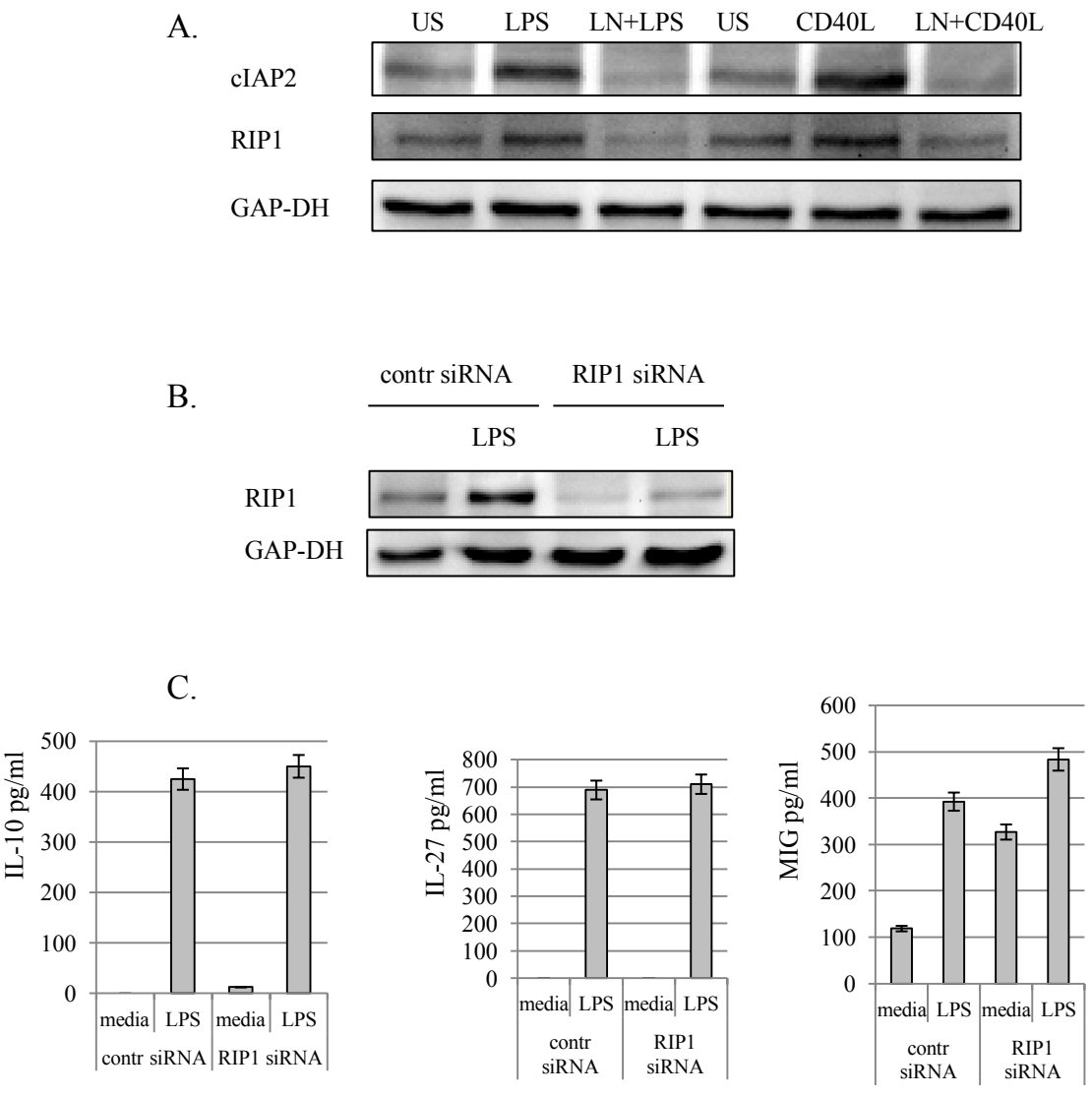

A.
US
LPS
LN+LPS
US
CD40L
LN+CD40L
cIAP2
RIP1
GAP-DH
B.
contr siRNA
RIP1 siRNA
LPS
LPS
RIP1
GAP-DH
C.
IL-10 pg/ml
500
400
300
200
100
0
media
LPS
media
LPS
contr siRNA
RIP1 siRNA
IL-27 pg/ml
800
700
600
500
400
300
200
100
0
media
LPS
media
LPS
contr siRNA
RIP1 siRNA
MIG pg/ml
600
500
400
300
200
100
0
media
LPS
media
LPS
contr siRNA
RIP1 siRNA

**Figure 35**

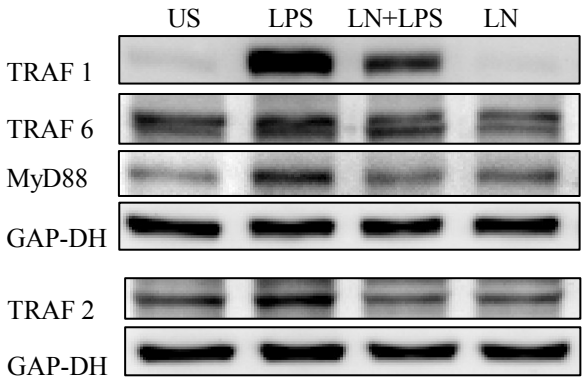
**Effect of cIAPs ablation on TLR4 signaling proteins**

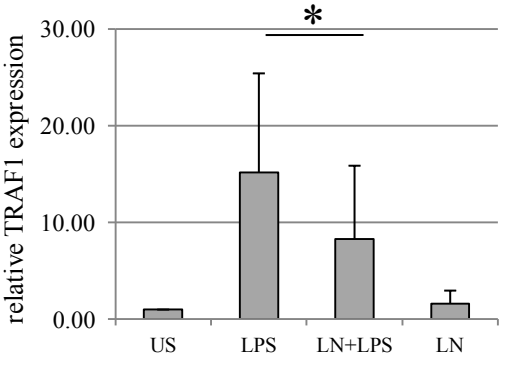
To elucidate the mechanism of SMM inhibition of LPS signaling, I investigated the effect of SMM on proteins involved in TLR4 signaling. LPS binding to TLR4 triggers two separate signaling cascades according to different adaptor molecules recruited to the receptor, MyD88 and TRIF signaling, that culminate with cytokine production and interferon responses respectively (323). TNF receptor associated factors (TRAFs) are signal transduction molecules involved in multiple transduction pathways, such as TLR and TNF receptor signaling. Of all six TRAF molecules, TRAF6 is implicated in TLR4 signaling downstream of MyD88, where it serves as ubiquitin ligase that activates IκB kinase (IKK) and classical NFκB signaling (324). TRAF2 is mainly involved in TNF signaling, while the role of TRAF1 is to recruit cIAP1 and 2 to TRAF2, thereby forming a cIAP1/2-TRAF1/2 heterocomplex (325).

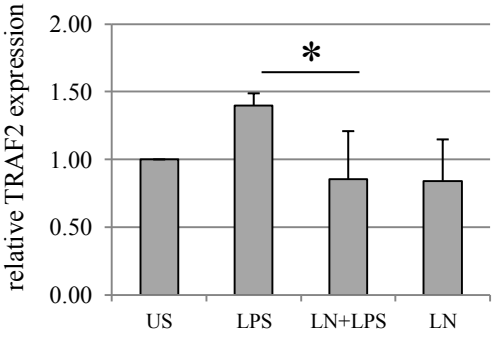
Initially, I evaluated if SMM affects TRAF1, 2, 6 and MyD88 expression levels. LN treatment inhibited LPS-induced TRAF1, TRAF2 and MyD88, with no effect on TRAF6 (Fig. 36) suggesting that these adaptor proteins may be involved in SMM-mediated inhibition of cytokine secretion. To determine the involvement of TRAF1 and TRAF2 on IL-10, IL-27 and MIG secretion, I have used siRNA for both TRAF1 and 2, which induced specific knockdown of their targets (Fig. 37A). There was impaired IL-10 (Fig. 37B) and IL-27 (Fig. 37C) secretion in response to LPS in cells transfected with TRAF2 siRNA compared to controls and no such effect was observed with TRAF1 knockdown. In contrast, downregulation of both TRAF1 and 2 prevented LPS-induced MIG secretion (Fig. 37D), suggesting that TRAF2 is important for IL-10 and IL-27 production, whereas both TRAF1

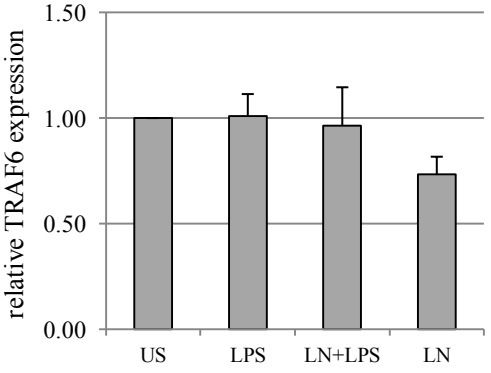
**Figure 36. Smac mimetic LN prevents LPS induction of TRAF1, TRAF2 and MyD88**

MDMs were treated with LPS in the presence or absence of LN pretreatment. Whole protein lysates were evaluated for TRAFs and MyD88 expression by Western blotting. GAP-DH was used as a loading control. Based on the densitometric analysis, the bar graphs show the mean ± SD of relative expression from three different donors.


