

Selective Induction of Cell Death by Smac Mimetics in Primary Human Proinflammatory and Anti-inflammatory Macrophage Subsets

**By
Hamza Ali**

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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa



uOttawa

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ABSTRACT

The inflammatory and anti-inflammatory macrophages have been implicated in many diseases including rheumatoid arthritis, inflammatory bowel disease and chronic rhinosinusitis. Recent studies suggest targeting macrophage function and activation may represent a potential target to treat these diseases. Herein, I investigated the effect of second mitochondria-derived activator of caspases (SMAC) mimetics (SMs), the inhibitors of apoptosis (IAPs) proteins, on the killing of normal human pro- and anti-inflammatory macrophage subsets. It has been shown that human monocytes are highly susceptible to the cytotoxic effects of SMs, however, differentiated macrophages (M0) develop resistance to the cytotoxic abilities of SMs. Whether human macrophage subsets are also resistant to the cytotoxic effects of SM remains unknown. My results show that differentiation of M0 macrophages towards M1 state rendered them highly susceptible to SM-induced cell death, whereas M2a, M2b and M2c differentiated subsets were resistant, with M2c being the most resistant. SM-induced cell death in M1 macrophages was mediated by apoptosis as well as necroptosis and activated both extrinsic and intrinsic pathways of apoptosis. The susceptibility of M1 macrophages to SM-induced cell death was attributed to the IFN- γ -mediated polarization as JAK inhibitor reversed their susceptibility. In contrast, M2c and M0 macrophages experienced cell death through necroptosis pathway following simultaneous blockage of the IAPs pathways by SM-LCL161 and the caspase pathways by the pan-caspase inhibitors (zVAD.fmk).

I investigated the molecular mechanism governing SM-induced cell death in M1 macrophages. My results show that in contrast to the cancer cell lines, SM-induced cell death in M1 macrophages is independent of endogenously produced TNF- α , the canonical and non-canonical NF- κ B pathways. The susceptibility of M1 macrophages to SM-induced cell death was

found to be dependent on IFN- γ -mediated differentiation through the JAK-STAT pathway and subsequent activation of IRF-1. In addition, the selective cell death in SM-treated M1 macrophages is mediated by simultaneous degradation of cellular IAP-2 (cIAP-2) and RIPK-1/3 through the activation of mTORC signaling pathway. Overall, the results suggest that survival of human macrophages is critically linked to the activation of the IAPs pathways. Moreover, agents blocking cIAP-1/2, mTORC and IRF-1 can be exploited therapeutically to address inflammation-related diseases. These observations hold a promising therapeutic strategy to limit the activation of proinflammatory M1 macrophages and eventually controlling the M1-associated diseases.

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TABLE OF CONTENTS

ABSTRACT	II
ACKNOWLEDGEMENTS.....	IV
TABLE OF CONTENTS.....	V
LIST OF FIGURES	VIII
LIST OF TABLES	X
LIST OF ABBREVIATIONS	XI
CHAPTER 1: General Introduction	1
1.1 Monocyte/Macrophage origins and functions.....	1
1.1.1 Monocyte-derived macrophages	1
1.1.2 Yolk sac and fetal liver-derived macrophages	2
1.2 Organ/Tissue distribution of macrophages	3
1.3 Macrophage polarization.....	4
1.3.1 Classically activated macrophages (M1) and their role in disease	5
1.3.2 Alternatively activated macrophages (M2).....	9
1.4 Molecular determinants of M1/M2 macrophage polarization	10
1.5 M2 macrophages subsets and their role in disease.....	11
1.5.1 M2a macrophages subset	11
1.5.2 M2b macrophages subset.....	13
1.5.3 M2c macrophages subset	14
1.5.4 Tissue-associated macrophages (M2d macrophages subset).....	15
1.6 Macrophage polarization manipulation as a therapeutic strategy	16
1.7 Overview of apoptosis.....	16
1.7.1 Caspases	17
1.7.2 Bcl-2 proteins	18
1.7.3 The mitochondrial intrinsic pathway	19
1.7.4 Death receptor-the extrinsic pathway of apoptosis	20
1.8 Inhibitor of apoptosis proteins (IAPs).....	24
1.8.1 Structure and function of IAP proteins	24
1.8.2 Regulation of Apoptosis by IAPs.....	28
1.8.3 Role of IAPs in NF- κ B signalling pathway	32
1.8.4 Endogenous IAP Antagonists	36
1.8.5 Smac mimetics:	36
1.9 Necroptosis- an alternative form of cell death	37
1.9.1 Necroptosis effector proteins and their structures.....	37
1.9.2 Necroptosis initiation and necrosome assembly	38
1.9.3 Necroptosis cell death in diseases	39
1.10 Interferon- γ signaling and its role in disease.....	42
1.11 mTORC pathway	46
1.11.1 PI3K/Akt/TSC/mTOR signaling in macrophage polarization	50
1.12 RATIONALE	53
1.13 HYPOTHESIS	54
1.14 OBJECTIVES	54

CHAPTER 2: Materials and Methods	55
2.1 Isolation of human primary monocytes and generation of monocyte-derived macrophages and the different macrophage subsets	55
2.2 Generation of mouse bone marrow-derived macrophages (BMDM) and SNB75 cell line	56
2.3 Cell surface marker analysis by flow cytometry	57
2.4 Treatment of MDMs and BMDM subsets with chemical inhibitors.....	58
2.5 Pre- and Post-M1 polarization treatment with chemical inhibitors	60
2.6 Evaluation of cell death by intracellular Propidium Iodide (PI) staining and flow cytometry	60
2.7 Evaluation of cell death by YOYO-1 Iodide staining and IncuCyte™ Zoom	61
2.8 Determination the involvement of apoptosis pathway in SM-treated human macrophage subsets	61
2.8.1 Determination of caspase-3/7 activity by caspase-3/7 reagent and IncuCyte™ Zoom:	61
2.8.2 Determination of caspase-8 activity by caspase-8 reagent and flow cytometry:.....	62
2.9 Western immunoblot analysis	63
2.9.1 Lysate preparation.....	63
2.9.2 Total protein quantification.....	63
2.9.3 SDS-PAGE and Western immunoblotting.....	63
2.10 TNF- α analysis by ELISA	67
2.11 Human recombinant TNF- α treatment of different macrophage subsets.....	68
2.12 Blocking TNF- α by anti-TNF- α neutralizing antibodies in MDMs and SNB-75 cell line	68
2.13 Transfection of macrophages with small interfering RNA	69
2.14 Statistical analysis	69
2.15 Ethics statement	70
CHAPTER 3: Selective killing of human M1 macrophages by Smac mimetics alone and M2 macrophages by Smac mimetics and caspase inhibition	71
3.1 INTRODUCTION.....	72
3.2 RESULTS	76
3.2.1 Human macrophage subsets differentially express distinct cell surface markers.....	76
3.2.2 Blocking IAPs signaling pathway by SM-LCL161 selectively causes cell death in human M1 macrophages, whereas M2 macrophages are resistant to SM-induced cell death.....	80
3.2.3 SM-induced cell death in M1 macrophages is mediated by both extrinsic and intrinsic pathways of apoptosis	85
3.2.4 SM-induced cell death in M1 macrophages involves both apoptosis and necroptosis pathways.....	93
3.2.5 Reversal of M0 to M1 differentiation by blockage of JAK/STAT pathway reverses susceptibility of M1 macrophages to SM-induced apoptosis	97
3.2.6 Blockage of caspases and IAPs signaling selectively cause cell death in M0 and M2c macrophages through the necroptosis pathway	100
3.2.7 Human and mouse M1 and M2c macrophages behave similarly with respect to sensitivity towards SM-induced cell death.....	108
3.2.8 M1 and M2c macrophages are plastic and repolarization of M1 into M2c and of M2c into M1 subset reverses the effects of SM-LCL161 on cell death and expression of cell surface markers.....	108
3.3 DISCUSSION	118

CHAPTER 4: The molecular mechanism governing SM-induced cell death in M1 macrophages.	125
4.1 INTRODUCTION.....	126
4.2 RESULTS	128
4.2.1 The IAPs antagonist, SM-LCL161 selectively induces cell death in human M1 macrophage subset.....	128
4.2.2 Molecular mechanism governing SM-induced cell death in M1 macrophages.....	129
4.2.3 TNF- α failed to regulated cell death seen in SM-treated M1 macrophage subset.....	132
4.2.4 SM-LCL161 does not activate either canonical or non-canonical NF- κ B:	145
4.2.5 NF- κ B pathway is not involved in SM-induced cell death in M1 macrophages.....	149
4.2.6 Involvement of IFN- γ activated pathway in SM-induced cell death of M1 macrophage.....	152
4.2.7 Silencing IRF-1 regulates SM-induced cell death in M1 macrophages.....	155
4.2.8 PKR inhibition does not regulate SM-induced cell death in M1 macrophages.....	158
4.2.9 Involvement of PI3K/Akt-mTORC pathway in SM-induced cell death.....	161
4.2.10 SM-induced cell death during M1 differentiation is regulated by mTORC1-S6K pathway ..	166
4.2.11 SM-induced cell death in differentiated M1 is regulated by mTORC2.....	170
4.2.12 MAPK pathways do not affect SM-induced cell death of M1 macrophages.....	173
4.2.13 Analysis of proapoptotic and anti-apoptotic proteins involved in SM induced cell death in M1 macrophages:.....	179
4.2.14 SM-induced cell death in M1 macrophages is regulated by cIAP-2 and RIPK-1/3 through the activation of mTORC	187
4.3 DISCUSSION	191
CONCLUDING REMARKS AND FUTURE DIRECTIONS	202
REFERENCES.....	208
CURRICULUM VITAE.....	234
RIGHTS AND PERMISSION	237
PUBLISHED ARTICLE	246

LIST OF FIGURES

Figure 1.1: Macrophage polarization and main functions.	7
Figure 1.2: The intrinsic and extrinsic pathways of apoptosis.....	23
Figure 1.3: IAPs members and structures.	27
Figure 1.4: Schematic representative of the effect of IAPs.	31
Figure 1.5: Canonical and non-canonical NF- κ B pathway.....	35
Figure 1.6: Initiation of necroptosis pathway.	41
Figure 1.7: IFN- γ signaling.	45
Figure 1.8: PI3K/Akt/TSC/mTOR pathway.	49
Figure 3.1: Phenotypic characterization of undifferentiated M0 and polarized M1, M2a, M2b and M2c macrophages.	79
Figure 3.2: M1 and M2c are the most sensitive and resistant macrophages subsets to SM-LCL161 induced cell death, respectively.....	82
Figure 3.3: Kinetics of cell death in SM-treated M0, M1 and M2c macrophages as determined by IncuCyte™ automated machine.	84
Figure 3.4: Effect of SM-LCL161 on the expression of anti-apoptotic and proapoptotic protein.	87
Figure 3.5: SM-LCL161 activates caspase-3/7, -8 and -9 in M1 macrophages.....	92
Figure 3.6: SM-induced cell death in M1 macrophages is mediated by apoptosis and necroptosis.....	96
Figure 3.7: JAK pathway regulates SM-induced cell death in M1 macrophages.....	99
Figure 3.8: SM induce cell death in M0 and M2c following blockage of caspases.	102
Figure 3.9: SM-induced cell death in M0 and M2c cells following caspase blockage is mediated by necroptosis.	105
Figure 3.10: Necrosulfonamide (NSA) alone does not cause cell death in M1 macrophages but reduces cell death in zVAD.fmk and SM-LCL161 treated M0 and M2c macrophages.....	107
Figure 3.11: M1 mouse BMDMs are highly sensitive whereas M2c macrophages are resistant to SM- induced cell death.	110
Figure 3.12: Repolarization of M1 into M2c and of M2c into M1 macrophage subsets reverses the effect of SM-LCL161.....	114
Figure 3.13: Repolarization of M1 into M2c and of M2c into M1 macrophage subsets reverses the effect of SM-LCL161 on the effector cell death molecules.....	117
Figure 4.1: M1 macrophages are sensitive whereas M2c macrophages are resistant to SM-induced cell death.	131
Figure 4.2: M0, M1 and M2c macrophages produce TNF- α spontaneously and following SM-LCL161 treatment.....	134
Figure 4.3: SM-LCL161 does not induce cell death in LPS-treated M0, M1 and M2c macrophages.	137
Figure 4.4: Recombinant human TNF- α (<i>rh</i> TNF- α) does not induce cell death in SM-treated M0, M2c or M1 macrophages.	140
Figure 4.5: Endogenously produced TNF- α does not mediate SM-induced cell death in M1 macrophages.....	144

Figure 4.6: SM-LCL161 does not affect canonical or non-canonical pathway of NF- κ B activation in human macrophages.	148
Figure 4.7: Blocking NF- κ B either before or after M1 polarization does not inhibit cell death in SM-treated M1 macrophages.....	151
Figure 4.8: SM-mediated cell death in M1 macrophages is mediated by the JAK-STAT pathway.....	154
Figure 4.9: IRF-1 regulates SM-induced cell death in M1 macrophages.	157
Figure 4.10: Blocking PKR did not reduce cell death in SM-treated M1.....	160
Figure 4.11: Inhibition of PI3K pathway either before or after M1 polarization does not affect SM-induced cell death.	163
Figure 4.12: Akt inhibition either before or after M1 polarization does not affect SM-induced cell death.	165
Figure 4.13: Blocking mTORC1/2 and S6K pathway during M1 differentiation inhibits SM-induced cell death in M1 macrophages.....	169
Figure 4.14: Blocking mTORC1/2 pathway after M1 polarization by Torin alone inhibits SM-induced cell death in M1 macrophages.....	172
Figure 4.15: Blocking of P38, ERK or JNK MAPK pathway before M1 polarization does not inhibit SM-induced cell death in M1 macrophages.	176
Figure 4.16: Blocking of P38, ERK or JNK MAPK pathway after M1 polarization does not inhibit SM-induced cell death in M1 macrophages.	178
Figure 4.17: SM-LCL161 causes degradation of cIAP-1/2 in M0, M1 and M2c macrophages.....	181
Figure 4.18: SM-LCL161 does not inhibit Bcl-2 family of anti-apoptotic proteins (Bcl-2, Mcl-1 and Bcl-xL), pro-apoptotic (Bak), FADD, FAS or TRAIL proteins.....	184
Figure 4.19: SM-LCL161 causes degradation of RIPK-1, RIPK-3, MLKL, cFLIP and upregulation of Bax in M1 but not in M0 and M2c macrophages.....	186
Figure 4.20: Blocking mTORC1/2 by Torin prevents cIAP-2, RIPK1/3 degradations.	190

LIST OF TABLES

Table 2.1: Summary of the chemical inhibitors and their concentrations used in this study.....	59
Table 2.2: SDS-PAGE gel solution components and their concentrations.....	65
Table 2.3: Summary of Western immunoblotting primary antibodies used in this study	66

LIST OF ABBREVIATIONS

4E-BPs	Eukaryotic translation initiation factor 4E binding proteins
4HB	Four-helix bundle
AKT	Protein kinase B (PKB)
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor-1
ARG-1	Arginase-1
ASK-1	Apoptosis signal-regulating kinase 1
AVPI	Smac N-terminal tetrapeptides (Alanine-Valine-Proline-Isoleucine)
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated protein X
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra-large
BH	Bcl-2 homology domains
Bim	Bcl-2-like protein 11
BIR	Baculovirus-IAP-Repeat
BMDMs	Bone marrow derived macrophages
CARD	Caspase recruitment domain
Caspase	Cysteine-dependent aspartate proteases
CDP	Common dendritic cell precursors
cFILP	Cellular FLICE-inhibitory protein
cIAP-1/2	Cellular inhibitor of apoptosis protein 1/2
CLP	Common lymphoid progenitors
cMOP	Committed monocyte precursors
CNS	Central nervous system
CYLD	Cylindromatosis
DAI	DNA-dependent activator of IFN-regulatory factors, IRFs
DD	Death domain
DISC	Death-inducing signal complex
DR	Death receptor
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
eIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular regulated kinases
FADD	Fas-associated death domain protein
Fas	First apoptotic signal
FasL	Fas ligand
FBS	Fetal bovine serum
FCGR	Fc portion of immunoglobulin G receptor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GAS	Gamma-activated sequence
GMP	Granulocyte and macrophage progenitors
HIV	Human immunodeficiency virus
HME	Human monocytic ehrlichiosis
HRP	Horseradish peroxidase
HSCs	Hematopoietic stem cells
IAPs	Inhibitor of apoptosis proteins
IBMs	IAP binding motifs
ID	Intermediary domain
IFN- γ	Interferon gamma
IL	Interleukin
IL-1ra	IL-1R receptor antagonist
IL-1RII	The decoy IL-1beta type II receptor
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
ISGs	Interferon stimulated genes
JAK	Janus-associated kinase
JNK	JUN N-terminal kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LUBAC	Linear ubiquitin chain assembly complex
M-CSF	Macrophage colony-stimulating factor
MAPKs	Mitogen-activated protein kinases
Mcl-1	Myeloid cell leukemia 1
MDMs	Monocytes-derived macrophages
MDP	Monocyte-macrophage/dendritic cell precursors
MEP	Megakaryocyte and erythrocyte progenitors
MLKL	Mixed-lineage kinase domain-like pseudokinase
MMR	Mannose receptor
MOMP	Mitochondrion outer membrane permeabilization
MPP	Multipotent progenitors
MRSA	<i>methicillin-resistant S. aureus</i>
mTORC1/2	Mammalian target of rapamycin complex 1/2
Necrostatin-1	Nec-1;5-((1H-indol-3-yl)methyl)-3-methyl-2-thioxoimidazolidin-4-one
NEMO	NF- κ B essential modulator
NF- κ B2	NF- κ B precursor protein p100
NF- κ B	Nuclear factor kappa-light-chain enhancer of activated B cells
NIK	NF- κ B-inducing kinase
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
NSA	Necrosulfonamide
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PDK	Phosphoinositide-dependent kinase 1
PI	Propidium iodide

PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
pJAK-1	Phosphorylated janus-associated kinase
PKA	Protein kinase A
PKC- α	Protein kinase C- α
PKR	Protein kinase R
PPAR γ	Peroxisome proliferator-activated receptor- γ
Puma	p53 upregulated modulator of apoptosis
Raptor	Regulatory-associated protein of mTOR
Rheb	Small GTPase Ras homolog enriched in brain
RHIM	RIP-homotypic interaction motif
Rictor	Rapamycin-insensitive companion of mTOR
RIPK-1	Receptor-interacting serine/threonine-protein kinase-1
RIPK-3	Receptor-interacting serine/threonine-protein kinase 3
RNIs	Reactive nitrogen intermediates
ROIs	Reactive oxygen intermediates
RUX	Ruxolitinib
S6K	Ribosomal protein S6 kinase
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SGK1	Serum and glucocorticoid-induced protein kinase 1
siRNAs	Small interfering RNAs
SLE	Systemic lupus erythematosus
SMs	Smac mimetics
Smac	Second mitochondrial activator of caspases
SOCS	Suppressor of cytokine signalling
STAT	Signal transducers and activators of transcription
TAB	TAK-binding proteins
TAK1	TFG β activated kinase 1
TAMs	Tumour-associated macrophages
TGF- β	Transforming growth factor beta
TLR	Toll like receptor
TNF-R1/2	TNF receptor 1/2
TNF- α	Tumor necrosis factor alpha
TRADD	TNF-R1-associated death domain
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis inducing ligand
TSC	Tuberous sclerosis complex
UBC	Ubiquitin-conjugating domain
VEGF	Vascular endothelial growth factor
VPR	Viral Protein R
XIAP	X-chromosome-linked IAP
YOYO-1	1,1'-[1,3-propanediylbis[(dimethylimino)-3,1-propanediyl]]-bis[4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]]-quinolinum tetraiodide
ZBP1	Z-DNA-binding protein 1
zVAD.fmk	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

CHAPTER 1: General Introduction

1.1 Monocyte/Macrophage origins and functions

Since their first discovery almost a century ago, the study of macrophages is still a very motivating topic in the field of immunology. Elie Metchnikoff first introduced the term “macrophage,” meaning large eaters, and described their phagocytic ability (1, 2). These big cells are involved in different immunological aspects, and they are considered as an integral component of innate immunity (3). Macrophages are a heterogeneous population of innate myeloid cells, representing the most functionally diverse cells of the hematopoietic system (4). These cells have multiple functions, which include their ability to phagocyte different cells such as infected, senescent, dead cells as well as pathogens and cellular debris. They also link the innate and adaptive immune system by antigen processing and presentation (5, 6). They are considered one of the significant cytokine producers such as TNF- α , IL-1 and IL-6. Additionally, they are involved in the induction or resolution of inflammation and tissue repair (5–7).

1.1.1 Monocyte-derived macrophages

Monocyte-derived macrophages are the most-well studied form of macrophages due to the easy access to human blood monocyte and the convenience of *in vitro* differentiation techniques. Monocytes originate from hematopoietic stem cells (HSCs) in the bone marrow, which generates a heterogeneous population of multipotent progenitors (MPP) (8). MPP further differentiates into either common lymphoid progenitors (CLP) that give rise to different lymphoid cells or common myeloid progenitors (CMP). CMP can differentiate into megakaryocyte and erythrocyte progenitors (MEP) responsible for the production of platelets and red blood cells or granulocyte and macrophage progenitors (GMP). GMP is responsible for granulocytic lineage development or further differentiate into monocyte-macrophage/dendritic cell precursors (MDP). MDP can either

give rise to common dendritic cell precursors (CDP) or committed monocyte precursors (cMoP). The cMoP develop into monoblasts, which can be recognized morphologically in the bone marrow. Monoblasts give rise to promonocytes and finally into mature monocytes (8). Monocytes represent 5-10% of all circulating leukocytes in humans and commonly considered to be responsible for replenishing tissue-resident macrophage in a steady state or during inflammation (9, 10). After a short circulatory life in the blood (1-2 days), they migrate from the bloodstream into various tissues/organs and subsequently differentiate into tissue-resident macrophages (9, 11).

1.1.2 Yolk sac and fetal liver-derived macrophages

For the last 40 years, macrophages have been thought to be originating from circulating peripheral blood monocytes (12). However, several recent studies have shown that monocytes are not the sole source of tissue-resident macrophages. Macrophages can arise in the fetal liver or even in the yolk sac during embryonic development *in utero* (13–15). These primitive macrophages represent a distinct population that can persist, self-renew into adulthood and maintained independently from monocyte recruitment/replenishment. Bone marrow transplantation studies revealed insufficient ability to replenish tissue macrophage population. Some tissue-resident macrophages are mainly unaffected in patients who suffer from monocytopenia (16). Tissue macrophage can self-renew to sustain the macrophage population. Ajami *et al.*, demonstrated a local expansion of microglia cells in their chimeric models. These self-renewal microglia maintained their function independently of hematopoietic stem cells (HSCs) throughout adult life (17). These studies and others suggested a dual origin of tissue macrophages, one from the classical bone marrow HSCs and another from an early time at the embryonic stage (18–20).

Several studies have defined and tracked the origin of these tissue-resident macrophages and their persistence into adulthood (15). Genetic fate-mapping techniques to permanently label cells

and their subsequent progeny facilitated the precise differentiation of embryonic macrophages from the classical macrophages originated from HSCs. For example, it has been shown that microglia are predominantly derived from yolk sac progenitors, while Langerhans cells can arise from the fetal liver as well as the yolk sac progenitors (14, 21). Moreover, Schulz *et al.*, demonstrated that the transcription factor *Myb* was necessary for the development of HSCs macrophages, in contrast, yolk sac-derived macrophages developed in several tissues independently of *Myb* and persist in adulthood without HSCs input (22). Furthermore, during early embryonic development, yolk sac derived mouse macrophages express CX3CR1^{hi}, F4/80^{hi} and CD11b^{lo} while in wild type mice, macrophages that are derived from HSCs exhibited F4/80^{lo}, and CD11b^{hi} (22). Thus, the current understanding of the source and origin of macrophages need to be expanded to include primitive macrophages.

1.2 Organ/Tissue distribution of macrophages

Once monocytes successfully enter the different tissues, they are exposed to various stimuli such as local growth factors, cytokines, or microbial products. These stimuli promote monocytes differentiation to fully mature and specialized tissue-resident macrophages (9, 23). Depending on the microenvironment they reside in and the functional phenotypes, macrophages are divided into several subpopulations. For instance, in the lungs, macrophages exist as alveolar macrophages, which are responsible for the clearance of microparticles and pathogens (24, 25). Microglia in the central nervous system (CNS) play essential roles in tissue remodelling, CNS maintenance and intracellular communication (26–29). There are other macrophages in the brain, such as perivascular, choroid plexus and meningeal macrophages, that are involved in brain hemostasis and function (30). Kupffer cells in the liver are essential for pathogens clearance, detoxification, elimination of harmful substances, and metabolic functions such as iron recycling (11, 31, 32).

Osteoclasts in the bone play a critical role in bone resorption and formation of long bones during embryonic development (33, 34). In the intestine, several macrophage subpopulations with distinct phenotypes and functions collaborate to maintain mucosal homeostasis, regulation of immune response and clearance of enteric pathogens (35, 36). Two types of macrophage subpopulations coexist in the skin: Langerhans cells located in the epidermal layer and dermal macrophages predominantly located in the dermis. They are involved in immune surveillance, T lymphocytes interaction, cancer regression, skin homeostasis and wound repair (37–39). Alveolar macrophages, microglia, Kupffer cells and Langerhans cells all have been reported to be derived from primitive origins such as yolk sac and/or fetal liver and maintain their production by self-renewal or peripheral blood precursors replenishment (11, 40).

1.3 Macrophage polarization

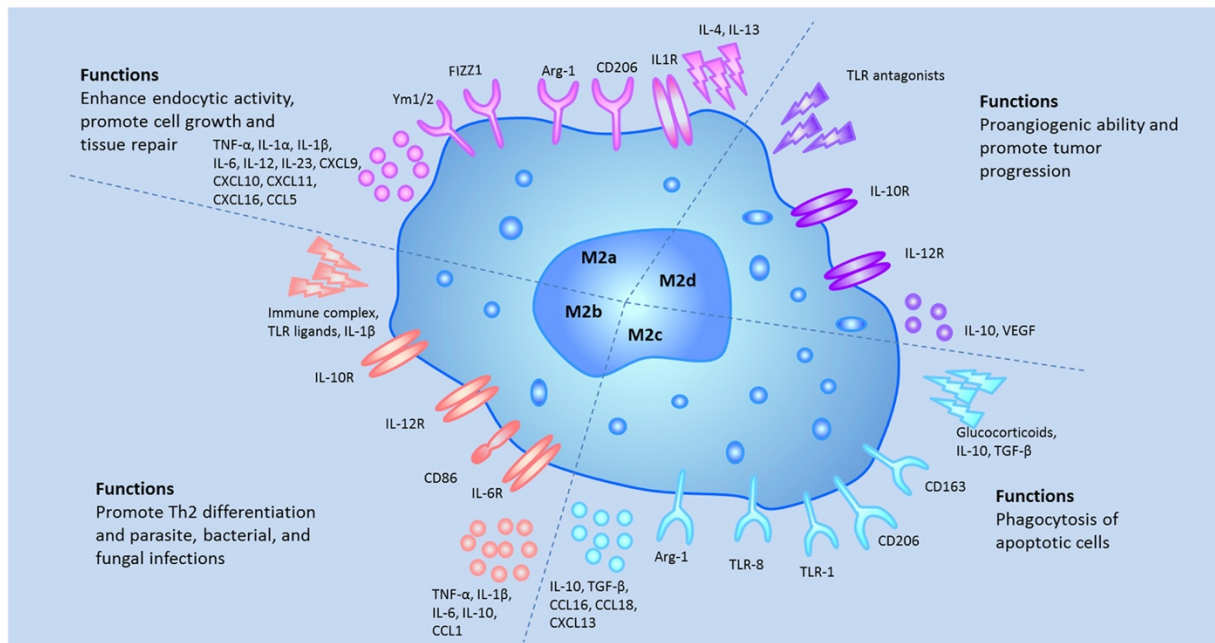
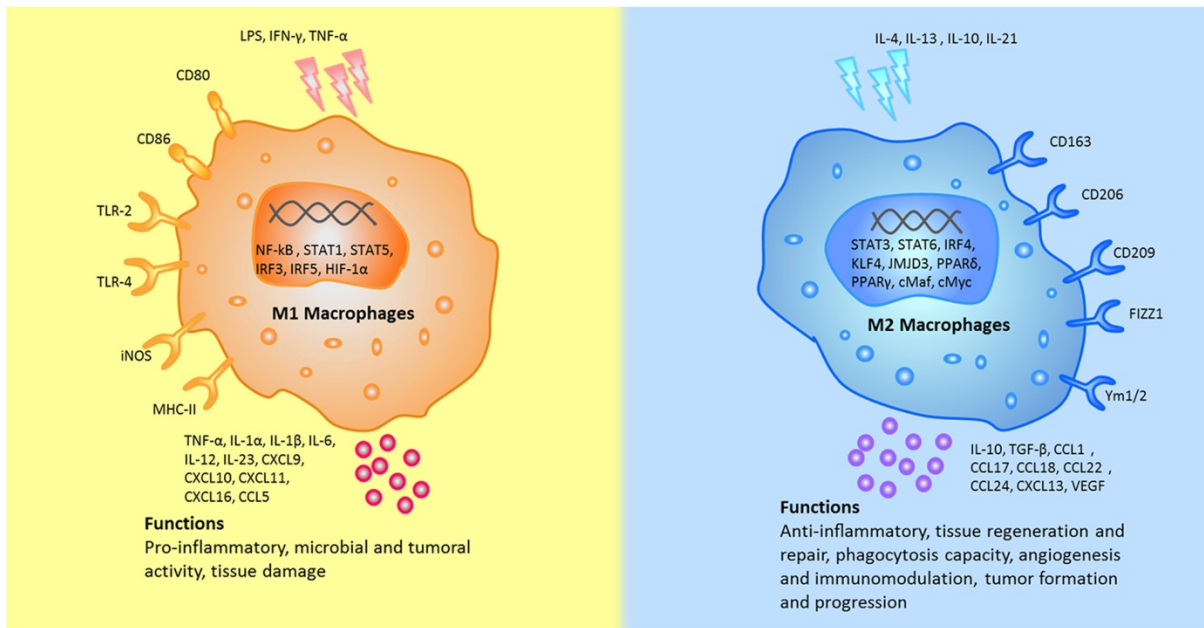
At the tissue level, macrophages are exposed to and are profoundly affected by exogenous and endogenous factors including, tissue-specific immune-modulating cytokines, chemokines and microbial products, which allow them to express multifunctional and different properties (7, 41, 42). There are several subpopulations of macrophages that have been reported in humans and mice based on their surface markers, specific factors production and biological activities (5, 43, 44). Broadly speaking, macrophages can be activated to adopt two opposite polarized states. The classically activated inflammatory macrophages, and the alternatively-activated anti-inflammatory macrophages (7, 42).

In 2000, Mills *et al.*, proposed the term M1 and M2 macrophages phenotypes in murine models. They further suggested that M1/M2 phenotypes are not merely considered as activated or inactivated macrophages, but rather cells with distinct metabolic activities (45). In their study, the main difference between M1 and M2 activation was based on the macrophage L-arginine

metabolism pathway. M1 produces high levels of inducible nitric oxide synthase (iNOS), whereas M2 produces arginase-1. M1 macrophage metabolizes amino acid L-arginine and oxygen to nitric oxide (NO) and L-citrulline via the enzyme iNOS (46). Citrulline helps in efficient NO synthesis via citrulline–NO cycle. In contrast, M2 macrophages hydrolyze L-arginine via the arginase-1 enzyme to produce L-ornithine and urea. The arginase-1 itself limits L-arginine availability for NO synthesis, and L-ornithine can be further converted to polyamine and proline syntheses, which are essential for collagen synthesis, cellular proliferation and tissue repair (46). These findings and others set the primary fundamental role of macrophage plasticity, which allows to conflict of pathogens, plays an immunomodulatory role and maintains tissue integrity (42, 47, 48).

1.3.1 Classically activated macrophages (M1) and their role in disease

M1 macrophages can be induced by T helper 1 cell-derived cytokines such as IFN- γ which is mainly produced by T helper-1 (Th1) lymphocytes, or TNF- α . Intracellular pathogens and bacterial cell wall components such as lipopolysaccharide (LPS) can also induce M1 macrophages activation (42–44). M1 are characterized by a) enhanced secretion of pro-inflammatory cytokines such as TNF- α , IL-12, TNF- α , IL-6, IL-1 α , and IL-1 β . B) Produce a large number of toxic molecules such as iNOS-dependant reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) (49). C) Exhibit high phagocytic capacity and antigen presentation (7, 42, 50). Phenotypically, M1 macrophages express monocyte chemoattractant protein-1 (MCP-1; also known as CCL2), Th1 cell attracting chemokines MIG (CXCL9) and the IFN- γ -inducible chemokine IP-10 (CXCL10), and other chemokines such as CXCL1-3, CXCL5 and CXCL8 (51). They also express high levels of major histocompatibility complex class-II (MHC-II), and co-stimulatory molecules (CD80 and CD86) (50). Thus, M1 have potent anti-microbial, anti-tumoral activities, and mediate inflammation and Th1 responses (**Figure 1.1A**).



Yao et al., 2019. (52)

Figure 1.1: Macrophage polarization and main functions.

(A) M1 macrophages are induced by IFN- γ , TNF- α or microbial lipopolysaccharide (LPS) and characterized by expressing several surface markers including MHC-II, CD80, CD86 and TLRs. Their main functions include antitumorigenic and microbicidal activities as well as secretion of pro-inflammatory cytokines and toxic mediators. (B) M2 macrophages are induced by several cytokines such as IL-4, IL-13, IL-10 and TFG- β . They express different surface markers and well defined by their anti-inflammatory and immunomodulating functions. (C) M2 macrophages are more heterogeneous and can be divided into M2a, M2b, M2c and M2d based on stimulus. M2 subsets are involved in tissue repair and remodeling, immunosuppression and production of anti-inflammatory cytokines (52).

M1 macrophages play an important role as a first line of defense against intracellular pathogens. During infection, monocytes are recruited, infiltrated, and differentiated into resident-tissue macrophages. The microenvironment changes due to microbial infection drive M1 macrophages polarization. M1 macrophages exhibit a high level of phagocytic activity to engulf and destroy the microbes. However, excessive or inability to resolve inflammatory responses may cause damage of the surrounding microenvironment and might contribute to several human diseases such as cancer, chronic infection, or inflammatory autoimmune diseases (5, 51). For example, M1 macrophages have been shown to be the predominant macrophage phenotype in human colon carcinoma (53). Additionally, M1 macrophages have been shown to be involved in human inflammatory bowel disease and rheumatoid arthritis progression (54–56).

An imbalance in recruitment or differentiation to M1 macrophages have been shown to negatively affect successful intracellular pathogens clearance. For example several pathogens including bacteria, viruses and parasites inhibit M1 macrophage differentiation to ensure their survival (57, 58). In acute pathogen infection, M1 macrophage polarization is induced to express high levels of proinflammatory cytokines, thus providing microbicidal properties to clear infection. However, some pathogens such as *Leishmania donovani* and *Mycobacterium tuberculosis* have been shown to disrupt IFN- γ signalling pathway. *Leishmania donovani* impaired IFN- γ signaling by inhibiting tyrosine phosphorylation of JAK-STAT1 pathway (59), while *Mycobacterium tuberculosis* does not affect the JAK-STAT1 pathway, but rather inhibit the transcription of IFN- γ responsive genes by disrupting the association of STAT1 to the basal transcriptional apparatus and RNA polymerase II (60, 61). The opportunistic fungus *Candida albicans* has also been shown to inhibit the inflammatory response in human macrophages by inducing the M1 to M2 switch and blocking NO production by induction of host arginase activity (62, 63). M1 polarization signature

has also been associated with other bacterial infections including typhoid fever, tuberculoid leprosy, and *Helicobacter pylori* gastritis (64–66). Gastric biopsies from infected patients with *H. pylori* demonstrated increased M1 macrophage markers such as iNOS and TNF- α compared to uninfected healthy human (65). Moreover, the transcriptome analysis of circulating cells from patients with acute typhoid fever, revealed enrichment of M1 genes (67).

1.3.2 Alternatively activated macrophages (M2)

M2 macrophages are widely known as an anti-inflammatory, wound healing, and tissue repair macrophages. They are induced by several stimulatory factors, including fungal cells, immune complexes, complement, apoptotic cells and cytokines (68). Among the cytokines, IL-4, IL-13, IL-10 and transforming growth factor-beta (TGF- β) are potent activators of M2 phenotypes (68, 69). They have high phagocytosis activity and produce chemotactic factors that help recruit other immune cells to the site of injury. Phenotypically, M2 macrophages are characterized by the production of anti-inflammatory cytokines such as IL-10, IL-1 receptor antagonist (IL-1ra), type II IL-1 decoy receptor and TGF- β . Additionally, they are characterized by low IL-12 and IL-23 productions (7, 68). They also express several surface receptors including c-type mannose receptor 1 (Mrc1, also known as CD206), hemoglobin-haptoglobin scavenger receptor (CD163), chitinase 3-like protein 3 (Chi3l3, also known as Ym1), resistin-like- α (also known as Found in inflammatory Zone 1; FIZZ1), CXCR1, CXCR2 and arginase-1 (Arg-1). These markers are involved in immune regulation and tissue remodelling and repair (7, 68, 70). They also express MHC-II and co-receptors molecules in low amounts (71). Additionally, they secrete various chemokines involved in the regulation the response of Th2, T regulatory cells (Tregs), eosinophils and basophils (**Figure 1.1B**) (68, 72, 73). Therefore, M2 macrophages differ from M1 in their inadequate capacity to present antigens, produce oxygen and nitrogen radicals, their ability to kill

intracellular pathogens, as well as mediate tissue remodelling and repair.

1.4 Molecular determinants of M1/M2 macrophage polarization

Macrophage polarization is tightly regulated at the signal transduction, transcriptional and post-transcriptional levels (7). Several transcription factors play a major role in M1, and M2 polarization including signal transducers and activators of transcription (STAT), Interferon regulatory factor (IRF), NF- κ B and peroxisome proliferator-activated receptor γ (PPAR γ) and PPAR δ . These factors are involved in different signalling pathways and interact with each other in a very complex way to drive macrophage cells to adopt a particular phenotype. For instance, IFN- γ activates STAT1 through JAK/STAT pathway, leading to the dimerization and translocation of STAT1 into the nucleus. Subsequently, it mediates the transcription of M1-associated genes and induce the pro-inflammatory cytokines (7). In contrast, IL-4 and IL-13 promote STAT6 activation through IL-4 receptor alpha (IL-4R α) (7). Additionally, IL-10 activates STAT3 through IL-10R1 and IL-10R2 (74, 75). Activation of STAT3 and STAT6 drives the M2 activation by induction of the transcription of genes involved in anti-inflammatory and tissue repair responses (7, 74, 76).

The suppressor of cytokine signalling (SOCS) family also regulates STAT-mediated activation of macrophages. IL-4 upregulates SOCS1, which in turn inhibits the action of STAT1. On the other hand, IFN- γ with TLR stimulation upregulates SOCS3 and inhibits STAT3 (77, 78). Moreover, SOCS3 activates NF- κ B and phosphatidylinositol-3-kinase (PI3K) pathways and drives the production of pro-inflammatory IL-6 and nitric oxide, while restricting the expression of IL-10 (79). The nuclear receptor PPAR γ and PPAR δ are activated by STAT6 and mediates M2 macrophage polarization (80, 81). Bouhrel *et al.*, demonstrated that PPAR γ activation primes human monocytes to M2 differentiation *in vivo* and *in vitro* (82). Therefore, the balance between the activation of STAT1 and STAT3/6 plays an essential role in regulating M1/M2 macrophage

polarization.

In the M1 phenotype, interferon regulatory factor 5 (IRF5) is upregulated and induces the production of pro-inflammatory cytokines such as IL-12, IL-23 and TNF- α , promotes Th1, Th17 responses and inhibits the production of IL-10, hence promoting M1 polarization (83). In contrast, IL-4 activates the transcription factor *c-myc*, which in turn enables IRF-4 to promote M2 activation and further inhibit IRF-5 induced M1 polarization (84, 85) (**Figure 1.1A and B**).

1.5 M2 macrophages subsets and their role in disease

M2 macrophages are characterized by their high phenotypic heterogeneity (68). Several reports have suggested the presence of macrophages exhibiting a wide range of phenotypic properties that intermediate between the two extremes, i.e. M1 and M2 macrophages (72, 86). M1/M2 classification is generally used to describe the two extremes forms of macrophages activation and thus is considered as an oversimplified classification. In M2 macrophages, despite sharing similar general functional properties such as low IL-12, immunoregulation, and tissue repair, different forms/subsets of M2 macrophages have been proposed (72). Based on the different stimuli and their induced transcriptional changes, Mantovani *et al.*, in 2004, described various M2 macrophages sub-classes. He suggested that M2 macrophages can be divided into several subsets, including M2a, M2b, M2c subtypes (72). Additionally, he demonstrated that tumor-associated macrophages (TAMs) that infiltrate into tumor tissue as an M2 polarized macrophage population (87). Later, TAMs were reported as a novel M2 subset and named M2d (88). All M2 subsets share similar anti-inflammatory cytokine profiles characterized by high production of IL-10 and low production of IL-12 (72, 89) (**Figure 1.1C**).

1.5.1 M2a macrophages subset

M2a macrophages are induced by IL-4 or IL-13 stimulation. Th2 cells, basophils,

eosinophils and mast cells are the primary producers of these cytokines (89–91). IL-4 and IL-13 share overlapping secondary structures, thus they bind to the same receptor complex that consists of IL-4R α /IL-13 α heterodimers (92). They downregulate pro-inflammatory mediators such as TNF- α , IL-6 and IL-12. They also downregulate the biological activity of IL-1 β by enhancing the production of IL-1 receptor antagonist (IL-1Ra) and the decoy IL-1 β type II receptor (IL-1RII) or by downregulation of caspases-1 which is responsible for pro-IL-1 β cleavage and activation (89). M2a macrophages express several surface receptors including CD206, CD163, Ym1, FIZZ1, CXCR1, CXCR2 and Arginase-1 (Arg-1). Arg-1 is involved in polyamine and proline synthesis through the arginine metabolic pathway. These by-products are essential for cell proliferation, collagen formation and tissue repair (93). However, human M2a polarized macrophages do not either express Arg-1 or Ym1 (94). Due to the limited amount of pro-inflammatory cytokines and mediators in M2a macrophages, they show less microbicidal ability to clear intracellular pathogens (89, 95). Additionally, M2a macrophages secrete high levels of chemokines including CCL17 (TARC), CCL18 (AMAC-1), MDC (CCL22), and eotaxin-2 (CCL24) involved in the regulation of Th2, T regulatory cells (Tregs), eosinophils and basophils responses (**Figure 1.1C**) (68, 72, 89).

Similar to M1 polarized macrophage, M2a macrophage polarization is a tightly regulated process. Excessive, or unnecessary M2a polarization may promote intercellular pathogens survival and disease progression (5, 57, 65, 96). For example, nasal tissue analysis from chronic rhinosinusitis patients demonstrated increased M2a macrophage polarization and that was correlated with decreased *S. aureus* clearance and chronic disease development (97). Whipple's disease is a chronic infection caused by *Tropheryma whipplei*. Macrophages isolated from Whipple's disease patient's samples demonstrated increased M2a macrophage phenotypes population and were associated with a lack of local inflammation and *T. whipplei* clearance (98).

In murine pulmonary infection model, *Cryptococcus neoformans* was shown to induce IL-13 production associated with increased M2a macrophage population, pulmonary fungal load and reduced mice survival time compared to mice deficient in IL-13 expression (99). IL-4 needed for M2a polarization has also been shown to induce the susceptibility of murine cryptococcosis (100). Additionally, a M1 to M2a phenotype switch has been implicated in the development of susceptible *Leishmania major* infection. Macrophage-specific IL-4R α deficient mice were able to control leishmaniasis and significantly delayed disease progression (101).

1.5.2 M2b macrophages subset

M2b, induced by exposure to combinations of immune complexes recognized by receptors of the Fc portion of immunoglobulin G (FCGR) with lipopolysaccharide (LPS) or IL-1 β through Toll-like receptor-4 (TLR4) or IL-1 receptor (89, 102). M2b are involved in antigen presentation and immunoregulation of Th2 and B cell responses. They are characterized by low IL-12 and high IL-10 production (5, 103). They differ from M2a by their ability to produce significant amounts of TNF- α , IL-6 and IL-1 β . They also secrete CCL1(I-309) that binds to CCR8 receptors expressed on Th2 and Tregs and thus promote their recruitments (**Figure 1.1C**) (103).

M2b macrophages, similar to M2a macrophages, display high phagocytic capacity, but they exhibit low microbicidal activities. They increase the susceptibility and persistence of infection by dampening the immune response and facilitate immune evasion. Some bacteria, parasites and viruses can directly induce polarization into M2b macrophages to promote their persistence (103). For example, upon *Leishmania infantum* parasitic infection, macrophage displayed M2b phenotype characterized by high production of pro-inflammatory cytokine profile such as TNF- α , IL-1 β , IL-6 and combined with the production of anti-inflammatory cytokines such as IL-10, IL-1RA and TGF- β (104). Another study demonstrated an increase in M2b polarization and

permissive replication in *Leishmania major* infected mice (105). M2d macrophages were also shown to increase the susceptibility of opportunistic bacterial infections such as *Enterococcus faecalis* and *Klebsiella pneumonia* in mice with chronic alcohol consumption (CAC) model. In these mice, macrophages produced IL-10 and express mRNAs for CD163, and chemokine ligand (CCL)1. Treatment with CCL1 antisense oligodeoxynucleotides (ODN), an M2b macrophages inhibitor, reverted these macrophages into M1 phenotype and significantly diminished the bacterial infection (106).

1.5.3 M2c macrophages subset

M2c macrophages are induced after exposure to IL-10, TGF- β or glucocorticoids. Similar to M2b, M2c macrophages are characterized by high IL-10 and TGF- β productions. Additionally, M2c are characterized by high expression of scavenging molecules such as CD163 and other receptors and chemokines such as CD14, CD206 (MMR), CCL16, CCL18 and CXCL13 (71, 72). Functionally, they are essential for limiting immune responses by downregulating MHC-II and costimulatory molecules or chemokines involved in immune cell recruitments such as CXCL11, CCL5 and CCL24 (**Figure 1.1C**). They also downregulate various pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8, IL-12 and inflammatory mediators such as iNOS and cyclooxygenase-2 (5, 107). Additionally, they play a crucial role in tissue remodelling, matrix deposition and phagocytosis of apoptotic cells (5, 6, 89). Zizzo *et al.*, reported that M2c macrophages demonstrate enhanced ability to clear apoptotic cells compared to other macrophage subsets such as M1 or M2a and further suggested that M2c polarization could have a therapeutic utility for human diseases with defective apoptotic cells clearance such as lupus nephritis (108). Moreover, some viruses and bacteria drive M2c polarization to repress immune responses and ensure the persistence of infection. For example, human cytomegalovirus (CMV) encodes viral

IL-10, a homolog of human IL-10 that drove M2c macrophage polarization and decreased Th cells activation (109). Similarly, *Haemophilus ducreyi* induces the production of IL-10 in infected macrophages through activation of mitogen-activated protein kinase (MAPK) and PI3K pathway to suppress M1 and drove M2c macrophage polarization (110). M2c macrophages have been shown to play an essential role in reduce renal inflammation and injury by reducing Th cells infiltration in chronic kidney diseases. M2c were more effective than M2a in reducing renal fibrosis and promote epithelial and vascular repair in murine nephrosis (111, 112).

1.5.4 Tissue-associated macrophages (M2d macrophages subset)

TAMs are the most predominant immunosuppressive cells in the tumor microenvironment (87). Due to their role in promoting angiogenesis, tissue repair and suppressing immune responses, they are usually defined as the M2 subclass (87, 113). TAMs are characterized as high IL-10 and low IL-12 producers, but they do not express Ym1, FIZZ1 or CD206 (114). They are named M2d compared to M2a-c classification (**Figure 1.1C**) (113). Induction of M2d macrophage population has been linked to poor prognosis in various solid tumors including breast, cervical and ovarian cancers (113, 115, 116). M2d macrophages participate in tumor growth and progression by releasing cytokines which promote tumor growth or by mediating angiogenesis and production of angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (87). VEGF produced by M2d macrophages have been proposed to play a role in peritumoral lymphangiogenesis and the formation of lymphatic metastasis (117). In lung tumors, M2d macrophages release PDGF, which promotes angiogenesis and tumor stroma formation (87, 89). M2d also produce tumor-inducing chemokines such as CCL2, which contribute to tumor progression in different human organs such as bladder, cervix, lung and breast (118). IL-4 and IL-13 or IFN- γ , can play a crucial role in regulating the tumor growth and progression. The IL-4 and

IL-13 ensure constant M2 macrophage nourishment in the tumor microenvironment and suppress the immune response (119). While IFN- γ reverse the immunosuppressive properties of M2d macrophages, induces M1 polarization and recruits T cells to stimulate anti-tumor responses (113).

1.6 Macrophage polarization manipulation as a therapeutic strategy

Recently, targeting macrophages has been suggested to be a potential therapeutic strategy such as recruitment of M2 macrophages, inducing a class switch from M1 to M2 subsets and downregulation of pro-inflammatory pathways within the macrophage subsets to control cancer, chronic infections, atherosclerosis, autoimmune and allergic diseases (7, 120, 121). Several specific macrophage-related therapies and therapeutic agents such as PPAR γ agonists, statins and zoledronic acid have been found to affect murine and human macrophage activation and polarization (122–124). The effect of these agents on macrophages recruitment, polarization and differentiation have been studied in vivo mouse models as well as in humans (7). Therefore, it is of utmost importance to identify agents that specifically induce macrophage polarization or target M1 and/or M2 macrophages for killing by induction of different cell death mechanisms within these macrophages. These approaches might provide potential therapeutic strategies to control diseases associated with pro- and anti-inflammatory phenotype.

1.7 Overview of apoptosis

Apoptosis is one of the most extensively studied pathways of cell death (125). It serves a critical role for successful organ development, maintenance of tissue homeostasis and prevents the growth of malignancy (126, 127). During embryogenesis, the excessive or damaged cells are programmed to undergo apoptosis. This process is essential in the interdigit formation, such as the fingers and toes, sex differentiation, and immune system development (128). This type of cell

death also occurs to maintain a healthy physiological tissue balance between the number of newly generated cells and senescent, dysfunctional or infected cells (129–131).

Apoptosis is a non-inflammatory and silent process, characterized by distinct morphological and biochemical changes. Some examples including shrinkage of both nucleus and cytoplasm, plasma membrane blebbing (apoptotic bodies), the activation of a series of cysteine-aspartic proteases known as caspases, disruption mitochondrial potential, exteriorization of phosphatidylserine on the cell surface (eat me signal), DNA fragmentation and chromatin condensation (126, 132, 133). The process of apoptosis orchestrating a series of events that are well conserved within multi-cellular organisms (132). The two most important groups of proteins that control the process of apoptosis are caspases and the B-cell lymphoma 2 (Bcl-2) family of proteins (134, 135). Both protein families are involved through the activation of several proteins that participate in the different steps of the apoptosis process.

1.7.1 Caspases

Caspases are a large family of cysteine-dependant endoproteases proteins that recognize and cleave their target proteins at specific tetrapeptide motifs (P4-P3-P2-P1)(135). They specifically break any peptide bond after the amino acid aspartic acid (position P1). Thus, they are called cysteine aspartyl proteases or simply caspases (136). In healthy cells, most caspases are produced as inactive precursors (zymogens) known as procaspase with little to no protease activity and require specific proteolysis for activation (137). The procaspase consists of a large subunit, a small subunit, and an N-terminal prodomain (137). Specific cleavages at two positions in procaspase remove the prodomain at the N-terminus and facilitate the homodimerization to another procaspase to generate a biologically active protein. The active caspase consists of two large, two small subunits ($\alpha_2\beta_2$) and two identical active sites. The active sites contain a large pocket to

accommodate the P4-P1 residues of its substrate (135, 137, 138). Caspases activation can also lead to a positive feedback effect on other procaspases, which lead to further amplification of caspases activation and apoptotic signalling (135, 137).

Currently, there are 12 human and 11 mice caspases that have been identified, which differ in their substrate recognition and cleavage efficiency (137). Their roles in apoptosis and inflammation generally classify most caspases into three main groups a) the upstream initiator caspases (caspase-8 and -9); b) the downstream executioner caspases (caspase-3, -6, and -7) and c) inflammatory caspases (caspases-1, -4, -5, -11, and -12L (132, 135, 138). Furthermore, other caspases have been identified, for example, caspase-2, -10, -12S and -14. They remain unclassified and do not belong to groups as mentioned earlier either because they share structural elements with initiator and executioner caspases such as caspase-2 or because their role has not been fully elucidated yet (135, 138).

1.7.2 Bcl-2 proteins

The protein of the Bcl-2 family has long been identified for its role in regulating apoptosis (139). The members of the Bcl-2 family are categorized due to their Bcl-2 homology (BH) domains and their effects in regulating apoptosis (126, 139). There are three main subfamilies of Bcl-2 proteins. The anti-apoptotic subfamily that contain four Bcl-2 homology domains (BH1, BH2, BH3 and BH4) such as B-cell lymphoma-2 (Bcl-2), B-cell lymphoma extra-large (Bcl-xL), induced myeloid leukemia cell differentiation protein (Mcl-1). The second subfamily is the pro-apoptotic BH-3 only members such as BH3 interacting-domain death agonist (Bid), Bcl-2-like protein 11 (Bim), Bcl-2-associated death promoter (Bad), p53 upregulated modulator of apoptosis (Puma), and Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa). The third subfamily is the pro-apoptotic effector molecules that lack the BH4 domain such as Bcl-2-associated protein X

(Bax), and Bcl-2 homologous antagonist killer (Bak) (126, 138, 140). Different therapeutic strategies to selectively induce apoptosis in cancers have been proposed, such as inhibiting the anti-apoptotic Bcl-2 proteins or directly activate the pro-apoptotic proteins such as Bax (141, 142). Thus, highlight the importance of these Bcl-2 proteins members in regulating apoptosis.

1.7.3 The mitochondrial intrinsic pathway

Apoptosis involves two main distinct signalling pathways that ultimately lead to the activation of the executioner caspases, and promote cell death (128, 143). The intrinsic pathway, also known as a mitochondrial pathway of apoptosis, depends on factors released from the mitochondria to initiate the intrinsic pathway (**Figure 1.2A**). Different factors are responsible for the activation of the intrinsic pathway including intracellular stress signals by cytokines, hormones, hypoxia, toxins, DNA damage or various cytotoxic agents (138, 144). In response to such stimuli, the pro-apoptotic (BH3-only) of Bcl-2 family protein inhibits the effect of the anti-apoptotic Bcl-2 family proteins. This inhibitory effect on the anti-apoptotic Bcl-2 proteins leads to activation and aggregation of the pro-apoptotic effector proteins, Bax and Bak on the outer membrane of the mitochondria. Also, some BH3-only proteins, such as PUMA and Bim, can directly interact and activate Bax and Bak (144, 145). Once the effector pro-apoptotic proteins are activated, they aggregate and form a channel, causing mitochondrion outer membrane permeabilization (MOMP) which facilitates the release of mitochondrial apoptotic factors such as cytochrome-C and second mitochondrial-derived activator of caspases (SMAC) into the cytosol (138, 141). The release of cytochrome-C rapidly binds to the adaptor protein apoptotic protease activating factor-1 (Apaf-1) (146). Apaf-1 is usually folded in such a manner where its caspase recruitment domain (CARD domain) is blocked and unable to bind to its counterpart CARD domain that presents in procaspase-9. Binding of cytochrome-C to apaf-1 monomers results in

ATP-dependent conformational changes, which lead to unfold and exposure of the CARD domain and the oligomerization of apaf-1. Thus allowing the assembly of several apaf-1 and pro-caspases-9 proteins in a heptameric complex known as apoptosome, and this stimulates caspase-9 activation (147, 148). The activated caspase-9 triggers the activation of the executioner caspases (caspase-3, -6 and -7) (128) which in turn, cleave hundreds of proteins leading to the distinct apoptosis morphological changes and cell death (**Figure 1.2A**).

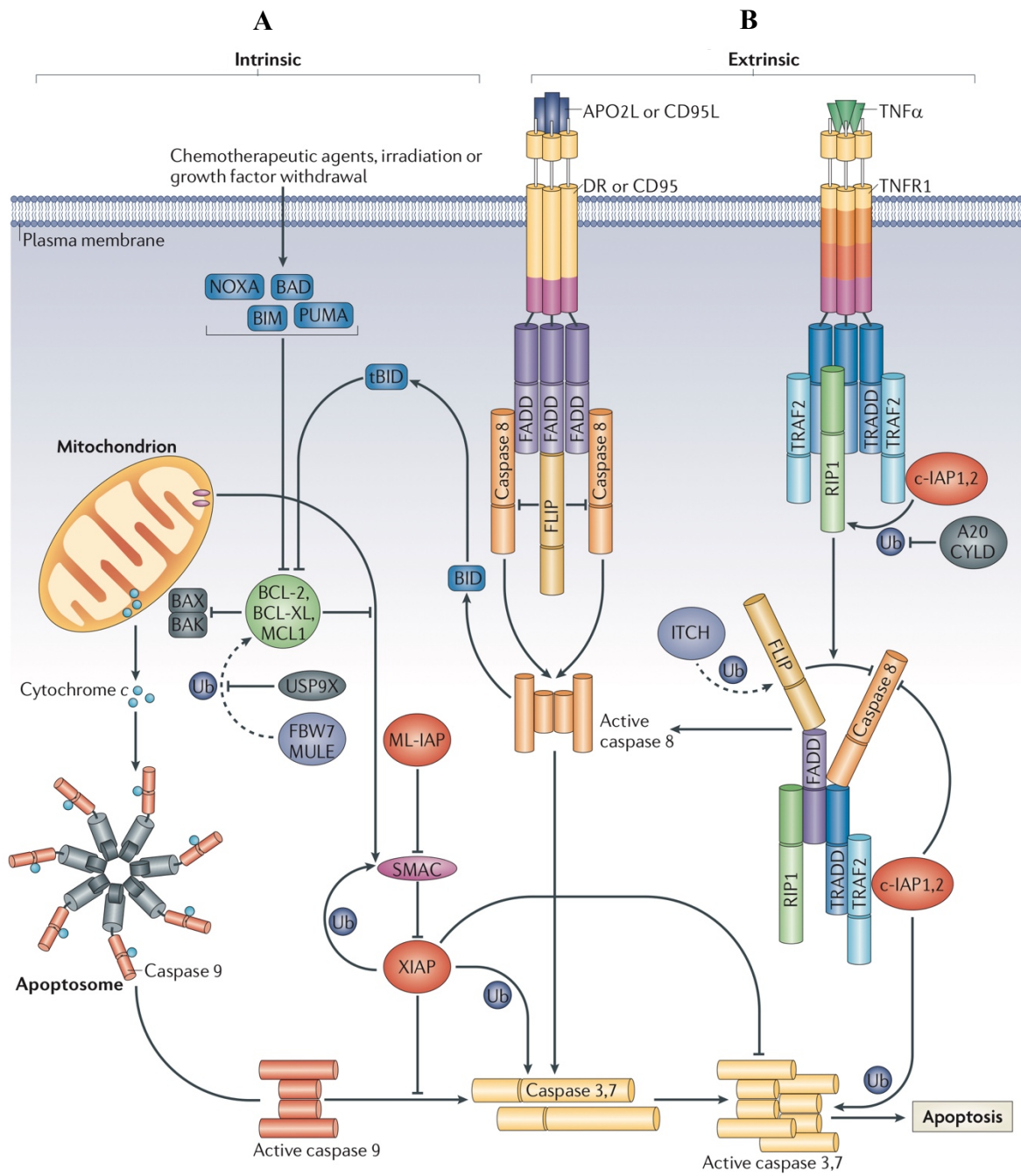
1.7.4 Death receptor-the extrinsic pathway of apoptosis

The extrinsic pathway is triggered by death receptors (DR)-ligand interactions on the surface of death-targeted cells. DRs are members of the tumor necrosis factor (TNF) receptor family, where each DR has its cognate death ligands (143, 149). The best-characterized death receptors in terms of apoptosis are the first apoptotic signal (Fas, also known as APO-1 or CD95) that can be activated by Fas ligand (FasL also known as CD95-L) (126), TNF-related apoptosis-inducing ligand receptor-1 (TRAIL-R1; DR4), TRAIL-R2 (DR5) that can be activated by TRAIL (also known as Apo2-L) (132, 150) and TNF receptor-1 (TNF-R1; also known as p55 or p60) that can be activated by TNF- α (126, 151) (**Figure 1.2B**).

Binding of Fas or TRAIL-Rs to their cognate death ligands induce a conformational change in the cytosolic tail of DRs to recruit a complex composed of Fas-associated death domain (FADD), procaspase-8 and cellular FLICE-inhibitory protein (cFLIP) (126, 151, 152). This complex is known as death-inducing signal complex (DISC), where FADD facilitates the interaction of the procaspase-8 monomers, while cFLIP regulates the activation of caspases-8. If the formation of DISC and the activation of caspase-8 were sufficient, it could directly lead to the activation of executioner caspases (caspase-3, -6 and -7). In some scenarios, the weak formation of DISC, low amount of active caspase-8 or the presence of IAPs could inhibit the direct activation of

executioner caspases (153, 154). However, the low amount of active caspase-8 can still cleave Bid, a BH3-only protein member of the Bcl-2 family. The truncated Bid translocates to the outer mitochondrial membrane where it mediates MOMP, which causes the release of cytochrome-C and induction in cell death (156, 157).

Similar to Fas and TRAIL-Rs, the activation of TNFR-1 by its specific ligand TNF- α , leads to downstream signalling activation and recruitment of different adaptor proteins including TNFR1-associated death domain (TRADD), the TNFR-associated factor 2 (TRAF2), receptor-interacting serine/threonine-protein kinase-1 (RIPK-1), cellular inhibitor of apoptosis proteins 1 and 2 (cIAP-1 and cIAP-2; will be discussed below in more details) and the linear ubiquitin chain assembly complex (LUBAC complex) (**Figure 1.2B**) (158, 159). This complex of different recruited proteins known as complex-I and does not affect apoptosis. The cIAPs trigger ubiquitination of RIPK-1 and TGF- β activated kinase 1 (TAK1) containing complexes thus, provide survival signaling by activating the mitogen-activated protein kinases (MAPKs) and NF- κ B signaling pathway (159–161). However, when cIAPs are absent or inhibited by pharmacological inhibitors such as Smac mimetic compounds (SMs) or cylindromatosis (CYLD) cause deubiquitination of RIPK-1, and prevent NF- κ B activation and dissociation of RIPK-1 from the plasma membrane complex (162). Subsequently, the deubiquitinated RIPK-1 binds to FADD and caspase-8 and forms complex-IIa. If NF- κ B signalling is inactivated, it does not induce the production of proteins such as cFLIP. Thereby, complex-IIa is formed in the cytoplasm and activates caspase-8 and initiates apoptosis (**Figure 1.2B**) (163, 164).



Vucic et al., 2011. (155)

Figure 1.2: The intrinsic and extrinsic pathways of apoptosis.

Apoptosis pathway is executed via intrinsic or extrinsic pathways. **(A)** The intrinsic pathway (or mitochondrial) is induced by intracellular stress signals leading to the activation of different Bcl-2 proteins and the release the cytochrome-C from the mitochondria. The released cytochrome-C mediates apoptosome formation and activation of the initiator caspase-9. **(B)** The extrinsic pathway is activated after death receptor-ligand interaction which facilitates the aggregation of several adaptor proteins and activation of the initiator caspases-8. Subsequently, caspases-9 or -8 activates the executioner caspases-3 and -7 and induced apoptosis cell death (155).

1.8 Inhibitor of apoptosis proteins (IAPs)

Inhibitor of apoptosis (IAPs) proteins play a direct suppressing role of pro-apoptotic signaling and activation of executioner caspases (165). IAP proteins are a family of structurally related anti-apoptotic proteins. The human genome encodes for eight different IAP protein members: mammalian neuronal apoptosis inhibitory protein (NAIP), cellular inhibitor of apoptotic protein 1 (cIAP-1), cellular inhibitor of apoptotic protein 2 (cIAP-2), X-linked chromosomal inhibitor of apoptosis (XIAP), Survivin (also known as TIAP), Apollon (also known as BRUCE), melanoma-IAP (ML-IAP; also known as livin) and IAP-like protein 2 (ILP2) (125, 166). XIAP, cIAP-1 and cIAP-2 are the most critical and well-studied members of IAPs (**Figure 1.3**) (125, 167, 168).

1.8.1 Structure and function of IAP proteins

IAP proteins are defined by the presence of one to three specific domains called Baculovirus-IAP-Repeat (BIR) domains that mediate protein-protein interactions (169). These BIRs domains consist of 70-80 amino acids and categorized based on their number and functions into type-I (only BIR1) or type-II BIRs (BIR2 and BIR3). NAIP, cIAP-1, cIAP-2 and XIAP contain three BIR domains (BIR1, BIR 2, and BIR 3), while Survivin, Apollon, ML-IAP, ILP2 have only one BIR domain (**Figure 1.3**) (170). Type-I and type-II BIR proteins differ in their ability to bind to a conserved tetrapeptide motif called IAP binding motifs (IBMs) that are present in some caspases and the extreme N-terminus end of IAP antagonists such as SMAC (170, 171). Type-I BIR (BIR1 domain) does not exhibit IBM binding capability (170) while the type-II BIRs (BIR2 and BIR3) can form a hydrophobic groove with distinct binding properties to IBMs. The BIR2 domain is essential for the anti-apoptotic properties of IAPs and show a potent inhibitory activity for caspases-3 and caspase-7 (172, 173). The BIR3 domain of XIAP primarily targets caspases-9 by

preventing dimerization of procaspase-9 and its catalytic activity (172, 173). In contrast, BIR1 interacts with proteins involved in cell signaling pathways such as TRAFs and TAK1-binding proteins 1 (TAB1). For example, cIAP-1 and cIAP-2 use their BIR1 domain to bind to TRAF2 (174). Additionally, XIAP activates NF- κ B by interacting of its BIR1 domain with TGF β -activated kinase 1 binding protein 1 (TAB1) (175).

Five of the 8 IAPs also possess another important domain at the C-terminal known as RING (really interesting new gene) zinc-finger motif. RING motif displays a ubiquitin E3 ligase activity able to ubiquitinate associated proteins such as caspases (166, 176). Apollon lacks a RING domain but, it contains a ubiquitin-conjugating domain (UBC) at the C-terminal region which mediates ubiquitylation, thus facilitates proteasomal degradation of bound proteins (177). XIAP, cIAP-1, cIAP-2 also contain a ubiquitin-associated-binding domain (UBA), which is important for their signaling function (178). However, only cIAP-1 and cIAP-2 carry the caspase recruitment domain (CARD) that can mediate homotypic protein interactions such as FADD, TRADD and caspase-8 (179, 180). Survivin is the smallest IAP proteins and contains only one BIR. Additionally, it contains coiled-coil domain (CC), that plays an important role in chromosomal segregation, cytokinesis and cell division (181). NAIP, have a nucleotide-binding oligomerization domain (NOD) and a leucine-rich repeat (LRR) domain involved in regulation of innate immune signalling and microbial pathogen recognition (**Figure 1.3**), (166, 182).

BIRC1 (also known as NAIP)



BIRC2 (also known as cIAP1)



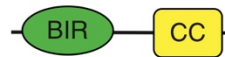
BIRC3 (also known as cIAP2)



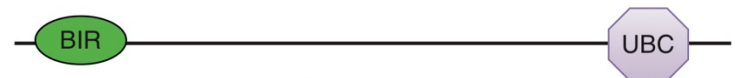
BIRC4 (also known as XIAP)



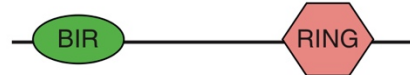
BIRC5 (also known as Survivin)



BIRC6 (also known as BRUCE or Apollon)



BIRC7 (also known as Livin or ML-IAP)



BIRC8 (also known as Ts-IAP or ILP2)



Key:



Baculovirus
IAP repeat



Leucine rich
repeats



Ubiquitin-
associated domain



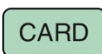
Coiled-coil



Nucleotide-binding and
oligomerization domain



Ubiquitin-
conjugase-like



Caspase recruitment
domain



Really interesting
new gene

Beug et al., 2012. (169)

Figure 1.3: IAPs members and structures.

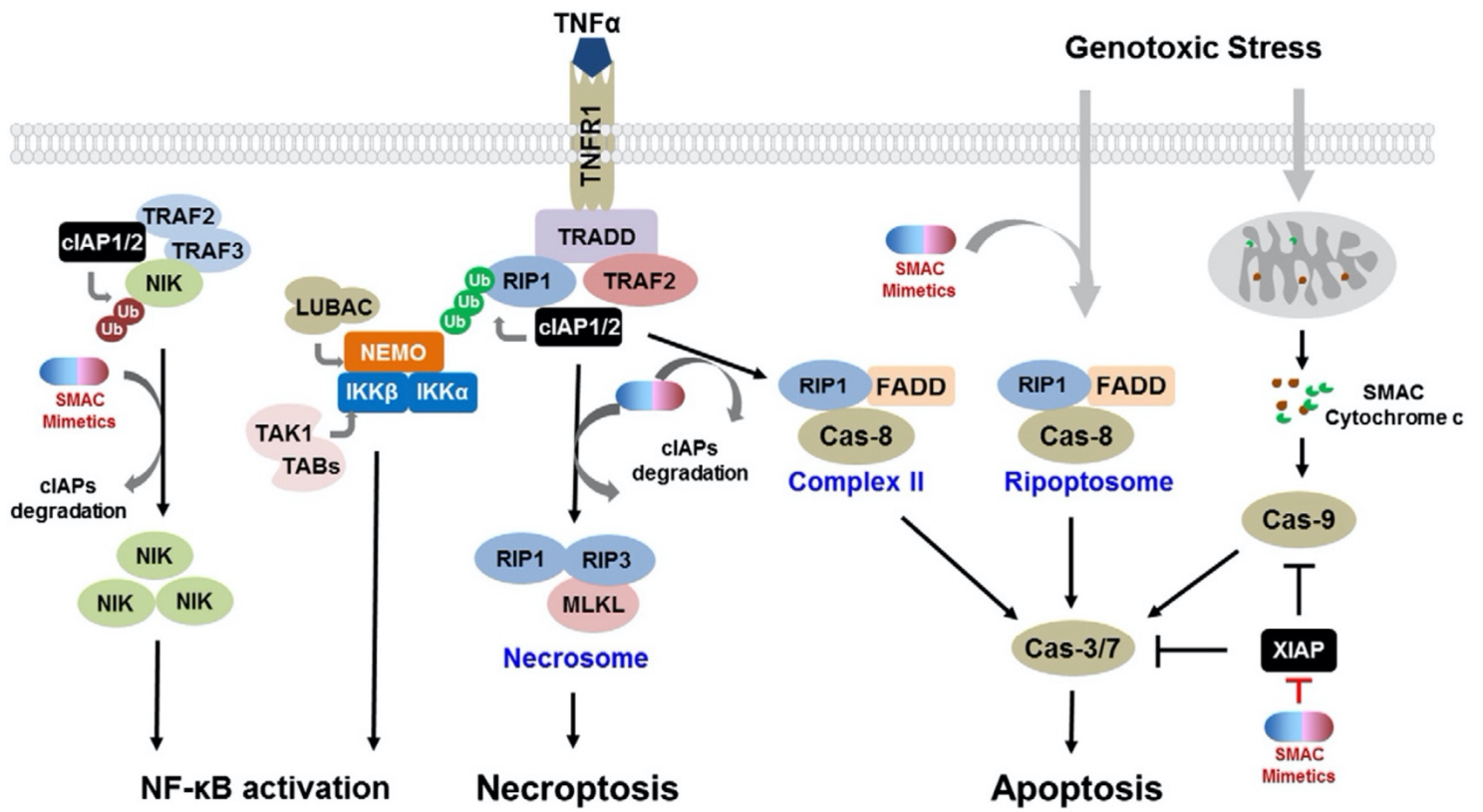
IAP proteins are a family of structurally related anti-apoptotic proteins. The human genome encodes for eight different IAP protein members, among them XIAP, cIAP1 and cIAP2 are the most critical and well-studied IAPs in cell death. IAP proteins are defined by the presence of one to three BIR domains and other various domains including CARD, RING, UBA, CC, NOD and UBC. Type-I BIR consists of BIR1 only domain that responsible for binding to TRAFs and other mediator proteins. Type-II BIR consists of BIR2 and BIR3 domains that binds to caspases and IAP antagonist (169).

1.8.2 Regulation of Apoptosis by IAPs

IAPs including XIAP, cIAP-1 and cIAP-2 directly or indirectly prevent apoptosis by binding and inhibiting several initiators and executioner caspases such as caspases-9, -3 and -7. However, several studies suggested that IAPs differ in regulating apoptosis. XIAP is the most well-studied IAP with a potent direct caspase-9 inhibition (183). The BIR3-binding groove of XIAP binds to IBM located at the N-terminus of active caspase-9, thereby prevents caspase-9 dimerization and sequesters caspase-9 in an inactive monomeric state. Therefore, XIAP prevents the catalytic activity of caspase-9 and the activation of downstream executioner caspases (171, 183). On the other hand, BIR2 of XIAP binds to IBM of the large subunits of active caspase-3 and -7. It prevents the substrate-binding pocket from binding to target substrates, thus limiting their enzymatic activities (172, 173). However, inhibition of caspase-3 and -7 does not seem to be essential for the anti-apoptotic function of XIAP *in vivo*. Silke *et al.*, introduced several mutations into full-length XIAP, which lead up to a 500-fold reduction in XIAP's ability to inhibit caspase-3 activity but retained full ability to block ultra-violet-induced apoptosis (173).

Human cIAP-1 and cIAP-2 share high degree of similarity with the potent caspase inhibitor XIAP and are considered the closest paralogs IAP members of XIAP. Therefore, cIAPs have often been suggested to inhibit apoptosis similarly as XIAP, i.e. by directly inhibiting caspases activation (184). Indeed, cIAP-1 and cIAP-2 can bind/interact with caspase-3, -7, and -9, through their type II BIRs. However, the inability of cIAPs to inhibit the enzymatic activities of these caspases is not clear. It has been suggested that differences in the IBM-interacting sites on cIAP-1/2 and XIAP, play a critical role in binding and inhibiting caspase-7 and -9 (185, 186). Residues substitutions in cIAPs with residues on the XIAP BIR domain, promotes caspases inhibition by cIAPs (185). Although cIAPs are unable to inhibit the caspases activities, their RING domains located at the C-

terminal can regulate the stability of the processed caspases. The RING domains mediate E3 ubiquitin ligase activation of caspases-3 and -7. These ubiquitinated caspases then undergo proteasomal degradation and prevent induction of apoptosis (187, 188). Independently of death receptors stimulation or mitochondrial pathways, another complex called ripoptosome can be assembled, in the presence of genotoxic stress-induced IAPs depletion such as DNA-damage or IAP antagonist SMAC. The cytoplasmic RIPK-1 binds to FADD and pro-caspase-8, thus stimulates caspase-8 mediated apoptosis or even caspase-8 independent cell death also known as necroptosis (**Figure 1.4**) (189, 190).



Bai et al., 2014. (191)

Figure 1.4: Schematic representative of the effect of IAPs.

cIAP1/2 ubiquitinate RIPK-1 which mediates the recruitment of downstream adaptor proteins and the formation of complex-I to activate the canonical NF- κ B pathway. IAP antagonist, SMAC bind/interact with cIAP-1 and cIAP-2 and trigger their proteasomal degradation, the loss of cIAPs leads to the formation of complex-II involved in both apoptosis and necroptosis cell death. Smac mimetics (SMs) directly antagonize XIAP which leads to the release of XIAP from its inhibitory function against caspase-3, -7 and -9. SMs can also induce ripoptosome formation which subsequently, leads to apoptosis. Additionally, SMs increases NIK protein accumulation and mediates the activation of non-canonical NF- κ B (191).

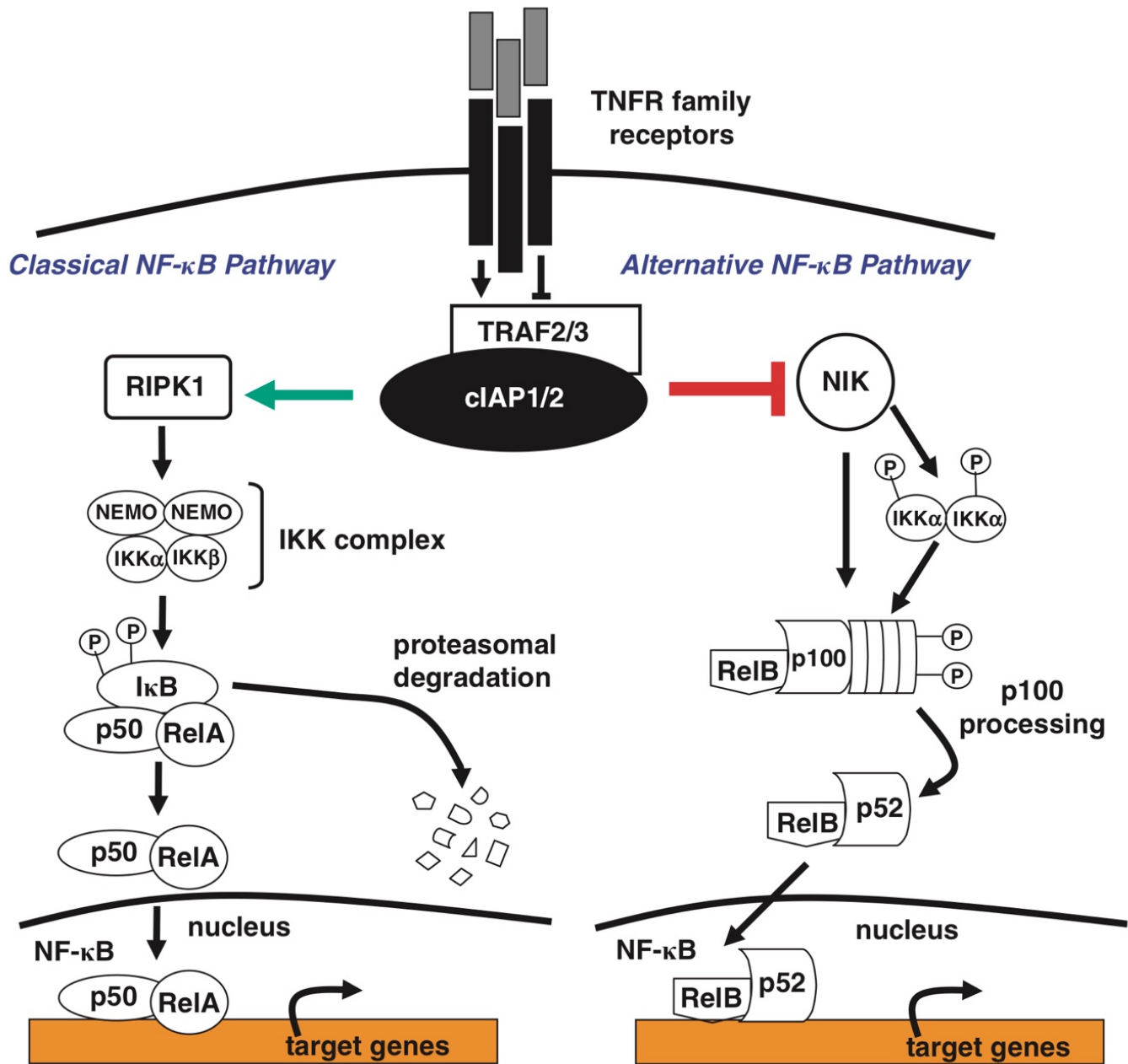
1.8.3 Role of IAPs in NF- κ B signalling pathway

In addition to their roles in direct caspase regulation, cIAPs play an essential regulatory role in the activation of important signalling pathways such as the pro-survival NF- κ B signalling pathway and the MAPK pathway (192). One of the best-characterized roles of cIAPs is initiated just downstream of TNFR-1 stimulated with TNF- α (193). TNF- α stimulation does not necessarily mean cell death in fact, most cells do not die following TNF- α stimulation. However, they die when TNF- α is unable to induce NF- κ B activation due to disruption in the recruitment or activation of the adaptor proteins (194). NF- κ B is a transcriptional protein complex that responds to several stimuli, including TLRs or cytokine stimulation. NF- κ B activation leads to their translocation to the nucleus and promotes gene transcriptions of several proteins involved in inflammation, proliferation and cell survival (193–195).

cIAP-1 and cIAP-2 play an essential role in NF- κ B activation. cIAPs are recruited into complex-I, that is assembled after TNF- α ligation with TNFR-1. Within complex-I, TRAF2 is the primary adaptor protein that facilitates the recruitment of cIAP-1 and cIAP-2 to the complex through their BIR1 and UBA domains to bind to TRAF2 protein (125). Subsequently, cIAPs through their RING domains, conjugate K11-, K48-, K63-linked ubiquitin chains to themselves and other components of complex-I, most importantly RIPK-1 (**Figure 1.5**) (196). Ubiquitylated RIPK-1 works as a scaffold for the recruitment of kinase complexes such as; the linear ubiquitin chain assembly complex (LUBAC), the TAK1-TAB kinase complex, and the trimeric inhibitor of κ B kinase (IKK) complex, which consists of IKK α and IKK β subunits and the regulatory subunit IKK γ (also known as NF- κ B essential modulator; NEMO)(197, 198). LUBAC, in turn, linearly ubiquitylates and activates these proteins and further stabilizes the complex-I (199). Thus, the poly-ubiquitylated RIPK-1 by cIAPs facilitates the activation of IKK containing complex (IKK α ,

IKK β and IKK γ) directly or through the activation of TAK-1 (200). Subsequently, the activated IKK β phosphorylates I κ B α , which in non-stimulated cells inhibits the activation of NF- κ B by sequestering its main components; p50 and p62/RelA (198). The phosphorylation of I κ B α promotes its proteasomal degradation and allows the translocation of NF- κ B transcription factors (p50 and p65/RelA heterodimer) to the nucleus and regulate the transcription of targeted genes. Also, TAK-1 activation leads to MAPK pathway activation (192). TAK-1 targets the apoptosis signal-regulating kinase 1 (ASK-1) for k48-lined ubiquitination and proteasomal degradation. Subsequently, it triggers the activation of JUN N-terminal kinase (JNK) and p38 MAPK (200, 201).

cIAPs are also involved in regulating the non-canonical NF- κ B activation. cIAP-1 and cIAP-2 conjugate K48 ubiquitylation chain to the NF- κ B-inducing kinase (NIK); thus, ensure continuous proteasomal degradation of NIK protein (125, 202). In the absence of cIAPs, NIK protein accumulates, then phosphorylates and activates IKK α (203). The activated IKK α phosphorylates NF- κ B precursor protein p100 (NF- κ B2) and leads to partial proteasomal processing of p100 subunit to generates p52 NF- κ B (204). Subsequently, p52 binds to RelB, the P52/RelB heterodimer translocates to the nucleus to activate the non- canonical NF- κ B and induce the expression of the targeted genes (**Figure 1.5**) (205).



LaCasse et al., 2008. (206)

Figure 1.5: Canonical and non-canonical NF- κ B pathway.

cIAP-1 and cIAP-2 regulate the canonical (classical) and non-canonical (alternative) NF- κ B pathway. cIAPs ubiquitylate RIPK-1 which works as a scaffold for the recruitment and activation of the inhibitor of κ B kinase IKK containing complex (IKK α , IKK β and NEMO). Subsequently, the activated IKK β phosphorylates I κ B α , promotes its proteasomal degradation. The NF- κ B transcription factors (p50 and p65/RelA heterodimer) are then translocate to the nucleus and regulate the transcription of targeted genes. In the non-canonical NF- κ B, cIAP-1 and cIAP-2 continuously degrade the NF- κ B-inducing kinase (NIK). In the absence of cIAPs, NIK protein accumulation, mediates the phosphorylates and activation of IKK α . The activated IKK α phosphorylates NF- κ B precursor protein p100 and leads to partial proteasomal processing of p100 subunit to generates p52 NF- κ B. Subsequently, p52 binds to RelB, and translocates to the nucleus to activate the non-canonical NF- κ B and induce the expression of the targeted genes (206).

1.8.4 Endogenous IAP Antagonists

Smac/Diablo is one of the best characterized endogenous IAP antagonists (207, 208). Smac is a proapoptotic protein that resides between the inner and outer membranes of mitochondria (207). Once the cell is triggered to undergo apoptosis, the pores formed within the mitochondrial membranes facilitate the release of this protein. Upon releasing, Smac undergoes a maturation process that leads to the expression of IBM at the N-terminus of the protein. Subsequently, they bind to the IBM grooves of different members of the IAPs family and inhibit their activity (209, 210). Smac can antagonize XIAP by binding to BIR2 and BIR3 domains, which leads to the release of XIAP from its inhibitory function against caspase-3, -7 and -9 (125, 211). Smac also bind/interact with cIAP-1 and cIAP-2 and trigger their proteasomal degradation, by stimulating the E3 ubiquitin ligase activity of cIAPs proteins (212).

1.8.5 Smac mimetics

The identification of Smac and its potential capacity to antagonize the inhibitory effect of IAPs led to the development of several small peptides known as Smac mimetics (SMs) to selectively target different IAPs (**Figure 1.4**)(213–215). Several studies have reported the use of SMs to induce cells death in various tumors cells as well as increased susceptibility of resistant cells to chemotherapies (216–218). Various SMs compounds are currently undergoing evaluation in phase I/II clinical trials as anticancer therapy, including SM-LCL161, AT-406/Debio-1143, CUDC-427/GDC-0917, Birinapant and HGS1029/AEG40826 (219). SMs compounds mimic the N-terminal end of the endogenous Smac protein, which contains the AVPI tetrapeptide (220). SMs can be grouped into two categories: bivalent and monovalent SMs, depending on the presence of one or two AVPI tetrapeptide mimetic residues in the structure (220). Bivalent SMs have been shown to be more effective than monovalent form in the induction of cIAP-1/2 degradation in

MDA-MB-231 breast cancer cell line and was 1,000 times more potent in binding to XIAP and inducing apoptosis compared to monovalent SMs (221). The variation of these chemically distinct SMs in their potency to inhibit specific IAP proteins may lead to differences in their effectiveness to regulate the cell death.

1.9 Necroptosis- an alternative form of cell death

In early 2005, a novel form of cell death that morphologically similar to necrosis yet highly controlled was identified and termed necroptosis (222). Necroptosis is characterized by necrotic cell death morphology including cellular rounding and swelling, increased cytoplasmic granularity, membrane rupturing and releasing the cellular contents (223–225). The apoptosis and necroptosis share some degree of similarity. Both pathways share common cell death triggers and recruit identical adaptor proteins. However, the intracellular signalling pathways differ and sometimes might intersect during the signalling transduction process. For instance, both apoptosis and necroptosis can be activated by ligand-receptor interactions, which eventually lead to cell death by caspase-8 activation and initiation of the apoptosis process. However, in necroptosis, caspase-8 prevent RIPK-1 and RIPK-3 interactions, thus prevent the initiation of necroptosis (226, 227). Therefore, the initiation of necroptosis is heavily dependent on the inactivation of caspase-8, in addition to the essential role of both RIPK-1 and RIPK-3. More importantly, necroptosis generates more inflammatory responses by releasing intracellular contents (228). However, in apoptosis the intact plasma membrane and the rapid clearance of apoptotic buddies prevent the stimulation of inflammatory responses (229).

1.9.1 Necroptosis effector proteins and their structures

At the molecular level, RIPK-1 and RIPK-3 are central effector molecules regulate the necroptosis process (230). RIPK-1 comprises three major domains by which it regulates different

cellular pathways: a) an N-terminal serine/threonine kinase domain, which is required to initiate necroptosis as well as RIPK-1 dependant apoptosis induced by TNF- α . B) An intermediary domain (ID) which helps in MAPK and NF- κ B activation through recruitment and activation of TGF- β activated kinase 1 (TAK1) and IK β kinase (IKK). At the C-terminal part of this domain, there is a RIP-homotypic interaction motif (RHIM) that plays an essential role in the recruitment and binding to other RHIM-containing proteins, most importantly to RIPK-3 (231). RHIM also binds to Z-DNA-binding protein 1 (ZBP1, also known as DNA-dependent activator of IFN-regulatory factors, IRFs; DAI) which stimulate IFN production and NF- κ B activation (232, 233). Finally, C) a C-terminal death domain (DD) that is required to recruit other death domain-containing adaptor proteins such as TRADD and FADD, thus induces caspase-8-dependant apoptosis (234, 235).

RIPK-3 contains both the N-terminal kinase domain and the intermediate domain that has an RHIM motif, but it lacks the C-terminal death domain. Therefore, RIPK-3 cannot directly initiate FADD-caspase-8 dependant apoptosis observed after RIPK-1 activation (235, 236). The effector protein human mixed-lineage kinase domain-like pseudokinase (MLKL) is located downstream of RIPK-1/3 signaling and is considered the main executioner protein in the necroptosis process (237). It contains the N-terminal four-helix bundle (4HB) domain, which exerts the action of MLKL and C-terminal pseudokinase domain that keeps MLKL in an inactive state (237, 238). RIPK-3 phosphorylates MLKL, which undergo conformational changes that leads to the exposure of the 4HB. Subsequently, MLKL migrates to the cell membrane and cause necroptosis (235, 239).

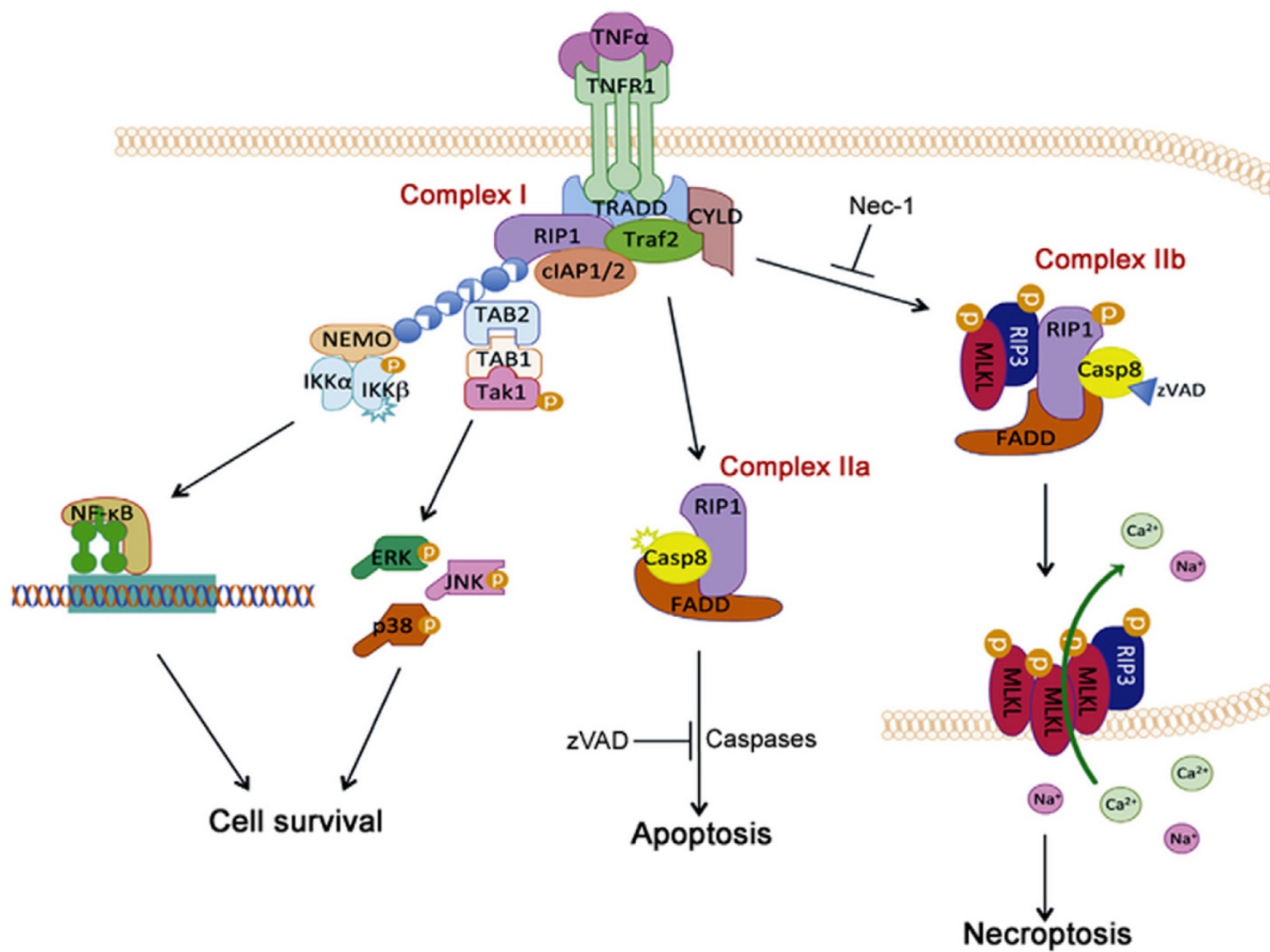
1.9.2 Necroptosis initiation and necrosome assembly

The activation of the death receptor TNFR-1 by TNF- α cytokine is the most well-studied pathway in necroptosis activation (**Figure 1.6**) (240). In dying cells, TNF- α binds TNFR-1 and

promote the assembly of complex-IIa and leads to apoptosis by activating caspase-8 (240). However, in the absence of caspase-8 activation, overexpression of RIPK-3 or cFLIP, necroptosis process is initiated and lead to the assembly of complex IIb (241, 242). In these cases, RIPK-1 recruits and phosphorylates RIPK-3. The phosphorylated RIPK-3 oligomerizes and forms the complex IIb also known as a necrosome. Upon assembly of necrosome, RIPK-3 recruits and phosphorylates MLKL (237). Phosphorylated MLKL in turns, oligomerizes and translocate from the cytoplasm to the cell membrane. Activated MLKL binds to phosphatidylinositol phosphate (PIPs) phospholipids, the mitochondrial specific lipid cardiolipin or ion channels causing necrotic membrane disruption, pores formation, osmotic pressure and ion influx (239, 243, 244). The disrupted membrane results in the release of intracellular contents, proteolytic enzyme, cell swelling, inflammation and subsequent cell death (132, 243, 245).

1.9.3 Necroptosis cell death in diseases

Necroptosis has been implicated in several diseases including; Huntington's disease (246), HIV (247), multiple sclerosis (248), neurodegenerative diseases such as Alzheimer's disease (249), inflammatory bowel disease (250) rheumatoid arthritis (251) and Parkinson's disease (252). However, necroptosis is not always a hurtful process; it could be beneficial in some pathological settings; for example, some cancer types, viral or bacterial infection, can inhibit or evade apoptosis in infected cells. Thus, necroptosis induced cell death prevents the spread of infected or cancers cells, and protects against disease progression or even promotes tissue repair (253–255).



Pan et al., 2019. (192)

Figure 1.6: Initiation of necroptosis pathway.

The activation of death receptors of the TNF superfamily induces a conformational change downstream of the receptor and recruits several adaptor proteins. In the absence of cIAP1/2 and caspase-8, the deubiquitinated RIPK-1 phosphorylates RIPK-3. The phosphorylated RIPK-3 recruits and phosphorylates MLKL which in turns, oligomerizes and translocates from the cytoplasm to the cell membrane. Activated MLKL binds to ion channels causing necrotic membrane disruption, pores formation (192).

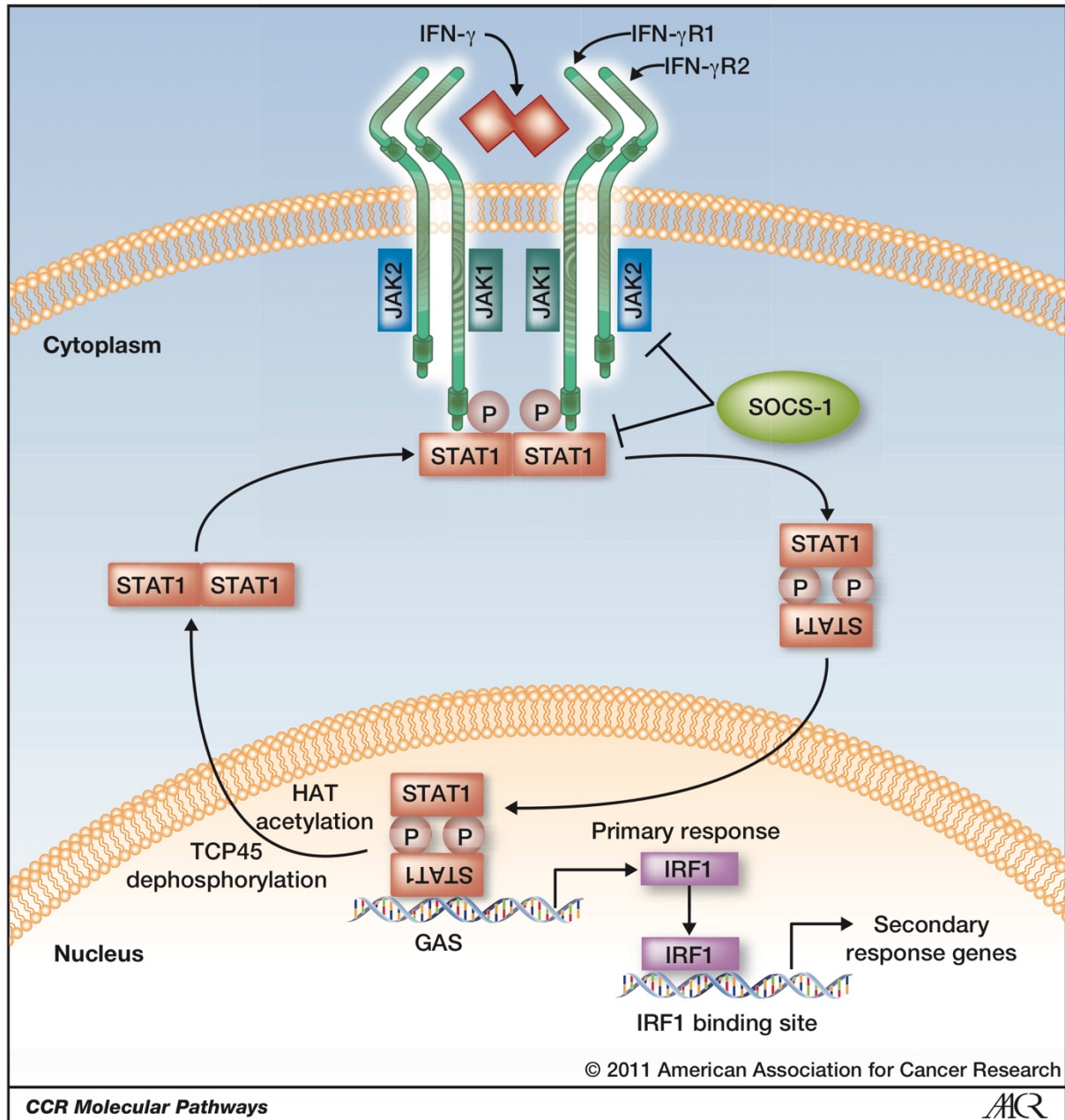
1.10 Interferon- γ signaling and its role in disease

Interferons (IFNs) were originally described as antiviral agents that exhibited ability to interfere with influenza virus replication (256). IFN- γ is the only member that belongs to type-II IFNs. NK cells and Th1 cells are the major producers of IFN- γ . NK cells cannot sustain a prolonged IFN- γ production, thus NK cells are important in early host defense against infection, whereas Th1 cells are the major source of IFN- γ in the adaptive immune response to sustain host defense against pathogens (257). Other cells including, B cells, dendritic cells and macrophages also have been shown to secrete IFN- γ (257). IFN- γ binds to its own specific receptor, IFNGR, that consists of two ligand-binding high affinity IFNGR1 chains and two signal-transducing IFNGR2 chains (258). Upon binding of IFN- γ to IFNGR, a conformational change allows the recruitment and phosphorylation of two nonreceptor tyrosine kinases Janus-activated kinases (JAKs): JAK1 and JAK2 to the IFNGR1 and IFNGR2 chains, respectively. The activation of JAK1/2 induce a conformational change that creates binding sites for STAT1 (259). Activated JAK1/2 facilitates binding of STAT1 to the IFN- γ -IFNGR complex as well as phosphorylate STAT1 to form a homodimer (260). The phosphorylated STAT1 dimer is then translocated to the nucleus where it binds with high affinity to DNA sequences known as the gamma-activated sequence (GAS) sites and induces transcription of interferon stimulated genes (ISGs) such as the interferon regulator factor 1 (IRF-1) and IRF-9, which in turn activate number of secondary IFN- γ regulated genes as shown in (**Figure 1.7**) (257, 258).

IFN- γ induce hundreds of genes that regulate several biological processes such as inflammation, host defense, the cell growth, apoptosis, and immune responses (258, 261). For example, IFN- γ promotes antiviral and antibacterial defense mechanisms by activating macrophages, induction of phagocytosis and production of cytokines and pro-inflammatory

mediators such as oxygen and nitrogen radicals to limit the replication and the spread of pathogens (258). Several studies reported the involvement of IFN- γ in host resistance to pathogens. For example, mice lacking IFN- γ or IFNGR1 expression demonstrated increase susceptibility to *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, mycobacteria species and vaccinia virus (262–265). In humans, genetic mutation in IFN- γ pathways such as mutation in IFNGR1, IFNGR2 or deficient IFN- γ production have also been implicated in increased suitability to intracellular bacterial infection (266–268).

Cell growth, proliferation and apoptosis are also tightly affected by IFN- γ . Several studies demonstrate that IFN- γ provides a survival signal by arresting macrophages cell cycle, while other suggested a pro-apoptotic role of IFN- γ in macrophages to prevent colonization by intracellular pathogens (257). For example, IFN- γ downregulates *c-myc* which is required for cell cycle progression from G1 to S phase, while upregulates Mad1 which antagonizes *c-myc*, thereby inhibiting proliferation (257). IFN- γ induces cell death by upregulating various proapoptotic molecules. For example, IFN- γ induces the expression of IRF-1, a tumor-suppressor gene that induces the transcription of several IFN- γ regulated genes (257). IRF-1 induces the expression of caspase-1 which is involved in macrophages and cancer cells apoptosis (269). Recently, it has been shown that IFN- γ upregulates the mRNA and protein levels of MLKL, an effector molecule in necroptosis pathway (270). Inhibition of IRF-1 or STAT1 significantly reduced IFN- γ -mediated expression of MLKL in various cancer cell lines and experimental autoimmune arthritis mice (251, 271). Additionally, it has been shown that IFN- γ synergies with SMs to induce necroptosis in different cancer cell lines by a mechanism that requires IRF-1 activation (272). This synergism also induces apoptosis in lung cancer cells in a TNF- α -independent manner (273). INF- γ may also



Zaidi and Merlino, 2011. (261)

Figure 1.7: IFN- γ signaling.

IFN- γ binds to IFNGR and induces a conformational change that mediates the recruitment of JAK1 and JAK2. Activated JAK1/2 recruits and phosphorylates STAT1. The phosphorylated STAT1 is then translocated to the nucleus and binds to the gamma-activated sequence (GAS) sites and induces transcription of interferon stimulated genes (ISGs) such as IRF-1. IRF-1 activates number of secondary IFN- γ regulated genes (261).

induce PKR expression, an antiviral enzyme that inhibit viral protein synthesis, to induce TNF- α and Fas-induced apoptosis (274, 275). When apoptosis pathway is inhibited, PKR can also induce cell death by mediating RIPK-1/RIPK-3 mediated necroptosis (276).

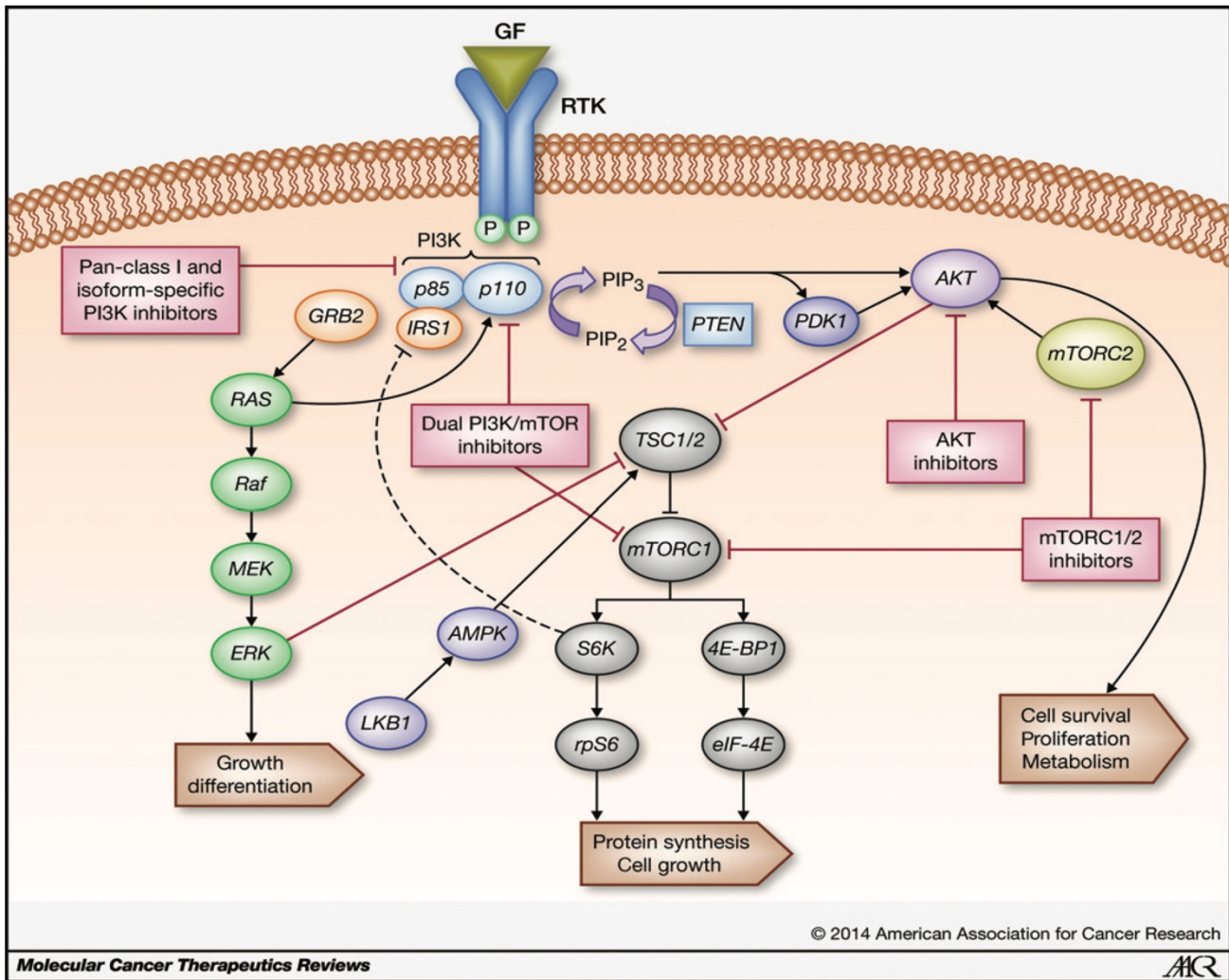
IFN- γ is also well known for its immunomodulatory activity and mediates the recruitment and stimulation of immune cells. IFN- γ is involved in upregulation of MHC which assist in antigen presentation, promote activation and differentiation of several innate and adaptive immune cells such as NK cells, macrophages, T and B cells (277). IFN- γ plays a critical role in M1 macrophage polarization and the production of pro-inflammatory cytokines. However, overexpression of IFN- γ has been implicated in the development of several chronic inflammatory and autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease and some cancers (278).

1.11 mTORC pathway

The mammalian target of rapamycin (mTOR), is a large conserved serine/threonine protein kinase belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family (279). mTOR pathway plays a central regulatory role in many cellular processes such as cell survival, differentiation, proliferation, immune responses and autophagy (279, 280). mTOR senses the surrounding environmental signals such as growth factors, stress, energy, oxygen and amino acids. mTOR responds to these stimuli via various receptors, including growth factor receptors, pattern-recognition receptors and cytokines receptors and drives intercellular metabolic responses accordingly (279, 280). mTOR interacts with several proteins to form two structurally and functionally distinct complexes, mTOR complex1 (mTORC1) and mTORC2 (280). The mTORC1 complex composed of six proteins, whereas the mTORC2 complex is composed of seven proteins. Some of these proteins are shared between the two complex, however, the presence of the

regulatory-associated protein of mTOR (Raptor) in mTORC1 and the rapamycin-insensitive companion of mTOR (Rictor) in mTORC2 are the key components in these complexes (281, 282).

Both mTOR complexes differ in their upstream signals and downstream target proteins. The PI3K/Akt pathway is the most well-known upstream pathway involved in mTORC activation. Activated receptors trigger activation of PI3K which facilitates the conversion of phosphatidylinositol (3,4)-biphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (**Figure 1.8**). PIP3 recruits Akt and mTORC2 at the plasma membrane, allowing phosphoinositide-dependent kinase 1 (PDK-1) to phosphorylate and partially activate Akt (283). Akt activation has also been reported in PI3K-independent manner through activation of the protein kinase A (PKA), but the exact mechanism is not completely clear (284). mTORC2 phosphorylates Akt and promote full Akt activation (285). The activated Akt phosphorylates and inactivates the heterodimer tuberous sclerosis complex (TSC1/2), a key upstream negative regulator of mTORC1 activation. Inhibition of TSC1/2 complex subsequently activate mTORC1 via inactivating Ras homolog enriched in brain (Rheb) (279, 286). Activated mTORC1 induces downstream signals as well as mediates a feedback inhibition of both mTORC2 and Akt activity (287, 288).



Dienstmann et al., 2014. (289)

Figure 1.8: PI3K/Akt/TSC/mTOR pathway.

Ligand-receptor interactions trigger activation of PI3K subunits p85 and p110. PI3K subunits mediate the conversion of phosphatidylinositol (3,4)-biphosphate (PIP₂) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). PIP₃ recruits PDK-1, Akt and mTORC2 at the plasma membrane and subsequently activates Akt. The activated Akt phosphorylates and inactivates the heterodimer tuberous sclerosis complex (TSC1/2). Inhibition of TSC1/2 complex leads to mTORC1 activation. Activated mTORC1 induces downstream signals and phosphorylates ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) to mediate protein synthesis. Sensing intracellular nutrients signals can also affect mTORC activation such as through the interaction with RAS and LKB1/AMPK pathways. Liver Kinase B1 (LKB1), AMP-activated protein kinase (AMPK), growth factor (GF), growth factor receptor-bound protein 2 (GRB2), insulin receptor substrate 1 (IRS1), phosphoinositide-dependent kinase 1 (PDK1) (289).

Activated mTORC1, targets different downstream proteins and transcription factors involved in cell cycle progression, cell growth, protein and lipid synthesis, mitochondrial metabolism/ biogenesis, inflammation responses and autophagy (**Figure 1.6**) (279, 280, 290). For instance, mTORC1 phosphorylates ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) which are involved in protein synthesis (291). mTORC1 also regulates the sterol regulatory element-binding protein 1/2 (SREBP1/2), involved in lipid and cholesterol synthesis. The mTORC2 complex controls several protein kinases such as serum and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase C- α (PKC- α) that contributes to cell survival, metabolism, cytoskeleton organization, immune cells differentiation and polarization (279, 280, 288, 292, 293).

1.11.1 PI3K/Akt/TSC/mTOR signaling in macrophage polarization

The PI3K/Akt/mTOR signalling network plays an important role in macrophages activation and polarization status. Responding to different metabolic and inflammatory signals, PI3K/Akt/mTORC pathway mediates downstream responses and genes expressions at both transcriptional and translational levels (294, 295). The PI3K/Akt/mTORC signaling has been shown to regulate macrophages polarization and cytokine secretion. Activation or overexpression of PI3K, Akt or their adaptor kinases promotes M2-anti-inflammatory phenotype activation whereas, inhibition of PI3K/Akt signalling mediates M1-proinflammatory activation (295–297). For example, inhibition of Akt downregulates genes associated with M2 phenotype and upregulates iNOS, IL-12 β as well as increased LPS-induced inflammatory responses (298, 299). Inhibition of PI3K mediates NF- κ B activation and the production of iNOS expression associated with M1 phenotype activation (300, 301). Additionally, several anti-inflammatory cytokines such as TGF-

β , IL-4 and IL10 have been shown to utilize PI3K/Akt signalling pathway to promote M2 polarization (302–304).

PI3K is composed of two subunits; p110 subunit and a regulatory subunit p85 α and each of these subunits has several isoforms (305). Several studies demonstrated that various isoforms of PI3K or their regulatory subunits mediate M1 macrophages polarization instead of M2 polarization, thereby providing a contradictory role of PI3K/Akt pathway in promoting anti-inflammatory responses. For instance, Fordham *et al.*, demonstrated that overexpression of miR-24, a negative regulator of M1, in human macrophages reduced the expression of p110 δ isoform of PI3K, which is shown to be responsible in LPS-induced TNF- α , IL-12 and IL-6 secretion (306). Additionally, macrophages lacking the regulatory subunit p85 α of PI3K demonstrated non-functional iNOS activity, and impaired nitric oxide (NO) production in response to LPS and IFN- γ stimulation, and further exhibited increased intracellular bacterial infections (307).

The Akt isoforms have also been shown to regulate macrophages polarization (295, 308). Akt2 deficient macrophages expressed high levels of M2 macrophages markers such as Arg-1, Ym1 and Fizz1 and produced high levels of IL-10 in response to LPS stimulation (295, 308, 309). In contrast, Akt 1 has been shown to restrict inflammation and promotes M2 polarization. Deletion of Akt1 has been reported to upregulate iNOS and IL-12 production, and improved bacterial clearance by macrophages (298, 308).

Downstream of PI3K/Akt pathway, TSC and mTOR signaling have also been reported to modulates macrophages activation with contrasting results. One study demonstrated that, deletion of TSC1 in macrophages, promotes LPS-stimulated proinflammatory cytokines secretions such as IL-6 and TNF- α -associated with M1 phenotype and impaired M2 polarization stimulated by IL-4 or IL-10 demonstrated by downregulation of Arg-1, YM1, FIZZ1 and IL-10 expression (287). In

contrast, another study demonstrated that TSC1 deficient macrophages enhanced both M1 and M2 polarization in response to LPS and IL-4 stimulation respectively, compared to wild type macrophages (310). TSC2 has also shown to modulate macrophages into more inflammatory status. TSC2 knockdown human macrophages (THP-1) reversed the proinflammatory M1 cytokine productions upon LPS stimulation to anti-inflammatory status with enhanced IL-10 (311).

Similarly, the effector mTORCs molecules in regulating macrophages polarization is also controversial. Several studies have shown that mTORCs activation drives M2 macrophages phenotype while others demonstrated contradictory results. For example, inhibition of mTORC1 promotes enhanced M1 polarization associated with induction in proinflammatory cytokines in response to LPS stimulation in both human and mouse macrophages during infection and cancer (311–313). Additionally, mTORC1 activation during *Leishmania donovani* infection induced M2 polarization, characterized by increased expression of arginase-1, IL-10, TGF- β and CD163 and rapamycin treatment reduced *leishmania* survival (314). Moreover, mTORC2 deficient macrophages isolated from mice model demonstrated M1 phenotype associated with a reduction in IL-10 production (315). In contrast, mTORC1 has also been shown to drive M1 macrophages polarization. Mice bone marrow derived macrophages infected with *Ixodes ovatus ehrlichia*, an intercellular bacterium responsible for developing human monocytic ehrlichiosis (HME), demonstrated high expression of MHC II and enhanced M1 polarization via mTORC1 activation (316).

1.12 RATIONALE

Macrophages are the most heterogeneous and diverse population of innate myeloid cells of the hematopoietic system (4). The microenvironmental signals affect their polarization status into pro- and anti-inflammatory macrophages and allow them to exert a wide range of innate and adaptive immunological responses (7, 41, 44). The inflammatory and anti-inflammatory macrophages have been implicated in many diseases (5, 51, 65). Recent studies suggest that modulating macrophage polarization and activation status may represent a potential target to treat these diseases (7, 120, 121, 124).

During myeloid differentiation, macrophages develop resistance to spontaneous and induced apoptosis that is crucial to maintain survival of macrophages during the immune response against extracellular and intracellular viral and bacterial pathogens (9, 317). The mechanism underlying enhanced resistance of macrophages may relate to the differential expression of pro- and anti-apoptotic genes/proteins including Bcl-2, Bcl-xL and inhibitors of apoptosis (IAPs) family members (317–321). The role of IAP family members has been studied by employing second mitochondria-derived activator of caspases (SMAC) mimetics (SM) (213, 214). We have previously described the role of IAPs in the resistance of macrophages to HIV-Vpr-induced apoptosis (319, 320). We and others have also shown that SMs do not induce cell death on differentiated normal macrophages (319, 322). Whether SMs affect survival of normal human proinflammatory M1 macrophages and anti-inflammatory macrophages M2a, M2b and M2c subsets remains unknown. Herein, I investigated the impact of smac mimetics on the survival of human macrophage subsets and the mechanism involved. My studies provide another approach to modulate survival and selective induction of cell death in pro- and anti-inflammatory macrophages by SM that could be exploited for potential therapeutic benefits in regard to macrophage associated inflammatory or anti-inflammatory diseases.

1.13 HYPOTHESIS

The IAPs are critically linked to the survival of human macrophages. Thus, smac mimetics (SMs) exert differential effects on the killing of proinflammatory and anti-inflammatory human macrophage subsets that eventually may have potential therapeutic benefits in regard to macrophage associated inflammatory or anti-inflammatory disorders.

1.14 OBJECTIVES

- 1 Generation and characterization of macrophage subsets.
- 2 Determining the effect of SMs on the survival of M0, M1, M2a, M2b and M2c macrophage subsets.
 - a. The involvement of apoptosis pathway.
 - b. The involvement of necroptosis pathway.
- 3 Investigate the macrophage plasticity in terms of SM-induced cell death and the associated apoptotic signaling kinases.
- 4 Determine the molecular mechanism(s) by which SMs induce cell death in human macrophage subsets
 - a. The role of TNF- α in regulating SM-induced cell death.
 - b. The involvement of IFN- γ -activated signaling pathways such as JAK/STAT, IRF-1 and PKR in SM-induced cell death in M1 macrophages.
 - c. The involvement of signaling pathways including MAPKs and mTORC-PI3K/Akt-S6 kinase pathways in SM-induced cell death.
 - d. The involvement of IAPs associated signaling kinases, transcription factors and apoptosis associated genes/gene products in SM-induced cell death

CHAPTER 2: Materials and Methods

2.1 Isolation of human primary monocytes and generation of monocyte-derived macrophages and the different macrophage subsets

Blood was collected from healthy donors that did not show any sign of acute or chronic infection at the time of signing the consent form and as per the protocol approved by the Ethics Review Committee of The Ottawa Hospital. The isolation of human primary monocytes and the generation of Monocyte-derived macrophages (MDMs;M0) was previously described (43, 319, 320, 323, 324). Briefly, blood was collected in 60 ml heparinized syringes and subjected to density gradient centrifugation using Lymphoprep™ solution (Stem Cell technologies, Vancouver, BC, CA) and centrifuged at 1600 rpm (515 xg) for 45 min. The buffy coat ring was collected to isolated the peripheral blood mononuclear cells (PBMC) and suspended in serum-free media (Iscome's Modified DMEM 1X; Wisent Inc., QC, CA) at a concentration of 2.5×10^6 cells/ml/well into 12 well polystyrene plates (Thermo Scientific, Rochester, NY, USA). Cells were cultured for 3 hrs to facilitate the adherence of monocytes to the plate surface while non-adherent cells were washed off using Iscome's Modified DMEM 1X media.

To generate monocyte derived macrophages (MDMs;M0), adherent monocyte cells were cultured for 7 days in complete media which consists of iscome's modified DMEM 1X supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) 1% penicillin and streptomycin (Pen-Strep; R&D Systems, Minneapolis, MN, USA) along with 10 ng/ml of human recombinant M-CSF (R&D Systems). On the 4th day, the adherent cells were washed, and fresh complete media was added. The purity of macrophages was assessed on day 8 by measuring CD14⁺ expression by flow cytometry. M0 macrophages thus generated contained less than 2% T cells, B cells and NK cells.

To generate the different macrophage subsets, M0 macrophages were washed and polarized for 48 hrs by adding fresh complete media containing the following cytokines as previously described (43). Briefly, M1 macrophages subset was generated by stimulating the M0 macrophages with IFN- γ (20 ng/mL; Thermo Scientific, Rochester, NY, USA) for 48 hrs, M2a macrophages by IL-4 stimulation (20 ng/mL), (R&D Systems) for 48 hrs. LPS (1 μ g/mL; Sigma-Aldrich) and IL-1 β (10 ng/mL; R&D Systems) were used to generate M2b macrophages, whereas IL-10 (10 ng/mL; R&D Systems) was used to generate M2c macrophage subset for 48 hrs. For repolarization experiments, M1 and M2c macrophages were washed twice with serum free media and rested for 48 hrs. M1 macrophages were repolarized with IL-10 (10 ng/mL) and M2c macrophages were repolarized with IFN- γ (20 ng/mL) for another 48 hrs to generate M1 > M2c and M2c > M1 repolarized macrophages, respectively

2.2 Generation of mouse bone marrow-derived macrophages (BMDM) and SNB75 cell line

BMDM isolation and culture were kindly done by Dr. Edana Cassol's Lab member and were performed based on a previously described protocol (325, 326). Briefly, total bone marrow cells were collected from the tibias and femurs of 6-13 week- old C57BL/6 mice, resuspended in a solution mixture contains of 90% FBS and 10% DMSO and cryopreserved until use. Cells were then suspended in a complete media consists of DMEM 1X media, 10% fetal bovine serum 10% FBS and 1% Pen-Strep, in addition to 15% L929 fibroblast cell-conditioned medium that contains M-CSF and cultured for ten days in sterile petri dishes at a concentration of 5×10^6 cells/dish. On the third day, 10 mL of complete fresh media were added, and cells were left to adhere to the surface of the petri dish. On day 10, cells were detached using cold PBS, counted and centrifuged at 1600 rpm (515 xg) for 10 min. BMDMs were then resuspended in complete media and seeded to 12 well polystyrene plate at a concentration of 0.5×10^6 cells/well and incubated overnight. Next

BMDM were washed with PBS and left untreated, or stimulated with mouse recombinant IFN- γ (20 ng/mL; Stem Cell technologies) or with mouse recombinant IL-10 (10 ng/mL; Stem Cell technologies) in for 48 hrs. All animal procedures involved in this work were approved by the Carleton University Animal Care Committee and were conducted in accordance with the guidelines provided by the Canadian Council for Animal Care.

The cell line SNB 75 was kindly provided by Dr. Bob Korneluk's Lab. Cells were cultured in DMEM 1X supplemented with 10% FBS and 1% Pen-Strep and cultured in petri-dishes until reach 90% confluency. Cells were then detached and resuspended in media and seeded into 12 wells culture plates and incubated overnight. Next day, cells were used to conduct the experiment.

2.3 Cell surface marker analysis by flow cytometry

Cell surface staining was performed based on a previously described protocol (43). Briefly, the polarized and re-polarized macrophages were washed twice with PBS and the cells were dislodged using 500 μ L of Accutase™ (Stem Cell technologies) for 45 min at 37 °C. Cells were then collected and centrifuged at 2200 rpm (974 xg) for 5 min, followed by two times wash with PBS. The cells were incubated with human FcR blocking buffer (Milteny Biotec Inc. Auburn, CA, USA) for 20 mins at 4 °C followed by staining using conjugated antibodies for CD14 (Cat. #347497), CD80 (Cat. #557227), CD86 (Cat. #560958), CD163 (Cat. #333618), CD200R (Cat. # 329305) and HLA-DR (Cat. # 361708), (BD Biosciences, San Jose, CA, USA) for 1 hr at 4 °C in the dark. The cells were washed with PBS and centrifuged to remove any excess antibodies. 200 μ L of PBS were added to each tube and the fluorescence was measured using PE (CD14, CD200R, and CD80), FITC (CD86 and CD163) and PE/Cy7 (HLA-DR) channels in Flow cytometer-BD LSRFortessa™ cell analyzer (BD Life Sciences, Mississauga, Ontario, CA). All samples tested where compared to M0 samples. The histograms and MFI were analyzed and plotted using FlowJo

Software version 10.0.7 (BD Biosciences, San Jose, CA, USA) and GraphPad Prism 5.0 software (San Diego, CA, USA).