US
LPS
LN+LPS
LN
TRAF 1
TRAF 6
MyD88
GAP-DH
TRAF 2
GAP-DH


relative TRAF1 expression
30.00
20.00
10.00
0.00
*
US
LPS
LN+LPS
LN


relative TRAF2 expression
2.00
1.50
1.00
0.50
0.00
*
US
LPS
LN+LPS
LN


relative TRAF6 expression
1.50
1.00
0.50
0.00
US
LPS
LN+LPS
LN

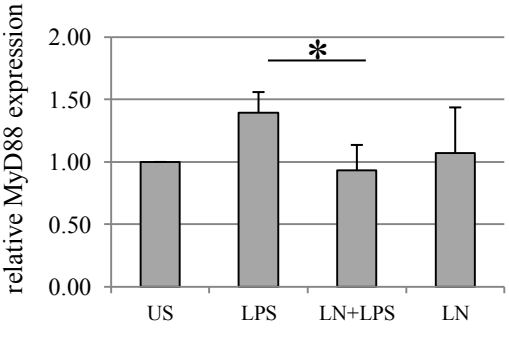

relative MyD88 expression
2.00
1.50
1.00
0.50
0.00
*
US
LPS
LN+LPS
LN

**Figure 36**

**Figure 37. TRAF2 mediates LPS induction of IL-10, IL-27 and MIG production in MDMs**

MDMs were transfected with TRAF1 siRNA, TRAF2 siRNA or control siRNA for 24 h before adding LPS for another 24 h. After LPS treatment whole protein lysates were evaluated for TRAF1 and TRAF2 knockdown by Western blotting (A) and supernatants were used to measure IL-27 by ELISA (C) and IL-10 (B) and MIG (D) by flow cytometry. Graph bars show the mean ±SD of three experiments with different donors.

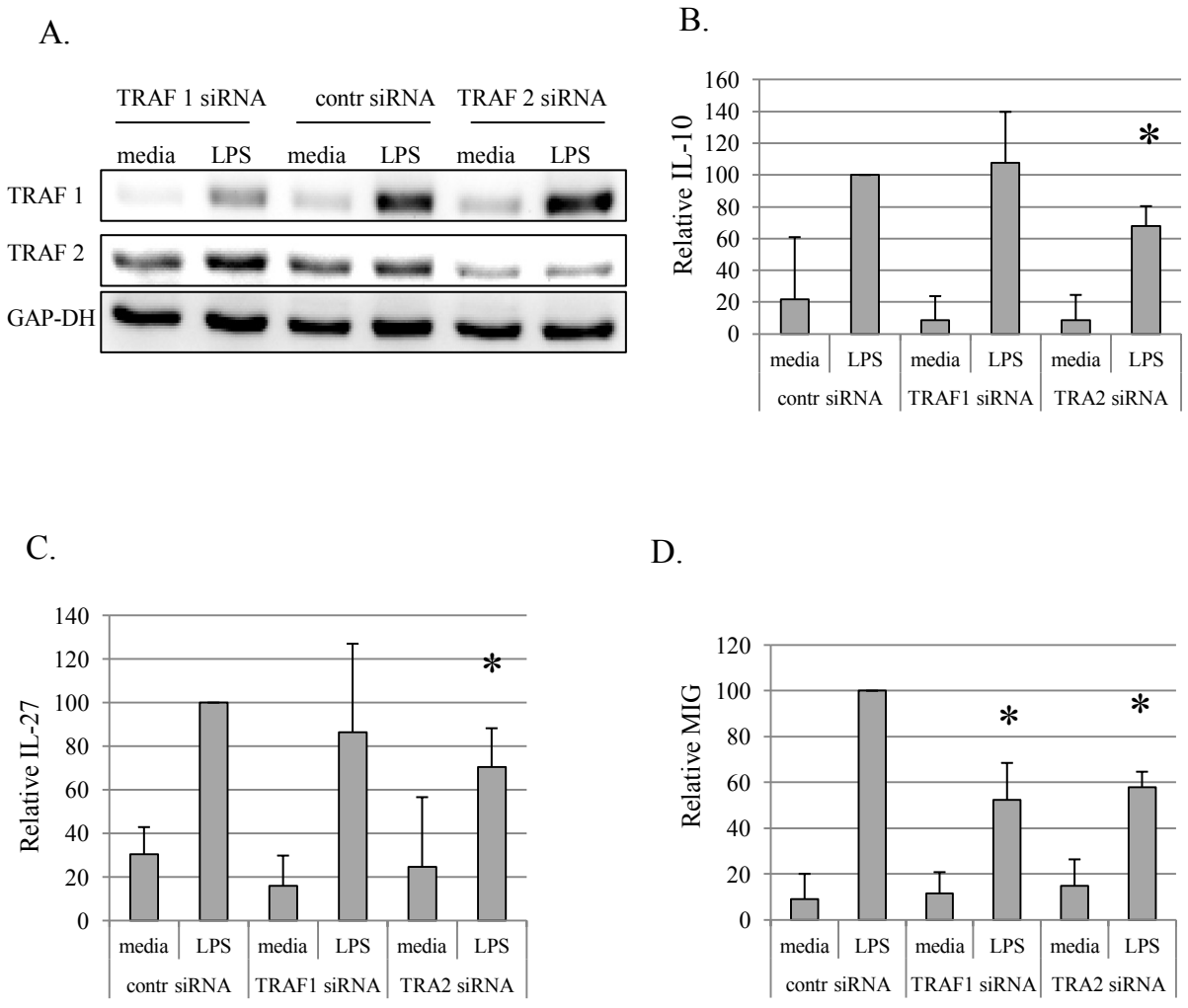

A.
TRAF 1 siRNA
contr siRNA
TRAF 2 siRNA
media
LPS
media
LPS
media
LPS
TRAF 1
TRAF 2
GAP-DH
B.
Relative IL-10
160
140
120
100
80
60
40
20
0
*
media
LPS
media
LPS
media
LPS
contr siRNA
TRAF1 siRNA
TRA2 siRNA
C.
Relative IL-27
140
120
100
80
60
40
20
0
*
media
LPS
media
LPS
media
LPS
contr siRNA
TRAF1 siRNA
TRA2 siRNA
D.
Relative MIG
120
100
80
60
40
20
0
*
*
media
LPS
media
LPS
media
LPS
contr siRNA
TRAF1 siRNA
TRA2 siRNA

**Figure 37**

and 2 are involved in MIG secretion. In conclusion, SMM treatment may inhibit cytokine production by downregulating vital molecules for TLR signaling.

**cIAPs mediate LPS-induced macrophage maturation**

SMM treatment alone using compound BV6 has been shown to induce maturation of DCs (326). However, the effect of SMM on human macrophages is not known. Since SMM treatment interfered with cytokine production in response to LPS, I determined if SMMs affect LPS-induced maturation of MDMs. As shown in Fig. 38A, both LN and SM significantly inhibited LPS-induced CD80 and CD86 expression on MDMs. Moreover, these compounds also prevented LPS-induced CD86 expression in DCs (Fig. 38B ) suggesting that the inhibitory effect of SMMs on maturation is not limited to one cell type, but has a broader effect on immune cells.