2.4 Treatment of MDMs and BMDM subsets with chemical inhibitors

All chemical inhibitors used in this study were reconstituted and stored based on the recommendation from the manufacturers (manufactural protocol). **Table 2.1** summarizes all chemical inhibitors used for this study. M0 were left unpolarized or polarized into different macrophage subsets for 48 hrs. On the day of the experiment, human macrophage subsets M1, M2a M2b and M2c in addition to unpolarized M0 were washed with serum free media. Each chemical inhibitor was diluted to different concentrations in a complete media master-mix. Next, cells were left untreated (complete media) or treated with different concentrations of the specific inhibitor and incubated at 37°C with 5% CO₂ for 1 hr or otherwise stated. The macrophage subsets were then treated with different concentrations of SM-LCL161 and incubated for another 48 hrs. Lastly, cells were gently scraped and collected for cell death assessment by intracellular PI staining and flow cytometry.

In case of multiple treatment with different inhibitors, cells were treated with a master-mix media containing a mixture of inhibitors, added to each well and incubated at 37°C with 5% CO₂. One hour later, the SM-LCL161 was added directly to each well, mixed very well and incubated again for 48 hrs. Finally, cells were collected by gently scraping each well and cell death was assessed by intracellular PI staining and flow cytometry analysis. For BMDM treatment, M1 and M2c mice macrophage subsets were washed with 1 mL of PBS two times. Cells were then left untreated or treated with 0.2, 0.5 and 1 µM of SM-LCL161 for 48 hrs, followed by cell collection and assessment of cell death by intracellular PI staining and flow cytometry

Table 2.1: Summary of the chemical inhibitors used in this study.

Cat. #	Chemical inhibitor	Description	Source
A-1147	SM-LCL161; SMs	Smac mimetics	Active Biochem Ltd (Kowloon, Hong Kong)
A1902	zVAD.fmk	Caspase inhibitor	ApexBio (Houston, Texas, USA)
A4213	Necrostatin-1; Nec-1	RIPK-1 inhibitor	
A3012	Ruxolitinib; RUX	JAK inhibitor	
A8192	Staurosporine; STS	Protein kinase inhibitor	
P6624	PS-1145	NF- κ B-IKK inhibitor	Sigma-Aldrich (St. Louis, Missouri, USA)
R0395	Rapamycin	mTORC inhibitor	
PZ0143	PF-4708671	S6kinase inhibitor	
513000	PD-98059	MAPK-ERK inhibitor	Calbiochem (La Jolla, California, USA)
420119	SP-600125	MAPK-JNK inhibitor	
559389	SB-203580	MAPK-P38 inhibitor	
527450	PKR inhibitor; C16	PKR inhibitor	
440202	LY294002	PI3K inhibitor	
4247	Torin	mTORC inhibitor	Tocris Bioscience (Oakville, Ontario, CA)
5025	Necrosulfonamide; NSA	MLKL inhibitor	R&D Systems (Minneapolis, Minnesota, USA)
11593	MK-2206	AKT inhibitor	Cayman Chemicals (Ann Arbor, Michigan, USA).

2.5 Pre- and Post-M1 polarization treatment with chemical inhibitors

For the pre- and post-polarization experiments, each chemical inhibitor was added either before (Pre-polarization) or after M1 polarization (Post-polarization) step. For the pre-M1 polarization treatment, the unpolarized M0 were washed with serum-free media and then left untreated (complete media only) or treated with different concentrations of the desired chemical inhibitor and incubated at 37°C with 5% CO₂ for 1 hr. Next, M0 were polarized by adding IFN- γ (20 ng/mL) directly to each well and incubated for 48 hrs. Next, SM-LCL161 (1 μ M) was added to each well and incubated for another 48 hrs. Lastly, cells were gently scraped and collected for assessment of cell death by intracellular PI staining and flow cytometry. The post-M1 Polarization treatment was performed after generating M1 macrophage subset. M1 macrophage subset was polarized in the presence of IFN- γ for 48 hrs then washed with serum free media and left untreated or treated with different concentrations of the desired chemical inhibitor for 1 hr. The M1 macrophage subsets were then treated with SM-LCL161 for 48 hrs. Next, cells were collected for cell death evaluation by intracellular PI staining and flow cytometry analysis.

2.6 Evaluation of cell death by intracellular Propidium Iodide (PI) staining and flow cytometry

Cell death was determined by intracellular Propidium Iodide (PI) staining as described previously (319). Briefly, treated cells were gently scraped using sterile non-toxic polystyrene cell scraper 16cm (Nümbrecht, Germany) and transferred along with the supernatant containing dead cells into 15 mL tubes. Cells were centrifuged at 2200 rpm (974 xg) for 5 min, supernatants were removed, and cell pellets were washed with 500 μ L PBS, centrifuged again followed by removal of supernatant. Methanol (350 μ L) was added to each pellet, vortexed for few seconds and incubated for 15 min at 4°C in the dark. Cells were centrifuged again and washed before adding a

mixture of 25 μ L PI solution (Sigma-Aldrich) and 25 μ L RNase (Roche Diagnostics, Laval, Québec, CA) to each pellet followed by incubation for 1 hr at 4°C in the dark. Cells were washed one last time to remove any excessive PI staining and 300 μ L of PBS was added. PI stained cells were analyzed using PI channel in Flow cytometer-BD LSRFortess™ cell analyzer (BD Life Sciences, Mississauga, Ontario, CA). All samples tested were compared to untreated samples. The histograms were analyzed and plotted using FlowJo Software version 10.0.7 (BD Biosciences, San Jose, CA, USA) and GraphPad Prism 5.0 software (San Diego, CA, USA).

2.7 Evaluation of cell death by YOYO-1 Iodide staining and IncuCyte™ Zoom

Kinetic live cell imaging was carried out with exogenous YOYO-1 iodide (Cat. #Y3601, Thermo Fisher Scientific) and monitored using IncuCyte™ Zoom live cell imaging (ESSEN Bioscience, Michigan, USA). The instrument allows visualization of the fluorescent signals and quantifies the intensity of signal/cell while the cells are still in culture at 37°C. Cells were washed with serum free media and YOYO-1 iodide was added at 1 μ L:1 mL ratio. Plates were then incubated in IncuCyte™ Zoom and images were automatically taken every 2 hrs for 48 hrs. Images were collected, processed and analyzed by IncuCyte™ Zoom software and data were presented using GraphPad Prism 5.0 software.

2.8 Determination the involvement of apoptosis pathway in SM-treated human macrophage subsets

2.8.1 Determination of caspase-3/7 activity by caspase-3/7 reagent and IncuCyte™ Zoom:

IncuCyte™ caspase-3/7 green apoptosis assay reagent (Cat. #4440) were purchased from Essen Bioscience (Michigan, USA). The reagent contains activated caspase-3/7 recognition motif (DEVD) bonded to a DNA binding dye (NucView™ 488). In the apoptotic cells, this non-

fluorescent substrate freely crosses the cell membrane, where it is cleaved by the activated caspase 3/7 at the DEVD recognition motif, and thus release the green DNA binding dye that fluorescently labels the nuclear DNA of cells undergo apoptosis. In this study, M0 were washed with serum free media and a master-mix of complete media and 5 μ M/mL of caspase-3/7 reagent was added to each well. The cells were then left untreated or treated with different concentrations of SM-LCL161 and incubated in IncuCyte™ Zoom device for 48 hrs. Images were automatically taken every 2 hrs, collected, processed and analyzed by IncuCyte™ Zoom software and data were presented using GraphPad Prism 5.0 software.

2.8.2 Determination of caspase-8 activity by caspase-8 reagent and flow cytometry:

Caspase-8 was determined by using caspase-8 (active) FITC staining kit (Cat. #ab65614, Abcam Inc., Toronto, ON, CA) as per the manufacturer's protocol. The FITC-IETD-FMK substrates in the caspase 8 reagent irreversibly binds to activated caspase 8 within the cell thus allow the visualization of cell with activated caspase-8 by flow cytometry. Macrophage subsets (M0, M1 and M2c) were washed with serum-free media and then left untreated or treated with SM-LCL16. After 10 hrs of incubation at 37°C, 1 μ L of caspase-8 reagent was added to each well and incubated for 1 hr at 37°C with 5% CO₂. Cells were then collected by gentle scraping, centrifuged at 300 rpm (18 xg) for 5 min and washed using wash buffer provided in the kit before analyzing by flow cytometry using FL-1 channel. Caspase-8 activity was assessed by flow cytometer-BD LSRFortessa™ cell analyzer (BD Life Sciences, Mississauga, Ontario, CA). The histograms were analyzed using FlowJo software version 10.0.7 and data were presented using GraphPad Prism 5.0 software.

2.9 Western immunoblot analysis

2.9.1 Lysate preparation

To make total protein lysates from cultured cells, macrophage subsets were scraped using sterile non-toxic polystyrene cell scraper 16 cm. Cells along with the supernatant were collected into 1.5 mL tubes and kept on ice. Fresh cold lysis buffer (2xIP lysis buffer with protease inhibitor tablet (Roche Diagnostics, Laval, Québec, CA) was prepared and 50-70 μ L was added to each tube. Samples were kept on ice with constant agitation every 10 min for 45 min. Sample extracts were then centrifuged for 30 min at 1480 rpm (21.1 xg) at 4°C, followed by collection the supernatant and store at -80°C until used.

2.9.2 Total protein quantification

To determine samples protein concentration, the colorimetric Bradford assay was performed. Briefly, each sample was diluted 1:200 in PBS (2 μ L sample in 198 μ L PBS). In addition, albumin standard was serially diluted for the standard curve preparation based on the manufacture protocol (Thermo Scientific, Rochester, NY, USA). Next, 50 μ L of Bio-Rad protein assay dye reagent concentrate (Cat. # 5000006, Bio-Rad Laboratories, Mississauga, Ontario, CA) was added to each sample in addition to the diluted standards. All samples were then mixed, and 200 μ L of each sample was transferred to flat-bottom 96 well microplate. The microplate was read at 595 nm using iMark Microplate reader (Bio-Rad Laboratories) analyzed, and the final concentrations were calculated by microplate manager 6 software.

2.9.3 SDS-PAGE and Western immunoblotting

Western immunoblotting was carried out using a previously described (319). Briefly, 40-50 μ g/ μ L of each sample was mixed with 2x Laemmli sample buffer (Cat. # 161-0737; Bio-Rad) in 1:1 ratio then heated up at 95°C for 5 mins. Samples were then centrifuged for few second and

loaded onto 10% SDS-PAGE gel along with loading control protein ladder. **Table 2.2** shows the chemical compounds and concentrations used to make 10% SDS-PAGE. The gel was run in a running buffer at 120V for 120 mins. Next, the gel was transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) and run in a transfer buffer at 110V for 1 hr. All membranes were then blocked with 5% skim milk for 30 mins followed by 3 times washing with 1x TBST each wash for 5 mins. Membranes were then probed with the desired primary antibody and incubated at 4°C overnight. Next day, membranes were washed again 3 times with 1xTBST, followed by adding the goat anti-rabbit or anti-mouse secondary antibodies IgG conjugated to horseradish peroxidase (Cat. #172-1019, Cat. #170-6516; Bio-Rad Laboratories) and incubated at room temperature for 1 hr with consistent agitation. Protein bands were developed using the Amersham ECL Western blotting detection reagent (Cat. #RPN2235; Cedarlane, Burlington, Ontario, CA), visualized using Syngene Chemi Genius 2 Bio Imaging System (Syngene; Frederick, Maryland, USA) and processed in GeneSnap software, version 6.08 (Syngene). For total loading proteins evaluation, membranes were striped using stripping buffer (Cat. #21059; Fisher) and probed with either anti- β -Actin or anti-GAPDH antibodies (Cell Signaling Technology). **Table 2.3** summarizes all the primary antibodies and the working concentrations used in this study.

Table 2.2: SDS-PAGE gel solution components and their concentrations

10% SDS-PAGE Gel		
Solution Components	Lower Gel Volume (Volume adjusted for 2 gels)	Upper Gel Volume (Volume adjusted for 2 gels)
H₂O	9.9 mL	6.8 mL
30 % acrylamide mix	8.3 mL	1.7 mL
--Lower gel-- 1.5 M Tris (pH 8.8)	6.3 mL	-----
-- Upper gel --1.5 M Tris (pH 8.8)	-----	1.25 mL
10 % SDS	250 µL	100 µL
10 % ammonium persulfate (APS)	250 µL	100 µL
TEMED	20 µL	20 µL

Table 2.3: Summary of Western immunoblotting primary antibodies used in this study.

Cat. #	Antibody	Size (kDa)	Source
3331	Phospho-Jak1	130	Cell Signaling Technology (Danvers, Massachusetts, USA)
4882	NF- κ B p100/p52	52, 120	
9532	PARP	89,116	
2697	Phospho-IKK α / β	87	
4926/3493	RIPK-1	78	
65746	Phospho-RIPK-1	78	
3076	Phospho-PKR	74	
9234	Phospho-S6 Kinase	70	
3103	cIAP-2	70	
7065	cIAP-1	62	
13526	RIPK-3	46-62	
93654	Phospho-RIPK-3	46-62	
4056	Phospho-Akt	60	
9746	Caspase-8	43, 57	
56343	cFLIP	55	
14993	MLKL	54	
91689	Phospho-MLKL	54	
9502	Caspase-9	35, 37, 47	
4668	Phospho-JNK MAPK	46	
8478	IRF-1	45	
4970/8457	β -Actin	45	
9101	Phospho-ERK1/2 MAPK	44	
9215	Phospho-P38 MAPK	43	
5453	Mcl-1	40	
4233	Fas	40	
4812/9242	I κ B α	39	
2118	GAPDH	37	
9662	Caspase-3	19,35	
2764	Bcl-xL	30	
2782	FADD	28	
4223	Bcl-2	26	
6947	Bak	25	
5023	Bax	20	
3219	TRAIL	28	

2.10 TNF- α analysis by ELISA

The supernatant of different macrophage subsets (M0, M1 and M2c) before and after treatment with SM-LCL161 (0.2, 0.5 or 1 μ M and/or LPS 1 μ g/mL were collected and subjected to ELISA quantification of TNF- α production. Human TNF- α DuoSet[®] ELISA (Cat. # DY210-05; R&D System, Minneapolis, MN, USA) was used to measure human Tumor Necrosis Factor alpha (TNF- α) production as per the manufacturer protocol. Briefly, a 96 well-culture microplate (Corning Incorporated, Corning, NY) was coated with 100 μ L/well anti-human TNF- α capture antibody, sealed and incubated overnight at room temperature. The following day, wells were washed 3 times with 400 μ L/well washing buffer (0.05% Tween[®] 20 in PBS) using Elx50[™] Microplate Washer (Biotek, Winooski, Vermont, USA) and blocked with 300 μ L/well of reagent diluent (0.2 μ m filtered 1% Bovine serum albumin (BSA) in PBS) followed by 1 hr incubation at room temperature. Next, the plates were washed as previously described and 100 μ L of sample and the reconstituted standards TNF α (15.6-1000 pg/mL) were added. The plate was then sealed with adhesive strip and incubated overnight at room temperature. Next, the plates were washed as previously mentioned and 100 μ L/well of detection antibody (Biotinylated Goat Anti-Human TNF- α Detection Antibody (400 ng/mL) in 1% BSA in PBS was added to each well, sealed again and incubated for 2 hrs. A washing step was performed before adding 100 μ L/well of working dilution (Streptavidin-HRP in 1% BSA in PBS, covered and incubated in dark for 20 min. Next, last washing step was performed again and 100 μ L substrate solution (1:1 H₂O₂ and Tetramethylbenzidine) was added. Lastly, 50 μ L/well of BioFX[®] 450 nm liquid stop solution (Cat. # LSTP-1000-01; Surmodics IVD, Inc., MN, USA) was added to stop the enzymatic reaction. The microplate was read at 490 nm

using iMark Microplate reader (Bio-Rad, Mississauga, Ontario, CA) and analyzed by microplate manager 6 software.

2.11 Human recombinant TNF- α treatment of different macrophage subsets

Human recombinant TNF- α protein was purchased from R&D systems (Cat. # 210-TA), reconstituted in PBS containing at least 0.1% human or bovine serum albumin and stored at -80 until used as per the manufacturer's instructions. On the day of the experiment, M0, M1 and M2c macrophage subsets were washed with serum free media and then left untreated or treated with different concentrations of human recombinant TNF- α (5, 10, 20 ng/mL) and incubated at 37°C with 5% CO₂ for 1 hr. Next, cells were treated with SM-LCL161 (1 μ M) and incubated for another 48 hrs. Cells were then gently scraped and the cell death was assessed by intracellular PI staining and flow cytometry. Data were analyzed using FlowJo software version 10.0.7 and presented using GraphPad Prism 5.0 software.

2.12 Blocking TNF- α by anti-TNF- α neutralizing antibodies in MDMs and SNB-75 cell line

LEAFTM Purified anti-human TNF- α antibody was purchased from Biolegend (Cat. # 502804; San Diego, California, USA) and diluted based on the manufacturer protocol. Macrophage subsets (M0, M1 and M2c) were first washed with serum free media. Next, complete media containing different concentrations of anti-TNF- α antibody was added to different wells and incubated for 1 hr, followed by adding SM-LCL161 (1 μ M) for 48 hrs. A control IgG (LEAFTM Purified Mouse anti-human IgG1, κ Isotype Ctrl; Cat. # 400153, Biolegend) was also added to eliminate non-specific binding. The cell line SNB-75 were also treated with complete media containing different concentrations of *rh*TNF- α , anti-TNF- α

antibody or a combined treatment of both antibodies for 1 hr before treatment with SM-LCL161 (3 μ M) for 48 hrs. Macrophage subsets and SNB75 cells were then collected by a gentle scrape or trypsinization, respectively. The cell death was then assisted by intracellular PI staining as previously mentioned. Data were analyzed using FlowJo software version 10.0.7 and presented using GraphPad Prism 5.0 software.

2.13 Transfection of macrophages with small interfering RNA

IRF-1 siRNA and the negative control siRNA were purchased from Thermo Fisher Scientific (Cat. # 4392420; Cat. # 4390843). To prepare macrophage for transfection, wells containing M0 cells were washed with serum free media. A 600 μ L antibiotic-free media (X1 DMEM supplemented with 10% FBS) was added and the cells were then incubated at 37°C with 5% CO₂ for at least 3 hrs. Next, IRF1 siRNA and the transfection reagent Lipofetamine™ RNAiMax (Thermo Fisher Scientific Invitrogen, Cat. # 13778075) were diluted in 200 μ L of transfection medium Opti-MEM. The diluted siRNA and the diluted Lipofetamine™ RNAiMax reagent were then mixed in a 1:1 ratio and incubated for 5 mins at room temperature to allow formation of siRNA complex. The siRNA mixtures were then added to cells and incubated for 24 hrs. Next, cells were stimulated with IFN- γ for 48 hrs and then washed and replaced with complete media containing SM-LCL161 (1 μ M). After 48 hrs cells were collected and cell death was assessed by intracellular PI staining and flow cytometry.

2.14 Statistical analysis

All statistical analyses were determined using the GraphPad Prism 5.0 software (San Diego, CA, USA). Data from all experiments were analyzed using unpaired Student's t-test and values with $p < 0.05$ were considered to be statistically significant. Data are presented in

graphical form as mean values $\pm$ SD or otherwise stated. P values are represented as not significant (ns), *P< 0.05, ** P< 0.01, *** P< 0.001 and **** P< 0.0001.

2.15 Ethics statement

Healthy participants involved in the study gave informed written consent and the protocol for obtaining blood samples was approved by the Review Ethics Board of the Ottawa General Hospital and the Children's Hospital of Eastern Ontario, Ottawa, ON, Canada. All animal procedures were approved by the Carleton University Animal Care Committee and were conducted in accordance with the guidelines provided by the Canadian Council for Animal Care.

CHAPTER 3:

Selective killing of human M1 macrophages by Smac mimetics alone and M2 macrophages by Smac mimetics and caspase inhibition

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3.1 INTRODUCTION

Macrophages, a heterogeneous population of innate myeloid cells, play a key role in innate immunity and in the pathogenesis of several chronic and inflammatory diseases by induction or resolution of inflammation and tissue repair (4, 9, 41). At the tissue level, macrophages are exposed to different exogenous and endogenous factors including cytokines, chemokines and microbial by-products which allows them to functionally polarize and express distinct surface markers, and cytokines (7, 41, 44). In general, macrophages can be activated to adopt two opposite polarized states, the “classically activated” M1 macrophages and the “alternatively activated” M2 macrophages (4, 23). M1 macrophages are induced by intracellular pathogens, bacterial cell wall components such as lipopolysaccharide (LPS), lipoproteins, TNF- α , or IFN- γ (7, 23). These macrophages are characterized by their high capacity to present antigen, produce nitric oxide or reactive oxygen intermediates, and secrete high levels of pro-inflammatory cytokines such as, IL-12, IL-23, TNF- α , IL-6, IL-1 β (7, 43, 65). In contrast, M2 macrophages exhibit anti-inflammatory phenotype generated following stimulation with IL-4, IL-13, IL-10, or TGF- β , and play a key role in wound healing, and tissue repair (5, 43, 68, 91). The M2 macrophages can be further sub-classified into M2a, M2b and M2c subsets, depending on their functions, cytokine and chemokine profiles and relative expression of surface receptors (43, 89, 95). M2a subset can be generated following treatment with IL-4 and/or IL-13, M2b subset can be induced by stimulation with immune complexes and TLR-agonists or IL-1 receptor ligands (43, 86, 89), while M2c subset can be induced by glucocorticoids and/or IL-10 (43, 89, 108). M2a and M2c subsets are anti-inflammatory whereas M2b secrete both pro and anti-inflammatory cytokines (43, 86, 89, 108). We have previously characterized human macrophage subsets based on the expression of phenotypic

cell surface markers, and production of basal levels of pro- and anti-inflammatory cytokines and following stimulation with TLR ligands. Each polarization state revealed a significant upregulation/downregulation of macrophage-specific cell surface markers and cytokines profile (43).

Recent evidence obtained by microarray data and conventional approaches employed in *in vivo* studies show that M1 polarization signature is associated with bacterial infections including typhoid fever, tuberculoid leprosy, active tuberculosis and *Helicobacter pylori* gastritis (64–66, 96), as well as chronic autoimmune and inflammatory diseases such as rheumatoid arthritis, atherosclerosis, type-2 diabetes and cancer (5, 51). On the other hand, M2 polarization program is observed in lepromatous leprosy, Whipples disease and chronic rhinosinusitis (65, 96–98). Recently, targeting macrophages has been suggested to be a potential therapeutic strategy such as recruitment of M2 macrophages, inducing a class switch from M1 to M2 subsets and downregulation of pro-inflammatory pathways within the macrophage subsets (7, 120, 121) to control cancer, chronic infections, atherosclerosis and autoimmune and allergic diseases (7). Several clinically approved therapeutic agents such as peroxisome proliferator-activated receptor- γ (PPAR γ) inhibitors, statins, and zoledronic acid have been shown to impact the functional status of murine macrophages (7, 122–124). Therefore, it is of utmost importance to identify agents that specifically target M1 and/or M2 macrophages as a potential therapeutic strategy to control diseases associated with pro- and anti-inflammatory phenotype.

During myeloid differentiation, macrophages develop resistance to spontaneous and induced apoptosis that is crucial to maintain survival of macrophages during the immune response against pathogens and in particular intracellular viral and bacterial pathogens

including HIV and tuberculosis (9, 317). Infection of macrophages may enhance their viability, cause dissemination of intracellular pathogens and modulate the surrounding environment by over expression of proinflammatory cytokines (318, 319, 327, 328). The mechanism underlying enhanced resistance of macrophages may relate to the differential expression of pro- and anti-apoptotic genes/proteins including Bcl2 and inhibitors of apoptosis (IAPs) family members (317–321). We have shown that phosphatidyl-inositol-3 kinase (PI3K)/Akt pathway regulates the survival of macrophages by activating NF κ B through the increased expression of the anti-apoptotic protein, Bcl-xl (321). The inhibitors of apoptosis (IAPs) family encompasses XIAP, cellular IAPs (cIAP-1), and cIAP-2 that tightly regulates cell survival downstream of tumor necrosis factor receptor-1 (TNFR1) and other death receptors (165, 168). The role of IAP family members has been studied by employing second mitochondria-derived activator of caspases (SMAC) mimetics (SMs) (213, 214) . SMs are small molecule antagonists of IAPs, which competitively inhibit IAPs interactions with effector proteins and thus repress anti-apoptotic functions of IAP proteins (213, 214). We have previously described the role of IAPs in the resistance of macrophages to HIV-Vpr-induced apoptosis (319, 320). CpG-induced protection against apoptosis and mitochondrial depolarization in monocytic cells was shown to be mediated by cIAP-2 induction (320, 323). Moreover, down regulation of cIAP-1/2, by siRNAs and SMs sensitized macrophages to Vpr-induced apoptosis (319). However, we and others have shown that SMs do not exhibit any apoptotic effect on differentiated normal macrophages (319, 322). In this study, we investigated the effect of SM-LCL161, a monovalent form of SMs, on normal human proinflammatory M1 macrophages and anti-inflammatory macrophages M2a, M2b and M2c subsets. Our results show that SM-LCL161 selectively killed M1 macrophages through both apoptosis and necroptosis whereas other subsets including M0, M2a M2b and M2c were highly

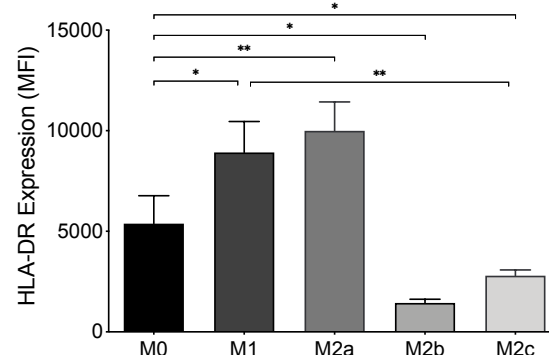
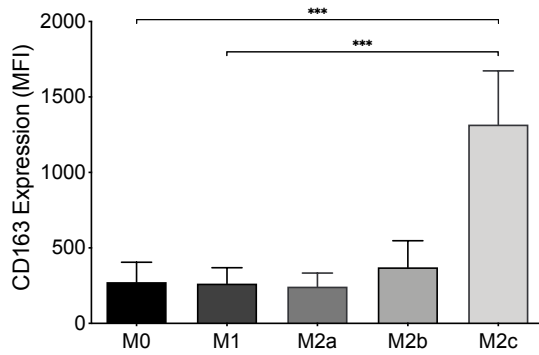
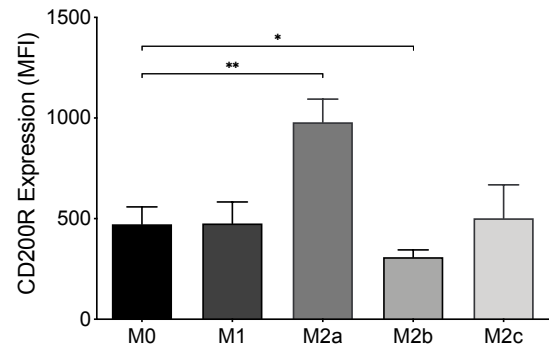
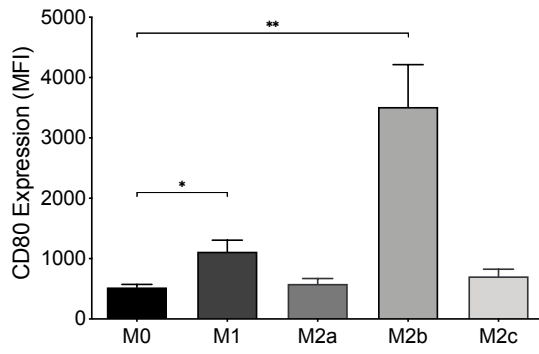
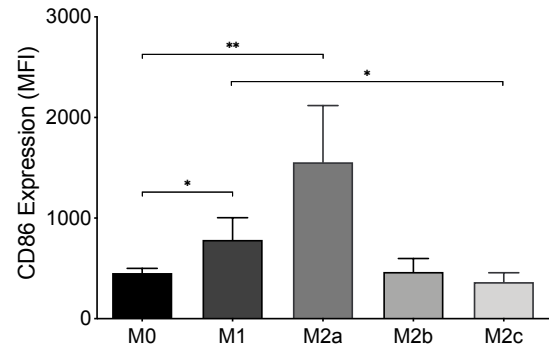
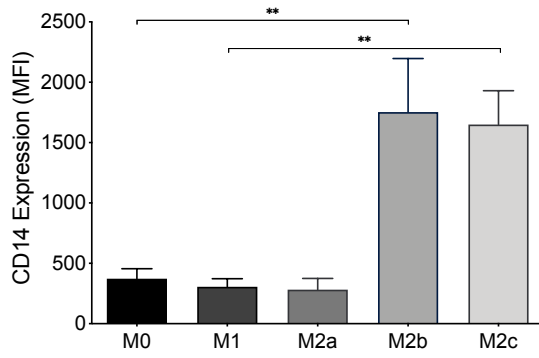
resistant to SM-induced apoptosis. However, M2c macrophages were selectively killed by SM-LCL161 in conjunction with caspase inhibitors through necroptosis. These observations suggest that agents blocking the cIAP-1/2 and/or caspases maybe potentially effective therapeutic agents to control inflammation related disorders.

3.2 RESULTS

3.2.1 Human macrophage subsets differentially express distinct cell surface markers

The macrophage subsets were characterized by analyzing the expression of cell surface markers that are generally present on macrophages and are involved in their activation including CD80, CD86, HLA-DR, CD14, CD163 and CD200R (43, 329–331). M0 macrophages expressed all the above markers. The expression of surface markers on macrophage subsets was defined by setting the expression levels in unpolarized M0 as the base line. The surface markers expressed at statistically significant high levels compared to the M0 macrophage subsets were designated as “high” and selected as characteristic for that macrophage subset. Thus, M1 macrophages differentially expressed high levels of CD80, CD86 and HLA-DR compared to the M0 macrophages (**Figure 3.1A**) and were designated as CD80^{high}, CD86^{high}, HLA-DR^{high}. Similarly, M2a macrophages were defined as CD86^{high}, CD200R^{high}, HLA-DR^{high}; M2b macrophages were defined as CD14^{high}, CD80^{high} and CD163^{high}; and M2c were characterized as CD14^{high}, CD163^{high}, CD200R^{high}. The representative histogram for each of these expression markers on macrophages from an individual donor are shown in **Figure 3.1B**. Overall, macrophage subsets could be distinguished by the differential upregulation of these surface markers.

A



B

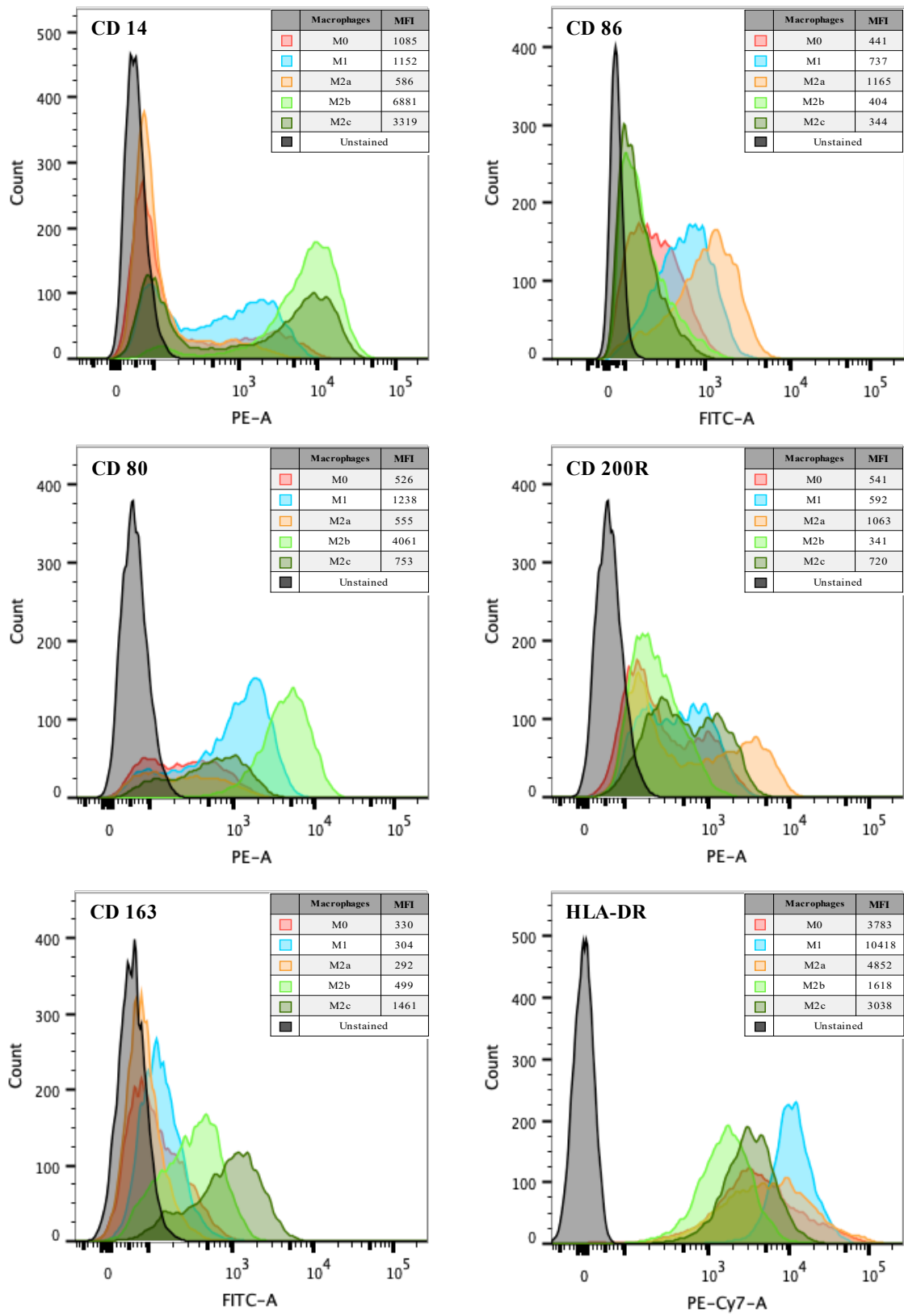


Figure 3.1: Phenotypic characterization of undifferentiated M0 and polarized M1, M2a, M2b and M2c macrophages.

(A) MDMs (M0) were polarized using indicated stimuli for 48 hrs: IFN- γ (20 ng/mL) for M1 macrophages, IL-4 (20 ng/mL) for M2a macrophages, LPS (1 μ g/mL) and IL-1 β (10 ng/mL) for M2b macrophages and IL-10 (10 ng/mL) for M2c macrophages. Polarized macrophages were stained with antibodies against the mentioned cell surface markers and fluorescence was measured by flow cytometry. MFI values were obtained using FlowJo software and potted using GraphPad Prism 5. The results shown are mean $\pm$ SD of three independent experiments from three different donors. (B) Overlay histograms from one representative experiment are shown.

3.2.2 Blocking IAPs signaling pathway by SM-LCL161 selectively causes cell death in human M1 macrophages, whereas M2 macrophages are resistant to SM-induced cell death

We have previously shown that human monocytes are highly susceptible to the apoptotic effects of SMs whereas differentiated macrophages (MDMs; M0) are resistant (317, 319). To determine whether human macrophage subsets M1, M2a, M2b and M2c generated *in vitro* from M0 are also resistant to the SM-induced cell death, we first determined the involvement of IAPs signaling pathway on the cell survival of different macrophage subsets by using IAPs antagonist, SM-LCL161. The macrophage subsets M0, M1, M2a, M2b and M2c were treated with different concentrations of SM-LCL161 for 48 hrs followed by analysis of cell death by intracellular PI staining. The dose of SM-LCL161 was selected based on previous studies (332, 333). Cell death below 10% were considered as background/basal levels of cell death. The results show that M1 is the most susceptible subset to SM-induced cell death at all concentrations (**Figure 3.2**). In contrast, M2c was the most resistant subset and did not show increased cell death after SM-LCL161 treatments at any concentration. Therefore, M1 and M2c in addition to the un-polarized M0 were selected for further investigations. Using the automated IncuCyte™-YOYO-1 staining, we confirmed that M1 is highly sensitive to SM-induced cell death. SM-induced cell death in M1 cells was detectable as early as 10 hrs and reached a peak by 20 hrs following treatment with all the concentrations of SM-LCL161 (**Figure 3.3**). In contrast, M2c subsets did not show increased levels of cell death at any time and at any concentration until 48 hrs post-SM-LCL161 treatment. Furthermore, M1 cells treated with SM-LCL161 exhibited increased level of YOYO-1 fluorescent intensity compared to M2c macrophages (**Figure 3.3** left panel). However, M0 macrophages exhibited scattered

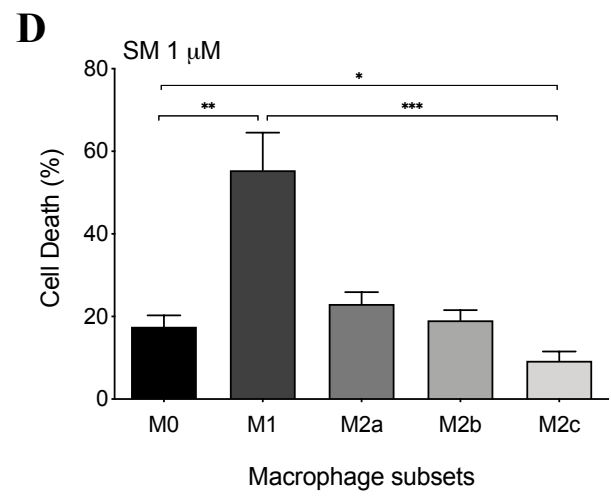
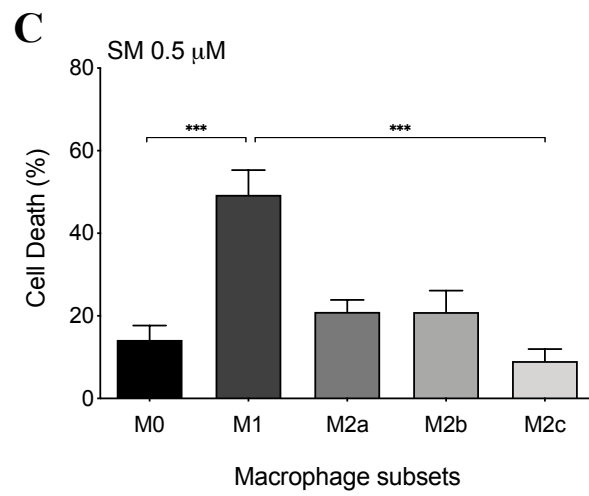
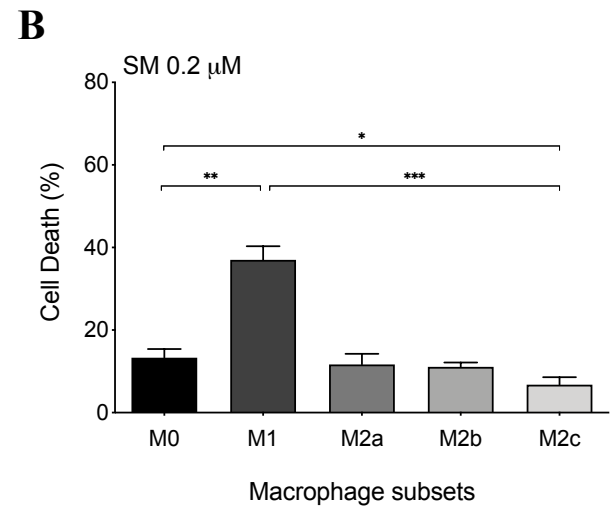
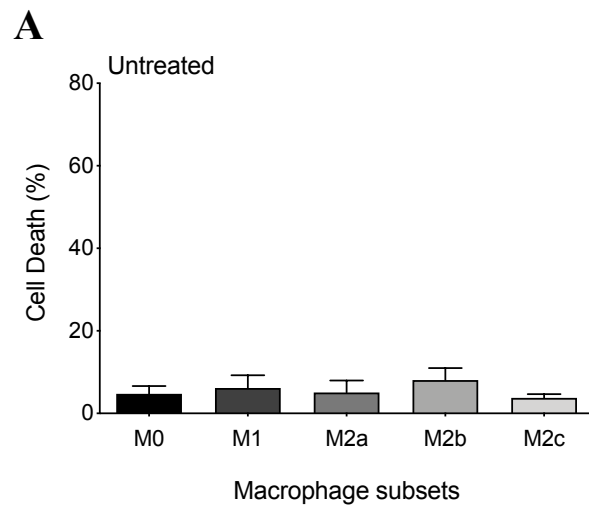


Figure 3.2: M1 and M2c are the most sensitive and resistant macrophages subsets to SM-LCL161 induced cell death, respectively.

M0, M1, M2a, M2b and M2c macrophage subsets were left untreated (**A**) or treated with SM-LCL161 (SM) 0.2 μ M (**B**), 0.5 μ M (**C**) or 1 μ M (**D**) for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The results shown in A-D are mean $\pm$ SD of three independent experiments from three different donors.

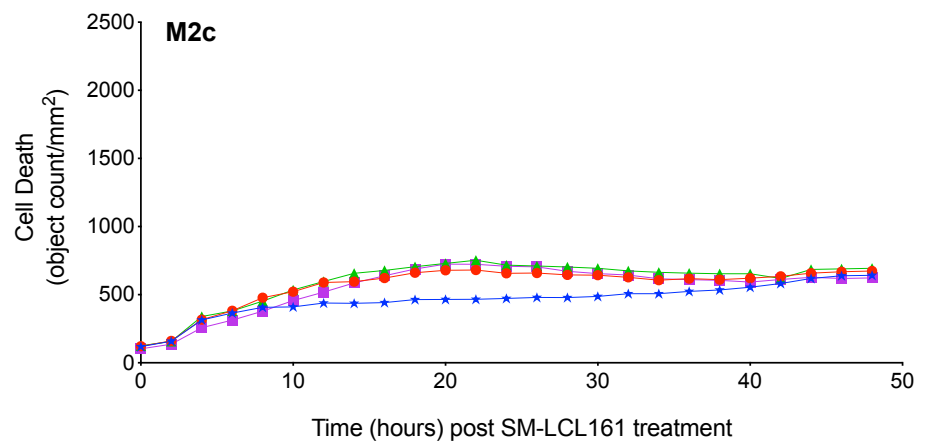
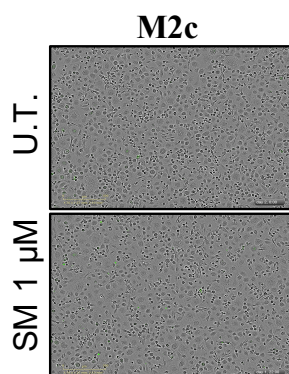
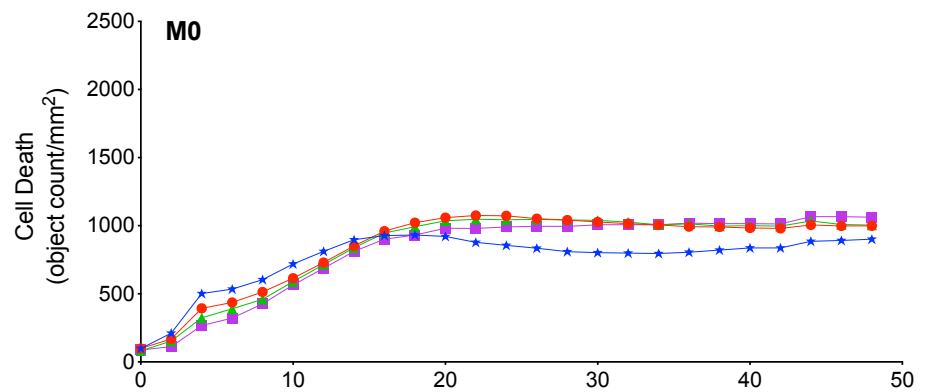
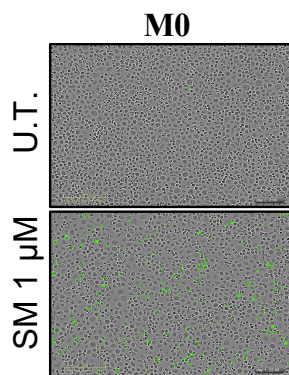
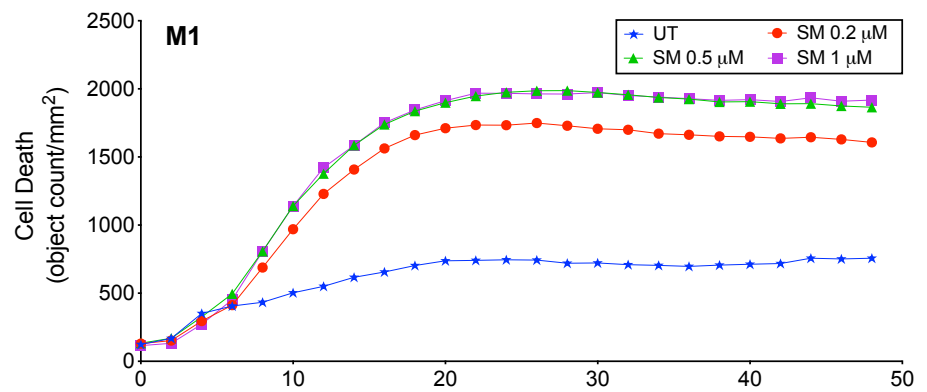
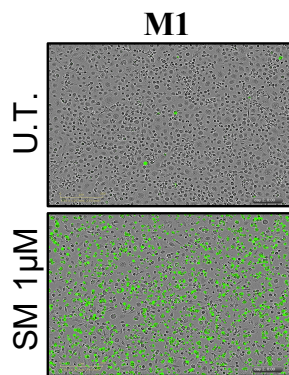


Figure 3.3: Kinetics of cell death in SM-treated M0, M1 and M2c macrophages as determined by IncuCyte™ automated machine.

Cell death stain (YOYO-1 iodide) was added to each well containing M1, M0 or M2c macrophage subsets 1 hr prior to SM-LCL161 (SM) treatment. Cells were treated with SM at concentrations of 0.2 μ M, 0.5 μ M and 1 μ M and incubated in IncuCyte™ machine for 48 hrs. Images were recorded every 2 hrs for 48 hrs. Left panel shows live pictures taken at 48 hrs of IncuCyte™ incubation and the green fluorescent represents dead cells. The result shown is a representative of three independent experiments from three different donors.

levels of YOYO-1 staining perhaps because M0 macrophages are heterogeneous and contain population of all subsets including M1 macrophages.

Next, we determined whether there is any association between the levels of IAP proteins expressed in various subsets and SM-induced cell death. Although SM-LCL161 caused selective cell death in M1 macrophages, SM-LCL161 caused degradation of both cIAP-1 and cIAP-2 in M0, M1 and M2c subsets and as early as 5 hrs after SM-LCL161 treatment (**Figure 3.4A, and B**). The untreated M0, and M2c macrophages expressed similar levels of cIAP-1 and cIAP-2 while untreated M1 macrophages expressed significantly high levels of cIAP-1 and cIAP-2 compared to M0 and M2c macrophages (**Figure 3.4A and B**) suggesting the lack of association between the levels of IAP proteins expressed in various subsets and SM-induced cell death. In addition, the effect of SM-LCL161 on cIAP-1 and cIAP-2 was specific as it did not impact other proteins, including Fas, TRAIL, and anti-apoptotic protein, Mcl-1. (**Figure 3.4C**). However, SM-LCL161 treatment as expected resulted in down regulation of the anti-apoptotic protein cFLIP and upregulation of pro-apoptotic protein Bax expressions in M1 macrophages but not in M0 and M2c macrophages compared to untreated macrophages (**Figure 3.4C**).

3.2.3 SM-induced cell death in M1 macrophages is mediated by both extrinsic and intrinsic pathways of apoptosis

Apoptosis involves proteolytic processing and activation of caspases, the key effector molecules of apoptosis pathway (164). The extrinsic pathway of apoptosis is mediated by the activation of caspase-8 while the intrinsic pathway is mediated by caspase-9, both of which activate effector caspases-3/7 leading to apoptosis (143, 164). To determine whether SM-LCL161 induced cell death is regulated by the intrinsic or the extrinsic pathways, we measured

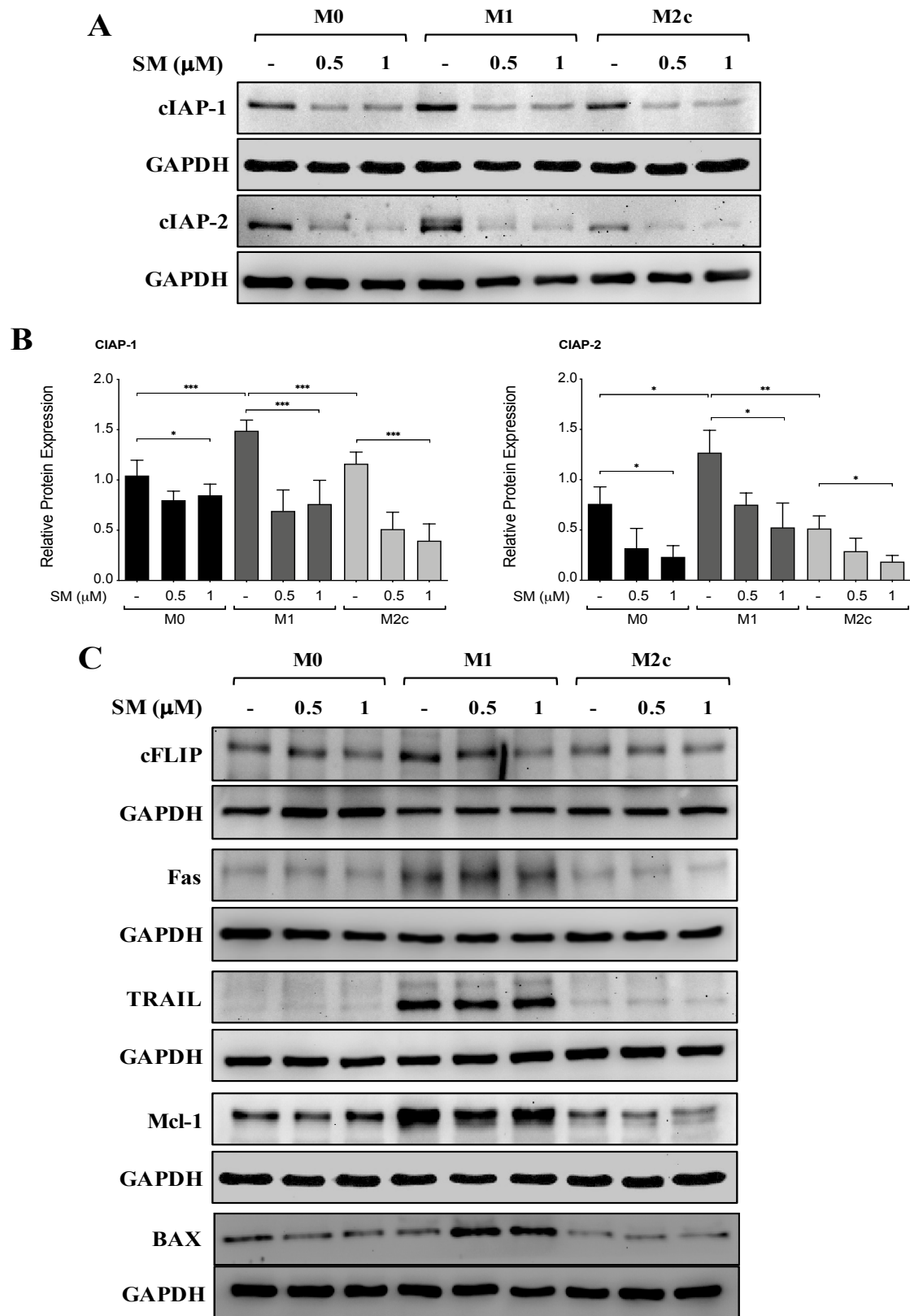
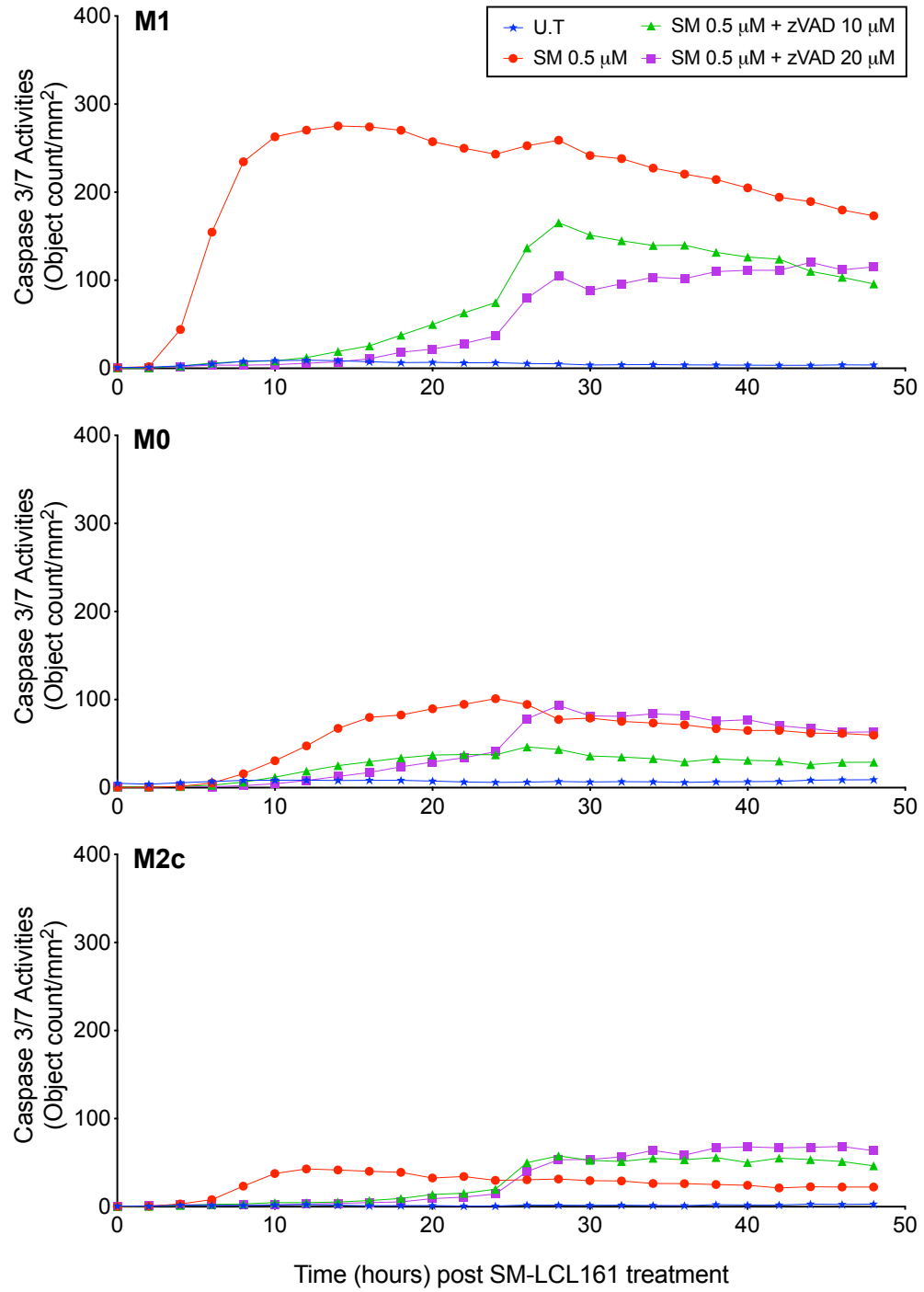
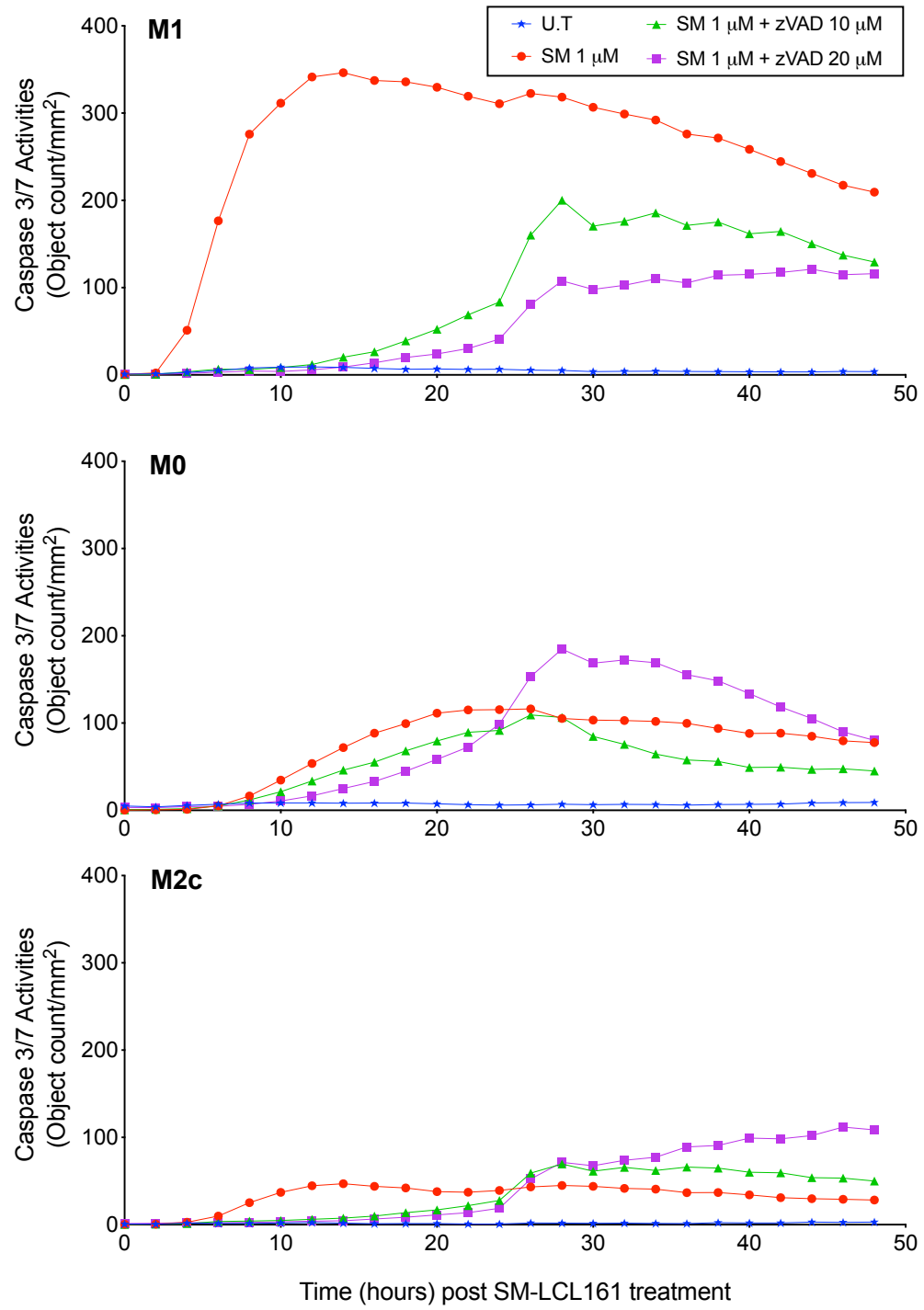


Figure 3.4: Effect of SM-LCL161 on the expression of anti-apoptotic and proapoptotic protein.

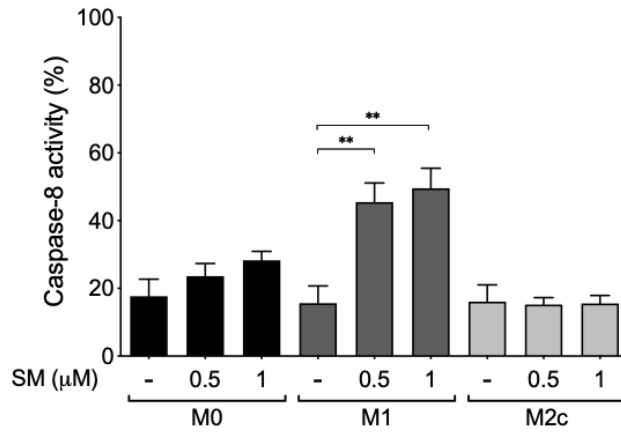
(A) Total proteins lysates from M0, M1 and M2c macrophages treated with 0.5 μ M and 1 μ M of SM-LCL161 for 5 hrs were subjected to Western immunoblotting for the expression of cIAP-1 (62 kDa) and cIAP-2 (70 kDa). (B) Relative protein expression of cIAP-1, cIAP-2 and with and without treatment with SM-LCL161 0.5 μ M or 1 μ M for 5 hrs. (C) Total proteins lysates from M0, M1 and M2c macrophages treated with SM-LCL161 0.5 μ M and 1 μ M for 5 hrs and were subjected to Western immunoblotting for the expression of cFLIP (55 kDa), Fas (40 kDa), TRAIL (28 kDa), Mcl-1 (40 kDa) and Bax (20 kDa). The immunoblot shown is a representative of three independent experiments from three different donors.

the activation of caspase-3/7, -8 and -9 in SM-treated macrophage subsets. M1, M0 and M2c macrophages were treated with either 0.5 μ M or 1 μ M of SM-LCL161 for 48 hrs followed by analysis of caspase-8, -9, and -3/7 by flow cytometry, immunoblotting, and IncuCyte™ Live cell imaging, respectively. There was a significant increase in caspase-3/7 activities in M1 macrophages upon treatment with 0.5 μ M (**Figure 3.5A**) or 1 μ M (**Figure 3.5B**) of SM-LCL161 compared to M0 and M2c macrophage subsets as measured by the IncuCyte™ live imaging. This increase in caspase-3/7 activity in M1 macrophages was detected as early as 4 hrs and reached a peak by 10 hrs post SM-LCL161 treatment (**Figure 3.5A and B**). Moreover, this induction was reduced in a dose-dependent manner after treatment with a pan-caspase inhibitor, zVAD.fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) at 10 and 20 μ M concentrations (**Figure 3.5A and B**). The caspase-3/7 expression was poorly detected in the presence or absence of SM-LCL161 in both M0 and M2c macrophages. The caspase-8 activity was assessed by measuring its cleavage activity by flow cytometry. M1 macrophages treated with either 0.5 μ M or 1 μ M of SM-LCL161 revealed significantly higher caspase-8 activity compared to the untreated cells, whereas SM-treated M0 or M2c macrophages did not show cleavage activity following SM-LCL161 treatment and their levels were similar to the untreated macrophages (**Figure 3.5C**). The representative flow histogram showing caspase-8 activity in M0, M1 and M2c subsets is shown in **Figure 3.5D**. Staurosporine was used as a positive control to induce caspase-8 activity in all three subsets. For caspase-9, the Western immunoblots showed increase in cleaved caspase-9 bands upon treatment with 0.5 μ M and 1 μ M of SM-LCL161 in M1 macrophages (**Figure 3.5E**). These results suggest the involvement of both intrinsic and extrinsic apoptosis pathways in SM-induced apoptosis in M1 macrophages.

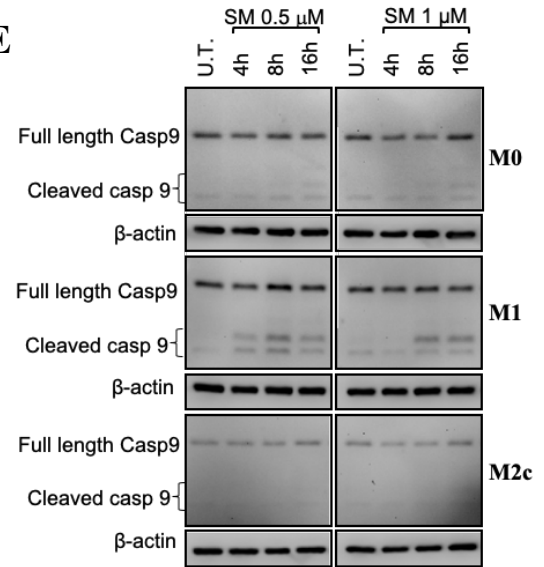
A

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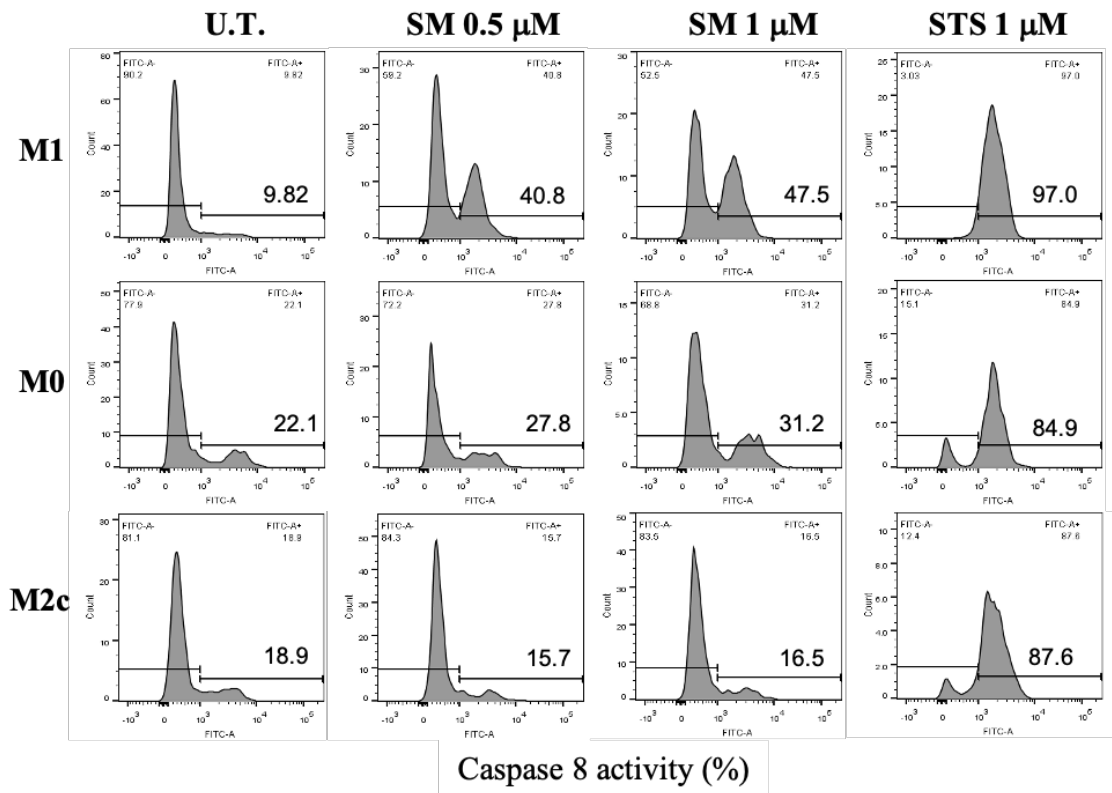


Figure 3.5: SM-LCL161 activates caspase-3/7, -8 and -9 in M1 macrophages.

M1, M0 and M2c subsets were treated with IncuCyte™ caspase-3/7 reagent in the presence or absence of zVAD.fmk (10 μ M and 20 μ M) 1 hr prior to treatment with (A) 0.5 μ M or (B) 1 μ M of SM-LCL161 and incubated in IncuCyte™ machine for 48 hrs. The caspase-3/7 activity was recorded every two hrs for 48 hrs. (C) M0, M1 and M2c macrophages were treated with SM-LCL161 (0.5 μ M, 1 μ M) and cultured for 10 hrs following which caspase-8 reagent was added for 1 hr before assessment of caspase-8 activity. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (D) A representative figure of M0, M1 and M2c macrophages treated with SM-LCL161 (0.5 μ M and 1 μ M) or Staurosporine (STS; 1 μ M) determined by flow cytometry analysis. (E) M0, M1 and M2c macrophages were treated with either 0.5 μ M or 1 μ M of SM-LCL161 for 4, 8 and 16 hrs. Total protein lysates were subjected to Western immunoblotting for caspase-9 expression. The immunoblots show full length and cleaved caspase-9 (47, 37 and 35 kDa). The results shown are representative figures of three independent experiments from three different donors.

3.2.4 SM-induced cell death in M1 macrophages involves both apoptosis and necroptosis pathways.

To confirm that SM-induced cell death was associated with caspase activation, we examined whether blocking these caspases would inhibit SM-induced apoptosis. For this, M1 macrophages were pretreated with either 10 μ M or 20 μ M of zVAD.fmk, for 1 hr followed by treatment with either 0.5 μ M or 1 μ M of SM-LCL161. Evaluation of apoptosis by intracellular PI staining and flow cytometry revealed that zVAD.fmk alone did not cause an increase in cell death. Cell death below 10% were considered as background/basal levels of cell death. M1 macrophages treated with SM-LCL161 demonstrated an increased cell death as expected. SM-induced cell death was significantly reduced upon combined treatment with either concentrations of zVAD.fmk (**Figure 3.6A**). Although blocking apoptosis by zVAD.fmk treatment significantly reduced SM-induced apoptosis, it reduced cell death by approximately 50% suggesting that the residual cell death seen in M1 macrophages after blocking caspases might be due to cell death mechanisms other than apoptosis.

Another key pathway by which cells die is by activation of necroptosis. Necroptosis is a programmed form of necrosis, or inflammatory cell death (223, 224). We surmised that the residual SM-induced cell death seen following caspase inhibition may be due to necroptosis pathway. Necroptosis has been examined by chemical inhibitors, Necrostatin-1 and Necrosulfonamide (NSA). Necrostatin-1 targets the receptor-interacting protein kinase 1 (RIPK-1) activity, while NSA targets the mixed lineage kinase domain-like protein (MLKL) (222, 334). To understand the involvement of necroptosis, M1 macrophages were treated with different concentrations of necrostatin-1 (0.5 μ M or 1 μ M) (**Figure 3.6B**) or NSA (0.5 μ M to 7 μ M) (**Figure 3.6C**) for 1 hr prior to treatment with 0.5 μ M or 1 μ M of SM-LCL161. After

48 hrs, cell death was assessed by intracellular PI staining/flow cytometry. Both Necrostatin-1 and NSA significantly reversed SM-induced cell death in M1 macrophages in a dose dependent manner after treatment with either 0.5 μ M or 1 μ M of SM-LCL161. Interestingly, similar to zVAD.fmk, blocking necroptosis pathway by either Necrostatin-1 or NSA inhibited SM-induced cell death by approximately 50%. Therefore, we hypothesized that SM-induced cell death in M1 macrophages may be mediated by both apoptosis as well as necroptosis. To determine whether SM-induced cell death involves both apoptosis and necroptosis, M1 macrophages were pretreated with zVAD.fmk and NSA together followed by treatment with 0.5 μ M or 1 μ M of SM-LCL161. SM-induced cell death was significantly inhibited and reduced to basal levels upon combined treatment of M1 macrophages with zVAD.fmk and NSA (**Figure 3.6D**). Moreover, treatment of M1 macrophages with Necrostatin-1 alone (**Figure 3.6B**) or NSA alone at 4 μ M or 5 μ M concentration (**Figure 3.6D**) or combined treatment with both zVAD.fmk and NSA (5 μ M; **Figure 3.6D**) did not cause an increase in cell death suggesting that M1 macrophages are not more sensitive to toxic compounds. These results suggest the involvement of both apoptosis and necroptosis pathways in SM-induced cell death in M1 macrophages.

RIPK-1, RIPK-3 and MLKL have emerged as the key effectors molecules in apoptosis and necroptosis (143, 223, 241, 243). Thus, we also examined the effect of SM-LCL161 on the expression levels of RIPK-1, RIPK-3 and MLKL following treatment of M1 macrophages with SM-LCL161 in the presence and absence of Necrostatin-1 and NSA. The results show that SM-LCL161 degraded RIPK-1, RIPK-3 and MLKL that was restored following Necrostatin-1 and/or NSA treatment. Similarly, SM-LCL161 induced phosphorylation of

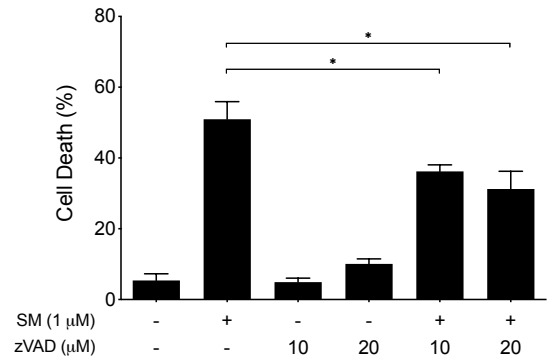
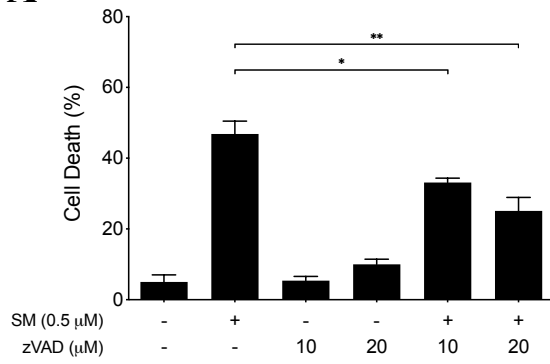
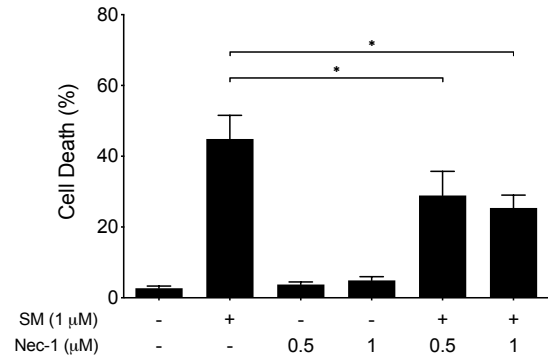
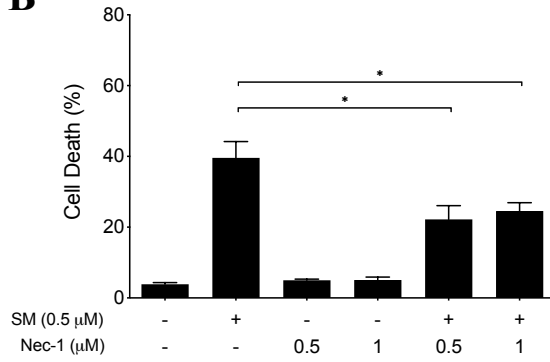
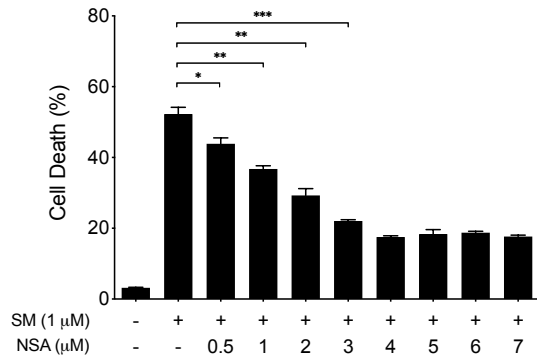
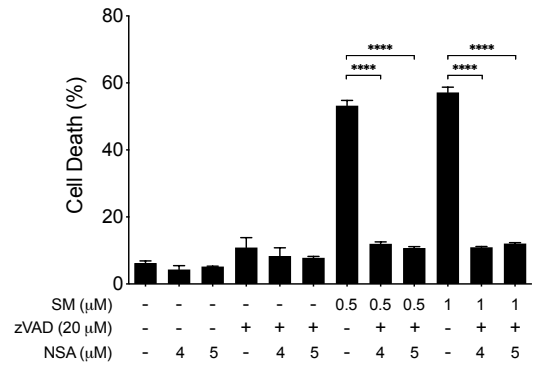
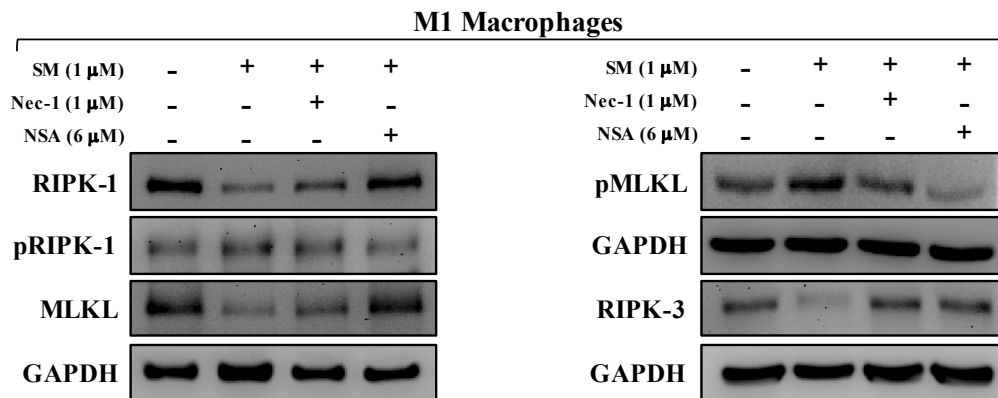
A**B****C****D****E**

Figure 3.6: SM-induced cell death in M1 macrophages is mediated by apoptosis and necroptosis.

M1 macrophages were treated with either SM-LCL161 0.5 μ M (left panels in A and B) or SM-LCL161 1 μ M (right panels in A and B) in the presence or absence of (A) zVAD.fmk (10 μ M, or 20 μ M), (B) Nec-1 (0.5 μ M, or 1 μ M), (C) NSA (0.5 μ M to 7 μ M) or (D) a combination of zVAD.fmk (20 μ M) and NSA (4 μ M or 5 μ M). All chemical inhibitors were added 1 hr prior to SM-LCL161 treatment. Cells were incubated for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SEM of four (A), five (B) and three (C and D) independent experiments from different donors. (E) Total protein lysates from M1 macrophages treated with 1 μ M of SM-LCL161 alone or combined treatment with Necrostatin-1 1 μ M or NSA 6 μ M for 5 hrs were subjected to Western immunoblotting for the expression of RIPK-1 (78 kDa), pRIPK-1 (78 kDa), RIPK-3 (46-62 kDa), MLKL (54 kDa) and pMLKL (54 kDa). The immunoblot shown is a representative of three independent experiments from three different donors.

RIPK-1 and MLKL that was significantly reduced in the presence of Necrostatin-1 or Necrosulfonamide (NSA) (**Figure 3.6E**).

3.2.5 Reversal of M0 to M1 differentiation by blockage of JAK/STAT pathway reverses susceptibility of M1 macrophages to SM-induced apoptosis

JAK/STAT pathway is a principal signaling mechanism for a wide range of cytokines including IFNs. Activation of JAK lead to the phosphorylation of downstream targets including the STATs (259). We determined if IFN- γ -mediated differentiation of M0 into M1 macrophages plays a role in regulating the susceptibility of M1 macrophages to SM-induced cell death. This was investigated by blocking JAK activation using JAK inhibitor, Ruxolitinib (RUX) (335). M0 macrophages were treated with various concentrations of RUX for 2 hrs prior to IFN- γ stimulation for 48 hrs. Cells were treated with SM-LCL161 for another 48 hrs followed by the measurement of cell death by PI staining and flow cytometry. The results show that JAK inhibitor reversed susceptibility of M1 macrophages to SM-induced cell death in a dose-dependent manner to levels equivalent to the basal levels seen in unstimulated M0 macrophages (**Figure 3.7A**). The biological activity of RUX was confirmed by inhibition of IFN- γ -induced JAK-1 phosphorylation (**Figure 3.7B**).

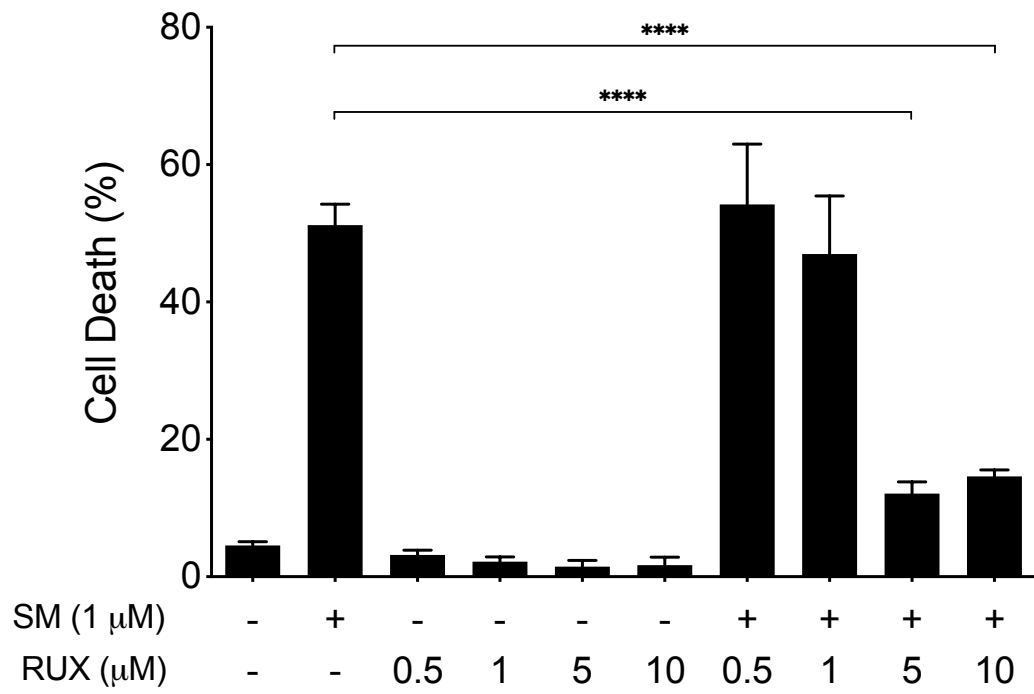
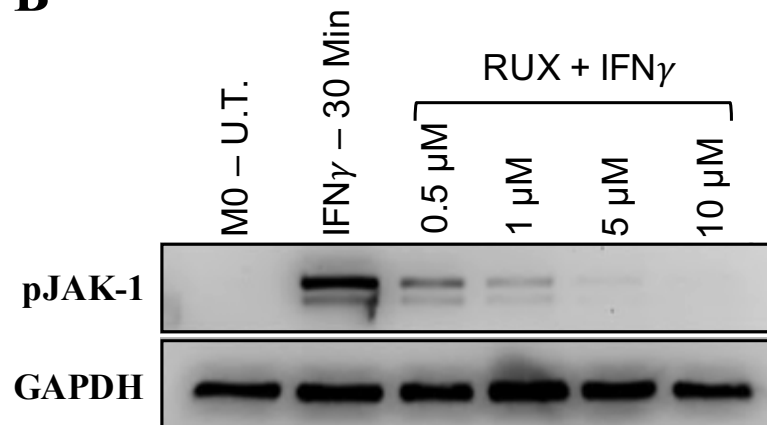
A**B**

Figure 3.7: JAK pathway regulates SM-induced cell death in M1 macrophages.

(A) M0 macrophages were treated with various concentrations of JAK inhibitor (Rux), for 2 hrs prior to stimulation with IFN- γ for 48 hrs. Cells were then treated with SM-LCL161 1 μ M for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (B) M0 macrophages were treated with various concentrations of Rux for 2 hrs prior to stimulation with IFN- γ for 30 min. Total protein lysates were analyzed for pJAK-1 (130 kDa) by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

3.2.6 Blockage of caspases and IAPs signaling selectively cause cell death in M0 and M2c macrophages through the necroptosis pathway.

In contrast to M1 macrophages, M0 and M2c macrophages did not show a significant increase in cell death following treatment with SM-LCL161 (**Figure 3.2**). Blocking of caspases by the pan-caspase inhibitor zVAD.fmk in the absence of IAPs signaling pathway has been shown to induce cell death in cancer cells (336–339). To determine if SM-LCL161 can induce cell death in M0 and M2c cells following blockage of caspase activity, M0 and M2c cells were treated with zVAD.fmk for 1 hr followed by treatment with SM-LCL161 for 48 hrs. SM-LCL161 at concentrations of 0.5 μ M or 1 μ M alone did not result in induction of cell death in M0 or M2c macrophages (**Figure 3.8**). Interestingly, upon blocking caspase activities by zVAD.fmk at both concentrations of 10 μ M or 20 μ M, SM-LCL161 treatment at both concentrations of 0.5 μ M and 1 μ M, led to significant increase in cell death in both M0 and M2c (**Figure 3.8A and B**) macrophages.

This induction in cell death after blocking caspases might be due to the activation of necroptosis pathway (336–339). Therefore, it is possible that blocking of caspases in SM-treated M0 and M2c macrophages selectively activates the necroptosis pathway. Similar to SM-treated M1 macrophages, Necrostatin-1 (0.5 μ M or 1 μ M) was used to determine necroptosis in zVAD.fmk and SM-treated M0 and M2c macrophages. The two subsets were treated with Necrostatin-1 and zVAD.fmk for 2 hrs followed by treatment with SM-LCL161 (**Figure 3.9**: left panel, 0.5 μ M; right panel, 1 μ M)

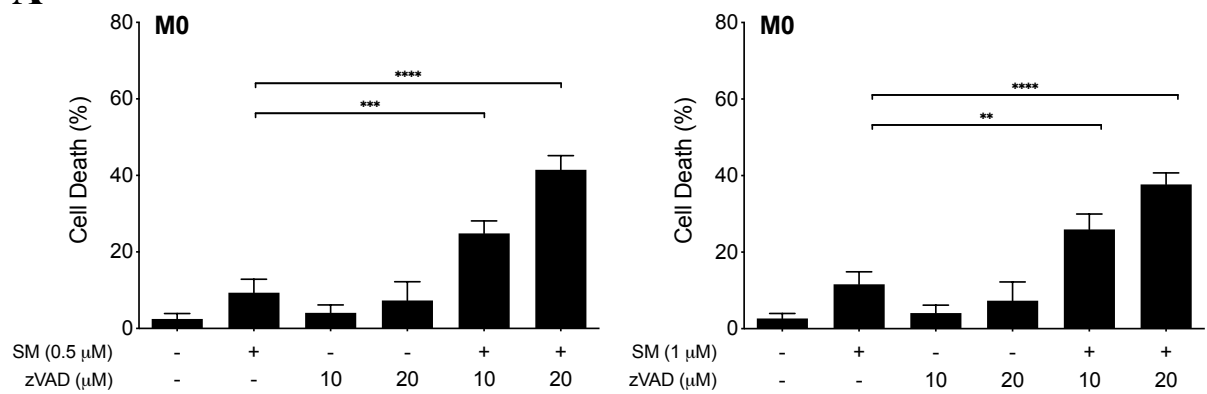
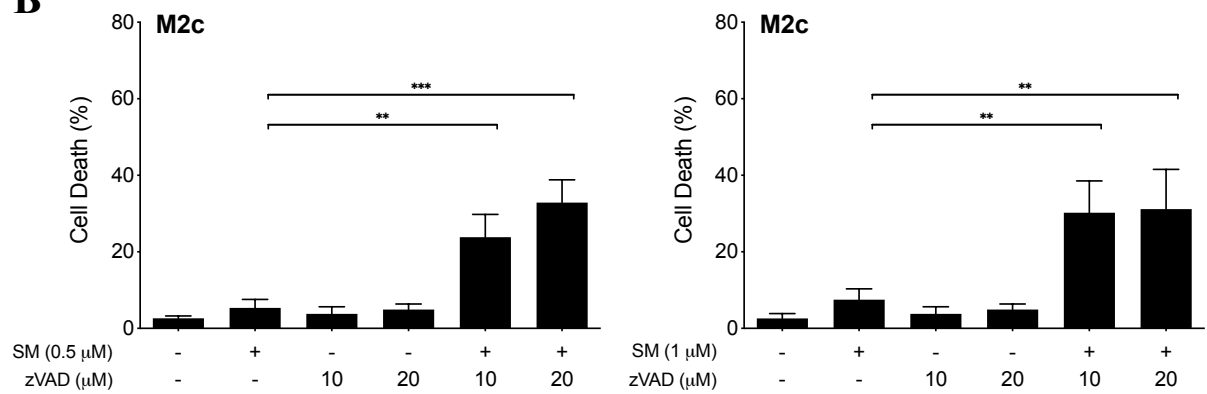
A**B**

Figure 3.8: SM induce cell death in M0 and M2c following blockage of caspases.

(A) M0 and (B) M2c macrophage subsets were treated with either SM-LCL161 0.5 μ M (left panels) or SM-LCL161 1 μ M (right panels) in the presence or absence of 10 μ M, or 20 μ M zVAD.fmk. The inhibitor zVAD.fmk was added 1 hr prior to SM-LCL161 treatment. Cells were then incubated for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown in A and B are a mean $\pm$ SD of four independent experiments from four different donors.

for 48 hrs. Treatment with Necrostatin-1 significantly inhibited SM-LCL161 and zVAD.fmk-induced cell death in a dose-dependent manner (**Figure 3.9A and B**).

First, I confirm the cytotoxicity of NSA treatment alone in M1 (**Figure 3.10A**). NSA treatment even at high concentration of 7 μ M did not affect cell death in M1 without SM-LCL161. Blocking necroptosis pathway by NSA (2 μ M) in M0 and M2c macrophages following combination treatment of SM-LCL161 and zVAD.fmk demonstrated similar results to Necrostatin-1 as seen in a representative histogram (**Figure 3.10B**).

Similar to the M1 macrophages, the effect of SM-LCL161 and zVAD.fmk on the expression levels of RIPK-1, RIPK-3 and MLKL of M0 and M2c macrophages following treatment with Necrostatin-1 and NSA was evaluated. The results show that SM-LCL161 and zVAD.fmk combined treatment inhibited RIPK-1, RIPK-3 and MLKL expressions that was restored following prior treatment with Necrostatin-1 and NSA. Similarly, SM-LCL161 and zVAD.fmk induced the phosphorylation of pRIPK-1 and pMLKL that was dephosphorylated in the presence of Necrostatin-1 and NSA (**Figure 3.9C**).

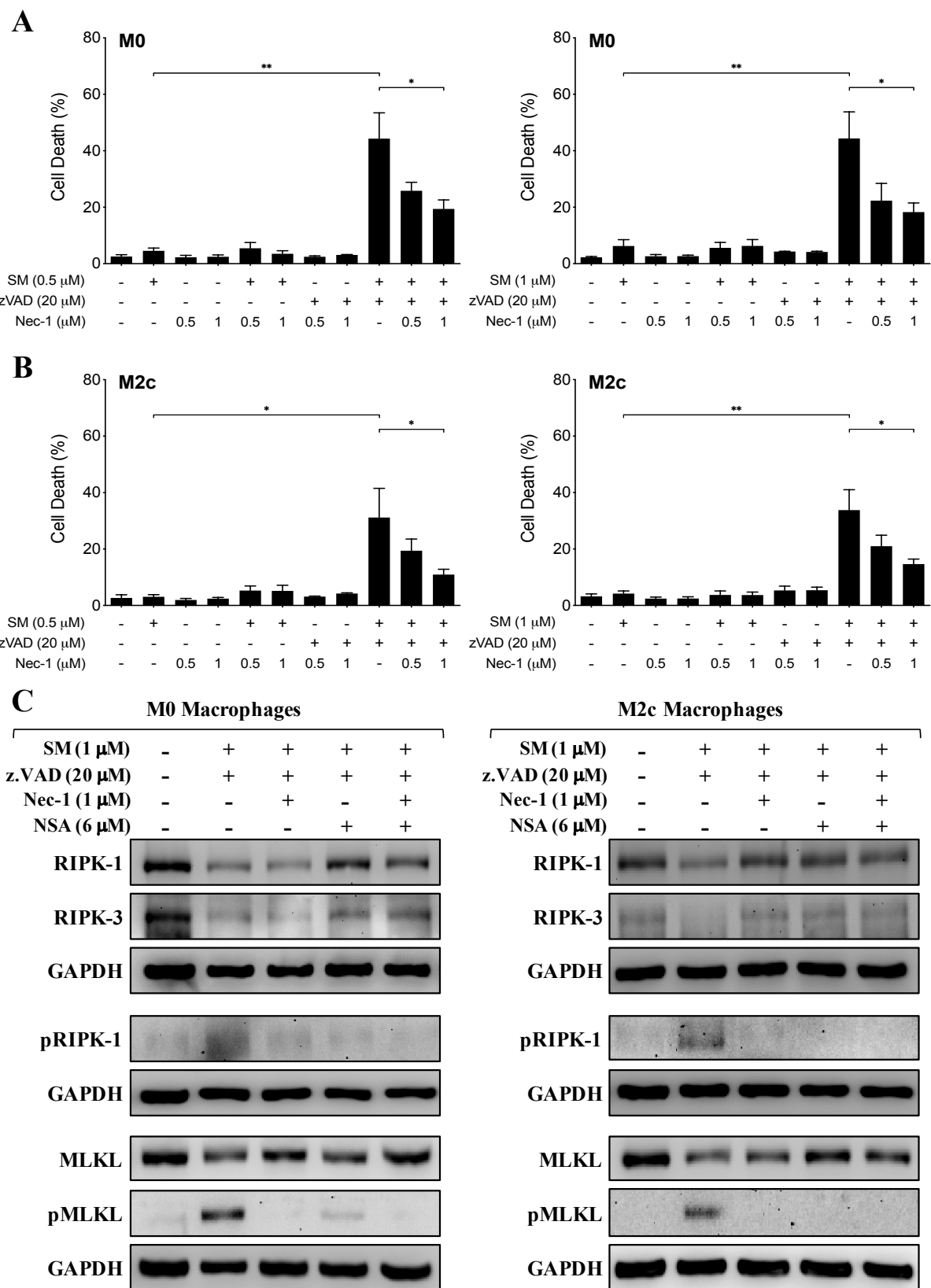
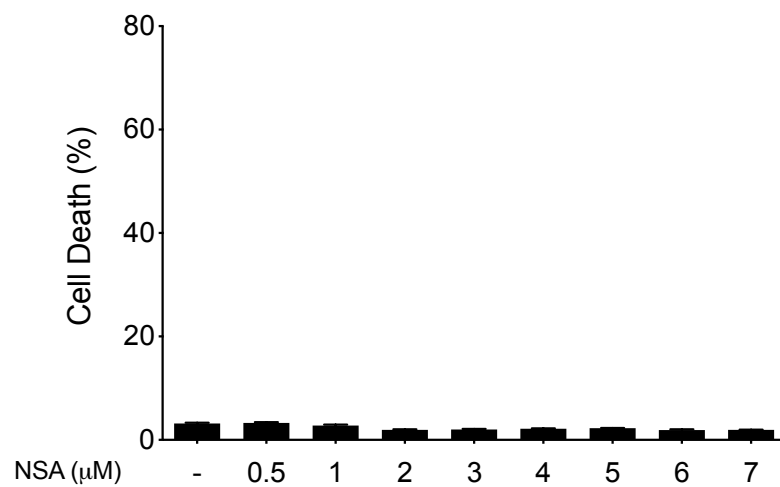


Figure 3.9: SM-induced cell death in M0 and M2c cells following caspase blockage is mediated by necroptosis.

(A) M0 and (B) M2c macrophages were treated with either SM-LCL161 0.5 μ M (left panels) or SM 1 μ M (right panels) in the presence or absence of 20 μ M zVAD.fmk and/or Necrostatin-1 (Nec-1; 0.5 μ M and 1 μ M). The inhibitors were added 1 hr prior to SM-LCL161 treatment. Cells were then incubated for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown in A and B are a mean $\pm$ SEM of four and three independent experiments from different donors, respectively. (C) Total protein lysates from M0 and M2c macrophages treated with 1 μ M of SM-LCL161 and 20 μ M zVAD.fmk or combined treatment with Necrostatin-1 (1 μ M) or Necrosulfonamide (6 μ M) for 5 hrs were subjected to Western immunoblotting for the expression of RIPK-1 (78 kDa), pRIPK-1 (78 kDa), RIPK-3 (46-62 kDa), MLKL (54 kDa) and pMLKL (54 kDa). The result shown is a representative of three independent experiments from three different donors.

A



B

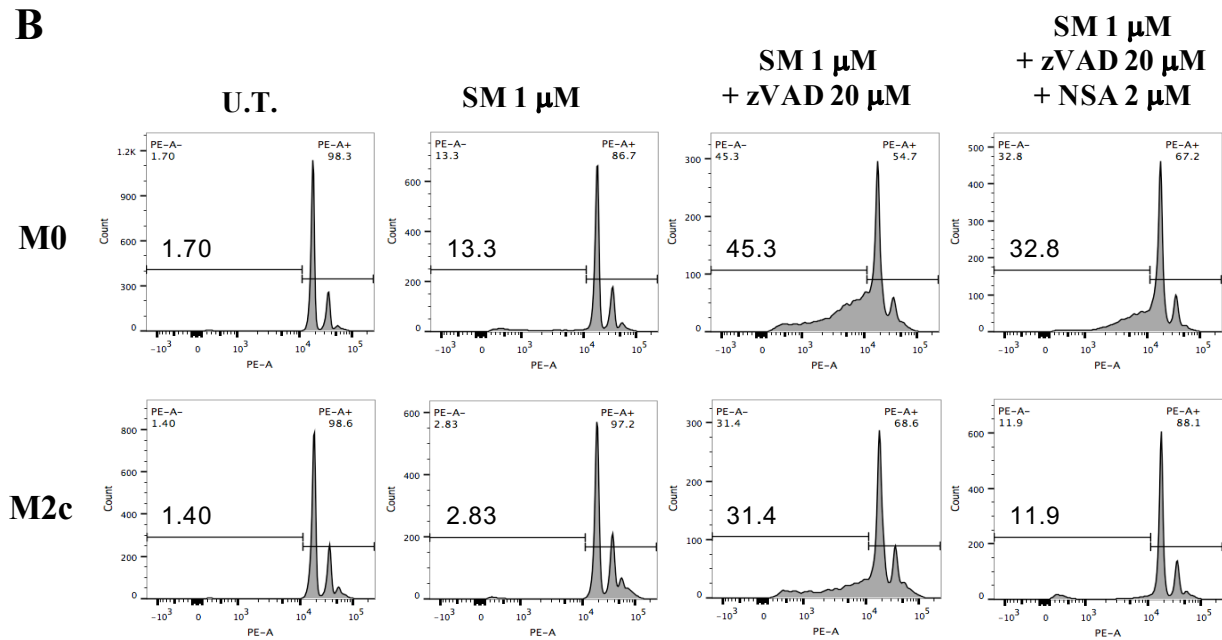


Figure 3.10: Necrosulfonamide (NSA) alone does not cause cell death in M1 macrophages but reduces cell death in zVAD.fmk and SM-LCL161 treated M0 and M2c macrophages.

(A) M1 macrophages were treated with different concentrations of NSA (0.5 μ M to 7 μ M) for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments. (B) A representative figure of M0 and M2c macrophages treated with either SM-LCL161 (1 μ M) alone or zVAD.fmk (20 μ M) or a combined treatment of zVAD.fmk (20 μ M) and NSA (2 μ M). The chemical inhibitors zVAD.fmk and NSA were added 1 hr prior to SM-LCL161 treatment. Cells were incubated for 48 hrs and the % cell death was determined by intracellular PI staining and flow cytometry.

3.2.7 Human and mouse M1 and M2c macrophages behave similarly with respect to sensitivity towards SM-induced cell death

To investigate whether mouse M1 and M2c macrophages would behave in a manner similar to human macrophages with respect to the SM-induced cell death, BMDMs were cultured for 10 days followed by stimulation with either mouse *rIFN- γ* or *rIL-10* for 48 hrs for the generation of M1 and M2c macrophages, respectively. M1 and M2c mouse macrophages were treated with different concentrations of SM-LCL161 followed by assessment of cell death. Results show that mouse M1 macrophages are highly susceptible whereas M2c macrophages are resistant to SM-LCL161-induced cell death (**Figure 3.11**) suggesting that both human and mouse M1 and M2c macrophages behave similarly with respect to sensitivity towards SM-induced cell death.

3.2.8 M1 and M2c macrophages are plastic and repolarization of M1 into M2c and of M2c into M1 subset reverses the effects of SM-LCL161 on cell death and expression of cell surface markers

Plasticity is one of the characteristic features of macrophages (5, 7). Therefore, we hypothesized that repolarization of M1 into M2c macrophages and M2c into M1 macrophages would reverse the effect of SM-LCL161-induced cell death. For this, M1 macrophages were rested for 48 hrs followed by stimulation with IL-10 for 48 hrs to generate M2c macrophages from M1 macrophages (M1>M2c). Similarly, M2c macrophages were rested for 48 hrs and then stimulated with IFN- γ to generate M1 macrophages from M2c macrophages (M2c>M1). M1 macrophages as expected were highly susceptible to SM-LCL161-induced cell death. However, M1 macrophages when repolarized into M2c macrophages by IL-10 (M1>M2c) lost

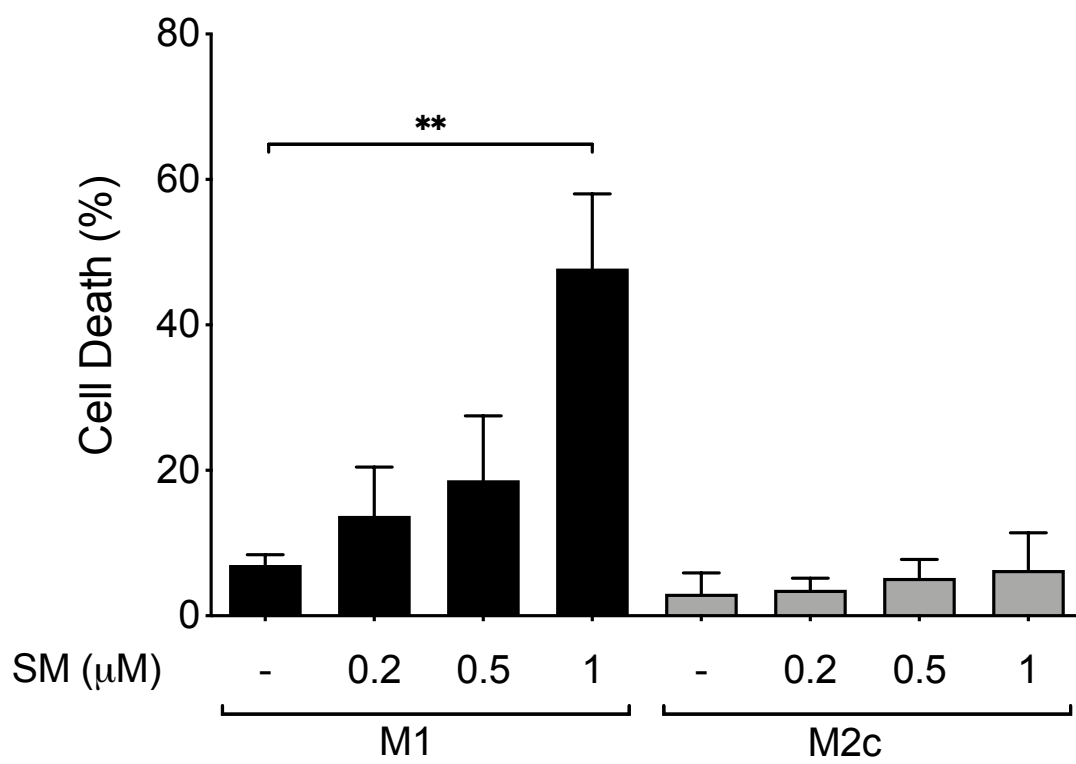
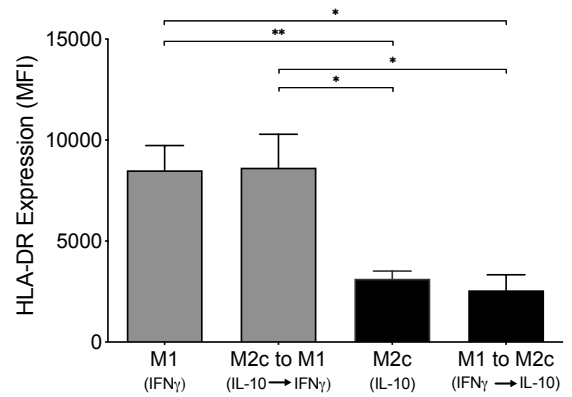
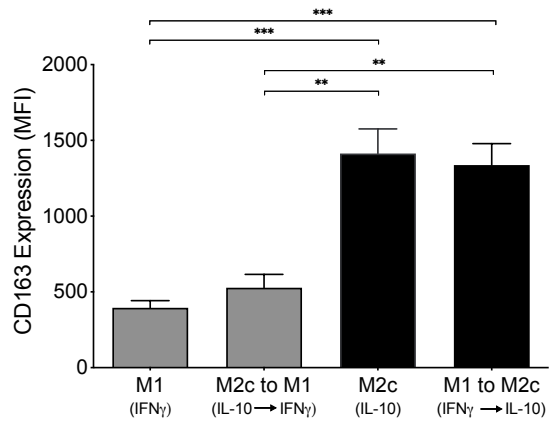
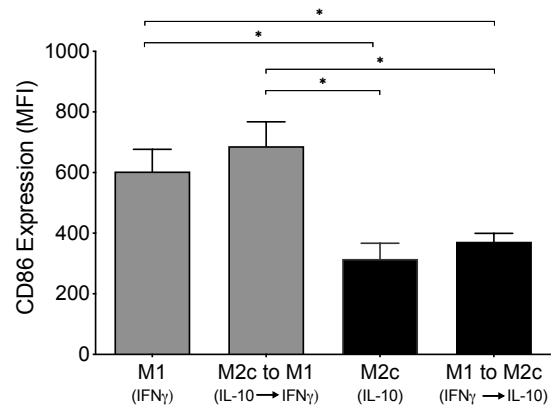
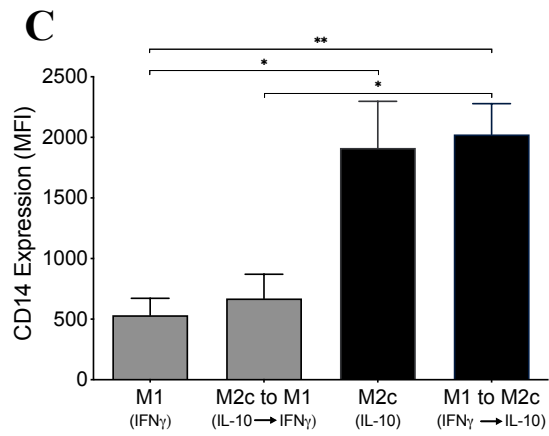
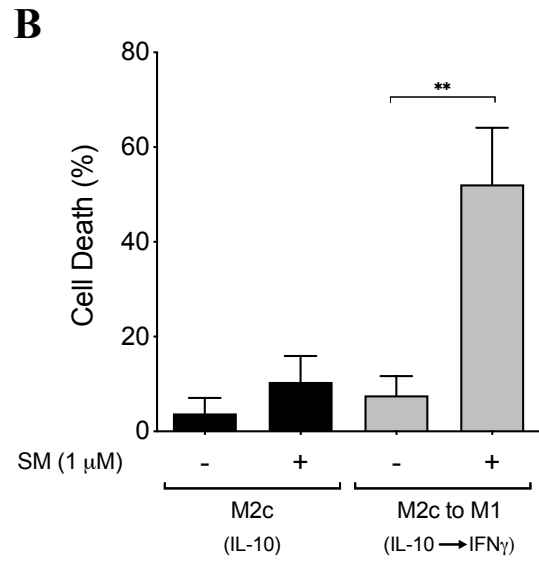
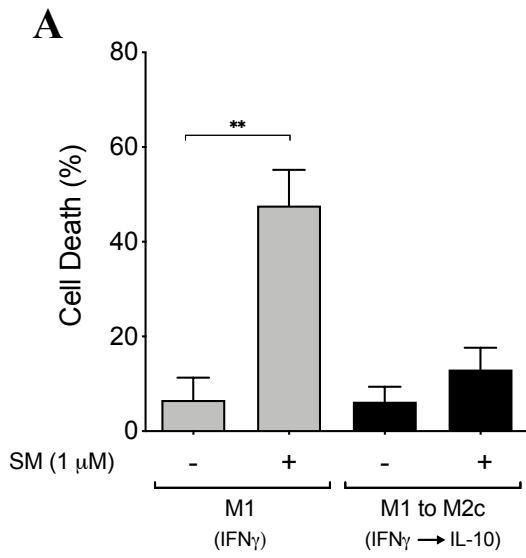


Figure 3.11: M1 mouse BMDMs are highly sensitive whereas M2c macrophages are resistant to SM-induced cell death.

BMDMs were stimulated with mouse *r*IFN- γ (M1) or *r*IL-10 (M2c) for 48 hrs. M1 and M2c mouse BMDMs were then treated with 0.2, 0.5 and 1 μ M of SM-LCL161 for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The results shown are a mean $\pm$ SD of three independent experiments from three different BMDMs.

that susceptibility (**Figure 3.12A**). On similar lines, M2c macrophages were resistant to SM-LCL161-induced cell death as expected. However, M2c macrophages when differentiated into M1 macrophages by IFN- γ (M2c>M1) developed susceptibility to SM-LCL161-induced cell death (**Figure 3.12B**).

Subsequently, we determined whether repolarization of M1 into M2c subset and of M2c into M1 subset reverses the expression of cell surface markers characteristic for M1 and M2c macrophages (**Figure 3.12C and D**). As above, M1 macrophages were repolarized into M2c macrophages (M1>M2c) and M2c were repolarized into M1 macrophages (M2c>M1) followed by analysis of cell surface markers characteristic for M1 and M2c macrophages namely CD14, CD86, CD163 and HLA-DR. The M2c macrophages expressed significantly high levels of CD14 and CD163 and significantly low levels of CD86 and HLA-DR compared to the M1 macrophages. Following repolarization of M2c into M1 macrophages (M2c>M1), repolarized M1 macrophages expressed significantly low levels of CD14 and CD163 and significantly high levels of CD86 and HLA-DR similar to the levels expressed by M1 macrophages. Similarly, M1 macrophages expressed significantly high levels of CD86 and HLA-DR and significantly low levels of CD163 and CD14 compared to the M2c macrophages. Following repolarization of M1 into M2c macrophages (M1>M2c), repolarized M2c macrophages expressed significantly low levels of CD86 and HLA-DR and significantly high levels of CD163 and CD14 similar to the levels expressed by M2c macrophages (**Figure 3.12C**). The representative histograms for surface marker expression on polarized and repolarized macrophages subsets are shown in **Figure 3.12D**.



D

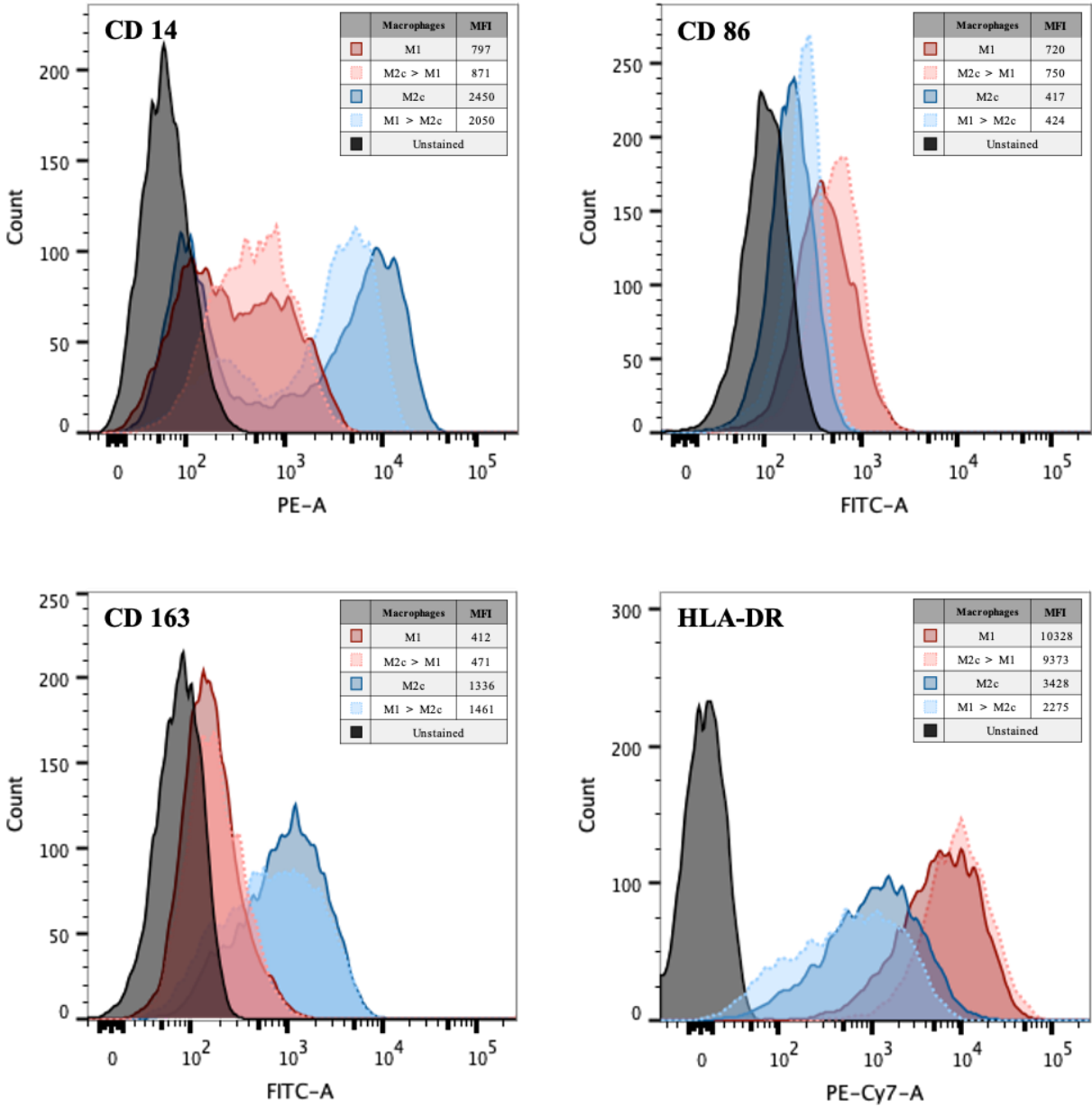


Figure 3.12: Repolarization of M1 into M2c and of M2c into M1 macrophage subsets reverses the effect of SM-LCL161.

Human M0 macrophages were stimulated for 48 hrs with (A) IFN- γ , or (B) IL-10. Cells were then washed, rested for 48 hrs and then restimulated with (A) IL-10, or (B) IFN- γ for another 48 hrs. Cells were treated with SM-LCL161 (1 μ M) for 48 hrs following IFN- γ or IL-10 stimulation and after restimulation with either IL-10 or IFN- γ . The % cell death was determined by intracellular PI staining and flow cytometry. (C) Cell surface markers expression on M1, M2c, M2c>M1 and M1>M2c macrophages. Cells were stained with antibodies against the mentioned cell surface markers and fluorescence was measured by flow cytometry. MFI values were obtained using FlowJo software. The result shown are a mean $\pm$ SD of three independent experiments from three different donors. (D) Overlay histograms from one representative experiment are shown.

Next, we examined whether repolarization of M1 into M2c subset and of M2c into M1 subset reverses the expression of caspase-3 and caspase-8, PARP, RIPK-1 and RIPK-3, the key effectors molecules of apoptosis and necroptosis pathways, following treatment with SM-LCL161. As above, M1 macrophages were repolarized into M2c macrophages (M1>M2c) and M2c were repolarized into M1 macrophages (M2c>M1) followed by treatment with SM-LCL161 and analysis of caspase-3 and caspase-8, PARP, RIPK-1 and RIPK-3 by Western immunoblotting. Treatment of repolarized M1 to M2c macrophages (M1>M2c) with SM-LCL161 did not affect the expression of full length or cleaved caspase-8, caspase-3 or PARP or degradation of RIPK-1 and RIPK-3 (**Figure 3.13**). Interestingly and as expected, treatment of repolarized M2c to M1 macrophages (M2c>M1) with SM-LCL161 did cause the degradation of full-length caspase-8, caspase-3 and PARP, accumulation of cleaved caspase-3 and caspase-8, accumulation of Bax and degradation of RIPK-1 and RIPK-3 (**Figure 3.13**). Overall, these results suggest that repolarization of M1 into M2c subset and of M2c into M1 subset reversed the effect of SM-LCL161 on cell death that was associated with the reversal in the expression of cell surface markers and the effectors of the cell death pathways such as caspases, Bax, PARP, RIPK-1 and RIPK-3.

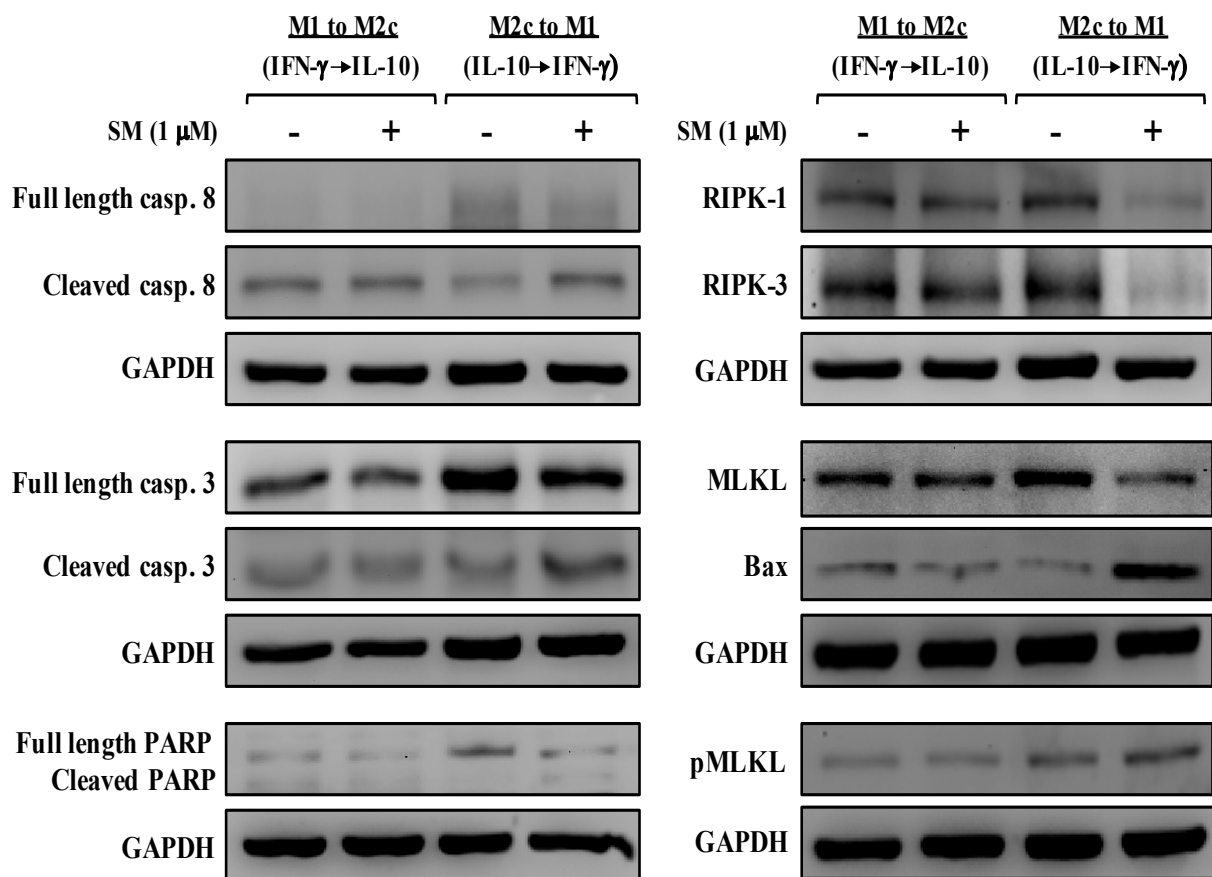


Figure 3.13: Repolarization of M1 into M2c and of M2c into M1 macrophage subsets reverses the effect of SM-LCL161 on the effector cell death molecules.

Total protein lysates from M1>M2c and M2c>M1 repolarized macrophages treated with 1 μ M of SM-LCL161 for 5 hrs were subjected to Western immunoblotting for the expression of caspases-8 (43 and 57 kDa), caspase-3 (19 and 35 kDa), PARP (89 and 116 kDa), RIPK-1 (78 kDa), RIPK-3 (46-62 kDa), MLKL (54 kDa), Bax (20 kDa) and pMLKL (54 kDa). The immunoblot shown is a representative of three independent experiments from three different donors.

3.3 DISCUSSION

SMAC mimetics have been actively studied for their apoptosis-inducing abilities in cancer cells (206, 220). However, the role of IAPs in regulating immune responses in primary cells and in particular primary human macrophages is not well understood. Primary human monocytes are highly susceptible to the cytotoxic effects of SMs (319). However, monocytic cells following differentiation into macrophages (M0) develop resistance to the cytotoxic abilities of SMs (317). Whether normal human macrophage subsets are also resistant to the cytotoxic effects of SMs remains unknown. Herein, we show that polarization of M0 macrophages towards M1 state rendered them highly susceptible to cell death in response to SM-LCL161 whereas M2a, M2b and M2c differentiated subsets, like their parent undifferentiated M0 cells, were resistant to SM-induced cell death, M2c being the most resistant. Furthermore, SM-induced cell death in M1 macrophages exhibited the characteristics of apoptosis as well as necroptosis and activated both extrinsic and intrinsic pathways of apoptosis. In contrast, M0 and M2c macrophages experienced cell death following simultaneous blockage of the IAPs pathways by SM-LCL161 and the caspase pathways by the pan-caspase inhibitors, and this cell death was attributed to necroptosis.

Phenotypic characterization revealed that macrophage subsets could be distinguished by the differential upregulation of cell surface markers. The results confirm our previous observations that pro-inflammatory M1 macrophages expressed high levels of CD80, CD86 and HLA-DR (43). CD80 and CD86 play key roles in Th1 and Th2 cell differentiation and generation of immune responses (340). The anti-inflammatory M2a macrophages exhibited a distinctly higher CD200R expression compared to M0. CD200-CD200R interactions in endothelial cells and macrophages delivers an inhibitory signal and inhibit their activation

(43). The viral homologues of CD200, such as herpesvirus 8K14 protein (341), cytomegalovirus e127 protein (342) and myxoma virus M141R (343) following interaction with CD200R on macrophages may inhibit macrophage function and may contribute to viral pathology. M2c macrophages expressed high levels of CD163 (43, 344). CD163 plays a role in preventing tissue inflammation (345, 346) that may also highlight the role of M2c in preventing tissue inflammation. M2b and M2c macrophages characteristically exhibited low levels of HLA-DR that may also be involved in dampening the immune responses mediated by stress, such as trauma, burns, surgery and sepsis (347). The expression of high levels of CD14, and CD80 on M2b macrophages may well explain pro-inflammatory nature of M2b macrophages (43, 86, 89, 108).

Initially described in the context of apoptosis regulation as promoting cell survival, the IAPs proteins have recently emerged as key regulators of innate immune signaling that promote inflammation (165). The role of IAPs family members has been studied by employing SMs (214, 215, 220). Several commercially available SMs are in clinical trials as anti-cancer agents (214). SM-LCL161, is a monovalent form of SMs that mimics the AVPI tetrapeptide binding motif present in natural SMAC (348). Therefore, SMs following binding to different IAPs promote their proteasomal degradations leading to IAPs downregulation and apoptosis particularly in cancer cells (214, 215, 348). We have previously shown that IAPs play a key role in the resistance of macrophages to HIV-Vpr-induced apoptosis and mitochondrial depolarization (319, 320). Herein, we show that although SM-LCL161 caused degradation of IAPs in all macrophage subsets tested, M1 macrophages only, were most sensitive to SM-induced cell death. Furthermore, SM-induced cell death was not attributed to the relative levels of cIAP-1 and cIAP-2 expressed in M0, M1 and M2c macrophages as M1 macrophages expressed

highest levels of cIAP-1 and cIAP-2 yet were highly susceptible to SM-induced cell death. On similar lines, although M2c macrophages expressed lowest levels of cIAP-1 and cIAP-2 yet were highly resistant to the SM-induced cell death. Our results also show that IAPs-mediated macrophage survival is determined by their state of differentiation. The susceptibility of macrophages to SM-induced apoptosis was attributed to the IFN- γ -mediated M1 differentiation as JAK inhibitor reversed the susceptibility of M1 macrophages to SM-induced cell death. Similarly, the resistance of macrophages to SM-induced apoptosis was attributed to the IL-10-mediated differentiation towards M2c state. This was further confirmed by reversal of susceptibility to SM-induced cell death following repolarization of M1 into M2c and of M2c into M1 subsets.