**Signaling pathways regulating CD80 and CD86 expression in macrophages**

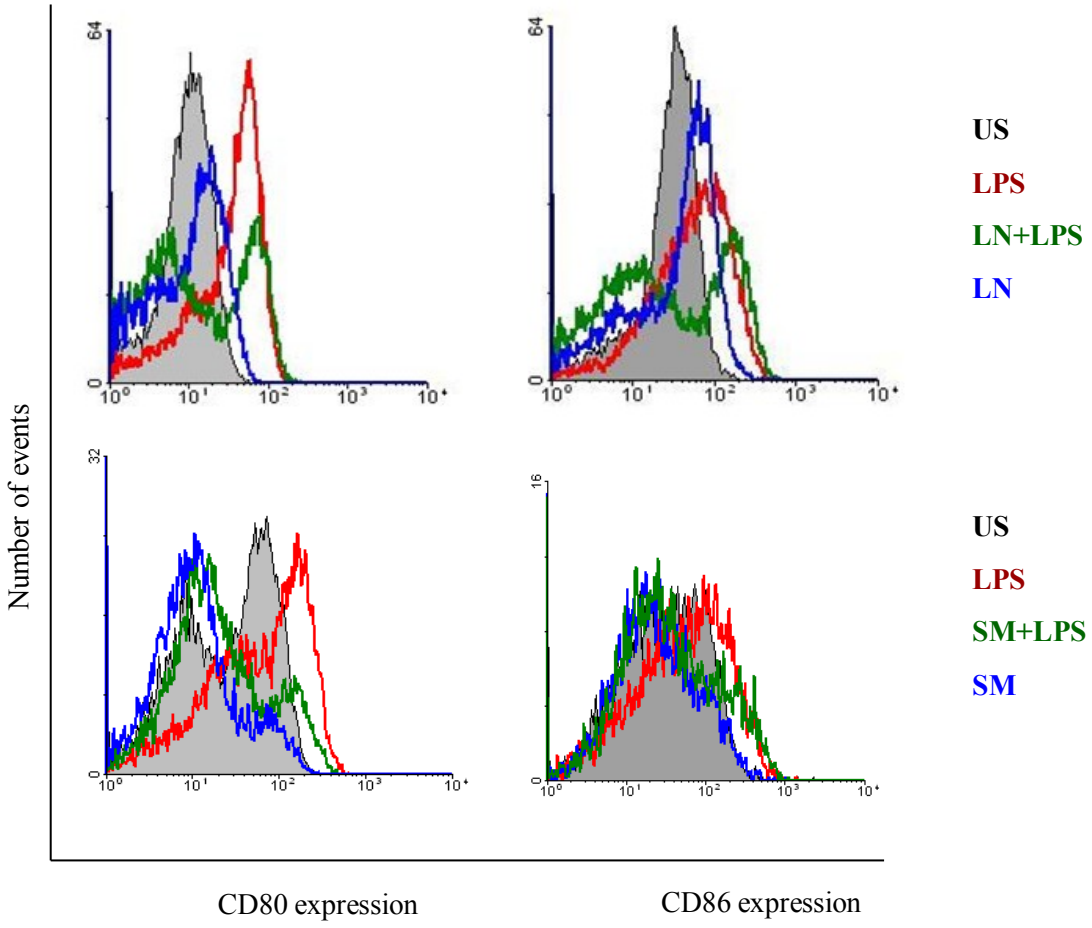
To determine if the effect of SMM treatment on macrophage maturation could also be attributed to inhibition of MAPKs and NFκB, I studied their involvement in LPS-induced expression of costimulatory CD80 and CD86 molecules by using pharmacological inhibitors. The regulation of CD80 and CD86 has been in investigated in primary monocytes (327) and dendritic cells (328), but not in human MDMs. Therefore I first investigated the involvement of MAPKs and NFκB in the regulation of CD80 and CD86 in human MDMs. Inhibition of p38 with SB resulted in a reduced expression of CD80 in response to LPS, whereas inhibition of JNK, ERK or NFκB had no such effect (Fig. 39). In contrast, CD86 expression did not change with any of the inhibitors. This suggests that the SMM inhibition of macrophage maturation could be attributed, at least in part, to impaired p38 signaling. Taken

together, these results indicate that the effect on IL-10, IL-27 and MIG (regulated by JNK, p38 and NFκB) and impaired maturation (regulated in part by p38) seem to constitute separate effects of cIAPs depletion, with p38 acting as a potentially overlapping mechanism.

**Figure 38. Smac mimetics inhibit maturation of macrophages and dendritic cells in response to LPS**

MDMs and dendritic cells were treated as in Fig.4. Following LPS treatment, cells were collected and stained for surface expression of CD80 and/or CD86, as described in Materials and methods. Fluorescence intensities were measured by flow cytometry using a FACSCanto flow cytometer (BD Biosciences) equipped with the FACSDiva software. Histograms shown are representative for 4 donors for MDMs and for 2 donors for dendritic cells.

**A. MDMs**




**B. DCs**

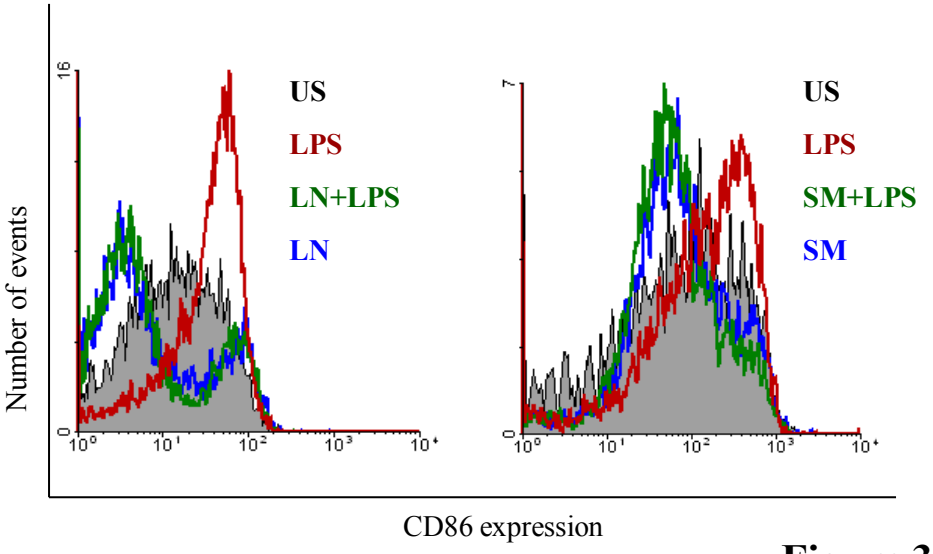



**Figure 38**

**Figure 39. Blocking p38 prevents LPS-induced CD80 expression**

Cells treated as in Fig.10 were collected and stained for surface expression of CD80 and CD86, as described in Materials and methods. Fluorescence intensities were measured by flow cytometry using a FACSCanto flow cytometer (BD Biosciences) equipped with the FACSDiva software. Histograms shown are representative for at least 3 donors for each inhibitor.

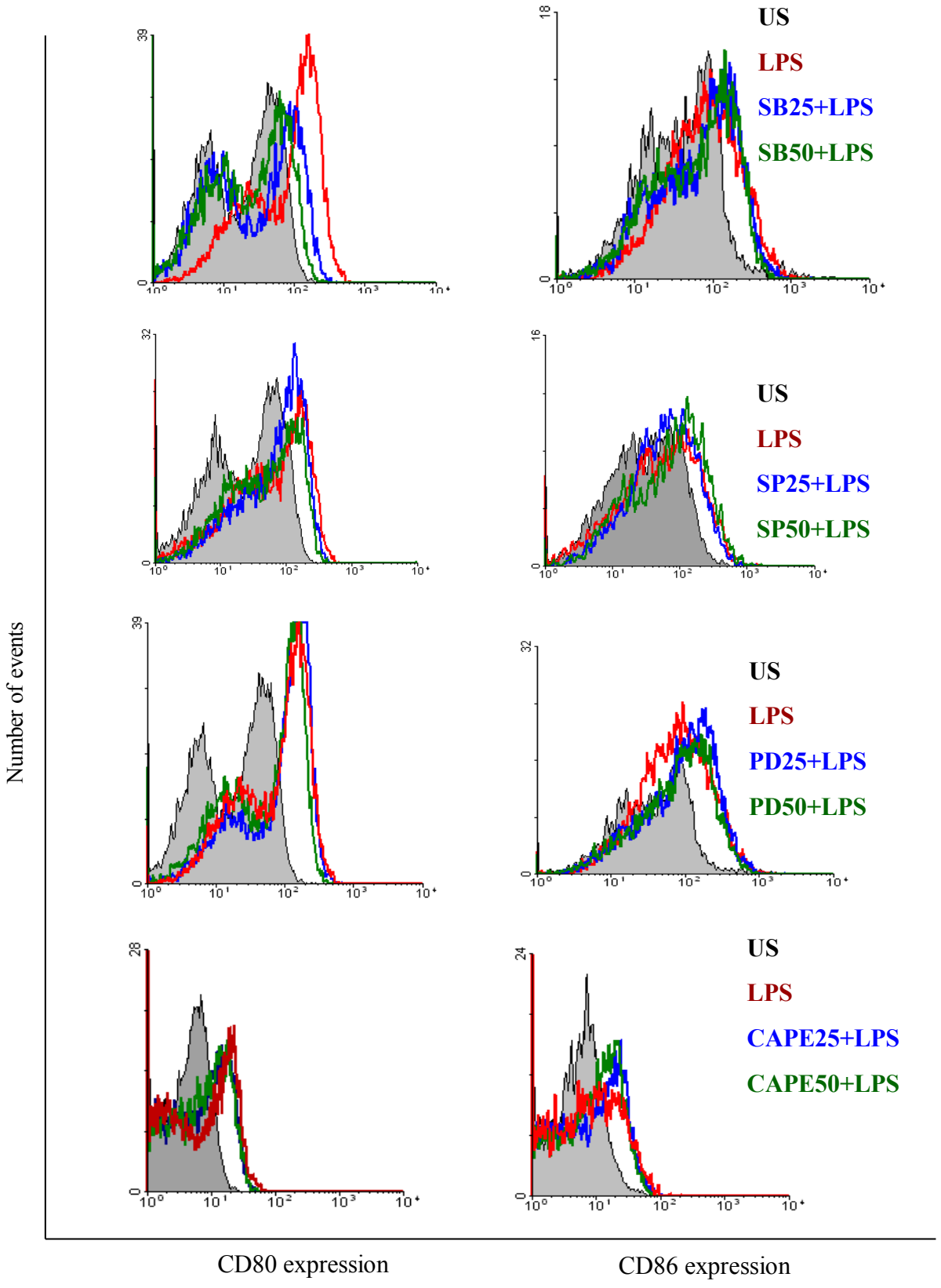

US
LPS
SB25+LPS
SB50+LPS
US
LPS
SP25+LPS
SP50+LPS
US
LPS
PD25+LPS
PD50+LPS
US
LPS
CAPE25+LPS
CAPE50+LPS
Number of events
CD80 expression
CD86 expression