Our results show that SM-LCL161 induced apoptosis in M1 macrophages through activation and cleavage of caspase-3/7, -8 and -9, and blockage of caspases by pan-caspase inhibitors significantly reduced SM-induced cell death. SMs generally induce apoptosis in SM-sensitive tumor cell lines through the secretion of TNF- α in response to IAPs ablation and consequently become susceptible to TNF- α -induced apoptosis (216, 349, 350). cIAP-1 and cIAP-2 associate with the TNF- α receptor complex-I to mediate TNF- α -induced activation of the canonical NF- κ B pathway (165) and at the same time inhibit the non-canonical NF- κ B pathway by promoting constant degradation of NF- κ B-inducing kinase (NIK), the regulatory kinase of non-canonical NF- κ B signaling (125, 202, 206). The IAPs degradation by SMs facilitates TNF- α -stimulated cell death by promoting the assembly of RIPK-1 together with complex-IIa, comprising of proteins such as non-ubiquitinated RIPK-1, Fas-associated protein with death domain (FADD) and caspase-8 resulting in caspase-dependent apoptosis (192). The

IAPs degradation can also promote the formation of complex IIb, the necrosome complex containing RIPK-1 and RIPK-3, leading to necroptosis following blockade of caspase activation (192, 223, 242). The precise mechanism governing selective apoptosis and necroptosis in M1 macrophages following SMs treatment is under investigation.

Since SM-LCL161 caused the activation and cleavage of caspase-9, the caspase responsible for intrinsic apoptotic pathway, and caspase-8, the caspase responsible for extrinsic pathway of apoptosis (143, 164) suggest that SM-induced apoptosis involves the activation of both intrinsic and extrinsic pathways in M1 but not in M0 and M2c macrophages. The extrinsic pathway is activated via the release of TNF- α or the activation of Fas (143, 164). We have previously shown that macrophages subsets produce endogenous TNF- α in the absence of SMs treatment (43), thus, suggesting that TNF- α or other factors may be involved in activating the extrinsic pathway in M1 macrophages. SM-LCL161 treatment did not affect the expression of either Fas or TRAIL suggest that SM-induced apoptosis is not regulated by Fas or TRAIL activation in M1 macrophages. However M1 macrophages expressed higher levels of these proteins compared to M0 and M2c. The activation of the intrinsic pathway following SM-LCL161 treatment in M1 macrophages may be due to synergistic effect of the competitive inhibition of SMAC to the IAPs and the upregulation of these death receptors in M1 macrophages. The mechanism governing the activation of both extrinsic and intrinsic apoptotic pathways of apoptosis in M1 macrophages and not in M0 and M2c macrophages remains to be investigated.

The fact that zVAD.fmk, the pan-caspase inhibitor, did not fully restore cell survival in SM-treated M1 macrophages clearly suggested that SM-induced cell death in M1 macrophages may involve other cell death mechanisms most likely necroptosis, which works as a backup

mechanism in certain cells when apoptosis is inhibited (223). The signaling pathway involved in necroptosis observed following TNF- α stimulation has been studied. TNF- α binding to its receptor leads to the recruitment of TNFR-associated death protein (TRADD) and TNF receptor-associated factor-2 (TRAF2) and activation of RIPK-1. In the absence of caspase activation, activated RIPK-1 recruits RIPK-3 to form the necrosome (242, 351). Phosphorylation of MLKL by necrosome drives oligomerization of MLKL, causes plasma membrane to rupture and eventually leading to necroptosis (237, 242, 243). Since necroptosis inhibitors Necrostatin-1 (222) and NSA (237) inhibited SM-induced cell death suggested that SM-induced cell death is mediated by necroptosis. SMs have been shown to trigger necroptosis in various tumor cell lines including pancreatic (339), acute leukemic (336, 338), and breast cancer (337) cell lines in the presence of caspase inhibitors (zVAD.fmk and/or caspase-8 inhibitors). Similar to the cancer cell lines, M0 and M2c macrophages exhibited SM-induced necroptosis in the presence of zVAD.fmk. In contrast, we show for the first time that SM-LCL161 alone induced necroptosis in primary human M1 macrophages in the absence of caspase inhibitors. Moreover, simultaneous addition of inhibitors for both apoptosis and necroptosis completely prevented SM-induced cell death as well as signalling molecules RIPK-1, RIPK-3 and MLKL clearly indicated that SM-induced cell death in M1 macrophages is mediated by both apoptosis and necroptosis. Whether both processes are operative in the same cell, or different cells are undergoing different death mechanisms independently of each other is not clear and remains to be investigated. Moreover, the molecular mechanism(s) by which SMs alone induce necroptosis in M1 but not in M0/M2c macrophages is not known and needs further investigation.

Plasticity is one of the key features of macrophages (5, 7, 121). The results of our study show that M1 macrophages lose the susceptibility to SM-induced cell death once the same cells are cultured with IL-10, the stimuli for generating M2c macrophages. Inversely, M2c macrophages undergo cell death when stimulated with IFN- γ , the M1 generating stimulus. In addition, repolarization of M1 into M2c and of M2c into M1 subsets reversed the effect of SM-LCL161 with respect to expression of cell surface markers and signalling pathways associated with apoptosis and necroptosis pathways such as caspase-8, -3, PARP, Bax, RIPK-1, RIPK-3 and MLKL. These observations suggest that macrophage repolarization in response to their respective cytokines impacts their IAPs signaling and their responsiveness to SM-induced cell death. Furthermore, macrophage subsets are not an end stage of differentiation as their susceptibility to SM-induced cell death can be modulated by changing the cytokine environment. The M1 macrophage ability to undergo SM-induced cell death is transient and this transient status can last as long as these cells are exposed to the specific stimuli, IFN- γ .

In summary, the results suggest that survival of human macrophages is critically linked to the activation/blockage of the IAPs pathways and differentiation towards either the pro-inflammatory state by IFN- γ or the anti-inflammatory state by IL-10. Moreover, in addition to the existing therapeutic strategies such as to reprogram M1 macrophages or induce subsets switching (7, 121), selective induction of cell death in M1 macrophages by SM-LCL161 alone or in M0/M2c macrophages by SM-LCL161 in conjunction with caspase inhibitors would have potential therapeutic benefits in regards to macrophage associated inflammatory or anti-inflammatory diseases, respectively. Inflammatory M1 macrophages have been implicated in many diseases including rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis (5, 7, 352–355), whereas anti-inflammatory M2 macrophages have been associated

with lepromatous leprosy, Whipples disease and chronic rhinosinusitis (65, 96–98). Since SM-LCL161 effect on cell death is conserved across mouse and human M1 macrophage subsets, mouse model can be a useful experimental tool to study the cytocidal effect of SMs on several inflammatory diseases.

CHAPTER 4:

The molecular mechanism governing SM-induced cell death in M1 macrophages

Selectively induction of cell death in human M1 macrophages by Smac mimetics is mediated by cIAP-2 and RIPK-1/3 through the activation of mTORC

4.1 INTRODUCTION

Macrophages can be generally described as either “classically activated” M1 macrophages or “alternatively activated” M2 macrophages based on their distinct inductive stimuli, phenotypic and functional characteristics (4, 42–44, 89). These broad categories of polarized macrophages represent the two extreme ends of macrophages polarization. M1 macrophages can be induced by cytokines such as TNF- α , IFN- γ or by intracellular pathogens, and bacterial lipopolysaccharide (LPS) (7, 23, 42). M1 macrophages exhibit enhanced phagocytic capacity and antigen presentation ability, secrete high levels of pro-inflammatory cytokines such as TNF- α , IL-12, IL-6, IL-1 β and toxic molecules such as iNOS-dependent reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) (7, 42, 49, 50). In contrast, IL-4, IL-13, IL-10 or TGF- β induce macrophage polarization toward anti-inflammatory phenotype known as alternatively activated M2 macrophages (43, 68, 69). Depending upon the specific stimuli, M2 macrophages can be further classified into M2a, M2b, or M2c subsets (43). In general, IL-4 and/or IL-13 polarize macrophages toward M2a subset whereas M2b subset can be generated following stimulation with immune complexes and TLR-agonists or IL-1 receptor ligands (43, 86, 89). Lastly, M2c subset can be generated by glucocorticoids and/or IL-10 (43, 89, 108).

Although macrophage subsets are not the end-stage differentiated macrophages and are highly plastic as shown earlier in chapter 3 and others (5, 7, 121), distinct macrophage subsets have been shown to play a role in disease pathogenesis (5, 356). Macrophages play an important role as a first line of defense against intracellular pathogens and bacterial infection (23, 50, 65). In proinflammatory diseases, the changes in the tissue microenvironment drives macrophages polarization toward M1 phenotype to control the microbial infection and further

induce inflammatory response (5, 357). After a successful microbial clearance, the microenvironment drives macrophages toward M2 anti-inflammatory stage of macrophages, which helps in resolving the inflammatory responses and promote wound healing (5, 51). However, excessive or inability to resolve the inflammatory responses may cause damage to the surrounding microenvironment and could lead to several human diseases such as cancer, chronic infection, or autoimmune diseases. For example; M1 polarization signature have been shown to be associated with human colon carcinoma, inflammatory bowel disease, rheumatoid arthritis (53–56), typhoid fever, tuberculoid leprosy, active tuberculosis and *Helicobacter pylori* gastritis (64–66, 96). Conversely, M2 polarization program is observed in lepromatous leprosy, Whipples disease and chronic rhinosinusitis (96–98).

We have previously shown that M1 macrophages can selectively be killed by targeting the inhibitor of apoptosis proteins 1 and 2 (cIAP1 and cIAP2) using Smac mimetic, SM-LCL161 (SM) through apoptosis as well as necroptosis. In contrast, unpolarized M0 and M2c macrophages were highly resistant to SM-induced cell death (**Figure 3.2**). Herein, we extended these observations by investigating the molecular mechanism governing SM-induced cell death in M1 macrophages. My results show that SM-induced cell death in M1 macrophages is independent of TNF- α , as well as canonical and non-canonical NF- κ B pathways. SM-induced cell death in M1 macrophages was shown to be regulated by the mTORC pathway, RIPK-1 and RIPK-3 kinase degradation and IRF-1 transcription factor. These observations hold a promising therapeutic strategy to reduce the proinflammatory M1 macrophages and control diseases associated with increased inflammatory responses.

4.2 RESULTS

4.2.1 The IAPs antagonist, SM-LCL161 selectively induces cell death in human M1 macrophage subset

I have previously characterized human M0, M1, M2a, M2b and M2c macrophages by using a non-exhaustive panel of surface markers (**Figure 3.1**). The pro- and anti-inflammatory cytokines production by these subsets has also been assessed (43). I have shown that M1 subset is characterized by high expression of CD80, CD86, and a low expression of CD163. Moreover, M1 macrophages produced high levels of IFN- γ , IL-12, TNF- α and IL-23 following stimulation with TLR-2, TLR-3 and TLR-4 ligands. M2a subset expressed low levels of CD14, CD163, high levels of HLA-DR, CD 200R, CD 86 surface markers and IL-10 production. M2b subset expressed high levels of CD14, CD80 and low levels of CD200R, HLA-DR and produced IL-6. On the other hand, M2c subset expressed high levels of CD163, CD 14 and low levels of CD86, HLA-DR and produced high levels of IL-10 (**Figure 3.1**) (43). To investigate whether these macrophage subsets also differed in regulating IAPs signaling pathway and their survival, we used the monovalent form of IAPs antagonist SM-LCL161 (SM). Differentiation of M0 macrophages towards M1 state rendered them highly susceptible to SM-induced cell death, whereas M2a, M2b and M2c differentiated subsets were resistant, with M2c being the most resistant (**Figure 3.2**). Herein, I have confirmed our previous findings with more emphasis on M1, M0 and M2c macrophages subsets. MDMs were polarized into M1, M2c subsets with IFN- γ and IL-10, respectively or left unpolarized (M0). After 48 hrs of polarization, these macrophage subsets were treated with different concentrations of SM-LCL161 for another 48 hrs followed by analysis of cell death by intracellular PI staining and flow cytometry as previously described (**Chapter 3**). Herein, the results confirm my previous

findings that M1 is highly sensitive to SM-LCL161 treatment with nearly 60% reduction in cell survival after SM-LCL161 treatment compared to untreated (**Figure 4.1**). In contrast, M0 and M2c subsets remained resistant to SM-induced cell death. This result suggests that IAPs signaling pathway is differentially regulated in macrophages subsets and that blocking IAPs signaling selectively induces cell death in M1 macrophage subset.

4.2.2 Molecular mechanism governing SM-induced cell death in M1 macrophages

I have previously shown that SM-induced cell death in M1 macrophages was mediated by apoptosis as well as necroptosis and activated both extrinsic and intrinsic pathways of apoptosis (**Figure 3.5 and 3.6**). The susceptibility of M1 macrophages to SM-induced cell death was attributed to the IFN- γ -mediated polarization as JAK inhibitor reversed their susceptibility (**Figure 3.7**). In contrast, M2c and M0 macrophages experienced cell death through necroptosis pathway following simultaneous blockage of the IAPs pathways by SM-LCL161 and the caspase pathways by the pan-caspase inhibitors (**Figure 3.8 and 3.9**). In the next series of my investigations, I will elucidate as below the role of TNF- α , signaling pathways such as PI3K-Akt-mTOR pathway, MAPKs and transcription factors NF- κ B, IRF-1 and PKR and the pro- and anti-apoptotic genes in SM-induced apoptosis in M1 macrophages.

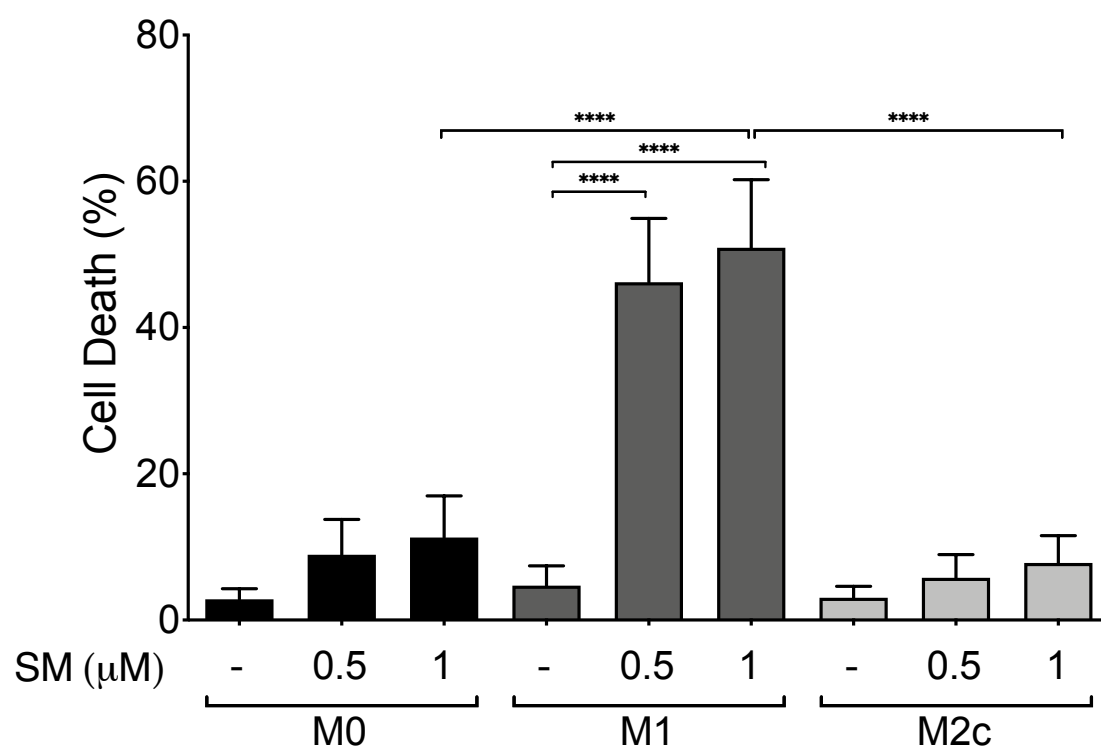


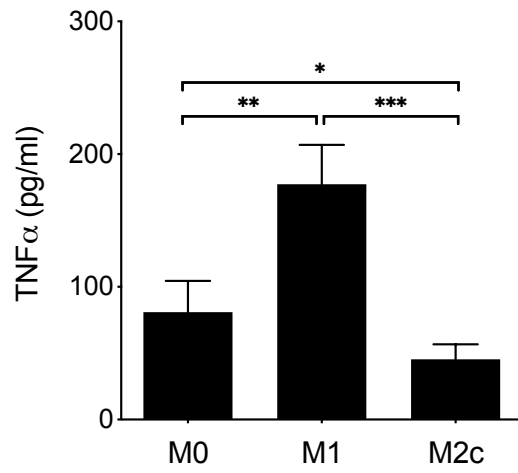
Figure 4.1: M1 macrophages are sensitive whereas M2c macrophages are resistant to SM-induced cell death.

M0, M1, and M2c macrophage subsets were left untreated or treated with 0.5 μ M and 1 μ M of SM-LCL161 for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of eleven independent experiments from different donors.

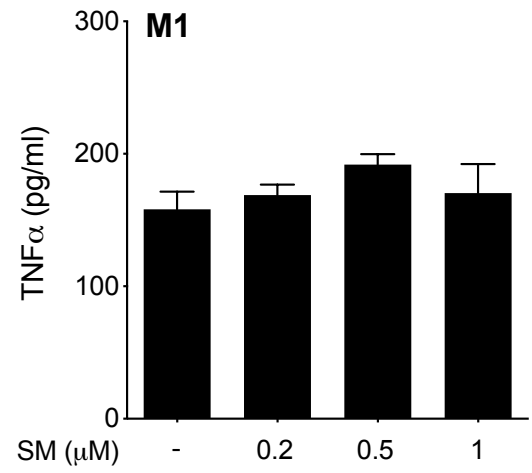
4.2.3 TNF- α failed to regulate cell death seen in SM-treated M1 macrophage subset

Endogenously produced TNF- α has been shown to mediate SM-induced cell death in various cells especially tumor cell lines (216, 349, 350, 358). SMs induce the production of TNF- α that, synergizes with SMs to induced cell death in various cancer cells and cell lines (202, 216, 350, 359, 360). Therefore, I first determined whether M1 macrophages produce higher levels of TNF- α compared to that produced by M0 and M2c macrophages either spontaneously or following treatment with SM-LCL161. To determine the endogenous levels of TNF- α before and after SM treatment, M0, M1 and M2c were polarized for 48 hrs and the supernatants were collected and subjected to ELISA for measuring TNF- α production. At the same time, M0, M1 and M2c macrophages were treated with various concentrations of SM-LCL161 for 48 hrs followed by collection of supernatants and TNF- α level measurement. My results show that all three subsets produced different levels of endogenous TNF- α spontaneously after 48 hrs of polarization (**Figure 4.2A**). Interestingly, M1 macrophages produced significantly higher levels of TNF- α compared to the M0 and M2c subsets. Moreover, SM-LCL161 at any concentration did not induce the production of TNF- α compared to that produced by untreated macrophages (**Figure 4.2B, C and D**). Although M1 macrophages produced higher levels of TNF- α , SM-LCL161 did not induce higher levels of TNF- α after treatment (**Figure 4.2B**). These results suggest that contrary to the tumor cell lines, SM-LCL161 did not induce TNF- α production in any subset of primary human macrophages.

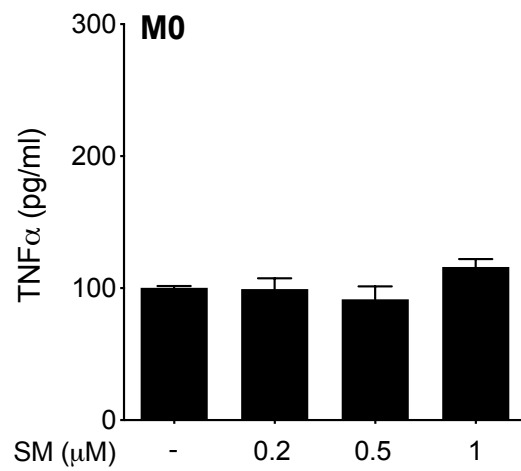
A TNF- α after 2 days of polarization



B TNF- α after SM treatment



C TNF- α after SM treatment



D TNF- α after SM treatment

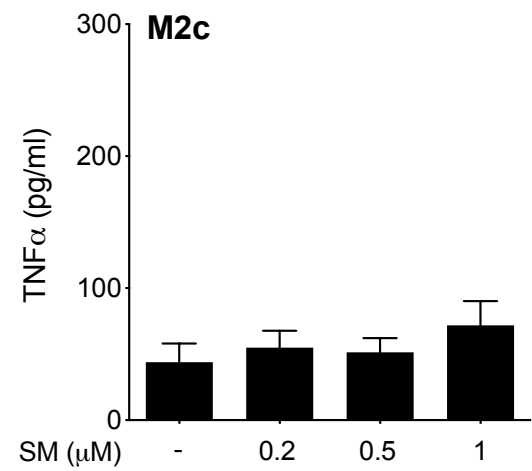


Figure 4.2: M0, M1 and M2c macrophages produce TNF- α spontaneously and following SM-LCL161 treatment.

(A) TNF- α production by different macrophage subsets after 48 hrs of polarization determined by ELISA assay. TNF- α production by (B) M1, (C) M0, and (D) M2c macrophage subsets treated with SM-LCL161 (0.2, 0.5 and 1 μ M) for 48 hrs. The data represent a mean $\pm$ SD of three independent experiments of three different donors.

Since M0 and M2c produced lower levels of TNF- α compared to M1 subset, I investigated whether enhancing TNF- α production by LPS activation would affect their sensitivity to SM-LCL161 treatment. I stimulated M0, M1 and M2c macrophages with LPS for 24 hrs followed by collection of the supernatants and assessment of TNF- α production. As seen in (**Figure 4.3A**), LPS stimulation induced high levels of TNF- α production (>1000 pg/mL) in all the three subsets. However, M1 still produced the highest amounts of TNF- α (~ 3000 pg/mL) that was twice the levels produced by M0 and M2c macrophages. To determine whether enhancing TNF- α production by LPS would affect SM-induced cell death, M0, M2c and M1 were stimulated with LPS for 24 hrs and then treated with different concentrations of SM-LCL161 followed by assessment of cell death. My results show that LPS-stimulated M0 and M2c macrophages were still resistant to cell death induced by any concentrations of SM-LCL161 (**Figure 4.3B and C**). Furthermore, as expected treatment with SM-LCL161 alone induced cell death in M1 macrophages. However, stimulation of M1 macrophages with LPS did not lead to a significant increase in cell death at any concentration of SM-LCL161 treatment (**Figure 4.3D**).

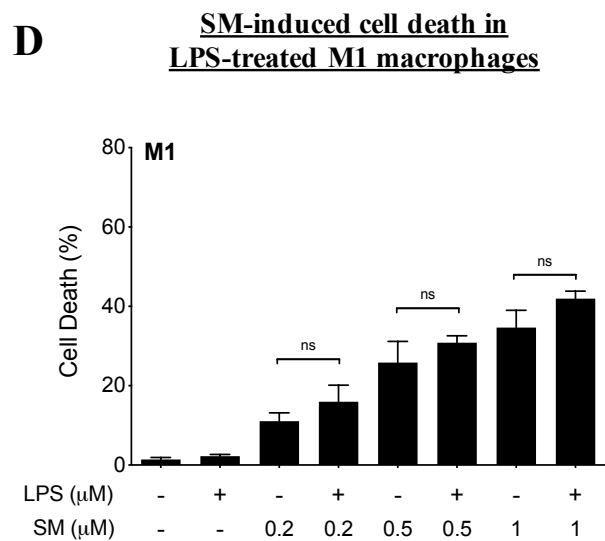
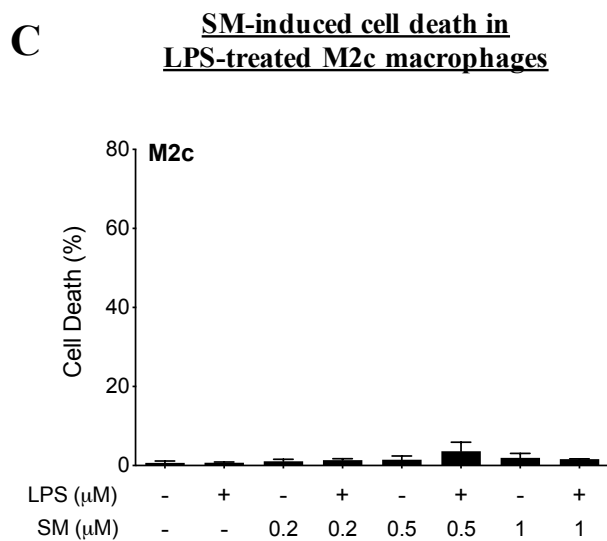
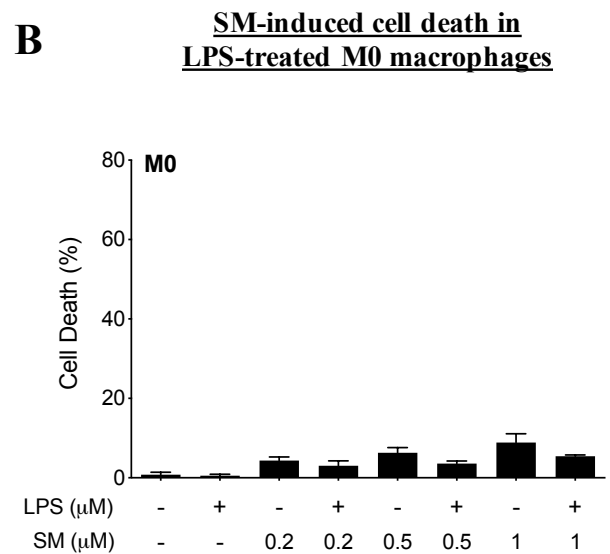
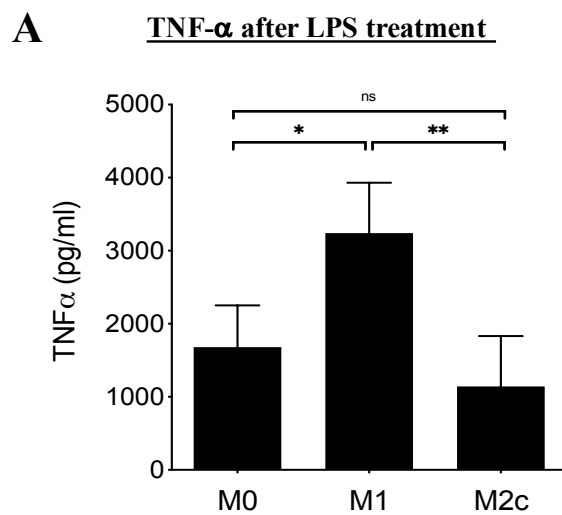


Figure 4.3: SM-LCL161 does not induce cell death in LPS-treated M0, M1 and M2c macrophages.

(A) TNF- α production of M0, M1 and M2c macrophage subsets after LPS stimulation for 24 hrs determined by ELISA assay. (B) M0 and (C) M2c (D) M1 macrophage subsets were left untreated, treated with SM-LCL161 (0.2, 0.5 and 1 μ M), LPS (10 ng/mL) or SM and LPS combined treatment for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The data represent a mean $\pm$ SD of three independent experiments of three different donors.

LPS stimulation affects the activation status of macrophages that may be responsible for resistance to cell death in M0 and M2c macrophage subsets. Therefore, to rule out the involvement of macrophage activation after LPS treatment, and to directly determine the involvement of TNF- α , M0, M2c and M1 macrophage subsets were directly treated *ex vivo* with different concentrations of human recombinant TNF- α (*rh*TNF- α) an hour prior to SM-LCL161 treatment (**Figure 4.4**). My results show that *rh*TNF- α -treated M0 and M2c were still resistant to SM-induced cell death (**Figure 4.4A and B**). As expected, SM-LCL161 treatment induced cell death in M1 macrophages. However, addition of any concentration of *rh*TNF- α (5-20 ng/mL) to SM-treated M1 macrophages failed to significantly increase cell death compared to the cell death observed following SM-LCL161 treatment alone. (**Figure 4.4C**). These results suggested that SM-induced cell death may occur independently of TNF- α in primary human M1 macrophages.

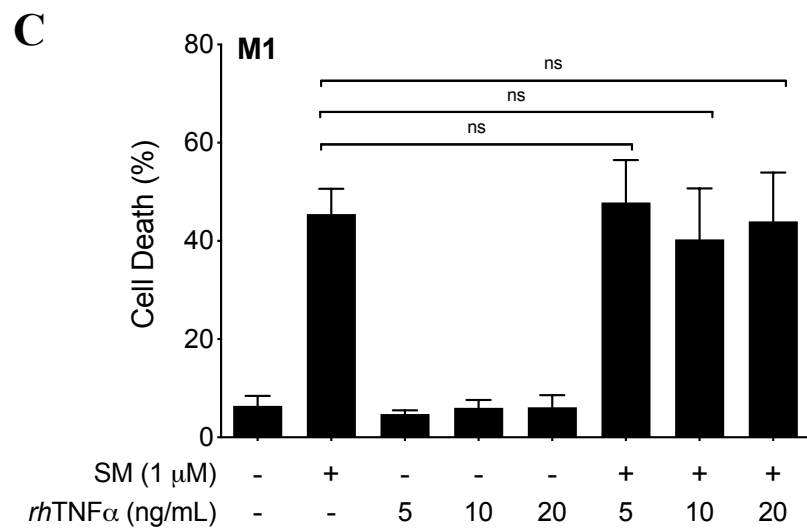
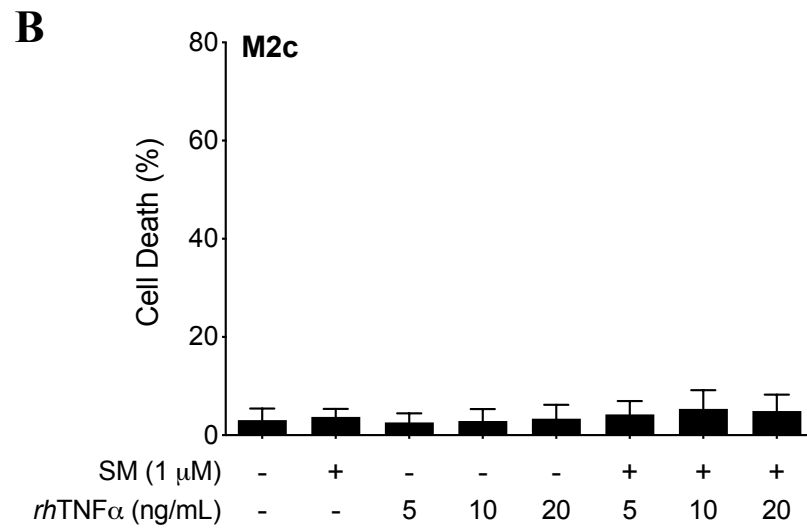
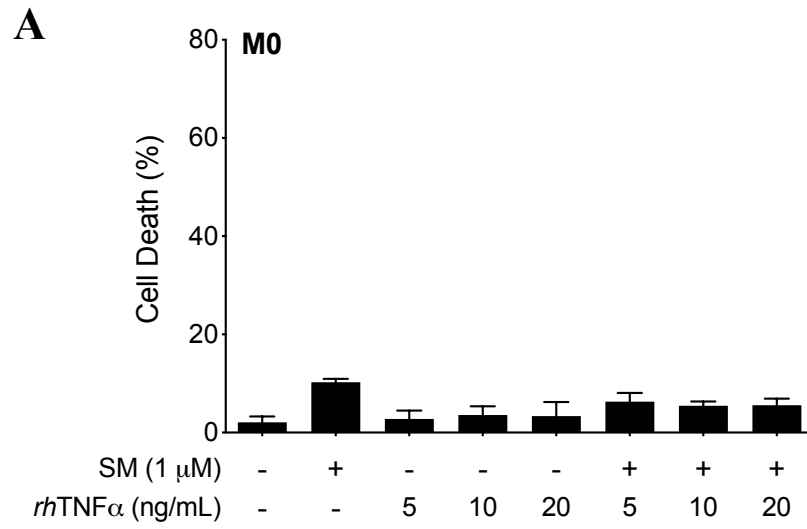
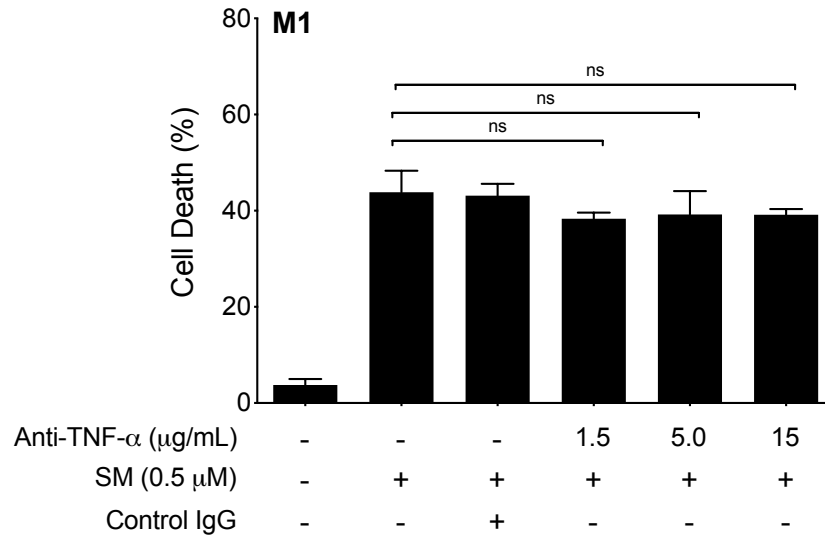
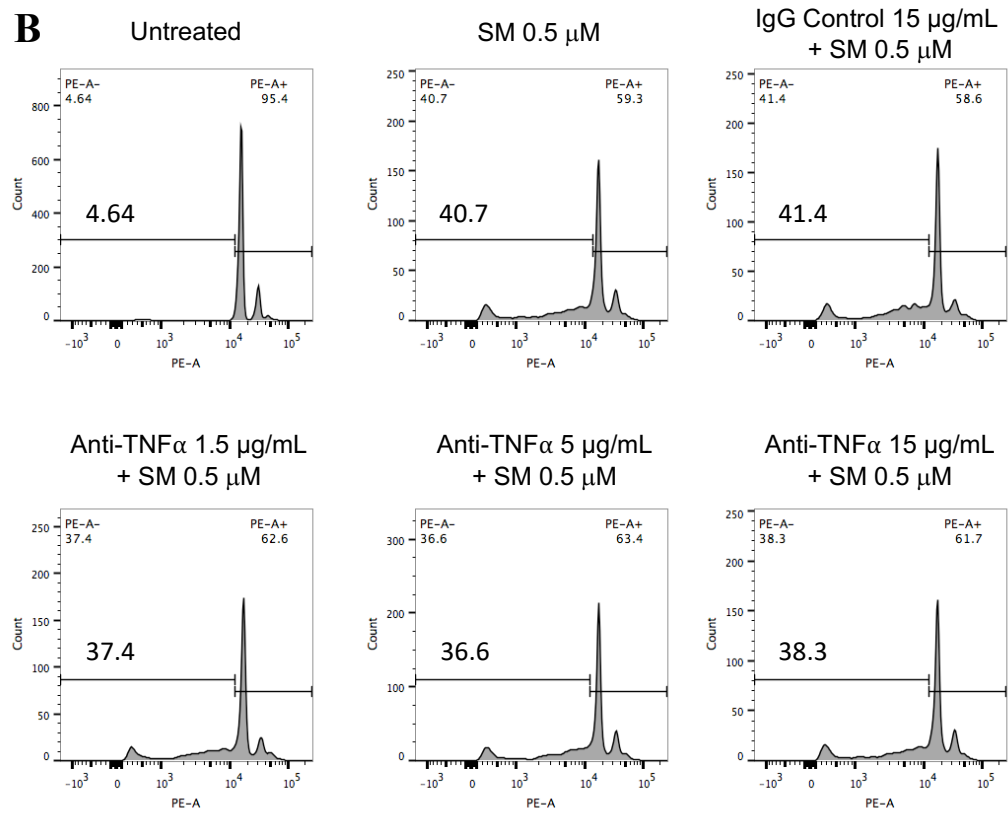


Figure 4.4: Recombinant human TNF- α (*rh*TNF- α) does not induce cell death in SM-treated M0, M2c or M1 macrophages.

(A) M0 (B) M2c (C) M1 macrophage subsets were left untreated, treated with *rh*TNF- α (5, 10 and 20 ng/mL), SM-LCL161 (1 μ M) alone or combined treatment of *rh*TNF- α and SM-LCL161. The % cell death was determined by intracellular PI staining and flow cytometry. The data represent a mean $\pm$ SD of three independent experiments of three different donors.

To further rule out the involvement of TNF- α in SM-induced cell death of M1 macrophages, I neutralized the effect of TNF- α in SM-treated M1 macrophage using anti-TNF- α neutralizing antibodies. M1 macrophages were treated with various concentrations of anti-TNF- α neutralizing antibodies for 2 hrs prior to treatment with 1 μ M of SM-LCL161. M1 macrophages were still sensitive to SM-induced the cell death (**Figure 4.5A and B**). The addition of anti-TNF- α antibodies at any concentration ranging from 1.5 to 15 μ g did not significantly reduce SM-induced cell death in M1 macrophages compared to the cell death observed with isotype matched control antibodies (**Figure 4.5A**). The representative histograms showing the effect of anti-TNF- α antibodies on SM-induced cell death of M1 macrophages (**Figure 4.5B**).

The biological activity of the neutralizing anti-TNF- α antibodies was determined by using standard SNB-75 cell line. SNB-75 is a human SNB-75 glioblastoma cell line that was used to confirm the ability of the antibody to neutralize the effect of TNF- α . SM-LCL161 was able to induce cell death in this cell line only when it was combined with *rh*TNF- α . SM-LCL161 alone or TNF- α alone (3 ng/mL) did not induce cell death in SNB-75 cells. The addition of anti-TNF- α antibodies (15 μ g/mL) to SM-treated SNB-75 cells significantly reduced cell death comparable to the cell death observed in the presence of isotype control antibodies (15 μ g/mL) and SM-LCL161 (**Figure 4.5C**). Collectively, these data suggest that SM-LCL161 does not affect the production of endogenous TNF- α levels in primary human M1 macrophages. Moreover, SM-induced cell death in M1 macrophages is TNF- α independent.

A**Anti-TNF- α neutralizing antibody****B**

C

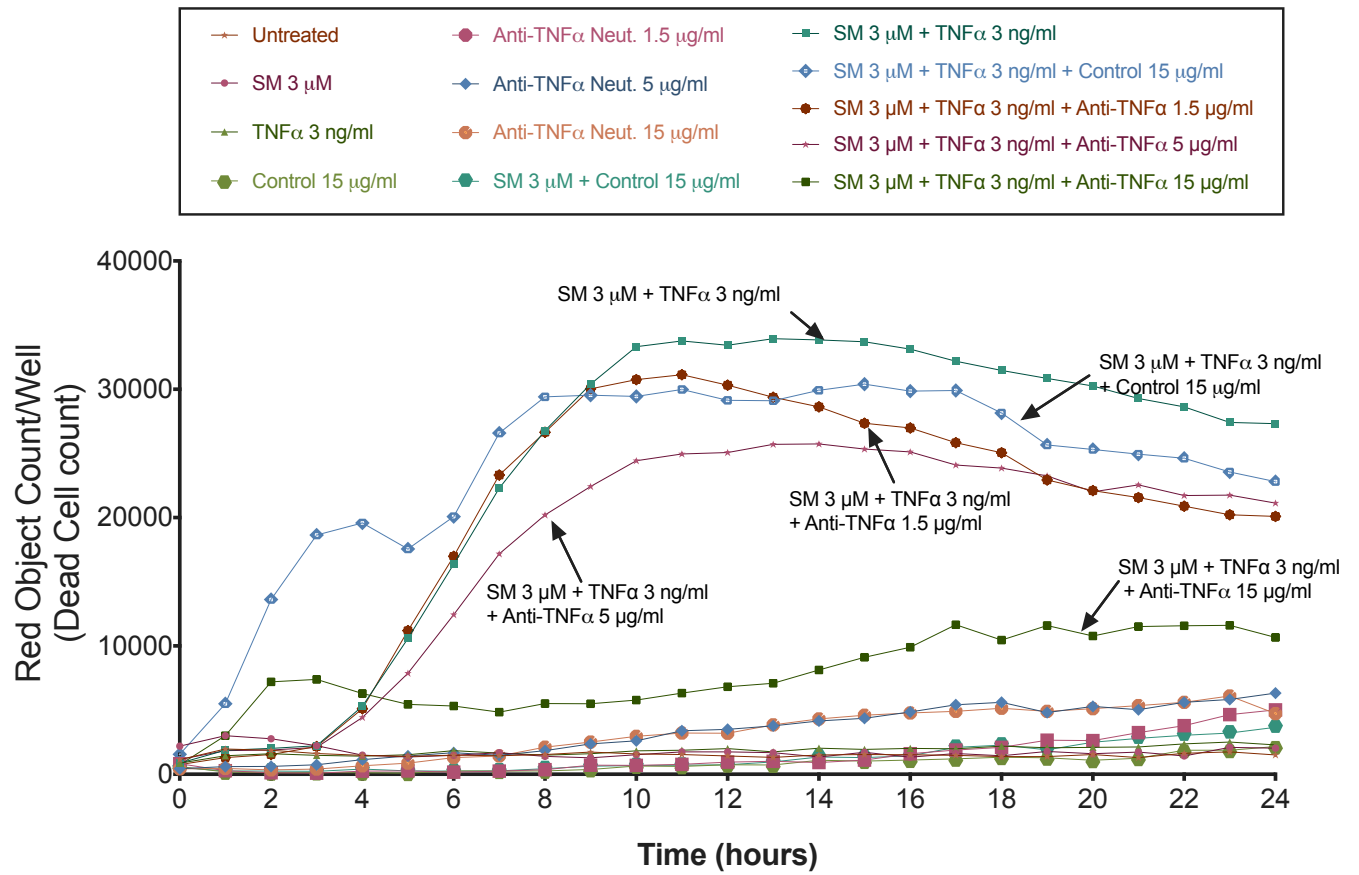


Figure 4.5: Endogenously produced TNF- α does not mediate SM-induced cell death in M1 macrophages.

(A) M1 macrophages were treated with anti-TNF- α neutralizing antibodies (1.5, 5 and 15 $\mu\text{g/mL}$) for 2 hrs prior to SM-LCL161 (0.5 μM) treatment for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The data represent a mean $\pm$ SD of three independent experiments of three different donors. (B) A representative figure of flow cytometry analysis of M1 macrophages treated with different concentrations of anti-TNF- α neutralizing antibodies. (C) SNB 75 cell line treated with TNF- α (3 $\mu\text{g/mL}$), SM-LCL161 (3 μM), anti-TNF- α neutralizing antibodies (1.5, 5 and 15 $\mu\text{g/mL}$), control IgG (15 $\mu\text{g/mL}$) or combined treatments. Red objective count per well determined by IncuCyteTM automated machine and collected every 2 hrs.

4.2.4 SM-LCL161 does not activate either canonical or non-canonical NF- κ B

Since SMs are well known to degrade cIAP1/2, and IAPs regulate NF- κ B signaling (206, 361), I evaluated if SM-LCL161 induce cell death through NF- κ B signaling in M1 macrophages. cIAPs ablation by SMs has been shown to promote canonical pathway of NF- κ B activation in cancer cells due to increased TNF- α secretion, which promotes degradation of I κ B α protein and subsequent release of p65 and p50 subunits of NF- κ B (202, 359). To determine the effect of SM-LCL161 on canonical pathway of NF- κ B activation in human M1 macrophages, we measured the level of I κ B α protein as an indirect indicator of the canonical NF- κ B activity. For this, M0 macrophages were treated with 0.5 and 1 μ M of SM-LCL161. LPS stimulation was used as a positive control for canonical NF- κ B activation. Cells were assayed for I κ B α expression by Western immunoblotting. In contrast to previous results obtained with cancer cells (359, 362), SM-LCL161 alone did not affect I κ B α expression in human macrophages. As expected, LPS stimulation decreased I κ B α levels, indicative of NF- κ B activation. However, LPS-induced degradation of I κ B α was not affected by prior treatment of macrophages with SM-LCL161 (**Figure 4.6A**), suggesting that SM-LCL161 do not affect the canonical pathway of NF- κ B activation. Although LPS-induced NF- κ B activation is dependent on cIAPs in human macrophages, SM-LCL161 do not modulate the LPS-activated NF- κ B pathway in these cells.

Non-canonical regulation of NF- κ B depends on NF- κ B-inducing kinase (NIK), which promotes partial processing of the p100 subunit to its p52 mature form (204, 363). In resting cells, this pathway is subdued through continuous NIK degradation performed by cIAP1/2 (125, 202). SMs treatment results in NIK accumulation and subsequent activation of non-

canonical NF- κ B pathway in cancer cells (202, 203, 359, 364). To determine the effect of SMs on non-canonical NF- κ B signaling in human macrophages, M0 macrophages were treated with 0.5 and 1 μ M of SM-LCL161 as above. LPS stimulation was again used as a positive control. Cells were assayed for p100 to p52 conversion by Western immunoblotting. As expected, LPS stimulation induced the expression of p100 and its subsequent processing to p52. SM-LCL161, in contrast to cancer cells (202, 203, 359, 364), did not affect p100 processing in macrophages suggesting that SM-LCL161 does not activate non-canonical pathway of NF- κ B activation. **(Figure 4.6B)**. Collectively, the results demonstrated that SM-LCL161 treatment does not activate canonical or non-canonical pathways of NF- κ B.

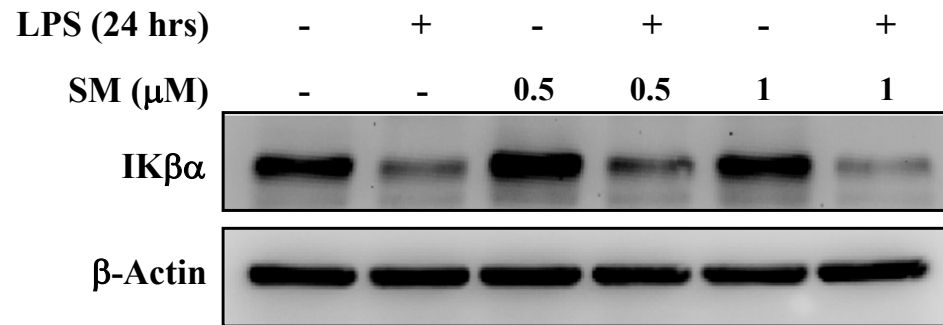
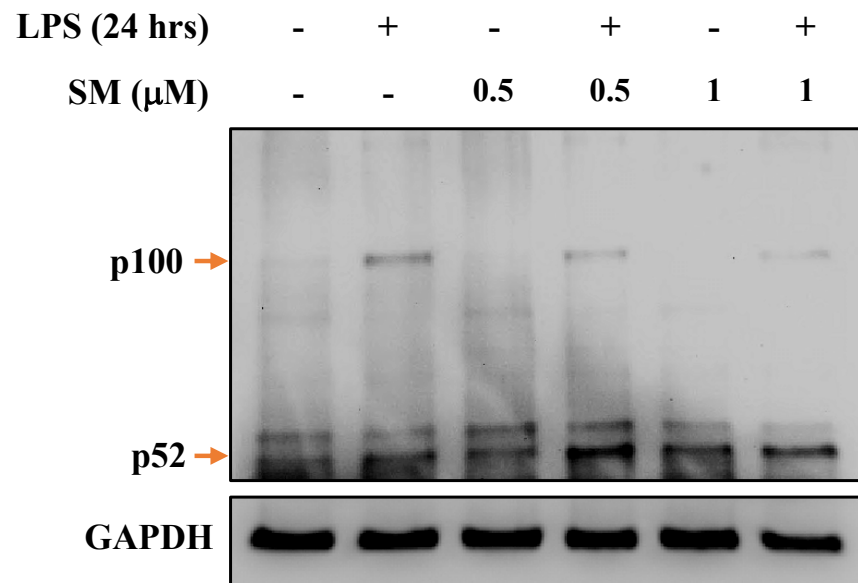
A**B**

Figure 4.6: SM-LCL161 does not affect canonical or non-canonical pathway of NF- κ B activation in human macrophages.

M0 macrophages were left untreated or treated with LPS (24 hrs), SM-LCL161 (0.5 and 1 μ M) or LPS and SM-LCL161 combined treatment. Cells were collected after 5 hrs and the total protein lysates were analyzed for (A) IK β α (39 kDa), or (B) p100 and p52 (120/52 kDa) expressions by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

4.2.5 NF- κ B pathway is not involved in SM-induced cell death in M1 macrophages

Next, we investigated if blocking NF- κ B pathway would rescue SM-treated M1 macrophages. M0 macrophages were treated with various concentrations of NF- κ B inhibitor PS-1145, that inhibits the I κ B kinase (IKK) for 2 hrs followed by IFN- γ stimulation for 48 hrs (pre-polarization). Alternatively, M1 macrophages first polarized by IFN- γ for 48 hrs were treated with PS-1145 for 2 hrs (post-polarization). Subsequently, M1 macrophages were treated with SM-LCL161 for another 48 hrs followed by assessment of cell death. My result show that blocking NF- κ B pathway by PS-1145 either prior to or after polarization into M1 macrophages did not rescue SM-LCL161 treated M1 macrophages (**Figure 4.7A and B**). The biological activity of PS-1145 was confirmed by inhibition of IFN- γ induced phosphorylation of IKK β expression (**Figure 4.7C**). Overall, the data suggest that NF- κ B pathway is not involved in SM-induced cell death in M1 macrophages.

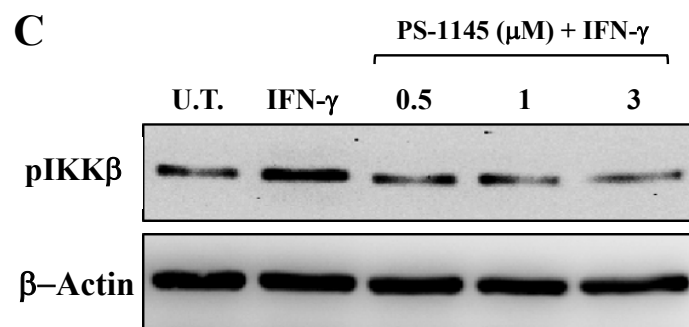
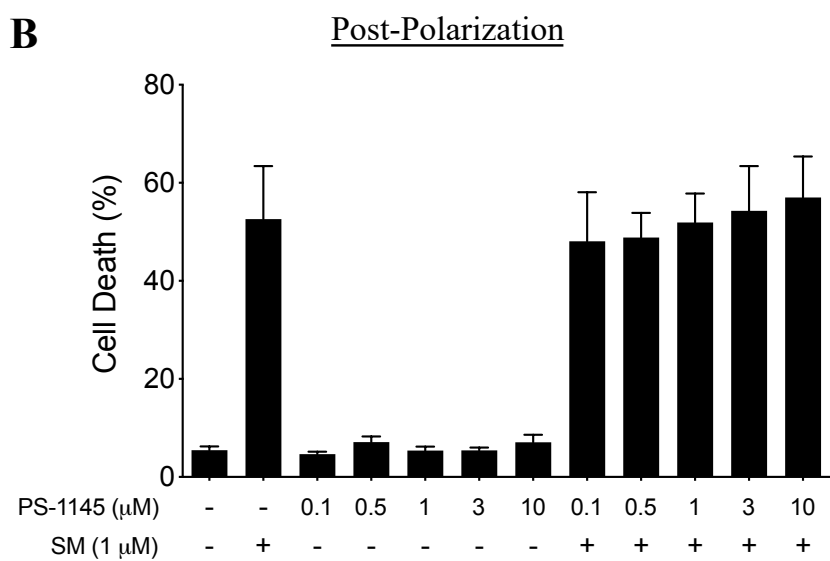
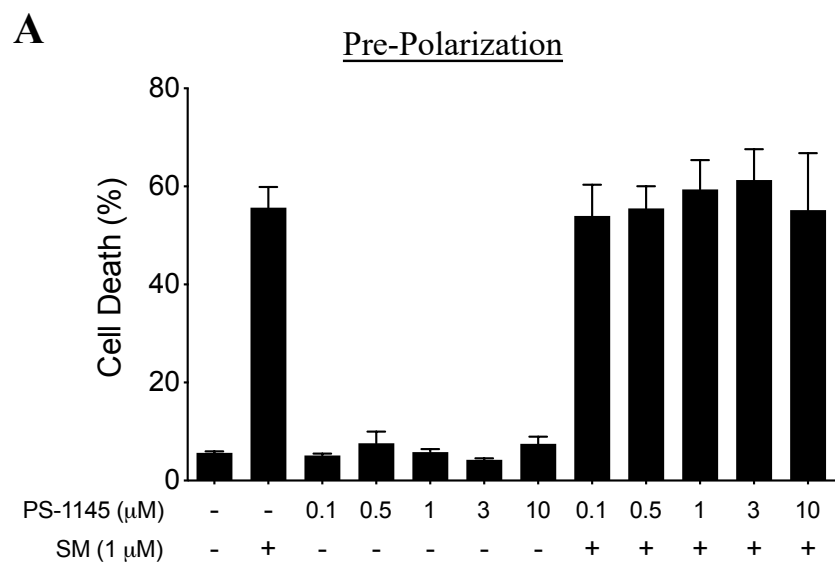


Figure 4.7: Blocking NF- κ B either before or after M1 polarization does not inhibit cell death in SM-treated M1 macrophages.

(A) M0 macrophages were treated with various concentrations of NF- κ B inhibitor (0.1, 0.5, 1, 3 and 10 μ M) for 2 hrs prior to stimulation with IFN- γ for 48 hrs (pre-polarization). (B) For post-polarization, M1 macrophages were treated with same concentrations of NF- κ B inhibitor for 2 hrs after 48 hrs stimulation with IFN- γ . Cells were then left untreated or treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (C) M0 macrophages were left untreated or treated with NF- κ B inhibitor (0.5, 1 and 3 μ M) for 2 hrs prior to stimulation with IFN- γ for 30 min. Total protein lysates were analyzed for pIKK β expression (87 kDa) by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

4.2.6 Involvement of IFN- γ activated pathway in SM-induced cell death of M1 macrophage

IFN- γ mediates the polarization of M0 into M1 macrophage subset. Therefore, we hypothesized that IFN- γ -induced differentiation to M1 macrophages confer the sensitivity of M1 cells to SM-induced cell death and blocking IFN- γ signaling would reverse the effect SM-induced cell death. IFN- γ mediates its activity through the activation of JAK/STAT signaling pathway (260, 261). To determine the involvement of JAK/STAT pathway, M1 macrophages were treated with varying concentrations of JAK inhibitor (Ruxolitinib; RUX), for 2 hrs prior to stimulation with IFN- γ . As seen in **Figure 4.8A** and in **Figure 3.7 A**, blocking JAK signaling by RUX significantly reduced cell death in SM-treated M1 macrophages in a dose dependent manner. RUX alone did not affect cell survival at any concentration in the absence of SM-LCL161. **Figure 4.8B** and **Figure 3.7B** shows the biological activity of RUX in M1 cells by Western immunoblotting. IFN- γ induced JAK-1 phosphorylation that was significantly inhibited by RUX in a dose-dependent manner.

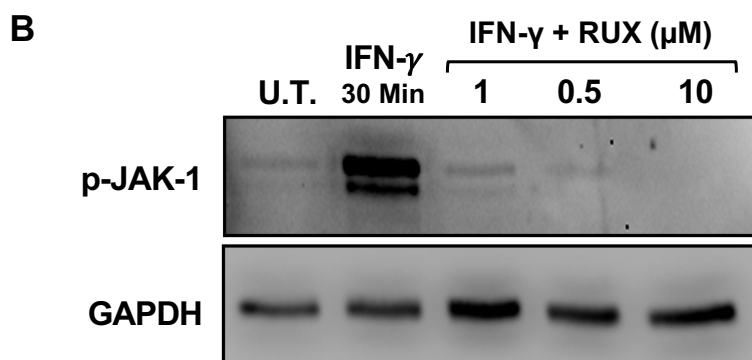
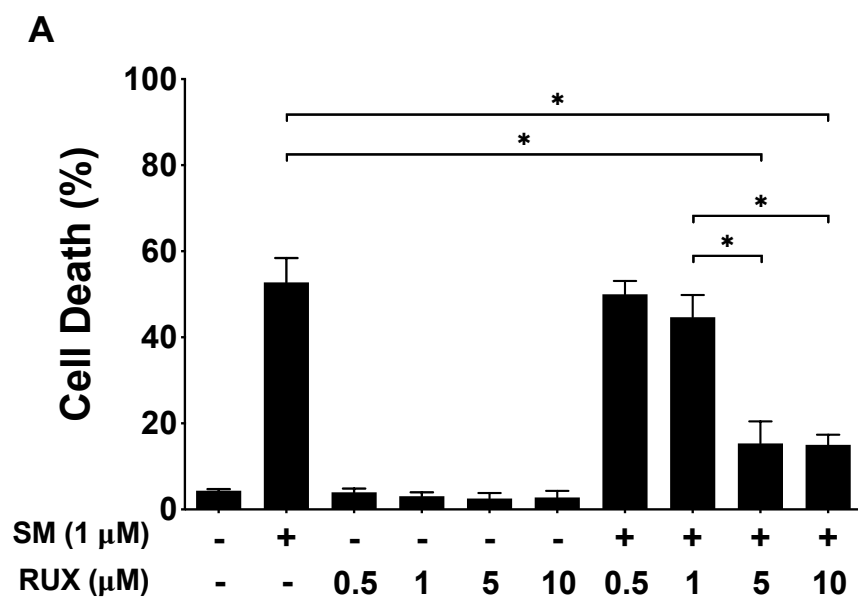


Figure 4.8: SM-mediated cell death in M1 macrophages is mediated by the JAK-STAT pathway.

(A) M0 macrophages were treated with various concentrations of JAK inhibitor (Rux; 0.5, 1, 5, 10 μ M), for 2 hrs prior to stimulation with IFN- γ for 48 hrs. Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (B) M0 macrophages were treated with various concentrations of Rux for 2 hrs prior to stimulation with IFN- γ for 30 min. Total protein lysates were analyzed for pJAK-1 (130 kDa) by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

4.2.7 Silencing IRF-1 regulates SM-induced cell death in M1 macrophages

IFN- γ induces the expression of transcription factor interferon regulatory factor-1 (IRF-1) that plays a key role in IFN- γ -induced cell death in various cancer cells (272, 365, 366). Therefore, to investigate whether IRF-1 is involved in SM-induced cell death in M1 macrophage, I first, determined the expression of IRF-1 in M0, M1 and M2c macrophage subsets and whether SM-LCL161 affects IRF-1 expression. Interestingly, M1 macrophages demonstrated increased IRF-1 expression compared to M0 and M2c subsets. Although SM-LCL161 treatment did not affect the expression of IRF-1 at any concentration (**Figure 4.9A**), I hypothesized that high IRF-1 expression in M1 macrophages may be responsible for increased cell death after SM-LCL161 treatment. To understand the role of IRF-1, I silenced its expression in M1 macrophages using siRNA. M0 macrophages were transfected with specific IRF-1 siRNA followed by IFN- γ stimulation and then SM-LCL161 treatment for 48 hrs and analysis of cell death by PI staining and flow cytometry. My results show that IFN- γ -stimulated M0 macrophages treated with SM-LCL161 in the presence or absence of non-targeting control siRNAs induced cell death. Interestingly, IRF-1 silencing of macrophages followed by SM-LCL161 treatment rescued M1 macrophage from SM-induced cell death compared to the macrophages transfected with non-targeting siRNA (**Figure 4.9B**). The effect of IRF-1 silencing was confirmed by showing downregulation of IRF-1 protein in M1 macrophages compared to the un-transfected M1 macrophages or M1 macrophages transfected with the control non-targeting siRNAs by Western immunoblotting (**Figure 4.9C**). These results suggested that IRF-1 mediates SM-induced cell death in M1 macrophages.

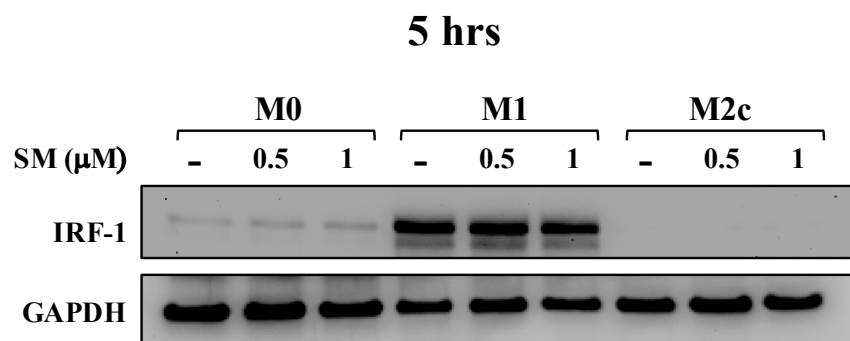
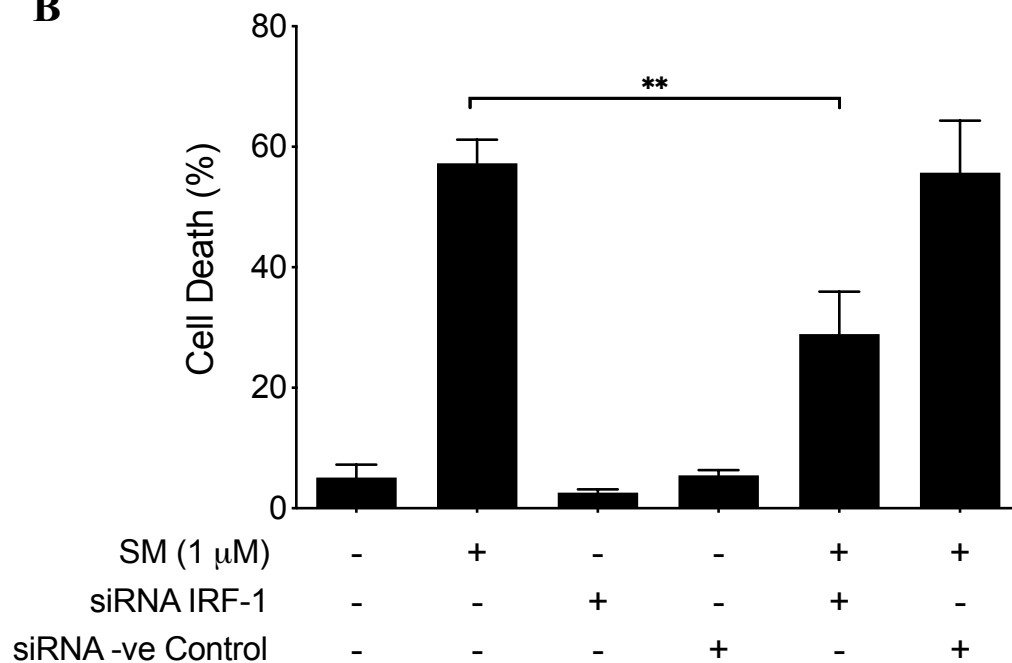
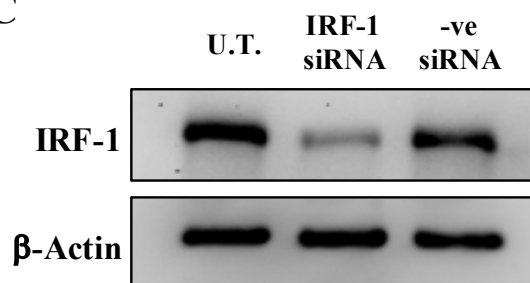
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Figure 4.9: IRF-1 regulates SM-induced cell death in M1 macrophages.