**Figure 39**

## Discussion

This report shows for the first time that cIAPs have a broad effect on innate immunity. Using two different Smac mimetic compounds, LN and SM, my results show that cIAP ablation inhibits IL-10, IL-27 and MIG secretion in human macrophages in response to LPS and CD40L stimulation. Other effects of IAPs ablation include prevention of LPS induced maturation of macrophages and dendritic cells. The inhibitory effect of SMM on cytokine production can be attributed to cIAPs roles in the signaling pathways required for IL-10, IL-27 and MIG induction in response to LPS. In an attempt to investigate the signaling pathways by which cIAPs regulate LPS-induced IL-10, IL-27 and MIG production, my results show that downstream of TLR4 cIAPs positively regulate the expression of MyD88 and TRAF2 and activation of p38, JNK and NFκB, all of which are necessary for LPS-induced production of IL-10, IL-27 and MIG in human macrophages. In addition, cIAPs are also required for TRAF1 induction, which is also necessary for MIG production. The effect on macrophage maturation could be partly attributed to defective p38 signaling, since my results also show that this pathway is responsible for regulating CD80 expression in macrophages. It remains to be determined how cIAPs regulate CD86 expression in this cell type.

While SMM compounds have been actively studied in the literature for the apoptosis inducing abilities in cancer cells, their effects on human primary cells with respect to the development of immune responses remain largely unknown. We (206, 233) and others (326) have shown that cIAP ablation does not influence steady state survival of primary human cells and this result is in agreement with mouse study data that show a similar lack of sensitivity to death inducing activities of SMM (315). One recent report also shows that

cIAP1 and 2 prevent necroptosis of mouse macrophages, since cIAP ablation via SMM treatment resulted in RIP1-dependent necroptotic cell death (329). However, in this study higher doses in the μM range were used. I have shown that nM concentrations of SMM compound are enough to cause IAPs degradation in human macrophages and these concentrations are also being used in other studies (94, 108, 315, 330). These reports may suggest that at higher concentrations, SMM have a toxic effect in primary macrophages and the mode of cell death may be necroptosis.

The role of IAPs in regulating immune responses in primary cells is poorly understood. One report from mouse studies indicates that cIAPs mediate the production of proinflammatory cytokines in response to LPS, without affecting IFN responses. As a result, cIAPs ablation using SMM resulted in decreased IL-6, TNF-α and IL-12 production, with no effect on IL-10 (108). I show that in human macrophages SMM selectively inhibit LPS/CD40L-induced IL-10, IL-27 and MIG production. My results are in contrast to the findings in mouse studies, suggesting that SMM effects may differ across species.

Of all the cytokines and chemokines tested, cIAPs seem to selectively regulate only IL-10, IL-27 and MIG secretion in response to LPS. The exact mechanism of this process requires further study. Remarkably, all three cytokines seem to have common regulatory pathways that involve TRAF2, p38, JNK and NFκB. The other tested cytokines may have redundant signaling pathways that control their production when MAPKs and NFκB signaling are impaired, which may explain why their levels where not changed following cIAPs depletion.

I also show that SMM treatment affects TLR4 signaling by inhibiting TRAF1, TRAF2 and MyD88 expression, which contributes to its effect on cytokine secretion. LPS

binding to TLR4 recruits two adaptor molecules with Toll-interleukin 1 receptor (TIR) domains, myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). MyD88 recruits IRAK1/4, followed by TRAF6, which ultimately mediates IκB kinase (IKK) complex activation and NFκB dependent secretion of inflammatory cytokines (331). The importance of TRAF6 in LPS signaling was demonstrated in macrophages from TRAF6-deficient mice, which displayed decreased TNF and IL-6 production in response to LPS (332).

TRAF1/2 bind cIAP1/2 to form a heterocomplex with important functions in TNF receptor signaling (333), whereas TRAF6 has been shown to contribute to MyD88-dependent TLR4 signaling in mice (332). I show that in human macrophages cIAPs maintain TRAF1/2 and MyD88, rather than TRAF6 expression. Although more studies are needed to dissect the exact associations, my results suggest that in human macrophages cIAP1/2 associate with TRAF1/2 and MyD88, resulting in the formation of a signaling complex that promotes downstream p38 and JNK activation and classical NFκB activation, which culminates with IL-10, IL-27 and MIG production in response to LPS.

I found that in addition to TRAF1 and 2, MyD88 levels were also inhibited in cells treated with SMM. I can speculate that by affecting MyD88, SMM treatment targets the MyD88-dependent NFκB activation. However, SMM impact on TRIF levels and TRIF-dependent signaling remains to be evaluated. Also, siRNA experiments are needed to confirm the roles of cIAP1, cIAP2 and MyD88 in LPS-induced IL-10, IL-27 and MIG production in human MDMs.

Pull down assays of SMM treated cancer cells have shown that SMM binds specifically to cIAP1 and 2 and their interacting proteins, TRAF1 and 2 (93), and causes

specific degradation of cIAPs without affecting TRAF levels (91). Unexpectedly, my results indicate that SMM effects on LPS signaling may involve TRAF1 and 2, molecules classically associated with TNF-receptor signaling, rather than TRAF6. My results show that SMM treatment inhibited LPS-induced TRAF1 and 2 levels, with no effect on TRAF6. The mechanism of TRAF1 and 2 inhibition may be an indirect effect of NFκB. Similarly to cIAP1 and 2, TRAF1 and 2 are also gene targets of NFκB transcriptional activity (334). Therefore, by inhibiting LPS-induced classical NFκB pathway, SMM treatment may indirectly prevent TRAF1 and 2 induction.

SMM induced inhibition of TRAF2 also correlates with SMM activation of noncanonical NFκB signaling. TRAF2 is a dual regulator of NFκB that promotes activation of the classical pathway, but inhibits the noncanonical pathway (335) by inducing degradation of NIK kinase (336). Therefore, I can speculate that SMM induced downregulation of TRAF2 also potentiates its effects on NFκB pathways, promoting alternative NFκB signaling and inhibiting the classical one.

Reports from human cell lines have shown that cIAP ablation activates both classical and alternative NFκB pathways by promoting TNF-α secretion and stabilizing NIK, respectively (92). In contrast, I show that SMM treatment of human macrophages has divergent effects on LPS-induced NFκB signaling, causing inhibition of classical NFκB and activation of alternative NFκB pathway. The different results obtained in cell lines and primary cells on the classical pathway activation may be attributed to different TNF-α secretion patterns in response to SMM. SMM sensitive cell lines secrete TNF-α in response to cIAP ablation and consequently become susceptible to TNF-α-induced apoptosis (92, 93). In contrast, I failed to detect any TNF-α secretion in response to SMM treatment alone (Fig.

31). In addition, SMM treatment had no effect on TNF-α induction in response to LPS. LPS-induced TNF-α levels were associated with decreased TRAF1 and TRAF2 expression, which may indicate defective TNF signaling. These results may explain why SMM treatment inhibited NFκB activation in the presence of TNF-α.

The effect on alternative NFκB pathway can be explained by cIAP ability to bind and degrade NIK, the regulatory kinase of this pathway. NIK degradation requires the formation of a heterocomplex of cIAP1, cIAP2, TRAF2 and TRAF3 that is recruited to NIK (315). Within this complex, cIAP1 and 2 serve as ubiquitin ligases for NIK, whereas TRAF2 mediates cIAPs recruitment to the TRAF3-NIK complex (315, 337). Although the levels of TRAF3 were not evaluated, I have shown that in human macrophages SMM treatment downregulates cIAP1, cIAP2 and TRAF2 levels. I can speculate that in the absence of these proteins, NIK regulatory complex formation is impaired and NIK accumulates, which leads to increased p100 to p52 processing, as observed.

**cIAPs involvement in IL-10 production**

IL-10 is a potent anti-immune and anti-inflammatory cytokine, produced in a wide variety of cell types, including Th2 and regulatory T cells, monocytes and macrophages (338). The effects of IL-10 on macrophages include inhibition of IFN responses (339), inhibition of maturation and reduced cytokine production (338). During LPS stimulation, IL-10 is produced in the second phase of the immune response and serves to limit the inflammatory effects that characterize the initial host response to infection. In doing so, IL-10 prevents immune over-activation that would otherwise kill the host (340).

Regulation of IL-10 production involves distinct signaling pathways in various cell types. Mouse studies have indicated that both MyD88 and TRIF-dependent pathways contribute to IL-10 induction to LPS (341). Pharmacological inhibitors data have also shown a requirement for NFκB, ERK and p38 MAPKs for IL-10 induction in mouse macrophages (342, 343). In human monocytes p38 MAPK (319, 344), ERK MAPK (318) and SP-1 transcription factor (319) were shown to regulate IL-10 production, whereas STAT3 was shown to mediate this effect in human macrophages (345). However, the involvement of NFκB and MAPKs in regulating IL-10 production in human macrophages has not been yet investigated.

My results show that cIAPs ablation via SMM compounds caused a significant inhibition of LPS-induced IL-10 production in both macrophages and monocytes, suggesting that this effect is not limited to one cell type. It is yet to be determined if cIAPs regulate IL-10 production in dendritic cells, another major source of IL-10 (341). In addition, my results show for the first time that LPS-induced IL-10 production in human macrophages is regulated by a novel signaling pathway comprising of a signaling complex that includes cIAP1/2 and TRAF2, activation of p38 and JNK MAPKs and NFκB-dependent IL-10 secretion. Of all three MAPKs, p38 and JNK regulate IL-10 production in response to LPS in macrophages, this being the first report on JNK involvement in this process. However, the involvement of JNK and p38 in the regulation of IL-10 production in human macrophages needs to be confirmed by employing siRNAs specific for p38 and JNK MAPKs.

Although the role of MyD88 in IL-10 signaling needs to be confirmed in future experiments, I can speculate that SMM induced downregulation of MyD88 may also contribute to its effect on IL-10 production. It remains to be investigated whether TRIF

dependent pathway is involved in IL-10 production in human macrophages and whether cIAPs are also involved in this pathway.

In summary, this is the first report that shows an involvement of cIAPs, NFκB, p38 and JNK in LPS-induced IL-10 production. In addition, siRNA studies indicate for the first time a requirement for TRAF2 in IL-10 responses via TLR-4 signaling. It was previously shown that TRAF2 is required for JNK activation and protection against TNF-induced apoptosis (346). TRAF2 ablation can also potentiate TNF-induced apoptosis because as part of the TNF-R1 signaling complex, TRAF2 is a target of cIAP1 induced ubiquitination (347, 348). Whether TRAF2-mediated JNK activation results in LPS-induced IL-10 production and by extension IL-27 production in human macrophages needs to be confirmed.

**cIAPs involvement in IL-27 production**

IL-27 is a heterodimeric cytokine of the IL-12 family, composed of Epstein-Barr virus–induced gene 3 (EBI3) and p28, similar to p40 and p35 subunits of IL-12. It is secreted mainly by antigen-presenting cells in the early stages of bacterial infections (349). Multiple functions have been described for IL-27, both on adaptive and innate immunity. Initially reported as promoting Th1 differentiation (350), immunoregulatory functions of IL-27 include inhibition of Th1, Th2 and Th17 cells (349). Both pro- and anti-inflammatory effects have been described for IL-27 in innate immunity. Thus, IL-27 augmented inflammatory cytokine production in response to TLRs and attenuated IL-10 secretion in human monocytes (351), but it displayed anti-inflammatory properties in human macrophages, where it suppressed TNF-α and IL-1β responses (352).

Regulation of IL-27 production in response to TLR signaling is dependent on type I IFN responses in both mouse dendritic cells (353, 354) and human macrophages (355).

Transcription factors involved in IL-27 expression include IRF-1 (353, 355, 356) and IRF-3 (353, 354). NFκB is also required for LPS-induced IL-27 p28 gene induction in mouse macrophages (356), yet the involvement of NFκB in IL-27 production in human macrophages is not determined.

I show for the first time that cIAPs positively regulate IL-27 production in response to LPS in MDMs. Treatment of primary monocytes with SM resulted in inhibition of LPS-induced IL-27 production although this inhibition was not significant. Further studies are needed to confirm these results in primary monocytes. Whether IAPs regulate LPS-induced IL-27 production in dendritic cells also remains to be investigated. To elucidate the mechanism of cIAPs involvement in IL-27 production, I have studied the signaling pathways required for IL-27 induction in MDMs. My results show for the first time that LPS-induced IL-27 is regulated by a novel signaling complex, comprising cIAPs and TRAF2, followed by p38 and JNK activation and NFκB signaling. However, the involvement of JNK and p38 in the regulation of IL-27 production in human macrophages needs to be confirmed by employing siRNAs specific for p38 and JNK MAPKs. Given the requirement for MyD88 dependent signaling for IL-27 production (356) and SMM-mediated downregulation of MyD88 expression in our model, this effect may also contribute to IL-27 inhibition. Taken together, these pathways may constitute a novel mechanism by which SMM treatment inhibits IL-27 production.

Since type I IFN responses regulate IL-27 production in other cell types, including human macrophages (355), it remains to be determined if cIAPs have any impact on IFN-α and IFN-β produced following TLR4 activation and if this effect may contribute to decreased IL-27 production. It would also be of interest to determine if cIAPs impact on IL-

27 production and by extension IL-10 production in response to other TLRs activators, such as TLR3, TLR7 and TLR8 that have been shown to induce IL-27 in human macrophages (355).

**cIAPs involvement in MIG production**

MIG is a chemokine of the CXC family that specifically acts on activated T cells and uses CXCR3 as a receptor. Apart from T cell chemotaxis and activation, MIG functions also include inhibition of angiogenesis, tumor growth in mouse models and protection during parasitic infections (357). MIG was initially identified in the monocytic U937 cell line treated with IFN-γ (358) and IFN-γ alone is a potent inducer of MIG. Mouse studies have shown that IFN-γ can synergize with TLR ligands such as LPS (TLR4) and CpG (TLR9) to promote maximal MIG secretion (359, 360). However, in these studies TLR ligation alone was ineffective in inducing MIG in the absence of IFN-γ. Similarly, LPS alone does not induce MIG secretion of human neutrophils, but potentiates IFN-γ responses (361).

The mechanism of MIG induction in response to IFN-γ involves STAT1 activation (359). However, the synergistic effect of LPS and IFN-γ on MIG induction is dependent on NFκB activation in mouse cells (359). Moreover, NFκB is required for full MIG induction and STAT-1 activation in response to IFN-γ alone (362).

I show here that in primary human macrophages both LPS and CD40L stimulate robust MIG secretion in the absence of IFN-γ stimulation, albeit LPS was a more potent inducer, with a mean response of about 10 ng/ml. This would indicate that during infections with Gram negative bacteria, LPS stimulation initially induces MIG secretion in macrophages, with the role of attracting activated T cells. MIG induction is further increased once IFN-γ is produced by NK cells or T cells, thus creating a positive feedback loop.

Further studies are necessary to investigate the effect of IFN-γ alone or in combination with LPS and other TLR ligands to produce MIG in macrophages, primary monocytes and DCs and the molecular mechanism governing its regulation in these cells.

Of the chemokines tested, only MIG induction was prevented by SMM treatment and no effect was observed on MIP-1α, MIP-1β and IL-8. Although the exact molecular mechanism of this selective effect is yet to be determined, I have investigated the signaling pathways involved in MIG signaling that may explain SMM effect. I show for the first time that cIAPs positively regulate MIG production in response to LPS in MDMs. I have also demonstrated that NFκB (Fig. 11E), p38 and JNK mediate LPS-induced MIG secretion (Fig. 10C) and that both TRAF1 and 2 are required for this effect (Fig. 15D). Although my results clearly show the involvement of p38 and JNK in MIG regulation by using pharmacological inhibitors, the involvement of JNK and p38 in the regulation of MIG production in human macrophages needs to be confirmed by employing specific siRNAs.

In summary, my results show for the first time that LPS alone can induce MIG in the absence of IFN-γ in human macrophages and that this effect is mediated by cIAPs, TRAF1/2, NFκB, p38 and JNK activation. This is also the first report on TRAF1 and 2 requirements for MIG induction. My findings correlate with a previous report on the role of TRAF1 in promoting inflammatory responses, which showed a requirement of TRAF1 for T cell recruitment and expression of proinflammatory cytokines in the lungs following LPS inhalation (363). Given its specific effect on MIG, SMM inhibition could be a possible therapeutic target in diseases where MIG is a factor of pathogenesis, such as lupus (364) and multiple sclerosis (365).

**cIAPs involvement in the regulation of CD80/CD86**

CD80 (B7.1) and CD86 (B7.2) are part of the B7 family of costimulatory molecules expressed on antigen presenting cells that provide the secondary signal required for an immune response, independently of the specific antigen. The receptors for B7 molecules are CD28 and CTLA-4, which provide a stimulatory and an inhibitory signal, respectively, for T cell activation and proliferation (366).

In human monocytes both JNK and p38 regulate CD86 expression in response to LPS (367), whereas JNK but not p38 is involved in CD80 upregulation (327). Similar results were obtained in dendritic cells, where JNK (368) and p38 (369) regulate both CD80 and CD86 expression induced by LPS. Transcription factors involved in gene expression of CD80 and CD86 include IRF-7 (327) in monocytes and PU.1 (328) in dendritic cells. NFκB is involved in CD86 regulation in B cells (370) and dendritic cells (371). However, the signaling pathways and transcription factors that regulate CD80 and CD86 expression in human macrophages are yet unknown.

My results indicate for the first time that cIAPs are involved in upregulation of CD80 and CD86 in macrophages in response to LPS and in CD86 expression in dendritic cells. It is yet to be determined if SMM treatment affects CD80 expression in dendritic cells. Investigating the mechanism of this effect, I also show that p38 regulates CD80 expression in macrophages. Since SMM also inhibited LPS-induced p38 activation, this may constitute one mechanism that could explain cIAPs involvement in CD80 regulation. In contrast, none of the tested signaling pathways (MAPKs or NFκB) was involved in regulating CD86 expression, therefore it remains to be determined how cIAPs are involved in this process.