(A) M0, M1 and M2c macrophage subsets were left untreated or treated with SM-LCL161 (0.5 and 1 μ M) for 5 hrs. Cells were then collected and the total protein lysates were analyzed for expressions of IRF-1 (45 kDa) by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors. (B) M0 macrophages were transfected with IRF1 siRNA or negative control siRNA for 24 hrs. cells were then stimulated with IFN- γ for 48 hrs and then left untreated or treated with SM-LCL161 (1 μ M) for 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (C) M0 macrophages were left untreated, transfected with IRF1 siRNA or negative control siRNA for 24 hrs then stimulated with IFN- γ . Total protein lysates were analyzed for IRF-1 expressions (45 kDa) by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

4.2.8 PKR inhibition does not regulate SM-induced cell death in M1 macrophages

Interferon-induced, double-stranded RNA-activated protein kinase also known as protein kinase R (PKR), contains an N-terminal dsRNA binding domain (dsRBD) and a C-terminal kinase domain, that gives it pro-apoptotic functions (274, 367, 368). INF- γ induces the expression of PKR to inhibits viral protein synthesis, and mediates TNF- α and Fas-induced apoptosis or even RIPK-1/RIPK-3 mediated necroptosis (274–276, 367, 369). Therefore, I hypothesized that IFN- γ -induced differentiation of M0 macrophages into M1 state may induce PKR activity that may mediate SM-induced cell death in M1 macrophages. The involvement of PKR was investigated by its specific inhibitor C16 that is known to inhibit apoptotic PKR/eIF2 α signaling pathway (368, 370). M0 macrophages were treated with various concentrations of C16 for 2 hrs prior to stimulation with IFN- γ for 48 hrs followed by treatment with SM-LCL161 for another 48 hrs and determination of cell death by PI staining and flow cytometry (**Figure 4.10A**) and by IncuCyte™ PI staining. (**Figure 4.10B**). Inhibition of the PKR pathway by C16 at any concentration did not rescue SM-induced cell death in IFN- γ -stimulated M0 macrophages as assessed by either PI intracellular straining/flow cytometry or by IncuCyte™ PI staining. The biological activity of C16 was confirmed by the Western immunoblotting (**Figure 4.10C**) and shows reduction in the expression of p-PKR by C16 in a dose-dependent manner. Overall, my results suggest that SM-induced cell death in IFN- γ -stimulated M0 macrophages is regulated by the JAK/STATS pathway through the activation of IRF-1 transcription factor and independently of PKR activation.

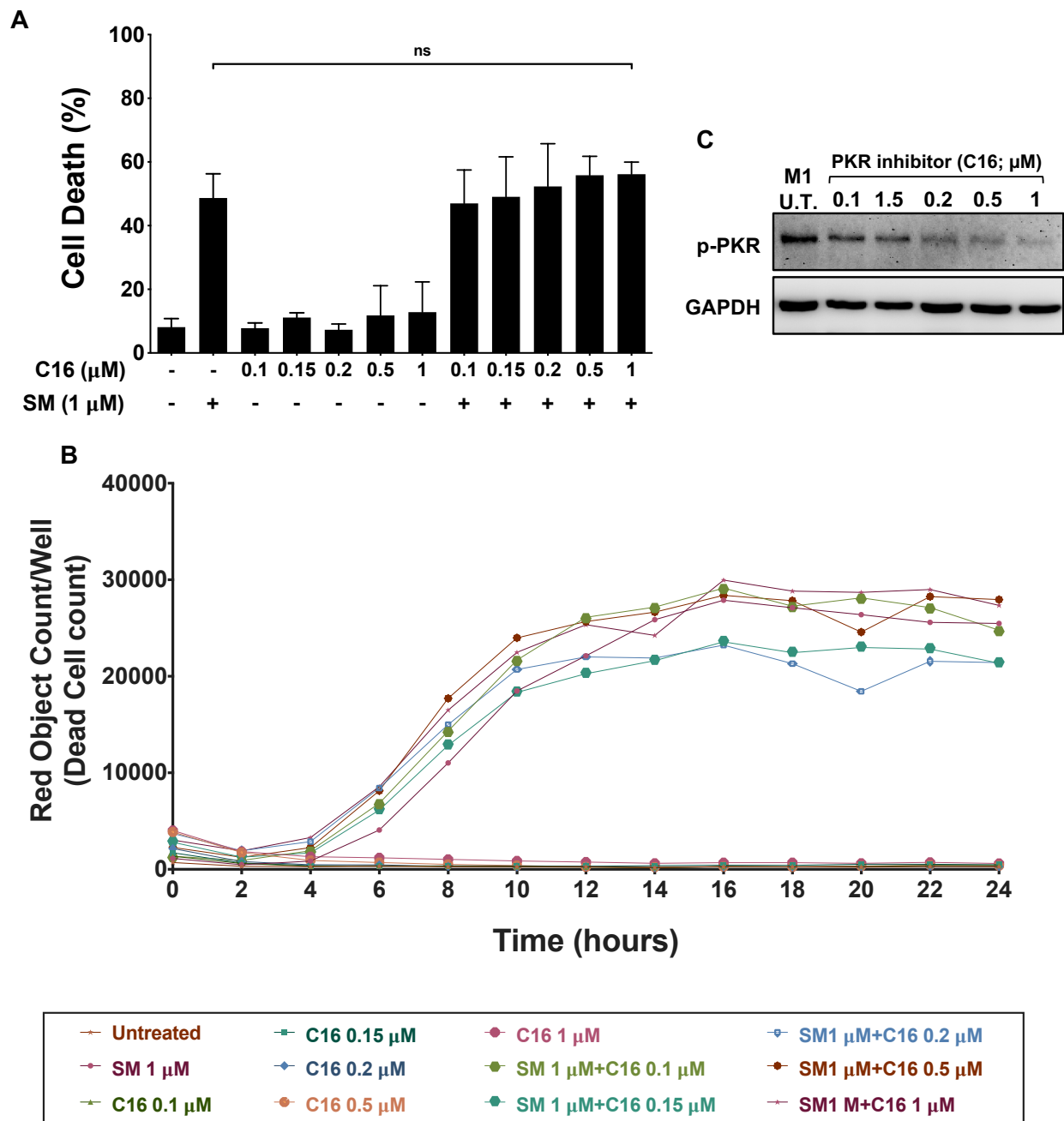


Figure 4.10: Blocking PKR did not reduce cell death in SM-treated M1.

M0 macrophage were left untreated or treated with different concentrations of PKR inhibitor (C16; 0.1, 0.15, 0.2, 0.5 and 1 μ M) for 2 hrs prior to stimulation with IFN- γ for 48 hrs. Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and (A) flow cytometry or (B) IncuCyte™ automated machine. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (C) Total protein lysates of M1 macrophages treated with different concentration of PKR inhibitor were analyzed for p-PKR (74 kDa) expression by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

4.2.9 Involvement of PI3K/Akt-mTORC pathway in SM-induced cell death

To further elucidate the mechanism by which INF- γ contributes to SM-induced cell death in M1 macrophages, we investigated the involvement of PI3K/Akt-mTOR pathway in SM-induced cell death. The PI3K-Akt cascade was investigated by employing PI3K and Akt inhibitors, LY294002 and MK0266, respectively. M0 macrophage subset was treated with either LY294002 or MK0266 for 2 hrs followed by INF- γ stimulation for 48 hrs (pre-polarization). Alternatively, M0 macrophages were first polarized into M1 state by stimulation with INF- γ for 48 hrs and then treated with either LY294002 or MK0266 inhibitors for 2 hrs (post-polarization). Subsequently, M1 macrophages treated with these inhibitors either prior to INF- γ differentiation (pre-polarization) or after 48 hrs of differentiation with INF- γ (post-polarization) were challenged with SM-LCL161 for another 48 hrs. Finally, cell death was assessed by intracellular PI staining and flow cytometry. My results show that neither PI3K inhibitor (**Figure 4.11A and B**) nor the Akt inhibitor (**Figure 4.12A and B**) added either prior to polarization or post-polarization at any concentration affected SM-induced cell death of INF- γ -stimulated M0 macrophages. The activity of PI3K inhibitors was determined by showing inhibition of LPS-induced Akt phosphorylation in M0 macrophages by Western immunoblotting (**Figure 4.11C**). The activity of Akt inhibitor was determined by showing inhibition of INF- γ -induced Akt phosphorylation in M0 macrophages by Western immunoblotting (**Figure 4.12C**). These data suggest that PI3K/Akt pathway is not involved in SM-induced cell death in M1 macrophages.

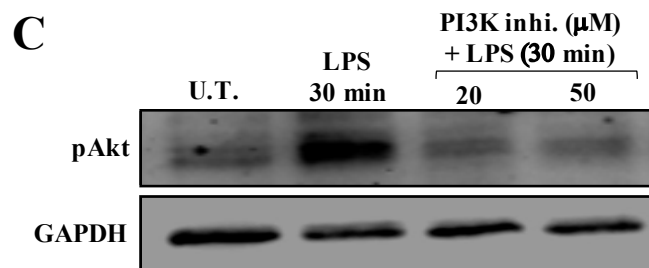
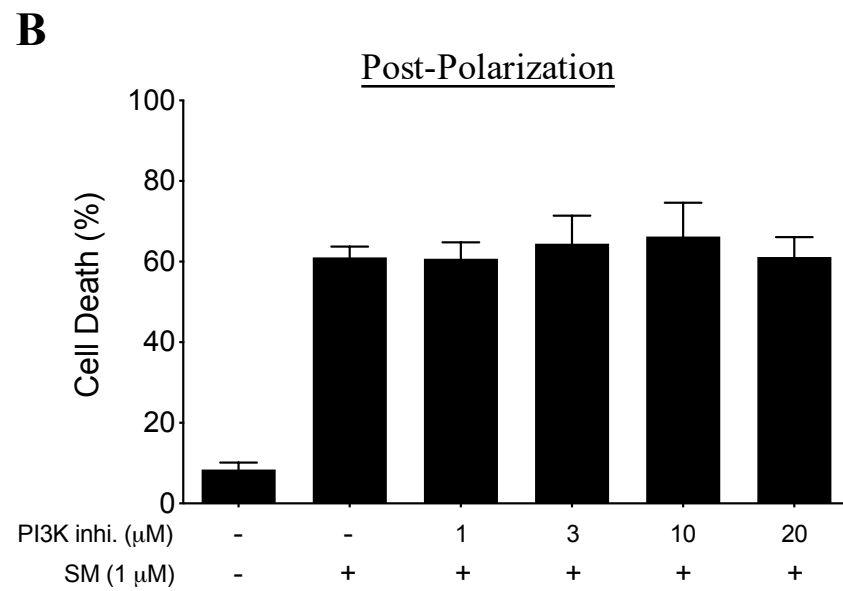
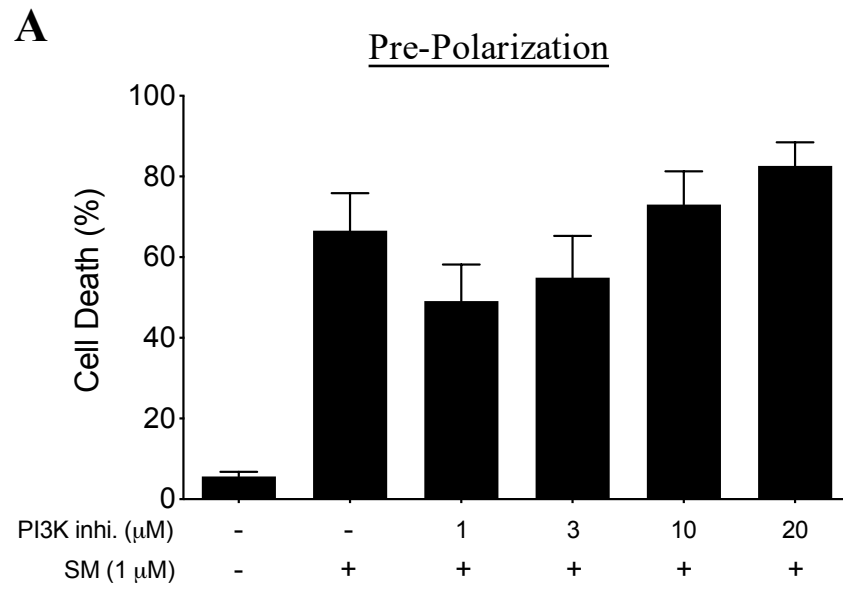


Figure 4.11: Inhibition of PI3K pathway either before or after M1 polarization does not affect SM-induced cell death.

(A) M0 macrophages were treated with various concentrations of PI3K inhibitor (1, 3, 10 and 20 μ M) for 2 hrs prior to stimulation with IFN- γ for 48 hrs (pre-polarization). (B) For post-polarization, M1 macrophages were treated with same concentrations of PI3K inhibitor for 2 hrs after stimulation with IFN- γ for 48 hrs. Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (C) M0 macrophages were left untreated or treated with PI3K inhibitor (20 and 50 μ M) for 2 hrs prior to stimulation with LPS for 30 min. Total protein lysates were analyzed for pAkt (60 kDa) by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

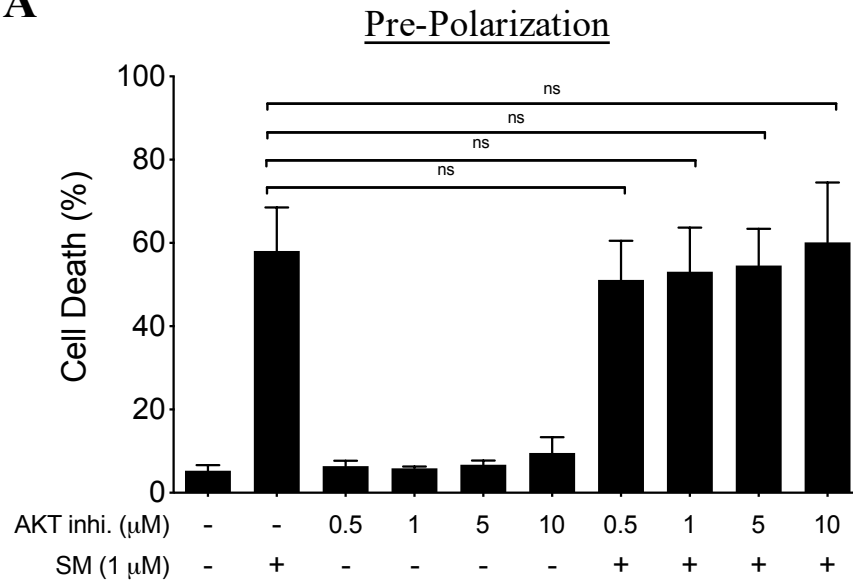
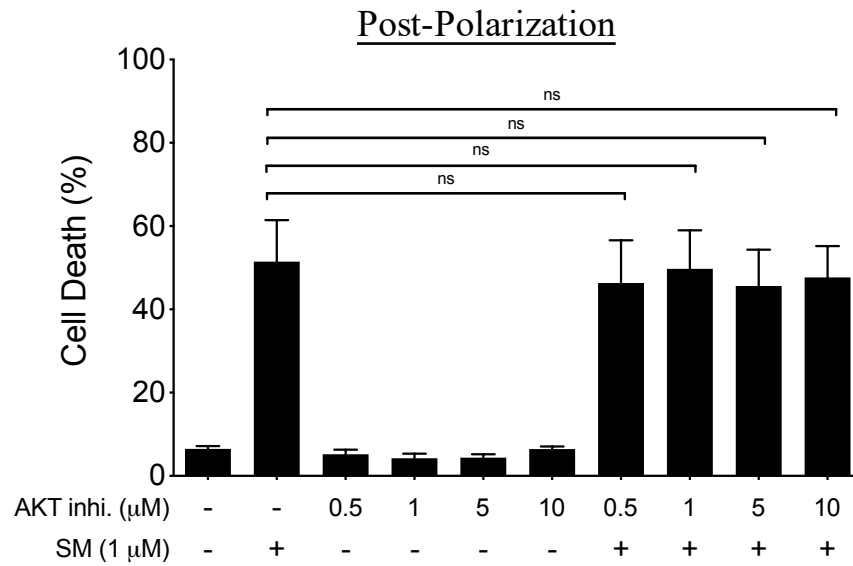
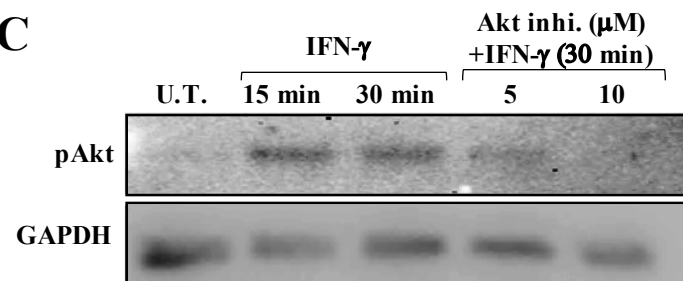
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Figure 4.12: Akt inhibition either before or after M1 polarization does not affect SM-induced cell death.

(A) M0 macrophages were treated with various concentrations of Akt inhibitor (0.5, 1, 5 and 10 μ M) for 2 hrs prior to stimulation with IFN- γ for 48 hrs (pre-polarization). (B) For post-polarization, M1 macrophages were treated with same concentrations of Akt inhibitor for 2 hrs after stimulation with IFN- γ for 48 hrs. Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (C) M0 macrophages were left untreated or treated with Akt inhibitor (5 and 10 μ M) for 2 hrs prior to stimulation with IFN- γ for 30 min. Total protein lysates were analyzed for pAkt (60 kDa) by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

4.2.10 SM-induced cell death during M1 differentiation is regulated by mTORC1-S6K pathway

The mTOR pathway plays a central regulatory role in many cellular processes such as cell survival, differentiation, proliferation, and immune responses (279, 280). The mTOR protein complex is a serine/threonine kinase that acts downstream of phosphoinositide-dependent kinase-1 in the PI3K pathway and functions as a nutrient/energy sensor and controls protein synthesis and cell growth (279, 280). mTOR links with other proteins and serves as a core component of two distinct protein complexes, mTORC-1 and mTORC-2, which regulate different cellular processes (279, 281, 282, 292). Rapamycin inhibits mTORC1 whereas Torin inhibits both complexes (371). Since p70S6 kinase is a downstream target of mTORC-1 complex (290, 372), it was of interest to investigate the role of p70S6K in the SM-induced apoptosis in M1 macrophages as well. Therefore, I used ribosomal protein S6 kinase (S6K) inhibitor (PF-4708671) that targets S6K downstream of mTORC1 responsible for protein synthesis (373). To determine the involvement of mTORC-1/2-S6K pathway, I first examined the dose and the ability of the mTORC inhibitors; Torin and Rapamycin, and S6K inhibitors (PF-4708671) to block the activation of mTORC pathway in macrophage. Macrophages were treated with different concentrations of these inhibitors and then stimulated with IFN- γ or LPS for 30 min followed by Western immunoblotting for the expression of S6K phosphorylation. The biological activities of Torin (**Figure 4.13D**), Rapamycin (**Figure 4.13E**) and S6K inhibitor (**Figure 4.13F**) confirmed by showing the inhibition of S6K phosphorylation in a dose dependent manner as compared to the IFN- γ or LPS-stimulated macrophages in the absence of the inhibitor.

To determine the involvement of mTORC pathway in SM-induced cell death in M1 macrophages, M0 macrophages (pre-polarization) were left untreated or treated with various concentrations of mTORC pathway inhibitors, Torin or Rapamycin, or S6K inhibitor for 2 hrs and then stimulated with IFN- γ to generates M1 macrophages. After 48 hrs, these macrophages were treated with SM-LCL161 for another 48 hrs, followed by assessment of cell death by intracellular PI staining and flow cytometry. My results show that blocking of mTORC pathway by Torin or Rapamycin (**Figure 4.13A and B**) and of the S6K pathway by S6K inhibitor (**Figure 4.13C**) prior to IFN- γ stimulation significantly reduced SM-induced cell death. Since Rapamycin mostly inhibits mTORC-1 and Torin inhibits both mTORC-1 and mTORC-2, these results suggested that the SM-induced cell death during differentiation into M1 macrophages is mediated by the activation of mTORC pathway and its downstream S6K signaling pathway.

Pre-Polarization

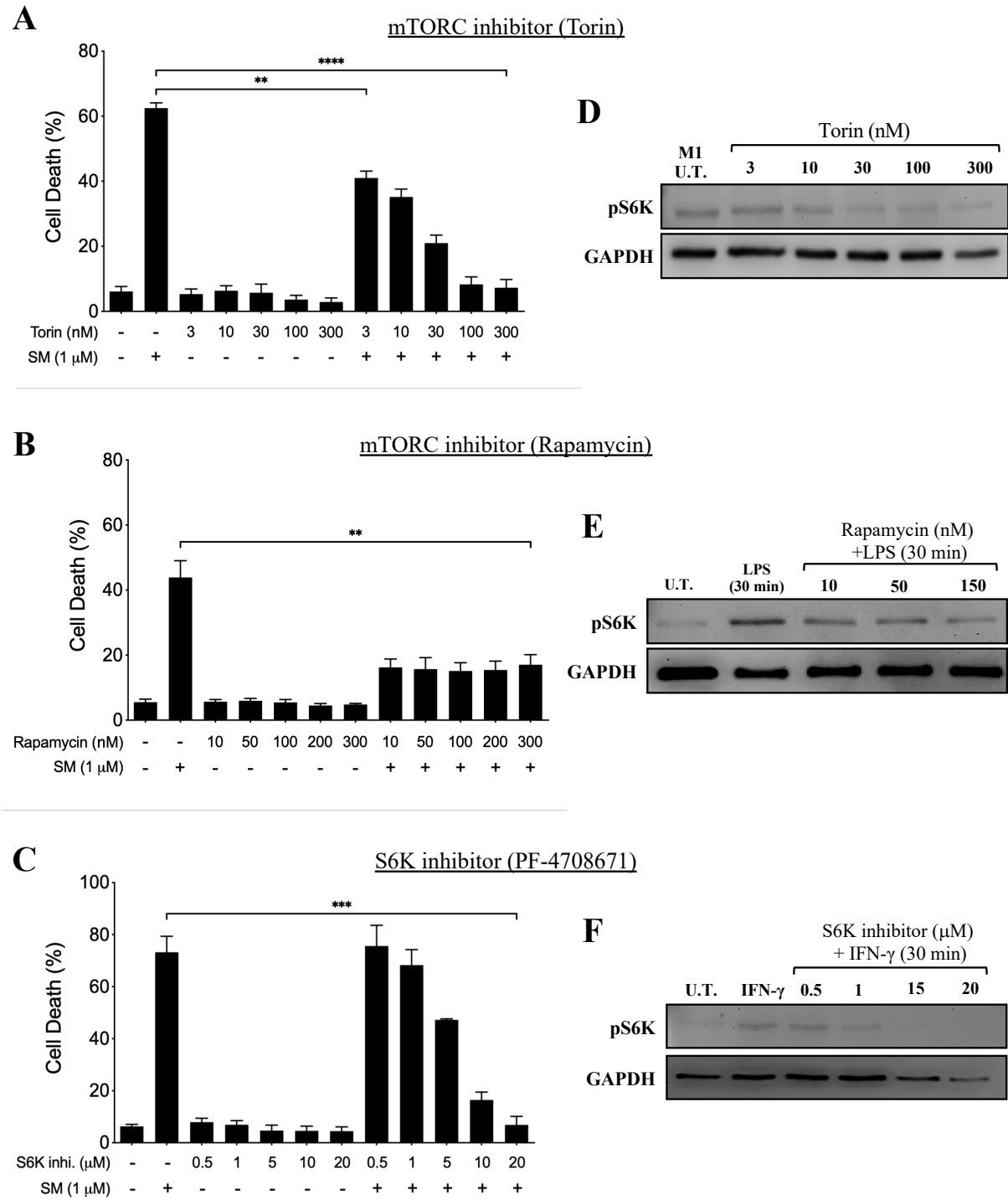


Figure 4.13: Blocking mTORC1/2 and S6K pathway during M1 differentiation inhibits SM-induced cell death in M1 macrophages.

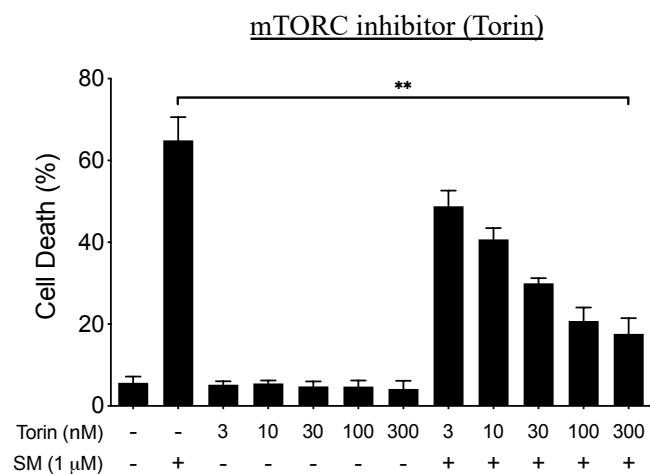
M0 macrophages were treated with various concentrations of (A) Torin inhibitor (3, 10, 30, 100 and 300 nM), (B) Rapamycin inhibitor (10, 50, 100, 200 and 300 nM) or (C) S6K inhibitor (0.5, 1, 5, 10 and 20 μ M) for 2 hrs prior to stimulation with IFN- γ for 48 hrs (pre-polarization). Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. (D) M1 macrophages were left untreated or treated with Torin inhibitor (3, 10, 30, 100 and 300 nM). M0 macrophages were left untreated or treated with (E) Rapamycin inhibitor (10, 50, and 150 nM) or (F) S6K inhibitor (0.5, 1, 15 and 20 μ M) for 2 hrs prior to stimulation with either LPS or IFN- γ for 30 min. Total protein lysates were analyzed for pS6K (70 kDa) expressions by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

4.2.11 SM-induced cell death in differentiated M1 is regulated by mTORC2.

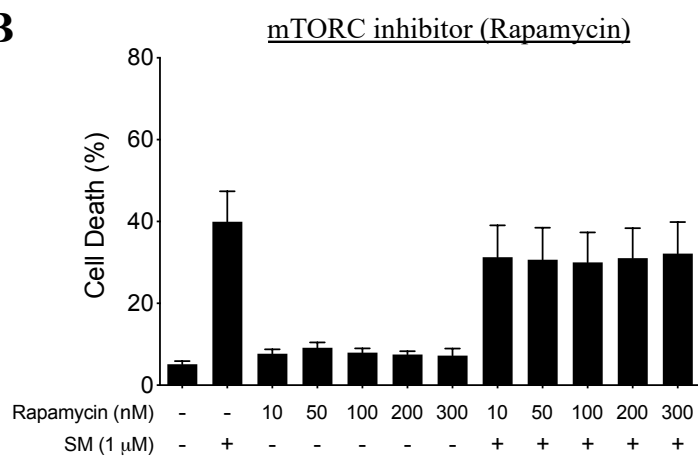
To determine the signaling pathways involved in SM-induced cell death post-M1 differentiated macrophages, as stated earlier, M0 macrophages were first polarized into M1 state by stimulation with IFN- γ for 48 hrs and then either left untreated or treated with various concentrations of mTORC pathway inhibitors, Torin or Rapamycin, or S6K inhibitor for 2 hrs (post-polarization). Subsequently, M1 macrophages treated with these inhibitors were treated with SM-LCL161 for another 48 hrs followed by assessment of cell death. My results show that only Torin was able to protect differentiated M1 macrophages when mTORC pathway was blocked after IFN- γ stimulation (post-polarization) (**Figure 4.14A**). Both Rapamycin and S6Kinase inhibitors involved mTORC1 inhibition failed to regulate SM-induced cell death in post-M1 differentiated macrophages (**Figure 4.14B and C**). Since Rapamycin failed to rescue SM-induced cell death, the results suggest that SM-induced cell death in M1 differentiated macrophages may be regulated by the mTORC-2 pathway and independent of the S6K pathway.

Post-Polarization

A



B



C

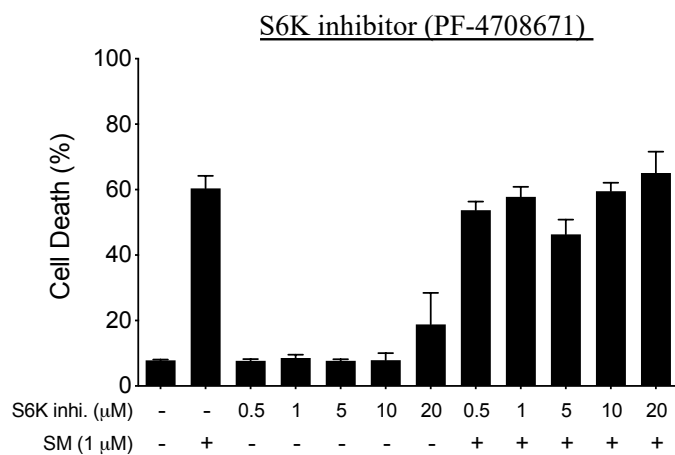


Figure 4.14: Blocking mTORC1/2 pathway after M1 polarization by Torin alone inhibits SM-induced cell death in M1 macrophages.

M1 macrophages were treated with various concentrations of (A) Torin inhibitor (3, 10, 30, 100 and 300 nM), (B) Rapamycin inhibitor (10, 50, 100, 200 and 300 nM) and (C) S6K inhibitor (0.5, 1, 5, 10 and 20 μ M) for 2 hrs (Post-polarization). Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors.

4.2.12 MAPK pathways do not affect SM-induced cell death of M1 macrophages

cIAPs have been shown to be critical regulators of MAPK signaling pathways activation (362). MAPKs regulate important cellular processes such as proliferation, stress responses, apoptosis and immune defense (374–376). There are three well-known MAPK pathways in mammalian cells: the ERK1/2, the c-JUN N-terminal kinase 1, and 2 (JNK1/2), and the p38 MAPK pathways. The role of ERK, JNK and p38 pathways was determined by employing their specific inhibitors PD-98059, SP-600125 and SB-203580, respectively (377). First, I determined the biological activity of these inhibitors. For this, M0 macrophages were treated with different concentrations of ERK, JNK or P38 inhibitors for 2 hrs followed by IFN- γ or LPS stimulation for 30 min to induce the phosphorylation of ERK, JNK and P38 MAPKs expressions followed by Western immunoblotting. As shown in **Figure 4.15B, D and F**, these inhibitors were able to inhibit the phosphorylation of the targeted proteins namely JNK following IFN- γ stimulation, and ERK and p38 following LPS stimulation compared to untreated IFN- γ /LPS-stimulated macrophages.

Next, I investigated whether blocking MAPK pathways would rescue cell death in SM-treated M1 macrophages. As described earlier for PI3K/Akt inhibitors, M0 macrophage subset were treated with either PD-98059, SP-600125 or SB-203580 the inhibitors specific for the ERK, JNK and p38 pathways, respectively, for 2 hrs followed by IFN- γ stimulation for 48 hrs (pre-polarization). Alternatively, M0 macrophages were first polarized into M1 state by stimulation with IFN- γ for 48 hrs and then treated with the above inhibitors for 2 hrs (post-polarization). Subsequently, M1 macrophages treated with these inhibitors either prior to IFN- γ differentiation (**Figure 4.15A, C and E**) or after 48 hrs of differentiation with IFN- γ (**Figure**

4.16A, B and C) were treated with SM-LCL161 for another 48 hrs followed by assessment of cell death. Treatment of macrophages (pre-polarization) (**Figure 4.15A, C and E**) or differentiated M1 macrophages (post-polarization; **Figure 4.16A, B and C**) with MAP kinase inhibitors at any concentration did not rescue SM-induced cell death in M1 macrophages. These results suggest that the MAPKs do not regulate SM-induced apoptosis in M1 macrophages.

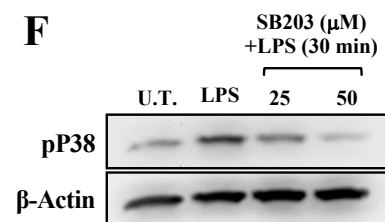
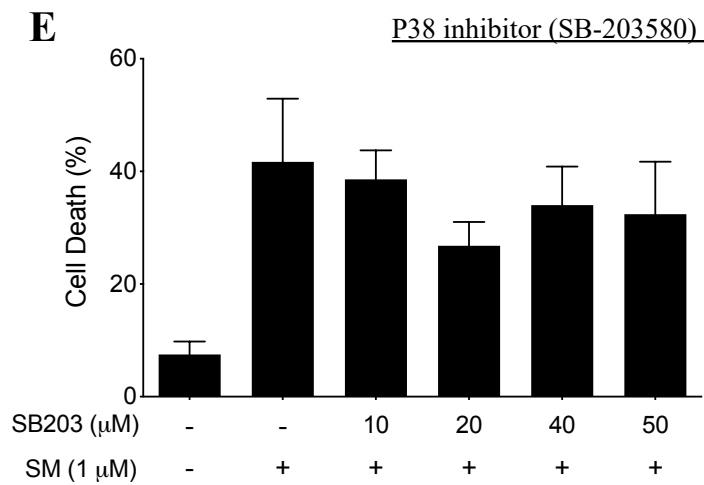
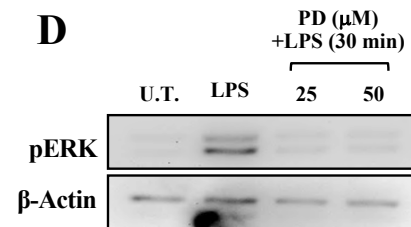
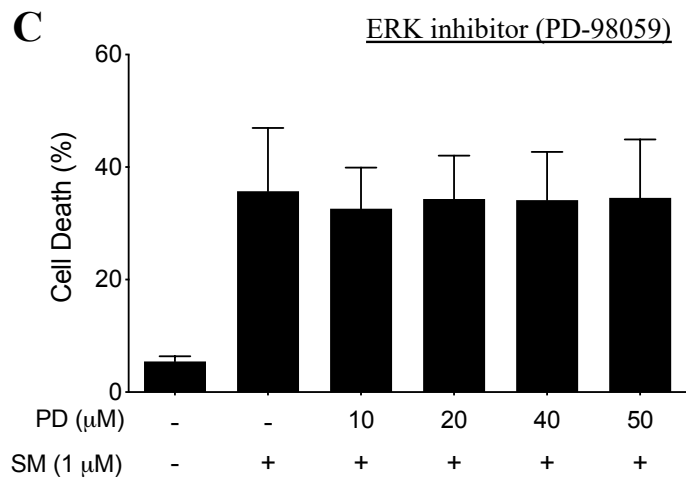
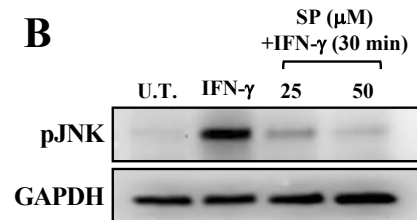
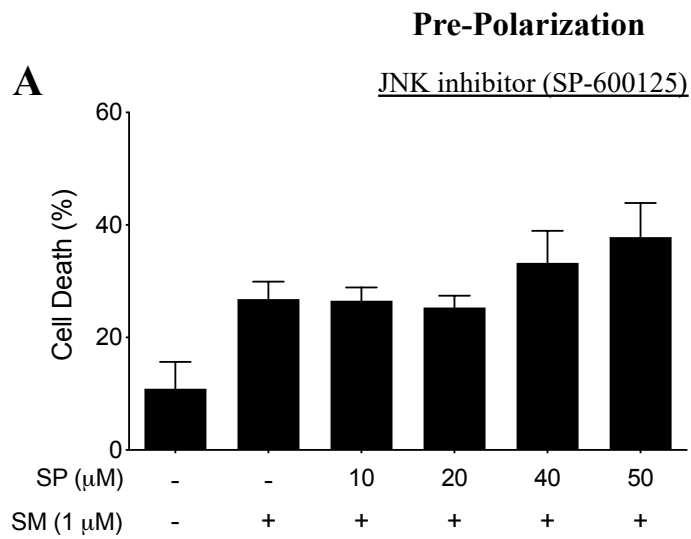
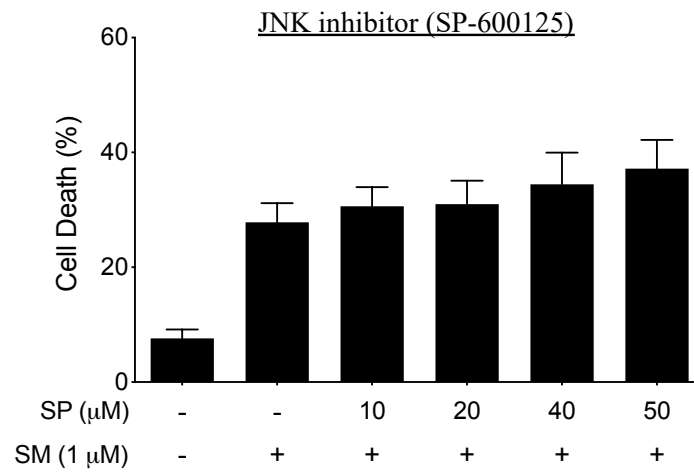


Figure 4.15: Blocking of P38, ERK or JNK MAPK pathway before M1 polarization does not inhibit SM-induced cell death in M1 macrophages.

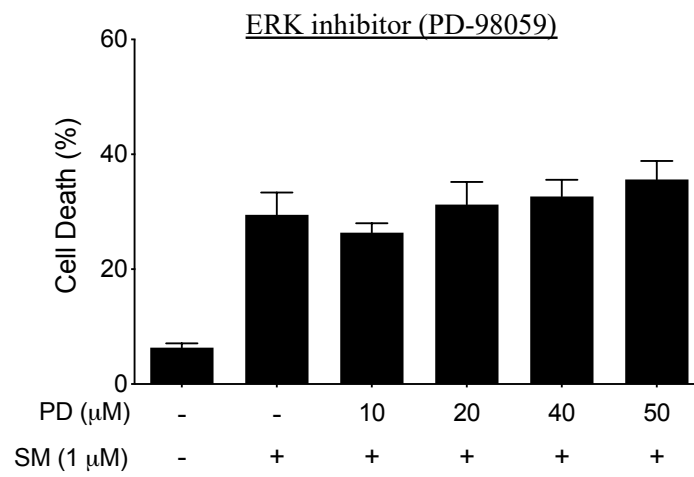
M0 macrophages were treated with various concentrations of (A) JNK inhibitor (SP-600125; 10, 20, 40 and 50 μ M), (C) ERK inhibitor (PD-98059; 10, 20, 40 and 50 μ M) or (E) P38 inhibitor (SB-203580; 10, 20, 40 and 50 μ M) for 2 hrs prior to stimulation with IFN- γ for 48 hrs (pre-polarization). Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors. M0 macrophages were left untreated or treated with (B) JNK inhibitor (25 and 50 μ M), (D) ERK inhibitor (25 and 50 μ M) or (F) P38 inhibitor (25, and 50 μ M) for 2 hrs prior to stimulation with either IFN- γ or LPS for 30 min. Total protein lysates were analyzed for pJNK (46 kDa), pERK (44 kDa) and pP38 (43 kDa) expressions by Western immunoblotting. The result shown is a representative of three independent experiments from three different donors.

Post-Polarization

A



B



C

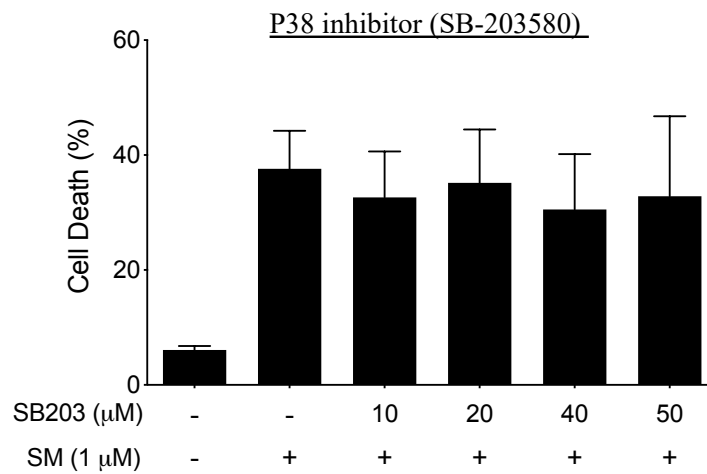


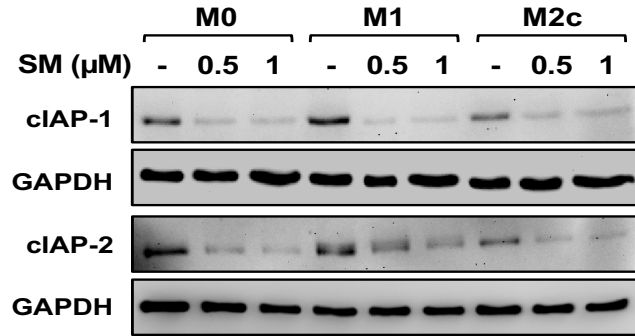
Figure 4.16: Blocking of P38, ERK or JNK MAPK pathway after M1 polarization does not inhibit SM-induced cell death in M1 macrophages.

M1 macrophages were treated with various concentrations of (A) JNK inhibitor (SP-600125; 10, 20, 40 and 50 μ M), (B) ERK inhibitor (PD-98059; 10, 20, 40 and 50 μ M) or (C) P38 inhibitor (SB-203580; 10, 20, 40 and 50 μ M) for 2 hrs (Post-polarization). Cells were then treated with SM-LCL161 (1 μ M) for another 48 hrs. The % cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of three independent experiments from three different donors.

4.2.13 Analysis of proapoptotic and anti-apoptotic proteins involved in SM induced cell death in M1 macrophages:

SM-induced cell death in M1 macrophages is regulated by the simultaneous degradation of cIAP-1/2 and RIPK-1/3. SM-induced cell death in M1 macrophages and the contrasting resistance of M0/M2c macrophages to SM-LCL161 may be due to the differential expression of proapoptotic and anti-apoptotic proteins in M1 and M0/M2c macrophages. Therefore, I first determined the differential expression of anti-apoptotic cIAP1/2 proteins, the targeted proteins of SM-LCL161, in the presence and absence of SM-LCL161 treatment in M0, M2c and M1 macrophages. M0, M1 and M2c macrophages were treated with SM-LCL161 for 15 hrs followed by analysis of cIAP1/2 protein expression by Western immunoblotting analysis. The untreated M0, and M2c macrophages expressed similar levels of cIAP-1 and c-IAP-2, while untreated M1 macrophages expressed significantly high levels of cIAP-1 and cIAP-2 compared to M0 and M2c macrophages (**Figure 4.17A, B and C**). Moreover, SM-LCL161 treatment caused significant degradation of cIAP1/2 proteins in all M0, M1 and M2c macrophage subsets. Similar findings were also obtained after 5 hrs of SM-LCL161 treatment (**Figure 3.4A and B**). High levels of IAP-1/2 proteins in M1 macrophages and equal degradation of these genes by SM-LCL161 does not explain the resistance of M0/M2c subsets and susceptibility of M1 macrophages to SM-induced cell death. My results further suggest that SM-induced degradation of cIAP1/2 proteins alone may not be enough to cause cell death in M1 macrophages and may require the involvement of other pro-apoptotic, anti-apoptotic or IAP associated proteins.

A



B

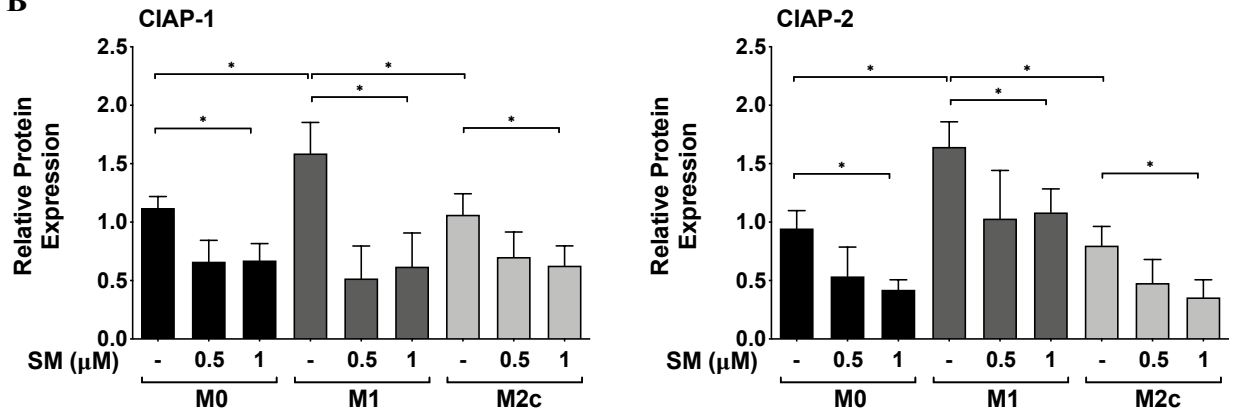


Figure 4.17: SM-LCL161 causes degradation of cIAP-1/2 in M0, M1 and M2c macrophages.

(A) M0, M1 and M2c macrophages were left untreated or treated with SM-LCL161 (0.5 and 1 μ M) for 15 hrs. The total protein lysates were subjected to Western immunoblotting for the expression of cIAP-1 (62 kDa) and cIAP-2 (70 kDa). The immunoblot shown is a representative of three independent experiments from three different donors. (B) Relative protein expression of cIAP-1 and cIAP-2.

Therefore, I investigated the expression of proapoptotic Bak, Bax, Fas and FADD proteins and anti-apoptotic Bcl-2 family of proteins namely Bcl-2, Mcl-1, and Bcl-xL proteins in M0 M1 and M2c macrophages. As expected, SM-LCL161 at various concentrations causes degradation of both cIAPs proteins (**Figure 4.18A**). However, SM-LCL161 did not affect the expression of anti-apoptotic Bcl-2, Bcl-xL or Mcl-1 in M0, M1 and M2c macrophages, although M1 macrophages exhibit higher expression of these proteins (**Figure 4.18A**). Similarly, SM-LCL161 did not affect the expression of Fas, or TRAIL proteins in M0, M1 and M2c macrophage subsets (**Figure 4.18A**). Moreover, SM-LCL161 did not affect the expression of proapoptotic Bak and FADD in M0, M1 and M2c macrophages treated with SM-LCL161 (1 μ M) for 5, 10 and 15 hrs (**Figure 4.18B**).

Interestingly, analysis of the IAP associated proteins RIPK-1 and RIPK-3 revealed that SM-LCL161 treatment caused degradation of RIPK-1 and RIPK-3 as well as the unphosphorylated MLKL protein in M1 macrophages whereas the expression of these proteins was not modulated in M0 and M2c macrophage subsets (**Figure 4.19A**). As expected, SM-LCL161 caused degradation of anti-apoptotic cFLIP protein, in addition, SM-LCL161 caused the accumulation of pro-apoptotic Bax protein in M1 macrophages but not in M0 and M2c macrophages (**Figure 4.19B**). These results suggest that SM-LCL161 induced cell death in M1 macrophages may be associated with the simultaneous degradation of cIAP-1/2 and RIPK-1 and RIPK-3 proteins.

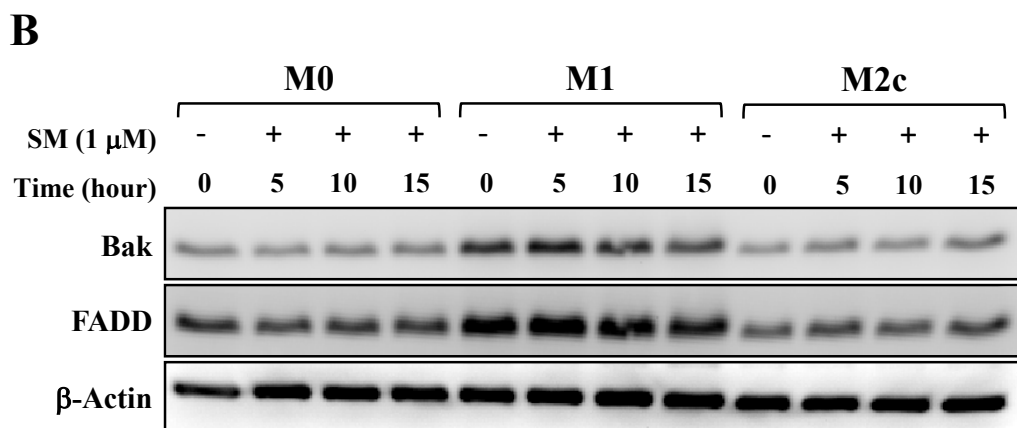
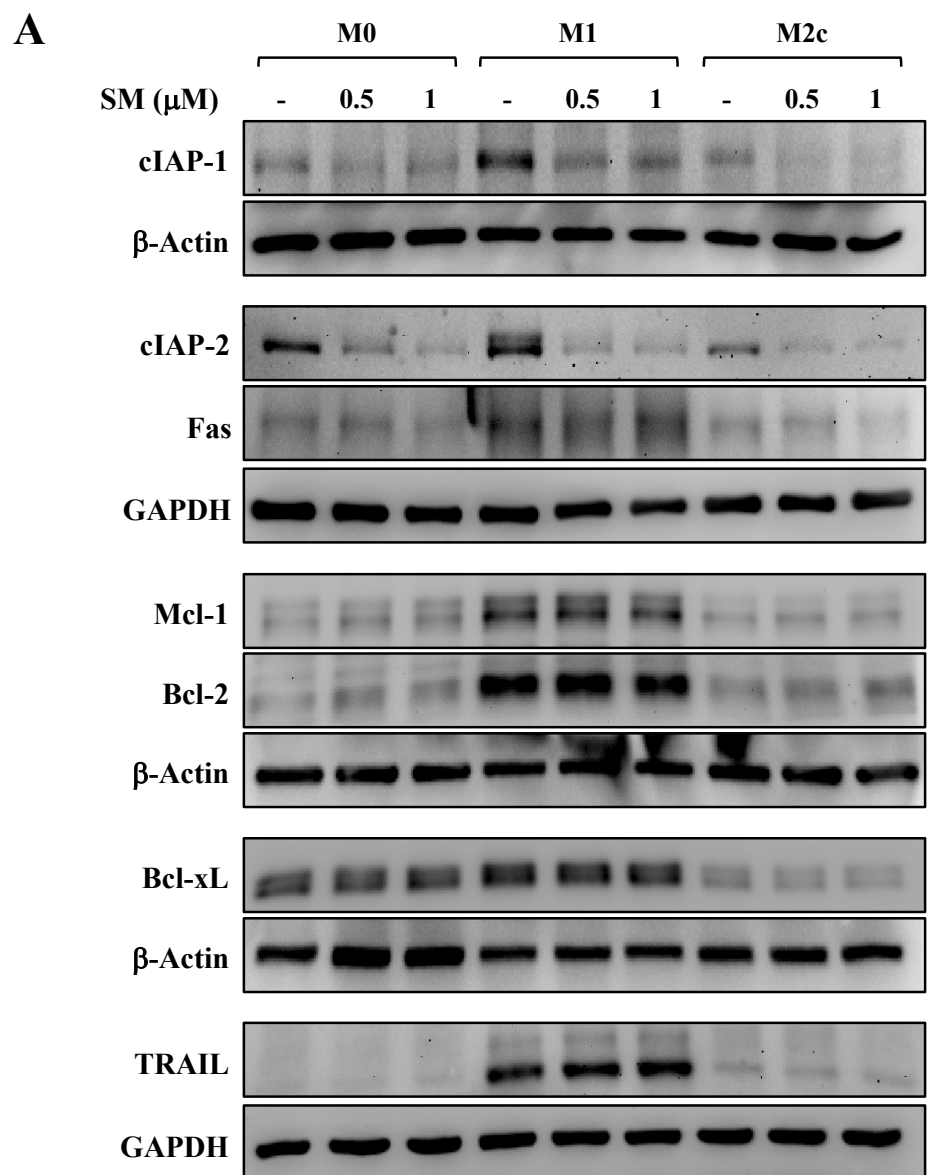


Figure 4.18: SM-LCL161 does not inhibit Bcl-2 family of anti-apoptotic proteins (Bcl-2, Mcl-1 and Bcl-xL), pro-apoptotic (Bak), FADD, Fas or TRAIL proteins.

(A) M0, M1 and M2c macrophages subsets were treated with SM-LCL161 (0.5 and 1 μ M) for 5 hrs. The total protein lysates were subjected to Western immunoblotting for the expression of cIAP-1 (62 kDa), cIAP-2 (70 kDa), Fas (40 kDa), Mcl-1 (40 kDa), Bcl-2 (26 kDa), Bcl-xL (30 kDa) and TRAIL (28 kDa). (B) M0, M1 and M2c macrophages subsets were treated with SM-LCL161 (1 μ M) for 5,10 and 15 hrs. The total protein lysates were subjected to Western immunoblotting for the expression of Bak (25 kDa) and FADD (28 kDa) proteins expression. The result shown is a representative of three independent experiments from three different donors.

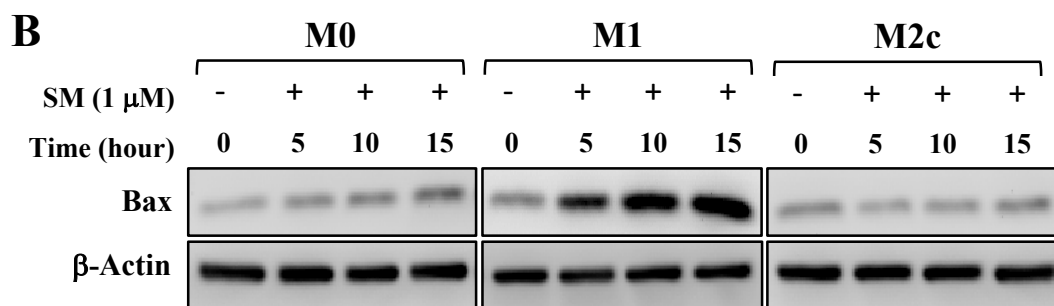
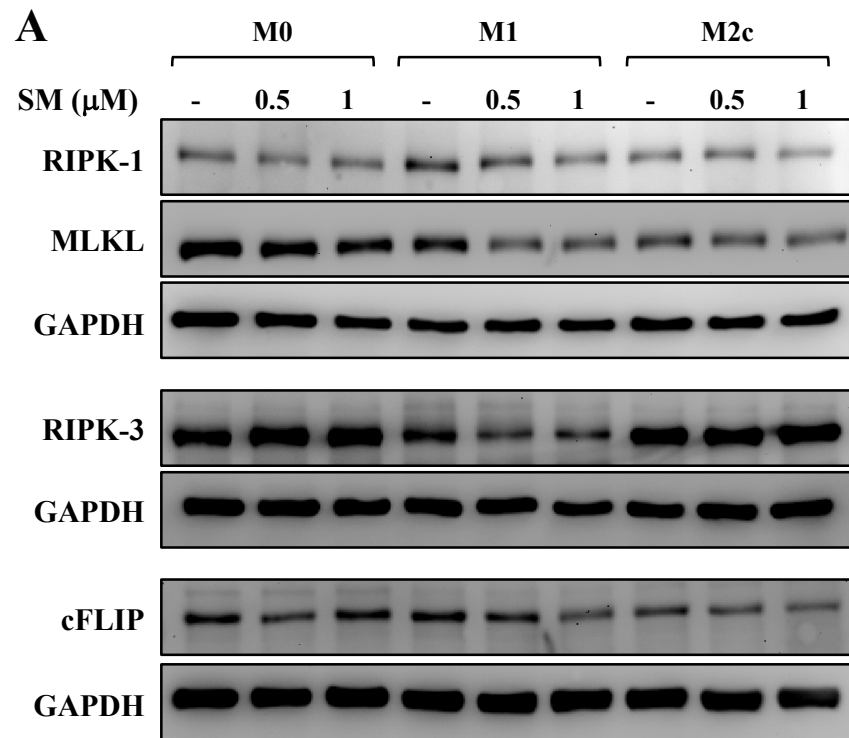


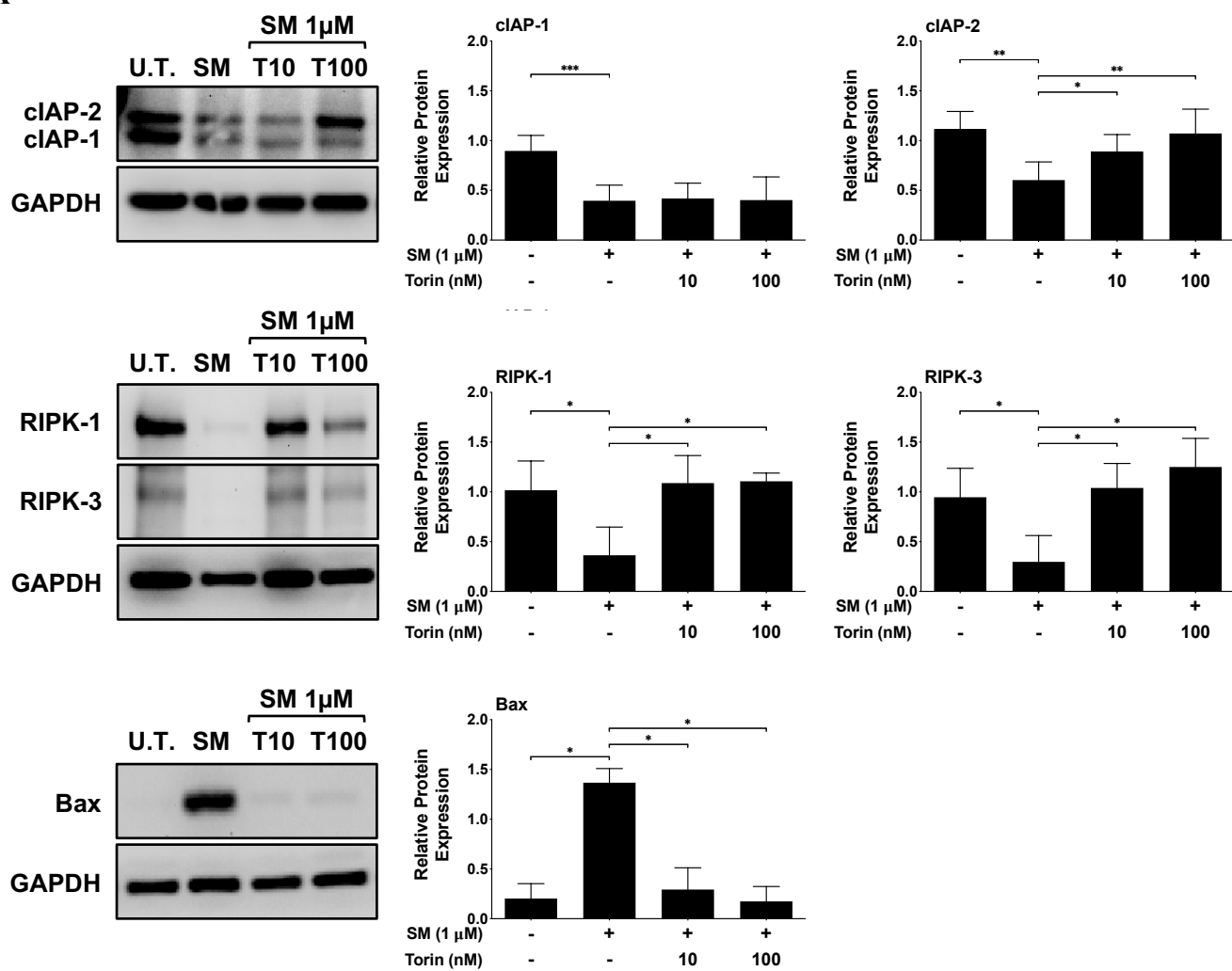
Figure 4.19: SM-LCL161 causes degradation of RIPK-1, RIPK-3, MLKL, cFLIP and upregulation of Bax in M1 but not in M0 and M2c macrophages.

(A) M0, M1 and M2c macrophage subsets were treated with SM-LCL161 (0.5 and 1 μ M) for 5 hrs. The total protein lysates were subjected to Western immunoblotting for the expression of RIPK-1 (78 kDa), MLKL (54 kDa), RIPK-3 (46-62 kDa), and cFLIP (55 kDa). (B) M0, M1 and M2c macrophages subsets were treated with SM-LCL161 (1 μ M) for 5,10 and 15 hrs. The total protein lysates were subjected to Western immunoblotting for the expression of Bax (20 kDa) proteins expression. The result shown is a representative of three independent experiments from three different donors.

4.2.14 SM-induced cell death in M1 macrophages is regulated by cIAP-2 and RIPK-1/3 through the activation of mTORC

Since mTORC inhibitor Torin rescues SM-induced cell death, I hypothesized that SM-induced cIAP-1/2 and RIPK-1/3 degradation would be restored following treatment of M0 with Torin. Therefore, whether blocking mTORC pathway reverses the effect of SM-induced cell death in polarized M1 macrophages. I investigated the expression of both cIAP-1 and cIAP-2 as well as other effector molecules including RIPK-1, RIPK-3 and Bax after blocking mTORC by Torin. As seen in **Figure 4.20A and B**, SM-LCL161 treatment caused degradation of both cIAP-1 and cIAP-2 as well as RIPK-1 and RIPK-3. Blocking mTORC either pre- or post-polarization by the chemical inhibitor Torin prevented the degradation of cIAP-2, RIPK-1 and RIPK-3. However, cIAP-1 was not affected by blocking mTORC compared to SM-LCL161 treated M1 macrophages. As expected SM-LCL161 treatment enhanced the expression of pro-apoptotic Bax protein in M1 macrophages. Prior treatment of M1 macrophages during differentiation or in polarized M1 macrophages with Torin, inhibited Bax expression in SM-treated M1 macrophages to levels similar to untreated M1 macrophages. Overall the results suggest that SM-induced cell death in M1 macrophages is regulated by cIAP-2/RIPK-1/3 through the activation of mTORC pathway.

A



B

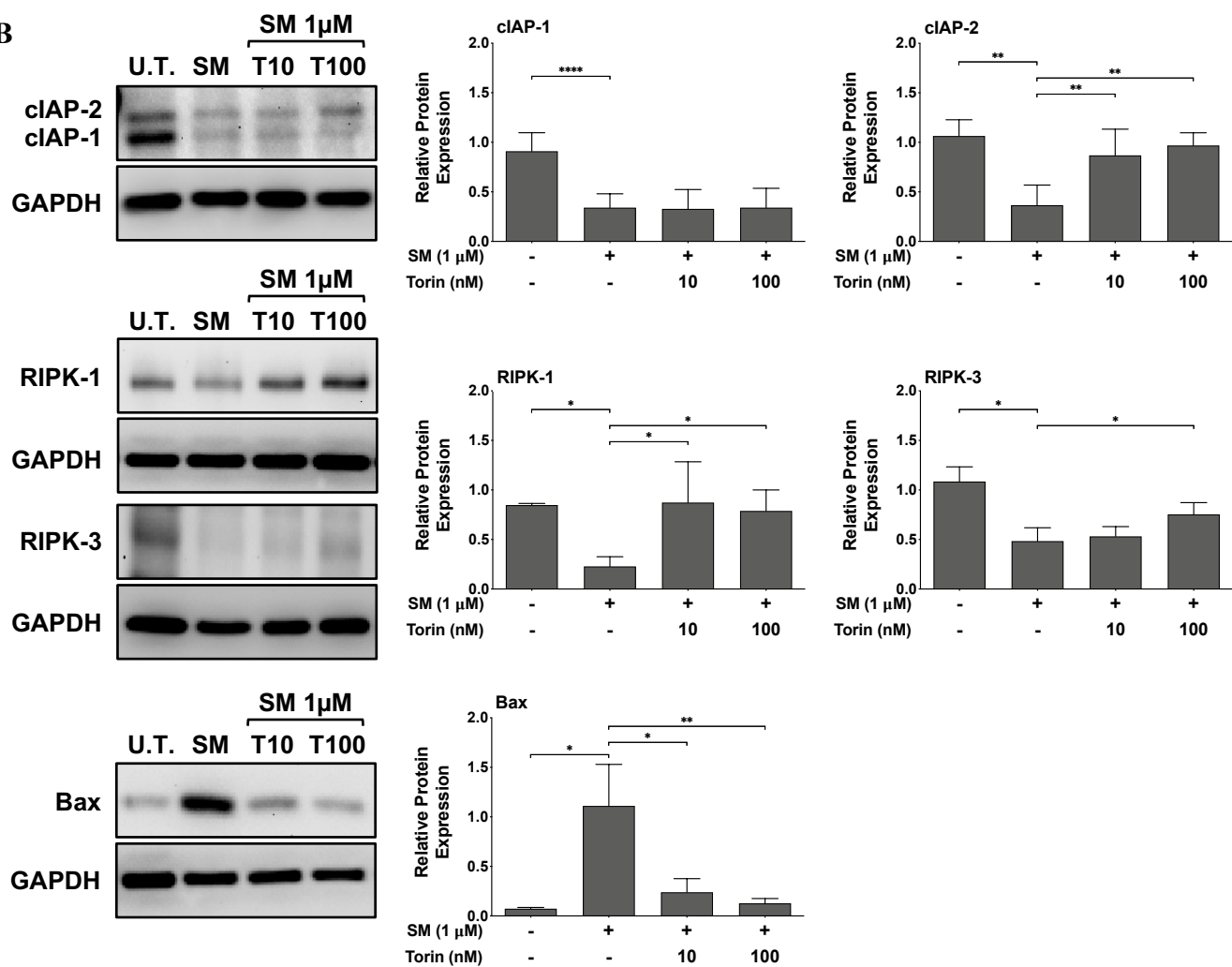


Figure 4.20: Blocking mTORC1/2 by Torin prevents cIAP-2, RIPK-1/3 degradations.

(A) M0 macrophages were treated with Torin inhibitor (10 and 100 nM) for 2 hrs prior to (pre-polarization) stimulation with IFN- γ for 48 hrs. Cells were then left untreated or treated with SM-LCL161 (1 μ M) alone or Torin and SM-LCL161 combined treatment for another 24 hrs. The total protein lysates were analyzed for cIAP-1 (62 kDa), cIAP-2 (70 kDa), RIPK-1 (78 kDa), RIPK-3 (46-62 kDa) and Bax (20 kDa) expressions by Western immunoblotting. Right panels are relative protein expressions of cIAP-1, cIAP-2, RIPK-1, RIPK-3 and Bax normalized to their respective GAPDH. The result shown is a mean $\pm$ SD of three independent experiments from different donors. (B) M0 macrophages were treated with Torin inhibitor (10 and 100 nM) for 2 hrs after (post-polarization) stimulation with IFN- γ for 48 hrs. Cells were then left untreated or treated with SM-LCL161 (1 μ M) alone or Torin and SM-LCL161 combined treatment for another 24 hrs. The total protein lysates were analyzed for cIAP-1 (62 kDa), cIAP-2 (70 kDa), RIPK-1 (78 kDa), RIPK-3 (46-62 kDa) and Bax (20 kDa) expressions by Western immunoblotting. Right panels are relative protein expressions of cIAP-1, cIAP-2, RIPK-1, RIPK-3 and Bax normalized to their respective GAPDH. The result shown is a mean $\pm$ SD of three independent experiments from different donors.

4.3 DISCUSSION

We have previously shown that primary human monocytes are highly sensitive to SM-induced cell death while, *in vitro* differentiated macrophages (M0) are resistant to the cytotoxic effect of SMs (317, 319, 320). I extended these studies and investigated the effect of SMs on the survival of normal human proinflammatory M1 macrophages and anti-inflammatory macrophages M2a, M2b and M2c subsets and the mechanisms involved. My results in the previous chapter show that SMs selectively killed proinflammatory M1 macrophages through both apoptosis and necroptosis whereas the M0 macrophages and the anti-inflammatory M2a M2b and M2c were highly resistant to SM-induced apoptosis. In this study, I investigated the molecular mechanism governing SM-induced cell death in M1 macrophages. My results show that susceptibility of M1 macrophages to SM-induced cell death is very much dependent on IFN- γ -mediated differentiation through the JAK-STAT pathway and subsequent activation of IRF-1. In addition, the selective cell death in SM-treated M1 macrophages is mediated by the simultaneous degradation of cIAP-2, RIPK-1 and RIPK-3 through the activation of mTORC signaling pathway. In contrast to the cancer cells. SM-induced cell death in M1 macrophages is independent of endogenously produced TNF- α and the canonical and non-canonical NF- κ B pathways. These observations hold a promising therapeutic strategy to kill proinflammatory M1 macrophages and eventually controlling the M1-associated diseases.

SM-mediated killing has been attributed to endogenous TNF- α in cancer cells (216, 349, 350, 358, 378). However, it has also been reported to be independent of TNF- α in some cancer cells (272, 379, 380). Given that macrophages produce high levels of TNF- α , the possibility that SM-mediated killing of M1 macrophages could be attributed to endogenously produced TNF- α was investigated. TNF- α is a pro-inflammatory cytokine that regulate cell survival,

inflammation and induction of cell death (381, 382). The degradation of cIAP-1 and cIAP-2 by SMs inhibit TNF- α -induced activation of the pro-survival NF- κ B pathway, and promotes the assembly of cell death complexes and induction of cell death (165, 349, 351). Indeed, my results demonstrate higher levels of endogenous TNF- α in M1 macrophages compared to M0 and M2c macrophages. However, SM-LCL161 did not show direct inductive effect on TNF- α production or synergistic effect with exogenous *rh*TNF- α on cell death in M0, M1 or M2c subsets. Since LPS stimulation is known to induce significant amount of TNF- α production, I investigated whether enhancing TNF- α production by LPS activation would affect their sensitivity to SM-LCL161 treatment. Interestingly, LPS stimulation as well did not affect the susceptibility of any of the macrophage subsets to SM-induced cell death. The fact that neither addition of *rh*TNF- α to M0 and M2c macrophages nor neutralizing the endogenous levels TNF- α by anti-TNF- α antibodies in M1 macrophages reversed the effect of SM-LCL161 on cell death suggested that SM-LCL161 does not affect or synergize with TNF- α in primary human M1 macrophages. Overall, the selective induction of cell death in M1 macrophages is regulated by a mechanism independent of endogenously produced TNF- α .

IFN- γ plays a key role in immunity against intracellular pathogens and cancer (257). Mice genetically deficient in IFN- γ or its receptor are highly susceptible to several microbial pathogens (383). It activates macrophages by up-regulating their lysosomal enzymatic activity and increasing the level of reactive oxygen species (384). It is primarily produced by activated Th cells and NK cells (385). IFN- γ mediates the polarization of M0 into M1 macrophage subset (42, 43). Thus, IFN- γ produced by immune cells may cause differentiation of macrophages into proinflammatory M1 macrophages *in vivo*. The molecular mechanism by which M1

macrophages following IFN- γ induced differentiation develop susceptibility to SM-induced cell death remains unknown. Binding of IFN- γ to its receptors (IFNGR) mediates the recruitment and activation of JAK/STAT signaling pathway which subsequently induces the transcription of interferon stimulated genes (ISGs) to regulate several biological processes involved in inflammation, host defense, the cell cycle, apoptosis, and immune responses (258, 260, 261). My results show that susceptibility of M1 macrophages to SM-induced cell death is very much dependent on IFN- γ -mediated differentiation as blocking its differentiation by inhibiting the IFN- γ signaling pathway using the Jak inhibitor and silencing of IRF-1 reversed the susceptibility of M1 macrophages to SM-induced cell death.

IFN- γ is known to induce the expression of IRF-1 that plays a key mediator in IFN- γ induced cell death in various cancer cells (272, 365, 366). IRF-1 has been shown to be a critical mediator of apoptosis and necroptosis induction in several apoptosis sensitive cancer cell lines following treatment with SMs in combination with IFN- γ (272). In agreement with this, evaluation of IRF-1 expression demonstrated an upregulation of IRF-1 in M1 macrophages compared to M0 and M2c macrophages suggesting that IFN- γ following priming induced the expression of IRF-1 in M1 macrophages. However, SM-LCL161 treatment did not affect the expression of IRF-1 in M1 macrophages. Interestingly, silencing IRF-1 expression by IRF-1-siRNAs demonstrated enhanced survival or reduction in SM-induced cell death in M1 macrophages. These observations suggest that high expression of IRF-1 may contribute to SM-induced cell death in M1 macrophages. The fact that SMs-LCL161 induced apoptosis and necroptosis in M1 macrophages (**Chapter 3**) and blocking IRF-1 provided protection from

SM-induce cell death, strongly suggests an important role for IRF-1 in SM-induced cell death in M1 macrophages.

PKR is an IFN-induced kinase that plays a key role in the innate immunity for defense against viral infection (276, 368) and has been shown to promote cell death through IFN- γ and Fas-induced apoptosis (431–434). It also mediates necroptosis by regulating RIPK-1/RIPK-3 interactions (283). Therefore, I hypothesized that IFN- γ -induced differentiation of M0 macrophages into M1 macrophages may be mediated by the induction of PKR activity and inhibition of this activity may reduce SM-induced cell death. Using the PKR specific inhibitor (C16), my results clearly show that PKR is not involved in the regulation of SM-induced cell death in M1 macrophages.

In addition to the JAK/STAT pathway, IFN- γ is also known to regulates several other signaling pathways including PI3K-mTORC and MAPK pathways and transcription factors (386–388). Thus, to elucidate the mechanism by which INF- γ contributes to SM-induced cell death in M1 macrophages, I investigated the involvement of several intracellular pathways in SM-induced cell death including PI3K/Akt, mTORC, MAPKs and NF- κ B by employing specific chemical inhibitors for each signaling pathway. These signaling pathways were targeted for inhibition either prior to polarization with IFN- γ or post-IFN- γ polarization.

In response to different metabolic and inflammatory signals, PI3K/Akt/mTORC pathway mediates downstream responses and gene expression which has been shown to regulate macrophage polarization, activation, and cytokines secretion (452, 460). However, several studies demonstrated contradictory roles of PI3K/Akt and mTORC on macrophage polarization (295). PI3K/Akt was shown to promote M2 macrophages polarization (446, 460–

463), while others reported its association with M1 phenotype activation (472–475). My results show that blocking of either the PI3K or Akt pathway by their respective inhibitors either prior to or post-M1 differentiation did not affect SM-induced cell death in M1 macrophages, thus, suggesting that SM-LCL161-induced cell death in M1 macrophages occurs independent of PI3K/Akt signaling pathway. The PI3K and Akt inhibitors used were biologically active as they inhibited the activation of their respective pathways following stimulation with either IFN- γ or LPS. However, adopting similar approach to block mTORC pathway revealed very interesting results. The mTORC pathway was targeted for inhibition using two common chemical inhibitors; Rapamycin that mainly targets mTORC1 and Torin that targets both mTORC1 and mTORC2. Additionally, I also used S6K inhibitor that works on S6K proteins downstream of mTORC1 (290). I have shown that IFN- γ activated the mTORC pathway by demonstrating the induction of S6K phosphorylation. All three inhibitors when applied on M0 macrophages prior to M1 differentiation significantly protected IFN- γ -stimulated M0 macrophages from the cytotoxic effect of SM-LCL161. Since both Rapamycin, the mTORC-1 inhibitor, and Torin, the inhibitor for both mTORC-1 and 2, inhibited SM-induced cell death, it was inferred that mTORC-1 was sufficient to mediate SM-induced cell death. These observations clearly suggested a strong involvement of mTORC-1-S6K signaling pathway independent of the PI3K/Akt pathway in regulating SM-induced cell death in M1 macrophages possibly by inhibiting IFN- γ -induced M1 differentiation. Whether rapamycin inhibited IFN- γ -induced M1 differentiation needs to be investigated.

Interestingly, only Torin when applied on differentiated M1 macrophages, was able to rescue M1 macrophages consistently and significantly from the cytotoxic effect of SM-LCL161. Incidentally, the S6K inhibitor did not rescue M1 macrophages from the cytotoxic

effect of SM-LCL161 under similar conditions in contrast to when it was applied prior to M1 polarization. Since both Rapamycin, the mTORC-1 inhibitor and the S6K inhibitors failed to inhibit SM-induced cell death, it was inferred that SM-induced cell death in differentiated M1 macrophages is regulated by a distinct mTORC-2 pathway independent of the mTORC-1-S6K pathway involved in SM-induced cell death in macrophages prior to M1 differentiation. These results suggest a differential requirement of mTORC1, mTORC-2 and S6K in SM-induced cell death occurring in differentiated M1 macrophages and the one occurring in macrophages prior to M1 differentiation. Further studies are needed to understand the effect of Torin on the M1 differentiation process and the precise mechanism of differential involvement of mTORC1, mTORC-2 and S6K in SM-induced cell death observed in differentiated M1 macrophages and the one observed in macrophages prior to M1 differentiation.

MAPKs are involved in several cellular processes such as stress, cell proliferation and cell death (374–376). cIAPs have been shown to be critical regulators of MAPK activation (362). Hence, I investigated the involvement of MAPK pathways in SM-induced cell death in M1 macrophages using their specific inhibitors. My results showed consistently that none of the p38, ERK or JNK MAPKs were involved in SM-induced cell death occurring in differentiated M1 macrophages or in macrophages prior to M1 differentiation. The MAPK inhibitors used were biologically active as they inhibited the activation of their respective pathways following stimulation with either IFN- γ or LPS.

The IAPs proteins are the key regulators of apoptosis, cell survival and innate immune responses (191, 210, 214). We have previously shown that IAPs play a key role in the resistance of macrophages to HIV-Vpr-induced apoptosis and mitochondrial depolarization (214)(320). In **chapter 3**, I demonstrated that M1 macrophages are highly sensitive to the

cytotoxic effects of SM whereas M0 and M2c macrophages are resistant. Herein, I investigated the involvement of IAPs in the differential regulation of SM-induced cell death in M1, M0 and M2c macrophages. I have shown that although SMs caused similar levels of IAPs degradation in M0, M1 and M2c macrophages, and M1 macrophages expressed higher levels of cIAP-1 and cIAP-2 compared to M0 and M2c macrophages, M1 macrophages were highly susceptible to SM-induced cell death. Conversely, M0 and M2c macrophages were highly resistant to SM-induced cell death although these macrophages expressed lower levels of cIAP-1 and cIAP-2. These results clearly suggested that SM-induced cell death in M1 macrophages is not attributed to the expression levels of IAPs alone and require the involvement of other IAP-associated signaling molecules or other members of pro-or anti-apoptotic family. Expression of the Bcl2 family members including Bcl2, Mcl-1, Bak, Bcl-xL and other mediator proteins such as FADD, Fas and TRAIL did not reveal any differences in their expression levels following SM-LCL161 treatment in either of M0, M1 or M2c macrophages. As expected, the levels of proapoptotic Bax were increased whereas that of anti-apoptotic cFLIP were decreased in M1 macrophages following SM-LCL161 treatment in contrast to the M0 and M2c macrophages wherein their levels were not affected. These results suggested that the anti-apoptotic Bcl-2 family members do not mediate the differential regulation of SM-induced apoptosis in M0, M2c and M1 macrophages.

RIPK-1 and RIPK-3 belong to the receptor interacting protein (RIP) kinases family. These enzymes play a key role in a variety of cellular processes including cell survival, apoptosis and necroptosis (389). RIPK1 associates with IAP proteins and is involved in the activation of several signalling pathways including NF- κ B, PI3K-Akt, and MAPKs (235, 351). In the context of TNF- α , the most studied stimulus in the context of RIPK activation, its

interaction with the TNF-R leads to the recruitment of the adaptor, TRADD, which recruits RIPK-1 and two ubiquitin ligases TRAF2 and cIAP-1 which eventually form the TNFR complex-I (240). Complex-I is then modified by the IAPs and the LUBAC leading to its ubiquitination and activation of NF- κ B and cFLIP (158, 159). cFLIP then binds to caspase-8, leading to caspase-8 inactivation and prevention of apoptosis (351). In the absence of IAPs and NF- κ B function, cFLIP is not produced. This results in the assembly of caspase-8 with FADD, and RIPK-1 in the cytosol forming a complex-IIa that eventually culminates in apoptosis (235, 351). In the absence of IAPs and the caspases, complex-IIb is assembled in the cytosol. This new complex contains the caspase-8-FLIP heterodimer as well as RIPK-1 and RIPK-3 (235, 351). Caspase inhibition within this complex allows RIPK-1 and RIPK-3 to auto-transphosphorylate each other, leading to the formation of necrosome (235, 351). The necrosome recruits MLKL which is phosphorylated by RIPK-3, the complex translocates to the plasma membrane, leading to pore formation and eventually necroptosis (239, 243).

We have shown for the first time that SM-LCL161 alone in the absence of caspase inhibitors induced apoptosis as well as necroptosis in M1 macrophages as simultaneous addition of inhibitors for both apoptosis and necroptosis completely prevented SM-induced cell death. Interestingly, evaluation of IAPs and IAP-associated kinases in M0, M1 and M2c macrophages revealed that cIAP-1 and cIAP-2, RIPK-1, RIPK-3 and MLKL were degraded in M1 macrophages following SM-LCL161 treatment. Prior treatment of M1 macrophages with Necrostatin-1 or NSA prevented their degradation. These results clearly suggest that RIPK-1 and RIPK-3 proteins may co-operate with IAPs in the differential regulation of SM-induced cell death in M0, M2c and M1 macrophages. The fact that mTORC inhibitor, Torin, restored the expression of cIAP-2 and RIPK-1 and RIPK-3 following SM-LCL161 treatment further

suggested that SM-induced cell death in M1 macrophages is regulated by cIAP-2 and RIPK-1/3 through the activation of mTORC1/2. However, the involvement of IRF-1 in the regulation and degradation of cIAP-1/2 and the RIPK-1/3 is under investigation. The precise mechanism by which mTORC1/2 and IRF-1 regulate the expression of cIAP-2 and RIPK-1/3 is not clear at present and is being investigated. My results also show that SM-induced cell death in M1 macrophages is also regulated by the JAK-STAT pathway. The relationship between the JAK-STAT pathway and the mTORC-1/2 pathway in the regulation of SM-induced cell death is not clear. Whether these two pathways cross talk with each other or run parallel and independent of each other is under investigation.

cIAP-1 and cIAP-2 also play an essential role in NF- κ B activation (202, 362). Following interaction of TNF- α with TNF- α receptor, cIAP-1/2, and several other mediator proteins such as TRAFs, RIPK-1 are recruited to form a complex that eventually leads to the activation of the canonical NF- κ B pathway (2). Simultaneously, cIAPs inhibit the activation of non-canonical NF- κ B pathway by promoting constant degradation of NF- κ B-inducing kinase (NIK) (17). cIAPs ablation by SMs has been shown to promote NIK accumulation and subsequent activation of non-canonical NF- κ B pathway in various cell types (203, 364) leading to the gene transcription for several proteins involved in inflammation, proliferation and cell survival (193–195). In tumor cell lines, SMs alone activate both canonical and non-canonical NF- κ B pathways by promoting TNF- α secretion and stabilizing NIK, respectively (202, 203, 359). In this study, we show that NF- κ B activation by SMs differ significantly in human macrophages and tumor cell lines. SMs alone did not activate either canonical or non-canonical NF- κ B pathway in human M1 macrophages. As expected, when using LPS as a control, my results show that LPS stimulation induced NF- κ B signaling, causing activation of

the canonical NF- κ B pathway and the non-canonical NF- κ B pathway, manifested by reduced I κ B degradation and increased p100 processing, respectively. Moreover, SM enhanced LPS-induced activation of non-canonical NF- κ B pathway as indicated by enhanced p100 processing and accumulation of p52. The different results on SM-induced TNF- α production, and NF- κ B pathways obtained in cancer cell lines and primary macrophages may be due to different cell types and TNF- α secretion patterns in response to SMs. SMs-sensitive cell lines secrete TNF- α in response to cIAPs ablation and consequently become susceptible to TNF- α -induced apoptosis (350). However, we did not detect enhanced TNF- α secretion in M1 macrophages following SMs treatment either alone or in response to LPS stimulation. This result is in keeping with the fact that SMs treatment causes apoptosis of sensitive cancer cells (349, 350, 358) but not in primary macrophages (319, 320). These results were in conformity with our observations using the NF- κ B inhibitor clearly indicating that NF- κ B did not mediate SM-induced cell death in M1 macrophages.

In summary, my results show that susceptibility of M1 macrophages to SM-induced cell death is dependent on IFN- γ -mediated differentiation through the JAK-STAT pathway and subsequent activation of IRF-1. In addition, SM-induced cell death in M1 macrophages occurs through the simultaneous degradation of cIAP-2, RIPK-1 and RIPK-3 through the activation of mTORC signaling pathway. Moreover, unlike cancer cells, SM-induced cell death in M1 macrophages is not regulated by endogenously produced TNF- α and the canonical and non-canonical NF- κ B pathways. The inflammatory M1 macrophages have been implicated in many diseases including rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis (5, 7, 55, 56, 352–355). Selective induction of cell death in M1 macrophages by agents blocking

the IAPs and/or RIPK-1/3 would have potential therapeutic benefits in regard to macrophage associated inflammatory diseases.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Primary macrophages once polarized, express different levels of macrophage-specific surface markers expression that can be used to differentiate various macrophages subsets *in vitro*. Since the main aim of my research project was to study the effects of the smac mimetics (SMs), the inhibitors of IAPs, on the survival or killing of proinflammatory and anti-inflammatory human macrophages, I, first, generated and phenotypically characterized all four polarization states in human macrophages. Each polarization state exhibited a unique surface marker profile which can be used to characterize human macrophage populations in inflammation and inflammation related disorders and understand disease pathogenesis.

Subsequently, I studied the role of IAPs in regulating immune responses in primary human macrophages and their subsets. I have shown for the first time that blocking IAPs signaling pathway by SM-LCL161 selectively induced cell death in proinflammatory M1 macrophages through both extrinsic and intrinsic pathways. SM-induced cell death in M1 macrophages is mediated by both apoptosis and necroptosis pathways as blocking caspases by a pan-caspase inhibitor (zVAD.fmk) and necroptosis inhibitors (NSA) completely prevented SM-induced apoptosis in M1 macrophages. Furthermore, evaluation of apoptosis/necroptosis associated proteins in SM-treated macrophages revealed induction in phosphorylation of effector proteins including RIPK-1, RIPK-3 and MLKL. The exact molecular mechanism by which SM-LCL161 alone induced necroptosis in M1 macrophages in the presence of activated caspases most importantly caspase-8 need to be investigated. Moreover, whether apoptosis and necroptosis are operative in the same cell or different cells are undergoing different death mechanisms independently of each other is not clear and remains to be investigated.

In contrast to M1 macrophages, M0 and M2c macrophages experienced cell death following simultaneous blockage of the IAPs pathways by SM-LCL161 and the caspase pathways by pan-caspase inhibitors, and this cell death was attributed to necroptosis. SM-LCL161 and zVAD.fmk combined treatment demonstrated induction of RIPK-1, MLKL phosphorylation as well as reduction of unphosphorylated RIPK-1, RIPK-3 and MLKL. The precise mechanism by which M0/M2c macrophages are resistant to SM-LCL161 alone compared to M1 macrophages is not known and needs further investigation. Since M2c macrophages are generated following IL-10 stimulation, the role and molecular mechanism by which IL-10 leads to induction of necroptosis in M2c macrophages needs further investigation.

Macrophage polarization is not an end stage of differentiation as the expression of cell surface markers, signaling mediator proteins as well as the susceptibility to SM-induced cell death can be modulated by changing the cytokine environment. The M1 macrophage ability to undergo SM-induced cell death is transient and this transient status can last as long as these cells are exposed to the specific stimuli. Additionally, I have shown that SM-LCL161 effect on cell death is conserved across mouse and human M1 macrophage subsets. Thus, mouse model can be a useful experimental tool to study the cytotoxic effect of SMs on several inflammatory diseases.

Next, I examined the molecular mechanism by which SM-LCL161 induces cell death in M1 macrophages. I have demonstrated that selective induction of cell death in SM-treated M1 macrophages is independent of endogenously produced TNF- α as well as canonical and non-canonical NF- κ B pathways. Despite higher levels of endogenous TNF- α in M1 macrophages compared to M0 and M2c macrophages, SM-LCL161 did not show any direct inductive effect on cell death in M1 macrophages or synergistic effect with exogenous *rh*TNF-

α on cell death of M0 or M2c macrophages. Moreover, SM-LCL161 did not show activation of either canonical or non-canonical NF- κ B pathway in human M1 macrophages.

The susceptibility of M1 macrophages to SM-induced cell death was attributed to the IFN- γ -mediated differentiation as blocking its differentiation by inhibiting the IFN- γ signaling pathway using the Jak inhibitor or silencing of IRF-1 reversed the susceptibility of IFN- γ -stimulated M0 macrophages to SM-induced cell death. IFN- γ induces the expression of IRF-1 in M1 macrophages through JAK/STAT signaling pathway. I observed upregulation of IRF-1 in M1 macrophages compared to M0 and M2c macrophages, suggesting that high expression of IRF-1 may contribute to SM-induced cell death in M1 macrophages.

My results show that blocking of the MAPKs, PI3K or Akt pathway by their respective inhibitors either prior to or post-M1 differentiation did not affect SM-induced cell death, thus, suggesting that SM-LCL161-induced cell death in M1 macrophages occurs independent of MAPKs and PI3K/Akt signaling pathway. I have also demonstrated that selective induction of cell death in SM-treated M1 macrophages is mediated by the status of macrophages polarization and the mTORC-1/2 pathway. Inhibition of mTORC-1/2 or S6Kinase during M1 polarization or prior to M1 polarization rescued M1 macrophages from SM-induced cell death. Since treatment with Rapamycin, the mTORC-1 inhibitor, prior to M1 polarization completely inhibited SM-induced cell death, mTORC-1 was thought to be sufficient enough to mediate SM-induced cell death. The results clearly suggested a strong involvement of mTORC-1-S6K signaling pathway independent of the PI3K/Akt pathway in regulating SM-induced cell death in M1 macrophages possibly by inhibiting IFN- γ -induced M1 differentiation. Whether Rapamycin inhibited IFN- γ -induced M1 differentiation needs to be investigated.

However, the mechanism governing SM-induced cell death in polarized M1 macrophages was quite different than SM-induced cell death observed in M1 macrophages during differentiation. My results suggest a differential requirement of mTORC1, mTORC-2 and S6K in SM-induced cell death occurring in polarized M1 macrophages and the one occurring in macrophages prior to M1 differentiation. Interestingly, only Torin when applied on differentiated M1 macrophages, was able to consistently and significantly rescue M1 macrophages from the cytotoxic effect of SM-LCL161. Since both Rapamycin, the mTORC-1 inhibitor and the S6K inhibitors failed to inhibit SM-induced cell death, it was inferred that SM-induced cell death in differentiated M1 macrophages is regulated by a distinct mTORC-2 pathway independent of the mTORC-1-S6K pathway involved in SM-induced cell death in M1 macrophages during differentiation. Further studies are needed to understand the effect of Torin on the M1 differentiation process and the precise mechanism of differential involvement of mTORC1, mTORC-2 and S6K in SM-induced cell death observed in differentiated M1 macrophages and the one observed in macrophages prior to M1 differentiation.

Investigation on the involvement of IAPs in the differential regulation of SM-induced cell death in M1, M0 and M2c macrophages revealed that SM-induced cell death in M1 macrophages is not attributed to the expression levels of IAPs alone and require the involvement of other IAP-associated signaling molecules or other members of pro-or anti-apoptotic family. My results suggested that the anti-apoptotic Bcl2 family members do not mediate the differential regulation of SM-induced apoptosis in M0, M2c and M1 macrophages. There was no difference in the expression levels of the Bcl-2 family members including Bcl-2, Mcl-1, Bak, Bcl-xL and other mediator proteins such as FADD, Fas and TRAIL following SM-LCL161 treatment in either of M0, M1 or M2c macrophages. However, as expected, the

levels of pro-apoptotic Bax were increased whereas that of anti-apoptotic cFLIP were decreased in M1 macrophages following SM-LCL161 treatment in contrast to the M0 and M2c macrophages wherein their levels were not affected.

I have shown for the first time that SM-LCL161 alone in the absence of caspase inhibitors induced apoptosis as well as necroptosis in M1 macrophages as simultaneous addition of inhibitors for both apoptosis and necroptosis completely prevented SM-induced cell death. Interestingly, evaluation of IAPs and IAP-associated kinases in M0, M1 and M2c macrophages revealed that cIAP-1 and cIAP-2, MLKL, RIPK-1 and RIPK-3 were degraded in M1 macrophages following SM-LCL161 treatment. Prior treatment of M1 macrophages with Necrostatin-1 or Necrosulfonamide prevented their degradation.

Investigations on the expression of apoptosis and necroptosis associated kinases in SM-induced cell death in M1 macrophages suggested that RIPK-1 and RIPK-3 proteins may co-operate with IAPs in the differential regulation of SM-induced cell death in M0, M2c and M1 macrophages. As mentioned earlier, cIAP-1 and IAP-2, RIPK-1 and RIPK-3 were degraded in M1 macrophages following SM treatment. Prior treatment of M1 macrophages with Necrostatin-1 or Necrosulfonamide prevented their degradation. The fact that mTORC inhibitor, Torin, restored the expression of cIAP-2 and RIPK-1 and RIPK-3 following SM-LCL161 treatment further suggested that SM-induced cell death in M1 macrophages is regulated by cIAP-2 and RIPK-1/3 through the activation of mTORC1/2. However, the involvement of IRF-1 in the regulation and degradation of cIAP-1/2 and the RIPK-1/3 is under investigation. The precise mechanism by which mTORC1/2 and IRF-1 regulate the expression of cIAP-2 and RIPK-1/3 is not clear at present and is being investigated.

My results also show that SM-induced cell death in M1 macrophages is also regulated by the JAK-STAT pathway. The relationship between the JAK-STAT pathway and the mTORC-1/2 pathway in the regulation of SM-induced cell death is not clear. Whether these two pathways cross talk with each other or run parallel and independent of each other is under investigation.

In summary, the results show that survival of human macrophages is critically linked to the activation / blockage of the IAPs pathways and differentiation towards either the pro-inflammatory state by IFN- γ or the anti-inflammatory state by IL-10. My results suggest that agents blocking the IAPs and/or caspases can be exploited therapeutically to address inflammation-related disorders. Inflammatory M1 macrophages have been implicated in many diseases including rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis, whereas anti-inflammatory M2 macrophages have been associated with lepromatous leprosy, Whipples disease and chronic rhinosinusitis. Since SM-LCL161 effect on cell death is conserved across mouse and human M1 macrophage subsets, mouse model can be a useful experimental tool to study the cytotoxic effect of SMs on several inflammatory diseases.

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Nature 517:311–320.

CURRICULUM VITAE

HAMZA ALI

Education:

- 2014-Present **PhD Candidate. Department of Microbiology and Immunology**
University of Ottawa, Ottawa, Canada
Supervisor: Dr. Ashok Kumar.
- 2009- 2012 **M.Sc. Student. Department of Microbiology and Immunology**
University of Ottawa, Ottawa, Canada
Thesis Title: “*Growth and Biofilm Formation of Bacteria Isolated from Contaminated Platelet Units*”
Supervisor: Dr. Sandra Ramirez-Arcos
- 2003-2007 **B.Sc. Student. Faculty of Applied Medical Sciences**
King Abdulaziz University, Jeddah, Saudi Arabia
Thesis Title: “The Effect of Smoking on the Concentration of Secretory IgA”
Grade: Very Good, GPA 80.70%

Work Experience:

- 2014- Present **Student - Researcher, Children Hospital of East Ontario, and Department of Microbiology and Immunology.**
University of Ottawa, Ottawa, Canada
- Involved in studying and conducting experiments to selectively induce killing of HIV infected macrophages by targeting the apoptosis machinery using SMAC and PI3K inhibitors.
- 2007- 2014 **Teaching Assistant, Bacteriology Department, Faculty of Applied Medical Sciences.**
Taibah University, Medina, Saudi Arabia
- **Courses taught:**
 - Bacteriology/Microbiology, for third year Faculty of Applied Health Sciences students
 - Laboratory Instrumentation, for third year Faculty of Applied Health Sciences students
- 2009- 2012 **Student - Researcher, Canadian Blood Services, and Department of Microbiology and Immunology,**
University of Ottawa, Ottawa, Canada
- Involved in studying and conducting experiments on biofilm formation of different staphylococcus species in platelet concentrates
 - The research had an emphasis on the analysis and identification of 29 coagulase-negative staphylococcus isolates

Publications:

- 1- **Hamza Ali**, Ramon Caballero, Simon Dong, Niranjala Gajnayaka, Duale Ahmed, Salma Iqbal, Edana Cassol, and Ashok Kumar. 2021. Selective killing of proinflammatory human M1 macrophages by SMAC mimetics and anti-inflammatory M2 macrophages by SMAC mimetics and caspase inhibitors. *Journal of Leukocyte Biology* DOI: 10.1002/JLB.4A0220-114RR.
- 2- Salma Iqbal, Ananda Mookerjee, Niranjala Gajnayaka, **Hamza Ali**, Faria Ahmed, Angela M Crawley and Ashok Kumar 2020. IFN- γ enhances Toll-like receptors-2, -3 and -4-mediated synthesis of IFN- γ in human M1 macrophages through the activation of S6K1-S6 pathway. **Submitted J. Immunology**.
- 3- Simon X.M. Dong, Frederick S. Vizeacoumar, Kalpana K. Bhanumathy, Nezeka Alli, Cristina Gonzalez-Lopez, Niranjala Gajanayaka, Ramon Caballero, **Hamza Ali**, Franco J. Vizeacoumar, and Ashok Kumar 2020. A genome-wide screen identified novel gene targets to selectively induce the apoptosis of HIV infected macrophages. **Submitted Molecular Systems Biology**.
- 4- **Hamza Ali**, Simon Dong, Niranjala Gajnayaka, Ramon Caballero, Edana Cassol, Jonathan Angel and Ashok Kumar. 2021. Selective induction of cell death in human M1 macrophages by Smac mimetics is mediated by cIAP-2 and RIPK-1/3 through the activation of mTORC. **Submitted J. Immunology**.
- 5- Pearl, D., Katsumura, S., Amiri, M., Tabatabaei, N., Zhang, X., Vinette, V., **Ali, H.**,... & Robichaud, N. (2020). 4E-BP–Dependent Translational Control of Irf8 Mediates Adipose Tissue Macrophage Inflammatory Response. *The Journal of Immunology*, 204(9), 2392-2400.
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- 8- **Ali H.**, Rood I.G., de Korte D, Ramírez-Arcos S. (2012) Strict anaerobic *Staphylococcus saccharolyticus* isolates recovered from contaminated platelet concentrates fail to multiply during platelet storage. *Transfusion*, 52(4):916-7.
- 9- Greco-Stewart V, **Ali H.**, Kumaran D, Kalab M, Rood IG, de Korte D, Ramírez-Arcos S. (2013 Apr 4). Biofilm Formation by *Staphylococcus capitis* strains isolated from contaminated platelet concentrates. *J Med Microbiol*. 2013. [Epub ahead of print]. PMID:23558135
- 10- **Ali H.**, Greco-Stewart VS, Jacobs MR, Yomtovian RA, Rood IG, de Korte D, Ramírez-Arcos S.(2014). Growth dynamics and biofilm formation of *S. epidermidis* strains isolated from contaminated platelet units. *J Med Microbiol*. 2014 Jun;63(Pt 6):884-91.PMID:24667769

Oral and Poster Presentations:

- 2018 **Ali, H.**, Caballero, R., Gajanayaka, N., Dong, S., Iqbal, S., Kumar, A., “*Involvement of IAPs Pathway in Survival of Human Macrophages Subsets*”. BMI Department Seminar day
University of Ottawa, ON (Oral Presentation)
- 2017 **Ali, H.**, Caballero, R., Gajanayaka, N., Dong, S., Iqbal, S., Kumar, A., “*Involvement of IAPs Pathway in Survival of Human Macrophages Subsets*”. BMI Department poster day
University of Ottawa, ON (Poster Presentation)
- 2016 **Ali, H.**, Caballero, R., Gajanayaka, N., Dong, S., Iqbal, S., Kumar, A., “*The Cellular Mechanisms of SMAC Treatment on Different Polarized Macrophage Subsets*”. BMI Department Seminar day
University of Ottawa, ON (Oral Presentation)
- 2015 **Ali, H.**, Iqbal, S., Kumar, A., “*selective induction of apoptosis in macrophages subsets*”. BMI Department poster day
University of Ottawa, ON (Poster Presentation)
- 2010 **Ali, H.**, Uzicanin, S., Jacobs, M., Yomtovian, R., Rood, I., de Korte, D., and Ramirez-Arcos, S., “*Characterization of coagulase negative staphylococci isolated from contaminated platelet concentrates*”.
31th Meeting of the Canadian Society for Transfusion Medicine. Vancouver, BC. May 13-16, 2010 (Oral Presentation)
- 2009 **Ali, H.**, Uzicanin, S., Jacobs, M., Yomtovian, R., Rood, I., de Korte, D., and Ramirez-Arcos, S., “*Growth and Biofilm Formation of Bacteria Isolated from Contaminated Platelet Units*”. BMI Department poster day
University of Ottawa, ON (Poster Presentation)

Awards and Scholarships:

- 2008-Present **Full scholarship for graduate studies (MSc/PhD)**, offered by Taibah University, Madinah, Saudi Arabia.
- 2007 **First poster award**, Second Microbiology Symposium, King Abdulaziz University, Saudi Arabia. \$500
- 2006-2007 **Academic excellence award**, Faculty of Applied Medical Sciences, King Abdulaziz University, Saudi Arabia
- 2005-2006 **Best microbiologist student**, Microbiology Laboratory, King Abdulaziz University Hospital, Saudi Arabia. \$1500

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Figure1.1 : Macrophage polarization and main functions.



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Figure 1.2: The intrinsic and extrinsic pathways of apoptosis.

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Figure 1.3: IAPs members and structures.

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Figure 1.4: Schematic representative of the effect of IAPs.

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Figure 1.5: Canonical and non-canonical NF- κ B pathway.

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Figure 1.6: Initiation of necroptosis pathway.

Pan, P., Cai, Z., Zhuang, C., Chen, X., & Chai, Y. (2019). Methodology of drug screening and target identification for new necroptosis inhibitors. *Journal of Pharmaceutical Analysis*, 9 (2), 71-76.

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Figure1.7: IFN- γ Signaling.

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Figure1.8: PI3K/Akt/TSC/mTOR pathway.

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ARTICLE

Selective killing of human M1 macrophages by Smac mimetics alone and M2 macrophages by Smac mimetics and caspase inhibition

Hamza Ali^{1,4}  | Ramon Caballero⁴ | Simon X.M. Dong⁴ | Niranjala Gajayaka⁴ |
Agatha Vranjkovic⁵ | Duale Ahmed⁶ | Salma Iqbal⁴ | Angela M. Crawley^{1,3,5,6} |
Jonathan B. Angel^{1,3,5}  | Edana Cassol⁷ | Ashok Kumar^{1,2,4} 

¹Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, ON, Canada

²Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, ON, Canada

³Department of Medicine, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada

⁴Apoptosis Research Centre, Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON, Canada

⁵Chronic Disease Program, The Ottawa Hospital Research Institute, Ottawa, ON, Canada

⁶Department of Biology, Carleton University, Ottawa, ON, Canada

⁷Department of Health Sciences, Carleton University, Ottawa, ON, Canada

Correspondence

Ashok Kumar, Division of Virology, Apoptosis Research Centre, Research Institute, Children's Hospital of Eastern Ontario and University of Ottawa, 401 Smyth Road, Ottawa, Ontario, Canada, K1H 8L1.
Email: akumar@uottawa.ca

Abstract

The inflammatory and anti-inflammatory M ϕ s have been implicated in many diseases including rheumatoid arthritis, multiple sclerosis, and leprosy. Recent studies suggest targeting M ϕ function and activation may represent a potential target to treat these diseases. Herein, we investigated the effect of second mitochondria-derived activator of caspases (SMAC) mimetics (SMs), the inhibitors of apoptosis (IAPs) proteins, on the killing of human pro- and anti-inflammatory M ϕ subsets. We have shown previously that human monocytes are highly susceptible whereas differentiated M ϕ s (M0) are highly resistant to the cytotoxic abilities of SMs. To determine whether human M ϕ subsets are resistant to the cytotoxic effects of SMs, we show that M1 M ϕ s are highly susceptible to SM-induced cell death whereas M2a, M2b, and M2c differentiated subsets are resistant, with M2c being the most resistant. SM-induced cell death in M1 M ϕ s was mediated by apoptosis as well as necroptosis, activated both extrinsic and intrinsic pathways of apoptosis, and was attributed to the IFN- γ -mediated differentiation. In contrast, M2c and M0 M ϕ s experienced cell death through necroptosis following simultaneous blockage of the IAPs and the caspase pathways. Overall, the results suggest that survival of human M ϕ s is critically linked to the activation of the IAPs pathways. Moreover, agents blocking the cellular IAP1/2 and/or caspases can be exploited therapeutically to address inflammation-related diseases.

KEYWORDS

apoptosis, human M ϕ subsets, inhibitors of apoptosis, necroptosis

Abbreviations: Bak, Bcl-2 homologous antagonist/killer; Bcl-2, B cell lymphoma 2; BMDM, bone marrow derived M ϕ s; cIAP1/2, cellular inhibitor of apoptosis protein 1/2; FADD, Fas-associated protein with death domain; Fas, Fas cell surface death receptor; HIV, human immunodeficiency virus; JAK, Janus Kinase; MDMs, monocyte-derived M ϕ s; MLKL, mixed lineage kinase domain-like protein; NIK, NF- κ B-inducing kinase; NSA, necrosulfonamide; PI, propidium iodide; PPAR γ , peroxisome proliferator-activated receptor- γ ; RIPK-1/3, receptor-interacting protein kinase-1/3; RUX, Ruxolitinib; SM, Smac mimetic; Smac, second mitochondrial activator of caspases; TNFR, TNF receptor; TRADD, TNFR-associated death protein; TRAF2, TNF receptor-associated factor-2; TRAIL, TNF-related apoptosis inducing ligand; xIAP, X-chromosome-linked IAP; YOYO-1, 1,1'-[1,3-propanediyl]bis[4-(dimethylimino)-3,1-propanediyl]-bis[4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]]-quinolinum tetraiodide; zVAD.fmk, Carbobenzoyl-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

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1 | INTRODUCTION

M ϕ s, a heterogeneous population of innate myeloid cells, play a key role in innate immunity and in the pathogenesis of several chronic and inflammatory diseases.^{1,2} At the tissue level, M ϕ s are exposed to different exogenous and endogenous factors including cytokines, chemokines, and microbial by-products that allow them to functionally polarize and express distinct surface markers, and cytokines.^{2–4} In general, M ϕ s can be activated to adopt two opposite polarized states, the “classically activated” M1 and the “alternatively activated” M2 M ϕ s.^{1,5} M1 M ϕ s are induced by intracellular pathogens, bacterial cell wall components such as LPS, IFN- γ , or TNF- α .^{3,5} These M ϕ s have a

high capacity to present Ag, produce nitric oxide or reactive oxygen intermediates, and secrete high levels of pro-inflammatory cytokines such as, IL-12, IL-23, TNF- α , IL-6, and IL-1 β .^{3,6,7} In contrast, M2 M ϕ s exhibit anti-inflammatory phenotype, generated following stimulation with IL-4, IL-13, IL-10, or TGF- β , and play a key role in wound healing, and tissue repair.^{8–10} The M2 M ϕ s can be further subclassified into M2a, M2b, and M2c subsets, depending on their functions, cytokine and chemokine profiles, and relative expression of surface receptors.^{6,11,12} M2a subset can be generated following treatment with IL-4 and/or IL-13, M2b subset can be induced by stimulation with immune complexes and TLR-agonists or IL-1 receptor ligands,^{6,12,13} whereas M2c subset can be induced by glucocorticoids and/or IL-10.^{6,12,14} M2a and M2c subsets are anti-inflammatory, whereas M2b secrete both pro- and anti-inflammatory cytokines.^{6,12–14}

Recent evidence obtained by microarray data and conventional approaches employed in *in vivo* studies show that M1 polarization signature is associated with bacterial infections including typhoid fever, tuberculosis leprosy, active tuberculosis, and *Helicobacter pylori* gastritis,^{7,15–18} and chronic autoimmune and inflammatory diseases such as rheumatoid arthritis, atherosclerosis, type-2 diabetes, and cancer.^{9,19} In contrast, M2 subset is associated with lepromatous leprosy, Whipple's disease, chronic rhinosinusitis, and allergic diseases.^{7,16,20–22} Recently, targeting M ϕ s has been suggested to be a potential therapeutic strategy such as recruitment of M2 M ϕ s, inducing a class switch from M1 to M2 subsets and downregulation of pro-inflammatory pathways within the M ϕ subsets to control cancer, chronic infections, autoimmune, and allergic diseases.^{3,23,24} Several clinically approved therapeutic agents such as peroxisome proliferator-activated receptor- γ (PPAR γ) inhibitors, statins, and zoledronic acid have been shown to impact the functional status of murine M ϕ s.^{3,25–27} Therefore, it is of utmost importance to identify agents that specifically target M1 and/or M2 M ϕ s as a potential therapeutic strategy to control diseases associated with pro- and anti-inflammatory phenotype.

During myeloid differentiation, M ϕ s develop resistance to spontaneous and induced apoptosis that is crucial to maintain survival of M ϕ s during the immune response against various pathogens.^{28,29} Infection of M ϕ s may enhance their viability and modulate the surrounding environment by over-expression of proinflammatory cytokines.^{30–32} The mechanism underlying enhanced resistance of M ϕ s may relate to the differential expression of pro- and anti-apoptotic genes/proteins including Bcl-2 and inhibitors of apoptosis (IAPs) family members.^{29,32,33} The IAP family encompasses XIAP, cellular IAPs (cIAP-1), and cIAP-2 that tightly regulates cell survival downstream of TNF receptor-1 (TNFR1) and other death receptors.^{34,35} The role of IAP family members has been studied by employing second mitochondria-derived activator of caspases (SMAC) mimetics (SMs).^{36,37} SMs are small molecule antagonists of SMAC, which competitively inhibit SMAC-cIAP-1/2 interaction and thus repress anti-apoptotic functions of IAP proteins.^{36,37} We have described the role of IAPs in the CpG-induced protection of M ϕ s to HIV-Vpr-induced apoptosis.^{32,33} Moreover, cIAP-2 down-regulation by siRNAs and SMs sensitized M ϕ s to Vpr-induced apoptosis.^{32,33,38} However, we and others

have shown that SMs do not exhibit any apoptotic effect on differentiated normal M ϕ s.^{32,39} Herein, we investigated the effect of SM-LCL161, a monovalent form of SMs, on normal human proinflammatory M1 M ϕ s and anti-inflammatory M ϕ s M2a, M2b, and M2c subsets.

2 | MATERIALS AND METHODS

2.1 | Generation of human monocyte derived M ϕ s subsets and mouse bone marrow derived M ϕ s *in vitro*

Blood was collected from healthy donors in heparinized 60 mL syringes. Monocyte-derived M ϕ s (MDMs; MO) were generated and differentiated as previously described.^{6,32} Briefly, PBMCs were collected by density gradient centrifugation using LymphoprepTM at 1600 rpm for 45 min (Stem Cell technologies, Vancouver, BC, CA). PBMCs were suspended in serum-free media (Isocove's Modified DMEM 1X; Wisent Inc., QC, CA) at a concentration of 2.5×10^6 cells/mL/well into 12 well polystyrene plates (Thermo Scientific, Rochester, NY, USA). Cells were cultured for 3 h to facilitate the adherence of monocyte to the plate surface, whereas non-adherent cells were washed off. To generate MDMs (MO), adherent cells were cultured for 7 days in complete media supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin, and streptomycin (R&D Systems, Minneapolis, MN, USA) along with 10 ng/mL of human recombinant M-CSF (R&D Systems). On day 4, cells were washed and fresh complete media with M-CSF was added. On day 8, purity of M ϕ s was assessed by measuring CD14⁺ expression by flow cytometry. MO M ϕ s thus generated contained <2% T cells, B cells, and NK cells. The MO M ϕ s were washed and M ϕ subsets were generated by adding the following cytokines in the fresh complete media for 48 h as previously described.⁶ M1 subsets were generated using IFN- γ (20 ng/mL; Thermo Scientific, Rochester, NY, USA), IL-4 (20 ng/mL; R&D Systems) was used to generate M2a M ϕ s, LPS (1 μ g/mL; Sigma-Aldrich) and IL-1 β (10 ng/mL; R&D Systems) was used to generate M2b M ϕ s whereas IL-10 (10 ng/mL; R&D Systems) was used to generate M2c M ϕ s. For repolarization studies, M1 and M2c M ϕ s were washed twice with serum-free media and rested for 48 h. M1 were then treated with IL-10 and M2c M ϕ s were treated with IFN- γ for another 48 h to generate M1 to M2c and M2c to M1 repolarized M ϕ s, respectively.

Bone-marrow derived M ϕ s (BMDM) were isolated and cultured based on a previously described protocol.⁴⁰ Briefly, cells were cultured in sterile Petri dishes (5×10^6 cells/dish) with a complete media (DMEM + 10% FBS + 1% penicillin and streptomycin) in the presence of 15% L929 conditioned medium that contains murine M-CSF and incubated for 10 days. On day 3, 10 mL of complete fresh media were added, and cells were left to adhere to the surface of Petri dish. On day 10, cells were detached using cold PBS, counted, and centrifuged at 1200 rpm for 10 min. Cells were then resuspended in 12-well polystyrene plate at a concentration of 0.5×10^6 cells/well and incubated overnight. Next, cells were washed with PBS and left untreated or treated with mouse recombinant IFN- γ (20 ng/mL; Stem Cell technologies), or mouse recombinant IL-10 (10 ng/mL; Stem Cell technologies) in a complete media for 48 h.

2.2 | Cell surface marker analysis by flow cytometry

The polarized and re-polarized Mφs were washed twice with PBS and the cells were dislodged using 500 µL of Accutase™ (Stem Cell technologies) for 45 min at 37°C. Cells were then collected and centrifuged at 2000 rpm for 5 min, followed by 2 times wash with PBS. The cells were incubated with human FcR blocking buffer (Milteny Biotec Inc. Auburn, CA, USA) for 20 min at 4°C followed by staining using PE-conjugated Abs for CD14 (Cat# 347497), CD80 (Cat# 557227), CD86 (Cat# 560958), CD163 (Cat# 333618), CD200R (Cat# 329305), and HLA-DR (Cat# 361708), (BD Biosciences, San Jose, CA, USA) for 1 h at 4°C in the dark as previously described.⁶ The cells were washed with PBS and centrifuged to remove any excess Abs. Next, 200 µL of PBS were added to each tube and the fluorescence was measured using PE (CD14, CD200R, and CD80), FITC (CD86 and CD163), and PE/Cy7 (HLA-DR) channels in Flow cytometer-BD LSRFortessa™ cell analyzer (BD Life Sciences, Mississauga, ON, CA). All samples tested were compared to M0 samples. The histograms and the mean fluorescence intensity (MFI) were analyzed and plotted using FlowJo Software version 10.0.7 (BD Biosciences, San Jose, CA, USA) and GraphPad Prism 5.0 software (San Diego, CA, USA).

2.3 | Cytokine quantification

The concentrations of pro- and anti-inflammatory cytokines in culture supernatants were quantified using multiplexing immunobead assays: IL-1β, IL-6, IL-10, IL-12p70, IFN-γ, and TNF-α were assayed using the Human Cytokine Panel A Kit, whereas IL-23 was assayed using the Human Cytokine Panel 2 Kit (Milliplex®, Millipore Sigma). The assays were performed as per the manufacturer's instructions. Briefly, cytokines standards were prepared in a series of dilutions to generate standard curves. Fifty microliters of the resuspended standard cytokine or culture supernatant were pipetted and incubated for 30 min at room temperature with a mixture of beads having specificity for each cytokine. The beads were washed several times with wash buffer. Biotinylated secondary Ab mixture was added and incubated for 30 minutes, washed 3 times and finally incubated with streptavidin-conjugated R-PE. Beads were washed 3 times and resuspended in 125 µL assay buffer, acquired and analyzed using the BioRad Luminex machine and BioPlexManager software (Bio-Rad Laboratories, Hercules, CA, USA).

2.4 | Signaling pathway inhibitors

SM-LCL161 was purchased from Active Biochem Ltd. (Kowloon, Hong Kong) diluted in DMSO and stored at -20°C before use. On the day of the experiment, cells were washed with serum-free media and different concentrations of SM-LCL161 (0.5 µM or 1 µM) were added or as indicated. The caspase inhibitor (zVAD.fmk; Cat#A1902), the receptor-interacting protein kinase 1 (RIPK-1) inhibitor (Necrostatin-1; Nec-1, Cat#A4213), and the JAK inhibitor (Ruxolitinib; RUX, Cat#A3012) were purchased from ApexBio (Houston, TX, USA), and the mixed lineage kinase domain-like protein (MLKL) inhibitor (Necrosulfonamide; NSA, Cat#5025) was purchased from R&D systems.

2.5 | Determination of cell death and caspase-8 activity by flow cytometry

Cell death was determined by intracellular propidium iodide (PI) staining as described previously.³² Briefly, cells were gently scraped and transferred along with the supernatant containing dead cells. Cells were centrifuged at 2200 rpm for 5 min, supernatants were removed, and cells were washed with 500 µL PBS, centrifuged, and followed by removal of supernatant. Methanol (350 µL) was added to each pellet and incubated for 15 min at 4°C in the dark. Cells were centrifuged again and washed before adding a mixture of 25 µL PI solution (Sigma-Aldrich) and 25 µL RNase (Roche Diagnostics, Laval, QC, CA) to each pellet followed by incubation for 1 h at 4°C in the dark. Cells were washed to remove excessive PI and 300 µL of PBS was added.

Caspase-8 was determined by using caspase-8 (active) FITC staining kit (Abcam Inc., Toronto, ON, CA, Cat # AB65614) as per the manufacturer's protocol. Briefly, Mφs were treated with SM-LCL161 followed by addition of caspase-8 reagent (FITC-IETD-FMK) for 1 h at 37°C. Cells were centrifuged and washed before analyzing by flow cytometry using FL-1 channel. Cell death and caspase-8 activity was assessed by flow cytometry using a BD LSRFortessa™ X20 flow cytometer. The histograms were plotted using FlowJo version 10.0.7 software.

2.6 | Determination of cell death and caspase-3/7 activity by IncuCyte™ Zoom

Kinetic live cell imaging was carried out with exogenous YOYO-1 iodide (Thermo Fisher Scientific Cat # Y3601) or caspase-3/7 reagent (ESSEN Bioscience, MI, USA, Cat# 4440) and monitored using IncuCyte™ Zoom (ESSEN Bioscience). The caspase 3/7 reagent directly binds to cells undergoing apoptosis by intrinsic and extrinsic pathways. The instrument allows visualization of the fluorescent signals and quantifies the intensity of signal/cell, whereas the cells are still in culture at 37°C. The IncuCyte™ live cell imaging was taken every 2 h for 48 h.

2.7 | Western blot analysis

Western immunoblotting was performed as described previously.³² Briefly, total cell proteins were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Mississauga, ON, CA). Membranes were treated with the following primary Abs: rabbit anti-caspase-9 (Cat# 9502), anti-caspase-8 (Cat# 9746), anti-caspase-3 (Cat# 9662), anti-PARP (Cat# 9532), anti-cFLIP (Cat# 56343), anti-Fas (Cat# 4233), anti-TRAIL (Cat# 3219), anti-pJAK-1 (Cat# 3331), anti-clAP-1 (Cat# 7065), anti-clAP-2 (Cat# 3130), anti-Mcl-1 (Cat# 5453), anti-Bax (Cat# 2772), anti-Bak (Cat# 6947), anti-FADD (Cat# 2782), anti-RIPK-1 (Cat# 4926), anti-pRIPK-1 (Cat# 65746), anti-RIPK-3 (Cat# 13526) anti-MLKL (Cat# 14993), and anti-pMLKL (Cat# 91689), Abs, all purchased from Cell Signaling Technology (Danvers, MA, USA). Membranes were then washed and probed with goat secondary Abs conjugated to HRP for 1 h (Bio-Rad Laboratories). For total loading proteins, membranes were restriped and probed with either anti-β-Actin (Cat# 8457) or anti-GAPDH

(Cat# 2118) Abs (Cell Signaling Technology). Protein bands were visualized using the Amersham ECL western blotting detection system (GE Healthcare Buckinghamshire, UK) and GeneSnap software.

2.8 | Statistical analysis

Bar graphs were generated using the GraphPad Prism 5.0 software (San Diego, CA, USA). Data were compared between the 2 groups from all experiments using Mann-Whitney test and values with $P < 0.05$ were considered to be statistically significant. Data are presented in graphical form as mean $\pm$ SD.

2.9 | Ethics statement

Healthy participants involved in the study gave informed written consent and the protocol for obtaining blood samples was approved by the Review Ethics Board of the Ottawa General Hospital and the Children's Hospital of Eastern Ontario, Ottawa, ON, Canada. All animal procedures were approved by the Carleton University Animal Care Committee and were conducted in accordance with the guidelines provided by the Canadian Council for Animal Care.

3 | RESULTS

3.1 | Human M ϕ subsets differentially express distinct cell surface markers and pro- and anti-inflammatory cytokines

The M ϕ subsets were characterized by analyzing the expression of cell surface markers that are generally present on M ϕ s and are involved in their activation including CD80, CD86, HLA-DR, CD14, CD163, and CD200R.^{6,41–43} M0 M ϕ s expressed all the above markers. The expression of surface markers on M ϕ subsets was defined by setting the expression levels in unpolarized M0 M ϕ s as the base line. The surface markers expressed at substantially high levels compared to the M0 M ϕ subsets were designated as "high" and selected as characteristic for that M ϕ subset. Thus, M1 M ϕ s differentially expressed high levels of CD80, CD86, and HLA-DR compared to the M0 M ϕ s (Fig. 1A) and were designated as CD80^{high}, CD86^{high}, and HLA-DR^{high}. Similarly, M2a M ϕ s were defined as CD86^{high}, CD200R^{high}, and HLA-DR^{high}; M2b M ϕ s were defined as CD14^{high} and CD80^{high}; whereas M2c M ϕ s were characterized as CD14^{high}, and CD163^{high} (Fig. 1A). Overall, M ϕ subsets could be distinguished by the differential up-regulation of these surface markers.

The expression of cytokines namely IL-1 β , IL-6, IL-10, IL-12p70, IFN- γ , and TNF- α by the above M ϕ subsets was determined following their stimulation with LPS. The results show that M1 M ϕ s produced significantly high levels of pro-inflammatory IFN- γ , IL-23, IL-12p70, and TNF- α and significantly low levels of anti-inflammatory IL-10 (Fig. 1B). M2c subset on the other hand primarily produced significantly high levels of IL-10 and significantly low levels of pro-inflammatory IFN- γ , IL-23, IL-12p70, and TNF- α . M2b produced both

pro-inflammatory IL-6, IL-1 β , TNF- α , and anti-inflammatory IL-10 cytokines. However, M2b did not produce IFN- γ , IL-12p70, and IL-23. M2a M ϕ s as expected did not produce any of the pro-inflammatory IFN- γ , IL-1 β , IL-23, IL-12p70 and produced high levels of anti-inflammatory IL-10 compared to that produced by M1 M ϕ s (Fig. 1B). Overall, phenotypic and functional characterization of the human M ϕ subsets suggest that M1 M ϕ s are pro-inflammatory whereas and M2c M ϕ s are anti-inflammatory in nature and confirm our previous observations.⁶

3.2 | Blocking IAPs signaling pathway by SM-LCL161 selectively causes cell death in human M1 M ϕ s, whereas M2 M ϕ s are resistant to SM-induced cell death

We have previously shown that human monocytes are highly susceptible to the apoptotic effects of SMs whereas differentiated M ϕ s (MDMs; M0) are resistant.^{29,32} To determine whether human M ϕ subsets namely M1, M2a, M2b, and M2c generated in vitro from M0 are also resistant to the SM-induced cell death, we first determined the involvement of IAPs signaling pathway on the cell survival of different human M ϕ subsets by using IAPs antagonist, SM-LCL161. The M ϕ subsets M0, M1, M2a, M2b, and M2c were treated with different concentrations of SM-LCL161 for 48 h followed by analysis of cell death by intracellular PI staining and flow cytometry. The dose of SM-LCL161 was selected based on previous studies.^{44,45} The results show that M1 is the most susceptible subset to SM-induced cell death at all concentrations (Fig. 2A–D). In contrast, M2c was the most resistant subset and did not show increased cell death after SM-LCL161 treatment at any concentration. Therefore, M1, M2c in addition to the unpolarized M0 were selected for further investigations. Using the automated IncuCyte™-YOYO-1 staining, we confirmed that M1 is highly sensitive to SM-induced cell death. SM-induced cell death in M1 cells was detectable as early as 10 h and reached a peak by 20 h following treatment with all the concentrations of SM-LCL161 (Fig. 3). In contrast, M2c subsets did not show increased levels of cell death at any time and at any concentration until 48 h post-SM-LCL161 treatment. Furthermore, M1 cells treated with SM-LCL161 exhibited increased level of YOYO-1 fluorescent intensity compared to M2c M ϕ s (Fig. 3 left panel). However, M0 M ϕ s exhibited scattered levels of YOYO-1 staining perhaps because M0 M ϕ s are heterogeneous and contain population of all subsets including M1 M ϕ s.