Moreover, the transcription factors that control CD80 and CD86 gene expression in macrophages are yet to be determined.

In summary, since costimulatory molecules are critical for optimal T cell activation and proliferation and cIAP ablation downregulated their expression in macrophages, cIAPs may also impact T cell responses, with broader effects on adaptive immunity. Given the constant interest for administration of Smac mimetics as cancer treatment, it is important to study their effects on primary human macrophage function as a way to evaluate their impact on innate immunity. My results indicate that IAP ablation via SMM treatment may promote a pro-inflammatory response in human macrophages, in contrast to their effects in mouse models (372).

## Concluding remarks and future directions

Resistance to apoptosis is a defining characteristic of macrophages that develops as part of the differentiation process from monocyte precursors. My research aimed to understand the molecular mechanisms and signaling pathways that mediate this resistance, with an emphasis on the main families of antiapoptotic proteins, IAPs and Bcl2, which may mediate this process. My research consists of three objectives, the main findings of which are summarized below and depicted in figures 40 and 41.

1. The first objective was to investigate the signaling pathways that mediate the development of resistance to apoptosis during the differentiation process by utilizing human MDMs and THP1-MACs as models. I have shown that regulation of antiapoptotic Bcl2 proteins is one of the main mechanisms by which PI3K/Akt pathway contributes to survival of human macrophages. However, the molecular mechanisms of PI3K/Akt-induced survival are different during and after macrophage differentiation, with Bcl-xL expression being dependent on Akt activity during, but not after differentiation. In mature macrophages Akt regulates cell survival by maintaining Mcl-1 expression, suggesting that Akt explores different survival pathways according to the developmental stage of the cell. Moreover, inhibition of PI3K/Akt during differentiation leads to cell death via both caspase-dependent and –independent pathways. The involvement of other proapoptotic molecules such as apoptosis inducing factor (AIF) or Smac in inducing caspase-independent cellular demise following PI3K/Akt blockage remains to be addressed in future studies.

The exact molecular mechanisms by which antiapoptotic Bcl-xL and Mcl-1 maintain cellular viability needs to be addressed. Taking into consideration that the proposed mode of action for both Bcl-xL and Mcl-1 include formation of heterodimers with proapoptotic Bcl2

**Figure 40. Summary of findings on the role of Bcl2 proteins and IAPs in the survival and function of human macrophages**

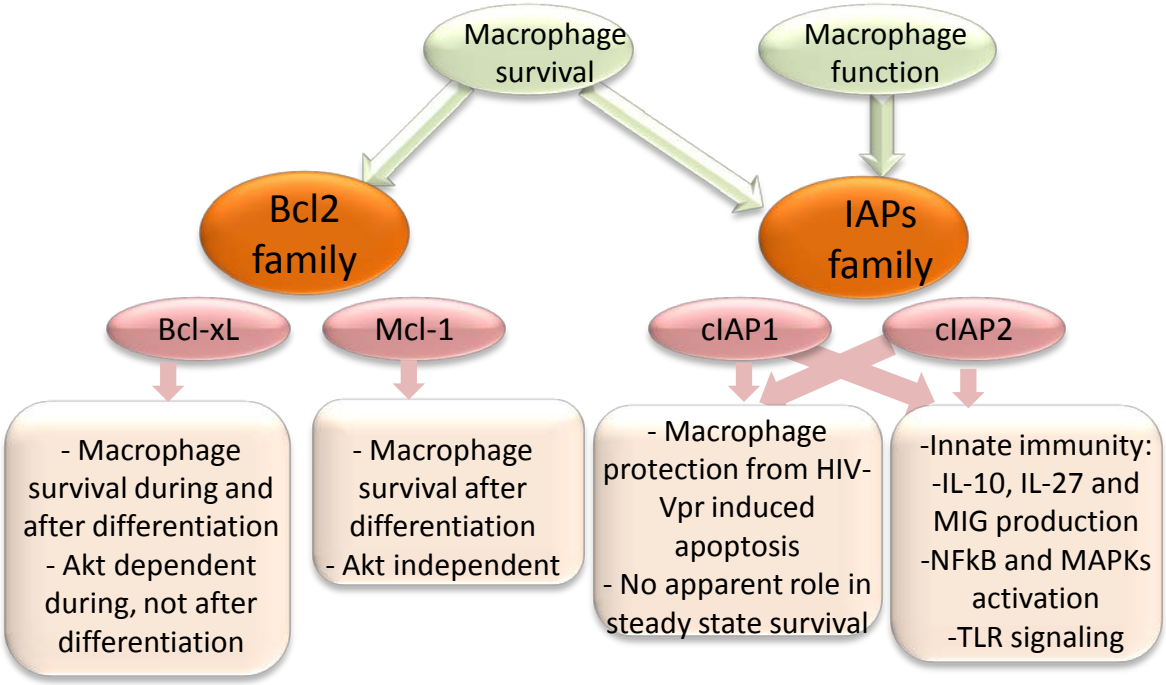

Macrophage survival
Macrophage function
Bcl2 family
IAPs family
Bcl-xL
Mcl-1
cIAP1
cIAP2
- Macrophage survival during and after differentiation
- Akt dependent during, not after differentiation
- Macrophage survival after differentiation
- Akt independent
- Macrophage protection from HIV-Vpr induced apoptosis
- No apparent role in steady state survival
-Innate immunity:
-IL-10, IL-27 and MIG production
-NFkB and MAPKs activation
-TLR signaling

**Figure 40**

members such as Bax and Bak, it would be of interest to determine whether PI3K/Akt inhibition also affects the expression levels of these molecules. Moreover, Akt has been shown to phosphorylate and thus inactivate proapoptotic Bad (258), a "BH3-only" member of the Bcl2 family with regulatory function that can initiate activation of proapoptotic proteins (32). The involvement of Bad in inducing cell death following Akt inhibition of differentiating macrophages is yet to be determined.

The results also indicate that PI3K/Akt inhibition could be a valuable therapeutic target, since it seems to be involved in survival of macrophages both during and after differentiation. PI3K inhibitors, currently evaluated for cancer therapy (373), could have applications in treatment of chronic inflammatory conditions such as arthritis and Crohn's disease, where ablation of both mature and newly differentiated macrophages would be desirable (374). The newly developed Bcl-xL (13) and Mcl-1 inhibitors (312) would allow a more targeted approach to eliminate newly differentiating cells or mature macrophages respectively. Also, PI3K/Akt activation would be desirable in diseases with accumulation of monocytic precursors, such as monocytic leukemia, which would promote the differentiation of immature progenitors.

2. Since resistance to apoptosis is a characteristic that enables macrophages to serve as viral reservoirs during HIV infection, in my second objective I have studied how macrophage differentiation influences survival in an HIV environment by using HIV accessory protein Vpr as a model. Despite its apoptotic effects in other cell types, including monocytes, my results show that human macrophages are resistant to Vpr-induced cell death. Using siRNA to knockdown antiapoptotic proteins, I have shown that Bcl2 proteins are involved in steady state survival, but not in macrophage protection in the presence of

Vpr. In contrast, downregulating cIAPs via siRNA or by using a Smac mimetic compound that targets cIAPs for degradation abolished macrophage protection and sensitized them to Vpr-induced apoptosis.

The challenge in eradicating HIV reservoirs is to eliminate infected macrophages with minimal toxicity for uninfected cells. Therefore, it would be interesting to determine if this could be accomplished by targeting antiapoptotic genes. I have evaluated the ability of Smac mimetics, which target IAPs for degradation, to eliminate HIV infected cells. However, as single agents, Smac mimetics did not cause apoptosis in either uninfected (Fig. 24B) or infected macrophages (data not shown). It would seem that downregulation of IAPs alone is not sufficient to cause cell death and that additional apoptotic stimuli, such as Vpr, are needed for this effect. The challenge at this point is to try and find apoptotic stimuli that Smac mimetics may sensitize infected macrophages to. Potential candidates are members of the TNF family, such as TNF-α and TRAIL, which have been shown to be secreted during HIV infection in macrophages (208). TNF-α has the ability to cause cell death through the extrinsic pathway, an action that IAPs counteract. It is reasonable to hypothesize that in the presence of Smac mimetics that degrade IAPs, increased TNF-α produced during HIV infection may selectively sensitize infected cells to cell death.

Keeping in view the results of objective 1, showing the importance of PI3K/Akt pathway in regulating Bcl2 proteins to promote survival of macrophages, it would be of interest to determine how HIV infection impacts this pathway or the expression of antiapoptotic proteins. PI3K inhibitors, either alone or in the presence of Smac mimetics, could also be evaluated as death inducing agents in HIV infected macrophages.