Next, we determined whether there is any association between the levels of IAP proteins expressed in various subsets and SM-induced cell death. Although SM-LCL161 caused selective cell death in M1 M ϕ s, SM-LCL161 caused degradation of both cIAP-1 and cIAP-2 in M0, M1, and M2c subsets and as early as 5 h after SM-LCL161 treatment (Fig. 4A and B). The untreated M0, and M2c M ϕ s expressed similar levels of cIAP-1 and cIAP-2, whereas untreated M1 M ϕ s expressed significantly high levels of cIAP-1 and cIAP-2 compared to M0 and M2c M ϕ s (Fig. 4A and B) suggesting the lack of association between the levels of IAP proteins expressed in various subsets and SM-induced cell death. In addition, the effect of SM-LCL161 on cIAP-1 and cIAP-2 was

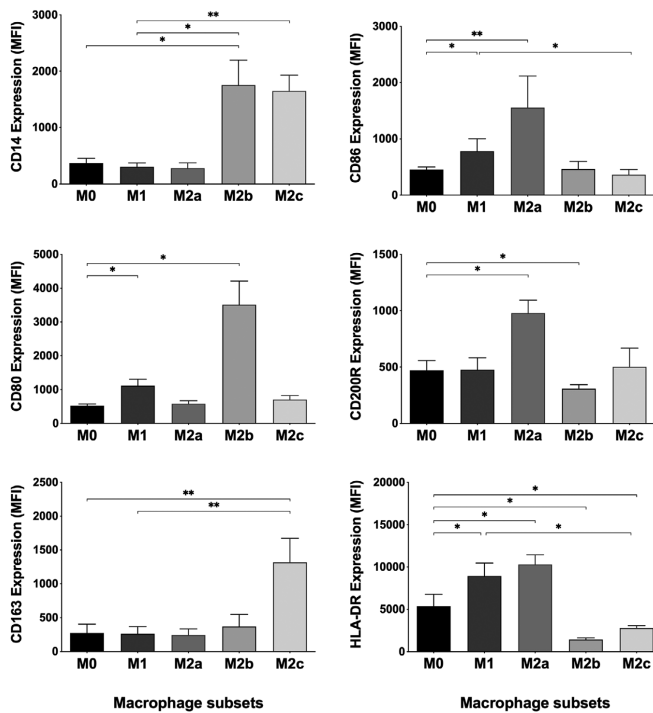


FIGURE 1 Phenotypic (A) and functional (B) characterization of undifferentiated M0 and polarized M1, M2a, M2b and M2c Mφs. (A) MDMs (M0) were polarized using indicated stimuli for 2 days: IFN- γ (20 ng/mL) for M1 Mφs, IL-4 (20 ng/mL) for M2a Mφs, LPS (1 μ g/mL) and IL-1 β (10 ng/mL) for M2b Mφs and IL-10 (10 ng/mL) for M2c Mφs. Polarized Mφs were stained with Abs against the mentioned cell surface molecules and fluorescence was measured by flow cytometry. The mean fluorescence intensity (MFI) values were obtained using FACSDiva software. The number (*n*) of different donors for analysis of CD14, CD80, and CD163 were 4 each, for CD86 and CD200R were 5 each and HLA-DR were 3. The results shown are mean $\pm$ SD. (B) Cytokines levels in the supernatants were measured using multiplexing immunobead assays as described in Section 2. Cytokines production by various subsets was reported as percentage of highest level of cytokines produced by M1 Mφs for IFN- γ , IL-12p70, IL-23, and TNF- α . M2c Mφs for IL-10 and M2b for IL-1 β and IL-6. The number (*n*) of different donors for analysis of IFN- γ , IL-1 β , and IL-23 were 4 each and for TNF- α , IL-10, IL-6, and IL-12p70 were 3 each. The results shown are mean $\pm$ SD.

specific as it did not impact other pro-apoptotic proteins, Fas, TRAIL, and Bak, and anti-apoptotic protein, Mcl-1 or the adaptor protein, FADD. (Fig. 4C, Supplementary Fig. 1). However, SM-LCL161 treatment at 1 μ M concentration resulted in up-regulation of pro-apoptotic protein Bax and down-regulation of the anti-apoptotic protein cFLIP expression in M1 Mφs but not in M0 and M2c Mφs compared to untreated Mφs (Fig. 4C).

3.3 | SM-induced cell death in M1 Mφs is mediated by both extrinsic and intrinsic pathways of apoptosis

Apoptosis involves proteolytic processing and activation of caspases, the key effector molecules of apoptosis pathway.⁴⁶ The extrinsic pathway of apoptosis is mediated by the activation of caspase-8 while the intrinsic pathway is mediated by caspase-9, both of which activate caspases-3/7 leading to apoptosis.^{46,47} To determine whether SM-LCL161 induced cell death is regulated by the intrinsic or the extrinsic pathways of apoptosis, we measured the activation of caspase-3/7, -8, and -9 in SM-treated Mφ subsets. M1, M0, and M2c Mφs were treated with either 0.5 μ M or 1 μ M of SM-LCL161 for 48 h followed by analysis of caspase-8, -9, and -3/7 by flow cytometry, immunoblotting, and IncuCyte™ Live cell imaging, respectively. There was a significant increase in caspase-3/7 activities in M1 Mφs upon treatment with 0.5 μ M of SM-LCL161 compared to M0 and M2c Mφ

subsets as measured by the IncuCyte™ live imaging (Fig. 5A). Increase in caspase-3/7 activities in M1 Mφs upon treatment with 1 μ M of SM-LCL161 is shown in Supplementary Fig. 2. This increase in caspase 3/7 activity in M1 Mφs was detected as early as 4 h and reached a peak by 10 h post SM-LCL161 treatment (Fig. 5A). Moreover, this induction was reduced in a dose-dependent manner after treatment with a pan-caspase inhibitor, zVAD.fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) at 10 and 20 μ M concentrations (Fig. 5A and Supplementary Fig. 2). The caspase-3/7 expression was poorly detected in the presence or absence of SM-LCL161 in both M0 and M2c Mφs. The caspase-8 activity was assessed by measuring its cleavage activity by flow cytometry. M1 Mφs treated with either 0.5 μ M or 1 μ M of SM-LCL161 revealed significantly higher caspase-8 activity compared to the untreated cells, whereas SM-treated M0 or M2c Mφs did not show cleavage activity following SM-LCL161 treatment and their levels were similar to the untreated Mφs (Fig. 5B). The representative flow histogram showing caspase-8 activity in M0, M1, and M2c subsets is shown (Supplementary Fig. 3). Staurosporine was used as a positive control to induce caspase-8 activity in all 3 subsets. For caspase-9, the Western immunoblots showed increase in cleaved caspase-9 bands upon treatment with 0.5 and 1 μ M of SM-LCL161 in M1 Mφs (Fig. 5C). These results suggest the involvement of both intrinsic and extrinsic apoptosis pathways in SM-induced apoptosis in M1 Mφs.

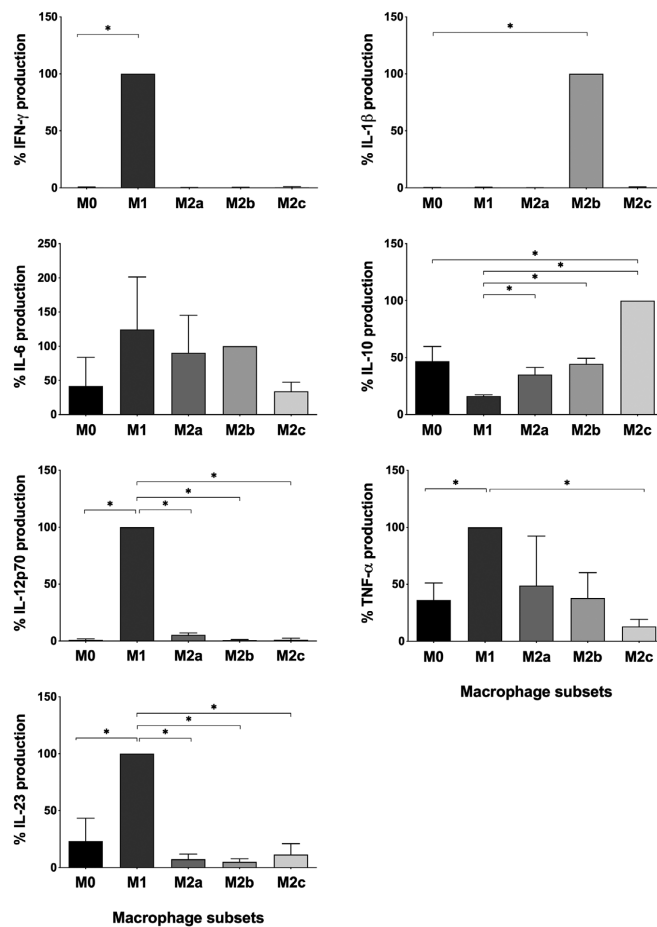


FIGURE 1 Continued

3.4 | SM-induced cell death in M1 M ϕ s involves both apoptosis and necroptosis pathways

To confirm that SM-induced cell death was associated with caspase activation, we examined whether blocking these caspases would inhibit SM-induced apoptosis. For this, M1 M ϕ s were pretreated with either 10 or 20 μ M of zVAD.fmk, for 1 h followed by treatment with either 0.5 or 1 μ M of SM-LCL161. Evaluation of apoptosis by intracellular PI staining and flow cytometry revealed that zVAD.fmk alone did not cause an increase in cell death. M1 M ϕ s treated with SM-LCL161 demonstrated an increased cell death as expected. SM-induced cell death was significantly reduced upon combined treatment with either concentrations of zVAD.fmk (Fig. 6A). Although blocking apoptosis by zVAD.fmk treatment significantly reduced SM-induced apoptosis, it reduced cell death by approximately 50% suggesting that the residual

cell death seen in M1 M ϕ s after blocking caspases might be due to cell death mechanisms other than apoptosis.

Another key pathway by which cells die is by necroptosis. Necroptosis is a programmed form of necrosis, or inflammatory cell death.^{48,49} We surmised that the residual SM-induced cell death seen following caspase inhibition may be due to necroptosis pathway. Necroptosis has been examined by chemical inhibitors, Necrostatin-1 and Necro-sulfonamide (NSA). Necrostatin-1 targets the receptor-interacting protein kinase 1 (RIPK-1) activity, whereas NSA targets the mixed lineage kinase domain-like protein (MLKL).^{50,51} To understand the involvement of necroptosis, M1 M ϕ s were treated with different concentrations of necrostatin-1 (0.5 or 1 μ M; Fig. 6B) or NSA (0.5 to 7 μ M; Fig. 6C) for 1 h prior to treatment with 0.5 μ M or 1 μ M of SM-LCL161. After 48 h, cell death was assessed by intracellular PI staining/flow cytometry. Both Necrostatin-1 and NSA significantly reversed SM-induced

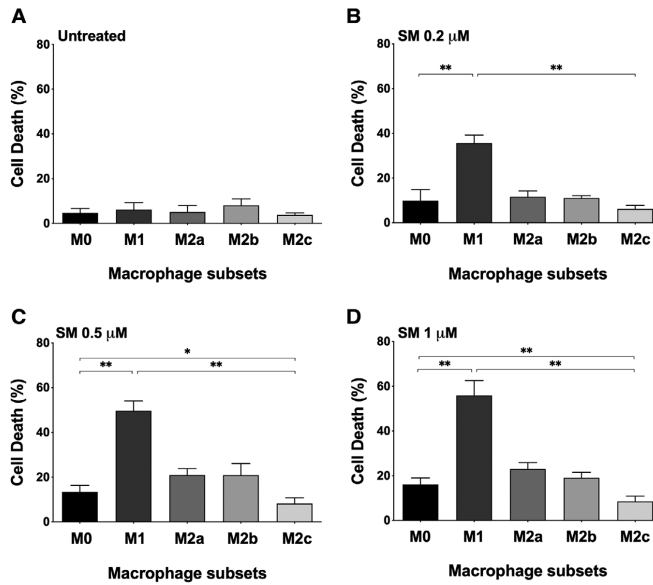


FIGURE 2 M1 and M2c are the most sensitive and resistant *Mφ*s subsets to SM-LCL161-induced cell death, respectively. M0, M1, M2a, M2b, and M2c *Mφ* subsets were (A) left untreated or treated with SM-LCL161 (SM) (B) 0.2 μ M, (C) 0.5 μ M, or (D) 1 μ M for 48 h. The percentage cell death was determined by intracellular PI staining and flow cytometry. The number (n) of different donors for A were 3 and for (B), (C), and (D) were 5 each. The results shown in (A-D) are mean $\pm$ SD

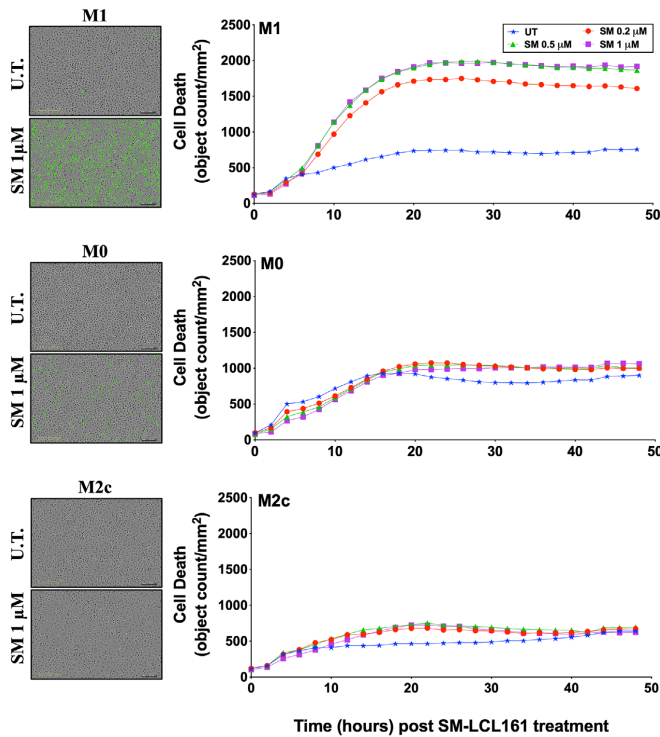


FIGURE 3 Kinetics of cell death in SM-treated M0, M1 and M2c *Mφ*s as determined by IncuCyte™ automated machine. Cell death stain (YOYO-1 iodide) was added to each well containing M1, M0, or M2c *Mφ*s 1 h prior to SM-LCL161 (SM) treatment. Cells were treated with SM-LCL161 at concentrations of 0.2 μ M, 0.5 μ M and 1 μ M and incubated in IncuCyte™ machine for 48 h. Cell death was recorded every 2 h for 48 h. Left panel shows live pictures taken at 48 h of IncuCyte™ incubation. Green fluorescent (left panels) represents dead cells. The result shown is a representative of 3 independent experiments from 3 different donors

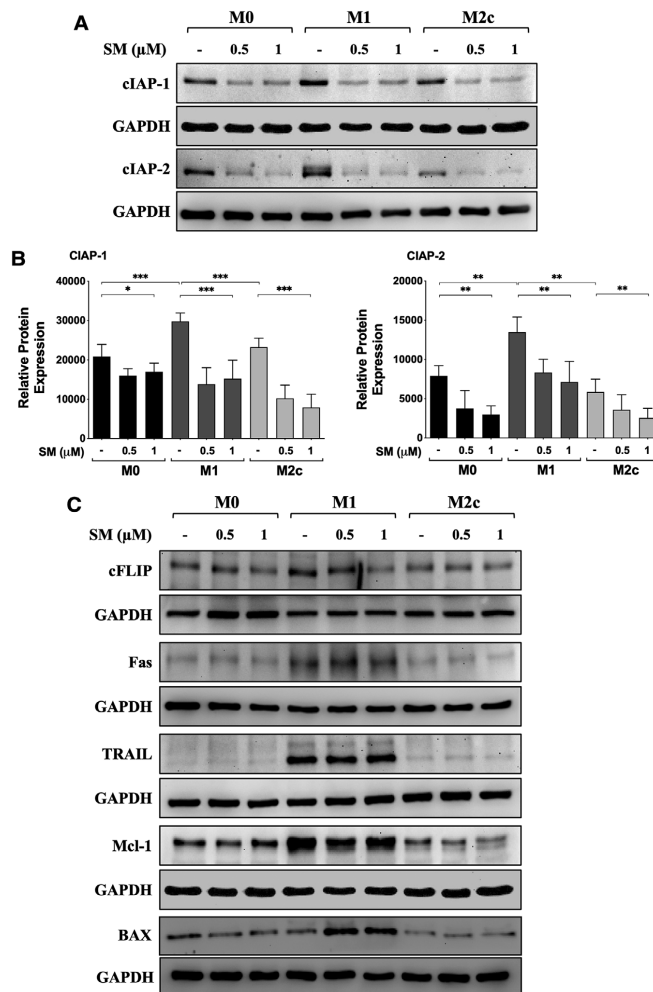


FIGURE 4 Effect of SM-LCL161 on the expression of anti-apoptotic and proapoptotic proteins. **(A)** Total protein lysates from M0, M1, and M2c Mφs treated with 0.5 and 1 μM of SM-LCL161 for 5 h were subjected to Western immunoblotting for the expression of cIAP-1 (62 kDa) and cIAP-2 (70 kDa). **(B)** Relative protein expression of cIAP-1, cIAP-2 in M0, M1, and M2c Mφs with or without treatment with SM-LCL161 (0.5 or 1 μM) for 5 h. The results shown are a mean $\pm$ SD from 6 experiments each. **(C)** Total protein lysates from M0, M1, and M2c Mφs treated with SM-LCL161 (0.5 and 1 μM) for 5 h and were subjected to Western immunoblotting for the expression of Fas (40 kDa), cFLIP (55 kDa), TRAIL (28 kDa), Bax (20 kDa), and Mcl-1 (40 kDa). The immunoblot shown is a representative of 3 independent experiments from 3 different donors

cell death in M1 Mφs in a dose-dependent manner after treatment with either 0.5 μM or 1 μM of SM-LCL161. Interestingly, similar to zVAD.fmk, blocking necroptosis pathway by either Necrostatin-1 or NSA inhibited SM-induced cell death by approximately 50%. Therefore, we hypothesized that SM-induced cell death in M1 Mφs may be mediated by both apoptosis as well as necroptosis. To determine whether SM-induced cell death involves both apoptosis and necroptosis, M1 Mφs were pretreated with zVAD.fmk and NSA together followed by treatment with 0.5 or 1 μM of SM-LCL161. SM-induced cell death was significantly inhibited and reduced to basal levels upon combined treatment of M1 Mφs with zVAD.fmk and NSA (Fig. 6D). Treatment with zVAD.fmk or NSA alone in the absence of SM-LCL161 did not affect cell death of M1 Mφs (Fig. 6D). These results suggest

the involvement of both apoptosis and necroptosis pathways in SM-induced cell death in M1 Mφs. Moreover, treatment of M1 Mφs with Necrostatin-1 alone (Fig. 6B) or NSA alone even at a very high concentration of 7 μM (Supplementary Fig. 4A) or even combined treatment with both zVAD.fmk and NSA (4 and 5 μM; Fig. 6D) did not cause an increase in cell death suggesting that M1 Mφs are not more sensitive to toxic compounds.

RIPK-1, RIPK-3, and MLKL have emerged as the key effectors molecules in apoptosis and necroptosis.^{47,52-54} Thus, we also examined the effect of SM-LCL161 on the expression levels of RIPK-1, RIPK-3, and MLKL following treatment of M1 Mφs with SM-LCL161 in the presence and absence of Necrostatin-1 and NSA. The results show that SM-LCL161 degraded RIPK-1, RIPK-3, and MLKL that was restored

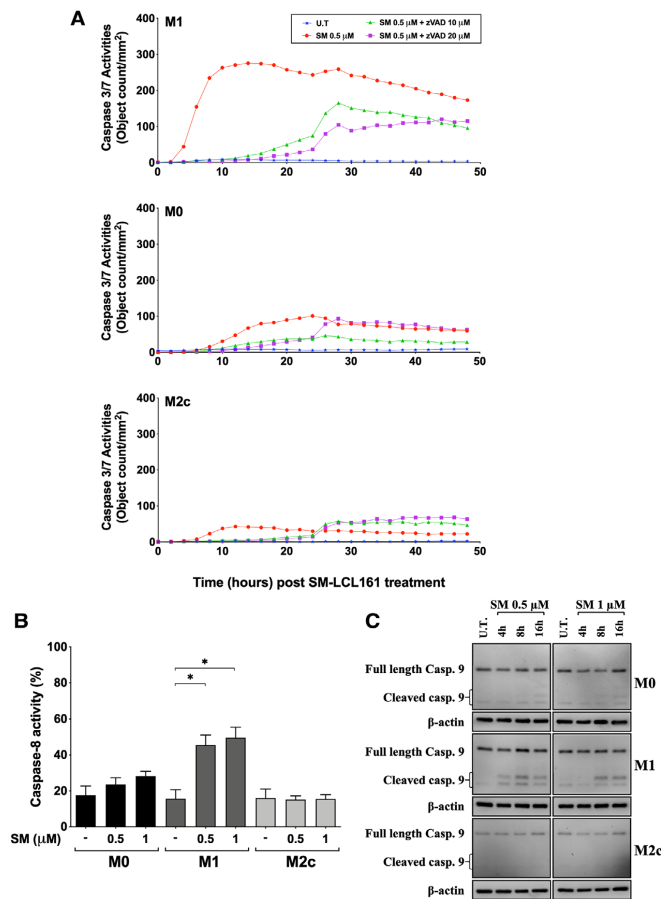


FIGURE 5 SM-LCL161 activates caspase 3/7, 8, and 9 in M1 M ϕ s. (A) M1, M0, and M2c subsets were treated with InCyteTM-casp3/7 reagent in the presence or absence of zVAD.fmk (10 and 20 μ M) 1 h prior to treatment with 0.5 μ M SM-LCL161 and incubated in InCyteTM machine for 48 h. The caspase-3/7 activity was recorded every 2 h for 48 h. (B) M0, M1, and M2c M ϕ s were treated with SM-LCL161 (0.5 μ M, 1 μ M) and cultured for 10 h following which caspase-8 reagent was added for 1 h before assessment of caspase-8 activity. The result shown is a mean $\pm$ SD of 3 independent experiments from 3 different donors. (C) M0, M1, and M2c M ϕ s were treated with either 0.5 or 1 μ M of SM-LCL161 for 4, 8, and 16 h. Total protein lysates were subjected to Western immunoblotting for caspase-9 expression. The immunoblots show full length and cleaved caspase-9 (47, 37, and 35 kDa). The result shown in (A) and (C) is a representative of 3 independent experiments from 3 different donors

following Necrostatin-1 and/or NSA treatment (Fig. 6E). Similarly, SM-LCL161 induced the phosphorylation of pRIPK-1 and pMLKL that was dephosphorylated in the presence of Necrostatin-1 and NSA (Fig. 6E).

3.5 | Reversal of M0 to M1 differentiation by blockage of JAK/STAT pathway reverses susceptibility of M1 M ϕ s to SM-induced apoptosis

JAK/STAT pathway is a principal signaling mechanism for a wide range of cytokines including IFNs. Activation of JAK leads to the phosphorylation of downstream targets including the STATs.⁵⁵ We determined if IFN- γ -mediated differentiation of M0 into M1 M ϕ s plays a role in regulating the susceptibility of M1 M ϕ s to SM-induced cell death. This was investigated by blocking JAK activation using JAK inhibitor, Ruxolitinib (RUX).⁵⁶ M0 M ϕ s were treated with various concentrations of RUX for 2 h prior to IFN- γ stimulation for 48 h. Cells were treated with SM-LCL161 for another 48 h followed by the measurement of cell

death by PI staining and flow cytometry. The results show that JAK inhibitor reversed susceptibility of M1 M ϕ s to SM-induced cell death in a dose-dependent manner to levels equivalent to the basal levels seen in unstimulated M0 M ϕ s (Fig. 7A). The biological activity of RUX was confirmed by inhibition of IFN- γ -induced JAK-1 phosphorylation (Fig. 7B).

3.6 | Blockage of caspases and IAPs signaling selectively cause cell death in M0 and M2c M ϕ s through the necroptosis pathway

In contrast to M1 M ϕ s, M0 and M2c M ϕ s did not show a significant increase in cell death following treatment with SM-LCL161 (Fig. 2). Blocking of caspases by the pan-caspase inhibitor zVAD.fmk in the absence of IAPs signaling pathway has been shown to induce cell death in cancer cells.^{57–60} To determine if SM-LCL161 can induce cell death in M0 and M2c cells following blockage of caspase activity, M0 and

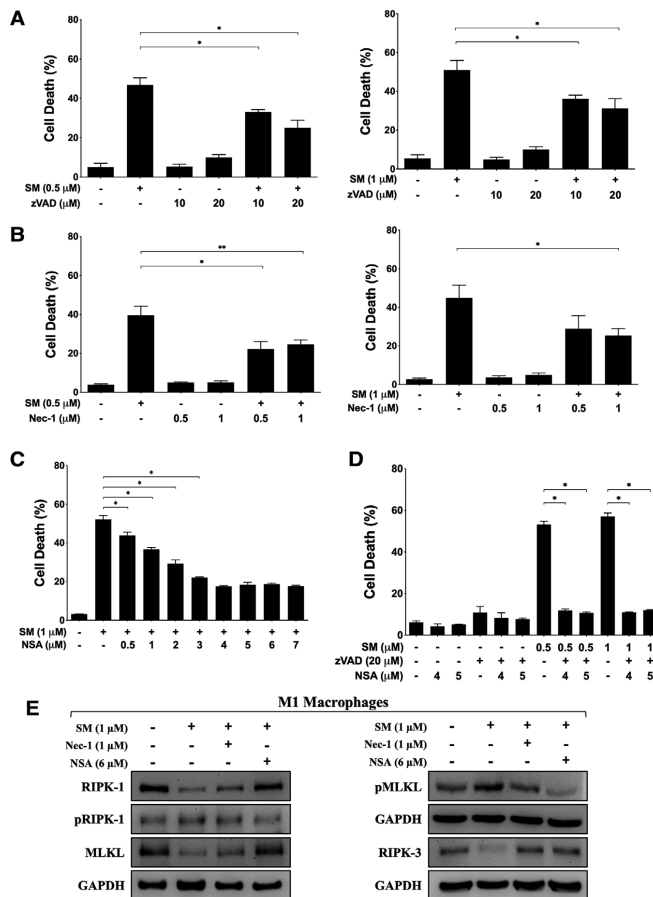


FIGURE 6 SM-induced cell death in M1 Mφs is mediated by apoptosis and necroptosis. M1 Mφs were treated with either SM-LCL161 (0.5 μM; left panels in A and B) or SM-LCL161 (1 μM; right panels in A and B) in the presence or absence of (A) zVAD.fmk (10 μM, or 20 μM), (B) Nec-1 (0.5 μM, or 1 μM), (C) NSA (0.5 μM to 7 μM) or (D) a combination of zVAD.fmk (20 μM), NSA (4 μM or 5 μM) and SM-LCL161. All chemical inhibitors were added 1 h prior to SM-LCL161 treatment. Cells were incubated for 48 h. The percentage cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean ± SD of 4 (A), 5 (B), and 3 (C and D) independent experiments from independent donors. (E) Total protein lysates from M1 Mφs treated with 1 μM of SM-LCL161 alone or combined treatment with Necrostatin-1 (1 μM) or NSA (6 μM) for 5 h were subjected to Western immunoblotting for the expression of RIPK-1 (78 kDa), pRIPK-1 (78 kDa), RIPK-3 (46–62 kDa), MLKL (54 kDa), and pMLKL (54 kDa). The result shown is a representative of 3 independent experiments from 3 different donors

M2c cells were treated with zVAD.fmk for 1 h followed by treatment with SM-LCL161 for 48 h. SM-LCL161 at concentrations of 0.5 or 1 μM alone did not result in induction of cell death in M0 or M2c Mφs (Fig. 8). Interestingly, upon blocking caspase activities by zVAD.fmk at both concentrations of 10 or 20 μM, SM-LCL161 treatment at both concentrations of 0.5 and 1 μM, led to significant increase in cell death in both M0 (Fig. 8A) and M2c (Fig. 8B) Mφs. A representative histogram showing cell death in M0 and M2c Mφs following combination treatment of SM-LCL161 and zVAD.fmk is shown in the Supplementary Fig. 4B.

This induction in cell death after blocking caspases might be due to the activation of necroptosis pathway.^{57–60} Therefore, it is possible that blocking of caspases in SM-treated M0 and M2c Mφs selectively activates the necroptosis pathway. Similar to SM-treated M1 Mφs, Necrostatin-1 (0.5 or 1 μM) was used to determine necroptosis in zVAD.fmk and SM-treated M0 and M2c Mφs. The 2 subsets were treated with Necrostatin-1 and zVAD.fmk for 2 h followed by treatment

with SM-LCL161 (Fig. 9: left panel, 0.5 μM; right panel, 1 μM) for 48 h. Treatment with Necrostatin-1 significantly inhibited SM-LCL161 and zVAD.fmk-induced cell death in a dose-dependent manner (Fig. 9A and B). Similar results were obtained following blockage of the necroptosis pathway by NSA (2 μM) as seen in a representative histogram showing reversal of cell death in NSA-treated M0 and M2c Mφs following combination treatment of SM-LCL161 and zVAD.fmk (Supplementary Fig. 4B).

Similar to the M1 Mφs, the effect of SM-LCL161 and zVAD.fmk on the expression levels of RIPK-1, RIPK-3, and MLKL of M0 and M2c Mφs following treatment with Necrostatin-1 and NSA was evaluated. The results show that SM-LCL161 and zVAD.fmk combined treatment inhibited RIPK-1, RIPK-3, and MLKL expressions that was restored following prior treatment with Necrostatin-1 and NSA in M0 and M2c Mφs (Fig. 9C). Similarly, SM-LCL161 and zVAD.fmk induced the phosphorylation of pRIPK-1 and pMLKL that was dephosphorylated in the presence of Necrostatin-1 and NSA in both M0 and M2c Mφs (Fig. 9C).

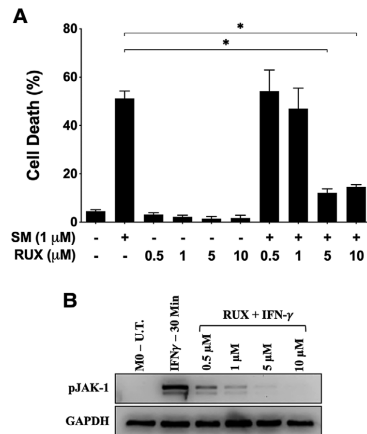


FIGURE 7 JAK pathway regulates SM-induced cell death in M1 Mφs. (A) M0 Mφs were treated with various concentrations of JAK inhibitor (Rux), for 2 h prior to stimulation with IFN- γ for 48 h. Cells were then treated with SM-LCL161 (1 μ M) for another 48 h. The %cell death was determined by intracellular PI staining and flow cytometry. The result shown is a mean $\pm$ SD of 3 independent experiments from 3 different donors. (B) M0 Mφs were treated with various concentrations of Rux for 2 h prior to stimulation with IFN- γ for 30 min. Total protein lysates were analyzed for pJAK-1 (130 kDa) by Western immunoblotting. The result shown is a representative of 3 independent experiments from 3 different donors

3.7 | Human and mouse M1 and M2c Mφs behave similarly with respect to sensitivity toward SM-induced cell death

To investigate whether mouse M1 and M2c Mφs would behave in a manner similar to human Mφs with respect to the SM-induced cell death, mouse BMDMs were cultured for 10 days followed by stimulation with either mouse rIFN- γ or rIL-10 for 48 h for the generation of M1 and M2c Mφs, respectively. M1 and M2c mouse MDMs were treated with different concentrations of SM-LCL161 for another 48 h followed by assessment of cell death by intracellular PI staining and flow cytometry. Results show that mouse M1 Mφs are highly susceptible whereas M2c Mφs are resistant to SM-LCL161-induced cell death (Fig. 10) suggesting that both human and mouse M1 and M2c Mφs behave similarly with respect to sensitivity toward SM-induced cell death.

3.8 | M1 and M2c Mφs are plastic and repolarization of M1 into M2c and of M2c into M1 subset reverses the effects of SM-LCL161 on cell death, expression of cell surface markers, cytokines, and the effectors of the apoptosis pathway

Plasticity is one of the characteristic features of Mφs.³ Therefore, we hypothesized that repolarization of M1 into M2c Mφs and M2c into M1 Mφs would reverse the effect of SM-LCL161-induced cell death. For this, M1 Mφs were rested for 48 h followed by stimulation with

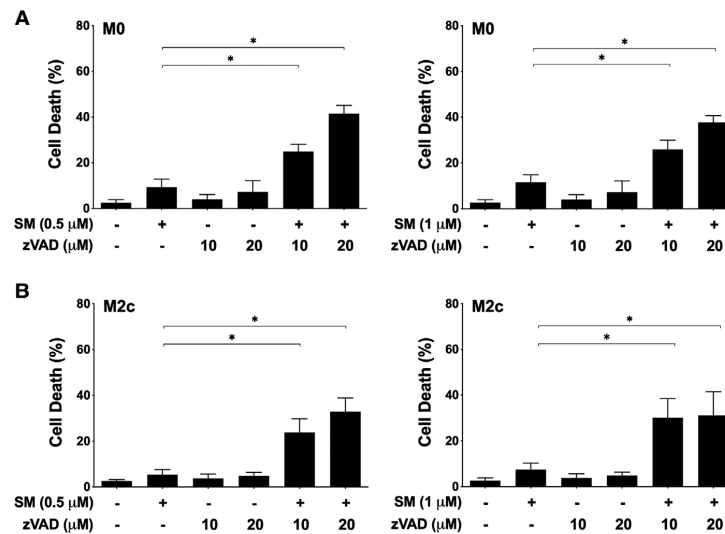


FIGURE 8 SM-LCL161 induces cell death in M0 and M2c following blockage of caspases. (A) M0 and (B) M2c Mφ subsets were treated with either SM-LCL161 (0.5 μ M; left panels) or SM-LCL161 (1 μ M; right panels) in the presence or absence of 10 μ M, or 20 μ M zVAD.fmk. The inhibitor zVAD.fmk was added 1 h prior to SM-LCL161 treatment. Cells were then incubated for 48 h. The percentage cell death was determined by intracellular PI staining and flow cytometry. The result shown in (A) and (B) are a mean $\pm$ SD of 4 independent experiments from 4 different donors

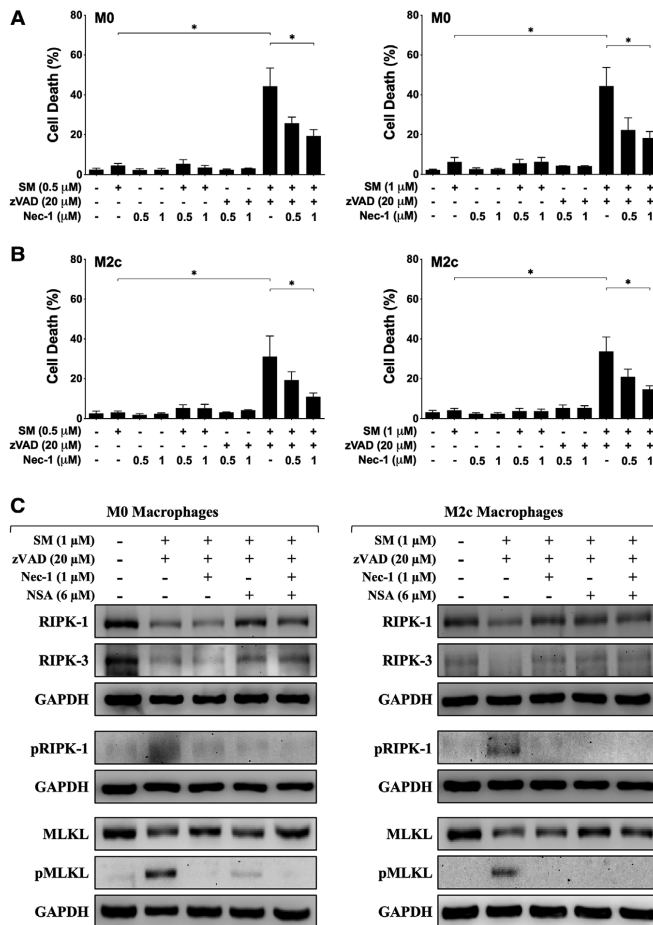


FIGURE 9 SM-induced cell death in M0 and M2c cells following caspase blockage is mediated by necroptosis. (A) M0 and (B) M2c *Mφ*s were treated with either SM-LCL161 (0.5 μ M; left panels) or SM-LCL161 (1 μ M; right panels) in the presence or absence of 20 μ M zVAD.fmk and/or Nec-1 (0.5 μ M or 1 μ M). The inhibitors were added 1 h prior to SM-LCL161 treatment. Cells were then incubated for 48 h. The percentage cell death was determined by intracellular PI staining and flow cytometry. The results shown in (A) and (B) are mean $\pm$ SD of 4 and 3 independent experiments from different donors, respectively. (C) Total protein lysates from M0 and M2c *Mφ*s treated with 1 μ M of SM-LCL161 and 20 μ M zVAD.fmk or combined treatment with Necrostatin-1 1 μ M or NSA 6 μ M for 5 h were subjected to Western immunoblotting for the expression of RIPK-1 (78 kDa), pRIPK-1 (78 kDa), RIPK-3 (46–62 kDa), MLKL (54 kDa), and pMLKL (54 kDa). The result shown is a representative of 3 independent experiments from 3 different donors.

IL-10 for 48 h to generate M2c *Mφ*s from M1 *Mφ*s (M1 > M2c). Similarly, M2c *Mφ*s were rested for 48 h and then stimulated with IFN- γ to generate M1 *Mφ*s from M2c *Mφ*s (M2c > M1). M1 *Mφ*s as expected were highly susceptible to SM-LCL161-induced cell death. However, M1 *Mφ*s when repolarized into M2c *Mφ*s by IL-10 (M1 > M2c) lost that susceptibility (Fig. 11A). On similar lines, M2c *Mφ*s were resistant to SM-LCL161-induced cell death as expected. However, M2c *Mφ*s when differentiated into M1 *Mφ*s by IFN- γ (M2c > M1) developed susceptibility to SM-LCL161-induced cell death (Fig. 11B).

Subsequently, we determined whether repolarization of M1 into M2c subset and of M2c into M1 subset reverses the expression of cell surface markers characteristic for M1 and M2c *Mφ*s. As above, M1 *Mφ*s were repolarized into M2c *Mφ*s (M1 > M2c) and M2c were repolarized into M1 *Mφ*s (M2c > M1) followed by analysis of cell surface markers characteristic for M1 and M2c *Mφ*s namely CD14, CD86, CD163, and HLA-DR. The M2c *Mφ*s expressed significantly high lev-

els of CD14 and CD163 and significantly low levels of CD86 and HLA-DR compared to the M1 *Mφ*s. Following repolarization of M2c into M1 *Mφ*s (M2c > M1), repolarized M1 *Mφ*s expressed significantly low levels of CD14 and CD163 and significantly high levels of CD86 and HLA-DR similar to the levels expressed by M1 *Mφ*s (Fig. 11C). Similarly, M1 *Mφ*s expressed significantly high levels of CD86 and HLA-DR and significantly low levels of CD163 and CD14 compared to the M2c *Mφ*s (Fig. 11C). Following repolarization of M1 into M2c *Mφ*s (M1 > M2c), repolarized M2c *Mφ*s expressed significantly low levels of CD86 and HLA-DR and significantly high levels of CD163 and CD14 similar to the levels expressed by M2c *Mφ*s (Fig. 11C).

We also determined whether repolarization of M1 into M2c subset and of M2c into M1 subset reverses the expression of pro- and anti-inflammatory cytokines following stimulation of M1 and M2c *Mφ*s with LPS. M1 *Mφ*s as expected produced significantly high levels of proinflammatory cytokines IFN- γ , IL-6, IL-12p70, TNF- α ,

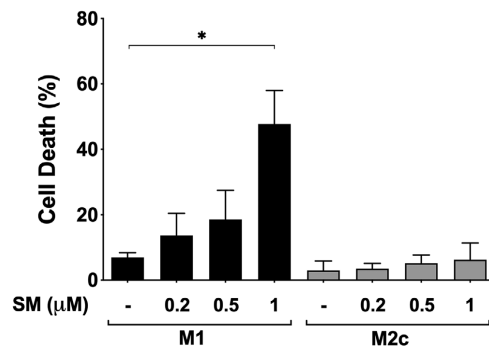


FIGURE 10 M1 mouse BMDMs are highly sensitive whereas M2c Mφs are resistant to SM-induced cell death. BMDMs were stimulated with mouse rIFN- γ (M1) or rIL-10 (M2c) for 48 h. M1 and M2c mouse BMDMs were treated with 0.2, 0.5, and 1 μ M of SM-LCL161 for 48 h. The percentage cell death was determined by intracellular PI staining and flow cytometry. The results shown are mean $\pm$ SD of 3 independent experiments from 3 different BMDMs

and IL-23 compared to the M2c Mφs. Repolarization of M1 into M2c abrogated the production of these inflammatory cytokines that was equal to the levels produced by M2c Mφs (Fig. 11D). Similarly repolarization of M2c into M1 significantly enhanced the production of abovementioned pro-inflammatory cytokines that was equivalent to the cytokines produced by M1 Mφs (Fig. 11D). On the other hand, M2c Mφs produced high levels of anti-inflammatory IL-10 compared to M1 Mφs. Repolarization of M2c into M1 significantly decreased IL-10 production that was equivalent to that produced by M1 Mφs. Similarly repolarization of M1 into M2c significantly enhanced IL-10 production that was equivalent to that produced by M2c Mφs (Fig. 11D).

Next, we examined whether repolarization of M1 into M2c subset and of M2c into M1 subset reverses the expression of caspase-3 and caspase-8, PARP, RIPK-1, RIPK-3, MLKL, pMLKL, and Bax, the key effectors molecules of apoptosis and necroptosis pathways, following treatment with SM-LCL161. As above, M1 Mφs were repolarized into M2c Mφs (M1 > M2c) and M2c were repolarized into M1 Mφs (M2c > M1) followed by treatment with SM-LCL161 and analysis of caspase-3 and caspase-8, PARP, RIPK-1, and RIPK-3 by Western immunoblotting. Treatment of repolarized M1 to M2c Mφs (M1 > M2c) with SM-LCL161 did not affect the expression of full length or cleaved caspase-8, caspase-3, or PARP or degradation of RIPK-1, RIPK-3 and MLKL, pMLKL, or Bax (Fig. 11E). Interestingly, treatment of repolarized M2c to M1 Mφs (M2c > M1) with SM-LCL161 caused the degradation of full-length caspase-8, caspase-3, and PARP, accumulation of cleaved caspase-3, caspase-8, pMLKL, and Bax, and degradation of RIPK-1, RIPK-3, and MLKL (Fig. 11E). Overall, these results suggest that repolarization of M1 into M2c subset and of M2c into M1 subset reversed the effect of SM-LCL161 on cell death that was associated with reversal in the expression of cell surface markers, pro- and anti-inflammatory cytokines, and the effectors of the cell death pathways such as caspases, PARP, RIPK-1, and RIPK-3 and MLKL.

4 | DISCUSSION

Smac mimetics have been actively studied for their apoptosis-inducing abilities in cancer cells.^{61,62} However, the role of IAPs in regulating cell death in primary cells and in particular primary human Mφs is not well understood. Human monocytes are highly susceptible to the cytotoxic effects of SMs³² whereas differentiated Mφs (M0) develop resistance to the cytotoxic abilities of SMs.²⁹ Whether normal human Mφ subsets are resistant to the SM-induced cytotoxic effects remains unknown. Herein, we show that polarization of M0 Mφs toward M1 state selectively rendered them highly susceptible to cell death in response to SM-LCL161 whereas M2a, M2b, and M2c differentiated subsets, like their parent undifferentiated M0 cells, were resistant, M2c being the most resistant. Furthermore, SM-induced cell death in M1 Mφs exhibited the characteristics of apoptosis as well as necroptosis and activated both extrinsic and intrinsic pathways of apoptosis. In contrast, M0 and M2c Mφs experienced cell death following simultaneous blockage of the IAPs pathway by SM-LCL161 and the caspase pathway by the pan-caspase inhibitors, and this cell death was attributed to necroptosis.

The results on phenotypic and functional characterization confirm our previous observations that M1 Mφs expressed high levels of CD80, CD86, and HLA-DR and produced high levels of pro-inflammatory IFN- γ , IL-23, IL-12p70, and TNF- α .⁶ CD80 and CD86 play key roles in Th1/Th2 cell differentiation and generation of immune responses.⁶³ M2a Mφs exhibited higher CD200R expression and produced high levels of IL-10. CD200-CD200R interactions in endothelial cells and Mφs delivers an inhibitory signal and inhibit their activation. The viral homologs of CD200, such as herpesvirus 8K14 protein,⁶⁴ cytomegalovirus e127 protein,⁶⁵ and myxoma virus M141R⁶⁶ following interaction with CD200R may inhibit Mφ function. M2c Mφs also expressed high levels of CD163 that plays a role in preventing tissue inflammation that may explain the role of M2c in preventing tissue inflammation.⁶⁷ M2b and M2c Mφs did not produce any of the pro-inflammatory IFN- γ , IL-23, and IL-12p70. These cells characteristically exhibited low levels of HLA-DR that may also be involved in dampening the immune responses mediated by stress, such as trauma, burns, surgery, and sepsis.⁶⁸ The expression of high levels of CD14, and CD80, and production of both pro-inflammatory IL-6, IL-1 β , TNF- α , and anti-inflammatory IL-10 cytokines on M2b Mφs may explain both pro- and anti-inflammatory nature of M2b Mφs.^{6,12,69}

Initially described in the context of apoptosis as promoting cell survival, the IAPs proteins serve as key regulators of innate immune signaling and inflammation.³⁵ The role of IAPs has been studied by SMs.^{37,58,61} Several commercially available SMs are in clinical trials as anticancer agents³⁷ (<https://www.cancer.gov/about-cancer/treatment/clinical-trials/intervention/smac-mimetic-lcl161>). SM-LCL161, a monovalent form of SMs, mimics the AVPI tetrapeptide binding motif present in natural SMAC.⁷⁰ SMs following binding to IAPs promote their proteasomal degradations leading to IAPs down-regulation and apoptosis particularly in cancer cells.^{37,58,61,70} We have previously shown that IAPs play a key role in the resistance of Mφs to HIV-Vpr-induced apoptosis and mitochondrial depolarization.^{32,33}

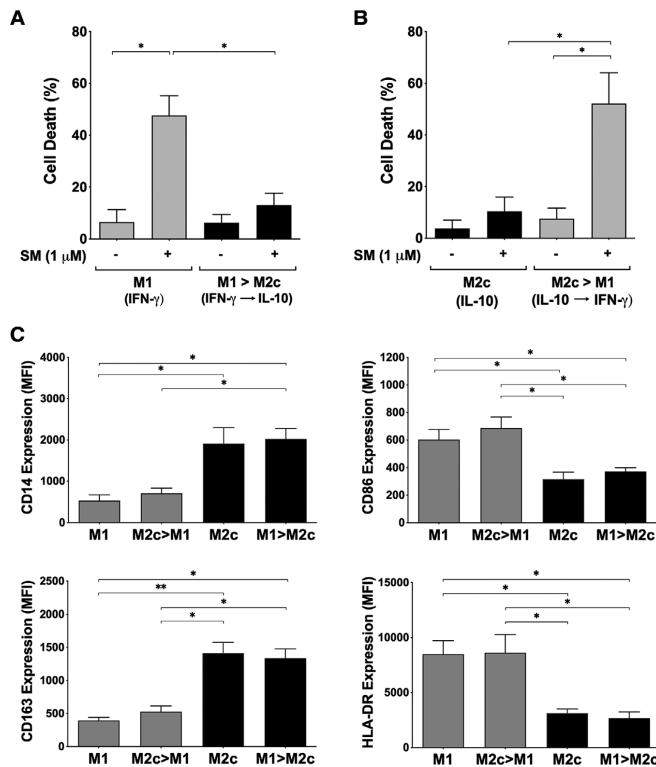


FIGURE 11 Repolarization of M1 into M2c and of M2c into M1 M ϕ subsets reverses the effect of SMs on cell death, cell surface markers, cytokines expression, and apoptosis/necroptosis-associated molecules. M0 M ϕ s were stimulated for 48 h with (A) IFN- γ , or (B) IL-10 to generate M1 and M2c M ϕ s. Cells were then washed, rested for 48 h, and then restimulated with (A) IL-10, or (B) IFN- γ for another 48 h. Cells were treated with SM-LCL161 (1 μ M) for 48 h following IFN- γ or IL-10 stimulation and after restimulation with either IL-10 (M1 to M2c) or IFN- γ (M2c to M1). The percentage cell death was determined by intracellular PI staining and flow cytometry. (C) Cell surface markers expression on M1, M2c, M2c > M1 and M1 > M2c M ϕ s. Cells were stained with Abs against CD14, CD86, CD163, and HLA-DR cell surface molecules and fluorescence was measured by flow cytometry. The MFI values were obtained using FlowJo software. The results shown are mean $\pm$ SD of 3 independent experiments from 3 different donors. (D) Cytokines production in M1, M2c > M1, M2c, and M1 > M2c M ϕ s. M1, M2c > M1, M2c, and M1 > M2c M ϕ s were stimulated with LPS for 24 h followed by measurement of IFN- γ , IL-6, IL-12p70, IL-23, IL-10, and TNF- α . Cytokines levels in the supernatants were measured using multiplexing immunobead assays as described in Section 2. The results shown are mean $\pm$ SD of 3 independent experiments from 3 different donors. (E) Total protein lysates from M1 > M2c and M2c > M1 repolarized M ϕ s treated with 1 μ M of SM-LCL161 for 5 h were subjected to Western immunoblotting for the expression of caspases-8 (43 and 57 kDa), caspase-3 (19 and 35 kDa), PARP (89 and 116 kDa), RIPK-1 (78 kDa), RIPK-3 (46-62 kDa), MLKL (54 kDa), pMLKL (54 kDa), and Bax (20 kDa). The immunoblot shown is a representative of 3 independent experiments from 3 different donors.

Herein, we show that although SM-LCL161 caused degradation of IAPs in all M ϕ subsets, M1 M ϕ s only were sensitive to SM-induced cell death. Furthermore, SM-induced cell death was not attributed to the relative levels of cIAP-1 and cIAP-2 expressed in M0, M1, and M2c M ϕ s as M1 M ϕ s expressed highest levels of cIAP-1 and cIAP-2, yet were highly susceptible to SM-induced cell death. On similar lines, although M2c M ϕ s expressed lowest levels of cIAP-1 and cIAP-2, yet were highly resistant to the SM-induced cell death. Our results also show that IAPs-mediated M ϕ survival is determined by IFN- γ -mediated M1 state of differentiation as JAK inhibitor reversed the susceptibility of M1 M ϕ s to SM-induced cell death. Similarly, the resistance of M ϕ s to SM-induced apoptosis was attributed to the IL-10-mediated differentiation toward M2c state. This was confirmed by reversal of susceptibility to SM-induced cell death following repolarization of M1 into M2c and of M2c into M1 subsets.

Our results show that SM-LCL161 induced apoptosis in M1 M ϕ s through activation and cleavage of caspase-3/7, -8, and -9, and blockage of caspases by pan-caspase inhibitors significantly reduced SM-induced cell death. SMs generally induce apoptosis in SM-sensitive tumor cell lines through the secretion of TNF- α in response to IAPs ablation and consequently become susceptible to TNF- α -induced apoptosis.⁷¹⁻⁷³ cIAP-1 and cIAP-2 associate with the TNF- α receptor complex I to mediate TNF- α -induced activation of the classical NF- κ B pathway³⁵ and at the same time inhibit the alternative NF- κ B pathway by promoting constant degradation of NF- κ B-inducing kinase (NIK), the regulatory kinase of noncanonical NF- κ B signaling.⁷⁴ The IAPs degradation by SMs facilitates TNF- α -stimulated cell death by promoting the assembly of ripoptosome containing the core components of complex-IIa, comprising of proteins such as non-ubiquitinated RIPK-1, Fas-associated protein with death domain (FADD), and caspase-8

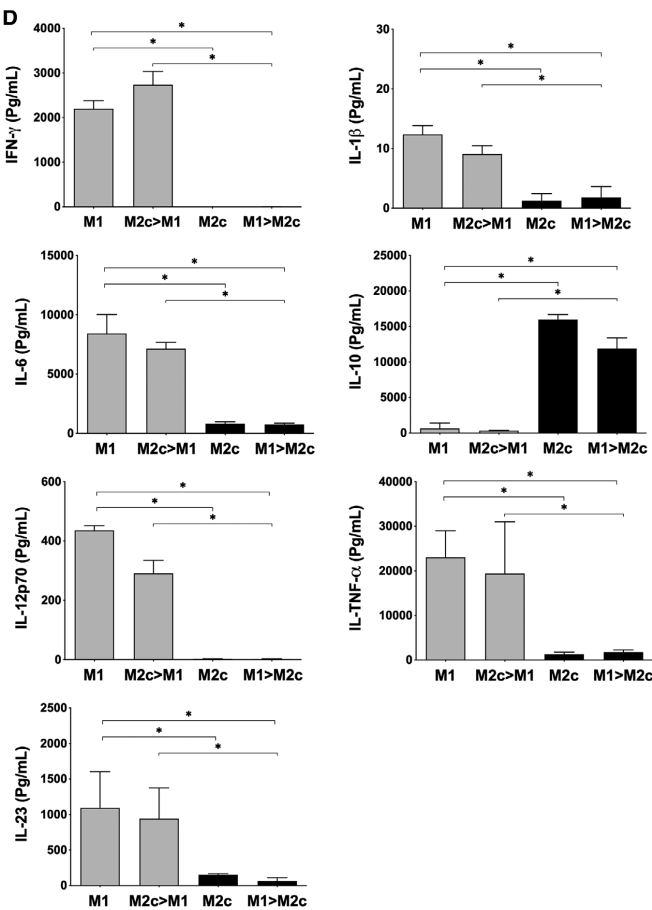


FIGURE 11 Continued

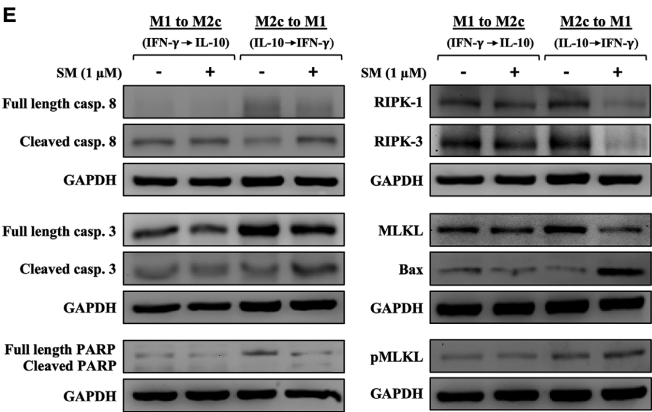


FIGURE 11 Continued

resulting in caspase-dependent apoptosis. However, IAPs degradation by SMs and blockade of caspase activation, especially of caspase-8, can promote the formation of complex IIb, the necrosome complex containing RIPK-1, RIPK-3, and MLKL, leading to necroptosis.^{48,52,74}

Since SM-LCL161 treatment up-regulated Bax and caused activation and cleavage of caspase-9, the molecules involved in the intrinsic apoptotic pathway, and caspase-8, the caspase responsible for extrinsic pathway of apoptosis^{46,47} suggest that SM-induced apoptosis involves the activation of both intrinsic and extrinsic pathways in M1 but not in M0 and M2c M ϕ s. The extrinsic pathway is activated via the release of TNF- α or the activation of Fas.^{46,47} Since SMs are known to induce TNF- α production in cancer cells, it is possible that SM-induced cell death in M1 M ϕ s may involve TNF- α /TNF- α R extrinsic pathway in addition to apoptosis mediated by the intrinsic pathway. SM-LCL161 treatment did not affect the expression of either Fas or TRAIL suggest that SM-induced apoptosis is not regulated by Fas or TRAIL activation in M1 M ϕ s despite expressing higher levels of these proteins compared to M0 and M2c. Although Mcl-1 expression was not affected by SM treatment, the involvement of other members of the Bcl-2 family proteins and the mitochondrial membrane potential, the apical components in the intrinsic pathway,^{32,34,37} in SM-induced cell death remains to be investigated.

The fact that zVAD.fmk, the pan-caspase inhibitor, did not fully restore cell survival in SM-treated M1 M ϕ s suggested that SM-induced cell death in M1 M ϕ s may involve other cell death mechanisms most likely necroptosis, which works as a backup mechanism in certain cells when apoptosis is inhibited.⁴⁸ The signaling pathway involved in necroptosis observed following TNF- α stimulation has been studied. TNF- α binding to its receptor leads to the recruitment of TNFR-associated death protein (TRADD) and TNF receptor-associated factor-2 (TRAF2) and activation of RIPK-1 that recruits RIPK-3 forming the ripoptosome.^{74,75} Phosphorylation of MLKL by the ripoptosome drives oligomerization of MLKL, causes plasma membrane to rupture, and eventually leading to necroptosis.^{76,77} Since necroptosis inhibitors necrostatin-1⁵¹ and NSA⁷⁷ inhibited SM-induced cell death suggested that SM-induced cell death in M1 M ϕ s is also mediated by necroptosis. SMs have been shown to trigger necroptosis in various tumor cell lines including pancreatic,⁶⁰ acute leukemic,^{57,59} and breast cancer⁵⁸ cell lines in the presence of caspase inhibitors (zVAD.fmk and/or caspase-8 inhibitors). Similar to the cancer cell lines, M0 and M2c M ϕ s exhibited SM-induced necroptosis in the presence of zVAD.fmk. This was evident by the degradation of RIPK-1, RIPK-3, MLKL, and up-regulation of phospho-RIPK-1 and phosphor-MLKL following SM treatment and their prevention by necrostatin-1 and NSA. In contrast, we show for the first time that SM-LCL161 alone induced necroptosis in M1 M ϕ s in the absence of caspase inhibitors. Moreover, simultaneous addition of inhibitors for both apoptosis and necroptosis completely prevented SM-induced cell death. The observation that SM-induced cell death is mediated by apoptosis and necroptosis was evident by the degradation of RIPK-1, RIPK-3, caspase-8, and MLKL in M1 M ϕ s that was prevented by necrostatin-1. Whether both processes are operative in the same cell, or different cells are undergoing different death mechanisms independently of each other is not clear.

Plasticity is one of the key features of M ϕ s.^{3,9,24} M ϕ subsets are not an end stage of differentiation as their susceptibility to SM-induced cell death can be modulated by changing the cytokine environment. M1 M ϕ s lose the susceptibility to SM-induced cell death once these cells are cultured with IL-10, the stimuli for generating M2c M ϕ s. Inversely, M2c M ϕ s undergo cell death when stimulated with IFN- γ , the M1 generating stimulus. In addition, repolarization of M1 into M2c and of M2c into M1 subsets reversed the effect of SM-LCL161 with respect to expression of cell surface markers, cytokines, and signaling pathways associated with apoptosis and necroptosis. These observations suggest that M ϕ repolarization in response to their respective cytokines impacts their IAPs signaling and their responsiveness to SM-induced cell death.

In summary, the survival of human M ϕ s is critically linked to the activation/ blockage of the IAPs pathways and differentiation towards either the pro-inflammatory state by IFN- γ or the anti-inflammatory state by IL-10. Moreover, selective induction of cell death in M1 M ϕ s by SM-LCL161 alone or in M0/M2c M ϕ s by SM-LCL161 in conjunction with caspase inhibitors would have potential therapeutic benefits in regards to M ϕ associated inflammatory or anti-inflammatory diseases, respectively, in addition to the existing therapeutic strategies such as to reprogram M1 M ϕ s or induce subsets switching.^{3,24} Inflammatory M1 and anti-inflammatory M2 M ϕ s have been associated with many diseases including rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis,^{7,9,19,78} lepromatous leprosy, Whipples disease, and chronic rhinosinusitis.^{16,20,21} It may be pointed out that killing of cancer cells by SMs in diseases like cancer or alternatively, killing of M0/M2c M ϕ s by caspase inhibitors and SMs in chronic diseases may adversely impact their ability to mount an effective innate response and worsen immune deficiency. Since SM-LCL161 effect on cell death is conserved across mouse and human M1 M ϕ s, mouse model can be a useful experimental tool to study the cytotoxic effect of SMs on several inflammatory diseases.

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AUTHORSHIP

H.A. designed and performed the experiments and analyzed all data. R.C. performed some of the confirmatory experiments. N.G. performed experiment shown in Fig. 5B. S.I. initiated the project. D.A. generated the mouse M ϕ s. S.D. helped in performing experiment shown in Fig. 1A, A.V. and A.C. performed cytokine analysis shown in Fig. 1B and 11D. S.D., N.G., and R.C. contributed in blood collection, monocyte isolation,

and generation of human M ϕ s. J.B.A. provided blood samples and discussed the results. E.C. provide the mouse M ϕ s, and helped in writing the manuscript. A.K. designed the experiments, supervised the study, and helped in data interpretation and writing of the manuscript.

DISCLOSURE

The authors declare no conflicting financial interests.

ORCID

Hamza Ali  <https://orcid.org/0000-0002-6215-5409>

Jonathan B. Angel  <https://orcid.org/0000-0003-3102-4462>

Ashok Kumar  <https://orcid.org/0000-0002-3615-8681>

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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