3. Given the involvement of IAPs in NFκB and TLR signaling in mouse models and cell lines and the resistance of macrophages to IAPs ablation, I hypothesized that downregulating IAPs using Smac mimetics treatment may impact macrophage function. The results from my third objective have shown that IAPs contribute to a number of macrophage functions that are important in innate immunity, such as cytokine secretion and expression of co-stimulatory CD80/CD86 molecules in human macrophages. Downregulation of IAPs selectively decreased LPS induction of IL-10, IL-27 and MIG in MDMs and primary monocytes. In trying to elucidate how cIAPs regulate LPS-induction of these cytokines in human macrophages, I investigated the role in NFκB, MAPKs and TLR4 pathways and the specific contribution of these pathways to IL-10, IL-27 and MIG secretion. The results indicate a signaling pathway consisting of TRAF1/2 recruitment of cIAPs, p38 and JNK activation and NFκB-dependent production of IL-10, IL-27 and MIG in response to LPS in human macrophages. A summary of cIAPs involvement in TLR4 signaling is illustrated in fig 41.

Another interesting possibility that needs to be addressed in future studies is that SMM effect on LPS signaling is indirect and dependent on TNF-α that is being secreted in response to LPS stimulation. My results show that SMM treatment did not affect LPS-induced TNF-α secretion in MDMs, in contrast to effects observed in cell lines, where TNF-α induction following cIAP ablation led to NFκB activation and apoptosis through the extrinsic pathway (92). However, TNF-α signaling may be blocked because of SMM induced degradation of cIAP1/2 and TRAF1/2. This mechanism would be similar to SMM-induced cell death on cancer cells, which is dependent on autocrine TNF-α signaling (93).

**Figure 41. Proposed model for the role of IAPs in TLR4 signaling in human macrophages**

Following LPS binding to TLR4 two parallel signaling cascades are initiated, depending on the adaptor protein recruited to the receptor. MyD88 signaling culminates with inflammatory cytokine secretion, while TRIF is involved in type I interferon responses. TRAF6 recruits TAK1 and TAB2/3 to promote IKK complex activation and IκBα phosphorylation, with release of NFκB and transcription of NFκB dependent genes, IL-10, IL-27 and MIG. IAPs are part of the MyD88-dependent pathway, where they maintain TRAF2 and MyD88 expression, with no effect on TRAF6. In addition, IAPs positively regulate MAPKs activation, which also impacts on cytokine secretion. Red arrows indicate activatory actions, question marks represent effects that need further investigation.

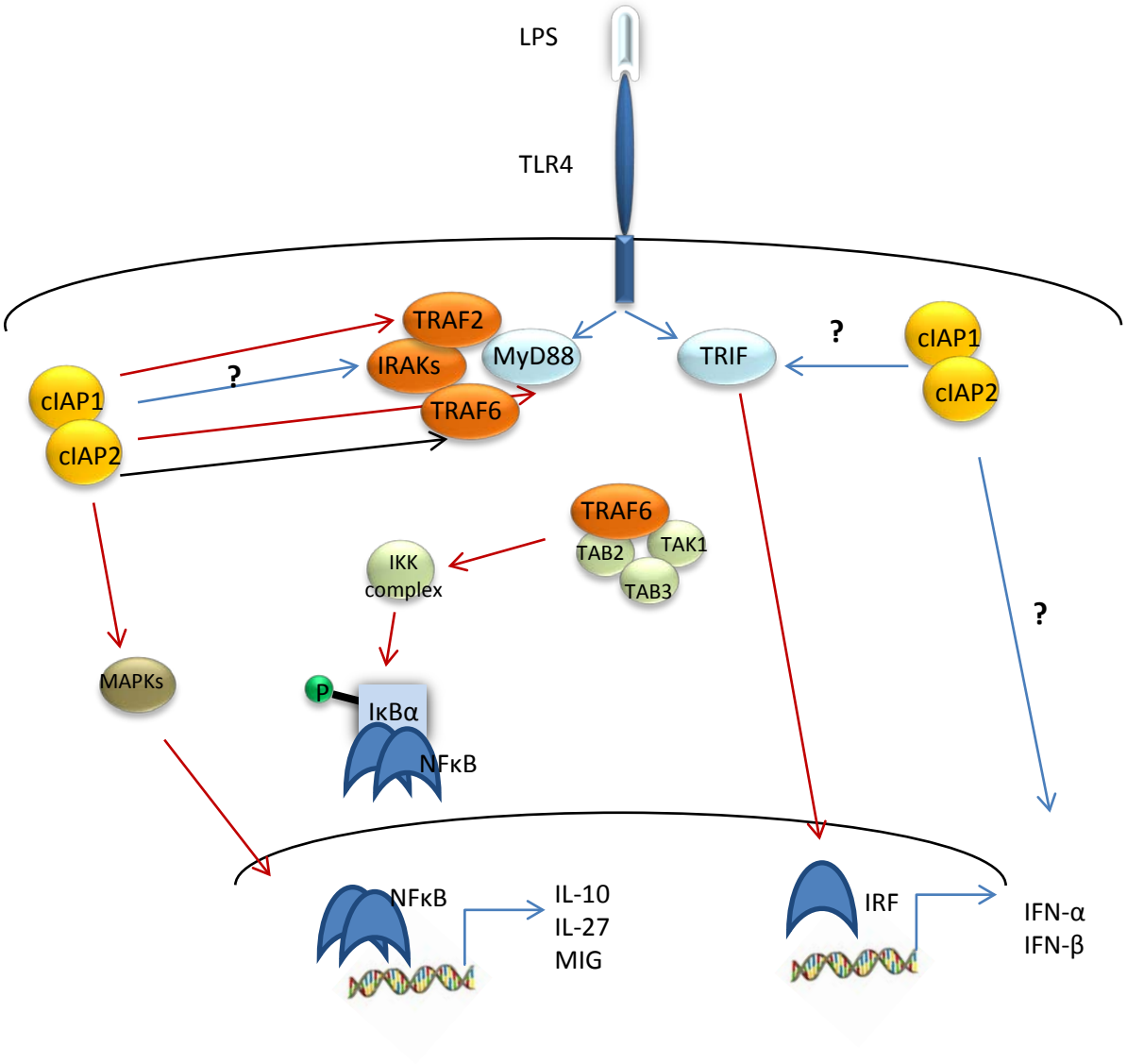

LPS
TLR4
TRAF2
IRAKs
MyD88
TRIF
?
cIAP1
cIAP2
?
TRAF6
cIAP1
cIAP2
TRAF6
TAB2
TAK1
TAB3
IKK
complex
?
MAPKs
P
IκBα
NFκB
NFκB
IL-10
IL-27
MIG
IRF
IFN-α
IFN-β

**Figure 41**

During my investigations, I have also evaluated the impact of SMM treatment on RIP1, an adaptor protein with kinase activity that has been implicated in inflammatory responses, cell survival and apoptosis (320). RIP1 is the adaptor protein that mediates TRIF-dependent TLR3 and TLR4 NFκB activation (322, 375) by binding and activating downstream adaptor TRIF (322).

In cancer cells RIP1 is constitutively ubiquitinated by cIAPs, a process that is blocked following SMM treatment (94). As a result, RIP1 binds and activates caspase-8, thereby sensitizing cells to autocrine TNF-α signaling (376). I have observed that in contrast to cancer cells, where SMM treatment does not affect the expression level of RIP1, but only prevents its ubiquitination (94), RIP1 induction was prevented in human macrophages, in a similar manner as cIAP2 expression. However, siRNA experiments indicated that RIP1 does not contribute to SMM effect on IL-10, IL-27 or MIG. Given the role of RIP1 in TRIF-dependent signaling, it would be of interest to determine if SMM treatment also impacts TRIF dependent TLR activation by inhibiting RIP1.

TRIF is required for all signaling by TLR3 and shares TLR4 signaling with MyD88. TRIF signaling can activate NFκB (MyD88 independent pathway) to promote cytokine synthesis and IRF-3, resulting in type I IFN responses (323). Therefore, TRIF represents the divergent point for TLR signaling, by inducing both NFκB and IRF-3 activation (377). Although RIP1 is only involved in TRIF-dependent NFκB responses, with no effect on IFN secretion (322), the involvement of cIAPs in IRF-3 activation and IFN responses cannot be excluded and needs further investigation.

TRIF recruitment of the adaptor proteins TRAF3 is essential for the induction of both type I IFN and IL-10, as shown by defective IL-10 production and overproduction of pro-

inflammatory cytokines in TRAF3-deficient macrophages (378). It remains to be investigated whether MyD88 or TRIF dependent pathway is involved in IL-10 production in human macrophages and the requirement for TRAF3 in these pathways. Other reports on regulation of IL-10 production in mouse models have shown that type I IFN secretion is necessary for successful IL-10 induction (379, 380). Since TRIF is the adaptor protein that mediates TLR-induced interferon responses, future studies could investigate TRIF contribution to IL-10 induction via IFN production (323). This reiterates the need for further study of the effect of cIAP ablation on TRIF signaling.

Another possibility to be investigated is that cIAPs effect on IL-10 is an indirect result of IL-27 suppression. It has been shown recently that IL-27 signaling is necessary for IL-10 induction by LPS in mouse macrophages, where IL-10 is the result of a two-step induction process that includes LPS-induced IFN type I and IFN-induced IL-27 (355, 379). However, it is yet to be determined whether IL-27 can induce IL-10 secretion in human macrophages. Interestingly, in human monocytes the reverse has been reported, with IL-27 suppressing TLR-induced IL-10 production (351).

Studying the involvement of IAPs in regulation of innate immune responses in cIAP1/2 knockout mice (105, 107) constitutes another interesting possibility to be addressed in future experiments. The results of objective 3 show that cIAPs regulate LPS-induced IL-10 production in human MDMs and primary monocytes. This observation could potentially be expanded to other cell types that produce IL-10, such as dendritic cells. cIAP1/2 knockout mice may also provide a valuable study model for the role of IAPs in IL-10 regulation, although the lack of a double knockout may constitute an obstacle to replicating the results obtained with SMM treatment.

cIAPs ablation via SMM treatment also inhibited LPS-induced expression of co-stimulatory CD80/CD86 molecules. However, the mechanism of this effect and the signaling pathways that regulate CD80/CD86 expression in human MDMs require further study.

IAP inhibition via SMM treatment is currently studied as a therapeutic option for cancer treatment (381). Due to IAPs effects in modulating NFκB signaling, SMM treatment has also been proposed as a potential anti-inflammatory therapy (382). However, my preliminary results show an overall pro-inflammatory effect of SMM in human primary cells and indicate potential immunological hazards of IAP ablation, due to inhibition of IL-10. Since IL-10 is required to limit chronic inflammation, to induce peripheral tolerance and to protect against autoimmunity (340), SMM treatment may have unwanted adverse effects that may limit its use. Although further studies are needed to fully understand the role of IAPs in immune activation and their contribution to innate immunity, my results suggest that their role extends well beyond cell survival and may contribute to vital biological processes, such as cytokine production in human macrophages.

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## Contributions of collaborators

Experiments depicted in Fig. 14, upper right panel (monocytes results), Fig. 16A, upper panel (monocytes) and Fig. 17 were performed by Mansi Saxena, author of the paper

*Critical role for antiapoptotic Bcl-xL and Mcl-1 in human macrophage survival and cellular IAP1/2 (cIAP1/2) in resistance to HIV-Vpr-induced apoptosis*, Journal of Biological Chemistry. 2012 Apr 27;287(18), Busca A, Saxena M, Kumar A

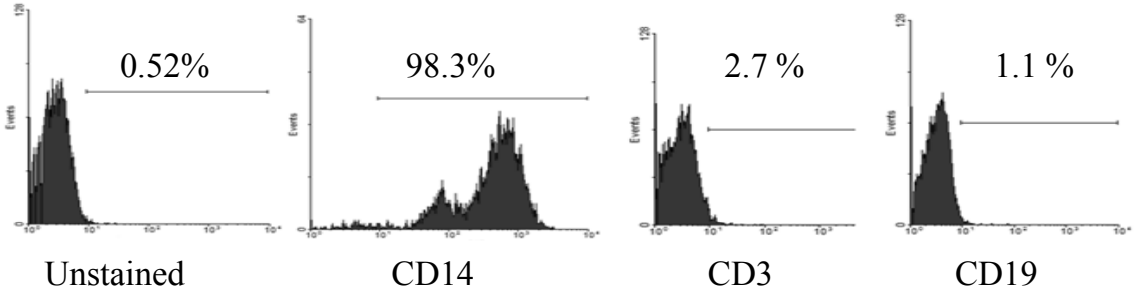
# Appendices

## Supplementary figure

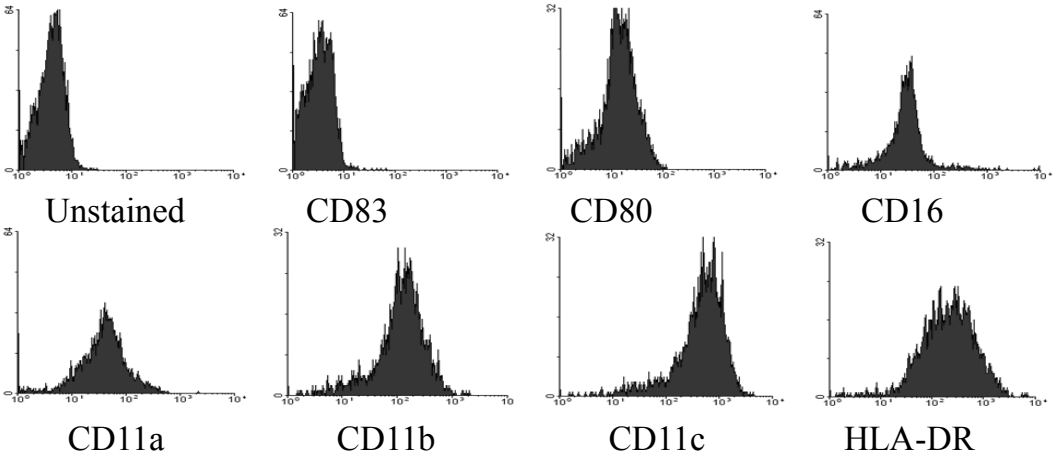
**A** and **B.** Following differentiation with M-CSF, MDMs were stained with antibodies against the indicated surface molecules and evaluated by flow cytometry. Histograms in A show MDMs purity on day 6 following differentiation, using CD14 as a macrophage marker, CD3 as a T cell marker and CD19 as B cell marker. Histograms in B show the MDMs phenotype on day 6 following M-CSF differentiation.

**C.** THP1 cells were differentiated with PMA for 2 days, then evaluated for surface markers by flow cytometry. Histograms in C show the THP1-MACs phenotype on day 2 following PMA differentiation.

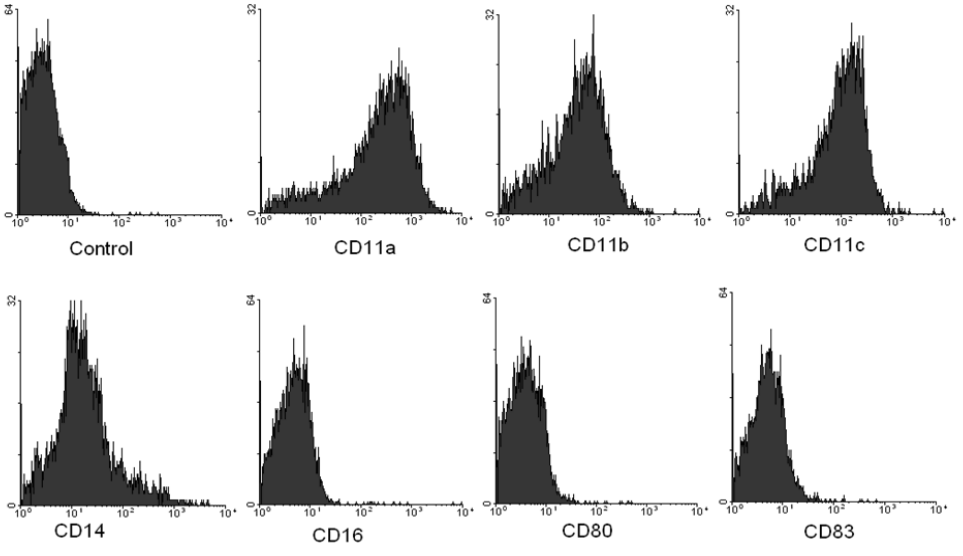
**A. MDMs**




**B. MDMs**




**C. THP1-MACs**




**Supplementary figure**

## Authors permissions

**Figure 2. Bcl2 family of proteins**

**Richard Youle, PhD**

Subject:Re: request for permission
From: Youle, Richard (NIH/NINDS) [E]
Date: Friday, August 10, 2012 5:41 PM

Aurelia, Yes, you have my permission to use that Figure in your thesis. best wishes, richard

On Aug 10, 2012, at 4:55 PM, aurelia busca wrote:

Dear Dr Youle,

I am writing to request your permission to use one of your figures in my PhD thesis. It's figure 1 on Bcl2 proteins structures from your 2008 review "The BCL-2 protein family: opposing activities that mediate cell death".
I am currently writing my PhD thesis on the role of Bcl2 and IAP proteins in survival and immune function of human macrophages. While writing my introduction, I enjoyed reading your review and was hoping I could include your figure.

Thank you.
With consideration,
Aurelia

Aurelia Busca, MD

**Figure 3. IAP family of proteins**

**Colin Duckett, PhD**

Subject:Re: request for permission
From: Duckett, Colin
Date: Friday, August 10, 2012 4:30 PM

Of course! Flattered!

Best

Colin Duckett.

Sent from my iPhone; my apologies for errors or brevity.

On Aug 10, 2012, at 4:25 PM, "aurelia busca" wrote:

Dear Dr Duckett,

I am writing to request your permission to use one of your figures in my PhD thesis. It's figure 1 on IAPs from your 2002 review "Apoptosis: IAP proteins: blocking the road to death's door".
I am currently writing my PhD thesis on the role of IAPs in survival and immune function of human macrophages. While writing my introduction, I enjoyed reading your review and was hoping I could include your figure on IAPs.

Thank you.
With consideration,
Aurelia

Aurelia Busca, MD